Phytochemistry, 1980, Vol. 19, pp. 1463–1466. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0701-1463 $02.00/0

BIOGENESIS OF THE *C*-GLYCOSIDE LEUCODRIN IN *LEUCADENDRON ARGENTEUM*

C. W. GLENNIE and G. W. PEROLD

Department of Chemistry, University of the Witwatersrand, Johannesburg, South Africa

(*Received* 8 *May* 1979)

Key Word Index—*Leucadendron argenteum*; Proteaceae; leucodrin; ascorbigens; biosynthesis.

Abstract—Biogenetic pathways for leucodrin formation in *Leucadendron* spp. are discussed and were tested by feeding labelled D-galactose, D-glucose and L-ascorbic acid to cut stem ends of *L. argenteum*. It was shown that the two aldohexoses are better precursors and preferentially incorporated into that portion of leucodrin derivable from *p*-coumaric acid. When this route is suppressed by feeding the labelled sugars in the presence of a large amount of phenylalanine) label is predominantly incorporated in the carbohydrate part of leucodrin. The biogenesis of leucodrin therefore may proceed via coupling of L-galactono-γ-lactone and *p*-coumaric acid.

INTRODUCTION

Leucodrin (**1**, R=H) [1–3] occurs in all of the 55 species of the genus *Leucadendron* (Proteaceae) so far examined and is generally accompanied by its catechol analogue, leudrin (**1**, R=OH [4]). In the closely related genus *Leucospermum* of the same family, its diastereoisomer, conocarpin (**2**) has been isolated together with the analogous B-ring-opened hydroxy acid conocarpic acid (**3**, R=H), and the corresponding (probably artefactual) methyl ester, reflexin (**3**, R=Me) [5, 6].

The biogenesis of leucodrin has been postulated [3] to be via a Michael condensation of *p*-coumaric acid with L-galactono-γ-lactone. The subsequent discovery of the diastereoisomeric series of conocarpin compounds [5, 6] made this an even more attractive proposal which was fully developed [7] in terms of attack by L-galactono-γ-lactone on *trans*-*p*-coumaric acid from either side in the genera *Leucadendron* and *Leucospermum* respectively (Scheme 1).

A different biogenetic scheme for leucodrin is the concept of leucodrin as a (reduced) ascorbigen which could arise from the coupling of ascorbic acid with 3-hydroxy-3-*p*-hydroxyphenylpropionic acid, followed by (stereospecific) reduction (Scheme 2) [8]. A related approach is that of Synge [9], whereby leucodrin could arise from the reductive C–C coupling of *p*-coumaric acid with ascorbic acid (Scheme 3).

It should be noted that, since leucodrin (**1**) and conocarpin (**2**) differ from each other only in the chirality of C-4, the stereospecific reduction required at the carbonyl group at C-9 of the initial coupling product would then have to be such as to afford the same chirality for the secondary alcohol group at C-9 of the final products (**1** and **2**) in the two genera.

The origin of the carbohydrate portion of leucodrin produced by *Leucadendron* was studied by feeding labelled carbohydrates to freshly cut stems. The extent of incorporation was determined in each case and the labelled leucodrin molecules were degraded following procedures previously established [10, 11] to yield fragments containing: (a) the three carbon atoms C-8, C-10, C-11 (glyceraldehyde), and (b) the six aromatic carbon atoms C-12 to C-17 together with C-2 to C-5 (*p*-hydroxyphenylsuccinic acid).

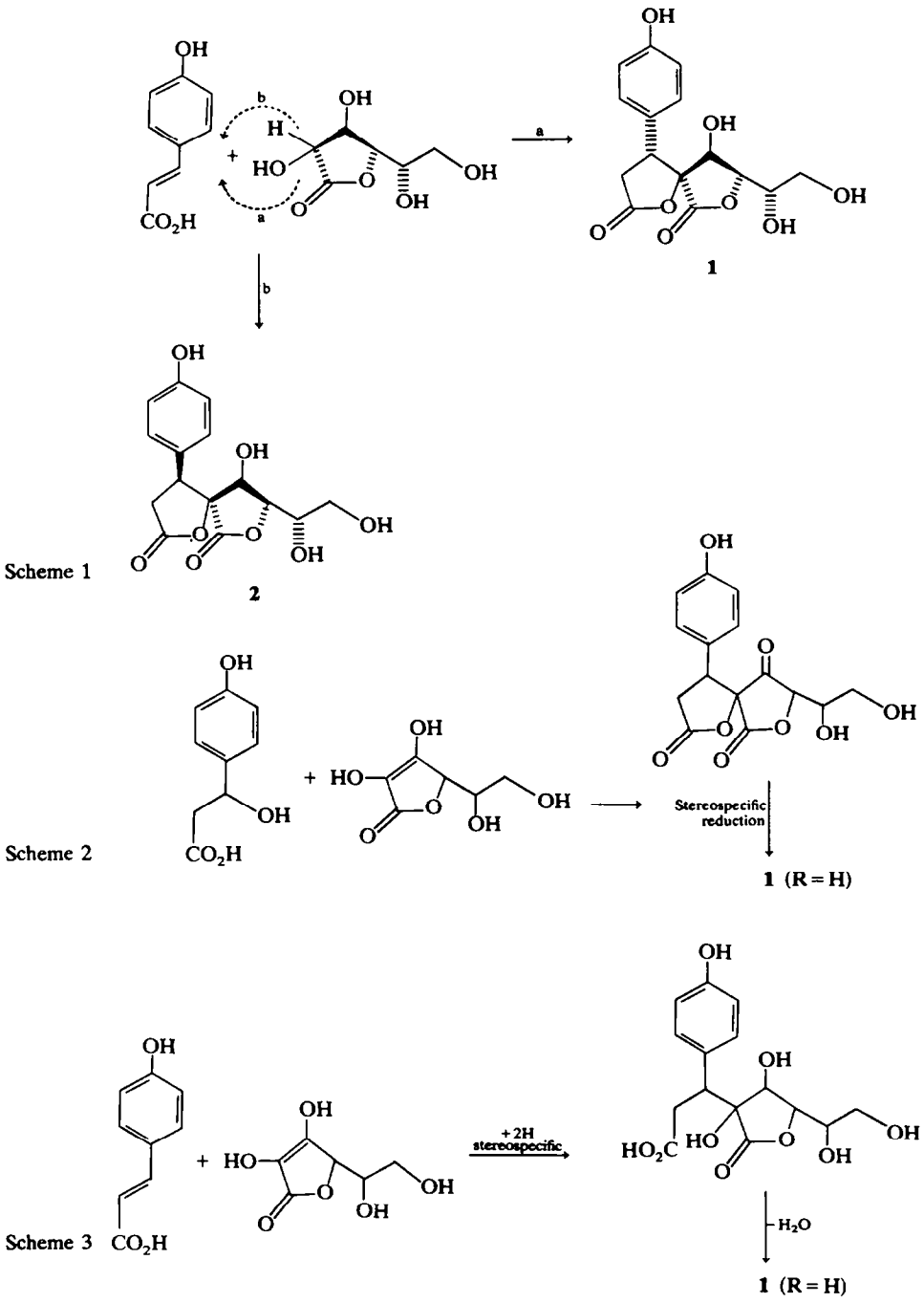
RESULTS AND DISCUSSION

Leucadendron species are hard woody shrubs or trees and it is difficult to feed labelled compounds to them. However, *L. salignum* (ex *adscendens*) which contains up to 20% of leucodrin in the dried leaves [12] was tested for incorporation studies. Doses of 5μCi of [1-^{14}C]-D-galactose and of [1-^{14}C]-D-glucose were administered per 10 g cut stem ends of this plant (see Experimental) and leucodrin recovered after 24 hr. In neither case did leucodrin show any radioactivity. The same two compounds were then fed through the roots to small plants of *L. salignum*. The leucodrin, recovered after 6 days, was radioactive but as expected the label was extensively scrambled after this period of metabolism.

However, *L. argenteum*, which only contains 1% of leucodrin, was found to incorporate labelled carbohydrates fed via cut stem ends into the product satisfactorily. In early experiments, cut stem ends were fed 1 μCi of labelled carbohydrate per 10 g of fresh plant material over 24 hr and leucodrin extracted and purified by column and preparative paper chromatography. The leucodrin samples were then recrystallized to constant specific activity. The results over 6 months from November to May are summarized in Table 1.

The level of incorporation was low, but did suggest that D-galactose and D-glucose were incorporated better than L-ascorbic acid.

It was apparent, however, that the 24 hr period of metabolism led to randomization of the label in the leucodrin. Thus when [1-^{14}C]-D-galactose was

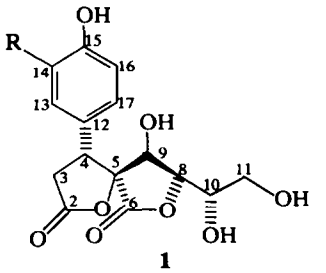


Schemes 1–3. Possible biogeneses of leucodrin and conocarpin.

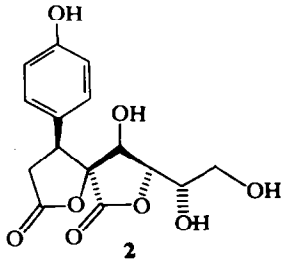
metabolized for 24 hr, and the recovered labelled leucodrin cleaved to isolate carbon atoms C-8, C-10 and C-11, it was found that the proportion of the label they contained ranged (for 6 experiments) from 12 to 40% of the activity of the total; when [1-^{14}C]-D-glucose was similarly fed, a value of 22% was found.

In order to reduce the extent of randomization of label the time of feeding was reduced to 1 hr, and the dosage of labelled carbohydrates was increased ten-fold. This showed that the hexoses were preferentially incorporated into the aromatic part of the leucodrin molecule (*p*-hydroxyphenylsuccinic acid). Thus with [1-^{14}C]-D-galactose 73–86% of the label was found in that portion of leucodrin (Table 2). However with [1-^{14}C]-L-ascorbic acid for 1 hr, the specific activity of the leucodrin was very low, and the level of incorporation about 100 times lower than for D-galactose. (Table 2). These findings clearly show therefore that D-galactose is much better incorporated into leucodrin in *L. argenteum* than L-ascorbic acid.

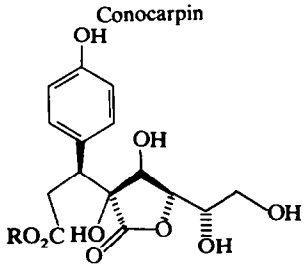
In order, however, to demonstrate more decisively that D-galactose and D-glucose may serve as fairly direct precursors of the carbohydrate portion of the leucodrin molecule (C-5 to C-11), it was necessary to



1
Leucodrin (R = H)
Leudrin (R = OH)



2
Conocarpin



3
Conocarpic acid (R = H)
Reflexin (R = Me)

suppress the (rapid) incorporation of these aldohexoses into the biogenesis of the *p*-coumaric acid portion (C-2 to C-4 and C-12 to C-17). This was achieved by feeding the labelled aldohexoses in the presence of a large excess of unlabelled phenylalanine. Incorporation studies with labelled aldohexoses in 0.05 M aqueous phenylalanine solution [13, 14] gave normal yields of leucodrin but the level of incorporation of labelled carbohydrate was depressed *ca* 100-fold. The results (Table 3) show that under these conditions D-galactose and D-glucose are preferentially incorporated into the carbohydrate portion of the leucodrin molecule.

The clear effect of added phenylalanine in depressing the incorporation of these aldohexoses into leucodrin shows the importance of phenylalanine as an intermediate in leucodrin biogenesis. These results therefore support the proposal that *p*-coumaric acid, for which phenylalanine is known to be a precursor [14], is an intermediate; they do not, however, as such serve to rule out the possible participation of alternative C_9-units (Scheme 2). The very large difference in the rate of incorporation found between D-galactose and L-ascorbic acid (Table 2) does make it unlikely that the latter is a precursor and clearly supports Scheme 1 as the most likely biogenetic pathway for leucodrin.

EXPERIMENTAL

Radioactivity measurements. All samples counted were recrystallized to a high degree of purity. Counting for ^{14}C was effected in Insta Gel (Packard Instruments Ltd.) mixture using liquid scintillation. Labelled precursors [1-^{14}C]-D-galactose, [1-^{14}C]-D-glucose, [6-^{14}C]-D-glucose and [1-^{14}C]-L-ascorbic acid were all from Amersham and had radiochemical purities of 99, 99, 95 and 97%, respectively. The three products counted were leucodrin, *p*-hydroxyphenylsuccinic acid and the dimedone derivative of glyceraldehyde [2, 11, 12] and each prepn provided its own identification by way of its TLC and PC behaviour.

Incorporation of precursors into L. argenteum. Five non-flowering detached stem ends of *ca* 10 g each of *Leucadendron argenteum* from one tree were equilibrated, under a 200 W incandescent lamp in a slight draught in a fume cupboard, in a beaker containing tap water (or 0.05 M aq. phenylalanine soln) for 5 hr and then the liquid replaced by labelled carbohydrate (1 or 10 μCi) in 1 ml aq. soln (or 0.05 M aq. phenylalanine soln) which was taken up in about 30 min, and followed by 0.5 ml portions of water until no activity remained in the beaker. After a suitable period of metabolism, the plant material was ground for several min in a blender with MeOH.

Isolation of pure leucodrin. The ground plant material was extracted (Soxhlet) with the MeOH for 16 hr. The extract was dried *in vacuo*, the residue was shaken with MeOH (10 ml per g), centrifuged and the supernatant dried *in vacuo* on Si gel (twice the mass of the solid obtained). This mixture was chromatographed over Si gel with C_6H_6–EtOAc–MeOH (5:3:2) and spots were made visible with Pauly's reagent [15]. Column fractions containing leucodrin (and leudrin) were combined and leucodrin was separated from leudrin by PC on Whatman 1 paper impregnated with a 10% soln of glycerol in MeOH. Descending elution was with *n*-BuOH–toluene (1:1) saturated with H_2O, after equilibration for 12 hr. Leucodrin was obtained free of entrained glycerol by chromatography over Si gel as before, using C_6H_6–Me_2CO (1:2). The solid product was crystallized either 4 or 5× from H_2O to constant mp 217–218° (lit. [1] 216°) and constant sp. act. About 250 mg of pure labelled leucodrin was typically so obtained from 50 g fresh plant material.

Degradation of labelled leucodrin. The degradation sequences previously reported were optimized for maximum yields from 50 mg amounts of (labelled) leucodrin [10, 11].

Table 1. Incorporation of ^{14}C into leucodrin over 24 hr

Source	No. of experiments	% Incorporation	Sp. act. of leucodrin (dpm/mM)
[1-^{14}C]-D-galactose	11	0.015	1975
[1-^{14}C]-D-glucose	5	0.012	1240
[6-^{14}C]-D-glucose	5	0.014	1810
[1-^{14}C]-L-ascorbic acid	5	0.009	820

Table 2. Incorporation and labelling pattern with 10 μCi ^{14}C fed in 1 hr per 10 g cut stem ends of *L. argenteum*

Label	% Incorporation	Sp. act. leucodrin (dpm/mM)	% Label in *p*-hydroxyphenyl-succinic acid	% Label in glyceraldehyde derivative
[1-^{14}C]-D-galactose	0.034	18 900	74.3 70.9	— —
[1-^{14}C]-D-galactose	0.014	6 770	83.6 87.9	2.5 2.5
[1-^{14}C]-L-ascorbic acid	0.0004	180	—	—

(a) *Preparation of glyceraldehyde dimedone derivative.* Labelled leucodrin (50 mg) and dry $CuSO_4$ (500 mg) were boiled in dry Me_2CO (20 ml) for 4 hr. The filtrate was dried *in vacuo* and the residue (the acetal) dissolved in M NaOH (0.6 ml) and added to a soln of $NaIO_4$ (138 mg) in H_2O (2.3 ml) and kept at room temp. for 1 hr (pH 7). Pb subacetate (1.8 ml of a 20% soln in H_2O) was added and the mixture filtered after 10 min. The pH of the soln was adjusted to pH 1–2 (0.5 M H_2SO_4), $PbSO_4$ was filtered and the filtrate kept at 50° for 1 hr. The soln was cooled, neutralized by adding Dowex resin 1 × 8 (HCO_3^- form, *ca* 4 g), filtered and evapd *in vacuo* to a vol. of 1.5 ml. The pH was adjusted to pH 4 with 0.5 M H_2SO_4, dimedone (380 mg in 0.38 ml EtOH) added and the soln kept at 40° for 4 hr and left at room temp. overnight. The whole reaction mixture was dried on Si gel (200 mg) and chromatographed over Si gel (2 g) eluting with C_6H_6–EtOAc–MeOH (5:3:2). Fractions containing the dimedone derivative (from TLC monitoring) were combined and gave the product which was crystallized 4× from MeOH to constant mp 205–206° (lit. [10] 202–206°) and sp. act.; yield, ~25 mg.

(b) *p-Hydroxyphenylsuccinic acid.* Labelled leucodrin (50 mg) in M NaOH (0.6 ml) was added to $NaIO_4$ (230 mg) in H_2O (5.4 ml), the soln kept at room temp. for 3 hr when 0.5 M H_2SO_4 (0.6 ml) was added (pH 2), and then kept at room temp. for 3 hr again. The soln was cooled in ice, SO_2 gas was passed through to reduce free I_2 to I^- and the soln continuously extracted with Et_2O (16 hr). The Et_2O extract was dried *in vacuo* on Si gel (0.2 g) and chromatographed over Si gel (2 g) eluting with C_6H_6–Me_2CO–HOAc (7:1:1). Fractions monitored by TLC were combined to yield the *p*-hydroxyphenylsuccinic acid which was then crystallized 5× from H_2O to constant mp 189–191° (lit. [11] 180–184°) and sp. act.; yield, ~20 mg.

Acknowledgements—Helpful discussions with Professor C. F. Cresswell, Department of Botany and Microbiology, plant identification by Mrs. L. E. Davidson, Head of the Moss Herbarium, and financial support by the Council of Scientific and Industrial Research are gratefully acknowledged.

Table 3. Short-time incorporation and labelling pattern with 10 μCi ^{14}C fed in 0.05 M phenylalanine solution per 10 g of *L. argenteum*

Label	Time of incorporation (min)	% Incorporation	Sp. act. of leucodrin (dpm/mM)	% Label in *p*-hydroxyphenyl-succinic acid	% Label in glyceraldehyde derivative
[1-^{14}C]-D-galactose	55	0.0003	180	—*	70
[1-^{14}C]-D-glucose	23	0.0003	160	—*	40
[6-^{14}C]-D-glucose	55	0.0007	550	13	—*

* Results too low to be statistically valid against background values.

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