New Steroidal Antiinflammatory Antedrugs: Steroidal [16α,17α-*d*]-3′-Carbethoxyisoxazolines

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Novel steroidal antiinflammatory antedrugs, 11β,20-dihydroxy-3,20-dioxo-3′-(ethoxycarbonyl)-isoxazolino[16,17-*d*]pregna-1,4-diene (**2a**) and 9-fluoro-11β,20-dihydroxy-3,20-dioxo-3′-ethoxycarbonylisoxazolino[16,17-*d*]pregna-1,4-diene (**2b**) were prepared in 97% yield via 1,3-dipolar cycloaddition of carbethoxyformonitrile (CEFNO) to 11β,21-dihydroxy-3,20-dioxopregna-1,4,-16-triene (**1a**) and 11β,21-dihydroxy-3,20-dioxo-9-fluoropregna-1,4,16-triene (**1b**), respectively, which were prepared *via* five steps from prednisolone and 9-fluoroprednisolone, respectively. The treatment of steroids **2a** and **2b** with acetic anhydride in pyridine led to the corresponding 21-acetates **3a** and **3b**, respectively, in 95% yield. Dose–response profiles of the croton oil-induced ear edema bioassay in rats were used to calculate the following ID_{50} values (nmol/ear resulting in a 50% reduction of edema): prednisolone (**P**), 540 nmol; **2b**, 135 nmol; and **3b**, 101 nmol. Inhibition of edema did not exceed 50% following application of either **2a** or **3a**. Relative potency calculations indicated that **2b** was 4-fold and **3b** 5.3-fold more potent than the parent compound **P** when applied topically. No significant adverse systemic effects were seen following treatments with **3b**. These results suggest that C-9-fluorination, side-chain hydroxy group esterification, and [16α,17α-*d*]-3′-carbethoxyisoxazoline additions to the conventional steroid **P** improve topical antiinflammatory activity without concomitant increases in adverse systemic activity.

Introduction

The predominant setback in the use of antiinflammatory steroids is their systemic suppressive effects on pituitary function and the immune system. In an effort to circumvent the adverse clinical systemic effects, new steroidal antiinflammatory antedrugs that act locally at the site of application but are easily transformed into inactive metabolites upon entry into the systemic circulation are being synthesized and tested.[1–7] Our most recent efforts toward acheiving this goal include the synthesis of 11β,20-dihydroxy-3,20-dioxo-3′-(ethoxycarbonyl)isoxazolino[16,17-*d*]pregna-1,4-diene (**2a**) and 9-fluoro-11β,20-dihydroxy-3,20-dioxo-3′-(ethoxycarbonyl)isoxazolino[16,17-*d*]prednisolone (**2b**), respectively. Treatment of steroids **2a** and **2b** with acetic anhydride in pyridine led to the corresponding 21-acetates **3a** and **3b**, respectively. Novel compounds were then screened for antiinflammatory activity and adverse systemic effects using both single and multiple dose experimental paradigms of the croton oil-induced ear edema bioassay.

Although topical application of steroids reduces adverse systemic effects, long-term usage or application of large doses result in toxic systemic side effects.[3] Much effort has been devoted to structural modification of cortisol with hopes of increasing its potency as well as minimizing the well-documented adverse effects of glucocorticoids. The prototypic steroidal antedrugs, ester derivatives of steroid-21-oic acids, prepared by modifying the 17β-ketol side chain of prednisolone have been shown to retain the significant local antiinflammatory activity seen following application of the parent compound but are devoid of prednisolone-like adverse systemic effects.[8–10] The antedrug concept has been further applied by incorporating a metabolically labile group such as a carboxy ester or carboxamide at strategic positions on the steroid nucleus.[1,2,6]

Syntheses of the steroidal [16α,17α-*d*]-3′-carbethoxyisoxazolines and results of their pharmacologic screening using single- and multiple-dose paradigms of the croton oil-induced ear edema bioassay in rats are presented in this report.

Discussion

Results of pharmacologic screening assays employing the croton oil-induced edema bioassay indicate that, of the novel antedrugs tested, the 9-fluoro derivatives **2b** and **3b** showed marked improvement in antiinflammatory activity when compared with the parent compound **P**. Calculation of relative potencies with prednisolone defined as 1 indicated that **2b** was 4-fold more potent than **P** while **3b** was 5.3-fold more potent than **P**. It is well-documented that fluorination of the steroidal molecule, particularly at the 9α-position, enhances antiinflammatory activities of glucocorticoids. We have previously demonstrated that 9α-fluorination of the antedrug DP16CM (methyl 11β,21-dihydroxy-3,20-dioxo-1,4-pregnadiene-16α-carboxylate) doubled its antiinflammatory potency when applied topically in the croton oil-induced ear edema bioassay.[7] Results of the present results demonstrate an even greater increase in potency of the steroidal [16α,17α-*d*]-3′-carbethoxyisoxazoline which had been 9α-fluorinated.

Esterification of the 21- and/or 17-hydroxyl groups of corticosteroids, which increase physicochemical properties such as lipophilicity and solubility, has been employed to improve topical antiinflammatory activity of glucocorticoids.[4] Results of the present investigations demonstrate that side-chain esterification of the novel fluorinated derivative (**3b**) further increased its topical antiinflammatory potency.

Multiple topical applications of equiactive ID_{50} doses of the two novel fluorinated derivatives **2b** and **3b** and **P** in the subacute croton oil edema assay yielded results

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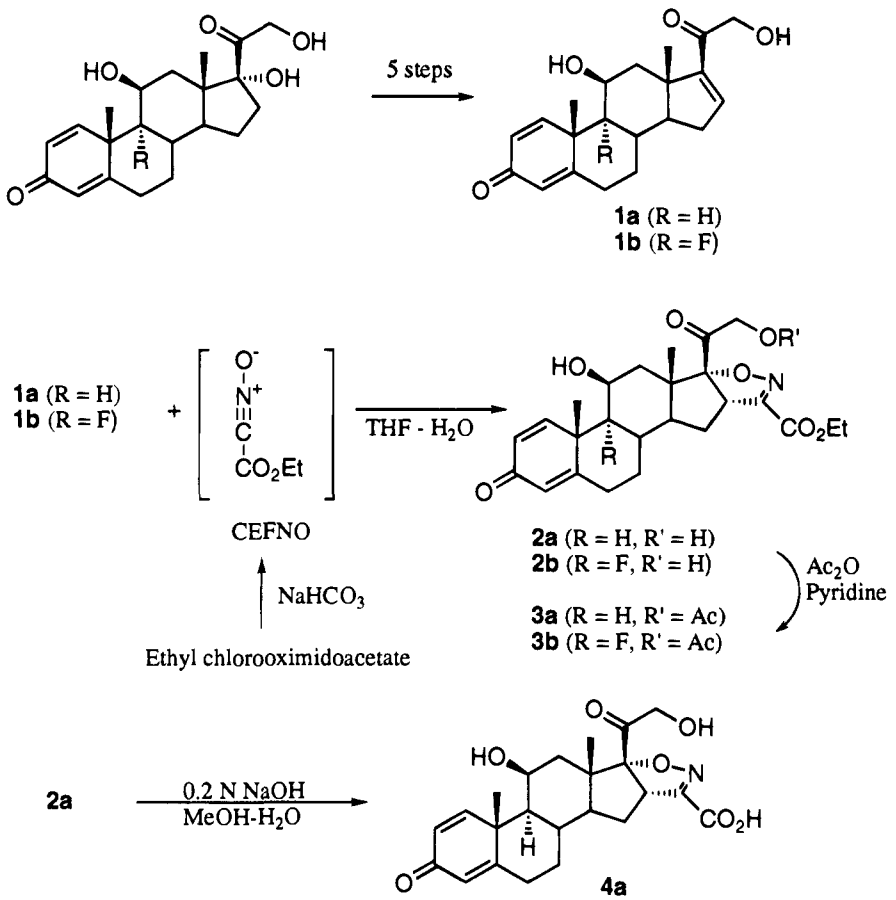
Scheme 1



suggesting that only **P** exhibited untoward effects as measured by less body weight gain, decreased thymus weights, and lowered plasma corticosterone levels. Novel derivative **3b**, both esterified and fluorinated, exhibited no significant adverse systemic effects. In contrast, **2b**, its fluorinated but non-esterified counterpart, did significantly decrease plasma corticosterone levels and showed significant inhibition of edema in the contralateral untreated ears. This is taken to indicate that some systemic absorption of **2b** or an active metabolite was exerting adverse effects.

Taken together, results of these investigations suggest that the [16α,17α-*d*]-3′-carbethoxyisoxazoline moeity, in combination with 9α-fluorination and side-chain esterification of **P**, improve antiinflammatory activity following topical application. This compound shows favorable separation of antiinflammatory activity from pituitary–adrenal axis suppression and thymic atrophogenic effects following multiple topical applications.

Chemistry

11β,21-Dihydroxy-3,20-dioxopregna-1,4,6-triene (**1a**) and 11β,21-dihydroxy-3,20-dioxo-9-fluoropregna-1,4,6-triene (**1b**) were prepared in five steps starting from prednisolone and 9-fluoroprednisolone, respectively, by a method established in this laboratory.[1] 1,3-Dipolar cycloaddition of carbethoxyformonitrile oxide (CEFNO, generated *in situ* by the treatment of ethyl chlorooximidoacetate with aqueous $NaHCO_3$ solution)[11] to an α,β-unsaturated enone **1a** or **1b** gave a single adduct **2a** or **2b**, respectively in 97% yield. The 16,17-double bond reacted with CEFNO, but the cross-conjugated dienone system in ring A was inert to the employed reaction condition. ^1H NMR spectra for **2a** showed that the vinyl hydrogen peaks due to ring A were still present but 16-vinyl hydrogen peak at δ 6.70 disappeared with the appearance of new 16-methine hydrogen peak at δ 4.26. Green *et al.* found similar results from the 1,3-dipolar cycloaddition of nitrones to analogous steroid system.[12] The regiospecificity of 1,3-dipolar cycloaddition of CEFNO to an α,β-unsaturated enone[13] and the stereospecificity of the cycloadditon to 16-ene steroid system with 17-acetyl side chain[14] are known. The treatment of **2a** or **2b** with acetic anhydride in pyridine gave corresponding acetates **3a** or **3b**, respectively, in 95% yield. It was difficult to determine the splitting constants for the 16-C*H* of **2a** due to the overlapping of other peaks such as one hydrogen (δ 4.30) of 21-CH_2 and 3′-$CO_2CH_2CH_3$ (δ 4.33), whereas the splitting constants for the 16-C*H* of the acetate **3a** could be easily obtained (dd, J = 9.6, 2.9) since the peaks of 21-CH_2 shifted downfield (δ 4.99, 4.71) and the peak of 16-C*H* shifted upfield (δ 4.15). Similar results were obtained for the compounds **2b** and **3b**. A putative metabolite, acid **4a**, was obtained from the basic hydrolysis of **2a**.

Pharmacology

Dose–response data of the inhibitory effects of prednisolone, **2a**, **3a**, **2b**, and **3b** in the croton oil-induced ear edema bioassay in the rat are depicted in Figure 1 and Table 1. Following a single topical application, treatment with all compounds resulted in dose-dependent inhibition of edema. From the dose–response profiles, the following ID_{50} values (nmol resulting in a

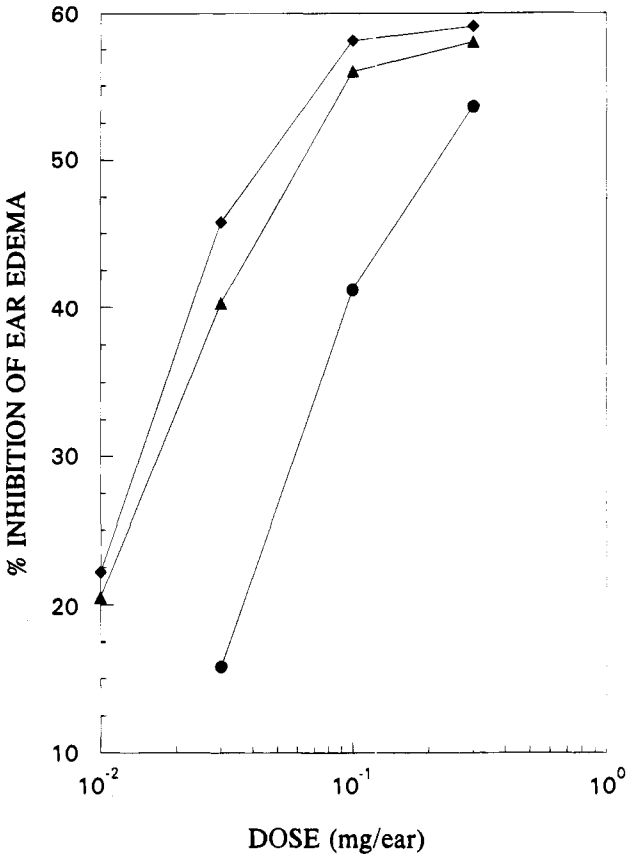


Figure 1. Inhibition of croton oil-induced ear edema in rats following a single topical application of prednisolone (●), **2b** (▲), or **3b** (◆). Each point represents the mean ± SEM of five animals. Control mean ear thickness changes ($\times 10^2$) were the following: **P**, 29.1 ± 1.7 mm; **2b**, 26.8 ± 0.1 mm; and **3b**, 36.5 ± 1.5 mm.

Table 1. Inhibitory Effects of **2a** and **3a** in the Croton Oil-Induced Ear Edema Bioassay[a]

	% inhibition of ear edema	
dose (mg/ear)	**2a**	**3a**
0.01	31.3	14.9
0.03	44.0	21.4
0.1	44.0	35.7

[a] Control mean ear thickness changes ($\times 10^2$) were as follows: **2a**, 15.0 ± 0.7 mm, and **3a**, 17.1 ± 0.8 mm; five animals per group.

50% reduction of edema) were calculated: **P**, 540 nmol; **2b**, 135 nmol; and **3b**, 101 nmol. Dose–response results following applications of **2a** or **3a** were remarkable as inhibition of edema did not exceed the 50% level. A putative antedrug metabolite, **4a**, was also examined in the croton oil-induced ear edema bioassay. At the highest dose tested, 0.5 mg/ear, only 19% inhibition was measured, and this was not statistically significant. These results indicated that when applied topically, addition of the [16α,17α-*d*]-3′-cabethoxyisoxazoline to prednisolone, as in **2a**, did not improve antiinflammatory activity. Likewise, side-chain esterification, as in **3a**, did not improve antiinflammatory activity. Marked improvements in antiinflammatory activity were noted with both 9-fluoro derivatives. When relative potencies were calculated from ID_{50} doses, with prednisolone defined as 1, it was found that **2b** was 4-fold and **3b** 5.3-fold more potent than the parent compound when applied topically.

Calculated, equiactive ID_{50} doses of prednisolone, **2b**, and **3b** were topically applied for 5 consecutive days. Local and systemic antiinflammatory effects, body weight increases, thymic atrophy, and plasma corticosterone levels were assessed. These are symmarized in Table 2. All compounds tested did inhibit ear edema by 50% in the treated right ears. Significant inhibition of edema in the contralateral untreated ears, taken to reflect a systemic effect of absorbed steroid, was noted following treatments with prednisolone and **2b**. In contrast, no significant systemic antiinflammatory effects were noted following consecutive treatments with **3b**.

Significant adverse systemic effects were measured following treatments with prednisolone and included less body weight gain, thymolytic effects, and decreases in plasma corticosterone levels. No adverse systemic effects were seen following treatments with **3b** while some plasma corticosterone level supression was evident following administration of **2b**. These results suggest that C-9-fluorination, side-chain hydroxy group esterification, and [16α,17α-*d*]-3′-carbethoxyisoxazoline additions to the conventional steroid prednisolone improve topical antiinflammatory activity without concomitant increases in adverse systemic activity.

Experimental Section

The syntheses of compounds **1a** and **1b** have been previously reported.[1] Prednisolone and 9-fluoroprednisolone were obtained from the Upjohn Co (Kalamazoo, MI). Ethyl chlorooximidoacetate was purchased from Aldrich Co. Melting points were determined on a Thomas capillary melting point apparatus and were uncorrected. The ^1H NMR spectra and IR spectra were obtained with a Bruker HX-270 spectrometer and Perkin-Elmer 1430 spectrometer, respectively. Coupling constants are given in hertz. Mass spectra were recorded on a Finnigan 4510 GCMS using positive chemical ionization. Silica gel (Merck, 70–230 mesh) was used for flash column chromatography. All pharmacological data are presented as mean values of five or six samples + SEM. The ANOVA analysis, followed by least squares differences between means subtest, was used to determine significant differences between groups at $p < 0.05$.

11β,20-Dihydroxy-3,20-dioxo-3′-(ethoxycarbonyl)isoxazolino[16,17-*d*]pregna-1,4-diene (2a). To a solution of compound **1a** (0.5 g, 1.47 mmol) and ethyl (chlorooximino)-acetate (0.66 g, 4.36 mmol) in 30 mL of a 1:1 mixture of THF and Et_2O was slowly added over 5 h a solution of sodium bicarbonate (0.42 g, 5.00 mmol) in 10 mL of water. The reaction mixture was diluted with 150 mL of ethyl acetate. The solution was washed with 5% HCl solution, washed with saturated NaCl solution, dried (Na_2SO_4), and evaporated *in vacuo* to give 1.04 g of white sticky solid. This solid was dissolved in a minimum amount of ethyl acetate. The ethyl acetate solution was added to 150 mL of hexane with stirring. The resulting white solid was filtered and purified by flash column chromatography on silica gel (1: 3 mixture of hexane–EtOAc) to give 0.65 g (97%) of pure **2a**: mp 184–186 °C; ^1H NMR ($CDCl_3$ DMSO-d_6) δ 7.22 (d, 1, J = 10.3, 1-C*H*), 6.27 (dd, 1, J = 10.3, 2.2, 2-C*H*), 6.02 (br s, 1, 4-C*H*), 4.65 (d, 1, J = 19.9, one of 21-CH_2), 4.53 (m, 1, 11-C*H*), 4.22–4.38 (m, 4, one of 21-CH_2, 16-C*H*, 3′-$CO_2CH_2CH_3$), 1.45 (s, 3, 18-CH_3), 1.35 (t, 3, J = 7.0, 3′-$CO_2CH_2CH_3$), 1.06 (s, 3, 19-CH_3); IR (cm^{-1}) 1775, 1665, 1625; MS $(M + H)^+$ 458.4.

9-Fluoro-11β,20-dihydroxy-3,20-dioxo-3′-(ethoxycarbonyl)isoxazolino[16,17-*d*]pregna-1,4-diene (2b) was prepared from compound **1b** in a manner similar to the preparation of compound **2a**: mp 260–270 °C dec; ^1H NMR ($CDCl_3$ + DMSO-d_6) δ 7.26 (d, 1, J = 10.3, 1-C*H*), 6.20 (dd, 1, J = 10.3, 2.2, 2-C*H*), 6.09 (br s, 1, 4-C*H*), 4.65 (d, 1, J = 19.9, one of 21-CH_2), 4.20–4.43 (m, 5, 11-C*H*, one of 21-CH_2, 16-C*H*, 3′-

Table 2. Effects of Prednisolone and Novel Derivatives in the Croton Oil Ear Edema Bioassay following Multiple Topical Applications

drug	dose (nmol/ear)	antiinflammatory activity: % inhibition[a] R[b]	L[b]	body weight change (g)	thymus weight change (g)	plasma corticosterone (ng/mL)
control				35.6 ± 2.4	609.4 ± 55.1	241.1 ± 57.3
P	540	66.7	49.6 ***	22.8 ± 2.1*	369.4 ± 19.5**	31.8 ± 5.2**
2b	135	56.9	24.4**	36.0 ± 2.1	561.6 ± 28.5	48.6 ± 5.5**
3b	101	51.2	8.9	38.0 ± 3.1	625.0 ± 26.4	134.2 ± 22.7

[a] Control mean change: right, 28.8 ± 1.2 mm (×10²); left, 27.0 ± 0.6 mm (×10²). [b] Right ears treated daily for 5 days with indicated doses of drug and croton oil; left ears treated daily with vehicle and croton oil. *p < 0.5, **p < 0.01, and ***p < 0.001, ANOVA followed by Bonferroni's posthoc test.

$CO_2CH_2CH_3$), 1.54 (s, 3, 18-CH_3), 1.35 (t, 3, J = 7.0, 3′-$CO_2CH_2CH_3$), 1.05 (s, 3, 19-CH_3); IR (cm^{-1}) 1775, 1665, 1625; MS $(M + H)^+$ 475.9.

20-Acetoxy-11β-hydroxy-3,20-dioxo-3′-(ethoxycarbonyl)isoxazolino[16,17-*d*]pregna-1,4-diene (3a). To a solution of **2a** (185 mg, 0.41 mmol) in 1 mL of pyridine was added 0.2 mL of acetic anhydride. After 1 h, the reaction mixture was diluted with ethyl acetate, washed with 5% HCl solution, washed with saturated NaCl solution, dried (Na_2SO_4), and evaporated *in vacuo* to give 220 mg of white solid, which was purified by flash column chromatography on silica gel (1:2 mixture of hexane–EtOAc) to give 192 mg (95%) of compound **3a**: mp 181–183 °C; ^1H NMR ($CDCl_3$) δ 7.22 (d, 1, J = 10.3, 1-C*H*), 6.26 (dd, 1, J = 10.3, 2.2, 2-C*H*). 6.00 (br s, 1, 4-C*H*), 4.99 (d, 1, J = 17.6, one of 21-CH_2), 4.71 (d, 1, J = 17.6, one of 21-CH_2), 4.51 (m, 1, 11-C*H*), 4.31 (q, 2, J = 7.0, 3′-$CO_2CH_2CH_3$), 4.15 (dd, 1, J = 9.6, 2.9, 16-C*H*), 2.15 (s, 3, 21-$OCOCH_3$), 1.43 (s, 3, 18-CH_3), 1.33 (t, 3, J = 7.0, 3′-$CO_2CH_2CH_3$), 1.07 (s, 3, 19-CH_3); IR (cm^{-1}) 1775, 1665, 1625; MS $(M + H)^+$ 518.2.

20-Acetoxy-9-fluoro-11β-hydroxy-3,20-dioxo-3′-(ethoxycarbonyl)isoxazolino[16,17-*d*]pregna-1,4-diene (3b) was prepared from compound **2b** in a manner similar to the preparation of compound **3a**: mp 228–230 °C; ^1H NMR ($CDCl_3$) 7.14 (d, 1, J = 10.3, 1-C*H*), 6.33 (dd, 1, J = 10.3, 2.2, 2-C*H*), 6.11 (br s, 1, 4-C*H*), 5.05 (d, 1, J = 17.6, one of 21-CH_2), 4.72 (d, 1, J = 17.6, one of 21-CH_2), 4.43 (br d, 1, J = 8.6, 11-C*H*), 4.33 (q, 2, J = 7.0, 3′-$CO_2CH_2CH_3$), 4.18 (br d, 1, J = 8.1 16-C*H*), 2.20 (s, 3, 21-$OCOCH_3$), 1.56 (s, 3, 18-CH_3), 1.36 (t, 3, J = 7.0, 3′-$CO_2CH_2CH_3$), 1.10 (s, 3, 19-CH_3); IR (cm^{-1}) 1775, 1665, 1625; MS $(M + H)^+$ 518.2.

11β,20-Dihydroxy-3,20-dioxo-3′-carboxyisoxazolino-[16,17-*d*]pregna-1,4-diene (4a). To solution of compound **2a** (59 mg, 0.13 mmol) in 5 mL of MeOH was added 20 mL of 0.2 N NaOH solution. After 0.5 h, the reaction mixture was acidified with 2 N HCl solution and extracted with ethyl acetate (3 × 25 mL). The ethyl acetate solution was washed with saturated NaCl solution, dried ($MgSO_4$), and evaporated *in vacuo* to give 50 mg (90%) of white solid: mp 200–210 °C dec; ^1H NMR ($CDCl_3$ + DMSO-d_6) 7.17 (d, 1, J = 10.3, 1-C*H*), 6.09 (dd, 1, J = 10.3, 2.2, 2-C*H*), 5.83 (br s, 1, 4-C*H*), 4.49 (d, 1, J = 19.9, one of 21-CH_2), 4.30 (m, 1, 11-C*H*), 4.18 (d, J = 19.9, one of 21-CH_2), 4.10 (dd, 1, J = 9.6, 2.9, 16-C*H*), 1.30 (s, 3, 18-CH_3), 0.90 (s, 3, 19-CH_3).

Biological Assays. Effects of topically applied steroids on edema formation were measured using the croton oil-induced ear edema bioassay of Tonneli *et al.* as described by Heiman *et al.*[2,4] Briefly, initial ear thicknesses of male Sprague–Dawley rats (Harlan Sprague–Dawley, Inc., Indianapolis, IN) were measured with a spring-loaded micrometer. Then, 25 μL of vehicle (acetone/DMSO) or drug solution was applied to surfaces of the ears. Thirty minutes later, 5% croton oil was applied in the same manner. Five hours later, at peak inflammation, ear thicknesses were measured. Percent inhibition of edema formation was determined by comparing the ear thickness of steroid-treated animals with that of control animals. The dose which inhibited ear edema by 50% (ID_{50}) was estimated from a plot of percent inhibition versus dose (μM). For multiple topical application studies, the drugs were applied as described above to the animals' right ears, once daily for 5 days, while left ears were treated with vehicle alone. Five hours following the final treatment, right and left ears were measured for local and systemic activities, respectively. Blood samples were obtained by cardiac puncture for plasma corticosterone measurements and relative thymus and body weights were assessed in order to monitor adverse systemic effects of the steroids. Plasma corticosterone levels were quantitated by RIA using the RSL Rat corticosterone kit (ICN Biomedicals Inc. Carson, CA).

Statistical Analysis. All data are presented as mean values of five samples ± SEM. Analysis of variance (ANOVA), followed by the Bonferroni posthoc test, was used to determine significant differences between groups at p < 0.05.

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