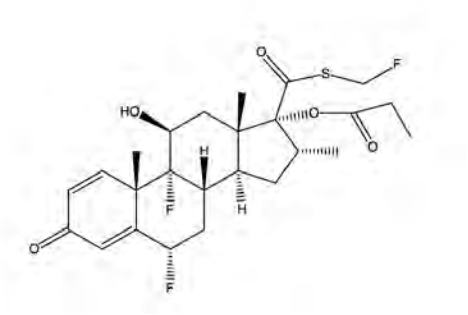
SAMPLE LITERATURE REPORT

Class ID #: 007

LITERATURE PROBLEM for FLUTICASONE PROPIONATE

1. **Structural Formula:**



Drawn using ChemDraw Prime 15.1

2. Compound Name

Flonase (commercial name)
Fluticasone Propionate (active compound)

3. **Molecular Formula**: $C_{25}H_{31}F_3O_5S$

4. Molecular Weight: 500.57

5. **Physical Properties:**

Pure, white crystalline solid.
Melting Point: 274-275° C [1]

6. **Spectral Properties:**

Infrared: Recorded in the range from 650-4000 cm^{-1} at 4 cm^{-1} spectral resolution: 3336 cm^{-1} (m, δ, O-H) 3069 cm^{-1} (vw, ν, C-H) 3051 cm^{-1} (vw, ν, C-H) 3024 cm^{-1}(vw, ν, C-H of five-membered ring) 2975 cm^{-1} (m, ν, C=C-H) 2964 cm^{-1} (m, νas, CH_3) 2942 cm^{-1} (m, νas, CH_2) 2881 cm^{-1} (m, νs, CH_2) 1774 cm^{-1} (s, ν, C-O) 1699 cm^{-1} (s, ν, S-C-O) 1661 cm^{-1} (vs, ν, C-O) 1616 cm^{-1} (s, ν, C=C) 1530 cm^{-1} (vw, δ, CH_3) 1455 cm^{-1} (m, δ, CH_3) 1395 cm^{-1} (mw, δ, CH_2) 1365 cm^{-1} (mw, δ, C-H) 1304 cm^{-1} (m, ν, C-C) 1271 cm^{-1} (m, ν, C-F) 1027 cm^{-1} (m, ν, F-C-S) 993 cm^{-1} (s, ρ, CH_3) 782 cm^{-1} (w, ρ, CH_2). [2]

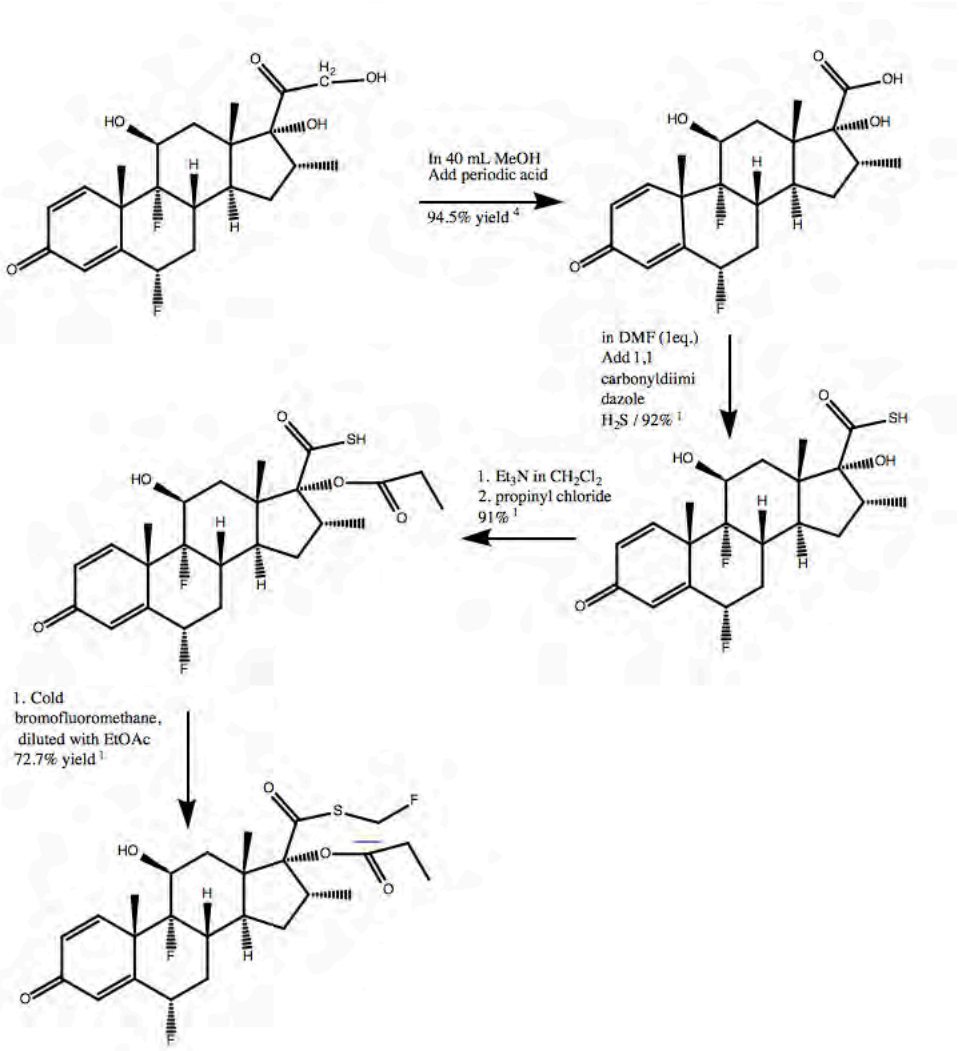
Proton NMR: ^{1}H (90 MHz) 0.96 (16α-CH_3, d, J = 7Hz), 1.06 (propionate CH_3, t, *J* = 7 Hz), 1.07 (13-CH_3, s), 1.56 (10-CH_3, s), 2.33 (propionate CH_2, q, *J* = 7 Hz), 4.32 (11α-H, broad m), 5.63 (11-OH, broad d, *J* = 4 Hz), *ca.* 5.75 (6-H, broad dm, *J* = 50 Hz), 6.00 (SCH_2F, d, *J* = 51 Hz), 6.19 (4-H, m), 6.37 (2-H, dd, *J* =10, 2 Hz), 7.33 (1-H, d, J = 10Hz). [1]

Carbon NMR: ^{13}C (101 MHz, DMSO-d6) δ 192.87 (s), 183.98 (s), 172.07 (s), 162.43 (d, J = 13.5 Hz), 151.54 (s), 128.91 (s), 119.25 (d, J = 12.1 Hz), 99.72 (d, J = 176.3 Hz), 95.94 (s), 86.62 (d, J = 178.0 Hz), 80.92 (d, J = 211.8 Hz), 69.97 (d, J = 37.2 Hz), 48.40 (s), 47.84 (d, J = 22.4 Hz), 42.84 (s), 35.74 (s), 35.10 (s), 33.73 (d, J = 19.4 Hz), 33.37 (s), 31.93 (m), 26.94 (s), 22.73 (s), 16.95 (s), 16.08 (s), 9.05 (s). [3]

Fluorine NMR: ^{19}F (376 MHz, CDCl3) δ − 165.35 (dd, J = 27.5, 8.5 Hz), − 187.00 (dd, J = 48.3, 13.8 Hz), − 191.35 (t, J = 49.6 Hz). [3]

Mass Spectra: MS (ESI+) *m/z* 501.0 $[M + H]^+$. [3]

7. **SYNTHETIC ROUTE:**



First compound is flumethasone, which is referred to in Kertesz as being one of four baseline "commercially available" steroid hormones that one can make thiol esters from.[4] Given that Flonase is sold for around 10-15$ for a total of 3000 mcg of active compound (0.003 grams), 1 gram of the active compound would provide ~333 bottles or 20,000 doses of the drug. Thus, 1 gram would equal, in a low estimate, about $3,300. Thus, I don't think it's unreasonable that the previously described "commercially available" precursor, flumethasone is priced at $1042 per gram, given as we're essentially producing, considering yields and lowballing, the active compound for 10,000 doses of a drug.

8. **SHORT SUMMARY**

Fluticasone Propionate (FP) is an androstane carbothioate glucocorticosteroid that is prescribed to treat inflammatory respiratory conditions such as asthma and allergic rhinitis. As opposed to other glucocorticosteroids, FP has a very low oral bioavailability and is measured to have approximately 100 times more concentration during its half-life in peripheral and 300-400 times higher in central lung tissue as opposed to serum. [5] It is also retained in lung tissue and has been found to saturate glucocorticosteroid receptors, binding with nearly full affinity.[6] For medicinal purposes, it is most commonly administered as a nasal spray with dosing from 50-2000μg per day in adults.[5]

9. **BIOLOGICAL MODE OF ACTION**

Fluticasone propionate is a glucocorticosteroid, and in this class of compounds, the main effect is a reduction of inflammation. This is generally achieved by many kinds of glucocorticoids by inhibiting the expression of various components of the immune and inflammatory systems such as cytokines, chemokines, cell-surface glucocorticoid receptors, adhesion molecules, tissue factor, degradative proteinases, and certain enzymes that produce inflammatory mediators.[7] When considering the specific example of glucocorticoids on lung tissue, the immediate decrease in inflammation seems to be caused by the reduction of mast cells (derived from the German *Mastzellen*, which means "well-fed cells" due to their high concentration of granules in their cytoplasm). Mast cells contain many inflammatory mediators such as histamine, proteases, and cytokines.[8] The reduction in mast cells seems to be caused by the subsequent reduction of IL-3, which is necessary for mast cells to survive.[9] Fluticasone propionate has also been shown to reduce levels of IL-4[10], but this would be indicative of the reduced number mast cells not releasing IL-4, as the release of inflammatory mediators does not seem to be decreased. Therefore, it is due to the decrease in the number of mast cells based on IL-3 that is a more likely model. As stated on the drug facts information for fluticasone propionate, the "the precise mechanism through which fluticasone propionate affects allergic rhinitis symptoms is unknown", but there are several models for how glucocorticoids reduce the production of inflammatory factors, mostly based on transcriptional interference. The mechanism for this would be that the glucocorticoid would bind to the glucocorticoid receptor, which then would directly bind to two transcription factors (activator protein-1 and nuclear factor κB), thus leaving them unable to bind to the DNA as a transcription factor and produce the associated protein for an inflammatory mediator (interleukins, cytokines, etc.). A second proposed mechanism is that the liganded glucocorticoid receptor inhibits the MAP kinase pathway by keeping the kinases dephosphorylated.[7] In more recent literature, these theories have continued to be supported, with the added discovery that there are different types of glucocorticoid receptor isoforms and that a given glucocorticoid will confer a different conformational change in the receptor that will *then* change either the binding of transcriptional factors to the DNA, effect a change on the chromatin of the strand, or influence a co-receptor.[11] In terms of fluticasone propionate, the discovery that the glucocorticoid receptor binds to NF-κB at relatively low glucocorticoid concentrations compared to the requirement to induce gene expression, or otherwise put, it is more likely that the mechanism occurs by repressing the expression of inflammatory mediators rather than inducing the expression of inflammatory inhibitors.[12]

10. **BIBLIOGRAPHY**

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HOWEVER, IF YOU PREFER, YOU MAY ONLY PROVIDE THE RELEVANT PAGE.

IN THIS SAMPLE REPORT, ALL THE REFERENCES ARE PROVIDED AND THE DATA IS HIGHLIGHTED

Synthesis and Structure–Activity Relationships in a Series of Antiinflammatory Corticosteroid Analogues, Halomethyl Androstane-17β-carbothioates and -17β-carboselenoates

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Received September 20, 1993®

The preparation and topical antiinflammatory potencies of a series of halomethyl 17α-(acyloxy)-11β-hydroxy-3-oxoandrosta-1,4-diene-17β-carbothioates, carrying combinations of 6α-fluoro, 9α-fluoro, 16-methyl, and 16-methylene substituents, are described. Key synthetic stages were the preparation of carbothioic acids and their reaction with dihalomethanes. The carbothioic acids were formed from 17β-carboxylic acids by initial reaction with dimethylthiocarbamoyl chloride followed by aminolysis of the resulting rearranged mixed anhydride with diethylamine, or by carboxyl activation with 1,1'-carbonyldiimidazole (CDI) or 2-fluoro-*N*-methylpyridinium tosylate (FMPT) and reaction with hydrogen sulfide, the choice of reagent being governed by the 17α-substituent. Carboxyl activation with FMPT and reaction with sodium hydrogen selenide led to the halomethyl 16-methyleneandrostane-17β-carboselenoate analogues. Antiinflammatory potencies were measured in humans using the vasoconstriction assay and in rats and mice by a modification the Tonelli croton oil ear assay. Best activities were shown by fluoromethyl and chloromethyl carbothioates with a 17α-propionyloxy group. *S*-Fluoromethyl 6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (fluticasone propionate, FP) was selected for clinical study as it showed high topical antiinflammatory activity but caused little hypothalamic–pituitary–adrenal suppression after topical or oral administration to rodents.

The successful development of corticosteroid analogues designed to show high potency on local application to inflamed tissue has been reviewed.[1-4] Although the avilable compounds showed only weak undesirable systemic side effects after topical administration, we continued to seek further improvement. In this paper we describe a series of potent and novel corticosteroidal halomethyl esters of androstane-17β-carbothioic acids with promising separations of activity.[5]

Earlier we reported that the normal two-carbon 17β-side chain of pregnanes was not necessary for corticoid activity, androstane-17β-carboxylates showing high topical antiinflammatory activity if both the 17α-hydroxy and 17β-carboxylic acid functions were esterified, with the greatest activity being shown by 17α-propionates as fluoromethyl carboxylates.[1,6-8]

We first explored the synthesis of 17α-unsubstituted 17β-carbothioates in making compounds with anaesthetic activity.[9,10] Kertesz and Marx[11] overcame many difficulties while employing carboxyl-activation procedures followed by reactions with alkanethiols in the synthesis of some alkyl 17α-acyloxy 17β-carbothioates which showed good topical antiinflammatory activity. Their synthetic methods could not, however, be applied to the preparation of halomethyl carbothioates (analogues of the potent 21-halopregnan-20-ones[1-3]) as halomethanethiols are not known and would be expected to be very unstable. We therefore devised new methods of preparing 17α-hydroxy and 17α-acyloxy 17β-carbothioic acids, which could then be esterified by reacting their salts with dihalomethanes. Furthermore, 17α-acylation of haloalkyl 17α-hydroxy 17β-carbothioates was likely to be unselective in the presence of an 11β-ol, so it would be preferable to 17α-acylate at the carbothioic acid stage, as with the carboxylic acids.[7,8] The antiinflammatory potencies of the halomethyl 17α-acyloxy 17β-carbothioates were in general high, as delineated in Table 3.

Chemistry

The 17α-hydroxyandrostane-17β-carboxylic acid intermediates **2** for the desired carbothioates were prepared by oxidative cleavage of 21-hydroxypregnan-20-ones **1** with periodic acid in aqueous dioxane or THF. They were readily 17α-acylated, without concomitant 11β-acylation, by reaction with excess acyl chloride and triethylamine followed by aminolysis of the resulting 17α-acyloxy 17β-carboxylic acid mixed anhydrides with diethylamine[1,7,8] to give the corresponding acids **3** (Scheme 1).

Our first synthesis of carbothioic acids resulted while studying the aminolysis of the mixed anhydride formed by reaction of the 17α-propionyloxy 17β-carboxylic acid **3d**[11] with dimethylthiocarbamoyl chloride in pyridine. The mixed anhydride was initially believed to be the thione **4a**, but aminolysis with diethylamine gave the 17β-carbothioic acid **6i** and it now has been assigned the rearranged structure **4bc**;[3] this was supported by its synthesis as the major product from the reaction of thioacid **6i** with dimethylcarbamoyl chloride and by the lack of a carbonyl IR absorption above 1740 cm^{-1}, in contrast to that observed (1783 cm^{-1}) for the oxygen

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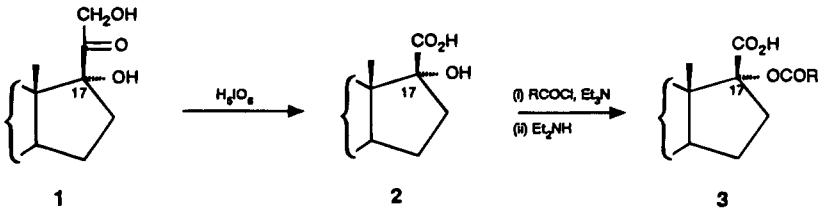
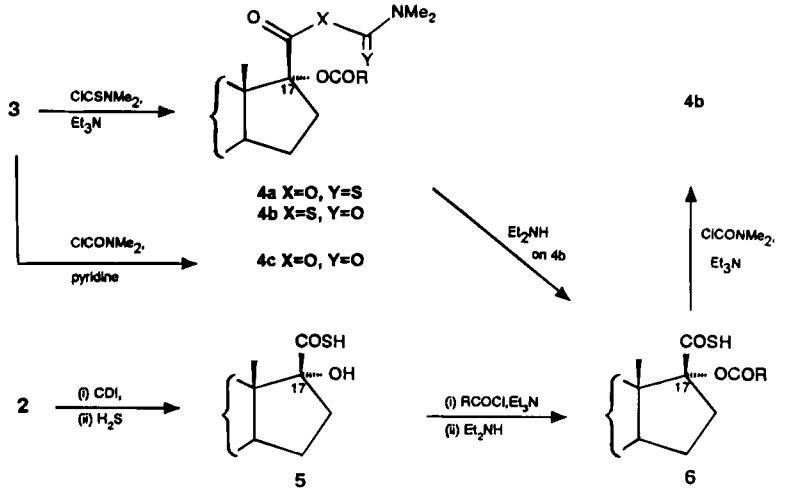
® Abstract published in *Advance ACS Abstracts*, September 15, 1994.

0022-2623/94/1837-3717$04.50/0

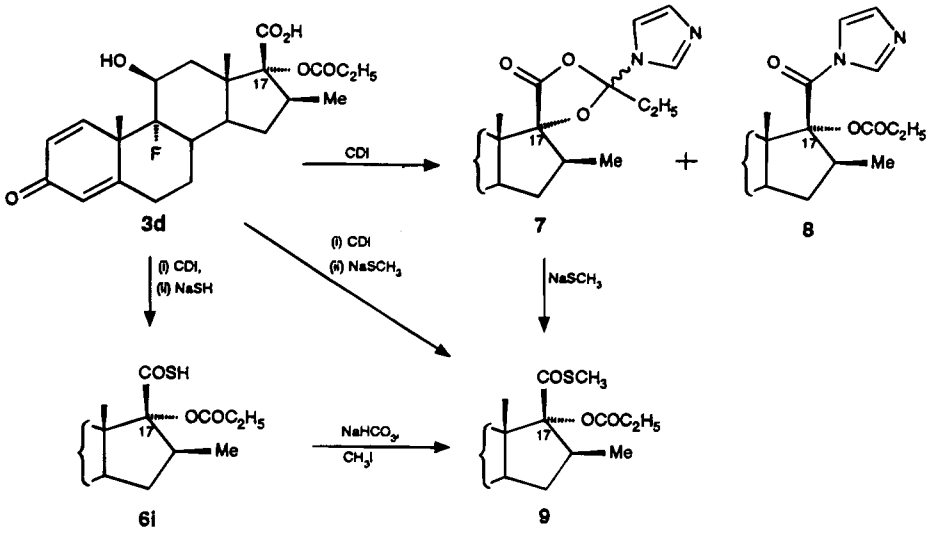
Scheme 1



Scheme 2



Scheme 3

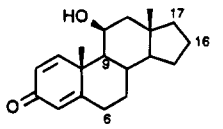


analogue **4ca** (see Scheme 2, also Table 1 for the definition of these compound letters).

Gais[12] reported that carboxylic acid imidazolides are accessible in almost quantitative yields from carboxylic acids and 1,1′-carbonyldiimidazole (CDI) and that they react rapidly with alphatic and aromatic thiols to give thioesters in high yields. Kertesz and Marx[11] applied this method to the synthesis of alkyl androstane-17β-carbothioates. We found that the imidazolides from 17α-hydroxy 17β-carboxylic acids also react with hydrogen sulfide to give 17α-hydroxy 17β-carbothioic acids **5**. Like the corresponding carboxylic acids, these were readily and selectively 17α-acylated without concomitant 11β-acylation (Scheme 2).

Kertesz and Marx[11] reported that CDI failed to activate 17α-acyloxy 17β-carboxylic acids, but we found that CDI in dimethylformamide at 22 °C activated the 17-propionate acid **3d** to reaction with NaSH (to give **6i**) or NaSMe (to give **9**) but not to reaction with H_2S. The reaction of H_2S with other carboxyl-activated species is known to be base-catalyzed.[13] The known thioester **9**[11] could also be prepared by methylation of **6i**. The nature of the activated species was studied by partitioning the reaction mixture between water (not acid) and ethyl acetate (Scheme 3). The two major components isolated by preparative TLC were the diastereoisomeric 17-spiro-2′-(1-imidazolyl)-1′,3′-dioxolan-4′-ones **7**. The structures of each were indicated by their characteristic IR absorption (1810 cm^{-1}) for the spirocyclic carbonyl and by their ^{13}C NMR spectra, where the spirocyclic ring carbon atoms were readily assigned. In particular the 2′-carbon resonances (112.1 and 112.0 ppm) in the two isomers showed no C–H coupling in the off-resonance spectra. These and the

Table 1. Physical Properties of Intermediates[a]



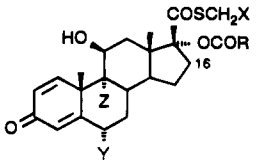
no.	6α	9α	16	17α	17β	mp, °C	$[\alpha]_D$ deg (dioxane)	formula	anal.[b]	UV, λ_{max} (EtOH) nm ($\epsilon \times 10^{-3}$)
2a	F	H	H	OH	CO_2H	241–248	+54	$C_{20}H_{25}FO_5$	C,H	242 (15.3)
2b	H	F	H	OH	CO_2H	289–293	+66[q]	$C_{20}H_{25}FO_5$	C,H	239.5 (14.5)
2c	F	F	=CH_2	OH	CO_2H	248–252[c]	−24	$C_{21}H_{24}F_2O_5$	C,H	238 (16.0)
3a	F	H	H	$OCOC_2H_5$	CO_2H	224–227	+3	$C_{23}H_{29}FO_6$	C,H	242 (14.3)
4ba	H	F	α-CH_3	$OCOC_2H_5$	$COSCONMe_2$	191–193	+82	$C_{27}H_{36}FNO_6S$	C,H,N,S	239 (18.7)
4bb	H	F	β-CH_3	$OCOCH_3$	$COSCONMe_2$	foam	+172	$C_{26}H_{34}FNO_6S$	C,H,N,S[e]	238 (19.6)
4bc	H	F	β-CH_3	$OCOC_2H_5$	$COSCONMe_2$	167–170	+185	$C_{27}H_{36}FNO_6S$	C,H,N	237.5 (19.8)
4bd	H	F	=CH_2	$OCOC_2H_5$	$COSCONMe_2$	189–192	+6	$C_{27}H_{34}FNO_6S$	C,H,N,S[f]	236.5 (19.2)
4ca	H	F	β-CH_3	$OCOC_2H5$	CO_2CONMe_2	200–203	+74	$C_{27}H_{36}FNO_7$	C,H,N	238 (15.9)
5a	H	F	H	OH	COSH	222–225	+116	$C_{20}H_{25}FO_4S$	C,H	244 (19.3)
5b	H	F	α-CH_3	OH	COSH	209–214	+104	$C_{21}H_{27}FO_4S$	C,H,S[g]	245 (17.6)
5c	F	F	α-CH_3	OH	COSH	230–232	+94	$C_{21}H_{26}F_2O_4S$	C,H,S	243 (21.0)
5d	H	F	β-CH_3	OH	COSH	198–201[c]	+189	$C_{21}H_{27}FO_4S$	C,H,S[h]	243 (19.5)
5e	F	F	=CH_2	OH	COSH	251–256[c]	+58	$C_{21}H_{24}F_2O_4S$	C,H,S	242 (20.5)
6a	F	H	H	$OCOC_2H_5$	COSH	189–193	+72	$C_{23}H_{29}FO_5S$	C,H,S	247 (18.1)
6b	H	F	H	$OCOC_2H_5$	COSH	136–138	+75	$C_{23}H_{29}FO_5S$	C,H,S	245 (18.4)
6c	H	F	α-CH_3	$OCOC_2H_5$	COSH	141–143	+30	$C_{24}H_{31}FO_5S$	C,H	243 (16.3)
6d	F	F	α-CH_3	$OCOCH_3$	COSH	175–177	−10	$C_{23}H_{28}F_2O_5S$	C,H,S	242.5 (18.0)
6e	F	F	α-CH_3	$OCOC_2H_5$	COSH	161–164	−27	$C_{24}H_{30}F_2O_5S$	C,H,S	243 (18.9)
6f	F	F	α-CH_3	$OCOC_3H_7$	COSH	155–157	+21	$C_{25}H_{32}F_2O_5S$	C,H,S	242.5 (18.4)
6g	H	H	β-CH_3	$OCOC_2H_5$	COSH	159–163	+113	$C_{24}H_{32}O_5S$	C,H,S	247 (17.8)
6h	H	F	β-CH_3	$OCOCH_3$	COSH	178.5–179	+98	$C_{24}H_{29}FO_5S$	C,H,S[h]	242 (17.8)
6i	H	F	β-CH_3	$OCOC_2H_5$	COSH	177–179	+110	$C_{24}H_{31}FO_5S$	C,H,S	242 (18.3)
6j	H	F	β-CH_3	$OCOC_3H_7$	COSH	175–176	+107	$C_{25}H_{33}FO_5S$	C,H,S[k]	244 (18.1)
6k	H	F	=CH_2	$OCOC_2H_5$	COSH	236–239	−71	$C_{24}H_{29}FO_5S$	C,H,S[f]	242 (17.7)
7a	H	F	β-CH_3	spiro($C_9H_8N_2O_3$)[i]		193–195	+96	$C_{27}H_{33}FN_2O_5$	C,H,N[e]	240 (14.3)
7b	H	F	β-CH_3	spiro($C_9H_8N_2O_3$)[j]		168–176	−8	$C_{27}H_{33}FN_2O_5$	C,H,N[l]	240 (14.5)
8	H	F	β-CH_3	$OCOC_2H_5$	$CO(C_3H_3N_2)$	190–203	+52	$C_{27}H_{33}FN_2O_5$	C,H,N[m]	240 (17.3)
10i	F	H	H	$OCOC_2H_5$	$COSCH_2Cl$	188–191	+48	$C_{24}H_{30}ClFO_5S$	C,H,Cl,S[n]	240 (18.7)
10j	H	F	α-CH_3	$OCOC_2H_5$	$COSCH_2Cl$	247–250	+50.5	$C_{25}H_{32}ClFO_5S$	C,H,Cl,S	238 (19.0)
10k	F	F	α-CH_3	$OCOCH_3$	$COSCH_2Cl$	280–283	+45	$C_{24}H_{29}ClF_2O_5S$	C,H,Cl,S	238.5 (19.8)
10l	F	F	α-CH_3	$OCOC_3H_7$	$COSCH_2Cl$	235–238	+49	$C_{26}H_{33}ClF_2O_5S$	C,H,Cl,S	238.5 (19.9)
11b	F	H	H	$OCOC_2H_5$	$COSCH_2I$	195–197	+18	$C_{24}H_{30}FIO_5S$	C,H,I,S	243.5 (20.7)
11c	H	F	H	$OCOC_2H_5$	$COSCH_2I$	175–178	+4	$C_{24}H_{30}FIO_5S$	C,H,I[o]	242 (17.9)
11d	F	F	α-CH_3	$OCOCH_3$	$COSCH_2I$	241–243	+78	$C_{24}H_{29}F_2IO_5S$	C,H,I,S	240 (20.2)
11e	F	F	α-CH_3	$OCOC_2H_5$	$COSCH_2I$	233–236[c]	+87	$C_{25}H_{31}F_2IO_5S$	C,H,I,S	241 (20.6)
11f	F	F	α-CH_3	$OCOC_3H_7$	$COSCH_2I$	210–212	+89	$C_{26}H_{33}F_2IO_5S$	C,H,I,S[m]	241 (20.7)
11g	H	F	β-CH_3	$OCOCH_3$	$COSCH_2I$	204–205	−29	$C_{24}H_{30}FIO_5S$	C,H,I,S	241 (20.6)
11h	H	F	=CH_2	$OCOC_2H_5$	$COSCH_2I$	191–199	−31	$C_{25}H_{30}FIO_5S$	C,H,I,S	241 (19.8)
16	H	F	β-CH_3	epoxide	$COSCH_2Cl$	246–251	+131	$C_{22}H_{26}ClFO_4S$	C,H,Cl	239 (20.5)
17	H	F	=CH_2	OH	$COSCH_2Cl$	242–243	+36	$C_{22}H_{26}ClFO_4S$	C,H	239.5 (19.4)
18a	H	F	β-CH_3	epoxide	$COSeCH_3$	297–299	+156	$C_{22}H_{27}FO_4Se$	C,H,Se	239 (16.3)
18b	H	F	β-CH_3	epoxide	$COSeCH_2Cl$	229–232[c]	+111	$C_{22}H_{26}ClFO_4Se$	C,H,Cl[h]	237.5 (16.5)
19a	H	F	=CH_2	OH	$COSeCH_3$	248–254[c]	+82.5	$C_{22}H_{27}FO_4Se$	C,H,Se	240.5 (15.9)
19b	H	F	=CH_2	OH	$COSeCH_2Cl$	237–239	+45.5	$C_{22}H_{26}ClFO_4Se$	C,H,Cl,Se	240.5 (16.3)
20a[d]	H	F	=CH_2	OH	$COSeCH_3$	225–235[c]	+124	$C_{22}H_{25}FO_4S$	C,H	238 (15.7)
20b[d]	H	F	=CH_2	OH	$COSeCH_2Cl$	209–210	+142	$C_{22}H_{24}ClFO_4Se$	C,H	237 (16.1)
21a[d]	H	F	=CH_2	$OCOC_2H_5$	$COSeCH_3$	209–211	−9	$C_{25}H_{29}FO_5Se$	C,H,Se	237 (16.0)
21b[d]	H	F	=CH_2	$OCOC_2H_5$	$COSeCH_2Cl$	156–158	−8	$C_{25}H_{28}ClFO_5Se$	C,H	237 (16.1)
22c	H	F	=CH_2	$OCOC_2H_5$	$COSeCH_2I$	174–175	−70	$C_{25}H_{30}FIO_5Se$	C,H,Se	238 (16.7)
22d	H	F	=CH_2	$OCOC_2H_5$	$COSeCH_2F$	216–218	−43.5	$C_{25}H_{30}F_2O_5Se$	C,H	239.5 (16.8)
24	H	F	β-CH_3	$OCOC_2H_5$	$COS]_2$[p]	234–235	+107	$C_{48}H_{60}F_2O_{10}S_2$	C,H,S[m]	238 (37.1)

[a] ^1H NMR and infrared spectra were obtained for all compounds, and data are in the Experimental Section for selected compounds. [b] Persistent solvation was confirmed spectroscopically. [c] Decomposition. [d] 11-Ketones. [e] Solvate (0.5EtOAc). [f] Solvate (0.25Me_2CO). [g] Solvate (1.0H_2O). [h] Solvate (0.25H_2O). [i] Isomer A. [j] Isomer B. [k] Solvate (0.16EtOAc). [l] Solvate (0.2EtOAc). [m] Solvate (0.5H_2O). [n] Solvate (0.33H_2O). [o] Solvate (0.6MeOH). [p] Disulfide.

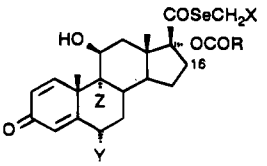
C-17 resonances (92.1 and 91.1 ppm) were structurally characteristic, and the ^1H NMR confirmed that the ethyl groups in each isomer were no longer part of an ester function; the configurations of the two isomers were not established. From a further experiment, in which the reaction mixture was treated with dimethylamine, the same dioxolanones **7** were isolated together with a minor third component of intermediate polarity, identified as the imidazolide **8**, ν_{max} 1742 cm^{-1}. The dioxolanones **7** each reacted with NaSMe to give **9**, but not with NaSH. There was insufficient **8** to test its reactivity.

Chloromethyl carbothioates **10** were prepared from carbothioate salts by alkylation with bromochloromethane, or chloroiodomethane, in dimethylacetamide. The chloromethyl thioesters **10** reacted with sodium iodide to give iodomethyl thioesters **11**, which in turn reacted with sodium bromide or silver fluoride

Table 2. Biologically-Assayed Halomethyl Androstane-17β-carbothioates and -17β-carboselenoates (Physical Properties[a])



10,11,12,13

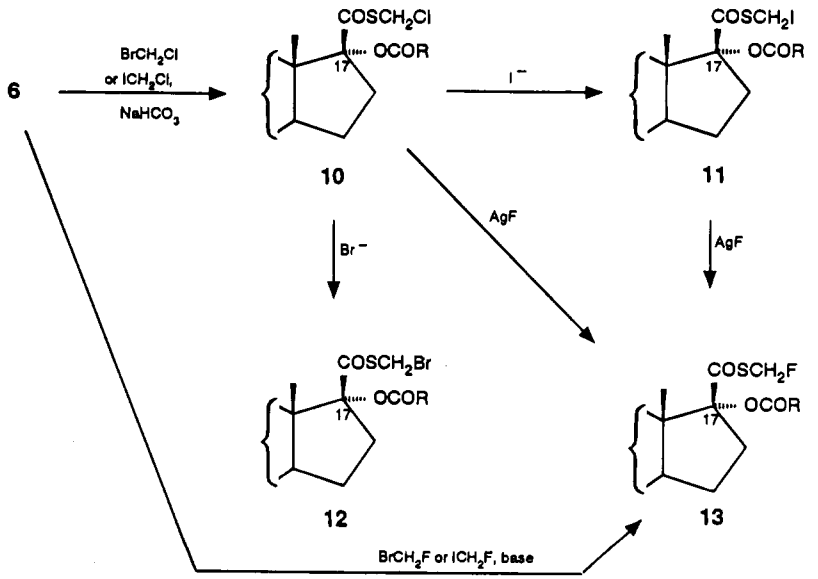


22

no.	Z	Y	X	R	16	mp, °C	$[\alpha]_D$, detg (dioxane)	formula	anal.	UV, λ_{max} (EtOH) nm ($\epsilon \times 10^{-3}$)
13a	H	F	F	C_2H_5	H	224–225	+70	$C_{24}H_{30}F_2O_5S$	C,H,F,S	238.5 (17.4)
10a	F	H	Cl	C_2H_5	H	196–199	+38	$C_{24}H_{30}ClFO_5S$	C,H,S	238 (16.9)
13b	F	H	F	C_2H_5	H	207–211	+70	$C_{24}H_{30}F_2O_5S$	C,H,F,S	237 (17.1)
13c	F	H	F	C_2H_5	α-CH_3	242–243	+37	$C_{25}H_{32}F_2O_5S$	C,H,S	237 (18.0)
10b	F	F	Cl	C_2H_5	α-CH_3	272–275	+49	$C_{25}H_{31}ClF_2O_5S$	C,H	238 (20.0)
13d	F	F	F	CH_3	α-CH_3	308–310	+29	$C_{24}H_{29}F_3O_5S$	C,H,S	236 (19.0)
13e	F	F	F	C_2H_5	α-CH_3	274–275	+32	$C_{25}H_{31}F_3O_5S$	C,H,F,S	236.5 (18.6)
13f	F	F	F	C_3H_7	α-CH_3	249–252	+32	$C_{26}H_{33}F_3O_5S$	C,H,F,S	237 (19.1)
10c	H	H	Cl	C_2H_5	β-CH_3	192–193	+65	$C_{25}H_{33}ClO_5S$	C,H,Cl,S	241 (17.8)
13g	H	H	F	C_2H_5	β-CH_3	223–225	+103	$C_{25}H_{33}FO_5S$	C,H,S	240 (16.3)
10d	F	H	Cl	CH_3	β-CH_3	220–223	+39.5	$C_{24}H_{30}ClFO_5S$	C,H,Cl,S	238 (18.4)
13h[b]	F	H	F	CH_3	β-CH_3	248–249	+101	$C_{24}H_{30}F_2O_5S$	C,H,F,S	237 (17.7)
10e	F	H	Cl	C_2H_5	β-CH_3	212–214	+44	$C_{25}H_{32}ClFO_5S$	C,H,Cl,S	238.5 (18.8)
12[c]	F	H	Br	C_2H_5	β-CH_3	186.5–187	+2	$C_{25}H_{32}BrFO_5S$	C,H,Br,S	241 (19.9)
11a[c]	F	H	I	C_2H_5	β-CH_3	196–197	−32	$C_{25}H_{32}FIO_5S$	C,H,I,S	242 (20.2)
13i	F	H	F	C_2H_5	β-CH_3	237–241	+98	$C_{25}H_{32}F_2O_5S$	C,H,F,S	237 (17.4)
10f	F	H	Cl	C_3H_7	β-CH_3	172–175	+46	$C_{26}H_{34}ClFO_5S$	C,H,Cl,S	239 (18.7)
10g	F	H	Cl	C_2H_5	=CH_2	212–221	−56	$C_{25}H_{30}ClFO_5S$	C,H,Cl,S	239 (19.5)
13j	F	H	F	C_2H_5	=CH_2	205–215	−58	$C_{25}H_{30}F_2O_5S$	C,H,F,S	237 (18.1)
10h	F	F	Cl	C_2H_5	=CH_2	242–245	−56	$C_{25}H_{29}ClF_2O_5S$	C,H,Cl,S	238.5 (19.9)
13k	F	F	F	C_2H_5	=CH_2	251–255[d]	−56	$C_{25}H_{29}F_3O_5S$	C,H,S	236.5 (19.1)
22a	F	H	H	C_2H_5	=CH_2	225–227	−37	$C_{25}H_{31}FO_5Se$	C,H,Se	239.5 (16.3)
22b	F	H	Cl	C_2H_5	=CH_2	212–214	−48	$C_{25}H_{30}ClFO_5Se$	C,H,Cl,Se	240.5 (16.6)

[a] ^{1}H NMR and infrared spectra were obtained for all compounds, and data are in the Experimental Section for selected compounds. [b] Solvated with 0.5 mol of H_2O. [c] Solvated with 0.33 mol of H_2O. [d] Decomposition.

Scheme 4

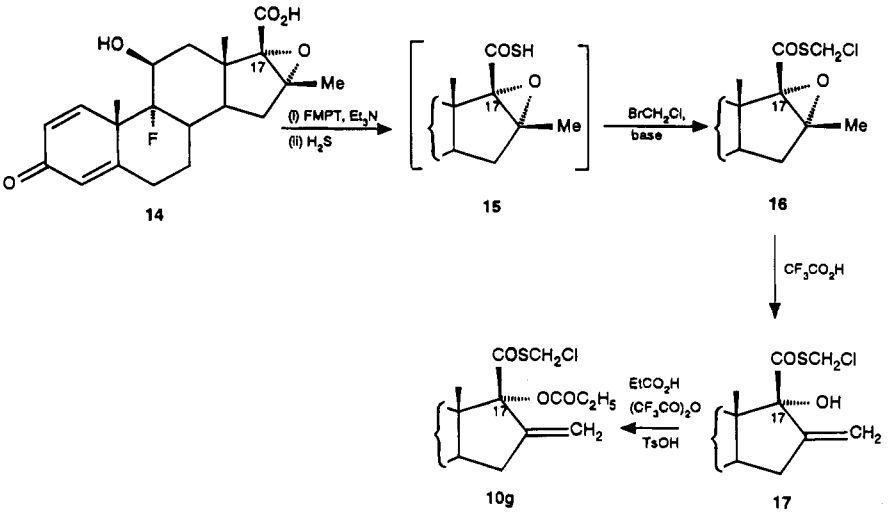


to give the bromomethyl and fluoromethyl thioesters **12** and **13**, respectively. The fluoromethyl thioester **13e** was also prepared directly from the potassium salt of the carbothioic acid **6e**, using fluoroiodomethane[14] or bromofluoromethane[15] (Scheme 4).

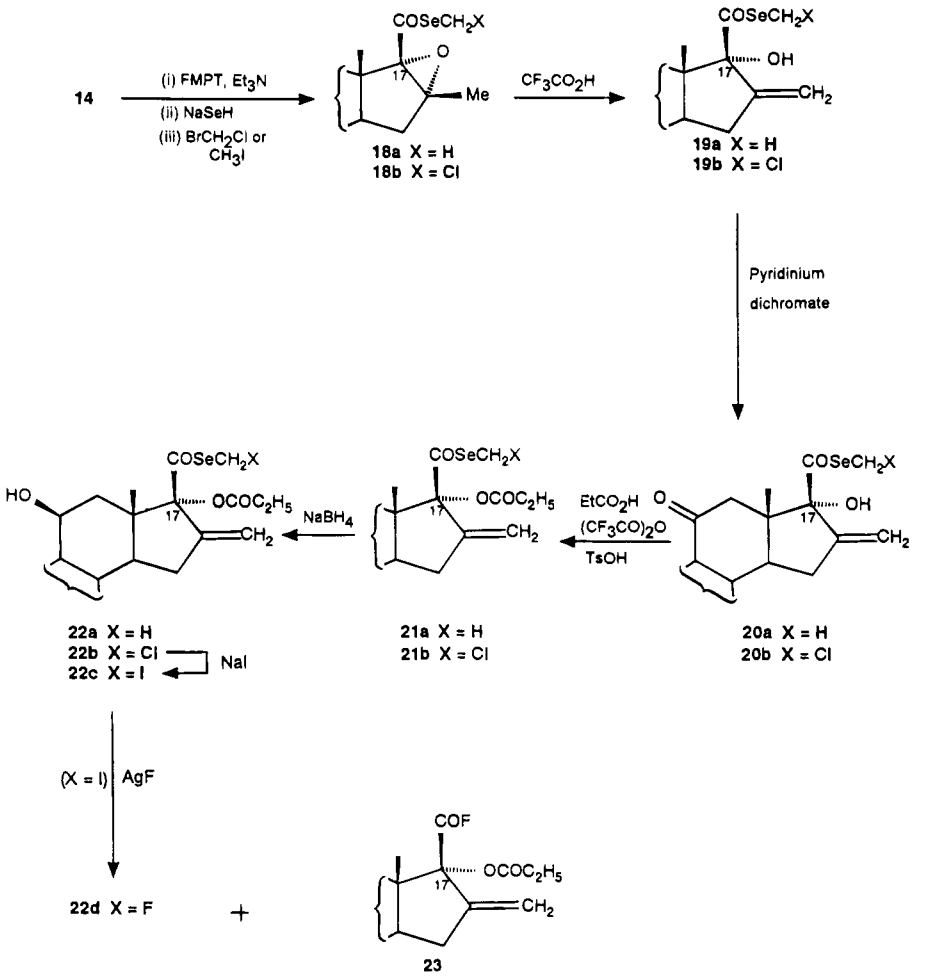
Kertesz and Marx[11] reported that 2-fluoro-*N*-methylpyridinium tosylate (FMPT) could be used for the activation of 17β-carboxylic acids in the presence of a 16α,17α-acetonide but that neighboring group participation dominated the chemistry of 17α-hydroxy or 17α-acyloxy compounds. FMPT also activated the 16α,17α-epoxy-16β-methyl 17β-carboxylic acids **14** to reaction with H_2S. The resulting carbothioic acid **15** was unstable but treatment with bromochloromethane *in situ* gave the chloromethyl carbothioate **16**. This could be rearranged with trifluoroacetic acid to the 16-methylene 17α-ol **17** which on propionylation in the presence of acid gave a mixture of the 17α-ester **10g** and the isomeric 11β-monoester (Scheme 5).

Activation of **14** with FMPT and reaction with NaSeH[16] gave the corresponding carboselenoic acid, which could be alkylated *in situ* to the methyl or

Scheme 5



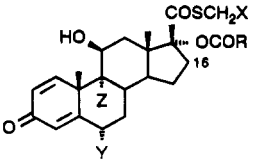
Scheme 6



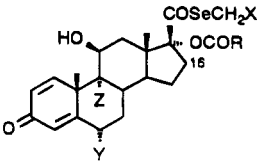
chloromethyl carboselenoates **18a,b**. Epoxide rearrangement (CF_3CO_2H) then gave the corresponding 11β-17α-diols **19a,b**. In this series, oxidation of **19a,b** to the 11-ketones **20a,b** prior to 17α-propionylation followed by selective reduction of 11-oxo esters **21a,b** to the 11β-alcohols **22a,b** avoided unwanted 11β-esterification (Scheme 6).

Reaction of the iodomethyl selenoester **22c** with silver fluoride in acetonitrile gave the corresponding fluoromethyl selenoester **22d** as a minor product (3%), but the major product (29%) was the 17β-acyl fluoride **23**, presumably formed by silver ion-assisted displacement of the selenide group by fluoride ion. While **23** was not analytically pure, the structure of the major component (83% by HPLC) was clearly revealed by its highly characteristic ^1H, ^{13}C, and ^{19}F NMR, infrared, and mass spectra. The same main fragment ions were observed in the chemical ionisation mass spectra of both **22d** and **23**, the initial losses being those of $HSeCH_2F$ and HF, respectively, from the major MH^+ molecular ions. The MH^+ peaks were confirmed by HRMS accurate mass measurements.

Table 3. Biological Activities of Halomethyl Androstane-17β-carbothioates and -17β-carboselenoates



10,11,12,13



22

							mouse		rat	
no.	Z	Y	X	R	16	human V^a	AIT[b]	HPA[c]	AIT[b]	HPA[c]
13a	H	F	F	C_2H_5	H	697 (230–2438)	56 (36–86)	>200 (G)	97 (62–156)	–
10a	F	H	Cl	C_2H_5	H	916 (471–1874)	20 (14–29)	100 (G)	–	–
13b	F	H	F	C_2H_5	H	1984 (1023–4013)	63 (39–105)	149 (103–219)	39 (27–56)	1.5 (G)
13c	F	H	F	C_2H_5	α-CH_3	653 (311–1395)	–	–	–	–
10b	F	F	Cl	C_2H_5	α-CH_3	124 (63–231)	56 (37–86)	0.04 (0.01–0.09)	29 (25–32)	<0.02 (G)
13d	F	F	F	CH_3	α-CH_3	392 (159–959)	76[d]	2.9 (1.4–5.2)	88[e]	3 (G)
13e	F	F	F	C_2H_5	α-CH_3	945 (551–1655)	113[f]	1.0 (0.5–2.1)	85[g]	1.5 (0.8–2.2)
13f	F	F	F	C_3H_7	α-CH_3	299 (98–953)	55 (34–87)	0.7 (G)	55 (35–86)	–
10c	H	H	Cl	C_2H_5	β-CH_3	295 (72–1148)	27 (17–44)	–	29 (19–44)	1.4 (0.5–3.2)
13g	H	H	F	C_2H_5	β-CH_3	800 (231–2773)	50 (35–71)	–	44 (30–63)	5.1 (1.8–11.8)
10d	F	H	Cl	CH_3	β-CH_3	544 (154–2047)	59 (33–103)	14.7 (8.8–24.6)	24 (G)	0.5 (G)
13h	F	H	F	CH_3	β-CH_3	1388 (374–5370)	67 (47–95)	–	40 (25–64)	13.5 (G)
10e	F	H	Cl	C_2H_5	β-CH_3	1469 (858–2541)	41 (27–64)	44 (G)	36 (24–53)	0.9 (0.5–1.7)
12	F	H	Br	C_2H_5	β-CH_3	254 (G)	–	–	–	–
11a	F	H	I	C_2H_5	β-CH_3	41 (G)	–	–	–	–
13i	F	H	F	C_2H_5	β-CH_3	1262 (597–2669)	89 (45–169)	450[h]	39 (30–52)	13 (8–24)
10f	F	H	Cl	C_3H_7	β-CH_3	143 (41–435)	–	–	–	–
10g	F	H	Cl	C_2H_5	=CH_2	365 (198–665)	42 (28–64)	2 (1–3)	12 (9–17)	0.8 (G)
13j	F	H	F	C_2H_5	=CH_2	1497 (575–4085)	41 (28–64)	44 (27–76)	20 (14–26)	2.5 (G)
10h	F	F	Cl	C_2H_5	=CH_2	170 (G)	108 (70–171)	>100 (G)	–	–
13k	F	F	F	C_2H_5	=CH_2	1048 (G)	197 (G)	>100 (G)	–	–
22a	F	H	H	C_2H_5	=CH_2	187 (85–415)	27 (16–44)	–	19 (12–29)	<0.2
22b	F	H	Cl	C_2H_5	=CH_2	200 (103–388)	40 (27–61)	2 (G)	16 (9–26)	–
(fluocinolone acetonide)[i]						100	100	100	100	100

[a] Human vasoconstrictor activity relative to fluocinolone acetonide (100). Numbers in parentheses are 95% confidence level intervals or G represents a graphical estimate. [b] Topical antiinflammatory activity relative to fluocinolone acetonide (100). Parentheses as for *a*. [c] Systemic corticosteroid activity after topical application relative to fluocinolone acetonide (100). Parentheses as for *a*. [d] Mean of two results: 35 (21–58) and 117 (81–167). [e] Mean of three results: 103 (60–176), 84 (47–158), and 76 (37–156). [f] Mean of two results: 134 (85–212) and 86 (46–150). [g] Mean of two results: 72 (57–90) and 97 (70–136). [h] Mean of two results: 530 (G) and 370 (G). [i] Standard.

The ^{1}H NMR spectra of carbothioic acids were best measured in deuteriochloroform if solubility was sufficient, as in Me_2SO-d_6 impurity signals gradually appeared due to oxidation to the neutral disulfide (e.g., with **6i**), a process complete in a longer time or at elevated temperature.

In each series of halomethyl 17α-acyloxy 17β-carbothioates the ultraviolet extinction coefficients increased from *ca.* 16 000 to *ca.* 20 000, and the positions of the maxima moved slightly to higher wavelength (*ca.* 239 to 244 nm) on progressing from fluoromethyl through to iodomethyl thioesters. Gradations of optical rotation at 589 nm were also noted, increasing when a 16α-methyl substituent was present but decreasing when a 16β-methyl or no substituent was present. Presumably the contribution due to the carbothioate chromophore is considerably influenced by its conformation, and this in turn depends on the nature of ring D and its substituents.

Biological Results and Discussion

Topical activity in humans was measured by the vasoconstriction assay according to the method of McKenzie and Atkinson.[17] Topical antiinflammatory activity was measured in rats and mice by modifications of the croton oil ear assay of Tonelli et al.[18] The undesired hypothalamic–pituitary–adrenal (HPA) function suppression was assessed in rats and mice by measuring reductions of levels of circulating corticosterone in response to ether stress, using the procedure of Zenker and Bernstein.[19] The results are shown in Table 3.

In the vasoconstriction assay, fluoromethyl carbothioates **13** were in general more active than their chloromethyl analogues **10** (**13h**, **10d**; **13g**, **10c**; **13e**, **10b**; **13b**, **10a**; **13j**, **10g**; **13k**, **10h**; six pairs); however, for another pair (**13i**, **10e**) the chloro analogue had the slightly greater potency. The bromomethyl and iodomethyl carbothioates **12** and **11a** were less active than the chloromethyl analogue **10e**. 17-Propionates **10e**, **13e** were better than the acetate (**10d**, **13d**) and butyrate (**10f**, **13f**) analogues, although the potency of the propionate (**13i**) was marginally less than that of the acetate (**13h**). A similar pattern was found in the mouse and rat antiinflammatory activities. A different pattern was found for HPA suppression where the 16α-methyl compounds **10b**, **13d**, and **13e** showed little effect in both mouse and rat. The most active of these in the vasoconstriction and antiinflammatory tests was **13e** (fluticasone propionate, FP), so this was chosen for more detailed examination. The carboselenoates **22a,b** showed moderate activity but were not examined further as it was considered unlikely that drugs containing selenium would be acceptable.

The fluoromethyl and chloromethyl carbothioates **13i** and **10e** were, respectively, more than 3.5 and 1.5 times as active than their oxygen analogues[1] in the vasoconstriction test, while the methyl carbothioate analogue of FP (**13e**) has been reported[11] to show topical activity

in the vasoconstriction test only in the order of that of the standard, fluocinolone acetonide.

FP did cause HPA suppression if given as a suspension in saline by the subcutaneous route in rats [1.2 (0.8–1.7)] and mice [0.7 (G)], compared with betamethasone (1.0); in rats the suppression was measured by weighing the adrenals. Given orally, however, it showed only weak adrenolytic activity in rats [0.13 (0.08–0.21)] and depression of corticosterone levels in mice [0.01 (0.004–0.03)] compared with betamethasone (1.0). Weak glucocorticoid activity after oral administration is particularly of value in the treatment of airway conditions where a high proportion of the dose is swallowed.[20]

FP has been reported[5] to be rapidly converted by liver homogenates (from mouse, rat, or dog) into the known carboxylic acid **3f**.[11] This acid has since been confirmed to be the principal metabolite in a study[20] of the human pharmacology of FP, arising from orally administered drug by first-pass conversion in the liver, **3f** showed negligible HPA suppression as measured by its adrenolytic activity in rats [<0.01 (G)] and by depression of corticosterone levels in mice [<0.001 (G)] compared with betamethasone (1.0) by the subcutaneous route. Formulations of FP have now received approval for the treatment of rhinitis (Flixonase[21]), asthma (in UK, Flixotide[21]), and steroid-responsive dermatoses (in USA, Cutivate[21]).

Experimental Section

Melting points were determined on a Kofler block and are uncorrected. Optical rotations were determined in dioxane at 20–22 °C; ^{1}H NMR spectra were determined in Me_2SO-d_6 (unless stated otherwise) at 60, 90, or 100 MHz on Perkin-Elmer R24B, R32, or JEOL MH100 spectrometers, respectively. The R32 instrument was also used at 84.68 MHz for ^{19}F NMR. A JEOL FX100 Fourier-transform spectrometer was used at 25.05 MHz for ^{13}C NMR or at 100 MHz for ^{1}H NMR. Chemical shifts are relative to $Me_3Si(CH_2)_3SO_3Na$ in Me_2SO-d_6 and $SiMe_4$ in $CHCl_3$-d for ^{1}H and ^{13}C NMR and to $CFCl_3$ for ^{19}F NMR, as internal standards. IR spectra were recorded for Nujol mulls, or in $CHBr_3$ where so indicated. Mass spectra were determined on a Finnigan MAT 4600 spectrometer using positive-ion chemical ionization with ammonia as the reagent gas. Accurate mass measurements were made on a Kratos Concept spectrometer by Mrs. V. Boote, Department of Chemistry, University of Manchester. Organic reaction extracts were routinely dried over magnesium sulfate prior to removal of the solvent by rotary evaporation at *ca.* 20 mmHg at or below 50 °C. Products were dried in vacuo at up to 50 °C. Analytical TLC was conducted on Merck Kieselgel 60 F_{254} plates, developed with chloroform:acetone (e.g., 4:1) for neutral compounds or chloroform:acetone:acetic acid (e.g., 30:8:1) for acidic compounds. Preparative-layer chromatography (PLC) was performed in the same solvent systems on Merck Kieselgel 60 $PF_{254+366}$. Products were usually detected at 254 nm and eluted with ethyl acetate. Physical properties of intermediates are given in Table 1; those of biologically-assayed compounds are in Table 2. ^{1}H NMR (in Me_2SO-d_6 unless stated otherwise) and infrared spectra (in Nujol unless stated otherwise) were taken for all compounds, and where solvation is tabulated this was confirmed from the spectra.

6α-Fluoro-11β,17α-dihydroxy-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (2a). A solution of 6α-fluoro-11β,17α,-21-trihydroxypregna-1,4-diene-3,20-dione (**1a**)[22] (4.99 g, 13.2 mmol) in tetrahydrofuran (50 mL) was stirred with a solution of periodic acid (10.0 g, 45 mmol) in water (24 mL) at 22 ‰C for 50 min. The tetrahydrofuran was removed *in vacuo* to leave an aqueous suspension which was filtered, and the solid was washed with water and dried to give **2a** (4.80 g, 100%). A portion (0.271 g) was crystallized from methanol to give the analytical sample of **2a** (0.171 g, 63%).

9α-Fluoro-11β,17α-dihydroxy-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (2b). A stirred suspension of 9α-fluoro-11β-17α,21-trihydroxypregna-1,4-diene-3,20-dione (**1b**)[23] (10.0 g, 26.4 mmol) in tetrahydrofuran (55 mL) was stirred at 22 °C for 2 h with a solution of periodic acid (9.0 g, 39.5 mmol) in water (90 mL) and then poured into water (150 mL) and crushed ice (250 mL). Filtration gave **2b** (9.42 g, 98%), a portion (0.100 g) of which was recrystallized from ethanol to give the analytical sample of **2b** (0.060 g; 59%).

6α,9α-Difluoro-11β,17α-dihydroxy-16-methylene-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (2c). 6α,9α-Difluoro-11β,17α,21-trihydroxy-16-methylenepregna-1,4-diene-3,20-dione (**1c**)[24] (3.6 g) was oxidized as in the preceding experiment to give **2c** (3.40 g, 98%). A portion (0.250 g) crystallized from aqueous methanol gave the analytical sample of **2c** (0.162 g, 64%).

6α-Fluoro-11β-hydroxy-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboxylic Acid (3a). A solution of **2a** (4.49 g, 12.3 mmol) and Et_3N (4.46 mL, 31.6 mmol) in CH_2Cl_2 (160 mL) at −5 °C was treated dropwise with stirring with propionyl chloride (2.80 mL, 32 mmol) in CH_2Cl_2 (5 mL) during 5 min. After 20 min the reaction mixture was diluted with CH_2Cl_2, washed with aqueous $NaHCO_3$ and then H_2O, dried, and evaporated to give the intermediate mixed anhydride as a solid (5.70 g). This was stirred in acetone (30 mL) with Et_2NH (4.6 mL, 44.3 mmol) for 30 min to give a clear yellow solution which was concentrated, diluted with water (150 mL), and washed with EtOAc. The aqueous phase was acidified to pH 2 with 2 N HCl (50 mL) and extracted with EtOAc. The extract was washed with water and evaporated to give a foam (5.82 g), a portion of which (0.304 g) was crystallized from EtOAc to give the analytical sample of **3a** (0.144 g, 47%) as plates.

Formation of 17β-[(*N,N*-dimethylcarbamoyl)thio]carbonyl Compounds 4b. General Method A. The steroid 17β-carboxylic acid **3** (1 equiv) stirred in CH_2Cl_2 (*ca.* 15 mL/g of **3**) was treated successively with Et_3N (1 equiv) and *N,N*-dimethylthiocarbamoyl chloride (2 equiv) under N_2 at 20 °C, the reaction being monitored by TLC until no further consumption of starting material was observed (usually 6–30 h). The reaction mixture was diluted with EtOAc (*ca.* 50 mL/g of **3**), washed with 1N HCl, 5% $NaHCO_3$, and water, dried, and evaporated to give the crude anhydride **4b**, purified by crystallization or by PLC then crystallization.

17β-[[(*N,N*-Dimethylcarbamoyl)thio]carbonyl]-9α-fluoro-11β-hydroxy-16α-methyl-17α-(propionyloxy)androsta-1,4-dien-3-one (4ba). 9α-Fluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboxylic acid (**3b**)[25] was treated by general method A, but with the addition of NaI (1 equiv) to the reaction mixture, to give crystalline **4ba** (63%) recrystallized twice from acetone to give the analytical sample: IR ($CHBr_3$) 3610 (OH), 1740, 1710 (w) (COSCON, propionate), 1670, 1632, 1618 ($\Delta^{1,4}$-3-one) cm^{-1}.

17α-Acetoxy-17β-[[(*N,N*-dimethylcarbamoyl)thio]carbonyl]-9α-fluoro-11β-hydroxy-16β-methylandrosta-1,4-dien-3-one (4bb). 17α-Acetoxy-9α-fluoro-11β-hydroxy-16β-methylandrosta-1,4-diene-17β-carboxylic acid (**3c**)[11] treated by general method A gave crude anhydride (87%, *ca.* 90% pure by TLC), purified by PLC to give the analytical sample of **4bb** as a foam: IR ($CHBr_3$) 3590 (OH), 1735 (COSCON, propionate), 1665, 1630, 1612 ($\Delta^{1,4}$-3-one) cm^{-1}.

17β-[[(*N,N*-Dimethylcarbamoyl)thio]carbonyl]-9α-fluoro-11β-hydroxy-16β-methyl-17α-(propionyloxy)androsta-1,4-dien-3-one (4bc). 9α-Fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboxylic acid (**3d**),[11] solvated with 0.75 mol of EtOAc (4.99 g, 9.96 mmmol) in pyridine (20 mL), was treated with dimethylthiocarbamoyl chloride (2.69 g, 21.8 mmol) for 19 h at room temperature. TLC indicated that only half of the starting material had been consumed. Isolations in general method A gave the crude anhydride (2.10 g, 40.5%), part of which was purified by PLC and crystallization from ether to give the analytical sample of **4bc**: IR ($CHBr_3$) 3670 (OH), 1740 (COSCON, propionate), 1670, 1636, 1618 ($\Delta^{1,4}$-3-one) cm^{-1}; ^{1}H NMR (90 MHz) 0.99 (13-CH_3 s) 1.07 (propionate CH_3, t, J = 7 Hz), 1.29 (16β-CH_3, *d*, *J*

= 6 Hz), 2.39 (propionate CH_2, q, J = 7 Hz), 2.99, 3.04 (NMe_2, singlets), 7.32 (1-H, d, J = 10 Hz).

In a second method, 9α-fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioic acid, **6i**, solvated with 0.75 mol of EtOAc (517 mg, 1.0 mmol) and Et_3N (0.49 mL, 3.5 mmol) in CH_2Cl_2 (10 mL), was treated with *N,N*-dimethylcarbamoyl chloride (0.37 mL, 4 mmol) and stirred under nitrogen at room temperature for 24 h. The mixture was diluted with EtOAc, washed successively with 1 N HCl, aqueous $NaHCO_3$, and H_2O, dried, and evaporated to a foam (674 mg). PLC gave solvated **4bc** as a solid (249 mg, 44%): $[\alpha]_D$ +168° (*c* 1.05); MS *m/e* 522 (MH^+), 417 ($MH^+ - Me_2NCOSH$), with mass, IR, and ^1H NMR spectra closely similar to those of the above. Anal. ($C_{27}H_{36}FNO_6S$·0.5EtOAc) C, H, N, S.

17β-[[(*N,N*-Dimethylcarbamoyl)thio]carbonyl]-9α-fluoro-11β-hydroxy-16-methylene-17α-(propionyloxy)androsta-1,4-dien-3-one (4bd). 9α-Fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboxylic acid (**3e**)[25] treated by general method A gave the crude anhydride (86%), a portion of which was purified by PLC and crystallization from acetone to give the analytical sample of **4bd** (43%): IR ($CHBr_3$) 3620 (OH), 1740, 1710 (COSCON, propionate), 1665, 1630, 1612 ($\Delta^{1,4}$-3-one) cm^{-1}.

17β-[[(*N,N*-Dimethylcarbamoyl)oxy]carbonyl]-9α-fluoro-11β-hydroxy-16β-methyl-17α-(propionyloxy)androsta-1,4-dien-3-one (4ca). 9α-Fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboxylic acid (**3d**),[11] solvated with 0.75 mol of EtOAc (491 mg 0.98 mmol) in pyridine (2 mL), was treated with dimethylcarbamoyl chloride (208 mg, 1.94 mmol) at room temperature for 2 h. Isolation as in general method A gave the crude anhydride (489 mg), which was purified by two recrystallizations from acetone to give the analytical sample of **4ca** (73%): IR ($CHBr_3$) 3625 (OH), 1783 (COOCON), 1740, 740 (propionate), 1673, 1634, 1618 ($\Delta^{1,4}$-3-one) cm^{-1}.

Formation of 17α-Hydroxy 17β-Carbothioic Acids 5. General Method B. A solution of the 17α-hydroxy 17β-carboxylic acid **2** (1 equiv) in DMF (*ca.* 20 mL/g of **2**) was treated with 1,1′-carbonyldiimidazole (2 equiv), and the mixture was stirred under nitrogen at room temperature for *ca.* 4 h. Hydrogen sulfide was bubbled into the reaction for 15–30 min. After 0.5–4 h the reaction mixture was poured into 2 N HCl and ice, and the precipitated acid **5** was collected by filtration.

9α-Fluoro-11β,17α-dihydroxy-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (5a). The carboxylic acid **2b** treated by general method B gave **5a** (97%), mp 219–222 °C. A portion (0.12 g) crystallized from ethanol gave the analytical sample **5a** (0.07 g, 57%).

9α-Fluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (5b). 9α-Fluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid (**2d**)[8] by general method B gave the analytical sample of **5b** (97%).

6α,9α-Difluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (5c). 6α,9α-Difluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid (**2e**)[11] by general method B gave the analytical sample of **5c** (92%): ^1H NMR (100 MHz) 0.85, (16α-CH_3, d, J = 6 Hz), 0.97 (13-CH_3, s), 1.50 (10-CH_3, s), 4.20 (11α-H, m), 5.35 (11β-OH, m), 6.10 (4-H, m), 6.26 (2-H, dd, J = 10, 2 Hz), 7.26 (1-H, broad d, J = 10 Hz).

9α-Fluoro-11β,17α-dihydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (5d). 9α-Fluoro-11β,17α-dihydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid (**2f**)[11] by general method B, except that the first stage was carried out at −5 °C for 18 h and the product was isolated by extraction with EtOAc and crystallized twice from EtOAc to give **5d** (60%): ^1H NMR (90 MHz) 1.02 (13-CH_3, s), 1.10 (16β-CH_3, d, J = 6 Hz), 1.56 (10-CH_3, s), 4.22 (11α-H, m), 4.09 (4-H, m), 6.28 (2-H, dd, J = 10, 2 Hz), 7.37 (1-H, d, J = 10 Hz).

6α,9α-Difluoro-11β,17α-dihydroxy-16-methylene-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (5e). The carboxylic acid **2c** by general method B gave **5e** (89%). A portion (0.25 g) crystallized from ethyl acetate gave the analytical sample of **5e** (0.10 g, 36%).

Formation of 17α-Acyloxy 17β-Carbothioic Acids 6. General Method C. The crude 17β-[(*N,N*-dimethylcarbamoyl)thio]carbonyl compound **4b** prepared by general method A was refluxed under nitrogen in Et_2NH (5–20 mL/g) for 3.5–6 h. The cooled reaction mixture was poured into 6 N HCl (*ca.* 60 mL/g) and ice (*ca.* 60 mL/g). The product was extracted into EtOAc, and the extract was washed with water before back-extraction into 5% Na_2CO_3 (*ca.* 120 mL/g). The aqueous layer was acidified with HCl to pH 1 and the product extracted into EtOAc, washed with water, dried, and evaporated to give **6**.

General Method D. The crude 17α-hydroxy 17β-carbothioic acid (**5**), prepared by general method B, with Et_3N (*ca.* 3.5 equiv) in CH_2Cl_2 (*ca.* 25 mL/g) at *ca.* 0 °C was treated dropwise with an acyl chloride (*ca.* 4.5 equiv) and then stirred for *ca.* 45 min. The solution was washed with 2 N Na_2CO_3, water, 2 N HCl, water, and brine, dried, and evaporated to leave a residue of the intermediate anhydride. This was dissolved in acetone (*ca.* 25 mL/g) and treated with Et_2NH (*ca.* 10 equiv) for *ca.* 1 h and then poured into 2 N HCl (*ca.* 40 mL/g) and ice (*ca.* 40 mL/g) to precipitate the carbothioic acid **5**.

6α-Fluoro-11β-hydroxy-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioic Acid (6a). The 17β-carboxylic acid **3a** was converted by general methods A and then C and the product crystallized from a mixture of acetone and petrol (bp 60–80 °C) to give **5a** (46%).

9α-Fluoro-11β-hydroxy-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioic Acid (6b). The 17β-carboxylic acid **2b** was converted by general methods B then D (using propionyl chloride) to give crude **6b** (93%), mp 118–120 °C. A portion (0.35 g) crystallized from EtOAc gave the analytical sample of **6b** (0.16 g, 44%).

9α-Fluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioic Acid (6c). 9α-Fluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboxylic acid (**3b**)[25] was converted by general methods A then C, and the product was crystallized twice from acetone to give **6c** (29%). The 17α-hydroxy compound **5b** also gave **6c** (90%), mp 134–137 °C, by general method D, using propionyl chloride.

17α-Acetoxy-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (6d). The 17α-hydroxy compound **5c** was converted by general method D using acetyl chloride to give **6d** (94%). Crystallization of a portion (0.40 g) from EtOAc gave the analytical sample of **6d** (0.28 g, 64%).

6α,9α-Difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioic Acid (6e). The 17α-hydroxy compound **5c** was converted by general method D using propionyl chloride to give **6e** (91%). Crystallization of a portion (0.40 g) from EtOAc gave the analytical sample of **6e** (0.29 g, 54%): ^1H NMR (90 MHz, in $CHCl_3$-*d*) 0.99 (16α-CH_3, d, J = 7 Hz), 1.15 (propionate CH_3, t, J = 7 Hz), 1.16 (13-CH_3, s), 1.54 (10-CH_3, s), 2.40 (propionate CH_2, q, J = 7 Hz), 4.46 (11α-H, broad m), 5.46 (6-H, broad dm, *ca.* J = 50 Hz), 6.40 (2-H, broad d, J = 10 Hz), 6.46 (4-H, m), 7.18 (1-H, d, J = 10 Hz). 6α,9α-Difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboxylic acid (**3f**)[11] also gave **6e** (25%) using general methods A then C.

17α-(Butyryloxy)-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (6f). The 17α-hydroxy compound **5c** was converted by general method D using butyryl chloride to give **6f** (89%). Crystallization of a portion (0.40 g) from EtOAc gave the analytical sample of **6f** (0.27 g, 60%).

11β-Hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioic Acid (6g). 11β-Hydroxy-16β-methyl-17β-(propionyloxy)-3-oxoandrosta-1,4-diene-17β-carboxylic acid (**3g**)[8] was converted by general methods A then C and crystallized from ethyl acetate to give **6g** (24%).

17α-Acetoxy-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (6h). Crude **4bb** was converted by general method C to give **6h** (51%). A

portion (0.150 g) crystallized twice from EtOAc gave the analytical sample of **6h** (0.127 g, 43%).

9α-Fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioic Acid (6i). Crude **4bc** was converted by general method C and crystallization to give **6i** (42%), mp 172–173 °C; a portion was recrystallized twice from acetone to give the analytical sample of **6i**: ^{1}H NMR (90 MHz) 1.00 (13-CH_3, s), 1.07 (propionate CH_3, t, J = 7 Hz), 1.29 (16β-CH_3, d, J = 6 Hz), 1.54 (10-CH_3, s), 2.37 (propionate CH_2, q, J = 7 Hz), 4.30 (11α-H, broad m), 5.45 (11β-OH, m), 6.07 (4-H, m), 6.27 (2-H, d, J = 10 Hz), 7.32 (1-H, d, J = 10 Hz) with signals for the disulphide developing with time; ^{1}H NMR (90 MHz in $CHCl_3$-*d*) 1.07 (13-CH_3, s), 1.17 (propionate CH_3, t, J = 7 Hz), 1.37 (16β-CH_3, d, J = 6 Hz), 1.57 (10-CH_3, s), 2.38 (propionate CH_2, q, J = 7 Hz), 4.46 (11α-H, broad m), 6.17 (4-H, m), 6.37 (2-H, d, J = 10 Hz), 7.27 (1-H, d, J = 10 Hz).

The 17α-hydroxy compound **5d** was also converted into **6i** (53%), mp 174–179 °C from EtOAc, using general method D with propionyl chloride. In a third method, the carboxylic acid **3d**[11] (0.70 g, 1.40 mmol) and CDI (0.473 g, 2.9 mmol) in DMF (26 mL) were stirred at 22 °C under N_2 for 19.5 h. A dark-blue solution, prepared by passing H_2S through sodium hydride (60% in oil, 0.235 g) in DMF (10 mL), was added, and stirring was continued for 5.5 h. The mixture was diluted with EtOAc (100 mL), washed with 2N HCl, water, and brine, dried, and evaporated *in vacuo* to give **6i** (0.186 g, 26%).

17α-(Butyryloxy)-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (6j). 17α-(Butyryloxy)-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid (**3h**)[8] was converted by general methods A then C and crystallization from EtOAc to give **6j** (34%).

9α-Fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioic Acid (6k). Crude **4bd** was converted by general method C to give crude **6k** (44%), purified by crystallization of a portion (0.20 g) from EtOAc to give the analytical sample of **6k** (0.137 g, 30%).

9α-Fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17(*R*)-spiro-5′-[2′ξ-ethyl-2′ξ-(imidazol-1-yl)-1′,3′-dioxolan-4′-one], Isomers 7a and 7b. A solution of **3d** (1.337 g, 2.67 mmol) in DMF (40 mL) was stirred under N_2 and treated with CDI (0.910 g, 5.6 mmol). After 5 h at 22 °C the reaction mixture was poured into water (250 mL) and extracted with EtOAc. The extract was washed with water, 3% Na_2CO_3, water, and brine, dried, and evaporated *in vacuo*. PLC gave the more-polar isomer **7a** (0.41 g, 32%), crystallized from EtOAc: IR 1810 (4′-one), 1660, 1620, 1604 ($\Delta^{1,4}$-3-one) cm^{-1}; ^{1}H NMR (90 MHz) 0.85 (ethyl CH_3, t, J = 8 Hz), 1.14 (13-CH_3, s), 1.17 (16β-CH_3 d, J = 7 Hz), 1.47 (10-CH_3, s), 2.31 (ethyl CH_2, q, J = 8 Hz), 7.02, 7.27, 7.81 (imidazole protons); ^{13}C NMR 8.6, 34.8 (ethyl), 17.1 (C18), 24.4 (C19), 71.4 (C11), 92.1 (C17), 102.1 (C9), 112.1 (C2′), 117.7, 130.8, 135.3 (imidazole), 153.8 (C1), 170.2 (C4′). The less-polar isomer **7b** (0.218 g, 17%) crystallized from EtOAc: IR 1810 (4′-one), 1667, 1629, 1604 ($\Delta^{1,4}$-3-one) cm^{-1}; ^{1}H NMR (90 MHz) 0.82 (ethyl CH_3, t, J = 8 Hz), 0.85 (16β-CH_3, d, J = 7 Hz), 1.25 (13-CH_3, s), 1.56 (10-CH_3, s), 2.27 (ethyl CH_2, q, J = 8 Hz), 7.02, 7.37, 7.87 (imidazole protons); ^{13}C NMR 8.6, 34.0 (ethyl), 17.2 (C18), 24.5 (C19), 71.6 (C11), 91.1 (C17), 102.4 (C9), 112.0 (C2′), 118.0, 131.0, 136.0 (imidazole), 154.0 (C1), 170.7 (C4′). In a repeat experiment on (0.455 g, 1.05 mmol) in which dimethylamine (1 mL) in DMF (9 mL) was added after 4 h, and stirring continued for 24 h, the isomers **7a** and **7b** were accompanied by a compound of intermediate polarity which crystallized from EtOAc–petroleum ether (bp 60–90 °C) to give **1-[(9α-fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-propionyloxyandrosta-1,4-dien-17β-yl)carbonyl]imidazole** (**8**, 0.039 g, 4%): IR 1742 (imidazole C=O and ester), 1669, 1625, 1605 ($\Delta^{1,4}$-3-one) cm^{-1}; ^{1}H NMR (90 MHz) 0.94 (propionate CH_3, t, J = 7 Hz), 1.08 (13-CH_3, s), 1.35 (16β-CH_3, d, J = 7 Hz), 1.54 (10-CH_3, s), 2.43 (propionate CH_2, q, J = 7 Hz), 7.08, 7.69, 8.31 (imidazole protons).

***S*-Methyl 9α-Fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (9).** A mixture of **6i** (0.277 g, 0.61 mmol) and $NaHCO_3$ (0.167 g, 1.99 mmol) in CH_3I (0.3 mL, 4.8 mmol) and dimethylacetamide (2 mL) was stirred at room temperature for 3 h, diluted with EtOAc (150 mL), washed with 10% sodium thiosulfate (50 mL), water, 1 N HCl, water, 5% $NaHCO_3$, and water, dried, and evaporated *in vacuo* to give a foam (0.221 g) which was crystallized twice from methanol to give **9** (0.191 g, 67%): mp 235–237 °C; λ_{max} (EtOH) 239 nm (ε 18 995) [lit.[11] mp 223–224 °C; λ_{max} (MeOH) 239 nm (ε 18 890]. Alternatively, **3d** (0.681 g, 1.36 mmol) and CDI (0.456 g, 2.8 mmol) were stirred under N_2 at 22 °C in DMF (26 mL) for 19.5 h. A solution made from NaH (60% in oil, 0.285 g) in DMF (10 mL) saturated with MeSH was added, and after 5.5 h EtOAc (100 mL) was added and the solution was washed with 2 N– HCl, water, 2 N– Na_2CO_3, H_2O, and brine, dried, and evaporated *in vacuo* to give **9** (0.306 g, 45%). Similarly, **7a** (0.131 g) with MeSNa in DMF for 1 h gave crude **9** as a foam (0.103 g, 81%) and **7b** (0.042 g) gave crude **9** (0.041 g, 104%).

Formation of *S*-Chloromethyl 17β-Carbothioates 10. General Method E. A solution of the carbothioic acid **6** (1 equiv) in dimethylacetamide (3.5–10 mL/g) was stirred with $NaHCO_3$ (2 equiv) and bromochloromethane (2–5 equiv) for 1–2 h, diluted with EtOAc (*ca* 200 mL/g), washed with 5% $NaHCO_3$ and water, dried, and evaporated *in vacuo* to give crude **10**.

***S*-Chloromethyl 9α-Fluoro-11β-hydroxy-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (10a).** General method E on **6a** and two crystallizations from methanol gave **10a** (52%).

***S*-Chloromethyl 6α,9α-Difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (10b).** General method E on **6e** and crystallization from EtOAc gave **10b** (67%): ^{1}H NMR (90 MHz) 0.94 (16α-CH_3, d, J = 7 Hz), 1.06 (13-CH_3, s), 1.06 (propionate CH_3, t, J = 7 Hz), 1.54 (10-CH_3, s), 2.40 (propionate CH_2, q, J = 7 Hz), 4.29 (11α-H, broad m), 5.22 (CH_2Cl, s), *ca.* 5.65 (6-H, broad dm, J = 50 Hz), 6.18 (4-H, m), 6.36 (2-H, broad d, J = 10 Hz), 7.31 (1-H, broad d, J = 10 Hz).

***S*-Chloromethyl 11β-Hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (10c).** General method E on **6g** and crystallization from EtOAc gave **10c** (76%).

***S*-Chloromethyl 17α-Acetoxy-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (10d).** General method E on **6h** and two crystallizations from EtOAc gave **10d** (76%).

***S*-Chloromethyl 9α-Fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (10e).** General method E on **6i** and two crystallizations from methanol gave **10e** (36%): ^{1}H NMR (90 MHz) 0.98 (13-CH_3, s), 1.06 (propionate CH_3, t, J = 7 Hz), 1.37 (16β-CH_3, d, J = 7 Hz), 1.53 (10-CH_3, s), 2.38 (propionate CH_2, q, J = 7 Hz), 4.28 (11α-H, broad m), 5.11 (CH_2Cl, s), 6.05 (4-H, m), 6.27 (2-H, broad d, J = 10 Hz), 7.29 (1-H, d, J = 10 Hz). A similar result was obtained with ICH_2Cl in place of $BrCH_2Cl$.

***S*-Chloromethyl 17α-(Butyryloxy)-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (10f).** General method E on **6j** and crystallization from EtOAc gave **10f** (53%).

***S*-Chloromethyl 9α-Fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (10g).** General method E on **6k** and PLC followed by two crystallizations from EtOAc gave **10g** (50%): ^{1}H NMR (90 MHz) 1.01 (propionate CH_3, t, J = 7 Hz), 1.01 (13-CH_3, s), 1.54 (10-CH_3, s), 2.32 (propionate CH_2, q, J = 7 Hz), 4.30 (11α-H, m), 5.21 (CH_2Cl, s), 5.50–5.71 (16=CH_2 and 11β-OH, m), 6.08 (4-H, m), 6.29 (2-H, dd, J = 10, 2 Hz), 7.32 (1-H, d, J = 10 Hz).

***S*-Chloromethyl 6α,9α-Difluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (10h).** General methods D then E on **5e**, with PLC and crystallization from aqueous methanol, gave **10h** (19.7%).

***S*-Chloromethyl 6α-Fluoro-11β-hydroxy-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (10i).** General method E on **6a** and crystallization from acetone and

petroleum ether (bp 60–80 °C) then EtOAc and petroleum ether (bp 60–80 °C) gave **10i** (81%).

***S*-Chloromethyl 9α-Fluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (10j).** General method E on **6c** and two crystallizations from acetone gave **10j** (36%).

***S*-Chloromethyl 17α-Acetoxy-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (10k).** General method E on **6d** and crystallization from acetone gave **10k** (74%).

***S*-Chloromethyl 17α-(Butyryloxy)-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (10l).** General method E on **6f** and two crystallizations from acetone gave **10l 50%**).

Formation of *S*-Iodomethyl 17β-Carbothioates 11. General Method F. The *S*-chloromethyl carbothioate **10** (1 equiv) and NaI (*ca.* 4 g/g, *ca.* 12 equiv) were refluxed in acetone (10–30 mL/g) for 3–7 h. EtOAc (150 mL/g) was added, and the solution was washed with water, 10% sodium thiosulfate or 5% sodium metabisulfate, 5% $NaHCO_3$, and water, dried, and evaporated *in vacuo* to give crude **11**.

***S*-Iodomethyl 9α-Fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (11a).** General method F on **10e** and PLC and then two crystallizations from acetone gave **11a** (41%): ^{1}H NMR (90 MHz in $CHCl_3$-*d*) 4.26 and 4.52 (CH_2I, dd, J = 11 Hz).

***S*-Iodomethyl 6α-Fluoro-11β-hydroxy-3-oxo-17α-(propionlyoxy)androsta-1,4-diene-17β-carbothioate (11b).** General method F on **10i** and two crystallizations from acetone and petroleum ether (bp 60–80 °C) gave **11b** (76%).

***S*-Iodomethyl 9α-Fluoro-11β-hydroxy-3-oxo-17β-(propionyloxy)androsta-1,4-diene-17β-carbothioate (11c).** General method F on **10d** and crystallization from methanol gave **11c** (81%).

***S*-Iodomethyl 17α-Acetoxy-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (11d).** General method F on **10k** and crystallization from EtOAc gave **11d** (82%).

***S*-Iodomethyl 6α,9α-Difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (11e).** General method F on **10b** and crystallization from EtOAc gave **11e** (85%): ^{1}H NMR (90 MHz) 4.63 (CH_2I, s).

***S*-Iodomethyl 17α-(Butyryloxy)-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (11f).** General method F on **10l** and crystallization from EtOAc gave **11f** (85%).

***S*-Iodomethyl 17α-Acetoxy-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (11g).** General method F on **10d** then PLC and two crystallizations from EtOAc gave **11g** (71%).

***S*-Iodomethyl 9α-Fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (11h).** General method F on **10g** then PLC and two crystallizations from acetone gave **11h** (74%).

***S*-Bromomethyl 9α-Fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (12).** A solution of **11a** (0.660 g, 1.12 mmol) in acetone (20 mL) was stirred with LiBr (0.972 g, 11.2 mmol) at room temperature for 5 d. The reaction mixture was diluted with EtOAc (150 mL), washed with 10% sodium thiosulfate, water, and brine, dried, and evaporated *in vacuo* to give a foam (0.624 g). This crystallized from acetone and petroleum ether (bp 40–60 °C) to give **12** (0.499 g, 82%): ^{1}H NMR (90 MHz in $CHCl_3$-*d*) 4.51 and 4.89 (CH_2Br, dd, J = 11 Hz).

Formation of *S*-Fluoromethyl 17β-Carbothioates 13. General Method G. The *S*-iodomethyl carbothioate (**11**, 1 equiv) and AgF (3–10 equiv) were stirred in the dark at room temperature in CH_3CN (12–75 mL/g) for 1–72 h. The highest reaction rates were achieved with finely pulverized AgF, obtained in granular form (from Ventron). The reaction mixture was diluted with EtOAc (*ca.* 150 mL/g) and filtered through kieselguhr. The filtrate was washed with water, dried, and evaporated *in vacuo* to give crude **13**.

***S*-Fluoromethyl 6α-Fluoro-11β-hydroxy-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (13a).** General method G on **11b** and two crystallizations from acetone and petroleum ether (bp 60–80 °C) gave **13a** (62%).

***S*-Fluoromethyl 9α-Fluoro-11β-hydroxy-3-oxo-17α-(propionyloxy)androstra-1,4-diene-17β-carbothioate (13b).** General method G on **11c** and two crystallizations from MeOH gave **13b** (58%).

***S*-Fluoromethyl 9α-Fluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (13c).** General methods F then G on **10j**, without purification of the intermediate *S*-iodomethyl carbothioate, PLC, and two crystallizations from acetone gave **13c** (43%).

***S*-Fluoromethyl 17α-Acetoxy-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (13d).** General method G on **11d** and recrystallization from EtOAc gave **13d** (70%).

***S*-Fluoromethyl 6α,9α-Difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (13e).** Small pieces of AgF (2.46 g, 19.4 mmol) were stirred in CH_3CN (320 mL, dried over molecular sieves) for 17 h. The *S*-iodomethyl ester **11e** (5.9 g, 9.7 mmol) was added, and the reaction mixture was stirred for 2 h. Finely powdered AgF (2.46 g, 19.4 mmol) was added, and after being stirred for a further 2 h the reaction mixture was concentrated to *ca.* 50 mL *in vacuo* and diluted with EtOAc (800 mL). After filtration through kieselguhr, The filtrate was washed with 2 N HCl (400 mL), water (3 × 400 mL), and brine (400 mL), dried over $MgSO_4$, and evaporated *in vacuo* to low volume when crystallization occurred. The white crystals (4.2 g, 87%) were collected and recrystallized from acetone to give **13e** (3.6 g, 74%): IR 3350 (OH), 1750 (propionate), 1708 (carbothioate), 1668, 1622, 1612 ($\Delta^{1,4}$-3-one) cm^{-1}; ^{1}H NMR (90 MHz) 0.96 (16α-CH_3, d, J = 7 Hz), 1.06 (propionate CH_3, t, J = 7 Hz), 1.07 (13-CH_3, s), 1.56 (10-CH_3, s), 2.33 (propionate CH_2, q, J = 7 Hz), 4.32 (11α-H, broad m), 5.63 (11-OH, broad d, J = 4 Hz), *ca.* 5.75 (6-H, broad dm, J = 50 Hz), 6.00 (SCH_2F, d, J = 51 Hz), 6.19 (4-H, m), 6.37 (2-H, dd, J = 10, 2 Hz), 7.33 (1-H, d, J = 10 Hz).

The fluoromethyl ester **13e** was also prepared from the carbothioic acid **6e** (0.5 g, 1.07 mmol) in DMF (2.05 mL) by first stirring it at −5 °C under nitrogen for 5 min with K_2CO_3 (0.118 g, 1.18 mmol). Cold (−60 °C) bromofluoromethane (0.138 g, 1.22 mmol) was added, and the mixture was stirred at 0 °C to −5 °C for 1 h and then diluted with EtOAc (6 mL). The mixture was washed with 5% Na_2CO_3, and the aqueous layer was extracted with EtOAc (6 mL). The combined organic extracts were washed with H_2O (4 mL) and concentrated *in vacuo* to *ca.* 1.5 mL. After the suspension was cooled to 0 °C and stirred for 1 h, the solid was collected by filtration and dried at 40 °C *in vacuo* for 16 h to give **13e** (0.370 g, 69.3%), with a second crop (0.050 g, 9.4%) by concentration of the mother liquors.

The fluoromethyl ester **13e** was also prepared from the carbothioic acid **6e** (47.9 g, 102 mmol) in DMF (190 mL) by first stirring it with $KHCO_3$ (11.26 g, 112 mmol) at room temperature for 10 min under N_2. Fluoroiodomethane (17.2 g, 107 mmol) was added over 3–4 min with cooling to keep the temperature at 22–25 °C. Isolation after 0.25 h as in the preceding experiment gave **13e** (67.1%), mp 184–185 °C, with IR and ^{1}H NMR spectra resembling those detailed above. The latter showed solvation with DMF (*ca.* 0.05 mol).

***S*-Fluoromethyl 17α-(Butyryloxy)-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (13f).** General method G on **11f** and crystallization from EtOAc gave **13f** (76%).

***S*-Fluoromethyl 11β-Hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (13g).** General methods F then G on **10c**, without purification of the intermediate *S*-iodomethyl carbothioate, and crystallization from EtOAc gave **13g** (23%).

***S*-Fluoromethyl 17α-Acetoxy-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (13h).** General method G on **11g** and two crystallizations from acetone gave **13h** (35%).

***S*-Fluoromethyl 9α-Fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboth-**

ioate (13i). General method G on **11a** with PLC and crystallization from MeOH then MeOH and Et_2O gave **13i** (33%).

The fluoromethyl ester **13i** was also prepared from the chloromethyl ester **10e** (0.377 g, 0.760 mmol) with AgF (0.669 g, 5.3 mmol) in CH_3CN (6 mL) in the dark for 38 days. Isolation as in general method G gave **13i** (0.070 g, 19.2%): ^1H NMR (90 MHz) 0.94 (13-CH_3, s), 1.06 (propionate CH_3, t, J = 7 Hz), 1.35 (16β-CH_3, d, J = 7 Hz), 1.52 (10-CH_3, s), 2.38 (propionate CH_2, q, J = 7 Hz), 4.27 (11α-H, m), 5.84 (CH_2F, d, J = 51 Hz), 6.04 (4-H, m), 6.24 (2-H, dd, J = 10, 2 Hz), 7.29 (1-H, d, J = 10 Hz); ^{19}F NMR (84.68 MHz) −187.5 (CH_2F, t, J = 51 Hz), −162 (9α-F, broad dd).

***S*-Fluoromethyl 9α-Fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothioate (13j).** General method G on **11h** with PLC and two crystallizations from acetone gave **13j** (22%).

***S*-Fluoromethyl 6α,9α-Difluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbothhioate (13k).** General methods F then G on **10h**, without purification of the intermediate *S*-iodomethyl carbothioate, PLC, and crystallization from aqueous acetone gave **13k** (79%): ^1H NMR 0.98 (13-CH_3, s), 1.03 (propionate CH_3, t, J = 7 Hz), 1.55 (10-CH_3, s), 2.34 (propionate CH_2, q, J = 7 Hz), 4.32 (11α-H, m), 5.63 (16=CH_2, broad m), *ca.* 5.7 (6-H, broad dm, J = 50 Hz), 5.95 (SCH_2F, d, J = 51 Hz), 6.18 (4-H, m), 6.35 (2-H, dd, J = 10, 2 Hz), 7.30 (1-H, broad d, J = 10 Hz).

***S*-Chloromethyl 16α,17α-Epoxy-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate (16).** A suspension of 16α,17α-epoxy-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid (**14**)[26] (0.753 g, 2.0 mmol) and FMPT (0.680 g, 2.4 mmol) in CH_2Cl_2 (7 mL) was treated dropwise at 0 °C with Et_3N (1.39 mL, 10 mmol) and then stirred at 0 °C for 1 h. H_2S was passed through the mixture for 15 min, and the resultant solution was stirred at 0 °C for 1 h. After addition of $BrCH_2Cl$ (0.26 mL, 4 mmol) the mixture was stirred for 1.5 h at room temperature, diluted with EtOAc (250 mL), washed with 2 N HCl, 5% $NaHCO_3$, and water, dried, and evaporated *in vacuo* to give a yellow solid (0.818 g). This on PLC and then crystallization from acetone gave **16** (51%).

***S*-Chloromethyl 9α-Fluoro-11β,17α-dihydroxy-16-methylene-3-oxoandrosta-1,4-diene-17β-carbothioate (17).** A solution of **16** (0.400 g, 0.91 mmol) in CF_3CO_2H (16 mL) was stirred for 5.5 h at room temperature, evaporated to near dryness *in vacuo*, and dissolved in EtOAc (100 mL). The solution was washed with 5% $NaHCO_3$ and water, dried, and evaporated *in vacuo* to give a foam (0.466 g), part of which on PLC and two crystallizations from acetone gave **17** (70%).

The 11β,17α-diol **17** (0.227 g, 0.52 mmol) in propionic acid (2.2 mL) and $(CF_3CO)_2O$ (0.7 mL) was treated with a dry solution of *p*-toluenesulfonic acid in $CHCl_3$ (0.044 mL, containing *ca.* 0.08 g/mL) and then stirred at room temperature for 6 h and at 3 °C for 16.5 h. The reaction mixture was diluted with EtOAc, washed with 5% $NaHCO_3$, H_2O, and brine, dried, and evaporated *in vacuo* to give a mixture of 11β- and 17α-monopropionates. Purification by PLC gave sample of **10g** (48%) containing *ca.* 7% of the 11β-monopropionate.

***Se*-Methyl 16α,17α-Epoxy-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboselenoate (18a).** The 17β-carboxylic acid **14** (0.376 g, 1.0 mmol) and FMPT (0.340 g, 1.2 mmol) were stirred under N_2 at 0 °C, and Et_3N (0.70 mL, 5.0 mmol) was added dropwise. After 70 min a solution of NaSeH [prepared under N_2 by the addition of EtOH (3 mL) to a mixture of $NaBH_4$ (0.063 g, 1.66 mmol) and powered Se (0.118 g, 1.5 mmol) at 0 °C followed by stirring for 20 min] was added, and the brown solution was stirred at 0 °C for 1.25 h. MeI (0.12 mL, 2.0 mmol) was added under N_2, and the yellow solution was stirred for 3.75 h at room temperature. The reaction mixture was diluted with EtOAc (200 mL), washed with 2 N HCl, 5% $NaHCO_3$, and water, dried, and evaporated *in vacuo* to give a solid (0.390 g). PLC and two crystallizations from acetone gave **18a** (0.174 g, 38.4%): IR (in $CHBr_3$) 1680 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz) 1.38 (13-CH_3, s), 1.54 and 1.58 (16β-CH_3 and 10-CH_3, singlets), 2.22 ($SeCH_3$, s).

***Se*-Chloromethyl 16α,17α-Epoxy-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboselenoate (18b).** The carboxylic acid **14** (0.376 g, 1.0 mmol) reacted as in the preceding experiment, using FMPT−NEt_3, NaSeH [from Se (2.0 mmol)] and $BrCH_2Cl$ (0.130 mL, 2.0 mmol) in place of MeI, to give, after crystallization from acetone, **18b** (29.5%): IR 1710 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz) 1.41 (13-CH_3, s), 1.54 and 1.58 (16β-CH_3 and 10-CH_3, singlets), 5.06 ($SeCH_2Cl$, s).

***Se*-Methyl 9α-Fluoro-11β,17α-dihydroxy-16-methylene-3-oxoandrosta-1,4-diene-17β-carboselenoate (19a).** The epoxide **18a** (0.574 g, 1.27 mmol) in CF_3CO_2H (25 mL) was stirred at room temperature for 2.5 h and then diluted with 5% $NaHCO_3$ (550 mL). The product was extracted into EtOAc, and the extract was washed with water, dried, and evaporated *in vacuo* to give a yellow foam (0.561 g). PLC and two crystallizations from acetone gave **19a** (0.326 g, 56.8%): IR 1700 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz) 0.91 (13-CH_3, s), 1.53 (10-CH_3, s), 2.06 ($SeCH_3$, s), 4.97 and 5.22 (16=CH_2, broad singlets), 6.38 (17α-OH, s).

***Se*-Chloromethyl 9α-Fluoro-11β,17α-dihydroxy-16-methylene-3-oxoandrosta-1,4-diene-17β-carboselenoate (19b).** The epoxide **18b** (1.332 g), in a manner similar to the preceding experiment, gave crystalline **19b** (0.276 g, 20.7%): IR 1720 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz) 0.96 (13-CH_3, s), 1.53 (10-CH_3, s) 5.01 ($SeCH_2Cl$, s), 5.00 and 5.25 (16=CH_2, broad singlets), 6.71 (17α-OH, s).

***Se*-Methyl 9α-Fluoro-17α-hydroxy-16-methylene-3,11-dioxoandrosta-1,4-diene-17β-carboselenoate (20a).** Pyridinium dichromate[27] (0.130 g, 0.345 mmol) was added to a solution of **19a** (0.125 g, 0.246 mmol) in DMF (1.4 mL), and the mixture was stirred at 0 °C for 6 h. More pyridinium dichromate (0.130 g, 0.345 mmol) was added, and stirring continued for 24 h at 3−4 °C. The mixture was diluted with water (20 mL), and the product was extracted into EtOAc. The extract was washed with water, dried, and evaporated *in vacuo* to a yellow gum (0.116 g) which, on PLC and two crystallizations from acetone, gave **20a** (0.050 g, 45.0%): IR 3390 (OH), 1718 (11-one), 1689 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz) 0.66 (13-CH_3, s), 1.53 (10-CH_3, s), 2.09 ($SeCH_3$, s), 5.04 and 5.29 (16=CH_2, broad singlets), 6.92 (17α-OH, s).

***Se*-Chloromethyl 9α-Fluoro-17α-hydroxy-16-methylene-3,11-dioxoandrosta-1,4-diene-17β-carboselenoate (20b).** Pyridinium dichromate (7.825 g, 20.8 mmol) was added to a stirred solution of **19b** (4.155 g, 8.32 mmol) in DMF (46 mL), and after 3.25 h the product was isolated as in the preceding experiment to give crude **20b** (3.00 g, 74.2%). Two crystallizations of a portion (0.150 g) from acetone gave the analytical sample of **20b** (0.056 g): IR 3350 (broad, OH), 1720 (11-one), 1709 (carboselenoate); ^1H NMR (90 MHz) 0.83 (13-CH_3, s), 1.54 (10-CH_3, s), 5.06 ($SeCH_2Cl$, s), 5.06 and 5.34 (16=CH_2, broad singlets), 7.26 (17α-OH, s).

***Se*-Methyl 9α-Fluoro-16-methylene-3,11-dioxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboselenoate (21a).** A solution of **20a** (1.451 g, 3.214 mmol) in propionic acid (14.5 mL) and $(CF_3CO)_2O$ (5.8 mL) was stirred and treated with a solution of *p*-toluenesulfonic acid (0.016 g) in $CHCl_3$ (0.20 mL) for 38 h at room temperature. The mixture was poured into 5% $NaHCO_3$ (400 mL), and the product was extracted into EtOAc (300 mL). The extract was washed with water, dried, evaporated *in vacuo*, and purified by PLC to give **21a** (1.095 g, 67%). A portion of (0.250 g) recrystallized twice from acetone gave the analytical sample of **21a** (0.198 g): IR 1740 (propionate), 1722 (11-one), 1678 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz) 0.71 (13-CH_3, s), 1.05 (propionate CH_3, t, J = 7 Hz), 1.55 (10-CH_3, s), 2.23 ($SeCH_3$, s), 2.39 (propionate CH_2, q, J = 7 Hz), 5.64 and 5.70 (16=CH_2, broad singlets).

***Se*-Chloromethyl 9α-Fluoro-16-methylene-3,11-dioxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboselenoate (21b).** Propionylation of **20b** (2.850 g, 5.87 mmol) as in the preceding experiment but for 6 days gave **21b** (2.380 g, 75%). A portion (0.150 g) crystallized twice from acetone gave the analytical sample of **21b** (0.070 g): IR 1744 (propionate), 1718 (11-one), 1688 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz)

0.77 (13-CH_3, s), 1.06 (propionate CH_3, t, J = 7 Hz), 1.55 (10-CH_3, s), 2.42 (propionate CH_2, q, J = 7 Hz), 5.21 ($SeCH_2Cl$, s), 4.30 (16=CH_2, broad).

***Se*-Methyl 9α-Fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboselenoate (22a).** A suspension of **21a** (0.841 g, 1.66 mmol) and $NaBH_4$ (0.069 g, 1.82 mmol) in EtOH (12 mL) was stirred at room temperature for 1.3 h, treated with acetone (3.5 mL), and concentrated to near dryness *in vacuo*. EtOAc (100 mL) was added, and the solution was washed with 1N HCl and water, dried, and evaporated *in vacuo* to give a white foam. Two crystallizations from acetone gave **22a** (0.657 g, 77.7%): IR 3340 (broad, OH), 1744, 1735 (propionate), 1718 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz) 0.96 (13-CH_3, s), 1.02 (propionate CH_3, t, J = 7 Hz), 1.54 (10-CH_3, s), 2.20 ($SeCH_3$, s), 2.32 (propionate CH_2, q, J = 7 Hz), 5.61 and 5.66 (16=CH_2, broad singlets).

***Se*-Chloromethyl 9α-Fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboselenoate (22b).** The 11-ketone **21b** (2.656 g, 4.90 mmol) was reduced as in the preceding experiment to give **22b** as a white foam (1.802 g, 67.7%). A portion (0.500 g) was recrystallized twice from EtOAc to give the analytical sample of **22b** (0.297 g): IR 3310 (broad, OH), 1740 (propionate), 1724 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz) 1.01 (13-CH_3, s), 1.01 (propionate CH_3, t, J = 8 Hz), 1.55 (10-CH_3, s), 2.33 (propionate CH_2, q, J = 8 Hz), 4.29 (11α-H, m) 5.19 ($SeCH_2Cl$, s), 5.60 (11β-OH, m), 5.62 (16=CH_2, broad).

***Se*-Iodomethyl 9α-Fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboselenoate (22c).** A mixture of the *Se*-chloromethyl selenoester **22b** (1.291 g, 2.37 mmol) and NaI (4.447 g, 29.7 mmol) in acetone (40 mL) was heated under reflux for 24 h. The mixture was cooled, diluted with EtOAc (200 mL), washed with water (60 mL), 10% sodium thiosulfate (2 × 60 mL), 5% $NaHCO_3$ (60 mL), and water (2 × 60 mL), dried, and evaporated to a foam (1.392 g). PLC gave colorless crystals (1.225 g, 81%), a portion (0.225 g) of which was recrystallized twice from acetone to give **22c** (0.201 g, 72%): IR 1744 (propionate), 1728 (carboselenoate) cm^{-1}; ^1H NMR (90 MHz) 1.00 (propionate CH3, t, J = 8 Hz), 1.02 (13-CH_3, s), 1.53 (10-CH_3, s), 1.00 (propionate CH_3, t, J = 8 Hz), 1.02 (13-CH_3 s), 1.53 (10-CH_3, s), 2.31 (propionate CH_2, q, J = 8 Hz), 4.43 ($SeCH_2I$, s), 5.53–5.71 (16=CH_2, 11-OH, broad).

Reaction of 22c with Silver Fluoride. 22c (1.0 g) and AgF (2.0 g, 10 equiv) in CH_3CN (16 mL) were stirred at ambient temperature in the dark for 24 h. The reaction mixture was diluted with EtOAc (300 mL), solid material was removed by filtration, and the filtrate was washed with water, dried, and concentrated to a foam (0.690 g). Silica gel chromatography eluting with $CHCl_3$ gave a white solid (0.027 g, 3%, mechanical loss) which was recrystallized from EtOAc–petroleum ether (bp 40–60 °C) to give colorless crystals (0.008 g) of ***Se*-fluoromethyl 9α-fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carboselenoate (22d)**: IR 3600–3000 (OH), 1738, 1708, 1695, (shoulder) cm^{-1}; ^1H NMR (100 MHz) δ 0.96 (13-CH_3, s), 1.00 (propionate CH_3, t, J = 7 Hz), 1.51 (10-CH_3, s) 2.32 (propionate CH_2, q, J = 7 Hz), 5.5–5.6 (16=CH_2, 11-OH, broad), 6.22 ($SeCH_2F$, d, J = 50 Hz); MS m/e (rel intensity) Se cluster MH^+ 531 (23.0), 529 (100), 527 (52), 526 (19.4), 525 (19.9), 523 (3.0), fragments 415 (14.7), MH − $SeCH_2F$), 359 (12.4), 313 (35.1), 293 (24.2). The MH^+ ion-cluster had relative peak intensities close to the calculated values for the seven Se isotopes, HRMS m/e calcd for $C_{25}H_{31}F_2O_5{}^{80}Se$ (MH^+) 529.1304, found 529.1269. Further elution gave a white foam (0.543 g, 80%), which was recrystallized twice from acetone to give colorless crystals (0.196 g, 29%) of 9α-fluoro-11β-hydroxy-16-methylene-3-oxo-17α-(propionyloxy)androsta-1,4-diene-17β-carbonyl fluoride **23**: 83% pure by HPLC; mp 193–195 °C; $[\alpha]_D$ −82° (c 0.93); λ_{max} 239 nm ($E_{1cm}{}^{1\%}$ 346); IR ($CHBr_3$) 3605 (OH), 1842 (COF), 1738 and 1262 (propionate), 1668, 1630, and 1612 ($\Delta^{1,4}$-3-one) cm^{-1}; ^1H NMR (90 MHz) 1.01 (propionate, t, J = 8 Hz), 1.10 (13-CH_3, s), 1.54 (10-CH_3, s), 2.38 (propionate, q, J = 8 Hz), 6.55 and 6.65 (16=CH_2, 11-OH, broad); ^{19}F NMR (84.68 MHz) +29 (COF, s), −161.7 (9α-F, m); ^{13}C NMR (25.05 MHz) 174.3 (s, propionate C=O), 159.3 (d, $^1J_{CF}$ = 365 Hz, COF), 145.4 (s, C16), 120.9 (s, 16=CH_2; off-resonance t), 87.8 (d, $^2J_{CF}$ = 50 Hz, C17), 10.1 (s, propionate CH_3, off-resonance q); MS m/e (rel intensity) 435 (98.7, MH^+), 415 (100, MH^+ − HF), 359 (35.7), 313 (16.7), 293 (15.8); HRMS m/e calcd for $C_{24}H_{29}F_2O_5$ (MH^+) 435.1983, found 435.1982. Anal. ($C_{24}H_{28}F_2O_5$) H, F; C calcd, 66.3; found, 65.3.

Bis[9α-fluoro-11β-hydroxy-16β-methyl-3-oxo-17α-(propionyloxy)androsta-1,4-dien-17β-yl)carbonyl] Disulfide. The carbothioic acid **6i** (0.104 g, 0.230 mmol) in DMSO (1 mL) was stirred and heated at 85 °C for 1.5 h. The solution was diluted with EtOAc (100 mL), washed with 5% $NaHCO_3$ and water, dried, and evaporated *in vacuo* to a solid (0.108 g). PLC and two crystallizations from acetone gave the title disulfide (0.068 g, 65.6%): ^1H NMR (90 MHz) 0.99 (13-CH_3, s), 1.06 (propionate CH_3, t, J = 7 Hz), 1.32 (16β-CH_3, d, J = 6 Hz), 1.52 (10-CH_3, s), 2.42 (propionate CH_2, q, J = 7 Hz), 4.33 (11α-H, m), 5.72 (11β-OH, m), 6.08 (4-H, s), 6.29 (2-H, d, J = 10 Hz), 7.36 (1-H, d, J = 10 Hz). Anal. ($C_{48}H_{60}F_2O_{10}S_2{\cdot}0.5H_2O$) C, H, S.

Test Methods. Human Vasoconstrictor Activity. The method used was a modification of that described by McKenzie and Atkinson.[17] Six male and six female subjects with six marked sites on the flexor surface of each forearm were treated with 12 test solutions, allocated by means of a 12 × 12 latin square. The test solutions (0.02 mL) were four graded doses of the standard fluocinolone acetonide (3, 12.5, 50, 200 ng) and of two test steroids in ethanol. They were pipetted on to the marked circular areas (ca. 2.25 cm^2) and spread as evenly as possible. The solvent was allowed to evaporate, and the forearms were enclosed in polythene tubing secured at each end by elastic surgical tape. The tubing was left in place overnight (*ca.* 16 h), and 1 h after its removal the arms were assessed for areas of vasoconstriction. The areas were scored as either positive or negative, and estimates of relative potency were obtained by analysis of the data using the method of Litchfield and Wilcoxon.[28]

Topical Antiinflammatory Activity in Rats. The method used was a modification of that described by Tonelli et al.[18] and used 7 groups of 10 rats (54–70 g) in each experiment. Croton oil soluton was prepared by mixing croton oil (5 vol), EtOH (20 vol), and Et_2O (75 vol). Graded doses of the standard fluocinolone acetonide and test steroid were dissolved in the croton oil solution such that the doses (standard steroid 0.06, 0.25, and 1 μg) were contained in 0.04 mL. The doses were applied to the inner aspect of both ears, the control group receiving croton oil solution without medication. Six hours later, the rats were sacrificed by CO_2 inhalation and both ears were removed and weighed separately. The metameters used for the analyses of variance and calculation of relative potencies were the logarithm of the applied dose and ear weights.

Topical Antiinflammatory Activity in Mice. The methodology was similar to that for rats except that a mixture of croton oil (2 vol), EtOH (20 vol), and Et_2O (78 vol) was used and the dose volume was 0.02 mL. Nine groups of 10 mice (22–28 g) were used, and doses of the standard fluocinolone acetonide were 0.015, 0.06, 0.25, and 1 μg.

Systemic Corticosteroid Activity after Topical Application to Rats. Seven groups of six rats (170–200 g at the beginning of the experiment) were used. The fur on the dorsal skin was removed by clipping, and a circular area (*ca.* 2 cm^2) was marked using an indelible marker. Graded doses of the standard fluocinolone acetonide and test steroid were dissolved in acetone such that the doses (0.5, 2, 8 μg of standard) were contained in 0.02 mL. The rats were treated once daily for seven consecutive days; one group which received unmedicated vehicle acted as controls. On the day after the final dose, the rats were stressed by exposure to Et_2O vapor for 1 min. Twenty minutes later, the rats were re-anaesthetised with intraperitoneal pentobarbitone, and blood was withdrawn by cardiac puncture into heparinized tubes. Plasma corticosterone levels were measured for each blood sample by a modification of the fluorimetric method of Zenker and Bernstein.[19] Alternatively, adrenal glands were removed, blotted, and weighed. The metameters used for the analyses of variance and calculation of relative potencies were the

logarithm of the daily dose and plasma corticosterone concentrations or adrenal weights.

Systemic Corticosteroid Activity after Topical Application to Mice. The methodology was similar to that for rats except that nine groups of 10 mice (25–30 g) were used and that, after stressing, the mice were re-anaesthetised with Et_2O. Daily doses of the standard fluocinolone acetonide were 0.47, 1.9, 7.5, and 30 μg. The blood from two mice was pooled to obtain sufficient for the corticosterone estimation.

Systemic Corticosteroid Activity after Subcutaneous or Oral Administration to Rats and Mice. The methods involved seven daily doses of the standard betamethasone (2.5, 10, and 40 μg to rats and 1.56, 6.25, and 25 μg to mice) and the test steroid given subcutaneously as a suspension in normal saline containing 0.5% Tween 80 by volume (0.2 mL for rats and 0.1 mL for mice). Adrenal weights in rats and corticosterone levels in mice were measured as for topical application above. Oral activities were similarly assessed.

Acknowledgment. We thank Miss P, J. McDonough for the elemental analyses, Dr. R. A. Fletton and C. J. Seaman for the NMR and IR measurements, and Dr. S. J. Lane for the mass spectra. We are indebted to Dr. P. J. May for discussions and help in the preparation of the paper, and finally, but not least to Miss L. Micklewright for her patience and skill in preparing the manuscript.

Supplementary Material Available: ^1H NMR data (5 pages). Ordering information is given on any current masthead page.

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Spectrochimica Acta Part A 72 (2009) 244–247

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Vibrational spectroscopic study of fluticasone propionate

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ARTICLE INFO

Article history:
Received 24 July 2008
Accepted 13 August 2008

Keywords:
Raman spectroscopy
Infrared spectroscopy
Fluticasone propionate
Respiratory pharmaceuticals
Ab initio structural calculations

ABSTRACT

Fluticasone propionate is a synthetic glucocorticoid with potent anti-inflammatory activity that has been used effectively in the treatment of chronic asthma. The present work reports a vibrational spectroscopic study of fluticasone propionate and gives proposed molecular assignments on the basis of *ab initio* calculations using BLYP density functional theory with a 6-31G* basis set and vibrational frequencies predicted within the quasi-harmonic approximation. Several spectral features and band intensities are explained. This study generated a library of information that can be employed to aid the process monitoring of fluticasone propionate.

1. Introduction

Fluticasone propionate (FP) is a highly potent, triflourinated glucocorticoid. Unlike most other corticosteroids, the structure of it is based on androstane, rather than a pregnane, corticosteroid nucleus (Fig. 1). The molecule is designed to maximize topical anti-inflammatory activity and minimise the unwanted systemic side effects associated with other glucocorticoids; this is because FP is rapidly metabolised to the inactive 17β-carboxylic acid metabolite [1–3]. The combination of potent topical anti-inflammatory and a low systemic side effect profile makes FP an ideal compound to treat asthma, seasonal and perennial rhinitis [4,5].

Raman spectroscopy has proved to be a simple and reliable method for the determination of the composition profile of solid pharmaceutical samples [6–10]. Due to its non-invasiveness, high sensitivity and good reproducibility, in addition to the requirement for little or no sample preparation, this technique is an important tool for the screening of drugs, once the unique fingerprint spectrum that is specific for each pharmaceutical compound has been identified.

In this paper, we present the first vibrational spectroscopic study of FP and propose a vibrational assignment of the experimental spectral features aided by *ab initio* calculations using BLYP density functional theory with a 6-31G* basis set and vibrational frequencies and intensities predicted within the quasi-harmonic approximation. The results thus obtained permit a rapid and unequivocal spectroscopic identification of FP in pharmaceutical preparation and processes.

2. Experimental

2.1. Materials

A specimen of fluticasone propionate was obtained from the Sigma–Aldrich Chemical Co. and used without further purification.

2.2. Raman spectroscopy

Fourier-transform Raman spectroscopy was carried out using a Bruker IFS 66 instrument with an FRA 106 Raman module attachment and a Nd^{3+}/YAG laser operating at 1064 nm in the near infrared. The powdered specimens were examined in aluminium cups. The spectra were recorded at 4 cm^{-1} spectral resolution and 500 spectral scans accumulated to improve signal-to-noise ratios. Laser powers were maintained at 100 mW at the sample.

2.3. Infrared spectroscopy

The IR spectra were recorded as KBr discs (1:200) using a Digilab Scimitar 2000 Series spectrometer. The spectra were recorded over the range of 650–4000 cm^{-1} at 4 cm^{-1} spectral resolution and 512 spectral scan accumulations.

Raman and infrared spectra were analysed using a GRAMS7 curve resolution package.

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doi:10.1016/j.saa.2008.08.004

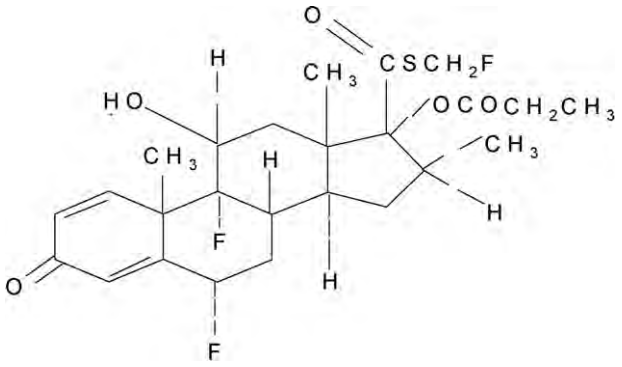

Fig. 1. The chemical structure of fluticasone propionate (androsta-1,4-diene-17-carbothioic acid, 6,9-difluoro-11-hydroxy-16-methyl-3-oxo-17-(1-oxopropoxy)-S(fluoromethyl) ester (6α,11β,16α,17α).

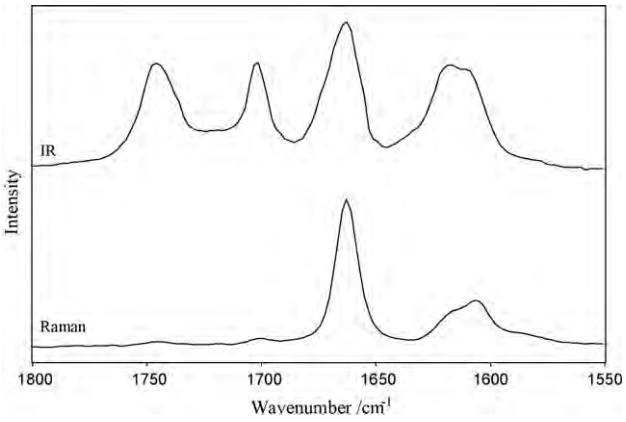

Fig. 3. FT-Raman and IR spectral stackplot of FP in the 1550–1800 cm^{-1} region.

2.4. Calculation details

Calculations were performed using GAMESS-UK [11] and ORCA software packages [12]. An initial structure (code DAXYUX) for the geometry optimisation was taken from the Cambridge Crystal structure Database (CSD) optimised using BLYP density functional theory [13,14]. Infrared and Raman spectra were calculated using the quasi-harmonic approximation, through diagonalisation of the mass-weighted Hessian matrix. Intensities for the infrared and Raman transitions were calculated using the dipole-moment derivatives and the polarisability derivatives respectively of the normal modes.

3. Results and discussion

The infrared and Raman spectra of fluticasone propionate were recorded over the wavenumber range 4000–600 cm^{-1} and 4000–100 cm^{-1}, respectively, using both transmission and attenuated total reflectance modes of operation in the former and a 1064 nm laser wavelength of excitation in the latter. The infrared and FT-Raman spectra are shown in Figs. 2–4. A complete assignment of the experimental vibrational features was carried out (Table 1) in the light of both the theoretical results presently performed and the vibrational spectroscopic data. Several calculated modes have been matched with the experimentally observed bands in the IR and Raman spectra, and these modes have been described. The vibrational spectra illustrate clearly the complexity of structural information that is provided from the infrared and Raman analysis; the OH stretching modes observed near 3500 cm^{-1} Fig. 2 in the infrared spectrum are not observed in the Raman spectrum, whereas bands in the low wavenumber region of the Raman spectrum that are normally characteristic of polymorphic crystalline pharmaceutical forms are not recorded in the lower infrared region Fig. 4. Although the infrared and Raman spectra have been reported previously [15,16], these reports provide neither spectral band assignments nor vibrational spectroscopic characterisation in conjunction with the molecular *ab initio* calculations.

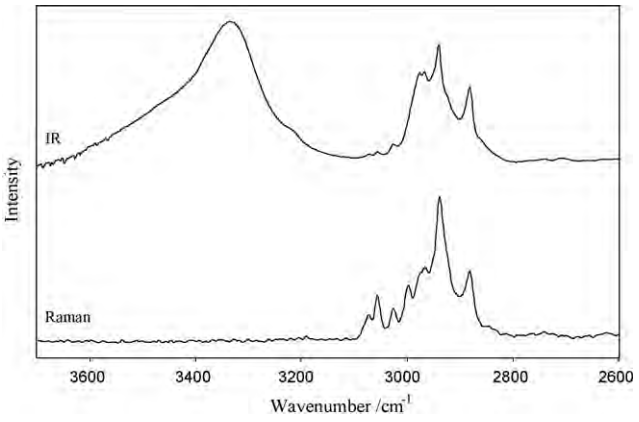

Fig. 2. FT-Raman and IR spectral stackplot of FP in the 2600–3700 cm^{-1} region.

The profile of the υ(OH) stretching band (Fig. 2) in the infrared spectrum at 3336 cm^{-1} suggests that external hydrogen bonding occurs in the crystalline FP. The CH stretching region comprises several features in the wavenumber range 3100–2800 cm^{-1} (Fig. 2 for the infared and Raman spectra); the CH stretching bands of the unsaturated, component of FP are shown more clearly in the Raman spectrum rather than in the infrared spectrum (Table 1) and can be assigned to the bands at 3075 and 3058 cm^{-1} in the Raman spectrum in addition to, the CH stretching band of the five-membered ring at 3023 cm^{-1}. The CH aliphatic stretching bands comprise both CH_3 and CH_2 symmetric and asymmetric stretching modes which have been assigned to the medium features between 2878 and 2965 cm^{-1}.

3.1. 1800–1550 cm^{-1} region

This is a very important spectroscopic region for structural studies, particularly for the C=O and C=C groups in the molecule. Expanded wavenumber regions of the infrared and Raman spectra are shown in Fig. 3. The infrared spectrum has one very strong band at 1661 cm^{-1} and four strong bands at 1744, 1699,1616 and 1611 cm^{-1} compared to one very strong band at 1663 cm^{-1}, one medium band at 1606 cm^{-1}, medium shoulder band at 1611 cm^{-1} and two weak bands at 1742 and 1698 cm^{-1} in the Raman spectrum. The strong IR bands at 1744 and 1699 cm^{-1} in the IR spectrum and the weak Raman spectral feature at 1742 and 1698 cm^{-1} can be attributed to the carbonyl attached to the aliphatic ring (*cf.* literature values for a five-membered aliphatic ring at 1750–1700 cm^{-1}, and dimethyl ketone at 1715 cm^{-1}) [17] and carbonyl attached to sulphur, respectively, this shift occurs because of force constant changes in the carbonyl bond [18], whereas that at 1661 and 1616 cm^{-1} can be attributed to the C=O and C=C stretching vibration in the quinonoid aromatic ring (*cf.* benzophenone at 1660 cm^{-1}), which couples together in the same moiety. This coupling explains the observed strength in both Raman and infrared at 1663 and 1661 cm^{-1}, respectively.

Table 1
The observed and calculated vibrational wavenumbers/cm^{-1} of fluticasone propionate. The calculated Raman and infrared intensities (I_{Raman} and I_{IR}) are relative to that of υ(C=O) at 1667 cm^{-1}, set arbitrarily equal to 1.

Observed		Calculated			Proposed assignment
Raman (cm^{-1})	IR (cm^{-1})	υ	I_{Raman}	I_{IR}	
	3336 m	3567	0.513	0.030	υ(OH)
3075 w	3069 vw	3123	0.186	0.035	υ(CH)
3058 mw	3051 vw	3082	0.465	0.069	υ(CH)
3023 mw	3024 vw	3027	0.229	0.120	υ(CH) of the five-membered ring
3005 mw	2975 m	2999	0.291	0.119	υ(C=CH)
2965 mw	2964 m	2968	0.138	0.080	υ_{as} (CH_3), υ_{as} (CH_2)
2935 m	2942 m	2944	0.308	0.155	υ_{as} (CH_2)
2878 mw	2881 m	2933	0.356	0.102	υ_{s} (CH_2)
1742 w	1744 s	1737	0.684	0.047	υ(C=O)
1698 w	1699 s	1695	0.118	0.525	υ(S—C=O)
1663 vs	1661 vs	1667	1.000	1.000	υ(C=O)
	1616 s	1639	0.036	0.155	υ(C=C)
1611 sh,m	1611 s				
1606 m		1607	0.054	0.039	δ(CH_3)
1492 mw	1530 vw	1493	0.071	0.019	δ(CH_3)
1453 mw	1455 m	1445	0.027	0.058	δ(CH_3)
1422 w	1421 mw	1425	0.004	0.024	Ring stretching
1387 w	1395 mw	1399	0.041	0.017	δ(CH_2)
1369 w	1376 mw	1372	0.028	0.045	
1334 w	1365 mw	1357	0.019	0.002	δ(CH)
1321 m	1319 m	1323	0.074	0.034	
1281 w	1304 m	1298	0.081	0.059	υ(C—C)
1233 mw	1271 m	1267	0.035	0.074	υ(C—F)
	1248 m	1244	0.018	0.055	
1194 mw	1226 m	1223	0.097	0.052	In-plane δ(CH)
	1214 m	1217	0.015	0.016	
1168 mw	1188 m	1177	0.017	0.022	
1146 mw	1147 w	1147	0.004	0.397	
1126 w	1124 m	1124	0.167	0.046	
1106 w		1109	0.021	0.016	
1067 w	1064 s	1070	0.039	0.090	
1058 w		1058	0.018	0.055	
1023 w	1027 m	1020	0.023	0.480	υ(F—C—S)
992 w	993 s	993	0.097	0.196	r(CH_3)
970 w		972	0.004	0.053	r(CH_3)
953 w	944 m	953	0.047	0.257	δ(C—C—C)
931 w	933 m	936	0.015	0.124	
887 w	895 ms	904	0.028	0.135	OOC/CCH aromatic deformation
	884 ms	877	0.011	0.094	OOC/CCH aromatic deformation
869 w	873 m	863	0.016	0.110	OOC/CCH aromatic deformation
843 w		835	0.033	0.028	υ(O=C—O—C)
	816 w	816	0.022	0.046	
799 vw	797 w	802	0.002	0.014	
773 vw	782 w	776	0.021	0.032	r(CH_2)
733 m	734 mw	751	0.008	0.009	
698 vw	707 w	706	0.003	0.013	CH wagging
650 vw		655	0.005	0.037	υ(C—S)
616 vw		626	0.015	0.012	υ(C—S)
584 w		581	0.005	0.016	
562 w		558	0.034	0.015	
549 w		541	0.004	0.084	
532 w		524	0.022	0.009	
519 w		512	0.019	0.012	Out-of-plane δ(CC)
505 w		508	0.002	0.014	
470 vw		484	0.019	0.016	
448 vw		448	0.008	0.004	
435 vw		425	0.009	0.007	
409 w		408	0.013	0.033	
387 vw		387	0.014	0.018	
370 w		372	0.015	0.025	
348 vw		348	0.013	0.014	
330 vw		324	0.007	0.203	
308 w		311	0.019	0.008	
286 vw		282	0.011	0.08	
264 w		264	9.6e-4	0.018	$CH_3\tau$
234 w		237	0.013	0.026	$CH_3\tau$
159 w		156	0.010	0.011	

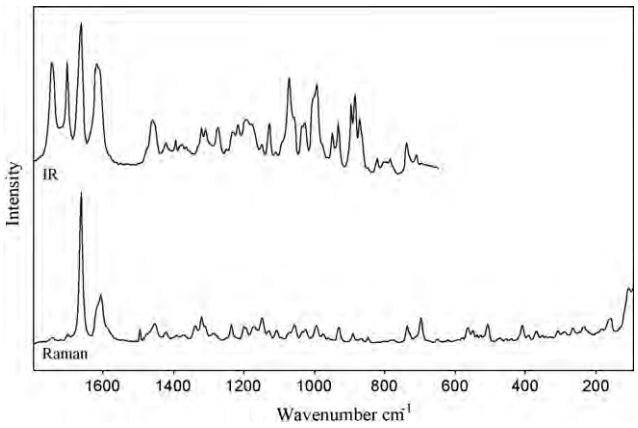

Fig. 4. FT-Raman and IR spectral stackplot of FP in the 100–1800 cm^{-1} region.

3.2. 1800–100 cm^{-1} region

Infrared and Raman spectra over this wavenumber range are shown in Fig. 4; the infrared region comprises a rich spectrum from the C=O and C=C functionalities, which has been already discussed in some detail above, along with several bands of medium and strong intensities, whereas the Raman spectrum consists mainly of the strong C=C features and other weaker features. In the range 1530–1330 cm^{-1}, where we expect CH_3, CH_2 and CH aliphatic deformation vibration to occur. Lower in wavenumber, we expect to find the aliphatic C—F stretching vibrations at 1271 cm^{-1} and 1233 cm^{-1}, of medium intensity in the infrared and of weaker strength in the Raman spectrum, respectively. The in-plane δ(CH) vibration occurs at 1226 cm^{-1} in the IR spectrum of medium intensity and at 1194 cm^{-1} in the Raman spectrum of medium weak intensity and the υ(F—C—S) band occurs at 1027 cm^{-1} in the IR spectrum of medium intensity and at 1023 cm^{-1} in the Raman spectrum of weak intensity.

Methyl rocking and CH_2 rocking modes extend over the range 970–995 cm^{-1}, the OOC/CCH aromatic deformation modes extend over the range 870–900 cm^{-1}, the (O=C—O—C) stretching mode occurs at 843 cm^{-1}, the CH wagging mode occurs near 700 cm^{-1} and the C—S stretching mode occurs in the range of 616–650 cm^{-1}.

Finally, the complex ring modes of CCO and COCOC vibrations and deformations in the range between 600 and 500 cm^{-1} occur in the low wavenumber region; in this region we should expect the aromatic quinonoid ring deformations near 600 cm^{-1}. In the Raman spectrum below 400 cm^{-1}, the methyl torsional mode at 234 and 264 cm^{-1} of weak intensity can be noted.

Even with the support of *ab initio* calculations, it is difficult to be certain about some molecular vibrational assignments because of the complexities of mode mixing; however, it is clear that the major spectroscopic features of FP have been reasonably assigned in this study.

4. Conclusions

A complete vibrational spectroscopic analysis has been carried out for fluticasone propionate. Raman, infrared and molecular *ab initio* calculations, have been used to explain the presence of the observed spectroscopic complexity in the C=C and C=O stretching region which will provide useful structural information when considering the complexation of fluticasone propionate.

Acknowledgement

Hassan R.H. Ali is grateful for the support of the Government of the Arab Republic of Egypt during which this work was carried out.

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Improved Synthesis of Fluticasone Propionate

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Supporting Information

ABSTRACT: A novel process for the preparation of fluticasone propionate (**1**), a corticosteroid, is reported. In this paper, compound **2** was used as starting material to prepare **6** by using NaClO or NaBrO which was much cheaper than H_5IO_6 as an oxidizing agent. Furthermore, toxic, expensive, and pollutive $BrCH_2F$ was replaced by $AgNO_3$ and Selectfluor in decarboxylative fluorination.

INTRODUCTION

Steroidal glucocorticoid agonists such as fluticasone propionate (**1**) are anti-inflammatory agents used widely against a broad spectrum of inflammatory diseases. Fluticasone propionate (**1**), synthesized by Glaxo Wellcome and launched in 1993, is a trifluorinated glucocorticosteroid. It shows good topical anti-inflammatory activity and is commonly used as a safe and effective inhaled treatment for asthma and allergic rhinitis.[1] The previous route for the synthesis of **6** from **2** was in the commercial scale. Compound **6** was synthesized from commercial grade flumethasone (**5**) by H_5IO_6 oxidation with a yield of 95.0%.[2a] Flumethasone (**5**) can be prepared from **2** in three steps with a unclear yield (Scheme 1).[3] According to the literature,[2a,3] we obtained **6** from **2** in a total yield of 45%.

The compound **7** was synthesized from **6** by propionyl chloride or propionic anhydride acylation. Compound **7** reacted with *N*, *N*-dimethylthiocarbamoyl chloride[4] in the presence of an iodide catalyst and Et_3N to produce **8**, followed by hydrolyzation with K_2CO_3,[2a] Et_2NH,[2b] or NaSH[4a] to obtain **9**. Alternatively, the combination of 1,1′-carbonyldiimidazole (CDI) with NaSH[2b] can also be used to prepare **9** from **7**. Fluticasone propionate (**1**) can be synthesized from **9** by using $BrCH_2F$,[2a] $ClCH_2F$,[5a] or *S*-(monofluoromethyl) diarylsulfonium tetrafluoroborate[5f,g] directly. Using $BrCH_2F$ can get an ideal yield; however, $BrCH_2F$ is costly and will destroy to the ozone layer. In addition, **9** reacted with $BrCH_2Cl$ or Br_2CH_2 and then by an anion exchange with AgF,[2c,5e] KF, or tetrabutylammonium fluoride[5b] to afford **1** in a low yield. Fluticasone propionate (**1**) could be obtained from **10**, in the presence of fluorodecarboxylating reagents such as XeF_2 and BrF_3.[5d] Unfortunately, XeF_2 is extremely expensive, and BrF_3 which should be stored in Teflon containers is a strong corrosive toxic liquid, which tends to react very exothermically with water and release poisonous vapours. Furthermore, the high toxicities and instabilities of XeF_2 and BrF_3 prevented practical applications of this method. According to the literature,[5c] Deoxo-Fluor or DAST can also be used as monofluoromethylation reagent to acquire **1** from **11** at −60 °C (Scheme 2). Considering the different literature sources, the highest overall yield for the previous synthesis of fluticasone propionate (**1**) from **2** was close to 30%.

The disadvantages of the above processes include safety issues, high expenses, and environmental problems, such as the use of costly H_5IO_6 as an oxidant and $BrCH_2F$, XeF_2, BrF_3, or Deoxo-Fluor as monofluoromethylation reagents. Considering these drawbacks, we have subjected this synthetic route to further researches and intended to develop an efficient, eco-friendly, and commercially feasible process for fluticasone propionate (**1**). In this article, we describe an improved process with an overall yield of 42.3% in method A and 54.5% in method B (Scheme 3).

RESULTS AND DISCUSSION

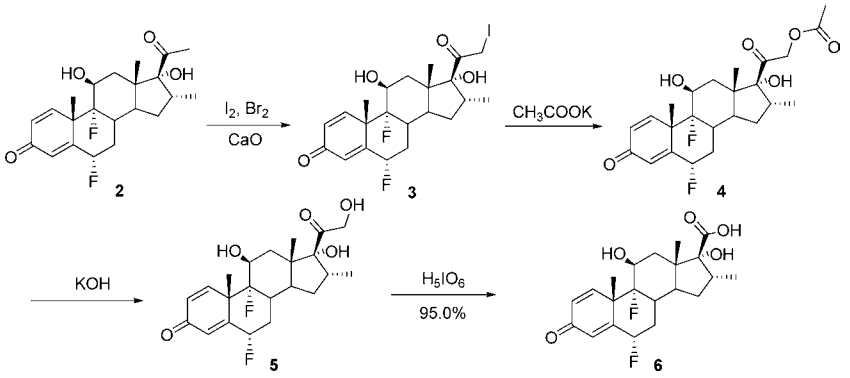
Preparation of 6α,9α-Difluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (6). As one of the oldest known organic reactions, the haloform reaction can be used to convert a terminal methyl ketone into appropriate carboxylic acid.[6] Applying this reaction to the synthesis of compound **6** from **2** could shorten reaction routes,[7] reduce the cost,[8] and improve the total yield compared with the traditional method in four steps. Luckily, we found that using NaClO in the presence of NaOH could successfully obtain compound **6** in room temperature with a yield of 63.7%,[9] and 11β,17α-dihydroxy and 1,4-diene were tolerated. A series of solvents such as dioxane/H_2O, THF/H_2O, dimethoxyethane/H_2O, and EtOH/H_2O, were screened in order to find an optimal solvent. The results showed that all of those mixed solvents did not work well to afford the desired product **6** except THF/H_2O/EtOH, which provided a homogeneous reaction system.

By contrast, the usage of NaBrO which has a stronger activity than NaClO could get a higher yield of 84.5% in a lower temperature (Scheme 3). To this reaction, dioxane/H_2O was proved to be the best solvent.[10] Other solvents such as THF/H_2O, dimethoxyethane/H_2O, EtOH/H_2O, and THF/H_2O/EtOH did not work well, and the reaction did not proceed in biphasic systems. When the reaction was finished, the remaining oxidizing agent (NaClO or NaBrO) was destroyed by the addition of excess sodium sulfite solution, and the

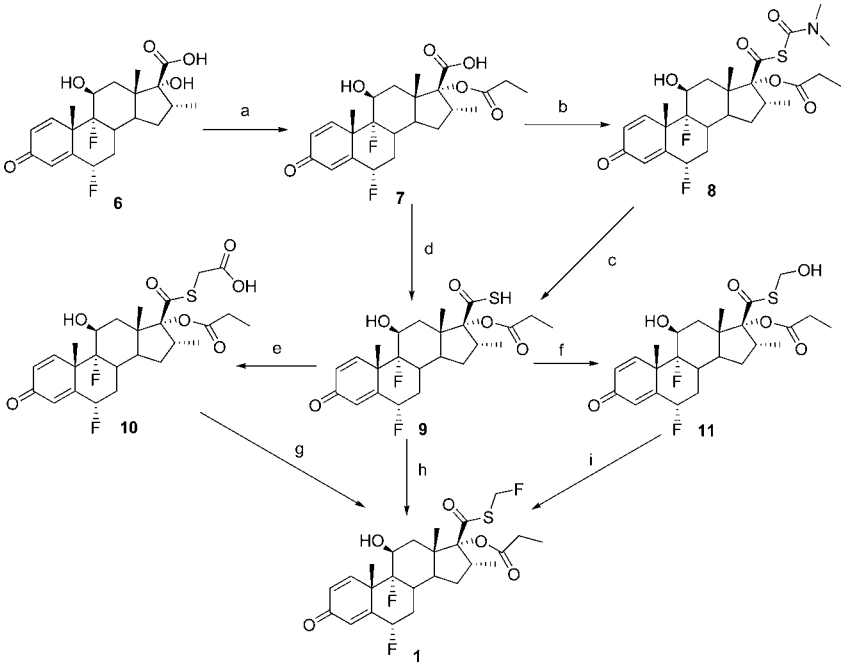
Received: April 14, 2014
Published: July 24, 2014

dx.doi.org/10.1021/op5001226 | *Org. Process Res. Dev.* 2014, 18, 928−933

Scheme 1. Previous route for the synthesis of 6



Scheme 2. Previous method for the synthesis of fluticasone propionate (1)[a]



[a]Reactions and conditions: (a) (i) propionic anhydride or propionyl fluoride/Et_3N; (ii) Et_2NH; (b) *N*,*N*-dimethylthiocarbamoyl chloride/Et_3N/NaI or tetrabutylammonium iodide; (c) K_2CO_3 or Et_2NH or NaSH; (d) CDI/NaSH; (e) $BrCH_2COOH/Et_3N$; (f) formaldehyde; (g) XeF_2/BrF_3; (h) $BrCH_2F$ or $ClCH_2F$ or *S*-(monofluoromethyl) diarylsulfonium tetrafluoroborate or $BrCH_2Cl$/AgF or $BrCH_2Cl$/KI/KF; (i) Deoxo-Fluor or DAST.

solvent THF/EtOH or dioxane was removed under reduced pressure. After extraction with ethyl acetate, the aqueous phase was acidified with hydrochloric acid to furnish a white precipitate of **6**, which was collected by filtration, washed with water, and dried.

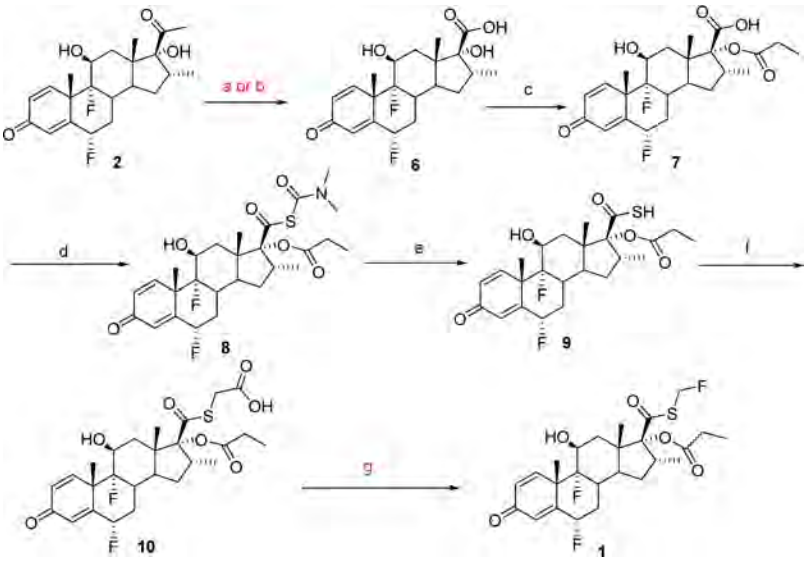
Preparation of 6α,9α-Difluoro-11β-hydroxy-16α-methyl-17α-propionyloxy-3-oxoandrosta-1,4-diene-17β-carbothioate (10). According to the literature,[2a,5d] the compound **10** can be synthesized from **6** in four steps including esterification, acylation, alcoholysis, and alkylation (Scheme 3). In the original methods,[5d] the product **10** was acquired from **9** by $BrCH_2COOH$ alkylation under the condition of using DCM as the solvent. In our improved process, lower toxic solvent acetone was used to replace DCM. When the reaction was finished, the compound **10** could obtained by filtration conveniently after acidification with 1 mol/L HCl.

Preparation of *S*-Fluoromethyl-6α,9α-difluoro-11β-hydroxy-16α-methyl-17α-propionyloxy-3-oxoandrosta-1,4-diene-17β-carbothioate (1). Recently reported N−F reagents, such as Selectfluor and NFSI, are commercially available, easy to use, and stable electrophilic fluorinating reagents that can be used to conduct decarboxylative fluorination.[11] In our improved process, fluticasone propionate (**1**) was prepared from **10** with $AgNO_3$/Selectfluor (Scheme 4).[12] According to the literature,[11a] the combination of $AgNO_3$ (20 mol %) with Selectfluor (2.5 equiv) shows a considerable decarboxylative fluorination ability (Table 1, entry 1). Other Ag(I) salts, such as AgOAc and AgOTf, exhibited a weaker catalytic activity (Table 1, entries 2 and 3), while no reaction occurred without the presence of a Ag(I) salt (Table 1, entry 4). Switching the electrophilic fluorinating reagent from Selectfluor to NFSI caused no reaction (Table 1, entry 5). In addition to Ag(I) ions, water also was turned out to be essential (Table 1, entry 6). Much of the experimental results was similar to the optimization done by the Li group.[11a]

The Ag(II)- or Ag(III)-mediated decarboxylation of carboxylic acids is well-documented.[13] According to the literature,[11a] a tentative mechanism of the decarboxylative

dx.doi.org/10.1021/op5001226 | Org. Process Res. Dev. 2014, 18, 928−933

Scheme 3. Improved process for the synthesis of fluticasone propionate (1)[a]



[a]Reactions and conditions: (a) NaClO/NaOH, THF/EtOH/H_2O, 25 °C, 63.7%; (b) NaBrO/NaOH, dioxane/H_2O, 0−5 °C, 84.5%; (c) (i) propionic anhydride/Et_3N, acetone, 15−25 °C; (ii) Et_2NH, 15−25 °C, 97.3%; (d) *N*,*N*-dimethylthiocarbamoyl chloride/Et_3N/NaI, acetone/H_2O, 30 °C, 96.0%; (e) K_2CO_3, CH_3OH, 25 °C, 95.0%; (f) $BrCH_2COOH/Et_3N$, acetone, 15−25 °C, 97.1%; (g) $AgNO_3$/Selectfluor, acetone/H_2O, 45 °C, 92.7%.

Scheme 4. Decarboxylative fluorination for the synthesis of 1

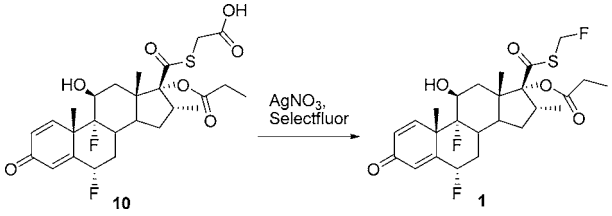


Table 1. Screening of Ag(I) catalyst and N−F reagent of decarboxylative fluorination of 10[a]

entry	catalyst (equiv)	N−F reagent (equiv)	solvent	time (h)	yield[b] (%)	purity[c] (%)
1	$AgNO_3$	Selectfluor	acetone/H_2O	3	92.7	92.6
2	AgOAc	Selectfluor	acetone/H_2O	8	64.2	74.2
3	AgOTf	Selectfluor	acetone/H_2O	8	48.8	79.5
4		Selectfluor	acetone/H_2O	8	0	
5	$AgNO_3$	NFSI	acetone/H_2O	8	0	
6	$AgNO_3$	Selectfluor	acetone	8	trace	

[a]Reagents and conditions: 1.0 equiv **10**, 0.2 equiv catalyst, 2.5 equiv N−F reagent, 45 °C, under nitrogen. [b]The isolated yield was calculated with **10**. [c]The purity was monitored by HPLC.

fluorination with $AgNO_3$/Selectfluor was proposed. The oxidation of Ag(I) by Selectfluor generates an Ag(III)−F intermediate, which initiated the decarboxylative fluorination of carboxylic acids (Figure 1).

Further optimization of the decarboxylative fluorination was carried out by screening of a range of temperatures, and of reagent stoichiometries, using $AgNO_3$ and Selectfluor (Table 2). As shown in (Table 2, entry 1), **10** was treated with $AgNO_3$ (20 mol %) and Selectfluor (2.5 equiv) at 30 °C for 8 h under a nitrogen atmosphere giving **1** in 80.1% yield, when raising the temperature to 45 °C improved the yield of **1** to 92.7% (Table 2, entry 2). However, more impurities were generated when the

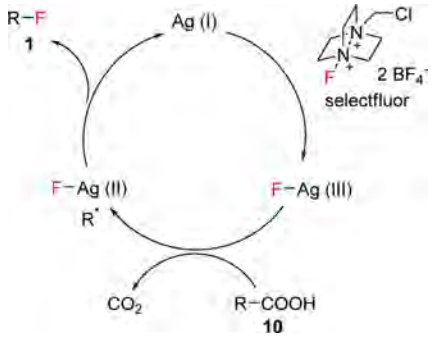


Figure 1. Proposed mechanism of silver-catalyzed decarboxylative fluorination.

reaction was performed at 55 °C (Table 2, entry 3). Unfortunately, using $AgNO_3$ (10 mol %)/Selectfluor (2.5 equiv) or $AgNO_3$ (20 mol %)/Selectfluor (2.0 equiv) as fluorodecarboxylating reagents provided **1** in only 75.2% and 70.3% yields, respectively (Table 2, entries 4 and 5).

The activity of decarboxylative fluorination was proved to be solvent-dependent, the mixed solvent of acetone/H_2O (2:1, v:v) exhibited the best activity (Table 2, entry 2). By contrast, a higher reaction temperature was needed in CH_3CN/H_2O (2:1, v:v) solution that led to more impurity **12** (Table 2, entry 6). No fluorodecarboxylation occurred in THF/H_2O (2:1, v:v) solution (Table 2, entry 7) or other biphasic systems.

The optimized reaction conditions (Table 2, entry 2), **10** was treated with $AgNO_3$ (20 mol %) and Selectfluor (2.5 equiv) in acetone/H_2O (2:1, v:v) solution at 45 °C for 3 h under a nitrogen atmosphere giving the expected product fluticasone propionate **1**. Dilution with water upon completion of the reaction and collection of the product by filtration followed by washing with water gave **1** in 92.7% isolated yield.

The major impurity **12** was obtained by preparative HPLC, the process of impurity formation was studied (Scheme 5). We suspected that fluticasone propionate (**1**) could be hydrolyzed

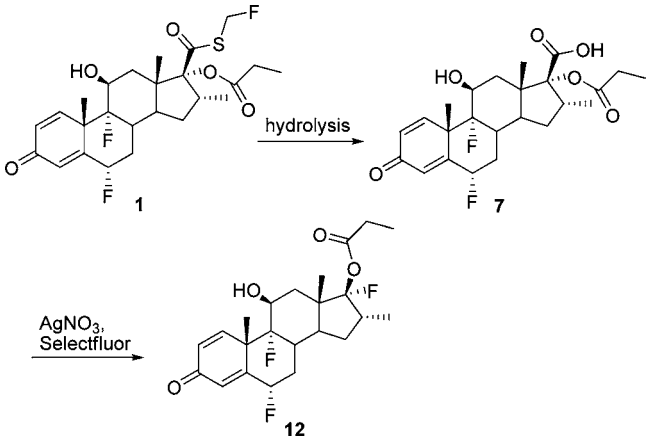
dx.doi.org/10.1021/op5001226 | *Org. Process Res. Dev.* 2014, 18, 928−933

Table 2. Optimisation of decarboxylative fluorination of 10

entry	$AgNO_3$ (equiv)	Selectfluor (equiv)	solvent	temp (°C)	time (h)	yield[a] (%)	purity[b] (%)
1	0.2	2.5	acetone/H_2O	30	8	80.1	85.0
2	0.2	2.5	acetone/H_2O	45	3	92.7	92.6
3	0.2	2.5	acetone/H_2O	55	3	92.0	79.7
4	0.1	2.5	acetone/H_2O	45	8	75.2	80.2
5	0.2	2.0	acetone/H_2O	45	8	70.3	83.1
6	0.2	2.5	MeCN/H_2O	55	8	80.5	67.0
7	0.2	2.5	THF/H_2O	55	8	0	

[a]The isolated yield was calculated with **10**. [b]The purity was monitored by HPLC.

Scheme 5. Process of impurity 12 generated



to **7** which might generate **12** through decarboxylative fluorination rapidly. The C-17 stereochemistry of compound **12** was established by X-ray crystallography. In order to confirm the speculation, we successfully using **7** as material to obtain **12** with $AgNO_3$/Selectfluor in acetone/H_2O solution at 45 °C.

CONCLUSION

In conclusion, an efficient, eco-friendly, and commercially viable process for the synthesis of fluticasone propionate (**1**) has been developed. In this method, compound **2** was used as the starting material, which was transformed to **1** in six steps including oxidation, esterification, acylation, alcoholysis, alkylation, and fluorodecarboxylation. Especially, compared to traditional flumethasone oxidation with H_5IO_6, application of haloform reaction to the synthesis of compound **6** for the first time could shorten reaction routes, reduce the cost, and improve the total yield dramatically. Furthermore, the usage of toxic, extremely costly, and pollutive $BrCH_2F$ was replaced by an efficient method with $AgNO_3$ and Selectfluor in a good yield.

EXPERIMENTAL SECTION

Compound **2** was provided by Zhejiang Xianju Junye Pharmaceutical Co., Ltd., and all other chemicals were purchased from commercial sources and were used without further purification. HPLC analysis for fluticasone propionate (**1**) was carried out on an Agilent HPLC system (series 1200, Agilent Technologies, Germany) equipped with Agilent ZORBAX SB-C18 reversed-phase column (250 mm × 4.6 mm, 5 μm). A mobile phase of methanol, acetonitrile, and buffer with 1.2 g/L of monobasic ammonium phosphate, a pH of 3.5 adjusted with phosphoric acid, (50:15:35) was used at a flow rate of 1.5 mL/min and a column temperature of 40 °C. The UV detector was set at 239 nm to analyze the column effluent. ^1H (400 MHz) NMR, ^{13}C (101 MHz) NMR, and ^{19}F (376 MHz) NMR spectra were recorded on a Varian spectrometer in $CDCl_3$ or DMSO-d_6 using tetramethylsilane (TMS) as internal standards.

6α,9α-Difluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (6). *Method A.* NaOH (25.0 g, 0.625 mol) was dissolved in H_2O (0.040 L), the mixture was diluted with 0.500 L of THF and 0.500L of EtOH. Into the solution, **2** (50.0 g, 0.126 mol) was added, then 10% NaClO (0.500 L) was added gradually at 25 °C. The reaction was kept at 25−30 °C for 6 h. When the reaction was finished, the remaining oxidizing agent (NaClO) was destroyed by the addition of excess 10% Na_2SO_3 solution. The solvent THF/EtOH was removed under reduced pressure. Ethyl acetate (0.500 L) was added to the solution; the layers were separated, and then acidification of the aqueous phase to pH = 1.0−2.0 by 3 mol/L HCl furnished a white precipitate of **6** (32.0 g), which was collected by filtration, washed with water, and dried. Yield: 63.7%; HPLC purity 96.0%.

Method B. Br_2 (140.0 g, 0.875 mol) was added slowly to a vigorously stirred solution of 130.0 g of NaOH in 1.170 L of H_2O while cooling in an ice-salt-bath. When all of the Br_2 had dissolved, the mixture was diluted with 0.600 L of cold dioxane, and the ice-cold NaBrO was added slowly to a stirred solution of 100.0 g of **2** in 1.400 L of dioxane which was maintained at a temperature below 8 °C throughout the oxidation. After 5 h, the remaining oxidizing agent (NaBrO) was destroyed by the addition of excess 10% Na_2SO_3 solution. The solvent dioxane was removed under reduced pressure. Ethyl acetate (1.000 L) was added to the solution; the layers were separated, then acidification of the aqueous phase to pH = 1.0−2.0 by 3 mol/L HCl furnished a white precipitate of **6** (85.0 g), which was collected by filtration, washed with water, and dried. Yield: 84.5%; HPLC purity 95.6%; ^1H NMR (400 MHz, DMSO-d_6) δ 12.45 (s, 1H), 7.24 (d, J = 10.3 Hz, 1H), 6.27 (dd, J_1 = 10.2 Hz, J_2 = 1.6 Hz, 1H), 6.08 (s, 1H), 5.70−5.54 (2m, 1H), 5.32 (s, 1H), 4.69 (s, 1H), 4.12 (d, J = 11.7 Hz, 1H), 2.88−2.80 (m, 1H), 2.50−2.35 (m, 2H), 2.25−1.96 (m, 3H), 1.70−1.50 (m, 2H), 1.49 (s, 3H), 1.12−1.05 (m, 1H), 0.99 (s, 3H), 0.86 (d, J = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 184.14 (s), 174.35 (s), 162.86 (d, J = 13.7 Hz), 151.86 (s), 128.85 (s), 119.23 (d, J = 12.9 Hz), 100.13 (d, J = 176.0 Hz), 86.83 (d, J = 178.0 Hz), 85.34 (s), 70.61 (d, J = 36.0 Hz), 48.14 (d, J = 19.5 Hz), 47.31 (s), 42.23 (s), 35.42 (s), 35.19 (s), 33.91 (d, J = 18.8 Hz), 32.30 (m), 31.99 (s), 22.86 (s), 16.93 (s), 15.45 (s); MS(ESI-) m/z 395.2 $[M - H]^+$.

6α,9α-Difluoro-11β-hydroxy-16α-methyl-17α-propionyloxy-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (7). To a suspension of **6** (85.0 g, 0.215 mol) in acetone (0.425 L) at 10−15 °C was added sequentially Et_3N (65.1 g,

dx.doi.org/10.1021/op5001226 | Org. Process Res. Dev. 2014, 18, 928−933

0.645 mol) and propionic anhydride (83.8 g, 0.645 mol). After stirring for 4 h at 25 °C, Et_2NH (31.4 g, 0.430 mol) was added dropwise at 10−15 °C and then stirred at 25 °C for 1 h. Thereafter, the reaction mixture was acidified to pH 1.0−1.5 with 1 mol/L HCl at 0 °C. The precipitated product **7** (93.3 g) was obtained by filtered, washed with water, and dried. Yield 96.0%; HPLC purity 97.0%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.24 (d, J = 10.3 Hz, 1H), 6.27 (dd, J = 10.1, 1.9 Hz, 1H), 6.09 (s, 1H), 5.70−5.54 (2m, 1H), 5.46 (s, 1H), 4.16 (m, 1H), 3.14 (m, 1H), 2.50 (m, 1H), 2.30 (q, J = 7.2 Hz, 2H), 2.23 (m, 1H), 2.05 (m, 2H), 1.82−1.68 (m, 2H), 1.49 (m, 4H), 1.18 (m, 1H), 1.01 (t, J = 7.2 Hz, 3H), 1.00 (s, 3H), 0.84 (d, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 184.03 (s), 171.96 (s), 169.79 (s), 162.59 (d, J = 13.5 Hz), 151.64 (s), 128.88 (s), 119.24 (d, J = 12.1 Hz), 99.96 (d, J = 175.9 Hz), 91.13 (s), 86.69 (d, J = 180.8 Hz), 70.23 (d, J = 36.1 Hz), 47.96 (d, J = 22.2 Hz), 47.64 (s), 42.63 (s), 35.41 (s), 35.30 (s), 33.86 (d, J = 18.9 Hz), 33.08 (s), 32.19 (m), 27.02 (s), 22.77 (s), 16.44 (s), 16.43 (s), 9.26 (s); MS(ESI+) m/z 475.4 $[M + Na]^+$.

6α,9α-Difluoro-11β-hydroxy-16α-methyl-17α-propionyloxy-3-oxoandrosta-1,4-diene-17β-carbothioic Acid (9). A solution of **7** (93.3 g, 0.206 mol) and *N*,*N*-dimethylthiocarbamoyl chloride (50.8 g, 0.449 mol) in acetone (1.866 L) at room temperature was cooled to 10−15 °C. It was sequentially treated with Et_3N (41.3 g, 0.413 mol), NaI (15.0 g, 0.080 mol), and water (9.330 mL, 10% w/w with **7**) at 10−15 °C. The solution was stirred for 6 h at 30 °C, then added DMF (0.466 L) and water (3.000 L). The resultant was cooled to 0 °C and stirred for 1 h. The precipitated product **8** (106.6 g) was obtained by filtration, washed with water, and dried. Yield 96.0%; HPLC purity 96.5%.

A suspension of **8** (106.6 g, 0.196 mol) and K_2CO_3 (54.1 g, 0.392 mol) in methanol (0.530 L) was stirred at 25 °C for 5 h under a blanket of nitrogen. Thereafter, water (0.530 L) was added to the reaction mixture, and the resultant clear solution was washed twice with toluene (0.212 L). The aqueous layer was acidified with 1 mol/L HCl until pH is 1.5 to 2.0. The precipitated product was filtered, washed with water, and dried to obtain **9** (87.1 g). Yield 95.0%; HPLC purity 96.0%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.25 (d, J = 11.2 Hz, 1H), 6.30 (dd, J = 10.1, 1.8 Hz, 1H), 6.11 (s, 1H), 5.80 (d, J = 5.0 Hz, 1H), 5.72−5.55 (2m, 1H), 4.27 (m, 1H), 3.30 (m, 1H), 2.64−2.54 (m, 1H), 2.40 (q, J = 7.5 Hz, 2H), 2.30−2.09 (m, 4H), 1.92−1.88 (m, 1H), 1.51 (m, 4H), 1.26 (m, 1H), 1.13 (s, 3H), 1.03 (t, J = 7.5 Hz, 3H), 0.87 (d, J = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 189.76 (s), 184.00 (s), 172.36 (s), 162.45 (d, J = 13.5 Hz), 151.38 (d, J = 10.9 Hz), 128.92 (s), 119.27 (d, J = 12.5 Hz), 99.73 (d, J = 176.4 Hz), 96.90 (s), 86.63 (d, J = 174.0 Hz), 69.85 (d, J = 36.1 Hz), 48.34 (s), 47.87 (d, J = 24.4 Hz), 42.90 (s), 36.63 (s), 35.44 (s), 33.76 (d, J = 18.8 Hz), 33.51 (s), 32.01 (m), 27.07 (s), 22.84 (s), 17.08 (s), 15.51 (s), 9.05 (s); MS(ESI−) m/z 467.1 $[M - H]^+$.

6α,9α-Difluoro-11β-hydroxy-16α-methyl-17α-propionyloxy-3-oxoandrosta-1,4-diene-17β-carbothioate (10). A solution of **9** (87.1 g, 0.186 mol), Et_3N (28.2 g, 0.279 mol), and $BrCH_2COOH$ (28.2 g, 0.205 mol) in acetone (0.871 L) was stirred at 25 °C for 5 h. Thereafter, water (0.871 L) was added, and the reaction mixture was acidified to pH 1.0−1.5 with 1 mol/L HCl at 0 °C. The precipitated product **10** (95.0 g) was obtained by filtered, washed with water, and dried. Yield 97.1%; HPLC purity 97.0%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.24 (d, J = 10.6 Hz, 1H), 6.28 (dd, J = 10.0, 1.6 Hz, 1H), 6.10 (s, 1H), 5.60 (d, J = 4.4 Hz, 1H), 5.70−5.53 (2m, 1H), 4.19 (m, 1H), 3.67 (s, 2H), 3.25 (m, 1H), 2.56−2.45 (m, 1H), 2.33 (q, J = 7.4 Hz, 2H), 2.24 (m, 1H), 2.08 (m, 2H), 1.91−1.86 (m, 2H), 1.48 (m, 4H), 1.24 (m, 1H), 1.02 (s, 3H), 1.00 (t, J = 7.6 Hz, 3H), 0.89 (d, J = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 194.93 (s), 184.01 (s), 171.81 (s), 169.19 (s), 162.49 (d, J = 13.4 Hz), 151.46 (d, J = 8.1 Hz), 128.90 (s), 119.25 (d, J = 12.8 Hz), 99.84 (d, J = 176.4 Hz), 95.63 (s), 86.68 (d, J = 180.7 Hz), 70.00 (d, J = 35.0 Hz), 48.65 (s), 47.90 (d, J = 19.9 Hz), 42.71 (s), 35.75 (s), 35.07 (s), 33.78 (d, J = 19.3 Hz), 33.40 (s), 31.98 (m), 31.47 (s), 27.09 (s), 22.77 (s), 17.03 (s), 15.83 (s), 9.10 (s); MS(ESI−) m/z 525.0 $[M - H]^+$.

***S*-Fluoromethyl-6α,9α-difluoro-11β-hydroxy-16α-methyl-17α-propionyloxy-3-oxoandrosta-1,4-diene-17β-carbothioate (1).** A solution of **10** (95.0 g, 0.180 mol), Selectfluor (159.3 g, 0.450 mol), and $AgNO_3$ (6.1 g, 0.036 mol) in acetone (1.900 L) and water (0.950 L) was stirred at 45 °C for 3 h under a blanket of nitrogen. Then water (1.900 L) was added to the solution; the resultant was cooled to 0 °C and stirred for 1 h. The precipitated product **1** (83.5 g) was collected by filtered, washed with water, and dried. Yield 92.7%; HPLC purity 92.6%.

Purification. The crude product **1** (83.5 g) was dissolved in ethyl acetate (0.835 L) and ethanol (3.340 L). The suspension was refluxed for 30 min, gradually cooled to 0 °C, and stirred for 1 h, and the soild was collected by filtration and dried at 40 °C under vacuum to provide 69.5 g (83%) of product **1**. HPLC purity 99.2%; residual silver content 0.04633 (μg/g).[14]

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.23 (d, J = 10.0 Hz, 1H), 6.28 (dd, J = 10.1, 1.6 Hz, 1H), 6.10 (s, 1H), 5.92 (J = 50.0 Hz, 2H), 5.70−5.54 (2m, 1H), 5.58 (d, J = 3.2 Hz, 1H), 4.20 (m, 1H), 3.28 (m, 1H), 2.36 (q, J = 7.2 Hz, 2H), 2.23 (m, 1H), 2.09 (m, 2H), 1.86 (m, 2H), 1.53 (m, 1H), 1.48 (s, 3H), 1.26 (m, 1H), 1.05 (m, 1H), 1.01 (t, J = 7.2 Hz, 3H), 0.99 (s, 3H), 0.89 (d, J = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 192.87 (s), 183.98 (s), 172.07 (s), 162.43 (d, J = 13.5 Hz), 151.54 (s), 128.91 (s), 119.25 (d, J = 12.1 Hz), 99.72 (d, J = 176.3 Hz), 95.94 (s), 86.62 (d, J = 178.0 Hz), 80.92 (d, J = 211.8 Hz), 69.97 (d, J = 37.2 Hz), 48.40 (s), 47.84 (d, J = 22.4 Hz), 42.84 (s), 35.74 (s), 35.10 (s), 33.73 (d, J = 19.4 Hz), 33.37 (s), 31.93 (m), 26.94 (s), 22.73 (s), 16.95 (s), 16.08 (s), 9.05 (s); ^{19}F NMR (376 MHz, $CDCl_3$) δ −165.35 (dd, J = 27.5, 8.5 Hz), −187.00 (dd, J = 48.3, 13.8 Hz), −191.35 (t, J = 49.6 Hz); MS(ESI+) m/z 501.0 $[M + H]^+$.

6α,9α,17α-Trifluoro-11β-hydroxy-16α-methyl-17β-propionyloxy-3-oxoandrosta-1,4-diene (12). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 (d, J = 10.1 Hz, 1H), 6.41 (s, 1H), 6.35 (dd, J = 10.1, 1.2 Hz, 1H), 5.45−5.29 (2m, 1H), 4.35 (m, 1H), 2.58−2.46 (m, 1H), 2.35 (q, J = 7.6 Hz, 2H), 2.28−2.23 (m, 1H), 2.05−1.68 (m, 6H), 1.52 (s, 3H), 1.35−1.28 (m, 1H), 1.18−1.07 (m, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.20 (s), 171.28 (s), 160.94 (d, J = 13.8 Hz), 150.23 (s), 130.10 (s), 121.93 (d, J = 250 Hz), 121.08 (d, J = 9.7 Hz), 98.53 (d, J = 177.4 Hz), 86.47 (d, J = 181.1 Hz), 71.76 (d, J = 35.1 Hz), 48.05 (d, J = 22.6 Hz), 47.08 (d, J = 20.6 Hz), 42.09 (s), 39.58 (s), 39.36 (s), 37.79 (s), 33.21 (m), 32.08 (s), 28.13 (s), 23.25 (s), 16.44 (s), 15.32 (d, J = 13.1 Hz), 9.04 (s); ^{19}F NMR (376 MHz, $CDCl_3$) δ −129.60 (d, J = 17.3 Hz), −165.39 (dd, J = 27.5, 8.3 Hz), −187.05 (dd, J = 48.3, 13.7 Hz); HRMS(ESI+): $C_{23}H_{30}F_3O_4$ $[M + H]^+$; calculated: 427.2091, found: 427.2089.

dx.doi.org/10.1021/op5001226 | Org. Process Res. Dev. 2014, 18, 928−933

■ ASSOCIATED CONTENT

Supporting Information

Safety assessment for the NaClO oxidation and fluorodecarboxylation, ^{1}H and ^{13}C NMR copies for compounds **6**, **7**, **9**, **10**, **1**, and **12**, ^{19}F NMR copies for compounds **1** and **12**, and X-ray crystallographic data for compound **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge financial support from National Natural Science Foundation of China (No. 21176222) and Collaborative Innovation Center of Yangtze River Delta Region Green Pharmaceuticals.

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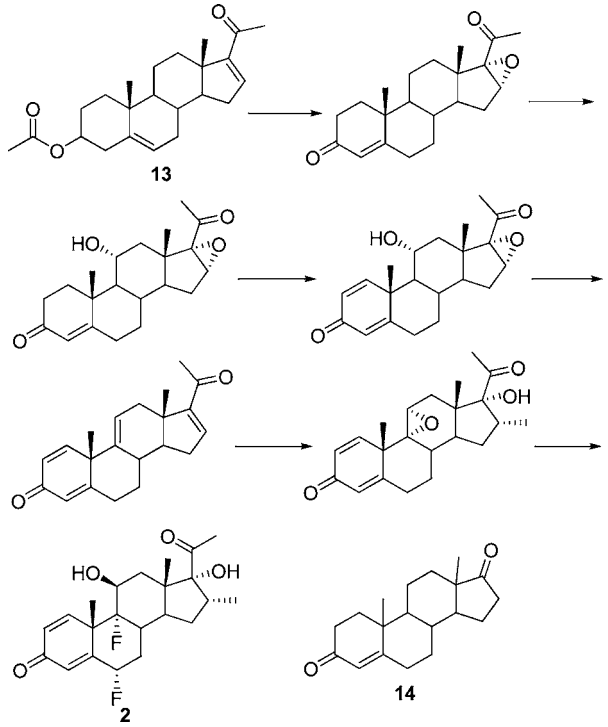
(7) Compound **13**, acquired by chemical degradation of Saponin, is a basic raw material for the synthesis of steroidal glucocorticoid drugs. Compound **14** (17-keto), acquired by biological selective degradation phytosterol side chain, is a basic raw material for the synthesis of sex hormones. It is difficult to synthesize 21-desoxy from 17-keto. Compound **2** could be prepared from **13** in several steps, and flumethasone (**5**) was prepared from **2.** In our improved process, applying haloform reaction to the synthesis of compound **6** from **2** could shorten reaction routes, reduce the cost, and improve the total yield (see scheme below).



(8) We have checked the prices for NaClO, Br_2, and H_5IO_6 on a tonne scale. 10% NaClO (96 $ per ton), Br_2 (288 $ per ton), and NaBrO could be prepared by Br_2 on the spot. H_5IO_6 needs 136085 $ per ton, and it was not friendly to the environment.

(9) The yield of NaClO oxidation (63.7%) was higher than previous synthesis in four steps (45%), even if it was lower than NaBrO oxidation (84.5%). Furthermore, the NaClO oxidation system was more environmentally friendly and suitable for industrialization.

(10) Dioxane could be recycled under reduced pressure, and the water in the recycled dioxane has no significant influence for the next halogen reaction.

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(12) In the previous routes, $BrCH_2F$ is used as the major electrophilic monofluoromethylation reagent. However, $BrCH_2F$ is costly (2500 $ per kg) and toxic and will destroy the ozone layer. The cost of Selectfluor (170 $ per kg)/$AgNO_3$ (500 $ per kg) is lower than $BrCH_2F$. Furthermore, Selectfluor/$AgNO_3$ is more environmentally friendly than $BrCH_2F$.

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(14) According to the ICH Q3D, we calculated the USP Inhalation Limit of Silver was close to 0.69 (μg/g). The residual silver content of compound **1** was 0.04633 (μg/g) by ICP-MS detection. In addition, the silver salts in the aqueous waste stream could be recycled. Silver salts reacted with HCl or NaCl could generate the AgCl precipitation which could be collected by filtration.

Thiol Esters from Steroid 17β-Carboxylic Acids: Carboxylate Activation and Internal Participation by 17α-Acylates[1]

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Received September 24, 1985

The chemistry of the steroid 17β-carboxylic acids derived from 16,17α-disubstituted corticosteroids was investigated with respect to thiol ester formation. Major quantities of 17-spiro byproducts were observed in the reactions of 16-methyl-17α-acyloxy acids, and the degree of 17-ester participation leading to these structures was dependent on the carboxylate activating group used and stereochemistry at C-16. Diethyl phosphate mixed anhydrides of these acids reacted with mercaptide salts to give mixtures of thiol esters with 17-spiro acylthio ortho esters, which predominated and were particularly stable in the case of 16β-methyl substrates; in addition, considerable reversion of 16α-methyl phosphate intermediates to starting acid was experienced. The use of diphenyl chlorophosphate as the activating agent greatly improved yields of thiol esters. Methanolysis of the phosphate adducts derived from 17α-acyloxy acids gave 17-spiro acyl ortho esters as the exclusive products. The reactions of 17α-acetoxy acids with 2-fluoro-*N*-methylpyridinium tosylate (FMPT) gave the novel 17-spiro acyl fluoro ketals **32–35**, whereas similar treatment of 17-hydroxy acids led to products of dehydration or of 18-methyl migration, including the novel 13,17-β-lactones **39** and **41**. Activation with carbonyldiimidazole followed by addition of mercaptans allowed the preparation of thiol ester products from 17-hydroxy acids, but the method was restricted to use with these substrates. Neighboring-group participation was not possible for the 16,17-acetonide acid **10**, and activation with either chlorophosphate diesters or FMPT followed by reaction with methanethiolate gave high yields of methylthio ester **17**.

Introduction

Dermatologically useful antiinflammatory activity in corticosteroids is compatible with a variety of modifications to the normal 20-keto-21-hydroxy side chain, as exemplified by the 21-deoxy, 21-deoxy-21-chloro, and steroid 17β-carboxylate ester classes of topical agents.[2] In the course of our investigations of structure–activity relationships in this area, we required the synthesis of thiol esters of steroid 17β-carboxylic (etianic) acids bearing either the 16α,17α-acetonide functionality or a 16-methyl in combination with a 17α-acyloxy or -hydroxy group.[3] Although the thiol esters of 16-unsubstituted-17-desoxyetianic acids were known,[4,5] there was no precedent for synthesis of the analogous 16,17α-disubstituted derivatives. The system of interest is particularly hindered, the 17β-carboxyl being flanked by a quaternary carbon (C-13), a tertiary carbon (C-16), and a 17α-oxy substituent. These features had a profound influence on the chemistry of activation and further reactivity of the 17β-carboxylate, particularly through participation by the neighboring 17α-acyloxy and -hydroxy groups, and led to the isolation of a novel series of 17-spiro dioxolanones and 18-methyl migration products. The elaboration of these findings is reported here.

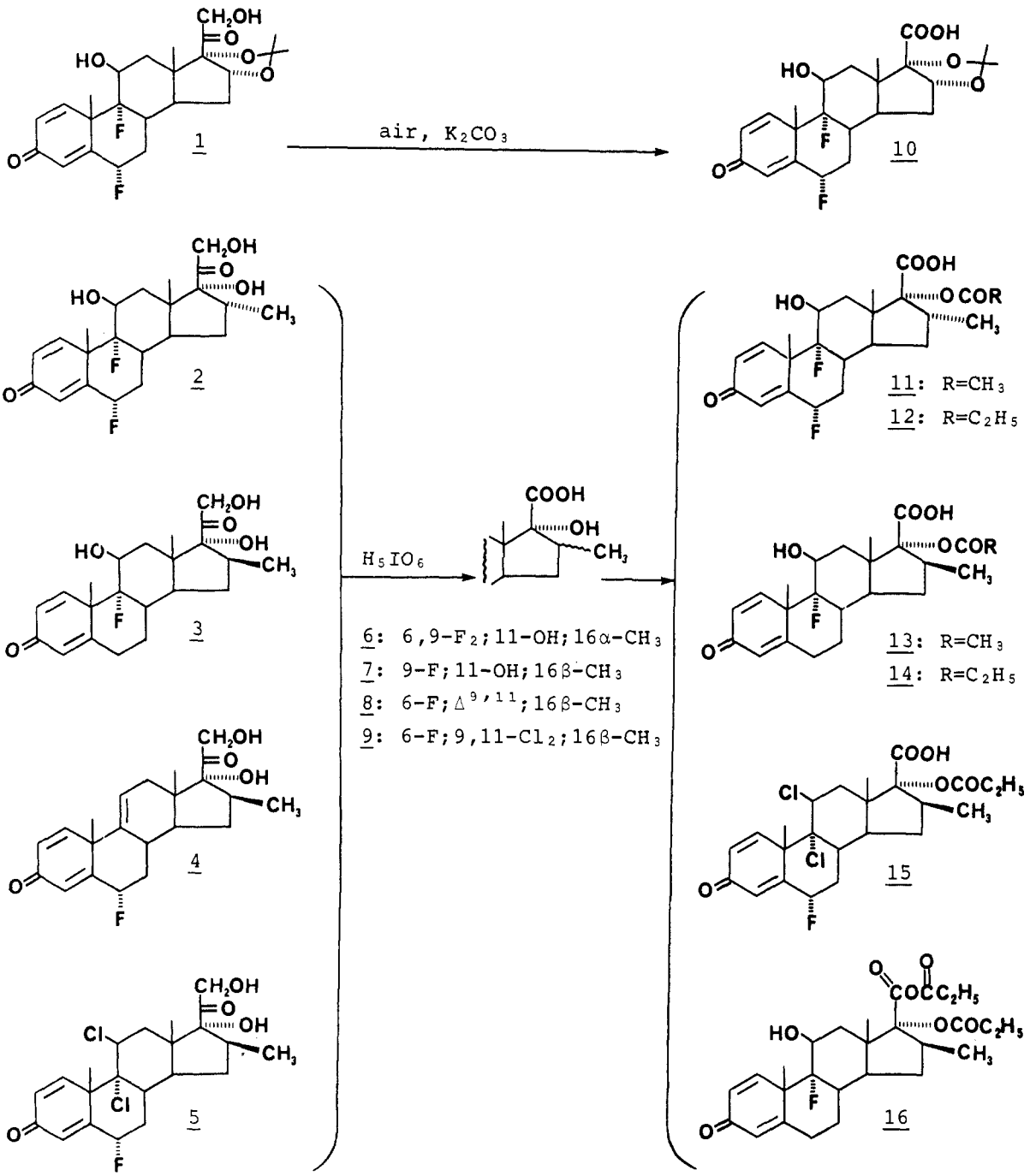
Carboxylic acid starting materials for the desired thiol esters were prepared from fluocinolone acetonide **1**, flumethasone **2**, betamethasone **3**, the $\Delta^{9,11}$-16β-methyl corticoid **4**, and the 9α,11β-dichloride **5** by oxidative cleavage of the corticoid side chain, usually followed by esterification of free 17α-alcohol groups (Scheme I). Thus, oxidation of the 17α-hydroxy substrates **2–5** with periodic acid[6] in aqueous methanol gave high yields of etianic acids **6–9**. The 16,17-acetonide **1** (and corticoid 17-esters) did not react under these conditions, showing an unexpected limitation to the use of periodic acid. A potassium carbonate catalyzed air oxidation was therefore developed based on reports by Velluz[7] and Herzig,[8] permitting cleavage of **1** to the 16,17-acetonide acid **10** in 75% yield. The 17α-hydroxyetianic acids **6**, **7**, and **9** were transformed into the corresponding acetates and propionates **11–15** in a one-pot procedure similar to that of Phillips et al.,[9,10] consisting of reaction with excess acetyl or propionyl chloride and triethylamine followed by the addition of diisopropylamine to cleave the resulting 17-acyloxy carboxylic acid mixed anhydrides. The mixed anhydrides of 16β-methyl acids were particularly stable, as demonstrated by the isolation of the dipropionyloxy compound **16**[9] in 72% yield from **7** when the use of diisopropylamine was eliminated. The ease with which 17-esters could be prepared from etianic acid substrates suggests that the free acid group was involved in the process,[11] probably through the initial formation of a mixed anhydride and transfer of the acyl group from the 17β-carboxylate to the 17α-alcohol.

Activation with Chlorophosphate Reagents. Thiol esters of 16,17-acetonide acid **10** were successfully prepared by forming diethyl phosphate intermediates[12] and treatment in situ with sodium mercaptide salts. Thus, reaction of **10** with diethyl chlorophosphate and triethylamine in THF followed by filtration of the amine hydrochloride salt

(1) Contribution No. 704 from the Institute of Organic Chemistry.
(2) (a) Wolff, M. E. In *Burger's Medicinal Chemistry*, 4th ed., Part III; Wolff, M. E., Ed.; Wiley-Interscience: New York, 1981; pp 1310–1311. (b) Phillips, G. H. In *Mechanisms of Topical Corticosteroid Activity*; Wilson, C.; Marks, R., Ed.; Churchill Livingstone: New York, 1976; pp 1–18.
(3) Edwards, J. A. U.S. Patent 4 188 835, 1980.
(4) Jeger, O.; Norymberski, J.; Szpilfogel, S.; Prelog, V. *Helv. Chim. Acta* **1946**, *29*, 684.
(5) Phillips, G. H.; Marshall, D. R. U.S. Patent 3 989 686, 1980.
(6) Reichstein, T.; Meystre, Ch.; von Euw, J. *Helv. Chim. Acta* **1939**, *22*, 1107.
(7) Velluz L.; Petit, A.; Pesez, M.; Berret, R. *Bull. Soc. Chim. Fr.* **1947**, 123; *Chem. Abstr.* **1947**, *41*, 5537.
(8) Herzig, P. Th.; Ehrenstein, M. *J. Org. Chem.* **1951**, *16*, 1050.
(9) Phillips, G. H.; Bain, B. M. U.S. Patent 4 093 721, 1978.
(10) Phillips, G. H.; May, J. M. U.S. Patent 3 828 080, 1974; *Chem. Abstr.* **1975**, *82*, 31453x.
(11) By contrast, the esterification of free 17-alcohols in etianic acid thiol esters required catalysis with DMAP at elevated temperatures, as in the preparations of **48** and **49** from **47** (vide infra). If an 11β-alcohol was present, it was also esterified under these conditions. Selective base hydrolysis of the 11-esters of the resulting 11,17-diesters was readily achieved with 16α-methyl substrates, but no distinction was possible in the presence of a 16β-methyl group: Alvarez, F.; Kertesz, D., unpublished results.
(12) (a) Masamune, S.; Kamata, S.; DiaKur, J.; Sugihara, Y.; Bates, G. S. *Can. J. Chem.* **1975**, *53*, 3693. (b) For a review of thiol ester forming methods, see: Haslam, E. *Tetrahedron* **1980**, *36*, 2409.

0022-3263/86/1951-2315$01.50/0

Scheme I. Preparation of Etianic Acids



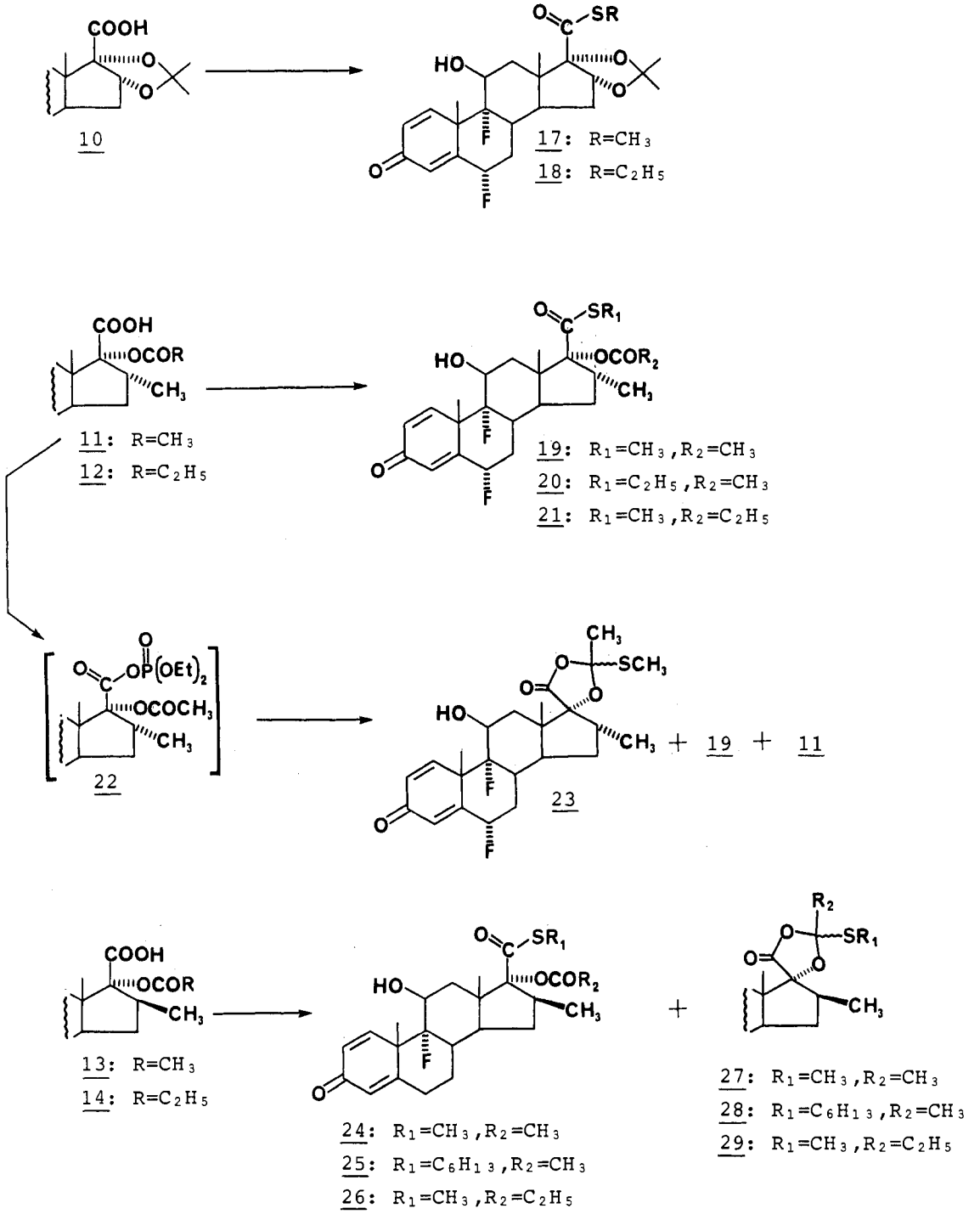
and addition of excess sodium methylthiolate in DMF gave 72% of methylthio ester **17** and 18% of unreacted **10**, whereas the use of sodium ethanethiolate gave 65% of **18** (Scheme II). However, application of this method to 16α-methyl-17α-acyloxy substrates gave uniformly low yields of the desired thiol esters accompanied by large quantities of recovered starting acids. For example, acetate **11** gave methylthio ester **19** (13%) and ethylthio ester **20** (26%) accompanied in each case by 46–48% of starting acid, while 17-propionate **12** afforded 33% of methylthio ester **21** and 25% of returned **12**.[13]

The reactions of etianic acids with chlorophosphate reagents produced single intermediates which could be observed by thin-layer chromatography (TLC), and when the intermediate formed from **11** (assumed to be phosphate **22**) was titrated to its disappearance with $NaSCH_3$, 12% of a new less polar material was formed in conjunction with 9% of thiol ester **19** and 34% of returned acid **11**. The new product showed a single absorption at 1790 cm^{-1} in the infrared in place of the 17-acylate and thiol ester peaks, and its identification as the novel 17-spiro acylthio orthoacetate **23** was supported by further spectral and analytical data. Although **23** was readily isomerized to **19** with excess thiolate and could be hydrolyzed to **11** with either acid or base, the isolated material was stable under neutral conditions. The formation of **23** by preferential attack of thiolate at the 17-acyloxy carbonyl has analogy in the chemistry of α-acetoxyisobutyryl chloride studied by Mattocks[14] and others,[15] and this form of neighboring-

(13) These results were representative of a series of reactions employing mercaptides as large as *n*-hexylthio in combination with 17-acylates as large as caproate.

(14) Mattocks, A. R. *J. Chem. Soc.* **1964**, 1918.

Scheme II. Thiol Esters via Phosphoric Mixed Anhydrides



group participation was noted with increasing frequency in further work.

When we applied the diethyl phosphate method of thiol ester formation to 17-acyloxy substrates in the 16β-methyl series, we found 17-spiro thio ortho esters to be the major products; the desired thiol esters were produced in low yields and amounts of recovered starting acids were insignificant. Moreover, chromatographic separation of the normal thiol ester and 17-spiro products derived from lower alkyl thiolates was difficult or impossible. Thus, treatment of the phosphate intermediate derived from 17-acetate **13** with $NaSC_6H_{13}$ gave 43% of spiro hexylthio orthoacetate **28** and 13% of the readily separable thiol ester **25**, whereas the reaction of similarly activated 17-propionate **14** with a stoichiometric amount of $NaSCH_3$ yielded 64% of an unresolvable mixture judged by IR and NMR[16] to be 3:1 of orthopropionate **29** with ester **26**. The

(15) (a) Greenberg, S.; Moffatt, J. G. *J. Am. Chem. Soc.* **1973**, *95*, 4016. (b) Alkoxydioxolanones (cyclic acyl ortho esters) are formed in the reactions of α-acetoxyisobutyryl chloride with alcohols. Treatment of this reagent with ethanethiol gave a mixture absorbing at 1800 cm^{-1} in the infrared, implying the formation of an alkylthiodioxolanone similar to **23**, but the product could not be isolated: Rüchardt, Ch.; Brinkmann, H. *Chem. Ber.* **1975**, *108*, 3224.

(16) In the absence of adequate guidance from TLC, comparison of the intensity of the 1790 cm^{-1} peak with that of the 17-ester carbonyl at 1730 cm^{-1} was useful for judging the composition of ortho ester–thiol ester product mixtures. A large downfield shift of the 18-methyl resonance (0.30–0.35 ppm from that of the thiol ester) was the most dramatic feature of cyclic ortho ester NMR spectra, and other differences were used to calculate proportions in isomeric mixtures: see the Experimental Section.

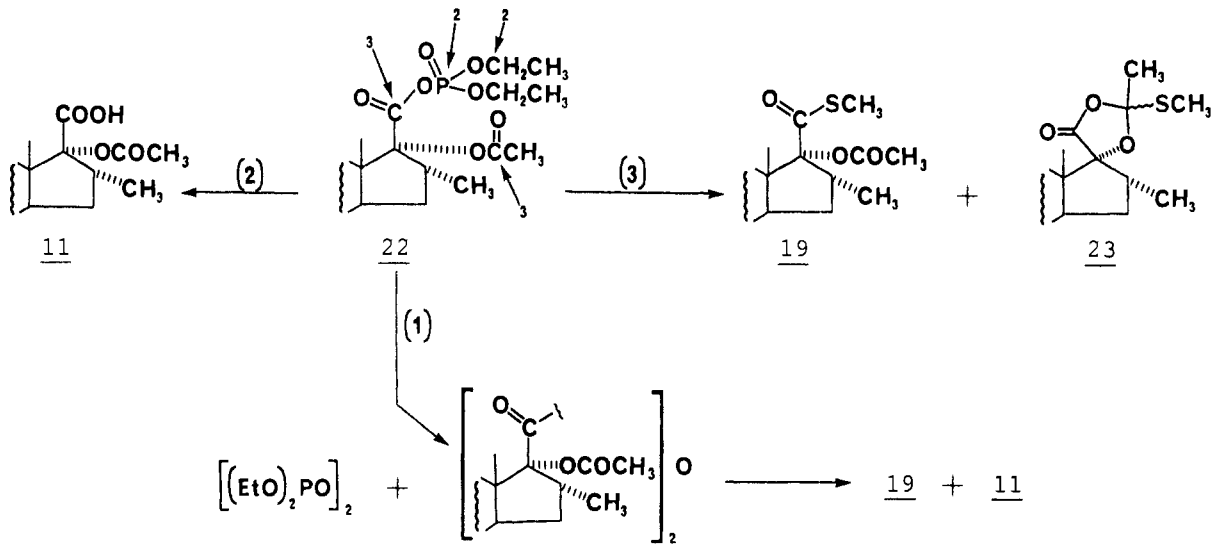


Figure 1.

conversion of ortho ester **29** to thiol ester **26** was completed by warming with an excess of $NaSCH_3$, as followed by disappearance of the 1790 cm^{-1} infrared absorption. Reaction of the diethyl phosphate adduct of acetate **13** with one equivalent of $NaSCH_3$ at 0 °C gave an estimated 9:1 mixture of cyclic orthoacetate **27** with thiol ester **24**, and a sample of pure **27** was obtained by painstaking chromatographic separation. The tendency of the 16β-methyl group to enhance the formation of 17-spiro thio ortho esters and increase their stability toward excess thiolate was a major feature of the chemistry of this series.[17]

Substitution of diphenyl chlorophosphate for the diethyl reagent markedly altered the course of subsequent reactions with thiolates; notably, reversion of intermediates derived from 16α-methyl acids to starting material did not occur and ortho ester formation was greatly reduced for 16β-methyl substrates, leading to increased yields of thiol esters in all cases. Thus, treatment of 16α-methyl propionate **12** with diphenyl chlorophosphate and TEA in dry THF for 1 h at 55 °C gave a single TLC visible intermediate, and subsequent addition of $NaSCH_3$ afforded an 89% isolated yield of **21**. Similarly, the reaction of the diphenyl phosphate adduct of 16β-methyl-17-propionate **14** with an equivalent of $NaSCH_3$ gave a mixture of more than 10:1 of thiol ester **26** with ortho ester **29**, and was readily driven on with excess thiolate and heat to give a 64% yield of pure **26**. Acetate **13** likewise gave 74% of **24**. The use of diphenyl chlorophosphate for activation of **10** gave a 75% of acetonide thiol ester **17**.

The thiol ester forming reactions of 16α-methyl acids activated with diethyl chlorophosphate were noteworthy for the considerable reversion to starting material experienced, and an attempt was made to relate this behavior to the nature of the actual intermediate. The similar quantities of thiol ester and returned acid obtained from reactions of thiolate with these diethyl phosphates could be rationalized in several ways (Figure 1). One possibility is that the true intermediates are symmetric anhydrides, resulting from a reported tendency of diethyl phosphate mixed anhydrides to "disproportionate"[12a] (path 1). Reversion of the mixed phosphate anhydride to carboxylic acid might also result from a partitioning of the intermediate by attack of thiolate on phosphorus[18] or the phosphate ethoxy α-carbons[21] (path 2) in preference to the 17β-carboxylate and 17α-acyloxy carbonyl carbons (path 3). Either the absence of alkoxy groups or a lower susceptibility of phosphorus to thiolate attack could then explain the superiority of diphenyl phosphate for thiol ester synthesis in this series. It was also considered possible that displacement of phosphate by chloride might occur during the activation procedure, with the resulting acid chlorides being the true intermediates in some of the phosphate-mediated chemistry. The possibility of acid chloride intermediates was discounted by investigation of the salt precipitates filtered from the intermediate-forming reactions, since formation of the presumed mixed anhydrides from **11** and **13** using either diethyl or diphenyl chlorophosphate was accompanied in each case by the virtual quantitative recovery (relative to carboxylic acid) of chloride ion as pure triethylammonium chloride. We found that acid chlorides could actually be formed and used without isolation,[24] and the reactions of **11** and **14** with thionyl chloride and TEA for 10 min at 0 °C followed by addition of $NaSCH_3$ thereby afforded 32 and 34% yields of respective methylthio esters **19** and **26**. Only small amounts of starting acids were recovered, and there were no detectable 17-spiro thio ortho ester products from these reactions.

An attempt was made to isolate and characterize a representative set of phosphate intermediates. Thus, reaction mixtures containing the 16α-methyl-17-acetoxy acid **11** and the 16β-methyl analogue **13** in combination with both diethyl and diphenyl chlorophosphate reagents were prepared as usual and subjected to aqueous workup pro-

(17) Facilitated access of thiolate to the 17α-acyloxy carbonyl and shielding of the resulting dioxolanone product carbonyl by the 16β,18-dimethyl system probably both contribute to this finding.

(18) Nucleophilic attack on phosphorus was suggested by Liu et al. to explain low yields of esters from the use of chlorophosphate diesters as activating agents (see ref 19). Low yields were also reported for thiol ester preparations using these reagents (see ref 20), but no difference between diethyl and diphenyl phosphate cases was noted in the cited work.

(19) Liu, H. J.; Chan, W. H.; Lee, S. P. *Tetrahedron Lett.* **1978**, 4461.

(20) Liu, H. J.; Sabesan, S. I. *Can. J. Chem.* **1980**, *58*, 2645.

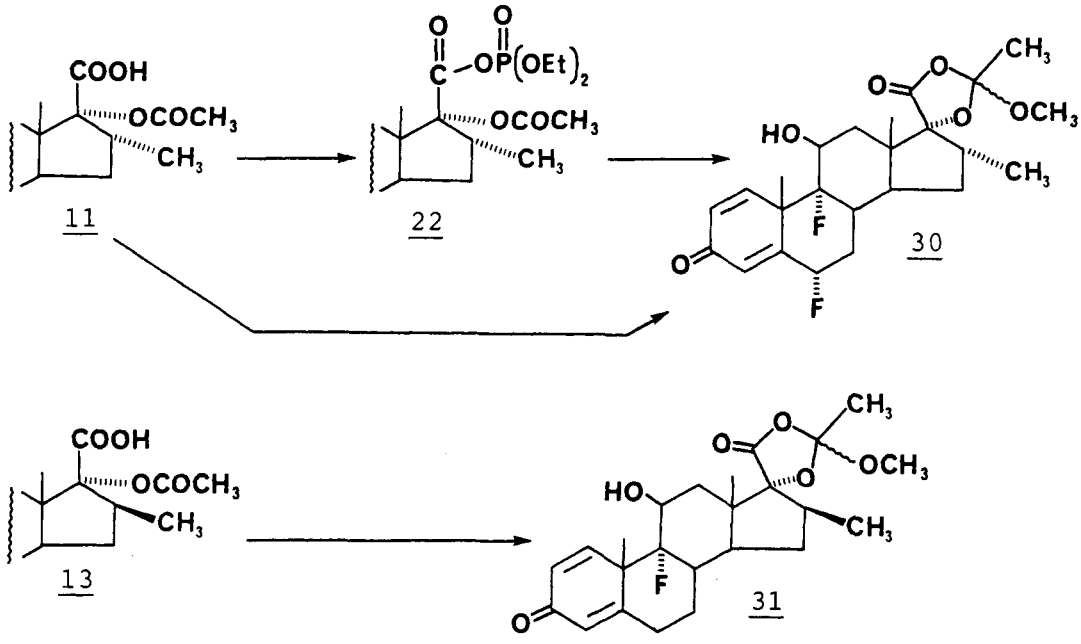
(21) Monodealkylation of the diethyl phosphate intermediate by sodium mercaptide (see ref 22) would give an anhydride salt, which could lead to free carboxylic acid by loss of metaphosphate or hydrolysis in the workup (see ref 23).

(22) Savignac, P.; Lavielle, G. *Bull. Chim. Soc. Fr.* **1974**, 1506.

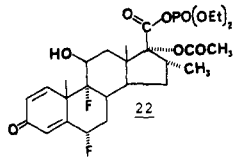
(23) Moon, M. W.; Khorana, H. G. *J. Am. Chem. Soc.* **1966**, *88*, 1798.

(24) The reactions of **11** and **14** with thionyl chloride gave TLC visible intermediates with very similar R_f's to those noted in reactions with diphenyl chlorophosphate, which at first suggested that acid chlorides might play a role in this chemistry.

Scheme III. Methanolysis of Phosphate-Activated 17α-Acetoxy Acids



cedures. Only the 16α-methyl diethyl chlorophosphate product **22** proved stable enough to be isolated in this



manner, with starting acid alone being isolated from the other reaction mixtures. Intermediate **22** was isolated in 84% yield; it survived purification by chromatography, and its identity was the desired diethyl phosphate mixed anhydride was confirmed by spectral and analytical data. To our surprise in a series of TLC experiments with mercaptide salts, the isolated phosphate **22** gave erratic yields of less than 10% of substitution products with $NaSCH_3$, these being roughly equal amounts of thiol ester **19** and spiro ortho ester **23**, and virtually no such products were obtained with $NaSC_2H_5$. In both cases the free acid **11** was the single major product. The inconsistent behavior of **22** toward thiolate before and after isolation is puzzling, but it is clear that reversion to free acid is a characteristic of the diethyl phosphate species itself, and is not a result of "disproportionation".

Although phosphate-activated etianic acids were found to be unreactive toward free mercaptans,[25] the solvolysis of these species with methanol proceeded with ease to give high yields of 17-spiro products. The products were those of 17-ester participation exclusively; no normal methyl ester products were detected. Thus, the 17-spiro acyl orthoacetate **30** was isolated in 71% yield from a solution of isolated phosphate **22** in methanol after heating at reflux for 1 h (Scheme III). Isolation of phosphate intermediates was not necessary to produce methoxydioxolanones, and the exchange of methanol for the THF in freshly filtered reaction mixtures containing either diethyl or diphenyl phosphate anhydrides and warming gave excellent yields of 17-spiro products in all cases. The 16α- and 16β-methyl orthoacetates **30** and **31** were prepared in this manner from **11** and **13** via the diethyl phosphates in 85% and 76% yields, respectively. Diethyl phosphates gave somewhat better yields than the diphenyl intermediates, and acid chloride preparations gave the same methanolysis products, but in poorer yields than phosphates.

The thiol esters of base-sensitive substrates and of free 17-hydroxy acids were inaccessable from phosphate intermediates. Thus, an attempted preparation of thiol ester **49** from **15** failed due to sensitivity of the 9,11-dichloride system to the required thiolate, and reactions of 17-hydroxy acids **6** and **7** with chlorophosphate reagents gave only polar materials presumed to be 17-phosphate esters.

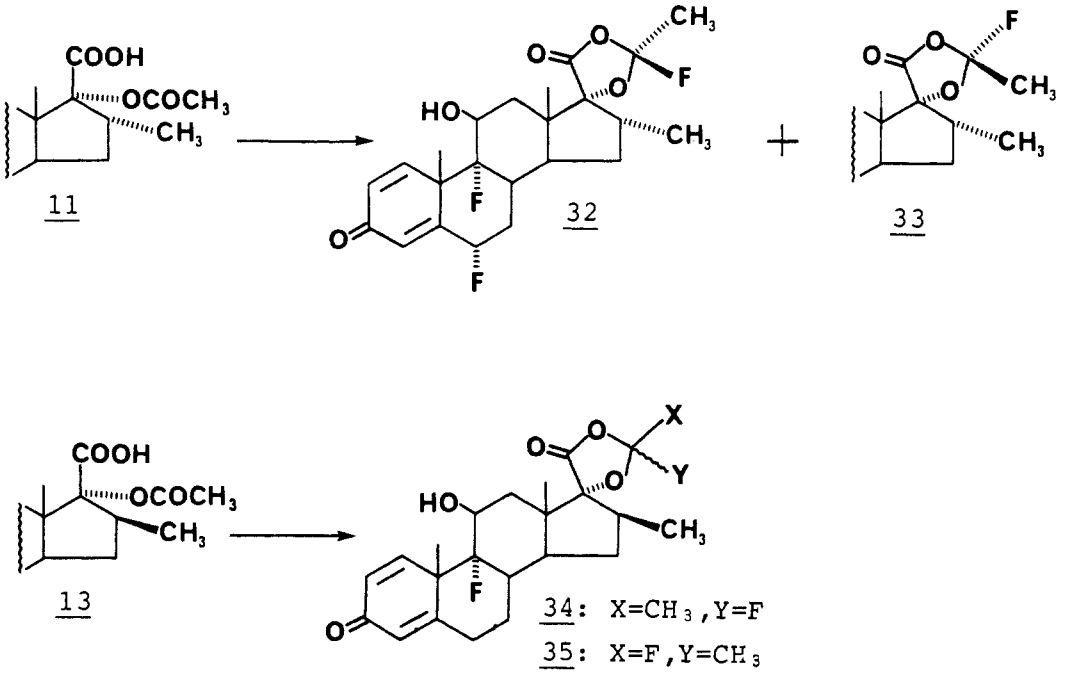
Reactions with 2-Fluoro-*N*-methylpyridinium Tosylate. In view of the limitations of chlorophosphate reagents, the use of 2-fluoro-*N*-methylpyridinium tosylate[26] (FMPT) for etianic acid activation was investigated. However, FMPT-derived intermediates did not react with free mercaptans, and neighboring group participation again dominated the chemistry. Thus, although the reaction of acetonide acid **10** with freshly prepared FMPT and TEA at −15 °C followed by addition of $NaSCH_3$ afforded a 92% yield of thiol ester **17**, the chemistry of substrates bearing 17α-acyloxy or -hydroxy groups was complex. Treatment of the 16α-methyl-17-acetoxyetianic acid **11** with FMPT and TEA gave complete conversion to a mixture of two compounds of similar R_f that were totally unreactive toward a large excess of $NaSCH_3$, even after 16 h at 110 °C. The products, isolated in a total yield of 64% by preparative TLC, were identified as the separate epimers **32** (34%) and **33** (30%) of a 17-spiro acylfluoro ketal (Scheme IV). Both cyclic ketals absorbed at 1805 cm^{-1} in the infrared and are related in structure and presumably in mechanism of formation to the previously noted acylorthoesters, but the complete incorporation of fluoride ion in this manner[27] and the stability of the resulting fluoroketal structures were quite unexpected. Proton NMR spectroscopy allowed the assignment of stereochemistry between the two fluoroketals, since the *S* epimer **32** showed splitting of the 16α-methyl by an adjacent fluorine,

(25) Carboxylate intermediates formed with phenyl dichlorophosphate are reported to be especially reactive toward free thiols (see ref 20), but the use of this reagent offered no advantage in the present work.

(26) (a) For a review, see: Mukaiyama, T. *Angew. Chem.* **1979**, *18*, 707. (b) For use of FMPT in thioester synthesis, see: Watanabe, Y.; Shoda, S.; Mukaiyama, T. *Chem. Lett.* **1976**, 741.

(27) The participation of fluoride ion in the absence of other nucleophiles is the basis of a method for the synthesis of acyl fluorides using FMPT: Mukaiyama, T.; Tanaka, T. *Chem. Lett.* **1976**, 303.

Scheme IV. Reactions of 17α-Acetoxy Acids with FMPT



whereas the *R* epimer **33** did not.[28] The 16β-methyl-17-acetoxy acid **13** was similarly converted into epimeric fluoroketals **34** and **35**, but the relative stereochemistry of this pair could not be assigned by NMR, presumably since the 16β-methyl was too far from the ketal fluorine to show a coupling.

The standard FMPT/$NaSCH_3$ conditions were applied to 16β-methyl-17-hydroxy acid **9** in the hope that the lower reaction temperature would spare the 9,11-dichloride system. The dichloride system did indeed survive, but in this case the major product isolated in 34% yield was that of dehydration, the Δ^{16}-carboxylic acid **36** (Scheme V). The 9,11-fluorohydrin 16α-methyl acid **6** behaved quite differently when used in the same reaction sequence. In this case, the reaction mixture yielded 30% of normal thiol ester **37**, 18% of the 18-nor-$\Delta^{13,17}$-17-methyl rearrangement product **38**, and 12% of an unstable compound to which the 13,17-lactone-17β-methyl structure **39** has been assigned. This labile material absorbed at 1810 cm^{-1} in the infrared, and was transformed quantitatively into **38** with apparent loss of carbon dioxide while drying at 100 °C. The properties of **39** are compatible with the assigned structure as well as its possible role as an intermediate in the formation of olefin **38**. Mercaptide anion was not essential to the formation of these rearrangement products, and reaction of **6** with FMPT and TEA alone gave **38** (16%) and **39** (11%) along with some starting acid (7%) as the only isolable products. The 17-methyl olefin substrate exemplified by **38** is familiar among products of the Miescher–Kägi rearrangement[29] of androstane 17-alcohols and tosylates,[30] and the formation of β-lactone **39** appears related to a known rearrangement of steroid 17-spiro oxetanones.[31,32]

Stereochemistry at C-16 was suspected of influencing the choice of reaction path between products of 18-methyl migration and 16,17-olefin formation. Therefore, two additional 16β-methyletianic acids were subjected to the FMPT/TEA reaction conditions. The 9,11-fluorohydrin **7** gave 48% of 17-methyl-$\Delta^{13,17}$-olefin **40** and 8% of the labile β-lactone **41**, in results which parallel those for 16α-methyl fluorohydrin **6**. Addition of $NaSCH_3$ to the reaction mixture did not change yields or give thiol ester products. The $\Delta^{9,11}$-acid **8** behaved much the same as 9,11-dichloride **9** and gave 31% of dehydration product acid **42**. A 15% yield of corresponding thiol ester **43** accompanied **42** when $NaSCH_3$ was added to the reaction mixture, whereas the Δ^{16}-acyl fluoride **44** was isolated[27] in place of **43** when thiolate was not used. It was not possible to detect any overlap of product types in the reported reactions: each 17-hydroxyetianic acid appeared to give either 18-methyl migration or Δ^{16}-products exclusively.

Thus, the difference in reactions of 17-hydroxy acids with FMPT was associated with the 9,11-substitution pattern rather than C-16 stereochemistry. Both the 16α and the 16β-methyl fluorohydrins **6** and **7** gave 18-methyl migration products, whereas dichloride **5** and 9,11-olefin **4** experienced 17-alcohol dehydration.[33] The influence of the C-ring substitution pattern on the choice of reaction path was most likely due to its effect on overall molecular conformation,[36] probably in determining the nature and strain energy of the actual intermediate species. The

(28) The use of long range coupling effects to determine the stereochemistry of fluoride substituents is precedented by studies of the interaction between the steroid 6β-fluorine and the 19-angular methyl group: Bhacca, N. S.; Williams, K. H. *Applications of NMR Spectroscopy in Organic Chemistry*; Holden-Day: San Francisco, 1964; pp 123–134, and references therein.

(29) (a) Kägi, H.; Miescher, K. *Helv. Chim. Acta* **1939**, *22*, 683. (b) Miescher, K.; Kägi, H. *Ibid* **1949**, *32*, 761.

(30) For reviews, see: (a) Wendler, N. L. In *Molecular Rearrangements Part 2*; de Mayo, P., Ed.; Wiley-Interscience: New York, 1964; pp 1020–1026. (b) Kirk, D. N.; Hartshorn, M. P. *Steroid Reaction Mechanisms*; Elsevier: New York, 1968; pp 269–272.

(31) (a) Herz, J. E.; Fried, J.; Grabowich, P.; Sabo, E. F. *J. Am. Chem. Soc.* **1956**, *78*, 4812. (b) Hirschmann, R.; Bailey, G. A.; Poos, G. I.; Walker, R.; Chemerda, J. M. *Ibid* **1956**, *78*, 4814.

(32) Rearrangement of 17,21-oxetanones to the isomeric 17β-methyl-13α,17α-keto oxides (see ref 31) is an example of 18-methyl migration with participation of a neighboring oxygen functionality (see ref 30a; also ref 30b, p 369).

(33) A similar divergence in reactivity reflecting C-ring substituent effects has been observed in acid-catalyzed reactions of 17α-hydroxy steroid 3,20-disemicarbazones, wherein 11-ketones gave Δ^{16}-products (see ref 34) and 11-alcohols underwent 18-methyl migration (see ref 35).

(34) Slates, H. L.; Wendler, N. L. *J. Org. Chem.* **1957**, *22*, 498.

(35) Taub, D.; Hoffsommer, R. D.; Wendler, N. L. *J. Org. Chem.* **1964**, *29*, 3486.

(36) The potential of C-ring substitution to markedly alter the preferred conformation of the steroid side chain was illustrated in a recent X-ray crystallographic study of a series of pregnanes: Duax, W. L.; Griffin, J. F.; Rohrer, D. C. *J. Am. Chem. Soc.* **1981**, *103*, 6705.

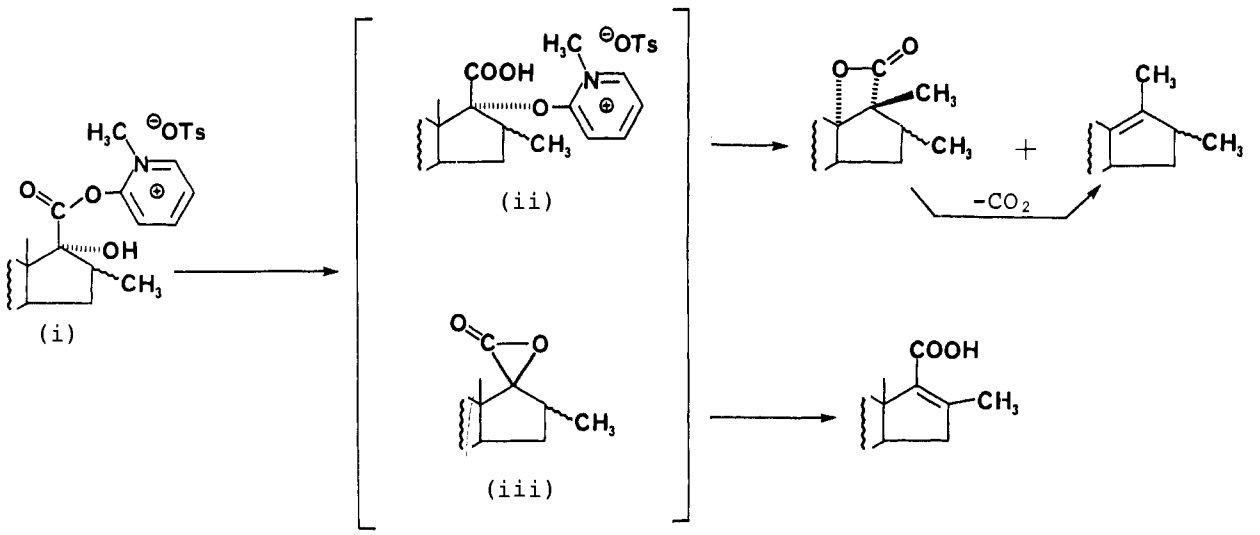
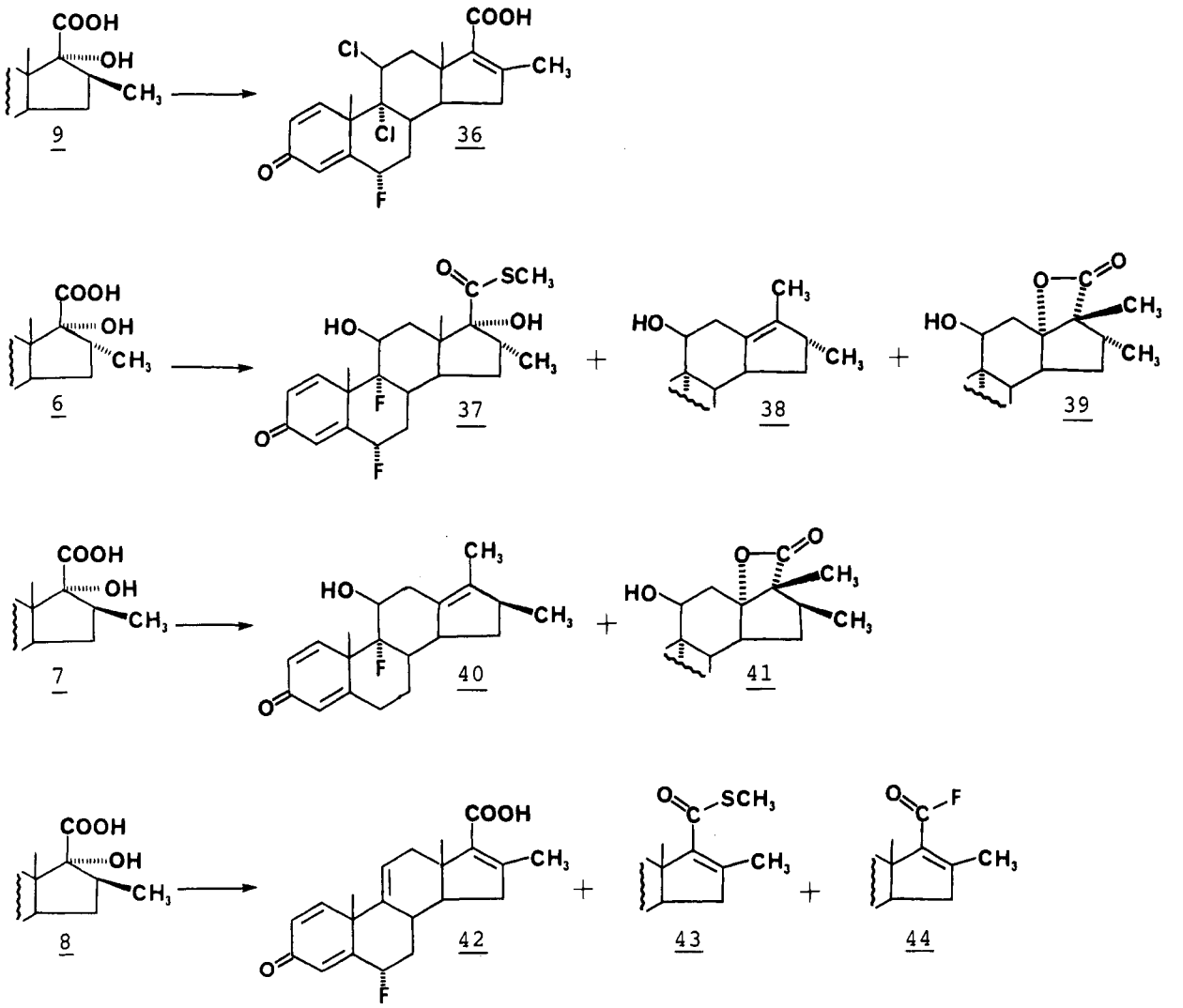


Figure 2.

Scheme V. Reactions of 17α-OH Acids with FMPT and $NaSCH_3$



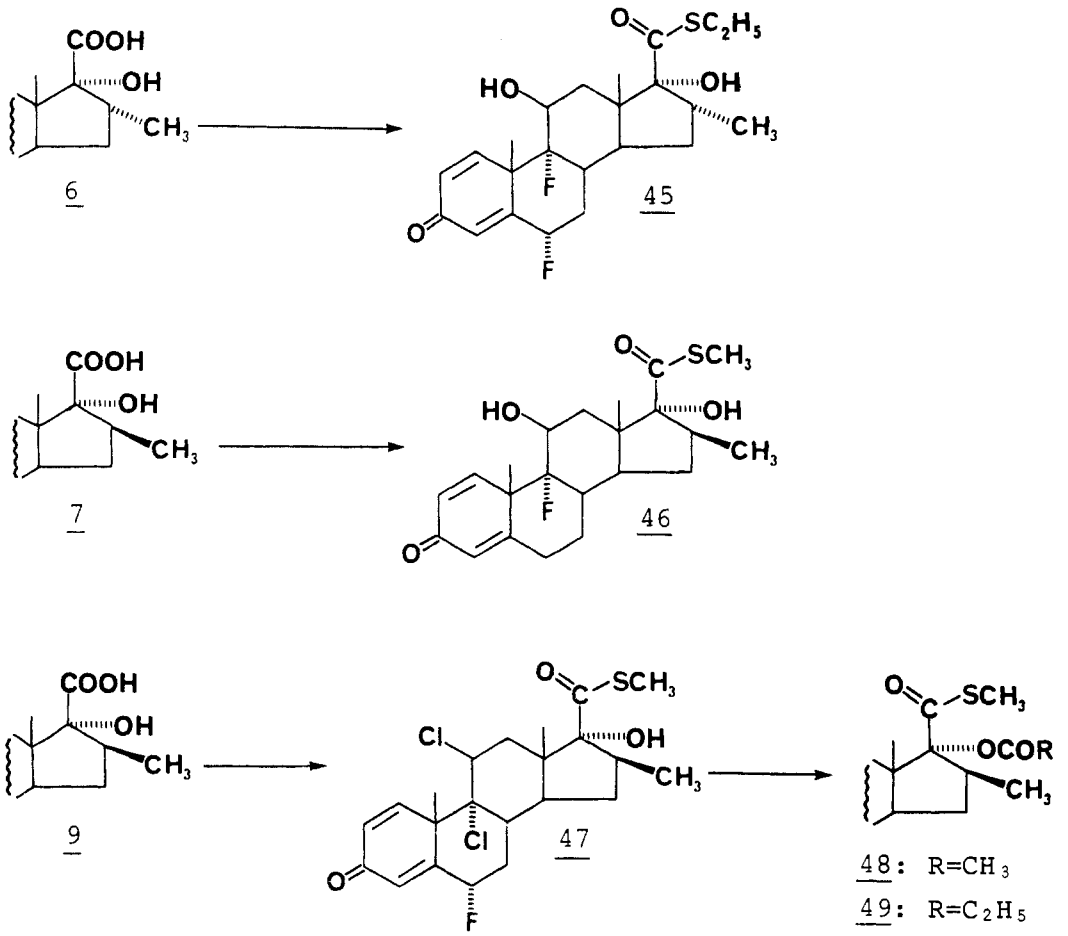
tendency of the resulting intermediate to rearrange would then depend on the degree of transcoplanarity between the 18-methyl and 17-leaving groups attained therein.[30] Both 17-oxo adduct (ii) and α-lactone[37] (iii) are potentially accessible from base-catalyzed transformations of initial pyridinium adduct (i), and either could serve as a transient intermediate in the subsequent reactions (Figure 2).

Activation with Carbonyldiimidazole. The use of carbonyldiimidazole[38] (CDI) as an activating agent pro-

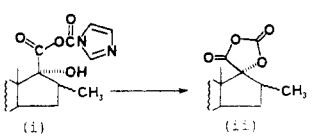
(37) Chapman, O. L.; Wotjkowski, P. W.; Adam, W.; Rodriguez, O.; Rucktäschel, R. *J. Am. Chem. Soc.* **1972**, *94*, 1365, and references therein.

(38) Gais, H. J. *Angew. Chem.* **1977**, *16*, 244.

Scheme VI. Thiol Esters of 17α-OH Acids via CDI



vided a solution to problems of thiol ester formation in the presence of both base-sensitive C-ring substituents and free 17-hydroxy groups. The intermediates formed in reactions of CDI with free 17-hydroxy acids comprised the only species encountered in the present work that was reactive toward free mercaptans. Thus, treatment of 16α-methyl-17-hydroxy acid **6** with CDI followed by addition of ethanethiol gave ethylthio ester **45** (40%), and 16β-methyl methylthio ester **46** (61%) was obtained by bubbling methyl mercaptan through the reaction mixture of CDI with 17-hydroxy acid **7** in DMF (Scheme VI). Syntheses of 9,11-dichloro-17-acetate methylthio ester **48** and propionate **49** were then accomplished by a similar preparation of 17-hydroxy thiol ester **47** from **9**, followed by dimethylaminopyridine (DMAP) catalyzed esterifications of the hindered 17-alcohol in mixtures of acetic or propionic anhydride with TEA at 80 °C. No reaction was observed when the 17-acetoxy acids **11** and **13** and the 16,17-acetonide **10** were treated with CDI followed by either mercaptan or mercaptide anion, presumably because reactive intermediates were not formed. The ability of CDI to activate 17-hydroxy acids towards thiol ester formation is remarkable in contrast to the failures with 17-derivatized substrates, and could result from steric factors alone or in combination with actual participation by the free 17-alcohol. In view of the ease with which 17-spirocyclic derivatives of the 17-hydroxy acids are formed, it seems reasonable to propose that the initial adduct (i) could give



the cyclic carbonate (ii) as the actual intermediate in these cases,[39] instead of an acylimidazolide.[38]

Many of the etianic acid thiol esters prepared in this work have proven to be extremely potent topical antiinflammatory agents, as measured in the rat[40] and in human vasoconstrictor[41] assays. A noteworthy separation between topical and systemic corticoid activity was also achieved, since most of the compounds were relatively inactive in the rat thymus involution and carrageenan-paw assays.[42] Thus, the 16α-methyl-17-propionyloxy methylthio ester **21** possessed topical activity in the order of fluocinolone acetonide (**1**) and the 16β-methyl 17-acetate **24** was approximately 1.3 times as potent, whereas both compounds demonstrated systemic activities of less than 5 times hydrocortisone.[45] A high ratio of topical to systemic activity such as that displayed by thiol esters **21** and **24** is of potential therapeutic value.[2b] All examples of 17-spiro acyl ortho esters and fluoro ketals were virtually inactive in both types of assay.

Experimental Section

General Methods. Melting points were obtained by using a Fisher-Johns apparatus and are uncorrected. Analytical TLC was

(39) We thank Dr. Arthur Kluge for this suggestion.
(40) Tonelli, G.; Thibault, L.; Ringler, I. *Endocrinology* **1965**, *77*, 625.
(41) Place, V. A.; Velasquez, J. G.; Burdick, K. H. *Arch. Dermatol.* **1970**, *101*, 531.
(42) Potencies were determined in intact rats using a combination of a two-day thymolytic assay (see ref 43) and a paw edema test (see ref 44).
(43) Dorfman, R. I.; Dorman, A. S.; Agnello, E. J.; Figdor, S. K.; Lauback, G. D. *Acta Endocrinol. (Copenhagen)* **1961**, *37*, 343.
(44) Winter, C. A.; Risley, E. A.; Nass, G. W. *Proc. Soc. Exp. Biol. Med.* **1962**, *111*, 544.
(45) The topical activity of hydrocortisone is approximately 0.001 times that of fluocinolone acetonide **1** when measured in our vasoconstrictor assays (see ref 41).

performed with Analtech 2.5 cm × 10 cm × 0.25 mm silica GF plates, and preparative TLC was done on Analtech silica GF 20 cm × 40 cm × 1 mm plates or with a Harrison Chromatotron centrifugal chromatography apparatus using 1 mm and 2 mm thick silica PF plates. Infrared spectra were recorded with a Perkin-Elmer Model 137 spectrophotometer, and frequencies are quoted to the nearest 5 cm^{-1}. Ultraviolet spectra were taken in methanol on a Cary 14 spectrophotometer. Proton NMR spectra were determined on Varian HA-100 (100 MHz), Bruker WH-90 (90 MHz), or Bruker WM-300 (300 MHz) instruments as solutions in hexadeuteriodimethyl sulfoxide (Me_2SO) or deuteriochloroform ($CDCl_3$) containing tetramethylsilane as an internal reference, and carbon-13 spectra were recorded on the Bruker WM-300 (75.5 MHz). Electron impact mass spectral data were obtained with Finnegan MAT CH-7 and 122-S direct inlet instruments at 70 eV, chemical ionization spectra were measured on the MAT 122-S at 90 eV, and high-resolution mass spectrometric measurements were made on a Finnigan MAT-311A. Elemental analyses were performed by the Syntex Analytical Services Group. All reactions were performed in heat-dried glassware under an atmosphere of nitrogen, unless otherwise noted, and sensitive reagents were introduced through septa by syringe.

Materials. Fluocinolone acetonide (**1**, 6α,9α-difluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide), flumethasone (**2**, 6α,9α-difluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione), and betamethasone (**3**, 9α-fluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione) are commercially available. The $\Delta^{9,11}$-corticoid **4** was kindly supplied by the Upjohn Company in the form of the 17,21-diacetate (17,21-dihydroxy-6α-fluoro-16β-methylpregna-1,4,9(11)-triene-3,20-dione 17,21-diacetate).[46] Chlorophosphate reagents, CDI, and DMAP were purchased from Aldrich; FMPT (prepared from 2-fluoropyridine,[27] Aldrich) is now commercially available. Tetrahydrofuran (THF) was purified by distillation from Na benzophenone. Standard solutions of $NaSCH_3$ (usually 0.83 N) were prepared by bubbling methyl mercaptan through a slurry of NaH (50% in oil, usually 2.0 g) in DMF (50 mL) until all solids dissolved, and solutions of other mercaptide salts were made similarly from liquid mercaptans and NaH in DMF or THF.

17,21-Dihydroxy-6α-fluoro-16β-methylpregna-1,4,9(11)-triene-3,20-dione (4). To a solution of 17,21-dihydroxy-6α-fluoro-16β-methylpregna-1,4,9(11)-triene-3,20-dione 17,21-diacetate[46] (1 g, 2.18 mmol) in MeOH (40 mL) was added K_2CO_3 (0.5 g) in water (5 mL), and the mixture was stirred at 20 °C for 1 h. The solution was acidified with 6 N HCl and diluted with ice water, after which the precipitate was filtered, washed, and dried to give 745 mg (91%) of crystalline **4**, mp 198–200 °C. A sample was recrystallized from acetone–hexane for analysis: mp 202–204 °C; UV 238 nm (ε 16400); IR (KBr) 1720 (20-C=O), 1665 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.73 (s, 3 H, 18-CH_3), 1.03 (d, 2 H, J = 6.4 Hz, 16-CH_3), 1.37 (s, 3 H, 19-CH_3), 4.18 (d, 1 H, J = 19.5 Hz, 21-H), 4.38 (d, 1 H, J = 19.5 Hz, 21-H), 5.60 (d, 1 H, J = 4.6 Hz, 11-H), 5.71 (br dm, 1 H, J = 45 Hz, 6-H), 6.07 (s, 1 H, 4-H), 6.20 (dd, 1 H, J_1 = 10 Hz, J_2 = 2 Hz, 2-H), 7.39 (d, 1 H, J = 10 Hz, 1-H); MS, m/e 374 (M^+). Anal. Calcd for $C_{22}H_{27}O_4F$: C, 70.57; H, 7.27. Found: C, 70.56; H, 7.17.

9α,11β-Dichloro-17α,21-dihydroxy-6α-fluoro-16β-methylpregna-1,4-diene-3,20-dione (5). A solution of 17,21-dihydroxy-6α-fluoro-16β-methylpregna-1,4,9(11)-triene-3,20-dione 17,21-diacetate[46] (1 g, 2.18 mmol) in a mixture of CCl_4 (20 mL) and pyridine[47] (3 mL) was purged with nitrogen for 5 min, then treated with a gentle stream of chlorine for 15 min, and flushed with nitrogen again for 5 min. The resulting mixture was diluted with EtOAc, washed with dilute HCl, water, and brine, dried (Na_2SO_4), and evaporated to dryness. The residue was recrystallized twice from acetone–hexane, giving 840 mg of the intermediate 9,11-dichloro-17,21-diacetate, which was then slurried in a mixture of MeOH (40 mL) and a solution of K_2CO_3 (300 mg) in water (4 mL) for 30 min. Ice water (30 mL) was added and the resulting precipitate was filtered, washed, and dried to give 590 mg (61%) of crystalline **5**: mp 215–217 °C; UV 236 nm (ε 15000); IR (KBr) 1710 (20-C=O), 1670 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 1.07 (s, 3 H, 18-CH_3), 1.08 (d, 3 H, J = 6 Hz, 16-CH_3), 1.71 (s, 3 H, 19-CH_3), 4.15 (d, 1 H, J = 19 Hz, 21-H), 4.39 (d, 1 H, J = 19 Hz, 21-H), 4.96 (br m, 1 H, 11-H), 5.58 (br dm, J = 48 Hz, 6-H), 6.10 (s, 1 H, 4-H), 6.30 (dd, 1 H, J_1 = 10 Hz, J_2 = 2 Hz, 2-H), 7.24 (d, 1 H, J = 11 Hz, 1-H); MS, m/e 426, 428 ($M^+ - H_2O$). Anal. Calcd for $C_{22}H_{27}O_4Cl_2F$: C, 59.33; H, 6.11. Found: C, 59.32; H, 5.98.

Oxidations with Periodic Acid. 6α,9α-Difluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (6). To a slurry of (**2**) (2 g, 5.11 mmol) in MeOH (40 mL) was added a solution of periodic acid (1.7 g, 7.46 mmol) in water (40 mL), and the mixture was stirred open to the air at 20 °C for 16 h. The volume was reduced to 60 mL by evaporation, ice water (80 mL) was added, and the precipitate was filtered, washed, and dried, giving 1.88 g (94.5%) of crystalline **6**, mp 285–289 °C. A sample was recrystallized from acetone–hexane for analysis:[48] mp 303–304 °C dec; UV 238 nm (ε 16300); IR (KBr) 1700 (COOH), 1660 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.87 (d, 3 H, J = 9 Hz, 16-CH_3), 1.00 (s, 3 H, 18-CH_3), 1.49 (s, 3 H, 19-CH_3), 4.15 (br d, 1 H, J = 10 Hz, 11-H), 5.64 (br dm, 1 H, J = 48 Hz, 6 H), 6.10 (s, 1 H, 4-H), 6.27 (dd, 1 H, J_1 = 10 Hz, J_2 = 2 Hz, 2-H), 7.26 (d, 1 H, J = 9.4 Hz, 1-H); MS, m/e 396 (M^+). Anal. Calcd for $C_{21}H_{26}O_5F_2$: C, 63.63; H, 6.61. Found: C, 63.69; H, 6.72.

17-Hydroxyetianic acids **7–9** were prepared by the same procedure.

11β,17α-Dihydroxy-9α-fluoro-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid (7) was prepared from **3** in 97% yield: mp 247–251 °C. A sample was purified by dissolving in dilute Na_2CO_3, washing with EtOAc, acidifying the aqueous phase with dilute HCl, and cooling to ice. The precipitate was collected, washed, dried, and recrystallized from EtOAc:[50] mp 254–256 °C (lit.[10] mp 256–258 °C); UV 239 nm (ε 21450); IR (KBr) 1750, 1720 (COOH), 1665 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 1.07 (s, 3 H, 18-CH_3), 1.14 (d, 3 H, J = 9 Hz, 16-CH_3), 1.50 (s, 3 H, 19-CH_3), 4.14 (br d, 1 H, J = 12 Hz, 11-H), 6.01 (s, 1 H, 4-H), 6.21 (dd, 1 H, J_1 = 10 Hz, J_2 = 2 Hz, 2-H), 7.29 (d, 1 H, J = 10 Hz, 1-H); MS, m/e 378 (M^+). Anal. Calcd for $C_{21}H_{27}O_5F$: C, 66.65; H, 7.19. Found: C, 66.40; H, 7.47.

6α-Fluoro-17α-hydroxy-16β-methyl-3-oxoandrosta-1,4,9(11)-triene-17β-carboxylic acid (8) was prepared from **4** in 92% yield: mp 200–201 °C dec. Analytical sample from acetone–hexane: mp 197–199 °C dec; UV 238 nm (ε 16200); IR (KBr) 1740, 1710 (COOH), 1655 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.86 (s, 3 H, 18-CH_3), 1.13 (d, 3 H, J = 6.6 Hz, 16-CH_3), 1.38 (s, 3 H, 19-CH_3), 5.61 (d, 1 H, J = 5.2 Hz, 11-H), 5.72 (br dm, 1 H, J = 48 Hz, 6-H), 6.07 (s, 1 H, 4-H), 6.20 (dd, 1 H, J_1 = 10 Hz, J_2 = 1.4 Hz, 2-H), 7.40 (d, 1 H, J = 10 Hz, 1-H); MS, m/e 360 (M^+). Anal. Calcd for $C_{21}H_{25}O_4F$: C, 69.98; H, 6.99. Found: C, 69.99; H, 7.24.

9α,11β-Dichloro-6β-fluoro-17α-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid (9) was prepared from **5** in 91% yield: mp 221–223 °C dec; UV 236 nm (ε 15200); IR (KBr) 1750, 1710 (COOH), 1665 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 1.14 (s, 3 H, 18-CH_3), 1.17 (d, 3 H, J = 8 Hz, 16-CH_3), 1.69 (s, 3 H, 19-CH_3), 4.96 (br m, 1 H, 11-H); MS, m/e 430, 432 (M^+). Anal. Calcd for $C_{21}H_{25}O_4Cl_2F$: C, 58.48; H, 5.84. Found: C, 58.59; H, 5.78.

Base-Catalyzed Air Oxidation. 6α,9α-Difluoro-11β,16α,17α-trihydroxy-3-oxoandrosta-1,4-diene-17β-carboxylic Acid 16,17-Acetonide (10). A stream of air was bubbled under the surface of a slurry of **1** (0.5 g, 1.10 mmol) in MeOH (25 mL) containing K_2CO_3 (0.5 g) for 10 min, after which the air flow was stopped and the mixture was stirred open to the air for another 16 h at 20 °C. The volume was reduced to 10 mL by evaporation, after which EtOAc (80 mL) was added and the mixture was extracted with water. The aqueous layer was acidified

(46) For preparation, see: Ayer, D. E.; Schlagel, C. A.; Flynn, G. L. *Ger. Patent* 2308731, 1973; *Chem. Abstr.* **1973**, *79*, 146739a.
(47) For a prior use of pyridine as a solvent for chlorination of steroids, see: Cutler, F. A., Jr.; Mandell, L.; Shew, D.; Chemerda. J. M. *J. Org. Chem.* **1959**, *24*, 1621.
(48) Full characterization was performed because the literature melting point of 333 °C (see ref 49) was unattainable.
(49) Anner, G.; Meystre, Ch., U.S. Patent 3636010, 1972.
(50) This compound has been reported, but spectral data were incomplete: cf. ref 10.

with dilute HCl and reextracted with EtOAc, the resulting organic layer being washed with water and brine, dried (Na_2SO_4), and evaporated to dryness. Recrystallization from acetone–hexane gave 370 mg (75%) of **10**: mp 301–303 °C dec; UV 237 nm (ε 15900); IR (KBr) 1725 (COOH), 1665 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 0.93 (s, 3 H, 18-CH_3), 1.14 and 1.29 (2 s, 6 H, $C(CH_3)_2$), 1.48 (s, 3 H, 19-CH_3), 4.15 (br d, 1 H, J = 10 Hz, 11-H), 4.96 (m, 1 H, 6-H); MS, m/e 438 (M^+). Anal. Calcd for $C_{23}H_{28}F_2O_5$: C, 63.00; H, 6.44. Found: C, 63.13; H, 6.24.

17α-Acetoxy-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (11). Acetyl chloride (2.5 mL) was added to an ice-cooled solution of **6** (1.5 g, 3.78 mmol) in CH_2Cl_2 (50 mL) and TEA (7.5 mL), and the mixture was stirred at 0 °C for 30 min, after which TLC (1% HOAc–35% acetone–hexane) indicated that starting material had been replaced by a mixture of **11** with a less polar material assumed to be the mixed anhydride of acetic acid with **11**. Diisopropylamine (7.5 mL) was added and the mixture was stirred for 2 h at 20 °C and then evaporated to dryness. The residue was dissolved in EtOAc and the solution washed with dilute HCl, water, and brine, dried (Na_2SO_4), and evaporated. The residue was triturated with hot CH_2Cl_2, affording 1.15 g (69%) of **11**. The analytical sample was recrystallized 4 times from acetone–hexane: mp 180–182 °C dec; UV 238 nm (ε 15800); IR (KBr) 1735 (br, COOH, ester), 1665 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.86 (d, 3 H, J = 7 Hz, 16-CH_3), 1.02 (s, 3 H, 18-CH_3), 1.49 (s, 3 H, 19-CH_3), 2.01 (s, 3 H, $COCH_3$); MS, m/e 418 (M^+ – HF). Anal. Calcd for $C_{23}H_{28}O_6F_2$: C, 63.00; H, 6.44. Found: C, 62.87; H, 6.28.

6α,9α-Difluoro-11β-hydroxy-17α-(propionyloxy)-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid (12) was prepared from **6** by using propionyl chloride in the above procedure. Recrystallization from acetone–hexane gave 94% of **12**, mp 217–218 °C; UV 238 nm (ε 16825); IR (KBr) 1730 (ester), 1700 (COOH), 1665 (3-C=O) cm^{-1}; ^1H NMR (100 MHz Me_2SO) δ 0.83 (d, 3 H, J = 7 Hz, 16-CH_3), 0.98 (t, 3 H, J = 7 Hz, CH_2CH_3), 0.99 (s, 3 H, 18-CH_3), 1.47 (s, 3 H, 19-CH_3), 2.26 (q, 2 H, J = 7 Hz, $COCH_2$); MS, m/e 350 (M^+ – COOH, COC_2H_5). Anal. Calcd for $C_{24}H_{30}O_6F_2$: C, 63.71; H, 6.68. Found: C, 63.74; H, 6.73.

17α-Acetoxy-9α-fluoro-11β-hydroxy-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (13). Acetyl chloride (6 mL) was added to a solution of **7** (4.60 g, 12.2 mmol) in CH_2Cl_2 (200 mL) and TEA (4 mL), and the mixture was stirred at 0 °C for 30 min. Diisopropylamine (25 mL) was added and the solution was heated at reflux for 1 h, after which the procedure was completed as described for **11**. Crystallization from acetone–hexane gave 4.76 g (93%) of **13**, mp 196–198 °C. Analytical sample from acetone–hexane:[50] mp 218–220 °C (lit.[10] 212–214 °C); UV 239 nm (ε 17900); IR (KBr) 1735 (br, COOH, ester), 1655 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.98 (s, 3 H, 18-CH_3), 1.31 (d, 3 H, J = 7 Hz, 16-CH_3), 1.50 (s, 3 H, 19-CH_3), 1.94 (s, 3 H, $COCH_3$); MS, m/e 361 (MH^+ – HOAc). Anal. Calcd for $C_{23}H_{29}O_6F$: C, 65.70; H, 6.95. Found: C, 65.73; H, 7.01.

9α-Fluoro-11β-hydroxy-17α-(propionyloxy)-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (14) and 9α-Fluoro-11β-hydroxy-17α-(propionyloxy)-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Propionic Anhydride (16). Propionate **14** was prepared in 84% yield from **7** by using propionyl chloride in the above procedure. The analytical sample was recrystallized three times from acetone–hexane:[50] mp 182–183 °C dec (lit.[10] mp 188–190 °C; UV 239 nm (ε 14000); IR (KBr) 1735 (br, COOH, ester), 1655 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 1.06 (s, 3 H, 18-CH_3), 1.06 (t, 3 H, J = 7 Hz, CH_2CH_3), 1.39 (d, 3 H, J = 7 Hz, 16-CH_3), 1.53 (s, 3 H, 19-CH_3), 2.25 (q, 2 H, J = 7 Hz, $COCH_2$); MS, m/e 435 (MH^+). Anal. Calcd for $C_{24}H_{31}O_6F$: C, 66.34; H, 7.19. Found: C, 66.15; H, 7.08. A second reaction using 75 mg (0.20 mmol) of **7** was worked up directly, without the diisopropylamine step, affording 80 mg (72%) of **16**, mp 170–173 °C. The analytical sample was recrystallized from acetone–hexane:[50] mp 177–178 °C dec (lit.[10] 180–182 °C); UV 239 nm (ε 16240); IR (KBr) 1815 (anhydride), 1730 (br, anhydride ester), 1660 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, $CDCl_3$) δ 1.10, 1.14 (2 t, 6 H, J's = 7 Hz, CH_2CH_3's), 1.11 (s, 3 H, 18-CH_3), 1.42 (d, 3 H, J = 7 Hz, 16-CH_3), 1.55 (s, 3 H, 19-CH_3), 2.30, 2.43 (2 q, 4-H, J's = 7 Hz, $COCH_2$'s); MS, m/e 470 (M^+ – HF). Anal. Calcd for $C_{22}H_{35}O_7F$: C, 66.11; H, 7.19. Found: C, 66.09; H, 7.16.

9α,11β-Dichloro-6α-fluoro-17α-(propionyloxy)-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid (15). Treatment of **9** (352 mg, 0.82 mmol) with propionyl chloride and TEA followed by diisopropylamine gave 229 mg (58%) of **15** (from acetone–hexane), mp 178–180 °C dec; UV 236 nm (ε 14400); IR (KBr) 1740 (ester), 1710 (COOH), 1665 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 1.00 (t, 3 H, J = 7.5 Hz, CH_2CH_3), 1.05 (s, 3 H, 18-CH_3), 1.35 (d, 3 H, J = 7 Hz, 16-CH_3), 1.72 (s, 3 H, 19-CH_3), 2.27 (q, 2 H, J = 7.5 Hz, $COCH_2$), 5.06 (d, 1 H, J = 2.5 Hz, 11-H); MS, m/e 487 (MH^+). Anal. Calcd for $C_{24}H_{29}O_5Cl_2$: C, 59.14; H, 6.00. Found: C, 59.11; H, 6.04.

General Methods for Synthesis of Thiol Esters from Acids 10–14. Preparation of 17β-[(Methylthio)carbonyl]-6α,9α-difluoro-11β,16α,17α-trihydroxyandrosta-1,4-dien-3-one 16,17-Acetonide (17). Method A. A slurry of **10** (351 mg, 0.80 mmol) in THF (10 mL) containing TEA (0.20 mL, 1.44 mm), was stirred at 20 °C for 30 min, during which time the acid dissolved and a precipitate assumed to be the triethylammonium salt was formed. Diethyl chlorophosphate (0.17 mL, 1.20 mmol) was added and stirring was continued, giving a new precipitate of triethylammonium chloride (Et_3N·HCl). After 2 h, TLC (3% MeOH–CH_2Cl_2) of a sample of reaction mixture worked up between EtOAc and dilute aqueous HCl showed that conversion of **11** to a neutral intermediate was complete. Filtration into a fresh flask gave 118 mg (0.85 mmol) of pure Et_3N·HCl (elemental analysis) which was discarded. An 0.83 N solution of $NaSCH_3$ in DMF (2.2 mL, 1.83 mmol) was added to the filtrate, and the mixture was stirred at 20 °C for 16 h, after which it was diluted with EtOAc and extracted with dilute aqueous Na_2CO_3. The organic layer was washed, dried (Na_2SO_4), and evaporated to dryness, giving the crude thiol ester as a very poorly soluble solid. Recrystallization from 1:2 MeOH/CH_2Cl_2 by boiling off the CH_2Cl_2 and cooling in ice gave 255 mg (68%) of pure **17**, mp >300 °C; UV 238 nm (ε 20415); IR (KBr) 1665 (br, 20-C=O, 3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 0.83 (s, 3 H, 18-CH_3), 1.13, 1.33 (2 s, 6 H, $C(CH_3)_2$), 1.46 (s, 3 H, 19-CH_3), 2.29 (s, 3 H, SCH_3); MS, m/e 453 (M^+ – CH_3). Anal. Calcd for $C_{24}H_{30}O_5FS$: C, 61.52; H, 6.45; S, 6.84. Found: C, 61.40; H, 6.56; S, 6.55. Acidification of the Na_2CO_3 extract, extraction with EtOAc, and recrystallization from acetone–hexane gave 64 mg (18%) of pure starting acid **10**.

Method B. Diphenyl chlorophosphate (116 μL, 0.5 mmol) was added to a solution of **10** (175 mg, 0.40 mmol) in THF (6 mL) and TEA (100 μL, 0.72 mmol), and the mixture was heated at 55 °C for 2 h, after which the TLC of a worked-up sample showed that formation of a neutral intermediate was complete. The mixture was cooled in ice, filtered to remove the Et_3N·HCl (52 mg, 0.38 mmol), and 0.83 N $NaSCH_3$/DMF (1.0 mL, 0.83 mm) was added. The mixture was stirred at 20 °C for 1 h and worked up as described in method A, affording (after recrystallization from MeOH–CH_2Cl_2) 141 mg (75%) of pure **17**, mp >300 °C.

Method C. To a slurry of FMPT (90 mg, 0.32 mmol) in CH_2Cl_2 (3 mL) at −15 °C was added a separately prepared solution of **10** (110 mg, 0.25 mmol) in a mixture of CH_2Cl_2 (3 mL) and TEA (58 μL, 0.42 mmol). The conversion of **10** to a nonpolar intermediate was complete in 20 min (TLC, 1% HOAc–40% EtOAc–hexane), after which a solution of $NaSCH_3$ (0.83 N in DMF, 1.0 mL, 0.83 mmol) was added and the mixture was maintained at −15 °C in a freezer for 16 h. Standard acid–base workup and recrystallization from MeOH then afforded 109 mg (93%) of pure **17** in two crops, mp >300 °C.

17β-[(Ethylthio)carbonyl]-6α,9α-difluoro-11β,16α,17α-trihydroxyandrosta-1,4-dien-3-one 16,17-Acetonide (18). Prepared from **10** (350 mg, 0.80 mmol) according to method A, using $NaSC_2H_5$ [from ethanethiol (0.20 mL) and NaH (50% in oil, 68 mg, 1.42 mmol) in THF (3 mL)]. Recrystallization from acetone–hexane gave 240 mg (65%) of **18** in two crops: mp >300 °C; UV 239 nm (ε 20145); IR (KBr) 1665 (br, 20-C=O, 3-C=O); ^1H NMR (100 MHz, Me_2SO) δ 0.85 (s, 3 H, 18-CH_3), 1.13, 1.32 (2 s, 6 H, $C(CH_3)_2$, 1.17 (t, 3 H, J = 7 Hz, CH_2CH_3), 2.83 (q, 2 H, J = 7, SCH_2); MS, m/e 467 (M^+ – CH_3). Anal. Calcd for $C_{25}H_{32}O_5F_2S$: C, 62.22; H, 6.68. Found: C, 62.11; H, 6.75.

17α-Acetoxy-6α,9α-difluoro-11β-hydroxy-17β-[(methylthio)carbonyl]-16α-methylandrosta-1,4-dien-3-one (19) and 6α,9α-Difluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid 17,20-(1′-Methyl-1′-(methylthio))methylene Ketal (23). A solution of the in-

termediate prepared from 11 (420 mg, 0.96 mmol) according to method A was treated with 0.83 N $NaSCH_3$/DMF (4 mL, 3.32 mmol) and stirred at 20 °C for 16 h. Workup gave 200 mg (48%, after recrystallization) of pure starting acid 11 and a neutral fraction which was recrystallized from acetone–hexane, affording 58 mg (13%) of thiol ester **19**. Analytical sample of **19** (from acetone–hexane): mp >300 °C; UV 238 nm (ε 20 100); IR (KBr) 1750 (ester), 1670 (20-C=O, 3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.91 (d, 3 H, J = 7 Hz, 16-CH_3), 0.97 (s, 3 H, 18-CH_3), 1.48 (s, 3 H, 19-CH_3), 2.01 (s, 3 H, $COCH_3$), 2.26 (s, 3 H, SCH_3); MS, m/e 469 (MH^+). Anal. Calcd for $C_{24}H_{30}O_5F_2S$: C, 61.52; H, 6.45. Found: C, 61.63; H, 6.38. The neutral mother liquor material consisted of a mixture of **19** with a slightly less polar material which was probably the spiro ortho ester **23**. In a separate experiment, a reaction mixture prepared from 11 (400 mg, 0.91 mmol), diethyl chlorophosphate (0.25 mL, 1.73 mmol) and TEA (0.20 mL, 1.43 mmol) in THF (10 mL) was treated with 0.4 mL increments of 0.83 N $NaSCH_3$/DMF at 15-min intervals until the TLC (3% MeOH–CH_2Cl_2, developed twice) of worked-up samples showed complete replacement of phosphate intermediate (R_f 0.34) by thiol ester **19** (R_f 0.47), ortho ester **23** (R_f 0.55), and acid 11 (R_f 0.0). A total of 2.8 mL (2.3 mmol) of $NaSCH_3$ solution was required. Acid–base workup gave 136 mg (34%) of acid 11, and crystallization of the neutral residue from acetone–hexane gave mixtures **19** and **23** consisting of a first crop (113 mg) enriched in **19** and a second crop (98 mg) enriched in **23**. The mixtures were separated by preparative TLC (30% EtOAc–hexane, eluted twice), and recrystallizations (acetone–hexane) then gave 40 mg (9%) of **19** and 52 mg (12%) of **23**: mp 201–203 °C; UV 237 nm (ε 16 670); IR ($CHCl_3$) 1790 (20-C=O), 1670 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.95 (d, 3 H, J = 7 Hz, 16-CH_3), 1.26 (s, 3 H, 18-CH_3), 1.50 (s, 3 H, 19-CH_3), 1.92 (s, 3 H, 1′-CH_3), 2.22 (s, 3 H, SCH_3); MS, m/e 469 (MH^+). Anal. Calcd for $C_{24}H_{30}O_5F_2S$: C, 61.52; H, 6.45. Found: C, 61.50; H. 6.37. Addition of a 5-fold excess of $NaSCH_3$ to a solution of **23** in THF gave complete conversion to **19** in 2 h at 20 °C.

Thiol ester **19** was also prepared as follows: thionyl chloride (40 μL, 0.56 mmol) was added to solution of 11 (70 mg, 0.16 mmol) in THF (5 mL) containing TEA (0.10 mL, 0.72 mmol) at 0 °C, and the mixture was filtered into a fresh flask after 10 min. Addition of 0.83 N $NaSCH_3$/DMF (0.8 mL, 0.66 mmol) to the filtrate and workup after 1 h at 20 °C gave 24 mg (32%) of **19**, mp >300 °C, and 11 mg (16%) of 11.

17α-Acetoxy-6α,9α-difluoro-17β-[(ethylthio)carbonyl]-11β-hydroxy-16α-methylandrosta-1,4-dien-3-one (20). Preparation from 11 (200 mg, 0.46 mmol) and $NaSC_2H_5$ according to method A gave 58 mg (26%) of thiol ester **20** and 92 mg (46%) of returned 11. The analytical sample was recrystallized twice from acetone–hexane: mp >300 °C; UV 238 nm (ε 19 595); IR (KBr) 1745 (ester), 1665 (br, 20-C=O, 3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 0.90 (d, 3 H, J = 7 Hz, 16-CH_3), 0.98 (s, 3 H, 18-CH_3), 1.16 (t, 3H, J = 7 Hz, CH_2CH_3), 1.46 (s, 3 H, 19-CH_3), 1.98 (s, 3 H, $COCH_3$), 2.84 (q, 2 H, J = 7 Hz, SCH_2); MS, m/e 421 ($M^+ - SC_2H_5$). Anal. Calcd for $C_{25}H_{32}O_5F_2S$: C, 62.23; H, 6.68. Found: C, 62.50; H, 6.63.

6α,9α-Difluoro-11β-hydroxy-17β-[(methylthio)carbonyl]-17α-(propionyloxy)-16α-methylandrosta-1,4-dien-3-one (21). The reaction of 12 (1.5 g, 3.32 mmol) with $NaSCH_3$ according to method A gave 376 mg (25%) of returned 12 and 525 mg (33%) of thiol ester 21 in two crops. Analytical sample from acetone–hexane: mp 285–287 °C dec; UV 238 nm (ε 19 565); IR (KBr) 1740 (ester), 1675 (20-C=O), 1665 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 0.89 (d, 3 H, J = 7 Hz, 16-CH_3), 0.93 (s, 3 H, 18-CH_3), 1.00 (t, 3 H, J = 7 Hz, CH_2CH_3), 1.46 (s, 3 H, 19-CH_3), 2.24 (s, 3 H, SCH_3), 2.29 (q, 2 H, J = 7 Hz, $COCH_2$); MS, m/e 482 (M^+). Anal. Calcd for $C_{25}H_{32}O_5F_2S$: C, 62.22; H, 6.68; S, 6.64. Found: C, 62.45; H, 6.82; S, 6.59. Method B proved superior for the preparation of 21. Thus, the reaction of 12 (200 mg, 0.44 mmol) with diphenyl chlorophosphate (168 μL, 0.81 mmol) and TEA at 55 °C followed by treatment with $NaSCH_3$/DMF (0.83 mmol) gave 189 mg (89%) of 21, mp 286–288 °C dec.

17α-Acetoxy-6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Diethyl Phosphoric Anhydride (22). Diethyl chlorophosphate (120 μL, 0.83 mmol) was added to a solution of 11 (219 mg, 0.50 mmol) in THF (6 mL) containing TEA (125 μL, 0.9 mmol) and the mixture was warmed at 50 °C. After 1 h, TLC (0.5% HAc–35% acetone–hexane) showed that all but a trace of 11 had been converted to the intermediate (R_f 0.32) seen in preparations of **19** by method A. The mixture was cooled, diluted with EtOAc, washed with dilute Na_2CO_3 and water, dried (Na_2SO_4), and evaporated to dryness. The residue was partially purified by dissolving in acetone (2 mL) containing a drop of TEA, adding hexane (20 mL), and decanting the supernatant. Vacuum drying of the precipitate gave 242 mg (84%) of **22** as an amorphous solid judged >95% pure by TLC. An analytical sample was purified by centrifugal TLC (0.1% TEA–20% acetone–CH_2Cl_2) and precipitation as before, affording a semi-crystalline solid, mp 100–110 °C dec; UV 238 nm (ε 16 100); IR ($CHCl_3$) 1780 (20-C=O), 1745 (ester), 1670 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.96 (d, 3 H, J = 7 Hz, 16-CH_3), 1.17 (s, 3 H, 18-CH_3), 1.36 (dt, 3 H, J_1 = 1 Hz, J_2 = 7 Hz, CH_2CH_3), 1.39 (dt, 3 H, J_1 = 1 Hz, J_2 = 7 Hz, CH_2CH_3), 1.54 (s, 3 H, 19-CH_3), 2.10 (s, 3 H, $COCH_3$), 4.21 (dq, 2 H, J_1 = 7 Hz, J_2 = 7 Hz, OCH_2), 4.35 (dq, 2 H, J_1 = 7 Hz, J_2 = 7 Hz, OCH_2); MS, m/e 575 (MH^+). Anal. Calcd for $C_{27}H_{37}O_9F_2P$: C, 56.44; H, 6.49. Found: C, 56.53; H, 6.57. Samples of **22** stored under argon at −20 °C remained 95–98% pure for 1–2 months, after which degradation (largely to 11) was observed.

17α-Acetoxy-9α-fluoro-11β-hydroxy-17β-[(methylthio)carbonyl]-16β-methylandrosta-1,4-dien-3-one (24) and 11β,17α-Dihydroxy-9α-fluoro-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid 17,20-(1′-Methyl-1′-(methylthio))methylene Ketal (27). The activation of **13** (168 mg, 0.40 mmol) by method B and reaction with $NaSCH_3$ (0.60 mmol) afforded (after recrystallization from acetone–hexane) 134 mg (74%) of **24**, mp 263–265 °C dec; UV 239 nm (ε 19 600); IR (KBr) 1745 (ester), 1700 (20-C=O), 1660 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 0.86 (s, 3 H, 18-CH_3), 1.28 (d, 3 H, J = 7 Hz, 16-CH_3), 1.49 (s, 3 H, 19-CH_3), 1.98 (s, 3 H, $COCH_3$), 2.14 (s, 3 H, SCH_3); MS, m/e 403 ($M^+ - SCH_3$). Anal. Calcd for $C_{24}H_{31}O_5FS$: C, 63.98; H, 6.94; S, 7.11. Found: C, 63.73; H, 7.07; S, 7.41. The reaction of **13** (210 mg, 0.50 mmol) with diethyl chlorophosphate (0.70 mmol) and $NaSCH_3$ (0.70 mmol) according to method A gave 180 mg (80%) of a mixture, shown by TLC (0.25% TEA–55% ether–cyclohexane, developed three times) to be approximately 9:1 of ortho ester **27** (R_f 0.38) with **24** (R_f 0.44). The tendency of **27** to isomerize to **24** on silica gel during chromatography was partially overcome by inclusion of TEA in the eluent. Thus, a sample of **27** was purified for analysis by centrifugal TLC (0.4% TEA–65% ether–cyclohexane, developed 3 times) and recrystallization from ether–pentane: mp 202–203 °C dec; UV (ε 16 000); IR (KBr) 1795 (20-C=O), 1665 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) 1.15 (d, 3 H, J = 7 Hz, 16-CH_3), 1.17 (s, 3 H, 18-CH_3), 1.51 (s, 3 H, 19-CH_3), 1.83 (s, 3 H, 1′-CH_3), 2.19 (s, 3 H, SCH_3); MS, m/e 451 (MH^+). Anal. Calcd for $C_{24}H_{31}O_5FS$: C, 63.98; H, 6.94. Found: C, 63.86; H, 7.03.

17α-Acetoxy-9α-fluoro-17β-[(n-hexylthio)carbonyl]-11β-hydroxy-16β-methylandrosta-1,4-dien-3-one (25) and 11β,17α-Dihydroxy-9α-fluoro-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid 17,20-(1′-Methyl-1′-(n-hexylthio))methylene Ketal (28). The reaction of **13** (220 mg, 0.5 mmol) with diethyl chlorophosphate and $NaSC_6H_{13}$ according to method A and separation of the product mixture by preparative TLC (4% acetone–CH_2Cl_2, developed twice) afforded 35 mg (13%) of thiol ester **25** (R_f 0.38), mp 168–172 °C, and 111 mg (43%) of ortho thio ester **28** (R_f 0.33). A sample of **25** was recrystallized from EtOAc–hexane for analysis: mp 176–178 °C; UV 240 nm (ε 19 025); IR (KBr) 1730 (ester), 1700 (20-C=O), 1660 (3-C=O) cm^{-1}; ^1H NMR (90 MHz, Me_2SO) δ 0.87 (br m, 6 H, 18-CH_3, CH_2CH_3), 1.28 (br m, 11 H, 16-CH_3, hexyl CH_2's), 1.49 (s, 3 H, 19-CH_3), 2.00 (s, 3 H, $COCH_3$); MS, m/e 403 ($M^+ - SC_6H_{13}$). Anal. Calcd for $C_{29}H_{41}O_5FS$: C, 66.89; H, 7.94. Found: C, 66.82; H, 8.07. The data for **28** (from acetone–hexane) were as follows: mp 129–131 °C; UV 237 nm (ε 16 310); IR (KBr) 1795 (20-C=O), 1675 (3-C=O) cm^{-1}, ^1H NMR (90 MHz, Me_2SO) δ 0.85 (br t, 3 H, J = 5 Hz, CH_2CH_3), 1.14 (d, 3 H, J = 7 Hz, 16-CH_3), 1.16 (s, 3 H, 18-CH_3), 1.50 (s, 3 H, 19-CH_3), 1.84 (s, 3 H, 1′-CH_3); MS, m/e 403 ($M^+ - SC_6H_{13}$). Anal. Calcd for $C_{29}H_{41}O_5FS$: C, 66.89; H, 7.93. Found: C, 67.01; H, 8.00.

9α-Fluoro-11β-hydroxy-17β-[(methylthio)carbonyl]-17α-(propionyloxy)-16β-methylandrosta-1,4-dien-3-one (26) and

the Isomeric 17,20-(1′-Ethyl-1′-methylthio)methylene Ketal (29). The activation of acid **14** (113 mg, 0.26 mmol) with diethyl chlorophosphate (1 mmol) and treatment with $NaSCH_3$ (2.5 mmol) according to method A gave 133 mg of a neutral residue which was purified by preparative TLC (2% CH_3OH–CH_2Cl_2, developed twice). The product was 77 mg (64%) of a chromatographically homogeneous solid, estimated to be a 3:1 mixture of ketal **29** with thiol ester **26** by comparing the integrals of the methylthio group ^1H NMR resonances (2.12 ppm for **29**, 2.17 ppm for **26**) and intensities of the major infrared absorptions (1790 for 17β-carbonyl of **29**, 1740 cm^{-1} for acetate of **26**). A sample of the mixture (64 mg, 0.14 mmol) in THF (8 mL) was treated with additional $NaSCH_3$/DMF (1.65 mmol), and the mixture was heated at 45 °C for 6 h. Acid–base workup and recrystallization from acetone–hexane then afforded 30 mg (30% overall yield) of pure **26**: mp 223–224 °C; UV 239 nm (ε 18890); IR (KBr) 1740 (ester), 1700 (20-C=O), 1665 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.88 (s, 3 H, 18-CH_3), 1.03 (t, 3 H, J = 7 Hz, CH_2CH_3), 1.31 (d, 3 H, J = 7 Hz, 16-CH_3), 1.50 (s, 3 H, 19-CH_3), 2.17 (s, 3 H, SCH_3), 2.34 (q, 2 H, J = 7 Hz, $COCH_2$); MS, m/e 464 (M^+). Anal. Calcd for $C_{25}H_{33}O_5FS$: C, 64.63; H, 7.16; S, 6.90. Found: C, 64.38; H, 7.34; S, 6.87. Thiol ester **26** was also prepared by reaction of **14** (5.7 g, 13.26 mmol) with diphenyl chlorophosphate (19.3 mmol) and $NaSCH_3$ (20.8 mmol) according to method B. Analysis of the reaction mixture after 1 h at 20 °C, indicated the product to be **26** containing 5–10% of **29**. Extra $NaSCH_3$ (5 mmol) was added and the mixture was heated at 60 °C for 2 h, after which recovery and recrystallization of the product afforded 3.9 g (64%) of pure **26**, mp 223–224 °C. A third sample of **26** (34%) was prepared via activation of 14 with thionyl chloride, using the procedure described for **19**.

6α,9α-Difluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid 17,20-(1′-Methyl-1′-methoxy)methylene Ketal (30). A solution of freshly prepared **22** (104 mg, 0.18 mmol) in MeOH (10 mL) was heated at reflux for 1 h, giving a 1:1 mixture of ketal isomers by TLC (R_f's 0.30, 0.37, 40% EtOAc–hexane). The solution was diluted with EtOAc, washed with dilute $NaHCO_3$ and water, dried (Na_2SO_4), and evaporated to dryness. Recrystallization from acetone–hexane afforded 58 mg (71%) of **30**, mp 200–202 °C dec; UV 238 nm (ε 16300); IR (KBr) 1795 (20-C=O), 1670 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.99, 1.04 (2 d, 3 H, J's = 7 Hz, 16-CH_3's); 1.30, 1.32 (2 s, 3 H, 18-CH_3's), 1.54 (s, 3 H, 19-CH_3), 1.71, 1.72 (2 s, 3 H, 1′-CH_3's), 3.39, 3.42 (2 s, 3 H, OCH_3's); MS, m/e 452 (M^+). Anal. Calcd for $C_{24}H_{30}O_6F_2$: C, 63.71; H, 6.68. Found: C, 63.89; H, 6.64. A second sample of **30** was prepared from **11** (175 mg, 0.40 mmol) by reaction with diethyl chlorophosphate (0.56 mmol) and TEA (0.72 mmol) in THF according to method A followed by filtration of the mixture into a flask containing MeOH (5 mL). The THF was evaporated and the solution was stirred at 20 °C for 16 h and at 50 °C for 1 h, after which isolation as above afforded 154 mg (85%) of **30**, mp 198–200 °C.

11β,17α-Dihydroxy-9α-fluoro-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid 17,20-(1′-Methyl-1′-methoxy)methylene Ketal (31). Following activation of acid **13** (168 mg, 0.40 mmol) with diethyl chlorophosphate (81 μL, 0.56 mmol) and TEA (100 μL, 0.72 mmol) and exchange of the THF solvent for MeOH as described above, the formation of **31** was complete in 16 h at 20 °C. The ketal isomers (R_f's 0.37) were not separable by TLC (40% EtOAc–hexane). Recovery and recrystallization of the product as described for **30** afforded 132 mg (76%) of **31**: mp 223–225 °C dec; UV 238 nm (ε 15400); IR (KBr) 1790, (20-C=O), 1665 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) 1.20, 1.22 (2 d, 3 H, J's = 7.5 Hz, 16-CH_3's), 1.27, 1.31 (2 s, 3 H, 18-CH_3's), 1.57 (s, 3 H, 19-CH_3), 1.65, 1.66 (2 s, 3 H, 1′-CH_3's), 3.31, 3.32 (2 s, 3 H, OCH_3's); MS, m/e 435 (MH^+). Anal. Calcd for $C_{24}H_{31}O_6F$: C, 66.34; H, 7.19. Found: C, 66.42; H, 7.34.

Reactions of Acids 6–9, 11, and 13 with FMPT: Method D. Preparations were carried out in CH_2Cl_2 containing TEA as described in method C, except that thiolate was not added. Reactions were worked up after 30 min to 2 h at −15 °C.

6α,9α-Difluoro-11β,17α-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid 17,20-(1′-Methyl-1′-fluoro)-(*S*)-methylene Ketal (32) and Isomeric (*R*)-Methyl Ketal (33). The treatment of **11** (219 mg, 0.50 mmol) with FMPT at −15 °C (method D) resulted in conversion to two less polar products (R_f's 0.40, 0.57, 1% HOAc–35% EtOAc–hexane). The reaction mixture was diluted with CH_2Cl_2 (50 mL), washed with dilute Na_2CO_3 and water, dried (Na_2SO_4), and evaporated to dryness, after which centrifugal TLC (45% EtOAc–hexane) and recrystallizations from acetone–hexane afforded 74 mg of ketal **32** (34%) and 66 mg of less polar isomer **33** (30%). The 13-Hz coupling of the 1′-methyl group in the ^1H NMR spectrum of each compound was consistent with the presence of a vicinal fluorine, and the additional 2-Hz coupling of the 16-CH_3 by a proximal fluorine was the basis for assignment of "*S*" stereochemistry to ketal **32**.[28] The data for **32** were as follows: mp 213–214 °C dec; UV 237 nm (ε 15770); IR (KBr) 1810 (20-C=O), 1665 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, $CDCl_3$) δ 1.03 (dd, 3 H, J_1 = 7 Hz, J_2 = 2 Hz, 16-CH_3), 1.30 (s, 3 H, 18-CH_3), 1.52 (s, 3 H, 19-CH_3), 1.82 (d, 3 H, J = 13, 1′-CH_3); MS, m/e 440 (M^+). Anal. Calcd for $C_{23}H_{27}O_5F_3$: C, 62.72; H, 6.18. Found: C, 62.67; H, 6.19. The data for **33** were as follows: mp 210–211 °C dec; UV 236 nm (ε 15570); IR (KBr) 1805 (20-C=O), 1670 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, $CDCl_3$) δ 0.95 (d, 3 H, J = 7 Hz, 16-CH_3), 1.31 (s, 3 H, 18-CH_3), 1.52 (s, 3 H, 19-CH_3), 1.90 (d, 3 H, J = 13, 1′-CH_3); MS, m/e 440 (M^+). Anal. Calcd for $C_{23}H_{27}O_5F_3$: C, 62.72; H, 6.18. Found: C, 62.53; H, 5.94. Attempts to convert fluoroketals **32** and **33** in a freshly prepared reaction mixture into thiol ester **19** failed. No reaction was observed following the addition of an excess of methanethiol in methylene chloride containing TEA, followed by addition of a 4-fold excess of 0.83 N $NaSCH_3$/DMF and warming at a reflux, followed by exchange of the solvent for toluene and heating at reflux for 16 h.

11β,17α-Dihydroxy-9α-fluoro-16β-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic Acid 17,20-(1′-Methyl-1′-fluoro)-methylene Ketal (34) and Isomeric Ketal (35). Treatment of **13** (210 mg, 0.50 mmol) with FMPT (method D) gave a 5:1 mixture of two neutral products (R_f's 0.42, 0.49, 40% EtOAc–hexane, developed twice). Workup and two recrystallizations of the crude product from acetone–hexane afforded 85 mg (40%) of less polar fluoroketal **34**, mp 185–186 °C dec; UV 238 nm (ε 15700); IR ($CHCl_3$) 1810 (20-C=O), 1665 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.19 (d, 3 H, J = 7.5 Hz, 16-CH_3), 1.26 (s, 3 H, 18-CH_3), 1.56 (s, 3 H, 19-CH_3), 1.80 (d, 3 H, J = 13 Hz, 1′-CH_3); MS, m/e 402 (M^+ − HF). Anal. Calcd for $C_{23}H_{28}O_5F_2$: C, 65.39; H, 6.68. Found: C, 65.53; H, 6.69. The mother liquor materials were recovered and purified by centrifugal TLC (0.2% TEA–40% EtOAc–hexane), and the separated products were recrystallized from acetone–hexane, which gave an additional 35 mg (17%) of pure **34** and 21 mg (10%) of more polar fluoro ketal **35**, mp 193–194 °C dec; UV 238 nm (ε 15300); IR ($CHCl_3$) 1810 (20-C=O), 1670 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.21 (d, 3 H, J = 7.5 Hz, 16-CH_3), 1.30 (s, 3 H, 18-CH_3), 1.56 (s, 3 H, 19-CH_3), 1.78 (d, 3 H, J = 13 Hz, 1′-CH_3); MS, m/e 402 (M^+ − HF). Anal. Calcd for $C_{23}H_{28}O_5F_2$: C, 65.39; H, 6.68. Found: C, 65.47; H, 6.40. Ketals **34** and **35** appeared more labile on silica gel than the 16α-methyl analogues, which necessitated the inclusion of TEA in the chromatographic eluent.

9α,11β-Dichloro-6α-fluoro-16-methyl-3-oxoandrosta-1,4,16-triene-17-carboxylic Acid (36). The preparation of thiol ester **47** from acid **9** (180 mg, 0.42 mmol) was attempted by method C. However, the major less polar product of reaction with FMPT (R_f 0.38, 1% HOAc–25% EtOAc–hexane, developed twice) remained unchanged after addition $NaSCH_3$/DMF (1.41 mmol) and warming to 20 °C, and workup then afforded 58 mg (34%) of Δ^{16}-acid **36** and a variety of minor neutral materials which were not further investigated. Analytical sample from acetone–hexane: mp 252–254 °C dec; UV 234 nm (ε 21040); IR (KBr) 1660 (br, COOH, 3-C=O) cm^{-1}; ^1H NMR (90 MHz, Me_2SO) δ 1.27 (s, 3 H, 18-CH_3), 1.74 (s, 3 H, 19-CH_3), 2.04 (s, 3 H, 16-CH_3), 4.98 (br m, 1 H, 11-H); MS, m/e 412, 414 (M^+). Anal. Calcd for $C_{21}H_{23}O_3Cl_2F$: C, 61.03; H, 5.61. Found: C, 61.12; H, 5.54.

6α,9α-Difluoro-11β,17α-dihydroxy-17β-[(methylthio)-carbonyl]-16α-methylandrosta-1,4-dien-3-one (37). The reaction of **6** (198 mg, 0.05 mmol) with FMPT and $NaSCH_3$ (method C) gave a mixture of three neutral products and 14 mg (7%) of returned **6**. Separation of the neutral components by centrifugal TLC (6% acetone–CH_2Cl_2) afforded 63 mg (30%) of **37** (R_f 0.25), along with 31 mg (18%) of triene **38** (R_f 0.40) and 22 mg (12%) of lactone **39** (R_f 0.15) (vide infra). Analytical sample of **37** from acetone–hexane: mp 275–277 °C dec; UV 239 nm (ε 19550); IR

(KBr) 1680 (20-C=O), 1665 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.83 (d, 3 H, J = 7 Hz, 16-CH_3), 0.92 (s, 3 H, 18-CH_3), 1.49 (s, 3 H, 19-CH_3), 2.17 (s, 3 H, SCH_3); MS, m/e 426 (M^+). Anal. Calcd for $C_{22}H_{28}O_4F_2S$: C, 61.95; H, 6.62. Found: C, 61.98; H, 6.74.

6α,9α-Difluoro-16α,17-dimethyl-11β-hydroxy-18-norandrosta-1,4,13(17)-trien-3-one (38) and 6α,9α-Difluoro-11β,13α-dihydroxy-16α,17β-dimethyl-3-oxo-18-norandrosta-1,4-diene-17α-carboxylic Acid 13-Lactone (39). The reaction of **6** (158 mg, 0.40 mmol) with FMPT according to method D followed by workup, preparative TLC, and recrystallizations from acetone–hexane gave 22 mg (16%) of **38** and 17 mg (11%) of **34** along with 11 mg (7%) of recovered **6** as the only isolable products. The data for **38** were as follows: mp 215–217 °C; UV 234 nm (ε 15 700); IR (KBr) 1670 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 0.92 (d, 3 H, J = 7 Hz, 16-CH_3), 1.39 (s, 3 H, 17-CH_3), 1.53 (s, 3 H, 19-CH_3); MS, m/e 334 (M^+). Anal. Calcd for $C_{20}H_{24}O_2F_2$: C, 71.83; H, 7.23. Found: C, 71.70; H, 7.05. The data for **39** were as follows: mp 154–156 °C dec; UV 236 nm (ε 15 800); IR ($CHCl_3$) 1815 (20-C=O), 1670 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, trace CD_3OD) δ 1.14 (d, 3 H, J = 7.5 Hz, 16-CH_3), 1.36 (s, 3 H, 17-CH_3), 1.43 (s, 3 H, 19-CH_3); ^{13}C NMR ($CDCl_3$, trace CD_3OD) δ 13.15, 13.19 (16-CH_3, 17-CH_3), 23.73 (19-CH_3), 40.28 (C-16), 45.35 (C-14), 66.24 (C-17), 66.76 (d, J_{CF} = 0.40 Hz, C-11), 86.45 (d, J_{CF} = 2.45 Hz, C-6), 90.57 (C-13), 97.54 (d, J_{CF} = 2.39 Hz, C-9), 173.61 (17-C=O), 185.73 (C-3); MS, m/e 378 (M^+); HRMS, m/e calcd for $C_{21}H_{24}O_4F_2$ 378.1643, found 378.1640. A satisfactory elemental analysis could not be obtained. A sample of **39** which had been dried in vacuo at 100 °C for 16 h was completely transformed into the triene **38** (presumably by loss of CO_2), as confirmed by TLC, loss of the 1815 cm^{-1} absorption in the infrared, and the ^1H NMR spectrum. Samples of **39** were subsequently dried at ambient temperature and proved to be reasonably stable when stored at −20 °C for up to 2 months, after which significant degradation to **38** was observed.

16β,17-Dimethyl-9α-fluoro-11β-hydroxy-18-norandrosta-1,4,13(17)-trien-3-one (40) and 11β,13α-Dihydroxy-16β,17β-dimethyl-9α-fluoro-3-oxo-18-norandrosta-1,4-diene-17α-carboxylic Acid 13-Lactone (41). Reaction of **7** (151 mg, 0.40 mmol) with FMPT (method D) gave 107 mg of a neutral product which proved to be a mixture of triene **40** (R_f 0.44) and the lactone **41** (R_f 0.24) by TLC (7% acetone–CH_2Cl_2). Recrystallization from acetone–hexane gave 30 mg (24%) of pure **40**, and centrifugal TLC of the mother liquor materials (same system) followed by recrystallizations from acetone–hexane gave a further 31 mg (24%) of **40** and 12 mg (8%) of **41**. Products and yields were not altered by addition of $NaSCH_3$ to the reaction mixture in a separate experiment. Data for **40** were as follows: mp 256–258 °C; UV 236 nm (ε 20 070); IR (KBr) 1660 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 1.00 (d, 3 H, J = 7 Hz, 16-CH_3), 1.39 (s, 3 H, 17-CH_3), 1.51 (s, 3 H, 19-CH_3); MS, m/e 316 (M^+). Anal. Calcd for $C_{20}H_{25}O_2F$: C, 75.92; H, 7.96. Found: C, 75.77; H, 7.92. Lactone **41** was reasonably stable in solution, but dry samples were more labile than the 16α-methyl analogue **39** and rapidaly degraded to triene **40** on storage. The ^{13}C NMR resonances for the 16- and 17-methyls of **41** were shifted related to those of lactone **39**, and were consistent with the assigned cis-dimethyl system. Data for **41** were as follows: mp 258–260 °C dec (from CH_2Cl_2–hexane); UV 237 nm (ε 15 200); IR ($CHCl_3$) 1815 (20-C=O) 1670 cm^{-1} (3-C=O); ^1H NMR (300 MHz, $CDCl_3$) δ 1.15 (d, 3 H, J = 7.5 Hz, 16-CH_3), 1.34 (s, 3 H, 17-CH_3), 1.45 (s, 3 H, 19-CH_3); ^{13}C NMR ($CDCl_3$) δ 11.89, 17.97 (16-CH_3, 17-CH_3), 23.63 (19-CH_3), 28.95 (C-6), 37.89 (C-16), 45.79 (C-14), 65.82 (C-17), 67.97 (d, J_{CF} = 0.42 Hz, C-11), 91.55 (C-13), 98.19 (d, J_{CF} = 2.38 Hz, C-9), 175.59 (17-C=O), 185.99 (C-3); MS, m/e 340 (M^+ − HF); HRMS, m/e calcd for $C_{21}H_{24}O_4$ (M^+ − HF) 340.1675, found 340.1699, calcd for $C_{20}H_{25}O_2F$ (M^+ − CO_2) 316.1839, found 316.1826.

6α-Fluoro-16-methyl-3-oxoandrosta-1,4,9(11),16-tetraene-17-carboxylic Acid (42) and 6α-Fluoro-17-[(methylthio)-carbonyl]-16-methylandrosta-1,4,9(11),16-tetraen-3-one (43). Reaction of **8** (170 mg, 0.47 mmol) with FMPT and $NaSCH_3$ (0.83 mmol) (method C) gave 77 mg of acidic material and 104 mg of neutral residue as an oil. Two crystallizations of the acid fraction from EtOAc–hexane gave 50 mg (30%) of **42**, mp 270–271 °C dec; UV 235 nm (ε 21 000); IR (KBr) 1715 (COOH), 1670 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 0.84 (s, 3 H, 18-CH_3), 1.36 (s, 3 H, 19-CH_3), 2.01 (s, 3 H, 16-CH_3), 5.57 (br d, 1 H, J = 4 Hz, 11-H); ^{13}C NMR ($CDCl_3$) δ 15.47 (18-CH_3), 17.48 (16-CH_3), 26.88 (19-CH_3), 38.27 (C-15), 40.12 (C-12), 40.35 (C-7), 45.85 (C-13), 50.93 (C-14), 87.31 (d, J_{CF} = 2.44 Hz, C-6), 119.66 (C-4), 122.74 (C-11), 127.75 (C-2), 135.67 (C-17), 141.23 (C-9), 158.79 (C-16), 161.89 (C-5), 170.19 (17-C=O), 185.39 (C-3); MS, m/e 342 (M^+). Anal. Calcd for $C_{21}H_{23}O_3F$: C, 73.66; H, 6.77. Found: C, 73.54; H, 6.67. A difficult purification of the neutral residue by preparative TLC (15% acetone–hexane, developed five times) and recrystallzation of the very soluble major component from pentane gave 26 mg (15%) of thiol ester **43**, mp 259–262 °C; UV 240 nm (ε 18 005); IR (KBr) 1700–1665 cm^{-1} (br, 20-C=O, 3-C=O); ^1H NMR (100 MHz, Me_2SO) δ 0.94 (s, 3 H, 18-CH_3), 1.37 (s, 3 H, 19-CH_3), 2.04 (s, 3 H, 16-CH_3), 2.25 (s, 3 H, SCH_3), 5.57 (br d, 1 H, J = 6, 11-H); MS, m/e 372 (M^+). Anal. Calcd for $C_{22}H_{25}O_2FS$: C, 70.94; H, 6.76. Found: C, 70.98; H, 6.50.

6α-Fluoro-16-methyl-3-oxoandrosta-1,4,9(11),16-tetraene-17-carbonyl Fluoride (44). The reaction of **8** (190 mg, 0.50 mmol) with FMPT alone (method D) gave 48 mg (28%) of acid **42** and 35 mg of a neutral fraction containing a single major product (R_f 0.58, 4% acetone–CH_2Cl_2). Centrifugal TLC (same system) of the labile neutral product and recrystallization from acetone–hexane gave 11 mg (8%) of **44**, mp 243–244 °C dec; UV 237 nm (ε 23 400); IR ($CHCl_3$) 1790 (20-C=O), 1670 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.94 (s, 3 H, 18-CH_3), 1.42 (s, 3 H, 19-CH_3), 2.19 (s, 3 H, 16-CH_3), 5.64 (d, 1 H, J = 6.6 Hz, 11-H); MS, m/e 344 (M^+); HRMS, m/e calcd for $C_{21}H_{22}O_2F_2$ 344.1588, found 344.1577. Anal. Calcd for $C_{21}H_{22}O_2F_2$: C, 73.24; H, 6.44. Found: C, 73.12; H, 6.50.

6α,9α-Difluoro-11β,17α-dihydroxy-17β-[(ethylthio)-carbonyl]-16α-methylandrosta-1,4-dien-3-one (45). To a solution of **6** (105 mg, 0.27 mmol) in DMF (7 mL) at −10 °C was added CDI (80 mg, 0.49 mmol) in DMF (3 mL) and the mixture was stored at −5 °C for 16 h, after which conversion to a slightly less polar material was complete (TLC, 0.25% HOAc–40% acetone–hexane). Ethyl mercaptan (0.2 mL, 2.7 mmol) was added, and the mixture was stirred at 20 °C for 16 h. The solution was evaporated to dryness, and the residue was purified by preparative TLC (10% acetone–benzene) to give 47 mg (40%) of **45**. Analytical sample, recrystallized 4 times from acetone–hexane: mp 253–256 °C dec; UV 238 nm (ε 19 730); IR (KBr) 1665 cm^{-1} (br, 20-C=O, 3-C=O); ^1H NMR (100 MHz, Me_2SO) δ 0.81 (d, 3 H, J = 7 Hz, 16-CH_3), 0.90 (s, 3 H, 18-CH_3), 1.14 (t, 3 H, J = 7 Hz, CH_2CH_3), 1.46 (s, 3 H, 19-CH_3), 2.72 (q, 2 H, J = 7 Hz, SCH_2); MS, m/e 440 (M^+). Anal. Calcd for $C_{23}H_{30}O_4F_2S$: C, 62.71; H, 6.86. Found: C, 62.49; H, 7.08.

11β,17α-Dihydroxy-9α-fluoro-17β-[(methylthio)-carbonyl]-16β-methylandrosta-1,4-dien-3-one (46). An ice-cooled solution of **7** (100 mg, 0.26 mmol) and CDI (80 mg, 0.49 mmol) in DMF (8 mL) was stirred for 2 h, after which a gentle stream of CH_3SH gas was introduced below the surface of the mixture for 1 h. The mixture was purged with nitrogen and evaporated to dryness, and the product was purified by centrifugal TLC (3% MeOH–CH_2Cl_2) and recrystallization from acetone–hexane to give 66 mg (61%) of pure **46**, mp 242–244 °C dec; UV 239 nm (ε 19 400); IR (KBr) 1690 (20-C=O), 1670 (3-C=O) cm^{-1}; ^1H NMR (100 MHz, Me_2SO) δ 0.95 (s, 3 H, 18-CH_3), 1.02 (d, 3 H, J = 7 Hz, 16-CH_3), 1.47 (s, 3 H, 19-CH_3), 2.04 (s, 3 H, SCH_3); MS, m/e 388 (M^+ − HF). Anal. Calcd for $C_{22}H_{29}O_4FS$: C, 64.68; H, 7.16. Found: C, 64.69; H, 7.20.

9α,11β-Dichloro-6α-fluoro-17α-hydroxy-17β-[(methylthio)carbonyl]-16β-methylandrosta-1,4-dien-3-one (47). Reaction of **9** (200 mg, 0.46 mmol) with CDI and CH_3SH as described for the preparation of **46** and recrystallization of the product from acetone–hexane gave 180 mg (84%) of **47**, mp 232–233 °C dec; UV 237 nm (ε 19 850); IR (KBr) 1675 cm^{-1} (20-C=O, 3-C=O); ^1H NMR (90 MHz, Me_2SO) δ 1.03 (s, 3 H, 18-CH_3), 1.07 (d, 3 H, J = 7 Hz, 16-CH_3), 1.70 (s, 3 H, 19-CH_3), 2.10 (s, 3 H, SCH_3), 4.99 (br d, 1 H, J = 4 Hz, 11-H); MS, m/e 461–465 (MH^+). Anal. Calcd for $C_{22}H_{27}O_3Cl_2FS$: C, 57.27; H, 5.90; Cl, 15.37. Found: C, 57.34; H, 5.93; Cl, 15.33.

17α-Acetoxy-9α,11β-dichloro-6α-fluoro-17β-[(methylthio)carbonyl]-16β-methylandrosta-1,4-dien-3-one (48). A solution of **47** (110 mg, 0.24 mmol) and DMAP (15 mg, 0.12 mmol) in TEA (2 mL) and acetic anhydride (2 mL) was heated at 80 °C

for 1.5 h and then stirred at 20 °C for 16 h. The mixture was diluted with EtOAc, washed with water, and evaporated to dryness. The product was purified by preparative TLC (20% acetone–hexane, eluted 4 times) and crystallization from acetone–hexane, giving 57 mg (47%) of **48**, mp 250–251 °C dec; UV 235 nm (ε 19345); IR (KBr) 1750 (ester), 1700 (20-C=O), 1675 (3-C=O) cm^{-1}; ^1H NMR (90 MHz, Me_2SO) δ 0.95 (s, 3 H, 18-CH_3), 1.34 (d, 3 H, J = 7 Hz, 16-CH_3), 1.71 (s, 3 H, 19-CH_3), 2.05 (s, 3 H, $COCH_3$), 2.20 (s, 3 H, SCH_3), 5.09 (br d, 1 H, J = 4 Hz, 11-H); MS, m/e 503–507 (MH^+). Anal. Calcd for $C_{24}H_{29}O_4Cl_2FS$: C, 57.26; H, 5.81. Found: C, 57.19; H, 5.85.

9α,11β-Dichloro-6α-fluoro-17β-[(methylthio)carbonyl]-17α-(propionyloxy)-16β-methylandrosta-1,4-dien-3-one (49). Freshly distilled propionic anhydride (0.3 mL, 2.34 mmol) was added to a solution of **47** (140 mg, 0.30 mmol) and DMAP (50 mg, 0.41 mmol) in TEA (3 mL), and the mixture was heated at 70 °C for 16 h. Purification of the product by centrifugal TLC (0.5% acetone–CH_2Cl_2) followed by crystallizations from MeOH and EtOAc–hexane gave 55 mg (35%) of **49**, mp 244–246 °C dec; UV 237 nm (ε 18995); IR (KBr) 1745 (ester), 1705 (20-C=O), 1670 (3-C=O) cm^{-1}; ^1H NMR (300 MHz, Me_2SO) δ 0.95 (s, 3 H, 18-CH_3), 1.05 (t, 3 H, J = 7 Hz, CH_2CH_3), 1.33 (d, 3 H, J = 7 Hz, 16-CH_3), 1.71 (s, 3 H, 19-CH_3), 2.19 (s, 3 H, SCH_3), 2.33 (q, 2 H, J = 7 Hz, $COCH_2$); MS, m/e 468–472 ($M^+ - HSCH_3$). Anal. Calcd for $C_{25}H_{31}O_4Cl_2FS$: C, 58.03; H, 6.04; Cl, 13.70. Found: C, 57.98; H, 6.04; Cl, 13.68.

Acknowledgment. We thank Dr. F. S. Alvarez,[51] Dr. J. Patterson, and Paul Wagner for their contributions to this work. We are indebted to Janis Nelson and Dr. M. Maddox for the measurement and interpretation of NMR spectra, and to Dr. I. J. Massey and Dr. L. Partridge for mass spectra. We also thank Dr. A. F. Kluge and Dr. K. A. M. Walker for many helpful discussions and suggestions.

Registry No. **1**, 67-73-2; **2**, 2135-17-3; **3**, 378-44-9; **4**, 101916-22-7; **4** diacetate, 50630-16-5; **5**, 101916-23-8; **5** diacetate, 101916-49-8; **6**, 28416-82-2; **7**, 37926-75-3; **8**, 101916-24-9; **9**, 101916-25-0; **10**, 65751-34-0; **11**, 101916-26-1; **12**, 65429-42-7; **13**, 37927-21-2; **14**, 37927-23-4; **15**, 101916-27-2; **16**, 37927-22-3; **17**, 74131-78-5; **18**, 101916-28-3; **19**, 101916-29-4; **20**, 74131-73-0; **21**, 73205-13-7; **22**, 101916-30-7; **23**, 101916-31-8; **24**, 79578-14-6; **25**, 74156-42-6; **26**, 79578-08-8; **27**, 101932-58-5; **28**, 101916-32-9; **29**, 101916-33-0; **30** (isomer 1), 101916-34-1; **30** (isomer 2), 101916-35-2; **31** (isomer 1), 101916-36-3; **31** (isomer 2), 101916-37-4; **32**, 101932-54-1; **33**, 101916-38-5; **34**, 101916-39-6; **35**, 101916-40-9; **36**, 101916-41-0; **37**, 74131-77-4; **38**, 101916-42-1; **39**, 101916-43-2; **40**, 101916-44-3; **41**, 101932-61-0; **42**, 101932-59-6; **43**, 101916-45-4; **44**, 101916-46-5; **45**, 74131-76-3; **46**, 87116-72-1; **47**, 101916-47-6; **48**, 101932-60-9; **49**, 101916-48-7; $NaSCH_3$, 5188-07-8; $NaSC_2H_5$, 811-51-8; $NaSC_6H_{13}$, 22487-02-1; CH_3SH, 74-93-1; acetyl chloride, 75-36-5; ethyl mercaptan, 75-08-1; propionyl chloride, 79-03-8.

(51) Deceased.

Organometallic Derivatives of Hormonal Steroids: 500-MHz One- and Two-Dimensional NMR Spectra of 17α-Propynylestra-1,3,5(10)-triene-3,17β-diol and Its $Co_2(CO)_6$ and $(C_5H_5)_2Mo_2(CO)_4$ Complexes

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Received October 16, 1985

Treatment of estrone with a propynyl Grignard reagent gives exclusively 17α-propynylestra-1,3,5(10)-triene-3,17β-diol. This 17α-alkynyl steroid reacts with $Co_2(CO)_8$ or $(C_5H_5)_2Mo_2(CO)_4$ to yield the cluster complexes $(RC{\equiv}CR')M_2$, where R = methyl, R′ is the steroidal moiety, and M = $Co(CO)_3$ or $(C_5H_5)Mo(CO)_2$. The cobalt complex of mestranol has likewise been prepared. The 500-MHz ^1H NMR spectra of these molecules are reported and are assigned by the two-dimensional COSY technique. The shifts of the 12α- and 14α-protons of the steroid are discussed in terms of the anisotropy in diamagnetic susceptibility of the alkyne linkage. ^{13}C spectra are also reported.

Introduction

The incorporation of organometallic moieties into biologically important molecules is a field of burgeoning importance. Typically, in steroid chemistry, $Fe(CO)_3$ fragments may be used as temporary protecting agents,[1] and allylpalladium[2] or $Cr(CO)_3$ units[3] have been exploited for synthetic purposes. Recently, advances in bioorganometallic chemistry have been directed toward immunology,[4] and we have described the use of steroidal hormones labeled with metal carbonyls to assay receptor sites.[5] This latter concept takes advantage of the strong infrared absorptions of metal carbonyls in the range 2100–1850 cm^{-1}—a window in which proteins do not absorb. Our goal is to monitor the hormone dependence of breast cancer while avoiding the use of radioactivity and its associated inconveniences.

In the particular case of estrogenic hormones, it has been reported that the 7α-, 11β-, and 17α-positions of estradiol (**I**) can tolerate substitution by bulky groups and still ex-

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0022-3263/86/1951-2328$01.50/0

Eur Respir J 1997; 10: 1496–1499
DOI: 10.1183/09031936.97.10071496
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European Respiratory Journal
ISSN 0903 - 1936

Distribution of inhaled fluticasone propionate between human lung tissue and serum *in vivo*

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Distribution of inhaled fluticasone propionate between human lung tissue and serum in vivo. *N. Esmailpour, P. Högger, K.F. Rabe, U. Heitmann, M. Nakashima, P. Rohdewald. ©ERS Journals Ltd 1997.*

ABSTRACT: High retention of inhaled glucocorticoids in the airways means prolonged anti-inflammatory action and low delivery into the serum. The objective of this study was to investigate the retention in and distribution of inhaled fluticasone propionate (FP) between central and peripheral human lung tissue and serum *in vivo.*

In 17 patients undergoing lung resection surgery, a single 1.0 mg dose of FP was inhaled at varying time-points (range 2.8–21.7 h) preoperatively. Peripheral and central lung tissue was obtained, and blood was drawn simultaneously.

FP concentrations in central lung tissue were approximately three to four times higher than peripheral lung tissue concentrations, which in turn, exceeded those found in serum by 10 times. FP was detectable up to 21 and 16 h, respectively, after inhalation, with drug levels falling almost in parallel in peripheral lung tissue and in serum.

The results of this study demonstrate that fluticasone propionate is retained in lung tissue for a long time. Serum concentrations after a single inhaled dose are low. Retention of high concentrations of fluticasone propionate in the airways may promote high topical anti-inflammatory activity.

Eur Respir J 1997; 10: 1496–1499.

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Keywords: Fluticasone propionate
glucocorticoid
inhalation
plasma concentration
tissue concentration *in vivo*

Received: July 30 1996
Accepted after revision April 20 1997

The investigation was supported by Glaxo-Wellcome.

The ideal corticosteroid for use by inhalation in the treatment of asthma should act effectively in the airways and produce a minimum of systemic effects within its dose range [1]. To achieve this, the compound should have a high intrinsic topical activity (anti-inflammatory potency) combined with low oral systemic bioavailability [2]. It is favourable for a corticosteroid to display high retention in the airways and low delivery into plasma after inhalation. Therefore, it is of interest to investigate the distribution of glucocorticoids between lung tissue and blood.

Potent glucocorticoids have a high affinity for the glucocorticoid receptor. Of all corticosteroids tested, fluticasone propionate (FP) has the highest *in vitro* affinity for the glucocorticoid receptor in human lung [3, 4]. Binding and retention studies in human lung *in vitro* have demonstrated high tissue concentrations for FP (similar to beclomethasone dipropionate and its active metabolite, but greater than budesonide, flunisolide and hydrocortisone), compared with blood plasma concentrations [5–7].

To demonstrate the clinical relevance of these data, we present the first investigation of the tissue-plasma distribution of FP in humans. In this study, the distribution of a single 1.0 mg dose of FP between human lung tissue and blood plasma was studied *in vivo*.

Materials and methods

Patients and study design

The study was approved by the local Ethics Committee. Informed consent to participate in the study was obtained from 17 patients (16 males and 1 female) with bronchial carcinoma, who were referred for pneumectomy or lobe resection. Their mean (±SD) age was 58±6 yrs (range 48–67 yrs). Squamous cell carcinomas were found in 11 patients, five patients had adenocarcinoma, and one patient had a carcinoid tumour. Vital capacity ranged 2.7–6.1 L, and forced expiratory volume in one second (FEV_1) 58–115% of predicted (table 1).

At varying times before surgery (range 2.8–21.6 h), 1.0 mg FP was administered *via* a large volume spacer, Volumatic™ (four puffs each of 250 μg), and the time of administration was recorded. Thirty to 45 min before intubation, 0.5 mg atropine sulphate and an analgesic were given to patients as premedication. Peripheral and central lung tissue free of tumour was obtained for analysis of FP. A blood sample was drawn intraoperatively at the time-point of ligation of the pulmonary artery for analysis. Whole blood was centrifuged and serum samples were frozen and stored until analysed at -20°C. The time after inhalation, identical with time

Table 1. – Patient characteristics, lung function parameters and medical data

Pt No.	Age yrs	Sex	Smoking status	Type of operation	Histology	VC L	FEV1 % pred	Time between inhalation and resection h	Comments
1	60	M	Smoker	PN	AC	3.6	80	4.7	-
2	61	M	Smoker	PN	AC	3.8	80	3.7	-
3	67	M	Ex	PN	AC	3.3	79	2.8	-
4	50	F	Smoker	LR	Carcinoid	2.7	61	14.0	Plasma only
5	51	M	Ex	PN	SC	5.2	104	3.3	-
6	59	M	Ex	PN	SC	4.7	94	12.3	-
7	61	M	Smoker	LR	AC	4.1	87	3.7	-
8	62	M	Smoker	PN	SC	4.4	93	12.7	Viscous mucus
9	60	M	Smoker	PN	SC	6.1	105	13.3	-
10	57	M	Smoker	PN	SC	3.2	58	17.5	-
11	50	M	Smoker	PN	SC	4.1	101	11.1	Viscous mucus
12	56	M	Ex	PN	SC	5.6	98	12.1	Viscous mucus
13	64	M	Ex	PN	SC	5.5	115	21.2	-
14	51	M	Smoker	PN	AC	4.2	70	16.3	-
15	58	M	Smoker	PN	SC	5.1	83	17.2	-
16	48	M	Smoker	PN	SC	3.5	67	13.3	-
17	66	M	Smoker	PN	SC	4.1	68	16.9	-

Pt: patient; M: male; F: female; Ex: ex-smoker; PN: pneumonectomy; LR: lobe resection; AC: adenocarcinoma; SC: squamous cell carcinoma; VC: vital capacity; FEV1: forced expiratory volume in one second; % pred: percentage of predicted value. FEV1 % pred for males: FEV1/(4.3 × (height/100))-(0.029 × age)-2.49) × 100. FEV1 % pred for females: FEV1/(3.95 × (height/100))-(0.025 × age)-2.6) × 100.

between inhalation and resection, has been defined as the time between inhalation and the ligation of the pulmonary artery.

Materials

Chemicals were purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany), activated charcoal Norit Gsx was from BDH (Wesel, Germany). Fluticasone-17-propionate (FP), tritiated FP (specific activity 55.4 $Ci \cdot mmol^{-1}$) and FP antiserum [8] were a generous gift from Glaxo Wellcome (Greenford, UK). Solid phase extraction cartridges, C_{18}-SPE-columns, were obtained from J.T. Baker (Philipsburg, USA), scintillation cocktail Aquasafe 500 Plus and 4 mL polyethylene vials were from Zinsser (Frankfurt, Germany), and 2 mL incubation tubes were obtained from Eppendorf (Hamburg, Germany).

Assay buffer (pH 7.4 at 4°C) consisted of 100 mM tricine, 150 mM sodium chloride, and 15 mM sodium azide. Tricine-ethanol buffer contained 5% (v/v) ethanol in assay buffer, and tricine-gelatine buffer contained 0.1% gelatine (w/v) in assay buffer. Dextran-coated charcoal suspension consisted of 0.5% (w/v) Norit Gsx and 0.05% (w/v) dextran. The working dilution of [^{3}H]-FP was 532.4 $pmol \cdot L^{-1}$, and the working solution of FP antiserum resulted in 50% binding of [^{3}H]-FP.

Quantification of FP in lung tissue

Tissue was pulverized and one part tissue was homogenized (Ultra-turrax, Jahnke Kunkel, Germany; Potter S, Braun Melsungen, Germany) with three parts of assay buffer. An aliquot (2.0 g) of the tissue suspension was frozen in liquid nitrogen and lyophilized. The pellet was extracted twice with 5.0 mL methanol, and an aliquot of the extract was evaporated at 50°C under a gentle nitrogen flow. Dry extracts were reconstituted in 0.1 mL tricine-ethanol buffer and 0.1 mL tricine-gelatine buffer. Samples were mixed with 0.1 mL of FP antiserum solution and incubated at 0°C for 30 min. For determination of nonspecific binding, antiserum solution was replaced by assay buffer. After 30 min, 0.1 mL of [^{3}H]-FP was added and incubation was continued for another 30 min at 0°C. The reaction was stopped by addition of 1.0 mL dextran-coated charcoal suspension. Samples were incubated for 10 min on ice and centrifuged. A 1.0 mL aliquot of the supernatant was counted for radioactivity. Mean (±SD) recovery was 90±3% (n=8) for tissue samples. Calibration curves were prepared with blank tissue (swine lungs). Specific binding was 75±4% for human lung tissue (n=8) and 80±2% for swine tissue (n=8). Thus, specific binding was not statistically significantly different between human and swine lung tissue. There was no detectable difference in calibration curves obtained with swine tissue compared to calibration curves obtained with blank human lung tissue. Curves were linear, the coefficient of correlation was r=0.99±0.01 (n=5). The lower limit of quantification was 1 $pg \cdot mg^{-1}$.

Quantification of FP in serum

Frozen serum samples were thawed. Solid phase extraction cartridges were equilibrated with 1 mL methanol, and subsequently with 1 mL assay buffer. A 1.0 mL aliquot of serum samples was applied to the column. The cartridges were washed with 1 mL assay buffer and 1 mL distilled water, and dried for 2 min under vacuum. FP was eluted with 1.0 mL methanol, and the eluate was evaporated at 50°C under a gentle nitrogen flow. Radioimmunoassay (RIA) was performed as described for tissue samples. Recovery was 95±0.8% (n=8) for serum samples. Calibration curves were prepared with blank human serum. Curves were linear and the coefficient of correlation was r=0.99±0.01 (n=6). The lower limit of quantification was 25 $pg \cdot mL^{-1}$.

Results

FP concentrations of 0–6 ng·g^{-1} (0–12 nmol·kg^{-1}) found in peripheral lung tissue, were approximately 100 times greater than the FP concentrations of 0–0.11 ng·mL^{-1} (0–0.22 nmol·L^{-1}) in serum, (figs. 1 and 2). Levels of FP in peripheral lung and serum fell almost in parallel with time after inhalation. Regression analysis of the two sets of data revealed high coefficients of correlation between concentration and time: r=0.70, p<0.01; and r=0.79, p<0.01, respectively.

The serum concentration of FP was generally below 0.1 ng·mL^{-1}, with the exception of one data point contributed by a patient with viscous airway mucus (this outlier was not used for regression analysis). Peripheral lung tissue concentrations of FP could be detected up to 16.3 h, and serum concentrations up to 13.3 h after inhalation. Concentrations of FP were generally higher (by three to four times) in central tissue, compared with concentrations in peripheral lung tissue (fig. 1), although

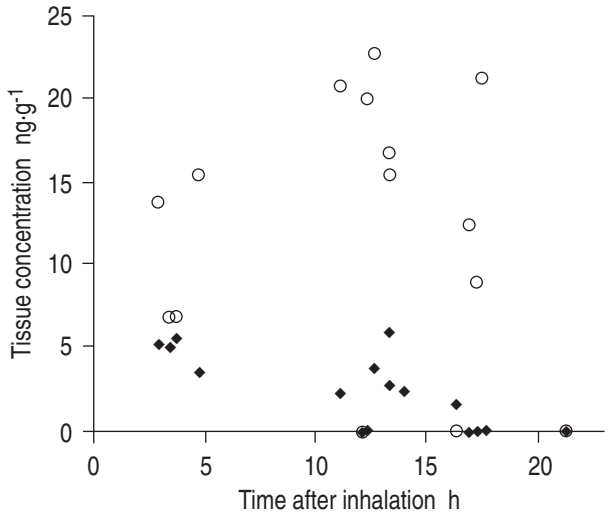

Fig. 1. – Concentration of fluticasone propionate (FP) in central and peripheral lung tissue after inhalation of 1 mg. Symbols represent single patient data. ○: FP concentration in central lung tissue; ◆: FP concentration in peripheral lung tissue

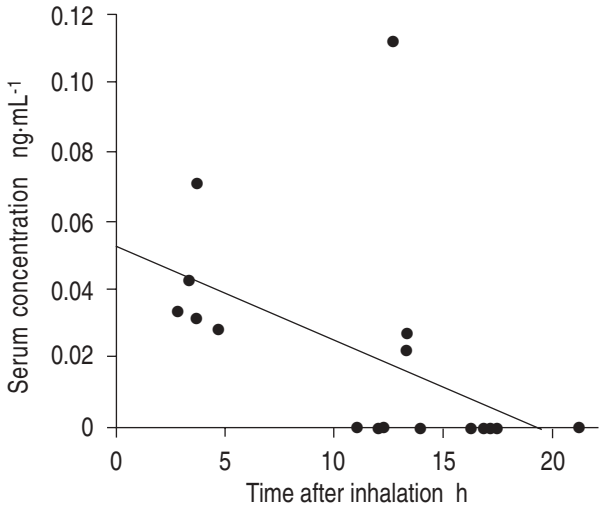

Fig. 2. – Concentration of fluticasone propionate (FP) in human serum after inhalation of 1 mg. Symbols represent single patient data. ●: FP concentration in serum.

interindividual variation in central lung tissue drug concentrations was high (range <1 ng·g^{-1} to 23 ng·g^{-1}). Concentrations of FP in central lung tissue persisted at a high level up to 17 h, with the exception of one patient.

Discussion

This study showed that FP concentrations in central lung tissue were about three to four times higher than in peripheral lung tissue, which in turn exceeded those found in serum by 100 times. No relevant information can be drawn from serum levels of inhaled steroids about the concentrations in the target organ. The present investigation gives more useful information than pharmacokinetic studies analysing serum levels only, because drug concentrations in the lung became directly available. The study design allowed the time course of drug concentrations to be described by combining single data from a sufficiently high number of patients.

Human *in vivo* data for the tissue-serum distribution of inhaled glucocorticoids and drug deposition in central and peripheral lung has not previously been investigated. Only the distribution of a glucocorticoid between the peripheral lung tissue and plasma of patients was studied after inhalation of 1.6 mg budesonide [9].

The higher FP concentrations in central lung tissue compared with peripheral lung tissue is not unexpected, since deposition of an inhaled drug is likely to decrease with greater distance from the trachea. This may also explain differences in drug deposition found in central lung tissue between patients where tissue samples were obtained from bronchi of different diameter. No close correlation was found for FP concentrations in the central airways and FEV1, vital capacity or the presence of mucus. However, in two of the three patients having viscous mucus in the upper airways, we found high FP concentrations of about 20 ng·g^{-1}, and another patient with 22 ng·g^{-1} FP in central lung tissue presented the lowest FEV1.

Systematic investigations of drug deposition and distribution to tissue in the central airways are needed, to obtain a better understanding of drug deposition as a function of the distance from the trachea as well as a function of airway diameter. The higher FP concentrations in central lung tissue persisted for more than 17 h, in contrast to the steadily declining concentrations in peripheral lung tissue. There are two probable reasons for this difference. Firstly, FP particles deposited in central lung tissue are likely to be of bigger mean size than those deposited in peripheral lung tissue. Secondly, the density of blood capillaries is higher in peripheral than in central tissue, so that the tissue-plasma exchange rate is higher in peripheral tissue and FP tissue concentrations decrease faster.

The lung tissue concentration of FP found in this study is estimated to be approximately 10–100 times higher than the concentration of FP previously calculated to occupy 50% of human glucocorticoid receptor *in vitro* [4]. This means that the tissue receptor binding sites should be 100% saturated with FP, still leaving a non-(receptor)-bound excess. Thus, FP can exhibit its anti-inflammatory action over a longer period of time, since

it is stored in lung tissue for a prolonged period in sufficiently high concentrations. The prolonged presence of FP in lung tissue, together with the high relative receptor affinity to the glucocorticoid receptor, helps to explain the high clinical efficacy of FP.

Under steady-state conditions it is to be expected that FP concentrations in lung tissue and plasma are higher than in the present study after a single dose, because plasma concentrations of FP under steady-state conditions are about 50% higher compared to single dosing [10].

In the present study, the FP concentrations in peripheral lung tissue following inhalation of a 1.0 mg dose were twice as high as those found for budesonide in a similar study [9]. Whereas linear regression analysis showed a statistically significant correlation between FP concentration and time in peripheral lung tissue ($r=0.74$; $p>0.001$), a significant correlation was not demonstrable with budesonide concentrations in lung parenchyma ($r=0.37$; p=NS). This might be due to high variation of data, or to the fact that lung tissue and serum concentrations were measured up to 4 h only. In contrast to tissue concentrations, VAN DEN BOSCH *et al.* [9] found that budesonide concentrations in plasma were considerably higher than FP serum concentrations.

The results of the present study and of the study with budesonide [9] confirm our *in vitro* data with human lung tissue [5–7]. After saturation of human lung tissue *in vitro* with glucocorticoids and subsequent incubation in serum, the total concentration of FP remaining in the human lung tissue was about three times higher than for budesonide after 1 h *in vitro,* whereas FP concentrations *in vivo* were twice as high as for budesonide.

The parallel decline of FP concentrations in peripheral lung tissue and serum indicates that the delivery of FP from tissue to serum is the rate-limiting step for the elimination of FP.

Whereas the correlation of *in vitro* with *in vivo* data was high if the study was performed with human lung tissue [5–7], *in vitro* data obtained with rat tissue [10] did not correspond with the results of this FP *in vivo* study or with the budesonide *in vivo* study [9], since MILLER-LARSSON *et al.* [11] found that 40–50% of budesonide and only 30–40% of FP was left in the trachea *in vitro* 2 h after administration of the glucocorticoid.

The preclinical results that we obtained with fluticasone propionate, such as its affinity to the human glucocorticoid receptor [3], the receptor kinetics [4], and the binding to and retention in human lung tissue *in vitro* [5–7] concurrently proved that fluticasone propionate is a long-acting and very potent glucocorticoid. The *in vivo* study presented here is in good accordance with these previous findings.

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Available online http://arthritis-research.com/content/4/3/146

Commentary

Glucocorticoids: do we know how they work?

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Received: 24 October 2001
Revisions requested: 8 November 2001
Revisions received: 22 November 2001
Accepted: 26 November 2001
Published: 21 January 2002

Arthritis Res 2002, **4**:146-150

(Print ISSN 1465-9905; Online ISSN 1465-9913)

Abstract

It is not known to what extent glucocorticoid hormones cause their anti-inflammatory actions and their undesirable side effects by the same or different molecular mechanisms. Glucocorticoids combine with a cytoplasmic receptor that alters gene expression in two ways. One way is dependent on the receptor's binding directly to DNA and acting (positively or negatively) as a transcription factor. The other is dependent on its binding to and interfering with other transcription factors. Both mechanisms could underlie suppression of inflammation. The liganded receptor binds and inhibits the inflammatory transcription factors activator protein-1 and NF-κB. It also directly induces anti-inflammatory genes such as that encoding the protein inhibitor of NF-κB. Recent work has shown that glucocorticoids inhibit signalling in the mitogen-activated protein kinase pathways that mediate the expression of inflammatory genes. This inhibition is dependent on *de novo* gene expression. It is important to establish the significance of these different mechanisms for the various physiological effects of glucocorticoids, because it may be possible to produce steroid-related drugs that selectively target the inflammatory process.

Keywords: glucocorticoid, inflammation, JNK, MAP kinase, NF-κB

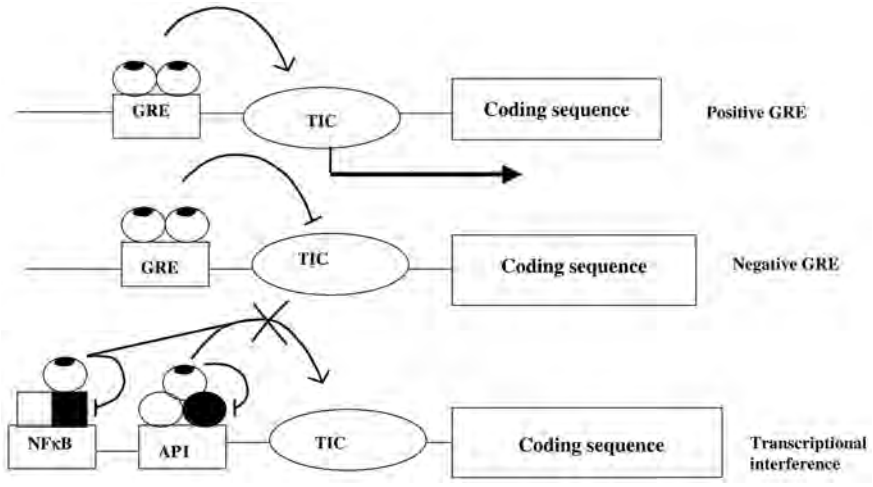
Introduction

The anti-inflammatory action of glucocorticoid hormones was discovered by Hench and colleagues over 50 years ago [1]. Hench had noted that the symptoms of rheumatoid arthritis were often improved in pregnancy and when a patient had jaundice, both situations in which, he reasoned, there was an increase in steroids in the body. The active substances of the adrenal cortex had then recently been isolated by Reichstein and Kendall and shown to be steroids. It seemed possible that these might alleviate inflammatory symptoms. Hench and his co-workers found that small doses of cortisone dramatically improved the symptoms of patients with rheumatoid arthritis. Hench, Kendall, and Reichstein were jointly awarded the Nobel Prize in physiology and medicine in 1950. Powerful synthetic glucocorticoids were then developed, which, despite their unwelcome side effects, became and remain mainstays of anti-inflammatory and immunosuppressive therapy.

The side effects of glucocorticoids that severely limited their use were osteoporosis, diabetes, hypertension, cataracts, thinning of the skin, and the characteristic appearance of Cushing's syndrome. They also suppressed the hypothalamic–pituitary–adrenal axis and arrested growth. Their potency in suppressing inflamma-

ACTH = adrenocorticotropic hormone; AP-1 = activator protein-1; CBP = CREB-binding protein; COX = cyclooxygenase; CREB = cAMP-response-element-binding protein; ERK = extracellularly regulated kinase; GR = glucocorticoid receptor; GRE = glucocorticoid response element; IL = interleukin; JNK = c-Jun N-terminal kinase; MAP = mitogen-activated protein; MKK = MAP kinase kinase; MKP = MAP kinase phosphatase; NF = nuclear factor.

Figure 1



Mechanisms of transcriptional regulation by glucocorticoids (adapted from M Karin [3]). Liganded GRs (ovals with patches) regulate transcription by direct binding to DNA elements (GREs) or by binding to other transcription factors (tethering). GRE-regulated and NF-κB/AP-1-regulated promoters are shown schematically with single binding sites; typically, there may be one or several sites. Interference with the activity of AP-1 (open and dark ovals) or NF-κB (open and dark squares) after tethering could be a direct effect on the factors' function, or it could be an interaction with coactivators or components of the TIC. AP-1 = activator protein-1; GR = glucocorticoid receptor; GRE = glucocorticoid response element; NF-κB = nuclear factor-κB; TIC = transcriptional initiation complex.

tion stimulated much investigation into their mechanism of action. Glucocorticoids inhibit expression of many of the genes involved in inflammatory and immune responses. These include those encoding cytokines, chemokines, cell-surface receptors, adhesion molecules, tissue factor, degradative proteinases, and enzymes such as cyclo-oxygenase (COX)-2 and inducible nitric oxide synthase, which produce inflammatory mediators.

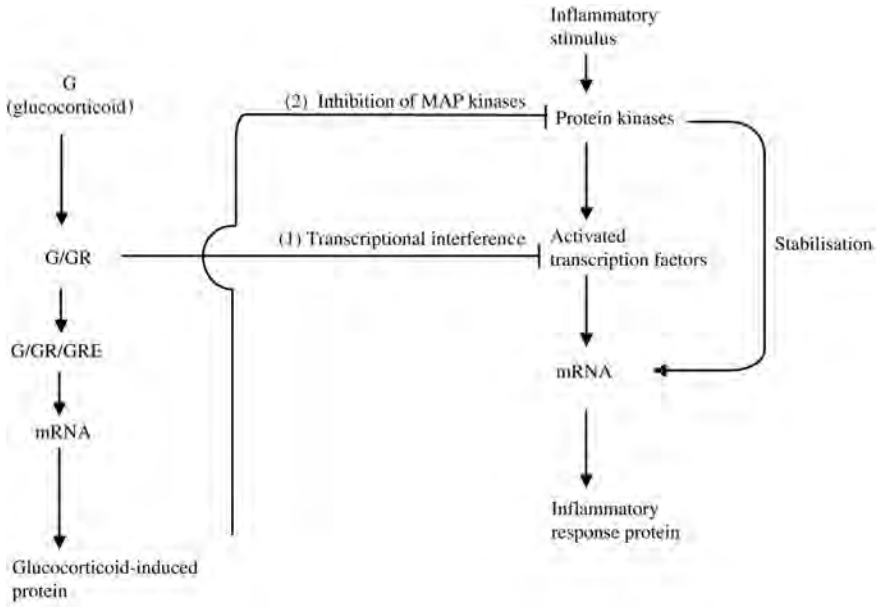
The glucocorticoid receptor (GR) [2] binds to specific DNA sequences – glucocorticoid response elements (GREs) – which may be positive or negative, either activating or repressing transcription, respectively (Fig. 1). In the absence of ligand, the receptor is cytoplasmic and is complexed with chaperones, including hsp90, hsp56 (immunophilin), and calreticulin. Upon ligand binding to the receptor, the chaperones are shed, exposing nuclear localisation signals. The receptor dimerises when it binds to a GRE. Genes that are positively regulated by GREs include those involved in gluconeogenesis, such as those for tyrosine aminotransferase, alanine aminotransferase, and phosphoenolpyruvate carboxykinase. Examples of genes negatively regulated by GREs are those for pro-opiomelanocortin (the ACTH precursor) and prolactin. The genes responsible for such side effects of glucocorticoids as osteoporosis, diabetes, and hypertension are largely unknown. Understanding the transcriptional basis of the different physiological effects is important because it may be possible to develop more selective agents, which target the inflammatory process.

Glucocorticoid receptors interact with inflammatory transcription factors

No genes responding positively to glucocorticoid hormones have been identified that explain the profound suppression of the inflammatory response. Furthermore, genes of the inflammatory response lack repressing GREs, so other explanations for the suppression of inflammation have been sought [3,4]. Currently, it is thought that a major mechanism is transcriptional interference (see Fig. 1). Inflammatory-response genes are typically regulated by the transcription factors NF-κB and activator protein-1 (AP-1). The liganded GR interacts with AP-1 complexes (generally c-Jun/c-Fos heterodimers) and prevents their transcriptional activity [3,4]. Similar tethering occurs with NF-κB [5,6]. This interference between transcription factors is reciprocal: not only can GR prevent the function of AP-1 and NF-κB, but also AP-1 and NF-κB can prevent transcriptional activation by GR. The physiological significance of these mechanisms is still speculative and the interactions between the endogenous proteins have been difficult to demonstrate *in vivo*. Another possible explanation for the functional competition between AP-1, NF-κB, and GR is that they compete for the transcriptional coactivators CREB-binding protein (CBP) and p300 [7,8]. However, this notion is controversial.

An additional mechanism of transcriptional interference has been recently proposed [9]. Corticosteroid interferes with IL-1-induced gene activation by inhibiting the histone acetylation that loosens chromatin structure, and enables

Figure 2



Glucocorticoids may act (1) by transcriptional interference and (2) by inhibiting MAP kinases. The left side represents glucocorticoid (G) combining with its receptor (GR) and inducing gene expression by binding to DNA glucocorticoid response elements (GREs). The right side shows an inflammatory stimulus (e.g. lipopolysaccharide, IL-1, tumour necrosis factor) activating protein kinase cascades (see Fig. 3) and inducing inflammatory response genes. (1) Transcriptional interference is due to the liganded GR directly binding the transcription factors activator protein-1 and nuclear factor-κB and inhibiting their action. (2) Glucocorticoid-induced genes, possibly MKPs, inhibit MAP kinase signalling pathways by keeping them in the dephosphorylated state. This would inhibit both transcriptional and post-transcriptional mechanisms underlying inflammatory gene expression. MAP = mitogen-activated protein; MKP = MAP kinase phosphatase.

access of transcription factors to their DNA binding sites [9]. The liganded GR bound to the transcriptional complexes inhibits their acetyltransferase activity. This could be an adjunct to the mechanism of tethering of transcription factors.

Glucocorticoids may also interfere with NF-κB activation by a mechanism dependent upon *de novo* gene expression. NF-κB is sequestered in the cytoplasm with an inhibitor, IκBα, whose degradation is induced by inflammatory stimuli. Production of this inhibitor is increased by dexamethasone [10,11]. This effect is slow and its physiological significance remains to be established.

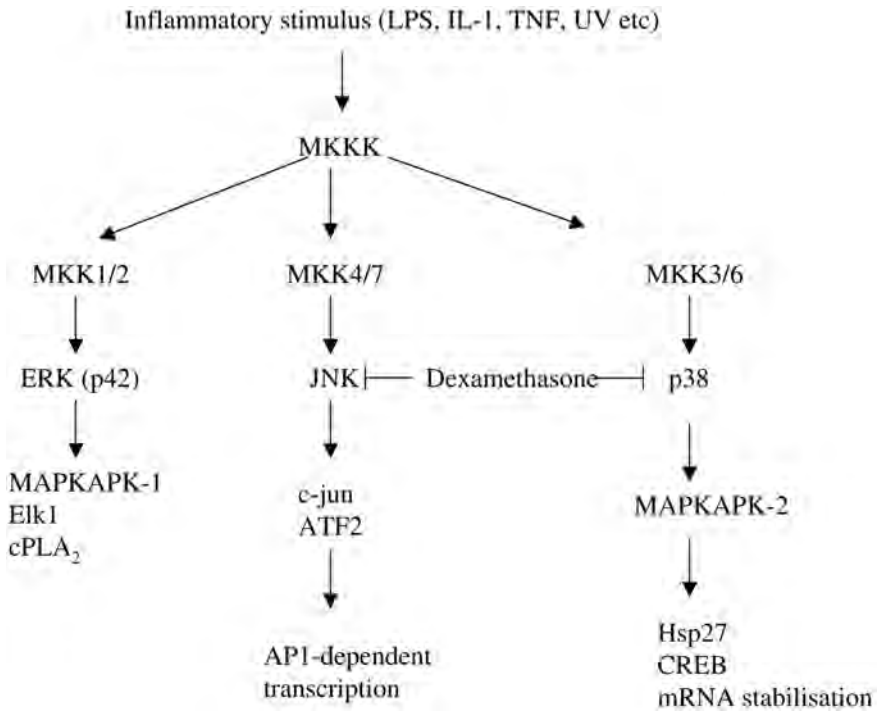
Dexamethasone interferes with pathways of mitogen-activated protein kinase

Recently, another possible mechanism for glucocorticoid action has become apparent: dexamethasone inhibits signalling in mitogen-activated protein kinase (MAP kinase) pathways, which are activated by inflammatory stimuli [12]. This suggests that glucocorticoids may block inflammatory signalling at a level above transcription factor activation. The effect, unlike transcriptional interference, requires gene induction, possibly of a MAP kinase phosphatase (MKP), and could inhibit both transcriptional and post-transcriptional mechanisms controlled by the MAP kinases (Fig. 2).

There are three types of MAP kinase [13] and they participate in distinct phosphorylation cascades (Fig. 3). The prototype MAP kinase is the extracellularly regulated kinase (ERK), or p42, which is activated by many stimuli. The other two, the c-Jun N-terminal kinase (JNK) and p38, are very strongly activated by inflammatory stimuli such as IL-1, tumour necrosis factor, and microbial products (e.g. lipopolysaccharide), and by cellular stress (e.g. UV). The MAP kinases are activated by their specific MAP kinase kinases (MKKs) by dual phosphorylation of a motif comprising a threonyl and a tyrosyl residue separated by a single amino acid. The MAP kinases phosphorylate various substrates, including other protein kinases. At the bottom of the cascades lie proteins controlling gene expression and other processes.

Serving to modulate the activity of MAP kinases and to switch off the pathways after the response to a stimulus, are MKPs [14]. These dual-specificity phosphatases remove phosphates from both the threonyl and tyrosyl residues in the MAP kinase activation motifs. More than 12 of these phosphatases are known to exist, differing in expression, subcellular localisation, and specificity for the different MAP kinases. Since removal of phosphate from either threonyl or tyrosyl residues inactivates the enzymes, they may also be regulated by phospho-

Figure 3



The MAP kinase cascades. This highly simplified scheme shows the three major MAP kinase pathways of mammalian cells. The MAP kinases are ERK, JNK, and p38. A few downstream substrates are shown. ATF = activating transcription factor; $cPLA_2$ = cytosolic phospholipase A_2; CREB = cAMP-response-element-binding protein; ERK = extracellularly regulated kinase; JNK = c-Jun N-terminal kinase; LPS = lipopolysaccharide; MAP = mitogen-activated protein; MAPKAPK = MAP kinase-activated protein kinase; MKK = MAP kinase kinase; MKKK = MAP kinase kinase kinase; TNF = tumour necrosis factor.

serine/phosphothreonine protein phosphatases and protein phosphotyrosine phosphatases.

The three MAP kinase pathways, in addition to the protein kinase system that activates NF-κB by phosphorylating the inhibitor IκB, are the major signalling systems through which genes of the inflammatory response are activated. Such genes are activated by several transcription factors and typically, as mentioned earlier, NF-κB and AP-1 are crucial. The JNK pathway is a major route for activation of c-Jun, and therefore of AP-1 complexes. The mRNAs of many cytokines and key regulators of the inflammatory response are unstable by virtue of clustered AUUUA motifs in their 3′ untranslated regions [15]. The p38 MAP kinase pathway stabilises such mRNAs [16–21]. It is presumed that downstream kinases regulate proteins that bind to the instability motifs.

The role of the p38 MAP kinase pathway has been elucidated by studying its effect on the function of the 3′ untranslated regions of the COX-2 [20] and IL-8 mRNAs [21]. Because dexamethasone destabilises some mRNAs, including the mRNA of COX-2 [22], its mechanism was investigated. It antagonised the stabilising action of the p38 MAP kinase on the COX-2 3′ UTR. This raised the possibility that dexamethasone was inhibiting p38 MAP kinase. There had been reports that glucocorticoids inhibited JNK activity in cells [23,24] and suppressed activity of the ERK cascade in mast cells [25]. Treating cells with dexamethasone for 1–2 hours before stimulation inhibited the activation of both p38 MAP kinase and JNK by UV light, by IL-1, or by bacterial lipopolysaccharide [12]. We know that this inhibition was at the level of the MAP kinase, because dexamethasone had no effect on the p38 activator, MKK6, and the inhibition could be circumvented by transfecting an active mutant of the substrate of p38, the MAP kinase-activated protein kinase-2 [12]. The effect of dexamethasone on the p38 MAP kinase was dependent upon GR and *de novo* mRNA synthesis.

An explanation for these findings is that steroids induce a protein phosphatase that keeps the p38 MAP kinase (and JNK) in the dephosphorylated state. Alternatively, the steroid could be inducing a molecule that inhibits the MKKs. It will be interesting to know the basis of the MAP kinase inhibition, its dependence on GRE-regulated genes, and its importance in inflammation relative to the mechanism of transcriptional interference.

Note added in proof

Glucocorticoid increasing expression of MKP-1 has recently been reported by Kassel *et al*. [26].

Acknowledgements

The author is grateful to Mrs Suzie Johns for preparation of the manuscript, to the many authors whose work has not been directly cited in this short review, and to the Arthritis Research Campaign and the Medical Research Campaign for financial support.

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Respiratory Medicine (2012) **106**, 9–14

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REVIEW

The role of mast cells in allergic inflammation

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Received 3 April 2011; accepted 20 September 2011
Available online 22 November 2011

KEYWORDS
Allergic asthma;
Mast cells;
Inflammation;
Cytokines;
Remodeling;
B- and T-lymphocytes

Summary
The histochemical characteristics of human basophils and tissue mast cells were described over a century ago by Paul Ehrlich. When mast cells are activated by an allergen that binds to serum IgE attached to their FcεRI receptors, they release cytokines, eicosanoids and their secretory granules. Mast cells are now thought to exert critical proinflammatory functions, as well as potential immunoregulatory roles, in various immune disorders through the release of mediators such as histamine, leukotrienes, cytokines chemokines, and neutral proteases (chymase and tryptase). The aim of this review is to describe the role of mast cells in allergic inflammation.

Mast cells interact directly with bacteria and appear to play a vital role in host defense against pathogens. Drugs, such as glucocorticoids, cyclosporine and cromolyn have been shown to have inhibitory effects on mast cell degranulation and mediator release. This review shows that mast cells play an active role in such diverse diseases as asthma, rhinitis, middle ear infection, and pulmonary fibrosis.

In conclusion, mast cells may not only contribute to the chronic airway inflammatory response, remodeling and symptomatology, but they may also have a central role in the initiation of the allergic immune response, that is providing signals inducing IgE synthesis by B-lymphocytes and inducing Th2 lymphocyte differentiation.

Abbreviations: AA, allergic asthma; APC, antigen presenting cell; BAL, bronchoalveolar lavage; CTMC, connective tissue mast cell; ECM, extracellular matrix; ELAM-1, endothelial-leukocyte adhesion molecule-1; FcεRI, high-affinity IgE receptors I; ICAM-1, intercellular adhesion molecule; INF-γ, interferon-gamma; IL, interleukin; LTs, leukotriences; MC, mast cell; MMC, Mucosal mast cell; PGs, prostaglandins, platelet-activating factor; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule.

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doi:10.1016/j.rmed.2011.09.007

Contents

Introduction

Mast cells are found in the skin and in all mucosal tissues at homeostasis, and numbers are elevated in asthmatics lungs[1] and gastrointestinal tract of inflammatory bowel disease. Mast cells were first described by Ehrlich in his 1878 doctoral thesis on the basis of their unique staining characteristics and large granules, that gave them their name, "Mastzellen" which means well-fed cells, because their cytoplasm was stuffed with granular material. Mast cells are now considered to be part of the immune system. The mast cell was identified as a mesenchymal cell which is stained metachromatically with some blue dyes and it was recognized several years later that these cells contained in their granules the majority of the body's histamine.[2] Mast cells play a central role in inflammatory and immediate allergic reactions. They are able to release potent inflammatory mediators, such as histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid that act on the vasculature, smooth muscle, connective tissue, mucous glands and inflammatory cells.[3] Histamine is not only released when the body encounters a toxic substance, it is also released when mast cells detect injury. It causes nearby blood vessels to dilate allowing more blood to reach the site of the injury or infection. Mast cells are localized in the connective tissue and do not usually circulate in the blood stream.

The aim of this review is to discuss the effects of different Th2 cytokines on mast cell development, and the contribution of these cells to the chronic airway inflammatory response, tissue remodeling and symptomatology, and also to understand the role of these cells at the initiation of the allergic immune response, where they provide signals inducing IgE synthesis by B-lymphocytes and Th2 lymphocyte differentiation.

Mast cell development and differentiation

Mast cells arise in the bone marrow where maturation is influenced by stem cell factor binding to the receptor c-kit and by other cytokines such as interleukin (IL)-3, IL-4, IL-9, and IL-10. These cytokines promote differentiation and proliferation of both human and mouse mast cells.[4–7] The SCF receptor (c-kit) plays an important role in the hematopoiesis during embryonic development. Mast cell is the only terminally differentiated hematopoietic cell that expresses the c-Kit receptor. In addition, SCF promotes mast cell adhesion, migration, proliferation, and survival.[8] SCF also promotes the release of histamine and tryptase, which are involved in the allergic response. Mast cell progenitors leave the bone marrow and settle in various tissues dependent of stimulation.[9] Two types of mast cells, mucosal and connective tissue mast cells, were reported in rodent tissue in the 1960's on the basis of histochemical and fixation characteristics that reflect, in part, whether heparin proteoglycan was present in secretory granules. Mucosal mast cell (MMC) granules stain blue with copper phthalocyanin dyes, such as Astra blue or Alcian blue, in a staining sequence with safranin, while connective tissue mast cell (MCTC) granules stain red.[10] MC(T) and MC(TC) types of human mast cells (MCs) are distinguished from one another on the basis of the protease compositions of their secretory granules, but their structural, functional differences and developmental relationships have been well characterized by other authors.[11,12] Mast cells are long-lived, surviving for month or even years, in the tissue. Evolutionary, mast cells existed and participated in host defense long before the development of cells of adaptive immune system. Increased numbers of MCT and MCTC mast cells are seen in fibrotic diseases whereas its numbers are relatively unchanged in allergic or parasitic diseases and in HIV infection.[13] The presence of these MCTC cells could help explain why patients with HIV infection continue to have allergic reactions. The MCTC mast cell, however, expresses tryptase, chymase. It tends to predominate in the respiratory tract, gastrointestinal tract as well as in skin, synovium, and subcutaneous tissue.

Mast cell activation and mediator production

The cytoplasm of mast cells contains organelles: lipid bodies where metabolism of arachidonic acid occurs and where the products of this metabolism, including leukotrienes, are stored.[14] Cytokines and histamine are other products found in mast cells organelles (Fig. 1). These organelles are prone to exocytosis and extracellular release of mediators. The release may be induced by: (a) chemical substances, such as toxins, venoms, and proteases; (b) endogenous mediators, including tissue proteases, cationic proteins derived from eosinophils and neutrophils; (c) immune mechanisms that may be IgE-dependent or IgE-independent. IgE-dependent degranulation is a consequence of the preferential production of IgE, in response to certain antigens (allergens). During an allergic response IgE release from B-cells will bind to mast

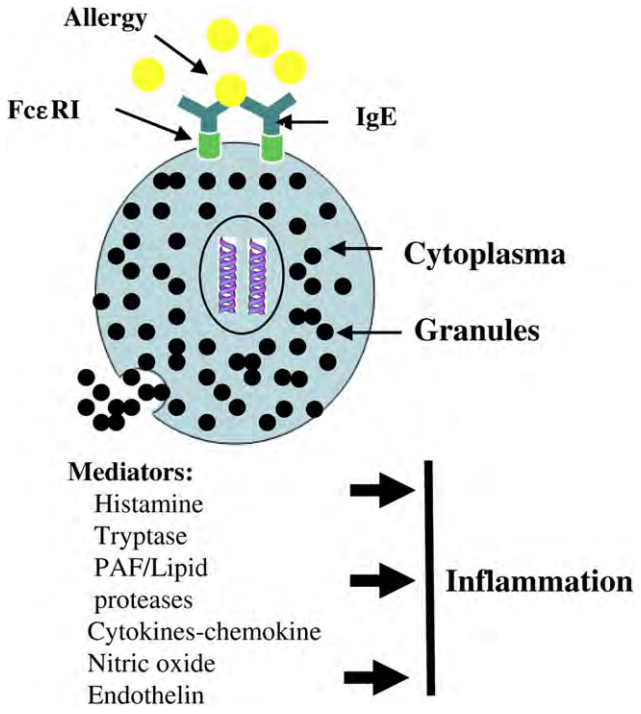

Figure 1 The IgE-primed mast cell releases granules and powerful chemical mediators, such as histamine, cytokines, granulocyte macrophage colony-stimulating factor (GM-CSF), leukotrienes, heparin, and many proteases into the environment. These chemical mediators cause the characteristic symptoms of allergy.

cells, blanketing the plasma membranes of these immune cells. Half a million IgE molecules coat the surface of mast cells, binding to the high-affinity IgE receptors (FcεRI) on membranes with the Fc portion. This leaves their Fab, or antigen binding segment, free to bind the antigen.[9,13,15,16] A subsequent exposure to the same allergen cross-links the cell-bound IgE and triggers the release of preformed prostaglandins, histamines and cytokines (Fig. 2).[9,14,17–19] Mast cell degranulation is preceded by increased Ca^{2+} influx, which is a crucial process; ionophores that increase cytoplasmic Ca^{2+} also promote degranulation, whereas agents which deplete cytoplasmic Ca^{2+} suppress degranulation (Fig. 2).[18,20] Additionally, in some cases, other ligand—receptor interactions, summarized in Fig. 1, can lead to mast cell degranulation.

Newly generated mediators, often absent in the resting mast cells, are as well produced during IgE-mediated activation, and consist of arachidonic acid metabolites, principally leukotriene C_4 (LTC_4), prostaglandin D_2 (PGD_2) and of cytokines.[21–23] Of particular interest in humans is the production of tumor necrosis factor (TNF-α, β), and interleukin (IL)-4, IL-5, IL-6, IL-1β and IL-13.[9,24–26] Those lipid mediators and cytokines and preformed histamine, can have profound effects on vascular endothelium, including the alteration of vascular permeability and adhesiveness. This can allow other circulating inflammatory cells to adhere to the endothelium and to migrate into the surrounding tissue. Cytokines and lipid mediators do as well elicit a direct influence on lymphocytes and macrophages in the murine system.[27] IL-4, IL-5 and IL-6 stimulates the proliferation and differentiation of activated B-cells, and induces class switch.[9,28,29] However, B-cells stimulated with IL-5 become plasma cells secreting IgA. IL-5 is also very important in stimulating growth and differentiation of eosinophils.[30–33] The production of cytokines by human mast cells has not been as extensively studied as in rodents, but several studies suggest that it has a similar pattern. For example, human mast cells have been shown to produce IL-4, IL-5, and IL-6.[30,31] In addition, mast cells produce several neutral proteases including tryptase and chymase that potentially damage and activate the bronchial epithelium, and may contribute to airway wall remodeling. Thus, mast cells are key players in host defense, with a role in immune surveillance, phagocytosis, and immune activation.

Functions of mast cells in physiological and pathological states

The biological function of mast cell neutral proteases remains to be fully clarified. In serum, elevated levels of tryptase are detected in systemic mast cell disorders, such as anaphylaxis and mastocytosis. Ongoing mast cell activation in asthma appears to be a characteristic of the chronic inflammatory nature of the disease. Activation is detected by elevated levels of tryptase and PGD_2 in bronchoalveolar lavage (BAL) and higher spontaneous release of histamine by mast cells obtained from the BAL of asthmatics than those obtained from non asthmatics.[21] Ultrastructural analysis of mast cells in lung tissue also shows that asthmatics have more degranulation than atopic nonasthmatics.[34] The number of the cells increases at sites of inflammation. To reach these areas, mast cell progenitors must migrate from the blood into tissue sites. A crucial step in this process is the adherence of cells to the endothelium. Cell adherence is mediated by several families of adhesion molecules and adhesion receptors on the surface of mast cells that can mediate binding to other cells and to extracellular matrix (ECM) glycoprotein. Upon stimulation, mast cells release cytokines, including TNF-α and IL-4 that can modulate adhesion molecules on endothelial cells. Activated endothelial cells express the intercellular adhesion molecule (ICAM-1), endothelial-leukocyte adhesion molecule-1 (ELAM-1) and vascular cell adhesion molecule (VCAM-1) on their cell surface.[35,36] Human mast cells express integrins as receptors for these molecules. Until recently, the effects of adherence on cell function were believed to result only from changes in cell shape and cytoskeletal organization. However, in addition to cell spreading, aggregated adhesion receptors transduce a variety of intracellular signals that regulate cell function. These signals include protein tyrosine phosphorylation, phosphoinositide hydrolysis and changes in intracellular pH or calcium concentration and the expression of several genes. The adhesion properties of the cells regulate their migration, localization, proliferation and phenotype. Recently, murine mast cells have been implicated in the mediation of inflammatory responses at long distances. TNF alpha containing particles released by the cells were found transported through draining lymphatic system activating cells in the lymph nodes.[37]

Different mechanisms could contribute to the increase in the number of mast cells at the sites of tissue injury: mast

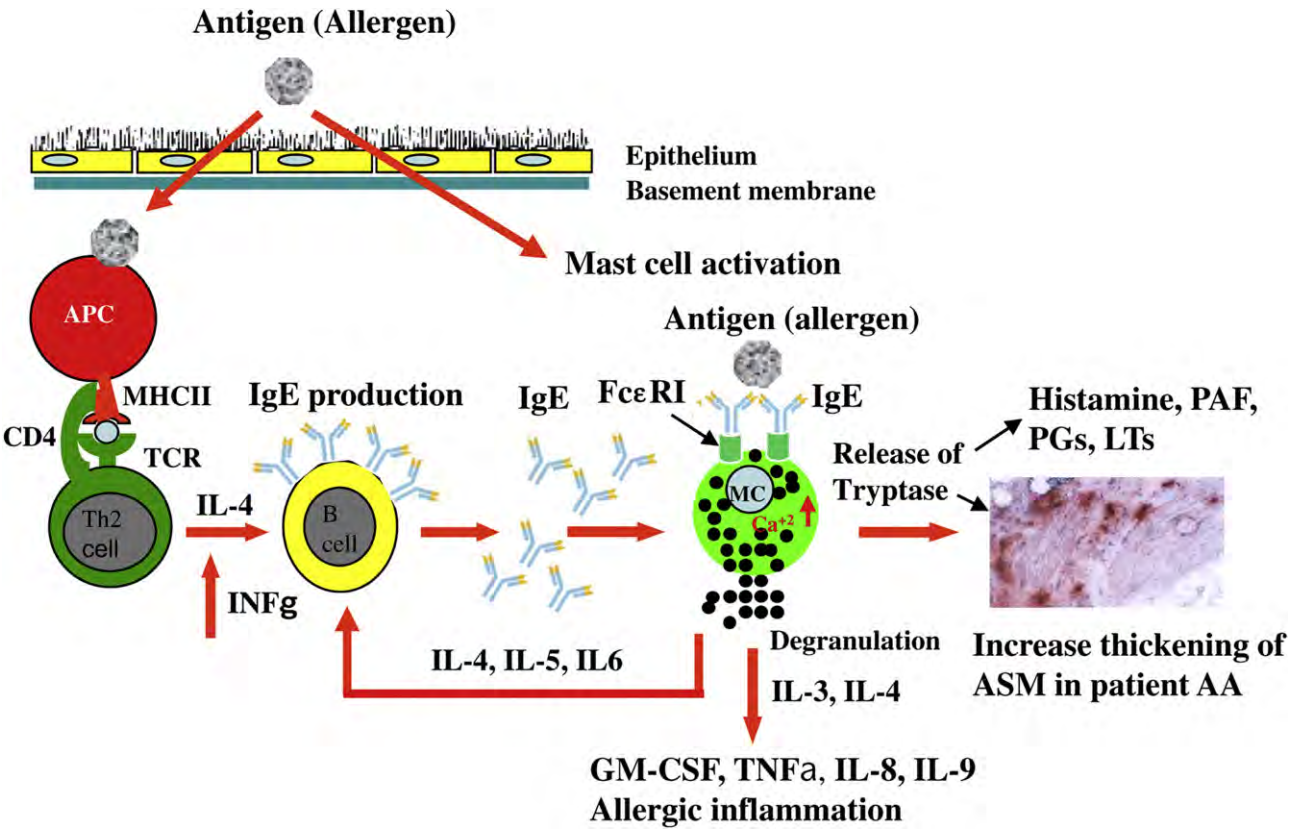

Figure 2 Induction and effector mechanisms in type I hypersensitivity.

cells or their progenitors could migrate to these sites; or resident mast cell precursors could proliferate. Adhesion receptors and their ligands also play a role in the localization and migration of mast cells in normal tissues. ECM proteins that are the ligands for adhesion receptors are chemotactic for mast cells. Adherence of mast cells to fibroblasts, other cells or to ECM proteins can transduce signals that affect cell growth and differentiation. The increase in the number of mast cells,[38,39] and the enhanced secretion at sites of inflammation, can accelerate the elimination of the cause of tissue injury or, paradoxically, may lead to a chronic inflammatory response. Thus, manipulating mast cell adhesion may be an important strategy in controlling the outcome of allergic and inflammatory responses.

In a previous study by us,[1] Mast cell numbers were increased in both allergic and non-allergic asthma. Similar to a more recent study, we found[17] infiltration of mast cells in of the bronchi of both allergic asthmatics and non-allergic asthmatics[40] but the accumulation of mast cells was more pronounced for the allergic asthmatics. We did also find that mast cells in the bronchial mucosa of the allergic asthmatics showed more signs of activation with extracellular deposition of tryptase than mast cells from non-allergic asthmatics.[1] Signs of mast cell activation in allergic asthma have been indicated by others.[40] However, in that study we could not observe any differences between allergic and non-allergic asthmatics.[40] This could be explained by limited number of non-allergic asthmatics included in the study. Specific allergens and their reaction with IgE on the mast cells might provide the mechanisms for activation in the bronchial mucosa in allergic asthma, especially since all those patients were sensitized to perennial allergens such as from pets.[41]

Also, other authors found inflammatory cells in bronchial mucosa in subjects with toluene Diisocyanate (TDI) induced asthma.[42] The mast cell, which is one of the inflammatory cells, plays an important role in TDI activation because the activation of mast cells is associated with TDI-induced early and late asthmatic reaction.[42,43]

Mast cells are increased in number in many fibrotic diseases and may play a crucial role in the development of fibrosis.[44,45] The percentage of human mast cells in BAL fluid from patients with sarcoidosis or interstitial fibrosis is greater than in BAL fluid from healthy individuals,[44,46] and patients with idiopathic interstitial pulmonary fibrosis show evidence of mast cell degranulation and elevated mast cell numbers.[47]

Concluding in a previous study show that Mast cells are located in connective tissue, including the lung, skin, the linings of the stomach and intestine, and other sites. They play an important role in helping defend these tissues from diseases. By releasing chemical such as histamine, mast cells attract other key players of the immune defense system to areas of the body where they are needed.

Mast cells and airway remodeling

Tissue remodeling is characteristic feature of asthma and other lung diseases. The mechanisms behind this relationship between mast cells and fibrosis/tissue remodeling are

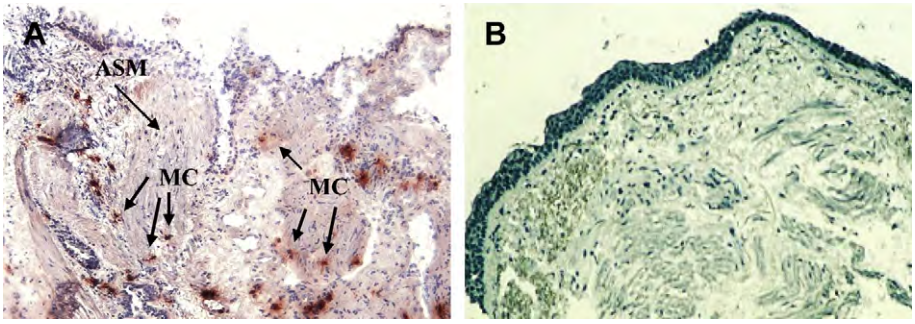

Figure 3 Staining of tissue mast cells with anti-tryptase antibody 1 (AA1) in (A) airway smooth muscle (arrow) of patients with allergic asthma and (B) control subjects, respectively. (Mayer's hematoxylin). Original magnification: ×40. The scale bars 50 μm for A and B are shown in the figures.

unclear. We have shown that mast cells may have a substantial effect on tissue remodeling, especially in the airway, on smooth muscle hypertrophy and on mucus hypersecretion, by releasing proteases such as tryptase, and growth factors.[17] These cells also have an effect on epithelial damage, and on basement membrane thickening in patients with allergic asthma,[1,48] allergic rhinitis,[49] and middle ear infection in allergy patients,[50,51] mast cells related to airway smooth muscle hypertrophy, compared to healthy controls.[17] Fig. 3 shows that mast cells specifically are localized within or close to airway smooth muscle bundles in patients with allergic asthma whereas little or no mast cells are found in the airways of healthy controls (Fig. 3). Tryptase and other proteases such as chymase are abundant in mast cell granules. Therefore, mast cells seem to play a crucial role in airway remodeling by releasing tryptase onto smooth muscle and epithelium, and may play a role in skin tissue remodeling by releasing chymase in an IgE-dependent manner in allergic diseases.

These data suggest multiple mechanisms and multiple levels in different organs in the human body, where mast cells can regulate tissue fibrosis and repair, and provide evidence for the direct involvement of mast cells in fibrosis and human connective tissue remodeling.

Conclusions

Mast cells are fascinating, multifunctional, bone marrow-derived, tissue-dwelling cells. They can be activated to degranulate in minutes, not only by IgE and antigen signaling via the high-affinity receptor for IgE, but also by a diverse group of stimuli. These cells can release a wide variety of immune mediators, including an expanding list of cytokines, chemokines, and growth factors. Mast cells have been shown to play roles in allergic inflammation and, more recently, they have been shown to modulate coagulation cascades, host defense, and tissue remodeling. The role of mast cells in asthma and other diseases is being actively studied.

This review suggests that mast cells may not only contribute to the chronic airway inflammatory response, airway remodeling and symptomatology, but may also have a central role at the initiation of the allergic immune response, that is, providing signals inducing B cell IgE synthesis and Th2 lymphocyte differentiation. Th-targeted therapy would be of considerable interest in controlling allergic asthma. Having more knowledge and resources about mast cells can lead to finding cures to diseases caused by the mast cells.

Acknowledgments

I am thankful for Professor Godfried M. Roomans, Professor Christer Jonson and Dr Jonas Bystrom for taking the time to read and give valuable suggestions to the review. I thank the following institution for kindly giving us permission to publish results obtained at their sites. Images of bronchial biopsies were obtained at the Department of Respiratory Medicine and Allergology and Clinical Chemistry at Uppsala Academic Hospital, Uppsala, Sweden. This study was supported by the foundation Lily and Ragnar Åkerhams foundation and grants from the Swedish Heart and Lung Foundation.

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CARFAX

Mediators of Inflammation, 7, 229–237 (1998)

Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma

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GLUCOCORTICOIDS are potent inhibitors of inflammatory processes and are widely used in the treatment of asthma. The anti-inflammatory effects are mediated either by direct binding of the glucocorticoid/glucocorticoid receptor complex to glucocorticoid responsive elements in the promoter region of genes, or by an interaction of this complex with other transcription factors, in particular activating protein-1 or nuclear factor-κB. Glucocorticoids inhibit many inflammation-associated molecules such as cytokines, chemokines, arachidonic acid metabolites, and adhesion molecules. In contrast, anti-inflammatory mediators often are up-regulated by glucocorticoids. *In vivo* studies have shown that treatment of asthmatic patients with inhaled glucocorticoids inhibits the bronchial inflammation and simultaneously improves their lung function. In this review, our current knowledge of the mechanism of action of glucocorticoids and their anti-inflammatory potential in asthma is described. Since bronchial epithelial cells may be important targets for glucocorticoid therapy in asthma, the effects of glucocorticoids on epithelial expressed inflammatory genes will be emphasized.

Key words: Glucocorticoids, Asthma, Bronchial epithelial cells, Inflammation

Introduction

Glucocorticoids are hormones synthesized in the adrenal cortex and secreted into the blood, where the levels of glucocorticoids fluctuate in a circadian mode. In humans, the naturally occurring glucocorticoid is hydrocortisone (cortisol), which is synthesized from its precursor cortisone.

The beneficial effects of glucocorticoids in asthmatic patients were first described in 1950.[1] Since then on, many studies have focused on the therapeutic potential of glucocorticoids. Several synthetic glucocorticoids, much more potent than cortisol and without the unwanted mineralocorticoid side effects, have been developed. Nowadays, glucocorticoids are powerful agents in the treatment of inflammatory diseases and are by far the most effective anti-inflammatory drugs used in the treatment of asthma.

Mechanism of Action

Although glucocorticoids have been known for a long period of time, their precise mechanism of action is still not completely understood. However, recent studies have increased our understanding of their complex mechanisms of action.

Glucocorticoid receptor

To exert their effects, glucocorticoids need to bind to a specific cytoplasmic glucocorticoid receptor (GR). Almost all cells of the body express the GR, but the number of receptors may vary between different cell types.[2] Cloning of the GR has revealed that the GR consists of approximately 800 amino acid residues, and that certain areas of the molecule show homology with other steroid receptors, receptors for thyroid hormones, and receptors for retinoic acid.[3–7] All members of the nuclear hormone receptor family share a characteristic three-domain structure, first described for the human GR. The C-terminal domain is equal in size in all nuclear receptors studied (about 250 amino acids) and its main function is to bind the steroid.[8] It also contains the binding sites for the heat shock proteins (hsp) 90.[9,10] Removal of the steroid-binding domain results in a constitutively active GR molecule, indicating that this part of the molecule acts as a repressor of the transcription-activation function. The most conserved central domain is involved in direct binding of the receptor to DNA. It contains two distinct loops of protein, each bound at their base via four cysteine residues to a single zinc ion, the so-called zinc fingers.[11] These zinc clusters are involved in binding of the GR to the major groove of the DNA double helix and play a role in dimeriza-

tion of two GR molecules.[12,13] In addition, the central DNA-binding domain has a transcription-activation function.[4,14] The steroid-binding and DNA-binding domains are separated by the 'hinge-region', which contains sequences that are important for nuclear translocation and dimerization.[9,10] The N-terminal domain is extremely variable in size (24–600 amino acids). Its precise role is still uncertain, but it is required in transcriptional activation.[15]

Two different forms of the human GR have been described.[3,16] These two highly homologous isoforms, termed GRα and GRβ, are generated by alternative splicing of the human GR pre-mRNA. The GRβ isoform differs from the GRα isoform only in its C-terminal domain, in which the last 50 amino acids of the latter are replaced by a unique 15 amino acid sequence. However, this replacement has dramatic functional consequences, since the GRβ isoform is unable to bind glucocorticoids and to transduce ligand-dependent transactivation. However, the physiological significance of the GRβ isoform remains questionable, since some recent studies indicate that this form is not conserved among species and no dominant negative inhibition of GRα activity could be found.[17,18] Nevertheless, abundant expression of GRβ protein can be found in the epithelial cells lining the terminal bronchioli of the lung.[19]

The expression of the GR may be regulated by numerous factors either at the transcriptional, translational or post-translational level.[20,21] Glucocorticoids have been shown to down-regulate the expression of the GR, both *in vitro* and *in vivo*.[22,23] In contrast, inflammatory mediators like interleukin (IL)-1β, IL-4, tumour necrosis factor (TNF)-α, lipopolysaccharide (LPS) and interferon (IFN)-γ have been shown to increase glucocorticoid binding *in vitro*.[24–28] However, the increase in GR numbers may be accompanied by a reduced affinity for glucocorticoids.[24,28] Analysis of GR localization in normal and asthmatic lung has not revealed differences in the level or sites of GR expression.[29]

Regulation of gene transcription

In the absence of glucocorticoids, the GR is present in the cytoplasm of the cell as a hetero-oligomer consisting of the GR itself, two molecules of hsp 90, one molecule hsp 70, and one molecule of hsp 56 (which probably does not interact with the GR itself, but interacts with hsp 90).[30–34] Glucocorticoids enter the cytoplasm of the cell by passive diffusion through the cell membrane. In the cytoplasm they bind to the GR complex, which subsequently undergoes conformational changes, resulting in the dissociation of the hsp 90 and hsp 56 molecules. Upon this activation, the glucocorticoid-GR complex passes the nuclear membrane, enters the nucleus, and the hsp 70 molecule is dissociated.

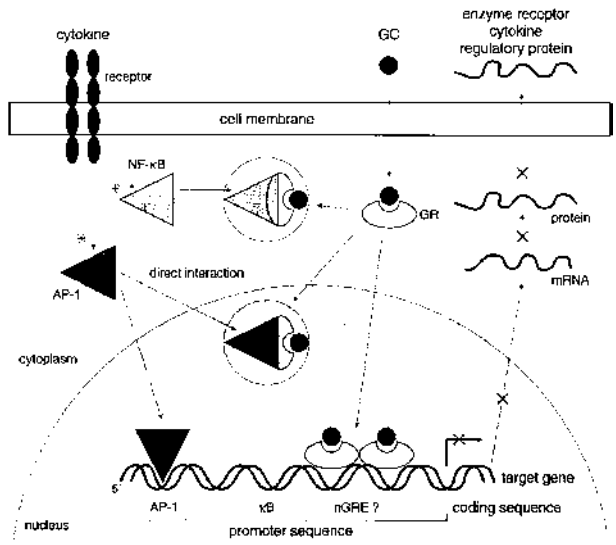

FIG. 1. Schematic representation of the cellular events after administration of glucocorticoids (adapted from Ref. 39).

Furthermore, in the nucleus liganded GR form homodimers (Fig. 1).

Within the nucleus, the GR homodimers may regulate gene transcription in several ways: (1) via binding of the glucocorticoid-GR complex to specific DNA sequences, thereby directly activating or repressing genes; (2) via interaction with other transcription factors; and (3) via modulating the stability of specific mRNA molecules.[35–39]

Binding to DNA sequences

Several steroid-responsive genes contain glucocorticoid responsive elements (GRE) in their promoter region.[35,40] Binding of GR homodimers to GRE may either result in transcriptional activation of the gene (via a *positive* GRE) or repression of the gene (via a *negative* GRE) (Fig. 1). The consensus sequence for (positive) GRE is the palindromic 15-base-pair sequence GGTACAnnnTGTTCT, whereas the negative GRE has a more variable sequence.[36] The rate of transcriptional regulation of steroid-responsive genes is dependent on both the numbers of GRE, the affinity of the glucocorticoid-GR complex to the GRE, and the position of the GRE relative to the transcriptional start site. Binding of the complex to GRE may result in conformational changes in the DNA and exposure of previously masked areas, resulting in increased binding of other transcription factors.[41–44]

Interaction with other transcription factors

Many steroid-responsive genes do not have GRE in their promoter region. However, binding sites for other transcription factors, including nuclear factor (NF)-κB, activating protein (AP)-1, and cAMP-responsive element binding protein (CREB), often can be found.[45]

AP-1, which is a dimer of two proto-oncogenes (members of the c-jun and c-fos family),[46,47] is involved in the regulation of several genes, including adhesion molecules and cytokines (reviewed in Ref. 47). Direct protein–protein interaction between AP-1 and the glucocorticoid-GR complex results in reciprocal repression of one another's transcriptional activation by preventing binding of the AP-1 and glucocorticoid-GR complex to AP-1 sites and GRE, respectively (Fig. 1).[37,48,49]

Comparable to AP-1, NF-κB (a heterodimer of p50 and p65 subunits[50,51]) regulates the transcription of several genes involved in inflammatory reactions.[50,52,53] In unstimulated cells, NF-κB is retained in the cytoplasm of the cells through the interaction with the inhibitors IκBα and IκBβ.[54–56] Upon cell stimulation, for example by IL-1β or TNF-α, IκB are rapidly phosphorylated, ubiquitinated, and consequently proteolysed.[53,57] The liberated NF-κB dimers translocate to the nucleus where they can activate target genes. Glucocorticoids may inhibit NF-κB-stimulated genes by a direct interaction between the glucocorticoid-GR complex and the p65 subunit of NF-κB, resulting in transrepression (Fig. 1).[51,55,58,59] Furthermore, glucocorticoids may indirectly antagonize NF-κB mediated transcription by up-regulating the synthesis of the inhibitory protein IκBα, which traps NF-κB in inactive cytoplasmic complexes.[39,54,55] A large number of immunoregulatory genes, whose expression is induced by a variety of pro-inflammatory mediators, contain NF-κB sites in their promoters/regulatory regions. Therefore, it is no wonder that glucocorticoids have been found to prevent the expression of these genes, including those coding for IL-1β, IL-2, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, RANTES (Regulated upon Activation, Normal T cell Expressed, and presumably Secreted), granulocyte macrophage colony-stimulating factor (GM-CSF), the IL-2 receptor, intercellular adhesion molecule (ICAM)-1, and E-selectin (reviewed in Ref. 45). Probably, interactions between glucocorticoids and NF-κB or AP-1 will explain most of the anti-inflammatory and immunosuppressive activities of glucocorticoids.

An interaction between CREB and the glucocorticoid-GR complex has also been suggested.[60,61] β-agonists, which are used as bronchodilators in the treatment of asthma, increase cAMP formation and subsequently activate CREB. Therefore, simultaneous treatment of asthmatic patients with glucocorticoids and β-agonists may result in reduced responsiveness of the airways for steroids.[61–63]

Modulation of mRNA stability.
A third mechanism by which glucocorticoids may regulate the synthesis of proteins is via enhanced transcription of specific ribonucleases which are able to degrade mRNA containing constitutive AU-rich sequences in the untranslated 3′-region.[64] Such glucocorticoid-mediated modulation of post-translational events (resulting in decreased mRNA stability and reduced half-life time) has been observed for IL-1β, IL-6 and GM-CSF.[65,66]

Table 1. Influence of glucocorticoids on the synthesis of proteins with inflammatory effects by bronchial epithelial cells

Protein	Glucocorticoid effect
Cytokines	
IL-1β, IL-6, IL-11, TNF-α, GM-CSF	↓
IL-10, LIF	?
G-CSF	=
Chemokines	
MCP-1, eotaxin, IL-8, RANTES, MIP-1α	↓
Receptors	
NK, GR	↑
IL-1R II, IL-6R, β_2-adrenergic receptor	↓
Enzymes	
iNOS, COX-2, $cPLA_2$	↓
NEP	↑
Adhesion molecules	
ICAM-1	↓
Inhibitory proteins	
Lc-1	=/↑
IL-1RA type I, SPLI	↑

Glucocorticoid Regulated Genes

Glucocorticoids are able to modulate the transcription of a variety of genes, including cytokines and chemokines, receptors, enzymes, adhesion molecules, and inhibitory proteins (Table 1). Since epithelial cells may be one of the most important targets for glucocorticoid therapy in asthma, the effects of glucocorticoids on epithelial expressed inflammatory genes will be emphasized in this review.

Cytokines and chemokines

Glucocorticoids inhibit the transcription of most cytokines and chemokines that are relevant in asthma, including IL-1β, TNF-α, GM-CSF, IL-3, IL-4, IL-5, IL-6, IL-8, IL-11, IL-12, IL-13, RANTES, eotaxin, and macrophage inhibitory protein (MIP)-1α.[45,66] In general, reduced synthesis of these mediators may result in a decreased recruitment and activation of leukocytes, also indirectly due to effects on adhesion molecules and cell survival. Since many cytokine gene promoters do not contain a negative GRE, the effects of glucocorticoids on cytokine and chemokine production are probably mediated via an effect on a critical transcription factor (especially NF-κB and AP-1).[67] Cross-signalling between NF-κB and AP-1 with glucocorticoid/GR complex have indeed been demonstrated in bronchial epithelial cells.[67]

Bronchial epithelial cells are capable of producing a variety of cytokines and chemokines that may contribute to the initiation and perpetuation of airway inflammation. Several studies have shown that cytokine-induced expression of eotaxin, IL-6, IL-8, GM-CSF, and RANTES can be diminished by glucocorticoids *in vitro*.[68–76] In contrast, glucocorticoids did not modulate the secretion of G-CSF by human bronchial epithelial cells.[76] *In vivo* studies have shown that treatment with inhaled steroids decreases both the expression of GM-CSF,[77] IL-1β,[78] IL-8,[79] and RANTES[80] by the bronchial epithelium, together with the number of activated eosinophils in the epithelium.

Receptors

Glucocorticoids may modulate the expression of several receptors. The expression of the neurokinin $(NK)_1$ receptor, which mediates many effects of substance P (SP) in the airways and is believed to be up-regulated in asthma,[81] is down-regulated by glucocorticoids.[82] Since the NK_1 receptor gene promoter region has no GRE but has an AP-1 response element, this effect probably will be mediated via an interaction of the glucocorticoid-GR complex with AP-1.

In contrast to NK_1 receptors, expression of the β_2-adrenergic receptor is increased by glucocorticoids.[83] Since the human β_2-adrenergic receptor gene contains three potential GRE, this effect of glucocorticoids probably is a direct one.[83] Up-regulation of β_2-adrenergic receptors by glucocorticoids may be relevant in asthma as it may prevent down-regulation in response to prolonged treatment with β_2-agonists.[84]

The IL-1 receptor type II, which functions as a decoy receptor,[85] may also be up-regulated by glucocorticoids, thereby reducing the functional activity of IL-1 agonists.[86,87] Soluble TNF-receptor type I (p55) release by human bronchial epithelial cells, both constitutive as well as IL-1β-induced, has been shown to be reduced by glucocorticoids.[88] In contrast, glucocorticoids up-regulate the expression of IL-6 receptors in rat hepatoma and human epithelial cells.[89,90] Thus far little is known about this process in human bronchial epithelial cells, which constitutively express these receptors.[91]

Glucocorticoids also modulate the expression of their own receptor. In a recent study it was shown that expression of the α-form (but not the β-form) of the GR in human bronchial epithelial cells was down-regulated in healthy subjects after 4 weeks of budesonide inhalation.[23]

Enzymes

Glucocorticoids inhibit the synthesis of several inflammatory mediators implicated in the pathogenesis of asthma through an inhibitory effect on enzyme induction. The synthesis of inducible nitric oxide synthase (iNOS) by human airway epithelial cells is inhibited by glucocorticoids, both *in vitro* and *in vivo*.[92–94] This effect seems to be mediated via inactivation of NF-κB.[95,96] Since nitric oxide (NO) may contribute to skewing of Th lymphocytes towards a Th2 phenotype, thereby promoting IgE production and eosinophil recruitment, inhibition of iNOS may be of importance in anti-inflammatory therapy in asthma.[97]

Glucocorticoids also inhibit the gene transcription of a cytosolic form of phospholipase A_2 induced by cytokines[98] and inhibit the gene expression of cyclooxygenase-2, resulting in reduced formation of prostaglandins and thromboxanes.[99]

In contrast to the enzymes mentioned above, glucocorticoids have been shown to increase the expression of neutral endopeptidase (NEP),[100–102] thereby potentially limiting neurogenic inflammatory responses.[103] In accordance with these results, it was found that the expression of NEP on bronchial epithelial cells was higher in asthmatics treated with steroids compared with nonsteroid-treated asthmatics.[104]

Endothelins

Endothelins are a family of highly homologous 21-amino acid peptides, characterized by two intrachain disulphide chains, a hairpin loop consisting of polar amino acids, and a hydrophobic C-terminal chain.[105] Human bronchial epithelial cells have been shown to produce ET-1,[106–108] which promotes the proliferation of smooth muscle cells, is a potent constrictor of both vascular and non-vascular smooth muscle cells, increases the secretion of mucus, and may activate inflammatory cells.[105,107,109] ET-1 also stimulates collagen gene expression and through its inhibitory actions on collagenase will promote airway wall collagen deposition, thereby contributing to airway wall thickening which underlies bronchial hyperresponsiveness.[110–112] Increased levels of ET-1-immunoreactivity were detected in airway epithelium and vascular endothelium of bronchial biopsy specimens from nonsteroid-treated asthmatics compared with healthy subjects.[106,113,114] In contrast, no increased ET-1 expression was found in the bronchial epithelium of asthmatic patients treated with glucocorticoids.[115]

Adhesion molecules

Adhesion molecules play an important role in the recruitment of inflammatory cells to the inflammatory locus. Expression of adhesion molecules on endothelial, epithelial or inflammatory cells is often

induced by cytokines, whereas glucocorticoids reduce surface expression of adhesion molecules. This effect may be due either to inhibition of cytokine synthesis or to a direct effect of glucocorticoids on adhesion molecule gene transcription. It has been shown that the expression of ICAM-1, endothelial leukocyte adhesion molecule (ELAM)-1, and E-selectin is down-regulated by steroids.[116] Basal and cytokine-stimulated ICAM-1 expression on human bronchial epithelial cell lines is inhibited by glucocorticoids.[117,118] However, inhaled glucocorticoids did not modulate ICAM-1 expression by bronchial epithelial cells from asthmatics *in vivo*.[119]

Eosinophil adhesion to cytokine-stimulated bronchial epithelial cells was shown to be inhibited by the synthetic glucocorticoid dexamethasone.[120] Although cytokine-activated epithelial cells showed increased expression of ICAM-1, this molecule did not seem to be involved in the decreased adhesion of eosinophils observed in the presence of dexamethasone.[120]

Inhibitory proteins

The anti-inflammatory effects of glucocorticoids may be mediated by increasing the production of inhibitory proteins, such as lipocortins. Lipocortins are members of a superfamily of proteins characterized by their ability to bind calcium and anionic phospholipids, now known as the 'annexins'.[121,122] In several cell types, but not all, glucocorticoids are inducers of lipocortins, which have an inhibitory effect on the activity of phospholipase A_2.[123,124] As a result, the synthesis of lipid mediators, including prostaglandins and eicosanoids, will be reduced. However, in human bronchial epithelial cells glucocorticoids do not seem to up-regulate the expression of lipocortins.[125] Furthermore, no significant difference was found between lipocortin-1 concentration in BAL fluid from asthmatic patients receiving inhaled glucocorticoid therapy and those who were not treated with glucocorticoids.[126]

Recently, glucocorticoids have also been shown to increase the expression of intracellular IL-1 receptor antagonist type I by human bronchial epithelial cells *in vitro*.[127] Increased production of this mediator may inhibit the effects of IL-1 agonists, thereby reducing inflammation. However, glucocorticoid treatment of asthmatic patients did not affect the expression of IL-1 receptor antagonist by the bronchial epithelium.[78]

To provide protection against potentially injurious agents, airway epithelial cells secrete a number of mediators, including antiproteases. Secretory leukocyte protease inhibitor (SLPI) is the predominant antiprotease in the airways. Its expression has been shown to be increased in airway epithelial cells after stimulation with glucocorticoids.[128]

Cellular and Clinical Effects of Glucocorticoids in Asthma

Several studies have determined the effects of inhaled glucocorticoids on bronchial inflammation, either by measurements in BAL fluid, sputum, or exhaled air, or by performing bronchial biopsies. Although differences can be observed between different trials, these studies have confirmed that glucocorticoid treatment of asthmatic patients reduces the number and activation of inflammatory cells in the airways, together with an improvement of lung function. Nowadays, the potent anti-inflammatory actions of glucocorticoids are thought to underlie the clinical efficacy of oral glucocorticoids.[129]

Effects of glucocorticoids on immunopathology

Inhaled glucocorticoids decrease the number and activation status of most inflammatory cells in the bronchus, including mast cells, dendritic cells, eosinophils, and T lymphocytes. Changes in cellular infiltration are accompanied by modulated expression of several cytokines. Inhaled glucocorticoids have been shown to decrease mRNA expression of GM-CSF, IL-13, IL-4, and IL-5, whereas mRNA levels of IL-12 and IFN-γ increased, suggesting a shift from a Th2- towards a more Th1-like environment.[77,130,131]

Glucocorticoid treatment is associated with a reduction in *mast cell* numbers in the bronchus[79,129,132–135] and with reduced mast cell associated mediators in BAL fluid.[135,136] This may be due to a reduction in IL-3 and stem cell factor production, which are necessary for the survival of mast cells in tissue. The (IgE-dependent) release of mediators from mast cells does not seem to be affected by glucocorticoid treatment.[137,138]

Dendritic cells play an important role in presenting antigens to (naive) T cells.[139,140] Inhaled glucocorticoids have been shown to reduce the number of dendritic cells in the human bronchial epithelium.[141]

Increased numbers of eosinophils are a prominent feature of asthmatic airways.[142–148] *In vitro* studies have shown that many eosinophil functions, including adherence and chemotaxis, are diminished following glucocorticoid treatment.[138] However, most data suggest that eosinophil responses to steroids are likely to be indirect, since eosinophil function is markedly affected by cytokines elaborated from T lymphocytes (IL-3, IL-4, IL-5, GM-CSF), endothelial cells (GM-CSF) and epithelial cells (GM-CSF).[149–153] *In vivo* studies indicate that treatment with inhaled steroids reduces the number of eosinophils and eosinophil-related mediators in BAL fluid[79,148,154] and the number of (activated) eosinophils in bronchial biopsies.[79,129,132,133,155] Recently, induced sputum has been suggested as a useful tool for evaluating the

effects of therapy on airway mucosal inflammation. Thus far, most studies have focused on the presence of eosinophils and eosinophil-related mediators. In accordance with the findings in BAL fluid and bronchial biopsies, glucocorticoid treatment was associated with a reduction in sputum eosinophil numbers, eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO).[156]

Glucocorticoids also reduce the number of activated T lymphocytes in bronchial biopsies and BAL fluid.[129,133,134,155,157] In addition, inhaled corticosteroids reduced the number of cells expressing mRNA for IL-4 or IL-5, and increased the number of cells expressing mRNA for IFN-γ,[131,132] thereby favouring the development of Th1 cells.[158]

In addition to the effects of glucocorticoids on epithelial cells described above, inhaled glucocorticoid therapy has been shown to reduce the shedding of epithelial cells.[155,159,160] No consistent effect of corticosteroids on the thickness of the basement membrane has been observed.[79,160,161]

Besides the suppressive effects on inflammatory cells, inhaled glucocorticoids have also shown to inhibit mucus secretion and microvascular leakage (as determined by the down-regulation of plasma proteins in BAL fluid).[160,162–166] At present it is not clear whether this is mediated via a direct effect of glucocorticoids on endothelial or mucous cells, or via a reduction of inflammatory mediators that increase mucus secretion and vascular leakage.

Effects of glucocorticoids on lung function

Treatment with glucocorticoids has been consistently shown not only to reduce the symptoms of asthma, but also bronchial hyperresponsiveness.[134,167–169] In contrast to the rapid inhibitory effects of β_2-agonists, glucocorticoids given in a single dose are not effective in preventing early allergen-invoked bronchoconstriction, but inhibition of the late response has been clearly demonstrated.[170,171] In contrast, chronic treatment with either oral or inhaled steroids attenuates even the early bronchoconstriction to allergen,[171–173] an effect that probably is mediated via the anti-inflammatory actions of glucocorticoids already described. Although inhaled glucocorticoids consistently reduce airway hyperreactivity in asthmatics,[169] even after several months of treatment responsiveness fails to return to the normal range. This may reflect persistence of structural changes that cannot be reversed by steroids (such as the thickening of the basement membrane), despite of suppression of the inflammatory and immunological processes.

Concluding Remarks

Glucocorticoids are widely used in the treatment of asthma and have anti-inflammatory effects. These effects are mediated either by direct binding of the glucocorticoid/GR complex to GRE in the promoter region of responsive genes, or by an interaction of this complex with transcription factors such as AP-1 and NF-κB. Glucocorticoids inhibit the expression of a large number of inflammation-associated molecules, including cytokines, chemokines, arachidonic acid metabolites, and adhesion molecules. These effects predominantly are mediated via inhibition of NF-κB activity. In contrast, anti-inflammatory mediators, such as NEP and IL-1 receptor antagonist, often are up-regulated by glucocorticoids. The beneficial effects of glucocorticoid therapy in asthma is demonstrated by *in vivo* studies showing that treatment of asthmatic patients with inhaled glucocorticoids inhibits the inflammation of the airways and simultaneously improves their lung function. These effects may be mediated in part by modulation of epithelial cell functions, since many studies, both *in vitro* and *in vivo*, have shown that glucocorticoids are able to modulate the inflammatory functions of bronchial epithelial cells. Further studies on the mechanism of action of glucocorticoids will eventually lead to the development of drugs which specifically inhibit the transcription of inflammatory genes without having negative side effects, and will contribute to a more efficient treatment of asthmatic patients.

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ACKNOWLEDGEMENTS. I acknowledge Mr T. M. van Os for preparing the figure.

Received 28 April 1998; accepted 7 May 1998

Immunology 1994 **82** 192–199

Topical glucocorticosteroid (fluticasone propionate) inhibits cells expressing cytokine mRNA for interleukin-4 in the nasal mucosa in allergen-induced rhinitis

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SUMMARY

Allergen-induced late nasal responses are associated with recruitment and activation of T lymphocytes and eosinophils and preferential mRNA expression for T-helper type 2 (Th2) cytokines. We tested the hypothesis that topical corticosteroids may inhibit late responses by inhibiting cells expressing mRNA for Th2 cytokines. A randomized double-blind placebo-controlled trial of topical corticosteroid (fluticasone propionate) was performed in 48 adult grass pollen-sensitive patients. Nasal biopsies were taken at baseline and repeated 24 hr after local nasal allergen provocation following 6 weeks treatment with either fluticasone propionate 200 μg or placebo nasal spray twice daily. Baseline mRNA expression for interleukin-4 (IL-4) ($P = 0{\cdot}01$) and IL-5 ($P = 0{\cdot}002$) was higher in the patients than in normal controls. Topical corticosteroid treatment significantly inhibited immediate nasal symptoms, with almost complete inhibition of the late response following allergen challenge. This was associated with a marked decrease in the allergen-induced increases in cells expressing mRNA for IL-4 ($P = 0{\cdot}002$) but not for IL-5. Inhibition of the late response was also accompanied by decreases in $CD25^+$ cells, presumed T lymphocytes and eosinophils. A significant correlation was observed between the decreases in IL-4 $mRNA^+$ cells and in eosinophils after treatment ($r = 0{\cdot}46$, $P < 0{\cdot}05$). These results suggest that prolonged treatment with topical corticosteroid inhibits allergen-induced early and late nasal responses and the associated tissue eosinophilia, and that, at least in part, this may result from inhibition of cells expressing mRNA for IL-4.

INTRODUCTION

Mosmann *et al.*[1] have described two types of murine T-lymphocyte clones according to their profile of cytokine release. T-helper cell type 1 cells produce predominantly interleukin-2 (IL-2) and interferon-γ (IFN-γ), whereas Th2-type cells express mainly IL-4 and IL-5. Recent studies have suggested the involvement of Th2-type cytokines in atopic allergic diseases. For example, IL-5 promotes the differentiation, prolongs survival, and selectively enhances vascular adhesion of eosinophils.[2–5] IL-4 induces isotype switching of B cells in favour of IgE synthesis.[6] Human allergen-specific T-cell clones derived from atopic donors have been shown to exhibit a Th2-type cytokine profile following allergen stimulation.[7,8] Although Th2-type cytokines were originally described as products of T lymphocytes, more recent studies have identified human mast cells,[9] basophils[10] and eosinophils as alternative sources.[11,12] We recently performed *in situ* hybridization studies of biopsies of human skin and nose obtained during allergen-induced late responses.[13,14] At 24 hr after allergen there were significant increases in cells expressing cytokine mRNA for IL-4, IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF). These observations support the view that Th2-type mRNA expression may be implicated in the development of late-phase responses.

Topical glucocorticosteroids are effective in the clinical management of allergic rhinitis and inhibit both early and late nasal responses and the associated eosinophilia[15] following allergen challenge, although their mode of action remains unknown. In this study we have examined the influence of a novel potent topical glucocorticosteroid (fluticasone propionate)[16] on early- and late-phase nasal responses, and cells and cytokine mRNA expression in the nasal mucosa in biopsies obtained 24 hr after allergen challenge.

Received 23 November 1993; revised 30 December 1993; accepted 1 February 1994.

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MATERIALS AND METHODS

Patients

Forty-eight non-smoking Timothy grass pollen-sensitive

patients were recruited from the out-patient clinic of the Asthma and Allergy Centre, Department of Medicine, Sahlgren Hospital, Gothenburg, Sweden. They were selected on the basis of (1) a clinical history of moderate to severe hayfever for at least 2 years; and (2) a positive skin prick test (wheal diameter greater than 5 mm) to Timothy grass pollen extract (*Phleum pratense*; Soluprick; ALK, Horsholm, Denmark). Patients were excluded if they gave a history of perennial allergy, birch pollen allergy, had previously received immunotherapy or were taking regular topical or oral medication.

Study design

The study was performed with the approval of the Ethics Committees of The Royal Brompton National Heart and Lung Hospital, London, U.K. and Sahlgren Hospital, Gothenburg, Sweden. All patients gave informed written consent. The 48 patients were stratified according to disease severity into 'moderate' and 'severe' groups on the basis of the clinical history and skin prick test results prior to randomization. One female patient dropped out because of pregnancy. Patients were randomized in double-blind fashion to receive 6 weeks treatment with either aqueous fluticasone propionate[16] (Flixonase, Glaxo, Sweden), two sprays (200 μg), or a matched placebo spray (containing the aqueous diluent) twice daily. Baseline nasal biopsies were performed out of season in all patients at a time when they were asymptomatic. None of the patients had received topical corticosteroids in the previous 6 months or had had an upper respiratory infection in the month prior to biopsy. After 6 weeks treatment all patients underwent local nasal provocation with a grass pollen extract followed by a second nasal biopsy at 24 hr after allergen.

Blood samples

Venous blood was taken for measurement of total and allergen-specific IgE concentrations by ELISA.

Nasal provocation

Nasal provocation was performed by the application of a 4-mm filter paper disk presoaked with 7 μl solution containing 1000 biological units (BU) of Timothy grass pollen extract to the undersurface of the inferior turbinate, 2 cm distal to its anterior insertion. The paper disk was held in position by an attached fine cotton thread which was taped to the cheek. The disk was removed after 10 min.

Nasal symptoms were recorded as number of sneezes, weight of nasal secretions and degree of nostril blockage between 0–30 min, 30–60 min, 1–4 hr, 4–10 hr and 10–24 hr. Data were summed for the early response (0–60 min) and late response (1–24 hr). Sneezes were recorded as the total number during a given interval. The patients recorded the degree of nostril blockage by gently applying the thumb to the opposite nostril without deforming the nose and inhaling and exhaling gently through the nose. The degree of blocking was recorded as 'no blocking' to 'complete blockage' on a scale of 0–4. Nasal secretions were collected by blowing into paper tissues which were supplied in preweighted resealable plastic bags. Bags were reweighed after 24 hr to determine the weight of nasal secretions. Secretions were expressed as weight in grams.

Nasal biopsy

Nasal biopsies were obtained at baseline and at 24 hr after allergen challenge. Local anaesthesia was achieved by applying a small cotton wool plug soaked in 3% cocaine and 0·025% adrenaline immediately below the inferior turbinate for 10 min. A 2·5-mm biopsy was taken using Gerritsma forceps.[17] Any local bleeding was controlled by cautery using a silver nitrate stick. After biopsy patients were kept under medical observation for 1 hr.

In situ *hybridization*

Riboprobes, both antisense (complementary RNA) and sense (having an identical sequence to mRNA), were prepared from cDNA for IL-2, IL-4, IL-5 and IFN-γ as described previously.[13,18,19] cDNA were inserted into different pGEM vectors and linearized with appropriate enzymes before transcription. Transcription was performed in the presence of [^{35}S]uridine triphosphate ([^{35}S]UTP) and the appropriate T7 or SP6 RNA polymerases. For *in situ* hybridization, 10-μM cryostat sections were processed on poly-L-lysine-coated slides. Cryostat sections were permeabilized with 0·3% solution of Triton X-100 in phosphate-buffered saline (PBS) for 15 min and then with proteinase K (1 μg/ml) in 0·1 M Tris containing 50 mM EDTA (pH 8) for 15 min at 37°. The reaction was terminated by immersion of the preparations in 4% paraformaldehyde for 5 min. In order to inhibit non-specific binding of ^{35}S, the preparations were treated with 10 mM iodoacetamide and 10 mM N-ethylmaleimide for 30 min, 0·5% acetic anhybride in 0·1 M triethanolamine for 10 min, and 0·1 M triethanolamine for 2 min. Prehybridization was carried out with 50% formamide and 2 × standard saline citrate (SSC) for 30 min at 40°. For hybridization, antisense or sense probes ($0{\cdot}5 \times 10^6$ c.p.m./section) diluted in hybridization buffer containing 100 mM dithiothrieol (DTT)[15] were used. Hybridization was performed in a humid chamber for 16 hr at 42°. Posthybridization washing was performed in a decreasing concentration of SSC (4 × SSC–0·1 × SSC) at 45°. Unhybridized single-stranded RNA were removed by treating the preparations with a 2 × SSC solution containing RNase A (20 μg/ml) for 30 min at 45°. After dehydration, the sections were immersed in K-5 emulsion (Ilford, U.K.), and exposed for 10 days. The autoradiographs were developed in Kodak D-19 and counterstained with haematoxylin.

Positive controls for IL-2, IL-4 and IL-5 were cytospins prepared from a peripheral blood T-lymphocyte clone obtained from a patient with the hyper-IgE syndrome. This clone was treated with concanavalin A and, after stimulation, expressed mRNA for IL-2, IL-4 and IL-5. Phytohaemagglutin in (PHA)-stimulated blood mononuclear cells were used for the IFN-γ controls. For the negative controls nasal biopsies were hybridized using sense probes for the relevant cytokines. In addition, sections were treated with RNase A solution before the prehybridization step and then hybridized with antisense probes.

Immunohistology

Immunohistology was performed on 6-μm cryostat sections (fixed for 7 min in 60 : 40 acetone : methanol) using the alkaline phosphatase–anti-alkaline phosphatase method, as described previously.[16] The monoclonal antibodies used were CD4 (T-helper lymphocytes), CD25 (IL-2-receptor-positive cells) (both from Becton Dickinson, Cowley, U.K.) and EG2 ('activated', eosinophil cationic protein-secreting eosinophils; Kabia Pharmacia, Milton Keynes, U.K.).

Quantification

For *in situ* hybridization and immunohistology sections were counted 'blind' in coded random order using an Olympus microscope with an eyepiece graticule at 200× magnification. The graticule (0·202 mm^2) was orientated beneath the epithelial basement membrane and counts were made along the whole length of the biopsy. The depth counted (0·45 mm) included the whole lamina propia where the majority of inflammatory cells are located. At least two sections were counted for each marker which produced counts from a minimum of six fields. Submucosal counts were expressed as mean counts per high power field (0·202 mm^2; HPF). The within observer coefficient of variation was < 5% for *in situ* hybridization counts and 8% for immunohistology counts.

Statistical analysis

Within-group comparisons were performed using the Wilcoxon matched-pairs signed-ranks test. Between-group comparisons were made using the Mann–Whitney *U*-test. Correlations were performed using Spearman's rank method. *P* values <0·05 were considered statistically significant. Statistical tests were performed using a standard computer package (Minitab^R Release 7; Minitab Inc., State College, PA).

RESULTS

Randomization resulted in 23 patients receiving fluticasone and 24 placebo nasal spray. After randomization, three of the fluticasone-treated patients and two placebo-treated patients dropped out for reasons unconnected to the clinical protocol. Fluticasone- and placebo-treated patients were well matched for age, gender, disease severity and for their degree of grass pollen sensitivity as determined by skin prick tests and allergen-specific IgE antibody concentration (Table 1). Total IgE levels for both groups were either normal or moderately increased, and there was no significant difference between the groups. Six weeks treatment with fluticasone resulted in highly significant inhibition of the size of both early and late nasal responses after grass pollen allergen challenge. There was an approximate 50% reduction in early sneezes ($P < 0·004$), degree of nostril blocking ($P < 0·0005$) and weight of nasal secretions ($P < 0·001$) in the actively treated group. During the late phase (1–24 hr) nostril blocking was similarly reduced ($P < 0·002$) and there was almost complete inhibition of late sneezing ($P < 0·0001$) and nasal secretions ($P < 0·0001$) (Fig. 1).

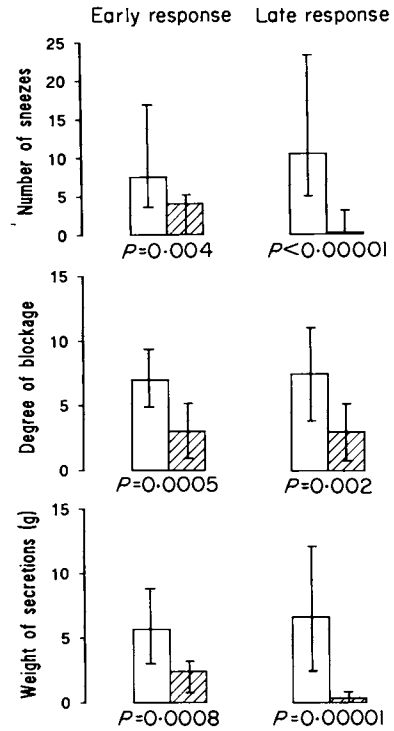

Figure 1. Effect of fluticasone on allergen-induced early and late nasal responses. Number of sneezes, degree of nostril blocking and weight of secretions during early (0–60 min) and late (1–24 hr) periods after grass pollen challenge, following 6 weeks treatment with fluticasone (hatched columns) or placebo (open columns) nasal spray. Median values and interquartile ranges. Mann–Whitney *U*-test *P* values are shown.

Nasal biopsies were performed at baseline in all 47 patients (*in situ* hybridization studies were performed on 42 patients for whom paired tissue samples were available) and in 12 non-atopic normal healthy control subjects (Table 2). When the number of cells in the nasal submucosa expressing positive hybridization signals for cytokine mRNA was compared, a small but significant increase in cytokine mRNA for IL-4

Table 1. Clinical data of patients who underwent *in situ* hybridization studies

	Treatment	
	Placebo	Fluticasone
Number of patients	22	20
Male : female ratio	12 : 10	11 : 9
Age (years mean ± SD)	32·5 ± 10·4	31·8 ± 9·4
RAST* score Timothy grass (0–5) (Mean ± SD)	3·0 ± 1·1	3·3 ± 0·9
Total IgE (IU/ml) (median ± interquartile ranges)	45·0 ± (18·7–93·7)	82·5 ± (41·3–197·5)

*Radioallergosorbant test.

Table 2. Number of cells in nasal submucosa/HPF expressing mRNA for cytokines in grass pollen-sensitive patients compared with normal healthy controls

Cytokine	Patients ($n = 42$)	Controls ($n = 12$)	*P* value
IL-4	2·1 (2·0, 3·2)*	0 (0, 1·7)	0·01
IL-5	2·2 (1·6, 3·0)	0 (0, 1·2)	0·002
IFN-γ	1·5 (0·2, 2·4)	1·0 (0, 2·7)	NS
IL-2	1·0 (0, 1·0)	0·5 (0, 4·6)	NS

*Values are medians (95% confidence intervals). Comparison was made using Mann–Whitney *U*-test. *P* values are shown.

NS, not significant.

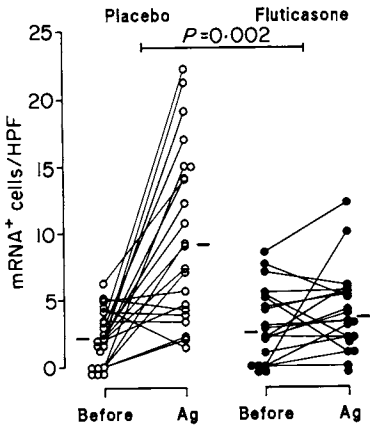

Figure 2. Effect of fluticasone on the number of cells in the nasal submucosa expressing mRNA for IL-4. Data are shown for baseline (before) and at 24 hr after allergen challenge (Ag) following 6 weeks treatment with fluticasone (closed circles) or placebo (open circles). Median values are represented by the horizontal bars. Median differences (Ag minus before) were compared using the Mann–Whitney *U*-test.

($P = 0{\cdot}01$) and IL-5 ($P = 0{\cdot}002$) was observed in rhinitics compared to controls. In contrast, no differences for IL-2 or IFN-γ were observed. At baseline, when biopsies from fluticasone- and placebo-treated patients were examined, the number of cytokine mRNA$^+$ cells was small (mean counts 1–2·5 cells/HPF) and no significant differences for any of the four cytokines were observed (data not shown).

Within the placebo-treated group, a significant increase in IL-4 mRNA$^+$ cells ($P = 0{\cdot}0001$) 24 hr after allergen was observed. In contrast, in the fluticasone-treated group no increase in IL-4 was observed. This difference was highly significant compared with the placebo group (Fig. 2; $P = 0{\cdot}002$). For IL-5, increases were observed 24 hr after allergen in both placebo ($P = 0{\cdot}0001$) and fluticasone ($P = 0{\cdot}0001$)-treated patients. Median counts for IL-5 mRNA$^+$ cells after allergen were reduced in the treated group, although not significantly when compared with the placebo group (Fig. 3). No changes in cytokine mRNA$^+$ cells for IL-2 or IFN-γ 24 hr after allergen were observed for either group of patients (Fig. 3). In general, cytokine mRNA$^+$ cells were identifiable as discrete, well-circumscribed areas of silver grains (Fig. 4). No hybridization signals were observed when the sense probe was employed or following pretreatment of sections with RNase.

Immunohistology of nasal biopsies from the same patients obtained 24 hr after allergen revealed fewer numbers of CD4$^+$ T-helper lymphocytes ($P = 0{\cdot}1$), CD25$^+$ cells, presumed activated T lymphocytes ($P = 0{\cdot}03$) and EG2$^+$ eosinophils ($P = 0{\cdot}02$) in the nasal submucosa in fluticasone-treated patients compared with the placebo group (Fig. 5).

In placebo-treated patients, significant correlations were found at allergen-challenged sites between the number of CD4$^+$ T lymphocytes and CD25$^+$ cells ($r = 0{\cdot}67$, $P < 0{\cdot}01$); between CD4$^+$ cells and IL-4 mRNA$^+$ cells ($P = 0{\cdot}45$, $P < 0{\cdot}05$) and between CD25$^+$ cells and IL-4 mRNA$^+$ cells ($r = 0{\cdot}67$, $P < 0{\cdot}01$). Within the fluticasone-treated group the magnitude of the changes in eosinophil (EG2$^+$) numbers after allergen correlated with changes in IL-4 mRNA$^+$ cells ($r = 0{\cdot}46$, $P < 0{\cdot}05$), i.e. the lower the number of IL-4 mRNA$^+$ cells after fluticasone treatment the lower the number of eosinophils. No significant associations were found between the numbers of submucosal CD4$^+$ T lymphocytes, eosinophils, or IL-4 mRNA$^+$ cells at the single time-point examined and the magnitude of either early or late nasal symptoms for either fluticasone- or placebo-treated patients.

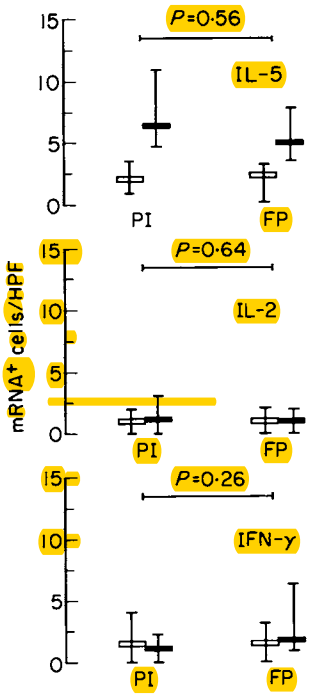

Figure 3. Effect of fluticasone on the number of cells in nasal submucosa expressing mRNA for IL-5, IL-2 and IFN-γ. Data are shown for baseline (open bars) and at 24 hr after local allergen provocation (closed bars) following 6 weeks treatment with placebo (Pl) or fluticasone (FP). Results expressed as medians and interquartile ranges. Median differences were compared using the Mann–Whitney *U*-test.

DISCUSSION

We have shown that 6 weeks treatment with topical glucocorticosteroid inhibited early and late nasal responses and decreased the number of IL-4 mRNA$^+$ cells in nasal biopsies at 24 hr after local allergen challenge. Glucocorticosteroid treatment also resulted in significantly lower numbers of CD25$^+$ cells (presumed T lymphocytes)[20] and activated eosinophils at allergen-challenged sites. CD4$^+$ T-lymphocyte numbers were also lower although not significantly when compared with placebo-treated patients. The magnitude of the decreases in IL-4 mRNA$^+$ cells correlated with the decreases in eosinophils. The findings confirm our previous observations that allergen-induced late nasal responses[14,21] and late responses occurring in the skin[13] and lung[22,23] are associated with recruitment of activated T lymphocytes and eosinophils and increased expression of mRNA for Th2-type cytokines. They also support the view that topical corticosteroids may inhibit *in vivo* human allergen-induced late-phase responses by

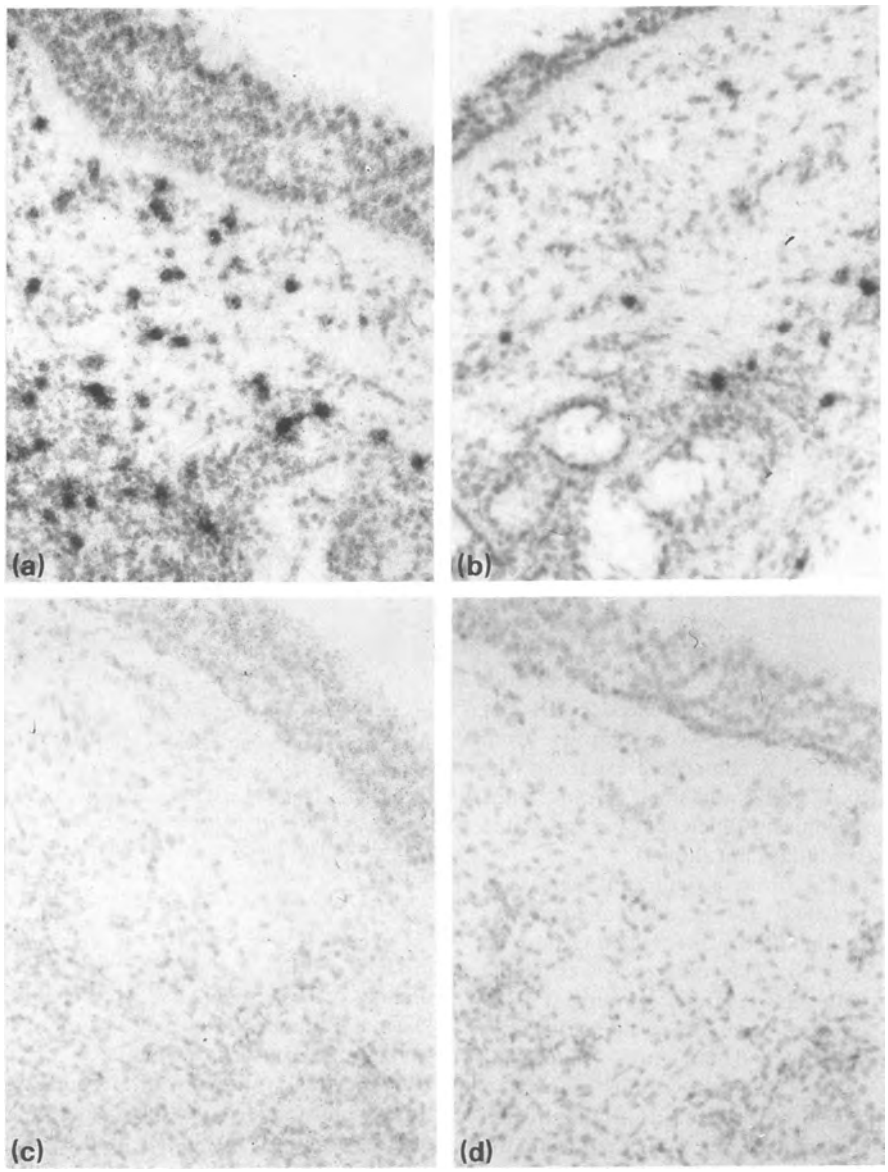

Figure 4. Autoradiographs of cryostat sections of nasal biopsies 24 hr after local allergen provocation from placebo (a) and fluticasone (b)-treated patients. Sections were hybridized with a ^{35}S-labelled antisense IL-4 probe. Autoradiographs of sections of nasal biopsies from a placebo-treated patient hybridized with a ^{35}S-labelled sense IL-4 probe (c), and following treatment with RNase before hybridization (d).

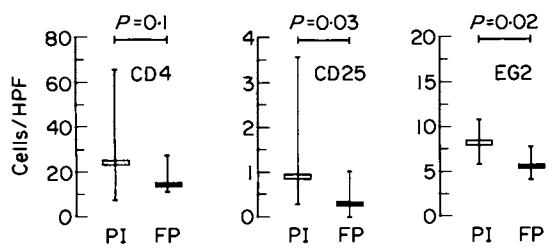

Figure 5. Cell infiltration (median cell counts ± interquartile ranges) at allergen-challenged sites in nasal biopsies from placebo (open bars) and fluticaseone (closed bars)-treated patients. Numbers of T-helper cells ($CD4^+$), activated cells ($CD25^+$) and eosinophils ($EG2^+$) are shown. *P* values are shown (Mann–Whitney *U*-test).

suppressing local eosinophilia, and raise the possibility that this is related to the suppression of IL-4 expression.

The biological properties of Th2-type cytokines are pertinent to the development of late responses. Thus late responses are known to be IgE-dependent[24–26] and IL-4 may regulate local antibody production by switching B cells to produce preferentially IgE. Tissue eosinophilia is characteristic of human late responses.[21] IL-5 stimulates eosinophil maturation,[2] activation,[3] and prolongs eosinophil survival in culture[5] and also, presumably, in tissues. IL-5 also promotes selective local adhesion of eosinophils to human vascular endothelium.[4] However, the predominant role of Th2-type cytokines during late responses, whether in enhancing local IgE or promoting tissue eosinophilia, is unclear.

Corticosteroids are potent inhibitors of late-phase responses. Our original hypothesis was that corticosteroids might act by suppressing IL-5. In this study, in placebo-treated patients, we confirmed our previous observation that late responses were accompanied by increases in the number of cells expressing IL-4 and IL-5. It turned out that in corticosteroid-treated patients the number of IL-5 mRNA$^+$ cells remained increased despite almost complete inhibition of the late response. In contrast, there was a marked inhibition of allergen-induced increases in IL-4 mRNA$^+$ cells in patients who had received topical corticosteroid. In addition, corticosteroid significantly reduced the number of activated eosinophils in the nasal mucosa.[27] There was no close correlation among cells, cytokines and the magnitude of early or late nasal symptoms. This is perhaps not surprising since, for ethical reasons, biopsy could only be performed at one time-point after allergen. However, it seems likely that the development of symptoms may also depend on other influences such as psychological or neurological factors which we have not examined. It is of interest that the single patient who had received fluticasone and demonstrated a large increase in IL-4 mRNA$^+$ cells also showed a large local eosinophilia and marked early and late nasal symptoms, in contrast to the remaining steroid-treated patients.

A disadvantage of the nasal biopsy technique is that it is not amenable to the performance of time–course studies. We chose the 24-hr time-point based on our previous observation of readily identifiable changes in T lymphocytes and eosinophils at this time.[21] Thus we may have missed an effect of corticosteroid on cytokine mRNA, including IL-5, at earlier time-points. For example, previous studies which have employed nasal lavage have shown that the peak time of release of mediators of hypersensitivity[28] and the appearance of cells,[29] particularly eosinophils, in nasal washings during late responses may vary between individuals. In these studies the release of mediators including histamine, TAME-esterase, prostaglandin D_2 (PGD_2) and bradykinin have been shown to be inhibited by topical corticosteroids.[30]

The expression of cytokine mRNA does not equate with protein release, which was not examined in our study. However, to our knowledge, Th2-type cytokines have not been measured in nasal lavage studies during late responses and the influence of topical steroids on secreted cytokines *in vivo* is yet to be determined. A further important question is the cell type responsible for the increase in mRNA expression which we have observed during late responses, and which cell is influenced by corticosteroids. Th2 T lymphocytes,[7,8] mast cells[9] and basophils[10,31] are all candidates for IL-4 mRNA expression in humans. Basophils represent an attractive candidate since several studies have supported participation of basophils during human late nasal responses.[32] Unfortunately, there is no specific monoclonal antibody marker directed against basophils for tissue studies. A very recent study which employed immunostaining of cytokines in nasal biopsies from patients with perennial rhinitis has shown the presence of IL-4 protein.[9] By use of double immunostaining, IL-4 was localized to mast cells using antibodies directed against human IL-4 and mast cell tryptase. Our own recent studies have focused on the cell source of IL-4 mRNA during late nasal responses.[33] By use of double immunocytochemistry/*in situ* hybridization of 10-micron cryostat sections, the majority of IL-4 mRNA$^+$ cells, approximately 80%, were T lymphocytes, whereas 20% of IL-4 mRNA$^+$ cells were mast cells. In contrast, no IL-4 mRNA was co-localized to either eosinophils or macrophages. In our hands, therefore, the principal cellular source of IL-4 transcripts during late responses was the T lymphocyte. It therefore seems likely that T lymphocytes express mRNA for IL-4 but, unlike the mast cell, do not store IL-4 and are therefore not amenable to specific immunostaining for IL-4 product. The precise cell expressing IL-4 mRNA which is modulated (or inhibited from migrating into late reaction sites) by corticosteroids has yet to be determined.

The influence of glucocorticosteroids on IL-4 production by T lymphocytes *in vitro* is dependent upon the species of origin. Previous studies showed, rather surprisingly, that IL-4 production by murine T lymphocytes *in vitro* was enhanced.[34] In contrast, the production of IL-4 and IL-5 by human T lymphocytes *in vitro* was inhibitable at both mRNA and protein levels by corticosteroids.[35–37] Our results support the view that corticosteroids also inhibit IL-4, at least at the level of mRNA expression, *in vivo* in humans. Furthermore, the inhibition of IL-4 which we observed after allergen was accompanied by marked inhibition of both early and late phase allergic responses and the associated tissue eosinophilia. As mentioned above, this effect of corticosteroids may be mediated by inhibition of local IgE-dependent events. However, IL-4 has recently been demonstrated to up-regulate VCAM-1 on human vascular endothelium.[38] Eosinophils, but not neutrophils, adhere to vascular endothelium through VLA-4–VCAM-1-dependent pathways. Thus an attractive alternative hypothesis may be that corticosteroids inhibit late responses by suppressing IL-4-induced up-regulation of VCAM-1.

In summary, we have shown that prolonged treatment with topical corticosteroids inhibited early and late nasal responses and the associated allergen-induced increases in IL-4 mRNA$^+$ cells in the nasal mucosa. Corticosteroid treatment was also associated with fewer numbers of CD25$^+$ cells and eosinophils. It is not possible to determine, *in vivo*, whether the inhibition of IL-4 mRNA resulted from corticosteroid-induced inhibition of transcription of mRNA or simple inhibition of recruitment of IL-4 mRNA$^+$ cells. The precise cell type involved and the potential IL-4-dependent pathways influenced by corticosteroids are yet to be determined.

ACKNOWLEDGMENTS

The study was supported by a grant from the Medical Research Council United Kingdom and financial assistance from Glaxo Group Research Limited. We are grateful to the Glaxo Institute of Molecular Biology S.A., Geneva, Switzerland, for their kind gifts of IL-2, IL-4 and IFN-γ plasmid constructs, to Dr Colin Sanderson for the IL-5 cDNA, and to Glaxo, Sweden for supplying fluticasone and placebo nasal sprays. T-cell clones from the hyper-IgE syndrome were kindly supplied by Dr Diana Quint, Glaxo, Greenford, U.K.

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Series editors: Joshua A. Boyce, MD, Fred Finkelman, MD, and William T. Shearer, MD, PhD

The biology of the glucocorticoid receptor: New signaling mechanisms in health and disease

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Glucocorticoids are primary stress hormones necessary for life that regulate numerous physiologic processes in an effort to maintain homeostasis. Synthetic derivatives of these hormones have been mainstays in the clinic for treating inflammatory diseases, autoimmune disorders, and hematologic cancers. The physiologic and pharmacologic actions of glucocorticoids are mediated by the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors. Ligand-occupied GR induces or represses the transcription of thousands of genes through direct binding to DNA response elements, physically associating with other transcription factors, or both. The traditional view that glucocorticoids act through a single GR protein has changed dramatically with the discovery of a large cohort of receptor isoforms with unique expression, gene-regulatory, and functional profiles. These GR subtypes are derived from a single gene by means of alternative splicing and alternative translation initiation mechanisms. Posttranslational modification of these GR isoforms further expands the diversity of glucocorticoid responses. Here we discuss the origin and molecular properties of the GR isoforms and their contribution to the specificity and sensitivity of glucocorticoid signaling in healthy and diseased tissues. (J Allergy Clin Immunol 2013;132:1033-44.)

Key words: *Glucocorticoid receptor, glucocorticoid, isoforms, glucocorticoid signaling*

Abbreviations used

ACTH:	Adrenocorticotropic hormone
AF:	Activation function
AP1:	Activator protein 1
β2AR:	β_2-Adrenergic receptor
CBG:	Corticosteroid-binding globulin
DBD:	DNA-binding domain
GPCR:	G protein–coupled receptor
GR:	Glucocorticoid receptor
GRE:	Glucocorticoid-responsive element
LBD:	Ligand-binding domain
MAPK:	Mitogen-activated protein kinase
NF-κB:	Nuclear factor κB
nGRE:	Negative glucocorticoid-responsive element
NTD:	N-terminal transactivation domain
SEGRA:	Selective glucocorticoid receptor agonist

Glucocorticoids are hormones essential for life that are synthesized and released by the ***adrenal cortex*** in a ***circadian*** manner and in response to stress. The secretion of these hormones is controlled by the hypothalamic-pituitary-adrenal axis (Fig 1). Internal and external signals trigger the hypothalamus to release corticotropin-releasing hormone, which acts on the anterior pituitary to stimulate the synthesis and secretion of adrenocorticotropic hormone (ACTH). ACTH then acts on the adrenal cortex to stimulate the production and secretion of glucocorticoids. Acting on nearly every tissue and organ in the body, glucocorticoids function to maintain homeostasis both in response to normal diurnal changes in metabolism and in the face of stressful perturbations. Glucocorticoids regulate a plethora of physiologic processes, including intermediary metabolism, immune function, skeletal growth, cardiovascular function, reproduction, and cognition.[1,2] In a classic negative feedback loop, glucocorticoids also target the hypothalamus and anterior pituitary to inhibit the production and release of corticotropin-releasing hormone and ACTH and thereby limit both the magnitude and duration of the glucocorticoid increase (Fig 1).

Because of their powerful anti-inflammatory and immunosuppressive actions, glucocorticoids are one of the most widely prescribed drugs in the world today.[3,4] Synthetic glucocorticoids have been indispensable over the last half century for treating inflammatory and autoimmune diseases, such as asthma, allergy, sepsis, rheumatoid arthritis, ulcerative colitis, and multiple sclerosis. They are also commonly prescribed to prevent organ transplant rejection and to treat cancers of the lymphoid system, such as leukemias, lymphomas, and myelomas. Unfortunately, the therapeutic benefits of glucocorticoids are limited by severe side effects that develop in patients chronically treated with these steroids.[3,5] Adverse effects include osteoporosis, skin atrophy, diabetes, abdominal obesity, glaucoma, growth retardation in children, and hypertension. In addition, patients receiving long-term glucocorticoid therapy frequently have tissue-specific glucocorticoid resistance. Understanding the factors at a molecular

From the Laboratory of Signal Transduction, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services.

Supported by the Intramural Research Program of the National Institutes of Health/ National Institute of Environmental Health Sciences.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication August 1, 2013; revised September 5, 2013; accepted for publication September 6, 2013.

Available online September 29, 2013.

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0091-6749

http://dx.doi.org/10.1016/j.jaci.2013.09.007

Terms in boldface and italics are defined in the glossary on page 1034.

level that control the cellular response to glucocorticoids is a critical goal of current research because progress in this area will facilitate the development of novel glucocorticoids with improved benefit/risk ratios.

The physiologic and pharmacologic actions of glucocorticoids are mediated by the glucocorticoid receptor (GR; NR3C1), a member of the nuclear receptor superfamily of ligand-dependent transcription factors.[6] After glucocorticoid binding, the GR induces or represses the transcription of target genes, which can comprise up to 10% to 20% of the human genome.[7-9] Consistent with the pleiotropic actions of glucocorticoids, the GR is expressed in nearly every cell of the body and is necessary for life after birth.[10] The cellular response to glucocorticoids is remarkably diverse, exhibiting profound variability in specificity and sensitivity.[11,12] For example, glucocorticoids induce the killing of thymocytes and osteoblasts but promote the survival of hepatocytes and cardiomyocytes. In addition, glucocorticoid sensitivity varies among subjects, within tissues of the same subject, and even within the same cell during different stages of the cell cycle.[13,14] Although cell type–specific alterations in ligand bioavailability and cofactor expression can modulate the glucocorticoid response, recent studies have also identified a major role for an expanding array of GR isoforms.[15,16]

The GR is derived from a single gene; however, multiple GR proteins exist because of ***alternative splicing*** and alternative translation initiation mechanisms. This large cohort of functionally distinct receptor subtypes are subject to various posttranslational modifications that further regulate their signaling properties. Consequently, the cellular response to glucocorticoids is determined in large measure by the expressed complement and composite actions of the individual GR isoforms. In this review we discuss the molecular heterogeneity of the GR and its potential contribution to the regulation and dysregulation of glucocorticoid signaling.

GR SIGNALING

Classic GR signaling pathway

The GR is a modular protein composed of 3 major domains: an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD; Fig 2).[17] The DBD and LBD are separated by a flexible region of the protein termed the hinge region. The DBD is the most conserved domain across the nuclear receptor family and contains 2 zinc finger motifs that recognize and bind target DNA sequences called glucocorticoid-responsive elements (GREs). The NTD houses a powerful transcriptional activation function (AF1) that interacts with coregulators and the basal transcription machinery and is the primary site for posttranslational modifications (Fig 2). The LBD, consisting of 12 α-helices and 4 β-sheets, forms a hydrophobic pocket for binding glucocorticoids and also contains an AF2 domain that interacts with coregulators in a ligand-dependent manner.[18] Two ***nuclear localization signals*** (NL1 and NL2) are located at the DBD/hinge region junction and within the LBD, respectively.

In the absence of hormone, the GR resides predominantly in the cytoplasm of cells as part of a large multiprotein complex that includes chaperone proteins (hsp90, hsp70, and p23) and immunophilins of the FK506 family (FKBP51 and FKBP52; Fig 3).[19,20] These proteins maintain the receptor in a conformation that is transcriptionally inactive but favors high-affinity ligand binding. Cortisol, the most abundant endogenous glucocorticoid in human subjects, is transported in the blood predominantly bound to corticosteroid-binding globulin (CBG). CBG not only facilitates cortisol distribution but also plays a role in its release to tissues. CBG-free cortisol passively diffuses across the plasma membrane; however, its bioavailability within the cell is controlled by 2 enzymes working in an opposing manner.[21] 11β-Hydroxysteroid dehydrogenase type 2 oxidizes cortisol into

GLOSSARY

ACETYLATION: The addition of an acetyl group (CH_3CO) to an organic compound.

ADRENAL CORTEX: An endocrine organ made up of specific zones that synthesizes 3 different classes of steroids: (1) glucocorticoids (cortisol), which are synthesized primarily in the fasciculata, the middle layer; (2) mineralocorticoids (aldosterone), which are generated in the glomerulosa, the outermost layer; and (3) sex steroids (estrogens and androgens), which are produced largely in the innermost layer, the reticularis.

ALTERNATIVE SPLICING: The process by which a given gene is spliced into more than 1 type of mRNA molecule.

ARACHIDONIC ACID: A polyunsaturated fatty acid derived from membrane phospholipids by the action of cytosolic phospholipase A_2.

β-ARRESTINS: A family of proteins that function to transduce and terminate (desensitize) G protein–coupled receptor signals.

CAVEOLAE: Latin for "little caves"; a membrane compartment at the surface of most cells capable of endocytosis and exocytosis, as well as compartmentalizing a variety of signaling activities. Caveolin-1 is the marker protein used to isolate caveolae by means of cell fractionation.

CIRCADIAN: Latin for "approximately a day"; biological processes under daily rhythmicity through an internal clock. Diurnal species demonstrate a gradual increase in plasma cortisol several hours before awakening. Plasma cortisol levels reach their nadir around midnight.

HISTONE: Proteins that are rich in the basic amino acids lysine and arginine and complexed with DNA in chromatin.

MIFEPRISTONE: A synthetic steroid that competitively binds to the glucocorticoid receptor and progesterone receptor, blocking their effects. Clinically, it can be used to terminate a pregnancy. At high doses, it blocks the effects of cortisol in patients with Cushing syndrome.

NUCLEAR LOCALIZATION SIGNALS: Factors necessary for nuclear import (eg, transcription factors, coactivators or corepressors, DNA repair enzymes, ribosomal proteins, and mRNA processing factors) that allow molecules to pass through nuclear pore complexes through shuttling receptors, such as importins.

POLYMORPHISM: One of 2 or more variants of a particular DNA sequence. The most common type of polymorphism involves variation at a single base pair (single nucleotide polymorphism). Polymorphisms can also involve long stretches of DNA.

SEX-SPECIFIC DIFFERENCE: The female/male ratio among patients with rheumatoid arthritis is 2:1 to 3:1, and that among patients with systemic lupus erythematosus is 9:1.

SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION FAMILY: Transcription factors that are substrates for the Jak family of tyrosine kinases. STATs are activated by Jak-dependant tyrosine phosphorylation and form dimers that then translocate into the nucleus.

The Editors wish to thank Daniel A. Searing, MD, for preparing this glossary.

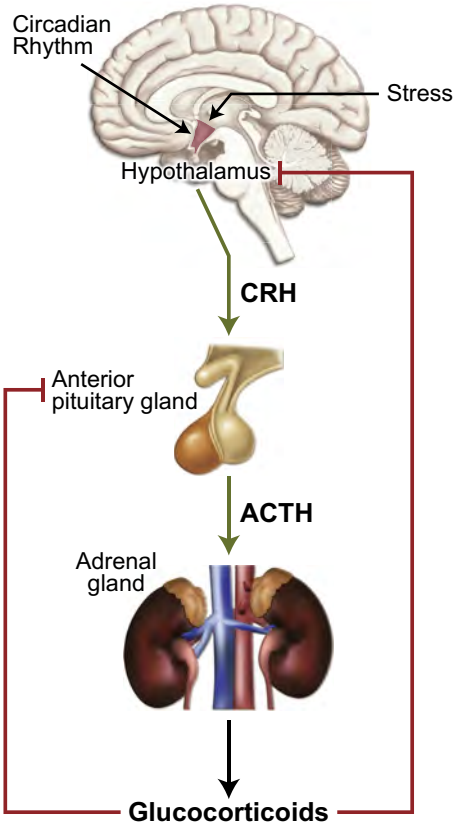

FIG 1. Regulation of glucocorticoid hormone secretion by the hypothalamic-pituitary-adrenal (HPA) axis. *CRH,* Corticotropin-releasing hormone.

the inactive metabolite cortisone, whereas 11β-hydroxysteroid dehydrogenase type 1 converts cortisone to cortisol (Fig 3). Changes in the expression level or activity of these enzymes can contribute to cellular differences in glucocorticoid sensitivity. In fact, inhibitors of 11β-hydroxysteroid dehydrogenase type 1 have been designed to limit the adverse metabolic side effects of increased endogenous glucocorticoids.[22] In contrast to cortisol, most synthetic glucocorticoids do not bind CBG and are not metabolized by 11β-hydroxysteroid dehydrogenase type 2. On binding glucocorticoids, the GR undergoes a conformational change, resulting in the dissociation of the associated proteins. This structural rearrangement exposes the 2 nuclear localization signals, and the GR is rapidly translocated into the nucleus through nuclear pores.

Once inside the nucleus, the GR binds directly to GREs and regulates the expression of target genes (Fig 3).[23,24] The consensus GRE sequence GGAACAnnnTGTTCT is an imperfect palindrome that is comprised of two 6-bp half sites. The GR binds this element as a homodimer, with each half site occupied by 1 receptor subunit. The 3-nucleotide spacing between the 2 half sites is strictly required for the GR to dimerize on this element. The GRE has been shown to mediate the glucocorticoid-dependent induction of many genes and therefore is often referred to as an activating or positive GRE. However, recent genome-wide analyses have revealed that GR occupancy of the canonical GREs can also lead to the repression of target genes.[25] These findings suggest a critical role for factors outside the GRE sequence, such as epigenetic regulators and chromatin context, in determining the polarity of the transcriptional response. A negative glucocorticoid-responsive element (nGRE) that mediates glucocorticoid-dependent repression of specific genes has also been recently described.[26] The consensus nGRE sequence CTCC(n)$_{0\text{-}2}$GGAGA is palindromic but differs from the classic GRE in sequence, in having a variable spacer that ranges from 0 to 2 nucleotides, and in being occupied by 2 GR monomers that do not homodimerize.[27] These nGREs are abundant throughout the genome; however, more work is needed to clarify the extent this motif is used by the GR for directly repressing target genes and whether this element can also be used in the activation of gene expression.

Global GR recruitment assays indicate that only a small fraction of GREs are actually occupied by the receptor, and the specific sites of GR binding vary in a tissue-specific manner because of differences in chromatin accessibility and exposure of the GRE.[28] These findings suggest that the widely varying effects of glucocorticoids on different tissues can be attributed in part to cell type–specific differences in the chromatin landscape that influence which GREs are accessible for GR binding. In addition, the concentration of glucocorticoids at which the GR binds GREs and regulates gene expression varies throughout the genome.[29] Some GREs are occupied by the GR at very low concentrations of glucocorticoids (hypersensitive), whereas others require high doses of ligand for GR binding to occur. Both chromatin accessibility and other DNA-binding proteins appear to govern the sensitivity of specific GREs. The identification of hypersensitive GREs suggests that low-dose glucocorticoid therapy might provide a novel treatment option for regulating specific sets of genes that avoids the deleterious side effects of high exogenous glucocorticoids.

Genome-wide analyses have also found that the majority of GR-binding sites are located outside the promoter of glucocorticoid-responsive genes in intergenic or intragenic regions often far removed from the transcription start site.[30] For example, glucocorticoid induction of ***β-arrestin 1*** and repression of ***β-arrestin 2*** occurs through an intron 1 GRE and an intron 11 nGRE, respectively.[31] Both β-arrestin isoforms play critical roles in the termination and transduction of G protein–coupled receptor (GPCR) signals. By altering the ratio of β-arrestin 1 and β-arrestin 2, glucocorticoids shift the balance of G protein– and β-arrestin–dependent signaling responses for a given GPCR.[31] This redirection of the GPCR signaling profile might account for the superior clinical efficacy of the glucocorticoid/β_2-adrenergic receptor (β2AR) agonist combination therapies currently used for treating asthma and chronic obstructive pulmonary disease. The β2AR is preferentially desensitized by β-arrestin 2. Therefore the glucocorticoid-dependent reduction in β-arrestin 2 should counteract β2AR desensitization and allow agonist-bound β2AR to promote a greater or more sustained relaxation response in airway smooth muscle cells. In addition, lower levels of β-arrestin 2 in lung epithelial cells should impair the proinflammatory effects of β2AR agonists that depend on the signaling function of β-arrestin 2. Another example of a GR-binding site located a great distance from the transcription start site is the intragenic nGRE recently identified in exon 6 of the GR gene that mediates homologous downregulation of GR expression, a mechanism that might limit therapeutic responses to glucocorticoids.[32] The location of GREs and nGREs at great distances from the transcription start site suggests that these response elements can loop to the promoter areas of target genes to regulate transcription.

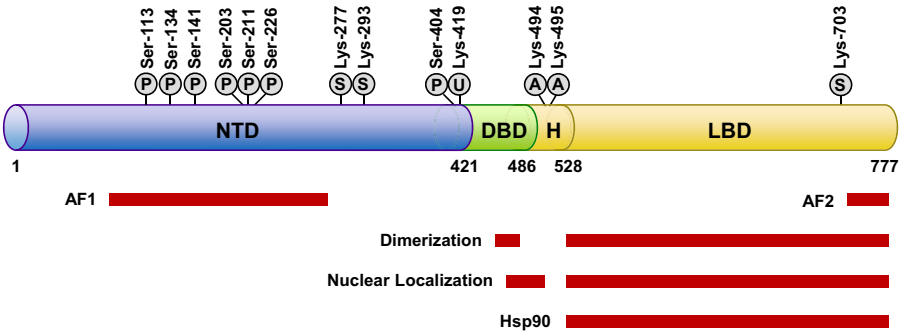

FIG 2. GR domain structure and sites of posttranslational modification. Shown are the domains of the GR and regions of the receptor involved in transactivation (AF1 and AF2), dimerization, nuclear localization, and hsp90 binding. Also depicted are the amino acid residues modified by phosphorylation *(P)*, sumoylation *(S)*, ubiquitination *(U)*, and acetylation *(A)*. *Numbers* are for human GR.

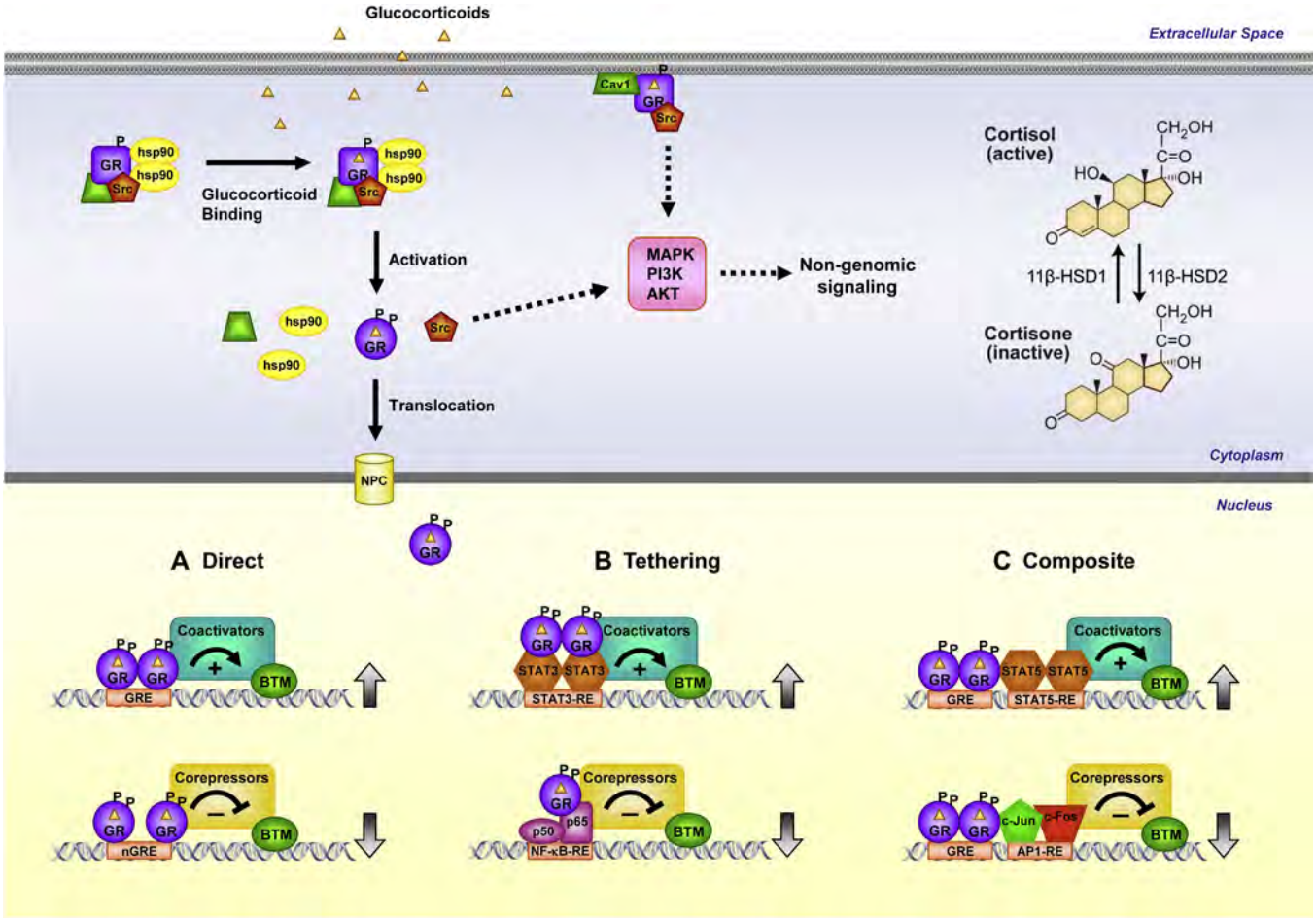

FIG 3. GR signaling pathways. The glucocorticoid-activated GR regulates gene expression in 3 primary ways: binding directly to DNA **(A)**, tethering itself to other DNA-bound transcription factors **(B)**, or binding directly to DNA and interacting with neighboring DNA-bound transcription factors **(C)**. The GR can also signal in a nongenomic manner through alterations in the activity of various kinases. *BTM*, Basal transcription machinery; *PI3K*, phosphoinositide 3-kinase; *STAT*, signal transducer and activator of transcription.

The interaction of the GR with DNA is highly dynamic, with the GR cycling between bound and unbound states every few seconds.[33] Once bound to the GRE, the receptor undergoes additional conformational changes that lead to the recruitment of coregulators and chromatin-remodeling complexes that modulate gene transcription rates by affecting the activity of RNA polymerase II.[34-36] Cofactors that mediate transcriptional activation include steroid receptor coactivators, the ***histone*** acetyltransferase CBP/p300, and the nuclear methylase coactivator-associated arginine methyltransferase. Nuclear receptor corepressor 1 and

silencing mediator of retinoid and thyroid hormone receptors are established corepressors that are recruited to the GR bound to nGREs. The specific cofactors assembled and their subsequent activity are dictated by both the nature of the glucocorticoid ligand and the specific GRE sequence bound by the receptor.[37,38]

The GR can also regulate the transcription of target genes by physically interacting with other transcription factors (Fig 3). The association of the GR with specific members of the ***signal transducer and activator of transcription family***, either apart from or in conjunction with GRE binding, has been shown to enhance the transcription of responsive genes.[39] In contrast, the interaction of the GR with the proinflammatory transcription factors activator protein 1 (AP1) and nuclear factor κB (NF-κB) antagonizes their activity and is considered to be a primary mechanism through which glucocorticoids suppress inflammation. The GR directly binds the Jun subunit of AP1 and the p65 subunit of NF-κB and interferes with the transcriptional activation function of these 2 proteins.[40,41] For some genes, the repression is accomplished by the GR tethering itself to these DNA-bound proteins without itself directly interacting with the DNA. For other genes, however, the GR functions in a composite manner, binding directly to a GRE and physically associating with AP1 or NF-κB bound to a neighboring site on the DNA. GR-dependent recruitment of the glucocorticoid receptor–interacting protein 1, a transcriptional coregulator of the p160 family, is important for this inhibition.[42] Interestingly, recent work has demonstrated that the anti-inflammatory actions of glucocorticoids differ in a sex-specific manner.[43] Glucocorticoids regulate a greater number of inflammatory genes and elicit a greater anti-inflammatory response in male compared with female rats. Sex-specific expression profiles of coregulatory molecules might underlie the sexual dimorphism by modulating the repressive actions of the GR on AP1 and NF-κB. The ***sex-specific differences*** in the anti-inflammatory actions of glucocorticoids not only provide a mechanistic basis for the predisposition of female subjects to autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, but also suggest that therapeutic doses of glucocorticoids should be optimized according to the sex of the patient.

Nonclassical GR signaling pathway

Although the principal effects of glucocorticoids are mediated by transcriptional responses that occur in minutes to hours, a growing body of evidence suggests that the GR can also act through nongenomic mechanisms to elicit rapid cellular responses that occur within a few seconds to minutes and do not require changes in gene expression (Fig 3).[44,45] Multiple mechanisms appear to be involved in these signaling events that ultimately impinge on the activity of various kinases, such as phosphoinositide 3-kinase, AKT, and mitogen-activated protein kinases (MAPKs). For example, the glucocorticoid-dependent release of accessory proteins associated with unliganded GR in the cytoplasm, such as the nonreceptor tyrosine kinase c-Src, can mediate nongenomic actions of glucocorticoids. When liberated from the GR complex, c-Src activates multiple kinase cascades that lead to the phosphorylation of annexin 1, inhibition of cytosolic phospholipase A2 activity, and impaired release of ***arachidonic acid***.[46,47] The GR has also been reported to localize at the plasma membrane in ***caveolae*** through an interaction with caveolin 1.[48] Glucocorticoid activation of this membrane-associated GR regulates gap junction intercellular communication and neural progenitor cell proliferation through a mechanism that requires c-Src activity and rapid MAPK-dependent phosphorylation of connexin-43.[49] The existence of nongenomic signaling adds greater complexity and diversity to glucocorticoids and their biological actions and raises the possibility that selective modulators of GR-dependent genomic or nongenomic pathways might be therapeutically advantageous.

GR polymorphisms

The capacity of the GR to function as a transcriptional activator or repressor is affected by several ***polymorphisms*** in the GR gene that alter the amino acid sequence of the encoded receptor. The ER22/23EK GR polymorphism occurs in approximately 3% of the population and results in an arginine to lysine change at position 23 within the NTD.[50,51] GR with the ER22/23EK polymorphism exhibits reduced transcriptional activity on both glucocorticoid-responsive reporters and endogenous genes and has been associated with glucocorticoid insensitivity. Persons with the ER22/23EK polymorphism have a lower tendency to have impaired glucose tolerance, type 2 diabetes, and cardiovascular disease, suggesting the relative resistance of ER22/23EK carriers to endogenous glucocorticoids might result in a more favorable metabolic profile.

The N363S polymorphism occurs in approximately 4% of the population and results in an asparagine to serine substitution in the NTD of the GR.[50] In contrast to ER22/23EK, the presence of the N363S polymorphism enhances the transcriptional activity of the GR and is associated with glucocorticoid hypersensitivity. In addition, a genome-wide microarray analysis has revealed a unique pattern of gene regulation for the N363S polymorphism.[52] N363S carriers have been reported to have an increased body mass index and a tendency toward decreased bone mineral density.[51]

These GR polymorphisms can account, at least in part, for the variability in the glucocorticoid response observed among subjects treated with these steroids.

GR SPLICE VARIANTS

The human GR gene is composed of 9 exons. The GR NTD is encoded primarily by exon 2, the DBD is encoded by exons 3 and 4, and the hinge region and LBD are encoded by exons 5 to 9 (Fig 4). Alternative splicing in exon 9 near the end of the GR primary transcript generates 2 receptor isoforms, termed GRα and GRβ (Fig 4).[53,54] GRα is derived from the end of exon 8 being joined to the beginning of exon 9, whereas GRβ results from the end of exon 8 being joined to downstream sequences in exon 9. These 2 proteins are identical through amino acid 727 but then diverge. GRα, the classic GR protein that mediates the actions of glucocorticoids, contains an additional 50 amino acids. The splice variant GRβ contains an additional, nonhomologous 15 amino acids. The unique GRβ carboxyl-terminal sequence confers several distinct properties to this receptor isoform. GRβ does not bind glucocorticoid agonists, resides constitutively in the nucleus of cells, and by itself is inactive on glucocorticoid-responsive reporter genes.[55,56] However, when coexpressed with GRα, GRβ functions as a dominant negative inhibitor and antagonizes the activity of GRα on many glucocorticoid-responsive target genes. Competition for GRE binding, competition for transcriptional coregulators, and formation of inactive GRα/GRβ heterodimers have each been proposed to underlie the antagonism mediated by GRβ.

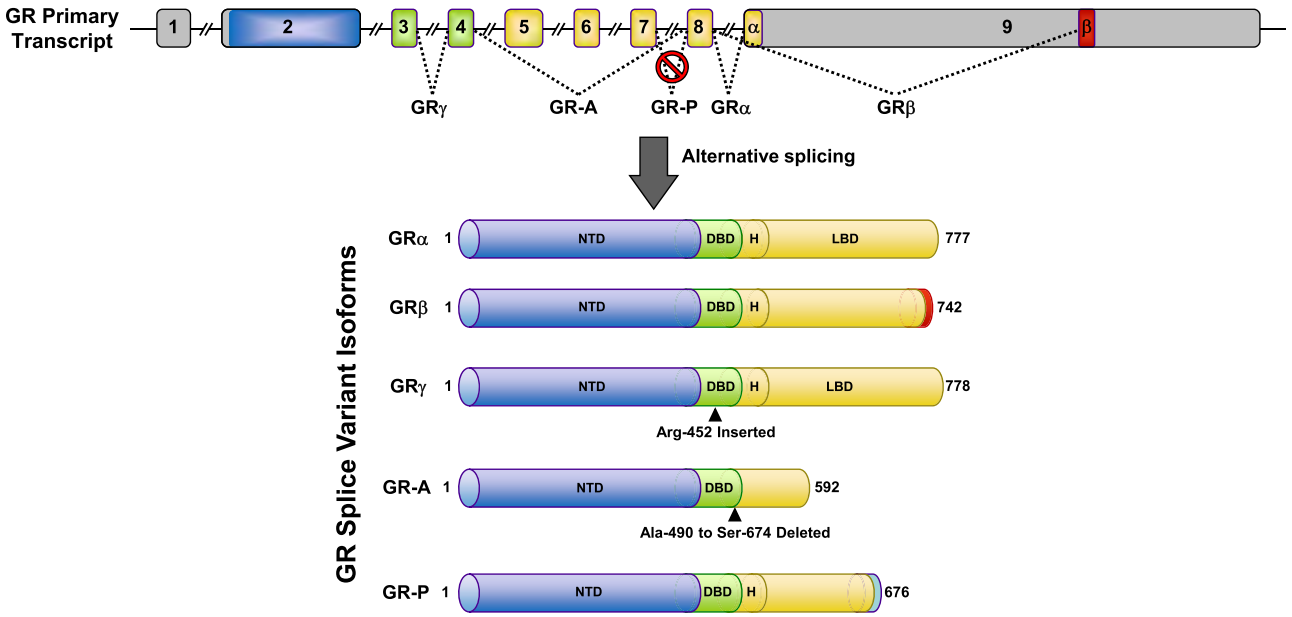

FIG 4. GR splice variants. The GR primary transcript is composed of 9 exons. Exon 2 encodes the NTD, exons 3 to 4 encode the DBD, and exons 5 to 9 encode the hinge region *(H)* and LBD. GRα results from splicing exon 8 to the beginning of exon 9. GRβ, GRγ, GR-A, and GR-P are generated by the depicted alternative splicing events.

The ability of GRβ to inhibit the activity of GRα suggests that high levels of GRβ might lead to glucocorticoid resistance. GRβ is prevalent in many cells and tissues but generally is found at lower levels than GRα. However, GRβ is abundant in certain cell types, such as neutrophils and epithelial cells. Moreover, the expression of GRβ can be selectively increased by proinflammatory cytokines and other immune activators and lead to reduced glucocorticoid sensitivity.[57-59] Increased GRβ levels have been associated with glucocorticoid resistance in a variety of inflammatory diseases, including asthma, rheumatoid arthritis, ulcerative colitis, nasal polyposis, systemic lupus erythematosus, sepsis, acute lymphoblastic leukemia, and chronic lymphocytic leukemia.[56] Upregulation of GRβ and diminished GRα signaling are also observed in erythroid cells expanded from patients with polycythemia vera.[60] The molecular factors that control GRβ expression are poorly understood, but several studies have implicated the involvement of the splicing factor SRp30c.[61-63] Treatment of cells with agents that induce SRp30c expression leads to a selective increase in GRβ levels and consequent glucocorticoid insensitivity. A naturally occurring polymorphism (A3669G) in the 3′ untranslated region of the GRβ mRNA also leads to increased GRβ expression.[64,65] The polymorphism disrupts an mRNA destabilization motif (AUUUA), resulting in a prolonged half-life of the GRβ mRNA. A3669G carriers have an increased risk for pathologies with known inflammatory underpinnings, such as autoimmune disease, myocardial infarction, coronary artery disease, and heart failure.[66-68] These findings suggest that the increase in GRβ levels in persons with the A3669G polymorphism might attenuate the beneficial immunosuppressive and anti-inflammatory actions of GRα.

Recent discoveries suggest an even broader role for GRβ in cell signaling and physiology. Using genome-wide microarrays on cells engineered to selectively express GRβ, multiple laboratories have shown that GRβ directly induces and represses the expression of a large number of genes independent of its dominant negative activity on GRα.[69,70] The ability of GRβ to recruit histone deacetylases and close local chromatin structures appears to be involved in its repression of certain genes, such as IL-5 and IL-13.[71,72] GRβ can also bind the glucocorticoid antagonist ***mifepristone*** (RU486).[70] In the presence of RU486, many of the GRβ-mediated changes in gene expression are abolished. These findings indicate that GRβ can function as a *bona fide* transcription factor, and they raise the possibility that GRβ modulates glucocorticoid responses through genomic actions distinct from its antagonism of GRα. Indeed, GRβ has recently been shown to directly reduce the expression of histone deacetylase 2 and promote glucocorticoid insensitivity in bronchoalveolar lavage cells from patients with steroid-resistant asthma.[73]

Another interesting finding has been the discovery of GRβ in zebrafish, mice, and rats.[74-76] These isoforms are similar in structure and function to human GRβ but originate from a mechanism of alternative splicing that differs from that in human subjects. Knockout of GRβ in these species promises to shed new light on the physiologic and pathologic importance of this splice variant.

In addition to GRβ, alternative splicing of the GR gene gives rise to other receptor isoforms with distinct signaling properties (Fig 4). The use of an alternative splice donor site in the intron separating exons 3 and 4 results in a receptor isoform that contains an insertion of a single arginine residue between the 2 zinc fingers of the GR DBD.[77] This receptor variant, termed GRγ, is widely expressed and binds glucocorticoids and DNA in a manner similar to GRα. However, GRγ is impaired in its ability to regulate glucocorticoid-responsive reporter genes and exhibits a transcriptional profile distinct from GRα on a subset of endogenous genes regulated by glucocorticoids. GRγ expression is associated with glucocorticoid resistance in patients with small cell lung carcinoma, corticotroph adenomas, and childhood acute lymphoblastic leukemia.[77-79] Two GR splice variants missing large regions of the LBD were initially discovered in

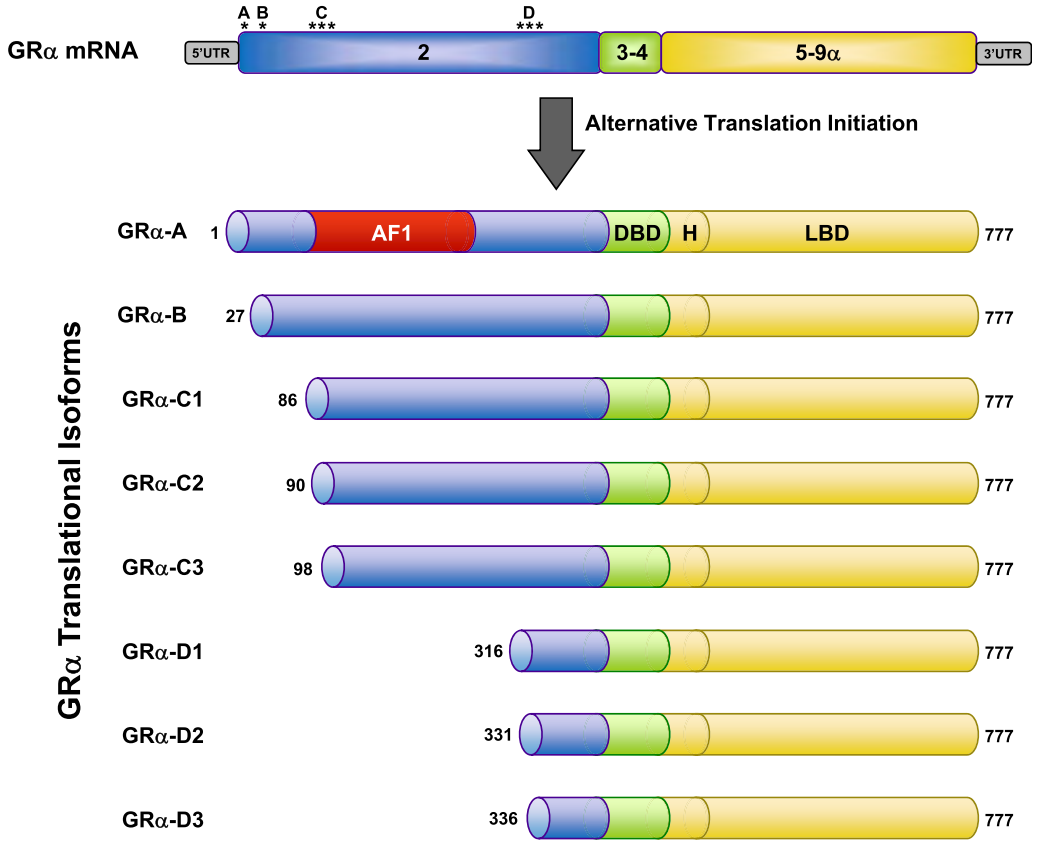

FIG 5. GR translational isoforms. Translation initiation from 8 different AUG start codons in the single GRα mRNA (location of the AUG start codons indicated by an *asterisk*) produces 8 receptor isoforms with progressively shorter amino NTDs. The *numbers* for the initiator methionines and AF1 region (amino acids 77-262) are for the human GRα protein. *UTR*, Untranslated region.

glucocorticoid-resistant multiple myeloma cells.[80] GR-A is missing middle exons 5 to 7, which encode the amino-terminal half of the LBD, and GR-P is missing the terminal exons 8 to 9, which encode the carboxyl-terminal half of the LBD. As expected from these changes in the LBD, GR-A and GR-P do not bind glucocorticoids. Little is currently known about GR-A; however, GR-P has been shown to modulate the transcriptional activity of GRα in a cell type–specific manner.[81-83] GR-P is found in many tissues and appears to be the predominant receptor variant expressed in glucocorticoid-resistant cancer cells.

GR TRANSLATIONAL ISOFORMS

Alternative translation initiation from the single GRα mRNA transcript produces an additional cohort of diverse GR proteins (Fig 5). Eight AUG start codons derived from exon 2 of the GR gene give rise to 8 GRα subtypes with progressively shorter NTDs (GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D3).[15,84] All 8 sites are highly conserved across species. GRα-A is the full-length receptor that is generated from the first initiator codon. Production of the truncated isoforms from internal start codons involves both ribosomal leaky scanning and ribosomal shunting mechanisms. The mRNA species encoding the splice variants discussed above (GRβ, GRγ, GR-A, and GR-P) also contain the identical set of start codons and would therefore be expected to give rise to a similar complement of translational isoforms.

The GRα translational isoforms display a similar affinity for glucocorticoids and a similar capacity to interact with GREs after ligand activation; however, they show marked differences in other properties.[8,15] The GRα-A, GRα-B, and GRα-C isoforms are localized in the cytoplasm of cells in the absence of hormone and translocate to the nucleus on glucocorticoid binding. In contrast, the GRα-D isoforms reside constitutively in the nuclei of cells. In addition, nuclear localized GRα-D associates with GRE-containing promoters of specific target genes independent of glucocorticoid treatment. Most striking, however, is that each GRα subtype possesses a distinct gene regulatory profile. When the individual isoforms are expressed at similar levels in U2OS osteosarcoma or Jurkat T lymphoblastic leukemia cells, they each regulate a unique set of genes.[8,85] Less than 10% of the genes were commonly regulated by all the subtypes, indicating that the vast majority of genes were selectively regulated by different GRα isoforms. These isoform-unique gene regulatory profiles lead to functional differences in glucocorticoid-induced apoptosis.[8,85,86] Cells expressing GRα-C3 were the most sensitive to the cell-killing effects of glucocorticoids, whereas cells expressing GRα-D3 were the most resistant. The heightened activity of the GRα-C3 isoform has also been observed on glucocorticoid-responsive reporters and has been linked to an N-terminal motif (residues 98-115) that is sterically hindered in the larger receptor isoforms.[15,87] When unobstructed in GRα-C3, this motif increases the activity of the AF1 domain, presumably through enhanced recruitment of various coregulators.

The complete absence of the AF-1 domain likely underlies the reduced transcriptional activity of the GRα-D isoforms. GRα-D3 does not efficiently repress the expression of multiple antiapoptotic genes because of deficits in its ability to interact with the p65 subunit of NF-κB and to be recruited to the promoters of these glucocorticoid-responsive genes.[86]

The discovery that each GRα translational isoform regulates a unique transcriptome suggests that the cellular response to glucocorticoids will be governed by the expressed complement of receptor subtypes. The GRα-A and GRα-B isoforms are the most abundant GR proteins in many cell types. However, trabecular meshwork cells from the human eye preferentially express the GRα-C and GRα-D isoforms.[88] In addition, immature dendritic cells predominantly express the GRα-D isoforms, whereas mature dendritic cells predominantly express the GRα-A subtype.[89] In rodents levels of GRα-C isoforms are highest in the pancreas and colon, and levels of the GRα-D subtypes are highest in the spleen and lung.[15] Recent studies have also demonstrated that the composition of GRα subtypes in a given cell or tissue can change in response to different conditions. Treatment of differentiated murine skeletal muscle cells with a selective estrogen-related receptor β/γ agonist induced a preferential increase in levels of the GRα-D isoforms,[90] and levels of the GRα-C isoforms are selectively upregulated after mitogen activation of human primary T cells.[85] In the human brain alterations in the expression of the GR subtypes were observed during development and during the ageing process.[91] Moreover, a selective increase in GRα-D levels was measured in specific regions of the brain in patients with schizophrenia and bipolar disorder.[92,93] Because preferential increases in the expression of the GRα-C or GRα-D isoforms might lead to glucocorticoid hypersensitivity or hyposensitivity, respectively, an important goal of future studies will be to define the operative cellular mechanisms that control the expressed complement of translational isoforms. Currently, the efficiency with which a particular start codon is used has been reported to be influenced by the ER22/23EK polymorphism in the GR gene and heterogeneity in the 5′ untranslated region of the GRα mRNA.[94,95] Posttranslational modifications that differentially affect the half-life of the various receptor subtypes might also contribute to alterations in the expression profile of the GRα isoforms.

POSTTRANSLATIONAL MODIFICATION OF GR ISOFORMS

Each GR isoform originating from alternative processing of the GR gene is subject to a variety of posttranslational modifications that further modulate its function and expand the repertoire of receptor subtypes available for glucocorticoid signaling. The first identified and most extensively studied covalent modification of GR is phosphorylation.[96-98] Human GRα is phosphorylated on at least 7 serine residues (Ser-113, Ser-134, Ser-141, Ser-203, Ser-211, Ser-226, and Ser-404), all of which are located in the NTD of the receptor (Fig 2). The major kinases that phosphorylate GRα include MAPKs, cyclin-dependent kinases, casein kinase II, and glycogen synthase kinase 3β. Many of the sites exhibit a low level of basal phosphorylation and become hyperphosphorylated after the binding of glucocorticoids.[99] The structure of the bound glucocorticoid can influence both the pattern and extent of GRα phosphorylation.[100] Ser-134 is unique in that it is phosphorylated in a glucocorticoid-independent manner by stress-activating stimuli.[101]

One of the major effects of GRα phosphorylation is that it changes the transcriptional activity of the receptor. Early studies demonstrated that phosphorylation-deficient GRα mutants were impaired in their ability to activate some glucocorticoid-responsive promoters but not others.[102] Subsequent reports showed that phosphorylation at Ser-211 correlated with increased transcriptional activity of GRα, whereas phosphorylation at Ser-226 decreased the signaling capacity of the receptor.[99,103,104] A deficiency in Ser-211 phosphorylation might contribute to the resistance to glucocorticoid-induced apoptosis that develops in malignant lymphoid cells.[105,106] Conversely, increased phosphorylation at Ser-226 might account for the impaired glucocorticoid signaling in the pathophysiology of depression.[107]

Phosphorylation of Ser-404 also has major consequences on glucocorticoid responses because the ability of GRα to both activate and repress target genes is diminished by phosphorylation at this site.[108] In cells expressing a GRα mutant incapable of Ser-404 phosphorylation, the global transcriptional response to glucocorticoids is redirected to favor the activation of distinct signaling pathways.

Consistent with many of the phosphorylation sites residing within the AF1 domain, differences in cofactor recruitment appear to be responsible for the transcriptional alterations that accompany GRα phosphorylation. GRα recruitment of the coactivator MED14 is enhanced by glucocorticoid-dependent phosphorylation at Ser-211,[104] whereas the interaction of GRα with the coactivator p300/CBP and the p65 subunit of NF-κB are both diminished by phosphorylation at Ser-404.[108]

Phosphorylation alters other properties of GR that effect the profile of glucocorticoid signaling. Degradation of the GRα protein is enhanced by glucocorticoid-dependent phosphorylation of the receptor because phosphorylation-deficient mutants are stabilized in the presence of glucocorticoids.[102] In addition, the ligand-free GRα is protected from degradation by its association with the tumor-suppressor gene TSG101, which preferentially interacts with the nonphosphorylated receptor.[109] The cellular distribution of GRα is also altered by receptor phosphorylation. GRα phosphorylated on Ser-203, Ser-226, or Ser-404 spends less time in the nuclear compartment because of greater cytoplasmic retention, enhanced nucleocytoplasmic transport, or both. As a consequence, GRα phosphorylated on either of these residues exhibits reduced transcriptional activity on glucocorticoid-responsive target genes.[99,103,108,110]

Additional posttranslational modifications of the GR have been described that regulate the function of the receptor. Ubiquitin is a 76-amino-acid protein that, when attached to specific lysine residues, marks proteins for proteasomal degradation. Ubiquitination of GRα at a conserved lysine residue (Lys-419) has been shown to target the receptor for turnover by the proteasome (Fig 2).[111,112] Mutant receptors that cannot be ubiquitinated at this residue are resistant to ligand-dependent downregulation and exhibit potentiated transcriptional activity on glucocorticoid-responsive reporter genes. Moreover, changes in the expression of an E3 ubiquitin ligase for GRα leads to alterations in both receptor levels and cellular sensitivity to glucocorticoids.[113]

GRα is also posttranslationally modified by sumoylation, a process in which SUMO (small ubiquitin-related modifier) peptides are covalently attached to specific lysine residues (Lys-277, Lys-293, Lys-703) within the receptor (Fig 2). The addition of SUMO peptides to the receptor occurs in the absence of ligand and is increased by the binding of glucocorticoids. Depending on

the site of sumoylation, the transcriptional activity of GRα can be enhanced or repressed through alterations in the recruitment or activity of specific coregulators.[114-119]

Finally, it has been suggested that GRα becomes acetylated on lysine residues (Lys-494 and Lys-495) located within the hinge region in response to glucocorticoid binding (Fig 2).[120] Deacetylation of the GR by histone deacetylase 2 was reported to be required for the receptor to efficiently repress the transcriptional activity of NF-κB, suggesting that ***acetylation*** of the GR limits the inhibitory actions of glucocorticoids on NF-κB signaling. More recently, studies have shown that the clock transcription factor acetylates GRα and attenuates its ability to both activate and repress glucocorticoid responsive genes, conferring glucocorticoid insensitivity to target tissues.[121]

Clearly, posttranslational modifications can regulate multiple aspects of GRα function, providing cells with additional receptor heterogeneity for controlling glucocorticoid responses. An important goal of future research will be to determine to what extent the various splicing and translational isoforms of the GR are subject to and regulated by these posttranslational modifications.

SUMMARY AND FUTURE PERSPECTIVES

Glucocorticoids act through the GR to regulate numerous physiologic processes, and synthetic derivatives of these hormones are widely prescribed for treating inflammatory diseases, autoimmune disorders, and various cancers. A challenge to clinicians and patients alike is that cell, tissue, and individual responses to glucocorticoids are markedly different and can change over time and in response to various extracellular cues. The discovery that multiple GR isoforms arise from a single gene has advanced our understanding of the molecular basis for the diversity in glucocorticoid signaling. GR isoforms with unique expression, gene-regulatory, and functional profiles are generated by alternative splicing of the primary transcript, alternative translation initiation of the mature mRNA, and posttranslational modifications of the encoded protein. The potential for these GR subtypes to undergo various posttranslational modifications and to function as monomers, homodimers, and heterodimers provides cells with a wealth of possibilities for generating diverse glucocorticoid responses with fine-tuned precision. Alterations in the relative levels of the GR subtypes might underlie pathologies characterized by hyposensitivity or hypersensitivity to glucocorticoids.

The cellular response to glucocorticoids will depend not only on the GR isoform composition but also on the glucocorticoid that binds and activates the receptor, as well as the concentration of the administered steroid. Not all glucocorticoids are created equal because structurally different but similarly potent steroids used in the clinic regulate both common and unique sets of genes.[88,122] The distinct transcriptional signature of these glucocorticoids suggests that their binding confers unique conformations on the GR that lead to differences in DNA binding, chromatin remodeling, and/or coregulator recruitment. Recent discoveries also suggest that the concentration of glucocorticoids needed to achieve the desired transcriptional response can vary in a context-specific manner. For example, GR repression of proinflammatory genes through tethering to NF-κB occurs at much lower glucocorticoid concentrations than the induction of gene expression.[123] Global GR recruitment assays have revealed that different GREs and their associated target genes also exhibit profound differences in their sensitivity to glucocorticoids.[29] In addition, dose-response studies in the livers of male and female rats have discovered several genes in female rats that are 10- to 100-fold less sensitive to glucocorticoid regulation than they are in male rats.[43] Remarkably, even the polarity of the transcriptional response of certain GR-regulated genes can vary depending on the dose of glucocorticoid.[124] Collectively, these findings suggest that as the specific genes underlying various pathologies become identified, careful consideration of the structure and dose of the used glucocorticoid might help fine-tune the glucocorticoid response and lead to improved benefit/risk ratios for patients undergoing steroid therapy.

Glucocorticoid resistance remains a major barrier to the effective treatment of a variety of immune and inflammatory diseases.[125,126] Advances in our understanding of glucocorticoid signaling have uncovered a variety of mechanisms that contribute to reduced glucocorticoid responsiveness, including increased expression of the GRβ and GRα-D isoforms, changes in GR phosphorylation, and homologous downregulation of the receptor. Understanding the molecular mechanisms of resistance permits not only the prediction of patient responsiveness to glucocorticoids but also the design of novel therapeutic strategies for combating the insensitivity. For instance, altered patterns of GR phosphorylation thought to contribute to glucocorticoid resistance in patients with severe asthma can be reversed by coadministration of long-acting β2AR agonists or p38 MAPK inhibitors.[125] Development of glucocorticoid analogues deficient in their ability to downregulate the expression of GR might also prove to be an effective strategy for overcoming steroid resistance.

The harmful side effects of medicinal glucocorticoids also remain a frustration for clinicians and their patients. Therefore intense efforts have been made over the last decade to develop novel GR ligands, termed dissociated or selective glucocorticoid receptor agonists (SEGRAs), that retain the negative regulation of gene expression thought to be important for many of the anti-inflammatory actions of glucocorticoids but lose the positive regulation of gene expression thought to underlie many of their adverse effects. A number of SEGRAs have been developed, but few have made it to clinical trials, and several recent studies have challenged both the premise and utility of these analogues as therapeutic agents.[127,128] One potential shortcoming of SEGRAs is that mounting evidence indicates that glucocorticoid-dependent activation of many genes also plays a critical role in the anti-inflammatory mechanisms of glucocorticoids. Clearly, more research is needed to unravel the molecular details by which glucocorticoids repress the inflammatory response and induce undesirable side effects. A greater understanding of the heterogeneity in GR signaling responses in both healthy and diseased tissues will aid in the development of safer and more effective glucocorticoid therapies.

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British Journal of Pharmacology (1999) 127, 1003–1011
http://www.stockton-press.co.uk/bjp

Ligand-induced differentiation of glucocorticoid receptor (GR) trans-repression and transactivation: preferential targetting of NF-κB and lack of I-κB involvement

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1 Glucocorticoids are highly effective in controlling chronic inflammatory diseases, such as asthma and rheumatoid arthritis, but the exact molecular mechanism of their anti-inflammatory action remains uncertain. They act by binding to a cytosolic receptor (GR) resulting in activation or repression of gene expression. This may occur *via* direct binding of the GR to DNA (transactivation) or by inhibition of the activity of transcription factors such as AP-1 and NF-κB (transrepression).

2 The topically active steroids fluticasone propionate ($EC_{50} = 1.8 \times 10^{-11}$ M) and budesonide ($EC_{50} = 5.0 \times 10^{-11}$ M) were more potent in inhibiting GM-CSF release from A549 cells than tipredane ($EC_{50} = 8.3 \times 10^{-10}$ M), butixicort ($EC_{50} = 3.7 \times 10^{-8}$ M) and dexamethasone ($EC_{50} = 2.2 \times 10^{-9}$ M). The anti-glucocorticoid RU486 also inhibited GM-CSF release in these cells ($IC_{50} = 1.8 \times 10^{-10}$ M).

3 The concentration-dependent ability of fluticasone propionate ($EC_{50} = 9.8 \times 10^{-10}$ M), budesonide ($EC_{50} = 1.1 \times 10^{-9}$ M) and dexamethasone ($EC_{50} = 3.6 \times 10^{-8}$ M) to induce transcription of the β_2-receptor was found to correlate with GR DNA binding and occurred at 10–100 fold higher concentrations than the inhibition of GM-CSF release. No induction of the endogenous inhibitors of NF-κB, IκBα or I-κBβ, was seen at 24 h and the ability of IL-1β to degrade and subsequently induce IκBα was not altered by glucocorticoids.

4 The ability of fluticasone propionate ($IC_{50} = 0.5 \times 10^{-11}$ M), budesonide ($IC_{50} = 2.7 \times 10^{-11}$ M), dexamethasone ($IC_{50} = 0.5 \times 10^{-9}$ M) and RU486 ($IC_{50} = 2.7 \times 10^{-11}$ M) to inhibit a $3 \times \kappa$B was associated with inhibition of GM-CSF release.

5 These data suggest that the anti-inflammatory properties of a range of glucocorticoids relate to their ability to transrepress rather than transactivate genes.

Keywords: Glucocorticoids; inflammation; GM-CSF; NF-κB; cross-coupling; RU486

Abbreviations: AP-I, activator protein-1; Bud, budesonide; Dex, dexamethasone; ECL, enhanced chemiluminescence; FCS, foetal calf serum; FP, fluticasone propionate; GCs, glucocorticoids; GM-CSF, granulocyte-macrophage colony stimulating factor; GR, glucocorticoid receptor; I-κBα, inhibitor of NF-κB; NF-κB, nuclear factor-κB; P/CAF, p300/CBP associated protein; PAO, phenylarsine oxide; PBS, phosphate buffered saline; PCIP, p300/CBP co-integrator protein; SRC-1, steroid-receptor coactivator-1; STAT, signal transduction and activation of transcription; TNFα, tumour necrosis factor-α; TRE, TPA-response element

Introduction

Cytokines, such as IL-1β, tumour necrosis factor-α (TNFα) and granulocyte-macrophage colony stimulating factor (GM-CSF), are released in a co-ordinate network and play an important role in chronic inflammation. As such, the pattern of cytokine expression largely determines the nature and persistence of the inflammatory response (Barnes & Adcock, 1993). Cytokines produce their cellular effects by activation of various transcription factors such as activator protein-1 (AP-I), nuclear factor-κB (NF-κB), and the signal transduction and activation of transcription (STAT) family. Furthermore, the expression of many of these cytokines and their receptors are also upregulated by these transcription factors. The increased expression of some of these factors may be responsible for the prolonged inflammation seen in asthma. AP-1 and NF-κB can also be induce, and be induced by, numerous other mediators such as NO, histamine, and eicosanoids (Barnes & Adcock, 1993).

Glucocorticoids (GCs) are the most effective anti-inflammatory therapy for the treatment of asthma and act by reducing airway hyper-responsiveness and suppressing the airway inflammatory response (Barnes, 1995). However, their exact mechanisms and cellular targets in the lung are uncertain. Glucocorticoid receptors (GR) are predominantly localized to the airway epithelium and endothelium (Adcock *et al*., 1996), therefore, these are likely to be important sites of the anti-inflammatory action of steroids, especially when delivered by the inhaled route. Airway epithelial cells do not act solely as a physical barrier but act as important regulators of the inflammatory reaction, responding to various inflammatory mediators by the production of a wide range of cytokines, chemokines and other inflammatory mediators (Levine, 1995).

Classically GCs act by binding to, and activating, the cytosolic GR. Upon activation the GR dimerizes and translocates to the nucleus. Within the nucleus GR binds to specific DNA elements (GREs) in the promoters of responsive genes resulting in modulation of transcription (Beato *et al*., 1996; Strahle *et al*., 1988). Important genes induced by

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glucocorticoids include the β_2-receptor (Collins *et al*., 1988) and lipocortin-1 (Flower & Rothwell, 1994). Lipocortin inhibits the activation of cytosolic phospholipase A_2 and thus may inhibit the synthesis of leukotrienes and prostanoids, although there is some doubt whether this mechanism is important in inhibiting asthmatic inflammation (Davidson *et al*., 1990).

The mechanisms involved in GR-mediated gene repression are less well understood. Experiments involving overexpression of the subunits of AP-1 and NF-κB along with deletion mutants of specific DNA binding moieties have indicated that the predominant mechanism of glucocorticoid down-regulation of inflammatory genes requires a direct protein-protein interaction with these, or related, transcription factors (Karin, 1998). An additional mechanism of repression by glucocorticoids on NF-κB mediated transcription has been reported in cultured monocytes and T-lymphocytes (Auphan *et al*., 1995; Scheinman *et al*., 1995a). Following steroid treatment a rapid induction of the inhibitor of NF-κB (I-κBα) mRNA and protein synthesis occurs. Newly synthesized I-κBα interacts with NF-κB heterodimers within the cytoplasm, and possibly the nucleus (Zabel *et al*., 1993) thereby inhibiting NF-κB DNA binding and transcriptional activation by cytokines. This mechanism does not appear to occur in all cells examined (Brostjan *et al*., 1996; Heck *et al*., 1997).

We have, therefore, investigated the role of these various mechanisms in mediating the ability of the glucocorticoids to inhibit IL-1β-induced release of GM-CSF, an NFκB-inducible gene (Kochetkova & Shannon, 1996), from a human lung epithelial-like cell line (A549). We have also examined the GR-induced inhibition of NFκB and AP-1 activity and the ability of GR to bind to its DNA binding motif and induce the β_2-receptor and IκBα and β proteins. In addition, we have examined the relative abilities of a number of topically acting steroids to activate or repress gene transcription to investigate whether current steroids can differentiate between these two modes of glucocorticoid action.

Methods

Drugs and chemicals

Fluticasone propionate, budesonide, tipredane and butixicort were kindly supplied by Dr M. Johnson (Glaxo-Wellcome, U.K.). RU486 (Mifepristone) was supplied by Dr A. Phillibert (Rousel-Uclaf, France). Enzymes were obtained from Promega (Cambridge, U.K.) and all other reagents, except where stated, were obtained from Sigma (Dorset, U.K.).

Cell culture

A549 cells were grown to confluence in Dulbecco's modified medium containing 10% foetal calf serum (FCS) before incubation for 72 h in serum-free media, as previously described (Newton *et al*., 1996). Cells were then used for the analysis of glucocorticoid action on GM-CSF release, β_2-receptor, I-κB expression and transcription factor activity.

GM-CSF release

A549 cells were cultured for 24 h with 1 ng ml^{-1} IL-1β in the presence or absence of various concentrations of steroids. After this time the culture supernatant was removed and stored at -70°C until samples were analysed. GM-CSF concentrations were measured using a specific ELISA calibrated with human recombinant GM-CSF (0–200 pg ml^{-1}, PharMingen, Lugano, Switzerland). Coating solution (0.1 M $NaHCO_3$, pH 8.2) containing 2 μg ml^{-1} rat anti human GM-CSF monoclonal antibody was used to coat an enhanced protein binding ELISA plate and incubated overnight at 4°C. The plate was washed three times in 0.05% Tween-20 in phosphate buffered saline (PBS) before blocking with 10% FCS in PBS for 2 h at 18°C. Plates were washed again and incubated with samples and standards (diluted in 10% serum in PBS) overnight at 4°C before washing again with 0.05% Tween-20 in PBS. 1 μg ml^{-1} biotinylated rat anti human GM-CSF diluted in 0.05% Tween-20 in PBS was incubated for 45 min at 18°C before extensive washing. The signal was detected following a 30 min incubation at 18°C with a 1:400 dilution of 1 mg ml^{-1} avidin-peroxidase solution (Sigma), extensive washing and final addition of ABTS substrate solution containing 1 μl ml^{-1} H_2O_2. The colour reaction products were read at 405 nm.

Electrophoretic mobility shift assay

Nuclear and cytosolic proteins were extracted from A549 cells as previously described (Adcock *et al*., 1995). Briefly, cells were collected and lysed in 200 μl of Buffer A 10 (mM) HEPES $MgCl_2$ 1.5, KCl 10, DTT 0.5, 0.1% Nonidet P40, and incubated at 4°C for 15 min. After microcentrifugation for 10 s and collection of the cytoplasmic fraction, the nuclear pellet was lysed with 20 μl of Buffer B (mM) HEPES 20, $MgCl_2$ 1.5, NaCl 0.42, DTT 0.5, 25% glycerol, PMS 0.5, EDTA 0.2. The subsequent soluble fraction was mixed with 100 μl of buffer C (mM) HEPES 20, KCl 50, DTT 0.5, PMSF 0.5, EDTA 0.2. 2 μg nuclear protein from each sample was pre-incubated at 4°C for 30 min in binding buffer (mM) Tris HCl 10, pH 7.5 $MgCl_2$ 1, EDTA 0.5, DTT 0.5, NaCl 50, 4% glycerol, 0.1 μg μl^{-1} salmon sperm DNA). Double-stranded oligonucleotides encoding the specific sequence of GRE (5′-TCGACTGTACAGGATGTTCTAGCTACT-) (Promega) were end-labelled with [γ^{-32}P]-ATP and T_4 polynucleotide kinase. Each sample was then incubated with 50,000 c.p.m. of labelled oligonucleotide for 40 min at 4°C. Protein-DNA complexes were separated on a 6% polyacrylamide gel using 0.25 × Tris-Borate-EDTA running buffer. Specificity was determined by the addition of excess unlabelled double stranded oligonucleotides.

Western blotting

Total cellular proteins were extracted from A549 cells by freeze-thawing samples in lysis buffer (mM) HEPES 20, $MgCl_2$ 1.5, NaCl 0.42, DTT 0.5, 25% glycerol, PMSF 0.5, EDTA 0.2 (Adcock *et al*., 1996). 30–50 μg total soluble protein extracts were size fractionated on 10% PAGE gels and transblotted onto Nitrocellulose-ECL membranes (Amersham International, Amersham, U.K.). Membranes were blocked overnight with 2% casein prior to incubation with either 1:1000 rabbit anti-human I-κBα antibody (Santa Cruz Biotechnology Inc., Devizes, U.K.), 1:1000 rabbit anti-human I-κBβ antibody (Santa Cruz Biotechnology Inc) or 1:600 rabbit anti-human human β_2 receptor (kindly donated by Dr J. MacDermot, RPMS, U.K.) at 18°C for 3 h. After washing (3 × 20 min in PBS-Tween), bound antibody was detected using 1:7000 sheep anti-rabbit antibody ($F(ab')_2$ fragment) linked to horseradish peroxidase (Amersham International, U.K.) and bound complexes detected using Enhanced Chemiluminescence (ECL, Amersham International) (Adcock *et al*., 1996).

Immunoprecipitation

Cells were treated for 30 min with 1 μM dexamethasone or fluticasone propionate in the presence of 1 ng ml^{-1} IL-1β. After incubation the cells were washed three times with fresh media before extraction in Buffer A above and two cycles of freeze-thaw. At the end of this time, soluble proteins (100 μg) were incubated for 18 h at 4°C with either an anti-human p65 antibody, an anti-human GR antibody or pre-immune serum (Santa Cruz). Immune complexes were precipitated with protein A-sepharose and loaded onto 10% PAGE gels. Following electrophoretic separation, proteins were electroblotted and transferred to ECL-nitrocellulose membranes and probed for the presence of p65 or GR.

Transient transfection and luciferase assay

Sense and antisense oligonucleotides encoding 6 copies of the consensus DNA binding site for AP-1 (TRE, 5′-CGCTTGATGAGTCAGCCGGAA-) were annealed by allowing to cool slowly to room temperature after incubation at 95°C for 2 min. The double stranded oligonucleotide was then inserted into pGL3-Basic at the *Sma*I site. The number of incorporated TRE sites and the direction of incorporation were confirmed by sequencing.

Cells were grown to confluence and then treated for 2 days in serum-free media. pGM-CSF(−123)-Luc (containing the −123 to +1 sequence of the human GM-CSF promoter), pGL-3κBLuc or pGL-6 × TRE-Luc were incubated with 2.5 μl Tfx−50 reagent (Promega) μg^{-1} DNA ml^{-1} of serum free media for 15 min at room temperature. Cells were transfected by the addition of 1 ml of media containing DNA-Tfx−50 (5 μl Tfx−50 μg−1 DNA) for 1.5 h before washing in fresh media and incubation in 1 ml serum free media. All cells were transfected with 1 μg pSV-β-gal vector (Promega) to control for transfection efficiency. After 18 h the media was changed and cells stimulated with varying concentrations of glucocorticoids in the presence or absence of 1 ng ml^{-1} of IL-1β. Cells were harvested by scraping and resuspended in 1 × reporter Lysis Buffer (Promega). After incubation at room temperature for 15 min lysates were vortexed for 10 s and subjected to one freeze-thaw cycle. Cellular debris was pelleted and total protein was measured. Luciferase assays were performed using 20 μl of extract and 50 μl Luciferase Assay Reagent (Promega) and luminescence measured with a TD 20/20 Luminometer (Turner Designs, Hemel Hempstead, U.K.). Relative luminescence readings were normalized to β-galactosidase expression and expressed as a percentage of activation relative to control or IL-1β-stimulated release.

Results

Effects of glucocorticoids on IL-1β-stimulated GM-CSF release

IL-1β caused a concentration-dependent increase in GM-CSF release after 24 h in A549 cells ($EC_{50} = 0.3$ ng ml^{-1}). IL-1β (1 ng ml^{-1}) stimulated the production of approximately 80% maximal levels of GM-CSF (22.5 ± 4.5 ng ml^{-1}). No detectable GM-CSF was found in the supernatant of control untreated cells. Co-incubation with fluticasone propionate, budesonide or dexamethasone all produced a concentration-dependent inhibition of IL-1β-stimulated GM-CSF release over this time period. Fluticasone propionate ($EC_{50} = 1.8 \times 10^{-11}$ M) and budesonide ($EC_{50} = 5.0 \times 10^{-11}$ M) produced a 122 fold and a 44 fold greater inhibition of GM-CSF release respectively than dexamethasone ($EC_{50} = 2.2 \times 10^{-9}$ M) (Figure 1a and Table 1). The anti-glucocorticoid RU486 also caused a concentration-dependent inhibition of IL-1β-stimulated GM-CSF release from A549 cells with an $EC_{50} = 1.8 \times 10^{-10}$ M. At concentrations greater than 10^{-7} M RU486 was less effective at inhibiting IL-1β-induced GM-CSF release (Figure 1b). Furthermore, the ability of 10^{-10} M fluticasone propionate, budesonide or dexamethasone to inhibit IL-1β-stimulated GM-CSF release was not inhibited by 10^{-8} M RU486 (Figure 1c). Both tipredane ($EC_{50} = 8.3 \times 10^{-10}$ M) and butixicort ($EC_{50} = 3.7 \times 10^{-8}$ M) also caused a concentration-dependent inhibition of IL-1β-stimulated GM-CSF release. The ability of IL-1β to stimulate GM-CSF release was markedly attenuated by the NF-κB inhibitor phenylarsine oxide (PAO) in a concentration-dependent manner with a maximal effect at 10 μM (Figure 1d). The proteosome inhibitor CBZ-leucine-leucine-leucinal (LLLal) caused a concentration-dependent inhibition of IL-1β (1 ng ml^{-1})-stimulated GM-CSF release ($EC_{50} = 30$ μM).

Effect of glucocorticoids on DNA binding activity

The ability of fluticasone propionate and dexamethasone to stimulate DNA binding was assessed by EMSA. Fluticasone propionate and dexamethasone produced a concentration-dependent increase in DNA binding. Fluticasone propionate ($EC_{50} = 5.2 \times 10^{-9}$ M) and budesonide ($EC_{50} = 6.4 \times 10^{-9}$ M) caused a marked increase in DNA binding with an approximately 10 fold higher potency than that seen for dexamethasone ($EC_{50} = 4.6 \times 10^{-8}$ M) (Table 1). Moreover the maximal level of DNA binding induced by fluticasone propionate was greater (30 fold stimulation) than that seen with dexamethasone (5 fold stimulation). At higher concentrations of fluticasone propionate (10^{-6} M) the inducibility of DNA binding was much reduced possibly reflecting the relative partial agonist activity of this drug. RU486 gave no consistent increase in DNA binding (Figure 2). Confirmation that the correct band was detected was obtained by using an excess of unlabelled oligonucleotide and also by the use of a specific GR antibody that supershifted the retarded band.

Effects of glucocorticoids on the induction of the β_2-adrenoceptor

In order to ensure that the glucocorticoids were able to induce gene expression in these cells the ability of these glucocorticoids to induce the expression of the β_2-adrenoceptor after 24 h was investigated by Western blotting (Figure 3). The concentration-dependent ability of fluticasone propionate ($EC_{50} = 1.0 \times 10^{-9}$ M), budesonide ($EC_{50} = 1.1 \times 10^{-9}$ M) and dexamethasone ($EC_{50} = 3.6 \times 10^{-8}$ M) to induce a 2–3 fold increase in the expression of the β_2-receptor at 24 h correlated with the induction of GR DNA binding. This occurred at 10–100 fold higher concentrations than that which repressed IL-1β-stimulated GM-CSF release (Figure 3). Fluticasone propionate caused a greater increase in β_2-receptor expression than either budesonide or dexamethasone. RU486 had no effect on β_2-adrenoceptor expression at any time or at any concentration tested (Table 1).

Effects of glucocorticoids on I-κBα and β expression

The ability of glucocorticoids to affect the induction of the cytoplasmic inhibitor of NF-κB, I-κBα, was also investi-

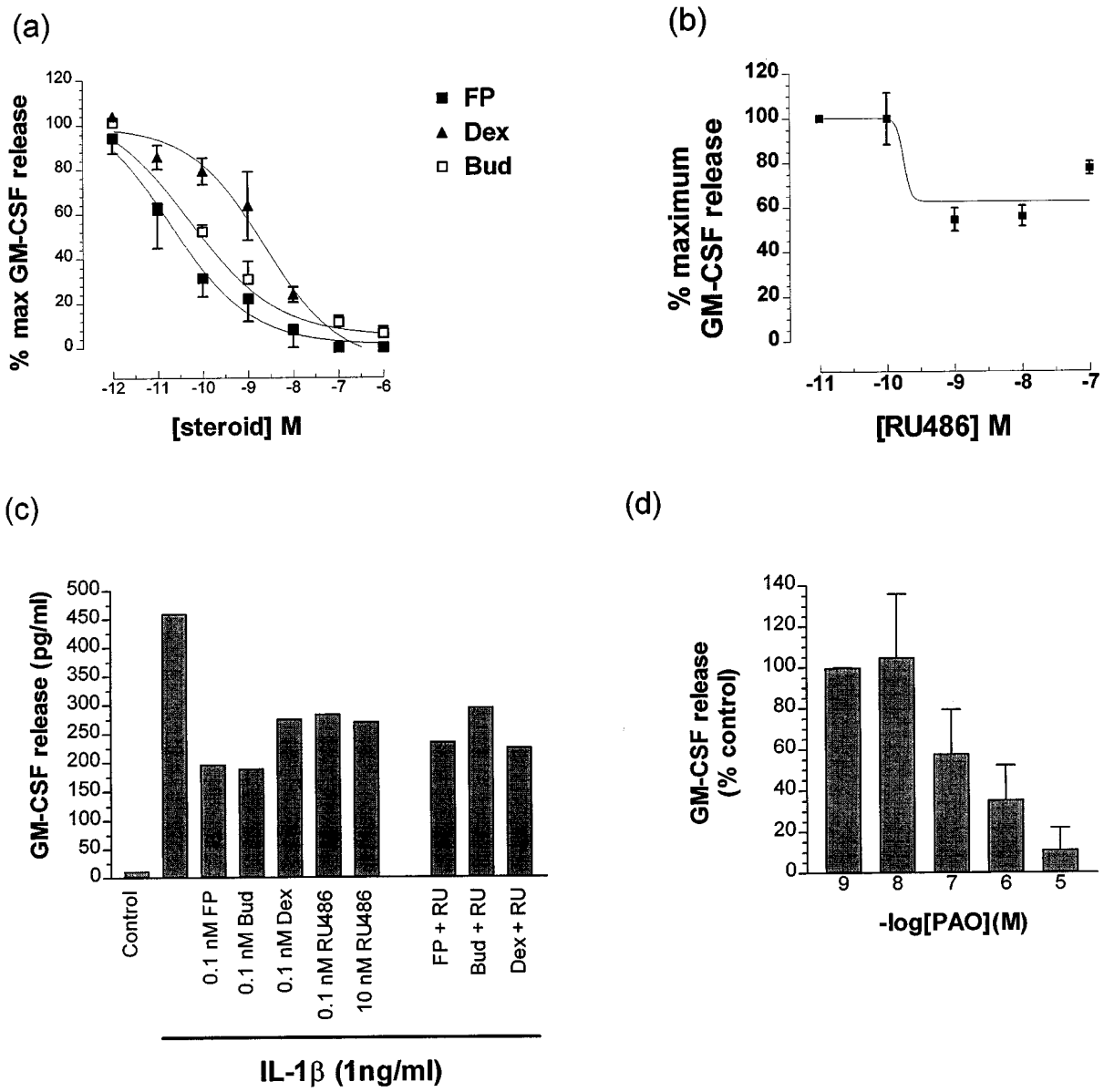

Figure 1 (a) Concentration-dependent inhibition of interleukin (IL)-1β (1 ng ml^{-1})-stimulated granulocyte-macrophage colony stimulating factor (GM-CSF) release into the media from A549 cells at 24 h following fluticasone propionate (FP), budesonide (Bud) and dexamethasone (Dex) treatment. (b) Concentration-dependent inhibition of IL-1β (1 ng ml^{-1})-stimulated GM-CSF release from A549 cells at 24 h following treatment with the anti-glucocorticoid RU486. (c) The effect of low concentration (10^{-9} M) RU486 (RU) treatment on the inhibition of IL-1β-stimulated GM-CSF release by 10^{-10} M FP, Bud and Dex. (d) The effects of increasing concentrations of phenylarsine oxide (PAO) on IL-1β (1 ng ml^{-1})-stimulated induction of GM-CSF release into culture medium at 24 h. Results are plotted as the means$\pm$s.e.means of the percentage of maximal IL-1β-stimulated GM-CSF release in the absence any drug. $n=4-7$ for each data point except in (c) where results are the mean of two independent experiments.

Table 1 Glucocorticoid effects on transactivation and transrepression in A549 cells

	FP IC_{50}	*Bud IC_{50}*	*Dex IC_{50}*	*RU486 IC_{50}*
GM-CSF release*	1.8×10^{-11} M	5.0×10^{-11} M	2.2×10^{-9} M	1.8×10^{-10} M
β_2 Receptor	1.0×10^{-9} M	1.1×10^{-9} M	3.2×10^{-8} M	No induction
GRE Binding	5.0×10^{-9} M	2.4×10^{-9} M	4.6×10^{-8} M	No induction
I-κBα	No induction	No induction	No induction	No induction
I-κBα degradation	No effect	No effect	No effect	No effect
I-κBβ	No induction	No induction	No induction	No induction
I-κBβ degradation	No effect	No effect	No effect	No effect
κB activity (*stim)	1.8×10^{-11} M	2.7×10^{-11} M	0.8×10^{-9} M	8.0×10^{-11} M
κB activity (basal)	0.5×10^{-11} M	2.7×10^{-11} M	0.5×10^{-9} M	2.5×10^{-11} M
TRE activity (*stim)	1.7×10^{-10} M	ND	0.9×10^{-9} M	ND
TRE activity (basal)	1.1×10^{-10} M	1.0×10^{-10} M	0.3×10^{-9} M	7.1×10^{-11} M
GM-CSF promotor*	0.6×10^{-11} M	ND	1.3×10^{-9} M	ND

FP, fluticasone propionate; Bud, budesonide; Dex, dexamethasone; GM-CSF, granulocyte macrophage colony stimulating factor; GRE binding, glucocorticoid receptor DNA binding. *After stimulation with interleukin (IL)-1β (1 ng ml^{-1}). ND, experiment not performed.

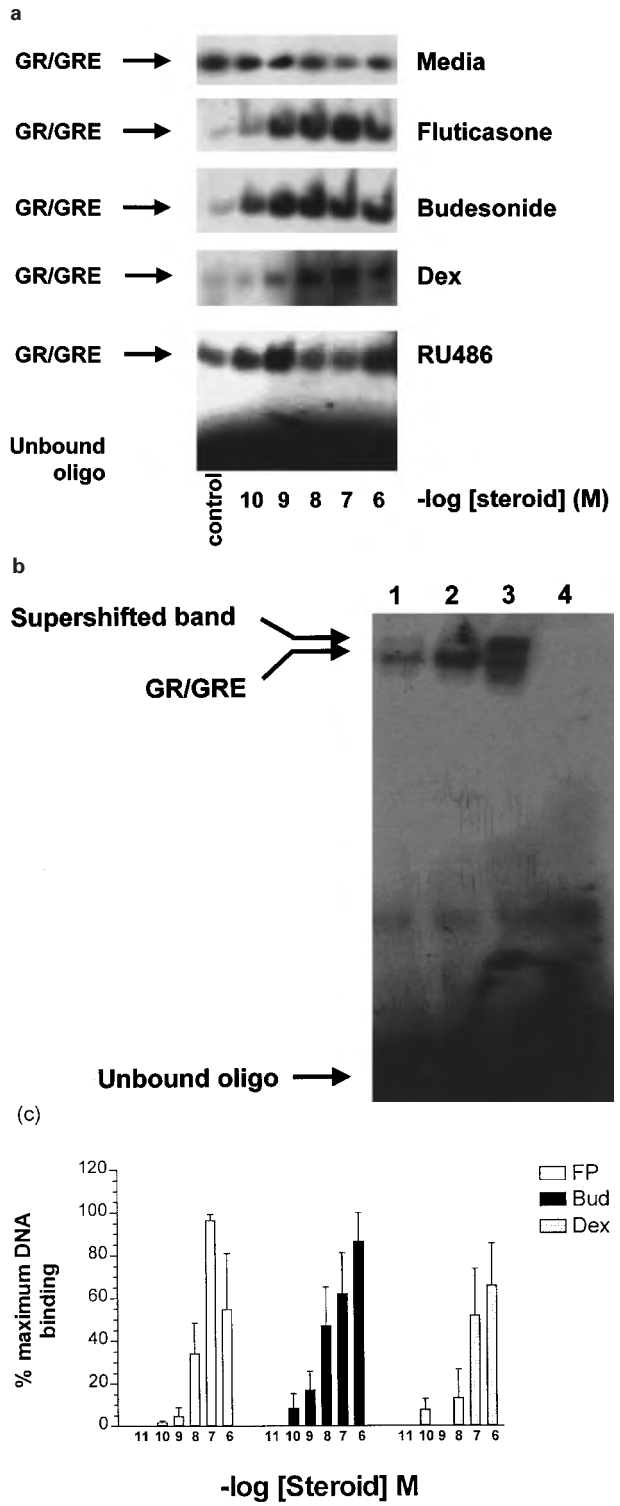

Figure 2 (a) Representative electrophoretic mobility shift assay showing the concentration-dependent effect of fluticasone propionate (FP), Budesonide (Bud) and dexamethasone (Dex) on glucocorticoid receptor (GR)-induced activation as represented by increased DNA binding (GRE binding) (arrowed) within the nucleus after 2 h incubation. (b) Supershift assay of dexamethasone (10^{-6} M)-stimulated GR DNA binding. Increased DNA binding is seen following dexamethasone treatment (lane 2). Pre-incubation of retarded complexes with an anti-GR antibody (lane 3) shows specific enhanced retardation of GR/GRE band. Specificity of binding was indicated by the addition of 100 fold excess unlabelled oligonucleotide (lane 4). Unbound oligonucleotide is indicated by an arrow at the bottom of the gel. (c) Densitometric analysis of the retarded bands in (a) and corrected for maximal band intensity showing the concentration-dependent increase in GRE binding following 2 h incubation with FP, Bud and Dex within the nucleus as a percentage of the maximal increase observed.

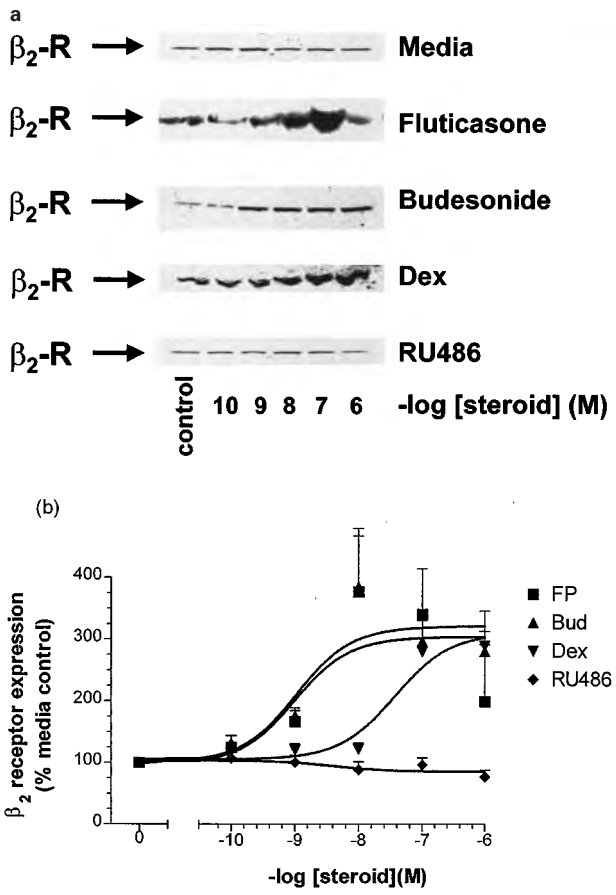

Figure 3 (a) Western blot analysis of β_2-receptor (β_2R) expression at 24 h following increasing concentrations of fluticasone propionate (FP), budesonide (Bud), dexamethasone (dex) or RU486. The single 47 kD band representing the β_2-receptor is indicated by the arrow. Incubation with control media does not affect β_2-receptor expression. (b) Graphical representation of the results shown in (a). Results are shown as the percentage change in β_2-receptor band density compared to control untreated cells and are representative of four individual experiments and are reported as the means ± s.e.means.

gated in these cells by Western blot analysis. Cells were incubated with glucocorticoids (10^{-12}–10^{-6} M) for various time periods of up to 24 h. None of the glucocorticoids investigated (fluticasone propionate, budesonide, dexamethasone or RU486) had any effect on the expression of I-κBα protein at any concentration tested in these cells (Figure 4a). IL-1β (1 ng ml^{-1}) stimulation caused a rapid phosphorylation and subsequent degradation of I-κBα which was inhibited by the proteosome inhibitor CBZ-leucine-leucine-leucinal (LLLal, 50 μM) (Figure 4b). This was followed at 60–90 min by the induction of *de novo* synthesized I-κBα (Figure 4c). We further examined the effects of these glucocorticoids on this degradation and reappearance of I-κBα within the cytoplasm of these cells. IL-1β caused a total loss of I-κBα protein from the cytoplasm within 2–5 min. The expression of I-κBα protein returned to control levels between 90 and 120 min. None of the drugs tested had any effect on the time course of I-κBα degradation or synthesis (Figure 4c). Glucocorticoids may affect NF-κB activation by altering the level of the I-κB associated with longer term induction of NF-κB, I-κBβ in these cells. None of the glucocorticoids tested had any effect on the induction of I-κBβ at 24 h. In contrast to I-κBα, IL-1β did not cause a rapid degradation of I-κBβ but

caused a decrease in I-κBβ levels at 4–6 h before returning to control levels which was not affected by glucocorticoids (Figure 4d and Table 1).

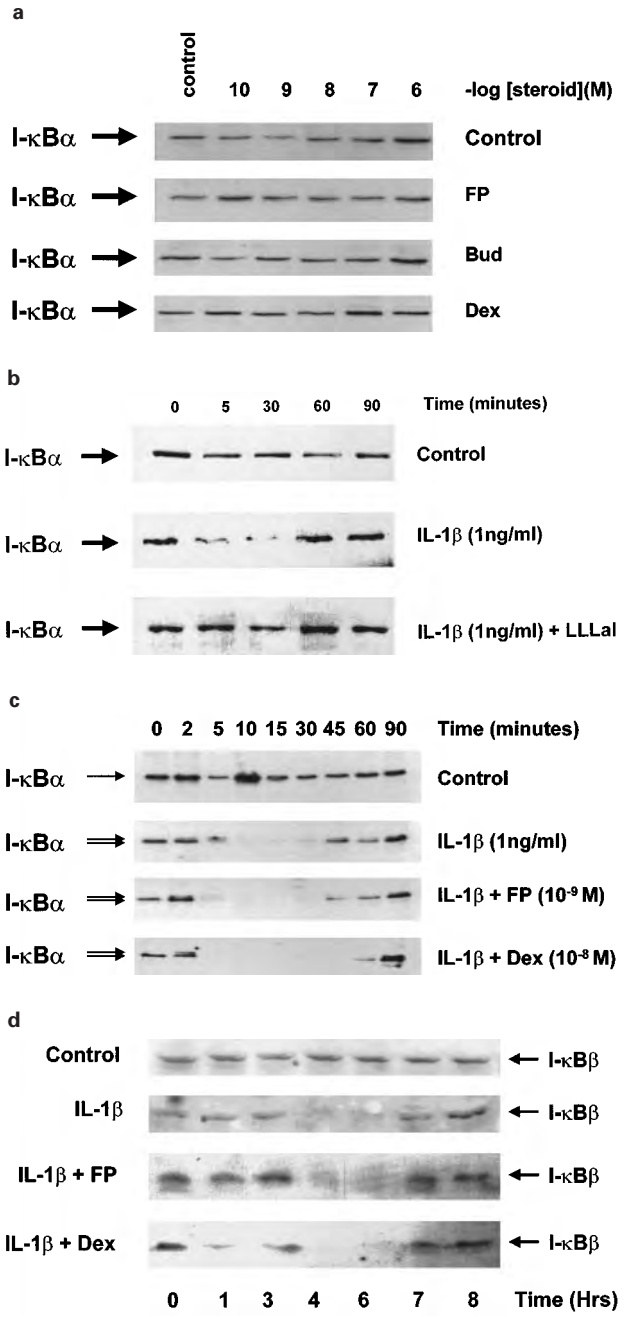

Figure 4 Western blot analysis of the time course of I-κBα expression following 24 h treatment with various concentrations of fluticasone propionate (FP), budesonide (Bud) and dexamethasone (Dex). Concentrations are reported as −log of Molar concentrations. (b) Western blot analysis of the time course of I-κBα expression following up to 90 min treatment with IL-1β (1 ng ml^{-1}) in the presence and absence of the proteosome inhibitor CBZ-leucine-leucine-leucinal (LLLal) (50 μM) I-κBα is indicated by the arrow. (c) Western blot analysis of the time course of I-κBα expression following up to 90 min treatment with IL-1β (1 ng ml^{-1}). The effects of fluticasone propionate (FP, 10^{-10} M), budesonide (Bud, 10^{-10} M) and dexamethasone (Dex, 10^{-9} M) on IL-1β-stimulated I-κBα phosphorylation, degradation and subsequent induction are shown. I-κBα and the slower migrating phosphorylated form of I-κBα are indicated by the arrows. (d) The lack of effect of fluticasone propionate (FP, 10^{-10} M) budesonide (Bud, 10^{-10} M) and dexamethasone (Dex, 10^{-9} M) on IL-1β-stimulated I-κBβ phosphorylation, degradation and subsequent induction over 0–8 h are shown. Results are representative of four individual experiments.

GR/p65 immune complex

Immunoprecipitation studies showed that the p65 subunit of NF-κB was associated with GR, either directly or within a complex, within A549 cells. Western blot analysis of the immunoprecipitated p65 complex showed the presence of a specific GR band. In the reverse experiment the immunoprecipitated GR complex showed the presence of p65 (Figure 5). In contrast, immunoprecipitation with pre-immune serum showed no binding of GR or p65.

NF-κB- and TRE-driven reporter gene constructs

IL-1β (1 ng ml^{-1}) stimulation produced a significant 3 fold induction in luciferase activity with both the pGM-CSF(−123)-Luc and the pGL-3κBLuc vectors. IL-1β-stimulated 3×κB-activated luciferase activity was inhibited in a concentration-dependent manner by fluticasone propionate (FP, $IC_{50} = 1.8 \times 10^{-11}$ M), budesonide (Bud, $IC_{50} = 2.7 \times 10^{-11}$ M), dexamethasone (Dex, $IC_{50} = 0.8 \times 10^{-9}$ M) and RU486 ($IC_{50} = 8 \times 10^{-11}$ M) (Figure 6a). The basal expression of pGL-3κB-Luc was also modulated by glucocorticoids. Fluticasone propionate ($IC_{50} = 0.5 \times 10^{-11}$ M), budesonide ($IC_{50} = 2.7 \times 10^{-11}$ M) and dexamethasone ($IC_{50} = 0.5 \times 10^{-9}$ M) caused a 50–70% decrease in luciferase activity compared to those seen in control unstimulated cells. RU486 also caused a concentration-dependent inhibition of κB-driven luciferase activity ($IC_{50} = 2.5 \times 10^{-11}$ M) but the maximal reduction seen was 50% of basal levels. IL-1β-stimulated GM-CSF-promoter driven luciferase activity was inhibited in a concentration-dependent manner by fluticasone propionate ($IC_{50} = 0.6 \times 10^{-11}$ M) and dexamethasone ($IC_{50} = 1.3 \times 10^{-9}$ M) (see Table 1).

IL-1β (1 ng ml^{-1}) caused a 2.4 fold increase in a 6×TRE promoter-driven luciferase activity after 18 h of stimulation. IL-1b-stimulated TRE-promoter driven luciferase activity was inhibited in a concentration-dependent manner by fluticasone propionate ($IC_{50} = 1.7 \times 10^{-11}$ M) and dexamethasone ($IC_{50} = 0.9 \times 10^{-9}$ M) (Figure 6b). These glucocorticoids also reduced basal expression of TRE activity, fluticasone propionate ($IC_{50} = 1.1 \times 10^{-10}$ M), budesonide ($IC_{50} = 1.0 \times 10^{-10}$ M) and dexamethasone ($IC_{50} = 0.3 \times 10^{-9}$ M) all reducing levels to less than 50% of those seen in control unstimulated cells. RU486 also caused a concentration-dependent inhibition of TRE activity ($IC_{50} = 7.1 \times 10^{-11}$ M) but the maximal reduction seen was 50% of basal levels. Inhibition of TRE activity occurred at approximately 10 fold lower concentrations than that which caused inhibition of κB-driven luciferase activity and GM-CSF release (see Table 1).

Discussion

Fluticasone propionate and budesonide were more potent as inhibitors of GM-CSF release and NF-κB activity than dexamethasone. Although all these ligands were acting through the same receptor, fluticasone propionate and budesonide were approximately five times more potent at these targets than would be predicted purely by ligand binding affinity. In contrast, the ability of these drugs to modulate AP-1 reporter gene activity was more closely related to their GR ligand binding affinity (see Brattsand & Linden (1996)). This suggests that altered conformational changes in the GR monomer may alter the ability to repress gene expression in a ligand dependent manner. This was further suggested by the ability of low concentration, but not high concentration, RU486 to suppress IL-1β-stimulated GM-CSF release and κB activity.

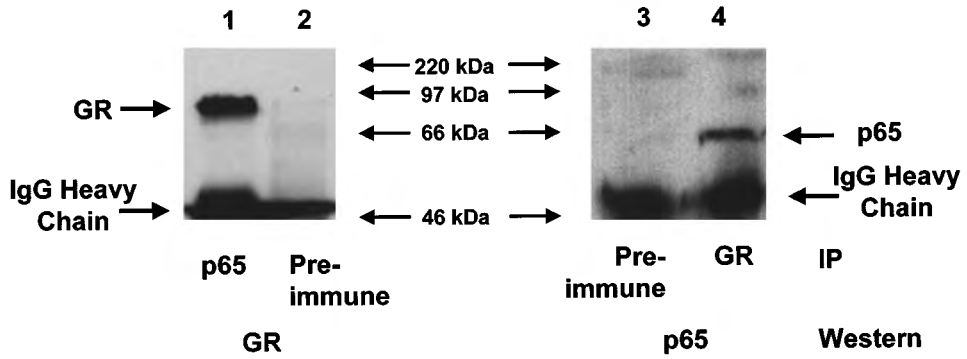

Figure 5 Western blot analysis of immunoprecipitated p65 and GR complexes. Cells were treated for 30 min with a combination of IL-1β (1 ng ml^{-1}) and dexamethasone (10^{-6} M) before cell were lysis. Total cell extracts were immunoprecipitated with an anti-human p65 antibody (lane 1), an anti-human GR antibody (lane 4) or with pre-immune serum (lanes 2 and 3) before separation by 10% PAGE and detection of bands by either anti-human GR antibody (lanes 1 and 2) or anti-human p65 antibody (lanes 3 and 4). The specific GR or p65 bands are indicated arrows. The 50 kDa IgG heavy chain is detected in all samples and is also arrowed. Molecular weight markers are as indicated. The results are representative of three independent experiments.

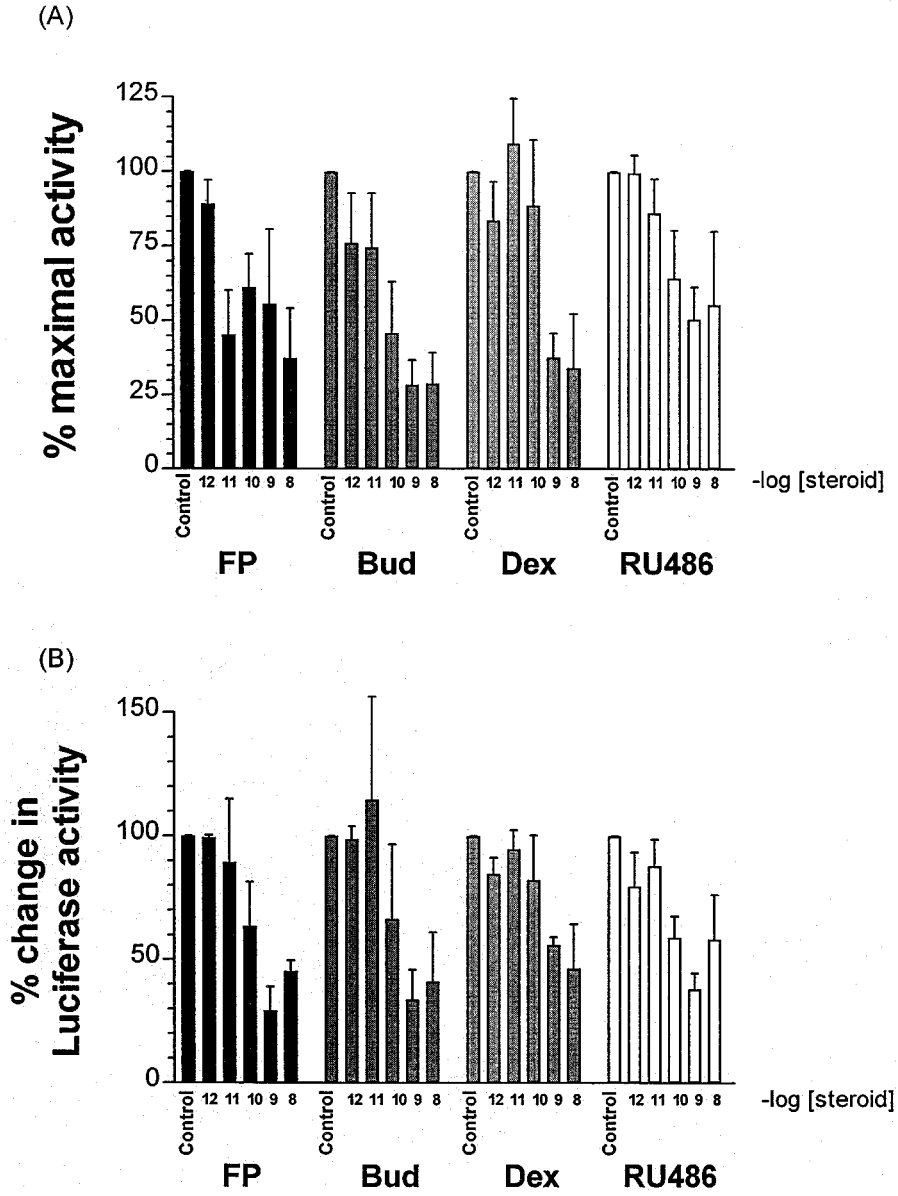

Figure 6 (a) Inhibition of an IL-1β (1 ng ml^{-1})-stimulated 3 × κB-driven luciferase reporter gene by fluticasone propionate (FP), budesonide (Bud), dexamethasone (Dex) and RU486. Results are expressed as relative light units/unit β-galactsidase activity (means ± s.e.mean) and represent the results of at least four independent experiments. (b) Inhibition of IL-1β (1 ng ml^{-1})-stimulated 6 × TRE-Luc reporter gene by fluticasone propionate (FP) and dexamethasone (Dex). Results are expressed as relative light units/unit β-galactsidase activity (means ± s.e.mean) and represent the results of at least four independent experiments.

In order to examine the potential mechanisms for this repression of GM-CSF release we examined the ability of these drugs to increase the expression of the β_2-adrenoceptor and the inhibitor of NF-κB-driven transcription, I-κB. The ability of these glucocorticoids to induce the β_2-adrenoceptor was found to correlate with GR/GRE binding and occurred at 10–100 fold higher concentrations than the inhibition of GM-CSF release (see Table 1). These results are similar to those for fluticasone and dexamethasone suppression of TNFα-induced E-selectin expression (Ray *et al*., 1997). No induction of I-κBα, was seen in these cells by any steroid at concentrations up to 1 μM for time periods up to 24 h. Furthermore, the ability of IL-1β to cause I-κBα or I-κBβ degradation and subsequent induction was not affected by steroids. This confirms results obtained in several other cell types where no effect of dexamethasone was observed (Heck *et al*., 1997; Brostjan *et al*., 1996; Ray *et al*., 1997) and suggest that induction of gene transcription by the activated glucocorticoid receptor was not required for inhibition of GM-CSF release.

The possibility of a direct interaction between activated GR and NF-κB in the suppression of NF-κB activated gene transcription was indicated by the ability of these glucocorticoids to inhibit a reporter gene construct containing three κB sites alone and was confirmed by immunoprecipitation experiments. This interaction is likely to occur through a leucine charged domain in the p65 subunit (Heery *et al*., 1997). The inhibition of luciferase activity correlated with inhibition of GM-CSF release suggesting that this is indeed an important mechanism in regulating GR actions in these cells. However only a 50–60% inhibition of luciferase activity was seen at concentrations of steroid at which GM-CSF release was completely inhibited. This suggests that although repression of gene transcription plays a major role in the suppression of GM-CSF release in these cells other post-transcriptional events may also be important in glucocorticoid-repression of GM-CSF release.

The data presented here suggests that the anti-inflammatory properties of a range of glucocorticoids relate to their ability to transrepress rather than transactivate genes. Furthermore, the results seen with RU486 suggests that repression of NF-κB and AP-1 activity by GR does not require the transactivation function of GR. The results also suggest that transrepression occurs at approximately 10 fold lower concentrations than that required for transactivation of genes such as the β_2-receptor. The ability of the more modern inhaled glucocorticoids fluticasone propionate and budesonide, but not dexamethasone, to inhibit NF-κB activity appears to correlate more closely with GM-CSF release than the ability to inhibit AP-1 activity. This suggests that NF-κB may be a more important target for glucocorticoid actions, at least in the regulation of inflammatory genes in A549 cells, than AP-1. However, in the context of other diseases and other cells AP-1 may be a more important target. The importance of inhibiting both NF-κB and AP-1 within these cells may be relevant in the control of inflammatory responses since these transcription factors are important for the expression of many genes and often act in concert with each other (Stein *et al*., 1993).

Similar results have been demonstrated for the interaction between GR and AP-1 (Heck *et al*., 1994). In these studies using overexpression vectors, a variety of GR mutants and a choice of glucocorticoid ligands, it has been possible to dissociate the transrepressive and transactivation functions of GR on AP-1 mediated reporter gene activities. Moreover, DNA binding and activation of glucocorticoid-regulated promoters require GR dimerisation, whereas AP-1, and probably NF-κB, repression may be mediated by GR monomers (Heck *et al*., 1994). Increased levels of either AP-1 or NF-κB, raised by overexpression of cDNAs, also have profound effects on the ability of GR to inhibit transcription driven by these genes. Expression of GR at levels that cause marked inhibition of either AP-1 or NF-κB alone fails to repress either factor when both are activated together (Scheinman *et al*., 1995b).

Recent results from several laboratories have provided similar but slightly different results regarding the exact mechanisms of glucocorticoid suppression of gene transcription. These differences may be due to the level of expression of GR in each system since some depend upon over-expression of GR, some occur in the presence of serum and others in the absence of serum. We have recently found that the removal of serum (or the use of charcoal-stripped serum) results in markedly up-regulated expression of GR at 48 h (Adcock *et al*., unpublished observations).

Studies of transcription factor interactions may have therapeutic potential in the control of inflammatory disease. Glucocorticoids exert their anti-inflammatory effects largely by interference with the ability of transcription factors that have been activated by inflammatory cytokines to induce transcription of inflammatory genes. This interaction between GR and transcription factors may be either direct or through an integrator molecule such as CBP or associated co-activators such as p300/CBP associated protein (P/CAF), steroid-receptor coactivator-1 (SRC-1) or p300/CBP co-integrator protein (pCIP) (Janknecht & Hunter, 1996). Interaction of these factors with CBP allows interaction, modulation of histone acetylation and subsequent activation of the basal transcription initiation complex. Although not shown here for NF-κB, it is possible that this is the mechanism by which glucocorticoids interfere with NF-κB-driven gene transcription (Perkins *et al*., 1997; Sheppard *et al*., 1998). These interactions may affect transactivation by the pro-inflammatory transcription factor due to effects on DNA-binding, association with the integrator molecules or activation of RNA polymerase II to varying degrees. The exact contribution of each mechanism may vary between cell types and depend upon the cell stimulus. The differences in activity of different glucocorticoids did not correlate solely with ligand binding but may reflect differential interaction of the same receptor with DNA or proteins according to the activating ligand. Similar ligand-induced differentiation of transrepression and transactivation activities has been reported previously for the interaction between retinoic acid and its receptors (RARα) (Yang Yen *et al*., 1991). Thus, there is potential for the development of novel glucocorticoids with enhanced transrepressive and reduced transactivation actions. Other drugs that regulate the activity of specific transcription factors may also be developed in the future.

In conclusion, both trans-repression and trans-activation by GR may be affected by the ligand in a manner that reflects not only ligand affinity but also the differential abilities of ligands to influence GR interaction with DNA, other transcription factors, integrator molecules or the basal transcription apparatus. The differentiation of these two activities (trans-repression and trans-activation) leads to the possibility of the development of more specific glucocorticoids. Indeed, these results suggest that current drugs used in asthma therapy may already have been selected for an enhanced ability to transrepress rather than activate gene transcription.

This work was funded by the Medical Research Council (U.K.), The National Asthma Campaign, The European Union Biomed II programme and Glaxo-Wellcome.

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(Received January 25, 1999
Revised March 18, 1999
Accepted March 23, 1999)