Pergamon

Tetrahedron Vol. 51, No. 11, pp. 3095-3102, 1995

0040-4020/95 $9.50+0.00

0040-4020(95)00071-2

4-Hydroxy-3-Nitrosobenzamide and Its Ferrous Chelate from *Streptomyces murayamaensis*

Martha C. Cone, Chris R. Melville, John R. Carney, Makarand P. Gore, and Steven J. Gould*

Department of Chemistry, Oregon State University, Corvallis, Oregon 97331-4003

Abstract: Two new colored metabolites of *Streptomyces murayamaensis* have been isolated and characterized as 4-hydroxy-3-nitrosobenzamide and its ferrous chelate. These structures have been confirmed by synthesis via a modified Baudisch reaction. A sample of 3-amino-4-hydroxy[2-^{2}H]benzoic acid was fed to *S. murayamaensis*, affording a sample of the 4-hydroxy-3-nitrosobenzamide ferrous chelate deuterated at C-2 (28.5% enrichment, 3.2% incorporation).

Streptomyces murayamaensis produces a variety of aromatic polyketides based on benzo[*b*]fluorenes (kinamycins,[1] kinafluorenone[2]), benz[*a*]anthraquinones (dehydrorabelomycin, **1**[3]), phenanthraquinones (murayaquinone,[4] murayalactone[5]), and benzo[*b*]phenanthridines (phenanthroviridin aglycone[6]), as well as metabolites based in part on aliphatic polyketides (antimycins[7]). A number of these were first detected in extracts of *S. murayamaensis* UV-derived mutants[2,6] that were prepared as part of our program to study kinamycin biosynthesis.[2,8] One target in this effort has been a presumed *seco*-benzo[*a*]anthraquinone **2**, expected to be involved in the conversion of **1** to the benzo[*b*]fluorene skeleton (e.g. **3**) leading to kinamycin D, **4** (Scheme 1). A red acidic metabolite with a UV/vis spectrum (Figure 1) similar to WS 5995C, **5**,[9,10] was first transiently observed in *S. murayamaensis* fermentations and later detected more reproducibly in fermentations of mutant MC2. A new fermentation medium was developed that increased the accumulation of this new compound and greatly diminished accumulation of kinamycins. We now report the isolation, structure, and synthesis of this compound, 4-hydroxy-3-nitrosobenzamide, **6**, and of its iron-complex.

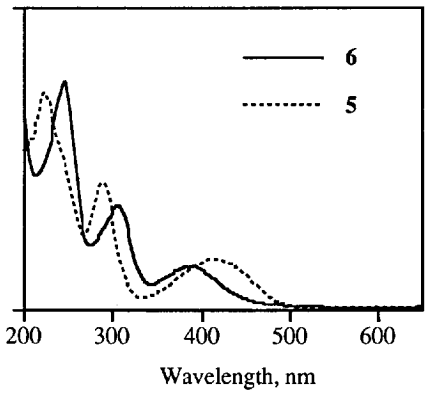
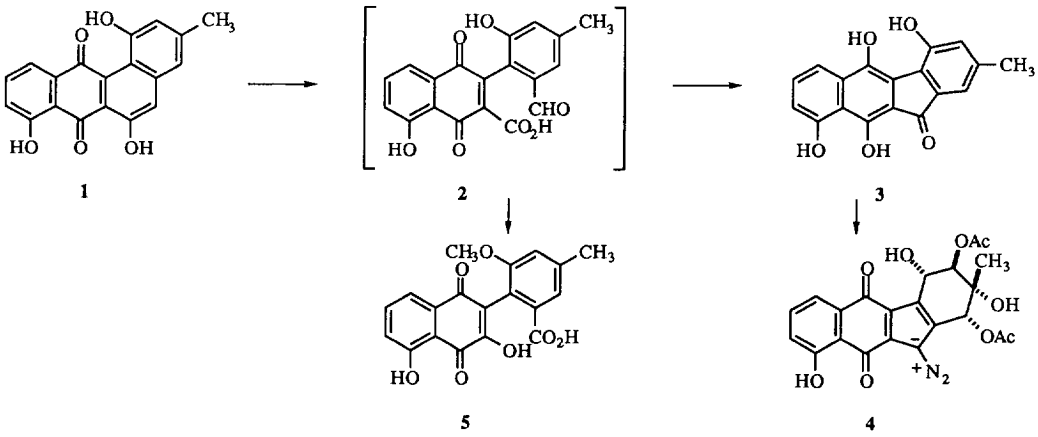


Figure 1. UV/vis Spectra of Antibiotic WS 5995C, **5**, and 4-Hydroxy-3-nitrosobenzamide, **6**.



Scheme 1

RESULTS AND DISCUSSION

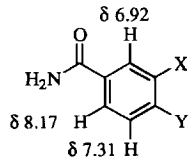
An oatmeal-trace metals medium had been found to be advantageous for accumulating a number of kinamycin biosynthetic intermediates,[11] as well as a variety of other, uncharacterized colored metabolites. However, the acidic red compound was not always observed. Changing to a farina (wheat)-based medium led to reproducible accumulation of the red metabolite, typically peaking after two days of fermentation. Colored metabolites were first extracted into ethyl acetate from the acidified broth and, after the volume was reduced, acidic components were extracted into 1% sodium bicarbonate. This solution was applied to a column of Q-Sepharose and eluted with 0.05 M NaCl. Fractions containing the target metabolite (red-orange in base and greenish-yellow in acid) were combined, adjusted to pH 3, saturated with sodium chloride, and extracted with ethyl acetate. Typically, 50 mg/L of fermentation were obtained as a light yellow solid.

While both TLC and HPLC suggested a pure compound, initial NMR spectra in dimethylsulfoxide contained numerous aromatic resonances in varying proportions, suggesting a mixture. At other times NMR spectra with half as many resonances were obtained, and these changes proved to be pH-dependent. This could be controlled by using methanol as the solvent and adding either acid or base. High resolution chemical ionization mass spectrometry afforded a formula of $C_7H_6N_2O_3$. Due to its acidic behavior, the compound was expected to contain a carboxylic acid function. The IR spectrum, however, indicated the presence of an amide (3467, 3358, and 1652 cm^{-1}). 1D- and 2D NMR spectra (COSY, HMQC, and HMBC) yielded partial structure **7**, requiring that the remaining hydrogen, nitrogen and two oxygens be placed at "X" and "Y". A phenol and a nitroso substituent were the only arrangement consistent with the observed chemical shifts.

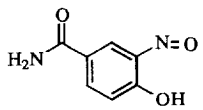
In the course of other work with *S. murayamaensis*, a green, water soluble metabolite was observed. After extraction of the less polar kinamycins with ethyl acetate, the aqueous layer was re-extracted with *n*-

BuOH to afford a bright green solid. Purification on Sephadex LH-20 provided the pure metabolite. It was subsequently found that this compound is consistently produced in fermentations of *S. murayamaensis* mutants MC3 and MC11, in glycerol-asparagine or glycerol-ammonium sulfate media, both of which contain 20 mg Fe^{+2}/L.[11] In the farina medium ,which contains 0.4 mg Fe^{+2}/L, the red metabolite predominated.

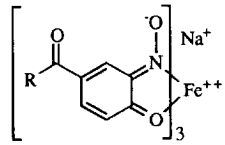
A 3,4-disubstituted benzamide substructure was again recognized from the IR spectrum and the 1D- and 2D-NMR spectra. The UV/vis spectrum of the green metabolite was unaltered by addition of acid or base, and a similar spectrum had been reported for ferroverdin, **8**, also produced by a *Streptomyces.*[12-14]



7



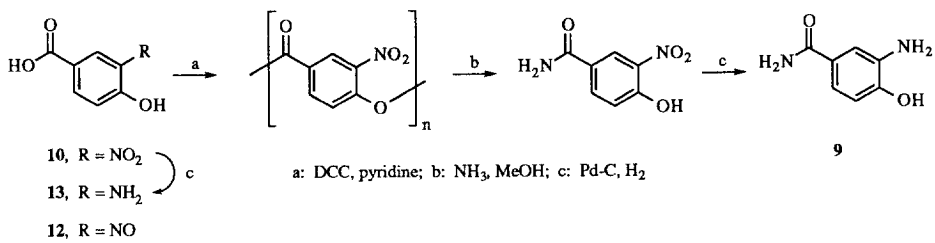
6



8, R = O

11, R = NH_2

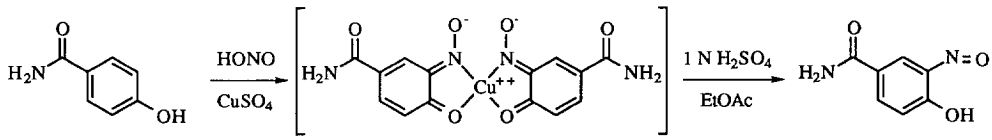
Iron, but no other transition metal, was detected by electron microprobe analysis of the green compound. This explained the presence of an *m/z* 386 ion ($C_{14}H_{10}FeN_4O_6$) in the CIMS (negative mode), which would correspond to an iron chelate of the red compound. Indeed, treating a sample of the red metabolite with $FeSO_4$ in water yielded the same material. Catalytic reduction of either compound gave 3-amino-4-hydroxybenzamide **9**, identical to an authentic sample synthesized from 4-hydroxy-3-nitrobenzoic acid,[17,18] **10**, as shown in Scheme 2. Thus, the structure of the green metabolite is **11**. Consistent with chelate **8**, **11** appears to exist in the quinone-oxime form, as evidenced from the ^{13}C NMR values for C-4 (δ 179.9) and C-3 (δ 158.7).



9

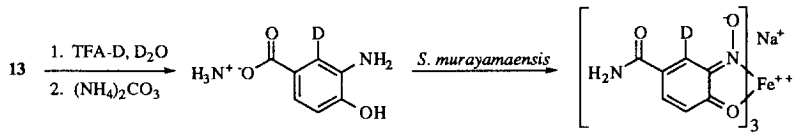
Scheme 2

To further confirm the structures of these compounds, **6** has been synthesized *via* its copper chelate by a modification of the Baudisch reaction (Scheme 3). The literature procedure[15] for removal of the copper required substantial quantities of boiling dilute sulfuric acid, but this could be circumvented by treating the chelate with Chelex-100 resin, affording a 92% yield of **6**. Substituting $FeSO_4$ for $CuSO_4$ yielded the ferrous chelate **11** directly.



Scheme 3

Only one other nitrosophenol has been isolated from a *Streptomyces*: ferroverdin, **8**, is the ferrous chelate of the *p*-vinylphenyl ester of 3-nitroso-4-hydroxybenzoic acid, **12**.[12-14] Both **6** and **11** may be derived biosynthetically from **12**, and **12** derived either by oxidation of the corresponding amino acid, **13**, or by reduction of the nitro acid, **10**. A deuterium-exchanged sample of **13a** was fed to a culture of *S. murayamaensis* mutant MC11 and the derived **11a** isolated. ^{2}H NMR analysis of **11a** showed a strong resonance centered at δ 7.5. Comparison of the integrations of this resonance and the solvent signal yielded a 28.5% enrichment at H-2, corresponding to a 3.2% incorporation of the **13a** fed (Scheme 4). The presence and location of the deuterium label in **11a** was also readily apparent from the ^{13}C NMR spectrum, which showed ^{2}H-induced β-isotope shifts for the C-1 and C-3 resonances (0.05 ppm and 0.09 ppm, respectively), as shown in Figure 2.



Scheme 4

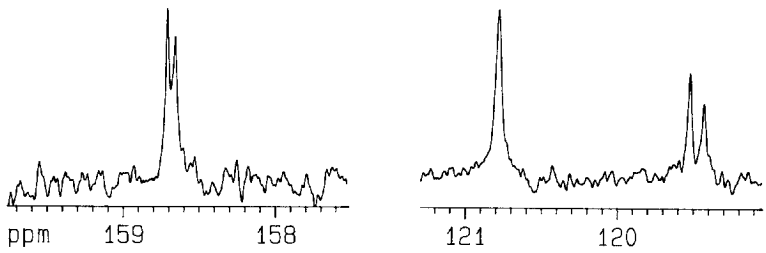


Figure 2. Partial ^{13}C NMR spectrum of **11a** showing resonances for C-5 (δ 120.8), C-1 (δ 119.6), and C-3 (δ 158.7). The latter two carbons show additional signals due to deuterium enrichment at C-4 (β-isotope shifts).

Many microbial siderophores have been characterized, but ferroverdin has represented the sole example of a nitrosophenol. The discovery of *o*-nitrosophenols in a second *Streptomyces* raises the possibility that the biosynthesis of **6**, and its precursor **13**, may be more widespread than has been realized. This question, as well

as the nature of the pathway leading to **13** are currently being addressed. Recently, synthetic aromatic nitroso compounds have been shown to have cytotoxic and antiviral activity – in particular, anti-HIV activity.[16] The bioactivity of **6** is currently under investigation.

EXPERIMENTAL SECTION

General. NMR spectra were obtained on Bruker AC 300 and AM 400 spectrometers. HPLC samples were analyzed on a Waters NovaPak C_{18} radial compression column (0.8 x 10 cm) eluting at 1.50 mL/min over 20 min with a linear gradient from 5 to 95% acetonitrile in H_2O (each contained 0.1% HOAc) with a Waters 600E chromatograph. A Waters 990+ photodiode array detector provided absorbance spectra from 200 to 650 nm with 2 nm resolution of all analyte peaks, and allowed comparisons with an in-house database of metabolites. Mycelia were disrupted using a Heat Systems-Ultrasonics, Inc. Model W225R sonicator.

*Production and isolation of **6** from Streptomyces murayamaensis strain MC2.* Frozen agar plugs of *Streptomyces murayamaensis* mutant strain MC2 were inoculated into a seed medium of Kinako soybean-glucose broth.[11] The production medium consisted of 200 mL of 4% farina and trace metals solution (2 ml/liter)[11] in a 1 L Erlenmeyer flask, and this was inoculated with a 3 day seed culture (5% v/v). After 2 d incubation in a rotary shaker at 27 °C and 290 rpm, the cultures from 5 flasks were harvested by centrifugation, and the orange supernantant was adjusted to pH 3.0 with 1 N HCl and extracted with EtOAc (2 x 350 mL). The aqueous layer was brought to near saturation with NaCl and extracted again with EtOAc. The combined EtOAc extracts were concentrated by rotary evaporation to a volume of ~50 mL and then extracted with a minimum volume of 1% $NaHCO_3$. This extract (from 1 L of culture) was applied to a column of Q-Sepharose (2.5 x 12 cm, HCO_3^-). The column was rinsed with 1 L of water and **6** was then eluted with 0.05 M NaCl while collecting 200-mL fractions (monitored by HPLC and by TLC). The combined column fractions containing **6** were adjusted to pH 3 with 1 N HCl, brought to near saturation with NaCl, and extracted twice with an equal volume of EtOAc. After drying, concentration yielded **6** as a light yellow solid (~50 mg/L of culture), which could be crystallized from EtOH/hexane: mp 145 °C (dec); IR (KBr) 3467, 3358, 2931, 1652, 1613, 1552 cm^{-1}; UV_{max} (MeOH) (ε) 233 (11,000), 280 (sh, 5,300), 373 (2,950) nm; (MeOH/0.1 N NaOH) 200 (10,800), 267 (13,000), 449 nm (4,450); (MeOH/0.1 N HCl) 232 (11,300), 288 (4,600), 372 nm (3,060); ^1H NMR (DMSO-d_6) δ 8.17 (dd, 1H, J = 8.8 Hz, 2.2 Hz), 7.95 (br s, 1H, exch), 7.31 (d, 1H, J = 8.9 Hz), 7.29 (br s, 1H, exch), and 6.92 (d, 1H, J = 2.1 Hz); ^{13}C NMR (DMSO-d_6) δ 109.1, 121.6, 123.9, 137.9, 157.4, 164.8, and 166.7; CIMS (positive mode) calcd for $C_7H_7N_2O_3$ (M+H), 167.0457, found 167.0457.

*Production and isolation of **11** from Streptomyces murayamaensis.* Glycerol-asparagine production medium[11] (400 mL in 2 L Erlenmeyer flasks) was inoculated (5% v/v) with the seed culture. After 36 h, 1.6 L of fermentation broth was adjusted to pH 2.8, sonicated, and filtered through celite. The broth was extracted with EtOAc (4 x 250 mL), filtered, and then extracted with *n*-BuOH (4 x 200 mL). The organic extracts were washed with H_2O (100 mL), evaporated to dryness, and the resulting solids (320 mg) sequentially washed with $CHCl_3$ and EtOAc (2 x 10 mL each) to give 140 mg crude **11**. This was applied to a Sephadex LH-20 column (2.5 x 15 cm) prepared and eluted in MeOH. A brown band eluted, followed by a green band which

was collected and concentrated to give 46 mg. Rechromatography in the same manner but on a column prepared and eluted with 5% H_2O/MeOH gave 35 mg pure **11**: mp >300 °C; IR (KBr) 3426, 3388, 1656, 1599 cm^{-1}; UV_{max} (ε) 203 (33,600), 272 (24,400), 292 (21,400), 434 (5,300), 688 nm (5,700); ^1H NMR (300 MHz, DMSO-d_6) δ 7.93 (dd, 1H, J = 9.1, 2.2 Hz), 7.85 (br s, 1H), 7.53 (d, 1H, J = 2.1 Hz), 7.10 (br s, 1H), 6.97 (d, 1H, J = 9.2 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.9, 167.5, 158.7, 135.7, 120.8, 119.6, 110.2; CIMS (negative mode) 386.1.

*Production and isolation of **11** from Streptomyces murayamaensis from mutant MC3 or MC11.* The whole broth (400 mL) from fermentations using glycerol-ammonium sulfate production medium[11] was diluted with an equal volume of EtOAc. After sonication and filtration, the aqueous layer was adjusted to pH 2-3 and re-extracted with EtOAc (2 x 200 mL). Extraction of the resulting aqueous layer with *n*-BuOH (2 x 200 mL) gave crude **11**, which was chromatographed on Sephadex LH-20 (2.5 x 15 cm, prepared and eluted with MeOH:H_2O = 19:1), typically providing ~8 mg of **11**.

4-Hydroxy-3-nitrobenzamide.[17] 4-Hydroxy-3-nitrobenzoic acid (3.46 g, 18.89 mmol) was dissolved in DMF (27.0 mL) and cooled in an ice water bath. Pyridine (3.10 mL, 38.33 mmol), followed by DCC (3.97 g, 19.24 mmol) were added and the suspension allowed to warm to rt overnight. Dilution with Et_2O (50 mL) and filtration with an EtOH rinse gave 3.40 g of poly-4-hydroxy-3-nitrobenzoic acid as a white powder. A portion of this material (1.25 g) was added to MeOH (100 mL) which had been presaturated with ammonia gas.[18] Additional NH_3 was passed through the suspension for 0.1 h, stirred for 36 h, and then concentrated under a stream of air. Recrystallization from EtOH (110 mL) gave the ammonium salt as a yellow solid, which was dissolved in 0.5 M HCl (100 mL) and extracted into EtOAc (3 x 50 mL) to provide after concentration 0.855 g (68%) of 3-amino-4-hydroxybenzamide as a beige solid: mp 184.2-185.8 °C (lit. 185.5-187.5 °C); IR 1673, 1615, 1531 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.42 (d, 1H, J = 2.2 Hz), 8.02 (dd, 1H, J = 8.7, 2.2 Hz), 7.33 (br t, 1H, J = 10 Hz), 7.16 (d, 1H, J = 8.7 Hz), and 6.99 (br t, 1H, J = 10 Hz); ^{13}C NMR (NH_4 salt, DMSO-d_6) δ 168.0, 167.4, 136.0, 132.6, 127.3, 125.7, and 114.1; EIMS *m/z* (rel intensity) 182.1 (0.3%), 166.1 (1.5%), 100.1 (12%), 84.1 (100%); HREIMS calcd for $C_7H_6N_2O_4$ 182.0328, found 182.0328.

*3-Amino-4-hydroxybenzamide, **9**.* **A.** A solution of 3-nitro-4-hydroxybenzamide (0.853 g, 4.68 mmol) in MeOH (10.0 mL) was treated with 10% Pd-C (3.2 mg) and stirred 1 h under an atmosphere of H_2. The mixture was then filtered and concentrated to give 705.4 mg **9** (99%) as a tan solid: mp 167.2-170.0 °C; IR (KBr) 3360, 1655, 1585, 1403, 1295 cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.54 (br s, 1H), 7.14 (d, 1H, J = 2.1 Hz), 6.98 (dd, 1H, J = 8.2, 2.1 Hz), 6.90 (br s, 1H), 6.64 (d, 1H, J = 8.2 Hz), 4.68 (br s, 2H), and 3.41 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 168.6, 146.9, 136.1, 125.7, 116.6, 114.1, and 113.3; CIMS *m/z* (rel intensity) 152.0 (94%, M^+), 136.0 (74%), 84 (100%); HREIMS calcd for $C_7H_8N_2O_2$ 152.0586, found 152.0586. **B.** MeOH (5 mL) was added to a mixture of **6** (12 mg) and10% Pd-C (20 mg), and the mixture stirred under an atmosphere of H_2. The suspension was filtered, and concentration afforded **9** (10 mg), identical to authentic sample by ^1H- and ^{13}C NMR, CIMS, and by HPLC. **C.** A solution of **11** (1.4 mg) in MeOH (2 mL) was stirred with 10% Pd-C (0.6 mg) under an atmosphere of H_2. HPLC analysis of the colorless solution showed **9** as the only product.

4-Hydroxy-3-nitrosobenzamide, 6. 4-Hydroxybenzamide (685 mg, 5 mmol) was added to NaOAc buffer [glacial HOAc (1.5 mL) and H_2O (2.5 mL) with the addition of NaOAc to pH 4.2] and dissolved by boiling. After cooling, this solution was added to an aqueous solution (25 mL) of $NaNO_2$ (865 mg, 12.5 mmol) and $CuSO_4.5H_2O$ (625 mg, 2.3 mmol), and the deep green solution started to turn purple within a few min. After 3 d and an additional 14 d, the dark purple copper complex was recovered by filtration yielding a total of 623 mg (65%). A portion of the insoluble copper complex (133 mg) was suspended in 0.01 M ammonium bicarbonate (25 mL) and the suspension sonicated in an ultrasonic water bath to disperse it finely. Chelex-100 resin (~10 ml) was added and the suspension was stirred overnight. The orange-red solution was decanted and the resin rinsed with H_2O. The combined solutions were brought to near saturation with NaCl, overlaid with 0.5 vol of EtOAc, acidified and extracted with additional EtOAc to yield 53 mg of **6**. Further extraction of the resin with MeOH (50 mL) yielded an additional 50 mg of **6** (total yield 60%): IR (KBr) 3467, 3358, 2931, 1652, 1613, 1552 cm^{-1}; UV_{max} (MeOH) 233, 280 (sh), 373 nm; ^1H NMR (DMSO-d_6) δ 8.17 (dd, 1H, J = 8.8 Hz, 2.2 Hz), 7.95 (br s, 1H, exch), 7.31 (d, 1H, J = 8.7 Hz), 7.29 (br s, 1H exch), and 6.92 (d, 1H, J = 2.2 Hz); ^1H NMR (methanol-d_4) δ 8.13 (dd, 1H, J = 8.8, 2.1 Hz), 7.50 (d, 1H, J = 2.1 Hz), 7.26 (d, 1H, J = 8.8 Hz); ^1H NMR (methanol-d_4 + DCl) δ 8.15 (dd, 1H, J = 8.8, 2.2 Hz), 7.50 (d, 1H, J = 2.1 Hz), 7.29 (d, 1H, J = 8.9 Hz); ^1H NMR (methanol-d_4 + NaOD) δ 7.75 (dd, 1H, J = 9.4, 2.7 Hz), 7.27 (d, 1H, J = 2.2 Hz), 6.86 (d, 1H, J = 9.4 Hz); ^{13}C NMR (DMSO-d_6) δ 109.1, 121.6, 123.9, 137.9, 157.4, 164.8, and 166.7.

Sodium tris(4-hydroxy-3-nitrosobenzamide) ferrate (1-), 11. 4-Hydroxybenzamide in NaOAc buffer was prepared as above and added to 2% HOAc (25 mL) containing $NaNO_2$ (865 mg) and $FeSO_4.7H_2O$ (700 mg). The solution turned deep red within a few min, and was stirred overnight. The deep green, soluble iron complex was adsorbed onto a column of ODS (3.3 x 14.0 cm, Baker) equilibrated with H_2O, and the column was washed first with H_2O to remove inorganic salts, and then with MeOH to elute the organic materials. The green organic eluent was concentrated to dryness. A portion of the product (50 mg) was dissolved in 5% aqueous MeOH and chromatographed on Sephadex LH-20 (2.7 x 29 cm, same solvent), affording **11** (23.5 mg), identical with the natural product (HPLC retention time, and UV and ^1H NMR spectra).

Ammonium 3-amino-[2-^2H]-4-hydroxybenzoate, 13a. 3-Amino-4-hydroxybenzoic acid (526 mg, 3.43 mmol) was dissolved in trifluoroacetic acid-*d* (10.0 g) and deuterium oxide (10.0 g). This solution was heated at reflux for 1 d, then diluted with Et_2O (20.0 mL), filtered, and rinsed with Et_2O. The purple trifluoroacetate salt thus obtained was dissolved in ammonium bicarbonate (25 mL, 1 M, pH 8.5) and lyophilized to give 585 mg **13a** (100%) as a brown solid: 73% ^2H at H-2 (^1H NMR); EIMS *m/z* (rel intensity) 154.1 (100%), 153.1 (37%), 137.1 (25%); HREIMS calcd for $C_7H_6{}^2HNO_3$ 154.0489, found 154.0489.

Incorporation of ammonium 3-amino-[2-^2H]-4-hydroxybenzoate, 13a. A solution of **13a** (206.4 mg) in H_2O (10 mL) was added aseptically in equal aliquots by syringe to two 400-mL cultures of strain MC11. Additions were made 12, 15, 18, 21, and 24 h after inoculation, and the cultures harvested at 36 h. HPLC analysis (254 nm) at this point indicated the presence of 18 mg **11a**. Extraction gave 72 mg of crude material, which after purification on Sephadex LH-20 yielded 11.2 mg of **11a**: ^2H NMR (DMSO, chemical shift

reference and deuterium quantitation) δ 2.5, 7.5 (28.5% enrichment); partial ^{13}C NMR (DMSO-d_6) δ 119.51 and 119.60 (C-1), 158.65 and 158.70 (C-3).

ACKNOWLEDGEMENT

This research was supported by U.S. Public Health Service Grant GM 31715 to S.J.G. The N. L. Tartar Charitable Trust to Oregon State University provided partial support for C.R.M. Professor A. Echavarren (Universidad Autonoma de Madrid) is thanked for a sample of synthetic antibiotic WS 5995C. Professor R. Nielsen (Oregon State University Department of Oceanography) is thanked for the electron microprobe analysis. Ms. Karen Halley, Dr. Donna Minott, and Dr. Nuria Tamayo are thanked for help at various stages of this work. The Bruker AM 400 NMR spectrometer was purchased in part through grants from the National Science Foundation (CHE-8216190) and from the M. J. Murdock Charitable Trust to Oregon State University, and the Bruker AC 300 spectrometer was purchased in part through grants from the Public Health Service Division of Research Resources (RR04039-01) and the National Science Foundation (CHE-8712343) to Oregon State University.

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(*Received in USA* 18 *October* 1994; *accepted* 20 *January* 1995)