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Synthesis and biological activity of new quinoxaline antibiotics of echinomycin analogues☆

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Abstract—Novel quinoxaline antibiotics having the methylenedithioether bridge as an analogue of echinomycin have been synthesized by insertion of methylene moiety between –S–S– bond. The compound **1a** shows remarkable cytotoxicities against human tumor various cell lines, and is active VRE (vancomycin-resistant enterococci) within MIC range 0.5–8 μg/mL. According to the eukaryotic or prokaryotic data, **1a** might be a first analogue to replace echinomycin.

The quinoxaline antibiotics of bicyclic octadepsipeptide[1] show activity against gram-positive bacteria[2] and certain animal tumors,[3] and also are potent inhibitors of RNA synthesis.[4] The mechanism of action apparently occurs by binding to DNA in which they function as bifunctional intercalating agents.[5] Two antibiotic families of the antibiotic echinomycin,[4,5] and the triostins,[6] are well known. Both series are similar in composition, consist of two quinoxaline-2-carboxylic acid moieties attached to a cyclic octadepsipeptide containing a sulfur cross-linkage. Echinomycin contain a thioacetal cross bridge. Few reports[7] have appeared in the synthetic studies on the quinoxaline antibiotics. In our earlier work on echinomycin, sulfonium salts of echinomycin showed stronger antitumor activity than that of echinomycin.[8]

However echinomycin or such an analogue of echinomycin having a methylenedithioether moiety has never been reported yet. In order to study the biological activity of echinomycin analogues, a series of new quinoxaline antibiotics (**1**–**4**) containing thioether, sulfoxide and sulfone moiety have been prepared. We wish to describe herein an efficient synthesis of a quinoxaline antitumor antibiotic having a djenkolic acid moiety as a sulfur cross bridge (**1a**,**b**). Djenkolic acid-[3,3′-(methylenedithio)dialanine], isolated from the djenkol bean, has a unique methylene dithioether structure corresponding a monocarba analogue of the biscystein trisulfide.[9]

The **6a** and **6b** have been achieved basically according to the method for preparing triostins, which involves the preparation of tetradepsipeptides (**5a**,**b**) as a key intermediate. Tetradepsipeptides represents one-half of the symmetrical octadepsipeptides portions of **1a** and **1b**. Fragment coupling of free C-terminal and free N-terminal tetradepsipeptide each prepared from **5a** and **5b** by removal of appropriate protecting group, gave linear octadepsipeptide possessing the complete amino acid sequence of **1a** and **1b**. Further transformations involve disulfide formation, cyclization, methylene insertion and introduction of quinoxaline chromophore provided **1a** and **1b** (Fig. 1).

The Cbz-D-Ser-Opa[10] was coupled with Boc-L-MeVal-OH[11] by using DCC-HOBt (*N*-hydroxy benzotriazole) in pyridine to give didepsipeptide [Cbz-D-Ser-Opa-Boc-L-Val], in 82% yield. After removal of the Boc group with CF_3CO_2H (TFA) in CH_2Cl_2, resulting didepsipeptides were coupled with Boc-L-MeCys(Bam)-OH[12] or Boc-L-Cys(Bam)-OH using DCC-HOBt to give tridepsipeptide [Cbz-D-Ser-Opa-L-MeVal-Boc-L-MeCys (Bam)], in 74% yield. The Boc group was removed from tridepsipeptide and then reacted with $(Boc\text{-}L\text{-}Ala)_2O$ to give tetradepsipeptide [Cbz-D-Ser-Opa-L-MeVal-L-MeCys(Bam)-Boc-L-Ala] (**5a**). The protected tetradepsipeptides (**5a**)

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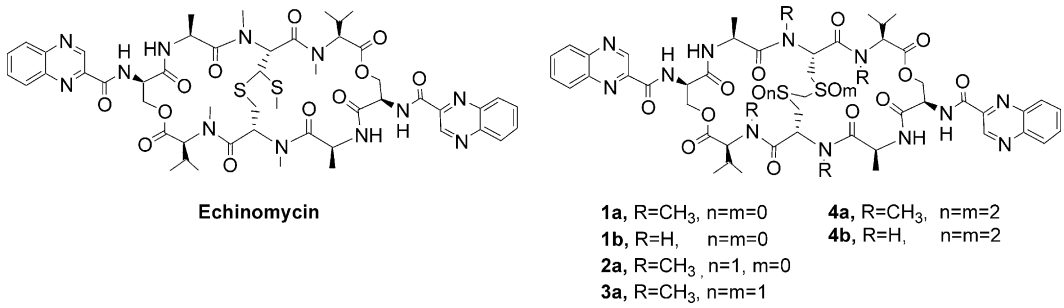
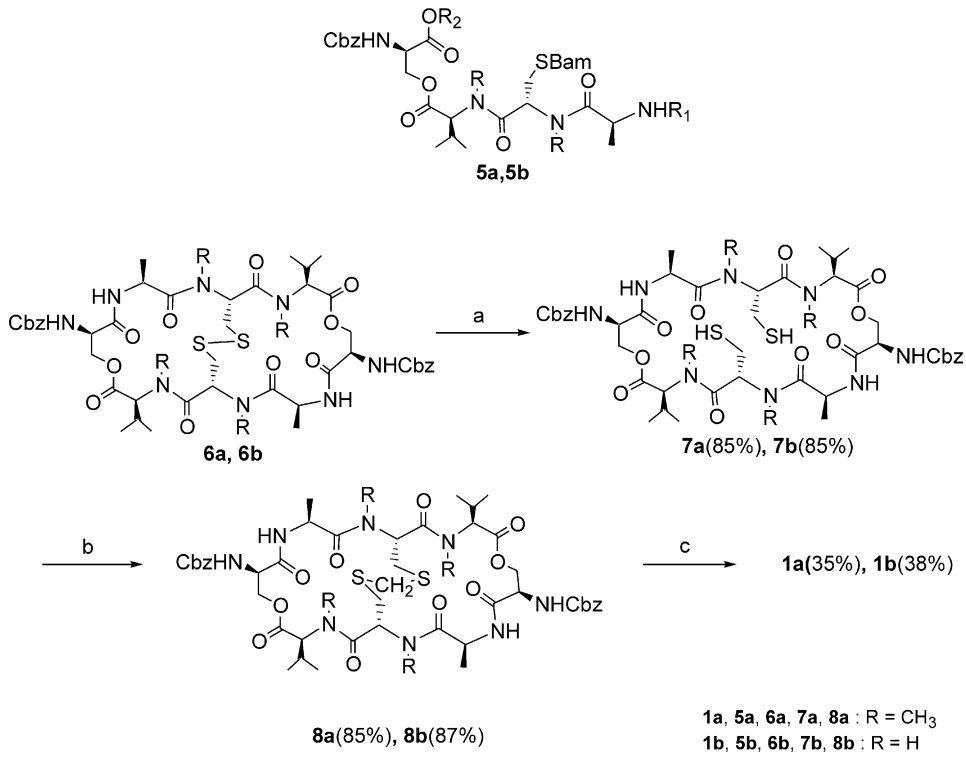


Figure 1. Structure of echinomycin and new quinoxaline antibiotics.



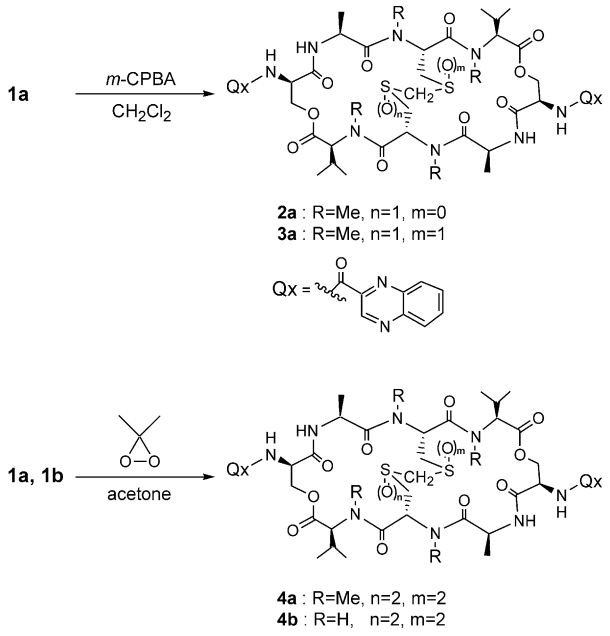
Scheme 1. (a) NaSeH, EtOH, 0 °C; (b) TBAF–xH$_2$O, CH_2Cl_2; (c) (i) 30% HBr in AcOH; (ii) QxCl, Et_3N, DMF.

was treated with Zn in 90% aqueous acetic acid effected reductive cleavage of the phenylacyl ester function to provide tetradepsipeptide[Cbz-D-Ser-L-MeVal-L-MeCys (Bam)-Boc-L-Ala], having a free C-terminal carboxyl group. Tetradepsipeptide [Cbz-D-Ser-Opa-L-Val-L-MeCys(Bam)-L-Ala] was prepared by treatment of **5a** with TFA-CH_2Cl_2 as the trifluoroacetate salt. The coupling of free C-terminal and free N-terminal tetradepsipeptide with DCC-HOBt in THF afforded the linear octadepsipeptide [Cbz-D-Ser-Opa-L-MeVal-L-MeCys(Bam)-Boc-L-Ala-Cbz-D-Ser-L-MeVal-L-MeCys (Bam)-Boc-L-Ala] in 65% yield. Cyclization of linear octadepsipeptide to provide the 26-membered cyclic octadepsipeptides (**6a**) with ring closure was accomplished through four steps sequential Pa ester deprotection (Zn, 90% aqueous AcOH, 0 °C, 4 h),[13] disulfide bond formation of free C-terminal linear octadepsipeptide (disulfide-linkage octadepsipeptide, I_2, CH_2Cl_2–MeOH, 25 °C),[14] Boc deprotection, and cyclization was followed by treatment with [Cbz-D-Ser-L-MeVal-L-MeCys(Bam)-Boc-L-Ala-Cbz-D-Ser-L-Me-Val-L-MeCys(Bam)-L-Ala] [10.0 equiv of 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDCI), HOBt, CH_2Cl_2, 0 °C, 24 h].

The synthesis of novel quinoxaline antibiotic was achieved through four steps from **6a**. Reductive cleavage of the disulfide gave dithiol (**7a**) by ethanolic NaSeH.[15] A methylene insertion to construct an S–CH_2–S bridge between two *N*-Me cysteine residue was performed by using tetrabutylammonium fluoride hydrate in CH_2Cl_2 (**8a**).[16] Removal of the benzyloxycarbonyl group (HBr in acetic acid) and acylation with 2-quinoxalyl chloride[17] gave **1a**. The **1b**, **7b**, and **8b** were prepared by the same method described for **1a** and their yields are shown in Scheme 1.

Oxidation of **1a** and **1b** with *m*-CPBA or dimethyldioxirane provided the corresponding monosulfoxide (**2a**), disulfoxide (**3a**) and disulfone (**4a**,**b**) (Scheme 2).

Anticelluar activities of new compounds were evaluated in vitro against various cell lines.[18] The results are summarized in Table 1.



Scheme 2. Oxidation of **1a** by *m*-CPBA and **1a**,**b** by dimethyldioxirane.

Table 1. MTT assay for IC_{50}[a] values of novel antibiotics on various cell lines

	1a	1b	2a	3a	4a	4b	Echinomycin
HT-29 (colon)	5.0	>20	>20	>20	>20	>20	2.2
PANC-1 (pancreas)	4.0	>20	>20	>20	>20	>20	1.8
BeWO (placenta)	2.7	>20	>20	>20	>20	>20	1.
B16 (mouse myeloma)	1.4	>20	>20	>20	>20	>20	0.4

[a] IC_{50} was defined as the concentration that caused 50% inhibition of cell growth (unit: μg/mL).

As expected the new echinomycin analogue **1a** shows a remarkable IC_{50} effect. However, others of **1b**, **2a**, **3a**, **4a** and **4b** show low biological activities.

Novel compounds **1–4** were designed to circumvent echinomycin's hydrophobicity as well as to attenuate immune cell toxicity.[19] Of novel analogues, **1a** clearly enabled to induce apoptosis of HT-29 cells (Table 2). The signaling mechanism exerted by echinomycin or **1a** is differential in inducing apoptosis of cancer cells (data not shown). It is noteworthy that **1a** had comparable cytotoxicity against solid cancer cells compared to echinomycin via novel signaling pathway.

Moreover, **1a** is active VRE (vancomycin-resistant enterococci) within MIC range 0.5–8.0 μg/mL (cf. echinomycin: 0.25 μg/mL). Echinomycin and related compounds owe their antitumor and antimicrobial activities to the binding ability to DNA which they do by the mechanism of bifunctional intercalation[20] as well as signaling inhibition. This mechanism of action would suggest the plausible antimicrobial actions against VRE. However, clinical trials of echinomycin raised the need to broaden the therapeutic margins as well as to reduce the toxicity. This disadvantage of echinomycin may be overcome by **1a**, analogues of echinomycin.

Table 2. Apoptosis induced in HT-29 by various stimuli using FACScan

Stimuli	Percentage of apoptotic cells
Control	9.6±1.1
Echinomycin	47.5±0.9
1a	28.9±0.8

HT-29 cells were treated with echinomycin (2 μg/mL) and **1a** (10 μg/mL) for 24 h (each concentration is the lowest one to initiate the apoptosis of HT-29 cell).
The percentage of apoptotic cells was assessed by flow cytometry.
Results are expressed as mean±SEM of at least three separate experiments.

In summary, the synthesis of new compounds **1a**, **1b**, **2a**, **3a**, **4a** and **4b** were successfully achieved. According to the eukaryotic or prokaryotic data, the novel compound **1a** might be a first analogue to replace echinomycin.

Acknowledgements

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18. Drug effect on cellular viability was evaluated using an assay based on the cleavage of the yellow dye MTT to purple formazan crystals by dishydrogenase activity in mitochondria, a conversion that occurs only in living cells. Exponential growing cells were inoculated to $2\times10^4\sim5\times10^4$ cells/well using 96-well plates supplemented with 200 μL RPMI-1640 medium. After the cell treated with drug, cells were incubated for 72 h, 20 μL MTT (5 mg/mL, sigma) was added and the plates were incubated at 37 °C for 4 h. To dissolve formazan, 150 μL DMSO was added and the plates were incubated at rt and subjected to measurement at 540 nm by spectrophotometer. The IC_{50} values were determined by plotting the logarithm of the drug concentration versus the growth rate of the treated cells.
19. The **1a** and **1b** were synthesized for attenuating immune cell toxicity, and then **2–4** were synthesized for increasing hydrophobicity (**1a**,**b**).
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