Journal of Natural Products
Vol. 50, No. 3, pp. 463-467, May-Jun 1987

NEW HYDROXYLATION PRODUCTS OF PROGESTERONE WITH *MUCOR GRISEOCYANUS*

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ABSTRACT.—Hydroxylation products of progesterone, not previously reported, from incubation of progesterone with *Mucor griseocyanus* ATCC 1207a (+), have been isolated and characterized. Besides the known major component, 14α-hydroxypregn-4-ene-3,20-dione, 7α-hydroxy- and 9α-hydroxypregn-4-ene-3,20-dione, and the dihydroxylated metabolites, 6β,14α-dihydroxypregn-4-ene-3,20-dione, 7α,14α-dihydroxypregn-4-ene-3,20-dione together with an epoxide, 14,15α-epoxypregn-4-ene-3,20-dione, and an unsaturated component, 8β-hydroxypregna-4,14-diene-3,20-dione have been isolated and identified by ^{1}H- and ^{13}C-nmr spectroscopic techniques.

In synthetic studies on progesterone derivatives with potential activity on the digitalis receptor of cardiac muscle (1), 14α-hydroxypregn-4-ene-3,20-dione (14α-hydroxyprogesterone) was required as a starting material. *Mucor griseocyanus* (ATCC 1207), both in the spore and vegetative culture, has been reported to hydroxylate progesterone (2,3) predominantly in the 14α-position. The formation of the 11ξ-OH derivative (4) and unidentified more polar metabolites has also been reported (3). Incubation of C-6, C-16, and C-21 substituted progesterone derivatives with the *Mucor* has led to hydroxylation at the 6β,7α,9α-,11ξ-positions as well as 14α-hydroxylation (3-6). In this report, we show hydroxylation of progesterone to occur at the 6β-, 7α-, and 9α-positions as well as the 14α-position with the *Mucor*. Unusual minor products, 14,15α-epoxypregn-4-ene-3,20-dione and 8β-hydroxypregna-4,14-diene-3,20-dione, were also identified.

TABLE 1. ^{1}H-nmr Assignments for Progesterone Metabolites[a]

Proton	Chemical Shift in ppm; *J* and *W*½ in Hz						
	8βOH, 14-ene	14α,15α-epoxide	14αOH	9αOH	7αOH	7αOH, 14αOH	6βOH, 14αOH
C-13CH_3	1.13	0.84	0.79	0.68	0.67	0.76	0.83
C-10CH_3	1.36	1.22	1.20	1.33	1.20	1.22	1.40
C-20CH_3	2.16	2.11	2.12	2.13	2.13	2.12	2.13
C-4H	5.78, m W½=3.8	5.73, d *J*=1.7	5.74, d *J*=1.5	5.88, d *J*=1.8	5.81, d *J*=2.2	5.81, d *J*=1.8	5.82 d *J*=1
C-17H	2.93, dd *J*=10.2, 7.0	2.54, dd *J*=10.1, 7.1	3.22, t *J*=8.4	2.60, t *J*=8.9	2.58, t *J*=9 Hz	3.21, t *J*=8.5	3.20, t *J*=8.6
Other					4.00, m W½=7 (7-H)	3.61 (14OH) 2.32, d *J*=3.8 (7-OH) 4.30, m W½=8 (7-H)	4.43, m W½=6 (6-H)

[a]Spectra are recorded in $CDCl_3$; TMS internal standard.

RESULTS AND DISCUSSION

^{1}H- and ^{13}C-nmr spectra were recorded for all of the metabolites of progesterone (Tables 1, 2, and 3). ^{13}C-resonances were classified into CH_3, CH_2, CH, and C signals with the DEPT (7) sequence. The ^{13}C assignments were aided by reference to the previously reported ^{13}C spectra of progesterone and 6β-hydroxypregn-4-ene-3,20-dione and the substituent shift values reported by Blunt and Stothers (8). Difference-double-resonance (DDR) (9), homonuclear correlation spectroscopy (COSY) (10), and heteronuclear correlation spectroscopy (11) were used as necessary. These techniques were sufficient for the unambigous identification of the metabolites previously isolated from other sources. For the new metabolites, 8β-hydroxypregna-4,14-diene-3,20-dione and 14,15α-epoxypregn-4-ene-3,20-dione a more extensive nmr analysis was required.

TABLE 2. ^{13}C nmr Assignments for Progesterone Metabolites[a,b]

Carbon no.	Chemical shift in ppm						
	8βOH, 14-ene	14α,15α-epoxide	14αOH	9αOH	7αOH	7αOH, 14αOH	6βOH, 14αOH[c]
1	36.8	35.5	35.8	28.5	35.5	35.6	37.3
2	33.7	33.9	34.0	(34.0)	34.0	34.0	(34.2)
3	199.5	199.2	199.4	198.9	198.7	198.6	195.5
4	124.1	124.3	124.4	127.1	127.0	127.2	126.4
5	171.1	169.5	170.2	168.1	167.1	166.2	167.2
6	28.8	31.9	32.6	31.7	41.1	41.3	73.4
7	37.5	25.8	27.2	25.4	68.3	69.7	(33.9)
8	71.3	32.1	38.3	37.4	39.7	39.6	33.2
9	54.9	49.6	46.4	76.4	45.2	40.5	46.4
10	48.0	38.5	38.6	44.5	38.5	38.6	38.1
11	18.6	21.0	21.4	26.8	20.9	21.6	21.5
12	42.5	34.6	33.4	(34.2)	38.3	33.1	32.6
13	39.0	42.0	47.9	43.7	43.9	48.1	48.1
14	152.0	72.1	85.2	49.5	50.7	85.2	83.7
15	121.2	57.9	31.0	24.2	23.8	30.9	31.0
16	30.8	27.8	20.2	22.9	22.9	20.0	20.1
17	66.3	56.7	59.4	63.3	63.4	59.2	59.5
18	20.7	15.9	(17.3)	12.5	13.2	(17.2)	17.1
19	18.8	17.6	(17.3)	19.9	17.0	(17.1)	19.4
20	208.7	208.6	210.2	209.2	209.2	210.5	206.0
21	31.3	31.3	31.5	31.5	31.5	31.5	31.2

[a]Spectra were recorded in $CDCl_3$; TMS internal standard.
[b]Numbers in parenthesis in a column are either overlapping or interchangeable.
[c]Run at 57°.

The ^{1}H-nmr spectrum of the 8β-OH, 14-ene compound showed a high field doublet (δ 0.85, J=1.4) without one-bond ^{13}C-satellites, a vinylic proton (δ 5.55, dd, J=3.2 and 1.6), 4-H (δ 5.78, $W½$=3.8), 17-H (δ 2.93, dd, J=10.2 and 7.0), C-10 CH_3 (δ 1.36), C-13 CH_3 (δ 1.13), and C-20 CH_3 (δ 2.16), as well as the band of peaks between δ 1.2 and δ 2.5 typical of a steriod. These data, along with the ^{13}C-nmr spectrum, suggested that the progesterone skeleton was intact with the addition of one tertiary hydroxyl and one tri-substituted double bond. Irradiation of 4-H in a DDR experiment revealed the 6α-H, 6β-H, and 2β-H, while irradiation of C-10 CH_3 revealed 1α-H and 2β-H. Examination of these multiplets showed that there was no modification of ring A and no substitution at C-6 or C-7. The lack of ^{13}C satellites, high field

TABLE 3. ^{1}H-nmr Data for 8β-Hydroxypregna-4,14-diene-3,20-dione and 14,15α-Epoxypregn-4-ene-3,20-dione (excluding data in Table 1)

Proton	Chemical shifts in ppm (1st Order J values in Hz)	
	8βOH,14-ene	14α,15α-epoxide
1α	1.64[a] (J1α,1β=−13.2;[a] J1α,2α=4.8;[a] J1α,2β=14.6;[a] J1α,C-10CH_3=0.4)	1.79[a] (J1α,1β=−13.2;[a] J1α,2α=6.1;[a,b] J1α,2β=13.2;[a,b] J1α,C-10CH_3=0.4)
1β	2.12[c] (J1β,2α=2.7; J1β,2β=5.2)	2.09 (J1β,2α=3;[a] J1β,2β=5[a])
2α	2.36 (J2α,2β=−17.2; J2α,4=0.6)	2.4[b,c] (J2α,2β=−17;[a] J2α,4=0.6)
2β	2.50	2.4[b,c]
4	5.78 (J4,6α<1;[a] J4,6β=1.9)	5.74 (J4,6α=0.5;[a] J4,6β=2.0[a])
6α	2.21[a] (J6α,6β=−14.2;[a] J6α,7α=4.0;[a] J6α,7β=2.7[a])	2.27[a] (J6α,6β=−14.4; J6α,7α=4.6;[a] J6α,7β=2.5[a])
6β	2.94 (J6β,7α=11.3; J6β,7β=4.8)	2.40[a] (J6β,7α=14.4;[a] J6β,7β=5.5[a])
7α	1.79[a] (J7α,7β=−14.2;[a,b] J7α,8-OH=0.3[a])	1.38[c,d] (J7α,7β=−13;[c] J7α,8=11.4[e])
7β	2.12[d]	1.51 (J7β,8=3.9[3])
8	0.85 (J8-OH,9=1.5)	2.31[e] (J8-H,9=11.4[e])
9	1.17[a] (J9,11α=2.5;[a] J9,11β=12.3[a])	1.43[c] (J9,11α=3;[c] J9,11β=16[c])
11α	2.08[c,d] (J11α,11β=−13;[c] J11α,12α=4;[a] J11α,12β=3.5)	1.75[d] (J11α,11β=−13;[b] J11α,12α=4.2;[a] J11α,12β=3.5)
11β	1.62[d] (J11β,12α=13;[d] J11β,12β=4[b,d])	1.57 (J11β,12α=12.5;[a,b] J11β,12β=3.7)
12α	1.65[d] (J12α,12β=−13.0[b])	1.82[d] (J12α,12β=−12.5[b,d])
12β	2.32	2.06[d]
15	5.55 (J15,16α=1.6; J15,16β=3.2)	3.45 (J15β,16α=1; J15β,16β=1)
16α	2.21 (J16α,16β=−16.2; J16α,17=7.0)	1.98[a] (J16α,16β=−14.4;[a] J16α,17=7.1[a])
16β	2.83[a] (J16β,17=10.2[a])	2.06[a] (J16β,17=10.2[a])

[a]Value obtained from difference-double-resonance (DDR) experiment.
[b]Strong coupling. Reported values are approximations only.
[c]Value obtained from heteronuclear correlation spectrum.
[d]Value obtained from COSY spectrum.
[e]Value obtained from nOe difference experiment.

shift, and small coupling constants suggested that the signal at δ 0.85 was due to a non-hydrogen bonded tertiary hydroxyl. Irradiation of this signal in a DDR experiment revealed a multiplet at δ 1.17 (dd, J=12.3 and 2.5) and a multiplet at δ 1.79 (ddd, J=14.2, 11.3, and 4.0). The COSY spectrum showed that the low field multiplet was coupled to 6α-H and 6β-H. Because this multiplet shows axial coupling, it was assigned to the 7α-H and the hydroxyl signal to the 8β-OH. The high field shift and coupling constants characteristic of an axial cyclohexyl proton implied that the other multiplet was due to 9-H rather than 14-H. Irradiation of the vinylic signal at δ 5.55 in a DDR experiment revealed multiplets at δ 2.83 (dd, J=16.2 and 10.2) and δ 2.21 (dd, J=16.2 and 7.0). These multiplets were shown by the COSY spectrum to be both coupled to 17-H and to each other and were, therefore, assigned to 16β-H and 16α-H, respectively. The large (and presumably negative) geminal coupling confirms the proximity of a π system. Careful examination of the COSY and resolution enhanced 1D spectra allowed all protons to be assigned (shift and coupling data reported in Tables 1 and 3). The ^{1}H assignments were used to confirm the ^{13}C assignments reported in Table 2 via the heteronuclear correlation experiment. These data clearly identified this metabolite as 8β-hydroxypregna-4,14-diene-3,20-dione.

The ^{1}H-nmr spectrum of the 14α,15α-epoxide showed a narrow triplet (δ 3.45, J=1.1) with one-bond ^{13}C satellites. Signals from 4-H (δ 5.73, bd, J=1.8), C-10 CH_3

(δ 1.22), C-13 CH_3 (δ 0.84), C-20 CH_3 (δ 2.11), and 17-H (δ 2.54, dd, J=1.0 and 7.1) could also be identified by inspection. From these data it was concluded that the progesterone skeleton was intact and that the signal at δ 3.45 was not due to a hydroxyl proton. DDR experiments from C-10 CH_3 and 4-H showed an unmodified ring A and no substitution at C-6 or C-7. Irradiation of the δ 3.45 signal in a DDR experiment revealed two multiplets (δ 1.98, dd, J=14.1 and 7.1; δ 2.06, dd, J=14.1 and 10.1) that the COSY spectrum showed to be coupled to 17-H and to each other. It was, therefore, concluded that these multiplets resulted from the 16α-H and 16β-H and that the signal at δ 3.45 was the single proton on C-15. A nuclear Overhauser effect difference spectroscopy (NOEDS) (9,12) experiment from C-13 CH_3 confirmed the low field signal as 16α-H. NOEDS from C-13 CH_3 also showed enhancement of 8-H, 9-H, 11β-H, and 17-H, while NOEDS from C-10 CH_3 showed enhancement of 8-H, 9-H, and 11β-H. Because 8-H lacked the expected diaxial coupling to 14-H, it followed that this metabolite was either the 14α,15α-epoxide or the 14α,15α-diol. The unusually small 15β-16 coupling constants, the lack of observable OH signals, and eims (molecular ion *m/z* 328) confirmed the structure as the epoxide. Careful examination of the COSY, heteronuclear correlation, and resolution enhanced 1D spectra allowed the assignment of all proton resonances with the results reported in Tables 1 and 3. ^{13}C assignments (Table 2) were confirmed via the heteronuclear correlation experiment. The coupling constants and nOe experiments confirmed the 14α,15α stereochemistry of the epoxide.

Compared with progesterone the ^1H-nmr spectrum of 14α-hydroxyprogesterone shows a downfield shift (0.12 ppm) in the C-13 CH_3 group with no significant change to the C-10 CH_3. The 17α-proton which is assigned to a triplet at δ 2.53 (J=9.0 Hz) in progesterone is shifted to δ 3.22 consistent with a 1,3-diaxial type interaction with the14α- hydroxyl group.

With the exception of the 6β-alcohol, where hydroxylation may take place through a different mechanism (13), hydroxylation has occurred on the α-face at the 7α-, 9α-, and 14α-axial positions. The formation of the 14α,15α-epoxide, and the 8β-hydroxy C-14 olefin suggests that the C-14 olefin is an intermediate probably resulting from dehydration of the major metabolite, 14α-hydroxyprogesterone. Analogous conversions at C-5 by *Mucor* have been reported (4). Formation of the 14α,15α-epoxide from the C-14 olefin is also consistent with the early observation by Bloom and Shull (14) regarding the regioselectivity and stereochemistry of oxidizing enzymes that "a microorganism capable of introducing an axial hydroxy-function at C-n of a saturated steroid will also effect the introduction of an epoxide grouping axial at C-n in the corresponding unsaturated steroid."

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^1H nmr and ^{13}C nmr were recorded (Tables 1-3) on a Bruker AM 300 instrument in $CDCl_3$. The mass spectrum was recorded on a Finnigan Quadrupole Model 1015 instrument at 70 eV using a direct probe. Tlc was conducted on SiO_2 gel (Merck type 60 H) in 75% EtOAc/C_6H_{14} (Rf values) and first visualized under uv (254 nm) followed by spraying with 4% H_2SO_4/EtOH and heating (5-10 min) at 110°. Hplc was carried out on a Waters μ-Porasil (10 μ) semipreparative column (7.8 mm×30 cm) using a Waters M45 instrument (injection volumes were 10 mg in 0.1 μl). Melting points were uncorrected. Elemental analyses (C,H) were performed by W. Baldeo, School of Pharmacy, University of London, England.

ISOLATION.—A fermentation medium consisting of the following was used: progesterone (1.5 g), D-glucose (100 g), $MgSO_4.7H_2O$ (5 g), KOAc (4 g), and H_2O (to 1 liter). The pH of the medium was adjusted to 6.8 with HOAc. For each fermentation 3.3 liters of medium was used in a 5-liter Erlenmeyer flask. The innoculum consisted of 50 ml of a 72 h culture of *M. griseocyanus* ATCC 1207a (+) grown in Sabourand-dextrose broth. The fermentation was allowed to proceed for 3 weeks at 20° while being stirred with a magnetic stirrer at 200 rpm. Without the innoculum only progesterone was recovered from incuba-

tion of the medium as described. The cells and other solids were collected by filtration and extracted repeatedly with CH_2Cl_2 (100-200 ml) until no further material was obtained (total volume ca. 1 liter). The combined extracts were dissolved in CH_2Cl_2 and passed through a column of SiO_2 gel (Merck type 60 H for tlc) (100 g). Elution with CH_2Cl_2, 1-2% Me_2CO/CH_2Cl_2 gave progesterone (1.63 g) (Rf 0.41), and further fractions were collected with the addition of 1% increments of Me_2CO. Fractions were monitored by tlc. Elution with 3-4% Me_2CO/CH_2Cl_2 gave a residue (100 mg) containing two components and was subjected to hplc separation in 1% Me_2CO/CH_2Cl_2 that yielded 8β-hydroxypregna-4,14-diene-3,20-dione (21 mg) (retention volume 75 ml) (Rf 0.37) mp 249-251° from Me_2CO (Found: C, 76.77; H, 8.61, $C_{21}H_{28}O_3$ requires C, 76.79; H, 8.59%) and 14,15α-epoxypregn-4-ene-3,20-dione (47 mg) (retention volume 92 ml) (Rf 0.28) mp 166-170° from Me_2CO, eims *m/z* 328 (M^+) (Found: C, 76.71; H, 8.59, $C_{21}H_{28}O_3$ requires C, 76.79; H, 8.59%). Elution with 4-5% Me_2CO/CH_2Cl_2 yielded fractions (1.9 g) which on recrystallization from CH_2Cl_2/EtOAc gave 14α-hydroxypregn-4-ene-3,20-dione (1.4 g) (Rf 0.24) mp 204.5-206° [lit. (3) mp 198-200°]; later fractions (50 mg) contained a second component separated by hplc in 1% EtOH/CH_2Cl_2 to give fractions (37 mg) (retention volume 53 ml), which on recrystallization from CH_2Cl_2/EtOAc gave 9α-hydroxypregn-4-ene-3,20-dione (Rf 0.21) mp 186-188° [lit. (15) 189-192°]. Elution with 10% Me_2CO/CH_2Cl_2 gave fractions (147 mg), which on recrystallization from CH_2Cl_2/MeOH gave 7α-hydroxypregn-4-ene-3,20-dione (Rf 0.17) mp 234-235° [lit. (16) mp 227-231°]. Similarly, 12-14% Me_2CO/CH_2Cl_2 gave 7α,14α-dihydroxypregn-4-ene-3,20-dione (264 mg) (Rf 0.13) mp 245-250° [lit. (17) mp 252-255°] from CH_2Cl_2/MeOH and 25% Me_2CO/CH_2Cl_2 gave 6β,14α-dihydroxypregn-4-ene-3,20-dione (112 mg) mp 241-245° [lit. (18) mp 242-249°] from CH_2Cl_2/Me_2CO. In subsequent experiments, a further portion (ca. 10%) of metabolites was extracted with ether from the aqueous filtrate.

ACKNOWLEDGMENTS

We thank the Manitoba Heart Foundation for financial support and Diane Smith for assistance with the hplc.

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Received 20 October 1986