Organic LETTERS

Letter

pubs.acs.org/OrgLett

Succinylated Apoptolidins from *Amycolatopsis* sp. ICBB 8242

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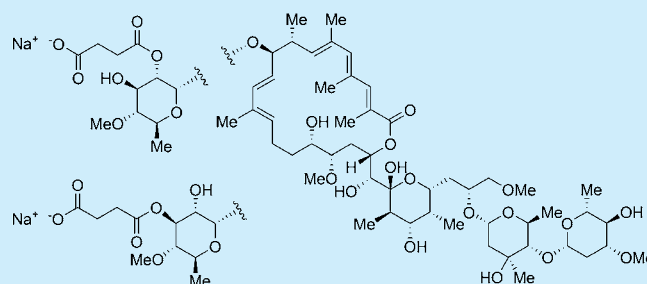
Supporting Information

ABSTRACT: Two new apoptolidins, 2′-*O*-succinyl-apoptolidin A (**11**) and 3′-*O*-succinyl-apoptolidin A (**12**), were isolated from the culture broth of an Indonesian *Amycolatopsis* sp. ICBB 8242. These compounds inhibit the proliferation and viability of human H292 and HeLa cells. However, in contrast to apoptolidin A (**1**), they do not inhibit cellular respiration in H292 cells. It is proposed that apoptolidins are produced and secreted in their succinylated forms and **1** is the hydrolysis product of **11** and **12**.



Apoptolidin A (**1**, Figure 1), the first member of the apoptolidin family, was isolated from the cultures of a *Nocardiopsis* sp. by Seto and co-workers in 1997. Remarkably, this macrolide antibiotic induced apoptosis in rat glia cells transformed with adenovirus E1A oncogene (IC_{50} = 11 ng/mL), but did not show cytotoxicity in normal glia cells (IC_{50} > 100 μg/mL).[1,2] Studies by the National Cancer Institute involving 37 000 substances against the 60 human cancer cell lines concluded that **1** was among the top 0.1% of the most selective cytotoxic agents known at that time.[3] Further mechanistic studies revealed that **1** is an inhibitor of the eukaryotic mitochondrial F_0F_1-ATP synthase, which is associated with its apoptotic activity.[3,4] However, subsequent investigations suggested that the inhibition of mitochondrial F_0F_1-ATP synthase was not the only determinant of the potent antiproliferative activity of **1** and its analogues.[5] We have recently determined that although apoptolidins A and C are potent inhibitors of mitochondrial function they differ from the commonly known ATP synthase inhibitor oligomycin A in the way they trigger acute metabolic stress in several cancer cells.[6]

Following the discovery of **1**, which was isolated as the major compound from culture broths of a *Nocardiopsis* sp. (150 mg/L), various minor apoptolidin analogues (2−7 mg/L), namely apoptolidin B−F (**2**−**6**, Figure 1), have also been identified.[7−9] In addition, during isolation and purification steps, apoptolidins may also undergo various isomerization events. Isoapoptolidin A (**7**, Figure 1), isolated as the second major compound, is believed to be derived from **1** through an acyl migration from C19 to the C20 hydroxy group.[10] On the other hand, apoptolidin G (**10**, Figure 1), which was isolated from the same cultures, may be derived from **1** by a light induced olefin isomerization.[11] In addition to the above natural products and their artifacts, a number of synthetic or semisynthetic derivatives of apoptolidins have also been generated, opening up opportunities for exploring the structure−activity relationships and mechanism of action of this group of natural products.[12−14]

As part of our ongoing drug discovery efforts, we investigated bioactive natural products from microorganisms isolated from a unique black water ecosystem on the island of Borneo in Indonesia. Based on preliminary screenings using mass spectrometry (MS) and a bioactivity assay of extracts, we identified a number of strains of *Amycolatopsis* that produce apoptolidins. Among them is *Amycolatopsis* sp. ICBB 8242, which produces the apoptolidins in high yields. Similar to the original producer *Nocardiopsis* sp., this strain produced apoptolidin A (**1**) and isoapoptolidin A (**7**) as major products, along with apoptolidins B (**2**), C (**3**), and D (**4**) as minor products.[6] Interestingly, MS analysis of the *n*-BuOH extract of this strain showed signals with significant intensity at *m*/*z* 1169 $[M + Na]^+$ and 1251 $[M + Na]^+$, indicating the presence of apoptolidins having molecular masses higher than any known naturally occurring apoptolidin analogues. The *n*-BuOH extract of the culture broth was consecutively subjected to solid phase extraction (SPE) C_{18} column and Sephadex LH-20 column chromatography, followed by HPLC to give two new apoptolidin analogues (compounds **11** and **12**, Figure 2) with an identical *m*/*z* value of 1251 ($[M + Na]^+$), which is 100 atom mass units (amu) higher than that of **1** (*m*/*z* 1151, $[M + Na]^+$). Moreover, another pure compound (**13**) with an *m*/*z* value of 1169 ($[M + Na]^+$), 18 amu higher than **1**, was also isolated. We

Received: April 12, 2015
Published: May 6, 2015

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DOI: 10.1021/acs.orglett.5b01055
Org. Lett. 2015, 17, 2526−2529

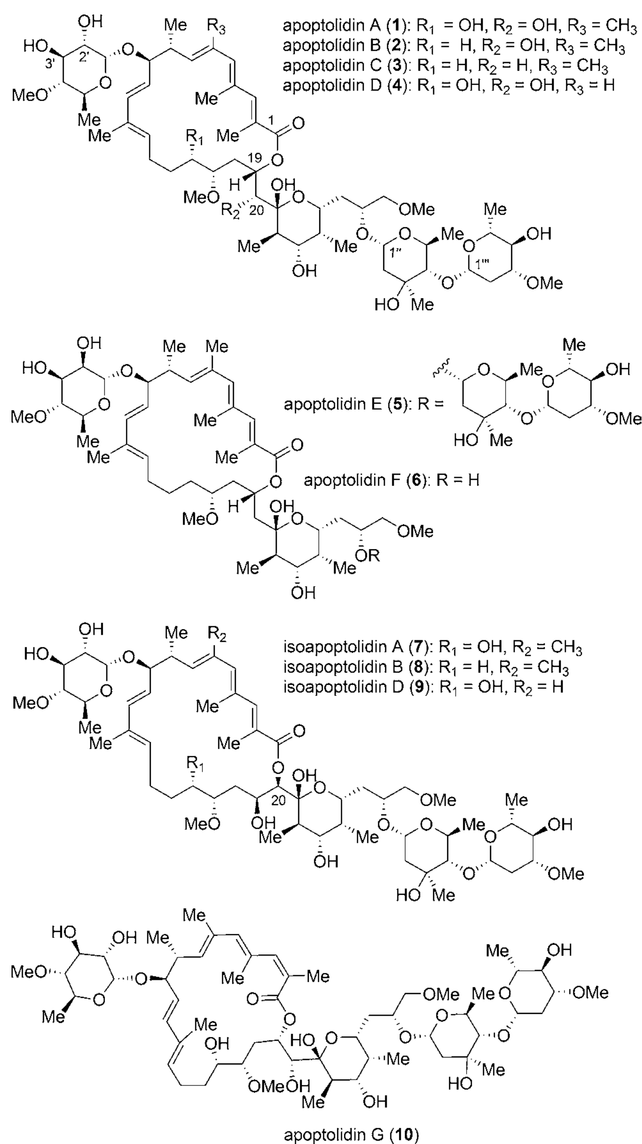

Figure 1. Chemical structures of apoptolidins isolated from *Nocardiopsis* sp. and *Amycolatopsis* sp. ICBB 8242.

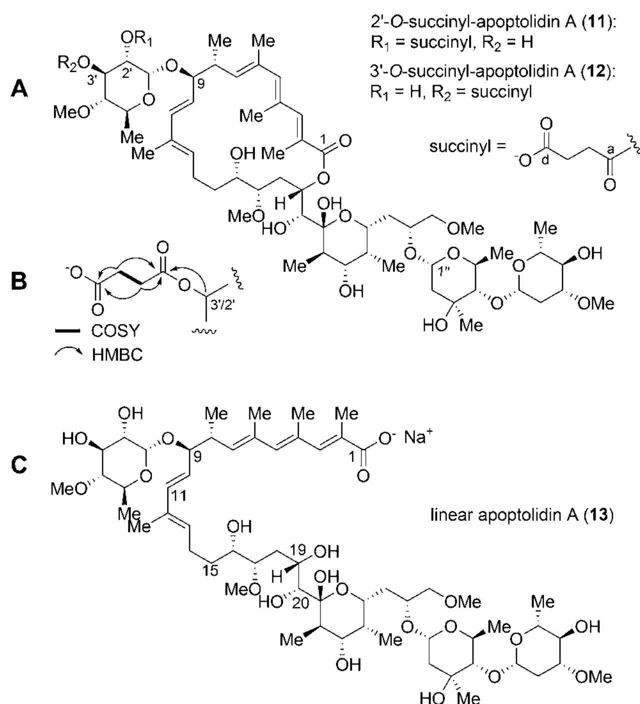

Figure 2. Chemical structures of the new apoptolidin analogues isolated from *Amycolatopsis* sp. ICBB 8242. (A) Structures of succinylated apoptolidins. (B) COSY and HMBC correlations of the succinyl moiety in **11** and **12**. (C) A linear analogue of apoptolidin A (**13**).

discovered that compounds **11** and **12** are relatively sensitive to pH, as they may be hydrolyzed to **1** under HPLC conditions with trifluoroacetic acid containing solvent systems.

The molecular formula of compound **11** ($[\alpha]^{21}_{D}$ −72 (*c* 0.3, MeOH), UV (MeOH) λ_{max} (log ε) 234 (4.46), 319 (4.22) nm) was determined by HR-ESI-MS to be $C_{62}H_{100}O_{24}$ (*m/z* 1251.6539, $[M + Na]^+$; calcd for $C_{62}H_{100}O_{24}Na$ 1251.6497). The ^1H NMR spectrum of **11** is very similar to that of **1** in terms of chemical shifts and coupling constants except that extra resonances were observed around 2.6 ppm. Also similar to the spectrum of **1**, six methyl doublets and five methyl singlets in the aliphatic region, four methoxy singlets, and sugar as well as sp^2 proton signals were observed. The ^{13}C and DEPT-135 spectra of **11** also showed extensive similarity to those of **1**. However, there are three carbonyl carbons present in **11** (δ_C 176.2, 173.9, and 172.8) instead of one carbonyl carbon in **1** (δ_C 172.8) and in other known apoptolidin analogues. Moreover, in addition to seven methylene carbons found in **1**, compound **11** possesses two additional methylene carbons (δ_C 30.2 and 30.1 ppm). These confirmed the presence of 62 carbons in **11**, instead of 58 in **1**. Based on the molecular formula and detailed analysis of two-dimensional (2D) NMR experiments (COSY, HSQC, HMBC, TOCSY, ROESY) compound **11** was determined to be a succinylated apoptolidin A. The two additional carbonyl carbons and two methylene carbons in **11** were assigned to the succinyl moiety. The methylene protons [δ_H 2.69, 2.62, 2.58 (2H) ppm] showed HMBC correlations to both carbonyl carbons (δ_C 176.2, 173.9). The shift of the H-2′ signal from 3.40 ppm in **1** to 4.55 ppm in **11** and the HMBC correlation between H-2′ (δ_H 4.55 ppm) and one of the succinyl carbonyl carbons (δ_C 173.9) indicate that the succinyl moiety is attached to the C-2′ hydroxy group of **1**. This was confirmed by comparisons of the MS/MS fragments of **11** and **1** (Figures S31 and S33 in the Supporting Information).

Compound **12** ($[\alpha]^{21}_{D}$ +17 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε) 235 (4.38), 319 (4.18) nm) has a molecular formula of $C_{62}H_{100}O_{24}$ (*m/z* 1227.6586, $[M - H]^-$; calcd for $C_{62}H_{99}O_{24}$ 1227.6532). The ^1H, ^{13}C and DEPT-135 spectra of **12** are highly similar to those of **11**, including the presence of resonances for three carbonyls (δ_C 177.2, 174.1, 172.8) and nine methylenes. Based on extensive 2D NMR studies, **12** was also identified as a succinylated apoptolidin A. Interestingly, unlike **11**, in which the succinyl moiety is attached to the C-2′ hydroxy group, **12** has the succinyl moiety linked to the C-3′ hydroxy group, as indicated by an HMBC correlation between H-3′ and one of the succinyl carbonyl carbons (δ_C 174.1). Consequently, the resonance for H-3′ appears at 5.26 ppm, deshielded 1.54 ppm from that in **1**. Interestingly, compound **12** has a significantly different specific rotation (+17) than compound **11** (−72). Although the reason for this significant divergence is unclear, it may be due to conformational/rotational differences of the succinylated sugar moieties between the two compounds.

DOI: 10.1021/acs.orglett.5b01055
Org. Lett. 2015, 17, 2526−2529

Compound **13** ($[\alpha]^{21}_{D}$ −180 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 236 (4.41), 288 (4.09) nm) has a molecular formula of $C_{58}H_{98}O_{22}$ (*m*/*z* 1169.6447, $[M + Na]^+$; calcd for $C_{58}H_{98}O_{22}Na$ 1169.6442). The molecular mass of **13** is 18 amu higher than **1**, indicating the formal addition of one water molecule. Based on its ^{13}C and DEPT-135 data, compound **13** has, among others, one carbonyl carbon and 10 olefinic carbons, including four quaternary ones, suggesting that the 18 amu difference was not a result of water addition to a double bond. Detailed analysis of the 2D NMR spectra of **13** revealed significant differences in the proton and carbon resonances at and around the C-19 position. The ^1H NMR signal for H-19 in **13** is 4.32 ppm, shifted from 5.29 ppm in **1**, suggesting that compound **13** lacks an ester functionality at this position. In addition, no HMBC correlation between H-19 and C-1 was observed. Considered together the above data suggest that compound **13** is the ester hydrolysis product of **1**, bearing a hydroxy group at C-19 and a free carboxylic acid moiety at C-1 (isolated as the sodium salt). To confirm the presence of a free carboxylic acid in **13**, the compound was subjected to methylation with trimethylsilyldiazomethane. ESI-MS analysis of the reaction mixture showed a product with an *m*/*z* value of 1183 ($[M + Na]^+$), which is 14 amu higher than **13** ($[M + Na]^+$, 1169), consistent with the addition of a methyl group (Figure S28a, S28b). Compound **1** was also treated with trimethylsilyldiazomethane under the same conditions. However, no product was observed in this reaction, indicating that no other functionalities in **13** can be methylated under the reaction conditions used (Figure S28c). The ^1H NMR spectrum of the purified methylated product of **13** is almost identical to that of **13**, except for the presence of an additional methyl group resonance at 3.76 ppm (Figure S29). Moreover, a significant hypsochromic shift of λ_{max} from 320 nm in **1** to 288 nm in **13** confirmed the distinctive structural backbone of **1**, which has a conjugated ester chromophore, and **13**, which possesses a conjugated acid.

To investigate the biological activities of the new apoptolidins, we tested the ability of the compounds to inhibit the viability of human NCI-H292 (H292) lung and HeLa cervical cancer cells. The viability of H292 and HeLa cells was assessed after continuous exposure to increasing concentrations of the compounds for 6 days, using a 3 day + 3 day strategy to avoid nutrient deprivation.[6] The results show that **11** and **12** can inhibit the viability of both cancer cell lines tested with similar activity; **11** and **12** were 4- to 6-fold less potent than **1**, whereas **13** did not show any activity up to 1 μM (Table 1, Figure S34), suggesting that an intact macrocyclic ring is important for biological activity. We next used a target-based assay to examine the effects of the compounds on mitochondrial function in intact cells. Real-time analysis of cellular respiration in H292 cells (Seahorse XF 24 Analyzer, Seahorse Bioscience, Billerica, MA) showed that only **1** was affecting the oxygen consumption rate (OCR) of living cells following short-term exposure, whereas **11**, **12**, and **13** produced no change in cellular respiration (Figure 3). Although the result for the linear analogue **13** was consistent with that of the cell viability assays, the lack of activity of **11** and **12** was seemingly contradictory to their activity at nanomolar concentrations in standard end point assays and potentially indicative of an alternate biological mechanism of action.

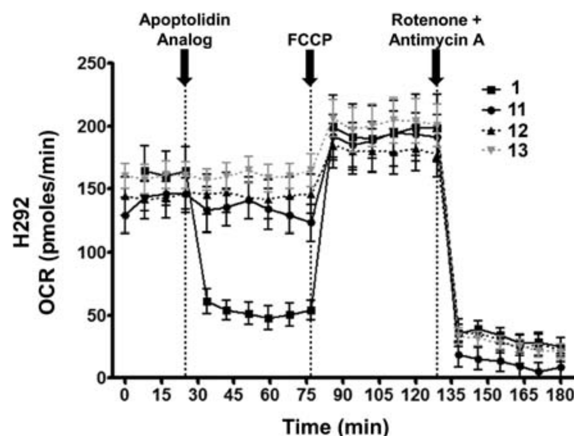

Figure 3. Real-time analysis of cellular respiration in human H292 lung cancer cells in response to apoptolidins plus target-specific inhibitors of the electron transport chain. OCR, oxygen consumption rate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone.

In light of the relatively unstable nature of compounds **11** and **12**, we hypothesized that hydrolysis of **11** and **12** to the more active product **1** may have occurred during the time course of the cell viability assay, and the observed activities were not for **11** and **12** but for the hydrolytic product **1**. To test this hypothesis, we incubated compounds **11** and **12** individually in RPMI-1640 mammalian cell culture medium in a humidified cell culture incubator at 37 °C with 5% CO_2 and analyzed their potential conversion to **1** by ESI-MS after 2 and 72 h. We observed a significant conversion (up to 30%) of the compounds to **1** after 72 h of incubation but not at 2 h (Figures S35). Taken together our findings indicate that **11** and **12** are not acute inhibitors of mitochondrial function. However, as hydrolysis is anticipated to be higher inside the cells due to nonspecific cellular esterase enzymes,[15] it is likely that **1** contributes to the antiproliferative activities of **11** and **12** and that **11** and **12** may act as prodrugs of **1**.

The production of succinylated apoptolidins by *Amycolatopsis* sp. ICBB 8242 suggests that succinylation may play a role in self-resistance and/or as an export mechanism. In addition to compounds **11** and **12** (*m*/*z* 1251, $[M + Na]^+$), we observed other minor metabolites with *m*/*z* values consistent with those for succinylated apoptolidin analogues, such as succinylated apoptolidin B (*m*/*z* 1235 $[M + Na]^+$) and a related succinylated apoptolidin (*m*/*z* 1221 $[M + Na]^+$) in the ESI-MS spectrum of the EtOAc extract of the culture broths (Figure S36). This observation further suggests that all known natural apoptolidins may be produced and secreted in their succinylated forms and then hydrolyzed to the apoptolidins during fermentation and/or the isolation process. To test this hypothesis, we produced cell-free extracts of *Amycolatopsis* sp. ICBB 8242 by treating the cells (from 5 day cultures) with lysozyme followed by sonication and centrifugation to remove

Table 1. Effect of Apoptolidin A (1) and the New Analogues (11−13) on the Viability of Human Cancer Cell Lines

compound	H292 cell IC_{50} (nM)	HeLa cell IC_{50} (nM)
apoptolidin A (**1**)	22	39
2′-*O*-succinyl-apoptolidin A (**11**)	91	240
3′-*O*-succinyl-apoptolidin A (**12**)	82	260
linear apoptolidin A (**13**)[a]	NA	NA

[a]Compound **13** was not active up to 1 μM.

DOI: 10.1021/acs.orglett.5b01055
Org. Lett. 2015, 17, 2526−2529

the cell debris. The cell-free extracts were lyophilized and extracted with MeOH. ESI-MS analysis of the MeOH extracts revealed the presence of **11** and/or **12** as predominant products (Figure S37), supporting the notion that natural apoptolidins are produced and secreted in their succinylated forms by *Amycolatopsis* sp. ICBB 8242. Inspection of the biosynthetic gene cluster of the apoptolidins in *Nocardiopsis* sp. FU40 did not provide clear indication of the presence of genes that may encode proteins for the succinylation reaction.[16] Therefore, the question of whether or not succinylation occurs in the *Nocardiopsis* producer, and if this modification is important in self-resistance and/or secretion, remains unclear and warrants further investigation.

■ ASSOCIATED CONTENT

Supporting Information

General experimental procedures, NMR data table, NMR and MS spectra of **1** and **11−14**, cytotoxicity assay. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01055.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the OSU College of Pharmacy General Research Funds.

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DOI: 10.1021/acs.orglett.5b01055
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