# Biosynthesis of Diazepinomicin/ECO-4601, a *Micromonospora* Secondary Metabolite with a Novel Ring System

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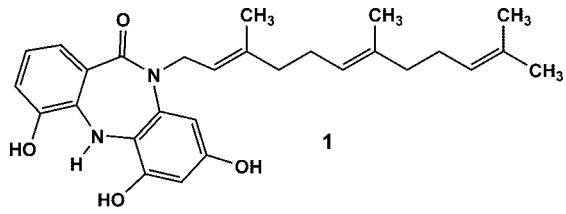
The novel microbial metabolite diazepinomicin/ECO-4601 (**1**) has a unique tricyclic dibenzodiazepinone core, which was unprecedented among microbial metabolites. Labeled feeding experiments indicated that the carbocyclic ring and the ring nitrogen of tryptophan could be incorporated via degradation to the 3-hydroxyanthranilic acid, forming ring A and the nonamide nitrogen of **1**. Genomic analysis of the biosynthetic locus indicated that the farnesyl side chain was mevalonate derived, the 3-hydroxyanthranilic acid moiety could be formed directly from chorismate, and the third ring was constructed via 3-amino-5-hydroxybenzoic acid. Successful incorporation of 4,6-$D_2$-3-hydroxyanthranilic acid into ring A of **1** via feeding experiments supports the genetic analysis and the allocation of the locus to this biosynthesis. These studies highlight the enzymatic complexity needed to produce this structural type, which is rare in nature.

Diazepinomicin/ECO-4601 (**1**) was isolated more or less simultaneously by different research groups at Wyeth and Ecopia BioSciences, Inc. (now Thallion Pharmaceuticals, Inc.), respectively.[1,2] The research group at Wyeth used antibacterial activity-guided fractionation to isolate diazepinomicin from the fermentation extracts of a *Micromonospora* sp., DPJ12, a strain that was obtained from an ascidian, *Didemnum proliferum* Kott., collected at Shishijima Island, Japan. Researchers at Ecopia discovered ECO-(0)4601 from a *Micromonospora* sp., 046Eco-11, by the use of their proprietary DECIPHER discovery platform. This producer was obtained from a soil sample collected in Montréal, Canada. ECO-(0)4601 and several derivatives were subsequently patented[3] by Ecopia.




**1**

The dibenzodiazepinone core found in **1** is exceedingly rare among natural products and to date has only been reported to be produced by several *Micromonospora* strains.[4] Hence, the biosynthesis of **1** is of considerable interest. Our approach to elucidate the biosynthesis of **1** has been via both labeled precursor feeding studies and genomic analysis. Discovery and analysis of the biosynthetic pathway responsible for the production of this novel molecular class enables the identification of related compounds in various microorganisms via the DECIPHER technology and moreover provides valuable insights for future mutasynthetic studies aimed at producing variants of this compound by genetic manipulation of strains.

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## Results and Discussion

**Feeding Experiments.** The only other known secondary metabolites with any structural relationship to the dibenzodiazepinone core of **1** are the anthramycins. Their biosynthesis has been studied extensively by labeled substrate feeding experiments,[5] which indicated that the methylhydroxyanthranilic acid moiety originates from tryptophan. Accordingly, when tryptophan, labeled at both nitrogens, was fed to *Micromonospora* strain DPJ15, $^{15}N$ was incorporated specifically into the N-5 of **1**, whereas feeding of tryptophan-$d_5$ (deuterium at each of the aromatic positions) led to the isolation of **1** with three deuteriums incorporated on ring A of the tricyclic core. This, and the genomic analysis below, indicated that ring A was assembled via the shikimate biosynthetic pathway. To confirm this finding, incorporation of 4,6-$D_2$-3-hydroxyanthranilic acid was attempted next. Independent studies, feeding 4,6-$D_2$-3-hydroxyanthranilic acid to *Micromonospora* strain 046Eco-11 in both media HI and Bennett's as well as strain DPJ15 in Bennett's, revealed that 3-hydroxyanthranilic acid served as a precursor in the biosynthesis of the dibenzodiazepinone core. The $^1H$ NMR spectrum of the purified product (**1**), isolated from HI medium, showed an approximately 40% reduction of the intensities of the signals at $\delta$ 7.17 and 6.83 (Figure S1, Supporting Information), corresponding to the H-1 and H-3 protons of the dibenzodiazepinone core (ring A) (Table 1). Moreover, the mass spectrum of **1** obtained during LC-MS analysis revealed additional pseudomolecular ions at *m/z* 465.3 (2 amu higher than the regular *m/z* 463.3), corresponding to the incorporation of two deuterium atoms into **1**. Incorporation of 4,6-$D_2$-3-hydroxyanthranilic acid using Bennett's medium was even higher (60%, as determined from $^1H$ NMR analysis of the purified sample). Feeding 4,6-$D_2$-3-hydroxyanthranilic acid to *Micromonospora* strain DPJ15 in Bennett's medium resulted in more than 80% incorporation based on the MS analyses. However, **1** was not isolated from this fermentation extract due to the rather low-yielding production under these conditons.

Although significant incorporation of the label was demonstrated in these experiments, unlabeled 3-hydroxyanthranilic acid showed apparent feedback inhibition. The production of **1** in fermentations not supplemented with 3-hydroxyanthranilic acid was 47.2 mg/L. However, the yield was reduced to 19.0 and 10.8 mg/L, respectively, in cultures supplemented with labeled or unlabeled 3-hydroxyanthranilic acid (Table 1). Likewise, yield reduction was also observed when tryptophan was fed to DPJ15.

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**Table 1.** Results of Feeding Experiments with Labeled and Unlabeled 3-Hydroxyanthranilic Acid in Both HI and Bennett's Media

| *Micromonospora* strain | medium | 3-hydroxyanthranilic acid | yield (mg/L) | incorporation |
|---|---|---|---|---|
| 046-ECO11 | Bennett's | labeled (4,6-dideutero) | 1.3 | ~40% |
| 046-ECO11 | Bennett's | unlabeled | 1.1 | |
| 046-ECO11 | Bennett's | none | 0.8 | |
| 046-ECO11 | HI | labeled (4,6-dideutero) | 19.0 | ~60% |
| 046-ECO11 | HI | unlabeled | 10.8 | |
| 046-ECO11 | HI | none | 47.2 | |
| DPJ15 | Bennett's | labeled (4,6-dideutero) | <1.0 | >80% |

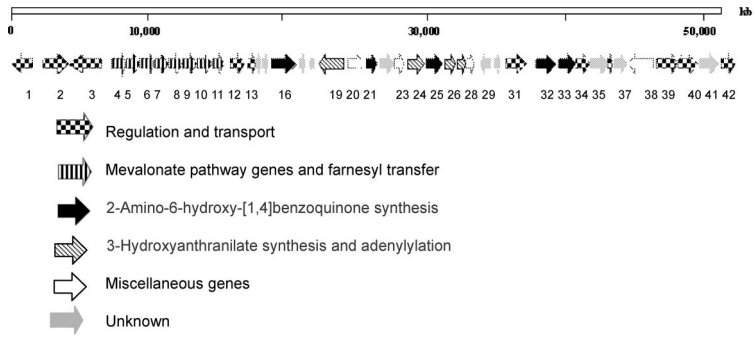
**Genomic Analyses.** From the genomic analysis, five distinct biosynthetic gene clusters were identified in 046Eco-11 as coding for secondary metabolite biosynthesis. Among these clusters, one designated locus A contained a gene-cassette comprising enzymes involved in the mevalonate pathway for isoprenoid production. Although actinomycetes produce a plethora of natural products, very few of these compounds contain an isoprenoid substructure. In those that do, the isoprenoid unit is often cyclized through the action of specific cyclases. Interestingly, locus A did not contain any isoprenoid cyclase gene, indicating that the compound produced by this locus would contain a linear isoprenoid unit.

Locus A (Figure 1) comprised an approximately 50 kb genomic DNA fragment and specified expression of 42 genes including genes putatively required for isoprenoid, 3-hydroxyanthranilic acid, and 3-amino-5-hydroxybenzoic acid synthesis.

**Genes Directing Biosynthesis of the Isoprenoid Moiety.** A complete gene-cassette for isoprenoid synthesis via the mevalonate pathway is present in locus A. ORF 10 is similar to hydroxymethylglutaryl-CoA synthase from *Streptomyces* sp. CL190 (67% identity).[6] This enzyme catalyzes an aldol addition of acetoacetyl-CoA onto acetyl-CoA to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This compound is subsequently reduced through the action of ORF 9 to form mevalonic acid. ORF 9 shows 82% identity in amino acid composition to HMG-CoA reductase from *Streptomyces* sp. CL190. In the mevalonate pathway for isoprenoid synthesis, mevalonate is doubly phosphorylated through the action of two kinases that use ATP as the phosphate donor and generate 5-phosphomevalonate followed by 5-diphosphomevalonate (DPMVA). ORFs 5 and 7 are similar to mevalonate kinase (46% identity) and phosphomevalonate kinase (50% identity), respectively, present in an isoprenoid gene cluster in *Streptomyces* sp. CL190. The next step in the biosynthesis of isoprenoids is the decarboxylation of DPMVA to produce isopentenyl diphosphate (IPP). ORF 6 is similar to the diphosphomevalonate decarboxylase (58% identity) found in *Streptomyces* sp. CL190 and thought to catalyze this reaction. Isopentenyl diphosphate is further converted to dimethylallyldiphosphate (DMAD) through the action of ORF 8, which is similar to isopentenyl diphosphate isomerase found in *Enterococcus faecalis* (66% identity). The concluding step in the biosynthesis of isoprenoids is the condensation of one molecule of DMAD with one or two molecules of IPP to form geranyl phosphate or farnesyl phosphate, respectively. ORF 4 is similar to BAD07374, the farnesyl diphosphate synthase (FDPSase) involved in the biosynthesis of the terpenoid antibiotic BE-40644 in *Actinoplanes* sp. A40644.[7] Catalytic sites important for enzymatic activity are conserved between FDPSases and ORF 4. The first Asp-rich motif (FARM: DDXXD), conserved in all FDPSases, which participates in the formation of C−C bonds between IPP and DMAD, is also present in ORF 4 (DDIID). However, in this ORF, the last aspartic acid in the second Asp-rich domain has been replaced with a glycine (SARM:DDLLG). Site-directed mutagenesis performed on the rat FDPSase clearly demonstrated that the last position of the SARM motif is not crucial for activity, as modifications in that position only slightly affect catalytic activity.[8] Additional amino acid residues important for activity are also conserved between FDPSases and ORF 4. The lack of isoprenoid cyclase catalytic activity in the gene cluster suggests that the isoprenoid unit remains linear (Figure 2).

**Genes Directing Biosynthesis of the 3-Hydroxyanthranilic Acid Moiety.** A number of genes present in locus A share degrees of similarity with those encoding enzymes of the chorismate pathway. ORF 19 is similar (53% identity) to PhzE (AAC18904), the anthranilate synthase gene present in the phenazine biosynthetic locus in *Pseudomonas fluorescens* 2-79.[9] Like PhzE, ORF 19 encodes an N-terminal isochorismate binding domain and a C-terminal domain that shares high similarity with class I glutamine amidotransferases (GATs). The catalytic triad forming the active site of type I GATs, as defined by 3D structure analysis, is composed of cysteine, histidine, and glutamine residues that are located at positions 536, 621, and 623, respectively. Through the action of ORF 19, chorismic acid would be converted to aminodeoxyisochorismic acid (ADIC) in a transamination reaction using glutamine as the amine source. ADIC would then be converted to 3-hydroxyanthranilic acid through the action of ORF 27. Indeed, this ORF shares a high degree of similarity (54% identity) with PhzD (AAC18903), from the phenazine biosynthetic locus in *P. fluorescens* 2-79, which possesses isochorismatase activity and converts ADIC to pyruvate and 3-hydroxyanthranilic acid. Orf 26 is likely to be involved in an intermediary step in the generation of 3-hydroxyanthranilic acid, as it shares similarity (57% identity) with MxcC (AAG31126) present in the myxochelin biosynthetic locus in *Stigmatella aurantiaca* Sg a15. MxcC catalyzes the conversion of 5,6-dihydroxycyclohexa-1,3-diene carboxylic acid to 2,3-dihydroxybenzoic acid. Similarly, ORF 26 would catalyze the conversion of 6-amino-5-hydroxycyclohexa-1,3-dienecarboxylic acid to 3-hydroxyanthranilic acid. ORF 24 is an adenylate ligase, as it shares 66% sequence identity with coenzyme F390 synthetase (ZP_00568228) from *Frankia* sp. EAN1pec. This enzyme was shown to adenylate aromatic compounds, namely, coenzyme F420. Protein sequence alignments of ORF 24 to related proteins show that ORF 24 contains a conserved motif (SSGTAGK) found in numerous ATP-binding proteins and would interact with the pyrophosphate leaving group generated during the adenylation reaction so as to catalyze the adenylation of 3-hydroxyanthranilic acid to form 3-hydroxyanthranilate adenylate (Figure 3).

**Genes Directing Biosynthesis and Subsequent Modification of 3-Amino-5-hydroxybenzoic Acid.** Locus A contains genes that resemble those encoding enzymes involved in the shikimate and aminoshikimate pathways. Indeed, ORF 33 shares 44% identity with PhzC (AAC64491), from the phenazine biosynthetic cluster in *Pseudomonas fluorescens* 2-79, which catalyzes formation of 3-deoxy-D-arabinoheptulosonic acid 7-phosphate (DAHP), and 42% identity with AnsI (AAD31838) present in the ansatrienin A gene cluster in *Streptomyces collinus* Tü1892, which catalyzes formation of 4-amino-3,4-dideoxy-D-arabinoheptulosonic acid 7-phosphate (aminoDAHP). DAHP and aminoDAHP are direct precursors of shikimate and 3-amino-5-hydroxybenzoic acid (AHBA), respectively.[10] The two enzymes that catalyze these reactions, DAHP and aminoDAHP synthases, share high sequence similarity, but differ in that aminoDAHP additionally catalyzes transamination of erythrose 4-phosphate using glutamine as the nitrogen donor. The catalytic residues for the transaminating activity have not been yet defined, which makes the distinction between the two enzymatic activities unclear. ORF 21 shares similarity with fructose biphosphate aldolases that generate D-glyceraldehyde 3-phosphate, which is a precursor for the synthesis of erythrose 4-phosphate. Locus A lacks additional enzymatic activities required for the formation of shikimate or AHBA. Similarly, other known gene clusters, such as the naphthomycin gene cluster in *S. collinus* Tü1892 and the geldanamycin gene cluster in *S. hygroscopicus*, do not contain the full panel of enzymes required to produce AHBA. In the case of geldanamycin, a gene cluster encoding enzymes for AHBA




| ORFs | Amino acids | most homologous proteins and their accession numbers |
|---|---|---|
| ORF1 | 571 | ABC transporter of *Corynebacterium efficiens* (45% identity, NP_736627) |
| ORF2 | 689 | regulator of *Lechevalieria aerocolonigenes* (36%, CAC93719) |
| ORF3 | 895 | regulator of *Lechevalieria aerocolonigenes* (28%, CAC93719) |
| ORF4 | 362 | farnesyl diphosphate synthase of *Actinoplanes* sp. A40644 (72%, BAD07374) |
| ORF5 | 354 | mevalonate kinase of *Streptomyces* sp. CL190 (46%, BAB07790) |
| ORF6 | 346 | diphosphomevalonate decarboxylase of *Streptomyces* sp.(58%, BAB07791) |
| ORF7 | 369 | phosphomevalonate kinase of *Streptomyces* sp. CL190(50%, BAB07792) |
| ORF8 | 360 | isopentenyl diphosphate isomerase of *Enterococcus faecalis* (66%, Q9KWF6) |
| ORF9 | 351 | HMG-CoA reductase *Streptomyces* sp. (82%, BAA70975) |
| ORF10 | 391 | HMG-CoA synthase of *Streptomyces* sp. CL190 (67%, BAB07795) |
| ORF11 | 290 | prenyltransferase of *Streptomyces* sp. CL190 (38%, BAE00106) |
| ORF12 | 370 | two-component sensor *Streptomyces coelicolor* A3(2) (54%, AAM78435) |
| ORF13 | 220 | regulatory protein *Streptomyces coelicolor* A3(2) (67%, NP_630508) |
| ORF14 | 131 | no hit with known sequences |
| ORF15 | 154 | sodium/calcium exchanger protein *Drosophila melanogaster* (38%, CG1090-PB) |
| ORF16 | 661 | multicopper oxidase of *Geobacillus kaustophilus* HTA426 (32%, BAD75189) |
| ORF17 | 129 | bleomycin resistance protein family *of Sinorhizobium meliloti* (60%, NP_437360) |
| ORF18 | 178 | unknown protein of *Streptomyces griseus* (29%, AAQ08932) |
| ORF19 | 661 | anthranilate/para-aminobenzoate synthase *P. aeruginosa* (51%,P_00137697) |
| ORF20 | 427 | aspartate kinase of *Rubrobacter xylanophilus* DSM 9941(45%, EAN36347) |
| ORF21 | 274 | fructose-bisphosphate aldolase of *Frankia* sp. CcI3 (62%, ZP_00549318) |
| ORF22 | 367 | alternative 3-dehydroquinate synthase of *Frankia* sp. CcI3 (64%, YP_483283) |
| ORF23 | 253 | metal-dependent hydrolase of *Pyrococcus fu*riosus DSM 3638 (31%, NP_577771) |
| ORF24 | 367 | coenzyme F390 synthetase of *Frankia* sp. EAN1pec (66%, ZP_00568228) |
| ORF25 | 253 | salicylate 1-monooxygenase *Frankia* sp. CcI3 (52%, ZP_00548930) |
| ORF26 | 261 | 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase *B. subtilis* (56%, NP_391080) |
| ORF27 | 224 | isochorismatase of *Frankia* sp. EAN1pec (58%, ZP_00568222) |
| ORF28 | 233 | short-chain dehydrogenase/reductase *Anaeromyxobacter dehalogenans* 2CP-C (33%, YP_466337) |
| ORF29 | 246 | hypothetical protein of *Streptomyces coelicolor* (29%, S18541) |
| ORF30 | 111 | membrane protein of *Thermobifida fusca* (36%, ZP_00058149) |
| ORF31 | 559 | Na+/solute symporter of *Thermobifida fusca* YX (35%, AAZ56895) |
| ORF32 | 532 | 2,4-dihydroxybenzoate monooxygenase of *Sphingobium chlorophenolicum* (39%, AAM96655) |
| ORF33 | 423 | hypothetical protein of *Streptomyces hygroscopicus* (54%, T03226); DAHP synthase of *Pseudomonas aeruginosa* UCBPP-PA14 of (45%, ZP_00137693); PhzC of *Pseudomonas aeruginosa* (44%, AAC64491); PlmI of *Streptomyces* sp. HK803 (46%, AAQ84163); DAHP synthetase, classII of *Frankia* sp. EAN1pec (39%, ZP_00569555); DAHP synthase of *Streptomyces collinus* Tu 189 (42%, AAD31838) |
| ORF34 | 340 | regulator protein of *Streptomyces kasugaensis* (46%, BAC53615) |
| ORF35 | 493 | hypothetical protein AcidDRAFT_4187 of *Solibacter usitatus* Ellin6076 (33%, ZP_00522772) |
| ORF36 | 112 | hypothetical protein of *Streptomyces coelicolor* A3(2) (48%, NP_627088) |
| ORF37 | 325 | hypothetical protein CC3409 of *Caulobacter crescentus* CB15 (39%, NP_422203) |
| ORF38 | 663 | putative acyl-CoA oxidase of *Nocardia farcinica* IFM 10152 (54%, BAD57326) |
| ORF39 | 537 | ABC transport protein of *Mycobacterium smegmatis* (37%, T14162) |
| ORF40 | 596 | exiT protein of *Mycobacterium smegmatis* (40%, AAC32046) |
| ORF41 | 507 | methyltransferase of *Bacillus cereus* (24%, NP_831570) |
| ORF42 | 232 | transporter, LysE family of *Bacillus cereus* (22%, NP_830809) |

**Figure 1.** ECO-4601 (**1**) biosynthetic gene cluster in *Micromonospora* sp. 046Eco11.

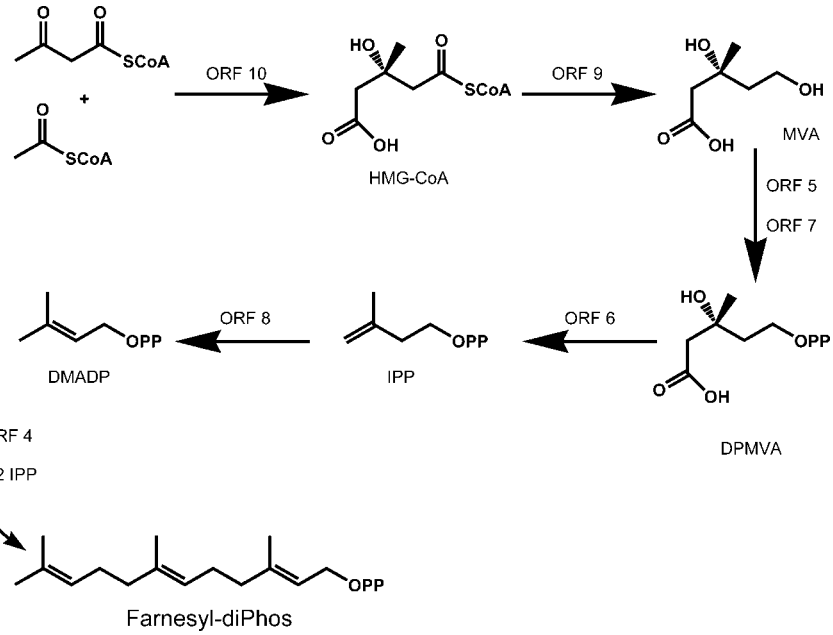



**Figure 2.** Putative mevalonate pathway predicted for generating polyprenyl diphosphate (farnesyl diphosphate) in *Micromonospora* sp. 046Eco11.

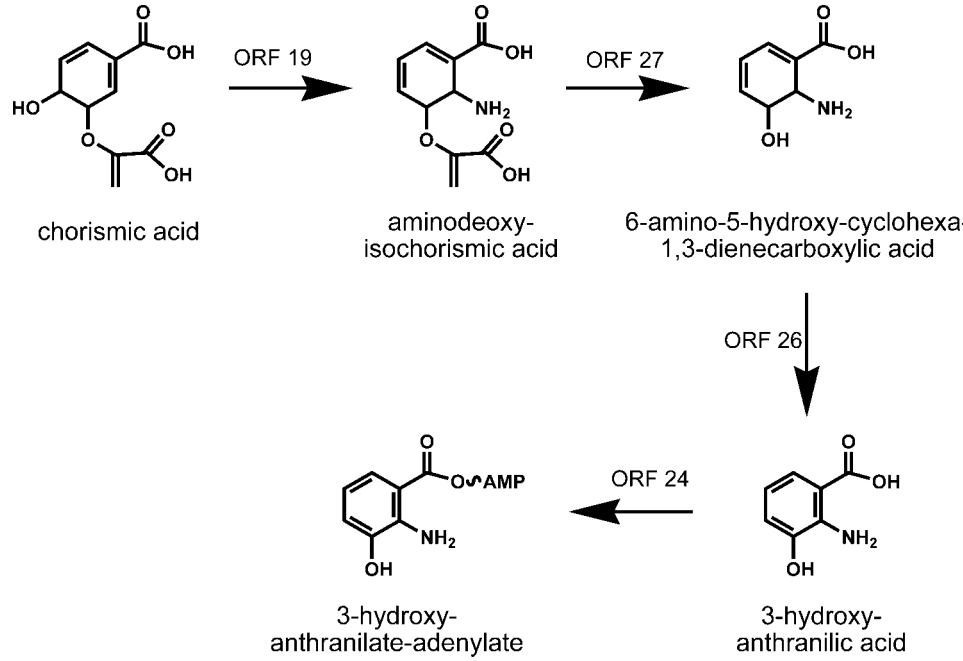



**Figure 3.** Proposed pathway for biosynthesis and activation of 3-hydroxyanthranilate in *Micromonospora* sp. 046Eco11.

biosynthesis and shown to be essential for geldanamycin production was found to be more than 30 kb away from the geldanamycin gene cluster.[11] The structure of **1** strongly indicates that the C-ring component is likely to be derived from AHBA rather than shikimate. Additional enzymatic activities present in locus A would modify further the AHBA component. ORF 25 shares 52% identity with salicylate 1-monooxygenase (ABD13555) in *Frankia* sp. CcI3. This family of enzymes is composed of NADH-oxygen-dependent oxidoreductases that catalyze decarboxylative hydroxylation of phenolic acids. ORF 25 is likely to encode for enzymes that decarboxylate AHBA, generating 5-aminobenzene-1,3-diol. The product of ORF 32 shares similarity (39% identity) with the 2,4-dihydroxybenzoate monooxygenase (AAM96655) of *Sphingobium chlorophenolicum* as well as other monooxygenases and is likely to further hydroxylate the 5-aminobenzene-1,3-diol component to form an aminobenzenetriol. The product of ORF 16 shares similarity (32% identity) with the multicopper oxidase (BAD75189) of *Geobacillus kaustophilus*. Therefore, ORF 16 is likely to further oxidize the aminobenzenetriol intermediate to form an aminohydroxybenzoquinone (Figure 4).

**Assembly of the Final Product.** The adenylate of 3-hydroxyanthranilate is activated to react and self-condense with the aminohydroxy[1,4]benzoquinone to form the dibenzodiazepinone core. ORF 11 is similar to genes encoding isoprenoid transferases from different species. It displays 49% identity to the geranyl transferase (BAE78975) genes present in the furaquinocin gene cluster in *Streptomyces* sp. strain KO-3988 and 25% identity to CloQ (AAN65239), the geranyl transferase genes involved in chlorobiocin biosynthesis in *Streptomyces roseochromogenes*. This gene product putatively transfers the farnesyl group to the core structure, resulting in the final structure, **1** (Figure 5).

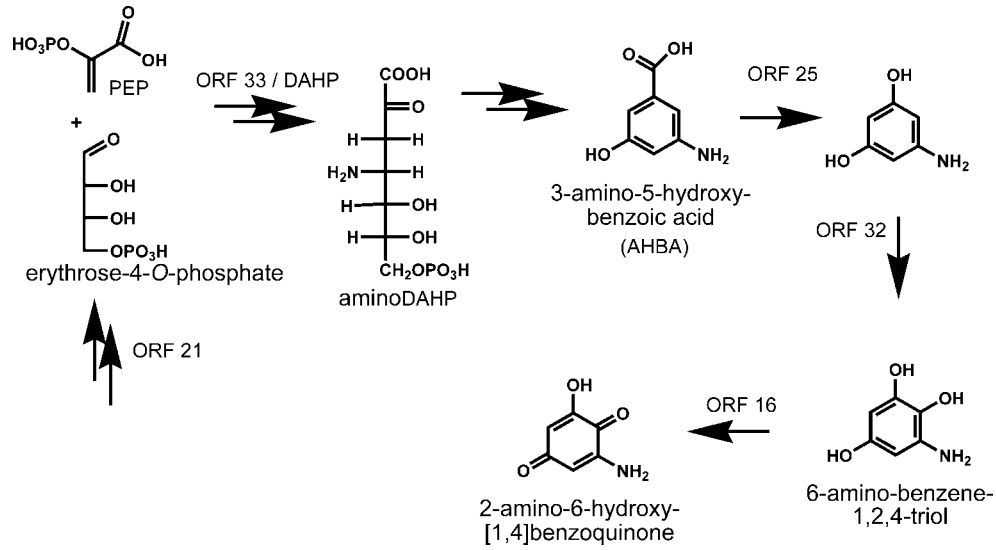



**Figure 4.** Proposed biosynthetic pathway of 2-amino-6-hydroxybenzoquinone in *Micromonospora* sp. 046Eco11.

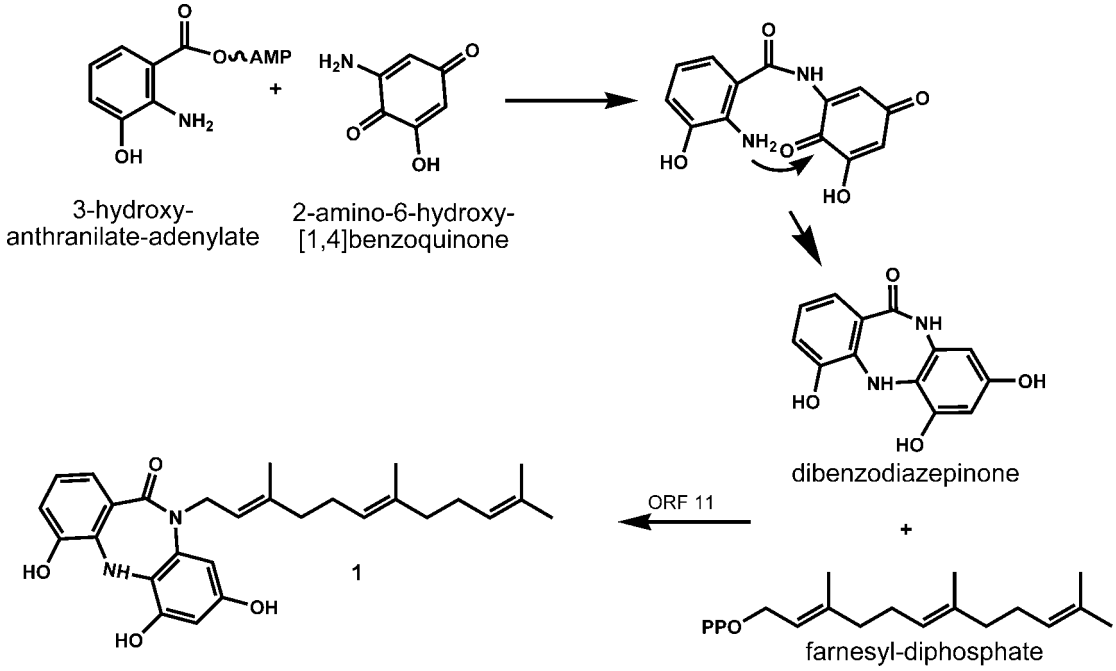



**Figure 5.** Proposed final steps in the biosynthesis of **1** in *Micromonospora* sp. 046Eco11 based on genomic information and the chemical structure.

**Regulators and Transporters.** Several genes present in locus A encode regulators and transport proteins. ORFs 2, 3, 12, 13, and 36 share similarity with genes encoding diverse transcriptional regulators and sensors, whereas ORFs 1, 15, 30, 31, 39, 40, and 42 present similarities to membrane transporter genes from a variety of microorganisms. The presence of several regulators and transporters in the biosynthetic locus of **1** suggests that expression of this compound is highly regulated. Indeed, only a small percentage of fermentation conditions investigated induced its expression.

Of the 42 open reading frames in the biosynthetic gene cluster, seven are assigned to synthesis of the farnesyl chain via the mevalonate pathway and one to its transfer to the core. Three are assigned to the biosynthesis of 3-hydroxyanthranilate moiety and one to its activation as the adenylate, and five are assigned to the synthesis of 2-amino-6-hydroxy-[1,4]benzoquinone via 3-amino-5-hydroxybenzoic acid. Eleven show homology to genes encoding regulatory proteins in other bacteria, and four show homology to genes encoding proteins with specific functions in other bacteria, for which a comparable function has not been assigned here. Ten short ORFs are homologous to ORFs in other bacteria, but for which no function has been ascribed. Compound **1** exhibited potent activity against a broad spectrum of tumor cell lines and efficacy in animal models.[12] It is currently under clinical development as an antitumor agent.

## Experimental Section

**General Experimental Procedures.** Analytical HPLC was carried out on a Waters Alliance 2695 instrument equipped with a Micromass ZQ electrospray source and 996 diode array UV detector. Semipreparative HPLC was carried out on a Waters Delta 600 instrument with a Waters 2996 diode array UV detector. NMR spectra were measured either in MeOH-$d_4$ or in DMSO-$d_6$ on a Varian Unity INOVA 500 MHz spectrometer. HRMS were measured by ESI on an Ionspec Ultima 7T Fourier transform mass spectrometer from a solution in 50% aqueous acetonitrile and 0.1% formic acid. HPLC grade solvents were used for both analytical and preparative work and were from J.T. Baker. Peptone agar, glucose, potato dextrin type IV, NZ Amine, $CaCO_3$, formic acid,

glycerol, bacto-peptone, and oceanic salt were purchased from Sigma-Aldrich. Yeast extract and beef extract were from BD Biosciences.

**Genome Scanning.** The genome of *Micromonospora* sp. 046Eco-11, a strain in the Thallion collection, was analyzed by the genome scanning technique as described.[13] The DNA that comprises the ECO-046D gene cluster is available from the deposited *E. coli* DH10B vectors, each harboring a cosmid clone (designated as 046KM and 046KQ, respectively) of a partial biosynthetic locus for **1** and together spanning the full biosynthetic locus. The strains containing cosmid clones designated 046KM and 046KQ were assigned deposit accession numbers IDAC 250203-06, and IDAC 250203-07, respectively, at the International Depositary Authority of Canada, Bureau of Microbiology, Health Canada, 1015 Arlington St., Winnipeg, Manitoba, Canada R3E 3R2, on February 25, 2003.

**Feeding Experiments.** *Micromonospora* sp. 046Eco-11 was maintained as frozen glycerol stock at −80 °C. Inoculum for fermentation was obtained by streaking freshly thawed frozen stock onto glucose yeast peptone agar (Sigma G-9663) and incubating at 30 °C until sporulation was apparent (15 to 20 days). Four loopfuls of vegetative mycelium and spores were homogenized in 25 mL of KH medium [10 g/L glucose, 20.0 g/L potato dextrin type IV, 5.0 g/L yeast extract, 5.0 g/L NZ Amine, and 1.0 g/L $CaCO_3$ in tap water, with the pH adjusted to 7.0 before the addition of $CaCO_3$]. The seed (0.5 mL) was used to inoculate either modified Bennett's medium [1.0 g/L yeast extract, 1.0 g/L beef extract, 2.0 g/L NZ Amine A, 15.0 g/L glucose, 34.4 g/L oceanic salt in tap water] (25 mL × 4), in 125 mL flasks supplemented with 5% activated HP20, or in HI medium [30.0 g/L glycerol, 20.0 g/L potato dextrin, 2.5 g/L bacto-peptone, 8.3 g/L yeast extract, 1.0 g/L $CaCO_3$ in tap water (pH 7.0 adjusted before the addition of $CaCO_3$)] (25 mL × 4) in 125 mL flasks. Each medium was supplemented with 4,6-$D_2$-3-hydroxyanthranilic acid at 0.5 mg/mL before inoculating with the vegetative culture at 2%. Control cultures, with or without 3-hydroxyanthranilic acid (0.5 mg/mL), were also prepared in order to determine any feedback inhibition of **1** production by 3-hydroxyanthranilic acid. The culture was incubated either 7 days for HI medium or 9 days for Bennett's medium at 28 °C on a rotary shaker (250 rpm).

The labeled precursor feeding experiments conducted at Wyeth were performed with *Micromonospora* sp. DPJ15, a related strain to DPJ12, also isolated from *Didemnum proliferum* Kott. For fermentation seed inoculum, cultures of DPJ15 were streaked onto YPSS agar (4.0 g/L yeast extract, 0.5 g/L $K_2HPO_4$, 10.0 g/L Difco soluble starch, 15.0 g/L Bacto agar, 500 mL of artificial seawater, 500 mL of distilled water, pH 7.2) from frozen stock. After confluent growth appeared on the agar plates (about 7 days), spores and mycelia of DPJ15 were scraped into several 25 × 150 mm seed tubes each containing 7 mL of YPSS broth. After 3 days of incubation at 28 °C with agitation (200 rpm on a rotary shaker), the seed stages were combined into one vessel and used to inoculate the production fermentation at 2.5% volume-to-volume.

All precursor feedings at Wyeth were performed in 100 mL of Bennett's medium (10.0 g/L dextrose, 0.77 g/L beef extract, 1.0 g/L yeast extract, 2.0 g/L NZ Amine A, in distilled water, pH 7.3) per 500 mL Erlenmeyer shake flask. The following labeled compounds were added at 500 μg/mL preautoclaving: L-tryptophan-$^{15}N_2$, L-tryptophan-2′,4′,5′,6′,7′-$D_5$ (indole-$D_5$), anthranilic acid-$^{15}N$ (Cambridge Isotope Laboratories, Inc.), and 4,6-$D_2$-3-hydroxyanthranilic acid. Gentle heating in a 50 °C water bath was used to completely dissolve the precursors, and the pH was readjusted to 7.3. Activated sterile HP20 resin in water was added to each flask at 5% volume-to-volume post autoclaving. The fermentations were incubated at 28 °C with agitation.

To follow the incorporation of the labeled precursors, 1 mL was sampled from each fermentation on the third, fourth, and seventh days of incubation. After centrifugation to pellet the cells and resin, the supernatants were decanted and the pellets were extracted with methanol. Extracts concentrated to 10× were analyzed by LC-MS.

**Synthesis of 4,6-$D_2$-3-Hydroxyanthranilic Acid.** The 4,6-$D_2$-3-hydroxyanthranilic acid was synthesized according to the published protocol.[14] In brief, 3-hydroxyanthranilic acid (1.08 g, 7.1 mmol) was placed in a thick-walled screw-capped tube, and deuterated water ($D_2O$, 20 mL) was added to the tube after flushing with nitrogen. Potassium *tert*-butoxide (1.54 g, 13.7 mol) was added to the tube; the cap was screwed on tightly and the tube was heated at 100 °C for 3 days. The reaction mixture was then cooled to room temperature and acidified to pH 6 with 6 N HCl. The colorless precipitate, which appeared during acidification, was separated by filtration and dried under vacuum, to yield 4,6-$D_2$-3-hydroxyanthranilic acid (0.75 g, 4.8 mmol). The $^1H$ NMR spectrum [$\delta_H$ 7.37, 6.80, 6.47; integration ratio 1:1:8] of the product suggested that it was a mixture of 4,6-$D_2$-3-hydroxyanthranilic acid and partially deuterated and nondeuterated 3-hydroxyanthranilic acid, which was used for the feeding experiment described above.

**Extraction.** At harvest, cultures of each experiment (four flasks) were pooled and stored overnight at −20 °C after adjusting to pH 3 with 20% $H_2SO_4$. The culture broth was centrifuged at 3000 rpm (15 min) in order to separate the mycelial cake, which was then extracted with methanol (3 × 35 mL). The combined methanolic extracts were dried under reduced pressure and resuspended in MeOH (10 mL). The suspension was filtered through a 0.45 μm × 13 mm Acrodisc GHP syringe, and the filtrate was subjected to isolation and liquid chromatography mass spectrometry (LC-MS) analysis.

**LC-MS Analyses.** The LC-MS analyses of the MeOH extracts (50 μL) described above were performed on a Waters Alliance 2695 instrument with electrospray source using a Symmetry $C_{18}$ 5 μm, 60 Å, 4.6 × 150 mm column (Waters). A linear gradient of water/acetonitrile containing 0.2% formic acid (0.2% formic acid in acetonitrile/0.2% formic acid in water, 50:50−100:0, 0−9 min; acetonitrile alone up to 14 min; 100:0−50:50, 14−16 min, 50:50 run up to 20 min) was used as eluent, in which **1** eluted at 6.24 min. A standard calibration curve (concentration 0.2−5.0 μg vs area under UV at 290 nm) was plotted in order to calculate the concentration of **1** in each culture described above.

**Isolation of 1.** The MeOH extracts from the labeled experiments in both HI and Bennett's medium described above were subjected to semipreparative HPLC purification using a Waters Delta 600 HPLC coupled with a diode array UV detector. Multiple injections of no more than 500 μL samples on a Waters RCM Nova-Pak HR $C_{18}$ 6 μm, 60 Å, 25 × 200 mm column (water/acetonitrile 80:20−30:70, 0−8 min; 30:70−0:100, 8−18 min at 20 mL/min) afforded **1** ($t_R$ 13.5 min), 1.5 mg/L and 0.5 mg/L in HI and Bennett's medium, respectively (Figure S2, Supporting Information).

**1:** UV (MeOH) $\lambda_{max}$ (log ε) 220 (4.6), 240 (4.4), 290 (3.8) nm; MS (ESI in positive mode) *m/z* 463.36 $(M + H)^+$, 271.15, 243.31; MS (ESI in negative mode) *m/z* 461.21 $(M - H)^-$, 255.99. The $^1H$ and $^{13}C$ NMR data are in Table S1, Supporting Information.

**Supporting Information Available:** $^1H$ and $^{13}C$ NMR assignments for **1**. HPLC (UV scan) of the MeOH extract from the label incorporation experiment with 4,6-$D_2$-3-hydroxyanthranilic acid in HI medium. Downfield portion of the $^1H$ NMR spectra of labeled and unlabeled **1**. This information is via the Internet free of charge at http://pubs.acs.org.

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