isomer, a compound that is not available in pure form, is unknown. It appears unlikely that the trans isomer is responsible for the biphasic character of the dissociation curves since the corresponding biotin derivatives behave similarly. In conclusion, ligands that stimulate glucose oxidation in the presence of a large excess of avidins are promising tools for insulin receptor isolation.

Registry No. Streptavidin, 9013-20-1; insulin, 9004-10-8; biotinylinsulin, 89889-04-3; biotinyl-A1-insulin, 89889-05-4; biotinyl-A2-insulin, 89889-07-6; dethiobiotinyl-A1-insulin, 89889-06-5; dethiobiotinyl-A2-insulin, 89889-08-7; dethiobiotinyl-A1-DPA-insulin, 89889-09-8; dethiobiotin, 533-48-2; *N*-hydroxysuccinimido dethiobiotinate, 80750-24-9.

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18,19-Dihydroxydeoxycorticosterone, a New Metabolite Produced from 18-Hydroxydeoxycorticosterone by Cytochrome P-450$_{11\beta}$. Chemical Synthesis and Structural Analysis by ^{1}H NMR†

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ABSTRACT: A new metabolite was produced from 18-hydroxydeoxycorticosterone by the cytochrome P-450$_{11\beta}$ linked hydroxylase system purified from bovine adrenocortical mitochondria. It was identified as 18,19-dihydroxydeoxycorticosterone by chemical synthesis on the basis of high-performance liquid chromatography, gas chromatography–mass spectrometry, and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, and detailed structural analysis of it was performed by ^{1}H NMR spectroscopy. The methylene protons at the C-19 position of the steroid were nonequivalent and coupled with each other, having a coupling constant of 10.6 Hz. These protons had different coupling constants, 6.7 and 3.4 Hz, for the hydroxy proton at the C-19 position. Due to these couplings, the signals of the methylene protons were observed around 3.9 ppm as two double doublets. The methylene protons at the C-21 position were also nonequivalent, having a coupling constant of 11.1 Hz. Coupling constants between these methylene protons and the hydroxy proton at the C-21 position were 8.2 and 4.2 Hz, respectively. These results indicate that both hydroxymethyl groups at the C-19 and C-21 positions do not freely rotate in chloroform solution. The signals of hydroxy protons at the C-19 and C-21 positions were found at 1.25 and 1.87 ppm, respectively, by means of decoupling of the corresponding methylene protons. The hydroxy proton at the C-18 position was found to scarcely couple with any proton. This fact suggests that this hydroxy group is linked to the C-20 position, making a hemiketal bridge between the C-18 and the C-20.

It has been recently reported that the urinary excretion of 19-nordeoxycorticosterone was elevated in adrenal regeneration hypertensive rats (Gomez-Sanchez et al., 1979) and in spontaneously hypertensive rats in connection with the development of hypertension (Dale et al., 1982). This steroid has also been shown to be a potent mineralocorticoid and to have higher sodium-retaining activity than deoxycorticosterone (Kagawa & Van Arman, 1957). It has been speculated that 19-nordeoxycorticosterone was produced from 19-hydroxydeoxycorticosterone (Gomez-Sanchez et al., 1982; Dale et al., 1982). In fact, Gomez-Sanchez et al. (1982) have found that rat adrenal glands have the enzymes required to convert deoxycorticosterone to 19-hydroxydeoxycorticosterone, 19-oxodeoxycorticosterone, and 19-carboxydeoxycorticosterone. These facts, together with the fact that 18-OH-deoxycorticosterone[1] can also produce hypertension (Melby et al.,

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[1] Abbreviations: NMR, nuclear magnetic resonance; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; δ, chemical shift relative to internal chloroform (7.27); 18-OH-deoxycorticosterone, 18,21-dihydroxy-4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; 18-OH-corticosterone, 11β,18,21-trihydroxy-4-pregnene-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; 18,19-(OH)$_2$-deoxycorticosterone, 18,19,21-trihydroxy-4-pregnene-3,20-dione; 18,19-(OH)$_2$-progesterone, 18,19-dihydroxy-4-pregnene-3,20-dione; 18-OH-19-nordeoxycorticosterone, 18,21-dihydroxy-19-nor-4-pregnene-3,20-dione; 19-nordeoxycorticosterone, 21-hydroxy-19-nor-4-pregnene-3,20-dione; Tris, tris(hydroxymethyl)aminomethane.

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1972; Rapp et al., 1973; Oliver et al., 1973), prompted endocrinologists to pay attention to the possible biological activity of the 19-nor- and 19-OH- derivatives of 18-OH-deoxycorticosterone.

We showed in previous papers that cytochrome P-450$_{11\beta}$ purified from bovine adrenocortical mitochondria produced a hitherto unknown steroid from 18-OH-deoxycorticosterone in addition to the well-known metabolite 18-OH-corticosterone (Momoi et al., 1983; Okamoto et al., 1982). It was confirmed by the experiment with 18-OH-[1,2-^{3}H]deoxycorticosterone as a substrate that the new steroid was derived from 18-OH-deoxycorticosterone. The fact that the new metabolite was produced by the cytochrome P-450$_{11\beta}$ catalyzed reaction was ascertained by the following experiments. At various steps of purification of cytochrome P-450$_{11\beta}$, the amounts of corticosterone produced from deoxycorticosterone, 18-OH-corticosterone from 18-OH-deoxycorticosterone, and the new metabolite from 18-OH-deoxycorticosterone were always in a constant ratio. When 18-OH-deoxycorticosterone was used as a substrate, generation of the new metabolite as well as that of 18-OH-corticosterone had a linear relationship with the concentration (0–60 μM) of the purified cytochrome P-450$_{11\beta}$, and the ratio of the formation of the new metabolite to that of 18-OH-corticosterone was constant. Besides, in an experiment on time course of formation of the new metabolite and 18-OH-corticosterone, the ratio between the two products was again constant. When 18-OH-deoxycorticosterone was used as a substrate at the concentration of 100 μM, the rates of production of the new metabolite and 18-OH-corticosterone were estimated to be approximately 5 and 7 nmol min^{-1} (nmol of P-450)$^{-1}$, respectively.

In the previous papers (Momoi et al., 1983; Okamoto et al., 1982), the structure of the new steroid, in spite of the limited amount of sample available for research, was tentatively assigned as 18,19-dihydroxydeoxycorticosterone on the basis of the observations with physicochemical methods (GC–MS, ^{1}H NMR, etc.). In the present paper, we wish to report that the proposed structure of the new metabolite was completely established by chemical synthesis and refined NMR spectral observation.

Materials and Methods

Chemicals. 18-OH-deoxycorticosterone was purchased from Makor Chemicals (Jerusalem, Israel). For reference, 18,19-(OH)$_2$-progesterone was chemically synthesized in this work (Scheme I). Deuterated chloroform (D ≥ 99.8%) was obtained from Merck and used for NMR measurements without further purification. Other chemicals were of the highest grade available from commercial sources.

Purification of Enzymes. Cytochrome P-450$_{11\beta}$ was purified from adrenal mitochondria according to the method of Suhara et al. (1972) with some modification. The detailed procedure was described in a previous paper (Momoi et al., 1983). The fractions of cytochrome P-450$_{11\beta}$ with absorbance ratios, A_{393}/A_{280}, exceeding 0.8 were collected, and the enzyme was used in the cytochrome P-450$_{11\beta}$ linked hydroxylase system. The cytochrome P-450$_{11\beta}$ used showed a single band after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Adrenodoxin and adrenodoxin reductase were purified from bovine adrenal mitochondria according to methods published previously (Suhara et al., 1972; Sugiyama & Yamano, 1975).

Purification of the New Metabolite Produced by Cytochrome P-450$_{11\beta}$. The new metabolite was prepared from 18-OH-deoxycorticosterone in the following system. 18-OH-deoxycorticosterone (1000 nmol dispersed in 100 μL of ethanol) was incubated with 2 μM cytochrome P-450$_{11\beta}$ in 5 mL of Tris-HCl buffer (50 mM, pH 7.4) containing NADPH (0.1 mM), glucose 6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (2.5 units), $MgCl_2$ (3 mM), adrenodoxin (10 μM), and adrenoxin reductase (0.3 μM). After incubation for 30 min at 37 °C, the reaction was terminated by adding 5 mL of ethanol. The reaction mixture was extracted with dichloromethane, and the solvent was evaporated under nitrogen. For purification of the metabolite, high-performance liquid chromatography was performed with an LDC liquid chromatograph system (Riviera Beach, FL). A Chemcosorb ODS-H column (particle size 7 μm) was employed as a reversed-phase column. Elution was carried out with two kinds of solutions, methanol–water solution (v/v, 1:1) or acetonitrile–water solution (v/v, 1:3). The isolated new metabolite showed a single peak on chromatograms with various columns of TSK-Gel LS 300 (particle size 5 μm), Chemcosorb Si-7 (particle size 7 μm), and Chemcosorb ODS-H (particle size 7 μm), with various eluents. Its methyloxime–trimethylsilyl ether derivative also shows a single peak on a gas chromatogram with a 2-m OV-1 (2%) column. It was confirmed by these results that the isolated new metabolite was pure.

GC–MS Analyses. For gas chromatography–mass spectrometry, a JEOL JGC 20K gas chromatograph/JMS D 300 mass spectrometer was used with a 2-m OV-1 (2%) column. The temperature was raised at 2 °C/min from 260 to 280 °C. Mass spectra were obtained at an ionization energy of 24 eV and an emission current of 300 μA.

NMR Measurement. ^{1}H NMR spectra were obtained at 200 MHz with a Varian XL-200 spectrometer with a standard 5-mm probe. For NMR measurements, the observed radiofrequency pulse used was 60°, and the recycle time was 2.8 s. The metabolite eluted from a reversed-phase column was concentrated with a TC-8F Taiyo concentrator and further dried in a desiccator connected to a vacuum pump. For NMR measurements, the chemically synthesized steroid was treated in the same way as the enzymic product. The probe temperature was kept at 24 ± 0.5 °C. Chemical shifts were determined in ppm by taking the residual $CHCl_3$ in the solvent as the internal standard (δ = 7.27).

Results

We showed in the previous study (Momoi et al., 1983) that cytochrome P-450$_{11\beta}$ purified from bovine adrenocortical mitochondria produced a new metabolite from 18-OH-deoxycorticosterone. This result was further confirmed by the following experiments.

In the experiment using 18-OH-[1,2-^{3}H]deoxycorticosterone, it was observed on a chromatogram of HPLC that cytochrome P-450$_{11\beta}$ produced the new radioactive metabolite in addition to 18-OH-[1,2-^{3}H]corticosterone from the substrate (Momoi et al., 1983). In the reaction system without cytochrome P-450$_{11\beta}$ or NADPH, the radioactivity was detected only in a fraction of the substrate, 18-OH-deoxycorticosterone. These results indicate that the new metabolite was not produced through a steroid interconversion or a nonenzymatic reaction.

When purified cytochrome P-450$_{11\beta}$ was preincubated for 10 min at 100 °C, cytochrome P-450$_{11\beta}$ had no activities of 11β- and 18-hydroxylations for deoxycorticosterone. This inactivated cytochrome P-450$_{11\beta}$ produced no new metabolite from 18-OH-deoxycorticosterone as shown in Table I. When cytochrome P-450$_{11\beta}$ was preincubated at 37 °C, its hydroxylation activities decreased concomitantly with the time course of the preincubation, and the amount of the new metabolite produced from 18-OH-deoxycorticosterone decreased. As shown in Table I, cytochrome P-450$_{11\beta}$ preincubated at 37

Table I: Effect of Heat Treatment of Cytochrome P-450$_{11\beta}$ on Formation of the New Metabolite[a]

treatment[b]	18-OH-deoxycorticosterone[c]		deoxycorticosterone[c]	
	18-OH-corticosterone[d]	new metabolite[d]	corticosterone[d]	18-OH-deoxycorticosterone[d]
without treatment	6.52 (90.6)	5.18 (71.9)	20.50 (1423)	1.09 (76.0)
	7.63 (106.0)	5.48 (76.1)	20.74 (1440)	1.11 (77.1)
at 100 °C for 10 min	nd[e]	nd	nd	nd
	nd	nd	nd	nd
at 37 °C for 10 min	3.46 (48.1)	2.89 (40.1)	8.21 (570.5)	0.62 (42.8)
	3.87 (53.7)	2.96 (41.1)	8.28 (575.1)	0.56 (38.7)

[a] The reaction was carried out for 4 min at 37 °C in the reconstituted system of 0.5 mL of Tris-HCl buffer (50 mM, pH 7.4) with 50 nmol of 18-OH-deoxycorticosterone or 100 nmol of deoxycorticosterone. The reconstituted system was as follows: 0.018 mg of cytochrome P-450$_{11\beta}$ (220 pmol), 30 μM adrenodoxin, 1 μM adrenodoxin reductase, 0.1 mM NADPH, 10 mM glucose 6-phosphate, 0.5 unit of glucose-6-phosphate dehydrogenase, and 3 mM $MgCl_2$. The amounts of products were estimated from chromatograms monitored at 254 nm (Momoi et al., 1983). [b] The treatment was carried out in 50 mM phosphate buffer (pH 7.4) containing 22 μM cytochrome P-450$_{11\beta}$, 0.3% Tween 20, 0.3% sodium cholate, and 10 μM deoxycorticosterone. [c] Substrate. [d] Nanomoles of product formed; numbers in parentheses indicate nanomoles of product formed per minute per milligram of protein. [e] nd, not detected.

°C for 10 min produced half as much of the new metabolite, compared with cytochrome P-450$_{11\beta}$ without the preincubation. The cytochrome P-450$_{11\beta}$ preincubated at 37 °C also had half hydroxylation activities at 11β- and 18-positions for deoxycorticosterone. These results are in good agreement with the fact that this preincubation converted a half-amount of cytochrome P-450 to inactive cytochrome P-420. That is, the amount of the new metabolite produced was well correlated with hydroxylation activities at 11β- and 18-positions by cytochrome P-450$_{11\beta}$. These results, together with the previous study (Momoi et al., 1983), clearly show that cytochrome P-450$_{11\beta}$ produces the new metabolite from 18-OH-deoxycorticosterone.

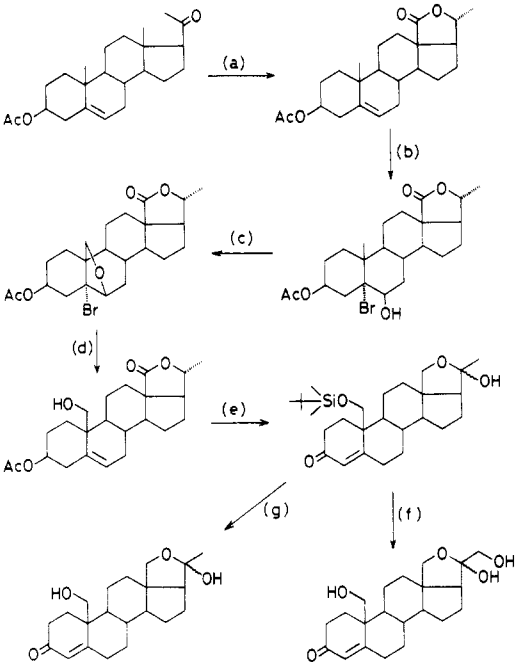
Chemical Synthesis of 18,19-(OH)$_2$-deoxycorticosterone. In order to establish the struture of the new metabolite, we chemically synthesized 18,19-(OH)$_2$-deoxycorticosterone by starting with pregnenolone acetate as outlined in Scheme I. Each chemical synthetic process was as follows.

Process a. This process involved the well-known two-step synthesis (Heusler et al., 1965). 3β-Acetoxy-20β-hydroxy-5-pregnen-18-oic acid 18,20-lactone was synthesized with a 41% overall yield.

Process b. To a stirred solution of 1.862 g of the lactone in 19 mL of ether and 8 mL of tetrahydrofuran containing 2.12 mL of water and 0.25 mL of 70% $HClO_4$ was added in portions 1.035 g of *N*-bromoacetoamide at 15 °C (Kalvoda et al., 1963). Stirring was continued at room temperature for a further 0.5 h, and then 15 mL of 1% sodium thiosulfate solution was added under ice cooling. The mixture was extracted with chloroform–methanol (v/v, 10:1). The extract was washed with saturated $NaHCO_3$ solution and aqueous saline and then dried (Na_2SO_4). Evaporation of the solvent in vacuo below 45 °C left a crystalline residue, which was triturated with dichloromethane, giving 1.692 g of the bromohydrin (72.1% yield), mp 185–188 °C.

Process c. A stirred suspension of 4.647 g of the bromohydrin, 500 mL of cyclohexane, 25.6 g of lead tetraacetate, 11.7 g of calcium carbonate, and 5.47 g of iodine was heated under reflux by irradiation with a 500-W lamp. After 1 h, the mixture was cooled to room temperature, filtered through celite (ca. 3 g), and rinsed with cyclohexane, and then the filtrate was extracted with 3:1 ether–dichloromethane. The extract was washed successively with 10% sodium thiosulfate solution and aqueous saline and then dried (Na_2SO_4). Evaporation of the solvent in vacuo below 45 °C left a crystalline residue. Two recrystallizations from dichloromethane–acetone gave 2.312 g of the pure bromo ether (50.0%), mp 267–271 °C. The mother liquor residues were combined and purified by preparative HPLC on silica gel

Scheme I: Outline of Chemical Synthesis of 18,19-(OH)$_2$-deoxycorticosterone and 18,19-(OH)$_2$-progesterone



(Merck, Lobar size Bx2, 20:1 $CHCl_3$–acetone and then 10:1 benzene–ethyl acetate) to obtain an additional 0.869 g of the bromo ether (18.8%), mp 267–270 °C.

Process d. Zinc powder (18 g) was added in portions to a stirred solution of 2.71 g of the above bromo ether in 95 mL of acetic acid and 5 mL of water warmed at 45 °C. The mixture was stirred at the same temperature for 40 min and then cooled to room temperature. The excess zinc was filtered off, and the filtrate was concentrated in vacuo below 50 °C. The residue was extracted with 4:1 ether–dichloromethane, and then the extract was washed with water, aqueous $NaHCO_3$ solution, and finally aqueous saline followed by the usual workup.[2] The crude product was crystallized from dichloromethane–ether to give 2.136 g of the alcohol (94.8%), mp 198.5–200 °C.

Process e. To a stirred solution of 777 mg of the alcohol in 30 mL of dry dimethylformamide were added 603 mg of *tert*-butyldimethylsilyl chloride and 766 mg of imidazole at 0 °C. After 5 min, the solution was allowed to stand at room

[2] Usual workup means washing extracts with water and then brine, drying (sodium sulfate), filtering, and evaporating in vacuo.

temperature overnight. The reaction mixture was diluted with 3:1 ether–dichloromethane, washed with water and aqueous saline, dried (Na_2SO_4), and then evaporated in vacuo below 40 °C. The crude silyl ether (3β-acetoxy-19-[(*tert*-butyldimethylsilyl)oxy]-20β-hydroxypregn-5-en-18-oic acid 18,20 lactone) was dissolved in 60 mL of dry ether, and 228 mg of lithium aluminium hydride was added in portions under ice cooling. The mixture was gently refluxed for 1 h, cooled, and then quenched by addition of a small amount of ethyl acetate and successively cold aqueous sodium sulfate solution. Extraction with chloroform followed by the usual workup furnished the triol (19-[(*tert*-butyldimethylsilyl)oxy]-3β,18,20β-trihydroxypregn-4-ene) (963 mg). The crude material was dissolved in 100 mL of dry toluene containing 7.4 mL of 1-methyl-4-piperidone. The solution was heated under reflux in a Dean-Stark apparatus containing a 4-Å molecular sieve. The first 20 mL of distillate was discarded, and then 408 mg of aluminium isopropoxide was added in portions. After the refluxing had been continued for 4 h, the solution was cooled and then shaken twice with 1 N HCl. The aqueous layer was further extracted with chloroform. The combined organic layer was washed with saturated $NaHCO_3$ and then worked up as usual. The residue (960 mg) was subjected to preparative HPLC on silica gel (Merck, Lobar size B, 9:1 $CHCl_3$–acetone) to obtain 430.5 mg of the hemiketal (46.7%). Recrystallization from acetone–pentane afforded the pure hemiketal, mp 153–155 °C.

Process f. To a stirred solution of 257 mg of the hemiketal obtained above in 5.6 mL of dry acetic acid was added 272 mg of lead tetraacetate in portions. Stirring was continued for 1 h at room temperature. The reaction mixture was poured into ice–water and extracted with dichloromethane. The extract was washed with water, saturated $NaHCO_3$, and finally aqueous saline followed by the usual workup to give the acetate. The crude material (295 mg) was dissolved in 5.6 mL of dioxane, and 0.56 mL of 2 N KOH was added. The mixture was stirred under argon gas for 3 h at room temperature and then poured into ice–water. Extraction with dichloromethane followed by the usual workup gave the alcohol as a foam. The crude product (273 mg) was dissolved in 4.2 mL of dry tetrahydrofuran (THF) containing 438 mg of tetra-*n*-butylammonium fluoride. The solution was allowed to stand at room temperature for 15 h. The mixture was extracted with chloroform. The usual workup furnished a crystalline residue (223 mg), which was crystallized from $CHCl_3$–THF–CH_2Cl_2, giving 110.8 mg of the triol (54.8%) as a crystalline solid, mp 178–180 °C. The mother liquor was further purified by preparative HPLC (Merck, Lobar size A, 1:1 $CHCl_3$–acetone containing 0.1% pyridine) to isolate an additional 17.6 mg of the triol (8.7%) as a crystalline solid.

Process g. A solution of 129 mg of the silyl ether in 2.5 mL of dry tetrahydrofuran containing 220 mg of tetra-*n*-butylammonium fluoride was stirred at room temperature for 4 h. The mixture was poured into ice–water and extracted with chloroform followed by the usual workup. The residue was purified by preparative HPLC on silica gel (Merck, Lobar size A, 2:1 chloroform–acetone containing 0.1% pyridine), affording a total of 67.6 mg of the diol as a crystalline solid. Recrystallization from the same solvent provided the pure product.

Thus, 18,19-(OH)$_2$-deoxycorticosterone and 18,19-(OH)$_2$-progesterone were chemically synthesized. The ^{1}H NMR spectrum of the chemically synthesized 18,19-(OH)$_2$-deoxycorticosterone is shown in Figure 1b. This spectrum was completely identical with that of the enzymic product (cf. Figure 1a). On the reversed-phase column with acetonitrile–water (v/v, 1:3) as eluate, the retention time of the chemically synthesized 18,19-(OH)$_2$-deoxycorticosterone was identical with that of the enzymic product. The methyloxime–trimethylsilyl ether of the chemical product was prepared according to the method described in the previous paper (Momoi et al., 1983). The GC–MS spectrum of the derivative is shown in Figure 1c. Its spectrum was the same as that of the derivative of the enzymic product. With the results mentioned above, the new steroid produced by cytochrome P-450$_{11\beta}$ was definitely identified as 18,19-(OH)$_2$-deoxycorticosterone.

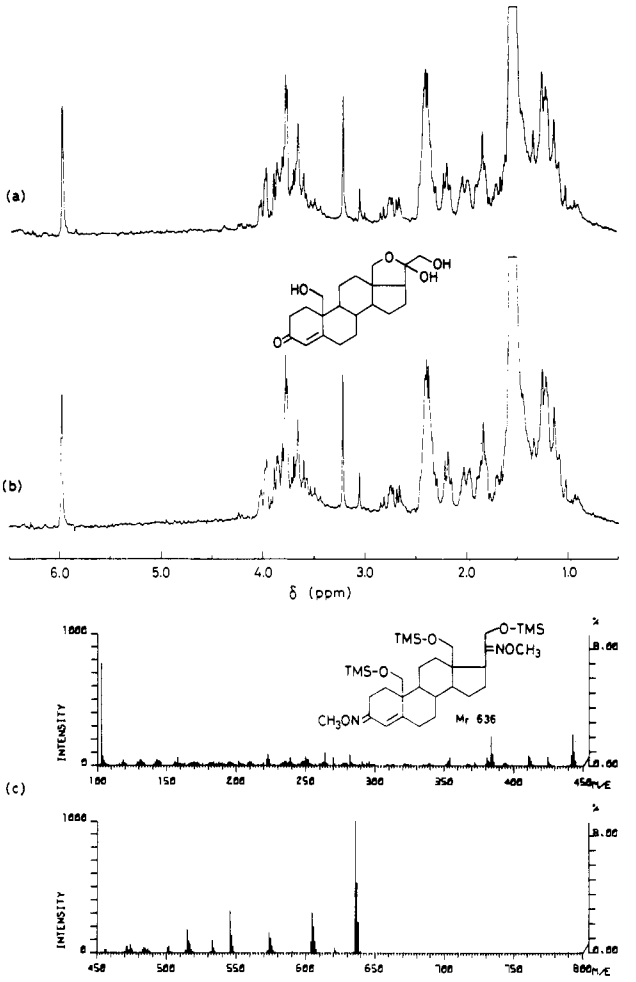


FIGURE 1: ^{1}H NMR spectra of the new metabolite from 18-OH-deoxycorticosterone and chemically synthesized 18,19-(OH)$_2$-deoxycorticosterone, and the GC–MS spectrum of the methyloxime–trimethylsilyl ether derivative of the chemically synthesized product. (a) ^{1}H NMR spectrum of the new metabolite taken in deuterated chloroform solution at 200 MHz. The concentration was 0.2 mg/0.5 mL. (b) ^{1}H NMR spectrum of the chemically synthesized product, at a concentration of 0.1 mg/0.5 mL. (c) GC–MS spectrum of the methyloxime–trimethylsilyl ether derivative. In the ^{1}H NMR spectra, the resonance peak at δ = 1.56 is due to the trace of water in the solvent.

Assignment of ^{1}H NMR Signals and Structural Analysis of the New Metabolite. Detailed structural analysis of the new metabolite, 18,19-(OH)$_2$-deoxycorticosterone, was carried out by ^{1}H NMR spectroscopy. For reference, ^{1}H NMR spectra of 18-OH-deoxycorticosterone, the substrate in the reaction, and 18,19-(OH)$_2$-progesterone were obtained (Figure 2). In the region from 3.4 to 4.1 ppm, the ^{1}H NMR spectrum of the new steroid was almost equal to that of 18-OH-deoxycorticosterone superimposed on the signals of methylene protons at the C-19 position of 18,19-(OH)$_2$-progesterone. This result strongly suggests that the signals of the methylene

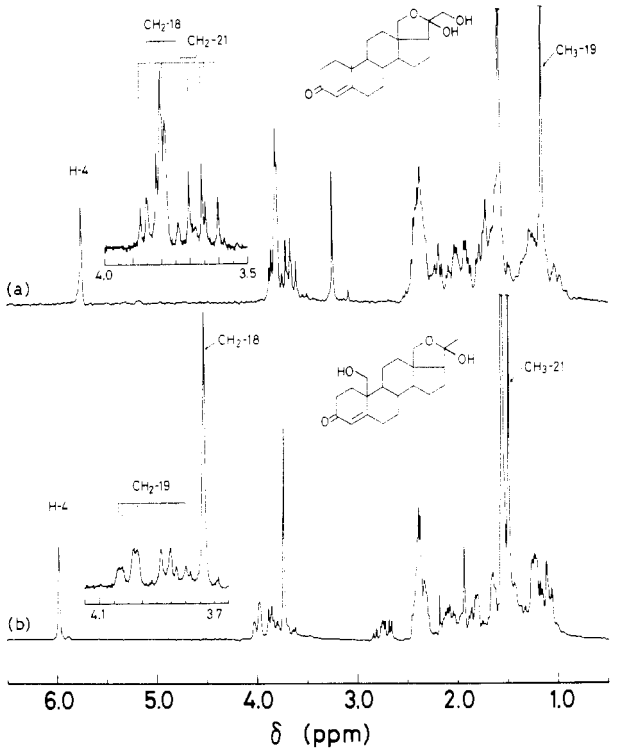


FIGURE 2: ^{1}H NMR spectra of 18-OH-deoxycorticosterone and 18,19-$(OH)_2$-progesterone taken in deuterated chloroform solution at 200 MHz: (a) 18-OH-deoxycorticosterone, 0.4 mg/0.5 mL; (b) 18,19-$(OH)_2$-progesterone, 0.4 mg/0.5 mL.

protons at the C-18, C-19, and C-21 positions of the new steroid can be assigned in comparison with those of 18-OH-deoxycorticosterone and 18,19-$(OH)_2$-progesterone.

As for 18-OH-deoxycorticosterone, it was reported that the methylene protons at the C-18 and C-21 positions appeared as a singlet and a quartet, respectively, in a 100-MHz ^{1}H NMR spectrum (Genard et al., 1975). Our spectrum for 18-OH-deoxycorticosterone obtained at 200 MHz (Figure 2a) shows that the methylene protons at the C-18 position are nonequivalent. The rise in magnetic field strength must have made this nonequivalency detectable. The methylene protons at the C-21 position were also nonequivalent. However, these protons did not give a quartet signal but a more complicated signal. This is due probably to the facts that the methylene protons couple with the hydroxy proton at the C-21 position and that their coupling constants are different from each other. The methylene protons at the C-18 position seemed not to couple with any proton other than the methylene protons. This result indicates that a hemiketal bridge is formed between the C-18 and the C-20 in chloroform solution. That is, due to the formation of the hemiketal bridge, the hydroxy group is rearranged, and there is no proton to couple with the methylene protons at the C-18 position. It has been reported for some steroids, having hydroxy groups at the C-18 position and carbonyl groups at the C-20 position, that the hemiketal forms are the predominant forms in $CDCl_3$ solution (Genard et al., 1975, 1978; Arison et al., 1982).

The spectrum of 18,19-$(OH)_2$-progesterone shows that the methylene protons at the C-19 position are nonequivalent but that the methylene protons at the C-18 position are equivalent. As for the equivalency of methylene protons at the C-18 position of the other steroids, the methylene protons of 18-OH-corticosterone were nonequivalent, whereas those of 18-OH-progesterone were equivalent. These results indicate that the hydroxy group at the C-21 position has an influence on the environment of methylene protons at the C-18 position in

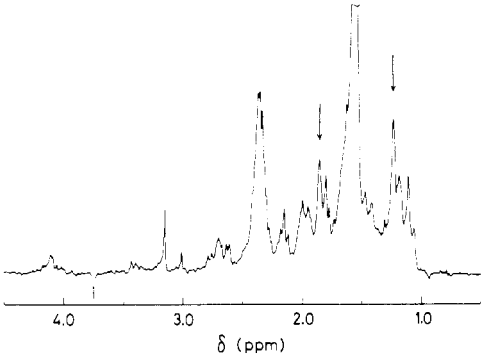


FIGURE 3: ^{1}H NMR spectrum of the new metabolite upon application of a decoupling radio-frequency pulse around δ = 3.75. The arrows indicate the increasing peaks with the decoupling.

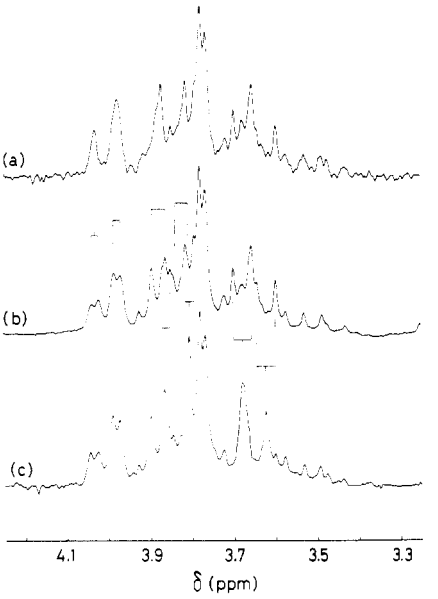


FIGURE 4: ^{1}H NMR spectra of the new metabolite in the region of the methylene protons of the hydroxymethyl groups: (a) upon application of a decoupling radio-frequency pulse at δ = 1.25; (b) without decoupling; (c) upon application of a decoupling radio-frequency pulse at δ = 1.87.

a hemiketal conformation between C-18 and C-20. The spectrum also indicates that the methylene protons at the C-19 position couple with the hydroxy proton at the C-19 position, as described above for the methylene protons at the C-21 position of 18-OH-deoxycorticosterone. The nonequivalency of methylene protons of the hydroxymethyl group at the C-19 position has also been reported for 19-OH-3,17-androstenedione (Osawa et al., 1975). We also observed that in 19-OH-3,17-androstenedione the methylene protons were nonequivalent and had different coupling constants, 6.3 and 2.7 Hz, for the hydroxy proton at the C-19 position (spectrum not shown).

Since the methylene protons at the C-19 and C-21 positions of the new steroid were expected to couple with hydroxy protons as mentioned above, we irradiated the methylene protons in order to explore the signals of these hydroxy protons. The irradiated spectrum is shown in Figure 3. Figure 3 clearly shows that the two peaks at δ = 1.25 and at δ = 1.87 increased with the decoupling (cf. Figure 1a). In other parts of the spectrum, little change was observed. These results indicate that two hydroxy protons couple with methylene protons. Irradiation of the decoupling radio frequency at δ = 1.25 simplified the signal of the methylene protons at the C-19 position observed around δ = 3.9, as shown in Figure 4a. These results indicate that the peak observed at δ = 1.25 on

Table II: Chemical Shifts[a] and Coupling Constants[b] of 18-OH-deoxycorticosterone, 18,19-(OH)$_2$-progesterone, and 18,19-(OH)$_2$-deoxycorticosterone

	18-OH-deoxycorticosterone	18,19-(OH)$_2$-deoxyprogesterone	18,19-(OH)$_2$-deoxycorticosterone
CH_2-18	3.71 (9.1)	3.73	3.75 (8.9)
	3.81 (9.1)		3.79 (8.9)
CH_2-19		3.84 (10.6, 6.8)	3.85 (10.6, 6.7)
		3.99 (10.6, 2.5)	3.99 (10.6, 3.4)
CH_2-21	3.65 (11.1, 8.9)		3.65 (11.1, 8.8)
	3.83 (11.1, 4.4)		3.82 (11.1, 4.2)
CH_3-19	1.15		
CH_3-21		1.50	
H-4	5.75	5.97	5.97

[a] Relative to internal $CHCl_3$, in ppm. [b] Coupling constants are indicated in parentheses, in hertz.

the decoupling of the methylene protons originates from the hydroxy proton at the C-19 position. As for 18,19-(OH)$_2$-progesterone, the hydroxy proton at the C-19 position was also observed at δ = 1.25 by using the same decoupling method (at a concentration of 0.4 mg/0.5 mL). Upon irradiation at δ = 1.87, the signal of the methylene protons at the C-21 position was simplified as shown in Figure 4c. This result indicates that the peak observed at δ = 1.87 comes from the hydroxy proton at the C-21 position. The hydroxy proton at the C-21 position of 18-OH-deoxycorticosterone was also found at δ = 1.87 by using the decoupling. From these results, it was clearly confirmed that the methylene protons at the C-19 and C-21 positions couple with the corresponding hydroxy protons.

The nonequivalency of each methylene proton indicates that the hydroxymethyl groups at the C-19 and C-21 positions do not freely rotate in chloroform solution. The difference of coupling constants between the methylene protons and the hydroxy proton at the C-19 or the C-21 position shows that the hydroxy groups also do not freely rotate. The hindrance of rotation of the hydroxymethyl groups and of the hydroxy groups is perhaps due to some interaction with other groups or to steric hindrance. The candidates for the groups interacting with the hydroxymethyl groups at the C-19 and C-21 positions are the carbonyl group at the C-3 position and the hydroxy group at the C-20 position, respectively.

The third hydroxy proton of the new steroid gives signals at δ = 3.24 and δ = 3.08 as sharp singlet peaks (Figure 1a). These two resonances were also observed for 18-OH-deoxycorticosterone (Figure 2a). As for 18,19-(OH)$_2$-progesterone, the signal of the hydroxy proton was observed at δ = 1.94 (Figure 2b). These peaks disappeared upon the addition of a small amount of deuterated methanol. These results support that these peaks originated from the hydroxy proton at the C-20 position, which was exchanged for a deuteron of deuterated methanol. The total area of the two peaks at δ = 3.24 and δ = 3.08 was identical with that of the peak of the C-4 proton, suggesting that the two peaks come from one hydroxy proton in different conformers. These different conformers may be produced upon the formation of the hemiketal bridge between C-18 and C-20; that is, these conformers maybe correspond to *R* and *S* configurations at the C-20 position. From each peak area of the two peaks, the fraction of the minor structure was estimated to be about 20% in the new steroid, while it was about 10% in 18-OH-deoxycorticosterone.

In the spectral region from δ = 3.4 to δ = 4.1, some small peaks appeared other than the large peaks mentioned above, but they remain unassigned. These peaks did not decrease with purification of the steroid. The small peaks of the new steroid were larger than that of 18-OH-deoxycorticosterone. This result may reflect the fact that the fraction of the minor structure of the new steroid was larger than that of 18-OH-deoxycorticosterone. The implication may be that the small peaks in the region from δ = 3.4 to δ = 4.1 possibly come from the minor conformer. Thus, the peaks of the new metabolite were assigned, and the results are summarized in Table II.

Discussion

It has been reported that 19-OH-deoxycorticosterone was isolated from bovine and hog adrenal glands (Mattox, 1955; Neher & Wettstein, 1956) and that it was produced from deoxycorticosterone or progesterone in adrenal glands of some animals (Hayano & Dorfman, 1955; Levy & Kushinsky, 1955; Dale et al., 1980). Suhara et al. (1978) have reported that cytochrome P-450$_{11\beta}$ hydroxylated 4-androstene-3,17-dione at the C-19 position. Watanuki et al. (1978) have also demonstrated that the cytochrome can catalyze hydroxylations of deoxycorticosterone at the C-18 as well as the C-11β position. In this paper and previous papers (Momoi et al., 1983; Okamoto et al., 1982), we clearly demonstrated that cytochrome P-450$_{11\beta}$ hydroxylated 18-OH-deoxycorticosterone, an adrenal steroid, at the C-19 position and that the structure of the product was 18,19-(OH)$_2$-deoxycorticosterone. These results indicate that cytochrome P-450$_{11\beta}$ can catalyze hydroxylations at the C-19 position as well as the C-11β and C-18 positions of steroids.

Two methyl groups at the C-19 and C-18 positions lie close to the C-11β position on the β-face of steroids. If the catalytic center of cytochrome P-450$_{11\beta}$ is slightly shifted around the C-11β position upon binding of a substrate, cytochrome P-450$_{11\beta}$ can easily catalyze the hydroxylations at the C-11β, C-18, or C-19 position. We showed that cytochrome P-450$_{11\beta}$ hydroxylated 18-OH-deoxycorticosterone at the C-11β and C-19 positions. It was also shown in the previous papers that these two hydroxylations were catalyzed at rather similar rates. When corticosterone was used as a substrate in a reconstituted system of cytochrome P-450$_{11\beta}$, we did not find a major product other than 18-OH-corticosterone. These results suggest that when cytochrome P-450$_{11\beta}$ binds with various substrates, its catalytic site comes close to different parts of the substrates depending on their structures. The activities of these hydroxylations are thought to vary depending on the structures of substrates and on the structures of catalytic and binding sites of cytochrome P-450$_{11\beta}$. A study on these activities for various substrates would provide information on the structure of catalytic and binding sites of cytochrome P-450$_{11\beta}$.

Also of great interest is the problem of whether or not 18,19-(OH)$_2$-deoxycorticosterone has some pharmacological activity. Some groups have reported that 19-nordeoxycorticosterone is a potent mineralocorticoid with a high affinity for a mineralocorticoid receptor and that it has higher sodium-retaining activity than deoxycorticosterone (Gomez-Sanchez et al., 1982; Dale et al., 1982). This mineralocorticoid

was speculated to be produced from 19-OH-deoxycorticosterone. These facts lead us to pay attention to the problem of whether 18,19-$(OH)_2$-deoxycorticosterone and its derivative take part in the etiology of mineralocorticoid-induced hypertension.

In a preliminary experiment, 18,19-$(OH)_2$-deoxycorticosterone did not show a remarkably high affinity for a mineralocorticoid receptor from rat kidney cytosol. This preliminary result was not unexpected, because it is known that 19-nordeoxycorticosterone has higher sodium-retaining ability than deoxycorticosterone, whereas 19-OH-deoxycorticosterone has much weaker sodium-retaining ability than deoxycorticosterone. Duax et al. (1982) have proposed from X-ray analysis of steroids that the A-ring end of the steroid is primarily responsible for initiating and maintaining receptor binding and D-ring variation governs the agonist–antagonist response. This proposition may suggest that the hydroxymethyl group at the C-19 position above the A-ring weakens the binding affinity for the receptor and the removal of the methyl group at the C-19 position enhances the affinity. The hemiketal bridge between C-18 and C-20 near the D-ring may affect the agonist–antagonist response. Therefore, 18-OH-19-nordeoxycorticosterone, which has not yet been found as a natural product, may be a more interesting steroid to examine for biological properties. This aspect of the research is currently under investigation in our laboratory.

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Registry No. Cytochrome P-450, 9035-51-2; 18,19-$(OH)_2$-deoxycorticosterone, 84356-52-5; 18-OH-deoxycorticosterone, 379-68-0; pregnenolone acetate, 1778-02-5; 3β-acetoxy-20β-hydroxy-5-pregnen-18-oic acid 18,20-lactone, 3020-10-8; *N*-bromoacetamide, 79-15-2; 3β-acetoxy-5α-bromo-6β,20β-dihydroxypregnan-18-oic acid 18,20-lactone, 89637-77-4; 3β-acetoxy-5α-bromo-6β,19-epoxy-20β-hydroxypregnan-18-oic acid 18,20-lactone, 89637-78-5; 3β-acetoxy-19,20β-dihydroxy-5-pregnen-18-oic acid 18,20-lactone, 89637-79-6; *tert*-butyldimethylsilyl chloride, 18162-48-6; 3β-acetoxy-19-[(*tert*-butyldimethylsilyl)oxy]-20β-hydroxypregn-5-en-18-oic acid 18,20-lactone, 89637-80-9; 19-[(*tert*-butyldimethylsilyl)oxy]-3β,18,20β-trihydroxypregn-14-ene, 89637-81-0; 18,20-epoxy-19,20,21-trihydroxypregn-4-en-3-one, 89637-83-2; 19-[(*tert*-butyldimethylsilyl)oxy]-18,20-epoxy-20-hydroxypregn-4-en-3-one, 89637-82-1; 18,20-epoxy-19,20-dihydroxypregn-4-en-3-one, 89637-84-3.

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