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Nocardiopsins: New FKBP12-Binding Macrolide Polyketides from an Australian Marine-Derived Actinomycete, *Nocardiopsis* sp.**

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Abstract: A marine-derived actinomycete, *Nocardiopsis* sp. (CMB-M0232), obtained from a sediment sample collected at a depth of 55 m off the coast of Brisbane, Australia, yielded two new macrolide polyketides. Structures for nocardiopsins A and B were assigned by detailed spectroscopic analysis, degradation and chemical derivatization. A Marfey's analysis revealed an unexpected acid-mediated partial racemization of the L-pipecolic acid incorporated within the nocardiopsins. The scope of this racemization was assessed against a selection of natural and synthetic *N*-acyl pipecolic acids. While the nocardiopsins are not antibacterial, antifungal or cytotoxic, they do exhibit low-micromolar binding to the immunophilin FKBP12, consistent with their structural and biosynthetic relationship to the immunosuppressive agents FK506 and rapamycin. The nocardiopsins represent a new point of entry into what has been a valuable, exclusive and reclusive region of bioactive chemical space—that surrounding the FK506/rapamycin pharmacophore.

Keywords: immunochemistry · macrolides · marine actinomycetes · natural products · polyketides

Introduction

Over the last seventy years, terrestrial bacteria and fungi have been a mainstay of the pharmaceutical and agrochemical industries. The challenge to discover, develop, manufacture, and deliver microbial metabolites to the marketplace sparked and fuelled a revolution in global science, economics, industry, and healthcare. These successes notwithstanding, late last century saw investment in microbial biodiscovery wane, as the cost, time and commercial risk for new discoveries increased.[1] However, recent technological advances in the science of natural products chemistry (chromatography and spectroscopy for structure elucidation) and biology (high-throughput and high-content bioassays for detecting bioactive compounds) provided the means to accelerate discovery and reduce costs. With similar advances in microbiological techniques, it is now possible to cultivate many previously inaccessible marine-derived bacteria and fungi, opening up a whole new frontier in molecular discovery. This enhanced technical capability, together with the emergence of marine-derived microorganisms as a new source of bioactive metabolites, has reignited academic (and industrial) interest in microbial biodiscovery.

Inspired by these recent advances, we have commenced a program investigating the chemical diversity of marine-derived microorganisms collected from various locations around the coast of Australia. During this program, we isolated an actinomycete (designated CMB-M0232), from a sediment sample obtained at a depth of 55 m off the coast of Brisbane, Australia. Preliminary chemical profiling of this actinomycete, identified as a *Nocardiopsis* sp., revealed a rich diversity of secondary metabolites, including novel polyketides and diketopiperazines. Our investigations into the diketopiperazines are ongoing, and will be reported at a later date. In this report we describe the isolation, characterization, structure elucidation and bioassay of two novel polyketides, nocardiopsins A and B.

Results and Discussion

Preliminary HPLC-DAD-MS analysis of a small-scale (100 mL) saline liquid fermentation of the *Nocardiopsis* sp.

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[**] FKBP12: FK506 (macrolide isolated from *Streptomyces tsukubaensis*)-Binding Protein 12

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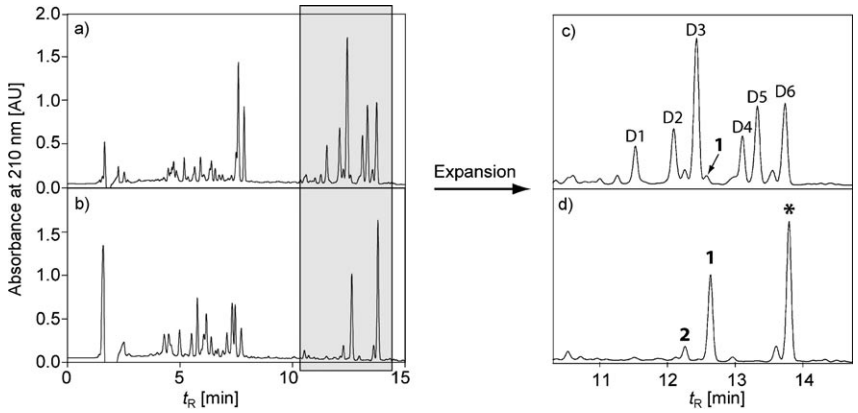
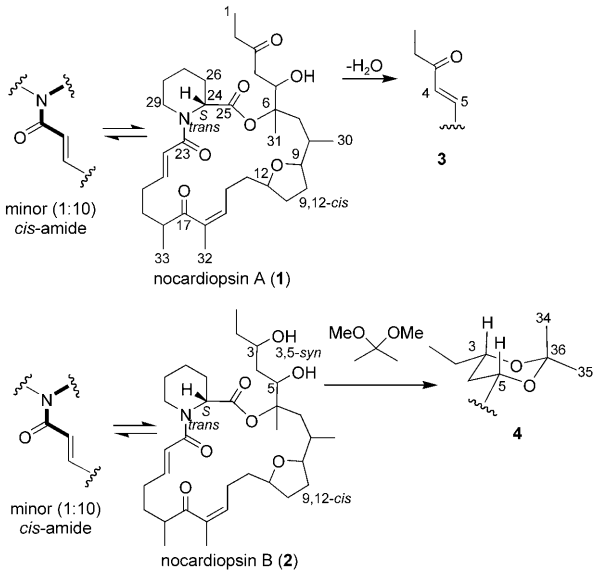


Figure 1. HPLC traces (210 nm) of EtOAc extracts of *Nocardiopsis* sp. cultivated in a) salt water medium and b) fresh water medium. c) Expansion of HPLC trace from salt water medium showing the presence of six diketopiperazines (D1–D6) with M_W 369, 383, 482, 466, 468 and 452, respectively, as well as a small amount of nocardiopsin A (**1**) with M_W 573. d) Expansion of HPLC trace from fresh water medium showing the absence of diketopiperazines and a significant increase in the production of nocardiopsin A (**1**), nocardiopsin B (**2**) and an as yet unidentified compound with M_W 252 (*).

revealed two noteworthy minor metabolites (M_W 573 and 575) almost completely obscured by co-eluting major metabolites (M_W 482 and 383) (Figure 1). ^{1}H NMR analysis of an HPLC fraction enriched with these metabolites suggested that the former were polyketides and the latter diketopiperazines, with both sets of metabolites being unreported in the natural products literature. In an attempt to improve access to these polyketides, we were delighted when a small-scale (100 mL) non-saline liquid fermentation resulted in considerably enhanced polyketide production (>30-fold relative increase for the same cultivation volume and conditions) and complete suppression of the co-eluting diketopiperazines (Figure 1). Empowered by this adjustment to metabolite expression, we undertook a larger-scale (2 L) non-saline liquid fermentation, followed by solvent extraction, partitioning and HPLC fractionation, to yield two macrolide polyketides, nocardiopsins A (**1**) and B (**2**) (Scheme 1).

HRESI(+)MS analysis of nocardiopsin A (**1**) returned a molecular formula ($C_{33}H_{51}NO_7$) requiring nine double bond equivalents (DBE). The NMR ([D_4]MeOH) data (Table 1) revealed resonances for two ketones (δ_C 208.4 and 213.2), two ester/amide carbonyl groups (δ_C 170.2 and 173.1), and two double bonds (δ_C 123.9, 137.8, 145.4 and 147.5), requiring that **1** be tricyclic. 2D NMR correlations defined the sequence from the C-1 (δ_H 1.03 and δ_C 8.1) through the ketone C-3 (δ_C 213.2) to the oxy quaternary C-6 (δ_C 85.9) with pendant methyl C-31 (δ_H 1.12 and δ_C 22.4), and extending through the diastereotopic methylene C-7 (δ_H 1.72/1.81 and δ_C 43.0) to the oxymethine C-12 (δ_H 4.93 and δ_C 76.0), and incorporating the C-30 secondary methyl (δ_H 0.96 and δ_C 14.9) and the oxymethine C-9 (δ_H 4.05 and δ_C 78.8). A comparable set of correlations defined the sequence from the ester/amide carbonyl C-23 (δ_C 170.2) through *E* $\Delta^{21,22}$ ($J_{21,22}$ 15.0 Hz) to the ketone C-17 (δ_C 208.4), incorporating the C-33 secondary methyl (δ_H 1.01 and δ_C 18.7), and on to a $\Delta^{15,16}$ (δ_H 6.67 and δ_C 145.4/137.8) bearing a C-32 methyl (δ_H 1.73 and δ_C 11.9) and the diastereotopic methylene C-14 (δ_H 2.14/2.34 and δ_C 29.9). ROESY correlations between H-15 and H_3-32 defined the *Z* $\Delta^{15,16}$ configuration, while correlations between H-9 and H-12 supported ring closure to a *cis*-disubstituted tetrahydrofuran. Although the overlapping ^{1}H NMR resonances for H_2-13 precluded a definitive COSY correlation to either H-12 or H_2-14, HMBC correlations from H_2-14 to both C-13 and C-11 permitted assembly of the C-1 to C-23 structure fragment. The remaining NMR resonances were suggestive of a pipecolic acid residue, confirmed as L by acid hydrolysis and C_3 Marfey's analysis[2] (see below). Key HMBC correlations from H-24 to the amide carbonyl C-23 and lactone carbonyl C-25 positioned the L-pipecolic acid residue and completed the C-1 to C-25 linear structure fragment. Lactone closure from C-25 to C-6 was determined by diagnostic NMR ($CDCl_3$) resonances for a 5-OH (δ_H 3.52), with COSY correlations to H-5 (δ_H 4.00). Attempts at assigning absolute configuration about C-5 using the Mosher approach failed, due to dehydration under the reaction conditions to yield the *E* $\Delta^{4,5}$ analogue **3** ($J_{4,5}$=16.0 Hz). The structure for **1** with partial absolute (15*Z*,21*E*,24*S*) and relative (9,12-*cis*) configuration is as shown.



Scheme 1. Structure of nocardiopsin A (**1**), nocardiopsin B (**2**), dehydration product (**3**) of nocardiopsin A and isopropylidene derivative (**4**) of nocardiopsin B.

Table 1. NMR (600 MHz, [D_4]MeOH) data for nocardiopsin A (**1**).

Pos.	δ_H *trans*, mult (*J* in Hz)	δ_C *trans*	COSY	ROESY	^{1}H,^{13}C HMBC	δ_H *cis*, mult (*J* in Hz)[a]	δ_C *cis*[a]
1	1.03, t (7.2)	8.1	2		2, 3		
2	2.58, m	37.4	1		1, 3	2.56, m	41.8
3		213.2					
4a	2.68, dd (15.6, 2.4)	46.2	4b, 5	31	3, 5, 6		
4b	2.49, dd (15.6, 10.1)		4a, 5	31	3, 5, 6		
5	4.00, dd (2.4, 10.2)	75.1	4a/b	7a, 30, 31	3, 6, 7, 31	3.93[e]	75.4
6		85.9					
7a	1.72, m[b]	43.0	7b, 8	5, 30	5, 6, 9, 30, 31		
7b	1.81, m[c]		7a, 8	31	5, 6, 9, 30, 31		
8	2.54, m	37.2	7a/b, 9, 30	31	7, 10, 30	2.46, m	37.4
9	4.05, ddd (10.6, 7.2, 3.4)	78.8	8, 10a/b	12, 30, 31		3.97, ddd (10.6, 7.2, 3.4)	78.6
10a	1.74, m[b]	37.1	9, 10b, 11				
10b	1.54, m		9, 10a, 11	30			
11	1.46, m[d]	25.9	10a/b, 12	30			
12	4.93, m	76.0	11, 13a	9		5.14, m	75.3
13a	1.72, m[b]	34.0	12				
13b	1.45, m[d]				15		
14a	2.34, m	29.9	14b, 15	32	13		
14b	2.14, m		14a, 15	32	13		
15	6.67, dd (10.2, 4.6)	145.4	14a/b, 32	18	17, 32	6.59, dd (10.2, 4.6)	145.1
16		137.8					
17		208.4					
18	3.51, m	37.6	19a/b, 33	15, 21	17, 19, 20, 33	3.27, m	40.2
19a	1.82, m[c]	34.4	18, 19b, 20a/b				
19b	1.57, m		18, 19a, 20a/b	33			
20a	2.34, m	30.7	19a/b, 20b, 21		18, 19		
20b	2.14, m		19a/b, 20a, 21	22			
21	6.76, ddd (15.2, 10.1, 4.7)	147.5	20a/b, 22	18	19, 20, 23	6.80, ddd (15.2, 10.1, 4.7)	148.1
22	6.29, d (15.2)	123.9	21	20b, 29a/b	20, 23	6.54, d (15.2)	122.3
23		170.2					
24	5.23, dd (6.0, 1.2)	54.5	26a/b		23, 25, 26, 27, 29	5.05, m	57.6
25		173.1					
26a	2.27, m	27.5	24, 26b, 27a/b				
26b	1.69, m[b]		24, 26a, 27a/b				
27a	1.71, m[b]	21.8	26a/b, 27b, 28a/b				
27b	1.30, m		26a/b, 27a, 28a/b				
28a	1.77, m	26.0	27a/b, 28b, 29a/b				
28b	1.46, m[d]		27a/b, 28a, 29a/b				
29a	3.91, m[e]	45.5	28a/b, 29b	22	24, 27, 28	4.49, m	41.6
29b	3.21, ddd (16.2, 12.7, 3.0)		28a/b, 29a	22		2.54, m	
30	0.96, d (7.0)	14.9	8	5, 7a, 9, 10b, 11	7, 8, 9		
31	1.12, s	22.4		4a/b, 5, 7b, 8, 9	5, 6, 7		
32	1.73, s	11.9	15	14a/b	15, 16, 17	1.84, s	9.4
33	1.01, d (7.2)	18.7	18	19b	17, 19		

[a] Only selected resonances were observed for the minor *cis* isomer due to overlapping with the major *trans* isomer. [b]–[e] Overlapping signals.

HRESI(+)MS analysis of nocardiopsin B (**2**) returned a molecular formula ($C_{33}H_{53}NO_7$) consistent with a dihydro analogue of **1**. The principal difference in the NMR ([D_4]MeOH) data for **2** (Supporting Information, Table S1) over **1** was conversion of the C-3 carbonyl in **1** (δ_C 213.2) to a hydroxymethine in **2** (δ_H 3.83 and δ_C 73.8). Confirmation of the L-pipecolic acid residue was again achieved by C_3 Marfey's analysis (see below), while treatment with 2,2-dimethoxypropane yielded isopropylidene **4**, which was assigned the 3,5-*cis* configuration on the basis of a large diaxial $J_{4\alpha,5}$ (11.2 Hz) and ^{13}C NMR shifts C-34 and C-35 (20.4 ppm and 31.1 ppm).[3] Thus, the structure for **2** with partial absolute (15*Z*,21*E*,24*S*) and relative (3,5-*syn*;9,12-*cis*) configuration is as shown.

Further analysis of the ^{1}H NMR data for nocardiopsins revealed major and minor (10:1) amide conformers, for which ROESY correlations established the major as *trans* (H-22 to H_2-29), and the minor as *cis* (H-22 to H-24) (Supporting Information, Figure S10). Having attributed the doubling of NMR resonances to amide conformers, we were surprised when a C_3 Marfey's analysis of both nocardiopsins revealed a 2:1 ratio of L versus D-pipecolic acid, choosing to interpret this as evidence of partial racemization during acid hydrolysis rather than being indicative of natural diastereomers. To test this hypothesis, we undertook acid hydrolysis and C_3 Marfey's analysis of other pipecolic acid-containing microbial natural products (see below), including FK506 and rapamycin (polyketide macrocycles that incorporate an *N*-2-oxoacyl-L-pipecolic lactone residue) and apicidin A (a cyclic lipopeptide that incorporates an *N*-Ile-D-pipecolic amide residue). Authentic L-pipecolic acid was also subjected to the same acid treatment and C_3 Marfey's analysis. Interest-

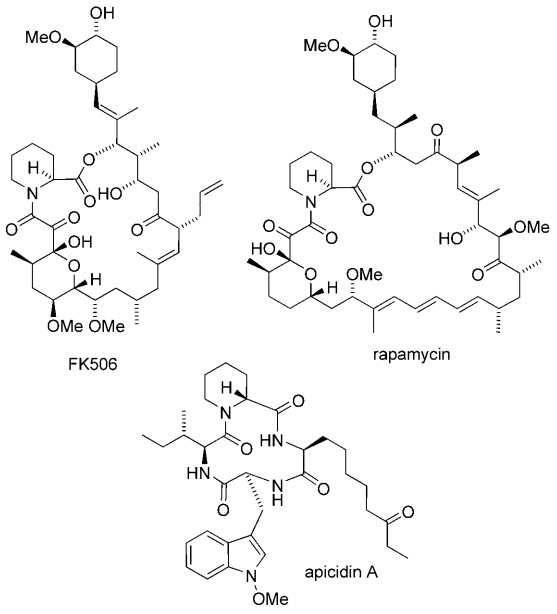
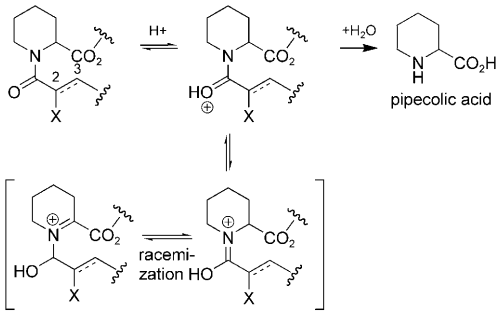


Table 2. Plausible mechanism for acid-catalyzed partial racemization of pipecolic acid derivatives.



Compound	X	C2–C3	Ratio L/D
nocardiopsin A (**1**)	H,H	=	2:1
nocardiopsin B (**2**)	H,H	=	2:1
N-crotonyl-L-Pip	H,H	=	2:1
N-butanoyl-L-Pip	H,H	–	4:1
FK506	=O	–	10:1
rapamycin	=O	–	10:1
apicidin A	NH,H	–	no racemization

ingly, the pipecolic acid recovered from hydrolysis of both FK506 and rapamycin had undergone partial racemization (10:1), whereas the pipecolic acid recovered from apicidin A and acid-treated authentic L-pipecolic acid remained unchanged (see Supporting Information).

The observations described above reveal, for the first time, that *N*-acyl substituted pipecolic acid residues are susceptible to acid hydrolysis mediated partial racemization, and that the nature of the *N*-acyl substituent can influence the extent to which racemization occurs. In an attempt to better define this process, we prepared and subjected the *N*-crotonyl and *N*-butanoyl amides of L-pipecolic acid to hydrolysis and C_3 Marfey's analysis, confirming partial racemizations of 2:1 and 4:1, respectively (Supporting Information). A plausible mechanism for the racemizations (Table 2) features competing pathways involving initial protonation of the *N*-acyl carbonyl (step A) followed by either hydrolysis to yield pipecolic acid (step B) or ii) enolization of the acyl oxonium to yield equilibrating imines that racemize the pipecolic acid (step C) prior to hydrolysis (step B). The *N*-acyl substituent common to nocardiopsins displays the highest level of "imine racemization", compared with the more readily hydrolyzed *N*-acyl substituent present in apicidin A. Given these observations, exposure of *N*-acyl pipecolic residues to acids (i.e., acidic hydrolysis, extraction, chromatography), where the *N*-acyl group is not an amino acid, should be performed with caution, being alert to the possibility of partial racemization.

The closest known natural product analogues to the nocardiopsins are the polyketide immunosuppressant agents FK506, from a Japanese soil isolate of *Streptomyces tsukubaensis*,[4] and rapamycin from an Easter Island soil isolate of *Streptomyces hygroscopius*.[5] The latter polyketides exert their immunosuppressive effect by forming a binding complex with the cytosolic protein FKBP12 that in turn inhibits key intracellular signaling cascades.[6] SAR studies have confirmed the rare α-ketoacyl pipecolic residue as key to the immunosuppressive pharmacophore.[7] The only other known natural product members of this structure class are meridamycin, from a Venezuelan isolate of *Streptomyces hygroscopius*,[8] and the antascomicins, from a Chinese soil *Micromonospora* sp.,[9] which also bind to FKBP12 but are not immunosuppressive. Recent literature reports note that FKBP-binding polyketides can exhibit separate and distinct immunosuppressive and neurotrophic pharmacologies,[10] with non-immunosuppressive inhibitors of FKBPs having potential application against important neurological disorders such as multiple sclerosis,[11] traumatic spinal cord injury[12] and Parkinson's disease.[13] In the absence of new natural product members of this structure class, exploration of the pharmacophore defined by FK506 and rapamycin has relied on virtual screening,[14] genetic engineering[15] and precursor-directed biosynthesis, the latter manipulating polyketide synthases (PKS) to incorporate unnatural starter unit carboxylic acids,[16] chain units[17] and terminating pipecolic acid mimics.[10,18] As a new entry into this molecular landscape, the nocardiopsins are the first natural product examples of this biosynthetic structure class to be reported in over a decade (Figure 2).

Nocardiopsins A and B displayed no growth inhibitory or cytotoxic activity in antibacterial or antifungal disc diffusion assays (up to 50 μg per disc) against *Escherichia coli* (ATCC 11775), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6051) and *Candida albicans*, nor did they display cytotoxicity (IC_{50} > 30 μM) in MTT assays against AGS (gastric adenocarcinoma), SH-SY5Y (neuroblastoma) and HT-29 (colorectal adenocarcinoma) cancer cell lines. In contrast to rapamycin, the nocardiopsins do not induce complex formation between EGFP-FKBP12 and Cherry-FRB fusion proteins as determined by measuring FRET-between fluorescent molecules (Supporting Information, Figure

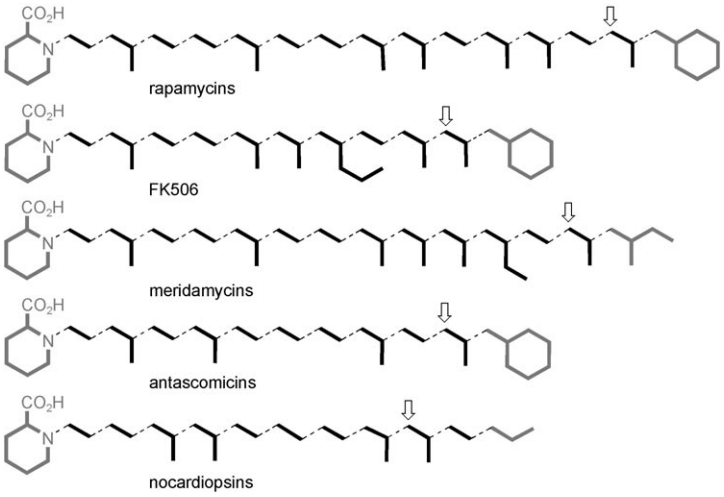


Figure 2. Comparison of linear carbon skeletons for macrocyclic polyketides showing proposed polyketide synthase starter units, acetate/propionate assembly units and pipecolic acid terminating units, with arrows indicating points of macrolactonization.

S15A). The apparent affinity for interaction of EGFP-FKBP12 with Cherry-FRB in the presence of rapamycin was found to be 0.3 ± 0.07 μM (Supporting Information, Figure S15B). To determine the affinities between individual proteins and polyketides we performed isothermal titration calorimetry (ITC) experiments, establishing that **1** and **2** bound EGFP-FKBP12 with K_d values of 2.9 ± 0.3 μM and 5.0 ± 0.4 μM, respectively (Figure 3a and b). In contrast, rapamycin bound to EGFP-FKBP12 with a K_d of 0.10 ± 0.01 μM (Figure 3c), which is ~100-fold lower than previously reported,[19] indicating that fusion of FKBP12 to EGFP domain may have reduced binding affinity. A larger change in enthalpy was observed for the rapamycin-EGFP-FKBP12 interaction (45 kcal mol^{-1}) than for the interactions with **1** (1.0 kcal mol^{-1}) and **2** (1.5 kcal mol^{-1}). This may reflect a larger number of interactions between residues in rapamycin and EGFP-FKBP12 than for the nocardiopsins. No detectable binding of nocardiopsins to FRB was observed.

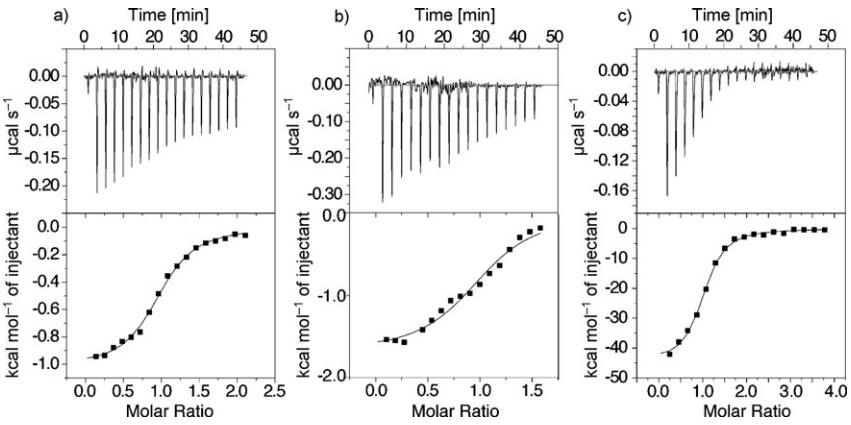


Figure 3. Thermodynamics of interaction between polyketides and EGFP-FKBP12 as determined by ITC. a) Titration of nocardiopsin A (**1**; 45 μM) with EGFP-FKBP12 (470 μM). b) Titration of nocardiopsin B (**2**; 45 μM) with EGFP-FKBP12 (470 μM). c) Titration of rapamycin (1.6 μM) with EGFP-FKBP12 (30 μM). Data obtained were fitted to a 1:1 binding model.

Conclusion

In this report, we described our investigations into an Australian marine-derived actinomycete, *Nocardiopsis* sp. (CMB-M0232), leading to the discovery of two new polyketide macrolides, nocardiopsins A and B. During the course of this study we also detected, documented and proposed a mechanism for a previously unknown acid mediated partial racemization of *N*-acyl pipecolic acid residues, including those found in the nocardiopsins, FK506 and rapamycin. Although structurally distinct from the commercially successful FK506 and rapamycin, the nocardiopsins nonetheless represent the first new natural examples of this biosynthetic genre to be described in over a decade. More significantly, despite lacking structural elements previously deemed critical to FKBP12 binding, the nocardiopsins display modest binding to FKBP12. These considerations, together with an appreciation that potent non-immunosuppressive inhibitors of FKBPs other than FKBP12 have potential application against important neurological disorders, encourages the view that the nocardiopsins are deserving further attention. We believe our account of the nocardiopsins will encourage synthetic chemists to take up the challenge of total synthesis, to confirm the proposed structure, assign unresolved relative and absolution configurations, and generate analogue libraries to probe what has historically been an extremely valuable but nevertheless exclusive and reclusive region of bioactive chemical space.

Experimental Section

General experimental details: Chiroptical measurements ($[\alpha]_D$) were obtained on a JASCO P-1010 polarimeter in a 100×2 mm cell at 22 °C. NMR spectra were obtained on a Bruker Avance DRX600 spectrometer, in the solvents indicated and referenced to residual ^{1}H signals in deuterated solvents. Electrospray ionization mass spectra (ESIMS) were acquired using an Agilent 1100 Series separations module equipped with an Agilent 1100 Series LC/MSD mass detector in both positive and negative ion modes under the following conditions (Zorbax C_8 column, 150×4.6 mm, eluting with 1.0 mL min^{-1} 90% H_2O/MeCN to MeCN (with isocratic 0.05% HCO_2H modifier) over 15 min, then held for 5 min with detection at 210 and 254 nm). High resolution ESIMS measurements were obtained on a Bruker micrOTOF mass spectrometer by direct infusion in MeCN at 3 μL min^{-1} using sodium formate clusters as an internal calibrant. HPLC was performed using an Agilent 1100

Series diode array and/or multiple wavelength detectors and Agilent 1100 Series fraction collector, controlled using ChemStation Rev.9.03 A and Purify version A.1.2 software. Fluorescence spectra and long-time FRET measurements were performed in 1 mL quartz cuvettes (Hellma) with continuous stirring at 25 °C on a Spex Fluoromax-4 spectrofluorometer (Jobin Yvon Inc., Edison, NJ). ITC was performed on an iTC_{200} Isothermal Titration Calorimetry system (Microcal, Northampton, MA). DMEM, RPMI and FBS were obtained from Invitrogen (USA). FK506, rapamycin and apicidin A were obtained from BioAustralis (Sydney, Australia).

Isolation and identification of strain CMB-M0232: The marine-derived actinomycete strain CMB-M0232 was isolated from a sediment sample collected in December 2007 at a depth of ~55 m by a sediment corer near South Molle Island, Brisbane. The fresh sediment sample was transferred to the laboratory in a sealed 50 mL Falcon tube at room temperature (16 h), where it was stored in the dark at −30 °C for one week. Approximately 1 g of the thawed sediment sample was suspended in 4 mL of Ocean Nature seawater (Aquasonic, Australia) and then subjected to heat-shock at 55 °C for 8 min after which 50 μL was transferred on to agar plates (comprising 25 mL of 1% starch, 0.4% yeast extract, 0.2% peptone, 1.8% agar, and 0.0005% rifampicin). The resulting agar plate was incubated at 27 °C for four weeks. Pure strains of individual colonies were obtained by standard microbiological techniques, and were grown to dense colonies on a single agar plate. One such strain (CMB-M0232) was identified as *Nocardiopsis* sp. on the basis of a 16S rRNA gene sequence showing 99% homology with other members of this genus (NCBI website using the BLAST Database).

Chemical profiling: A single colony of CMB-M0232 was sub-sampled into seawater medium (100 mL of Ocean Nature seawater, 1% starch, 0.4% yeast extract and 0.2% peptone) and incubated at 27 °C for 7 d at 190 rpm. The culture was extracted with EtOAc (100 mL), and the organic phase concentrated in vacuo to yield an extract that was subsequently analyzed by HPLC-DAD-ESI(±)MS and NMR. This procedure was then repeated using deionized water in place of seawater. Comparative HPLC traces are shown in Figure 1.

Large scale cultivation and extraction of strain CMB-M0232: A seed culture was prepared by inoculating a 100 mL flask containing liquid medium (15 mL deionized water containing 1% starch, 0.4% yeast extract and 0.2% peptone) with a single colony of CMB-M0232 followed by shaking at 200 rpm for 7 d at 26.5 °C. Aliquots of the seed culture (2.8 mL) were transferred to four 2 L Fernbach flasks, each containing the same liquid medium (500 mL), and the flasks were shaken at 200 rpm for 7 d at 26.5 °C. The resulting cultures were each extracted with EtOAc (400 mL) and the combined organic phases concentrated in vacuo to yield an extract (132 mg). The extract was sequentially triturated with 25 mL each of hexane, CH_2Cl_2 and MeOH to afford, after concentration in vacuo, 30.7, 87.6 and 3.9 mg fractions, respectively. Each fraction was analyzed by HPLC-DAD-ESI(±)MS and NMR. The CH_2Cl_2 fraction, which was found to be rich in the target polyketides, was subsequently subjected to semi-preparative reversed-phase HPLC (Zorbax C_8 250 × 9.4 mm, 5 μm column, 4 mL min^{-1} gradient elution from 90% H_2O/MeCN to 100% MeCN over 40 min with a hold at 100% MeCN for 5 min), to yield nocardiopsin A (**1**) (t_R = 27.8 min, 7.1 mg, 5.4%) and nocardiopsin B (**2**) (t_R = 27.3 min, 5.3 mg, 4%). [Note: % yields are mass-to-mass calculations against the EtOAc extract.]

Nocardiopsin A (1): Colorless oil; $[\alpha]_D$ = −28 (c = 0.1, MeOH); UV (MeOH): λ_{max} (ε) = 219 nm (2290); ^{1}H and ^{13}C NMR (600 MHz, [D$_4$]MeOH) see Table 1. HRESI(+)MS: m/z: calcd for $C_{33}H_{51}NO_7Na$: 596.3564; found: 596.3561 $[M+Na]^+$.

Nocardiopsin B (2): Colorless oil; $[\alpha]_D$ = −72 (c = 0.1, MeOH); UV (MeOH): λ_{max} (ε) = 218 nm (3105); ^{1}H and ^{13}C NMR (600 MHz, [D$_4$]MeOH): see Supporting Information, Table S1; HRESI(+)MS: m/z: calcd for $C_{33}H_{53}NO_7Na$: 598.3720; found: $[M+Na]^+$.

Nocardiopsin A Mosher dehydration product (3): A solution of **1** (3.0 mg, 5.2 μmol), *S*-(−)-MTPA acid (10 mg, 42.7 μmol), *N*,*N*-dicyclohexylcarbodiimide (DCC) (3.4 mg, 16.2 μmol) and 4-dimethylaminopyridine (2.0 mg, 16.2 μmol) in anhydrous CH_2Cl_2 (1 mL) was stirred at room temperature for 24 h. The solvent was then removed under a stream of nitrogen and the crude product purified by reversed-phase HPLC (Zorbax column, C_8, 250 × 9.4 mm, 5 μm, 4 mL min^{-1}, gradient from 90% H_2O/MeCN to 100% MeCN over 20 min with a hold at 100% MeCN for 5 min) to yield the nocardiopsin dehydration product (**3**) as a colorless oil (0.6 mg, 20%). $[\alpha]_D$ = −12.9 (c = 0.05, MeOH); NMR (600 MHz, [D$_4$]MeOH): see Supporting Information, Table S2; HRESI(+)MS m/z: calcd for $C_{33}H_{50}NO_6$: 556.3638; found: 556.3633 $[M]^+$.

Nocardiopsin B isopropylidene derivative (4): A solution of **2** (2.5 mg, 4.3 μmol), pyridinium-*p*-toluenesulfonate (4.5 mg, 18 μmol) and 2,2-dimethoxypropane (1 mL) in MeOH (1 mL) was stirred at 0 °C for 3 h. The reaction was then quenched with 5% aqueous $NaHCO_3$ (1 mL), and extracted twice with CH_2Cl_2. The combined organic layer was reduced to dryness in vacuo and the residue purified by HPLC (Zorbax C_8 column, 5 μm, 250 × 9.4 mm, 4 mL min^{-1}, starting from 90% H_2O/MeCN to 100% MeCN over 20 min with a hold at 100% MeCN for 5 min) to yield the isopropylidene derivative **4** as a colorless oil (1.4 mg, 56%). $[\alpha]_D$ = −1.6 (c = 0.08, MeOH); NMR (600 MHz, [D$_6$]benzene): see Supporting Information, Table S3; HRESI(+)MS m/z: calcd for $C_{36}H_{58}NO_7$: 616.4213; found: 616.4208 $[M]^+$.

Crotonic acid NHS ester: A solution of crotonic acid (200 mg, 2.3 mmol), DCC (479 mg, 2.3 mmol) and *N*-hydroxysuccinimide (NHS) (267 mg, 2.3 mmol) in MeCN (25 mL) was stirred at room temperature for 18 h, during which time a white precipitate formed. The reaction mixture was then cooled to −20 °C, filtered, and the filtrate reduced to dryness in vacuo to give crotonic acid NHS ester as a white solid (130 mg, 65%). ^{1}H NMR (600 MHz, [D$_4$]MeOH): δ = 7.24 (dq, J = 15.8, 7.0 Hz, 1H, =CH), 6.00 (d, J = 15.8 Hz, 1H, =CH), 2.80 (s, 4H, 2 × NCOC*H*$_2$), 1.95 ppm (d, J = 7.0 Hz, 3H, C*H*$_3$).

Butyric acid NHS ester: Prepared as for crotonic acid NHS ester as a viscous yellow liquid (125 mg, 63%). ^{1}H NMR (600 MHz, [D$_4$]MeOH): δ = 2.83 (s, 4H, 2 × NCOC*H*$_2$), 2.60 (t, J = 7.5 Hz, 2H, CH$_2$CO), 1.75 (m, 2H, CH$_3$C*H*$_2$), 1.04 ppm (t, J = 7.5 Hz, 3H, C*H*$_3$CH$_2$).

Synthesis of *N*-crotonyl and *N*-butanoyl amides of L-pipecolic acid: A solution of L-pipecolic acid (10 mg, 78 μmol), NHS ester (1 equiv) and potassium carbonate (3 equiv) in THF (75 mL) was refluxed for 4–5 h. The reaction mixture was diluted with water (50 mL) and extracted with *n*-butanol (2 × 50 mL). The combined organic layer was reduced to dryness in vacuo and further purified by reversed phase HPLC (Zorbax C_8 250 × 9.4 mm column, 5 μm, 4 mL min^{-1} gradient elution from 90% H_2O/MeOH to 100% MeOH over 20 min with a hold at 100% MeOH for 5 min).

***N*-Crotonyl-L-pipecolic acid**: Colorless oil (4.6 mg, 46%); $[\alpha]_D$ = −62.7 (c = 0.10, MeOH); ^{1}H NMR (600 MHz, [D$_4$]MeOH; see also Supporting Information, Table S4): δ_{trans} = 6.73 (m, 1H, =CH), 6.47 (d, J = 15.1 Hz, 1H, COC*H*=), 5.14 (m, 1H, NC*H*CO$_2$H), 3.93 (d, J = 13.2 Hz, 1H, NCH$_2$), 3.45 (dt, J = 13.2, 2.8 Hz, 1H, NCH$_2$), 2.37 (m, 1H, NCHC*H*$_2$), 1.88 (dd, J = 7.0, 1.5 Hz, 3H, CH$_3$), 1.67 (m, 1H, NCH$_2$C*H*$_2$), 1.67 (m, 1H, CH$_2$), 1.52 (m, 1H, NCHC*H*$_2$), 1.51 ppm (m, 1H, CH$_2$); δ_{cis} = 6.69 (m, 1H, =CH), 6.31 (d, J = 15.1 Hz, 1H, COC*H*), 4.53 (m, 1H, NC*H*CO$_2$H), 4.43 (d, J = 13.2 Hz, 1H, NCH$_2$), 3.03 (dt, J = 13.2, 2.8 Hz, 1H, NCH$_2$), 2.37 (m, 1H, NCHC*H*$_2$), 1.85 (dd, J = 7.0, 1.6 Hz, 3H, CH$_3$), 1.67 (m, 1H, NCH$_2$C*H*$_2$), 1.67 (m, 1H, CH$_2$), 1.58 (m, 1H, NCHC*H*$_2$), 1.51 (m, 1H, CH$_2$), 1.37 ppm (m, 1H, NCH$_2$C*H*$_2$); HRESI(+)MS: m/z: calcd for $C_{10}H_{15}NO_3Na$: 220.0905; found: 220.0944 $[M+Na]^+$.

***N*-Butanoyl-L-pipecolic acid**: Colorless oil (4.9 mg, 49%). $[\alpha]_D$ = −40.7 (c = 0.10, MeOH); ^{1}H NMR (600 MHz, [D$_4$]MeOH; see Supporting Information, Table S5): δ_{trans} = 5.11 (m, 1H, NC*H*CO$_2$H), 3.76 (d, J = 12.7 Hz, 1H, NCH$_2$), 3.41 (dt, J = 12.7, 2.7 Hz, 1H, NCH$_2$), 2.43 (m, 1H, COCH$_2$), 2.36 (m, 1H, COCH$_2$), 2.34 (m, 1H, NCHC*H*$_2$), 1.68 (m, 1H, NCH$_2$C*H*$_2$), 1.63 (m, 1H, CH$_2$), 1.62 (m, 2H, CH$_3$C*H*$_2$), 1.48 (m, 1H, NCHC*H*$_2$), 1.47 (m, 1H, CH$_2$), 1.42 (m, 1H, NCH$_2$C*H*$_2$), 0.98 ppm (t, J = 7.3 Hz, 3H, CH$_3$); δ_{cis} = 4.41 (m, 1H, NCH$_2$), 4.39 (m, NC*H*CO$_2$H), 2.93 (dt, J = 12.7, 2.7 Hz, 1H, NCH$_2$), 2.37 (m, 1H, NCHC*H*$_2$), 2.35 (m, 1H, COCH$_2$), 2.26 (m, 1H, COCH$_2$), 1.66 (m, 1H, NCH$_2$C*H*$_2$), 1.63 (m, 1H, CH$_2$), 1.59 (m, 1H, NCHC*H*$_2$), 1.47 (m, 1H, CH$_2$), 1.34 (m, 1H, NCH$_2$C*H*$_2$), 0.96 ppm (t, J = 7.3 Hz, 3H, CH$_3$); HRESI(+)MS: m/z: calcd for $C_{10}H_{17}NO_3Na$: 222.1106; found: 222.1101 $[M+Na]^+$.

C_3 Marfey's analysis: A sealed vial containing **1** (100 μg) and 6 M HCl (200 μL) was heated at 105 °C for 16 h, after which the reaction mixture was dried under a stream of nitrogen, dissolved in H_2O (50 μL), and treated with 1 M sodium bicarbonate (20 μL) and a 1% solution of L-1-fluoro-2,4-dinitrophenyl-5-alanine amide (100 μL) (L-FDAA). After 60 min at 40 °C, the reaction mixture was quenched by the addition of 1 M HCl (20 μL) followed by dilution with acetonitrile (500 μL), to yield an analysis solution. This process was repeated using D-1-fluoro-2,4-dinitrophenyl-5-alanine amide (100 μL) (D-FDAA) to provide a second independent analyte. This process described above, using both D-FDAA and L-FDAA, was repeated for samples of **2**, FK506, apicidin A, rapamycin, L-pipecolic acid and the *N*-crotonyl and *N*-butanoyl amides of L-pipecolic acid. An aliquot (5 μL) of each analyte was injected onto a Zorbax StableBond C_3 HPLC column (150×4.6 mm, 5 μm) maintained at 50 °C with a 1 mL min^{-1} gradient elution profile, 85% H_2O/MeOH to 40% H_2O/MeOH, while maintaining 5% of acetonitrile and 1% formic acid (v/v) over 55 min. Individual analyte retention times were compared by co-injection with authentic standards.

MTT cytotoxicity assay: Cells were seeded in a 96-well microtiter plate at 1×10^5 to 4×10^5 cells per mL in either 100 μL of DMEM supplemented with 10% FBS (for AGS and HT-29 cells) or 100 μL of RPMI supplemented with 15% FBS (for SH-SY5Y cells), and the plate was incubated for 3–5 h (37 °C; 5% CO_2). Compounds to be tested were dissolved in DMSO (to 1 mg mL^{-1}) and diluted from 300 μM–300 nM with 10% aqueous DMSO. Aliquots (10 μL) of each dilution (or of 10% aqueous DMSO for control wells) were added the plate in duplicate and the plate was incubated for 24 h (37 °C; 5% CO_2). A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) in PBS was added to each well to a final concentration of 0.5 mg mL^{-1} and the plate was incubated for a further 4 h (37 °C; 5% CO_2). The medium was then carefully aspirated from each well and precipitated formazan crystals were dissolved in DMSO (100 μL). Finally, the absorbance of each well at 600 nm was measured spectrophotometrically.

Fluorescence measurements: Fluorescence titrations were carried out in titration buffer (50 mM HEPES pH 7.4, 100 mM NaCl, and 1 mM DTT). Protein concentration was 100 nM for each FRET partner. The EGFP-FKBP was excited at 445 nm (slit 5 nm) and the FRET to Cherry-FRB was monitored at 590 nm (slit 5 nm). The K_d value was obtained by drawing a fit to the data to binding models using the program Graphit 5.0 (Erithacul Software Ltd).

Isothermal titration calorimetry (ITC) measurements: Binding affinities of nocardiopsin A and B to EGFP-FKBP or FRB were determined using ITC. All proteins were kept in buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM DTT. Concentrations of the proteins in the syringe were at least 10-fold higher than the concentration of the ligand in the cell (usually 470 μM for EGFP-FKBP and 730 μM for FRB) in the cell. The titration experiments were carried out at 25 °C in triplicates. The heat of dilution was subtracted from the data and titration curves fitted to a 1:1 binding model (Supporting Information) using the MicroCal-ITC implementation of the Origin7 software package. EGFP-FKBP Cherry-FRB were expressed in *E. coli* BL21 codon plus RIL, purified on Ni-chelating Sepharose followed by gel filtration on Superdex 75.

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