

Further Studies on the 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 Ltd., Osaka

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Incorporation of doubly labelled tyrosine into stizolobinic acid and stizolobic acid by the etiolated seedlings of *Stizolobium hassjoo* was studied. The retention of tritium activity in stizolobinic acid was 55%, while that in stizolobic acid was only 4.4% in average. The results strongly suggest that the heterocyclic rings of these two amino acids may be derived from 3,4-dihydroxyphenylalanine (DOPA) by extradiol cleavage of the aromatic ring.

Introduction

Stizolobinic acid, L- β -(6-carboxy- α' -pyron-3-yl)-alanine, and stizolobic acid, L- β -(6-carboxy- α' -pyron-4-yl)-alanine, occur in *Stizolobium*, *Mucuna*¹ and *Amanita* species².

These two heterocyclic amino acids are supposed to be originated from 3,4-dihydroxyphenylalanine (DOPA) by an extradiol type of ring cleavage and subsequent closure to α -pyrone-6-carboxylic acid forms³. In a preceding study we have shown that DOPA is practically utilized to the formation of these novel compounds⁴. Tyrosine is also used as the precursor but less efficiently. To confirm the hypothesis of biosynthetic mechanism of stizolobinic and stizolobic acids from the aromatic amino acids, incorporation of doubly labelled tyrosine into these heterocyclic amino acids was studied in this work.

Materials and Methods

Materials

L-[3,5-³H]Tyrosine (60.3 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. L-[U-¹⁴C]Tyrosine (118.8 mCi/mmol) was obtained from Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan. Locally harvested seeds of *Stizo-*

lobium hassjoo were used in this work and the seedlings were obtained as described previously⁴.

Administration of dually labelled tyrosine

The epicotyls were separated from 4-day-old etiolated seedlings. The length of an epicotyl was 3.8 cm and the fresh weight was 0.3 g in average. One hundred epicotyls were placed in a small vial, containing an aqueous solution of labelled tyrosine (L-[U-¹⁴C]tyrosine; 1.58 μ Ci and L-[3,5-³H]tyrosine; 1.56–31.18 μ Ci), and were kept in darkness for 24 hours at 27 °C. To insure complete uptake of the fed precursors, distilled water was added at intervals and was allowed to be absorbed by the epicotyls for a successive few hours.

Isolation and purification of stizolobinic acid and stizolobic acid

After 24 hours of incubation the epicotyls were repeatedly extracted with hot 80% methanol (11–13 times, each time for 20–30 min). The combined methanol extracts were concentrated and filtered, and the resulting filtrate was then passed through Amberlite IR-45 (HCOO⁻-form) and washed with distilled water. Absorbed radioactive compounds were displaced by 0.5 M formic acid and purified chromatographically with the use of an ascending one dimensional technique in the following solvent systems: upper or lower layer of *n*-butanol/acetic acid/water (4:1:5, by vol.); *tert*-butanol/formic acid/water (4:1:2, by vol.); *iso*-propanol/formic acid/water (20:1:5, by vol.); phenol saturated with water; *n*-butanol/ethanol/water (10:3:7, by

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



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vol., upper phase). The R_F -values in each solvent were: stizolobinic acid = 0.16; 0.88; 0.65; 0.24; 0.34; 0.09, stizolobic acid = 0.12; 0.89; 0.62; 0.19; 0.23; 0.11, respectively. Chromatographically purified stizolobinic acid or stizolobic acid was then recrystallized from *n*-propanol/water (6 : 4, by vol.).

Determination of specific radioactivity

Radioactivity was counted in a Beckman liquid scintillation spectrometer (model LS 250) using two channels. The contribution of ^{14}C counts in the tritium channel (window A) was calculated from a calibration curve. The concentrations of stizolobinic acid and stizolobic acid were determined spectrophotometrically with an ultraviolet VIS spectrophotometer (Hitachi Perkin-Elmer, type 139; λ_{max} in distilled water, stizolobinic acid = 303 nm, stizolobic acid = 301 nm).

Results and Discussion

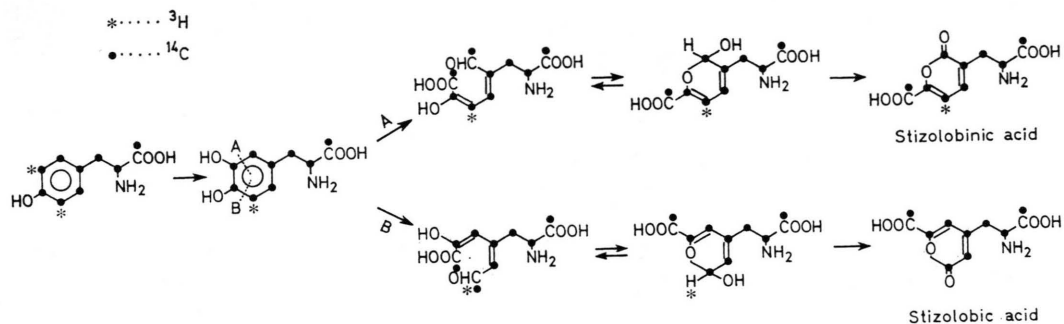
To establish the proposed synthetic mechanism for α -pyrone rings of both stizolobinic acid and stizolobic acid, doubly labelled tyrosine was fed to the etiolated epicotyls of *S. hassjoo* and the relative radioactivity levels of ^3H and ^{14}C were examined in isolated heterocyclic amino acids. Since L-[5- ^3H]-DOPA was not available, L-[3,5- ^3H]tyrosine was exclusively used in this work as a feeding substrate. It has been shown that when L-[3,5- ^3H]tyrosine is enzymatically hydroxylated, one tritium atom is lost from the 3-position resulting in the formation of L-[5- ^3H]DOPA (55% tritium activity retention) ⁵.

Therefore, when L-[3,5-³H]- and L-[U-¹⁴C]labeled tyrosine are administered, about half of ³H/¹⁴C ratio in fed tyrosine would be expected in produced stizolobinic acid, while isolated stizolobic acid should be almost free from tritium activity, if the conversion of DOPA into α -pyrone moieties of both amino acids takes place via extradiol cleavage of the aromatic ring (Scheme 1).

The results from doubly labelled tracer experiments carried out with different $^3\text{H}/^{14}\text{C}$ ratios in fed precursors are summarized in Table I. The isolated stizolobinic acid had about half $^3\text{H}/^{14}\text{C}$ ratio as that of the fed precursor, indicating that approximately 55% of the tritium was retained (Table I), whereas only poor retention level of tritium activity (4.4% in average) in stizolobic acid was observed. These findings support the postulation that stizolobinic acid and stizolobic acid may be formed from DOPA by extradiol cleavage of the aromatic ring.

Table I. Incorporation of doubly labelled tyrosine into stizolobinic and stizolobic acids by the etiolated seedlings of *S. hassjoo*.

Expt. No.	L-Tyrosine			Stizolobinic acid				Stizolobic acid							
	3,5- ³ H [μCi]	U- ¹⁴ C [μCi]	³ H/ ¹⁴ C	³ H [μCi/ μmol]	Spec. Act. ¹⁴ C [μCi/ μmol]	³ H [%]	Incorp. ¹⁴ C [%]	³ H/ ¹⁴ C	³ H reten- tion [%]	³ H [μCi/ μmol]	Spec. Act. ¹⁴ C [μCi/ μmol]	³ H [%]	Incorp. ¹⁴ C [%]	³ H/ ¹⁴ C	³ H reten- tion [%]
1	1.56	1.58	0.99	0.022	0.042	0.021	0.040	0.52	52.5	0.0010	0.0110	0.004	0.089	0.09	9.1
2	3.12	1.58	1.97	0.043	0.039	0.021	0.038	1.10	55.8	0.0013	0.0125	0.003	0.102	0.10	5.1
3	15.59	1.58	9.87	0.216	0.041	0.021	0.039	5.27	53.4	0.0022	0.0129	0.002	0.104	0.17	1.7
4	31.18	1.58	19.73	0.789	0.071	0.038	0.068	11.11	56.3	0.0062	0.0193	0.003	0.157	0.32	1.6



Scheme 1. Possible biosynthetic pathways leading to stizolobinic acid and stizolobic acid in *S. hassjoo*.

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⁵ G. Guroff, J. W. Daly, D. M. Jerina, J. Renson, B. Witkop, and S. Udenfriend, *Science* **157**, 1524–1530 [1967].