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BIOSYNTHESIS OF THE BUTENOLIDE RING OF CARDENOLIDES IN *DIGITALIS PURPUREA*

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Key Word Index—*Digitalis purpurea*; Scrophulariaceae; foxglove; cardenolide; biosynthesis; digitoxin; gitoxin; norcholanoic acids.

Abstract—Administration of labelled 3β,20ξ-dihydroxy-23-norchol-5-enoic acid, 3-oxo-20ξ-hydroxy-23-norchol-4-enoic acid, 3β,20ξ-dihydroxy-23-nor-5β-cholanoic acid, 3β,14β,20ξ-trihydroxy-23-nor-5β-cholanoic acid, 3β-hydroxy-23-norchola-5,20(22)*E*-dienoic acid, 3-oxo-23-norchola-4,20(22)*E*-dienoic acid and 3β-hydroxy-23-nor-5β-chol-20(22)*E*-enoic acid to *Digitalis purpurea* intact plants produced labelled digitoxin and gitoxin. The incorporation results indicate the existence of an alternative pathway, via norcholanoic acid derivatives, for the biosynthesis of the butenolide ring of cardenolides.

INTRODUCTION

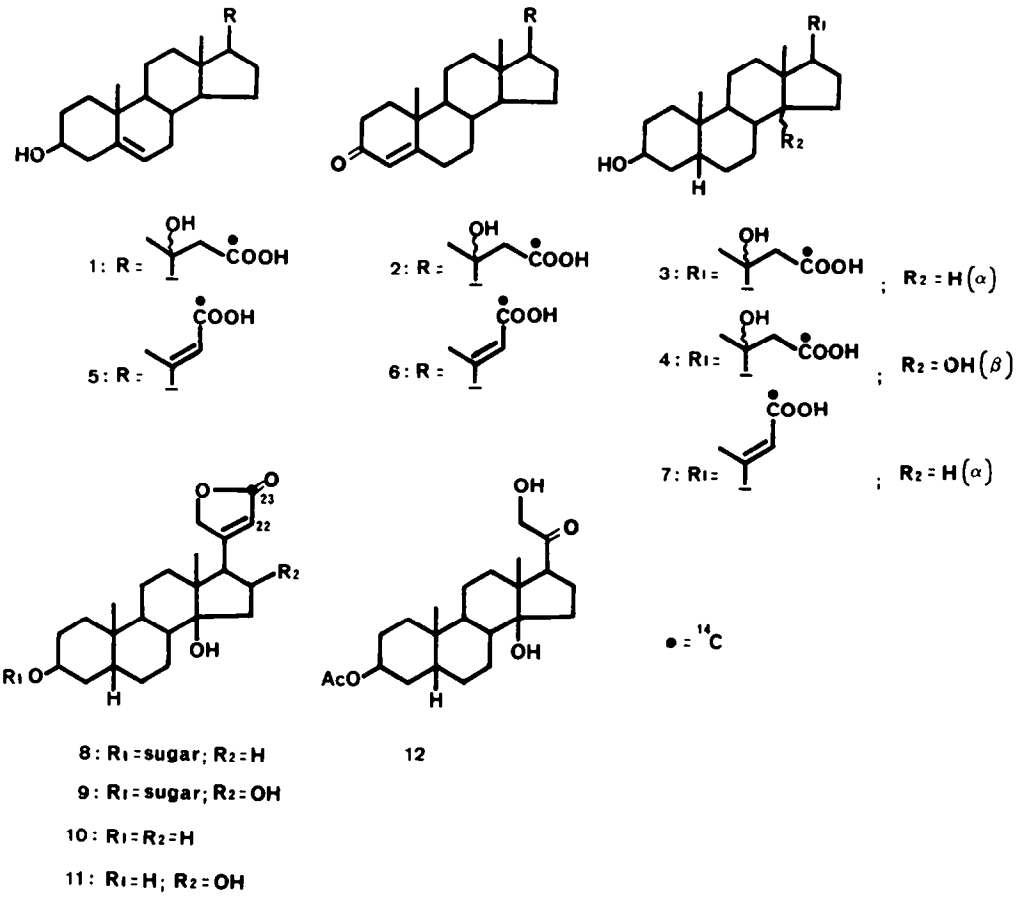
The biosynthesis of cardenolides has been the subject of several investigations using a variety of labelled intermediates which were administered in different ways and during different lapses of time to the known cardenolide-producing plants *Digitalis purpurea*, *Digitalis lanata* and *Strophanthus kombé*. The results obtained until now have indicated that mainly pregnenolone [1–3] and progesterone [4–6] but also 5β-pregnan-3,20-dione [7], 3β-hydroxy-5β-pregnan-20-one [7], 3β,14β-dihydroxy-5β-pregnan-20-one [8] and 3β,14β,21-trihydroxy-5β-pregnan-20-one [9] were incorporated into cardenolides to an extent which supports their participation in the biosynthetic pathway to the cardioactive genins. The role of deoxycorticosterone as a precursor of cardenolides was found to be of little significance [10]. The formation of the lactone ring, characteristic of cardenolides, was found to be produced by condensation of an acetate unit on a 20-keto-pregnane derivative [11, 12]. As plausible precursors having a formed butenolide ring were not incorporated into cardenolides [8] it was proposed that the condensation of the acetate should be one of the final steps in the biosynthetic pathway. According to these results, Tschesche [13, 14] proposed a sequence in which the formation of the butenolide ring should be produced after hydroxylation at C-14β and at C-21 of the 20-keto-pregnane intermediate. On the other hand, the condensation of the acetate unit could have been performed on a partially hydroxylated 20-keto pregnane derivative as pregnenolone, progesterone, 3β-hydroxy-5β-pregnan-20-one, etc. leading to cardenolides by an alternative route. This pathway, which could be called 'of the norcholanoic acids' may be operative in the plant as well as the tested route 'of the pregnanes' indicated above.

In order to investigate the proposed alternative pathway, *Digitalis purpurea* plants were fed in separate experiments with several norcholanoic acid derivatives labelled at C-24 which were synthesized for this purpose [15, 16].

RESULTS AND DISCUSSION

[24-^{14}C]-3β,20ξ-Dihydroxy-23-norchol-5-enoic acid (**1**), [24-^{14}C]-3-oxo-20ξ-hydroxy-23-norchol-4-enoic acid (**2**), [24-^{14}C]-3β,20ξ-dihydroxy-23-nor-5β-cholanoic acid (**3**), [24-^{14}C]-3β,14β,20ξ-trihydroxy-23-nor-5β-cholanoic acid (**4**), [24-^{14}C]-3β-hydroxy-23-norchola-5,20(22)*E*-dienoic acid (**5**), [24-^{14}C]-3-oxo-23-norchola-4,20(22)*E*-dienoic acid (**6**) and [24-^{14}C]-3β-hydroxy-23-nor-5β-chol-20(22)*E*-enoic acid (**7**) were administered to 5-month-old *Digitalis purpurea* plants growing in soil by application of solutions of the labelled compounds on the upper surface of the leaves. To check the efficiency of the administration procedure [1,2,6,7-^{3}H]progesterone was used in parallel experiments. The plants were exposed to indirect sunlight for 14 hr/day. After 14 days the plants were harvested and processed as described elsewhere [17, 18]. To facilitate the isolation and purification of the glycosides, non-radioactive digitoxin (**8**) and gitoxin (**9**) were added at the time of the extraction of the fresh leaves. In all cases the crude glycosides were isolated by prep. TLC, diluted with authentic material and recrystallized to constant specific activity. The results are summarized in Table 1. Mild acid hydrolysis produced the respective genins, digitoxigenin (**10**) and gitoxigenin (**11**) which maintained the total radioactivity of the glycosides indicating that the label was exclusively located at the steroid moiety of the products. The results obtained in the experiment with labelled progesterone were similar to those previously reported [5]. In one case radioactive digitoxin was degraded, as reported elsewhere [12] leading to the isolation of 3β-acetoxy-14β,21-dihydroxy-5β-pregnan-20-one (**12**) and glycolic acid; the results (Table 2) indicated that the label was located at the glycolic acid formed from the original C-22 and C-23 of the genin.

From the figures presented in Table 1 it is clear that all the norcholanoic acid derivatives are actively metabolized and incorporated into the glycosides, supporting the existence in *D. purpurea* of a system capable of converting



C_{23} precursors into cardenolides. Our results, however, differ from the observations from other research groups which support the complete stereochemical modifications and introduction of functional groups in the tetracyclic steroid system of a pregnane before the introduction of the remaining two carbons needed for the construction of the lactone ring of cardenolides.

From Table 1 it can be observed that Δ^{22} compounds **5**, **6** and **7** were better precursors than their 20-hydroxylated counterparts **1**, **2** and **3** respectively. The somewhat higher incorporation of the Δ^{22} compounds may be significant and can be rationalized on the basis that 20-hydroxy compounds **1**, **2** and **3** are mixtures of epimers at C-20 [19] while the unsaturated derivatives **5**, **6** and **7** have the correct stereochemistry for forming the lactone ring.

An unexpected feature of these experiments was that compounds having a pregnenolone-type structure resulted in better precursors than those having progesterone

Table 1. Data of administration of tracers to *Digitalis purpurea* plants

Precursor fed	Activity $\left(\frac{\text{dpm}}{\text{mmol}} \times 10^9\right)$	Amount (mg)	Activity absorbed after 14 days (dpm × 10^7)	Digitoxin Total act. (dpm × 10^4)	Digitoxin Inc.* (%)	Gitoxin Total act. (dpm × 10^4)	Gitoxin Inc.* (%)
1	2.60	1.8	0.26	1.63	0.62	0.61	0.23
2	2.33	3.2	0.30	0.71	0.24	0.33	0.11
3	2.22	2.8	0.25	0.72	0.29	0.37	0.15
4	2.18	3.3	0.15	1.45	0.96	0.77	0.51
5	2.29	2.0	0.38	11.46	3.00	10.87	2.86
6	1.38	1.4	0.11	0.52	0.47	0.55	0.50
7	2.42	2.5	0.06	0.32	0.53	0.29	0.48

*Incorporation is defined as the total radioactivity present in the isolated glycoside divided by the total radioactivity absorbed by the plants.

Table 2. Degradation of digitoxin from the experiment with tracer **3**

Compound	Sp. act. $\left(\frac{dpm}{mmol} \times 10^6\right)$	Rel. sp. act. (%)
Digitoxin	1.49	100
Digitoxigenin	1.50	100
Aqueous phase (digitoxose)	< 0.01	0
3-Acetyldigitoxigenin	1.52	101
Compound **12**	< 0.01	0
Glycolic acid	1.50	100

and 5β-saturated systems. In this respect it is notable that compound **5** is the preferred substrate for the biosynthetic process.

Although at this time it is not possible to explain the more efficient incorporation of **5** into the glycosides **8** and **9**, these results suggest that in the case of C_{23} precursors the introduction of the hydroxyl group at C-14β precedes the reduction of the Δ^5 double bond; moreover, this finding may indicate that the presence of a double bond in ring B might be necessary for the hydroxylation at C-14 with inversion of configuration, a mechanism still unknown.

Compound **4**, a 14β hydroxylated substrate but also having a hydroxyl group at C-20, gave more efficient incorporation than the remaining 20-hydroxylated compounds and in this respect was less important than compound **5**. Taking into account that **4** is an epimeric mixture at C-20, its incorporation value was almost comparable to that of **5**.

Although plants may utilize non-specific substrates for the biosynthesis of secondary metabolites [20] the present results would support the hypothesis that in *D. purpurea* plants the formation of the butenolide ring of cardenolides may follow different pathways, namely, the 'route of pregnanes' already established and the 'route of norcholanoic acids' here reported. A direct comparison between both mechanisms at different stages of the process is under way in our laboratory.

EXPERIMENTAL

Analytical TLC was performed on silica gel G; prep. TLC on silica gel F_{254}. Labelled compounds **1**–**7** were obtained by synthetic procedures already reported [15, 16]. Labelled progesterone was purchased from New England Nuclear. Radioactivity was measured by liquid scintillation counting.

Feeding of tracers and isolation of the glycosides. The experiments were conducted on 5-month-old *Digitalis purpurea* plants growing in soil. The leaf wax was removed from the upper surface of the leaves by wiping with cotton wool moistened with EtOAc. Solns of tracers (CH_2Cl_2–MeOH, 7:3) were applied with a glass capillary. After evaporation of the solvent, the leaves were sprayed with 10% silicone oil in petrol. After 14 days the plants were harvested, the leaves were washed with CH_2Cl_2–MeOH (7:3) and the washings were concd, analysed by TLC and measured for radioactivity. In all cases the recovered radioactivity corresponded to the administered tracer. The fresh leaves were ground with sand and extracted with boiling 70% MeOH; the MeOH was removed *in vacuo* and the remaining aq. soln was extracted with CH_2Cl_2 (5 × 10 ml). As each extract showed the presence of digitoxin and gitoxin (TLC), pure glycosides (2 mg of each) were added to the extracts. Isolation of the glycosides was performed by prep. TLC (CH_2Cl_2–MeOH–H_2O, 90:10:0.25). Each glycoside was diluted with authentic material (10 mg) and recrystallized from MeOH–Et_2O to constant specific activity.

Degradation of digitoxin. Labelled digitoxin from the feeding experiment with tracer **3** (7.9 mg, 1.49×10^6 dpm/mmol) was hydrolysed in MeOH (0.5 ml) containing 0.1 N H_2SO_4 (0.5 ml) by refluxing for 40 min. The digitoxigenin (5.4 mg) isolated as described [12] was acetylated (Ac_2O–pyridine) affording acetyldigitoxigenin (3.5 mg, 1.52×10^6 dpm/mmol). This was dissolved in CH_2Cl_2 (2 ml), cooled to $-60°$ and ozone was bubbled through the soln until disappearance (TLC) of the starting material (20 min). Decomposition of the ozonide by addition of Zn powder (13 mg) and HOAc (0.01 ml) and stirring for 20 hr afforded a product that was hydrolysed in MeOH (0.2 ml) and 10% aq. $NaHCO_3$ (0.08 ml). Addition of H_2O (3 ml), removal of MeOH and extraction with CH_2Cl_2 (4 × 1 ml) yielded inactive compound **12** (2.6 mg). The remaining aq. phase was worked up as described [12]. The glycolic acid had a sp. act. of 1.50×10^6 dpm/mmol.

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