

3-Normeridamycin: A Potent Non-Immunosuppressive Immunophilin Ligand is Neuroprotective in Dopaminergic Neurons

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Abstract 3-Normeridamycin (**1**), isolated from fermentation extracts of the soil actinomycete *Streptomyces* sp. LL-C31037, demonstrated potent neuroprotective activity. When challenged with the neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺), known to induce parkinsonism, **1** restored functional dopamine uptake in a concentration-dependent manner, with an EC₅₀ of 110 nM in dopaminergic neurons. The structure of **1** was determined *via* spectroscopic methods, and the immunosuppressive and immunophilin binding properties of the compound were also measured.

Keywords immunophilin ligand, neurodegeneration, neuroprotection, Parkinson's disease, structure determination

Introduction

Therapeutic agents that slow or reverse the effects of neurodegenerative diseases such as Parkinson's disease (PD) would fulfill a major and as yet unmet medical need. In an effort to expand upon the reported neuroprotective and neuroregenerative effects of natural product immunophilin ligands such as FK506 and rapamycin we herein report the isolation, characterization, and preliminary *in vitro* biological activity of a new compound, 3-normeridamycin (**1**), obtained from fermentation extracts

of the soil actinomycete *Streptomyces* sp. LL-C31037.

PD is a progressive neurodegenerative disease characterized by the selective degeneration of dopaminergic (DAergic) neurons of the substantia nigra. Current PD medications treat symptoms, whereas none prevent or retard DAergic neuron degeneration [1]. However, a number of clinical trials evaluating neuroprotective agents are in progress [2]. Immunophilins are proteins with *cis/trans*-proline isomerase enzymatic activity that act as receptors for immunosuppressant drugs. More specifically, rapamycin (**2**) and FK506 (**3**) bind to the immunophilin FK506-binding-protein-12 (FKBP12). The immunophilin-3 complex then binds to calcineurin to inhibit cell proliferation, while in contrast, the immunophilin-2 complex binds to mammalian target-of-rapamycin (mTOR) to induce cell cycle arrest [3]. Studies indicate immunophilins are expressed 10~50 fold more highly in central nervous system (CNS) and peripheral nervous system (PNS) tissue than in immune tissue [4]. These findings prompted the evaluation of immunosuppressive immunophilin ligands on nervous system tissue [5]. Subsequent studies, spanning the last decade, revealed potent neuroprotective and neuroregenerative properties of immunophilin ligands in both culture and animal models [6]. However, the mechanisms underlying these trophic effects are still largely unknown, though the role of FKBP52 has been implicated in a number of studies [7].

In the course of screening microbial extracts for biological activity, the actinomycete designated as LL-C31037 was identified as producing a compound having FKBP12 binding activity, as well as neuroprotective activity.

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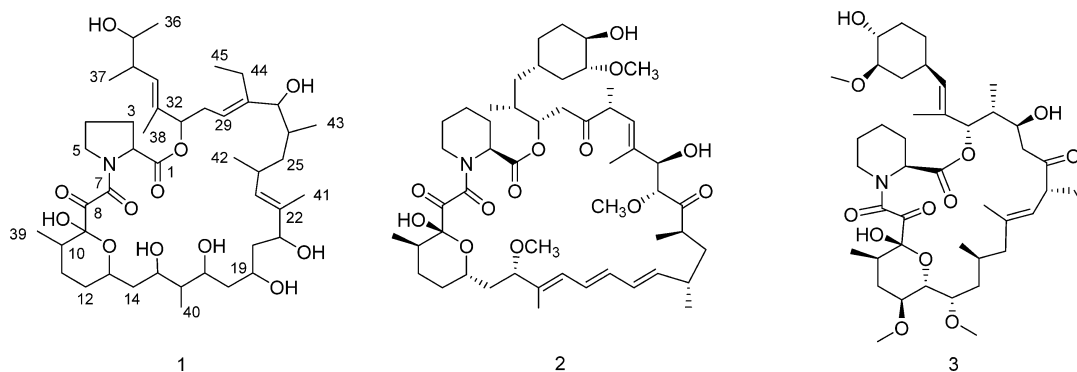


Fig. 1 Structures of 3-normeridamycin (**1**), rapamycin (**2**), and FK506 (**3**).

Materials and Methods

General

HPLC was performed on a Varian ProStar preparative HPLC system equipped with a photo diode array (PDA) detector. LCMS was acquired on an Agilent 1100 LC system with PDA detector, coupled to a ThermoFinnigan LCQ-DECA ion trap mass spectrometer. ^1H , ^{13}C , COSY, MEHSQC, HMBC and TOCSY experiments were performed on a Bruker Avance 500 MHz spectrometer. NMR spectra were recorded in $\text{MeOH}-d_4$ solutions, and the chemical shifts were referenced relative to the corresponding solvent signals ($\delta_{\text{H}}=3.30$ for ^1H NMR, $\delta_{\text{C}}=49.1$ for ^{13}C NMR). High resolution mass spectral data were acquired on a Bruker-Daltonics Apex II Fourier transform mass spectrometer equipped with a Bruker-Magnex 9.4 T superconducting magnet, and an Analytica electrospray source. The ESI source was operated in the nanoelectrospray mode. Typically, $5\ \mu\text{l}$ sample was loaded into the nanoelectrospray tip (New Objective, Woburn, MA) and a high voltage of about $-700\ \text{V}$ was applied between the tip and the glass capillary. Infrared spectra were recorded on a Nicolet Nexus 470 instrument in attenuated total reflectance (ATR) mode.

Fermentation

A seed medium of the following formulation was prepared by combining: dextrose (added after autoclaving), 1.0%; soluble starch, 2.0%; yeast extract, 0.5%; N-Z amine type A (Sheffield), 0.5%; calcium carbonate, 0.1%; pH 7.0. Ten ml of seed medium in a $25\times 150\ \text{mm}$ glass tube was inoculated with two loopfuls of cell mass of LL-C31037 cultured on ATCC agar medium #172. Sufficient inoculum from the agar culture was used to provide a turbid seed after 72 hours of growth. The primary seed tube was incubated for 72 hours at 28°C , at 200 rpm using a gyro-

rotary shaker with a 2-inch orbit. The primary seed (7.0 ml) was then used to inoculate a 250-ml Erlenmeyer flask containing 30 ml of seed medium. This secondary seed flask was incubated for 24 hours at 28°C , 200 rpm using a gyro-rotary shaker (2-inch orbit). Material from this seed fermentation was stored as a frozen stock in 10% glycerol and used as inoculum for the tank production medium (see below). A seed medium of the following formulation was prepared by combining: dextrose (added after autoclaving), 2.0%; soluble starch, 2.0%; yeast extract (Difco), 0.3%; wheat hydrolysate WGE80M (DMV International), 0.5%; soy hydrolysate SE50MAF (DMV International), 1.5%; pH 6.8 to 7.0. One ml of frozen seed culture was inoculated into 1.0 liter of seed medium in a 4-liter Erlenmeyer flask. This seed flask was incubated for 72 hours at 30°C at 250 rpm using a gyro-rotary shaker (2-inch orbit). A fermentation production medium of the following formulation was prepared by combining: dextrose (added after autoclaving), 2.0%; maltrin M500, 8.0%; nutrisoy (GPC), 1.0%; yeast extract (Difco), 0.6%; Gamaco (CaCO_3), 0.1%; Macol P2000, 0.2%; pH 6.8 to 7.0.

Neuroprotective Activity

Mesencephalic DAergic neuron cultures were prepared [8] and treated with compound. Cultures were then exposed to the neurotoxin MPP^+ , known to induce parkinsonism [9], and subsequent measurement of high-affinity ^3H -dopamine uptake [10] provided a functional readout for putative neuroprotection of compounds that may be relevant to PD. For dopamine uptake experiments, single-cell suspensions in complete media were seeded on poly-L-ornithine and laminin coated 24-well plates. The cultures were maintained for seven days prior to experimentation. Cultures were pretreated with various concentrations of **1** for 24 hours, and then exposed to $10\ \mu\text{M}$ of the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) for 1 hour to assess the neuroprotective effect of **1** in cell culture. Following the

1-hour incubation, media was exchanged three times and fresh compound was added for an additional 48 hours. Cultures were washed with pre-warmed phosphate-buffered saline (PBS) containing 5.6 mM glucose and 1 mM ascorbic acid. Cultures were then incubated for 15 minutes at 37°C with 50 nM ^3H -dopamine (31 Ci/mmol, Du Pont-NEN, Wilmington, DE, U.S.A.). The cultures were washed twice with buffer and lysed with 0.5 N NaOH. The lysate was transferred to a scintillation vial containing Ultima Gold scintillation cocktail and radioactivity was determined with a liquid scintillation counter.

Results

Taxonomy of the Producing Organism

Strain LL-C31037 was isolated from a soil sample collected in Eagle River, Wisconsin, USA in 1980. Cultures of *Streptomyces* sp. LL-C31037 on ATCC agar medium No. 172 display tan-colored substrate mycelia and produce neither aerial mycelium nor soluble pigment. The 16S rDNA sequence was amplified and aligned to yield phylogenetic trees that supported classification of the strain in the genus *Streptomyces*. A viable culture of this microorganism has been deposited with the Northern Regional Research Laboratory (NRRL), U.S. Department of Agriculture, Peoria, IL 61604, and added to its permanent collection under accession number 30721.

Isolation

Cells from 20 liters of culture of C31037 were pelleted by centrifugation. 5% Diaion HP20 (Mitsubishi) in water was added to the supernatant, and this was stirred at room temperature overnight. The HP20 was washed with methanol and the filtrate collected. To the cell pellet, 80 : 20 ethyl acetate methanol was added. This was repeatedly shaken and centrifuged, and the supernatant collected. The cell supernatant and the broth methanol filtrate were combined and concentrated *in vacuo*. The crude extract was adsorbed onto silica (32-63, 60A), and run by VLC. The compound was eluted with 5% methanol in dichloromethane. This extract was concentrated *in vacuo*, adsorbed onto silica (32-63, 60A) and loaded onto a flash silica column (60 mm×250 mm). The compound was eluted with 2% methanol in dichloromethane. The extract was concentrated *in vacuo* and loaded onto Sephadex LH-20 (60×400 mm, methanol). The column was initially washed with 300 ml methanol and 30 ml fractions were collected and monitored by LCMS. Early fractions which contained the compound of interest were collected and concentrated. This semi-pure material

was chromatographed by preparative HPLC (YMC ODS-A 50×250 mm 10 μ column, A: water B: methanol, gradient: 55%B to 70%B in 200 minutes, 30 ml/minute). 3-Normeridamycin was identified through LCMS analysis of fractions. These fractions of interest were pooled, concentrated *in vacuo* to afford pure 3-normeridamycin (t_R =130 minutes, 138 mg).

Physico-chemical Properties

3-Normeridamycin (**1**) was obtained as an amorphous white solid; $[\alpha]_D^{25}$ -1.1° (c 1.0, MeOH); UV (MeOH): end absorbance; IR (attenuated total reflectance) ν_{max} : 3391, 2931, 1726, 1677, 1635, 1449, 1179, 1132, 800 cm^{-1} ; FT-ICRMS: m/z =830.50021 for $\text{C}_{44}\text{H}_{73}\text{NO}_{12}$ ($\text{M}+\text{Na}^+$) (calculated: 830.50305; Δ mmu= -2.8); ^1H NMR (500 MHz, MeOH- d_4): see Table 1; ^{13}C NMR (125 MHz, MeOH- d_4): see Table 1.

Structure Elucidation

The high resolution FT-ICR mass spectrum of **1** gave a molecular ion at m/z =830.50021 for the ($\text{M}+\text{Na}$) $^+$ adduct, yielding a molecular formula of $\text{C}_{44}\text{H}_{73}\text{NO}_{12}$. Extensive analysis of the two-dimensional NMR spectral data for **1** confirmed the compound's identity. Structurally, **1** is related to the previously described non-immunosuppressive immunophilin ligand, meridamycin [11]. Although the NMR assignments of **1** were complicated by the two conformational populations observed due to *cis*-/*trans*-isomerization of the amide bond, as is the case for rapamycin [12], all resonances for the major conformer were assigned and are in good agreement with the literature assignments for meridamycin [11]. The presence of a proline residue in the macrocycle was confirmed by the observation of HMBC correlations from the ring carbons (C-3 to C-5) into 2-H, as well as correlations from C-1 into 3-H $_2$ and extensive COSY correlations between the proton resonances of the five-membered ring, (*vide infra*). The six olefinic carbons were identified from HSQC correlations, and from the ^{13}C spectrum. In particular, there is an HMBC correlation from a hydroxylated methine with proton at δ 3.75 into a quaternary olefinic carbon at δ 147.8. A methyl group at δ 1.05 has an HMBC correlation into this olefinic carbon and a COSY correlation into a two methylene protons at δ 1.86 and 2.34. These methylene protons also have HMBC correlation into the olefinic carbon at δ 147.8.

Two methylene protons at δ 2.39 and 2.62 also show correlations in the HMBC spectrum to the δ 147.8 carbon. However, these methylene protons have HMBC and COSY correlations to the adjacent olefinic methine at δ 5.26 securing its position on the other side of the double bond. This series of correlations confirms the presence of the

Table 1 ^1H and ^{13}C Chemical shift assignments for 3-normeridamycin **1** (MeOH- d_4 , 500 MHz)

Position	$\delta^{13}\text{C}$	$\delta^1\text{H}$ (mult, J Hz)
1	171.8	
2	59.9	4.44 (dd, 5.5, 9.0)
3	30.2	1.92 (m), 2.20 (m)
4	25.5	1.97 (m), 2.00 (m)
5	48.2	3.48 (m), 3.66 (m)
7	167.7	
8	199.5	
9	100.8	
10	36.2	2.25 (m)
11	28.2	1.55 (m), 1.68 (m)
12	33.4	1.42 (m), 1.62 (m)
13	69.5	3.81 (m)
14	43.3	1.67 (m)
15	73.3	3.82 (m)
16	43.3	1.61 (m)
17	72.5	4.14 (m)
18	43.5	1.54 (m), 1.70 (m)
19	68.1	3.89 (m)
20	44.0	1.53 (m), 1.70 (m)
21	74.1	4.14 (m)
22	134.0	
23	131.2	5.16 (m)
24	31.0	2.55 (m)
25	44.2	1.63 (m), 1.73 (m)
26	36.7	1.54 (m)
27	80.4	3.75 (m)
28	147.8	
29	120.3	5.26 (m)
30	31.7	2.39 (m), 2.62 (m)
31	82.4	5.00 (dd, 5.6, 9.0)
32	133.5	
33	134.7	5.30 (m)
34	41.4	2.37 (m)
35	72.9	3.47 (m)
36	21.7	1.11 (m)
37	17.3	1.00 (m)
38	13.0	1.70 (d, 0.91)
39	15.5	0.85 (m)
40	12.2	0.94 (m)
41	13.6	1.66 (m)
42	22.7	0.96 (m)
43	14.5	0.87 (m)
44	23.6	1.86 (m), 2.34 (m)
45	14.3	1.05 (m)

allylic ethyl group (44- H_2 and 45- H_3) at C-28, therefore ruling out the possibility of an allylic methyl at this position (to account for the formal deficit of 14 mass units

relative to meridamycin).

The macrolactone linkage was confirmed *via* the observation of HMBC correlations from both the H-31 methine proton at δ 5.00 and from the H-2 methine proton at δ 4.44 to the ester carbonyl (C-1) at 171.8. Moreover, the chemical shift of this proton (H-2) is consistent with an amino acid α -proton. There are additional HMBC correlations from 3- H_2 to the δ 171.8 carbonyl. Subsequent analysis of COSY correlations and HMBC correlations around and across the ring confirm the presence of only two additional methylenes (4- H_2 and 5- H_2), indicating that the ring is a 5-membered proline, not the 6-membered pipercolic acid. The important COSY and HMBC correlations herein described are diagrammed in Figure 2.

Extensive overlap in both the ^1H and ^{13}C NMR spectra of **1**, particularly in the C-15 to C-21 region, precluded the possibility of using *J*-configuration analysis [13] to assign the relative stereochemistry of **1**. The absolute stereochemistry of meridamycin has been reported [14], and while it is biosynthetically logical to assume an identical configuration for **1**, experiments are underway to obtain a crystalline sample of **1** for X-ray diffraction analysis.

Biological Activity

3-Normeridamycin (**1**) exhibited potent restoration of dopamine uptake in MPP⁺ challenged neurons, with statistical significance down to low nanomolar concentrations [15]. Relative to a maximum recovery obtained using the growth factor GDNF, EC_{50} of all compounds are given in Table 2. The biological activity of **1** stands in marked contrast to the related natural product immunophilin ligands FK506 [16] and rapamycin [17]. Whereas **1** is non-immunosuppressive, as measured in a human mixed lymphocyte reaction (MLR) [18], both **2** and **3** are potent inhibitors of immune response, though by different mechanisms [3]. FKBP12 binding was measured per standard procedure [19].

Discussion

3-Normeridamycin is closely related to the non-immunosuppressive macrolide meridamycin, isolated from *S. hygroscopicus*. Meridamycin, in contrast to the macrolides FK506 and rapamycin, is one of only two non-immunosuppressive natural product immunophilin ligands in this structural class. Previous work on rapamycin has shown that the pipercolate incorporating enzyme can accommodate a number of substrate analogs [20], in particular proline to produce a proline containing nor-

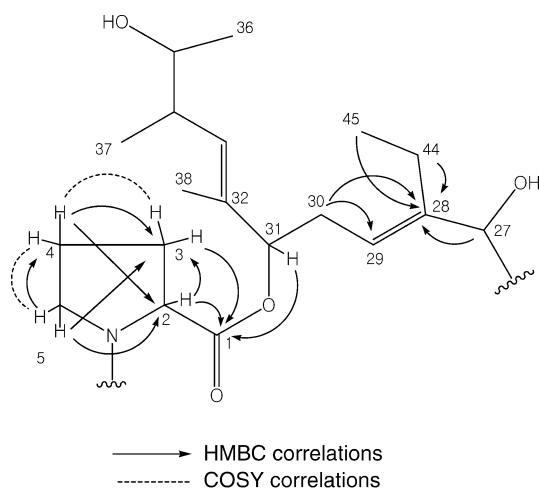


Fig. 2. 3-Normeridamycin selected 2D NMR correlations.

rapamycin [21]. The occurrence of a naturally occurring normeridamycin is therefore in agreement with literature precedent, suggesting that the biosynthetic pathway of meridamycins may be similar to that of rapamycin and FK506 [22].

Meridamycin is known to inhibit binding of FK506 to FKBP12, and to antagonize immunosuppression in FK506 and rapamycin. 3-Normeridamycin is also non-immunosuppressive and exhibits potent neuroprotective activity against the neurotoxin MPP⁺. In particular, it was found to have low nanomolar activity in restoring dopamine uptake in MPP⁺ treated neurons. Based on these findings, 3-normeridamycin shows potential utility as a therapeutic agent in the treatment of Parkinson's disease.

In conclusion, we herein report the isolation and characterization of a new neuroprotective non-immunosuppressive immunophilin ligand, 3-normeridamycin (**1**). The compound exerts a profound neuroprotective effect in DAergic neurons after challenge with MPP⁺, thus representing a potential new lead for therapeutic applications in the treatment of Parkinson's disease.

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Table 2 Neuroprotective activities of compounds **1–3**

Compound	³ H DA uptake EC ₅₀ (nM)	FKBP12 binding ED ₅₀ (nM)	MLR IC ₅₀ (nM)
1	110	50	>10000
2	>10000	0.2	1.1
3	49	0.4 ^a	0.22 ^a

^a Data taken from reference 16.

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