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Nocardioazines: A Novel Bridged Diketopiperazine Scaffold from a Marine-Derived Bacterium Inhibits P-Glycoprotein

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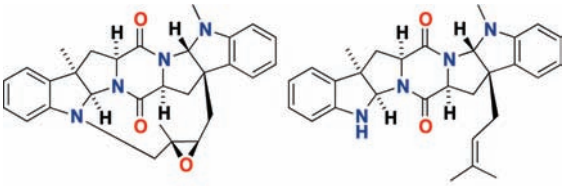
ABSTRACT



An Australian marine sediment-derived isolate, *Nocardiopsis* sp. (CMB-M0232), yielded a new class of prenylated diketopiperazine, indicative of the action of a uniquely regioselective diketopiperazine indole prenyltransferase. The bridged scaffold of nocardioazine A proved to be a noncytotoxic inhibitor of the membrane protein efflux pump P-glycoprotein, reversing doxorubicin resistance in a multidrug resistant colon cancer cell.

During our recent investigations into marine-derived bacteria isolated from coastal sediment samples, we have reported a range of new structure classes, including the dimeric diketopiperazine nasesazines A–B,[1] farnesylated nitropyrrole heronapyrroles A–C,[2] macrolactam polyene heronamides A–C[3] and polyketide macrolide nocardiopsins A–B.[4] The latter metabolites, isolated from a nonsaline liquid culture of a *Nocardiopsis* sp. (CMB-M0232) recovered from a sediment sample (−55 m) off South Molle Island, near Brisbane, Australia, were the first new natural examples of the FK506/rapamycin class of FKBP binding macrolides to be described in over a decade. In the course of that study, we also noted that saline liquid cultures of CMB-M0232 were devoid of polyketides but rich in unidentified diketopiperazines (DKPs). This report revisits that observation to reveal two unprecedented prenylated DKPs, nocardioazines A (**1**) and B (**2**), accompanied by *cyclo*-(L-Trp-L-Trp) and *cyclo*-(L-Trp-D-Trp) (Figure 1).

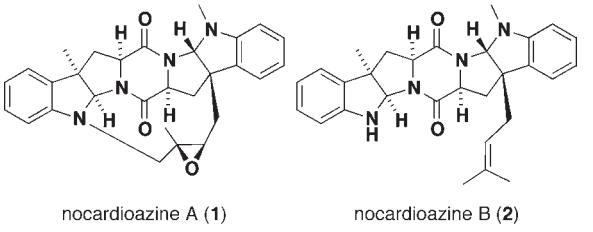


Figure 1. Nocardioazines A (**1**) and B (**2**).

An EtOAc extract obtained from a 3 L saline liquid cultivation of *Nocardiopsis* sp. (CMB-M0232) was concentrated *in vacuo* and subjected to sequential trituration to yield hexane, CH_2Cl_2 and MeOH soluble materials. Preliminary chemical analysis (HPLC-DAD-MS and ^{1}H NMR) suggested that the CH_2Cl_2 solubles (40.2 mg) were rich in DKPs,

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with reversed phase HPLC fractionation yielding nocardioazine A (**1**) (0.6 mg), nocardioazine B (**2**) (0.4 mg), *cyclo*-(L-Trp-L-Trp) (0.5 mg) and *cyclo*-(L-Trp-D-Trp) (0.5 mg). Literature comparisons[5,6] and C_3 Marfey's analysis[7] permitted identification of the latter two known DKPs, while detailed spectroscopic analysis (below) identified **1** and **2**.

HRESI(+)MS analysis of **1** returned a pseudomolecular ion $(M + H)^+$ consistent with a molecular formula ($C_{29}H_{30}N_4O_4$, Δmmu -1.0) requiring seventeen double bond equivalents (DBE). The ^{13}C NMR ($CDCl_3$) data revealed 2 ester/amide carbonyls (δ_C 169.2 and 169.8) and a further 12 sp^2 resonances (δ_C 105.5 to 148.9), accounting for 8 DBE and requiring 9 rings. Consideration of the 1D and 2D NMR data (Supporting Information, Table S1) revealed two ortho-disubstituted benzene rings [COSY correlations from H-4 to H-7 extended by HMBC correlations to C-3a and C-7a defined ring A, while COSY correlations from H-4′ to H-7′ extended by HMBC correlations to C-3a′ and C-7a′ defined ring A′] (Figure 2a). Further examination of the ^1H and COSY NMR data documented an additional 9 isolated spin systems, indicative of 3 deshielded tertiary methyls (3-Me, 1′N-Me and 3″-Me), 3 CH_2−CH moieties (H_2-8 to H-9; H_2-8′ to H-9′; H_2-1″ to H-2″), 2 methines (H-2 and H-2′), and a methylene (H_2-4″) (Figure 2A). HMBC correlations from (i) 3-Me to C-3, C-3a and C-8, (ii) H_2-8 to C-3 and C-2, (iii) H-2 to C-8 and C-9, and (iv) H-9 to C-10, defined a heterocyclic indoline system incorporating rings A, B and C, extending across N-1 (through C-2 to C-10) to N-11 (Figure 2A). Similar HMBC correlations from (i) H_2-8′ to C-3′, C-2′ and C-10′, (ii) H-2 to C-8 and C-9, and (iii) 1′N-Me to C-7a′ and C-2′, defined a heterocyclic indoline system incorporating rings A′, B′ and C′, extending across N-1′ (through C-2′ to C-10′) to N-11′.

The remaining structure fragment in **1** was determined to be an epoxy-isopentanyl, spanning the two heterocyclic indoline residues (N-1 to C-3′). Evidence for this fragment was apparent in HMBC correlations from 3″-Me to C-2″, C-3″ and C-4″, which extended the C-1″/C-2″ subunit (previously identified from the COSY data) and established the carbon framework for the isopentanyl moiety. The deshielded character of C-2″ (δ_C 62.4) and C-3″ (δ_C 61.4), combined with the need to incorporate an oxygen atom, suggested a C-2″ to C-3″ epoxide. HMBC correlations from H_2-4″ to C-2 and C-7a, and from H_2-8′ to C-1″, positioned the epoxy-isopentanyl unit as indicated, pendant to N-1 and C-3′. To accommodate the remaining DBE, the planar structure for **1** was formulated as the DKP shown in Figure 2A.

Analysis of the ROESY NMR data for **1** (Figure 2B) revealed diagnostic correlations between 3-Me, H-2 and H-9, unambiguously establishing the relative configuration about the ring A−B−C heterocyclic system. Likewise,

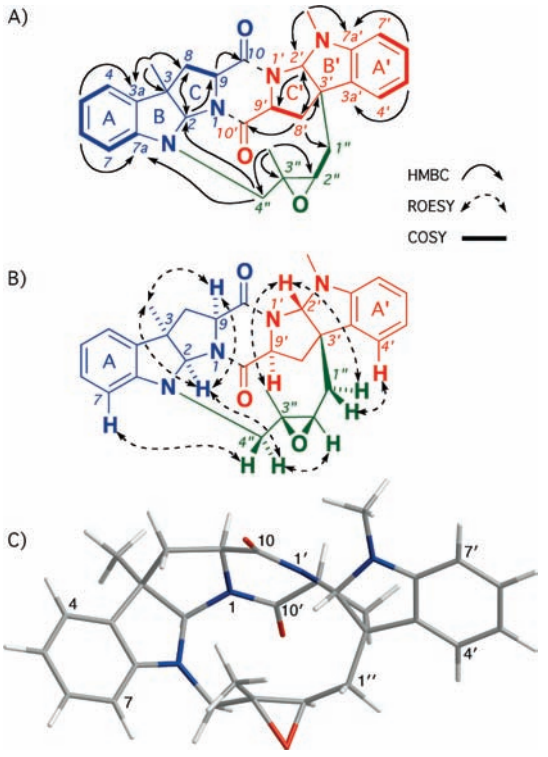

Figure 2. (A) HMBC, COSY and (B) ROESY NMR correlations and (C) energy-minimized (MM2) model of nocardioazine A (**1**).

ROESY correlations between H-2′ and H_2-1″ defined a relative configuration in which these protons occupied the same face of the ring A′−B′−C′ heterocyclic system, while the absence of a ROESY correlation between H-2′ and H-9′ was suggestive that H-9′ occupied the opposite face (contrary to the situation between H-2 and H-9). ROESY correlations between H_b-4″ and H-2″ established a *trans*-epoxide configuration, while a correlation between H-2′ and 3″-Me linked the relative configuration across the ring A′−B′−C′ heterocyclic with the epoxy-isopentanyl system as indicated (Figure 2B). Unfortunately, overlapping ^1H NMR ($CDCl_3$) chemical shifts for H-9 and H-9′ precluded consideration of a ROESY correlations, and prevented assignment of a complete relative configuration. While the ^1H NMR (C_6D_6) data for **1** (Supporting Information, Table S2) did provide adequate resolution of resonances for H-9 and H-9′, the corresponding ROESY data was crowded and ambiguous with respect to relative configuration. Fortunately, assignment of the H-9/H-9′ relative configuration in **1** was achieved by comparison to the biosynthetic precursor and cometabolite **2** (see below). The unprecedented bridged DKP scaffold proposed for **1** was further validated by an energy-minimized (MM2) 3D model (Figure 2C).

HRESI(+)MS analysis of **2** returned a pseudomolecular ion $(M + H)^+$ consistent with a molecular formula ($C_{29}H_{32}N_4O_2$, Δmmu -0.2) requiring 16 DBE. The 1D and 2D NMR ($CDCl_3$) data for **2** (Supporting

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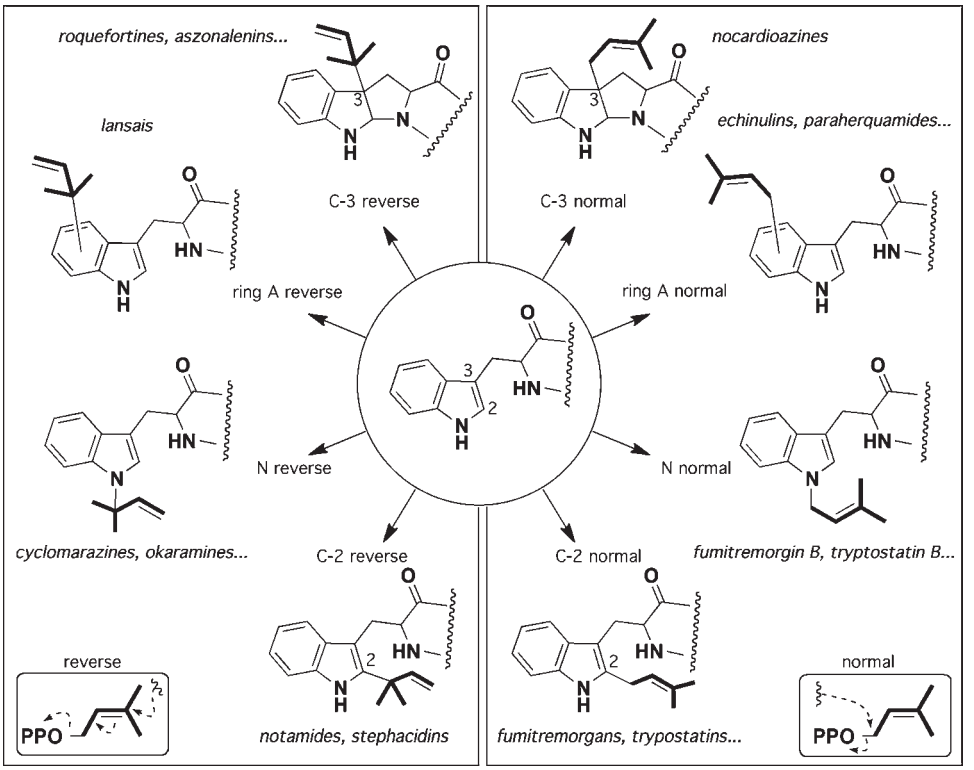

Figure 3. Indole prenylation patterns for DKPs incorporating tryptophan.

Information Table S3) indicated a bis-indoline diketopiperazine motif in common with **1**, spanning N-1 to C-10, and N-1′ to C-10′, inclusive of C-3 and N-1′ methyl substitution, and C-3′ isoprenylation. Key ^{1}H NMR differences between **2** and **1** included an H-1 (δ_H 5.18, s) resonance, as well as resonances consistent with a terminal isopentenyl moiety (δ_H 2.35, m, H_2-1″; 4.98, m, H-2″; 1.63, H_3-4″; 1.48, 3″-Me). Assembly of the complete relative stereostructure for **2** was achieved by analysis of the 2D NMR correlations (Supporting Information, Figure S12). Of particular note was a ROESY correlation between H-9 and H-9′.

With structures for the four DKP cometabolites in hand, we propose that *cyclo*-(L-Trp-L-Trp) serves as a key biosynthetic precursor, undergoing alkylation (prenylation and methylation) and cyclization to yield **2**, followed by enzymatic oxidative activation of the prenyl moiety and intramolecular cyclization (N-1 to C-4″) to yield **1** (Supporting Information, Figure S16). This biosynthetic proposal permits assignment of absolute configuration to **1** and **2**. The nocardioazines are biosynthetically noteworthy in that they represent the first C-prenylated DKPs to be reported from a marine-derived bacterium and the first C-3 *normal* prenylated DKPs to be reported from any source and provide the first example of a new class of bridged DKPs scaffold (i.e., **1**). In reviewing the natural products literature, we noted that known DKP chemical diversity include examples of *normal* and *reverse* C-2, C-3, ring-A and N indole prenylation (Figure 3), with the single exception of *normal* C-3 prenylation (unique to the nocardioazines). As fungal[8,9] and bacterial[10,11] DKP indole prenyltransferases have recently attracted attention as highly regioselective, chemically mild, green reagents for chemoenzymatic synthesis, the indole prenyltransferase in *Nocardiopsis* sp. (CMB-M0232) represents a potentially attractive chemoenzymatic reagent, that if isolated could find application in the synthesis of uniquely prenylated and bridged DKP scaffold.

The DKP motif is a valuable template employed by medicinal chemists for *de novo* drug design and development, with the choice of synthetically engineered DKP targets often being informed by knowledge of naturally occurring bioactive DKPs. In this regard, prenylated DKP natural products are well-known for their diverse and valuable properties, including the acyl-CoA:cholesterol

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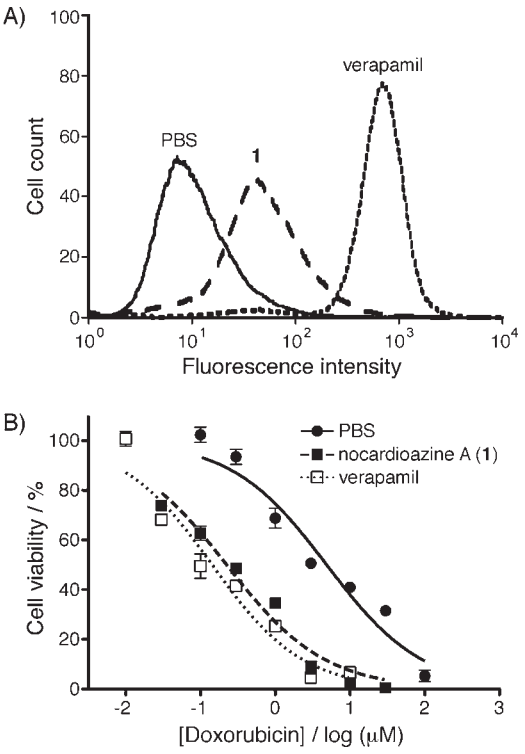

Figure 4. (A) Cell flow cytometry of calcein AM-treated P-gp overexpressing colon cancer cells (SW620 Ad300) and (B) cytotoxicity (MTT) of doxorubicin to SW620 Ad300 cells, in the presence of PBS (negative control), 20 μM nocardioazine A (1) and 10 μM verapamil (positive control).

acyltransferase (ACAT) inhibitor gypsetin,[12] the vasodilator amauromine,[13] the antiparasitic paraherquamides,[14,15] and the anticancer notoamides[16] and stephacidins.[17]

Although the nocardioazines were not cytotoxic against a selection of Gram positive and negative bacteria and human cancer cell lines (Supporting Information, Figures S17 and S18), nocardioazine A revealed a very promising biological property. The membrane efflux pump P-glycoprotein (P-gp) is overexpressed in many multidrug resistant cancer cell lines and is an important mechanism whereby cancer cell lines acquire resistance to clinically important anticancer agents. Inhibitors of P-gp represent a potential strategy to overcome this resistance, and in an effort to discover such inhibitors, we employed a P-gp overexpressing colon cancer cell line (SW620 Ad300) assay in which cancer cells were exposed to calcein AM, a nonfluorescent P-gp substrate. In the absence of an inhibitor, calcein AM undergoes rapid efflux,[18] whereas in the presence of an inhibitor, calcein AM is retained in the cell where it undergoes hydrolysis by endogenous esterases to the fluorescent dye calcein, which is not a P-gp substrate. Inhibitors are detected by an increase in intracellular calcein fluorescence as measured by cell flow cytometry. Figure 4A displays the results from such a study, which revealed **1** as a P-gp inhibitor (with the known synthetic P-gp inhibitor verapamil as the positive control). Figure 4B provides evidence that coincubation of SW620 Ad300 cells with **1** and doxorubicin, reversed resistance to doxorubicin. Cometabolite **2** did not exhibit P-gp inhibitory activity, consistent with a discrete structure activity relationship focused around the novel bridged DKP scaffold.

In conclusion, the nocardioazines provide valuable insights into new structurally and biosynthetically diverse chemical space, with the bridged DKP scaffold in **1** being representative of a new class of noncytotoxic P-gp inhibitor, capable of reversing doxorubicin resistance in a P-gp overexpressing drug resistant colon cancer cell line.

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Supporting Information Available. Full details of the collection, cultivation and taxonomy of strain CMB-M0232 and of the isolation, purification, characterization and bioassay of the nocardioazines.This material is available free of charge via the Internet at http://pubs.acs.org.

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