ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 8, Issue 6, 2015

ISSN - 0974-2441

Research Article

DESIGN, SYNTHESIS, AND CYTOTOXICITY EVALUATION OF NOVEL OPEN-CHAIN ANALOGUES OF ANTIMYCIN A_3 AS POTENTIAL ANTI-COLORECTAL CANCER AGENTS

ADE ARSIANTI[1]*, FADILAH FADILAH[1], KUSMARDI KUSMARDI[2], HIROKI TANIMOTO[3], KIYOMI KAKIUCHI[3]

[1]Department of Medical Chemistry, Faculty of Medicine, University of Indonesia, Jalan Salemba Raya 6, Jakarta 10430, Indonesia.
[2]Department of Anatomical Pathology, Faculty of Medicine, University of Indonesia, Jalan Salemba Raya 6, Jakarta 10430, Indonesia.
[3]Synthetic Organic Chemistry Laboratory, Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma, Nara, Japan. Email: arsi_ade2002@yahoo.com

Received: 09 July 2015, Revised and Accepted: 09 September 2015

ABSTRACT

Objective: Colorectal cancer is the third most common diagnosed cancer in the world. The aim of this work was to design, to synthesize, and to evaluate the novel open-chain analogues of antimycin A_3 as highly potent anti-colorectal cancer agents.

Methods: Our analogue synthesis was designed by modifying the nine-membered dilactone moiety of antimycin A_3 with a simple open-chain moiety, as well as introducing the stereocenter, and the hydroxyl groups on the side chain of the ester group. The synthesis was conducted through a sequence of reactions from Boc-L-threonine by esterification, amidation, and sharpless asymmetric dihydroxylation. After completion the synthesis, cytotoxicities of the analogues were evaluated as inhibitors of colorectal HCT-116 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay.

Results: Novel open-chain analogues of antimycin A_3 were successfully synthesized in a good yield. The analogues exhibited a greater anticancer activity against colorectal HCT-116 cells than the original antimycin A_3 with 50% inhibitory concentrations ranging of 35.0-47.0 μM. The results indicated that the presence of stereocenter and a hydroxylated open-chain moiety in the analogues were successfully improved its anti-colorectal cancer activity.

Conclusion: Our results clearly demonstrate that the opened-chain analogues of antimycin A_3 as a promising candidates of new anti-colorectal cancer agents.

Keywords: Design, Synthesis, Open-chain, Analogue, Antimycin A_3, Anti-colorectal cancer.

INTRODUCTION

Colorectal cancer is the third most common diagnosed cancer in the world with nearly 1.4 million new cases in 2012. It is estimated that worldwide the number of colorectal cancer cases will raise to 2.44 million by 2035 with approximately 394,000 people would die annually. Chemotherapy is a standard method which can alter tumor growth and improves survival rates in a patient with colorectal cancer. However, cancer chemotherapy is often failure due to the development of resistance by cancer cells against current anti-colorectal cancer agents [1,2]. Consequently, there is a significant need for new agents which more effective, safe, and potentially extend the survival of colorectal cancer patients.

Antimycin A_3 (1) is an active agent isolated from *Streptomyces* sp. in 1949 that inhibit the electron transfer activity of ubiquinol cytochrome *c* oxidoreductase, induce apoptosis of cancer cells, as well as shows a strong growth inhibitory activity against human colorectal cancer COLO201 [3]. The unique biological activity of antimycin A_3 inspired us to carry out the synthesis of its novel analogues, as well as evaluated its cytotoxicity against HCT-116 cells of colorectal cancer. Antimycin A_3 consists of a nine-membered dilactone core which is similar to antifungal antibiotic UK-3A that was also isolated from *Streptomyces* sp. in 1997 [3,4]. Previously, in 2010, we have reported the synthesis of novel 2-hydroxynicotinoyl-serine-butyl esters related to antibiotic UK-3A, which showed a strong antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus* [5]. Subsequently, in 2012, we succeeded in synthesizing polyhydroxylated 18-membered analogue of antimycin A_3 which strongly inhibited the growth of HeLa cells, breast MDA-MB-231 cells and prostate PC-3 cells [6]. More recently, in 2014, we have simulated some antimycin A_3 analogues as inhibitors of anti-apoptotic Bcl-2 of breast cancer by computational molecular docking [7]. In this research, as the continuing research to develop antimycin A_3 analogues as anticancer agents, we conduct the synthesis of open-chain analogues of antimycin A_3.

Structure-activity relationship (SAR) studies of antimycin A_3 by Miyoshi *et al.* in 1995 had shown that the anticancer activity of antimycin A_3 highly depend on the presence of hydroxyl group, amide bond and 3-formamido group. Whereas, the nine-membered dilactone core in antimycin A_3 was less necessary for anticancer activity compared to 3-formamidosalicylyl moiety [8]. These reports suggesting that the nine-membered dilactone core in antimycin A_3 can be modified by another active core in order to increase its anticancer activity. To date, there are several reports on the analogue synthesis of antimycin A_3. However, studies on the synthesis of open-chain analogues of antimyin A_3 are still limited. Therefore, in this work, we conducted the synthesis of antimycin A_3 analogues by modifying the nine-membered dilactone core of antimycin A_3 with an open-chain moiety in our desired analogue 1 and analogue 2 (Fig. 1).

It has been reported that introduction of hydroxyl groups into biologically active compound resulted in increasing of its biological activity due to the enhancement of its solubility in water, which is the one of important factors influencing the efficacy of drugs [9]. Thus, the introduction hydroxyl group in the open-chain moiety of the analogues is expected to greatly improve its anticancer activity. Furthermore, to study whether the stereochemistry influences the anticancer activity of the analogues; we designed hydroxyl group with bottom facial stereochemistry on the open-chain moiety of analogue 1, in contrast to those analogue, with the top facial stereochemistry in analogue 2.

METHODS

General experimental method

Unless otherwise noted, all reactions were performed in oven-dried glassware, sealed with a rubber septum under nitrogen atmosphere. Anhydrous tetrahydrofuran (THF) and CH_2Cl_2 were purchased from Kanto Chemical Co., dimethyl formamide (DMF) and *t*-butyl alcohol was distilled prior to use. Methanol, ethyl acetate (EtOAc), n-hexane, chloroform, Et_2O and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries. Boc-L-threonine, allyl bromide, $NaHCO_3$, K_2CO_3, *N,N*-4-dimethylaminopyridine, *N*-methylmorpholine (NMM), 1-hydroxybenzotriazole (HOBt), 1-ethyl-3(3-diaminopropyl) carbodiimide hydrochloride (EDCI), iodomethane (MeI), benzylbromide, formamide and osmium (VIII) oxide (OsO_4) were obtained from Wako Pure Chemical Industries. 3-Aminosalicylic acid was obtained from Tokyo Chemical Industries Co. Ltd. Diisopropylcarbodiimide, dihydroquinine phthalazine $(DHQ)_2$ PHAL, dihydroquinidine phthalazine (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 pre-coated 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).^{1}H nuclear magnetic resonance (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 GCMS QP-5000 or JEOL JMS-AX 700 spectrometers. Specific rotation, (α)D, were measured on JASCO DIP-1000 digital polarimeter. Cytotoxicity evaluation of the synthesized products was carried out in Department of Anatomical Pathology, Faculty of Medicine, University of Indonesia. The tested colon cancer HCT-116 cells are the culture collection of Anatomical Pathology Department, Faculty of Medicine, University of Indonesia.

Design and synthesis

Retrosynthetic analysis

Synthesis of target analogues was designed by retrosynthetic approach through several steps chemical reaction. Starting from the target analogue 1 and 2, break it down by series of disconnection, interconversion of functional group, and modification into the starting material (8). Retrosynthetic analysis of the analogues is outlined in Scheme 1. As shown, analogue 1 can be built by hydrogenolysis of the benzyl group of amide 3, whereas analogue 2 can be derived from hydrogenolysis of the benzyl group of amide 4. Amide 3 and amide 4 can be prepared from sharpless asymmetric dihydroxylation (AD) [10,11] of amide 5 in the presence of $(DHQ)_2$PHAL ligand and $(DHQD)_2$PHAL ligand, respectively. Amide **5** can be constructed from amidation of ester 6 with 3-formamido-2-benzyloxy-benzoic acid (7) which can be prepared from 3-formamidobenzoic acid according to known procedure as reported previously by Pettit *et al.* [12]. Ester 6 can be synthesized from esterification of commercially available Boc-L-threonine (8) followed by Boc deprotection.

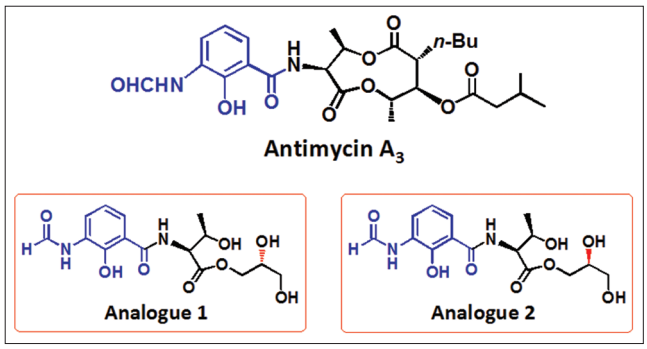


Fig. 1: Structure of antimycin A_3, analogue 1 and analogue 2

Synthesis of Boc-L-threonine-Allyl ester (9)

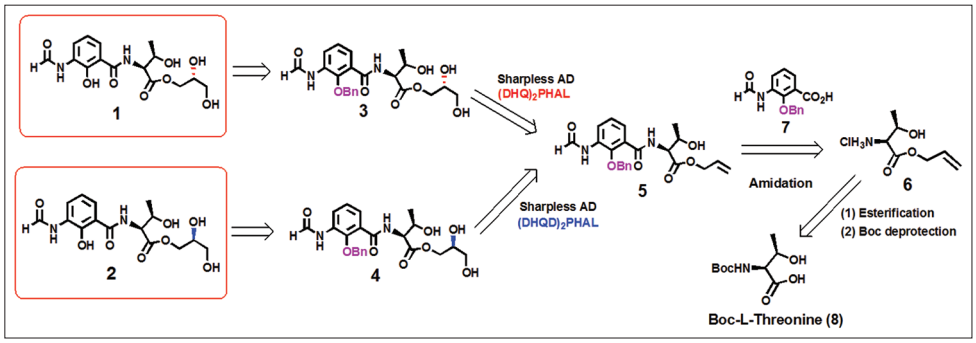
To a stirred solution of Boc-L-threonine (2.0 g, 9.12 mmol) in DMF (50 ml) was added Na_2CO_3 (1.93 g, 4.56 mmol), followed by allyl bromide (0.93 ml, 10.94 mmol) and water (1.6 ml). The resulting mixture was stirred for 40 h at room temperature. The solvent was removed from the reaction mixture in vacuo 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}H NMR (500 MHz, $CDCl_3$): δ 5.92-5.85 (m, 1H, CH=CH_2), 5.34-5.22 (m, 3H, Thr-NH + CH=CH_2), 4.65-4.63 (m, 2H, OCH_2CH=), 4.29-4.24 (m, 2H, THr-Hα + Thr-Hβ), 2.13 (br s, 1H, Thr-OH), 1.43 (s, 9H,^{t}Bu), 1.23 (d, 3H, *J*=6.2 Hz, CH_3);^{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.

Synthesis of L-threonine-allyl ester ammonium chloride (6)

A round-bottomed flask was charged with ester 9 (5 g, 19.2 mmol) dissolved in EtOAc (200 ml). 35% (w/v) of HCl (20 ml) was added to this solution and the mixture was stirred at room temperature for 10 hrs. After the reaction was complete, the solvent was evaporated in vacuo, and the crude was purified by flash column chromatography on silica gel (gradient elution 20:1 to 1:1, $CHCl_3$:CH_3OH) gave an ammonium chloride salt of 6 (3437 mg, 91%) as pale yellow solid. R_f=0.23 (5:1 $CHCl_3$:CH_3OH); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.46 (s, 3H), 5.97-5.91 (m, 1H), 5.69-5.60 (m, 1H), 5.41 (d, *J*=17.1 Hz, 1H), 5.27 (d, *J*=10.2 Hz, 1H), 4.69 (d, *J*=5.0 Hz, 1H), 4.16 (s, 1H), 3.98 (s, 1H), 1.22 (d, *J*=6.0 Hz, 3H);^{13}C NMR (125 MHz, DMSO-d_6): δ 167.8 (s), 131.7 (d), 118.4 (t), 65.8 (t), 65.1 (d), 57.8 (d), 19.9 (q); HRMS ESI$^+$ calcd for$C_7H_{13}NO_3Na$ [M+Na]$^+$: 182.0793, found: 182.0791.

Synthesis of amide 5

EDCI (0.3 ml, 1.83 mmol) and NMM (2.5 ml, 22.95 mmol) were added to a mixture solution of L-threonine-alyll-ester ammonium chloride 6 (0.3 g, 1.53 mmol), 3-formamido-2-benzyloxybenzoic



Scheme 1: Retrosynthetic analysis of open-chain analogue 1 and 2

acid 7 (0.51 g, 1.83 mmol) and HOBt (0.31 g, 2.295 mmol) in DMF (22 ml). The mixture was stirred at room temperature for 17 hrs. The mixture was then diluted by addition of EtOAc (200 ml), and washed repeatedly by water (4 × 75 ml) and saturated NaCl (2 × 75 ml). EtOAc phase was dried over $MgSO_4$ anhydrous and evaporated the residue was flash chromatographed on silica gel (gradient elution 6:1 to 3:1, hexane:EtOAc), gave amide 5 (535.1 mg, 85%) as pale yellow oil.

R_f=0.56 (1:1 hexane:EtOAc); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.74 (s, 1H), 8.47 (d, J=8.0 Hz, 1H), 8.31 (d, J=6.0 Hz, 1H), 8.17 (d, J=8.0 Hz, 1H), 7.50-7.44 (m, 2H), 7.39-7.31 (m, 4H), 7.20 (t, J=8.0 Hz, 1H), 5.88-5.85 (m, 1H), 5.34 (dd, J=18.9 and 5.4 Hz, 1H), 5.19 (dd, J=10.9 and 5.4 Hz, 1H), 5.04-4.94 (dd, J=17.2 and 5.7 Hz, 2H), 4.59-4.56 (m, 1H), 4.53-4.51 (m, 1H), 4.25-4.15 (m, 1H), 1.22 (d, J=6.3 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 170.2 (s), 165.9 (s), 160.4 (d), 146.2 (s), 135.9 (s), 132.3 (s), 131.6 (s), 129.0 (d), 128.9 (d), 128.2 (d), 128.0 (d), 124.9 (d), 124.3 (d), 124.1 (d), 117.7 (t), 76.15 (t), 66.27 (d), 64.96 (t), 58.62 (d), 20.37 (q); HRMS ESI$^+$ calcd for $C_{22}H_{24}N_2O_6Na$ [M+Na]$^+$: 435.1532, found: 435.1529.

Synthesis of amide 3

To a solution of amide 5 (0.3 g, 0.73 mmol), $(DHQ)_2PHAL$ (57 mg, 10 mol%) and N-methylmorpholine-N-oxide (NMO) (256 mg, 2.18 mmol) in t-BuOH:THF:H_2O (6:6:1.2) was added OsO_4 (19 mg, 10 mol%). The resulting mixture was stirred at room temperature and monitoring by thin-layer chromatography (TLC) until disappearance of starting material 5 (4 hrs). The reaction was quenched with addition of Na_2SO_3 (0.3 g) and water (7 ml). 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 (gradient elution 20:1 to 8:1, $CHCl_3$:CH_3OH) gave a colorless oil of 10:1 dr of dihydroxylated amide products (0.26 g, 64%), with **3** as major diastereomer. This inseparable diastereomeric mixture was then used for the next step. R_f=0.41 (4:1 $CHCl_3$:CH_3OH) for mixture of two diastereomers.^{1}H NMR (500 MHz, acetone-d_6): δ 8.28 (dd, J=6.9 and 3.2 Hz, 1H), 8.04 (s, 1H), 7.83-7.78 (m, 2H), 7.60 (d, J=8.0 Hz, 1H), 7.33-7.24 (m, 5H), 7.19-7.11 (m, 3H), 5.14 (d, J=10.3 Hz, 1H), 4.88-4.81 (m, 1H), 4.74 (d, J=9.2 Hz, 1H), 4.57-4.36 (m, 3H), 3.84 (br s, 1H), 3.73-3.62 (m, 1H), 3.59-3.45 (m, 2H), 1.20 (d, J=6.5 Hz, 3H); ^{13}C NMR (125 MHz, acetone-d_6): δ 171.5 (s), 166.5 (s), 160.5 (d), 147.0 (s), 136.7 (s), 132.9 (s), 132.8 (d), 132.0 (d), 129.9 (d), 129.0 (d),128.7 (d), 126.1 (d), 124.9 (s), 78.1 (t), 70.5 (d), 67.8 (d), 67.1 (t), 63.7 (t), 59.4 (d), 20.7 (q); HRMS ESI$^+$ calcd for $C_{22}H_{26}N_2O_8Na$ [M+Na]$^+$:469.1587, found: 469.1582; $[\alpha]_D$ = +2 (c=0.42, CH_3OH, 26°C).

Synthesis of amide 4

To a solution of amide 5 (0.3 g, 0.73 mmol), $(DHQD)_2PHAL$ (57 mg, 10 mol%) and NMO (256 mg, 2.18 mmol) in t-BuOH:THF:H_2O (6:6:1.2) was added OsO_4 (19 mg, 10 mol%). The resulting mixture was stirred at room temperature and monitoring by TLC until disappearance of starting material 5 (4 hrs). The reaction was quenched with addition of Na_2SO_3 (0.3 g) and water (7 ml). 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 (gradient elution 20:1 to 8:1, $CHCl_3$:CH_3OH) gave a colorless oil of 10:1 dr of dihydroxylated amide products (0.26 g, 68%), with 4 as major diastereomer. This inseparable diastereomeric mixture was then used for the next step. R_f=0.41 (4:1 $CHCl_3$:CH_3OH) for mixture of two diastereomers.^{1}H NMR (500 MHz, acetone-d_6): δ 8.41 (dd, J=6.3 and 3.2 Hz, 1H), 8.38 (s, 1H), 7.61 (d, J=8.0 Hz, 1H), 7.55-7.50 (m, 2H), 7.33-7.30 (m, 3H), 7.22 (t, J=8.0 Hz, 1H), 7.55-7.51 (m, 2H), 7.36-7.30 (m, 3H), 7,23 (t, J=8.0 Hz, 1H), 5.25 (d, J=11.5 Hz, 1H), 5.03 (d, J=10.5 Hz, 1H), 4.74-4.69 (m, 1H), 4.47 (s, 1H), 4.31-4.13 (m, 2H), 3.92-3.84 (m, 1H), 3.55 (d, J=5.0 Hz, 2H), 1.22 (d, J=6.5 Hz, 3H);^{13}C NMR (125 MHz, acetone-d_6): δ 171.5 (s), 166.5 (s), 160.5 (d), 146.9 (s), 136.7 (s), 133.0 (s), 132.9 (d), 130.0 (d), 129.2 (d), 129.1 (d), 126.1 (d), 125.3 (d), 124.9 (s), 79.1 (t), 78.2 (d), 70.5 (d), 67.1 (t), 63.7 (t), 59.4 (d), 20.7 (q); HRMS ESI$^+$ calcd for $C_{22}H_{26}N_2O_8Na$ [M+Na]$^+$: 469.1587, found: 469.1586; $[\alpha]_D$ = −9 (c=0.71, CH_3OH, 26°C).

Synthesis of analogue 1

Mixture of 10:1 dr of hydroxylated amide products with amide 3 as the major product (170 mg) and 10% Pd/C (255 mg) in methanol (22 ml) were stirred under 1 atm H_2 atmosphere at room temperature for 4 hrs. After reaction was complete, the solution phase was filtered through celite and the solid phase washed with 1:1 EtOAc-MeOH (40 ml). The combined solvent filtrate and washings was evaporated and the residue was flash chromatographed on silica (gradient elution 20:1 to 9:1, $CHCl_3$:CH_3OH) gave a mixture of 10:1 dr of the corresponding Bn-deprotected products. This mixture was separated by medium pressure liquid chromatography afforded major diastereomer 1 (37.3 mg, 74%) as a pale brown oil. R_f=0.29 (4:1 $CHCl_3$:CH_3OH);^{1}H-NMR (500 MHz, acetone-d_6): δ 9.16 (s, 1H), 8.51 (s, 1H), 8.47 (d, J=7.5 Hz, 1H), 8.13 (d, J=9.0 Hz, 1H), 7.71 (d, J=8.5 Hz, 1H), 6.90 (t, J=8.0, 1H), 4.80-4.76 (m, 1H), 4.53-4.45 (m, 1H), 4.32-4.14 (m, 2H), 3.91-3.89 (m, 1H), 3.75-3.72 (m, 1H), 3.59-3.55 (m, 2H), 3.29 (s, 2H), 1.24 (d, J=6.0 Hz, 3H);^{13}C NMR (125 MHz, acetone-d_6): δ 171.2 (s), 170.8 (s), 160.8 (d), 151.6 (s), 128.3 (s), 124.9 (d), 122.1 (d), 119.1 (d), 114.4 (s), 70.5 (d), 67.9 (d), 67.1 (t), 63.6 (t), 59.0 (d), 20.3 (q); HRMS ESI$^+$ calcd for $C_{15}H_{20}N_2O_8Na$ [M+Na]$^+$: 379.1117, found: 379.1118; $[\alpha]_D$ = +1 (c=0.59, CH_3OH, 22°C).

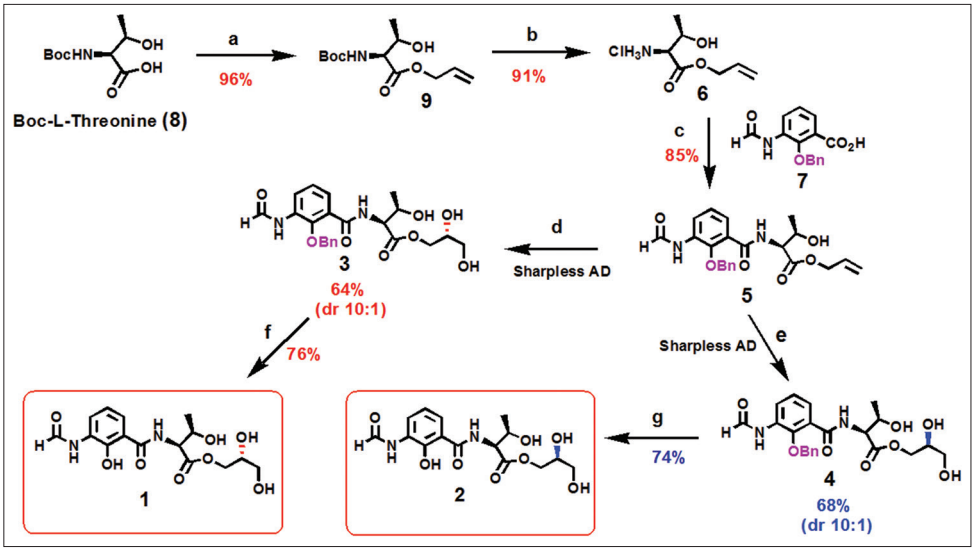
Synthesis of analogue 2

Mixture of 10:1 dr of hydroxylated amide products with amide 4 as the major product (90 mg) and 10% Pd/C (135 mg) in methanol (11 ml) was stirred under 1 atm H_2 atmosphere at room temperature for 4 hrs. After reaction was complete, the solution phase was filtered through celite and the solid phase washed with 1:1 EtOAc-MeOH (40 ml). The combined solvent filtrate and washings was evaporated and the residue was flash chromatographed on silica (gradient elution 99:1 to 90:10, $CHCl_3$:CH_3OH) gave a mixture of 10:1 dr of the corresponding Bn-deprotected products. This mixture was separated by medium pressure liquid chromatography afforded major diastereomer 2 (38.3 mg, 76%) as a brown oil. R_f=0.32 (4:1 $CHCl_3$:CH_3OH); ^{1}H-NMR (500 MHz, acetone-d_6): δ 9.16 (s, 1H), 8.51 (s, 1H), 8.47 (d, J=7.5 Hz, 1H), 8.13 (d, J=9.0 Hz, 1H), 7.71 (d, J=8.5 Hz, 1H), 6.90 (t, J=8.0, 1H), 4.80-4.76 (m, 1H), 4.53-4.45 (m, 1H), 4.32-4.14 (m, 2H), 3.91-3.89 (m, 1H), 3.75-3.72 (m, 1H), 3.59-3.55 (m, 2H), 3.29 (s, 2H), 1.24 (d, J=6.0 Hz, 3H); ^{13}C NMR(125 MHz, acetone-d_6): δ 171.2 (s), 170.8 (s), 160.8 (d), 151.6 (s), 128.3 (s), 124.9 (d), 122.1 (d), 119.1 (d), 114.4 (s), 70.5 (d), 67.9 (d), 67.1 (t), 63.6 (t), 59.0 (d), 20.3 (q); HRMS ESI$^+$ calcd for $C_{15}H_{20}N_2O_8Na$ [M+Na]$^+$: 379.1117, found: 379.1118; $[\alpha]_D$ = −2 (c=0.43, CH_3OH, 27°C).

***In vitro* cytotoxicity assay**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [13,14] is performed to measure the anti-proliferation effects of synthesized amide 5, amide 3, amide 4, analogue 2, analogue 1 and the original antimycin A_3 on the colon cancer HCT-116 cells. Synthesized amide 5, amide 3, amide 4, analogue 2, analogue 1 and antimycin A_3 are diluted and added to target cells in triplicates with final concentrations at 51.2, 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4 µg/ml. The cells are incubated for 48 hrs and 20 µl of 5 mg/ml solution of MTT in phosphate-buffered saline is added to triplicate samples and the plates are incubated for additional 4 hrs. The plates are then centrifuged and the medium is removed. 200 µl of DMSO is added to each well to dissolve the purple blue sediment, the absorbance is determined at 590 nm on a microplate reader (Model 550, Bio-Rad, USA). The 50% inhibitory concentrations (IC_{50}) of the 48 hrs are calculated with Bliss assay. The inhibition rate is calculated as follows:

$$\text{Inhibition rate}(\%) = 1 - \left(\frac{\text{Absorbance of treatment group}}{\text{Absorbance of control group}} \right) \times 100\%$$



Scheme 2: Synthesis pathway of desired analogue 1 and analogue 2. Reagents and conditions: (a) Allyl bromide, Na_2CO_3, dimethyl formamide (DMF), rt; (b) Concd HCl, ethyl acetate, rt; (c) carbodiimide hydrochloride, 1-hydroxybenzotriazole, N-methylmorpholine, DMF, rt; (d) osmium (VIII) oxide (OsO_4), N-methylmorpholine-N-oxide (NMO), Diisopropylcarbodiimide, dihydroquinine phthalazine, rt; (e) OsO_4, NMO, dihydroquinidine phthalazine, rt; (f) 10% Pd/C, H2, MeOH, rt; (g) 10% Pd/C,H2, MeOH, rt

RESULTS AND DISCUSSION

Chemistry

Scheme 2 outlines the synthesis of analogue 1 and 2. Starting from esterification of Boc-L-threonine (8) with allyl bromide under basic condition, which was conducted according to the procedure provided by Wu *et al*. [15], afforded Boc-L-threonine-allyl ester (9) in 96% yield. Subsequently, removal of Boc protecting the group from ester 9 was completed in the presence of 20 equivalents of HCl for 10 hrs, afforded 91% yield of ester 6 as ammonium chloride salts. With ester 6 in hands, our subsequent plan was to conduct the amidation of 6 with 3-formamidobenzoic acid (7) to form amide intermediate 5. The formation of amide intermediate 5 is the key step in this work, and it has accomplished by performing the reaction using the base NMM and the combination of EDCI/HOBt with DMF as a solvent, gave key intermediate amide 5 in 85% yield. In the next step, there are two synthetic pathways available which applying sharpless AD. In our initial attempt, introducing the stereocenter as well as addition of two hydroxyl groups at the terminal olefin of amide 5 was carried out by NMO-based catalytic dihydroxylation system in the presence of OsO_4, but in the absence of $(DHQ)_2PHAL$ or $(DHQD)_2PHAL$ ligand, to give hydroxylated amide product with very poor diastereoselectivity. Subsequently, the addition of 10 mol% of $(DHQ)_2PHAL$ or $(DHQD)_2PHAL$ ligand into this system was affected to significantly improved the diastereomeric diastereoselectivity. These results revealed that $(DHQ)_2PHAL$ or $(DHQD)_2PHAL$ ligand was very important to control stereoselectivity of sharpless dihydroxylation of amide 5, in order to obtain the product with satisfactory selectivity. Thus, in the first pathway, sharpless dihydroxylation of amide 5 in the presence of 6 equivalents of NMO with 10 mol % of both OsO_4 and $(DHQ)_2PHAL$ ligand, proceeded smoothly to give inseparable diastereomeric mixture of hydroxylated amide products in 64% yield with satisfactory diastereoselectivity (dr=diastereomeric ratio = 10:1), while 3 as a major diastereomer. As the final step, hydrogenolysis of this diastereomeric mixture with 10% Pd/C resulted in cleavage of Bn group, and afforded a 10:1 diastereomeric mixture of the corresponding Bn deprotected hydroxylated amide products, which was successfully separated in this step by medium pressure liquid chromatography to give a pure major analogue 1 in 76% yield. In the second pathway, sharpless dihydroxylation of amide 5 with 6 equivalents of NMO and 10 mol% of both OsO_4 and $(DHQD)_2PHAL$ gave hydroxylated amide products in 68% yield as a 10:1 inseparable diastereomeric mixture. Hydrogenolysis of this diastereomeric mixture with 10% Pd/C afforded a 10:1 diastereomeric mixture of the corresponding Bn deprotected hydroxylated amide products, which was separated by medium pressure liquid chromatography to give a pure major analogue 2 in 74% yield.

Table 1: Cytotoxicities of amide 5, amide 4, amide 3, analogue 2, analogue 1, and antimycin A_3 against colorectal HCT-116 cells

Compound	IC_{50} (μM)*
Amide 5	>200±2.5
Amide 4	156.8±1.8
Amide 3	106.5±2.7
Analogue 2	47.0±2.1
Analogue 1	35.0±1.6
Antimycin A_3	77.2±2.0

*IC_{50} is the 50% half maximal inhibitory activity in μM, expressed in mean value (n=3) ±SD. SD: Standard deviation

Cytotoxicity

After completion of the synthesis, cytotoxicities of the analogue 1, analogue 2, and three intermediate products, amide 5, amide 4 and amide 3 were evaluated as inhibitors of cancer cell growth versus colorectal cancer HCT-116 cells. The result is summarized in Table 1. As shown in Table 1, amide 5 with IC_{50} over 200 μM showed no cytotoxicity against HCT-116 cells. In contrast to amide 5, amide 3 and amide 4 which two additional of hydroxyl groups showed the improvement in cytotoxicity with concentration 106.5 μM and 156.8 μM against HCT 116, respectively. The cytotoxicity of amide 3 and amide 4 are greatly improved by the presence of the hydroxyl groups compared to that of amide 5. This fact suggested that the hydroxyl groups are very important for the anti-colorectal cancer activity. Compared to amide 4, amide 3 which possess hydroxyl group with bottom facial stereochemistry, showed stronger cytotoxicity, indicating that introduction hydroxyl group with bottom facial stereochemistry was potentially responsible for the increase in its anticancer activity.

Compared to amide 3 and amide 4, analogue 1 and analogue 2 which have hydroxyl group instead of benzyloxy group on 3-formamidosalicylyl moiety, showed greater cytotoxicity, suggesting that the presence of hydroxyl group on 3-formamido-salicylyl moiety in both of analogue 1 and analogue 2 was very necessary to enhance its anticancer activity against HCT-116 cells. Moreover, analogue 1 and analogue 2 which contains open-chain moiety exhibited a greater anticancer activity

than that of the original antimycin A_3 on HCT-116 cells of colorectal cancer, with IC_{50}: 35 μM and 47 μM, respectively. These results indicated that modifying the nine-membered dilactone core of antimycin A_3 with a hydroxylated open-chain moiety in analogue 1 and analogue 2 was successfully improved its anticancer activity. Furthermore, analogue 1 contains bottom facial stereocenter showed greater anti-colorectal cancer activity compared to analogue 2 which has top facial stereocenter. These results revealed that bottom facial stereocenter was more effective for anti-colorectal cancer activity than top facial stereocenter. Thus, analogue 1 which strongly inhibited the growth of colorectal HCT-116 cells should be considered as a promising candidate for the treatment of human colorectal cancer.

CONCLUSION

We synthesized novel open-chain analogues of antimycin A_3 from Boc-L-threonine through esterification, amidation and sharpless AD. Analogue 1 and analogue 2 showed greater anticancer activity against colorectal HCT-116 cells compared to the original antimycin A_3. The results and findings in this work are expected to be helpful to the medicinal chemist to develop synthesis strategy of antimycin A_3 analogues, as well as to give a more thorough understanding of the SAR between the analogues and the original antimycin A_3.

ACKNOWLEDGMENTS

We wish to express our gratitude to Directorate of Research and Public Service University of Indonesia for the Research Cluster Grant, and to the Graduate School of Materials Science, Nara Institute of Science and Technology (NAIST), Japan, for International Research Collaboration Program (NAIST Global Initiative Program).

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61(2):69-90.
2. Gorlick R, Bertino JR. Drug resistance in colon cancer. Semin Oncol 1999;26(6):606-11.
3. Ueki M, Kusumoto A, Hanafi M, Shibata K, Tanaka T, Taniguchi M. UK-3A, a novel antifungal antibiotic from *Streptomyces* sp. 517-02: Fermentation, isolation, structural elucidation and biological properties. J Antibiot (Tokyo) 1997;50(7):551-5.
4. Ueki M, Taniguchi M. The mode of action of UK-2A and UK-3A, novel antifungal antibiotics from *Streptomyces* sp. 517-02. J Antibiot (Tokyo) 1997;50(12):1052-7.
5. Arsianti A, Hanafi M, Saepudin E, Morimoto T, Kakiuchi K. Synthesis and biological activity of 2-hydroxynicotinoyl-serine-butyl esters related to antibiotic UK-3A. Bioorg Med Chem Lett 2010;20(12):4018-20.
6. Arsianti A, Tanimoto H, Morimoto T, Bahtiar A, Takeya T, Kakiuchi K. Synthesis and anticancer activity of polyhydroxylated 18-membered analogue of antimycin A_3. Tetrahedron 2012;68(13):2884-91.
7. Arsianti A, Fadilah F, Kusmardi K, Tanimoto H, Morimoto, T, Kakiuchi K. Design and molecular docking study of antimycin A_3 analogues as inhibitors of anti-apoptotic Bcl-2 of breast cancer. Open J Med Chem 2014;4:79-86.
8. Miyoshi H, Tokutake N, Imaeda Y, Akagi T, Iwamura H. A model of antimycin A binding based on structure-activity studies of synthetic antimycin A analogues. Biochim Biophys Acta 1995;1229(2):149-54.
9. Thomas G. Fundamentals of Medicinal Chemistry. 3rd ed. Chichester: John Wiley & Sons Ltd.; 2003.
10. Xu D, Crispino GA, Sharpless KB. Selective asymmetric dihydroxylation (AD) of dienes. J Am Chem Soc 1992;114(19):7570-1.
11. Gobel T, Sharpless KB. Temperature effects in asymmetric dihydroxylation: Evidence for a stepwise mechanism. Angew Chem Int Ed Engl 1993;32(9):1329-31.
12. Pettit GR, Smith TH, Feng S, Knight JC, Tan R, Pettit RK, *et al.* Antineoplastic agents 561. Total synthesis of respirantin. J Nat Prod 2007;70(7):1073-83.
13. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnol Annu Rev 2005;11:127-52.
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1-2):55-63.
15. Wu Y, Yang YQ. An expeditious enantioselective synthesis of antimycin A3b. J Org Chem 2006; 71(11): 4296-301.