Biosynthesis of Stizolobinic Acid and Stizolobic Acid in the Etiolated Seedlings of *Stizolobium hassjoo*

Koshi Saito and Atsushi Komamine

Department of Botany, Faculty of Science, University of Tokyo, Tokyo

and

Siro Senoh

Central Research Institute, Suntory Ltds., Osaka

(Z. Naturforsch. **30 c**, 659–662 [1975]; received June 19, 1975)

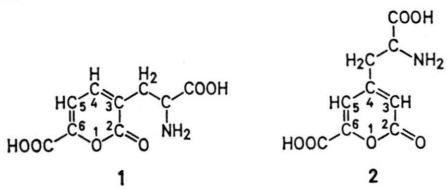
Stizolobium hassjoo, Biosynthesis, Stizolobinic Acid, Stizolobic Acid

Incorporation of L-[U-^{14}C]phenylalanine, L-[U-^{14}C]tyrosine and DL-[β-^{14}C]3,4-dihydroxyphenylalanine (DOPA) into stizolobinic acid, L-β-(6-carboxy-α'-pyron-3-yl)-alanine, and stizolobic acid. L-β-(6-carboxy-α'-pyron-4-yl)-alanine, was investigated as test precursors for these new types of amino acids in the epicotyls of etiolated seedlings of *Stizolobium hassjoo*. Radioactive stizolobinic acid and stizolobic acid resulted from administering labelled precursors were identified by colour reactions, radioautography and/or recrystallization to constant specific radioactivity. Radiolabelled stizolobic acid was subjected to alkaline hydrolysis and distribution pattern of the radioactivity was further examined in a degradation fragment of the amino acid.

Proposed biosynthetic routes to stizolobinic or stizolobic acid from DOPA were empirically confirmed and the results are further discussed in relation to the possibility that phenylalanine may also be introduced into reaction sequences leading to the synthesis of both heterocyclic amino acids.

Introduction

Stizolobinic acid and stizolobic acid, new types of heterocyclic non-protein amino acids were isolated for the first time in our laboratory from the sap of the etiolated epicotyl tips of *Stizolobium hassjoo* [1]. The structure of these amino acids was shown to be α-pyrone-6-carboxylic acid derivatives with the alanyl side-chain in the 3- or 4-position, respectively [2, 3] (Fig. 1). Although L-3,4-dihydroxyphenylalanine (DOPA) has been postulated as a possible precursor of the amino acids [3], no work has yet been done on the biosynthesis of these novel compounds.



1

2

Fig. 1. Structures of stizolobinic acid (**1**) and stizolobic acid (**2**).

The present paper reports an investigation on the incorporation of labelled precursors into the amino acids in the etiolated seedlings of *S. hassjoo* to confirm the postulated biosynthetic pathways of these two novel amino acids.

Requests for reprints should be sent to K. Saito, Department of Botany, Faculty of Science, University of Tokyo, Hongo, *Tokyo 113*, Japan.

Materials and Methods

Materials

L-[U-^{14}C]Phenylalanine (382 mCi/mmol), L-[U-^{14}C]tyrosine (118.8 mCi/mmol) were obtained from Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan. DL-[β-^{14}C]DOPA (58 mCi/mmol) was purchased from CEA, Gif-sur-Yvette, France. Scintillation-counting chemicals: PPO; 2,5-diphenyloxazole and POPOP; 1,4-bis-[2-(5-phenyloxazolyl)]-benzene from Wako Pure Chemical Ind. Ltd., Tokyo, Japan. Bis-MSB; *p*-bis-(*o*-methylstyryl)-benzene from Packard Instr. Co. Inc., Downer Grove, Ill., U.S.A. Double coated medical X-ray film (Fuji KX, 25.4 × 30.5 cm) was supplied from Fuji Photo Co. Ltd., Tokyo, Japan. The seeds of *S. hassjoo* were locally harvested and the seedlings were obtained by germinating semisterile seeds in the dark at 27 °C in trays with moist Vermiculite.

Administration of the radioactive test precursors

Uniform plants with epicotyls (3.0 – 3.5 cm, 0.25 – 0.30 g fr. wt.) were selected from 4-day-old etiolated seedlings. Epicotyls were cut under water and held in small vials. Their cut ends were immersed in aqueous solutions containing radioactive compounds (0.2 – 3.0 μCi/ml). The cuttings were allowed to metabolize the fed compounds for 70 – 72

hours in the dark at 27 °C. Water was supplied at intervals as required for complete uptake of the administered precursors.

Extraction and separation of stizolobinic and stizolobic acids

The epicotyls were repeatedly extracted with hot 80% methanol until the final extract was no longer coloured. The cooled extracts were filtered and concentrated *in vacuo* with a rotary evaporator at 60 °C. The remaining liquid was layered on an ion-exchange column (Amberlite IR-45, $HCOO^-$-form) and washed with distilled water for successive several hours. Adsorbed radioactive compounds were eluted with 0.4 M formic acid and taken to a small volume under reduced pressure at 60 – 65 °C. In small scale feeding experiments with L-[U-^{14}C]-phenylalanine, L-[U-^{14}C]tyrosine and DL-[β-^{14}C]-DOPA, a given volume of the concentrated solution from each sample was spotted respectively on Whatman No. 1 papers and stizolobinic and stizolobic acids were separated by the use of an ascending two-dimensional technique in *n*-butanol/acetic acid/water (4:1:5, v/v/v, upper phase) and then in phenol saturated, v/v/v, upper phase) and then in phenol of DL-[β-^{14}C]DOPA, radioactive stizolobic acid was separated and purified by PC (on Whatman No. 3 MM) with following solvents: *n*-butanol/acetic acid/water (4:1:5, v/v/v, upper or lower layer); methyl-ethylketone/pyridine/water/acetic acid (70:15:15:2, v/v/v/v); *tert*-butanol/formic acid/water (4:1:2, v/v/v); *iso*-propanol/formic acid/water (20:1:5, v/v/v); phenol saturated with water; *n*-butanol/ethanol/water (10:3:7, v/v/v, upper layer). In above described solvent systems the following R_F-values were found: 0.12; 0.88; 0.00; 0.62; 0.18; 0.23; 0.11 at 20 °C.

Recrystallization of stizolobic acid

Stizolobic acid was recrystallized from *n*-propanol/water (6:4, v/v) after adding the authentic amino acid (32 mg) dissolved in a minimum amount (3 – 4 ml) of hot solvent and allowing the solution to stand overnight in a cold room. The crystals (specific activity 4.2×10^3 dpm/μmol) were washed with ice-cold solvent and recrystallization were further repeated until the compounds show constant specific radioactivity (9 – 12 times).

Degradation of stizolobic acid

Radioactive crystals (10 mg, specific activity 2.3×10^2 dpm/μmol) were dissolved into a small volume (2 – 3 ml) of 3 M NaOH and the basic liquid was gently refluxed for 3 hours under nitrogen atmosphere at 100 °C.

Isolation and recrystallization of glutamic acid

The degradation product was cooled on ice, taken up in water (10 ml) and the solution was freed from alkali by passing it through a column of Amberlite IRC-50 (H^+-form).

The alkali free solution was concentrated and chromatographed (on Whatman No. 3 MM) in following solvents, successively: phenol saturated with water; pyridine/acetic acid/water (50:35:15, v/v/v); *tert*-butanol/formic acid/water (4:1:2, v/v/v); *n*-butanol/acetic acid/water (4:1:5, v/v/v, upper phase). The R_F-values were: 0.32; 0.57; 0.67; 0.23. The spot corresponding to glutamic acid on papers was cut out, eluted with distilled water, evaporated to small volume and filtered. Carrier glutamic acid (30 mg) was added to the filtrate and the mixture (specific activity 1.6×10^2 dpm/μmol) was recrystallized from 80% methanol till the radioactive glutamic acid showed a constant specific radioactivity (4 – 6 times).

Determination of radioactivity

Measurement of radioactivity was carried out in a liquid scintillation spectrometer (Beckman, model LS 250). The dioxane scintillation fluid contained 4 g of PPO, 0.2 g of POPOP, 60 g of naphthalene, 100 ml of absolute methanol, 20 ml of ethylene glycol per litre of dioxane (counting efficiency 83 – 85%). The radioactivity on paper chromatograms from small scale feeding experiments was determined after combustion of the marked radioactive spots corresponding to stizolobinic or stizolobic acid with a sample oxidizer (Packard Tri-Carb, model 305). A mixture of 15 g of PPO and 1 g of bis-MSB dissolved in 1 litre of toluene was used as the scintillant (counting efficiency 51 – 69%). Radioautographs of paper chromatograms were obtained conventionally by exposing the papers to X-ray films.

The concentrations of stizolobinic acid and stizolobic acid were determined spectrophotometrically (λ_{max} 303 nm and 301 nm in distilled water, respectively). Glutamic acid concentration was determined according to the procedure described by Balis [4].

Results

Incorporation of radioactivity from DL-[β-^{14}C]-DOPA into stizolobic acid by excised epicotyls of *S. hassjoo* was examined at given intervals of time.

The results are shown in Fig. 2. It can be seen that maximum value for total radioactivity transferred from DOPA is at about 70 hours after feeding of the radioactive precursor. At the end of the incubation period (72 hours) stizolobinic acid and stizolobic acid were extracted from the seedlings and separated by paper chromatography. Radioactive spots, revealed on radiochromatograms, were identical with the authentic samples of stizolobinic or stizolobic acid (Fig. 3 *). To further confirm the chromatographic evidence that radiolabelled stizolobic acid was formed from labelled DOPA, radioactive spots corresponding to the amino acid were cut out from paper chromatograms, eluted with water and the eluates were evaporated to a small volume. Authentic sample of stizolobic acid was added to the eluate and the mixture was recrystallized repeatedly many times. The recrystallized stizolobic acid showed a constant specific radioactivity (3.7×10^3 dpm/μmol, average specific activity).

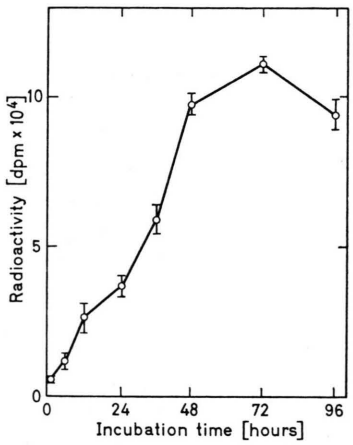


Fig. 2. Time-course of incorporation of DL-[β-^{14}C]DOPA into stizolobic acid.

Stizolobinic and sticolobic acids are postulated to be formed directly from DOPA by oxidative cleavage of the aromatic ring and subsequent ring closure to α-pyrone form [3]. If such an intramolecular rearrangement could occur limitedly within aromatic ring, the side-chain of the DOPA would be introduced intact into those two amino acid moieties. Purified stizolobic acid originated from DL-[β-^{14}C]-DOPA was subjected to alkaline hydrolysis and distribution pattern of the radioactivity was further examined in glutamic acid separated from degradation products. The glutamic acid contained, as expected, sufficient radioactivity (1.3×10^2 dpm/μmol, average specific activity).

L-[U-^{14}C]Phenylalanine, L-[U-^{14}C]tyrosine and DL-[β-^{14}C]DOPA were fed to excised epicotyl of *S. hassjoo*. After incubation for 72 hours in darkness labelled stizolobinic and stizolobic acids were separated and their radioactivities were measured (Tables I and II). It is evident from the results that DOPA is the most efficient precursor for stizolobinic and stizolobic acids among the compounds that were tested as shown by its higher value of radioactivity incorporation. Tyrosine comes next, while phenylalanine is the poorest among three precursors used.

Table I. Incorporation of radioactivity from labelled compounds administered as precursors of stizolobinic acid in *S. hassjoo*.

Experiment No.	Compound fed	Activity [μCi]	Activity found Specific activity [μCi/μmol]	Activity found Incorporation [%]
	L-[U-^{14}C]Tyrosine			
1		0.82	0.144	0.18
2		0.82	0.153	0.15
3		0.82	0.162	0.21
4		0.82	0.149	0.15
	DL-[β-^{14}C]DOPA			
5		0.85	0.320	0.41
6		0.85	0.302	0.23
7		0.85	0.306	0.30
8		0.85	0.333	0.29

Table II. Incorporation of radioactivity from labelled compounds administered as precursors of stizolobic acid in *S. hassjoo*.

Experiment No.	Compound fed	Activity [μCi]	Activity found Specific activity [μCi/μmol]	Activity found Incorporation [%]
	L-[U-^{14}C]Phenylalanine			
9		0.84	0.003	0.06
10		0.84	0.004	0.06
11		0.84	0.004	0.06
	L-[U-^{14}C]Tyrosine			
12		0.82	0.015	0.32
13		0.82	0.021	0.33
14		0.82	0.015	0.27
	DL-[β-^{14}C]DOPA			
15		0.85	0.046	0.65
16		0.85	0.041	0.56
17		0.85	0.043	0.62

* Fig. 3 see Table on page 662 a.

Discussion

The results presented above provide the first direct evidence that DOPA as well as tyrosine can serve as efficient precursors of both stizolobinic acid and stizolobic acid in *S. hassjoo*. These findings also strongly support the postulation drawn from earlier investigation [3].

Stizolobinic and stizolobic acids are contained in the seeds and vegetative parts of *S. hassjoo*. Though the synthetic mechanism remains obscure, they would be derived from DOPA by oxidative cleavage of the aromatic ring between 2 and 3 or 4 and 5 carbon atoms subsequent ring closure to α-pyrone-6-carboxylic acid forms.

Many works have been done on the biosynthesis of betacyanins or betaxanthins and the dihydropyridine moiety of these pigments is shown to be originated from DOPA by extradiol ring fission and subsequent recyclization involving the amino group [5, 6]. Recently Musso and his collaborators suggested the possibility that muscaflavin in *Hygrocybe* species may also be formed from DOPA by closely related mechanism as mentioned above [7]. Oxygenases which catalyze the cleavage of dihydroxyaromatic compounds are known to occur in bacteria [8, 9] and it may not be impossible to expect that analogous enzymes operate in higher plants.

The incorporation of tyrosine into the heterocyclic amino acids would indicate its direct conversion to DOPA in the plant tissue, possibly catalyzed by a tyrosine hydroxylase. The *o*-hydroxylation of tyrosine by a specific tyrosine hydroxylase from a variety of sources has been extensively studied [10–12]. Moreover, the incorporation of tyrosine into DOPA has been reported in *S. hassjoo* [13] and other plant materials [14].

Although phenylalanine is not effective precursor for stizolobic acid, some radioactivities were found in the amino acid when labelled phenylalanine was fed. This fact suggests that phenylalanine may be converted to tyrosine, DOPA and then stizolobic acid in the epicotyls of *S. hassjoo*. It has been pointed out that the conversion of phenylalanine to tyrosine is a severely limited process in plants that have been investigated [15, 16]. Although Nair and Vining have described a phenylalanine hydroxylase in spinach leaf extracts [17], Fritz and Aman were unable to obtain evidence for the hydroxylation of phenylalanine *in vivo* in experiments specifically designed for that purpose [18]. Similar attempts by Kindl to demonstrate the enzyme in *Astilbe chinensis* were negative although in this case the apparent absence of the hydrocylase was explained by the low levels of *p*-hydroxyphenolic acids that were observed [19].

Verifing study on the conversion of phenylalanine to tyrosine in *S. hassjoo* is in progress.

[1] S. Hattori and A. Komamine, Nature **183**, 1116–1117 [1959].
[2] S. Senoh, S. Imamoto, Y. Maeno, T. Tokuyama, T. Sakan A. Komamine, and S. Hattori, Tetrahedron Lett. **46**, 3431–3436 [1964].
[3] S. Senoh, S. Imamoto, Y. Maeno, K. Yamashita, M. Matsui, T. Tokuyama, T. Sakan, A. Komamine, and S. Hattori. Tetrahedron Lett. **46**, 3437–3444 [1964].
[4] M. E. Balis, Methods Biochem. Anal. **20**, 103–133 [1971].
[5] N. Fischer and A. S. Dreiding, Helv. Chim. Acta **55**, 649–658 [1972].
[6] G. Impellizzeri and M. Piattelli, Phytochemistry **11**, 2499–2502 [1972].
[7] R. von Ardenne, H. Döpp, H. Musso, and W. Steglich, Z. Naturforsch. **29 c**, 637–639 [1974].
[8] O. Hayaishi and K. Hashimoto, J. Biochem. (Tokyo) **37**, 371–374 [1950].
[9] Y. Kojima, N. Itada, and O. Hayaishi, J. Biol. Chem. **236**, 2223–2228 [1961].
[10] R. H. Kenten, Biochem. J. **67**, 300–307 [1957].
[11] D. A. Robb, L. W. Mapson, and T. Swain, Phytochemistry **4**, 731–740 [1965].
[12] R. Shiman, M. Akino, and S. Kaufman, J. Biol. Chem. **246**, 1330–1340 [1971].
[13] A. Komamine, Bot. Mag. (Tokyo) **75**, 228–236 [1962].
[14] I. Liss, Flora **151**, 351–367 [1961].
[15] E. Leete, R. M. Bowman, and M. F. Manuel, Phytochemistry **10**, 3029–3033 [1971].
[16] G. H. N. Towers and P. V. Subba Rao, Recent Advances in Phytochemisry (V. C. Runeckles and J. E. Watkins, eds.), **vol. 4**, pp. 1–43, Appleton Century Crofts, New York 1972.
[17] P. M. Nair and L. C. Vining, Phytochemistry **4**, 401–411 [1965].
[18] G. Fritz and F. Aman, Plant Physiol. **41**, XIV (Supplement) [1966].
[19] H. Kindl, Eur. J. Biochem. **7**, 340–347 [1969].

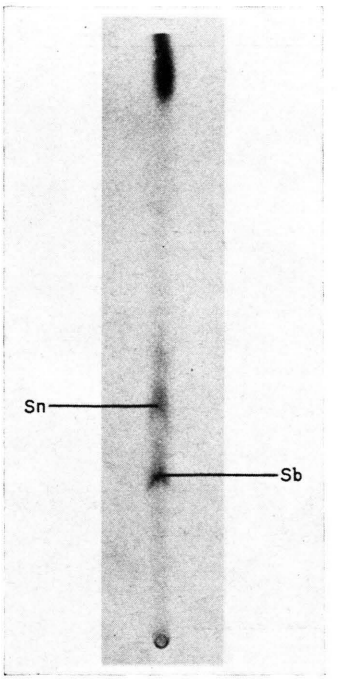


Fig. 3. Radioautogram of chromatographed stizolobinic acid and stizolobic acid fraction from the etiolated seedlings of *S. hassjoo* administered with DL-[β-^{14}C]DOPA. (The ascending chromatography was carried out two times successively in phenyl saturated with water. Sn = stizolobinic acid, Sb = stizolobic acid.)