NOTES

1100-50, a Novel Nematocide from *Streptomyces lavendulae* SANK 64297

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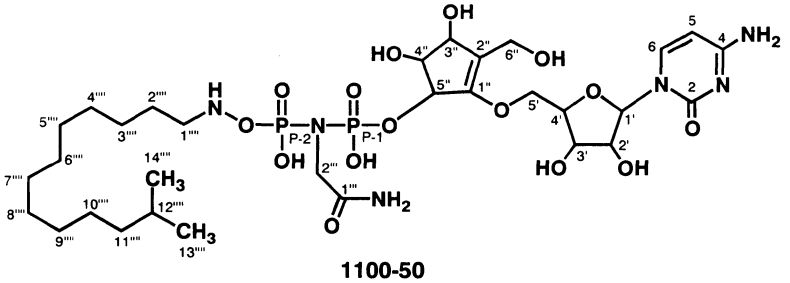
Commercially available pesticides made by chemical synthesis are now widely employed for agricultural use. Although antibiotics were directly applied in the field historically, such use is now limited. Recently, increase in antibiotic-resistant pathogenic microorganisms is of concern and has become a serious clinical problem. For example, in Europe, the prominence of vancomycin-resistant enterococci (VRE) is believed to be derived in some cases from the usage of glycopeptide antibiotics as a feed additive for livestock for growth promotion.[1~3] Thus, the necessity to seek out new antibiotics primarily for field use has arisen.

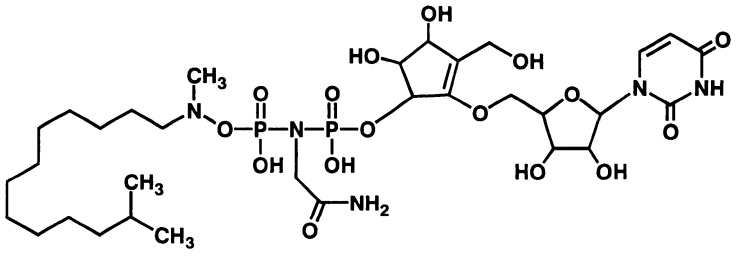
Although plant infecting nematodes cause heavy damage to roots and significant yield loss in crops, few nematocides are put to practical use. In the course of our screening for inhibitors to nematode infection of plants, a novel compound, 1100-50 (**1**, Fig. 1), was found and isolated from the culture of the strain SANK 64297.

The producing strain of SANK 64297 was isolated from a soil sample collected in Maruseppu-cho, Hokkaido, Japan. SANK 64297 belongs closely to the species *Streptomyces lavendulae* based on its taxonomical characteristics. Among the 1493 base pairs of 16S rDNA, 99.7% of the codon usage was found to be identical with

Fig. 1. Structures of 1100-50 and EM2487.



1100-50



EM2487

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the type strain *Streptomyces lavendulae* subsp. *lavendulae* IFO12789. Consequently, the producing strain of SANK 64297 was identified as *Streptomyces lavendulae* SANK 64297 and deposited as FERM BP-7163 at the National Institute of Advanced Industrial Science and Technology, Japan.

One loopful of the producing strain was inoculated into 100 ml of a medium consisting of maltose 1.5%, glucose 0.5%, Pharmamedia 1%, yeast extract 0.3%, K_2HPO_4 0.02%, NaCl 0.05% and silicon KS-66 (Nihon Yushi Co.) 0.01%, pH 7.2, in a 500-ml Sakaguchi flask. The fermentation was carried out for 5 days at 27°C on a rotary shaker (150 rpm).

Acetone (3 liters) was added to the harvested culture (3.3 liters) from 50 flasks and the active substance was extracted for 30 minutes by stirring. The concentrated filtrate (1.6 liters) was applied to a Diaion HP-20 column (800 ml), which was washed with deionized water (3 liters), and the active substance was eluted with 90% aqueous MeOH (1 liter). Just after the eluate was concentrated *in vacuo* to dryness, methanol (100 ml) was added to the oil and the suspension was filtered to remove insoluble material. The filtrate was evaporated to 16 ml and half the amount of the concentrate was applied to a Sephadex LH-20 column (180 ml) equilibrated with MeOH. The chromatography was developed with the same solvent. This process was repeated for the remaining concentrate. The active fractions of the two trials were combined (370 ml) and concentrated *in vacuo* to dryness. The material was washed with acetone (100 ml) and air-dried to yield a crude substance (2.2 g). After the substance was dissolved into 10% MeCN in 20 mM phosphate buffer at pH 6.8 (50 ml), half the amount of the solution was applied to a Cosmosil 140C18OPN column (100 ml) equilibrated with the same solvent. The column was washed with 10% and 20% MeCN in 20 mM phosphate buffer at pH 6.8 (each 200 ml) followed by elution of the active substance with 39% MeCN in 20 mM phosphate buffer at pH 6.8 (400 ml). The process was repeated for the remaining substance. The eluates of the two trials were combined and concentrated *in vacuo* to 20 ml. The concentrate was desalted with a Diaion CHP-20P column (5 ml). The column was washed with 0.06 N NH_4OH (20 ml) and elution of the active substance was performed with 20% acetone in 0.06 N NH_4OH (20 ml). The obtained eluate was concentrated *in vacuo* to 5 ml and the pH was adjusted to 9 with conc. NH_4OH. Then, the solution was freeze-dried to obtain partially purified active substance (55 mg). The substance was dissolved in 1 ml of 35% MeCN in 20 mM phosphate buffer at pH 6.8 and a 20 μl portion was charged to a preparative HPLC column (Senshupak ODS H-4251, 10 mm×250 mm) equilibrated with 20% MeCN in 20 mM phosphate buffer at pH 6.8. The HPLC separation was performed with the same solvent system at a flow rate of 5 ml/minute and the peak was eluted from 10.5 minutes to 11.5 minutes under UV detection at 210 nm. Repeating the HPLC purification fifty-one times yielded pure active substance, and following the desalting process as described above gave 21 mg of **1** as a colorless powder.

Water-soluble compound **1**, $[\alpha]_D^{22}$ −3.8° (*c* 1.0, 0.02 N NaOH), is stable only in basic conditions. The solution easily foams suggesting that **1** is amphiphilic. As shown in Table 1, two doublet phosphorus signals with the coupling constant, $J_{P\text{-}1,P\text{-}2}$ (16.3 Hz), comparable to a 2-bond coupling were observed in the ^{31}P NMR spectrum. The molecular formula was determined to be $C_{31}H_{56}N_6O_{15}P_2$ mainly based on the observed de-protonated molecular ion (found: *m/z* 813.3224 $[M-H]^-$, calcd.: 813.3200) by negative ion mode high resolution FAB-MS analysis with supporting data from ^1H, ^{13}C and ^{31}P NMR. The UV spectrum in 0.02 N NaOH exhibited absorption maximum at 271 nm (ε 5,090) that is indicative of the presence of cytosine. Acid hydrolysis revealed the presence of cytidine (TFA aq. at pH 3.0, amb., 30 minutes), glycine and 12-methyltridecylamine (12 N HCl - AcOH, 1 : 11, 105°C, 15 hours) and glycinamide (0.5 N HCl, 90°C, 1 hour). These physico-chemical properties as well as the results of the various acid hydrolysis experiments mentioned above suggest that **1** is a nucleoside antibiotic composed of two phosphate groups and one cytidine, one glycinamide and one 12-methyltridecylamine substructure.

Compound **1** showed very broad ^1H NMR signals in D_2O. This problem was solved when the observation was performed in basic or diluted conditions below 4 mg/ml. Overlapping signals in the *O*-methine region were resolved successfully by the addition of deuterated methanol. Finally, **1** (2 mg) was dissolved in 0.5 ml of 0.02 N NaOD -CD_3OD (1 : 1) solution for further NMR studies. The ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) signal assignments from these studies are listed in Table 1.

The substructures described above were confirmed by the ^1H-^1H spin couplings observed in pulse-field gradient double quantum filtered COSY (PFG-DQF-COSY) spectrum[4], ^{13}C-^1H couplings in PFG-heteronuclear single quantum coherence (PFG-HSQC) spectrum[5] and ^{13}C-^1H long-range couplings in PFG-heteronuclear multiple quantum coherence (PFG-HMBC) spectrum[6]. As shown in Fig. 2, ^{13}C-^1H long-range coupling between H-5′ (δ 4.44) and C-1″ (δ 155.0) revealed the connection site of cytidine and cyclopentene. Observed ^1H-^{31}P long-range couplings

Table 1. ^{13}C, ^{1}H and ^{31}P signal assignments of 1100-50 (**1**).

Position	δ_C [a]	δ_H [b]	δ_p [c]
2	167.4	-	-
4	158.6	-	-
5	97.0	6.00 (1H, d, J = 7.5 Hz)	-
6	142.6	7.86 (1H, d, J = 7.5 Hz)	-
1'	91.2	5.88 (1H, d, J = 3.6 Hz)	-
2'	76.0	4.14 (1H, dd, J = 3.6, 5.2 Hz)	-
3'	70.0	4.29 (1H, br. t, J = 5.6 Hz)	-
4'	83.7	4.17 (1H, m)	-
5'	69.5	4.17 (1H, m)	-
		4.44 (1H, br. dd, J = 4.0, 11.9 Hz)	-
1"	155.0 (d, J_{c-p} = 8.0 Hz)	-	-
2"	121.4	-	-
3"	71.8	4.61 (1H, dd, J = 1.4, 6.0 Hz)	-
4"	76.6	4.11(1H, dd, J = 4.2, 6.0 Hz)	-
5"	80.7 (d, J_{c-p} = 4.9 Hz)	5.28(1H, m)	-
6"	55.2	4.06 (1H, dd, J = 1.4, 12.5 Hz)	-
		4.23 (1H, d, J = 12.5 Hz)	-
1'''	179.1	-	-
2'''	51.3 (br. t, J_{c-p} = 2.9 Hz)	3.80 (1H, m), 3.87 (1H, m)	-
1''''	53.7 (d, J_{c-p} = 4.7 Hz)	2.98 (2H, br. t, J = 7.2 Hz)	-
1''''-NH [d]	-	6.47 (1H, m)	-
2''''	27.4	1.43 (2H, m)	-
3''''	28.0	1.23 (2H, m)	-
4'''' - 8''''	30.3	1.19 (10H, m)	-
9''''	30.5	1.19 (2H, m)	-
10''''	28.1	1.19 (2H, m)	-
11''''	39.9	1.08 (2H, m)	-
12''''	28.8	1.43 (2H, m)	-
13''''	23.1	0.79 (3H, d, J = 6.8 Hz)	-
14''''	23.1	0.79 (3H, d, J = 6.8 Hz)	-
P-1	-	-	0.03 (J_{p-p} = 16.3 Hz)
P-2	-	-	4.69 (J_{p-p} = 16.3 Hz)

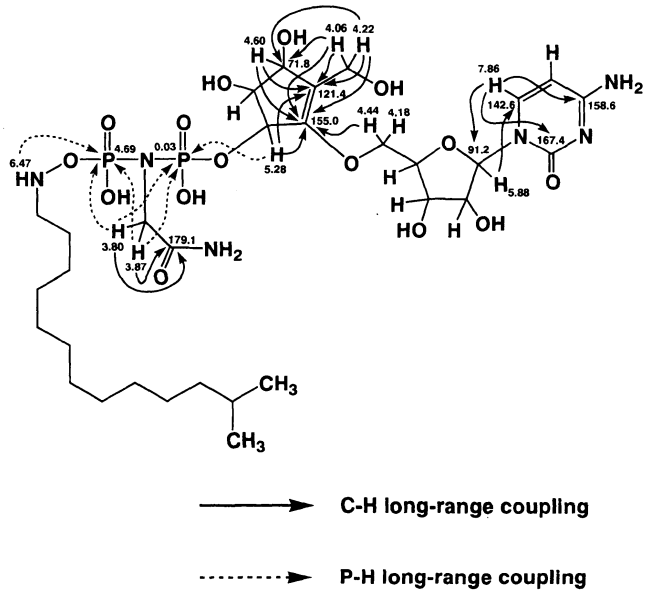
a) Two mg of 1100-50 was dissolved in 0.02 N NaOD-CD_3OD (1:1), and a CHD_2OD signal was used as the internal standard (49.1 ppm). Multiplicity and ^{13}C-^{31}P coupling constants are shown in parentheses.

b) Two mg of 1100-50 was dissolved in 0.02 N NaOD-CD_3OD (1:1), and an HOD signal was used as the internal standard (4.75 ppm). The number of protons, multiplicity and ^{1}H-^{1}H coupling constants are shown in parentheses.

c) Two mg of 1100-50 was dissolved in 0.02 N NaOD-CD_3OD (1:1), and an H_3PO_4 signal in D_2O-CD_3OD (1:1) was used as the external standard (0 ppm). ^{31}P-^{31}P coupling constants are shown in parentheses.

d) Two mg of 1100-50 was dissolved in 0.02 N NaOH-CD_3OH (1:1). An H_2O signal was used as the internal standard (4.75 ppm) and was suppressed by WET.[8, 9]

Fig. 2. C–H and P–H long-range couplings.



between H-5″ (δ 5.28) and P-1 (δ 0.03), H-2‴ (δ 3.80 and 3.87) and P-1 (δ 0.03), H-2‴ (δ 3.80 and 3.87) and P-2 (δ 4.69), and NH-1⁗ (δ 6.47) and P-2 (δ 4.69) in the ^{1}H-^{31}P WET (water suppression enhanced through T_1 effects)-PFG-HMBC spectrum[7~9] clarified the sequential linkage from cyclopentene to 12-methyltridecylamine *via* phosphate, glycinamide and phosphate moieties (Fig. 2). As shown in Table 1, ^{13}C-^{31}P long-range couplings at C-1″ (δ 155.0), C-5″ (δ 80.7), C-2‴ (δ 51.3) and C-1⁗ (δ 53.7) signals in the ^{13}C NMR spectrum also confirmed the linkage. Therefore, the planar structure of **1** was elucidated as shown in Fig. 1. The structure fully explains the instability that is derived from its vinylether moiety.

Although the structure of **1** is related to EM2487 (**2**)[10,11] as shown in Fig. 1, **1** is distinct in that the nucleoside substructure is not a uridine but a cytidine. A further distinction is the absence of an *N*-methyl group at the 1⁗ position.

Twenty-four hour pre-treatment of *Meloidgyne hapla* secondary larvae by **1** exhibited potent inhibition of root-knot formation in a cucumber pot test. The minimal effective concentration of **1** was below 0.05 ppm. Compound **2** was reported to possess anti-HIV activity derived from the inhibitory activity of Tat protein biosynthesis at a transcriptional level.[10,11] Accordingly, the anti-nematode activity of **1** may be derived from the inhibition of RNA synthesis.

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