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TRITIUM-LABELLED CARDIAC GLYCOSIDES: DIGOXIN-[12α-^{3}H][1]

A. v. WARTBURG, F. KALBERER and J. RUTSCHMANN*

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Abstract—The partial synthesis of digoxin-[12α-^{3}H] with a specific activity of 50 mc/mM is described.

A NUMBER of publications dealing with the use of ^{3}H-labelled cardiac glycosides in biochemical and pharmacological investigations[2–11] have appeared in recent years. It seems fitting, therefore, to point out some problems encountered in the preparation of such compounds and to report a method of synthesising ^{3}H-digoxin in which such difficulties have been overcome or lessened.

In principle there are three ways of preparing radioactively labelled natural substances:

1. Biosynthesis by intact plants or by parts of plants with the aid of a labelled precursor (e.g. $^{14}CO_2$,[12]^{14}C-acetate or ^{14}C-mevalonate).[13–16]
2. Exchange labelling with $^3H_2$ by the Wilzbach method,[17, 18] or with the aid of catalysts.[6]
3. Introduction of ^{14}C or ^{3}H by partial synthesis.

The first two methods have at least one of the following disadvantages: only relatively low specific activities are usually obtained; the radioactive starting materials are poorly utilised; random labelling occurs and the chemical and metabolic stability of the marker varies in the different positions; the purification of the desired product from highly active contaminants can be very difficult with concomitant low yields. The latter is undoubtedly the most serious of these objections. Numerous studies[19, 20] have shown that saturation of C—C double bonds occurs in the Wilzbach method and probably to a still greater extent in catalytic exchange labelling with tritium. In the case of aliphatic and alicyclic olefins hydrogenation may be the main reaction.[20, 21] Therefore on treating cardiac glycosides with tritium gas (or ^3H_2O and catalysts), hydrogenation of the unsaturated lactone ring has to be expected giving rise to greater or lesser amounts of dihydro derivatives whose specific activity is by several orders of magnitude greater than that of the desired products of the exchange reaction.

Since the preparative separation of cardiac glycosides from their dihydro-derivatives is, in our experience, fraught with considerable difficulties,[22] the greatest caution is called for in establishing that the products labelled by the exchange method are radiochemically homogeneous. Unfortunately in many of the papers cited, the data on the question of radiochemical purity are incomplete. Thus it is not possible to decide

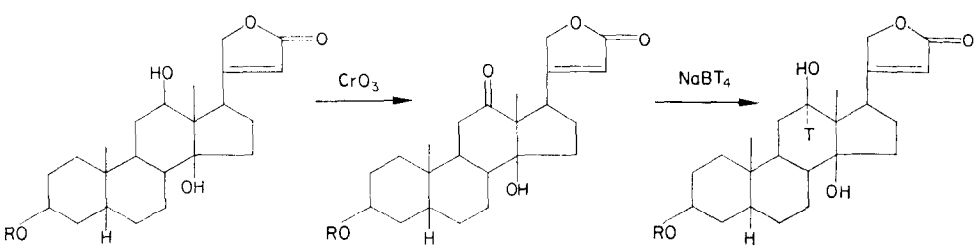
* Research Laboratories for Pharmaceutical Chemistry, Sandoz Ltd, Basle, Switzerland.

whether the preparations employed in the experiments were entirely satisfactory in this respect.

Influenced by considerations such as these and by a few unsatisfactory experiments of our own with the Wilzbach method, we thought to develop a procedure, permitting the introduction of the tracer into a specific position of a representative cardiac glycoside. This was accomplished, as described below, by reacting tetra-*O*-acetyl-12-dehydrodigoxin with $NaBT_4$ and saponifying the reaction product to digoxin-[12α-^{3}H].[24] This synthesis avoids the formation of highly active impurities, and the specific activity of the product is limited, in principle, only by that of the reducing agent.

As starting material we employed acetyldigoxin-α which is readily obtainable by the enzymatic degradation of lanatoside C.[25] We found that, under suitable conditions acetyldigoxin-α (1) is selectively oxidised by means of chromic acid in acetic acid, the sugar chain remaining largely intact. On chromatography of the oxydation products, a crystalline compound $C_{43}H_{64}O_{15}$, m.p. 201°; $(\alpha)_D = +63°$ (in methanol) was isolated and identified as 12-dehydroacetyldigoxin-α (2). The u.v. spectrum displayed maxima at 216 mμ and 285 mμ (log $\epsilon = 4{\cdot}18$ and $1{\cdot}66$, respectively); the i.r. spectrum had characteristic bands at 1780, 1740 and 1730 cm^{-1} (butenolide ring), at 1705 (six-ring ketone), at 1730 (shoulder) and at 1240 cm^{-1} (acetate). Hydrolytic cleavage of 12-dehydroacetyl-digoxin-α (2) yielded an aglycone $C_{23}H_{32}O_5$ (3) with a double m.p. 210°/270°, identical with 12-dehydrodigoxigenin (3).[26] Gentle saponification of the acetyl group converted 2 into the crystalline 12-dehydrodigoxin (4) $C_{41}H_{62}O_{14}$ which on acid hydrolysis also yielded 12-dehydrodigoxigenin (3). Compound 4 has u.v. and i.r. spectra such as would be expected for a 12-*oxo*-cardenolide (see Experimental).

On reacting 12-dehydro-acetyldigoxin-α (2) with acetic anhydride in pyridine, a mixture of tri- and tetra-*O*-acetyl-12-dehydrodigoxin is obtained; if allowed to proceed,



1 Acetyldigoxin-α (R = -Di-Di-AcDi)

2 12-Dehydro-acetyl-digoxin-α (R = -Di-Di-AcDi)

3 12-Dehydrodigoxigenin (R = H)

4 12-Dehydrodigoxin (R = -Di-Di-Di)

5 Tetra-O-acetyl-12-dehydrodigoxin (R = -AcDi-AcDi-Ac_2Di)

4 Digoxin-[12α-^{3}H] (R = -Di-Di-Di)

7 Digoxigenin-[12α-^{3}H] (R = H)

Di = β,D-Digitoxopyranosyl-
AcDi = 3-0-Acetyl-β,D-digitoxopyranosyl-
Ac_2Di = 3,4-Di-*O*-acetyl-β,D-digitoxopyranosyl-
Concerning the structure of the sugar chain vide M. Kuhn *et al.*[23]

the reaction favours the formation of the tetra-*O*-acetyl derivative $C_{49}H_{70}O_{18}$ (5). Chromatographic fractionation yields 5 in a homogeneous, though amorphous form: m.p. 142–146°; $[\alpha]_D = +92°$ (in chloroform). Preliminary attempts to reduce tetra-*O*-acetyl-12-dehydrodigoxin (5) with $NaBH_4$ or with $NaBT_4$ followed by careful deacetylation furnished a mixture of digoxin and two unidentified by-products. In the actual preparation, 5 was first reduced with $NaBT_4$ and was then treated with $NaBH_4$. The product so obtained was subjected to gentle deacetylation with dilute sodium carbonate solution in methanol and was then chromatographed on silicagel.[27] A continuous control by thin layer chromatography was maintained during fractionation. After crystallisation of the appropriate fractions from 75-per cent ethanol, chemically and radiochemically homogeneous digoxin-[12-α-^{3}H] was obtained, as shown by thin layer and paper radiochromatographies. The product was identical in all its chemical properties with an inactive test substance of highest purity. The specific activity hitherto attained in our investigations was 50 mc/mM.

To confirm the position of the tritium atom, digoxin-[12-^{3}H] (6) was hydrolysed. The sugar proved inactive, the total activity being found in the aglycone (7). Proof that the compound was specifically labelled in position 12, was obtained by oxidising digoxin-[12α-^{3}H] with chromic acid and hydrolysing the product to yield 12-dehydrodigoxigenin (3). This resulted in practically complete elimination of the ^{3}H, the product containing merely 1·7 per cent of the activity of the starting material.

Having regard to papers in the literature on the metabolic degradation of digitoxin and digoxin,[28–32] it may be assumed that the ^{3}H atom in position 12 of the glycoside is metabolically stable. Thus our preparation is a ^{3}H-labelled glycoside which meets all the requirements with regard to position of tracer and radiochemical homogeneity that must be imposed on material of this kind, if it is to furnish valid biochemical and pharmacological results.

EXPERIMENTAL*

12-*Dehydro-acetyldigoxin-α* (*2*)

823 mg acetyldigoxin-α (1) (1 mM) was dissolved in 30 ml glacial acetic acid with warming; after cooling to 20° 5 ml of a 2 per cent solution (w/v) of CrO_3 in acetic acid was added with stirring over 2 hr. 10 ml of methanol were added to the reaction mixture which was then left to stand for 1 hr whereupon it was evaporated to dryness *in vacuo* at 20°. The greenish-blue residue was taken up in 150 ml of chloroform and shaken with 2N H_2SO_4 and with water. The chloroform phase dried over Na_2SO_4 yielded 800 mg of colourless oxidation product on evaporation.

By way of purification, 3·2 g of crude product (from 4 oxidation batches) were chromatographed on 400 g of silica gel (Merck, granule size 0·05–0·2 mm). The fractions eluted with anhydrous ethyl acetate were analysed by thin layer chromatography (silica gel; developing solvent: isopropyl acetate + 5 per cent methanol, or chloroform +10 per cent methanol) and yielded 2·5 g homogeneous 12-dehydro-acetyl-digoxin-α (2). Crystallisation from ethyl acetate: colourless prisms, m.p. 201–204°; $[\alpha]_D^{20} =$

* All melting points were determined on the *Kofler* block. The samples employed for measuring the optical rotation were dried in high vacuum at 80° for 1 hr. All substances analysed and intermediates were tested by thin-layer chromatography, the latter being carried out by the method of E. STAHL.[33] The spots were made visable with antimony trichloride, 2, 4, 2′, 4′-tetranitrodiphenyl/NaOH or a 1% solution of ceric ammonium nitrate in 50% H_2SO_4. Radioactivity was determined by combustion in oxygen flasks and counting with the aid of a liquid scintillation counter as described previously.[34]

+ 62·4° (c = 0·481 in methanol), u.v. spectrum: λ_{max} at 216 mμ and at 285 mμ (log ε = 4·18 and 1·66, respectively); i.r. spectrum in CH_2Cl_2: 3550 (OH), 1780, 1740, 1630 (butenolide ring), 1730 (shoulder), 1240 (acetate), 1705 cm^{-1} (6-ring ketone).

$C_{43}H_{64}O_{15}$	Calculated	C 62·9, H 7·9, O 29·2 per cent.
(820·94)	Found	C 62·9, H 7·8, O 29·1 per cent.

12-*Dehydrodigoxigenin* (*3*)

A solution of 411 mg 12-dehydro-acetyldigoxin-α (2) in 20 ml methanol was heated with 20 ml O·1N H_2SO_4 under reflux for 30 min. The product was diluted with 10 ml of water, and the methanol was removed by concentration *in vacuo*. 173 mg of the crude aglycone crystallised from the aqueous concentrate. A further aglycone fraction of 82 mg was recovered from the mother liquor by extraction with chloroform. On recrystallisation from acetone, the genin (3) was obtained in the form of colourless prisms: double m.p. 210–213°/270–275°; $[\alpha]_D^{20}$ = + 115·9° (c = 0·500 in chloroform). u.v. spectrum: λ_{max} at 216 mμ and 282 mμ (log ε = 4·20 and 1·78, respectively); i.r. spectrum in CH_2Cl_2: 3580 (OH), 1780, 1742, 1640 (butenolide ring), 1700 cm^{-1} (12-ketone). The compound is identical with 12-dehydrodigoxigenin (3).*

$C_{23}H_{32}O_5$	Calculated	C 71·1, H 8·3, O 20·6 per cent.
(388·49)	Found	C 71·2, H 8·1, O 20·5 per cent.

12-*Dehydrodigoxin* (*4*)

370 mg 12-dehydro-acetyldigoxin-α (2) was dissolved in 92·5 ml methanol, and the solution was mixed with a solution of 370 mg $KHCO_3$ in 18·5 ml water and allowed to stand for ten days at 20°. The reaction solution was then concentrated to 15 ml *in vacuo* at 20°, whereupon 257 mg of crude product separated out. Recrystallisation from methanol yielded homogeneous 12–dehydrodigoxin (4), m.p. 277–279°; $[\alpha]_D^{20}$ = + 47·9° (c = 0·480 in absolute pyridine), u.v. spectrum: λ_{max} at 217 mμ and 282 mμ (log ε = 4·18 and 1·72, respectively), i.r. spectrum in CH_2Cl_2: 3575, 3475 (OH), 1785, 1745, 1630 (butenolide ring), 1705 cm^{-1} (12-ketone). Thin layer chromatography (silica gel; developing solvent: ethyl acetate/pyridine/water (5:1:4) (upper phase) or ethyl acetate + 6 per cent alcohol) showed the product to be homogeneous.

$C_{41}H_{62}O_{14}$	Calculated	C 63·2, H 8·0, O 28·8 per cent.
(778·91)	Found	C 62·9, H 8·2, O 29·1 per cent.

Tetra-O-acetyl-12-*dehydrodigoxin* (*5*)

2·08 g 12-dehydro-acetyldigoxin-α (2) were sealed in an ampoule with 20 ml absolute pyridine and 20 ml acetic anhydride and allowed to stand for six days at 20°. The contents were heated to 60° for 30 min and the solvent was then evaporated off *in vacuo*. The residue was taken up in 200 ml chloroform and shaken successively with 2N HCl, saturated $KHCO_3$ solution and water. The chloroform phase was dried over Na_2SO_4 and yielded 2·16 g of acetylation product. 1·58 g tetra-*O*-acetyl-12-dehydrodigoxin (5) was isolated by chromatography on alumina and elution with 4:1 and 7:3 benzene/chloroform mixtures. The amorphous substance was shown by thin-layer chromatography (silica gel; developing solvent: water-saturated isopropyl acetate +

* Reference sample was prepared by YAMADA's method[23] and had m.p. 270–275°; $(\alpha)_D^{20}$ = + 114·9° (c = 0·583 in chloroform). YAMADA reports the following values: m.p. 269–272°C; $(\alpha)_D^{20}$ = + 113° (in chloroform).

0·5 per cent methanol) to be homogeneous. Its m.p. was 142–146°; $[\alpha]_D^{20} = +92{\cdot}4°$ c = 0·557 in chloroform). The i.r. spectrum in CH_2Cl_2: 3575, 3450 (OH), 1780, 1740, 1630 (butenolide ring), 1705 (six-ring ketone), 1740, 1245 (acetyl) cm^{-1}.

$C_{49}H_{70}O_{18}$	Calculated	C 62·1, H 7·5, O 30·4, CH_3CO 18·2 per cent
(947·05)	Found	C 61·9, H 7·5, O 30·1, CH_3CO 18·2 per cent

Digoxin-[12α-^3H] (6)

A solution of 970 mg tetra-*O*-acetyl-12-dehydrodigoxin in 10 ml 75 per cent dioxane/water mixture was added dropwise over 15 min to 43 mg $NaBT_4$* (200 mc) in 5 ml 75 per cent dioxane/water contained in a flask with side-arm. The contents of the flask were then stirred with a magnetic stirrer for 18 hr at 20°, whereupon 100 mg $NaBH_4$ in 5 ml 75 per cent dioxane were added, the stirring being continued for a further 6 hr. The solution was next frozen in liquid air, the flask was evacuated via the side-arm, and when the contents had thawed they were acidified with 20 per cent acetic acid. After the reaction solution had again been frozen, the hydrogen-tritium mixture liberated by acidification was transferred to an ampoule with the aid of a Toepler pump. The solvent was removed by freeze-drying and the residue was taken up in 10 ml water and again freeze dried. The residue was dissolved in 10 ml water and exhaustively extracted with chloroform, the combined chloroform extracts being washed with water and dried over Na_2SO_4. The solvent was removed by evaporation and the reaction product taken up three times in 10 ml portions of methanol, the latter being removed each time by evaporation *in vacuo*. This yielded 930 mg crude tetra-*O*-acetyl-digoxin-[12α-^{3}H] with a specific activity of 40·8 mc/mM.

The crude product was dissolved in 60 ml methanol and this solution was stirred for two hours with 30 ml 0·25 N sodium carbonate solution. The solution was then adjusted to pH 5·5 with 0·25 H_2SO_4 and concentrated to 10 ml at 25° *in vacuo*. 20 ml water was added to the crystal slurry and the solution was again concentrated to ~10 ml. The solution was then extracted with 9:1 chloroform/butanol and the extract was washed with water, dried over Na_2SO_4 and the solvent mixture evaporated off. The residue (860 mg) was taken up in chloroform/methanol 9:1 and chromatographed on 400 g silica gel (Merck, granule size 0·05–0·2 mm) by Duncan's method,[27] using the same mixture of solvents. After a first run of 600 ml, 100 fractions each of 13 ml were collected. Fractions 30–80 were evaporated separately to dryness and 100 μg of each sample were tested by thin-layer chromatography (silica gel; developing solvent: chloroform/methanol 9:1, spray-reagent: 1 per cent solution of ceric ammonium nitrate in 50 per cent H_2SO_4). Recrystallisation of fractions 57–70 (237 mg) twice from alcohol/water yielded 197 mg digoxin-[12α-^{3}H] (6), m.p. 218–247°, specific activity 47·3 mc/mM. This product was chemically identical with a test preparation of the highest purity and was free from radioactive contaminants (thin-layer chromatogram, see above; for the paper chromatogram, the paper was impregnated with 8:2 acetone/formamide; developing solvent: methyl-ethylketone/*m*-xylene/formamide (100:100:8). The mother liquors as well as fractions 50–56 and 71–80 (221 mg) were again chromatographed on 400 g silica gel in the same manner. Two recrystallisations furnished addi-

* The preparation used was one that had been procured two years earlier from the Radiochemical Center, Amersham, England. It had been kept at room temperature and was employed without prior purification. Some deterioration with a decrease of tritium available for reduction must be assumed.

tional 98 mg radiochemically pure digoxin-[12α-^{3}H] (6) with a specific activity of 46·9 mc/mM.

Hydrolytic cleavage of digoxin-[12α-^{3}H] (6) to digoxigenin[12α-^{3}H] (7) and digitoxose

128 mg digoxin-[12α-^{3}H] (a sample of the above preparation diluted with inactive material to 281 μc/mM) was dissolved in 20 ml methanol and warmed with 20 ml 0·1 N H_2SO_4 for 45 min under reflux. The solution was then concentrated to ~20 ml *in vacuo* and exhaustively extracted with chloroform. The extract was washed with water and dried, and the solvent evaporated off. The residue (60 mg) was recrystallised from alcohol/water. The digoxigenin-[12α-^{3}H] (7) so obtained was identical in m.p. (205–209°), in thin-layer and paper chromatograms with a standard substance and had a specific activity of 280 μc/mM.

The combined aqueous phases were neutralised with a suspension of 880 mg finely powdered barium carbonate in 6 ml water. The barium salt was centrifuged off and the solution evaporated to dryness *in vacuo*. The residue was taken up in water and again evaporated to dryness in vacuo. The digitoxose (70 mg) so obtained had a specific activity of 1·9 μc/mM.

Oxidation and hydrolysis of digoxin-[12α-^{3}H] to 12-dehydrodigoxigenin (3).

450 mg digoxin-[12α-^{3}H] (60·5 μc/mM) was dissolved in 70 ml glacial acetic acid with warming, and 9 ml of a 2 per cent solution of CrO_3 in glacial acetic acid was added dropwise over 45 min at 20° with stirring. After 90 min 10 ml methanol was added to the green solution which was allowed to stand overnight and then concentrated by evaporation *in vacuo*. The syrupy residue was taken up in acetic ester and shaken in turn with ice-cold N H_2SO_4, saturated $KHCO_3$ solution and water. The organic phase was dried over Na_2SO_4 and evaporated to dryness *in vacuo*. For hydrolysis the white, amorphous residue (333 mg) was taken up in 50 ml methanol, and 50 ml 0·1 N H_2SO_4 was added to the solution which was then heated under reflux for 45 min. The solution was then concentrated to 30 ml and shaken with ethyl acetate. The crude aglycone (150 mg) obtained after drying and evaporating the solution, was recrystallised from acetone/ether. The 12-dehydrodigoxigenin (3) obtained had a specific activity of 1·8 μc/mM and a double melting point of 210–213°/270–277°; $[\alpha]_D^{20} = +113°$ (c = 0·528 in chloroform).

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