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Steroids

Effect of chirality at C-20 of methyl 11β,17α,20-trihydroxy-3-oxo-1,4-pregnadien-21-oate derivatives on antiinflammatory activity

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Abstract

In an effort to determine the C-20 chirality effect on the antiinflammatory activity of 17β-glycolate esters, methyl 11β,17α,20-trihydroxy-3-oxo-1,4-pregnadien-21-oate and its 9α-fluoro analog, their acetonide and their carbonate derivatives were synthesized and evaluated. The agents were tested for their binding potency to the macrophage glucocorticoid receptor, and their effect on LPS-induced nitric oxide generation in RAW 264.7 cells. The acetonide derivatives showed the highest binding affinity while the triols and carbonates bound rather poorly to the receptors. With the exception of the triols, the α-isomer in each pair of the agents exhibited higher binding affinity to the receptor than its corresponding β-isomer, clearly indicating that C-20 chirality has a significant effect on antiinflammatory activity. In addition, the α-isomers of the acetonides showed substantially higher binding affinity than the parent compound, prednisolone. In contrast to the high binding activity exhibited by some of the acetonides, all of the agents showed weak inhibitory effect on NO generation. Metabolic inactivation during assessment of NO inhibition may play a role in the divergence noted between receptor affinity and the measured biologic activity resulting from the binding.

Keywords: Anti-inflammation; Steroid methyl ester; Acetonide; Chirality; Antedrug

1. Introduction

Our continuing search for new potent antiinflammatory steroids devoid of systemic side effects has been based on the antedrug concept. An antedrug is a compound that exerts desirable local effects and rapidly biotransforms to an inactive metabolite by a predictable enzymatic reaction upon entry into the circulation. The chief strategy involved has been incorporation of metabolically labile alkyl carboxylates, at various positions of corticosteroids [1–3]. It had been observed in the mid-1980s that chirality at C-20 of methyl 11β,17α,20-trihydroxy-3-oxo-1,4-pregnadien-21-oate, synthesized from prednisolone, has an influence on the antiinflammatory activity [4]. The two triols showed moderate antiinflammatory activities, with the 20α-isomer [or 20(R)-epimer], weaker than the 20β-isomer [or 20(S)-epimer]. However, the acetonides synthesized from these triols were more active, and acetonide of the α-isomer was substantially stronger than that of the β-isomer. The experiment suggested that the α-acetonide might have a much more complementary structure with the receptor, and thus a much higher binding affinity than its β-epimer. A binding study of these epimers, as well as similar acetonides derived from other steroids, thus became highly desirable. It was also very interesting to see if carbonates made from the triols, which would render similar structural rigidity to the acetonides, would show any α/β differentiation in activity. A plan was therefore made to compare the antiinflammatory activity of the α/β isomers of the acetonides, the carbonates and the triols derived from both prednisolone and 9α-fluoroprednisolone.

2. Experimental

2.1. Synthesis

Prednisolone and isoflupredone acetate were purchased from The Upjohn Company, Kalamazoo, MI. All other reagents were ordered from Aldrich chemical company, Milwaukee, WI. Solvents used, for reaction, extraction and chromatography, were obtained from Fisher Scientific, Fair

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Lawn, NJ. NMR spectra were recorded on a Brucker HX-270 spectrometer and the chemical shifts in parts per million (ppm) down field from tetramethylsilane as an internal standard. Mass spectra were obtained on a Finnigan 4510 GCMS spectrometer, using positive chemical ionization. Elemental analysis was carried out by Galbraith Laboratories, Knoxville, TN. Melting points were determined on a Thomas Hoover Capillary Melting Point Apparatus and uncorrected.

*2.1.1. Methyl 11β,17α,20-trihydroxy-3-oxo-1,4-pregnadien-21-oate (**4α,β**)*

A mixture of prednisolone (12.5 g, 34.5 mmol) and cupric acetate monohydrate (8.2 g, 40 mmol) in anhydrous methanol (850 ml) was stirred under a drierite tube for 2 days. An aqueous EDTA disodium (6 g in 200 ml) was added, and the resultant mixture condensed via rotary evaporation to a volume of about 100 ml. More water (200 ml) was then added and the new aqueous suspension was filtered. The solid was washed with water (50 ml), and then mixed with 2% aqueous NaOH (150 ml), and stirred for 1 h. The reaction mixture was acidified with 10% aqueous HCl to pH 2, and then filtered to give a white solid, a mixture of 20(R) (**3α**), and 20(S)-epimer (**3β**). The filtrate was extracted with ethyl acetate (3 × 150 ml) and the combined organic solution condensed to yield more white solid. The combined solid was dissolved in ethyl acetate (300 ml), and treated with excess diazomethane in diethyl ether. The methyl ester reaction was complete within a few minutes of stirring and its crude products, the 20(R) (**4α**) and 20(S)-epimer (**4β**), were obtained by condensation of the solution to yield a slightly yellow solid. The solid was subjected to repeated recrystallizations from 3:1 EtOAc/methanol to afford **4α** as a white solid (6.9 g, 51%). The combined mother liquors were condensed and then chromatographed through silica gel with 3:2 chloroform/acetone solvent system to yield **4β** as a while solid (1.4 g, 10%). A mixture of roughly 1:1 ratio of the epimeric isomers (1.5 g, 11%) remained after the separation procedure. **4α**: mp 265–267°C (lit [5]. 254–255°C). Proton NMR ($CDCl_3$): 7.197 (1H, d, J = 10.1 Hz, H-1), 6.231 (1H, dd, J = 10.1, 1.9 Hz, H-2), 5.991 (1H, dd, J = 1.9, 1.3 Hz, H-4), 4.406 (1H, m, H-11), 4.1675 (1H, d, J = 9.6 Hz, H-20), 3.787 (3H, s, OMe), 3.086 (1H, d, J = 9.6 Hz, OH-20), 1.426 (3H, s, H-19), 1.111 (3H, s, H-18). **4β**: mp 174–176°C (lit [5]. 171–173°C). Proton NMR ($CDCl_3$): 7.244 (1H, d, J = 10.1 Hz, H-1), 6.240 (1H, dd, J = 10.1, 1.9 Hz, H-2), 5.987 (1H, br s, H-4), 4.421 (1H, m, H-11), 4.339 (1H, d, J = 5.8 Hz, H-20), 3.778 (3H, s, OMe), 3.162 (1H, d, J = 5.8 Hz, OH-20), 1.435 (3H, s, H-19), 1.141 (3H, s, H-18).

*2.1.2. Methyl 11β,17α,20-trihydroxy-3-oxo-1,4-pregnadien-21-oate 17,20-acetonide (**5α,β**)*

The triol (**4α** or **4β**, 0.6 g, 1.5 mmol) dissolved in anhydrous DMF (3 ml), and *p*-toluenesulfonic acid monohydrate (100 mg, 0.5 mmol) and 2,2-dimethoxypropane (5 g, 47 mmol) were added. The resultant mixture was heated to 43°C for 2 days under a reflux condenser and then diluted with 1:1 EtOAc/Hexanes (250 ml). The solution was washed with saturated aqueous $NaHCO_3$ (20 ml) and water (20 ml). The organic solution was then dried over sodium sulfate and condensed. The residue was purified by flash chromatography (3:2 EtOAc/Hexanes) to yield the 20(R)-epimeric acetonide **5α** (355 mg, 54%) as a slightly yellow solid after vacuum drying. Part of the solid was recrystallized from MeOH/water (3/1) to afford needles for melting point measurement. **5α**: mp 197–199°C (lit [6]. 199–200°C). Proton NMR ($CDCl_3$): 7.266 (1H, d, J = 10.2 Hz, H-1), 6.261 (1H, dd, J = 10.2, 1.8 Hz, H-2), 6.006 (1H, br s, H-4), 4.567 (1H, s, H-20), 4.445 (1H, m, H-11), 3.774 (3H, s, MeO), 1.495 (3H, s, Me), 1.440 (3H, s, Me), 1.368 (3H, s, Me), 0.945 (3H, s, H-18). 20(S)-Epimeric acetonide **5β**: 48%, mp 187–189°C (lit [6]. 188–190°C). Proton NMR (DMSO-d6): 7.362 (1H, d, J = 10 Hz, H-1), 6.152 (1H, br d, J = 10 Hz, H-2), 5.907 (1H, br s, H-4), 4.512 (1H, s, H-20), 4.408 (1H, m, H-11), 3.746 (3H, s, MeO), 1.463 (6H, s, 2× Me), 1.298 (3H, s, Me), 1.041 (3H, s, H-18).

*2.1.3. Methyl 11β,17α,20-trihydroxy-3-oxo-1,4-pregnadien-21-oate 17,20-carbonate (**6α,β**)*

The triol (**4α** or **4β**, 140 mg, 0.35 mmol) was mixed with carbonyldiimidazole (300 mg, 1.85 mmol) in ethyl acetate (30 ml). The mixture was heated to reflux for 2 h and then diluted with more EtOAc (100 ml). The solution was then washed with 3% aqueous HCl (2 × 25 ml) and water (25 ml). The solution was dried over sodium sulfate and then condensed to remove the solvent. The residue was triturated with EtOAc to give the 20(R)-epimeric carbonate **6α** (142 mg, 94%) as a white solid: mp 266–268°C. Proton NMR ($CDCl_3$): 7.189 (1H, d, J = 10.1 Hz, H-1), 6.189 (1H, dd, J = 10.1, 1.8 Hz, H-2), 5.952 (1H, dd, J = 1.8, 1.3 Hz, H-4), 4.801 (1H, s, H-20), 4.357 (1H, m, H-11), 3.805 (3H, s, MeO), 1.390 (3H, s, H-19), 1.116 (3H, s, H-18). ^{13}C NMR (75.5 MHz, $CDCl_3$): 186.38, 169.55, 166.96, 155.91, 152.97, 127.79, 122.49, 96.09, 78.83, 68.86, 54.70, 52.80, 51.90, 44.59, 43.79, 38.36, 36.99, 33.39, 31.71, 30.67, 22.64, 21.02, 15.47. Anal. Calc. for $C_{23}H_{28}O_7$: C 66.33%, H 6.78%. Found: C 66.21%, H 6.92%. 20(S)-Epimeric carbonate **6β** (92%): mp 257.5–260°C. Proton NMR ($CDCl_3$): 7.203 (1H, d, J = 10.1 Hz, H-1), 6.240 (1H, dd, J = 10.1, 1.9 Hz, H-2), 5.998 (1H, br s, H-4), 4.925 (1H, s, H-20), 4.502 (1H, m, H-11), 3.822 (3H, s, MeO), 1.434 (3H, s, H-19), 1.130 (3H, s, H-18). C-13 NMR ($CDCl_3$, 68 MHz): 186.41, 169.49, 167.49, 155.80, 153.03, 127.93, 122.58, 95.28, 75.35, 69.20, 54.79, 52.97, 50.22, 46.87, 43.85, 38.38, 33.44, 31.77, 31.37, 30.36, 23.27, 21.04, 17.04. Anal. Calc. for $C_{23}H_{28}O_7$: C 66.33%, H 6.78%. Found: C 66.19%, H 6.98%.

The fluorinated derivatives of triols, acetonides and carbonates were synthesized by the same procedures as their non-fluorinated counterparts and their data are as follows.

Methyl 11β,17α,20α-trihydroxy-9α-fluoro-3-oxo-1,4-

pregnadien-21-oate (**F-4a**): white solid, mp 280–282°C (lit [7]. 279–281°C). Proton NMR (DMSO-d6): 7.266 (1H, d, J = 10.2 Hz, H-1), 6.194 (1H, dd, J = 10.2, 1.8 Hz, H-2), 5.987 (1H, br s, H-4), 5.214 (1H, m, H-11), 5.139 (1H, d, J = 7.8 Hz, H-20), 4.147 (1H, s, OH-17), 4.052 (1H, d, J = 7.8 Hz, OH-20), 3.610 (3H, s, MeO), 1.495 (3H, s, H-19), 1.036 (3H, s, H-18). C-13 NMR (DMSO-d6, 75.47 MHz): 185.28, 172.80, 167.22, 152.90, 128.87, 124.03, 101.26 (d, J = 174 Hz, C-9), 84.42, 73.64, 70.60 (d, J = 37 Hz, C-11), 51.02, 47.92 (d, J = 24 Hz), 45.08, 44.01, 34.84, 34.60, 33.41 (d, J = 18 Hz), 30.30, 27.35, 23.04, 22.98, 22.41, 16.21. Methyl 11β,17α,20β-trihydroxy-9α-fluoro-3-oxo-1,4-pregnadien-21-oate (**F-4b**): white solid, mp 257–260°C (lit [8]. 259–261°C). Proton NMR: ($CDCl_3$): 7.196 (1H, d, J = 10.2 Hz, H-1), 6.306 (1H, dd, J = 10.2, 1.8 Hz, H-2), 6.092 (1H, br s, H-4), 4.30 (2H, m, H-11 & H-20), 3.774 (3H, s, MeO), 3.308 (1H, br s, OH), 1.526 (3H, s, H-19), 1.137 (3H, s, H-18).

Methyl 11β,17α,20α-trihydroxy-9α-fluoro-3-oxo-1,4-pregnadien-21-oate 17,20-acetonide (**F-5α**): mp 278–281°C. Proton NMR ($CDCl_3$): 7.203 (1H, d, J = 10.2 Hz, H-1), 6.326 (1H, dd, J = 10.2, 1.8 Hz, H-2), 6.110 (1H, br s, H-4), 4.573 (1H, s, H-20), 4.358 (1H, m, H-11), 3.780 (3H, s, MeO), 1.532 (3H, s, Me), 1.498 (3H, s, Me), 1.371 (3H, s, H-19), 0.938 (3H, s, H-18). C-13 NMR ($CDCl_3$, 75.47 MHz): 186.54, 170.18, 166.14, 152.28, 129.68, 125.04, 108.91, 100.12 (d, J = 177 Hz, C-9), 93.84, 78.18, 71.84 (d, J = 38 Hz, C-11), 52.05, 48.18 (d, J = 23 Hz), 45.42, 44.85, 36.59, 36.46, 34.03 (d, J = 19 Hz), 31.05, 27.09, 26.54, 25.85, 23.01, 22.94, 22.73, 16.34. Anal. Calc. for $C_{25}H_{33}FO_6$: C 66.95%, H 7.42%, F 4.23%. Found: C 66.81%, H 7.61%, F 3.92%.

Methyl 11β,17α,20β-trihydroxy-9α-fluoro-3-oxo-1,4-pregnadien-21-oate 17,20-acetonide (**F-5β**): mp 222–224.5°C. Proton NMR ($CDCl_3$): 7.243 (1H, d, J = 10.2 Hz, H-1), 6.340 (1H, dd, J = 10.2, 1.6 Hz, H-2), 6.127 (1H, br s, H-4), 4.563 (1H, s, H-20), 4.430 (1H, m, H-11), 3.786 (3H, s, MeO), 1.570 (3H, s, Me), 1.520 (3H, s, Me), 1.348 (3H, s, H-19), 1.078 (3H, s, H-18). C-13 NMR ($CDCl_3$, 75.47 MHz): 186.66, 171.63, 166.43, 152.30, 129.73, 125.03, 111.40, 100.50 (d, J = 180 Hz, C-9), 94.56, 77.62, 72.15 (d, J = 38 Hz, C-11), 52.14, 48.31 (d, J = 23 Hz), 45.76, 44.34, 36.19, 34.46 (d, J = 20 Hz), 33.47, 31.05, 27.75, 27.38, 27.15, 23.50, 22.99, 22.90, 17.33. Anal. Calc. for $C_{25}H_{33}FO_6$: C 66.95%, H 7.42%, F 4.23%. Found: C 66.86%, H 7.56%, F 3.88%.

Methyl 11β,17α,20α-trihydroxy-9α-fluoro-3-oxo-1,4-pregnadien-21-oate 17,20-carbonate (**F-6α**): mp 277–279°C. Proton NMR (DMSO-d6): 7.240 (1H, d, J = 10.2 Hz, H-1), 6.214 (1H, dd, J = 10.2, 1.6 Hz, H-2), 6.014 (1H, br s, H-4), 5.505 (1H, m, H-11), 5.419 (1H, s, H-20), 3.813 (3H, s, MeO), 1.479 (3H, s, H-19), 1.076 (3H, s, H-18). C-13 NMR (75.47 MHz, DMSO-d6): 185.16, 167.34, 166.57, 152.90, 152.41, 129.05, 124.22, 100.62 (d, J = 178 Hz, C-9), 96.32, 77.75, 69.67 (d, J = 37 Hz, C-11), 52.87, 47.64 (d, J = 22 Hz), 45.42, 43.51, 35.92, 34.34, 32.88 (d, J = 19 Hz), 30.05, 26.96, 23.01, 22.93, 21.77, 14.71. Anal. Calc. for $C_{23}H_{27}FO_7$: C 63.58%, H 6.26%, F 4.37%. Found: C 63.42%, H 6.54%, F 4.31%.

Methyl 11β,17α,20β-trihydroxy-9α-fluoro-3-oxo-1,4-pregnadien-21-oate 17,20-carbonate (**F-6β**): mp 252.5–254.5°C. Proton NMR ($CDCl_3$): 7.187 (1H, d, J = 10.2 Hz, H-1), 6.301 (1H, dd, J = 10.2, 1.8 Hz, H-2), 6.098 (1H, br s, H-4), 4.944 (1H, s, H-20), 4.390 (1H, m, H-11), 3.824 (3H, s, MeO), 1.536 (3H, s, H-19), 1.125 (3H, s, H-18). C-13 NMR ($CDCl_3$, 75.47 MHz): 186.56, 167.41, 165.97, 153.03, 152.04, 129.74, 125.12, 99.84 (d, J = 177 Hz, C-9), 95.17, 75.28, 71.14 (d, J = 38 Hz, C-11), 52.97, 48.09 (d, J = 22 Hz), 46.41, 43.81, 34.80, 34.37 (d, J = 20 Hz), 30.82, 30.44, 26.98, 23.01, 22.96, 16.59. Anal. Calc. for $C_{23}H_{27}FO_7$: C 63.58%, H 6.26%, F 4.37%. Found: C 63.33%, H 6.44%, F 4.72%.

2.2. Bioassays

2.2.1. Cell culture

RAW 264.7 murine macrophages were purchased from American Type Tissue Collection (Rockville, MD) (ATCC TIB-71) and grown in monolayer culture in 100 mm Corning tissue culture dishes containing DMEM supplemented with 10% newborn calf serum (NCS) and pen/strep. At confluence, cells were passaged by scraping, washing in sterile Hank's Balanced Salts solution and seeding at 0.25×10^6 viable cells/ml. Viability was accessed by trypan blue exclusion. Cells were incubated at 35°C in an environment of 5% CO_2 and 95% relative humidity.

2.2.2. Whole cell receptor binding assays

Whole cell glucocorticoid receptor binding assays were carried out using RAW 264.7 cells seeded at 0.2×10^6 cells/well in 96-well plates and allowed to attach and grow for 24 h in the medium described above. Cell monolayers were washed with cold HBSS then incubated for 2 h in Ham's F-10 with 0.1% lactalbumin hydrolysate (200 μl/well). Medium was aspirated and fresh culture medium containing 5 nM dexamethasone and various concentrations (1–1000 nM) of unlabeled competing steroids were added (250 μl/well). Nonspecific binding was determined in the presence of 1000-fold excess of [^{3}H]dexamethasone. Cells were then incubated at 35°C, 5% CO_2 for 3 h, then washed five times with 250 μl/well cold HBSS with a 2 min soak between washes using a microplate washer. Following aspiration of the final wash, cells were solubilized by adding 80 μl 0.1 N NaOH/well. Contents of 6 wells were pooled for each data point and a 0.2 ml aliquot counted by scintillation spectroscopy. Total cellular protein was quantitated by the Lowry method using bovine serum albumin (BSA) as standard.

2.2.3. Generation and measurement of NO

RAW 264.7 macrophages were seeded into 24 well cluster plates at densities of 0.8×10^6 cells/well and allowed to

attach and grow overnight. Culture medium was aspirated and replaced with DMEM without phenol red and supplemented with 2.5% NCS to which indicated concentrations of steroids were added in triplicate. Following a 3 h incubation period, cells were stimulated to generate NO by addition of 30 ng/ml LPS (*Escherichia coli* serotype 0111: B4). Following incubation for the indicated times, the amount of stable nitrite, the end product of NO generation by activated cells, was determined by a modification of the Griess reaction. Briefly, 50 μl of culture supernatants from control or stimulated macrophages were transferred to 96-well microtiter plates. Supernatants were mixed with 50 μl of 1% sulfanilamide in 5% phosphoric acid, incubated for 10 min at room temperature, protected from light, followed by 50 μl of 0.1% aqueous N-1-naphthethylenediamine dihydrochloride for another light-protected 10 min incubation. Absorbances at 540 nm were determined on a Bio-Tek microtiter plate reader. Nitrite accumulation (nmol/50 μl) was determined by extrapolation from a sodium nitrite standard curve. Data from concentration-response time course experiments were expressed as nmol nitrite/10^6 cells, and data from steroid treatment experiments were expressed as percent untreated controls [9].

3. Results and discussions

3.1. Synthesis

The literature procedure to prepare methyl 11β,17α,20-trihydroxy-3-oxo-1,4-pregnadien-21-oate was time consuming and gave very low yield [5,10]. Thus a modified procedure based on the research of Lewbard and Maddox [11,12] was developed: cupric oxidation of prednisolone in methanol to the 21-hemiacetal (**2** & **F-2**), followed by NaOH initiated Cannizzaro rearrangement to 20-hydroxy-21-oic acid (**3*α,β*** & **F-3*α,β***), and methyl ester formation (**4*α,β*** & **F-4*α,β***) by treatment with diazomethane. Separation of the ester epimers was achieved by fractional recrystallization, rather than the HPLC method as described in the literature [5,10]. The 20α-isomers (**4*α,*** **F-4*α***) were obtained pure after the recrystallizations, while the 20β-isomers (**4*β,*** **F-4*β***) needed further purification through silica gel column chromatography. A combined yield of the two methyl ester epimers of about 70% was obtained from the starting material, with an α/β ratio of approximately 3.5/1.

Preparation of the acetonides and carbonates went smoothly. Thus treatment of the triols with 2,2-dimethoxypropane under the catalysis of *p*-toluenesulphonic acid in DMF gave the desired acetonide derivatives (**5*α,β*** & **F-5*α,β***) in moderate yields. And reaction of the triols with carbonyldiimidazole in ethyl acetate afforded the carbonates in high yields (**6*α,β*** & **F-6*α,β***).

The stereochemistry at C-20 was confirmed by melting point comparison with literature data, and NOE experiment with irradiation at the proton on C-20 of the carbonate

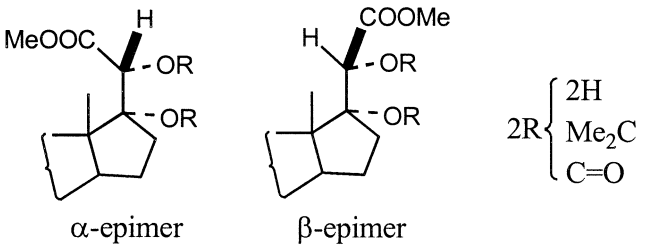


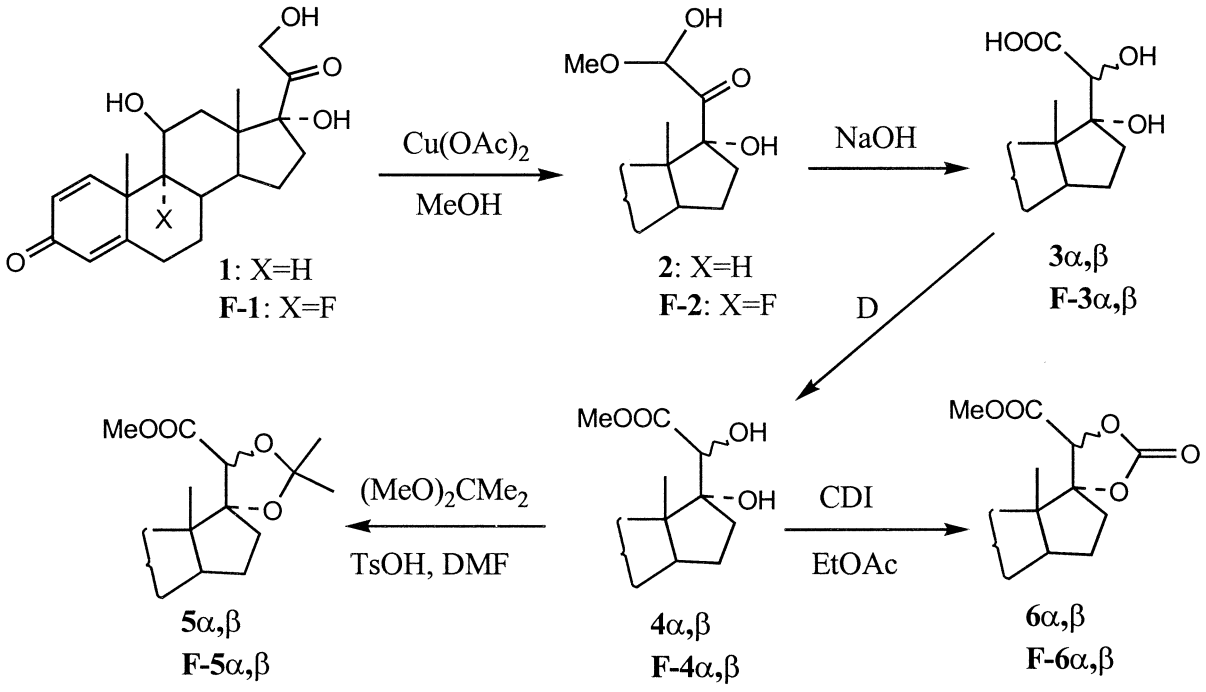
Fig. 1. 20α and 20β epimer

epimers in each pair. While H-18 of the α-isomer showed a small NOE response ($\sim$ 1%), that of the β-isomer gave a significantly stronger signal enhancement of about 4%.

3.2. Screening bioassays

Both receptor binding in a whole cell glucocorticoid receptor binding assay and inhibition of nitric oxide (NO) production in lipopolysaccharide-stimulated RAW 264.7 murine macrophages were used to assess anti-inflammatory activity of the agents. The rationale for these tests is based on reports that isoforms of nitric oxide synthase (NOS) are found in endothelial cells, macrophages and other cells involved in inflammatory responses. NO generated by the inducible NOS isoform is considered an important modulator of inflammatory responses and macrophages may be one of the effector cells in this process. Following induction of inducible NOS by treatment with bacterial products and/or proinflammatory cytokines, macrophages generate high sustained levels of NO which can be inhibited by pretreatment of cells with glucocorticoids [13–15]. Recently, our laboratories reported that new 9α-fluoroprednisolone anti-inflammatory antedrugs showed high affinity for macrophage glucocorticoid receptors and potent inhibition of NO generation [9]. In contrast, results of another study demonstrated that anti-inflammatory steroids with a spiro ring structure exhibited low affinity for macrophage glucocorticoid receptors and no significant inhibition of NO generation [16].

Results of macrophage glucocorticoid receptor binding of the present series of agents are depicted in Table 1 which also includes the previously reported ear edema inhibition activity [4]. All members of the two pairs of triols, **4*α*/4*β*** and **F-4*α*/F-4*β***, showed much weaker receptor affinity than prednisolone. These results are consistent with their inhibitory activity in the rodent croton oil ear edema bioassay. Similarly, the two carbonate pairs, **6*α*/6*β*** and **F-6*α*/F-6*β*** showed weak binding affinity with the α-isomer of each pair exhibiting slightly higher affinity than the β-derivative. Of the compounds tested, the acetonides, **5*α*/5*β*** and **F-5*α*/F-5*β*** competitively displaced [^{3}H]dexamethasone from the macrophage glucocorticoid receptors with each α-isomer displaying much greater affinity than its β-counterpart. These results are consistent with the in vivo inhibition of croton oil induced ear edema. It is noteworthy that the



9-fluorine substitution did not significantly improve glucocorticoid receptor binding as exhibited by IC_{50} values of 35 nM and 33 nM for **5*α*** and **F-5*α***, respectively, and that affinities of the acetonide derivatives equal that of 34 nM obtained with 9α-fluoroprednisolone.

Biologic activity resulting from glucocorticoid receptor occupancy was assessed as inhibition of NO generation in LPS-stimulated macrophages. Results are shown in Table 2. With the exception of **F-5*α***, all agents were very weak inhibitors of NO which were assessed as the accumulation of nitrites in cell culture supernatants. IC_{50} calculated from concentration-response curves were 342 nM, 186 nM and 75 nM for **F-5*α***, **Pred** and **F-Pred**, respectively. There are, however, several similarities in the data of the two tables. The acetonides are more active than the other derivatives, and results suggest that the α-isomer in each of the acetonide pairs is more potent than its β-counterpart.

Table 1
Competitive displacement of [^{3}H]dexamethasone from Raw 264.7 cell glucocorticoid receptors[a]

Steroid	[^{3}H]Dex bound 300 nM steroid	[^{3}H]Dex bound 1000 nM steroid	IC_{50} (nM)[b]	Ear edema ID_{50}[d]
4*α*	75.7	51.2	>1000	32.8
4*β*	66.0	39.1	622	5.9
F-4*α*	80.5	47.1	901	
F-4*β*	83.1	84.4	>1000	
5*α*	16.7	11.1	35.1	1.4
5*β*	33.4	11.7	211	3.0
F-5*α*	25.9	9.7	32.6	
F-5*β*	47.4	30.2	81.5	
6*α*	78.2	56.9	>1000	
6*β*	80.9	62.7	>1000	
F-6*α*	69.0	46.1	972	
F-6*β*	93.4	82.1	>1000	
Pred	18.4	—	85.5[c]	0.4
FPred	13.9	—	34.1[c]	

[a] Percent of [^{3}H]dexamethasone bound after competitive displacement. Specific [^{3}H]dexamethasone binding was 8853 ± 286 dpms/mg protein.
[b] IC_{50} is the concentration of competing steroid which displaces 50% of specifically bound [^{3}H]dexamethasone.
[c] IC_{50} for **Pred** and **FPred** were calculated from the concentration-response ranges of 3–300 nM.
[d] The dose (μmol/ear) which inhibited ear edema by 50% was estimated from a plot of percent inhibition versus dose.

Novel anti-inflammatory steroids including **4*β***, **5*α*/5*β***, **F-5*α*/F-5*β*** displaced [^{3}H]dexamethasone from the glucocorticoid receptors in the competitor range of 10–1000 nM, thus IC_{50} values were calculated. With the exception of **F-5*α***, these agents did not inhibit NO generation in LPS-stimulated macrophages in comparable concentration ranges. These differences may be explained, at least in part, by differences in time required to complete binding versus NO generation assays and the intracellular events required for the assay endpoints. Whole cell receptor competition assays are completed in 3 h. The endpoint of the competitive binding assay is binding of the agent to the cognate cytoplasmic glucocorticoid receptor, the first of a long series of events which culminate in the biologic activity resulting from receptor occupancy. NO generation was assessed by utilizing the Griess reaction which detects the amounts of stable nitrite, an endproduct of NO generation by activated cells. Results of time course studies with RAW 264.7 macrophages have shown that colorimetric detection of nitrite requires approximately 12 h with levels continuing to

Table 2
Effects of steroids on LPS-induced nitric oxide generation in Raw 264.7 cells[a]

Steroid	% NO Inhibition–100 nM	% NO Inhibition–300 nM	% NO Inhibition–1000 nM	IC_{50} (nM)[c]
4α	NI[b]	NI	NI	>1000
4β	NI	NI	NI	>1000
F-4α	NI	NI	NI	>1000
F-4β	NI	NI	NI	>1000
5α	35.8	39.8	44.9	>1000
5β	8.9	14.8	17.8	>1000
F-5α	48.0	50.5	53.1	342[d]
F-5β	19.1	11.4	12.7	>1000
6α	NI	NI	6.7	>1000
6β	NI	7.3	8.6	>1000
F-6α	NI	1.6	4.6	>1000
F-6β	NI	NI	NI	>1000
Pred	41.5	54.5	69.2	186[d]
FPred	57.8	69.2	80.6	75.4[d]

[a] Attached cells (1×10^6 cells/well) in 24-well plates were treated with LPS (10 ng/ml) in DMEM/5% fetal bovine serum 3 h after treatment with 3–1000 nM steroids. Supernatant nitrite, the stable end product of NO generation, was assayed by the Griess reaction at 18 h. Experiments were conducted in triplicate. Untreated controls generated 12.9 ± 0.89 nmol NO/1×10^6 cells.

[b] NI = no inhibition.

[c] IC_{50} is the concentration of steroid which inhibits generation of NO by 50%.

[d] IC_{50}s for **F-5α** and **Pred** were calculated from the concentration-response range of 10–1000 nM, and the IC_{50} for **FPred** was calculated from the range of 3 to 300 nM.

increase up to 72 h following addition of the stimulant [9]. In contrast to **Pred** and **F-Pred**, the steroidal antedrugs being screened in these investigations may not be stable enough to endure the 18 h treatment. Substantial hydrolysis of the methyl ester group may have occurred within this time, thus resulting in a loss of NO inhibition. This is supported by results of recent investigations on inhibition of macrophage NO production by arachidonate-cascade inhibitors. Inhibitory activity of the classic anti-inflammatory steroid dexamethasone was much higher in continuous exposure experiments than in glucocorticoid pulsing experiments [17]. To exert steroid-regulated biologic effects, the current working model involves binding of steroid to the cognate receptor, activation and/or transformation of the receptor-steroid complex, nuclear translocation and dimerization, complex binding to the hormone response element, recruitment of coactivators vs corepressors and interaction with auxiliary transcription factors followed by a series of events which initiate or inhibit gene transcription [18,19]. These are the intracellular events which precede measurement of the ability of glucocorticoids to inhibit NO generation in stimulated cells. Diverse mechanisms of steroid-receptor complex actions have been partially clarified as a consequence of discovery of steroid receptor coactivators and corepressors which function as transcriptional power boosters or inhibitors, respectively [20]. Effective interactions of coactivators and corepressors have been studied using classic glucocorticoids and are thought to occur exclusively with steroid-bound glucocorticoid receptors where their associations result in further conformational differences. The current emerging picture suggests coactivators/corepressors are flexible, but precise, coordinators of complex and dynamic networks in which transcriptional regulation by glucocorticoid receptor complexes and other nuclear receptors is linked to the final signaling pathway [21]. Subtly different conformational changes induced by binding of the novel antedrugs to the glucocorticoid receptors could influence the associations of either coactivators or corepressors and markedly alter receptor-mediated transcriptional activity.

Taken together, these results suggest that the α-isomers of the acetonides (**5α**, **F-5α**) which bind to glucocorticoid receptors with high affinity and possess a metabolically labile methyl ester group appear to provide a solid lead to ideal anti-inflammatory steroid antedrugs. Structural modifications which retain their binding affinity while modestly enhancing the ester stability against metabolic hydrolysis could yield very potent anti-inflammatory agents with greatly reduced systemic side effects.

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