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ORIGINAL ARTICLE

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Isolation and Identification of Three New 5-Alkenyl-3,3(*2H*)-furanones from Two *Streptomyces* species using a Genomic Screening Approach

Arjun H. Banskota, James B. McAlpine, Dan Sørensen, Mustapha Aouidate, Mahmood Piraee, Anne-Marie Alarco, Satoshi Ōmura, Kazuro Shiomi, Chris M. Farnet, Emmanuel Zazopoulos

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Abstract Analyses of biosynthetic gene clusters derived from *Streptomyces aculeolatus* NRRL 18422 and *Streptomyces* sp. Eco86 indicated that both microorganisms have similar type I polyketide synthase (PKS) gene clusters with relatively few genes encoding post-PKS elaborative enzymes. However both gene clusters included a sequence coding for a relatively uncommon oxidative enzyme related to Baeyer-Villiger, flavin-type monooxygenases. Screening of culture extracts for compounds with the predicted physicochemical properties of the end products from these loci, led to the isolation of three 5-alkenyl-3,3(*2H*)-furanones, one (E-837, **1**) from the former and two (E-492, **2**, E-975, **3**) from the latter strain. The structures, confirmed by spectral analyses including MS, and 1D and 2D NMR experiments, were in accord with those predicted by genomic analyses. Baeyer-Villiger type oxidation is postulated to be involved in the formation of the furanone moieties in these molecules. All three new compounds were tested for their electron transport inhibitory activities. They had IC_{50} values of 1∼4 μg/ml against *Ascaris suum* NADH-fumarate reductase and 1∼12 μg/ml against bovine heart NADH oxidase.

Keywords *Streptomyces aculeolatus*, *Streptomyces* sp., E-837, E-492, E-975, Baeyer-Villiger monooxygenase

Introduction

Natural products play an important role in drug discovery and have been used for the treatment of various diseases for decades. They constitute a leading source of novel molecules for the development of new drug candidates to treat life threatening infections and other human disorders [1]. To identify such potential drug candidates from nature, different methods have been developed and routinely used in natural product discovery laboratories. The genomics of secondary metabolite biosynthesis has recently evolved to the point where analysis of the genome of an organism can define its secondary metabolic capabilities. A genome scanning technique has been developed in our laboratories to greatly reduce the amount of sequencing required to define this capability [2, 3]. This approach not only ascertains the potential of a producing organism, but it provides the scientist with a handle to identify, isolate and structurally define a specific metabolite. We have demonstrated this approach in the isolation and structural determination of an antifungal agent, ECO-02301 from *Streptomyces aizunensis* [4].

In our continuing effort to find novel secondary metabolites from actinomycetes, we observed that two different streptomycetes, *Streptomyces aculeolatus* NRRL 18422 (a producer of the semi-naphthaquinone antibiotic,

J. B. McAlpine (Corresponding author)**, A. H. Banskota, D. Sørensen, M. Aouidate, M. Piraee, A.-M. Alarco, C. M. Farnet, E. Zazopoulos:** Ecopia BioSciences Inc., 7290 Frederick-Banting, Montréal, Québec, H4S 2A1, Canada,
E-mail: mcalpine@ecopiabio.com

S. Ōmura, K. Shiomi: The Kitasato Institute for Life Sciences, Kitasato University, and The Kitasato Institute, 9-1, Shirokane 5-Chome, Minato-ku, Tokyo 108-8641, Japan

A80915A) [5] and *Streptomyces* sp. Eco86 (a proprietary Ecopia strain) had similar gene clusters for type I polyketides with relatively few post-polyketide elaborative enzymes, but including an uncommon Baeyer-Villiger type monooxygenase. Analyses of these gene clusters led us to conclude that these microorganisms produce members of a group of very closely related secondary metabolites. Here, we report the isolation and identification of three new 5-alkenyl-3,3(*2H*)-furanones from these two streptomycetes [6].

Results and Discussion

Streptomyces aculeolatus NRRL 18422 was reported to produce a complex of Gram-positive antibiotics related to tetronomycin, napyradiomycin and the semi-naphthaquinone antibiotic, A80915 [5]. Genomic analysis of this organism identified 7 gene clusters, including those for the biosynthesis of tetronomycin and A80915 and for several new secondary metabolites. *Streptomyces* sp. Eco86, a soil isolate, contained 5 gene clusters identified as coding for secondary metabolite biosynthesis. These included a polyether antibiotic- and a terpenoid-specifying gene cluster. Among the clusters encoding biosynthetic machinery for novel metabolites, two loci, A and B, one from each organism, were very similar. They each encoded a type I PKS and an unusual Baeyer-Villiger flavin monooxygenase.

The biosynthetic locus A of *Streptomyces aculeolatus* NRRL 18422 spans approximately 50,000 base pairs of DNA and encodes 8 proteins. The order, relative position and orientation of the open reading frames (ORFs) representing the proteins of the biosynthetic locus are illustrated schematically in Fig. 1. The type I PKS system is composed of 5 ORFs and specifies 9 modules. Analysis of domain active sites shows that the ketosynthase (KS) domain in module 1 contains a glutamine residue in the place of the conserved cysteine residue in the catalytic site, indicating that this module is the loading module of the PKS. Moreover, analysis of the catalytic sites of the domains in the reducing loops of the PKS system shows that the dehydratase (DH) domain in module 7 and the ketoreductase (KR) domain in module 8 are inactive.

The biosynthetic locus B of *Streptomyces* sp. Eco86 spans approximately 65,000 base pairs of DNA. As illustrated in Fig. 2, the locus comprises 16 ORFs, including a type I PKS system composed of 4 ORFs containing a total of 9 modules. Analysis of the active sites of the various domains shows that module 1 is the loader, as the KS domain present in this module specifies a glutamine residue instead of the conserved cysteine residue in the catalytic site. Analysis of active sites of reducing domains showed that three domains, namely DH in module

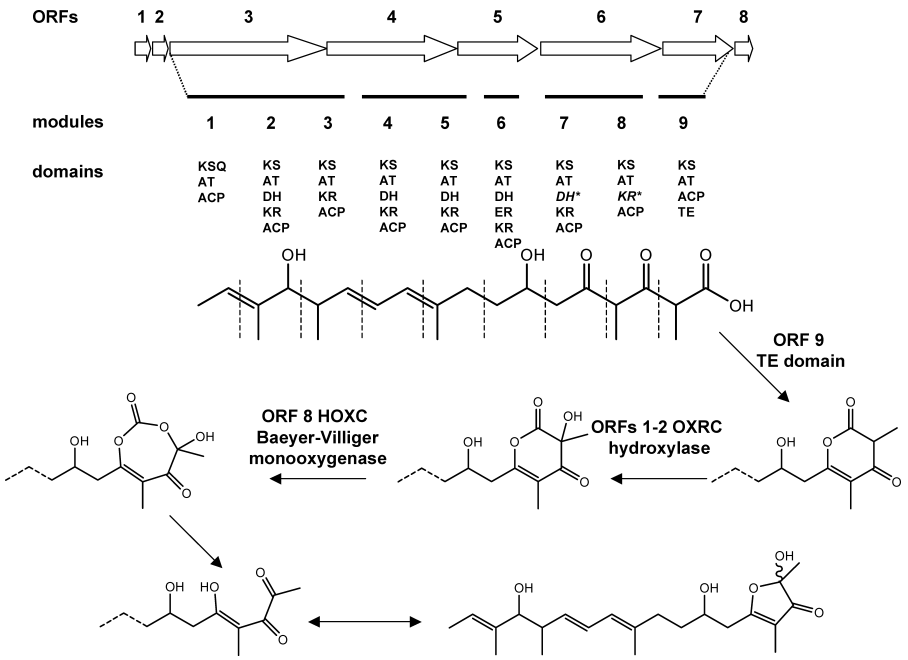

Fig. 1 Gene cluster A of *Streptomyces aculeolatus* NRRL 18422 and predicted biosynthetic pathway leading to E-837. Domains in italics and bearing an asterisk are predicted to encode inactive enzymes.

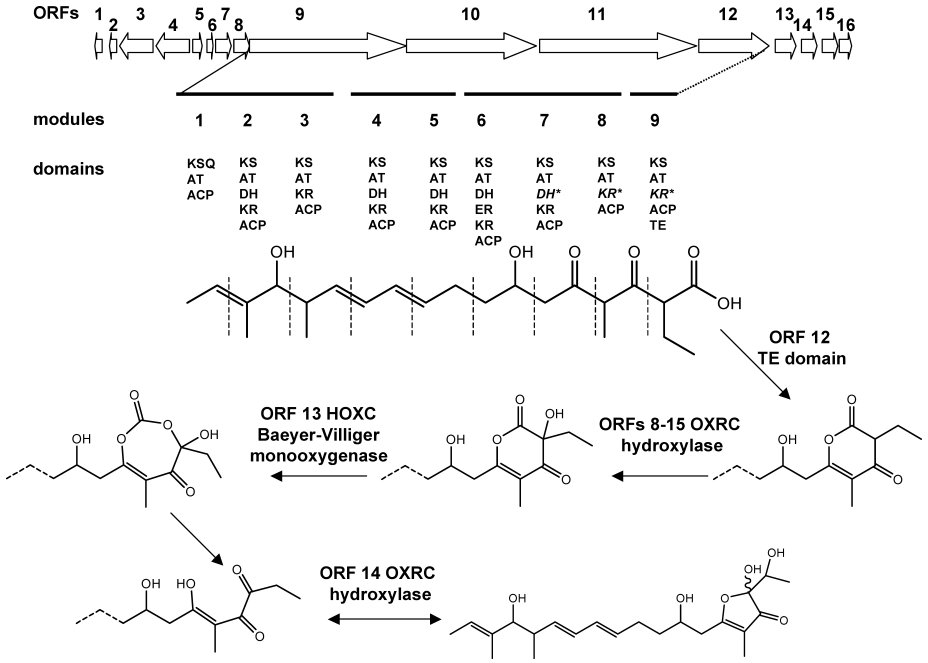

Fig. 2 Gene cluster B of *Streptomyces* sp. Eco86 and predicted biosynthetic pathway leading to E-492 and E-975. Domains in italics and bearing an asterisk are predicted to encode inactive enzymes.

7 and KR in modules 8 and 9 are inactive.

Extensive analysis of genes present in these two biosynthetic loci and determination of their function was performed using Ecopia's Decipher® database, that contains genetic information from over 1,600 gene clusters involved in the biosynthesis of secondary metabolites, from a variety of microorganisms. Automated correlations made between gene clusters encoding known metabolites and their chemical structures allow prediction of chemical structures of compounds encoded by newly discovered gene clusters.

Phylogenetic analyses and comparisons of acyltransferase (AT) domains present in the polyketide synthase systems of loci A and B with AT domains of known specificities allow determination of acyl units incorporated in the polyketide core structures. Analysis of AT domains as shown in Fig. 3A demonstrates that 9 AT domains from loci A and B specify the incorporation of a malonate extender unit as they cluster with a malonyl-CoA-specifying AT domain from the nystatin biosynthetic cluster [7]. Similarly, it is demonstrated that 8 AT domains specify the incorporation of methylmalonate extender unit as they cluster with a methylmalonyl-CoA-specifying AT domain from the nystatin biosynthetic cluster [7]. The ultimate module of the PKS system present in locus B contains an AT domain that incorporates ethylmalonate as it clusters with an ethylmalonate-CoA-specifying domain present in the oligomycin biosynthetic gene cluster [8]. Based on this bioinformatics analysis, predictions on the polyketide core structures encoded by loci A and B were made (Figs. 1 and 2).

To determine whether the polyketide structures would be in a linear or macrolactone form, the two terminal thioesterase (TE) domains were closely examined. Phylogenetic analysis and comparison of the TE domain sequences from both loci with thioesterases from gene clusters encoding metabolites of defined chemical structure, predicted the formation of a terminal 6-membered ring structure (Fig. 3B). Indeed, TE domains from loci A and B were closely related to each other as well as to TE domains derived from gene clusters encoding polyketides that contain terminal 6-membered ring structures such as phoslactomycin [9] and aureothin [10]. Closer analysis of the polyketide backbone suggests an intra-molecular lactonization of the carboxy-terminus of the polyketide with formation of a pyran-2-one moiety. This structure is likely to be the substrate for two types of oxidoreductases (OXRC and HOXC) present in both loci. HOXC presents amino acid sequence similarity to the oxygenase MtmOIV involved in the biosynthesis of the antitumor antibiotic, mithramycin [11]. This enzyme is predicted to catalyze a Baeyer-Villiger monooxygenation reaction using NADPH as a cofactor to give a seven-membered oxygenated cyclic carbonate which will undergo spontaneous hydrolytic cleavage with a loss of one

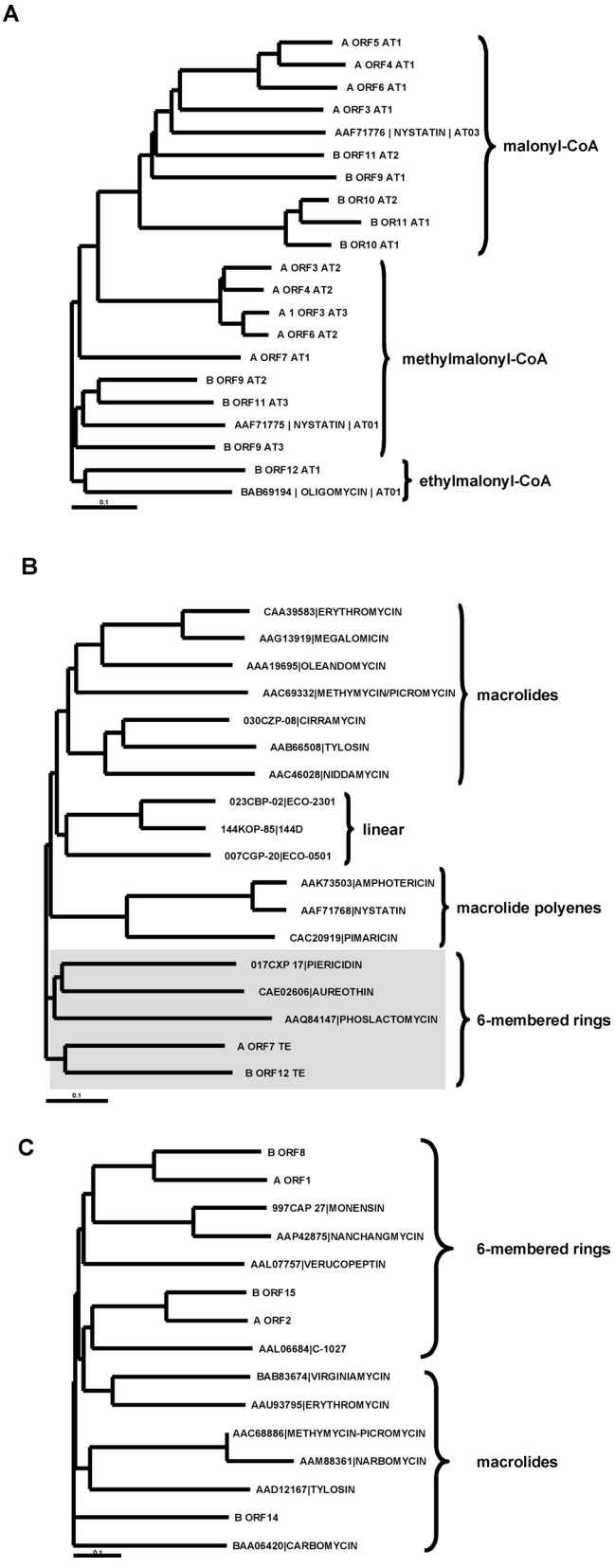

Fig. 3 Phylogenetic relatedness of acyltransferase domains (A), thioesterase domains (B) and hydroxylases (C) present in gene clusters A and B.

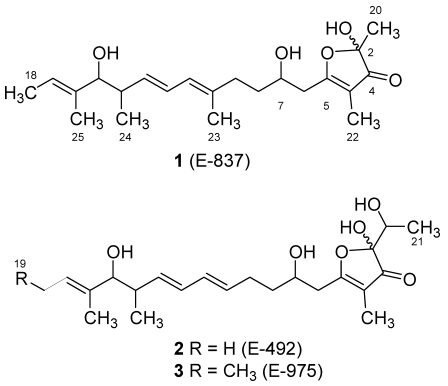

Fig. 4 The structures of **1** (E-837), **2** (E-492) and **3** (E-975).

carbon unit (CO_2) to give the 5-alkenyl-3,3(*2H*)-furanone framework after hemiketalization. Phylogenetic analysis of the 5 hydroxylases (OXRC) present in loci A and B indicated that four of them are closely related to each other, namely ORF 1 in locus A to ORF 8 in locus B and ORF 2 in locus A to ORF 15 in locus B. All four ORFs are related to enzymes that hydroxylate 6-membered ring structures such as sgcD3 in the C-1027 gene cluster that hydroxylates carbon 5 of the 3-hydroxy-anthranilic acid component [12]. A possible function for these ORFs would be hydroxylation, most likely at position 3 of the pyran-2-one moiety, the 6-membered ring intermediate structure, formed through the action of the terminal thioesterase. Locus B contains an additional hydroxylase, ORF 14, that is absent in locus A suggesting a second hydroxylation event in the biosynthesis of the compound specified by this locus. This ORF is closely related to enzymes that hydroxylate macrolides. A possible site for the action of this enzyme would be the ethyl group of the pyran-2-one or of the 5-alkenyl-3,3(*2H*)-furanone moieties. Full analysis of the gene clusters led to the prediction of two polyketides of structures **1** and **2** (Fig. 4). Additional ORFs present solely in locus B correspond to transcriptional regulators and to membrane proteins and are likely to regulate expression of compounds of structures **2** and **3**.

To obtain expression of these gene clusters, both *S. aculeolatus* NRRL 18422 and *Streptomyces* sp. Eco86 were grown in shaken flasks in a dozen different media designed for the production of secondary metabolites. At harvest, an equal volume of MeOH was added to the broths, which were then vortexed and centrifuged. The supernatant liquid was then drawn off and concentrated to dryness. The resulting residue was re-suspended in MeOH and subjected to HPLC/MS/UV analyses. The extracts of fermentations of *S. aculeolatus* NRRL 18422 in six of the twelve media yielded a compound with UV absorption λ_{max} at 236 and

Table 1 ^{1}H and ^{13}C NMR data of compounds **1**∼**3**

Position	**1**		**2**		**3**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2		103.0		104.3 (104.5)		104.3 (104.4)
3		204.1		204.6 (203.1)		204.6 (203.1)
4		109.2		111.4 (110.9)		111.3 (110.9)
5		185.8		186.3 (186.8)		186.7 (186.1)
6	2.73, 2.68	37.1	2.78, 2.72	37.1 (36.9)	2.78, 2.72	37.2 (37.0)
7	3.96	68.4	4.07 (4.00)	68.5 (68.2)	4.07 (4.00)	68.5 (68.2)
8	1.70, 1.64	35.5	1.70, 1.64	36.9 (36.8)	1.70, 1.64	37.0 (36.9)
9	2.24, 2.16	35.6	2.26, 2.21	28.5 (28.6)	2.26, 2.21	28.7 (28.6)
10		135.5	5.62	131.3	5.62	131.3
11	5.87	125.7	6.08	130.5	6.08	130.5
12	6.31	126.8	6.08	131.4	6.08	131.3
13	5.55	135.3	5.59	135.3	5.59	135.2
14	2.35	40.7	2.31	40.5	2.32	40.4
15	3.64	82.3	3.65	82.3	3.64	82.3
16		136.7		136.8		135.3
17	5.44	121.1	5.44	121.7	5.36	129.4
18	1.63	11.9	1.63	11.9	2.07	20.9
19					0.98	13.3
20	1.44 (1.45)	21.0	3.92	68.8 (69.7)	3.92	68.8 (69.7)
21			1.31 (1.19)	15.5 (15.1)	1.31 (1.19)	15.6 (15.1)
22	1.68	4.6	1.67 (1.66)	4.6 (4.5)	1.67 (1.66)	4.7 (4.6)
23	1.76	15.4				
24	0.86	16.9	0.86	16.7	0.87	16.8
25	1.60	9.8	1.63	9.8	1.60	10.1

The ^{1}H NMR was measured at 500 MHz and ^{13}C NMR was measured at 125 MHz in methanol-d_4. The chemical shifts on parentheses are belongs to the second diastereomer.

283 nm, and MS peaks at *m*/*z* 401 (in positive mode) and 377 (in negative mode). By comparison the extracts of nine of the 12 fermentations of *Streptomyces* sp. Eco86 yielded one or two compounds having similar retention times to the *S. aculeolatus* metabolite and with identical UV absorption, λ_{max} at 231 and 284 nm, and differing from one another in 14 amu in their mass with positive ions at *m*/*z* 417 for the earlier eluting compound and 431 for the later eluting one. Larger scale fermentations of each organism were carried out in the most productive media and the target compounds were then isolated by a series of fractionations followed by reversed phase HPLC.

The ^{1}H NMR spectrum of **1** displayed four olefinic protons (δ 6.31, 5.87, 5.55 and 5.44) together with two oxygenated methine protons (δ 3.64 and 3.96) and six methyl groups (δ 1.76, 1.68, 1.63, 1.60, 1.44 and 0.86). The analyses of COSY and HSQC experiments indicated that the presence of a further methine proton at δ 2.35 and three methylene groups (δ 2.24, 2.16; 2.73, 2.68; and 1.70, 1.64). A quaternary carbon signal at δ 103.0 having long-range correlation with a methyl group at δ 1.44 in a CIGAR spectrum* [13] suggested the presence of methyl ketal function. Moreover, the long range correlation between the methyl group at δ 1.44 and the ketone at δ 204.1 and the methyl group at δ 1.68 with both the ketone at δ 204.1 and the carbon at δ 185.8 suggested that the presence of 5-alkenyl-3,3(*2H*)-furanone moiety in the molecule. The partial structures deduced from COSY and HSQC were connected by the long-range correlations observed in a CIGAR spectrum and the structure was confirmed as **1** (Fig 4). Selected COSY and NOESY cross peaks and 2- and 3-bond H-C couplings from the CIGAR experiment are shown in Fig. 5. The NOESY data is sufficient to define the stereochemistry to the extent presented. The structure was as predicted by genomic

* See reference for a description of the advantages of CIGAR over more classical HMBC pulse sequences.

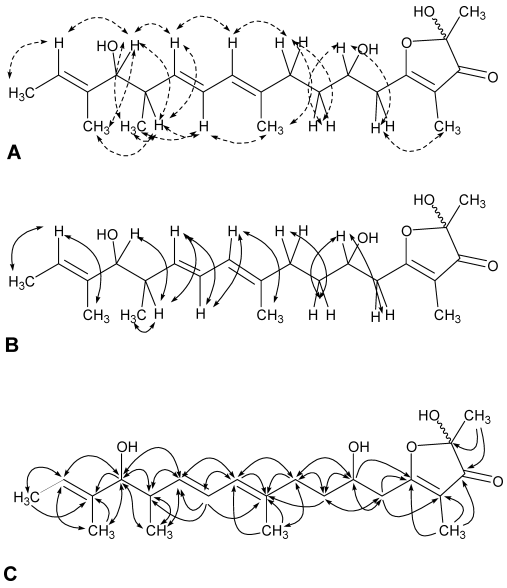

Fig. 5 Compound **1**; A, NOE interactions as read from a NOESY experiment; B, ^{1}H-^{1}H couplings from a COSY experiment; C, 2- and 3-bond ^{1}H-^{13}C couplings as read from a CIGAR experiment.

Arrows point from H to C. Two-headed arrows indicate that both possible interactions were seen.

analysis. The two singlet peaks for methyl groups at δ 1.45 and δ 1.44 suggested that compound **1** is an equilibrium mixture of 2*R* and 2*S* anomers. This was confirmed by measuring the ^{1}H NMR in benzene-d_6, where these two methyl signals occurred at a very different ratio.

The ^{1}H NMR spectrum of **2** was similar to that of **1**. Significant differences included an additional olefinic proton signal at δ 5.52 and an oxymethine proton signal at δ 3.92, replacing the methyl signal at δ 1.76 as in the spectrum of **1**. The additional olefinic proton was assigned to H-10, because of its COSY correlations with another olefinic proton at δ 6.08 and methylene protons at δ 2.26 and δ 2.21. The COSY correlation between the methyl group signal at δ 1.31 and the methine at δ 3.92 indicated that the additional oxymethine should be assigned to H-20. This was confirmed by the long range correlations observed between the oxymethine proton at δ 3.92 and carbons at δ 15.5, 104.3 and 204.6 in a gHMBC experiment. The final structure was in accord with that predicted (Fig. 4). Compound **3**, on the other hand, had an additional –CH_2 in the chain, which was supported by the high field shift of the methyl group to δ 0.98 and additional methylene proton signals at δ 2.07 having COSY correlations with the δ 0.98 methyl protons and olefinic proton at δ 5.36. Similar COSY, NOESY and CIGAR data, to that shown in Fig. 5 for compound **1** were also obtained for both compounds **2** and **3**. (Data not shown.)

Table 2 The electron transfer activities of compounds **1**~**3**

	IC_{50} (μg/ml)	
	NADH-fumarate reductase (complexes I+II) from *Ascaris suum*	NADH oxidase (complexes I+III+IV) from bovine heart
E-492	1.9	6.5
E-837	1.8	11.1
E-975	3.7	1.8
Nafuredin	0.0051	0.15

Despite the relative simplicity of the structures of compounds **1** to **3** they have few known close structural relatives. An analog of **1** in which a shorter side-chain is fully branched and fully reduced, AS-183, was isolated from a *Scedosporium* sp. as an inhibitor of acyl-CoA: cholesterol acyl transferase (ACAT) [14]. There is a closer structural resemblance, at least in size, if not in oxidation state to nafuredin-γ, a degradation product of another fungal metabolite from *Aspergillus niger* FT-0554, and shown to selectively inhibit helminth NADH-fumarate reductase [15]. Perhaps the closest structural relatives are the aurafurons A, *E*B and *Z*B isolated from the myxobacteria *Stigmatella aurantiaca* and *Archangium gephyra* [16]. These compounds exhibited modest activity against certain filamentous fungi.

E975 did not display inhibitory activity against ACAT at 10 μM.

Compounds' E-975 and E-837 antifungal activities were also evaluated. Each compound had a minimal inhibitory concentration (MIC) of 64 μg/ml in microbroth dilution assays against a *Saccharomyces cerevisiae* strain deleted of genes *pdr1*, *pdr3* and *erg6*. In addition, compound E-975 displayed antifungal activity against the filamentous fungus *Aspergillus fumigatus* using an agar zone of inhibition assay. Compounds E-837 and E-492 were inactive in this assay.

All three compounds were tested for their electron transport enzymes inhibition. They showed IC_{50} values of 1~4 μg/ml against *Ascaris suum* NADH-fumarate reductase complex and 1~12 μg/ml against bovine heart NADH oxidase (Table 2). Though E-837 exhibited a 6-fold selectivity in inhibition against the helminth complex compared to the bovine heart complex, nafuredin had a 30 fold selectivity and was many times more potent. These three streptomycete metabolites appear to be non-selective

electron transport complex inhibitors of moderate potency.

Experimental

General

The NMR spectra were measured on a Varian Unity Inova 500 MHz spectrophotometer and are referenced to TMS. The analytical HPLC was carried out with a Waters Alliance 2690 instrument equipped with a Micromass ZQ electrospray source and Waters 996 diode array UV detectors. Semi-preparative HPLC was done on a Waters 1525 instrument with Waters 2996 diode array UV detector. High resolution mass spectra were measured at the University of Iowa, Department of Chemistry on a Micromass Autospec instrument in the positive electrospray mode with a methanol/water 1 : 1 eluent and polyethylene glycol as the calibrant.

Genome Scanning

The genome of *Streptomyces aculeolatus* NRRL 18422 and *Streptomyces* sp. Eco86 were analyzed by genome scanning technique as described previously by Zazopoulos *et al.* [2]. The DNA and protein sequences that comprise E-837, E-492 and E-975 gene clusters are deposited in GeneBank under accession numbers DQ272520 (E-837) and DQ272521, DQ272522 (two contigs from the E-492/E-975 producer).

Fermentation and Isolation of 1 from *S. aculeolatus* NRRL 18422

S. aculeolatus NRRL 18422 was obtained from the Agricultural Research Service Culture Collection (1815 N. University Street, Peoria, IL 61604, USA). To prepare a vegetative culture, *S. aculeolatus* NRRL 18422 was grown on ISP2 agar (Difco) for 5 to 7 days, and the surface growth from the agar plate was homogenized and transferred to a 125 ml flask containing three glass beads (5 mm diameter), and 25 ml of sterile medium ITSB composed of 30 g trypticase soy broth (Bacto), 3.0 g yeast extract, 2.0 g $MgSO_4$, 5.0 g glucose, 4.0 g maltose to which one liter distilled water was added. This vegetative culture was incubated at 28°C for about 70 hours on a shaker set at 250 rpm.

From the vegetative culture, 10 ml was used to inoculate 2-liter baffled flasks each containing 500 ml of sterile production medium consisting of 35 g malt extract, 30 g corn starch, 15 g corn steep liquor, 15 g Pharmamedia and 2.0 g calcium carbonate ($CaCO_3$) per liter of distilled water. The medium was adjusted at pH 7.3 before sterilization. The fermentation batches were incubated aerobically on a rotary shaker (250 rpm) at 28°C for a 4-day period.

The mycelia and broth of the culture media (5 to 8 liters) was separated by centrifugation (3000 rpm, 15 minutes). The mycelia cake was extracted consecutively with methanol (200 ml) and acetone (200 ml) to produce an organic cell extract. The organic content of the broth was adsorbed (slurry-mode) on Dianion HP-20 resin (120 ml), which was subsequently washed with water (200 ml) and eluted with a step gradient of 60 : 40 methanol/water, methanol, and acetonitrile (200 ml). The two latter fractions were combined with the organic cell extract and evaporated to produce the total crude extract. The solid crude extract was extracted twice with ethyl acetate (500 ml portions). The combined extracts were evaporated and the residue was resuspended in methanol/water (90 : 10, 50 ml). This methanolic phase was defatted by extraction with *n*-heptane (50 ml) and the methanolic layer was evaporated to produce an HPLC-compatible sample.

The sample was dissolved in a minimal amount of dimethyl sulfoxide (DMSO) and filtered through a 0.45 μm 13 mm Acrodisc GHP syringe filter. Multiple injections of no more than 500 μl samples on a Waters RCM Nova-Pak HR C18 6 μm 60 Å 25×200 mm column (water/acetonitrile 70 : 30～43 : 57, 0～12 minutes+43 : 57～0 : 100, 12～16 minutes at 20 ml/minute) afforded E-837 as a semi-pure solid (2～3 mg/liter broth). A final step of normal phase HPLC was needed to purify compound **1**. A Waters Nova-Pak Silica 6 μm 19×300 mm column eluted with chloroform/methanol 98 : 2 under isocratic conditions at 20 ml/minute yielded pure compound **1** as a syrup (0.9 mg/liter broth).

1: UV λ_{max}^{MeOH} (log ε): 236 (4.2), and 283 (3.4) nm, MS (ESI in positive mode) *m*/*z*: 401.36 $(M+Na)^+$, 379.35 $(M+H)^+$, 361.35 $(M+H-H_2O)^+$, 343.35 $(M+H-2H_2O)^+$; MS (ESI in negative mode) *m*/*z*: 377.29 $(M-H)^-$, 345.20 $(M-H-2H_2O)^-$; HRMS *m*/*z*: 401.2297 ($C_{22}H_{34}O_5Na$ requires 401.2304). The ^1H and ^{13}C NMR data are in Table 1.

Fermentation and Isolation of 2 and 3 from *Streptomyces* sp. Eco86

Streptomyces sp. Eco86 is a soil isolate and was deposited at the International Depositary Authority of Canada (IDAC), Bureau of Microbiology, Health Canada (1015 Arlington Street, Winnipeg, Manitoba, Canada R3E 2R2) under IDAC accession number 130204-04. The strain Eco86 was cultivated and the vegetative culture was prepared under identical conditions and media to those described above. From the vegetative culture 10 ml aliquots were used to inoculate 2-liter baffled flasks each containing 500 ml of sterile production medium consisting of 60 g

molasses, 20 g soluble starch, 20 g fish meal, 0.1 g copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), 0.5 mg sodium iodide (NaI) and 2.0 g calcium carbonate ($CaCO_3$), to which 1.0 liter distilled water was added. The fermentation batches were incubated aerobically on a rotary shaker (250 rpm) at 28°C for a period of 7 days.

The culture broth (2.0 liters) was treated with EtOAc (1.2 liters), shaken for 30 minutes and centrifuged at 3000 rpm for 20 minutes. The organic layer was collected and dried over anhydrous $MgSO_4$, filtered and the filtrate was evaporated under reduced pressure leading a viscous EtOAc extract. The extract was then suspended in methanol/water (9 : 1; 50 ml) and defatted by extraction with *n*-heptane (50 ml×3). The resulting MeOH/water fraction was subjected to solid phase extraction on Strata C18-E (10 g, Phenomenex) eluted with a MeOH/water step-wise gradient (100 ml fractions) and led to six fractions: MeOH/water (20 : 80), MeOH/water (40 : 60), MeOH/water (60 : 40), MeOH/water (80 : 20), MeOH and MeOH with 0.1% trifluoroacetic acid. The MeOH/water (80 : 20) fraction was concentrated to dryness, and the residue was dissolved in MeOH (500 μl) and filtered through a 0.45 μm 13 mm Acrodisc GHP filter. Compounds **2** (7.34 mg) and **3** (2.49 mg) were isolated as syrups after multiple injections (50 ml each) of material from the MeOH/water (80 : 20) fraction onto a Waters RCM Nov-Pak HR C18 6 μm 60A 25×200 mm column (20 ml/minute, gradient H_2O/CH_3CN 75 : 25 to 25 : 75, 0～20 minutes; 25 : 75 to 0 : 100, 20～22 minutes) with retention times of 15.5 and 17.9 minutes respectively.

2: UV λ_{max}^{MeOH} (log ε): 231 (4.5) and 284 (3.9) nm; MS (ESI in positive mode) *m*/*z*: 417.14 $(M+Na)^+$, 395.16 $(M+H)^+$, 377.22 $(M+H-H_2O)^+$, 359.22 $(M+H-2H_2O)^+$; MS (ESI in negative mode) *m*/*z*: 393.13 $(M-H)^-$; HRMS *m*/*z*: 417.2264 ($C_{22}H_{34}O_6Na$ requires 417.2253). The ^1H and ^{13}C NMR data are in Table1.

3: UV λ_{max}^{MeOH} (log ε): 231 (4.3) and 284 (3.4) nm; MS (ESI in positive mode) *m*/*z*: 431.17 $(M+Na)^+$, 409.18 $(M+H)^+$, 391.18 $(M+H-H_2O)^+$, 373.24 $(M+H-2H_2O)^+$; MS (ESI in negative mode) *m*/*z*: 407.09 $(M-H)^-$; HRMS *m*/*z*: 431.2405 ($C_{23}H_{36}O_6Na$ requires 431.2410). The ^1H and ^{13}C NMR data are in Table 1.

Assay Methods

NADH-fumarate Reductase

NADH-fumarate reductase activity was assayed using a mitochondrial fraction of *Ascaris suum*. *Ascaris suum* muscle (0.5 g) was homogenized in 1.5 ml of buffer (120 mM sodium phosphate, pH 7.0) and centrifuged at 3,000×*g* for 10 minutes to remove cell debris. The supernatant was further centrifuged at 10,000×*g* for 20 minutes and the resulting mitochondrial precipitate was resuspended in the same buffer. The assay solution (120 mM sodium phosphate, 0.35 mM NADH, and 7.2 mM disodium fumarate, pH 7.0, 80 μl) and 10 μl of 50% DMSO solution of test compound were preincubated for 5 minutes at 37°C. The reaction was initiated by the addition of 10 μl of the mitochondrial fraction (0.3 mg protein/ml), and the incubation was carried out for 3 minutes at 37°C. The NADH-fumarate reductase activity was measured spectrophotometrically by monitoring the oxidized NADH at 340 nm.

NADH Oxidase

NADH oxidase activity was assayed using a mitochondrial fraction of bovine heart. Bovine heart muscle (1 g) was homogenized in 5 ml of buffer (120 mM sodium phosphate, pH 7.0) and centrifuged at 1,000×*g* for 10 minutes to remove cell debris. The supernatant was further centrifuged at 10,000×*g* for 20 minutes and the resulting mitochondrial precipitate was resuspended in 2 ml of the same buffer. This was treated with ultrasonic irradiation and centrifuged at 100,000×*g* for 30 minutes. The precipitate was suspended in 20 ml of the buffer as the mitochondrial fraction. The assay solution (120 mM sodium phosphate, 0.7 mM NADH, pH 7.0, 80 μl) and 10 μl of 50% DMSO solution of test compound were preincubated for 3 minutes at 37°C. The reaction was initiated by the addition of 10 μl of the mitochondrial fraction, and the incubation was carried out for 3 minutes at 37°C. The NADH oxidase activity was measured spectrophotometrically by monitoring the oxidation of NADH at 340 nm.

Antifungal Activity Determination

Broth microdilution assays were performed according to NCCLS recommendations [17]. *Saccharomyces cerevisiae pdr1Δ/pdr3Δ/erg6Δ* strain was a gift from the Dr. Julian Simon's laboratory (Fred Hutchinson Cancer Research Center, WA, USA).

Agar zone of inhibition assays were performed according to Arikan *et al.* [18]. Briefly, an aliquot of test article (10 μl; 32 μg) prepared in 100% DMSO was deposited on the surface of a 150 mm agar plate containing *Aspergillus fumigatus* fungal spores. Following incubation for 48 hours at 35°C, antifungal activity was defined by the presence of a zone of inhibition of fungal growth surrounding the aliquoted test article. The *Aspergillus fumigatus* strain was provided by Dr. Louis de Repentigny (University of Montréal, Qc, Canada).

ACAT Assays

Acetyl CoA-cholesterol acyltranferase assays were

performed according to Largis *et al.* [19] and Sliskovic and White [20].

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