3β,11α,17-TRIHYDROXY-5-PREGNEN-20-ONE 11-HEMISUCCINATE AS A HAPTENE FOR RIA OF "17α-HYDROXYPREGNENOLONE"*,**

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6β,17-Dihydroxy-3α,5-cyclo-5α-pregnan-20-one (*VII*) and 17-hydroxy-6β-methoxy-3α,5-cyclo--5α-pregnan-20-one (*IX*) were hydroxylated into the position 11α using *Rhizopus nigricans* fungi culture, and then selectively converted into haptene *XIII* for the RIA determination of 3β,17-dihydroxypregn-5-en-20-one (*IV*).

Recently, many hormonal disturbances can be easily identified using quantitative analysis[1] of individual steroid biosynthetic intermediates in the patient's body. In this context, chemical endocrinological laboratories needed another diagnostic tool enabling radioimmunoassay of "17α-hydroxy-pregnenolone" (3β,17-dihydroxypregn--5-en-20-one, *IV*). In this case, a compound with succinic acid moiety in position 11α seemed to be the haptene of choice because the bond of a steroid to albumin, mediated by this bridge, has been successfully used many times when a highly specific antibody, discriminating the substitution in the rings A and D, was required.

The preparation of the desired product (see Scheme 1) started from acetate *I*, accessible by alkaline epoxidation of 20-oxopregna-5,16-dien-3β-yl acetate according to Julian[2]. It appeared that of the methods used so far for the reduction of the 16α,17α-oxide to the 17α-hydroxy derivatives the later Julian's[3] method, consisting in hydrogenolysis of the corresponding bromohydrin *II*, gave the highest yields; preparatively advantageous was also the direct[4] hydride reduction of the 16,17--epoxide grouping after protection of the 20-keto group (dioxolane *V*).

However, the thus-obtained 3β,17-dihydroxy-5-pregnen-20-one (*IV*) could not be directly subjected to hydroxylation, e.g. by use of the fungus *Rhizopus nigricans* which with Δ^5-olefins proceeds preferentially[5] in the position 7. Nevertheless, for achieving the desired 11α-hydroxylation a simple method has been described[6-8]:

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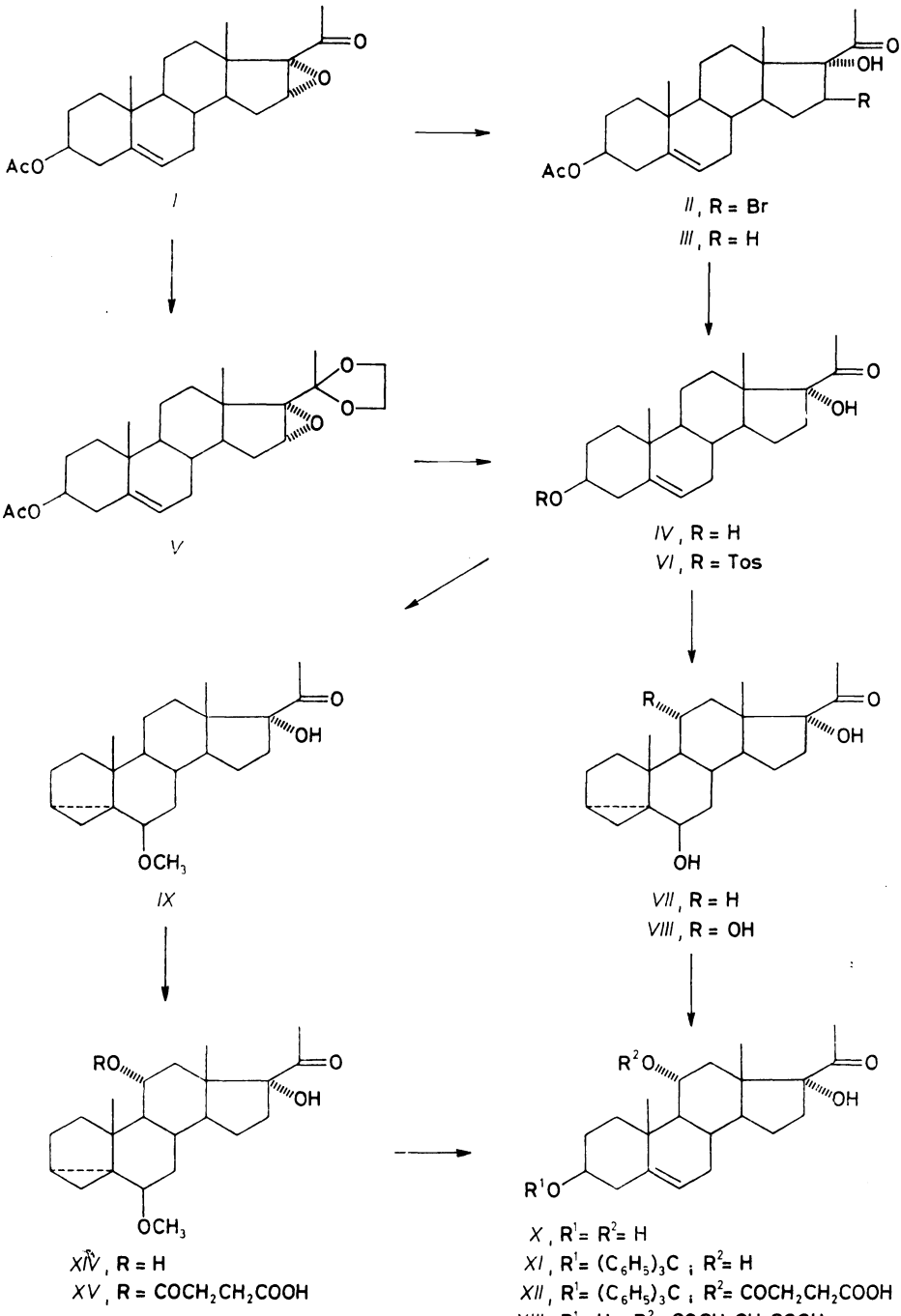
AcO
O
O
I
OH
R
II, R = Br
III, R = H
IV, R = H
VI, R = Tos
RO
V
OCH3
IX
VII, R = H
VIII, R = OH
XIV, R = H
XV, R = COCH2CH2COOH
R2O
R1O
X, R1 = R2 = H
XI, R1 = (C6H5)3C ; R2 = H
XII, R1 = (C6H5)3C ; R2 = COCH2CH2COOH
XIII, R1 = H ; R2 = COCH2CH2COOH

SCHEME 1

prior to hydroxylation, 3β-hydroxy-Δ^5-olefins of the type *IV* were converted into 6β-hydroxy-3α,5α-cyclo derivatives of the type *VII* which had been used in the steroid chemistry already many times as their synthetic equivalent. In this manner we prepared 6β,11α,17-trihydroxy derivative *VIII* which was epimerized directly to the known 3β,11α,17-trihydroxypregn-5-en-20-one[8] (*X*), containing two secondary hydroxy groups. The more reactive 3β-hydroxyl was preferentially etherified[9] with triphenylmethyl chloride and the obtained compound *XI* was reacted at the 11α--hydroxyl with succinic anhydride in pyridine. Finally, the trityl protecting group in position 3β of the product *XII* was removed by treatment with formic acid[10]. The structure of the hemisuccinate *XIII* has been confirmed by IR and ^{1}H NMR spectroscopy (see Experimental). The yield of this pathway was low, mainly because tritylation of the triol *X* was incomplete or, when working at higher temperatures with an excess of trityl chloride, the selectivity was lost and also the 11α-hydroxyl was tritylated.

For this reason, we worked out an alternative and more advantageous approach to compound *XIII*. In this case, the 3α,5α-cyclo derivative, used as the fermentation substrate, was not the 6β-hydroxy derivative *VII* but the methoxy compound[11] *IX* which also was hydroxylated into the position 11α by the fungus *Rhizopus nigricans* in a buffered medium. ^{1}H NMR spectrum of the product (*XIV*) showed the typical signal of an axial 11β-proton interacting with one equatorial (12β) and two axial (9β and 12β) protons. Compound *XIV* could be directly acylated with succinic anhydride to give the hemisuccinate *XV*. We have found conditions under which the protecting "i-steroid" (6β-methoxy-3α,5α-cyclosteroid) grouping in *XV* was opened without substantially endangering the 11α-ester bond or the 17α-ketol chain (short treatment with perchloric acid in an aprotic solvent). Suitable choice of the substrate for microbiological hydroxylation led thus to a higher yield and also the work-up of the subsequent chemical reactions was simpler.

Compound *XIII* was bound to albumin and the conjugate was used in preparation of the antibody for RIA of "17α-hydroxypregnenolone". The pertinent results will be published elsewhere.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. Optical rotations and IR spectra (Zeiss UR 20) were measured in chloroform solutions unless stated otherwise (wavenumbers in cm^{-1}). ^{1}H and ^{13}C NMR spectra were obtained in deuteriochloroform with tetramethylsilane as internal standard at 23°C either on a Tesla BS 497 spectrophotometer (100 MHz for ^{1}H, FT mode) or on a Varian XL-200 instrument (200 MHz for ^{1}H, 50·31 MHz for ^{13}C, FT mode). The latter instrument was also used for measurements of $^{13}C\{^1H\}$ spectra of solutions in deuteriochloroform with tetramethylsilane as internal standard. The chemical shifts are given in ppm (δ-scale), coupling constants (J) and half-height widths ($W_{1/2}$) in Hz: all parameters were obtained by analysis of the 1st order. Mass spectra were measured on a VG-ZAB-EQ

spectrometer (relative intensities referenced to the base peak and assignments are given in parentheses). The identity of compounds prepared by different procedures was proven by mixture melting point determination and comparison of their IR spectra. The reaction course and purity of the samples were followed by thin-layer chromatography on silica gel (TLC, Woelm DC, detection with sulfuric acid and heating), separation of compounds was performed using flash chromatography[12] on silica gel Silpearl (Kavalier, Votice) or by preparative TLC on silica gel (PLC) on 200 × 200 × 0·7 mm plates (Woelm DC, inspection at 254 nm after spraying with 0·02% solution of morin in methanol). *Rhizopus nigricans* culture was cultivated in a medium, containing corn-steep (5 g), glucose (11 g), $K_2HPO_4.3\ H_2O$ (0·52 g) and $MgSO_4.7\ H_2O$ (0·4 g) in 1 l of water, in 500 ml flasks on an orbital shaker at 25°C. After 48 h the culture was filtered, washed with distilled water and resuspended in a buffer (according to Sörensen, pH 7·4, prepared from aqueous solutions of KH_2PO_4 and $Na_2HPO_4.12\ H_2O$). A solution of the steroid in dimethyl sulfoxide (30 mg of the steroid in 0·5 ml of dimethyl sulfoxide into 100 ml of the buffer) was added into the flasks. The mixture was incubated at 25°C for 48 h with shaking on an orbital shaker.

16α,17-Oxido-20-oxopregn-5-en-3β-yl Acetate (*I*)

The compound was prepared according to Julian[2] by reaction of hydrogen peroxide (30%; 50 ml) and potassium hydroxide (5·5 g; 98 mmol) with 20-oxopregna-5,16-dien-3β-yl acetate (12 g; 33·7 mmol) in a mixture of methanol (800 ml) and water (24 ml). The product was reacetylated; yield after crystallization from methanol 10·3 g (82%), m.p. 156–158°C (reported[13] m.p. 156–158°C).

20,20-Ethylenedioxy-16α,17-oxidopregn-5-en-3β-yl Acetate (*V*)

The title compound was prepared according to ref.[4] from epoxide *I* (18 g; 48·3 mmol), ethylene glycol (20 ml; 0·36 mol) and *p*-toluenesulfonic acid monohydrate (1·6 g; 8·4 mmol) as catalyst in boiling benzene (800 ml) using a Dean–Stark apparatus. After 4 h, the mixture was worked up by crystallization of the products from methanol and then from toluene; yield 11·1 g (55%) of compound *V*, m.p. 195–198°C, which was used in the next experiment.

17-Hydroxy-20-oxopregn-5-en-3β-yl Acetate (*III*)

A) From bromohydrin *II* and tributyltin hydride: a solution of tributyltin hydride (1 mol l^{-1}) in toluene (0·6 ml) and 2,2′-azobis(2-methylpropionitrile) (5 mg; 0·04 mmol) were added to a solution of compound *II* (60 mg; 0·13 mmol) in toluene (2 ml). The mixture was heated to 80°C for 5 h, the solvent was evaporated and the dry residue was diluted with saturated aqueous solution of potassium fluoride (5 ml). The organic layer was extracted with ethyl acetate, the solvent was evaporated and the residue applied onto 2 preparative layers of silica gel which were developed with chloroform–ether–toluene (1 : 1 : 1) mixture. The zone of the principal product (R_F 0·6) was continuously extracted with boiling ether to give 34 mg (69%) of the product *III*, m.p. 230–234°C (reported[3] m.p. 232–235°C).

B) From bromohydrin *II* under conditions of catalytic hydrogenation. A mixture of bromohydrin *II* (2·5 g; 5·5 mmol), ammonium acetate (6 g), methanol (50 ml) and palladium on carbon (5%; 2·5 g) was shaken with hydrogen for 2 h. The catalyst was filtered off and the filtrate was partially concentrated in water. The product was precipitated by addition of water, filtered, washed with water and crystallized from methanol, m.p. 233–236°C; yield 1·8 g (87%).

3β,17-Dihydroxypregn-5-en-20-one (*IV*)

A) A mixture of epoxide *V* (1·1 g; 2·64 mmol) and solution of lithium aluminium hydride (about 200 mg) in tetrahydrofuran (25 ml) was refluxed for 2 h in a nitrogen atmosphere. After the usual work-up procedure, the ethylenedioxy protecting group was removed by boiling with a solution of sulfuric acid (2·5 ml; 46·9 mmol) in aqueous methanol (83%; 60 ml) for 1 h. The hot mixture was mixed with water (40 ml), cooled, and after standing at 0°C for 2 h, the product was filtered; yield 0·58 g (66%), m.p. 266–271°C (reported[4] m.p. 270–272°C).

B) An aqueous solution of potassium carbonate (10%; 6 ml) was added to a solution of acetate *III* (1·5 g; 4 mmol) in methanol (45 ml) and the mixture was refluxed for 1 h. The excess base was destroyed with acetic acid and the solution was concentrated to crystallization. Yield 1.1 g (83%), m.p. 260–264°C.

17-Hydroxy-20-oxopregn-5-en-3β-yl *p*-Toluenesulfonate (*VI*)

p-Toluenesulfonyl chloride (2·6 g; 13·6 mmol) was added to a solution of diol *IV* (1·5 g; 4·5 mmol; dried by distillation with toluene) in pyridine (40 ml); after standing at 20°C for 23 h, the solution was poured into a stirred saturated aqueous solution of sodium chloride and the flask was rinsed with methanol (2 × 4 ml). The mixture was left aside at −18°C for 1 h, the separated product was filtered and washed with water and light petroleum. Yield 1·8 g (82%), m.p. 147–149°C (reported[8] m.p. 138–140°C). ^{1}H NMR spectrum (100 MHz): 0·71 s, 3 H (3 × H-18); 0·97 s, 3 H (3 × H-19); 2·25 s, 3 H (3 × H-21); 2·44 m, 3 H (CH_3-arom); 4·30 mt, 1 H (H-3, $W_{1/2}$ = = 29); 5·31 mt, 1 H (H-6, $W_{1/2}$ = 9); 7·3–8·0 m, 4 H (H-arom).

6β,17-Dihydroxy-3α,5-cyclo-5α-pregnan-20-one (*VII*)

The compound was prepared according to ref.[8] by refluxing a mixture of tosylate *VI* (5 g; 10·3 mmol), aqueous acetone (90%, 100 ml) and potassium acetate (5 g; 51 mmol) in an argon atmosphere for 6 h. The mixture was concentrated in vacuo, diluted with water, the separated product was collected and crystallized from a mixture of ethyl acetate and acetone; yield 2·1 g (61%) of product *VII*, m.p. 216–225°C (reported[8] m.p. 226–229°C). ^{1}H NMR spectrum (100 MHz): 0·77 s, 3 H (3 × H-18); 1·04 s, 3 H (3 × H-19); 2·25 s, 3 H (3 × H-21); 3·23 m, 1 H (H-6, $W_{1/2}$ = 7).

17-Hydroxy-6β-methoxy-3α,5-cyclo-5α-pregnan-20-one (*IX*)

A solution of tosylate *VI* (1·8 g; 3·7 mmol) in methanolic sodium methoxide solution (0·43 mol . l^{-1}; 150 ml; 65 mmol) was refluxed under argon for 2 h. The solution was concentrated in vacuo, the product was precipitated with water and the mixture was set aside at −18°C for 1 h. The precipitate was filtered, washed with light petroleum and crystallized from toluene; yield 1·12 g (91%), m.p. 223–225°C (reported[11] m.p. 210–213°C for a sample prepared by another route). ^{1}H NMR spectrum (200 MHz): 0·45 dd, 1 H (H-4β, J(gem) = −4, J(4β, 3β) = 8); 0·66 dd, 1 H (H-4α, J(gem) = −4, J(4α, 3β) = 5·3); 0·78 s, 3 H (H-18); 1·03 s, 3 H (H-19); 2·28 s, 3 H (H-21); 2·80 dd, 1-H (H-6α, J(6α, 7β) = 3·1, J(6α, 7α) = 5·5); 3·34 s, 3 H (OCH_3).

3β,11α,17-Trihydroxypregn-5-en-20-one (*X*)

A solution of 6β-hydroxy derivative *VII* (60 mg; 0·18 mmol) in dimethyl sulfoxide (1 ml) was treated with a culture of *Rhizopus nigricans* in two flasks. After 48 h, the content of the flasks was filtered, the mycelium washed with water and the filtrate extracted with ethyl acetate. The

product (60 mg) consisting of i-steroid *VIII* and Δ^5-unsaturated compound *X*, was dissolved in a chloroform–acetone mixture (1 : 1; 4 ml) containing perchloric acid (4 drops). After 8 min the mixture was concentrated in vacuo to a quarter of the original volume, diluted with chloroform and washed with potassium hydrogen carbonate solution and water, dried over sodium sulfate and the solvent was evaporated. The residue (42 mg) was crystallized from toluene–methanol yielding 25 mg (40%) of triol *X*, m.p. 253–256°C (reported[8] m.p. 250–254°C). ^{1}H NMR spectrum (CD_3OD, residual methanol signal as standard, δ 3·55): 0·81 s, 3 H (3 × H-18); 1·35 s, 3 H (3 × H-19); 2·39 s, 3 H (3 × H-21); 3·60 m, 1 H (H-3, $\sum J$ 29·0); 4·19 ddd, 1 H (J(11β, 12β) = 5·2, J = 11·0, J' = 10·2); 5·61 bd, 1 H (J(6, 7β) = 5·2, J(6, 7α) = 1·4).

11α,17-Dihydroxy-6β-methoxy-3α,5-cyclo-5α-pregnan-20-one (*XIV*)

The 5β-methoxy derivative *IX* (0·6 g; 1·73 mmol) was hydroxylated similarly as described in the preceding experiment in 20 flasks containing each 100 ml of the buffer and *Rhizopus nigricans* culture. The mixture was worked up as in the preceding example; ethyl acetate used for the extraction was freshly shaken with aqueous solution of potassium hydrogen carbonate. The product (0·6 g), consisting of *XIV* and *IX* (9 : 1) (TLC in ether–light petroleum 6 : 4), on repeated crystallization from toluene afforded compound *XIV*, m.p. 214–216°C (toluene). IR spectrum (KBr): 3 390, 3 345 (OH); 3 070, 3 020 (cyclopropane); 2 830, 1 079, 1 069 (OCH_3); 1 708 (CO). ^{1}H NMR spectrum (200 MHz, C_4D_5N): 0·48 dd, 1 H (4β-H, J(gem) = −4·9, J(4β, 3β) = 7·9); 0·84 s, 3 H (3 × H-18); 1·37 t, 1 H (9α-H, J(8β, 9α) = 10·3, J(11, 9α) = 10·2); 1·51 s, 3 H (3 × H-19); 2·22 dd, 1 H (H-12β, J(12α, 13β) = −11·8, J(11, 12β) = 4·7); 2·38 s, 3 H (H-21); 2·65 bt, 1 H (H-12α, J(12α, 12β) = −11·8, J(11, 12α) = 11·2); 2·75 t, 1 H (H-6, $\sum J$ = 5·4); 3·35 s, 3 H (OCH_3); 4·31 ddd, 1 H (H-11, J(11, 12β) = 4·7, J(11, 12α) = 11·2, J(11, 9α) = 10·2). ^{13}C NMR spectrum (C_5D_5N, referenced to α-atom signals of the solvent, δ 144·9): 13·4 (C-4), 16·61 (C-3), 20·40 (C-18), 22·83 (C-19), 56·55 (OCH_3), 27·22, 29·74, 50·90, 53·54, 69·92, 82·57 (C-6, C-8, C-9, C-11, C-14, C-21), 24·16, 25·35, 33·63, 35·34, 36·09, 43·49, 43·93, 47·92, 56·27, 90·25 (C-1, C-2, C-5, C-7, C-10, C-12, C-13, C-15, C-16, C-17), 210·89 (C-20). For $C_{22}H_{34}O_4$ (362·5) calculated: 72·89% C, 9·45% H; found: 72·10% C, 9·44% H.

11α,17-Dihydroxy-3β-triphenylmethoxypregn-5-en-20-one (*XI*)

4-Dimethylaminopyridine (5 mg; 40 μmol) and triethylamine (0·15 ml; 1·08 mmol) were added at room temperature to a stirred suspension of triol *X* (75 mg; 0·22 mmol) and triphenylmethyl chloride (150 mg; 0·54 mmol) in dichloromethane (0·75 ml). After standing for 60 h, the solution was diluted with chloroform, washed with potassium carbonate solution and water and dried over anhydrous magnesium sulfate. The residue was subjected to preparative thin-layer chromatography on silica gel (2 preparative plates pretreated with ammonia vapours) in toluene–ether 1 : 1. The principal product (R_F 0·3) was extracted with ether to give 54 mg (42%) of *XI*, m.p. 142–144°C (toluene–heptane). IR spectrum: 3 605, 1 058 (OH); 1 709, 1 366, 1 358 ($COCH_3$); 1 601, 1 495, 1 450, 915 (C_6H_5). ^{1}H NMR spectrum (100 MHz): 0·69 s, 1 H (3 × H-18); 1·09 s, 3 H (3 × H-19); 2·25 s, 3 H (3 × H-21); 3·40 m, 1 H (H-3, $W_{1/2}$ = 28); 4·02 ddd, 1 H (H-11, J(11, 12β) = 5, J(11, 12α) = 11, J(11, 9α) = 10); 4·95 bd, 1 H (H-6, J = 5); 7·30–7·63 m, 15 H (H-arom). For $C_{40}H_{46}O_4$ (590·8) calculated: 81·32% C, 7·85% H; found: 81·26% C, 8·02% H.

The more polar part (R_F 0·10) consisted in the starting triol *X* (30 mg; 40%), whereas the more lipophilic portion (130 mg) contained triphenylmethanol, triphenylmethyl chloride and another triphenylmethoxy steroid (which upon detritylation also afforded the starting *X*, 10·7%). When the reaction was performed at higher temperature, not only the starting compound but also the monotritylation product disappeared and the amount of the ditritylation product increased.

17-Hydroxy-3β-triphenylmethoxy-20-oxopregn-5-en-11α-yl Hydrogen Butanedioate (*XII*)

A mixture of the trityl ether *XI* (50 mg; 0·084 mmol), succinic anhydride (100 mg; 0·85 mmol), 4-dimethylaminopyridine (2 mg; 16 μmol) and pyridine (1 ml) was heated under argon in a sealed ampoule for 2 h (bath temperature 140–150°C). After evaporation in vacuo, the dry residue was heated with acetone (about 1 ml) and the acetone extract was subjected to PLC (2 plates 200 × 200 ml) on silica gel in ether. The sharp zone of R_F 0·6 was eluted with ether, the extract was concentrated and rechromatographed on thin layer of silica gel in ether. This time the zone of R_F 0·6 contained the neutral unreacted compound *XI* (19 mg; 38%) whereas the desired ester *XII* (36 mg; 52·7%) was obtained from the polar zone (R_F 0·0–0·2), m.p. 187–191°C (acetone–heptane). IR spectrum: 3 610 (OH); 3 515, 1 722 (COOH); 1 600, 1 443, 712 (C_6H_5); 1 060 (–O–); 1 722 (C=O, ester). ^{1}H NMR spectrum (100 MHz): 0·76 s, 3 H (3 × H-18); 1·01 s, 3 H (3 × H-19); 2·23 s, 3 H (3 × H-21); 2·61 m, 4 H ($COCH_2CH_2CO$); 3·35 m, 1 H (H-3, $W_{1/2}$ = = 27); 4·87 d, 1 H (H-6, J = 5); 5·21 m, 1 H (H-11, $W_{1/2}$ = 26); 7·49 m, 15 H(H-arom.). For $C_{44}H_{50}O_7$ (690·8) calculated: 76·49% C, 7·30% H; found: 76·12% C, 7·51% H.

17-Hydroxy-6β-methoxy-20-oxo-3α,5-cyclo-5α-pregnan-11α-yl Hydrogen Butanedioate (*XV*)

The compound was prepared as described in the preceding experiment. Compound *XIV* (50 mg; 0·14 mmol) was esterified with succinic anhydride (180 mg; 1·52 mmol) in pyridine (1·8 ml) at 150°C. The crude product in acetone solution was purified by TLC on silica gel (2 plates 200 × 200 mm) in ether–light petroleum (7 : 11). The zone of R_F 0·40 was eluted with ether; the extract (83 mg) still contained traces of the starting compound *XIV* (the purity was checked by TLC in the same solvent system which had been shaken with aqueous ammonia: the neutral starting compound had R_F 0·25, the acidic product R_F 0·00). Repeated TLC afforded 57 mg (89%) of *XV*, m.p. 172–174°C (acetone–toluene). Mass spectrum: 462 (M^+), 312 ($M^+ - CH_3COO - CH_3OH$), 256, 213, 167, 149. For $C_{26}H_{38}O_7$ (462·6) calculated: 67·51% C, 8i28% H; found: 67·33% C, 8·49% H.

3β,17-Dihydroxy-20-oxopregn-5-en-11α-yl Hydrogen Butanedioate (*XIII*)

A) A solution of perchloric acid (72%; 4 drops) in a mixture of acetone and chloroform (1 : 1; 4 ml) was added to compound *XV* (50 mg; 0·11 mmol) and the mixture was intensively shaken at room temperature for 8 min. After dilution with chloroform (20 ml) and concentration in vacuo to 1/4 of the original volume, the residue was diluted with saturated sodium chloride solution and made alkaline with potassium hydroxide (10%; 18 drops). The solution was extracted with chloroform (3 × 30 ml), the extract was washed with water, dried over sodium sulfate, concentrated, and subjected to TLC on silica gel in ether containing 1% of acetic acid. The zone of R_F 0·35 was eluted with boiling ether; yield 33 mg (68%) of *XIII*, m.p. 236–242°C (acetone). ^{1}H NMR spectrum (200 MHz): 0·80 s, 3 H (3 × H-18); 1·08 s, 3 H (3 × H-19); 2·26 s, 3 H (2 × H-21); 3·16 m, 1 H (H-3, $\sum J$ = 23); 5·31 ddd, 1 H (H-11, $J(9\alpha, 11\beta)$ = 10·2, $J(11\beta, 12\alpha)$ = 10·2, $J(11\beta, 12\beta)$ = 5·8); 5·42 bd, 1 H (H-6, $J(6, 7\beta)$ = 5). For $C_{25}H_{36}O_7$ (448·5) calculated: 66·94% C, 8·09% H; found: 66·50% C, 8·21% H.

B) Compound *XII* (38 mg) was dissolved in a mixture of ether and formic acid (2 : 3; 1 ml) and the solution was set aside for 10 min. After concentration in vacuo, the residue was chromatographed on thin layer of silica gel (200 × 200 mm plate) in chloroform–acetone (4 : 1) and the product-containing zone was eluted as described above. Yield of ester *XIII* was 25 mg (92%).

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