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New Biotransformation Products of Nemadectins

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Selected nemadectins (formerly LL-F28249 series[1]) have been fed to a panel of microorganisms with the aim of generating new derivatives. In addition to products resulting from the oxidation of the terminal methyl group (C-29), a unique phosphorylated nemadectin was isolated. The phosphate group was determined to be at C-23 by HMBC between phosphorus and H-23. Milbemycin or nemadectin derivatives with natural substituents involving the 23-hydroxyl group were hitherto unknown.

The nemadectins were first isolated from cultures of *Streptomyces cyaneogriseus* ssp. *noncyanogenus*[2]. They belong to a family of macrocyclic lactones that have been recognized for their potent antiparasitic activity against a broad spectrum of endo- and ectoparasites of mammals. The nemadectins[2,3] differ from the avermectins[4] and milbemycins[5] by their unique, unsaturated side chain at C-25, and typically, by a hydroxyl group at C-23. The chief component, nemadectin α (**1**) is currently being used as starting material for the production of commercial moxidectin (Cydectin®), employed in veterinary medicine.

As part of a program to provide natural precursors that could perhaps substitute nemadectin α as the starting material in the production of moxidectin, or to supply new derivatives for antiparasitic use or as synthetic intermediate, microbial transformation experiments were conducted. Previously, microbial hydroxylations of related macrolides to yield new antiparasitic and anthelmintic compounds have been described by various reseachers[6~9].

Results and Discussion

A panel of cultures known to be capable of transforming xenobiotics was screened for microbial transformation products of **1**. The studies were carried out using a 24 well array plate. Individual wells were inoculated with selected microorganisms and **1**, dissolved in DMF, was added to yield a final concentration of 1 mg/ml fermentation broth. Samples were taken over a 14-day period and analyzed by HPLC. Two cultures, *Streptomyces griseus* NRRL 3242 and *Mucor ramannianus* 1839, were found to generate components with altered retention times, but having UV spectra identical to that of **1**. LC/MS analysis revealed that the compound transformed by *Mucor ramannianus* had a MW of 692 (**2**=**1**+80) and those generated by *Streptomyces griseus* had MW's of 628 (**3**=**1**+16) and 642 (**4**=**1**+30), respectively.

Preparative scale biotransformation of **1** by *M. ramannianus* (yielding **2**) and *S. griseus* (yielding **3** and **4**) was carried out in two 250-ml culture flasks, each containing 50 ml of biotransformation medium. A total of 100 mg of **1** (in 4.0 ml of DMF) was evenly distributed over the mash after inoculating with a 24 hour old culture.

After 14 days of incubation, the mash was stirred with three volumes of MeOH for 2 hours and then centrifuged. The resulting supernatant was concentrated to remove the bulk of MeOH, and subsequently extracted five times with one volume of EtOAc. The combined EtOAc extracts were concentrated, loaded onto a silica gel column, and eluted with a mixture of hexane and EtOAc (4 : 6). The fractions containing the nemadectin metabolites were further purified

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by preparative HPLC. Each purification step was monitored by analytical HPLC.

Structure Elucidation of **2** (**1**+80)

High resolution Fourier transform ion cyclotron resonance mass spectrometry (HRFTICR-MS) indicated a molecular adduct ion of *m/z* $[M+H]^+$ 693.34139, predicting a molecular formula of $C_{36}H_{54}O_{11}P$, (error −1.6 mmu) which suggested that **1** was conjugated to a phosphate group. ES-MS/MS experiments on the $[M+Na]^+$ ion (*m/z* 715 amu) of **2**, produced only one significant fragment (base peak) ion of *m/z* 617 for $[M+Na-98]^+$, suggestive of the elimination of H_3PO_4. Assuming that no other modification had occurred, the phosphate group was likely to be conjugated to either the C-5 or C-23-hydroxyl group. Analysis of data obtained from ^{13}C, ^1H, and HMQC NMR experiments, revealed chemical shift changes for position 23 (from 70.8/3.81 ppm in **1** to 76.0/4.48 ppm in **2**), position 24 (from 37.1/1.60 ppm in **1** to 36.7/1.84 ppm in **2**), and position 25 (from 78.2/3.81 ppm in **1** to 77.3/3.96 ppm in **2**). In addition, line broadening and C–P couplings were observed for C-23 (J=5 Hz), and C-24 (J=6 Hz). Correlation between H-23 and the phosphorus atom as observed in the ^1H-^{31}P HMBC (heteronuclear multiple bond correlation) further established the attachment site at position 23. All other assignments, as given in Tables 1 and 2, were corroborated by COSY and HMBC and are entirely consistent with the proposed structure of **2**. Therefore, *M. ramannianus* is capable of biotransforming **1** to **2** by adding a phosphate group at C-23.

Table 1. ^{13}C-NMR data and assignment.

Carbon #	**1** (d_4-MeOH)	**3** (d_4-MeOH)	**2** (d_4-MeOH)	**4** ($CDCl_3$)	**1** ($CDCl_3$)
1	173.6	173.6	173.7	173.8	173.4
2	47.1	47.1	47.2	46.0	45.7
3	120.3	120.3	120.3	118.3	118.0
4	137.2	137.2	137.2	137.7	137.3
4a	19.9	19.9	19.9	20.1	19.9
5	69.0	69.0	69.0	67.9	67.4
6	82.4	82.4	82.4	79.5	79.3
7	81.9	81.9	81.9	80.5	80.2
8	141.5	141.5	141.4	139.6	139.4
8a	68.7	68.7	68.8	68.7	68.4
9	121.8	121.8	121.9	120.6	120.3
10	125.1	125.2	125.1	123.6	123.3
11	143.5	143.5	143.6	143.2	142.8
12	37.3	37.3	37.3	36.3	36.0
12a	23.0	23.0	23.0	22.5	22.2
13	49.7	49.6	49.7	48.7	48.4
14	138.9	138.9	138.6	138.2	137.4
14a	15.9	15.9	15.9	15.8	15.3
15	121.9	121.9	122.5	120.4	120.3
16	35.8	35.8	35.7	34.9	34.7
17	70.3	70.3	69.7	69.0	68.5
18	37.3	37.3	37.5	36.6	36.1
19	69.5	69.5	70.1	67.9	67.8
20	42.2	42.3	41.0	40.9	40.7
21	101.2	101.2	99.4	100.0	99.7
22	42.3	42.1	42.8	41.1	41.1
23	70.8	70.8	76.0	69.5	69.3
24	37.1	37.0	36.7	36.2	35.9
24a	14.4	14.5	14.1	13.9	13.9
25	78.2	78.2	77.3	76.5	76.7
26	132.5	135.4	132.6	136.6	130.6
26a	11.3	11.7	11.2	11.7	11.0
27	136.6	133.7	138.3	127.9	137.2
28	28.1	36.5	28.1	38.3	26.8
28a	23.4	17.6	23.4	17.7	22.8
29	23.4	68.0	23.4	178.6	22.8

Structure Elucidation of **3** (**1**+16) and **4** (**1**+30)

LC/MS analysis of the biotransformation products suggested that **3** had a MW of 628, whereas that of **4** was indicated to be 642. HRFTICR-MS of **3** registered a molecular adduct ion of *m/z* 651.35089 for $[M+Na]^+$, implying a formula of $C_{36}H_{52}O_9Na$, (error −0.53 mmu), while the value for **4** was found to be *m/z* 665.32830 for $[M+Na]^+$, indicating the formula $C_{36}H_{50}O_{10}Na$, (error +1.3 mmu). Therefore, **1** was converted to **3** through the addition of one oxygen atom and transformed to **4** by the addition of two oxygen atoms with the concomitant extraction of two hydrogen atoms. MS/MS experiments indicated that both of these modifications occurred on the right-hand side of the nemadectin molecule as shown in Figure 1, because compounds **1**, **3**, and **4** produced fragment ions of *m/z* 465. This ion represents the portion of the molecule that comprises the entire macrolide, but without the side chain. As the C-22 to C-29 chain is eliminated due to bond cleavages between C-22 and C-23 as well as between C-21 and O-7, it was obvious that the oxidations occurred in this region of the molecule. Analysis of the purified biotransformtion products by NMR [based on ^{13}C, ^1H, and HMQC (heteronuclear multiple quantum correlated) NMR experiments], revealed that the signal for C-29 had changed from 22.8 ppm in **1** to 178.6 ppm in **4**, while in the proton resonance spectrum the doublet signal representing one methyl group at around 1 ppm was lacking. Additional chemical shift changes for 28a (22.8/1.05 in **1** to 17.7/1.37 in **4**), 28 (26.8/2.58 in **1** to 38.3/3.47 in **4**), 26 (130.6 in **1** to 136.6 in **4**), and 27 (137.2/5.20 in **1** to 127.9/5.41 in **4**) provided further evidence that the C-29 methyl group of **1** converted to the carboxyl group in **4**. All other NMR assignments, as shown

Table 2. ^{1}H-NMR data and assignment.

Proton # (position)	signal multipl.	1 (d_4-MeOH)	3 (d_4-MeOH)	2 (d_4-MeOH)	4 ($CDCl_3$)	1 ($CDCl_3$)
2	q	3.22	3.20	3.21	3.29	3.27
3	s	5.42	5.40	5.40	5.42	5.41
4						
4a	s	1.82	1.81	1.81	1.88	1.87
5	d	4.23	4.21	4.22	4.32	4.29
6	d	3.77	3.74	3.77	3.96	3.95
7,8						
8a	d	4.61	4.61	4.65	4.68	4.68
9	m	5.81	5.78	5.79	5.78	5.75
10	m	5.86	5.85	5.84	5.78	5.73
11	dd	5.41	5.41	5.39	5.35	5.35
12	m	2.45	2.48	2.47	2.45	2.45
12a	d	1.01	1.00	1.01	1.01	1.00
13	d,t	1.93,2.23	1.90, 2.21	1.91, 2.22	1.90, 2.20	1.90, 2.20
14						
14a	s	1.57	1.54	1.54	1.54	1.53
15		5.08	5.06	5.02	4.97	4.95
16	d	2.25	2.23	2.25	2.25	2.22
17	m	3.67	3.66	3.60	3.63	3.62
18		0.88, 1.99	0.86, 1.98	0.89, 1.94	0.88, 1.85	0.88, 1.85
19	m	4.97	4.95	4.97	5.36	5.32
20		1.33, 2.26	1.28, 2.24	1.74, 2.28	1.41, 2.07	1.40, 2.04
21						
22		1.78, 1.96	1.76, 1.94	1.23, 2.20	1.74, 2.05	1.70, 1.99
23	d	3.81	3.80	4.48	3.83	3.80
24		1.60	1.69	1.84	1.64	1.60
24a	d	0.80	0.80	0.80	0.80	0.80
25	d	3.81	3.83	3.96	3.87	3.74
26						
26a	s	1.65	1.68	1.64	1.70	1.61
27	d	5.24	5.21	5.19	5.41	5.20
28	q	2.63	2.63	2.60	3.47	2.58
28a	d	0.98	1.03	0.96	1.37	1.05
29	d	1.04	3.36 (dq)	1.02	-	0.95

in Tables 1 and 2, and established by COSY, HMQC and HMBC, fully support the proposed structures of **3** and **4**. These structures suggest that *Streptomyces griseus* is able to oxidize **1** at its side chain where apparently the terminal methyl group is converted to the hydroxymethyl group of **3** which is then further transformed to yield the carboxyl group of **4** (for NMR assignments see Tables 1 and 2).

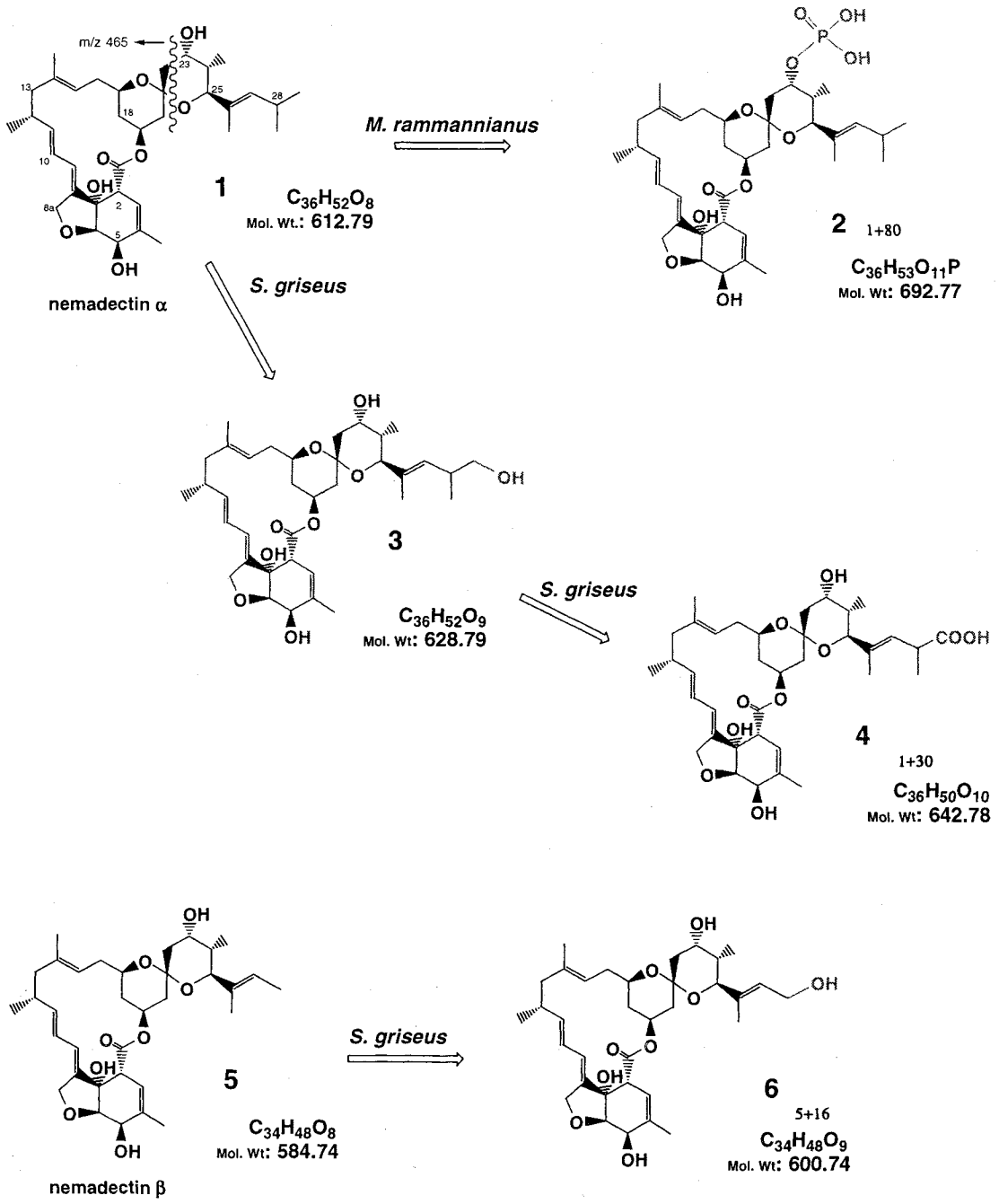
Structure of **6** (**5**+16)

When nemadectin β (**5**) was subjected to the same biotransformation conditions with *Streptomyces griseus* as **1**, a new conversion product (MW=600, observed mass $[M+Na]^+$ 623.31985, formula $C_{34}H_{48}O_9Na$, error -0.79 mmu) was isolated. MS/MS experiments indicated that oxidation of **5** occurred also at the side chain of the molecule suggesting that, similar to **1**, the corresponding hydroxylation took place at C-28. Proton NMR data were sufficient to show the lack of a methyl doublet at 1.67 ppm.

Biological (Nematocidal) Activity

Nemadectin α (**1**) and its derivatives (**2**~**4**), obtained by biotransformation, were tested for biological activity against *Caenorhabditis elegans* larvae and adults, and observations were made at 4 and 24 hours. **1** was fully active even at the lowest concentration tested (1.15 μg/ml), but the derivatives were not quite as active. **2** and **3** were similarly effective down to levels of 2~5 μg/ml, but **4** was significantly less effective. Its lowest activity was recorded at concentrations of 30~40 μg/ml.

Fig. 1. Microbial transformation products of nemadectins.



Experimental

LC Conditions for Screening

Column: YMC ODS-AQ (4.6×150 mm, 5 μm, 120 Å). Flow rate: 1.0 ml/minutes. Detection: 244 nm. Gradient, 0~10 minutes (50% B), 10.0~10.1 minutes (50~95% B), 10.1~18.0 minutes (95% B), 18.0~18.1 minutes (95~50% B), 18.1~20.0 minutes (50% B). Solvent A: H_2O/0.025% TFA or HCOOH; Solvent B: CH_3CN/0.025% TFA or HCOOH.

Sample Preparation

From each well 200 μl culture broth was taken, which was mixed with 600 μl of MeOH, shaken vigorously for 0.5

hours, and centrifuged. The supernatants were then analyzed by HPLC.

Preparative Scale Transformation of Nemadectins by *S. griseus* and Isolation of **3**, **4** and **6**

S. griseus (NRRL 3242) was grown in two 250-ml culture flasks, each containing 50 ml of biotransformation medium, consisting of 20 g glucose, 5 g NaCl, 5 g K_2HPO_4, 5 g yeast extract, and 5 g peptone per liter of distilled water (pH 7). A total of 100 mg of nemadectin component (in 4.0 ml of DMF) was evenly distributed among a 24-hour-old culture and incubated for 14 days. The content of the two flasks were then combined and stirred overnight with 3 vol of MeOH. The supernatant obtained after centrifugation was concentrated to remove the bulk of MeOH, and extracted by 5×1 vol of EtOAc. The combined EtOAc extract was concentrated, loaded onto a silica gel column, and eluted with a mixture of hexane and EtOAc (4 : 6). The fractions containing new metabolites were subsequently purified by preparative HPLC. Each purification step was monitored by analytical HPLC.

Preparative HPLC Conditions

Column, YMC-Pack ODS-A (250×20 mm, 10 μm, 120 Å). Flow rate: 6 ml/minutes. Detection: 244 nm. Gradient: 0~60 minutes (50% B), 60~70 minutes (95% B), 70~100 minutes (95% B). Solvent A, 0.1% TFA in water; Solvent B, 0.1% TFA in acetonitrile. Yields: **3**, 4.6 mg (4.6%); **4**, 5.8 mg (5.8%), **3**, 4.6 mg (4.6%), **6**, 2.0 mg (2%)

Preparative scale transformation of nemadectin α by *M. rammannianus* and isolation of **2** were carried out under identical conditions to that of *S. griseus* except that the supernatant was adjusted to pH 3 prior to extraction with EtOAc. After preparative HPLC purification, **2** was obtained in 5.5% yield (5.5 mg).

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