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Microbial Production of Tenuazonic Acid Analogues

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The fungus *Alternaria tenuis* normally produces tenuazonic acid (3-acetyl-5-secbutyltetramic acid). On supplementation of the culture substrate with L-valine and L-leucine, the organism formed two new tetramic acids, 3-acetyl-5-isopropyltetramic acid and 3-acetyl-5-isobutyltetramic acid, respectively. L-Phenylalanine was not utilized by the organism as a tetramic acid precursor.

It has been possible in only a very few cases to change qualitatively the production of a microorganism. The best known example is the influence of side-chain precursors on penicillin fermentations. In this paper, we describe the biological formation of two tenuazonic acid analogues obtained by additions of appropriate precursors to growing cultures of *Alternaria tenuis*. Tenuazonic acid was first isolated by Rosett et al. from *A. tenuis* (3). Its structure was established by Stickings (5), who also showed that it is biosynthetically derived from L-isoleucine and acetate (6). As tenuazonic acid and some synthetic congeneric tetramic acids show certain biological activities (1, 4), it is of interest to achieve microbial production of tetramic acids other than tenuazonic acid. The biosynthetic pattern of tenuazonic acid and the stimulation of its production by the addition of L-isoleucine to the fermentation suggested the possibility of obtaining tetramic acids with different side chains at the 5-position by growing the organism in media supplied with various L-amino acids. The production of the 5-isopropyl and the 5-isobutyl derivatives could be demonstrated by using ^{14}C-carboxyl-labeled L-valine and L-leucine, respectively, as additives to the culture media. The radioactive tetramic acids were isolated and identified by isotopic dilution with nonlabeled synthetic tetramic acids as carriers. In this way, it was also shown that phenylalanine could not be utilized by the organism as a tetramic acid precursor.

Microorganism and culture conditions. *A. tenuis* CMI 89343 was used in this study. Stock cultures were maintained on agar slants of malt extract (1.0%), yeast extract (0.4%), and glucose (0.4%), *p*H 7.3. The fermentation experiments were carried out in 500-ml conical flasks containing 150 ml of modified Czapek-Dox medium of the following composition (g/liter): glucose, 40.0; $NaNO_3$, 1.0; NH_4Cl, 0.25; KH_2PO_4, 1.0; KCl, 0.25; NaCl, 0.25; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; and yeast extract, 1.0; *p*H 5.5. The flasks were incubated at 28 C on a rotary shaker operating at 240 rev/min with a 2.5-cm stroke. Amounts of 50 μCi (0.022 mmole) of aqueous solutions of ^{14}C-carboxy-labeled L-amino acids (valine, leucine, and phenylalanine) were added to the cultures after 48 hr of incubation. After 24-hr exposure to the labeled compound, the mycelium was filtered off and the tetramic acid was isolated from the filtrate.

Isolation and identification of L-valine-derived tetramic acid. The culture filtrate was acidified with HCl and extracted with ether. The ether phase was evaporated to dryness and redissolved in aqueous $Cu(NO_3)_2$. Addition of nonlabeled Cu salt of 3-acetyl-5-isopropyltetramic acid (100 mg), synthesized according to the method described by Harris et al. (2), and reisolation of the Cu salt gave a radioactive product. The 2,4-dinitrophenylhydrazone of 3-acetyl-5-isopropyltetramic acid was obtained by dissolving the Cu salt in a small volume of methanol and adding an excess of 2,4-dinitrophenylhydrazine in diluted HCl. The solution was heated on a steam bath for 30 min and then left for crystallization overnight. The yellow needles were recrystallized from methanol-water with all radioactivity retained, m^+/e, 363, melting point 222 to 224 C. (Found: C, 49.64; H, 4.86; N, 18.99. Calculated for $C_{15}H_{17}N_5O_6$: C, 49.66; H, 4.72; N, 19.28.)

The radioactivity was determined in a liquid scintillation spectrometer on $BaCO_3$ suspended with Cabosil in a toluene solution of Omnifluor (0.4%). The $BaCO_3$ was obtained by wet combustion of the hydrazone and by trapping the

CO_2 formed in aqueous $Ba(OH)_2$. The incorporation of labeled valine into 3-acetyl-5-isopropyltetramic acid was found to be 0.5%.

Isolation and identification of L-leucine-derived tetramic acid. An alcoholic solution of 100 mg of synthesized nonlabeled 3-acetyl-5-isobutyltetramic acid was added to the ether solution obtained on extraction of the culture filtrate. The semicarbazone was prepared by adding an excess of semicarbazide hydrochloride in ethanol-water to the ether solution. The ether was driven off by heating the mixture, and the residue was boiled for a few minutes. The semicarbazone crystallized overnight, m^+/e 254, melting point 204 to 206 C (7). Its specific radioactivity was retained on recrystallization from ethanol-water, and 0.5% of the labeled leucine was found to be incorporated into the tetramic acid. For radioactive measurements, the semicarbazone dissolved in methanol was added to the scintillator solution. (Found: C, 51.84; H, 7.08; N, 21.99. Calculated for $C_{11}H_{18}N_4O_3$: C, 51.90; H, 7.14; N, 22.02.)

Experiment with phenylalanine as a tetramic acid precursor. Nonlabeled 3-acetyl-5-benzyltetramic acid used as carrier was prepared as described by Harris et al. (2). By treatment of the Cu salt with 2,4-dinitrophenylhydrazine as above, the hydrazone was obtained. This compound lost its radioactivity on recrystallization, melting point 246 to 248 C (decomposition), m^+/e 411. (Found: C, 55.34; H, 4.32; N, 16.89. Calculated for $C_{19}H_{17}N_5O_6$: C, 55.40; H, 4.17; N, 17.04.)

In another experiment, the semicarbazone of the tetramic acid was prepared, melting point 202 to 203 C (reported as 191 to 192 C [7]). This compound also lost its radioactivity on recrystallization, indicating that phenylalanine cannot be used by the organism as a 3-acetyltetramic acid precursor.

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