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HYDROXYLATION OF 19-NORANDROSTENEDIONE BY ADRENAL CORTEX MITOCHONDRIAL *P*-450$_{11\beta}$

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Summary—The activity of purified bovine adrenocortical *P*-450$_{11\beta}$ on the C_{18}-steroid, 4-estrene-3,17-dione (19-norandrostenedione), is described. The major steroid products were separated by HPLC and identified by GC–MS, and ^{1}H- and ^{13}C-NMR as 11β-, 18- and 6β-hydroxylated derivatives of 19-norandrostenedione. The turnover numbers of the 11β-, 18- and 6β-hydroxylase reactions were 45, 7.5 and 1.9 (mol/min/mol of *P*-450$_{11\beta}$), respectively, with a common K_m of 44 μM. All of these activities required the presence of the electron donating system consisting of NADPH, adrenal ferredoxin (adrenodoxin) and its reductase. These findings provide additional insights into the versatile catalytic roles of *P*-450$_{11\beta}$ in the adrenal cortex, in which it may act on C_{18}-19-nor-steroids in addition to its known activities on C_{21}- and C_{19}-steroids.

INTRODUCTION

P-450$_{11\beta}$ is a heme-thiolate monooxygenase (EC 1.14.15.4) which has been made available in homogeneous form from adrenal cortex mitochondria [1–3]. Upon reconstitution with the adrenal electron donating system consisting of NADPH, adrenodoxin and adrenodoxin reductase, it catalyzes an interesting variety of monooxygenase reactions at the 11β-, 18- and 19-positions of C_{21}- and C_{19}-steroids. Thus, C_{21}-steroids are converted to important hydroxylated derivatives, glucocorticoids, corticosterone and cortisol, and mineralocorticoid aldosterone [2, 4, 5]. We have found that C_{19}-steroids are also efficient substrates of *P*-450$_{11\beta}$ which are converted to 11β- and 19-hydroxylated derivatives [4]. It is also shown that one of the products, 19-hydroxyandrostenedione, which itself is known to be an aldosterone amplifier [6], is further converted to the C_{18}-steroid estrone, estrogen, and 19-norandrostenedione [7–9], another mineralocorticoid amplifier [10]. In a preliminary experiment, we have also found that 19-norandrostenedione could further be converted to an unidentified compound(s) which yields a peak(s) at a shorter retention time by reverse phase HPLC [9]. The purpose of this study was to identify these products in an effort to understand the molecular basis of the catalytic roles played by *P*-450$_{11\beta}$ on steroid hormone metabolism in the adrenal cortex.

EXPERIMENTAL

The following were purchased: estradiol, 19-norandrostenedione and 19-nortestosterone (Sigma); 4-estrene-3β,17β-diol, 6β-hydroxy-19-norandrostenedione and 19-norepitestosterone (Steraloids); and estrone (Merck). The solvents used were HPLC grade and all other chemicals were of the highest available grades. *P*-450$_{11\beta}$, crystalline adrenodoxin and purified NADPH:adrenodoxin reductase were preparations from bovine adrenal cortex mitochondria [3, 11, 12]. *P*-450$_{SCC}$ [3] and *P*-450$_{C21}$ [13] were prepared from bovine adrenal cortex. *P*-450$_{17\alpha}$ was prepared from porcine testis microsomes [14], and NADPH:*P*-450 reductase, from porcine liver microsomes [14]. Their assay systems were reconstituted according to the respective published methods and incubation was carried out for 15 min at 37°C [3, 13, 14].

For the measurement of *P*-450$_{11\beta}$-dependent conversion of 19-norandrostenedione, the reaction mixture contained 100 nmol of NADPH, 0.2 unit of NADPH:adrenodoxin reductase, 1.2 nmol of

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Abbreviations: *P*-450$_{SCC}$ and *P*-450$_{11\beta}$ (mitochondrial forms of *P*-450 which catalyze the cleaving of the side chain of cholesterol between the 20- and 22-positions, and the hydroxylation of C_{21}- and C_{19}-steroids at the 11β-, 18- and 19-positions, respectively); *P*-450$_{C21}$ and *P*-450$_{17\alpha}$ (endoplasmic forms of steroidogenic *P*-450 which catalyze the hydroxylation of C_{21}-steroids at the 21-position, and the hydroxylation of C_{21}-steroids at the 17α-position cleaving the side chain of the 17α-hydroxylated products between the 17- and 20-positions, respectively); 19-norandrostenedione (4-estrene-3,17-dione); TMS (tetramethylsilane); HPLC (high-performance liquid chromatography); GC–MS (gas chromatography–mass spectrometry); NMR (nuclear magnetic resonance); and δ (chemical shift relative to internal TMS, 0.00, with ^{1}H-NMR and to chloroform, 77.0, with ^{13}C-NMR).

adrenodoxin, 50 pmol of P-450$_{11\beta}$, and 50 nmol of 19-norandrostenedione in 0.3 ml of 30 mM phosphate buffer, pH 6.5. After a 2-min preincubation at 37°C, the reaction was started with the addition of P-450$_{11\beta}$ and the medium was incubated for 3 min at 37°C. Reactions were terminated by the addition of 1.6 ml of chloroform:methanol (1:1, v/v) and 0.8 ml of water was added. The mixture was separated after 10 nmol of 11β-hydroxyandrostenedione was added as an internal standard, and the organic phase was collected and dried by flushing with N_2 gas at room temperature. The sample was then dissolved in 100 μl of methanol and an aliquot (10 μl) was injected into an HPLC column (MCI GEL, ODS-IHU, 4.6 × 250 mm, Mitsubishi Chemical Industries Ltd). Elution of the products from the column was performed with methanol:water (52:48, v/v) at a flow rate of 0.6 ml/min at 27°C, and absorption was monitored at 240 nm.

In order to obtain a sufficient quantity of the products for GC–MS and NMR analyses, reaction mixtures from hundreds of the same experiments were pooled and the product was subjected to TLC on silica gel 60 F-254 plates (Merck) with a solvent system of benzene:acetone (3:1, v/v) at room temperature. The product bands were excised from the plates and final purification of the product steroids was done by HPLC (Chemcosorb 5-ODS-H column, 2.1 × 150 mm) with acetonitrile:water (36:64, v/v) at a flow rate of 0.2 ml/min at 35°C.

GC–MS was carried out as described previously for 19-norandrostenedione [9]. High resonance GC–MS was performed with perfluorokerosene as a standard. ^{1}H- and ^{13}C-NMR spectra were obtained at 400 MHz with a JEOL JNM-GX400 Spectrometer using a standard 5-mm probe. For ^{13}C-NMR analysis, ^{1}H complete decoupling spectra were measured. Chemical shifts were determined in ppm in $CDCl_3$. As internal standards, TMS was added for ^{1}H-NMR, and $CDCl_3$ in the solvent as employed for ^{13}C-NMR (TMS at ^{1}H-NMR; $\delta = 0.00$ and $CDCl_3$ at ^{13}C-NMR; $\delta = 77.0$).

RESULTS

Formation and separation of new products from 19-norandrostenedione

Purified and reconstituted P-450$_{11\beta}$ monooxygenase system from bovine adrenal cortex produced three metabolites from 19-norandrostenedione (referred to as A-1, A-2 and A-3) which were separated by reverse-phase HPLC (Fig. 1). The retention times were 17.6, 16.7 and 15.6 min for A-1, A-2 and A-3, respectively. The reactions were absolutely dependent on each component of the mitochondrial electron donating system (NADPH, NADPH:adrenodoxin reductase and adrenodoxin) which have been shown to be essential for all other P-450$_{11\beta}$-mediated monooxygenase reactions of various C_{21}- and C_{19}-steroids at the 11β-, 18- and 19-positions [4, 7–9].

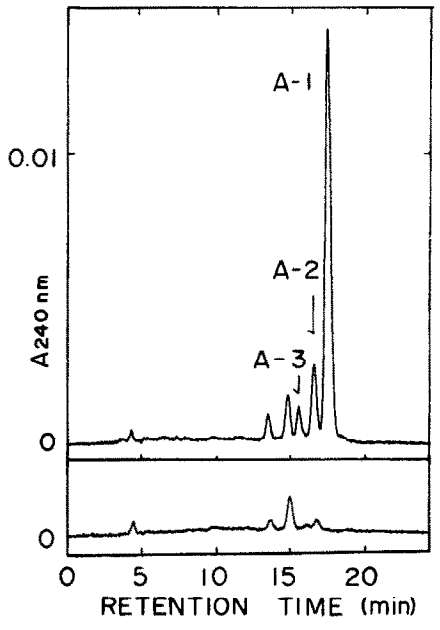

Fig. 1. Upper; HPLC elution profile of the reaction products of P-450$_{11\beta}$-dependent conversion of 19-norandrostenedione as monitored at 240 nm. Lower; the control experiments in which the reaction was run similarly but without P-450$_{11\beta}$, which was added immediately after the reaction was terminated. After extraction and being concentrated under N_2 gas, the product steroids were injected into the HPLC column (MCI GEL, ODS IHU, Mitsubishi Chemical Industries Ltd). Other conditions are as described in Experimental.

Figure 2 shows that the product formation was proportional to the concentration of P-450$_{11\beta}$ in a range up to 100 pmol in the presence of saturated amounts of the electron donating components. The apparent K_m value for 19-norandrostenedione was found to be 44 μM and the optimum pH value of 6.5 was obtained for all of the three reactions. Assuming that the molar absorption coefficients (ϵ) for A-1 and

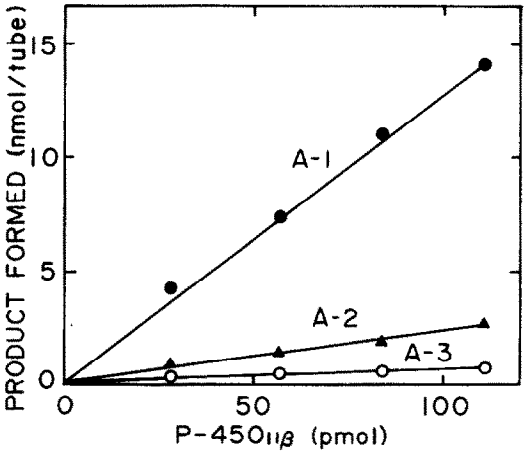

Fig. 2. Effect of increasing concentrations of P-450$_{11\beta}$ on A-1, A-2 and A-3 formation from 19-norandrostenedione. All reactions were carried out under the conditions as described in 'Experimental' in which the amounts of P-450$_{11\beta}$ varied.

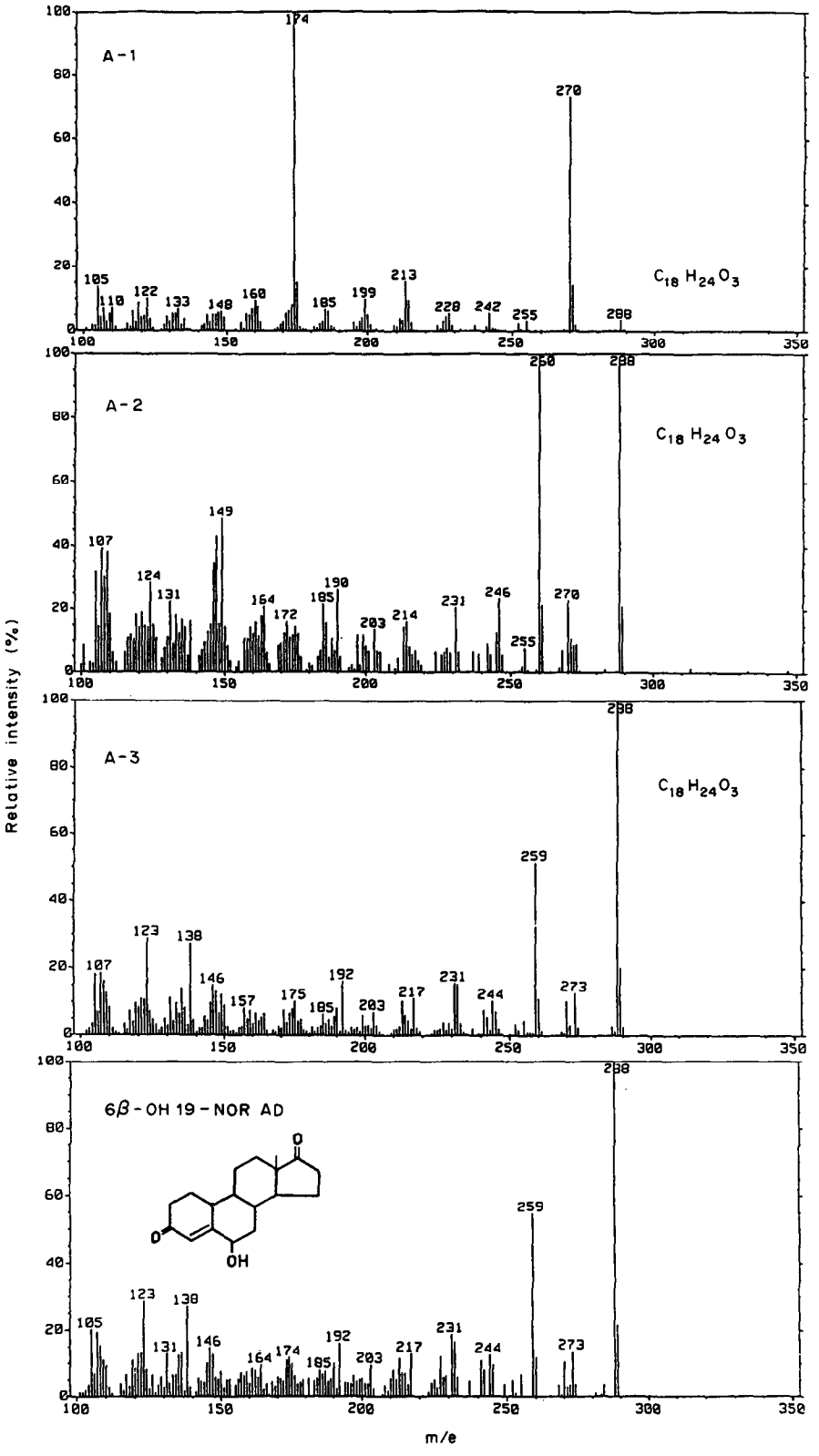

Fig. 3. GC–MS analyses of the three products described in Fig. 1. Conditions are as indicated in 'Experimental'.

A-2 are virtually the same as that for 19-norandrostenedione (16.2×10^3 at 240 nm), and that for A-3 is the same as that for 6β-hydroxy-19-norandrostenedione (11.4×10^3 at 233 nm), the turnover numbers (mol of product formed/min/mol of *P*-450) with correction for recovery of the internal standard were shown to be 45, 7.5 and 1.9 for A-1, A-2 and A-3 formation, respectively. The ratio between the rates

of deoxycorticosterone 11β-hydroxylase reactions and 19-norandrostenedione hydroxylase reaction was found to be constant during the time-course of the reaction which was linear for 3 min under these assay conditions (data not shown). These observations provide further evidence that a single $P\text{-}450_{11\beta}$ is involved in the hydroxylase reactions of both deoxycorticosterone and 19-norandrostenedione. Deoxycorticosterone, a typical and the most efficient substrate of $P\text{-}450_{11\beta}$, was found to affect the formation of hydroxylase products from 19-norandrostenedione. The half-inhibition concentration of 80 μM of deoxycorticosterone was obtained for both of the hydroxylase reactions. No interconversion between A-1 and A-2, nor conversions from A-1 or A-2 to A-3 could be observed under similar conditions.

Identification of the products

The pooled steroid extracts were separated on TLC followed by HPLC (Chemcosorb, 5-ODS-H) as specified under Experimental. The TLC R_f values were 0.33, 0.42, 0.28 for A-1, A-2 and A-3, and the HPLC retention times were 7.7, 7.4 and 6.4 min, respectively. Each steroid was separated and concentrated at room temperature, and the residue was dissolved in the desired solvent. The absorption maxima of A-1, A-2 and A-3 in acetonitrile were found to be 237, 230 and 233 nm. The three products equally gave the same molecular ion peaks (m/e 288) in GC–MS analyses (Fig. 3), exhibiting a common elementary composition of $C_{18}H_{24}O_3$ by high resonance GC–MS. Thus, all three products are monohydroxylated derivatives of 19-norandrostenedione but at different positions. Among these, A-3 was found to give an identical GC–MS profile to that produced by the authentic sample of 6β-hydroxyandrostenedione (Fig. 3). Since authentic samples for the anticipated products, 11β- and 18-hydroxy-19-norandrostenedione, were not commercially available, their purified preparations of A-1 and A-2 were subjected to NMR analyses in order to establish the position of the hydroxyl group. Figure 4 shows the ^{1}H-NMR spectrum for A-1 in $CDCl_3$. A signal due to the proton on the hydroxyl-bearing carbon atom appeared at $\delta = 4.28$ and the calculated number of proton of one showed A-1 to be a secondary alcohol. The resonance peaks at $\delta = 1.16$ and $\delta = 5.89$ were assigned as due to the 13-methyl- and 4H-protons of the steroid. The chemical shifts of these signals from 19-norandrostenedione were +0.22 and +0.04, which corresponded respectively to the values between androstenedione and 11β-hydroxyandrostenedione (Table 1). In addition, these values indicate the 11β-position to be a hydroxyl group [15]. A-1 was further analyzed by ^{13}C-NMR, in which the signal due to C11 of A-1 had been shifted from $\delta = 26.6$ of 19-norandrostenedione to $\delta = 66.6$ (Fig. 5). This is typical in the reported results of hydroxylation at the 11(β or α)-position of a steroid [16]. Taken together, the position of the hydroxyl group of A-1 was deduceed to be at 11β. Also shown in Table 1, the ^{1}H-NMR of A-2 showed that the peaks due to 13-methyl-H protons disappeared and those corresponding to the proton on the hydroxyl-bearing carbon atom and 4H-proton appeared at $\delta = 3.66$ and $\delta = 5.81$, respectively. The calculated number of protons of the hydroxyl-bearing carbon

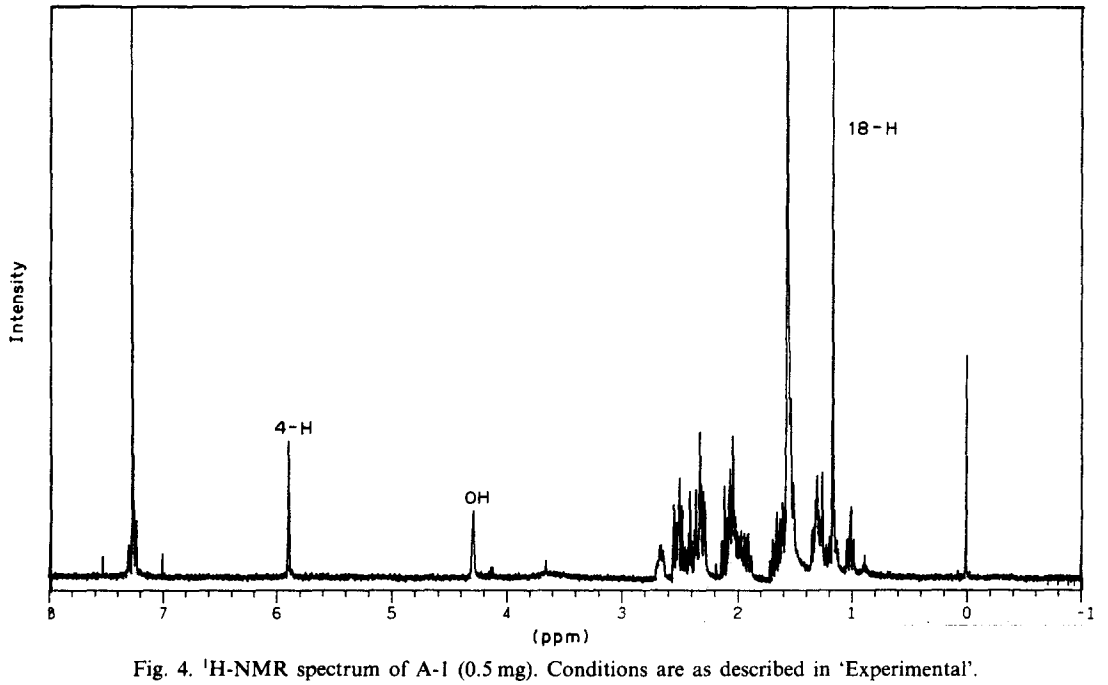

Fig. 4. ^{1}H-NMR spectrum of A-1 (0.5 mg). Conditions are as described in 'Experimental'.

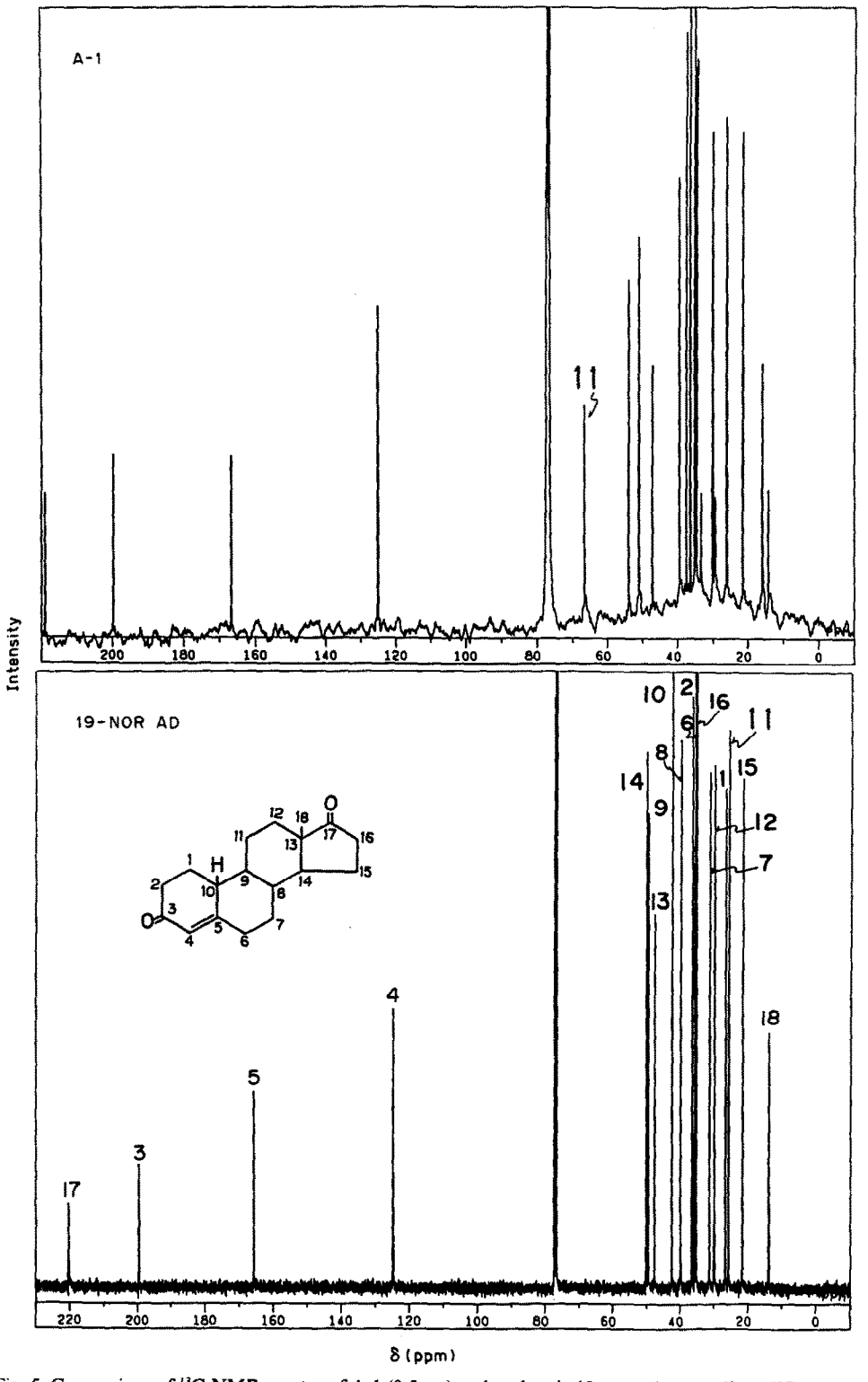

Fig. 5. Comparison of ^{13}C-NMR spectra of A-1 (0.5 mg) and authentic 19-norandrostenedione (27.6 mg). Conditions are as described in 'Experimental', and the carbon positions are assigned as described by Blunt and Stothers [16].

Table 1. ^{1}H-NMR spectra of androstenedione, 19-norandrostenedione and its derivatives in deuteriochloroform

Steroid	Signal (ppm) 19-H	18-H	4-H	-OH
Androstenedione	1.22	0.92	5.70	—
11β-Hydroxyandrostenedione	1.47	1.17	5.75	4.47
	(+0.25)	(+0.25)	(+0.05)	
19-Norandrostenedione		0.94	5.85	—
A-1		1.16	5.89	4.28
		(+0.22)	(+0.04)	
A-2		—	5.81	3.66
			(−0.04)	

Figures in parentheses show the chemical shift of each proton in $CDCl_3$ (plus sign represents an upfield shift).

indicated that the steroid was a primary alcohol. The chemical shift, −0.04, of the signal due to 4H-proton from 19-norandrostenedione corresponded to that of an 18-hydroxylated derivative. From these results the position of the hydroxyl group of A-2 was interpreted to be at the 18-position. Further analysis of A-2 has not been performed by ^{13}C-NMR owing to the unavailability of sufficient quantities.

Reactivity of P-450$_{11\beta}$ on other C$_{18}$-steroids besides 19-norandrostenedione

19-Nortestosterone and 19-norepitestosterone were also subjected to similar *P*-450$_{11\beta}$-mediated hydroxylase reactions, although they were only 1/4–1/10th as active as 19-norandrostenedione. The products from 19-nortestosterone could be separated into two bands by thin-layer chromatography which corresponded to those of the 11β- and 18-hydroxylated derivatives of 19-norandrostenedione, but neither of the products was definitely identified. No third product corresponding to the 6β-derivative of these steroids, as was the case for 19-norandrostenedione, was seen by TLC. Estrone, estradiol and 4-estrene-3β,17β-diol were equally inactive. No other bovine adrenal *P*-450 species such as *P*-450$_{scc}$ (56 pmol), *P*-450$_{c21}$ (8 pmol) or pig testis *P*-450$_{17\alpha}$ (23 pmol) catalyzed the hydroxylation of 19-norandrostenedione.

DISCUSSION

P-450$_{11\beta}$ plays an essential role in the adrenal cortex in generating gluco- and mineralocorticoids catalyzing the bioconversion of deoxy-C$_{21}$-steroids to their 11β- and 18- modified, hormonally active derivatives such as corticosterone, cortisol and aldosterone [2,4,5]. It has also been demonstrated that C$_{19}$-steroids are good substrates for *P*-450$_{11\beta}$, producing 11β- and 19-hydroxyderivatives [4,7–9]. The 19-hydroxylated derivative, 19-hydroxyandrostenedione may be further metabolized by the same *P*-450$_{11\beta}$ through a series of 19-monooxygenase reactions to form estrone and 19-norandrostenedione [8,9]. All of these observations suggest that *P*-450$_{11\beta}$ exhibits regio- and stereoselectivities on one side, and is capable of catalyzing of variety of reactions on the other. Accordingly, considering the known specificities of *P*-450$_{11\beta}$, there is good reason to assume that *P*-450$_{11\beta}$ also acts on 19-norandrostenedione, a C$_{18}$-steroid in which the 4-en-3-one structure of corticosteroids and androgens is preserved, to form 11β- and 18-hydroxylated derivatives.

In this study, we have obtained the results anticipated showing that *P*-450$_{11\beta}$ efficiently converted 19-norandrostenedione to form 11β-hydroxy-19-norandrostenedione and 18-hydroxy-19-norandrostenedione with turnover numbers of 45 and 7.5, respectively. These values are comparable to those of 60 and 10 for deoxycorticosterone 11β- and 18-hydroxylase reactions, respectively. *P*-450$_{11\beta}$ was also active with the same substrate to form a third product 6β-hydroxy-19-norandrostenedione, but to a lesser degree. This is comparable to the results of our observations that the C$_{19}$-steroid, androstenedione, is significantly hydroxylated by *P*-450$_{11\beta}$ at the 6β-position to form 6β-hydroxy-androstenedione in parallel with the formation of 11β-hydroxyandrostenedione and 19-hydroxyandrostenedione (unpublished data).

The present study, taken together with previously established works, elucidates the versatile enzymatic activities of the purified *P*-450$_{11\beta}$ which is responsible for the conversions of steroids to a variety of important hormones. As shown above, high reaction rates of 19-norandrostenedione hydroxylation with a moderate K_m value of 44 μM were observed, allowing further insight into the physiological functions of *P*-450$_{11\beta}$ in steroid biosynthesis and its control in the modification of 19-norandrostenedione in the adrenal cortex. In fact, the substrate used in the present study, 19-norandrostenedione is the product of a *P*-450$_{11\beta}$-catalyzed reaction [9] and has been shown to be a potent amplifier of aldosterone [10]. Its production in the adrenal gland [17,18] as well as in the ovary [19–21] has also been reported.

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