Paralithocins, Antimicrobial Peptides with Unusual Disulfide Connectivity from the Red King Crab, *Paralithodes camtschaticus*

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ABSTRACT

As part of an ongoing exploration of marine invertebrates as a source of new antimicrobial peptides, hemocyte extracts from the red king crab, *Paralithodes camtschaticus* were studied. Three cationic cysteine (Cys)-rich peptides, named paralithocins 1-3, were isolated by bioassay guided purification and their amino acid sequences determined by Edman degradation and expressed sequences tag analysis. Disulfide bond mapping was performed by high resolution tandem mass spectrometry. The peptides (38-51 amino acids in length) share a unique Cys motif composed of eight Cys, forming four disulfide bridges with a bond connectivity of (Cys relative position) Cys*1*-Cys*8*, Cys*2*-Cys*6*, Cys*3*-Cys*5*, and Cys*4*-Cys*7*, a disulfide arrangement that has not been previously reported among antimicrobial peptides. Thus, paralithocins 1-3 may be assigned to a previously unknown family of antimicrobial peptides within the group of Cys-rich antimicrobial peptides. Although none of the isolated peptides displayed antimicrobial activity against the target strains *Escherichia coli*, *Pseudomonas aeruginosa* or *Staphylococcus aureus*, they inhibited the growth of several marine bacterial strains with minimal inhibitory concentrations (MIC) in the 12.5-100 µM range. These findings corroborate the hypothesis that marine organisms are a valuable source for discovering bioactive peptides with genuine structural motifs.

Antimicrobial peptides (AMPs) are oligopeptides that inhibit bacterial growth *in vitro* under physiological conditions. They are considered to be an important part of the innate immune system in all species, including vertebrates and invertebrates.[1, 2] Due to their lack of T- and B-cell repertoire, invertebrates depend on AMPs and other innate immune factors/mechanisms to combat invading pathogens. The importance of AMPs for survival during microbial challenge has been well documented in several invertebrate species, including the fruit fly, *Drosophila melanogaster*,[3] the whiteleg shrimp, *Litopenaeus vannamei*,[4-6] and the Pacific oyster *Crassostrea gigas*.[7] Upon a microbial challenge, increased levels of AMPs are due to either increased ribosomal translation or cleavage of larger proteins (already present, having other physiological functions) of which the AMPs are an integral part.[8, 9] Beside their function as genetically coded antimicrobials, evidence suggests that AMPs may alter membrane properties or interact with receptors to influence e.g. cytokine release, chemotaxis, wound healing, angiogenesis, and antigen presentation.[10-12]

Most AMPs share some common features; they constitute less than 100 amino acids (aa), are positively charged, and amphipathic.[13] A finer classification is challenging, but some broad groups exist: 1) amphipathic peptides with a linear α-helix structure; 2) peptides with β–sheets and/or extended or loop structures, often stabilized by disulfide bridges; 3) peptides with a high proportion of one or more aa (most often Pro, Arg, Gly, Trp, and/or His); and 4) peptides containing one or more D-aa and or modified aa (e.g. brominated Trp).[10, 14] Marine invertebrates have proven to be a promising source for discovery of bioactive peptides and numerous AMPs have been characterized from crustacean hemolymph/hemocytes, most of them Cys-rich.[15, 16] *Crustacea* is an arthropod subphylum comprising a diverse group of animals, including species with both ecological and economic importance. In terms of AMPs, there has been a particular emphasis on the characterization of the AMP families of penaeidins and crustins.[15, 17] The

penaeidins, consisting of a Pro-Arg rich N-terminal region and a cysteine-containing C-terminal region, have only been detected in shrimp (Penaidae) species.[18] The C-terminal part contains three disulfide bridges having a Cys motif of Cys*1*-Cys*3*, Cys*2*-Cys*5*, and Cys*4*-Cys*6* (the italic numbers corresponds to the relative positions of Cys throughout the peptide chain).[19, 20] The crustin peptide family is defined as AMPs of ca. 7-14 kDa in size, containing 8-12 Cys, with a characteristic four-disulfide core-containing whey acidic protein (WAP) domain in the C-terminal part. In contrast to the penaeidins, the crustins seem to be widespread throughout the entire crustacean subphylum,[21] and even present in other arthropod subphyla.[22] In addition to penaeidins and crustins, numerous other AMPs have been discovered in crustaceans.[15] Recently, panusin, a β-defensin-like AMP containing six Cys with Cys*1*-Cys*5*, Cys*2*-Cys*4*, and Cys*3*-Cys*6* connectivity was discovered.[23]

Although the disulfide bridges are not essential for biological activity in some disulfide-containing AMPs,[24-26] a stable tertiary structure is usually imperative for proper biological function and stability of disulfide-rich peptides.[27] A peptide with *n* Cys have *i* possible arrangements for disulfide bridge connectivity:

$$i = \frac{n!}{(n/2)!2^{n/2}}$$

Determining the correct Cys pair bridging is crucial to predict the correct peptide tertiary structure. Three methods are commonly used in order to determine the disulfide connectivity; NMR analysis,[28] X-ray crystallography,[29] and Edman degradation,[30] however, these techniques usually require a few mg of the sample material and peptides obtained from marine invertebrates are rarely isolated in such high amounts. Mass spectrometry (MS) is therefore a suitable technique for disulfide bridge connectivity studies of polypeptides, although the

structure elucidation is more cumbersome compared to NMR and X-ray analysis, because manual interpretation of the spectra is required, especially if the peptide constitutes a undescribed Cys-rich motif. Several strategies can be used for disulfide connectivity determination by MS analysis either used alone or in combination with other techniques, as thoroughly reported by Tsai et al.:[31] 1) peptide profiles are compared before and after reduction; 2) one or more partial reduction(s) and alkylation(s); 3) data analysis based on *in silico* algorithms; 4) in line reduction of Cys-Cys, and 5) labeling with disulfide selective reagents.

In a previous study,[32] we detected antibacterial activity in various tissues and the hemocytes of the red king crab, *Paralithodes camtschaticus*. Later, the primary structure of a putative crustin AMP was described in the same species.[16] Herein we report on the isolation and structure elucidation of three new AMPs, all containing four disulfide bridges, from the hemocytes of *P. camtschaticus*. The peptides were isolated through bioassay-guided purification and tested for activity against Gram negative and Gram positive bacteria. Their primary structures were partially elucidated through Edman amino acid sequencing and totally verified by comparison of mass spectrometry (MS/MS) data analysis of intact peptides with sequences of putative peptides derived from an expressed sequence tag (EST) library. Finally, the disulfide connectivity was elucidated by partial reduction and alkylation followed by high resolution tandem MS (HRMS/MS) analysis.

RESULTS AND DISCUSSION

Isolation of Antimicrobial Peptides from *P. camtschaticus* Hemocytes. A previous screening for antibacterial activity in the red king crab, *P. camtschaticus*, showed that hemocyte extracts contain proteinaceous compounds that possess antibacterial activity *in vitro*.[32] Herein we describe the purification and characterization of three new antibacterial peptides from the hemocytes of the king crab. An extract prepared from dried hemocytes was subjected to solid phase extraction (SPE). Elution was successively performed with 10, 40, and 80% solutions of MeCN in acidified H_2O. Based on our previous results,[32] and taking into account the amount of available material, we focused our attention on the 40% SPE fraction. This fraction, containing a total of 206 mg protein, was further fractionated by preparative reversed-phase high performance liquid chromatography (RP-HPLC) using a linear gradient of 0-40% MeCN. Several fractions (peaks) showed antibacterial activity against the Gram-positive bacterium *Corynebacterium glutamicum* and Gram-negative bacterium *Listonella (Vibrio) anguillarum*. The active peptides designated as P23, P30 and P34 (Figure 1) were purified to homogeneity and subjected for further analysis. As shown in Figure 1, these peptides (especially P23) constitute a major quantitative part of the compounds present in the 40% SPE eluate. Distinct UV absorbance at 280 nm during RP-HPLC (data not shown) suggests that the peptides contain tyrosine and/or tryptophan residues.

Peptide Sequencing, Amino Acid Analysis and Mass Analysis. Partial primary structure determination was performed by Edman degradation. The partial N-terminal sequences for P23 (30 residues: WQQPSXSSIXDYSXGKSAXISYSGRXGXXA), P30 (30 residues: RSPPQXQYTNXAAVLXPAVYXANAYTPPXG), and P34 (29 residues:

RSQPGPTXPSSVQAILXDNRXGRSAXSYY) revealed that all peptides contained several unidentified amino acids (denoted X), most likely Cys, but otherwise no apparent homology between each other. According to amino acid analyses (Table S1), all peptides contained Cys and more basic amino acids than negatively charged amino acids, i.e. they are cationic of nature. In addition, P23 was devoid of Thr, Val, His, Phe, and Leu, but rich in Ser (ca. 23 mol%). P30 was devoid of His and Phe but rich in Pro (ca. 21 mol%), whereas P34 was devoid of Phe and relatively rich in Pro and Ser (ca. 11-16 mol% of both). The monoisotopic masses of the three peptides were determined by HRMS to be 4075.6248 (P23), 5045.1705 (P30), and 5559.4226 Da (P34), respectively. However, in each of these peptide fractions, a mass 0.98 Da above the reported value (<10% abundance compared to the main peptide) was also observed. The peptides were named paralithocin 1 (P23), paralithocin 2 (P30), and paralithocin 3 (P34) after the species from which they were isolated.

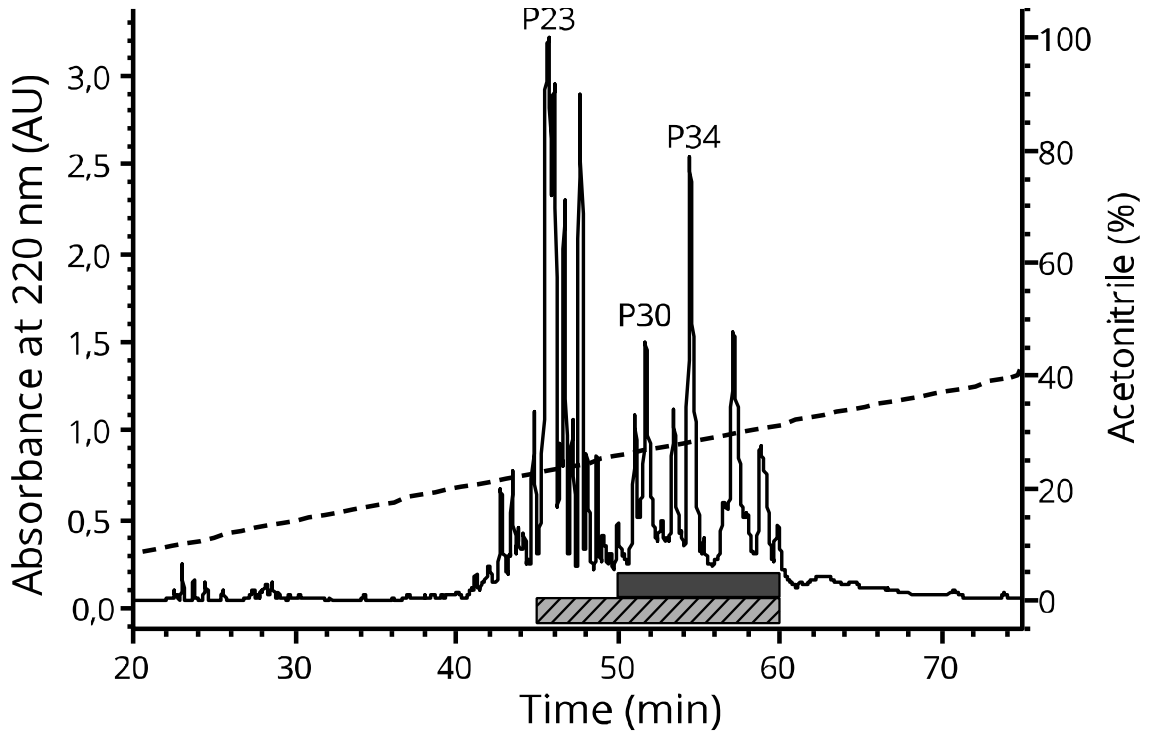

Figure 1. RP-HPLC profile of the 40% SPE eluate of a hemocyte extract from *P. camtschatica*. Peaks containing the paralithocins are labeled in the chromatogram. The dotted line shows the linear MeCN gradient (0-40%) dissolved in 0.1% TFA. The horizontal bars indicate fractions with antibacterial activity against *L. anguillarum* (dark grey) and *C. glutamicum* (hatched).

Identification of Genes and Complete Primary Structures. To identify the cDNA gene of the peptides, an EST library of the hemocytes was constructed and 384 randomly picked clones were sequenced and data from 374 were subjected to bioinformatic analyses. Cap3 sequence analyses revealed that five of 16 contigs were more redundant than the other (Table S2). The longest cDNA clones of three of the contigs (contig 2, 16 and 11) were sequenced forward and backward and consensus sequences were established (Figure S1). All three sequences coded for a signal peptide of 23 amino acids and mature peptides containing eight Cys (Figure 2). The deduced mature peptides corresponded with the partial amino acid sequences of the isolated peptides. Consensus for contig 2 (40% redundancy) and contig 16 (8% redundancy) contained open reading frames (ORF) of 62 and 71 amino acids, respectively (Figure S1A and B).

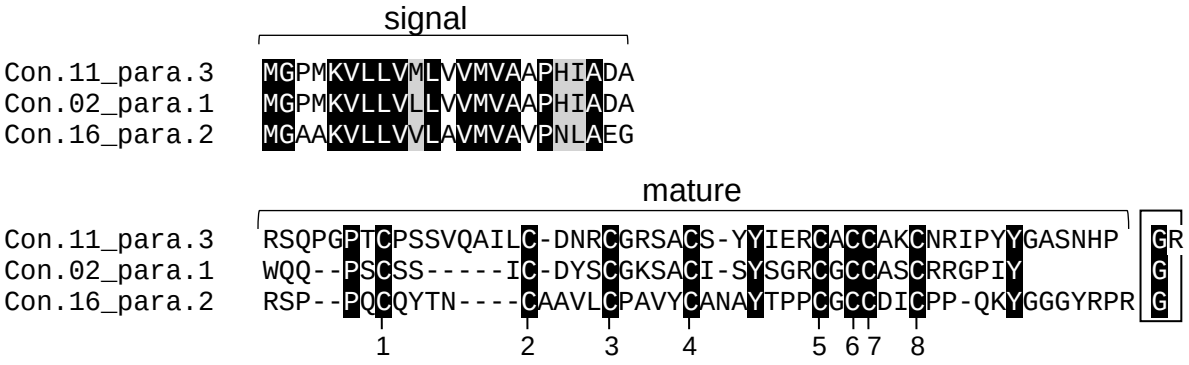

Figure 2. Alignment of the deduced peptide sequences from the EST library (contigs 11, 2 and 16) from hemocytes of the red king crab, *P. camtschatica*. The mature part of the sequences are identified as

paralithocins 3, 1 and 2, respectively. The alignment is done according to the eight cysteine arrays as indicated. Identical and strongly similar amino acids are shaded in black or grey, respectively. Pre-sequences are marked as signal-sequences and the C-terminal amino acids not present in the mature paralithocins (G and GR) are boxed.

The putative mature peptides coded were 39 and 48 aa long with theoretical monoisotopic masses of 4133.63 and 5103.17 Da (with all Cys in oxidized forms), respectively. These values deviates from the measured masses of the isolated paralithicins 1 and 2 by +58 Da, indicating the presence of additional modifications. Both sequences contain a C-terminal Gly residue, a target for peptidylglycine α-amidating monooxygenase (PAM), an enzyme contributing to C-terminal amidation of peptides.[33, 34] Cleavage of the C-terminally Gly residue and subsequent C-terminal amidation of the deduced sequences, leads to theoretical monoisotopic masses of 4075.62 and 5045.17 Da, which corresponds to the measured masses of paralithocin 1 and 2, respectively. Contig 11 (4% redundancy) contained an ORF of 76 amino acids (Figure S1C), coding for a putative mature peptide of 53 aa with a calculated monoisotopic mass of 5773.53 Da. This mass is about 214 Da above the measured mass of paralithocin 3. However, enzymatic cleavage of the C-terminal residues Gly-Arg, first by an exopeptidase and secondly by PAM, leads to a C-terminally amidated peptide with a theoretical monoisotopic mass (5559.42 Da) that corresponds completely to the experimental monoisotopic mass of paralithocin 3. Post-translational cleavage of dipeptides C-terminally (like Gly-Arg in paralithocin 3) and subsequent C-terminal amidation has been described previously in various invertebrate AMPs.[35-38] A top down MALDI-TOF MS/MS analysis approach confirmed that the native amino acid sequences were identical to the putative mature peptides of the consensus contigs. Hence, this also confirmed that the C-terminal extended

amino acids were not present in the native peptides. Masses observed at +0.98 Da can be explained by paralithocin analogues having a free acid C-terminally (Table S3). Natural mixtures of C-terminally amidated and non-amidated AMPs have been described before.[39, 40] Although the primary sequence and peptide length differ between the three mature peptides, there are some similarities. Firstly, all three peptides contain eight cysteine residues with a common cysteine pattern (C-C-C-C-C-CC-C) and sequence motif of CXCCXXC, indicating identical Cys connectivity. The isolated peptides are all cationic with isoelectric points (pI) of 8.51 (paralithocin 1), 8.50 (paralithocin 2) and 8.73 (paralithocin 3). BLAST and homology searches of the obtained oligopeptide and nucleotide sequences resulted in no overall sequence similarity to other known peptides/proteins. Comparison of the Cys pattern in the primary structure of paralithocins with other AMPs containing eight Cys residues (searches performed against various antimicrobial peptide databases) revealed a unique Cys location pattern within the paralithocins (Table 1).

Table 1. Antimicrobial Peptides Containing Four Disulfide Bridges. Adjacent Cysteines in the Cysteine Pattern Are Marked in Bold.

Cysteine-connectivity	Cysteine pattern	Representative peptide	# aa	Origin	UniProt code/ref.
1-2, 3-7, 4-8, 5-6	C-C-**CC**C-C-C	Sillucin	30	Fungus	P02885
1-3, 2-4, 5-7, 6-8	C-C-C-C-C-C-C-C	Gambicin	61	Insect	Q9XZN6
1-4, 2-5, 3-6, 7-8	C-C-**CC**-C-C-C-C	PN-AMP1 (Hevein)	41	Plant	P81591
1-5, 2-6, 3-4, 7-8	C-C-C-C-C-C-C-C	Antifungal protein	73	Fungus	P17737
1-5, 2-6, 3-7, 4-8	C-C-C-C-C-C-C-C	MGD-1	38	Mussel	P80571
1-6, 2-3, 4-7, 5-8	C-C-**CC**-C-C-C-C	LTP-1	91	Plant	P07597
1-6, 2-5, 3-7, 4-8	C-C-C-C-C-C-C-C	Hydramacin-1	60	Cnidarian	B3RFR8
1-6, 2-7, 3-5, 4-8	C-C-C-C-**CC**-C-C	Nawaprin	50	Snake	P60589

1-7, 2-6, 3-5, 4-8	C-C-C-C-C-C-C-C	Locustin	55	Insect	P83428
1-8, 2-4, 3-6, 5-7	C-**CC**-**CC**-C-**CC**	Hepcidin	25	Human	P81172
1-8, 2-5, 3-6, 4-7	C-C-C-C-C-C-C-C	Drosomycin	44	Insect	P41964
1-8, 2-6, 3-5, 4-7	C-C-C-C-C-**CC**-C	Paralithocin 1	38	Crustacean	This study
1-8, 2-7, 3-6, 4-5	**CC**-C-C-C-C-C-C	Leaf-specific thionin	46	Plant	P09617

Paralithocin 1 showed 55.8% and 63.2% identity to paralithocin 2 and 3, respectively, at the cDNA level. The paralithocin transcripts have been submitted to NCBI GenBank with the accession numbers MF919584 (paralithocin 1), MF919585 (paralithocin 2), and MF919586 (paralithocin 3), respectively.

Determination of Disulfide Bridge Connectivity. Peptides containing intact disulfide bridges produce y- and b-ions upon collision induced dissociation (CID) through cleavage of peptide bonds of amino acids not enveloped within the disulfide knot. Occasionally, b- and y-ions from Cys-Cys are observed as their [b/y ± H/SH] ions, but b- and y-ions formed by peptide bond cleavage of amino acids that exist within the disulfide knot are rarely observed. The strategy used in the present work was to conduct a partial reduction of the disulfide bonds and alkylation with N-ethylmaleimide (Nem), and to conduct CID experiments of the resulting peptides containing four, three, two, one, and no intact disulfides. The retention times of the partially reduced and alkylated peptides increased with their number of reduced and alkylated S–S bridges. This may be rationalized by the fact that the hydrophobic amino acids occupy the less water exposed interior of the intact peptides, but following reduction and alkylation, they were more exposed to the aqueous mobile phase used in reversed-phase chromatography, resulting in an increased non-polar surface area. Additionally, the alkylation itself introduces a slightly hydrophobic moiety to the peptide.

The total ion chromatograms (TIC) of native, partially, and totally reduced and alkylated paralithocin 1 is shown in Figure S2 and only one isomer of each reduced and alkyled peptide was observed. For native paralithocin 1, CID of $[M+5H]^{5+}$ (*m/z* 816.3287, Δm = –0.7 ppm) y_1^+-y_6^+ ions were observed, in addition to $(y_7+SH)^+$, which is in accordance with Cys^{32} (Figure 3a).

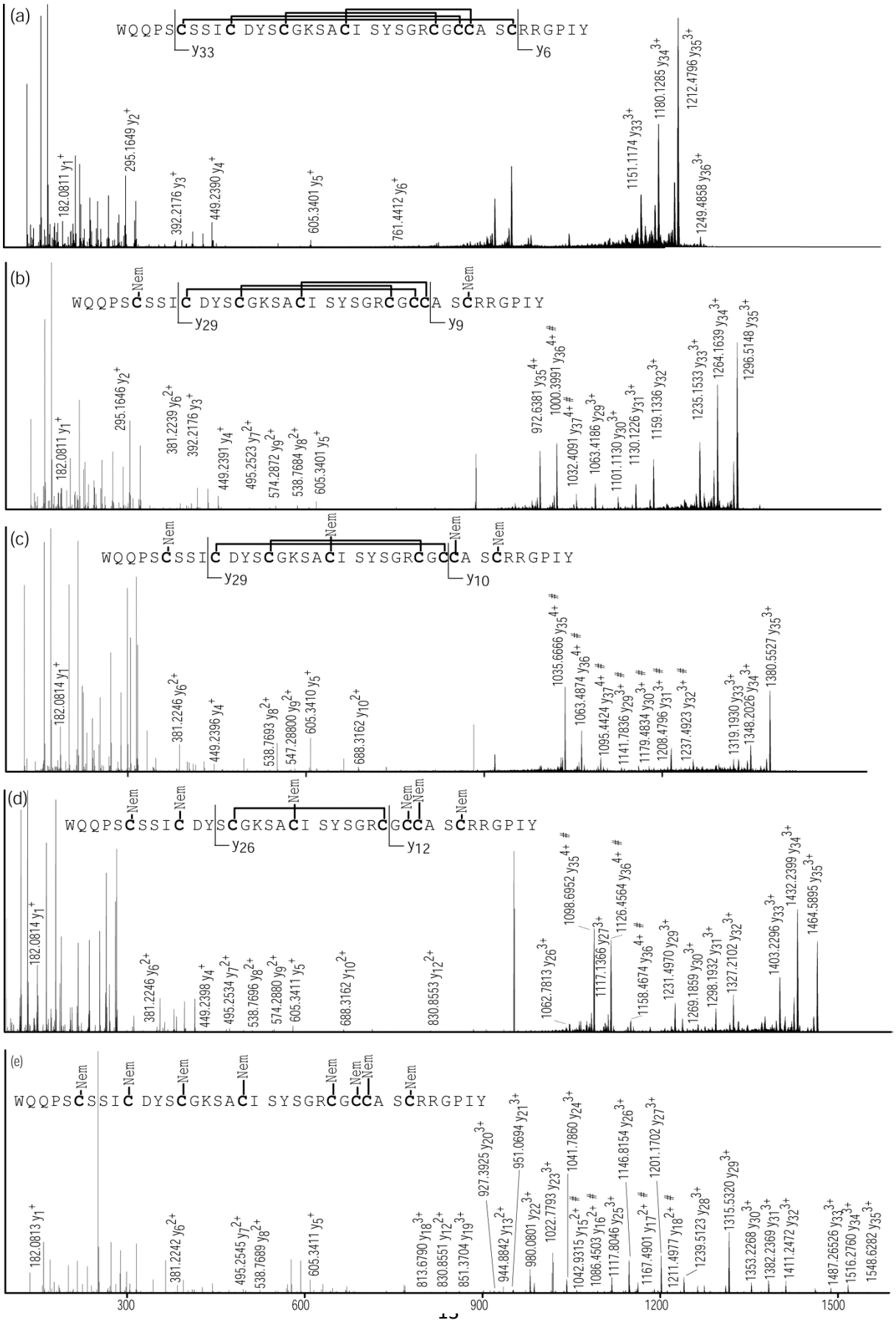
(a)
WQQPSCSSICDYSCGKSACISYSGRCGCCASCRRGPIY
y33
y6
182.0811 y1+
295.1649 y2+
392.2176 y3+
449.2390 y4+
605.3401 y5+
761.4412 y6+
1151.1174 y33 3+
1180.1285 y34 3+
1212.4796 y35 3+
1249.4858 y36 3+
(b)
WQQPSCSSICDYSCGKSACISYSGRCGCCASCRRGPIY
Nem
y29
y9
182.0811 y1+
295.1646 y2+
381.2239 y6 2+
392.2176 y3+
449.2391 y4+
495.2523 y7 2+
574.2872 y9 2+
538.7684 y8 2+
605.3401 y5+
972.6381 y35 4+
1000.3991 y36 4+ #
1032.4091 y37 4+ #
1063.4186 y29 3+
1101.1130 y30 3+
1130.1226 y31 3+
1159.1336 y32 3+
1235.1533 y33 3+
1264.1639 y34 3+
1296.5148 y35 3+
(c)
WQQPSCSSICDYSCGKSACISYSGRCGCCASCRRGPIY
Nem
y29
y10
182.0814 y1+
381.2246 y6 2+
449.2396 y4+
538.7693 y8 2+
547.28800 y9 2+
605.3410 y5+
688.3162 y10 2+
1035.6666 y35 4+ #
1063.4874 y36 4+ #
1095.4424 y37 4+ #
1141.7836 y29 3+ #
1179.4834 y30 3+ #
1208.4796 y31 3+ #
1237.4923 y32 3+ #
1319.1930 y33 3+
1348.2026 y34 3+
1380.5527 y35 3+
(d)
WQQPSCSSICDYSCGKSACISYSGRCGCCASCRRGPIY
Nem
y26
y12
182.0814 y1+
381.2246 y6 2+
449.2398 y4+
495.2534 y7 2+
538.7696 y8 2+
574.2880 y9 2+
605.3411 y5+
688.3162 y10 2+
830.8553 y12 2+
1062.7813 y26 3+
1098.6952 y35 4+ #
1117.1366 y27 3+
1126.4564 y36 4+ #
1158.4674 y36 4+ #
1231.4970 y29 3+
1269.1859 y30 3+
1298.1932 y31 3+
1327.2102 y32 3+
1403.2296 y33 3+
1432.2399 y34 3+
1464.5895 y35 3+
(e)
WQQPSCSSICDYSCGKSACISYSGRCGCCASCRRGPIY
Nem
182.0813 y1+
381.2242 y6 2+
495.2545 y7 2+
538.7689 y8 2+
605.3411 y5+
813.6790 y18 3+
830.8551 y12 2+
851.3704 y19 3+
927.3925 y20 3+
944.8842 y13 2+
951.0694 y21 3+
980.0801 y22 3+
1022.7793 y23 3+
1041.7860 y24 3+
1042.9315 y15 2+ #
1086.4503 y16 2+ #
1117.8046 y25 3+
1146.8154 y26 3+
1167.4901 y17 2+ #
1201.1702 y27 3+
1211.4977 y18 2+ #
1239.5123 y28 3+
1315.5320 y29 3+
1353.2268 y30 3+
1382.2369 y31 3+
1411.2472 y32 3+
1487.26526 y33 3+
1516.2760 y34 3+
1548.6282 y35 3+
300
600
900
1200
1500

Figure 3. HRMS/MS spectra of (a) native paralithocin 1 (P23) $[M+5H]^{5+}$ at *m/z* 816.3287, (b) paralithocin 1 with one reduced and alkylated Cys-Cys bond $[M+5H]^{5+}$ at *m/z* 866.7509, (c) paralithocin 1 with two reduced and alkylated Cys-Cys bonds $[M+5H]^{5+}$ at *m/z* 917.1749, (d) paralithocin 1 with three reduced and alkylated Cys-Cys bonds $[M+5H]^{5+}$ at *m/z* 967.5953, and (e) paralithocin 1 with all Cys-Cys bonds reduced and alkylated $[M+5H]^{5+}$ at *m/z* 1018.0175.

Furthermore, $y_{36}^{3+\#}$, y_{35}^{3+}, y_{34}^{3+}, and y_{33}^{3+} were observed ($^{\#}$ indicates additional loss of ammonia), as well as $(y_{32}+SH)^{3+}$, all in support for a Cys^{6}. More ions were recorded upon CID of paralithocin 1 where one Cys-Cys was reduced and alkylated ($[M+5H]^{5+}$ was observed at *m/z* 866.7509; Figure 3b). A complete series of y_1^{+} to y_5^{+} and y_6^{2+} to y_9^{2+} ions were recorded, indicating a reduction/alkylation of Cys^{32} (Cys^{32}-Nem). From the *N*-terminal end, the $y_{37}^{4+\#}$-$y_{35}^{4+\#}$, and y_{35}^{3+}-y_{29}^{3+} ions were all observed, which indicated Cys^{6}-Nem. Thus, these observations suggest a Cys^{6}-Cys^{32} (Cys*1*-Cys*8*) disulfide bridge in the native peptide. Paralithocin 1 containing four Nem groups (two reduced Cys-Cys), was observed as its $[M+5H]^{5+}$ ion at *m/z* 917.1731 (Figure 3c). Its CID spectrum had peaks corresponding to y_1^{+}, y_4^{+}, y_5^{+}, y_6^{2+}, and y_8^{2+}-y_{10}^{2+}, which was due to Cys^{29}-Nem. However, the observation of $y_{37}^{4+\#}$ - $y_{35}^{4+\#}$, and y_{35}^{3+}-y_{29}^{3+}, suggest that Cys^{10} was still disulfide bonded, leaving the remaining Cys-Nem at either of Cys^{14}, Cys^{19}, or Cys^{26} (as no ions indicative of Cys^{28}-Nem were observed). The final partial reduced paralithocin 1, containing six Cys-Nem, was observed as its $[M+5H]^{5+}$ ion at *m/z* 967.5953 (Figure 3d). An ion series of y_1^{+}, y_4^{+}, y_5^{+}, y_6^{2+}-y_{10}^{2+}, and y_{12}^{2+} was observed, suggesting Cys^{28}-Nem. Furthermore, the ions y_{35}^{3+}-y_{29}^{3+}, as well as y_{27}^{3+} and y_{26}^{3+} were observed, indicating Cys^{10}-Nem. To summarize, the observed ions indicate Cys^{6}-Cys^{32} and Cys^{10}-Cys^{28} in native paralithocin 1, but as no ions were observed upon CID of paralithocin 1 with six Cys-Nem indicating the presence of either Cys^{14}-Nem or Cys^{26}-Nem, it was deduced

that the triply reduced/alkylated peptide contained an intact Cys^{14}-Cys^{26} (Cys*3*-Cys*5*) disulfide bridge. As Cys^{29}-Nem was observed upon CID of the four Cys-Nem containing paralithocin 1, it was further deduced that a Cys^{19}-Cys^{29} (Cys*4*-Cys*7*) disulfide existed in the native peptide. The fully reduced and alkylated paralithocin 1 ($[M+5H]^{5+}$ at *m/z* 1018.0175) produced a spectrum where several y-ions were recorded upon CID (Figure 3e). Thus, the coupling pattern of disulfide bridges in paralithocin 1 was Cys*1*-Cys*8*, Cys*2*-Cys*6*, Cys*3*-Cys*5*, and Cys*4*-Cys*7*.

The native paralithocin 2 ($[M+5H]^{5+}$ at *m/z* 1010.0416), produced b_1^+-b_5^+ and y_6^{2+}-y_{12}^{2+} ions upon CID, in accordance with intact disulfide bridges at Cys^6 and Cys^{35}, respectively. The chromatograms of paralithocin 2 with one, two or three reduced and alkylated Cys-Cys bond all suggest that minor disulfide scrambling has occurred, as two peaks are observed in each chromatogram. Following partial reduction, paralithocin 2 + 2×Nem ($[M+5H]^{5+}$ at *m/z* 1060.4630) produced b_2^+ to b_{10}^+ upon CID, which indicated Cys^6-Nem. Singly (y_1 to y_7) and doubly (y_8 to y_{15}) charged y-ions were also recorded, which is in accordance with Cys^{35}-Nem (Table S4). Hence, in the native paralithocin 2 there was a Cys^6-Cys^{35} (Cys*1*-Cys*8*) disulfide bridge. The reduction and alkylation of two Cys-Cys in paralithocin 2 gave a compound observed as $[M+5H]^{5+}$ at *m/z* 1110.8844. Upon CID, b_2^+ to b_{10}^+ ions were recorded, which indicated the previously identified Cys^6-Nem and an intact disulfide at Cys^{11} (Cys*2*), and the series y_6^{2+} to y_{16}^{2+}, which indicated Cys^{32}-Nem (Cys*7*). The position of the second Cys-Nem introduced in this reduction and alkylation step was not identified, but its location at Cys^{11} and Cys^{31} can be excluded, leaving Cys^{16}, Cys^{21} and Cys^{29} as the possible alkylation sites. Next, paralithocin 2 with three Cys-Cys bonds reduced and alkylated ($[M+5H]^{5+}$ at *m/z* 1161.3082) underwent CID. The spectrum contained peaks corresponding to b_2^+ to b_{14}^+ ions, strongly indicating a Cys^{11}-Nem, and peaks corresponding to y_7^{2+} to y_{18}^{2+} ions, which indicated a Cys^{31}-

Nem, yielding a Cys*2*-Cys*6* in the native peptide. There were no peaks in the spectrum that corresponded to reduction and alkylation of any of Cys^{16} or Cys^{29}, we therefore deduced that an intact Cys^{16}-Cys^{29} (Cys*3*-Cys*5*) disulfide bridge remained in triply reduced and alkylated paralithocin 2. The disulfide coupling pattern of native peptide was therefore Cys^{6}-Cys^{35} (Cys*1*-Cys*8*), Cys^{11}-Cys^{31} (Cys*2*-Cys*6*), Cys^{16}-Cys^{29} (Cys*3*-Cys*5*), and Cys^{21}-Cys^{32} (Cys*4*-Cys*7*). A large number of ions were recorded upon CID of the fully reduced and alkylated paralithocin 2, covering almost the entire sequence (Table S4).

The final peptide, paralithocin 3, is 51 aa long, has four disulfide bridges, and has the same motif as paralithocin 1 and paralithocin 2, that is, CXCCXXC, and it was expected that this peptide had the same disulfide bridge coupling pattern as the former peptides. It was, however, difficult to record CID spectra of paralithocin 3 with the same quality, that is, a vast number of ions indicative of the structure, as was obtained upon CID of paralithocins 1 and 2. Native paralithocin 3 ($[M+7H]^{7+}$ at *m/z* 795.3528) underwent CID and b_2^+ to b_7^+ and y_{50}^{6+} to y_{44}^{6+} were observed, indicating Cys^{8} (Table S5). Ions providing evidence for Cys^{39} were also recorded, that is, y_2^+ to y_{10}^+, y_{11}^{2+}, and y_{12}^{2+}. The reduction and alkylation of one Cys-Cys in paralithocin 3 yielded a $[M+7H]^{7+}$ ion at *m/z* 831.3688. Its CID spectrum showed peaks indicating Cys^{8}-Nem (Cys*1*) by the recorded b_8^+, b_{10}^{2+}, y_{46}^{5+} to y_{37}^{4+}, and y_{35}^{4+} ions. Furthermore, the recorded y_2^+ to y_9^+, y_{11}^{2+}, y_{12}^{2+}, and y_{14}^{2+} ions are indicative of Cys^{39}-Nem, suggesting a Cys*1*-Cys*8* bridge in the native peptide. The CID spectra of paralithocin 3 where two or more disulfide bridges are reduced and alkylated were hard to interpret, as very few ions useful for structure characterization were recorded, most ions were of low abundance (Table S5). For (paralithocin 3 + 4×CysNem), the CID spectrum showed peaks corresponding to y_{10}^+ and y_{14}^{2+} ions, weakly indicating Cys^{36}-Nem (Cys*7*). A b_{15}^{2+} was also recorded, but this is not sufficient evidence to claim a disulfide constituting Cys^{17} (Cys*2*). A series of low abundant y-

ions was observed upon CID of (paralithocin 3 + 6×CysNem), that is, y_2^+-y_{10}^+ and y_{12}^{2+}-y_{18}^{2+}. These ions suggest Cys[39]-Nem, Cys[36]-Nem, and Cys[35]-Nem, and if the CXCCXXC motif is taken into consideration, this implicates an identical Cys-Cys bridging pattern in paralithocin 3 as for paralithocin 1 and 2. Unfortunately, no b- or y-ions were observed that could add evidence to the anticipated Cys[17]-Nem, except the y_{33}^{4+} ion, however, this ion had a mass accuracy of $\Delta m = -7.9$ ppm.

In a peptide having eight cysteines, and where all of them have formed disulfide bridges, there are 105 coupling possibilities. The bridging in paralithocins 1 and 2 were identical, both having a bond connectivity of Cys*1*-Cys*8*, Cys*2*-Cys*6*, Cys*3*-Cys*5*, and Cys*4*-Cys*7*, and there is evidence to claim that this also applies to paralithocin 3 (Figure 4). Unwanted disulfide scrambling products were not observed during the fragmentation analysis of the paralithocins. However, paralithocin 1 was observed almost solely as N-terminal acid during the analysis, whereas the amide-carboxylic acid ratio was approximately 8:1 for paralithocin 2 and 2:1 for paralithocin 3. As these peptides contain a consensus CXCCXXC motif, it was anticipated an identical bridging in all three. Disulfide bridges are found to be remarkably conserved between homologous proteins and they are not produced randomly.[41] The folding and correct bridge formation requires the involvement and aid of several proteins. For an organism to express peptides possessing the same motif, it would presumably be more demanding than beneficial to have more than one folding and cysteine oxidation apparatus simultaneously running. Cys-rich AMPs are widely distributed in animals and plants, but although having the same number of cysteine residues, the paralithocins show a cysteine arrangement pattern and disulfide connectivity different from any known Cys-rich AMPs (Table 1). Disregarding the flanking cysteines (Cys*1* and Cys*8*) of the paralithocins, the remaining cysteines fold into a Cys*1*-Cys*5*, Cys*2*-Cys*4*, and Cys*3*-Cys*6* connectivity, identical to the lobster panusin[23] and the vertebrate

β-defensins and the invertebrate big defensins.[42] However, homology searches and sequence alignment with selected peptides obtained from these classes reveal no similarities to the paralithocins. The paralithocins 1-3 may therefore be assigned to a new family of antimicrobial peptides within the group of Cys-rich antimicrobial peptides.

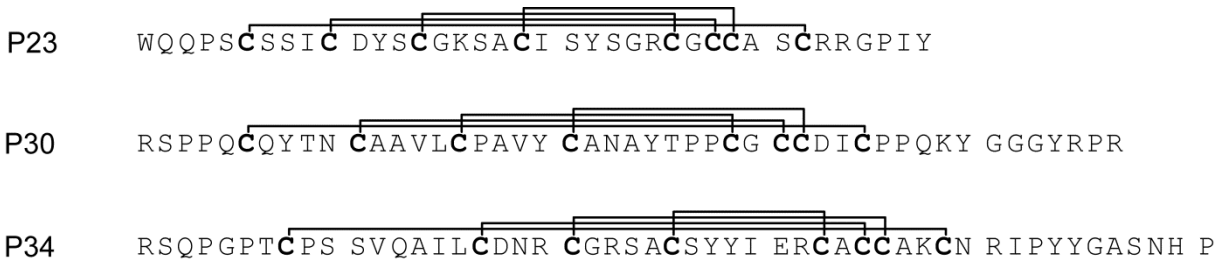

Figure 4. Final Cys coupling pattern of paralithocins 1 (P23), 2 (P30), and 3 (P34).

Antibacterial Properties of the Isolated Peptides. The native peptides isolated from *P. camtschaticus* were screened for antibacterial activity against a panel of terrestrial and marine bacterial strains. The paralithocins display in general a low to moderate activity against microorganisms under the testing conditions used (Table 2).

In general, paralithocin 3 was the most potent peptide with minimal inhibitory concentration (MIC) values as low as 12.5 μM against *C. glutamicum* and some Gram-positive marine bacterial strains. Paralithocin 1 was the least active peptide with MIC values of 50 μM and upwards. This fact might explain why the peptides (especially paralithocin 1) are present in relatively high concentrations in the hemocyte extracts. According to the UV-Vis chromatogram, the paralithocins are among the major components (with paralithocin 1 being the dominant peptide) in the extract. Furthermore, 52.7% of the EST contigs subjected to bioinformatics analyses corresponded to the paralithocins, with 40% of them corresponding for paralithocin 1 alone. None of the paralithocins displayed any growth inhibition of the terrestrial

strains *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* at a concentration of 100 μM. Most natural AMPs are considered to kill or inhibit bacteria by damaging the outer and/or cytoplasmic membranes. Differences in membrane architecture and/or lipid composition might therefore affect the efficacy of AMPs, resulting in varying AMP susceptibility of different strains. As a response to low temperatures, marine bacteria optimize their membrane fluidity by incorporating long-chain polyunsaturated fatty acids in their membranes,[43] a membrane composition that might make the marine bacteria more susceptible to the paralithocins. Furthermore, due to lower growth rate for marine bacteria at low temperatures, a longer incubation period is necessary before any antimicrobial activity can be evaluated *in vitro*. This longer incubation period (72 h at 12 °C versus 24 h at 37 °C), where the AMPs are given time to interact with its target(s), might also lead to lower MIC values against the marine Gram-positive bacteria. Although the paralithocins display low to moderate antibacterial activity in the present study, they might still have an antimicrobial function *in vivo*. The paralithocin producer, the red king crab, is a cold-loving species tolerating water temperatures of –1.7 to +11 °C [44] and immature crabs have in laboratory studies shown a temperature preference of below 6 °C.[45] It remains to be studied whether the paralithocins are more potent at temperatures lower than 12 °C.

As shown by MS analysis, the ratio for the C-terminal amidated versus C-terminal acid forms of paralithocin 1 and 3 was reduced during processing and/or storage. The increased proportion of the acid form (with reduced charge) might be responsible for the low activity detected for (especially) paralithocin 1. A net positive charge of AMPs is known to facilitate electrostatic interactions with the negatively charged membrane and/or cell wall structures of bacteria.[46] For many other peptides, a C-terminal amide is even required for full biological activity.[33] The paralithocins might also act synergistically to fight infectious pathogens, as

shown for other arthropods.[47] SPE extracts from *P. camtschaticus* hemocytes have previously shown to be antibacterial at protein concentrations as low as 31.25 µg/ml, even against *E. coli*.[48] Assuming that the average molecular weight of the peptides in the extract were around 5 kDa, the observed MIC is close to 6 µM, a more than 16-fold lower MIC compared to the most potent paralithocin.

The reference AMP, cecropin P1, was active against all test bacteria with MIC values ranging from 0.8 to 100 µM. In contrast to the disulfide-rich paralithocins, cecropin P1 is a linear, α-helical AMP with no disulfide bridges, known to affect bacterial membranes (Kjuul et al., 1999). Of note, whereas the paralithocins show increased activity against the marine Gram-positive bacteria, cecropin P1 display a reduced and moderate activity, indicating different mechanism(s) of action between the two types of peptides.

Although the antimicrobial activity of the paralithocins are moderate-low, the peptides are present (and expressed) in relatively high concentrations indicating they are of importance for the animal. As shown for many other AMPs, such molecules may also act as immune effectors *in vivo*.[49] AMPs containing eight Cys residues have also shown to have other functions. For instance, the insect-derived drosomycin and the structurally related plant defensins, all having the cysteine-connectivity of Cys*1*-Cys*8*, Cys*2*-Cys*5*, Cys*3*-Cys*6*, and Cys*4*-Cys*7*, display multiple biological activities. Although some of them display antibacterial activity, their antimicrobial activity is mainly directed against fungi[50, 51] and drosomycin also display antiparasitic activity.[52] In addition, some of the plant defensins inhibit α-amylases[53] and proteases,[54] and some are shown to block calcium,[55] potassium,[56] and sodium channels.[57]

Table 2. Antibacterial Activities of the Native Paralithocins and the Reference Peptide Cecropin P1 Against Terrestrial and Marine Bacteria.

Minimal inhibitory concentrations, MIC (µM)[a]											
Bacterial strains[b]	**Gram-negative**					**Gram-positive**					
	Terrestrial		**Marine**			**Terrestrial**		**Marine**			
	E.c.	***P.a.***	***Y.r.***	***L.a.***	***A.s.***	***C.g.***	***S.a.***	***C.ma.***	***C.mo.***	***C.d.***	***C.f.***
Paralithocin 1	>100	>100	>100	>100	200	50	>100	200	100	200	200
Paralithocin 2	>100	>100	n.t.[c]	>100	>100	12.5	>100	50	50	50	25
Paralithocin 3	>100	>100	100	50	100	12.5	>100	25	12.5	25	12.5
Cecropin P1	1.6	1.6	0.8	1.6	3.1	1.6	100	50	6.3	25	25

[a] MIC was determined as the lowest concentration of peptide causing an optical density less than 50% of the growth control. [b] *E.c.*, *Escherichia coli*; *P.a.*, *Pseudomonas aeruginosa*; *Y.u.*, *Yersinia ruckeri*; *L.a.*, *Listonella anguillarum*; *A.s.*, *Aeromonas salmonicida*; *C.g.*, *Corynebacterium glutamicum*; *S.a.*, *Staphylococcus aureus*; *C.ma.*, *Carnobacterium maltaromaticum*; *C.mo.*, *Carnobacterium mobile*; *C.d.*, *Carnobacterium divergens*; *C.f.*, *Carnobacterium funditum*. [c] n.t., not tested.

In summary, the three paralithocin peptides are the first AMPs isolated from the red king crab. The three peptides are all cationic and contain eight cysteines, which are engaged in four intramolecular disulfide bridges in the native configuration. Although these peptides only possess weak antibacterial activity *in vitro*, the concentrations at sites of infections in the animal might be much higher. In addition, the high redundancy of the EST in the library indicates their importance in response to bacterial LPS. Nevertheless, their primary structure and disulfide arrangement is unique and could serve as a template for finding other, more potent AMPs in other species. Because these peptides seem to be produced in relatively high concentrations *in vivo*, we hypothesize that they play an important role in the animal´s host defense, and have other biological functions which remain to be explored. Due to their unique cysteine motifs and disulfide arrangement, the paralithocins may be assigned to a previously unknown family of AMPs. Further studies should be directed towards studying other biological functions (such as antifungal, chitin-binding, and protease inhibitor activity) of these peptides. Furthermore, the

evolutionary relationship between the paralithocins and other Cys-rich peptides should be elucidated.

EXPERIMENTAL SECTION

Collection of Animals and Sample Preparation. Live specimens (22 adult animals, average weight ca. 1500 g, all males) of the red king crab, *Paralithodes camtschaticus*,[58] were collected using crab pots in Varangerfjord, Finnmark, Norway. The animals were kept in tanks with circulating seawater until hemolymph collection. Hemolymph was collected from 10 animals by entering the unsclerotized membrane at the base of the chelipeds and pereiopods, as previously described.[32] The hemolymph (1925 mL in total) was immediately centrifuged at 800 x g at 4 °C for 20 min to separate the hemocytes from the plasma. The hemocytes were subsequently frozen at –80 °C, lyophilised, pooled and kept frozen at –20 °C until peptide extraction and purification. Six animals were used for nucleic acid extractions. Lipopolysaccharide (LPS), purified from *Aeromonas salmonicida*,[59] was diluted to a concentration of 1 mg/mL in PBS buffer, adjusted to 1000 mOsmol/kg, and injected into four animals with a dose of 200 ng/g crab. The injection was done with a G syringe through the unsclerotized membrane at the base of the chelipeds and pereiopods. Two control animals were treated identically, but injected with only PBS. The hemolymph was collected 42 h after injection and the hemocytes were isolated as described above.

Extraction and Purification of Antimicrobial Peptides. Freeze-dried hemocytes (9.92 g) were extracted as previously described.[32] Briefly, the material were extracted with 10 volumes (v/w) of 60% (v/v) MeCN (HPLC-grade, SDS) containing 0.1% trifluoroacetic acid

(TFA; Fluka Chemie AG) for 24 h at 4 °C. The supernatant was collected, stored at 4 °C, and the residue was extracted once again using the same conditions. The combined supernatants were incubated at –20 °C for 1-2 h to allow an organic and an aqueous phase to be partitioned. Due to high salt content (from the sample) and high MeCN content, the extraction medium precipitates high molecular weight proteins and proteolytic enzymes. The aqueous phase was collected, and dried in a vacuum centrifuge (Maxi Dry Lyo, Heto Laboratories). The material (3.58 g) was solubilised in 0.05% TFA to a concentration of 100 mg/mL. The extract was applied onto a reversed-phase solid phase extraction (SPE) cartridge,[32] and the peptides were eluted with MeCN-aqueous TFA (0.5%; 40:60, v/v). The peptide containing eluate was dried under vacuum, resuspended in 0.05% TFA, and subjected to preparative reversed-phase high performance liquid chromatography (RP-HPLC). The HPLC system (Waters Associates) consisted of a 600E pump, a 717 autosampler, a 2996 photodiode array (PDA) detector, and a SunFire Prep C_{18} (90Å; 5 µm; 10 x 250 mm) column. Elution was performed with a linear gradient running from 0 to 40% MeCN in 0.05% TFA over 75 min, with a flow rate of 2 mL/min. Absorbance was recorded in the range of 200-400 nm, and peak fractions were collected manually. Fractions were dried under vacuum and reconstituted in 200 µL distilled H_2O, and aliquots (50 µL) of the fractions were tested for antibacterial activity against *Listonella anguillarum* and *Corynebacterium glutamicum*. Active fractions were analyzed by electrospray ionization MS (ESI-MS) to determine their purity. Fractions with impurities were subjected to a second round of chromatography on a Symmetry 300 C18 column (Waters; 300 Å; 5 µm; 4.6 x 250 mm) and eluted under the same experimental conditions as described in step 2, but with a flow rate of 1.0 mL/min. Fractions were collected manually and submitted to ESI-MS analysis.

Mass Spectrometry Analysis. Peptide purity was analyzed using a Waters Micromass ZQ single quadrupole instrument equipped with an ESI ion source (Micromass Ltd) controlled by the MassLynx v4.1 software (Micromass). Samples were dissolved in H_2O and MeOH (50:50 v/v) containing 0.02% formic acid. Spectra were recorded in positive ion mode with a spray voltage of 3 kV and a cone voltage of 30 V. Samples were infused with a flow rate of 10 μL/min. Nitrogen was used as desolvation gas (flow 1200 L/h) and cone gas (flow 40 L/h), and the ionization temperature was set to 110 °C. The spectra were recorded in the continuum mode of acquisition and the quadrupole was scanned from *m/z* 100 to 2000 at 4 s per scan. MALDI-TOF analyses of reduced, alkylated and enzyme digested native peptides were done at the Department of Cell and Molecular Pharmacology, Medical University of South Carolina, United States. The top down MS/MS analysis was combined with the ProSightPTM program made by the Kelleher Group of the Department of Chemistry, University of Illinois, USA.[60, 61]

Amino Acid Analysis and Sequencing. The HPLC peak fractions P23, P30, and P34 were dissolved in 6 M HCl and hydrolyzed in vacuum for 20 h at 110 °C. Phenylisothiocyanate was added for amino acid derivatization, and phenylthiocarbamyl residues were separated and analyzed on an automatic amino acid analyzer (Model 421, Applied Biosystems, Perkin Elmer). The instrument was calibrated with 100 pmol of phenylthiohydantoin amino acid standards. Edman degradation of native and alkylated (4-vinyl-pyridine) peptides was done at the Biotechnology Centre of Oslo (University of Oslo, Norway), and was performed on a protein micro sequencer model 477A with a 120A PTH analyzer (Applied Biosystems) and a HP 241 Protein Sequencer (Hewlett-Packard).

Tissue Collection and RNA Extraction. The hemocytes from LPS challenged and non-challenged king crabs were sampled and subjected to RNA extraction (see below). Briefly, one mL of hemolymph was centrifuged at 13.200 rpm for 1 min, the serum was discarded and the pelleted hemocytes suspended in 1 ml of Qiazol (Qiagen). To ensure complete lysis, the hemocytes were squeezed through a 0.8 mm x 4.0 mm needle and left on ice for 5 min. RNA was purified according to the manufacturer's protocol (Qiagen), and dissolved in 50 mL 1mM Na-citrate, pH 6.4 (Ambion). RNA quantity, purity and integrity was verified spectrophotometrically (A260/A280) and by 1% agarose gel electrophoresis. The two samples containing the highest RNA concentration, were pooled and stored at –80 °C until used.

cDNA Library Construction and Sequence Analysis. A hemocyte cDNA library from LPS-injected animals was constructed with pooled total RNA from hemocytes of two king crabs using the SMART cDNA library constructions kit (Clontech) following the manufacturer´s instructions for long distance PCR. The cDNA was ligated into the λTriplEx2 arms and packaged, using the Gigapack III Gold packaging system (Stratagene). *Escherichia coli* strain BM25.8 was used to convert the λTriplEx2 phage into plasmid pTripleEx2 by mass excision and circularization. The converted library with the highest titer (originated from LPS injected animals) was then plated and grown overnight at 31 °C on 10 LB agar plates containing 50 μg/mL carbenicillin each. Three hundred and eighty-four randomly selected clones from this library were individually picked (Genetix colony picker robot), cultured in microtiter plates and subsequently sequenced from the 5′ end with the Clontech pTriplEx2 forward primer (5′-AGCTCCGAGATCTGGACGAGC-3′). The library was amplified in B25.8 following the manufacturer´s protocol (SMART cDNA library construction kit) and stored with 0.3 % $CHCl_3$ at 4 °C and added 7% DMSO to the tubes for storage at -80 °C.

Determination of Disulfide Connectivity

Chemicals and Equipment. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 0.5 M in H_2O), citric acid monohydrate, trisodium citrate dihydrate, *N*-ethylmaleimide (Nem), MeOH, and formic acid were purchased from Sigma-Aldrich. Fraction collection (T-piece 9:1 flow splitting post column) and HRMS/MS was conducted on an Accela 1250 HPLC pump and Accela Open autoinjector coupled though an ESI ion source to a QExactive mass spectrometer from Thermo, controlled by XCalibur 2.3. The mass spectrometer was operated in full scan mode (*m/z* 750-1250) during semipreparative HPLC, with spray voltage 3 kV, desolvation temperature 275 °C, and desolvation gas (nitrogen) at 40 L/h. A Polaris C18A (50 x 2 mm) from Agilent reversed-phase HPLC column was used for all separations. The mobile phase consisted of A: 0.1% formic acid in H_2O, B: MeOH, and a linear gradient 5-30% B over 45 min was used to elute the peptides.

Partial Reduction and Alkylation. The peptides, 50 μL of an approximately 50 μg/mL solution, were incubated with 10 μL TCEP (1 mM) in 0.25 M citric acid buffer (pH 3.0) at 40 °C for 5 min, then 10 μL Nem (40 mM in the same buffer) was added and the reaction proceeded for 30 min at rt.[62] The sample was then immediately subjected to semipreparative HPLC (V_{inj}: 25 μL) Fractions were collected manually, and fractions containing peptides with four, three, two, one, and zero disulfide bridges were collected. The partial reduction sometimes resulted in one major and one or more minor fractions of the partially reduced peptides. In these cases, all fractions were collected into separate vials. The most abundant fractions were used for further mass spectrometric investigations. The fractions were diluted with MeOH to consist of approximately 50:50 MeOH-aqueous 0.1% formic acid.

Tandem Mass Spectrometry Experiments. High resolution MS/MS was done by infusing the peptide fractions at 5 μL/min. A resolution of 140,000 (@ *m/z* 200) was used in all experiments. For full scan experiments (determination of MW and MI), 30 spectra were recorded, and 100-250 spectra were recorded per sample for MS/MS experiments. A normalized collision energy (NCE) of 30% was applied in the higher collision dissociation (HCD) cell for $[M + 5H]^{5+}$ precursor ions, NCE = 25% for $[M + 6H]^{6+}$ and NCE = 20% for $[M + 7H]^{7+}$. The quadrupole was operated with an isolation width of 4 Th.

Data File Conversion and Data Interpretation. The recorded .raw files were converted to mzXML by MassMatrix Mass Spectrometric Data File Conversion Tools 3.9 (www.massmatrix.org). To interpret all spectra, mMass 4.5.1 was used.[63-65] The program was slightly rewritten in Python 2.7 (www.python.org) to enable the identification of ions resulting from S-S cleavages, that is, $[y_n$ +/– H/SH] and $[b_n$ +/– H/SH] ions. As the most abundant isotopomers all were introduced to the HCD cell, this was exploited in structure characterization. The *m/z* of a monoisotopic ion was calculated by mMass, and during the interpretation of the spectra the presence of isotopomers (and the isotope distribution pattern), along with the recorded mass accuracy of the monoisotopic ion, was used to determine whether a recorded ion was due to an actual dissociation.

Bacterial Strains and Antibacterial Activity Testing. A total of 11 bacterial strains were tested for their susceptibility to the isolated peptides. The Gram-positive bacteria *C. glutamicum* (ATCC 13032) and *Staphylococcus aureus* (ATCC 9144), and the Gram-negative bacteria *E. coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 25853) were maintained

in culture at 37 °C. Three marine Gram-negative and fish pathogenic strains, namely *L. anguillarum* (AL104), *A. salmonicida* (AL2020), and *Yersinia ruckeri* (CCUG 14190), and five marine Gram-positive *Carnobacterium* strains; *C. maltaromaticum* (CCUG 34645), *C. mobile*, *C. divergens* and *C. funditum* (CCUG 34644) were all grown at 12 °C. All bacteria were grown in Mueller Hinton Broth (Difco Laboratories), except the marine Gram-positive Carnobacteria strains which were grown in modified Tryptic soy broth with added glucose (5%) and 2% NaCl. *L. anguillarum* and *A. salmonicida* were provided by the Norwegian Veterinary Institute (Oslo, Norway), whereas the marine Gram-positive strains were a gift from Prof. E. Ringø, Norwegian College of Fishery Science, UiT The Arctic University of Norway (Tromsø, Norway).

The antibacterial activities of the obtained HPLC fractions against two bacterial strains (*L. anguillarum*, and *C. glutamicum*) were determined as previously described.[66] Briefly, 50 µL of test material were incubated with an equal volume of actively growing bacteria in 96 well Nunc-plates. The starting concentration of bacteria were 5×10^3 cells per well, and the test strains were incubated at 37 °C for 24 h, whereas the marine bacteria were incubated at 12 °C for 72 h. Bacterial growth was monitored using a Bioscreen C microbiology reader (Labsystems Oy). Cecropin P1[67] (0.5 µg/mL) was used as a positive control for all strains, while distilled H_2O was added in the growth control. Antibacterial activity was determined when the optical density (OD) of the growth control (bacteria plus water) reached an absorbance of approximately 0.3. Fractions were regarded as active when the OD was less than 50% of the growth control. Serial two-fold dilutions (concentrations ranging from 1.6 to 100 µM) of purified, native peptides were made in double distilled H_2O and tested for activity against four terrestrial and eight marine bacterial strains according to the method described above. For selected marine strains, the peptide concentration of P23 was raised to 200 µM. The peptide content was determined by weighing mg amounts of purified samples. The minimal inhibitory

concentration (MIC) was defined as the lowest concentration of peptide causing an OD less than 50% of the growth control after 24 and 72 h of incubation for terrestrial and marine bacteria, respectively.

Bioinformatics and Sequence Analysis. Sequences were submitted to BLASTX and BLASTN[68] searches against GenBank provided by the NCBI server (www.ncbi.nlm.nih.gov/BLAST). Sequence redundancy was determined by contig analysis using CAP3[69] through the GOCART tool available at www.marinegenomics.org.[4] AMP similarity searches were performed against APD3, the Antimicrobial Peptide Database (aps.unmc.edu/AP/main.html);[70] DBAASP, Database of Antimicrobial Activity and Structure of Peptides (dbaasp.org/home);[71] DRAMP, Data Repository of Antimicrobial Peptides (dramp.cpu-bioinfor.org);[72] and $CAMP_{R3}$, Collection of Anti-Microbial Peptides (www.camp.bicnirrh.res.in/index.php).[73] Isoelectric points was calculated using the Expert Protein Analysis System (ExPASy; www.expacy.org) proteomics server of the Swiss Institute of Bioinformatics whereas theoretical monoisotopic masses were calculated using the ChemCalc online prediction software (http://www.chemcalc.org/).[74] Signal peptides was predicted by the SignalP 4.1 Server (www.cbs.dtu.dk/services/SignalP).[75] Nucleic acid sequences were edited and analyzed with the BioEdit program (www.mbio.ncsu.edu/BioEdit/bioedit.html) and uploaded to NCBI GenBank.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is free of charge on the ACS Publications website at DOI: xxxx.

This material includes data from amino acid, MS and MS/MS analysis, overview of contigs obtained from the EST library, nucleotide sequences, and peptide sequences deduced thereof (PDF).

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Notes

The authors declare no competing financial interest.

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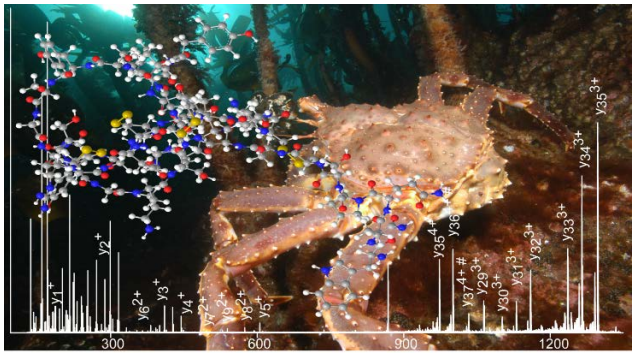
TOC Graphic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Supporting information

Paralithocins, Antimicrobial Peptides with Unusual Disulfide Connectivity from the Red King Crab, *Paralithodes camtschaticus*

Running title: *Cysteine arrangements and antimicrobial activity of peptides from the red king crab*

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Table S1. Amino Acid Composition of P23, P30, and P34

Table S1. Amino Acid Composition of P23, P30, and P34, Antimicrobial Peptides isolated from *P. camtschaticus* Haemocytes. Values shown are in Molecular Percentage.

Amino acid	Composition (mol %) of		
	P23	P30	P34
Aspartic acid/asparagine[a]	4.0	7.5	8.8
Threonine	0.0	4.3	2.3
Serine	22.5	4.3	11.0
Glutamic acid/glutamine[a]	7.0	8.1	8.9
Proline	5.7	21.2	16.1
Glycine	13.6	11.4	9.1
Alanine	6.1	12.3	10.7
Valine	0.0	4.3	2.9
Cysteine[b]	18.9	3.8	4.5
Methionine[b]	0.0	0.0	0.0
Isoleucine	8.0	3.1	5.8
Leucine	0.0	2.9	3.0
Tyrosine[b]	1.0	6.1	0.0
Phenylalanine	0.0	0.0	0.7
Lysine	3.3	2.7	3.1
Histidine	0.0	0.6	2.6
Arginine	9.9	7.4	10.5
Tryptophan[c]	-	-	-

[a] These amino acids could not be differentiated one from the other. [b] Partially destroyed during acid hydrolysis. [c] Not determined due to destruction during acid hydrolysis.

Table S2. Overview and Redundancy of Contigs obtained from an EST Library

Table S2. Overview and Redundancy of Contigs obtained from an EST Library originated from Hemocytes of two Red King Crabs 42 h post injection of *A. salmonicida* LPS. Contigs 2, 11 and 16 were selected for further Analysis and are High lightened in grey.

Contigs	Redundancy of 374 EST	Percent (%)	Expected redundancy of 10 000 EST
1	3	0,8	80,2
2	150	40,1	4010,7
3	4	1,1	107,0
4	2	0,5	53,5
5	18	4,8	481,3
6	2	0,5	53,5
7	17	4,5	454,5
8	3	0,8	80,2
9	3	0,8	80,2
10	2	0,5	53,5
11	16	4,3	427,8
12	3	0,8	80,2
13	2	0,5	53,5
14	4	1,1	107,0
15	2	0,5	53,5
16	31	8,3	828,9
Singletons	112	29,9	2994,7
	374	100,0	10000,0

Table S3. Masses of the Native Paralithocins

Table S3. Masses and calculated Properties of the Native Paralithocins.

Peptide	C-term	# aa	Monoisotopic mass (Da)			Net charge[d]
			Calcd.[a]	Exp.[b]	Δ m[c]	
Paralithocin 1	$-NH_2$	38	4075.6231	4075.6248	0.0017	+4.0
	-COOH		4076.6072	4076.6044	0.0028	+3.0
Paralithocin 2	$-NH_2$	47	5045.1668	5045.1705	0.0037	+4.0
	-COOH		5046.1508	5046.1480	0.0028	+3.0
Paralithocin 3	$-NH_2$	51	5559.4239	5559.4226	0.0013	+5.1
	-COOH		5560.4080	5560.4052	0.0028	+4.1

[a]Values calculated based on cysteines in oxidized forms. [b]Masses calculated from monoisotopic m/z $[M+nH]^{n+}$ values obtained from ESI-Q-TOF MS spectra. [c]The absolute difference between the calculated and experimental monoisotopic mass. [d]Net charge at pH 7 was calculated using Innovagen's peptide property calculator (http://pepcalc.com).

Table S4. Collision induced dissociation (CID) fragments obtained by MS/MS sequencing of native and partially reduced/alkylated Paralithocin 2

Table S4. Collision induced dissociation (CID) Fragments obtained by MS/MS sequencing of Native and Partially reduced/alkylated Paralithocin 2. (A) Native, intact Paralithocin 2, (B) Partially reduced and alkylated with 2 x NEM, (C) Partially reduced and alkylated with 4 x NEM, (D) Partially reduced and alkylated with 6 x NEM, (E) Completely reduced and alkylated with 8 x NEM.

(A) Paralithocin 2. Native. 4 cystine bridges. $[M+5H]^{5+}$ @ *m/z* 1010.0416

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
352.7021	352.7012	0.0010	2.7	1.15	2	y6
381.2132	381.2119	0.0013	3.5	13.92	2	y7
426.5682	426.5669	0.0013	3.1	3.95	3	y11
427.2904	427.2888	0.0016	3.8	0.20	1	y3
438.2474	438.2459	0.0015	3.4	0.80	1	b4
458.9193	458.9178	0.0015	3.2	11.56	3	y12
462.7452	462.7436	0.0016	3.5	30.58	2	y8
526.7929	526.7911	0.0018	3.4	15.66	2	y9
566.3066	566.3045	0.0021	3.7	28.47	1	b5
590.8221	590.8203	0.0018	3.1	10.60	2	y10
639.3488	639.3467	0.0021	3.3	44.22	2	y11
647.3759	647.3736	0.0023	3.5	0.24	1	y5
687.8752	687.8731	0.0021	3.1	83.33	2	y12
704.3973	704.3951	0.0023	3.2	1.72	1	y6
761.4194	761.4165	0.0029	3.8	14.80	1	y7
924.4835	924.4799	0.0037	4.0	15.53	1	y8
1052.5787	1052.5748	0.0039	3.7	2.30	1	y9
1072.2041	1072.1967	0.0075	6.9	0.13	4	b40
1277.6921	1277.6862	0.0060	4.7	0.36	1	y11
1289.5283	1289.5208	0.0075	5.8	0.12	3	b37
1374.7423	1374.7389	0.0034	2.5	0.59	1	y12

(B) Paralithocin 2. 2 x Nem, 3 cystine brigdes. $[M+5H]^{5+}$ @ *m/z* 1060.4630

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
174.1355	174.1349	0.0006	3.2	0.08	1	y1
219.6273	219.6266	0.0007	3.0	1.37	2	b4
244.1413	244.1404	0.0008	3.4	41.60	1	b2
271.1886	271.1877	0.0009	3.2	17.91	1	y2
283.6567	283.6559	0.0008	3.0	1.68	2	b5
295.6809	295.6797	0.0012	4.0	0.09	2	y4
341.1943	341.1932	0.0011	3.2	4.34	1	b3
352.7024	352.7012	0.0012	3.5	0.89	2	y6
381.2134	381.2119	0.0014	3.8	7.17	2	y7
397.6857	397.6843	0.0013	3.4	1.53	2	b6

426.5684	426.5669	0.0015	3.5	2.80	3	y11
427.2903	427.2888	0.0015	3.4	0.64	1	y3
438.2474	438.2459	0.0015	3.4	1.66	1	b4
458.9194	458.9178	0.0016	3.4	9.38	3	y12
461.7153	461.7136	0.0017	3.6	0.04	2	b7
462.7452	462.7436	0.0016	3.4	13.57	2	y8
526.7929	526.7911	0.0019	3.5	11.29	2	y9
534.9387	534.9368	0.0019	3.5	0.74	3	y13
543.2476	543.2453	0.0023	4.2	0.17	2	b8
566.3067	566.3045	0.0022	3.9	31.44	1	b5
590.3541	590.3521	0.0022	3.7	0.26	1	y4
590.8223	590.8203	0.0020	3.4	6.21	2	y10
639.3490	639.3467	0.0023	3.6	30.56	2	y11
647.3760	647.3736	0.0024	3.8	0.73	1	y5
687.8757	687.8731	0.0026	3.7	58.86	2	y12
704.3977	704.3951	0.0026	3.7	2.38	1	y6
761.4193	761.4165	0.0027	3.6	12.54	1	y7
794.3635	794.3614	0.0021	2.7	3.64	1	b6
801.9043	801.9015	0.0028	3.4	17.43	2	y13
858.4463	858.4436	0.0027	3.2	22.47	2	y14
915.9602	915.9570	0.0032	3.5	4.98	2	y15
922.4229	922.4200	0.0029	3.1	5.89	1	b7
924.4834	924.4799	0.0035	3.8	13.76	1	y8
1028.9493	1028.9458	0.0035	3.4	0.06	4	y38
1052.5789	1052.5748	0.0040	3.8	4.46	1	y9
1054.2040	1054.2077	-0.0037	-3.5	0.03	4	y39
1085.4866	1085.4833	0.0033	3.0	0.91	1	b8
1180.6386	1180.6334	0.0052	4.4	1.26	1	y10
1186.5263	1186.5310	-0.0047	-4.0	0.31	1	b9
1277.6915	1277.6862	0.0054	4.2	3.90	1	y11
1300.5794	1300.5739	0.0055	4.2	0.55	1	b10
1374.7435	1374.7389	0.0046	3.3	4.16	1	y12
1715.8849	1715.8799	0.0050	2.9	0.06	1	y14

(C) Paralithocin 2. 4 x Nem, 2 cystine brigdes. $[M+5H]^{5+}$ @ *m/z* 1110.8844

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
219.6272	219.6266	0.0006	2.9	1.77	2	b4
244.1412	244.1404	0.0008	3.2	42.76	1	b2
271.1885	271.1877	0.0008	3.1	17.88	1	y2
283.6567	283.6559	0.0008	2.9	2.73	2	b5
341.1942	341.1932	0.0010	3.0	4.50	1	b3
352.7022	352.7012	0.0010	2.9	0.05	2	y6
381.2132	381.2119	0.0013	3.4	7.67	2	y7
397.6857	397.6843	0.0013	3.4	3.41	2	b6
438.2476	438.2459	0.0017	3.8	0.98	1	b4
458.9193	458.9178	0.0015	3.2	2.34	3	y12
462.7451	462.7436	0.0015	3.2	10.51	2	y8
526.7927	526.7911	0.0017	3.2	7.56	2	y9
543.2471	543.2453	0.0018	3.3	0.90	2	b8
566.3066	566.3045	0.0021	3.6	60.41	1	b5

590.8218	590.8203	0.0015	2.5	1.61	2	y10
593.7713	593.7691	0.0022	3.7	0.26	2	b9
639.3489	639.3467	0.0022	3.5	18.73	2	y11
650.7958	650.7906	0.0053	8.1	0.09	2	b10
687.8754	687.8731	0.0023	3.4	47.31	2	y12
761.4196	761.4165	0.0030	4.0	8.66	1	y7
794.3644	794.3614	0.0030	3.8	11.76	1	b6
801.9045	801.9015	0.0030	3.8	7.37	2	y13
858.4462	858.4436	0.0027	3.1	16.96	2	y14
915.9605	915.9570	0.0035	3.8	0.35	2	y15
922.4226	922.4200	0.0027	2.9	25.51	1	b7
924.4832	924.4799	0.0034	3.6	8.72	1	y8
1029.9889	1029.9855	0.0034	3.3	0.34	2	y16
1052.5785	1052.5748	0.0037	3.5	1.60	1	y9
1085.4872	1085.4833	0.0039	3.6	4.15	1	b8
1186.5369	1186.5310	0.0059	5.0	0.48	1	b9
1277.6880	1277.6862	0.0018	1.4	0.64	1	y11
1300.5763	1300.5739	0.0024	1.8	1.59	1	b10
1374.7430	1374.7389	0.0041	3.0	2.52	1	y12

(D) Paralithocin 2. 6 x Nem, 1 cystine brigdes. $[M+5H]^{5+}$ @ *m/z* 1161.3082

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
171.1007	171.1002	0.0005	2.8	0.20	2	b3
219.6273	219.6266	0.0007	3.0	0.24	2	b4
244.1412	244.1404	0.0007	3.0	2.51	1	b2
271.1885	271.1877	0.0008	3.0	0.96	1	y2
283.6568	283.6559	0.0009	3.1	0.33	2	b5
295.6806	295.6797	0.0009	2.9	0.00	2	y4
324.1915	324.1904	0.0011	3.4	0.02	2	y5
341.1942	341.1932	0.0010	3.0	0.51	1	b3
352.7023	352.7012	0.0011	3.1	0.07	2	y6
381.2130	381.2119	0.0011	2.9	0.60	2	y7
397.6857	397.6843	0.0014	3.5	0.44	2	b6
426.5685	426.5669	0.0016	3.6	0.05	3	y11
427.2902	427.2888	0.0014	3.3	0.06	1	y3
438.2475	438.2459	0.0015	3.5	0.21	1	b4
458.9194	458.9178	0.0016	3.4	0.16	3	y12
461.7152	461.7136	0.0016	3.5	0.10	2	b7
462.7452	462.7436	0.0016	3.5	0.81	2	y8
526.7929	526.7911	0.0019	3.5	0.56	2	y9
534.9380	534.9368	0.0012	2.3	0.01	3	y13
543.2473	543.2453	0.0020	3.6	0.29	2	b8
566.3066	566.3045	0.0021	3.6	4.45	1	b5
590.3540	590.3521	0.0019	3.2	0.01	1	y4
590.8222	590.8203	0.0019	3.2	0.20	2	y10
593.7712	593.7691	0.0021	3.5	0.20	2	b9
639.3490	639.3467	0.0023	3.6	0.91	2	y11
647.3762	647.3736	0.0026	4.0	0.06	1	y5
650.7927	650.7906	0.0021	3.2	0.02	2	b10
687.8750	687.8731	0.0019	2.8	2.77	2	y12

704.3974	704.3951	0.0023	3.3	0.10	1	y6
761.4193	761.4165	0.0027	3.6	0.57	1	y7
764.8217	764.8190	0.0026	3.4	0.33	2	b11
794.3638	794.3614	0.0025	3.1	1.01	1	b6
801.9039	801.9015	0.0023	2.9	0.70	2	y13
835.8585	835.8561	0.0024	2.9	0.05	2	b13
858.4465	858.4436	0.0029	3.4	1.37	2	y14
880.7227	880.7211	0.0015	1.7	0.01	3	y21
915.9606	915.9570	0.0036	3.9	0.15	2	y15
922.4232	922.4200	0.0032	3.5	1.92	1	b7
924.4829	924.4799	0.0031	3.3	0.54	1	y8
980.9217	980.9197	0.0020	2.1	0.00	4	y32
1029.9890	1029.9855	0.0036	3.4	0.04	2	y16
1085.4869	1085.4833	0.0037	3.4	0.62	1	b8
1144.0194	1144.0139	0.0055	4.8	0.00	2	y17
1172.5284	1172.5246	0.0038	3.2	0.03	2	y18
1180.6378	1180.6334	0.0044	3.7	0.02	1	y10
1186.5349	1186.5310	0.0039	3.3	0.30	1	b9
1277.6907	1277.6862	0.0045	3.5	0.14	1	y11
1300.5784	1300.5739	0.0045	3.4	0.37	1	b10
1320.5806	1320.5781	0.0025	1.9	0.02	2	y21
1374.7440	1374.7389	0.0051	3.7	0.41	1	y12
1528.6370	1528.6308	0.0062	4.1	0.00	1	b11
1599.6748	1599.6679	0.0069	4.3	0.01	1	b12
1602.8013	1602.7958	0.0055	3.4	0.00	1	y13
1670.7105	1670.7050	0.0055	3.3	0.04	1	b13
1715.8866	1715.8799	0.0067	3.9	0.25	1	y14
1769.7773	1769.7734	0.0039	2.2	0.00	1	b14
1960.8214	1960.8321	-0.0107	-5.4	0.00	2	y32

(E) Paralithocin 2. 8 x Nem. $[M+5H]^{5+}$ @ *m/z* 1211.7411

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
421.2210	421.2194	0.0016	3.8	0.04	3	y11 -NH3
421.2210	421.2194	0.0016	3.8	0.04	1	b4 -NH3
426.8958	426.8949	0.0009	2.2	0.04	3	y11
438.2470	438.2459	0.0010	2.4	2.17	1	b4
453.1998	453.2003	-0.0006	-1.3	0.26	2	b7 -NH3
454.2325	454.2303	0.0023	5.0	0.06	2	y8 -H2O
461.7144	461.7136	0.0008	1.7	2.62	2	b7
504.2116	504.2116	-0.0000	-0.0	0.18	3	b11 -H2O
511.2428	511.2397	0.0030	5.9	0.11	4	y16 -H2O
518.2792	518.2778	0.0015	2.8	1.31	2	y9 -H2O
534.7344	534.7320	0.0024	4.4	0.66	2	b8 -NH3
543.2453	543.2453	0.0000	0.0	12.89	2	b8
566.3046	566.3045	0.0001	0.2	100.00	1	b5
568.2488	568.2540	-0.0052	-9.1	6.63	4	y17 -H2O
573.3213	573.3256	-0.0043	-7.5	1.47	1	y4 -H2O
584.7647	584.7638	0.0009	1.5	3.35	2	b9 -H2O
593.7694	593.7691	0.0003	0.5	9.63	2	b9
595.2786	595.2736	0.0050	8.4	0.20	4	b19

628.2875	628.2907	-0.0032	-5.1	0.74	3	b15
630.3470	630.3471	-0.0001	-0.1	4.31	1	y5 -H2O
630.8339	630.8335	0.0005	0.8	5.98	2	y11 -H2O
631.3238	631.3255	-0.0017	-2.6	1.28	2	y11 -NH3
641.7860	641.7853	0.0007	1.1	5.37	2	b10 -H2O
648.3592	648.3576	0.0016	2.4	0.24	1	y5
650.7913	650.7906	0.0007	1.0	2.87	2	b10
687.3691	687.3685	0.0006	0.9	8.17	1	y6 -H2O
688.0541	688.0499	0.0042	6.1	0.07	4	y21 -H2O
744.3936	744.3900	0.0036	4.8	42.82	1	y7 -H2O
755.8141	755.8137	0.0004	0.5	3.76	2	b11 -H2O
756.3132	756.3057	0.0074	9.8	11.01	2	b11 -NH3
764.8189	764.8190	-0.0001	-0.1	38.73	2	b11
777.3334	777.3348	-0.0015	-1.9	0.90	1	b6 -NH3
791.3333	791.3323	0.0010	1.3	0.03	2	b12 -H2O
791.8319	791.8243	0.0076	9.6	7.26	2	b12 -NH3
793.8842	793.8803	0.0039	4.9	2.19	2	y13 -NH3
794.3673	794.3614	0.0059	7.5	36.40	1	b6
800.3389	800.3376	0.0013	1.6	15.54	2	b12
804.8503	804.8503	-0.0000	-0.0	0.14	4	y25
818.3477	818.3530	-0.0053	-6.5	2.11	4	y26 -NH3
826.8532	826.8509	0.0024	2.9	1.26	2	b13 -H2O
827.3449	827.3429	0.0021	2.5	15.28	2	b13 -NH3
835.8576	835.8561	0.0015	1.8	13.46	2	b13
849.9321	849.9303	0.0018	2.1	1.87	2	y14 -H2O
867.3752	867.3805	-0.0052	-6.0	0.06	4	b28 -NH3
876.8831	876.8771	0.0060	6.8	0.09	2	b14 -NH3
884.7107	884.7132	-0.0025	-2.8	0.06	3	y20 -H2O
885.3905	885.3903	0.0002	0.2	1.67	2	b14
890.7249	890.7167	0.0082	9.2	1.61	3	y20
904.4077	904.4094	-0.0017	-1.9	0.08	1	b7 -H2O
905.3963	905.3934	0.0029	3.2	6.63	1	b7 -NH3
907.4545	907.4533	0.0012	1.3	52.18	1	y8 -H2O
922.4205	922.4200	0.0006	0.6	72.85	1	b7
923.7407	923.7358	0.0049	5.3	6.36	3	b21
925.4683	925.4639	0.0045	4.8	9.89	1	y8
985.4260	985.4291	-0.0031	-3.2	0.14	3	b23
1030.4789	1030.4775	0.0014	1.4	5.21	2	y16
1035.5514	1035.5483	0.0031	3.0	26.21	1	y9 -H2O
1053.5686	1053.5588	0.0098	9.3	7.34	1	y9
1085.4873	1085.4833	0.0040	3.7	42.32	1	b8
1110.4989	1110.4882	0.0107	9.6	0.06	4	y35 -H2O
1135.5178	1135.5006	0.0172	15.1	0.06	2	y17 -H2O
1144.5046	1144.5059	-0.0013	-1.1	8.39	2	y17
1163.6060	1163.6069	-0.0009	-0.8	8.01	1	y10 -H2O
1164.0214	1164.0114	0.0100	8.6	0.19	2	y18 -H2O
1168.5203	1168.5204	-0.0001	-0.1	10.35	1	b9 -H2O
1173.0278	1173.0166	0.0112	9.5	15.29	2	y18
1186.5319	1186.5310	0.0009	0.8	25.58	1	b9
1260.6680	1260.6596	0.0084	6.6	31.09	1	y11 -H2O
1278.0499	1278.0398	0.0101	7.9	0.17	2	y19 -H2O
1279.0353	1279.0392	-0.0039	-3.1	0.48	4	b39 -H2O

1283.5565	1283.5523	0.0042	3.3	7.28	3	y30
1283.5565	1283.5474	0.0092	7.1	7.28	1	b10 -NH3
1300.5770	1300.5739	0.0031	2.4	37.07	1	b10
1326.5774	1326.5662	0.0112	8.5	8.19	2	y20 -H2O
1335.5758	1335.5715	0.0044	3.3	28.14	2	y20
1357.7184	1357.7124	0.0060	4.4	95.16	1	y12 -H2O
1367.0819	1367.0738	0.0082	6.0	0.33	4	b43
1375.0896	1375.0926	-0.0030	-2.1	19.08	2	y21 -H2O
1375.7140	1375.7229	-0.0090	-6.5	31.63	1	y12
1376.0977	1376.0948	0.0029	2.1	26.16	2	b21 -H2O
1384.1110	1384.0978	0.0131	9.5	53.98	2	y21
1385.1094	1385.1000	0.0093	6.7	37.30	2	b21
1425.6257	1425.6164	0.0093	6.6	0.09	2	y22 -H2O
1426.1162	1426.1084	0.0078	5.5	0.40	2	y22 -NH3
1429.6122	1429.6168	-0.0047	-3.3	0.11	3	y33
1429.6122	1429.6095	0.0027	1.9	0.11	4	y44
1434.6309	1434.6217	0.0092	6.4	11.36	2	y22
1507.6509	1507.6401	0.0108	7.2	0.27	2	y23 -NH3
1516.1659	1516.1533	0.0126	8.3	8.59	2	y23
1528.6432	1528.6308	0.0124	8.1	4.06	1	b11
1551.6848	1551.6719	0.0129	8.3	5.91	2	y24
1585.7723	1585.7692	0.0031	2.0	32.45	1	y13 -H2O
1599.6768	1599.6881	-0.0113	-7.1	7.53	2	y25 -H2O
1599.6768	1599.6679	0.0089	5.6	7.53	1	b12
1603.7935	1603.7798	0.0137	8.5	9.47	1	y13
1608.7046	1608.6934	0.0112	7.0	3.46	2	y25
1644.2090	1644.2119	-0.0029	-1.8	2.31	2	y26
1645.2081	1645.2141	-0.0060	-3.6	1.59	2	b26
1653.6863	1653.6784	0.0078	4.7	1.32	1	b13 -NH3
1670.7111	1670.7050	0.0061	3.6	21.38	1	b13
1698.8556	1698.8533	0.0023	1.3	28.23	1	y14 -H2O
1752.7544	1752.7469	0.0075	4.3	0.11	1	b14 -NH3
1758.2520	1758.2404	0.0116	6.6	4.37	2	y27
1769.7780	1769.7734	0.0046	2.6	7.68	1	b14
1813.8782	1813.8803	-0.0021	-1.1	3.76	1	y15 -H2O
1814.8768	1814.8643	0.0126	6.9	3.69	1	y15 -NH3
1882.8635	1882.8575	0.0061	3.2	5.29	1	b15
1973.3547	1973.3512	0.0036	1.8	0.97	2	y31
2042.9064	2042.9211	-0.0148	-7.2	0.28	1	y16 -NH3
2326.9951	2327.0154	-0.0203	-8.7	0.37	1	y18 -H2O
2328.0144	2327.9995	0.0150	6.4	0.51	1	y18 -NH3
2556.0708	2556.0563	0.0145	5.7	0.16	1	y19 -NH3
2652.1387	2652.1251	0.0136	5.1	1.30	1	y20 -H2O
2653.1245	2653.1091	0.0154	5.8	3.97	1	y20 -NH3
2749.1677	2749.1778	-0.0101	-3.7	2.77	1	y21 -H2O
2750.1609	2750.1618	-0.0010	-0.3	7.94	1	y21 -NH3
2751.1641	2751.1823	-0.0182	-6.6	7.32	1	b21 -H2O
2752.1448	2752.1663	-0.0215	-7.8	3.01	1	b21 -NH3

Table S5. Collision induced dissociation (CID) fragments obtained by MS/MS sequencing of Native and partially alkylated Paralithocin 3

Table S5. Collision induced dissociation (CID) fragments obtained by MS/MS sequencing of Native and partially alkylated Paralithocin 3. (A) Native, intact paralithocin 3, (B) Partially Reduced and alkylated with 2 x NEM, (C) Partially reduced and alkylated with 4 x NEM, (D) Partially reduced and alkylated with 6 x NEM, (E) Completely reduced and alkylated with 8 x NEM.

(A) Paralithocin 3. Native. 4 cystine brigdes. $[M+7H]^{7+}$ @ *m/z* 795.3528

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
244.1404	244.1404	0.0000	0.0	33.89	1	b2
253.1296	253.1295	0.0001	0.4	0.14	1	y2
291.6350	291.6352	-0.0002	-0.7	0.00	2	y6
367.1731	367.1724	0.0006	1.7	0.54	1	y3
372.1994	372.1990	0.0004	1.0	100.00	1	b3
454.2051	454.2045	0.0006	1.3	1.78	1	y4
469.2523	469.2518	0.0005	1.1	0.23	1	b4
503.2255	503.2249	0.0006	1.2	0.03	2	y9
525.2424	525.2416	0.0009	1.6	0.49	1	y5
526.2737	526.2732	0.0005	1.0	3.84	1	b5
559.7674	559.7669	0.0005	0.9	0.00	2	y10
582.2642	582.2631	0.0011	1.9	0.92	1	y6
623.3262	623.3260	0.0002	0.3	0.05	1	b6
724.3743	724.3737	0.0007	0.9	0.06	1	b7
745.3275	745.3264	0.0011	1.5	0.20	1	y7
807.1765	807.1809	-0.0044	-5.5	0.00	6	y44
824.0258	824.0222	0.0037	4.5	0.01	6	y45
840.2001	840.1976	0.0025	3.0	0.13	6	y46
849.7040	849.7012	0.0028	3.3	0.01	6	y47
865.8793	865.8767	0.0026	3.0	0.29	6	y48
887.2219	887.2198	0.0021	2.4	0.02	6	y49
901.7289	901.7251	0.0039	4.3	0.10	6	y50
968.4193	968.4156	0.0037	3.8	0.21	5	y44
988.6295	988.6251	0.0044	4.4	1.03	5	y45
1008.0393	1008.0357	0.0036	3.5	3.52	5	y46
1019.4434	1019.4400	0.0035	3.4	1.31	5	y47
1038.8538	1038.8505	0.0032	3.1	0.86	5	y48
1064.4596	1064.4622	-0.0026	-2.5	0.20	5	y49
1081.8683	1081.8687	-0.0003	-0.3	0.00	5	y50
1210.2623	1210.2677	-0.0053	-4.4	0.18	4	y44
1235.5309	1235.5296	0.0013	1.0	1.67	4	y45
1259.7921	1259.7928	-0.0007	-0.5	0.35	4	y46
1274.0514	1274.0482	0.0033	2.5	0.04	4	y47

(B) Paralithocin 3. Native 2 x Nem, 3 cystine brigdes. $[M+7H]^{7+}$ @ *m/z* 831.3688

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
252.1462	252.1455	0.0007	2.6	3.79	1	y2
366.1894	366.1884	0.0010	2.7	2.45	1	y3
453.2217	453.2205	0.0013	2.8	10.48	1	y4
524.2593	524.2576	0.0018	3.4	4.04	1	y5
581.2808	581.2790	0.0018	3.1	9.16	1	y6
623.3289	623.3260	0.0029	4.6	0.07	1	b6
637.3276	637.3255	0.0021	3.3	0.06	2	y11
724.3757	724.3737	0.0021	2.9	6.26	1	b7
744.3446	744.3424	0.0022	3.0	1.53	1	y7
873.3792	873.3809	-0.0016	-1.9	0.05	5	y38
895.3891	895.3905	-0.0014	-1.5	0.06	5	y39 -H2O
907.4108	907.4057	0.0051	5.7	0.89	1	y8
915.4001	915.4009	-0.0009	-0.9	0.16	5	y40 -NH3
936.2140	936.2127	0.0013	1.4	0.14	5	y41
953.6178	953.6191	-0.0013	-1.3	0.14	5	y42
973.0302	973.0296	0.0005	0.6	0.66	5	y43
991.6641	991.6726	-0.0085	-8.5	0.02	4	y34
1004.4619	1004.4585	0.0035	3.4	0.66	1	y9
1012.9246	1012.9163	0.0083	8.2	0.06	4	y35 -NH3
1018.6485	1018.6410	0.0075	7.4	0.05	5	y44
1038.8565	1038.8505	0.0059	5.7	1.28	5	y45
1040.9324	1040.9413	-0.0089	-8.6	0.04	4	y36 -H2O
1040.9489	1040.9413	0.0075	7.2	0.03	4	y36 -H2O
1041.1919	1041.1873	0.0046	4.4	0.10	4	y36 -NH3
1054.6627	1054.6590	0.0038	3.6	1.30	5	y46 -H2O
1054.8629	1054.8558	0.0072	6.8	0.84	5	y46 -NH3
1058.2634	1058.2611	0.0024	2.2	0.41	5	y46
1066.0648	1066.0633	0.0016	1.5	0.07	5	y47 -H2O
1066.2626	1066.2601	0.0025	2.4	0.03	5	y47 -NH3
1069.6692	1069.6654	0.0038	3.6	0.15	5	y47
1073.7122	1073.7150	-0.0028	-2.6	0.12	4	y37
1086.9716	1086.9716	-0.0001	-0.0	0.23	4	y38 -H2O
1087.2208	1087.2176	0.0032	3.0	0.35	4	y38 -NH3
1089.0775	1089.0759	0.0016	1.5	0.17	5	y48
1091.4764	1091.4743	0.0022	2.0	0.24	4	y38
1114.6854	1114.6876	-0.0022	-2.0	0.77	5	y49
1118.9945	1118.9863	0.0083	7.4	0.28	4	y39 -H2O
1119.2328	1119.2323	0.0005	0.5	0.23	4	y39 -NH3
1123.4876	1123.4889	-0.0013	-1.2	0.34	4	y39
1128.4807	1128.4919	-0.0112	-9.9	0.20	5	y50 -H2O
1148.2504	1148.2560	-0.0056	-4.9	0.64	4	y40
1165.7555	1165.7574	-0.0019	-1.6	0.24	4	y41 -NH3
1170.0042	1170.0140	-0.0099	-8.4	0.34	4	y41
1187.2623	1187.2694	-0.0070	-5.9	0.17	4	y42 -H2O

1187.5159	1187.5154	0.0005	0.4	0.62	4	y42 -NH3
1191.7651	1191.7720	-0.0069	-5.8	0.36	4	y42
1216.0248	1216.0352	-0.0104	-8.6	0.09	4	y43
1268.5492	1268.5468	0.0024	1.9	0.55	4	y44 -H2O
1268.7992	1268.7928	0.0064	5.1	0.51	4	y44 -NH3
1273.0386	1273.0494	-0.0108	-8.5	0.83	4	y44
1293.8019	1293.8087	-0.0068	-5.3	0.13	4	y45 -H2O
1294.0581	1294.0547	0.0034	2.6	0.44	4	y45 -NH3
1298.3079	1298.3113	-0.0035	-2.7	0.57	4	y45
1318.0647	1318.0719	-0.0072	-5.5	0.15	4	y46 -H2O
1318.3242	1318.3179	0.0063	4.8	0.05	4	y46 -NH3
1322.5747	1322.5745	0.0002	0.1	0.26	4	y46
1332.5729	1332.5733	-0.0004	-0.3	0.06	4	y47 -NH3
1349.9044	1349.8913	0.0131	9.7	0.03	3	y35 -H2O
1388.5953	1388.6051	-0.0098	-7.0	0.03	4	y49 -H2O
1410.5984	1410.6091	-0.0107	-7.6	0.25	4	y50 -NH3
1448.9473	1448.9597	-0.0125	-8.6	0.22	3	y38 -H2O
1449.2797	1449.2877	-0.0081	-5.6	0.08	3	y38 -NH3
1454.9528	1454.9632	-0.0105	-7.2	0.15	3	y38
1491.6421	1491.6459	-0.0038	-2.6	0.26	3	y39 -H2O
1491.9761	1491.9739	0.0022	1.4	0.35	3	y39 -NH3
1497.6445	1497.6494	-0.0049	-3.3	0.33	3	y39
1524.6593	1524.6687	-0.0094	-6.2	0.67	3	y40 -H2O
1524.9911	1524.9967	-0.0056	-3.7	0.67	3	y40 -NH3
1530.6560	1530.6722	-0.0162	-10.6	0.07	3	y40
1553.6655	1553.6794	-0.0139	-8.9	0.04	3	y41 -H2O
1559.6643	1559.6829	-0.0186	-11.9	0.13	3	y41
1583.0164	1583.0181	-0.0017	-1.1	0.04	3	y42 -NH3
1615.3523	1615.3690	-0.0167	-10.3	0.63	3	y43 -NH3
1621.0283	1621.0445	-0.0162	-10.0	0.12	3	y43
1691.0415	1691.0599	-0.0184	-10.9	0.04	3	y44 -H2O
1691.3829	1691.3880	-0.0050	-3.0	0.03	3	y44 -NH3

(C) Paralithocin 3. Native 4 x Nem, 2 cystine brigdes. $[M+7H]^{7+}$ @ *m/z* 867.3836

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
244.1411	244.1404	0.0007	2.8	3.38	1	b2
252.1463	252.1455	0.0006	2.5	0.12	1	y2
291.1435	291.1432	0.0004	1.3	0.06	2	y6
366.1895	366.1884	0.0011	3.0	0.06	1	y3
453.2218	453.2205	0.0014	3.0	0.85	1	y4
524.2590	524.2576	0.0014	2.7	0.11	1	y5
581.2806	581.2790	0.0016	2.8	1.61	1	y6
817.3525	817.3542	-0.0018	-2.1	0.12	5	y33 -H2O
836.8597	836.8689	-0.0093	-11.1	0.08	6	y42
843.9658	843.9617	0.0040	4.8	0.07	5	y34
864.3596	864.3621	-0.0025	-2.8	0.05	5	y35
872.4246	872.4228	0.0017	2.0	0.13	2	y14
923.8069	923.8031	0.0038	4.1	0.10	5	y38
932.9103	932.9073	0.0030	3.2	0.06	4	y30
969.2258	969.2285	-0.0026	-2.7	0.17	5	y40

986.6293	986.6349	-0.0055	-5.6	0.17	5	y41
997.4394	997.4329	0.0065	6.5	0.10	4	y32
1004.0408	1004.0413	-0.0005	-0.5	0.56	5	y42
1023.4531	1023.4518	0.0013	1.2	2.27	5	y43
1025.9440	1025.9436	0.0004	0.3	0.05	4	y33
1069.0602	1069.0632	-0.0030	-2.8	0.18	5	y44
1089.2671	1089.2727	-0.0056	-5.2	0.13	5	y45
1108.4686	1108.4717	-0.0031	-2.8	0.12	4	y36
1108.6882	1108.6833	0.0050	4.5	0.31	5	y46
1116.6857	1116.6823	0.0034	3.1	0.29	5	y47 -NH3
1117.5442	1117.5425	0.0017	1.5	0.06	1	y10
1136.7422	1136.7427	-0.0005	-0.5	0.07	4	y37
1139.5096	1139.4981	0.0115	10.1	0.08	5	y48
1154.5021	1154.5020	0.0001	0.1	0.35	4	y38
1165.1079	1165.1098	-0.0019	-1.7	0.29	5	y49
1182.0232	1182.0140	0.0092	7.8	0.08	4	y39 -H2O
1182.5153	1182.5163	-0.0010	-0.8	0.27	5	y50
1243.5349	1243.5406	-0.0057	-4.6	0.10	3	y30
1250.5458	1250.5431	0.0027	2.1	0.45	4	y42 -NH3
1274.7946	1274.8063	-0.0118	-9.2	0.87	4	y43 -NH3
1329.5679	1329.5748	-0.0069	-5.2	0.40	3	y32
1336.0717	1336.0772	-0.0055	-4.1	0.16	4	y44
1361.3304	1361.3391	-0.0086	-6.3	0.05	4	y45
1533.3361	1533.3247	0.0113	7.4	0.07	3	y38 -NH3

(D) Paralithocin 3. Native 6 x Nem, 1 cystine brigdes. $[M+7H]^{7+}$ @ *m/z* 903.4006

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
252.1462	252.1455	0.0007	2.7	3.36	1	y2
366.1892	366.1884	0.0008	2.2	0.53	1	y3
453.2218	453.2205	0.0013	2.9	8.70	1	y4
524.2592	524.2576	0.0017	3.1	3.13	1	y5
581.2807	581.2790	0.0017	2.9	7.52	1	y6
694.3500	694.3469	0.0031	4.4	0.20	2	y12
744.3445	744.3424	0.0022	2.9	2.93	1	y7
781.3436	781.3470	-0.0033	-4.3	0.16	3	y18
808.3781	808.3754	0.0027	3.3	1.53	2	y13
872.4262	872.4228	0.0034	3.9	3.20	2	y14
907.4108	907.4057	0.0051	5.7	0.35	1	y8
907.9435	907.9414	0.0022	2.4	0.36	2	y15
914.7839	914.7842	-0.0003	-0.3	0.16	5	y35
937.3944	937.4010	-0.0067	-7.1	0.14	5	y36
960.0270	960.0178	0.0091	9.5	0.17	5	y37
974.2256	974.2253	0.0003	0.3	0.33	5	y38
999.8365	999.8370	-0.0005	-0.5	0.56	5	y39
1004.4616	1004.4585	0.0031	3.1	0.89	1	y9
1019.6416	1019.6507	-0.0091	-8.9	0.65	5	y40
1028.9529	1028.9468	0.0061	6.0	0.22	4	y32
1054.4618	1054.4635	-0.0017	-1.6	0.84	5	y42
1057.4611	1057.4575	0.0036	3.4	0.63	4	y33
1073.8770	1073.8740	0.0029	2.7	0.65	5	y43

1117.5435	1117.5425	0.0009	0.8	0.18	1	y10
1119.4868	1119.4854	0.0014	1.3	1.16	5	y44
1139.6845	1139.6949	-0.0105	-9.2	0.22	5	y45
1143.2332	1143.2284	0.0047	4.1	0.20	4	y35
1159.1096	1159.1055	0.0041	3.6	0.54	5	y46
1170.4981	1170.5098	-0.0117	-10.0	0.18	5	y47
1171.4967	1171.4995	-0.0028	-2.3	0.46	4	y36
1171.5087	1171.5168	-0.0081	-6.9	1.11	2	y18
1171.5087	1171.4995	0.0092	7.9	1.11	4	y36
1189.9171	1189.9203	-0.0032	-2.7	0.19	5	y48
1214.5282	1214.5182	0.0100	8.2	0.17	3	y28
1215.5332	1215.5320	0.0012	1.0	13.22	5	y49
1217.5265	1217.5298	-0.0033	-2.7	0.28	4	y38
1249.5415	1249.5444	-0.0029	-2.3	1.10	4	y39
1266.5474	1266.5519	-0.0046	-3.6	1.79	3	y29
1274.3113	1274.3115	-0.0002	-0.2	0.37	4	y40
1285.5657	1285.5591	0.0066	5.1	0.20	3	y30
1296.0689	1296.0695	-0.0007	-0.5	0.25	4	y41
1317.8182	1317.8275	-0.0093	-7.0	0.38	4	y42
1319.5518	1319.5596	-0.0078	-5.9	0.21	3	y31
1365.0852	1365.0893	-0.0041	-3.0	1.29	2	y21
1371.5885	1371.5933	-0.0048	-3.5	0.48	3	y32
1371.5890	1371.5933	-0.0043	-3.1	1.46	3	y32
1399.0994	1399.1049	-0.0056	-4.0	0.26	4	y44
1409.6051	1409.6076	-0.0025	-1.7	0.54	3	y33
1424.3574	1424.3668	-0.0094	-6.6	0.25	4	y45
1599.3710	1599.3582	0.0128	8.0	0.21	3	y37
1623.0260	1623.0372	-0.0112	-6.9	0.20	3	y38
1665.7230	1665.7234	-0.0004	-0.2	0.29	3	y39

(E) Paralithocin 3. Native 4 x Nem. $[M+7H]^{7+}$ @ *m/z* 939.4169

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation
116.071	116.071	0.001	4.3	2.03	1	y1
253.130	253.130	0.001	2.2	0.15	1	y2
367.173	367.172	0.001	2.4	0.16	1	y3
454.209	454.204	0.005	10.1	0.73	1	y4
525.245	525.242	0.003	6.4	0.21	1	y5
551.254	551.254	0.000	0.6	0.01	2	y10 -NH3
582.265	582.263	0.002	2.6	0.40	1	y6
582.284	582.279	0.005	8.8	0.53	3	y14
639.262	639.266	-0.004	-6.2	0.00	4	y19 -NH3
678.293	678.291	0.002	3.5	0.01	4	y20 -NH3
745.328	745.326	0.002	2.5	0.12	1	y7
893.390	893.381	0.009	10.0	0.02	5	y33 -NH3
908.393	908.390	0.003	3.4	0.07	1	y8
908.442	908.433	0.009	9.8	0.04	2	y15
916.388	916.387	0.001	1.5	0.01	5	y34 -NH3
921.136	921.142	-0.006	-6.8	0.01	4	y27
947.074	947.066	0.008	8.5	0.01	3	y21 -NH3
972.407	972.417	-0.010	-10.5	0.01	6	y44 -NH3

981.930	981.926	0.004	4.4	0.03	4	y29
991.931	991.924	0.007	6.6	0.02	4	y30 -NH3
1005.443	1005.442	0.001	0.6	0.03	1	y9
1005.443	1005.434	0.009	9.2	0.03	6	y46 -NH3
1013.957	1013.949	0.008	7.9	0.01	2	y16 -NH3
1014.932	1014.937	-0.005	-5.0	0.03	6	y47 -NH3
1017.768	1017.775	-0.007	-7.1	0.02	6	y47
1024.851	1024.844	0.007	6.6	0.01	5	y38
1033.943	1033.951	-0.007	-6.9	0.04	6	y48
1050.466	1050.456	0.010	9.7	0.01	5	y39
1052.464	1052.456	0.008	7.7	0.02	6	y49 -NH3
1066.961	1066.961	-0.000	-0.0	0.02	6	y50 -NH3
1069.807	1069.799	0.008	7.9	0.01	6	y50
1070.265	1070.270	-0.005	-4.2	0.01	5	y40
1087.679	1087.676	0.003	3.0	0.01	5	y41
1105.081	1105.082	-0.002	-1.6	0.02	5	y42
1120.740	1120.731	0.008	7.5	0.06	4	y33
1122.483	1122.481	0.002	2.2	0.11	3	y25 -NH3
1124.493	1124.493	0.000	0.3	0.04	5	y43
1127.987	1127.977	0.010	8.6	0.03	2	y17 -NH3
1136.492	1136.490	0.002	1.4	0.02	2	y17
1149.477	1149.488	-0.011	-9.9	0.01	4	y34
1163.502	1163.496	0.007	5.9	0.04	2	y18 -NH3
1172.010	1172.009	0.001	1.2	0.04	2	y18
1190.323	1190.314	0.009	7.5	0.02	5	y45
1204.183	1204.175	0.008	6.9	0.01	3	y26
1206.330	1206.319	0.011	8.7	0.01	5	y46 -NH3
1217.734	1217.723	0.011	9.1	0.01	5	y47 -NH3
1221.138	1221.129	0.009	7.7	0.01	5	y47
1222.188	1222.179	0.009	7.7	0.01	3	y27 -NH3
1227.854	1227.854	-0.000	-0.2	0.01	3	y27
1234.783	1234.773	0.010	7.8	0.03	4	y36
1240.545	1240.539	0.006	4.6	0.13	5	y48
1263.054	1263.044	0.010	7.8	0.09	4	y37
1266.154	1266.151	0.003	2.4	0.05	5	y49
1280.803	1280.804	-0.001	-0.7	0.05	4	y38
1283.566	1283.557	0.008	6.3	0.10	5	y50
1303.228	1303.223	0.005	3.7	0.02	3	y29 -NH3
1312.824	1312.818	0.006	4.4	0.04	4	y39
1322.229	1322.230	-0.001	-0.6	0.03	3	y30 -NH3
1327.897	1327.906	-0.008	-6.1	0.02	3	y30
1337.575	1337.585	-0.010	-7.3	0.04	4	y40
1359.340	1359.343	-0.004	-2.7	0.01	4	y41
1364.091	1364.088	0.004	2.7	0.01	2	y20
1381.102	1381.101	0.001	0.7	0.01	4	y42
1398.259	1398.249	0.010	7.0	0.02	3	y31 -NH3
1401.106	1401.108	-0.002	-1.2	0.02	4	y43 -NH3
1428.595	1428.609	-0.014	-9.5	0.01	2	y21 [31-51]
1450.283	1450.283	0.000	0.3	0.03	3	y32 -NH3
1455.953	1455.958	-0.005	-3.6	0.07	3	y32
1476.636	1476.638	-0.001	-0.9	0.03	2	y22 -NH3
1488.308	1488.297	0.011	7.3	0.03	3	y33 -NH3

1493.985	1493.973	0.013	8.5	0.01	3	y33

Figure S1. Consensus nucleotide and amino acid sequences of contigs

(A) Contig 2 - Paralithocin 1

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1    CATCAGTAAGAAAGAATCCAGTCGGGACTCAGGACAGAACTCAGCTACTATTTCCACTCT 60

61   TACAGGTTGTGTTGACAGTTAACCATGGGTCCCATGAAGGTGTTGTTGGTTCTGTTGGTG 120
                               M  G  P  M  K  V  L  L  V  L  L  V

121  GTCATGGTGGCTGCACCACACATTGCAGATGCTTGGCAGCAACCGTCCTGTAGTTCCATC 180
      V  M  V  A  A  P  H  I  A  D  A  W  Q  Q  P  S  C  S  S  I

181  TGCGACTATAGCTGCGGCAAATCCGCCTGTATCTCCTACAGTGGTAGATGCGGCTGTTGC 240
      C  D  Y  S  C  G  K  S  A  C  I  S  Y  S  G  R  C  G  C  C

241  GCTTCCTGCCGCAGAGGACCGATTTACGGTTGACGCTGAAGGAGGAGGATCCACCCCCAG 300
      A  S  C  R  R  G  P  I  Y  G  *

301  GAGAACCCCCAGGAGAACTGACCCACTCACTATACACCGTTCACATTAGCTTGTGCTTTA 360
361  ATGTACCCCTTTGCTTACGAGCAACCTTTTTCTGAGCTTGAGATAAAATCGTCTAATGTT 420
421  AGCCATTGAACAAATCAATCGTGGCTGCATAACAGGGTCAGTATCTCTGACGTTCATTAT 480
481  GAAGCATATCCATTTGTATTTTTGAAGTCCCTTTAAGCAGTATTATATAAGATATATCAT 540
541  GATATAGAATACCCCTGTAGTCTAACGTCTATTTACTTTATCATGTGCTTTAATAAAACT 600
601  TATCATGTACGGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 644
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(B) Contig 16 - Paralithocin 2

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1    GACACCAGTAAGAAAGAATCCAGTTGGGAGTCAGGACAGAGCTCTCTGGAACTTCCACTC 60

61   TTACAGATTGTGTTGATATTTGAACCATGGGAGCCGCGAAGGTGTTGTTGGTTGTGTTGG 120
                               M  G  A  A  K  V  L  L  V  V  L

121  CGGTCATGGTGGCTGTACCTAACCTTGCAGAGGGTAGGTCTCCACCACAATGCCAATATA 180
     A  V  M  V  A  V  P  N  L  A  E  G  R  S  P  P  Q  C  Q  Y

181  CTAACTGCGCTGCTGTGTTATGTCCAGCCGTCTACTGTGCAAACGCCTACACACCCCCGT 240
     T  N  C  A  A  V  L  C  P  A  V  Y  C  A  N  A  Y  T  P  P

241  GTGGCTGCTGTGACATCTGCCCTCCACAGAAATACGGAGGTGGCTACCGGCCTAGAGGCT 300
     C  G  C  C  D  I  C  P  P  Q  K  Y  G  G  G  Y  R  P  R  G

301  GAAGGAGGAGGACCCACCCCCAGGMGAACCCCCAGGAGAACTGACCCATTCACTATACAC 360
     *

361  CGTACACATTAACTGGACAAATTTACTTCTCCTAGCAGTTAGTTATAATTAGCTGAAACT 420
421  GTATTAATCTATTCACATATTGCTGCTAGCCAATCACATTCATTCGTAGCTGCTCGCCAG 480
481  GATAAAATCGACACCTAAATGGCACGAAAGGTCTTAAAATTGACAGAATGGGGCGGAAAT 540
541  TCTTATGCTTATTATTATTAAAATATAATAATCTTATACCTAAAGGTTTGGAATGATTCA 600
601  ACGGTTAATAATGAACTTGGACTTTATTCAATAAACCCTTCTGATCTGAGTACTKHRAAA 660
661  AAAAAAAAAAAAAAAAAAAAAAAAAA 688
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(C) Contig 11 - Paralithocin 3

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1    GAGTTCAGTGAAGAGCAAATCAGGACGAGATACACTGTTGCTTTAATTTTCAAGGTTGCG 60

61   TTGTGTGGTCAGTTAACCATGGGTCCCATGAAGGTGTTGTTGGTTATGTTGGTGGTCATG 120
                         M  G  P  M  K  V  L  L  V  M  L  V  V  M

121  GTGGCTGCTCCCCACATCGCAGATGCTAGGAGTCAACCAGGACCAACCTGTCCATCCTCT 180
       V  A  A  P  H  I  A  D  A  R  S  Q  P  G  P  T  C  P  S  S

181  GTCCAGGCCATCCTCTGCGACAATAGGTGTGGTAGATCAGCCTGTTCATACTACATAGAG 240
       V  Q  A  I  L  C  D  N  R  C  G  R  S  A  C  S  Y  Y  I  E

241  CGATGCGCCTGTTGTGCTAAATGCAACAGAATACCGTATTACGGAGCTAGCAACCATCCT 300
       R  C  A  C  C  A  K  C  N  R  I  P  Y  Y  G  A  S  N  H  P

301  GGACGCTGAAGGAGGAGGACCCACCCCCAGGAGAACTGACCCACTCACTATACACCGTTC 360
       G  R  *

361  ACATTAACTGGACAAATTAACTCTTTCAAAACCTGCATAGATTGTATTCAACTTCGAAAA 420
421  TACCTAAAGAGGGTAAATTGAGATATGTATGACCCTAAGATACTAAGAGGTCCTTCCTAA 480
481  GCTGAGTATAATAACAAGTAAATAGCATTAATAACTTATGAATGTAGTGATTCTGCTTAA 540
541  AGAGTTGAACGTTTCCTCACAGTAACTCCTGAATGGTTCAATGTATGTCAATCTGTTCAC 600
601  ATAATACAACAAACGTTCCTTATTATTCAACTACAAGGGACACATCACACCATACTGCTA 660
661  ACCACTCATAAGCCCAGGACTTACTGCCATGTTAATATGAACGATGTATAGTGCGGTCCC 720
721  GGGAGCTTGTGCTTTAATGTACCCTTTGCTTACGAGCAACCTTTTGCTGAGCTTGAGATA 780
781  AAAATCGTCTAATGTTAGCCATTGAACAAATCAATCGTGGCTGCATAACTGGGTCAGTAT 840
841  CTCTAACGTTCATTATGAAGCATAGCCATTTGTATTTTTGAATTCACTTTAAGCAGTACG 900
901  GTATTATATAAGAAATATCATGATATAGAATACTCTTATAGTCTAAGGTCCATTTAATTA 960
961  ATTTTATCATGTGTTTTAATAAAACGTATCATGTACGGAAAAAAAAAAAAAAAAAAAAA
1021 AAAA 1024
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Figure S1. Consensus nucleotide and amino acid sequences of contigs from EST library of king crab hemocytes; (A) contig 2 corresponded to paralithocin 1, (B) contig 16 corresponded to paralithocin 2 and (C) contig 11 corresponded to paralithocin 3. Pre-sequences are underlined and putative mature amino acid sequences are in bold. Cys residues are shaded in grey. Start and stop codons are doubled underlined and polyadenylation signal sequence (aataa) are shaded in black. The C-terminal amino acids cleaved off in the mature peptides are boxed. The sequences have been submitted to the Gene Bank with accession numbers MF919584 (paralithocin 1), MF919585 (paralithocin 2), and MF919586 (paralithocin 3).

Figure S2. TIC of paralithocin 1 (P23):

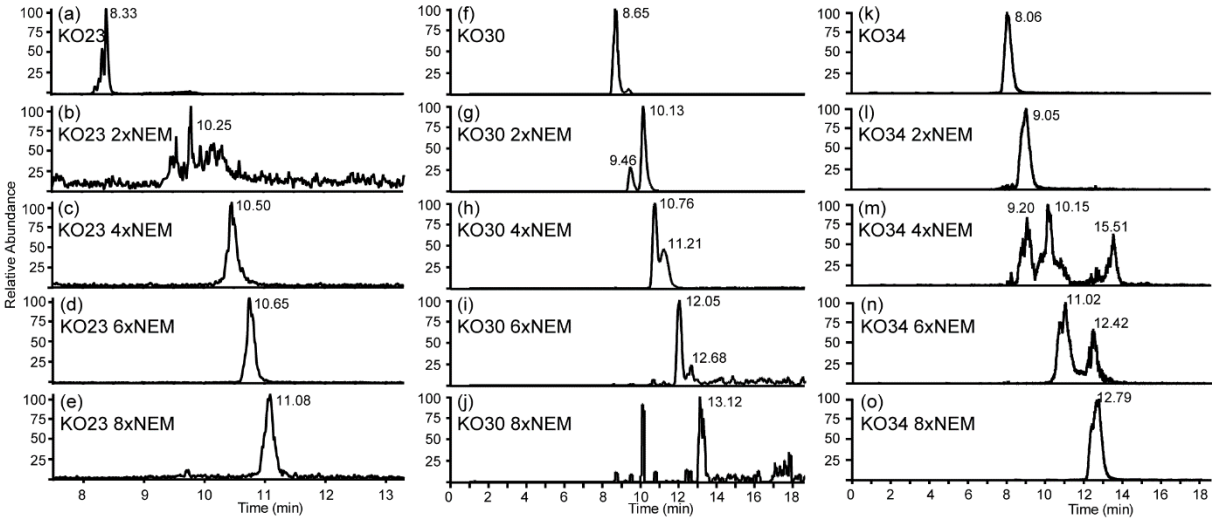

Figure S2. TIC of paralithocin 1 (P23): (a) intact, (b) one reduced Cys-Cys, (c) two reduced Cys-Cys, (d) three reduced Cys-Cys, (e) all reduced Cys-Cys; paralithocin 2 (P30): (f) intact, (g) one reduced Cys-Cys, (h) two reduced Cys-Cys, (i) three reduced Cys-Cys, (j) all reduced Cys-Cys; paralithocin 3 (P34): (k) intact, (l) one reduced Cys-Cys, (m) two reduced Cys-Cys, (n) three reduced Cys-Cys, (o) all reduced Cys-Cys. Regarding monoreduced paralithocin 1 (b) a contaminant of *m/z* 867.3, which is similar in mass to [M + 5H[$^{5+}$ of paralithocin 1, was observed throughout the chromatographic run, which explain the poorly defined shape of the peak in question (t_R 10.25 min). The TICs were recorded by an API4000 triple quadrupole mass spectrometer from ABSciex.