60–80 mesh) kept at a 150°, and a flow rate of 150 cc./min. of helium was used. The first (and largest) peak was collected. The material was reinjected in 10-μl. fractions into a 5 ft. × 0.25 in. column containing 20% Cyanowax on Chromosorb P (60–80 mesh) at 150°, and a flow rate of 80 cc./min. of helium was used. The center portion of the peak was collected and a total of 25 mg. of 90% pure cuprenene **2** was obtained: $\nu_{max}^{CCl_4}$ 822 cm.$^{-1}$; λ_{max}^{EtOH} 271 mμ (ε 6350); n.m.r., τ 4.49 (2H, vinyl, broad singlet), 8.29 (3H, allylic methyl, singlet), 9.01 (6H, 2 methyl, singlet), and 9.20 (3H, methyl, singlet).

Anal. Calcd. for $C_{15}H_{24}$: C, 88.16; H, 11.84. Found: C, 88.29; H, 11.71.

On prolonged contact with air or when oxygen was bubbled through the oil for 3 hr., cuparene was formed.

Synthesis of Cuprenene 4 from Cuparene (5).—Following the general procedure of Johnson,[6] a solution of 338 mg. of cuparene in 65 ml. of absolute ethanol was added cautiously to 75 ml. of liquid ammonia. Lithium metal (5 g.) was added in portions with vigorous stirring along with 50 ml. of ethanol and 75 ml. of ammonia; the reaction mixture contained a bronze-colored phase. The addition required about 20 min. The stirring was continued until all the lithium had dissolved, and then the ammonia was allowed to evaporate. The solid residue was diluted with water and the mixture was extracted with ether. The ethereal layer was washed with water, dried over magnesium sulfate, and percolated through a short column containing 5 g. of Woelm alumina. The solvent was evaporated, and the 347 mg. of remaining colorless oil possessed infrared, ultraviolet, and n.m.r. spectra identical with cuprenene **4** isolated from Hiba wood oil. On standing in air the product was converted back to cuparene.

Conversion of Cuprenene 4 to Cuprenene 2.—A mixture of 43 mg. of cuprenene **4**, 321 mg. of potassium *t*-butoxide, 5 ml. of dimethyl sulfoxide, and 5 ml. of benzene was heated under a nitrogen atmosphere at 67° for 3 hr. The reaction mixture was cooled to room temperature, diluted with water, and extracted with petroleum ether. The organic phase was washed with water and dried over magnesium sulfate. The solvent was evaporated to yield a yellow oil containing a 9:1 mixture of cuprenenes **2** and **4**, respectively, and sulfur compounds. To obtain pure cuprenene **2**, the oil was injected in 10-μl. portions into a 5 ft. × 0.25 in. column containing 20% DEGS on Chromosorb W, and the peak corresponding to cuprenene **2** was collected. The material was dissolved in a small volume of pentane, and the solution was filtered through a short column of neutral Woelm alumina. The ether was evaporated, and 31 mg. of cuprenene **2** in 95% purity was obtained as a colorless oil: $\nu_{max}^{CCl_4}$ 823 cm.$^{-1}$; λ_{max}^{EtOH} 271 mμ (ε 6500); n.m.r. identical with natural material reported earlier; $[\alpha]_D$ +99° (*c* 0.82, $CHCl_3$).

Anal. Found: C, 88.18; H, 11.82.

On standing in air, the compound was converted into cuparene.

Isolation of Widdrol.—Portions (3 μl.) of the "alcohol fraction" were injected into a 5 ft. × 3/8 in. column containing 20% DEGS on Chromosorb W at 145°, and a flow rate of 80 cc./min. of helium was used. The material from the largest peak was collected, the liquid was cooled in a refrigerator, and the solid which formed was removed. This solid possessed an infrared spectrum identical with that of authentic widdrol.

Isolation of Terpinen-4-ol.—Portions (300 μl.) of distillation fraction 1 were injected into a 10 ft. × 3/8 in. column containing a packing of 20% Carbowax-20 M on Chromosorb P (HMDS, 60–80 mesh) kept at 200°, and a flow rate of 150 cc./min. of helium was used. The main peak was collected, and the collected material was a solid at 4° but melted by 10°. The infrared spectrum fits well that of (+)-terpinene-4-ol.[10] The material distilled at 60° (0.5 mm.), $n^{20}D$ 1.4792, $[\alpha]^{20}D$ +39.8° (*c* 2.90, $CHCl_3$) [lit.[11] $n^{20}D$ 1.4778, $[\alpha]^{20}D$ +41.9° (*c* 20, $CHCl_3$)].

Anal. Calcd. for $C_{10}H_{18}O$ (154.24): C, 77.86; H, 11.76. Found: C, 77.88; H, 11.84.

The n.m.r. spectrum had the following bands: τ 4.84 (1H, broad, vinyl H), 8.11 (4H, multiplet, allylic methylenes), 8.43 (3H, singlet, vinyl methyl), 9.15 (3H, doublet, J = 2 c.p.s., *sec*-methyl), and 9.25 (3H, doublet, J = 2 c.p.s., *sec*-methyl).

(10) J. Plíva, M. Horák, V. Herout, and F. Sörm, "Collection of Spectra and Physical Properties of Terpenes," Academie-Verlag, Berlin, 1960, Spectrum M-22.

(11) Y. R. Naves and P. Tullen, *Bull. soc. chim. France*, 2123 (1960).

Anisomycin. II.[1] Biosynthesis of Anisomycin

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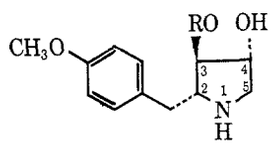
Anisomycin is a basic antibiotic which shows good biological activity against *Trichomonas vaginalis* and *Entamoeba histolytica.*[2] It is obtained by fermentation of various *Streptomyces* species,[3] and its structure has been shown to be 2-*p*-methoxybenzyl-3-*trans*-acetoxy-4-hydroxypyrrolidine[1] (I).



I, R = CH_3CO
II, R = H

The antibiotic may be extracted with chloroform from filtered broth at pH 9.0. It is recovered as white crystalline solid, and is usually accompanied by some deacetylanisomycin (II). Since the acetoxy function of anisomycin is readily hydrolyzed, it was not immediately clear whether the deacetylanisomycin (II) which accompanied the antibiotic was a product of hydrolysis during the isolation procedure or a prior fermentation product. However, a study of the fermentation showed the deacetylanisomycin (II) accumulated in the broth during the logarithmic phase of growth (Figure 1) and was converted into anisomycin during the phase of assimilation. Most of the anisomycin was produced during the third and fourth days of the fermentation when acetic acid had begun to accumulate in large quantities in the medium.

The structure of anisomycin (I) suggests that it might be derived from either tyrosine, phenylalanine, or proline. Labeled amino acids and aliphatic acids were added to the fermentation medium and specific activity of the isolated antibiotic was determined. Usually, the crude antibiotics isolated from the culture medium were hydrolyzed to deacetylanisomycin (II) which was then purified by partition chromatography. Occasionally it was advantageous to isolate anisomycin (I).

The results of these fermentations are shown in Table I. We conclude that the pyrrolidine ring of anisomycin is not derived from proline, but that the major precursor is tyrosine. Analysis of the soy meal, which was the only source of amino acids for the fermentation, showed that it contained 3.1% by weight of tyrosine, whereas phenylalanine was present in 4.8%. Thus, although the quantities of these amino acids in the fermentation medium are approximately the same, the tyrosine was incorporated 40 times more efficiently

(1) Paper I: J. J. Beereboom, K. Butler, F. C. Pennington, and I. A. Solomons, *J. Org. Chem.*, **30**, 2334 (1965).

(2) J. E. Lynch, A. R. English, H. Bank, and H. Deligianis, *Antibiot. Chemotherapy*, **4**, 844 (1954).

(3) B. A. Sobin and F. W. Tanner, *J. Am. Chem. Soc.*, **76**, 4053 (1954).

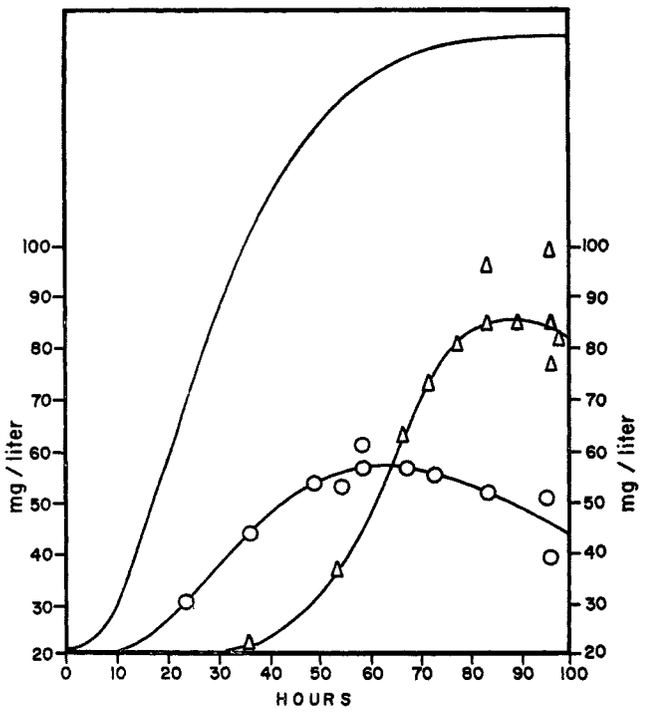


Figure 1.—Growth curve of *Streptomyces griseolus*, ———; yield of anisomysin, —Δ—Δ—; and yield of deacetylanisomycin, —O—O—.

than the phenylalanine. Other amino acids incorporated in significant amounts were glycine and methionine. Labeled acetate was utilized, but the degree of incorporation was erratic, probably owing to dilution by the large amounts of acetic acid produced in the fermentation. Malonate was not utilized to any significant degree.

TABLE I

Labeled precursor[a]	Specific activity, μcuries/mmole: Anisomycin (I)	Deacetylanisomycin (II)
[3-^{14}C]DL-Phenylalanine	*b*	0.244
[3-^{14}C]DL-Tyrosine	*b*	7.13
[2-^{14}C]DL-Tyrosine	3.01	...
[1-^{14}C]D-Tyrosine	0.313	...
[1-^{14}C]L-Tyrosine	*b*	0.004
[U-^{14}C]L-Proline	*b*	0.047
[2-^{14}C]Glycine	*b*	0.655
[Me-^{14}C]L-Methionine	*b*	7.60
[1-^{14}C]Acetate[c]	0.511	0.302
[2-^{14}C]Acetate	*b*	0.073
[2-^{14}C]Malonate	*b*	0.016

[a] 50 μcuries of each precursor was used for 1 l. of fermentation medium. [b] Not determined. [c] 100 μcuries of sodium acetate-1-C^{14} was used per 1 l. of medium.

Degradations of the radioactive antibiotics by the methods described earlier[1] were carried out to locate the positions of the labeled carbon atoms. The results of experiments involving the antibiotics obtained from labeled tyrosine and phenylalanine are presented in Table II.

The crude antibiotic isolated from the fermentation in the presence of labeled phenylalanine was shown by paper chromatography to be a mixture of anisomycin (I) and deacetylanisomycin (II). Hydrolysis converted all the material into deacetylanisomycin which was purified by partition chromatography on acid-washed Supercel. The specific activity of the deacetyl-

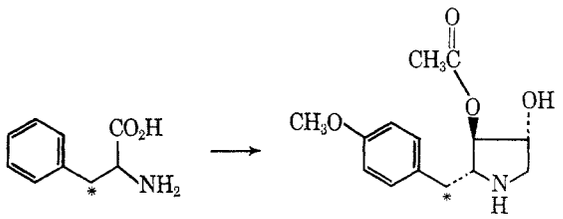
TABLE II

Labeled precursor	Specific activity of degradation products, μcuries/mmole: I	II	III[a]	V[a]	VI	VII	VIII
[3-^{14}C]DL-Phenylalanine	...	0.244	0.298	0.278	...	...	...
[3-^{14}C]DL-Tyrosine	...	7.13	6.39	6.80	...	...	6.91
[2-^{14}C]DL-Tyrosine	3.01	...	2.80	...	3.14	3.42	0.00
[1-^{14}C]L-Tyrosine	...	0.004	...	...	...	...	...
[1-^{14}C]D-Tyrosine	0.313	0.084	0.015	...	...	...	...

[a] Isolated as the dinitrophenylhydrazone derivative.

anisomycin obtained was 0.244 μcuries/mmole. A sample of this material was degraded[1] to *p*-methoxycinnamaldehyde (III) (see Chart I) which was isolated as the 2,4-dinitrophenylhydrazone. The specific activity of this aldehyde was determined by planchet counts to be 0.298 μcurie/mmole.

Ozonolysis of the *p*-methoxycinnamaldehyde gave anisaldehyde (V) which was isolated as the dinitrophenylhydrazone (specific activity, 0.278 μcurie/mmole). Hydrolysis of anisaldehyde dinitrophenylhydrazone produced methyl bromide which was distilled from the reaction mixture and was trapped in methanol. No radioactivity was detected in the methyl bromide solution. Thus, we concluded that fermentation of [3-^{14}C]DL-phenylalanine gives rise to anisomycin labeled at the benzylic carbon atom.

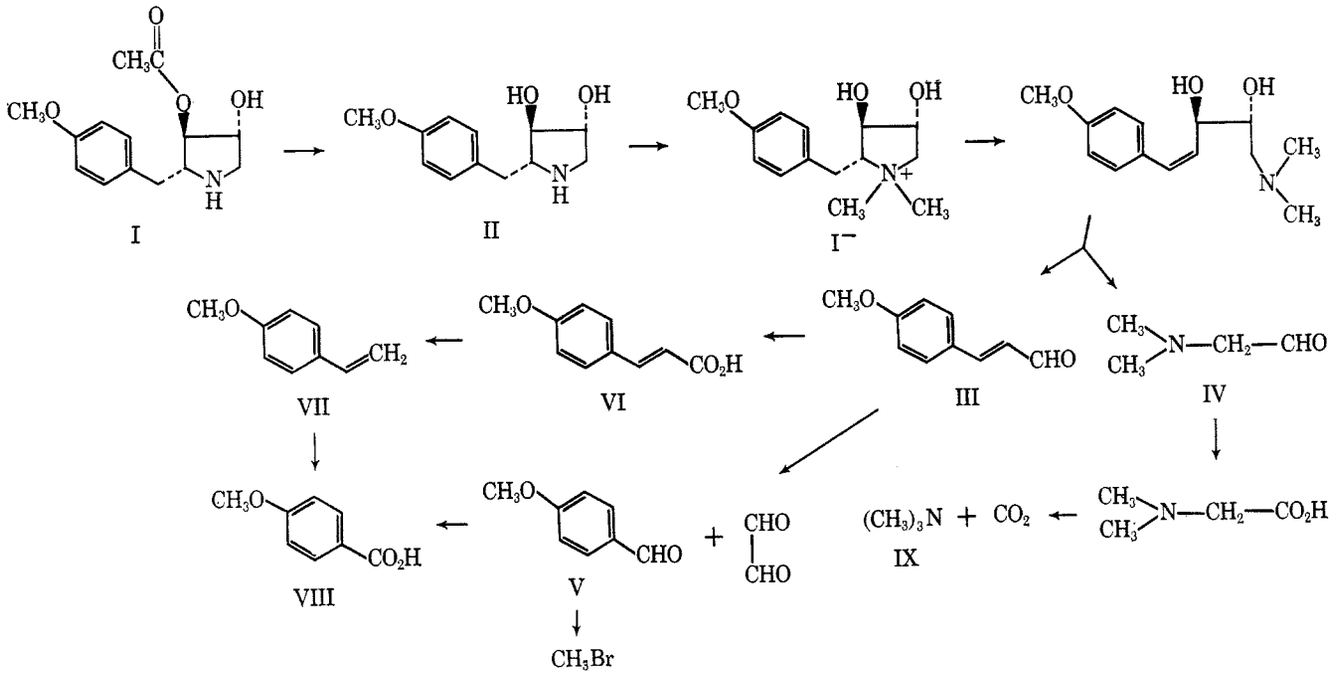


In the same fashion, we degraded the deacetylanisomycin obtained from the fermentation of [3-^{14}C]DL-tyrosine. The close correlation of the specific activities of the degradation products shown in Table II proves that the anisomycin was labeled at the benzylic carbon atom, as was also the case in the phenylalanine experiments described above. One extra step was added to this degradation process; the anisaldehyde (V) was oxidized to anisic acid (VIII) which was purified by sublimation. This enabled us to determine the specific activity of this fragment by liquid-scintillation counting, rather than by planchet counts which were necessary for the insoluble and highly colored dinitrophenylhydrazones.

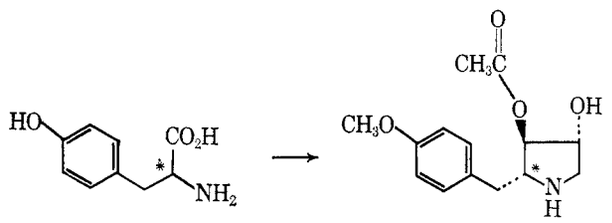
Degradation of material obtained from [2-^{14}C]DL-tyrosine required a modified process. We expected the antibiotic in this case to be labeled at C-2 of the pyrrolidine ring. Ozonolysis of *p*-methoxycinnamaldehyde removes two carbon atoms as glyoxal, which represents carbon atoms 2 and 4 of the pyrrolidine ring. This is unsatisfactory for our purposes and furthermore, the glyoxal is difficult to isolate on such a small scale. Consequently, the *p*-methoxycinnamaldehyde (III) was oxidized to *p*-methoxycinnamic acid (VI),[4] which was then decarboxylated to produce *p*-methoxystyrene.[5] The *p*-methoxystyrene (VII) showed a tendency to polymerize, but it was steam distilled from the reaction mixture and extracted from the distillate

(4) Doufresne, *Compt. rend.*, **145**, 873 (1907).
(5) Perkin, *J. Chem. Soc.*, **31**, 409 (1887); **33**, 215 (1878).

CHART I



with ether. Specific activities of the degradation products (Table II) showed that the labeled carbon atoms of the anisomycin (I) were still retained in the *p*-methoxystyrene (VII). Vigorous oxidation of VII produced anisic acid (VIII) which was sublimed and shown to be devoid of all radioactivity. Hence, the anisomycin derived from [2-^{14}C]DL-tyrosine is labeled at C-2 of the pyrrolidine ring.



Carbonyl-labeled tyrosine gave anomalous results. Thus, [1-^{14}C]L-tyrosine was not incorporated into the antibiotic to any significant degree, but [1-^{14}C]D-tyrosine gave rise to anisomycin (I) with a specific activity of 0.313 μcurie/mmole. This would seem to indicate that the D isomer is incorporated rather than the L isomer. However, if such were the case, the pure optical isomer should have been incorporated twice as efficiently as the DL-tyrosine and would have produced specific activities greater than those observed for the [2-^{14}C]- and [3-^{14}C]-labeled DL-tyrosine.

Hydrolysis of the anisomycin from [1-^{14}C]D-tyrosine gave deacetylanisomycin (II) having 0.084 μcurie/mmole, and further degradation to *p*-methoxycinnamaldehyde (III) reduced the level of radioactivity to 0.015 μcurie/mmole. Apparently, the [1-^{14}C]D-tyrosine must undergo decarboxylation during the fermentation, and the labeled carbon dioxide enters the pool of one-carbon fragments and is incorporated into acetic acid and possibly other metabolic products. These metabolites are then utilized in the biosynthetic scheme of anisomycin to introduce labeled carbon atoms in the acetoxy side chain and somewhere in the C-4–C-5 fragment of the pyrrolidine ring. This would explain why the radioactivity was scrambled and why the pattern of distribution of the ^{14}C atoms resembles that observed for labeled acetate (see below). It is obvious that [1-^{14}C]D-tyrosine was metabolized more readily than the L isomer, but we do not know whether the larger fragments of the D-tyrosine molecule were incorporated into the anisomycin. It would be imprudent to draw any further conclusions without conducting experiments with pure optical isomers of tyrosine labeled at two or more positions.

Degradations of the antibiotics obtained from labeled acetate, glycine, and methionine are summarized in Table III. Radioactive deacetylanisomycin (II) from [2-^{14}C]glycine had a specific activity of 0.655 μcurie/mmole. Hofmann degradation followed by glycol cleavage gave *p*-methoxycinnamaldehyde (III) which

TABLE III

Labeled precursor	Specific activity in degradation products, μcuries/mmole I	II	III[a]	V[a]	IX	MeBr[b]
[2-^{14}C]Sodium acetate	...	0.073	0.008	...	...	None
[1-^{14}C]Sodium acetate	0.511	0.302	...	...	...	...
[2-^{14}C]Glycine	...	0.655	0.285	0.250	0.402	91
[Me-^{14}C]L-Methionine	...	7.6	7.8	...	...	33

[a] Isolated as the dinitrophenylhydrazone derivative. [b] Figures in this column represent the percentage of the radioactivity of III recovered as methyl bromide.

was extracted from the mixture with ethyl acetate, and dimethylaminoglycine (IV) which remained in the aqueous liquors. The specific activity of the *p*-methoxycinnamaldehyde was 0.285 μcurie/mmole. Only 43.5% of the radioactivity of the glycine had been incorporated into the portion of anisomycin which gave rise to methoxycinnamaldehyde. The other 56.5% of the glycine must, therefore, be in the dimethylaminoacetaldehyde representing C-4 and -5 of the pyrrolidine ring. Unfortunately, we were unable to isolate any pure dimethylaminoacetaldehyde to determine its specific activity. Oxidation of the dimethylamino-

acetaldehyde fraction was accomplished with alkaline potassium permanganate, and the presence of N,N-dimethylglycine in the products was confirmed by paper chromatography. The total crude mixture containing the N,N-dimethylglycine was absorbed onto calcium oxide and pyrolized, and the trimethylamine produced was collected in methanolic hydrogen chloride. A sample of trimethylamine picrate was prepared to confirm the identity of the amine. Specific activity of the amine salt was 0.402 μcurie/mmole (61% of the activity of II). Hence, 61% of the carbon-14 was incorporated into C-5 of the pyrrolidine ring adjacent to the nitrogen atom. Ozonolysis of *p*-methoxycinnamaldehyde (III, 0.285 μcurie/mmole) gave anisaldehyde, hydrolysis of which, with hydrobromic acid, produced methyl bromide, which was distilled from the reaction mixture into methanol. The methyl bromide solution represented 91% of the radioactivity of III. Thus, [2-^{14}C]-glycine is incorporated into two parts of the anisomycin molecule. Some of it is metabolized to formic acid and formaldehyde and enters the pool of one-carbon fragments and is subsequently used to methylate the phenolic hydroxyl of the tyrosine, and some glycine becomes incorporated into the pyrrolidine ring. The location of the labeled carbon atom from glycine at C-5 of the pyrrolidine ring suggests that the adjacent carbon atom at C-4 is derived from the glycine carboxyl group.

[Me-^{14}C]L-Methionine gave rise to deacetylanisomycin (II) with a specific activity of 7.6 μcuries/mmole. Degradation of II gave *p*-methoxycinnamaldehyde (III) containing 7.8 μcuries/mmole; hence, the dimethylaminoacetaldehyde (IV) could not be radioactive. Hydrolysis of the dinitrophenylhydrazone of III proceeded in a most unsatisfactory manner. The derivative was not very soluble in hydrobromic acid, and there was some loss of material through bumping despite the stream of nitrogen which was bubbled through the reaction mixture. The methyl bromide which was collected represented only 33% of the radioactivity of III. This low yield of radioactivity is probably due to bumping and low boiling point of methyl bromide (4.5°), since it is not plausible that 70% of the labeled methionine was incorporated into the unsaturated side chain of III. Indeed, we have already shown that two of these carbon atoms were derived from phenylalanine or tyrosine, and the third carbon atom has a higher oxidation state than would be expected for a group derived from the labeled methyl group of methionine. Methionine probably plays its normal role as a biological methylating agent in the biosynthesis of anisomycin and gives rise to the methoxy group of the aromatic ring.

Fermentations in the presence of labeled acetate usually produced anisomycin with a low specific activity. This was due to the dilution of isotope by the large amounts of acetic acid produced in the course of the fermentation. Sodium [1-^{14}C]acetate provided anisomycin (I) with a specific activity of 0.511 μcurie/mmole. Hydrolysis of this sample gave deacetylanisomycin (II) with 0.285 μcurie/mmole; thus, 44% of the radioactivity was present in the acetoxy group. Therefore, acetic acid, probably as acetyl coenzyme A, is used for the acetylation of deacetylanisomycin (II) to produce anisomycin (I).

Degradation of the labeled deacetylanisomycin from [1-^{14}C]acetate was not continued further. However, II from [2-^{14}C]acetate was degraded to *p*-methoxycinnamaldehyde which had no significant amount of radioactivity. Acetate appears to be incorporated into the pyrrolidine ring at C-4 and C-5. Further studies with labeled acetate were not possible because of the poor incorporation into the anisomycin owing to isotope dilution and the low yields of the antibiotic.

Thus, we have been able to demonstrate that tyrosine, glycine, methionine, and acetate are precursors for the biosynthesis of anisomycin. Phenylalanine is utilized to a limited degree. Tyrosine donates C-2 of the pyrrolidine ring, and C-4 and C-5 are derived from acetate or glycine. The origin of C-3 was not determined.

Presence of Sandaracopimaric and $\Delta^{8(9)}$-Isopimaric Acids in Pine Oleoresin

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Sandaracopimaric and $\Delta^{8(9)}$-isopimaric acids[2] were isolated from pine oleoresin.

Sandaracopimaric acid has previously been detected in a number of sources.[3] von Rudloff and Sato have detected it in trace amounts from the heartwood extracts of *Pinus banksiana*[4] and also in *Pinus resinosa* Ait.[5] The $\Delta^{8(9)}$-isopimaric acid has been detected in small amounts in the resins from *Tetraclinis* and *Callitris* species and in trace amounts in samples of isopimaric acid isolated from *Dacrydium biforme* Pilger. Genge[6] suggested that one of the peaks in their mass spectra indicated the presence of $\Delta^{8(9)}$-isopimaric acid in some rosins. This is the first report, however, of either acid as a constituent of pine oleoresin.

In our work on gas chromatographic analysis of pine gums and rosins several unidentified peaks were observed. The components of two of these peaks were collected in large enough quantities for identification. The first peak off the gas chromatograph (see Table I) was identified as $\Delta^{8(9)}$-isopimaric acid. The fourth peak off the column was identified as sandaracopimaric acid.

Since the $\Delta^{8(9)}$-isopimaric acid was known to be an acid isomerization product of isopimaric acid,[7] it was suspected that the acid used to stimulate the flow of gum from the tree might be causing this isomeriza-

(1) One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.
(2) Steroid numbering.
(3) L. J. Gough, *Chem. Ind.* (London), 2051 (1964).
(4) E. von Rudloff and A. Sato, *Can. J. Chem.*, **41**, 2165 (1963).
(5) A. Sato and E. von Rudloff, *ibid.*, **42**, 635 (1964).
(6) C. A. Genge, *Anal. Chem.*, **31**, 1750 (1959).
(7) O. E. Edwards and R. Howe, *Can. J. Chem.*, **37**, 760 (1959).