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Artificial lantipeptides from *in vitro* translations†

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We have devised a protocol for enzyme-free insertion of dehydroalanine, dehydrobutyrine and thioether crosslinks into translated peptides. *In vitro* **translation using 4-selenalysine and 4-selenoisoleucine as substitutes for lysine and isoleucine yields peptides that can be converted to polycyclic structures using mild chemistry in water. This methodology presents a gateway for exploring the potential of artificial lantipeptides as scaffolds for drug development.**

Many gram-positive bacteria secrete highly modified peptides containing dehydroamino acids and thioether crosslinks (lanthionines). The maturation of these compounds (lantipeptides) requires enzymatic post-translational dehydration of β-hydroxy amino acids and formation of lanthionines by Michael-type addition of cysteine side chains onto dehydro residues.[1–3] As a result, lantipeptides assume a rigid and stable structure which is paramount for their biological function. For example, the first two macrocycles of nisin inhibit bacterial cell wall synthesis by docking to the pyrophosphate moiety of lipid II.[4,5] Specific recognition of this relatively small molecule by a short peptide motif is a strong indication that peptides with rigid three dimensional structures may have a broader functional scope than currently appreciated. Initial efforts to exploit such potential for the design of therapeutic peptides have shown that lanthionine forming enzymes can also transform unrelated sequences allowing either chemo-enzymatic[6–8] or *in vivo* production of modified peptides.[9–11] In order to generate large and unbiased libraries of lantipeptides we and others have worked towards an enzyme-free protocol for dehydration and cyclization of ribosomally translated peptides.[12–15] In this account we extend our methodology to access peptides containing a combination of dehydroalanines (Dh), dehydrobutyrines (Dhb) and lanthionine bridges. We also demonstrate that this protocol is robust enough to produce peptides with a structural complexity approaching those found in naturally evolved lantipeptides.

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Fig. 1 (A) Chemical synthesis of selenoisoleucine. (i) HBTU, TEA, CH_2Cl_2; (ii) LiCl, THF, reflux; (iii) $NaBH_4$, EtOH, $(MeSe)_2$; (iv) THF, reflux; (v) HBr, HOAc, diethyl ether. (B) Ribosomal synthesis of a selenopeptide and oxidative elimination. For ribosomal translation methionine, lysine and isoleucine were replaced with norleucine (M_N), 4-selenalysine (K_{Se}) and 4-selenoisoleucine (I_{Se}). Treatment of the translated peptide with hydrogen peroxide induced selenoxide elimination as inferred by MALDI-TOF (1, sequence: $_{formyl}M_N RK_{Se}I_{Se}LEH_6$, m/z calcd 1753.6, found 1751; 2, sequence: $_{formyl}M_N R$**DhDhb**LEH_6, m/z calcd 1532.7, found 1530).

We started with the idea that 4-selenoisoleucine (2*R*,3*R*-2-amino-3-methyl-4-seleno-pentanoic acid, Fig. 1) may be an efficient substitute for isoleucine in ribosomal peptide synthesis. Because of the selenium atom in the side chain this residue may be converted to Dhb by oxidative elimination.[16] A similar approach has been successful for the preparation of Dh containing peptides.[12–15] In contrast to Dh, the reaction of Dhb with thiols is slow[17] and usually requires elevated temperatures[15] or enzyme catalysis.[1–3] Consequently, spontaneous cyclization is expected to occur preferentially between a cysteine and Dh rather than Dhb.

Such regioselectivity should allow the design of peptides and peptide libraries with predictable cyclization patterns despite the presence of multiple dehydroamino residues.[18] This aspect is of particular interest given that the activity of most naturally evolved lantipeptides relies on well defined polycyclic topologies.

To test these ideas we synthesized 4-selenoisoleucine (Fig. 1),[19] and demonstrated that this molecule indeed substitutes for isoleucine in ribosomal peptide synthesis but does not

interfere with the incorporation of other amino acids (Fig. S4, ESI†).[20] Using a synthetic model peptide (Scheme S3, ESI†) we followed hydrogen peroxide induced conversion of 4-selenoisoleucine to Dhb by ¹H-NMR and found that only one of the two possible isomers E-Dhb and Z-Dhb is formed (Fig. S2 and S3, ESI†).[16] Since selenoxides are known to undergo *syn*-elimination[21] we conclude that oxidation of the 2R,3R isomer of 4-selenoisoleucine leads to E-Dhb (Fig. S2, ESI†). This presents a subtle difference to biosynthesized lantipeptides which contain Z-Dhb. Introducing Z-Dhb by our method would require the synthesis of the 2R,3S-isomer which is less similar to isoleucine but also a likely substrate for ribosomal peptide synthesis.[22] In the next experiment we treated ribosomally translated peptides containing 4-selenalysine and 4-selenoisoleucine with 50 mM hydrogen peroxide at pH 7.2 for 30 min which produced the corresponding dehydropeptide containing both Dh and E-Dhb (Fig. 1).

With E-Dhb as a new building block at our disposal we aimed at the synthesis of artificial lantipeptides with programmed cyclization patterns. Specifically, we prepared two peptides, both with a central cysteine flanked by one of each dehydro-amino acid in different orders (peptide 1: Dh–C–Dhb; peptide 2: Dhb–C–Dh, Fig. 2). The translated peptides were immobilized on NTA-agarose and treated with 50 mM oxidized glutathione at room temperature for 20 min. This step converted the cysteine side chain to a mixed disulfide as a protection against irreversible damage during subsequent oxidative selenoxide elimination. The resulting dehydropeptides were treated with 1 mM tris(carboxyethyl)phosphine (TCEP) at pH 8.0 for 20 min to deprotect cysteine and induce the formation of a thioether bond. MALDI-TOF analysis showed identical masses for peptides 1 and 2 consistent with proper installation of all three modifications (Fig. 2). However, tryptic digest of the two peptides revealed a different cyclization pattern which is consistent with the idea that cysteine reacts with Dh but not with the less electrophilic E-Dhb. This chemoselectivity presents a valuable tool to encode important aspects of the three dimensional peptide structure in the primary sequence.[18] Furthermore, inspection of the tryptic fragment of peptide 2 shows that the trypsin cleavage site within the macrocycle was significantly protected from hydrolysis, which corroborates the notion that lanthionines can stabilize peptides against proteolytic degradation (Fig. S5, ESI†).[23]

Finally, we constructed a mimic of a naturally evolved structure, namely the first two rings of nisin (Fig. 3). The N-terminus of the structural peptide of nisin consists of the sequence ITSISLCTPGCK where serine and threonine serve as precursors for enzymatic formation of the dehydroamino acids (structure A, Fig. 3).[1–3] We designed a peptide approximating this sequence by replacing serine and threonine with 4-selenalysine and 4-selenoisoleucine to allow for chemical posttranslational modification. Translation and installation of a total of six modifications yielded a single product as inferred by MALDI-TOF (Fig. 3). Because the linear peptide contains four electrophilic centers and two cysteines cyclization could in theory produce 12 different regioisomers. However, the distribution of Dh and E-Dhb predicts that the final peptide adopts structure B (Fig. 3),[17,24–26] although, we cannot formally rule out the alternative structure C. We should also
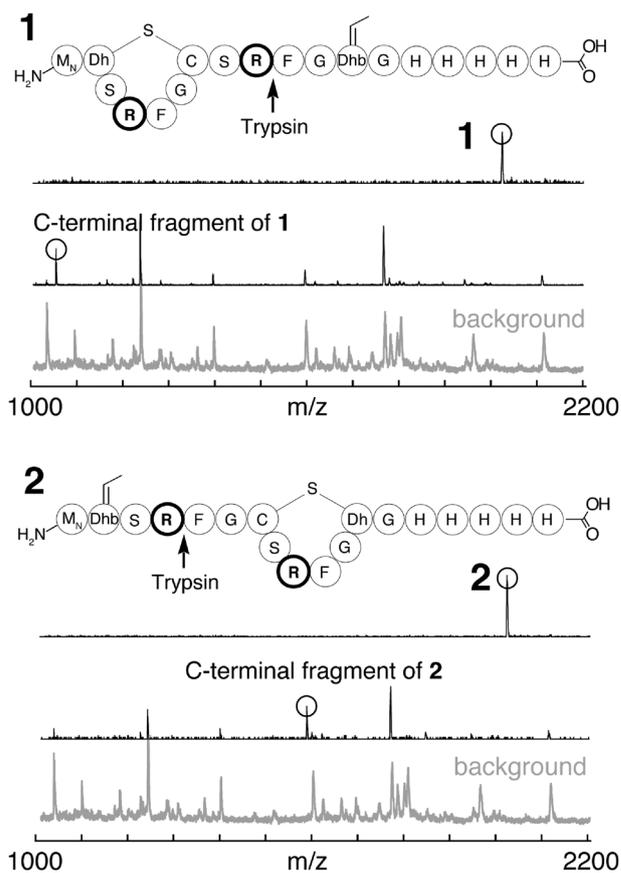
Fig. 2 Regiospecificity of thioether bond formation in peptides 1 and 2 (1, sequence: M_N**Dhb**SRFGCSRFG**Dhb**GH_5, *m/z* calcd 2023.9, found 2023, 2, sequence: M_N**Dhb**SRFGCSRFG**Dh**GH_5, *m/z* calcd 2023.9, found 2024). The tryptic cleavage sites are indicated with an arrow. The tryptic fragments observed by MALDI-TOF indicate cleavage after arginine 12 in peptide 1, and cleavage after arginine 4 in peptide 2 (C-terminal fragment of peptide 1, *m/z* calcd 1584.6, found 1585, C-terminal fragment of peptide 2, *m/z* calcd 1049.5, found 1050). The N-terminal fragments which lack a histidine tag are lost during sample preparation on NTA-agarose. In gray: tryptic digest in the absence of peptide 1 or 2.

mention that Michael-type addition onto a planar dehydro residue introduces a novel stereocenter. While enzyme catalyzed cyclization results in an *R*-configuration,[1–3] the spontaneous reaction is usually unspecific. As an exception, Dh–X_2–C or Dhb–X_2–C motifs cyclize with enzyme like selectivity[17,24–26] suggesting that the second ring in structure B contains a *meso*-lanthionine as does its natural model, the second ring of nisin (structure A, Fig. 3).

We have presented a methodology for the production of lantipeptides featuring multiple dehydroamino acids and lanthionines. The availability of residues with differential electrophilicities allows for regiospecific formation of polycyclic peptides. These molecules can be made using the unmodified translation apparatus of *Escherichia coli* in combination with chemical conditions at room temperature that should be well tolerated by proteins and nucleic acids. A similar approach to lantipeptides has been published before,[15] but this methodology requires tRNA precharged with vinylglycine and incubation at 95 °C to convert this residue to dehydrobutyrine and/or

Fig. 3 The N-terminal domain of nisin consists of two macrocycles which are tailored by lanthionine forming enzymes (structure A). An enzyme-free process is able to produce a similar structure by installing six posttranslational modifications resulting in a peptide with the structure B or, less likely C (B/C, sequence: $_{formyl}$M$_N$**DhbDh**V**Dhb**LC**Dh**PGCRLH$_6$, m/z calcd 2257.0, found 2256). Please note that natural lantipeptides (exemplified by structure A) contain Z-Dhb while the chemically modified peptides contain E-Dhb as indicated by the different orientation of the side chain methyl group.

methyllanthionine. Under these conditions regiospecificity is probably minimal which limits this protocol to constructing monocyclic peptides. In addition to these enzyme-free protocols, *in vitro* reconstituted lanthionine forming enzymes may also be used to construct artificial lantipeptides and libraries thereof.[6–8,27] While the chemical modification protocols do not require leader peptides and are highly sequence independent, enzymatic cyclization is stereoselective and does not require unnatural amino acids. Collectively these methodologies present a promising start towards the development of artificial lantipeptides with tailormade activities for therapeutic and analytical applications.

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