8.8 (d, 3, side chain CH_3), 8.7–8.2 (b, 2, CH_2), 7.8 (s, 3, ring CH_3), 7.6–7.4 (b, 3, CHCOO, ring CH_2), 6.22, 6.12 (s, 6, OCH_3).

Ubihydroquinone-7-disulfuric Acid (XXXII). A solution of ubihydroquinone-7 (99 mg) (Morimoto *et al.*, 1965) in pyridine (1 ml) and SO_3–triethylamine complex (51 mg) was similarly worked up, and then the band of the desired compound (R_F 0.85) was extracted with methanol. The extracts were evaporated to dryness to give XXXII as a yellow oil: yield 62 mg (50.4%); λ_{max}^{EtOH} 276, 282 mμ; nuclear magnetic resonance spectrum (CD_3SOCD_3) 8.46 (s, 18, *trans* C=CCH_3), 8.39 (s, 3, *cis* C=CCH_3), 8.30 (s, 3, *trans* C=CCH_3), 8.04 (b, 24, C=CCH_2), 7.92 (s, 3, ring CH_3), 6.33, 6.21 (s, 6, OCH_3) 4.95 (b, 7, C=CH).

Acknowledgments

The authors wish to thank Messrs. T. Shima and A. Ouchida, and Misses F. Kasahara, S. Noto, and I. Otsubo of the same Research Laboratories for the measurement of physicochemical data and elemental analysis.

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Biosynthesis of Echinulin by *Aspergillus amstelodami* from Cyclo-L-alanyl-L-tryptophyl-^{14}C*

George P. Slater, James C. MacDonald, and Ruka Nakashima†

ABSTRACT: *Aspergillus amstelodami* PRL 1698 synthesized echinulin when it was grown on molasses medium to which was added cyclo-L-alanyl-L-tryptophyl or cyclo-L-alanyl-D-tryptophyl (both labeled with ^{14}C in the methylene carbon of the tryptophyl moiety). The organism incorporated radioactivity from cyclo-L-alanyl-L-tryptophyl-^{14}C into echinulin in a specific way, and to a greater extent than from cyclo-L-alanyl-D-tryptophyl-^{14}C. There was greater incorporation of radioactivity from cyclo-L-alanyl-L-tryptophyl-^{14}C into echinulin than into mycelial tryptophan, and the incorporation into echinulin was not significantly lessened by adding unlabeled tryptophan to the medium. Therefore, cyclo-L-alanyl-L-tryptophyl-^{14}C was not hydrolyzed to tryptophan-^{14}C before incorporation of radioactivity into echinulin, and the cyclic dipeptide itself appears to be an intermediate in the biosynthesis of echinulin.

Studies with ^{14}C-labeled tryptophan, alanine, and mevalonic lactone have shown that these compounds are precursors of echinulin (Figure 1a) synthesized by *Aspergillus amstelodami*. Tryptophan-^{14}C was incorporated exclusively into the echinin (Figure 2) moiety of the diketopiperazine ring of echinulin (Birch and Farrar, 1963; MacDonald and Slater, 1966). Mevalonic lactone-^{14}C and alanine-^{14}C were primarily

* From the Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Saskatchewan, Canada. *Received February 24, 1970.* Issued as N.R.C. No. 11361.

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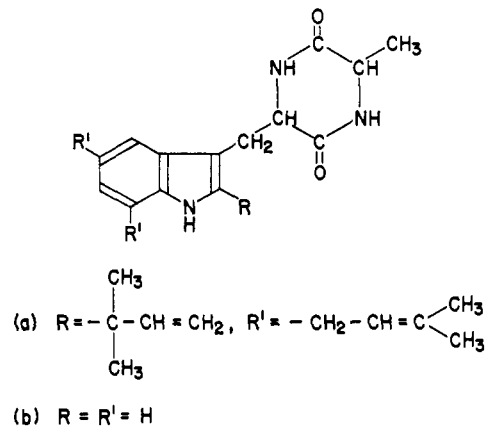

FIGURE 1: The structure of (a) echinulin and (b) cycloalanyltryptophyl.

incorporated, respectively, into the isoprenoid side chains of echinin and the alanyl moiety of the diketopiperazine ring of echinulin (Birch *et al.*, 1961).

Cycloalanyltryptophyl isomers (isomers of 3-(indol-3-yl methyl)-6-methyl-2,5-piperazinedione, Figure 1b) structurally resemble echinulin and could be intermediates in the biosynthesis of echinulin from tryptophan, mevalonic acid, and alanine. Because the diketopiperazine ring of echinulin has two asymmetric carbon atoms of L configuration (Westley *et al.*, 1968; Nakashima and Slater, 1969), cyclo-L-alanyl-L-tryptophyl is more likely to be an intermediate than another isomer, such as cyclo-L-alanyl-D-tryptophyl. The present work tests this possibility.

Materials and Methods

Growth Conditions and Echinulin Isolation. Organism maintenance, inoculum preparation, and preparation of sterile medium (with or without the addition of unlabeled tryptophan) have been described (MacDonald and Slater, 1966). Dimethyl sulfoxide or solutions of ^{14}C-labeled compounds in it were sterilized by filtration and added to cold sterile medium (see footnote *a*, Table I). Five flasks were used for expt 1a and 1c; and three flasks for 1b, 1d, and 2 (Table I).

A. amstelodami PRL 1698 was grown in stationary culture for 15–17 days and echinulin was isolated (MacDonald and Slater, 1966). The echinulin was recrystallized once from ethanol to obtain the yield, and then several times more to obtain a product which showed no impurities by thin-layer chromatography (Nakashima and Slater, 1969).

Isotopic Compounds and ^{14}C Assay. Carbon-14 was assayed with a Nuclear-Chicago Mark 1 liquid scintillation counter. Bray's solution (1960) or a Triton X mixture (3 ml of Triton X-100 + 6 ml of toluene + 45 mg of butyl PBD) was used as the scintillant. Triton X-100 and butyl PBD were obtained from Beckman Instruments, Inc.

Cyclo-L-alanyl-L-tryptophyl-^{14}C and cyclo-L-alanyl-D-tryptophyl-^{14}C were synthesized (Nakashima and Slater, 1969) from methylene-^{14}C-labeled L- and D-tryptophan, respectively. Echinulin-^{14}C from expt 1b (Table I) was diluted with carrier, hydrogenated, and hydrolyzed with alkali

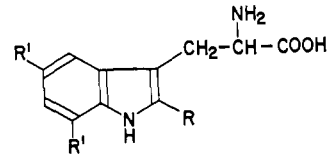

FIGURE 2: The structure of echinin. R and R' as in Figure 1a.

to yield hexahydroechinin (MacDonald and Slater, 1966) which was assayed for ^{14}C.

Isolation of Fungal Tryptophan and Cycloalanyltryptophyl Isomers from the Culture. Mycelium was extracted and hydrolyzed with alkali and tryptophan was isolated from the hydrolysate (MacDonald and Slater, 1966). The tryptophan was recrystallized several times from ethanol–water without addition of carrier, and then was assayed for ^{14}C. The presence of unutilized tryptophan in the culture filtrate from expt 2 (Table I) was revealed by paper chromatography (MacDonald and Slater, 1966).

In the reisolation and purification of cyclo-L-alanyltryptophyl isomers from culture filtrates, the fractions or extracts which contained the most ^{14}C were assumed to contain most of the isomers, and other fractions or extracts were discarded. This method of following the effect of purification steps was necessary because impurities sometimes markedly changed the solubility properties of the isomers. Evaporations were carried out at reduced pressure with a rotary evaporator, or under a stream of nitrogen on a warm water bath, and were followed by drying at 20 Torr with concentrated sulfuric acid as the desiccant. Sublimations were done with an apparatus similar to that of Gettler *et al.* (1950). The apparatus was used in a horizontal position without a cellulose insert, and the joint was lubricated with high-vacuum silicone grease.

For each experiment worked up, the culture filtrate and water washings of the mycelium were combined, and most of this solution was extracted with a Kutscher–Steudal (1903) apparatus continuously with 1-butanol for 2 days. The butanol extract was cooled overnight at 4°, filtered, and evaporated to give a syrup, which was dissolved in ethanol. The ethanol solution was chromatographed on an aluminum oxide column (30 cm high, 2.4-cm diameter, packed in ethanol) with ethanol as the eluent. Portions of the eluate which contained ^{14}C were combined and evaporated to yield an oily solid. The solid was washed three times with 5 ml of chloroform and then 3 times with 2 ml of water at 0°. The washed solid from expt 1c (Table I) contained more ^{14}C than did the washings; the chloroform washings from expt 1a and 1b contained more ^{14}C than did the water washings or washed solid.

The washed solid from expt 1c was dried and sublimed at 200° and 0.03 Torr overnight. The sublimate was identified as cycloalanyltryptophyl by its ultraviolet spectrum in ethanol, and it was assayed for ^{14}C.

The chloroform washings from expt 1a and 1b were evaporated to yield an oil which was partially distilled with the sublimation apparatus for 5 hr at 110° and 0.03 Torr. The distillate was discarded and the undistilled residue was washed 3 times with 2 ml of chloroform, then dissolved in hot ethanol, and filtered. The ethanol solution was evaporated to yield a solid which was sublimed at 200° and 0.03

TABLE I: Incorporation of Cyclo-L-alanyltryptophyl-methylene-^{14}C Isomers into Echinulin.[a]

Expt	Compounds Added, mg/flask	% Derivation[b] from ^{14}C Compounds of: Echinulin	Mycelial Tryptophan	Reisolated Cycloalanyltryptophyl
1a	Cyclo-L-alanyl-L-tryptophyl-^{14}C, 28.8	9.9		88
1b	Cyclo-L-alanyl-L-tryptophyl-^{14}C, 57.6	16.2	1.2	92
1c	Cyclo-L-alanyl-D-tryptophyl-^{14}C, 28.8	0.12		101
1d	Cyclo L-alanyl-D-tryptophyl-^{14}C, 57.6	0.20	0.9	
2	Cyclo L-alanyl-L-tryptophyl-^{14}C, 57.6, and L-tryptophan, 210	15.5	0.2	

[a] Cyclo L-alanyl-L-tryptophyl-^{14}C (1.02 mCi/mole) or cyclo-L-alanyl-D-tryptophyl-^{14}C (0.581 mCi/mole) at concentrations of 115.2 mg/ml of dimethyl sulfoxide were added to each 500-ml erlenmeyer flask (containing 50 ml of sugar beet molasses medium) prior to incoulation with *Aspergillus amstelodami*. [b] % Derivation (or incorporation) = 100 (mCi/mole in isolated compound)/(mCi/mole in ^{14}C compound added).

Torr overnight. The sublimate was further purified by washing once with 0.7 ml of chloroform and twice with 0.7 ml of ethanol. The undissolved portion was identified as cycloalanyltryptophyl by its ultraviolet spectrum, and was dried and assayed for ^{14}C.

Thin-layer chromatography (Nakashima and Slater, 1969) was used to distinguish between *cis* isomers (cyclo-L-alanyl-L-tryptophyl and cyclo-D-alanyl-D-tryptophyl) and *trans* isomers (cyclo-L-alanyl-D-tryptophyl and cyclo-D-alanyl-L-tryptophyl). Detection of these compounds was easier when Merck silica gel GF 254 (containing fluorescent indicator) was used. The compounds then showed up as dark spots on a light background when illuminated by short-wavelength ultraviolet light.

Results

Use of Dimethyl Sulfoxide. To completely dissolve the cyclo-L-alanyltryptophyl-^{14}C isomers in medium and prevent their possible decomposition by heating with medium, the isomers were dissolved in warm (60°) dimethyl sulfoxide and added to cold medium prior to inoculation with *A. amstelodami*. Dimethyl sulfoxide was chosen because the isomers were more soluble in it than in other polar solvents such as water, acetone, or ethanol. The molasses medium was too dark to determine visually whether the isomers remained in solution or precipitated, but the following solubility test indicated that the isomers remained mostly in solution. Labeled cyclo-L-alanyl-L-tryptophyl or cyclo-L-alanyl-D-tryptophyl was added to medium as described for expt 1b or 1d (Table I) except that sucrose was used in place of sugar beet molasses. During 17-days incubation of these uninoculated solutions, less than 0.5 mg of these isomers precipitated.

For comparative experiments, the amount of isomer that could be added was limited. Cyclo-L-alanyl-D-tryptophyl has limited solubility in dimethyl sulfoxide and the solution used (Table I) was almost saturated; also, dimethyl sulfoxide can inhibit echinulin production. Addition of 1 or 2 ml of dimethyl sulfoxide to 50 ml of medium slowed the growth of the organism, which nevertheless formed a mycelial mat of normal appearance, and completely inhibited the production of echinulin. With medium containing less dimethyl sulfoxide (0.5 or 0.25 ml/50 ml), echinulin yields (86–100 mg/flask) were similar to those obtained with medium alone (114–125 mg/flask), with the exception of expt 1d of Table I (47 mg/flask). The yields of echinulin in the experiments of Table I were, however, all adequate for ^{14}C studies.

Incorporation of Cyclo-L-alanyltryptophyl-^{14}C Isomers. It is unlikely that the ^{14}C content of echinulin (Table I), recrystallized several times from ethanol, resulted from contamination by cyclo-L-alanyltryptophyl-^{14}C isomers, because this recrystallization would remove these isomers. For example, when 500 mg of echinulin plus 50 mg of either isomer was recrystallized once from ethanol, the recrystallized echinulin contained no amount of isomer detectable by thin-layer chromatography (less than 0.5%).

The echinulin isolated from the experiments of Table I had optical rotatory dispersion curves (Nakashima and Slater, 1969) the same as that of authentic echinulin; the presence of the cyclic dipeptides therefore did not change the configuration of echinulin.

Carbon-14 from cyclo-L-alanyl-D-tryptophyl-^{14}C was incorporated poorly into echinulin whereas that from cyclo-L-alanyl-L-tryptophyl-^{14}C was incorporated much better, even when 5 moles of unlabeled tryptophan was added per mole of cyclo-L-alanyl-L-tryptophyl-^{14}C (Table I).

The ^{14}C in echinulin derived from cyclo-L-alanyl-L-tryptophyl-^{14}C (expt 1b, Table I) was mostly (95%) in the echinin moiety of the molecule, so the precursor was incorporated into echinulin in a specific way without extensive randomization of ^{14}C.

Cycloalanyltryptophyl samples reisolated from culture medium (Table I) had ultraviolet spectra quantitatively the same as the pure isomer added to the medium. However, the ultraviolet spectra of all four isomers of this compound are the same. Each of the reisolated compounds was shown by thin-layer chromatography to contain both *cis* and *trans* isomers. The isomer present in the larger amount was the same (*cis* or *trans*) as that originally added to the medium.

In all cases, optical rotatory dispersion measurements showed that the rotation of the reisolated product was in the same direction as that of the compound added to the medium but was less intense.

An experiment in which cyclo-L-alanyl-L-tryptophyl (a *cis* isomer) was added to uninoculated medium and then reisolated by the same procedure used in expt 1a and 1b showed that only *cis* isomer (perhaps containing a trace of *trans*) was present at the stage of purification before sublimation, but both *cis* and *trans* isomers were present in the sublimate. Also, in the attempted reisolation of cyclo-L-alanyl-D-tryptophyl-^{14}C (a *trans* isomer) from the culture filtrate of expt 1c (Table I) only *trans* isomer was present prior to sublimation, but both *cis* and *trans* isomers were present in the sublimate. It seems most likely that the isomers present in the culture filtrate were the same as those originally added to the medium, and that the isolation procedure caused partial racemization of these compounds. In any case, at least some of the isomer added was still present in the culture at the time of harvesting.

The specific activities of the cycloalanyltryptophyl samples reisolated from the filtrate were not strikingly different from that of the isomer originally added to the medium (Table I). This indicates that there is either no equilibration with endogenously synthesized cycloalanyltryptophyl (expt 1c) or very little equilibration (expt 1a and 1b).

Discussion

Echinulin might be synthesized by *A. amstelodami* from the precursors tryptophan, alanine, and mevalonic acid by two different ways. First, echinin (Figure 2) might be synthesized from tryptophan and mevalonic acid and cyclized with alanine to form echinulin. Second, cycloalanyltryptophyl might be synthesized from alanine and tryptophan and then substituted with side chains derived from mevalonic acid to form echinulin. Our work provides evidence for the second pathway, and we think it unlikely that both pathways of biosynthesis occur.

The incorporation of ^{14}C from cyclo-L-alanyltryptophyl-^{14}C isomers into echinulin shows an intermediate–product relationship if the incorporation is good and represents incorporation of the whole molecule. For cyclo-L-alanyl-D-tryptophyl-^{14}C incorporation was poor and was less than that found in mycelial tryptophan, so this isomer is unlikely to be a direct precursor of echinulin. Poor incorporation was not due to rapid metabolism and exhaustion of the isomer in the culture prior to synthesis of echinulin; at least some of the isomer was present in the culture filtrate. Incorporation of cyclo-L-alanyl-L-tryptophyl-^{14}C into echinulin was good, when one considers that the medium was rich in alternative carbon sources for growth and biosynthesis, and that the amount of radioactive isomer added was relatively low and was not completely utilized by the organism.

Two experimental results indicate that cyclo-L-alanyl-L-tryptophyl-^{14}C was incorporated into echinulin without extensive prior hydrolysis to tryptophan-^{14}C. First, the cyclic dipeptide was incorporated into echinulin much better than into mycelial tryptophan. L-tryptophan-^{14}C is incorporated differently—incorporation of radioactivity into mycelial tryptophan was found to be twice the incorporation into echinulin (MacDonald and Slater, 1966). Second, the addition of 5 moles of unlabeled tryptophan per mole of ^{14}C cyclic dipeptide decreased the incorporation of ^{14}C into echinulin by only 5%, but caused a sixfold decrease of incorporation of ^{14}C into mycelial tryptophan. Although the presence of ^{14}C in mycelial tryptophan suggests that some hydrolysis of the cyclic dipeptide to tryptophan takes place, the above results indicate that the major amount of ^{14}C entering echinulin represents incorporation of intact molecules of cyclo-L-alanyl-L-tryptophyl-^{14}C.

Various antibiotics and toxins (mycelianamide, sporidesmin, and gliotoxin) have structures which suggest they may be biosynthesized from cyclic dipeptides. Our experiments show that such a pathway of biosynthesis is feasible.

Acknowledgments

We thank Mr. J. Dyck for ^{14}C determinations, and Mr. L. R. Hogge and Mr. D. M. Tenaschuk for technical assistance.

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