Antisense-Guided Isolation and Structure Elucidation of Pannomycin, a Substituted *cis*-Decalin from *Geomyces pannorum*

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Antisense-based screening strategies can be used to sensitize a microorganism and selectively detect inhibitors against a particular cellular target of interest. A strain of *Staphylococcus aureus* that generates an antisense RNA against SecA, a central member of the protein secretion machinery, has been used to screen for novel antibacterials. Possible inhibitors of the SecA ATP-ase were selected with a high-throughput, two-plate agar-based whole cell differential sensitivity screen. After screening a library of over 115 000 natural products extracts with the SecA antisense strain, an extract of *Geomyces pannorum* was identified as providing increased activity against the sensitized strain as compared with the wild-type control. Bioassay-guided isolation of the active component from this fungal extract provided a new *cis*-decalin secondary metabolite, which we have named pannomycin.

New antibiotics with distinct mechanisms of action are a continuing need, as clinically significant bacterial pathogens not only have acquired some level of resistance to nearly all existing antibiotics but also increasingly exhibit multidrug resistance.[1,2] Screening strategies that target biological pathways or enzymes that have not been exploited previously for the development of antibacterial agents are more likely to lead to the discovery of antibiotics with novel structural scaffolds. One potential pathway to explore for the development of new antibiotics is protein secretion. Bacterial protein secretion is highly conserved among a wide range of species, but is distinct from its eukaryotic counterpart. This fact makes secretion an attractive target for antimicrobial chemotherapies that may have limited or no toxicity. The Sec machinery is the major pathway of protein translocation from the cytosol across, or into, the cytoplasmic membrane.[3,4] The Sec-dependent pathway of secretion is a multicomponent system consisting of at least seven proteins, five of which are essential for cell viability. The SecYEG heterotrimer forms an integral membrane channel for movement of a protein out of the cytosol. SecA is a loosely membrane-associated homodimeric ATP-ase that has high affinity for SecYEG.[5,6] The SecB chaperone binds to proteins that are tagged for secretion, stabilizes that peptide chain in the unfolded state, and escorts it to the SecA−SecYEG complex.[7] Cycles of ATP hydrolysis by SecA provide the required energy to move the protein through the channel and out of the cell. Homologues for several of these protein components have been identified in both Gram-negative and Gram-positive bacteria, and therefore, it is considered likely that an inhibitor of this pathway would have broad-spectrum activity.[4,8,9] The central role played by SecA in the protein translocase pathway makes it a particularly attractive target for the inhibition of secretion.[5,6] Bacterial viability should be significantly impaired by an inhibitor that prevents or decreases the ability of the cell to export proteins. In addition, there is no human counterpart of the SecA protein, with the closest human homologue having less than 6% identity.[4,6,8]

Antisense screening strategies in *Staphylococcus aureus* have recently been used to identify novel antibacterials.[10−14] In this method, selectively expressed antisense RNA binds to the targeted
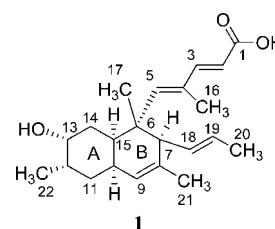
Figure 1. Structure and relative stereochemistry of pannomycin (**1**).

gene's mRNA, leading to degradation of that mRNA and a reduction in the translation of the corresponding gene product.[12] While still viable, these weakened strains are sensitized to an inhibitor that targets the depleted gene product. For example, the novel diterpene-derived natural product platensimycin was isolated from an antisense screen for inhibitors of FabF, the condensation enzyme that plays a central role in bacterial type II fatty acid synthesis.[15,16] With antisense methodology, the essentiality of SecA in *S. aureus* has been demonstrated.[17] We have used a strain with an inducible SecA antisense construct to screen for natural products that are potential inhibitors of SecA and protein translocation. To selectively screen for a compound that targets SecA, we employed a two-plate agar-based differential sensitivity assay comparing the SecA antisense strain to a vector control strain. Antibacterial activity against an antisense-sensitized *S. aureus* strain will be greater than that against the wild-type organism. Screening of a collection of microbial natural product extracts using this assay led to the identification of a new secondary metabolite, pannomycin (**1**, Figure 1), from a fungal strain, *Geomyces pannorum*, isolated from leaf litter. This *cis*-decalin natural product demonstrated wild-type antibacterial activity and was structurally similar to a known inhibitor of SecA.[18]

Results and Discussion

A library of over 115 000 natural product extracts derived from both actinomycetes and fungi was screened in a SecA two-plate differential sensitivity antisense assay. Extracts were tested at a single concentration and designated as active if the sample provided a zone differential of at least 5 mm. The primary hit rate for this screen was 0.1%. One of the most striking differentials in this assay was observed for an extract of a fungus (*G. pannorum*) isolated from leaf litter collected from Toledo, Spain. The fungal extract

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Table 1. ^{1}H and ^{13}C NMR Data for Pannomycin (**1**)^a

| position	δ_C	mult		δ_H (J in Hz)	HMBC^b	ROESY^c
1	167.8	qC				
2	115.7	CH		5.68, d (15.5)	1, 4	16
3	151.2	CH		7.09, d (15.5)	1, 2, 4, 5, 16	5
4	131.7	qC				
5	154.4	CH		5.84, s	3, 6, 7, 15, 16, 17	3, 7, 10, 15
6	41.4	qC				
7	55.0	CH		2.45, bd (9.5)	5, 6, 8, 9, 15, 18, 19	5, 16, 17, 18, 19, 21
8	133.9	qC				
9	126.5	CH		5.18, s	7, 10, 15, 21	10, 11β, 12, 21
10	33.4	CH		2.32, m	6	5, 9, 11α, 11β, 15
11	33.2	CH$_2$	α	1.57, m	12	10, 11β
			β	1.22, m		9, 10, 11α, 22
12	31.7	CH		1.31, m		9, 13, 22
13	68.5	CH		3.57, bs		12, 14α, 14β, 22
14	31.4	CH$_2$	α	1.61, m		13, 14β, 15, 17, 19
			β	1.40, td (13.0, 1.5)		13, 14α, 18, 20
15	34.7	CH		2.14, dt (13.0, 4.0)	9	5, 10, 14α, 16, 17
16	13.6	CH$_3$		1.84, s	3, 4, 5	2, 7, 15, 17
17	22.5	CH$_3$		1.04, s	5, 6, 7, 15	7, 14α, 15, 16, 19, 20
18	132.0	CH		5.30, ddd (14.5,9.5, 1.0)	19, 20	7, 14β, 20, 21
19	128.4	CH		5.44, dq (14.5, 6.0)	7, 18, 20	7, 14α, 17, 20
20	17.7	CH$_3$		1.64, d (6.0)	18, 19	14β, 17, 18, 19
21	23.1	CH$_3$		1.59, s	8, 9	7, 9, 18
22	18.6	CH$_3$		0.80, d (6.5)	11, 12, 13	11β, 12, 13
13-OH				4.15, b		
COOH				12.0, b		

^{a} Recorded in DMSO-d_6. ^{1}H NMR spectra were referenced to the residual solvent peak for DMSO-d_5 at δ 2.49. Carbon spectra were referenced to the DMSO-d_6 septet at δ 39.51. ^{b} HMBC correlations are from the proton stated to the indicated carbon. ^{c} ROESY correlations are from the proton stated to the indicated proton.

was significantly more potent against the antisense-sensitized strain than against the control strain, exhibiting a zone differential of 8−9 mm. This suggested that the fungal extract contained an inhibitor of the SecA ATP-ase.

The antibacterial component responsible for the differential activity against the SecA antisense strain was isolated with a two-step process. After extraction of the active component from the mycelium with acetone, the organic solvent was removed and the sample was adjusted with acid to pH 2.5. This feed sample was loaded onto a styrene−divinylbenzene polymeric resin, CHP20Y, and the active component was fully retained. After gradient elution from this resin, the fractions with a differential in the SecA two-plate assay were pooled. This sample was then concentrated and further purified by preparative C18 HPLC at pH 6. After this final chromatography step, the purified active component was obtained, and this material maintained the antibacterial activity in the SecA differential assay that was observed in the original extract.

The molecular formula of the active component (**1**) was $C_{22}H_{32}O_3$ (MW 366) based on HRMS analysis (obsd m/z 367.2246, calcd for $[C_{22}H_{32}O_3 + H]^+$ 367.2249). The UV absorbance spectrum of **1** had absorbance maxima at 196 and 266 nm. A full NMR data set (Table 1) was obtained for **1** in DMSO-d_6. ^{1}H and ^{13}C NMR spectra were fully consistent with the proposed molecular formula. Of the 22 carbon atoms in **1**, DEPT identified five methyl groups, two methylenes, and 11 methines, leaving four quaternary carbons. One-bond C−H connectivities were assigned by HSQC analysis and are provided in Table 1. COSY correlations identified an extended spin system that corresponded to H-10 to H-15, including the methyl group at C-22, thereby completing the connectivity of the decalin A ring (Figure 1). A smaller spin system was identified from H-7 and extending into the propenyl side chain. The only additional COSY correlation observed was that corresponding to the *trans*-disubstituted olefin at C-2−C-3, with H-2 resonating at δ_H 5.68 and H-3 at δ_H 7.09.

HMBC correlations (Table 1) were used to complete the structure elucidation of **1**. Key correlations were observed from H-5, H-7, H-9, and H-22. The connectivity of the dienoic acid moiety at C-6 could be confirmed by correlations from H-5 to C-7 and C-15. Correlations between the vinylic H-2 and H-3 and the C-1 carbonyl
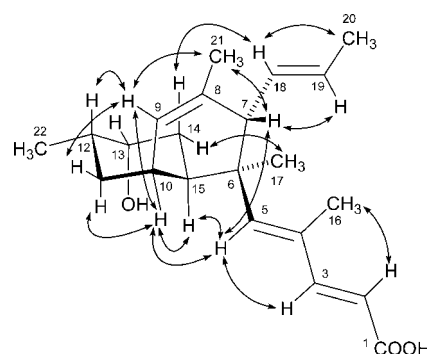

Figure 2. Three-dimensional representation of **1** with key ROESY correlations.

(δ_C 167.8) placed the carboxylic acid at the terminus of this side chain. The position of the propenyl side chain was clarified by the correlations of H-7 to C-5, C-8, C-9, and C-15. Vinylic proton H-9 showed correlations to C-7, C-10, C-15, and C-21, thereby supporting the structure of the decalin B ring (Figure 1). Correlations from H_3-22 to C-11 and C-13 supported the previously identified decalin A ring assignments.

The relative configuration of the decalin ring system (Figure 2) was established by ROESY as well as with an analysis of vicinal proton coupling constants (Table 1). There are six stereogenic centers in the decalin system of **1**. The *cis* ring juncture was confirmed by the ROESY correlation between H-10 and H-15. In addition, the coupling constant between these protons was small (J = 4.0 Hz). The large coupling constant of H-15 (J = 14.0 Hz) at δ_H 2.14 is due to H-14β, since these two protons are disposed in a *trans*-diaxial orientation. ROESY correlations between H-5 of the dienoic acid moiety and both ring juncture protons (H-10 and H-15) placed that side chain also on the α face. Further confirmation of the relative configuration at C-6 was the ROESY correlations of H_3-17 to H-14α. The propenyl side chain was placed on the β face of the decalin since ROESY showed a correlation between H-18 (δ_H 5.30) and H-14β (δ_H 1.40). The remaining stereogenic centers in need of configurational assignment were the A ring
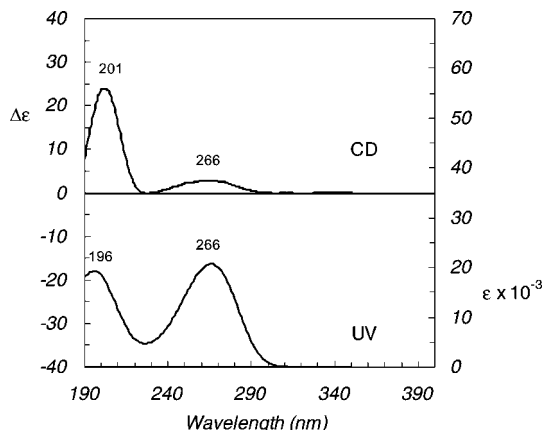
Figure 3. Circular dichroism and UV absorbance spectra of **1**.
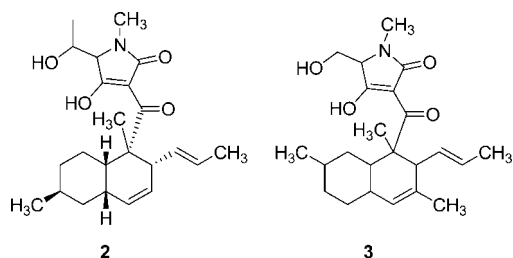
Figure 4. Structure of cissetin (**2**) and SecA inhibitor CJ-21,058 (**3**).

methyl- and hydroxy-bearing carbons, C-12 and C-13, respectively. Carbinol H-13 (δ_H 3.57) showed ROESY correlations with both protons at C-14, the methine proton H-12, and the H_3-22 methyl group. These data were consistent with H-13 and the 13-hydroxy group being in an equatorial and axial orientation, respectively. A ROESY correlation between H-9 (δ_H 5.18) and the C-12 methine proton indicated that that proton was on the β face of the A ring in an axial orientation.

All three exocyclic double bonds were assigned *E*-configuration based on the large coupling constants between H-2 and H-3, H-18 and H-19, and the ROESY correlations for H-5 and H_3-16 for the trisubstituted olefinic C-4–C-5 bond (Figure 2). After analysis of all through-bond and through-space correlations, the conformation of **1** in which an A ring chair is *cis*-fused to a B ring half-chair conformation was established (Figure 2). This conformation disposed the dienoic acid and propenyl side chains in opposite directions with an angle of approximately 180°. In this case, no strong exciton coupled circular dichroism interaction would be expected.[19] Consistent with this conformation, the CD spectrum of **1** (Figure 3) in acetonitrile had only weak positive Cotton effects at 201 and 266 nm.

Compound **1** was tested against a selected panel of microorganisms in order to assess its antibacterial activity. In a liquid media assay, only weak antibacterial activity was observed against *S. aureus* Smith strain (MIC 1.4 mM), *Enterococcus faecalis* (1.4 mM), and *Bacillus subtilis* (0.4 mM). No activity was observed at the concentration tested (up to 1.4 mM) against the following microorganisms: *Streptococcus pneumoniae*, *Hemophilus influenzae*, *Escherichia coli*, and *Candida albicans*. While limited antibacterial activity was observed with **1**, the possibility that this component targets the SecA ATP-ase makes it an attractive starting point for the possible development of a more potent antibacterial agent. The most closely related natural products with *cis*-decalin core structures include cissetin (**2**, Figure 4),[20] a tetramic acid derivative with anti-MRSA activity (MIC ~10 μM), phomopsidin,[21] an inhibitor of microtubule assembly, and antifungal polyketides isolated from a *Penicillium* sp.[22] Of these natural products, only cissetin has the

analogous quaternary carbon that is present in **1** at C-6, but the configuration of that quaternary center relative to the ring fusion protons is opposite that in **1**. Interestingly, this quaternary center also is present in known SecA inhibitor **3** (MRSA MIC 12 μM, Figure 4).[18] The more potent antibacterial activity of **2** and **3** is attributed to the presence of a tetramic acid in both of those compounds. The strong structural similarity between **1** and **3**, which is comprised of a decalin linked to a tetramic acid, suggests a minimal structural unit required for SecA inhibition. The structure elucidation of **3** did not fully resolve the relative configuration of the decalin substituents and, in particular, whether the ring junction was *cis* or *trans*.[18] It would be of interest to clarify this stereochemical point, allowing for a fuller examination of the pharmacophore required for the inhibition of the SecA translocase. Provided that the ring fusion for **3** is *cis* as in compound **1**, this decalin scaffold containing a quaternary carbon center analogous to C-6 could be used as a starting point for the development of more potent analogues that target SecA and bacterial protein secretion.

Experimental Section

Producing Organism. The producing organism is a fungus isolated from unidentified plant litter collected in Ontígola (Toledo, Spain). The fungus was isolated using a particle-washing technique previously described.[23] The isolate was identified as *Geomyces pannorum* (Myxotrichaceae) based on its micromorphological characteristics in potato dextrose agar (Difco) culture, i.e., thallic one-celled and truncate conidia, born terminally on verticillate branches, and intercalary conidia born on the outer branches of the verticillate hyphae.[24] This identification was supported by sequence analysis of the internal transcribed spacers (ITS) of the rDNA, performed as previously described.[25] The comparison with ITS sequences stored in GenBank revealed a high similarity corresponding to *Geomyces* and *Pseudogymnoascus* spp., e.g., 99% with *G. pannorum* (DQ888720) and 98% with a *Pseudogymnoascus roseus* strain (AY608922). The strain has been deposited in the Merck Culture Collection (Rahway, NJ) with the accession code MF7016.

Fermentation of *Geomyces pannorum* MF7016. Fermentation conditions for the production of pannomycin (**1**) in F202 medium is set forth below. The strain was maintained on agar plugs in vials containing sterile glycerol 10% stored at 80 °C until ready for use. The seed culture was inoculated by aseptically transferring four agar plugs into a 250 mL Erlenmeyer flask containing 60 mL of seed medium (KFA) of the following composition (in g/L): corn steep powder, 2.5; tomato paste, 40; oat flour, 10; glucose, 10; agar, 4; and trace elements solution, 10 mL/L. The trace elements solution consisted of the following components (in g/L): $FeSO_4 \cdot 7H_2O$, 1; $MnSO_4 \cdot 4H_2O$, 1; $CuCl_2 \cdot 2H_2O$, 0.025; $CaCl_2 \cdot 2H_2O$, 0.1; H_3BO_3, 0.056; $(NH_4)_6MoO_{24} \cdot 4H_2O$, 0.019; and $ZnSO_4 \cdot 7H_2O$, 0.2. The seed medium was prepared with distilled H_2O. The pH was adjusted to 6.8 by adding NaOH, and the medium was dispensed into 250 mL Erlenmeyer flasks and capped with cellulose plugs before being autoclaved at 121 °C for 20 min. The seed culture was incubated at 22 °C on a gyratory shaker (220 rpm) for 5–8 days prior to the inoculation of the fermentation flasks.

The F202 production medium was formulated as follows (per 250 mL flask): millet 14 g and base liquid 30 mL (base liquid composition (in g/L): yeast extract, 16.5; sucrose, 16.65; alfalfa, 16.65; sodium tartrate, 3.33; corn oil, 3.33 mL/L; and $FeSO_4 \cdot 7H_2O$, 0.33). The production medium was prepared with distilled H_2O, and no adjustment was made to the pH. It was dispensed into 250 mL Erlenmeyer flasks and capped with cellulose plugs before being autoclaved at 121 °C for 20 min. Fermentation flasks were inoculated with 2 mL of vegetative seed growth and were incubated at 22 °C, 70% humidity for 28 days.

After the end of the fermentation period, the whole broth was extracted directly with an equal volume of acetone.

Two-Plate Differential Sensitivity SecA Assay. S*taphylococcccus aureus* cells (RN450) carrying plasmid S1-163B bearing an antisense sequence to SecA regulated by a xylose promoter (*SecA* AS-RNA strain) or vector (control strain) were inoculated in Miller's LB broth (Invitrogen) containing 34 μg/mL chloramphenicol and incubated overnight at 37 °C with shaking at 220 rpm. Each culture was diluted to a final optical density at 600 nm of 0.003 into a flask containing Miller's LB broth supplemented with 1.2% Select agar (Invitrogen), 0.2% glucose, 15 μg/mL chloramphenicol, and 9.4 mM xylose (only for the antisense strain). Two assay plates, one seeded with the SecA AS-RNA strain (AS plate) and the other seeded with the control strain (control plate), were prepared by pouring 100 mL of each of the above mixtures into a Nunc plate. Immediately, well-caster templates were placed into the agar and the agar was allowed to solidify at room temperature for 30 min. Then, 20 μL of test samples were added to the wells on both plates, and then they were incubated at 37 °C for 18 h. Any difference in zone size between the AS plate and the control plate was measured in millimeters, with an active sample showing a differential of 5 mm or more in the diameter of inhibition on the S1-163B strain than the outermost zone of inhibition for the control strain.[26]

Isolation of Pannomycin (1). Whole broth fermentation of *G. pannorum* MF7016 (120 mL) was extracted at room temperature with an equal volume of acetone with stirring. After filtration through Celite to separate the mycelium, the extract was concentrated to remove most of the acetone. The extract pH was lowered from 5.2 to 2.5 with 0.2 N HCl and then loaded onto CHP20Y resin (5 mL) at a flow rate of 2 mL/min. The resin was eluted with a gradient of acetonitrile in 10 mM pH 2.5 KH_2PO_4 at 1.25 mL/min. Fractions were assayed in the two-plate SecA differential assay, as described above, in order to guide the isolation of the active component. Active fractions were pooled, concentrated to remove the organic solvent (6 mL), brought to 12 mL of 50% acetonitrile, and adjusted to pH 6.0 with 0.2 N KOH. This sample was used as a feed for further purification by preparative HPLC (Waters Symmetry300 C18, 300 Å, 300 × 19 mm, 7 μm, 8 mL/min, 25 °C; gradient elution 30−80% ACN (30 min) in pH 6 10 mM KH_2PO_4). Fractions that maintained the differential in the SecA antisense-based assay were analyzed by HPLC (Waters C18 Symmetry300, 300 Å, 4.6 × 50 mm, 5 μm, isocratic elution 40:60 ACN/pH 6, 10 mM KH_2PO_4, 2 mL/min, t_R = 1.5 min), and those containing the purified active component were combined. After removal of the organic solvent, the aqueous was adjusted to pH 3 with 0.2 N HCl, extracted with EtOAc, and concentrated to dryness, providing 18.6 mg of a colorless oil (from 50 mL of whole broth equivalents). The overall titer of the original whole broth was 650 mg/L of **1**, as calculated from a standard solution: $[\alpha]_D$ +127 (c 1.0, CH_2Cl_2); UV (acetonitrile) λ_{max} 266 (ϵ = 20 700 M^{-1} cm^{-1}), 196 (ϵ = 19 300 M^{-1} cm^{-1}); CD (acetonitrile) λ_{max} 266 ($\Delta\epsilon$ = +2.9 M^{-1} cm^{-1}), 201 ($\Delta\epsilon$ = +24.0 M^{-1} cm^{-1}); FTIR (ZnSe) ν_{max} 2927, 1693, 1616, 1377, 1182 cm^{-1}; full NMR assignments for **1** are in Table 1; HRESIFTMS 367.2246 (calcd for $C_{22}H_{32}O_3$ + H 367.2249).

Determination of MICs. Strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, and *Escherichia coli* were tested in cation-adjusted Mueller-Hinton broth (CAMHB), inoculation = 10^5 cfu/mL, incubated at 35 °C for 20 h. *Streptococcus pneumoniae* was tested in CAMHB with 2.5% lysed horse blood, inoculation = 10^5 cfu/mL, incubated at 35 °C for 20 h. *Haemophilus influenzae* was tested in Haemophilus Test medium, inoculation = 10^5 cfu/mL, incubated at 35 °C for 20 h. *Candida albicans* was tested in RPMI 1640, inoculation = 10^3 cfu/mL, incubated at 35 °C for 24 h.

Supporting Information Available: ^{1}H, ^{13}C, COSY, HSQC, HMBC, and ROESY NMR spectra of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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