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ORIGINAL ARTICLE

Isolation and characterization of NAI-802, a new lantibiotic produced by two different *Actinoplanes* strains

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Lantibiotics are biologically active peptides produced by Gram-positive bacteria. Starting from fermentation broth extracts preselected from a high-throughput screening program for discovering cell-wall inhibitors, we successfully isolated a new lantibiotic produced by *Actinoplanes* sp., designated as NAI-802. MS and NMR analysis together with explorative chemistry established that NAI-802 consists of 21 amino acids, 19 of which are identical to those present in the class II lantibiotic actagardine. Interestingly, NAI-802 carries one extra alanine and one extra arginine at the N- and C-termini, respectively. As expected from the overall higher positive charge, NAI-802 was slightly more active than actagardine against staphylococci and streptococci. Further improvement of its antibacterial activity was achieved by adding one additional positive charge through conversion of the C-terminal carboxylate into the corresponding basic amide. NAI-802 thus represents a novel promising candidate for treating Gram-positive infections caused by multidrug-resistant pathogens.
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INTRODUCTION

The lantibiotic NAI-107 (previously known as 107891 or microbisporicin), produced by *Microbispora* sp.,[1] displays potent activity against most Gram-positive pathogens of medical importance[2] and is effective in experimental models of infection.[3] This compound was isolated after high-throughput screening (HTS) of ca. 120 000 microbial broth extracts through a program for identification of compounds active on bacterial cell wall. Unexpectedly, this assay system turned out to significantly enrich for lantibiotics.[4] We thus decided to further investigate the samples positive in that screening program and report here the isolation, structure elucidation and preliminary biological profiling of a new lantibiotic, designated NAI-802.

Our proprietary Natural Products Library database contains information regarding strains, their growth conditions (media, growth temperatures and harvest times) and extracts (preparation procedure). It also reports data obtained with extracts in the different HTS programs run at Biosearch Italia/Vicuron Pharmaceuticals in the 1996–2006 period. Overall, it covers about 70 000 strains, 160 000 extracts and over 20 HTS programs. Details of the screening project for identification of compounds active on bacterial cell wall have been described elsewhere.[4] Briefly, the program involved the HTS microbial extracts to identify those able to inhibit *Staphylococcus aureus* growth but ineffective against its isogenic L-forms (protoplast-type cells that are able to replicate under appropriate osmotic conditions despite the lack of a functional cell wall and are thus insensitive to peptidoglycan synthesis inhibitors). Only those extracts were further retained whose activity against *S. aureus* was not affected by incubation with β-lactamases or with excess ε-amino-caproyl-D-alanyl-D-alanine. The extracts generated from the actinomycete strains IDs 104802 and 104771 were among those fulfilling the screening selection criteria.

RESULTS

Taxonomy of the producing strains

We report here on the analysis of the actinomycete strains reported as ID104802 and ID104771, and classified as *Actinoplanes* on the basis of morphological features and formation of motile spores. *Actinoplanes* strains ID104802 and ID104771 were deposited as *Actinoplanes* sp. DSM24057 and DSM25201, respectively, in Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). When grown on S1 medium, both strains formed dark-orange colonies with whitish aerial mycelium. A brown–green pigment was released in the medium upon ageing of the cultures. Nearly complete sequences (1439 bp) of the 16 S ribosomal RNA gene showed that the two strains are closely related having almost identical sequences (>99.9%). Comparison of the sequences with those maintained in GenBank showed >98% identity with the 16 S ribosomal RNA gene sequences of several *Actinoplanes* species (highest identity with *A. teichomyceticus*), thus confirming the original classification.

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Accession numbers for the 16 S sequences of strains ID104771 and ID104802 are JX680596 and JX680597, respectively.

Fermentation, isolation and purification of NAI-802

For metabolite analysis, the strains were grown in M8 medium for 168 h and the corresponding cultures assayed for antimicrobial activity. After HPLC fractionations of the broth filtrates, both strains yielded a single fraction endowed with activity against *Bacillus subtilis* 168, eluting at 17.6 min. Upon HR-MS, the 17.6-min fraction from both strains showed a double protonated ion at *m/z* 1058.9707, together with a triple protonated ion at *m/z* 706.3151, thus corresponding to a molecular mass of 2116 a.m.u. and suggesting a molecular formula of $C_{90}H_{141}N_{25}O_{26}S_4$. No matches were found between this mass and known molecules reported in the proprietary ABL database, which contains UV properties and mass data on ~29 000 microbial metabolites published up to 2006.[5] Further experiments using the production media M8 and KC4 indicated that the 2116 a.m.u. compound was detectable after about 48 h, reaching maximum production after 72–96 or 96–120 h in media KC4 and M8, respectively, for both strains. Production by both strains in medium KC4 was ca. three times higher than in M8, as estimated from the relative area of the HPLC peaks. After growing strain ID104802 in KC4 medium for 72 h in a 20-l fermentor, the culture was harvested and the mycelium was separated from the supernatant broth by filtration. HPLC analysis showed that the mass 2116 peak was equally distributed between the broth filtrate and the mycelium, and so was the activity against *B. subtilis* 168. The work-up procedure involved methanol extraction of the mycelium and HP20 resin adsorption of the broth filtrate, followed by reversed-phase chromatography, as described under Experimental Procedure. This procedure allowed the recovery and purification of pure NAI-802 **1**. During the recovery process, we also observed minor amounts of a related congener designated Ala(0)-NAI-802 **2**, as described below.

Structure determination and physico-chemical properties

The structure of **1** (Figure 1) was elucidated by spectroscopic analysis and chemical studies. Its UV spectrum exhibits two shoulders at 225 and 280 nm, as usually found in peptides, while its ^{1}H NMR spectrum displayed a typical peptide pattern (Supplementary Figure 1). The correlation of ^{1}H NMR signals to the corresponding ^{13}C-carbon atoms was carried out in a HSQC NMR experiment, followed by analysis of the COSY and HMBC spectra. These data indicated the absence of dehydrated residues such as dehydroalanine or dehydrobutyrine, while the presence of a spin system attributed to one Trp residue could be easily established by NOE and HMBC contacts from the β-methylene group to the indole ring. The presence of one Leu, two Val and two Ile residues could be readily assigned by their characteristic ^{1}H and ^{13}C chemical shifts, while the presence of one Ser was assigned by the characteristic ^{13}C of the β-methylene group in the HSQC spectrum. One Arg was clearly identified from its particular spin system. As observed for actagardine,[6] the HSQC spectra revealed unusual ^{13}C chemical shifts of the β- and γ-carbons of one of the β-methyl-lanthionine (Me-Lan) bridges, suggesting the presence of one oxidized Me-Lan bridge in **1**. To partially assign the peptide sequence, we investigated short-range NOESY cross peaks between the CH_α, CH_β or amide proton of residue *i* and the amide proton of the adjacent *i+1* residue in the peptide sequence, as described.[7] Accordingly, we were able to sequentially assign part of the spin systems establishing an overall sequence identity with actagardine while the C-terminal position of the Arg residue in **1** was highlighted by the NOESY correlation between Arg-NH at 8.29 and Lan-20 CH_α at 5.14. The assignments of protons and carbons in the NMR spectra of NAI-802 are summarized in Table 1.

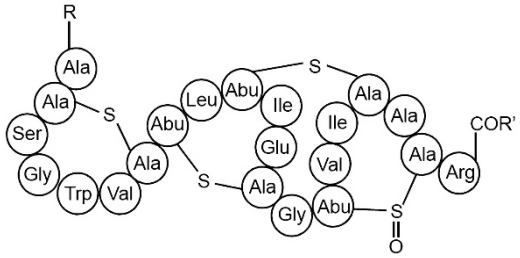

Figure 1 Structures of NAI-802 and related compounds: NAI-802 (R = H, R′ = OH) (**1**); Ala (0)-NAI-802 (R = Alanine, R′ = OH) (**2**); NAI-802 monoamide with ethylenediamine (R = H, R′ = $NHCH_2CH_2NH_2$) (**3**).

MS analyses were performed on the hydrolyzed and intact compound. Analysis of the acid hydrolysate showed the presence of Lan(1), Me-Lan (3), Ala (2), Arg (1), Gly (2), Ser (1), Trp (1), Val (2), Glu (1), Leu (1) and Ile (2), consistent with NMR analyses. The HR-mass spectrum of intact **1** showed the presence of two intense signals at *m/z* 1058.9707 and 706.3151 corresponding to the double- $[M+2H]^{2+}$ and triple- $[M+3H]^{3+}$ charged ions, respectively. Upon MS/MS fragmentation of the *m/z* 1059 $[M+2H]^{2+}$ ion at several collision energy values, the double protonated ion at *m/z* 1050, resulting from a single dehydration, was initially formed, followed by two major monoprotonated ions at *m/z* 673 and 1445, consistent with residues 1–7 and 8–21 of the assigned structure (Table 2). Ions at *m/z* 870 and 1248, consistent with residues 1–9 and 10–21, were also formed, together with ions *m/z* 1856 and 1786, consistent with dehydrated residues 1–19 and 1–18 (or 2–19), respectively (Table 2), thus confirming the C-terminal position of the Arg residue. The structure established by spectroscopic means was confirmed by selected chemical analyses. Edman degradation of **1** established Ala as the N-terminal residue. Reaction with ethanethiol at neutral pH excluded the presence of dehydrobutyrine, dehydroalanine or disulfide bridges,[8] whereas the same reaction at alkaline pH suggested the presence of four Lan bridges from the addition of four mercaptoethanol moieties to **1**[9] after reduction of the sulfoxide, which in turn is expected to occur under alkaline conditions as previously reported for actagardine.[6] Moreover, two carboxylic acids, associated with the single Glu and the C-terminal Arg residues, were detected following amidation of **1** with benzylamine and isolation of the resulting diamide derivative. However, when ethylendiamine replaced benzylamine, the amidation reaction occurred regioselectively at the C-terminus, yielding the monoamidated derivative **3**, as indicated by the observation of two intense signals at *m/z* 1080 and 720, corresponding to the double $[M+2H]^{2+}$ and triple $[M+3H]^{3+}$ charged ions, respectively, of **3** (Figure 2 Supplementary data) and by the fragmentation pattern (Table 2). Careful inspection by LC-MS of partially purified samples of **1** highlighted the presence of an impurity coeluting with **1** at 17.6 min. Its HR-mass (*m/z* 1094.4889 $[M+2H]^{2+}$) was consistent with the formula $C_{93}H_{146}N_{26}O_{27}S_4$ and the presence of an additional Ala residue, while the MS/MS fragmentation yielded major peaks at m/z 745-1445 and 942-1248 (Table 2). These results suggested that this impurity is actually a minor congener of **1** carrying an extra Ala at the N-terminus, and was thus designated Ala(0)-NAI-802 **2**. Both *Actinoplanes* sp. ID104802 and *Actinoplanes* sp. ID104771 produced **1** and **2** in an approximate ratio of 20:1. An Ala(0)-congener has also been reported for actagardine.[10]

Table 1 ^{13}C and ^{1}H NMR chemical shift data for 1

	Residues	*NH*	*Hα*	*Hβ*	*Hβ′*	*Others*
^{1}H chemical shifts						
1	Ala	—	4.45	1.8	—	
2	Lan	9.10	4.9	3.25	3.5	
3	Ser	8.61	4.7	4.1	—	
4	Gly	8.59	3.92; 4.34	—	—	
5	Trp	8.27	4.88	3.59	3.49	2H 7.52; 4H 7.91; 5H 7.36; 6H 7.44; 7H 7.70
6	Val	7.75	4.5	2.31	—	γ 1.04; γ 1.09
7	Lan	8.39	5.01	3.05	3.25	
8	Abu		5.16	4.05	—	γ 1.50
9	Leu		4.75	1.8	1.97	γ 1.75; δ 1.12; δ′ 1.16
10	Abu	8.41	4.88	4.1	—	γ 1.66
11	Ile		4.42	2.1	—	γ—CH_3 1.20; γ,γ′ 1.44,1.84; δ 1.13
12	Glu	9.25	4.47	2.35	2.55	γ,γ′ 2.75
13	Lan	7.92	5.17	2.99	3.46	
14	Gly	8.23	4.13	—	—	
15	Abu	8.72	4.99	3.69	—	γ 1.50
16	Val	8.49	4.35	2.29	—	γ 1.21; γ 1.25
17	Ile		3.82	2.33	—	γ—CH_3 1.20; γ,γ′ 1.55,1.63; δ 1.18
18	Lan	8.10	4.93	3.54	(3.25)	
19	Ala	8.50	4.37	1.63	—	
20	Lan	8.77	5.14	3.5		
21	Arg	8.29	4.72	2.12	1.97	γ,γ′ 1.84; δ,δ′ 3.43; NH chain 7.48
	Residues	*C′*	*Cα*	*Cβ*	*Cγ*	*Others*
^{13}C chemical shifts						
1	Ala		49.66	17.14		
2	Lan		53.54	33.8		
3	Ser		55.97	61.3		
4	Gly		43.2	—		
5	Trp		55.32	27.66		C2 124.72; C4 118.89; C5 119.54; C6 122.13; C7 112.1
6	Val		58.88	30.89	18.92;17.95	
7	Lan		53.54	33.97		
8	Abu		57.42	44.64	20.38	
9	Leu		53.38	40.92	25.07	δ 21.51; δ′ 22.8
10	Abu		59.04	43.83	19.08	
11	Ile		54.83	37.2	15.36	γ′ 25.55; δ 10.99
12	Glu		54.35	24.91	31.38	
13	Lan		55	34.42		
14	Gly		44	—		
15	Abu		54.83	56.34	6.95	
16	Val		61.63	30.9	18.92;15.36	
17	Ile		60.76	35.58	15.36	γ′ 29.6; δ 10.51
18	Lan		51.76	33.64		
19	Ala		50.95	16.33		
20	Lan		48.2	51.92		
21	Arg		53.38	29.11	25.07	δ 41.08

Biological properties

The *in vitro* activities of **1** and **3** against a panel of microbial strains is reported in Table 3. **1** was active against staphylococci and streptococci, with MICs ranging from 0.5 to 32 μg ml^{-1}, while the MICs against *Enterococcus* sp. were 128 μg ml^{-1} or higher. The compound was not active against *Escherichia coli* or *Candida albicans*. Overall, **1** showed a better activity than actagardine, with MIC values consistently 2–4 fold lower against *Staphylococcus* and *Streptococcus* sp. Compound **3** exhibited an improved antimicrobial activity, with MIC values reduced 4–8 fold in comparison to **1**, leading also to

Table 2 MS/MS fragmentation patterns of 1, 2 and 3

	Peptide segment					
Compound	*1–7*	*1–9*	*Dehydrated 1–18 or 2–19*	*Dehydrated 1–19*	*8–21*	*10–21*
1	673	870	1786	1856	1445	1248
2	745	942	1858	1927	1445	1248
3	673	ND	1786	1858	1488	1291

Numbers refer to the *m/z* values for the $[M+H]^+$ ions.

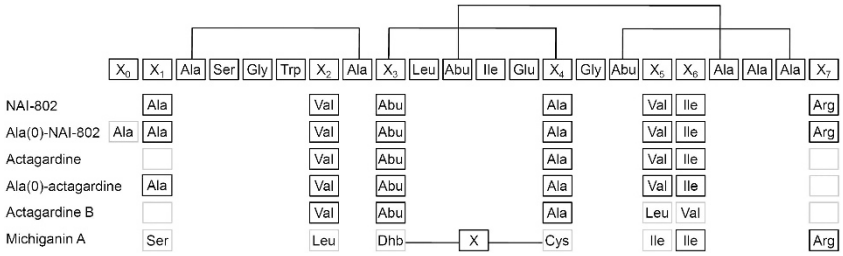

Figure 2 Comparison of the peptide sequences of **1**, **2**, actagardine, Ala(0)-actagardine, actagardine B and michiganin A. Thioether bridges are indicated by a black line. The symbol 'X' denotes lack of a thioether. For simplicity, sulfoxides are not illustrated. Gray lines denote differences from NAI-802.

Table 3 MICs ($\mu g\,ml^{-1}$) against aerobic bacteria

	Compound no.		*Compounds*	
Microrganism[a]	***1***	***3***	*Actagardine*	*Vancomycin*
S. aureus Met^S ATCC 6538P	8	4	32	0.25
+30% bovine serum	32	4	64	1
S. aureus Met^S ATCC 19636	16	2	32	0.5
+30% bovine serum	32	4	64	1
S. aureus Met^R L1400	32	8	64	0.5
S. pyogenes SKF13400	0.5	⩽0.125	2	0.25
S. pneumoniae L44	8	2	16	0.5
E. faecium VanS L568	>128	32	128	2
+30% bovine serum	>128	32	>128	4
E. faecium VanA L569	>128	16	128	>128
E. faecalis VanS L559	128	32	128	1
+30% bovine serum	128	32	128	2
E. faecalis VanA L560	128	32	128	>128
E. coli ATCC 25922	>128	>128	>128	>128
C. albicans ATCC 90028	>128	>128	>128	>128

[a]All strains except Met^S *S. aureus* and *S. pyogenes* came from the NAICONS collection and are indicated by an L-prefix.

Table 4 MICs ($\mu g\,ml^{-1}$) against anaerobic bacteria

	Compounds		
Microrganism[a]	***1***	***3***	*Vancomycin*
C. difficile L4013	0.25	⩽0.125	0.25
C. difficile L1365	1	0.25	0.5
C. difficile L4015	2	1	0.25
C. butyricum L4008	0.25	1	1
C. perfringens L4053	2	4	1
C. perfringens L3607	2	8	1
Peptostreptococcus asaccharolyticus L521	1	0.5	0.5
Bacteroides fragilis ATCC 25285	>128	>128	32

[a]All strains except *B. fragilis* came from the NAICONS collection and are indicated by an L-prefix.

measurable MICs against enterococci. When measurable, the MICs of **1**, **3** and actagardine were not significantly affected by the presence of 30% bovine serum in the media or by the resistant phenotypes of the strains. Compounds **1** and **3** showed a significant activity also against Gram-positive anaerobic bacteria, such as *Clostridium difficile*, *C. butyricum, C. perfringens* and *Peptostreptococcus asaccharolyticus* with MIC ranges of 0.25–2 and $\leqslant 8\,\mu g\,ml^{-1}$ for **1** and **3**, respectively. These MIC values are comparable to vancomycin's (Table 4). As expected, no activity was observed against the Gram-negative anaerobe *Bacteroides fragilis*. It should be noted that, while **3** was 2–4 times more active than **1** against the three *C. difficile* strains, it was 2–4 less active against the three other tested *Clostridium* spp. (Table 4).

DISCUSSION

In the last two decades the ribosomally synthesized lantibiotics have aroused interest for their antibacterial activity, particularly against the worrisome methicillin-resistant *S. aureus*.[11,12] Approximately 60 lantibiotics, mostly produced by strains belonging to the phylum *Firmicutes*, have been discovered since 1927 when the first representative, nisin, was discovered,[13] but relatively few from the *Actinobacteria*, possibly because of the abundance of many other classes of lower MW antibiotics produced by this phylum. Recent investigations are, however, demonstrating that lantipeptides (Lan-containing peptides called lantibiotics when they posses antibiotic activity) can be frequently found in other bacterial phyla, including the *Actinobacteria*, expanding the chemical diversity and the biological activities of this family of ribosomally synthesized peptides.[14] In the present study, we show that two *Actinoplanes* strains, ID104802 and ID104771, produce the new lantibiotic NAI-802 **1**, along with minor amounts of its Ala(0)-congener **2**. Compound **1** is highly related to the class II lantibiotic actagardine and thus represents a further addition in this family (Figure 2), which includes: actagardine and Ala(0)-actagardine, produced by *Actinoplanes liguriae* ATCC 31048;[15] actagardine B, produced by *A. liguriae* NCIMB41362;[16] michiganin A, produced by *Clavibacter michiganensis*;[17] NAI-802 and Ala(0)-NAI-802, as described here. All these lantibiotics share three conserved thioether bridges within a core 19-aa peptide consisting of 14 invariant residues. Actually, the number of invariant residues would be 16 if those present in the precursor peptide were taken into account, as michiganin lacks one of the central Lan bridges despite possessing the amino-acid residues (dehydrobutyrine and Cys) required for its formation (Figure 2). Among the natural variants described so far, variable positions accommodate hydrophobic residues only. However, many positions can be manipulated by appropriately engineering the structural gene encoding the precursor peptide of actagardine in the producing strain.[16] Despite the high conservation of the central core in the natural variants, there appears to be some flexibility at the N- and C-terminal ends of this lantibiotic family. This feature, which had been adumbrated by the discovery of Ala(0)-actagardine,[10] became apparent with the discovery of michiganin A, which carries extra Ser and Arg residues at the N- and C-termini, respectively,[17,18] and has further been confirmed in this study with **1**, where Ala and Arg are found, respectively, and its Ala(0)-congener **2**. It remains to be determined whether N- and/or C-terminally extended variants could be easily generated by appropriately engineering the actagardine structural gene. Considering its high structural similarity to

actagardine, **1** is likely to exert its antimicrobial action through a similar mechanism by binding to the peptidoglycan intermediate lipid II, as does actagardine.[19] A conserved Glu residue (Figure 2) is present in many class II lantibiotics (such as mersacidin, lacticin 481, lacticin 3147 A1 and haloduracin-α) and has been demonstrated to be essential for antibiotic activity in mersacidin.[20] In comparison with actagardine, **1** carries one additional positive charge at physiological pH due to the C-terminal Arg, a feature that might explain its improved antimicrobial activity (Table 3). Positively charged residues have indeed been suggested to have an important role in the interaction of lantibiotics with the negatively charged bacterial membranes.[20] The antibacterial activity of actagardine was improved after transforming the negatively charged C-terminal carboxylate into a positively charged basic amide.[21] The same result is observed here with the basic amide derivative **3**, indicating an additive effect on antimicrobial activity of the basic amide and of the Arg side chain. Compound **3** resulted particularly active against *Streptococcus* sp. It is worth recalling that, despite the presence of two carboxylates, only the C-terminus reacted with a diamine, giving the corresponding monoamide. An identical behavior was observed with actagardine under similar conditions.[21] Apparently, di-carboxamide derivatives of actagardine and **1** can be obtained only with a monoamine but not with a diamine, even under a stechiometric excess of reactants (data not shown). Those outcomes suggest that a different conformation of NAI-802, depending on the reactive amine, allows or prevents reaction on the glutamic residue. Yet deeper studies should be necessary to clarify the reason of this behavior.

In conclusion, NAI-802 and its derivative **3** obtained by regio-selective amidation represent promising class II lantibiotics for treating infections caused by multidrug-resistan Gram-positive pathogens. Indeed, a related compound NVB302, developed for treating *C. difficile*-associate diarrhaea, is undergoing formal preclinical studies.[22]

EXPERIMENTAL PROCEDURE

General experimental conditions

HPLC chromatography employed a Shimadzu Series 10 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), equipped with a Lichrosphere C18 4.6×100 mm column (Merck, Darmstadt, Germany) and a diode-array detector (190–800 nm), using a linear gradient of 0.05 M ammonium formate-acetonitrile (from 5 to 90% of organic phase in 30 min) at a flow rate of 1 ml min^{-1}. ESI-MS data were recorded on an Ion Trap ESQUIRE 3000 Plus spectrometer (Bruker, Karlsruhe, Germany) equipped with an LC Agilent 1100 Diode-Array Detector (Agilent Technologies, Waldbronn, Germany), using an Ascentis express Supelco RP18, 2.7 μ (50×4.6 mm) column kept at 40 °C, eluting at 1 ml min^{-1} with a 7-min linear gradient from 95:5 phase A (0.05% (v/v) trifluoroacetic acid in water):phase B (0.05% (v/v) trifluoroacetic acid in acetonitrile) to 100% phase B. HR-MS spectra were acquired in fourier transform mode from 400 to 2500 *m/z* (high-mass range mode, 1×10^6 resolution, automatic gain control (AGC) scan target 5×10^5). Spectra acquired during 30 min were averaged. Samples were infused by syringe at 8 μl min^{-1} into a LTQ XL Orbitrap (Thermo, West Palm Beach, FL, USA) interfaced with an ESI source. ^1H-,^{13}C-, 1- and 2-D NMR spectra (COSY, TOCSY, NOESY, HSQC, HMBC) were measured in CD_3CN-D_2O at 25 °C using an AMX 600 MHz spectrometer (Bruker). Chemical shifts are reported relative to D_2O (δ 4.79) and MeCN. Sequencing of the 16S ribosomal RNA gene was performed following published procedures.[23]

Actinomycete strains and growth conditions

Actinoplanes strains ID104802 and ID104771 were grown on S1 plates for 10–15 days at 30 °C. S1 (oatmeal, 60 g l^{-1}; agar, 18 g l^{-1}; $FeSO_4 \times 7$ H_2O, 0.001 g l^{-1}; $MnCl_2 \times 4$ H_2O, 0.001 g l^{-1}; $ZnSO_4 \times 7$ H_2O, 0.001 g l^{-1}) was prepared by boiling oatmeal in 1 l distilled water for 20 min, filtering it through cheesecloth, adding the remaining components adjusting volume to 1 l with distilled water and pH to 7.2 with 0.5 M NaOH before sterilization at 121 °C for 20 min. For metabolite production, the microbial strain was scraped from a 90-mm agar plate and inoculated into a 500 ml Erlenmeyer flask containing 100 ml of seed medium (dextrose monohydrate, 20 g l^{-1}; yeast extract, 2 g l^{-1}; soybean meal, 8 g l^{-1}; NaCl, 1 g l^{-1}; calcium carbonate, 4 g l^{-1}; pH 7.3 by 0.5 M NaOH), which was incubated at 30 °C in an orbital shaker set at 200 r.p.m. After 48–72 h, 5 ml of the culture were transferred into 100 ml of fresh medium in a new 500-ml flask. After further 48 h, a 5% inoculum was made into production media M8 (soluble starch, 20 g l^{-1}; glucose, 10 g l^{-1}; yeast extract, 2 g l^{-1}; casein hydrolysate, 4 g l^{-1}; meat extract, 4 g l^{-1}; $CaCO_3$, 3 g l^{-1}; pH 7.2 with 0.5 M NaOH) or KC4 (Pharmamedia) (Traders Protein, Memphis, TN, USA), 30 g l^{-1}; maize dextrin, 40 g l^{-1}; yeast extract, 5 g l^{-1}; glucose monohydrate, 10 g l^{-1}; $CaCO_3$, 2 g l^{-1}; NaCl, 1 g l^{-1}; pH 7.0 with 0.5 M NaOH). For bioreactor experiments a 20-l BioFlo 415 fermenter (New Brunswick Scientific, Enfield, CT, USA) was used, containing 15 l of production medium KC4, with 400 r.p.m. agitation and 0.4-v.v.m. aeration at a temperature of 30 °C. For monitoring metabolite production, methanol extracts were prepared by withdrawing 0.45 ml of culture, thoroughly mixing it with 2 vol methanol: acetic acid 92:8 for 1 h at 50 °C and 1400 r.p.m., followed by centrifugation. The resulting methanol extract was either assayed by HPLC, as described above, or dried for antimicrobial assays, as described below.

Metabolite purification

A 15-l, 72 h culture of strain ID104802 in KC4 medium was filtered through a paper filter and the collected 3 l of mycelium was treated with one volume methanol. The resulting organic phase was concentrated under vacuum to about 1 l and then shaken with 113 ml Diaion HP20 resin (Supelco, Bellefonte, PA, USA) for 3 h at 30 °C. After washing twice with 500 ml 35% MeOH, the resin was eluted with 1.5 l 90% MeOH. After evaporating under vacuum, samples were resuspended in 5 ml of H_2O-dimethylformamide 1:1 and purified by reversed-phase medium pressure liquid chromatography on a Combiflash system (Teledyne ISCO, Lincoln, NE, USA), using an RP18 86 g column, a 20-min linear gradient of 0.05 M ammonium formate-MeOH (from 35 to 95% eluent B) at 60 ml min^{-1} with a 214-nm detection wavelength. Fractions eluted with 85% eluent B, which exhibited activity against *S. aureus* ATCC 6538P, were combined and, after removing methanol under vacuum, were lyophilized, affording 907 mg **1**. The broth filtrate obtained from the fermentation run was also absorbed onto the HP20 resin (using 10 g l^{-1}) and processed as above, yielding 568 mg **1**.

Chemistry

Edman degradation: One milligram of **1** was dissolved in 500 μl of 1 M Na_2CO_3 (pH 8) and phenylisothiocyanate (1 μl) was added. The reaction was stirred at 60 °C for 1 h before MS analysis. Amino acid composition analysis: **1** (3 mg) was hydrolyzed in 1 ml 6 M HCl at 160 °C for 5 min under microwave irradiation. The hydrolyzed sample was evaporated to dryness, resuspended in 1 ml H_2O-acetonitrile 1:1 and treated with 4-(4-isothiocyanate-phenyl)-azo-*N*,*N*-dimethyl aniline (5 mg) and Triethylamine (6 μl). The reaction mixture was stirred for 2 h at 60 °C and then extracted twice with petroleum ether: dichloromethane 8:2 (3 ml). The organic phase was evaporated to dryness, redissolved in 1 ml water-acetonitrile 1:1 and analyzed by HPLC-MS. Ethanethiol derivatization (alkaline pH):[9] One milligram **1** was dissolved in 200 μl dimethylformamide and then 200 μl of the derivatization mixture were added (780 μl ethanol, 560 μl water, 180 μl of 5 M NaOH and 168 μl ethanethiol). The mixture was kept for 1 h at 60 °C and then directly analyzed by LC-MS. Ethanethiol derivatization (neutral pH):[8] 1 mg **1** was dissolved in acetonitrile - 0.1 M ammonium acetate 1:1 (800 μl) and then 3 μl of ethanethiol were added. The mixture was kept for 1 h at 60 °C and then directly analyzed by LC-MS. Amidation with benzylamine: to a stirred solution of 2 mg **1** in 1 ml dimethylformamide, 2 μl of benzylamine (20 Eq.) and 9.4 mg PyBOP (20 Eq.) were added; the reaction was kept under stirring for 20 min at room temperature before LC-MS analysis. Amidation with ethylenediamine: to a stirred solution of 30 mg **1** in 2 ml dimethylformamide, 5.5 μl of ethylendiamine (6 Eq.) and 16.5 mg of PyBOP (2 Eq.) were added and the reaction was kept under stirring for 20 min at room temperature. The reaction product was purified on a 4.3 g reverse-phase C18 RediSep Column (Teledyne

ISCO) by using the Combiflash System. The resin was eluted at 18 ml min^{-1} with a linear gradient from 5 to 90% of phase B in 18 min. Phase A was 0.05 M ammonium formate whereas phase B was acetonitrile. After LC-MS analysis fractions containing the monoamide were pooled, concentrated under vacuum and lyophilized, yielding 12 mg of **3**.

Physico-chemical properties

NAI-802 (**1**): white powder, UV (in $CH_3CN:H_2O = 50:50$ with 0.05 % of trifluoroacetic acid) 225, 280 nm. ^{1}H- and ^{13}C-NMR (600 MHz, CD_3CN-D_2O) (Table 1 and Supplementary information). (+)-HR-ESI MS (*m/z*) 1058.9707 $[M+2H]^{2+}$, 706.3151 $[M+3H]^{3+}$ calculated for $C_{90}H_{143}N_{25}O_{26}S_4$ 1058.9754 $[M+2H]^{2+}$. MS/MS fragmentation (Table 2).

Ala(0) NAI-802 (**2**): white powder, UV (in $CH_3CN:H_2O = 50:50$ with 0.05 % of trifluoroacetic acid) 225, 280 nm. (+)HR-ESI MS (*m/z*) 1094.4889 $[M+2H]^{2+}$ 729.9937 $[M+3H]^{3+}$ calculated for $C_{93}H_{148}N_{26}O_{27}S_4$ 1094.4940 $[M+2H]^{2+}$. MS/MS fragmentation (Table 2).

NAI-802 monoamide with ethylenediamine (**3**): white powder, UV (in $CH_3CN:H_2O = 50:50$ with 0.1 % of trifluoroacetic acid) 225, 280 nm. (+)HR-ESI MS (*m/z*) 1080.0120 $[M+2H]^{2+}$, 720.3453 $[M+3H]^{3+}$ calculated for $C_{92}H_{149}H_{27}O_{25}S_4$ 1080.0045 $[M+2H]^{2+}$. MS/MS fragmentation (Table 2).

Antimicrobial assays

After drying, methanol extracts or purified fractions were resuspended in 100 µl 10% dimethyl sulfoxide and 20 µl were deposited on 4-mm thick Müller Hinton Agar (Difco Laboratories, Detroit, MI, USA) plates, inoculated with 10^5 c.f.u. ml^{-1} *S. aureus* ATCC 6538P. Plates were incubated overnight at 37 °C before scoring inhibition halos.

MIC assays were performed by the broth microdilution methodology in sterile 96-well microtiter plates according to Clinical and Laboratory Standards Institute (CLSI) guidelines;[24] media used were Müller Hinton Broth containing 20 mg l^{-1} $CaCl_2$ and 10 mg l^{-1} $MgCl_2$ for all strains, except for *Streptococcus* spp. and *C. albicans*, which were grown in Todd Hewitt Broth (Difco Laboratories) and in RPMI 1640 (Sigma Aldrich, St Louis, MO, USA) supplemented with 0.165 M MOPS (pH 7), respectively. When indicated, bovine serum was added to the medium at 30% (w/v). Bacteria were inoculated at 5×10^5 c.f.u. ml^{-1}, while *C. albicans* at 5×10^4 c.f.u. ml^{-1}. After 24-h incubation (48 h for *C. albicans*) at 37 °C, MIC was defined as the lowest drug concentration causing complete suppression of visible growth. Compounds were dissolved in dimethyl sulfoxide except for vancomycin, which was dissolved in water. Appropriate dilutions were made with the required culture medium immediately before testing.

MICs for anaerobic bacteria were determined by the broth dilution method in Brucella broth supplemented with 5 µg ml^{-1} hemin, 1 µg ml^{-1} vitamin K1, 5% lysed horse blood and 1:25 (v/v) Oxyrase (Mansfield, OH, USA), as described.[25] Inocula were prepared by suspending few colonies from a 48-h agar plate in Brucella broth to an OD_{625} of 0.8, then diluting 1:10 with Brucella broth to achieve a final titer of about 10^5 CFU ml^{-1}. Cultures were incubated for 48 h at 37 °C under 80% N_2, 10% CO_2 and 10% H_2, using a GasPak EZ anaerobe container system (Becton Dickinson, Buccinasco, Milano, Italy).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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