On the Biosynthesis of Tenuazonic Acid in *Alternaria tenuis*

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Tenuazonic acid (Fig. 1) was first isolated by Rosett *et al.*[1] from *Alternaria tenuis*. Its structure was established by Stickings who also showed that it is biosynthetically derived from L-isoleucine and acetate.[2,3] In an earlier publication [4] we were able to demonstrate the formation of the corresponding tetramic acids from L-valine and L-leucine after feeding the organism with the respective amino acid. Under similar conditions L-phenylalanine did not give rise to a tenuazonic acid analogue.

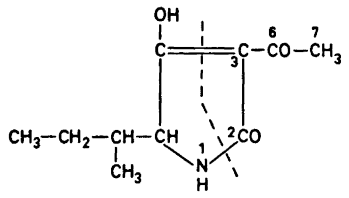

Fig. 1. Tenuazonic acid.

Very few natural tetramic acids have so far been found. One example, however, is erythroskyrine [5] isolated from *Penicillium islandicum*. This compound is derived from L-valine and acetate. In this case acetate participates in the formation of the complex side chain.

A group of substances formed according to the same biosynthetic principle as tenuazonic acid is the tetronic acids produced by *Penicillium charlesii*. In the biosynthesis of these acids the amino acids occurring in the formation of the tetramic acids are substituted with a C_4-dicarboxylic acid, presumably malate. Recently Bentley *et al.*[6] showed that the condensation partner of the C_4-acid is formed from acetate-malonate in the regular way of fatty acid synthesis.

The participation of malonate in the biosynthesis of tenuazonic acid and the possible formation of an initially long side chain which has been oxidatively degraded by the organism to the acetyl group will be considered in this publication.

There are two theoretical alternatives for the sequence of condensations between the active forms of L-isoleucine, and the appropiate β-keto acid. In one of them the carboxyl group of isoleucine first condenses with the α-methylene group of the β-keto acid and then follows the ring closure by the amide formation between the α-amino group of isoleucine and the carboxyl group of the β-keto acid. In the other alternative the amide formation precedes the reaction between the isoleucine carboxyl group and the α-methylene group of the β-keto acid which now completes the tetramic acid structure. Experiments that support the first alternative will be presented.

Experimental. The fermentation of *Alternaria tenuis* CMI 89343 was carried out in modified Czapek-Dox medium as described earlier.[4]

Incorporation of malonate-1-^{14}C. To each of two flasks 40 mg of L-isoleucine was added 68 h after incubation of the organism. After another 3 h 10 mg of L-isoleucine and 25 μCi of sodium malonate-1-^{14}C in trace amounts were added. The organism was exposed to the radioactive precursor for 24 h before the tenuazonic acid was isolated as the copper salt. The labeled product was diluted with 220 mg cold carrier by recrystallization to constant specific radioactivity from methanol/water. Yield 160 mg. Specific radioactivity of Cu-tenuazonate 2.1×10^5 dpm/mmol; Incorporation 0.2 %.

Incorporation of acetate-1-^{14}C and butyrate-1-^{14}C. Samples of radioactive tenuazonic acid were prepared as described above from 25 μCi of sodium acetate-1-^{14}C and 50 μCi of sodium butyrate-1-^{14}C, respectively.

Degradation of labeled tenuazonic acid. Specific radioactivities of the acids were determined on $BaCO_3$ obtained by trapping in $Ba(OH)_2$ the CO_2 evolved on wet-combustion according to van Slyke and Folch.[7] The radioactivities were measured in a liquid scintillation spectrometer (Packard model 3375) in toluene solution of PPO and POPOP after suspending the $BaCO_3$ with the aid of Cabosil gel. The labeled acids were hydrolysed by refluxing in M H_2SO_4 for 5 h as described by Stickings.[3] The CO_2 obtained from position 2 was trapped in $Ba(OH)_2$. The acetic acid derived from C-6 and C-7 was isolated by steam distillation and neutralization with NaOH. By decarboxylation of the sodium acetate in the Schmidt reaction [8] and transferring the CO_2 into $BaCO_3$ the

Table 1. Distribution of radioactivity in labeled samples of tenuazonic acid.

Labeled precursor	Tenuazonic acid	Dpm/mmol C-2	C-6	C-6/C-2
Malonate-1-^{14}C	10.4×10^4	5.1×10^4	4.8×10^4	0.94
Acetate		2.2×10^5	2.0×10^5	0.91
Butyrate-1-^{14}C		9.3×10^6	2.5×10^6	0.27
Butyrate-1-^{14}C		2.3×10^6	0.5×10^6	0.22

radioactivity of the C-6 position was determined. The specific radioactivities of the tenuazonic acid samples actually used for degradation, and the various degradation products, are shown in Table 1.

Trapping of radioactive N-*acetoacetyl-*L-*isoleucine.* To each of two fermentation flasks 40 mg of L-isoleucine and 100 μCi of aqueous sodium acetate were added. After 8 h exposure to the radioactive precursor the mycelium was separated from the culture medium by filtration. Acidification and subsequent extraction of the filtrate with ether yielded a radioactive solution which was dried over anhydrous Na_2SO_4 and then evaporated to dryness. The mycelium was ground with sand in acetone. The acetone solution was evaporated to dryness and the residue pooled with the extract from the culture medium. This mixture together with 100 mg of *N*-acetoacetyl-L-isoleucine (synthesized as described by Harris *et al.*[9]) were dissolved in hot benzene, treated with active carbon and filtered while still hot. The *N*-acetoacetyl derivative was recrystallized to constant specific radioactivity (3220 dpm/mg). The incorporation of radioactivity into isolated *N*-acetoacetyl-L-isoleucine was 0.07 %.

The described experiment was repeated with *N*-acetyl-L-isoleucine, instead of *N*-acetoacetyl-L-isoleucine. In this case the radioactivity was rapidly lost on recrystallizations.

Results and discussion. If the carbon atoms in positions 2 and 3 were derived from malonate and those in positions 6 and 7 from acetate an uneven labelling would be expected in positions 6 and 2 when malonate-1-^{14}C is fed to the organism. From Table 1 is seen that the specific radioactivities in these positions are almost equal which indicates that malonate as such is not involved in the biosynthesis of tenuazonic acid unless a very active malonate decarboxylase system is present in the organism. The slightly lower radioactivity in C-6 compared to C-2 depends on the experimental conditions in the degradation procedure as it is also observed in tenuazonic acid obtained from labeled acetate.

The incorporation of the intact butyric acid molecule into tenuazonic acid confirms the conclusion that malonate is not involved in the biosynthesis as well as it indicates that the acetyl group at 3 position has not been part of a longer side chain. Acetoacetyl-coenzyme A formed in the thiolase reaction is the suggested origin of the positions C-2, C-3, C-6, and C-7 of the tenuazonic acid. It is surprising that the added butyric acid appears without cleavage to the observed extent in the tenuazonic acid structure. The localizations of the involved reactions inside the cell become an intriguing question.

The demonstration of the occurrence of *N*-acetoacetyl-L-isoleucine in the organism indicates that the initial step in the biosynthesis of tenuazonic acid is *N*-acetoacetylation of L-isoleucine followed by formation of the five-membered ring.

This investigation was supported by a research grant from *The Swedish Natural Science Research Council.*

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Received May 18, 1973.

Crystallographic and Structural Data of Three Thallium(I) Compounds

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The crystal structures of diethylthioselenophosphinatothallium(I), [Tl(Et_2PSeS)], and diethyldithiophosphinatothallium(I), [Tl(Et_2PS_2)], have been determined by three-dimensional X-ray methods. Space group and unit cell have further been determined for diethyldiselenophosphinatothallium(I), [Tl(Et_2PSe_2)].

The crystals of [Tl(Et_2PSeS)] (I) are colourless prisms elongated along the *c* axis. The structure is monoclinic with cell dimensions $a = 10.342(3)$ Å, $b = 9.116(3)$ Å, $c = 10.154(2)$ Å, $\beta = 101.98(2)°$, and $Z = 4$. Possible space groups were $C2$, Cm, and $C2/m$.

The intensity data of 997 reflections greater than background were recorded by means of a Siemens AED-1 single crystal diffractometer, using Mo$K\alpha$-radiation. The crystal structure was solved by a three-dimensional Patterson synthesis. The map could only be interpreted in terms of a dimeric complex. In the centric space group, $C2/m$, which was the first choice and which proved to be the correct one, the dimers occupy special twofold positions with the Tl atoms on a twofold axis parallel to *b* and the Se, S, and P atoms lying in a mirror plane halfway between the two Tl atoms at right angles to *b*. The structure was refined by full-matrix least squares methods to an *R*-value of 0.088. Attempts to refine the structure in the non-centric alternative space groups were not successful.

The crystals of [Tl(Et_2PS_2)] (II) are thin colourless plates with $a = 9.026(3)$ Å, $b = 12.134(2)$ Å, $c = 8.468(3)$ Å, and $Z = 4$. The crystals are orthorhombic with the space group $Pcca$, which requires that the molecules occupy fourfold special positions.

Intensities were estimated visually from Weissenberg photographs taken with Ni-filtered Cu$K\alpha$-radiation using the multiple film technique. 376 out of 507 independent reflections from the five layers $hk0 - hk3$ and $0kl$ were observed and measured. The structure was solved by three-dimensional Patterson and Fourier syntheses. The Tl and P atoms lie on a twofold axis. The atomic parameters were refined by least squares methods to an *R*-value of 0.094.

The crystals of [Tl(Et_2PSe_2)] form extremely thin colourless monoclinic prisms extended along the *b* axis, with $a = 11.731(7)$ Å, $b = 6.741(3)$ Å, $c = 13.091(4)$ Å, $\beta = 111.71(3)°$, and $Z = 4$. The space group is $P2_1/c$. During exposure to X-rays, the crystal surface became gradually covered by a yellow powder. This happened also to the two former complexes, but to a lesser degree. This effect together with the crystal size made a structure solution difficult.

Thallium (I) compounds have a tendency to occur as polymers in the solid state.

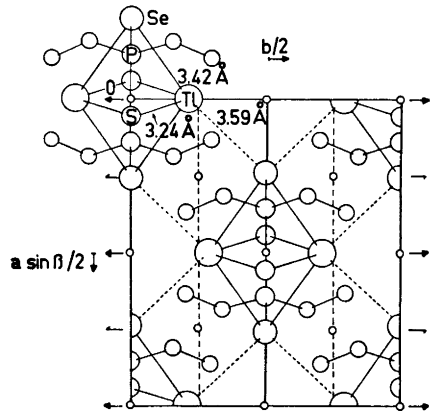

Fig. 1. The arrangement of the [Tl(Et_2PSeS)]$_2$ dimers in the unit cell as seen along the *c* axis.