*Phytochemistry*, Vol. 28, No. 1, pp. 109–111, 1989.
Printed in Great Britain.

0031-9422/89 $3.00 + 0.00


# BIOSYNTHESIS OF DIGITOXIN IN *DIGITALIS PURPUREA*

MÓNICA E. DELUCA, ALICIA M. SELDES and EDUARDO G. GROS

Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, 1428 Buenos Aires, Argentina

(*Received in revised form* 11 *May* 1988)

**Key Word Index**—*Digitalis purpurea*; Scrophulariceae; cardenolide; digitoxin; biosynthesis; [21-$^3$H]-3$\beta$, 20$\xi$-dihydroxy-23-nor-5$\beta$-cholanoic acid; [21-$^{14}$C]-3$\beta$-hydroxy-5$\beta$-pregnan-20-one.

**Abstract**—Simultaneous administration of a mixture of [21-$^3$H]-3$\beta$, 20$\xi$-dihydroxy-23-nor-5$\beta$-cholanoic acid and [21-$^{14}$C]-3$\beta$-hydroxy-5$\beta$-pregnan-20-one to *Digitalis purpurea* intact plants produced double labelled digitoxin. The $^3$H:$^{14}$C ratio of the cardenolide was considerably higher than the $^3$H:$^{14}$C ratio of the administered substrates. This result indicates that in the main biosynthetic pathway from a 20-keto-pregnane to a cardenolide the introduction of an acetate unit at C-20 should occur before the hydroxylation at C-21.

## INTRODUCTION

It has been established by feeding experiments with labelled acetate, mevalonate and pregnenolone that the biological formation of the butenolide ring of cardenolides in plants of the genus *Digitalis* proceeds by the condensation of an acetate unit on to C-20 of a 20-keto-pregnane derivative [1–3].

Our results led us to propose that the biosynthetic pathway would involve, after the condensation of the acetate unit, a dehydration of the 20-hydroxy intermediate to a $\Delta^{20(22)}$-unsaturated side chain which upon allylic hydroxylation at C-21 would produce the unsaturated lactone ring [3].

Following experiments conducted with labelled pregnane derivatives bearing hydroxyl groups at C-14$\beta$ and at C-21, Tschesche *et al.* [4, 5] proposed that the formation of the butenolide ring should occur after hydroxylations at C-14$\beta$ and at C-21 of the 20-keto-pregnane precursor.

We have recently demonstrated that various 23-norcholanoic acid derivatives can act as precursors of the butenolide ring of cardenolides in the plant *Digitalis purpurea* [6]. These results indicated that the condensation of the acetate unit on a 20-keto-pregnane to form the lactone ring of cardenolides could preceed the hydroxylations at C-21 and at C-14$\beta$ of the pregnane intermediate.

In order to confirm that a 23-norcholanoic acid is a real biosynthetic intermediate between a 20-keto-pregnane and a cardenolide, *Digitalis purpurea* plants were fed with a mixture of [21-$^3$H]-3$\beta$,20$\xi$-dihydroxy-23-nor-5$\beta$-cholanoic acid and [21-$^{14}$C]-3$\beta$-hydroxy-5$\beta$-pregnan-20-one.

Labelled digitoxin was isolated and the $^3$H:$^{14}$C isotopic ratio was determined to draw some conclusions regarding the relative importance of the biosynthetic roles of the two steroids.

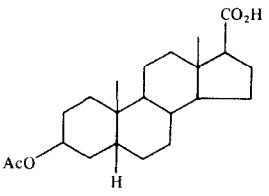
## RESULTS

[21-$^3$H]-3$\beta$, 20$\xi$-Dihydroxy-23-nor-5$\beta$-cholanoic acid (**4**) was synthesized by a sequence that starts with 3$\beta$-acetoxy-5$\beta$-androstane-17$\beta$-carboxylic acid (**1**) which was converted into [21-$^3$H]-3$\beta$-acetoxy-5$\beta$-pregnan-20-one (**2**) following the procedure described for the radiocarbon labelled compound [7] but using [$^3$H]-methyl iodide instead of the radiocarbon-labelled compounds. Treatment of compound **2** with ethyl bromoacetate in Reformatsky conditions as reported elsewhere [8] afforded ethyl [21-$^3$H]-3$\beta$-acetoxy-20$\xi$-hydroxy-23-nor-5$\beta$-cholanoate (**3**) which was saponified to yield compound **4**. On the other hand, [21-$^{14}$C]-3$\beta$-hydroxy-5$\beta$-pregnan-20-one (**5**) was prepared as already described [7].

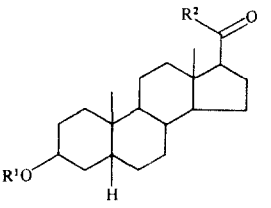
Five specimens of six-month-old *Digitalis purpurea* plants growing in soil were fed in parallel experiments with a mixture of both labelled compounds **4** ($9.51 \times 10^6$ dpm) and **5** ($1.42 \times 10^6$ dpm) with a $^3$H:$^{14}$C ratio of 6.70, by application of a solution of the mixture on the upper surface of the leaves. The plants were exposed to sunlight for 10 hr/day. After seven days the plants were harvested, the leaves were washed to recover the unabsorbed labelled steroids, and the washings were analysed by TLC confirming that both labelled compounds had not been degraded. In five parallel experiments the average $^3$H:$^{14}$C ratio in the washing of leaves after administration of compounds **4** and **5** ($^3$H:$^{14}$C ratio 6.70) was $6.67 \pm 0.51$. The washed leaves were extracted as already reported [9]. To facilitate the isolation and purification of the glycoside, non-radioactive digitoxin (**6**) was added at the time of extraction. In all cases the crude digitoxin was isolated by preparative TLC, diluted with authentic material and recrystallized to constant $^3$H:$^{14}$C ratio. The results are summarized in Table 1.

## DISCUSSION

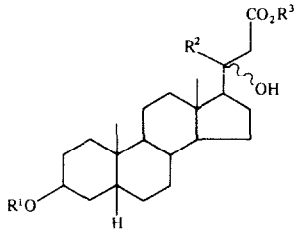
Since the experiment reported by Tschesche *et al.* [2], it has been shown that pregnenolone is a good precursor of cardenolides. This result was followed by positive results using progesterone [10–12] and other hydroxylated $C_{21}$-steroids [4, 5]. We demonstrated [6] that 23-norcholanoic acids can also act as precursors of cardenolides, supporting the postulated existence in the plant of a

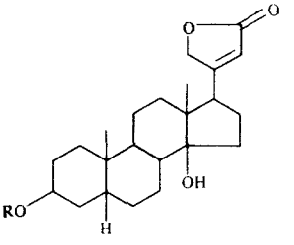



**1**




**2** $R^1$ = Ac, $R^2$ = $C^3H_3$
**5** $R^1$ = H, $R^2$ = $^{14}CH_3$




**3** $R^1$ = Ac, $R^2$ = $C^3H_3$, $R^3$ = Et
**4** $R^1$ = $R^3$ = H, $R^2$ = $C^3H_3$

**6** R = sugar

Table 1. Radioactivity values and $^3H$:$^{14}C$ ratio of digitoxin recovered after administration of [21-$^3$H]-3$\beta$, 20$\xi$-dihydroxy-23-nor-5$\beta$-cholanoic acid (9.51 × $10^6$ dpm) and [21-$^{14}$C]-3$\beta$-hydroxy-5$\beta$-pregnan-20-one (1.42 × $10^6$ dpm) ($^3H/^{14}C$ ratio = 6.70) to five *Digitalis purpurea* plants in parallel experiments

| Experiment | | A | B | C | D | E |
|---|---|---|---|---|---|---|
| Amount of digitoxin (mg) | | 15 | 12 | 12 | 13 | 10 |
| Activity of digitoxin (dpm) | $^3H$ (x $10^4$) | 8.27 | 3.06 | 8.56 | 9.44 | 3.14 |
| | $^{14}C$ (x $10^3$) | 5.30 | 2.46 | 4.15 | 5.53 | 2.61 |
| Specific activity (dpm/mg) | $^3H$ ( × $10^3$) | 5.51 | 2.55 | 7.13 | 7.26 | 3.14 |
| | $^{14}C$ ( × $10^2$) | 3.53 | 2.05 | 3.46 | 4.25 | 2.61 |
| First recryst. (dpm/mg) | $^3H$ ( × $10^3$) | 5.63 | 2.43 | 7.29 | 7.18 | 3.02 |
| | $^{14}C$ ( × $10^2$) | 3.42 | 1.94 | 3.65 | 4.13 | 2.68 |
| Second recryst. (dpm/mg) | $^3H$ ( × $10^3$) | 5.58 | 2.51 | 7.30 | 7.12 | 3.07 |
| | $^{14}C$ ( × $10^2$) | 3.45 | 1.96 | 3.70 | 4.20 | 2.72 |
| $^3H$:$^{14}C$ ratio | | 16.2 | 12.8 | 19.7 | 17.0 | 11.3 |

system capable of converting $C_{23}$-steroids into cardenolides [3].

To check the precursor capability of a 20-keto-pregnane derivative against a 23-norcholanoic acid derivative on the biosynthesis of the cardenolide digitoxin in plants *Digitalis purpurea*, our approach was to administer simultaneously a mixture of [21-$^3$H]-3$\beta$, 20$\xi$-dihydroxy-23-nor-5$\beta$-cholanoic acid and [21-$^{14}$C]-3$\beta$-hydroxy-5$\beta$-pregnan-20-one, and to evaluate the relative extents of incorporation of the two tracers into digitoxin.

The label at C-21 of the 20-keto-pregnane derivative was chosen to be carbon-14 instead of tritium because the latter could be partially lost during the feeding experiment through a keto–enol equilibrium in the intracellular media. A mixture of both labelled products was administered to healthy plants as described in the Exper-

imental, and after seven days the plants were harvested.

At this point, it is appropriate to remark that the administration of a mixture of substrates of different structures constitutes a complicated biosynthetic problem. It seems probable that the relative rate of absorption, transport and metabolism of the exogenous steroids will differ, and therefore, the conclusions drawn from a study of this type should be treated with caution. However, the distribution of the two isotopes in the washings of the leaves (Table 1) did not show considerable variations, remaining fairly close to the $^3H:^{14}C$ ratio of the fed substrates. Therefore, in spite of the limitations discussed above, these results indicate that the relative rate of absorption of both precursors did not differ to such an extent as to introduce considerable errors in the final results; the probable differences in transport and metabolism of the exogenous steroids remain unknown.

The washed leaves were extracted and processed as reported elsewhere [9]. Digitoxin was purified by recrystallization to constant $^3H:^{14}C$ ratio. All the ratio values presented in Table 1, although showing variations among the different experiments, are indicative of a greater incorporation of the tritium-labelled 23-norcholanoic acid derivative **4**; the difference in incorporation between both precursors could be even greater than the observed values taking into account that compound **4** was an epimeric mixture at C-20.

The present results strongly indicate that *D. purpurea* plants have the capacity to incorporate a $C_{23}$-steroidal precursor into cardenolides more efficiently than a $C_{21}$-compound. Moreover, the $^3H:^{14}C$ ratio determined for digitoxin (mean value 15.4) was more than twice the value of the initially administered mixture of steroids (6.7) and this suggests a competition between two pathways, i.e. hydroxylation of **5** at C-21 or introduction of the acetate unit at C-20 of the same precursor **5**. Considering the two possibilities, the latter mechanism, through a 23-norcholanoic acid, appears significantly more important than the former one. The 20-keto-pregnane must be a more active metabolite and it may enter different metabolic pathways. Among them, it can be hydroxylated at C-21 en route to cardenolides, but in view of the aforementioned results this pathway should be of secondary importance. This conclusion is supported by the work of Caspi *et al.* [13] who found that the hydroxylation at C-21 of a 20-keto pregnane such as progesterone was not of major importance in the biosynthesis of cardenolides.

Hence, the condensation of an acetate unit onto a 20-keto-pregnane intermediate should be the main biosynthetic pathway from this intermediate to cardenolides. It may be inferred that dehydration to a $\Delta^{20(22)}$-unsaturated intermediate and allylic introduction of the C-21 oxygen function would follow the condensation reaction.

## EXPERIMENTAL

Analytical TLC was performed on silica gel G; prep. TLC on silica gel $F_{254}$. [$^3$H]-Methyl iodide was purchased from New England Nuclear. Radioactivity was measured by liquid scintillation counting.

[21-$^3$H]-*3β-Acetoxy-5β-pregnan-20-one* (**2**). 3β-Acetoxy-5β-androstane-17β-carboxylic acid (**1**, 97 mg) was treated as previously described [7] with [$^3$H]-methyl iodide (96.0 mCi/mmol, 25 mCi) diluted with inactive MeI. Compound **2** was purified by CC (silica gel, $CHCl_3$) and recrystallized from EtOH yielding 27 mg of **2**, sp. act. 11.1 mCi/mmol and IR spectrum identical to that of an authentic standard.

*Ethyl* [$^3$H]-*3β-acetoxy-20ξ-hydroxy-23-nor-5β-cholanoate* (**3**). A soln of **2** (25 mg) in dry $C_6H_6$ (0.5 ml) was treated, under a $N_2$ atmosphere, with Zn powder (37 mg) and a few crystals of iodine. The mixture was heated under reflux while ethyl bromoacetate (0.075 ml) was added dropwise. After 30 min the reaction mixture was poured into dil. HCl kept at 0°, and extracted with $Et_2O$. The organic layer was washed with $H_2O$ and dried ($MgSO_4$). The solvent was removed and the residue was purified by CC (silica gel, petrol–EtOAc, 9:1) and recrystallized from EtOH. Compound **3** (12 mg, 10.9 dpm/mmol) had an IR spectrum identical to that of an authentic standard [8].

[21-$^3$H]-*3β, 20ξ-Dihydroxy-23-nor-5β-cholanoic acid* (**4**). Compound **3** (11.5 mg) was dissolved in MeOH (2 ml), a 10% soln of KOH (0.15 ml) was added, and the soln was refluxed for 2 hr. The reaction was diluted with $H_2O$, neutralized with dil. HCl, and extracted with EtOAc. The organic soln was washed with $H_2O$ and dried. Evapn of the solvent gave compound **4** (8.8 mg from $Me_2CO$) of sp. act. 10.9 mCi/mmol. Its IR spectrum was identical to that of authentic material.

*Feeding of tracers and isolation of digitoxin.* The experiments were conducted on 6-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 $Me_2CO$. Soln of tracers (EtOH) was applied with a glass capillary. After 7 days the plants were harvested, the leaves were washed with EtOH, and the washings were concd, analysed by TLC and measured for radioactivity. In all cases the recovered radioactivity corresponded to the administered tracers.

The leaves were ground with sand and extracted with EtOH (6 × 15 ml). The solvent was evaporated, the residue was redissolved in EtOH (1 ml) containing pure digitoxin (4 mg) and evapd again. Isolation of the glycoside was performed by prep TLC ($CHCl_3$–EtOH 93:7). The isolated digitoxin was diluted with authentic material (15 mg) and recrystallized from EtOH–$H_2O$ to constant $^3H:^{14}C$ ratio.

*Acknowledgements*—We thank CONICET and The Organization of the American States for partial financial support.

## REFERENCES

1. Euw, J. von and Reichstein, T. (1964) *Helv. Chim. Acta* **47**, 711.
2. Tschesche, R. and Lilienweiss, G. (1964) *Z. Naturforsch.* **19b**, 265.
3. Leete, E., Gregory, H. and Gros, E. G. (1965) *J. Am. Chem. Soc.* **87**, 3475.
4. Tschesche, R., Hulpke, H. and Scholten, H. (1967) *Z. Naturforsch.* **22b**, 677.
5. Tschesche, R., Becker, R. and Hombach, R. (1968) *Z. Naturforsch.* **23b**, 1615.
6. Maier, M. S., Seldes, A. M. and Gros, E. G. (1986) *Phytochemistry* **25**, 1327.
7. Garraffo, H. M., Deluca, M. E., Seldes, A. M. and Gros, E. G. (1985) *J. Lab. Compds. Radiopharm.* **22**, 673.
8. Maier, M. S., Seldes, A. M. and Gros, E. G. (1985) *J. Lab. Compds. Radiopharm.* **22**, 467.
9. Deluca, M. E., Seldes, A. M. and Gros, E. G. (1987) *Z. Naturforsch.* **42c**, 77.
10. Caspi, E. and Lewis, D. O. (1967) *Science* **156**, 519.
11. Bennett, R. D., Sauer, H. H. and Heftmann, E. (1968) *Phytochemistry* **7**, 41.
12. Caspi, E. and Hornby, G. M. (1968) *Phytochemistry* **7**, 423.
13. Caspi, E., Wickramasinghe, J. A. F. and Lewis, D. O. (1968) *Biochem. J.* **108**, 499.