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Synthesis and anticancer activity of polyhydroxylated 18-membered analogue of antimycin A_3

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ABSTRACT

A novel polyhydroxylated 18-membered analogue of antimycin A_3 was synthesized. Our synthesis commenced with Boc-L-threonine and was achieved by way of one-pot homocoupling/ring-closing olefin metathesis reaction cascade and Sharpless asymmetric dihydroxylation. The analogue exhibited a greater anticancer activity against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells compared to the original antimycin A_3.

1. Introduction

The search for new anticancer agents has become a major challenge for current research in medicinal chemistry, especially with an increase in the incidence of cancer drug resistance exhibited by tumor cells that seriously reduce the effectiveness of anticancer therapy.[1,2] The recent drug discovery process shows that useful anticancer drugs were derived not only from natural sources but also came from synthetic chemistry processes.[3] Considering that research and development of new synthetic anticancer drugs with complex structures is difficult, expensive, and time consuming, analogue synthesis of anticancer drugs has emerged as a promising strategy to seek new anticancer agents that are more effective and responsive in anticancer therapy. Analogue synthesis can have two advantages. First, it has short duration and high probability of yielding more active compounds by modifying the structure. Second, it gives information about the structure–activity relationship between the analogue and the original compound.

Antimycin A_3 was isolated from *Streptomyces* sp. in 1949 and in later, this was revealed as mixture of two molecules, A_{3a} and A_{3b}.[4] Antimycin A_3 is one of the most active agents that inhibits the electron transfer activity of ubiquinol-cytochrome *c* oxidoreductase and prevents the growth of human cancer cells. Antimycin A_3 was also found to induce apoptosis of cancer cells by selectively killing cancer cells that expressed high levels of anti-apoptotic Bcl-2 and Bcl-$_{XL}$.[5–8] The structure of antimycin A_3 consisting of a nine-membered dilactone is similar to the antifungal antibiotic UK-3A that was also isolated from *Streptomyces* sp. in 1997.[9,10] We recently reported the synthesis of novel 2-hydroxynicotinoyl-serine-butyl esters related to antibiotic UK-3A, which demonstrated significant activity as a growth inhibitor of *Bacillus subtilis* and *Staphylococcus aureus*.[11] In this research, we focused on the synthesis of antimycin A_3 analogue (**1**) and examined its anticancer activity (Fig. 1). The total and formal syntheses of antimycin A_3 have been accomplished by some researchers,[4,12] however, studies on its analogue synthesis by specifically modifying the dilactone core are still limited. Therefore, in this work, we carried out the synthesis of antimycin A_3 analogue, which are more focus on the modification of the nine-membered dilactone core. Studies on the structure–activity relationship of antimycin A_3 by Miyoshi et al. in 1995 revealed that the hydroxyl group and 3-formamide group in the salicylyl moiety, as well as the amide bond were necessary for

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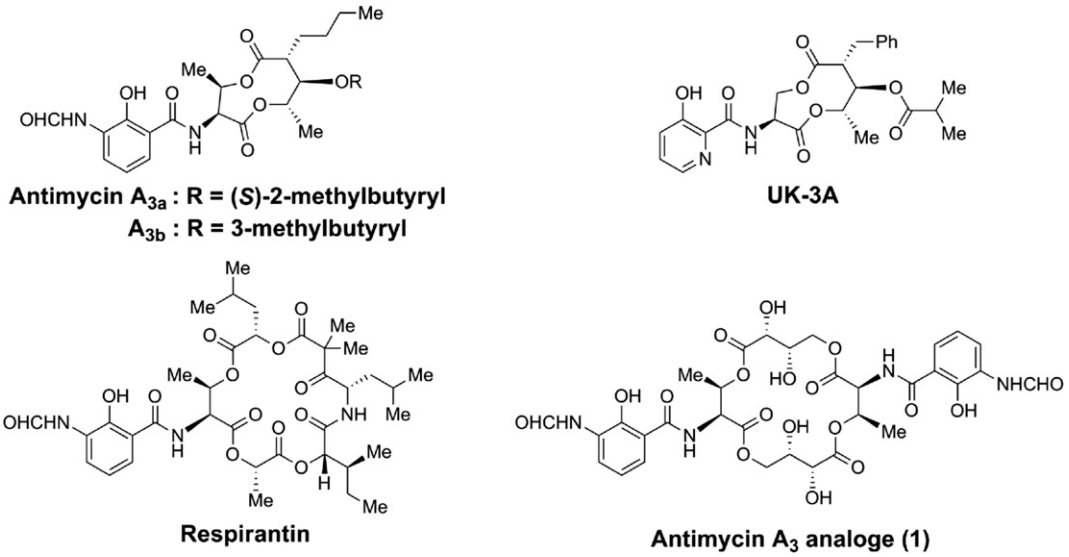
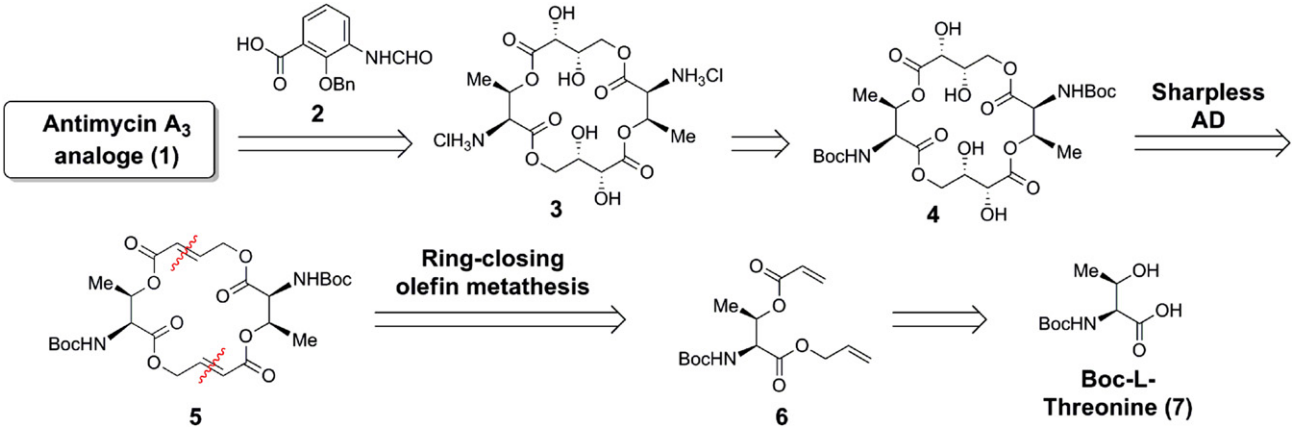


Fig. 1. Structure of antimycin A_3, UK-3A, respirantin, and desired antimycin A_3 analogue (**1**).

anticancer activity. On the other hand, the nine-membered dilactone core in antimycin A_3 was found less effective for anticancer activity than 3-formamidosalicylyl moiety.[13] These results indicated that, it is quite possible to carry out the synthesis of antimycin A_3 analogue by replacing the nine-membered dilactone core with another active core that contributes to the improvement of its anticancer activity. Therefore in this work, the nine-membered dilactone core of antimycin A_3 was replaced by a polyhydroxylated 18-membered tetralactone core in our analogue. It has been reported that the 18-membered ring of polylactone, such as respirantin, which has a similar structure with antimycin A_3 showed potent antitumor activity against human cancer cell lines with IC_{50} range 0.00018–0.47 μg/mL. Furthermore, respirantin with IC_{50}: 0.0037 μg/mL, showed a stronger cytotoxicity than antimycin A_3 (IC_{50}: 0.015 μg/mL) against mouse Leukemia P-388 cells.[14] It is also known that the presence of hydroxyl groups in biologically active compound significantly increases their biological activity due to the enhancement of solubility in water, which is the one of important factors influencing the efficacy of drugs.[3,15] Thus, the polyhydroxylated 18-membered tetralactone core in our analogue is expected to be active as an anticancer agent. Moreover, introduction of the 3-formamidosalicylyl group into this core, is expected to synergistically improve its anticancer activity compared to that of the original compound antimycin A_3. The retrosynthetic analysis of the antimycin A_3 analogue is outlined in Scheme 1. As shown, analogue **1** can be built by amidation of tetraol **3** with 3-formamidobenzoic acid moiety (**2**), followed by hydrogenolysis of the benzyl group. Tetraol **3** can be prepared from Boc deprotection of **4**, whereby **4** can be derived from 18-membered tetralactone **5** by way of Sharpless dihydroxylation.[16,17] Tetralactone **5** can be constructed from one-pot homocoupling ring-closing olefin metathesis[18,19] of diester **6**, and **6** can be synthesized from two-step esterification of commercially available Boc-L-threonine (**7**).

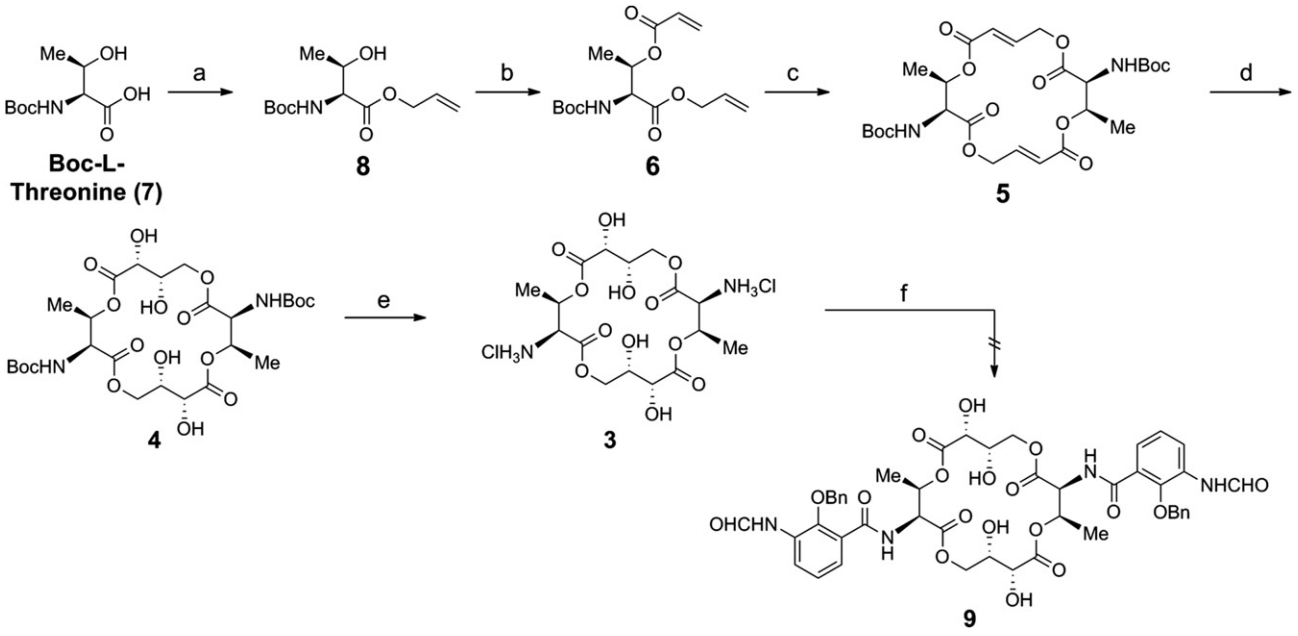


Scheme 1. Retrosynthetic analysis of antimycin A_3 analogue (**1**).

2. Results and discussion

2.1. Synthesis of tetraol 3

Scheme 2 outlines the synthesis of 18-membered tetraol **3**. Starting from esterification of Boc-L-threonine (**7**) with allyl bromide under basic condition, the synthesis was conducted according to Wu et al.,[20] and afforded Boc-L-threonine allyl ester (**8**) in 96%



Scheme 2. Synthesis of 18-membered tetraol **3**. Reagents and conditions: (a) Allyl bromide, Na_2CO_3, DMF, rt (96%); (b) Acrylic acid, DIC (diisopropyl carbodiimide), DMAP, CH_2Cl_2, 0 °C to rt (92%); (c) 4 mol % Zhan catalyst 1B, CH_2Cl_2, 45 °C (73%); (d) OsO_4, $K_3Fe(CN)_6$, K_2CO_3, $NaHCO_3$, $(DHQ)_2PHAL$, $MeSO_2NH_2$, *t*-BuOH/H_2O, 0 °C (61%); (e) concd HCl, EtOAc, rt (81%); (f) **2**, DIC/DMAP or EDCI/DMAP or EDCI/HOBt or EDCI/HOSu, NMM.

yield. Condensation of ester **8** with acrylic acid employing the combination of DIC and DMAP occurred easily to afford 92% yield of diester **6**. In the next step, Ru-catalyzed homocoupling ring-closing metathesis (RCM)[18,19] was used to transform diester **6** into an 18-membered tetralactone **5**. Three kinds of Ru-catalyst, Grubbs first generation, Grubbs second generation, and Zhan catalyst 1B,[21] were tested in this metathesis reaction (Fig. 2). Homocoupling RCM of diester **6** with the less reactive Grubbs first catalyst failed to form tetralactone **5**, while the use of the more reactive Grubbs second catalyst provided **5** in only 32% yield. The best result, 73% yield of **5** was achieved by performing the reaction with 4 mol % of Zhan catalyst 1B in concentration 0.1 M at 45 °C. Subsequently, Sharpless asymmetric dihydroxylation of **5** with OsO_4, $(DHQ)_2PHAL$ ligand, and $K_3Fe(CN)_6$ as a co-oxidant gave 67% yield of a 12:1 diastereomeric mixture of tetraol products. This mixture could be separated by medium pressure liquid chromatography to afford 61% yield of pure major tetraol **4**, which was then treated with with concd HCl in EtOAc to afford the polyhydroxylated 18-membered tetralactone **3** in 81% yield as a white solid. The X-ray crystallographic analysis of **3**[22] clarified its stereochemistry (Fig. 3A).

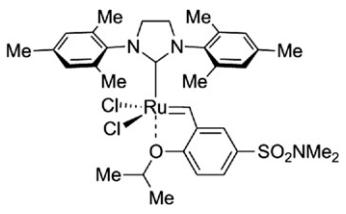


Fig. 2. Structure of Zhan catalyst 1B.

With key intermediate **3** in hand, our subsequent plan was to conduct the amidation of tetraol **3** with benzylated 3-formamidobenzoic acid **2**, which was prepared according to Pettit et al.,[14] to give tetraol diamide **9**. Unfortunately, after extensive attempts with a variety of amidation reagents, such as DIC/DMAP, EDCI/DMAP, EDCI/HOBt, and EDCI/HOSu under many reaction conditions, the desired tetraol diamide **9** was not obtained (step f in Scheme 2). We postulated that not only the amine groups but also four hydroxyl groups of **3** can react with carboxylic acid **2**, and might cause byproducts instead of the desired tetraol diamide **9**. To solve this problem, we abandoned this strategy, and proposed a new one.

2.2. Synthesis of tetralactone 11 and analogue 1

The revised synthetic strategy for the synthesis of antimycin A_3 analogue **1** is summarized in Scheme 3. We planned to carry out the amidation of non-hydroxylated tetralactone **11** with acid **2** to give diamide **10** in the initial stage, and then introduce the hydroxyl groups into diamide **10** by Sharpless asymmetric dihydroxylation followed by deprotection of benzyl groups in the late stage, to generate desired the analogue **1**. With this method, the amidation of non-hydroxylated tetralactone **11** with acid **2** was expected to proceed well without the formation of byproducts, which might be obtained from the esterification of a polyhydroxylated tetralactone with acid **2**. The non-hydroxylated tetralactone **11** derived from Boc deprotection of 18-membered tetralactone **5**, which we had already prepared in the former strategy.

As shown in Scheme 4, tetralactone **11** as diammonium dichloride salts was formed from Boc deprotection of **5** with concd HCl. Amidation of **11** with acid **2** could be performed using the base NMM and the combination of EDCI/HOBt in THF as a solvent afforded diamide **10** in 45% yield. In the next step, Sharpless dihydroxylation of **10** in the presence of 6 equiv of NMO with 50 mol % of both OsO_4 and $(DHQ)_2PHAL$ proceeded smoothly to give tetraol diamide products in 66% yield as a 4:1 inseparable diastereomeric mixture, with **9** as a major diastereomer. As the final step, hydrogenolysis of this diastereomeric mixture with 10% Pd/C resulted in simultaneous cleavage of both Bn group, and afforded a 4:1 diastereomeric mixture of the corresponding Bn deprotected tetraol diamide products, which was successfully separated in this step by medium pressure liquid chromatography to give a pure major

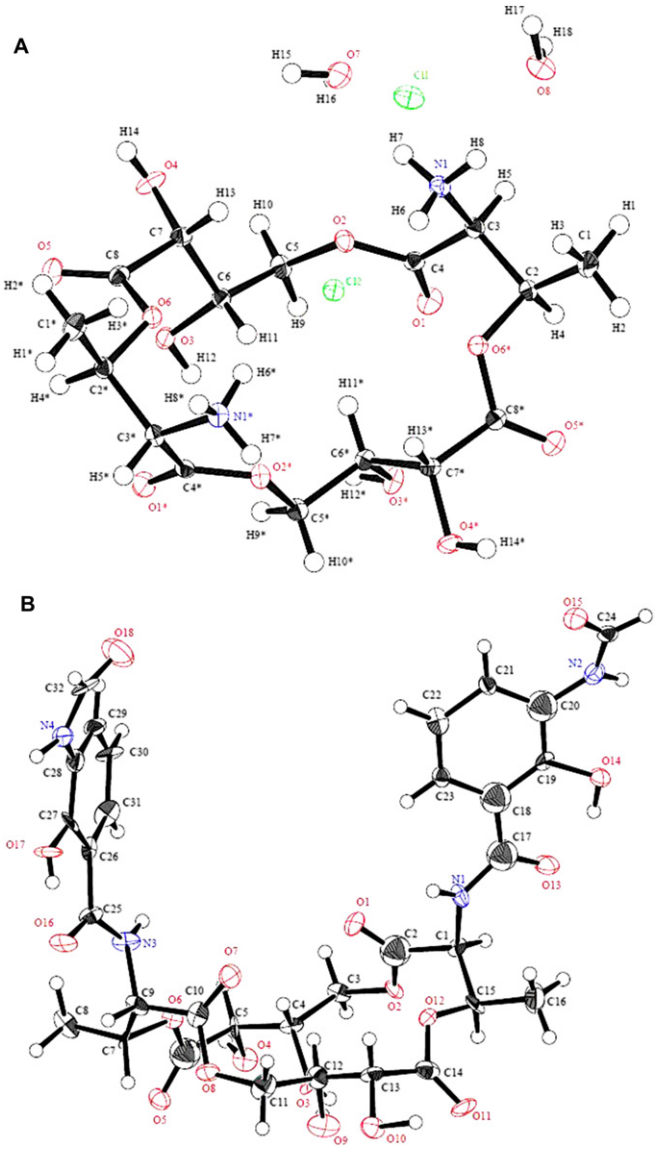


Fig. 3. (A) ORTEP drawing of **3**·2H_2O, (B) ORTEP drawing of the analogue **1**.

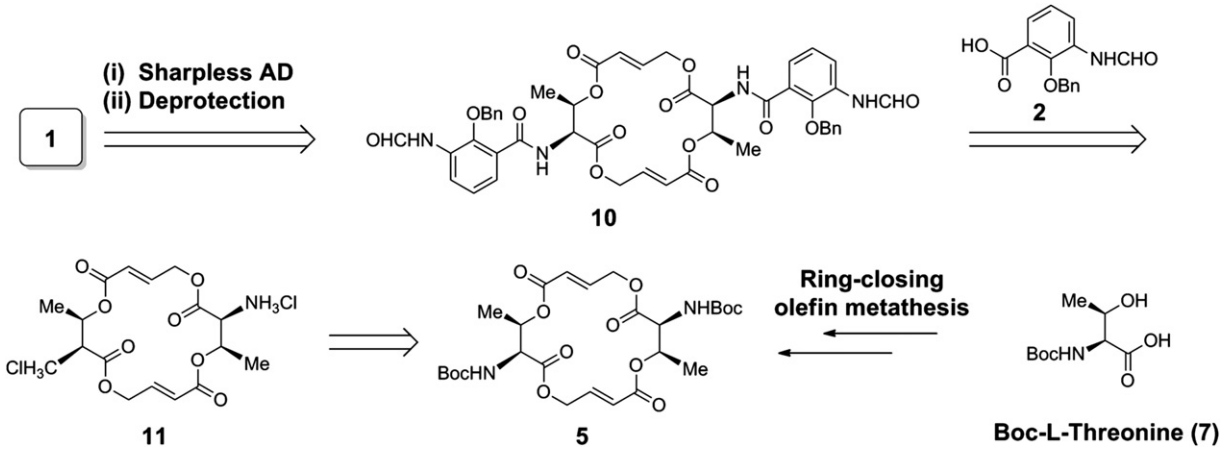
analogue **1** in 51% yield. Subsequently, the analogue **1** could be recrystallized readily by methanol. The X-ray crystallographic analysis of **1**[23] has its stereochemistry assigned, as shown in Fig. 3B.

2.3. Cytotoxicity of tetralactone 11, tetraol 3, and analogue 1

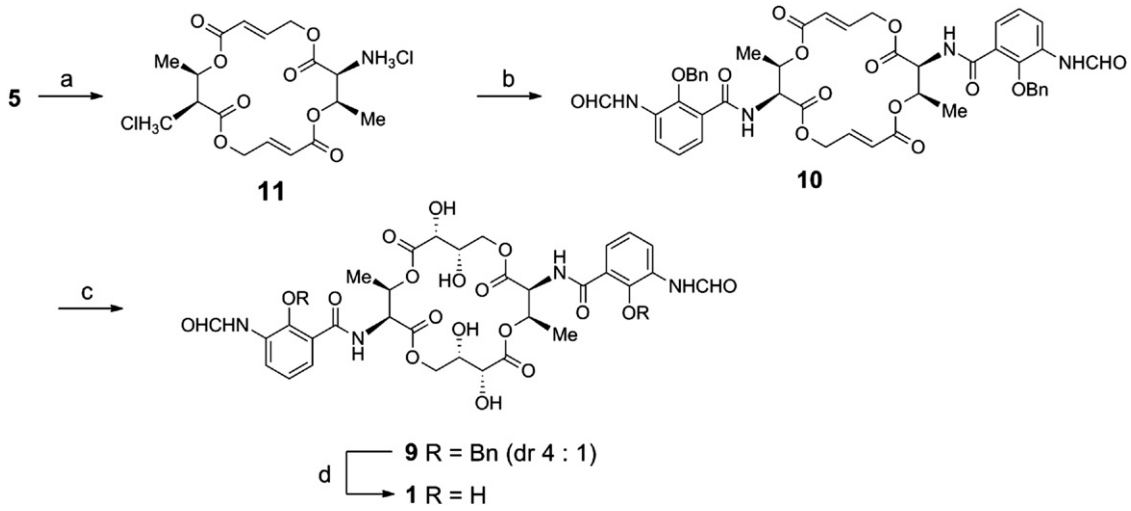
After completion of the synthesis, cytotoxicities of the analogue **1** and two intermediate products, 18-membered ring tetraol **3** and 18-membered ring tetralactone **11** were evaluated as inhibitors of cancer cell growth versus HeLa cells, breast cancer MDA-MB-231 cells, and prostate cancer PC-3 cells (Table 1). Tetralactone **11** with IC_{50} over 4000 μM showed no cytotoxicity against all tested cancer cells. In contrast to tetralactone **11**, tetraol **3**, which has four hydroxyl groups showed the cytotoxicity in concentration 250 μM, 112 μM, and 205 μM against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells, respectively. The cytotoxicity of tetraol **3** was greatly improved by the presence of the hydroxyl groups compared to that of tetralactone **11**. This suggested that the hydroxyl groups are very important for the anticancer activity. Compared to analogue **1**, tetraol **3**, which does not possess 3-formamidosalicylyl group, showed less cytotoxicity, suggesting that the introduction of 3-formamidosalicylyl group on 18-membered ring tetraol moiety in analogue **1** was potentially responsible for the increase in its anticancer activity. Moreover, analogue **1**, which contains a polyhydroxylated 18-membered ring core and two parts of 3-formamidosalicylyl groups exhibited greater anticancer activity than that of the original antimycin A_3 on all cancer cells tested, with IC_{50}: 39, 40, and 43 μM against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells, respectively. These results indicated that introducing two parts of formamidosalicylyl groups and replacing the nine-membered dilactone ring of antimycin A_3 with a polyhydroxylated 18-membered ring in analogue **1** successfully improved its anticancer activity. Thus, analogue **1**, which strongly inhibited the growth of HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells, should be considered as a promising candidate for the treatment of human cervical, breast, and prostate cancers.

3. Conclusion

In conclusion, we synthesized a novel analogue of antimycin A_3 (**1**) from Boc-L-threonine through olefin metathesis cascade. Analogue **1** showed greater anticancer activity against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells compared to the original antimycin A_3.



Scheme 3. The revised retrosynthetic analysis of antimycin A_3 analogue (**1**).



Scheme 4. Completion of the synthesis of antimycin A_3 analogue (**1**). Reagents and conditions: (a) concd HCl, EtOAc, rt (90%); (b) **2**, EDCI, HOBt, NMM, THF, rt (45%); (c) OsO_4, NMO, $(DHQ)_2PHAL$, *t*-BuOH/THF/H_2O, rt (66%, 4:1 dr); (d) 10% Pd/C, H_2, MeOH, rt (51%).

Table 1
Cytotoxicities (IC_{50}, μM) of tetralactone **11**, tetraol **3**, analogue **1**, and antimycin A_3 against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells

Compound	Cytotoxicity (IC_{50}, μM)		
	HeLa	Breast MDA-MB-231	Prostate PC-3
Tetralactone **11**	4514	4289	4002
Tetraol **3**	250	112	205
Analogue 1	**39**	**40**	**43**
Antimycin A_3	60	79	50

The values in bold represent the best results for the analogue compound.

4. Experimental section

4.1. General experimental methods

Unless otherwise noted, all reactions were performed in an oven-dried glassware, sealed with a rubber septum under nitrogen atmosphere. Anhydrous THF, CH_2Cl_2, and toluene were purchased from Kanto Chemical Co., DMF and *tert*-butyl alcohol were distilled prior to use. Methanol, ethyl acetate, *n*-hexane, chloroform, Et_2O, and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries. Grubbs catalysts and Zhan catalyst 1B were purchased from Stream Chemicals. Boc-L-Threonine, allyl alcohol, allyl bromide, acrylic acid, *p*-toluenesulfonic acid monohydrate, $NaHCO_3$, K_2CO_3, $K_3Fe(CN)_6$, DMAP (*N*,*N*-4-dimethylaminopyridine), NMM (*N*-methylmorpholine), HOBt (1-hydroxybenzo-triazole), HOSu (*N*-hydroxysuccinimide), EDCI (1-ethyl-3-(3-diaminopropyl) carbodiimide hydrochloride), MeI, Benzylbromide, formamide, and OsO_4 (osmium (VIII) oxide) were obtained from Wako Pure Chemical industries. 3-Aminosalicylic acid was obtained from Tokyo Chemical Industries Co. Ltd. DIC (diisopropyl carbodiimide), $(DHQ)_2PHAL$, $(DHQD)_2PHAL$, and antimycin A_3 were purchased from Sigma–Aldrich Chemical Company. Flash column chromatography was carried out using Merck silica gel 60 (spherical/40–63 μm). Reactions and chromatography fractions were analyzed employing precoated silica gel 60 F_{254} plates (Merck). Compounds were visualized using an ultraviolet lamp (254 nm) and/or by staining with ninhydrin (in EtOH), *p*-anisaldehyde (in EtOH), and ammonium molybdate (in 10%H_2SO_4). All melting points were determined on Yanaco micro melting point apparatus and uncorrected. IR spectra were measured on a JASCO FT/IR-420 spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded on JEOL JNM-ECP500 (500 MHz) spectrometers with tetramethylsilane (δ 0), $CHCl_3$ (δ 7.26), CH_3OH (δ 3.30) or DMSO (δ 2.49) as an internal standard. Mass spectra were recorded on Shimadzu GC–MS QP-5000 or JEOL JMS-AX 700 spectrometers. X-ray crystallography was performed on Rigaku RAXIS-RAPID imaging plate diffractometer. Specific rotation, $[\alpha]_D$, were measured on JASCO DIP-1000 digital polarimeter. Cytotoxicity evaluations of the synthesized products were carried out in Molecular Oncology Laboratory of the Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST). The tested cancer cells: HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells are the culture collections of Molecular Oncology Laboratory, Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST).

4.2. Boc-L-threonine-allyl ester 8

To a stirred solution of Boc-L-Threonine (2.0 g, 9.12 mmol) in DMF (50 mL) was added Na_2CO_3 (1.93 g, 4.56 mmol), followed by allyl bromide (0.93 mL, 10.94 mmol) and water (1.6 mL). The resulting mixture was stirred for 40 h at rt. The solvent was removed from the reaction mixture in vacuo and water (50 mL) was added. The aqueous layer was extracted with EtOAc (3×50 mL) and the combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo, followed by purification by column chromatography on silica gel (gradient elution 20:1 to 18:2, hexane/EtOAc) to give ester **8** (2.09 g, 96%) as colorless liquid. R_f 0.63 (1:1 hexane/EtOAc); IR (neat) 3440, 2979, 2935, 2362, 1718, 1507, 1367, 1165, 1067, 989 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 5.92–5.85 (m, CH=CH_2, 1H), 5.34–5.22 (m, Thr-NH+CH=CH_2, 3H), 4.65–4.63 (m, OCH_2CH=, 2H), 4.29–4.24 (m, Thr-Hα+Thr-Hβ, 2H), 2.13 (br s, Thr-OH, 1H), 1.43 (s, *t*-Bu, 9H), 1.23 (d, J=6.2 Hz, CH_3, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.3, 156.3, 131.6, 118.7, 80.0, 68.0, 66.0, 59.0, 28.3, 19.9. HRMS FAB^+ calcd for $C_{12}H_{22}NO_5$ $[M+H]^+$: 260.1498, found: 260.1506.

4.3. Diester 6

To a solution of ester **7** (0.2 g, 0.77 mmol) in dry CH_2Cl_2 (15 mL) was added acrylic acid (0.16 mL, 2.31 mmol) and DIC (0.36 mL, 2.31 mmol) stirred at 0 °C. The mixture was stirred at the same temperature 0 °C for 1 h before DMAP (9.4 mg, 0.077 mmol) was introduced. The stirring mixture was then continued at 0 °C for 6 h and then allowed to warm to rt until TLC showed disappearance of the starting material Boc-L-Threonine allyl ester (24 h). The reaction mixture was diluted with Et_2O (20 mL), filtered and evaporated in

vacuo. The crude product was purified by flash column chromatography on silica gel with hexane/EtOAc 20:1 to afford diester **6** (222.7 mg, 92%) as colorless oil. R_f 0.63 (2:1 hexane/EtOAc); IR (neat) 3359, 2980, 2938, 1725, 1637, 1507, 1406, 1367, 1267, 1168, 1063, 987 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 6.38 (d, J=17.7 Hz, 1H), 6.06 (dd, J=17.1 and 10.4 Hz, 1H), 5.87–5.83 (m, 2H), 5.49 (m, 1H), 5.31 (d, J=17.1 Hz, 1H), 5.23 (d, J=10.4 Hz, 2H), 4.61 (m, 2H), 4.50 (dd, J=9.8 and 2.4 Hz, 1H), 1.47 (s, 9H), 1.35 (d, J=6.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.8 (s), 164.8 (s), 155.8 (s), 131.5 (t), 131.4 (d), 127.9 (d), 119.1 (t), 80.3 (s), 70.9 (d), 66.3 (t), 57.3 (d), 28.3 (q), 16.9 (q); HRMS FAB$^+$ calcd for $C_{15}H_{24}NO_6$ $[M+H]^+$: 314.1604, found: 314.1606.

4.4. 18-Membered tetralactone 5

A round-bottomed flask was charged with diester **6** (0.25 g, 0.80 mmol) dissolved in CH_2Cl_2 (8 mL). To this solution Zhan catalyst 1B (23.4 mg, 4 mol %) was added. The mixture was refluxed under nitrogen atmosphere for 21 h. After reaction completed, the solvent was evaporated and the crude was purified by column chromatography on silica gel (gradient elution 20:1 to 15:5, hexane/EtOAc) gave tetralactone **5** (0.17 mg, 73%) as colorless liquid. R_f 0.35 (2:1 hexane/EtOAc); IR (neat) 3383, 2980, 2937, 1729, 1665, 1504, 1453, 1368, 1308, 1278, 1165, 1065, 987 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 6.88 (dt, J=15.9 and 7.3 Hz, 1H), 5.90 (d, J=15.9 Hz, 1H), 5.52 (m, 1H), 5.23 (d, J=9.8 Hz, 1H), 4.83 (s, 2H), 4.59 (d, J=9.8 Hz, 1H), 1.50 (s, 9H), 1.34 (d, J=6.7 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.1 (s), 164.1 (s), 155.8 (s), 141.8 (d), 122.2 (d), 80.6 (s), 71.4 (d), 62.9 (t), 57.3 (d), 28.3 (q), 16.3 (q); HRMS FAB$^+$ calcd for $C_{26}H_{38}N_2O_{12}Na$ $[M+Na]^+$: 593.2322, found: 593.2327.

4.5. Tetraol 4

To a stirred solution of tetralactone **5** (0.1 g, 0.18 mmol) in t-BuOH/H_2O (1:1) was added $K_3Fe(CN)_6$ (0.28 g, 0.90 mmol), K_2CO_3 (0.12 g, 0.90 mmol), $NaHCO_3$ (0.074 g, 0.90 mmol), $MeSO_2NH_2$ (25 mg, 0.27 mmol), and $(DHQ)_2PHAL$ (54.5 mg, 40 mol %). The resulting mixture was stirred at rt for 5–10 min and then this solution was cooled to 0 °C. To this solution was added OsO_4 (18 mg, 40 mol %), the resulting solution was stirred at 0 °C and monitoring by TLC until disappearance of starting material **5** (6 h). The reaction was quenched by addition Na_2SO_3 and H_2O. The resulting mixture was extracted by CH_2Cl_2. The combined CH_2Cl_2 layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was then purified by column chromatography on silica gel (gradient elution 100:0 to 97:3, $CHCl_3$/CH_3OH) and gave a diastereomeric mixture of tetraol products (75.1 mg, 67%), which were separated by medium pressure liquid chromatography and afforded major diastereomer **4** (68.3 mg, 61%) as a white solid. R_f 0.61 (5:1 $CHCl_3$/CH_3OH); mp 112–115 °C; IR (KBr disc) 3845, 2989, 1731, 1668, 1493, 1365, 1302, 1278, 1091, 1065 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 5.39 (dq, J=6.1 and 1.8 Hz, 1H), 5.33 (d, J=9.8 Hz, 1H), 4.58 (dd, J=9.8 and 1.8 Hz, 1H), 4.48 (m, 2H), 4.31 (s, 1H), 4.14 (t, J=12.2 Hz, 1H), 4.05 (m, 2H), 3.80 (d, J=8.6 Hz, 1H), 1.48 (s, 9H), 1.41 (d, J=7.3 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.6 (s), 170.1 (s), 155.9 (s), 80.6 (s), 74.6 (d), 70.8 (d), 69.2 (d), 66.5 (t), 56.7 (d), 28.3 (q), 16.4 (q); HRMS FAB$^+$ calcd for $C_{26}H_{42}N_2O_{16}Na$ $[M+Na]^+$: 661.2432, found: 661.2437.

4.6. Tetraol 3

A round-bottomed flask was charged with tetraol **4** (0.16 g, 0.24 mmol) dissolved in EtOAc (10 mL). 35% (w/v) of HCl (0.63 mL, 6.0 mmol) was added into this solution and the mixture was stirred at rt for 12 h. After reaction was complete, the solvent was evaporated in vacuo, and the crude was purified by flash column chromatography on silica gel (gradient elution 10:1 to 6:4, $CHCl_3$/CH_3OH) gave an ammonium chloride salt of **3** (101 mg, 81%) as a white solid. Subsequently, the white crystalline solid of **3** was obtained as dihydrate after recrystallization from methanol/EtOAc. R_f 0.12 (3:1 $CHCl_3$/CH_3OH); mp 126–130 °C; IR (KBr disc) 3432, 2979, 1750, 1509, 1455, 1392,1369, 1278, 1209, 1165, 1131, 1091, 1063 cm^{-1}; ^1H NMR (500 MHz, CD_3OD): δ 5.38 (d, J=6.1 Hz, 1H), 4.54 (d, J=11.0 Hz, 1H), 4.43 (s, 1H), 4.31–4.25 (m, 3H), 4.16–4.12 (m, 2H), 1.45 (d, J=6.1 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD): δ 171.9 (s), 168.4 (s), 72.0 (d), 70.6 (d), 69.0 (t), 57.3 (d), 49.8 (d), 16.2 (q); HRMS FAB$^+$ calcd for $C_{16}H_{26}N_2O_{12}Na$ $[(M-72)+Na]^+$: 461.1383, found: 461.1375; $[\alpha]_D^{30}$ −9 (c 1.0, CH_3OH).

4.7. Tetralactone 11

To a stirred solution of tetralactone **5** (0.53 g, 0.92 mmol) in EtOAc (25 mL) was added 35% (w/v) of HCl (1.44 mL, 13.8 mmol) and the mixture was stirred at rt for 10 h. After reaction was complete, the solvent was evaporated and the crude was purified by column chromatography on silica gel (gradient elution 6:1 to 3:1, $CHCl_3$/CH_3OH) gave diammonium chloride salts of **11** (0.37 g, 90%) as a white solid. R_f 0.38 (5:1 $CHCl_3$/CH_3OH); mp 187–190 °C; IR (KBr disc) 3440, 2926, 1755, 1664, 1631, 1517, 1438, 1391, 1311, 1179, 1031 cm^{-1}; ^1H NMR (500 MHz, CD_3OD): δ 6.95–6.88 (m, 1H), 6.15 (d, J=15.9 Hz, 1H), 5.63 (dq, J=9.8 and 2.4 Hz, 1H), 5.13–4.80 (m, 2H), 4.52 (d, J=2.4 Hz, 1H), 1.43 (d, J=7.3, 3H); ^{13}C NMR (125 MHz, CD_3OD): δ 167.4 (s), 165.1 (s), 143.0 (d), 124.2 (d), 69.3 (d), 65.1 (t), 57.7 (d), 16.3 (q); HRMS FAB$^+$ calcd for $C_{16}H_{23}N_2O_8$ $[(M-72)+H]^+$: 371.1454, found: 371.1453; $[\alpha]_D^{28}$ −12 (c 1.0, CH_3OH).

4.8. Diamide 10

NMM (1.0 mL, 9.0 mmol) was added to a solution of **11** (0.2 g, 0.45 mmol) in THF (20 mL). The mixture was stirred at rt for 1.5 h before 3-formamidobenzoic acid **2** (0.25 g, 0.9 mmol), EDCI (0.17 g, 0.9 mmol) and HOBt (0.18 g, 1.35 mmol) were successively introduced. Stirring of mixture continued at rt for 10 h. The reaction was terminated by addition of saturated $KHSO_4$ (20 mL) and extracted with EtOAc (3×15 mL). The extract was dried and evaporated, the residue was flash chromatographed on silica gel (gradient elution 100:0 to 100:0.3, $CHCl_3$/CH_3OH) to give diamide **10** (0.18 g, 45%) as a pale yellow oil. R_f 0.78 (9:1 $CHCl_3$/CH_3OH); IR (neat) 3389, 3035, 2937, 1730, 1667, 1520, 1427, 1381, 1309, 1277, 1179, 1067, 984, 914 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): δ 9.77 (s, 2H), 8.92–8.83 (m, 2H), 8.30 (s, 2H), 8.18 (d, J=7.9 Hz, 2H), 7.43 (d, J=7.9 Hz, 2H), 7.33–7.28 (m, 6H), 7.24–7.14 (m, 4H), 6.89 (dt, J=11.0 and 5.5 Hz, 2H), 5.98 (d, J=15.3 Hz, 2H), 5.37–5.31 (m, 2H), 5.06 (dd, J=8.6 and 4.3 Hz, 2H), 4.97 (s, 4H), 4.86 (d, J=15.9 Hz, 2H), 4.63 (d, J=15.9 Hz, 2H), 1.18 (d, J=6.7 Hz, 6H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.7 (s), 167.1 (s), 164.5 (s), 160.5 (d), 145.6 (s), 143.7 (d), 136.5 (s), 131.6 (s), 130.3 (s), 128.4 (d), 128.2 (d), 128.1 (d), 124.4 (d), 124.1 (d), 123.6 (d), 119.9 (d), 75.8 (t), 70.3 (d), 62.9 (t), 55.3 (d), 15.7 (q); HRMS ESI$^+$ calcd for $C_{46}H_{44}N_4O_{14}Na$ $[M+Na]^+$: 899.2752, found: 899.2751.

4.9. Tetraol diamide 9

To a solution of diamide **10** (0.37 g, 0.42 mmol), $(DHQ)_2PHAL$ (164 mg, 50 mol %), and NMO (297 mg, 2.52 mmol) in t-BuOH/THF/H_2O (7.5:7.5:1.5) was added OsO_4 (53.6 mg, 50 mol %). The resulting mixture was stirred at rt and monitored by TLC until disappearance of starting material **10**. The reaction was quenched with addition of Na_2SO_3 (0.63 g). The resulting mixture was extracted by CH_2Cl_2 (3×15 mL). The combined CH_2Cl_2 layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by column chromatography on silica gel (gradient elution 99:1 to 96:4, $CHCl_3$/CH_3OH) to give a white solid of 4:1 dr of

tetraol diamide products (0.26 g, 66%), with **9** as major diastereomer. This inseparable diastereomeric mixture was then used for the next step. R_f 0.43 (8:1 $CHCl_3/CH_3OH$); mp 135–138 °C; IR (KBr disc) 3370, 2934, 1752, 1684, 1605, 1583, 1524, 1427, 1396, 1268, 1205, 1123, 1081, 973, 915 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$), based on **9**: δ 8.37 (d, J=7.9 Hz, 2H), 8.22 (d, J=8.6 Hz, 2H), 7.92 (s, 2H), 7.77 (d, J=7.9 Hz, 2H), 7.39–7.18 (m, 12H), 5.49–5.47 (m, 2H), 5.29 (d, J=12.2 Hz, 2H), 5.20 (dd, J=8.6 and 4.3 Hz, 2H), 4.85–4.76 (m, 4H), 4.41–4.32 (m, 2H), 4.17–4.06 (m, 4H), 3.97 (br s, 2H), 3.84 (d, J=7.3 Hz, 2H), 1.94 (br s, 2H), 1.39 (d, J=6.7 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$), based on **9**: δ 171.5 (s), 169.7 (s), 165.8 (s), 158.6 (d), 146.2 (s), 135.4 (s), 131.5 (s), 129.3 (d), 129.1 (d), 128.9 (d), 126.4 (d), 125.9 (s), 125.2 (d), 125.1 (d), 78.6 (t), 74.1 (d), 70.8 (d), 69.2 (d), 66.9 (t), 55.5 (d), 16.7 (q); HRMS ESI$^+$ of 4:1 dr of tetraol diamide products:calcd for $C_{46}H_{48}N_4O_{18}Na$ $[M+Na]^+$: 967.2861, found: 967.2860.

4.10. Analogue 1

Mixture of 4:1 dr of tetraol diamide products (90 mg) and 10% Pd/C (135 mg) in methanol (12 mL) were stirred under H_2 atmosphere (balloon-pressure) at room temperature for 3 h. After reaction was complete, the solution phase was filtered through Celite and the solid phase washed with 1:1 EtOAc/MeOH (50 mL). The combined solvent filtrate and washings was evaporated and the residue was flash chromatographed on silica gel (gradient elution 99:1 to 90:10, $CHCl_3/CH_3OH$) to give a mixture of 4:1 dr of the corresponding Bn-deprotected products. This mixture was separated by medium pressure liquid chromatography afforded major diastereomer **1** (37.3 mg, 51%) as a white solid. The pure product **1** was then obtained as white crystalline solid after recrystallization from methanol. R_f 0.31 (5:1 $CHCl_3/CH_3OH$); mp 142–145 °C; IR (KBr disc) 3350, 2924, 2852, 1745, 1679, 1642, 1540, 1434, 1364, 1259, 1210, 1128, 1086, 1014, 752; ^1H NMR (500 MHz, CD_3OD), δ 8.33 (s, 2H), 8.28 (d, J=7.9 Hz, 2H), 7.75 (d, J=7.9 Hz, 2H), 6.87 (t, J=8.6 Hz, 2H), 5.50–5.45 (m, 2H), 5.17 (d, J=2.4 Hz, 2H), 4.55–4.49 (m, 2H), 4.25 (s, 2H), 4.19–4.16 (m, 4H), 1.40 (d, J=6.7 Hz, 6H); ^{13}C NMR (125 MHz, CD_3OD), δ 172.5 (s), 172.1 (s), 170.8 (s), 162.1 (d), 142.9 (s), 128.2 (s), 126.4 (d), 124.1 (d), 119.3 (d), 115.8 (s), 73.9 (d), 72.3 (d), 71.1(d), 68.3 (t), 56.9 (d), 16.7 (q); HRMS ESI$^+$ calcd for $C_{32}H_{36}N_4O_{18}Na$ $[M+Na]^+$: 787.1922, found: 787.1923; $[\alpha]_D^{31}$ +8 (c 0.25, CH_3OH).

4.11. Cytotoxicity evaluation of antimycin A3 analogues

4.11.1. Cell culture. The human cervical adenocarcinoma HeLa cell, human breast MDA-MB-231 cells and prostate PC-3 cells were maintained in humidified room air containing 5% CO_2 at 37 °C. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM), breast MDA-MB-231 cells were in DMEM (low glucose), and Prostate PC-3 cells were in RPMI-1640. These cells were supplemented with 10% fetal bovineserum (FBS) and 1% penicillin-streptomycin (GIBCO BRL, Grand Island, NY). Cells were routinely grown in 100 mm plastic tissue culture dishes and harvested with a solution of trypsin-EDTA when they reached 80–90% of confluence. Cells were maintained with these culture conditions for all experiment.

4.11.2. Reagent and tested sample. Antimycin A_3 from Sigma–Aldrich Chemical Co. was dissolved in ethanol at 2×10^{-2} M as a stock solution. The stock solution was wrapped in foil and kept at 4 °C or −20 °C. The tested samples, tetralactone **11**, tetraol **3**, and analogue **1** were prepared as previously described in this chapter. Tetralactone **11** and tetraol **3** were dissolved in water at the variety of concentration 0–100 mM, whereas analogue **1** was dissolved in DMSO at the variety of concentration 0–100 mM.

4.11.3. Cytotoxicity assay.[24,25] The effect of the tested compounds and antimycin A_3 on the cell proliferation of HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells was determined by CCK-8 (Cell Counting Kit-8), which is a very convenient assay utilizing Dojindo's highly water soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-2*H*-tetrazolium, monosodium salt]. WST-8 is reduced by dehydrogenase in cells to give a yellow-colored WST-8 formazan. The amount of WST-8 formazan dye generated by the activity of dehydrogenase in cells was directlyproportional to the number of living cells.

4.11.4. Procedure of cytotoxicity evaluation of the analogue. Cell suspension (100 μL) (5000 cells/well) of HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells, respectively, was dispensed to a 96-well plate. The plate was pre-incubated for 24 h in a humidified incubator containing 5% CO_2 at 37 °C. The various concentration of tested samples and antimycin A_3 (0–100 mM) were added into the culture media in the plate. The plate was incubated for an appropriate length of time (72 h) in the incubator. 10 μL of CCK-8 solution was then added to each well of the plate, and the plate was subsequently incubated for 1–4 h in the incubator. This mixture was then measured for absorbance at 450 nm using a microplate reader. Each plate contained multiple wells at a given experimental condition and multiple control wells. This procedure was replicated for 2–4 plates/condition. The half maximal inhibitory (IC_{50}) was determined using the probit method by plotting the concentration of the sample against percentage (%) of living cells.

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Supplementary data

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23. Crystallographic data for analogue 1 is deposited with the Cambridge Crystallographic Data Centre (Supplementary Publication No: CCDC 813907) and can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (email: deposit@ccdc.cam.ac.uk).
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