PREPARATION OF 3-CARBOXYPROPANOATES OF DIGITOXIN AND DIGOXIN AND THEIR CONJUGATES WITH L-TYROSINE*

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Received April 16th, 1985

Digitoxin and digoxin 4‴-(3-carboxypropanoates) (4‴-hemisuccinates) *III* and *X* were prepared by an indirect method, using dicyclohexylcarbodiimide-induced condensation of the cardioglycoside with 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid in dichloromethane in the presence of 4-dimethylaminopyridine or pyridine followed by removal of the 2,2,2-trichloroethyl group with zinc in a mixture of tetrahydrofuran, acetic acid and water. The hemisuccinates *III* and *X* were condensed with L-tyrosine methyl ester in tetrahydrofuran, using N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as the coupling reagent. Structure of the products was proved by ^{1}H NMR and ^{13}C NMR spectra. The conjugates *IV* and *XI* were labelled with [^{125}I] in the L-tyrosine moiety. The obtained radioligands *V* and *XII* are designed as a part of the RIA system for cardioglycoside analyses.

The determination of cardiotonic steroids in serum and urine is of considerable importance because these compounds belong to the most toxic drugs used in the human medicine and cause the highest number of lethal intoxications (about 0·2%) in the clinical practice. Among the methods of determination of cardiotonic steroids[1-4] radioimmunoassay (RIA) occupies a prominent position[4,5]. This method often uses the tyrosine derivative of digoxigenin 3-(3-carboxypropanoate)[5,6] in which the succinate unit mediates the bond between the genin and tyrosine. The compound is labelled with sodium [^{125}I]iodide under standard conditions[7-10]. Since use of the genin instead of the natural glycoside may affect the interaction with the antibody, we focused our attention on the preparation of a digoxin derivative with hemisuccinate moiety in the sugar part and on its coupling with tyrosine as a substrate for radioiodination[11]. A different labelling of digoxin starting from the isocyanate derived from tyrosine is described in the patent literature[12].

The crucial problem in our synthesis appeared to be the preparation of the cardioglycoside hemisuccinate since classical methods of preparing hemisuccinates[13-15]

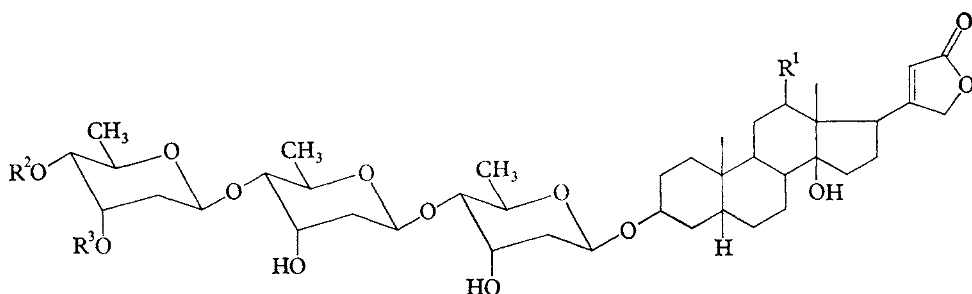
* Part CCCXVII in the series On Steroids; Part CCCXVI: This Journal *50*, 2457 (1985).

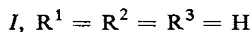
gave no applicable results. We have therefore made use of the indirect method described recently[16]. Our preliminary experiments were performed with digitoxin (*I*) which does not contain the interfering 12-OH group and should be acylated in the sugar part of the molecule. The reaction of digitoxin (*I*) with 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid (2 equivalents) was carried out in dichloromethane using dicyclohexylcarbodiimide (1·18 equivalent) as coupling reagent and 4-dimethylaminopyridine as catalyst. A part of digitoxin remained unreacted despite further addition of the reaction components which only decreased the amount of the isolated succinate *II*. Column chromatography on silica gel afforded the succinate *II* (69% yield) as the only isolable esterification product. It was characterized by the IR spectrum which exhibited hydroxyl bands at 3 610 and 3 560 cm^{-1}, a lactone and an ester band at 1 746 cm^{-1} and a double bond band at 1 622 cm^{-1}. The structure was further confirmed by the ^1H NMR and ^{13}C NMR spectra.

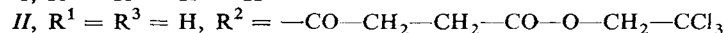
The 2,2,2-trichloroethyl protecting group in the succinate *II* was removed by reduction with powdered zinc in a tetrahydrofuran–acetic acid–water (15 : 4 : 4) mixture at 0°C to give, after chromatography on silica gel, the hemisuccinate *III* in 85% yield. Its IR spectrum exhibited bands due to hydroxyl at 3 610 and 3 530 cm^{-1}, free carboxyl at 3 300–2 500 cm^{-1}, lactone and ester at 1 745 cm^{-1} and double bond at 1 622 cm^{-1}; also the ^1H NMR spectrum corresponded to the structure *III*. The hemisuccinate *III* was condensed with L-tyrosine methyl ester in tetrahydrofuran using N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) as coupling reagent[17] to give the tyrosine derivative *IV* in 16% yield. Its structure was confirmed by bands due to unsaturated γ-lactone (1 785 sh, 1 745 cm^{-1}) and secondary amide (3 425, 1 668 and 1 521 cm^{-1}) in the IR spectrum. A convincing structural proof was provided by the ^1H NMR and ^{13}C NMR spectra.

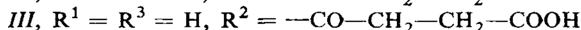
The presence of the 12-hydroxy group in digoxin (*VI*) complicates the situation. Reaction of *VI* with 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid under the same conditions as described for digitoxin (*I*) led to a complex mixture of products containing a substantial amount of the starting compound. Therefore a further portion (about one third of the original amount) of 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid, dicyclohexylcarbodiimide and 4-dimethylaminopyridine was added. The reaction mixture contained at least six compounds, including the starting *VI* (for the HPCL analysis see Table I). After removal of N,N'-dicyclohexylurea by chromatography on silica gel, the products were separated by high performance liquid chromatography on silica gel which afforded two monoacyl derivatives *VII* and *VIII* and one diacyl derivative *IX* in the pure state. The products were shown by ^1H NMR and ^{13}C NMR spectra to be digoxin 4'''-(4-(2,2,2-trichloroethoxy)-4-oxobutanoate) (*VII*), digoxin 3'''-(4-(2,2,2-trichloroethoxy)-4-oxobutanoate) (*VIII*) and digoxin 4''',12-bis(4-(2,2,2-trichlorethoxy)-4-oxobutanoate) (*IX*).

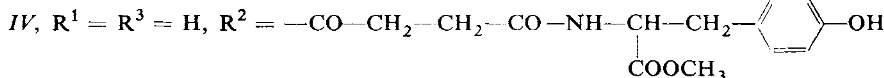
The principal products are the 4'''-monoacyl derivative *VII* and the diacyl derivative *IX*, each isolated in 23% yield. The 3'''-monoacyl derivative *VIII* was isolated

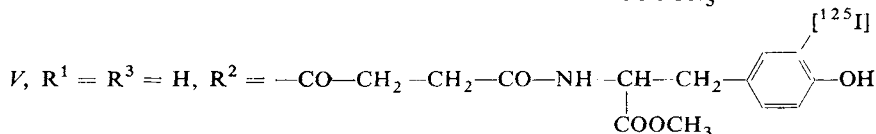



I, $R^1 = R^2 = R^3 = H$

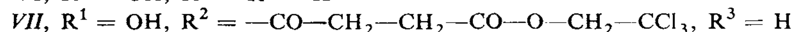

II, $R^1 = R^3 = H$, $R^2 = —CO—CH_2—CH_2—CO—O—CH_2—CCl_3$


III, $R^1 = R^3 = H$, $R^2 = —CO—CH_2—CH_2—COOH$

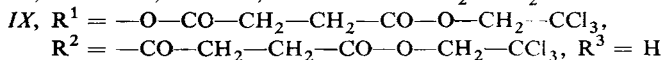

IV, $R^1 = R^3 = H$, $R^2 = —CO—CH_2—CH_2—CO—NH—CH(COOCH_3)—CH_2—C_6H_4—OH$

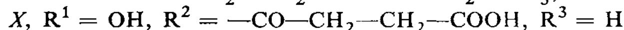

V, $R^1 = R^3 = H$, $R^2 = —CO—CH_2—CH_2—CO—NH—CH(COOCH_3)—CH_2—C_6H_3([^{125}I])—OH$

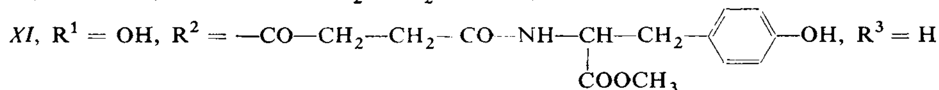
VI, $R^1 = OH$, $R^2 = R^3 = H$

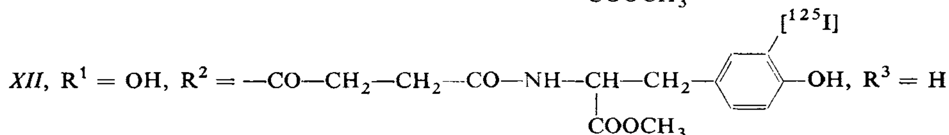

VII, $R^1 = OH$, $R^2 = —CO—CH_2—CH_2—CO—O—CH_2—CCl_3$, $R^3 = H$

VIII, $R^1 = OH$, $R^2 = H$, $R^3 = —CO—CH_2—CH_2—CO—O—CH_2—CCl_3$


IX, $R^1 = —O—CO—CH_2—CH_2—CO—O—CH_2—CCl_3$,
$R^2 = —CO—CH_2—CH_2—CO—O—CH_2—CCl_3$, $R^3 = H$


X, $R^1 = OH$, $R^2 = —CO—CH_2—CH_2—COOH$, $R^3 = H$


XI, $R^1 = OH$, $R^2 = —CO—CH_2—CH_2—CO—NH—CH(COOCH_3)—CH_2—C_6H_4—OH$, $R^3 = H$


XII, $R^1 = OH$, $R^2 = —CO—CH_2—CH_2—CO—NH—CH(COOCH_3)—CH_2—C_6H_3([^{125}I])—OH$, $R^3 = H$

only as a minor component, as well as higher-acylated derivatives which were not studied further. In order to optimize the yield of the 4‴-monoacyl derivative *VII*, a series of experiments under various conditions (temperature, solvent, catalyst) was carried out. The best results were obtained when digoxin (*VI*) was treated with 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid and dicyclohexylcarbodiimide in dichloromethane and pyridine at room temperature. Under these conditions the formation of higher-acylated products was suppressed. Although some unreacted digoxin still remained in the mixture, chromatographic separation of the desired monoacyl derivative was easier and the substantially longer reaction time was compensated by the higher yield (38%).

The 2,2,2-trichloroethyl protecting group from the succinate *VII* was removed by powdered zinc in an ice-cold tetrahydrofuran–acetic acid–water (10 : 1 : 1) mixture. Since significant amount of the 14-dehydro derivative was formed during evaporation on a rotatory evaporator, we used lyophilization during the work-up procedure. Chromatography on a silica gel column afforded pure hemisuccinate *X*, together with a substantial amount (about 20%) of its salt. Its purity was confirmed by comparison of intensity of the carboxyl band at 3 300–2 500 cm^{-1} with that of the carboxylate band at 1 575 cm^{-1} in the IR spectra.

The hemisuccinate *X* was condensed with L-tyrosine methyl ester using N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline in tetrahydrofuran, which afforded the tyrosine derivative *XI* in 24% yield. Its structure was proved by the ^{1}H NMR and ^{13}C NMR spectra and confirmed by the presence of IR bands of unsaturated lactone (1 780 sh, 1 745 cm^{-1}), and secondary amide (3 440, 1 671 and 1 510 cm^{-1}).

The radioiodination of both the tyrosine derivatives *IV* and *XI* with labelled sodium iodide (^{125}I) of activity 18·5 MBq (0·5 mCi) per 2 μg of the substrate was performed in the usual manner[18], the radiochemical yields of compounds *V* and *XII* being 37% and 50%, respectively. The radioligand has been successfully used in the RIA system employed in the common digoxin kit.

TABLE I

Retention times (t_R), capacity factors (k') and relative retentions for HPLC in methanol–water mixtures[a]

Compound	Methanol–water (8 : 2)			Methanol–water (7 : 3)		
	t_R	k'	relative retention	t_R	k'	relative retention
I	2·44	1·29	2·28	7·30	6·50	5·49
II	6·63	5·21	6·20	[b]	—	—
III	1·32	0·24	1·23	3·59	2·69	2·70
VI	1·31	0·23	1·22	1·75	0·80	1·32
VII	2·53	1·37	2·36	10·43	9·72	7·84
VIII	2·17	1·03	2·03	7·81	7·02	5·87
IX	9·00	7·43	8·41	[b]	—	—
X	1·07	0·003	1·00	1·33	0·37	1·00

[a] Solvent flow rate 2·25 ml/min, pressure 13·27 MPa in methanol–water (8 : 2) and 15·34 MPa in methanol–water (7 : 3), stainless steel column 250 × 4 mm, packing Separon Si C_{18} (10 μm), 5–10 μl samples applied in dichloromethane–methanol solutions (1 : 1), concentration 10 mg/ml, detection at 230 nm; [b] conditions not suitable for separation of the less polar compounds *II* and *IX*.

NMR *Spectra*

Combination of the ^{1}H NMR spectra (compounds *II*–*IV* and *VII*–*XI*) and ^{13}C NMR spectra (compounds *IV* and *VI*–*XI*) proved not only the number and position of the succinate residues but also preservation of the original cardioglycoside skeleton during the synthesis. ^{1}H NMR spectrum of the monoacyl derivative *II* (Table II) exhibits a series of overlapping signals at δ 3·2–4·5 due to the protons of the trisaccharide residue which have been partly assigned by careful decoupling experiments.

Irradiation of the doublet at δ 1·18 (6‴-H_3) results in a collapse of the doublet of quartets at δ 4·04 to a doublet corresponding to the signal of the neighbouring 5‴-H atom. Similarly, irradiation of the six-proton doublet at δ 1·21 (6′-H_3 and 6″-H_3) converts the overlapped doublets of quartets at δ 3·74 and δ 3·84 into two distinct doublets (5′-H and/or 5″-H). Irradiation of the 5′-H + 5″-H multiplet affects the two-proton multiplet at δ 3·21 and δ 3·24 which splits into two distinct doublets due to the 4′-H and 4″-H protons. On the other hand, irradiation of the 5‴-H signal

TABLE II

^{1}H NMR Spectral parameters of 4-(2,2,2-trichloroethoxy)-4-oxobutanoates of digitoxin and digoxin in deuteriochloroform

Signal[a]	*II*	*VII*	*VIII*	*IX*
18-H_3	0·87 s	0·80 s	0·79 s	0·89 s
19-H_3	0·92 s	0·92 s	0·93 s	0·92 s
3-H	4·01 m	4·03 m[b]	4·02 m[b]	4·03 m[b]
12-H	[c]	3·35 m	3·38 m[b]	4·69 m
22-H	5·86 dd[d]	5·96 dd[d]	5·90 dd[d]	5·94 dd[d]
3′-H and 3″-H	4·25 m[b]	4·26 m[b]	4·25 m[b]	4·26 m[b]
4′-H[e]	3·21 dd[f]	3·21 m	3·21 m	3·21 m
4″-H[e]	3·24 dd[f]	3·25 m	3·25 m	3·25 m
5′-H[e]	3·74 dq[g]	3·77 dq[g]	3·73 dq[g]	3·74 m
5″-H[e]	3·84 dq[g]	3·84 dq[g]	3·84 m	3·85 m
6′-H_3 and 6″-H_3	1·21 d[h]	1·22 d[h]	1·23 d[h]	1·22 d[h]
3‴-H	4·25 m[b]	4·26 m[b]	5·34 m	4·26 m[b]
4‴-H	4·53 dd[i]	4·53 dd[i]	3·38 m[b]	4·53 dd[i]
5‴-H	4·04 dq[j]	4·03 m[b]	4·02 m[b]	4·06 m[b]
6‴-H	1·18 d[h]	1·18 d[h]	1·21 d[h]	1·19 d[h]
$COOCH_2CCl_3$	4·75 [k]	4·76[k]	4·76 s	4·77 s[l]

[a] For other conditions see Experimental; [b] overlapped signals; [c] undeterminable value; [d] $J = 2·1 + 1·7$; [e] tentative assignment; [f] $J = 9·3 + 3·0$; [g] $J_d = 9·3$, $J_q = 6·5$; [h] $J = 6·5$; [i] $J = 10·0 + 2·8$; [j] $J_d = 10·0$, $J_q = 6·5$; [k] AB system; [l] 4 H integral.

decouples the doublet of doublets at δ 4·53 (4‴-H) into a doublet (J = 2·8 Hz). Further, we identified the 3′-H, 3″-H and 3‴-H protons which form an unseparated three-proton multiplet at δ 4·25, unaffected by irradiation of 5′-H, 5″-H or 5‴-H. The coupling constants between the protons in the first and the second sugar unit are: $J_{3',4'} = J_{3'',4''} = 3{\cdot}0$, $J_{4',5'} = J_{4'',5''} = 9{\cdot}2$, and $J_{5',6'} = J_{5'',6''} = 6{\cdot}5$ Hz. For the terminal unit, bearing the succinate moiety in the position 4‴, the values are $J_{3''',4'''} = 2{\cdot}8$, $J_{4''',5'''} = 10{\cdot}0$ and $J_{5''',6'''} = 6{\cdot}5$ Hz, comparable with those reported[19] for methyl β-L-digitoxoside (3·0, 10·0 and 6·0 Hz, respectively).

The *in situ* acylation of compound *II* with trichloroacetyl isocyanate[20] (TAI) shifts the three-proton multiplet of 3′-H, 3″-H and 3‴-H markedly downfield (from δ 4·25 to δ 5·52, see Table III), which indicates that the oxygen functionalities in these positions belong to free hydroxyl groups. Consequently, compound *II* contains one succinate residue in position 4‴. Also the two-proton signal of the 2,2,2-trichloroethoxy group (AB system, δ 4·76, $|J_{gem}|$ = 11 Hz) indicates the presence of only one succinate residue.

The structure of the deblocked hemisuccinate *III* was confirmed by the ^{1}H NMR spectrum (Table IV). The spectrum showed essentially the same structural elements as in the protected ester *II*, *i.e.* an acyloxy group in position 4‴ (doublet of doublets at δ 4·46), an intact butenolide ring (signals at δ 5·85 and δ 4·83) and a preserved 14β-hydroxyl (absence of an olefinic proton). Disappearance of the 2,2,2-trichloroethoxy protecting group signal (δ 4·76) proved deblocking to the free hemisuccinate.

The spectrum of the tyrosine derivative *IV* is very similar to that of the hemisuccinate *III* (Table IV); the position of the 4‴-H signal (δ 4·39) indicates that no migration of the succinate moiety took place. The spectrum also displays signals characteristic of the L-tyrosine residue: two multiplets of aromatic protons (δ 6·91 and δ 6·75), a doublet of the amidic proton (δ 6·82; $J_{CH,NH}$ = 8·0 Hz), a singlet of the

TABLE III

TAI Acylation shifts in ^{1}H NMR spectrum of compound *II* in deuteriochloroform

Signal	Acylation shift, ppm
18-H$_3$	+0·12
19-H$_3$	+0·19
22-H	+0·01
3′-H, 3″-H, 3‴-H	+1·27
4′-H, 4″-H	+0·17
4‴-H	+0·07

methyl ester group (δ 3·72) and a multiplet of the methylene linked to the aromatic nucleus (δ 2·97).

The ^{1}H NMR spectrum of the digoxin derivative *VII* resembles that of the analogous derivative *II* (Table II). The doublet of doublets at δ 4·53 confirms again that the acylation occurs in the position 4‴ and the shifts of the protons 12-H (δ 3·35), 3′-H, 3″-H, and 3‴-H (δ 4·26) prove that the corresponding hydroxyl groups are not acylated. This is consistent with the presence of a two-proton AB system of the 2,2,2-trichloroethoxy group (δ 4·76, $|J_{gem}|$ = 11·0 Hz) whose integral corresponds to one acyl group in the molecule.

No doublet of doublets characteristic of derivatives acylated in position 4‴ is present in the spectrum of the minor acylation product *VIII*. On the other hand, the multiplet of 3′-H and 3″H (δ 4·25) consists of only two protons and a new signal appears at δ 5·34 which well corresponds to the expected acylation shift of the 3‴-H

TABLE IV
^{1}H NMR Data for digitoxin and digoxin derivatives

Signal[a]	*III*	*IV*[b]	*X*	*XI*[c]
18-H_3	0·91 s	0·86 s	0·78 s	0·78 s
19-H_3	0·93 s	0·91 s	0·95 s	0·93 s
3-H	4·02 m	4·02 m	4·05 m	4·02 m
12-H	[d]	[d]	3·36 m	[d]
21-H_2	4·83[e]	[f]	[f]	[f]
22-H	5·85 m	5·87 m	5·92 m	5·93 m
3′-H + 3″-H + 3‴-H	4·25 m	4·26 m	4·26 m	4·24 m
4′-H[g]	3·22 dd[h]	3·20 dd[h]	3·22 m	3·22 dd[h]
4″-H[g]	3·24 dd[h]	3·23 dd[h]	3·27 dd[h]	3·24 dd[h]
5′-H[g]	3·76 dq[i]	3·77 dq[i]	3·85 m[j]	3·80 dq[i]
5″-H[g]	3·85 dq[i]	3·83 dq[i]	3·85 m[j]	3·86 dq[i]
6′-H_3 + 6″-H_3	1·21 d[k]	1·22 d[k]	1·24 d[k]	1·23 d[k]
4‴-H	4·46 dd[l]	4·39 dd[l]	4·48 dd[l]	4·40 dd[l]
5‴-H	4·08 dq[m]	4·06 dq[m]	4·10 m	4·06 dq[m]
6‴-H_3	1·17 d[k]	1·19 d[k]	1·20 d[k]	1·18 d[k]
$OOCCH_2CH_2CO$	2·68 m	2·57 m	2·66 m	2·57 m

[a] Measured in C^2HCl_3 (compound *IV*), in a C^2HCl_3—$C^2H_3COC^2H_3$ mixture (compound *III*), or in a C^2HCl_3—$C^2H_3O^2H$ mixture (compound *X* and *XI*); [b] other signals: 6·91 m and 6·75 m (arom-H), 6·82 d J = 8 (CONH), 3·72 s ($COOCH_3$), 3·00 m (arom-CH_2); [c] other signals: 7·04 d J = 7·0 (CONH), 6·95 m and 6·75 m (arom-H), 3·57 s ($COOCH_3$), 2·97 m (arom-CH_2); [d] undeterminable value; [e] AB system $|J_{gem}|$ = 17·6; [f] the signal si obscured by the 1′-H, 1″-H, and 1‴-H signals; [g] tentative assignment; [h] J = 3 + 9·5; [i] J_q = 6·5, J_d = 9·5; [j] overlapped signals; [k] J = = 6·5; [l] J = 3 + 10; [m] J_q = 6·5, J_d = 10·0.

signal. The acylation in this position is also manifested by significant downfield shift of the 4‴-H signal (for digoxin δ 3·00) which coincides with the 12-H signal (δ 3·38). All other structural features of the digoxin skeleton in compound *VIII* are preserved (Table II).

The ^{1}H NMR spectrum of compound *IX* (Table II) differs from those of the mono-acyl derivatives *VII* and *VIII*. The downfield shift of the 12-H and 18-H_3 signals is characteristic (3·35→4·69 and 0·80→0·89, respectively). Also here the doublet of doublets at δ 4·53 indicates the acylation in position 4‴. The four-proton singlet (δ 4·77) due to two 2,2,2-trichloroethoxy groups also agrees with the 12,4‴-diacyl digoxin structure.

The spectrum of the hemisuccinate *X* confirms the preservation of all structural features except the 2,2,2-trichloroethyl protecting group (Table IV). No acyl migration from the 4‴ position to another hydroxyl group occured during the deprotection as shown by the signals of 12-H (δ 3·36), 3′-H, 3″-H, 3‴-H (δ 4·26), and 4‴-H (δ 4·48). The absence of an olefinic proton signal proves that the 14-hydroxyl group was not eliminated during the work-up procedure.

The tyrosine derivative *XI* has a spectrum very similar to that of the hemisuccinate *X* (Table IV). The doublet of doublets at δ 4·40 is characteristic of 4‴-acyl derivatives of digoxin. The tyrosine methyl ester gives rise to two multiplets of aromatic protons (δ 6·95 and δ 6·75), a doublet of the amide proton (δ 7·04; $J_{NH,CH}$ = 7·0 Hz), a singlet of the methyl ester group (δ 3·57) and a multiplet of the methylene bonded to the aromatic nucleus (δ 2·97).

The ^{13}C NMR spectra were interpreted by correlating the observed chemical shifts with those of model compounds representing different parts of the complex systems in question, *e.g.* digoxigenin (*XIII*), digitoxigenin[22] (*XIV*) (Tables V and VI), methyl β-L-digitoxoside[19], 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid (*XV*), and N-acetyl-L-tyrosine ethyl ester (*XVI*). In most cases (*IV*, *VI*, *VIII*, *IX*, *XI*) the assignment of the ^{13}C signals was corroborated by "attached proton test" spectra[21] (APT). The assignment of ^{13}C signals corresponding to the trisaccharide moiety is based on the published spectra of digoxin (*VI*) and its 3‴-O-acetyl derivative, measured in ($^{2}H_6$)dimethyl sulfoxide[23]. Due to the similarity of ^{13}C chemical shifts of the trisaccharide methines and some methylenes coming from the steroid, saccharide and oxobutanoate parts, the assignment is rather tentative in regions of δ 65 – 70 and δ 29 – 32 ppm.

The chemical shifts of carbon nuclei belonging to the steroid part of the molecule correlate with those of the corresponding genins *XIII* and *XIV*. The presence of the carbohydrate moiety linked to oxygen at C–3 affects the chemical shift of C–3 ($\Delta\delta$ = +5·6 – 6·5 ppm), C–2 and C–4 ($\Delta\delta$ = –1·1 to –4·0 ppm). In the spectrum of the 4‴, 12-bis acyl derivative *IX* there is a marked acylation shift of C–12 ($\Delta\delta$ = = +2·9 ppm), C–11 ($\Delta\delta$ = –1·4 ppm), C–13 ($\Delta\delta$ = –2·4 ppm) and C–18 ($\Delta\delta$ = +1·4 ppm).

TABLE V

^{13}C NMR Parameters for digoxin and digitocin derivatives (steroid and sugar parts)

Carbon[a]	*VI*	*XIII*[b]	*VII*	*VIII*	*IX*	*X*	*XI*	*IV*	*XIV*[b]
1	30·1[c]	30·0	30·2[c]	29·5[c]	30·0[c]	30·0[c]	30·1[c]	30·2	30·0
2	26·8[d]	27·9	26·5[d]	26·2	26·4	26·8	26·3[d]	26·6	28·0
3	73·1	66·6	72·5	72·2	72·3	73·2	72·6	72·6	66·8
4	30·6[c]	33·3	29·8[c]	29·7[c]	29·7[c]	29·6[c]	29·3[c]	31·0	33·5
5	36·8	36·4	36·2	36·0	36·1	36·7	36·2	36·2	35·9
6	26·9[d]	26·9	26·6[d]	26·2	26·4	26·8	26·4[d]	26·6	27·1
7	22·0	21·9	21·7	21·3	21·4	21·9	21·6	21·1	21·6
8	41·3	41·3	41·4	40·9	41·0	41·3	41·0	41·7	41·9
9	32·8	32·6	32·6	32·2	32·2	32·8	32·4	35·7	35·8
10	35·4	35·5	35·0	34·7	35·0	35·3	34·9	35·1	35·8
11	30·0[c]	30·0	30·2[c]	29·9[c]	28·6	30·5[c]	30·3[c]	21·4	21·7
12	74·9	74·8	75·0	74·4	77·7	74·9	74·6	40·0	40·4
13	56·4	56·4	55·6	55·4	54·0	56·3	55·8	49·7	50·3
14	85·9	85·8	85·9	85·4	85·6	85·9	85·5	85·6	85·6
15	33·0	33·0	33·2	32·7	32·9	33·0	32·7	33·1	33·0
16	27·7	27·9	27·4	27·1	27·2	27·6	27·3	26·9	27·3
17	46·1	46·1	45·6	45·3	45·6	46·0	45·6	50·9	51·5
18	9·4	9·4	9·0	8·8	10·8	9·3	8·9	15·8	16·1
19	23·8	23·8	23·5	23·3	23·3	23·7	23·4	23·6	23·9
20	177·2	177·1	175·0	175·5	174·5	177·1	176·2	176·2	177·1
21	74·6	74·6	73·8	73·6	73·4	74·6	74·0	73·7	74·5
22	117·3	117·0	117·5	116·9	117·8	117·2	117·1	117·5	117·4
23	176·5	176·3	174·9	175·0	173·9	176·5	175·8	175·0	176·3
1′[k]	95·8	—	95·4	95·1	95·3	95·8	95·3	95·3	—
1″	99·4[e]	—	98·2[e]	98·0[d]	98·1[d]	99·1[d]	98·5[e]	98·2[c]	—
1‴	99·5[e]	—	98·5[e]	98·2[d]	98·3[d]	99·3[d]	98·8[e]	98·6[c]	—
2′[k]	37·6[f]	—	37·5[f]	36·8[e]	37·4[c]	37·5[e]	37·5[f]	37·4[d]	—
2″	37·3[f]	—	37·1[f]	36·5[e]	37·0[c]	37·2[e]	37·0[f]	37·1[d]	—
2‴	38·4	—	36·7	35·6	36·6	38·1	36·7	36·9	—
3′[k]	66·9[g]	—	66·4	66·1[f]	66·3	66·9[f]	66·4	66·5	—
3″	67·1[g]	—	66·4	66·2[f]	66·3	67·0[f]	66·4	66·5	—
3‴	68·1	—	65·5	71·1	65·3	65·2	64·5	64·6	—
4′[k]	82·7[h]	—	82·3[g]	82·0[g]	82·1[f]	82·7[g]	82·3[g]	82·2[e]	—
4″	83·0[h]	—	82·6[g]	82·2[g]	82·4[f]	83·0[g]	82·6[g]	82·5[e]	—
4‴	73·3	—	75·6	71·1	75·4	75·8	75·7	75·9	—
5′[k]	68·6[i]	—	68·1[h]	67·8[h]	67·9[g]	68·5[h]	68·1[h]	68·1[f]	—
5″	68·7[i]	—	68·2[h]	67·9[h]	68·0[g]	68·6[h]	68·2[h]	68·3[f]	—
5‴	70·1	—	67·1	70·2	67·0	67·6	67·1	67·2	—
6′[k]	18·3[j]	—	18·2[i]	17·9	18·0[h]	18·2[i]	18·0[i]	18·2[g]	—
6″	18·3	—	18·2	17·9	18·0	18·2	18·0	18·2	—
6‴	18·2[j]	—	17·9[i]	17·9	17·8[h]	18·0[i]	17·8[i]	18·0[g]	—

An assignment of the ^{13}C signals belonging to the D-digitoxose (2,6-dideoxy--D-*ribo*-hexopyranose) moieties is very difficult, especially as far as the respective positions of the three pyranose rings are concerned. The acetal methines, C−1′, C−1″, and C−1‴, appear at a low-field region (δ 95·1−99·4) of the sp^3 part of the spectrum. Of these the signal of C−1′ is distinctly shifted downfield by 2·9−3·6 ppm, compared with the signals of C−1″ and C−1‴. This is clearly due to the different type of the O-aglycone at C−1′ (steroid) and at C−1″ and C−1‴ (hexopyranose). The signals of the methyl groups C−6′, C−6″, and C−6‴ (δ 17·8−19·2) mostly coincide, although in some cases one methyl signal (perhaps C−6‴) is shifted upfield. The chemical shifts of the C−2 methylenes are affected by both the aglycone linked to the oxygen at C−1 and the O-substituents at C−3 and C−4 of the pyranose ring. On the basis of the published data on digoxin[23] (*VI*), we have assigned the low-field methylene signal at δ 38·4 to C−2‴. The signal of C−2′ (δ 37·6) appears at a slightly lower field than that of C−2″ (δ 37·3), probably due to different substituents at the adjacent carbons C−1′ and C−1″. Of the three C−2 methylenes, the signal of C−2‴ is most sensitive to the substituents at C−3‴ and C−4‴ in the derivatives *VII*−*XI*. The upfield shift of C−2‴ in the 3‴ acylated derivative *VIII*, due to the β-effect, is generally greater than that in the 4‴-acylated derivatives *IX*−*XI* due to the γ-effect. As expected, the digitoxin derivative *IV* shows very similar chemical shifts of the carbohydrate carbons to those of the corresponding digoxin derivatives.

The chemical shifts of C−3′ and C−3″ are very similar, but compared with β-L-digitoxoside (C−3, δ 68·0), they are shifted upfield by 1 ppm, probably due to the presence of another carbohydrate unit at C−4′ and C−4″. In the spectra of *IV*, *VII*, and *IX*−*XI* the signals of C−3‴ appear at a higher field, compared with digoxin *VI*. With the 3‴-acyl derivative *VIII*, the α-effect shifts the signal of C−3‴ downfield ($\Delta\delta = +3$ ppm). The acylation shifts are also visible in the 4‴-derivatives *IV*, *VII* and *IX*−*XI* in which the signals of C−4‴ are shifted downfield by α-effect ($\Delta\delta = +2{\cdot}1-2{\cdot}6$ ppm). Acylation at C−3‴ (*VIII*) causes an upfield shift of C−4‴ (β-effect, $\Delta\delta = -2{\cdot}2$ ppm). The signals of C−4′ and C−4″ appear at a lower field (δ 82·0−83·0) and they are insensitive to acylation of the terminal pyranose unit.

The ordering of the C−5′, C−5″, and C−5‴ signals parallels that of C−3′, C−3″, and C−3‴, but the former group appears at a lower field (δ 67·0−68·3). Acylation in position 3‴ (*VIII*) shifts the signal of C−5‴ downfield with respect to those of C−5′ and C−5″. The same order of chemical shifts is observed for digoxin (*VI*).

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[a] Measured in C^2HCl_3 (*IV, VII, VIII, IX*) or in a C^2HCl_3-$C^2H_3O^2H$ mixture (*VI, X, XI*) (δ C^2HCl_3 77·1), for mixtures with tetradeuteriomethanol δ $C^2H_3O^2H$ 49·0; [b] taken from ref.[22], digoxigenin (*XIII*), digitoxigenin (*XIV*); [c−j] values in the columns can be interchanged; [k] for comparison, the shifts for methyl β-L-digitoxoside[19] are: 99·0, 37·7, 68·0, 73·2, 69·9, 18·1 (in deuteriochloroform).

Assignment of the ^{13}C signals belonging to the succinate, 2,2,2-trichloroethoxy and tyrosine carbons is based on chemical shifts of the corresponding carbon atoms in the model compounds *XV* and *XVI*. Difficulties were encountered with the signals of the succinate methylenes (denoted as 2*S* and 3*S*) that fell into the complex region of δ 29·0–30·5, where the signals of steroid carbons C–1, C–4, and C–11 appear. These assignments are therefore only tentative.

EXPERIMENTAL

Melting points were determined on a Boetius micro melting point apparatus (GDR), optical rotations on a Perkin-Elmer 141 MC polarimeter. IR spectra were taken on a UR-20 (Zeiss, Jena) spectrophotometer; wavenumbers in cm^{-1}. NMR spectra were measured at 23°C on a Varian XL-200 spectrometer (200·058 MHz for ^{1}H and 50·309 MHz for ^{13}C), in the FT mode, solvents are given in the Tables. Chemical shifts are given in ppm (δ-scale) from tetramethylsilane as internal standard, coupling constants J in Hz, all parameters were obtained by first order analysis. Preparative chromatography was carried out on columns of silica gel according to Pitra, 60–120 μm (Service Laboratories of this Institute), thin-layer chromatography (TLC) was performed on silica gel G according to Stahl (Woelm). Spots were detected by spraying

TABLE VI
^{13}C NMR Spectral parameters for digoxin and digitoxin derivatives — 2nd part

Carbon[a]	*XV*[a]	*XVI*[a]	*VII*	*VIII*	*IX*[b]	*X*	*XI*[c]	*IV*[c]
1S	178·1	—	171·4[d]	171·4[d]	171·2 (171·7)[d]	177·1	172·1[d]	171·9[d]
2S	28·7[d]	—	29·0	28·7[e]	28·9 (28·9)	29·9	29·7	29·8
3S	28·6[d]	—	29·0	28·8[e]	28·9 (28·9)	29·9	29·7	29·8
4S	170·5	—	171·0[d]	170·6[d]	170·7 (170·8)[d]	172·6	172·0[d]	171·7[d]
1C	74·2	—	74·3	73·8	74·0 (74·1)	—	—	—
2C	94·7	—	94·6	94·4	94·5 (94·6)	—	—	—
1A	—	170·7	—	—	—	—	172·2[d]	172·0[d]
2A	—	53·6	—	—	—	—	53·6	53·5
3A	—	37·0	—	—	—	—	36·7	36·7
1P	—	126·8	—	—	—	—	126·6	126·8
2P, 6P	—	130·3	—	—	—	—	130·1	130·3
3P, 5P	—	115·5	—	—	—	—	115·3	115·6
4P	—	155·9	—	—	—	—	155·8	155·5

[a] See Table V, 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid (*XV*), N-acetyl-L-tyrosine ethyl ester (*XVI*); 1S-4S are succinate carbon atoms, free succinate carboxyl (if present) is denoted as 1S; 1C and 2C denote the 2,2,2-trichloroethanol part; the amino acid moiety is numbered as 4-hydroxyphenylalanine; 1A is carboxyl carbon atom, 4P is the carbon atom, bearing the hydroxyl; [b] values in parentheses correspond to the second succinate residue in the 4‴,12-dissuccinate; [c] signal of the methyl carbon in the methyl ester: for *XI* at 52·5 and for *IV* at 52·3; [d,e] the values in the columns can be interchanged.

with sulfuric acid and subsequent heating. Solutions were dried over anhydrous sodium sulfate and taken down on a rotatory evaporator at bath temperature 40—50°C and pressure 2—2·5 kPa. Analytical samples were dried over phosphorus pentoxide at 40—50°C/25 Pa for 24 h.

Digitoxin 4'''-(4-(2,2,2-Trichloroethoxy)-4-oxobutanoate) (*II*)

To a suspension of digitoxin (*I*; 765 mg, 1 mmol) in dichloromethane (70 ml) were successively added 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid[24] (500 mg; 2 mmol), dicyclohexylcarbodiimide (243 mg; 1·18 mmol) and 4-dimethylaminopyridine (8 mg). After stirring at room temperature for 2 h, the mixture was poured into water (100 ml) and extracted with dichloromethane (3 × 50 ml). The organic extracts were combined, evaporated *in vacuo* and the residue was chromatographed on a column of silica gel (75 g) in dichloromethane–methanol (95 : 5). Crystallization of the main fraction from dichloromethane–methanol–ether–benzene afforded 687 mg (69%) of the succinate *II*, m.p. 201—203°C. IR spectrum (chloroform): 3 610, 3 560 (OH), 1 746, 1 633, 1 622 (unsaturated γ-lactone), 1 746 (COOR). For $C_{47}H_{69}Cl_3O_{16}$ (996·4) calculated: 56·66% C, 6·98% H, 10·67% Cl; found: 56·93% C, 7·12% H, 11·02% Cl.

Digitoxin 4'''-(3-Carboxypropanoate) (*III*)

Water (4 ml) and acetic acid (4 ml) were added to a solution of the succinate *II* (600 mg; 0·60 mmol) in tetrahydrofuran (15 ml). Zinc powder (50 mg) was added under cooling with ice and the ice-cooled mixture was stirred for 12 h. During this time zinc was added in 20 mg portions at 30 min intervals. The mixture was filtered, the solid washed with methanol and the filtrate taken down *in vacuo*. The residue was dried in an exsiccator over potassium hydroxide and chromatographed on a silica gel column (70 g). Elution with dichloromethane–methanol (49 : 1 to 1 : 1) afforded 307 mg (59%) of the hemisuccinate *III*, m.p. 196—199°C (dichloromethane–ether–light petroleum). IR spectrum (chloroform): 3 610, 3 530 (OH), 3 300—2 500 (COOH), 1 745, 1 631, 1 622 (unsaturated γ-lactone), 1 745 (COOR). For $C_{45}H_{68}O_{16}$ (865·0) calculated: 62·48% C, 7·92% H; found: 62·77% C, 8·23% H.

4'''-(4-(1-Methoxycarbonyl-2-(2*S*)-(4-hydroxyphenyl)ethyl)-amino-4-oxobutanoyl)digitoxin (*IV*)

L-Tyrosine methyl ester (1·54 g; 7·88 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1·95 g; 7·87 mmol) were added to a solution of the hemisuccinate *III* (1·58 g; 1·82 mmol) in tetrahydrofuran (35 ml). After stirring at room temperature for 68 h, the mixture was diluted with dichloromethane (400 ml), washed with 5% aqueous citric acid (2 ×) and water (2 ×) and evaporated. The residue (2·42 g) was chromatographed in dichloromethane–methanol (99 : 1 to 33 : 1), yielding 310 mg (16%) of the amorphous *IV*. IR spectrum (chloroform): 1 785 sh, 1 745 (unsaturated γ-lactone), 1 745 (COOR), 3 600 (OH), 3 425, 1 668, 1 521 (CONH). For $C_{55}H_{79}NO_{18}$ (1 042·2) calculated: 63·38% C, 7·64% H, 1·34% N; found: 63·25% C, 7·55% H, 1·45% N.

4'''-(4-(1-Methoxycarbonyl-2-(2*S*)-(4-hydroxy-3-[^{125}I]-iodophenyl)ethyl)amino-4-oxobutanoyl)digitoxin (*V*)

A solution of compound *IV* in dioxane (10 μl, *c* 0·2 mmol/ml) was mixed in a 5 ml test-tube with a buffer solution (10 μl; a mixture of 19 ml of 0·2 mol l^{-1} aqueous sodium dihydrogen phosphate and 81 ml of 0·2 mol l^{-1} aqueous disodium hydrogen phosphate made up to 400 ml; pH 7·4—7·6). A solution of $Na^{125}I$ in water (18·5 MBq (0·5 mCi)) followed by sodium salt

of N-chloro-4-methylbenzenesulfonamide in the above-mentioned phosphate buffer (10 µl, *c* 5 mg/ml) was added and the mixture was shaken on a vibration shaker for 90 s. A solution of sodium hydrogen sulfite in the above-described phosphate buffer (10 µl, *c* 5 mg/ml) was then added and the mixture was extracted with ethyl acetate (2 × 100 µl) in a glass tube drawn into a capillary. The combined organic phases were chromatographed on a Silufol UV 254 plate (20 × 20 cm, pre-washed with the eluent) in benzene–ethanol (7 : 3). The zone of the applied compound was narrowed by a threefold partial developing in methanol. The chromatography was evaluated by means of autoradiograms. The zone containing the main product was cut out and the product was eluted with methanol. The labelled derivative *V* was obtained in a radiochemical yield of 37%.

Acylation of Digoxin

A) Digoxin (*VI*; 5·46 g; 7 mmol; dried over phosphorus pentoxide at 50°C/13 Pa) was dissolved in dichloromethane (500 ml). To this solution were added successively 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid[24] (3·5 g; 14·3 mmol), dicyclohexylcarbodiimide (1·7 g; 8·26 mmol) and 4-dimethylaminopyridine (70 mg). After stirring at room temperature for 2 h, another portion of the reagents was added (1·05 g, 4·2 mmol; 0·51 g, 2·48 mmol; and 20 mg, respectively) and the mixture was stirred for further 2 h. The reaction was followed by thin-layer chromatography in dichloromethane–methanol (10 : 1) and by high performance liquid chromatography (HPLC) on a reversed phase (see Tables I and II). The mixture was filtered, the filtrate applied on a column of silica gel (400 g) and eluted with dichloromethane–methanol (60 : 1). Evaporation of the product-containing fractions gave a product (5 g) which, according to TLC (silica gel dichloromethane–methanol 10 : 1), contained several minor components of R_F ~0·7 and two principal products of R_F ~0·5. Preparative HPLC (in five 1 g portions, column Jobling 350 × 27 mm i.d., silica gel according to Pitra, 20 µm) in dichloromethane–methanol (gradient 0—6%, flow rate 4 ml/min) gave, after separation of higher-acylated or unsaturated derivatives, the following products: *1*) The 4‴,12-disuccinyl derivative *IX*, solid foam, 1·96 g (23%), $[\alpha]_D$ +43° (*c* 0·27, pyridine). IR spectrum (chloroform): 1 750, 1 730 sh, 1 157 (ester and lactone), 3 615, 3 605 (—OH). For $C_{53}H_{74}Cl_6O_{20}$ (1 243·9) calculated: 51·18% C, 6·00% H, 17·10% Cl; found: 51·42% C, 6·27% H, 16·89% Cl. *2*) 4‴-Monosuccinate *VII*, solid foam, 1·6 g (23%), $[\alpha]_D$ +32° (*c* 0·27, pyridine). IR spectrum (chloroform): 3 610, 3 560 (OH), 1 747, 1 789 sh, 1 157 (lactone), 1 747, 1 157 (ester). For $C_{47}H_{69}Cl_3O_{17}$ (1 012·4) calculated: 55·76% C, 6·87% H, 10·51% Cl; found: 55·50% C, 6·85% H, 10·88% Cl. *3*) 3‴-Monosuccinate *VIII*, glass, 65 mg (1%). IR spectrum (chloroform): 3 615, 3 610 (OH), 1 746, 1 153 (ester and lactone). For $C_{47}H_{69}Cl_3O_{17}$ (1 012·4) calculated: 55·76% C, 6·87% H, 10·51% Cl; found: 55·92% C, 6·97% H, 10·32% Cl. For the isolation of the product *VIII* a further separation of the combined last fractions by HPLC was necessary (steel column 500 × 12·6 mm i.d., silica gel 10 µm, dichloromethane–2-propanol 10 : 1, flow rate 4 ml/min).

B) A solution of digoxin (*VI*; 1 g; 1·28 mmol; pre-dried as described under *A*) in a mixture of pyridine (2·5 ml) and dichloromethane (15 ml) was cooled in an ice-bath. Dicyclohexylcarbodiimide (317 mg; 1·54 mmol) in dichloromethane (5 ml), followed by 4-(2,2,2-trichloroethoxy)-4-oxobutanoic acid[24] (479· mg; 1·92 mmol) in dichloromethane (5 ml), was added with stirring. After 1 h, the mixture was allowed to attain room temperature, stirred for 72 h and again cooled. Water (0·1 ml) was added, dichloromethane was evaporated and traces of pyridine were removed by evaporation with toluene in vacuo (several times). The residue was suspended in a dichloromethane–methanol mixture (10 : 1, 10 ml), filtered through a layer of silica gel (7 g) and eluted with the same mixture (100 ml). The filtrate was taken down and the residue was dissolved in dichloromethane–2-propanol (20 : 1). The solids were filtered off, the solution was applied

on a HPLC column (silica gel 20 μm, see procedure *A*) and eluted with dichloromethane–2-propanol (30 : 1). Besides some higher acylated products (259 mg; predominantly the diester *IX*), the procedure gave 494 mg (38%) of the desired succinate *VII*, sufficiently pure for further work.

Digoxin 4'''-(3-Carboxypropanoate) (*X*)

A solution of the succinate *VII* (1·6 g; 1·58 mmol) in tetrahydrofuran (30 ml) was mixed with water (3 ml) and acetic acid (3 ml). Zinc powder was added in portions (20 mg every 30 min) during 10 h with stirring and cooling with ice. The mixture was filtered through cotton wool which was then washed with acetic acid (5 ml) and aqueous acetic acid (1 : 1; 10 ml) and the combined filtrates were freeze-dried (12 h). The residue was chromatographed on a column of silica gel (50 g) in dichloromethane–methanol (9 : 1). The product *X* (950 mg; 68%) was obtained as a solid foam, $[\alpha]_D$ +23° (*c* 0·41, pyridine). IR spectrum (chloroform): 3 200—2 500, 1 728 (COOH), 1 739, 1 631 (unsaturated γ-lactone). For $C_{45}H_{68}O_{17}$ (881·0) calculated: 61·13% C, 7·78% H; found: 61·31% C, 7·82% H. In addition to the main product, some starting succinate *VII* was isolated and the most polar fractions gave the salt of succinate *X*, characterized by its IR spectrum.

4'''-(4-(Methoxycarbonyl-2-(2*S*)-(4-hydroxyphenyl)ethyl)amino-4-oxobutanoyl)digoxin (*XI*)

L-Tyrosine methyl ester (195 mg; 1 mmol), followed by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (247 mg; 1 mmol), was added to a solution of the hemisuccinate *X* (200 mg; 0·23 mmol) in tetrahydrofuran (5 ml). The mixture was stirred at room temperature for 65 h, diluted with chloroform (60 ml) and washed with 5% aqueous solution of citric acid (2×) and water (2×). After evaporation, the residue (300 mg) was chromatographed on a silica gel column (30 g) in dichloromethane–methanol (95 : 5). The obtained product (100 mg) was further purified by preparative HPLC (steel column 500 × 12·6 mm, silica gel 10 μm, dichloromethane–2-propanol 10 : 1), affording 58 mg (24%) of compound *XI* as a solid foam. IR spectrum (chloroform): 1 780 sh, 1 745 (unsaturated γ-lactone), 1 745 (COOR), 3 605 (OH), 3 440, 1 671, 1 510 (CONH). For $C_{55}H_{79}NO_{19}$ (1 058·2) calculated: 62·43% C, 7·52% H, 1·32% N; found: 62·57% C, 7·77% H, 1·12% N.

4'''-(4-(1-Methoxycarbonyl-2-(2*S*)-(4-hydroxy-3-[^{125}I]-iodophenyl)ethyl)amino-4-oxobutanoyl)-digoxin (*XII*)

The title compound was prepared from the derivative *XI* in the same manner as described for *V* in a radiochemical yield of 50%.

Our thanks are due to Dr V. Machán for testing the activity of XII in the RIA-kit (Košice, Czechoslovakia). Analyses were carried out in the Analytical Laboratory of this Institute (Dr J. Horáček, Head), IR spectra were taken by Mrs H. Kapičková and Mrs K. Matoušková and interpreted by Dr J. Smoliková. We are indebted to Dr M. Buděšínský for the NMR measurements of compounds IV, VI, XI, XV, and XVI and for helpful discussions.

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Translated by M. Tichý.