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CHEMISTRY OF THE NEOPOLYOXINS, PYRIMIDINE AND IMIDAZOLINE NUCLEOSIDE PEPTIDE ANTIBIOTICS

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Abstract—New chitin synthetase inhibitors, neopolyoxins A, B, and C were isolated from the culture filtrate of *Streptomyces cacaoi* subsp. *asoensis*. Their absolute structures have been established on the basis of chemical and spectroscopic evidence. They are structural analogs of the polyoxins. As a nucleobase, neopolyoxin C possesses uracil, while neopolyoxins A and B contain the imidazoline moiety. A ring contraction reaction of pyrimidine nucleoside into imidazoline nucleoside played a key role in the structure determination of the nucleoside moiety. A similar transformation was suggested for the biosynthesis of neopolyoxins A and B.

An inhibitor of microbial cell wall biosynthesis is expected to have selective toxicity. For example, polyoxins[1] are potent and selective inhibitors of fungal cell wall chitin synthetase and have been used as excellent agricultural fungicides in Japan because of the low toxicity to plants and animals. During the course of the screening program for inhibitors of fungal cell wall biosynthesis, a newly-isolated streptomycete, which belongs to *Streptomyces cacaoi* subsp. *asoensis*,[2] was found to produce chitin synthetase inhibitors which are different from the polyoxins. Three compounds were isolated and named neopolyoxins A, B, and C. This paper is concerned with the isolation and structure determination of these antibiotics. Preliminary reports on isolation and structures have been published.[2,3]

Isolation and properties

Neopolyoxins A **1**, B **2**, and C **3** were produced in the fermentation broth of *Streptomyces cacaoi* subsp. *asoensis* and isolated as outlined in Scheme 1. The antibiotic complex was obtained by adsorption on Dowex 50W X-8 (H-form) from the culture broth and elution from the resin by dilute NH_4OH followed by chromatography on charcoal. Separation of each component was carried out by column chromatography using Dowex 50W X-4 buffered with pridine–acetic acid, and cellulose powder with a solvent system of butanol–methanol–water. Each antibiotic was obtained as a white powder by precipitation with ethanol–ether.

Neopolyoxins A, B, and C are all amphoteric compounds which are unstable under acid and alkaline conditions. They have characteristic UV absorption spectra. The following molecular formulae were established on the basis of the elemental analyses, titration equivalents, and ^{13}C NMR spectra: A, $C_{20}H_{25}N_5O_{10}$; B, $C_{20}H_{25}N_5O_{11}$; C, $C_{20}H_{25}N_5O_{10}$. The ^1H and ^{13}C NMR chemical shifts with assignments are listed in Tables 1 and 2, respectively. Other physiochemical properties are described in the previous report.[2]

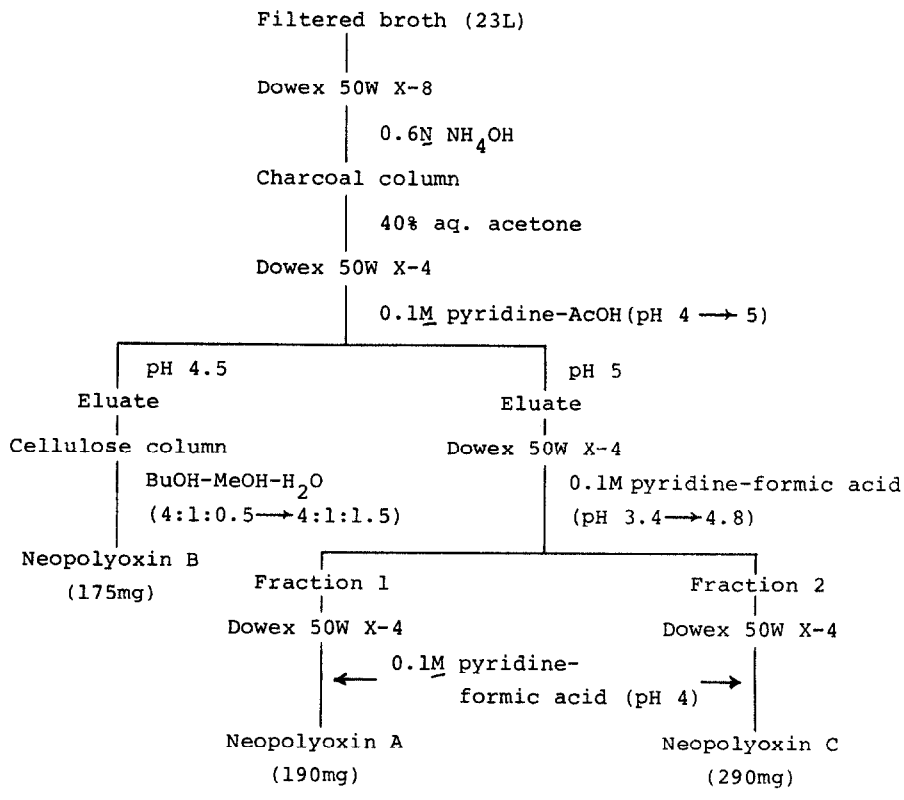
Neopolyoxins show inhibitory activity against phytopathogenic fungi such as *Pyricularia oryzae*, *Rhizoctonia solani*, *Botrytis cinerea*, *Cochliobolus miyabeanus*, and *Candida albicans* at concentrations of 0.05–50 μg/ml. The antifungal spectrum is shown in Ref. 2. Like polyoxins, neopolyoxins show selective inhibitory activity against phytopathogenic fungi. However, neopolyoxins A and C exhibit higher antifungal activity than the known polyoxins and are effective against *Candida albicans*. Polyoxin-resistant *Alternaria mali* showed cross resistance to neopolyoxins. Inhibition of chitin synthetase prepared from *Pyricularia oryzae* by neopolyoxins A, B, and C (ID_{50}) were 0.83, 41.7, and 1.7 μg/ml, respectively. Acute toxicities of these antibiotics in mice were low (LD_0, >250 mg/Kg by intravenous injection).

Structure determination

The ^1H and ^{13}C NMR spectra of neopolyoxin A **1** suggested the presence of an aldehyde group: $\delta_H^{D_2O}$ 9.20 (1H, s); $\delta_c^{D_2O}$ 180.4(d). It was hydrolyzed by leucine aminopeptidase[4] to afford a nucleoside **4** and an amino acid **7**. Mild alkaline hydrolysis of **1** gave a base moiety in addition to **4** and **7**. The base was positive to an *o*-dianisidine test and showed characteristic absorption maxima in the UV spectrum: $\lambda_{max}^{0.05N\ HCl}$ 284 nm, $\lambda_{max}^{0.05N\ NaOH}$ 315 nm; $M^+ = m/z$ 112.

Neopolyoxin B **2** was hydrolyzed by leucine aminopeptidase to afford two products, a nucleoside **5** and an amino acid identical with **7**.

Neopolyoxin C **3** was hydrolyzed with 0.5*N* NaOH to



Scheme 1. Isolation procedure of neopolyoxins.

give uracil, a nucleoside **6** ($C_{10}H_{13}N_3O_7$), and an amino acid **7**. The nucleoside **6** was identified as uracilpolyoxin C[1] on the basis of its ^1H NMR spectrum and mobility on TLC.

The nucleosides **4** and **5** obtained by hydrolysis of **1** and **2**, were trimethylsilylated to afford penta- and hexa-trimethylsilyl (TMS) derivatives, respectively. A number of structural features of the nucleosides derived from **1** and **2** were corroborated by high resolution mass spectrometry of their TMS derivatives, established by this and other means as structures **8** and **9**. Their mass spectra are shown in Figs. 1 and 2, and the corresponding fragmentation schemes in Figs. 3 and 4, respectively. The structural assignments indicated are supported in every case by appropriate mass shifts from -Si(CD$_3$)$_3$ derivatives, and are based in part on the known fragmentation behavior of silylated nucleosides,[5] including those bearing the basic 6-carbon polyoxin sugar.[6] All assignments in Figure 4 are confirmed by high resolution measurements of exact mass.

Elemental compositions of $C_{10}H_{13}N_3O_7$ for **4** and

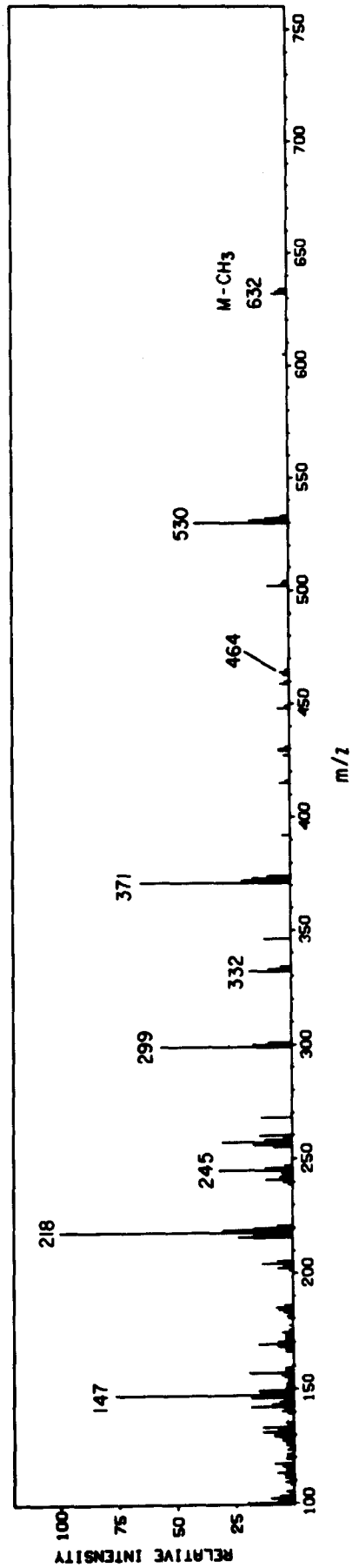


Fig. 1. Mass spectrum of 8.

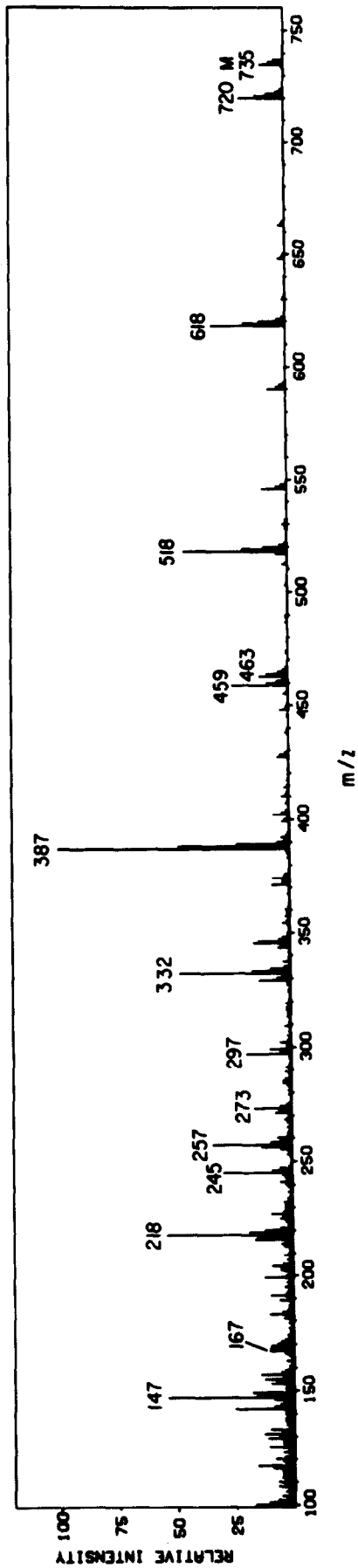


Fig. 2. Mass spectrum of 9.

Table 1. Assignment of ^1H chemical shifts (δ_H) of neopolyoxins in D_2O

	1		2		3	
	δ_H	J(Hz)	δ_H	J(Hz)	δ_H	J(Hz)
C_5-H	7.73(1H,s)		6.99(1H,s)		5.79(1H,d)	8
C_6-H	9.20(1H,s)				7.61(1H,d)	8
$C_{1'}$-H	5.63(1H,d)	5	5.56(1H,d)	4	5.81(1H,d)	4.8
$C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H, $C_{5'}$-H, $C_{2''}$-H	4.2 – 4.6 (5H)		4.3 – 4.7 (5H)		4.2 – 4.6 (5H)	
$C_{3''}$-H	2.67(1H,m)		2.74(1H,m)		2.65(1H,m)	
$C_{4''}$-H	5.10(1H,d)	2.5	5.33(1H,d)	2	5.13(1H,d)	2.8
$C_{6''}$-H, $C_{7''}$-H	7.37(2H)		7.65(2H)		7.40(2H)	
$C_{9''}$-H	8.00(1H,bs)		8.11(1H,bs)		8.00(1H,bs)	
$C_{10''}$-H	0.79(3H,d)	7	0.77(3H,d)	7	0.75(3H,d)	7

Table 2. Assignment of ^{13}C chemical shifts (δ_c) of neopolyoxins in D_2O

Carbon No.	1	2	3
2	153.9	153.5 *3	151.8
4	124.3	120.4	166.1
5	125.9	114.0	102.6
6	180.4	165.8	142.1
1'	86.6	86.2	89.2
2'	73.2 *1	72.3 *1	73.0 *1
3'	70.5	70.1	70.3
4'	84.4	84.0	84.1
5'	56.8	56.9	56.8
6'	169.0	168.7	168.7
1"	173.6	173.6	173.7
2"	56.8	56.9	56.8
3"	39.6	39.7	39.5
4"	74.3 *1	72.8 *1	73.7 *1
5"	149.8	147.8	149.3
6"	122.8 *2	124.3 *2	123.1 *2
7"	125.9 *2	130.7 *2	127.1 *2
8"	153.9	154.8 *3	153.8
9"	135.6	131.2	134.4
10"	6.8	6.5	6.6

* The assignments may be interchanged.

$C_{10}H_{13}N_3O_8$ for **5** were supported by measurements of exact molecular mass, after correction for presence of 5 TMS groups in **8** and 6 TMS groups in **9** as determined by deuterium labeling. Cleavage of the glycosidic bond accompanied by characteristic rearrangement of H or

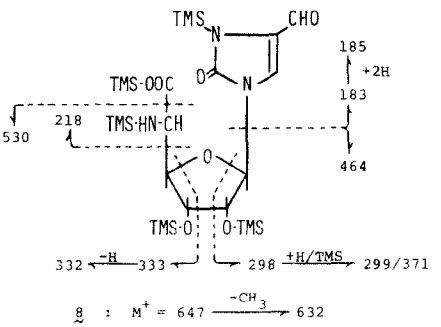


Fig. 3. Structure assignments from the mass spectrum of **8**.

TMS groups from the sugar moiety yields an ion series which shows the base in **8** to have the composition $C_4H_2N_2O_2$ (TMS), and $C_4HN_2O_3$ $(TMS)_2$ in **9**, which support the structures shown, or alternatively, the TMS

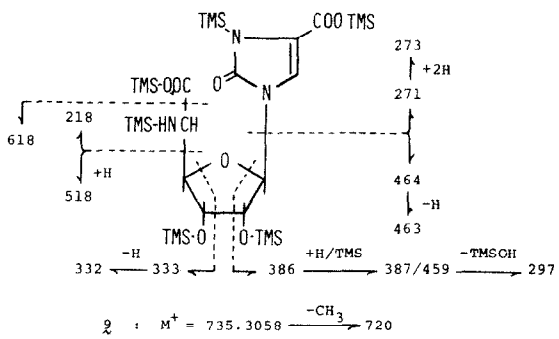


Fig. 4. Structure assignments from the mass spectrum of **9**.

derivative of uracil and 5-(or 6-) hydroxyuracil, respectively. In the former case, it was confirmed that the mass spectrum of the base moiety (*m/z* 112, 84, 56) obtained by alkaline hydrolysis of **1** was not the same as that of uracil. In the latter case, evidence for the presence of a carboxy ester function in the base is given by loss of -OTMS from the base +H ion (*m/z* 272), which produces a well-stabilized enone carbonium ion *m/z* 183, and its daughter ion 167 by elimination of CH_4. Loss of -OTMS from the alternative hydroxyuracil moiety is energetically unfavourable, and is not observed in the spectra of silylated 5- or 6-hydroxyuridine.

The sugar fragment *m/z* 464 and its common daughter products,[5] as well as *m/z* 530 (or 618 in **9**) and 218 in **8** and **9** which represent cleavage unique to the polyoxin sugar,[6] show the sugar composition to be $C_6H_6NO_5(TMS)_4$ in both TMS derivatives. The ribose cleavage products *m/z* 333, 299 in **8** and 333, 387 in **9**, and related ions, plus the C-5', 6' fragmentation products,[6] requires the overall sugar structure shown, exclusive of steric orientation of the groups.

In addition to the UV spectra of **2**, support for the 2 - oxo - 4 - imidazoline - 4 - carboxylic acid structure **5** over the alternative and biologically reasonable hydroxyuracil isomer was gained from acid-catalyzed ^{18}O-exchange experiments. Under the conditions employed, the C-6 carboxyl group should undergo extensive exchange of both oxygens, resulting in a mass spectrometric shift of +4 mass units. The hydroxyuracil base undergoes only 9% exchange of ^{18}O (shift of +2) under these conditions as determined from exchange using 5-hydroxyuridine as a model. This result is in accord with earlier work involving uridine derivatives in which much more drastic conditions were employed.[7] The hydroxyuracil-containing polyoxin would therefore be expected to principally incorporate 2 atoms of ^{18}O, and a small amount of 3 atoms of ^{18}O. By contrast, the imidazoline structure would be expected to accommodate maximum exchange of 2 oxygens in the base (shift of +4), or a maximum allowable shift of +8 mass units for the molecule. The experimentally found ^{18}O distributions, given in Table 3, show maximum incorporation of 4 atoms of ^{18}O (shift of +8) with substantial incorporation of 3 atoms of ^{18}O, clearly in support of the imidazoline structure. Although the extent of exchange in the present case was limited by degradation of the sample during heating, the method appears generally useful for microscale characterization of oxygen-containing functional groups in nucleoside analogs.[8]

The ^1H and ^{13}C NMR spectra of **4** obtained by hydrolysis of **1** indicated that the nucleoside **4** has an aldehyde group: $\delta_H^{3\%DCl}$ 9.21 (1H, s); $\delta_c^{3\%DCl}$ 180.9 (d, J_{CH} = 184.3 Hz). The UV spectrum exhibited a characteristic bathochromic shift in alkaline solution: $\lambda_{max}^{0.05N\ HCl}$ 286 nm (ϵ 9000); $\lambda_{max}^{0.05N\ NaOH}$ 309 nm (ϵ 7330). Oxidation of **4** with silver oxide afforded the corresponding carboxylic acid **5** ($C_{10}H_{13}N_3O_8$). Similarly, **1** was oxidised with silver oxide followed by mild alkaline hydrolysis to give an oxidised base in low yield, which was identical with 2 - oxo - 4 - imidazoline - 4 - carboxylic acid on the basis of its UV spectrum and mobility on tlc.

Fox and his co-workers reported chemical transformation of uridine into 1 - (β - D - ribofuranosyl) - 2 - oxo - 4 - imidazoline - 4 - carboxylic acid.[9] Similarly, uracilpolyoxin C **6** was treated with bromine followed by sodium bicarbonate to afford a crystalline product. The UV, IR, and CD spectra were in good accordance with those of **5**. Since the absolute configuration of **6** has been firmly established,[1] this is an unambiguous proof for the structure of **5**. Consequently, the absolute structure of **4** was determined as 1 - β - (5 - amino - 5 - deoxy - D - allofuranosyl uronic acid) - 2 - 4 - imidazoline - 4 - carbaldehyde.

Table 3. Extent of ^{18}O-exchange in neopolyoxin B nucleoside (**5**)

no. of ^{18}O atoms incorporated	relative abundance*
0	6
1	25
2	40
3	23
4	6
5	0

*Measured from the M-CH_3 peaks in the mass spectrum; corrected for contributions from naturally occurring heavy isotopes, and from ^{16}O in reaction solvent.

The nucleoside moiety obtained by hydrolysis of neopolyoxin B **2** was found to be identical with the imidazoline nucleoside **5** in their IR spectra, CD curves, and R_f values on tlc.

The assignments of the chemical shifts in the ^1H and ^{13}C NMR spectra of **4**, **5**, and **6** are listed in Tables 4 and 5.

The isolated side chain amino acid **7**, obtained by hydrolysis of all of the neopolyoxins, has an aromatic chromophore. In the ^1H NMR spectrum, an ABX type coupling was observed in the aromatic region: $\delta_H^{3\%DCl}$ 7.93 (C_3–H, J = 0.8, 9Hz), 8.07 (C_4–H, J = 2, 9Hz), 8.31 (C_6–H, J = 0.8, 2Hz). The UV and ^{13}C NMR spectra showed close similarity to those of authentic 3-hydroxy-6-methylpyridine: $\lambda_{max}^{H^+}$ 223, 289 nm, $\lambda_{max}^{OH^-}$ 238, 305 nm; $\delta_c^{3\%DCl}$ 18.4(q), 128.0(d), 128.7(d), 132.8(d), 144.6(s), 154.5(s). In the proton decoupling experiment, the irradiation of a doublet at δ5.12 (C_4–H) converted a multiplet at δ2.84(C_3–H) into a double quartet (J = 2, 7Hz). This indicated a partial structure $-\overset{|}{\underset{O}{CH}}-\overset{CH_3}{\overset{|}{CH}}-\overset{|}{\underset{N}{CH}}-$.

Thus, structure **7**, 2 - amino - 4 - hydroxy - 4 - (5 - hydroxy - 2 - pyridyl) - 3 - methylbutyric acid, was proposed, and was supported by the ^{13}C NMR assignments as shown in Fig. 5.

The amino acid **7** was trimethylsilylated to afford the *O*-tris(TMS) derivative. The mass spectrum, which was analogous to the tetrasilyl derivative,[10] also supported the assigned structure, which was ascertained by deuterium labeling (-Si$(CD_3)_3$) and measurements of exact mass (Fig. 6): M^+ = *m/z* 442 (0.13%, rel. int.), 427.1932 (2.3%), 296.1533 (21%), 269.1259 (100%), 235.1231 (78%).

To determine the stereostructure of **7**, it was converted to a cyclic compound. Treatment of **7** with acetic anhydride in methanol yielded a crystalline γ-lactone diacetate **10** ($C_{14}H_{16}N_2O_5$). The structure **10** was sup-

Table 4. Assignment of ^1H chemical shifts (δ_H) of the nucleosides in 3% DCl in D_2O

	4	5	6
C_5-H	7.58(s)[a]	7.37(s)	5.85(d) $J_{5,6}$ = 8
C_6-H	9.21(s)		7.48(d)
$C_{1'}$-H	5.59(d) $J_{1'2'}$ = 4[b]	5.65(d) $J_{1'2'}$ = 4	5.72(d) $J_{1'2'}$ = 3.7
$C_{2'}$-H	4.48(dd) $J_{2'3'}$ = 6	4.53(dd) $J_{2'3'}$ = 6	4.43(dd) $J_{2'3'}$ = 6.5
$C_{3'}$-H	4.70(dd) $J_{3'4'}$ = 6	4.77(dd) $J_{3'4'}$ = 6	4.68(dd) $J_{3'4'}$ = 6.5
$C_{4'}$-H	4.38(dd) $J_{4'5'}$ = 2.5	4.45(dd) $J_{4'5'}$ = 2.5	4.35(dd) $J_{4'5'}$ = 2.8
$C_{5'}$-H	4.55(d)	4.64(d)	4.59(d)

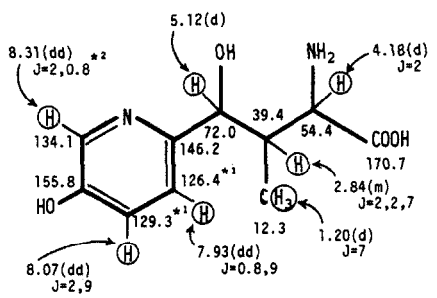
a, Multiplicities: s,singlet; d,doublet; dd,double doublet.

b, J values are in Hz.

Table 5. Assignment of ^{13}C chemical shifts (δ_C) of the nucleosides in 3% DCl in D_2O

Carbon No.	4	5	6 *
2	153.9	153.5	151.6
4	124.9	115.6	166.2
5	126.3	118.4	102.8
6	180.9	162.1	143.3
1'	88.7	87.8	92.2
2'	73.2	72.6	72.5
3'	70.4	69.8	69.3
4'	81.8	81.1	80.9
5'	54.7	54.1	53.9
6'	169.0	168.4	168.6

* Data from ref. 15.



*1, The assignments may be interchanged.

*2, J Values are in Hz.

Fig. 5. Assignment of ^1H and ^{13}C chemical shifts of **7** in 3% DCl in D_2O.

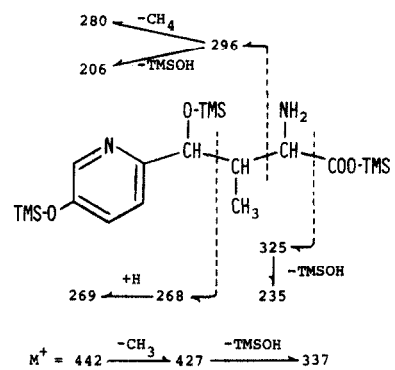


Fig. 6. Fragmentation pattern of the *O*-tris (TMS) derivative of **7**.

ported by its IR and ^1H NMR spectra (Fig. 7A): ν_{max}^{KBr} 1780 (γ-lactone), 1760 (ester), 1655 (amide) cm^{-1}; $\delta_H^{CDCl_3}$ 1.27 (3H, d, J = 7Hz; C_3-CH_3), 2.07 (3H, s; $OCOCH_3$), 2.34 (3H, s; $NHCOCH_3$), 2.53 (1H, m; C_3-H), 4.56 (1H, dd, J = 8, 10Hz; C_2-H), 5.03 (1H, d, J = 10Hz; C_4-H), 6.18 (1H, d, J = 8Hz; NHCO), 7.48 (2H; aromatic-H), 8.33 (1H; aromatic-H) ppm. The relative configuration of **10** was determined by NOE experiments as already described.[3] As shown in Fig. 7B–E, NOE enhancements of proton signals upon irradiation of specific protons were observed from the difference spectra. Thus, *trans* configuration for the substituents at C-2, C-3, and C-4 was established as shown in **10** (Fig. 8).

To determine the absolute configuration of **7**, hydrogenation of the pyridine ring to a piperidine ring was attempted. Amino acid **7** was hydrogenated over 5% rhodium on alumina in an aqueous solution. A diastereomeric mixture was obtained after purification of the hydrogenated products. For characterization, the purified product was trimethylsilylated and analyzed by gas chromatography-mass spectrometry. Several peaks were detected by gas chromatography. Two main peaks gave the same mass spectrum for the penta(TMS) derivative. Two intense ions at *m/z* 475 and 244 supported the structure **11** as shown in Fig. 9. The observed positive CD band ($[\theta]_{214} = +4090$, in 0.5N HCl) of the hydrogenated amino acid indicated the L (i.e. 2S) configuration. Therefore, configuration of **7** has been determined as (2S, 3S, 4S). The 4R configuration reported in our

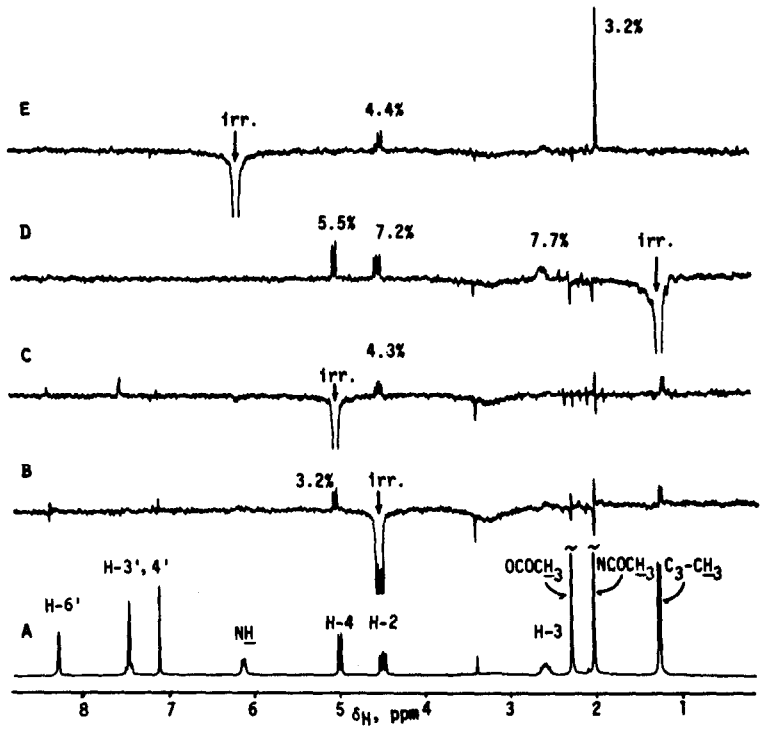


Fig. 7. Difference spectra in the NOE experiments of **10**: A, original ^1H NMR spectrum; B, irradiation at H-2; C, irradiation at H-4; D, irradiation at C_3–CH_3; E, irradiation at NH. In the difference spectra B-E, resonance signals were multiplied by 16.

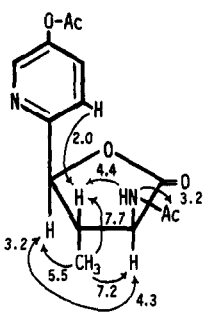


Fig. 8. The relative configuration determined from NOE experiments. The figures are values (%) of NOE enhancement calculated from difference spectra as shown in Fig. 7.

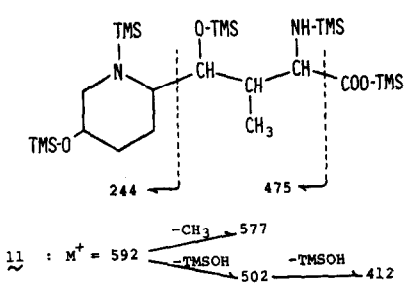


Fig. 9. Fragmentation pattern of **11**.

previous paper was due to misassignment in nomenclature.[3]

Neopolyoxins A, B, and C were treated with nitrous acid and deaminated products were hydrolyzed with 0.5*N* NaOH. The nucleosides **4**, **5** and **6** were detected, respectively, but **7** was not detected in each case. Therefore, the amino group of acid **7** is unsubstituted in **1**, **2** and **3**. Structures **1**, **2** and **3** are thus proposed for neopolyoxins A, B and C, respectively.

The position of the amide bond accounts reasonably for the alkaline lability of the nucleoside bond[1] and acid lability of the amide bond.[3]

Potentiometric titration of **1** showed pKa' values of 2.9 (-COOH), 4.3 (pyridine), 7.3(-NH_2), 8.7 (phenolic OH), and 10.3 (base). The pKa' of **3** indicates similar values except for that of the base moiety (2.7, 4.1, 7.1, 8.5, 9.7). In the case of **2**, the presence of the additional carboxyl group is obscured because of the low pKa' value: 3.0 (-COOH), 4.4 (pyridine), 7.7 (-NH_2), 9.1 (phenolic OH), 11.1 (base). The acidity of the base moiety in **2** is considered to be reduced by the carboxylate group.

DISCUSSION

The structures of the neopolyoxins reported here indicate that they are structural analogs of the polyoxins. It should be noted that both are produced by the same species of *Streptomyces*. In the biosynthesis of the polyoxins, which have the same sugar moiety as neopolyoxins, it was shown that the pyrimidine nucleoside moiety is biosynthesized by condensation of uridine and phosphoenolpyruvate.[11] It may be considered that, in neopolyoxin biosynthesis, the nucleoside moiety of

neopolyoxin C **3** is formed first from uridine, and then subsequent ring contraction of uracil to the imidazoline ring occurs at the nucleoside level. Indeed, this benzilic acid rearrangement-type reaction discovered by Fox *et al.* in 1969[9] played a key role in the determination of the structures of nucleosides **4** and **5**. By this transformation, it was possible to establish the base structure and the glycosyl position, as well as the absolute configuration of the sugar moiety. It should be noted that we have isolated polyoxin N,[12] a new member of the polyoxin family, which contains 2 - oxo - 4 - imidazoline - 4 - carbaldehyde. Neopolyoxin B **2** should not be an artifactual oxidation product formed during isolation, because extensive air oxidation of the nucleoside **4** in water gave no indication of the appearance of the nucleoside **5**. Also, air oxidation of neopolyoxin A **1** did not give **2**, although some hydrolysis occurred.

Recently, Zähner *et al.* reported[13] the structures of nikkomycins X and Z, which were isolated from *Streptdomyces tendae*. Their planar structures correspond to those of neopolyoxins A **1** and C **3**, respectively, although no proof is given for the glycosyl position in their paper. More recently, König *et al.* reported[14] the absolute configuration of the side chain amino acid **7** of nikkomycins on the basis of X-ray analysis and a CD curve of the *N*-ethylthio(thiocarbonyl) derivative of **7**. The absolute configuration is the same as reported herein. The configuration of the nucleoside moiety of nikkomycins is not yet known, although they stated[10] that the sugar moiety of nikkomycins is not identical with that of the polyoxins, i.e. that of the neopolyoxins.

EXPERIMENTAL

All m.ps were taken on a Yanagimoto micro melting point apparatus and were uncorrected. UV spectra were measured on a Hitachi 124 spectrophotometer and IR spectra on a Shimadzu IR-27G recording infrared spectrophotometer. Optical rotations were determined on a Perkin-Elmer 241MC polarimeter. ^{1}H NMR spectra at 100 MHz and ^{13}C NMR spectra at 25.05 MHz were obtained on a JEOL FX-100 FT NMR spectrometer. Chemical shifts were converted to the δ scale in ppm downfield from TMS in $CDCl_3$ as an external standard unless otherwise stated. NOE experiments were carried out by a Bruker WH-270 FT NMR spectrometer on $CDCl_3$ solution at 23°. CD curves were run on a JASCO J-20A automatic recording spectropolarimeter. Low resolution mass spectra were recorded on a Hitachi RMU-6MG GC/MS spectrometer and high resolution mass spectra were acquired using a JEOL-01SG spectrometer and a Varian MAT 731 instrument at 70 eV ionizing energy. High performance liquid chromatography was carried out by a Hitachi 635A liquid chromatograph. Tlc were developed on Avicel SF microcrystalline cellulose and Merck precoated cellulose plates. Solvent systems used were as follow: A, butanol–acetic acid–water (4:1:2); B, butanol–methanol–water (4:1:2); C, propanol-1*N* NH_4OH (7:3).

Isolation and separation of neopolyoxins A, B, and C.

Steptomyces cacaoi subsp. *asoensis* was fermented in a culture medium consisting of glucose (1%), soluble starch (9%), soybean flour (2%), dried yeast (4%), sodium nitrate (0.2%), and dipotassium hydrogen phosphate (0.2%) in a jar fermentor at 28° for 160 h. Filtered broth (23L) was passed through a column of Dowex 50W X-8 (50-100 mesh, H form 3.6L) and the antibiotics were eluted with 0.6*N* NH_4OH. After neutralization with dilute HCl, the eluate was passed through a charcoal column (2L). After washing with water, active components were eluted with 40% aqueous acetone. The eluate was concentrated *in vacuo* to a small volume, to which acetone and ether were added to precipitate an antibiotic complex (15g). The crude material was dissolved in 40 ml of a buffer of 0.1*M* pyridine–acetic acid (pH 4.0) and the soln was loaded on a column of Dowex 50W X-4 (100-200 mesh, 1.5L) buffered with the same buffer. The elution was carried out stepwise with the same buffer with pH gradient from 4.0 to 5.0 (16L), and separated into 20 ml fractions. A fraction (No. 237–272) eluted with a buffer of pH 4.5, which contained neopolyoxin B, was concentrated to a small volume. By adding ethanol and ether to the soln, and filtration, a crude powder was obtained (400 mg). It was further purified by cellulose column chromatography (18 × 700 mm) with a solvent system of butanol–methanol–water (4:1:0.5–4:1:1.5). Biologically active fractions were collected and evaporated to a small volume, to which ethanol and ether were added to afford purified neopolyoxin B (175 mg) as a white powder.

A fraction (No. 601–771) eluted with a buffer of pH 5.0 was concentrated to a small volume, to which ethanol and ether were added to precipitate a mixture of neopolyoxins A and C (1.2g). It was chromatographed on a column of Dowex 50W X-4 (100-200 mesh, 30 × 600 mm) buffered with 0.2*M* pyridine–formic acid (pH 3.4) and the elution was made stepwise from pH 3.4 to pH 4.8. The first biologically active fractions (No. 225–273), which showed positive ninhydrin and UV tests, were collected and rechromatographed on a Dowex 50W X-4 column using the same buffer (pH 4.0) in the similar manner described above. Pure neopolyoxin C was obtained from the active fractions as a white powder (290 mg).

The second biologically active fractions (No. 281–310), which showed positive UV, ninhydrin, and *o*-dianisidine tests, were collected and rechromatographed on a Dowex 50W X-4 column in the similar procedure to neopolyoxin C. Pure neopolyoxin A was obtained as a white powder (190 mg).

Enzymatic hydrolysis of neopolyoxin A **1**. To a soln of neopolyoxin A **1** (200 mg) in phosphate buffer (pH 6.4, 5 ml), leucine aminopeptidase (100 mg) was added and the soln. was incubated at 37° for 6 days in a water bath with gentle shaking. The reaction mixture was loaded on a charcoal column (15 × 150 mm). After washing with water, the hydrolysates were eluted with 40% aq acetone. The eluate was evaporated to dryness and the residual material (150 mg) was dissolved in a small amount of a buffer (0.2*M* pyridine–formic acid, pH 3.4). It was chromatographed on a column of Dowex 50W X-2 (100–200 mesh, 18 × 600 mm) buffered with the same buffer. The fractions, which were eluted with a buffer of pH 3.4 and showed positive UV, ninhydrin, and *o*-dianisidine tests, were collected and concentrated to afford crude crystals of **4**. After filtration, it was recrystallised from aq EtOH to obtain 45 mg of the nucleoside **4** as colourless needles: m.p. > 190° (dec.); $\lambda_{max}^{0.05N\ HCl}$ 286 nm (ϵ 9000), $\lambda_{max}^{0.05N\ NaOH}$ 309 nm (ϵ 7330); CD, $[\theta]_{285} = -4895$ (H_2O). Found: C, 41.66; H, 4.59; N, 14.37. Calc. for $C_{10}H_{13}N_3O_7$: C, 41.81; H, 4.56; N, 14.63%.

The ninhydrin- and UV-positive fractions eluted with a buffer of pH 4.0 were collected and concentrated to a small volume. The crystalline powder of **7** (67 mg) was obtained by addition of ethanol and ether and filtration. It was recrystallised from aq EtOH to yield 50 mg of crystalline **7**: m.p. 230° (dec.); $\lambda_{max}^{H_2O}$ 219, 256, 279, 317 nm (ϵ 8660, 1880, 3460, 560), $\lambda_{max}^{0.05N\ HCl}$ 205, 228, 289 nm (ϵ 13930, 5420, 6250), $\lambda_{max}^{0.05N\ NaOH}$ 242, 302 nm (ϵ 12130, 4070); CD, $[\theta]_{220} = +13058$, $[\theta]_{257} = +542$, $[\theta]_{284} = -763$ (H_2O). Found: C, 51.72; H, 6.21; N, 11.99%. Calc. for $C_{10}H_{14}N_2O_4 \cdot 1/2H_2O$: C, 51.06; H, 6.43; N, 11.91%.

Alkaline hydrolysis of neopolyoxin A **1**

Neopolyoxin A **1** (50 mg) was dissolved in 0.5*N* NaOH (2 ml) and the soln. was heated on a steam bath for 1 h in a sealed tube. The hydrolysates were passed through a column of 5 ml of Dowex 50W X-4 (100-200 mesh). The effluent was evaporated to dryness and the residue was purified by preparative tlc with solvent C. The UV absorbing band at R_f value of 0.66 was extracted with water and lyophilised giving 0.3 mg of the base moiety: $\lambda_{max}^{0.05N\ HCl}$ 284 nm, $\lambda_{max}^{0.05N\ NaOH}$ 315 nm: $M^+ = m/z$ 112.

The eluate from the resin with 0.5*N* NH_4OH was concentrated to dryness. It was analyzed by tlc. The nucleoside **4** and the amino acid **7** were detected on tlc. R_f values: For **4**, 0.20 (solvent A), 0.18 (B), 0.18(C); for **7**, 0.50(A), 0.53(B), 0.37(C).

Enzymatic hydrolysis of neopolyoxin B **2**

To a soln of **2** (30 mg) in phosphate buffer (pH 6.4, 1 ml),

leucine aminopeptidase (15 mg) was added and the soln. was incubated at 37° for 9 days in a water bath with gentle shaking. The reaction mixture was passed through a charcoal column (12 × 100 mm) and the column was eluted with 40% aqueous acetone. The eluate was evaporated to dryness and purified by preparative tlc with butanol–methanol–water (4:1:2). The amino acid portion was extracted with water from a UV absorbing band at R_f value of 0.54, and lyophilised to afford as a white powder (5 mg). It was identified with **7** on tlc. Similarly, the nucleoside moiety was obtained from a UV absorbing band at R_f 0.15 and recrystallised from water to obtain 6 mg of **5** as colourless needles: m.p. 212°C; $\lambda_{max}^{H_2O}$ 252 nm (ε 7720), $\lambda_{max}^{0.05N\ HCl}$ 263 nm (ε 8360), $\lambda_{max}^{0.05N\ NaOH}$ 256 nm (ε 6690); CD, $[\theta]_{252} = -5320$ (H_2O). Found: C, 39.12; H, 4.53; N, 13.49. Calc. for $C_{10}H_{13}N_3O_8$: C, 39.61; H, 4.32; N, 13.86.

Alkaline hydrolysis of neopolyoxin C **3**

A soln of **3** (70 mg) in 0.5*N* NaOH (3 ml) was heated on a steam bath for 1 h in a sealed tube. The hydrolysates were purified by the similar procedure to that used for neopolyoxin A **1**. The base moiety (0.5 mg), obtained from the effluent portion of the resin, was identical with uracil by comparison of mass spectra and mobility on tlc. The eluate from the resin with 0.5*N* NH_4OH was evaporated to dryness and subjected to preparative tlc with solvent B.

The UV absorbing band at R_f 0.5 was extracted with water and lyophilised to afford a white powder (5 mg), which was identical with **7** on tlc (solvent A, B, and C). The other UV absorbing band at R_f 0.14 was extracted with water and lyophilised to obtain 7 mg of a white powder. The ^1H NMR spectrum was identical with that of uracilpolyoxin C **6**.

Acid catalyzed ^{18}O exchange reaction with **5**

A soln of **5** (50 μg) in 90 atom% $H_2{}^{18}O$ (10 μl) 1*N* HCl was heated at 70° for 6 h in a sealed glass melting point capillary. The sample was withdrawn and dried in a separate screwcapped vial. It was dissolved in 50 μl of anhyd pyridine, *N,O*-bis(trimethylsilyl)-trifluoroacetamide, and trimethylchlorosilane (9:90:1) and heated at 100° for 1 h. An aliquot of the reaction soln. was used for mass spectrometric measurement of ^{18}O content.

Oxidation of the nucleoside **4** *from neopolyoxin A* **1**

To a soln of **4** (20 mg) and Na_2CO_3 (27 mg) in water (3 ml), freshly prepared wet silver oxide (about 70 mg) was added. The soln in a sealed tube was heated at 70° in a water bath for 2 h. Water was added to the reaction soln. which was centrifuged at 3000 rpm. The supernatant soln. was passed through 6 ml of Dowex 50W X-4 (100–200 mesh). The oxidised product was eluted with 0.1*N* NH_4OH, and the eluate was evaporated to afford crystals of **5**. After recystallisation from water, 5 mg of **5** was obtained as colourless needles. It was identical with the nucleoside **5** obtained from hydrolysis of **2** on the basis of the UV, IR, CD spectra and R_f values on tlc with solvents A, B, and C.

2 - Oxo - 4 - imidazoline - 4 - carboxylic acid from oxidized neopolyoxin A

To a soln of **1** (10 mg) and Na_2CO_3 (14 mg) in water (2 ml) was added freshly prepared wet silver oxide (about 40 mg). The soln was heated at 70° in a water bath in a sealed tube for 2 h. The reaction soln. was passed through a charcoal column and the oxidized product was eluted with 30% aqueous acetone. The eluate was evaporated to dryness and the residue was dissolved in 0.5 N NaOH (1 ml). The soln was heated on a steam bath for 1 h and passed through a column of 3 ml of Dowex 50W X-4 (100-200 mesh). The effluent was concentrated and purified by preparative tlc with solvent B. The UV absorbing band at R_f 0.44 was extracted and lyophilised to obtain 0.2 mg of a white powder. The UV spectrum and mobility on tlc were identical with those of authentic 2 - oxo - 4 - imidazoline - 4 - carboxylic acid.

Conversion of uracilpolyoxin C **6** *into imidazoline nucleoside* **5**

To a soln of **6** (30 mg) in water (3 ml), a small excess of Br_2 water was added. The excess Br_2 was removed by aeration, and $NaHCO_3$ (84 mg) was added. The soln was diluted to 4 ml and refluxed for 20 h. The reaction mixture was passed through a column of Dowex 50W X-4 (8 ml, 100-200 mesh). The reaction product was eluted with 0.6*N* NH_4OH and the eluate was evaporated to dryness. It was further purified by preparative tlc with solvent A. The UV absorbing band at R_f 0.17 was extracted with water and the soln was concentrated to dryness. It was further purified by preparative tlc with solvent A. The UV absorbing band at R_f 0.17 was extracted with water and the soln was concentrated to dryness. The residue was crystallised from hot water to afford 3 mg of crystals: m.p. 210°; CD, $[\theta]_{252} = -4813$ (H_2O); $\lambda_{max}^{H_2O}$ 252 nm (ε 6710), $\lambda_{max}^{0.05\ N\ HCl}$ 263 nm (ε 8120), $\lambda_{max}^{0.05N\ NaOH}$ 256 nm (ε 5680). It was identical to **5** as determined from the above data, IR spectrum, and TLC mobility (solvents A, B, and C).

Acetylation of the amino acid **7**

To a soln of **7** (80 mg) in MeOH (10 ml), was added Ac_2O (1 ml) and the soln was allowed to stand overnight at room temp with stirring. After addition of water (2 ml), the reaction soln was concentrated to dryness. The residue was crystallised from chloroform-hexane to afford crystalline diacetate, which was recrystalised from the same solvent system to obtain 82 mg of **10** as colourless prisms: m.p. 206–208°; $[\alpha]_D^{24}$ +13.9° (*c*0.5, MeOH); λ_{max}^{MeOH} 265, 270(sh) nm (ε 4330, 3700); MS, $M^+ = m/z$ 292.1023 (m/z Calc. for $C_{14}H_{16}N_2O_5$, 292.1058). Found: C, 57.51; H, 5.53; N, 9.55. Calc.: C, 57.53; H, 5.52; N, 9.59.

NOE of γ-lactone diacetate **10**

A soln of **10** (4 mg) in $CDCl_3$ (0.5 ml) was degassed 3 times by the freeze-thaw method and sealed in a NMR tube under N_2 gas. NOE enhancements were determined by applying a 20-s low power saturating pulse at the appropriate peak position, followed by a high power 90° observing pulse after 0.2-s waiting time. Off-resonance control spectra were measured in the same way, except that the presaturating pulse was offset 1000 Hz higher field from TMS so that no solute resonances were perturbed. On-resonance and off-resonance spectra were the sum of 128 scans.

Rhodium hydrogenation of amino acid **7**

A soln of **7** (80 mg) in water (20 ml) was hydrogenated over 5% rhodium on alumina (100 mg) at 3 kg/cm² for 16 h. After filtration, the filtrate was evaporated to dryness. The hydrogenated material was chromatographed on a cellulose column using butanol–acetic acid–water (4:1:1) as a solvent system. The fractions, which showed positive ninhydrin test and no UV absorption, were collected and evaporated to dryness (25 mg). The residue was purified by preparative tlc with solvent A. The band between R_f 0.15 and 0.25 was extracted with water and lyophilised to afford a white powder (17 mg). It was further purified by hplc using reverse-phase column (Nucleosil $5C_{18}$, 6 × 25 mm) with acetonitrile–methanol (4:1) as developing solvent. By collecting the effluent corresponding to the main peak, the purified material (3 mg) was obtained as a colourless solid on removal of solvent. For characterisation, the purified material (200 μg) was dissolved in anhyd. pyridine (0.1 ml) and *N,O*-bis (trimethylsilyl) acetamide (0.3 ml), and the soln in a sealed tube was heated in a waterbath at 70° for 30 min. The reaction soln was analyzed by gas chromaography-mass spectrometry.

Deamination and subsequent hydrolysis of neopolyoxins. An ice-cooled soln of $NaNO_2$ (4 mg) in water (0.1 ml) was added dropwise to a soln. of neopolyoxin A, B, or C (2 mg) and AcOH (0.02 ml) in water (0.2 ml) in an ice-bath. The reaction soln was allowed to stand for 18 h in a refrigerator. It was passed through a charcoal column (8 × 50 mm) and the product was eluted with 20% aq acetone. It was negative to the ninhydrin test on tlc. The eluate was concentrated to dryness and the deaminated material was hydrolyzed with 0.5*N* NaOH in a sealed tube on a steam bath for 1 h. The reaction soln was passed through a column of Dowex 50W X-4 (1.5 ml). The effluent and the eluate with 0.5*N* NH_4OH were lyophilised to obtain dried materials. Both of them were analyzed by tlc.

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