JOURNAL OF NATURAL PRODUCTS

Volume 66, Number 12 December 2003

Full Papers

Isolation and Structure Elucidation of Sch 642305, a Novel Bacterial DNA Primase Inhibitor Produced by *Penicillium verrucosum*

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Received May 16, 2003

A novel primase inhibitor, Sch 642305 (**1**), was isolated from the fermentation broth of the fungal culture *Penicillium verrucosum*. The structure of **1** was elucidated on the basis of MS and NMR spectroscopic data as a new and unusual bicyclic 10-membered macrolide. The absolute configuration of the asymmetric centers was determined by X-ray crystallographic analysis of the *p*-bromobenzoate derivative (**3**). Compound **1** exhibited inhibitory activity against bacterial DNA primase enzyme with an EC_{50} of 70 μM.

Bacterial infections due to the rapid emergence of antibiotic-resistant strains have become a serious threat to public health worldwide.[1] Concerns of antibiotic resistance issues have brought an urgent sense to the discovery and development of new classes of antibacterial drugs. Mechanism-based drug discovery approaches are being explored to identify novel antimicrobial agents that may potentially provide alternative treatments for infectious diseases. Bacterial DNA primase (DnaG) is a DNA-dependent RNA polymerase that is required for the replication of chromosomal DNA.[2,3] Genetic validation of DnaG indicated that inhibition of bacterial primase causes a rapid cidal response of bacteria.[4,5] Interaction of primase, encoded by DnaG, with the single-stranded DNA template is mediated by contacts with the replicative DNA helicase encoded by DnaB.[6,7] In the cell, the helicase progresses along the duplex DNA, mediating unwinding to a single-stranded DNA template. The DNA primase associates with the helicase and intermittently initiates synthesis of ss RNA primers, which are required for de novo DNA synthesis. While replication of the leading-strand DNA requires only one RNA primer, replication of the lagging-strand DNA requires >2000 primer sites. Interruption of this process would clearly cause a catastrophic event in bacterial chromosome replication. Therefore, the primase enzyme is an attractive target for antibacterial drug discovery. In our continuing search for primase inhibitors as potential leads from microbial sources, a large number of extracts were tested by high-throughput screening (HTS). As a result of the screening process, a novel primase inhibitor, Sch 642305 (**1**), was discovered from fermentation broth of the fungus *Penicillium verrucosum* (culture ILF-16214). We report herein the isolation, structure elucidation, and biological activity of **1**.

Results and Discussion

Characterization of the microorganism *P. verrucosum* was accomplished on the basis of the sequence data analysis of the 28S rDNA taxonomic identifications at the D2 region. The fermentation broth of *P. verrucosum* (4 L) was extracted with 8 L of acetonitrile (ACN) by adding 200 g of NaCl to separate organic layer from aqueous portion. The organic layer was concentrated in vacuo to generate ~130 mg of crude extract. The ACN extract was purified by preparative reversed-phase HPLC to yield ~6 mg of pure **1** as a white solid {mp = 143−145 °C, $[\alpha]_D$ +67.44° (*c* 0.50, CH_3OH)} after crystallization in acetone−H_2O.

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10.1021/np0302302 CCC: $25.00
Published on Web 11/15/2003

Table 1. ^{1}H and ^{13}C NMR Spectral Data for Sch 642305 (**1**) and Its Acetate (**2**)[a]

	1 (CD_3OD)		**2** ($CDCl_3$)	
	^{13}C (δ)	^{1}H (δ)	^{13}C (δ)	^{1}H (δ)
1	202.6 s[b]		199.2 s	
2	130.7 d	5.96 d, J = 9.9[c]	132.4 d	6.10 d, J = 9.9
3	149.6 d	7.03 dd, J = 5.6, 9.9	142.1 d	6.98 dd, J = 5.8, 9.9
4	67.2 d	4.22 dd, J = 3.7, 5.6	68.8 d	5.32 dd, J = 3.6, 5.8
5	37.9 d	2.82 dddd, J = 2.4, 3.7, 11.5, 11.5	34.9 d	3.01 dddd, J = 3.6, 3.6, 10.3, 11.5
6	47.8 d	2.65 dt, J = 3.7, 3.7, 11.5	47.2 d	2.70 dt, J = 3.6, 3.6, 11.5
7	24.2 t	1.08, 2.16 m, m	22.7 t	1.12, 2.23, m, m
8	22.6 t	1.33, 1.55 m, m	21.2 t	1.27, 1.61, m, m
9	24.1 t	1.23, 1.83 m, m	22.7 t	1.27, 1.81, m, m
10	30.8 t	1.39, 2.10 m, m	29.2 t	1.37, 2,12, m, m
11	74.8 d	5.05 m	73.4 d	5.10 m
12	173.9 s		170.7 s	
13	39.9 t	2.53 dd, J = 11.5, 16.8 2.68 dd, J = 2.4, 16.8	38.4 t	2.50 dd, J = 10.3, 16.8 2.55 dd, J = 3.6, 16.8
14	18.7 q	1.27 d, J = 6.6	18.1 q	1.27 d, J = 6.6
CO			170.4 s	
CH_3			20.7 q	2.08 s

[a] Recorded at 500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively. [b] Multiplicity was determined by APT data. [c] Coupling constants in Hz.

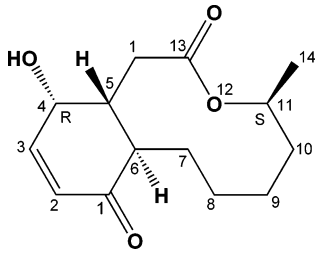

Figure 1. Structure of Sch 642305 (**1**).

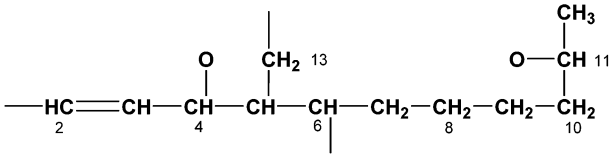

Figure 2. Partial structure of **2** established on the basis of COSY/HSQC data.

The molecular mass of **1** was determined to be 252 Da based on APCI-MS data that showed a protonated molecular ion at m/z 253 $(M + H)^+$. It was further confirmed by negative mode ionization data that showed a deprotonated molecular ion at m/z 251 $(M - H)^-$ and a bimolecular ion at 503 $(2M - H)^-$. The molecular formula of **1**, $C_{14}H_{20}O_4$, was assigned on the basis of HRFABMS analysis (found 253.1440 for $C_{14}H_{21}O_4$, calcd 253.1448). UV absorption at 217 nm (end absorption) indicated no significant conjugated chromophore, suggesting the presence of a macrolide moiety. Analysis of the ^{1}H NMR data (Table 1) revealed two olefinic signals (H2 and H3), two oxygenated methine multiplets (H4 and H11), two other methine multiplets (H5 and H6), and one methyl doublet (H14). The ^{13}C NMR spectrum displayed a total of 14 carbon signals, including two resonances for carbonyl carbons, two vinylic methines, two oxygenated methines, two aliphatic methines, five methylenes, and one methyl carbon.

To obtain additional structural information and generate better quality spectra for assignments, an acetate derivative (**2**) was formed by treatment of **1** with acetic anhydride−pyridine. Furthermore, the *p*-bromobenzoate derivative (**3**) {mp = 138−139 °C, $[\alpha]_D$ +236.87° (c 0.50, $CHCl_3$)}, which would in addition be suitable for X-ray diffraction analysis to determine the absolute stereochemistry, was prepared by treatment of **1** with *p*-bromobenzoyl chloride−triethylamine. Extensive 2D NMR experiments and all detailed assignments, therefore, were focused on **2** and **3**. The ^{1}H NMR spectrum of **2** (Table 1) clearly indicated that the acetylated oxymethine proton at the 4-position was shifted downfield to δ 5.32 from δ 4.22 in comparison with **1**. A partial structure of **2** was established on the basis of COSY and HSQC-TOCSY data analyses, which provide one-bond ^{1}H−^{1}H and ^{1}H−^{13}C connectivities as shown in Figure 2.

Assignments of all carbon and proton connections were completed on the basis of analysis of long-range ^{1}H−^{13}C coupling data from an HMBC experiment with **3**, which are summarized in Figure 3. Correlations of H6 to C1, C4, and C5, H3 to C1, C4, and C5, and H2 to C4 and C6 indicated the presence of a hexenone ring. On the basis of correlations of H6 to C5, C7, C8, and C13, and H13 to C5, C6, and C12, as well as the requirement of unsaturation for matching the molecular formula, a 10-membered lactone ring was assigned to the remaining part of the molecule. HMBC correlations from H13 to C4, C5, and C6, H4 to C5, C6, and C13, and H6 to C1, C5, and C7 further suggested that the 10-membered lactone ring is fused with the hexenone ring to form a bicyclic ring system. The presence of a hydroxyl group at the 4-position in **1** was also confirmed by the observation of a three-bond correlation between H4 and C7′ in **3**. Therefore, the structure of **3** was proposed. The relative stereochemistry of the three chiral centers at C4, C5, and C6 was established on the basis of coupling constant and NOESY data analyses. A large coupling constant between H5 and H6 ($J_{H5,H6}$ = 11.4 Hz) suggested the *trans* configuration of H5 and H6. H4 and H5 were assigned a *cis* configuration due to a relatively small coupling ($J_{H4,H5}$ = 3.4 Hz). These assignments were further confirmed by NOESY data that showed only correlation between H4 and H5 and the lack of H4 and H6 as well as H5 and H6 couplings. NMR experiments, however, were unable to reveal the stereochemistry at C11 due to the remote distance from the ring junction. To explore the stereochemistry of all chiral centers and obtain the absolute configuration, **3** was crystallized with an acetone−H_2O solvent mixture to obtain an orthorhombic crystal suitable for X-ray diffraction analysis. X-ray crystallographic study of **3** not only confirmed the proposed structure and relative stereochemistry at C4, C5, and C6 but also established the absolute configuration for the entire molecule. An ORTEP diagram showing the solid-state conformation is presented in Figure 4.

An example of a 10-membered macrolide fused with an aromatic system has been reported.[8] The nonaromatic

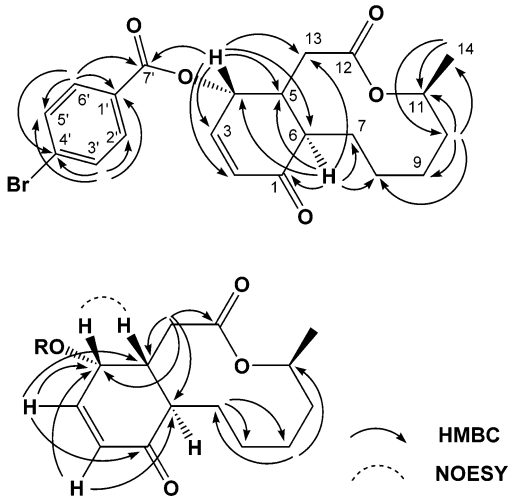

Figure 3. HMBC and NOESY data of *p*-bromobenzoate derivative **3**.

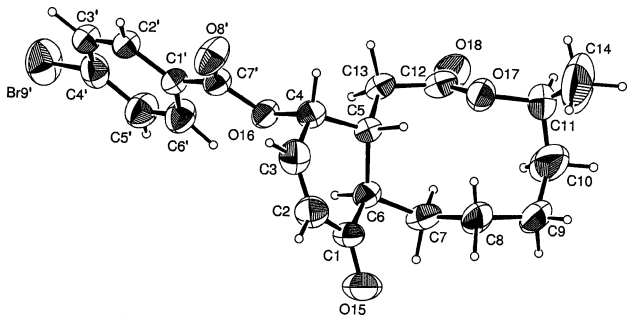

Figure 4. ORTEP diagram showing the structure and solid-state conformation of **3**. Small circles represent hydrogen atoms.

bicyclic macrolide **1**, however, consisting of decalactone with a fused 4-hydroxyhexenone ring, has not been previously reported in any natural source and represents a new bicyclic ring system.

Compound **1** demonstrated inhibitory activity against *E. coli* bacterial DNA primase with an $EC_{50} = 70\ \mu M$.[9] In a cell-based assay, compound **1** showed antibacterial activity, MIC of 40 μg/mL, against an *E. coli* strain with a defective lipopolysaccharide layer and disruption of the efflux pump *acrAB*.

Experimental Section

General Experimental Procedures. Melting points were recorded on a MEL-TEMP apparatus (Laboratory Devices, Cambridge, MA) and are uncorrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. LC-APCI-MS analyses were performed on a triple-stage quadrupole TSQ-7000 mass spectrometer equipped with APCI (atmospheric pressure chemical ionization)/ESI (electrospray ionization) interfaces (Finnigan MAT, San Jose, CA) linked to a Hewlett-Packard HP-1090 Series X system. HRFABMS data were produced by a VG ZAB-SE mass spectrometer in a glycerol−thioglycerol matrix. ^1H and ^{13}C NMR spectra were recorded using a Varian Inova-500 instrument operating at 500 and 125 MHz, respectively.

Microorganism. The producing microorganism was isolated from a soil sample collected in desert area near Tuscan, AZ. The fungal microbe was characterized and determined as *Penicillium verrucosum* on the basis of taxonomic analyses, including colony morphology, as well as the sequence data of the D2 region of the 28S rDNA taxonomic identifications. The 28S rDNA sequence data were further confirmed by internal BLASTs (basic local alignment search tool) with the sequencing company Accugenix as well as by BLASTs with National Center for Biotechnology Information (NCBI).[10]

Fermentation and Isolation. Stock cultures were maintained as frozen whole broths at −80 °C in a final concentration of 10% glycerol. Fermentation was carried out in shake flasks. The inoculum medium contained (g/L) proteus peptone 5, NaCl 5, KH_2PO_4 5, yeast extract 3, cerelose 20, soybean grits 5, antifoam 1 mL, and tap H_2O to 1 L. The pH (5.2) was adjusted to 7.2 prior to autoclaving. A 250 mL Erlenmeyer flask containing 70 mL of this medium was inoculated with 2.0 mL of the stock culture. The flasks were incubated at 24 °C on a rotary shaker at 250 rpm for 96 h. Approximately 2.5 mL of this seed culture was used to inoculate another 250 mL Erlenmeyer flask containing 70 mL of the same seed medium, and the flask was incubated, as above, for 96 h.

Five percent of the second germination was used to inoculate the fermentation medium containing (g/L) oat flour 25, potato starch 20, soluble starch 6, glucose 5, yeast extract 3, instant ocean 1, $CaCO_3$ 1, K_2HPO_4 0.5, and tap H_2O to 1 L. The fermentation was carried out in a 500 mL Erlenmeyer flask containing 100 mL of the fermentation medium. The flasks were incubated at 24 °C on a rotary shaker at 250 rpm for 120 h.

Isolation of **1** was accomplished by acetonitrile extraction followed by HPLC purification. The culture broth (4 L) under harvest pH = 5.2 was mixed with 8 L of ACN, then 200 g of NaCl was added into the mixture solution and stirred for 10 min. The ACN layer was separated, and the solvent was removed in vacuo. The crude ACN extract (130 mg) was purified by reversed-phase C-18 HPLC (YMC-ODS semi-preparative column 250 × 20 mm, S-5, 120 Å with a guard column 50 × 20 mm) using 5−50% ACN in water with a linear gradient for 25 min, then 50−100% ACN−H_2O in 7 min, 15 mL/min flow rate, UV detection at 220 nm. Two HPLC runs were performed with 65 mg for each injection to obtain a total of 6 mg of pure **1**.

Derivatizations. To a mixture of **1** (6 mg, 0.024 mmol), pyridine (0.2 mL), and CH_2Cl_2 (8 mL) at room temperature was added acetic anhydride (0.1 mL, excess). The reaction mixture was stirred for 5 h at 25 °C, and solvent was removed in vacuo. The residue (10 mg) was purified by reversed-phase HPLC (YMC-ODS semipreparative column 250 × 20 mm, S-5, 120 Å with a guard column 50 × 20 mm) using 5−50% ACN in water with a linear gradient for 20 min, then 50−100% ACN−H_2O in 10 min, 15 mL/min flow rate, UV detection at 220 nm. About 5 mg of pure **2** was obtained after lyophylization (see Table 1 for NMR spectral data). The FABMS data showed the protonated molecular ion at *m*/*z* 295 $(M + H)^+$ to confirm the molecular mass of **2** as 294 Da.

To obtain more material of **1** to make its *p*-bromobenzoate derivative **3**, multiple batches of fermentation were performed. The isolation of combined extracts was carried out under the same conditions as described above to afford ∼109 mg of pure **1**. To a mixture of **1** (109 mg, 0.43 mmol) and triethylamine (TEA, 0.6 mL) in dry CH_2Cl_2 (5 mL) was added *p*-bromobenzoyl chloride (268 mg, 1.22 mmol), and the mixture was stirred at room temperature overnight. MeOH (5 mL) was added to quench the reaction and precipitate the *p*-bromobenzoic acid−TEA salt. The supernatant was separated from solid and dried in vacuo. The residue was dissolved in 6 mL of EtOAc and washed with water. After removal of EtOAc, the crude product (∼120 mg) was purified on semipreparative HPLC (YMC-ODS semipreparative column 250 × 20 mm, S-5, 120 Å with a guard column 50 × 20 mm, 30−60% ACN in water with a linear gradient for 15 min, then 60−100% ACN/H_2O in 15 min, 15 mL/min flow rate, UV detection at 220 nm) to afford 70 mg of pure **3** as a white powder (see Table 2 for NMR spectral data). The molecular mass of 435 Da for **3** was confirmed by FABMS data that showed the protonated molecular ion at *m*/*z* 436 $(M + H)^+$. Since recrystallization of **3** with acetone−H_2O did not provide crystals suitable for X-ray analysis, recrystallization again with ACN yielded platelike crystals suitable for X-ray crystallographic study.

X-ray Crystallographic Analysis of Sch 642305 *p*-Bromobenzoate (3). Crystal data: $C_{21}H_{23}BrO_5$, MW = 435.32, orthorhombic, space group $P2_12_12_1$, $a = 11.659(2)$ Å, $b = 28.002(4)$ Å, $c = 6.459(1)$ Å, $V = 2109(1)$ Å^3, $Z = 4$, $D_{calcd} =$

Table 2. NMR Data for *p*-Bromobenzoate Derivative (**3**)[a]

	^{13}C (δ)	^{1}H (δ)	HMBC
1	198.9 s[b]		
2	132.7 d	6.15 d, J = 9.9[c]	C4, C6
3	141.8 d	7.09 dd, J = 9.9, 5.8	C1, C4, C5
4	69.7 d	5.56 dd, J = 5.8, 3.4	C2, C3, C5, C6, C13, C7′
5	35.3 d	3.14 dddd, J = 2.8, 3.4, 11.4, 11.4	
6	47.4 d	2.82 dt, J = 3.7, 3.7, 11.4	C1, C4, C5, C7, C8, C13
7	22.9 t	1.18, 2.29, m, m	C8, C9
8	21.3 t	1.29, 1.64, m, m	
9	22.8 t	1.29, 1.84, m, m	C7, C10
10	29.4 t	1.39, 2.13, m, m	C8, C9, C11, C14
11	73.5 d	5.10 m	
12	170.5 s		
13	38.6 t	2.57 dd, J = 11.4, 16.8 2.64 dd, J = 2.8, 16.8	C4, C5, C6, C12
14	18.2 q	1.28 d, J = 6.6	C10, C11
1′	128.1 s		
2′,6′	131.2 d	7.84 d, J = 8.5	C1′, C4′, C5′, C7′
3′,5′	132.0 d	7.59 d, J = 8.5	C1′, C2′, C4′, C5′
4′	128.8 s		
7′	165.1 s		

[a] Recorded at 500 and 125 MHz for ^{1}H and ^{13}C NMR in $CDCl_3$, respectively. [b] Multiplicity was determined by APT data. [c] Coupling constants in Hz.

1.371 g cm^{-3}, Cu Kα radiation λ = 1.5418 Å, absorption coefficient μ = 28.9 cm^{-1}, crystal dimensions 0.40 × 0.40 × 0.06 mm. Intensity data (+h, +k, +l, 2505 reflections, θ_{max} = 75°) were recorded on an Enraf-Nonius CAD-4 diffractometer at 296 K. The crystal structure was solved by direct methods. Full matrix least-squares refinement of atomic positional and thermal parameters (anisotropic Br, C, N; calculated H positions) converged (max. shift:esd = 0.03σ) at R = 0.050 (R_w = 0.068) over 1500 reflections with $I > 2.0\sigma(I)$. The absolute configuration was determined by use of anomalous dispersion effects (see Supporting Information). Crystallographic calculations were performed by use of the Enraf-Nonius Structure Determination Package (SDP 3.0).[11]

Bacterial DNA Primase Assay. DNA primase activity was measured by following the incorporation of ^{3}H-rNTPs into a polymerized product using a single-stranded DNA template and both DNAB and DNAG proteins at equal molar concentrations. Final counts incorporated following a 1 h assay period were used to calculate the EC_{50} values.

Bacterial MIC Assay. *E. coli* strain HS294 (W3110 Δ*acrAB* Δ*emrAB lpxC** Δ*ompT lacZ*:: (GmR::T7RNAP:: *ampC*P99::*lacIQ*::EmR) and other uncharacterized mutations) was used for MIC determinations. Overnight cultures were adjusted to OD 540 nm = 0.1 in sterile saline (~1 × 107 CFU/mL). A 1:400 dilution of the saline suspension was made into TB media to prepare the final inoculum. For growth inhibition experiments, 0.1 mL of the inoculum was added to the wells of a 96-well microtiter plate containing the appropriate dilutions of antibacterial compounds. The plates were incubated for 18 h at 37 °C. Growth inhibition was assessed by visual readout. Vancomycin was used as a positive control in the MIC assay with an MIC value of 16 μg/mL against *E. coli* strain HS294.

Acknowledgment. The authors are grateful to Mr. P. Bartner for high-resolution mass spectral data, and Ms. D. Walsifer for the preparation of the manuscript.

Supporting Information Available: Tables of X-ray crystallographic data (crystal data and structure refinement, atomic positional and thermal parameters, bond lengths and angles) for **3**, and ^{1}H and ^{13}C NMR spectra of Sch 642305 (**1**) and Sch 642305 *p*-bromobenzoate (**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) An inorganic molecule, cadmium chloride ($CdCl_2$), was utilized as a standard reference for inhibition of primase activity, since there was no organic compound being found as a primase inhibitor, including any known antibiotic that could be used as a positive control in the assay. $CdCl_2$ displayed weak inhibitory activity against bacterial DNA primase enzyme with EC_{50} = 500 μM.
(10) The producing culture was acquired from a private collection and has not been deposited in the collection of the American Type Culture Collection.
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NP0302302