Phytochemistry, Vol. 24, No. 6, pp. 1215–1218, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

CYCLOPENTENYLGLYCINE, A PRECURSOR OF DEIDACLIN IN *TURNERA ULMIFOLIA*

INGRID TOBER and ERIC E. CONN

Department of Biochemistry and Biophysics, University of California, Davis, CA 95616, U.S.A.

(*Revised received* 12 *November* 1984)

Key Word Index—*Turnera ulmifolia*; Turneraceae; cyclopentenylglycine; deidaclin; cyanogenic glycosides.

Abstract—When [2-^{14}C]cyclopentenylglycine was synthesized and fed to seedlings of *Turnera ulmifolia*, the label was incorporated into the nitrile group of the cyanogenic glycoside deidaclin. The amino acid cyclopentenylglycine was also found to occur naturally in *Turnera ulmifolia*. These findings indicate that cyclopentenyl cyanogenic glycosides are synthesized from the corresponding amino acids by the same pathway utilized in the biosynthesis of other cyanogenic glycosides.

INTRODUCTION

More than 30 cyanogenic glycosides have been reported in higher plants; they are glycosidic derivatives of 2-hydroxynitriles. The biosynthetic origin for the aglycone in many of these glycosides has been shown to be one of the following amino acids: valine, leucine, isoleucine, phenylalanine or tyrosine [1]. One group of cyanogenic glycosides, however, possesses aglycones with a cyclopentenyl structure (Fig. 1). These include gynocardin, deidaclin, tetraphyllins A and B, epi-tetraphyllin B and some derivatives of these (sulphate, disaccharide) [2]. Their occurrence appears to be restricted to the families Passifloraceae, Turneraceae, Flacourtiaceae and Malesherbiaceae, all in the order Violales [3]. The cyclopentenylglycosides may be chemotaxonomic markers in these plants [4].

If cyclopentenyl cyanogenic glycosides are synthesized as are the other cyanogenic glycosides [5], the precursor of deidaclin and its epimer tetraphyllin A should be the amino acid cyclopentenylglycine [6] (Fig. 1). The precursors of tetraphyllin B, epi-tetraphyllin B (and the sulphates) would be the corresponding hydroxylated cyclopentenylglycines unless the hydroxylation occurs at a later stage. Of these amino acids, only the unsubstituted cyclopentenylglycine has been reported to occur naturally. It has been isolated from seeds of *Hydnocarpus anthelminthica* (Flacourtiaceae) and shown to be the precursor of cyclopentenyl fatty acids [7, 8]. Deidaclin has been found only in *Deidamia clematoides* (Passifloraceae) [6], *Tetrapathea tetranda* (Passifloraceae) [9] and *Turnera ulmifolia* (Turneraceae) [10]. The biosynthetic studies described in this paper were carried out with *Turnera ulmifolia*.

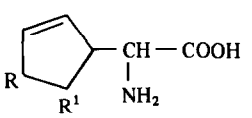
RESULTS AND DISCUSSION

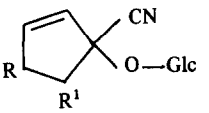
Leaves and stems of fully grown plants of *T. ulmifolia* contain four cyclopentenyl cyanogenic glycosides; viz. deidaclin and its epimer tetraphyllin A; tetraphyllin B and epi-tetraphyllin B. These tissues (200 g) were extracted and worked-up as described in the Experimental, to yield cyanogenic fractions which were then combined and further purified by HPLC. A major HPLC fraction, identified as cyanogenic by the Feigl–Anger test, was shown by ^{1}H NMR spectroscopy to consist of deidaclin and its epimer, tetraphyllin A in the ratio 12:1. A second, smaller (5% of the first) cyanogenic fraction, obtained by HPLC, was shown (^{1}H NMR) to be a mixture of epi-tetraphyllin B and tetraphyllin B in the ratio 3:1. Although only deidaclin had previously been reported in *Turnera ulmifolia* [10], K. C. Spencer (private communication) has now observed all four compounds in *Turnera* extracts. All four compounds also co-occur in *Tetrapathea tetranda* [9].

During the purification and identification procedures just described, cyanogenic compounds were detected by the release of hydrogen cyanide [11, 12] on treatment of the fractions with β-glucosidase preparations. Initially, a mixture of almond emulsin and flax seed linamarase was used to release the hydrogen cyanide and reaction times with Feigl–Anger paper varied from *ca* 2 hr for larger concentrations of cyanogenic glycoside to 24 hr for traces. Later, an enzyme extract prepared from *T. ulmifolia* leaves liberated hydrogen cyanide from the same fractions much more rapidly (10 min–2 hr) providing, thereby, evidence for the presence of an enzyme more specific for the endogenous cyanogens in the *Turnera* plants. This is in agreement with observations made for many other cyanogenic plants, as reviewed by Hösel and Conn [13].

In order to find suitable conditions for biosynthetic studies, the increase of cyanogenic material in *T. ulmifolia* seedlings after germination was examined. It was found that the seeds themselves released very low amounts (0.7 nmol/seed) of hydrogen cyanide on treatment with the β-glucosidase preparation described in the Experimental. During the early stages of germination, a lag phase was observed. From day 5 on, however, the amount of cyanogenic glycosides increased until at 11 days, individual seedlings contained *ca* 40 nmol.

Seedlings, 6- or 7-day-old, were chosen for feeding experiments utilizing [2-^{14}C]cyclopentenylglycine. Results of three series of experiments, each of which were carried out with a different batch of seedlings, are





1 $R = R^1 = H$ Cyclopentenylglycine

2 $R = R^1 = H$ Deidaclin, Tetraphyllin A

3 $R = OH; R^1 = H$ Tetraphyllin B, epi-Tetraphyllin B

4 $R = OSO_3^-; R^1 = H$ Tetraphyllin B sulphate, epi-Tetraphyllin B sulphate

5 $R = R^1 = OH$ Gynocardin

Fig. 1.

summarized in Table 1. Although quite different amounts of radioactivity were incorporated into the cyanogenic glycoside fractions in the different experiments, the ^{14}C-label was incorporated into the cyano group in every experiment in yields ranging from 81 to 93%. This specific incorporation confirms the precursor–product relationship between cyclopentenylglycine and deidaclin/tetraphyllin A (the epimers do not separate under the conditions used; in addition, the synthetic precursor contains all possible isomers).

The epi-tetraphyllin B/tetraphyllin B peaks obtained after HPLC of the glycoside extracts from series II and III of the feeding experiments were incubated with enzyme as well. However, less than 2% of the radioactivity in the HPLC fraction was recovered in the sodium hydroxide trap. This demonstrates that, in this case, the label is not incorporated directly into the cyano group. Since the amount of cyanogenic material in the epi-tetraphyllin B/tetraphyllin B fraction was only 5% of that in the deidaclin fraction, the absolute amount of incorporation was very small. The fact that cyclopentenylglycine is incorporated into deidaclin/tetraphyllin A and not into epi-tetraphyllin B/tetraphyllin B suggests that the hydroxylation takes place during the biosynthesis of the amino acid itself. The biosynthesis of cyclopentenylglycine has been studied [14]; it is derived from the α-ketodicarboxylic acid α-ketopimelate.

In 1974 Zilg and Conn [15] examined the ability of flax (*Linum usitatissimum*) seedlings to convert cyclopentenylglycine into deidaclin/tetraphyllin A. This experiment was performed because it was observed that the enzyme system in flax responsible for synthesis of linamarin/lotaustralin from valine and isoleucine could use other, unnatural amino acids related to valine and convert them to the corresponding cyanogens not normally found in that plant. As noted [15], the synthesis of deidaclin/tetraphyllin A from cyclopentenylglycine was not observed in flax seedlings.

Since cyclopentenylglycine can serve as a precursor of deidaclin in *T. ulmifolia*, extracts of leaves of this plant were examined for the amino acid. Qualitative evidence of its occurrence in the free amino acid fraction was readily obtained by 2D TLC. From an aliquot of the amino acid fraction, cyclopentenylglycine was further concentrated by prep. TLC [16] and HPLC. Co-migration of the purified compound with an authentic sample of synthetic cyclopentenylglycine was observed on TLC and HPLC. Dansyl derivatives of the isolated amino acid and standard compound also co-migrated on TLC [17].

A quantitative amino acid analysis was performed on total free amino acids isolated from 10-day-old seedlings of *T. ulmifolia*. Before analysis the extract was treated with 6 M hydrochloric acid for 12 hr at 110°, conditions under which cyclopentenylglycine decomposes in a repro-

Table 1. Incorporation of [2-^{14}C]cyclopentenylglycine into cyanogenic glycosides of *Turnera ulmifolia*

		Deidaclin/tetraphyllin A			Epi tetraphyllin B/tetraphyllin B*		
Series	Experiment No.	HPLC fraction (total cpm)	HCN (nmol)	HCN (% cpm)	HPLC fraction (total cpm)	HCN (nmol)	HCN (% cpm)
I	1	9120	388	93.0	No assay	No assay	No assay
II	1	3460	353	81.4	7020	19.6	1.9
	2	4860	568	81.4	5540	25.6	1.3
	3	8050	640	93.6	5480	25.5	1.5
III	1	3380	408	84.5	13560	17.5	0.8
	2	7670	593	91.0	2820	31.9	4.0

The amount of [2-^{14}C]cyclopentenylglycine available was: in Series I, 0.8 μCi for 15 seedlings per experiment, 7-days-old; in Series II, 1.0 μCi for 20 seedlings per experiment, 6-days-old; in Series III, 0.8 μCi for 20 seedlings per experiment, 7-days-old.

*The HPLC fraction epi-tetraphyllin B/tetraphyllin B was somewhat contaminated with unknown compound(s), since the HPLC system had been optimized for separating deidaclin/tetraphyllin B from all other peaks only.

ducible manner and can be quantitated as the ninhydrin complex of one of the decomposition products [7]. Cyclopentenylglycine constituted 7.1 mol% of the total free amino acids in the seedlings. The absolute amount was 2.4 μmol/g fr. wt, a value less than that of glutamic acid (15.9 μmol/g) and aspartic acid (3.7 μmol/g), but more than valine (2.2 μmol/g) and serine (1.6 μmol/g). In comparison, levels of cyclopentenylglycine in *Hydnocarpus anthelminthica* seeds were reported to range between 4 and 6 mol% of total free amino acids and, in leaves of *Caloncoba echinata*, a value of 2 mol% was found [7].

Because of the precursor–product relationship between cyclopentenylglycine and cyclopentenyl fatty acids in some Flacourtiaceae [8, 18] and because cyclopentenyl cyanogenic glycosides have been found to co-exist with cyclopentenyl fatty acids in *Carpotroche brasiliensis* (Flacourtiaceae) [19], lipids from both leaf material and seeds of *T. ulmifolia* were extracted and analysed. However, only straight-chain fatty acids were found in this plant.

EXPERIMENTAL

Chemicals. Unlabelled cyclopentenylglycine was synthesized according to the procedure of ref. [7]. A standard of cyclopentenylglycine was the gift of Professor F. Spener, Münster, West Germany. For the synthesis of [2-^{14}C]cyclopentenylglycine, a slightly different procedure than that described earlier [8] had to be followed since diethylacetamido [2-^{14}C]malonate was used as a starting material. In the first step of the synthesis, diethylacetamido[2-^{14}C]malonate (Amersham), 250 μCi (sp. act. 8 μCi/μmol after dilution with unlabelled material) was condensed with (2-cyclopentenyl)bromide, freshly prepared and distilled [20]. Hydrolysis of the intermediate was then accomplished in two steps, first with conc. NH_4OH (8 hr, 110°) and then, after evaporating the NH_3 in a stream of N_2, with 2 M HCl (2 hr; 110°) [8, 21]. The crude product was initially purified by TLC with BuOH–HOAc–H_2O (2:1:1) on cellulose. After filtration through a Sep-Pak C_{18} cartridge, the amino acid was separated by HPLC on a Whatman Partisil-10 column (ODS-3, 10 μm, 0.9 × 50 cm) with 10% acetonitrile at a flow rate of 3 ml/min; detection at 190 nm. The yield was 45 μCi [2-^{14}C]cyclopentenylglycine, or 18% overall from the labelled malonate. Chromatographic and radiochemical purity in TLC and HPLC under various conditions was *ca* 98%.

Tetraphyllins A and B, which were employed as reference compounds, were gifts from G. B. Russell and P. F. Reay.

Plant material. Turnera ulmifolia seeds, leaf and stem material were collected from fully grown plants kept in the greenhouse. Since young tissue is usually most active in the biosynthesis of cyanogenic glycosides, seedlings were chosen for biosynthetic studies. The seeds were soaked in 10^{-3} M gibberellic acid containing 10^{-2} M KNO_3, aerated for 24 hr and germinated on vermiculite at 27° in the dark. Seedlings were harvested when 6- or 7-days-old; the average size was 1–3 cm.

Extraction and analysis of free amino acids. Plant material was ground in liquid N_2 and extracted with 75% EtOH; the EtOH was evaporated and free amino acids purified by treatment of the aq. extracts with charcoal at 90° for 10 min, filtration, pH adjustment to 4.0 and ion exchange chromatography on Dowex 50-W, H^+. Qualitative analysis was carried out by 2D TLC on cellulose–silica gel G layers [22] with BuOH–HOAc–H_2O (2:1:1) in the first direction and *t*-AmOH–HOAc–H_2O (15:1:15, upper phase) in the second [7, 16]. Cyclopentenylglycine was detected as a yellowish-brown spot with a ninhydrin–collidine reagent [23]. Quantitative determination was performed according to ref. [7].

Extraction of lipids and analysis of fatty acids. After grinding the plant material in liquid N_2, lipids were extracted according to established procedures [24]. Fatty acids were obtained as methyl esters and analysed by GC (OV-3, 170° isothermal) [8].

Extraction and analysis of cyanogenic glycosides. Leaves and stems or seedlings of *T. ulmifolia* were ground in liquid N_2 and added to boiling 80% MeOH. After filtration, re-extraction of the residue and concn of the combined extracts [10] the residue was re-dissolved in MeOH–$CHCl_3$–H_2O (12:5:3), two phases were separated by addition of $CHCl_3$ and H_2O and the aq. layer evaporated until MeOH and $CHCl_3$ were removed. The sample was then treated with charcoal (20 min, 80°), filtered while hot and evaporated to a small vol. The cyanogenic glycosides and amino acids contained in this soln were separated by cellulose chromatography with Me_2CO–H_2O (5:1), either on TLC plates or in dry-packed columns. Small aliquots of the fractions obtained were tested for cyanogenic glycosides with Feigl–Anger paper [11, 12] and for the amino acids with a ninhydrin–collidine reagent [23]. The cyanogenic fractions were combined, further purified and analysed by HPLC on a C_{18}-reversed phase column (Whatman Partisil 10, ODS-3 10 μm, 0.46 × 25 cm or 0.9 × 50 cm) with 10% MeCN and UV detection at 190 nm. Aliquots of the cyanogenic peaks were converted into TMS derivatives and further analysed by GC (3% SP-2250, 2 mm × 1.85 m, temp. program 200–270° at 5°/min) and by ^{1}H NMR (360 MHz).

Enzyme preparation. Leaf material of *Turnera ulmifolia* (220 g) was ground and extracted (× 3) with cold Me_2CO (600 ml each) and the residue air-dried (yield 66 g). The Me_2CO powder was used as a source of a β-glucosidase preparation which readily hydrolysed deidaclin. The powder (15 g) was extracted with 400 ml 0.02 M Pi buffer, pH 6.8; the slurry was filtered and centrifuged and the supernatant concd and dialysed in an Amicon ultrafiltration cell (PM 30 membrane). The Amicon supernatant was either used directly to hydrolyse samples containing deidaclin, or it was freeze-dried and stored at −20° (yield 1.9 g from 15 g Me_2CO powder). The freeze-dried protein was also used as a source of β-glucosidase by dissolving (2 mg/ml) in 0.02 M Pi buffer, pH 6.8.

Feeding experiments. Seedlings (20) of *T. ulmifolia*, 6–7-days-old and 1–3 cm length, were immersed in aq. solns of [2-^{14}C]cyclopentenylglycine. After a period of metabolism (10 hr bright light, 14 hr dark) during which the solns were replenished with H_2O, the seedlings were ground in liquid N_2 and extracted with boiling MeOH, 2 × 4 ml [25]. The MeOH was evaporated in a stream of N_2 and the residues re-dissolved in 1 ml MeOH–$CHCl_3$–H_2O (12:5:3). Two phases were separated by addition of 1 ml H_2O and 0.5 ml $CHCl_3$. The $CHCl_3$ layer was washed with 0.5 ml H_2O and the combined aq. solns evaporated to *ca* 100 μl and applied to cellulose TLC plates (5 × 20 cm, 0.25 mm coating). The plates were developed in Me_2CO–H_2O (5:1) and the cyanogenic fractions scraped off and extracted with H_2O (3 + 1 + 1 ml). The eluates were filtered through Sep-Pak C_{18} cartridges, concd and further fractionated by HPLC (Whatman Partisil 10, ODS-3, 10 μm, 0.46 × 25 cm; 10% MeCN; 190 nm). HCN was determined after incubation of aliquots of HPLC fractions with the β-glucosidase preparation described above. The HCN released was trapped in 1 M NaOH and determined colorimetrically [26]. ^{14}C activity in the HCN was measured by liquid scintillation counting in a Triton–toluene scintillation cocktail [27]. In the same expt, the cyclopentenone released by action of the β-glucosidase could be distilled into a second trap containing a satd soln of 2,4-dinitrophenylhydrazine in 1 M H_2SO_4. The resulting hydrazone was applied to a TLC

plate and scanned for ^{14}C activity, since liquid scintillation counting was not possible due to quenching effects.

Acknowledgements—This work was supported in part by NSF Grant PCM-8104497 (EEC). We wish to thank Alan J. Smith for the quantitative amino acid analysis and Dr. K. C. Spencer for measuring and interpreting ^1H NMR spectra.

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