Metabolites of a Blocked Chloramphenicol Producer

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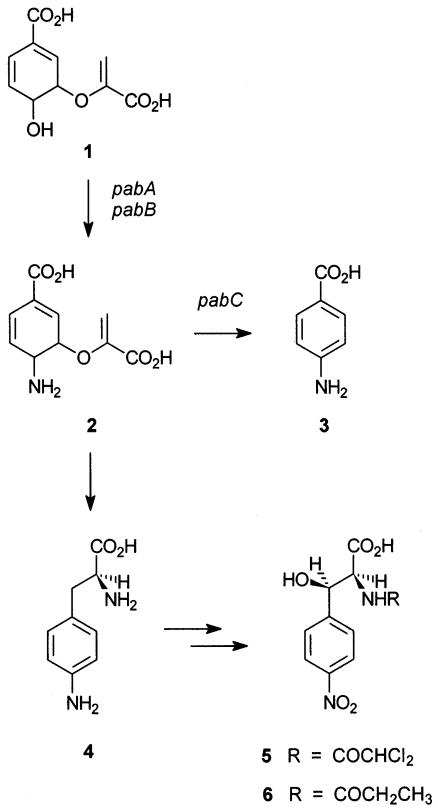
Received July 11, 2002

Addition of *p*-aminophenylalanine (**4**), an advanced biosynthetic precursor of the antibiotic chloramphenicol (**5**), to a *Streptomyces venezuelae pabAB* mutant (VS629) restored chloramphenicol production and led to formation of the non-chlorinated analogue corynecin II (**6**) and four acetanilide derivatives: *p*-(acetylamino)phenylalanine (**7**), *p*-(acetylamino)benzyl alcohol (**13**), *p*-(acetylamino)benzoic acid (**14**), and *p*-(acetylamino)phenol (acetaminophen, **16**). Metabolite structures were deduced from NMR and MS-MS data and established by chromatographic and spectroscopic comparisons with authentic samples. Reference compound **13** was synthesized by reducing the acid chloride of **14**. Shunt pathways are proposed to account for the formation of the metabolites from *p*-aminophenylalanine.

In *Streptomyces venezuelae* (ISP5230), the aromatic metabolites *p*-aminobenzoic acid (**3**, PABA) and chloramphenicol (**5**) are derived from the shikimate pathway[1] via chorismic (**1**) and 4-amino-4-deoxychorismic acid (**2**, ADC).[2] The aromatic ring in **3** is created by a pyridoxal phosphate-catalyzed elimination of the pyruvyl side chain in **2**,[3] whereas alternative reactions catalyzed by the enzyme system arylamine synthase[4] lead to multistep synthesis of L-*p*-aminophenylalanine (**4**). The plausible sequence of functional group transformations proposed for conversion of **4** to chloramphenicol (**5**) is supported by molecular genetic evidence[5] and incorporation of several isotopically labeled substrates, including L-*p*-aminophenylalanine.[6,7]

Molecular genetic studies have determined that two enzyme complexes, one (consisting of PabA and PabB) for primary metabolism and another (the fused *pabAB* gene product) for secondary metabolism, can catalyze conversion of **1** to ADC (**2**) in *S. venezuelae*.[8] The product of *pabC* associates with ADC synthase to supply the lyase step in the PABA synthase complex catalyzing *p*-aminobenzoic acid (**3**) biosynthesis.[9] Involvement of an ADC synthase in chloramphenicol (**5**) biosynthesis was tested by disrupting the fused *pabAB* gene in *S. venezuelae* ISP5230.[10] The mutant (*S. venezuelae* VS629) was able to produce chloramphenicol only when supplied with *p*-aminophenylalanine (**4**), but high-performance liquid chromatography (HPLC) of the supplemented cultures detected several additional metabolites. The structures of these metabolites, determined in the present investigation, revealed shunt pathways of secondary metabolism in *S. venezuelae*.

Scheme 1. Steps in the Biosynthesis of *p*-Aminobenzoic Acid (**3**) and Chloramphenicol (**5**)



Results and Discussion

Chloramphenicol (12−15 mg/L) was detected by bioassay and HPLC analysis in cultures of the *S. venezuelae* mutant VS629 grown in glucose-isoleucine production medium supplemented with 1 mM *p*-aminophenylalanine. The identity of the antibiotic was verified by purification from EtOAc extracts of cultures and comparison with an authentic sample by co-chromatography (TLC and HPLC). The ability of *p*-aminophenylalanine to restore approximately 40% of wild-type chloramphenicol production in cultures of a blocked mutant in which *pabAB* had been disrupted confirmed the role of *p*-aminophenylalanine in production of the antibiotic by the pathway predicted from isotopic labeling (Scheme 1). Chromatographic analyses of the supplemented cultures indicated that additional metabolites similar in some respects to chloramphenicol were produced by the mutant cultures in yields related to the amount of supplement provided, as well as to the time of addition. To identify these metabolites, the products extracted with EtOAc from cultures (1 L) grown for 120 h were fractionated initially by flash chromatography on silica gel. Elution with a stepwise gradient of MeOH in $CHCl_3$ yielded, in addition to chloramphenicol (14.1 mg), a second *p*-nitrophenylserinol derivative (**6**) identified as

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10.1021/np020306e CCC: $25.00
Published on Web 12/13/2002

corynecin II[11] from its UV absorption maximum at 273 nm, absence of acidic or basic functions, and chromatographic behavior matching an authentic specimen. The yield varied markedly with the age of the vegetative culture used as inoculum for production cultures; with the standard inoculum it was 2.8−3.1 mg/L, but if producing cultures were initiated from young, vigorously growing mycelium, it was as high as 15 mg/L, slightly above that for chloramphenicol. Fractions from flash chromatography collected immediately after elution of corynecin II contained predominantly a mixture of two substances. These were separated and purified by chromatography on silica C_{18} to give **13** (2.6 mg, t_R 4.6 min) and **16** (2.7 mg, t_R 4.15 min).

Examination by HPLC (detector at 246 nm) of the culture filtrate after extraction with EtOAc showed metabolites with t_R 3.40 and 4.00 min. The HPLC signal at t_R 4.0 min was due to an unresolved mixture of **14** and **16**, which was separated and purified by preparative TLC on silica gel. The extracted culture filtrate was acidified with HCl to pH 3, concentrated 20-fold, and fractionated by chromatography on a silica C_{18} column to yield **7** (33 mg, t_R 3.40 min).

The ^{1}H NMR spectra of metabolites **7**, **13**, **14**, and **16** showed an AA′BB′ pattern in the aromatic region and a singlet at about δ 2.1 integrating in a 4:3 ratio, suggesting a *p*-disubstituted aromatic ring and a methyl group bonded to a carbonyl carbon. ^{13}C NMR signals between δ 115 and 135 and at approximately δ 24 supported this conclusion, but maximum UV absorption at 242 or 246 nm indicated that **7**, **13**, and **16** differed from chloramphenicol and corynecin II in not possessing the nitrobenzene chromophore. A loss of 42 mass units observed in the MS-MS spectrum for each compound is consistent with the generation of ketene on fragmentation of an *N*-acetyl group,[12] and the presence of a nitrogen fits with the odd molecular masses. An acetylamino group and a para-disubstituted benzene ring are common to each metabolite, indicating that **7**, **13**, **14**, and **16** differ in the second substituent on the ring.

Metabolite **7** gave a typical purple color reaction with ninhydrin, and a fluorescence-quenching zone (R_f 0.32) by TLC on silica gel F with BuOH−HOAc−H_2O. These together suggested an aromatic amino acid, and an ABX pattern at chemical shifts typical for a $CHCH_2$ unit in an α-amino acid was evident in the ^{1}H NMR spectrum. ESI-MS analysis of **7** revealed $[M + H]^+$ at *m/z* 223; under MS-MS conditions, loss of ketene was observed along with neutral losses of NH_3, H_2O, and CO characteristic of an aromatic α-amino acid,[13] such as *p*-acetylaminophenylalanine (**7**).

Metabolites **13** and **16** were not extracted from EtOAc solution by either aqueous $NaHCO_3$ or HCl, indicating that carboxylic acid and amino functional groups were not present. For **13**, however, additional signals at δ 4.55 (s, 2H) and 64.5 in the ^{1}H and ^{13}C NMR spectra, respectively, suggested a CH_2OH group. An $[M + H]^+$ ion at *m/z* 166, and sequential fragmentations consistent with losses of formaldehyde (*m/z* 136) and ketene (*m/z* 94) further supported *p*-(acetylamino)benzyl alcohol as metabolite **13**.

The ^{1}H NMR spectra of **14** and **16** in CD_3OD displayed only a methyl singlet at δ 2.1 and the aromatic AA′BB′ pattern and suggested a second substituent lacking nonexchangeable protons. Simulated spectra indicated that electron-withdrawing (CO_2H) and electron-donating (OH) substituents are most consistent with the chemical shifts of the aromatic protons in **14** and **16**, respectively. The ^{1}H and ^{13}C NMR data collected for **14** are consistent with values published for *p*-(acetylamino)benzoic acid,[14,15] and UV absorption at 264 nm is consistent with extended conjugation provided by the carboxyl group. The APCI mass spectrum of **14** displayed a strong signal for $[M + H]^+$ at *m/z* 180, and sequential loss of ketene and CO_2 indicated by peaks at *m/z* 138 and 94 in the MS-MS spectrum is consistent with a carboxyl group in **14**.

A hydroxyl group as the second substituent of **16** was indicated in the APCI mass spectrum by $[M + H]^+$ at *m/z* 152, only 18 mass units higher than the $C_6H_4NHCOCH_3$ partial structure, and by extraction from EtOAc into aqueous Na_2CO_3. This implied that **16** is the well-known analgesic acetaminophen, a conclusion supported by agreement between the ^{13}C NMR data for **16** and values published for acetaminophen,[16] and the prominent loss of ketene in the MS-MS spectrum of **16** and the published MS-MS fragmentation of acetaminophen.[17]

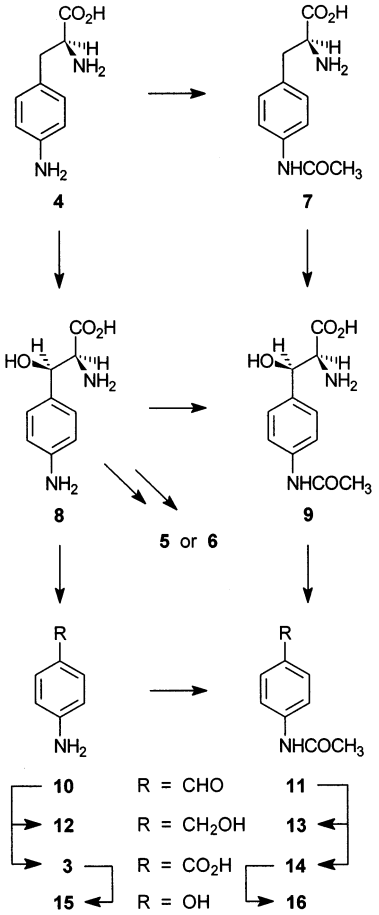
NMR, UV, and mass spectra were obtained for authentic samples of **7**, **13**, **14**, and **16**. A sample of **7** was synthesized previously,[18] and a ^{1}H−^{1}H NOESY experiment demonstrated that the acetyl and aromatic protons are in close proximity. A reference sample for **13** was prepared by sequentially treating *p*-(acetylamino)benzoic acid (**14**) with thionyl chloride and $NaBH_4$ on alumina. Mixtures of sample and reference compounds eluted as a single HPLC peak, and spectra of metabolites and standards were identical, establishing **7** as *p*-(acetylamino)phenylalanine, **13** as *p*-(acetylamino)benzyl alcohol, **14** as *p*-(acetylamino)-benzoic acid, and **16** as acetaminophen.

The dependence of metabolite formation on the presence of *p*-aminophenylalanine (**4**) in the culture and its sensitivity to supplement concentration (vide supra) indicate that the metabolites are derived from the amino acid. Supporting this are common structural features among the metabolites, the role of **4** as an intermediate in both chloramphenicol (**5**)[7] and corynecin (**6**)[19] biosynthesis (Scheme 1), and the formation of both products on addition of **4** to the *pabAB* mutant (VS629). The pathway after the step blocked in the *pabAB* mutant retains all of the necessary reactions, including hydroxylation at the benzylic position to yield *p*-aminophenylserine (**8**), another recognized intermediate in chloramphenicol biosynthesis.[7] The later steps of the corynecin pathway in *Corynbacterium hydrocarboclastus* have not been elucidated; however, the structural similarities suggest a close parallel to the chloramphenicol pathway with introduction of a propionyl unit instead of a dichloroacetyl group by acylation.

A metabolic grid incorporating steps of the chloramphenicol pathway and showing plausible reactions converting **4** to the acetylated metabolites **7**, **13**, **14**, and **16** is presented in Scheme 2 as alternative parallel pathways connected by acetylation steps. Formation of the four *p*-*N*-acetyl metabolites is consistent with acetyl transfer by an unspecific enzyme acting on more than one of the possible substrates shown on the left-hand side of Scheme 2, or with acetylation as an early step in the catabolism of *p*-aminophenylalanine (i.e., **7** → **9** → **11**, **13**, **14**, and **16**). The formation of **7**, as well as chloramphenicol and corynecin II, indicates that **4** is a substrate for both acetylation and hydroxylation reactions and that each potential route is exploited. The ability of a chloramphenicol-producing *Streptomyces* strain to acetylate the *p*-amino group of a biosynthetic intermediate was implied earlier by the isolation of 2-(*S*)-dichloroacetyl-3-(*p*-acetamidophenyl)alaninol.[18]

Formation of **13**, **14**, and **16** from **4** requires cleavage of the aliphatic side chain, which becomes more feasible after hydroxylation at the benzylic position. Both *p*-aminophen-

Scheme 2. Proposed Pathways for the Metabolism of *p*-Aminophenylalanine (**4**)



ylserine (**8**), an intermediate of the chloramphenicol pathway, and its acetylated analogue **9** are potential substrates for retro-aldol reaction, or after oxidation of the secondary alcohol, a retro-Claisen reaction. A similar degradation of *p*-aminophenylalanine involving benzylic hydroxylation before cleavage has been proposed to account for the incorporation of a C_6C_1 unit into the nitroaromatic moiety of the antibiotic aureothin in *Streptomyces thioluteus*.[20] Also, a catalytic antibody-catalyzed retro-aldol cleavage of *p*-acetaminophenylserine has been reported,[21] and an aldolase acting upon a range of phenylserine derivatives has been detected in *Streptomyces tendae*[22] and isolated from *Streptomyces amakusaensis*.[23,24] The enzyme is specific for the 2*S*,3*R* configuration of the substrate, the same absolute configuration as that of chloramphenicol (**1**) and its advanced precursors.

An aldehyde (**11**) formed by a retro-aldol cleavage would readily undergo reduction or oxidation to form metabolites **13** and **14**. Alternatively, a retro-Claisen cleavage would generate a coenzyme A ester,[25] from which **14** could be obtained by hydrolysis and **13** by reduction. The formation of 4-nitrobenzyl alcohol from 4-aminobenzoic acid in a chloramphenicol-producing *Streptomyces*[26] supports the feasibility of carboxyl group reduction. The formation of acetaminophen (**16**) by hydroxylation and acetylation of aniline has been demonstrated in several species of *Streptomyces*.[27,28] In *S. venezuelae* ATCC10712, however, aniline was only acetylated.[29] Alternatively, oxidative decarboxylation of a benzoic acid derivative, as in the flavin monooxygenase-catalyzed conversion of *p*-aminobenzoic acid (**3**) to *p*-aminophenol (**15**) by the mushroom *Agaricus bisporus*,[30,31] would account for the formation of **16**.

The degradative route in Scheme 2 parallels a proposed mechanism for resistance to chloramphenicol of *S. venezuelae* 13s in physiological environments where the genes for chloramphenicol biosynthesis are not expressed.[32] After prolonged exposure to such conditions, the organism develops the ability to metabolically inactivate exogenous chloramphenicol. Evidence to date supports the existence of a chloramphenicol hydrolase[33,34] hydrolyzing chloramphenicol (**5**) to *p*-nitrophenylserinol, which is then *N*-acetylated,[35] or degraded by other competing reactions to *p*-nitrobenzoic acid and *p*-nitrobenzyl alcohol.[32,34] These products, in addition to *p*-aminobenzoic acid (**3**) and 3-hydroxy-4-acetamidobenzoic acid, were among the degradation products characterized from cultures of a soil bacterium growing on chloramphenicol as the sole carbon and nitrogen source.[36]

Experimental Section

General Experimental Procedures. NMR spectra were acquired on Bruker AC250F and AMX 400 spectrometers using standard Bruker spectral acquisition parameters. Chemical shifts (δ) in ^{1}H and ^{13}C NMR spectra are reported relative to the central lines of CHD_2OH in CD_3OD (3.31 and 49.00 ppm, respectively), HOD in D_2O (4.80 ppm), or an acetone spike in D_2O (30.89 ppm).[37] Positive ion atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) mass spectra were acquired on a Micromass Quattro triple quadrupole mass spectrometer by flow injection using a syringe pump. Sample solutions were introduced through a Rheodyne valve equipped with a 10 μL sampling loop. The collision energy for collision-induced dissociation experiments (CID) was provided by argon gas at a nominal pressure of 1×10^{-4} Torr. UV−vis spectra were recorded in 1 cm cuvettes using a Hewlett-Packard model hp8452a diode array spectrophotometer. For HPLC a Beckman System Gold instrument was used. Melting points (uncorrected) were measured in open capillaries using a Gallenkamp melting point apparatus. Chromatography was performed using TLC-grade silica gel H (Merck) and Bakerbond C_{18} silica gel (40 μm, J. T. Baker). $NaBH_4$-Alox reagent[38] was prepared by mixing $NaBH_4$ (1 g in 1 mL of H_2O) with a stirred sample of solid alumina (10 g, 80−200 mesh, Fisher). The solid was dried under reduced pressure for 3 days and stored over phosphorus pentoxide.

Microorganisms, Media, and Incubation Conditions. *Streptomyces venezuelae* ISP5230 (wild-type chloramphenicol producer)[39] and VS629 (*pabAB* mutant blocked in chloramphenicol production)[10] were maintained at −20 °C as stock spore suspensions in 20% glycerol. Spores dispersed in H_2O (10 mL) from agar cultures (MYM agar for ISP5230 and TO agar for VS629) were filtered to remove mycelial fragments pelleted by centrifugation and resuspended in 20% aqueous glycerol. For detection or isolation of metabolites, cultures of *S. venezuelae* VS629[10] were grown in glucose-isoleucine medium[40] supplemented with 1 mM *p*-aminophenylalanine. Shaken cultures (25 mL) were started from a 1% (v/v) washed vegetative inoculum grown in GNY medium[32] and incubated for 7−10 days at 27 °C and 200 rpm in 250 mL Erlenmeyer flasks. Samples of culture broth were removed aseptically and assayed by HPLC.

HPLC Analysis. Samples (20 μL) were injected onto a C_{18} column (45 × 4.6 mm, Beckman Ultrasphere ODS) and routinely eluted with stepped linear gradients from 0 to 50% (4 min) and 50 to 100% (2 min) of MeOH in 0.1 M sodium acetate buffer (pH 4.75). This was followed by 100% MeOH (1 min), a gradient returning to 100% buffer (1 min), and 100% buffer alone (2 min) to reequilibrate the column before the next sample was injected. In some early HPLC analyses a slightly modified gradient was used, and H_2O replaced the buffer.

A chloramphenicol reference sample (200 μg/mL in 25% aqueous MeOH) was injected as a control with each batch of samples. Its retention time varied as the method and HPLC

solvents were altered, but was kept between 6.0 and 7.0 min; the peak area at 273 nm was used to estimate the chloramphenicol concentration in each sample. Unknown metabolites were estimated as chloramphenicol equivalents by comparing their peak areas with that of the reference. For some samples, the detector wavelength differed from 273 nm.

TLC Analysis. Metabolites extracted from cultures with EtOAc, fractions collected by column chromatography, and reference compounds were applied to 1 mm layers of silica gel 60 F254 coated on 5 × 20 or 20 × 20 cm glass plates (Merck). The chromatograms were developed with either $CHCl_3$−MeOH (9:1, v/v) or butanol−acetic acid−H_2O (12:3:5, v/v/v) and air-dried. The plates were viewed under UV light (254 nm) to detect fluorescence-quenching zones and heated at 80 °C after spraying with ninhydrin to detect amino acids.

Extraction and Isolation. Cultures (typically 500 mL total volume) were filtered to remove the mycelium, and the filtrate at pH 7.5 was extracted with EtOAc (3 × 200 mL). After evaporation to dryness, the extract was taken up in a minimum volume of $CHCl_3$ and applied to a dry column (4 × 2.3 cm) of silica gel in a 15 mL sintered glass funnel;[41] solvent was drawn through the column, and substances were eluted in fractions by successive 5 mL solvent additions: $CHCl_3$ (6 fractions) was followed by 95:5 $CHCl_3$−MeOH (12 fractions) and then 90:10 $CHCl_3$−MeOH (4 fractions). Separation of metabolites in the extract was monitored by HPLC analysis of each fraction after it had been evaporated and redissolved in a measured volume of H_2O−MeOH (3:1). Fractions containing the same metabolite or metabolite mixture were combined.

Chloramphenicol was the predominant metabolite in fractions 11−13 and was purified by successive crystallization from $CHCl_3$ and H_2O (colorless needles, mp 152 °C; unchanged on admixture with an authentic specimen). Corynecin II was the main component in fractions 14−16, but was mixed with the trailing edge of the preceding chloramphenicol band and the leading edge of another metabolite (see later). For further purification of corynecin II in pooled fractions 14−16, reversed-phase chromatography on a column (95 × 1.0 cm) of silica C_{18} was used with a linear gradient of MeOH in H_2O as the eluting solvent. Fractions (3.0 mL) were collected, monitored for absorbance at 273 nm, and examined by HPLC. The metabolite recovered from a well-resolved absorbance peak (fractions 44−45) by evaporation of the solvent in vacuo co-chromatographed with corynecin II (HPLC, t_R 5.5 min: TLC, R_f 0.29) in the standard systems used.

Monitoring the eluates from flash chromatography by HPLC with the detector at 246 nm showed two dominant peaks (t_R 4.15 and 4.6 min) in each of fractions 17−21, with a steady decrease in the relative intensity of the peak at t_R 4.15. Fractions 17−21 were pooled, evaporated to dryness, and applied in the minimum volume of H_2O−MeOH (3:1) to a 95 × 1.0 cm silica C_{18} column. Elution with a linear gradient of MeOH in H_2O and evaporation of fractions 34−35 in vacuo gave metabolite **16** (2.7 mg; t_R 4.15 min), while fractions 38−39 gave metabolite **13** (2.6 mg; t_R 4.6 min).

Metabolites retained in culture filtrates extracted with EtOAc at neutral pH were recovered by acidifying the aqueous phase with HCl to pH 3, concentrating 20-fold in vacuo, and fractionating the clarified concentrate by chromatography on a silica C_{18} column (20 × 1.6 cm). Fractions (5 mL) were collected during elution with a shallow gradient of MeOH−H_2O (3:1) in H_2O. Metabolites in the eluate were monitored by UV absorbance and HPLC (detector at 246 nm). Fractions 21−31 were evaporated in vacuo to give metabolite **14** (24.6 mg; t_R 4.00 min). Fractions 41−60 were evaporated in vacuo to give metabolite **7** (33.6 mg; t_R 3.35 min). Metabolites **7** and **14** were also isolated from the acidified culture filtrate concentrate by preparative TLC on silica gel GF_{254} using repeated irrigation with the solvent mixture toluene−acetic acid−H_2O (20:5:1) to separate components (R_f values for **7**, 0.02; **16**, 0.05; **13**, 0.08; **14**, 0.13).

***p*-(Acetylamino)phenylalanine (7):** UV (H_2O) λ_{max} 242 nm; ^{1}H NMR (D_2O, 250.1 MHz) δ 7.40−7.27 (4H, AA′BB′ system, major splittings of 8.2 Hz), 3.96 (1H, X of ABX, J_{AX} = 5.2 Hz, J_{BX} = 7.6 Hz), 3.25 and 3.09 (2H, AB of ABX, J_{AX} = 5.2 Hz, J_{BX} = 7.6 Hz, J_{AB} = 14.6 Hz), 2.15 (3H, s); partial ^{13}C NMR (D_2O spiked with acetone-d_6, 100.6 MHz) δ 173.7, 138.0 (C1′), 133.4 (C4′), 131.4 (C2′,C6′), 123.1 (C3′,C5′), 57.4 (C2), 37.4 (C3), 24.5 (CH_3); ESI$^+$MS (MeOH−H_2O, 1:1, trace HCO_2H, 30 μL/min, 15 V cone) *m*/*z* 223 [M + H]$^+$; CID of *m*/*z* 223 (30 eV) *m*/*z* 206 (44), 177 (100), 164 (41).

Standard *p*-(acetylamino)-L-phenylalanine was prepared previously:[18] UV (H_2O) λ_{max} 244 nm; ^{1}H NMR (D_2O, 250.1 MHz) δ 7.40−7.27 (4H, AA′BB′ system, major splittings of 8.5 Hz), 3.94 (1H, X of ABX, J_{AX} = 5.2 Hz, J_{BX} = 7.9 Hz), 3.24 and 3.08 (2H, AB of ABX, J_{AX} = 5.2 Hz, J_{BX} = 7.9 Hz, J_{AB} = 14.6 Hz), 2.15 (3H, s); ^{13}C NMR (D_2O spiked with acetone-d_6, 100.6 MHz) δ 175.7, 173.8, 137.8 (C1′), 133.8 (C4′), 131.4 (C2′,C6′), 123.3 (C3′,C5′), 57.6 (C2), 37.7 (C3), 24.3 (CH_3); ^{1}H−^{1}H NOESY correlation (D_2O, 250.1 MHz) δ 2.15 (s, 3H)/7.39 apparent d, 1H) and 7.29 (apparent d, 1H); ESI$^+$MS (MeOH−H_2O, 1:1, trace HCO_2H, 30 μL/min, 15 V cone) *m*/*z* 223 [M + H]$^+$; CID of *m*/*z* 223 (30 eV) *m*/*z* 206 (41), 177 (100), 164 (48).

***p*-(Acetylamino)benzyl Alcohol (13).** UV (MeOH) λ_{max} 246 nm; ^{1}H NMR (CD_3OD, 250.1 MHz) δ 7.53−7.28 (4H, AA′BB′ system, major splittings of 8.6 Hz), 4.55 (2H, s), 2.12 (3H, s); partial ^{13}C NMR (CD_3OD, 100.6 MHz) δ 128.6 (C2,C6), 121.1 (C3,C5), 64.9 (CH_2OH), 23.8 (CH_3); APCI$^+$MS (MeOH, 50 μL/min, 15 V cone) *m*/*z* 166 [M + H]$^+$; CID of *m*/*z* 166 (30 eV) *m*/*z* 148 (9), 136 (40), 106 (24), 94 (100), 77 (12), 43 (19), 31 (15); HREIMS *m*/*z* 165.0791 (calcd for $C_9H_{11}NO_2$, 165.0790).

Synthesis of 13. Thionyl chloride (0.052 mL, 0.7 mmol) was added to a mixture of *p*-acetamidobenzoic acid (0.105 g, 0.58 mmol), DMF (5 μL, 0.06 mmol), and THF (15 mL) under N_2 in a 10 mL flask fitted with a $CaCl_2$ drying tube. After stirring at ambient temperature overnight, the mixture was concentrated by vacuum distillation. The pale yellow, solid residue of 4-(acetylamino)benzoyl chloride was dissolved in THF (5 mL) and added to a suspension of $NaBH_4$-Alox[38] (1 g) in THF (15 mL) in a flask fitted with a $CaCl_2$ drying tube. The reaction mixture was stirred at ambient temperature overnight and filtered. The solid residue was washed with THF (10 mL). The wash and the filtrate from the reaction mixture were combined, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was sublimed, yielding a white solid (40 mg, 42%): mp 118.5−119 °C [lit.[42] 119−122 °C]; UV (MeOH) λ_{max} 246 nm; ^{1}H NMR (CD_3OD, 250.1 MHz) δ 7.53−7.27 (4H, AA′BB′ system, major splittings of 8.6 Hz), 4.55 (2H, s,), 2.11 (3H, s); ^{13}C NMR (CD_3OD, 62.9 MHz) δ 171.6 (NHC═O), 139.1, 138.5, 128.6 (C2,C6), 121.1 (C3,C5), 64.9 (CH_2), 23.8 (CH_3); APCI$^+$MS (MeOH, 0.2 mL/min, 15 V cone) *m*/*z* 166 [M + H]$^+$; CID of *m*/*z* 166 (35 eV) *m*/*z* 148 (1), 136 (33), 106 (26), 94 (100), 77 (13), 43 (13), 31 (17).

***p*-(Acetylamino)benzoic Acid (14).** The isolated metabolite was purified by preparative-layer chromatography (silica gel, toluene−acetic acid−H_2O, 20:6:0.2). After development, the fluorescence-quenching band at R_f 0.25 was eluted into dry CH_3CN, which was removed in vacuo to yield a white solid: UV (MeOH) λ_{max} 264 nm; ^{1}H NMR (CD_3OD, 250.1 MHz) δ 7.99−7.65 (4H, AA′BB′ system, major splittings of 8.9 Hz), 2.15 (3H, s); partial ^{13}C NMR (CD_3OD, 100.6 MHz) δ 131.7 (C2,C6), 120.1 (C3,C5), 24.0 (CH_3); APCI$^+$MS (MeOH, 0.2 mL/min, 25 V cone) *m*/*z* 180 [M + H]$^+$; CID of *m*/*z* 180 (30 eV) *m*/*z* 138 (14), 94 (100), 77 (41), 43 (45)); HREIMS *m*/*z* 179.0588 (calcd for $C_9H_9NO_3$, 179.0582).

Standard 4-(acetylamino)benzoic acid was the product of an undergraduate laboratory experiment:[43] mp 252−253.5 °C (H_2O) [lit.[43] 250−252 °C]; UV (MeOH) λ_{max} 264 nm; ^{1}H NMR (CD_3OD, 250.1 MHz) δ 7.98−7.65 (4H, AA′BB′ system, major splittings of 8.9 Hz), 2.15 (3H, s); ^{13}C NMR (CD_3OD, 100.6 MHz) δ 171.9, 169.6, 144.3 (C4), 131.7 (C2,C6), 127.1 (C1), 120.1 (C3,C5), 24.0 (CH_3); APCI$^+$MS (MeOH, 0.2 mL/min, 25 V cone) *m*/*z* 180 [M + H]$^+$; CID of *m*/*z* 180 (30 eV) *m*/*z* 138 (10), 94 (100), 77 (49), 43 (45).

***p*-(Acetylamino)phenol (16):** UV (MeOH) λ_{max} 246 nm; ^{1}H NMR (CD_3OD, 250.1 MHz) δ 7.32−6.70 (4H, AA′BB′ system, major splittings of 8.9 Hz), 2.08 (3H, s); partial ^{13}C NMR (CD_3OD, 100.6 MHz) δ 123.4 (C3,C5), 116.2 (C2,C6), 23.5 (CH_3); APCI$^+$MS (MeOH, 50 μL/min, 20 V cone) *m*/*z* 152 [M + H]$^+$;

CID of *m*/*z* 152 (35 eV) *m*/*z* 110 (75), 93 (41), 65 (56), 43 (100); HREIMS *m*/*z* 151.0638 (calcd for $C_8H_9NO_2$, 151.0633).

Standard *p*-(acetylamino)phenol (Eastman Chemicals): mp 166−167 °C (H_2O); UV (MeOH) λ_{max} 248 nm; ^{13}C NMR (CD_3OD, 62.9 MHz) δ 171.4 (C=O), 155.3 (C1), 131.6 (C4), 123.4 (C3,C5), 116.2 (C2,C6), 23.5 (CH_3); APCI$^+$MS (MeOH, 50 μL/min, 20 V cone) *m*/*z* 152 $[M + H]^+$; CID of *m*/*z* 152 (35 eV) *m*/*z* 110 (68), 93 (35), 65 (67), 43 (100).

Acknowledgment. We thank the Natural Sciences and Engineering Research Council of Canada for financial support; Drs. M. Lumsden and D. L. Hooper for NMR spectra from the Atlantic Region Magnetic Resonance Centre; and Dr. J. S. Grossert for the mass spectra.

References and Notes

(1) Emes, A.; Floss, H.-G.; Lowe, D. A.; Westlake, D. W. S.; Vining, L. C. *Can. J. Microbiol*. **1974**, *20*, 347−352.
(2) Teng, C.-Y. P.; Ganem, B.; Doktor, S. Z.; Nichols, B. P.; Bhatnagar, R. K.; Vining, L. C. *J. Am. Chem. Soc.* **1985**, *107*, 5008−5009.
(3) Green, J. M.; Nichols, B. P. *J. Biol. Chem*. **1991**, *266*, 12971−12975.
(4) Francis, M. M.; Westlake, D. W. S. *Can. J. Microbiol*. **1979**, *25*, 1408−1415.
(5) He, J.; Magarvey, N.; Piraee, M.; Vining, L. C. *Microbiology* **2001**, *147*, 2817−2829.
(6) Siddiqueullah, M.; McGrath, R.; Vining, L. C.; Sala, F.; Westlake, D. W. S. *Can. J. Biochem*. **1967**, *45*, 1881−1889.
(7) McGrath, R.; Vining, L. C.; Sala, F.; Westlake, D. W. S. *Can. J. Biochem*. **1968**, *46*, 587−594.
(8) Chang, Z.; Sun, Y.; He, J.; Vining, L. C. *Microbiology* **2001**, *147*, 2113−2126.
(9) Green, J. M.; Nichols, B. P.; Matthews, R. G. In *Escherichia coli and Salmonella Cellular and Molecular Biology*, 2nd ed.; Neidhardt, F. C., Ed.; ASM Press: Washington, 1996; Vol. 1, Chapter 41, pp 665−673.
(10) Brown, M. P.; Aidoo, K. A.; Vining, L. C. *Microbiology* **1996**, *142*, 1345−1355.
(11) Shirahata, K.; Hayashi, T.; Deguchi, T.; Suzuki, T.; Matsubara, I. *Agric. Biol. Chem*. **1972**, *36*, 2229−2232.
(12) Tu, Y.-P.; Harrison, A. G. *J. Am. Soc. Mass Spectrom*. **1998**, *9*, 454−462.
(13) Dookeran, N. N.; Yalcin, T.; Harrison, A. G. *J. Mass Spectrom*. **1996**, *31*, 500−508.
(14) Bennett, J.; Delmas, M.; Maire, J. C. *Org. Magn. Reson*. **1969**, *1*, 319−327.
(15) Rossi, C.; Picchi, M.; Tiezzi, E.; Corbini, G.; Corti, P. *Magn. Reson. Chem*. **1990**, *28*, 348−354.
(16) Newmark, R. A.; Hill, J. R. *J. Magn. Reson*. **1976**, *21*, 1−7.
(17) Ayrton, J.; Plumb, R.; Leavens, W. J.; Mallett, D.; Dickins, M.; Dear, G. J. *Rapid Commun. Mass Spectrom*. **1998**, *12*, 217−224.
(18) Wat, C.-K.; Malik, V. S.; Vining, L. C. *Can. J. Chem*. **1971**, *49*, 3653−3656.
(19) Nakano, H.; Tomita, F.; Suzuki, T. *Agric. Biol. Chem*. **1976**, *40*, 207−212.
(20) Cardillo, R.; Fuganti, C.; Ghiringhelli, D.; Giangrasso, D.; Grasselli, P.; Santopietro-Amisano, A. *Tetrahedron* **1974**, *30*, 459−461.
(21) Tanaka, F.; Oda, M.; Fujii, I. *Tetrahedron Lett*. **1998**, *39*, 5057−5060.
(22) Evans, D. R.; Herbert, R. B.; Baumberg, S.; Cove, J. H.; Southey, E. A.; Buss, A. D.; Dawson, M. J.; Noble, D.; Rudd, B. A. M. *Tetrahedron Lett*. **1995**, *36*, 2351−2354.
(23) Bycroft, M.; Herbert, R. B.; Ellames, G. J. *J. Chem. Soc., Perkin Trans. 1* **1996**, 2439−2442.
(24) Herbert, R. B.; Wilkinson, B.; Ellames, G. J. *Can. J. Chem*. **1994**, *72*, 114−117.
(25) Schnitzler, J.-P.; Madlung, J.; Rose, A.; Seitz, H. U. *Planta* **1992**, *188*, 594−600.
(26) Siddiqueullah, M.; McGrath, R.; Vining, L. C.; Sala, F.; Westlake, D. W. S. *Can. J. Biochem*. **1968**, *46*, 9−14.
(27) Jin, H. J.; Park, A. K.; Lee, S. S. *Arch. Pharm. Res*. **1992**, *15*, 41−47.
(28) Theriault, R. J.; Longfield, T. H. *Appl. Microbiol*. **1967**, *15*, 1431−1436.
(29) Lee, S. S.; Jin, H. J.; Son, M. W. *Arch. Pharm. Res*. **1992**, *15*, 30−34.
(30) Tsuji, H.; Oka, T.; Kimoto, M.; Hong, Y.-M.; Natori, Y.; Ogawa, T. *Biochim. Biophys. Acta* **1996**, *1309*, 31−36.
(31) Tsuji, H.; Ogawa, T.; Bando, N. Sasaoka, K. *J. Biol. Chem*. **1986**, *261*, 13203−13209.
(32) Malik, V. S.; Vining, L. C. *Can. J. Microbiol*. **1970**, *16*, 173−179.
(33) Malik, V. S.; Vining, L. C. *Can. J. Microbiol*. **1971**, *17*, 1287−1290.
(34) Mosher, R. H.; Ranade, N. P.; Schrempf, H.; Vining, L. C. *J. Gen. Microbiol*. **1990**, *136*, 293−301.
(35) Gottlieb, D.; Robbins, P. W.; Carter, H. E. *J. Bacteriol*. **1956**, *72*, 153−156.
(36) Lingens, F.; Eberhardt, H.; Oltmanns, O. *Biochim. Biophys. Acta* **1966**, *130*, 345−354.
(37) Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. *J. Org. Chem*. **1997**, *62*, 7512−7515.
(38) Santaniello, E.; Farachi, C.; Manzocchi, A. *Synthesis* **1979**, 912.
(39) Stuttard, C. *J. Gen. Microbiol*. **1982**, *128*, 115−121.
(40) Doull, J.; Ahmed, Z.; Stuttard, C.; Vining, L. C. *J. Gen. Microbiol*. **1985**, *131*, 97−104.
(41) Harwood, L. M. *Aldrichim. Acta* **1985**, *18*, 25.
(42) Byramova, N. E.; Mochalova, L. V.; Belyanchikov, I. M.; Matrosovich, M. N.; Bovin, N. V. *J. Carbohydr. Chem*. **1991**, *10*, 691−700.
(43) Kremer, C. B. *J. Chem. Educ*. **1956**, *33*, 71−72.

NP020306E