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ENZYME MEDIATED REDUCTION OF 21-DEHYDROCORTICOSTEROIDS AT C-20: ISOLATION AND CHARACTERIZATION OF DERIVATIVES CONTAINING THE 20β-HYDROXY-21-ALDEHYDE SIDE CHAIN

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SUMMARY

A method for the stereospecific reduction of 21-dehydrocorticosteroids with bacterial 20β-hydroxysteroid dehydrogenase to form 20β-hydroxy-21-aldehyde derivatives is described. These products have been given the trivial designation "isocorticosteroids". The isocorticosteroid homologues of 11-deoxycorticosterone, corticosterone, 11-deoxycortisol and cortisol were prepared in yields of 84–96%. The new compounds have been characterized by physcial and chemical criteria.

INTRODUCTION

We recently discovered that the side chains of corticosteroids are oxidatively metabolized in humans and other species to 21-oic acids [1–4]. These C_{21} acids represent a significant fraction of the total products of corticosteroid catabolism. We suggested a pathway which included a 20-ol-21-al intermediate to account for the formation of the steroidal α-hydroxy acid metabolites. Further studies on the metabolism of [4-^{14}C-21-^{3}H]-corticosteroids led us to conclude that cortisol was reduced to a certain extent to cortols and cortolones by a route which also utilizes the hypothetical 20-ol-21-al intermediate [5]. The relevant sequences which emphasize the pivotal position of the steroid 20-hydroxy-21-al, to which we have given the trivial class name "isocorticosteroid", is shown in Fig. 1. Direct reduction of cortisol (I) to cortols (II) proceeds *via* step (1). Isomerization of the ketol side chain (I) through step (2) leads to a derivative with the 20-ol-21-al side chain (III) which may then be oxidized to α-hydroxy acid (IV) (step (4)) or reduced to 17,20,21-triol (II) (step (3)).

In order to test whether isocorticosteroids are indeed physiological intermediates, we devised a procedure for their synthesis in high yield. The procedure described here is based on the observation of Furfine and White [6] that 21-dehydrocorticosteroids are good substrates for the 20β-hydroxysteroid dehydrogenase of Streptomyces hydrogenans.

Procedures and Results. 21-Dehydrocorticosteroids were prepared from their corresponding corticosteroids according to a modification [7] of the method described by Lewbart and Mattox [8] or were purchased from Steraloids Corp., Pawling, N.Y. Reduced nicotinamide adenine dinucleotide (disodium salt) was purchased from Sigma Chemical Co. 20β-Hydroxysteroid dehydrogenase (Streptomyces hydrogenans) was from Boehringer Co. t.l.c. was run on 0.25 mm thick Polygram silica gel G sheets with fluorescent indicator (Macherey–Nagel & Co.). Three solvent systems were used for chromatography. I) Chloroform–methanol–water (90:10:1, by vol.); II) Benzene–absolute ethanol (88:12, v/v); III) ethyl acetate. The oxidation of NADH during enzymatic incubations was followed on a Gilford model 2000 multiple sample recording spectrophotometer. Optical rotations were measured on a Zeiss 0.01° circle polarimeter against a solvent blank. Dilution to half the original concentration confirmed the assignment of a positive rotation to the isocorticosteroids. Melting points obtained on a Thomas–Hoover capillary melting point apparatus are corrected. Infrared data was obtained on a Perkin–Elmer model 221 infrared spectrophotometer. Samples were embedded in potassium

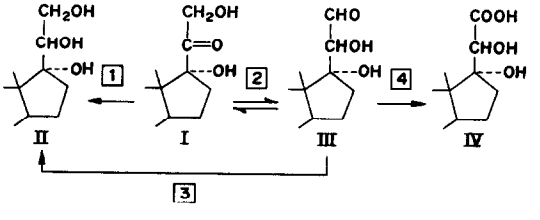

Fig. 1

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bromide. Nuclear magnetic resonance data was determined on a Varian HA-100 spectrometer. Mass spectra were obtained on a DuPont model 21-492 B mass spectrometer with a 21-094 computerized data system. Elemental analysis was performed by Galbraith Laboratories, Inc. Absorption spectra of isocorticosteroids and of their azine derivatives were measured in a Cary Model 15 recording spectrophotometer. Reaction of the 20-hydroxy-21-aldehyde derivatives with N-methyl-benzo-thiazolone hydrazone hydrochloride (MBTH) was run according to the procedure of Furfine and White[6], except that a 7 min boiling period was routinely used. The products were scanned from 420 nm to 200 nm.

Isomerization of isosteroids in basic media.

A modification of earlier procedures [9–11] was used. About 200 μg of isocorticosteroid and 0.7 ml of dry pyridine were placed in a side arm tube through which nitrogen was admitted. The tube was closed with a cold finger condenser. The mixture was heated in a paraffin bath kept at 110–112°. Under the conditions of base catalysis (pyridine, 112°), the hydroxyaldehydes readily isomerized to the corresponding ketols (Fig. 2). Isomerization proceeded smoothly, with yields in excess of 85% as determined by chromatography on thin layer plates in solvent systems I and III.

Preliminary studies were instituted to determine if 21-dehydrocorticosteroids were substrates for 20β-hydroxysteroid dehydrogenase. It is evident from Table 1 that 21-dehydrosteroids are more rapidly reduced than are other known substrates of 20β-hydroxysteroid dehydrogenase. The overall reaction proceeds preferentially in the direction of reduction with good yield to produce the desired 20β-hydroxy-21-aldehyde side chain. The 21-dehydrosteroids were

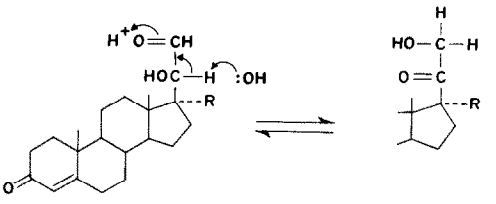


Fig. 2

reduced 2 to 70 times faster than the parent ketols. The pH optima of all substrates were from 6.9 to 7.2.

A summary of substrates used and overall reaction is presented in Fig. 3.

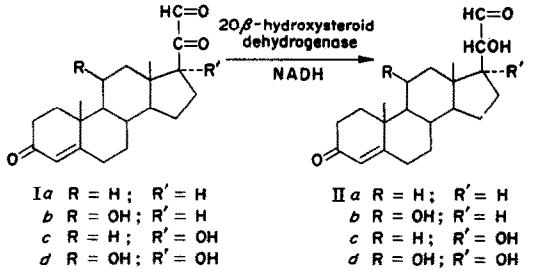
20β-hydroxy-3-oxo-4-pregnen-21-al (IIa) from 3,20-dioxo-4-pregnen-21-al (Ia).

A solution of 50.4 mg (0.154 mmol) of Ia in 5 ml of methanol was brought to 100 ml with 0.1 M phosphate buffer, pH 6.6. Half of the required NADH was added (0.077 mmol). The reaction was started by the addition of 11 units of 20β-hydroxysteroid dehydrogenase. The decrease in absorbancy at 340 nm was compared to that of a control in which all components were present except enzyme. A second aliquot of NADH (0.077 mmol) was added when the absorbancy became constant. The reaction went to completion in 1 h and 40 min although neither (Ia) nor (IIa) was completely soluble in the methanol-buffer system used. The product was extracted into ethyl acetate (5 extractions of 0.2 vol. each). The extract was washed twice with 0.2% EDTA (0.2 vol.), then with water, and dried over sodium sulfate. Best yields were obtained when all aqueous phases were saturated with sodium chloride during the extraction procedure. The solvent was evaporated under vacuum

Table 1. Substrate specificity of 20β-hydroxysteroid dehydrogenase*

Substrate	Rate of Reduction nmol/ml/min	21-dehydrosteroid / corticosteroid
11-deoxycorticosterone	0.57 ± 0.01	
21-dehydro-11-deoxycorticosterone	8.51 ± 0.17	14.9
corticosterone	0.057 ± 0.029	
21-dehydrocorticosterone	3.86 ± 0.09	67.7
11-deoxycortisol	1.53 ± 0.00	
21-dehydro-11-deoxycortisol	6.13 ± 0.25	4.00
cortisol	0.18 ± 0.14	
21-dehydrocortisol	2.63 ± 0.02	14.6
cortisone	3.21 ± 0.02	
21-dehydrocortisone	5.15 ± 0.10	1.65
Progesterone	1.92 ± 0.01	

* The incubation system contained (1.00 ml final vol) 90 μmol of phosphate buffer, pH 7.0, 141 nmol of NADH, 289 nmol of substrate, and 1 μg of enzyme. temp, 27° ± 1°.



Ia R = H; R' = H
b R = OH; R' = H
c R = H; R' = OH
d R = OH; R' = OH

IIa R = H; R' = H
b R = OH; R' = H
c R = H; R' = OH
d R = OH; R' = OH

Fig. 3

and the product transferred with chloroform to a small tube. All but 0.3 ml of solvent was removed under a stream of nitrogen. About 1.5 ml of heptane was added. A flocculent white precipitate of II*a* was recovered. The dry product weighed 46.5 mg (92% yield). *Anal.* calc'd for $C_{21}H_{30}O_3$: C 76.32%, H 9.15%; found C 76.10%, H 9.36%; n.m.r., δ 0.89 (*s*, 3, 18-C$\underline{H}_3$); 1.22 (*s*, 3, 19-C$\underline{H}_3$); 4.13 (*m*, *d*(D_2O), 1, J = 10 Hz, 20-C$\underline{H}$); 5.71 (*s*, 1, 4-C$\underline{H}$); 9.71 (*s*, 1, 21-C$\underline{H}$). t.l.c. (R_f values): system I: 11-deoxycorticosterone (DOC), 0.48; I*a*, 0.52; II*a*, 0.37; system II: DOC, 0.40; I*a*, 0.48; II*a*, 0.38; system III: DOC, 0.36; I*a*, 0.39; II*a*, 0.44; λ_{max} of azine derivatives (MBTH reaction): DOC, 338 nm, I*a*, 378 nm; II*a*, 312 nm; $(\alpha)_D^{25}$, + 93.6° (*c*, 1.0, ethanol) lit., + 84° ± 2° (*c*, 0.96, dioxane) [ref 12]; I.R. (KBr), cm^{-1}: 3400, 2700 (shoulder), 1730, 1670, 1620 $\lambda_{max}^{methanol}$ 241 nm: ϵ 15,900; m.p. 115° (phase transition), 135–138°; isomerization to 11-deoxycorticosterone in pyridine: 90% in 4.5 hr; mass spectrum (70 EV) *m/e* (relative intensity) 330 (8.9) M^+; 331 (2.6), $M^+ + 1$; 299 (100), M^+-(CHO + 2H); 300 (25.4); 301 (52.9), M^+-(CHO); 272 (54.9), M^+-(CHOHCHO − H).

*11β, 20β-dihydroxy-3-oxo-4-pregnen-21-al (II*b*) from 11β-hydroxy-3,20-dioxo-4-pregnen-21-al (I*b*).*

The reduction of I*b* (50.8 mg, 0.148 mmol) with 11 units of 20β-hydroxysteroid dehydrogenase and 0.074 mmol NADH in a total vol. of 100 ml in 5% methanol-0.1 M sodium phosphate buffer, pH 6.6, was identical to that of I*a*. A second aliquot of NADH (0.074 mmol) was added when the reaction rate slowed. Total incubation was 3 h. I*b* and II*b* were not completely soluble in the reaction medium. The extraction procedure for II*b* was identical to that of II*a*. Hexane was used to precipitate the product from the concentrated chloroform solution. Recovery, 49.3 mg (96% yield). *Anal.* calc'd for $C_{21}H_{30}O_4$: C, 72.80%; H, 8.73%; found: C, 72.59%; H 8.56% n.m.r.: δ 1.13 (*s*, 3, 18-C$\underline{H}_3$); 1.47 (*s*, 3, 19-C$\underline{H}_3$); 4.13 (*d*, J = 10 Hz, $\underline{H}$C-OH); 4.32 (*m*, 1, 11-C$\underline{H}$); 5.66 (*s*, 1, 4-C$\underline{H}$); 9.7 (*s*, 1, 21-C$\underline{H}$O). t.l.c. (R_f values): System I: corticosterone (B), 0.40; I*b* 0.45; II*b* 0.29; system II: B, 0.23; I*b*, 0.32; II*b*, 0.21; System III: B, 0.25; I*b*, 0.31; II*b*, 0.34; λ_{max} of azine derivatives (MBTH reaction): B, 338 nm; I*b* 375 nm; II*b* 312 nm; $(\alpha)_D^{27}$, + 118.6° (*c*, 1.0, ethanol); I.R. (KBr), cm^{-1}: 3400, 2700 (shoulder), 1730, 1665, 1615; $\lambda_{max}^{methanol}$ 242 nm: ϵ 14,200; m.p., 168°–175° with phase transition. Isomerization to corticosterone in pyridine was 90% in 6.5 h; mass spectrum (70 EV): *m/e* (relative intensity) 346 (11.4) M^+; 347 (6.4) $M^+ + 1$; 328 (11.4) M^+-H_2O; 315 (100) M^+-(CHO + 2H); 316 (30.4); 317 (22.4); 299 (24) M^+-(CHO + H_2O); 287 (6.2) M^+-(CHOHCHO); 269 (77.2) M^+-(CHOHCHO + H_2O).

*17,20β-dihydroxy-3-oxo-4-pregnen-21-al (I*c*) from 17-hydroxy-3,20-dioxo-4-pregnen-21-al.*

II*c* was prepared from 52.6 mg (0.153 mmol) of I*c* with 15 units of 20β-hydroxysteroid dehydrogenase and 2 additions at 30 min intervals of 0.077 mmol of NADH to the 5% methanol-0.1 M sodium phosphate buffer, pH 6.6, mixture. I*c* was completely soluble in the reaction mixture. Reaction was complete in 1.5 h. The product was extracted into ethyl acetate and precipitated with heptane. Recovery 44.4 mg (84% yield). *Anal.* calc'd for $C_{21}H_{30}O_4 \cdot 0.5\ H_2O$; C, 70.95%; H, 8.79%; found: C, 71.40; H, 9.05; n.m.r.: δ 0.95 (*s*, 3, 18-C$\underline{H}_3$) 1.21 (*s*, 3, 19-C$\underline{H}_3$); 4.36 (*s*, 1, 20-C$\underline{H}$); 5.72 (*s*, 1, 4-C$\underline{H}$) 9.74 (*s*, 1, 21-C$\underline{H}$O); t.l.c. (R_f values): System I: 11-deoxycortisol (S), 0.42; I*c*, 0.54; II*c*, 0.50; System II: S, 0.22; I*c*, 0.34; II*c*, 0.27; λ_{max} of azine derivatives (MBTH reaction): S, 340 nm; I*c*, 388 nm; II*c*, 313 nm; $(\alpha)_D^{25}$, + 88° (*c*, 1.0, ethanol); I.R. (KBr) cm^{-1}: 3450, 2725, 1730, 1667, 1613; $\lambda_{max}^{methanol}$ 242 nm: ϵ 13,050; m.p. 86°–101° with phase transition; isomerization to 11-deoxycortisol in pyridine was 90% in 5.5 h; mass spectrum (70 EV), *m/e* (relative intensity) 346 (15.2) M^+; 347 (3.8) $M^+ + 1$; 348 (1.9); 316 (15.8) M^+-(CHO + H); 288 (22.9) M^+-(CHOHCHO − H); 287 (100) M^+-(CHOHCHO), 286 (16.7) M^+-(CHOHCHO + H); 269 (16.3) M^+-(CHOHCHO + H_2O).

*11β,17,20β-trihydroxy-3-oxo-4-pregnen-21-al (II*d*) from 11β,17-dihydroxy-3,20-dioxo-4-pregnen-21-al (I*d*)*

II*d* was prepared by incubating 50.8 mg (0.141 mmol) of I*d* with 15 units of 20β-hydroxysteroid dehydrogenase and 2 sequential additions of 0.071 mmol of NADH at 1 h intervals in 5% methanol-0.1 M sodium phosphate, pH 6.6. The reaction was complete in 3.7 h. The extraction procedure was the same as described above except that dichloromethane was the solvent. Recovery, 44 mg (86%). *Anal.* calc'd for $C_{21}H_{30}O_5$: C, 69.58%; H, 8.61%; found: C 69.42%, H 8.64%; n.m.r.: δ 1.20 (*s*, 3, 18-C$\underline{H}_3$), 1.46 (*s*, 3, 19-C$\underline{H}_3$), 4.38 (*s*, 1, 20-C$\underline{H}$), 4.41 (*m*, 11-C$\underline{H}$OH), 5.65 (*s*, 1, 4-C$\underline{H}$), 9.72 (*s*, 1, 21-C$\underline{H}$O); t.l.c. (R_f values): system I: cortisol, 0.23; I*d*, 0.34; II*d*, 0.29; system II: cortisol 0.17; I*d*, 0.27; II*d*, 0.19; System III: cortisol 0.27; I*d*, 0.30, II*d*, 0.32. λ_{max} of azine derivatives (MBTH reaction): cortisol, 339 nm; I*d*, 338 nm; II*d*, 312 nm, $(\alpha)_D^{27.5}$, + 95.4° (*c*, 1.0, ethanol); I.R. (KBr) cm^{-1}: 3420, 2720, 1727, 1665, 1615; $\lambda_{max}^{methanol}$ 242 nm: ϵ 14,450; m.p., phase transition at 155°, melt at 176°–178°; isomerization to cortisol in

pyridine was 100% after 5.5 h. Mass spectrum (70 EV) *m/e* (relative intensity) 362 (47.8), M^+; 363 (13.9), $M^+ + 1$; 344 (31.3), M^+-H_2O; 332 (28.6), M^+-(CHO + H); 303 (47.7), M^+-(CHOHCHO); 302 (72.3), M^+-(CHOHCHO + H); 285 (76.7), M^+-(CHOHCHO + H_2O); 242 (56.7), $C_{17}H_{22}O^+$; 227 (67.7), $C_{16}H_{19}O^+$; 163 (100), M^+-199.

DISCUSSION

Corticosteroid homologues containing the hydroxyaldehyde side chain were first synthesized by von Euw and Reichstein[10, 11, 13] in response to the proposal by Kendall *et al.*[14] that these steroids were the physiologically essential products of adrenal cortex secretion. Reichstein and his coworkers showed that the corticosteroids secreted by the adrenal cortex were in fact 17-ketols, and Kendall's suggestion was consequently dismissed from further consideration. We have recently revived the possiblity that the 20-hydroxy-21-aldehydes are intermediates of corticosteroid metabolism. In order to explore the physiological role of this class of intermediate, we developed procedures for its synthesis. The enzymic procedure described in this paper is easy and rapid, and results in the formation of pure product in good yield. The chemical methods attempted by others [6, 11, 12, 15–18] have invariably proved troublesome and gave low yields. We have recently also developed a procedure for the efficient chemical synthesis of steroid hydroxyaldehydes [19].

The rapid reduction of the steroidal 20-keto-21-aldehydes by the S. hydrogenans 20β-hydroxysteroid dehydrogenase is noteworthy. This class of substrate is reduced by the enzyme faster than any other that we have tested, including 20-keto-21-oic acids, 20-keto-21-ol, 20-keto-21-methyl, 20-keto-21-methylamine, 20-keto-21-oxime (Monder, unpublished observations). Although the ketoaldehydes are consistently reduced faster than the corresponding corticosteroids, their rates of reduction varied over a wide range, presumably reflecting the effects of variations in the ring substituents. The remarkably rapid and efficient reduction of 21-dehydro corticosteroids by 20 hydroxysteroid dehydrogenase may reflect an important role for this reaction in corticosteroid metabolism.

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