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Biosynthesis of IC202C, a New Siderophore with Immunosuppressive Activity

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In the course of our screening program for low molecular weight immunosuppressive compounds, IC202A (**1**), B (**2**) and C (**3**) (Fig. 1) have been isolated from the culture filtrate of *Streptoalloteichus* sp. 1454-19.[1~3] They are structurally characteristic in terminal moieties such as butylidene-*N*-oxide, nitro and aldoxime groups, respectively.

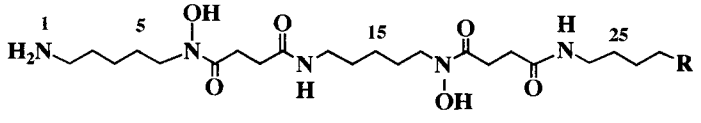
Of these compounds, IC202C (**3**) showed the most potent immunosuppressive activity and was produced as the major compound in the fermentation broth. However, the amounts of IC202s were very low for the evaluation of *in vivo* experiments. Thus, it is necessary to know the optimum fermentation condition and biosynthetic route for the increment of IC202s.

There are many microbial ferrioxamines, which are composed of alternative repeating unit of cadaverine and succinic acid. However, the ferrioxamine biosynthesis[4] is only partly understood. Especially, the *N*-hydroxylation process is unclear, although it may occur at the level of either L-lysine or cadaverine which is biosynthesized from L-lysine by decarboxylation[5]. Subsequently, it is supposed that the terminal moieties of IC202s would be converted to each component after biosynthesis of the skeleton. However, little is known about the nature of the microbe enzymes concerned with each conversion. There are two possibilities for the biosynthesis of IC202C. First, aldehyde product would be formed and then converted into aldoxime by hydroxyamination. Second, amine product would be formed and then oxidized. To verify these possibilities, we searched these putative precursors in the fermentation broth.

As the result, we found a new compound IC202D (**4**) and proferrioxamine G_{1t} (**5**)[4]. IC202D and proferrioxamine G_{1t} were produced by the same procedure for IC202s[1,3]. The isolation procedure of these compounds is shown in Fig. 2. IC202D was isolated as a hygroscopic powder and the physico-chemical properties are summarized in Table 1.

The molecular formula of IC202D (**4**) was determined to be $C_{23}H_{43}N_5O_7$ by HRFAB-MS; *m/z* 502.3257 $(M+H)^+$, calcd. for $C_{23}H_{44}N_5O_7$; *m/z* 502.3241. It gave a positive reaction with 2,4-dinitrophenylhydrazine, suggesting the presence of aldehyde group. It also gave a positive reaction with $FeCl_3$ suggesting the presence of hydroxamate moiety.

Fig. 1. Structures of IC202A (**1**), B (**2**), C (**3**), D (**4**) and proferrioxamine G_{1t} (**5**) isolated from *Streptoalloteichus* sp. 1454-19.



R		
	$-CH_2\overset{\uparrow O}{N}=CH(CH_2)_2CH_3$	IC202A (**1**)
	$-CH_2NO_2$	IC202B (**2**)
	$-CH{=}N{-}OH$	IC202C (**3**)
	$-CHO$	IC202D (**4**)
	$-CH_2NHOH$	proferrioxamine G_{1t} (**5**)

Fig. 2. Purification of IC202D and proferrioxamine G_{1t}.

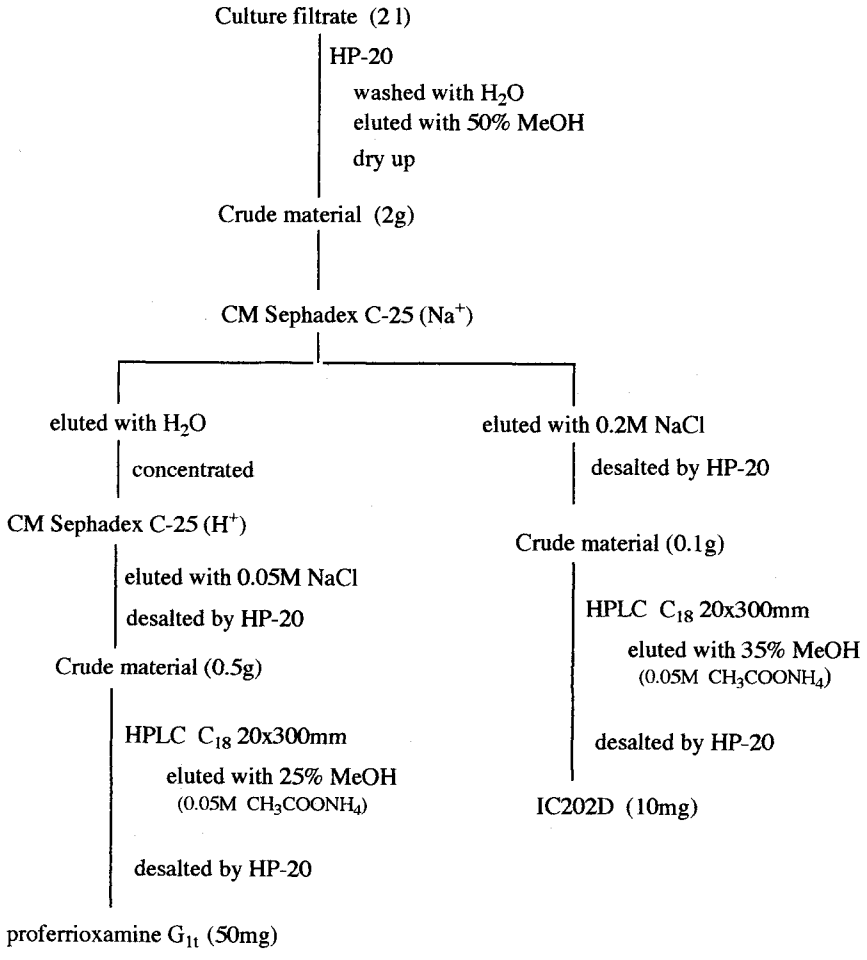


Table 1. Physico-chemical properties of IC202D and proferrioxamine G_{1t}.

	IC202D	proferrioxamine G_{1t}
Appearance	hygroscopic powder	white powder
Molecular formula	$C_{23}H_{43}N_5O_7$	$C_{23}H_{46}N_6O_7$
ESI-MS/MS (*m/z*)	502, 401, 319, 283, 201, 184	519, 401, 319, 283, 201 183, 154
HRFAB-MS (*m/z*)		
Calcd.	502.3241 for $C_{23}H_{44}N_5O_7$	519.3506 for $C_{23}H_{47}N_6O_7$
Found	502.3257 : $(M+H)^+$	519.3523: $(M+H)^+$
UV;	No maxima above 210 nm	No maxima above 210 nm
IR; cm^{-1} (KBr)	3310, 3000, 2930, 2860, 1620, 1565, 1460, 1430, 1400, 730	3310, 3000, 2930, 2860, 1620, 1565, 1460, 1430, 1400, 730

Table 2. ^{13}C-NMR analysis of **3** (DMSO-d_6, 100 MHz) enriched by incorporation of isotopic precursors.

Carbon No.	δc(ppm)	Enrichment ratio	Carbon No.	δc(ppm)	Enrichment ratio
1-N			17	47.1	1.20
2	39.5	1.00	18-N		
3	28.9	1.14	19	171.9	2.47*
4	23.0	1.24	20	27.6	0.98
5	25.8	1.07	21	30.0	1.20
6	46.9	1.08	22	171.4	2.73*
7-N			23-N		
8	171.9	2.47*	24	38.2	1.14
9	27.6	0.98	25	28.6	0.93
10	30.0	1.44	26	23.6	0.86
11	171.4	2.72*	27	24.3	1.54
12-N				28.7	0.90
13	38.4	1.41	28	150.2	1.69
14	28.8	1.39		149.4	1.69
15	23.5	1.25	29-N		
16	26.0	1.20			

* Enrichment of ^{13}C was observed.

Compound **4** showed almost the same IR spectrum as those of IC202s. In the ESI-MS/MS spectrum of **4**, empirical fragment ions at *m/z* 201, 319, and 401 were observed. These fragment ions were characteristic of IC202s.[2,3]

On the other hand, ^{1}H and ^{13}C-NMR spectra were very complicated perhaps due to the presence of alkanolamine structure coexisting of aldehyde and amine moieties. Furthermore, overlapping peaks in ^{1}H and ^{13}C-NMR due to the repeated diaminopentane moieties obstructed complete assignments of **4**. Thus, the chemical modification of **4** was examined to confirm the structure.

Treatment of **4** with hydoxylamine in methanol at room temperature for 2 hours gave an aldoxime (**3**, *syn* and *anti* isomers) quantitatively. The physico-chemical properties of **3** were completely identical with IC202C[3] which was obtained from the fermentation broth. ESI-MS; *m/z* 517 $(M+H)^+$, ^{1}H-NMR (D_2O); δ_H 1.1~1.3 (m, 4H), 1.3~1.45 (m, 6H), 1.45~1.6 (m, 6H), 2.1~2.25 (m, 2H), 2.3~2.4 (m, 4H), 2.5~2.7 (m, 4H), 2.8~2.9 (m, 2H), 3.0~3.1 (m, 4H), 3.4~3.6 (m, 4H), 6.72 (0.5H, t, J=5.4 Hz), 7.38 (0.5H, t, J=5.8 Hz).

The immunosuppressive activity of IC202D (11.2 μg/ml) was extremely low comparing with those of IC202A, B, and C. This may be partly due to the imine formation by amine and aldehyde moieties between intra- or intermolecular interaction in an assay system.

Compound **5** was also isolated as a white powder and the physico-chemical properties are summarized in Table 1. The mass fragmentation pattern of **5** was identical with the reported proferrioxamine G_{It}[4].

To explore the biosynthesis of IC202C, we examined feeding experiments using ^{15}N-L-lysine, the precursor of cadaverine, and ^{13}C-succinic acid. After 24 hours cultivation, 25 mg of [ε-^{15}N]-L-lysine dihydrochloride (Cambridge Isotope Laboratories, USA) or 25 mg of [*1,4*-^{13}C]-succinic acid (Aldrich Chemical Company Inc., USA) were added into the growing culture (100 ml) of *Streptoalloteichus* sp. 1454-19. After cultivation for 5 days, labeled IC202C was purified according to the method[3]. Enriched signals obtained by the feeding experiments were measured by ^{15}N [CH_3NO_2/$CDCl_3$ (1 : 1) at 379.6 ppm] and ^{13}C-NMR spectra. The enrichment ratios were calculated from the relative signal intensity of C-2 as 1.0. As expected, the feeding expriments of ^{13}C-succinic acid resulted in the signal enrichment of all carbonyl carbons (C-8, C-11, C-19, C-22) as depicted in Table 2, although the incorporation ratios were low. The reason of low incorporation was thought that succinic acid was metabolized by the

Fig. 3. ^{15}N-NMR spectrum of **3** (D_2O, 40 MHz) enriched by the feeding of [ε-^{15}N]-L-lysine.

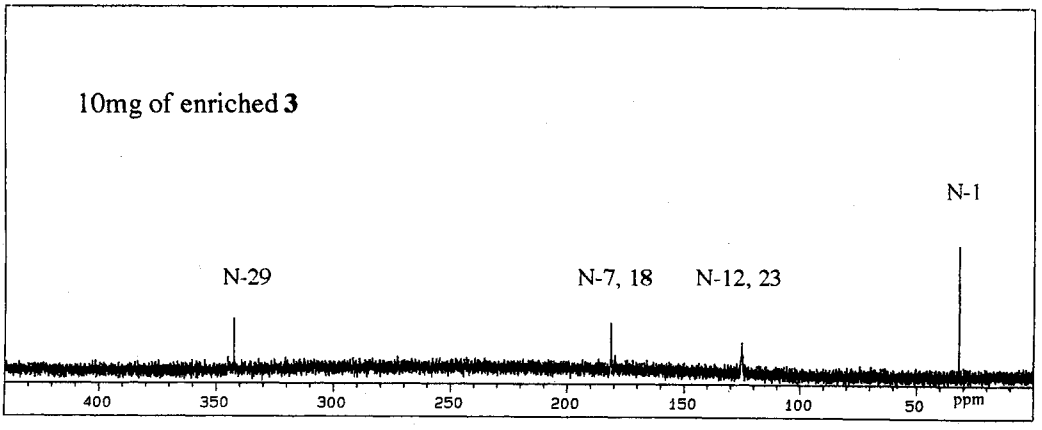
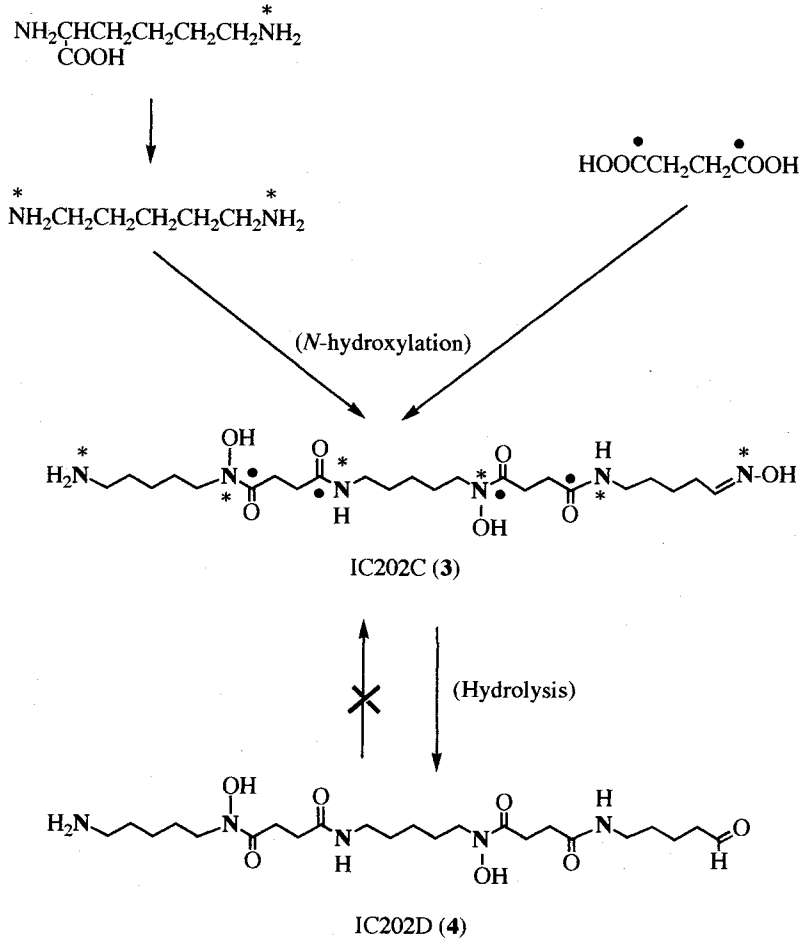


Fig. 4. Proposed biosynthetic scheme for IC202C.



producing strain. On the other hand, the feeding experiment of ^{15}N-lysine to IC202C showed that six nitrogen signals including aldoxime nitrogen were observed in ^{15}N-NMR spectrum as shown in Fig. 3.

This indicates that aldoxime **3** was not derived from aldehyde **4** by hydroxyamination in cultivation. It is likely

that **3** would be derived from terminal amine precursors, which was condensed by three moles of cadaverine and two moles of succinic acid. This does not necessarily mean that proferrioxamine G_{1t} is a certain precursor of IC202C. Because, *N*-hydroxylation of primary amines is a common bioconversion reaction[6], although primary amine product can not yet be detected in the fermentation broth. The ^{15}N signal enrichment of aldoxime moiety was unexpected. Because, it is well known that various amine oxidases[7] (amine dehydrogenases) in microbes catalyze the conversion of various polyamines including cadaverine to corresponding aldehyde derivatives. Subsequent oxime formation is a common reaction of aldehyde.

In conclusion, the following remarks can be made. (1) L-Lysine and succinic acid were well incorporated into IC202C. (2) *N*-Hydroxylation occurred after the decarboxylation of L-lysine since ^{15}N was incorporated into both amide and hydroxamate. (3) ^{15}N was also incorporated into the aldoxime moiety. This implies that the aldoxime **3** was not derived from the aldehyde **4**. Thus, we propose the biosynthetic pathway of IC202C as shown in Fig. 4.

Further studies on the converting enzymes on IC202C as well as the mode of biological actions of IC202C are now under investigation in our laboratories.

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