Biosynthesis of Pulcherriminic Acid

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1. *Candida pulcherrima* was grown on a complex medium to which various compounds had been added to determine their effect on the biosynthesis of pulcherriminic acid. Most of the pulcherriminic acid synthesized by *C. pulcherrima* PRL2019 was derived from the L-[1-¹⁴C]leucine added to the medium. 2. The cyclic dipeptide of L-leucine (cyclo-L-leucyl-L-leucyl) was shown, by trapping experiments involving cycloleucyl-leucyl isomers, to be synthesized by strain PRL2019. Cyclo-L-leucyl-L-leucyl was derived from L-leucine and was converted into pulcherriminic acid. Cyclo-L-leucyl-L-leucyl was a precursor of pulcherriminic acid in strain PRL2007 also. 3. The results supported the hypothesis that pulcherriminic acid is derived from L-leucine and that cyclo-L-leucyl-L-leucyl is an intermediate in the biosynthesis.

Pulcherrimin is a red pigment synthesized by certain strains of *Candida pulcherrima* grown on media containing ferric salts (Kluyver, Van Der Walt & Van Triet, 1953). Cook & Slater (1954) showed that this pigment was the ferric salt of a dibasic acid which they named 'pulcherriminic acid'. Although the properties of the pulcherriminic acid isolated by MacDonald (1963) were not the same as those of pulcherriminic acid isolated by Cook & Slater (1954, 1956), the structure for pulcherriminic acid proposed by MacDonald (1963) was essentially the same as the structure proposed by Cook & Slater (1956). A compound of this structure (3,6-dihydroxy-2,5-di-isobutylpyrazine 1,4-dioxide) has been synthesized by Ohta (1964) and was shown to be identical with a sample of pulcherriminic acid supplied by MacDonald.

Miller (1961) suggested that pulcherriminic acid might be biosynthesized from leucine with the cyclic dipeptide of leucine as an intermediate (Scheme 1). The present work gives the first experimental support for this postulate.

MATERIALS AND METHODS

In the isolation procedures, degradations and syntheses described below, the procedures were modified for different quantities of materials by proportionately changing the amounts of solvents and reagents.

Organisms. The source of the two strains of *C. pulcherrima*, PRL2019 and PRL2007, and their maintenance on agar slants were as described by MacDonald (1963). The slants were conserved by storage at 10° under sterile mineral oil. *C. pulcherrima* PRL2019 lost its ability to produce high yields of pulcherrimin when it was transferred frequently, and it was found advisable to use slants only a few transfers removed from the slant supplied by Dr Cook.

Production of pulcherrimin and conversion into pulcherriminic acid. The liquid medium and the conditions of growth in shake cultures were as described by MacDonald (1963). Radioactive leucine was added to 100 ml. of medium before autoclaving. Cycloleucyl-leucyl isomers were autoclaved separately in 500 ml. Erlenmeyer flasks and 100 ml. of autoclaved medium was added aseptically to these flasks before inoculation. A loopful of cells from a slant was dispersed in 10 ml. of sterile water and 2 drops were used to inoculate each flask. The organism was grown for 4 days at 28° (strain PRL2019) or 25° (strain PRL2007) and pulcherrimin

Scheme 1.

isolated by a modification of the method of Cook & Slater (1954). Centrifugation was used wherever practicable in preference to filtration to separate solids (including yeast cells) from suspensions, and washings were done by suspending the solid in the liquid, centrifuging and removing the liquid. The yeast from two flasks was centrifuged off and washed once or twice with 25 ml. of methanol, and the washings were discarded. The yeast was then washed with 25 ml. of methanolic KOH [22.5 ml. of methanol plus 2.5 ml. of aq. 50% (w/w) KOH] and then with 25 ml. of methanol. Pulcherrimin was precipitated from these two washings as described by Cook & Slater (1954). The crude pulcherrimin was washed successively with water, ethanol and water, and then was converted into pure pulcherriminic acid by a modification of the procedure of MacDonald (1963). Two washings of 2 ml. of 2*N*-NaOH were used to convert the wet pulcherrimin, obtained from two culture flasks, into Fe(OH)₃ and the soluble sodium salt of pulcherriminic acid. After removal of the Fe(OH)₃, the solution was acidified with HCl and the crude pulcherriminic acid washed thoroughly with water and then dissolved by adding a few drops of 5*N*-NH₃. Then 3 vol. of morpholine was added to obtain the morpholinium salt, which was collected, purified and washed with ether (MacDonald, 1963). After removal of most of the ether by a stream of N₂, the salt was dissolved in a minimum amount of *N*-NH₃. The solution was acidified with HCl, and the pulcherriminic acid washed thoroughly with water and dried in a vacuum.

Isolation of cycloleucyl-leucyl isomers from cultures. After growth of the organism on liquid medium, the contents of two flasks were combined, shaken with 100 ml. of chloroform and centrifuged. The chloroform was drawn off and the extraction with chloroform repeated twice more. The extracted cultures were used for the isolation of pulcherriminic acid from the yeast as described above. The combined chloroform extracts were evaporated to dryness on a rotary evaporator; the solid residue was taken up in a minimum amount of chloroform and chromatographed on a column (25 cm. × 2.5 cm. diam.) of alumina (Shawinigan reagent grade; McArthur Chemical Co., Montreal, Canada), which had been packed as a slurry in chloroform. The column was eluted in sequence with approx. 500 ml. of each of the following solvents: chloroform, and then chloroform containing 1% (v/v) of ethanol, 2% (v/v) of ethanol and 3% (v/v) of ethanol. Fractions containing 20 ml. of eluent were collected and evaporated to dryness. Cyclo-D-leucyl-L-leucyl was eluted first from such columns, usually with chloroform or 1% ethanol in chloroform, and cyclo-D-leucyl-D-leucyl, cyclo-L-leucyl-L-leucyl, or a mixture of these, was eluted next. Before determination of optical rotation or radioactivity, these compounds were purified either by sublimation at 150–170° and 0.02 mm. Hg (no appreciable racemization occurred), or by crystallization from ethanol–water (1:1, v/v).

Detection and isolation of leucine. The presence of leucine in the medium was determined by circular chromatography on Whatman no. 4 filter paper, with the solvent system butan-2-ol–aq. 3% (w/v) NH₃ (3:1, v/v), followed by spraying with 0.2% (w/v) ninhydrin in acetone. If leucine appeared to be present, the result was checked by descending chromatography on Whatman no. 4 filter paper, with 2-methylbutan-2-ol saturated with water as the solvent. The latter procedure separated leucine and isoleucine, whereas the former did not.

In one experiment, the cells remaining after the extraction of pulcherrimin were further extracted with methanol overnight in a Soxhlet apparatus. The hydrolysis of the cells and isolation of leucine from the hydrolysate were done as described by MacDonald (1961) for the isolation of leucine from mycelium.

Degradation procedures for radioactive compounds. Pulcherriminic acid was reduced to cycloleucyl-leucyl, which in turn was hydrolysed to leucine (MacDonald, 1963). Leucine was decarboxylated with ninhydrin by using the macro-analysis conditions of Van Slyke, MacFadyen & Hamilton (1941), but the apparatus was modified so that N₂ bubbled through the heated reaction mixture, passed through a trap containing a calculated excess of 2,4-dinitrophenylhydrazine in 4*N*-HCl, and then through a trap containing Ba(OH)₂–BaCl₂ solution. The 2,4-dinitrophenylhydrazone and BaCO₃ were filtered off, washed with water, dried and used for the determination of radioactivity.

Source of compounds. L-[1-¹⁴C]Leucine was purchased from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., and D,L-[1-¹⁴C]leucine from the Commercial Products Division, Atomic Energy of Canada, Ottawa. The D-[1-¹⁴C]leucine was prepared by MacDonald (1961). The leucine content (presumably L-leucine) of the batch of yeast extract used had been determined by MacDonald (1962), and amounted to 0.091 m-mole of total leucine/flask, including acid-hydrolysable leucine in peptides, or 0.081 m-mole of free leucine/flask. The latter value was added to the m-moles of L-leucine added to give the values presented in Expts. 1 and 5 in Table 1.

Racemic cycloleucyl-leucyl was synthesized from 500 mg. of D,L-[1-¹⁴C]leucine by the method of Dunn, Newbold & Spring (1949), except that a reflux time of 9 hr. was used instead of 90 min. Cyclo-D-[1-¹⁴C]leucyl-L-[1-¹⁴C]leucyl (132 mg.) was isolated from the racemic cycloleucyl-leucyl by chromatography on alumina and crystallization, as described above.

Cyclo-L-[1-¹⁴C]leucyl-L-leucyl was synthesized from L-[1-¹⁴C]leucine by a method involving the cyclization of the methyl ester of leucine at 50° in the presence of a trace of acetic acid (Micetich & MacDonald, 1965). Cyclo-D-leucyl-D-leucyl was synthesized from D-leucine by the same method and further purified by chromatography on alumina as described above.

Other methods. Optical rotation of compounds was determined with an optical polarimeter (model 127; O. C. Rudolph and Sons, Caldwell, N.J., U.S.A.). The standard deviation was calculated from five or more sample readings. Since volumetric errors could lead to absolute errors of ±1%, the standard deviation is stated only where it exceeded 0.5%.

Proton-magnetic-resonance (p.m.r.) spectra were determined with a Varian A-60 instrument with trifluoroacetic acid as the solvent and tetramethylsilane as the internal standard. It was found advisable to run the spectra in the same batch of solvent and on the same day. Where different batches of solvent and widely different times of analysis were used, there was sometimes a difference in the absolute values of the NH peaks, although their positions relative to each other did not change.

For determination of radioactivity, BaCO₃ was converted into CO₂ with conc. H₂SO₄, and other compounds (1–10 mg.) were oxidized to CO₂ (Burr, 1954). Radioactivity was determined with a 250 ml. ionization chamber and vibrating-

Table 1. *Effect of various compounds on the biosynthesis of pulcherrimin or pulcherriminic acid by C. pulcherrima grown on a complex medium*

Strain PRL2019 was used in Expts. 1-5, and strain 2007 in Expt. 6. For the calculation of 'percentage derivation' see the Materials and Methods section. Ten flasks were used in Expt. 1, five in Expt. 2, four in Expt. 6, and two in other experiments.

Expt. no.	Compound added			Compounds isolated from culture			
	Compound	m-moles/flask	$\mu\text{C}/\text{m-mole}$	Compound	m-mole/flask	$\mu\text{C}/\text{m-mole}$	Percentage derivation from ^{14}C -labelled compound added
1	L-[^{14}C]Leucine	3.13	0.348	Pulcherriminic acid	0.130	0.587	84
2	D-[^{14}C]Leucine	0.76	0.652	Pulcherriminic acid	0.104	0.129	27
3	Cyclo-L-[^{14}C]leucyl-L-leucyl	0.110	1.99	Pulcherriminic acid; mycelial leucine	0.135	0.674	34
4a	None			Crude pulcherrimin	0.150	< 0.006	
4b	Cyclo-L-[^{14}C]leucyl-L-leucyl	0.221	3.52	Crude pulcherrimin; pulcherriminic acid;	0.239	1.44	
				cyclo-L-leucyl-L-leucyl	0.091	1.95	55
4c	Cyclo-D-[^{14}C]leucyl-L-[^{14}C]leucyl	0.221	4.87	Crude pulcherrimin; cyclo-D-leucyl-L-leucyl;	0.075	1.86	53
				cyclo-L-leucyl-L-leucyl;	0.061	< 0.002	< 0.04
4d	Cyclo-D-leucyl-D-leucyl	0.221	0	cyclo-L-leucyl-L-leucyl Crude pulcherrimin;	0.217	4.83	99
				cyclo-D-leucyl-D-leucyl + cyclo-L-leucyl-L-leucyl	0.022	< 0.02	< 0.4
				Crude pulcherrimin;	0.004		
5	L-[^{14}C]Leucine	3.10	0.381	Pulcherriminic acid;	0.246	0.718	94
	Cyclo-L-leucyl-L-leucyl	0.222	0	cyclo-L-leucyl-L-leucyl	0.065	0.616	81
6	Cyclo-L-[^{14}C]leucyl-L-leucyl	0.0661	3.52	Pulcherriminic acid; cyclo-L-leucyl-L-leucyl	0.192	1.68	48
					0.0207	1.55	44
					0.0488		

reed electrometer (model 6000 Dynacon electrometer; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.), and duplicate samples agreed within $\pm 5\%$, except where little radioactivity was present. Radioactive samples generally contained 2–54 μC , and the instrument background current corresponded to 0.09 μC . For samples containing less than 1 μC , the higher value of two samples was used in calculations, and the result expressed as less than the calculated value (see Table 1).

The percentage derivation (Table 1) of radioactive compounds isolated after the fermentation, from radioactive compounds added to the medium, was calculated by assuming the conversions: 2 mol. of leucine $\rightarrow$ 1 mol. of cycloleucyl-leucyl $\rightarrow$ 1 mol. of pulcherriminic acid or pulcherrimin (see Scheme 1). The percentage derivation was therefore calculated by the function $100(A/nB)$, where A was the specific activity ($\mu\text{C}/\text{m-mole}$) of the C_{12} compound isolated after the fermentation and B the specific activity of the compound added before the fermentation. Where B referred to leucine, $n=2$, and where B referred to cycloleucyl-leucyl, $n=1$.

RESULTS AND DISCUSSION

Incorporation of leucine isomers into pulcherriminic acid. Preliminary experiments showed that, when *C. pulcherrima* PRL 2019 was grown on the liquid medium, the small amount of free leucine in the yeast extract was used in the first day and that approx. 2.3 m-moles of L-leucine or 0.2 m-mole of D-leucine could be utilized/flask in 4 days. Little pigment was produced during the first day of fermentation, and the pulcherrimin was formed largely during the next 3 days. Since the objective of isotope experiments was to test leucine as a precursor for the biosynthesis of pulcherrimin or pulcherriminic acid, it was necessary that the medium contain leucine during the latter days of the fermentation. Accordingly, a sufficient amount of labelled leucine was used in Expts. 1 and 2 (Table 1) to provide an excess throughout the fermentation.

The results of Expt. 1 show that radioactivity was incorporated from L-[1- ^{14}C]leucine into pulcherriminic acid. This pulcherriminic acid, of specific activity 0.587 $\mu\text{C}/\text{m-mole}$, was degraded chemically to DL-leucine of specific activity 0.297 $\mu\text{C}/\text{m-mole}$. The latter value is close to that expected, for 1 mol. of pulcherriminic acid theoretically yields 2 mol. of leucine in this degradation. Of the radioactivity in the DL-leucine, 93% was recovered from the carboxyl group and no detectable amount was found in the rest of the molecule. This result indicates that the carbon skeleton of leucine was incorporated intact into pulcherriminic acid. The specific activity of the pulcherriminic acid was approx. 1.7 times that of the L-[1- ^{14}C]leucine originally present in the medium. This finding, together with the absence of randomization of labelled carbon, indicates that 2 mol. of L-leucine was used in the biosynthesis of 1 mol. of pulcherri-

minic acid. The small dilution of radioactivity may result from endogenous synthesis of leucine. Although L-[1- ^{14}C]leucine may be degraded to smaller fragments containing radioactivity, it is unlikely that such fragments constituted the major source of carbon for the biosynthesis of pulcherriminic acid. If such were the case one would expect extensive dilution of radioactivity by metabolites from other nutrients (glucose, yeast extract, lactate and citrate) in the medium, and that the pulcherriminic acid synthesized would be randomly labelled and of low specific activity.

The results of Expt. 2 in Table 1 show that radioactivity from D-[1- ^{14}C]leucine was incorporated into pulcherriminic acid. Chemical degradation of the latter gave leucine, 0.065 $\mu\text{C}/\text{m-mole}$, in which 92% of the activity was present in the carboxyl group and less than 2% in the rest of the molecule. Although this indicates that the carbon skeleton of D-leucine was used intact in the biosynthesis of pulcherriminic acid, D-leucine was a poorer precursor of pulcherriminic acid than was L-leucine (compare percentage derivations in Expts. 1 and 2 in Table 1). It seems possible that D-leucine was converted into L-leucine at some stage in the biosynthesis of pulcherriminic acid. This possibility is supported by further evidence presented below, which indicated that cyclo-L-leucyl-L-leucyl participated in the biosynthesis of pulcherriminic acid, whereas the cycloleucyl-leucyl isomers containing D-leucyl did not participate.

Incorporation of cyclo-L-[1- ^{14}C]leucyl-L-leucyl into pulcherriminic acid. The results of Expt. 3 in Table 1 show that radioactivity from cyclo-L-[1- ^{14}C]leucyl-L-leucyl was incorporated by *C. pulcherrima* PRL 2019 into pulcherriminic acid. The latter was degraded to give DL-leucine, 0.333 $\mu\text{C}/\text{m-mole}$, in which 99% of the radioactivity was found in the carboxyl group and none in the rest of the molecule. Very little radioactivity was found in the leucine obtained from the hydrolysate of mycelium, which indicates that the cyclic dipeptide was not hydrolysed to free leucine before its incorporation into pulcherriminic acid. The lack of randomization of labelled carbon in pulcherriminic acid, together with the evidence against extensive hydrolysis of cyclo-L-[1- ^{14}C]leucyl-L-leucyl, indicates that the latter compound was incorporated into pulcherriminic acid without prior breakdown into smaller fragments. Only a trace of cycloleucyl-leucyl was isolated from the medium at the end of the fermentation and the amount was just sufficient to determine its identity by infrared spectrum. The specific activity of the pulcherriminic acid was considerably lower than that of the cyclo-L-[1- ^{14}C]leucyl-L-leucyl added to the medium. Experiments described below indicate that this dilution of radioactivity may be due to endogenous synthesis of non-radioactive

cyclo-L-leucyl-L-leucyl, which could dilute the radioactive cyclic dipeptide during its conversion into pulcherriminic acid.

Identification of cycloleucyl-leucyl isomers. The cyclic dipeptide of leucine can be obtained in three isomeric forms (Beilstein, 1944). These isomers are cyclo-D-leucyl-L-leucyl, which is the *trans* form, and cyclo-D-leucyl-D-leucyl and cyclo-L-leucyl-L-leucyl, both of which are *cis* forms. It was found (see below) that the *cis* and *trans* forms could be separated by chromatography on alumina and identified by the location of the NH peak on p.m.r. spectra by comparison with samples of known optical configuration.

The p.m.r. spectrum of racemic cycloleucyl-leucyl prepared by the method of Dunn *et al.* (1949) gave a peak at 1.55 τ and one at 1.76 τ . This sample, when chromatographed on alumina, separated into two fractions, both optically inactive, which were obtained in approximately equal amounts. The p.m.r. spectrum of the second fraction off the column had one peak at 1.56 τ , as did authentic cyclo-D-leucyl-D-leucyl, cyclo-L-leucyl-L-leucyl or a mixture of these compounds. Such a mixture of *cis* forms could not be separated by chromatography on alumina. The p.m.r. spectrum of the first fraction off the column had one peak at 1.76 τ , and the compound was therefore cyclo-D-leucyl-L-leucyl, since it had an infrared spectrum differing only slightly from that of the *cis* forms and was shown by rechromatography not to be the *cis* form.

In some experiments described below, cycloleucyl-leucyl isomers were isolated from cultures. The isomer was identified as the *trans* or *cis* form primarily by its behaviour on chromatography and secondarily from its infrared spectrum (solid in potassium bromide). The decision as to which isomers were present in the *cis* form was made on the basis of the specific rotation of the sample, with acetic acid as the solvent. The highest optical rotation obtained for cyclo-L-leucyl-L-leucyl prepared from L-leucine was $[\alpha]_D^{25} - 45.7^\circ$ (*c* 2.05). The one sample of cyclo-D-leucyl-D-leucyl prepared had $[\alpha]_D^{25} + 44.4^\circ$ (*c* 2.05), s.d. 1.9%. Samples of the *cis* form were assumed to be cyclo-L-leucyl-L-leucyl if their specific rotation was between -41° and -45.7° and are designated as such in Table 1. In Expt. 4d in Table 1, the *cis* form had $[\alpha]_D^{25} + 11.2^\circ$ (*c* 2.23), s.d. 1.2%, and hence must have been a mixture of cyclo-D-leucyl-D-leucyl and cyclo-L-leucyl-L-leucyl, with the former predominating.

Effect of cycloleucyl-leucyl isomers on the synthesis of pulcherrimin. The fermentations in Expt. 4 in Table 1 were done simultaneously. The crude pulcherrimin, unless it contained appreciable radioactivity, was not converted into pulcherriminic acid, because the purification procedure involved unknown and variable loss of material. The crude

pulcherrimin was probably about 74% pure, as judged from the specific activity calculated for it in Expt. 4b in comparison with the specific activity in the pure pulcherriminic acid purified from it in the same experiment. However, the yield of pulcherrimin in Expt. 4 was based solely on the weight of crude pulcherrimin and a molecular formula of $C_{12}H_{18}Fe_{0.67}N_2O_4$. As compared with the control (Expt. 4a) the presence of cyclo-D-leucyl-D-leucyl almost completely inhibited the production of pulcherrimin, whereas cyclo-D-leucyl-L-leucyl apparently inhibited production slightly, and cyclo-L-leucyl-L-leucyl apparently increased production slightly.

In Expt. 4a and similar experiments, it was found that no cycloleucyl-leucyl was present in cultures of *C. pulcherrima* PRL2019 that had been grown on the unsupplemented medium for 4 days. However, when cycloleucyl-leucyl isomers were added to the medium, they apparently trapped cyclo-L-leucyl-L-leucyl synthesized by the organism. In Expt. 4b there was dilution of radioactivity of the cyclo-L-leucyl-L-leucyl during the course of the fermentation and the reisolated compound had a specific activity of 53% of that of the compound originally present in the medium. The cyclic dipeptide had a specific activity close to that of the pulcherriminic acid, as might be expected if cyclo-L-leucyl-L-leucyl were an intermediate in the biosynthesis of pulcherriminic acid. In Expt. 4c, the presence of cyclo-D-[1- ^{14}C]leucyl-L-[1- ^{14}C]leucyl caused the accumulation of a small amount of cyclo-L-leucyl-L-leucyl. The latter contained no radioactivity and therefore was not derived from the former compound but must have been synthesized by the organism from unlabelled precursors. The cyclo-D-[1- ^{14}C]leucyl-L-[1- ^{14}C]leucyl was isolated almost quantitatively and without dilution of isotope, and, since it was not metabolized or synthesized to any extent by the organism, it is not surprising that the pulcherrimin isolated in this experiment contained essentially no radioactivity. In Expt. 4d, the cyclo-D-leucyl-D-leucyl not only inhibited the synthesis of pulcherrimin but caused the accumulation of cyclo-L-leucyl-L-leucyl.

The above results support the idea that cyclo-L-leucyl-L-leucyl was the only cycloleucyl-leucyl isomer that was either synthesized by the organism or used in the biosynthesis of pulcherrimin.

Incorporation of L-leucine into cyclo-L-leucyl-L-leucyl. In Expt. 5 in Table 1 sufficient L-[1- ^{14}C]leucine and unlabelled cyclo-L-leucyl-L-leucyl were added to the medium to provide an excess throughout the fermentation. The reisolated cyclo-L-leucyl-L-leucyl contained radioactivity (0.616 μC /m-mole) and was chemically degraded to leucine, 0.313 μC /m-mole, in which 96% of the radioactivity was found in the carboxyl group and no detectable amount in

the rest of the molecule. Since the specific activity of the reisolated cyclo-L-leucyl-L-leucyl was about 1.6 times that of the L-[1-¹⁴C]leucine originally present in the medium, and since there was no randomization of labelled carbon, most of this cyclic dipeptide isolated from the medium was probably synthesized from 2 mol. of L-leucine. The specific activity of the pulcherriminic acid was slightly higher than that of the cyclo-L-leucyl-L-leucyl reisolated from the medium, but the results are still consistent with the idea that cyclo-L-leucyl-L-leucyl was the precursor of pulcherriminic acid. If L-[1-¹⁴C]leucine were converted through the intermediate cyclo-L-leucyl-L-leucyl into pulcherriminic acid inside the yeast cells, and if the non-radioactive cyclo-L-leucyl-L-leucyl supplied in the medium equilibrated well, but not completely, with the radioactive cyclic dipeptide in the cells, one would expect results similar to those obtained in Expt. 5.

Effect of cyclo-L-leucyl-L-leucyl on the synthesis of pulcherriminic acid by C. pulcherrima PRL2007. *C. pulcherrima* PRL2019, used in the experiments described above, produced the pigment, pulcherrimin, when grown on the unsupplemented liquid medium, whereas strain PRL2007 did not. The latter strain became pigmented when the medium was supplemented with cyclo-L-leucyl-L-leucyl but not when it was supplemented with L-leucine. The amount of pigmentation increased with the amount of cyclo-L-leucyl-L-leucyl up to a concentration of approx. 0.066 m-mole/100 ml. of medium. In Expt. 6 in Table 1 *C. pulcherrima* PRL2007 was grown on medium containing 0.066 m-mole of cyclo-L-[1-¹⁴C]-leucyl-L-leucyl/100 ml. of medium. The specific activity of the cyclo-L-leucyl-L-leucyl isolated after the fermentation was 44% of that of the compound originally added to the medium, indicating that there was endogenous synthesis of this compound from unlabelled precursors. The specific activity of the pulcherriminic acid was similar to that of the reisolated cyclo-L-leucyl-L-leucyl. The percentage derivation of pulcherriminic acid from cyclo-L-[1-¹⁴C]leucyl-L-leucyl was similar to that obtained with strain PRL2019 in Expt. 4b in Table 1, even

though the amount of pulcherriminic acid produced was different. It seems likely that the cyclic dipeptide was the precursor of pulcherriminic acid in both these strains. Although strain PRL2007 could synthesize cyclo-L-leucyl-L-leucyl (as shown by the dilution of radioactivity in Expt. 6) it required an exogenous supply of this compound before it would synthesize pulcherrimin. Other experiments showed that cyclo-L-leucyl-L-leucyl had to be added to the medium at the time of inoculation to obtain maximum pigment production. When it was added after the culture had grown for 1 day there was little pigment production, and when it was added after 2 or 3 days there was no pigment production. Exogenous cyclo-L-leucyl-L-leucyl may therefore play a role in the induction of the synthetic steps leading to pigment production, although one cannot say what this role might be.

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