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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

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(54) Title: BIOCATALYTIC CYCLIZATION METHOD FOR PREPARATION OF CYCLIC PEPTIDES

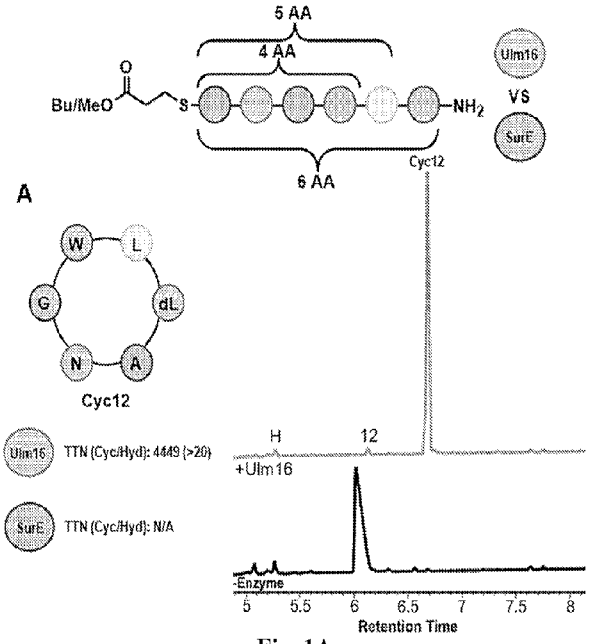


Fig. 1A

(57) Abstract: A method of biocatalytic cyclization for the preparation of cyclic peptides or salts thereof using Ulm16, a penicillin-binding protein (PBP)-type class of thioesterase (PBP-TE) enzyme.

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[Continued on next page]

SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

BIOCATALYTIC CYCLIZATION METHOD FOR PREPARATION OF CYCLIC PEPTIDES

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. provisional patent application no. 63/524,777, which was filed July 3, 2023, and which is hereby incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under GM138002, CA 275390, CA 23168 awarded by the National Institutes of Health and DGE-1842166 awarded by the National Science Foundation. The government has certain rights in the invention.

TECHNICAL FIELD

[0003] The present disclosure relates to a method of biocatalytic cyclization for the preparation of small cyclic peptides using penicillin-binding protein (PBP)-type thioesterases (PBP-TEs). In particular, the disclosure relates to a method of biocatalytic cyclization for the preparation of small cyclic peptides using Ulm16, a PBP-TE.

SEQUENCE LISTING

[0004] A computer-readable form (CRF) of the Sequence Listing is submitted with this application. The sequence listing is entitled 69959-02_SEQ_LISTING.xml, was generated on June 24, 2024, and is 4000 bytes in size. The entire content of the sequence listing is incorporated herein by reference in its entirety.

BACKGROUND

[0005] Peptides as therapeutic candidates are gaining interest due to their lack of immunogenicity as well as high potency, selectivity, and reasonable manufacturing costs. However, linear peptides often do not display these selectivities and drug-like properties without additional modifications (Tsomaia, N, Eur. J. Med. Chem. 2015, 94, 459-470). Macrocyclic peptides represent promising scaffolds for chemical tools and potential therapeutics. They often enable excellent membrane permeability and have increased potency, specificity, and proteolytic stability. However, their synthesis is very challenging since macrocyclization has often been hampered by epimerization of the C-terminal residue, oligomerization, and conformational rigidity, which precludes remote

residue coupling, leading to poor yields and scalability issues (White, C. J. et al., Nature Chemistry, 2011, 3, 509–524).

[0006] These issues become more challenging as the ring size in peptides decreases, particularly for small cyclic peptides such as tetrapeptides, which have high levels of ring strain. The ground-state (E)-geometry of the peptide bond prevents them from attaining a conformation favorable towards cyclization, making the synthesis and derivatization of pharmaceutically and industrially relevant cyclic tetrapeptides impossible. While several strategies have been employed to circumvent this issue, they often require certain amino acids to be present in the peptide sequence.

[0007] The enzymatic cyclizations that are suitable for the scalable production of cyclic peptides have been sought to overcome the challenges associated with macrocyclization. Nature can efficiently catalyze the production of linear peptide precursors using multimodular enzyme complexes known as nonribosomal peptide synthetases (NRPSs), which are frequently implicated in cyclic tetrapeptides and pentapeptides. Offloading from the complex and cyclization are typically catalyzed by C-terminal thioesterase (TE) domains or condensation termination (C_T) domains of NRPSs. While these domains can efficiently catalyze the cyclization of strained cyclic peptides *in vivo* and have been extensively studied as biocatalysts, they have failed to reach widespread use as biocatalysts, since they often display low substrate promiscuity, an inability to predict cyclization modes (e.g., head-to-tail) accurately, and strictly require peptidyl carrier protein (PCP) tethered substrate in the case of C_T domains.

[0008] Hence, there is an unmet need for a method of cyclizing small peptides that can be scaled for production and can efficiently use a biocatalyst. It is an object of the present disclosure to provide such a method. This and other objects and advantages, as well as inventive features, will be apparent from the detailed description.

SUMMARY

[0009] Provided is a method of biocatalytic cyclization for the preparation of a cyclic peptide or its salt, which method comprises reacting a substrate comprising a linear peptide or its salt with Ulm16, a penicillin-binding protein (PBP)-type class of thioesterase (PBP-TE).

[0010] The method comprises:

(a) reacting a substrate comprising a linear peptide or its salt with Ulm16, a penicillin-binding protein (PBP)-type class of thioesterase (PBP-TE) to obtain a reaction mixture;

(b) incubating the reaction mixture of step (a);

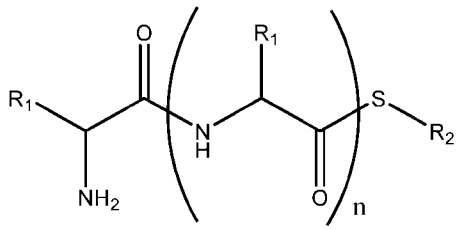
(c) quenching and centrifuging the reaction mixture; and

(d) isolating the cyclic peptide.

[0011] The step (a) is carried out in the presence of an aqueous solvent comprising one or more buffers and optionally further comprising an organic solvent. Desirably, the buffer used is Tris buffer. Examples of organic solvents include, but are not limited to, dimethyl sulfoxide (DMSO), acetonitrile, methanol, or dimethyl formamide (DMF).

[0012] In some embodiments, the concentration of Ulm 16 used is about 1 nM to about 250 nM.

[0013] The substrate comprising a linear peptide is represented by the formula (I):



(I)

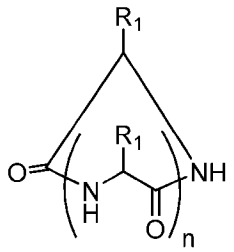
wherein n is 3-10;

each R_1 is independently a natural or unnatural amino acid side chain; and

R_2 is C_1-C_6 alkyl C_1-C_6 alkanoate or N-C_1-C_6 alkanoyl amino C_1-C_6 alkyl.

[0014] In some embodiments, R_2 is C_1-C_6 alkyl acetate or N-acetyl amino C_1-C_6 alkyl. In some embodiments, S-R_2 in formula (I) is N-acetylcysteamine (-SNAC), methyl 3-mercaptopropionate (-SMMP), or butyl 3-mercaptopropionate (-SBMP). Desirably, S-R_2 is butyl 3-mercaptopropionate (-SBMP).

[0015] The cyclic peptide obtained by the biocatalytic reaction is represented by the formula (II):



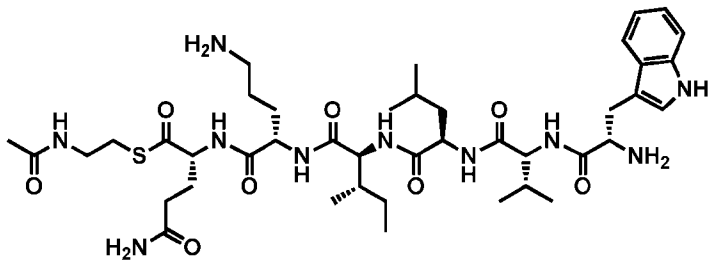
(II)

wherein n is 3-10;

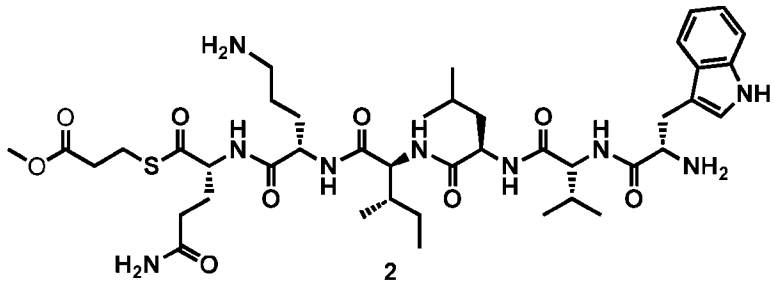
each R_1 is independently a natural or unnatural amino acid side chain.

[0016] In some embodiments, n is 4-6. The cyclic peptides obtained are tetrapeptides, pentapeptides, or hexapeptides, or a mixture of two or more of the foregoing. Desirably, the cyclic peptides are tetrapeptides.

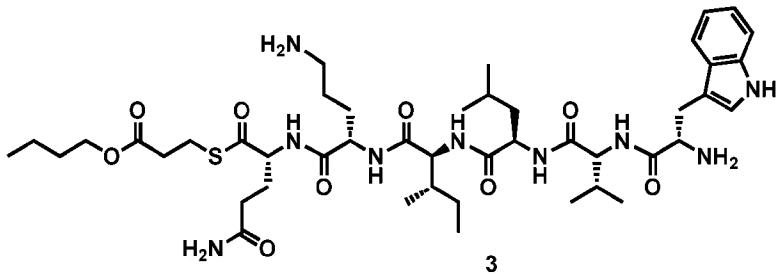
[0017] In some embodiments, the linear peptide of formula (I) is selected from:



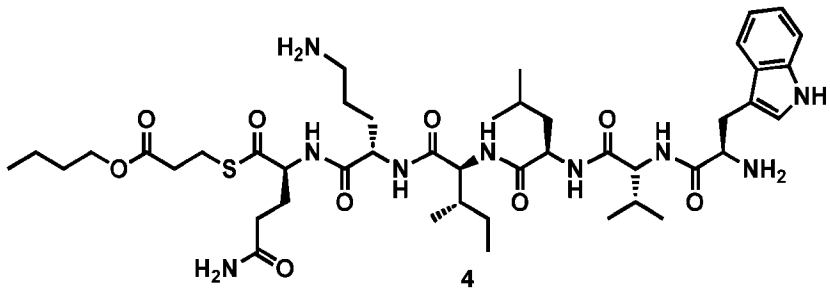
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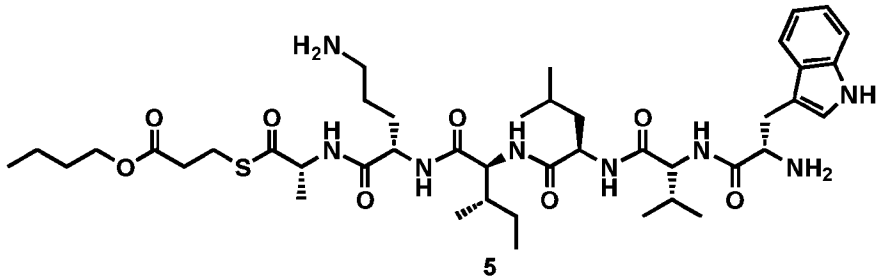
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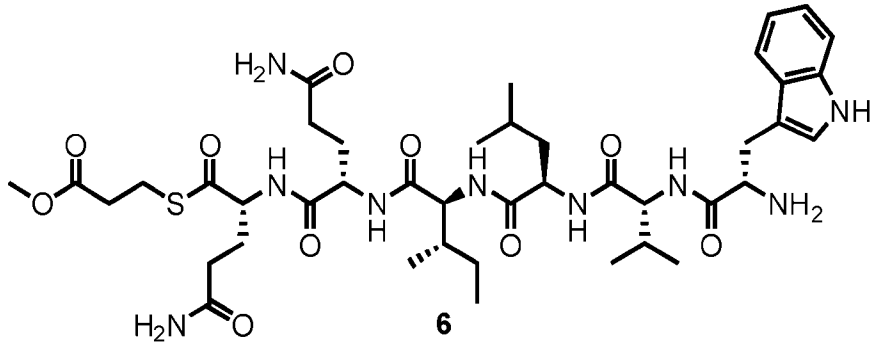
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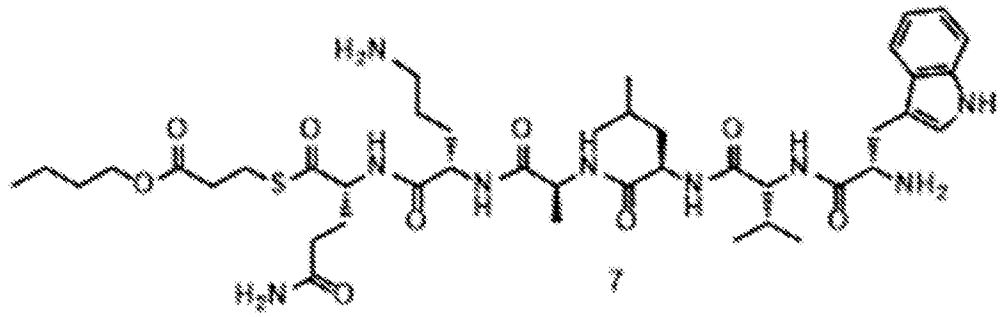
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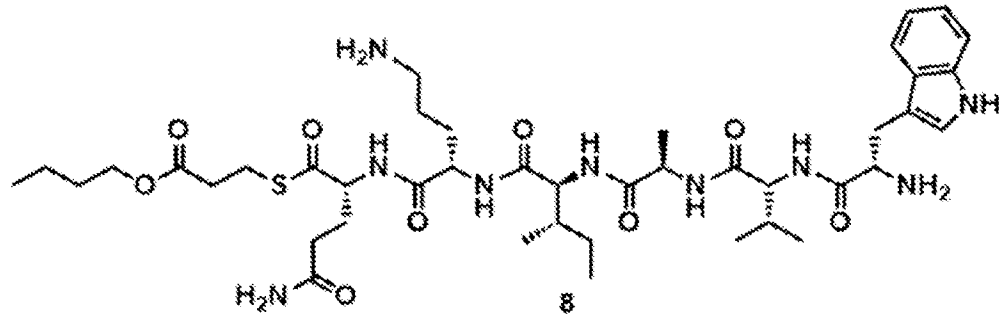
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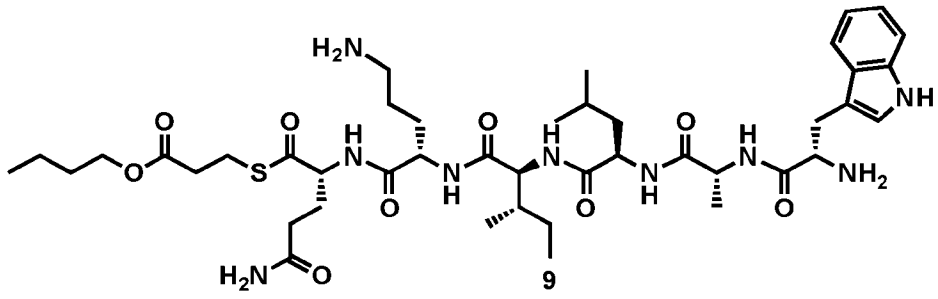
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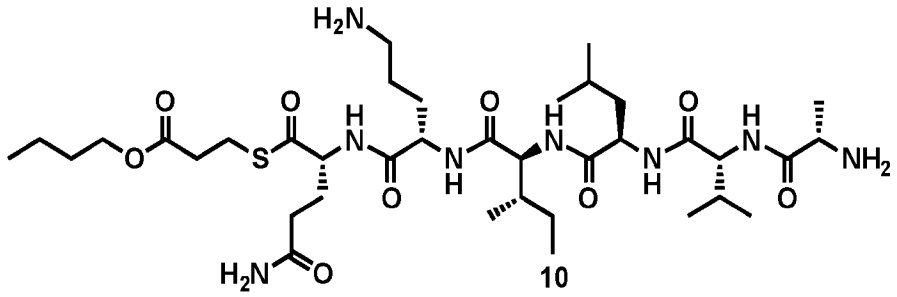
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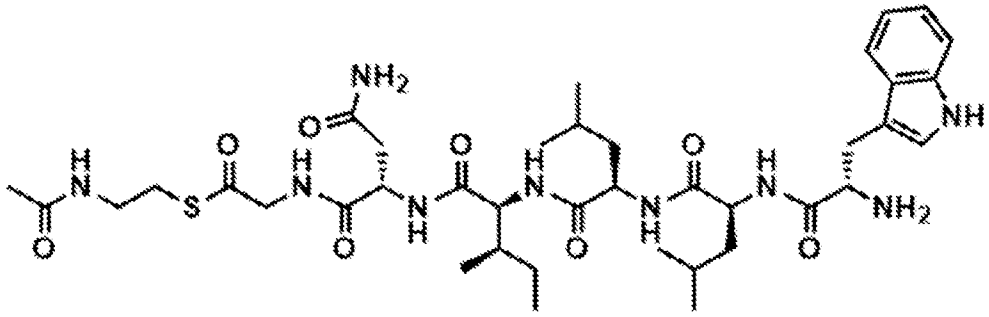
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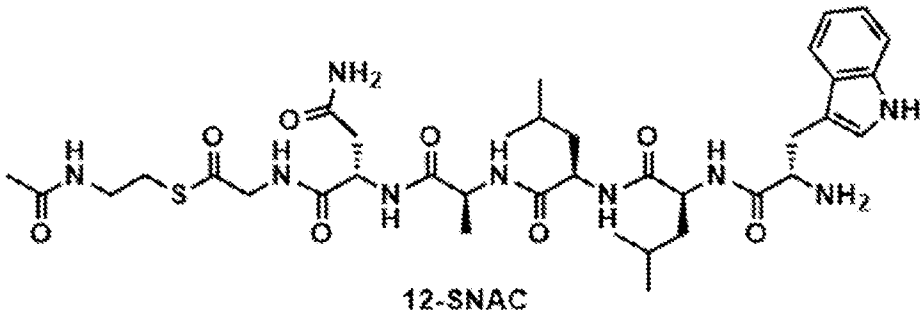
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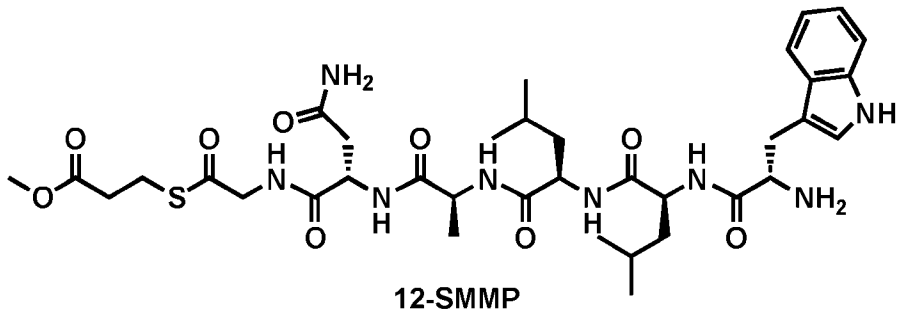
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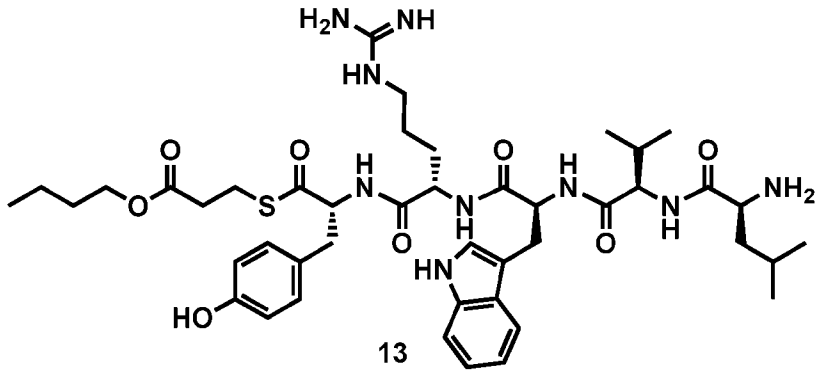
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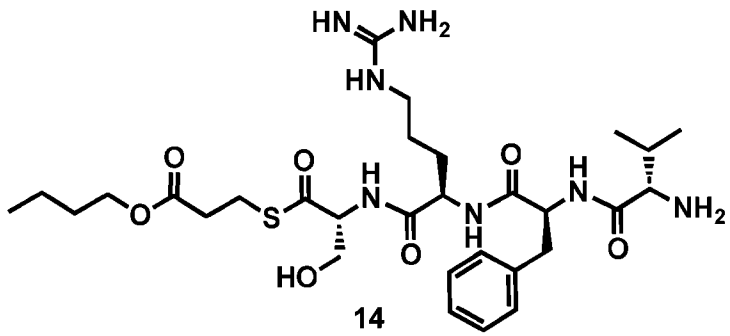
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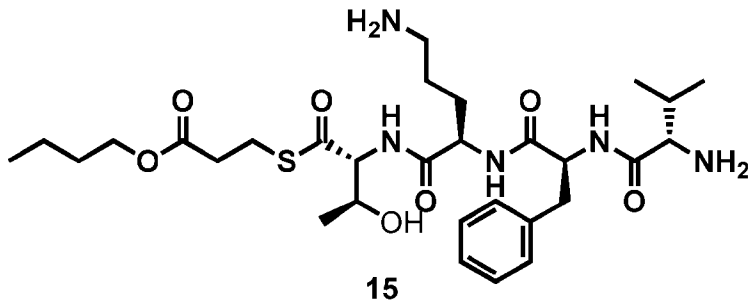
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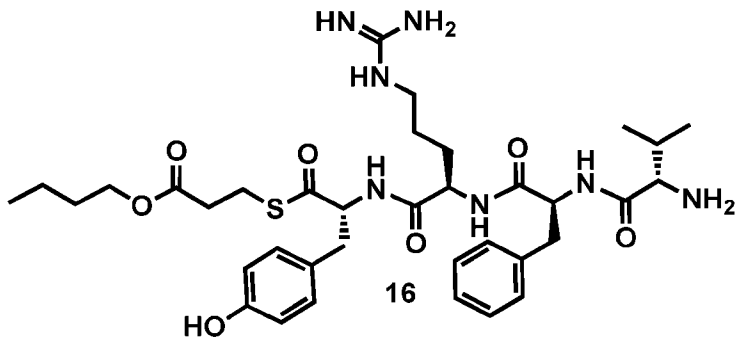
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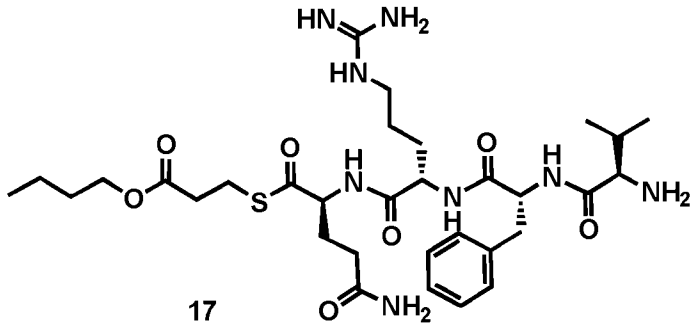
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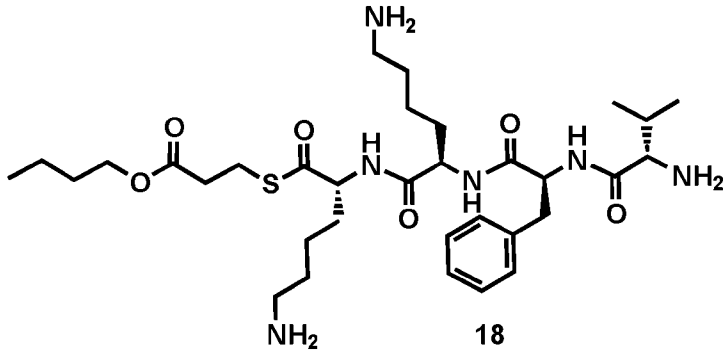
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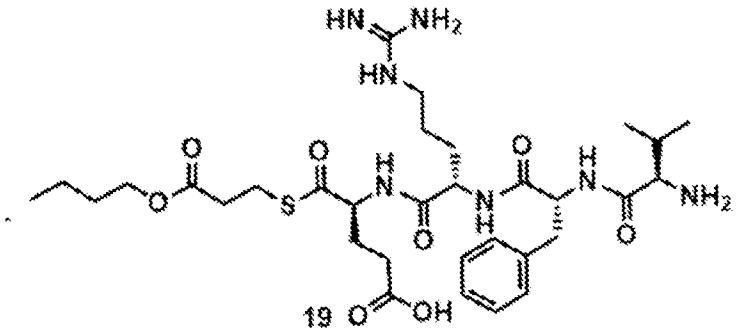
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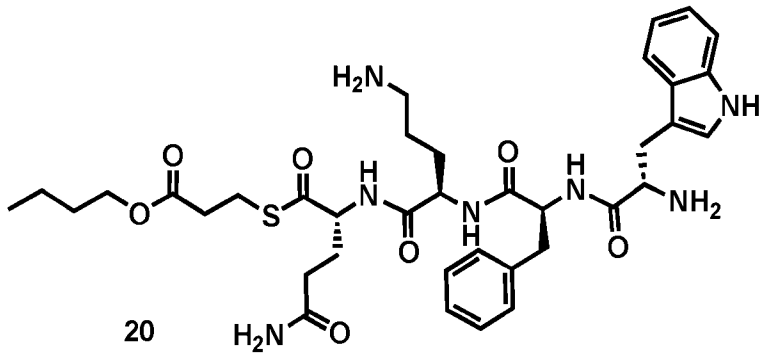
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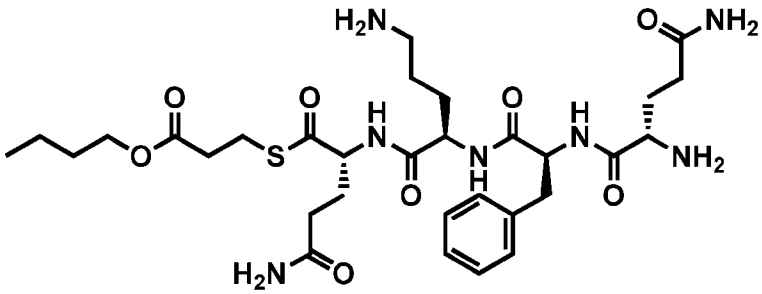
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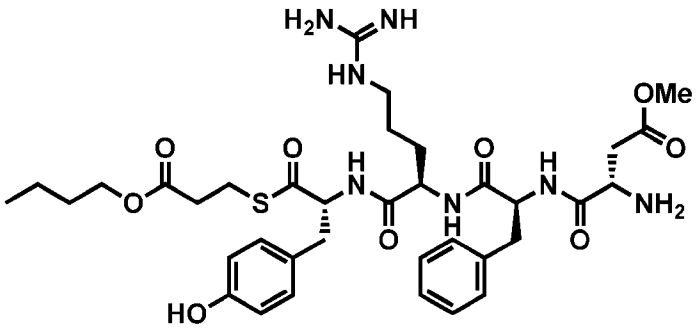
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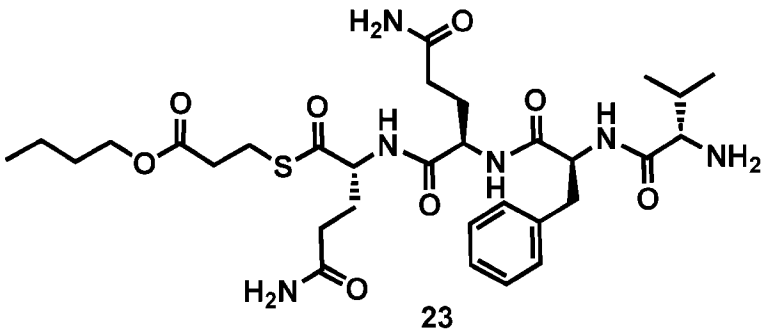
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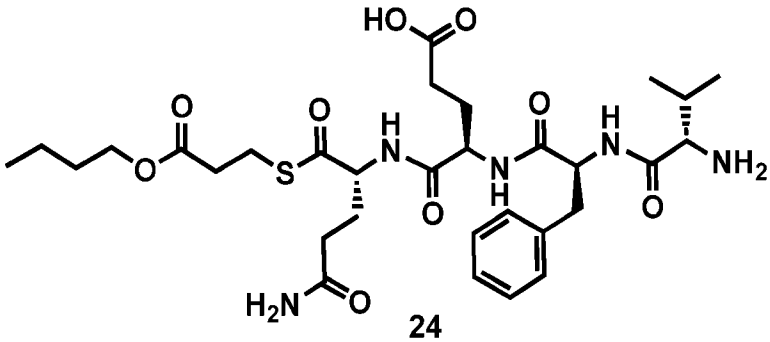
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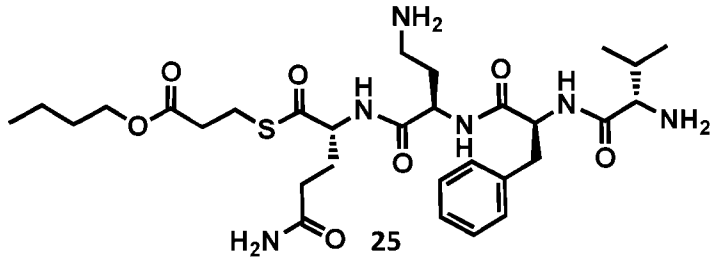
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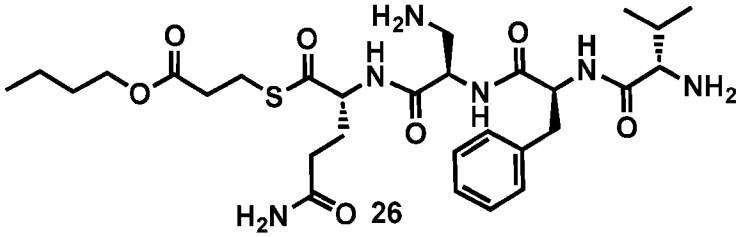
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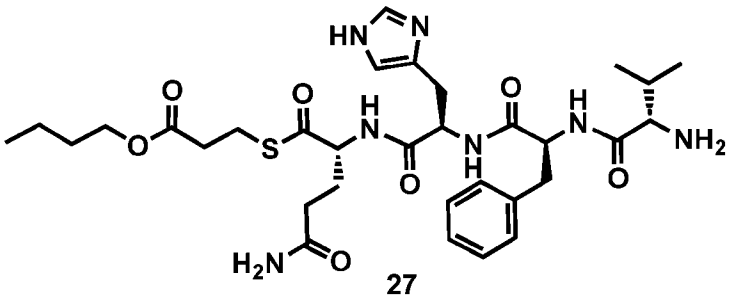
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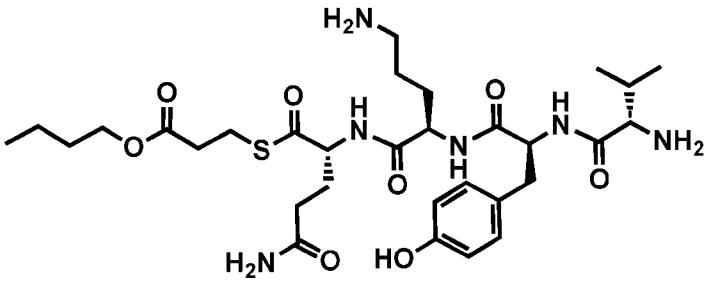
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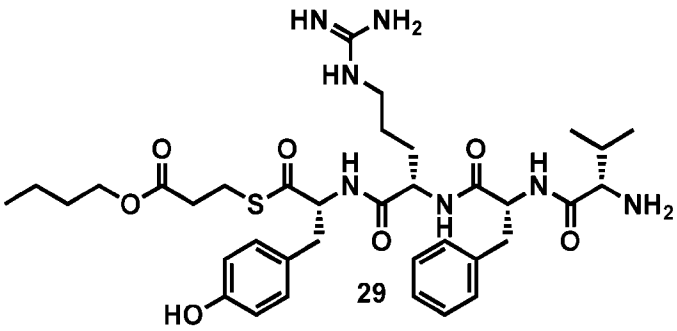
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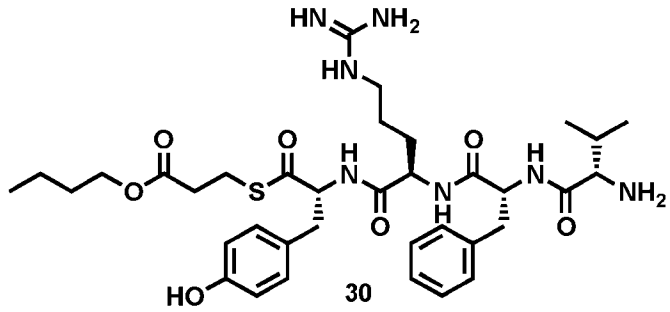
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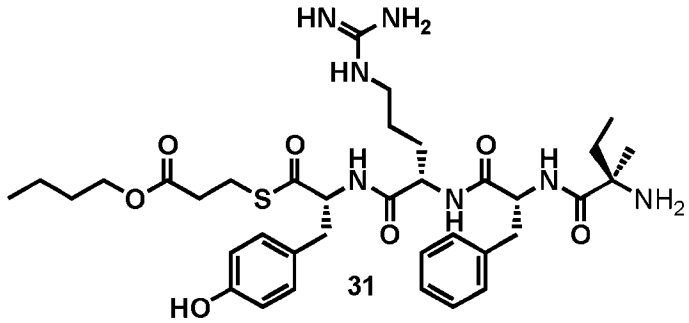
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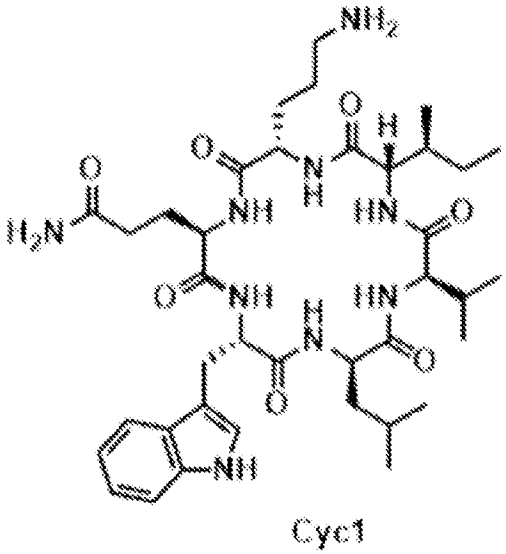
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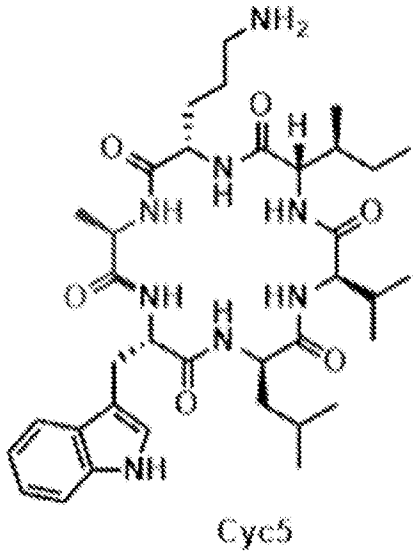
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or salt thereof.

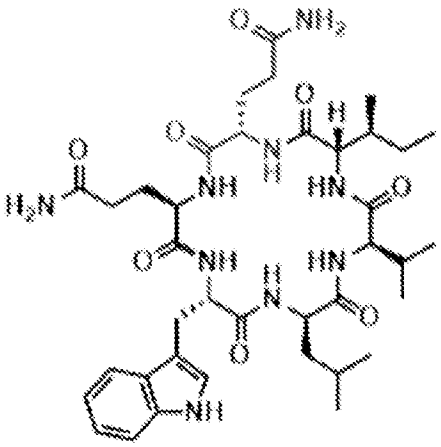
[0018] In some embodiments, cyclic peptide of formula (II) is one or more of:



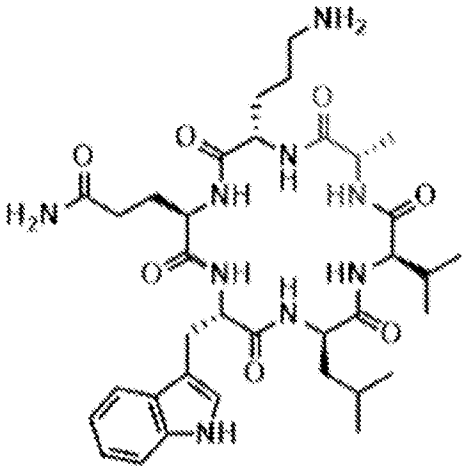
Cyc1



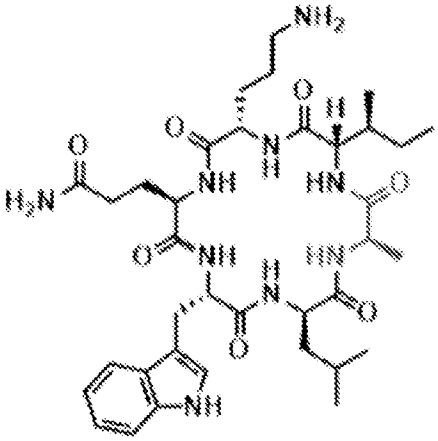
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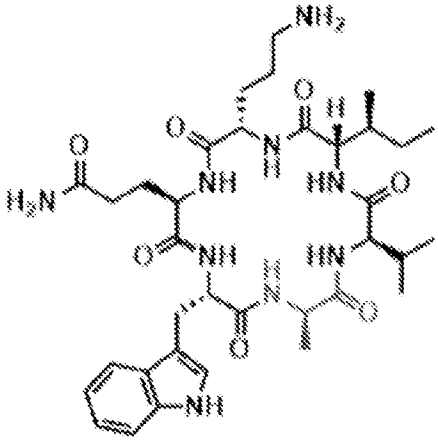
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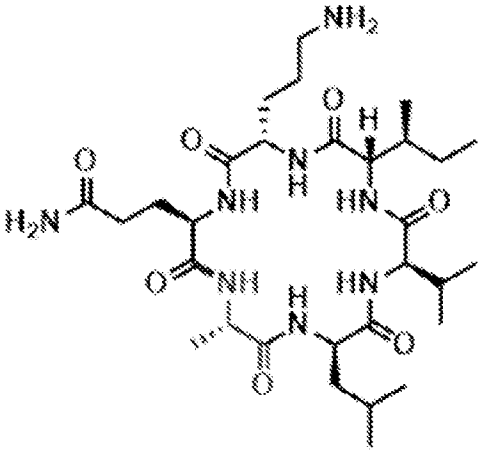
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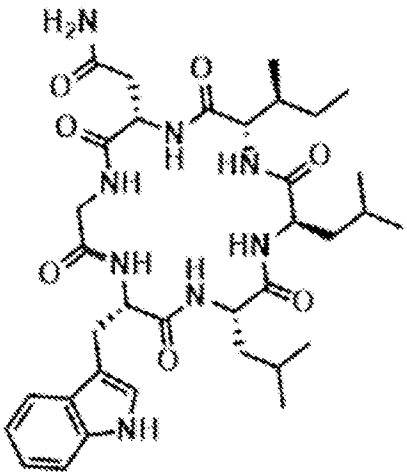
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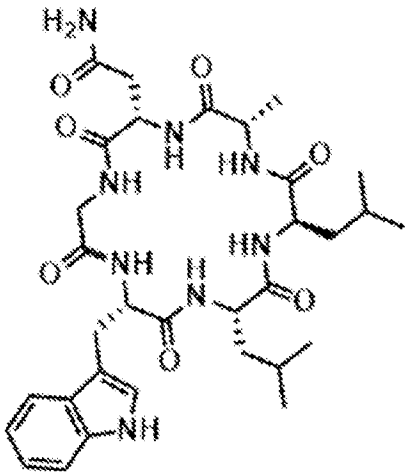
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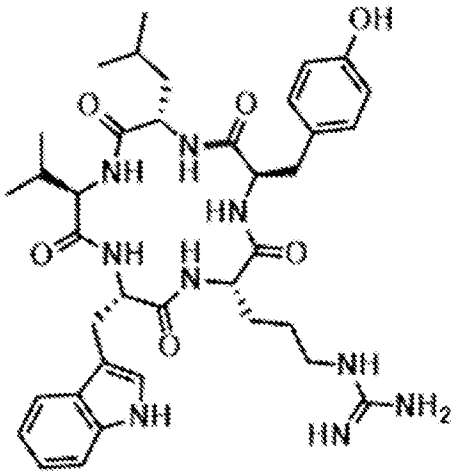
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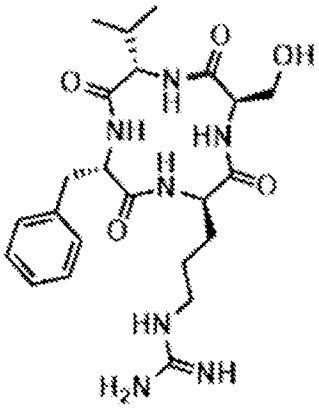
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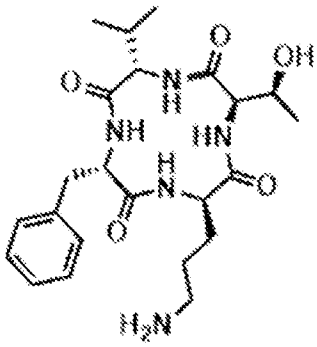
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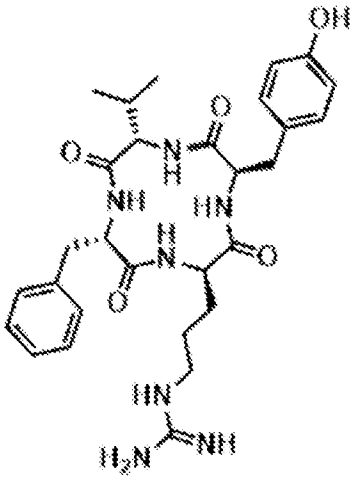
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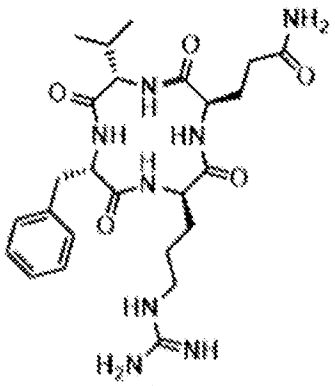
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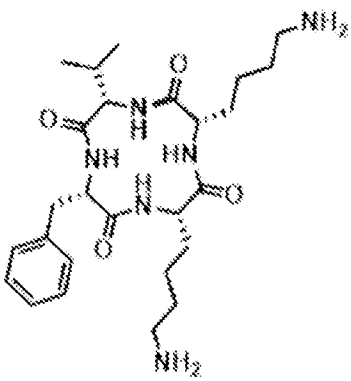
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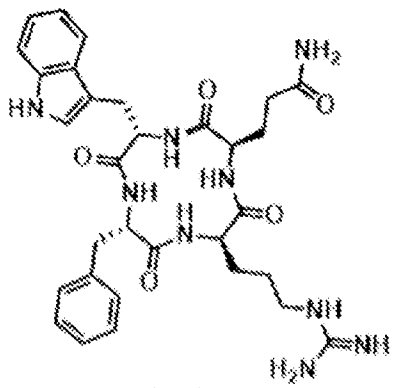
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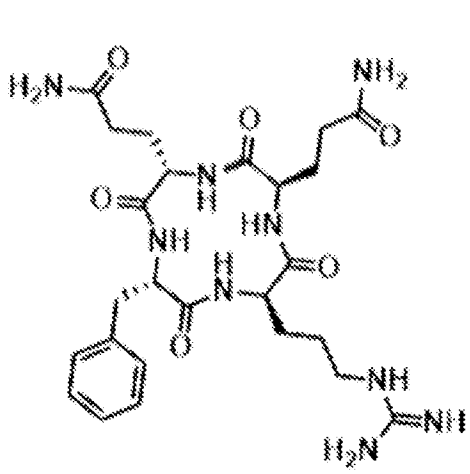
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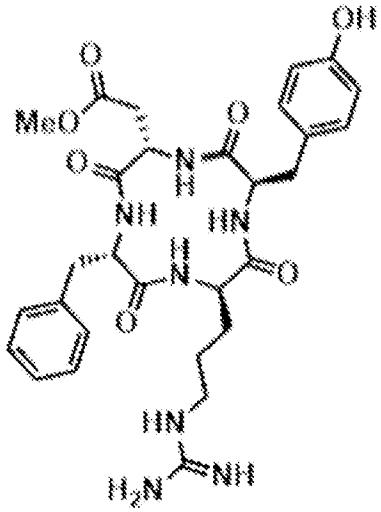
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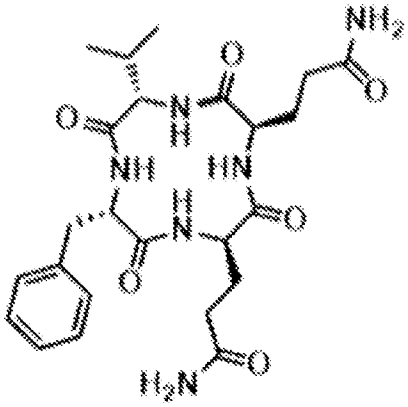
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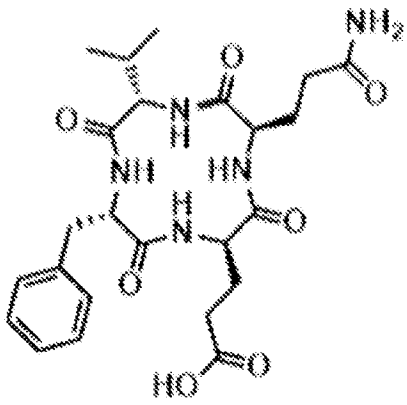
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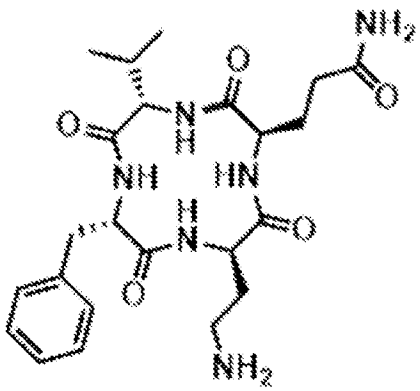
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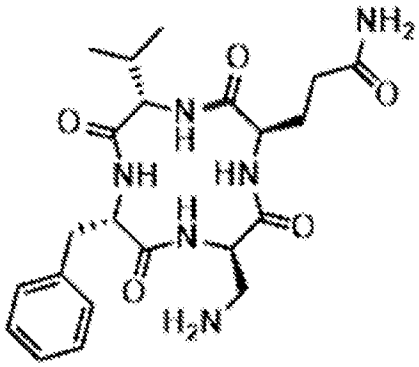
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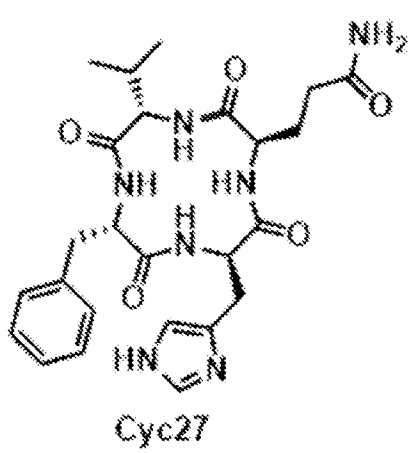
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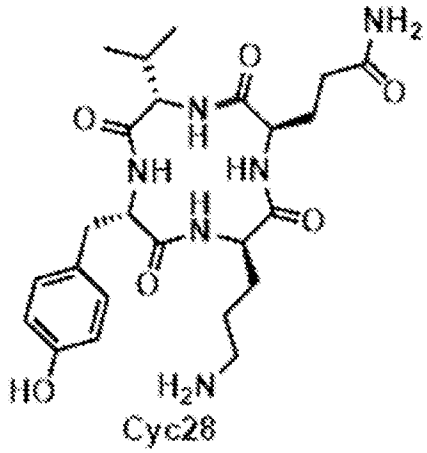
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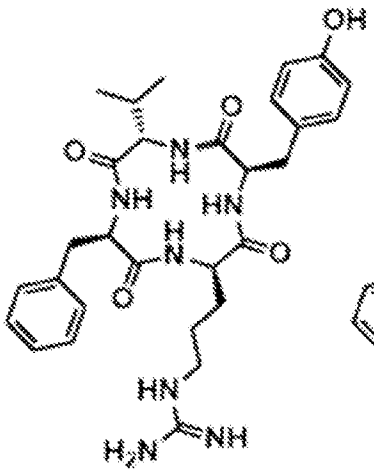
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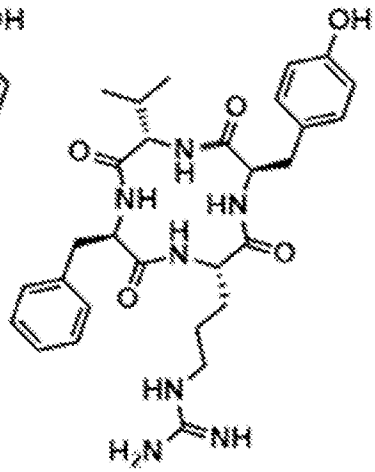
Cyc27



Cyc28



Cyc29



Cyc30

or a salt of any of the foregoing.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] The disclosed embodiments and other features, advantages, and aspects contained herein, and the matter of attaining them, will become apparent in light of the following detailed description of various exemplary embodiments of the present disclosure. Such detailed description will be better understood when taken in conjunction with the accompanying drawings.

[0020] Fig. 1A illustrates ultraviolet (UV) traces (214 nm) from ultra-performance liquid chromatography (UPLC) analysis of cyclization of hexapeptide **(12)** by Ulm16 (top, 70 nM,) or no enzyme control (bottom) after 4 hours of incubation. Ulm16 cyclized hexapeptides In the substrate scope comparison of Ulm16 and SurE.

[0021] Fig. 1B illustrates UV traces (214 nm) from UPLC analysis of cyclization of pentapeptide (**13**) by SurE (top, 240 nM), Ulm16 (middle, 17 nM), or no enzyme (bottom) control after 4 hours of incubation. Both SurE and Ulm16 can cyclize pentapeptide (**13**), but Ulm16 does so more efficiently and with significantly less enzyme.

[0022] Fig. 1C illustrates UV traces (214 nm) from UPLC analysis of cyclization of tetrapeptide (**16**) by SurE (top, 240 nM), Ulm16 (middle, 17 nM), or no enzyme control (bottom, after 4 hours of incubation. Ulm16 completely cyclized tetrapeptide (**16**), while SurE showed barely detectable cyclization.

[0023] Fig. 1D illustrates total turnover numbers (TTNs) of thiopeptide substrates. The top axis shows the TTN and the bottom axis shows the ratio of cyclized to hydrolyzed product formed. Values are averages of at least three replicates.

[0024] Fig. 1E illustrates a representation of the Ulm16 tetrapeptide substrate scope showcasing the tolerated amino acid substitutions (>5:1 Cyclic:Hydrolysis) tested at each position.

[0025] Fig. 2 shows Michaelis-Menten plots of Ulm16 kinetics for three peptide thioesters, such as Ulm-SNAC, Ulm-SMMP, and Ulm-SBMP. The data is summarized in **Table 2**. The plots represent the mean of triplicate experiments, and the error bars indicate the standard error of the mean (S.E.M).

[0026] Fig. 3 shows Michaelis-Menten plots of Ulm16 kinetics for three alanine scans of the 'Ulm' peptide. The substrates used were dQ1dA(**5**), 13A(**7**), dL4dA (**8**), dV5dA (**9**), and W6A(**10**). and The data is summarized in **Table 2**. The plots represent the mean of triplicate experiments, and the error bars indicate the S.E.M.

[0027] Fig. 4 shows **Table 1**, which displays details on Ulm16 tetrapeptide and pentapeptide Cyclization studies.

[0028] Fig. 5 shows **Table 3**, which shows Ulm16 mutant total turnover assay data.

DETAILED DESCRIPTION

[0029] For the purposes of promoting an understanding of the principles of the present disclosure, reference will now be made to the embodiments illustrated in the drawings, and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the claimed invention is thereby intended.

[0030] The terms "macrocyclic peptides" and "cyclic peptides" are used interchangeably.

[0031] The present disclosure is predicated, at least in part, on the discovery that a new class of

thioesterases, penicillin-binding protein (PBP)-type, which share a sequence homology to the penicillin-binding protein enzyme family of peptide cyclases, rather than the canonical thioesterases domains, and can act as biocatalysts for head-to-tail peptide macrocyclization. Unlike traditional TE and C_T domains, which act *in cis* to the nonribosomal peptide synthetases (NRPS), penicillin-binding protein-thioesterases (PBP-TEs) are physically discrete from the assembly line and act *in trans*.

[0032] Provided is a method of biocatalytic cyclization for the preparation of a cyclic peptide or its salt, which method comprises reacting a substrate comprising a linear peptide or its salt with Ulm16, a PBP-TE. The method comprises:

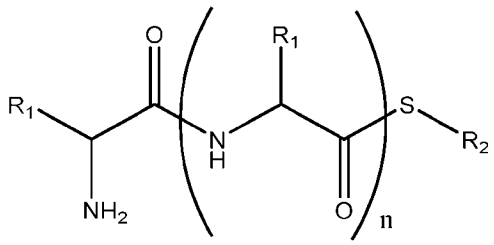
(a) reacting a substrate comprising a linear peptide or its salt with Ulm16, a PBP-TE to obtain a reaction mixture;

(b) incubating the reacting mixture of step (a);

(c) quenching and centrifuging the reaction mixture of step (b); and

(d) isolating the cyclic peptide.

[0033] In some embodiments, the substrate comprising a linear peptide is represented by the formula (I):



(I)

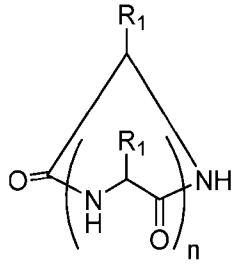
wherein n is 3-10;

each R_1 is independently selected a natural or unnatural amino acid side chain; and

R_2 is C_1-C_6 alkyl C_1-C_6 alkanoate or N- C_1-C_6 alkanoyl amino C_1-C_6 alkyl.

[0034] In some embodiments, R_2 is C_1-C_6 alkyl acetate or N- acetyl amino C_1-C_6 alkyl. R_2, together with sulfur to which it is attached, forms a S-R_2 group. In some embodiments, S-R_2 is N-acetylcysteamine (-SNAC), methyl 3-mercaptopropionate (-SMMP), or butyl 3-mercaptopropionate (-SBMP). Desirably, the S-R_2 is butyl 3-mercaptopropionate (-SBMP).

[0035] In some embodiments, the substrate of formula (I), upon reaction with Ulm16, forms the cyclic peptide represented by the formula (II):



(II)

wherein n is 3-10; and

each R_1 is independently a natural or unnatural amino acid side chain.

[0036] In some embodiments, provided is a method of biocatalytic cyclization for the preparation of a cyclic peptide of formula (II) or its salt, which method comprises reacting a substrate comprising a linear peptide represented by formula (I) or its salt with Ulm16, PBP-TE in the presence of an aqueous solvent.

[0037] In some embodiments, the linear peptides can comprise 4-10 amino acids when n is 4-10. Desirably, the linear peptides can comprise 4-6 amino acids when n is 4-6. The method involves head-to-tail cyclization of cyclic peptides using a biocatalyst, such as Ulm16. The cyclic peptides can be small cyclic peptides. In some embodiments, the small cyclic peptides are tetrapeptides, pentapeptides, or hexapeptides or a mixture of two or more of the foregoing. In some embodiments, the small cyclic peptides are tetrapeptides.

[0038] Ulm16, a peptide cyclase enzyme belonging to the PBP-TEs that can carry out biocatalytic head-to-tail macrolactamization of nonribosomal peptides. This enzyme obtained from *Streptomyces* sp. KCB13F003 can efficiently catalyze the production of linear peptide precursors using multimodular enzyme complexes known as nonribosomal peptide synthetases (NRPSs), which are frequently implicated in the production of cyclic peptides *in vivo*.

[0039] Cyclic peptides of formula (II) can be prepared by biocatalytic cyclization of corresponding linear peptide precursors of formula (I) wherein cyclic peptide ring closure can be effected, preferably by the formation of an amide bond catalyzed by Ulm16. A method involves reacting Ulm16 enzyme with a substrate comprising a linear peptide, wherein an amino acid on the one terminal of the liner peptide has an activated thioester functional group, and the other terminal of the peptide has an amino acid with a free amine in the presence of an aqueous solvent to form the cyclic

peptide.

[0040] Ulm16 can effectively cyclize the liner peptides comprising 4-6 amino acids (see **Table 2, Fig. 1**). In some embodiments, Ulm16 can cyclize various non-native peptides comprising 4-6 amino acids with catalytic efficiencies of up to 3 x 10^6 $M^{-1}s^{-1}$. In some embodiments, the cyclic peptides comprising 4-6 amino acids are tetrapeptides, pentapeptides, or hexapeptides. **Fig. 1C** illustares that Ulm16 cyclizes the tetrapeptide consisting of N-terminal L-valine and C-terminal D-serine (**14**) with very little hydrolysis observed (>20:1 cyclic:hydrolyzed). **Fig. 1E** illustrates the substrate scope of Ulm16, indicating that it can accommodate a diverse range of amino acids at positions 1-3 of the tetrapeptide sequence. Thus, Ulm16 can have broad specificity for substrate recognition and also can have selectivity for certain amino acid residues.

[0041] In some embodiments, the biocatalytic cyclization can be carried out in an aqueous medium that optionally includes one or more buffers or an organic solvent. A suitable organic solvent can be used. In some embodiments, the organic solvent is dimethyl sulfoxide (DMSO), acetonitrile, methanol, or dimethyl formamide (DMF). The desirable organic solvent is DMSO.

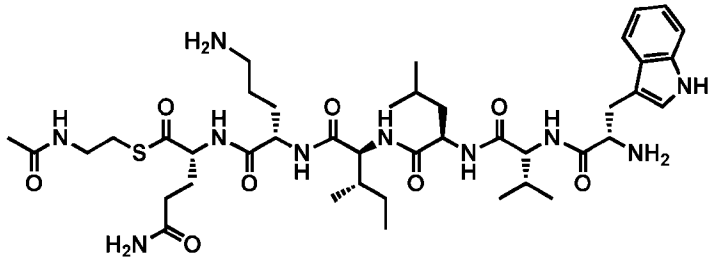
[0042] The amount of organic solvent used is about 0% to about 25%, such as about 0 % to 25%, or 0% to about 25% or 0% to 25%. The buffers used for the reaction can have a pH of about 6 to about 9, such as about 6 to 9, or 6 to about 9 or 6 to 9. Desirably, the reaction mixture has a pH of about 8 (such as 8). A suitable buffer that can maintain pH of about 6 to about 9 can be used. Desirably, the buffer is Tris buffer. The concentration of catalyst used depends upon the rate of catalysis for a particular substrate and the reaction volume. The concentration of Ulm16 used is about 1 nM to about 250 nM for about 50 μM to about 500 μM of the substrate, such as about 1 nM to 250 nM, or 1 nM to about 250 nM, or 1 nM to 250 nM, for about 50 μM to 500 μM, or 50 μM to about 500 μM or 50 μM to 500 μM of the substrate. Desirably, the concentration of Ulm16 used is about 10 nM to about 100 nM, such as about 10 nM to 100 nM, or 10 nM to about 100 nM, or 10 nM to 100 nM for about 250 μM to about 400 μM of the substrate, such as about 250 μM to 400 μM, or 250 μM to about 400 μM or 250 μM to 400 μM of the substrate. More desirably, the concentration of Ulm16 used is about 10 nM to about 70 nM for the about 280 μM to about 320 μM of the substrate (such as 10 nM to 70 nM for 280 μM to 320 μM).

[0043] The cyclization can be performed at about 10 °C to about 40 °C, such as about 10 °C to 40 °C or 10 °C to about 40 °C or 10°C to 40 °C. Preferably, the cyclization can be carried out at about room temperature, i.e., about 20 °C to about 25 °C, such as about 20 °C to 25 °C or 20° C to about

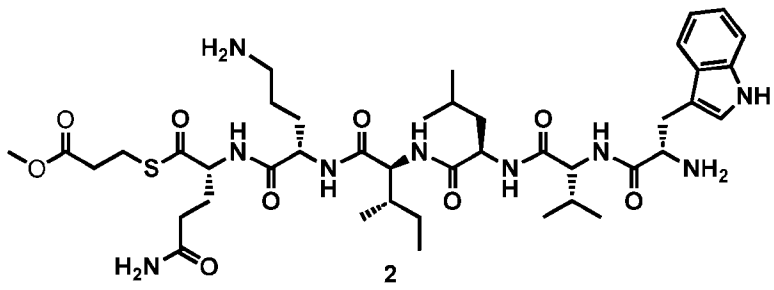
25 °C or 20 °C to 25 °C. The cyclization reaction can be completed in about 2 minutes to about 6 hours. Desirably, the cyclization reactions can be completed in about 4 hours (such as 4 hours).

[0044] Further provided is a method for biocatalytic cyclization for the preparation of a tetrapeptide or its salt using Ulm16, in which the method comprises reacting a substrate that comprises a linear peptide comprising four amino acids with a Ulm16 in the presence of an aqueous solvent.

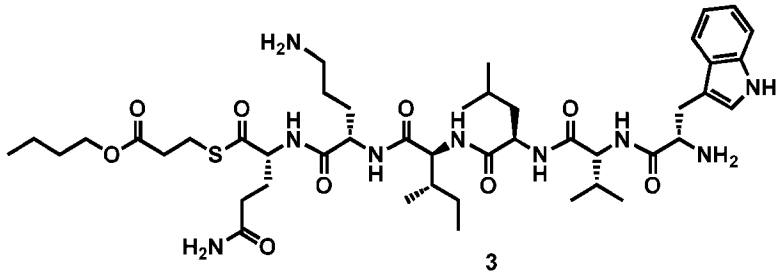
[0045] In some embodiments, the linear peptide of formula (I) is selected from:



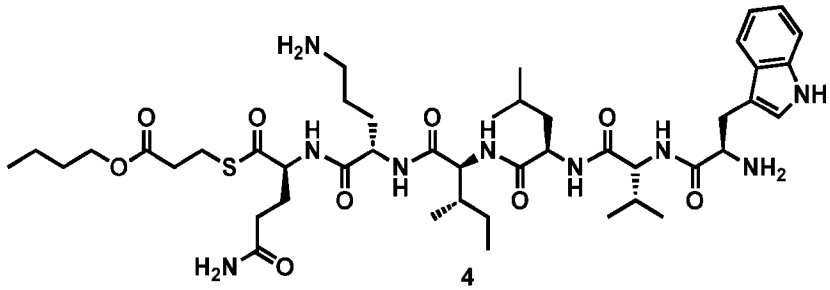
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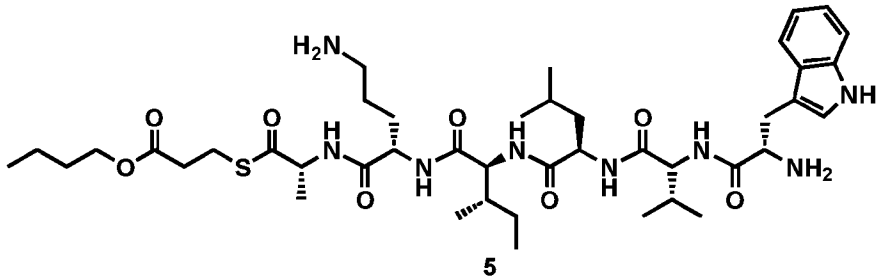
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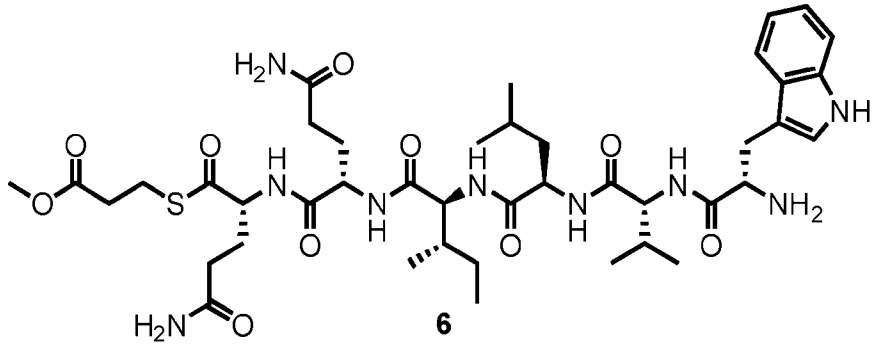
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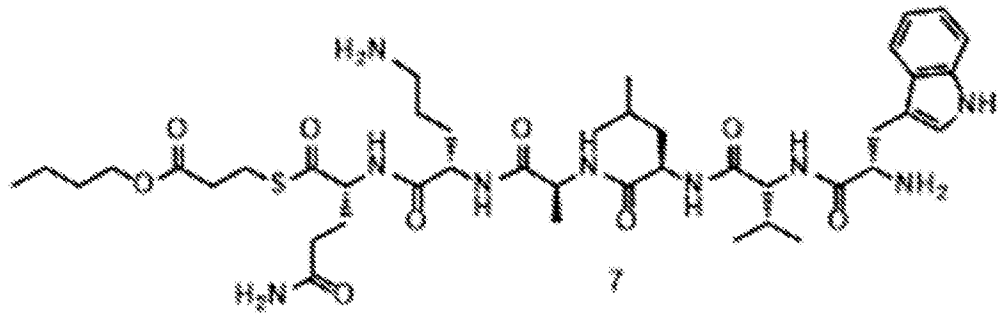
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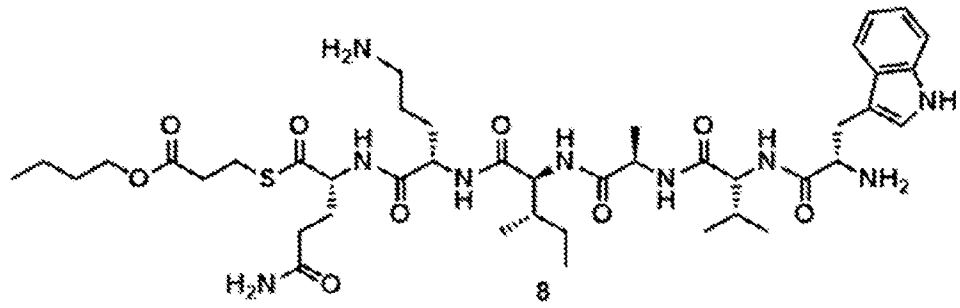
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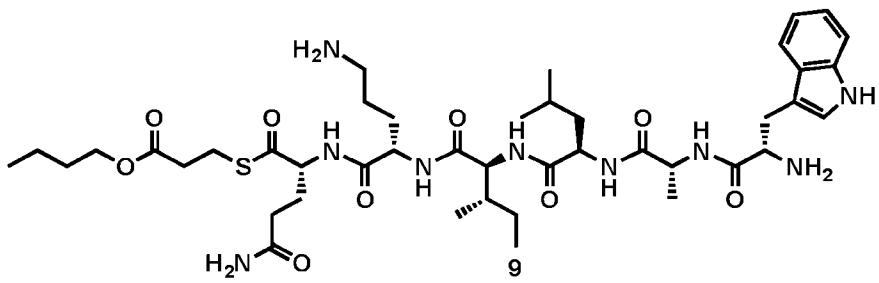
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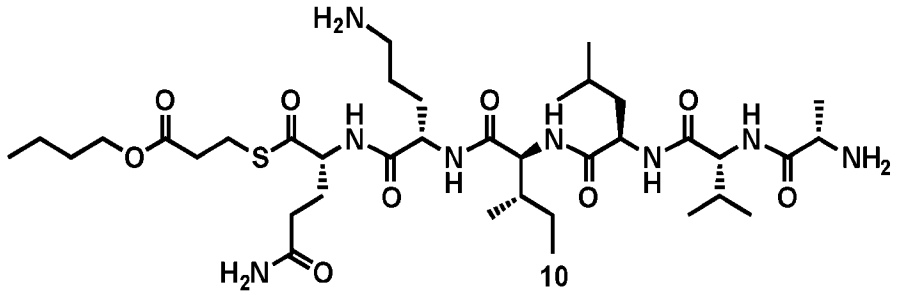
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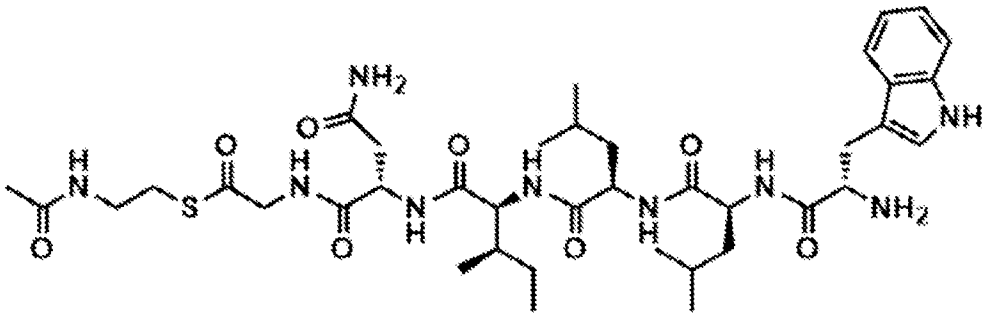
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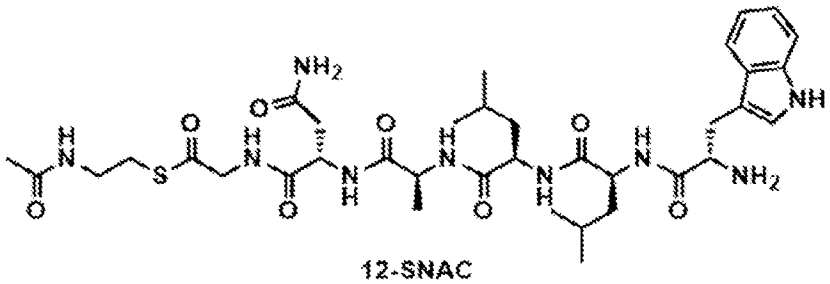
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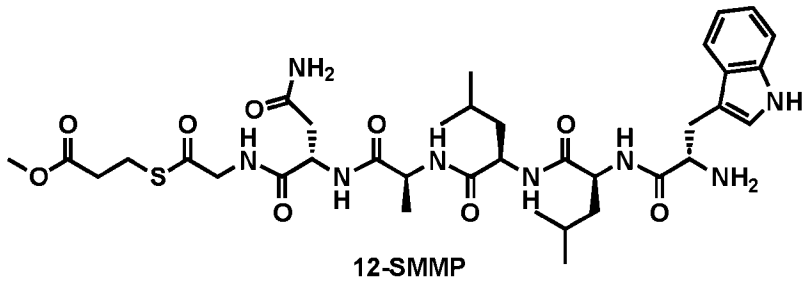
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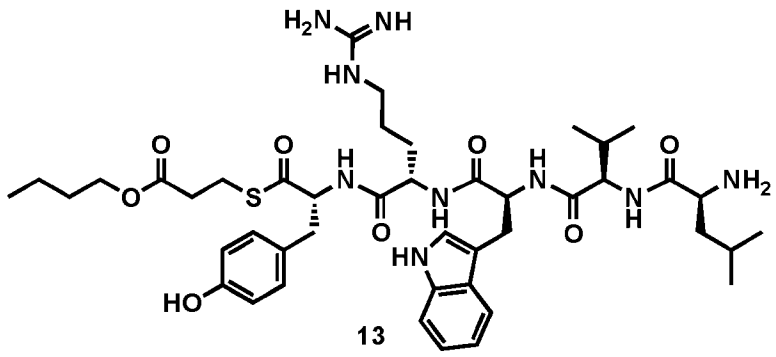
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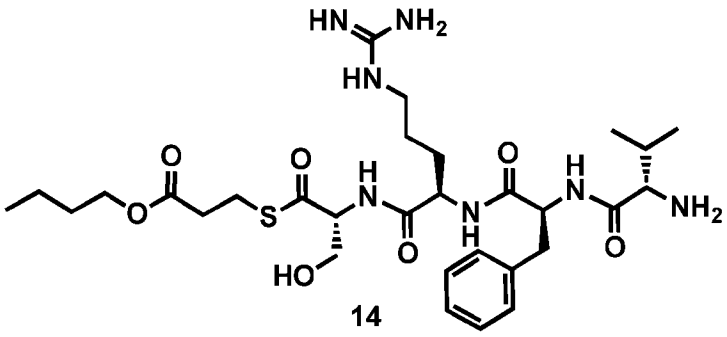
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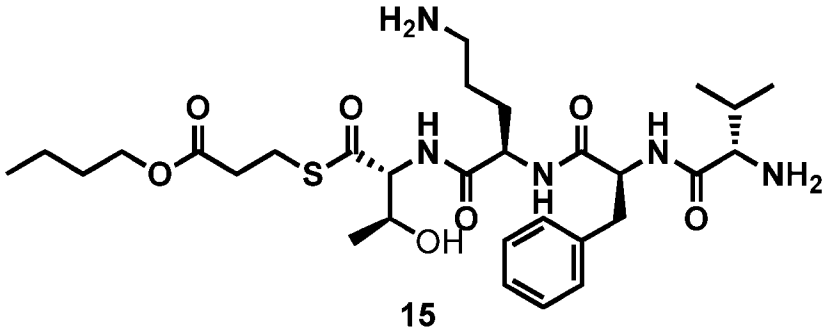
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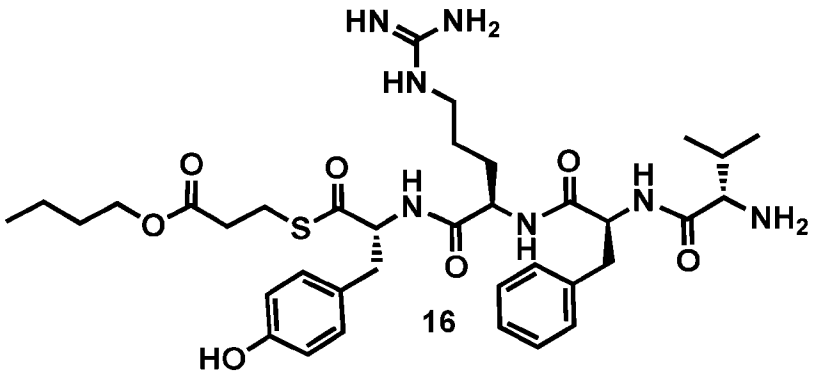
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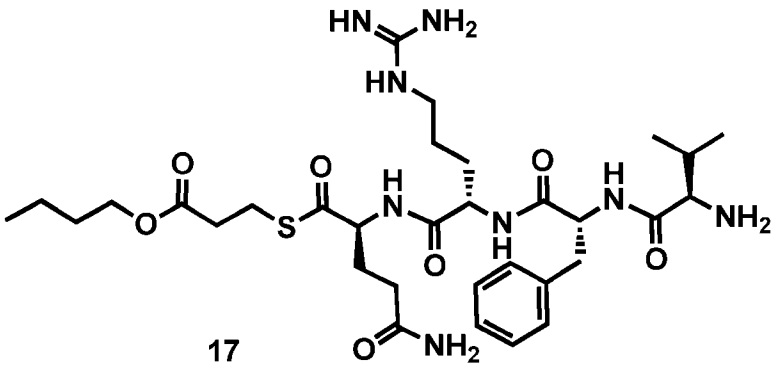
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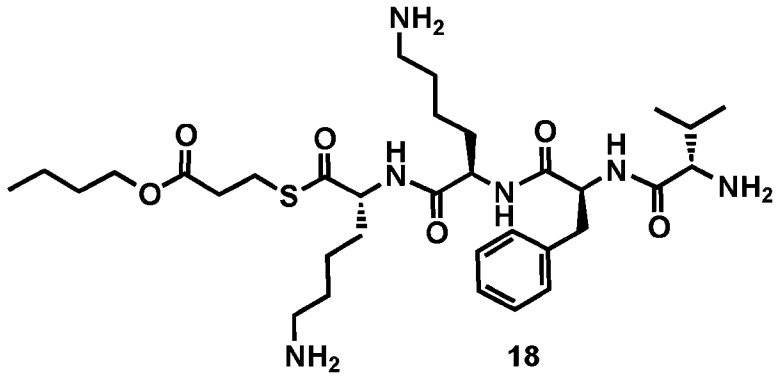
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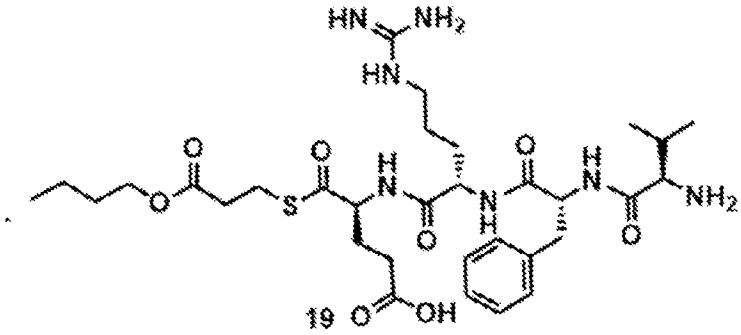
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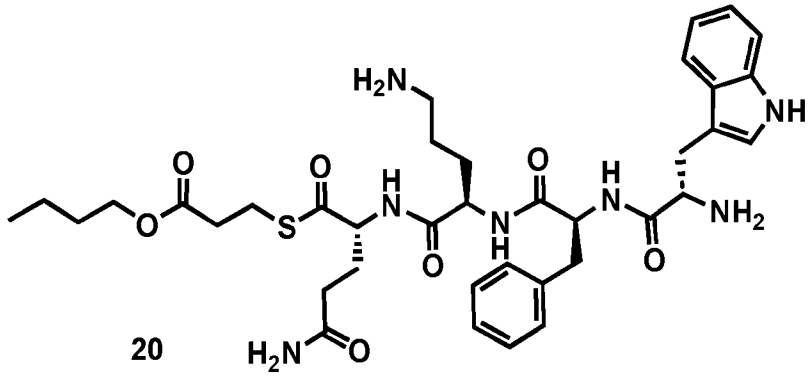
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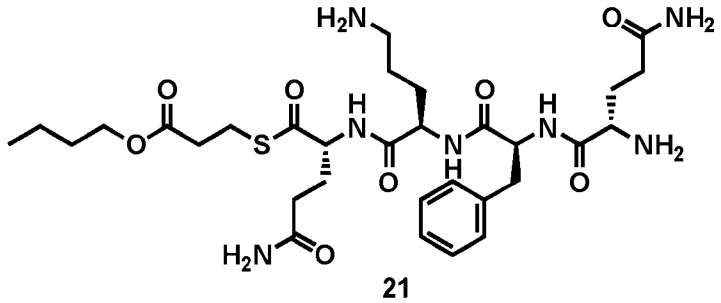
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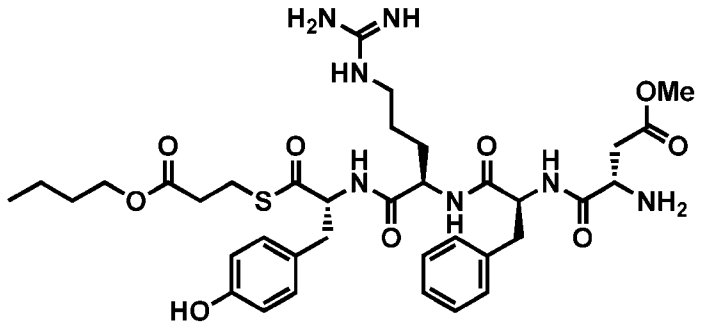
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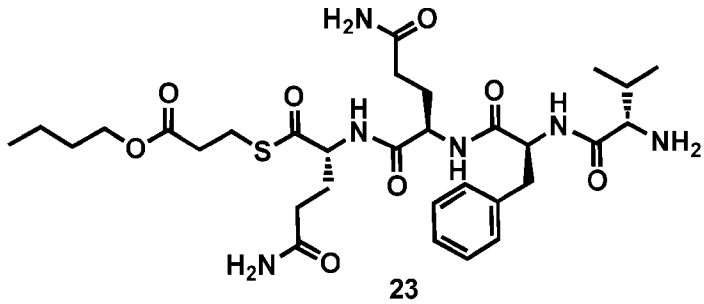
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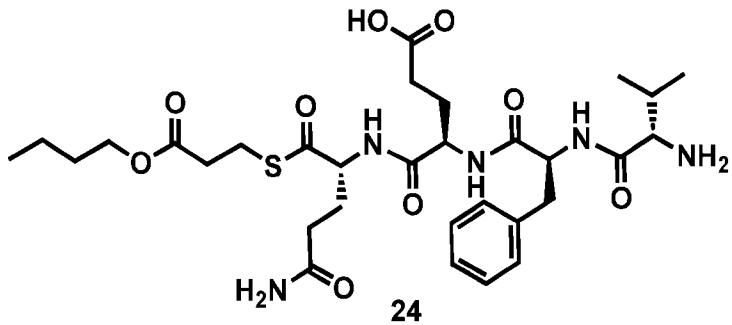
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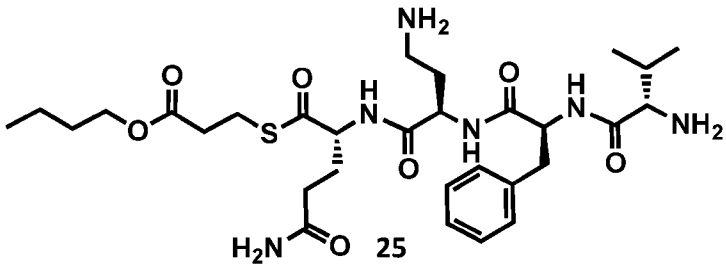
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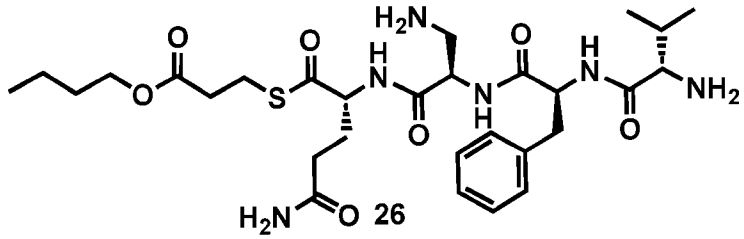
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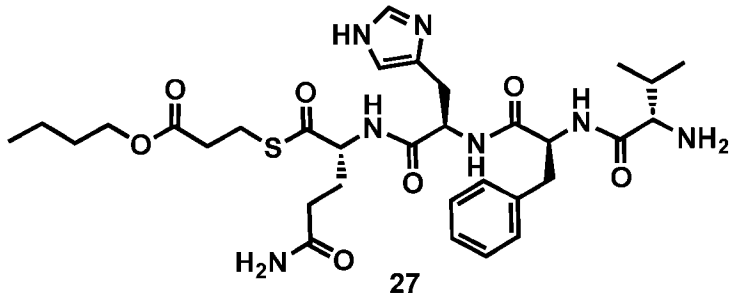
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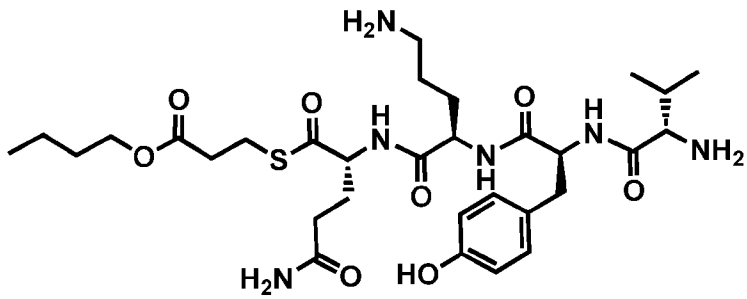
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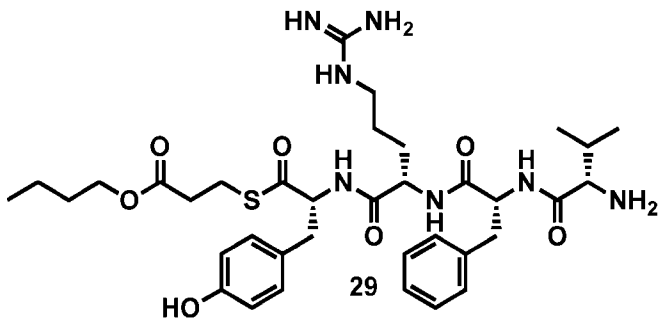
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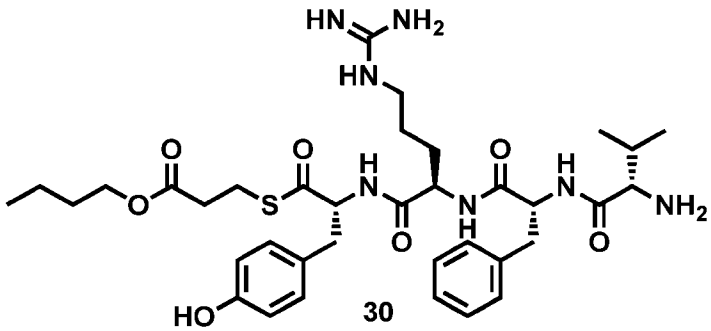
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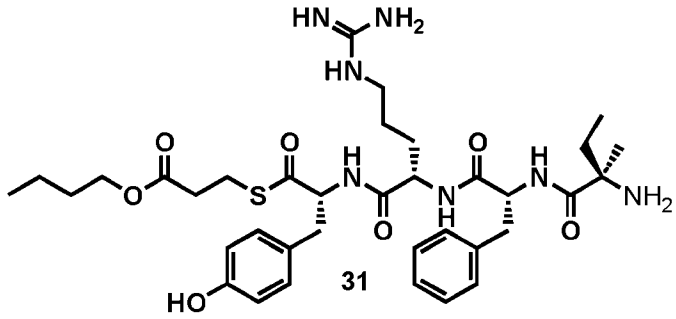
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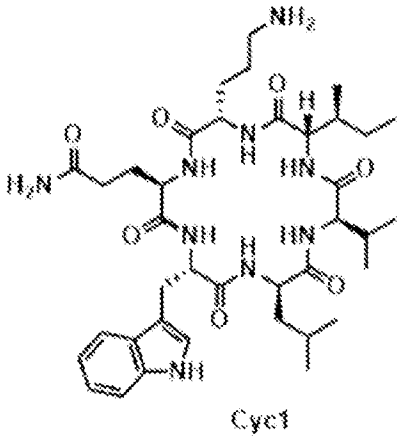
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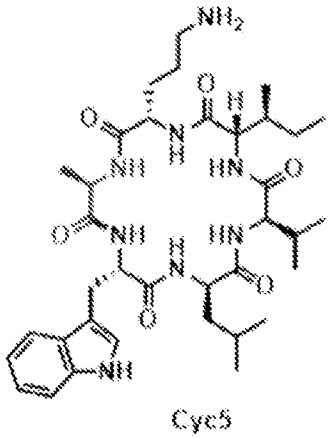
31

or its salt.

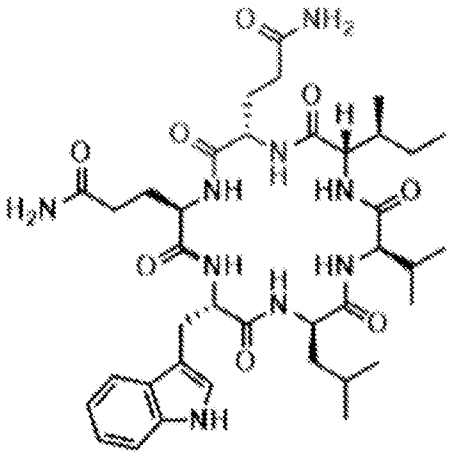
[0046] In some embodiments, the cyclic peptide of formula (II) is one or more of:



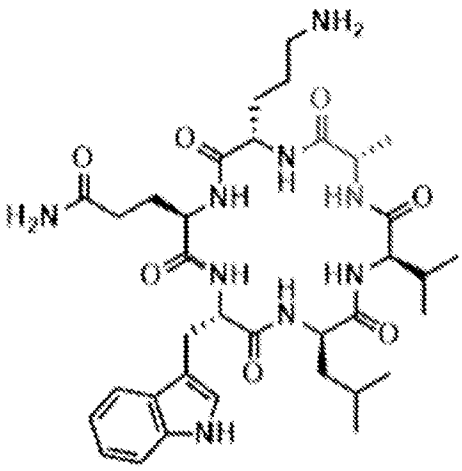
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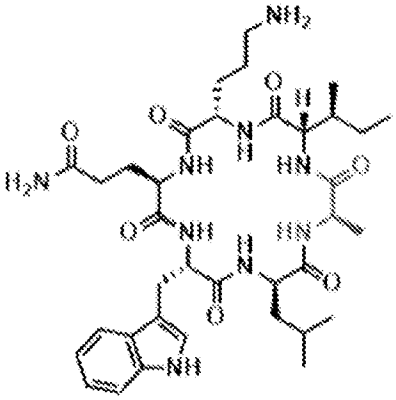
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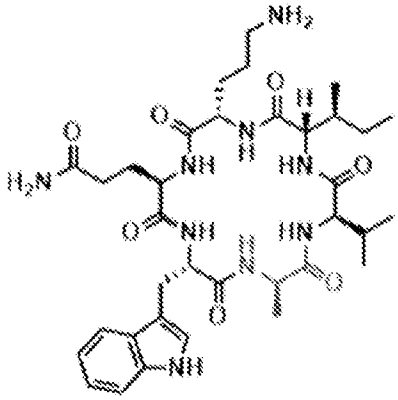
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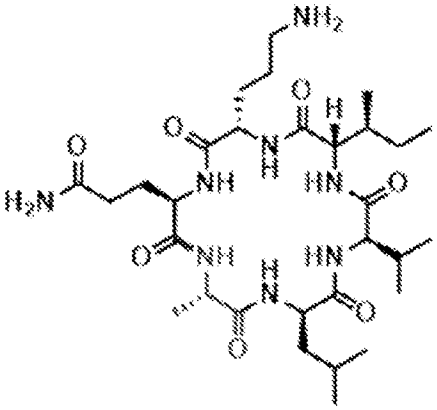
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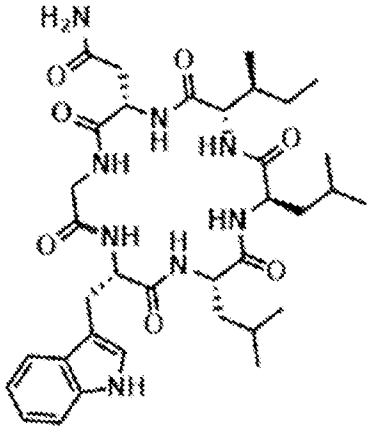
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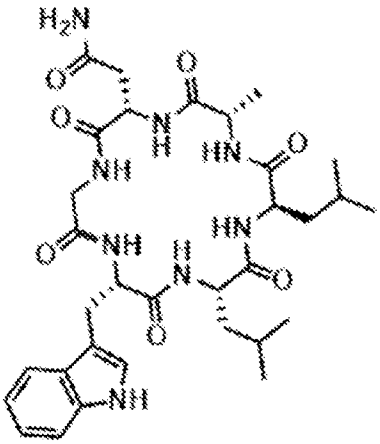
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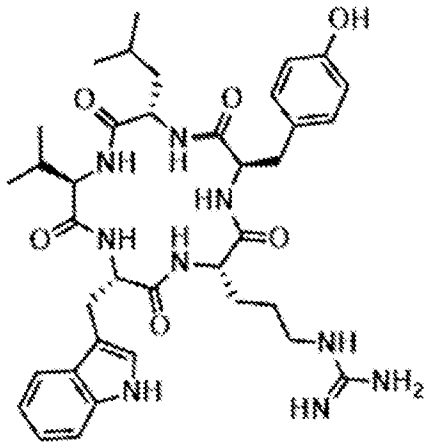
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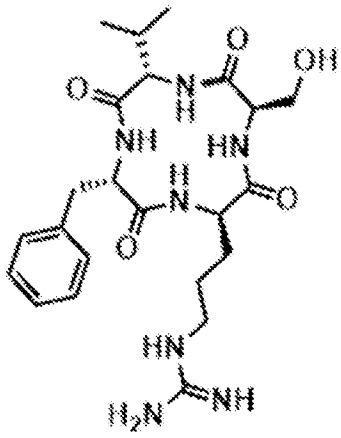
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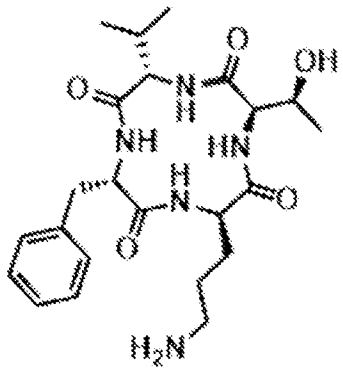
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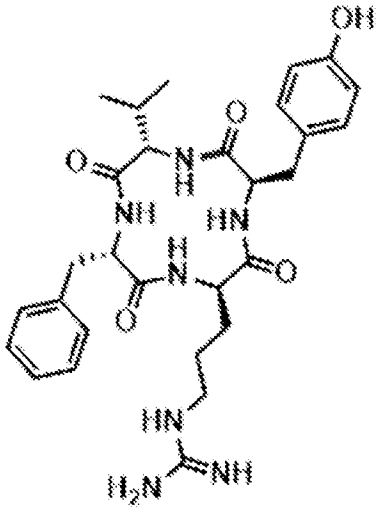
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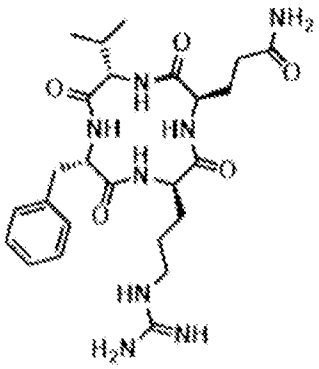
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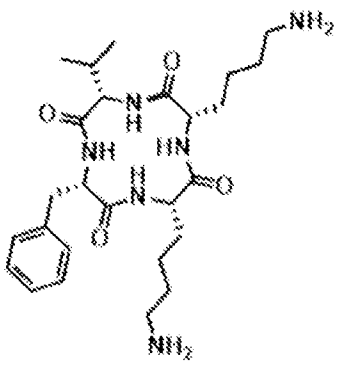
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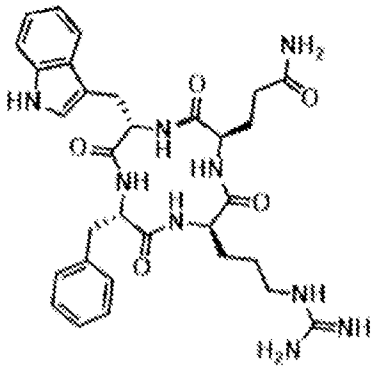
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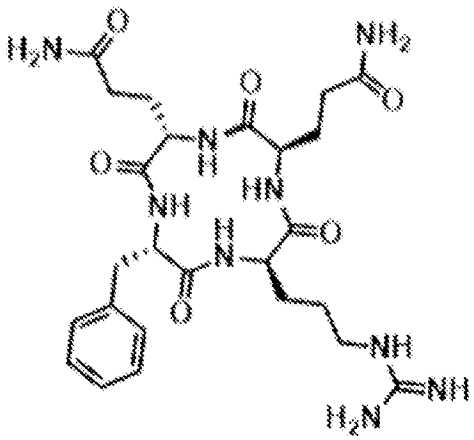
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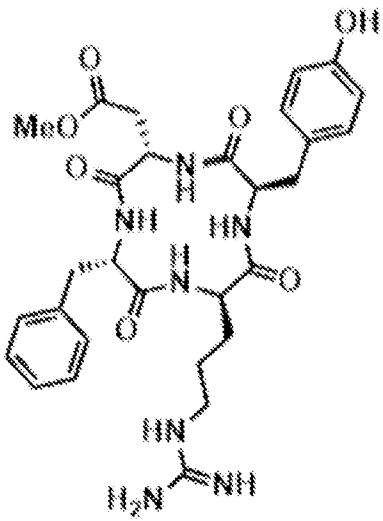
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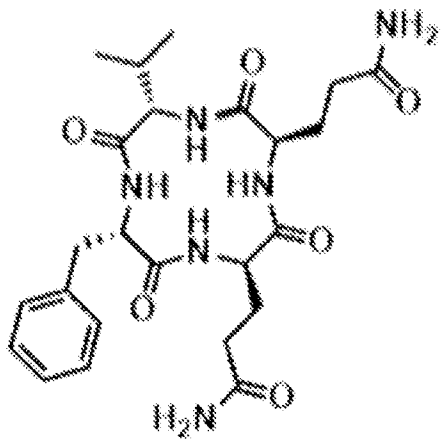
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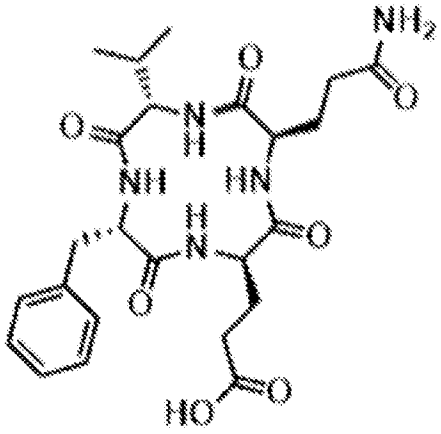
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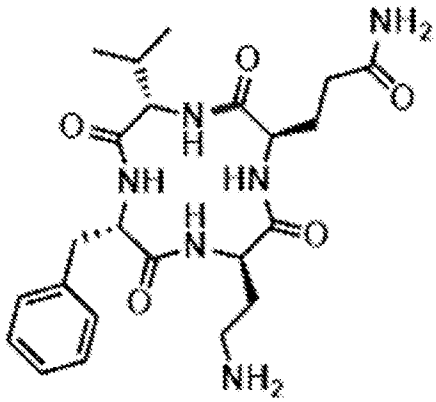
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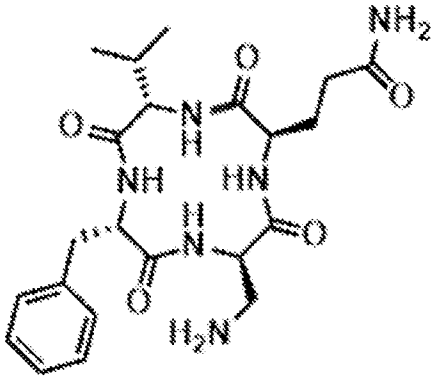
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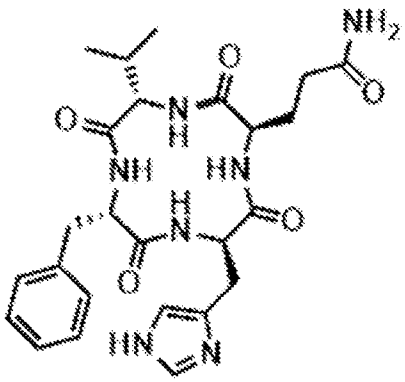
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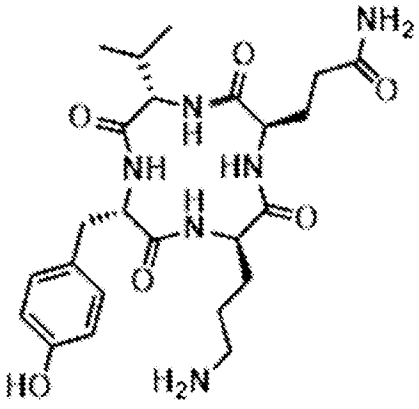
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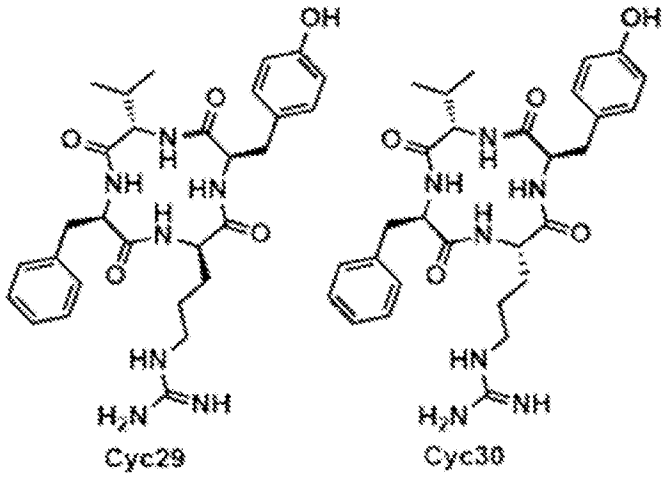
Cyc26



Cyc27



Cyc28



or a salt of any of the foregoing.

[0047] In some embodiments, cyclic peptides prepared by the method disclosed herein can have useful pharmaceutical applications that include but are not limited to use as antibiotics, antitumor agents, cholesterol-lowering drugs, and immune suppressants. Other applications and molecules with other biological activity profiles can also be suitable.

[0048] Bioinformatic analysis, X-ray crystal structure, and docking studies were conducted to explain the structural basis behind substrate promiscuity and increased catalytic activity of Ulm 16. Multiple sequence alignments were conducted to identify the region of substrate selectivity of Ulm 16 and known PBP-TEs, such as SurE, PenA, and WolJ and other predicted PBP-TEs from known BGCs, such as destoamides (DsaJ), Wollamides (WolJ), mannopeptimycin (MppK), cyclofaulknamycin (FlkO), and logicatenamide (Lon18) .Ulm16 displayed three notable features that distinguished it from its homologues: First, it had a remarkably shorter loop region above the active site c left, which is 35 amino acids shorter than the longest (PenA) and 21 amino acids shorter than the shortest (MppK). Ulm16 also displays a 16 amino acid loop insertion and a shorter loop region in the lipocalin domain , both of which were unique among the known PBP-TEs. Further bioinformatics analyses of the top 500 proteins most similar to Ulm16 did not reveal any other predicted PBP-TEs missing the first loop. The absence of a loop region in Ulm16 alters the peptide's binding trajectory and enhances its ability to bind and catalyze cyclization with high efficiency. A missing loop region can cause the C-terminal residue of the peptides to bind to a hydrophobic pocket beneath the activity site serine, resulting in a different trajectory than the other catalyst. Due to the unique sequence, Ulm16 can efficiently catalyzes the production of cyclic hexa, penta, and tetrapeptides beyond what has been previously reported for any thioesterase in the literature.

[0049] The term "substituted"(e.g., as in "optionally substituted") refers to a functional group in which one or more hydrogen atoms contained therein are replaced by one or more non-hydrogen atoms. The term "functional group" or "substituent" refers to a group that can be or is substituted onto a molecule. Examples of substituents or functional groups include, but are not limited to, a halogen (e.g., F, Cl, Br, and I); an oxygen atom in groups such as hydroxyl groups, alkoxy groups, aryloxy groups, aralkyloxy groups, oxo(carbonyl) groups, and carboxyl groups including carboxylic acids, carboxylates, and carboxylate esters; a sulfur atom in groups such as thiol groups, alkyl and aryl sulfide groups, sulfoxide groups, sulfone groups, sulfonyl groups, and sulfonamide groups; a nitrogen atom in groups such as amines, azides, hydroxylamines, cyano, nitro groups, N-oxides, hydrazides, and enamines; and other heteroatoms in various other groups.

[0050] The term " alkyl" refers to substituted or unsubstituted straight chain and branched alkyl groups and cycloalkyl groups having from 1 to about 20 carbon atoms (*e.g.*, C_1-C_{20}), 1 to 12 carbons (*e.g.*, C_1-C_{12}), 1 to 8 carbon atoms (*e.g.*, C_1-C_8), or, in some embodiments, from 1 to 6 carbon atoms (*e.g.*, C_1-C_6). Examples of straight chain alkyl groups include those with from 1 to 8 carbon atoms such as methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, n-heptyl, and n-octyl groups. Examples of branched alkyl groups include, but are not limited to, isopropyl, iso-butyl, sec-butyl, tert-butyl, neopentyl, isopentyl, and 2,2-dimethylpropyl groups. The term "alkyl" encompasses n-alkyl, isoalkyl, and anteisoalkyl groups as well as other branched chain forms of alkyl. Representative substituted alkyl groups can be substituted one or more times with any of the groups listed herein, for example, amino, hydroxy, cyano, carboxy, nitro, thio, alkoxy, and halogen groups.

[0051] The term "alkanoyl" refers to an acyl group (*e.g.*, -(C=O)-alkyl), in which carbon atoms are in a linear or branched alkyl arrangement and where attachment is through the carbon of the keto group. Alkanoyl groups have the indicated number of carbon atoms, with the carbon of the keto group being included in the numbered carbon atoms. For example a C_2-alkanoyl group is an acetyl group having the formula -(C=O)CH_3. Alkanoyl groups include, for example, C_2-C_8alkanoyl, C_2-C_6alkanoyl and C_2-C_4alkanoyl groups, which have from 2 to 8, from 2 to 6 or from 2 to 4 carbon atoms, respectively. "C_1-alkanoyl" refers to -(C=O)H, which (along with C_2-C_8 alkanoyl) is encompassed by the term "C_1-C_8alkanoyl.

[0052] The term "alkylalkanoate" refers to an ester unit which is one comprising up to 20 carbon atoms as a backbone and wherein the carbonyloxy component can be located anywhere along the 20-carbon backbone. The main chain especially can be replaced by C_1-C_6 alkyl or C_1-C_6 alkoxy.

[0053] The term "alkenyl" refers to substituted or unsubstituted straight chain and branched divalent alkenyl and cycloalkenyl groups having from 2 to 20 carbon atoms(C2-C20), 2 to 12 carbons (C2-C12), 2 to 8 carbon atoms (C2-C8) or, in some embodiments, from 2 to 4 carbon atoms (C2-C4) and at least one carbon-carbon double bond. Examples of straight chain alkenyl groups include those with from 2 to 8 carbon atoms such as -CH=CH-, -CH=CHCH$_2$-, and the like. Examples of branched alkenyl groups include, but are not limited to, -CH=C(CH3)- and the like.

[0054] An alkynyl group is a substituent, which contains an open point of attachment on a carbon atom that would form if a hydrogen atom bonded to a triply bonded carbon is removed from the molecule of an alkyne. The term "hydroxyalkyl" refers to alkyl groups as defined herein and substituted with at least one hydroxyl (-OH) group.

[0055] The term "cycloalkyl" refers to substituted or unsubstituted cyclic alkyl groups such as, but not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl groups. In some embodiments, the cycloalkyl group can have 3 to about 8-12 ring members, whereas in other embodiments the number of ring carbon atoms range from 3 to 4, 5, 6, or 7. In some embodiments, cycloalkyl groups can have 3 to 6 carbon atoms (C3-C6). Cycloalkyl groups further include polycyclic cycloalkyl groups such as, but not limited to, norbornyl, adamantyl, bornyl, camphenyl, isocamphenyl, and carenyl groups, and fused rings such as, but not limited to, decalinyl, and the like.The term "acyl" refers to a group containing a carbonyl moiety wherein the group is bonded via the carbonyl carbon atom. The carbonyl carbon atom is also bonded to another carbon atom, which can be part of a substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, heterocyclyl, heterocyclylalkyl, heteroaryl, heteroarylalkyl group or the like. In the special case wherein the carbonyl carbon atom is bonded to a hydrogen, the group is a "formyl" group, an acyl group as the term is defined herein. An acyl group can include 0 to about 12-40, 6-10, 1-5 or 2-5 additional carbon atoms bonded to the carbonyl group. An acryloyl group is an example of an acyl group. An acyl group can also include heteroatoms within the meaning herein. A nicotinoyl group (pyridyl-3-carbonyl) is an example of an acyl group within the meaning herein. Other examples include acetyl, benzoyl, phenylacetyl, pyridylacetyl, cinnamoyl, and cryloyl groups and the like. When the group containing the carbon atom that is bonded to the carbonyl carbon atom contains a halogen, the group is termed a "haloacyl" group. An example is a trifluoroacetyl group.

[0056] The term "aryl" refers to substituted or unsubstituted cyclic aromatic hydrocarbons that do not contain heteroatoms in the ring. Thus, aryl groups include, but are not limited to, phenyl,

azulenyl, heptalenyl, biphenyl, indacenyl, fluorenyl, phenanthrenyl, triphenylenyl, pyrenyl, naphthacenyl, chrysenyl, biphenylenyl, anthracenyl, and naphthyl groups. In some embodiments, aryl groups contain about 6 to about 14 carbons (C6-C14) or from 6 to 10 carbon atoms (C6-C10) in the ring portions of the groups. Aryl groups can be unsubstituted or substituted, as defined herein. Representative substituted aryl groups can be mono-substituted or substituted more than once, such as, but not limited to, 2-, 3-, 4-, 5-, or 6- substituted phenyl or 2-8 substituted naphthyl groups, which can be substituted with carbon or non-carbon groups such as those listed herein.

[0057] The terms "aralkyl" and "arylalkyl" refer to alkyl groups as defined herein in which a hydrogen or carbon bond of an alkyl group is replaced with a bond to an aryl group as defined herein. Representative aralkyl groups include benzyl and phenylethyl groups and fused (cycloalkylaryl)alkyl groups such as 4-ethyl-indanyl. Aralkenyl groups are alkenyl groups as defined herein in which a hydrogen or carbon bond of an alkyl group is replaced with a bondto an aryl group as defined herein.

[0058] The term "heterocyclyl" refers to substituted or unsubstituted aromatic and non-aromatic ring compounds containing three or more ring members, of which one or more is a heteroatom such as, but not limited to, B, N, O, and S. Thus, a heterocyclyl can be a cycloheteroalkyl, or a heteroaryl, or if polycyclic, any combination thereof. In some embodiments, heterocyclyl groups include 3 to about 20 ring members, whereas other such groups have 3 to about 15 ring members. In some embodiments, heterocyclyl groups can include 3 to 8 carbon atoms (C_3-C_8), 3 to 6 carbon atoms (C_3-C_6) or 6 to 8 carbon atoms (C_6-C_8).

[0059] A heteroaryl ring is an embodiment of a heterocyclyl group. The phrase "heterocyclylgroup" includes fused ring species including those that include fused aromatic and non-aromatic groups. Representative heterocyclyl groups include, but are not limited to, pyrrolidinyl, azetidinyl, piperidynyl, piperazinyl, morpholinyl, chromanyl, indolinonyl, isoindolinonyl, furanyl, pyrrolidinyl, pyridinyl, pyrazinyl, pyrimidinyl, triazinyl, thiophenyl, tetrahydrofuranyl, pyrrolyl, oxazolyl, oxadiazolyl, imidazolyl, triazyolyl, tetrazolyl, benzoxazolinyl, benzthiazolinyl, and benzimidazolinyl groups.

[0060] The term "heterocyclylalkyl" refers to alkyl groups as defined herein in which a hydrogen or carbon bond of an alkyl group as defined herein is replaced with a bond to a heterocyclyl group as defined herein. Representative heterocyclylalkyl groups include, but are not limited to, furan-2-yl methyl, furan-3-yl methyl, pyridine-3-yl methyl, tetrahydrofuran-2-yl methyl, and indol-2-ylpropyl.

[0061] The term "heteroarylalkyl" refers to alkyl groups as defined herein in which a hydrogen or carbon bond of an alkyl group is replaced with a bond to a heteroaryl group as defined herein.

[0062] The term "alkoxy" refers to an oxygen atom connected to an alkyl group, including a cycloalkyl group, as are defined herein. Examples of linear alkoxy groups include but are not limited to methoxy, ethoxy, propoxy, butoxy, pentyloxy, hexyloxy, and the like. Examples of branched alkoxy include but are not limited to isopropoxy, sec-butoxy, tert-butoxy, isopentyloxy, isohexyloxy, and the like. Examples of cyclic alkoxy include but are not limited to cyclopropyloxy, cyclobutyloxy, cyclopentyloxy, cyclohexyloxy, and the like. An alkoxy group can further include double or triple bonds and can also include heteroatoms. For example, an allyloxy group is an alkoxy group within the meaning herein. A methoxyethoxy group is also an alkoxy group within the meaning herein, as is a methylenedioxy group in a context where two adjacent atoms of a structure are substituted therewith.

[0063] The term "amine" refers to primary, secondary, and tertiary amines having, e.g., the formula N(group)3 wherein each group can independently be H or non-H, such as alkyl, aryl, and the like. Amines include but are not limited to R-NH2, for example, alkylamines, arylamines, alkylarylamines; R2NH wherein each R is independently selected, such as dialkylamines, diarylamines, aralkylamines, heterocyclylamines and the like; and R3N wherein each R is independently selected, such as trialkylamines, dialkylarylamines, alkyldiarylamines, triarylamines, and the like. The term "amine" also includes ammonium ions as used herein.

[0064] The term "amino group" refers to a substituent of the form $-NH_2$, -NHR, $-NR_2$, $-NR_3$ +, wherein each R is independently selected, and protonated forms of each, except for -NR3 +, which cannot be protonated. Accordingly, any compound substituted with an amino group can be viewed as an amine. An "amino group" within the meaning herein can be a primary, secondary, tertiary, or quaternary amino group. An "alkylamino" group includes a monoalkylamino, dialkylamino, and trialkylamino group.

[0065] The terms "optionally substituted" and "optional substituents" are used to describe groups, which are either unsubstituted or substituted with one or more of the substituents specified. When the groups in question are substituted with more than one substituent, the substituents can be the same or different. The terms "independently" "independently are" and "independently selected from" mean that the groups in question may be the same or different. Certain of the defined groups or

substituents can occur more than once in the structure, and upon such occurrence each group or substituent shall be defined independently of the other.

[0066] The term "aminoalkyl" as used herein refers to both secondary and tertiary amines where the point of attachment is through the nitrogen-atom and the alkyl groups are optionally substituted. The alkyl groups can be the same or different.

[0067] The term "amino acid" generally refers to an organic compound comprising both a carboxylic acid group and an amine group. The term "amino acid" includes both "natural" and "unnatural" or "non-natural" amino acids. Additionally, the term amino acid includes O-alkylated or N-alkylated amino acids, as well as amino acids having nitrogen or oxygen-containing side chains (such as Lys, Orn, or Ser) in which the nitrogen or oxygen atom has been acylated or alkylated. Amino acids may be pure L or D isomers or mixtures of L and D isomers, including racemic mixtures. In general, amino acids are represented by the residue of Formula V.

[0068] The term "natural amino acid" and equivalent expressions refer to L-amino acids commonly found in naturally occurring proteins. Examples of natural amino acids include, without limitation, alanine (Ala), cystein (Cys), aspartic acid (Asp), glutamic acid (Glu), phenylalanine (Phe), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), asparagine (Asp), proline (Pro), glutamine (Gin), arginine (Arg), serine (Ser), threonine (Thr), valine (Val), tryptophan (Trp), tyrosine (Tyr), β-alanine (β-ALA), and γ-aminobutyric acid (GABA).

[0069] The term "unnatural amino acid" refers to any derivative of a natural amino acid including D forms, and α- and β-amino acid derivatives. The terms "unnatural amino acid" and "non-natural amino acid" are used interchangeably herein and are meant to include the same moieties. It is noted that certain amino acids, e.g., hydroxyproline, that are classified as a non-natural amino acid herein, may be found in nature within a certain organism or a particular protein. Amino acids with many different protecting groups appropriate for immediate use in the solid phase synthesis of peptides are commercially available.

EXAMPLES

[0070] The following examples serve to illustrate the present disclosure. The examples are not intended to limit the scope of the claimed invention in any way.

Materials and Methods

[0071] Instrumentation: ultra-performance liquid chromatography (UPLC) and/or analytical high-performance liquid chromatography (HPLC) were utilized to assess the purity of the peptide prior to assay or purification by monitoring absorbance at 214 nm. The analytical HPLC analysis was performed on a Luna Omega 5 µm Polar C18 100 Å 150x4.6 mm (Phenomenex) column, while the UPLC analysis was carried out on a CORTECS T3 Column, 120Å, 1.6 µm, 2.1 mm X 50 mm (Waters) column. For purification, semi-preparative HPLC was employed using a Luna Omega 5 µm Polar C18 100 Å 150x21.2 mm (Phenomenex) column.

[0072] Reagents of the purest grade available were purchased from commercial sources and used without further purification: DMF, CH_2Cl_2, CH_3CN (HPLC grade) from Fisher Scientific, N-Methyl-2-pyrrolidone (NMP) from Alfa Aesar. All Amino acids were purchased from Chem-Impex or AAPTECC, and all other reagents used were purchased from Sigma-Aldrich unless stated otherwise.

[0073] AlphaFold2 Modeling

Protein models were generated using AlphaFold2 multimer. Ulm16 (Accession ATU31793.1), CppA (QQY97180.1), PenA (WP_158102277), SurE (BBZ90014.1), MppK (AAU34204.1), Lon18 (QUJ09165.1), FlkO (AGI87381.1), WolJ (UNO41476.1), and DsaJ (AJW76712.1) were used as inputs for the modeling and carried out utilizing the standard settings in ColabFold v1.5 (Mirdita M. et al., Bioinformatics 2009, 25, 1189–1191).

[0074] Multiple Sequence Alignment

Ulm16 (Accession ATU31793.1), CppA (QQY97180.1), PenA (WP_158102277), SurE (BBZ90014.1), MppK (AAU34204.1), Lon18 (QUJ09165.1), WolJ (UNO41476.1), and DsaJ (AJW76712.1) were used as inputs for a MUSCLE sequence alignment. The alignment was performed in JalView using MUSCLE v3.8.31.

[0075] Biosynthetic Gene Cluster Alignments

[0076] Biosynthetic gene clusters for ulleungmycin (BGC0001814), desotamide (BGC0001196), surugamide (BGC0001792), and mannopeptimycin (BGC0000388) were downloaded from MiBIG (Terlouw et al., Nucleic Acids Res. 2023, 51, D603–D610). The biosynthetic gene clusters for wollamide, longicatenamide, and pentaminomycin were determined by searching for the PBP-TE gene on NCBI and then expanding 20,000 bp on either side, then extending until the NRPS was not at the end of the region. The alignments were then generated using Clinker.

[0077] Phylogeny Analysis

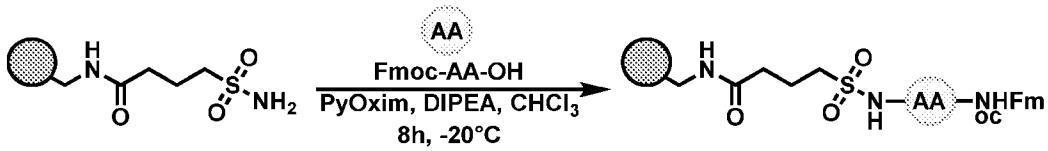
[0078] Ulm16 (Accession ATU31793.1). The top 500 sequences, along with CppA (QQY97180.1), PenA (WP_158102277), SurE (BBZ90014.1), MppK (AAU34204.1), Lon18 (QUJ09165.1), WolJ (UNO41476.1), and DsaJ (AJW76712.1) and the outlier AmpC (beta-lactamase from *E. coli*). MEGA-X was then used to perform a MUSCLE alignment followed by tree generation using the Maximum Likelihood method (100 bootstraps). Images were generated using MEGA-X.

[0079] The gene sequence for Ulm16:

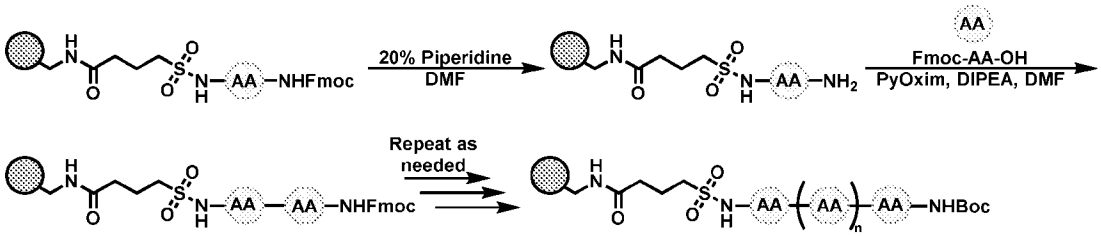
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TGCGCCGCTGGCTCACCGGCTTCCTGGCACTGCCGGTCGGCCCCTGGCCCGCACCCGG
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ACCGGACTGACCTGGTCCGAGGCGGTACGGGACTTCCTGCTGCACCCGCTGGGCACCG
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CGCCGCCGACCTGGTACGGCTGGGCCGGCTGCATCTGGACGAGCCCGGTGACCCGGA
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CGCCGACCCCTTCGGGCTGGCGGACGGCTGGGGACCGGGCCTGGGCCGGTTCGGCCC
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TCCACCGCCCCGCGCCCCCGCCTGCCATCGCCGCGGCGGCCTTCGCGGACTGTACGGG
TACCTACCGCAACGGCGACCTGGCGGTGACGGTCGGCATCGACGGCCCGTACCTCGTA
CTTGAACTCCCCGGCGGCGCACGGGAGTTGGCGCAGCCACTGGCTCACCGGACGTTCT
CCTCGCGGGGGGCCGGCTTCCTCGGGCGCTTTGTCACCGACGCGCGCTCCGACGCCGT
CCACGCCCTCCAGTACAGCGGACGCACCCTCCTGCGGGAAGCGGGAGAGTCCCGCCG
CGCACGGTCGCACTGA (SEQ ID NO: 1)

General procedure for the synthesis of linear peptide

[0080] Conventional peptide thioester synthesis at the sulfonamide "safety-catch" resin

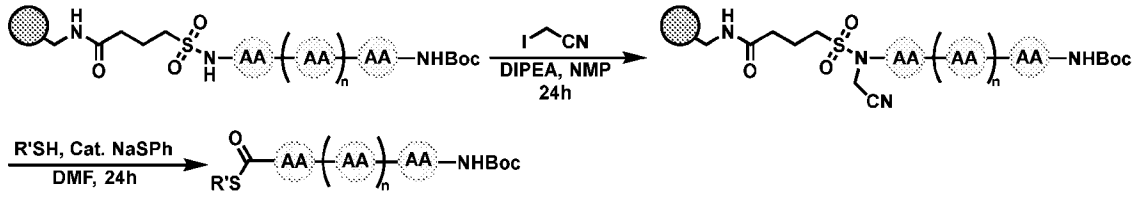


[0081] *Attachment of the first amino acid:* 0.4 g (0.37 mmol) of 4-sulfamylbutyryl AM resin (Novabiochem) and an Fmoc-protected amino acid (1.47 mmol) were used to initiate the reaction. The resin was placed in a 5 mL fritted polypropylene syringe (Torviq), washed with $CHCl_3$, and cooled to -20 °C. The resin was then swelled for 15 minutes. After that, the Fmoc-protected amino acid was dissolved in $CHCl_3$ (3 mL), and i-Pr_2EtN (1.84 mmol) was added to the resin. The mixture was allowed to mix for 10 minutes at -20 °C. Once cooled, PyOxim (1.47 mmol) was added to the reaction mixture, and the mixture was left to stand at -20 °C for 8 hours with occasional shaking. The resin was then washed with CH_2Cl_2 (3 x 5 mL) and MeOH (3 x 5 mL) and dried under vacuum for 1 hour. To determine the resin loading, an aliquot (1-3 mg) of the dried resin was treated with piperidine-DMF (1:4). The UV absorbance of the piperidine-dibenzofulvene adduct was observed at 301 nm (ε = 7800 M^{-1} cm^{-1}).

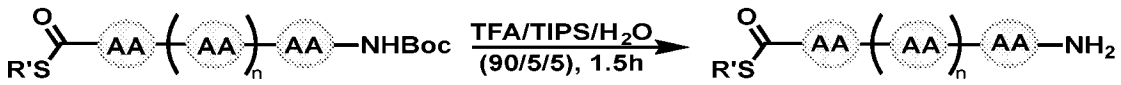


[0082] *Solid-phase peptide synthesis (SPPS):* All manual SPPS and cleavage steps were carried out using 5 mL fritted polypropylene syringes (Torviq) as reaction vessels. Pre-loaded 4-sulfamylbutyryl AM resin (0.05 mmol) was swelled in DMF for 15 minutes, drained, and treated with piperidine-DMF (1:4, 3 mL, 1 x 15 minutes). The resin was then filtered and washed with DMF (2 x 3 mL) and CH_2Cl_2 (2 x 3 mL). In a separate flask, DIC (0.25 mmol) was added to a solution of Fmoc-AA-OH (0.25 mmol) and Oxyma Pure (0.25 mmol) in DMF (1 mL). After a 5-minute preactivation period, the resulting solution was added to the resin, and the mixture was agitated for 1 hour. The resin was then filtered and washed with DMF (3 x 2 mL) and CH_2Cl_2 (3 x 2 mL). The Kaiser ninhydrin test was performed to determine reaction completion. Deprotection and coupling cycles were repeated

until the desired peptide sequence was complete. The last coupled amino acid was N-Boc-protected to block the N-terminus until final peptide cleavage. For amino acids following an arginine, PyOxim (0.25 mmol) and i-Pr_2EtN (0.3 mmol) were used in place of oxyma/DIC.

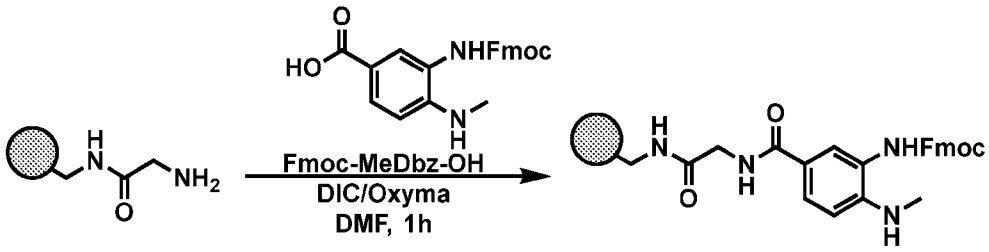


[0083] *Activation and thiolysis of acyl-sulfonamide bond:* After the last amino acid was attached, the resin (0.05 mmol) was washed with NMP (4 x 5 mL) and allowed to swell in NMP for 15 minutes. The resin was then filtered, and a solution of NMP, i-Pr_2EtN (0.75 mmol), and iodoacetonitrile (1.5 mmol), previously filtered through an alumina basic plug, was added to the resin. The mixture was allowed to shake for 24 hours. The resin was then washed with NMP (4 x 5 mL) and DMF (5 x 3 mL). The resulting activated N-acylsulfonamide resin was swollen in DMF for 15 minutes, filtered, and then treated with a 50:50 thiol (2.5 mmol) DMF solution containing catalytic sodium thiophenolate (0.025 mmol) for 24 hours. The resin was filtered and washed with DMF (3 x 1 mL). The combined filtrate and washes were collected in a 20 mL scintillation vial, and the solvent was removed by rotary evaporation followed by lyophilization.



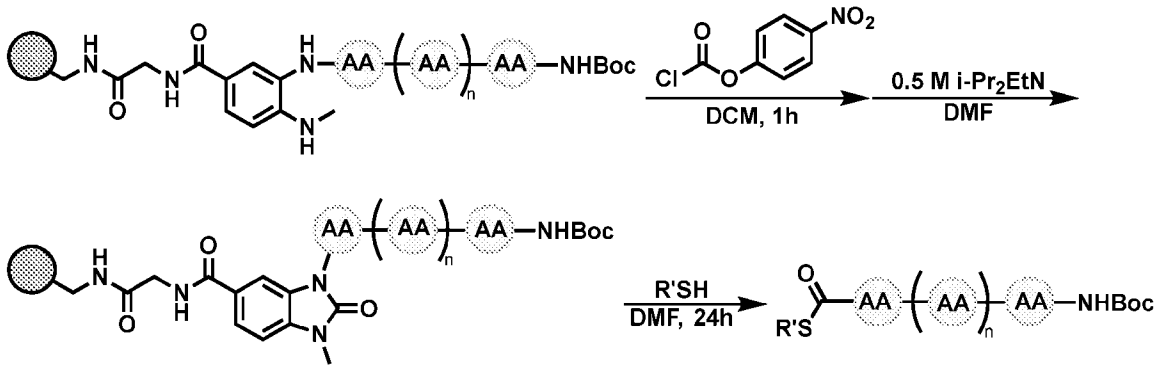
[0084] *Global deprotection of peptide thioesters:* the crude peptide was subjected to global deprotection by treating it with a mixture of trifluoroacetic acid (TFA), water, and triisopropylsilane (TIPS) in a ratio of 90:5:5 for 1.5 hours. The TFA solution was then removed by a stream of air, and the peptide was precipitated by adding diethyl ether at -20 °C. The resulting precipitate was collected by centrifugation, and the supernatant was discarded. The peptide was then lyophilized to obtain a dry powder. The crude product was characterized by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) or high-performance liquid chromatography (HPLC). If the purity of the crude product was >90%, as determined by monitoring the absorbance at 214 nm, it was used in the cyclization assay without further purification.

[0085] Conventional peptide thioester synthesis at the NDbz "safety-catch" resin



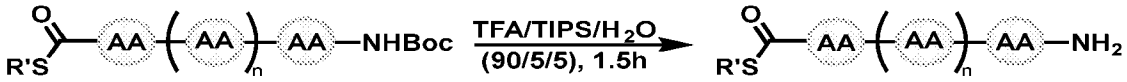
[0086] *Attachment of Fmoc-MeDbz-OH:* A 5 mL fritted polypropylene syringe containing 0.05 mmol of Fmoc-Gly Rink amide resin (Chem-Impex) was washed with DMF (3 x 5 mL) and allowed to swell for 15 minutes. A solution of Fmoc-MeDbz-OH (synthesized according to a known procedure (Blanco-Canosa et al., Journal of the American Chemical Society **137,** 7197–7209 (2015)) (0.25 mmol), Oxyma (0.25 mmol), and DIC (0.25 mmol) in 3 mL of DMF were added to the resin and allowed to shake for 1 hour. The resin was filtered and washed with CH_2Cl_2 (3 x 5 mL) and DMF (3 x 5 mL). Loading efficiency was assumed to be 100% based on a negative ninhydrin test, and the resin was subsequently used in solid-phase peptide synthesis.

[0087] *Solid-phase peptide synthesis:* Manual SPPS was carried out as previously described for the sulfonamide "safety-catch" resin.



[0088] *Activation and thiolysis:* The resin was washed with CH_2Cl_2 (3 x 5 mL) and allowed to swell for 15 minutes. The resin was then treated with 1 mL of 0.5 M 4-nitrophenyl chloroformate (TCI America) in CH_2Cl_2 and shaken for 2 hours. The resin was subsequently washed with CH_2Cl_2 (3 x 5 mL) and DMF (3 x 5 mL). A solution of 3 mL of 0.5 M i-Pr_2EtN in DMF was added to the resin and allowed to react for 15 minutes. The resin was then filtered, washed with DMF (1 x 5 mL), and exposed to fresh 0.5 M i-Pr2EtN in DMF solution, repeating this process until the solution no longer

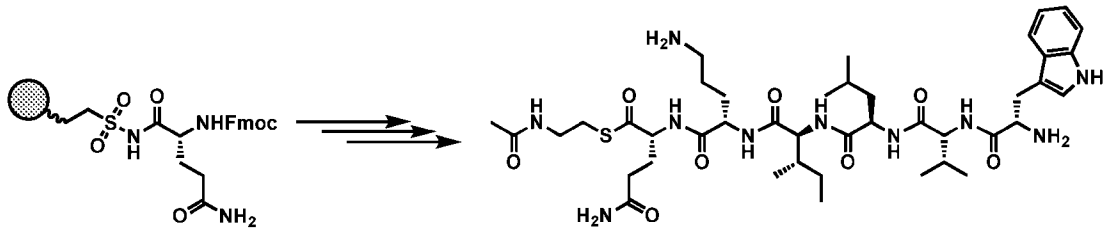
turned yellow. The resin was then swollen in DMF for 15 minutes, filtered, and treated with a 50:50 solution of thiol (2.5 mmol) in DMF, and shaken for 24 hours. The resin was filtered and washed with DMF (3 x 1 mL). The combined filtrate and washes were collected in a 20 mL scintillation vial and subjected to rotary evaporation and lyophilization.



[0089] *Global deprotection of peptide thioesters:* deprotection and UPLC-MS or HPLC analysis was carried out as described above.

Synthesis of Peptide Thioesters

[0090] Synthesis of liner peptide 1

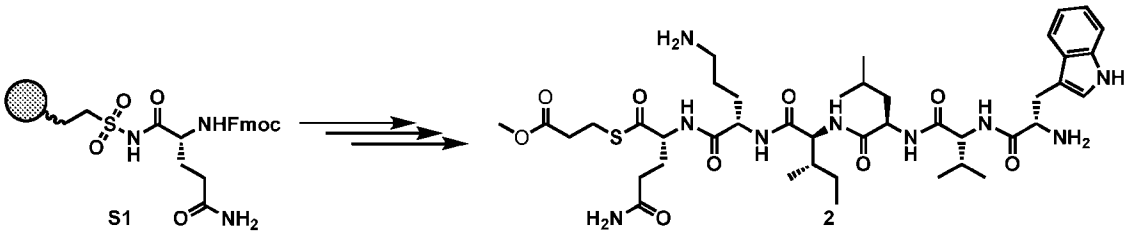


Peptide **1** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from 4-sulfamylbuyryl resin preloaded with Fmoc-D-Gln(Trt)-OH **(S1)** (0.05 mmol) using the following amino acids (Fmoc-L-Orn(Boc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Leu-OH, Fmoc-D-Val-OH, Boc-L-Trp(Boc)-OH) and N-Aceytlcysteamine as the cleaving thiol. The crude peptide was obtained as an off-white solid (30.3 mg, 55% yield) analyzed via RP-UPLC and was used in further enzyme assays without further purification (purity 96%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 11.03 (s, 1H), 8.64 (q, *J* = 6.8 Hz, 2H), 8.20 (dd, *J* = 8.2, 8.2 Hz, 2H), 8.07 (t, *J* = 6.1 Hz, 1H), 7.91 – 7.68 (m, 6H), 7.36 (d, *J* = 7.5 Hz, 1H), 7.27 (s, 1H), 7.23 (s, 1H), 7.09 (t, *J* = 7.5 Hz, 1H), 7.01 (t, *J* = 8.2 Hz, 1H), 6.81 (s, 1H), 4.40 (p, *J* = 5.4 Hz, 2H), 4.34 – 4.26 (m, 2H), 4.23 (t, *J* = 7.5 Hz, 1H), 4.15 (t, *J* = 5.6 Hz, 1H), 3.31 (q, *J* = 8.2 Hz, 1H), 3.25 (dd, *J* = 6.8, 5.4 Hz, 1H), 3.11 (q, *J* = 7.0 Hz, 2H), 3.02 (q, *J* = 9.6 Hz, 1H), 2.87 – 2.73 (m, 4H), 2.17 – 2.05 (m, 2H), 1.97 (h, *J* = 6.1 Hz, 1H), 1.92 (h, *J* = 5.4 Hz, 1H), 1.82 – 1.65 (m, 7H), 1.62 – 1.49 (m, 4H), 1.46 (t, *J* = 4.9 Hz, 2H), 1.42 – 1.35 (m, 1H), 1.05 (hept, *J* = 6.1 Hz, 1H), 0.87 (d, *J* = 7.5 Hz,

3H), 0.83 (d, J = 6.8 Hz, 3H), 0.79 (d, J = 5.6 Hz, 3H), 0.78 – 0.71 (m, 9H). **^{13}C NMR** (201 MHz, DMSO) δ 200.6, 173.2, 171.7, 171.6, 170.8, 170.4, 169.3, 136.3, 127.1, 124.9, 121.1, 118.7, 118.3, 111.4, 107.2, 58.7, 57.6, 56.5, 52.8, 51.7, 51.2, 42.2, 41.1, 38.5, 38.1, 38.0, 37.3, 36.8, 30.9, 30.9, 29.1, 28.3, 27.6, 27.0, 24.2, 24.1, 23.5, 23.0, 22.6, 22.5, 21.5, 19.1, 17.8, 15.3, 10.9.**Mass spec:** expected neutral mass for $C_{42}H_{68}N_{10}O_8S$ (Da): 872.4942, observed neutral mass (Da): 872.4967, mass error (ppm) 2.8.

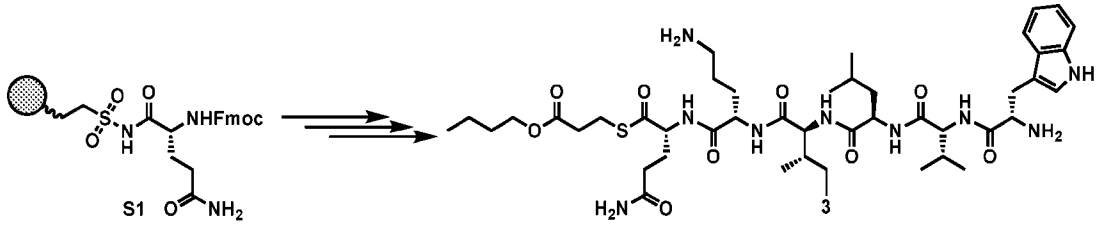
[0091] Synthesis of liner peptide 2



Peptide **2** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from 4-sulfamylbuyryl resin preloaded with Fmoc-D-Gln(Trt)-OH **(S1)** (0.05 mmol) using the following amino acids (Fmoc-L-Orn(Boc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Leu-OH, Fmoc-D-Val-OH, Boc-L-Trp(Boc)-OH) and methyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (27.2 mg, 49% yield) analyzed via RP-UPLC and used in further enzyme assays without further purification (purity 96%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 11.03 (s, 1H), 8.64 (dd, J = 335.2, 4.7 Hz, 2H), 8.20 (dd, J = 9.1, 9.1 Hz, 2H), 7.83 (d, J = 9.1 Hz, 2H), 7.74 (d, J = 9.1 Hz, 6H), 7.34 (d, J = 7.5 Hz, 1H), 7.26 (d, J = 24.4 Hz, 2H), 7.09 (t, J = 7.5 Hz, 1H), 7.00 (t, J = 6.3 Hz, 1H), 6.81 (s, 1H), 4.40 (p, J = 7.9 Hz, 2H), 4.33 – 4.26 (m, 2H), 4.24 (t, J = 7.5 Hz, 1H), 4.14 (t, J = 9.1 Hz, 1H), 3.60 (s, 4H), 3.24 (dd, J = 6.3, 5.9 Hz, 1H), 3.04 – 2.92 (m, 4H), 2.79 (t, J = 9.4 Hz, 2H), 2.56 (q, J = 5.9 Hz, 2H), 2.11 (h, J = 5.9 Hz, 2H), 1.99 – 1.89 (m, 2H), 1.77 – 1.66 (m, 4H), 1.61 – 1.50 (m, 5H), 1.46 (t, J = 7.5 Hz, 3H), 1.42 – 1.35 (m, 1H), 1.05 (h, J = 4.7 Hz, 1H), 0.90 – 0.69 (m, 24H).**^{13}C NMR** (201 MHz, DMSO) δ 200.6, 173.1, 171.7, 171.6, 171.6, 170.8, 170.4, 136.4, 127.1, 124.9, 121.1, 118.7, 118.3, 111.4, 107.3, 58.6, 57.6, 56.5, 52.8, 51.7, 51.6, 51.2, 41.1, 38.5, 36.8, 33.5, 30.9, 30.8, 29.1, 28.3, 26.9, 24.2, 24.1, 23.5, 23.2, 23.0, 21.5, 19.1, 17.8, 15.3, 10.9.**Mass spec:** expected neutral mass for $C_{42}H_{67}N_9O_9S$ (Da): 873.4782, observed neutral mass (Da): 873.4809, mass error (ppm) 3.1.

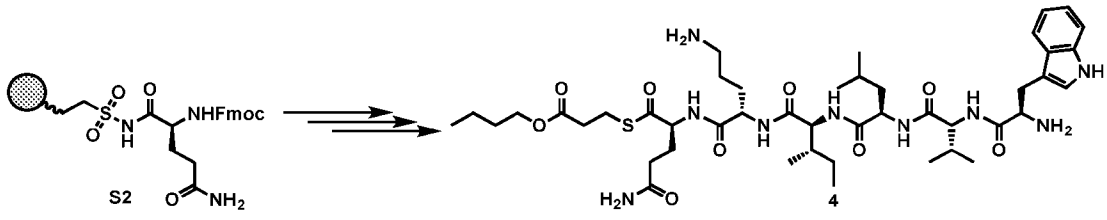
[0092] Synthesis of liner peptide 3



Peptide **3** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from 4-sulfamylbuyryl resin preloaded with Fmoc-D-Gln(Trt)-OH (**S1**) (0.05 mmol) using the following amino acids (Fmoc-L-Orn(Boc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Leu-OH, Fmoc-D-Val-OH, Boc-L-Trp(Boc)-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (22.3 mg, 39% yield) analyzed via RP-UPLC and was used in further enzyme assays without further purification (purity 99%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 10.87 (s, 1H), 8.66 (d, J = 8.4 Hz, 1H), 8.45 (s, 1H), 8.20 (dd, J = 6.2, 4.3 Hz, 3H), 7.98 (d, J = 7.3 Hz, 1H), 7.56 (d, J = 7.3 Hz, 1H), 7.38 (s, 1H), 7.33 (d, J = 7.3 Hz, 1H), 7.17 (s, 1H), 7.05 (t, J = 6.5 Hz, 1H), 6.97 (t, J = 6.2 Hz, 1H), 6.77 (s, 1H), 4.41 – 4.33 (m, 2H), 4.31 – 4.25 (m, 1H), 4.20 (t, J = 8.6 Hz, 1H), 4.14 (t, J = 6.5 Hz, 1H), 4.02 (t, J = 6.5 Hz, 2H), 3.60 (t, J = 5.4 Hz, 1H), 3.10 (dd, J = 6.5, 5.4 Hz, 1H), 3.00 – 2.90 (m, 2H), 2.74 – 2.65 (m, 3H), 2.54 (dd, J = 962.0, 6.2 Hz, 2H), 2.16 – 2.06 (m, 2H), 2.00 – 1.92 (m, 2H), 1.79 – 1.67 (m, 3H), 1.61 – 1.43 (m, 9H), 1.42 – 1.35 (m, 1H), 1.31 (dd, J = 181.8, 7.5 Hz, 2H), 1.07 (p, J = 8.4 Hz, 1H), 0.88 (t, J = 7.3 Hz, 7H), 0.83 (d, J = 7.3 Hz, 4H), 0.79 (q, J = 6.5 Hz, 8H), 0.77 (t, J = 2.2 Hz, 6H).**^{13}C NMR** (201 MHz, DMSO) δ 201.15, 174.93, 173.48, 172.19, 171.49, 171.38, 171.21, 166.30, 136.66, 127.70, 124.16, 121.25, 118.82, 118.57, 111.70, 111.01, 70.17, 64.29, 59.08, 58.02, 57.04, 55.46, 52.10, 51.66, 41.26, 39.00, 36.73, 34.02, 31.23, 30.91, 30.51, 29.59, 27.27, 24.62, 23.62, 23.37, 21.89, 19.61, 18.98, 18.41, 15.74, 13.94, 11.24.**Mass spec:** expected neutral mass for $C_{45}H_{73}N_9O_9S$ (Da): 915.5250, observed neutral mass (Da): 915.5249, mass error (ppm) 0.3.

[0093] Synthesis of liner peptide 4

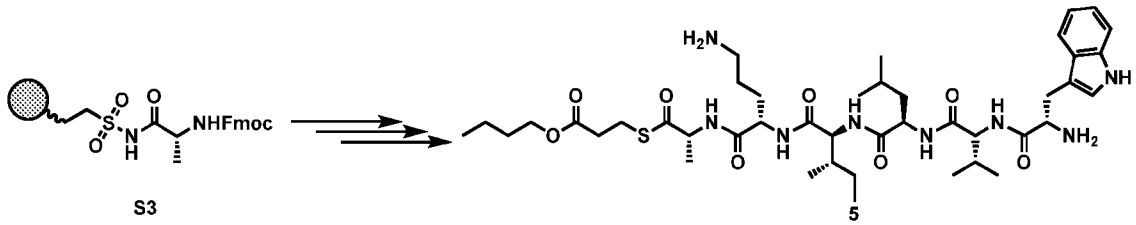


Peptide **4** was synthesized using the sulfonamide "safety-catch" resin following the general protocol described earlier. The synthesis was started from 4-sulfamylbuyryl resin preloaded with Fmoc-L-Gln(Trt)-OH (**S2**) (0.05 mmol) and utilized the following amino acids: Fmoc-L-Orn(Boc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Leu-OH, Fmoc-D-Val-OH, and Boc-D-Trp-OH, with Butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.1% formic acid (A) and acetonitrile+0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 55% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (8.1 mg, 16% yield, Purity 99%, HPLC) .

^{1}H NMR (800 MHz, DMSO) δ 10.91 (s, 1H), 8.72 (s, 1H), 8.45 (s, 2H), 8.34 – 7.97 (m, 6H), 7.56 (s, 1H), 7.33 (s, 3H), 7.19 (s, 2H), 7.06 (d, J = 8.0 Hz, 1H), 6.97 (d, J = 6.9 Hz, 2H), 6.78 (s, 1H), 6.16 (t, J = 56.3 Hz, 2H), 4.33 (d, J = 42.5 Hz, 5H), 4.20 (dd, J = 136.6, 17.8 Hz, 4H), 4.02 (d, J = 5.7 Hz, 3H), 3.60 – 3.43 (m, 2H), 3.17 – 3.02 (m, 1H), 2.97 (d, J = 6.3 Hz, 3H), 2.74 (d, J = 6.9 Hz, 5H), 2.55 (d, J = 7.5 Hz, 3H), 2.13 (d, J = 7.7 Hz, 3H), 1.95 (s, 3H), 1.87 – 1.67 (m, 5H), 1.66 – 1.17 (m, 29H), 1.06 (s, 1H), 0.94 – 0.58 (m, 42H).**^{13}C NMR** (201 MHz, DMSO) δ 200.8, 174.1, 173.2, 171.8, 171.7, 171.2, 171.0, 171.0, 166.0, 136.3, 118.2, 117.4, 116.2, 115.0, 111.3, 110.4, 63.9, 58.8, 57.2, 56.6, 55.1, 51.6, 51.4, 40.8, 38.2, 36.3, 33.6, 31.0, 30.8, 30.6, 30.1, 28.4, 27.1, 24.3, 24.2, 23.9, 23.2, 22.9, 21.5, 20.5, 20.4, 20.2, 19.2, 18.6, 17.9, 15.4, 13.5, 10.8.

Mass spec: expected neutral mass for $C_{45}H_{73}N_9O_9S$ (Da): 915.5251, observed neutral mass (Da): 915.5215, mass error (ppm) 6.0.

[0094] Synthesis of liner peptide 5

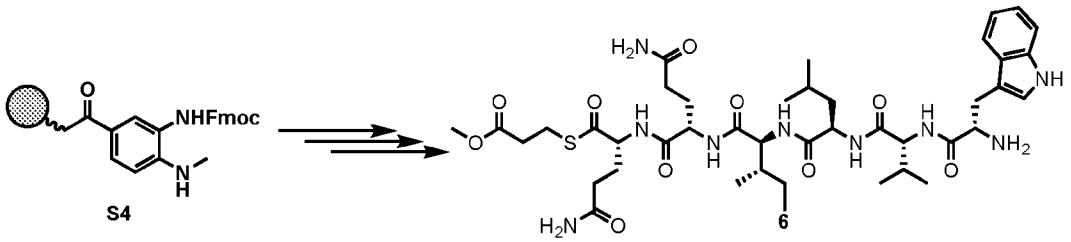


Peptide **5** was synthesized using the sulfonamide "safety-catch" resin according to the general protocol described earlier. The synthesis was initiated from 4-sulfamylbutryl resin preloaded with Fmoc-D-Ala-OH (**S3**) (0.05 mmol) and utilized the following amino acids: Fmoc-L-Orn(Boc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Leu-OH, Fmoc-D-Val-OH, and Boc-L-Trp(Boc)-OH, with butyl 3-

mercaptopropionate used as the cleaving thiol. The crude peptide was obtained as an off-white solid (15.5 mg, 29% yield) and was analyzed via RP-UPLC. It was used in further enzyme assays without undergoing further purification (purity 96%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 11.04 (s, 1H), 8.68 (dd, *J* = 36.9, 8.6 Hz, 2H), 8.21 (dd, *J* = 24.9, 7.9 Hz, 2H), 8.06 (bs, 3H), 7.85 (bs, 3H), 7.82 (d, *J* = 8.5 Hz, 2H), 7.77 (d, *J* = 7.9 Hz, 1H), 7.37 (d, *J* = 7.9 Hz, 1H), 7.24 (s, 1H), 7.09 (t, *J* = 7.3 Hz, 1H), 7.01 (t, *J* = 7.2 Hz, 1H), 4.42 – 4.31 (m, 4H), 4.29 (d, *J* = 7.2 Hz, 1H), 4.25 (t, *J* = 7.6 Hz, 1H), 4.19 (s, 1H), 4.02 (t, *J* = 6.3 Hz, 2H), 3.35 (bs, 3H), 3.26 (dd, *J* = 14.3, 5.6 Hz, 1H), 3.05 (dd, *J* = 14.6, 8.9 Hz, 1H), 3.00 – 2.92 (m, 2H), 2.78 (s, 2H), 2.54 (t, *J* = 6.6 Hz, 2H), 2.50 (s, 2H), 1.93 (h, *J* = 6.9 Hz, 1H), 1.74 (s, 1H), 1.69 (s, 1H), 1.61 – 1.49 (m, 8H), 1.46 (t, *J* = 6.9 Hz, 2H), 1.38 (s, 1H), 1.32 (h, *J* = 7.3 Hz, 3H), 1.26 (d, *J* = 7.3 Hz, 4H), 1.05 (hept, *J* = 7.3 Hz, 1H), 0.88 (quint, *J* = 7.2, 4.3 Hz, 8H), 0.84 (d, *J* = 6.3 Hz, 4H), 0.81 – 0.71 (m, 16H). **^{13}C NMR** (201 MHz, DMSO) δ 201.2, 171.6, 171.3, 171.1, 170.8, 170.3, 168.7, 136.4, 127.0, 125.0, 121.1, 118.7, 118.3, 111.4, 106.9, 63.9, 57.6, 56.4, 54.7, 52.6, 51.7, 51.2, 41.0, 38.5, 36.9, 33.6, 31.0, 30.1, 29.0, 28.0, 24.2, 24.1, 23.5, 23.2, 23.0, 21.6, 19.1, 18.6, 17.8, 17.3, 15.3, 13.5, 10.9.**Mass spec:** expected neutral mass for $C_{43}H_{70}N_8O_8S$ (Da): 858.5037, observed neutral mass (Da): 858.5063, mass error (ppm) 3.0.

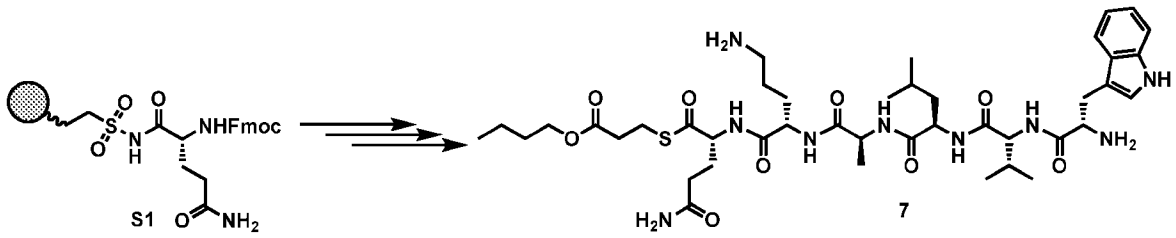
[0095] Synthesis of liner peptide 6



Peptide **6** was synthesized following the general protocol outlined above for the NDbz "safety-catch" resin. The synthesis was initiated from **(S4)** (0.15 mmol) using the following amino acids (Fmoc-L-Gln(Trt)-OH, Fmoc-L-Ile-OH, Fmoc-D-Leu-OH, Fmoc-D-Val-OH, Boc-L-Trp(Boc)-OH) and methyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.1% formic acid (A) and acetonitrile+0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 60% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (82.5 mg, 53% yield, purity 96%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 11.04 (s, 1H), 8.68 (d, *J* = 8.4 Hz, 1H), 8.52 (d, *J* = 9.5 Hz, 1H), 8.20 (d, *J* = 8.4 Hz, 1H), 8.14 (d, *J* = 8.1 Hz, 1H), 8.07 (s, 3H), 7.81 (d, *J* = 8.4 Hz, 1H), 7.76 (d, *J* = 8.1 Hz, 1H), 7.36 (d, *J* = 8.1 Hz, 1H), 7.26 (t, *J* = 25.5 Hz, 3H), 7.09 (dd, *J* = 50.4, 7.0 Hz, 1H), 7.01 (t, *J* = 8.1 Hz, 1H), 6.79 (d, *J* = 6.7 Hz, 2H), 4.40 (q, *J* = 7.0 Hz, 1H), 4.35 – 4.27 (m, 3H), 4.26 – 4.18 (m, 2H), 3.59 (s, 3H), 3.26 (dd, *J* = 5.3, 5.3 Hz, 1H), 3.06 (q, *J* = 6.7 Hz, 1H), 3.01 – 2.92 (m, 2H), 2.55 (t, *J* = 6.7 Hz, 2H), 2.17 – 2.03 (m, 4H), 2.01 – 1.91 (m, 2H), 1.89 – 1.83 (m, 1H), 1.82 – 1.70 (m, 3H), 1.58 (h, *J* = 8.1 Hz, 1H), 1.51 – 1.42 (m, 2H), 1.42 – 1.36 (m, 1H), 1.06 (h, *J* = 7.0 Hz, 1H), 0.87 (d, *J* = 8.1 Hz, 3H), 0.82 (d, *J* = 7.0 Hz, 3H), 0.82 – 0.70 (m, 13H).**^{13}C NMR** (201 MHz, DMSO) δ 201.07, 174.08, 173.57, 172.03, 171.94, 171.15, 170.66, 169.10, 136.74, 127.43, 125.41, 121.53, 119.09, 118.71, 111.81, 107.29, 59.07, 58.04, 56.90, 52.96, 52.68, 51.94, 51.65, 41.36, 36.99, 33.85, 31.83, 31.35, 31.20, 28.53, 28.36, 27.28, 24.58 (d, *J* = 14.8 Hz), 23.60, 23.29, 22.03, 19.44, 18.11, 15.69, 11.23.**Mass spec:** expected neutral mass for $C_{45}H_{71}N_9O_{10}S$ (Da): 887.4575, observed neutral mass (Da): 887.4599, mass error (ppm) 2.7.

[0096] Synthesis of liner peptide 7

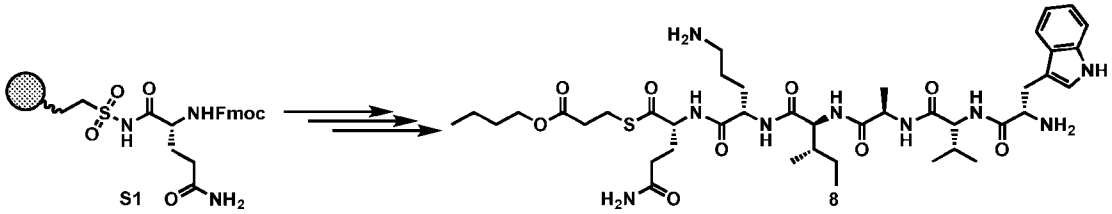


Peptide **7** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from (**S1**) (0.05 mmol) using the following amino acids (Boc-L-Trp(Boc)-OH, Fmoc-D-Val-OH, Fmoc-D-Leu-OH, Fmoc-L-Ala-OH, Fmoc-L-Orn(Boc)-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (26.9 mg, 49% yield), analyzed via RP-UPLC, and used in further enzyme assays without further purification (purity 97%).

^{1}H NMR (800 MHz, DMSO) δ 10.99 (s, 1H), 8.59 (d, *J* = 7.2 Hz, 1H), 8.49 (s, 1H), 8.16 (d, *J* = 8.6 Hz, 1H), 8.09 (d, *J* = 7.8 Hz, 1H), 7.94 (d, *J* = 7.8 Hz, 1H), 7.71 (d, *J* = 5.6 Hz, 1H), 7.35 (d, *J* = 8.8 Hz, 1H), 7.32 (d, *J* = 7.2 Hz, 1H), 7.25 (s, 1H), 7.21 (s, 1H), 7.08 (t, *J* = 5.6 Hz, 1H), 7.00 (t, *J* = 7.8 Hz, 1H), 6.80 (s, 1H), 4.38 – 4.27 (m, 4H), 4.23 (t, *J* = 5.4 Hz, 1H), 4.01 (t, *J* = 4.8 Hz, 3H), 3.20 (d, *J* = 10.2 Hz, 1H), 3.07 (s, 1H), 3.00 – 2.91 (m, 3H), 2.79 (t, *J* = 6.4 Hz, 2H), 2.55 (t, *J* = 6.2 Hz, 2H), 2.10 (hept, *J* = 7.2 Hz, 2H), 2.01 – 1.91 (m, 2H), 1.78 – 1.69 (m, 2H), 1.62 – 1.50 (m, 6H), 1.50 –

1.40 (m, 2H), 1.31 (h, J = 4.8 Hz, 2H), 1.18 (d, J = 6.2 Hz, 3H), 1.10 (s, 2H), 0.87 (t, J = 6.2 Hz, 6H), 0.84 (d, J = 7.0 Hz, 3H), 0.76 (dd, J = 8.6, 6.2 Hz, 6H).**^{13}C NMR** (201 MHz, DMSO) δ 201.0, 173.6, 172.4, 172.0, 171.9, 171.5, 171.0, 136.7, 127.5, 125.0, 121.5, 119.0, 118.7, 111.8, 64.3, 59.1, 58.1, 53.7, 52.2, 51.6, 49.1, 48.4, 41.1, 38.9, 34.0, 31.2, 31.2, 30.5, 29.3, 29.3, 27.3, 27.2, 24.6, 23.9, 23.6, 23.4, 21.9, 19.5, 19.0, 18.8, 18.2, 13.9.**Mass spec:** expected neutral mass for $C_{42}H_{67}N_9O_9S$ (Da): 873.4782, observed neutral mass (Da): 873.4791, mass error (ppm) 1.0.

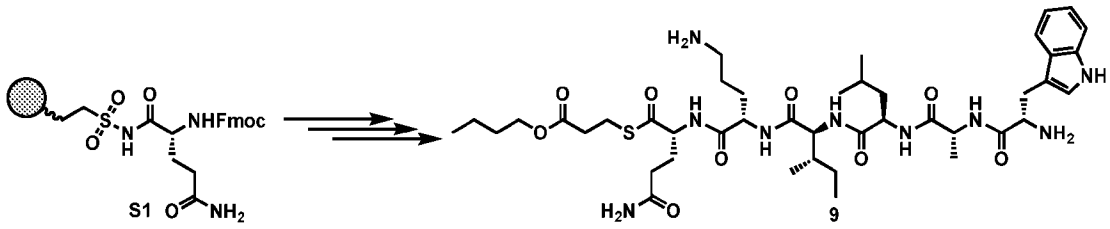
[0097] Synthesis of liner peptide 8



Peptide **8** was synthesized using the sulfonamide "safety-catch" resin according to the general protocol described earlier. The synthesis was initiated from **(S1)** (0.05 mmol) and utilized the following amino acids: Fmoc-L-Orn(Boc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Ala-OH, Fmoc-D-Val-OH, and Boc-L-Trp(Boc)-OH, with butyl 3-mercaptopropionate used as the cleaving thiol. The crude peptide was obtained as an off-white solid (22.48 mg, 41% yield) and was analyzed via RP-UPLC. It was used in further enzyme assays without undergoing further purification (purity 97%).

^{1}H NMR (800 MHz, DMSO) δ 11.00 (s, 1H), 8.64 (d, J = 5.1 Hz, 1H), 8.54 (d, J = 5.9 Hz, 1H), 8.29 (d, J = 5.1 Hz, 1H), 8.18 (d, J = 7.5 Hz, 1H), 7.80 (d, J = 8.6 Hz, 1H), 7.71 (d, J = 6.9 Hz, 1H), 7.48 (s, 3H), 7.35 (d, J = 6.9 Hz, 1H), 7.22 (d, J = 30.9 Hz, 2H), 7.08 (t, J = 6.4 Hz, 1H), 7.00 (t, J = 8.1 Hz, 1H), 6.80 (s, 1H), 4.41 (p, J = 4.5 Hz, 1H), 4.35 (p, J = 5.1 Hz, 1H), 4.33 – 4.21 (m, 3H), 4.02 (t, J = 6.4 Hz, 3H), 3.22 (dd, J = 5.3, 3.5 Hz, 1H), 3.07 – 2.92 (m, 3H), 2.79 (t, J = 6.3 Hz, 2H), 2.57 – 2.52 (m, 2H), 2.23 – 2.07 (m, 2H), 1.97 (h, J = 5.1 Hz, 1H), 1.90 (h, J = 5.7 Hz, 1H), 1.78 – 1.64 (m, 3H), 1.60 – 1.46 (m, 5H), 1.41 – 1.35 (m, 1H), 1.32 (h, J = 7.5 Hz, 2H), 1.22 (d, J = 6.9 Hz, 3H), 1.05 (h, J = 7.5 Hz, 1H), 0.88 (t, J = 6.9 Hz, 3H), 0.82 – 0.72 (m, 13H).**^{13}C NMR** (201 MHz, DMSO) δ 200.6, 173.1, 171.8, 171.6, 171.1, 170.8, 170.4, 136.3, 127.1, 124.7, 121.1, 118.6, 118.3, 111.4, 107.8, 63.9, 58.7, 57.5, 56.4, 53.2, 51.6, 48.3, 38.5, 37.1, 33.7, 30.8, 30.8, 30.1, 29.2, 28.7, 26.9, 24.1, 23.6, 23.2, 19.1, 18.6, 17.9, 15.3, 13.5, 11.0.**Mass spec:** expected neutral mass for $C_{42}H_{67}N_9O_9S$ (Da): 873.4782, observed neutral mass (Da): 873.4759, mass error (ppm) 2.7.

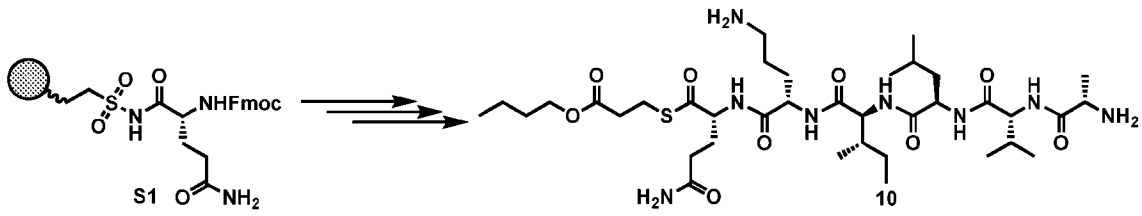
[0098] Synthesis of liner peptide 9



Peptide **9** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from (**S1**) (0.05 mmol) using the following amino acids (Fmoc-L-Orn(Boc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Leu-OH, Fmoc-D-Ala-OH, Boc-L-Trp(Boc)-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (21.4 mg, 41% yield), analyzed via RP-UPLC, and used in further enzyme assays without further purification (purity 96%).

^{1}H NMR (800 MHz, DMSO) δ 10.98 (s, 1H), 8.61 (d, *J* = 6.8 Hz, 1H), 8.49 (d, *J* = 6.8 Hz, 1H), 8.15 (dd, *J* = 9.1, 7.9 Hz, 2H), 7.84 (d, *J* = 6.8 Hz, 1H), 7.61 (d, *J* = 8.4 Hz, 1H), 7.34 (d, *J* = 7.9 Hz, 1H), 7.25 (s, 2H), 7.19 (d, *J* = 1.8 Hz, 2H), 7.08 (t, *J* = 7.9 Hz, 2H), 6.99 (t, *J* = 6.2 Hz, 1H), 6.80 (s, 1H), 4.41 (q, *J* = 6.1 Hz, 1H), 4.36 – 4.26 (m, 3H), 4.23 (t, *J* = 6.7 Hz, 1H), 4.02 (t, *J* = 6.2 Hz, 2H), 3.87 (t, *J* = 6.2 Hz, 1H), 3.16 (dd, *J* = 6.2, 6.2 Hz, 1H), 3.01 – 2.90 (m, 3H), 2.79 (t, *J* = 7.3 Hz, 2H), 2.58 – 2.52 (m, 2H), 2.11 (hept, *J* = 8.5 Hz, 2H), 1.96 (h, *J* = 4.6 Hz, 1H), 1.79 – 1.64 (m, 3H), 1.61 – 1.50 (m, 7H), 1.49 – 1.42 (m, 3H), 1.42 – 1.35 (m, 1H), 1.32 (h, *J* = 6.8 Hz, 3H), 1.11 (d, *J* = 4.6 Hz, 5H), 1.05 (p, *J* = 6.1 Hz, 1H), 0.88 (t, *J* = 7.3 Hz, 7H), 0.83 (d, *J* = 5.6 Hz, 4H), 0.81 – 0.73 (m, 8H).**^{13}C NMR** (201 MHz, DMSO) δ 200.6, 173.1, 172.0, 171.8, 171.6, 171.1, 170.8, 136.2, 127.2, 124.5, 121.0, 118.5, 118.3, 111.4, 64.7, 63.9, 58.7, 56.5, 53.5, 51.7, 51.3, 48.7, 48.2, 40.8, 38.5, 36.8, 33.6, 30.8, 30.1, 29.1, 26.9, 26.8, 24.3, 24.1, 23.6, 23.2, 23.0, 21.5, 18.8, 18.3, 17.6, 17.5, 15.3, 13.5, 10.9.**Mass spec:** expected neutral mass for $C_{43}H_{69}N_9O_9S$ (Da): 887.4939, observed neutral mass (Da): 887.4965, mass error (ppm) 2.9.

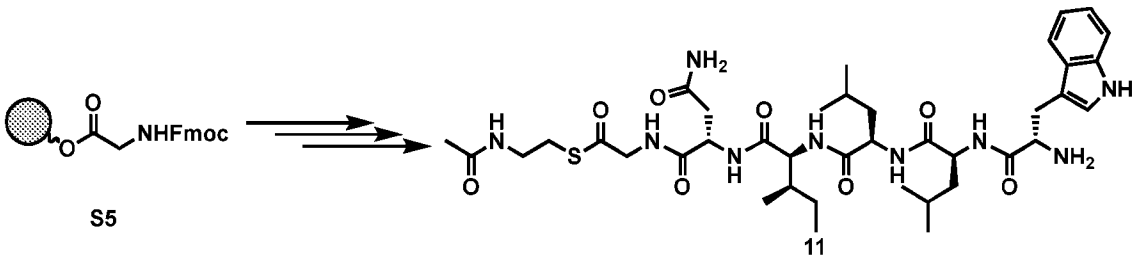
[0099] Synthesis of liner peptide 10



Peptide **10** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from **(S1)** (0.05 mmol) using the following amino acids (Fmoc-L-Orn(Boc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Leu-OH, Fmoc-D-Val-OH, Boc-L-Ala-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (10.6 mg, 21% yield) analyzed via RP-UPLC and was used in further enzyme assays without further purification (purity >99%).

^{1}H NMR (800 MHz, DMSO) δ 8.64 (d, *J* = 7.3 Hz, 1H), 8.47 (d, *J* = 8.5 Hz, 1H), 8.21 (d, *J* = 6.7 Hz, 1H), 8.15 (d, *J* = 7.3 Hz, 1H), 7.96 (s, 5H), 7.84 (d, *J* = 8.6 Hz, 2H), 7.25 (s, 1H), 6.80 (s, 1H), 4.39 (p, *J* = 4.1 Hz, 2H), 4.33 (q, *J* = 7.0 Hz, 1H), 4.29 (q, *J* = 5.7 Hz, 1H), 4.24 (t, *J* = 7.6 Hz, 1H), 4.02 (t, *J* = 6.7 Hz, 2H), 3.94 (q, *J* = 5.7 Hz, 1H), 3.00 – 2.91 (m, 2H), 2.79 (t, *J* = 6.1 Hz, 2H), 2.58 – 2.51 (m, 2H), 2.11 (h, *J* = 8.2 Hz, 2H), 2.03 – 1.92 (m, 2H), 1.77 – 1.66 (m, 3H), 1.60 – 1.49 (m, 7H), 1.45 (t, *J* = 6.0 Hz, 3H), 1.37 (d, *J* = 7.0 Hz, 5H), 1.32 (h, *J* = 8.0 Hz, 3H), 1.04 (hept, *J* = 7.6 Hz, 1H), 0.90 – 0.73 (m, 27H).**^{13}C NMR** (201 MHz, DMSO) δ 200.6, 173.1, 171.6, 171.1, 170.8, 170.4, 169.6, 63.9, 58.7, 57.4, 56.4, 51.6, 51.2, 48.2, 41.0, 38.5, 36.8, 33.6, 31.1, 30.8, 30.1, 29.2, 26.9, 24.2, 24.1, 23.5, 23.2, 23.0, 21.5, 19.2, 18.6, 17.8, 17.7, 15.3, 13.5, 10.9.**Mass spec:** expected neutral mass for $C_{37}H_{68}N_8O_9S$ (Da): 800.4830, observed neutral mass (Da): 800.4830, mass error (ppm) 1.2.

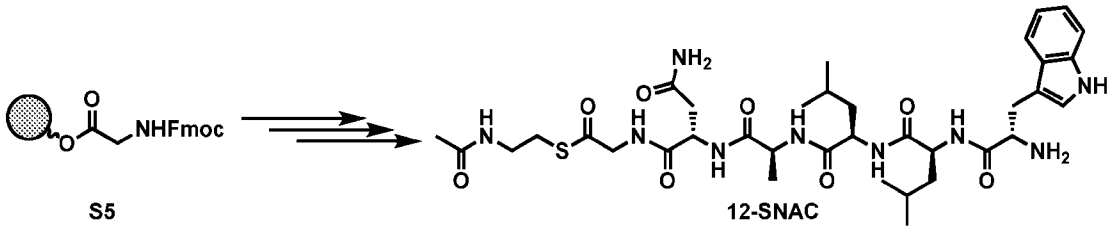
[00100] Synthesis of liner peptide 11



Peptide **11** was synthesized following a previously described method (Matsuda, K. et al. Nature. Catalyst. 2020, 3, 507–515). Fmoc-Gly-OH (3 eq) and i-Pr_2EtN (6 eq) were loaded onto 2-chlorotritylchloride (2-CTC) resin in DMF. After 2 hours, unreacted resin was capped with MeOH, and solid-phase peptide synthesis (SPPS) was initiated from **(S5)** (0.05 mmol) using Fmoc-L-Asn(Trt)-OH, Fmoc-L-Ile-OH, Fmoc-D-Leu-OH, Fmoc-L-Leu-OH, and Boc-L-Trp(Boc)-OH. To cleave the peptide from the 2-chlorotrityl resin, a solution of 25% hexafluoroisopropanol (HFIP) in DCM (3.0 mL) was added to the resin and shaken for 30 minutes at room temperature. The reaction mixture was filtered, and the filtrate was washed with DCM (10 mL), concentrated, redissolved in a

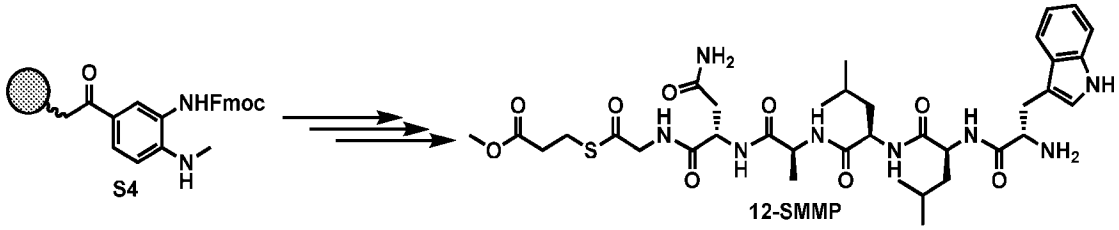
mixture of acetonitrile-water, frozen, and lyophilized. The crude linear peptide was used in the next reaction without further purification. For the next step, the crude peptide was dissolved in DCM/DMF, and N-Aceytlcysteamine (10 equiv.), 2,6 Lutidine (5 equiv.), and HCTU (5 quiv.) were added. The reaction was stirred overnight at room temperature, quenched with 40 mL EtOAc, and washed with saturated aqueous NH_4Cl (2x 15 mL), saturated aqueous $NaHCO_3$ (2x 10 mL), and brine (3x 20 mL). The product was dried over sodium sulfate, filtered, and concentrated. A cleaving cocktail of TFA/TIPS = 90:10 (3 mL) was added to remove protecting groups, and the mixture was shaken for 90 minutes. The volatiles were removed by a stream of air, and the peptide crashed out with 25 mL -20°C Et_2O. The product was centrifuged at 3200xg for 10 minutes at 4 °C, and the Et_2O layer was removed by decantation. This procedure was repeated twice. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.1% Formic acid (A) and acetonitrile+0.1% Formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 10% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 10% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (3.7 mg, 9% yield, purity 99%). **Mass spec:** expected neutral mass for $C_{39}H_{61}N_9O_8S$ (Da): 815.4364, observed neutral mass (Da): 815.4375, mass error (ppm) 1.3.

[00101] Synthesis of 12-SNAC



Peptide **12-SNAC** was synthesized following an identical protocol followed for Peptide **(11)** however, Fmoc-L-Leu-OH was substituted with Fmoc-L-Ala-OH. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.1% formic acid (A) and acetonitrile+0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 10% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 10% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (2.1 mg, 5% yield, purity 91%). **Mass spec:** expected neutral mass for $C_{36}H_{55}N_9O_8S$ (Da): 773.3894, observed neutral mass (Da): 773.3904, mass error (ppm) 1.2.

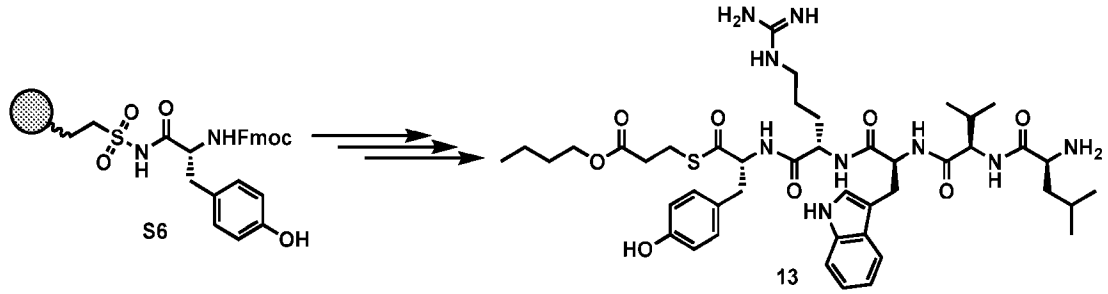
[00102] Synthesis of liner peptide 12-SMMP



Peptide **12-SMMP** was synthesized following the general protocol outlined above for the NDbz "safety-catch" resin. The synthesis was initiated from **(S4)** (0.10 mmol) using the following amino acids (Fmoc-Gly-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Ala-OH, Fmoc-D-Leu-OH, Fmoc-L-Leu-OH, and Boc-L-Trp(Boc)-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.05% Trifluoroacetic acid (A) and acetonitrile+0.05% Trifluoroacetic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (24.8 mg, 28% yield, purity >99%).

^{1}H NMR (800 MHz, DMSO) δ 10.97 (s, 1H), 8.76 (d, J = 6.5 Hz, 1H), 8.42 – 8.32 (m, 2H), 8.21 (d, J = 8.5 Hz, 1H), 8.11 (d, J = 8.5 Hz, 1H), 8.02 (s, 3H), 7.73 (d, J = 7.4 Hz, 1H), 7.37 (t, J = 7.4 Hz, 2H), 7.19 (s, 1H), 7.10 (t, J = 7.4 Hz, 1H), 7.01 (t, J = 8.2 Hz, 1H), 6.90 (s, 1H), 4.62 (q, J = 6.2 Hz, 1H), 4.44 (q, J = 6.2 Hz, 1H), 4.39 – 4.34 (m, 1H), 4.30 (p, J = 6.2 Hz, 1H), 4.06 (s, 1H), 3.97 (dd, J = 7.4, 4.2 Hz, 1H), 3.89 (dd, J = 7.4, 5.3 Hz, 1H), 3.60 (s, 3H), 3.28 (dd, J = 5.1, 4.2 Hz, 1H), 3.04 – 2.94 (m, 3H), 2.60 – 2.53 (m, 3H), 2.46 (q, J = 8.5 Hz, 1H), 1.63 (h, 1H), 1.58 (hept, J = 5.3 Hz, 1H), 1.54 – 1.42 (m, 4H), 1.21 (d, J = 6.2 Hz, 3H), 0.93 (d, J = 6.2 Hz, 3H), 0.88 (q, J = 7.4 Hz, 6H), 0.82 (d, J = 5.1 Hz, 3H).**^{13}C NMR** (201 MHz, DMSO) δ 198.44, 172.11 (d, J = 24.3 Hz), 171.59, 168.82, 136.74, 127.41, 125.50, 121.54, 118.87 (d, J = 24.3 Hz), 111.91, 107.17, 52.88, 51.96, 51.43, 49.99, 49.33, 48.53, 41.54, 40.00, 39.89, 39.79, 37.17, 33.81, 27.75, 24.59 (d, J = 21.8 Hz), 23.48, 23.40, 23.33, 22.17, 21.72, 18.65. **Mass spec:** expected neutral mass for $C_{36}H_{54}N_8O_9S$ (Da): 774.3734, observed neutral mass (Da): 774.3759, mass error (ppm) 3.2.

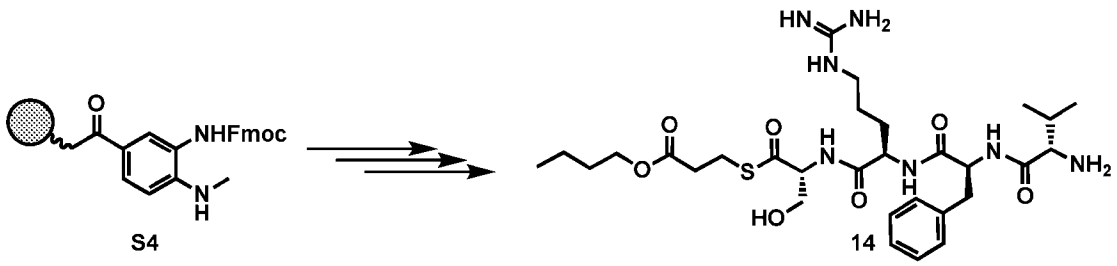
[00103] Synthesis of liner peptide 13



Peptide **13** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from **(S6)** (0.05 mmol) using the following amino acids (Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Trp(Boc)-OH, Fmoc-D-Val-OH, Boc-L-Leu-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (23.7 mg, 43% yield) analyzed via RP-HPLC and was used in further enzyme assays without further purification (purity >99%).

^{1}H NMR (800 MHz, DMSO) δ 10.82 (s, 1H), 9.37 (d, *J* = 528.2 Hz, 1H), 8.70 (d, *J* = 7.9 Hz, 1H), 8.46 (d, *J* = 7.5 Hz, 1H), 8.29 (d, *J* = 8.6 Hz, 1H), 8.19 (d, *J* = 8.1 Hz, 1H), 7.92 (s, 1H), 7.66 (dd, *J* = 8.1, 7.5 Hz, 3H), 7.42 – 7.22 (m, 4H), 7.14 (s, 1H), 7.03 – 6.82 (m, 4H), 6.65 (d, *J* = 8.1 Hz, 2H), 4.66 (d, *J* = 10.3 Hz, 1H), 4.37 (ddt, *J* = 8.5, 5.7, 5.1 Hz, 2H), 4.06 – 3.87 (m, 2H), 3.78 (s, 1H), 3.51 (s, 1H), 3.44 – 3.28 (m, 1H), 3.15 (d, *J* = 13.8 Hz, 1H), 3.07 – 2.91 (m, 5H), 2.87 (t, *J* = 13.2 Hz, 1H), 2.75 – 2.57 (m, 1H), 2.55 (t, *J* = 6.3 Hz, 1H), 1.71 – 1.58 (m, 2H), 1.56 – 1.37 (m, 8H), 1.37 – 1.03 (m, 5H), 0.85 (d, *J* = 5.7 Hz, 9H), 0.50 (d, *J* = 5.7 Hz, 2H), 0.42 (d, *J* = 6.9 Hz, 3H).**^{13}C NMR** (201 MHz, DMSO) δ 200.6, 172.2, 172.0, 171.5, 170.6, 157.4, 156.5, 136.6, 130.5, 129.0, 128.1, 127.5, 127.3, 125.9, 124.5, 121.1, 118.3, 116.8, 116.6, 115.5, 111.5, 110.3, 70.1, 64.3, 61.4, 57.7, 53.5, 52.5, 51.5, 41.7, 40.6, 36.5, 33.9, 31.2, 30.5, 29.5, 28.5, 25.3, 24.1, 23.8, 23.1, 22.1, 19.3, 18.9, 17.6, 13.9.**Mass spec:** expected neutral mass for $C_{44}H_{65}N_9O_8S$ (Da): 879.4677, observed neutral mass (Da): 879.4673, mass error (ppm) 0.4.

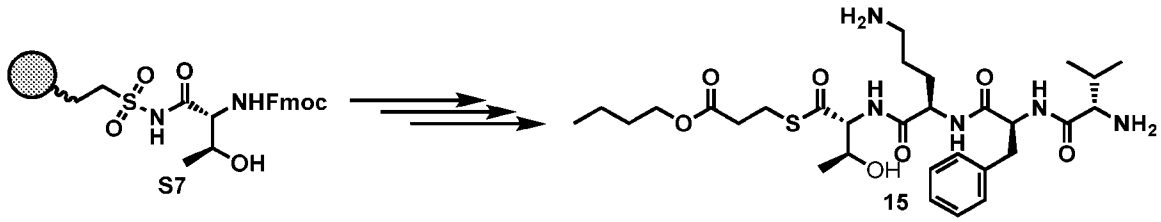
[00104] Synthesis of liner peptide 14



Peptide **14** was synthesized following the general protocol outlined above for the NDbz "safety-catch" resin. The synthesis was initiated from (**S4**) (0.10 mmol) using the following amino acids (Fmoc-D-Ser(tBu)-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-L-Phe-OH, and Boc-L-Val-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.05% trifluoroacetic acid (A) and acetonitrile+0.05% trifluoroacetic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (17.8 mg, 20% yield, purity 90.3%).

^{1}H NMR (800 MHz, DMSO) δ 8.66 (d, *J* = 7.0 Hz, 1H), 8.56 (d, *J* = 7.3 Hz, 1H), 8.33 (d, *J* = 7.3 Hz, 1H), 8.07 (s, 4H), 7.77 (s, 1H), 7.44 (s, 2H), 7.32 – 7.06 (m, 10H), 4.70 (p, *J* = 4.6 Hz, 1H), 4.41 (d, *J* = 38.4 Hz, 2H), 4.01 (dd, *J* = 220.7, 6.1 Hz, 3H), 3.72 (p, *J* = 8.2 Hz, 1H), 3.62 (s, 3H), 3.07 – 2.90 (m, 7H), 2.90 – 2.71 (m, 2H), 2.53 (t, *J* = 4.9 Hz, 2H), 2.08 (d, *J* = 4.9 Hz, 1H), 1.68 (d, *J* = 6.1 Hz, 1H), 1.53 (t, *J* = 7.0 Hz, 3H), 1.45 (p, *J* = 7.3 Hz, 1H), 1.31 (p, *J* = 8.2 Hz, 5H), 0.97 – 0.76 (m, 13H).**^{13}C NMR** (201 MHz, DMSO) δ 199.9, 172.7, 171.6, 169.8, 169.7, 157.4, 138.7, 138.2, 129.7, 129.2, 128.6, 126.6, 70.2, 64.3, 62.8, 61.6, 60.7, 56.0, 55.4, 34.5, 34.1, 30.5, 24.7, 24.3, 23.6, 19.8, 19.0, 17.3, 17.0, 13.9.**Mass spec:** expected neutral mass for $C_{30}H_{49}N_7O_7S$ (Da): 651.3414, observed neutral mass (Da): 651.3411, mass error (ppm) 0.5.

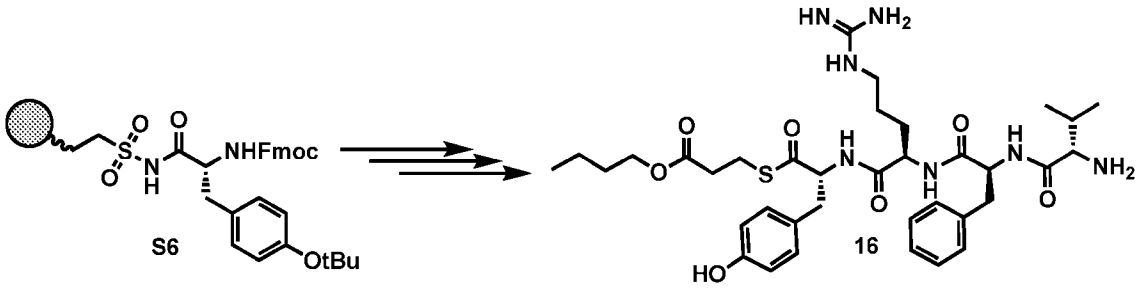
[00105] Synthesis of liner peptide 15



Peptide **15** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from (**S7**) (0.05 mmol) using the following amino acids (Fmoc-D-Orn(Boc)-OH, Fmoc-L-Phe-OH, Boc-L-Val-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (30.4 mg, 70% yield) analyzed via RP-HPLC and was used in further enzyme assays without further purification (purity 96%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 8.63 (d, *J* = 8.3 Hz, 1H), 8.41 (dd, *J* = 9.1, 9.1 Hz, 2H), 7.96 (s, 6H), 7.34 – 7.23 (m, 4H), 7.19 (t, *J* = 6.8 Hz, 1H), 5.12 (s, 1H), 4.74 (q, *J* = 6.0 Hz, 1H), 4.54 (h, *J* = 6.8 Hz, 1H), 4.31 (dd, *J* = 3.1, 3.1 Hz, 1H), 4.22 (s, 1H), 4.02 (t, *J* = 6.0 Hz, 2H), 3.60 (d, *J* = 3.9 Hz, 1H), 3.02 – 2.92 (m, 3H), 2.85 (q, *J* = 8.3 Hz, 1H), 2.73 (h, *J* = 7.6 Hz, 2H), 2.55 (t, *J* = 6.0 Hz, 2H), 2.07 (h, *J* = 7.0 Hz, 1H), 1.81 – 1.74 (m, 1H), 1.56 – 1.42 (m, 5H), 1.32 (h, *J* = 6.8 Hz, 2H), 0.98 (d, *J* = 6.0 Hz, 3H), 0.91 – 0.86 (m, 6H), 0.84 (d, *J* = 6.0 Hz, 3H).**^{13}C NMR** (201 MHz, DMSO) δ 200.6, 172.6, 171.6, 171.0, 168.2, 137.8, 129.6, 128.5, 126.8, 66.4, 64.9, 64.3, 57.4, 54.3, 51.6, 38.7, 38.4, 34.0, 30.5, 30.3, 29.0, 23.8, 23.7, 20.5, 19.0, 18.7, 17.6, 13.9.**Mass spec:** expected neutral mass for $C_{30}H_{49}N_5O_7S$ (Da): 623.3352, observed neutral mass (Da): 623.3350, mass error (ppm) 0.4.

[00106] Synthesis of liner peptide 16

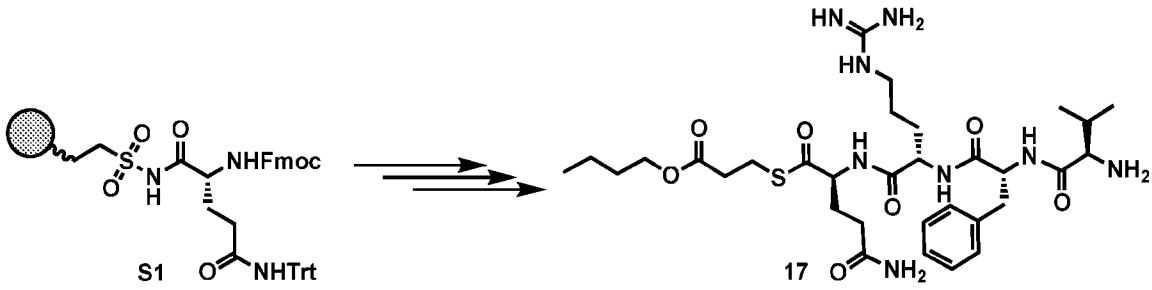


Peptide **16** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from 4-sulfamylbutyryl resin preloaded with Fmoc-D-Tyr(tBu)-OH (**S6**) (0.05 mmol) using the following amino acids (Fmoc-D-Arg(Pbf)-OH, Fmoc-L-Phe-OH, Boc-L-Val-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (29.6 mg, 61% yield) analyzed via RP-UPLC and was used in further enzyme assays without further purification (purity 96%, HPLC).

^{1}H NMR (800 MHz, DMSO) δ 9.32 (s, 1H), 8.68 (s, 1H), 8.54 (d, *J* = 4.5 Hz, 1H), 8.27 (s, 1H), 7.98 (s, 2H), 7.83 (s, 1H), 7.44 (s, 2H), 7.26 (d, *J* = 2.6 Hz, 6H), 7.19 (s, 2H), 6.99 (d, *J* = 5.2 Hz, 3H), 6.64 (d, *J* = 5.9 Hz, 2H), 4.68 (d, *J* = 7.1 Hz, 1H), 4.42 (s, 1H), 4.29 (s, 1H), 3.97 (t, *J* = 7.0 Hz, 2H), 3.58 (t, *J* = 4.0 Hz, 1H), 3.38 (s, 1H), 3.03 – 2.89 (m, 8H), 2.87 (t, *J* = 10.3 Hz, 1H), 2.81 (t, *J* = 10.3 Hz, 1H), 2.06 (d, *J* = 5.7 Hz, 1H), 1.64 (d, *J* = 7.1 Hz, 1H), 1.54 (t, *J* = 7.1 Hz, 3H), 1.43 (dd, *J* = 27.3, 6.4 Hz, 1H), 1.39 (s, 1H), 1.32 (q, *J* = 9.0 Hz, 3H), 1.23 (s, 3H), 0.92 – 0.82 (m, 12H).**^{13}C NMR** (201 MHz, DMSO) δ 200.5, 171.7, 171.5, 170.7, 168.5, 157.3, 156.5, 137.5, 130.3, 129.6, 128.5, 128.1, 127.1, 126.8, 125.8, 115.5, 64.3, 61.4, 57.6, 54.6, 52.1, 40.7, 38.2, 36.5, 34.0, 30.5,

30.4, 29.3, 25.1, 23.7, 19.0, 17.7, 13.9.**Mass spec:** expected neutral mass for $C_{36}H_{53}N_7O_7S$ (Da): 727.3727, observed neutral mass (Da): 727.3748, mass error (ppm) 2.9.

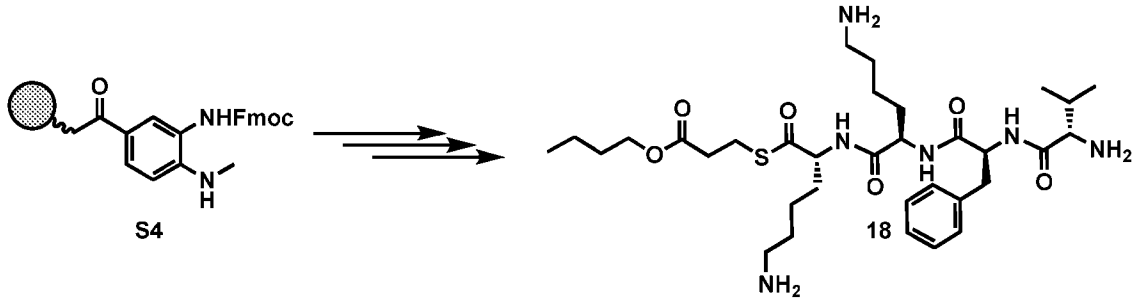
[00107] Synthesis of liner peptide 17



Peptide **17** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from **(S1)** (0.05 mmol) using the following amino acids (Fmoc-D-Arg(Pbf)-OH, Fmoc-L-Phe-OH, Boc-L-Val-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H2O+0.05% Trifluoroacetic acid (A) and acetonitrile+0.05% Trifluoroacetic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 ml/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (7.8 mg, 17% yield, purity 98%, UPLC)).

^{1}H NMR (800 MHz, DMSO) δ 9.31 (s, 1H), 8.67 (s, 1H), 8.53 (s, 1H), 8.26 (s, 1H), 7.97 (bs, 3H), 7.81 (s, 1H), 7.43 (bs, 2H), 7.24 (d, J = 54.6 Hz, 11H), 6.99 (s, 3H), 6.63 (s, 3H), 4.66 (s, 1H), 4.41 (s, 1H), 4.28 (s, 1H), 4.01 (s, 3H), 3.57 (s, 1H), 3.36 (s, 1H), 3.01 – 2.74 (m, 13H), 2.03 (s, 1H), 1.62 (s, 1H), 1.52 (s, 3H), 1.48 – 1.18 (m, 9H), 0.87 (s, 15H). **^{13}C NMR** (201 MHz, DMSO) δ 200.5, 171.7, 171.5, 170.7, 168.5, 157.3, 156.5, 137.5, 130.3, 129.6, 128.5, 128.1, 127.1, 126.8, 125.8, 118.2, 116.7, 115.5, 114.2, 70.2, 65.1, 64.3, 64.0, 61.4, 57.6, 54.6, 52.1, 39.6, 38.2, 36.5, 34.0, 30.4, 29.3, 25.1, 19.0, 17.7, 13.9.**Mass spec:** expected neutral mass for $C_{32}HC_{52}N_8O_7S$ (Da): 692.3679, observed neutral mass (Da): 692.3686, mass error (ppm) 0.9.

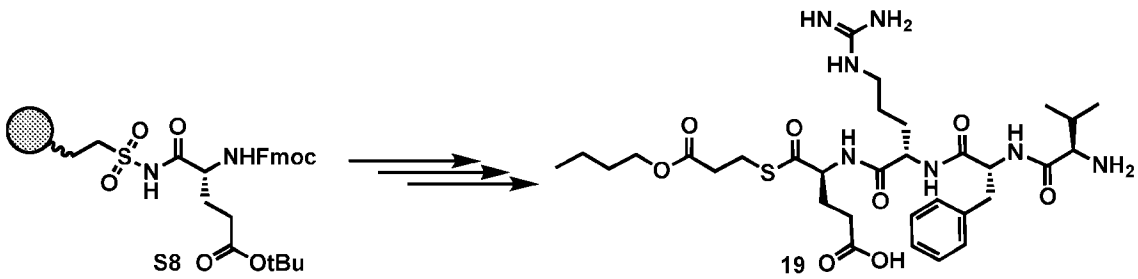
[00108] Synthesis of liner peptide 18



Peptide **14** was synthesized following the general protocol outlined above for the NDbz "safety-catch" resin. The synthesis was initiated from (**S4**) (0.15 mmol) using the following amino acids (Fmoc-D-Lys(Boc)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-L-Phe-OH, and Boc-L-Val-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.05% Trifluoroacetic acid (A) and acetonitrile+0.05% Trifluoroacetic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (2.31 mg, 2% yield).

^{1}H NMR (800 MHz, DMSO) δ 8.64 (d, J = 8.0 Hz, 1H), 8.29 (dd, J = 8.9, 8.3 Hz, 1H), 8.03 (s, 2H), 7.92 – 7.65 (m, 6H), 7.27 (d, J = 5.6 Hz, 3H), 7.21 (s, 2H), 4.68 (p, J = 7.5 Hz, 1H), 4.30 – 4.19 (m, 1H), 4.13 (s, 1H), 4.02 (q, J = 6.2 Hz, 1H), 3.65 – 3.51 (m, 1H), 3.51 (d, J = 505.9 Hz, 1H), 2.99 – 2.89 (m, 2H), 2.87 (d, J = 253.5 Hz, 1H), 2.76 – 2.57 (m, 3H), 2.54 (d, J = 386.2 Hz, 1H), 2.04 (h, 1H), 1.65 – 1.60 (m, 1H), 1.60 – 1.55 (m, 1H), 1.55 – 1.47 (m, 4H), 1.43 (h, 3H), 1.37 – 1.12 (m, 6H), 1.09 – 0.94 (m, 2H), 0.92 – 0.75 (m, 9H).**^{13}C NMR** (201 MHz, DMSO) δ 201.31, 158.55, 158.40, 137.52, 129.62, 128.52, 126.83, 118.40, 116.91, 70.17, 64.29, 59.40, 57.48, 54.47, 52.22, 39.07, 39.02, 38.85, 38.40, 34.02, 31.42, 30.76, 30.50, 30.33, 27.04, 26.83, 23.64, 22.50, 22.40, 18.97, 18.73, 17.79, 13.94.**Mass spec:** expected neutral mass for $C_{33}H_{56}N_6O_6S$ (Da): 664.3982, observed neutral mass (Da): 664.3973, mass error (ppm) 1.3.

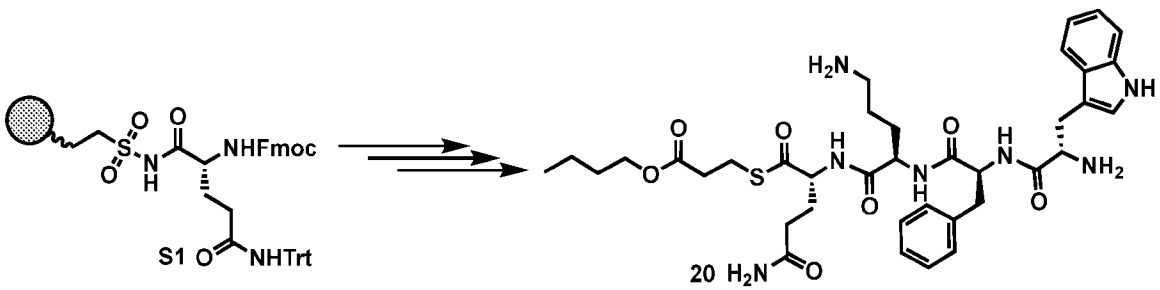
[00109] Synthesis of liner peptide 19



Using the general protocol described above for the sulfonamide "safety-catch" resin, Peptide **19** was synthesized by initiating the synthesis from **(S9)** (0.05 mmol) and coupling the following amino acids in the order of Fmoc-D-Arg(Pbf)-OH, Fmoc-L-Phe-OH, and Boc-L-Val-OH. Butyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was obtained as an off-white solid with a yield of 36% (16.4 mg) and was analyzed via RP-HPLC. The peptide was used in further enzyme assays without additional purification (purity >99%).

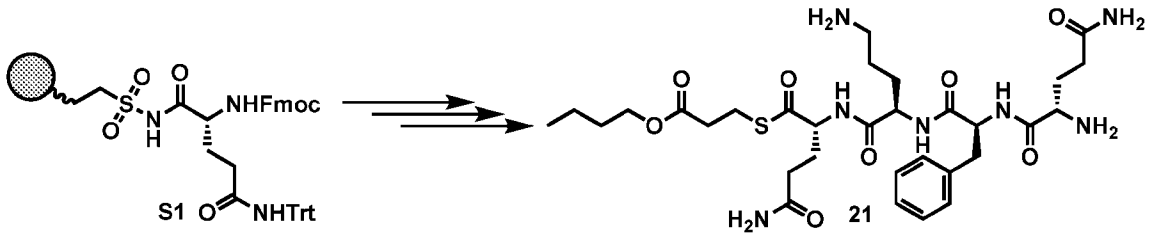
^{1}H NMR (800 MHz, DMSO) δ 8.66 (d, *J* = 7.9 Hz, 1H), 8.60 (d, *J* = 6.1 Hz, 1H), 8.33 (d, *J* = 8.2 Hz, 1H), 7.80 (s, 1H), 7.26 (t, *J* = 6.1 Hz, 5H), 7.21 – 7.18 (m, 1H), 7.15 (s, 1H), 4.67 (q, *J* = 7.9 Hz, 1H), 4.36 – 4.30 (m, 1H), 4.30 – 4.23 (m, 1H), 4.02 (t, *J* = 6.8 Hz, 2H), 3.58 – 3.46 (m, 1H), 3.07 – 2.90 (m, 5H), 2.87 (q, *J* = 8.2 Hz, 1H), 2.54 (t, *J* = 7.1 Hz, 2H), 2.31 – 2.20 (m, 2H), 2.05 – 1.92 (m, 2H), 1.82 – 1.74 (m, 1H), 1.74 – 1.66 (m, 1H), 1.53 (p, *J* = 6.1 Hz, 2H), 1.47 – 1.35 (m, 2H), 1.32 (hept, *J* = 7.5 Hz, 2H), 1.25 (p, *J* = 6.8 Hz, 2H), 0.90 – 0.86 (m, 6H), 0.84 (d, *J* = 6.7 Hz, 3H).**^{13}C NMR** (201 MHz, DMSO) δ 200.6, 173.9, 171.7, 171.1, 170.4, 168.6, 156.9, 137.1, 129.2, 128.1, 126.4, 63.9, 58.6, 57.5, 54.2, 51.7, 40.3, 37.9, 33.6, 30.1, 30.1, 29.9, 28.6, 26.4, 24.8, 23.3, 18.6, 18.5, 17.3, 13.5.**Mass spec:** expected neutral mass for $C_{32}H_{51}N_7O_8S$ (Da): 693.3520, observed neutral mass (Da): 693.3529, mass error (ppm) 1,3.

[00110] Synthesis of liner peptide 20



Peptide **20** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from **(S1)** (0.05 mmol) using the following amino acids Fmoc-D-Orn(Boc)-OH, Fmoc-L-Phe-OH, Boc-L-Trp(Boc)-OH and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.05% Formic Acid (A) and acetonitrile+0.05% Formic Acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (3.5 mg, 8% yield, purity 98%, UPLC).**Mass spec:** expected neutral mass for $C_{37}H_{51}N_7O_7S$ (Da): 737.3571, observed neutral mass (Da): 737.3567, mass error (ppm) 0.5.

[00111] Synthesis of liner peptide 21

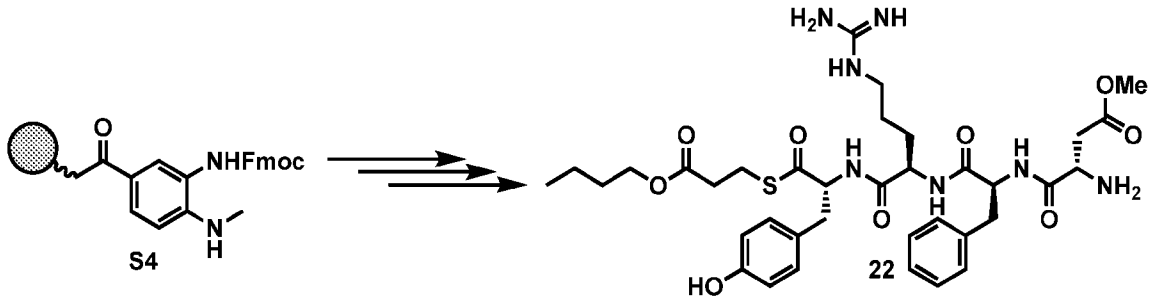


Peptide **21** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from **(S1)** (0.05 mmol) using the following amino acids Fmoc-D-Orn(Boc)-OH, Fmoc-L-Phe-OH, Boc-L-Gln(Trt)-OH, and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (28.1 mg, 62% yield) analyzed via RP-HPLC and was used in further enzyme assays without further purification (purity 98%).

^{1}H NMR (800 MHz, DMSO) δ 8.79 (d, *J* = 8.4 Hz, 1H), 8.65 (d, *J* = 7.6 Hz, 1H), 8.54 (d, *J* = 8.4 Hz, 1H), 7.99 (s, 5H), 7.43 (s, 1H), 7.36 – 7.07 (m, 6H), 7.00 (s, 1H), 6.81 (s, 1H), 4.65 (q, *J* = 3.7 Hz, 1H), 4.37 (dt, *J* = 37.4, 5.5 Hz, 2H), 4.02 (t, *J* = 6.8 Hz, 2H), 3.77 (d, *J* = 5.5 Hz, 1H), 3.09 – 2.86 (m, 3H), 2.82 (dd, *J* = 195.6, 14.4 Hz, 1H), 2.74 (s, 2H), 2.55 (t, *J* = 6.8 Hz, 2H), 2.33 – 2.18 (m, 2H), 2.15 (s, 2H), 2.00 – 1.81 (m, 3H), 1.78 (q, *J* = 4.7 Hz, 2H), 1.53 (p, *J* = 7.0 Hz, 5H), 1.31 (p, *J* = 7.8 Hz, 2H), 1.10 (s, 1H), 0.88 (t, *J* = 7.6 Hz, 3H).**^{13}C NMR** (201 MHz, DMSO) δ 200.8, 173.9, 173.4, 171.7, 171.2, 170.9, 168.6, 137.5, 129.3, 128.2, 126.5, 69.8, 63.9, 58.9, 54.5, 51.7, 51.6, 38.5, 37.8, 33.7, 30.8, 30.5, 30.2, 28.8, 27.2, 27.0, 26.9, 23.5, 23.3, 18.6, 13.6.

Mass spec: expected neutral mass for $C_{31}H_{49}N_7O_8S$ (Da): 679.3363, observed neutral mass (Da): 679.3366, mass error (ppm) 0.4.

[00112] Synthesis of liner peptide 22

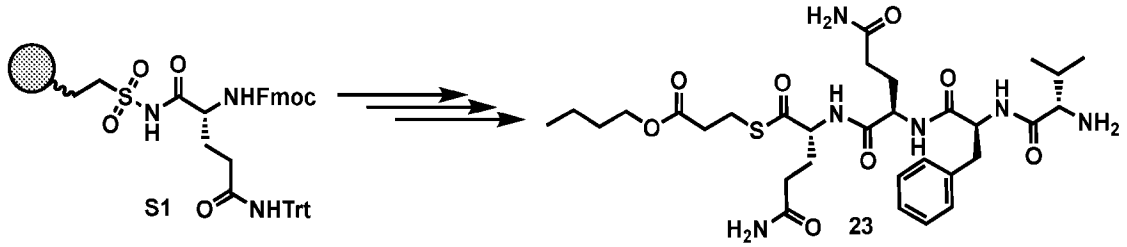


Peptide **22** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH loaded resin (**S4**) (0.15 mmol) and involved the following amino acids: Fmoc-D-Tyr(tBu)-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-L-Phe-OH, Boc-L-Asp(OMe)-OH. Butyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile+0.05% TFA (B) and H_2O+0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 minutes after and 1 minute before the gradient to yield an off-white solid (65.2 mg, 44% yield, purity 96%).

^{1}H NMR (800 MHz, DMSO) δ 9.32 (s, 1H), 8.73 (d, J = 8.7 Hz, 1H), 8.58 (d, J = 7.3 Hz, 1H), 8.30 (d, J = 8.8 Hz, 1H), 8.22 (s, 2H), 7.80 (t, J = 6.7 Hz, 1H), 7.44 (s, 1H), 7.28 – 7.24 (m, 4H), 7.21 – 7.18 (m, 1H), 7.00 (d, J = 8.1 Hz, 2H), 6.66 (d, J = 8.8 Hz, 2H), 4.65 (q, J = 8.1 Hz, 1H), 4.43 (q, J = 8.1 Hz, 1H), 4.32 (h, J = 5.1 Hz, 1H), 4.09 (dd, J = 4.5, 3.7 Hz, 1H), 4.02 (t, J = 6.7 Hz, 2H), 3.61 (s, 3H), 3.12 – 2.84 (m, 7H), 2.84 – 2.75 (m, 3H), 2.52 (t, J = 6.7 Hz, 2H), 1.73 – 1.64 (m, 1H), 1.58 (p, J = 7.3 Hz, 2H), 1.46 (h, J = 9.6 Hz, 1H), 1.36 – 1.21 (m, 4H), 0.88 (t, J = 6.7 Hz, 3H).**^{13}C NMR** (201 MHz, DMSO) δ 200.53, 171.82, 171.53, 170.58, 170.01, 167.86, 157.27, 156.49, 137.57, 130.38, 129.58, 128.52, 127.08, 126.82, 115.48, 64.30, 61.45, 54.80, 52.41, 52.07, 49.04, 40.77, 38.20, 36.58, 35.81, 33.99, 30.51, 29.46, 25.16, 23.68, 18.97, 13.92.

Mass spec: expected neutral mass for $C_{36}H_{51}N_7O_9S$ (Da): 757.3469, observed neutral mass (Da): 757.3480, mass error (ppm) 1.5.

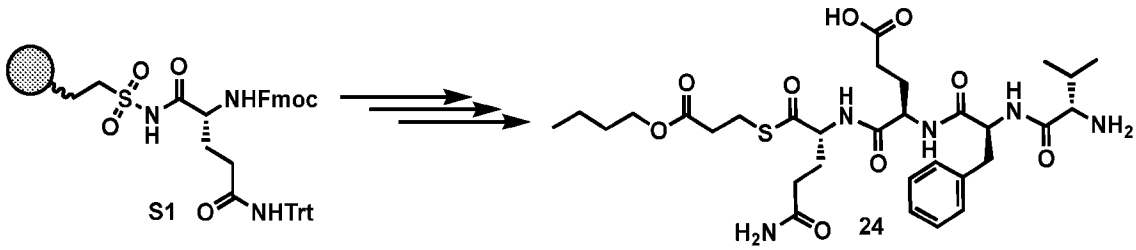
[00113] Synthesis of liner peptide 23



Peptide **23** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from **(S1)** (0.05 mmol) using the following amino acids Fmoc-D-Gln(Trt)-OH, Fmoc-L-Phe-OH, Boc-L-Val-OH and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (18.5 mg, 48% yield) analyzed via RP-HPLC and was used in further enzyme assays without further purification (purity >99%).

^{1}H NMR (800 MHz, DMSO) δ 8.65 (d, *J* = 7.8 Hz, 1H), 8.53 (d, *J* = 5.6 Hz, 1H), 8.36 (d, *J* = 7.0 Hz, 1H), 7.94 (s, 2H), 7.27 (t, *J* = 7.1 Hz, 5H), 7.16 (d, *J* = 29.0 Hz, 2H), 6.75 (d, *J* = 43.0 Hz, 2H), 4.64 (dd, *J* = 490.4, 4.0 Hz, 1H), 4.29 (s, 2H), 4.01 (t, *J* = 6.2 Hz, 2H), 3.59 (s, 1H), 3.50 (s, 1H), 3.35 (s, 1H), 3.07 – 2.93 (m, 3H), 2.87 (q, *J* = 7.0 Hz, 1H), 2.54 (t, *J* = 5.4 Hz, 2H), 2.20 – 2.02 (m, 3H), 1.94 (t, *J* = 16.4 Hz, 4H), 1.80 (s, 1H), 1.67 (t, *J* = 7.0 Hz, 1H), 1.54 (q, *J* = 7.8 Hz, 2H), 1.31 (p, *J* = 5.4 Hz, 2H), 0.99 – 0.64 (m, 10H).**^{13}C NMR** (201 MHz, DMSO) δ 200.8, 173.6, 173.4, 171.7, 171.2, 170.4, 168.2, 137.3, 129.3, 128.2, 126.5, 69.8, 63.9, 58.9, 57.2, 54.4, 52.0, 37.7, 33.7, 31.3, 31.0, 30.1, 30.0, 27.5, 27.1, 23.3, 18.6, 18.5, 17.3, 13.6, 13.6.**Mass spec:** expected neutral mass for $C_{31}H_{48}N_6O_8S$ (Da): 664.3254, observed neutral mass (Da): 664.3247, mass error (ppm) 1.1.

[00114] Synthesis of liner peptide 24

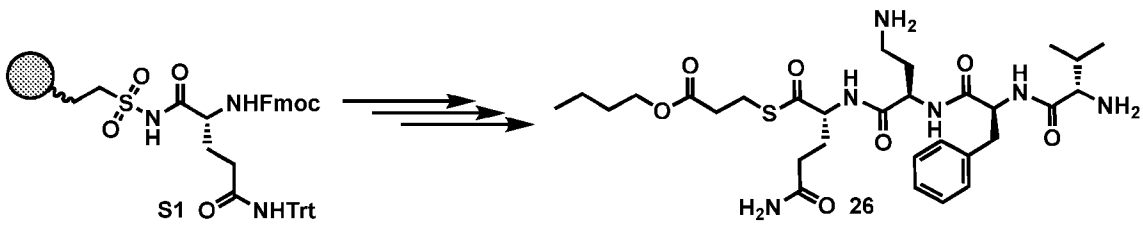


Peptide **24** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from **(S1)** (0.05 mmol) using the following amino

acids Fmoc-D-Glu(tBu)-OH, Fmoc-L-Phe-OH, Boc-L-Val-OH and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (11.2 mg, 28% yield) analyzed via RP-HPLC and was used in further enzyme assays without further purification (purity 99%).

^{1}H NMR (800 MHz, DMSO) δ 8.64 (s, 1H), 8.51 (d, *J* = 48.7 Hz, 2H), 7.26 (d, *J* = 57.6 Hz, 6H), 6.82 (s, 1H), 4.61 (s, 1H), 4.30 (d, *J* = 19.3 Hz, 2H), 4.01 (d, *J* = 3.8 Hz, 2H), 3.55 (d, *J* = 31.7 Hz, 2H), 2.97 (d, *J* = 59.3 Hz, 4H), 2.54 (d, *J* = 3.6 Hz, 2H), 2.13 (s, 2H), 2.03 (s, 3H), 1.93 (s, 2H), 1.81 (s, 1H), 1.69 (s, 1H), 1.52 (t, *J* = 10.2 Hz, 4H), 1.31 (dd, *J* = 334.3, 5.3 Hz, 2H), 0.88 (d, *J* = 30.4 Hz, 9H).**^{13}C NMR** (201 MHz, DMSO) δ 201.2, 174.4, 173.8, 171.9, 171.5, 170.7, 169.0, 137.6, 129.6, 128.5, 126.8, 70.2, 64.3, 59.3, 57.8, 54.8, 52.0, 38.0, 34.0, 31.4, 30.5, 30.4, 27.4, 27.2, 23.6, 19.0, 18.9, 17.6, 13.9. **Mass spec:** expected neutral mass for $C_{31}H_{47}N_5O_9S$ (Da): 665.3094, observed neutral mass (Da): 665.3090, mass error (ppm) 0.7.

[00115] Synthesis of liner peptide 25

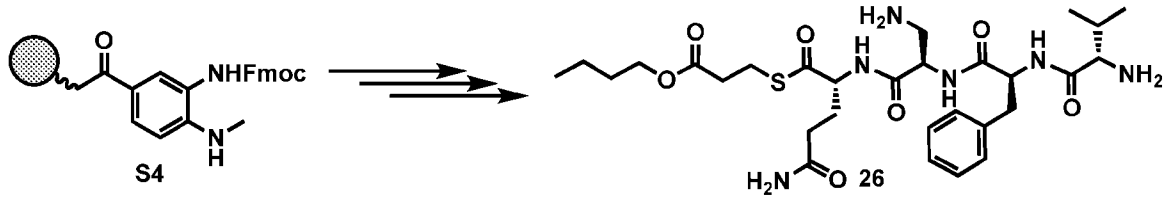


Peptide **25** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from **S1** (0.05 mmol) using the following amino acids (Fmoc-D-Dab(Boc)-OH, Fmoc-L-Phe-OH, Boc-L-Val-OH) and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was obtained as an off-white solid (23.3 mg, 54% yield) analyzed via RP-HPLC and was used in further enzyme assays without further purification (purity 99%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 8.75 (t, *J* = 6.5 Hz, 1H), 8.56 (t, *J* = 9.4 Hz, 2H), 7.98 (s, 5H), 7.34 – 7.11 (m, 6H), 6.83 (s, 1H), 4.61 (p, *J* = 6.8 Hz, 1H), 4.39 (p, *J* = 4.6 Hz, 1H), 4.29 (p, *J* = 3.6 Hz, 1H), 4.02 (q, *J* = 5.6 Hz, 2H), 3.61 (t, *J* = 2.7 Hz, 1H), 2.97 (d, *J* = 3.9 Hz, 3H), 2.91 – 2.82 (m, 1H), 2.66 – 2.57 (m, 1H), 2.57 – 2.51 (m, 2H), 2.21 – 2.08 (m, 2H), 2.06 (h, *J* = 4.8 Hz, 1H), 2.02 – 1.88 (m, 2H), 1.87 – 1.78 (m, 1H), 1.78 – 1.68 (m, 1H), 1.56 – 1.41 (m, 3H), 1.31 (h, *J* = 6.5 Hz, 2H), 1.06 (d, *J* = 3.9 Hz, 1H), 0.91 (t, *J* = 5.8 Hz, 2H), 0.88 (t, *J* = 6.8 Hz, 6H).

^{13}C NMR (201 MHz, DMSO) δ 200.5, 173.4, 171.1, 170.9, 170.7, 168.3, 137.1, 129.2, 128.2, 126.6, 64.0, 59.1, 57.2, 54.5, 50.0, 48.7, 37.5, 36.0, 33.6, 31.0, 30.1, 30.0, 29.6, 27.1, 26.8, 23.2, 18.6, 18.4, 17.4, 13.6. **Mass spec:** expected neutral mass for $C_{30}H_{48}N_6O_7S$ (Da): 636.3305, observed neutral mass (Da): 636.3310, mass error (ppm) 0.8.

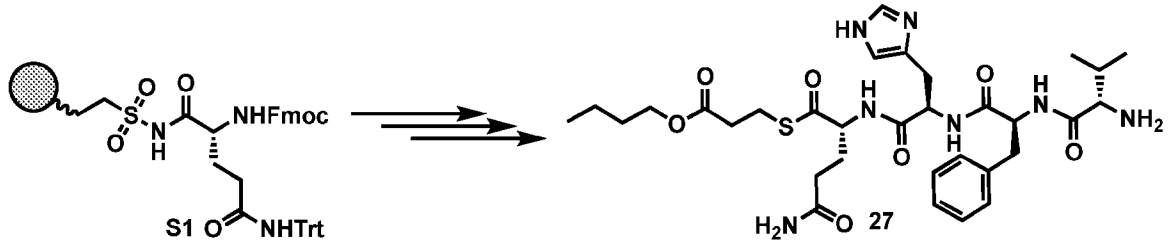
[00116] Synthesis of liner peptide 26



Peptide **26** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH loaded resin (**S4**) (0.1 mmol) and involved the following amino acids: Fmoc-D-Gln(Trt)-OH, Fmoc-D-Dap(Boc)-OH, Fmoc-L-Phe-OH, and Boc-L-Val-OH. Butyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.1% formic acid (A) and acetonitrile+0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (16.3 mg, 19% yield, purity 96%).

^{1}H NMR (800 MHz, DMSO) δ 8.87 (d, J = 6.6 Hz, 2H), 8.40 (d, J = 7.1 Hz, 1H), 8.08 (s, 6H), 7.34 – 7.25 (m, 4H), 7.22 (dd, J = 2056.0, 7.1 Hz, 1H), 6.89 (s, 1H), 4.63 (p, J = 6.1 Hz, 1H), 4.49 (h, J = 4.8 Hz, 1H), 4.32 – 4.21 (m, 1H), 4.02 (t, J = 6.6 Hz, 2H), 3.60 (s, 1H), 3.23 (dd, J = 5.2, 3.3 Hz, 2H), 3.01 – 2.90 (m, 2H), 2.90 – 2.75 (m, 2H), 2.60 – 2.52 (m, 1H), 2.24 – 1.81 (m, 5H), 1.53 (p, J = 8.7 Hz, 2H), 1.31 (h, J = 7.9 Hz, 2H), 0.95 – 0.68 (m, 9H).**^{13}C NMR** (201 MHz, DMSO) δ 200.77, 174.16, 171.85, 171.60, 170.00, 169.25, 137.91, 129.55, 128.67, 126.95, 70.20, 64.39, 59.62, 57.47, 55.63, 50.52, 39.96, 36.69, 34.05, 31.40, 30.57, 30.47, 27.43, 23.67, 19.04, 18.86, 17.89, 13.99. **Mass spec:** expected neutral mass for $C_{29}H_{46}N_6O_7S$ (Da): 622.3148, observed neutral mass (Da): 622.3152, mass error (ppm) 0.5.

[00117] Synthesis of liner peptide 27



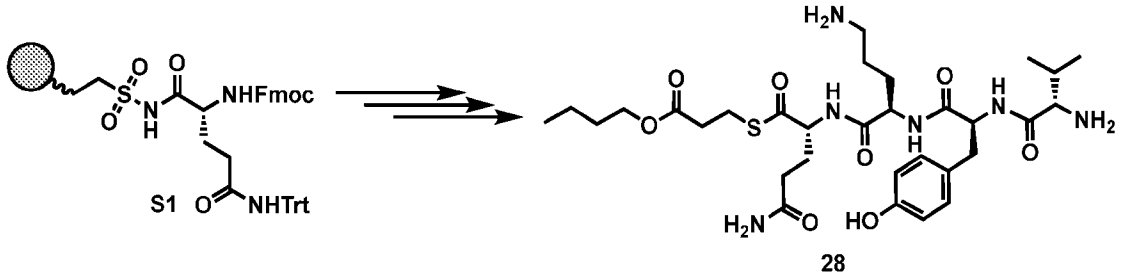
The synthesis of Peptide **27** was initiated from **S1** (0.05 mmol) using the following amino acids in the order of Fmoc-D-His(Trt)-OH, Fmoc-L-Phe-OH, and Boc-L-Val-OH, with butyl 3-mercaptopropionate used as the cleaving thiol, following the general protocol outlined above for the sulfonamide 'safety-catch' resin The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.1% formic acid (A) and acetonitrile+0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (12.1 mg, 27%) (purity 98%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 8.96 (s, 1H), 8.65 (dd, J = 42.6, 6.3 Hz, 3H), 8.05 (s, 2H), 7.39 – 7.05 (m, 7H), 6.83 (s, 1H), 4.64 (dt, J = 66.4, 5.7 Hz, 2H), 4.30 (s, 1H), 4.01 (t, J = 6.2 Hz, 2H), 3.59 (s, 1H), 3.13 (d, J = 13.7 Hz, 1H), 2.95 (s, 2H), 2.84 (q, J = 7.4 Hz, 2H), 2.73 (t, J = 8.1 Hz, 1H), 2.54 (s, 4H), 2.23 – 2.00 (m, 3H), 2.00 – 1.94 (m, 1H), 1.91 – 1.73 (m, 1H), 1.49 (t, J = 7.4 Hz, 2H), 1.31 (q, J = 6.2 Hz, 2H), 0.90 (d, J = 5.7 Hz, 3H), 0.87 (s, 6H).

^{13}C NMR (201 MHz, DMSO) δ 200.84, 173.72, 171.51, 171.00, 170.69, 168.50, 137.47, 134.18, 129.51 (d, J = 16.1 Hz), 128.47, 126.84, 117.26, 64.30, 59.44, 57.43, 54.69, 51.58, 40.81, 37.71, 33.95, 31.29, 30.49, 30.33, 27.40, 27.15, 23.60, 18.95, 18.76, 17.71, 13.90.

Mass spec: expected neutral mass for $C_{32}H_{47}N_7O_7S$ (Da): 673.3257, observed neutral mass (Da): 673.3257, mass error (ppm) < 0.0.

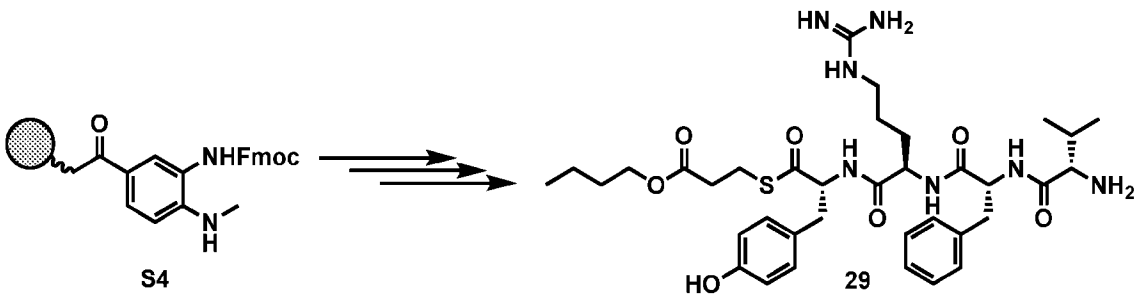
[00118] Synthesis of liner peptide 28



Peptide **28** was synthesized following the general protocol outlined above for the sulfonamide "safety-catch" resin. The synthesis was initiated from **S1** (0.05 mmol) using the following amino acids Fmoc-D-Orn(Boc)-OH, Fmoc-L-Tyr(tBu)-OH, Boc-L-Val-OH and butyl 3-mercaptopropionate as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O+0.05% TFA (A) and acetonitrile+0.05% TFA (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 ml/min. The column was equilibrated with 0% mobile phase B for 1 minute before and 5 minutes after the gradient to yield an off-white solid (13.0 mg, 29% yield) (purity 97%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 9.32 (s, 1H), 8.60 (d, J = 6.5 Hz, 1H), 8.52 (d, J = 4.7 Hz, 1H), 8.39 (d, J = 7.3 Hz, 1H), 8.04 (s, 3H), 7.83 (s, 3H), 7.26 (s, 1H), 7.08 (d, J = 7.1 Hz, 2H), 6.82 (s, 1H), 6.67 (d, J = 4.7 Hz, 2H), 4.58 (s, 1H), 4.31 (d, J = 42.2 Hz, 2H), 4.02 (s, 2H), 3.60 (s, 1H), 2.97 (s, 2H), 2.87 (d, J = 12.8 Hz, 1H), 2.54 (d, J = 33.4 Hz, 7H), 2.13 (d, J = 46.2 Hz, 3H), 1.94 (s, 1H), 1.80 (d, J = 39.9 Hz, 2H), 1.54 (s, 2H), 1.51 – 1.37 (m, 3H), 1.32 (d, J = 4.7 Hz, 2H), 1.01 – 0.60 (m, 9H). **^{13}C NMR** (201 MHz, DMSO) δ 201.09, 173.72, 171.95, 171.56, 171.10, 168.41, 156.37, 130.58, 127.67, 115.34, 64.29, 59.28, 57.44, 55.11, 51.74, 40.81, 38.86, 37.31, 34.01, 31.34, 30.50, 30.36, 27.49, 23.78, 23.60, 18.97, 18.79, 17.68, 13.93. **Mass spec:** expected neutral mass for $C_{31}H_{50}N_6O_8S$ (Da): 666.4311, observed neutral mass (Da): 666.3410, mass error (ppm) 0.1.

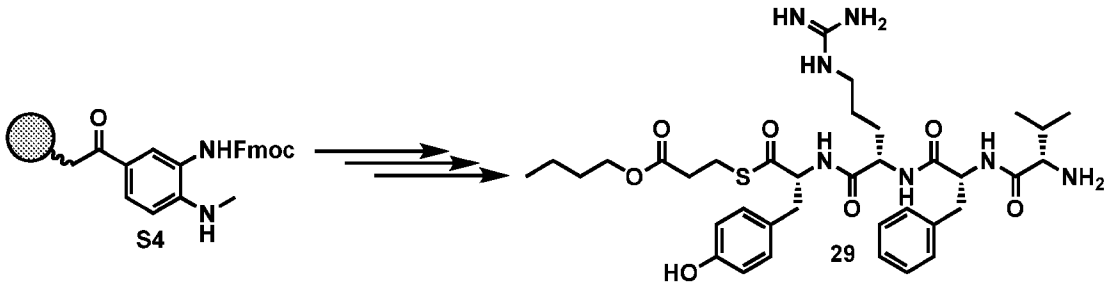
[00119] Synthesis of liner peptide 29



Peptide **29** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH loaded resin **S4** (0.15 mmol) and involved the following amino acids: Fmoc-D-Tyr(tBu)-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Phe-OH, Boc-L-Val-OH. Butyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile+0.05% trifluoroacetic Acid (B) and H2O+0.05% trifluoroacetic Acid (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 minutes after and 1 minute before the gradient to yield an off-white solid (42.4 mg, 30% yield) (purity 98%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 8.65 (q, *J* = 7.7 Hz, 2H), 8.49 (d, *J* = 7.7 Hz, 1H), 7.94 (d, *J* = 3.6 Hz, 3H), 7.90 (t, *J* = 5.4 Hz, 1H), 7.48 (s, 1H), 7.26 (d, *J* = 8.0 Hz, 2H), 7.23 (t, *J* = 8.0 Hz, 2H), 7.15 (t, *J* = 8.0 Hz, 2H), 7.01 (d, *J* = 8.1 Hz, 2H), 6.65 (d, *J* = 8.4 Hz, 2H), 4.78 (td, *J* = 5.7, 3.9 Hz, 1H), 4.52 – 4.47 (m, 1H), 4.36 (q, *J* = 8.1 Hz, 1H), 4.02 (t, *J* = 6.5 Hz, 2H), 3.59 (t, *J* = 5.4 Hz, 1H), 3.17 – 3.08 (m, 2H), 3.06 (dd, *J* = 3.1, 2.7 Hz, 1H), 3.02 – 2.93 (m, 3H), 2.81 (q, *J* = 9.5 Hz, 1H), 2.66 (t, *J* = 11.9 Hz, 1H), 2.54 (td, *J* = 3.1, 2.7 Hz, 4H), 1.81 (h, *J* = 5.7 Hz, 1H), 1.78 – 1.70 (m, 1H), 1.60 – 1.44 (m, 5H), 1.31 (h, *J* = 6.9 Hz, 2H), 0.88 (t, *J* = 6.9 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H), 0.39 (d, *J* = 6.9 Hz, 3H). **^{13}C NMR** (201 MHz, DMSO) δ 200.53, 171.95, 171.53, 171.26, 167.90, 157.31, 156.46, 137.88, 130.36, 129.55, 128.37, 127.14, 126.69, 115.44, 64.30, 61.25, 57.54, 54.04, 52.58, 40.81, 38.62, 36.44, 33.99, 30.50, 30.01, 29.47, 25.58, 23.74, 18.96, 18.55, 16.72, 13.92. **Mass spec:** expected neutral mass for $C_{36}H_{53}N_7O_7S$ (Da): 727.3727, observed neutral mass (Da): 727.3740, mass error (ppm) 1.8.

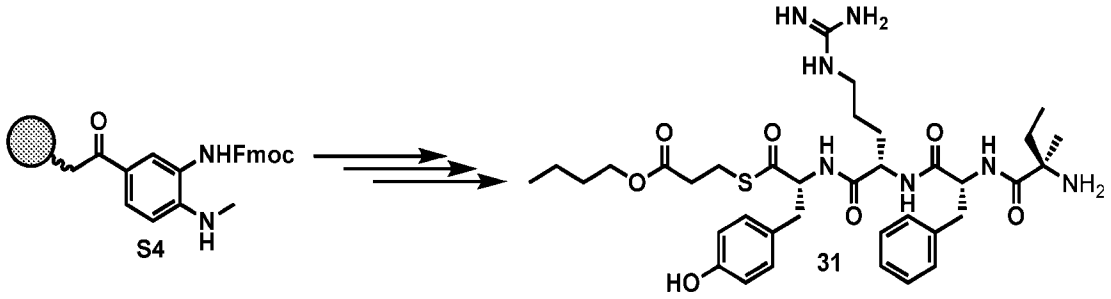
[00120] Synthesis of liner peptide 30



Peptide **30** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH loaded resin **S4** (0.15 mmol) and involved the following amino acids: Fmoc-D-Tyr(tBu)-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-D-Phe-OH, Boc-L-Val-OH. Butyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile+0.05% TFA (B) and H_2O+0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 ml/min. The column was equilibrated with 0% mobile phase B for 5 minutes after and 1 minute before the gradient to yield an off-white solid (49.1 mg, 34% yield) (purity 99%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 8.71 (t, *J* = 8.8 Hz, 2H), 8.45 (d, *J* = 8.4 Hz, 1H), 7.94 (d, *J* = 4.2 Hz, 3H), 7.73 (t, *J* = 6.0 Hz, 1H), 7.25 (d, *J* = 8.1 Hz, 2H), 7.23 (dd, *J* = 39.7, 7.3 Hz, 2H), 7.16 (t, *J* = 7.7 Hz, 1H), 7.01 (d, *J* = 8.3 Hz, 2H), 6.66 (d, *J* = 8.4 Hz, 2H), 4.93 – 4.86 (m, 1H), 4.54 – 4.49 (m, 1H), 4.40 (h, *J* = 4.5 Hz, 1H), 4.03 (t, *J* = 6.3 Hz, 2H), 3.59 (t, *J* = 5.2 Hz, 1H), 3.04 – 2.91 (m, 6H), 2.70 (t, *J* = 10.5 Hz, 2H), 2.61 – 2.52 (m, 2H), 1.81 (h, *J* = 2.4 Hz, 1H), 1.55 (p, *J* = 6.9 Hz, 2H), 1.43 – 1.37 (m, 1H), 1.32 (h, *J* = 7.7 Hz, 2H), 1.29 – 1.23 (m, 1H), 1.18 (p, *J* = 6.6 Hz, 2H), 0.88 (t, *J* = 6.9 Hz, 3H), 0.67 (d, *J* = 7.7 Hz, 3H), 0.42 (d, *J* = 7.0 Hz, 3H). **^{13}C NMR** (201 MHz, DMSO) δ 200.35, 171.85, 171.59, 170.92, 167.92, 157.25, 156.52, 137.64, 130.54, 129.63, 128.36, 127.07, 126.73, 115.43, 64.35, 61.19, 57.56, 53.95, 51.88, 40.63, 39.29, 36.83, 33.94, 30.51, 30.13, 29.99, 24.96, 23.82, 18.98, 18.64, 16.82, 13.93. **Mass spec:** expected neutral mass for $C_{36}H_{53}N_7O_7S$ (Da): 727.3727, observed neutral mass (Da): 727.3733, mass error (ppm) 0.9.

[00121] Synthesis of liner peptide 31



Peptide **31** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH loaded resin **S4** (0.15 mmol) and involved the following amino acids: Fmoc-D-Tyr(tBu)-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-L-Phe-OH, Boc-D-isoVal-OH. Butyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile+0.05% TFA (B) and H_2O+0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 50% over 22 minutes at a flow rate of 20 ml/min. The column was equilibrated with 0% mobile phase B for 5 minutes after and 1 minute before the gradient to yield an off-white solid (65.7 mg, 46% yield) (purity 97%, UPLC).

^{1}H NMR (800 MHz, DMSO) δ 9.35 (s, 1H), 8.76 (d, J = 7.2 Hz, 1H), 8.39 (dd, J = 9.7, 9.1 Hz, 2H), 7.98 – 7.88 (m, 4H), 7.47 (s, 1H), 7.32 (d, J = 7.2 Hz, 2H), 7.23 (t, J = 7.2 Hz, 3H), 7.16 (t, J = 7.9 Hz, 1H), 7.02 (d, J = 7.9 Hz, 2H), 6.65 (d, J = 9.1 Hz, 2H), 4.80 (td, J = 3.9, 3.9 Hz, 1H), 4.49 – 4.42 (m, 2H), 4.03 (t, J = 6.5 Hz, 2H), 3.13 – 3.03 (m, 3H), 2.98 (t, J = 7.2 Hz, 2H), 2.92 (dd, J = 5.9, 5.2 Hz, 1H), 2.80 (h, J = 7.2 Hz, 2H), 2.53 (t, J = 6.5 Hz, 2H), 1.79 – 1.73 (m, 1H), 1.67 (h, J = 7.9 Hz, 1H), 1.52 (h, J = 6.5 Hz, 4H), 1.50 – 1.40 (m, 5H), 1.32 (h, J = 7.9 Hz, 2H), 0.88 (t, J = 7.9 Hz, 3H), 0.24 (t, J = 6.5 Hz, 3H). **^{13}C NMR** (201 MHz, DMSO) δ 200.62, 171.94, 171.53, 171.19, 170.68, 157.33, 156.52, 138.22, 130.39, 129.59, 128.37, 127.08, 126.65, 115.51, 64.31, 61.46, 60.47, 54.73, 51.86, 40.78, 37.96, 36.57, 34.01, 30.51, 30.30, 29.93, 25.28, 23.70, 22.63, 18.97, 13.92, 7.45. **Mass spec:** expected neutral mass for $C_{36}H_{53}N_7O_7S$ (Da): 727.3727, observed neutral mass (Da): 727.3774, mass error (ppm) 6.4.

[00122] <u>Ulm16 Hexapeptide Cyclization</u>

A 50 µL reaction mixture containing 20 mM Tris-HCl (pH 8.0), 5% DMSO, 50 nM Ulm16, and 280 µM substrate was incubated at 30°C for 4 hours. The reaction mixtures were quenched with 50 µL of acetonitrile and centrifuged at 21,000g for 10 minutes. Five microliters of the resulting solution

were loaded onto a CORTECS T3 Column, 120Å, 1.6 µm, 2.1 mm X 50 mm (Waters) and separated by UPLC with monitoring at 214 nm. The mobile phases used were H_2O+0.1% FA and acetonitrile+0.1% FA, and the samples were eluted by gradient mode: 0% to 40% for mobile phase B in 11 minutes with a flow rate of 0.5 ml min^{-1}. All reactions were carried out in triplicate.

[00123] Ulm16 Tetrapeptide and Pentapeptide Cyclization

A 100 µL reaction mixture containing 20 mM Tris-HCl (pH 8.0), 5% DMSO, and a concentration of Ulm16 and substrate-dependent on the peptide (see **Table 1** of **Fig. 4** for concentrations) was incubated at 30 °C for 4 hours. The reaction mixtures were quenched with 100 µL of acetonitrile and centrifuged at 21,000 g for 10 minutes. Five microliters of the resulting solution were loaded onto a CORTECS T3 Column, 120 Å, 1.6 µm, 2.1 mm × 50 mm (Waters) and separated by UPLC with monitoring at 214 nm. The mobile phases used were H_2O + 0.1% FA and acetonitrile + 0.1% FA. Tetra- and pentapeptides were eluted by gradient mode: 0% to 40% mobile phase B in 11 minutes at a flow rate of 0.5 mL min−1. Unless stated otherwise, all reactions were carried out in triplicate.

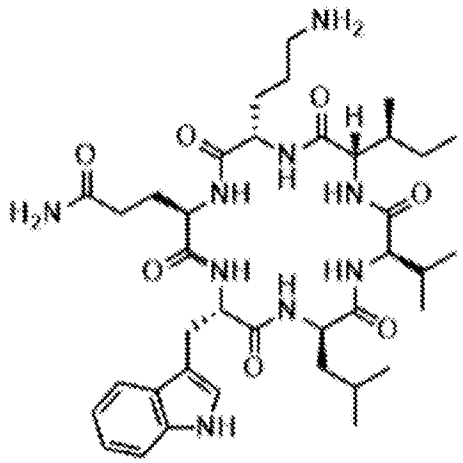
[00124] Total Turnover Number (TTN) Assays

TTN was calculated by the formula below.

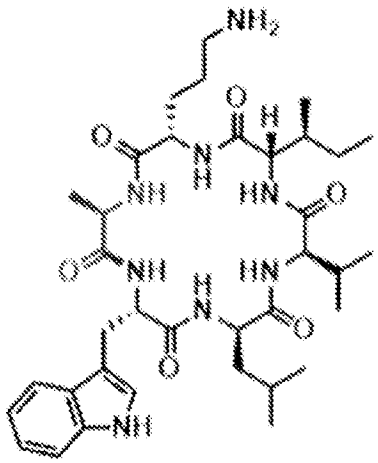
$$TTN = \frac{AREA_{Product}}{AREA_{Product} + AREA_{Hydrolysis} + Area_{Starting\ Material}} \times \frac{[Substrate]_{Total}}{[PBPTE]_{Total}}$$

Enzymatic reactions were conducted in 20 mM Tris at pH 8.0 with 5% DMSO for 4 hours at 30°C using various concentrations of peptides and Ulm16 as indicated by [Peptide] and [Ulm16], respectively (see **Table 1**). The areas of the enzymatic products and starting material were determined by analyzing the UPLC UV Trace at 214 nm. Total turnover numbers were calculated using the above formula. Numbers 19 and 31 indicate substrates that were neither cyclized nor hydrolyzed. **Table 1 (Fig. 4)** illustrates Ulm16 total turnover assays.

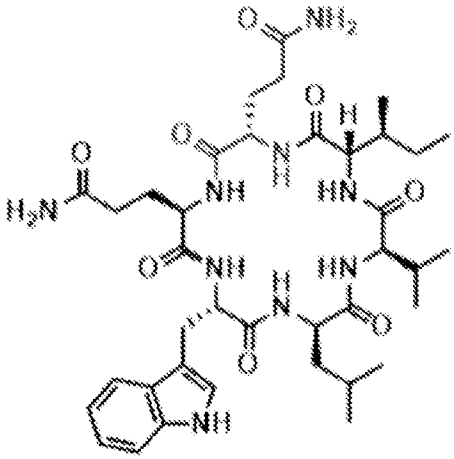
[00125] Examples of Cyclic peptides. Following cyclic peptides were synthesized and identified by MS.



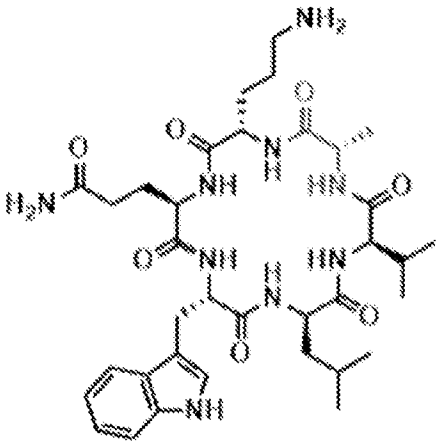
Cyc1



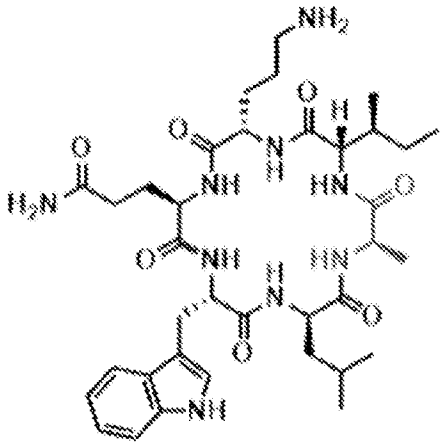
Cyc5



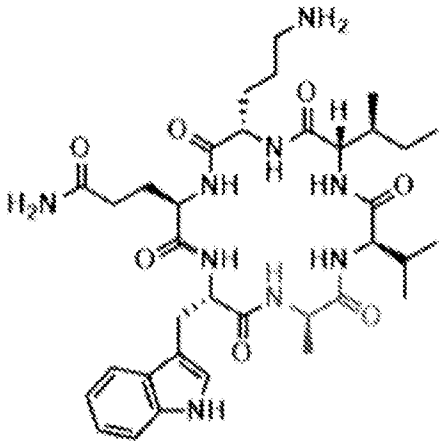
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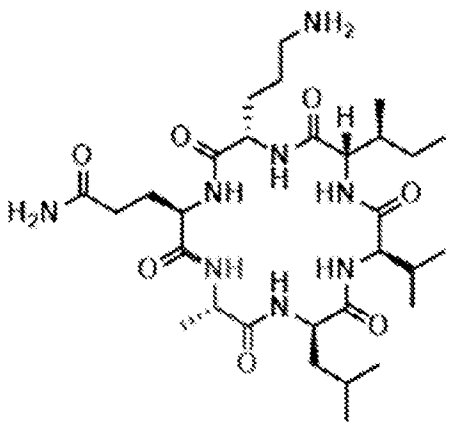
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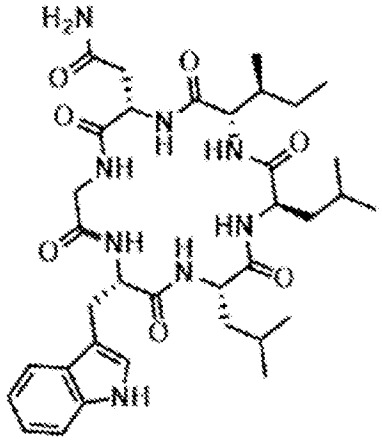
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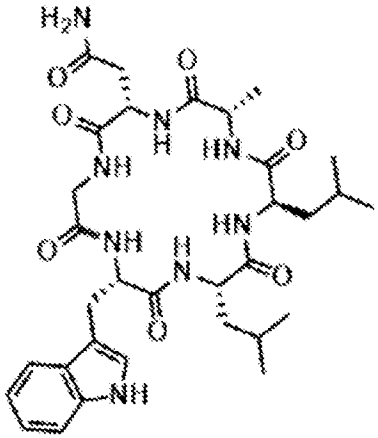
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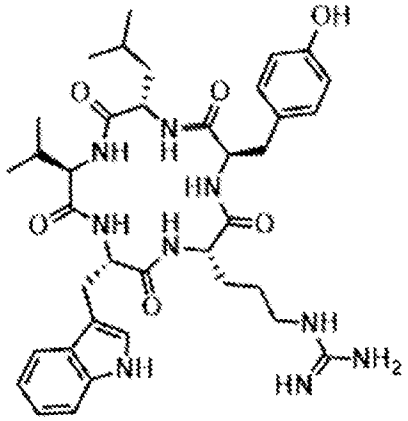
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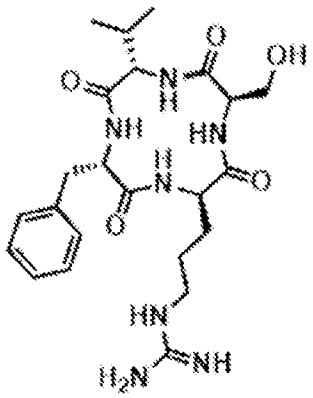
Cyc11



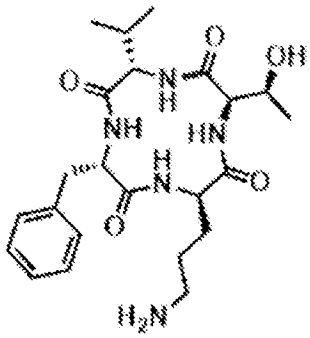
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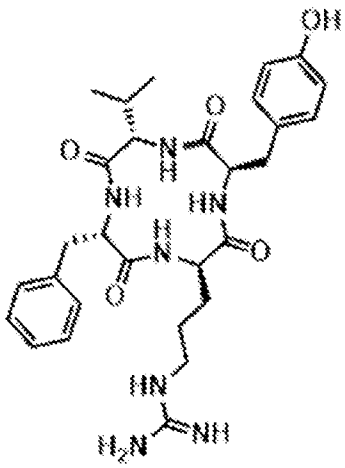
Cyc13



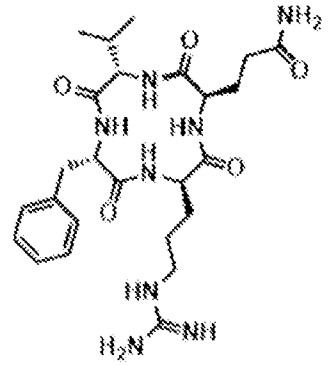
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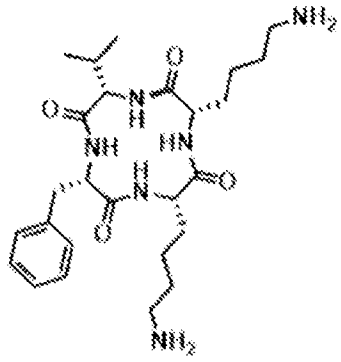
Cyc15



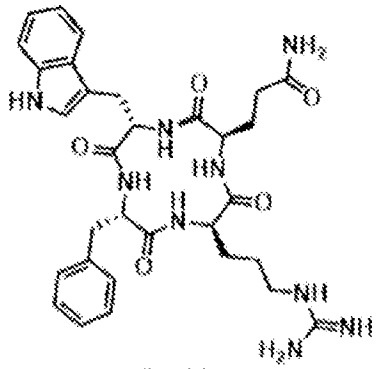
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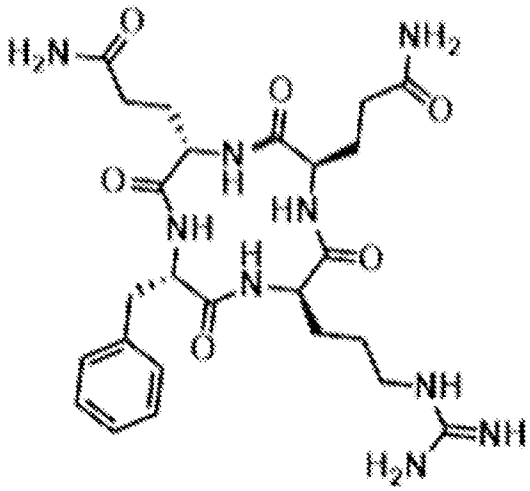
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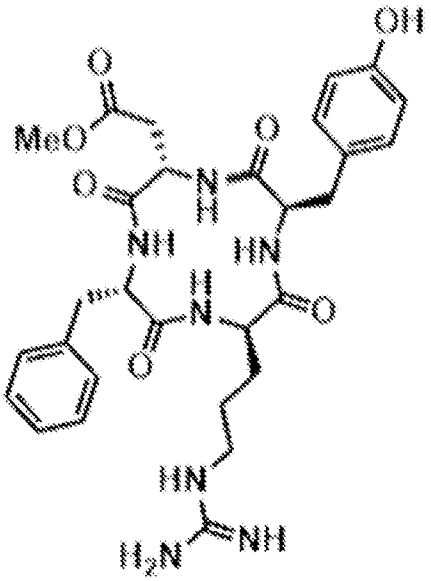
Cyc18



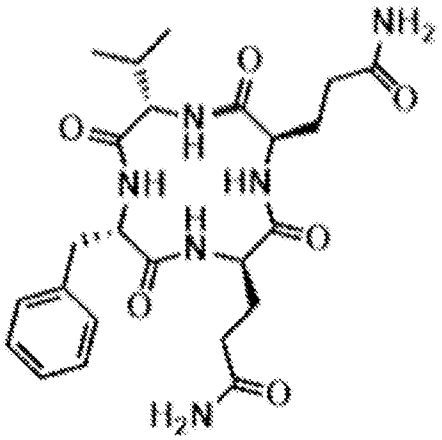
Cyc20



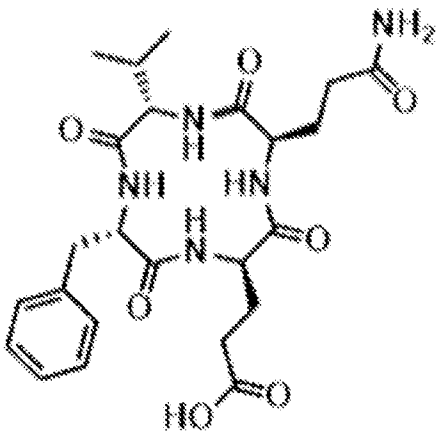
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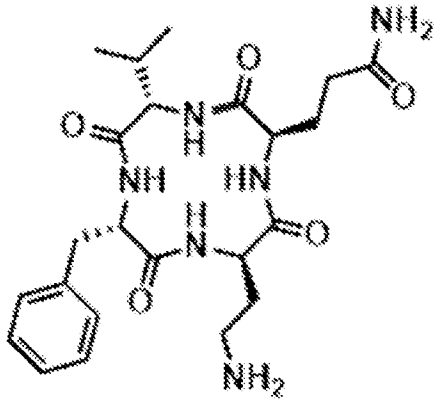
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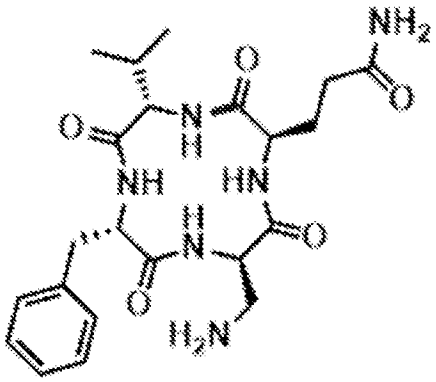
Cyc23



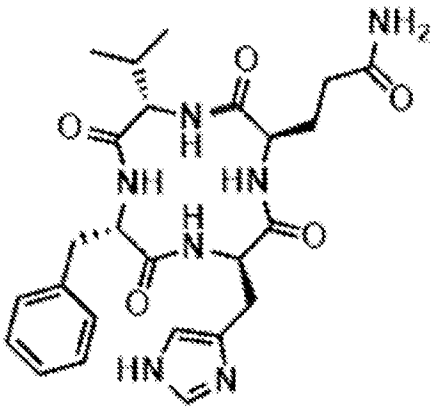
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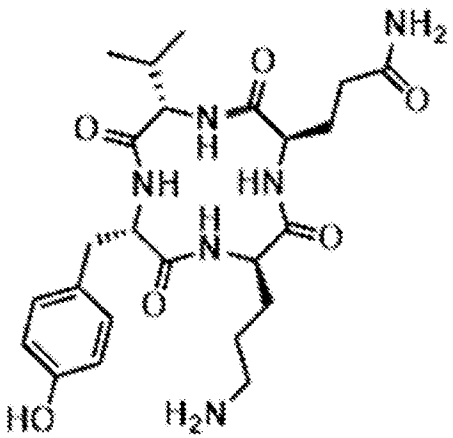
Cyc25



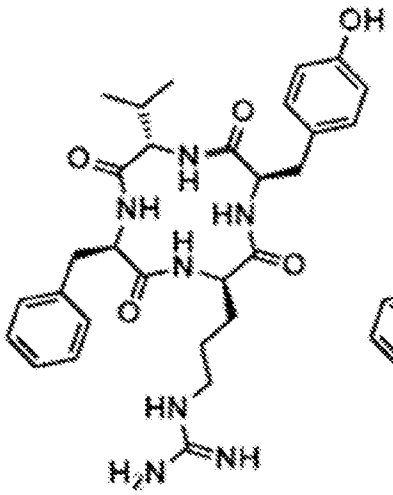
Cyc26



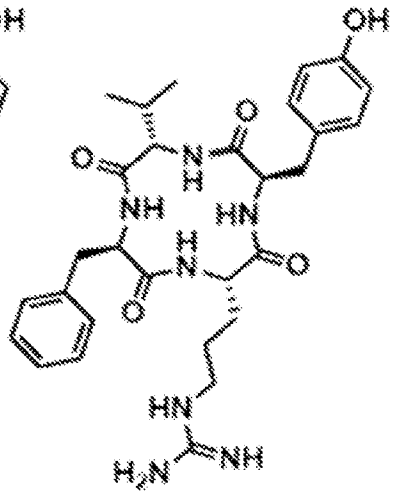
Cyc27



Cyc28



Cyc29

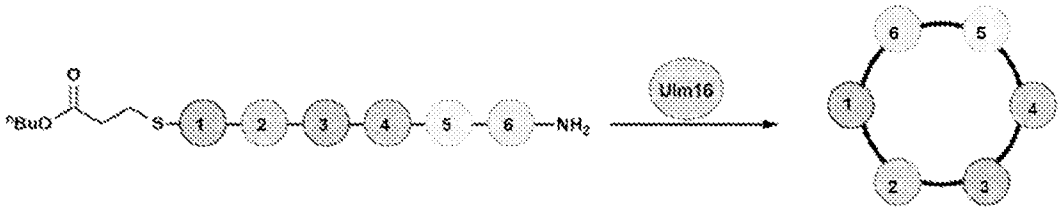


Cyc30

[00126] Ulm16 and SurE Kinetic Assays

To determine the kinetic parameters of Ulm16, a 50 µL reaction mixture containing 20 mM Tris-HCl (pH 8.0) and 5% DMSO, 24 nM Ulm16, and 12-800 µM substrate was incubated for 2 minutes at 30 °C. For reaction mixtures containing dL3dA (7) and lW1lA (10), 40 nM enzyme was incubated with the substrate at 30°C for 2 and 3 minutes, respectively. The reaction mixture was quenched with 50 µL acetonitrile and centrifuged at 21,000g for 10 minutes. Then, 5 µL was loaded onto a CORTECS T3 Column, 120Å, 1.6 µm, 2.1 mm x 50 mm (Waters) and samples were separated by UHPLC with monitoring at 214 nm. The mobile phases A and B were H_2O+0.1% formic acid (FA) and acetonitrile+0.1% FA, respectively, and samples were eluted by gradient mode: 0% to 40% for mobile phase B in 11 minutes with a flow rate of 0.5 ml min−1. The peptide concentrations were estimated based on the extinction coefficient ε (214nm) of a synthetic standard. The ε (214nm) values for all SBMP variants were assumed to be equal except for W1A (10), PenA Substrate (13), and dTyrTP (16), for which their initial velocity was calculated assuming that ε (214 nm) values are equal for SBMP substrate and corresponding enzymatic product. All reactions were carried out in triplicate. Kinetic parameters were estimated using the Michaelis-Menten equation and the curve-fitting program PRISM 9.

[00127] **Table 2** Kinetic parameters of Ulm16 against thioester substrates



Liner peptide/ Substrate	k_{Cat} (min^{-1}) (±s.e.m.)	k_{cat}/K_m ($M^{-1}s^{-1}$) (±s.e.m.)	k_{cat}/K_m ratio to (12-SBMP)
12-SNAC	1545 (± 71)	0.21 (± 0.03) x10^6	0.31
12-SMMP	2175 (± 109)	0.43 (± 0.03) x10^6	0.64
12-SBMP	1921 (± 121)	0.67 (± 0.18) x10^6	1.00
4	No Reaction		
5	2079 (±163)	0.39 (± 0.09) x10^6	0.58
6	TTN: 9055		
7	1900 (± 83)	0.54 (± 0.09) x10^6	0.81
8	441 (± 31)	0.13 (± 0.03) x10^6	0.19
9	1364 (± 80)	0.56 (± 0.15) x10^6	0.84
10	90.1 (± 2.7)	0.39 (± 0.05) x10^5	0.058
13	3200 (± 390)	2.0 (± 1.2) x10^6	2.99
16	1160 (± 63)	3.0 (± 1.2) x10^6	4.48

[00128] **Ulm16 hexapeptide cyclization**. To investigate the substrate scope of Ulm16, a derivative of ulleungymcicin A was synthesized, in which the native but commercially inaccessible amino acids D-threo-β-hydroxy asparagine, L-allo-isoleucine, D-homoleucine, and 5-chloro-L-tryptophan were substituted for D-glutamine, L-isoleucine, D-Leucine, and L-tryptophan, respectively (designated

'Ulm'). D-Asparagine, a better substitute for D-threo-β-hydroxy asparagine, was not used due to its propensity to rapid asparatamide formation at the C-terminus. While SurE and PenA have previously been examined using peptide thioesters that contained N-Acetylcysteamine (SNAC), a mimic of the phosphopantetheine arm utilized by the PCP domain, recent studies utilizing excised thioesterase domains have shown that other thiols not related to the phosphopantetheine backbone can improve enzyme activity and turnover. As a result, the modified Ulm16 peptide was synthesized with three different C-terminal thiols: N-acetylcysteamine (12-SNAC), methyl 3-mercaptopropionate (12-SMMP), and butyl 3-mercaptopropionate (12-SBMP), using safety catch resin to avoid issues with epimerization that necessitate and complicate purification. Notably, the SBMP-thioester was found to be three times more efficient in processing than the commonly used and expensive SNAC-thioester (see **Table 2**). For this reason, SBMP-thioesters were used for the rest of the substrate scope unless otherwise noted.

[00129] Ulm16 was a highly efficient biosynthetic enzyme, exhibiting a catalytic efficiency that is over 100-fold higher than that reported for SurE $k_{cat}/K_M = 1.7 \times 10^3$ $M^{-1}s^{-1}$ for its SNAC substrate ((Matsuda, K. et al. Nature. Catalyst. 2020, 3, 507–515). An alanine scan was performed to determine sites where modification was tolerated (**Table 1**). Substitution of internal residues 5 (D-valine) and 3 (L-isoleucine) with D-alanine and L-alanine, respectively, were well tolerated, displaying approximately 80% of the activity as the initial substrate. D-alanine substitution at position 4 was not as well tolerated (~20% of the activity of the initial substrate). Unfortunately, alanine substitution at site two ornithine resulted in an insoluble peptide. To investigate modifications at site two, L-ornithine was replaced with the uncharged L-glutamine. While both the SBMP and SMMP thioester for this molecule were too insoluble to obtain full kinetics, the SMMP thioester showed sufficient solubility to determine that the total turnover number (TTN) for **(6)** was 9055 with a greater than 20:1 ratio for cyclized over hydrolyzed (**Fig. 1D**). While the TTN was lower than that of the initial peptide (TTN for **(2)** = 22545), indicated that an uncharged residue at this site is well tolerated by the enzyme. Ulm16 tolerates substitution at the C-terminus very well, displaying approximately 60% of the activity observed when the C-terminal D-glutamine is substituted with D-alanine. This contrasts with previously investigated SurE and PenA, which have drastically reduced abilities to cyclize peptides with C-terminal alterations of their native residues. Modification at the N-terminus was less well tolerated, with an L-alanine substitution displaying only 6% of the catalytic efficiency of the native L-tryptophan. However, its efficiency is still approximately 20-fold better than that of SurE, and little hydrolysis is observed. Similarly, to other PBP-TEs, Ulm16 cannot tolerate an L-

amino acid at the C-terminus. Switching the chirality at the C-terminus (**4**) resulted in a molecule that was not processed by the enzyme. Ulm16 was able to tolerate glycine at the C-terminus, a property that has only been previously validated with WolJ and SurE. Specifically, both (**11**) and (**12**) had good TTNs (**Fig. 1D**) and showed selectivity for cyclized over hydrolyzed, while SurE showed no activity. Thus, a C-terminal D-chirality was not absolutely essential for Ulm16 activity.

[00130] Ulm16 cyclizes small rings

[00131] With the knowledge that Ulm16 can cyclize a wide range of hexapeptides, different ring sizes were explored. Ulm16 efficiently cyclized both pentapeptides and tetrapeptides (**Table 1** and **Fig. 1A**). Specifically, it was able to cyclize a modified PenA substrate where the C-terminal D-phenylalanine was substituted for a D-tyrosine, displaying nearly 3-fold better catalytic efficiency compared to its modified native substrate. This modified peptide was also used to directly compare Ulm16 to SurE, since SurE had previously been shown to cyclize the PenA substrate ((Matsuda K. et al., Journal of Industrial Microbiology Biotechnology, 2021, 48). While SurE also had very good catalytic efficiency with this substrate (1.7×10^5 $M^{-1}s^{-1}$), Ulm16 remains the more efficient enzyme with approximately 10-fold better activity and approximately 15-fold greater total turnover.

[00132] Ulm16 to cyclize a tetrapeptide consisting of N-terminal L-valine and C-terminal D-serine (**14**). The peptide was efficiently cyclized, with very little hydrolysis observed (>20:1 cyclic: hydrolyzed) (**Fig. 1C**). These results were particularly noteworthy, as no PBP-TEs studied to date have been shown to cyclize tetrapeptides. To confirm that the product was indeed the tetrapeptide, mass spectrometry analysis was conducted and compared it to a synthetic standard of the cyclic tetrapeptide. The substrate scope of the tetrapeptide by modifying each position and determining TTN for each substrate was investigated. Ulm16 was able to tolerate a wide range of substitutions at the C-terminal residue as long as the D-chirality was maintained (peptides **15-18**, **Fig. 1D**). The D-Serine substitution for D-Threonine (**15**) resulted in no significant change in Cyclized/Hydrolyzed ratio or TTN. However, substitution with a D-Tyrosine (**16**) produced a substrate with over 4-fold better catalytic efficiency than the native hexapeptide substrate (**Table 1**) and a TTN over 10-fold better than the initial tetrapeptide hit (**Fig. 1D**). Additionally, this product was once again confirmed as the desired tetrapeptide by comparison with the tetrapeptide standard. Ulm16 also accepted other polar amino acids such as D-Gln (**17**) (with an increased observance in hydrolysis 6:1) and a polar charge D-lysine (**18**) with low variability in TTN. However, the substitution of D-serine with a negatively charged D-glutamate (**19**) resulted in a substrate that could not be processed by Ulm16. As seen with the hexapeptides, modifications of the N-terminal modifications were more varied.

Substituting the N-terminal L-valine with L-tryptophan (**20**) had little effect on TTN and displayed good selectivity for cyclized over hydrolyzed products. However, substituting with polar L-glutamine (**21**) significantly reduced activity (~4-fold reduced TTN compared to L-valine) and completely abolished selectivity for cyclic versus hydrolyzed products. Anecdotally, a negative charge (L-asparate) at the N-terminus results in hydrolysis, consistent with the observation a polar amino acid increases hydrolysis. However, blocking the negative charge with a methyl group (**22**) resulted in a substrate that could be efficiently cyclized by Ulm16. Interestingly, position 2 showed a preference for polar residues with at least two methylenes between the α-carbon and the polar side chain atom. Substituting D-ornithine with D-glutamine (**23**), D-glutamic acid (**24**), or D-2,4-diamino butyric acid (**25**) resulted in predominantly cyclic products with good TTNs. However, substitution with D-2,3-diaminopropionic acid (**26**) or D-histidine (**27**) resulted in higher levels of linear products. Only one substitution was tested at the position, where L-phenylalanine was replaced with polar L-tyrosine (**28**). Although the turnover number (TTN) decreased by approximately 2-fold, the overall ratio of cyclic to hydrolysis remained unchanged (>20:1). Other polar substitutions at this position were explored, as the linear peptide eluted out in the solvent front of the UPLC. **Fig. 1E** illustrates the substrate scope of Ulm16, indicating that it can accommodate a diverse range of amino acids at positions 1-3 of the tetrapeptide sequence. However, the N-terminal amino acid shows a pronounced preference for non-polar amino acids. Overall, this suggests that Ulm16 has broad specificity for substrate recognition but also exhibits selectivity for certain amino acid residues.

[00133] Stereochemical preorganization into a cyclic conformation can have a significant impact on a peptide's ability to undergo cyclization. To explore the ability of Ulm16 to cyclize peptides with different stereochemistry, the stereochemistry of tetrapeptide (**16**) was modified, which exhibited the highest total turnover and catalytic efficiency in our study. Three new peptides (peptides **29-31**) were synthesized, with D-D-D-L, D-L-D-L, and D-D-L-D stereochemistry, respectively. Peptide **31** was synthesized with D-isovaline to prevent epimerization during the final amino acid coupling. Surprisingly, we found that Ulm16 was able to efficiently cyclize the D-D-D-L peptide (peptide **29**), with TTNs and good selectivity for cyclic over hydrolyzed peptide (~12:1). However, the D-L-D-L stereochemistry (peptide **30**) was more prone to hydrolysis, with a cyclization-to-hydrolysis ratio of ~1:1.4. The D-D-L-D stereochemistry (peptide **31**) was not efficiently processed by Ulm16, and only hydrolyzed peptide was observed. These results are consistent with previous reports that PBP-TEs require a D C-terminal amino acid and L N-terminal amino acid for efficient cyclization activity.

[00134] To rationalize Ulm16's ability to cyclize peptides with high catalytic efficiency, we solved its crystal structure to a resolution of 2.06 Å and performed covalent docking studies with multiple substrates that displayed varying degrees of cyclization efficiency and cyclization to hydrolysis ratios. Interestingly, the missing loop region seems to have caused the C-terminal residue of the peptides to bind to a hydrophobic pocket beneath the activity site serine, resulting in a different trajectory than what has been previously reported in docking studies with SurE (Matsuda, K. et al., Nature Catalyst. 2020, 3, 507–515). Additionally, the differing position of the lipocalin domain from SurE appears to play a role in organizing the substrate, with hydrophobic residues forming a binding pocket for the N-terminal residues of the peptides docked/tested, and R436 hydrogen bonding with backbone carbonyls of the peptide possibly aiding in the trans-cis conversion needed for cyclization. This provided insight into the structural basis of the unique ability of Ulm16 to efficiently cyclize small peptides, specifically tetrapeptides. Through crystal structure determination and covalent docking studies, it was observed how the absence of a loop region in Ulm16 alters the peptide binding trajectory and enhances its ability to bind and catalyze cyclization with high efficiency.

[00135] Mutagenesis studies of Ulm16

[00136] Studied site-directed mutagenesis of specific residues within alpha-beta hydrolase domain and lipocalin domain of Ulm16. These mutants were subsequently assessed using four tetrapeptides such as—**(14)** and **(16)**, along with **(29)** and **(30)** tetrapeptides, all of which had varying TTNs and cyclized to hydrolyzed ratios. Additionally, a hexapeptide (**12**-MMP) was also tested as the covalent docking predicted it was predisposed to forming a cyclic conformation . Earlier investigations on PBP-TEs, along with the docking studies, suggest that the lipocalin domain is responsible for the ability of the enzyme to induce a cyclic conformation of the peptide. Initially, the lipocalin domain was removed completely and created the Ulm16 1-344 construct. In the absence of this domain, Ulm16 produces very small amounts of cyclic peptide in most cases. However, the TTNs are orders of magnitude lower compared to the wild-type enzyme, suggesting that the domain is very important, if not essential, for cyclization. To gain deeper insights into the effect of the lipocalin domain on cyclization, Ulm16 mutants were expressed and purified targeting residues believed to aid substrate preorganization and effective cyclization. These positions (Arg431, Tyr428, and Ser429) are highly conserved throughout PBP cyclases. Notably, docking analyses consistently positioned Arg431 within hydrogen bonding distance of carbonyl groups on the peptide backbone, suggesting a role in preorganizing the peptide into a cyclic conformation

[00137] The substitution of arginine with alanine (Arg431 with Ala) resulted in an enzyme with a greatly reduced cyclized to hydrolyzed ratio (>14-fold change), highlighting the importance of Arg431 in cyclization. The remaining two mutants (Tyr428Ala and Ser429Ala) yielded variable outcomes. While Ser429Ala exhibited little effect on cyclization—an unexpected result considering its largely conserved nature across other PBP-TEs — Tyr428Ala led to a minor increase in dimerized peptide formation when tested with the tetrapeptide substrates. Notably, the sole peptide greatly affected by this mutation was the hexapeptide 12-SMMP, as $Ulm16^{Y428A}$ lost its ability to efficiently cyclize it.

[00138] For the PBP domain residues, we hypothesized two residues could be playing a role in substrate selectivity: Leu300, which adds increased bulk around the active site, and Asp297, which could be inhibiting the ability of Ulm16 to cyclize peptides with acidic terminal residues. These residues were mutated to glycine and asparagine, respectively, to match MppK, a close homolog of Ulm16. These mutants were then tested with the same 5 peptides mentioned previously. The Asp297Asn mutant was also tested on peptide **19,** a peptide containing a C-terminal glutamic acid. $Ulm16^{L300G}$ overall had lower turnover compared to the wild type protein. However, when tested with peptide **30**, an approximately 3-fold increase in cyclized to hydrolyzed ratio was observed. This could result from the decreased bulk in the active site allowing for the peptide to orientate itself into a more favorable conformation. For Asp297Asn, Ulm16 was still unable to perform any catalysis on the peptide containing a C-terminal acidic amino acid, suggesting a more complicated rationale for the substrate scope observed. While further experimental validation through co-crystallization is needed, these computational and mutational studies provide some rationale for the tetrapeptide substrate scope of Ulm16 and its unique ability to constrain linear peptide substrates into preorganized cyclic conformations for catalysis.

[00139] **Table 3 (Fig. 5)** shows Ulm16 Mutant total turnover assays. Enzymatic reactions were conducted in 20 mM Tris at pH 8.0 with 5% DMSO for 4 hours at 30°C using various concentrations of peptides and mutated Ulm16 as indicated by [Peptide] and [Ulm16], respectively. The areas of the enzymatic products and starting material were determined by analyzing the UPLC UV Trace at 214 nm. Total turnover numbers were calculated using a formula outlined in the methods section. In some cases, Tris from the reaction buffer was conjugated to the peptide and is indicated by 'Area Tris Peptide'. For Ulm16 R431A peptides (**14**) and (**29**) did not cyclize and the hydrolyzed peptide was used for TTN and are indicated with an asterisk.

[00140] As used herein, the following terms and phrases shall have the meanings set forth below. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art.

[00141] The term "about" can allow for a degree of variability in a value or range, for example, within 10%, within 5%, or within 1% of a stated value or of a stated limit of a range.

[00142] The term "substantially" can allow for a degree of variability in a value or range, for example, within 90%, within 95%, or within 99% of a stated value or of a stated limit of a range.

[00143] The terms "a," "an," or "the" are used to include one or more than one unless the context clearly dictates otherwise. The term "or" is used to refer to a nonexclusive "or" unless otherwise indicated. In addition, the phraseology or terminology employed herein, and not otherwise defined, is for the purpose of description only and not of limitation. Any use of section headings is intended to aid reading of the document and is not to be interpreted as limiting. Further, information that is relevant to a section heading may occur within or outside of that particular section. The terms "including" and "having" are defined as comprising (i.e., open language).

[00144] It will be appreciated by persons skilled in the art that the present disclosure is not limited by what has been particularly shown and described herein above. Rather the scope of the present disclosure includes both combinations and sub-combinations of the various features described hereinabove as well as variations and modifications which would occur to persons skilled in the art upon reading the specification and which are not in the prior art.

[00145] All patents, patent application publications, journal articles, textbooks, and other publications mentioned in the specification are indicative of the level of skill of those in the art to which the disclosure pertains. All such publications are incorporated herein by reference to the same extent as if each individual publication were specifically and individually indicated to be incorporated by reference.

WE CLAIM:

1. A method of biocatalytic cyclization for the preparation of a cyclic peptide or its salt, which method comprises reacting a substrate comprising a linear peptide or its salt with Ulm16, a penicillin-binding protein (PBP)-type class of thioesterase (PBP-TE).

2. The method of claim 1, which method comprises:

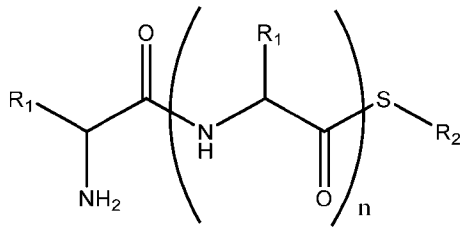
(a) reacting a substrate comprising a linear peptide or its salt with Ulm16, a penicillin-binding protein (PBP)-type class of thioesterase (PBP-TE) to obtain a reaction mixture;

(b) incubating the reaction mixture of step a);

(c) quenching and centrifuging the reaction mixture; and

(d) isolating the cyclic peptide.

3. The method of claim 1 or 2, wherein the substrate comprising a linear peptide is represented by the formula (I):



(I)

wherein n is 3-10;

each R_1 is independently a natural or unnatural amino acid side chain; and

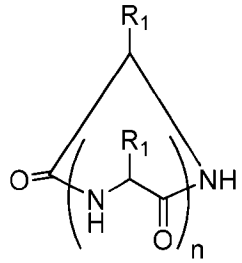
R_2 is C_1-C_6 alkyl C_1-C_6 alkanoate or N-C_1-C_6 alkanoyl amino C_1-C_6 alkyl.

4. The method of claim 3, wherein R_2 is C_1-C_6 alkyl acetate or N-acetyl amino C_1-C_6 alkyl.

5. The method of claim 3 or 4, wherein S-R_2 is N-acetylcysteamine (-SNAC), methyl 3-mercaptopropionate (-SMMP), or butyl 3-mercaptopropionate (-SBMP).

6. The method of claim 5, wherein S-R_2 group is butyl 3-mercaptopropionate (-SBMP).

7. The method of claim 1 or 2, wherein the cyclic peptide is represented by the formula (II):



(II)

wherein n is 3-10;

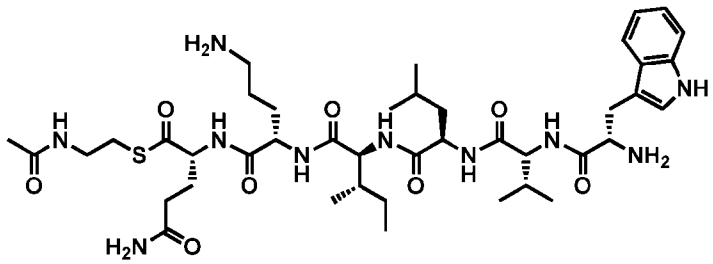
each R_1 is independently a natural or unnatural amino acid side chain.

8. The method of claim 3 or 7, wherein n is 4-6.

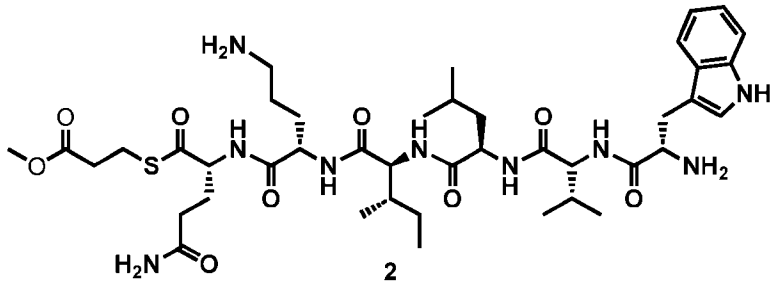
9. The method of claim 8, wherein the cyclic peptides are tetrapeptides, pentapeptides, or hexapeptides, or a mixture of two or more of the foregoing.

10. The method of claim 8 or 9, wherein the cyclic peptides are tetrapeptides.

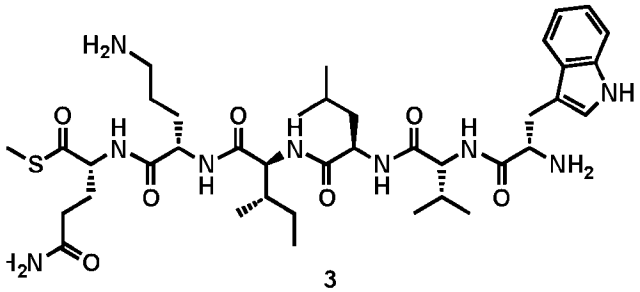
11. The method of claim 3, wherein the linear peptide of formula (I) is selected from:



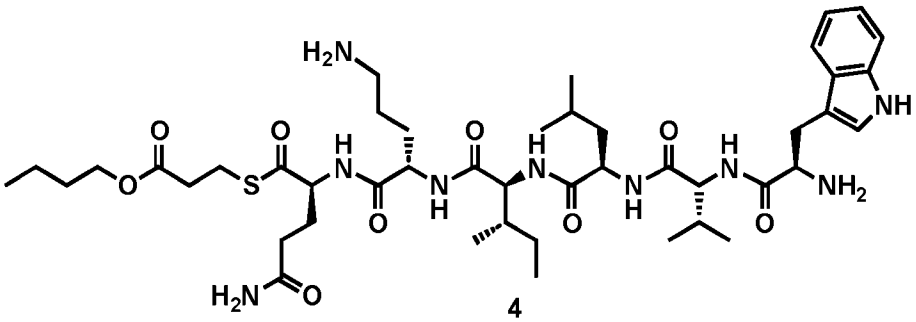
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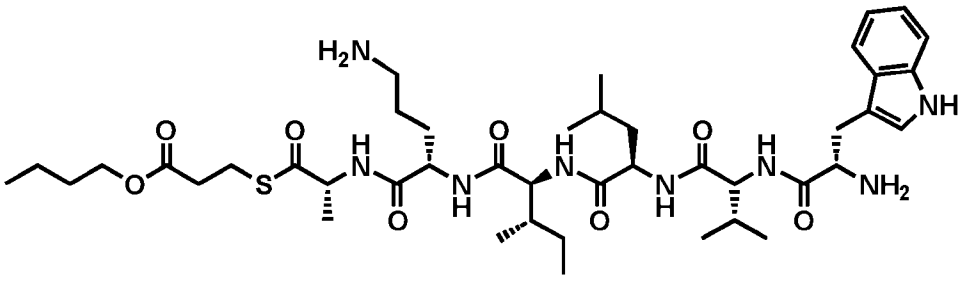
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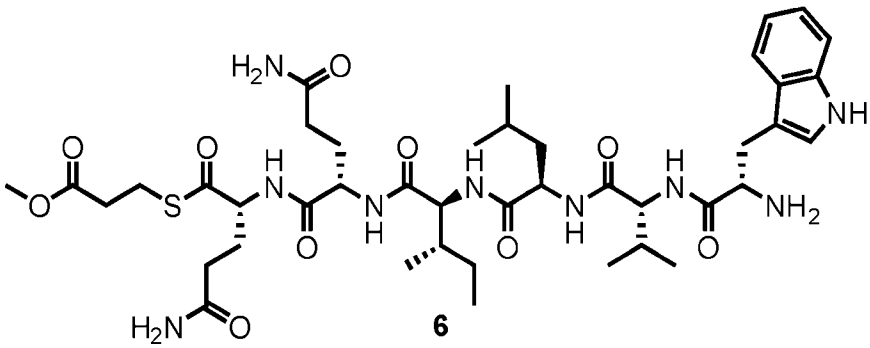
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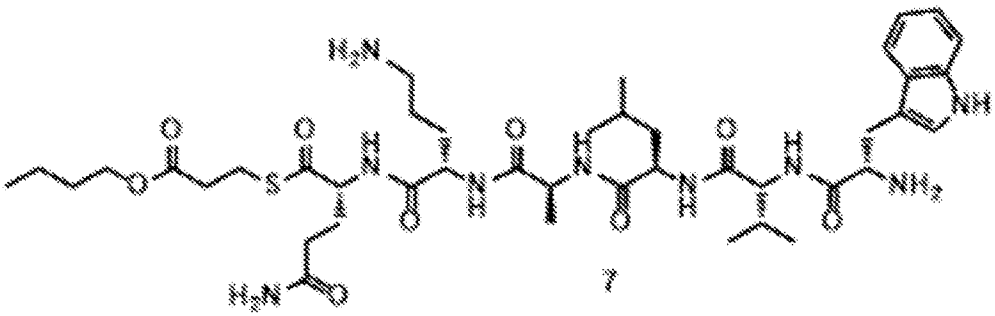
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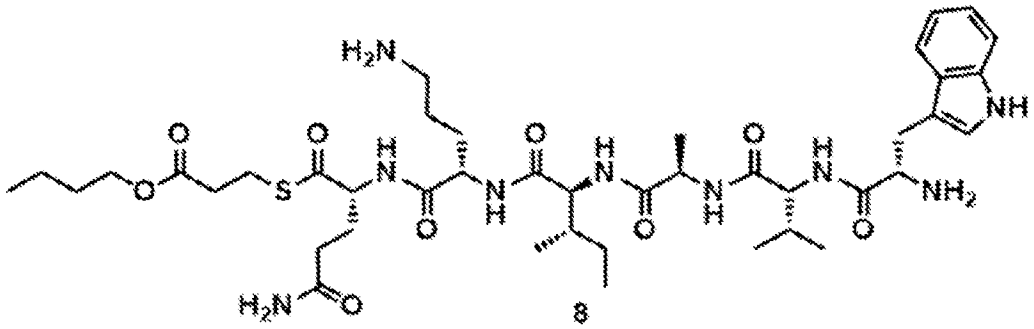
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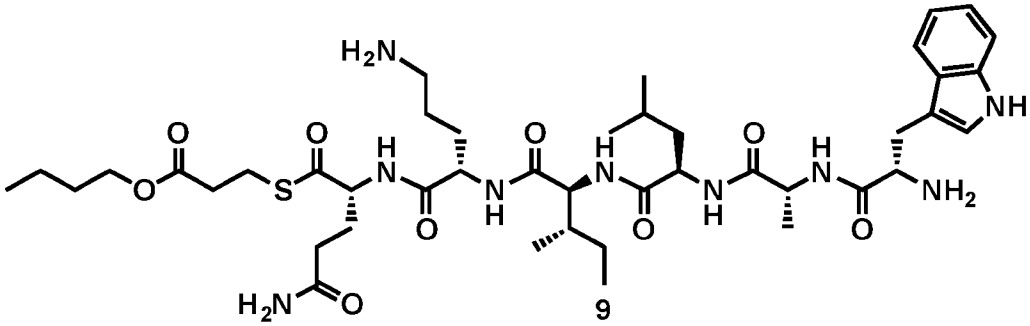
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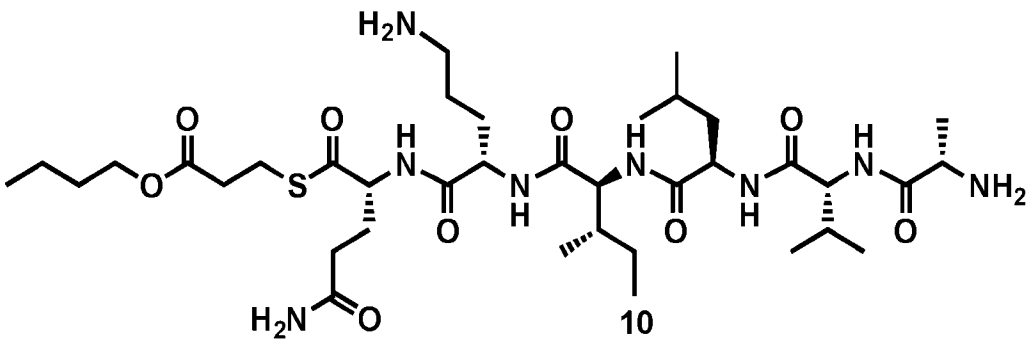
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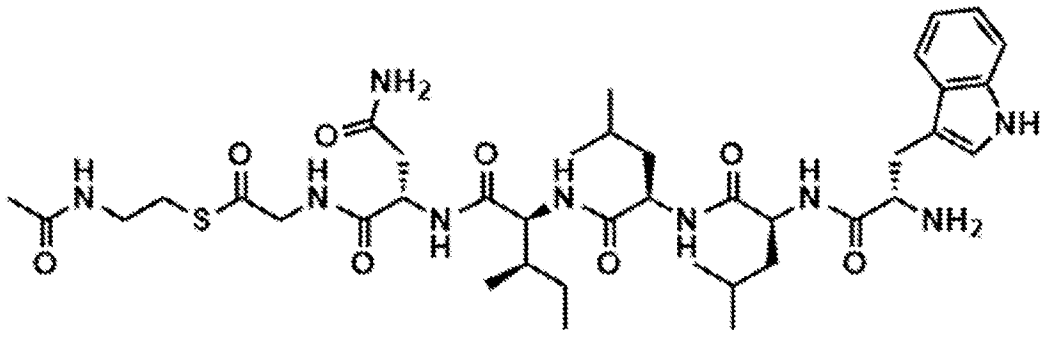
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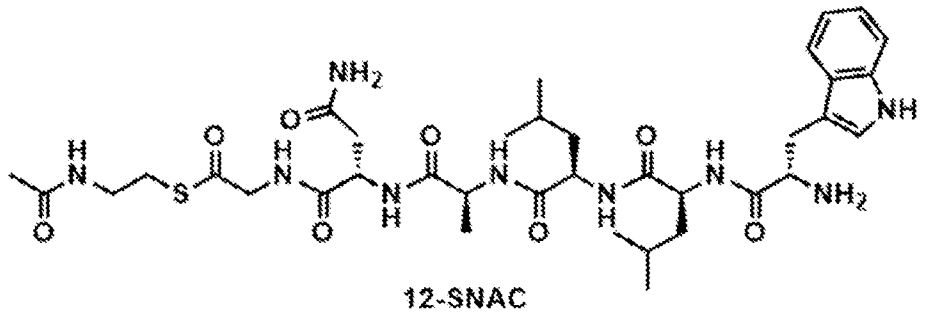
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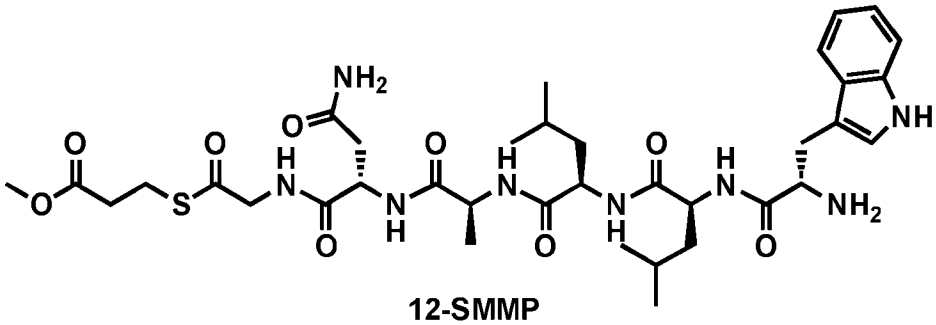
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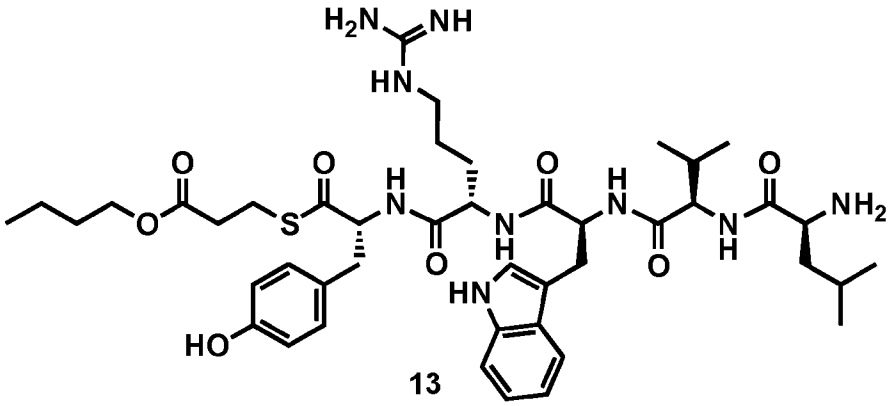
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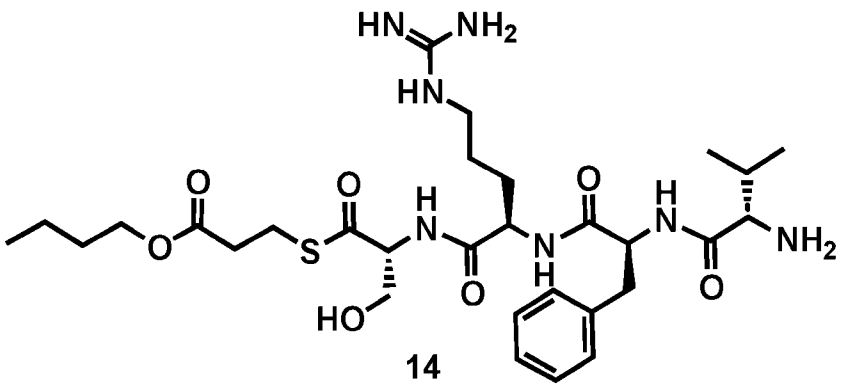
12-SNAC



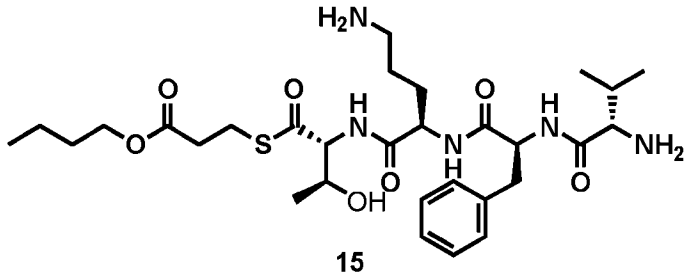
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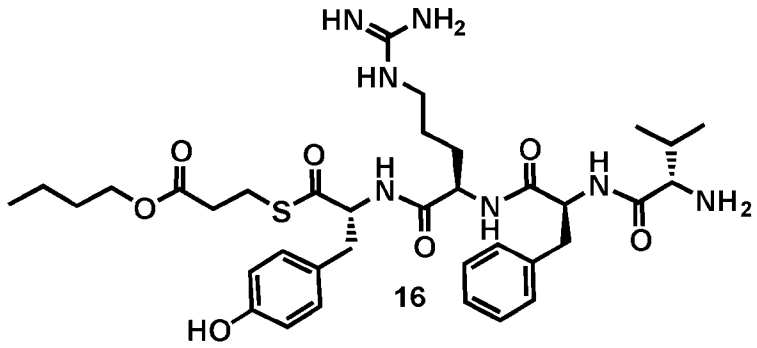
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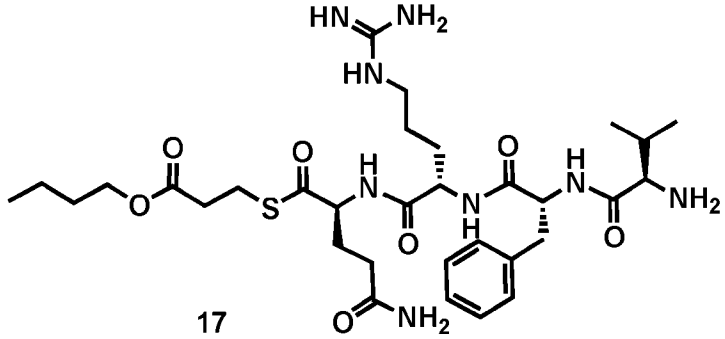
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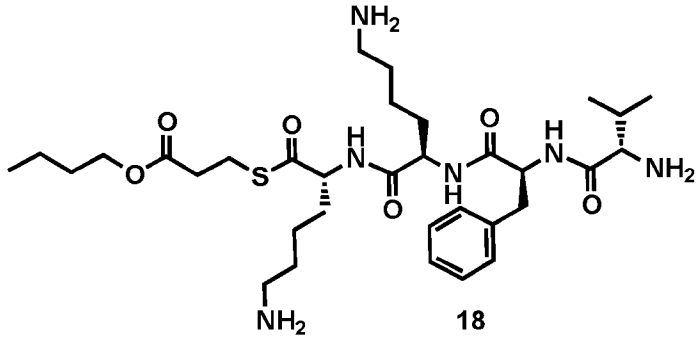
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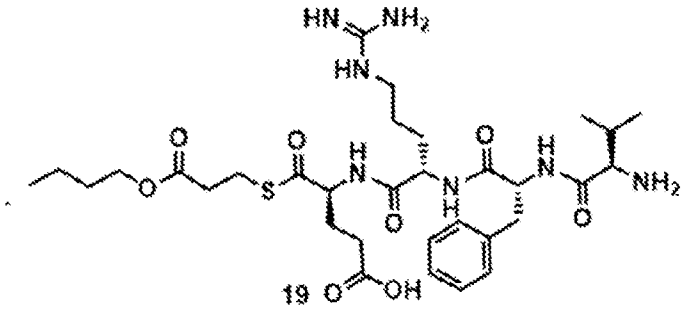
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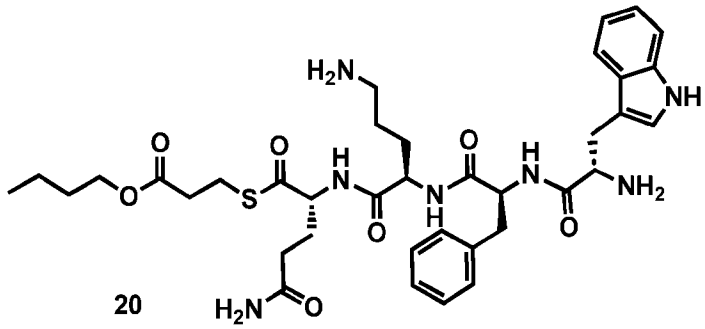
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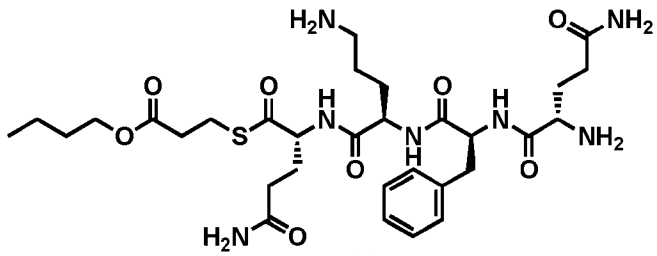
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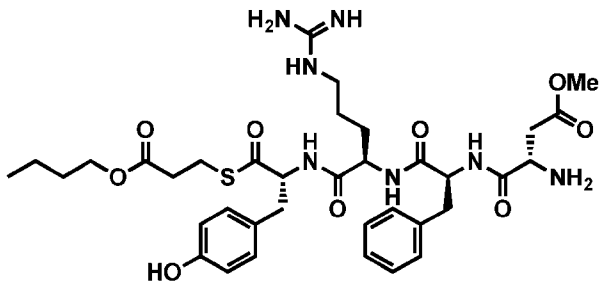
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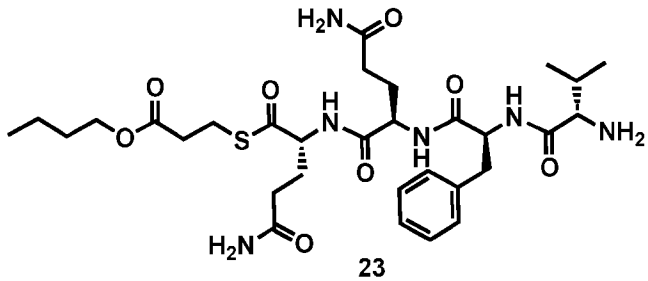
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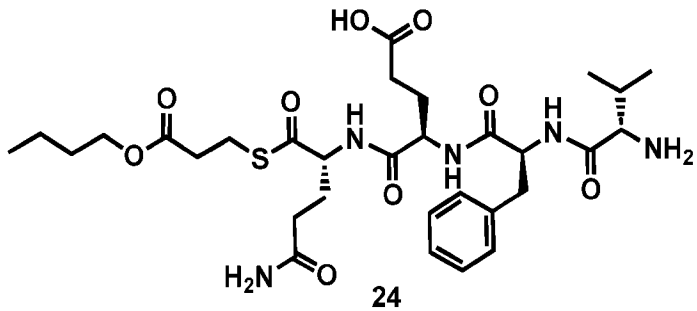
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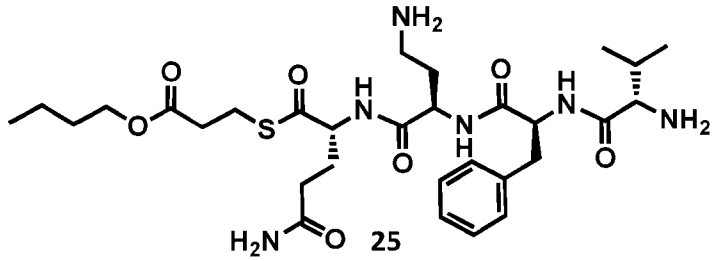
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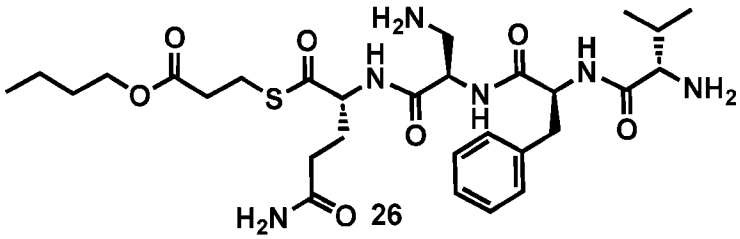
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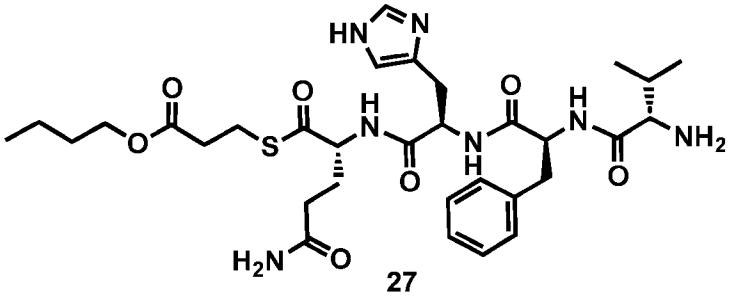
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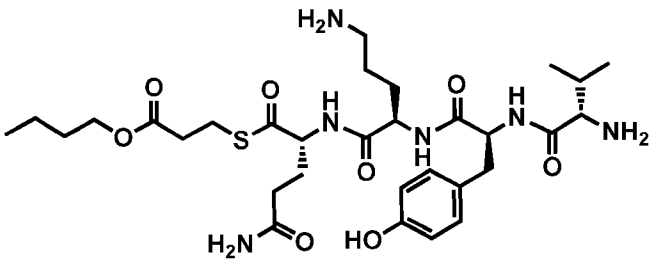
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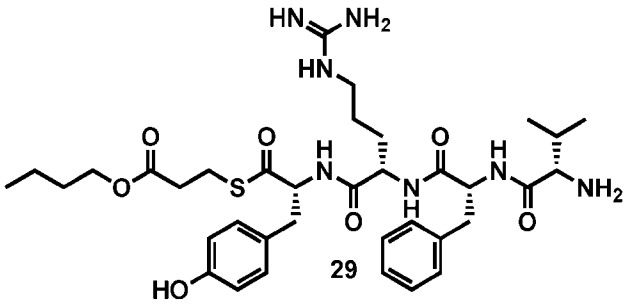
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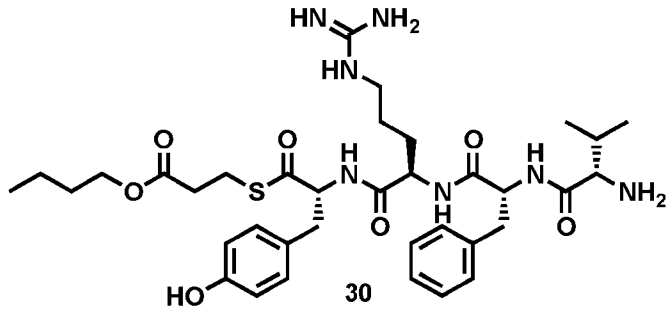
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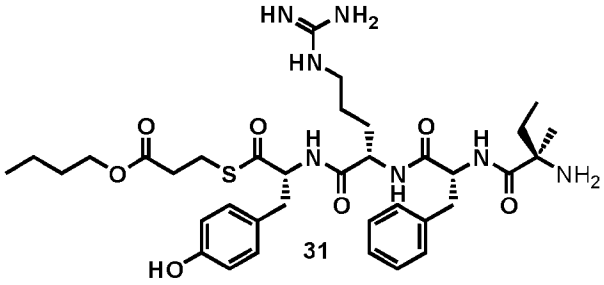
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29



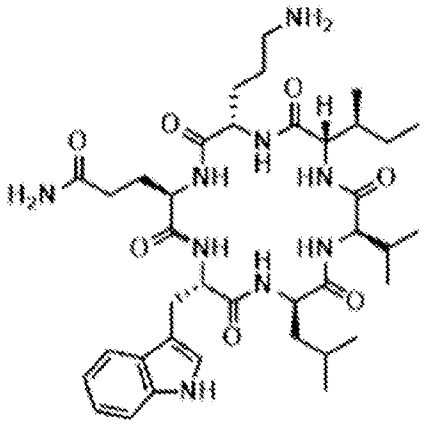
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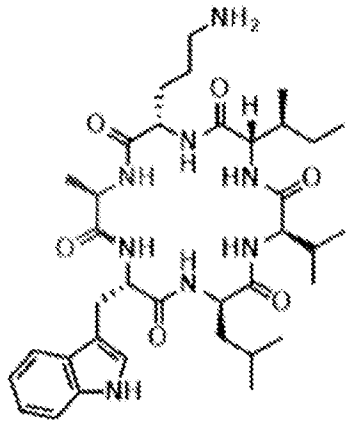
31

or salt thereof.

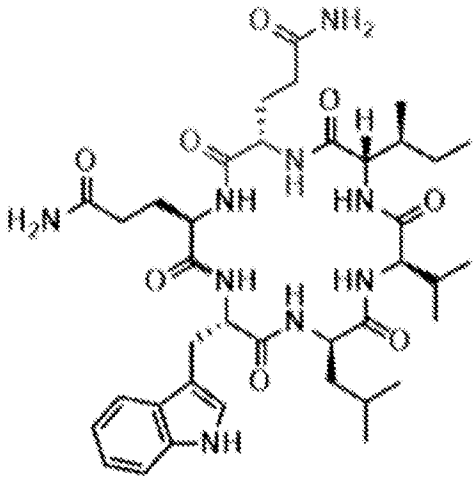
12. The method of claim 7, wherein the cyclic peptide of formula (II) is one or more of:



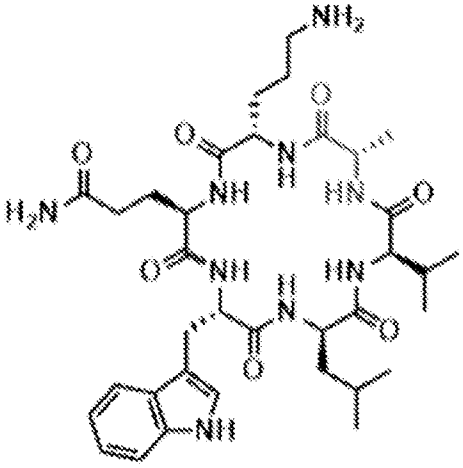
Cyc1



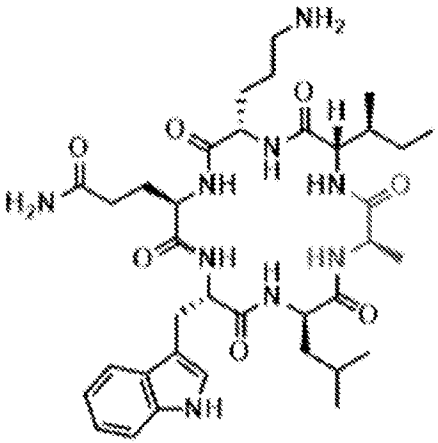
Cyc5



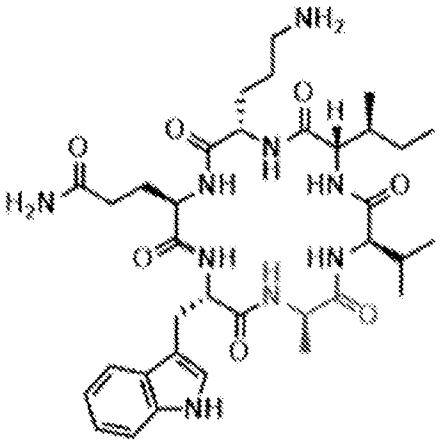
Cyc6



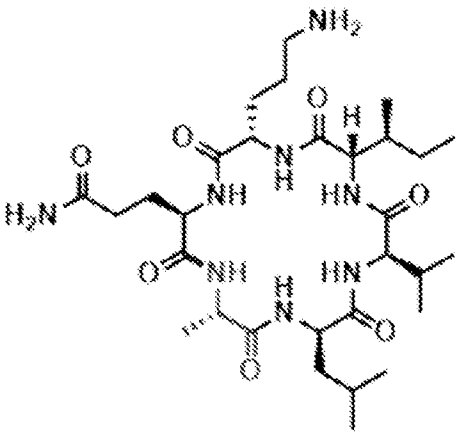
Cyc7



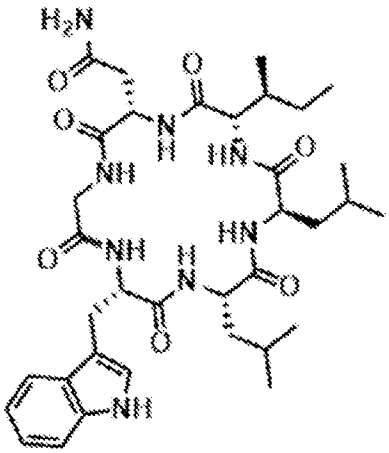
Cyc8



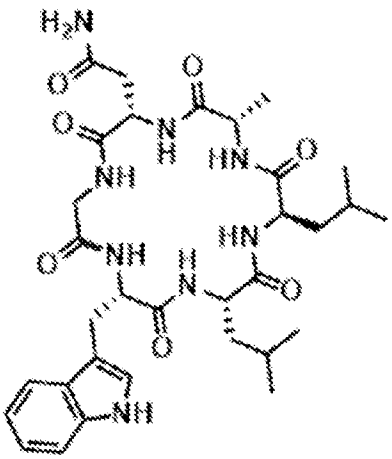
Cyc9



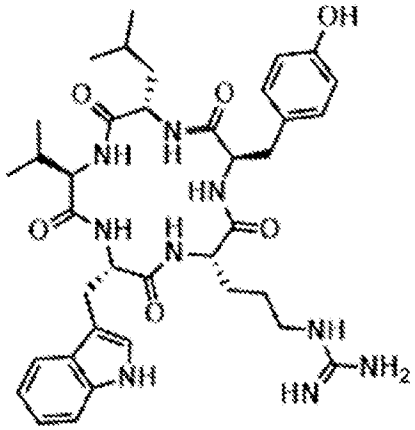
Cyc10



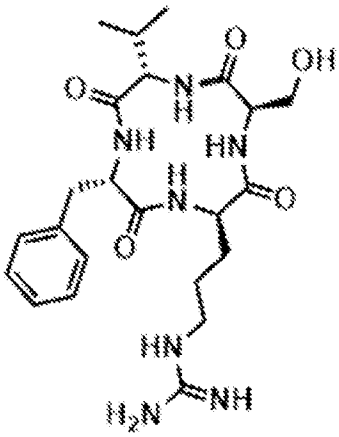
Cyc11



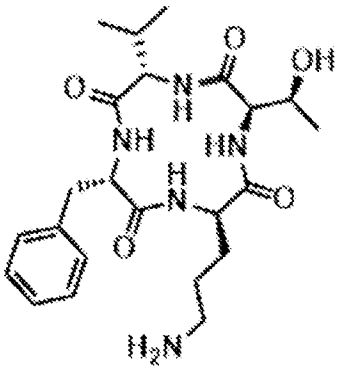
Cyc12



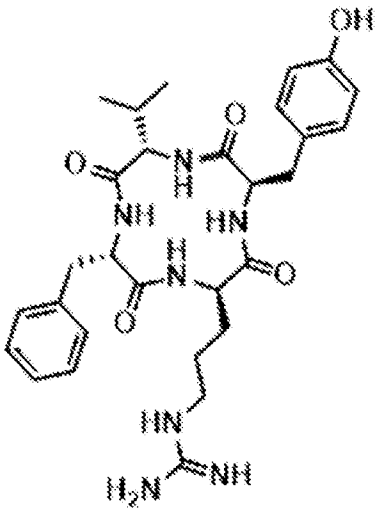
Cyc13



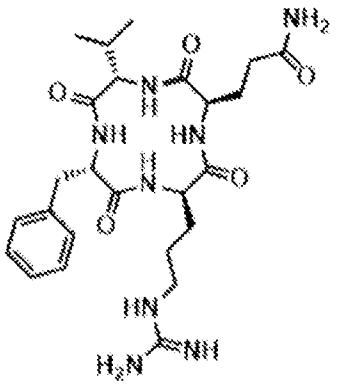
Cyc14



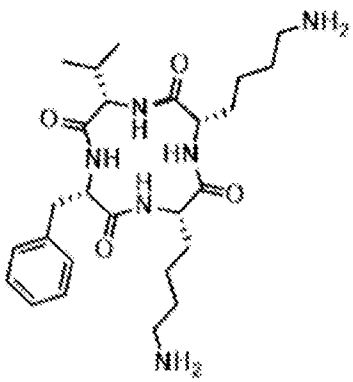
Cyc15



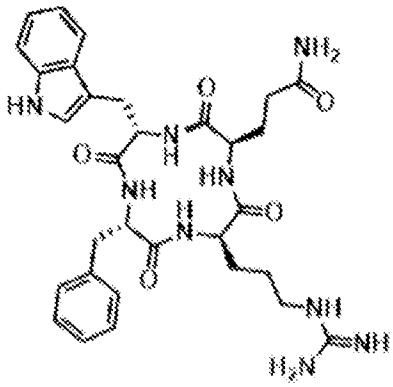
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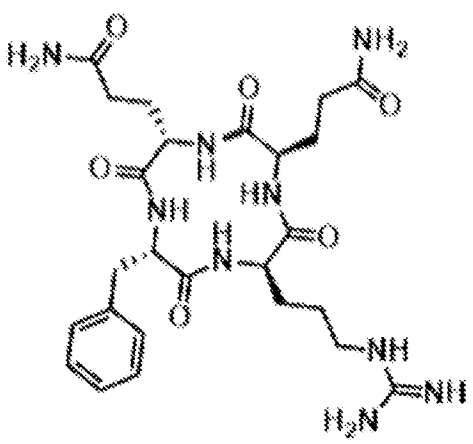
Cyc17



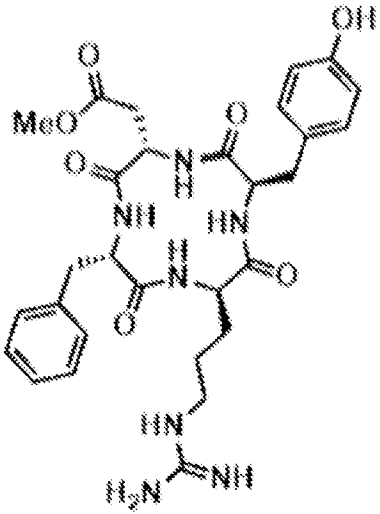
Cyc18



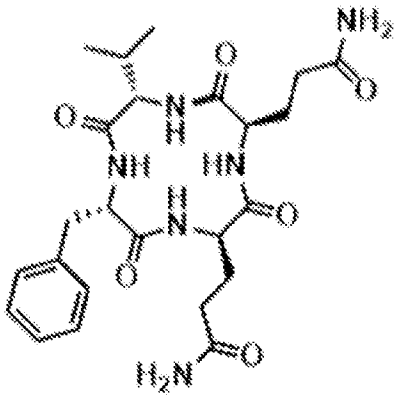
Cyc20



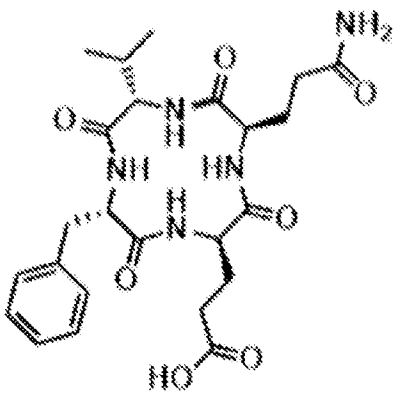
Cyc21



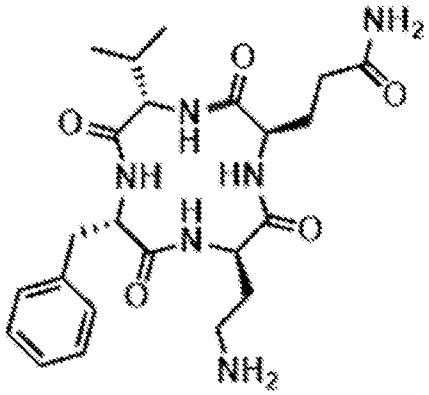
Cyc22



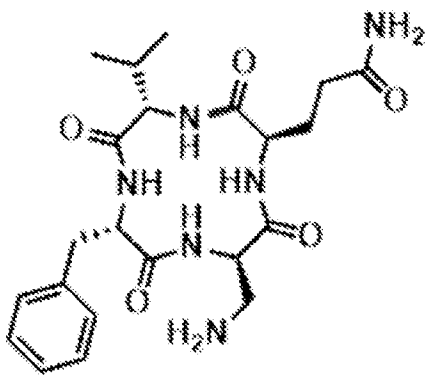
Cyc23



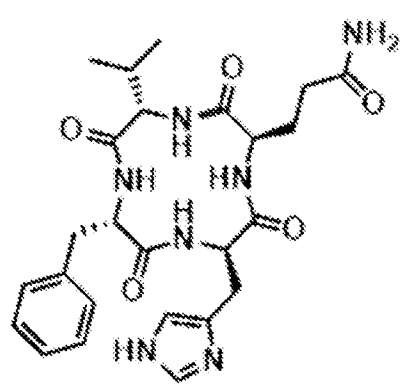
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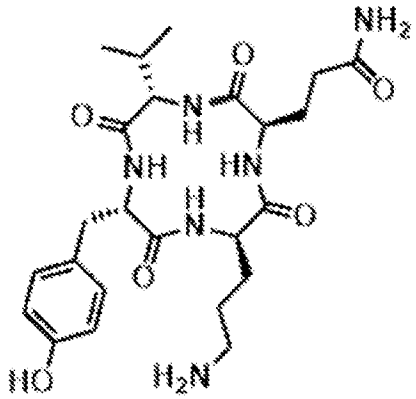
Cyc25



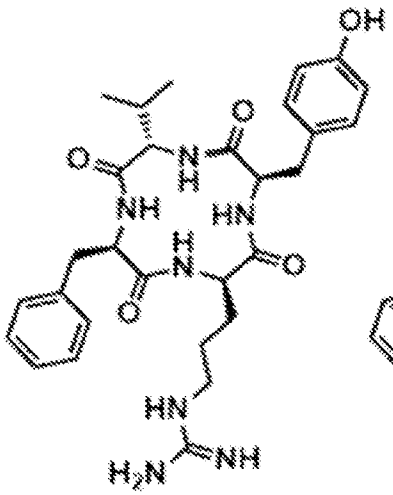
Cyc26



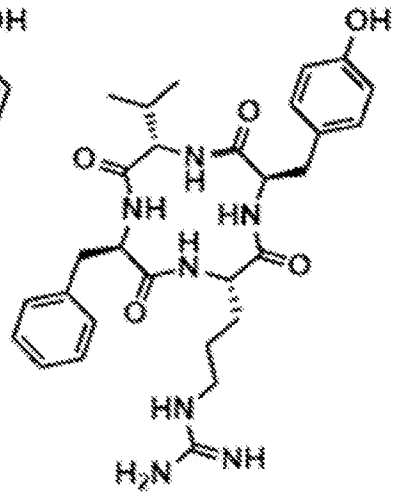
Cyc27



Cyc28



Cyc29



Cyc30

or a salt of any of the foregoing.

13. The method of claim 2, wherein step (a) is carried out in the presence of an aqueous solvent comprising one or more buffers and optionally further comprising an organic solvent.

14. The method of claim 13, wherein the buffer is Tris buffer.

15. The method of claim 13 or 14, wherein the organic solvent is dimethyl sulfoxide (DMSO), acetonitrile, methanol, or dimethyl formamide (DMF).

16. The method of claim 1 or 2, wherein the concentration of Ulm 16 is about 1 nM to about 250 nM for about 50 μM to about 500 μM of the substrate.

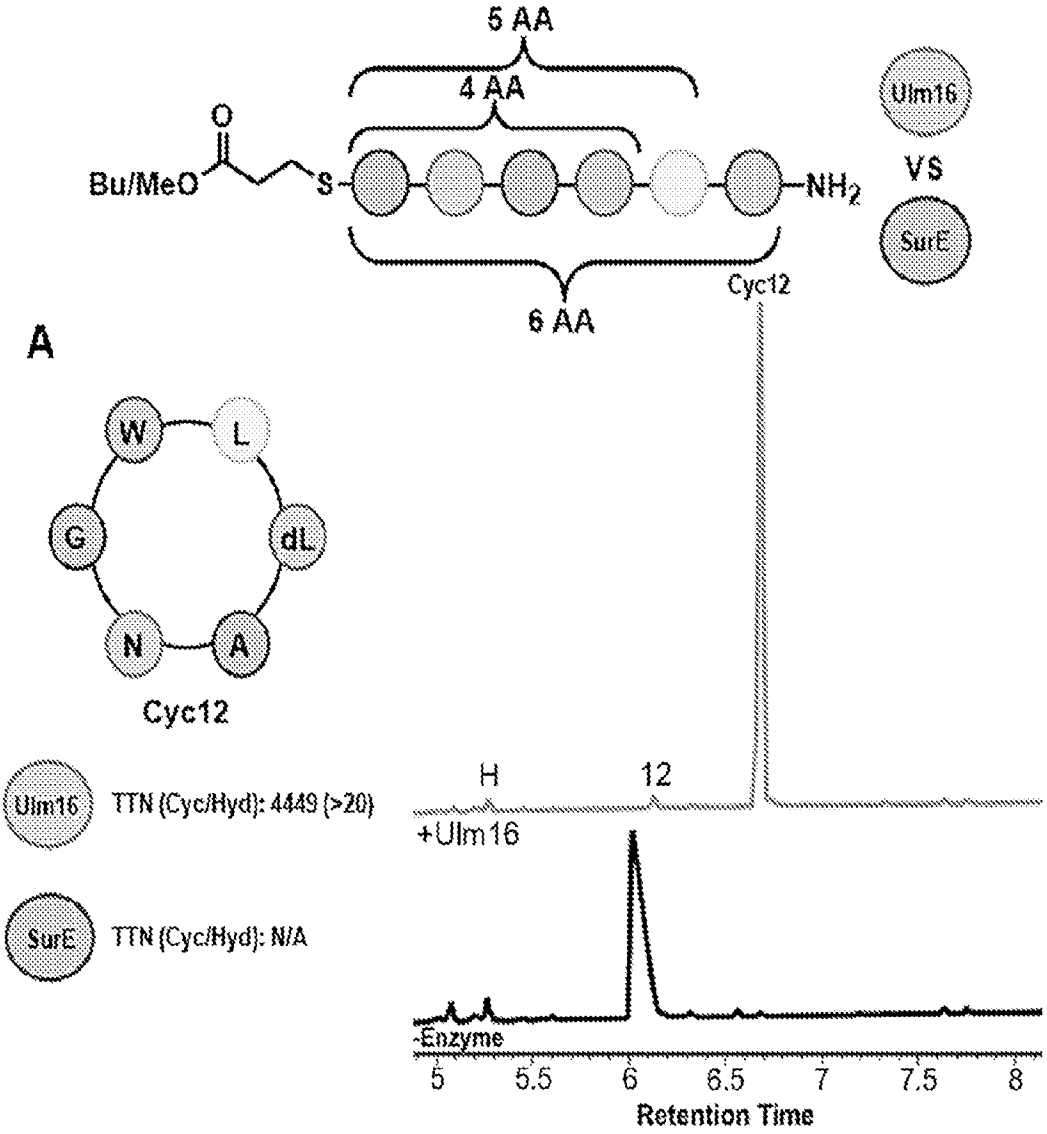

5 AA
4 AA
Bu/MeO
S
NH2
6 AA
Cyc12
Ulm16
VS
SurE
A
W
L
G
dL
N
A
Cyc12
Ulm16 TTN (Cyc/Hyd): 4449 (>20)
SurE TTN (Cyc/Hyd): N/A
H
12
+Ulm16
-Enzyme
5
5.5
6
6.5
7
7.5
8
Retention Time

Fig. 1A

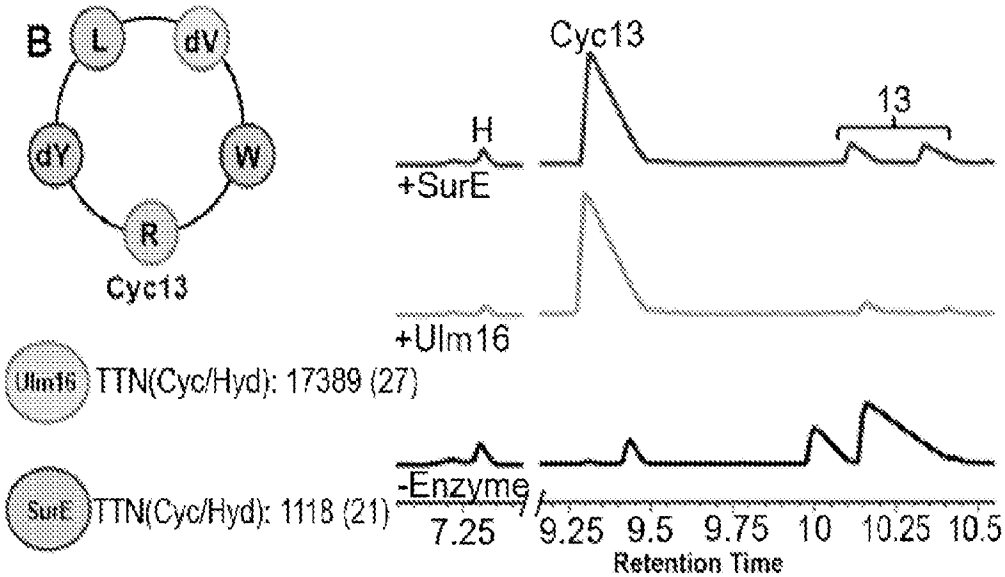

B
L
dV
dY
W
R
Cyc13
Cyc13
H
13
+SurE
+Ulm16
Ulm16 TTN(Cyc/Hyd): 17389 (27)
SurE TTN(Cyc/Hyd): 1118 (21)
-Enzyme
7.25
9.25
9.5
9.75
10
10.25
10.5
Retention Time

Fig. 1B

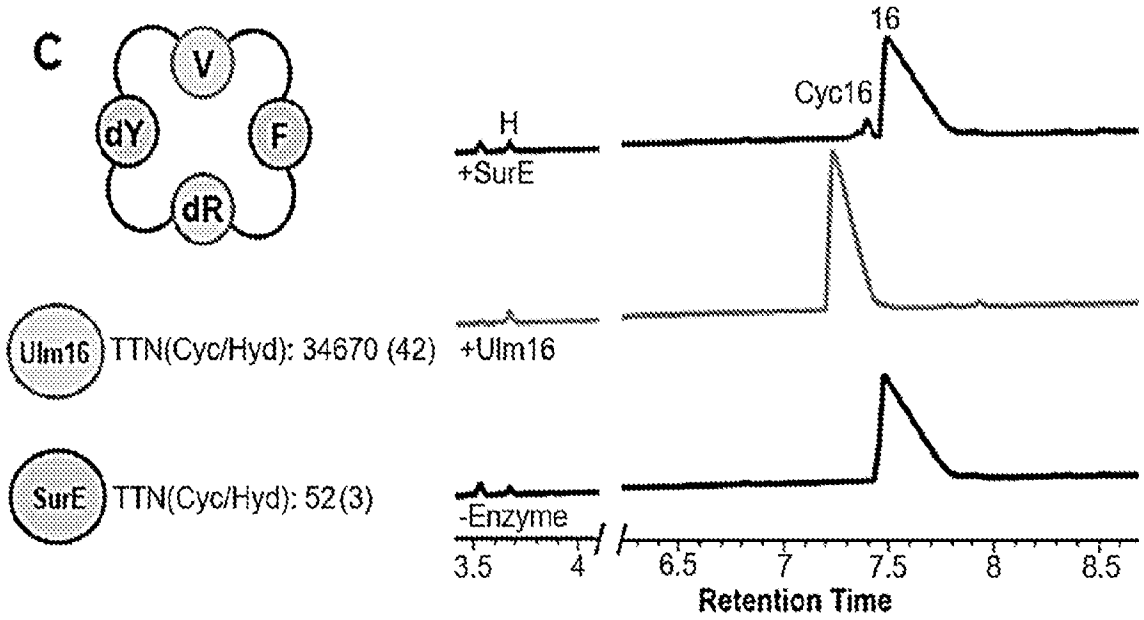

C
V
dY
F
dR
Ulm16
TTN(Cyc/Hyd): 34670 (42)
SurE
TTN(Cyc/Hyd): 52(3)
H
+SurE
16
Cyc16
+Ulm16
-Enzyme
3.5
4
6.5
7
7.5
8
8.5
Retention Time

Fig. 1C

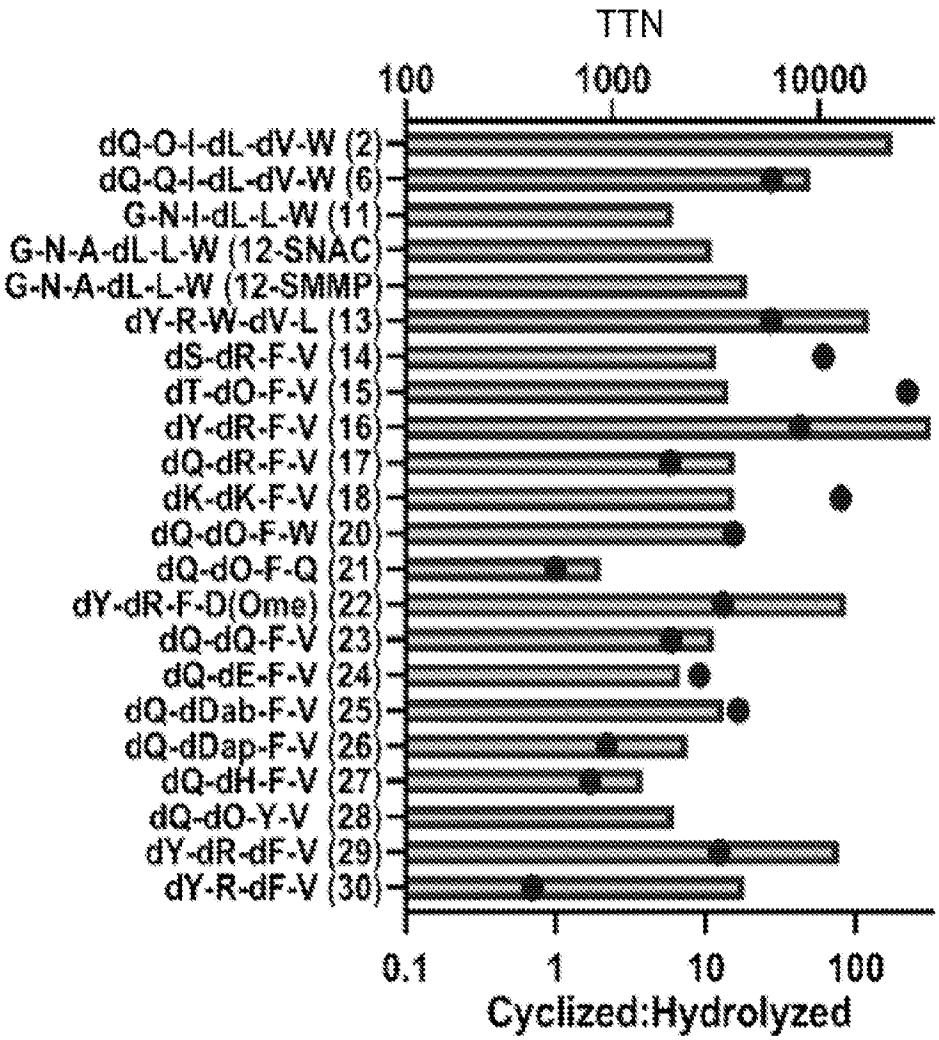

TTN
100
1000
10000
dQ-O-I-dL-dV-W (2)
dQ-Q-I-dL-dV-W (6)
G-N-I-dL-L-W (11)
G-N-A-dL-L-W (12-SNAC)
G-N-A-dL-L-W (12-SMMP)
dY-R-W-dV-L (13)
dS-dR-F-V (14)
dT-dO-F-V (15)
dY-dR-F-V (16)
dQ-dR-F-V (17)
dK-dK-F-V (18)
dQ-dO-F-W (20)
dQ-dO-F-Q (21)
dY-dR-F-D(Ome) (22)
dQ-dQ-F-V (23)
dQ-dE-F-V (24)
dQ-dDab-F-V (25)
dQ-dDap-F-V (26)
dQ-dH-F-V (27)
dQ-dO-Y-V (28)
dY-dR-dF-V (29)
dY-R-dF-V (30)
0.1
1
10
100
Cyclized:Hydrolyzed

Fig. 1D

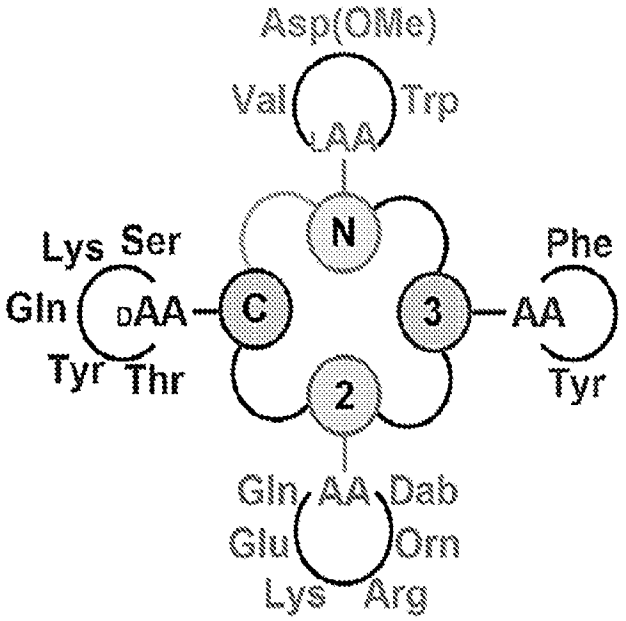

Asp(OMe)
Val
Trp
LAA
N
Lys
Ser
Gln
DAA
C
3
AA
Phe
Tyr
Thr
2
Tyr
Gln
AA
Dab
Glu
Orn
Lys
Arg

Fig. 1E

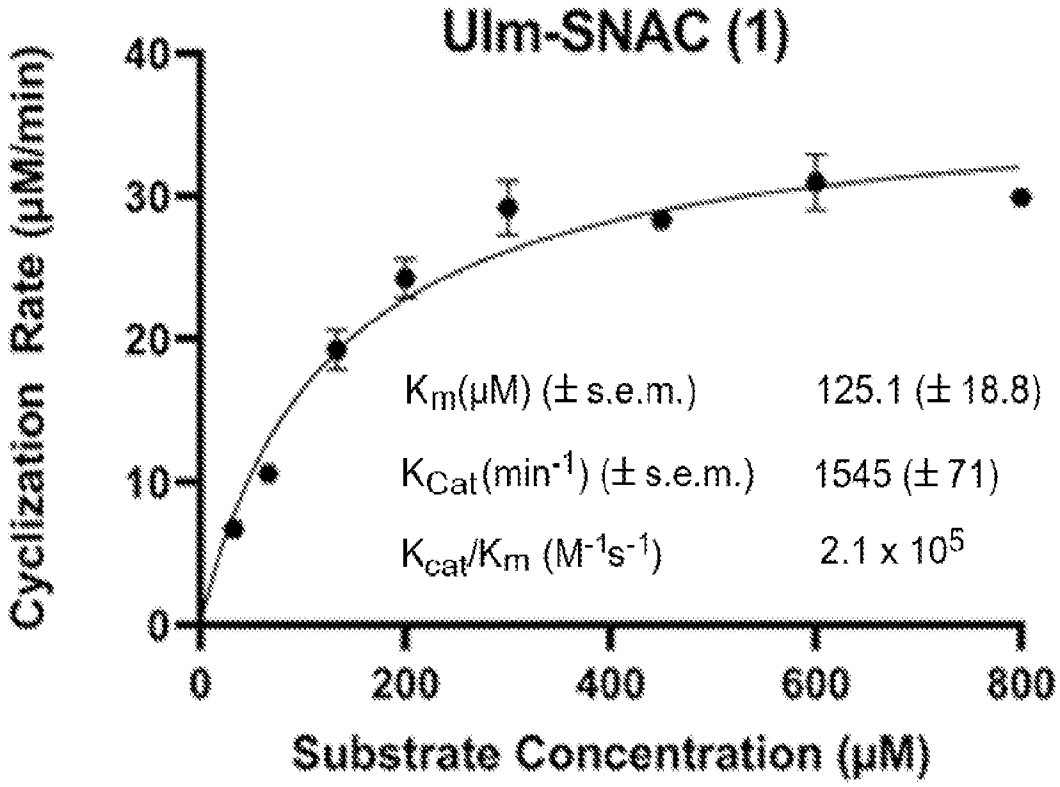

UIm-SNAC (1)
Cyclization Rate (μM/min)
40
30
20
10
0
0
200
400
600
800
Substrate Concentration (μM)
Km(μM) (± s.e.m.) 125.1 (± 18.8)
KCat(min-1) (± s.e.m.) 1545 (± 71)
Kcat/Km (M-1s-1) 2.1 x 10^5

Fig. 2

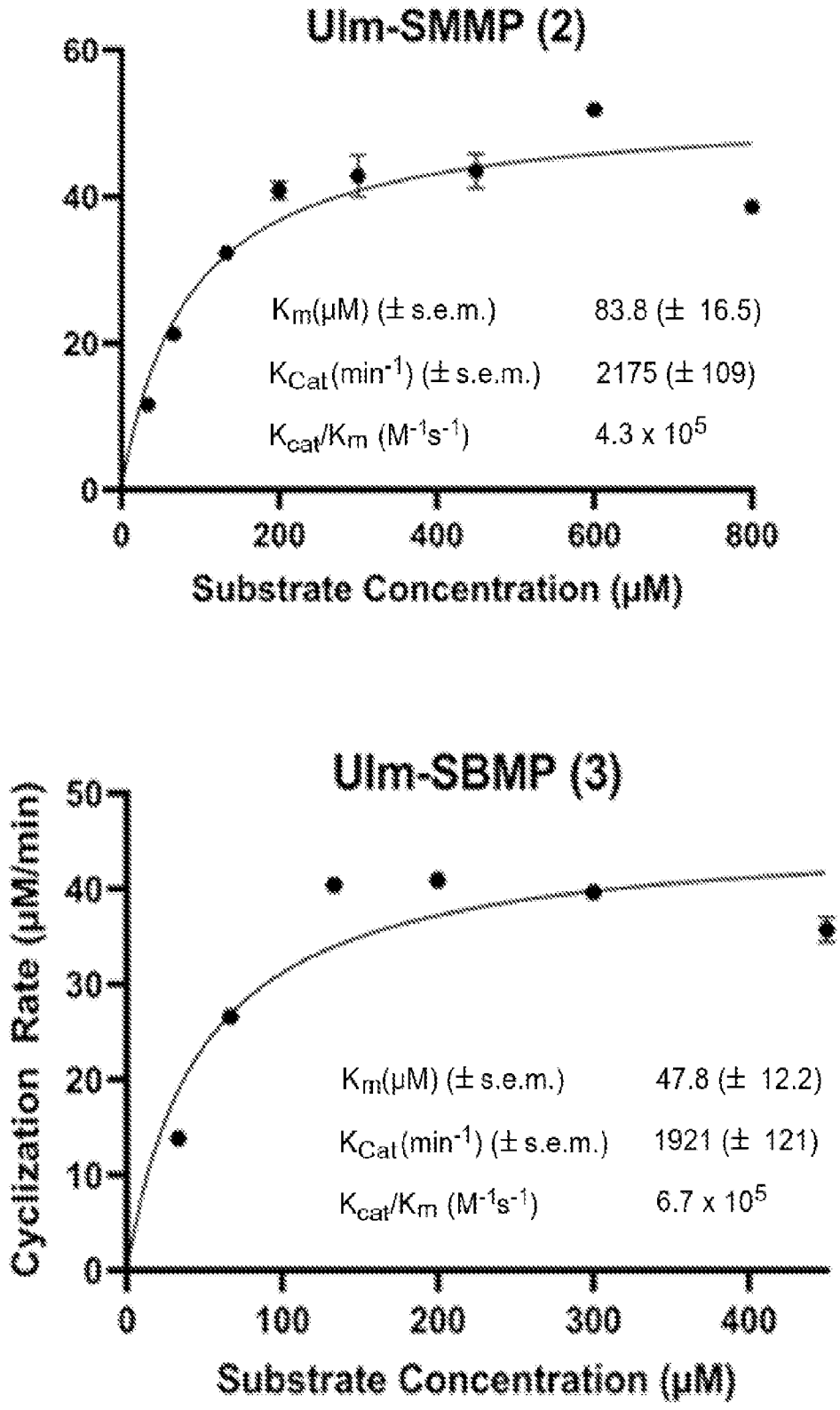

UIm-SMMP (2)
60
40
20
0
0
200
400
600
800
Substrate Concentration (μM)
Km(μM) (± s.e.m.) 83.8 (± 16.5)
KCat(min-1) (± s.e.m.) 2175 (± 109)
Kcat/Km (M-1s-1) 4.3 x 10^5
UIm-SBMP (3)
Cyclization Rate (μM/min)
50
40
30
20
10
0
0
100
200
300
400
Substrate Concentration (μM)
Km(μM) (± s.e.m.) 47.8 (± 12.2)
KCat(min-1) (± s.e.m.) 1921 (± 121)
Kcat/Km (M-1s-1) 6.7 x 10^5

Fig. 2 cont.

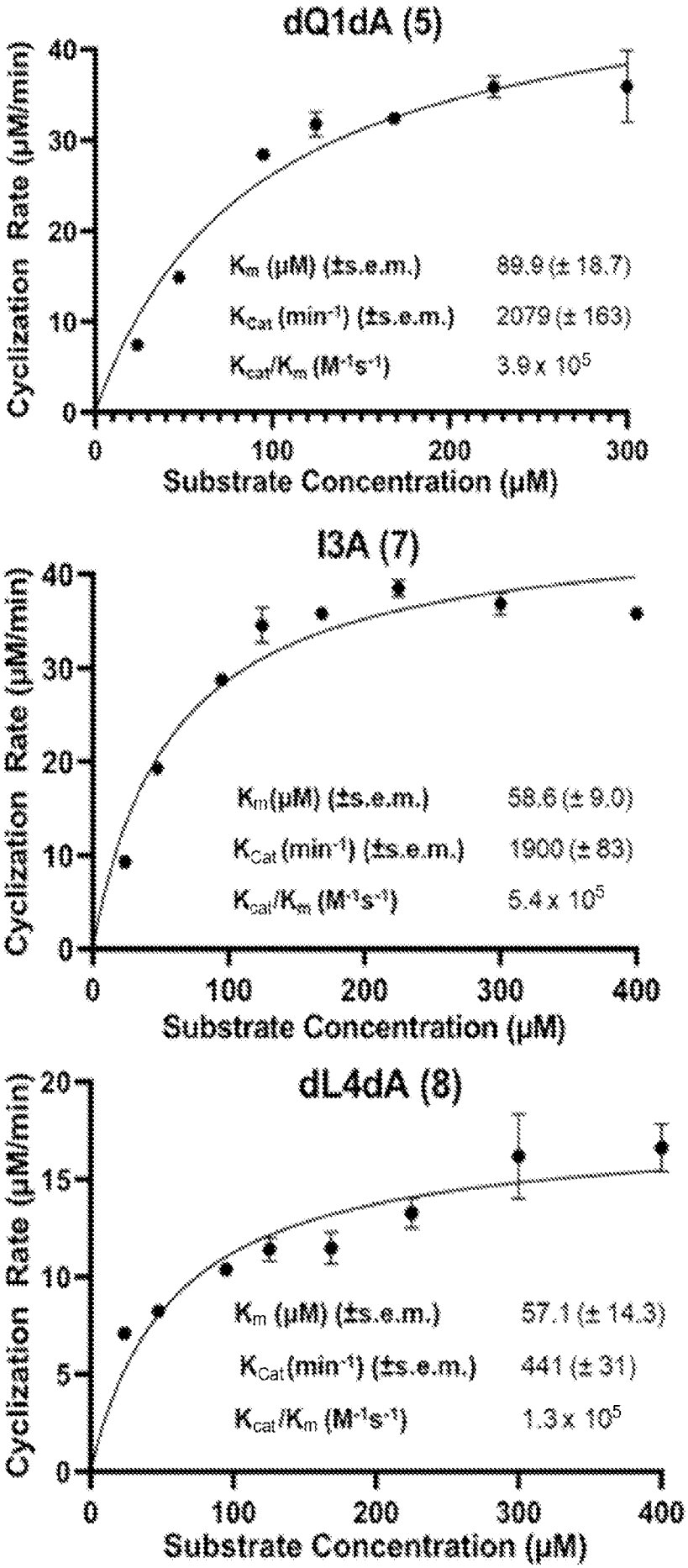

dQ1dA (5)
Cyclization Rate (μM/min)
Substrate Concentration (μM)
Km (μM) (±s.e.m.) 89.9 (± 18.7)
Kcat (min-1) (±s.e.m.) 2079 (± 163)
Kcat/Km (M-1s-1) 3.9 x 10^5
I3A (7)
Cyclization Rate (μM/min)
Substrate Concentration (μM)
Km(μM) (±s.e.m.) 58.6 (± 9.0)
KCat (min-1) (±s.e.m.) 1900 (± 83)
Kcat/Km (M-1s-1) 5.4 x 10^5
dL4dA (8)
Cyclization Rate (μM/min)
Substrate Concentration (μM)
Km (μM) (±s.e.m.) 57.1 (± 14.3)
KCat (min-1) (±s.e.m.) 441 (± 31)
Kcat/Km (M-1s-1) 1.3 x 10^5

Fig. 3

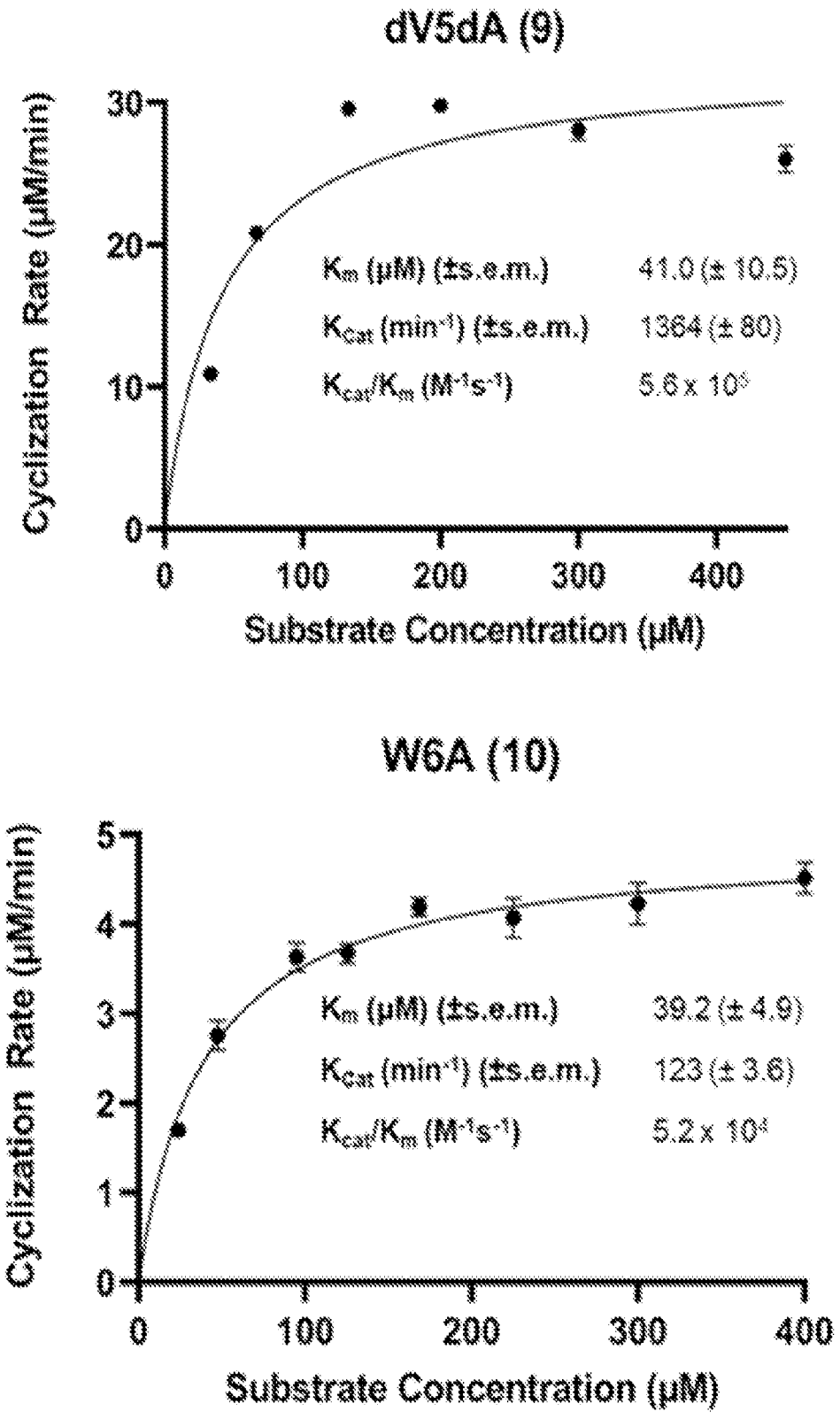

dV5dA (9)
Cyclization Rate (μM/min)
30
20
10
0
0
100
200
300
400
Substrate Concentration (μM)
K_m (μM) (±s.e.m.) 41.0 (± 10.5)
K_{cat} (min^{-1}) (±s.e.m.) 1364 (± 80)
K_{cat}/K_m (M^{-1}s^{-1}) 5.6 x 10^5
W6A (10)
Cyclization Rate (μM/min)
5
4
3
2
1
0
0
100
200
300
400
Substrate Concentration (μM)
K_m (μM) (±s.e.m.) 39.2 (± 4.9)
K_{cat} (min^{-1}) (±s.e.m.) 123 (± 3.6)
K_{cat}/K_m (M^{-1}s^{-1}) 5.2 x 10^4

Fig. 3 cont.

Table 1

Substrate	[Ulm16]	[Peptide]	Area Cyclic Peptide	Area Peptide Thioester	Area Hydrolyzed Peptide	Area Hydrolyzed Dimer	Area Cyclic Dimer	Sum	TTN	Cyc/SM	Cyc/H
2	14 nM	320 µM	2684660	37204	*			2721764	22545	72	>20:1
6	17 nM	320 µM	855364	891865	30967			1778196	9055	1.0	27.6
11	70 nM	280 µM	903688	927094	*			1830782	1974	1.0	>20:1
12-SNAC	70 nM	280 µM	1614745	512012	*			2126758	3037	3.2	>20:1
12-SMMP	70 nM	320 µM	2242929	61585	*			2304514	4449	36	>20:1
13	17 nM	320 µM	2133202	97416	78504			2309122	17389	21.9	27.2
14	70 nM	280 µM	584777	138590	9594			732961	3191	4.2	61
15	70 nM	280 µM	620130	56328	2801			679259	3652	11	221.4
16	11.5 nM	280 µM	1922450	320576	45581	15733	9476	2313817	34679	6.0	42.2
17	45 nM	280 µM	426879	176018	73213			676110	3929	2.4	5.8
18	70 nM	280 µM	439897	7240	5536			452673	3887	60.8	79.5
19	70 nM	280 µM									
20	70 nM	280 µM	1214431	14538	79313			1308282	3713	83.5	15.3
21	70 nM	280 µM	54373	138754	53697			246824	881	0.4	1.0
22	17 nM	320 µM	697840	227450	53826			979116	13416	3.1	13.0
23	70 nM	280 µM	175012	21341	29731			226084	3096	8.2	5.9
24	70 nM	280 µM	357393	277874	39241			674508	2119	1.3	9.1
25	70 nM	280 µM	346723	32301	21140			400764	3461	10.5	16.4
26	70 nM	280 µM	243137	67031	112373			422541	2302	3.6	2.2
27	70 nM	280 µM	142639	404619	86311			633569	1401	0.4	1.7
28	45 nM	280 µM	138785	296882	N.D			435668	1982	0.5	-
29	17 nM	320 µM	1078614	444170	86863	28754	0	1638401	12392	2.4	12.4
30	17 nM	320 µM	178195	270391	262105	57793	7509	775992	4323	0.7	0.7
31	17 nM	320 µM									

Fig. 4

Table 3

Substrate	[Ubi16]	Mutant	[Peptide]	Area Cyclic Peptide	Area Peptide Thioester	Area Hydrolyzed Peptide	Hydrolyzed Dimer	Area Tris Peptide	Sum	TTN	Cyc/SM	Cyc/H
12-MMP	70 nM	R431A	280 µM	76920	1297157	55670	6952	384405	1821105	169	0.1	1.4
12-MMP	70 nM	S429A	280 µM	2243756	463979	330492	-	-	3038226	2757	5	7
12-MMP	70 nM	Y428A	280 µM	337847	2114642	476929	-	-	2929418	431	0.2	0.7
12-MMP	500 nM	1-344	280 µM	34981	1914589	398195	118632	-	2466397	8	0.0	0.1
12-MMP	70 nM	L300G	280 µM	352754	1333933	73655	9596	-	1769938	797	0.3	4.8
12-MMP	70 nM	D297N	280 µM	639759	889575	25572	15839	-	1570745	1629	0.7	25.0
14	70 nM	R431A	280 µM	0	359874	40992	-	-	391866	391*	8.6*	-
14	70 nM	S429A	280 µM	421988	68621	15890	-	-	506499	3110	6	26.6
14	70 nM	Y428A	280 µM	263996	283678	14688	-	-	566362	1753	1	18.1
14	500 nM	1-344	280 µM				-	-				
14	70 nM	L300G	280 µM	92709	320930	14295	-	-	427935	800	0.3	6.5
14	70 nM	D297N	280 µM	46591	325366	13582	-	-	385539	451	0.1	3.4
16	32.5 nM	R431A	280 µM	206670	21343	451015	69447	-	748474	2379	9.7	0.3
16	32.5 nM	S429A	280 µM	1676219	16367	21117	-	-	1713703	8427	102	79
16	32.5 nM	Y428A	280 µM	1077523	510451	37788	26261	-	1652023	5619	2.1	28.5
16	500 nM	1-344	280 µM	32923	1428907	20947	-	-	1482676	12	0	1.6
16	32.5 nM	L300G	280 µM	676744	1097879	17546	-	-	1792168	3253	0.6	38.6
16	32.5 nM	D297N	280 µM	936327	819680	21794	-	-	1777802	4538	1.1	43
29	32.5 nM	R431A	280 µM	-	12064	506230	279574	-	797868	8485*	23.2*	-
29	32.5 nM	S429A	280 µM	1316148	7336	95074	44703	-	1463262	7749	179	14
29	32.5 nM	Y428A	280 µM	1139426	15322	99508	78596	-	1332852	7365	74	11.5
29	500 nM	1-344	280 µM	15295	546699	54107	-	-	616101	14	0	0.3
29	32.5 nM	L300G	280 µM	831196	626389	41292	-	-	1498876	4778	1.3	20.1
29	32.5 nM	D297N	280 µM	1013803	379441	111736	-	-	1504979	5804	2.7	9.1
30	32.5 nM	R431A	280 µM	17557	43403	53460	98024	313789	1008232	150	0.4	0.02
30	32.5 nM	S429A	280 µM	283097	12587	183397	206969	-	686050	3555	22.5	1.5
30	32.5 nM	Y428A	280 µM	293075	11015	280007	122283	-	708380	3589	26.8	1.1
30	500 nM	1-344	280 µM	22817	1153654	58062	-	-	1234532	10	0	0.4
30	32.5 nM	L300G	280 µM	501905	13196	246999	76568	-	838668	5156	38	2
30	32.5 nM	D297N	280 µM	127879	11739	380577	78431	74063	672789	1638	11	0.3

Fig. 5