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ORIGINAL ARTICLE

Sinefungin VA and dehydrosinefungin V, new antitrypanosomal antibiotics produced by *Streptomyces* sp. K05-0178

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Two new nucleotide antibiotics, named sinefungin VA and dehydrosinefungin V, were separated by cation exchange column chromatography and purified by HPLC from the culture broth of *Streptomyces* sp. K05-0178, together with the known antibiotics, sinefungin, dehydrosinefungin and KSA-9342. The structures of the two novel sinefungin analogs were elucidated by spectroscopic studies, including various NMR and advanced peptide chemical methods. Sinefungin VA consists of adenosine and ornithylvalylalanine, whereas dehydrosinefungin V consists of 4′,5′-dehydroadenosine and ornithylvaline. Sinefungin VA showed potent antitrypanosomal activity with an IC_{50} value of 0.0026 μg ml^{-1} in vitro without cytotoxicity against MRC-5 cells. Dehydrosinefungin V showed moderate antitrypanosomal activity (IC_{50}=0.15 μg ml^{-1}).
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Keywords: antitrypanosomal antibiotics; dehydrosinefungin V; sinefungin VA; *Streptomyces*

INTRODUCTION

Human African trypanosomiasis, also known as sleeping sickness, is regarded as one of the major neglected tropical diseases. It is a complex disease caused by infection with protozoan *Trypanosoma* spp. parasites, transmitted through the bites of infected tsetse flies. There are two forms of the disease, each having two distinct stages, and without treatment the disease is fatal. Today, some 70 million people are exposed to the threat of human African trypanosomiasis.[1] It was estimated that, in 2004, approximately 50 000–70 000 new cases of human African trypanosomiasis were occurring annually.[1] Currently, only four drugs, pentamidine, suramin, melarsoprol and eflornithine, are registered for the treatment of human African trypanosomiasis. All four are unsatisfactory, as they cannot be administered orally and are hampered by severe toxicity and increasing resistance of the parasites. Although there is a clear and urgent need for new classes of antitrypanosomal compounds, and despite the recent introduction of a new treatment using a combination of two existing drugs, no effective new drugs have been introduced into the field for 20 years.

During our screening program to discover new antitrypanosomal antibiotics, two novel sinefungin analogs, sinefungin VA (**1**) and dehydrosinefungin V (**2**), and three recognized structurally related compounds, sinefungin (**3**), dehydrosinefungin (**4**) and KSA-9342 (**5**), were isolated from a culture broth of *Streptomyces* sp. K05-0178 (Figure 1). In this paper, the fermentation, isolation, physicochemical properties and structure elucidation and biological properties of **1** and **2** are described.

RESULTS

Taxonomy of the producing strain K05-0178

The strain K05-0178 was isolated from a soil sample collected at Minato-ku, Tokyo, Japan. The vegetative mycelia developed well on yeast extract–malt extract agar, oatmeal agar and inorganic salts–starch agar and did not show fragmentation into coccoid forms or bacillary elements. The color of vegetative mycelia showed red. Aerial mycelia were produced abundantly on inorganic salts–starch agar and the aerial mass color showed pink. The strain formed verticillate sporophores bearing terminal umbels of spore chains (Figure 2). The mature spore chain had 5–10 spores per chain. The spores were cylindrical in shape, 1.0×0.5 μm in size and had a smooth surface. Brownish soluble pigment was produced on yeast extract–malt extract agar. The isomer of diaminopimelic acid in whole-cell hydrolysates of strain K05-0178 was determined to be the LL-form. An almost complete 16S rDNA sequence (1400 nucleotides) was determined, and the result of the identification analysis by EzTaxon.org server version 2.1 (http://147.47.212.35:8080/) showed the highest similarity

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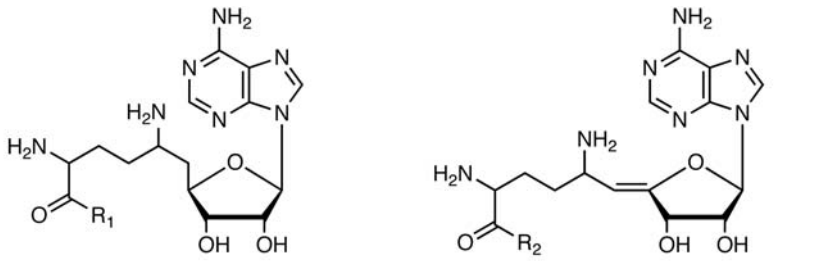


	R_1
Sinefungin VA (**1**)	L-Val-L-Ala
Sinefungin (**3**)	OH

	R_2
Dehydrosinefungin V (**2**)	L-Val
Dehydrosinefungin (**4**)	OH
KSA-9342 (**5**)	L-Val-L-Ala

Figure 1 Structures of sinefungin VA (**1**), dehydrosinefungin V (**2**), sinefungin (**3**), dehydrosinefungin (**4**) and KSA-9342 (**5**).

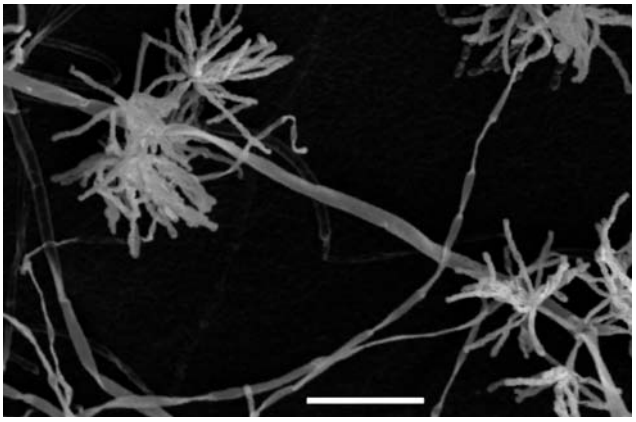


Figure 2 Scanning electron micrograph of the strain K05-0178 grown on 1/10 V-8 juice agar at 27 °C for 18 days. Bar represents 10 μm.

value with *Streptomyces coerulescens* NBRC 12758^T (AB184122, 99.6%) and *Streptomyces abikoensis* NBRC 13860^T (AB184537, 99.6%). On the basis of the morphological and cultural properties and 16S rDNA sequence similarity, the strain was identified with whorl-forming *Streptomyces* species and designated *Streptomyces* sp. K05-0178.[2,3]

Isolation

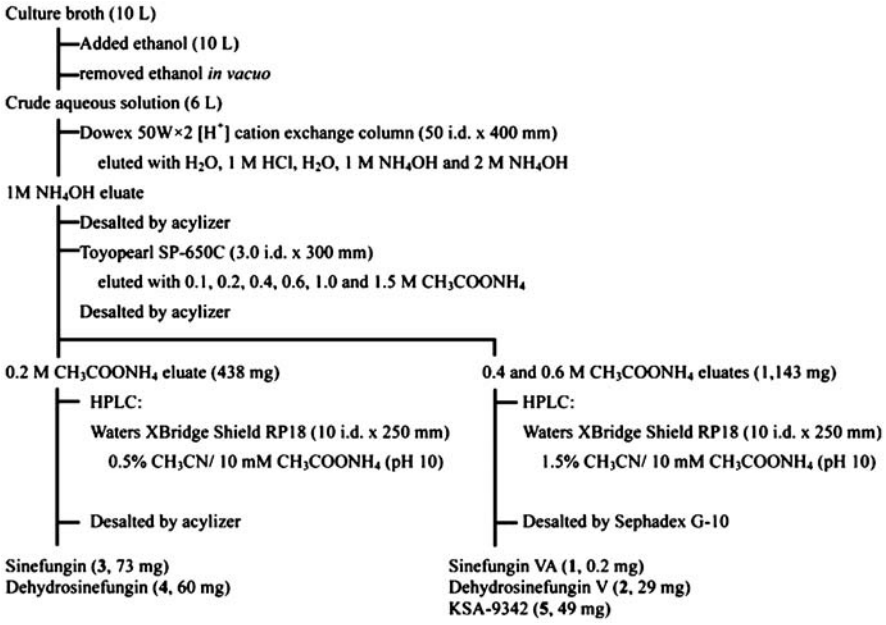
The procedure for isolation of sinefungin analogs is summarized in Scheme 1. The 6-day-old culture broth (10 l) was extracted with 10 l of ethanol and filtered through a paper to separate into supernatant and mycelia. The supernatant was concentrated under reduced pressure to remove ethanol and then passed through a Dowex 50 W×2 [H^+] column (50 i.d. ×400 mm, Dow Chemical, Midland, MI, USA) previously activated. After washing with water (600 ml), the active materials were eluted with 1.0 M NH_4OH (600 ml), followed by neutralization by HCl and desalted by a Micro Acylizer S3 (ASTOM, Tokyo, Japan). The whole eluate was applied on a Toyopearl SP-650C column (3.0 i.d. ×300 mm, Tosoh Co., Tokyo, Japan) previously equilibrated with 0.1 M CH_3COONH_4 buffer (pH 3.8). After washing with 0.1 M CH_3COONH_4 buffer (pH 3.8, 180 ml), the active materials were eluted with 0.2, 0.4 and 0.6 M CH_3COONH_4 buffers (pH 3.8, 180 ml each). The 0.4 and 0.6 M CH_3COONH_4 fractions were neutralized by NH_4OH, desalted by the Micro Acylizer and concentrated *in vacuo* to dryness to yield a brown material (1143 mg). The material was dissolved in a small amount of water and purified by HPLC on an XBridge Shield RP18 column (10 i.d. ×250 mm, Waters Milford, MA, USA) with 1.5% CH_3CN/10 mM CH_3COONH_4 (pH 10) at 4.0 ml min^{-1} detected at UV 210 nm. The retention times of sinefungin VA (**1**), dehydrosinefungin V (**2**) and KSA-9342 (**5**) were 33, 42 and 55 min, respectively (Figure 3a). Each solution was neutralized by HCl, desalted by Sephadex G-10 (GE Healthcare, Buckinghamshire, UK) gel filtration chromatography and concentrated *in vacuo* to dryness to afford **1** (0.2 mg), **2** (29 mg) and **5** (49 mg) as pale brown powders. On the other hand, the 0.2 M CH_3COONH_4 fraction was neutralized, desalted by the Micro Acylizer and concentrated *in vacuo* to dryness to yield a brown material (438 mg). It was dissolved in a small amount of water and purified by HPLC on an XBridge Shield RP18 column (10 i.d. ×250 mm) with 0.5% CH_3CN/10 mM CH_3COONH_4 (pH 10) at 4.0 ml min^{-1} detected at UV 210 nm. The retention times of sinefungin (**3**) and dehydrosinefungin (**4**) were 9 and 13 min, respectively (Figure 3b). Each solution was neutralized by HCl, desalted by the Micro Acylizer and concentrated *in vacuo* to dryness to afford **3** (73 mg) and **4** (60 mg) as pale brown powders.

Physicochemical properties

The physicochemical properties of sinefungin VA (**1**) and dehydrosinefungin V (**2**) are summarized in Table 1. They are both readily soluble in water, methanol and acetone. They showed absorption maxima at 206 and 260 nm in UV spectra. The broad IR absorption at 3300 cm^{-1} suggested the presence of hydroxyl groups. The similarity in physicochemical properties strongly suggested that they are structurally related to sinefungin (**3**).

Identification of sinefungins, dehydrosinefungin and KSA-9342

The molecular formulae of **3**–**5** were elucidated by HR-FAB-MS to be $C_{15}H_{23}N_7O_5$, $C_{15}H_{21}N_7O_5$ and $C_{23}H_{35}N_9O_7$, respectively (Table 1). The analysis of ^1H, ^{13}C and 2D NMR spectra data led to the identification of **3**–**5** as sinefungin,[4] dehydrosinefungin[5] and KSA-9342,[6] respectively. As the assignments of their chemical shifts have not been reported, the data are shown in Table 2.



Scheme 1 Purification of sinefungin analogs.

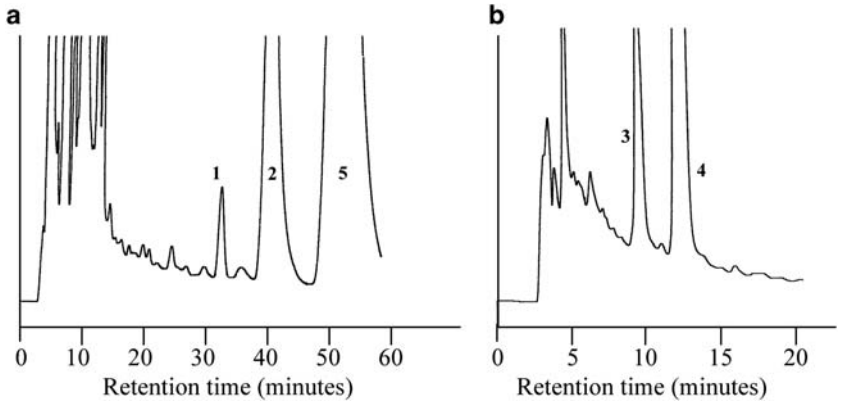


Figure 3 Chromatographic profile of (**a**) sinefungin VA (**1**), dehydrosinefungin V (**2**) and KSA-9342 (**5**) and (**b**) sinefungin (**3**) and dehydrosinefungin (**4**) by preparative HPLC.

Structure elucidation of sinefungin VA (1)

The molecular formula of **1** was elucidated by HR-FAB-MS to be $C_{23}H_{37}N_9O_7$, requiring 10 degrees of unsaturation. The ^1H and ^{13}C NMR spectral data of **1** are listed in Table 2. The ^{13}C NMR and heteronuclear multiple quantum coherence (HMQC) spectra indicated 23 carbons, which were classified into one carboxyl carbon at δ_c 181.3, two amide carbonyl carbons, five olefinic carbons, one hemiaminal carbon at δ_c 88.8, three oxygenated sp^3 methine carbons, four nitrogenated sp^3 methine carbons, one sp^3 methine carbon, three sp^3 methylene carbons and three methyl carbons. The characteristic UV spectra and fragment ion (*m/z* 135) in FAB-MS indicated the presence of an adenine moiety. Moreover, the spin systems from H-2 to H-10, deduced by the COSY, and the correlations from H-2 to C-1 and C-3, from H-5 to C-7, from H-8 and H-9 to C-10, and from H-10 to C-11 and C-15 of the adenine moiety in the heteronuclear multiple-bond correlation (HMBC) spectra, revealed **1** was a sinefungin analog (Figure 4). This was confirmed by comparison with the NMR spectra of sinefungin (Table 2). The spin systems from H-2′ to H_3-5′ and from H-2″ to H_3-3″ by the COSY and the correlations from H-2′ and H-2″ to C-1′ and from H_3-3″ to C-1″ in the HMBC spectra indicated the presence of the valylalanine moiety. The correlations from H-2′ to C-1 in HMBC revealed the C-1 of the sinefungin moiety was attached to the amino group of valylalanyl moiety, to form an amide bond. This was confirmed by fragment ions of *m/z* 463 $[M–Ala+H]^+$ and *m/z* 346 $[M–Val-Ala+H]^+$. The absolute configurations of amino acid residues of **1** were elucidated to be L-Val and L-Ala by HPLC analysis of the hydrolysates of **1** using a chiral column. Thus, the structure of **1** is shown in Figure 1, and the compound was designated as sinefungin VA.

Structure elucidation of dehydrosinefungin V (2)

The molecular formula of **2** was elucidated by HR-FAB-MS to be $C_{20}H_{30}N_8O_6$, requiring 10 degrees of unsaturation. The ^1H and ^{13}C NMR spectral data of **2** are listed in Table 2. The ^{13}C NMR and HMQC spectra indicated 20 carbons, which were classified into one carboxyl carbon at δ_c 178.4, one amide carbonyl carbon at δ_c 169.1, seven olefinic carbons containing one oxygenated olefinic carbon at δ_c 159.9, one hemiaminal carbon at δ_c 87.9, two oxygenated sp^3 methine carbons, three nitrogenated sp^3 methine carbons, one sp^3 methine carbon, two sp^3 methylene carbons and two methyl carbons. The

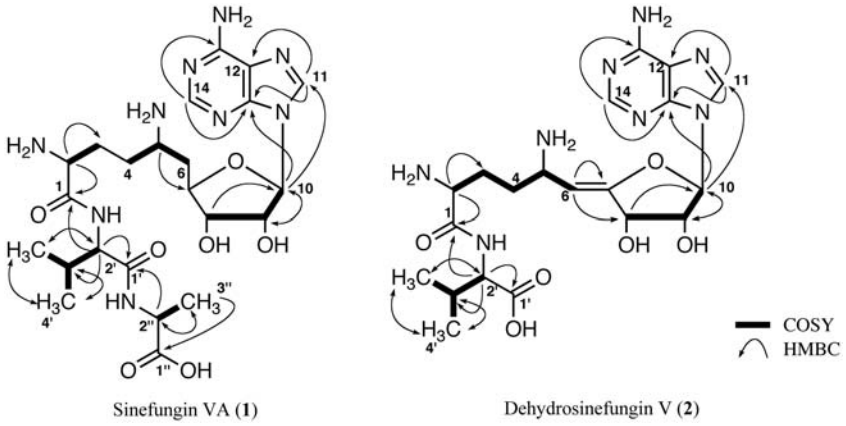
Table 1 Physicochemical properties of sinefungin analogs

	*Sinefungin VA (**1**)*	*Dehydrosinefungin V (**2**)*	*Sinefungin (**3**)*	*Dehydrosinefungin (**4**)*	*KSA-9342 (**5**)*
Appearance	Pale brown powder	Pale brown powder	Pale brown powder	Pale brown powder	Pale brown powder
Molecular formula	$C_{23}H_{37}N_9O_7$	$C_{20}H_{30}N_8O_6$	$C_{15}H_{23}N_7O_5$	$C_{15}H_{21}N_7O_5$	$C_{23}H_{35}N_9O_7$
Molecular weight	551	478	381	379	549
FAB-MS (m/z)					
Positive	552 $[M+H]^+$	479 $[M+H]^+$	382 $[M+H]^+$	380 $[M+H]^+$	550 $[M+H]^+$
	574 $[M+Na]^+$	523 $[M-H+2Na]^+$			572 $[M+Na]^+$
HR-FAB-MS (m/z)					
Calcd.	552.2894	523.2005	382.1839	380.1682	572.2557
Found	552.2889	523.2031	382.1843	380.1552	572.2555
UV $\lambda_{max}^{50\%MeOH}$ nm (ε)	206, 261	206, 259	206, 260	206, 259	206, 259
IR ν_{max}^{KBr} cm^{-1}	3459, 1652, 1402	3428, 1640, 1407	3434, 1650, 1405	3434, 1621, 1403	3428, 1644, 1400
Solubility					
Soluble	H_2O, MeOH, acetone	H_2O, MeOH, acetone	H_2O, MeOH, acetone	H_2O, MeOH, acetone	H_2O, MeOH, acetone
Insoluble	*n*-Hexane, $CHCl_3$	*n*-Hexane, $CHCl_3$	*n*-Hexane, $CHCl_3$	*n*-Hexane, $CHCl_3$	EtOAc, $CHCl_3$

Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; HR-FAB-MS, high-resolution FAB-MS; IR, infrared; UV, ultraviolet.

Table 2 ^1H and ^{13}C nuclear magnetic resonance spectral date of sinefungin analogs

	*Sinefungin VA (**1**)*		*Dehydrosinefungin V (**2**)*		*Sinefungin (**3**)*		*Dehydrosinefungin (**4**)*		*KSA-9342 (**5**)*	
Position	^{13}C	^1H *(J in Hz)*	^{13}C	^1H *(J in Hz)*	^{13}C	^1H *(J in Hz)*	^{13}C	^1H *(J in Hz)*	^{13}C	^1H *(J in Hz)*
1	169.7	—	169.1	—	173.8	—	174.0	—	171.0	—
2	52.7	4.17 (1H, m)	52.5	4.03 (1H, m)	53.8	3.62 (1H, t, 7.0)	53.9	3.80 (1H, t, 7.0)	52.7	4.02 (1H, m)
3	27.3	1.38 (1H, m)	27.6	1.17 (1H, m)	22.8	1.80 (2H, m)	26.8	1.88 (1H, m)	28.0	1.22 (1H, m)
		1.82 (1H, m)		1.77 (1H, m)				1.92 (1H, m)		1.84 (1H, m)
4	28.2	1.40 (1H, m)	28.2	1.61 (1H, m)	26.5	1.70 (1H, m)	28.7	1.90 (1H, m)	29.0	1.65 (1H, m)
		1.84 (1H, m)		1.79 (1H, m)		1.90 (1H, m)		1.95 (1H, m)		1.86 (1H, m)
5	49.0	3.58 (1H, m)	48.6	4.10 (1H, m)	48.8	3.41 (1H, m)	48.5	4.20 (1H, m)	48.5	4.12 (1H, m)
6	34.2	2.20 (2H, m)	99.5	4.92 (1H, d, 3.1)	34.0	2.06 (2H, m)	99.5	5.05 (1H, m)	99.5	4.80 (1H, m)
7	73.3	4.35 (1H, dt, 7.2, 7.1)	159.9	—	72.5	4.15 (1H, dt, 6.2, 6.0)	159.6	—	160.5	—
8	80.0	4.27 (1H, m)	67.4	4.94 (1H, d, 3.1)	79.5	4.20 (1H, dd, 6.2, 6.2)	67.2	5.10 (1H, d, 6.2)	67.3	4.67 (1H, m)
9	73.2	4.80 (1H, dd, 7.3, 6.2)	72.5	4.89 (1H, m)	72.3	4.60 (1H, dd, 7.0, 6.2)	72.3	5.00 (1H, dd, 7.0, 6.2)	72.4	4.99 (1H, dd, 7.5, 6.2)
10	88.8	6.05 (1H, d, 7.3)	87.9	6.20 (1H, d, 7.3)	88.5	5.88 (1H, d, 7.0)	87.8	6.23 (1H, d, 7.0)	88.0	6.17 (1H, d, 7.5)
Adenine										
11	141.0	8.25 (1H, s)	140.7	8.16 (1H, s)	140.5	8.10 (1H, s)	140.8	8.30 (1H, s)	141.0	8.26 (1H, s)
12	119.5	—	119.5	—	119.2	—	119.4	—	119.3	—
13	155.9	—	155.9	—	155.8	—	155.6	—	156.0	—
14	153.2	8.24 (1H, s)	153.1	8.05 (1H, s)	153.0	8.08 (1H, s)	152.7	8.24 (1H, s)	153.5	8.11 (1H, s)
15	149.2	—	149.0	—	148.5	—	150.6	—	149.2	—
Valine										
1′	172.2	—	178.4	—	—	—	—	—	172.0	—
2′	60.2	4.16 (1H, d, 7.0)	61.8	3.91 (1H, d, 7.0)	—	—	—	—	60.5	4.05 (1H, d, 7.0)
3′	30.3	2.15 (1H, m)	30.2	1.95 (1H, dqq, 7.2, 7.0)	—	—	—	—	30.0	2.07 (1H, m)
4′	18.6	0.96 (3H, d, 7.2)	18.9	0.81 (3H, d, 7.2)	—	—	—	—	17.7	0.88 (3H, d, 7.0)
5′	17.7	0.98 (3H, d, 7.2)	17.8	0.78 (3H, d, 7.2)	—	—	—	—	18.1	0.87 (3H, d, 7.0)
Alanine										
1″	181.3	—	—	—	—	—	—	—	179.2	—
2″	51.3	4.09 (1H, q, 7.1)	—	—	—	—	—	—	50.5	4.01 (1H, q, 7.2)
3″	17.9	1.31 (3H, d, 7.1)	—	—	—	—	—	—	17.5	1.22 (3H, d, 7.2)



Sinefungin VA (**1**) Dehydrosinefungin V (**2**)

Figure 4 COSY and HMBC studies of sinefungin VA (**1**) and dehydrosinefungin V (**2**).

Table 3 Antitrypanosomal activity and cytotoxicity of sinefungin analogs

	$IC_{50}(\mu g\,ml^{-1})$		
Compound	*Antitrypanosomal activity T. b. b. GUTat 3.1*	*Cytotoxicity MRC-5*	*Selectivity index MRC-5/T. b. b.*
Sinefungin VA (**1**)	0.0026	>5	>1923
Dehydrosinefungin V (**2**)	0.147	7.96	54
Sinefungin (**3**)	0.00021	79	376 000
Dehydrosinefungin (**4**)	0.0587	2.10	35
KSA-9342 (**5**)	0.186	3.91	21
Suramin[a]	1.58	>100	>63

Abbreviations: IC_{50}, inhibitory constant 50; *T.b.b.*, *Trypanosoma brucei brucei.*
[a]Suramin is a drug used to treat Human African Trypanosomiasis.

characteristic
UV spectrum and fragment ion (*m/z* 135) in FAB mass spectrum indicated the presence of an adenine moiety. Moreover, the spin systems from H-2 to H-6 and from H-8 to H-10, deduced by the ^{1}H-^{1}H COSY, and the correlations from H-2 to C-1 and C-3, from H-6 to C-7 and C-8, from H-8 to C-10 and from H-10 to C-11 and C-15 of the adenine moiety in the HMBC spectra, revealed that **2** was a dehydrosinefungin analog (Figure 4). This was confirmed by comparison with the NMR spectra of dehydrosinefungin (Table 2). The spin systems from H-2′ to H_3-5′ by the COSY and the correlations from H-2′ to C-1′ in the HMBC spectra indicated the presence of a valyl moiety. The correlations from H-2′ to C-1 in HMBC revealed the C-1 of dehydrosinefungin was attached to the amino group of the valyl moiety to form an amide bond. The absolute configuration of the valine residue was determined to be the L-form by HPLC analysis of the hydrolysates of **2** using a chiral column. The structure of **2** is shown in Figure 1, and the compound was designated as dehydrosinefungin V.

Biological activities

As shown in Table 3, sinefungin VA (**1**) showed potent activity against *Trypanosoma brucei brucei* GUTat 3.1 strain with an IC_{50} value of $2.60\times10^{-3}\,\mu g\,ml^{-1}$, which was 10-fold less than that of sinefungin (IC_{50} value of $2.10\times10^{-4}\,\mu g\,ml^{-1}$) but approximately 600-fold higher than that of the commonly used therapeutic drug, suramin. However, **1** exhibited significantly less cytotoxicity against MRC-5 cells than sinefungin. Dehydrosinefungin V (**2**) showed moderate antitrypanosomal activity with IC_{50} values of $0.14\,\mu g\,ml^{-1}$, which was similar to that of dehydrosinefungin (**4**) and KSA-9342 (**5**). A selectivity index (see Table 3), created to allow direct comparisons of effectiveness and cytotoxicity, indicated that sinefungin VA was almost 200-fold less potent than sinefungin but that, significantly, it was some 30-fold better than suramin.

DISCUSSION

Sinefungin and its analog, dehydrosinefungin, were isolated from a culture broth of *Streptomyces griseolus* and found to have antifungal and antitrypanosomal properties.[4,5] Sinefungin is a nucleoside antibiotic containing an ornithine residue linked by a C–C bond to the 5′ carbon of adenosine. The biosynthetic precursors of sinefungin were reported as being L-arginine and ATP, using incorporation studies as well as HPLC analysis,[7] although the details remain unclear. The isolation of sinefungin and its various analogs from the same culture broth in this study might provide a clue to help solve their biosynthesis pathway. Sinefungin is a structural analog of *S*-adenosylmethionine, a molecule that has an important role in the biosynthesis of polyamines and in transmethylations of proteins and lipids. Sinefungin and dehydrosinefungin are very potent inhibitors of *S*-adenosylmethionine-dependent transmethylations.[8] Sinefungin was also reported to exhibit strong antiviral, antileishmanial and antitumor activity.[8–10] In our study, compounds having a single bond between C-6 and C-7 of adenosine (sinefungin and sinefungin VA) showed potent antitrypanosomal activity as compared with compounds having a double bond (dehydrosinefungin, dehydrosinefungin V and KSA-9342). Many biological activities of sinefungin are believed to be related to the inhibition of *S*-adenosylmethionine-dependent methyltransferase enzymes.[11–16]

When administered intraperitoneally, sinefungin cures mice infected with *T. brucei brucei, Trypanosoma congolense* and *Trypanosoma vivax*.[17,18] It was well tolerated in mice, and when the dose per injection was 0.05–5 mg kg^{-1}, sinefungin was highly effective against African trypanosomes. Sinefungin and dehydrosinefungin were also leishmanicidal at concentrations ranging from 0.13 to 2.6 μM *in vitro*.[9] Our data confirm that sinefungin is both a potent antitrypanosomal and that it has a moderate cytotoxic effect against MRC-5 cells. Hitherto, sinefungin has not been developed because of its serious side effects (nephrotoxicity at subcurative levels in goats and dogs and toxicity for bone marrow cells).[19,20] However, synthetic studies for

some analogs have been reported to improve the therapeutic index.[21,22] Our results indicate that sinefungin VA retains potent antitrypanosomal properties but with a greatly reduced cytotoxicity, significantly outperforming suramin, indicating that it could be a promising candidate for development into a novel new antitrypanosomal drug.

METHODS

Taxonomic studies

We observed cultural and physiological characteristics of strain K05-0178 grown on yeast extract–malt extract agar, oatmeal agar and inorganic salts–starch agar, after incubation for 2 weeks at 27 °C. The morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL, Akishima, Japan) after cultivation on 1/10 V8 juice agar at 27 °C for 18 days. Isomers of diaminopimelic acid in whole-cell hydrolysates were determined by TLC, following the standard methods of Becker *et al.*[23] and Hasegawa *et al.*[24] 16S rDNA was amplified by PCR and sequenced directly on an ABI model 377A automatic DNA sequencer using a PRISM Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA).

Fermentation

The strain K05-0178 was grown and maintained on agar slants consisting of 1.0% starch, 0.3% NZ amine, 0.1% yeast extract, 0.1% meat extract, 1.2% agar and 0.3% $CaCO_3$. A loop of spores of strain K05-0178 was inoculated into 100 ml of seed medium, consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.4% $CaCO_3$ (adjusted to pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask. The flask was incubated on a rotary shaker (210 r.p.m.) at 27 °C for 3 days. A 1 ml portion of the seed culture was transferred to 500-ml Erlenmeyer flasks (total 100) containing 100 ml of production medium, consisting of 0.5% glucose, 0.5% corn steep powder, 1.0% oat meal, 1.0% Pharmamedia (Traders Protein, Lubbock, TX, USA), 0.5% K_2HPO_4, 0.5% $MgSO_4{\cdot}7H_2O$, 5.0×10^{-4}% $FeSO_4{\cdot}7H_2O$, 5.0×10^{-4}% $MgCl_2{\cdot}4H_2O$, 5.0×10^{-4}% $CuSO_4{\cdot}5H_2O$ and 5.0×10^{-4}% $CoCl_2{\cdot}6H_2O$ (adjusted to pH 7.0 before sterilization) and fermentation was carried out on a rotary shaker (210 r.p.m.) at 27 °C for 6 days.

General experiments

NMR spectra were measured using a Varian XL-400 spectrometer or a Varian Inova 600 spectrometer (Varian, Palo Alto, CA, USA), with ^{1}H NMR at 400 or 600 MHz and ^{13}C NMR at 100 or 150 MHz in D_2O. The chemical shifts are expressed in p.p.m. and are referenced to HDO (4.76 p.p.m.) in the ^{1}H NMR spectra and the end of both fields (0, 200 p.p.m.) in the ^{13}C NMR spectra. FAB-MS spectra were measured on a JEOL JMS AX-505 HA mass spectrometer. IR spectra (KBr) were taken on a Horiba FT-210 Fourier (Horiba, Kyoto, Japan) transform infrared spectrometer. UV spectra were measured with a Beckman DU640 spectrophotometer (Beckman, Fullerton, CA, USA). Optical rotation was measured on a JASCO model DIP-181 polarimeter (JASCO, Hachioji, Japan).

Amino acid analysis

Each of **1–3** (100 μg) was completely hydrolyzed in a gas phase of 6 M HCl (100 μl) at 110 °C for 18 h in a reaction vial in which air was replaced by N_2 gas, using the Pico-Tag work station (Waters). For elucidation of the absolute configuration of amino acids, the hydrolysates were dissolved in distilled water and subjected to HPLC analysis (Column, Sumichiral OA-5000 (4.6 i.d. ×150 mm, Sumika Chemical Analysis Service, Osaka, Japan); Mobile Phase, 2 mM $CuSO_4$; flow rate, 1.0 ml min^{-1}; detection, 254 nm; column temperature, 40 °C). Retention time (min): L-Ala 5.02, D-Ala 7.36, L-Val 12.2 and D-Val 22.1.

Antitrypanosomal activity *in vitro*

In vitro antitrypanosomal activities against *T. brucei brucei* strain GUTat 3.1 were measured, using the method described previously.[25] In brief, the strain GUTat 3.1 was cultured in Iscove's modified Dulbecco's medium (IMDM) with various supplements and 10% heat-inactivated fetal bovine serum at 37 °C, under 5.0% CO_2/95% air. Subsequently, 95 μl of the trypanosomes suspension ($2.0–2.5\times10^4$ trypanosomes ml^{-1}) was transferred in a 96-well microtiter plate and 5.0 μl of a test compound solution (dissolved in 5.0% DMSO) was added and incubated for 72 h at 37 °C (long incubation–low inoculation test). After that, 10 μl of the fluorescent dye alamer blue was added to each well. After incubation for 3–6 h, the resulting solution was read at 528/20 nm excitation wavelengths and 590/30 nm emission wavelengths by an FLx800 fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA). The IC_{50} values were determined using the fluorescent plate reader software (KC-4, BioTek). Successive subcultures were done in 24-well tissue culture plates under the same conditions.

Cytotoxic activity *in vitro*

Measurement of cytotoxic activity against human fetal lung fibroblast MRC-5 cells was carried out as described previously.[26]

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