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RESEARCH ARTICLE

In Silico Study and Cytotoxicity of the Synthesized Open-chain Analogues of Antimycin A_3 Against HEP-2 Laryngeal Cancer Cells

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Abstract: ***Background*:** Laryngeal cancers affect one quarter of all head and neck cancers. Chemotherapy is a standard method in treatment laryngeal carcinoma. However, cancer chemotherapy is often a failure due to the appearance of drug resistance. This fact suggests that the search for novel, safe, and more effective laryngeal cancer drugs are required. Antimycin A_3 is a fit ligand of anti-apoptotic Bcl-2. While Bcl-2 is known to be over-expressed in laryngeal cancer cell, it is quite reasonable to expect antimycin A_3 and its analogue to induce apoptosis in those cells.

***Methods*:** With this viewpoint, we decided to conduct research that is aimed to evaluate cytotoxic activity of the synthesized open-chain analogues of antimycin A_3 against HEP-2 laryngeal cancer cells, as well as to conduct *in silico* study of the analogues on receptor binding target Bcl-2 of laryngeal cancer.

***Results and Conclusion*:** Open-chain analogues of antimycin A_3 were successfully synthesized in a good yield from Boc-L-Threonine by esterification, amidation, and Sharpless asymmetric dihydroxylation.

Consistent with *in silico* study, the analogues exhibited a greater anticancer activity against laryngeal HEP-2 cells than the original antimycin A_3 with IC_{50} ranging of 31.6 μM to 46.3 μM. Our results clearly demonstrate that the open-chain analogues of antimycin A_3 as a promising candidates of new anti-laryngeal cancer agents.

Keywords: Synthesis, *in silico*, cytotoxicity, opened-chain analogue, antimycin A_3, HEP-2, laryngeal cancer.

1. INTRODUCTION

Laryngeal cancer is a significant entity of oncology. Approximately it causes 30% to 40% of all head and neck malignancies. The highest incidence of laryngeal cancer happens between the fifth and seventh decade of life. Several predisposing factors have been reported, the most important are smoking [1]. The relative risk for laryngeal cancer is greater than for smokers who have smoked for 40 years or more, and smokers who smoke 20 or more cigarettes per day. Moreover, other possible risk factors are viral infection with human papillomavirus (HPV) and Epstein-Barr virus (EBV), radiation, exposure to carcinogens in environment, nutrition, gastroesophageal reflux, and hereditary [2].

Cancer of the larynx is a predominantly male disease. The male to female ratio varies internationally from 30:1 to 5:1. The mortality from la-

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ryngeal cancer also varies depending on the region, with an increasing trend worldwide. For the year 2004, 89,000 deaths from cancer of the larynx in men and 12,000 in women are reported worldwide [2].

In Indonesia, the annual mortality rate per 100,000 population from larynx cancer has increased by 31.6 % since 1990, an average of 1.4 % a year. Cigarette-smoking and dietary risk were thought to be responsible for larynx cancer in Indonesia population during 2013 [3, 4]. Chemotherapy is a standard method in treatment laryngeal carcinoma. However, cancer chemotherapy is often a failure due to the appearance of drug resistance [5]. High mortality rate of larynx cancer in Indonesia along with developing of drug resistance has prompted us to search and discover novel laryngeal cancer drugs that are safer and more effective.

Antimycin A_3 (Fig. **1**), a nine-membered dilactone derived from *Streptomyces sp.,* is an active agent that prevents the electron transfer activity of ubiquinol-cytochrome c oxidoreductase and inhibit the growth of human cancer cells [6-8]. Antimycin A_3 interacts with the Bcl-2 homology domain 3 (BH3)-binding hydrophobic groove of Bcl-X_L. Antimycin A_3 and BH3 peptide both induce mitochondrial swelling and loss of mitochondrial membrane potential. These changes might follow the opening mitochondrial permeability transition pores. The Bcl-2 stabilized mitochondrial membrane has potential in induced cells to undergo apoptosis. In the absence of Bcl-2, apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF) are released from mitochondria. Aberrant overexpression of antiapoptotic members of the Bcl-2 protein family leads to malignant transformation and subsequent resistance to anticancer agents [9, 10].

Antimycin A_3 is a fit ligand of anti-apoptotic Bcl-2.While Bcl-2 is known to be expressed in laryngeal cancer cells [5, 11], it is quite reasonable to expect antimycin A_3 and its analogue to induce apoptosis in those cells. With this viewpoint, we decided to conduct research that is aimed to evaluate cytotoxicity of the synthesized open-chain analogues of antimycin A_3 (Fig. **1**) against HEP-2 laryngeal cancer cells, as well as to conduct *in silico* study of the analogues on receptor binding target Bcl-2 of laryngeal cancer. Open-chain analogue **1** and **2** used in this work (Fig. **1**) have been successfully synthesized in our recent research in 2015, which showed potent anti-colorectal cancer activity on HCT-116 cells [12]. Previously, in 2010, we have reported the synthesis of 2-hydroxynicotinoyl-serine-butyl esters related to antibiotic UK-3A which showed a strong inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus* [13]. In 2012, we have succeeded in synthesizing 18-membered analogue of antimycin A_3 which demonstrated a strong cytotoxicity against HeLa cells, Breast MDA-MB-231 cells and prostate PC-3 cells [14]. In 2014, we have simulated some antimycin A_3 analogues as inhibitors of anti-apoptotic Bcl-2 of breast cancer by *in silico* molecular docking [15]. In this work, as our continuing research to discover potential antibiotic anticancer agents, we focused on the *in silico* study and cytotoxicity evaluation of the synthezid open-chain analogues of antimycin A_3 against HEP-2 laryngeal cancer cells.

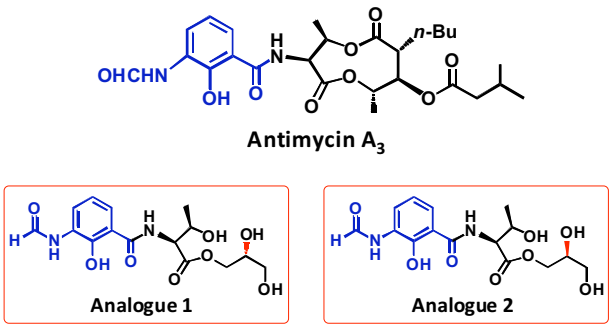
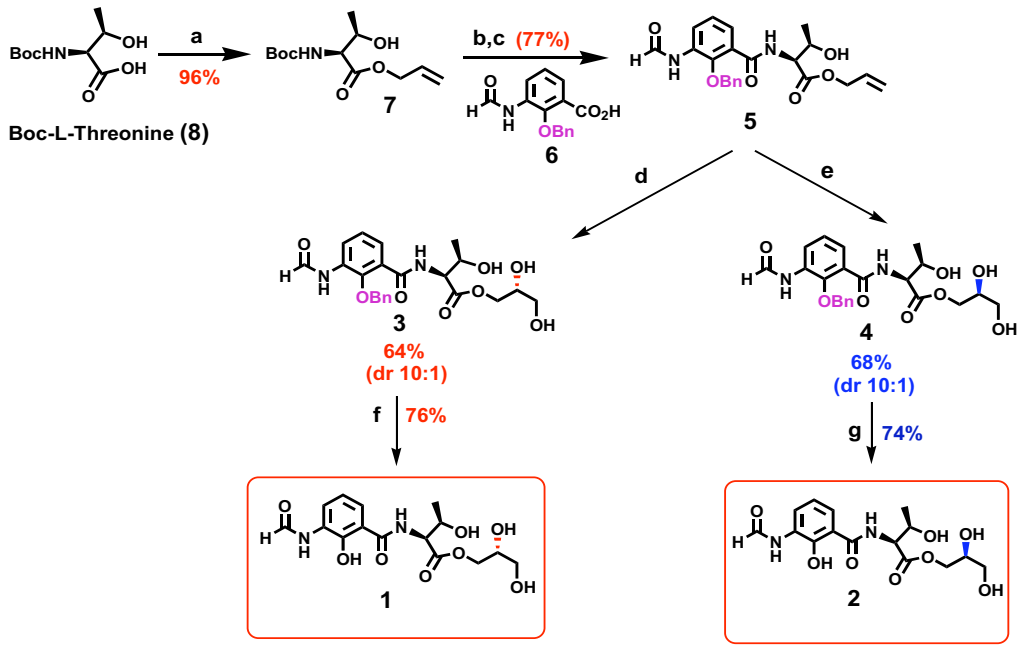


Fig. (1). Structure of antimycin A_3 and its analogues.

2. MATERIALS AND METHODS

2.1. Synthesis

Design and synthesis of the open-chain analogue **1** and analogue **2** have been reported in our previous work [12], as outlined in Scheme **1**. The preparation of **1** and **2** started from **8**. In this case, coupling of **8** with allyl bromide under basic condition as reported by Wu *et al.* [16], proceeded smoothly to give **7**. Boc deprotection of **7** followed by amidation with acid **6** provided **5**. Treatment of **5** with Sharpless reagent in the presence of $(DHQ)_2PHAL$ and $(DHQD)_2PHAL$ yielded corresponding hydroxylated amide **3** and **4** respectively, with diastereomeric ratio (dr) 10:1. Removal of the Bn group from **3** and **4** with H_2 and Pd/C followed by purification with medium pressure liquid chromatography completed the synthesis of analogue **1** and **2** in good yield.



Scheme 1. Synthesis pathway of open-chain analogue **1** and analogue **2** [9]. Reagents and conditions: (a) Allyl bromide, Na_2CO_3, DMF, rt; (b) Concd HCl, EtOAc, rt; (c) EDCI, HOBt, NMM, DMF, rt; (d) OsO_4, NMO, $(DHQ)_2PHAL$, rt; (e) OsO_4, NMO, $(DHQD)_2PHAL$, rt; (f) 10% Pd/C, H_2, MeOH, rt; (g) 10% Pd/C,H_2, MeOH, rt.

The synthesis procedure of compound **7**, amide **5**, hydroxylated amide **3**, hydroxylated amide **4**, analogue **1** and **2** has been described in our previous work [12].

2.2. Experimental Data

^{1}H NMR and ^{13}C NMR spectra were recorded on 500 MHz JEOL JNM-ECP500 spectrometers using tetramethylsilane (δ 0), $CDCl_3$ (δ 7.26), DMSO (δ 2.49) or acetone (δ 2.05) as an internal standard. Mass spectra were recorded on Shimadzu GCMS QP-5000 or JEOL JMS-AX 700 spectrometers.

2.2.1. Compound 7

^{1}H-NMR (500 MHz, $CDCl_3$): δ5.92-5.85 (m, 1H), 5.34-5.22 (m, 3H), 4.65-4.63 (m, 2H), 4.29-4.24 (m, 2H), 2.13 (br s, 1H), 1.43 (s, 9H), 1.23 (d, 3H, *J* = 6.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): δ171.3, 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.

2.2.2. Amide 5

^{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 dan 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.

2.2.3. Hydroxylated Amide 3

^{1}H-NMR (500 MHz, Acetone-d_6): δ8.28 (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.

2.2.4. Hydroxylated Amide 4

^{1}H-NMR (500 MHz, Acetone-d_6): δ8.41 (dd, *J* = 6.3 dan 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.

2.2.5. Analogue 1

^{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): δ171.2 (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.

2.2.6. Analogue 2

^{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): δ171.2 (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.

2.3. *In Silico* Study

In this research, we simulated open-chain analogue **1** and **2** based on their interactions with Bcl-2 using computer software applications (*Molecular* method) [17] to determine the best compounds [18]. Analysis and screening were based on Gibbs Free energy (ΔG) values, affinity, conformation of the structure, and hydrogen bonding interaction between compounds and the target proteins [19].

2.4. Sequence Alignment and Homology Modelling

Target protein sequences were selected and downloaded

From NCBI (http://www.ncbi.mlm.nih.gov/ genomes//). The multiple sequence alignment method was based on clustal W2 program (www.ebi.ac.uk/Tools/clustalw2/index.html). Homology modeling was performed using the Swiss Model which can be accessed through http://www.swissmodel.expasy.org/SWISS-MODEL.html. Swiss model showed that NS5B is structurally homologous to a target protein with template PDB code 1g5mA (target region 3-204, 88.00 % of sequence identity.

2.5. Structural Analysis of Target Protein

Validation of 3D structure from homology modeling was performed using the Protein Geometry program and superimposed using superpose program in MOE 2009.10 software. Based on superimposed the RMSD was calculated to find out structural similarity between template model mutated with 3D structure from homology modeling. Identification of binding site of protein target was conducted by using site finder program in MOE 2009.10 software.

2.6. Optimization and Minimization of 3D Structure

Optimization and minimization of three-dimensional structure of Bcl-2 were conducted using the software of MOE 2009.10. with addition of hydrogen atoms. Protonation was employed with protonate 3D programs. Furthermore, partial charges and force field were employed with MMFF94x. Solvation of enzymes was performed in the form of a gas phase with a fixed charge, RMS gradient of 0.05 kcal/A^0 mol, and other parameters using the standard in MOE 2009.10 software.

2.7. Preparation of Compounds

Antimycin A_3 analogues were designed using ACD Labs software. With this software, the analogues were built into three-dimensional structures. The three-dimensional shape was obtained by storing the derivative in the 3D viewer in ACDLabs. Furthermore, the output format was changed into Molfile MDL Mol format using the software Vegazz to confirm for the docking process. Compounds were in the wash with compute program, adjustments were made with the compound partial charge and partial charge optimization using MMFF94xforcefield. The conformation structure energy of compounds was minimized using the RMS gradient energy with 0,001 kcal/A^omol. Other parameters were in accordance with the default setting in the software.

2.8. Molecular Docking

The docking process started with the docking preparation using a docking program from MOE 2009.10 software. Docking simulations were performed with the Compute-Simulation dock program. The placement method was conducted using a triangle matcher with 1.000.000 repetition energy reading for each position and other parameters were in accordance with the default settings in the MOE software. Furthermore, scoring functions used London DG, refinement of the configuration repetition forcefield with 1.000 populations. The first repetition was done for 100 times and the second setting was conducted only for one of the best results.

2.9. *In Vitro* Cytotoxicity Assay

Cytotoxicity evaluation of the synthesized products was carried out in Department of Anatomical Pathology Department, Faculty of Medicine, University of Indonesia. The tested laryngeal cancer Hep-2 cells are the culture collection of Anatomical Pathology Department, Faculty of Medicine, University of Indonesia. MTT assay [20, 21] was performed to measure the antiproliferation effects of synthesized amide **5**, amide **3**, amide **4**, analogue **2**, analogue **1** and the original antimycin A_3 on the laryngeal cancer Hep-2 cells. Synthesized amide **5**, amide **3**, amide **4**, analogue **2**, analogue **1** and antimycin A_3 were 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 were incubated for 48 hours and 20 μl of 5 mg/ml solution of MTT in phosphate-buffered saline (PBS) was added to triplicate samples and the plates were incubated for additional 4 hours. The plates were then centrifuged and the medium was removed. Two hundred microliters (200 μl) of DMSO was added to each well to dissolve the purple blue sediment, the absorbance was determined at 590 nm on a microplate reader (Model 550, Bio-Rad, USA). The inhibition rate can be calculated as follows:

Inhibiton rate (%) = 1 – (absorbance of treatment group/absorbance of control group) x 100%.

The 50% inhibitory concentrations (IC_{50}) of the 48 hours were calculated with Bliss assay.

3. RESULTS AND DISCUSSION

3.1. *In silico* Study

Lipinski's rule based on the measurement of Gibbs energy (ΔG value), affinity (pKi), a number of hydrogen (H) donor and hydrogen (H) acceptor, was utilized for *in silico* study of analogue **1**, analogue **2** and antimycin A_3. The results are summarized in Table **1**.

As shown, analogue **1** and **2** which have ΔG value lower than antimycin A_3 on binding targets Bcl-2, showed a higher stability than antimycin A_3. pKi value of analogue **1** and **2** is higher than pKi

Table 1. ***In silico*** **docking data of analogue 1, analogue 2 and antimycin A_3 on Laryngeal cancer receptor Bcl-2.**

Compound	ΔG (Kcal/mol)	pKi (μM)	H Acceptor/H Donor Interaction
Antimycin A_3	- 7.6831	6.395	1
Analogue 1	- 12.4488	9.930	7
Analogue 2	- 11.6086	8.527	3

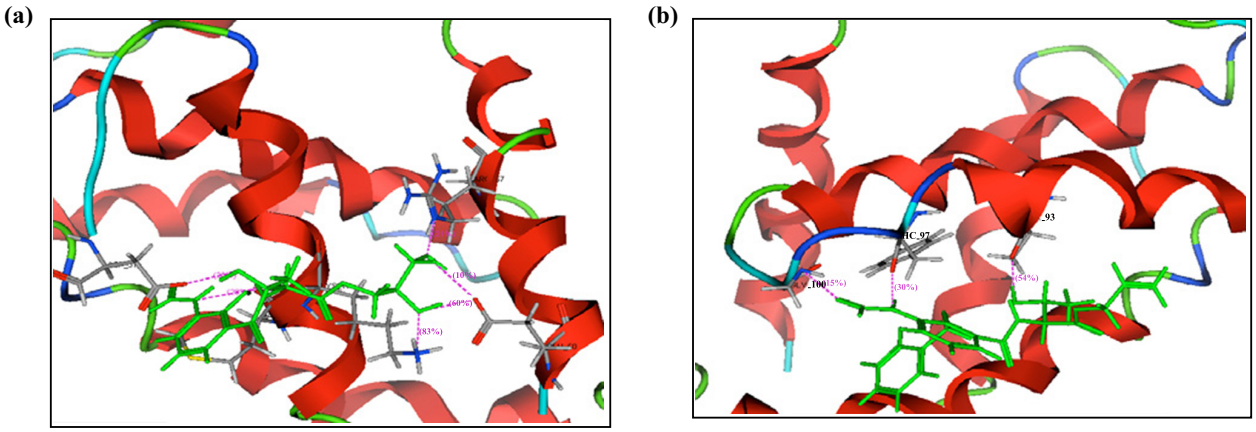


Fig. (2). Interaction of analogue **1** (**a**) And analogue **2** (**b**) On the catalytic site of Bcl-2.

value of antimycin A_3, suggesting that the analogue **1** and **2** showed better affinity than antimycin A_3. Moreover, analogue **1** and **2** have a number of Hydrogen donor-Hydrogen acceptor interactions more than antimycin A_3. Indicating that, compared to the original antimycin A_3, analogue **1** and **2** have more hydrogen bond interactions to the binding site of Bcl-2.

Ligand complex interaction of antimycin A_3, analogue **1** and analogue **2** with Bcl-2 are displayed in Fig. (**2**). Stability and affinity of the ligand complex with the active site was influenced by the bond distance and complex score. As shown in the figures and tables, analogue **1** and analogue **2** could change the conformation of all receptor target cavity, or in other words, it is able to enter the binding site of receptor target Bcl-2 of laryngeal cancer. Thus, the analogues have a stronger inhibitory activity on Bcl-2 of laryngeal cancer than antimycin A_3. These *in silico* docking suggest that it is quite possible to expect analogue **1** and analogue **2** as a potent and superior synthetic laryngeal cancer drugs.

3.2. Cytotoxicities of the Analogues

MTT cell proliferation assay was applied to determine the cytotoxicity of antimycin A_3, analogue 1, and analogue 2 on lary ngeal HEP-2 cells. The data are reported in Table **2**. Cytotoxicity is expressed by IC_{50}. The smaller the IC_{50} value, the stronger the anticancer activity.

As shown in Table **1**, amide 5 with IC_{50} over 100 μM showed no cytotoxicity against Hep-2 cells. In contrast to amide 5, the amide 3 and amide 4, which have two additional hydroxyl groups, showed improvement in cytotoxicity with concentration 59.3 μM and 84.8 μM against Hep-2 cells, respectively. The cytotoxicity of amide 3 and amide 4 was greatly improved by the presence of hydroxyl groups compared to that of amide 5. This fact suggested that the hydroxyl groups are very important for the anti-laryngeal cancer activity. Compared to amide 4, amide 3 which posses hydroxyl group with bottom facial stereochemistry, showed stronger cytotoxicity, indicating that introduction hydroxyl group with bottom facial stereochemistry was potentially responsible for increase in its anticancer activity.

Table 2. Cytotoxicities of amide 5, amide 4, amide 3, analogue 2, analogue 1, and antimycin A_3 against Hep-2 cell.

Compound	IC_{50} (μM)
Amide **5**	> 100
Amide **4**	84.8
Amide **3**	59.3
Analogue **2**	46.3
Analogue **1**	31.6
Antimycin A_3	56.8

Furthermore, 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-formamidosalicylyl moiety in both analogue **1** and analogue **2** is very necessary to

enhance its anticancer activity against Hep-2 cells. Moreover, analogue **1** and analogue **2** which contain open-chain moiety exhibited greater anticancer activity than that of the original antimycin A_3 on Hep-2 cells of laryngeal cancer, with IC_{50} : 31.6 μM and 46.3 μM, respectively. These results indicated that modifying the nine-membered dilactone core of antimycin A_3 with a hydroxylated opened-chain moiety in analogue **1** and analogue **2** successfully improved its anticancer activity.

Thus, analogue **1** and analogue **2** which strongly inhibited the growth of Hep-2 cells, should be considered as a promising candidate for the treatment of human laryngeal cancer.

CONCLUSION

Opened-chain analogues of antimycin A_3 have been synthesized from Boc-L-threonine through esterification, amidation and Sharpless asymmetric dihydroxylation. Consistent with *in sillico* study, analogue 1 and analogue 2 showed greater anticancer activity against laryngeal HEP-2 cells as compared to the original antimycin A_3.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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