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Permalink
https://escholarship.org/uc/item/1xx8n80r

Journal
ACS Chemical Biology, 9(2)

ISSN
1554-8929

Authors
Iorio, M
Sasso, O
Maffioli, SI
et al.

Publication Date
2014-02-21

DOI
10.1021/cb400692w

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Peer reviewed
A Glycosylated, Labionin-Containing Lanthipeptide with Marked Antinociceptive Activity

Marianna Iorio,†,∥ Oscar Sasso,‡∥ Sonia I. Maffioli,† Rosalia Bertorelli,‡ Paolo Monciardini,† Margherita Sosio,‡ Fabiola Bonezzi,‡ Maria Summa,‡ Cristina Brunati,† Roberta Bordoni,§ Giorgio Corti,§ Glauco Tarozzo,‡ Daniele Piomelli,‡ Angelo Reggiani,*∥ and Stefano Donadio*,†

†NAICONS Srl, Via Fantoli 16/15, 20138 Milano, Italy
‡Department of Drug Discovery and Development, Italian Institute of Technology, Via Morego 30, 16163 Genova, Italy
§Institute of Biomedical Technologies, National Research Council, Via Fratelli Cervi 93, 20090 Segrate, Italy
*Supporting Information

ABSTRACT: Among the growing family of ribosomally synthesized, post-translationally modified peptides, particularly intriguing are class III lanthipeptides containing the triamino acid labionin. In the course of a screening program aimed at finding bacterial cell wall inhibitors, we discovered a new lanthipeptide produced by an Actinoplanes sp. The molecule, designated NAI-112, consists of 22 amino acids and contains an N-terminal labionin and a C-terminal methyl-labionin. Unique among lanthipeptides, it carries a 6-deoxyhexose moiety N-linked to a tryptophan residue. Consistently, the corresponding gene cluster encodes, in addition to the LanKC enzyme characteristic of this lanthipeptide class, a glycosyl transferase. Despite possessing weak antibacterial activity, NAI-112 is effective in experimental models of nociceptive pain, reducing pain symptoms in mice in both the formalin and the chronic constriction injury tests. Thus, NAI-112 represents, after the labyrinthopeptins, the second example of a lanthipeptide effective against nociceptive pain.

Ribosomally synthesized, post-translationally modified peptides constitute a growing family of microbially synthesized compounds endowed with unique structural features. Among them, one of the best known groups is represented by the lanthipeptides, characterized by lanthionine (Lan) residues in which thioether bridges link cysteine and serine/threonine residues. Most of the lanthionine-containing peptides are also known as lantibiotics (e.g., nisin), since they were discovered through bioactivity-based screens for their antibacterial activities.

Lanthipeptides are currently divided into four classes on the basis of the enzyme(s) responsible for installing the lanthionine bridges. Among them, class III lanthipeptides are made by enzymes designated as LanKC, possessing an N-terminal lyase domain, a central Ser/Thr kinase domain, and a C-terminal cyclase domain. Particularly intriguing is the observation that some class III lanthipeptides, but not all, contain the unusual triamino acid labionin (Lab), which results from cyclodehydration of two serine and one cysteine residues in a mechanism similar to that used for lanthionine formation. In this case, however, the enolate intermediate is not quenched by protonation but undergoes a second Michael addition in situ with a second dehydroalanine, yielding the characteristic quaternary carbon of Lab residues. Since the discovery of the labyrinthopeptins, the first Lab-containing lanthipeptides, this unusual amino acid has been found in few other lanthipeptides.

In the course of a screening program for peptidoglycan inhibitors, we unexpectedly discovered a labionin-containing lanthipeptide, designated NAI-112, which possesses unique structural properties. Since labyrinthopeptins have been reported to exert potent antiallodynic effects in the spinal nerve injury model of neuropathic pain, we also assessed whether NAI-112 has antinociceptive/antiallodynic activity in two different experimental models of inflammatory pain.

RESULTS AND DISCUSSION

Discovery of NAI-112. As part of a systematic evaluation of fermentation broth extracts fulfilling the selection criteria for cell wall inhibitors in a phenotypic screen (growth inhibition of

Received: September 9, 2013
Accepted: November 5, 2013
Published: November 5, 2013
a *Staphylococcus aureus* strain; no inhibition of its isogenic L-form; and no reversion of antibacterial activity after incubation with either β-lactamases or excess d-alanyl-d-alanine), a one extract, derived from *Actinoplanes DSM24059*, produced an active peak 1, designated as NAI-112, along with minor amounts of the related compound 2 (Figure 1). Under the best conditions identified so far, NAI-112 production started during exponential growth and reached a maximum at ca. 120 mg mL⁻¹, in coincidence with the end of the growth phase (Supplementary Figure S1).

**Structure Elucidation of NAI-112.** High-resolution MS of 1 (Supplementary Figure S2A) pointed to the formula C₁₀₉H₁₄₇N₂₅O₃₁S₂ (calcd 1184.0146 [M + 2H]²⁺; found 1184.0231 [M + 2H]²⁺). Upon hydrolysis, the amino acids Gly, Trp, Ser, Pro, Val, Leu, and Phe were identified in the approximate ratio 1:2:3:3:2:1:1. Amidation with benzylamine and reaction with PhNCS indicated the presence of one free carboxylic acid and one amino group, respectively. Edman degradation stopped at the first cycle after Val elimination. The easy loss of 146 amu upon acid treatment, which transformed 1 into a more hydrophobic species, together with acetonide formation observed with the intact molecule, suggested the presence of a sugar moiety. Lack of reactions with dithiothreitol and iodoacetamide indicated the absence of disulfides and free cysteines, whereas the different reactivity observed with ethanethiol (EtSH) at basic and neutral pH indicated the presence of two thioethers. However, the many overlapping signals observed in the NMR spectrum of 1 prevented structure elucidation.

Luckily, partial hydrolysis of 1 afforded a small amount of compound 3 with m/z 1098 [M + H]⁺, whose NMR spectra (Supplementary Figures S3–S5) indicated the presence of the peptide sequence Val-X-Dhb-Leu-X-Val-Ser-Ser-Pro-X-Pro-Gly, where X and Dhb designate lanthionine-like and dehydrobutyrine residues, respectively (Supplementary Table S1). This sequence was then used to query a draft genome of *Actinoplanes DSM24059*, leading to the identification of a 43-aa Coding DNA Sequence (CDS) that included the segment established by NMR as VSSLVSSPCPG, preceded by a 21-aa leader and followed by 10 further residues. Furthermore, this CDS lies within a locus with genes typical of class III lanthipeptides (Figure 2A).

The 22-aa sequence predicted from genomic data was then used as a template to interpret the MS² (Supplementary Figure S2B) and NMR data (Supplementary Figures S6–S8) of 1. The presence in the MS of the glycosylated [1–13]-fragment and of an unmodified [11–22]-fragment indicated that the sugar should reside between Pro11 and Trp13 (Supplementary Table S1).

In conclusion, the structural data indicate that NAI-112 is a 22-aa, neutralized, glycosylated lanthipeptide containing N-terminal Lab and C-terminal MeLab residues separated by a 4-aa linker (Figure 2B). Each series of rings contains both dehydrated and unmodified Ser residues (Figure 2B). On the basis of MS data, compound 2 likely represents the deglycosylated [1–14] N-terminal segment of NAI-112 (Figure 2B). NAI-112 represents the first occurrence of a lanthipeptide containing a MeLab and an N-glycosylation. While N-glycosyl-indoles are encountered in microbial e.g., staurosporins, 16 rebeccamycins, 17 and neosidomycin 18] and plant e.g., vinosamides 19] metabolites, we are unaware of any natural product containing an N-glycosylated Trp residue. To our knowledge, the closest example is represented by chori
peroxidase from the mosquito *Aedes aegypti*, the main vector of dengue and yellow fever, in which α-1-mannose is covalently connected via the N-1 atom of the indole ring.\textsuperscript{20,21}

**NAI-112 Gene Locus.** The *Actinoplanes* DSM24059 locus includes seven genes likely involved in NAI-112 biosynthesis. The structural gene *labA* encodes a leader peptide with the ILELQE motif (Figure 2C), highly conserved in class III precursor peptides and essential for enzymatic processing.\textsuperscript{22} Upstream to *labA* are *labKC*, coding for the expected Ser/Thr kinase, lyase, and cyclase domains; *labH*, *labT1*, and *labT2*, coding for ABC transporters with putative immunity and secretion functions; and *labP*, encoding an aminopeptidase possibly involved in the leader peptide processing, which however does not show obvious relatedness to other lanthipeptide-associated proteases (Figure 2A). Consistent with NAI-112 being produced in a glycosylated form, a glycosyltransferase-encoding gene lies downstream to *labA*. As expected, the LabA core peptide contains the characteristic Ser/Ser/Cys and Ser/Thr/Cys motifs that serve as precursors for Lab and MeLab formation, respectively (Figure 2C).

In addition to the labyrinthopeptins and catenulipeptin, NAI-112 represents the third example of a lanthipeptide that is produced exclusively in (Me)Lab-containing form. This finding may help shed light on the role of the precursor peptide and/or the LabKC enzyme in directing post-translational modifications toward Lan or Lab.

**Antibacterial Activity.** The purified compound shows only modest antibacterial activity, with MIC values of 32–64 μg mL\textsuperscript{−1} against *Staphylococcus* and *Streptococcus* at standard inocula (10\textsuperscript{5} CFU mL\textsuperscript{−1}). Under the conditions of the screening assay (10\textsuperscript{4} CFU mL\textsuperscript{−1} and 18-h incubation),\textsuperscript{11} an impact on *S. aureus* growth could however be observed at 4–8 μg mL\textsuperscript{−1} (Supplementary Figure S9), consistent with the screening data, which indicated the presence of a single antibacterial activity in the original extract (Figure 1). However, such a weak antibacterial activity is in stark contrast with the properties of many lantibiotics\textsuperscript{23,24} and suggested that NAI-112 may possess additional activities.

Among the few Lab-containing lanthipeptides, in only two cases have data on bioactivity been reported: catenulipeptin lacks antibacterial activity but is able to restore aerial growth to a dried *Streptomyces coelicolor* mutant.\textsuperscript{16} Another labKC strain, however, does not show obvious relatedness to other lanthipeptide-associated proteases (Figure 2A). Consistent with NAI-112 being produced in a glycosylated form, a glycosyltransferase-encoding gene lies downstream to *labA*.

**Antinociceptive Activity.** We initially tested NAI-112’s ability to prevent pain-related behaviors elicited in mice by intraplantar injection of the chemical irritant formalin. The formalin test is a sonic model of continuous pain resulting from tissue injury. The first phase has been associated to acute nociceptive pain, while the second phase should mimic a more tonic (chronic) state. Systemic administration of NAI-112 (1–10 mg kg\textsuperscript{−1}, i.p.) caused a dose-dependent reduction of formalin-induced pain behavior (Figure 3A). Substantial activity was observed on both the first phase of formalin pain, which involves acute activation of sensory C fibers, and the second phase of formalin pain, in which sensory fibers activity is accompanied by inflammation and central sensitization. Similar effects were observed when NAI-112 (1–100 μg per animal) was injected into the hind paw together with formalin (Figure 3B). Also compound 2 tested at 10 mg kg\textsuperscript{−1} i.p. was effective in the formalin test, with efficacy comparable to that of NAI-112 (Figure 3C), although the effect on the second phase of formalin pain was less pronounced. When tested at 10 mg kg\textsuperscript{−1}, i.p., on the Rotarod assay, NAI-112 did not interfere with motor coordination up to 60 min after administration (Figure 4). Furthermore, in a separate set of animals, mice treated with NAI-112 at 30 μg/10 μL i.c.v., displayed normal behavior and no sign of toxicity up to 48 h.

Interestingly, the first phase of the antinociceptive effect of 10 mg kg\textsuperscript{−1} i.p. NAI-112 in the formalin test was prevented by the administration of AMG9810, a selective TRPV1 antagonist,\textsuperscript{25} whereas no prevention was seen with the CB\textsubscript{1} inverse agonist AM251 (Figure 5A),\textsuperscript{26} the opioid antagonist naloxone, or the α2 adrenergic antagonist yohimbine (Figure 5B). Thus, while NAI-112 is effective on both phases, indicating a broad antinociceptive efficacy, the first phase of antinociceptive effect can be prevented by a selective TRPV1 antagonist.
NAI-112 was then studied for its ability to alleviate established chronic pain condition. The compound was tested in the sciatic nerve chronic constriction injury (CCI) model of persistent hyperalgesia and allodynia in mice, a model that has both inflammatory and neuropathic pain components. NAI-112 (3, 10, and 30 mg kg\(^{-1}\), i.p.) was administered on day 7 after left sciatic nerve ligation, and pain readouts were measured 2 h after dosing. As shown in Figure 6, a single administration of NAI-112 was sufficient to reduce significantly both hyperalgesia (Figure 6A) and allodynia (Figure 6B) in a dose-dependent manner, with a full effect at 30 mg kg\(^{-1}\). NAI-112 is also effective on an established chronic pain state such as the CCI model, and thus our findings indicate that the antinociceptive profile of NAI-112 could be further expanded to chronic mixed inflammatory/neuropathic pain states.

The mechanism by which NAI-112 modulates pain behavior is unclear. Our findings indicate that NAI-112 does not act through opioid, cannabinoid, or descending adrenergic mechanisms. Interestingly, in the first phase of formalin assay the effect of NAI-112 was sensitive to nonanalgesic doses of the TRPV1 antagonist AMG9810, suggesting an engagement of the vanilloid pathway. However, NAI-112 has no TRPV1 antagonistic activity in vitro (by binding assay; data not shown), and thus NAI-112 might act, at least partially, via an as-yet unidentified mechanism on a vanilloid-sensitive pathway.

Conclusions. NAI-112 shares antiallodynic activities with labyrinthopeptins and natural peptides from spider toxins or cone snails. Although a direct comparison of the bioactivities of NAI-112 and labyrinthopeptins is not possible, since different animal models and route of administrations were used, it is striking that two lanthipeptides, unrelated in their amino acid sequence, exert similar bioactivities in vivo. Furthermore, the observation that the N-terminal portion of NAI-112 retains some activity in the formalin test is reminiscent of the mechanism of action of nisin and related lantibiotics. In that case, the two N-terminal rings are involved in binding to the lipid II target, while the C-terminal portion of the lantibiotic determines further events after this initial docking step.23 In any case, the results presented here suggest that lantipeptides possess interesting bioactivities beyond their well-established properties as antibacterial agents. This finding highlights the

Figure 4. Motor coordination after NAI-112 injection. The time spent by animals on the Rotarod was recorded 30 and 60 min after NAI-112 administration and expressed in seconds as mean ± SEM. Control animals received i.p. vehicle (10% PEG, 10% Tween 80 in saline).

Figure 5. Antinocifensive effect of NAI112 in the formalin test was challenged with the selective TRPV1 antagonist AMG9810 (1 mg kg\(^{-1}\); i.p.) and the CB1 inverse agonist AM251 (5 mg kg\(^{-1}\); i.p.) (A) or the α2 antagonist yohimbine (5 mg kg\(^{-1}\); i.p.) and the opioid antagonist naloxone (1 mg kg\(^{-1}\); i.p.) (B). The antagonists were injected 30 min before formalin challenge. Animals (n = 6) were immediately transferred to a transparent observation chamber, where nocifensive behavior (time spent licking and biting the injected paw) was continuously monitored for 45 min. Nocifensive response is expressed as mean ± SEM. Symbols indicate statistical significance as * p < 0.05, ** p < 0.01, and *** p < 0.001 vs vehicle; and ## p < 0.01 vs NAI-112.

Figure 6. Effect of NAI-112 in the CCI model. Intraperitoneal injection of NAI-112 (1−30 mg kg\(^{-1}\)) reduced both heat hyperalgesia (A) and mechanical allodynia (B). Hyperalgesia and alldynia were measured 2 h after treatment. Results are expressed as mean ± SEM (n = 6, each group). *** p < 0.001 vs vehicle; ### p < 0.001 vs sham-operated mice.
possibility of further exploring this class of microbial metabolites as drug leads for pain-related applications.

**METHODS**

**Analytical Procedures.** LC–MS was performed on an Agilent 1100 series liquid chromatograph equipped with an Agilent Zorbax SB C18 (4.6 mm × 50 mm) column eluted at 1 mL min⁻¹ at 40 °C using a multistep program: time = 0 (5% phase B); time = 6 min (95% phase B); time = 7 min (100% phase B). The flows rate was 1 mL min⁻¹.

**Chromatography.** Samples were injected on a C8 reversed phase column system (Thermo Fisher Scientific) equipped with an Ascentis express reversed phase column (150 mm × 2.1 mm, 5 μm, 2.6 Å pore size) and a linear gradient from 10% to 99% B in 15 min, followed by a 1-min linear gradient from 99% B to 99% A in 1 min, and then a 5-min linear gradient from 99% A to 99% B in 5 min. The pump was set at 0.8 mL min⁻¹.

**Exploratory Chemistry.** Amino Acid Analyses. Compound I (3 mg) dissolved in 1 mL of 6 M HCl at 160 °C for 5 min under microwave irradiation, was evaporated to dryness, redissolved in 1 mL of H2O/CH3CN 1:1, adjusted to pH 7.0 with triethylamine and treated with (R)-(−)-NBD-PyNCS (5 mg). The reaction mixture was stirred for 2 h at 60 °C and then extracted twice with 3 mL of petroleum ether/CH2Cl2 8:2. The organic phase was evaporated to dryness, redissolved in 1 mL of H2O/CH3CN 1:1 and analyzed by HPLC–MS.

**Reaction with PhNCS.** Compound I (3 mg) was dissolved in 200 μL of DMF, and 10 μL of PhNCS were added. After stirring at RT for 1 h, complete transformation was observed into a 4.9-min lipophilic peak with m/z 1254.2 [M + 2H]²⁺. After evaporation to dryness the residue was redissolved in 500 μL of water and extracted with CH3Cl/hexane 2:8 (2 × 2 mL). The aqueous phase was dried, dissolved in 50% TFA, and kept for 1 h at RT, when LC–MS showed the presence of m/z 1134.9 [M + 2H]²⁺ and 1062.4 [M + 2H]²⁺, corresponding to the Edman product after N-terminal Val removal with and without the sugar moiety, respectively.

Amidation. Compound I (7 mg) was dissolved in 200 μL of DMF, and 50 μL of N,N-dimethylthelylenediamine to pH 7 (pH tested after dilution: 1:10 in H2O), and reacted with PyBOP (5 mg) for 30 min at RT, when LC–MS analysis showed the formation of monoamidated compound with m/z 1218.7 [M + 2H]²⁺.

**Reaction with EiSH at pH 11.** Compound I (3 mg) was dissolved in 200 μL of DMF and added to 200 μL of a solution consisting of 780 μL of EtOH, 560 μL of H2O, 180 μL of 5 M NaOH, and 168 μL of EtSH. The mix was reacted for 2 h at 60 °C, when a peak was observed with m/z 1276.7 [M + 2H]²⁺, corresponding to addition of three EiSH molecules.

**Reaction with EiSH at pH 7.** Compound I (3 mg) was dissolved in 400 μL of MeCN and 400 μL of 0.1 M acetate buffer, pH 7. EiSH (3 μL) was added, and the solution allowed to react at 50 °C for 1 h, when a peak was observed with m/z 1215.8 [M + 2H]²⁺, corresponding to addition of one EiSH.

**Acetonide Formation.** Compound I (33 mg) was dissolved in 2 mL of DMF/acetone 1:9. p-Toluenesulfonic acid (5 mg) was added, and the solution was stirred for 5 h at 45 °C, when a peak was observed having m/z 1204.6 [M + 2H]²⁺. After solvent evaporation the residue was purified by medium pressure reversed chromatography, as above. NMR analysis of the purified compound showed a quaternary carbon at 110 ppm expected from a 1,2 diol acetonide derivative.

**Partial Hydrolysis.** Compound I (50 mg) was dissolved in 50% TFA and allowed to react for one week at 40 °C. After evaporation, the main hydrolysis product 3 (m/z 1098 [M + H]⁺) was purified by medium pressure liquid chromatography as above.

**Genome Sequence and Identification of the NAI-112 Gene Cluster.** High molecular weight gDNA was prepared from Actino planes sp. DSM24059 grown in seed medium for 4 days. A draft genome sequence was generated by the 454 technology, which yielded 1900 contigs for a total of 7.4 Mb. A CDS encoding the peptide sequence deduced from NMR data was found in a 10590-nt contig, which was then connected on the right-end side of Figure 2A to a 3350-nt contig by targeted PCR.

**In Vitro Assays.** MICs were determined by broth microdilution as described. Growth curves were measured using a 5 × 10⁶ CFU mL⁻¹ inoculum of S. aureus L100 in a 96-well microtiter plate, which was incubated at 37 °C in a Synergy 2 plate reader (BioTek) recording the optical densities at 595 nm (OD₅₉⁵) over 20 h. Binding assays were performed by CEREP SA.

**Animals.** Male CD1 mice weighing 25–30 g (Charles River) were used in accordance with the Ethical Guidelines of the International Association for the Study of Pain and in compliance with Italian and European Economic Community regulations (D.M. 116192; O.J. of
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E.C. L. 358/1 12/18/1986). Mice were housed in groups of 5 or 4, in ventilated cages containing autoclaved cellulose paper as nesting material, with free access to food and water. They were maintained under a 12-h light/dark cycle (lights on at 08:00 a.m.), at controlled temperature (21 ± 1 °C) and relative humidity (5 ± 10%). Behavioral testing was performed between 9:00 a.m. and 5:00 p.m.

**Mouse Formalin Test.** Ten microliters of 5% formalin was injected into the plantar surface of the left hind paw, and the time the animals spent licking the injected paw was measured. As expected, two distinct lasting of high licking activity were identified, an early phase lasting the first 5 min and a late phase lasting from 15 to 45 min after formalin injection. Nocifensive behavior was monitored (licking and biting of the injected paw) for 45 min in blocks of 5 min each. Compounds were dissolved in 10% PEG400/10% Tween 80 and 80% saline and administered intraperitoneally at 1, 3, and 10 mg kg⁻¹, 30 min before formalin injection.

**Chronic Constriction Injury (CCI).** Sciatic nerve ligations were performed as described. Adult male CD1 mice were anesthetized 30 min before formalin injection. Nocifensive behavior was monitored (licking and biting of the injected paw) for 45 min in blocks of 5 min each. Compounds were dissolved in 10% PEG400/10% Tween 80 and 80% saline and administered intraperitoneally at 1, 3, and 10 mg kg⁻¹, 30 min before formalin injection.

**ACKNOWLEDGMENTS**

This work was partially supported by grants to NAICONS from Regione Lombardia and from the European Commission (contract number 245066 for FP7-KBBE-2009-3).

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