NOTE

Additional congeners of the macrolide neaumycin: structure revision and biological activity

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INTRODUCTION

Analysis of whole-genomic sequences of actinomycetes has consistently indicated that each strain has the potential to produce several distinct families of specialized metabolites.¹ Whereas many of these molecules are not produced under normal laboratory conditions, a strong emphasis on bioassay-guided screening might have also resulted in compounds with modest activity having been overlooked in past studies.² Re-investigation of already-described strains often leads to the detection of previously unreported metabolites,^{3,4} without the need to use special triggers to activate expression of cryptic clusters. The macrolide neaumycin (**1**) was recently isolated from the soil actinomycete *Streptomyces* sp. NEAU-x211 and reported to consist of a 30-membered macrolide featuring a unique internal diester bridge (Figure 1a).⁵ No significant antimicrobial or cytotoxic activity was detected up to 1 μ M. While investigating *Actinoplanes* sp. ATCC 33076⁶ the producer of ramoplanin, a glycolipodepsipeptide antibiotic discovered in 1984 and highly active against multidrug-resistant Gram-positive bacteria,^{7,8} we observed that the strain also produced a family of unrelated compounds, one of which was established to be identical to neaumycin (**1**; Figure 1).

ISOLATION AND STRUCTURE ELUCIDATION

Here, we report the isolation and characterization of two novel neaumycin congeners, which led to a structural revision of the parent compound. While optimizing the conditions for ramoplanin production by *Actinoplanes* sp. ATCC 33076, we observed that the strain produced, under several different growth conditions, lipophilic HPLC peaks distinct from the ramoplanin complex. Upon adjusting the HPLC conditions (see Supplementary Information), these peaks could be resolved and one of them was identical by UV and MS to **1**, hereafter designated as neaumycin A (Figure 1).⁵ NMR analysis on the purified powder confirmed the identity showing ¹H and ¹³C signals undistinguishable (Supplementary Figures S16 and S17 and Supplementary Table S4) from that of the already-published neaumycin A. The other lipophilic peaks, designated neaumycin B (**2**), C (**3**), D and E (Supplementary Figure S2), possess the same UV spectrum as **1**. After cultivating *Actinoplanes* sp. ATCC 33076 in

medium AF-1 maximal production of the neaumycin complex was observed after 96 h with an approximate titer of 7 mg l⁻¹ (Supplementary Figure S3) showing comparable amounts of **1** and **2** and lower amounts of **3**. The neaumycin complex was purified (SI) leading to the isolation of **1**, **2** and **3** in sufficient amounts for structure elucidation. Compound **2**, having retention time of 18.72 min, showed *m/z* signals at 875.6 and 897.6, corresponding to the [M+H]⁺ and [M+Na]⁺ ions, respectively, highlighting a 112-amu difference from **1**. NMR analysis of **2** highlighted the absence of signals originating from the 3-methylfumaric residue of **1**. Yet, a further dehydration must have occurred in **2** to account for the observed mass difference between **1** and **2**. Upon NMR analysis only the methine group at position 13 showed a significant change in ¹H-chemical shift, from δ 4.95 in **1** to 3.48 in **2**, as expected from the absence of esterification with the fumaric moiety; smaller changes were also observed for the protons located at the vicinal positions 11, 12 and 14. No further relevant changes in chemical shifts were observed between **1** and **2** (Supplementary Table S1). In particular, the methine group at position 27, which is esterified in structure **1a** with the fumaric residue, maintains the same high chemical shift (4.88 and 4.8 ppm in **1** and **2**, respectively), suggesting that its diagnostic low field value is actually due to a chemical environment different from fumarate esterification, whereas a clear HMBC correlation between H-27 and the carboxyl signal at 166.1 ppm indicates its esterification with position 1 (Supplementary Figure S10). Moreover, the diagnostic differences in the chemical shifts of the diastereoisomeric methylene hydrogens at positions 24, 28, 30, 31 and 32 in **1**, which indicate a zone of low flexibility, are superimposable to the corresponding signals in **2**. This observation suggests that the rigidity of this part of the molecule is not because of the annulation resulting from diesterification of the fumaric moiety, but results instead from a different element of rigidity common to both molecules. Upon MS analysis, we observed that, whereas **1** was equally detected under negative and positive ionization, **2** could only be observed by positive ionization, suggesting that the two congeners have different ionizable groups. This was confirmed by testing the chemical reactivities of **1** and **2** toward amines in the presence of a condensing agent: whereas the latter

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Figure 1 Previously published (a) and revised (b) chemical structure of neaumycins.

compound remained unchanged, **1** yielded the amidated compound **4** having m/z 1071.7 $[\text{M}+\text{H}]^+$ (Supplementary Figure S4). Thus, **1** contains a free carboxyl group and the fumaric residue must be linked to position 13 only, whereas position 27 must be involved in a rigid moiety common to **1** and **2**. On the basis of the above observations, we propose that **1** actually has the structure **1b** (Figure 1).

Accordingly, neaumycin A consists of a spiroketal-containing macrocycle with a 3-methylfumaric moiety linked to position 13. The 28-membered macrocycle is formed by esterification of the carboxyl group at position 1 with the hydroxyl at position 27, consistent with the C-27 chemical shift remaining unmodified in **2**. The spiroketal moiety accounts for the necessary dehydration, results in agreement with the C-29 chemical shift and explains the rigid system observed for the methylenes at positions 24, 28, 30, 31 and 32, which remains unaltered in **2**. Further confirmation of structure **1b** came after alkaline hydrolysis of **1**, which readily generated **2** as the first product. The other purified congener neaumycin C (**3**), with retention time of 15.79 min and m/z 861.6 for $[\text{M}+\text{H}]^+$ and 883.9 $[\text{M}+\text{Na}]^+$, resulted closely related to **2**, by comparison of the ^1H and ^{13}C NMR spectra that clearly indicate the absence of the methyl group at position 7 in **2** (Supplementary Tables S1 and S2). Whereas NMR spectroscopic data suggest that compounds **1**, **2** and **3** share the same stereochemistry, it remains to be determined whether the spiroketal-containing fragment C25–C34 has the relative configuration proposed by Shen and co-workers⁹ or whether it resembles the structurally related molecules 21-hydroxyoligomycin A, neomaclafungins¹⁰ and rutamycin.¹¹ We also observed additional congeners related to **1**, named neaumycin D and E, with signals at m/z 868 $[\text{M}+\text{Na}]^+$ and 897.8 $[\text{M}+\text{Na}]^+$ and retention times of 17.12 and 18.07 min, respectively. Their m/z values suggest that all these congeners lack the mesaconate moiety, which is probably one of the

late steps during neaumycin biosynthesis. Moreover, neaumycin D, more lipophilic and lighter for 16 amu in respect of neaumycin C, probably lacks one of the hydroxyl groups. However, the small amounts of purified congeners D and E prevented elucidation of their structures.

BIOLOGICAL ACTIVITY

Little cytotoxic activity was observed for **1** and **2** at 1 or 10 μM against two different cell lines. However, at 100 μM both compounds fully inhibited cell proliferation. This result is consistent with previous work in which no cytotoxic activity of **1** was observed at 1 μM . Compound **1** showed a modest but measurable activity against a few Gram-positive bacteria, with MICs of 64 $\mu\text{g ml}^{-1}$ observed against *Staphylococcus aureus* and *Streptococcus pneumoniae* and 16 $\mu\text{g ml}^{-1}$ against *S. pyogenes*. No activity ($\text{MIC} > 128 \mu\text{g ml}^{-1}$) was observed against these strains using **2**. Both compounds were inactive against Gram-negative bacteria ($\text{MIC} = 178 \mu\text{g ml}^{-1}$) or *Candida albicans* ($\text{MIC} > 128 \mu\text{g ml}^{-1}$). The observation that **1** and related compounds were produced, under most conditions, together with ramoplanin, prompted us to investigate whether the combination of ramoplanin with either **1** or **2** would result in enhanced antibacterial activity. We thus employed a methicillin-resistant *Staphylococcus aureus* strain in a checkerboard dilution assay. The data are reported in Figure 2. Remarkably, the presence of either **1** or **2** significantly reduced the MIC of ramoplanin in a dose-dependent manner from 4 $\mu\text{g ml}^{-1}$ in the absence of neaumycin to 0.015 $\mu\text{g ml}^{-1}$ in the presence of either 4 $\mu\text{g ml}^{-1}$ **1** or 16 $\mu\text{g ml}^{-1}$ **2**. Thus, as little as 4 $\mu\text{g ml}^{-1}$ **1** (that is, a concentration 1/16th the MIC of **1**) was able to reduce the ramoplanin MIC by two orders of magnitude. A similar behavior was observed with **2**. No enhancement of the MIC was observed when similar checkerboard assays were performed with vancomycin. After

Figure 2 Interaction of compounds **1** and **2** with ramoplanin against MRSA.

structural revision, **1** bears a strong resemblance to the macrocyclic systems found in actinomycete metabolites such as maclafungin,¹² neomaclafungins,¹⁰ oligomycin¹³ and rutamycin.¹¹ Despite this similarity, the structural originality of neomycin noticed by Shen and co-workers⁵ persists in the following features: (i) a previously unreported 28-membered, spiroketal-containing macrocycle; (ii) a conjugated system containing three, instead of the usual two double bonds; (iii) a long lateral chain at position 33 carrying a unique epoxy group; and (iv) a 3-methylfumaric residue, which separates **1** from other metabolites carrying an unsubstituted fumaric moiety, such as hygrolidin,¹⁴ AH-758,¹⁵ PD-118.576¹⁶ and bafilomycin.¹⁷ The oligomycin-type macrolides reported above display a wide variety of activities, including anticancer, antifungal, nematocidal, insecticidal and immunosuppressive effects.¹⁰ In particular, oligomycin, apotolidin, cytovaricin, ossamycin and venturicin have been widely characterized as inhibitors of oxidative phosphorylation.¹⁸ The description of an additional but unusual member might provide further insights into structure activity relationships within this class of macrocycles. As a further advancement to the previous study,⁵ we also report that **1** is produced as a complex of related congeners, one of which has been characterized as likely biosynthetic precursors of neomycin lacking the fumarate moiety. Finally, we have shown that a modest antibacterial or cytotoxic activity can be observed for **1** and **2**. The significant enhancement of ramoplanin activity in the presence of either **1** or **2** is striking. However, as this enhancement is seen with a large molar excess of **1** or **2**, we refrain from considering it a synergistic effect. Further investigations will be required to establish the nature of this enhancement. We would like to make one additional observation from the present study. Because of its potent antibacterial activity, ramoplanin has been investigated as a potential drug for many years, reaching a phase 3 clinical study for the prevention of infections by vancomycin-resistant enterococci.¹⁹ Consequently, the *Actinoplanes*

ATCC 33076 strain has been thoroughly investigated in industrial laboratories. Nonetheless, the neomycin complex must have been systematically overlooked or deemed devoid of interest over all those years.^{6,20,21} We believe that this is not an exception and new compounds can be identified also by examining other 'thoroughly investigated' strains.

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CONFLICT OF INTEREST

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