ALKYLATION OF BRAIN CORTICOSTEROID ACETYLTRANSFERASE BY 17-HYDROXYPROGESTERONE 17-(9-OXO-10-CHLORODECANOATE) AND RELATED COMPOUNDS

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Received: 4/2/73

ABSTRACT

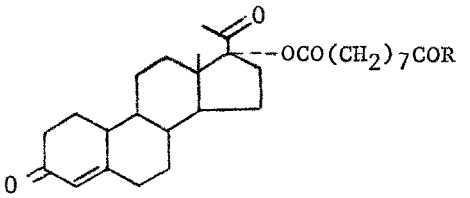
The addition of 17-hydroxyprogesterone 17-(9-oxo-10-chlorodecanoate) (1) at 1.5 μM to a partially purified preparation of corticosteroid acetyltransferase from the primate brain at pH 7.4, results in a 50% inhibition of enzymatic activity after 30 minutes at 37°. At this concentration the analogous 9-oxo-10-diazodecanoate, 8-carbomethyoxyoctanoate or 8-carboxyoctanoate esters show no effect on the activity of this enzyme. 9-Oxo-10-chlorodecanoate and its methyl ester are respectively 0.44 and 0.07-fold as effective inhibitors as 1. The inhibition by 1 has been shown to be non-competitive and irreversible. There is no reaction of 1 with amino acids, glutathione, or human or bovine serum mercaptalbumin in a pH 7.4 phosphate buffer at 37°, demonstrating the partial specificity of this alkylating agent.

INTRODUCTION

Solo and Gardner[1,2] have described the preparation and biological activity of a series of α-diazomethylketonic esters of 17-hydroxyprogesterone. They prepared these novel progestins in an effort to obtain receptor-directed alkylating agents for uterine tissue. Their design of these compounds was based upon principles delineated by Baker[3] and the knowledge that many 17-substituted progesterone derivatives are particularly active in the Clauberg assay.[4] Samples obtained through the courtesy of Dr. Solo were tested *in vitro* with a partially purified preparation of corticosteroid acetyltransferase (CoAc) from the primate brain.[5] They showed an increasing degree of irreversible inhibition of CoAc as the number

of methylene groups was increased in the acid moiety through compound 4.

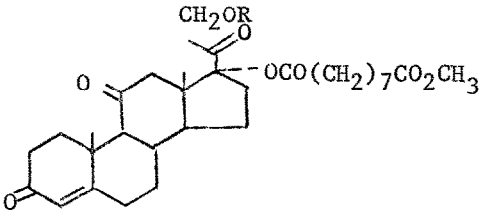
Examination of these compounds by thin-layer chromatography and infrared spectroscopy revealed that they had undergone partial decomposition since their original synthesis.[6]



CPD	
1	R = CH_2Cl
2	R = OCH_3
3	R = OH
4	R = CHN_2
5	R = $CH_2OSO_2\phi CH_3$
6	R = CH_2Br
7	R = CH_2OCOCH_3
8	R = CH_2OH

$R_1OCO(CH_2)_7COR_2$

CPD		
9	$R_1 = CH_3$,	$R_2 = CHN_2$
10	$R_1 = CH_3$,	$R_2 = CH_2Cl$
11	$R_1 = H$,	$R_2 = CH_2Cl$



12	R = $COCH_3$
13	R = H

When 4 was prepared via 2 and 3 and further purified to chromatographic homogeneity it had negligible activity as an inhibitor of CoAc. It was then observed that impure preparations of 4 contained a considerable amount of another product whose chromatographic mobility was less polar than 4. Its identity was subsequently established as compound 1. This chloromethyl ketone was found to be an effective irreversible inhibitor of CoAc activity. The analogous α-bromomethyl ketone, 6, was a less effective inhibitor than 1. The α-hydroxymethyl ketone substrate analog, 8, was not acetylated by CoAc in the presence of 1-^{14}C-acetyl-CoA to yield any detectable amount of ^{14}C-labeled 7. The x-ray crystallographic structures of 1 and 8 showed that their conformations were identical and that there were no significant molecular interactions.[7] The long side

chains of 1 and 8 are extended away from the steroid nucleus in the solid state. This suggested that the side chain of 1 was fitting into a hydrophobic crevice of CoAc normally occupied by the steroid nucleus of a substrate. In this report we further describe the preparation and properties of the non-steroidal chloromethyl ketones 10 and 11 and the substrate analog 13 which allowed us to test this concept.

MATERIALS AND METHODS

17-Hydroxyprogesterone 17-(8-carbomethoxyoctanoate)(2) and 17-hydroxyprogesterone 8-carboxyoctanoate (3) were prepared as described by Solo and Gardner.[2] Their m.p.'s, infrared, ultraviolet, and NMR spectra were identical with authentic samples received from Dr. Alan J. Solo.

17-Hydroxyprogesterone 17-(9-oxo-10-diazodecanoate)(4) was prepared from 3 which had been dried by azeotropic distillation with benzene. To a benzene solution (200 ml) of 3 (5 g) at 0° was added freshly distilled oxalyl chloride (23 ml) in dry benzene (60 ml), the mixture stirred at 0° for 45 min., warmed to room temperature for 30 min., and the benzene and excess oxalyl chloride removed in vacuo under a stream of nitrogen. The resulting residue was dried under high vacuum, dissolved in dry benzene (60 ml), and treated with a dry ethereal solution of diazomethane (8 g) at 0° with stirring for 30 min. The mixture was warmed to room temperature with stirring for 1 hr. and the excess diazomethane and solvents removed in vacuo under a stream of nitrogen. The crude product (6.5 g) was partially purified on a column of alumina (190 g, Woelm grade III) in benzene. Elution with benzene:ether (1:1) gave material (3.5 g) which was further purified by crystallization from ether-hexane (2.2 g, 43%), m.p. 73-75° (dec), reported[2] 60-62.5°. The infrared and NMR spectral parameters were identical with the published values.[2]

UV: λ_{max} 242 nm (ε = 24,200), 275 nm, shoulder (ε = 7,400)

17-Hydroxyprogesterone 17-(9-oxo-10-chlorodecanoate) (1) was synthesized from 4 (200 mg) via initial reaction with p-toluenesulfonic acid (200 mg) in benzene (6 ml) for 10 min at room temperature. After extraction of excess p-toluenesulfonic acid with aqueous $NaHCO_3$, the crude tosylate (5) was refluxed in acetone with tetramethylammonium chloride for 2 hours. The crude product (184 mg, 91%) was crystallized from ethanol, m.p.95-98°.

IR: ν_{max} 1720, 1670, 1655 (shoulder) and 1615 cm^{-1}.
UV: λ_{max} 241 nm (ε = 16,700).
NMR: δ 0.68 (S., 18-Me), 1.19 (S., 19-Me), 2.03 (S., 21-Me), 4.05 (S., 10-CH_2Cl), 5.75 (S., 4-H).

Anal. Calcd. for $C_{31}H_{45}ClO_5$: C, 69.84, H, 8.50; Cl, 6.64

Found: C, 69.28; H, 8.55; Cl, 6.55

17-Hydroxyprogesterone 17-(9-oxo-10-bromodecanoate)(6) was prepared from 4 (130 mg) via the tosylate (5), and subsequent refluxing with KBr in acetone. The crude product was crystallized from ethanol (92 mg, 65%), m.p. 87-91°.

IR: ν_{max} 1730, 1675, 1655 (shoulder) and 1615 cm^{-1}.
UV: λ_{max} 240 nm (ε = 15,200)
NMR: δ 0.69 (S., 18-Me), 1.21 (S., 19-Me), 2.04 (S., 21-Me), 3.88 (S., 10-CH_2Br), 5.77 (S., 4-H).

Anal. Calcd. for $C_{31}H_{45}BrO_5$: C, 64.46; H, 7.85; Br, 13.83

Found: C, 64.74; H, 8.05; Br, 12.68.

17-Hydroxyprogesterone 17-(9-oxo-10-acetoxydecanoate)(7) was prepared by heating 4 (250 mg) in glacial acetic acid at 90-95° for 30 min. The acetic acid was then removed under high vacuum and the residue crystallized from methanol-water (140 mg, 53%), m.p. 82-83°.

IR: ν_{max} 1740, 1720, 1665 and 1610 cm^{-1}.
UV: λ_{max} 240 nm (ε = 15,700)
NMR: δ 0.69 (S., 18-Me), 1.20 (S., 19-Me), 2.03 (S., 21-Me), 2.16 (S., 10-CO_2Me), 4.63 (S., 10-CH_2), 5.76 (S., 4-H).

Anal. Calcd. for $C_{33}H_{48}O_7$: C, 71.19; H, 8.69

Found: C, 71.14; H, 8.68.

17-Hydroxyprogesterone 17-(9-oxo-10-hydroxydecanoate)(8) was obtained by hydrolysis of 7 (106 mg) in a solution of potassium bicarbonate (106 mg) in aqueous 75% methanol for 3 hrs. at room temperature. The reaction mixture was then diluted with water, and the crude product (95 mg) obtained by extraction with ethyl acetate. This was purified by chromatography on silica gel (7.8 g) with elution by benzene:ether (1:1) to give 8 (47 mg, 48%) which was recrystallized from ether, m.p. 122-123°.

IR: ν_{max} 3430, 1710, 1665, 1610 cm^{-1}.
UV: λ_{max} 240 nm (ε = 15,500)
NMR: δ 0.69 (S., 18-Me), 1.21 (S., 19-Me), 2.04 (S., 21-Me), 4.23 (S., 10-CH_2), 5.76 (S., 4-H).

Anal. Calcd. for $C_{31}H_{46}O_6$: C, 72.34; H, 9.01

Found: C, 72.10; H, 9.18.

Methyl 9-oxo-10-diazodecanoate (9) was prepared from monomethylazelate (1 g) as described for 4. The crude product (950 mg, 85%) was obtained as a yellow oil which crystallized upon standing at 4°, m.p. 7.5-10.5°.

IR: ν_{max} (CCl_4) 2130, 1740, and 1655 cm^{-1}.

UV: λ_{max} 245 nm (ε = 15,000), 270 nm (ε = 13,000)
NMR: δ 2.33 (M., 2-CH_2, 8-CH_2), 3.67 (S., 1-CO_2Me), 5.27 (S., 10-CHN_2)

Anal. Calcd. for $C_{11}H_{18}N_2O_3$: C, 58.39; H, 8.02; N, 12.38

Found: C, 58.36; H, 8.09; N, 12.04.

Methyl 9-oxo-10-chlorodecanoate (10) was obtained from 9 (512 mg) as described for the preparation of 1. The crude product was crystallized from ethanol (384 mg, 72%) m.p. 50-52°, reported[6] 50.5-51°.

IR: ν_{max} 1730 cm^{-1}.
NMR: δ 2.48 (M., 2-CH_2, 8-CH_2), 3.67 (S., 1-CO_2Me), 4.06 (S., 10-CH_2).

9-Oxo-10-chlorodecanoic acid (11). Hydrolysis of 10 (100 mg) was effected with 1.2 N HCl in aqueous 50% tetrahydrofuran (16 ml). After remaining overnight at room temperature the solvents were removed in vacuo and the solid residue crystallized from acetone-hexane (72 mg, 77%), m.p. 73-74°.

IR: ν_{max} 2940, 2860, 1730, and 1695 cm^{-1}.
NMR: δ 2.48 (M., 2-CH_2, 8-CH_2), 4.06 (S., 10-CH_2), 10.68 (S., 1-CO_2H).

Anal. Calcd. for $C_{10}H_{17}ClO_3$: C, 54.42; H, 7.76; Cl, 16.07.

Found: C, 54.36; H, 7.75; Cl, 15.92.

21-Acetoxy-17-hydroxypregn-4-ene-3,11,20-trione 17-(8-carbomethoxyoctanoate) (12) was prepared by a modification of the procedure used to obtain 2. A mixture of 12 g of azelaic acid monomethyl ester and 9.9 ml of trifluoroacetic anhydride in 130 ml of dry benzene was refluxed for 15 min. After cooling, 6.0 g of cortisone 21-acetate and 3.0 g of sodium carbonate were added and reflux was continued for 48 hr. The solvent was removed in vacuo and the residue in 200 ml ether was washed 5 times with 200 ml portions of 0.05 M sodium phosphate buffer, pH 7.9. The resulting ether phase was washed with water and dried, yielding 10 g of a yellow oil. This oil was purified on a column of silica gel (350 g) in ethyl acetate:benzene (1:20). Elution with ethylacetate:benzene (1:7) gave material (2.6 g) which was crystallized from acetone-ether (1.1 g, 13%), m.p. 102-104°.

IR: ν_{max} 1745 (shoulder), 1730, 1700, 1670, and 1610 cm^{-1}
UV: λ_{max} 238 nM (ε = 15,900).
NMR: δ 0.74 (S., 18-Me), 1.41 (S., 19-Me) 2.16 (S., 21-OCOMe), 3.66 (S., 8-CO_2Me), 4.74 (S., 21-CH_2), 5.73 (S., 4-H).

17,21-Dihydroxypregn-4-ene-3,11,20-trione 17-(8-carbomethoxyoctanoate) (13). Hydrolysis of 12 (155 mg) in methanol (3 ml) was effected with 6 N HCl (0.45 ml) at room temperature for 22 hr. The reaction mixture was reduced in volume, diluted with water and the crude material (115 mg) obtained by extraction with ether was treated with a dry ethereal solution of diazomethane at 0° for 30 min. After removal of excess diazomethane the residue was chromatographed on silica gel in ether:benzene

(1:20). The desired product was eluted with ether:benzene (1:5) and crystallized from ether-pentane (18 mg, 13%), m.p. 141-142°.

IR: ν_{max} 1730, 1710 (shoulder), 1668, and 1618 cm^{-1}
UV: λ_{max} 238 nM (ε = 15,500).
NMR: δ 0.65 (S., 18-Me), 1.41 (S., 19-Me), 3.65 (S., 8-CO_2Me), 4.27 (S., 21-CH_2), 5.73 (S., 4-H).

Anal: Calcd. for $C_{31}H_{44}O_8$: C, 68.36; H, 8.14.

Found: C, 68.18; H, 8.03

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were determined in a potassium bromide disc, unless otherwise noted, using a Perkin Elmer Model 257 grating infrared spectrophotometer. Ultraviolet spectra were recorded in methanol using a Cary Model 11MS Spectrophotometer. NMR spectra were obtained with a Varian A-60A spectrometer in deuterochloroform at 37° and are reported in ppm from the internal standard of TMS. The microanalyses were carried out by Micro-Tech Laboratories, Skokie, Ill. and Galbraith Laboratories, Inc., Knoxville, Tenn.

The preparation and assay of CoAc from the primate brain have been described previously[5]. Solutions of compounds described in this report were prepared in dimethylsulfoxide and assayed in a 3.3% final concentration of this solvent. Analyses of amino acids and glutathione were performed with a Beckman Model 121 amino acid analyzer. Human and bovine serum mercaptalbumin were prepared by the procedure of Dintzis.[8] Benzene extracts of incubations carried out with compound 13 were purified by thin-layer chromatography on silica gel in choloroform: methanol (25:1) where compound 12 has an R_f of 0.6.

RESULTS AND DISCUSSION

Column chromatography and recrystallization were required for the preparation of the diazomethyl ketone 4 free from detectable impurities as judged by thin layer chromatography and NMR spectroscopy. The major impurity present in initial preparations[6] of 4 was the chloromethyl ketone 1. The latter compound was easily obtained from 4 via the tosylate, analogous to preparations of steroid 21-halomethyl ketones[9]. When compounds 1 and 4 were pre-incubated with CoAc at 10μM for 1 hour at 37°, followed by 1 hour assays after the addition of

50μM cortisol and 20μM 1-^{14}C-acetyl CoA, the recovery of CoAc activity was less than 5% and greater than 90% respectively of control incubations. The loss of CoAc activity as a function of the time of preincubation with a 10μM concentration of 1 is shown in Figure 1.

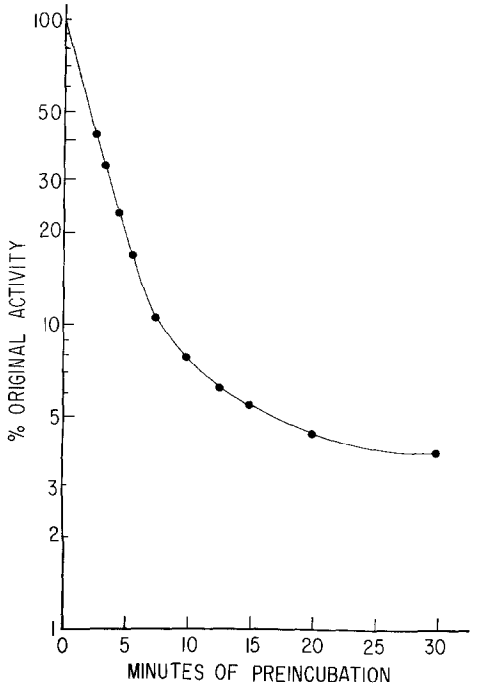


Figure 1. Loss of CoAc activity as a function of the period of preincubation with compound 1 (10μM) at 37° prior to assay with cortisol and 1-^{14}C-acetyl CoA as substrates.

There is pseudo first-order kinetics for the loss of the initial 90% of CoAc activity, followed by a slower decrease which is essentially complete at 30 minutes. We have therefore compared the effectiveness of inhibitors of CoAc by preincubation at 37° for 30 minutes, followed by 1 hour assays in the presence of 50μM cortisol. The results obtained with compounds 1, 2, 4, 10 and 11 as a function of the log of their concentration are shown in Figure 2.

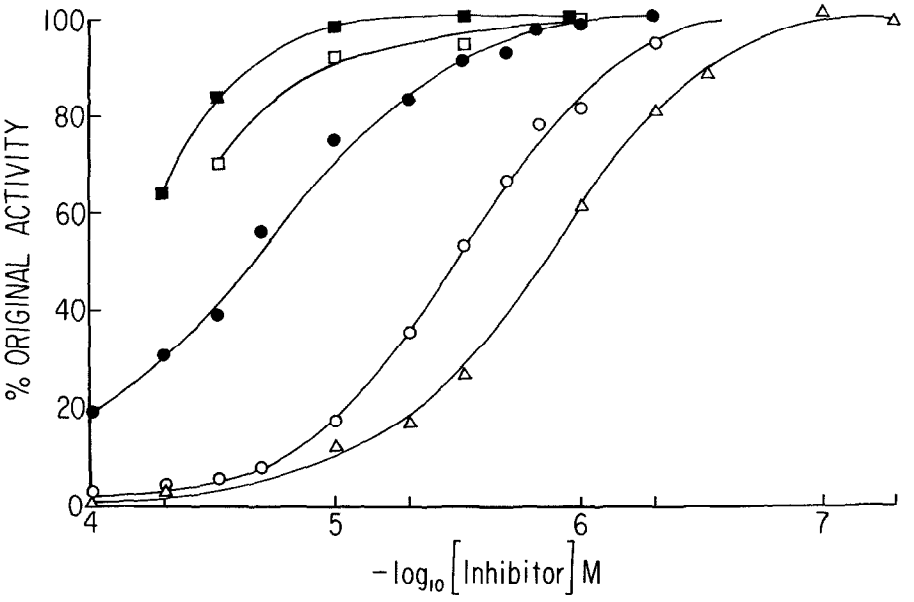


Figure 2. Loss of CoAc Activity as a function of $\log_{10}$ concentration of inhibitors preincubated with CoAc for 30 min at 37° followed by assay with cortisol and 1-^{14}C-acetyl CoA as substrates:
■—■ Compound 2, □—□ Compound 4,
●—● Compound 10, ○—○ Compound 11,
△—△ Compound 1.

The diazomethyl ketones 4 and 9 are poor inhibitors and were not examined further. It was anticipated that the steroidal chloromethyl ketone 1 would be considerably more effective than the straight chain alkylchloromethyl ketones 10 and 11. Comparing the concentrations required for 50% inhibition of CoAc activity, compounds 10 and 11 are respectively 0.07 and 0.44-fold as effective as compound 1. Based upon this one comparison between a simple alkyl ester and its carboxylic acid, we tentatively conclude that the carboxylate group of 11 is bound to the region of the enzyme surface which interacts with the partially charged C-20 carbonyl group of 1. The steroidal bromomethyl ketone 6, was also compared to 1 for its ability to inhibit CoAc activity. The effect of 6 (not shown in Figure 2) paralleled the curve for 1, with 50% inhibition of CoAc activity for 6 at 15μM as compared to 1.5μM for compound 1.

Since 1 was found to be the most effective inhibitor of CoAc in this series, further efforts were directed to determining the nature and specificity of its action. A kinetic analysis of the effect of 1 is compatible with non-competitive inhibition, as shown in Figure 3. To determine if the

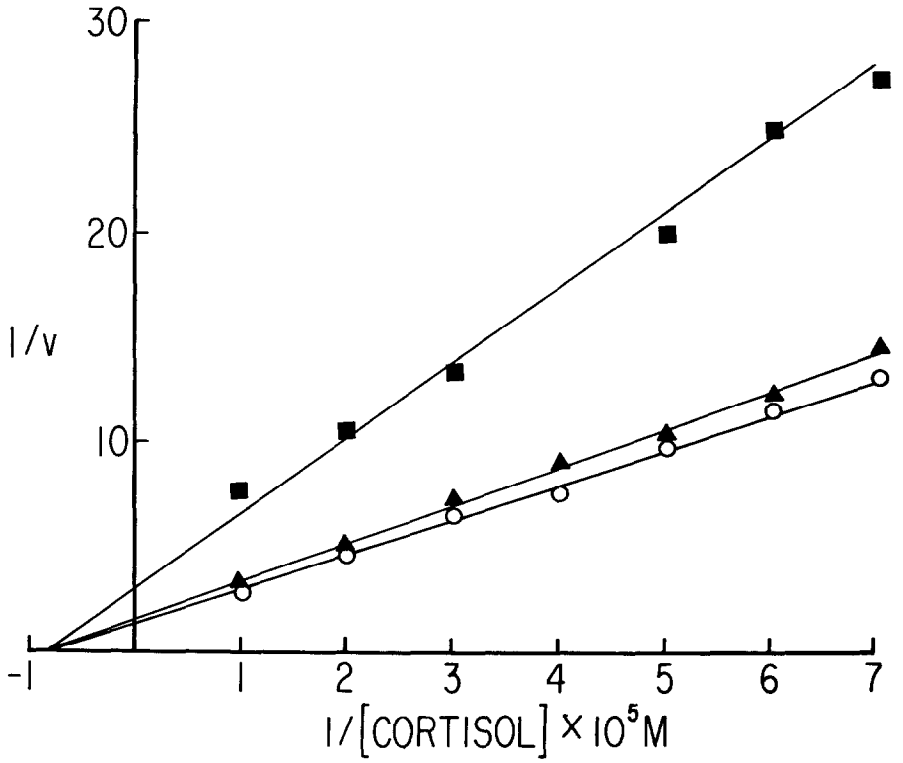


Figure 3. Lineweaver-Burk Plot for the inhibition of CoAc activity in the presence of Compound 1; O—O 0 μM, ▲—▲ 0.3μM, ■—■ 0.7μM.

inhibition of CoAc by 1 is irreversible, a solution of CoAc was prepared with 10μM 1 which showed 95% loss of activity. Extensive dialysis of this solution against large volumes of 0.01M tris buffer, pH 7.4 containing 0.15M NaCl and 0.5 mM EDTA at 4° failed to restore activity under conditions where CoAc is stable. Attempts to restore CoAc activity by passage through G-25 Sephadex columns or by millipore filtration in the presence of additional

buffer also failed. The above techniques are suitable for removing certain competitive inhibitors of CoAc[5]. It was concluded that 1 results in alkylation of CoAc and efforts are in progress to determine the nature of the amino acid residue(s) so involved.

The specificity of the alkylation of CoAc by 1 was studied by determining if it could react with amino acids and glutathione at pH 7.4 and 37°. Solutions in 10% dimethylsulfoxide were incubated for 1 hour at 50μM concentration of 1 and the other components. After acidification, amino acid analysis showed no reduction in the amounts of any of the normal amino acids or glutathione within an experimental error of 5%.

Incubations of 50μM 1 in the pH 7.4 buffer were also carried out with 100mg amounts of bovine and human serum mercaptalbumin. After remaining at 37° for 1 hour the solutions were extracted with benzene[5], the extracts dried in vacuo and the amount 1 in the extracts was measured by ultraviolet spectroscopy. In the case of the mercaptalbumin preparations, approximately 90% of 1 was recovered by such extraction, whereas less than 5% of 1 could be recovered from the incubation with CoAc. This result is also consistent with an alkylation of CoAc by 1.

Studies of the substrate specificity of CoAc have shown that only the 21-hydroxyl group in naturally occurring C-20 ketosteroids are acetylated[10]. Compound 8, the α-Ketol analog of 1 was prepared to determine if the primary alcohol group of 8 is also a substrate of CoAc. No detectable formation of the radioactive acetate, 7, of compound 8 was found when 2μCi of 1-^{14}C-acetyl CoA was employed in the incubation. The X-ray crystallographic structures of 1 and 8 are isomorphous with their ester side chains extended away from the steroid nucleus[7]. This suggests that the side chain of 1 may be bound to a hydrophobic crevice of CoAc which is normally occupied

by the steroid nucleus of a substrate. In the case of compound 8 the binding of this ester side chain with CoAc presumably does not permit stabilization of the structurally similar rate-controlling transition state of a 21-hydroxy steroid. A necessary consequence of this hypothesis would be that a 21-hydroxysteroid substrate also possessing a long ester side chain at C-17 would not be acetylated at C-21. We therefore prepared the cortisone derivative, compound 13. Incubation of 13 with CoAc and 1-^{14}C-acetyl CoA, followed by extraction and chromatography failed to demonstrate any radioactivity as the acetate, 12. This result supports the concept that long 17-ester side chains of C-21 steroids are bound to CoAc in a manner in which the side chain mimics the hydrophobic nature of the steroid nucleus.

ACKNOWLEDGMENTS

This work was supported by Grant NS 09915 from the National Institute of Neurological Diseases and Stroke and also in part by General Research Support Grant FR-05519 from the General Research Support Branch, Division of Research Facilities and Resources, National Institutes of Health. The authors are indebted to Connie F. Murguia, Chris M. Lancaster, Melvin C. Seffel and Bernard Haegelin for their excellent technical assistance. The authors are also grateful to Dr. Alan J. Solo for providing samples of compounds 2, 3 and 4 and to Dr. Alan H. Nathan of the Upjohn Company for supplying the 17-hydroxyprogesterone and cortisone acetate used in this work.

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