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Isolation and Biosynthesis of Cyclopentenylglycine, a Novel Nonproteinogenic Amino Acid in Flacourtiaceae[†]

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ABSTRACT: Evidence regarding the natural occurrence of cyclopentenylglycine in Flacourtiaceae and its biosynthesis in this family is reported. Two-dimensional thin-layer chromatography (TLC) of free amino acids from seeds of *Hydnocarpus anthelminthica* and leaves of *Caloncoba echinata* indicated the presence of cyclopentenylglycine. Pure cyclopentenylglycine was isolated from the free amino acids by ion exchange chromatography and fractionation on thick layers of cellulose. Mass spectral analysis established the cyclopentene and amino acid structures of the molecule. The *S* configuation of the α carbon was determined by enzymic reaction with α-amino acid oxidase. The ^{1}H NMR spectrum revealed that the ring double bond was located in the 2′ position, whereas the two-doublet character of the 2-H resonances exhibited the cooccurrence of (1′*R*,2*S*)- and (1′*S*,2*S*)-(2′-cyclopentenyl)glycine in *H. anthelminthica* seeds. Biosynthetic paths for cyclopentenylglycine envisioned a C_7 intermediate capable of cyclization. By incubation of *C. echinata* leaves and chloroplasts with ^{14}C-labeled substrates and determination of radioactivity incorporated into cyclopentenylglycine, we could examine three hypotheses: (1) biosynthesis via the shikimate path (glucose was not incorporated into cyclopentenylglycine); (2) condensation of a C_4 and a C_3 unit, in analogy to lysine biosynthesis (aspartate plus pyruvate, or α,ϵ-diaminopimelate were modest precursors); (3) C_1 chain elongations of C_5 and C_6 starter molecules (best results were obtained with glutamate plus acetate and α-aminoadipate plus acetate; up to 42% of the activity incorporated into free amino acids was found in cyclopentenylglycine). The results clearly favor the pathway involving stepwise elongation of α-ketodicarboxylic acids, i.e., the transamination products of substrate α-amino acids, to α-ketopimelate. Reduction to the semialdehyde may furnish the appropriate metabolite for the biogenesis of the five-membered carbon ring via intramolecular aldol condensation.

The natural occurrence of nonproteinogenic cyclopentenylglycine has been postulated solely by biochemical reasoning. This amino acid was assumed to already have the cyclopentene structure characteristic for the unusual cyclopentenyl fatty acid found in certain seeds and leaves of Flacourtiaceae (Mangold & Spener, 1977). Using synthetic cyclopentenyl[2-^{14}C]glycine, it was indeed shown that this compound served as a precursor for cyclopentenyl fatty acids after its conversion to cyclopentenyl carboxylic acid (Cramer & Spener, 1976). Cyclopentenylglycine has also been suggested to be a precursor of naturally occurring deidaclin,[1] a cyanogenic glycoside, containing the cyclopentene group as the aglycon (Clapp et al., 1970; Zilg & Conn, 1974).

Information on mechanisms for the formation of nonisoprenoid acyclic C_5 rings is rather scarce. Known examples are the biosyntheses of prostaglandins (Samuelsson et al., 1978) and of phytonoic acids (Vick & Zimmerman, 1979) by oxidative cyclization of a highly unsaturated fatty acid and the biosynthesis of terrein and related bacterial metabolites via polyketides (Hill et al., 1975; Holker & Young, 1975). In previous studies from this laboratory, it has been shown that neither mechanism is operative in the biosynthesis of cyclopentenyl fatty acids and hence of cyclopentenylglycine (Spener & Mangold, 1974; Cramer & Spener, 1977). It seemed reasonable to assume that cyclopentenylglycine may be generated from a C_7 compound equipped with functional groups suited for cyclization.

The present study reports on the search and final isolation of cyclopentenylglycine from seeds of *Hydnocarpus anthelminthica* and from leaves of *Caloncoba echinata*, tissues which are known to produce cyclopentenyl fatty acids (Cramer & Spener, 1976, 1977).

The biosynthetic investigations on cyclopentenylglycine, presented in this paper, focus on three working hypotheses, each involving the intermediate formation of C_7 metabolites: (1) biosynthesis via metabolites of the shikimate pathway; (2) the condensation of C_4 and C_3 building blocks ($C_4 + C_3$ hypothesis); (3) the extension of a C_5 to a C_7 unit via consecutive C_1 chain elongations ($C_5 + 2 \times C_1$ hypothesis). The latter two proposals are shown in Figure 1.

Experimental Section

Materials. Cyclopentene and ethyl acetamidocyanoacetate were obtained from Merck (Darmstadt, West Germany). DL-α-[6-^{14}C]Aminoadipic acid (46.9 Ci/mol) was purchased from CIS (Gif-sur-Yvette, France); all other radiochemicals (specific activities in curies/mol) were from New England Nuclear (Dreieichenhain, West Germany): [1-^{14}C]pyruvate (6.3); [3-^{14}C]pyruvate (20.5); [1-^{14}C]acetate (1.0); D-[2,3-^{14}C]glucose (7.7); D-[U-^{14}C]glucose (3.0); L-[U-^{14}C]aspartic acid (204.0); L-[U-^{14}C]glutamic acid (237.0); L-[U-^{14}C]lysine (306.0); α,ϵ-[1,7-^{14}C]diaminopimelic acid (29.4); D-[2,3,4,5-(n)-^{14}C]shikimic acid (80.0). Cofactors were obtained from Boehringer (Mannheim, West Germany), silica gels H and G were from Merck (Darmstadt, West Germany), cellulose MN 300 was from Macherey and Nagel (Düren, West Germany), and Ficoll 400 and Dextran T40 were from Pharmacia (Uppsala, Sweden). Standard amino acids were supplied by Serva (Heidelberg, West Germany).

Plants. Fresh seeds of *Hydnocarpus anthelminthica* were received about every 3 to 4 weeks during maturation from Foster Botanic Garden (Honolulu, HI), and fresh leaves of

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[1] Trivial names and abbreviations used: deidaclin, β-D-glucopyranoside of 1-hydroxy-1-cyano-2-cyclopentene (this compound has also been named tetraphyllin A (Russel & Reay, 1971)); terrein, 1-keto-3-*trans*-allyl-5,6-dihydroxy-2-cyclopentene; PPO, 2,5-diphenyloxazole; Me_2POPOP, dimethyl-1,4-bis[2-(5-phenyloxazolyl)]benzene.

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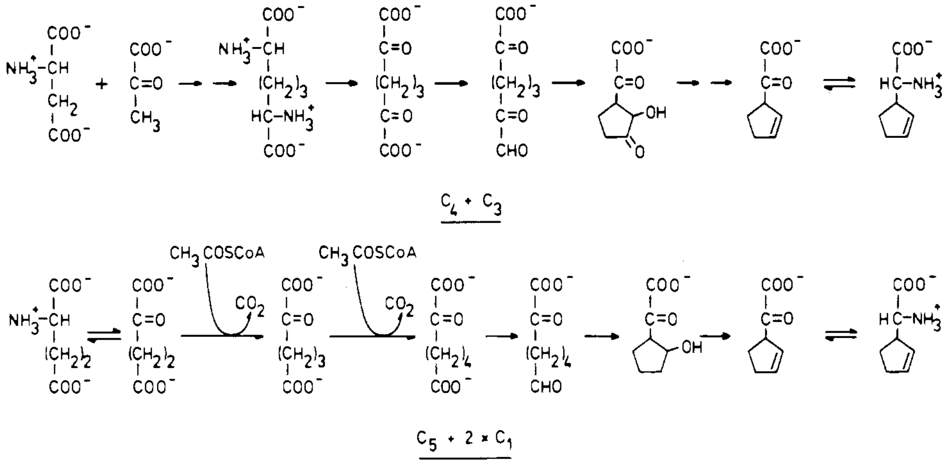

FIGURE 1: Proposed pathways for the biosynthesis of cyclopentenylglycine.

Caloncoba echinata were from the greenhouse of the Belmonte Arboretum (Wageningen, The Netherlands). *Corylus avellana* tissues were obtained locally.

Synthesis of (2-Cyclopentenyl)glycine. This compound was prepared earlier by Dennis et al. (1955) from acetamidomalonic ester and 2-cyclopentenyl chloride. With the goal of producing a radioactively labeled product in high yield (Cramer & Spener, 1977), we started from ethyl acetamidocyanoacetate and (2-cyclopentenyl) bromide. The latter compound (28% yield after fractional distillation under N_2, bp 46.5 °C (23 mm)) was synthesized from cyclopentene and *N*-bromosuccinimide according to Ziegler et al. (1942) and was used immediately for further synthesis.

Absolute ethanol (10 mL) and 70 mg (3.1 mg-atom) of Na were placed into a 25-mL two-necked flask, equipped with N_2-inlet tube, dropping funnel plus $CaCl_2$ tube, and magnetic stirrer. Ethyl acetamidocyanoacetate (497 mg, 2.9 mmol) was added at room temperature, and the solution was stirred for 1 h. To the slightly yellowish solution, placed in an ice bath, 0.38 mL (3.5 mmol) of (2-cyclopentenyl) bromide was added slowly (0.5 h). After 15 min a white precipitate of NaBr was formed, and stirring was continued for 2 h. The precipitate was removed by centrifugation, and the supernatant taken to dryness in vacuo at 45 to 50 °C. The residue was refluxed in 3 mL of 5 N NaOH for 24 h. After the solution had cooled to room temperature, the pH of the solution was lowered to 6.8 with 5 N H_2SO_4, which resulted in the formation of a voluminous precipitate. After we removed the precipitate by centrifugation, the supernatant was treated with charcoal. The clear solution was saturated with $(NH_4)_2SO_4$ to salt out (2-cyclopentenyl)glycine. The grayish flakes were suctioned off and recrystallized from 5 mL of 2-propanol/H_2O (1:1) (12 h at room temperature, 12 h at 4 °C), to afford white crystals of (2-cyclopentenyl)glycine (176 mg, 43% yield): mp 252–255 °C dec (lit. 252–255 °C (Dennis et al., 1955)); TLC on cellulose (1-butanol/acetic acid/H_2O (40:10:50)) R_f 0.60; paper chromatography (PC) on Whatman No. 3MM paper (95% methanol) R_f 0.56; ninhydrin/collidine (Von Arx & Neher, 1963) stained this amino acid yellowish brown to purple, characteristic of unsaturated amino acids (Fowden & Smith, 1968). Anal. Calcd for $C_7H_{11}NO_2$ (mol wt 141.18): C, 59.56; H, 7.85; N, 9.92. Found: C, 59.60; H, 7.97; N, 9.66.

^1H magnetic resonance spectra of cyclopentenylglycine in D_2O were obtained at 90 MHz on a Bruker WH 90 and at 270 MHz on a Bruker WH 270 spectrometer (internal standard sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propionate). The pH values are given as direct pH meter readings (pH), without correction for the deuterium isotope effect. Mass spectra (70 eV, source temperature 200 °C, direct injection) were recorded on a Varian CH 7 instrument.

Incubation of Leaves. Detached leaves were rinsed and wetted, and the substrate, dissolved in 0.5 mL of tap water, was taken up via the petiole under simultaneous illumination with incandescent light. In addition, 15 min each of pre- and postincubations with tap water were carried out. Leaves were then homogenized in 50–80 mL of 75% ethanol and processed for product isolation as described for chloroplasts.

Preparation and Incubation of Chloroplasts. Intact chloroplasts were isolated from 30–50-g portions of green leaves by the method of Kannangara & Stumpf (1972a,b), except that 845.1 mg of $MnSO_4 \cdot H_2O$ was added to 10 mL of suspension medium. The preparations were free of mitochondria, as shown by the lack of succinate:cytochrome *c* oxidoreductase activity (EC 1.3.99.1) (Massey, 1959). Chlorophyll was determined by the method of Bruinsma (1961). The chloroplast suspension (0.5 mL), stable for several hours at 4 °C, was combined with 0.5 mL of cofactor mix (ATP, 4 mM; coenzyme A, 1 mM; NADPH, 0.2 mM; NADH, 0.2 mM; NAD^+, 0.2 mM; bovine serum albumin, 2.2 mg/mL) and aqueous solutions of substrates were added. Incubations were carried out at 27 °C under exposure to incandescent light and with reciprocal shaking, and were stopped by the addition of 10 mL of 75% ethanol after 1 h.

Isolation of Products. For the isolation of free amino acids, the chloroplast suspension was extracted 3 times overnight with 10 mL of 75% ethanol each time. The combined ethanolic extracts were evaporated to dryness, and the residue was taken up in 25 mL of water and cleared with small amounts of charcoal. The aqueous solution was adjusted to pH 4.5 and applied to a Dowex 50-W (H^+) column (1.5 × 14 cm, 3 mL of resin). After the solution was washed with 25 mL of H_2O, total free amino acids were eluted (0.5 mL/min) with 50 mL of 2 N NH_3. The eluate was evaporated to remove NH_3, and the residue was dissolved in 1 mL of H_2O. Small amounts of carrier amino acids were added, and a 20-μL aliquot of the solution was applied to 0.25-mm air-dried layers of cellulose/silica gel G on 20 × 20 cm plates (Turner & Redgewell, 1966). Chromatograms were developed at 4 °C in 1-butanol/acetic acid/H_2O (50:25:25) in the primary direction and in *tert*-amyl alcohol/acetic acid/H_2O (15:1:15) (upper phase) in the secondary direction (Spener & Dieckhoff, 1973). Spots were visualized with ninhydrin and scraped into 10 mL of scintillation cocktail, and the radioactivity incorporated was determined with a Packard scintillation spectrometer. Water-soluble substances or TLC scrapings were counted in 10 mL of scintillation cocktail, composed of 5 g of PPO and

0.3 g of Me_2POPOP made up to 1 L with 1,4-dioxane.

Quantitative Amino Acid Analysis. The composition of free amino acids, extracted from seeds and leaves, was determined on a Beckman Unichrom amino acid analyzer, using M 72 and M 81 resins under standard conditions (Spackman et al., 1958). In this system cyclopentenylglycine was eluted together with leucine. In order to liberate cyclopentenylglycine, which possibly occurred as γ-glutamyl peptide, the extract was treated with 6 N HCl for 12 h 110 °C, prior to analysis. Under these conditions, cyclopentenylglycine decomposed into seven products in a reproducible manner, as has been verified in experiments with synthetic cyclopentenylglycine. After subjecting the hydrolysate to ion exchange chromatography, we could now quantitate cyclopentenylglycine as the ninhydrin complex of one of its decomposition products, which was clearly separated from all other amino acids.

Amino Acid Oxidase Assays. A modification of the method described by Bergmeyer (1974) was used. D-Amino acid oxidase (EC 1.4.3.3), 5 mg/mL H_2O, from hog kidney, L-amino acid oxidase (EC 1.4.3.2), 1 mg/mL H_2O, from *Crotalus terrificus terrificus*, and catalase (EC 1.11.1.6), 250 mg/12.5 mL H_2O, from beef liver, were obtained from Boehringer (Mannheim, West Germany). Catalase spray reagents were prepared by adding 125 μL of original catalase solution to 50 mL of 0.1 M phosphate buffer, pH 8.3 and 7.4, respectively. Aliquots (15 μL) of original oxidase solutions were applied as spots onto 0.3-mm layers of silica gel H and incubated with 1 to 3 drops of 0.1% solutions of individual amino acids. The plates were sprayed with the corresponding catalase reagent (pH 8.3 for D-oxidase and pH 7.4 for L-oxidase) and allowed to stand at 38 °C for 6 h in a water-saturated atmosphere. Finally, the plates were sprayed with ninhydrin/collidine in order to detect unreacted amino acids.

Results

Isolation of Cyclopentenylglycine. Since cyclopentenylglycine is considered a nonproteinogenic amino acid, it seemed quite logical to search for its occurrence in the pool of free amino acids in tissues that are known to synthesize cyclopentenyl fatty acids. Consequently, a method was elaborated that enabled a fast screening for the detection of cyclopentenylglycine in extracts of free amino acids from Flacourtiaceae. By two-dimensional chromatography on thin layers of cellulose/silica gel G, the solvent system described in Figure 2 clearly separated cyclopentenylglycine from all other proteinogenic amino acids. By using this method and with the aid of synthetic cyclopentenylglycine as a reference substance, we could identify naturally occurring cyclopentenylglycine in extracts of *H. anthelminthica* seeds and *C. echinata* leaves (Figure 2).

The presence of cyclopentenylglycine in the free amino acid pool of maturing *H. anthelminthica* seeds (maturation time from May to October) and of *C. echinata* leaves was further substantiated by quantitative ion exchange chromatography. In the seeds, the percentage of this nonproteinogenic amino acid ranged between 4 and 6 mol % of total free amino acids; in the leaves, the amount was around 2 mol %. A calculation showed cyclopentenylglycine to occur in maturing seeds in absolute amounts of 75 μg/g of fresh seed tissue and 180 μg/g of fresh leaf tissue. In view of the proposed biosynthetic schemes, the quantitative analysis indicated the presence of fairly large proportions (15–25 mol %) of each of the presumed precursors, i.e., aspartic and glutamic acids.

H. anthelminthica seeds were collected in the middle of the maturation time and shelled. The material obtained (85 g) was homogenized in 280 mL of hexane in a Virtis 23 homogenizer and completely delipidated with 800 mL of hexane for 12 h in a Soxhlet apparatus. The residue was extracted 3 times for 24 h, with 2 × 1 L of 75% ethanol and 1 L of H_2O, respectively. The combined extracts were evaporated to dryness, and the residue (4.23 g) was taken up in 1 L of H_2O. Charcoal (2 g) was added and the solution kept at 90 °C for 30 min. After filtration, the clear solution was brought to pH 4.5 and applied to an ion exchange column containing 30 mL of Dowex 50-W (H^+). Upon absorption of the amino acids, the column was washed with 2.4 L of H_2O to remove residual carbohydrates and other contaminants. Desorption of amino acids was achieved by elution with 500 mL of 0.2 N NH_3, giving a total of ca. 400 mg of free amino acids. The presence of amino acids in eluates was tested with ninhydrin.

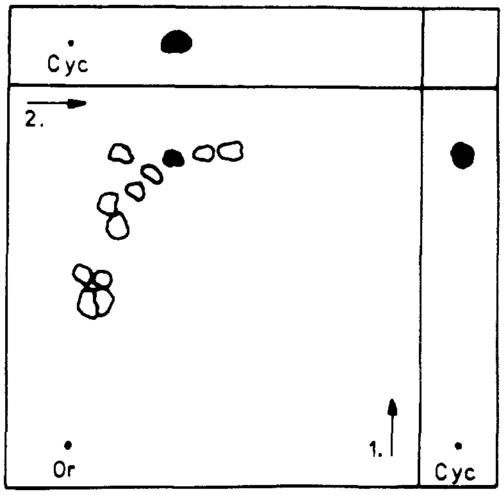

FIGURE 2: Separation of free amino acids from *H. anthelminthica* seeds on cellulose/silica gel G layers. Solvents: (1) direction 1-butanol/acetic acid/H_2O (50:25:25); (2) direction *tert*-amyl alcohol/acetic acid/H_2O (15:1:15) (upper phase); detection of amino acids was with ninhydrin–collidine. Synthetic cyclopentenylglycine served as a reference compound; Cyc, cyclopentenylglycine; Or, origin.

Cyclopentenylglycine was separated from other amino acids in a two-step chromatographic procedure on 1.5-mm cellulose layers (40 mg of sample/20 × 40 cm plate) as described in detail by Spener & Dieckhoff (1973). The total yield was 10 mg of pure cyclopentenylglycine, as judged by TLC. However, mass spectrometric analysis indicated cellulose and residual diethylamine as contaminants originating from the adsorbent and solvent, respectively. For final purification the sample was dissolved in 20 mL of H_2O and applied to a small column, filled with 10 mL of Dowex 1-X2 (OH^-), washed with 300 mL of H_2O, and eluted with 200 mL of 0.1 M acetic acid. Lyophilization of the eluate yielded 6.4 mg of pure cyclopentenylglycine.

Determination of Structure. Mass spectrometric analysis of synthesized and natural cyclopentenylglycine showed identical fragmentation patterns: base peak at m/e 75 (McLafferty rearrangement, $[(OH)_2C{=}CHNH_2]^+$), fragmentations adjacent to the α-carbon atom at m/e 67 ($C_5H_7^+$), 74 (NH_2CH^+COOH), and 96 ($C_5H_7CH^+NH_2$), thus establishing the cyclopentene structure and α-amino acid nature of the compound (Biemann & McCloskey, 1962). Additional prominent fragments were at m/e 39 and 41, and a small molecular ion was at m/e 141.

The reactivity of isolated cyclopentenylglycine toward amino acid oxidases allowed us to determine the configuration of the α carbon; control experiments were carried out with D- and L-isoleucine. As can be seen from Figure 3, cyclopentenylglycine was a substrate for the L-amino oxidase only, thus establishing the L configuration of the α carbon in naturally

Table I: ^{1}H NMR Data for Synthetic and Natural Cyclopentenylglycine

	proton resonances[a]				
	1′-H	2′-H[b,c]	3′-H[c]	4′,5′-H	2-H[b]
chemical shift					
at 90 MHz (ppm)	3.55–3.25	5.66; 5.57	6.07–5.98	2.48–1.55	3.84; 3.77[d]
at 270 MHz (ppm)[e]	3.46–3.32	5.69; 5.59	6.07–6.02	2.46–1.65	3.84; 3.77
number of protons	1	1	1	4	1
difference in chemical shift of the diastereomers					
at 90 MHz (Hz)		7.5			5.6
at 270 MHz (Hz)[e]		21.2			17.6

[a] Assignment of protons was as in Figure 4; spectra were obtained from solutions of cyclopentenylglycine in D_2O, pH 3.7. [b] Individual resonances of the diastereomers. [c] Tentative assignment of vinylic protons. [d] At pH 2.6, 4.07 and 4.02 ppm. [e] Synthetic compound only.

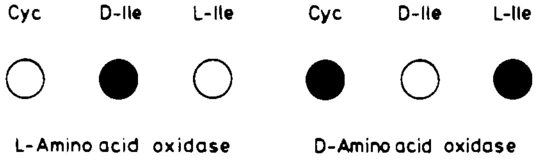

FIGURE 3: Spot test with amino acid oxidases: dark circles, ninhydrin positive; Cyc, cyclopentenylglycine isolated from *H. anthelminthica* seeds.

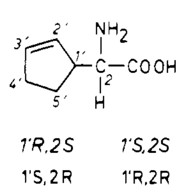

FIGURE 4: Structure of cyclopentenylglycine. Naturally occurring stereoisomers are italicized.

occurring cyclopentenylglycine.

Chemical synthesis of cyclopentenylglycine affords two pairs of enantiomers, each pair being the diastereomer of the other. The natural compound, having a fixed configuration at the α carbon, may also occur in two diastereomeric forms (Figure 4). To answer these structural and configurational questions, we have recorded the ^{1}H NMR spectra of synthetic and isolated cyclopentenylglycine at 90 MHz and supplemented these data with a 270-MHz spectrum of synthetic cyclopentenylglycine. Surprisingly, identical spectra were obtained of compounds synthesized and isolated. The relevant data are summarized in Table I.

The two diastereomeric forms can be recognized from the two doublets of the 2-H resonances at 3.84 and 3.77 ppm ($^3J_{2H\text{-}1'H}$ = 4.4 and 4.2 Hz, respectively). The "two-doublet" character of these signals is ascertained by a threefold separation on the frequency scale in the 270-MHz spectrum as compared to the 90-MHz spectrum. The presence of two diastereomeric forms was further substantiated by irradiation at 1′-H, which caused the appearance of two 2-H singlets. After hydrogenation of the sample, the spectrum showed one doublet as expected for the 2-H resonances. The resonances of vinylic protons at 6.0 and 5.6 ppm in connection with intensity measurements establish a 2′,3′ location of the ring double bond in agreement with reports on deidaclin and tetraphyllin A (Seigler, 1975). A threefold difference of the 2′-H chemical shift in the 270/90-MHz spectra was recognized. In the 270-MHz spectrum, the 2′-H resonances appeared as two sets of multiplets after expansion of this particular spectral part. Because a difference of the chemical shift was not detected for the 3′-H resonances of the cyclopentenylglycine diastereomers, we tentatively assigned these signals to the vinylic proton further away from the 1′-H and the two sets of multiplets to the proton vicinal to the 1′-H. The NMR data indicate that in seeds of *H. anthelminthica* cyclopentenylglycine occurs in two diastereomeric forms. It is important to state that, in view of cyclopentenyl fatty acid biosynthesis, the appropriate (1′*R*,2*S*)-cyclopentenylglycine is available as precursor for (1′*R*)-cyclopentenyl fatty acids.

Biosynthesis. The following criteria were considered promising to test the proposed schemes for cyclopentenylglycine biosynthesis in leaves of *C. echinata*. The occurrence of cyclopentenylglycine and of cyclopentenyl fatty acids (Spener & Mangold, 1975) in these leaves and their capacity to produce the cyclic fatty acids (Cramer & Spener, 1977) are well documented. In contrast to seeds, leaves of *C. echinata* were available from the greenhouse throughout the year. Thus, leaves and chloroplasts were incubated with radioactively labeled substrates, and the label incorporated into cyclopentenylglycine was quantitated. In control experiments with *C. avellana* leaves and chloroplasts, which do not contain compounds having the cyclopentene structure, unlabeled cyclopentenylglycine was incubated together with the radioactively labeled substrates. Regardless of the prospective precursors tested, the fraction of carrier cyclopentenylglycine never contained radioactivity after separation from the free amino acids isolated from tissues and media incubated.

After administration of D-[U-^{14}C]glucose or D-[3,4-^{14}C]-glucose to leaves and chloroplasts of *C. echinata*, label from these substrates was not incorporated into cyclopentenylglycine. Thus, a biosynthetic path via shikimate was not considered.

Emphasis was now directed toward the $C_4 + C_3$ and $C_5 + 2 \times C_1$ hypotheses; however, it soon became apparent that incubations of leaves and chloroplasts with a single substrate had a rather low effect on the biosynthesis of cyclopentenylglycine. The simultaneous application of two substrates in accordance with our working hypotheses greatly enhanced the incorporation of label into cyclopentenylglycine, demonstrating the ultimate necessity of at least two primary metabolites for the biogenesis of the cyclic compound. These experiments are described in more detail and summarized in Tables II and III.

Detached Leaf Experiments. The incorporation of substrate activity, expressed as nanomoles/gram of fresh leaves, from proteinogenic aspartate and glutamate into the pool of free amino acids was about one order of magnitude lower than that from α,ϵ-diaminopimelate and α-aminoadipate. This may reflect the dilution of the added substrate with endogenous amino acids combined with an enhanced turnover. After we administered $C_4 + C_3$ substrates, the radioactivity found in cyclopentenylglycine, as the percentage of radioactivity in free amino acids (Table II, last column), did not even reach the level of 2%, which is the level of endogenous cyclopentenylglycine. When unlabeled cyclopentenylglycine was applied together with the pair [U-^{14}C]aspartate + pyruvate to produce a "metabolic trap" effect, the percentage of radioactivity into

Table II: Incorporation of Labeled Substrates into Cyclopentenylglycine in Detached *Caloncoba echinata* Leaves[a]

substrates	amounts[b] (nmol)	incorpn into total free amino acids/g of fresh tissue		radioact. in cyclopentenylglycine (%)
		nmol	nmol %	
[U-^{14}C]aspartate	4.9	0.03	0.61	0.4
+pyruvate	4.9			
[U-^{14}C]aspartate	5.4	0.02	0.43	1.5
+pyruvate	5.4			
+*cyclopentenylglycine*	5.4			
α,ε-[1,7-^{14}C]diaminopimelate	34	0.14	0.41	0.7
[U-^{14}C]glutamate	4.2	0.01	0.26	2.3
+acetate	4.2			
α-[6-^{14}C]aminoadipate	21.3	0.11	0.52	2.3
+acetate	21.3			

[a] Mean of three determinations within ±5%. [b] Radioactivity in labeled substrates, 1 μCi each.

Table III: Incorporation of Labeled Substrates into Cyclopentenylglycine in *Caloncoba echinata* Chloroplasts[a]

substrates	amounts[b] (nmol)	incorpn into total free amino acids/mg of chlorophyll		radioact. in cyclopentenylglycine (%)
		nmol	nmol %	
α,ε-[1,7-^{14}C]diaminopimelate	34	2.87	8.44	1.0
[U-^{14}C]aspartate	5.4	0.02	0.35	1.8
+acetate	5.4			
[U-^{14}C]glutamate	4.2	0.01	0.18	6.7
+acetate	4.2			
α-[6-^{14}C]aminoadipate	21.3	0.29	1.35	3.7
+acetate	21.3			
[U-^{14}C]glutamate	4.2	0.24	5.69	22.1
+acetate	4.2			
+cyclopentenylglycine	4.2			
α-[6-^{14}C]aminoadipate	21.3	0.95	4.45	24.1
+acetate	21.3			
+cyclopentenylglycine	21.3			
[U-^{14}C]glutamate	4.2	0.41	9.76	42.2
+acetate	4.2			
+cyclopentenylglycine	4.2			
+isocitrate	1300			
α-[6-^{14}C]aminoadipate	21.3	1.27	5.95	29.0
+acetate	21.3			
+cyclopentenylglycine	21.3			
+isocitrate	1300			
[U-^{14}C]lysine	3.27	1.44	44.04	3.4
[U-^{14}C]lysine	3.27	0.66	20.89	0.9
+cyanide	1000			

[a] Mean of three determinations within ±5%. [b] Radioactivity in labeled substrates, 1 μCi each.

cyclopentenylglycine could be raised. The best incorporation values were obtained with substrates of the proposed $C_5 + 2 \times C_1$ path, i.e., glutamate + acetate and α-aminoadipate + acetate.

Chloroplast Experiments. It was considered that the randomization of label observed in experiments with detached leaves could be kept to a minimum, when working at the subcellular level. The plastid fraction has been shown to be a compartment actively synthesizing cyclopentenyl fatty acids (Rehfeldt et al., 1980), and other interfering fractions, e.g., mitochondria, were removed during the isolation of chloroplasts. The results of experiments with chloroplasts are summarized in Table III.

α,ε-Diaminopimelate, the presumed branching point and key C_7 intermediate of lysine biosynthesis in the $C_4 + C_3$ path, did not serve as a good substrate for cyclopentenylglycine. Surprisingly, lysine showed a much better result, whereas in the presence of cyanide the incorporation of radioactivity from this amino acid dropped sharply. We conclude that cyanide, an inhibitor of lysine decarboxylase (Sher & Malette, 1954), prevented the degradation of lysine to α-aminoadipate, a prospective substrate of the $C_5 + 2 \times C_1$ path.

Incorporations of label into cyclopentenylglycine from glutamate + acetate and α-aminoadipate + acetate were 6.4 and 3.7%, respectively, a first indication for C_1 chain elongations. A rather low incorporation was observed from aspartate + acetate, suggesting that in chloroplasts this mechanism is not very efficient with compounds shorter than C_5. When the pairs, glutamate + acetate and α-aminoadipate + acetate, were added to chloroplasts together with equimolar amounts of unlabeled cyclopentenylglycine, a substantial increase of radioactivity in cyclopentenylglycine was observed. The level of radioactivity incorporated into this nonproteinogenic amino acid from glutamate and α-aminoadipate was further raised to 42.4 and 29.9%, respectively, by supplementing the incubation medium with isocitrate. This metabolite competitively inhibits the reversible decarboxylation isocitrate $\rightleftharpoons$ α-ketoglutarate (Moyle, 1956) and may thus promote the condensation of acetyl-CoA with α-ketoglutarate, the transamination product of substrate glutamate.

Discussion

The discovery and successful isolation of cyclopentenylglycine corroborate its biogenetic relationship to the cyclopentenyl fatty acids demonstrated earlier (Cramer & Spener, 1977). Since cyclopentenylglycine was not detected after hydrolysis of seed protein, it can be classified as a truly nonproteinogenic amino acid, a group of natural substances numbering now well over 200 (Fowden, 1974). Apart from being integrated into fatty acids and cyanoglycosides, the characteristic cyclopentene ring serves a hitherto unknown physiological role in the amino acid as well. Cyclopentenylglycine, for instance, inhibits the growth of *Escherichia coli* (Dennis et al., 1955). A chemotaxonomic pattern of cyclopentenoid substances is difficult to discern because the cyclic fatty acids and the amino acid have so far been found in genera of Flacourtiaceae only, whereas the corresponding cyanogenic glycosides occur in Passifloraceae as well (Hegnauer, 1971).

The ^{1}H NMR spectrum of natural cyclopentenylglycine indicates the presence of two diastereomeric forms. As the known *S* configuration of the α carbon was not racemized under the mild isolation conditions applied, an inversion of the chiral center at $C_{1'}$ of cyclopentenylglycine is unlikely. We have to conclude that the two stereoisomers coexist in the same tissue. A precedent has been reported for a cyclopentenoid cyanoglycoside in fruits of *Adenia volkensii*, where the two stereoisomeric forms differ only at the chiral ring carbon bearing the nitrile and oxygen functions (Gondwe et al., 1978). The point of discrimination against the stereoisomer containing the 1′*S* carbon in the course of biosynthesis from (1′*R*,2*S*)- and (1′*S*,2*S*)-cyclopentenylglycine to the (1′*R*)-cyclopentenyl fatty acids is not known. One can only speculate that the metabolites of the 1′*R*,2*S* stereoisomer are channeled toward cyclic fatty acid biosynthesis, whereas the other or both stereoisomers can serve as precursors for cyanogenic glycosides not yet identified. The cyanogenicity of *H. anthelminthica* seeds have been reported (Power & Barrowcliff, 1905).

In view of the biosynthetic study, it should be noted that the amounts of substrate incorporated into leaves and chloroplasts depended on the quality of the leaves obtained from the greenhouse. Although higher incorporation values were observed with leaves than with chloroplasts, the leaf data were somewhat ambiguous due to the multibranched metabolism of the substrates administered. In the well-known path from glucose to shikimate, the intermediate C_7 metabolites may be a starting point for cyclopentenylglycine biosynthesis. In leaves and chloroplasts of *C. echinata*, labeled glucose was not incorporated into the amino acid. In seeds of *H. anthelminthica*, shikimate itself did not serve as a precursor either (Tober & Spener, 1980). The $C_4 + C_3$ and $C_5 + 2 \times C_1$ hypotheses tested propose that C_7 α-ketodicarboxylic acids are reduced to semialdehydes. Such reductions are known for aspartate and α-aminoadipate in the course of lysine biosynthesis and degradation (Kindl & Wöber, 1975). The cyclization to follow is envisaged as an intramolecular aldol condensation, a C–C coupling mechanism widely encountered in nature. This step creates the $C_{1'}$ chiral center, whose configuration depends on the site of nucleophilic attack of the β carbon on the aldehyde carbon either above or below the plane of the functional group.

According to the $C_4 + C_3$ hypothesis, the first steps are those of higher plant lysine biosynthesis, whereas the $C_5 + 2 \times C_1$ hypothesis proposes consecutive C_1-chain elongations of α-ketodicarboxylic acids with acetyl-CoA. Chain elongations of this kind are part of the citric acid cycle and of fungal lysine biosynthesis. Moreover, in higher plants, for example, methionine undergoes C_1 chain elongations to α-amino-6-methylthiocaproic acid (Löffelhardt & Kindl, 1975) and to higher homologues (Ettlinger & Kjaer, 1969). Our results clearly favor the $C_5 + 2 \times C_1$ path for cyclopentenylglycine biosynthesis, yet the $C_4 + C_3$ path cannot be ruled out with certainty, and several interconnections may exist. After transamination of substrate α-amino acids, the corresponding α-ketodicarboxylic acids are stepwise elongated by acetyl-CoA, thus being part of "clock spring" type cycles. Aspartate may enter the $C_5 + 2 \times C_1$ pathway via oxaloacetate or, together with pyruvate, via α-ketoadipate in the course of lysine degradation. As it is now established that pyruvate dehydrogenase is located in various forms of plastids (Yamada & Nakamura, 1975; Nothelfer et al., 1977; Reid et al., 1977), pyruvate may also enter the biosynthetic cycle via acetyl-CoA. When α-ketopimelate is reached in the course of the cycles, a reduction and cyclization step may lead to 2′-hydroxycyclopentylglyoxylate, which is dehydrated to cyclopentenylglyoxylate, the eventual precursor of cyclopentenylglycine and of cyclopentenyl fatty acids. Recently, we were able to show that α-[1,2-^{14}C]ketopimelate was indeed incorporated into these unusual cyclic compounds (Tober & Spener, 1980).

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Analysis of Cosolvent and Divalent Cation Effects on Association Equilibrium and Activity of Ribosomes[†]

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ABSTRACT: Interactions between *Escherichia coli* ribosomal subunits were studied as a function of divalent cations in water and various water–organic solvent solutions, and conditions favoring the association equilibrium of the two subunits were established. We show that the association constant, K_{assoc}, at fixed magnesium concentration, as determined by light scattering, was first stimulated and then inhibited when the concentration of organic solvent was raised, while the magnesium concentration $(\text{Mg}^{2+})_{1/2}$ yielding half-associated 70S species first decreased and then increased. Maximum K_{assoc} was obtained at higher concentrations (EGOH, 6.26 M; Me_2SO, 1.4 M) for hydrophilic solvents and at lower concentration (1-butanol, 0.33 M) for hydrophobic solvents. 1-Propanol (a linear alcohol) and *tert*-butyl alcohol (a branched alcohol) presented interesting cases since they exhibited no inhibitory effects till moderate concentrations (15% v/v or respectively 1.95 and 1.56 M); under these conditions, high stimulation of K_{assoc} ($\Delta K_{\text{assoc}} \gg 4$ units at 2 mM Mg^{2+}) and maximum decrease of $(\text{Mg}^{2+})_{1/2}$ ($\ll 0.5$ mM) were reached. These results are discussed in the light of experiments carried out on simpler systems taken as "models" (interactions of RNA, ATP, and GTP with Mg^{2+} and Ca^{2+}) and in the light of possible different physicochemical contributions of organic solvents. It is shown that changes in dielectric constant, p*K* values and solvation of divalent cations are not critical parameters in the perturbation of the equilibria. It is suggested that hydrophobic interactions between the cosolvent and nonpolar patches on the ribosomal protein surface might be initially responsible for the observed effect and possibly induce favorable conformation changes of ribosomal subunits. These structures are very stable, as shown by dialysis experiments against classical inactivating buffers, and very active, as shown by the corrected binding experiments of fMet-tRNA to 30 and 70 S.

Association of *Escherichia coli* ribosomal subunits 30 and 50 S to form the functional 70S particles is known to depend on the structure of the subunits and the ionic environment of the medium (Tissières & Watson, 1958; Tissières et al., 1958, 1959; Walters & Van Os, 1971; Debey et al., 1975; Noll & Noll, 1976). The degree of interaction between subunits as measured by the equilibrium association constant, K_{assoc}, at fixed (Mg^{2+}), should provide the basis for a most sensitive structural test of active ribosomes.

Because of the polyelectrolyte character of ribosomal RNA,[1] the behavior of ribosomal subunits is that of polyanions (Kliber et al., 1976). Analyzing experimental kinetics of divalent cation-binding data, Wishnia & Boussert (1977) showed that the main effects of divalent cations on K_{assoc} conform to the nonspecific charge-neutralization model. However, the need

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[1] Abbreviations used: EGOH, ethylene glycol; Me_2SO (DMSO in the figures), dimethyl sulfoxide; ATP, adenosine triphosphate; GTP, guanosine triphosphate; RNA, ribonucleic acid; poly(A,U,G), random copolymer of adenylic, uridylic, and guanylic acids.

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