Oviedomycin, an Unusual Angucyclinone Encoded by Genes of the Oleandomycin-Producer *Streptomyces antibioticus* ATCC11891

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Received November 5, 2001

Our investigations on the discovery of novel natural metabolites using type II polyketide synthase gene probes (*act*I/III) yielded an unusual angucyclinone, oviedomycin (**2**), when applied to the oleandomycin (**1**) producer *Streptomyces antibioticus* ATCC11891. The novel natural product was produced using *S. albus* R^-M^- as a host strain, into which a cosmid containing the oviedomycin gene cluster was transformed. Its structure was elucidated by NMR spectroscopy and mass spectrometry.

Genes that are unexpressed under normal conditions as well as genes from noncultivatable organisms are discussed in the context of drug discovery strategies. The search for such genes in bacteria of the genus *Streptomyces* seems profitable, since they not only produce the most biologically active natural products but are also known for their large genome, which often contains far more biosynthetic genes than necessary to encode the biosynthesis of the compounds normally produced by a certain strain. In particular, polyketide synthase (PKS) encoding genes are interesting, since polyketides represent perhaps the largest and structurally most diverse group of natural products, often displaying interesting biological and pharmaceutical activities.[1−5] PKS genes have even been found in bacterial strains that are not known to produce polyketides. For example, polyketide synthase genes were recently discovered in various *Mycobacteria*, and in one case an interesting polyketide natural product was already identified.[6−8] Thus, the search for PKS encoding genes and novel polyketides is promising. Among the various PKS, one may distinguish between (i) type I PKS, which typically catalyze the biosynthesis of modularly assembled macrolactones (e.g., erythromycin or oleandomycin) or polyethers;[2−5,9,10] (ii) type II PKS, which catalyze the synthesis of multicyclic, mostly aromatic, compounds that are often pigments (e.g., anthracyclines, tetracyclines, angucyclines);[2−5,11−15] and (iii) type III PKS,[16−18] which occur in plants (chalcone synthases) and were recently also discovered in bacteria. Usually, only one of these three PKS systems is described in context with a certain producer of natural compounds, although type II PKS genes that have been found to encode the biosynthesis of spore pigments of Streptomycetes[19] should exist in most strains of this genus.

Streptomyces antibioticus ATCC11891 is known as an oleandomycin (**1**) producer, which is a macrolide antibiotic, biosynthesized by a modular type I PKS. The oleandomycin gene cluster, which contains three genes for a modular polyketide synthase, has been cloned and characterized.[20−26] In this paper, we describe the identification of type II PKS genes in *S. antibioticus* ATCC11891, which code for a previously unknown, usually not produced, aromatic metabolite that may be a spore pigment.

To identify an aromatic polyketide gene cluster in *S. antibioticus* ATCC11891, a cosmid library of the total DNA of this microorganism,[26] constructed in the bifunctional (*Streptomyces*−*E. coli*) cosmid pKC505,[27] was hybridized with the *act*I/III probes from the actinorhodin gene cluster in *S. coelicolor*.[28] Both probes are part of a typical type II PKS gene cluster, with the *act*I probe containing part of the minimal polyketide synthase PKS genes and the *act*III probe containing a ketoreductase gene.[29] Two different clusters with putative type II PKS genes were identified: (a) Cluster I, cosmid clones that hybridize with the *act*I/III probes in a single 8 kb *Bam*HI band; five overlapping cosmid clones were isolated as part of this group, cosAB3, cosAB4, cosAB16, cosAB50, and cosAB51 (Figure 1); and (b) Cluster II, clones hybridizing with the *act*I probe in a 1.8 kb *Bam*HI fragment and with the *act*III region in a 8 kb *Bam*HI fragment. From this cluster, four overlapping cosmid clones were isolated, cosAB18, cosAB26, cosAB36, and cosAB38. All cosmids belonging to Clusters I and II were analyzed for their ability to produce aromatic polyketides. For this, they were used to independently transform protoplasts of *S. lividans* TK21[30] as well as *S. albus* R^-M^-.[31] Transformants were selected for their resistance to apramycin (25 μg/mL final concentration) on R5 (=R2YE)[30] medium and were screened for the production of pigments. Neither the control nor any of the cosmids belonging to Cluster II induced the production of a pigment in either of the *Streptomyces* hosts. However, in the case of cosmids belonging to Cluster I, some (cosAB3, cosAB4, cosAB16, and cosAB50) led to the production of a red-brown pigment both in *S. albus* and in *S. lividans*. While the pigment in those *Streptomyces* strains harboring cosAB16 seemed to remain in the colony, the pigment was diffusible in the case of those strains harboring cosAB3 or cosAB4 (more visible with cosAB3). The pigment produced by the strain harboring cosAB3 was isolated from bulk cultures to elucidate its structure.

The structure of the pigment, which was subsequently named oviedomycin, was elucidated using NMR and mass spectroscopy. Oviedomycin (**2**) has a molecular formula of

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10.1021/np010555n CCC: $22.00 © 2002 American Chemical Society and American Society of Pharmacognosy
Published on Web 04/25/2002

Figure 1. Schematic representation of overlapping cosmids from polyketide Cluster 1 region of *S. antibioticus*. B = *Bam*H1; shaded square = *act*I/III hybridizing region.

Table 1. NMR Data of Oviedomycin (**2**) (DMSO-d_6, 400 and 100.7 MHz, respectively)

position	δ_H mult. (J in Hz)	δ_C	H,H-COSY couplings	HMBC couplings
1		178.5		
OH-2	n.o.[a]	158.0 (181.0)[b]		
3	4.05 q (6)[c]	119.2		
4		182.9		
4a		139.4		
5	7.62 s	118.0		120.6 (^3J) 123.8 (^3J) 182.9 (^3J) 139.4 (^2J) 163.7 (^2J) 178.5 (^4J)[d] 189.9 (^4J)[d]
OH-6	n.o.[a]	163.7		
6a		120.6		
7		189.9		
7a		115.8		
OH-8	11.53 br s	160.5		
9	7.39 d (8)	123.7	7.50; 7.80	115.8 (^3J) 118.3 (^3J) 160.5 (^2J) 135.6 (^4J)[d] 189.9 (^4J)[d]
10	7.80 dd (8, 8)	137.5	7.39; 7.50	135.6 (^3J) 160.5 (^3J) 118.3 (^2J) 123.7 (^2J) 115.8 (^4J)[d] 186.3 (^4J)[d]
11	7.50 d (8)	118.3	7.39; 7.80	115.8 (^3J) 123.7 (^3J) 186.3 (^3J) 137.5 (^2J) 160.5 (^4J)[d] 189.9 (^4J)[d]
11a		135.6		
12		186.3		
12a		138.7		
12b		123.8		
13	1.93 s 1.25 d (6)[c]	8.3		158.0 (^3J) (181.0) (^3J) 182.9 (^3J) 119.2 (^2J)

[a] Not observed. [b] Due to keto–enol tautomerism. [c] Keto form. [d] Weak coupling signal.

$C_{19}H_{10}O_7$ (350.2874 g/mol) evident from the ^{13}C NMR data (19 carbons) and especially from the molecular ion peak of the HREIMS (*m/z* 350, 60%; calcd for $C_{19}H_{10}O_7$; *m/z* 350.0427, found *m/z* 350.0433). The ^1H NMR data revealed only an aromatic ABC system, an additional aromatic singlet, and a sp^2-bound methyl group. Only one OH signal was observed, although the structure (**2**, see below) demands three; two of them were evidently exchanged. The large number of quaternary carbons (14 out of 19) complicated the structure determination. However, the long-range couplings observed in the HMBC spectrum (see Table 1), particularly the long-range couplings of the diagnostic H-5 singlet (δ 7.72 s) with seven different carbons, permitted

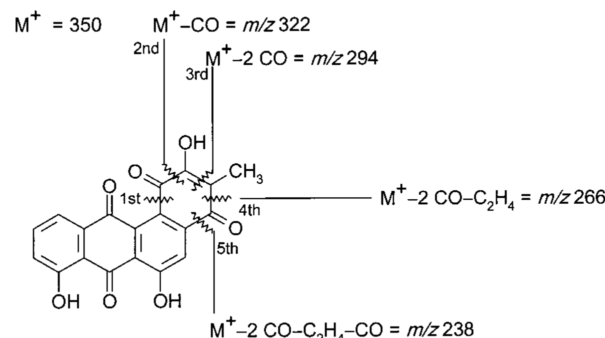


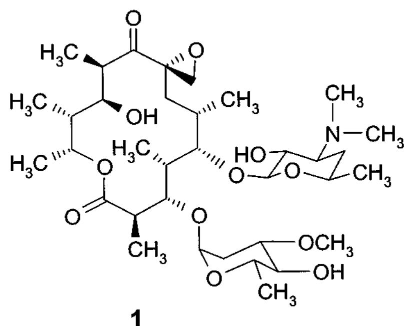
Figure 2. EIMS fragmentation of oviedomycin (**2**).

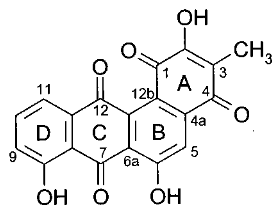
the combination of all determined structural elements as in structure **2**. For instance, the anellation of ring D followed from the fact that both H-5 and H-9 showed a small, diagnostic $^4J_{C-H}$ coupling to the same carbonyl (C-7, δ 189.9), and H-11 showed a large $^3J_{C-H}$ coupling to C-12 (δ 186.3) and a small $^4J_{C-H}$ coupling to C-7 (δ 189.9). Independent confirmation of the substitution pattern of ring A came from the EIMS, showing the consecutive fragmentation of the C-12b/C-1, C-1/C-2, C-2/C-3, C-3/C-4, and C-4/C-4a bonds. This fragmentation was verified by accurate mass determination of the resulting MS peaks (see Experimental Section and Figure 2). Thus, oviedomycin (**2**) is an angucyclinone with novel structural features, particularly the two quinone systems and the unusually substituted ring A. This oxygen-rich ring A bears three carbonyls, one of which (C-2) is predominantly enolized, although a small amount of the keto form (ca. 10%) was also visible in the NMR spectra, as indicated by several signals (e.g., δ_C of C-2: 158.0 and 181.0; δ_H of CH$_3$-3: 1.93 s and 1.25 d; δ_H of H-3: 4.05 q).

Considering the typical biosynthetic incorporation of acetate units into the angucyclinone backbone,[14,15] at least three of the oxygens (O-2, O-3, and O-12) may be introduced by oxygenases, while the remaining four might stem from acetate. Oxygenation at the C-4 position is unprecedented among angucyclinones. Thus, the investigation of the oviedomycin biosynthetic gene cluster, planned for the near future, will reveal novel genes, which may be useful for combinatorial biosynthetic studies.

The experiments described here show that it is profitable to screen and heterologously express type II PKS genes to stimulate the production of undiscovered novel multicyclic, aromatic compounds. The results suggest that oviedomycin (**2**) is a product of heterologously expressed *S. antibioticus* genes; however, it cannot be ruled out that the host strain provided some of the enzyme activities, which may have contributed to the formation of **2**. Additional experiments using various growth conditions showed that traces of oviedomycin (**2**) could also be detected in *S. antibioticus* when grown on solid agar using GAE medium,[32] and it seems that **2** confers a brown pigmentation to the colonies. However, this is hard to observe, since *S. antibioticus* also

produces large amounts of melanin that masks the oviedomycin pigmentation.





Experimental Section

General Experimental Procedures. UV spectra were recorded on a Beckman DU 650 spectrophotometer, and the IR spectrum was obtained from a pure sample on KCl disks in a Mattson Genesis II FT FT-IR spectrometer. NMR spectra were recorded in DMSO-d_6 on Varian Inova 400 and Bruker DMX 500 NMR instruments at 400 and 500 MHz, respectively, for ^{1}H and 100.6 and 125.7 MHz, respectively, for ^{13}C, using 1D and 2D homo- and heteronuclear correlation experiments (^{1}H, ^{13}C, DEPT, H,H-COSY, HSQC, and HMBC). Electron-impact mass spectrometry (EIMS) was carried out using a VG 70SQ double-focusing magnetic sector mass spectrometer with sample introduction by direct exposure probe and an ionization energy of 70 eV. HPLC was performed on a Waters HPLC system (Delta 600, M32 add-on single system, with a photodiode array detector model 996), using the columns and guard columns described below.

Bacterial Strains, Plasmids, and Gene Probes. For constructing a cosmid gene library of total DNA of *S. antibioticus*,[26] the bifunctional (*Streptomyces*–*E. coli*) cosmid, pKC505,[33] was used. The obtained cosmids were hybridized with the *act*I/III probes from the actinorhodin gene cluster in *S. coelicolor*.[28] The *act*I probe corresponds to a 2.2 kb *Bam*HI fragment from pIJ2345 that contains part of the polyketide synthase genes, and the *act*III probe corresponds to a 1.1 kb *Bam*HI fragment from pIJ2363 that contains the ketoreductase gene.[29] As host strains for transformation, *S. lividans* TK21 and *S. albus* R^-M^-[31] were used. Protoplast transformations were according to standard procedures.[31,34] In control experiments, cosmid pKC505[27] was transformed.

Cultivation and Fermentation. For the production of oviedomycin, the strain *Streptomyces albus* R^-M^- (cosAB3) was incubated in eight 2 L Erlenmeyer flasks each containing 400 mL of SCM medium,[34] with glycerol substituting for starch, and supplemented with 2.5 μg/mL of apramycin, for 3 days at 30 °C and 250 rpm.

Isolation of Oviedomycin (2). The cultures were centrifuged, and the supernatants were filtered, adjusted to pH 5.5 with formic acid, and applied to a solid-phase extraction cartridge (Supelclean LC-18; 10 g; Supelco). The cartridge was eluted with methanol and 0.1% trifluoroacetic acid in water, using a linear gradient from 0 to 100% methanol in 60 min, at 10 mL/min, taking fractions every 5 min. The eluate collected between 45 and 55 min contained most of the compound, as assessed by HPLC analysis of the fractions. The material in these fractions was dried in vacuo and redissolved in a small amount of dimethyl sulfoxide. The product was purified by preparative HPLC in a μBondapak C_{18} radial compression cartridge (PrepPak Cartridge; 25 by 100 mm; Waters). An isocratic elution with a mixture of acetonitrile and 0.1% trifluoroacetic in water (55:45), at 10 mL/min, allowed the separation of **2** as a pure peak, which was collected and dried in vacuo, yield 12.5 mg/L, obtained as an amorphous orange-red solid.[35]

Characterization of Oviedomycin (2): UV [MeOH] λ_{max} (ϵ) 473 (3600), 275 (12800), 218 (12300) nm; [MeOH–HCl] λ_{max} (ϵ) 433 (4400), 320 sh (4300), 277 (12800), 220 (12500) nm; [MeOH–NaOH] λ_{max} (ϵ) 522 (4100), 372 (6700), 279 (12700), 227 (12800) nm; IR ν_{max} (KCl) 3290, 2929, 2864, 7143sh, 1676, 1630, 1596, 1576, 1534, 1476, 1409, 1353, 1267, 1201, 1031 cm^{-1}; ^{1}H NMR, ^{13}C NMR, and observed COSY and HMBC couplings, see Table 1; HREIMS *m*/*z* 350 (M^+, 350.0433, calcd for $C_{19}H_{10}O_7$, 350.0427, 60), 322 (M – CO, 322.0478, calcd for $C_{18}H_{10}O_6$, 322.0477, 100), 294 (M – 2CO, 294.0525, calcd for $C_{17}H_{10}O_5$, 294.0528, 40), 266 (M – 2CO – C_2H_4, 266.0218, calcd for $C_{15}H_6O_5$, 266.0215, 25), 238 (M – 2CO – C_2H_4 – CO, 238.0267, calcd for $C_{14}H_6O_4$, 238.0266, 20); see also Figure 2.

Acknowledgment. This work was partly supported by grants from the U.S. Department of Defense, awarded to J.R., and the Spanish Ministry of Science and Technology (Plan Nacional en Biotecnología BIO2000-0274), awarded to J.A.S.

References and Notes

(1) Seow, K. T.; Meurer, G.; Gerlitz, M.; Wendt-Pienkowski, E.; Hutchinson, C. R.; Davies, J. *J. Bacteriol.* **1997**, *179*, 7360–7368.
(2) Hopwood, D. A. *Chem. Rev.* **1997**, *97*, 2465–2497.
(3) Hutchinson, C. R.; Fujii, I. *Annu. Rev. Microbiol.* **1995**, *49*, 201–238.
(4) Staunton, J.; Weissman, K. J. *Nat. Prod. Rep.* **2001**, *18*, 380–416.
(5) Tsoi, C. J.; Khosla, C. *Chem. Biol.* **1995**, *2*, 355–362.
(6) Rohr, J. *Angew. Chem., Int. Ed.* **2000**, *39*, 2847–2849.
(7) George, K. M.; Chatterjee, D.; Gunawardana, G.; Welty, D.; Hayman, J.; Lee, R.; Small, P. L. C. *Science* **1999**, *283*, 854–857.
(8) Gunawardana, G.; Chatterjee, D.; George, K. M.; Brennan, P.; Whittern, D.; Small, P. L. C. *J. Am. Chem. Soc.* **1999**, *121*, 6092–6093.
(9) Sherman, D. H. *Nature* **2000**, *403*, 571–572.
(10) Katz, L.; Donadio, S. *Annu. Rev. Microbiol.* **1993**, *47*, 875–912.
(11) Grimm, A.; Madduri, K.; Ali, A.; Hutchinson, C. R. *Gene* **1994**, *151*, 1–10.
(12) Rajgarhia, V. B.; Strohl, W. R. *J. Bacteriol.* **1997**, *179*, 2690–2696.
(13) Thomas, R. *ChemBioChem* **2001**, *2*, 612–627.
(14) Rohr, J.; Thiericke, R. *Nat. Prod. Rep.* **1992**, *9*, 103–137.
(15) Krohn, K.; Rohr, J. *Top. Curr. Chem.* **1997**, *188*, 127–195.
(16) Ferrer, J.-L.; Jez, J. M.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Nat. Struct. Biol.* **1999**, *6*, 775–783.
(17) Jez, J. M.; Ferrer, J.-L.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Biochemistry* **2000**, *39*, 890–902.
(18) Schroeder, J. In *Comprehensive Natural Products Chemistry*, vol. 1; *Polyketides and Other Secondary Metabolites Including Fatty Acids and Their Derivatives*; Sankawa, U., Ed.; Elsevier: Oxford, 1999; pp 749–771.
(19) Yu, T.-W.; Shen, Y.; McDaniel, R.; Floss, H. G.; Khosla, C.; Hopwood, D. A.; Moore, B. S. *J. Am. Chem. Soc.* **1998**, *120*, 7749–7759.
(20) Swan, D. G.; Rodriguez, A. M.; Vilches, C.; Méndez, C.; Salas, J. A. *Mol. Gen. Genet.* **1994**, *242*, 358–362.
(21) Olano, C.; Rodriguez, A. M.; Michel, J. M.; Mendez, C.; Raynal, M. C.; Salas, J. A. *Mol. Gen. Genet.* **1998**, *259*, 299–308.
(22) Rodríguez, A.; Olano, C.; Vilches, C.; Méndez, C.; Salas, J. A. *Mol. Microbiol.* **1993**, *8*, 571–582.
(23) Rodriguez, A. M.; Olano, C.; Méndez, C.; Hutchinson, C. R.; Salas, J. A. *FEMS Microbiol. Lett.* **1995**, *127*, 117–120.
(24) Olano, C.; Rodriguez, A. M.; Méndez, C.; Salas, J. A. *Mol. Microbiol.* **1995**, *16*, 333–343.
(25) Quiros, L. M.; Aguirrezabalaga, I.; Olano, C.; Mendez, C.; Salas, J. A. *Mol. Microbiol.* **1998**, *28*, 1177–1185.
(26) Aguirrezabalaga, I.; Olano, C.; Allende, N.; Rodriguez, L.; Braña, A. F.; Mendez, C.; Salas, J. A. *Antimicrob. Agents Chemother.* **2000**, *44*, 1266–1275.
(27) Richardson, M. A.; Kustoss, S.; Solenberg, P.; Shaus, N. A.; Rao, R. N. *Gene* **1987**, *61*, 231–241.
(28) Malpartida, F.; Hopwood, D. A. *Nature* **1984**, *309*, 462–464.
(29) Malpartida, F.; Hallam, S.; Kieser, H. M.; Motamedi, H.; Hutchinson, C. R.; Butler, M. J.; Sugden, D. A.; Warren, M.; McKillop, C.; Bailey, C. R.; Humpheys, G. O.; Hopwood, D. A. *Nature* **1987**, *235*, 818–821.
(30) Hopwood, D. A.; Bibb, M. J.; Chater, K. F.; Kieser, T.; Bruton, C. J.; Kieser, H. M.; Lydiate, D. J.; Smith, C. P.; Ward, J. M.; Schrempf, H. *Genetic Manipulation of Streptomyces. A Laboratory Manual*; The John Innes Foundation: Norwich, UK, 1985.

(31) Chater, K. F.; Wilde, L. C. *J. Gen. Microbiol.* **1980**, *116*, 323−334.
(32) Hardisson, C.; Manzanal, M. B.; Salas, J. A.; Suarez, J. E. *J. Gen. Microbiol.* **1978**, *105*, 203-214.
(33) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning. A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Plainview, NY, 1989; Vols. 1−3.
(34) Pereda, A.; Summers, R. G.; Stassi, D. L.; Ruan, X.; Katz, L. *Microbiol.* **1998**, *144*, 543−553.
(35) HPLC studies showed that the yield of oviedomycin (**2**) using constructs containing the other cosmids belonging to Cluster I is slightly lower compared to those reported for cosAB3, while the wild type strain normally produces no detectable **2** and only hardly detectable traces of **2** when grown on solid agar using GAE medium.

NP010555N