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ISOLATION OF *N*-ACETYL-3,4-DIHYDROXY-L-PHENYLALANINE FROM *STREPTOMYCES AKIYOSHIENSIS*

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ABSTRACT.—*N*-Acetyl-3,4-dihydroxy-L-phenylalanine (*N*-acetyl-L-DOPA) was isolated from cultures of *Streptomyces akiyoshiensis*. The structure was deduced from spectroscopic data and confirmed by comparison to a standard sample synthesized from acetic anhydride and L-DOPA. *N*-Acetyl-L-DOPA does not appear to be involved in the biosynthesis of 5-hydroxy-4-oxo-L-norvaline (HON), the major metabolite of *S. akiyoshiensis*.

A non-protein amino acid, 5-hydroxy-4-oxo-L-norvaline (HON), which possesses antitubercular (1) and antifungal (2) properties is produced by the soil bacterium *Streptomyces akiyoshiensis* (Streptomycetaceae) (3,4). Aspartate and acetate have been identified as the biosynthetic precursors of HON by isotopic feeding experiments (5), and ongoing investigations to obtain additional biosynthetic information are focused on mutants of *S. akiyoshiensis* unable to synthesize HON. Three groups of nonauxotrophic mutants have been obtained, and the relative order of the blocked steps has been determined by cross-feeding experiments; for example, culture supernatant obtained from mutant L138 stimulates HON synthesis in mutants L127 and L167 (6). Inasmuch as this effect could be explained by the accumulation in mutant L138 cultures of a biosynthetic intermediate (or a related compound) that is converted to HON by the other mutants, the metabolites accumulated by mutant L138 have been investigated.

Although HON is readily detected in culture supernatants by hplc using precolumn derivatization with *o*-phthalaldehyde (7), neither HON nor any other metabolite was detected by this method in culture supernatants of mutants L127, L138, and L167 over incubation for four days on casein-starch medium. Because this derivatization method is selective for primary amines, and intermediates of HON biosynthesis need not be free amino acids (5), a bioassay-directed purification of the metabolite(s) accumulated by mutant L138 was undertaken. Chromatography (Sephadex G-10) yielded three fractions that contained a small fraction of the mass applied and stimulated HON synthesis by mutant L127. Examination by ^{1}H-nmr spectroscopy of the lyophilized residues from these fractions revealed signals corresponding to both aromatic and aliphatic protons. Direct hplc analysis detected uv-absorbing material that was not retained on a reversed-phase column eluted with aqueous MeOH. When an ion-pairing hplc method (8) was employed to increase the retention of acidic substances on the reversed-phase column, two substances were detected at retention times of 2.7 (λ max 292 nm) and 3.5 (λ max 280 nm) min. The metabolite with R_t 3.5 min was present in both the wild-type organism and in mutants (L127, L138, and L167) presumed to be blocked at three different stages in the HON pathway (6). In the wild-type organism the metabolite accumulated over nine days of incubation on casein-starch medium, but production was more rapid during the first four to five days. It was isolated from acidified culture supernatant by *n*-BuOH extraction and successive chromatography on Amberlite XAD-2 and Sephadex G-10.

The ^{1}H- and COSY nmr spectra of

the hygroscopic product (12 mg) showed an ABX system at chemical shifts typical for the $CHCH_2$ unit of an amino acid, and a three-proton singlet near 2 ppm, most likely due to a methyl group attached to a carbonyl. Corresponding signals due to CH, CH_2, and CH_3 (DEPT analysis) at appropriate chemical shifts (59.1, 39.6, and 24.5 ppm, respectively) were present in the ^{13}C-nmr spectrum, along with two signals in the carbonyl region. A carboxylic acid (3500–2500 and 1728 cm^{-1}) and an amide (1654 cm^{-1}) were indicated by the ir spectrum; a derivatized nitrogen could account for the lack of peaks observed by hplc analysis of *o*-phthalaldehyde-treated samples. The molecular ion at *m/z* 239 was consistent with the presence of a nitrogen atom, and the loss of acetamide from the molecular ion was characteristic of *N*-acetyl aromatic amino acids (9).

An unsymmetrical, trisubstituted aromatic ring was supported by a three-proton pattern of resonances (6.6–6.9 ppm) in the ^{1}H-nmr spectrum, and by two groups of three signals in the ^{13}C-nmr spectrum corresponding to substituted (133–147 ppm) and unsubstituted (118–124 ppm) aromatic carbons. Two phenolic groups were indicated by the similar downfield shifts of two ^{13}C-nmr signals, and the major fragment ion in the mass spectrum (*m/z* 123) corresponding to a dihydroxytropylium ion. Of the six possible arrangements for two identical and one other substituent on an aromatic ring, the substitution pattern of 3,4-dihydroxyphenylalanine (DOPA) is most consistent with the ortho and meta couplings displayed in the ^{1}H-nmr spectrum by the upfield aromatic proton. A sample of *N*-acetyl-L-DOPA prepared by acetylation of L-DOPA in aqueous solution (10,11) provided nmr, ir, and mass spectral data identical to those of the isolated compound. Isolated and synthetic samples had identical R_fs by hplc and a mixture co-eluted as a single, symmetrical peak. The optical rotation of the synthetic sample after Sephadex G-10 chromatography (+68.2°) corresponded to that of the isolated sample (+67.2°), establishing that *N*-acetyl-L-DOPA is produced by *S. akiyoshiensis*.

Although L-DOPA is widely distributed in nature, previous reports on the natural occurrence of its acetylated derivative have been limited to microbial (12,13) and plant cell (14) cultures supplemented with *N*-acetyl-L-tyrosine. Also, *N*-acetyl-L-DOPA exhibited a dose-dependent cytotoxicity to melanoma cell lines and prolonged the life span of melanoma-bearing mice (11).

Because hplc analysis has shown that *N*-acetyl-L-DOPA accumulates in culture fluids of both the wild-type organism and each of the three mutants (L127, L138, and L167), and that synthetic *N*-acetyl-L-DOPA does not stimulate HON production in cultures of mutants L127 and L167, it is unlikely that this aromatic amino acid is an intermediate in HON biosynthesis. However, other acetylated amino acids are possible biosynthetic intermediates of the HON pathway (5), and acetyltranferase enzymes specific for L- (15) and D- (16) amino acids are known. The discovery of *N*-acetyl-L-DOPA provides a means to investigate amino acid acetylation in *S. akiyoshiensis*.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The nmr spectra were recorded on a Bruker AC 250F spectrometer using standard Bruker programs, and chemical shifts are reported in ppm referenced to HOD for ^{1}H-nmr spectra and to external dioxane for ^{13}C-nmr spectra. The ir spectra were obtained on a Nicolet 510P Ft-ir spectrometer in a N_2 atmosphere, and mass spectra were obtained by electron impact (70 eV) on a CEC 21-104 mass spectrometer. Optical rotations were measured on a Perkin-Elmer 141 polarimeter at 589 nm. Optical density measurements and uv spectra were obtained in 1-cm cuvettes on a Hewlett-Packard 8452A diode-array spectrophotometer. A Hewlett-Packard 1050 diode-array detector was connected to hplc equipment described previously (7). Maximum centrifugal forces are given.

MICROORGANISM.—*Streptomyces akiyoshiensis*

(ATCC 13480, American Type Culture Collection, Rockville, MD and mutants L127, L138, and L167 (6) were maintained at −20° as spore suspensions in 20% (w/v) glycerol with optical densities (640 nm) of 0.2 for a 1/50 dilution. Spore suspensions were prepared from cultures incubated for 7 days on tomato paste-oatmeal agar (Primo tomato paste, Heinz oatmeal pablum, and Difco Bacto agar, each 20.0 g/liter). Vegetative inocula were obtained by addition of spore suspension (50 μl) to a malt extract (10.0 g/liter), yeast extract (4.0 g/liter), and glucose (4.0 g/liter) medium (pH 6.0, 30 ml per 250-ml baffled flask), and incubation for 3 days at 27° and 220 rpm on a rotary shaker (3.8-cm eccentricity). For metabolite production by *S. akiyoshiensis*, casein-starch medium (300 ml per 2-liter Erlenmeyer flask) was inoculated with vegetative mycelium (3 ml), and incubated as described above. Casein-starch medium contained (per liter): casein (4.0 g, BDH), soluble starch (30.0 g, BDH), KH_2PO_4 (0.75 g), K_2HPO_4 (1.75 g), $MgSO_4$ (0.20 g), $FeSO_4 \cdot 7H_2O$ (9 mg), NaCl (10 mg), $CaCl_2$, (10 mg), H_3BO_3 (7.2 μg), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (45 μg), $CuSO_4 \cdot 5H_2O$ (45 μg), $MnCl_2 \cdot 4H_2O$ (45 μg), $ZnCl_2$ (180 μg), $FeCl_3 \cdot 6H_2O$ (540 μg). The pH was adjusted to 6.5 using 3 M HCl prior to autoclaving.

HPLC ANALYSIS.—Separations were achieved on a Beckman Ultrasphere ODS column (5 μm, 4.6×45 mm) fitted at the inlet with an Upchurch Scientific C-130B guard column (2×20 mm) packed with Perisorb RP-18 (30–40 μm). Samples taken from cultures were clarified by centrifugation (15,850 g, 5 min) prior to analysis, and chromatographic fractions were used directly. Amino acids were analyzed as fluorescent *o*-phthalaldehyde derivatives (7), and separations of uv-absorbing metabolites were achieved using a binary gradient (8) formed between solutions of KH_2PO_4 (20 mM)/tetrabutylammonium hydrogen sulfate (10 mM), and a mixture of KH_2PO_4 (40 mM)-tetrabutylammonium hydrogen sulfate (20 mM) and MeOH (1:1 v/v). The KH_2PO_4/tetrabutylammonium hydrogen sulfate solutions were adjusted to pH 5.0 with 5 M NaOH, filtered (0.45 μm), and degassed under reduced pressure (water aspirator). The absorbance of the hplc effluent was monitored simultaneously at 280 nm (4-nm bandwidth) and at 214 nm (4-nm bandwidth), each referenced to 500 nm (80-nm bandwidth).

N-ACETYL-3,4-DIHYDROXY-L-PHENYLALANINE (*N*-ACETYL-L-DOPA).—Cultures (96 h) from 14 2-liter Erlenmeyer flasks were centrifuged (23,400 g, 20 min), and the supernatant was filtered to remove floating debris. The filtrate (3,650 ml) was supplemented with NaCl (5 g/100 ml) and acidified (6 M HCl to pH 1.5). The resulting solution was divided into three equal portions, and each was extracted with *n*-BuOH (2×500 ml). The combined *n*-BuOH extracts were washed sequentially with 0.5 M HCl (150 ml), H_2O (2×300 ml), and 0.05 M K_2CO_3 (3×200 ml). The combined K_2CO_3 extracts were acidified (6 M HCl to pH 1.5), concentrated to 100 ml *in vacuo*, and freeze dried. The brown residue (8.09 g) was dissolved in H_2O (60 ml) and centrifuged (1,400 g, 5 min) to remove particulate material. The supernatant was acidified (6 M HCl to pH 1.5) and applied to an Amberlite XAD-2 column (2.5×48 cm) equilibrated in H_2O. The column was eluted with H_2O, and fractions (20 ml) were collected. Fractions containing metabolite (Nos. 56–77) were combined and freeze-dried. The residue (0.05 g) was dissolved in H_2O (3 ml). The solution was acidified (6 M HCl to pH 1.5) and applied to a second Amberlite XAD-2 column (1.5×59 cm). Fractions 20–38 (20 ml each) were combined, concentrated to about 50 ml *in vacuo*, and lyophilized. The resulting white hygroscopic solid (16.2 mg) was dissolved in H_2O (1 ml) and applied to a Sephadex G-10 column (1.5×87 cm). The column was eluted with H_2O, and fractions 37–53 (2.5-ml each) were combined. After concentration to 10 ml *in vacuo* and lyophilization, a white hygroscopic solid (12.0 mg), which gave a single spot (R_f 0.36) on tlc [Si gel, CH_2Cl_2-MeOH-AcOH (4.5:0.5:0.4), detected by I_2], was obtained: $[\alpha]^{24.5}D$ +67.2° (*c*=1.09, MeOH); uv (H_2O) λ max (ε) 198 (34,200), 224 (sh, 5,360), 280 nm (2,210); ir ν max (KBr) 3342, 3039, 2936, 2624, 1728, 1654, 1609, 1530, 1451, 1381, 1287, 1124 cm^{-1}; ^1H nmr (D_2O, 250 MHz) δ 6.87 (1H, d, *J*=8.2 Hz), 6.81 (1H, d, *J*=ca. 1.5 Hz), 6.72 (1H, dd, *J*=8.2 and ca. 1.5 Hz), 4.42 (1H, dd, *J*=8.7 and 4.7 Hz), 3.09 (1H, dd, *J*=14.0 and 4.9 Hz), 2.82 (1H, dd, *J*=13.9 and 8.7 Hz), 1.96 (3H, s); ^{13}C nmr (D_2O, 62.9 MHz) δ 180.88 (s), 176.06 (s), 146.47 (s), 145.29 (s), 133.20 (s), 124.30 (d), 119.60 (d), 118.84 (d), 59.12 (d), 39.61 (t), 24.54 (q); eims *m/z* 239 [M^+] (10), 222 (2), 221 (4), 181 (6), 180 (54), 124 (11), 123 (100).

Thermal decomposition of *N*-acetyl-L-DOPA occurred in the probe of the mass spectrometer; successive scans showed decreases in the relative intensities of the molecular ion peak and the peak at *m/z* 180 together with increases in the height of peaks at *m/z* 222 and 221. A similar, but much less pronounced, change in peak intensities was observed for *N*-acetyl-L-tyrosine. The lability of *N*-acetyl-DOPA was noted in an early synthetic investigation (17), and our samples colored rapidly at room temperature or on storage at 4° for several weeks.

ACETYLATION OF L-DOPA.—Ac_2O (2.0 ml) was added dropwise over 1 h to a stirred suspension of L-DOPA (0.20 g, 1.0 mmol, Aldrich) in H_2O (2.5 ml). The mixture was stirred for an additional 1 h at room temperature and concentrated *in vacuo* to yield a light yellow oil. The oil was dissolved in

H_2O (5 ml), reconcentrated *in vacuo* to remove HOAc, and chromatographed on an Amberlite XAD-2 column as described above to yield a slightly yellow hygroscopic solid (168 mg, 68%): $[\alpha]^{22}D$ +45.8° (*c*=1.10, MeOH) {lit. (10) $[\alpha]D$ +37.7° (*c*=0.82, MeOH)}. After Sephadex G-10 purification as above, a white hygroscopic solid, $[\alpha]^{19.5}D$ +68.2° (*c*=1.10, MeOH), with spectral properties matching those reported for the natural product, was obtained.

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