Phytochemistry, Vol 28, No 6, pp 1617–1620, 1989
Printed in Great Britain

0031-9422/89 $3.00+0.00

BIOSYNTHESIS OF RHIZOBITOXINE FROM L-ASPARTIC ACID AND L-*THREO*-HYDROXYTHREONINE BY *PSEUDOMONAS ANDROPOGONIS*

ROBIN E. MITCHELL

Division of Horticulture and Processing, Department of Scientific and Industrial Research, Private Bag, Auckland, New Zealand

(*Received* 9 *March* 1988)

Key Word Index—*Pseudomonas andropogonis*; rhizobitoxine, aspartic acid; hydroxythreonine; biosynthesis, L-[U-^{14}C]-aspartic acid; L-[^{14}C]-*threo*-hydroxythreonine.

Abstract—One strain of *Pseudomonas andropogonis* incorporated radioactivity from L-[U-^{14}C]-aspartic acid into L-*threo*-hydroxythreonine, and three strains incorporated radioactivity into both L-*threo*-hydroxythreonine and rhizobitoxine. L-[^{14}C]-*threo*-hydroxythreonine was isolated from a culture of *P. andropogonis* that had been provided with L-[U-^{14}C]-aspartic acid for 35 min, and purified. When this was fed to a rhizobitoxine-producing strain of *P. andropogonis*, <20% of the radioactivity supplied was removed from the medium in 5–6 hr. Radioactivity in the medium was found to be in hydroxythreonine and rhizobitoxine. A high level of the utilized radioactivity from hydroxythreonine had been incorporated into rhizobitoxine. These data support the proposal that biosynthesis of rhizobitoxine proceeds through L-aspartic acid and that hydroxythreonine is an intermediate en route to rhizobitoxine.

INTRODUCTION

Rhizobitoxine [L-2-amino-4-(2*R*-amino-3-hydroxypropoxy)-*trans*-but-3-enoic acid] is a phytotoxin produced in liquid cultures by the plant pathogenic bacterium *Pseudomonas andropogonis* (Smith 1911) Stapp 1928 [1, 2], and also by the root-nodulating organism *Bradyrhizobium japonicum* (Kirchner 1896) Jordan 1982 [3, 4]. The production of rhizobitoxine in seventeen strains of *P. andropogonis* was accompanied by the co-production of hydroxythreonine (L-*threo*-2-amino-3,4-dihydroxybutanoic acid) [1, 2], an uncommon amino acid little known in nature [5]. The structural homology between 2-amino-3,4-dihydroxybutanoic acid and the 2-amino-4-alkoxy-but-3-enoic acid moiety of rhizobitoxine has been noted, and is the basis of the suggestion that the two compounds may arise from the same biosynthetic pathway [1]. The present experiments were undertaken to determine whether the two compounds arise from a common precursor, and whether hydroxythreonine is an intermediate on the biosynthetic pathway to rhizobitoxine.

RESULTS AND DISCUSSION

The working hypothesis for experimentation was that the biosynthesis of hydroxythreonine involved a modification of the known pathway between aspartic acid and threonine, and that the biosynthesis of rhizobitoxine utilised hydroxythreonine as an intermediate. This later stage obviously requires carbon from some other source in the formation of the enol–ether structure of rhizobitoxine.

The first experiments therefore tested the incorporation of L-[U-^{14}C]-aspartic acid into both hydroxythreonine and rhizobitoxine by four different strains of *P. andropogonis*. After the exposure period, a mixture of the two metabolites was isolated from the culture-supernatant utilizing an ion-exchange resin and then the two were purified and separated by partition chromatography on LH20 Sephadex followed by anion-exchange chromatography on QAE Sephadex. The relative amounts of metabolite produced by each strain, and the recovery of purified components from the mixtures isolated, are given by the data in Table 1. It is likely that the ion-exchange method of isolation gives a 100% recovery of the products present in the culture-supernatant, but no data was sought to support this. The percentage incorporations of radioactivity into hydroxythreonine and rhizobitoxine from L-[U-^{14}C]-aspartic acid by each strain during the four hr exposure period are calculated from the radioactivity data in Table 2. Strain PDDCC 2809 incorporated 2.5% of the supplied radioactivity into hydroxythreonine; at the stage of growth of the culture (1.5 days), no rhizobitoxine was detectable, although strain 2809 has been recorded to produce a trace of rhizobitoxine in a six-day-old culture [2]. Substantial incorporations of ^{14}C into rhizobitoxine (2.0, 1.2%) were observed when L-[U-^{14}C]-aspartic acid was fed to cultures of PDDCC 7076 and 7856 respectively. With these two strains, moderate levels of ^{14}C were incorporated into hydroxythreonine at the same time (0.5, 0.6% respectively). This indicates that, assuming hydroxythreonine is an intermediate of rhizobitoxine, some hydroxythreonine is being excreted from the cell as it is synthesized, simultaneously with its intracellular utilization for rhizobitoxine synthesis. The alternative explanation that labelling of rhizobitoxine has occurred exclusively by way of extracellular ^{14}C-labelled hydroxythreonine is unlikely, after consideration of uptake data for hydroxythreonine (presented below, Table 3). The relative magnitudes of the percentage incorporations into rhizobitoxine and hydroxythreonine by strains 7076 and

Table 1. Recoveries of ^{14}C-hydroxythreonine and ^{14}C-rhizobitoxine isolated from *P andropogonis* cultures exposed for 4 hr to 7 5 μCi (2 μmol) of L-[U-^{14}C] aspartic acid

P. andropogonis strain PDDCC #	Quantity (ion exchange) ThrOH (mg)	rhizobitoxine (mg)	Quantity (purified) ThrOH (mg)	(μmol)	rhizobitoxine (mg)	(μmol)
2809	2.2	Nil	1 9	14 1	Nil	--
5980	1 0	ND	0 9	6.7	0 1	0 5
7076	ND	6 0	0.6	4 4	5 5	28 9
7856	ND	3 6	1 1	8 1	3 5	18 4

ND. Not determined

ThrOH Hydroxythreonine

Two μmol and 7.5 μCi of ^{14}C-aspartic acid were added to 400 ml cultures at 1 5 days (2809) or 2 0 days (5980, 7076) or 2 5 days (7856) of growth Cultures were harvested after an additional 4 hr growth. Quantities are for 400 ml of culture for each strain, and were determined by TLE analysis both after isolation by ion exchange, and after purification by LH20 Sephadex and QAE Sephadex chromatography

Table 2 Incorporation of radioactivity from L-[U-^{14}C] aspartic acid, by strains of *P andropogonis*, into hydroxythreonine and rhizobitoxine

P andropogonis strain PDDCC #	L-[U-^{14}C]asp added (cpm $\times 10^{-6}$)	Radioactivity isolated ThrOH (cpm $\times 10^{-4}$)	rhizobitoxine	Incorporation into ThrOH (%)	rhizobitoxine
2809	16 02	40 5	Nil	2.5	–
5980	16 37	13 1	3 3	0 8	0 2
7076	15 82	8 0	31 1	0 5	2 0
7856	15 08	9 0	17 8	0 6	1 2

Two μmol and 7 5 μCi of ^{14}C-aspartic acid were added to 400 ml cultures at 1.5 days (2809) or 2 0 days (5980, 7076) or 2 5 days (7856) of growth Cultures were harvested after an additional 4 hr of growth

Table 3 Uptake of ^{14}C-hydroxythreonine by *Pseudomonas andropogonis* and incorporation of ^{14}C into rhizobitoxine

L-[^{14}C]-ThrOH added (cpm $\times 10^{-6}$)	(μmol)	ThrOH isolated (cpm $\times 10^{-6}$)	ThrOH used (cpm $\times 10^{-6}$)	Rhizobitoxine isolated (cpm $\times 10^{-6}$)	Incorporation into rhizobitoxine (%)
1 523	4 6	1 110 (72.9%)	0 413	0 164	39.7
0 3049	0 93	0 2275 (74 6%)	0 0774	0 0182	23 5

Two different quantities of ^{14}C hydroxythreonine were added to 400 ml cultures of *P andropogonis* 7076 at 2 0 days of growth Cultures were harvested after an additional 5–6 hr growth and the hydroxythreonine and rhizobitoxine isolated and purified

7856 are likely to reflect the relative molar amounts of each compound secreted into the growth medium during the time period of the utilization of the ^{14}C-precursor. To some extent this is supported by the relative quantities (Table 1) of each compound for each strain which represent the net production over the complete growth period used.

The substantial incorporation of ^{14}C from L-[U-^{14}C] aspartic acid into hydroxythreonine by strain 2809 was utilized to prepare a purified sample of L-[^{14}C]-*threo*-hydroxythreonine In this experiment, the culture was six hr older than the previous experiment, and the higher incorporation rate obtained (2.7%) was probably because the rate of synthesis of hydroxythreonine increases with time at this stage of the culture (Mitchell, unpublished data). The purified product had no rhizobitoxine content, since strain 2809 does not synthesize rhizobitoxine at the stage of culture used, and furthermore, the purification method used completely separates hydroxythreonine from rhizobitoxine.

When L-[^{14}C]-*threo*-hydroxythreonine was added to a growing two-day-old culture of *P andropogonis* 7076, a

major proportion (>80%) of the ^{14}C supplied was recovered in the culture supernatant. Most of this radioactivity was subsequently determined to be in hydroxythreonine, this was designated as non-utilized hydroxythreonine for the analysis of the results of the experiment (Table 3). From the difference between the radioactivity of hydroxythreonine supplied and hydroxythreonine remaining, the radioactivity utilized during the exposure period was obtained. The percentage incorporation of radioactivity into rhizobitoxine, calculated from the utilized hydroxythreonine, was 39.7% and 23.5% in two separate experiments (Table 3). The results demonstrate that there is an uptake of hydroxythreonine by *P. andropogonis* cells, and, as there is a net increase of extracellular hydroxythreonine at this stage of the growth phase (R. E. Mitchell, unpublished data), that there is a two-way movement of hydroxythreonine between cells and the growth medium. The high percentage incorporations obtained are indicative of the highly specific usage of this compound by the cell and provide strong evidence that hydroxythreonine is an intermediate on the biosynthetic pathway to rhizobitoxine.

Collectively the results reported here suggest the course of biosynthesis of 4/7 of the carbon skeleton of rhizobitoxine to proceed via the aspartic acid biosynthetic pathway, with a branch from the normal route beyond aspartic acid which leads to the uncommon amino acid L-*threo*-hydroxythreonine. This, by way of an ether-forming reaction involving probably a three-carbon unit from a different origin, such as from glyceraldehyde-3-phosphate, could give rise to rhizobitoxine. Certainly the linkage of two units to form the enol ether structure of rhizobitoxine is indicative of the functioning of enzymes in a way that may be unique and characteristic of this system.

EXPERIMENTAL

Selection and culture of bacterial strains. A previous study [2] has shown a variation in metabolite production by *P. andropogonis* strains Some produced high levels of rhizobitoxine (up to 92 mg/l) and only low levels of hydroxythreonine, while other strains produced little or no rhizobitoxine but maintained high levels of hydroxythreonine production (up to 50 mg/l). For this reason the two hydroxythreonine producing strains PDDCC 2809 and 5980, and the two rhizobitoxine producing strains PDDCC 7076 and 7856 were selected for studies of incorporation of radioactivity into the respective metabolites.

Strains were obtained from the Plant Diseases Division Culture Collection (PDDCC) and recovered from lyophil by plating onto King's medium B [6]. A mixture of 5 colony-forming units was subcultured onto slants of King's medium B, and held at 4°. They were maintained throughout the course of these experiments by subculturing at 4–6 weekly intervals In all experiments liquid culture was undertaken as described in ref. [1] using the medium of ref. [7], but with 400 ml per 2 l flask instead of the usual 600 ml.

^{14}C-Labelling experiments. L-[U-^{14}C] Aspartic acid (*ca* 1 ml, 4.5–7.5 μCi, 2 μmol) was added to one flask for each of the four strains at the stage of growth detailed in Table 1, and the flask harvested after 4 hr. The timing of the addition of ^{14}C was chosen to coincide with the prior production of 10–20 μmol of hydroxythreonine or rhizobitoxine; the times varied for different strains because of different time lags in the commencement of production, and differing kinetics of production L-[^{14}C]-hydroxythreonine was added in 2 separate experiments (see Table 3) to strain 7076 after 2 days growth; cultures were grown for a further 5–6 hr then harvested.

At harvest, the culture (400 ml) was centrifuged at 0° and the supernatant immediately passed through an ion exchange column of Amberlite CG-120 resin in H^+ form. Products were recovered from the ion exchange column by elution with 5% NH_4OH, the products being in 12 ml, collection being started at the change of pH of the eluent. This fraction was evapd, and prior to purification procedures the quantity of hydroxythreonine and/or rhizobitoxine in it was estimated by thin layer electrophoresis (TLE), as described below.

Preparation of L-[^{14}C]-threo-*hydroxythreonine.* L-[U-^{14}C] Aspartic acid (4 ml, 105 μCi, 2 μmol) was added to *P andropogonis* 2809 after 1 75 days of growth. The radioactive culture was allowed to grow for 35 min and was then harvested. The L-[^{14}C]-*threo*-hydroxythreonine was isolated and purified, yielding 2.5 mg (determined as described below), and 2.87 μCi for 2.7% incorporation of ^{14}C from aspartic acid.

Purification of ^{14}C-products. The ion exchange fraction, after TLE quantitation for hydroxythreonine and rhizobitoxine, was further purified by chromatography on LH20 Sephadex, followed by ion exchange chromatography on QAE Sephadex as described by Mitchell *et al.* [1] except 12 5 g of Sephadex and 600 ml of 0 05 M–0.6 M $(NH_4)HCO_3$ gradient were used Quantities of each ^{14}C product for each strain were then again estimated by TLE The QAE Sephadex purification step completely separated rhizobitoxine from hydroxythreonine, and gave a good recovery of each purified product (Table 1) In earlier experiments (data not used here) prep. TLE was used to separate ^{14}C-hydroxythreonine and rhizobitoxine. This method resulted in a good recovery of hydroxythreonine, but in substantial losses (> 50%) of rhizobitoxine, probably because of the relatively long exposure times to air on the cellulose during manipulations with the plate and during autoradiography

Quantitation of hydroxythreonine and rhizobitoxine The quantities of rhizobitoxine and hydroxythreonine present in ion exchange fractions, and in each product after purification, were estimated by TLE in a similar way to the TLC method reported in ref. [2]. The use of TLE is quicker than TLC, and was possible in these experiments because the shorter growth times used gave ion-exchange fractions that had fewer contaminating ninhydrin-reacting products than obtained with the 6–7 day growth period In each sample, quantitation of hydroxythreonine and rhizobitoxine was undertaken separately, if both were present. An appropriate dilution of a sample was made, then 1 and 2 μl volumes were applied to 1.2 cm bands at the origin. Similarly, 0.5, 1.0, 1.5 and 2 0 μg quantities of standard (hydroxythreonine or rhizobitoxine, compounds purified as described in ref. [1]) were applied to the plate. After TLE (pH 2, 20 min) the plate was dried and then sprayed with a methanolic 1% (w/v) solution of ninhydrin containing 0.1% (w/v) $Cd(OAc)_2$ and 0.5% AcOH. The colour intensities of the resulting bands were compared visually. Then samples were re-run, after appropriate dilutions, until quantities were found at both application levels that gave ninhydrin colour-intensities that matched those of the standards. Quantities in the parent samples were then calculated.

Measurement of radioactivity. Radioactivity was measured on a Beckman LS 2800 liquid scintillation counter Samples were prepared for counting as a homogenate in 0 5 ml H_2O and 3.1 ml of ACS II aqueous counting scintillant (Amersham).

REFERENCES

1. Mitchell, R. E., Frey, E J and Benn, M H. (1986) *Phytochemistry* **25**, 2711.

2. Mitchell, R. E. and Frey, E. J. (1988) *Physiol. Molec. Plant Path.* **32**, 335.
3. Owens, L. D., Thompson, J. F., Pitcher, R. G. and Williams, T. (1972) *J. Chem. Soc. Chem. Commun.* 714.
4. La Favre, J. S. and Eaglesham, A. R. J. (1986) *Plant Soil* **92**, 443
5. Westley, J. W., Pruess, D. L., Volpe, L. A., Demny, T. C. and Stempel, A. (1971) *J. Antibiot.* **24**, 330.
6. King, E. O., Ward, M. K. and Raney, D. E. (1954) *J. Lab. Clin. Med.* **44**, 301.
7. Hoitink, H. A. J. and Sinden, S. L. (1970) *Phytopathology* **60**, 1236.