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IC202B and C, New Siderophores with Immunosuppressive Activity Produced by *Streptoalloteichus* sp. 1454-19

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IC202B (**1**) and C (**2**) were isolated from the culture filtrate of *Streptoalloteichus* sp. 1454-19. The structures were elucidated by various NMR spectral analyses including ^{1}H-^{15}N HMBC and FAB-MS experiments. IC202B and C showed immunosuppressive activity on a mixed lymphocyte culture reaction at the same IC_{50} value of 1.6 μg/ml.

In the course of our screening program for low molecular weight immunosuppressive compounds, IC202A, a new ferrioxamine related compound containing a butylidene *N*-oxide, has been isolated from the culture filtrate of *Streptoalloteichus* sp. 1454-19[1,2]. Further screening resulted in the isolation of two novel compounds, IC202B and C, which showed more potent activity than IC202A on a mixed lymphocyte culture reaction (MLCR). In this paper, we report the isolation, physico-chemical properties, structure elucidation and biological activities of IC202B and C.

Materials and Methods

Microorganism and Fermentation

The producing strain 1454-19 was isolated from a soil sample collected at Karuizawa-cho, Nagano prefecture, Japan. The taxonomic studies of producing strain and the fermentation procedure were reported in the previous paper[1].

General

MPs were determined on a Yanagimoto micro melting point apparatus. UV spectra were recorded on a Hitachi 228A spectrometer. IR spectra were recorded on a Horiba fourier transfer infrared spectrometer FT-200. ^{1}H, ^{13}C and ^{15}N NMR spectra were measured with a JEOL JNM-A500 spectrometer in DMSO-d_6 solution. ^{15}N-chemical shifts were given in ppm using $CH_3NO_2/CDCl_3$ (1 : 1) solution at 379.6 ppm as an external standard. ^{1}H and ^{13}C chemical shifts were given in ppm using TMS as an internal standard. FAB-MS spectra were measured on a JEOL JMS-SX102 spectrometer.

Mixed Lymphocyte Culture Reaction (MLCR) and Lymphocyte Blastogenesis

MLCR and lymphocyte blastogenesis were carried out according to the method described previously[3].

Results and Discussion

Production and Isolation

The production of IC202B and C was carried out by the method described for IC202A[1]. The culture filtrate (5 liters) was adjusted to pH 8.0 with 4% $NaHCO_3$ and was applied to a Diaion HP-20 column (500 ml). After washing with H_2O, the active substance was eluted with 50% MeOH and concentrated *in vacuo*. The crude material was dissolved in H_2O and applied to a CM Sephadex C-25 column (80 ml). The column was washed with H_2O (400 ml) and the fractions containing IC202B and C were eluted with 0.05 M NaCl. The active fractions were applied to a HP-20 column and eluted with 50% MeOH and concentrated *in vacuo*. Further purification was carried out

Fig. 1. Structures of IC202B (**1**), C (**2**) and A (**3**) isolated from *Streptoalloteichus* sp. 1454-19.

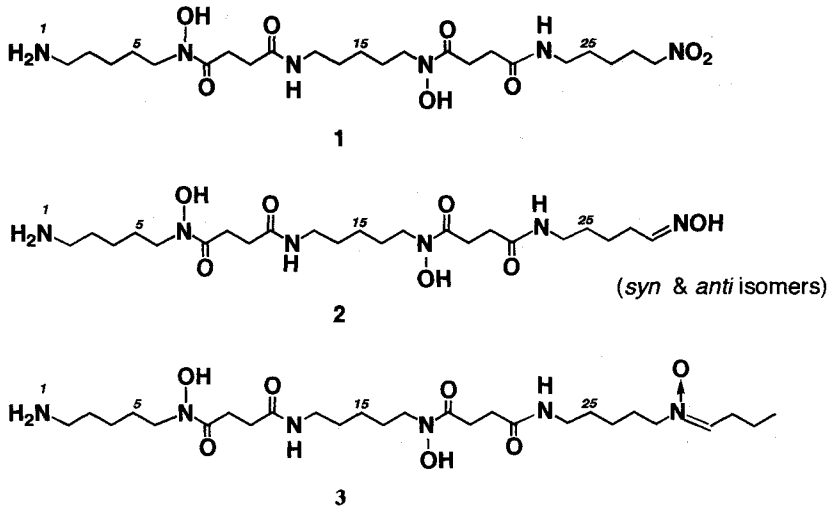


Table 1. Physico-chemical properties of IC202B and C.

	IC202B (**1**)	IC202C (**2**)
Appearance	pale yellow powder	white powder
MP	117-119°C	140-141°C
Molecular formula	$C_{23}H_{44}N_6O_8$	$C_{23}H_{44}N_6O_7$
HRFAB-MS (*m/z*) Calcd:	533.3299	517.3350
$(M+H)^+$ Found:	533.3306	517.3316
UV λ_{max} nm	No maximum above 210	No maximum above 210
	300 (sh)	
IR ν_{max} (KBr) cm^{-1}	3310,3000,2930,2860,	3310,3000,2930,2860,
	1620,1565,1560,1460,	1620,1565,1460, 1430,
	1430,1400,730	1400,730
TLC (Rf value)[a]	0.47[b]	0.41

[a] Crystalite cellulose TLC (Funakoshi, FC-2020):BuOH-BuOAc-MeOH-H_2O (2:2:1:1)

[b] Detected with I_2 vapor

by the HPLC (YMC-Pak ODS-SH, 20×300 mm; 4 ml/minute) using 35% MeOH containing 0.05 M ammonium acetate as a mobile phase. The fractions eluted at 50 minutes were collected and desalted by HP-20 chromatography and lyophilized to give a pale yellow powder (2 mg) of IC202B. The fractions eluted at 30 minutes were collected, and IC202C was obtained as a white powder (10 mg) by the same procedures.

Physico-chemical Properties

The physico-chemical properties of IC202B (**1**) and C (**2**) are summarized in Table 1. **1** and **2** are soluble in H_2O, DMSO, and MeOH, slightly soluble in EtOH, but insoluble in $CHCl_3$. **1** and **2** showed almost the same IR spectra, and the strong absorption at 1620 cm^{-1} and 1565 cm^{-1} suggested the presence of amide group. **1** and **2** showed positive response to iodine vapor and $FeCl_3$ reagent on a

cellulose TLC suggesting the presence of hydroxamate moiety. The molecular formulae of **1** and **2** were established to be $C_{23}H_{44}N_6O_8$ and $C_{23}H_{44}N_6O_7$, respectively, by HRFAB-MS spectra and ^{13}C NMR spectral information. These findings suggested that **1** and **2** are closely related compounds of IC202A (**3**).

Structure of IC202B (**1**)

The ^{13}C NMR spectrum of **1** in DMSO-d_6 showed 23 carbon signals, which were classified into nineteen methylene carbons and four carbonyl carbons by the analysis of DEPT spectra.

In the ^1H-NMR spectrum of **1** in DMSO-d_6, two NH protons were observed at δ_H 7.80 and δ_H 7.82. These protons correlated with two carbonyl carbons (δ_C 171.3 and δ_C 171.4) in the HMBC spectrum resulting in the presence of two amide moieties. Consequently, the presence of two hydroxamate moieties [–CO–N(OH)–] were indicated as the remaining carbonyl carbons (δ_C 171.9 and δ_C 172.0)

Table 2. ^1H, ^{13}C and ^{15}N NMR data of IC202B (**1**) and C (**2**) in DMSO-d_6.

	1			**2**		
position	^{13}C	^1H	^{15}N	^{13}C	^1H	^{15}N
1-N			35.0			26.0
2	38.4 t	2.72 (t,7.4)		39.5 t	2.60 (m)	
3	26.8 t	1.58 (m)		28.9 t	1.45 (m)	
4	22.9 t	1.25 (m)		23.0 t	1.25 (m)	
5	25.7 t	1.52 (m)		25.8 t	1.50 (m)	
6	46.8 t	3.49 (t,7.0)		46.9 t	3.45 (m)	
7-N			175.5			172.0
8	171.9 s[a]	-		171.9 s	-	
9	27.6 t	2.55 (m)		27.6 t	2.55 (m)	
10	30.0 t	2.25 (m)		30.0 t	2.55 (m)	
11	171.3 s[b]	-		171.4 s	-	
12-N		7.80 (br t)	117.5		7.80 (br t)	114.0
13	38.7 t	2.97 (m)		38.4 t	3.00 (m)	
14	28.8 t	1.35 (m)		28.8 t	1.35 (m)	
15	23.5 t	1.20 (m)		23.5 t	1.20 (m)	
16	26.0 t	1.50 (m)		26.0 t	1.60 (m)	
17	47.1 t	3.45 (t,7.0)		47.1 t	3.45 (m)	
18-N			175.5			172.0
19	172.0 s[a]	-		171.9 s	-	
20	27.6 t	2.55 (m)		27.6 t	2.55 (m)	
21	30.0 t	2.25 (m)		30.0 t	2.25 (m)	
22	171.4 s[b]	-		171.4 s	-	
23-N		7.82 (br t)	117.0		7.80 (br t)	114.0
24	38.0 t	3.00 (m)		38.2 t	3.00 (m)	
25	28.4 t	1.40 (m)		28.6 t	1.40 (m)	
26	23.0 t	1.28 (m)		23.6 t	1.35 (m)	
27	26.3 t	1.86 (m)		24.3 t[c]	2.20 (m)[c]	
				28.7 t[d]	2.07 (m)[d]	
28	75.4 t	4.50 (t,7.0)		150.2 d[c]	6.60 (t,5.4)[c]	
				149.4 d[d]	7.25 (t,5.8)[d]	
29-N			391.0			365.0[c]
						360.0[d]

[a,b]Chemical shifts with the same superscript may be transported.

[c]*anti* isomer

[d]*syn* isomer

based on the ^{13}C and ^{15}N chemical shifts (Table 2). The molecular formula suggested the presence of one nitrogen and two oxygen atoms as the remaining part.

Partial structures (*a*~*e*) shown in Fig. 2 were elucidated by the interpretation of ^{1}H-^{1}H COSY spectra and 1D-HOHAHA experiments. Two sets of overlapping peaks due to (*d*) $-CH_2(9)-CH_2(10)-$ and (*e*) $-CH_2(20)-CH_2(21)-$ were ascribed to the presence of two isolated $-CH_2-CH_2-$ based on the integration of two multiplet protons at δ_H 2.55 (4H) and δ_H 2.25 (4H).

Moreover, six methylenes (C-2, C-6, C-13, C-17, C-24, and C-28) were suggested to connect with nitrogen atoms based on the ^{1}H and ^{13}C chemical shifts.

The connectivity representing C-9 to C-13 and C-20 to C-24 were elucidated by the HMBC experiments, which revealed the long range couplings from amide protons NH-12 and NH-23 to relevant carbonyl carbons C-11 (δ_C 171.3) and C-22 (δ_C 171.4), respectively. Additionally, the long range couplings were observed from H-9, H-10, H-13 to C-11, and from H-20, H-21, H-24 to C-22, respectively.

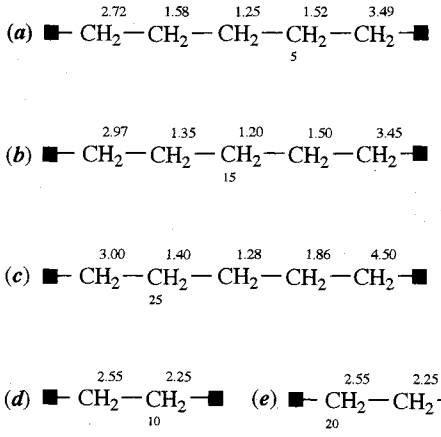
Two another carbonyl carbons (C-8 and C-19), which did not show any correlation with amide proton signals in the HMBC spectrum, revealed the long range couplings from H-6, H-9, H-10, and from H-17, H-20, H-21, respectively. Taking the molecular formula of **1** into consideration, two hydroxy groups must be attached to these N-7 and N-18 positions resulting in the hydroxamate moieties. Further structural elucidation around the amide linkages were performed by ^{1}H-^{15}N HMBC experiments.

As shown in Fig. 3, cross peaks were observed between methylene protons of H-3 (δ_H 1.58) and nitrogen at δ_N 35.0 (dotted arrow). This ^{15}N chemical shift was appropriate for a NH_2 nitrogen. Cross peaks were observed from H-5 (δ_H 1.52) and H-6 (δ_H 3.49) to δ_N 175.5, which was appropriate for a hydroxamate nitrogen.

Correlations were also observed from H-16 and H-17 to N-18 (δ_N 175.5) due to another hydroxamate nitrogen. In addition, correlations were observed from relevant methylene protons (H-13, 14) to N-12 (δ_N 117.0) and H-24, H-25 to N-23 (δ_N 117.5), respectively. These ^{15}N chemical shifts were appropriate for amide nitrogens. Finally, methylene protons (H-27, H-28) correlated to the NO_2 nitrogen at δ_N 390.0.

The structure of IC202B (**1**) thus obtained was further confirmed by a FAB-B/E linked scan spectrum, which revealed the characteristic fragment peaks as shown in Fig. 4. The prominent fragment ions at *m/z* 201, 319 and 401 were very characteristic for these compounds[4].

Fig. 2. Partial structures and chemical shifts (δ_H) in DMSO-d_6 of IC202B (**1**).



Structure of IC202C (**2**)

The molecular formula of IC202C (**2**) was elucidated as

Fig. 3. Structure of **1** elucidated by NMR analysis.

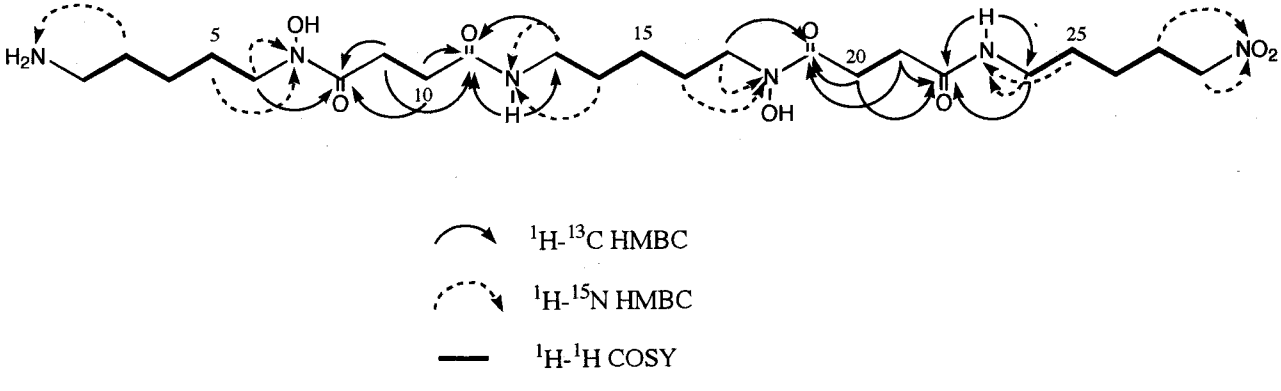


Fig. 4. FAB-B/E linked scan analysis of IC202B (**1**) and C (**2**).

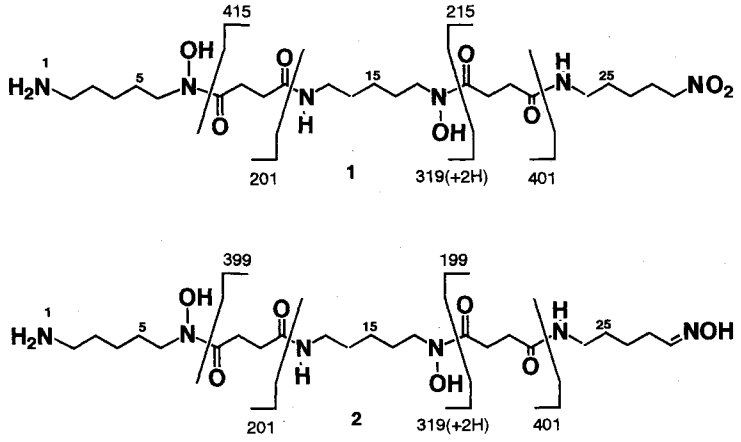


Table 3. Inhibitory effect of IC202B, C and A on MLCR and mitogen induced lymphocyte blastogenesis.

	IC_{50} (μg/ml)		
	MLCR	Lymphocyte blastogenesis	
		ConA	LPS
IC202B	1.6	2.5	4.4
IC202C	1.6	2.9	4.6
IC202A	3.6	9.6	11.3

$C_{23}H_{44}N_6O_7$ from HRFAB-MS, which lacked one oxygen atom from **1**. In the ^{13}C NMR spectrum of **2**, however, the total carbon number seemed to be more than that by HRFAB-MS spectral information. This discrepancy may be ascribed to the presence of two isomers based on the physico-chemical properties showing the same TLC and HPLC behaviors and molecular formula. Thus, we attempted to separate them. However, all attempts were unsuccessful. As a consequence, two sets of signals were predicted. Fortunately, however, the concomitant signals were limited and the most part of signals could be easily assignable.

The ^{13}C NMR spectrum of **2** was almost the same as that of **1** except for signals due to C-26 to C-28. The sp^2 methine carbons at C-28 (δ_C 149.4 and 150.2) for **2** were observed in place of the corresponding methylene carbon (δ_C 75.4) for **1**. In addition, methylene carbons at C-27 (δ_C 24.3 and 28.7) for **2** were shifted as compared with that for **1** (δ_C 26.3). These findings suggested the presence of $-CH_2-CH=N-$ group in **2** instead of $-CH_2-CH_2-NO_2$ in **1**. Moreover, the ^{15}N chemical shift of N-29 (δ_N 360.0 and 365.0) indicated the presence of aldoxime moiety (–CH=N–OH). From the results of ^1H-^{13}C and ^1H-^{15}N HMBC experiments, all assignments due to the isomeric aldoximes are shown in Table 2. The ratio of *syn* and *anti* isomers was almost 1 : 1 on the basis of the ^1H NMR integration (δ_H 7.25 and δ_H 6.60). Thus, the structure of **2** was concluded as shown in Fig. 1. A FAB-B/E linked scan spectral analysis (Fig. 4) also supported the conclusion.

Biological Activities

The immunosuppressive activity of IC202B and C was evaluated by MLCR and mitogen induced lymphocyte

blastogenesis. As shown in Table 3, IC202B and C showed more suppressive activity than IC202A on MLCR and lymphocyte blastogenesis. In the previous paper[1], we demonstrated that IC202A was remarkably reduced the immunosuppressive activity by the addition of $FeCl_3$ (100 μM). Then, we assessed the reduction of immunosuppressive activity of IC202B and C at 25 μg/ml by the addition (100 μM) of other metal salts (Fe^{3+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}). Among them, only $FeCl_3$ abrogated the suppressive effect of IC202B and C. Accordingly, the immunosuppressive activity of IC202A,B and C *in vitro* seemed to depend on the action of iron(Fe^{3+})-chelating as that by deferoxamine, which exibited to inhibit DNA synthesis but not RNA or protein synthesis on lymphocyte proliferation[5]. Studies on the other biological activities and biosynthesis of IC202A, B and C are in progress.

Acknowledgments

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