New Steroidal Diazo Ketones as Potential Photoaffinity Labeling Reagents for the Mineralocorticoid Receptor: Synthesis and Biological Activities

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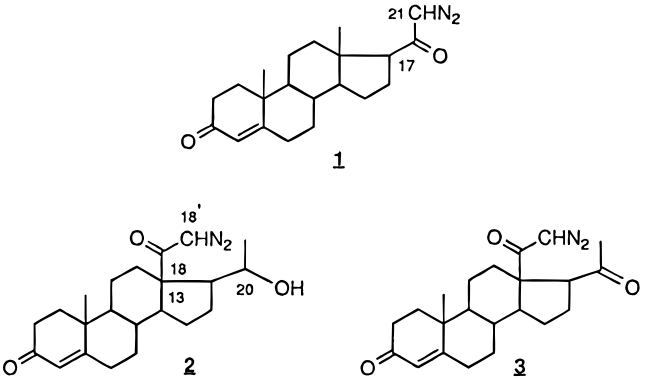
Three diazo ketones in the progesterone series were synthesized as potential photoaffinity reagents. The diazo ketone group was introduced at the C17 (21-diazopregn-4-ene-3,20-dione, **1**) or C13 (18-(diazomethyl)-20-hydroxypregn-4-ene-3,18-dione, **2**, 18-(diazomethyl)pregn-4-ene-3,18,20-trione, **3**) position of the pregnene skeleton. Whereas compound **1** could be easily obtained from the corresponding acid chloride, preparation of **2** and **3** required a less straightforward route involving reaction of tosyl azide on the formyl derivative of methyl ketone **5**. The affinity of the diazo ketones for the human mineralocorticoid receptor (hMR), expressed in Sf9 insect cells using the Baculovirus system, was estimated by competition experiments using [^{3}H]aldosterone as specific ligand. The affinity of **1** for hMR was almost identical with that of aldosterone. The affinities of **2** and **3** were 1 order of magnitude lower than that of aldosterone. The mineralocorticoid activity of the diazo ketones was measured in cis−trans cotransfection assays in CV-1 cells with the mouse mammary tumor virus as DNA target sequence. Compound **1** exhibits an agonist activity ($ED_{50} = 6 \times 10^{-9}$ M) with no antagonist activity. In contrast **2** and **3** behave as antagonists, displaying an IC_{50} of $\sim 10^{-6}$ M whether the substituent at the C20 position is a hydroxy (**2**) or an oxo (**3**) group.

Introduction

The first step in the action of aldosterone is its binding to an intracellular receptor, the mineralocorticoid receptor (MR), which is a member of the superfamily of ligand-induced transcription factors.[1,2] Despite recent advances in the knowledge of the MR heterooligomeric structure and the steps involved in the receptor activation,[3−11] very little is known about the precise mechanism by which the hormone interacts with the receptor. High-resolution structural data from crystallographic or NMR studies are lacking. Models have already been proposed for other steroid receptors.[12,13] The recent publication of the X-ray structure of the ligand-binding domain of several nuclear receptors of the same family (RXRα,[14] RARγ,[15] and TR[16]) should greatly help to refine these models. Identification of the amino acids residues involved in the ligand interaction and especially in agonist and antagonist discrimination will be of particular interest. This can be achieved by affinity or photoaffinity labeling (PAL), an approach that requires efficient and selective photoaffinity reagents with high affinity for the receptor. Previous works brought some structural information on the tolerance of steroid substitution for ligand−MR interaction. In particular, the nature as well as the bulk of the substituent at the C7, C11, C18, and C21 positions is crucial for a high binding affinity to MR.[17−20]

Progesterone binds to MR with a high affinity and acts as an antagonist derivative.[21] Recently, 18-substituted steroid derivatives designed as k_{cat} inhibitors were reported to be potent inhibitors of cytochrome $P450_{11\beta}$, a key enzyme in aldosterone biosynthesis.[22−28] These compounds exhibited also high affinities for the human and rat MR.[20,27,29] Taking into account these results, we designed three PAL reagents in the progesterone series where a diazomethyl group was introduced at either the C18 or C20 position. Diazo ketone functionality was chosen because it is one of the less bulky photolabile groups among the carbene precursors. PAL studies using diazo ketones as reagents have been applied to elucidate various structural aspects in the interaction of the hormone with the other steroidal receptors.[30−32]

Chart 1



In this paper, we describe the synthesis of three diazo ketones: 21-diazopregn-4-ene-3,20-dione (**1**), 18-(diazomethyl)-20-hydroxypregn-4-ene-3,18-dione (**2**), and 18-(diazomethyl)pregn-4-ene-3,18,20-trione (**3**) (Chart 1). Their binding affinities for the human MR (hMR) were measured using the recombinant hMR expressed in the Baculovirus system.[5] Their agonist/antagonist activities were measured using a cis−trans cotransfection assay,

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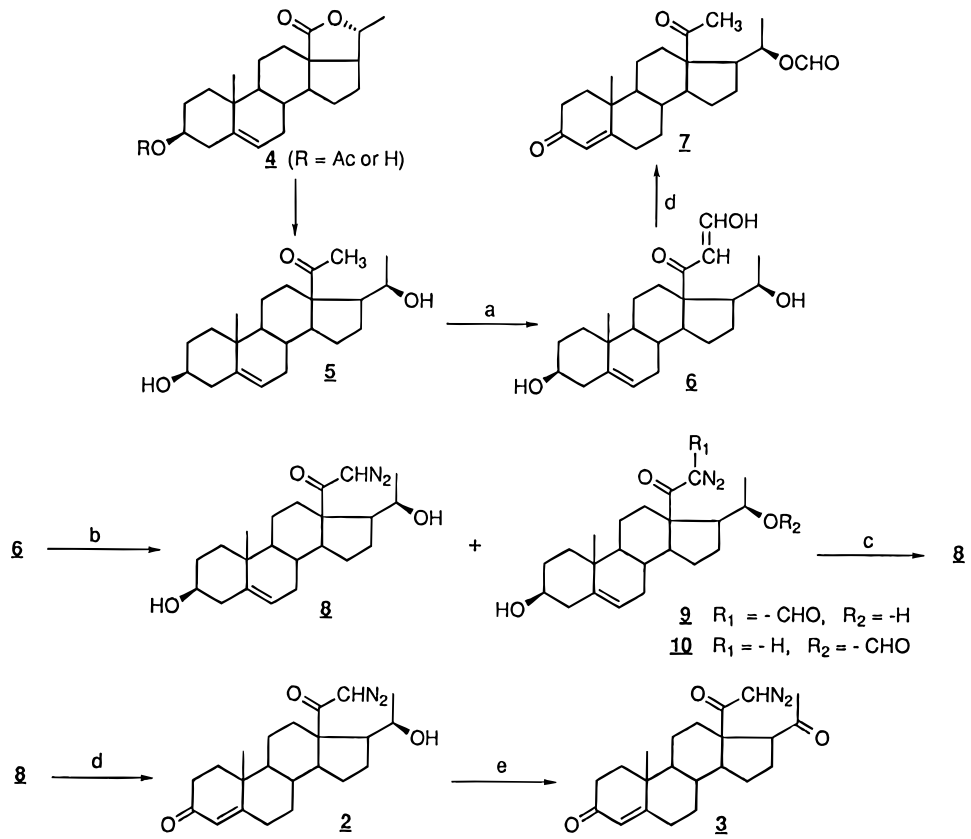
Scheme 1[a]



[a] Reagents: (a) NaH, C_2H_5OH, HCOOEt, THF; (b) $TosN_3$, NaH, C_2H_5OH, THF; (c) 1 N NaOH, MeOH, reflux; (d) $Al(OiPr)_3$, *N*-methyl-4-piperidone, reflux; (e) DMSO, $(COCl)_2$, −60 °C, NEt_3.

with the mouse mammary tumor virus (MMTV) promoter as the steroid receptor-inducible DNA target sequence.[2]

Results and Discussion

Synthesis of Diazo Ketones 1−3. 21-Diazoprogesterone (**1**) was prepared according to a well-described method[33,34] from 3-keto-4-etiocholenic acid. We attempted to synthesize diazo ketones **2** and **3** by the same strategy from the lactone **4** (R = Ac), but the corresponding acid chloride could never be obtained. They were thus synthesized by a different route as depicted in Scheme 1. The diazo group-transfer reaction[35,36] was applied to an active methylene compound, a β-keto aldehyde prepared from the known[37] 18-methyl ketone **5**. The formyl group was introduced into **5** with ethyl formate in the presence of sodium ethanolate, generated by sodium hydride and ethanol. The presence of the major enol form in equilibrium with the β-keto aldehyde was deduced from the ^1H NMR spectrum of **6**. Oxidation under Oppenauer conditions of the alcohol function at C3 of hydroxymethylene derivative **6** was first attempted at this step of the synthesis. It gave the only oxidation product **7**, characterized as 20β-hydroxy-18-methylpregn-5-ene-3,18-dione formate, coming from a transformylation reaction between the hydroxyl group at C20 and the hydroxymethylene group at C18.

In order to avoid this undesired reaction, we decided to introduce the diazo function before oxidation at C3. Hydroxymethylene compound **6** was first treated with triethylamine (dried on KOH pellets) and then tosyl azide, affording the desired diazo ketone **8** (23%) in a mixture containing also the lactone **4** (R = H, 26%) and two formylated diazo ketones, **9** and **10** (35% in a 38:62 ratio). Isolation of **9** and **10** showed that the cleavage of the formyl moiety was not complete in the course of the diazo group-transfer reaction and revealed again a transformylation reaction leading to the formate **10**. In order to decrease the formation of alcaline cleavage product **4**, we first added tosyl azide on **6** before base treatment. The use of sodium ethanolate instead of triethylamine led cleanly to a mixture of **8** and **10**. Saponification of the crude extract allowed the isolation of diazo ketone **8** (78%, total yield) in a pure and thermally stable state, as yellow crystals. Oxidation at C3 by Oppenauer procedure afforded diazo ketone **2** (88%), and oxidation at C20 was performed in Swern conditions to give diazo ketone **3** (61%).

Affinity of the Diazo Ketones 1−3 for the Recombinant hMR. The affinity of **1**−**3** for the hMR was assessed by competition experiments using [^3H]aldosterone as specific ligand and the recombinant hMR expressed in insect cells by the Baculovirus system.[5] In this model hMR is selectively expressed, allowing to discard cross-reactivity of the diazo ketones with other steroid receptors. The apparent dissociation constant values ($K_{d_{app}}$) of progesterone and diazo ketones are reported in Table 1. The order of potency of the derivatives in displacing [^3H]aldosterone binding was as follows: progesterone > **1** > aldosterone > **2** = **3**. The presence of a diazo group at the C21 (**1**) or C18′ (**2**, **3**) position induces a decrease in the affinity for hMR when compared to progesterone. These results confirm our previous report[20] indicating that the affinity of 18-substituted progesterone derivatives depends on the nature of the substitutents. Indeed, **2** and **3**, which have

Table 1. Binding Characteristics of the Diazo Ketones to the hMR and Mineralocorticoid Activity[a]

	$K_{d_{app}}$ (nM)	ED_{50} (nM)	IC_{50} (nM)
progesterone	0.39 ± 0.08		30
21-diazoprogesterone (**1**)	0.74 ± 0.14	6	
18-(diazomethyl)-20-hydroxy pregn-4-ene-3,18-dione (**2**)	20 ± 1		900
18-(diazomethyl)pregn-4-ene-3,18,20-trione (**3**)	20 ± 2		700

[a] The apparent dissociation constants at equilibrium ($K_{d_{app}}$) were calculated from the competition experiments as indicated in the Experimental Section. Values are the means ± SEM ($n = 3$). The ED_{50} values are the concentrations of the tested steroids required to induce half-maximum response in the cis−trans cotransfection assay (agonist activity). The IC_{50} values are the concentrations of the tested steroids that inhibited by 50% the activity induced by 10^{-9} M aldosterone. The ED_{50} and IC_{50} values were calculated from two separate cotransfection assays.

a similar affinity whatever the substituent at C20, have a lower affinity for hMR than progesterone, 18-vinylprogesterone, and 18-ethynylprogesterone.[20]

Mineralocorticoid Activity of the Diazo Ketones. The mineralocorticoid activity of **1**−**3** was analyzed using a cis−trans cotransfection assay in CV-1 cells. Under these conditions **1** behaved as a full agonist with an ED_{50} of 6×10^{-9} M (Table 1). Up to 10^{-5} M, **1** had no antimineralocorticoid activity (data not shown). **2** and **3** dose-dependently inhibited the aldosterone-induced receptor-mediated response with an IC_{50} of 9×10^{-7} and 7×10^{-7} M, respectively (Table 1). As a comparison progesterone displayed an IC_{50} of 3×10^{-8} M. **2** and **3** have an extremely low mineralocorticoid agonist activity, inducing at 10^{-6} M only 2% of the aldosterone-induced maximum response. Thus, the introduction of the diazo ketone function at the C13 position does not modify the antagonist feature of progesterone but lowers the activity. On the other hand, the introduction of a diazo group at C21 switched off the antagonist properties of progesterone, as did a hydroxyl group at that position in corticosterone.[38]

Taken together these results indicate that diazo ketones **1**−**3** displayed an affinity for hMR that is compatible with their use as photolabeling reagents. The availability of compounds with agonist (**1**) or antagonist (**2**, **3**) properties is of particular interest to determine the amino acids of the ligand-binding domain of hMR involved in the interaction with agonists and antagonists.

Experimental Section

Chemical Materials and Methods. Melting points (mp) were determined on a Kofler apparatus. ^{13}C and ^{1}H NMR spectra were recorded in $CDCl_3$, either on a JEOL 400 or a Brucker AC 200 spectrometer. Chemical shifts are expressed in ppm relative to TMS and coupling constants in Hz. IR spectra were recorded in $CHCl_3$ on a Perkin Elmer 1420 spectrometer. Optical rotations were measured in $CHCl_3$ with a Perkin Elmer 241 polarimeter. Mass spectra were carried out by the Centre de Spectrochimie de l'Université Paris VI and the Service de spectrométrie de masse de l'ENSCP. High-resolution mass spectra were obtained with a KRATOS MS 50 spectrometer. Microanalyses were carried out by the Centre de Microanalyse de l'Université Paris VI. Analytical TLC was carried out on 0.20 mm E. Merck precoated silica gel plates (60F-254) with detection by UV light or sulfuric acid (30%) spray followed by heating.

21-Diazopregn-4-ene-3,20-dione (21-diazoprogesterone) (1): synthesized from 3-oxoandrost-4-ene-17β-carboxylic acid as previously described;[33] recrystallization from CH_2Cl_2−isopropyl ether; mp 183−184 °C (lit.[33] mp 182−184 °C).

3β,20β-Dihydroxy-18-methylpregn-5-en-18-one (5): prepared from the lactone **4** (R = Ac) by the previously described method,[37] 64% yield; two recrystallizations; mp 192−194 °C (lit.[37] mp 187−190 °C).

3β,20β-Dihydroxy-18-(hydroxy-2′-ethylene)-pregn-5-en-18-one (6). Methyl ketone **5** (314 mg, 0.9 mmol) was dissolved in 6.3 mL of THF, and 3.15 mL of dry benzene was added. Sodium hydride (785 mg of a 80% dispersion in mineral oil, 26.1 mmol) and absolute ethanol (188 μL, 147 mg, 3.2 mmol) were transferred to the steroid solution kept on an ice bath. The flask was placed under argon atmosphere. The reaction mixture was stirred vigorously at room temperature for 25 min. Ethyl formate (1.1 mL, 1.0 g, 13.6 mmol) was added dropwise with vigorous stirring. After 40 min, 1.8 N CH_3COOH (25 mL) was added cautiously (frothing) until pH 6−7. Usual workup (AcOEt, H_2O, $MgSO_4$) gave 510 mg of crude product. Purification by silica gel chromatography (cyclohexane−AcOEt, 1:1.3) gave pure **6** as an oil (248 mg, 73%). Crystallization did not occur in any solvent system: $[\alpha]^{22}_D$ 5° (*c* 0.5, $CHCl_3$); IR ν_{max} 3518 (free OH), 3400 (br, bound OH), 1725 (C=O), 1690 (conjugated C=O), 1620 (C=C) cm^{-1}; ^1H NMR (200 MHz) δ 7.89 (d, 1H, $J = 4.6$ Hz, H-18″), 5.88 (d, 1H, $J = 4.6$ Hz, H-18′), 5.31 (bs, 1H, H-6), 3.48 (m, 2H, H-3 + H-20), 1.09 (d, 3H, $J = 6.1$ Hz, H-21), 0.84 (s, 3H, H-19); HRMS calcd for $C_{23}H_{34}O_4$ 374.2457, found 374.2457.

20β-Hydroxy-18-methylpregn-5-ene-3,18-dione Formate (7). The alcohol **6** (43 mg, 0.11 mmol) was dissolved in toluene (10 mL). *N*-Methylpiperidone (0.4 mL) was added. The mixture was heated to reflux under argon with a Dean−Stark apparatus, and the first 1.5 mL was discarded. Aluminum isopropoxide (56 mg, 0.27 mmol) was added and the mixture refluxed for 1 h. The reaction mixture was then acidified with 5% H_2SO_4, and after usual workup the crude product (22 mg) was purified by preparative TLC (CH_2Cl_2−acetone, 95:5) to give an analytical sample of **7** (6 mg): IR ν_{max} 1730 (O−C=O), 1680, 1660 (conjugated C=O), 1620 (C=C) cm^{-1}; ^1H NMR (400 MHz) δ 8.09 (s, 1H, −OCHO), 5.73 (s, 1H, H-4), 4.72 (m, 1H, H-20), 2.12 (s, 3H, $-COCH_3$), 1.24 (d, $J = 6.05$ Hz, 3H, H-21), 1.08 (s, 3H, H-19); ^{13}C NMR (100 MHz) δ 212.81, 199.36, 170.80, 160.48, 123.98, 73.21, 61.03, 57.12, 54.93, 53.96, 38.47, 36.27, 36.24, 35.69, 33.89, 32.61, 31.74, 29.78, 26.21, 24.59, 23.21, 20.03, 17.36; MS (EI) *m/z* 372, 344, 326, 283, 161.

18-(Diazomethyl)-3β,20β-dihydroxypregn-5-en-18-one (8). Compound **6** (578 mg, 1.5 mmol) was dissolved in 14.5 mL of THF. The flask was placed on an ice bath, and tosyl azide (1.2 mL, 6 mmol, 1.18 g) was added under argon atmosphere. Sodium hydride (230 mg of a 80% dispersion in mineral oil, 7.6 mmol) was added at 0 °C, and vigorous stirring was continued for 30 min. The reaction was quenched with ice (frothing). After evaporation of the solvent, the residue was dissolved in MeOH (5 mL), and 1 N NaOH (4 mL) was added. The reaction mixture was refluxed for 30 min. The solvent was evaporated, and the residue was extracted with AcOEt, washed with water, and dried ($MgSO_4$). The crude product (773 mg) was freed from excess tosyl azide by flash chromatography. Crystallization in cyclohexane−AcOEt (1:1) afforded **8** as a yellow product (451 mg, 78%): mp 197−198 °C; IR ν_{max} 3600 (free OH), 3440 (bound OH), 2100 (C=N=N), 1715, 1615 cm^{-1}; $[\alpha]^{22}_D$ −5.6° (*c* 0.5, $CHCl_3$); ^1H NMR (400 MHz) δ 5.74 (s, 1H, $-COCHN_2$), 5.28 (m, 1H, H-6), 3.57 (m, 1H, H-20), 3.45 (m, 1H, H-3), 1.09 (d, 3H, H-21), 0.85 (s, 3H, H-19). Anal. ($C_{22}H_{32}O_3N_2$) C, H, N.

18-(2′-Diazo-1′-oxo-ethyl)-3β,20β-dihydroxypregn-5-en-18-one (9) and 18-(Diazomethyl)-3β,20β-dihydroxypregn-5-en-18-one 20-Formate (10). Compound **6** was dissolved in dry THF (1.3 mL) and freshly distilled CH_2Cl_2 (10.5 mL). Triethylamine dried on KOH pellets (0.32 mL, 1.87 mmol) was added. The flask was placed under argon atmosphere, and tosyl azide (0.27 mL, 1.38 mmol) dissolved in distilled CH_2Cl_2 (0.8 mL) was added dropwise. The reaction mixture was stirred for 2 h. Usual workup (EtOAc, H_2O, $MgSO_4$) gave 631 mg of crude product. Purification by silica gel chromatography (cyclohexane−AcOEt, 1:10 gave the lactone **4** (46 mg, 26%), the diazo ketone **8** (46 mg, 23%), and the two formylated diazo

ketones **9** and **10** (76 mg, 35%). Analytical samples of **9** and **10** were further separated and purified by a second silica gel chromatography with CH_2Cl_2−10% acetone.

9: IR ν_{max} 3600 (free OH), 3460 (bound OH), 2120 (C═N═N), 1700, 1655, 1630 cm^{-1}; ^1H NMR (200 MHz) δ 10.11 (s, 1H, −CHO), 5.33 (s, 1H, H-6), 3.60 (m, 1H, H-20), 3.52 (m, 1H, H-3), 1.18 (d, J = 6.03 Hz, 3H, H-21), 0.87 (s, 3H, H-19); ^{13}C NMR (50 MHz) δ 193.92, 184.82, 140.52, 121.33, 71.64, 70.94, 62.78, 60.23, 58.59, 50.57, 42.13, 37.18, 36.44, 35.64, 32.76, 31.61, 31.49, 29.23, 26.71, 25.55, 24.46, 23.01, 19.37.

10: IR ν_{max} 3600 (free OH), 3400 (bound OH), 2100 (C═N═N), 1715, 1615 cm^{-1}; ^1H NMR (200 MHz) δ 8.10 (s, 1H, −OCHO), 5.34 (s, 1H, $COCHN_2$), 5.32 (bs, 1H, H-6), 4.86 (m, 1H, H-20), 3.50 (m, 1H, H-3), 1.22 (d, J = 6.18 Hz, 3H, H-21), 0.90 (s, 3H, H-19); MS (IC) m/z 401 (MH^+).

18-(Diazomethyl)-20-hydroxypregn-4-ene-3,18-dione (2). The diol **8** (125 mg, 0.33 mmol) dissolved in toluene (20 mL) and 1-methyl-4-piperidone (1.5 mL, 12.2 mmol) were heated to reflux under argon (Dean−Stark apparatus). The first 5 mL of distillate was discarded, aluminum isopropoxide (203 mg, 0.99 mmol was added under nitrogen atmosphere, and refluxing was continued for 2 h. The toluene solution was neutralized with 0.12 N HCl and ice, washed with water, dried with Na_2SO_4, and evaporated. Purification by flash chromatography with CH_2Cl_2−acetone (90:10) gave 110 mg of **2** as pure yellow crystals (88%): mp 165−167 °C; $[\alpha]^{22}_D$ 199° (c 0.5, $CHCl_3$); IR ν_{max} 3600 (free OH), 3460 (bound OH), 2095 (C═N═N), 1725, 1660, 1615 cm^{-1}; ^1H NMR (400 MHz) δ 5.90 (s, 1H, $-COCHN_2$), 5.71 (s, 1H, H-4), 3.68 (m, 1H, H-20), 1.16 (d, 3H, J = 6.1 Hz, H-21), 1.08 (s, 3H, H-19); ^{13}C (100 MHz) δ 199.55, 199.07, 171.27, 123.85, 70.47, 59.9, 58.69, 56.77, 55.51, 54.18, 38.54, 37.31, 36.24, 35.72, 33.91, 32.72, 31.86, 26.60, 24.97, 23.88, 22.81, 17.49. Anal. ($C_{22}H_{30}O_3N_2$) C, H, N.

18-(Diazomethyl)pregn-4-ene-3,18,20-trione (18-(diazomethyl)-18-oxoprogesterone) (3). Oxalyl chloride (27.5 μL, 0.31 mmol) was dissolved in anhydrous CH_2Cl_2 (0.61 mL) and cooled to −60 °C under argon. Anhydrous DMSO (44.2 μL, 0.62 mmol) diluted in CH_2Cl_2 (110 μL) was added dropwise, and the mixture was stirred at −60 °C for 15 min. The alcohol **2** (81 mg, 0.22 mmol) dissolved in CH_2Cl_2 (0.6 mL) and DMSO (44.2 μL) was added, and the mixture was stirred for 15 min at −60 °C. Et_3N (187.5 μL, 1.34 mmol) dried on KOH pellets was added, and stirring continued for 15 min at −60 °C. The reaction mixture was then allowed to warm to room temperature for 15 min. A saturated NaCl solution (5.8 mL) was added. Extraction with AcOEt and usual workup (NaCl solution, $MgSO_4$) gave 65 mg of crude product. Purification by flash chromatography on silica gel with CH_2Cl_2−10% acetone afforded 52 mg of **3** as yellow crystals (61% yield): mp 194−196 °C; $[\alpha]^{22}_D$ 205° (c 0.5 in $CHCl_3$); IR ν_{max} 2100 (C═N═N), 1700, 1660, 1615 cm^{-1}; ^1H NMR (200 MHz) δ 5.71 (s, 1H, H-4), 5.44 (s, 1H, $-COCHN_2$), 2.13 (s, 3H, H-21), 1.10 (s, 3H, H-19); ^{13}C NMR (100 MHz) δ 208.76, 199.27, 196.68, 170.63, 123.93, 63.30, 59.28, 57.05, 56.21, 53.94, 38.47, 36.09, 35.67, 35.52, 33.82, 32.52, 31.63, 30.46, 25.34, 24.86, 22.56, 17.44. Anal. ($C_{22}H_{28}O_3N_2$) C, H, N.

Biochemical Methods. Binding Characteristics of the Diazo Ketones to the Recombinant hMR. Sf9 insect cells were infected with the recombinant virus AcNPV-hMR which contains the full-length cDNA of the hMR.[5] At 48 h postinfection, the cells were rinsed with cold PBS and homogenized in TEGW buffer (20 mM Tris-HCl, 1 mM EDTA, 10% glycerol (v/v) 20 mM sodium tungstate, pH 7.4 at 20 °C) using a Teflon−glass homogenizor. The homogenate was centrifuged at 105.000g for 1 h at 4 °C, and the cytosol fraction was frozen in liquid nitrogen until further analysis. Cytosol was incubated for 4 h at 4 °C with 3 nM [^{3}H]aldosterone (40−60 Ci/mmol; Radiochemical Center, Amersham) in the absence or presence of increasing concentrations of unlabeled competitors (0.3−600 nM). Bound and unbound steroids were separated by the charcoal−dextran technique previously described.[3] The apparent dissociation constants at equilibrium ($K_{d_{app}}$) were calculated according to the formula: $K_{d_{app}} = (K_d)_A \times [X]_{50}/[A]_{50}$, where $[X]_{50}$ and $[A]_{50}$ are the concentrations of the competitor and aldosterone that induce a 50% displacement of [^{3}H]-aldosterone binding and $(K_d)_A$ is the mean value of the dissociation constant of aldosterone determined in a previous study (0.96 ± 0.08 nM).[5]

Mineralocorticoid Activity of the Diazo Ketones. CV-1 cells were seeded at a density of 0.5×10^6 cells/10 cm Petri dish in 10 mL of minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2. Four hours before and through out the transfection procedure, cells were maintained in medium supplemented with 10% charcoal-stripped FCS. Cells were transfected using the calcium phosphate precipitation method according to the standard procedure[39] with 5 μg of pRShMR, a plasmid which contains the entire hMR coding sequence, 5 μg of pFC31Luc which contains the MMTV promoter driving the luciferase gene, 5 μg of pCH110, which contains the gene coding for the β-galactosidase enzyme, and 5 μg of pSP72 as plasmid carrier. Sixteen hours after transfection, the cells were rinsed twice with PBS and incubated with MEM containing increasing concentrations (10^{-9}−10^{-6} M) of the tested steroid in the absence (agonist activity) or presence (antagonist activity) of 10^{-9} M aldosterone. After 24 h incubation the cells were harvested and the cell extracts assayed for luciferase[40] and β-galactosidase activity.[41] To standardize for transfection efficiency, the relative light units, obtained in the luciferase assay, were divided by the optical density obtained in the β-galactosidase assay.

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