Marmycins A and B, Cytotoxic Pentacyclic C-Glycosides from a Marine Sediment-Derived Actinomycete Related to the Genus *Streptomyces*

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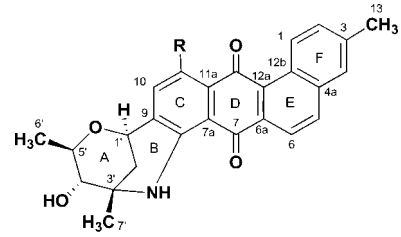
Two new cytotoxic quinones of the angucycline class, marmycins A and B (**1**, **2**), were isolated from the culture broth of a marine sediment-derived actinomycete related to the genus *Streptomyces*. The gross structures and absolute configurations of both compounds were determined by spectroscopic and crystallographic methods. Marmycin A (**1**) displayed significant cytotoxicity against several cancer cell lines, some at nanomolar concentrations; while compound **2**, a chloro analogue of **1**, was less potent. For marmycin A (**1**), tumor cell cytotoxicity appeared to coincide with induction of modest apoptosis and arrest in the G1 phase of the cell cycle.

In the past five years, it has become clear that phylogenetically unique actinomycete bacteria can be isolated from marine sediments.[1–4] Chemical studies of strains of *Salinispora*, the first obligate marine actinomycete genus to be recognized, led to the discovery of the potent proteasome inhibitor salinosporamide A,[5] as well as other unprecedented secondary metabolites.[6,7] In subsequent studies, another actinomycete group tentatively named *Marinispora* was isolated from marine sediments. We recently reported the isolation of the marinomycins, a series of antibacterial and melanoma-selective cytotoxic macrolides of a new structure class, from this group.[8] As part of a continuing program to explore the chemistry of marine-derived actinomycetes, we examined several sediment samples collected at the entrance to the Sea of Cortez, Baja California Sur, México. These sediments were found to contain a diversity of actinomycetes, including *Salinispora* and *Marinispora* strains, as well as several strains that fall broadly within the well-known genus *Streptomyces*. These strains are unique, however, as they are only distantly related to any previously described *Streptomyces* species for which 16S rRNA gene sequence data are available.

From one sediment sample, we isolated a new *Streptomyces*-related actinomycete, strain CNH990, the culture extract of which was observed to be significantly cytotoxic toward the human colon carcinoma cell line HCT-116. Subsequent fractionation of the extract, using *in vitro* inhibition of HCT-116 growth as a guide, resulted in the isolation of two new anthraquinone derivatives, marmycins A and B (**1**, **2**). The marmycins belong to the angucycline class of antibiotics because of their angular shape.[9]

Results and Discussion

Marmycin A (**1**), a red crystalline solid (mp 257–259 °C), showed $[\alpha]_D^{20}$ +520 (*c* 0.05, THF) and analyzed for the molecular formula $C_{26}H_{23}NO_4$ by HR-ESI-MS ($[M + H]^+$ *m/z* = 414.1659, Δ −11.2 ppm). This formula indicated that **1** possesses 16 degrees of unsaturation. The ^{13}C NMR spectrum of **1** (Table 1), showed 26 signals, consistent with the molecular formula. Two carbonyl resonances were observed at δ 185.7 and δ 186.4, which when combined with the observation of a sharp IR absorption band at 1652 cm^{-1} indicated the presence of a *p*-benzoquinone chromophore. Absorptions in the UV spectrum at λ_{max} (log ε) 220 (3.24), 239 (3.39), 290 (3.42), 318 (sh, 2.83), and 523 (2.45) nm were indicative of a complex anthraquinone moiety. Since no olefinic signals were observed between 5 and 7 ppm in the ^1H NMR spectrum, it was evident that the 16 downfield resonances seen in the ^{13}C NMR spectrum between 111.3 and 148.9 ppm were due to aromatic carbons. These data indicated the presence of three aromatic rings.



1. marmycin A, R = H
2. marmycin B, R = Cl

Comprehensive analysis of 2D NMR (COSY, HSQC, and HMBC) data facilitated the construction of the angucyclinone skeleton and the aminopyranose ring in marmycin A (**1**) (Table 1). Analysis of ^1H–^1H COSY spectroscopic data, and $^3J_{H\text{-}H}$ coupling constant information, revealed five isolated spin systems: the aromatic protons of C-1, C-2, C-13, and C-4; protons at C-5 and C-6; protons at C-10 and C-11; and aliphatic protons at C-4′, C-5′, and C-6′ as well as those at C-1′ and C-2′ of the pyranose ring (Figure 1). Key HMBC correlations of the angucyclinone aglycone included H-1 to C-3, C-4a, and C-12a; H-5 to C-6a and C-12b; H-6 to C-4a, C-7, and C-12a; H-10 to C-8 and C-11a; and H-11 to C-7b, C-9, and C-12. HMBC correlations from the pyranose ring consisted of H-1′ to C-3′ and C-5′; and NH to C-2′ and C-7′. The ^1H NMR downfield resonance at 9.53 ppm for H-1 suggested that it was positioned within the deshielding effects of the C-12 carbonyl oxygen. Interpretation of NMR data indicated that the pyranose ring was attached to C-8 of the angucyclinone aglycone via a C-glycosidic linkage. This was confirmed by HMBC correlations of the anomeric proton H-1′ to C-8 and C-10 (Figure 1) as well as a ROSEY cross peak from H-1′ to H-10 (Figure 2).

In order to satisfy the 16 degrees of unsaturation indicated by the molecular formula, it was apparent that the pyranose amino

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Table 1. NMR Spectroscopic Data for Marmycin A (**1**) in $CDCl_3$

C/H#	δ_C[a,b]	δ_H, mult (*J* in Hz)	HMBC	COSY	ROESY
1	128.2, CH	9.53, d (9.0)	C3, C4a, C12a	H2	H2
2	132.4, CH	7.54, d (9.0)	C4, C12b, C13	H1	H1, H13
3	138.9, C				
4	128.1, CH	7.64, s	C2, C5, C12b, C13	H13	H5, H13
4a	128.9, C				
5	134.5, CH	8.05, d (8.5)	C4, C6a, C12b	H6	H4, H6
6	122.5, CH	8.29, d (8.5)	C4a, C7, C12a	H5	H5
6a	134.8, C				
7	185.7, C				
7a	111.3, C				
8	148.9, C				
9	127.4, C				
10	136.0, CH	7.49, d (7.5)	C1′, C8, C11a	H11	H1′
11	116.2, CH	7.56, d (7.5)	C7a, C9, C12	H10	
11a	136.7, C				
12	186.4, C				
12a	136.4, C				
12b	128.4, C				
13	21.7, CH_3	2.52, s	C2, C3, C4	H4	H2, H4
$1'_{eq}$	69.4, CH	4.79, br s	C3′, C5′, C8, C10	$H2'_{ax}$, $H2'_{eq}$	$H2'_{ax}$, $H2'_{eq}$, H10
$2'_{eq}$	35.0, CH_2	1.83, d (13.3)	C3′, C4′, C7′	H1′	H1′
$2'_{ax}$		2.15, dd (13.3, 3.5)	C3′, C4′, C9	H1′	H1′, H4′
3′	51.7, C				
$4'_{ax}$	79.1, CH	3.17, d (9.3)	C5′, C6′, C7′	H5′	$H2'_{ax}$, H6′, H7′
$5'_{ax}$	69.3, CH	3.14, dq (9.3, 5.8)	C1′, C3′, C4′, C6′	H6′, H4′	H6′
6′	18.2, CH_3	1.22, d (5.8)	C1′, C4′	H5′	H4′, H5′
7′	24.9, CH_3	1.52, s	C2′, C3′, C4′		H4′, NH
NH		9.54, br s	C2′, C7′, C7a, C9		H7

[a] Numbers of attached protons were determined by DEPT experiments. [b] Recorded at 125 MHz.

group at C-3′ was involved in another ring closure to the highly deshielded C-8 aromatic carbon (148.9 ppm). This was supported by HMBC correlations from the amine proton to C-7a and C-9 of the benz[a]anthraquinone moiety. The relative stereochemistry of the amino sugar was deduced from analysis of proton NMR coupling constants between protons of the pyranose ring and

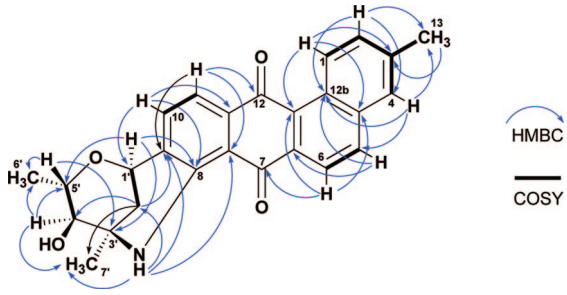

Figure 1. Observed COSY (bold bonds) and HMBC correlations (blue arrows) for marmycin A (**1**).

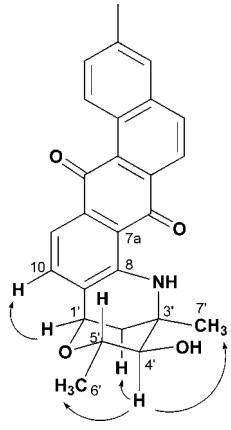

Figure 2. Selected ROESY NMR correlations for marmycin A (**1**).

through analysis of ROESY data (Figure 2). The sugar was found to be in a chair conformation, which allowed the anomeric proton H-1′ to be assigned as equatorial based on its multiplicity (br s) as well as ROESY correlations with $H\text{-}2'_{eq}$ and $H\text{-}2'_{ax}$. A *trans*-diaxial relationship between H-4′ (d, $J = 9.3$ Hz) and H-5′ (d, $J = 9.3$ Hz) allowed both the C-4′ hydroxyl and C-6′ methyl groups to be assigned in equatorial positions. ROESY correlations from H-4′ (δ 3.17) to $H\text{-}2'_{ax}$ (δ 2.15) and to the methyl protons H_3-7′ (δ 1.52) implied that both $H\text{-}2'_{ax}$ and the amino group at C-3′ were axial (Figure 2). The amino sugar in **1**, which has been seen in the angucyclinone antibiotics hedamycin, kidamycin, and pluromycin, and the saptomycins (as *N,N*-dimethyl derivatives),[10–13] can be assigned as 3-epi, 4-epi-vancosamine based on comparison of its ^1H and ^{13}C NMR data with those of vancosamine[14] and its related hydroxyl epimers.[15,16]

Confirmation of the structure assigned by NMR data, as well as the absolute configuration of **1**, was achieved by X-ray crystallography (Figure 3). Crystal growth of **1** proved difficult in single solvents as well as in mixed solvent pairs.[17] However, after many unsuccessful attempts, suitable crystals of **1** were obtained by a vapor diffusion protocol[18] using tetrahydrofuran (THF) and pentane. A light atom absolute configuration determination was carried out by the anomalous dispersion protocol with the more effective Cu Kα radiation[19,20] as opposed to the traditional Mo Kα radiation.[21] The Flack parameter was found to be −0.08 with a standard

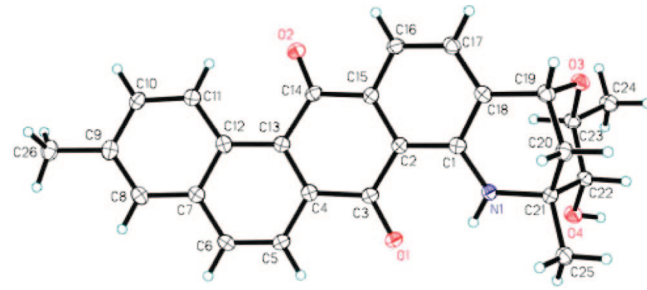

Figure 3. Final X-ray drawing for marmycin A (**1**) depicting its absolute configuration.

uncertainty of 0.09. The crystal of compound **1** is composed of an orthorhombic system with lattice constants $a = 6.9808(13)$, $b = 11.376(2)$, and $c = 24.129(5)$ and a $P2_12_12_1$ space group. The absolute configuration of marmycin A (**1**) is 1′*R*, 3′*R*, 4′*S*, 5′*S*, which further confirmed the presence of the L-amino sugar, is shown in Figure 3.

Marmycin B (**2**) was isolated as pink needles (THF/pentane), mp 291–293 °C dec., which showed $[\alpha]_D^{20}$ +600 (*c* 0.1, THF), and which analyzed for the molecular formula $C_{26}H_{22}{}^{35}ClNO_4$ based on HR-ESI-MS data ($[MH]^+$ $m/z = 448.1325$, Δ 2.1 ppm). The mass spectrum, which clearly showed the presence of a chlorine isotope, along with the highly analogous NMR spectroscopic data (Experimental Section and Supporting Information) in comparison with **1**, suggested that marmycin B (**2**) was a monochloro derivative. The absence of the H-11 aromatic proton resonance in the ^{1}H NMR spectrum of **2** suggested that the chlorine atom could be positioned at C-11 on the aromatic ring. This was supported by the observation of a singlet signal for H-10 (δ 7.60, s) in compound **2** as opposed to a doublet resonance (δ 7.49, d, $J = 7.5$ Hz) in the ^{1}H NMR spectrum of **1**. Comprehensive NMR analysis and comparison with data derived from marmycin A (**1**) allowed the full structure to be assigned.

Given that the cultivation of this *Streptomyces*-related strain was conducted in the presence of high levels of chloride (seawater-based medium), we questioned whether the substitution of a chlorine for the C-11 hydrogen in **1** during cultivation might be a feasible mechanism for the production of the minor metabolite **2**. Treatment of **1** with excess dilute HCl for 24 h at RT resulted in the production of very trace quantities of **2** as detected by LC-MS methods. Given this result, we cannot rule out the production of **2** by an acid-facilitated chloride substitution reaction.

Compounds **1** and **2** were evaluated for their bioactivity in a series of cancer and infectious disease bioassays. These compounds were initially tested for their cytotoxicity against the human colon tumor cell line (HCT-116), for their antibacterial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF), and finally against *Candida albicans* wild-type (CAW) and *C. albicans* amphotericin B resistant strain (CAAB). In the HCT-116 cytotoxic assay, compound **1** showed $IC_{50} = 60.5$ nM, which proved to be almost 18 times more potent than its halogenated congener **2** ($IC_{50} = 1.09$ μM). This result is the opposite of what has been generally observed, that chlorination usually plays a significant role in enhancing the pharmacological activity of a compound.[6,22] In testing with the drug-resistant human pathogens methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VREF), and amphotericin-resistant *Candida albicans* (CAAB), at doses as high as 250 μg/mL, neither marmycin A nor B exhibited any significant activity.

Because of its potent cancer cell activity and lack of antifungal activity, marmycin A (**1**) was further evaluated for its *in vitro* cytotoxicity against a panel of 12 human tumor cell lines (breast, prostate, colon, lung, leukemia). Marmycin A was observed to be quite potent, demonstrating a mean IC_{50} value of 0.022 μM (range 0.007 μM to 0.058 μM) after 72 h drug exposure. In these cell lines, consistent with our earlier observation, marmycin B (**2**) was significantly less potent with a mean IC_{50} value of 3.5 μM (range 1.0 μM to 4.4 μM). In order to gain additional insight into the cytotoxic properties of compound **1**, studies were carried out to examine if this compound induced apoptosis or altered cell cycle progression. As summarized in Table 2, modest induction of apoptosis in a human ovarian tumor A2780 cell line occurred after 24 h drug exposure with a corresponding loss of cells from G2 and arrest of cells in G1 phase. These results suggest that marmycin A is not a general cell toxin. Earlier studies with the related angucyclinone hedamycin showed that this cytotoxin is a DNA intercalator.[23] While our data are consistent with that mechanism, further work will be required to fully define the mechanism of action of this cytotoxic metabolite.

Table 2. Analysis of Apoptosis and Cell Cycle Progression in A2780 Cells after 24 h Exposure to Marmycin A (**1**)

compound	dose	% apoptosis	%<G1	%G1	%S	%G2	%>G2
DMSO	0.1%	0.6	0.8	37	42	19	0.5
marmycin A	20 nM	4.4	2.9	48	41	7.3	0.8
	60 nM	6.9	4.1	50	39	4.5	1.9
	200 nM	5.8	2.4	55	35	8.0	0.6

Members of the angucycline class are polyketide-derived tetracyclic benz[a]anthraquinones that exhibit a wide range of biological activities including antibacterial, enzyme-inhibitory, and cytostatic effects.[9] Most of the reported angucycline aglycones are O-desoxyglycosides, and a much smaller group are C-glycosides.[24] They are related to the linear anthracyclines and tetracyclines and have been the targets for synthesis due to their bioactivities and unusual structural features.[25,26] Marmycins A and B (**1**, **2**) are unique compared to the previously reported angucycline amino glycoside metabolites. Whereas C-glycoside linkages are quite common for some angucyclines, the presence of both the C- and N-glycoside bonds in **1** and **2**, which results in its hexacyclic framework, has not been previously observed. The biosynthetic origin of marmycin A (**1**) is hypothesized to originate in polyketide metabolism to produce a cyclized decaketide that is ultimately glycosylated with the L-amino sugar 3-epi, 4-epi-vancosamine.

Experimental Section

Biological Material. The actinomycete (strain number CNH990) was cultured from a sediment sample that had been air-dried and serially stamped onto medium A1 (1.0% starch, 0.4% yeast extract, 0.2% peptone, 1.6% agar, 100% seawater, 100 μg/mL cyclohexamide added after autoclaving). This sample was collected at a depth of 20 m at the entrance to the Sea of Cortez, ca. 5 km east of Cabo San Lucas, Mexico. Phylogenetic analysis of the nearly complete 16S rDNA gene indicated that strain CNH990 shares only 95.6% sequence identity with *S. sodiiphilus* (NCBI accession number AY236339), the most closely related *Streptomyces* species for which sequence data is available, suggesting that it may represent a new species. CNH990 (GenBank accession number EF058199) is closely related (>99% sequence identity) to other marine-derived, not formally described, *Streptomyces* spp. (GenBank accession numbers AY526525–AY526527), indicating that this taxon can be reproducibly recovered from marine samples.

Fermentation. Strain CNH990 was cultured in five 2.8 L Fernbach flasks, each containing 1 L of medium A1BFe + C: [A1 as above plus 1 g $CaCO_3$, 5 mL 21.3 mM $Fe_2(SO_4)_2 \cdot 4H_2O$, 5 mL 168 mM KBr]. The culture was grown for 5 days at 27 °C while shaking at 215 rpm.

Extraction and Isolation. The culture was extracted with Amberlite XAD-7 resin (20–30 gL^{-1}) and the resin was separated from the broth via vacuum filtration. The resin was eluted with Me_2CO (2 × 2 L) and the solvents were removed *in vacuo* to afford a dark reddish brown extract (356 mg). The broth was extracted with EtOAc (2 × 1000 mL) and the EtOAc was dried with anhyd Na_2SO_4, filtered, and concentrated *in vacuo* to yield 129 mg. The combined extract (485 mg/2 L) was subjected to medium-pressure column chromatography over Supelco Diaion HP-20SS using increasing amounts of Me_2CO in H_2O. The residue from the 75% Me_2CO in H_2O fraction was further purified on reverse phase HPLC (Dynamax C18, 250 × 10 nm, 80% aqueous MeCN, 3 mL/min, RI detection) to afford marmycins A (**1**, 4 mg) and B (**2**, 0.7 mg).

Marmycin A (**1**) was obtained as red needles (THF/*n*-C_5H_{12}): mp 257–259 °C dec.; $[\alpha]_D^{20}$ +520 (*c* 0.05, THF); UV (MeOH) λ_{max} (log ε) 220 (3.24), 239 (3.39), 290 (3.42), 318 (sh, 2.83), and 523 (2.45) nm; IR (NaCl) ν_{max} 3633, 3453, 1652, 1620, 1588, 1069 cm^{-1}; ^{1}H, ^{13}C and 2D NMR data of **1** are listed in Table 1; HRMS (ESI) *m/z* 414.1659 $[M + H]^+$ (calcd for $C_{26}H_{24}NO_4$, 414.1705).

Marmycin B (**2**) was obtained as pink needles (THF/*n*-C_5H_{12}): mp 291–293 °C dec.; $[\alpha]_D^{20}$ +600 (*c* 0.1, THF); UV (MeOH) λ_{max} (log ε) 203 (3.42), 221 (2.82), 235 (442), 285 (2.66), 300 (sh, 2.16), and 509

(2.16) nm; IR (NaCl) ν_{max} 3636, 3401, 1664, 1607, 1572, 1431, 1226, 1149 cm^{-1}; ^{13}C NMR data (DMSO-d_6) δ 127.9 (CH) C-1, 132.4 (CH) C-2, 138.9 (C) C-3, 128.4 (CH) C-4, 128.C) C-4a, 134.5 (CH) C-5, 122.5 (CH) C-6, 136.6 (C) C-6a, 184.3 (C) C-7, 111.6 (C) C-7a, 148.7 (C) C-8, 129.7 (C) C-9, 138.8 (CH) C-10, 133.1 (C) C-11, 119.2 (C) C-11a, 186.5 (C) C-12, 136.4 (C) C-12a, 127.4 (C) C-12b, 21.9 (CH_3) C-13, 68.0 (CH) C-1′, 35.0 (CH_2) C-2, 52.3 (C) C-3′, 69.1 (CH) C-5, 19.1 (CH_3) C-6′, 25.4 (CH_3) C-7. ^1H NMR data (DMSO-d_6): δ mult (*J* Hz) H#: 8.98 d (8.8) H-1, 7.65 d (8.8) H-2, 7.89 s H-4, 8.26 d (8.8) H-5, 8.19 d (8.8) H-6, 7.62 s H-10, 2.53 s H-13, 4.81 brs H-1′, 1.79 d (11.9) H-$2'_{eq}$, 2.09 dd (11.9, 2.5) H-$2'_{ax}$, 3.06 nr H-4′, 3.01 nr H-5′, 1.08 d (5.8) H_3-6′, 1.39 s H_3-7′, 9.72 brs NH. (HMQC, HMBC data are available in the Supporting Information); HRMS (ESI) *m*/*z* 448.1325 $[M + H]^+$ (calcd for $C_{26}H_{23}{}^{35}ClNO_4$, 448.1316).

Treatment of Marmycin A (1) with HCl. To marmycin A (**1**) (0.5 mg) dissolved in Me_2CO (4 mL) was slowly added 6 N HCl (4 mL). The reaction was stirred overnight at RT. Analysis of the reaction mixture by LC-MS showed trace amounts of a second compound which possessed the same mass, halogen isotope patern, UV profile, and retention time as marmycin B (**2**).

X-ray Crystal Structure of Marmycin A (1). A red plate crystal of **1** was obtained by vapor diffusion of *n*-C_5H_{12} into THF. Crystal data: Empirical formula = $C_{26}H_{23}NO_4$, M_r = 413.45, T = 90.0(2) K, crystal system = orthorhombic, space group = $P2_12_12_1$, unit cell dimensions: a = 6.9892(3) Å, b = 11.3987(5) Å, c = 24.2168(11) Å, V = 1929.30(15) Å^3, Z = 4, D_{calc} = 1.423 mg/m^3, λ = 1.54178 Å, μ(Cu Kα) = 0.776 mm^{-1}, F_{000} = 872, crystal size = 0.40 × 0.32 × 0.01 mm^3, theta range for data collection: θ = 3.65–67.90°, index ranges = $-8 \le h \le 8$, $-13 \le k \le 13$, $-29 \le l \le 29$, reflections collected = 25251, independent reflections = 3485 [R_{int} = 0.0548], completeness to theta = 67.90°, 99.5%, absorption correction = semi-empirical from equivalents, maximum and minimum transmission = 0.9923 and 0.7469, respectively. Refinement method: full-matrix least-squares on F^2, data/restraints/parameters = 3485/0/300, S = 0.997, final R indices [$I > 2\sigma(I)$]: $R1$ = 0.0318, w$R2$ = 0.0800; R indices (all data): $R1$ = 0.0340, w$R2$ = 0.0815, flack parameter = −0.08(9), largest difference peak and hole = 0.294 and −0.225 e.Å^{-3}.

Cytotoxicity Measurements for Marmycins A and B (1, 2). *In vitro* cytotoxicity against tumor cell lines was assessed by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2*H*-tetrazolium inner salt) MTS tetrazolium dye conversion assay after 72 h drug exposure as previously described.[27]

Evaluation of Marmycin A (1) Treatment on Apoptosis and Cell Cycle. Flow cytometry was used to assess cell cycle progression using propidium iodide staining for DNA content, while the induction of apoptosis was likewise analyzed using flow cytometry and the TUNEL assay (BD Biosciences) as previously described.[28] Analysis was carried out using a FACScalibur flow cytometer (Beckton and Dickinson).

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Supporting Information Available: Crystallographic information of **1**; ^1H, ^{13}C, and 2D NMR data of **1**; ^1H, HMQC, and HMBC data of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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