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BIOSYNTHESIS OF ANTIBIOTICS OF THE VIRGINIAMYCIN FAMILY, 5.[1] THE CONVERSION OF PHENYLALANINE TO PHENYLGLYCINE IN THE BIOSYNTHESIS OF VIRGINIAMYCIN S_1

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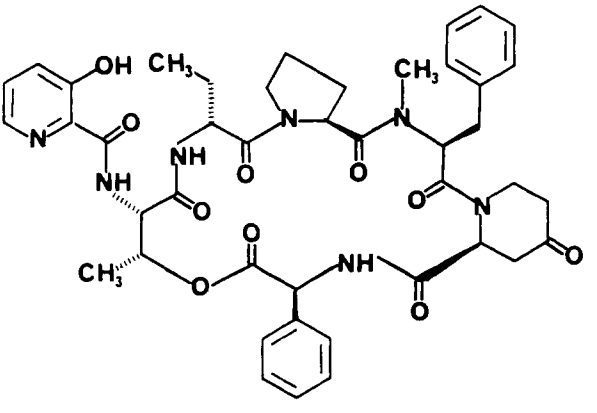
ABSTRACT.—Conversion of L-phenylalanine to L-phenylglycine in the biosynthesis of virginiamycin S_1 (**1**) can, in principle, take place with intramolecular nitrogen transfer or with intermolecular nitrogen transfer. A labeling experiment with DL-[3-^{13}C, ^{15}N]phenylalanine showed that the resulting L-phenylglycine contained no labeled nitrogen, indicating that the rearrangement proceeds via an intermolecular pathway.

Virginiamycin S_1 (**1**) is a macrocyclic peptidolactone antibiotic produced by *Streptomyces virginiae* and a few other closely related *Streptomyces* species (2-4). Its structure rests both on chemical degradations (4) and on an X-ray crystal structure (5), and its ^{1}H-nmr and ^{13}C-nmr spectra have been assigned as part of a study of its conformation in solution (6). It contains the protein amino acids L-threonine and L-proline, but its major constituents are the less common amino acids D-2-aminobutanoic acid, *N*-methyl-L-phenylalanine, 4-oxo-L-pipecolic acid, L-phenylglycine, and 3-hydroxypicolinic acid. In this paper we report the result of an experiment designed to elucidate the biosynthetic origin of the phenylglycine unit of virginiamycin S_1 from phenylalanine (**2**).



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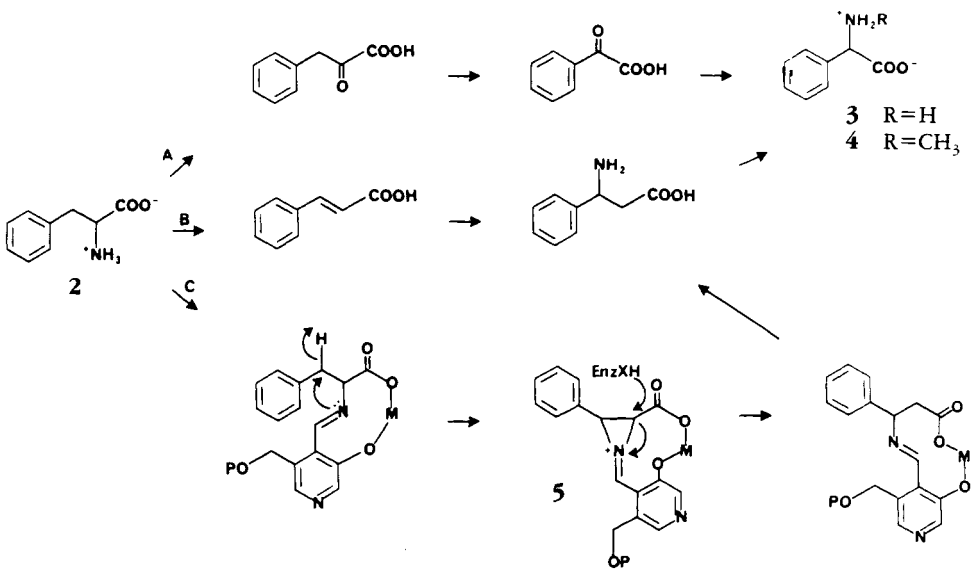
Although the phenylglycine unit (**3**) is relatively rare in natural products, its *N*-methyl derivative, L-α-phenylsarcosine (**4**), occurs in the related antibiotic etamycin, produced by *Streptomyces griseoviridus* (7,8). Biosynthetic results on this antibiotic support a pathway for the phenylalanine (**2**) to phenylsarcosine (**4**) conversion, which involves loss of the carboxyl group, migration of the amino group from the original α-carbon to the β-carbon, oxidation of the α-carbon to a carboxyl group, and *N*-methylation (9).

Although the broad outline of the pathway from phenylalanine to phenylsarcosine (**4**) or phenylglycine (**3**) is clear from the above work, there remain questions about the

[1]For Part 4, see LeFevre and Kingston (1).

details of the pathway. One question of interest to us was whether the migration of the amino nitrogen atom is intermolecular or intramolecular. Reasonable mechanistic pathways can be advanced for both pathways. Thus, the intermolecular pathway (Scheme 1) could involve transamination to phenylpyruvic acid, decarboxylation and oxidation to benzoylformic acid, and transamination to phenylglycine, with the amino group coming from an external donor such as glutamine (Pathway A). Alternatively, a phenylalanine ammonia-lyase catalyzed deamination to cinnamic acid followed by reamination would give β-phenylalanine in an analogous fashion to a proposal for the conversion of tyrosine to β-tyrosine (10); final conversion to phenylglycine would be by decarboxylation and oxidation (Pathway B). An intramolecular pathway could involve an aziridinium intermediate **5**, so that nitrogen always remains covalently bonded to the carbon skeleton. Opening of the ring by attack at the α-carbon would give a Schiff base, which would be converted to β-phenylalanine and thence to phenylglycine (pathway C). A similar pathway has been proposed for the biosynthesis of the β-lysine unit of streptothricin (11). Alternatively, pathways A or B could presumably occur in such a way that the originally eliminated nitrogen is returned without exchange. It should also be noted that although specific pathways are proposed for illustrative purposes, other plausible pathways exist.



SCHEME 1

In view of these reasonable possibilities for either type of reaction, we carried out experiments to test whether transformation of phenylalanine to phenylglycine proceeds by an intramolecular or intermolecular pathway.

RESULTS AND DISCUSSION

As a part of our investigation of the biosynthesis of virginiamycin S_1, we had already tested L-[U-^{14}C]phenylalanine as a precursor and shown it to be specifically incorporated into the *N*-methyl-L-phenylalanine and L-phenylglycine units of the antibiotic (12). In order to test the intermolecular or intramolecular nature of the phenylalanine to phenylglycine conversion, we elected to use the doubly labeled compound DL-[β-^{13}C, ^{15}N]phenylalanine hydrochloride. If rearrangement proceeds intermolecularly, the resulting phenylglycine would contain a ^{13}C-label at the α-position but not any ^{15}N-label. On the other hand, an intramolecular pathway would yield a phenylglycine in

which both the amino nitrogen and the α-carbon were labeled, and such labeling of adjacent atoms would be detectable by observation of a ^{13}C-^{15}N coupling of a few Hertz in its ^{13}C-nmr spectrum.

The synthesis of the doubly labeled precursor was achieved by standard methods, which were adapted to the case at hand (13-16). The ^{13}C-nmr spectrum of the labeled phenylalanine showed the expected resonances and couplings. Thus, C_β appeared as an intense singlet at 35.56 ppm, while C_α appeared as a doublet of doublets with $J=32.9$ and 6.5 Hz for the one-bond ^{13}C-^{13}C and ^{13}C-^{15}N couplings, respectively. The Ar-1 carbon also appeared as a doublet with $J=43.4$ Hz.

Growth of *S. virginiae* in a medium enriched with DL-[β-^{13}C, ^{15}N]phenylalanine yielded, as expected, an antibiotic in which both the *N*-methylphenylalanine and the phenylglycine units were enriched in ^{13}C. The ^{13}C-nmr spectrum of the labeled antibiotic showed intense resonances at 36.7 ppm and 56.1 ppm, corresponding to the β-carbon of *N*-methylphenylalanine and the α-carbon of phenylglycine, respectively (6). The resonance at 56.1 pm was examined carefully under high resolution conditions and was found to show no sign of any splitting due to ^{13}C-^{15}N coupling. This evidence thus indicates that rearrangement of phenylalanine to phenylglycine proceeds by an intermolecular pathway, unless transamination of phenylalanine and phenylglycine occurs so rapidly that the ^{15}N label is exchanged completely before incorporation into the antibiotic takes place.

Although it is not possible to determine the extent of transamination, if any, in phenylglycine, the extent of transamination in phenylalanine can be determined by an analysis of the ^{15}N content of the *N*-methylphenylalanine portion of virginiamycin S_1. A direct analysis by ^{13}C nmr was not possible since two-bond ^{13}C-^{15}N coupling constants are quite small, but analysis by mass spectrometry gave the desired information. Acid hydrolysis of the antibiotic followed by derivatization of the resulting amino acids yielded a mixture of *N*-trifluoroacetylamino acid butyl esters. Analysis of this mixture by gc/ms gave the data shown in Table 1 for *N*-trifluoroacetyl-*N*-methylphenylalanine butyl ester; the data shown are for the base peak due to the ion $[C_6H_5CH_2CHN(CH_3)COCF_3]^+$ and its isotopes, formed by loss of the carbobutoxy group. These data show that the ion in question (and hence the intact amino acid) consists of approximately 14% singly labeled species (either ^{13}C or ^{15}N) and 9% doubly labeled species, and thus, a substantial amount of ^{15}N label is retained in the *N*-methylphenylalanine portion of virginiamycin S_1.[2] A comparable retention of label in phenylglycine would have yielded a readily detectable splitting of the ^{13}C signal at 56.1 ppm in virginiamycin S_1,

TABLE 1. Relative Abundance for the Ions $[C_6H_5CH_2CHN(CH_3)COCF_3]^+$ from Labeled and Unlabeled *N*-Methyl-*N*-trifluoroacetylphenylalanine butyl ester[a]

m/z	Unlabeled	Labeled	Corrected labeled[b]	%
230	100	100	100	77
231	13.0	31.2	18.2	14
232	1.2	13.6	11.2	9

[a]Numbers given are an average of 6-8 runs.

[b]Corrected for the natural abundance contribution of ^{13}C.

[2]Similar measurements on the corresponding ion from *N*-trifluoroacetylphenylglycine butyl ester confirmed that essentially all of the ^{15}N label was lost in the formation of phenylglycine.

and thus, the observed absence of splitting confirms that the rearrangment of phenylalanine to phenylglycine occurs by an intermolecular pathway.

The rearrangement of phenylalanine to phenylglcyine in this stystem thus conforms to the type of pathway observed in the conversion of tyrosine to β-tyrosine (10), rather than the intramolecular pathway observed in the conversion of lysine to β-lysine (11).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were determined on an IBM WP-270 spectrometer. Gc/ms was carried out on a Finnigan-MAT 112 mass spectrometer coupled to a Varian 2100 gas chromatograph. Melting points were determined on a Kofler block and are uncorrected. Hplc was carried out on an apparatus consisting of a Waters M6000A pump, a Valco six-port injection valve, and a Waters 440 absorbance detector. Waters Nova-Pak Radial-Pak columns were used for analytical hplc, with acetonitrile-H_2O (40:60) containing 0.05% di-*n*-butylamine as solvent. Fermentation was carried out in a fermenter (New Brunswick Scientific).

[β-^{13}C,^{15}N]-α-ACETAMIDOCINNAMIC ACID AZLACTONE.—We heated [α-^{13}C]benzaldehyde (1.20 g, 11.2 mmol), [^{15}N]-*N*-acetylglycine (0.906 g, 768 mmol) (15), anhydrous NaOAc (0.470 g), and Ac_2O (1.9 g) under N_2 for 2 h. After cooling, the flask was stored at 4° overnight, and the contents were then treated with H_2O (2 ml). The solid product was broken up, filtered, and rinsed with two 1-ml portions of cold H_2O, yielding 1.062 g (73%) of crude product.

[β-^{13}C,^{15}N]-α-ACETAMIDOCINNAMIC ACID.—The crude azlactone (1.062 g) was heated under reflux for 6 h in Me_2CO (10 ml) and H_2O (4 ml). The Me_2CO was evaporated, 25 ml H_2O added, and the suspension boiled. The hot solution was filtered, the residue rinsed with a small amount of boiling H_2O, and the filtrate treated at 100° with 0.2 g Norit A for 5 min. The charcoal was filtered from the hot solution, and the filtrate was evaporated to 20 ml in vacuo and allowed to stand at 4° overnight. The white crystalline solid was filtered off and dried to give 0.977 g (84%) product, mp 193-194° [lit 193-194° (17)].

DL-[β-^{13}C,^{15}N]PHENYLALANINE HYDROCHLORIDE.—The acetamidocinnamic acid, (0.878 g) was hydrogenated in glacial HOAc (10 ml) over 5% palladium on carbon (0.25 g) for 2.5 h. at 25°. The catalyst was removed by filtration, the filtrate evaporated to dryness, and the residue heated under reflux in 20 ml 1 N HCl for 11 h. The mixture was evaporated to dryness in vacuo, and the residue dried in vacuo over NaOH to give 0.645 g (75%) of labeled phenylalanine hydrochloride, identical on tlc and co-tlc with an authentic sample. ^{13}C nmr (D_2O) δ 171.27 (s), 134.00 (d, J=43.4 Hz), 129.35 (d, J=3.1 Hz), 129.16 (d, J=3.7 Hz), 127.94 (s), 54.13 (dd, J_{CC}=32.9 Hz, J_{CN}=6.5 Hz), 35.50 (s).

CULTURE CONDITIONS.—The organism used in this work, *S. virginiae* strain 1830, was obtained from SmithKline Animal Health Products, West Chester, Pennsylvania. It was maintained on potato-glucose-agar slants of the following composition: filtrate from 200 g boiled potatoes, glucose 10 g, agar 20 g, H_2O to 1 liter. Newly inoculated slants were incubated at 28° for 7 days and stored at 4° until needed. A vegetative inoculum was prepared by transferring mycelium and spores from a slant to a 250 ml baffled Erlenmeyer flask containing 30 ml of vegetative medium STA-2 (corn steep liquor 36 g, peanut oil cake 8 g, glucose 50 g, $CaCO_3$ 5 g, $MnSO_4$ 0.01 g, H_2O to 1 liter). The vegetative inoculum was incubated at room temperature on a rotary shaker at 330 rpm for 72 h, and the contents were aseptically transferred to the fermenter vessel containing 1.5 liters of medium STA-14 (36 g corn steep liquor, 5 g yeast autolysate, 10 g peanut oil cake, 5 g $CaCO_3$, 10 g linseed oil, 5 g glucose, 25 g glycerol, H_2O to 1 liter). The broth was aerated at 1.25 liter/min and stirred at 450 rpm. Temperature was maintained at 21-22°. After 10 h, a solution of 0.29 g of DL-[β-^{13}C, ^{15}N]phenylalanine and 0.23 g L-threonine in 5 ml H_2O was introduced through a 0.45 μ filter. Fermentation was continued for a total of 48 h, with addition of sterile 1 N NaOH (5 ml) at 26 h and 36 h.

ISOLATION OF VIRGINIAMYCIN S_1.—The fermentation broth was filtered through Hyflo Super Cel and the filtrate extracted twice with one-third volume of hexane and then three times with a half volumes of EtOAc. The combined EtOAc extracts were dried ($MgSO_4$) and evaporated. The residue was dissolved in $CHCl_3$ (5 ml) and placed on a silica gel column, 25 mm×250 mm. Elution with $CHCl_3$-MeOH (99:1) and collection of 20 ml fractions yielded virginiamycin S_1 in fractions 14-16. The isolated material was shown to be >90% pure by analytical hplc and tlc (silica gel, $CHCl_3$-MeOH, (95:5); quantification by hplc gave a yield of 7.6 mg.

PREPARATION AND MASS SPECTROMETRIC ANALYSIS OF *N*-TRIFLUOROACETYLAMINO ACID BUTYL ESTERS.—Virginiamycin S_1 (30 mg) in 6 N HCl (2 ml) was heated at 104° for 24 h, and the mixture evaporated to dryness in vacuo. The residue was treated with 2 ml dry 3 N HCl in BuOH, heated at

110° for 3 h, and excess reagent removed in a stream of N_2. Treatment with trifluoroacetic anhydride (1 ml) in CH_2Cl_2 (1 ml) at room temperature for 24 h, and removal of excess reagent with N_2 yielded crude mixed *N*-trifluoroacetylamino acid butyl esters. Similar sequences were carried out on labeled virginiamycin S_1 (2.5 mg), and, without the initial hydrolysis step, on *N*-methyl-L-phenylalanine and on L-phenylglycine.

The mixed *N*-trifluoroacetylamino acid butyl esters were separated by gc on a 6 ft×2 mm i.d. glass column with 10% SP-2100 on Supelcoport (80-100 mesh), with a temperature program from 75° to 250° at 10°/min, and a helium flow of 20 ml/min. The gc peaks due to the derivatives of *N*-methyl-L-phenylalanine and L-phenylglycine were analyzed by ms, and the isotopic composition of the major fragment ion in each spectrum was determined on an average of 6-8 spectra by the method of Biemann (18).

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LITERATURE CITED

1. J.W. LeFevre and D.G.I. Kingston, *J. Org. Chem.*, **49**, 2588 (1984).
2. C. Cocito, *Microbiol. Rev.*, **43**, 145 (1979).
3. P. Crooy and R. de Neys, *J. Antibiot.*, **25**, 371 (1972).
4. H. Vanderhaeghe and G. Parmentier, *J. Am. Chem. Soc.*, **82**, 4414 (1960).
5. J.P. Declerq, G. Germain, M. Van Meerssche, S.E. Hull, and M.J. Irwin, *Acta. Cryst. Sect. B*, **34**, 3644 (1978).
6. M.J.O. Anteunis, R.E.A. Callens, and D.K. Tavernier, *Eur. J. Biochem.*, **58**, 529 (1975).
7. Q.R. Bartz, J. Standiford, J.D. Mold, D.W. Johannessen, A. Ryder, A. Maretzki, and T.H. Haskell, *Antibiotics Annual 1954/55*, 777 (1955).
8. J.C. Sheehan, H.G. Zachan, and W.B. Lawsan, *J. Am. Chem. Soc.*, **80**, 3349 (1958).
9. D.J. Hook and L.C. Vining, *Can. J. Biochem.*, **51**, 1630 (1973).
10. R.J. Parry and Z. Kurylo-Borowska, *J. Am. Chem. Soc.*, **102**, 836 (1980).
11. T.R. Thiruvengadam, S.J. Gould, D.J. Aberhart, and H.J. Lin, *J. Am. Chem. Soc.*, **105**, 5740 (1983).
12. A.A. Molinero, "The Biosynthesis of Virginiamycin S_1," M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 1982, p. 60.
13. A. Yoshitake, Y. Makari, K. Kawahara, and M. Endo, *J. Labelled Comp.*, **9**, 537 (1973).
14. R.M. Herbst and D. Shemin, *Org. Synth. Coll.*, **2**, 1 (1943).
15. H.B. Gillespie and H.R. Snyder, *Org. Synth. Coll.*, **2**, 489 (1943).
16. M. Calvin, "Isotopic Carbon," New York, John Wiley and Sons, 1960, p. 180.
17. R.C. Weast and M.J. Astle, eds., "CRC Handbook of Chemistry and Physics," CRC Press, Inc., Boca Raton, Florida, 1982, p. C-233.
18. K. Biemann, "Mass Spectrometry: Organic Chemical Applications," McGraw-Hill, New York, 1962, pp. 1-370.

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