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Cryptosphaerolide: A Cytotoxic Mcl-1 Inhibitor from a Marine-Derived Ascomycete Related to the Genus *Cryptosphaeria*

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Abstract

Examination of the saline fermentation products from the marine-derived ascomycete fungal strain CNL-523 (*Cryptosphaeria* sp.), resulted in the isolation of cryptosphaerolide (**1**). The new compound is an ester-substituted sesquiterpenoid related to the eremophilane class. The structure of the new compound was assigned by spectroscopic and chemical methods. Cryptosphaerolide was found to be an inhibitor of the protein Mcl-1, a cancer drug target involved in apoptosis. It also showed significant cytotoxicity against an HCT-116 human colon carcinoma cell line indicating that the compound may be of value in exploring the Mcl-1 pathway as a target for cancer chemotherapy.

As part of an NCI-supported collaborative program to identify target-specific agents for the treatment of cancer, we have screened numerous marine microbial extracts for the capacity to inhibit protein-protein interactions as regulatory mechanisms for apoptosis. Successful inhibition of anti-apoptotic proteins Bcl-2, Bcl-XL and their protein partners by small molecules has been reported and some compounds are showing positive clinical results. Mcl-1 (myeloid cell leukemia-1) is a Bcl-2 family member that selectively binds the pro-apoptotic proteins Bak, Bim, and Noxa, and is a critical negative regulator of apoptosis during myeloblastic leukemia cell differentiation.¹ Mcl-1 is over-expressed in many cancers and contributes to tumor progression and chemo-resistance by binding to and sequestering pro-apoptotic BH3-domain containing proteins. Disrupting Mcl-1/BH3 interactions with a small molecule is predicted to initiate apoptosis and/or sensitize cancer cells to cytotoxic inducers of apoptosis. Noteworthy is the fact that cancer cells with high levels of Mcl-1 tend to be refractory to Bcl-2 inhibitors whereas cancer cells with lower levels tend to be Bcl-2 inhibitor sensitive.^{2, 3} In addition, it was reported that Mcl-1 plays a critical role in melanoma cell resistance to apoptosis.²⁻⁴ Hence, inhibitors of Mcl-1 could play a significant role in cancer treatment by facilitating the onset of cancer cell apoptosis in several cancer types.

Chemical investigations of marine-derived fungi as sources of biologically active secondary metabolites have led to the discovery of a significant number of interesting natural products.⁵⁻⁸ As part of our investigations of microorganisms from marine environments, a fungal strain CNL-523 was identified as a species within the genus *Cryptosphaeria*. It was selected for further investigation based on significant cytotoxicity of its crude extract toward a HCT-116 colon carcinoma cell line. We have previously reported that when strain CNL-523 was cultivated in the presence of a marine α -proteobacterium (CNJ-328; co-culture) the

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biosynthetic induction of four new diterpenoids, the libertellenones, occurred.⁹ These diterpenoids were not observed in pure cultures of the fungus or the bacterium. In the current study only cryptosphaerolide was observed. The fungus was cultured in a nutrient medium prepared from sterile seawater, yeast, peptone, glucose, and crab meal. Subsequent separation and purification of the cytotoxic extract yielded cryptosphaerolide (**1**), a different class of terpenoid than what we previously observed from this strain in co-culture. The details of the structure elucidation, chemical conversions, and biological activities of this new fungal metabolite are described herein.

The molecular formula of cryptosphaerolide (**1**) was assigned as C₂₆H₄₂O₇ on the basis of combined NMR (in CDCl₃) and HREIMS data. This formula required six degrees of unsaturation. Analysis of ¹H and ¹³C NMR DEPT spectra suggested the presence of five methyl groups, an exocyclic terminal alkene, two oxygenated methylene groups, and three exchangeable protons. The observance of a carbon resonance at δ 174.9, in addition to an IR absorption at 1731 cm⁻¹ strongly indicated the presence of an ester carbonyl group. Three carbon resonances (δ 102.4, C-8; 150.9, C-11; 104.4, C-13) were assigned to one hemiketal carbon and one double bond. Given that the molecular formula afforded six degrees of unsaturation, and that the molecule contained an ester carbonyl and one double bond, the remaining degrees are satisfied by four ring systems. One-bond carbon-proton connectivities were determined by interpretation of HMQC data. Proton connectivities, based on the interpretation of COSY NMR data, could not be unambiguously assigned due to the presence of highly overlapping signals in the upfield portion of the ¹H NMR spectrum. However, analysis of HMBC data (Table 1) for **1** enabled the establishment of the C-1 substituent. In particular, the observation of signals corresponding to hydroxy protons in this side chain (OH-17 and -24 in DMSO-*d*₆), and HMBC correlations from these protons, along with HMBC correlations from the adjacent methyl protons, provided unambiguous data for this assignment. An HMBC correlation from H-1 to C-16 allowed the ester to be positioned at C-1. Having assigned the substructure of the aliphatic ester, three additional partially defined spin systems were established by interpretation of COSY, HMBC and HMQC data (H-7 to H₂-6, H-4 to

H₃-15, and H-1 to H₂-2). These substructures and some unassigned carbons were then connected on the basis of a number of key HMBC correlations. The adjacent six-membered rings were connected by HMBC correlations from methyl protons at δ_{H} 1.19 (H₃-14) to C-4, -5, -6, and -10. In addition, HMBC correlations of H₂-6 and H₂-12 to C-11 and C-8 allowed the formation of the tetrahydrofuran ring (hemiketal ether) in compound **1**. An unassigned methylene group (H₂-3) was linked to C-2 and C-4 on the basis of HMBC correlations of H₃-15 and H-1 to C-3. Based on the above assignments, HMBC correlations from H-1 to C-5 and C-10, and from H-9 to C-1, C-7, C-8, and C-10, completed the assignment of the tricyclic system in cryptosphaerolide (**1**). A hydroxy group was positioned at C-8, based on HMBC correlations of its proton (δ_{H} 6.22, DMSO-*d*₆) to C-7, C-8 and C-9. An epoxide ring was assigned to C-9 and C-10 based on characteristically shielded ¹H NMR shifts in addition to the need to account for one more degree of unsaturation.

Because of significant signal overlap, NOESY data for **1** failed to provide unambiguous stereochemical information. Therefore, the relative configuration of **1** was determined by interpretation of NOESY data derived from reaction products **3** and **4**. Synthetic derivative **2** was prepared from cryptosphaerolide by refluxing **1** with acidic Amberlite IR-120 in methanol solution.¹⁰ Next, the ester functionality in **2** was hydrolyzed to give the C-1 alcohol (**3**; Experimental Section). The alcohol **3** was then converted to Mosher esters **4** and **5** using established methods.^{11, 12}

NMR analysis of compound **3** was essential in completing the relative configuration of **1**. A broad singlet signal from H-1 suggested its equatorial position in the six-membered ring of the molecule. A strong NOESY correlation between H₃-14 and H₃-15 in addition to a correlation from H₃-14 to H-7 suggested that the two methyl groups are axial and equatorial, respectively. Also, NOESY correlations of H-9 with H-1 and the methoxy protons (OCH₃-8) indicated their spatial proximity in the molecule.

The modified Mosher's method was employed to determine the absolute configuration of the tetracyclic skeleton of cryptosphaerolide. The diastereomeric Mosher esters **4** and **5** were prepared from **3**, and the proton chemical shifts were examined for each compound (Figure 1). Although all of the protons for both diastereomers could be assigned on the basis of COSY and NOESY experiments, there were a few anomalies that had to be addressed. As shown in Figure 1, chemical shift differences between the (*R*)-MTPA ester and (*S*)-MTPA ester (**4** and **5**) did not in every instance yield the expected results. These data indicated the limitation of the modified Mosher's method for compounds possessing sterically hindered secondary hydroxy groups; such irregularities have been reported previously.^{13,14} Conventional molecular models of compounds **4** and **5** suggested that the phenyl and methoxy groups of the MTPA esters were in close proximity to the methoxy at C-8, suggesting that the tilting of MTPA plane toward C-2 occurs to avoid unfavorable steric interactions. This would explain the unexpected negative $\Delta\delta_{\text{H}}$ values for H-9 and methoxy protons at OCH₃-8. Otherwise, these data suggest the absolute configuration of C-1 to be *R*, thus allowing the assignment of absolute configuration for the remaining stereocenters in the fused ring system as 1*R*, 4*S*, 5*R*, 7*S*, 8*R*, 9*S*, 10*R*.

Evidence further supporting the proposed conformation of the tetracyclic ring system was obtained from interpretation of the NOESY data of **4**. A NOESY correlation of H-7 with the methoxy protons (OCH₃-8) led to the assignment of the C-7/C-8 ring fusion as *cis*. Similarly, the C-5/C-10 ring fusion was assigned as *trans* on the basis of a NOESY correlation of H-9 with H₃-14. We chose not to embark on determining the relative and absolute stereochemical features of the C-1 side chain. This endeavor, which would have required a significant investment involving producing synthetic models and utilizing numerous spectroscopic tools, was beyond the scope of this study.

Cryptosphaerolide falls in the diverse natural product class of eremophilane terpenoids. Though it is common for the functionalized decalin unit of this class to fuse with a furan ring to form the tricyclic system in **1**, there have been only a few reports of an exocyclic methylene moiety in this system.¹⁵⁻¹⁷ A saprobic fungal metabolite, berkleasmin A, containing an identical sesquiterpenoid skeleton was recently reported,¹⁷ and ¹H and ¹³C NMR spectroscopic data were nearly identical to those from the core 15-carbon skeleton of **1**. Most eremophilane terpenoids reported to date have been isolated from plants, although this structural type has been isolated once from an octocoral,¹⁸ and in a few cases from marine-derived fungi.¹⁹⁻²⁷

Cryptosphaerolide was found to exhibit in vitro cancer cell cytotoxicity with an IC₅₀ of 4.5 μM toward an HCT-116 colon carcinoma cell line. In subsequent screening, **1** was found to inhibit the Mcl-1 protein in the Mcl-1/Bak fluorescence resonance energy transfer assay (FRET) with an IC₅₀ of 11.4 μM. Methylated derivative **2** also exhibited similar bioactivity (IC₅₀ of 12.5 μM) in this assay. Interestingly, the alcohol derivative **3** was not cytotoxic and was inactive in the Mcl-1/Bak FRET assay, suggesting that the presence of the hydroxylated ester side chain is essential for these activities.

Experimental Section

General Experimental Procedures

Optical rotations were measured on a JASCO P-2000 polarimeter. UV spectra were measured on a Beckman Coulter DU800 spectrophotometer. IR spectra were recorded on a Perkin-Elmer 1600 FTIR spectrometer. NMR spectra were recorded on Varian Innova spectrometers at 300 MHz for ¹H and 100 MHz for ¹³C. All spectra were recorded in CDCl₃ or DMSO-*d*₆, and chemical shifts were referenced to either the corresponding solvent residual signal or tetramethylsilane. Numbers of attached protons to carbon were determined by DEPT experiments. HMBC and HMQC experiments were optimized for ⁿJ_{CH} = 8.0 Hz and ¹J_{CH} = 150.0 Hz, respectively. HRMS data were obtained at the Scripps Research Institute in La Jolla. Low-resolution LC/MS data were acquired using a Hewlett-Packard series 1100 system equipped with a reversed-phase C₁₈ column (Phenomenex Luna, 4.6 × 100 mm, 5 μm) at a flow rate of 0.7 mL/min. HPLC separations were performed using a Waters 600E system controller and pumps with a Model 480 spectrophotometer.

Fungal Isolation and Identification

The marine-derived ascomycete fungus, strain CNL-523, was isolated from an unidentified ascidian collected in the Bahamas in 1996 and first identified as a member of the genus *Libertella* based on morphological characteristics by the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl). Subsequent analysis of the 28S rDNA sequence from this strain revealed that it belonged to the ascomycete family Diatrypaceae with only 94% similarity to *Cryptosphaeria eunomia* (AY083826) suggesting it was a new species (GenBank accession # HM057167).

Isolation and Properties of Cryptosphaerolide (1)

The culture filtrate from the fermentation of CNL-523 (10 L fermentation) was extracted with EtOAc, and the organic phase was dried (MgSO₄) and concentrated to afford 1.5 g of brown residue. The extract was fractionated using reversed-phase C₁₈ flash column chromatography with a stepwise gradient of 0 to 100 % (v/v) CH₃OH in H₂O. The 80% CH₃OH in H₂O fraction was subjected to preparative reversed-phase C₁₈ HPLC using a gradient from 20 to 80% CH₃CN in H₂O over 60 min, followed by 100% CH₃CN for 10 min. (Waters Prep Nova C₁₈ HR-60 Å C₁₈ column, 6 μm particles, 3 × (25 × 100 mm); 10 mL/min; UV detection at 210 nm) to yield **1** (38.5 mg).

Cryptosphaerolide (1)—colorless oil; $[\alpha]_D^{25} +22.6$ (*c* 0.27 CHCl₃); UV (CH₃OH) $\lambda_{\max}$ (log ϵ): 202 (4.04), 256 (2.78) nm, IR 3404, 2946, 1731, 1456, 1374, 1203, 1145 cm⁻¹; ¹H NMR data (300 MHz, CDCl₃) Table 1; ¹H NMR data (300 MHz, DMSO-*d*₆) 6.23 (s, 1H, OH-8), 4.90 (br s, 1H, H-13), 4.72 (br s, 2H, OH-17 and OH-24), 4.33 (d, 1H, *J* = 12.9 Hz, H-12), 4.27 (d, 1H, *J* = 12.9 Hz, H-12), 4.19 (s, 1H, H-1), 3.52 (dd, 1H, *J* = 6.3, 12.5 Hz, H-24 α), 3.33 (dd, 1H, *J* = 6.3, 10.5, H-24 β), 3.04 (s, 1H, H-9), 2.42 (m, 1H, H-7), 1.69 (m, 2H, H-2), 1.60 (m, 1H, H-18 α), 1.56 (m, 1H, H-19), 1.47 (m, 1H, H-6 α), 1.42 (m, 1H, H-4), 1.36 (m, 2H, H-3), 1.33 (m, 1H, H-21), 1.32 (m, 1H, H-18 β), 1.26 (m, 1H, H-22 α), 1.11 (m, 1H, H-6 β), 1.10 (s, 3H, H-14), 0.99 (m, 1H, H-22 β), 0.88 (d, 3H, *J* = 6.3 Hz, H-19), 0.83 (d, 3H, *J* = 6.3 Hz, H-15), 0.80 (t, 3H, *J* = 9.0, H-23), 0.78 (d, 3H, *J* = 6.6 Hz, H-26); ¹³C NMR data (CDCl₃) Table 1; ¹³C NMR data (300 MHz, DMSO-*d*₆) 173.6 (C-16), 151.8 (C-11), 104.1 (C-13), 101.7 (C-8), 78.5 (C-17), 75.0 (C-1), 68.4 (C-12), 68.4 (C-24), 62.5 (C-10), 62.0 (C-9), 45.3 (C-20), 44.5 (C-7), 41.5 (C-18), 38.2 (C-4), 36.5 (C-6), 35.4 (C-5), 30.9 (C-21), 28.0 (C-2), 28.0 (C-22), 26.3 (C-19), 25.3 (C-3), 21.3 (C-25), 19.9 (C-26), 15.1 (C-14), 15.0 (C-15), 10.9 (C-23); HMBC data (CDCl₃) Table 1; HMBC data (DMSO-*d*₆) H-1 $\rightarrow$ C-3, 5, 10, and 16, H-6 $\rightarrow$ C-5 and 7, H-9 $\rightarrow$ C-1, 7, 8, and 10, H-13 $\rightarrow$ C-7 and 12, H-14 $\rightarrow$ C-4, 5, 6, and 7, H-15 $\rightarrow$ C-3, 4, and 5, H-23 $\rightarrow$ C-21 and 22, H-25 $\rightarrow$ C-18, 19, and 20, H-26 $\rightarrow$ C-20, 21, and 22, 8-OH $\rightarrow$ C-7, 8, 9, and 17-OH $\rightarrow$ C-16, 17, and 18; ESI MS $[M+Na]^+$ *m/z* 489, HR MALDI-FTMS (DHB matrix) *m/z* 489.2818 (calcd for C₂₆H₄₂O₇Na, 489.2823).

Preparation of 2

Cryptosphaerolide (**1**, 6.8 mg) was dissolved in CH₃OH (2.5 mL) and stirred. Amberlite IR-120 H⁺ (40 mg) was added to the stirred solution and heated at 70° C for 72 h to yield **2** in quantitative yield. Formation of **2** was monitored by TLC analysis and showed nearly identical ¹H NMR data with that of **1**, with the appearance of additional signal corresponding to a methoxy group (δ_H 8-OCH₃ = 3.37). ESI MS and ¹H NMR data confirmed this observation. The Amberlite was filtered and the compound was concentrated in vacuo. Compound **2** was sufficiently pure to be used in the next reactions without further purification.

Hydrolysis of 2

Compound **2** (5.0 mg, 10.4 μ mol) in dry THF (2 mL) was added to 2 mL of NaOH (0.5 N) solution, and heated at 50 °C for 3 h. The reaction mixture was concentrated, partitioned between CH₂Cl₂ and H₂O, and the organic layer was concentrated to give compound **3** (3.6 mg).

3: ¹H NMR (300 MHz, CDCl₃) 3.32 (m, 1H, H-1), 1.41 (m, 1H, H-2), 1.83 (m, 1H, H-2), 1.81 (m, 2H, H-3), 1.56 (m, 1H, H-4), 1.44 (m, 1H, H-6 α), 1.35 (m, 1H, H-6 β), 2.62 (m, 1H, H-7), 3.20 (s, 1H, H-9), 4.53 (ddd, 1H, *J* = 12.7, 4.1, 2.0 Hz, H-12), 4.42 (dddd, 1H, *J* = 12.7, 3.6, 1.2, 1.2 Hz, H-12), 4.95 (dd, 1H, *J* = 2.0, 2.0 Hz, H-13 α), 4.94 (dd, 1H, *J* = 2.0, 2.0 Hz, H-13 β), 1.12 (s, 3H, H-14), 0.87 (d, 3H, *J* = 6.9 Hz, H-15), 3.37 (s, 3H, OCH₃-8). ESI MS *m/z* 303 $[M+Na]^+$ (C₁₆H₂₄O₄Na).

Preparation of Mosher ester 4 (R)-MTPA

Compound **3** (2 mg) was dissolved in CH₂Cl₂ (0.5 mL) under argon. DMAP (1 mg), DIEA (35 μ L), and (*S*)-MTPA-Cl (20 μ L) were then added and the reaction mixture was stirred for 12 h. The reaction mixture was diluted with excess CH₂Cl₂, the organic layer was washed with brine and water, and the concentrated to dryness under vacuum. The product was purified by reversed-phase C₁₈ HPLC using a gradient of CH₃CN in H₂O to yield 3.4 mg of **4**.

4: ¹H NMR (300 MHz, CDCl₃) δ_H 7.49-7.52 (m, 2H, Ar-H), 7.41-7.44 (m, 3H, Ar-H), 4.95 (d, *J* = 2.1 Hz, 1H, H-13), 4.91 (d, *J* = 2.1 Hz, 1H, H-13), 4.69 (br s, 1H, H-1), 4.51 (d, *J* = 12.8 Hz, 1H, H-12), 4.41 (d, *J* = 12.8 Hz, 1H, H-12), 3.51 (s, 3H, OCH₃), 3.41 (s, 1H, H-9),

3.37 (s, 3H, 8-OCH₃), 2.56 (m, 1H, H-7), 1.94 (m, 1H, H-2 α), 1.86 (m, 1H, H-2 β), 1.60 (m, 1H, H-3 α), 1.56 (m, 1H, H-4), 1.43 (m, 1H, H-6 α), 1.42 (m, 1H, H-3 β), 1.33 (m, 1H, H-6 β), 0.88 (s, 3H, H-14), 0.80 (d, J = 6.6 Hz, 3H, H-15); HR MALDI-FTMS (DHB matrix) m/z 519.1962 [M + Na]⁺, (calcd for C₂₆H₃₁F₃O₇Na, 519.1965).

Preparation of Mosher ester **5** (S)-MTPA

Compound **5** was prepared from compound **3** and (*R*)-MTPA-Cl using the analogous procedure as above.

5: ¹H NMR (300 MHz, CDCl₃) δ _H 7.48-7.56 (m, 2H, Ar-H), 7.39-7.41 (m, 3H, Ar-H), 4.93 (d, J = 2.1 Hz, 1H, H-13), 4.88 (d, J = 2.1 Hz, 1H, H-13), 4.68 (br s, 1H, H-1), 4.49 (d, J = 12.8 Hz, 1H, H-12), 4.38 (d, J = 12.8 Hz, 1H, H-12), 3.60 (s, 3H, OCH₃), 3.49 (s, 1H, H-9), 3.38 (s, 3H, 8-OCH₃), 2.41 (m, 1H, H-7), 1.97 (m, 1H, H-2 α), 1.88 (m, 1H, H-2 β), 1.62 (m, 1H, H-3 α), 1.54 (m, 1H, H-4), 1.46 (m, 1H, H-3 β), 1.33 (m, 1H, H-6 α), 1.26 (m, 1H, H-6 β), 0.62 (s, 3H, H-14), 0.80 (d, J = 6.3 Hz, 3H, H-15); HR MALDI-FTMS (DHB matrix) m/z 519.1984 [M + Na]⁺, (calcd for C₂₆H₃₁F₃O₇Na, 519.1965).

HCT-116 Cytotoxicity Bioassay

Aliquot samples of HCT-116 human colon adenocarcinoma cells were transferred to 96-well plates and incubated overnight at 37° C in 5% CO₂/air. Test compounds were added to the plates in DMSO and serially diluted. The plates were then further incubated for 72 h, and at the end of this period, a CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega) was used to assess cell viability. Inhibition concentration (IC₅₀) values were deduced from the bioreduction of MTS/PMS by living cells into a formazan product. MTS/PMS was first applied to the sample wells, followed by incubation for 3 h. Etoposide (Sigma; IC₅₀ = 1.5–4.9 μ M) and DMSO (solvent) were used as the positive and negative controls in this assay. The quantity of the formazan product (in proportion to the number of living cells) in each well was determined by the Molecular Devices Emax microplate reader set to a wavelength of 490 nm. IC₅₀ values were calculated using the analysis program, SOFTMax.

Mcl-1 Bioassay

A FRET-based competition assay was developed to characterize antagonists of Mcl-1. The assay is based on the ability of antagonist molecules to compete with the biotin labeled BH3 peptide, BAK, for binding to His-tagged Mcl-1. TR-FRET is a proximity-based detection method that requires a donor label, Europium (Eu-W1024-Anti-6 $\times$ -His) and an acceptor label, APC (streptavidin-APC). In the absence of a competitive small molecule, the His-Mcl-1 fusion protein binds specifically to its natural ligand BAK, or in the context of this assay, to B-BAK (Biotin-LC-LC-PSSTMGQVGRQLAIGDDINRRYDSE-OH, Anaspec, Inc.). The subsequent addition of donor and acceptor labeled complexes results in Europium (via the anti-His:His-Mcl-1 interaction) and APC (via the Streptavidin:Biotin interaction) coming into proximity allowing fluorescence energy transfer. The FRET buffer used for all experiments was 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20. The Mut-BAK (Biotin-LC-LC-PSSTMGQVGRQAIIIGDDINRRYDSE-OH, Anaspec, Inc.) peptide containing an L78A substitution, which eliminates Mcl-1 binding, serves as a control for buffer effects and nonspecific binding contributions to the signal. His-tagged Mcl-1 (12.5 nM, final) was pre-incubated for 30 min with test compound (80 μ M-0.037 μ M; 3-fold serial dilution) and a master mix was then added containing B-BAK (20 nM, final), Eu-W1024-Anti-6 $\times$ -His (1 nM, final) and Streptavidin Surelight-APC (25 nM, final) and incubated for 1 h. Data were collected using the PerkinElmer EnVision 2103 Multilabel Reader using excitation and emission filters Europium 615 nM and APC 665 nM, respectively, and the optical module Lance Eu/APC Dual 452.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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R = (*R*) and (*S*)-MTPA

Figure 1.

$\Delta\delta_H$ values ($\Delta\delta_H = \delta_R - \delta_S$) for MTPA esters **4** and **5**.

Table 1NMR Spectroscopic Data for Cryptosphaerolide (**1**)

Position	δ_C^a	δ_H (J in Hz) ^b	HMBC ^c
1	76.7	4.62, br s	3, 5, 10, 16
2	28.3	1.87, m	--
3	25.6	1.46, m, 1.71 m	--
4	38.3	1.64, m	--
5	35.8	--	--
6	36.4	1.57, m; 1.37 m	5, 7, 8, 10
7	44.6	2.66, m	6, 8, 11
8	102.4	--	--
9	61.7	3.26, s	1, 7, 8, 10
10	62.9	--	--
11	150.9	--	--
12	70.0	4.55, d (12.9); 4.44, d (12.9)	7, 8, 11, 13
13	104.4	4.93, br s; 4.87, br s	7, 12
14	15.2	1.18, s	4, 5, 6, 10
15	15.1	0.90, d (5.4)	3, 4, 5
16	174.9	--	--
17	79.3	--	--
18	41.2	1.41, m; 1.68, m	16, 17, 24, 25
19	26.7	1.65, m	--
20	45.7	0.93, m; 1.18, m	19, 22, 25, 26
21	31.4	1.38, m	--
22	28.5	1.04, m; 1.38, m	--
23	11.1	0.83, t; (8.1)	21, 22
24	69.0	3.77, d (10.7); 3.57, d (10.7)	16, 17, 18
25	21.5	0.94, d (6.0)	18, 19, 20
26	19.9	0.80, d (6.6)	20, 21, 22

^aRecorded at 100 MHz at CDCl₃^bRecorded at 300 MHz in CDCl₃^cRecorded at 300 MHz (¹H-dimension); HMBC correlations are from the proton(s) stated to the indicated carbons.