PREPARATION AND PROPERTIES OF THREE IMMUNOGENS WITH A SPACER ON THE β-SIDE OF THE STEROID SKELETON AND THE EFFECT OF IMMUNOGEN STRUCTURE ON SPECIFICITY OF THE OBTAINED ANTISERA*

Alexander KASAL[a], Richard HAMPL[b], Zdeněk PUTZ[c], Ladislav KOHOUT[a] and Luboslav STÁRKA[b]

[a]*Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6*
[b]*Institute of Endocrinology, 116 94 Prague 1*
[c]*Institute of Endocrinology, 034 91 Lubochňa*

Received December 18, 1991
Accepted January 18, 1992

Three haptens for the determination of Reichstein's compound S and progesterone were prepared in which hemisuccinyloxy group was attached to the β-side of the steroid skeleton (compounds *VII*, *XIII* and *XV*). These haptens were bound to BSA and used for preparation of antisera. The specificity of these antisera was compared with that of antisera obtained with usual immunogens.

It is generally known that the position and steric orientation of the spacer by which a steroidal skeleton is bound to the protein carrier, as well as its chemical character and chain length, represent the decisive factors influencing the specificity of the obtained antisera[1].

So far, the preparation of immunogens utilized both natural functional groups (e.g. oxygen-containing groups in positions 3, 11, 17 or 21) and derivatives bound via positions that in natural steroids are usually unsubstituted. Of the latter group of compounds we may mention particularly derivatives with spacer in positions 6α, 6β, 7α, 7β, and 19, on which specific antisera have been based[2]. For practical reasons (low reactivity of the 11β-hydroxyl group) no haptens with spacer in position 11β have been prepared.

To steroids, for which preparation of sufficiently specific antisera is difficult, belongs also 11-deoxycortisol (Reichstein's compound S, 17,21-dihydroxy-4-pregnene-3,20-dione, *I*); with these antisera cross-reactions with related compounds, particularly with cortisol, frequently occur. So far, they have been prepared from immunogens with

* Part CCCLXV in the series On Steroids; Part CCCLXIV: Collect. Czech. Chem. Commun. *57*, 1928 (1992).

spacer in position 3 (refs[3,4]), 6 (ref[5]), 7 (ref.[6]), and 21 (refs[2,7]); the best antiserum was reported to be that derived from the not well accessible[6] derivative *II* in which the spacer is located on ring B of the steroid skeleton. For this reason we considered it useful to verify whether the easily accessible cortisol (*III*), with hydroxyl group on the ring C of the steroid skeleton, can serve as the starting material for the preparation of immunogen in which this 11β-hydroxyl group would be utilized for the construction of the desired spacer.

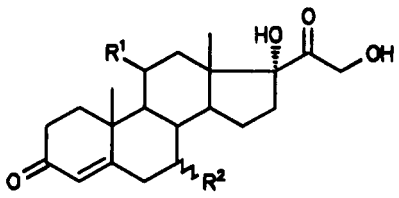
Cortisol 11β-hemisuccinate has not been prepared so far; the direct succinylation of cortisol leads only to the 21-hemisuccinate *IV* which has been used for RIA of cortisol[9] and also as an application form of cortisol, liberating the hormone proper under physiological conditions[10,11]. We found that the 11β-hydroxyl group can be acylated after protection of the cortisol primary hydroxyl as the 21-tert-butyldimethylsilyl ether *V*, if this acylation is performed under catalysis with 4-dimethylaminopyridine[12]. The acylation itself was performed indirectly[13,14] by treatment with 2-(trimethylsilyl)ethyl hydrogen butanedioate (*VIII*) under formation of the succinate *VI*. Both the silicone-containing protecting groups in the compound *VI* were removed by reaction with tetrabutylammonium tetrafluoride under formation of the desired 11β-hemisuccinyloxy derivative *VII*.

From the theoretical viewpoint, it was of interest to verify that the antiserum derived from 11β-hydroxyprogesterone exhibits the same specificity as that prepared from the currently used 11α-hydroxyprogesterone. Therefore, we subjected 11β-hydroxyprogesterone (*X*) to the same indirect hemisuccinylation using the compound *VIII* and the analogous reaction with compound *IX*. In the obtained products *XI* and *XII* we removed the protecting groups (in the latter case by treatment with zinc in acetic acid) and obtained the desired hemisuccinate *XIII*.

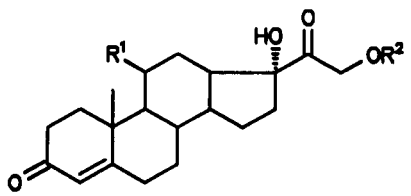
To verify the suitability of using haptens with spacer on the β-side of the steroidal skeleton we also prepared the hitherto undescribed 19-hydroxyprogesterone hemisuccinate (*XV*), in this case by standard acylation of hydroxy derivative *XIV* with succinic anhydride in pyridine.

The structure of the products follows from their preparation; moreover, it was confirmed by their spectral data (IR, MS, ^{1}H NMR, see Experimental) which proved that under the reaction conditions used no undesired reaction occured with the sensitive substrates (e.g. D-homoannulation at the expenses of the dihydroxyacetone side chain[15]).

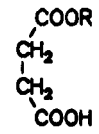
Compounds *VII*, *XIII*, and *XV* were bound to bovine serum albumin (BSA) using the method of mixed anhydrides according to Erlanger[16]. The antigens were used for immunization of rabbits[17], the molar ratios steroid : BSA being 16, 5, and 8 for compounds *VII*, *XIII*, and *XV*, respectively (as determined by UV spectra of conjugates and the derivatives).



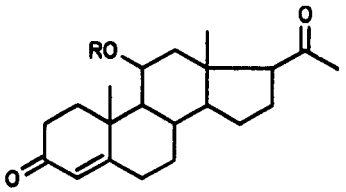
I, $R^1 = H$; $R^2 = H$
II, $R^1 = H$; $R^2 = CH_2COOH$
III, $R^1 = OH$; $R^2 = H$



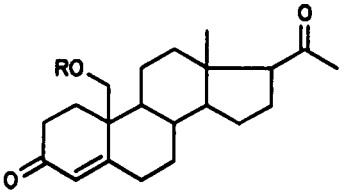
IV, $R^1 = H$; $R^2 = OCCH_2CH_2COOH$
V, $R^1 = H$; $R^2 = (CH_3)_3C(CH_3)_2Si$
VI, $R^1 = OCCH_2CH_2COOTse$;
$R^2 = (CH_3)_3C(CH_3)_2Si$
VII, $R^1 = OCCH_2CH_2COOH$; $R^2 = H$



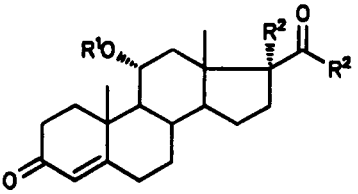
VIII, R = Tse
IX, $R = CH_2CCl_3$



X, R = H
XI, $R = OCCH_2CH_2COOTse$
XII, $R = OCCH_2CH_2COOCH_2CCl_3$
XIII, $R = OCCH_2CH_2COOH$



XVI, R = H
XV, $R = OCCH_2CH_2COOH$



XVI, $R^1 = OCCH_2CH_2COOH$; $R^2 = OH$
XVII, $R^1 = OCCH_2CH_2COOH$; $R^2 = H$

Tse = $CH_2CH_2Si(CH_3)_3$

Immunization of rabbits with the conjugate of compound *VII* with BSA afforded two antisera (working names 86 and 128) which at the experimental dilution 1 : 100 specifically bonded 5 – 10% of [^{3}H]11-deoxycortisol (*I*, 167 Bq per test tube). Therefore we prepared the bridge and position homologous [^{125}I]iodohistaminyl derivative of compound *VII*, as well as the corresponding [^{125}I]iodohistaminyl derivative of the 11α-epimeric compound *XVI* and both were tested as radioligands with the obtained antisera 86 and 128. In contrast to the tritiated radioligand, these antisera did not bind the iodinated radioligands at all.

Immunization with conjugates of progesterone derivatives *XIII* and *XV* afforded four antisera (97, 98 and 99, 100, respectively) which specifically bonded [^{3}H]progesterone at working dilutions at least 1 : 600. Nevertheless, neither of them bonded specifically the homologous [^{125}I]iodohistaminyl derivatives of compounds *XIII* and *XV*. In the case of 19-substituted hapten, the corresponding antisera bonded [^{3}H]progesterone which practically could not be expelled (obviously due to the bridge effect[1]) by an excess of unlabeled progesterone. As seen from Table I, the specificity of the obtained progesterone antisera is not higher than that of the currently used antiserum against 11α-hydroxyprogesterone hemisuccinate (*XVII*, see ref.[18]). The lower cross-reaction of the antisera toward the immunogen prepared via the position 19 (compound *XV*) by 11β-hydroxyprogesterone (*X*) may by of advantage only in special cases when greater amounts of this steroid are present in the analysed material: 11β-hydroxyprogesterone (*X*) cross-

TABLE I

Cross-reactions (%) of selected steroids with antisera against progesterone immunogens (*XIII*, *XV*, and *XVII*). The antisera (working dilution 1 : 4 000 for antiserum against compound *XVII*, 1 : 1 600 for 97, 98, and 1 : 600 for 99 and 100) were incubated at 0 – 4 °C with [^{3}H]progesterone (167 Bq per test tube) overnight, with or without excess (1 μmol/l) of unlabeled progesterone. Dextrane-coated charcoal was used for separation of bound from the free (radio)ligand

Steroid	*XVII*	*XIII*		*XV*	
		97	98	99	100
Progesterone	100	100	100	100	100
11α-Hydroxy-4-pregnene-3,20-dione	8.1	92	161	4.3	0.6
11β-Hydroxy-4-pregnene-3,20-dione	32.4	118	198	9.0	1.8
17α-Hydroxy-4-pregnene-3,20-dione	6.0	3.0	5.8	4.1	11
5α-Pregnane-3,20-dione	2.0	8.4	29	16	14
20α-Hydroxy-4-pregnen-3-one	5.9	5.1	1.7	3.4	1.7
20β-Hydroxy-4-pregnen-3-one	0.14	1.1	1.0	1.7	1.1
16α-Hydroxy-4-pregnene-3,20-dione	1.4	0	1.0	1.2	1.3
21-Hydroxy-4-pregnene-3,20-dione	7.1	2.0	2.1	4.6	3.7
Cortisol	0.2	0.3	0.07	0	0

reacted with antisera against the homologous (11β) as well as heterologous (11α) immunogen.

It seems therefore that the hemisuccinyloxy group, so successful with substrates with accessible OH groups, is not so effective with esters of 11β-hydroxy steroids, in which obviously no suitable distance is achieved between the protein carrier and the steroid bound to it.

EXPERIMENTAL

The melting points were determined on a Kofler block and are uncorrected. Unless otherwise stated, the IR spectra were measured in chloroform on a Zeiss UR 20 instrument (wavenumbers in cm^{-1}). The ^1H NMR spectra were taken in deuteriochloroform on Tesla BS-497 (FT mode, 100 MHz) or Varian XL-200 (FT mode, 200 MHz) instruments at 23 °C with tetramethylsilane as internal standard; chemical shifts are given in ppm (δ-scale), coupling constants (J) and halfwidths ($W_{1/2}$) in Hz. All the values were obtained by first order analysis. Thin-layer chromatography (200 × 200 × 0.7 mm) was performed on silica gel G according to Stahl (Woelm). The usual work-up means that solutions in organic solvents were washed, dried over sodium sulfate and concentrated in vacuo (about 2 kPa); analytical samples were dried over phosphorus pentoxide at 45 °C and 30 Pa for 1 h. [1,2,6,7-^3H]-Progesterone of specific activity 3.1 TBq/mmol (Radiochemical Centre, Amersham, Great Britain) and [1,2-^3H]-11-deoxycortisol of specific activity 1.5 TBq/mmol (NEN, U.S.A.) were purified by TLC on Alufol F_{254} (Merck, F.R.G.) in the solvent systems cyclohexane–ethyl acetate 3 : 1 or dichloromethane–methanol 97 : 3. Steroids used for testing the cross-reactions were purchased from Sigma (U.S.A.) and Steraloids (Wilton, N.H., U.S.A.). Epicortisol 11-hemisuccinate (*XVI*) was prepared according to ref.[19]. The hemisuccinates *VII*, *XIII*, and *XV* were attached to BSA using the mixed anhydride acylation method[16]; the molar ratio of hapten to the carrier protein was determined from the absorption differences at 254 nm. Rabbits were immunized by an emulsion of the conjugate (100 μg/dose) in a mixture of the complete Freund's adjuvans and physiological solution according to Vaitukaitis[17]. The separation of the free and bonded ligands in the antisera testing was performed on activated carbon with dextran.

21-Tert-butyldimethylsilyloxy-11β,17-dihydroxypregn-4-ene-3,20-dione (*V*)

Cortisol (*III*; 330 mg, 0.99 mmol) was dried by distillation with toluene and dissolved in N,N-dimethylformamide (3 ml). To the stirred solution were added imidazole (65 mg, 0.95 mmol) and tert-butyldimethylsilyl chloride (250 mg, 1.66 mmol). After 15 min the reaction was quenched by addition of water (3 ml) and the solution was allowed to stand for 18 h at −5 °C. The separated product was collected, washed with water, dried and crystallized from chloroform–light petroleum; m.p. 213 – 216 °C. Yield 415 mg (95%). $[\alpha]_D$ +113° (*c* 1.1, $CHCl_3$). IR spectrum: 3 615 (OH); 1 662, 1 619 (C=C–C=O); 1 710, 1 726 (C=O); 842 (Si–C). ^1H NMR spectrum (200 MHz): 0.12 s, 6 H (2 × CH_3–Si); 0.93 s, 9 H ($C(CH_3)_3$); 0.96 s, 3 H (3 × H-18); 1.44 s, 3 H (3 × H-19); 3.50 s, 1 H (OH); 4.46 m, 3 H (H-11 and 2 × H-21); 5.68 m, 1 H (C=CH). For $C_{27}H_{44}O_5Si$ (476.7) calculated: 68.02% C, 9.30% H; found: 67.71% C, 8.91% H.

21-Tert-butyldimethylsilyloxy-17α-hydroxy-3,20-dioxo-4-pregnen-11β-yl 2-(Trimethylsilyl)ethyl Butanedioate (*VI*)

A solution of compound *V* (1.0 g, 1.48 mmol) in toluene (40 ml) was distilled to remove 10 ml of the azeotropic distillate. To the stirred mixture was added 2-(trimethylsilyl)ethyl hydrogen butanedioate (*VIII*; 1.0 g, 4.58 mmol) in toluene (3 ml). After addition of N,N'-dicyclohexylcarbodiimide (1.0 g, 4.85 mmol) and 4-dimethylaminopyridine (30 mg, 0.25 mmol) the mixture was warmed to 40 °C for 64 h. Then another

portion of N,N'-dicyclohexylcarbodiimide (0.26 g, 1.26 mmol) and 4-dimethylaminopyridine (20 mg, 0.16 mmol) was added amd the heating was continued for further 64 h. Then N,N'-dicyclohexylcarbodiimide (0.26 g) and 4-dimethylaminopyridine (20 mg) were again added. After total 13 days the mixture was poured into an aqueous solution of potassium hydrogen carbonate, and the product was taken up in chloroform. The extract was washed with water, dried by filtration through a layer of anhydrous sodium sulfate, the solvent was evaporated in vacuo and the residue chromatographed on silica gel (60 ml). Elution with ether–benzene (1 : 20) afforded 200 mg (20%) of the starting compound *V* and 670 mg (47%) of product *VI*, m.p. 139 – 140 °C (chloroform–heptane). IR spectrum: 3 610 (OH); 1 726, 1 164 (COO); 1 712 (CO); 1 666, 1 620 (C=C–C=C); 861, 842 (Si–C). ^{1}H NMR spectrum (200 MHz): 0.82 s, 3 H (3 × H-18); 0.91 s, 9 H ($(CH_3)_3C$); 1.29 s, 3 H (3 × H-19); 2.49 s, 4 H ($OCCH_2CH_2CO$); 4.16 m, 2 H ($COOCH_2$; 4.38 AB system, 2 H (*J*(A,B) = 21, 2 × H-21); 5.50 dd, 1 H (*J* = 6.5 and 3, H-11); 5.70 s, 1 H (C=CH). Mass spectrum, *m/z*: 676 (M^+). For $C_{36}H_{60}O_8Si_2$ (677.0) calculated: 63.87% C, 8.93% H; found: 63.41% C, 8.59% H.

17α,21-Dihydroxy-3,20-dioxo-4-pregnen-11β-yl Hydrogen Butanedioate (*VII*)

A solution of tetrabutylammonium fluoride in tetrahydrofuran (2.5 ml of 1M solution) was added to a solution of derivative *VI* (450 mg, 0.66 mmol) in tetrahydrofuran (2 ml). After standing for 48 h at 20 °C the solvent was evaporated in vacuo, the residue was dissolved in ethyl acetate and washed three times with saturated aqueous solution of sodium chloride. The solution was concentrated in vacuo and applied onto a column of silica gel (10 ml). Elution with benzene–ether–acetic acid (90 : 10 : 1) mixture afforded 310 mg (67%) of compound *VII*, m.p. 160 – 162 °C (acetone–water). IR spectrum: 3 607 (OH); 3 575 – 2 620 (COOH); 1 727, 1 161 (COOR); 1 715 (CO), 1 662, 1 619 (C=C–C=O). ^{1}H NMR spectrum (200 MHz): 0.83 s, 3 H (3 × H-18); 1.29 s, H (3 × H-19); 2.17 s, 4 H ($OCCH_2CH_2CO$); 4.28 AB system, 2 H (2 × H-21, *J*(A,B) = 21); 5.54 m, 1 H ($W_{1/2}$= 5, H-11); 5.70 s, 1 H (H-4). For $C_{25}H_{34}O_8$ (462.5) calculated: 64.92% C, 7.41% H; found: 64.49% C, 7.62% H.

3,20-Dioxo-4-pregnen-11β-yl 2-(Trimethylsilyl)ethyl Butanedioate (*XI*)

A solution of 11β-hydroxypregn-4-ene-3,20-dione[20] (*X*; 1.0 g, 3.03 mmol) in toluene (200 ml) was distilled to remove 10 ml of the azeotropic mixture. To the remaining solution was added under stirring 2-trimethylsilylethyl hydrogen butanedioate (*VIII*; 1.0 g, 4.58 mmol) in toluene (3 ml). After addition of N,N'-dicyclohexylcarbodiimide (1.1 g, 5.33 mmol) and 4-dimethylaminopyridine (100 mg, 0.82 mmol) the mixture was heated to 80 °C for 48 h. Another portion (0.30 g, 1.45 mmol) of N,N'-dicyclohexylcarbodiimide and 4-dimethylaminopyridine (20 mg, 0.16 mmol) was added and heating was continued for further 48 h. The mixture was poured into aqueous solution of potassium hydrogen carbonate and the product was taken up in chloroform. The extract was washed with water, dried by filtration through a column of anhydrous sodium sulfate and the solvent was evaporated. The residue was chromatographed on silica gel (60 ml) in benzene–ether (20 : 1) to give 901 mg (90%) of the starting compound *X* and 150 mg (9%) of ester *XI*. ^{1}H NMR spectrum (200 MHz): 0.79 s, 3 H (3 × H-18); 1.30 s, 3 H (3 × H-19); 2.09 s, 3 H (3 × H-21); 2.5 m, 4 H ($OCCH_2CH_2CO$); 4.09 t, 2 H (*J* = 8, $COOCH_2$); 5.62 dd, 1 H (*J* = 6 and 3, H-11); 5.71 s, 1 H (H-4).

3,20-Dioxo-4-pregnen-11β-yl 2,2,2-Trichloroethyl Butanedioate (*XII*)

A solution of 11β-hydroxypregn-4-ene-3,20-dione[20] (*X*; 53 mg, 0.16 mmol) in toluene (8 ml) was distilled to obtain 5 ml of azeotropic mixture. To the remaining solution were added N,N'-dicyclohexylcarbodiimide (60 mg, 0.29 mmol), 2,2,2-trichloroethyl hydrogen butanedioate (*IX*; 100 mg, 0.40 mmol) and 4-dimethylaminopyridine (3 mg, 20 μmol). The mixture was stirred at 90 °C for 18 h and then another portion of N,N'-dicyclohexylcarbodiimide (30 mg, 0.15 mmol) was added and the heating was continued for further

24 h. After cooling, the mixture was diluted with toluene, washed with water and purified by chromatography on thin layer of silica gel in benzene–ether (1 : 1) to give 75 mg (83%) of compound *XII*. IR spectrum (tetrachloromethane): 1 760, 1 738, 1 150 (COOR); 1 711, 1 350 ($COCH_3$); 1 681, 1 622 (C=C–C=O). ^{1}H NMR spectrum (100 MHz): 0.80 s, 3 H (3 × H-18); 1.31 s, 3 H (3 × H-19); 2.10 s, 3 H (3 × H-21); 2.79 s, 4 H ($OCCH_2CH_2CO$); 4.76 s, 2 H ($COOCH_2CCl_3$); 5.50 dd, 1 H (J = 6 and 3, H-11); 5.70 s, 1 H (H-4). For $C_{27}H_{35}Cl_3O_6$ (561.9) calculated: 57.71% C, 6.28% H; found: 57.40% C, 6.49% H.

3,20-Dioxo-4-pregnen-11β-yl Hydrogen Butanedioate (*XIII*)

A) Zinc powder (180 mg, 2.75 mmol) was added at 0 °C in three portions to a stirred solution of derivative *XII* (53 mg, 0.09 mmol) in a mixture of tetrahydrofuran–acetic acid–water (2 ml, 10 : 10 : 1). After 5 h the mixture was filtered, the filtrate was concentrated to dryness and the residue subjected to preparative TLC on silica gel in ether–chloroform–2-propanol–acetic acid (49 : 49 : 1 : 1) to give 24 mg (59%) of compound *XIII*, m.p. 162 – 164 °C (acetone–ether); $[\alpha]_D$ +87° (*c* 0.8, acetone). IR spectrum: 3 600 – 2 400, 1 720, 1 164 (COO); 1 710 sh, 1 359 ($COCH_3$); 1 665, 1 621 (C=C–C=O). ^{1}H NMR spectrum (200 MHz): 0.78 s, 3 H (3 × H-18); 1.30 s, 3 H (3 × H-19); 2.08 s, 3 H (3 × H-21); 2.63 m, 4 H ($OCCH_2CH_2CO$); 5.48 dd, 1 H (J = 6 and 3, H-11); 5.72 s, 1 H (H-4). For $C_{25}H_{34}O_6$ (430.5) calculated: 69.74% C, 7.96% H; found: 69% C, 8.11% H.

B) A solution of 1M solution of tetrabutylammonium fluoride in tetrahydrofuran (2 ml) was added to a solution of derivative *XI* (50 mg, 0.09 mmol) in tetrahydrofuran (15 ml). After standing at 20 °C for 64 h, the solvent was evaporated in vacuo and the residue partitioned between ethyl acetate and saturated solution of sodium chloride. The extract was repeatedly washed with water, dried and, after evaporation, purified by preparative TLC on silica gel in chloroform–acetone–acetic acid (50 : 50 : 1) which afforded 40 mg (98%) of compound *XIII*, m.p. 162 – 164 °C (acetone–ether), without depression on admixture with the sample prepared by procedure *A*.

19-Hydroxypregn-4-ene-3,20-dione (*XIV*)

The title compound was prepared according to ref.[21]; m.p. 168 – 170 °C (acetone–water), $[\alpha]_D$ +168° (*c* 1.2, chloroform) (reported[21] m.p. 169 – 171 °C and $[\alpha]_D$ +182°). IR spectrum: 3 625, 1 035 (OH); 1 702, 1 359 (CH_3CO); 1 665, 1 621 (C=C–C=O).

3,20-Dioxo-4-pregnen-19-yl Hydrogen Butanedioate (*XV*)

A mixture of compound *XIV* (107 mg, 0.32 mmol), succinic anhydride (180 mg, 1.79 mmol) and pyridine (1.5 ml) was heated to 60 °C for 24 h in a sealed tube under argon. After cooling, the mixture was concentrated in vacuo to dryness and the residue was dissolved in a mixture of acetone and chloroform and chromatographed on a thin layer of silica gel in benzene–ether–acetic acid (50 : 50 : 1). The main product (112 mg, 80%) was crystallized from acetone and heptane, m.p. 148 – 150 °C. IR spectrum: 3 400 – 2 600, 1 718 (COOH); 1 735, 1 168 (COO); 1 710, 1 359 ($COCH_3$); 1 666, 1 623, (C=C–C=O). ^{1}H NMR spectrum (100 MHz): 0.67 s, 3 H (3 × H-18); 2.11 s, 3 H (3 × H-21); 2.59 s, 4 H ($OCCH_2CH_2CO$); 4.23 d, 1 H (J = 11, H-19); 4.67 d, 1 H (J = 11, H-19); 5.92 s, 1 H (H-6). For $C_{25}H_{34}O_6$ (430.5) calculated: 69% C, 7.96% H; found: 69.53% C, 7.71% H.

The authors are indebted to Mrs J. Neumannová for the technical assistance and to Dr J. Smolíková, Mrs J. Jelínková and Mrs M. Snopková for measurement and interpretation of the spectra. Their thanks are also due to the staff of the Analytical Department (Dr. V. Pechanec, Head).

REFERENCES

1. Fránek M.: J. Steroid Biochem. *28*, 95 (1987).
2. Hampl R., Putz Z., Stárka L. in: *Advances in Steroid Analysis '90* (S. Görög, Ed.), p. 83. Akadémiai Kiadó, Budapest 1991.
3. Perry L. A., Al-Dujaili E. A. S., Edwards C. R. W.: Steroids *39*, 115 (1982).
4. Brown J. R., Cavanaugh A. H., Farnsworth W. E.: Steroids *28*, 487 (1976).
5. Nishina T., Tsui A., Fukushima D.: Steroids *24*, 861 (1974).
6. Duval D., Prédine J., Emiliozzi R., Milgrom E.: Steroids *35*, 65 (1980).
7. Watanabe F., Tsubota N., Kobayashi Y., Miyata O., Ninomiya I., Miyai K.: Steroids *40*, 393 (1982).
8. Kobayashi Y., Mukai H., Tsubota N., Watanabe F.: J. Steroid Biochem. *20*, 913 (1984).
9. Fehér T., Bodrogi, L. Szabo P. E., Karpati G., Koranyi L.: Hu 38,411; Chem. Abstr. *105*, P170955s (1986).
10. Lawrence C. E., Wright J. M., Knight C. E.: Cell Biochem. Funct. *4*, 277 (1986).
11. Amidon G. L., Stewart B. H., Pogany S.: J. Controlled Release *2*, 13 (1985).
12. Kasal A., Smolíková J.: Collect. Czech. Chem. Commun. *49*, 2932 (1984).
13. Drašar P., Černý I., Pouzar V., Havel M.: Collect. Czech. Chem. Commun. *49*, 306 (1984).
14. Pouzar V., Drašar P., Černý I., Havel M.: Synth. Commun. *14*, 501 (1984).
15. Smith L. L., Ezell E. L.: Steroids *53*, 513 (1989).
16. Erlanger B. F., Borek F., Beiser S. M., Lieberman S.: J. Biol. Chem. *234*, 1090 (1959).
17. Vaitukaitis J. L. in: *Methods in Enzymology* (J. J. Langone and H. van Vunakis, Eds), Vol. 73, Part B, p. 46. Academic Press, New York 1983.
18. Langer L., Velemínský J., Hampl R., Stárka L., Holan J.: Radiochem. Radioanal. Lett. *34*, 267 (1978).
19. Kasal A., Pásztorová S.: Unpublished results.
20. Crowne C. W., Evans R. M., Green G. F. H., Long A. G.: J. Chem. Soc. *1955*, 4351.
21. Bowers A., Villoti R., Edwards J. A., Denot E., Halpern O.: J. Am. Chem. Soc. *84*, 3204 (1962).

Translated by M. Tichý.