Anthelmintic Macrolactams from *Nonomuraea turkmeniaca* MA7364

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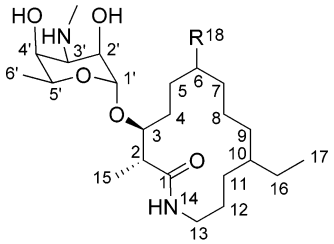
Two new macrolactams, 6-desmethyl-*N*-methylfluvirucin A_1 (**1**) and *N*-methylfluvirucin A_1 (**2**), have been isolated from the acetone extract of *Nonomuraea turkmeniaca* MA7364. These compounds were isolated by bioassay-guided fractionation as part of our search for new anthelmintics. The structures of these compounds were elucidated by comparison of their NMR and MS data to those of previously reported fluvirucins and confirmed by 2D NMR. Compound **1** exhibited *in vitro* activity (EC_{90} 15 ± 5 μg/mL) against *Haemonchus contortus* larvae, whereas compound **2**, while a bit less active *in vitro* (EC_{90} 29 ± 8 μg/mL), showed modest *in vivo* activity against a surrogate organism, *Heligmosomoides polygyrus* in mice, at 50 mg/kg.

Productivity in the livestock industry is significantly impacted by infections caused by internal parasites (e.g., *Haemonchus contortus*). A number of new classes of anthelmintic drugs have been approved for clinical practice since 1960 including the current classes of synthetic drugs such as the benzimidazoles and imidazothiazoles (tetramisole/levamisole). However, the discovery of the avermectin class of macrolactones in the early 1980s represented the most significant of discoveries not only as anthelmintic agents for animal health but also for treatment of parasitic infections in humans, particularly onchocerciasis (river blindness). This discovery led to the anthelmintic drugs ivermectin and doramectin, which have excellent broad-spectrum activity and superior potency. Unfortunately, resistance to all of these classes of drugs has been observed, however, leading to the continuing need of further research to discover new classes of anthelmintics, especially those with novel modes of action.[1]

To discover anthelmintic agents, our strategy has been to screen extracts of bacterial and fungal fermentations as well as plants for *in vitro* activity against *H. contortus*,[2] a prevalent parasitic worm species that infects livestock. *In vivo* activity was evaluated using *Heligmosomoides polygyrus* infected mice.[3] An acetone extract of *Nonomuraea turkmeniaca* MA7364 showed *in vitro* activity and was selected for further study. Bioassay-guided fractionation using the *in vitro* assay led to the isolation of two new macrolactams of the fluvirucin class, **1** and **2**. The isolation, structure, and biological activities of these compounds are herein described.



1: R = H
2: R = CH_3

The producing organism was obtained from a soil sample collected in Mexico and identified as a species closely related to *Nonomuraea turkmeniaca*. The fermentation broth generated from the producing organism was extracted with acetone and successively chromatographed on Amberchrome, Sephadex LH-20, and RP-HPLC, affording 14.1 mg (4.7 mg/L) and 8.3 mg (2.8 mg/L) of compounds **1** and **2**, respectively, as colorless, amorphous powders.

HRESIFTMS of compound **1** exhibited the molecular formula $C_{23}H_{44}N_2O_5$. The UV/vis spectrum showed only end absorption. The formula indicated an index of hydrogen deficiency of three. The carbon count was confirmed by the ^{13}C NMR spectrum (CD_3OD and C_5D_5N), which showed 23 signals. The DEPT spectrum indicated the presence of four methyl groups, 10 methylenes, and eight methines. One quaternary signal was present at δ_C 177.2, indicating a carbonyl group, which would account for one of the indices of hydrogen deficiency. Lack of resonances for additional sp or sp^2 carbons in the ^{13}C NMR spectrum suggested that compound **1** must contain two rings. Prominent features of the ^1H NMR spectrum include a methyl triplet at δ_H 0.88, methyl doublets at δ_H 1.18 and 1.25, and a methyl singlet at δ_H 2.74. The chemical shift of the latter signal suggested the presence of an *N*-methyl group. Numerous signals between δ_H 3.4 and 4.0 as well as a doublet at δ_H 4.90 were consistent with the presence of a sugar residue, which would be a source of one of the two rings present. Chemical shifts of most of these protons shifted significantly in C_5D_5N, indicating that they possess or were located near a free hydroxyl group. A multiplet present at δ_H 8.39 that showed no correlation in the HSQC spectrum was consistent with an amide proton; however, this is unusual because an amide proton would be expected to exchange with CD_3OD. By ^1H NMR, the amide proton does exchange, albeit very slowly. The presence of numerous overlapping protons in the δ_H 1.0−1.6 range indicated a high degree of saturation, consistent with the low index of hydrogen deficiency.

Comparison of the data described above with the corresponding literature data resulted in a very close match to a class of macrolactams isolated from the genus *Actinomadura*.[4−10] It should be noted that in 1998 numerous species of *Actinomadura* were reclassified as *Nonomuraea*, including *N. turkmeniaca*.[11] These compounds contain a branched 14-membered lactam and a mycosamine or 4-*epi*-mycosamine moiety and have exhibited antifungal[4−7] and antiviral activities.[8] The branching can vary from three methyl groups to three ethyl groups, as well as combinations of methyl, ethyl, and hydroxyethyl groups appropriately originating from acetate and propionate units. Positions C-2, C-6, and C-10 are always substituted with the three alkyl groups in this class of

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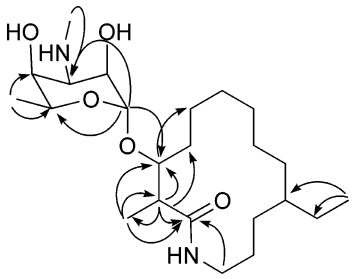

Figure 1. Selected HMBC correlations for **1**.

compounds. The amino sugar moiety is always attached to the macrocycle either at C-3 or at C-9. The C-3 sugar-substituted compounds are the fluvirucin "A" compounds (A_1 and A_2).[8−10] It was apparent from the COSY and the HMBC spectroscopic data (Figure 1) that compounds **1** and **2** had a sugar attached at C-3 like the fluvirucin A series. From coupling constant data, the sugar component of **1** and **2** was determined to be 4-*epi*-mycosamine, which is common with the fluvirucin A compounds. It is also apparent from the HMBC (Figure 1) and the COSY data that compounds **1** and **2** possess methyl substitution at C-2 and ethyl substitution at C-10. The major difference between **1** and **2** and the fluvirucins is the presence of an *N*-methyl singlet at δ_H 2.74. This *N*-methyl group could be attached to the amide nitrogen or to the sugar amine. The HMBC spectrum clearly shows a correlation from the *N*-methyl protons to C-3′ of the sugar (Figure 1). Another major difference between **1** and the other macrolactams of this class is the absence of an alkyl branch at C-6 in **1**.

Compound **2** showed a molecular formula of $C_{24}H_{46}N_2O_5$ indicating an index of hydrogen deficiency of three. This formula differs from **1** by an additional carbon and two additional hydrogens, which was attributed to a methyl group that resonated at δ_H 0.92, which was assigned to the methyl group substituted at C-6. The methyl protons exhibited HMBC correlations to C-5, C-6, and C-7. The ^{1}H and ^{13}C NMR spectra of **2** were practically identical to those of fluvirucin A_1,[8] except for the presence of an additional methyl singlet at δ_H 2.75, corresponding to an *N*-methyl group. The large coupling constant (∼10 Hz) for H-2 and H-3 for both compounds indicates an *anti* relationship between H-2 and H-3, which is consistent with the relative configuration of fluvirucins. The relative configurations at C-6 and C-10 of **1** and **2** could not be independently determined by NMR experiments but are likely to be the same as reported for the A and B series of fluvirucins. Both the A and B series of fluvirucins possess identical absolute configurations at the appropriate centers, consistent with their production as congeners by species of the genus *Nonomuraea*. The absolute configuration of fluvirucin A_1 was determined by a combination of Mosher ester and X-ray crystallography,[10] and the absolute configuration of fluvirucin B_1 was independently determined by X-ray crystallography of a *p*-bromobenzenesulfonamide derivative.[4] Unfortunately no specific rotations were reported for the fluvirucin A series, precluding the comparison of the specific rotation values. The NMR shift assignments were accomplished using the full suite of 2D NMR experiments including DQ-COSY, TOCSY, HMQC, gHMBC, and HSQC-TOCSY experiments, and data are summarized in the Experimental Section. The HSQC-TOCSY (600 MHz, C_5D_5N) in combination with HMBC was particularly useful in the assignment of carbon shifts connected to the protons with overlapping resonances in the ^{1}H NMR spectrum. Thus compound **1** was elucidated as 6-desmethyl-*N*-methylfluvirucin A_1 (3-[(3-methylamino-3,6-dideoxy-α-L-talopyranosyl)oxy]-2-methyl-10-ethyl-13-tridecanolactam), and **2** was elucidated as *N*-methylfluvirucin A_1 (3-[(3-methylamino-3,6-dideoxy-α-L-talopyranosyl)oxy]-2,6-dimethyl-10-ethyl-13-tridecanolactam).

Compounds **1** and **2** were first evaluated in the *in vitro H. contortus* assay as described earlier.[2] Both compounds **1** and **2** reduced motility of L3 larvae and showed EC_{90} (effective concentration of compounds that exhibited 90% reduction of larval motility) values of 15 ± 5 and 29 ± 8 μg/mL, respectively. These compounds were further evaluated in an *in vivo* mouse model infected with *H. polygyrus*.[3] Compounds were administered subcutaneously at a dose of 50 mg/kg. Three mice were used for evaluation of each compound, and data were averaged. Compound **1** showed no reduction in worm counts, while **2**, though less active *in vitro*, resulted in an average of 42 ± 15% reduction in worm counts. Ivermectin was used as a positive control, which was significantly more potent (EC_{90} = 0.18 μg/mL) in the *in vitro* assay as well as the *in vivo* assay (98−100% kill of larvae at 5−10 mg/kg dosed subcutaneously).

In summary, we have reported two new *N*-methyl derivatives of fluvirucin A_1. These compounds show *in vitro* anthelmintic activity, and **2** shows limited *in vivo* activity. This class of compound has not been previously reported to have anthelmintic activity.

Experimental Section

General Experimental Procedures. All reagents were obtained from Sigma-Aldrich and were used without further purification. NMR spectra were obtained on a Varian Inova 500 or 600 MHz spectrometer operating at 500 or 600 MHz for ^{1}H and 125 or 150 MHz for ^{13}C nuclei in CD_3OD and C_5D_5N. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter, and IR spectral data were obtained on a Perkin-Elmer Spectrum One spectrometer. High-resolution mass spectra were obtained on a Thermo Finnigan LTQ-FT with the standard Ion Max API source (without the sweep cone) and ESI probe. Ivermectin was obtained as the commercial product Ivomec injection (1%) for cattle and swine (Merial, Duluth, GA). Swiss Webster mice were obtained from Taconic Labs (Germantown, NY).

Producing Organism and Fermentation. The organism was obtained from a soil sample collected under banana trees in Las Tuxtlas, Veracruz, Mexico. The strain was isolated after pretreatment of the soil with benzethonium chloride and plating on NZ-amine-based medium.

The organism was grown on agar plates at 28 °C consisting of yeast extract (3 g/L), malt extract (10 g/L), dextrose (4 g/L), and agar (20 g/L), adjusted to pH 7. Agar plugs were used to inoculate 250 mL baffled flasks containing 50 mL of ATCC-2 medium, which consists of soluble starch (20 g/L), dextrose (10 g/L), NZ amine EKC (Kerry) (5 g/L), Difco beef extract (3 g/L), Bacto peptone (5 g/L), yeast extract (5 g/L), and $CaCO_3$ (1 g/L), adjusted to pH 7.0 with NaOH before addition of $CaCO_3$.

After 3-days incubation (220 rpm, 28 °C), these flasks were used to inoculate (2% inoculum) 1 L production flasks containing 225 mL of KHC medium consisting of dextrin (20 g/L), β-cyclodextrin (10 g/L), primary yeast (10 g/L), tomato paste (20 g/L), and $CoCl_2 \cdot 6H_2O$ (5 mg/L), adjusted to pH 7.2. Production flasks were incubated for 13 days (220 rpm, 28 °C). Total cultures were harvested and kept at −20 °C until thawed for metabolite extraction.

Identification of the Actinomycete Strain. The actinomycete strain was identified as a new member of the genus *Nonomuraea* on the basis of its complete 16S rDNA sequence aligned with nucleotide sequences from Genbank. The taxonomic position of the strain was determined by phylogenetic analysis of the aligned 16S rDNA sequences of validated species of the genus *Nonomuraea* and other members of the family Streptosporangiaceae. From the phylogenetic analysis based on the maximum parsimony method, the strain was closely related to the species *N. turkmeniaca*, a relationship highly supported by the bootstrap value (93%).

Extraction and Isolation. Three liters of fermentation broth was mixed with 3 L of acetone and shaken for 1 h on a platform shaker. The extract was filtered in a sintered-glass funnel using Celite as a filter aid. This extract was concentrated by rotary evaporation to aqueous (∼2.8 L). This concentrate was loaded onto Amberchrome CG 161M (∼150 mL of resin, column 1 in. diameter) at 15 mL/min. The column was then eluted with a 150 min 5−100% gradient of methanol/water at 10 mL/min followed by an additional 30 min with 100% methanol. Fractions were collected every 5 min, totaling 36 fractions. Fractions 30−32 were active in the *in vitro* assay and were combined and evaporated to give 1.54 g of a white solid. This active material was dissolved in 5 mL of 1:1 CH_2Cl_2/MeOH and loaded onto a Sephadex

LH-20 column using a 1 in. diameter column and approximately 275 mL of resin. The column was eluted with 1:1 CH_2Cl_2/MeOH at 2.5 mL/min. Fractions were collected every 2.5 min, 80 fractions total. Fractions 25−31 were active in the *in vitro* assay. They were combined and concentrated to give 202 mg of active material. This active material was fractionated using five equal injections by preparative RP-HPLC (Waters Symmetry C_{18}, 300 × 19 mm, 7 μm, 50 min gradient of 10−90% aqueous acetonitrile containing 0.1% TFA, 10 mL/min). Fractions 16−17 (48.1 mg) contained **1**, and fractions 18−20 (58.3 mg) contained **2**. Purification of these fractions by analytical HPLC using an Agilent Zorbax SB C_{18} column (250 × 4.6 mm, 5 μm, 1 mL/min, 20 min gradient of 10−90% aqueous acetonitrile + 0.1% TFA) followed by lyophilization afforded compound **1** (14.1 mg) and compound **2** (8.3 mg) as colorless, amorphous powders.

6-Desmethyl-*N*-methylfluvirucin A_1 (1): white solid; $[\alpha]_D^{23}$ +13.0 (*c* 0.9, methanol); IR (ZnSe film) ν_{max} 3320, 2947, 2917, 2857, 1674, 1625, 1548, 1465, 1436, 1203, 1134 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 8.39 (1H, dd, J = 8.0, 4.0 Hz, H-14), 4.90 (1H, d, J = 1.5 Hz, H-1′), 4.01 (1H, qd, J = 6.5, 1 Hz, H-5′), 3.92 (1H, dd, J = 3.0, 1.5 Hz, H-2′), 3.79 (1H, dd, J = 3, 1 Hz, H-4′), 3.77 (1H, m, H-3), 3.72 (1H, m, H-13a), 3.41 (1H, t, J = 3.0 Hz, H-3′), 2.74 (3H, s, *N*-CH_3), 2.58 (1H, m, H-13b), 2.53 (1H, dq, J = 10.5, 7.0 Hz, H-2), 1.60−1.00 (H-4, H-5, H-6, H-7, H-8, H-9, H-11, H-12), 1.33 (1H, m, H-16a), 1.25 (3H, d, J = 6.5 Hz, H-6′), 1.18 (3H, d, J = 6.5 Hz, H-15), 1.18 (1H, m, H-16b), 0.88 (3H, t, J = 7.0 Hz, H-17); ^{13}C NMR (CD_3OD, 125 MHz) δ 177.2 (C-1), 103.8 (C-1′), 84.5 (C-3), 67.6 (C-5′), 67.4 (C-4′), 66.5 (C-2′), 57.4 (C-3′), 46.3 (C-2), 40.0 (C-13), 39.7 (C-10), 32.9, 32.4, 30.1 (*N*-CH_3), 28.3, 28.2 (C-16), 27.9, 27.0, 25.0, 24.6, 20.8, 16.7 (C-6′), 16.6 (C-15), 12.2 (C-17); ^1H NMR (C_5D_5N, 500 and 600 MHz) δ 8.89 (1H, dd, J = 8.5, 4.0 Hz, H-14), 5.43 (1H, d, J = 1.5 Hz, H-1′), 4.67 (1H, dd, J = 3.0, 1.5 Hz, H-2′), 4.38 (1H, dd, J = 3, 0.5 Hz, H-4′), 4.33 (1H, dt, J = 10, 3.0 Hz, H-3), 4.27 (1H, dq, J = 0.5, 6.5 Hz, H-5′), 4.18 (1H, m, H-13a), 3.90 (1H, t, J = 3.0 Hz, H-4′), 3.02 (3H, s, *N*-CH_3), 2.92 (1H, dq, J = 10, 6.5 Hz, H-2), 2.67 (1H, m, H-13b), 1.98, 1.88 (2H, m, H-4), 1.55, 1.30 (2H, m, H-12), 1.30 (1H, m, H-10), 1.70−1.00 (H-5, H-6, H-7, H-8, H-9, H-11), 1.46 (3H, d, J = 6.5 Hz, H-6′), 1.25, 1.15 (2H, m, H-16), 1.39 (3H, d, J = 7.0 Hz, H-15), 0.85 (3H, t, J = 7.0 Hz, H-17); ^{13}C NMR (C_5D_5N, 125 MHz) δ 175.0 (C-1), 104.1 (C-1′), 84.2 (C-3), 67.8 (C-4′), 67.7 (C-5′), 66.4 (C-2′), 57.5 (C-3′), 46.1 (C-2), 39.5 (C-13), 39.0 (C-10), 32.6 (C-4), 32.1 (C-9), 30.12 (*N*-CH_3), 28.0 (C-16), 27.8 (C-11), 27.7 (C-7), 26.4 (C-6), 24.9 (C-12), 24.3 (C-8), 20.7 (C-5), 17.4 (C-6′), 17.1 (C-15), 12.6 (C-17); HRESIFTMS *m*/*z* 429.3322 (calcd for $C_{23}H_{44}N_2O_5$ + H, 429.3331).

***N*-Methylfluvirucin A_1 (2):** white solid; $[\alpha]_D^{23}$ +37.3 (*c* 0.4, methanol); IR (ZnSe film) ν_{max} 3325, 2948, 2856, 1675, 1625, 1548, 1466, 1204, 1136 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 8.41 (1H, dd, J = 8.0, 4.0 Hz, H-14), 4.90 (1H, d, J = 1.5 Hz, H-1′), 4.05 (1H, qd, J = 6.5, 1 Hz, H-5′), 3.94 (1H, dd, J = 3, 1.5 Hz, H-2′), 3.82 (1H, m, H-3), 3.80 (1H, dd, J = 3, 1 Hz, H-4′), 3.70 (1H, m, H-13a), 3.42 (1H, t, J = 3.0 Hz, H-3′), 2.75 (3H, s, *N*-CH_3), 2.58 (1H, m, H-13b), 2.55 (1H, dq, J = 10.5, 7.0 Hz, H-2), 1.80−1.00 (H-4−H-5, H-7−H-9, H-11−H-12), 1.72 (1H, m, H-6), 1.26 (3H, d, J = 7.0 Hz, H-6′), 1.18 (3H, d, J = 7.0 Hz, H-15), 0.92 (3H, d, J = 7.0 Hz, H-18), 0.87 (3H, t, J = 7.0 Hz, H-17); ^{13}C NMR (CD_3OD, 125 MHz) δ 177.2 (C-1), 104.0 (C-1′), 84.5 (C-3), 67.6 (C-5′), 67.5 (C-4′), 66.4 (C-2′), 57.4 (C-3′), 46.4 (C-2), 40.1 (C-13), 39.3 (C-10), 34.9, 33.4, 32.5 (C-6), 30.1 (*N*-CH_3), 28.2, 28.1, 27.9, 27.5, 24.6, 23.9, 21.1 (C-18), 16.7 (C-6′), 16.6 (C-15), 12.2 (C-17); ^1H NMR (C_5D_5N, 500 MHz) δ 8.92 (1H, dd, J = 7.5, 3.0 Hz, H-14), 5.42 (1H, d, J = 1.5 Hz, H-1′), 4.71 (1H, dd, J = 2.5, 1.5 Hz, H-2′), 4.46 (1H, dd, J = 2.5, 0.5 Hz, H-4′), 4.37 (1H, dt, J = 10, 3.0 Hz, H-3), 4.27 (1H, dq, J = 0.5, 6.5 Hz, H-5′), 4.14 (1H, m, H-13a), 3.92 (1H, t, J = 2.5 Hz, H-4′), 3.03 (3H, s, *N*-CH_3), 2.90 (1H, dq, J = 10, 6.5 Hz, H-2), 2.66 (1H, m, H-13b), 1.98, 1.65 (2H, m, H-4), 1.55, 1.30 (2H, m, H-12), 1.35 (1H, m, H-10), 1.70−1.00 (H-5, H-6, H-7, H-8, H-9, H-11), 1.49 (3H, d, J = 6.5 Hz, H-6′), 1.25, 1.15 (2H, m, H-16), 1.37 (3H, d, J = 7.0 Hz, H-15), 0.86 (3H, t, J = 7.0 Hz, H-17); ^{13}C NMR (C_5D_5N, 125 MHz) δ 175.1 (C-1), 104.4 (C-1′), 84.1 (C-3), 67.9 (C-4′), 67.7 (C-5′), 66.4 (C-2′), 57.5 (C-3′), 46.0 (C-2), 39.7 (C-13), 38.7 (C-10), 34.7 (C-7), 33.1 (C-9), 31.9 (C-6), 27.9 (C-16), 27.8 (C-11), 27.1 (C-4), 24.5 (C-12), 23.8 (C-8), 21.3 (C-18), 17.5 (C-6′), 17.0 (C-15), 11.8 (C-17); HRESIMS *m*/*z* 443.3478 (calcd for $C_{24}H_{46}N_2O_5$ + H, 443.3487).

Biological Assays. The *in vitro* assay against *H. contortus* was used as described in ref 2. The *in vivo* mouse assay was modified from ref 3 and performed as follows: Swiss Webster mice (approximately 30 g) were inoculated with 200−400 L3 *H. polygyrus* larvae. The mice were checked for infection around day 12 post inoculation, then dosed subcutaneously with test compound in triplicate. On day three post-treatment, mice were euthanized and the intestine was collected (from below stomach to above cecum), opened, and placed in 5 mL of sterile water. Mucosa was scraped and rinsed through a 200-mesh screen. The rinseate was then examined for the presence of worms, and worms were counted. Worm counts for treated mice were then compared to infected, untreated mice as negative controls, and infected mice treated with ivermectin (5−10 mg/kg) as positive controls.

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