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18,19-DIHYDROXYDEOXYCORTICOSTERONE; A NOVEL PRODUCT OF CYTOCHROME P-450$_{11\beta}$-CATALYZED REACTION

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After incubating 18-hydroxydeoxycorticosterone (18-OH-DOC) with cytochrome P-450$_{11\beta}$ in the reconstituted system, the products were analyzed with HPLC. There appeared two product-peaks on the chromatogram, one of which was identified as a peak of 18-hydroxycorticosterone (18-OH-B), an expected product of the 11β-hydroxylation. Another peak did not coincide with those of any known corticoids. This unidentified product was further purified, and the purified material was analyzed by gas chromatography-mass spectrometry (GC/MS). The mass spectrum showed that the unidentified product is one of the structural isomers of 18-OH-B. A further analysis with ^{1}H-NMR spectrometry indicated that a proton resonance peak of 19-CH_3 in 18-OH-DOC disappeared in the product and the methyl group of the substrate seemed to be converted to -CH_2OH. These results suggested that the unidentified product generated from 18-OH-DOC by P-450$_{11\beta}$-linked hydroxylase system may be 18,19-dihydroxydeoxycorticosterone (18,19,21-trihydroxypregn-4-ene-3,20-dione; 18,19-diOH-DOC), a hitherto unreported corticoid.

19-Hydroxydeoxycorticosterone (19-OH-DOC) and the related corticoids have recently received attention of endocrinologists because they are often found in the urinary excretion of hypertensive rats (1, 2). 18-OH-DOC has also been known to be one of the naturally occurring mineralocorticoids and can produce hypertension in rats (3, 4).

During the kinetic investigation of the pathway from deoxycorticosterone through 18-OH-B using bovine adrenocortical mitochondria, we have found a hitherto unidentified metabolite formed from 18-OH-DOC. We have also shown that this compound is a reaction product of the purified P-450$_{11\beta}$-linked hydroxylase system (a manuscript in preparation). An attempt has been made to elucidate the structure of this compound. In this paper we report that the structure of the compound may be 18,19-diOH-DOC, a novel corticoid whose structure is extremely interesting in the above-mentioned context.

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MATERIALS AND METHODS

Chemicals Most steroids were purchased from Makor Chemicals or Sigma. 16α,18-Dihydroxydeoxycorticosterone (16α,18-diOH-DOC) was a gift from Dr. D. N. Kirk of Westfield College, London. All other chemicals were obtained at the highest purity available from commercial sources.

Instrumentation For HPLC, a LDC liquid chromatograph system equipped with a 300 x 4 mm TSK-Gel LS 310 column was used. The mobile phase was dichloromethane:ethanol:water = 96:3.6:0.4 (v/v/v) with a flow rate of 1.2 ml/min. For the reversed phase HPLC, a Chemcosorb.ODS.H. column was used. The mobile phase was ethanol:water = 40:60 (v/v) with a flow rate of 0.4 ml/min.

^{1}H-NMR spectrum was obtained at 200 MHz with a Varian XL-200 spectrometer. Chemical shifts (δ) in $CDCl_3$ were given in ppm by making the residual $CHCl_3$ in the solvent as the internal standard (δ=7.27), and the chemical shifts in D_2O were determined downfield from the external TSP (sodium 3-trimethylsilylpropionate-2,2,3,3,-d_4).

For GC/MS, a JEOL JMS D300 Mass Spectrometer with a 2 m OV-1 column was used. The temperature was increased from 260 °C to 280 °C at 2 °C/min.

Enzymes $P\text{-}450_{11\beta}$, adrenodoxin, and adrenodoxin reductase were purified from bovine adrenocortical mitochondria as described elsewhere (5-7).

Preparation of the unidentified compound $P\text{-}450_{11\beta}$ (20 nmol) was incubated with 18-OH-DOC (1 μmol) for 1 h at 37 °C in 10 ml Tris-HCl (10 mM, pH 7.4) containing NADPH (1 μmol), glucose-6-phosphate (100 μmol), glucose-6-phosphate dehydrogenase (5 units), $MgCl_2$ (30 μmol), adrenodoxin (100 nmol), and adrenodoxin reductase (15 nmol). The reaction was terminated by addition of 10 ml ethanol. The reaction mixture was extracted three times with 30 ml dichloromethane. The solvent was evaporated under N_2. The dried extract was then injected into the chromatograph to purify the unidentified compound.

RESULTS

18-OH-DOC was incubated with the reconstituted system of $P\text{-}450_{11\beta}$ under aerobic conditions, and the products were analyzed with HPLC. There appeared two product-peaks on the chromatogram. On the basis of the comparative studies conducted on the authentic sample, the chemical nature of the first peak (appeared at 20 min) was identified as 18-OH-B, an expected product of the 11β-hydroxylation. The retention time of the second peak, 27 min, did not coincide with those of any other heretofore-reported corticoids. Because the amount of this unidentified product, as estimated from its UV absorbance, was not negligible by comparison with the amount of 18-OH-B produced, and because the polarity of the compound, as estimated from its retention time, suggested that its chemical property was of intermediate nature between those of 18-OH-B and 16α,18-diOH-DOC (8), the elucidation of chemical structure of the second peak-substance has become the subject of our study.

Fractions of the second peak were collected and concentrated under N_2. The concentrate was purified by the two successive HPLC on the same column. The

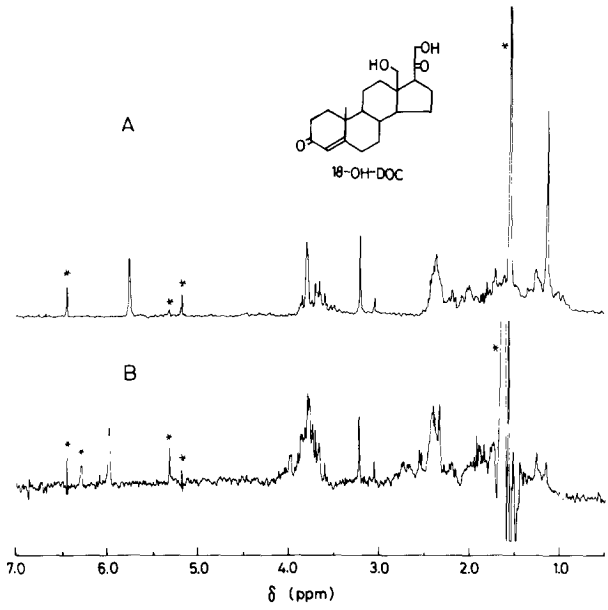

Figure 1. A. ^{1}H-NMR spectrum of 18-OH-DOC in $CDCl_3$. B. A difference ^{1}H-NMR spectrum between those of the unidentified compound and the HPLC column eluate from the background area. The solvent is $CDCl_3$. The peaks marked with asterisks are derived from the solvent.

eluate from the third HPLC was further purified by the reversed phase HPLC. A peak of the unidentified compound appeared from the column at 25 min, and its fractions were collected. After evaporating the solvent, the residue was converted to the methyloxime- and trimethylsilyl- (MO-TMS-) derivative and analyzed by GC/MS. The mass spectrum showed a prominent M^++1 ion peak (m/e,637), which is consistent with that of the MO-TMS-derivative of trihydroxypregnenedione. Although mass numbers of several peaks of fragment ions (M^+-31, M^+-90 and M^+-103) were the same as those of the MO-TMS-derivative of 18-OH-B, the relative peak intensity of those peaks was quite different from that of 18-OH-B, suggesting that the unidentified compound may be one of the structural isomers of 18-OH-B.

The next approach we used was ^{1}H-NMR spectroscopy. Fig. 1A shows the ^{1}H-NMR spectrum of 18-OH-DOC in $CDCl_3$. Several resonance peaks in the spectrum have been assigned by Genard et al. (9). On the basis of their assignment, the singlet peaks at δ=5.75 and 1.15 were identified as those arisen from the 4-H and 19-CH_3 group, respectively. The peaks around δ=3.80 and 3.66

were also assigned as those of the $-CH_2-$ groups at 18- and 21-positions. There appeared a prominent resonance peak at δ=1.56, which was attributed to a contaminant in the solvent.

^{1}H-NMR spectra of other steroids, corticosterone (B), 18-OH-B, androst-4-ene-3,17-dione (androstenedione) and 19-hydroxyandrost-4-ene-3,17-dione (19-OH-androstenedione), were also measured (data not shown). In the spectra of B, 18-OH-B and androstenedione, the peaks of 19-CH_3 were observed at δ=1.45, 1.43 and 1.23, respectively. The corresponding peak was missing in 19-OH-androstenedione.

Fig. 1B shows the difference ^{1}H-NMR spectrum between those of the unidentified compound and the HPLC eluate in the background region. As seen in the spectrum, the resonance peak of 19-CH_3, found at δ=1.15 in 18-OH-DOC, was missing. Unfortunately, the large peak at δ=1.59 was not perfectly canceled in the difference spectrum. In order to test a possibility that the resonance peak of 19-CH_3 in the unidentified compound was hidden in the large resonance at δ=1.59, the ^{1}H-NMR spectrum was measured in D_2O. There appeared no resonance peak to be ascribed to 19-CH_3 in this region, whereas in the spectra of 18-OH-DOC and B there appeared the resonance peaks of 19-CH_3 at δ=1.17 and 1.40, respectively. These observations suggest that the 19-CH_3 group in 18-OH-DOC was converted to another group in the unidentified compound.

As shown in Fig. 1B, resonance peaks around δ=3.77 increased their intensity compared to those of 18-OH-DOC. In the spectra of 18-OH-DOC and other steroids, the resonance peaks in this region have been identified as those originated from the $-CH_2OH$ group. Therefore, these findings strongly suggest that 19-CH_3 group in 18-OH-DOC was probably converted to 19-CH_2OH in the unidentified product.

A 4-H resonance, which was observed at δ=5.75 in 18-OH-DOC, was found at δ=5.98 in the unidentified compound. It is interesting to note that a quite similar shift of 4-H peak to lower magnetic field seems to take place generally in the NMR spectra of 19-CH_2OH-steroids compared to those of 19-CH_3-steroids, because 4-H resonance of androstenedione was observed at δ=5.76

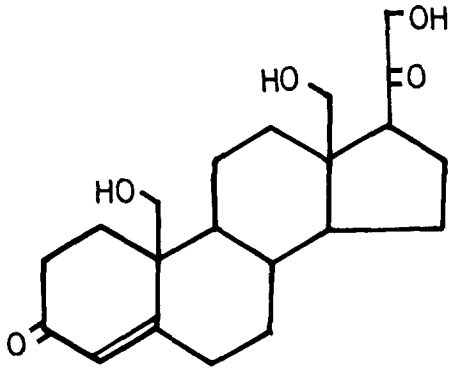

Figure 2. Proposed structure of the unidentified compound, 18,19-diOH-DOC.

whereas that of 19-OH-androstenedione was found at δ=5.98. This finding in the chemical shift of 4-H resonance again supports the previous implication that the unidentified product may be 18,19-diOH-DOC.

DISCUSSION

The GC/MS analyses as well as the ^{1}H-NMR studies conducted on the unidentified product of P-450$_{11\beta}$-catalyzed reaction strongly suggest that the chemical nature of the compound may be 18,19-diOH-DOC (Fig. 2). To our knowledge, this is the first report showing the production of 18,19-diOH-DOC by P-450$_{11\beta}$ system, although Sato et al. (10) have reported that the cytochrome can catalyze the 19-hydroxylation of androstenedione. Further details of the kinetics of this novel reaction pathway are now in preparation for publication.

19-OH-DOC and a further metabolite, 19-nor-deoxycorticosterone, have been reported as metabolites of rat adrenals (1), and Dale et al. have discussed the relationship between the urinary excretion of 19-nor-DOC and the onset of hypertension in spontaneously hypertensive rats (2). On the other hand, 18-OH-DOC has been reported to produce hypertension in rats and has been implicated in some forms of human hypertension (4,11). From these considerations, to investigate the biological properties of 18,19-diOH-DOC should be of extreme interest, and that prospect is now under investigation in our laboratory.

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