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CHEMICAL CONSTITUENTS OF HALOPHILIC FACULTATIVELY ANAEROBIC BACTERIA, 1

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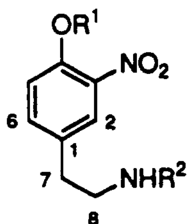
ABSTRACT.—Two new nitrotyramine derivatives, **1** and **2**, along with five known aromatic compounds, were isolated from the culture broth of a facultatively anaerobic, halophilic bacterium isolated from a sediment from the Great Salt Plains, Alfalfa County, Oklahoma. The structures of the new compounds were determined from spectral data and were confirmed by synthesis from tyramine hydrochloride. Compound **1** showed cytotoxicity against the murine leukemia P-388 cell line (IC_{50} 3 μg/ml).

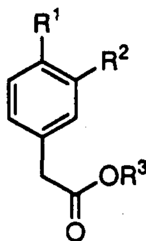
A program to culture halophilic anaerobic bacteria and evaluate their metabolites as sources of inhibitors of enzymes relevant in anticancer drug discovery has been initiated by our group. We were prompted to explore the potential of this class of bacteria as sources of antitumor metabolites because these microorganisms have not yet been studied for this purpose. Limited investigations of marine bacteria have yielded interesting biologically active compounds (1–7).

We have sampled marine sediments, tissues of marine organisms, and anaerobic saline environments such as the Great Salt Plains, Alfalfa County, Oklahoma, for both obligate and facultative anaerobes. We report here some metabolites from a bacterial strain initially selected for study because its extracts were weakly active in a tyrosine kinase $pp60^{src}$ inhibition assay. Although the source of this bioactivity has not been identified, we report here some metabolites of this bacterium, one of which inhibits the growth of P-388 murine leukemia cells.

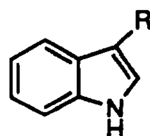
EtOAc extracts of the broth of the initial culture of this strain inhibited tyrosine kinase $pp60^{src}$ with an IC_{50} value of 210 μg/ml. The organism was recovered from cold storage (−20° in anoxic glycerol) and 8- and 18-liter recultures were grown. Extracts of both these cultures inhibited tyrosine kinase $pp60^{src}$. From the extract of the broth of the first bacterial culture (8 liters), small amounts of compounds **1–3** and **5** were isolated by conventional chromatographic procedures as outlined in the Experimental. From the 18-liter culture, compounds **3** and **5–8** were isolated. Thus, only **3** and **5** were common to both cultures. The remaining compounds were either truly unique to the specific batch or were pro-



1 R^1=H, R^2=C(O)CH(CH$_3$)$_2$
1a R^1=R^2=C(O)CH(CH$_3$)$_2$
2 R^1=H, R^2=C(O)CH$_2$CH(CH$_3$)$_2$
2a R^1=R^2=C(O)CH$_2$CH(CH$_3$)$_2$



	R^1	R^2	R^3
3	OH	NO_2	H
4	OMe	NO_2	Me
5	H	H	H



6 R=CHO
7 R=COOH
8 R=CN

duced in such different amounts that they were detected only in the separate batches. None of these pure metabolites inhibited tyrosine kinase $pp60^{src}$ and the active ingredient, apparently a very minor constituent, awaits further investigation. Cytotoxicity evaluation carried out in parallel with the enzyme assay revealed that **1** inhibited the growth of murine leukemia cells, P-388 (IC_{50} value of 3 μg/ml). We report herein the isolation and structural elucidation of these compounds.

Compounds **3**, **5**, **6**, **7**, and **8** were identified by comparison of spectral data (^{1}H-nmr, ms, ir) with literature values (8–14).

Compounds **1** and **2** were each obtained as fine needles, and both were deduced to have a nitro group from strong ir absorptions at ca. 1539 and ca. 1325 cm^{-1}. The molecular formula $C_{12}H_{16}N_2O_4$ was established for **1** by hrfabms, *m/z* 253.1179 $[M+H]^+$ (Δ 0.9 mmu). The ir spectrum indicated the presence of OH/NH groups (3290 cm^{-1}, br), a secondary amide (1645 cm^{-1}), and a nitro group (1325 and 1539 cm^{-1}); a carbonyl carbon nmr signal at δ 177.9 supported the amide assignment. The uv spectrum showed absorption maxima at 274 (ε 8860) and 357 (ε 3350) nm resembling those of *o*-nitrophenols (15–17). A 1,3,4-trisubstituted ring pattern was conclusively established by the aromatic three-proton nmr coupling pattern: δ 7.90 (1H, d, J=2.3 Hz, H-2), 7.09 (1H, d, J=8.5 Hz, H-5), and 7.42 (1H, dd, J=8.5 and 2.3 Hz, H-6). The relative position of the substituents in **1** follows from the fact that the chemical shifts correspond to those of 3-nitro-4-hydroxyalkylbenzenes (15–17). Attachment of an aminoethyl group to the benzene moiety was confirmed by its coupled spin-system: δ 5.45 (1H, br, exchangeable) coupled to δ 3.47 (2H, dt, J=6.4 and 7.0 Hz), coupled in turn to δ 2.80 (2H, t, J=7.0 Hz); the δ 2.80 signal was long-range coupled (evident in RCT-COSY) to aromatic proton signals H-2 and H-6. The remaining structural elements, a carbonyl group and an isopropyl group, δ 2.28 (1H, m) and 1.10 (6H, d, J=6.8 Hz) could only be added to give the isobutyryl moiety, thus leading to the final structure **1**, *N*-(2-methylpropionyl)-3-nitrotyramine. A HETCOR nmr experiment provided unambiguous assignment of all protonated carbon signals.

The molecular formula of compound **2**, established as $C_{13}H_{18}N_2O_4$ by hrfabms, *m/z* 267.1335 (Δ=1.0 mmu) for $[M+H]^+$, differs from that of **1** by CH_2. The ir and uv spectra of **2** were very similar to those of compound **1**. The ^{1}H-nmr spectrum of **2** possessed the same signals as **1** for the 3-nitrotyramine residue. ^{1}H-Nmr signals at δ 1.98 (2H, d, J=6.6 Hz), 2.05 (1H, m), and 0.90 (6H, d, J=6.6 Hz) combined with COSY data revealed that the remainder of the molecule consisted of a 3-methylbutanoyl moiety. Compound **2** was therefore determined to be *N*-(3-methylbutanoyl)-3-nitrotyramine.

Amides **1** and **2** were synthesized by reacting 3-nitrotyramine with 2-methylpropionyl and 3-methylbutanoyl chlorides, respectively. Spectral data for synthetic and natural **1** (^{1}H- and ^{13}C-nmr, uv) and **2** (^{1}H-nmr, uv) were identical.

Compounds containing a nitro functionality are relatively rare among natural products (16). Higher plants (17) and fungi (18–20) have been reported to produce nitro-containing secondary metabolites. Only a few nitro compounds have been isolated from marine organisms, e.g., bryozoans (15,16) and red alga (21). It has been suggested that the nitro-containing compounds isolated from both bryozoans and red algae may be of microbial origin (16). Indole-3-carboxaldehyde [**6**] has been reported from a sponge (22), algae (23,24), and a marine pseudomonad (25), while indole-3-carboxylic acid [**7**] is known from red (12,24) and brown algae (13). Indole-3-carbonitrile [**8**] is known as a synthetic product (14) but has not been reported as a natural product.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All nmr spectra were obtained on either Varian XL-300 or VXR-500 spectrometers; chemical shifts are referenced to solvent peaks. Ms were measured with either Hewlett-Packard 5985 B or VG ZAB mass spectrometers. Ir spectra were taken on a Bio-Rad 3240-spc Ft instrument and uv spectra on a Hewlett-Packard spectrophotometer. Prep. hplc was performed using a Spherex 5 C_{18} column (300×10 mm) with uv detection.

BACTERIAL MATERIAL.—Strain HNGS03 was isolated from a sediment collected in October, 1992, from the Great Salt Plains, Alfalfa Co., Oklahoma (26) on a glucose-nitrate medium containing 8% NaCl. Isolation was carried out at 30° using strict anoxic techniques (27). Strain HNGS03 was mass cultured under aerobic conditions on a medium containing (g/liter): NaCl, 80; KCl, 2; NH_4Cl, 1; $MgSO_4{\cdot}7H_2O$, 0.4; K_2HPO_4, 0.2; $CaCl_2{\cdot}2H_2O$, 0.2; $NaNO_3$, 4; *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 10; glucose, 2; sodium succinate, 2; yeast extract, 2; vitamin solution (28), 10 ml; trace metal solution (28), 5 ml. The final pH was 7.3. Cultures were incubated at 30° and held in stationary growth for at least 48 h prior to preparation of extracts.

Strain HNGS03 was characterized phenotypically (29) and was a nonfermentative, denitrifying, motile with peritrichous flagellation, halotolerant, non-spore-forming, Gram-variable rod (1×3 μm). Colonies were pigmented reddish-brown. Anoxic growth was dependent on nitrate. The strain was positive for nitrate, nitrite, ornithine, catalase, and oxidase, but negative for H_2S, indole, phenylalanine, lysine, MR, VP, citrate, gelatin, and urease. Review of the denitrifying bacteria (30) suggested that HNGS03 might be related to the genus *Bacillus*. Cells of HNGS03 were subjected to a fatty acid methyl ester (FAME) analysis by Microcheck, Inc. (Northfield, VT) and the results compared to the FAME profiles of bacteria in the MIDI database (31,32). The FAME analysis indicated that HNGS03 was a strain of *Bacillus marinus* (33). However, HNGS03 was readily distinguished from *B. marinus* on the basis of a number of phenotypic characters, including (character for *B. marinus*): endospore formation (positive); colony pigmentation (not pigmented); salt requirement (requires NaCl for growth); oxidase (negative); H_2S (positive); gelatin hydrolysis (positive); ornithine decarboxylase (negative) (33). Strain HNGS03 was readily differentiated from the other described species of denitrifying bacteria also based on the characters given above. Strain HNGS03 has been deposited in the marine bacteria culture collection in the Department of Botany and Microbiology at the University of Oklahoma, Norman, OK.

EXTRACTION AND ISOLATION.—The first batch (8 liters) of cultured bacterial broth was extracted twice with EtOAc (8 liters each), and the EtOAc layer was evaporated to dryness under reduced pressure to yield 1.68 g of residue. The residue was subjected to solvent partitioning as follows: hexane vs 10% aqueous MeOH; dilution to 30% aqueous MeOH and extraction with CH_2Cl_2; *n*-BuOH extraction of the H_2O layer remaining after vacuum evaporation of most of the MeOH. Evaporation of the solvents *in vacuo* left three fractions, hexane (55.5 mg), CH_2Cl_2 (626 mg), and *n*-BuOH (849 mg). The CH_2Cl_2-soluble fraction was chromatographed over Si gel eluting with a step gradient of MeOH in CH_2Cl_2. Seven fractions were collected. The third fraction was resolved by reversed-phase hplc (H_2O-MeOH, 35:65) to yield phenylacetic acid [**5**] (12.5 mg) as the major component in addition to small amounts of **1** (2.2 mg) and **2** (0.3 mg). Reversed-phase C_{18} hplc of fraction 4 using 50% H_2O in MeOH as eluent yielded a fraction consisting of ca. 85% 3-nitro-4-hydroxyphenylacetic acid [**3**] (10.8 mg), which was methylated by CH_2N_2, and then purified by Sep-pak Si gel (CH_2Cl_2 elution) to afford the pure derivative **4** (6.3 mg).

The broth (18 liters) from a second re-culture was extracted and the extract partitioned in the same manner as described above. The CH_2Cl_2 extract (1.03 g) was fractionated on an LH-20 column using MeOH as eluent to furnish eight fractions. Reversed-phase hplc of the fourth fraction using 45% H_2O in MeOH as eluent, yielded indole-3-carboxylic acid [**7**], indole-3-carbonitrile [**8**], and a mixture consisting of 3-nitro-4-hydroxyphenylacetic acid [**3**] and indole-3-carboxaldehyde [**6**], which was further separated by reversed-phase hplc using 60% H_2O in MeOH as eluent to give pure **3** (2 mg) and **6** (ca. 1.5 mg).

N-(2-Methylpropionyl)-3-nitrotyramine [**1**].—Fine needles from CH_2Cl_2 (2.2 mg); mp 99–100°; uv (EtOH) λ max (ε) 357 (3350), 274 (8860) nm; ir (neat) ν max 3290 (br), 1645, 1539, 1430, 1325, 1258, 1247, 1183 cm^{-1}; ^1H- and ^{13}C-nmr data, see Table 1; hrfabms found *m/z* $[M+H]^+$ 253.1179, calcd for $C_{12}H_{17}N_2O_4$ 253.1188 (Δ 0.9 mmu); lrfabms *m/z* $[2M+H]^+$ 505, $[M+H]^+$ 253; cims *m/z* $[M+NH_4]^+$ 270 (100%), [M+H] 253 (84%).

N-(3-Methylbutanoyl)-3-nitrotyramine [**2**].—(0.3 mg); uv (MeOH) λ max (ε) 358 (4360), 276 (9260) nm; ir (neat) ν max 3300 (br), 1639, 1583, 1535, 1320, 1260, 1170 cm^{-1}; ^1H-nmr data, see Table 1; hrfabms found *m/z* $[M+H]^+$ 267.1335, calcd for $C_{13}H_{19}N_2O_4$ 267.1345 (Δ 1.0 mmu); lrfabms *m/z* $[M+H]^+$ 267.

SYNTHESIS OF 3-NITRO-4-HYDROXYPHENETHYLAMMONIUM NITRATE.—Tyramine hydrochloride (500 mg) was dissolved in H_2O (3.5 ml) and treated at 0° with 56% HNO_3 (1.75 ml). The mixture was stirred at the same temperature for 6

TABLE 1. Nmr Data of Compounds **1** and **2** in $CDCl_3$.[a]

Proton	Compound			
	1		**2**	
	^1H	^{13}C	^1H	^{13}C
1		131.3 s[b]		131.3 s[b]
2	7.90 (d, J=2.3 Hz)	124.5 d	7.90 (d, J=2.2 Hz)	124.4 d
3		133.4 s[b]		133.3 s[b]
4		153.4 s		153.8 s
OH-4.....	10.47 (s)[c]		10.47 (s)[c]	
5	7.09 (d, J=8.5 Hz)	120.2 d	7.09 (d, J=8.5 Hz)	120.2 d
6	7.42 (dd, J=8.5 and 2.3 Hz)	138.3 d	7.43 (dd, J=8.5 and 2.2 Hz)	138.2 d
7	2.80 (t, J=7.0 Hz)	34.6 t	2.80 (t, J=7.0 Hz)	34.7 t
8	3.47 (dt, J=6.4 and 7.0 Hz)	40.2 t	3.48 (dt, J=6.2 and 7.0 Hz)	40.2 t
9	5.45 (br)[c]		5.41 (br)[c]	
10		177.9 s		172.6 s
11	2.28 (m)	35.7 d	1.98 (d, J=6.6 Hz)	46.0 t
12	1.10 (d, J=6.8 Hz)	19.6 q	2.05 (m)	26.1 d
13	1.10 (d, J=6.8 Hz)	19.6 q	0.90 (d, J=6.6 Hz)	22.4 q
14			0.90 (d, J=6.6 Hz)	22.4 q

[a]Measured at 500 MHz for ^1H- and 125 MHz for ^{13}C-nmr spectra; chemical shifts are referenced to solvent peaks: δ_H 7.24 for residual $CHCl_3$ and δ_C 77.0 for $CDCl_3$.

[b]Entries within a column with the same letter may be exchanged.

[c]CD_3OD exchangeable.

h and then kept at 4° overnight. The mixture was stirred at 0° again for 8 h, then the solid was filtered, washed with cold H_2O and MeOH, and dried *in vacuo*. Recrystallization from MeOH yielded 3-nitro-4-hydroxyphenethylammonium nitrate (460 mg) as yellow needles (MeOH): mp 215–216° (dec) [lit. (17) 215–216° dec]; uv (MeOH) λ max (log ε) 222 (4.08), 272 (3.78), 352 (3.50) nm; ^1H nmr (17); ^{13}C nmr (CD_3OD, 125 MHz) (assignments made with aid of HETCOR experiment) δ 155.1 (s, C-4), 139.0 (d, C-6), 136.2 (s, C-3), 130.5 (s, C-1), 126.6 (d, C-2), 122.0 (d, C-5), 42.0 (t, C-8), 33.7 (t, C-7); eims (70 eV) *m/z* $[M]^+$ 182 (20), 152 (5), 135 (100), 105 (40), 77 (59), 46 (74), 43 (17).

SYNTHESIS OF *N*-(2-METHYLPROPIONYL)-3-NITROTYRAMINE [**1**] AND *N*-(3-METHYLBUTANOYL)-3-NITROTYRAMINE [**2**].—A solution of 3-nitro-4-hydroxyphenethylammonium nitrate (147 mg, 0.6 mmol) in pyridine (0.5 ml) was kept in an ice bath while 0.1 ml of isobutyryl chloride was added slowly. The mixture was kept at room temperature for 15 min with occasional swirling. After partitioning between H_2O (3 ml) and CH_2Cl_2 (3 ml), the CH_2Cl_2 layer was subjected to Si gel chromatography (3 ml Sep-pak) (10% Me_2CO in CH_2Cl_2) and then reversed-phase hplc (H_2O-CH_3OH, 35:65) to yield compounds **1** and **1a**. Compounds **2** and **2a** were prepared and purified in the same manner as **1** and **1a**, except that the eluent of the reversed-phase hplc was H_2O-CH_3OH (25:75).

Compound **1**, fine needles from CH_2Cl_2, mp 99–100°; uv and ir data, see above; ^1H- and ^{13}C-nmr data, see Table 1.

Compound **1a** was obtained as fine needles (CH_2Cl_2): mp 116–117°; uv (MeOH) λ max (log ε) 260 (3.86) nm; ir (neat) ν max 3285 (NH), 1755 (C=O, ester), 1645 (C=O, amide), 1540 (NO_2), 1345 (NO_2), 1100 cm^{-1}; ^1H nmr ($CDCl_3$, 500 MHz) (assignments made by COSY) δ 7.85 (1H, d, J=2.1 Hz, H-2), 7.45 (1H, dd, J=8.2 and 2.1 Hz, H-6), 7.11 (1H, d, J=8.2 Hz, H-5), 5.92 (1H, br s, amide H), 3.45 (2H, dt, J=6.1 and 6.9 Hz, H-8), 2.86 (2H, t, J=6.9 Hz, H-7), -HNC(O)CH$(CH_3)_2$: 2.28 (1H, m), 1.07 (6H, d, J=6.9 Hz), -OC(O)CH$(CH_3)_2$: 2.83 (1H, m), 1.30 (6H, d, J=7.1 Hz); ^{13}C nmr ($CDCl_3$, 125 MHz) δ 142.5 (s, C-4), 141.5 (s, C-3), 138.2 (s, C-1), 134.9 (d, C-6), 125.6 (d, C-2), 125.1 (d, C-5), 40.0 (t, C-8), 34.7 (t, C-7); -HNC(O)CH$(CH_3)_2$: 174.6 (s), 35.4 (d), 19.5 (q); -OC(O)CH$(CH_3)_2$: 177.2 (s), 33.9 (d), 18.5 (q); hrfabms *m/z* $[M+H]^+$ 323.1604 (calcd for $C_{16}H_{23}N_2O_5$ 323.1607).

Compound **2** was obtained as fine needles (CH_2Cl_2), mp 88–89°; ir and uv data, see above; ^1H- and ^{13}C-nmr data, see Table 1.

Compound **2a**.—Powder (CH_2Cl_2): mp 66–67°; uv (MeOH) λ max (log ε) 260 (3.73) nm; ir (neat) ν max 3300 (NH), 1760 (C=O, ester), 1640

(C=O, amide) 1540 (NO_2), 1345 (NO_2), 1100 cm^{-1}; ^{1}H nmr ($CDCl_3$, 500 MHz) (assignment made by COSY) δ 7.86 (1H, d, J=2.3 Hz, H-2), 7.46 (1H, dd, J=8.2 and 2.3 Hz, H-6), 7.12 (1H, d, J=8.2 Hz, H-5), 5.87 (1H, br s, amide H), 3.47 (2H, dt, J=6.3 and 6.7 Hz, H-8), 2.87 (2H, t, J=6.7 Hz, H-7), -HNC(O)$CH_2CH(CH_3)_2$: 1.97 (2H, d, J=6.8 Hz), 2.04 (1H, m), 0.89 (6H, d, J=6.2 Hz), -OC(O)$CH_2CH(CH_3)_2$: 2.49 (2H, d, J=7.3 Hz), 2.22 (1H, m), 1.03 (6H, d, J=6.7 Hz); ^{13}C nmr ($CDCl_3$, 125 MHz) (assignment made with aid of HETCOR) δ 142.5 (s, C-4), 141.5 (s, C-3), 138.2 (s, C-1), 134.9 (d, C-6), 125.6 (d, C-2), 125.2 (d, C-5), 40.0 (t, C-8), 34.9 (t, C-7), -HNC(O)$CH_2CH(CH_3)_2$: 170.7 (s), 45.9 (t), 26.0 (d), 22.3 (q), -OC(O)$CH_2CH(CH_3)_2$: 172.8 (s), 42.7 (t), 25.3 (d), 22.3 (q); hrfabms m/z $[M+H]^+$ 351.1913 (calcd for $C_{18}H_{27}N_2O_5$ 351.1920).

Compounds **3–8** were identified by comparison of their spectral data (^{1}H-nmr, ms) with those of the authentic compounds (see text for literature citations).

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