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Biosynthesis of Echinulin. Isoprenylation of *cyclo*-L-Alanyl-L-tryptophanyl†

Charles M. Allen, Jr.

ABSTRACT: Crude ammonium sulfate fractions of a cell-free extract from *Aspergillus amstelodami* will catalyze the transfer of the 3,3-dimethylallyl moiety from 3,3-dimethylallyl pyrophosphate to *cyclo*-L-alanyl-L-tryptophanyl, forming a possible precursor of echinulin. The reaction product was formed using either a combination of [1-^{3}H]3,3-dimethylallyl pyrophosphate and unlabeled *cyclo*-L-alanyl-L-tryptophanyl or *cyclo*-[^{14}C]-L-alanyl-L-tryptophanyl (labeled on the methylene carbon of the tryptophanyl moiety) and unlabeled 3,3-dimethylallyl pyrophosphate as substrates. Tryptophan, alanyltryptophan, and tryptophanylalanine were not substrates. The chromatographic mobility and chemical reactivity of the enzymic product indicated that it was not echinulin. However, double-radioisotope experiments and ultraviolet and mass spectral analyses indicated that the product was a derivative of *cyclo*-L-alanyl-L-tryptophanyl with a single isoprene substitution on the tryptophanyl moiety. Possible structures of the reaction product are discussed.

A study of the biosynthesis of echinulin (I), a triisoprenylated cyclic dipeptide from *Aspergillus amstelodami*, permits the investigation of a number of unusual biochemical reactions. Although many large cyclic polypeptides have been described, little is known about the formation of cyclic dipeptides. In addition, the alkylation of a tryptophanyl moiety is unusual, particularly the substitution of the 1,1-dimethylallyl grouping at position 2.

Earlier studies (Birch *et al.*, 1961; Birch and Farrar, 1963) have clearly established that the biosynthetic precursors of echinulin are tryptophan, alanine, and mevalonic acid. It has been further established that the L isomer of tryptophan (MacDonald and Slater, 1966) and *cyclo*-L-alanyl-L-tryptophanyl (II)[1] (Slater *et al.*, 1970) are *in vivo* precursors of echinulin. However, no cell-free extracts which are active in catalyzing the incorporation of these precursors into echinulin or any intermediate in its biosynthesis have been described. It is likely that 3,3-dimethylallyl pyrophosphate, a direct prod-

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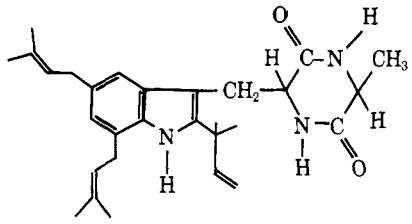
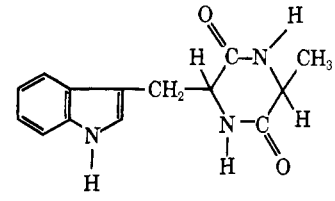
[1] We have chosen to use the name *cyclo*-L-alanyl-L-tryptophanyl for the diketopiperazine derivative of L-tryptophan and L-alanine instead of *cyclo*-L-alanyl-L-tryptophyl used previously (Slater *et al.*, 1970). The names 3,3-dimethylallyl pyrophosphate and isopentenyl pyrophosphate will be used for 3-methyl 2-butenyl-1-pyrophosphate and 3-methyl 3-butenyl-1-pyrophosphate, respectively.



I



II

uct of mevalonic acid metabolism in many systems, is the substrate in the alkylation of the tryptophanyl moiety. A study of the ability of cell-free extracts of *A. amstelodami* to catalyze the incorporation of the dimethylallyl moiety from 3,3-dimethylallyl pyrophosphate into a possible intermediate in echinulin biosynthesis is reported here.

Experimental Section

Materials. Silica gel for column chromatography (25–200 mesh) and silica gel N-HR precoated plastic tlc sheets with and without fluorescent indicator were purchased from the Brinkmann Instrument Co. Adsorbosil-1 was purchased from the Applied Science Laboratories. L-Tryptophan, carbobenzoxychloride, and dicyclohexylcarbodiimide were purchased from the Mann Chemical Co. The *p*-toluenesulfonate salt of L-alanine benzyl ester was purchased from the Nutritional Biochemicals Corp. L-Tryptophanyl-L-alanine was purchased from the Sigma Chemical Co. Tritiated $LiAlH_4$ (70 mCi/mmole) and L-[3-^{14}C]tryptophan (29 mCi/mmole) were purchased from New England Nuclear Corp. 3-Methyl-2-butenoic acid purchased from the Aldrich Chemical Co. was recrystallized and dried over KOH before use. All other reagents were reagent grade and used without further purification unless otherwise indicated. [1-^{14}C]Isopentenyl pyrophosphate (1 μCi/μmole) was a gift of Dr. Konrad Bloch.

[1-^{3}H]3,3-Dimethylallyl pyrophosphate was prepared by phosphorylation of [1-^{3}H]3-methyl-2-buten-1-ol. The tritiated alcohol was prepared by reduction of 2 g of 3-methyl-2-butenoic acid with 0.748 g of tritiated $LiALH_4$ (25 mCi, 1.27 mCi/mmole) according to the general methods described for 3-methyl-3-buten-1-ol synthesis (Yuan and Bloch, 1959) and phosphorylated by the general method previously described (Cornforth and Popjak, 1969). 3,3-Dimethylallyl pyrophosphate was isolated by chromatography on a silica gel column eluting the phosphorylated products with 1-propanol–concentrated NH_4OH–1% aqueous EDTA (6:3:1, v/v).

cyclo-L-Alanyl-L-tryptophanyl was prepared from the methyl ester of L-tryptophanyl-L-alanine. L-Tryptophanyl-L-alanine (500 mg) dissolved in 50 ml of methanol was added dropwise to 450 ml of a stirred ethereal solution of diazomethane at 0°. After stirring for 6 hr, dry nitrogen was passed through the solution for 2 more hr and the solvent then removed by flash evaporation. The residue was dissolved in 100 ml methanol and ammonia gas passed through the solution for 1.5 hr. The flask was then stoppered and allowed to stand 5 hr at room temperature. The degree of cyclization was monitored after thin-layer chromatography of the reaction product on silica gel N using isopropyl alcohol–concentrated NH_4OH–water (200:10:20, v/v) as solvent. The disappearance of the unreacted methyl ester was measured by the ninhydrin reaction. At the end of the reaction, nitrogen was passed through the solution and then the solvent removed by flash evaporation. The product was recrystallized from ethanol–ethyl acetate (1:1, v/v) by cooling to −20°, mp 280–282°, lit. (Nakashima and Slater, 1969) mp 282–284°.

Carbobenzoxy-L-[3-^{14}C]tryptophan was prepared by a modification of previous methods (Greenstein and Winitz, 1961). Carbobenzoxychloride (0.85 g) was added dropwise over 1 hr to a stirred solution of 1 g of L-tryptophan and 0.7 mg of L-[3-^{14}C]tryptophan (100 μCi) in 5% $NaHCO_3$ (20 ml) at room temperature. Unreacted carbobenzoxy chloride was removed by extraction of the reaction mixture with 100 ml of diethyl ether. Three back-extractions of this ether layer with fresh 5% $NaHCO_3$ were necessary to avoid undue loss of carbobenzoxytryptophan, which was finally precipitated from the pooled bicarbonate solution by acidification with 6 N HCl to pH 2.5. The acidified mixture was then extracted three times with 50 ml of ethyl acetate and the pooled extracts dried overnight with anhydrous $MgSO_4$. The product moved as a single radioactive spot (R_F 0.59) on silica gel tlc in benzene–butanol (40:10, v/v). To avoid undue losses of product, crystallization was not carried out. The ethyl acetate was removed from the dry product by flash evaporation and the resulting residue dissolved in dichloromethane (10 ml).

Carbobenzoxy-L-[^{14}C]tryptophanyl-L-alanine benzyl ester, L-[^{14}C]tryptophanyl-L-alanine, and *cyclo*-[^{14}C]-L-alanyl-L-tryptophanyl (all labeled in the methylene carbon of the tryptophanyl moiety) were prepared by minor modifications of earlier methods (Nakashima and Slater, 1969). The *p*-toluenesulfonate salt of L-alanine benzyl ester (1 g), dicyclohexylcarbodiimide (0.7 g), and redistilled triethylamine (0.5 ml) were added to the solution of carbobenzoxy-L-[3-^{14}C]tryptophan in dichloromethane and stirred at room temperature for 4 hr. Carbobenzoxy-L-[^{14}C]tryptophanyl-L-alanine benzyl ester was isolated (see Nakashima and Slater, 1969), but not crystallized. A solution of this ester in 100 ml of methanol containing 0.2 g of palladium black was treated with 2 atm of hydrogen by continuous shaking for 2 hr in a Parr hydrogenator. The reaction mixture was filtered and evaporated to dryness under reduced pressure to give the L-[^{14}C]tryptophanyl-L-alanine. The methyl ester of L-[^{14}C]tryptophanyl-L-alanine and radioactive cyclic dipeptide were synthesized in a manner similar to that described above for the unlabeled cyclic dipeptide. Chromatography of the radioactive product in chloroform–methanol–acetic acid (14:2:1, v/v) indicated a major product with R_F 0.63 and a minor contaminant (10%) with R_F 0.80. The minor component is probably an optical isomer resulting from racemization of the product during ring closure, although racemization was not observed in the earlier work (Nakashima and Slater, 1969).

Culturing and Enzyme Isolation. A. amstelodami (ATCC 10065) was grown at 30° by surface culture in 2-l. Fernbach flasks on 250 ml of Czapek-Dox broth supplemented with 270 g of sucrose/l. of added water. The fungus was grown for about 7 days, then the mat harvested by filtration and washed with distilled water. The wet mat, suspended in four volumes of buffer containing 10 mM each Tris (pH 7.0), $MgCl_2$, and mercaptoethanol, was sonicated for 4 min while cooled in an ice–water slurry. Subsequent steps were carried out at 0–4°. The sonicates were centrifuged at 8500*g* for 10 min. The enzyme was then isolated from the supernatant by precipitation

TABLE I: Substrate Specificity for Product Formation at 37°.

Substrate[a]		Radioactivity in Product[d] (dpm)
A[b]	B[c]	
CAT	[1-^{3}H]DMAPP	6250
L-Tryptophanyl-L-alanine	[1-^{3}H]DMAPP	57
L-Alanyl-L-tryptophan	[1-^{3}H]DMAPP	78
L-Tryptophan	[1-^{3}H]DMAPP	39
L-Alanine	[1-^{3}H]DMAPP	41
None	[1-^{3}H]DMAPP	58
[^{14}C]CAT	DMAPP	1202
[^{14}C]CAT	None	11
CAT	[1-^{14}C]IPP	75
None	[1-^{14}C]IPP	105

[a] Abbreviations used are CAT, *cyclo*-L-alanyl-L-tryptophanyl; DMAPP, 3,3-dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate. [b] Concentrations of unlabeled and labeled CAT were 0.97 and 0.49 mM (1.88×10^4 dpm), respectively. The concentration of all other amino acid derivatives was 1.0 mM. [c] Concentrations of unlabeled and labeled DMAPP were 3.1 and 0.039 mM (4.9×10^4 dpm), respectively. The concentration of [^{14}C]IPP was 14.8 μM (3.3×10^4 dpm). [d] In all reaction mixtures containing [^{3}H]-DMAPP and [^{14}C]IPP, enzyme in a AS_{40-70} fraction was used and product was isolated after 30-min incubation. In the reaction mixtures containing [^{14}C]CAT, enzyme from a AS_{50-70} fraction was used and product was isolated after 60-min incubation.

with ammonium sulfate. The most active fraction was precipitated between 40 and 70% (AS_{40-70}) saturation, although in some of the experiments cited here, enzyme precipitating between 0 and 50% (AS_{0-50}) saturation was used.

Assays. Two radiochemical methods were used for assaying the alkylating enzyme. The usual assay was carried out in a solution containing 100 mM Tris (pH 7.0), 10 mM $MgCl_2$, variable amounts of unlabeled *cyclo*-L-alanyl-L-tryptophanyl, and [1-^{3}H]3,3-dimethylallyl pyrophosphate (0.57 mCi/mmole) in a total volume of 1 ml. The enzyme was incubated with this mixture for 30 min at 37° (except as noted) and the reaction terminated by extraction with a 6-ml mixture containing 1 ml of ethanol and 5 ml of diethyl ether. The extraction step was repeated and the organic layers combined and washed with 3 ml of water. An aliquot from the organic layer was then pipetted into a scintillation vial, solvent removed by evaporation, and the sample counted in a scintillation counter. Cyclic dipeptide-dependent incorporation of radioactivity into the organic extract was taken as evidence for enzyme catalyzed alkylation.

In the second assay, *cyclo*-[^{14}C]-L-alanyl-L-tryptophanyl (0.0174 mCi/mmole) and unlabeled 3,3-dimethylallyl pyrophosphate were incubated with enzyme and the reaction mixture extracted as indicated above. The washed organic layer was then evaporated to dryness with a nitrogen stream and the residue dissolved in 5–8 drops of ethanol. This solution was applied to silica gel precoated tlc plastic sheets and the sheets developed with benzene–butanol (80:20, v/v). The areas corresponding to the enzymic product were cut out and counted in scintillation fluid. The scintillation fluid used in all these studies was a solution of 4 g of Omniflur (New England Nuclear) in 1 l. of a solution containing 750 ml of dioxane, 125 ml of anisole, and 125 ml of diethylene glycol dimethyl ether.

A similar procedure was carried out in the double-isotope experiments. The presence of the silica gel coated plastic sheets in the counting vials did not alter either the counting efficiency or isotope ratios in these experiments.

Isolation of Enzymic Product. Sufficient enzymic product to permit the determination of the ultraviolet and mass spectra was accumulated by pooling the organic extracts of many reaction mixtures containing 0.58 mM *cyclo*-L-alanyl-L-tryptophanyl and [1-^{3}H]3,3-dimethylallyl pyrophosphate (4.9×10^4 dpm, 0.39 mM) incubated with enzyme under the conditions of the general assay. The organic extracts were then concentrated and subjected to tlc on ethanol-washed Adsorbosil-1 in benzene–butanol (80:20, v/v) to separate starting cyclic dipeptide from the reaction product. That portion of the chromatogram which corresponded to the radioactive enzymic product was extracted with two 5-ml portions of ethanol. The ethanol was removed from the sample by a stream of nitrogen and the residue was extracted repeatedly with several aliquots of petroleum ether. The petroleum ether (bp 30–60°) washed residue was then either dissolved in ethanol for the uv spectral studies or transferred to a sample holder for injection into the mass spectrometer.

Detection of radioactive products on tlc sheets or plates was carried out with the use of a Packard Model 7201 radiochromatogram scanner. Ultraviolet spectra were determined with a Cary 14 spectrophotometer. Mass spectra were carried out on a Hitachi; Perkin-Elmer mass spectrometer (Model RMU 6-E).

Results

Reaction Definition. Incubation of *cyclo*-L-alanyl-L-tryptophanyl and 3,3-dimethylallyl pyrophosphate with crude ammonium sulfate fractions from *A. amstelodami* resulted in the formation of a product whose appearance was dependent on the addition of both substrates (Table I). This reaction can be measured by using either a combination of [1-^{3}H]3,3-dimethylallyl pyrophosphate and unlabeled *cyclo*-L-alanyl-L-tryptophanyl, or *cyclo*-[^{14}C]-L-alanyl-L-tryptophanyl and unlabeled 3,3-dimethylallyl pyrophosphate as substrates. When both [1-^{3}H]3,3-dimethylallyl pyrophosphate and cyclic dipeptide are incubated with boiled enzyme or without enzyme, no radioactive product is observed. The open chain dipeptides L-tryptophanyl-L alanine and L-alanyl-L-tryptophan, as well as L-tryptophan and L-alanine in the presence of [1-^{3}H]3,3-dimethylallyl pyrophosphate, were not substrates for this reaction. Furthermore, this crude extract was not capable of using the isomeric isoprene pyrophosphate, isopentenyl pyrophosphate, as a substrate.

The dependence of product formation on increase in time, *cyclo*-L-alanyl-L-tryptophanyl, and protein concentration were measured using unlabeled cyclic dipeptide and [1-^{3}H]3,3-dimethylallyl pyrophosphate as substrates. The results of these experiments are illustrated in Figures 1–3. In addition, the time-dependent formation of product was also measured using ^{14}C-cyclic dipeptide and unlabeled 3,3-dimethylallyl pyrophosphate as substrates (Figure 1).

Product Characterization. The product of the enzymic reaction chromatographed in four different solvent systems (benzene–butanol, 90:10, 80:20, 60:40, v/v, and butanol–acetic acid–water, 4:1:5, v/v) with R_F's of 0.17, 0.45, 0.74, and 0.83, respectively. In each case, its mobility was intermediate be-

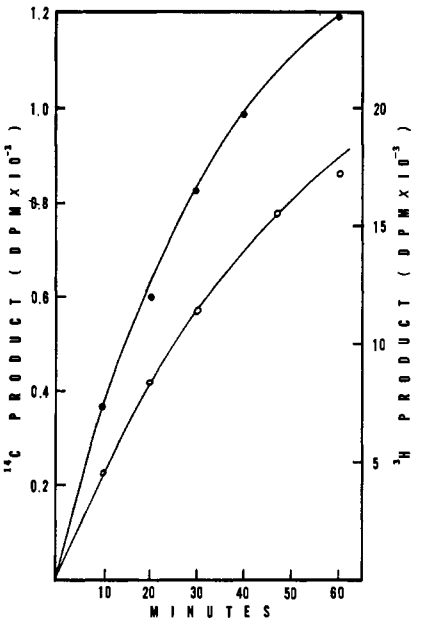


FIGURE 1: Dependence of product formation on time. Product formation was measured at 37° as described above using either the substrates [1-^{3}H]3,3-dimethylallyl pyrophosphate (1.82×10^5 dpm, 0.14 mM) and unlabeled *cyclo*-L-alanyl-L-tryptophanyl (1.1 mM) with 1.6 mg of enzyme from AS_{0-50} fraction, open circles; or with *cyclo*-[^{14}C]-L-alanyl-L-tryptophanyl (0.49 mM, 1.88×10^4 dpm) and unlabeled 3,3-dimethylallyl pyrophosphate (3.1 mM) with 3.6 mg of enzyme from a AS_{50-70} fraction, closed circles.

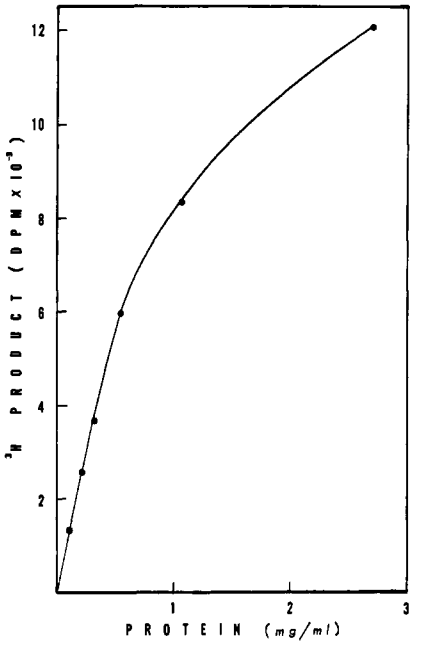


FIGURE 3: Dependence of product formation on protein concentration. Product formation was measured after 30-min incubation at 37° using the substrates, [1-^{3}H]3,3-dimethylallyl pyrophosphate (4.9×10^4 dpm, 0.039 mM) and unlabeled *cyclo*-L-alanyl-L-tryptophanyl (0.97 mM) as described in the Experimental Section. The enzyme used was the AS_{40-70} fraction prepared as described above.

tween that of *cyclo*-L-alanyl-L-tryptophanyl and the fully isoprenylated product, echinulin. Thus far, no echinulin has been identified as a product of these *in vitro* reactions. The use of either combination of labeled and unlabeled substrates always resulted in the formation of chromatographically identical enzymic products (Figure 4, IA and IB). This radioactive product gave a positive reaction with a chlorinating agent (Easley, 1965), sensitive to the amide bonds, and a slow, weak reaction with Ehrlich's reagent (blue color), whereas the substrate *cyclo*-L-alanyl-L-tryptophanyl gave fast, strong reactions with both reagents, with greater sensitivity toward the Ehrlich's reagent (purple color). The fungal product, echinulin was reactive with the chlorinating agent but not with Ehrlich's reagent.

In a control carried out under the same experimental conditions, *cyclo*-[^{14}C]-L-alanyl-L-tryptophanyl was incubated with enzyme in the absence of 3,3-dimethylallyl pyrophosphate.

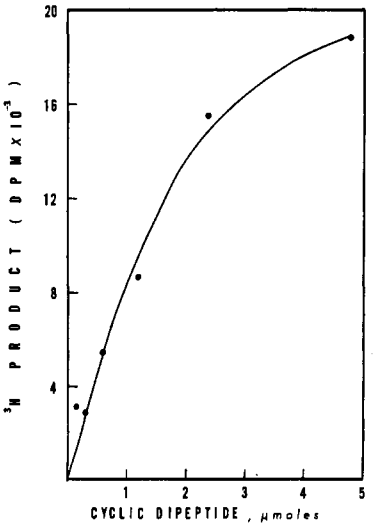


FIGURE 2: Dependence of product formation on cyclic dipeptide. Product formation was measured after 30-min incubation at 37° using [1-^{3}H]3,3-dimethylallyl pyrophosphate (1.54×10^5 dpm; 0.12 mM) with variable amounts of unlabeled *cyclo*-L-alanyl-L-tryptophanyl and 1.6 mg of enzyme from AS_{0-50} fraction as described in the Experimental Section.

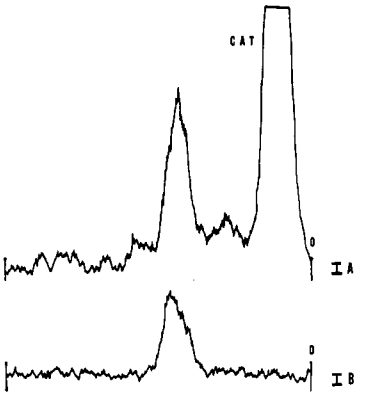


FIGURE 4: Radioscanning of chromatographed enzymic products. Chromatography was carried out on silica gel tlc plates in benzene–butanol (80:20, v/v). The chromatographic patterns represent the mobilities of the organic extracts obtained from enzymic reaction mixtures containing either 2.5×10^4 dpm (0.65 mM) of ^{14}C-cyclic dipeptide and 3.1 mM unlabeled 3,3-dimethylallyl pyrophosphate (IA) or 1.1 mM unlabeled cyclic dipeptide and 4.9×10^4 dpm, 0.039 mM [1-^{3}H]3,3-dimethylallyl pyrophosphate (IB) incubated at 37° for 30 min with 3.6 mg of AS_{50-70} fraction in the general assay procedure described above. CAT represents unreacted *cyclo*-[^{14}C]-L-alanyl-L-tryptophanyl.

TABLE II: Synthesis of Isoprenylated Product from *cyclo*-[^{14}C]-L-Alanyl-L-tryptophanyl and [1-^{3}H]3,3-Dimethylallyl Pyrophosphate.

	Substrate Added (μCi)		Radioactive Products[a]						
			^{3}H			^{14}C			
Expt	[^{3}H]-DMAPP, $\times 10^{2}$	[^{14}C]-CAT, $\times 10^{2}$	Dpm	μCi $\times 10^{+2}$	μmoles[b] $\times 10^{+2}$ (A)	Dpm	μCi[b] $\times 10^{+4}$	μmoles $\times 10^{+2}$ (B)	A:B
1	11.3	1.23	57,000	2.58	4.52	1585	7.14	4.10	1.10
2	5.6	1.23	32,500	1.47	2.58	934	4.21	2.42	1.07
3	11.3	2.46	66,600	3.00	5.26	1956	8.82	5.07	1.04
4	5.6	2.46	27,200	1.23	2.16	786	3.54	2.04	1.06
5		2.46				115			

[a] Each reaction mixture was 83 mM in Tris buffer (pH 7.0) containing a mixture of the tritium- and ^{14}C-labeled compounds (except expt 5) and 1.8 mg of enzyme in a 45–55% ammonium sulfate fraction in a total volume of 1.2 ml. This solution was incubated for 1 hr at 30°. The reaction products were extracted as in the normal assay procedure. The organic extract from each experiment was spotted on silica gel N for tlc and developed in benzene–butanol (80:20, v/v) to separate the enzymic product from ^{14}C-cyclic dipeptide (as in Figure 4, IA). The product was located by radioactive scanning, cut out, and counted. Experiment 5 was carried out in the same manner and that portion of the chromatogram corresponding to the known R_F of the reaction product was cut out for analysis. [b] The specific activities of the radioactive substrates used for these calculations are 0.57 μCi/μmole for [1-^{3}H]3,3-dimethylallyl pyrophosphate and 0.0174 μCi/μmole for the ^{14}C-cyclic dipeptide. These activities were determined from an analysis of the chemically synthesized products.

In this case, there was no radioactive product chromatographing like enzymic product and no material giving a positive test with the chlorinating agent having an R_F similar to the enzymic product. In these crude ammonium sulfate preparations, there was, however, an organic component present in the isolated protein, detectable on fluorescent tlc plates, that chromatographs nearly identical with the enzymic product. This material is not, however, reactive with either the Ehrlich's or chlorinating reagent.

Using a mixture of *cyclo*-[^{14}C]-L-alanyl-L-tryptophanyl and [1-^{3}H]3,3-dimethylallyl pyrophosphate, an attempt was made to estimate the number of isoprene units attached to the cyclic dipeptide. The result of these studies (Table II) indicated that the enzymic product contained about 1 μmole of the [^{3}H]dimethylallyl moiety per μmole of cyclic dipeptide.

The uv absorption spectra of echinulin, *cyclo*-L-alanyl-L-tryptophanyl, and the enzymic product are shown in Figure 5. The absorption maxima for the unsubstituted cyclic dipeptide are 289, 279, 273, and 220 nm. The triisoprenylated metabolite, echinulin, has a spectrum with a shoulder at 297 nm and max-

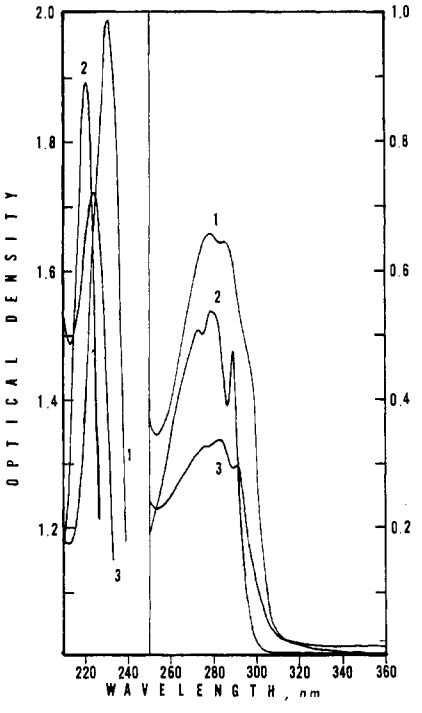


FIGURE 5: Ultraviolet spectra of various cyclic dipeptides. The spectra of echinulin (curve 1), *cyclo*-L-alanyl-L-tryptophanyl (curve 2) and enzymic product (curve 3) were obtained in ethanol. The optical density scale for wavelengths greater than 250 nm is 0–1.0 OD unit and below 250 nm 1–2.0 OD units. The concentration of compounds used for curves 1 and 2 was greater for that part of spectra above 250 nm than below 250 nm. For curve 3, the whole spectrum was obtained at one concentration of metabolite (47.6 μM, 90,430 dpm/1.5 ml) isolated as described in the Experimental Section.

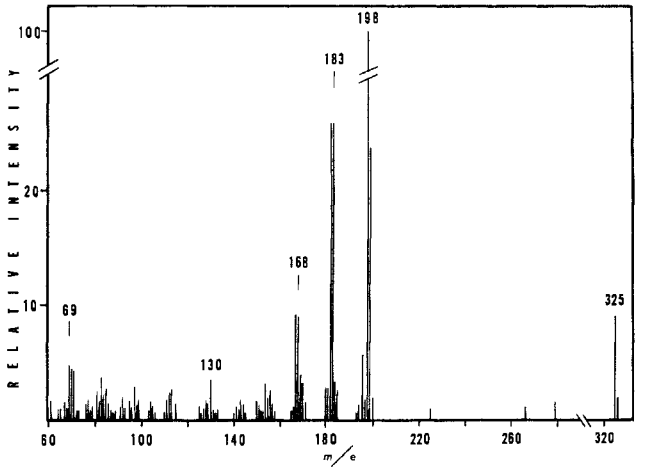
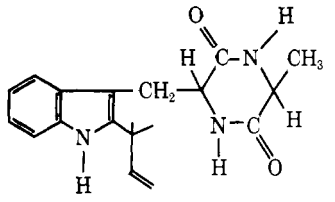


FIGURE 6: Mass spectrum of enzymic product. The enzymic product (about 0.15 μmole, 1.87×10^{5} dpm) was isolated as described in the Experimental Section. The intensities of the fragmentation products are given relative to the 198 peak.

ima at 285, 279, and 230 nm, indicating a general red shift due to alkylation. The spectrum of the enzymic product exhibits the general characteristics seen with most indole-containing compounds. It also shows a red shift compared to the unsubstituted cyclic depeptide, with maxima at 291, 283, and 224 nm, with a shoulder at 275 nm.

The mass spectrum of the enzymic product, Figure 6, exhibits a parent peak at *m/e* 325, which is consistent with a singly isoprenylated cyclic depeptide. The base peak at *m/e* 198, and other prominent peaks are listed in Table III with their relative intensities.

A comparison of this spectrum and its relative peak intensities to mass spectral data obtained on the chemically synthesized *cyclo*-L-alanyl-2(1,1-dimethylallyl)-L-tryptophanyl (III)[2] shows the same fragmentation pattern. A mass spectral



III

analysis of echinulin (I) showed, in addition to the parent peak at 461, a base peak at 334 ($M^+ - 127$) and prominent peaks at 266 ($M^+ - 195$) and 69 ($C_5H_9^+$), with other peaks at 278 ($M^+ - 183$), 319 ($M^+ - 142$), and 304 ($M^+ - 157$). An analysis of the spectrum of the unsubstituted cyclic dipeptide (II) gave in addition to the parent peak (257), the base peak at 130 ($M^+ - 127$) and prominent peaks at 103 ($M^+ - 154$) and 77 ($M^+ - 180$).

All four compounds give a base peak ($M^+ - 127$) representing the fragment ion resulting from the ready loss of the diketopiperazine moiety. Fragmentation in this manner is typical of alkylindoles (Budzikiewicz *et al.*, 1964). In addition, the enzymic product, *cyclo*-L-alanyl-2(1,1-dimethylallyl)-L-tryptophanyl, and echinulin all lose an isoprenoid unit (69), leaving a $M^+ - 195$ ($M^+ - 196 + H$) fragment. The spectrum of each of these compounds also shows the loss of one or two methyl groups, indicated by fragments at $M^+ - 142$ and $M^+ - 157$.

Discussion

The results of these experiments indicate that an isoprenylated cyclic dipeptide results from condensation of *cyclo*-L-alanyl-L-tryptophanyl and 3,3-dimethylallyl pyrophosphate. Product formation is a time, substrate, and protein dependent process and may be measured by an assay utilizing either of the two substrates labeled. This reaction is apparently not the result of isoprenylation of tryptophan, alanine, tryptophanylalanine, or alanyltryptophan. The isomer of 3,3-dimethylallyl pyrophosphate, isopentenyl pyrophosphate, is not a direct isoprene donor, and was not active, presumably because isopentenyl pyrophosphate: Δ^3,Δ^2-isomerase activity was low. Recently it has been shown that the isomerase is present in this fungus but is rather unstable and inhibited by ammonium sulfate (C. M. Allen, 1971, unpublished data).

The final enzymic product is clearly not echinulin; but from the results of the double radioisotopic labeling experiments and mass spectral data, it is apparent that 1 mole of isoprene unit is present per mole of cyclic dipeptide.

[2] The mass spectrum was kindly provided by J. E. Saxton.

TABLE III: Principal Peaks and Relative Intensities from Mass Spectrum of Enzymic Product.[a]

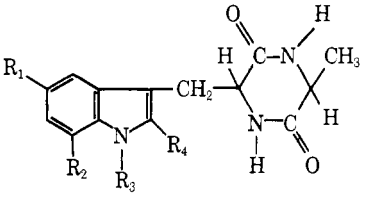
m/e	Peak Designation	Rel Intensity of Mass Peaks
325	M^+	9
198	$M^+ - 127$	100
183	$M^+ - 142$	26
168	$M^+ - 157$	9
154	$M^+ - 171$	3
143	$M^+ - 182$	2
130	$M^+ - 195$	4
69	$C_5H_9^+$	5

[a] See legend of Figure 6 and the Methods section for experimental details.

If the assumption is made that the enzymic product is on the pathway of echinulin biosynthesis, then there are at least seven plausible enzyme products (III–IX) which could result from single isoprene substitution.

The observed slow rate of reaction of Ehrlich's reagent with the enzymic product and the color of the reaction product suggests that the isoprene substitution probably is not located in positions 5 (IV) or 7 (V) of the indole nucleus. This conclusion is based on the assumptions that the rate of reaction of *p*-dimethylaminobenzaldehyde with IV and V should proceed as rapidly as the unsubstituted cyclic dipeptide and with a similarly colored product formed. Futhermore, mass spectral analysis indicates the loss of an entire isoprenoid unit during fragmentation, which is inconsistent with substitution at positions 5 or 7 since fragmentation with β cleavage is usually observed with alkyl substituents on aromatic systems.

The formation of N-substituted derivatives of the cyclic dipeptide such as VI and VII is possible. Compound VI has

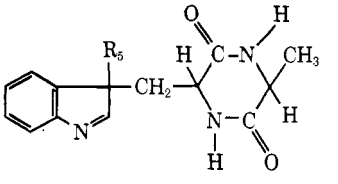


IV, R_2, R_3, R_4 = H; R_1 = $(CH_3)_2C{=}CH{-}CH_2$

V, R_1, R_3, R_4 = H; R_2 = $(CH_3)_2C{=}CH{-}CH_2$

VI, R_1, R_2, R_4 = H; R_3 = $(CH_3)_2C{=}CH{-}CH_2$

VII, R_1, R_2, R_4 = H; R_3 = $CH_2{=}CH{-}C(CH_3)_2$



VIII, R_5 = $(CH_3)_2C{=}CH{-}CH_2{-}$

IX, R_5 = $CH_2{=}CH{-}C(CH_3)_2$

been suggested as a precursor of echinulin, and its rearrangement to the 1,1-dimethylallyl derivative has been postulated by several workers (Jackson and Smith, 1965; Casnati and Pochini, 1970). Ehrlich's reagent might react with either VI or VII, although probably slowly because of steric inhibition due to the N substituent. A more important consideration here is the finding that N-substituted indoles usually do not show the small peak or inflection at 290 nm which is observed with indoles unsubstituted on nitrogen (Jackson and Smith, 1964, 1965). Since the purified enzymic product shows a distinct maximum at 291 nm, N substitution is not probable.

Although VIII has been considered a possible intermediate in echinulin biosynthesis (Jackson and Smith, 1965), results of model studies on its rearrangement to III make it an unlikely intermediate. The reported uv spectra of such indolenines, furthermore, would not be consistent with the spectrum reported here. Another indolenine, IX, has also been suggested (Bycroft and Landon, 1970) as a possible intermediate, but again on the basis of the uv spectral data, is not likely to be the enzymic product.

The present data are most consistent with the enzymic product being III. The LL isomer of III has been synthesized recently (Houghton and Saxton, 1969), and the ultraviolet spectrum reported indicates absorption maxima at 225, 283, and 291 nm. These correspond almost identically with maxima observed with the enzymic product.

The strongest evidence that the product is III comes, however, from a comparison of the mass spectral data for the two compounds. The same principal peaks, each with almost identical relative intensities, are seen for at least six of the major fragments from these compounds. It is clear from the mass spectra of the enzymic product, III, and echinulin, that the isoprenylated compounds undergo the expected major fragmentation with the loss of the diketopiperazine moiety to give a $M^+ - 127$ base peak. Fragmentations resulting in the loss of an isoprene unit or methyl groups in addition to the diketopiperazine group are seen with all three of these compounds.

The presence of mass fragments from the enzymic product containing both the indole and isoprenoid units ($M^+ - 127$), as well as the indole unit without the isoprenoid unit ($M^+ - 195$), strongly suggests that the isoprene unit is attached as a 1,1-dimethylallyl group to the indole nucleus. The fragmentation resulting in the loss of the methyl groups is also consistent with the presence of a 1,1-dimethylallyl side chain.

Studies on a cyclic dipeptide related to echinulin, neoechinulin, having a 1,1-dimethylallyl group at position 2 of the indole nucleus and a 3,3-dimethylallyl group at position 6 of the indole nucleus, also indicated loss of one isoprene and a methyl group as important peaks resulting from fragmentation in the mass spectrometer (Barbetta *et al.*, 1969).

Why the enzymic product gave a positive Ehrlich's reaction whereas echinulin gave no reaction is uncertain. One possible explanation which is being tested is that the enzymic product contains a minor contaminant which reacts with Ehrlich's reagent, but which is not separable from III by the analytical methods employed.

It is possible that the synthesized product is not a precursor of the dipeptide, echinulin, but of some other similar dipeptide. For example, neoechinulin, which has only two isoprene units, has been isolated and identified from *A. amstelodami* (Barbetta *et al.*, 1969) and is a possible end product. Also, studies in our laboratory (C. M. Allen, 1971, unpublished data) indicate that tryptophan, alanine, and mevalonic acid are metabolic precursors of a number of other as yet unidentified products in *A. amstelodami*.

Acknowledgments

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