The Journal of Antibiotics (2018) 71:535–537
https://doi.org/10.1038/s41429-018-0028-0

JARA Japan Antibiotics Research Association
The Society for Actinomycetes Japan

BRIEF COMMUNICATION

Dipyrimicin A and B, microbial compounds isolated from *Amycolatopsis* sp. K16-0194

Shoko Izuta[1] · Shohei Kosaka[2] · Makoto Kawai[2] · Rei Miyano[1] · Hirotaka Matsuo[1,3] · Atsuko Matsumoto[1,3] · Kenichi Nonaka[1,3] · Yōko Takahashi[3] · Satoshi Ōmura[3] · Takuji Nakashima[1,3]

Received: 20 September 2017 / Revised: 21 December 2017 / Accepted: 27 December 2017 / Published online: 20 February 2018

Abstract

In a search for compounds interacting with ergosterol resin, a new compound named dipyrimicin B was isolated from a rare actinomycete strain, *Amycolatopsis* sp. K16-0194. In addition, another analog, dipyrimicin A, which does not interact with the resin, was also discovered. The structures of the two dipyrimicins were established by comprehensive 1D and 2D NMR and MS analyses and found to contain a unique core structure, a 2,2′-bipyridine skeleton. Dipyrimicin A showed strong antimicrobial and cytotoxic activity, whereas dipyrimicin B displayed distinctly poor antimicrobial and cytotoxic activities.

Ergosterol is an essential component for fungal growth because it is included in fungal cell membranes and controls membrane fluidity. Antifungal drugs that target ergosterol, such as amphotericin B [1] and nystatin [2] (both polyene drugs), have been widely used to treat serious systemic fungal infections. In particular, amphotericin B has been used clinically for more than five decades due to its potent fungicidal activity, and relatively few examples of drug resistance to this antibiotic have been reported [3]. Amphotericin B has been a mainstay of antifungal therapy, despite there being problems with potential adverse events after intravenous administration and the drug's poor aqueous solubility [4]. New antifungal drugs will hopefully minimize or overcome these problems and improve antifungal therapy safety.

Based on this concept, we developed silica-binding ergosterol (Si-ES) resin and searched for natural products interacting with ergosterol from secondary metabolites of Actinomycetes. Physico–chemical properties of compounds interacting with Si-ES resin were analyzed by LC-UV and LC/MS as previously described [5]. First, a culture broth including amphotericin B produced by an Actinomycete strain was run through an Si-ES resin column. After washing with water, amphotericin B could be eluted by methanol. Polyenes as well as diketopiperazines and flavonoids without antifungal activity into the culture broths were also found to interact with Si-ES resin (data not shown). In our tests, a compound interacting with Si-ES resin from a culture broth of *Amycolatopsis* sp. K16-0194 was detected by HPLC (Figure S1). The MS and UV spectra were compared with those of known compounds using an existing database, the Dictionary of Natural Products (http://dnp.chemnetbase.com/), and the compound was deduced to be a new compound. An analog that does not interact with Si-ES resin was also found from the same cultured broth using data of MS analysis and UV spectra. These compounds possess a 2,2′-dipyridine core skeleton and were named dipyrimicin A (**1**) and B (**2**).

Si-ES resin was prepared via a two-step reaction: silica gel surface modification with 3-(trimethoxysilyl)propanethiol and thiol-ene reaction of thiolated silica gel with ergosterol. Si-ES resin was manufactured as follows; 20 g of silica gel (Chromatorex MB100-75/200, Fuji Silysia Chemical Ltd., Aichi, Japan) was put in a 500-ml separable flask and 300 ml of toluene was added. The slurry was

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41429-018-0028-0) contains supplementary material, which is available to authorized users.

✉ Takuji Nakashima
takuji@lisci.kitasato-u.ac.jp

[1] Graduate School of Infection Control Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

[2] Team Mirai Group, Fuji Silysia Chemical Ltd., 2-1846 Kozoji-cho, Kasugai, Aichi 487-0013, Japan

[3] Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

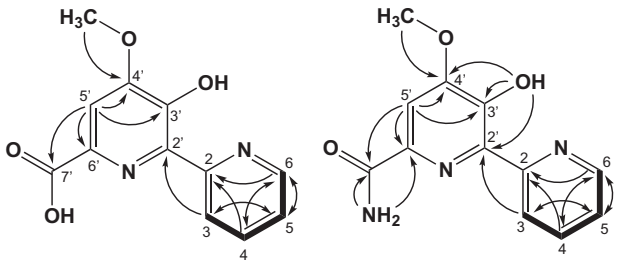

Fig. 1 Structures of dipyrimicin A (**1**) and B (**2**) isolated from a cultured broth of *Amycolatopsis* sp. K16-0194

agitated and reacted following the addition of 5.9 g of 3-(trimethoxysilyl)propanethiol for 8 h at 80 °C under a nitrogen gas atmosphere. After cooling, the reaction mixture was washed with toluene and methanol. The resin was harvested by drying at 80 °C for 12 h. The yield of resin with thiol groups on the surface of silica gel was 21.5 g. Subsequently, 20 g of silica gel with thiol groups was put in a 500 ml separable flask and mixed with 300 ml of 1,4-dioxane. The slurry was agitated with 4.0 g of ergosterol. Subsequently, 80 mg of azobisisobutyronitrile as a radical initiator was added and reacted for 14 h at 85 °C under a nitrogen gas atmosphere. After cooling, the resin was washed with toluene, tetrahydrofuran and methanol. The resin was harvested by filtration and dried at 80 °C for 12 h. The yield of resin with ergosterol on the surface of the silica gel was 20.7 g.

Fermentation, isolation and purification of **1** and **2** are shown in Scheme S1 of the Supplementary Information. *Amycolatopsis* sp. K16-0194 was cultured at 27 °C for 9 days with rotary shaking in 500 ml Erlenmeyer flasks each containing 100 ml of a production medium. The mycelial cake was extracted with MeOH and concentrated in vacuo. The MeOH extract was subjected to flash column chromatography on Diaion HP-20. The fractions with **1** were collected and concentrated in vacuo. Finally, **1** was purified by ODS flash column chromatography. The yield of **1** was 86.7 mg. The supernatant was extracted with EtOAc and concentrated in vacuo. The EtOAc extract was subjected to flash column chromatography on Si-ES resin. Compound **2** was easily purified using Si-ES resin and the yield of **2** was 16.6 mg.

The Physico-chemical properties of **1** and **2** are shown in Table S1. Compound **1** was isolated as a yellow powder ($[\alpha]_D^{25.0}$ −9.74, *c* 0.1, MeOH) that gave a $[M + H]^+$ ion at *m/z* 247.0714 in the HR-ESIMS. This was consistent with the molecular formula $C_{12}H_{10}N_2O_4$ (calculated for $C_{12}H_{11}N_2O_4$, 247.0719). Compound **2** was isolated as a yellow powder ($[\alpha]_D^{25.0}$ −2.74, *c* 0.1, MeOH) that gave a $[M + H]^+$ ion at *m/z* 246.0884 in the HR-ESIMS. This was consistent with the molecular formula $C_{12}H_{11}N_3O_3$ (calculated for $C_{12}H_{12}N_3O_3$, 246.0879). The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectral data of **1** and **2** are shown in Table S2. The ^{1}H NMR spectrum of **2** in DMSO-d_6 displayed five olefin protons from 8.96 to 7.55 ppm, one methoxy (δ_H 3.93), one hydroxyl group (δ_H 14.9), and one primary amide (δ_H 8.30 and 7.50 ppm). The ^{13}C NMR spectrum showed the resonances of 12 carbons, which were classified into one carbonyl carbon at 165.9 ppm, ten olefinic and aromatic carbons from 156.9 to 106.0 ppm, and one oxygenated sp^3 methyl carbon (δ_c 55.8 ppm) by HSQC spectra (Figures S9-S11). As shown in Fig. 1, the ^{1}H–^{1}H COSY of **2** indicated the presence of one partial structure from C-3 to C-6. The ^{1}H-^{13}C HMBC analysis confirmed the presence of a hydroxyl at the 3′ position, a methoxy at the 4′ position and an amide at the 6′ position. Finally, the structure of two pyridines linked to C-2 and C-2′ was confirmed by the HMBC correlation from H-3 to C-2′ (Figure S13) and the ^{1}H-^{15}N HMBC (Figure S14). In addition, the structure of **2** was supported by the chemical shifts of cyanogriside F that are compounds with a 2,2′-bipyridine moiety [6]. The low-field chemical shift (δ_H 14.9) of the hydroxyl proton at the 3′ position indicates the presence of strong intramolecular hydrogen-bonding of this hydroxyl group with the nitrogen atom, as well as cyanogriside [6] and caerulomycin B [7, 8].

The NMR chemical shifts (Figures S3-S8) and the physico-chemical properties (Table S2) of **1** were similar to those of **2**. The differences between compounds were determined by molecular formula and the HMBC correlation from the amino group of **2** (from δ_H 8.30 and 7.51 to δ_c 141.8 ppm). The ^{1}H NMR spectrum of **1** in DMSO-d_6 displayed five olefin protons from 8.73 to 7.58 ppm, one methoxy (δ_H 3.93) and two hydroxyl groups (δ_H 15.0 and 12.7). The ^{13}C NMR spectrum showed the resonances of 12 carbons, which were identified as one carbonyl carbon at 165.6 ppm, ten olefinic and aromatic carbons from 156.6 to 108.6 ppm, and one oxygenated sp^3 methyl carbon (δc 56.0 ppm) by HSQC spectra (Figures S3–S5). As shown in Fig. 1, the ^{1}H-^{1}H COSY of **1** indicated the presence of one partial structure from C-3 to C-6. The structure of two pyridines linked to C-2 and C-2′ was confirmed by the ^{1}H-^{13}C HMBC correlation from H-3 to C-2′ (Figure S7).

Antimicrobial and cytotoxic activities of dipyrimicin A (**1**) and B (**2**) were evaluated by the paper disk method and Cell Counting Kit-8 assays (see Supplementary Information S1. Biological activites). As shown in Table 1, dipyrimicin A (**1**) showed potent antimicrobial activity, except against *Aspergillus niger*, and was cytotoxic against all cell lines tested. Conversely, dipyrimicin B (**2**), with an amide group instead of the carboxy group of **1**, demonstrated remarkably poor antimicrobial and cytotoxic activity, with the being active only against *Escherichia coli* among the eight microbes tested. In addition, the cytotoxic effect of **2** on H1299 (a human non-small cell lung carcinoma cell line)

Table 1 Biological activities of dipyrimicin A (**1**) and B (**2**)

Antimicrobial activity				
Tested microorganisms	Dipyrimicin A (**1**)		Dipyrimicin B (**2**)	
	100 μg	30 μg	100 μg	30 μg
Candida albicans ATCC 64548	11[a]	–	–	–
Saccharomyces cerevisiae ATCC 9763	26	16	–	–
Aspergillus niger ATCC 6275	–	–	–	–
Mucor racemosus IFO 4581	18	–	–	–
Kocuria rhizophila ATCC 9341	27	18	–	–
Bacillus subtilis ATCC 6633	22	19	–	–
Escherichia coli NIHJ	23	16	12	–
Xanthomonas campestris pv. *oryzae* KB 88	27	21	–	–

Cytotoxic activity		
Cell lines	Dipyrimicin A (**1**)	Dipyrimicin B (**2**)
HeLa 3S	5.1 ± 0.5[b]	24.6 ± 13.9
HT29	6.2 ± 0.3	72.1 ± 27.0
A549	4.3 ± 0.2	>100
H1299	9.2 ± 0.5	6.8 ± 3.3
Panc1	9.4 ± 3.5	>100
THP-1	4.3 ± 0.6	>100
Jarkat	4.4 ± 0.5	>100
HL-60	3.9 ± 0.7	>100

[a] Numbers represent clear zone (mm) of growth inhibition (paper disk, φ 6 mm)

[b] IC_{50} values (μM)

was equal to or more than that of **1**. Although these structures were described in the SciFinder® chemical data base as CAS numbers 1235020-43-5 and 1332747-97-3, only structure details are present and no data on physico–chemical properties, NMR chemical shifts, bioactivity, etc. Therefore, we named them dipyrimicin A and B. A number of 2,2′-dipyridine compounds have been discovered as secondary metabolites of Actinomycetes, such as the caerulomycins, including glycosides, from *Streptomyces caeruleus* [7] and *Actinoalloteichus cyanogriseus* [6, 9], and the collismycins [10]. However, the 2,2′-dipyridine core structures are the first reported from secondary metabolites in *Amycolatopsis* species.

In conclusion, we isolated dipyrimicin B (**2**) which interacts with Si-ES resin from the culture broth of *Amycolatopsis* sp. K16-0194. Dipyrimicin A (**1**), which does not interact with the resin was also isolated from the same broth. Dipyrimicin A (**1**) and B (**2**) contain a unique 2,2′-dipyridine core skeleton. It was strongly suggested that an amide group with **2** facilitates the interaction with the Si-ES resin. Dipyrimicin A (**1**) showed potent antimicrobial and cytotoxic activities but dipyrimicin B (**2**) displayed only weak activities. It is suggested that the different in carboxyl and amide groups of dipyrimicins is the primary factor involved in the interaction with Si-ES resin and in bestowing antimicrobial and cytotoxic properties. Although the interaction mechanism between Dipyrimicin B (**2**) and Si-ES resin is unclear, we are continuing to search for new natural products with antifungal activity using Si-ES resin.

Acknowledgements This project was supported by JSPS KAKENHI Grant Number 17K08342 and the Institute for Fermentation, Osaka (IFO), Japan. We are grateful to Dr Masato Iwatsuki, Kitasato Institute for Life Sciences, Kitasato University, Mr Hirohumi Honda, Fuji Silysia Chemical Ltd., for helpful advice, and Dr Kenichiro Nagai and Ms Noriko Sato, School of Pharmacy, Kitasato University, for measurement of MS and NMR spectra.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Donovick R, Gold W, Pagano JF, Stout HA. Amphotericins A and B, antifungal antibiotics produced by *Streptomycetes*. I. In vitro studies. Antibiot Ann. 1955;3:579–86.
2. Raubitscheck F, Acker RF, Waksman SA. Production of an antifungal agent of the fungicidin type by *Streptomyces aureus*. Antibiot Chemother (North). 1952;2:179–83.
3. Cantón E, Pemán J, Gobernado M, Viudes A, Espinel-Ingroff A. Patterns of amphotericin B killing kinetics against seven *Candida* species. Antimicrob Agents Chemother. 2004;48:2477–82.
4. Kalepu S, Nekkanti V. Insoluble drug delivery strategies: review of recent advances and business prospects. Acta Pharm Sin B. 2015;5:442–53.
5. Nakashima T, Takahashi Y, Ōmura S. Search for new compounds from Kitasato microbial library by physicochemical screening. Biochem Pharmacol. 2017;134:42–55.
6. Fu P, et al. Acyclic congeners from Actinoalloteichus cyanogriseus provide insights into cyclic bipyridine glycoside formation. Org Lett. 2014;16:4264–7.
7. McInnes AG, Smith DG, Wright JLC, Vining LC. Caerulomycin B and C, new 2,2′-dipyridyl derivatives from *Streptomyces caeruleus*. Can J Chem. 1977;55:4159–65.
8. Mongin F, Trécourt F, Gervais B, Mongin O, Quéguiner G. First synthesis of caerulomycin B. A new synthesis of caerulomycin C. J Org Chem. 2002;67:3272–6.
9. Fu P, et al. Cytotoxic bipyridines from the marine-derived actinomycete *Actinoalloteichus cyanogriseus* WH1-2216-6. J Nat Prod. 2011;74:1751–6.
10. Gomi S, Amano S, Sato E, Miyadoh S, Kodama Y. Novel antibiotics SF2738A, B and C, and their analogs produced by *Streptomyces* sp. J Antibiot. 1994;47:1385–94.