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19-HYDROXYLATED STEROIDS, NEW METABOLITES PRODUCED BY THE Y1 ADRENAL CELL LINE

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Summary—The expression of 19-hydroxylase activity in the Y1 adrenal cell line is reported here for the first time. Two new metabolites from the incubation of deoxycorticosterone (DOC) with these cells, 19-hydroxy-20α-dihydroDOC and 19-hydroxy-20α-dihydrocorticosterone, have been identified. The most important of the two is the 11β,19-dihydroxylated metabolite, which is produced in smaller amounts than 18-hydroxy-20α-dihydrocorticosterone. A third 19-hydroxylated metabolite was identified as 19-hydroxy-20α-dihydroprogesterone, produced from the cholesterol in the serum supplemented medium. These results show that the cytochrome $P\text{-}450_{11\beta}$ of this cell line expresses 19-hydroxylase activity in addition to 11β- and 18-hydroxylase activities, as do those of other species.

INTRODUCTION

The mouse adrenal tumor cell line (Y1) is known to express most of the enzymatic activities needed for the synthesis of corticosteroids, and in particular a high 11β-hydroxylase activity [1, 2]. It has proven to be an interesting model for the study of the regulation of steroidogenesis *in vitro* [3]. Recently, we reported the expression of 18-hydroxylase activity in this model [4]. While conducting studies on the regulation of the hydroxylating metabolism of deoxycorticosterone (DOC)‡ and corticosterone (B) in this cell line, unknown metabolites appeared. They did not result from any of the hydroxylase activities known to be expressed by the Y1 cells. Further studies reported here, including the characterization of the unknown metabolites by gas chromatography and mass spectrometry and the synthesis of reference 19-hydroxylated steroids, demonstrated the presence of 19-hydroxylase activity in the Y1 cell line.

EXPERIMENTAL

Reagents

All solvents were of analytical or HPLC grade from Merck. *O*-methoxyamine hydrochloride was purchased from Pierce Chemical Co. (Rockford, Ill.), bis(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane from Supelco (Bellefonte, Pa.) and C_{28} and C_{34} *n*-alkanes from Fluka (Buchs, Switzerland). DOC was obtained from Ikapharm (Ramat-Gan, Israel), 19-hydroxyP from Sigma Chemical Co. (La Verpilliere, France).

Cell culture incubations and steroid analysis

Y1 cells were obtained from Flow Laboratories (Les Ulis, France) at the 61st to 63rd passage. They were routinely grown in Ham's F10 medium (Gibco, Grand Island, N.Y.) supplemented with 5% fetal calf serum (Gibco), 5% newborn calf serum (Gibco), 100 IU penicillin/ml and 100 μg streptomycin/ml as previously described [4]. Experiments were performed in culture medium containing 1, 5, 10, or 20% serum. The serum composition varied;

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‡The following trivial names and abbreviations are used: progesterone (P), 4-pregnene-3,20-dione; deoxycorticosterone (DOC), 21-hydroxy-4-pregnene-3,20-dione; corticosterone (B), 11β,21-dihydroxy-4-pregnene-3,20-dione; 19-hydroxyP, 19-hydroxy-4-pregnene-3,20-dione; 19-hydroxyDOC, 19,21-dihydroxy-4-pregnene-3,20-dione; 20α-dihydroxyP, 20α-hydroxy-4-pregnen-3-one; 20α-dihydroDOC, 20α,21-dihydroxy-4-pregnen-3-one; 20α-dihydroB, 11β,20α,21-trihydroxy-4-pregnen-3-one; 20α-dihydroA, 20α,21-dihydroxy-4-pregnene-3,11-dione; 11β-hydroxy-20α-dihydroP, 11β,20α-dihydroxy-4-pregnen-3-one; 19-hydroxy-20α-dihydroP, 19,20α-dihydroxy-4-pregnen-3-one; 19-hydroxy-20α-dihydroDOC, 19,20α,21-trihydroxy-4-pregnen-3-one; 19-hydroxy-20α-dihydroB, 11β,19,20α,21-tetrahydroxy-4-pregnen-3-one; 18-hydroxy-20α-dihydroB, 11β,18,20α,21-tetrahydroxy-4-pregnen-3-one.

either fetal calf serum, neonatal calf serum or horse serum (Inter Med, Strasbourg, France) was used alone or in a combination with another. Incubations were performed for 24, 48 or 72 h periods at 37°C under an air–CO_2 (95:5, v/v) atmosphere saturated with water. Steroids were extracted from the culture medium using Sep-Pak C_{18} cartridges [5], converted into methoxime-trimethylsilyl ether derivatives (MO-TMS), and analyzed by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) in electron impact mode [6, 7].

Preparation of reference steroids

(1) Conversion of 19-hydroxyP to 19-hydroxyDOC. Slices of six rat adrenals were incubated in 3 ml Tris buffer, 0.020 M and pH 7.4, containing 200 μg of 19-hydroxyP and 1 mg NADPH, for 3 h at 37°C. Steroids were extracted with ethyl acetate and dichloromethane and fractionated by thin-layer chromatography (TLC) using silica-gel type 60 plates with fluorescent indicator (Merck, Darmstadt, Germany) in chloroform–methanol (195:5, v/v). Steroids extracted from the different fractions were analyzed as MO-TMS derivatives by GC and GC–MS. Residual 19-hydroxyP was localized in the TLC fraction with an R_f of 0.26–0.33 and 19-hydroxyDOC in the fraction with an R_f of 0.15–0.22.

(2) Conversion of 19-hydroxyDOC to 19-hydroxy-20α-dihydroDOC and 19-hydroxy-20α-dihydroB. Purified 19-hydroxyDOC (about 30 μg) was incubated with the Y1 cells in a culture medium containing 0.5% fetal calf serum and 0.5% newborn calf serum for 48 h. In the same manner, 19-hydroxy-20α-dihydroP was prepared from 19-hydroxyP.

RESULTS

During the study of the metabolism of DOC and B by the Y1 adrenal cell line in serum supplemented culture medium, three unidentified metabolites appeared. Their chromatographic behavior and their mass spectra led us to synthesize reference 19-hydroxylated steroids for comparison.

Biosynthesis of reference 19-hydroxylated steroids

19-HydroxyDOC was obtained by conversion of 19-hydroxyP using the cytochrome $P\text{-}450_{C21}$ activity of rat adrenal slices. It was the most abundant metabolite produced from the incubation. The chromatographic profile of its MO-TMS derivative shows four peaks corresponding to the *syn*- and *anti*-isomers in positions 3 and 20 (Table 1), as is generally observed for MO-TMS derivatives of 3,20-dioxosteroids with a 21-hydroxyl group [6]. Its mass spectrum is comparable to that reported [9].

Table 1. Methylene unit values of the MO-TMS derivatives of reference 19-hydroxylated steroids

Steroids	M.U. values[a]
19-hydroxyP	30.70; 30.91[b]
19-hydroxy-20α-dihydroP	30.14; 30.30[b]
19-hydroxyDOC	31.64; 31.77[c] (32.10; 32.28)
19-hydroxy-20α-dihydroDOC	32.36; 32.45[b]
19-hydroxy-20α-dihydroB	33.97; 34.04[b]

[a]Methylene unit values are calculated with an oven temperature programmed at 1°C/min from 220°C using C_{28} and C_{34} *n*-alkanes [8].
[b]*Syn*- and *anti*-isomers of the MO derivative in position 3.
[c]*Syn*- and *anti*-isomers of the MO derivatives in position 3 and 20 (two major and two minor isomers; minor isomers in parentheses).

19-Hydroxy-20α-dihydroDOC and 19-hydroxy-20α-dihydroB were obtained by incubation of purified 19-hydroxyDOC with the Y1 adrenal cell line, known for its high 20α-reductase and 11β-hydroxylase activities (Fig. 1). The molecular ions of these two compounds at *m/z* 593 and *m/z* 681 were consistent with the MO-TMS derivatives of an oxopregnenetriol and an oxopregnenetetrol, respectively (Figs 2a and b). These mass spectra show several fragments which include the

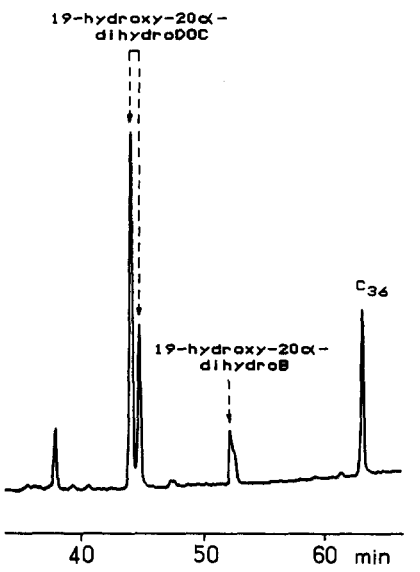

Fig. 1. GC analysis of the MO-TMS derivatives of reference 19-hydroxy-20α-dihydroDOC and 19-hydroxy-20α-dihydroB obtained from the incubation of 19-hydroxyDOC with the Y1 cell line. Analytical conditions were as follows: 25 m × 0.21 mm i.d. SE-30 open tubular glass column at 220°C, 1°/min.

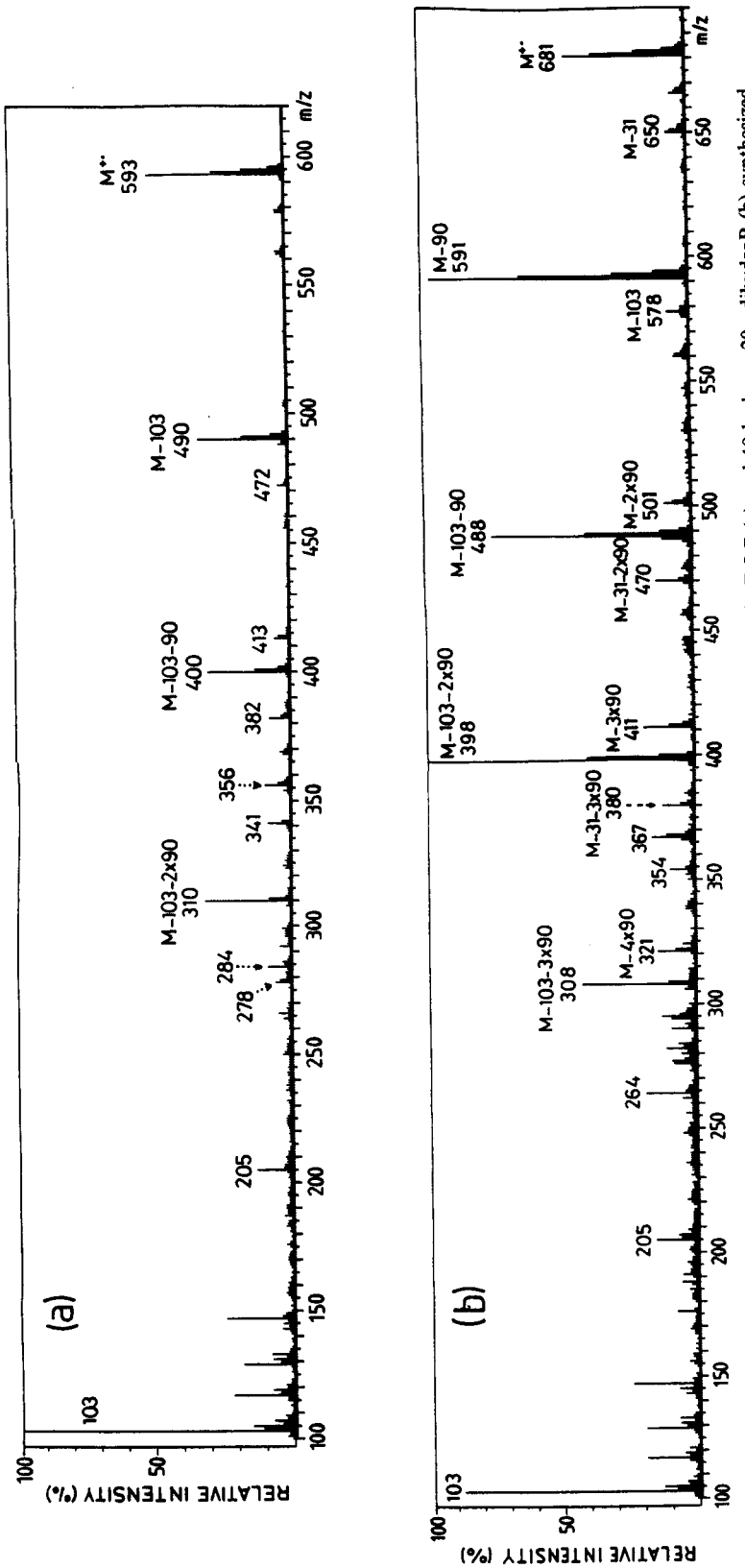

Fig. 2. Mass spectra of the MO-TMS derivatives of the reference compounds 19-hydroxy-20α-dihydroDOC (a) and 19-hydroxy-20α-dihydroB (b) synthesized from 19-hydroxyDOC using the Y1 cell line. These compounds were purified by TLC.

loss of 205 a.m.u., corresponding to the —CHOTMS—CH_2OTMS side chain. The fragment at m/z 103 indicates the presence of the trimethylsilyl derivative of a primary alcohol and corresponds to —CH_2OTMS. Several fragments which result from the loss of 103 a.m.u. (M-103, M-103-90, M-103-2 × 90...)$^+$ are abundant as usually observed for steroids with a —CHOTMS—CH_2OTMS side chain. Complementary characterization of the biosynthesized compounds is their chromatographic elution pattern. The degree of resolution of the two isomers formed from each steroid during the derivatization reaction (Table 1) is comparable to that observed from *syn*- and *anti*-isomers in position 3 [6]. The localization in position 11β of the additional hydroxyl of the oxopregnenetetrol was based on its chromatographic behavior and the fact that all precursors incubated with the Y1 cell line are converted principally into their 11β-hydroxylated metabolites.

19-Hydroxy-20α-dihydroP was obtained by incubation of 19-hydroxyP with the Y1 adrenal cell line. Its mass spectrum shows the fragments which characterize the presence of the —CHOTMS—CH_3 side chain (117 a.m.u.) and the —CH_2OTMS group (103 a.m.u.).

Identification of 19-hydroxylase activity in the Y1 cell line

The two unknown metabolites of DOC were compared to reference 19-hydroxylated steroids. The one metabolite was detected by a fragment ion current chromatogram at m/z 681, together with 18-hydroxy-20α-dihydroB (Fig. 3). It was identified as 19-hydroxy-20α-dihydroB on the basis of the similarity of its methylene unit (M.U.) values and mass spectrum (Fig. 4a) to those of the reference compound (Table 1, Fig. 2b). The other metabolite, eluted in the same peak as 20α-dihydroA, was detected by the fragment ion current of ions characteristic of hydroxy-20α-dihydroDOC. It was identified as 19-hydroxy-20α-dihydroDOC on the basis of the ions formed in mass spectrometry and of the elution pattern showing two peaks with M.U. values similar to those of the reference compound.

With the metabolites of DOC and B appeared endogenous steroids synthesized from cholesterol present in the serum-supplemented culture medium. In addition to the expected endogenous steroids, P, 20α-dihydroP and 11β-hydroxy-20α-dihydroP, there appeared other minor hydroxylated 20α-dihydroP. Among

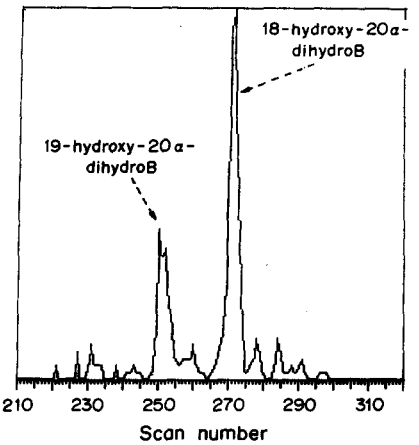

Fig. 3. GC–MS analysis of the MO-TMS derivatives of metabolites with a tetrahydroxy-4-pregnene-20-one structure: fragment ion mass choromatogram of the molecular ion at m/z 681. Analytical conditions as described in Materials and Methods.

these a metabolite was identified as 19-hydroxy-20α-dihydroP. Its mass spectrum (Fig. 4b) and its chromatographic elution pattern showing two peaks arising from the resolution of the *syn*- and *anti*-isomers in position 3 (Table 1) are comparable to those of the reference compound.

The time-course of the formation of 19-hydroxylated steroids, was examined. Incubations of DOC (50 μg/dish) were performed for 24, 48 and 72 h. The formation in relation with different proportions of serum in culture medium (1, 5, 10 and 20%) and in relation to the nature of the serum (horse serum, fetal calf serum or neonatal calf serum) was also examined. 19-hydroxylase activity was detected after 24 h of incubation of DOC in culture medium containing 5% fetal calf serum, after 48 h in culture medium containing 10% horse serum and after 72 h in culture medium containing 5% of fetal calf serum and neonatal calf serum in equal proportions (results not shown). It is thus in fetal calf serum supplemented culture medium that 19-hydroxylase activity is the strongest. It is weaker than 18-hydroxylase activity which was detected after 24 h of incubation in culture medium containing 5% fetal calf serum and neonatal calf serum in equal amounts. When 19-hydroxy-20α-dihydroDOC and 19-hydroxy-20α-dihydroB were present, the latter is present in much larger amounts.

DISCUSSION

In this paper, 19-hydroxylase activity in the Y1 cell line is reported for the first time. The structural determination of three novel 19-

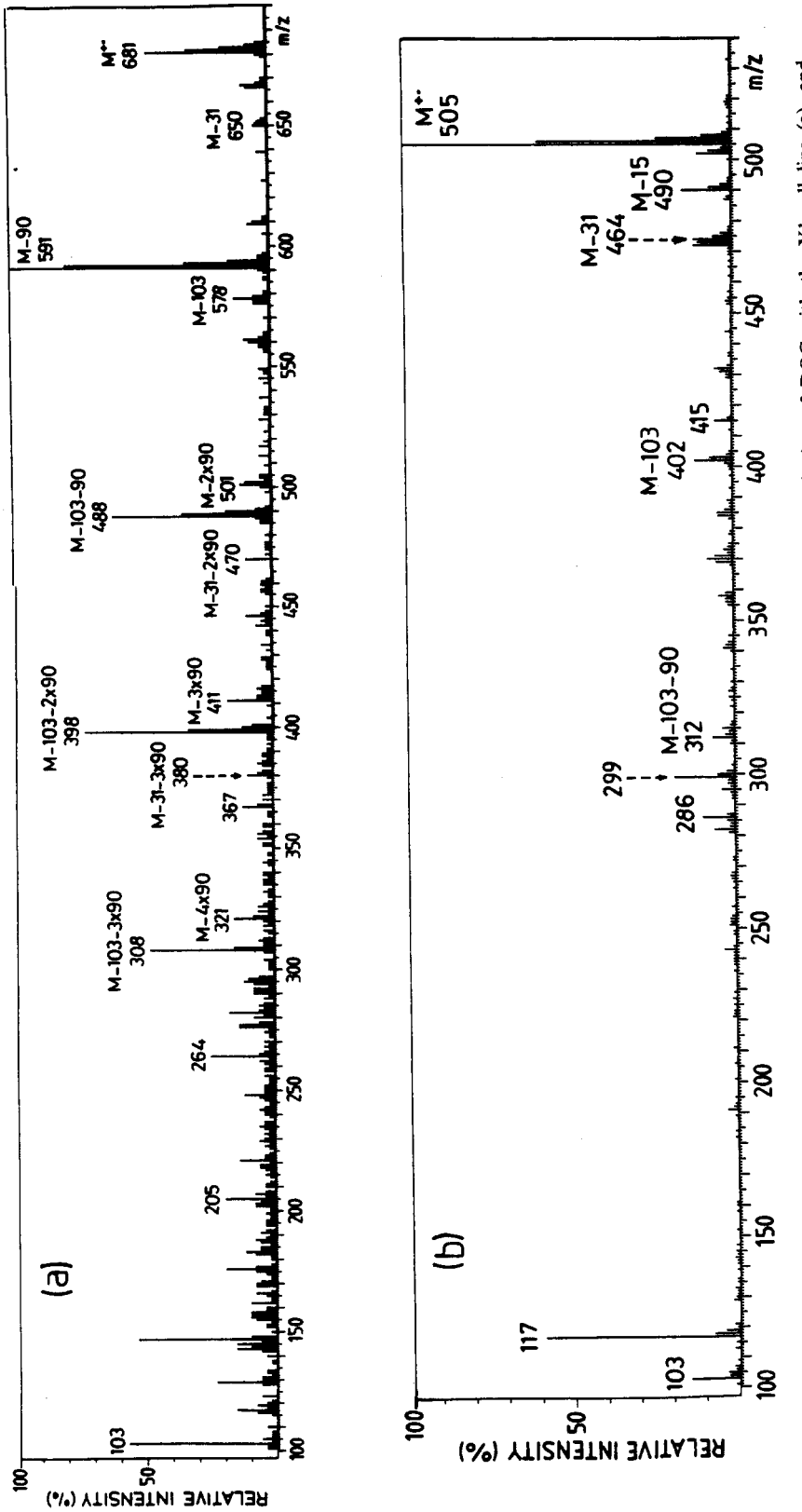

Fig. 4. Mass spectra of the MO-TMS derivatives of 19-hydroxy-20α-dihydroB produced from the incubation of DOC with the Y1 cell line (a), and 19-hydroxy-20α-dihydroP produced by Y1 cells during the metabolism of cholesterol from the serum-supplemented culture medium (b).

hydroxylated steroids produced by this cell line, 19-hydroxy-20α-dihydroP, 19-hydroxy-20α-dihydroDOC, and 19-hydroxy-20α-dihydroB is described. The first is an endogenous metabolite of cholesterol present in the serum supplemented medium. The other two are metabolites of exogenous DOC. These 19-hydroxysteroids are also 20α-reduced due to the very strong 20α-reductase activity in this cell line. Their formation, therefore, implies the 19-hydroxylation of 20α-dihydroP, 20α-dihydroDOC and 20α-dihydroB. A similar reaction sequence was previously reported for the formation of 18-hydroxylated-20α dihydrosteroids in the Y1 cell line [4].

19-Hydroxylase activity has been reported in human, bovine and rat adrenals [10–12]. Its expression by cytochrome $P\text{-}450_{11\beta}$ has been established [13–15]. The detection of 19-hydroxylase activity in the Y1 cell line is further proof of the similarity of cytochrome $P\text{-}450_{11\beta}$ activity in most species, that is, the catalysis of 11β, 18- and 19-hydroxylation reactions.

In the Y1 cell line, 19-hydroxylated steroids are produced in smaller amounts than 18-hydroxylated steroids. Therefore, in the mouse, as in the rat and the bovine, 19-hydroxylase activity is weaker than 18-hydroxylase activity. In contrast to these species, the gerbil [16] and the golden hamster [17], express very strong 19-hydroxylase activity, which replaces 18-hydroxylation.

Some 19-hydroxylated metabolites are implicated as substrates in the aromatization of androgens [18]. They are also implicated in the formation of 19-nor-steroids, which might be biologically active [19, 20]. The ability of the Y1 cell line to express 19-hydroxylase activity confers to this model a particular interest in the study of the regulation of the biosynthesis of steroid hormones derived from 19-hydroxylated precursors.

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