18-Substituted steroids. Part 18. Chemical synthesis and mineralocorticoid activity of 2α- and 2β-hydroxyaldosterone

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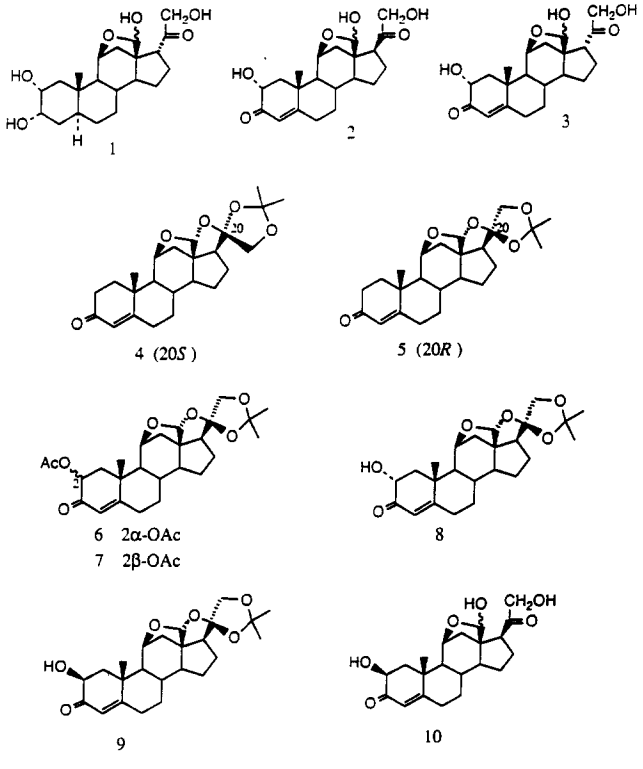
The 2α-hydroxy and 2β-hydroxy derivatives of aldosterone have been synthesized chemically from aldosterone, after the earlier identification of 2α-hydroxylated metabolites formed in liver. Both 2α- and 2β-hydroxyaldosterone are potent mineralocorticoids, with activities on the order of 1/10 that of aldosterone on the basis of a rat bioassay. (*Steroids* **58**:59–63, 1993)

Keywords: steroids; 2α-hydroxyaldosterone; 2β-hydroxyaldosterone; mineralocorticoid activity; liver metabolism

Introduction

We recently identified 11β,18-epoxy-2α,3α,18,21-tetrahydroxy-5α,17α-pregnan-20-one (**1**), the 2α-hydroxy derivative of 3α,5α-tetrahydroaldosterone in its 17-iso form, as a significant component of the mixture of neutral polar metabolites, which results from the incubation of aldosterone with the microsomal fraction of male rat liver[1] (see Scheme 1 for structures). The same compound has been recognized chromatographically among the metabolites formed by the kidney, and in the urine of male rats.[2]

The structural assignment was based on high-field, two-dimensional ^{1}H-nuclear magnetic resonance (^{1}H-NMR) analysis of fractions in which the compound had been concentrated by repeated high-performance liquid chromatography (HPLC). None of these fractions was chemically pure, and quantities were at submilligram levels, but the characteristic ^{1}H-NMR features (COSY) of the 2α,3α-dihydroxy-5α steroid system, as well as those of rings C and D and the side chain of aldosterone, were clearly recognizable. We also were able to observe NMR evidence for the pres-



Scheme 1

† It is with regret that we report the death of Professor David N. Kirk on October 7, 1992. This paper is dedicated to his memory.

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Table 1 ^{1}H-NMR data for 2-hydroxyaldosterones and intermediates in synthesis

	Acetonides		(20R)-Acetonide		(20R)-Acetonide		2α-OH-Aldo		2β-OH-Aldo	
Proton	(20S) **4**	(20R) **5**	2α-OAc **6**	2β-OAc **7**	2α-OH **8**	2β-OH **9**	18,20-hemiacetal **2a**	20-oxo form **2b**	18,20-hemiacetal **10a**	20-oxo form **10b**
1α	1.72	1.69			1.55	1.66	1.55	1.55	1.64	1.67
1β	2.18	2.17			2.51	2.52	2.53	2.51	2.52	2.50
2α	2.36	2.36	—	5.26	—	4.12	—	—	4.13	4.13
2β	2.49	2.49	5.50	—	4.31	—	4.32	4.32	—	—
4	5.71	5.72	5.74	5.79	5.77	5.80	5.79	5.79	5.81	5.82
6α	2.29	2.29			2.32	2.26	2.35	2.34	2.27	2.27
6β	2.45	2.45			2.43	2.56	2.45	2.41	2.57	2.51
7α	1.22	1.22			1.22	1.16	1.23	1.16	1.17	1.13
7β	1.97	2.02			2.04	2.14	2.04	1.97	2.16	2.07
8β	1.69	1.77			1.77	1.88	1.77	1.71	1.88	1.82
9α	1.07	1.05			1.06	1.46	1.07	1.02	1.47	1.41
11α	4.85	4.85	4.57	4.89	4.81	4.98	4.82	4.58	4.98	4.78
12α	1.45	1.44			1.42	1.46	1.42	1.48	1.47	1.52
12β	2.73	2.24			2.24	2.27	2.28	2.71	2.32	2.74
14α	1.64	1.50			1.48	1.49	1.48	1.48	1.48	1.48
15α	1.71	1.92			1.91	1.88	1.93	1.97	1.91	1.97
15β	1.52	1.44			1.42	1.43	1.43	1.43	1.43	1.43
16α	1.64	1.64			1.64	1.65	1.61	1.97	1.60	1.97
16β	2.08	2.17			2.17	2.17	2.20	2.30	2.21	2.30
17α	2.55	2.52			2.51	2.52	2.35	2.80	2.37	2.82
18	5.31	5.28	5.27	5.27	5.28	5.27	5.41	5.03	5.42	5.03
19	1.30	1.31	1.58	1.45	1.44	1.25	1.40	1.36	1.48	1.46
21	4.03/3.92	4.17/3.88	4.18/3.87	4.18/3.87	4.16/3.88	4.16/3.88	3.61/3.46	4.39/4.24	3.62/3.47	4.39/4.25
CMe_2	1.50/1.35	1.49/1.40	1.49/1.40	1.49/1.40	1.48/1.40	1.48/1.39				
OH					3.57	3.56				
OAc			2.18	2.16						

Values are changes in ppm.

ence of 2α-hydroxyaldosterone **(2)** or its 17-iso form **(3)** as a minor constituent of the same HPLC fractions.[1]

Separate experiments[1] have established that the conversion of aldosterone and its metabolites into derivatives of the 17-iso series occurs in contact with the liver microsomal material, but can be partly suppressed if incubations are conducted at pH 6.8 instead of pH 7.4, as in the main series of experiments. We therefore inferred that the primary product of 2-hydroxylation by the liver is 2α-hydroxyaldosterone **(2)** itself, although we cannot rule out the possibility that some or all of the 2α-hydroxytetrahydro metabolite **(1)** arises by 2α-hydroxylation of 3α,5α-tetrahydroaldosterone.

To explore the matter further, we undertook the chemical synthesis of 2α-hydroxyaldosterone, which is reported here.

Experimental

^{1}H-NMR spectra were recorded on a Bruker AM-500 or a Varian UNITY-600 Spectrometer at N.I.M.R., Mill Hill, London, in $CDCl_3$, using the chloroform signal (7.26 ppm) as internal standard. Table 1 lists assignments that, apart from those for the 2-acetoxy derivatives, are derived from two-dimensional ^{1}H homonuclear correlated spectra (COSY). Mass spectra were recorded on a Kratos MS 902 operating at 70 eV. Column chromatography was performed on silica gel (Sorbsil C 60, MPD 60 Å, 40–60 μm). Light petroleum refers to the fraction of bp 40–60 C. A molecular sieve was added to the reactions to guarantee dry conditions on a small scale.

(20S)- and (20R)-aldosterone-20,21-acetonides (4 and 5)

A mixture of toluene (40 ml) and 2,2-dimethoxypropane (40 ml) containing 4 Å molecular sieve, was heated to 85 C under N_2. Aldosterone (250 mg; 0.69 mmol) and pyridinium toluene-*p*-sulfonate (5 mg) were then added. The solution was stirred for a further 20 minutes, diluted with ethyl acetate (60 ml), and filtered. The organic phase was washed with saturated sodium hydrogen carbonate solution (2 × 30 ml), water (30 ml), and brine (30 ml), and then dried (Na_2SO_4). The solvents were evaporated under reduced pressure. The crude product was subjected to flash column chromatography (EtOAc–light petroleum, 1 : 9 → 3 : 7) giving (20S)-aldosterone-20,21-acetonide **(4)** (102 mg), (20R)-aldosterone-20,21-acetonide **(5)** (102 mg), and 26 mg of a mixture of **4** and **5**. Overall, 230 mg (83%) of acetonides were obtained. (20R)-Acetonide, melting point (mp) 136–138 C, ν_{max} (KBr) 1672, 1233, 1160, 1063, 1019, and 876 cm^{-1}. (20S)-Acetonide, mp 140–142 C, ν_{max} (KBr) 1667, 1234, 1045, 1088, and 874 cm^{-1}. [lit.[3] (for the unseparated mixture of isomers) mp 227–232 C, ν_{max} (Nujol) 1672, 1618, 1076, 1045, 1006, 985, 917, and 871 cm^{-1}].

(20R)-2α and 2β-acetoxyaldosterone-20,21-acetonides (6 and 7)

(20R)-Aldosterone-20,21-acetonide **(5)** (93.5 mg; 0.234 mmol) was dissolved in toluene (30 ml) containing molecular sieve.

Lead tetraacetate (7.78 g) in acetic acid (approximately 17 mmol) was added, and the mixture was heated under reflux for 7 hours under N_2, cooled, diluted with ethyl acetate (100 ml), and filtered. The organic phase was washed with aqueous 5% HCl (6 × 20 ml), water (1 × 20 ml), saturated sodium hydrogen carbonate solution (5 × 20 ml), water (20 ml), and brine (30 ml), and then dried (Na_2SO_4). The solvents were evaporated under reduced pressure. The crude product was purified by flash column chromatography (EtOAc–light petroleum, 1 : 9) resulting in 98.5 mg (92%) of mixed (20R)-2α- and 2β-acetoxyaldosterone-20,21-acetonides, which could not be separated. ^{1}H-NMR spectroscopy (Table 1) confirmed that acetoxylation had occurred, but the products were not further characterized at this stage.

(20R)-2α- and 2β-hydroxyaldosterone-20,21-acetonides (8 and 9)

The mixed 2α- and 2β-acetoxy derivatives, prepared as above (56 mg; 0.122 mmol), were added to a mixture of aqueous 0.1 M $NaHCO_3$ solution (2 ml) and methanol (2 ml). The mixture was stirred for 3 hours at room temperature in the dark, saturated with NaCl, and extracted with CH_2Cl_2 (6 × 10 ml). The combined organic phases were washed with brine and dried (Na_2SO_4). The solvent was evaporated under reduced pressure. The crude products were separated by flash column chromatography (EtOAc–light petroleum, 3 : 7 → 1 : 1) and yielded (20R)-2α-hydroxyaldosterone-20,21-acetonide **(8)** (14.0 mg), mp 90–92 C, ν_{max} (KBr) 3447 (br), 1682, 1616, 1229, 1062, and 875 cm^{-1}, and (20R)-2β-hydroxyaldosterone-20,21-acetonide **(9)** (19.3 mg) mp 148–149 C, ν_{max} (KBr) 3452 (br), 1678, 1618, 1228, 1061, and 880 cm^{-1} (total yield 33.3 mg, 66%).

2α-Hydroxyaldosterone (2)

(20R)-2α-Hydroxyaldosterone-20,21-acetonide **(8)** (14.0 mg; 0.034 mmol) was added to a dioxane/water solution (4 ml, 2 : 1; acidified with HCl to pH 1) and stirred for 1.5 hours at room temperature in the dark. The mixture was then saturated with NaCl and extracted with CH_2Cl_2 (6 × 10 ml). The organic phase was washed with brine and dried (Na_2SO_4). The solvent was evaporated under reduced pressure. Flash column chromatography (EtOAc) yielded 2α-hydroxyaldosterone **(2)** (5.4 mg) (42%), mp 136–138 C, ν_{max} (KBr) 3343 (br), 1689, 1619, 1270, 1084, and 865 cm^{-1}. [MS: $(M-H_2O)^+$. Found: 358.1820; $C_{21}H_{28}O_6-H_2O$ requires 358.1780. The M^+ ion was not observed].

2β-Hydroxyaldosterone (10)

(20R)-2β-Hydroxyaldosterone-20,21-acetonide **(9)** (3.6 mg; 0.009 mmol) treated as above yielded 2β-hydroxyaldosterone **(10)** (1.8 mg) (55%), mp 92–94 C, ν_{max} (KBr) 3420 (br), 1678, approximately 1625 (sh), 1076, and 872 cm^{-1}. [MS: $(M-H_2O)^+$. Found: 358.1814; $C_{21}H_{28}O_6-H_2O$ requires 358.1780].

Rat bioassay

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, USA) were bilaterally adrenalectomized under ether anesthesia at 6–8 weeks of age. Thereafter, the rats were allowed free access to 0.9% NaCl as drinking water at all times and to Purina rat chow (Ralston Purina, St. Louis, MO, USA) until 16 hours before experimentation. Rats (160–180 g body weight) were maintained in a temperature- and light-controlled room and were used 4–6 days postadrenalectomy.

On the morning of the test, rats were made to void their bladders; this was achieved with a whiff of ether and slight suprapubic pressure. Animals were then injected subcutaneously (s.c.), with either 2α-hydroxyaldosterone, 2β-hydroxyaldosterone, aldosterone, or vehicle. Aldosterone was administered at dosages of 0.01, 0.05, 0.25, and 1.0 μg/rat, whereas the 2-hydroxy derivatives were given at dosages of 1.0, 5.0, and 25.0 μg/rat. All steroids were dissolved in a mixture of 0.154 M NaCl/ethanol (9 : 1, v/v) and each compound was injected in a volume of 0.2 ml. In addition, all animals were injected s.c. with 3.0 ml of 0.154 M NaCl to ensure adequate diuresis for subsequent urine collection.

Urine was collected (again using ether) for the time periods 0–1, 1–3, and 3–4 hours postinjection of steroid (time zero) and analyzed for Na^+, K^+, and creatinine content as previously described.[4] Mean (± SE) urinary Na^+/creatinine (Na^+/Cr) and K^+/creatinine (K^+/Cr) ratios were computed for all experimental groups in each urine collection period. The results described here are those from the 1–3 hour urine collection period only; the various steroids used in these experiments did not significantly affect urinary electrolyte excretion in the 0–1 or 3–4 hour urine collection periods. The number of animals in each experimental group ranged from five to 10 (Figure 1).

Results and discussion

Chemical synthesis

The conversion of steroidal 4-en-3-ones into their 2-hydroxy derivatives has been achieved in various ways. A stereospecific route to the 2α-hydroxy isomer involves epoxidation with alkaline hydrogen peroxide to form the 4β,5β-epoxy-3-oxo derivative as the main product, followed by an acid-catalyzed rearrangement involving allylic substitution in the Δ^2-enolic form of the epoxy compound and elimination of the elements of water, which apparently affords the 2α-hydroxy-4-en-3-one directly.[5,6] If any 2β-hydroxylation occurs, the thermodynamically unstable 2β compound must be isomerized rapidly via the enol under the strongly acidic conditions. This process appeared unsuitable for aldosterone because it would require protection of the very labile side chain against both strongly alkaline and strongly acidic conditions. A possible alternative route[6] involving a preliminary 6β-bromination followed by allylic substitution with acetate ion, again via an enolic intermediate, also seemed likely to invite difficulties in the aldosterone series.

Direct acetoxylation of 4-en-3-ones with lead tetraacetate[6,7] or manganese triacetate[8] provides a route to mixtures of 2α- and 2β-acetoxy derivatives, which can be hydrolyzed under relatively mild conditions to form the 2α- and 2β-hydroxysteroidal 4-en-3-ones (Scheme 1). We chose to use this route, having first protected the aldosterone side chain, in its 18→20 hemiacetal form, as the 20,21-acetonides.[3]

Formation of the acetonides by reaction of aldosterone with 2,2-dimethoxypropane led to a separable (flash column chromatography) mixture of the (20S) and (20R) isomers of the acetonide (**4** and **5**, respectively) in equal proportions. The assignment of configuration at C-20 in the acetonides rests on nuclear Overhauser effect difference (n.O.e.d.) experiments in which the C-21 proton signals for the (20R)-isomer **(5)** were separately irradiated. Inspection of a molecular model showed that one of the C-21 protons lies close in space to the 17α- and 12β-protons. In the n.O.e.d.

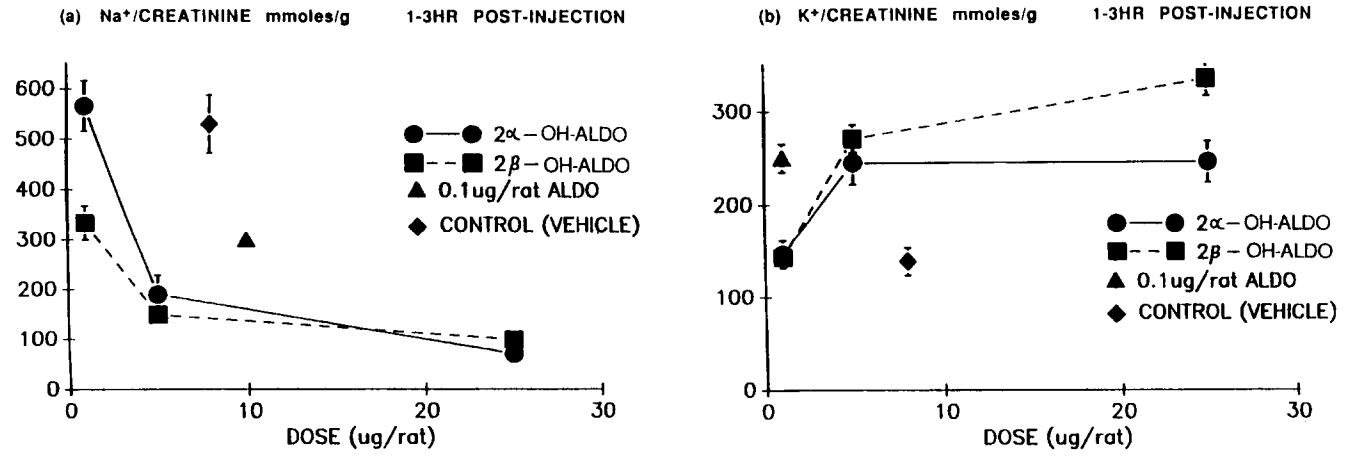

Figure 1 Mineralocorticoid activity of 2α- and 2β-hydroxyaldosterones.

experiment, irradiation of the C-21 proton at higher field (δ 3.88) caused strong enhancement of the 17α-H signal at δ 2.52, and weaker enhancement of the 12β-H signal at δ 2.24. Apart from the signal for the other C-21 proton, these were the only signals in the n.O.e.d. spectrum. Irradiation of the other C-21 proton (δ 4.17) caused no n.O.e. for ring protons, as it lies remote from the polycyclic structure. In accordance with this assignment, the 12β-H and 14α-H signals have larger chemical shifts for the (20S) isomer **(4)**, where they experience deshielding by the C-20 oxygen atom of the acetonide ring on the rear face of the molecule, in contrast to the (20R) acetonide. Conversely, the 16β-proton is more deshielded in the (20R) isomer **(5)**.

Reaction of the separated acetonides **(4** and **5)** with lead tetraacetate introduced acetoxy groups at C-2, giving the 2α- and 2β-isomers (**6** and **7**, respectively) in roughly equal proportions. We were unable to separate the C-2 isomers at this stage, so the acetoxy groups were hydrolyzed by sodium hydrogen carbonate to form a mixture of the corresponding 2-hydroxyaldosterone acetonides.

At this stage it proved possible to separate the 2α- and 2β-hydroxy derivatives **(8** and **9)** formed from the (20R)-acetonide, but unfortunately those formed from the (20S)-acetonide remained inseparable. In order to obtain pure samples of 2α-hydroxyaldosterone **(2)** and 2β-hydroxyaldosterone **(10)**, their (20R)-acetonides were separately deprotected by treatment with hydrochloric acid in aqueous dioxane, using the mildest conditions that would carry the reaction to completion (see Experimental), in order to minimize acid-catalyzed damage to the side chain, and possible acid-catalyzed isomerization of the 2β-hydroxy isomer to the 2α-configuration.

2α-Hydroxyaldosterone **(2)** obtained in this way was characterized by its mass spectrum and by a full analysis (COSY) of its 500-MHz ^{1}H-NMR spectrum, and comparison of the spectrum with those of aldosterone,[9] and of the 2α-hydroxy derivatives of other steroid hormones.[10] The spectrum of 2α-hydroxyaldosterone showed the expected peaks characteristic of both the 20-oxo and the 18→20-hemiacetal forms, with the latter, as usual,[9] predominant (ratio approximately 40 : 60).

2β-Hydroxyaldosterone **(10)** was similarly characterized as a mixture (approximately 40 : 60) of its 20-oxo and 18→20-hemiacetal forms.

Rat bioassay: effects on Na^+ excretion

The effects of increasing dosages of aldosterone on urinary Na^+ excretion are shown in Figure 1a. The effects of aldosterone are dose related; the smallest dosage of aldosterone, 0.01 μg/rat, did not lower the mean Na^+/Cr ratio compared with the control group, 550 ± 35 mmol/g), whereas dosages of 0.05, 0.25, and 1.0 μg/rat gave mean Na^+/Cr ratios of 430 ± 25, 200 ± 10, and 77 ± 8 mmol/g, respectively. Both 2α- and 2β-hydroxyaldosterone also lowered Na^+ excretion in a dose-dependent manner. 2α-Hydroxyaldosterone at dosages of 1, 5, and 25 μg/rat gave mean Na^+/Cr ratios of 566 ± 50, 190 ± 39, and 70 ± 12 mmol/g, respectively, whereas 2β-hydroxyaldosterone at the same dosages gave ratios of 334 ± 34, 149 ± 15, and 99 ± 13 mmol/g, respectively. Thus, it is evident that both 2α- and 2β-hydroxyaldosterone possess significant antinatriuretic activity, and that they are both approximately 1/10 as potent at aldosterone in adrenalectomized rats.

Effects on K^+ excretion

The effects of aldosterone and both 2α- and 2β-hydroxyaldosterone on urinary K^+ excretion are shown in Figure 1b. Aldosterone had no effect at 0.01 μg/rat, whereas 0.05, 0.25, and 1.0 μg/rat produced dose-dependent kaliuresis. It is evident from Figure 1b that 2α- and 2β-hydroxyaldosterone also possess significant kaliuretic activity, and that in a similar fashion to their antinatriuretic activity, these steroids are approximately 1/10 as potent at aldosterone in this assay.

Although it is not indicated on the figures, the antinatriuretic and kaliuretic effects of aldosterone (at dosages of 0.05, 0.25, and 1.0 μg/rat) and those of 2α- and

2β-hydroxyaldosterone (at dosages of 5 and 25 μg/rat) were statistically significant when compared with the control group (comparisons were made using analysis of variance and the Bonferoni *t* test).

Significant quantities of 5α- and 5β-ring A–reduced metabolites and several polar cytochrome P450–dependent hydroxylated neutral metabolites of aldosterone are present in the kidney during the latent period, before the expression of the effect of aldosterone on Na^+ and K^+.[11] In the kidney, several of the metabolites are synthesized locally, directly from aldosterone, whereas others are transported from the liver or are further renally modified hepatic derivatives of aldosterone. Of the 20 major peaks of polar hydroxylated neutral metabolites of aldosterone (present in the liver and kidney), resolved by HPLC into four major regions (A, B, C, and D) of increasing polarity, the least polar group of metabolites, designated region A, had earlier been shown to possess considerable mineralocorticoid activity (approximately 1/30 that of aldosterone) and to bind strongly to renal mineralocorticoid receptor.[4] This peak A material has now been resolved by HPLC into several region A metabolites, A_1–A_7, which consist of at least seven cytochrome P450–dependent hydroxylated metabolites. Many are both 5α-ring-A reduced and hydroxylated. Metabolite peaks A_2 and A_3 have been shown by high-resolution NMR to comprise 2-hydroxylated derivatives of aldosterone and 3α,5α-tetrahydroaldosterone,[1] and to possess 1/5 to 1/10 the mineralocorticoid activity of aldosterone; i.e., the 2-hydroxylated derivative of 3α-5α-tetrahydroaldosterone is more active than 3α,5α-tetrahydroaldosterone itself. Both 2α- and 2β-hydroxyaldosterone have now been chemically synthesized. Each has been shown to be approximately 1/10 as active as aldosterone when administered s.c. They migrate with retention times close to the products A_2 and A_3. These products are also synthesized when aldosterone or 3α,5α-tetrahydroaldosterone is incubated with rat kidney. Our experiments do not yet confirm the identities of region A metabolites produced in vivo in the kidney after administration of physiological quantities of [^{3}H]aldosterone; however, they do provide evidence that hydroxylation at the 2-position of aldosterone and/or its 5α-ring-A–reduced derivatives may play a significant role in the regulation of mineralocorticoid action in the kidney.

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