



(51) International Patent Classification:

C07K 7/50 (2006.01) A61K 38/12 (2006.01)

A61P 35/00 (2006.01)

(21) International Application Number:

PCT/US2022/046814

(22) International Filing Date:

17 October 2022 (17.10.2022)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/270,799 22 October 2021 (22.10.2021) US

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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,

(54) Title: CELL PERMEABLE MACROCYCLIC PEPTIDES USEFUL FOR EIF4E CAP-BINDING SITE INHIBITION

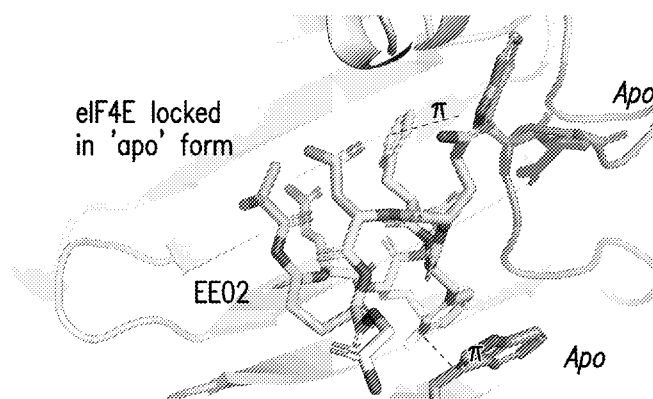


FIG. 1B

(57) Abstract: Several cell-permeable macrocyclic peptides that bind eIF4E on the cap-binding site via its "apo" form rather than its "closed" form which small molecules prefer, have been designed. These macrocyclic peptides were observed to effectively serve as m7GTP mimics, which inhibit eIF4E by preventing its phosphorylation, thus attenuating the translation of 'eIF4E-sensitive mRNAs' into proteins involved in the oncogenic pathways.



KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, 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.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, 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:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
- *with sequence listing part of description (Rule 5.2(a))*

CELL PERMEABLE MACROCYCLIC PEPTIDES USEFUL FOR EIF4E CAP-BINDING SITE INHIBITION

5 REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The instant application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. The XML file, created on September 1, 2022, is named 25281-WO-PCT_SL.XML and is 322 bytes in size.

10 BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to macrocyclic peptides that are cell-permeable and active in eIF4E cap-binding site inhibition. These macrocyclic peptides bind eIF4E in the “apo” or open conformation.

15

Description of Related Art

Translational control of mRNA is essential in the regulation and maintenance of gene expression in eukaryotes, which ultimately influences several cellular processes including, but not limited to, proliferation, apoptosis, differentiation and angiogenesis. The rate-limiting
20 initiation step of translation begins with the binding of the eukaryote trimeric initiation factor (IF) complex eIF4F to the 5' cap, m⁷GpppN (m⁷GTP), a structure common to all nuclear-transcribed mRNAs (Topisirovic et al., WIREs RNA 2: 277-298 (2011)). eIF4F consists of eIF4A – the ATP-dependent RNA helicase, eIF4G – the large scaffolding protein, and eIF4E – the cap-binding subunit. Since eukaryotic mRNAs are predominantly translated in a cap-dependent
25 manner, the regulation of eIF4E plays a paramount role in the expression of these mRNAs. To be more precise, eIF4E preferentially stimulates the aberrant translation of a subset of mRNAs labelled as ‘eIF4E-sensitive’, which primarily encode for proliferation and survival-promoting proteins such as cyclin D1 and D3, c-Myc, MDM2 (mouse double minute 2), VEGF (vascular endothelial growth factor), survivin, and Bcl-2 (B-cell lymphoma 2) (Bhat et al., Nature Reviews
30 Drug Discovery 14 (4), 261-278 (2015); Graff et al., Cancer Research 68: 631 (2008)). These mRNAs all share a commonality in their 5'-UTRs, which are long, G/C-rich, with the potential of forming stable secondary structures, hence exhibiting excessive dependence on eIF4E/eIF4A activity for efficacious translation (Payvar et al., J. Biol. Chem. 254: 7636-7642 (1979)).

A significant number of studies have proven that oncogenesis exerts modifications in the expression and activity of certain translation factors, especially those belonging to the initiation step (Siddiqui et al., *Biochem. Soc. Transactions* 43: 763-772 (2015)). There are multiple methodologies through which the functionality of eIF4E is regulated within the cells.

5 For instance, there is the regulation of the cap-binding complex through the use of nucleotide mimics (Soukarieh et al., *Eur. J. Med. Chem.* 124: 200-217 (2016); Chen et al., *J. Med. Chem.* 55: 3837-3851 (2012)). Unfortunately, most of these small molecule nucleotide mimics fail because of its low permeability and cellular activities. Thus, there remains a need for compounds that can effectively inhibit the functionality of eIF4E and therefore comprise effective therapies

10 for the treatment of cancers that overexpress eIF4E.

BRIEF SUMMARY OF THE INVENTION

The present invention provides compounds comprising macrocyclic peptides that may effectively serve as m7GTP mimics that can inhibit eIF4E activity in mRNA translation by preventing its phosphorylation, thereby attenuating the translation of eIF4E-sensitive mRNAs

15 into proteins involved in various oncogenic pathways. Unlike small molecule m7GTP mimics, which bind eIF4E in its “closed” form, the macrocyclic peptides of the present invention bind eIF4E in its “apo” or “open” form. The macrocyclic peptides of the present invention are useful for the treatment of cancers that overexpress eIF4E.

20 The present invention provides a macrocyclic peptide that can inhibit eIF4E by binding to eIF4E in its “apo” form, comprising 8 to 12 amino acids of which two adjacent amino acids thereof are aromatic amino acids, wherein from an N-terminal to C-terminal direction, a first aromatic amino acid comprising a first aromatic group that can interact with a first aromatic pocket of the eIF4E comprising amino acid residues W¹⁰², W¹⁶⁶, and H²⁰⁰ and a second aromatic

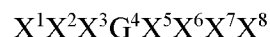
25 amino acid comprising a second aromatic amino acid group that can interact with a second aromatic pocket of the eIF4E comprising amino acid residues W⁵⁶ and F⁴⁸, and wherein the macrocyclic peptide is optionally linked to a cell penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane. In a further embodiment, the aromatic amino acid is phenylalanine or an analog thereof. In further embodiments, the first

30 aromatic amino acid is a phenylalanine analog and the second aromatic amino acid is a phenylalanine analog, wherein the phenylalanine analogs may be the same or different. In further embodiments, the first aromatic amino acid is pentafluorophenylalanine and the second aromatic amino acid is 2-fluorophenylalanine.

In a further embodiment, the macrocyclic peptide comprises 8 to 12 amino acid of which three amino acids thereof comprise from an N-terminal to C-terminal direction the amino acid sequence GX^1X^2 , wherein G is glycine, X^1 is a phenylalanine or phenylalanine analog, and X^2 is a phenylalanine or phenylalanine analog, and wherein the macrocyclic peptide is optionally
5 linked to a cell penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane. In a further embodiment, X^1 is a phenylalanine analog, and X^2 is a phenylalanine analog. In a further embodiment, the phenylalanine analog is N-methyl-phenylalanine, pentafluorophenylalanine, or 2-fluorophenylalanine. In further embodiments, X^1 is pentafluorophenylalanine and X^2 is 2-fluorophenylalanine.

10 In further embodiments, one or more of the 8 to 12 amino acids comprises a D-amino acid. In a further embodiment, each of the 8 to 12 amino acids of the aforementioned macrocyclic peptides comprises a D-amino acid.

The present invention further provides a macrocyclic peptide comprising the formula



wherein X^1 comprises a lysine in which the epsilon-amino group thereof and the alpha-carboxyl group at the C-terminus of X^8 are linked by an amide bond; X^2 comprises
20 glutamic acid, N-methyl-glutamic acid, or (S)-2-amino-4-(1H-tetrazole-5-yl)butanoic acid; X^3 comprises methionine, N-methyl-methionine, or selenomethionine; X^5 comprises phenylalanine, N-methyl-phenylalanine, or pentafluorophenylalanine; X^6 comprises phenylalanine, N-methyl-phenylalanine, or 2-fluorophenylalanine; X^7 comprises azidolysine or azidolysine linked to a cell
25 penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane; and, X^8 comprises aspartic acid or glutamic acid.

In particular embodiments of the macrocyclic peptide, X^5 comprises a pentafluorophenylalanine.

In particular embodiments of the macrocyclic peptide, X^5 comprises a pentafluorophenylalanine and X^6 comprises a 2-fluorophenylalanine.

30 In particular embodiments of the macrocyclic peptide, X^3 comprises selenomethionine.

In particular embodiments of the macrocyclic peptide, X³ comprises selenomethionine, X⁵ comprises a pentafluorophenylalanine, X⁶ comprises a 2-fluorophenylalanine, and X⁷ comprises azidolysine.

5 In particular embodiments of the macrocyclic peptide, X² comprises glutamic acid, X³ comprises selenomethionine, X⁵ comprises a pentafluorophenylalanine, X⁶ comprises a 2-fluorophenylalanine, X⁷ comprises azidolysine, and X⁸ comprises aspartic acid.

10 In particular embodiments of the macrocyclic peptide, X² comprises glutamic acid, X³ comprises selenomethionine, X⁵ comprises a pentafluorophenylalanine, X⁶ comprises a 2-fluorophenylalanine, X⁷ comprises azidolysine linked to a cell penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane, and X⁸ comprises aspartic acid.

In particular embodiments of the macrocyclic peptide, one or more of X¹-X⁸ comprises a D-amino acid. In particular embodiments of the macrocyclic peptide, each of X¹-X⁸ comprises a D-amino acid.

15 The present invention further provides a macrocyclic peptide comprising the formula



20 wherein X¹ is a lysine in which the epsilon amino group thereof and the carboxyl group at the C-terminus of X⁸ are linked by an amide bond; X² is glutamic acid, N-methyl-glutamic acid, or (S)-2-amino-4-(1H-tetrazole-5-yl)butanoic acid; X³ is methionine, N-methyl-methionine, or selenomethionine; X⁵ is phenylalanine, N-methyl-phenylalanine, or pentafluorophenylalanine; X⁶ comprises phenylalanine, N-methyl-phenylalanine, or 2-fluorophenylalanine; X⁷ is azidolysine or azidolysine linked to a cell penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane; and, X⁸ is aspartic acid or glutamic acid.

In particular embodiments of the macrocyclic peptide, X⁵ is a pentafluorophenylalanine.

30 In particular embodiments of the macrocyclic peptide, X⁵ is a pentafluorophenylalanine and X⁶ comprises a 2-fluorophenylalanine.

In particular embodiments of the macrocyclic peptide, X³ is selenomethionine.

In particular embodiments of the macrocyclic peptide, X³ is selenomethionine, X⁵ is a pentafluorophenylalanine, X⁶ is a 2-fluorophenylalanine, and X⁷ comprises azidolysine.

In particular embodiments of the macrocyclic peptide, X³ is selenomethionine, X⁵ is a pentafluorophenylalanine, X⁶ is a 2-fluorophenylalanine, and X⁷ comprises azidolysine
 5 linked to a cell penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane.

In particular embodiments of the macrocyclic peptide, X² comprises glutamic acid, X³ comprises selenomethionine, X⁵ is a pentafluorophenylalanine, X⁶ is a 2-fluorophenylalanine, X⁷ is azidolysine, and X⁸ is aspartic acid.

10 In particular embodiments of the macrocyclic peptide, one or more of X¹-X⁸ comprises a D-amino acid. In particular embodiments of the macrocyclic peptide, each of X¹-X⁸ comprises a D-amino acid.

In particular embodiments of the macrocyclic peptide, the azidolysine is linked to a cell-penetrating moiety comprising an alkyne group in a triazole linkage. In particular
 15 embodiments of the macrocyclic peptide, the cell-penetrating moiety comprises a cell-penetrating peptide (CPP).

In particular embodiments of any one of the aforementioned macrocyclic peptides, the CPP is a peptide selected from the group consisting of Tat (48–60), Tat (47–57), Tat (46–57), Tat (49–57), HIV-1 Rev (34–50), Penetratin (Antp), pVEC, M918, ARF(1–22),
 20 Mastoparan, TP10, NLS, LDP-NLS, LDP, hCT(9–32), DPV3, Secretin, LL-37, Lactoferrin sequences, RGD, Sweet arrow peptide (SAP), hLF, Bac7 (1–24), Buforin IIb, sC18, Protegrin-1, BPrPp (1–28), DPRSFL, VP22, Transcription factor (267–300), VP22, vT5, FGF, C105Y, p28, PFV, SG3, Pep-7, CyLoP-1, MK2i, Influenza HA-2, Influenza HA-2 (1–20) KALA sequence, p28, CPP-C, Bax-inhibiting peptides (BIP), PTD-5, q-NTD, FHV coat (35–49), KLA
 25 sequence, Translocation motif (TLM), Substance P, Crotamine, R9, ppTG1, KALA, Pen-Arg, R6H4, CADY, KAFK, Pep-1, ppTG20, BR2, R4, R6, R10, R12, MPG, HR9, Pep-3, 4K, MPG β, R8 (8-Arginine), 8-Lysine, 6K, 10K, 12K, 5RQ, 8RQ, 11RQ, MPGANLS, R15, H8R15, H16R8, NYAD-41, AcD4, RICK, WRAP, MAP, Chimeric dermaseptin S4, and SV40 (S413-PV), and Transportan.

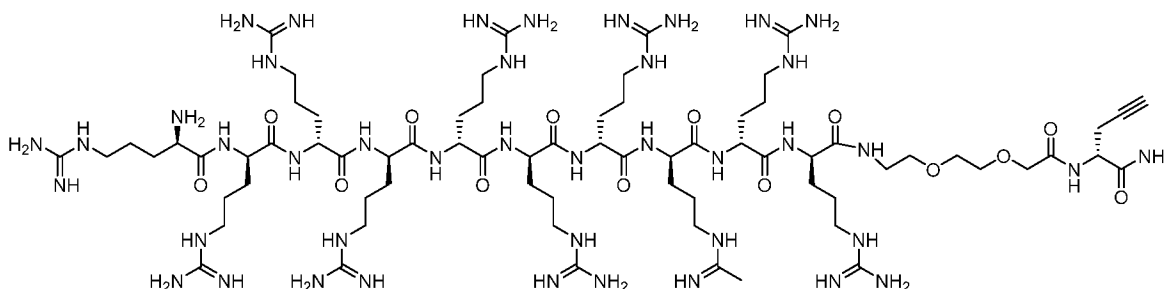
30 In further embodiments of any one of the aforementioned macrocyclic peptides, the CPP comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59,

SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 123, SEQ ID NO: 124, SEQ ID NO: 125, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, and SEQ ID NO: 138.

In a particular embodiment of any one of the aforementioned macrocyclic peptides, the CPP is a poly(Arg) polymer comprising 5 to 15 arginine residues (SEQ ID NO: 141). In a further embodiment, the poly(Arg) is a poly-L-arginine or poly-D-arginine polymer, which in particular embodiments, comprises 5 to 15 L-Arg (SEQ ID NO: 141) or D-Arg (SEQ ID NO: 142) residues, respectively. In a further embodiment, the poly(Arg) polymer comprises 10 L-Arg residues (SEQ ID NO: 113), 10 D-Arg residues (SEQ ID NO: 37), or a mixture of L-Arg and D-Arg residues.

In any one of the above embodiments of any one of the aforementioned macrocyclic peptides, the CPP comprises an alkyne group, optionally linked to the CPP by a linking moiety. In certain embodiments, the alkyne group is directly linked to the C-terminus of the CPP or indirectly linked to the C-terminus of the CPP by a linking moiety. In particular embodiments of the macrocyclic peptide, the C-terminus carboxy group of the poly-D-arginine polymer is covalently linked to the alkyne group by a linking moiety comprising a polyethylene glycol (PEG) linker. In particular embodiments of the macrocyclic peptide, the PEG linker comprises one to 10 PEG units. In particular embodiments, the alkyne group comprises proargylglycine.

In particular embodiments of any one of the aforementioned macrocyclic peptides, the cell penetrating moiety comprises the formula:



(SEQ ID NO: 37).

The present invention further provides a composition comprising of any one of the
5 macrocyclic peptides disclosed herein and a pharmaceutically acceptable carrier.

The present invention further provides a composition comprising a macrocyclic peptide disclosed herein that binds to eIF4E in the apo form linked to a cell-penetrating peptide disclosed herein and a pharmaceutically acceptable carrier. In particular embodiments, the macrocyclic peptide comprises the amino acid sequence GX^1X^2 , wherein G is glycine, X^1 is
10 phenylalanine or phenylalanine analog, and X^2 is phenylalanine or phenylalanine analog. In further embodiments, X^1 is pentafluorophenylalanine and X^2 is 2-fluorophenylalanine.

In further embodiments, the composition comprises a macrocyclic peptide comprising an amino acid sequence set forth in the group of amino acid sequences consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6,
15 SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34,
20 SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 38, and SEQ ID NO: 39, optionally linked to a cell-penetrating peptide disclosed herein and a pharmaceutically acceptable carrier.

In further embodiments, the composition comprises a macrocyclic peptide comprising an amino acid sequence set forth in the group of amino acid sequences consisting of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID
25 NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, and SEQ ID NO: 32, optionally linked to a cell-penetrating peptide disclosed herein and a pharmaceutically acceptable carrier.

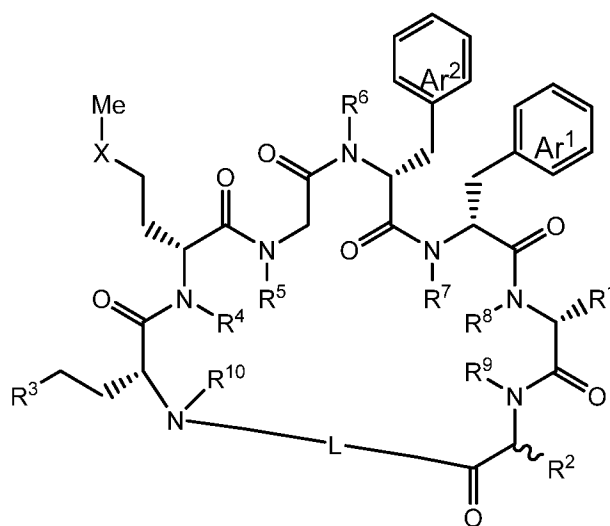
The present invention further provides a method for the treatment of a cancer comprising administering to an individual having a cancer a therapeutically effective amount of

any one of the foregoing embodiments of macrocyclic peptide or composition of said macrocyclic peptide and a pharmaceutically acceptable carrier to treat the cancer. In a further embodiment, the cancer is selected from the group of cancers that overexpress eIF4E. In a further embodiment, the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

The present invention further provides for use of a macrocyclic peptide of any one of the foregoing embodiments for the manufacture of a medicament for the treatment of cancer. In a further embodiment, the cancer is selected from the group cancers that overexpress eIF4E. In a further embodiment, the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

The present invention further provides any one of the foregoing embodiments of the macrocyclic peptide or a composition of said macrocyclic peptide for use in the treatment of a cancer. In a further embodiment, the cancer is selected from the group cancers that overexpress eIF4E. In a further embodiment, the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

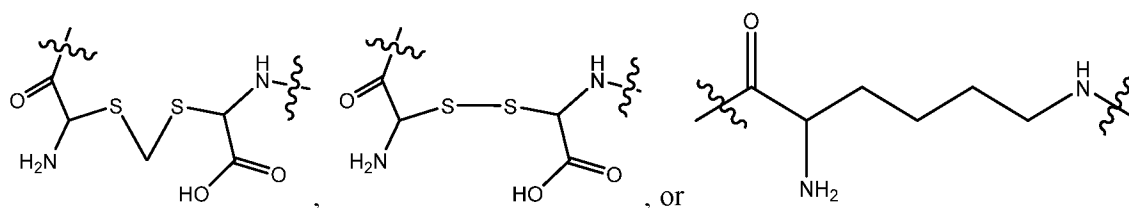
The present invention further provides a compound comprising Formula I



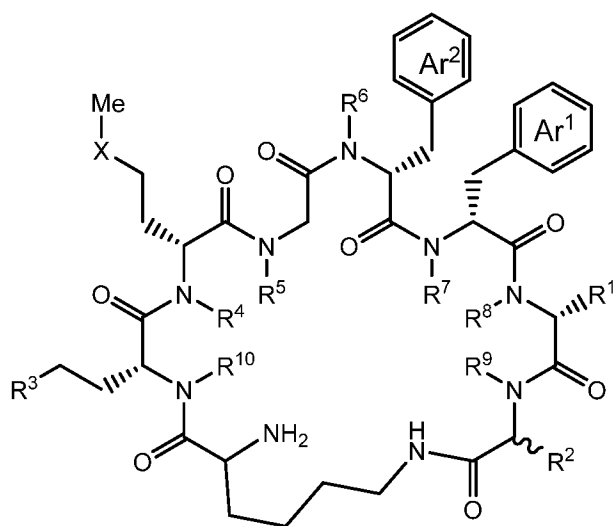
wherein R^1 comprises a C_1 - C_6 alkylene- R^{11} ; R^2 comprises a CH_2COOH , CH_2CH_2COOH , or $CH_2(5\text{-imidazole})$; R^3 is CH_2COOH ; $R^4 - R^{10}$ each independently comprises a CH_3 or H ; R^{11} comprises H , N_3 , alkyne, succinimide, maleimide, aryl-p-guanidino, 5-imidazole, or NH_2 ; X comprises sulfur (S) or selenium (Se); Ar^1 comprises a benzene, pentafluorobenzene, 2-fluoro-

benzene, hydroxybenzene, p-guanidino-benzene, or 3,4-fluoro-benzene; and Ar² comprises a benzene, pentafluorobenzene group, 2-fluoro-benzene, p-guanidino-benzene, or 3,4-fluoro-benzene; and, L comprises a linker; wherein the compound binds eIF43 in its open form; and wherein the compound is optionally covalently linked to a cell-penetrating moiety capable of being transported across a cell membrane. In particular embodiments, R¹¹ comprises an N₃. In particular embodiments, R¹ is a C₁-C₆ alkylene-R¹¹; R² is a CH₂COOH, CH₂CH₂COOH, or CH₂(5-imidazole); R³ is CH₂COOH; R⁴ – R¹⁰ each independently is a CH₃ or H; R¹¹ is H, N₃, alkyne, succinimide, maleimide, aryl-p-guanidino, 5-imidazole, or NH₂; X is sulfur (S) or selenium (Se); Ar¹ is a benzene, hydroxybenzene, pentafluorobenzene, 2-fluoro-benzene, p-guanidino-benzene, or 3,4-fluoro-benzene; and Ar² is a benzene, pentafluorobenzene group, 2-fluoro-benzene, p-guanidino-benzene, or 3,4-fluoro-benzene; and, L is a linker; wherein the compound binds eIF43 in its open form; and wherein the compound is optionally covalently linked to a cell-penetrating moiety capable of being transported across a cell membrane. In particular embodiments, R¹¹ is an N₃.

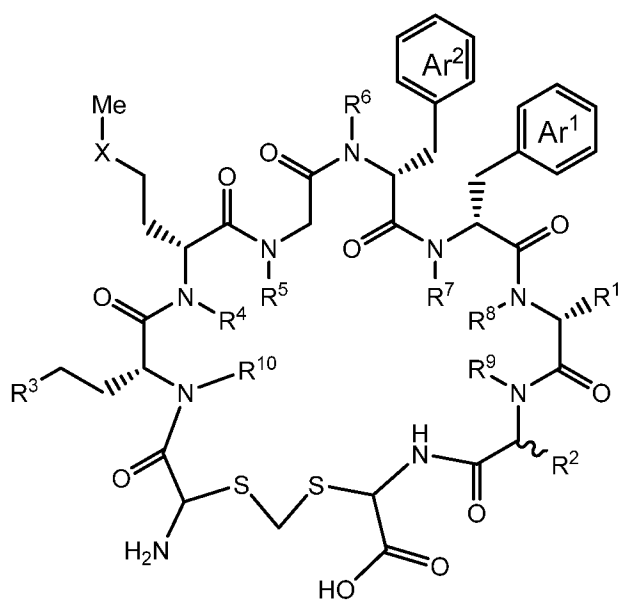
In particular embodiments of the compound, L has the formula



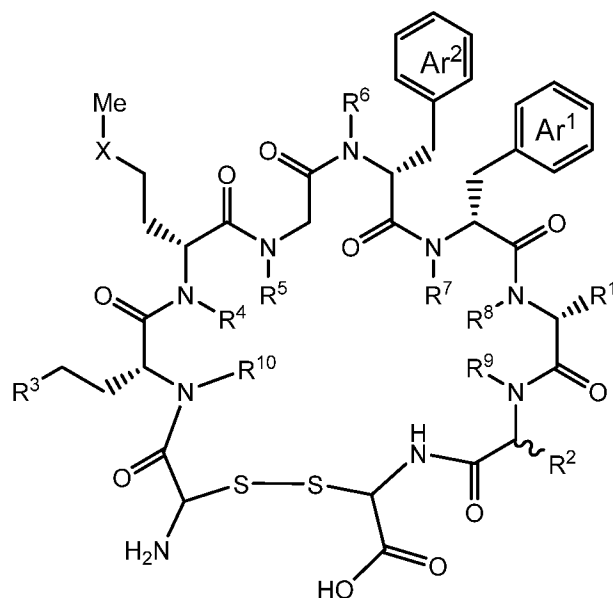
The present invention further provides a compound comprising Formula II



, Formula III



or Formula IV



In particular embodiments of the above compounds, R^1 comprises a C_1 - C_6 alkylene- R^{11} ; R^2 comprises a CH_2COOH , CH_2CH_2COOH , or $CH_2(5\text{-imidazole})$; R^3 is CH_2COOH ; $R^4 - R^{10}$ each independently comprises a CH_3 or H ; R^{11} comprises H , N_3 , alkyne, succinimide, maleimide, aryl-*p*-guanidino, 5-imidazole, or NH_2 ; X comprises sulfur (S) or selenium (Se); Ar^1 comprises a benzene, hydroxybenzene, pentafluorobenzene, 2-fluoro-benzene, *p*-guanidino-benzene, or 3,4-fluoro-benzene; and Ar^2 comprises a benzene, pentafluorobenzene group, 2-fluoro-benzene, *p*-guanidino-benzene, or 3,4-fluoro-benzene; wherein the compound binds eIF43 in its open form; and wherein the compound is optionally covalently linked to a cell-penetrating moiety capable of facilitating the transport of the compound across the cell membrane. In particular embodiments, R^{11} comprises an N_3 .

In particular embodiments, R^1 is a C_1 - C_6 alkylene- R^{11} ; R^2 is a CH_2COOH , CH_2CH_2COOH , or $CH_2(5\text{-imidazole})$; R^3 is CH_2COOH ; $R^4 - R^{10}$ each independently is a CH_3 or H ; R^{11} is H , N_3 , alkyne, succinimide, maleimide, aryl-*p*-guanidino, 5-imidazole, or NH_2 ; X is sulfur (S) or selenium (Se); Ar^1 is a benzene, hydroxybenzene, pentafluorobenzene, 2-fluoro-benzene, *p*-guanidino-benzene, or 3,4-fluoro-benzene; and Ar^2 is a benzene, pentafluorobenzene group, 2-fluoro-benzene, *p*-guanidino-benzene, or 3,4-fluoro-benzene; wherein the compound is optionally covalently linked to a cell-penetrating moiety capable of being transported across a cell membrane. In particular embodiments, R^{11} is an N_3 . In a further embodiment, the N_3 is linked to an alkyne of a cell-penetrating moiety in a triazole linkage.

In particular embodiments of the compound, R^1 comprises 1-azidobutane ($CH_2CH_2CH_2CH_2N_3$); R^2 comprises CH_2COOH or $CH_2(5\text{-imidazole})$; R^3 comprises

CH₂CH₂COOH; R⁴ – R¹⁰ each independently comprises CH₃ or H; X comprises sulfur (S) or selenium (Se); Ar¹ comprises pentafluorobenzene; and Ar² comprises 2-fluoro-benzene; wherein the 1-azidobutane is optionally linked to a cell-penetrating moiety.

In particular embodiments of the compound, R¹ is 1-azidobutane
 5 (CH₂CH₂CH₂CH₂N₃); R² is CH₂COOH or CH₂(5-imidazole); R³ is CH₂CH₂COOH; R⁴ – R¹⁰ each independently is CH₃ or H; X is sulfur (S) or selenium (Se); Ar¹ is pentafluorobenzene; and Ar² comprises 2-fluoro-benzene; wherein the 1-azidobutane is optionally linked to a cell-penetrating moiety.

In particular embodiments of the compound, R¹ is 1-azidobutane
 10 (CH₂CH₂CH₂CH₂N₃); R² is CH₂COOH; R³ is CH₂CH₂COOH; R⁴ – R¹⁰ each is H; X is selenium (Se); Ar¹ is pentafluorobenzene; and Ar² comprises 2-fluoro-benzene; wherein the 1-azidobutane is optionally linked to a cell-penetrating moiety.

In particular embodiments of the compound, the 1-azidobutane is linked to a cell-penetrating moiety comprising an alkyne group in a triazole linkage, wherein the cell-penetrating
 15 moiety is capable of facilitating the transport of the compound across the cell membrane. In particular embodiments of the compound, the cell-penetrating moiety comprises a cell-penetrating peptide (CPP).

In particular embodiments, the CPP is a peptide selected from the group consisting of Tat (48–60), Tat (47–57), Tat (46–57), Tat (49–57), HIV-1 Rev (34–50),
 20 Penetratin (Antp), pVEC, M918, ARF(1–22), Mastoparan, TP10, NLS, LDP-NLS, LDP, hCT(9–32), DPV3, Secretin, LL-37, Lactoferrin sequences, RGD, Sweet arrow peptide (SAP), hLF, Bac7 (1–24), Buforin IIb, sC18, Protegrin-1, BPrPp (1–28), DPRSFL, VP22, Transcription factor (267–300), VP22, vT5, FGF, C105Y, p28, PFV, SG3, Pep-7, CyLoP-1, MK2i, Influenza HA-2, Influenza HA-2 (1–20) KALA sequence, p28, CPP-C, Bax-inhibiting
 25 peptides (BIP), PTD-5, q-NTD, FHV coat (35–49), KLA sequence, Translocation motif (TLM), Substance P, Crotamine, R9, ppTG1, KALA, Pen-Arg, R6H4, CADY, KAFK, Pep-1, ppTG20, BR2, R4, R6, R10, R12, MPG, HR9, Pep-3, 4K, MPG β, R8 (8-Arginine), 8-Lysine, 6K, 10K, 12K, 5RQ, 8RQ, 11RQ, MPGΔNLS, R15, H8R15, H16R8, NYAD-41, AcD4, RICK, WRAP, MAP, Chimeric dermaseptin S4 and SV40 (S413-PV), and Transportan.

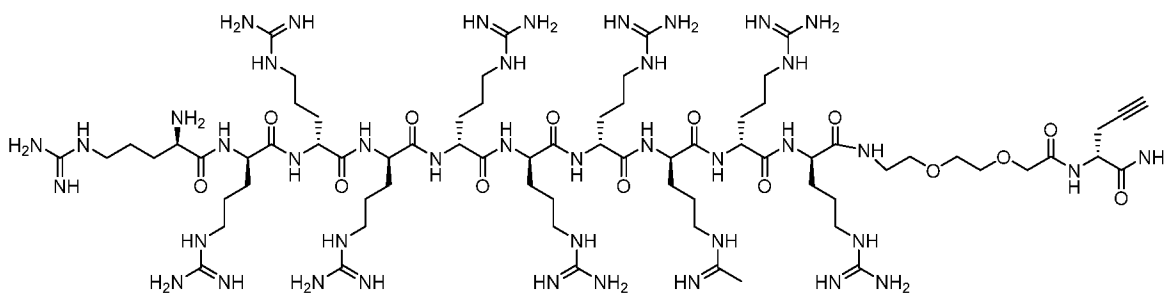
In further embodiments, the CPP comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ
 30 ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID

NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 123, SEQ ID NO: 124, SEQ ID NO: 125, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, and SEQ ID NO: 138.

In a particular embodiment, the CPP is a poly(Arg) polymer comprising 5 to 15 arginine residues (SEQ ID NO: 141). In a further embodiment, the poly(Arg) is a poly-L-arginine or poly-D-arginine polymer, which in particular embodiments, comprises 5 to 15 L-Arg (SEQ ID NO: 141) or D-Arg residues (SEQ ID NO: 142). In a further embodiment, the poly(Arg) polymer comprises 10 arginine residues (SEQ ID NO: 113), which in a further embodiment comprise all D-Arg residues.

In any one of the above embodiments, the CPP comprises an alkyne group, optionally linked to the CPP by a linking moiety. In certain embodiments, the alkyne group is directly linked to the C-terminus of the CPP or indirectly linked to the C-terminus of the CPP by a linking moiety. In particular embodiments of the compound, the C-terminus carboxy group of the poly-D-arginine polymer is covalently linked to the alkyne group by a linking moiety comprising a polyethylene glycol (PEG) linker. In particular embodiments of the compound, the PEG linker comprises one to 10 PEG units. In particular embodiments, the alkyne group comprises proargylglycine.

In particular embodiments of the compound, the cell penetrating moiety comprises the formula:



(SEQ ID NO: 37).

The present invention further provides a composition comprising any one of the foregoing embodiments of the aforementioned compounds and a pharmaceutically acceptable carrier.

The present invention further provides a method for the treatment of a cancer comprising administering to an individual having a cancer a therapeutically effective amount of any one of the foregoing embodiments of compound or composition of said compound to treat the cancer. In a further embodiment, the cancer is selected from the group cancers that overexpress eIF4E. In a further embodiment, the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

The present invention further provides for use of a of compound or composition of said compound for the manufacture of a medicament for the treatment of cancer. In a further embodiment, the cancer is selected from the group cancers that overexpress eIF4E. In a further embodiment, the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

The present invention further provides any one of the foregoing embodiments of the compound or composition of said compound for use in the treatment of a cancer. In a further embodiment, the cancer is selected from the group cancers that overexpress eIF4E. In a further embodiment, the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

The present invention further provides a compound comprising a macrocyclic peptide covalently linked to a cell-penetrating moiety comprising a poly-D-arginine polymer directly or indirectly covalently linked at the C-terminus carboxy group to an alkyne group. In particular embodiments of the macrocyclic peptide, the poly-D-arginine polymer comprises five to 15 D-arginine residues (SEQ ID NO: 142).

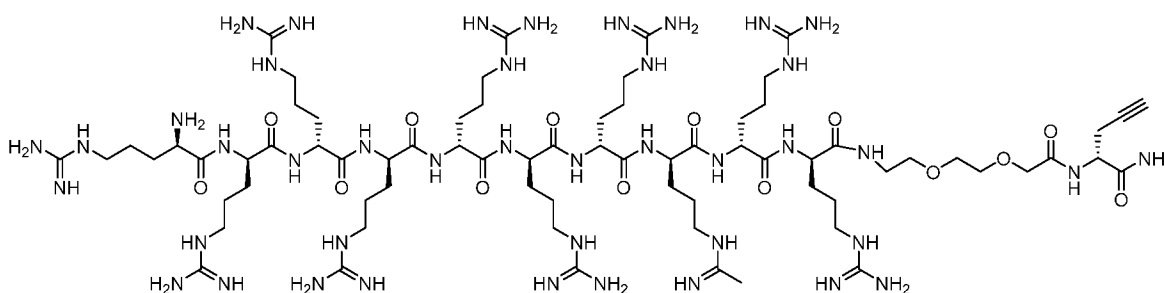
The present invention further provides a method for producing a compound that is cell permeable comprising: (a) providing a macrocyclic peptide comprising an azido group and a cell-penetrating moiety comprising a poly-D-arginine polymer directly or indirectly covalently linked at the C-terminus carboxy group to an alkyne group; and (b) conjugating the azido group of the macrocyclic peptide to the alkyne group to produce the cell permeable macrocyclic compound.

In particular embodiments of any one of the compounds disclosed supra, the cell-penetrating moiety comprises poly-D-arginine polymer directly or indirectly covalently linked at the C-terminus carboxy group to an alkyne group.

In particular embodiments of the compound, the poly-D-arginine polymer comprises five to 15 D-arginine residues (SEQ ID NO: 142).

In particular embodiments of the compound, the poly-D-arginine polymer comprises 10 D-arginine residues (SEQ ID NO: 37).

In any one of the above embodiments, the CPP comprises an alkyne group, optionally linked to the CPP by a linking moiety. In certain embodiments, the alkyne group is directly linked to the C-terminus of the CPP or indirectly linked to the C-terminus of the CPP by a linking moiety. In particular embodiments of the compound, the C-terminus carboxy group of the poly-D-arginine polymer is covalently linked to the alkyne group by a linking moiety comprising a polyethylene glycol (PEG) linker. In particular embodiments of the compound, the PEG linker comprises one to 10 PEG units. In particular embodiments, the alkyne group comprises proargylglycine. In particular embodiments of the compound, the cell penetrating moiety comprises:



(SEQ ID NO: 37).

The present invention provides a cell-penetrating moiety comprising a poly-D-arginine polymer directly linked or indirectly covalently linked at the C-terminus carboxy group to an alkyne group.

In particular embodiments of the compound, the poly-D-arginine polymer comprises five to 15 D-arginine residues (SEQ ID NO: 142).

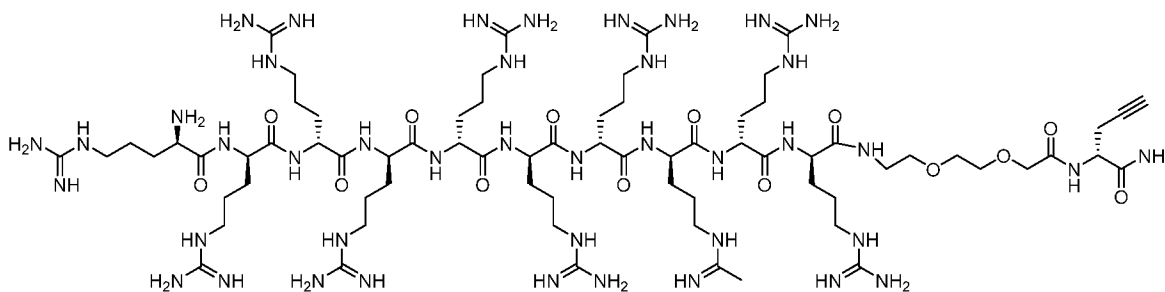
In particular embodiments of the compound, the poly-D-arginine polymer comprises 10 D-arginine residues (SEQ ID NO: 37).

In particular embodiments of the compound, the C-terminus carboxy group of the poly-D-arginine polymer is covalently linked to the alkyne group by a polyethylene glycol (PEG) linker.

In particular embodiments of the compound, the PEG linker comprises one to 10 PEG units.

In particular embodiments of the compound, the alkyne group comprises proargylglycine.

In particular embodiments of the compound, the cell penetrating moiety comprises the formula



(SEQ ID NO: 37).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A and **Fig. 1B** show a comparison between bound to m7GTP (**Fig. 1A**) and EE-02 (**Fig. 1B**) with the main changes to W⁵⁶, W¹⁰² and E¹⁰³ highlighted when going from ‘apo’ to ‘closed’ as shown by the arrows in panel A.

Fig. 2A, Fig. 2B, Fig. 2C, and Fig. 2D together show crystal structure coordinates for four cyclic peptide-protein complexes: EE-02, EE-48, EE-94 and EE-108, with the resolution of 2.35 Å, 2.70 Å, 2.10 Å, and 2.70 Å, respectively. All these cyclic peptides interact with the protein in a very similar manner, keeping the inter protein-peptide interactions

very similar. The main difference is found in EE-108 peptide with significant loop movement near residue Asn-50 caused by its interaction with the Linker. Linker effects on bi-molecular interactions between macrocyclic peptide ligand and eIF4E's N⁵⁰, R¹¹², R¹⁵⁷ and K¹⁶² shown by dotted lines. Interactions with eIF4E's F⁴⁸, W⁵⁶, S⁹², W¹⁰², W¹⁶⁶ and H²⁰⁰ remained constant in all cases.

Fig. 3A shows H-bond interaction of M⁴ on macrocyclic peptide with S⁹² on eIF4E.

Fig. 3B shows the interaction of F6 on macrocyclic peptide with aromatic pocket (W¹⁰², W¹⁶⁶ and H²⁰⁰) of eIF4E.

Fig. 3C shows the interaction of F⁷ on macrocyclic peptide with F⁴⁸ and W⁵⁶ of eIF4E, via edge-to-face and parallel displaced π -interactions, respectively.

Fig. 3D shows the water-mediated interaction between E³ on macrocyclic peptide with R¹¹² and K¹⁶² on eIF4E, which is present and conserved in all the co-crystal structures obtained.

Fig. 3E shows the unique interaction of EE-02's disulfide bond with γ -carbonyl of C², making it more delta-negative and, in turn, interacts stronger with R¹⁵⁷ (disulfide (S-S) bond donation into carbonyl induces stronger polar interaction with R157; this, in turn, stabilized S-S bonds in EE-02/44).

Fig. 4A shows an ¹H-¹⁵N TROSY-HSQC NMR study of EE-124 and EE-129 binding to ¹³C, ¹⁵N eIF4E; 200 μ M eIF4E with 200 μ M macrocyclic peptide ligands.

Fig. 4B shows that Chemical Shift Perturbation (CSP) analysis reveals the interacting residues (dark regions marked X and lighter regions marked Y), including the direct interaction region, as observed on crystal structures, and the distant contiguous region. The region of high positive charge (phosphate binding region of positively charged residues R¹⁵⁷, K¹⁵⁹ and K¹⁶²), which traditionally binds M⁷GTP was not observed to interact with EE-124 or EE-129 in the CSP analysis (region marked Z). Fluorinated phenyl groups in EE-129 were observed to affect interactions with (W¹⁰², G¹¹⁰ and E¹⁷¹, in bold). Refer to Example 10 for full study.

Fig. 5A shows Top: poly-D-Arg as CPP to carry EE-129 into cells, linked via a triazole linker. CPP and EE-129 were linked using 'click' chemistry between A8K(N₃) on macrocyclic peptide EE129-N₃ and (D-R)₁₀-PEG₂-(D-Pra) ("(D-R)10" disclosed as SEQ ID NO: 37). Bottom: EE-171 was found to be effective in inhibiting eIF4E phosphorylation when incubated with HEK293F cells for 1 and 4 hours, in 10% FCS.

Fig. 5B shows that for an N-methylation scan performed to identify potential sites of methylation – only the D9 position was tolerated. These were, however, not translatable onto future designs. Further evolutions were also carried out on the non-interacting residues (A8 and D9) using ALIS and computational modelling.

5 **Fig. 5C** shows a comparison of the cellular effects of EE-171 against the CPP poly(D-Arg)₁₀ (SEQ ID NO: 37) or dR₁₀ (SEQ ID NO: 37) alone, and other eIF4E phosphorylation inhibitors: (1) Mnk kinase inhibitor CGP4073 (Andersson et al., Cytokine 33: 52-57 (2006)), (2) eIF4G inhibitor 4EGI-1 (Moerke et al., Cell 128: 257-267 (2007)), (3) eIF4A inhibitor Silvestrol (Pelletier et al., Cancer Res 75: 250-263 (2015)), and (4) mTOR inhibitor
10 PP242 (Hoang et al., J. Biol. Chem. 287: 21796-21805 (2012)).

Fig. 6 shows that each residue/position is unique in each Aimed Rational Design (ARD) stage; Starting from EE-02 (top left), we begin the ARD process starting from Ala-scan and residue deletion to remove excess mass and polar groups without affecting binding affinity to obtain EE-44; next, we performed linker evolution to obtain EE-108; this was followed by
15 Structure-Activity Relationship (SAR) study to improve binding and obtain EE-129; finally, cellular permeability was designed using D9X mutations to obtain EE-233.

Fig. 7A shows the structures for eIF4E: in its ‘closed’ form, when bound to m⁷GTP as shown in panel A or to a published nucleotide mimic (e.g., Chen et al., J. Med. Chem. 55: 3837-51 (2012)) as shown in panel B.

20 **Fig. 7B** shows the structures for eIF4E: in its ‘apo’ form, when free in solution (panel C) and bound to EE-02 (panel D).

Fig. 7C shows a comparison between bound to m⁷GTP and EE-02 with the main changes to W⁵⁶, W¹⁰² and E¹⁰³ highlighted when going from ‘apo’ to ‘closed’ (as shown by the arrows).

25 **Fig. 8** shows a summary of initial EE-02 optimization to EE-44.

Fig. 9 shows one of the competitive FP experiments performed on EE-02 (left) and EE-44 (right).

Fig. 10 shows an example of results from a Differential scanning fluorimetry (DSF) experiment: a shift in melt curve with EE-2 (top) and EE-44 (bottom). DSF is also known
30 as ThermoFluor (TF) or Thermal Shift Assay.

Fig. 11 shows one of four ITC experiments performed to determine thermodynamic data and binding affinity of EE-02.

Fig. 12 shows one of four ITC experiments to determine thermodynamic data and binding affinity of EE-44.

Fig. 13 shows a summary of initial SAR results using native disulphide bond constraints.

5 **Fig. 14** shows Surface Plasmon Resonance (SPR) data from EE-44 and EE-48.

Fig. 15 shows one of the four ITC experiments performed to determine the thermodynamic data and binding affinity for the newly re-synthesized EE-48.

Fig. 16 shows a comparison of results between EE-44 and methylene bridged peptide, EE-48.

10 **Fig. 17** shows an X-ray crystal structure of EE-48 bound to eIF4E.

Fig. 18 shows one of four ITC experiments performed to determine thermodynamic data and binding affinity of EE-94.

Fig. 19 shows a summary of results and SAR for first set of HTC peptides, EE-91 to EE-94. Complete biophysical assay results for EE-94 shown in the center. X-ray crystal
15 structure of EE-94 bound to eIF4E shown on the extreme right.

Fig. 20 shows an X-ray crystal structure of EE-94 bound to eIF4E.

Fig. 21 shows evolution to EE-108 to increase flexibility of linker and to pick up extra interaction via the N-terminus. Figure discloses SEQ ID NOS 5 and 8, respectively, in order of appearance.

20 **Fig. 22** shows one of four ITC experiments performed to determine thermodynamic data and binding affinity for EE-108.

Fig. 23 shows an X-ray crystal structure of EE-108 bound to eIF4E.

Fig. 24 shows one of four ITC experiments to determine thermodynamic data and binding affinity of EE-124.

25 **Fig. 25** shows a summary of SAR development towards STC peptide EE-124. Figure also discloses in the middle SEQ ID NO: 226. The term nBu represents the side group of lysine wherein the epsilon amino group of a lysine is covalently linked to the C-terminus of D-Asp and the term nET represents the carboxyl group of the lysine linked to N-terminus of D-Glu, together which form a circular peptide.

30 **Fig. 26** shows an X-ray crystal structure of EE-124 bound to eIF4E.

Fig. 27 shows STC peptides evolved from EE-124. Rationalization for these modifications are based on crystal structures shown (summary of crystal structure data in top of figure); Selenomethionine (Semet) of EE-124 was rationalized to interact with S⁹² and D⁹⁰ of

eIF4E (top left); Phe⁶ of EE-124 was rationalized to loosely fit into an aromatic pocket with less-than-optimal pi-pi-stacking interactions with the surrounding aromatic groups (top, middle); Phe⁷ of EE-124 was rationalized to participate in an edge-to-face pi-pi-stacking event with F⁴⁸ of eIF4E, donating its *para*-phenyl proton to phenyl ring of F⁴⁸ (right, top). Figure discloses SEQ ID NOS 13 and 15-17, respectively, in order of appearance.

Fig. 28 shows a summary of results for TF and ITC experiments. for EE-124 to EE-129. Figure discloses SEQ ID NOS 11, 15, and 13, respectively, in order of appearance.

Fig. 29 shows one of four ITC experiments to determine thermodynamic data and binding affinity of EE-129.

Fig. 30 shows one of three ITC experiments to determine thermodynamic data and binding affinity of EE-130.

Fig. 31 shows one of two ITC experiments to determine thermodynamic data and binding affinity of EE-131.

Fig. 32 shows one of two ITC experiments to determine thermodynamic data and binding affinity of EE-133.

Fig. 33 shows crystal structures of EE44, EE108, EE48 and EE94 peptides with eIF4E proteins, as analyzed in computation modelling, are shown here. Protein eIF4E is in transparent grey cartoon representation. Peptides are shown as sticks. The polar interactions between protein-peptide are highlighted and shown by black dotted lines. Intra-peptide interactions are shown.

Fig. 34 shows an ¹H-¹⁵N TROSY-HSQC; Apo-eIF4E residues mapped. Key residues: W⁴⁶, F⁴⁸, K⁴⁹, N⁵⁰, D⁵¹, W⁵⁶, R⁶¹, G⁸⁸, C⁸⁹, S⁹², L⁹³, F⁹⁴, E⁹⁹, M¹⁰¹, W¹⁰², R¹⁰⁹, G¹¹⁰, R¹¹², G¹⁵¹, V¹⁵⁶, R¹⁵⁷, K¹⁶², W¹⁶⁶, T¹⁶⁸, C¹⁷⁰, E¹⁷¹, S¹⁹⁹, D²⁰², A²⁰⁴ & K²¹².

Fig. 35 shows an ¹H-¹⁵N TROSY-HSQC; Apo-eIF4E (black) versus eIF4E/EE-124 complex (grey).

Fig. 36 shows that interaction mapping and imaging showed extensive chemical shift perturbations in the cap-binding region & surrounding regions (dark and labeled X). eIF4E (grey) complex with EE-124. As shown the region of high positive charge is relatively unaffected by peptide binding, i.e., phosphate binding region.

Fig. 37 shows an ¹H-¹⁵N TROSY-HSQC study; Interaction between E³ on EE-124 with eIF4E's R¹¹² is much stronger than with R¹⁵⁷.

Fig. 38 shows an ¹H-¹⁵N TROSY-HSQC; Apo-eIF4E (black) versus eIF4E/EE-129 complex (grey).

Fig. 39 shows an interaction mapping comparison between EE-124 and EE-129 with eIF4E. While most interactions were similar, larger changes in chemical shifts were observed for EE-129 interaction with eIF4E via W¹⁰², G¹¹⁰ and E¹⁷¹.

Fig. 40 shows an ¹H NMR of EE-124 in d₆-DMSO.

Fig. 41 shows an ¹H NMR of EE-129 in d₆-DMSO.

Fig. 42 shows an ¹H NMR of EE-124 in d₆-DMSO with 20% D₂O.

Fig. 43 shows an ¹H NMR of EE-124 in d₆-DMSO with 20% D₂O.

Fig. 44 shows a ¹³C NMR of EE-124 in d₆-DMSO.

Fig. 45 shows a ¹³C NMR of EE-129 in d₆-DMSO.

Fig. 46 shows an ¹⁹F NMR of EE-124 in d₆-DMSO. Only the trifluoroacetate (TFA) counterion was observed.

Fig. 47 shows an ¹⁹F NMR of EE-129 with d₆-DMSO. TFA counterion, *ortho*-fluoro-*L*-phenylalanine and *L*-pentafluorophenylalanine were observed.

Fig. 48 shows an ¹⁹F NMR of EE-129 in d₆-DMSO with fluorinated phenylalanine areas expanded.

Fig. 49 shows a design concept for EE-171. "dR10" is disclosed as SEQ ID NO: 37.

Fig. 50 shows one of four ITC experiments to determine thermodynamic data and binding affinity of EE-155.

Fig. 51 shows one of three ITC experiments to determine thermodynamic data and binding affinity of EE-169 N₃.

Fig. 52 shows two of three ITC experiments to determine thermodynamic data and binding affinity of EE-171.

Fig. 53 shows two of three ITC experiments to determine thermodynamic data and binding affinity of EE-171.

Fig. 54 shows one of three ITC experiments to determine thermodynamic data and binding affinity of EE-233.

Fig. 55 shows one of four ITC experiments to determine thermodynamic data and binding affinity of EE-246.

Fig. 56 shows one of four ITC experiments to determine thermodynamic data and binding affinity of EE-249.

Fig. 57 shows a reaction scheme for solid phase peptide synthesis (SPPS). Examples are shown for HTC peptide EE-94. Figure discloses SEQ ID NOS 227 and 5, respectively, in order of appearance.

Fig. 58 shows a reaction scheme for EE-48. Figure discloses SEQ ID NOS 228 and 3, respectively, in order of appearance.

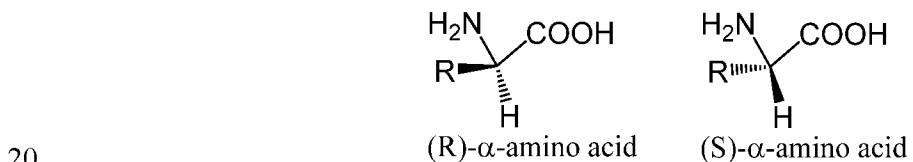
Fig. 59 shows Library 1 (D9X mutations of EE-129 N₃) consisting of L-residues. EE-129 N₃ parent peptide was present, along with internal standard D9G.

Fig. 60 shows Library 2 (D9X mutations of EE-129 N₃) consisting of mostly D-residues. D9G mutation serves as internal standard. Top hit was identified to be D9dH mutant, EE-233 N₃. BT = breakthrough – this means that effects of non-specific hydrophobic interactions may contribute to binding.

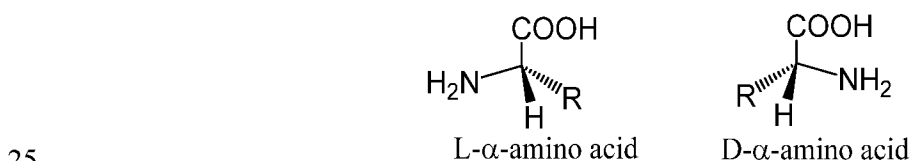
DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, " α -amino acid" or simply "amino acid" refers to a molecule containing both an amino group and a carboxyl group bound to a carbon, which is designated the α -carbon, attached to a side chain (R group) and a hydrogen atom and may be represented by the formula shown for (R) and (S) α -amino acids



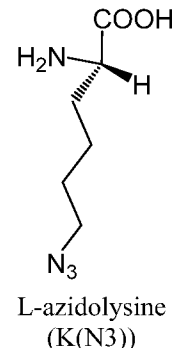
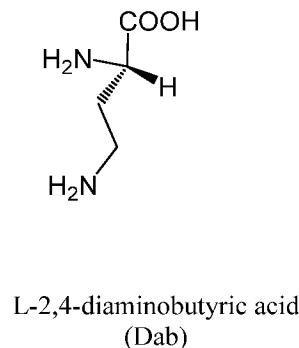
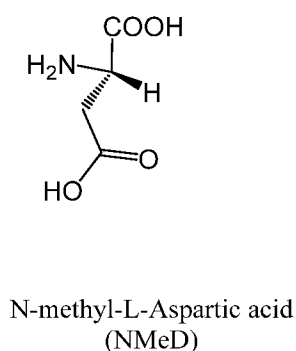
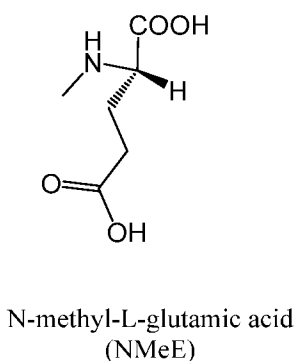
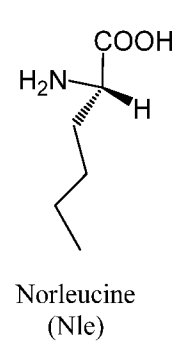
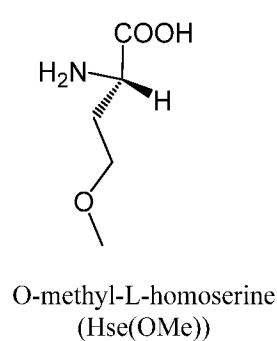
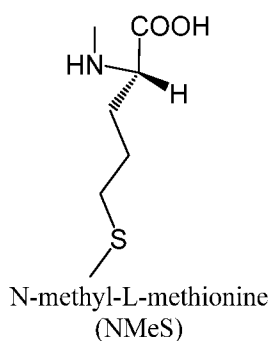
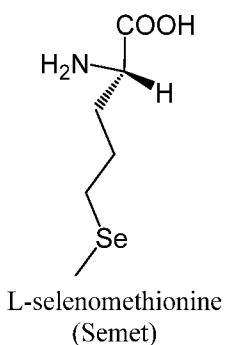
In general, L-amino acids have an (S) configuration except for cysteine, which has an (R) configuration, and glycine, which is achiral. The generalized structure of D and L amino acids may be represented by the formula

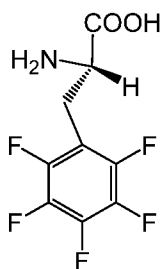
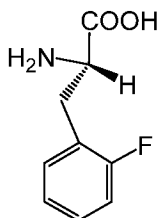
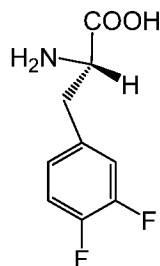
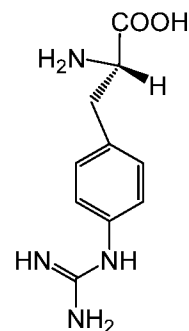
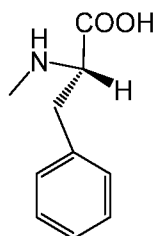
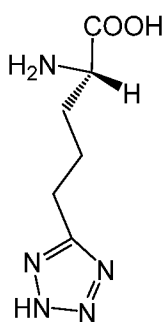
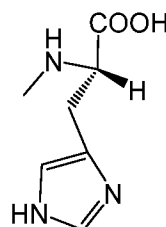
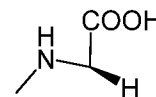


As used herein, D amino acids are denoted by the superscript "D" (e.g., ^DLeu or ^DL) or "d" preceding the amino acid (e.g., dLeu or dL or dL) or "D" (e.g., D-Leu) and L amino acids by "L" (e.g., L-Leu) or no L identifier (e.g., Leu). Unless indicated otherwise, an amino acid or analog as used herein is an L-amino acid. Unless the context specifically indicates otherwise, the term

amino acid, as used herein, is intended to include amino acid analogs, and depending on the context the term is used, the term amino acid or amino acid analog may be used to refer to the free amino acid or to an amino acid residue as a member of a peptide sequence.

As used herein, "amino acid analog" or "non-natural amino acid" refers to a molecule which is structurally similar to an amino acid and which can be substituted for an amino acid in the formation of a macrocyclic peptide. Amino acid analogs include, without limitation, compounds which are structurally identical to an amino acid, as defined herein, except for the inclusion of one or more additional methylene groups between the amino and carboxyl group (e.g., α -amino, β -carboxy acids), or for the substitution of the amino or carboxy group by a similarly reactive group (e.g., substitution of the primary amine with a secondary or tertiary amine, or substitution of the carboxy group with an ester). The specific amino acid analogs may include but are not limited to the following.



L-pentafluorophenylalanine
(Phe(F5) or F(F5))2-fluoro-L-phenylalanine
(Phe(2F) or F(2F))3,4-difluoro-L-phenylalanine
(Phe(3,4-F2) or F(3,4-Fs))4-(guanidino)-L-phenylalanine
(Phe(p-guanidino) or F(4-guanidino))N-methyl-L-phenylalanine
(NMeF)(S)-2-amino-4-
(1H-tetrazole-5-yl)butanoic acid
(E(T))N-methyl-L-histidine
(NMeH)Sarcosine or
N-methylglycine
(Sar)

As used herein, "amino acid side chain" refers to a moiety attached to the α -carbon in an amino acid. For example, the amino acid side chain for alanine is methyl, the amino acid side chain for phenylalanine is phenylmethyl, the amino acid side chain for cysteine is thiomethyl, the amino acid side chain for aspartate is carboxymethyl, the amino acid side chain for tyrosine is 4-hydroxyphenylmethyl, etc. Other non-naturally occurring amino acid side chains are also included, for example, those that occur in nature (e.g., an amino acid metabolite) or those that are made synthetically (e.g., an α,α -disubstituted amino acid).

As used herein, "aromatic group", also referred to "arene", "aryl", or "aromatic" are chemical structures that comprise a conjugated planar ring system accompanied by delocalized pi-electron clouds in place of individual alternating double and single bonds. Aromatics must satisfy Huckel's rule, which states that only planar, fully conjugated monocyclic polyenes having $4n + 2 \pi$ electrons, where n is an integer, that is, $n = 0, 1, 2, 3, 4$, etc., should possess aromatic stability. Thus, an aromatic group must be planar and contain a cyclic cloud of π electrons below and above the plane of the molecule. It contains SP^2 hybridized carbon atoms and must obey the Huckel's rule. Phenylalanine, tyrosine, and tryptophan are naturally-occurring

amino acids comprising an aromatic group. Phe(F5), Phe(2F), Phe(3,4-F2), and Phe(p-guanidino) are examples of non-natural amino acids comprising an aromatic group, i.e., phenylalanine analogs.

As used herein, "co-administer" means that each of at least two different biological active compounds are administered to a subject during a time frame wherein the respective periods of biological activity overlap. Thus, the term includes sequential as well as co-extensive administration. When co-administration is used, the routes of administration need not be the same. The biological active compounds include macrocyclic peptides, as well as other compounds useful in treating cancer, including but not limited to agents such as vinca alkaloids, nucleic acid inhibitors, platinum agents, interleukin-2, interferons, alkylating agents, antimetabolites, corticosteroids, DNA intercalating agents, anthracyclines, and ureas. Examples of specific agents in addition to those exemplified herein, include hydroxyurea, 5-fluorouracil, anthracycline, asparaginase, bleomycin, dactinomycin, dacarbazine, cytarabine, busulfan, thiotepa, lomustine, mechlorethamine, cyclophosphamide, melphalan, mechlorethamine, chlorambucil, carmustine, 6-thioguanine, methotrexate, etc. The skilled artisan will understand that two different macrocyclic peptides may be co-administered to a subject, or that a macrocyclic peptide and an agent, such as one of the agents provided above, may be co-administered to a subject.

As used herein, "combination therapy" as used herein refers to treatment of a human or animal individual comprising administering a first therapeutic agent and a second therapeutic agent consecutively or concurrently to the individual. In general, the first and second therapeutic agents are administered to the individual separately and not as a mixture; however, there may be embodiments where the first and second therapeutic agents are mixed prior to administration.

As used herein, "conservative substitution" as used herein refers to substitutions of amino acids with other amino acids having similar characteristics (e.g. charge, side-chain size, hydrophobicity/hydrophilicity, backbone conformation and rigidity, etc.), such that the changes can frequently be made without altering the biological activity of the protein. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (*see, e.g., Watson et al. Molecular Biology of the Gene*, The Benjamin/Cummings Pub. Co., p. 224 (4th Ed.) (1987)). In addition, substitutions of structurally or functionally similar amino acids are less likely to disrupt biological activity. Exemplary conservative substitutions are set forth in Table 1.

Table 1			
Original residue	Conservative substitution	Original residue	Conservative substitution
Ala (A)	Gly; Ser	Leu (L)	Ile; Val
Arg (R)	Lys; His	Lys (K)	Arg; His
Asn (N)	Gln; His	Met (M)	Leu; Ile; Tyr
Asp (D)	Glu; Asn	Phe (F)	Tyr; Met; Leu
Cys (C)	Ser; Ala	Pro (P)	Ala
Gln (Q)	Asn	Ser (S)	Thr
Glu (E)	Asp; Gln	Thr (T)	Ser
Gly (G)	Ala	Trp (W)	Tyr; Phe
His (H)	Asn; Gln	Tyr (Y)	Trp; Phe
Ile (I)	Leu; Val	Val (V)	Ile; Leu

As used herein, "dose", "dosage", "unit dose", "unit dosage", "effective dose" and related terms refer to physically discrete units that contain a predetermined quantity of active ingredient (e.g., macrocyclic peptide) calculated to produce a desired therapeutic effect (e.g., death of cancer cells). These terms are synonymous with the therapeutically-effective amounts and amounts sufficient to achieve the stated goals of the methods disclosed herein.

As used herein, "macrocyclic peptide" refers to a peptide having cyclic structure formed by cyclization of at least eight amino acids. A macrocycle may be head-to-tail cyclized (HTC) in which the N-terminal amino acid is covalently linked to the C-terminal amino acid, for example the N- and C-terminal amino acids are each cystine residues, which are covalently linked together by a disulfide bond or the N- and C-terminal amino acids are each beta-alanine residues, which are covalently linked together by an amide bond. A macrocycle may be a side-to-tail cyclized (STC) wherein the side chain of the amino acid at or near the N-terminus is covalently linked to the C-terminal amino acid, for example, the N-terminus amino acid may be a lysine residue and the epsilon amino group of the lysine residue is linked by an amide bond to the hydroxy group comprising the carboxyl group of the C-terminus amino acid. A macrocycle may be a side-to-tail cyclized (STC) wherein the side chain of the amino acid at or near the N-terminus is covalently linked to the side chain of a C-terminal amino acid, for example, the N-terminus amino acid side chain may comprise an azido group and the C-terminal amino acid side chain may comprise an alkyne group, both of which may be induced to cyclize by forming a triazole linkage between the azido and alkyne groups.

As used herein, "macrocyclization reagent" or "macrocycle-forming reagent" as used herein refers to any reagent which may be used to prepare a macrocyclic peptide of the invention by mediating the reaction between two reactive groups. Reactive groups may be, for example, an azide and alkyne, in which case macrocyclization reagents include, without
5 limitation, Cu reagents such as reagents which provide a reactive Cu(I) species, such as CuBr, CuI or CuOTf, as well as Cu(II) salts such as Cu(CO₂CH₃)₂, CuSO₄, and CuCl₂ that can be converted in situ to an active Cu(I) reagent by the addition of a reducing agent such as ascorbic acid or sodium ascorbate.

Macrocyclization reagents may additionally include, for example, Ru reagents
10 known in the art such as Cp*RuCl(PPh₃)₂, [Cp*RuCl]₄ or other Ru reagents which may provide a reactive Ru(II) species. In other cases, the reactive groups are terminal olefins. In such embodiments, the macrocyclization reagents or macrocycle-forming reagents are metathesis catalysts including, but not limited to, stabilized, late transition metal carbene complex catalysts such as Group VIII transition metal carbene catalysts. For example, such catalysts are Ru and Os
15 metal centers having a +2 oxidation state, an electron count of 16 and pentacoordinated. Additional catalysts are disclosed in Grubbs et al., "Ring Closing Metathesis and Related Processes in Organic Synthesis" Acc. Chem. Res. 1995, 28, 446-452, and U.S. Pat. No. 5,811,515. In yet other cases, the reactive groups are thiol groups. In such embodiments, the macrocyclization reagent is, for example, a linker functionalized with two thiol-reactive groups
20 such as halogen groups.

As used herein, "cell-penetrating moiety", refers to any compound or peptide that is capable of transporting a bioactive cargo across the cell membrane without clear toxicity. Cell-penetrating peptides are an example of a peptide that is capable of transporting itself and a bioactive cargo attached to it across a cell membrane.

As used herein, "cell-penetrating peptides" (CPPs), also known as protein
25 transduction domains (PTDs), membrane translocating sequences (MTS), Trojan peptides (TP) or membrane transduction peptides (MTPs), means any peptide that has the ability to translocate itself and bioactive cargo attached to it across the cell membrane without clear toxicity (See Xu et al., J. Controlled Release 309: 106-124 (2019) and Kardani et al, Expert Opin. On Drug
30 Discovery 16: 1227-1258 (2019) for a review). CPPs consist of 30 or less amino acids and are classified as either cationic or amphipathic in nature. The CPPsite 2.0 is an updated version of database CPPsite, which contains around 1,855 unique cell-penetrating peptides (CPPs), which may be useful for transporting conjugates comprising a CPP covalently linked to a macrocyclic

peptide disclosed herein across the cell membrane (see Agrawal et al., CPPsite 2.0: a repository of experimentally validated cell penetrating peptides. Nucleic Acids Research doi: 10.1093/nar/gkv1266 (2015); Gautam et al., CPPsite: a curated database of cell penetrating peptides. Database (Oxford). Mar 7;2012:bas015 (2012)).

5 As used herein, “bioactive cargo” refers to a chemical entity, compound, peptide, polypeptide, or protein that has or elicits a biological effect when administered to a cellular, a human, or an animal subject. The macrocyclic peptides of the present invention may be referred to as bioactive cargos.

 As used herein, “pharmaceutically acceptable carrier” refers to a carrier, inert or
10 active, capable of making a composition having a macrocyclic peptide disclosed herein especially suitable for diagnostic or therapeutic use *in vivo* or *ex vivo*. A "pharmaceutically acceptable carrier" does not cause undesirable physiological effects when administered to a subject. One or more solubilizing agents can be utilized as pharmaceutical carriers for delivery of an active agent. Pharmaceutically acceptable carriers include, but are not limited to,
15 biocompatible vehicles, adjuvants, excipients, stabilizers, additives, and diluents to achieve a composition usable as a dosage form. In specific embodiments, the pharmaceutically acceptable carrier is an aqueous pH buffered solution. Pharmaceutically acceptable carriers include, but are not limited to, buffers, such as phosphate, citrate, acetate, and other organic acids; antioxidants, such as ascorbic acid; low molecular weight (*e.g.*, fewer than about 10 amino acid residues)
20 polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulin; hydrophilic polymers, such as polyvinylpyrrolidone; amino acids, such as glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates, including glucose, mannose, or dextrans; chelating agents, such as EDTA; sugar alcohols, such as mannitol or sorbitol; salt-forming counterions, such as sodium; and/or nonionic surfactants, such as TWEENTM,
25 polyethylene glycol (PEG), and PLURONICSTM. Examples of other pharmaceutically acceptable carriers include colloidal silicon oxide, magnesium stearate, cellulose, and sodium lauryl sulfate. Examples of other pharmaceutically acceptable carriers include sterile liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. Water is an exemplary carrier
30 when a composition (*e.g.*, a pharmaceutical composition) is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Pharmaceutically acceptable carrier further includes excipients (*e.g.*, pharmaceutical excipients). Examples of generally used excipients include,

without limitation: saline, buffered saline, dextrose, water-for-infection, glycerol, ethanol, and combinations thereof, stabilizing agents, solubilizing agents and surfactants, buffers and preservatives, tonicity agents, bulking agents, lubricating agents (such as talc or silica, and fats, such as vegetable stearin, magnesium stearate or stearic acid), emulsifiers, suspending or viscosity agents, inert diluents, fillers (such as cellulose, dibasic calcium phosphate, vegetable fats and oils, lactose, sucrose, glucose, mannitol, sorbitol, calcium carbonate, and magnesium stearate), disintegrating agents (such as crosslinked polyvinyl pyrrolidone, sodium starch glycolate, cross-linked sodium carboxymethyl cellulose), binding agents (such as starches, gelatin, cellulose, methyl cellulose or modified cellulose such as microcrystalline cellulose, hydroxypropyl cellulose, sugars such as sucrose and lactose, or sugar alcohols such as xylitol, sorbitol or maltitol, polyvinylpyrrolidone and polyethylene glycol), wetting agents, antibacterials, chelating agents, coatings (such as a cellulose film coating, synthetic polymers, shellac, corn protein zein or other polysaccharides, and gelatin), preservatives (including vitamin A, vitamin E, vitamin C, retinyl palmitate, and selenium, cysteine, methionine, citric acid and sodium citrate, and synthetic preservatives, including methyl paraben and propyl paraben), sweeteners, perfuming agents, flavoring agents, coloring agents, administration aids, and combinations thereof. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. In specific embodiments, "pharmaceutically acceptable carrier" means any pharmaceutically acceptable salt, ester, salt of an ester, pro-drug or other derivative of a macrocyclic peptide disclosed herein, which upon administration to an individual, is capable of providing (directly or indirectly) a macrocyclic peptide disclosed herein. Particularly favored pharmaceutically acceptable derivatives are those that increase the bioavailability of the macrocyclic peptide disclosed herein when administered to an individual (e.g., by increasing absorption into the blood of an orally administered macrocyclic peptide disclosed herein) or which increases delivery of the active compound to a biological compartment (e.g., the brain or lymphatic system) relative to the parent species. Some pharmaceutically acceptable derivatives include a chemical group which increases aqueous solubility or active transport across the gastrointestinal mucosa. Additional suitable pharmaceutical carriers, as well as pharmaceutical necessities for their use, are described in Remington's Pharmaceutical Sciences.

As used herein, "polypeptide" encompasses two or more naturally or non-naturally-occurring amino acids joined by a covalent bond (e.g., an amide bond). Polypeptides as described herein include full length proteins (e.g., fully processed proteins) as well as shorter amino acid sequences (e.g., fragments of naturally-occurring proteins or synthetic polypeptide

fragments).

As used herein, "stability" refers to the maintenance of a defined secondary structure in solution by a macrocyclic peptide of the invention as measured by circular dichroism, NMR or another biophysical measure, or resistance to proteolytic degradation in vitro or in vivo.

5 Non-limiting examples of secondary structures contemplated in this invention are α -helices, β -turns, and β -pleated sheets.

As used herein, "therapeutically effective amount" or "therapeutically effective dose" as used herein refers to a quantity of a specific substance sufficient to achieve a desired effect in a subject being treated. For instance, this may be the amount of macrocyclic peptide of the present invention necessary to inhibit phosphorylation of eIF4E and thus, attenuate the translation of eIF4E-sensitive mRNAs into proteins involved in the oncogenic pathways. It may also refer to the amount or dose of another therapeutic agent including, but not limited to, a chemotherapy agent, anti-cancer antibody, immune modulating antibody, or radiation administered to a subject that has cancer that is commonly administered to a subject to treat the cancer.

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As used herein, "treat" or "treating" as used herein means to administer a therapeutic agent, such as a composition containing any of macrocyclic peptides of the present invention, internally or externally to a subject or patient having one or more disease symptoms, or being suspected of having a disease, for which the agent has therapeutic activity or prophylactic activity. Typically, the agent is administered in an amount effective to alleviate one or more disease symptoms in the treated subject or population, whether by inducing the regression of or inhibiting the progression of such symptom(s) by any clinically measurable degree. The amount of a therapeutic agent that is effective to alleviate any particular disease symptom may vary according to factors such as the disease state, age, and weight of the patient, and the ability of the drug to elicit a desired response in the subject. Whether a disease symptom has been alleviated can be assessed by any clinical measurement typically used by physicians or other skilled healthcare providers to assess the severity or progression status of that symptom. The term further includes a postponement of development of the symptoms associated with a disorder and/or a reduction in the severity of the symptoms of such disorder. The terms further include ameliorating existing uncontrolled or unwanted symptoms, preventing additional symptoms, and ameliorating or preventing the underlying causes of such symptoms. Thus, the terms denote that a beneficial result has been conferred on a human or animal subject with a

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disorder, disease or symptom, or with the potential to develop such a disorder, disease or symptom.

As used herein, "treatment" as it applies to a human or veterinary individual, as used herein refers to therapeutic treatment, which encompasses contact of a macrocyclic peptide of the present invention to a human or animal individual who is in need of treatment with the macrocyclic peptide of the present invention.

When any variable (e.g., R^3 , X, etc.) occurs more than one time in any constituent or formula herein, its definition on each occurrence is independent of its definition at every other occurrence. Combinations of substituents and/or variables are permissible only if such combinations result in stable compounds. In choosing compounds of the present disclosure, one of ordinary skill in the art will recognize that the various substituents, i.e., R^3 , X, etc., are to be chosen in conformity with well-known principles of chemical structure connectivity and stability. Unless expressly stated to the contrary, substitution by a named substituent is permitted on any atom in a ring (e.g., aryl, a heteroaryl ring, or a saturated heterocycloalkyl ring) provided such ring substitution is chemically allowed and results in a stable compound. A "stable" compound is a compound which can be prepared and isolated and whose structure and properties remain or can be caused to remain essentially unchanged for a period of time sufficient to allow use of the compound for the purposes described herein (e.g., therapeutic or prophylactic administration to a subject).

Macrocyclic peptides active in eIF4E cap-binding site inhibition

The present invention relates to macrocyclic peptides that are cell-permeable and active in eIF4E cap-binding site inhibition. These macrocycle-c peptides bind eIF4E in the 'apo' or open conformation.

As shown in **Fig. 1A**, the cap-binding site, is flexible and undergoes an induced-fit mechanism from its initial resting 'apo' form into its final cap-bound 'closed' form (**Fig. 1A**). Instead of re-designing a small molecule to fit into eIF4E's traditional 'closed' form as many have tried, we asked the question if we could develop a drug that selectively binds the 'apo' form, instead (**Fig. 1B**). Fundamentally, by targeting the 'apo' form we would also be accessing a novel chemical space without the need for structural rearrangement from eIF4E (in particular, adjustments in the S4-H4 loop – remote from either the mRNA cap or eIF4G binding sites) (Volpon et al., EMBO J 25: 5138-5149 (2006)), and since the 'apo' form presents a bigger

surface area for binding, we envisioned the use of a small macrocyclic peptide (less than 2 kDa) to bind this unique conformation.

Macrocyclic peptides have remarkable potential in drug development (Craik et al., Chem. Biol. & Drug Design 81: 136-147 (2013)). In comparison to linear analogues, these peptides are conformationally restricted resulting in improved bioavailability, resistance to peptidases, and are capable of selectively binding protein surfaces often involved in clinically important protein-protein interactions (PPIs) in a manner similar to antibody-based therapeutics. On the other hand, macrocyclic peptides also share some similarities to small molecules; they are synthetically accessible, and are thus amenable to lead optimizations via traditional medicinal chemistry efforts. These advantages make them exceptional tools for modulating biochemical pathways (Pelay-Gimeno et al., Angewandte Chemie International Edition 54: 8896-8927 (2015)). To-date, however, there have been few successes of macrocyclic peptides as drugs or biological tools to target intracellular proteins due to poor cellular penetration and stability (Otvos et al., Frontiers in Chem. 2: 62 (2014); Matsson et al., J. of Med. Chem. 60: 1662-1664 (2017)).

1.1 eIF4E:EE-02 crystal structure and the structure-based design process:

EE-02 was the first compound designed to interact with eIF4E's cap-binding site in the 'apo' form (**Fig. 1B**). Comparison of the interactions demonstrated that the binding profile differed significantly from reported nucleotide mimics (**Fig. 1A**) (Soukariéh et al., Eur. J. Med. Chem. 124: 200-217 (2016); Chen et al., J. Med. Chem. 55: 3837-3851 (2012); Fischer, P. M. Cell Cycle 8: 2535-2541 (2009)). Subsequently, this opened up opportunities for macrocyclic peptide drug development using our structure-based design process (**Figs. 2A-6**). In this work, we have used at least two of three orthogonal binding assays selected from ThermoFluor (TF), Competitive Fluorescent Polarization (FP) using 5- Fluorescein (FAM)-labelled EE-02, and Isothermal Calorimetry (ITC) to compare the K_D s and de-risk the development process (Ng et al., ACS Med. Chem. Letts. 11: 1993-2001 (2020)).

2. Alanine scan and sequence truncation:

The macrocyclic peptide EE-02, possessing the linear 11 amino acid sequence ACEMGFFQDCG (read from N- to C-terminus) (SEQ ID NO: 41), was constrained via a disulfide bond between C2 and C10 to provide a nine amino acid circular peptide (SEQ ID NO: 1). It was hypothesized from its co-crystal structure with eIF4E that the interacting region of EE-

02 consists only of the internal residues flanked by the Cys (C) residues within the macrocycle from which the disulfide bond originated. Residues outside of this 'macrocycle' are hence not necessary for binding. Furthermore, results from alanine-scanning revealed that substituting Gln-8 (Q-8) with Ala (A) improved binding affinity. Further optimization of SEQ ID NO: 41 provided EE-44 (SEQ ID NO: 2) having the linear sequence CEMGFFADC (SEQ ID NO: 42), in which the original EE-02 sequence was truncated to exclude the residues outside the macrocycle (see Table 5). These changes had the favorable effect of shortening the sequence and lowering the molecular weight from the 1204 Da of EE-02 to the 1019 Da of EE-44 while maintaining the binding affinity (K_D). See Table 2 entries 1 and 2 for comparisons of TF (5.1 nM vs 2.1 nM), FP (11 nM vs 11 nM) and ITC (59.7 nM vs 52.9 nM) results for EE-02 and EE-44, respectively.

Table 2					
Linker Study and Evolution (with supporting X-ray crystal structures)					
#	Name	Sequence and linker	Binding affinity (K_D , nM)		
			TF*	FP*	ITC*
1	EE-02	A ¹ C ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ Q ⁸ D ⁹ C ¹⁰ G ¹¹ -NH ₂ Disulfide: Cys-S-S-Cys (SEQ ID NO: 1)	5.1 (DT = 12.7K)	11.0	59.7 (±6.8)
2	EE-44	C ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ Q ⁸ D ⁹ C ¹⁰ -NH ₂ Disulfide: Cys-S-S-Cys (SEQ ID NO: 2)	2.9 (DT = 13.0K)	11.0	52.9 (±11.1)
3	EE-48	C ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ Q ⁸ D ⁹ C ¹⁰ -NH ₂ Methylene bridge: Cys-S-CH ₂ -S-Cys (SEQ ID NO: 3)	1721.3 (DT = 6.8K)	2355.0	1421 (±267)
4	EE-91	Cyclo[βA ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ A ⁸ D ⁹ bA ¹⁰) Head-to-Tail cyclized (HTC) (SEQ ID NO: 4)	3867.4 (DT = 4.4K)	-	-
5	EE-94	Cyclo[βA ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ A ⁸ D ⁹ G ¹⁰) Head-to-Tail cyclized (HTC) (SEQ ID NO: 5)	1133.6 (DT = 6.3K)	2980.0	2305 (±264)

6	EE-108	Cyclo[εK ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ A ⁸ D ⁹) εK-Side-to-Tail cyclized (STC) (SEQ ID NO: 8)	888.6 (DT = 8.0K)	1115.9	1130 (±153)
7	EE-106	βA ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ A ⁸ A ⁹ G ¹⁰ (HTC) (SEQ ID NO: 6)	5519.8 (DT = 4.0K)	-	-
8	EE-107	εK ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ A ⁸ A ⁹ (εK-STC) (SEQ ID NO: 7)	1550.2 (DT = 5.8K)	9247.0	-
βA = beta-alanine; εK = epsilon-lysine (lysine linked via its side-chain amino group to C-terminal amino acid in an amide linkage); Semet = selenomethionine *TF: Thermofluor, *FP: Fluorescence Polarization, *ITC: Isothermal Calorimetry					

3. Disulfide replacement campaign:

Disulfide bonds are frequently reduced in the cell's reducing environment. One example of a reducing enzyme is glutathione reductase, which catalyzes the reduction of glutathione disulfide to glutathione—which is itself reducing and would frequently reduce other surface-exposed disulfide bonds. Other enzymes reduce disulfide bonds by removing reactive oxygen species (ROS) and oxidation products from the cytosol. These include both thiol and non-thiol-based enzymes: for example, superoxide dismutase and thioredoxin-dependent alkyl peroxidases (Carmel-Harel, O. S., G. Annu. Rev. Microbiol. 54: 439–61 (2000)). For a macrocyclic peptide-based therapeutic, it is imperative that the integrity of the macrocycle be maintained. In the case of EE-44, this meant that the disulfide macrocyclic linker would have to be replaced. Although the disulfide bond was initially assumed to be non-participating in EE-44's binding to eIF4E, as reported for macrocyclic peptides in previous works (Verdine et al., Chapter one - Stapled Peptides for Intracellular Drug Targets. In Methods in Enzymology, Wittrup, K. D.; Verdine, G. L., Eds. Academic Press: 2012; Vol. 503, pp 3-33; Walensky et al., J. Med. Chem. 57: 6275-6288 2014); Iegre et al., Chemical Science 9: 4638-4643 (2018); Empting et al., Angewandte Chemie International Edition 50: 5207-5211 (2011)), it was also possible for macrocyclic peptide linkers to participate in interactions (Stewart et al., Nature Chem. Biol. 6: 595 (2010); Phillips et al., J. Amer. Chem. Soc. 133: 9696-9699 (2011); Baek et al., J. Amer. Chem. Soc. 134: 103-106 (2012); Chang et al., Proc. Natl. Acad. Sci. (USA) 110: E3445 (2013); Wright et al., J. American Chem. Soc. 139: 13063-13075 (2017); Williams et al., Organic & Biomolec. Chem. 13: 4059-4063 (2015); Slaninov et al., In Peptides for the New

Millennium: Proceedings of the 16th American Peptide Symposium June 26–July 1, 1999, Minneapolis, Minnesota, U.S.A., Fields, G. B.; Tam, J. P.; Barany, G., Eds. Springer Netherlands: Dordrecht, 2002; pp 632-633). Our initial study of the interactions acquired from the crystal structure of eIF4E with EE-02 provided insights into the main interacting regions (i.e., the interacting sequence E³M⁴G⁵F⁶F⁷; SEQ ID NO: 139). However, the role of the other residues – in this case C², C¹⁰, A⁸, D⁹, and the disulfide bond itself – remain unclear. This was further supported by the crystal structure, where no direct interaction of the S-S with the binding pocket was observed upon first inspection (**Fig. 2A**). The initial study to replace the linker thus ignored the role of the disulfide bond. However, we soon discovered that the disulfide bond plays an indirect but significant role (*vide infra*) (Kilgore et al., J. Amer. Chem. Soc. 140: 17606-17611 (2018)).

The first attempted replacement was a methylene bridge linchpin (EE-48, **Fig. 2B**), a single carbon unit extension to link the cysteines (C² and C¹⁰), with a binding affinity (K_D) of 1421 nM (ITC). However, we eventually abandoned this linker due to its thermal instability during storage. The second attempted replacement was via Head-to-Tail Cyclization (HTC) by employing β-Ala and/or Gly residues, which would achieve the replacement of disulfide bonds with fewer changes to the linker length. Another important reason for using HTC was the removal of both N- and C- termini, further reducing polarity, charges, and molecular weight – all of which favor the development of a cell-permeable drug candidate. This resulted in HTC peptides termed EE-91 and EE-94, with EE-94 displaying the better binding affinity (K_D of 2305 nM, ITC) relative to the rest of the HTC peptides (**Fig. 2C**). Given the challenges in linker replacement, we decided to choose EE-48 (methylene bridged macrocyclic) and EE-94 (HTC macrocyclic) for co-crystallization trials with eIF4E, in the hopes of obtaining empirical structural data to improve our rational design.

3.1 Comparison between EE-02/44, EE-48 and EE-94:

The co-crystallized compound was analyzed to determine the nature of the loss of binding due to the disulfide replacements in EE-48 and EE-94. Although most of the interacting regions remained constant in all cases, three striking differences were observed when the X-ray crystal structures of EE-02, EE-48, and EE-94 bound to eIF4E were compared (**Fig. 2A-2C**): (1) The first difference was that the Glu-3 (E³) residue in EE-94 moved by about 1.2 Å away from its position in EE-02 when interacting with the Arg¹¹² (R¹¹²) residue of eIF4E. This might indicate a conformation bias in EE-02 which facilitates closer interaction of E³ and R¹¹². EE-48, on the

other hand, had its E³ residue interacting with R¹¹² in the same position as EE-02; (2) The second difference was that the different linker types triggered changes to the intramolecular H-bond interactions. All the structures studied, however, achieved the obligatory i, i+3 hydrogen bond between the backbone atoms of E³ (carbonyl O) and F⁶ (amide N, See Example 9). This is important for maintaining the type-II β turn adopted by the peptides, based on the dihedral angles of residues M⁴ (-55, 121) and G⁵ (110, -24); (3) The third difference is the interaction of the γ -carbonyl of C² in EE-02 with Arg¹⁵⁷ (R¹⁵⁷) of eIF4E. This important interaction, present in EE-02 binding with eIF4E but absent in EE-94 binding with eIF4E, would be best explained by both linker flexibility and non-canonical interactions. A recent study by Kilgore et al., J. Amer. Chem. Soc. 140: 17606-17611 (2018) described the resonance cascade from a nearby disulfide bond into the π^* orbital of neighboring carbonyl C=O bond. Applied to our system, the resonance contribution of EE-02's disulfide lone pair into the π^* orbital of C² γ -carbonyl leaves it more polarized, which subsequently interacts with the positively charged Arg¹⁵⁷ (R¹⁵⁷) in eIF4E's cap-binding site (**Fig. 3E**). In terms of inter-atomic distance (between guanidinium-N of R¹⁵⁷ and carbonyl-O of C²), this translates to 3.2Å in EE-02 (**Fig. 2A**), 3.4Å in EE-48 (**Fig. 2B**), and 5.0Å in EE-94 (**Fig. 2C**), with the corresponding ITC binding affinities (K_D) of 60 nM, 1421 nM, and 2305 nM, respectively.

3.2 Evolution of HTC peptides – putting back the N-terminus:

One of the mysteries of the sequence discovered through phage-display was the seemingly ‘non-interacting’ residues Gln⁸ and Asp⁹ (Q⁸ and D⁹) in the sequence. While changes in Q⁸ were tolerated, with Q8A being the best mutation (as in EE-44), changes to the D⁹ residue were not tolerated. As shown in Table 1, the mutation of D⁹ in EE-94 with A⁹ in EE-106 caused an obliteration of binding (K_D of 6432 nM and ΔT of 3.8K by TF). One possibility was that D⁹ participated in important intramolecular H-bonds pre- and post-binding. To investigate this intramolecular interaction, an ϵ -Lys residue was used to replace the Gly- β Ala linkage in EE-94 to encourage an intramolecular H-bond interaction with D⁹, thus affording EE-108 – accessed via a Side-to-Tail cyclization (STC) strategy (**Fig. 2D**). This would provide an equivalent macrocyclic system with the same ring size; the only exception would be the addition of an ‘N-terminus’ from the ϵ -Lys residue, and the replacement of the Gly- β Ala amide linkage with a more flexible alkyl linkage. Binding studies of EE-108 revealed much stronger binding affinities (K_D of 889 nM and ΔT of 8K by TF, 1115.9 nM by FP, and 1130 nM by ITC) than EE-48 and EE-94. Furthermore, analysis of the crystal structure for EE-108 with eIF4E revealed a new interaction between the N-

terminus of EE-108 and N⁵⁰ of eIF4E (**Fig. 2D**). Similar to previous observation with EE-106, substituting the D⁹ in EE-108 with A⁹ in EE-107 (i.e., D9A) significantly attenuated binding (EE-107: K_D of 1797nM and ΔT of 5.7K by TF). X-ray crystal structures were obtained for EE-108 bound to eIF4E (**Fig. 3A-3E**).

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4. Structure-Activity Relationship (SAR) of main interacting residues:

Guided by X-ray crystal structures, computational modelling (*in silico*) and NMR experiments for Chemical Shift Perturbation (CSP) study (Williamson, Progress in Nuclear Magnetic Resonance Spectroscopy 73: 1-16 (2013)), we prepared analogues of different mutations of Glu³ (E³), Phe⁶ (F⁶), Phe⁷ (F⁷), and Met⁴ (M⁴) (Table 3).

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Table 3 Main Interacting Residues SAR				
Name	Sequence	Binding affinity (K _D , nM)		
		TF*	FP*	ITC*
EE-94	βA ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ A ⁸ D ⁹ G ¹⁰ (HTC) (SEQ ID NO: 5)	1133.6 (DT = 6.3K)	2980.0	2305 (±264)
EE-108	εK ² E ³ M ⁴ G ⁵ F ⁶ F ⁷ A ⁸ D ⁹ (εK-STC) (SEQ ID NO: 8)	888.6 (DT = 8.0)	1115.9	1130 (±153)
EE-122	εK ² E ³ Hse(OMe) ⁴ G ⁵ F ⁶ F ⁷ A ⁸ D ⁹ (εK-STC) (SEQ ID NO: 9)	10380.8 (DT = 4.1K)	51420	-
EE-123	εK ² E ³ Nle ⁴ G ⁵ F ⁶ F ⁷ A ⁸ D ⁹ (εK-STC) (SEQ ID NO: 10)	7587.2 (DT = 4.7K)	17340	-
EE-124**	εK ² E ³ Semet ⁴ G ⁵ F ⁶ F ⁷ A ⁸ D ⁹ (εK-STC) (SEQ ID NO: 11)	584.0 (DT = 9.1K)	530.8	622.1 (±109.7)
EE-130	εK ² E ³ Semet ⁴ G ⁵ F(F _S) ⁶ F ⁷ A ⁸ D ⁹ (εK-STC)	443.6 (DT = 10.3K)	643.1	360.8 (±48.7)

	(SEQ ID NO: 15)			
EE-131	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(F_5)^7 A^8 D^9$ (ϵK -STC) (SEQ ID NO: 16)	1913.0 (DT = 7.8K)	1330.0	828.7 (± 106.0)
EE-132	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^7 A^8 D^9$ (ϵK -STC) (SEQ ID NO: 17)	1082.5 (DT = 8.6K)	1380	-
EE-133	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(3,4-F_2)^7 A^8 D^9$ (ϵK -STC) (SEQ ID NO: 18)	1121.5 (DT = 8.8K)	1378	-
EE-129	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 A^8 D^9$ (ϵK -STC) (SEQ ID NO: 12)	130.3 (DT = 11.8K)	126.2	169.7 (± 13.6)
βA = beta-alanine; ϵK = epsilon-lysine (lysine linked via its side-chain amino group to C-terminal amino acid in an amide linkage); Semet = selenomethionine; F(F₅) = Phe with 5 fluoride atoms F; F(2-F) = Phe with fluoride on C2; Hse(OMe) = O-methyl-L-homoserine ; Nle = norleucine; *TF: Thermofluor, *FP: Fluorescence Polarization, *ITC: Isothermal Calorimetry **EE-124 (PDB: XYZ formats) X-ray co-crystal structure with eIF4E obtained				

Met⁴ (M⁴):

Analogues targeting the interactions between M⁴ and eIF4E cap-binding site's deep hydrophobic pocket were studied (**Fig. 3A**). From *in silico* analysis, M⁴ was postulated to interact with W⁴⁶, F⁴⁷, F⁴⁸, D⁹⁰, Y⁹¹ and S⁹². The M⁴ analogues studied thus used different atoms and groups as more favorable substitutes for the sulfur in methionine; namely an oxygen atom [O-methyl-L-homoserine (Hse(OMe)), EE-122, Table 2, entry 3], a methylene group (Nle, EE-123, Table 2, entry 4), and the more *in vivo* redox-stable selenium atom (Semet, EE-124, Table 2, entry 5) (Snider et al., Biochem. 52: 5472-5481 (2013); Reich et al., ACS Chem. Biol. 11: 821-841 (2016); Le et al., Biochem. 47: 6685-6694 (2008)). From this study, we discovered EE-124, the selenomethionine (Semet⁴) analogue of M⁴ in EE-108 (K_D of 584 nM and ΔT of 9.1 K by TF, 530.8 nM by FP, and 622 nM by ITC, Table 2, entry 5). The oxygen variant in EE-122, i.e., M⁴

to Hse(OMe) mutation, and the alkyl chain variant in EE-123, i.e., M⁴ to Nle mutation both obliterated binding—indicating a preference for ‘softer’ atoms such as sulfur and selenium that possess empty ‘d’-orbitals, presumably due to interactions with the Lewis basic oxygen (O) of S⁹² in eIF4E (Komatsu et al., Chem. Commun. 1999(2), 205-206 (1999); Zhang et al., J. Chem. Inform. Model. 55: 2138-2153 (2015)). This interaction with S⁹² was further corroborated in a ¹³C, ¹⁵N eIF4E ¹H-¹⁵N HSQC NMR experiment with EE-124 (see **Fig. 4A-4B** and Example 10). These NMR studies, however, did not support EE-124’s interaction with F⁴⁷, Y⁹¹ or D⁹⁰.

Phe⁶ (F⁶):

The aromatic pocket (bounded by W¹⁰², W¹⁶⁶ and H²⁰⁰) where F⁶ binds W¹⁰² could accommodate a slightly larger ring (**Fig. 3B**). Furthermore, the π - π interactions present in this pocket were imperfect. Perfluorination of the phenyl group in F⁶ would reverse the quadrupole moment and polarity of the phenyl ring—making novel interactions that were hypothesized to have improved binding. This led us to EE-130, which has a perfluorinated phenyl group on the F⁶ position, Phe(F₅), giving a K_D of 361 nM by ITC (Table 2, entry 6), a two-fold improvement from EE-124.

Phe⁷ (F⁷):

The aromatic pocket where F⁷ interacts with W⁵⁶ and F⁴⁸ is tight, and has an edge-to-face π - π interaction with F⁴⁸ (**Fig. 3C**). We hypothesized that employing a 2-fluoro-Phe [i.e. Phe(2-F)] on the F⁷ position would enhance the polarity of the aromatic ring and favor a buildup of positive charges (δ^+) on the remaining unsubstituted protons, especially the proton *para*- to the fluorine. This would enhance the edge-to-face π - π stacking interactions that already exist with F⁷. This presented us with EE-129 which has Phe(F₅) on the F⁶ position and Phe(2-F) on the F⁷ position, with a K_D of 130.3 nM and ΔT of 11.8K by TF, 126.2 nM by FP, and 169.7 nM by ITC (Table 2, entry 10), a further two-fold improvement from EE-130. The stronger binding affinity for EE-129 could be attributed to its interaction with eIF4E’s aromatic pocket, as observed on eIF4E ¹H-¹⁵N HSQC NMR experiments with EE-129 (see **Fig. 4A-4B** and Example 10).

Glu³ (E³) & Gly⁵ (G⁵):

Attempts to optimize binding residues E³ and G⁵ proved futile. For the E³ position (**Fig. 3D**), employing a longer homo-glutamic acid interrupted binding. For the G⁵ position, employing alanine and sarcosine mutations did not yield positive results (see **Table 4**).

¹H-¹⁵N HSQC SAR study:

Intriguingly, the ¹H-¹⁵N HSQC NMR experiments revealed that both EE-124 and EE-129 do not interact with R¹⁵⁷. This phenomenon was further corroborated by *in silico* studies, and might explain the weaker binding affinities of the STC peptides (EE-124 and EE-129) versus the disulfide linked peptides (EE-02 and EE-44), even after SAR evolution. In fact, no NMR chemical shifts in the traditional phosphate binding region (R¹⁵⁷, K¹⁵⁹ and K¹⁶²) was observed. Unfortunately, because the NMR conditions employs dithiothreitol (DTT), a reducing agent, we could not perform the same experiments on EE-02 and EE-44. Only R¹¹² was observed to interact with both EE-124 and EE-129, presumably via the E³ residue. Overall, from the NMR experiments, ¹H-¹⁵N HSQC perturbations were observed for W⁴⁶, F⁴⁸, K⁴⁹, N⁵⁰, W⁵⁶, G⁸⁸, C⁸⁹, S⁹², E⁹⁹, M¹⁰¹, W¹⁰², R¹⁰⁹, G¹¹⁰, R¹¹², G¹⁵¹, C¹⁷⁰, E¹⁷¹, S¹⁹⁹, D²⁰², A²⁰⁴ and K²¹² when equimolar amount of either EE-124 or EE-129 was introduced (see **Fig. 4A-4B** and Example 10).

5. Permeability evolution:

There were two separate goals for this aspect. The first was to use cell-penetrating peptides (CPPs) to prepare cell-active peptide conjugates as biological tool compounds, and positive controls in our cell assays (**Fig. 5A**). This is necessary due to the current lack of commercially available or published tool compounds for eIF4E's cap-binding site, which are both robust and cell-active. To conjugate our macrocyclic peptides onto CPPs, we opted for copper-catalyzed azide-alkyne cycloadditions (CuAAC) 'click' reactions. This was done by installing an azidolysine, or K(N₃), onto the more tolerant A⁸ position, i.e. A8K(N₃) mutation. Correspondingly, the CPP would carry a *D*-propargylglycine with a PEG₂ linker. For this study, we have tried various CPPs reported in literature (Copolovici et al., ACS Nano 8: 1972-1994 92014)), and found poly(*D*-Arg)₁₀ (SEQ ID NO: 37) or *d*R₁₀ (SEQ ID NO: 37) to be the CPP of choice, with the best IC₅₀ and the least amounts of LDH release in our cellular assays. This provided us EE-171 – derived from the conjugation of *D*-Pra-PEG₂-*d*R₁₀ (SEQ ID NO: 37) with EE-129-N₃ via 'click' chemistry (Table 4, entry 1) – which binds eIF4E with a K_D of 20nM by FP and 70.1nM by ITC (Table 4, entry 2). This improvement of binding affinity in EE-171 from the parent peptide EE-129 N₃ (K_D = 94nM by FP) indicated either extra interactions by the poly-*D*-Arg₁₀ (SEQ ID NO: 37) CPP conjugate, or better solubility.

Table 4 “Non-Interacting” Residues SAR & Permeability Development with Cell Assay							
Name	Sequence*	Binding affinity (K _D , nM)		NanoCLICK EC ₅₀ ratio**		NanoBIT IC ₅₀ (mM)****	
		FP*	ITC*	4h	18h	18h	LDH release
EE-129-N ₃	εK ² E ³ Semet ⁴ G ⁵ F(F ₅) ⁶ F(2-F) ⁷ K(N ₃) ⁸ D ⁹ (εK-STC) (SEQ ID NO: 13)	94	-	>1	>1	>100	>100
EE-171	εK ² E ³ Semet ⁴ G ⁵ F(F ₅) ⁶ F(2-F) ⁷ K[triazole- <i>d</i> Pra-PEG ₂ - <i>d</i> R ₁₀] ⁸ D ⁹ (εK-STC) (SEQ ID NO: 38)	20	70.1 (±15.6)	N.A.	N. A.	3.0	19.2
All- <i>D</i> -EE-171	εk ² e ³ <i>D</i> -Semet ⁴ <i>D</i> -G ⁵ <i>D</i> -F(F ₅) ⁶ <i>D</i> -F(2-F) ⁷ k[triazole <i>d</i> Pra-PEG ₂ - <i>d</i> R ₁₀] ⁸ d ⁹ (εk-STC) (SEQ ID NO: 39)	NB	-	N.A.	N. A.	79.7	16.9
EE-168-N ₃	εK ² E ³ Semet ⁴ G ⁵ F(F ₅) ⁶ F(2-F) ⁷ K(N ₃) ⁸ H ⁹ (εK-STC) (SEQ ID NO: 25)	99	-	>1	0.4 9	62.0	>100
EE-169-N ₃	εK ² E ³ Semet ⁴ G ⁵ F(F ₅) ⁶ F(2-F) ⁷ K(N ₃) ⁸ L ⁹ (εK-STC) (SEQ ID NO: 26)	426	384.1 (±189.9)	0.38	0.0 6	>100	>100
***EE-169-N ₃ +Endo-Porter	εK ² E ³ Semet ⁴ G ⁵ F(F ₅) ⁶ F(2-F) ⁷ K(N ₃) ⁸ L ⁹ (εK-STC) (SEQ ID NO: 26)	426	-	-	-	9.7	>100
EE-206-N ₃	εK ² E ³ Semet ⁴ G ⁵ F(F ₅) ⁶ F(2-F) ⁷ K(N ₃) ⁸ F(4-Guanidino) ⁹ (εK-STC)	4009	-	0.36	0.0 7	>100	>100

	(SEQ ID NO: 27)						
EE-209-N ₃	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 F(4\text{-Guanidino})^8 K(N_3)^9$ (ϵK -STC) (SEQ ID NO: 28)	Insol.	-	0.04	0.01	Insol.	Insol.
EE-233-N ₃	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 K(N_3)^8 h^9$ (ϵK -STC) (SEQ ID NO: 30)	123	86.5 (± 37.9)	0.93	0.20	45.1	>100
EE-231-N ₃	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 K(N_3)^8 \text{NMe-H}^9$ (ϵK -STC) (SEQ ID NO: 29)	1543	-	0.33	0.12	>100	>100
EE-244-N ₃	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 K(N_3)^8 \text{NMe-h}^9$ (ϵK -STC) (SEQ ID NO: 32)	NB	-	-	-	>100	>100
EE-242	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 F(4\text{-Guanidino})^8 G^9$ (ϵK -STC) (SEQ ID NO: 31)	36	-	N.A.	N.A.	>100	>100
EE-246	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 \text{Dab}^8 h^9$ (ϵK -STC) (SEQ ID NO: 33)	25	50.1 (± 14.3)	N.A.	N.A.	>100	>100
EE-249	$\epsilon K^2 E^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 A^8 h^9$ (ϵK -STC) (SEQ ID NO: 34)	44	104.0 (± 53.5)	N.A.	N.A.	>100	>100
EE-260	$\epsilon K^2 E(T)^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 F(4\text{-Guanidino})^8 G^9$ (ϵK -STC) (SEQ ID NO: 35)	846	-	N.A.	N.A.	>100	>100
EE-261	$\epsilon K^2 E(T)^3 \text{Semet}^4 G^5 F(F_5)^6 F(2-F)^7 \text{Dab}^8 h^9$ (ϵK -STC) (SEQ ID NO: 36)	NB	-	N.A.	N.A.	>100	>100
ϵK = epsilon-lysine (lysine linked via its side-chain amino group to C-terminal amino acid in an amide linkage); Semet = selenomethionine; F(F ₅) = Phe with 5 fluoride atoms F; F(2-F) =							

Phe with fluoride on C2; F(4-guanidino) = Phe with guanidino group on C-4; Hse(OMe) = O-methyl-L-homoserine; Dab = Diaminobutyric acid; E(T) = Amino-4-(1H-tetrazol-5-yl)butanoic acid; K(N3) = lysine with an epsilon azido group;

NMe-H = N-methyl-His; K[triazole-*d*Pra-PEG₂-*d*R₁₀ (SEQ ID NO: 37) = K(N₃) azido group in triazole linkage with alkyne group of *d*Pra-PEG₂-Arg₁₀ (SEQ ID NO: 37); lower case amino acids are D-amino acids;

*TF: Thermofluor; *FP: Fluorescence Polarization; *ITC: Isothermal Calorimetry; NB = No Binding; Insol. = solubility issues; lower case letters for *D*-amino acids;

**NanoCLICK is a target-agnostic permeability assay using ATSP-7041 as a positive control, with EC₅₀ ratio of 0.01; Higher EC₅₀ ratios indicate lesser cellular penetration, with a maximum ratio of 1.00 against the impermeable control EEE-ATSP-7041.

***Addition of external 8 μM Endo-Porter peptide-based delivery agent.

****NanoBIT assay developed from our previous work Frosi et al., BMC Biol. 17: 40 (2019). As a control for the experiment, we have also performed NanoBIT assays on digitonin-permeabilized cells (see Example 13).

5.1 Cellular activity of EE-171 tool compound:

EE-171 was found to be an effective tool compound for both our NanoBIT assay (IC₅₀ 18 hour = 3.0 μM, Table 4, entry 2) and in inhibiting eIF4E phosphorylation when

5 incubated with HEK293F cells for one and four hours, in 10% FCS (**Fig. 5A**). EE-171 was subsequently observed to have comparable effects on inhibiting eIF4E phosphorylation as with other drug compounds, such as (1) Mitogen-Activated Protein Kinase (MAPK) interacting protein kinase or Mnk kinase inhibitor CGP4073 (Andersson et al., Cytokine 33: 52-57 (2006)),³⁵ (2) eIF4G inhibitor 4EGI-1 (Moerke et al., Cell 128: 257-267 (2007)), (3) eIF4A inhibitor

10 Silvestrol (Pelletier et al., Cancer Res 75: 250-263 (2015)), and (4) Mammalian Target Of Rapamycin (mTOR) inhibitor PP242 (**Fig. 5C**) (Hoang et al., J. Biol. Chem. 287: 21796-21805 (2012)). In addition, LDH release assay indicated that EE-171 had a reasonable therapeutic window (LDH release EC₅₀18h = 19.2 μM). Cellular effects of EE-171 was also shown to be specific through the use of an all-*D*-EE-171 control compound ('click' chemistry conjugation

15 between all-*D*-EE-129 N₃ and *d*R₁₀ (SEQ ID NO: 37)) that does not bind eIF4E (IC₅₀18h = 79.7 μM, Table 3, entry 3).

5.2 Passive permeability development:

The second permeability evolution goal was to develop passive permeability. Besides the introduction of fluorinated aromatics in F⁶ and F⁷ positions of the macrocycle during the binding SAR (**Fig. 3B-3C**), which conveniently served to improve both binding and lipophilicity (Gillis et al., J. Med. Chem. 58: 8315-8359 (2015)), we additionally embarked on a focused campaign to further improve lipophilicity and hence cell membrane permeability. This involved: (1) an N-methylation scan, followed by (2) mutations of the non-interacting residues on positions A⁸ and D⁹, with the help of computational modelling, to identify modifications which would help improve passive permeability.

5.2.1 N-methylation scan:

Methylation of amides on the backbone is known to improve lipophilicity and oral bioavailability of macrocyclic peptides (Räder et al., Bioorg. & Med. Chem. 26: 2766-2773 (2018)). Thus, we have performed a scan of the different positions available for N-methylation, and found only the D⁹ position to be tolerant. In fact, the K_D was marginally improved for EE-155 (K_D: 151.1 nM and ΔT of 12.7K by TF, 56.1 nM by FP, and 150 nM by ITC), the D9NMeD mutation (**Fig. 5B**). However, while N-methylation of D⁹ was tolerated, mutating this to other residues (e.g. H⁹ and D-H⁹ in EE-231 and EE-244, respectively) caused the N-methylation to be less tolerated (Table 4). In summary, the N-methylation scan unfortunately revealed that most of the amide N-H in the backbone of the macrocyclic peptides were necessary for binding (whether directly or indirectly, *vide infra*).

5.2.2 Mutations of the non-interacting residues on positions A⁸ and D⁹:

To develop passive permeability, we focused on increasing the net charge of the macrocyclic peptides to zero or net-positive values. To identify residues which would retain binding but improve lipophilicity, we utilized an affinity selection–mass spectrometry (AS–MS) platform, Automated Ligand Identification System (ALIS), allowing us to study quickly libraries presenting mutations at each ‘non-interacting’ position (i.e., A8X and D9X libraries, see Example 20) (Annis et al., Curr. Opinion Chem. Biol. 11: 518-526 (2007); Annis et al., J. Amer. Chem. Soc. 126: 15495-15503 (2004); Kutilek et al., J. of Biomolecular Screening 21: 608-619 (2016)). To further complete the analysis, we also performed a protein titration experiment in which the concentration of the peptides is kept constant while the concentration of the protein is decreased thus making the competition between binders more stringent. At the lowest

concentration of the protein, only the highest affinity ligands remain bound to the protein. Results from the ALIS screening were then studied via computational modelling to identify peptides with the best binding and cell-permeability. A separate target-agnostic live HELA cell assay, termed NanoCLICK, was also performed to measure permeability directly via the azidolysine group [K(N₃)⁸] on the attached peptides (calculated as EC₅₀ ratios against an impermeable control, see Example 11, Table 4 entries 1 and 4-11).

Through these studies, we highlighted *D*-His (*d*H⁹) as a viable mutation to D⁹, giving us EE-233, a D9*d*H mutant with a K_D of 123 nM by FP and 86.5 nM by ITC, NanoCLICK EC₅₀ 18 hour ratio of 0.20 and cellular activity of 45 μM IC₅₀ (Table 4, entry 9). N-methylation of this residue (*d*H⁹) or the opposite enantiomer (H⁹), however, obliterated binding (EE-231 and EE-244, Table 3, entries 10 and 11). This is in contrast to what we observed previously with D⁹ in EE-155, where N-methylation of this position was tolerated (**Fig. 5B**). Replacing the azidolysine K(N₃)⁸ in EE-233 with A⁸ in EE-249, i.e., K(N₃)8A mutation, improved binding to 44 nM by FP and 104.0 nM by ITC (Table 4, entry 14), but completely obliterated cellular activities (with NanoBIT IC₅₀ greater than 100 μM). The same effect was found in EE-246, wherein the azidolysine K(N₃)⁸ was replaced with 2,4-diaminobutyric acid (Dab⁸), which has a stronger binding affinity (K_D) of 25nM by FP and 50.1nM by ITC (Table 4, entry 13) but has no cellular activity. This result indicated that the azido functionality in EE-233 confers enhanced permeability (perhaps through improved lipophilicity) to the macrocyclic peptide (Robins et al., J. Med. Chem. 32: 1763-8 (1989); Bliss et al., J. Chem. Soc., Perkin Transactions 1: 2217-2228 (1987)). The most permeable peptide with equivalent NanoCLICK EC₅₀18h ratio (0.01) as the positive control, ATSP-7041 (see WO2013123266; Chang et al., Proc. Natl. Acad. Sci. (USA) 110: E3445 (2013), was EE-209 (Table 4, entry 8). The key residue driving this drastic improvement in permeability was thought to be the *para*-guanidino-phenylalanine or F(*p*-guanidino), placed on position-8, with K(N₃) on position-9. When compared to EE-206 (Table 4, entry 7), wherein the same residues on positions 8 and 9 were swapped, the NanoCLICK EC₅₀18h ratio worsened sevenfold to 0.07. This was explained by modelling. These peptides, however, had no cellular activities due to a combination of solubility issues and weaker binding to eIF4E. When repeated on permeabilized cells (with digitonin), we observed modest cellular activities (NanoBIT_{digitonin} EC₅₀ 4 hour of 5647.6 nM and 4178.9 nM for EE-206 and EE-209, respectively, when compared to EE-233 with NanoBIT_{digitonin} EC₅₀ 4 hour of 701.5 nM). Replacing the K(N₃)⁹ group in EE-209 with glycine (G⁹) rescued binding (FP K_D = 36nM, Table

4, entry 12) but with no accompanying cellular activity on NanoBIT – again implying the role that azidolysine plays in imparting permeability.

Other hydrophobic peptides, such as EE-169, with poor solubilities (less than 5 μM in pH 7) were also carried into cells through the addition of formulation delivery vehicles, such as Endo-Porter (Table 4, entries 5-6); while EE-169 alone was not shown to be cell active on our NanoBIT assay (although it has NanoCLICK EC_{50} ratio of 0.06, with K_D of 426 nM by FP and 384.1 nM by ITC), it displayed an IC_{50} of 9.7 μM in our NanoBIT assay on HEK293F cells when used in-combination with 8 μM of Endo-Porter.

Last but not least, we attempted a final replacement of the glutamic acid (E^3) in EE-242 and EE-246 with 2-amino-4-(1H-tetrazol-5-yl)butanoic acid, or $\text{E}(\text{T})^3$, a tetrazole-substitute and known bioisostere of glutamic acid, affording EE-260 and EE-261, respectively. However, these attempts significantly attenuated binding affinities (Table 4, entries 15-16). In conclusion, only EE-233 performed modestly well in the NanoBIT assay. Other peptides require assistance from CPPs (EE-171) or formulation strategies (EE-169 + Endo-Porter). Furthermore, all peptides studied in this work had good cytosolic stability, as studied in assays using HELA cell extracts ($T_{1/2} > 6.213 \text{ h}$).

6. Cell-Penetrating moieties

The present invention further includes embodiments in which the macrocyclic peptides disclosed herein are conjugated to a cell-penetrating moiety, which enable the macrocyclic peptide to cross the cell and nuclear membranes. Cell-penetrating moieties may comprise a cell-penetrating peptide (CPP) that can naturally cross the lipid bilayer membrane that protects the cells. CPPs share common structural and physicochemical features: they contain a sequence length between 5 and 42 amino acids, (2) they are soluble in water and partially hydrophobic, (3) they are often cationic (positive charge at physiological pH) or amphipathic, and (4) they are rich in the arginine and lysine residues (Derakhshankhah & Jafari, *Biomed. Pharmacother* 108:1 090–1096 (2018); Milletti, *Drug Discov. Today*: 17: 850–860 (2012)). CPPsite 2.0 is an updated version of database CPPsite, it contains around 1855 unique cell-penetrating peptides (CPPs), which may be useful for transporting conjugates comprising a CPP covalently linked to a macrocyclic peptide disclosed herein across the cell membrane (see Agrawal et al., *CPPsite 2.0: a repository of experimentally validated cell penetrating peptides. Nucleic Acids Research* doi: 10.1093/nar/gkv1266 (2015); Gautam et al., *CPPsite: a curated database of cell penetrating peptides. Database (Oxford)*. Mar 7;2012:bas015 (2012)).

Exemplary CPPs are also disclosed in Deshayes et al., Cell. Mol. Life Sci. 62: 1839–1849 (2005); Xu et al., J. Controlled Release 309: 106-124 (2019); and, Hu et al., J. Phys. Chem. B 123: 2636–2644 (2019). Poly(Arg) polymer CPP have been disclosed in U.S. Patent Nos. 9,527,895; 9,220,744; 9,314,535; 10,494,412; 10,538,784; and 10,646,540.

- 5 Examples of CPPs that may be used in the present invention include, but are not limited to, those shown in Table 5 below.

Table 5			
CPP	Sequence	Ref.	SEQ ID NO:
Tat (48–60)	GRKKRRQRRRPPQ	69	48
Tat (47–57)	YGRKKRRQRRR	1	49
Tat (46–57)	CYGRKKRRQRRR	2	50
Tat (49–57)	RKKRRQRRR	3	51
HIV-1 Rev (34–50)	TRQARRNRRRRWRERQR	4, 68	52
Penetratin (Antp)	RQIKIWFQNRRMKWKK	5	53
pVEC	LLIILRRRIRKQAHASK	6	54
M918	MVTVLFRRRLRIRACGP PRVRV	71	55
ARF(1–22)	MVRRFLVTLRIRACG PPRVRV	7	56
Mastoparan	INLKALAALAKKIL-amide	9,10	57
TP10	AGYLLGKINLKALAALAKKIL	10	58
NLS	CGYGPKKKRKVGG	66	59
NLS	PKKKRKV	11	60
LDP-NLS	KWRRKLKKLRPKKKRKV	72	61
LDP	KWRRKLKKLR	72	62
hCT(9–32)	LGTYTQDFNKFHTFPQTAIGVGAP	12	63
DPV3	RKKRRRESRKKRRRES	13	64
Secretin	HSDGTFTSELSRLRDSARLQRLQLV	14	65
LL-37	LLGDDFRKSKEKIGKEFK RIVQRIKDFLRNLVPRTES	15	66
Lactoferrin sequences	SQPEATKCFQWQRNMRKVRGPPVSCI KRDSPIQI	16	67
RGD	GRGDSY	17	68
Sweet arrow peptide (SAP)	(VRLPPP)3	67	69
hLF	KCFQWQRNMRKVRGPPVSCI	67	70
Bac7 (1–24)	RRIRPRPPRLPRPRPLPFPRPG	18	71
Buforin IIb	RAGLQFPVG[RLLR]3	19	72
sC18	GLRKRLRKFRNKIKEK	20	73
Protegrin-1	RGGRLCYCRRRFCVCVGR	21, 79	74
BPrPp (1–28)	MVKSKIGSWILVLFVAMWSDVGLCK KRP	22	75
DPRSFL	DPRSFL	23	76
VP22	NAATATRGRSAASRPTQRPRAPARSA	24	77

	SRPRRPVQ		
Transcription factor (267–300)	DAATATRGRSAASRPTE	24	78
VP22	RPRAPARSASRPRRPVE		79
vT5	DPKGDPKGVTVTVTVTVTGKGDPKPD	25	80
FGF	PIEVCMYREP	26	81
C105Y	CSIPPEVKFNKPFVYLI	27	82
p28	LSTAADMQGVVTDGMASGLDKDYL KPDD	67	83
PFV	PFVYLI	28	84
SG3	RLSGMNEVLSFRWL	29	85
Pep-7	SDLWEMMMVSLACQY	30	86
CyLoP-1	CRWRWKCKK	31	87
MK2i	WLRRKAWLRRRIKALNRQLGVAA	32	88
Influenza HA-2	GLFGAIAGFIENGWEGMIDGWYG	33	89
Influenza HA-2 (1–20) KALA sequence	WEAKLAKALAKALAKHLAKALAKALK ACEA	34-37	90
p28	LSTAADMQGVVTDGMASGLDKDYL KPDD	38	91
CPP-C	PIEVCMYREP	26	92
Bax-inhibiting peptides (BIP)	VPTLK	65	93
PTD-5	RRQRRTSKLMKR	39	94
q-NTD	KGRKKRRQRRRPPQ	40	95
FHV coat (35–49)	RRRRNRTRNRNRVR-amide	4,68	96
KLA sequence	Acetyl-KLALKLALKALKAAALKLA-amide	35, 41-43	97
Translocation motif (TLM)	PLSSIFSRIQDP	73	98
Substance P and analogs	RPKPQQFGLM-amide	35, 44, 45	99
Crotamine	YKQSHKKGGKKGSG	46-48	100
R9	RRRRRRRRR	49	101
ppTG1	GLFKALLKLLKSLWKLKLLKA	76	102
KALA	WEAKLAKALAKALAKHLAKALAKAL KACEA	76	103
Pen-Arg	RQIRIWFQNRMRWRR	50	104
R6H4	RRRRRRHHHH	77	105
CADY	GLWRALWLLRSLWLLWRA	51	106
KAFK	KAFKLAARLYRKALARQLGVAA	52	107
Pep-1	KETWWETWWTEWSQPKKKRKV- Cysteamine	53	108
ppTG20	GLFRALLRLLRSLWLLLLRA	76	109
BR2	RAGLQFPVGRLLRLLR	54	110
R4	RRRR	4	111
R6	RRRRR	4	112
R10	RRRRRRRRR	4	113
R12	RRRRRRRRRRR	4	114

MPG	GALFLGWLGAAGSTMGAPKKRKV	55	115
HR9	CHHHHHRRRRRRRRHHHHC	56	116
Pep-3	ac-KWFETWFTWPKKRK-Cysteamine	57	117
4K	KKKK	58	118
MPG β	ALFLGFLGAAGSTMGAWSQPKKRKV	78	119
R8 (8-Arginine)	RRRRRRRR	3	120
8-Lysine	KKKKKKKK	58	121
6K	KKKKKK	58	122
10K	KKKKKKKKKK	70	123
12K	KKKKKKKKKKKK	58	124
5RQ	RRQRR	58	125
8RQ	RRQRRQRR	58	126
11RQ	RRQRRQRRQRR	58	127
MPG Δ NLS	GALFLGFLGAAGSTMGAWSQPKSKRK V	51	128
R15	RRRRRRRRRRRRRRR	59	129
H8R15	HHHHHHHHRRRRRRRRRRRRRRR	59	130
H16R8	HHHHHHHHHHHHHHHHRRRRRRRRR RRRRRR	59	131
NYAD-41	ISFDELLDYYGESGS	60	132
AcD4	GYGYGYGYGYGYGYKKRKKRKKRK KRKQKQKQKRRK	61	133
RICK	KWLLRWLSRLRLRLAR WLK	74	134
WRAP	LLWRLWRLWRLWRL	75	135
MAP	KLALKALKALKALKLA	62	136
Chimeric dermaseptin S4 and SV40 (S413-PV)	ALWKTLLKKVLKAPKKRKVC	63	137
Transportan	GWTLSAGYLLGKINLKALAALAKKIL	64	138

#	Table 5 - CPP References
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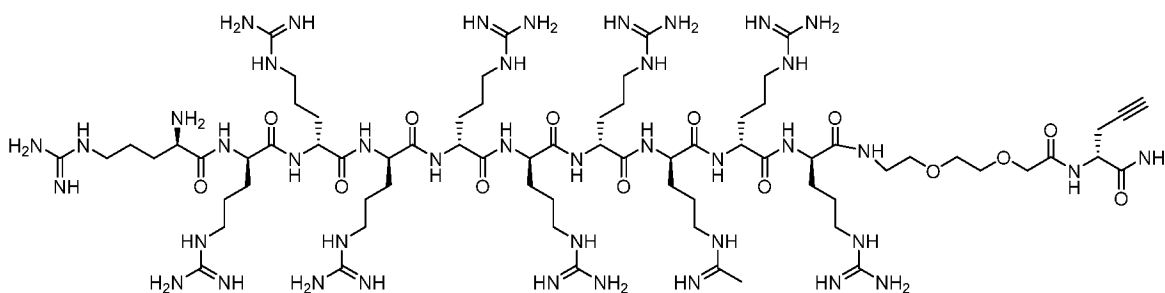
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An exemplary cell-penetrating moiety comprises a CPP covalently linked to a reactive moiety for conjugating to a reactive group comprising the macrocyclic peptide. For example, the reactive group covalently linked to the CPP may be an alkyne group and the reactive group linked to the macrocyclic peptide may be an azide group.

In particular embodiments, the CPP may further include an amino acid at the C-terminus with a side chain comprising an alkyne group, which may be linked via a triazole to an amino acid with a side chain comprising an azide group within a macrocyclic peptide. In particular embodiments, the C-terminus residue of the CPP may be linked via an amide bond to the N-terminal amino group of propargylglycine. In particular embodiments, the C-terminus residue of the CPP may be linked via an amide bond to a linker or spacer molecule, which is then linked via an amide bond to the N-terminal amino group of propargylglycine or other amino acid comprising a side chain comprising an azide or alkyne residue. In particular embodiments, the linker or spacer may comprise a polymer of amino acids, which in a further embodiment may be a poly(Gly) polymer comprising two to twenty glycine residues (SEQ ID NO: 143). In particular embodiments, the linker or spacer comprises polyethylene glycol (PEG), which in further embodiments may comprise two to 20 ethylene glycol units. Exemplary polyethylene glycol polymers, include but are not limited to, PEG2, PEG3, PEG4, PEG5, PEG6, PEG7, PEG8, PEG9, PEG10, PEG11, PEG12, PEG13, PEG14, PEG15, PEG16, PEG17, PEG18, PEG19, and PEG20.

In particular embodiments, the CPP comprises a poly(Arg) peptide polymer comprising five to 15 arginine residues (SEQ ID NO: 141), said poly(Arg) peptide polymer covalently linked at the C-terminus to a reactive moiety for conjugating to a reactive group comprising the macrocyclic peptide. For example, the reactive group covalently linked to the poly(Arg) peptide polymer may be an alkyne group and the reactive group linked to the macrocyclic peptide may be an azide group or the reactive group covalently linked to the poly(Arg) peptide polymer may be an azide group and the reactive group linked to the macrocyclic peptide may be an alkyne group. In particular embodiments, the poly(Arg) peptide polymer comprises about 8-10 arginine residues (SEQ ID NO: 144). In a further embodiment, the poly(Arg) peptide polymer comprises 10 arginine residues (SEQ ID NO: 113). In a further embodiment, the poly(Arg) peptide polymer consists of 10 arginine residues (SEQ ID NO: 113) covalently linked at the C-terminus to a linker or spacer comprising an alkyne reactive group to provide a cell-penetrating moiety capable of forming a triazole linkage with a macrocyclic peptide comprising an azide reactive group or the poly(Arg) peptide polymer consists of 10 arginine residues (SEQ ID NO: 113) covalently linked at the C-terminus to a linker or spacer comprising an azide reactive group to provide a cell-penetrating moiety capable of forming a triazole linkage with a macrocyclic peptide comprising an alkyne reactive group. In further embodiments of the cell-penetrating moiety, the poly(Arg) polymer comprises all D-arginine residues.

An exemplary cell penetrating moiety is dPra-PEG₂-dR₁₀ peptide (SEQ ID NO: 37) (*D*-Propargylglycine-PEG₂-Poly-*D*-Arg₁₀ (SEQ ID NO: 37) CPP) having the structure

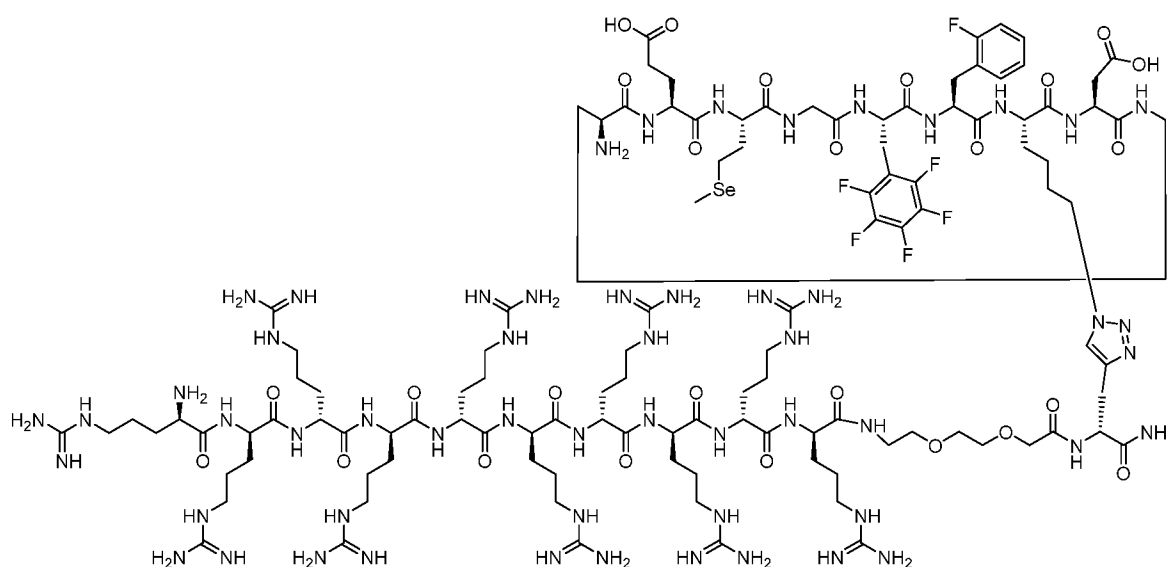


(SEQ ID NO: 37).

In particular embodiments, the macrocyclic peptide disclosed herein comprises an amino acid having a side chain terminated with an azide group and said macrocyclic peptide is conjugated to (*D*-Propargylglycine-PEG₂-Poly-*D*-Arg₁₀ (SEQ ID NO: 37) CPP) via a triazole linkage. In particular embodiments the amino acid having a side chain terminated with an azide

group is L-azidolysine (K(N₃)) comprising an azide group at the epsilon position on the sidechain.

- An exemplary macrocyclic peptide conjugate is *EE-171 peptide*: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(triazole-*d*Pra-PEG₂-*d*R₁₀)-D] (SEQ ID NO: 38), Side-to-Tail Cyclized (STC) and CuAAC-conjugated CPP wherein Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; K(N₃): *L*-azidolysine; and, PEG₂: 12-amino-4,7,10-trioxadodecanoic acid; *d*R: *D*-arginine.



(SEQ ID NO: 38).

10

Pharmaceutical Compositions

- The present invention also provides pharmaceutical compositions comprising a macrocyclic peptide of the present invention. The macrocyclic peptide may be used in combination with any suitable pharmaceutical carrier. Such pharmaceutical compositions comprise a therapeutically effective amount of one or more macrocyclic peptides, and pharmaceutically acceptable carrier(s). The specific formulation will suit the mode of administration. In particular aspects, the pharmaceutical acceptable carrier may be water or a buffered solution. Excipients included in the pharmaceutical compositions have different purposes depending, for example on the nature of the drug, and the mode of administration. Carriers are compounds and substances that improve and/or prolong the delivery of an active ingredient to a subject in the context of a pharmaceutical composition. Carrier may serve to prolong the *in vivo* activity of a drug or slow the release of the drug in a subject, using controlled-release technologies. Carriers may also decrease drug metabolism in a subject and/or

reduce the toxicity of the drug. Carriers can also be used to target the delivery of the drug to particular cells or tissues in a subject. Common carriers (both hydrophilic and hydrophobic carriers) include fat emulsions, lipids, PEGylated phospholipids, PEGylated liposomes, PEGylated liposomes coated via a PEG spacer with a cyclic RGD peptide c(RGD^DYK) (SEQ ID NO: 145), liposomes and lipospheres, microspheres (including those made of biodegradable polymers or albumin), polymer matrices, biocompatible polymers, protein-DNA complexes, protein conjugates, erythrocytes, vesicles, nanoparticles, and side-chains for hydro-carbon stapling. The aforementioned carriers can also be used to increase cell membrane permeability of the macrocyclic peptides of the invention. In addition to their use in the pharmaceutical compositions of the present invention, carriers may also be used in compositions for other uses, such as research uses in vitro (e.g., for delivery to cultured cells) and/or in vivo.

Pharmaceutical compositions adapted for oral administration may be presented as discrete units such as capsules or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids; or as edible foams or whips; or as emulsions). Suitable excipients for tablets or hard gelatin capsules include lactose, maize starch or derivatives thereof, stearic acid or salts thereof. Suitable excipients for use with soft gelatin capsules include for example vegetable oils, waxes, fats, semi-solid, or liquid polyols etc. For the preparation of solutions and syrups, excipients which may be used include for example water, polyols and sugars. For the preparation of suspensions oils, e.g. vegetable oils, may be used to provide oil-in-water or water in oil suspensions. In certain situations, delayed release preparations may be advantageous and compositions which can deliver the macrocyclic peptides in a delayed or controlled release manner may also be prepared. Prolonged gastric residence brings with it the problem of degradation by the enzymes present in the stomach and so enteric-coated capsules may also be prepared by standard techniques in the art where the active substance for release lower down in the gastro-intestinal tract.

Pharmaceutical compositions adapted for transdermal administration may be presented as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis as generally described in *Pharmaceutical Research*, 3(6):318 (1986).

Pharmaceutical compositions adapted for topical administration may be formulated as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. When formulated in an ointment, the active ingredient may be employed with

either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water cream base or a water-in-oil base. Pharmaceutical compositions adapted for topical administration to the eye include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent.

- 5 Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles and mouth washes.

Pharmaceutical compositions adapted for rectal administration may be presented as suppositories or enemas.

- 10 Pharmaceutical compositions adapted for nasal administration wherein the carrier is a solid include a coarse powder having a particle size for example in the range 20 to 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable compositions wherein the carrier is a liquid, for administration as a nasal spray or as nasal drops, include aqueous or oil solutions of the active ingredient.

- 15 Pharmaceutical compositions adapted for administration by inhalation include fine particle dusts or mists which may be generated by means of various types of metered dose pressurized aerosols, nebulizers or insufflators.

Pharmaceutical compositions adapted for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

- 20 Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injection solution which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation substantially isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. Excipients which may be used for injectable solutions
25 include water-for-injection, alcohols, polyols, glycerin and vegetable oils, for example. The compositions may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water or saline for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile
30 powders, granules and tablets. The pharmaceutical compositions may contain preserving agents, solubilizing agents, stabilizing agents, wetting agents, emulsifiers, sweeteners, colorants, odorants, salts (substances of the present invention may themselves be provided in the form of a pharmaceutically acceptable salt), buffers, coating agents or antioxidants. They may also contain

therapeutically-active agents in addition to the substance of the present invention.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, intratumor, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount
5 which is effective for treating and/or prophylaxis of the specific indication. In general, the pharmaceutical compositions are administered in an amount of at least about 0.1 mg/kg to about 100 mg/kg body weight. In most cases, the dosage is from about 10 mg/kg to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

Dosages of the macrocyclic peptides of the present invention can vary between
10 wide limits, depending upon the location, source, identity, extent and severity of the cancer, the age and condition of the individual to be treated, etc. A physician will ultimately determine appropriate dosages to be used.

The macrocyclic peptides may also be employed in accordance with the present invention by expression of the antagonists in vivo, i.e., via gene therapy. The use of the peptides
15 or compositions in a gene therapy setting is also considered to be a type of "administration" of the peptides for the purposes of the present invention.

Accordingly, the present invention also relates to methods of treating a subject having cancer, comprising administering to the subject a pharmaceutically-effective amount of one or more macrocyclic peptide of the present invention, or a pharmaceutical composition
20 comprising one or more of the antagonists to a subject needing treatment. The term "cancer" is intended to be broadly interpreted and it encompasses all aspects of abnormal cell growth and/or cell division. Examples include: carcinoma, including but not limited to adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, anaplastic carcinoma, large cell carcinoma, small cell carcinoma, and cancer of the skin, breast, prostate, bladder, vagina, cervix,
25 uterus, liver, kidney, pancreas, spleen, lung, trachea, bronchi, colon, small intestine, stomach, esophagus, gall bladder; sarcoma, including but not limited to chondrosarcoma, Ewing's sarcoma, malignant hemangioendothelioma, malignant schwannoma, osteosarcoma, soft tissue sarcoma, and cancers of bone, cartilage, fat, muscle, vascular, and hematopoietic tissues; lymphoma and leukemia, including but not limited to mature B cell neoplasms, such as chronic lymphocytic
30 leukemia/small lymphocytic lymphoma, B-cell prolymphocytic leukemia, lymphomas, and plasma cell neoplasms, mature T cell and natural killer (NK) cell neoplasms, such as T cell prolymphocytic leukemia, T cell large granular lymphocytic leukemia, aggressive NK cell leukemia, and adult T cell leukemia/lymphoma, Hodgkin lymphomas, and immunodeficiency-

associated lymphoproliferative disorders; germ cell tumors, including but not limited to testicular and ovarian cancer; blastoma, including but not limited to hepatoblastoma, medulloblastoma, nephroblastoma, neuroblastoma, pancreatoblastoma, leuopulmonary blastoma and retinoblastoma. The term also encompasses benign tumors.

5 In each of the embodiments of the present invention, the individual or subject receiving treatment is a human or non-human animal, e.g., a non-human primate, bird, horse, cow, goat, sheep, a companion animal, such as a dog, cat or rodent, or other mammal. In some embodiments, the subject is a human.

10 The invention also provides a kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention, such as a container filled with a pharmaceutical composition comprising a macrocyclic peptide of the present invention and a pharmaceutically acceptable carrier or diluent. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects
15 approval by the agency of manufacture, use or sale for human administration. In addition, the pharmaceutical compositions may be employed in conjunction with other therapeutic compounds.

Combination therapy comprising chemotherapy

20 The macrocyclic peptide of the present invention may be administered to an individual having a cancer in combination with chemotherapy. The individual may undergo the chemotherapy at the same time the individual is administered the macrocyclic peptide. The individual may undergo chemotherapy after the individual has completed a course of treatment with the macrocyclic peptide. The individual may be administered the macrocyclic peptide after
25 the individual has completed a course of treatment with a chemotherapy agent. The combination therapy of the present invention may also be administered to an individual having recurrent or metastatic cancer with disease progression or relapse cancer and who is undergoing chemotherapy or who has completed chemotherapy.

30 The chemotherapy may include a chemotherapy agent selected from the group consisting of

(i) alkylating agents, including but not limited to, bifunctional alkylators, cyclophosphamide, mechlorethamine, chlorambucil, and melphalan;

(ii) monofunctional alkylators, including but not limited to, dacarbazine, nitrosoureas, and temozolomide (oral dacarbazine);

(iii) anthracyclines, including but not limited to, daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone, and valrubicin;

5 (iv) cytoskeletal disruptors (taxanes), including but not limited to, paclitaxel, docetaxel, abraxane, and taxotere;

(v) epothilones, including but not limited to, ixabepilone, and utidelone;

(vi) histone deacetylase inhibitors, including but not limited to, vorinostat, and romidepsin;

10 (vii) inhibitors of topoisomerase i, including but not limited to, irinotecan, and topotecan;

(viii) inhibitors of topoisomerase ii, including but not limited to, etoposide, teniposide, and tafluposide;

15 (ix) kinase inhibitors, including but not limited to, bortezomib, erlotinib, gefitinib, imatinib, vemurafenib, and vismodegib;

(x) nucleotide analogs and precursor analogs, including but not limited to, azacitidine, azathioprine, fluoropyrimidines (e.g., such as capecitabine, carmofur, doxifluridine, fluorouracil, and tegafur) cytarabine, , gemcitabine, hydroxyurea, mercaptopurine, methotrexate, and tioguanine (formerly thioguanine);

20 (xi) peptide antibiotics, including but not limited to, bleomycin and actinomycin; a platinum-based agent, including but not limited to, carboplatin, cisplatin, and oxaliplatin;

(xii) retinoids, including but not limited to, tretinoin, alitretinoin, and bexarotene; and

25 (xiii) vinca alkaloids and derivatives, including but not limited to, vinblastine, vincristine, vindesine, and vinorelbine.

Selecting a dose of the chemotherapy agent for chemotherapy depends on several factors, including the serum or tissue turnover rate of the entity, the level of symptoms, the immunogenicity of the entity, and the accessibility of the target cells, tissue or organ in the individual being treated. The dose of the additional therapeutic agent should be an amount that provides an acceptable level of side effects. Accordingly, the dose amount and dosing frequency of each additional therapeutic agent will depend in part on the particular therapeutic agent, the severity of the cancer being treated, and patient characteristics. Guidance in selecting appropriate doses of antibodies, cytokines, and small molecules are available. *See, e.g., Wawrzynczak*

(1996) *Antibody Therapy*, Bios Scientific Pub. Ltd, Oxfordshire, UK; Kresina (ed.) (1991) *Monoclonal Antibodies, Cytokines and Arthritis*, Marcel Dekker, New York, NY; Bach (ed.) (1993) *Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases*, Marcel Dekker, New York, NY; Baert *et al.* (2003) *New Engl. J. Med.* 348:601-608; Milgrom *et al.* (1999) *New Engl. J. Med.* 341:1966-1973; Slamon *et al.* (2001) *New Engl. J. Med.* 344:783-792; Beniaminovitz *et al.* (2000) *New Engl. J. Med.* 342:613-619; Ghosh *et al.* (2003) *New Engl. J. Med.* 348:24-32; Lipsky *et al.* (2000) *New Engl. J. Med.* 343:1594-1602; Physicians' Desk Reference 2003 (Physicians' Desk Reference, 57th Ed); Medical Economics Company; ISBN: 1563634457; 57th edition (November 2002). Determination of the appropriate dose regimen may be made by the clinician, *e.g.*, using parameters or factors known or suspected in the art to affect treatment or predicted to affect treatment, and will depend, for example, the individual's clinical history (*e.g.*, previous therapy), the type and stage of the cancer to be treated and biomarkers of response to one or more of the therapeutic agents in the combination therapy.

The present invention contemplates embodiments of the combination therapy that include a chemotherapy step comprising platinum-containing chemotherapy, pemetrexed and platinum chemotherapy or carboplatin and either paclitaxel or nab-paclitaxel. In particular embodiments, the combination therapy with a chemotherapy step may be used for treating at least NSCLC and HNSCC.

The combination therapy may be used for the treatment any proliferative disease, in particular, treatment of cancer. In particular embodiments, the combination therapy of the present invention may be used to treat melanoma, non-small cell lung cancer, head and neck cancer, urothelial cancer, breast cancer, gastrointestinal cancer, multiple myeloma, hepatocellular cancer, non-Hodgkin lymphoma, renal cancer, Hodgkin lymphoma, mesothelioma, ovarian cancer, small cell lung cancer, esophageal cancer, anal cancer, biliary tract cancer, colorectal cancer, cervical cancer, thyroid cancer, or salivary cancer.

In another embodiment, the combination therapy may be used to treat pancreatic cancer, bronchus cancer, prostate cancer, pancreatic cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, testicular cancer, biliary tract cancer, small bowel or appendix cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, or cancer of hematological tissues.

In particular embodiments, the combination therapy may be used to treat one or more cancers selected from melanoma (metastatic or unresectable), primary mediastinal large B-

cell lymphoma (PMBCL), urothelial carcinoma, MSIHC, gastric cancer, cervical cancer, hepatocellular carcinoma (HCC), Merkel cell carcinoma (MCC), renal cell carcinoma (including advanced), and cutaneous squamous carcinoma.

5 *Additional Combination Therapies*

The macrocyclic peptides disclosed herein may be used in combination with other therapies. For example, the combination therapy may include a composition comprising a macrocyclic peptide co-formulated with, and/or co-administered with, one or more additional therapeutic agents, e.g., hormone treatment, vaccines, and/or other immunotherapies. In other
10 embodiments, the macrocyclic peptide is administered in combination with other therapeutic treatment modalities, including surgery, radiation, cryosurgery, and/or thermotherapy. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies.

By "in combination with," it is not intended to imply that the therapy or the therapeutic agents must be administered at the same time and/or formulated for delivery together, although these methods of delivery are within the scope described herein. The macrocyclic peptide may be administered concurrently with, prior to, or subsequent to, one or more other additional therapies or therapeutic agents. The macrocyclic peptide and the other agent or
20 therapeutic protocol may be administered in any order. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent. It will further be appreciated that the additional therapeutic agent utilized in this combination may be administered together in a single composition or administered separately in different compositions. In general, it is expected that additional therapeutic agents utilized in combination be utilized at levels that
25 do not exceed the levels at which they are utilized individually. In some embodiments, the levels utilized in combination will be lower than those utilized individually.

In certain embodiments, a macrocyclic peptide described herein is administered in combination with one or more check point inhibitors or antagonists of programmed death receptor 1 (PD-1) or its ligand PD-L1 and PD-L2. The inhibitor or antagonist may be an
30 antibody, an antigen binding fragment, an immunoadhesin, a fusion protein, or oligopeptide. In some embodiments, the anti-PD-1 antibody is chosen from nivolumab (OPDIVO, Bristol Myers Squibb, New York, New York), pembrolizumab (KEYTRUDA, Merck Sharp & Dohme Corp, Kenilworth, NJ USA), cetiplimab (Regeneron, Tarrytown, NY) or pidilizumab (CT-011). In

some embodiments, the PD-1 inhibitor is an immunoadhesin (e.g., an immunoadhesin comprising an extracellular or PD-1 binding portion of PD-L1 or PD-L2 fused to a constant region (e.g., an Fc region of an immunoglobulin sequence)). In some embodiments, the PD-1 inhibitor is AMP-224. In some embodiments, the PD-L1 inhibitor is anti-PD-L1 antibody such
5 durvalumab (IMFINZI, AstraZeneca, Wilmington, DE), atezolizumab (TECENTRIQ, Roche, Zurich, CH), or avelumab (BAVENCIO, EMD Serono, Billerica, MA). In some embodiments, the anti-PD-L1 binding antagonist is chosen from YW243.55.S70, MPDL3280A, MEDI-4736, MSB-0010718C, or MDX-1105.

The following examples are intended to promote a further understanding of the
10 present invention.

EXAMPLE 1

Binding modalities between small molecule (closed form) and peptide (apo form)

Fig. 7A shows the structures for eIF4E: in its ‘closed’ form, when bound to
15 m⁷GTP (panel A) or published nucleotide mimic, e.g., Chen et al., J. Med. Chem. 55: 3837-51 (2012) as shown in panel B). **Fig. 7B** shows the structures for eIF4E: in its ‘apo’ form, when free in solution (panel C) and bound to EE-02 (panel D). **Fig. 7C** shows a comparison between bound to m⁷GTP and EE-02 with the main changes to W⁵⁶, W¹⁰² and E¹⁰³ highlighted when going from ‘apo’ to ‘closed’ (as shown by the arrows).

EXAMPLE 2

Biophysical assay: descriptions and results for EE-02 and EE-44

Three biophysical assay experiments were performed throughout this work.

1. *Fluorescence polarization (FP)* is a method for determining K_D that relies on the change
25 in fluorescence polarization when a fluorophore is bound to protein. When free in solution, the fluorophore tumbles rapidly, and when excited by plane-polarized light, the light is depolarized and a low signal is observed. When bound to protein, the tumbling of the fluorophore is restricted, thus the plane-polarized light remains polarized and a higher fluorescence polarization signal is observed. This phenomenon can be exploited in a competitive assay using a FAM-
30 labelled peptide (tracer peptide) that binds the m⁷GTP binding site of eIF4E. Displacement of the tracer peptide by an unlabeled peptide that competes for binding to the m⁷GTP binding site results in a drop in signal, thus allowing for identification of peptides that bind the desired

interaction site, rather than other potential binding sites such as the eIF4E:eIF4G binding interface.

2. *Thermofluor*, also known as thermal shift assay, is a method for determining K_D that is based on the shift in a protein's melting temperature (T_m) in the presence of a ligand. In this assay, SYPRO orange dye, a dye whose fluorescence is quenched in aqueous solution, is mixed with protein and ligand, and the reaction mixture is heated. As the protein unfolds, hydrophobic regions are exposed, and SYPRO orange binds to these regions and the fluorescence is no longer quenched. Monitoring the change in fluorescence with increasing temperature allows a melt curve to be plotted, and for the shift in T_m to be determined. This method is useful for the rapid identification of peptides that bind to the target protein.

3. *Isothermal Titration Calorimetry (ITC)* is a quantitative technique used to study various biomolecular interactions, through direct measurement of the heat that is either released or absorbed during in intermolecular binding event. ITC is unique in that it is the only technique to simultaneously determine all the different binding parameters in a single experiment.

Furthermore, it requires no modification of biomolecular binding partners – i.e., no need for fluorescent tags (in FP) or immobilization (such as in Surface Plasmon Resonance or SPR experiments); ITC measures the direct binding affinity of partners in their native states. This enables accurate determination of binding constants (K_D), reaction stoichiometry (n), change in enthalpy (ΔH), and change in entropy (ΔS). Thus, providing a complete thermodynamic profile of the bi-molecular interaction. ITC may go beyond binding affinities and help to elucidate mechanisms underlying these interactions. This would, in turn, allow better understanding of SAR to enable more confident decision making in hit selection and lead optimization.

2.1 FP Results for EE-02 and EE-44:

Competitive FP experiments were also performed on EE-02, using a FAM-labelled version of EE-02 (EE2-FAM) as the tracer peptide. In this experiment, 0.625 μ M eIF4E was incubated with 50nM of EE2-FAM, and varying concentrations of EE-02 were added to displace the EE2-FAM from eIF4E (12-point titration, concentrations ranging from 0.005 to 20 μ M). This was carried out in PBS, 3% DMSO, 0.1% Tween 20. This experiment was repeated thrice to produce values shown in **Fig. 9** (left).

Competitive FP experiments were also performed on EE-44, using a FAM-labelled version of EE-02 (EE2-FAM) as the tracer peptide. In this experiment, 0.625 μ M eIF4E was incubated with 50nM of EE2-FAM, and varying concentrations of EE-44 were added to

displace the EE2-FAM from eIF4E (12-point titration, concentrations ranging from 0.005 to 20 μ M). This was carried out in PBS, 3% DMSO, 0.1% Tween 20. This experiment was repeated thrice to produce values shown in **Fig. 9** (right).

5 2.2 TF Results for EE-02 and EE-44:

TF results were obtained for EE-02 and EE-44 by comparing shifts in melting curves. Changes in melting temperatures for EE-02 and EE-44 were recorded as ΔT of 11.6 and 13.4K, respectively, and were in turn translated into K_D values of 97.8 and 2.3nM, respectively. An average of three experimental values were eventually taken to produce values shown in **Fig.**

10 **10.**

2.3 ITC results for EE-02 and EE-44:

Isothermal calorimetry (ITC) experiment was performed on EE-02 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a
15 buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (10 μ M) with the peptide ligand (100 μ M), at 25 °C (20 titrations of 2.5 μ L per injection with 300 s intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 59.7 ($\pm$ 6.82) nM, with an enthalpy change, ΔH , of $-35.4 (\pm 1.4)$ kJ/mol and entropy change, ΔS , of $19.6 (\pm 4.9)$ J/mol·K (**Fig. 11**).

20 ITC experiments were also performed on EE-44 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (10 μ M) with the peptide ligand (100 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 52.9 ($\pm$ 11.1) nM, with an enthalpy
25 change, ΔH , of $-49.4 (\pm 3.2)$ kJ/mol and entropy change, ΔS , of $-31.2 (\pm 8.3)$ J/mol·K (**Fig. 12**).

EXAMPLE 3

SAR using peptides with native disulphide constraints

Initial SAR for EE-02 used peptides purchased from Mimotopes, and all
30 possessed disulphide bond constraints. These macrocyclic peptides were used to probe the effect of residues substitutions, including Ala (A) scanning, on each position on the EE-02 and EE-44 sequence. These peptides were EE-03 to EE-47 and EE-49 to EE-75. Ala (A) scanning studies were done using peptides EE-80 to EE-85 and EE-88. Effects of acetylation of the N-terminus

was studied using peptide EE-11 and EE-89. The full breakdown is summarized below in Table 6.

Table 6, Part A														
Results for Fluorescence Polarisation (FP) competition assay – using EE-86 (5-FAM-EE-02) as ligand – and ThermoFluor (TF) assay with melting curve shift values (ΔT).														
		Peptide Amino Acid Position												SEQ ID NO:
EE	N-Ter	1	2	3	4	5	6	7	8	9	10	11	C-Ter	
2	NH ₂	A	C	E	M	G	F	F	Q	D	C	G	CONH ₂	229
3	NH ₂	A	C	E	L	G	Y	Y	N	D	C	G	CONH ₂	146
4	NH ₂	A	C	E	T	G	F	F	L	K	C	G	CONH ₂	147
5	NH ₂	A	C	E	L	G	F	Y	R	L	C	G	CONH ₂	148
6	NH ₂	A	C	E	T	G	F	F	L	R	C	G	CONH ₂	149
7	NH ₂	A	C	E	T	G	Y	F	S	Q	C	G	CONH ₂	150
8	NH ₂	A	C	E	T	G	F	Y	K	T	C	G	CONH ₂	151
8	NH ₂	A	C	E	M	G	Y	F	G	N	C	G	CONH ₂	152
10	NH ₂	A	C	I	H	S	P	T	S	L	C	G	CONH ₂	153
11	Ac	A	C	E	M	G	F	F	Q	D	C	G	CONH ₂	154
12	NH ₂		C	E	M	G	F	F	Q	D	C	G	CONH ₂	155
13	NH ₂	A	C	E	M	G	F	F	Q	D	C		CONH ₂	156
14	NH ₂	A	C	E	M	G	F	F	Q	D	C	G	G-CONH ₂	157
15	NH ₂	A	C	E	M	G	F	F	Q	D	C	G	GG-CONH ₂	158
16	NH ₂	A	C	E	M	G	F	F	D	C	G		CONH ₂	159
17	NH ₂	A	C	E	M	G	F	F	C	G			CONH ₂	160
18	NH ₂	A	C	E	M	G	F	F	hC	G			CONH ₂	161
19	NH ₂	A	C	D	M	G	F	F	Q	D	C	G	CONH ₂	162
20	NH ₂	A	C	E	I	G	F	F	Q	D	C	G	CONH ₂	163
21	NH ₂	A	C	E	L	G	F	F	Q	D	C	G	CONH ₂	164

22	NH ₂	A	C	E	Nl e	G	F	F	Q	D	C	G	CONH ₂	165
23	NH ₂	A	C	E	Cb a	G	F	F	Q	D	C	G	CONH ₂	166
24	NH ₂	A	C	E	E	G	F	F	Q	D	C	G	CONH ₂	167
25	NH ₂	A	C	E	D	G	F	F	Q	D	C	G	CONH ₂	168
26	NH ₂	A	C	E	F	G	F	F	Q	D	C	G	CONH ₂	169
27	NH ₂	A	C	E	M	βA	F	F	Q	D	C	G	CONH ₂	170
28	NH ₂	A	C	E	M	Sar	F	F	Q	D	C	G	CONH ₂	171
29	NH ₂	A	C	E	M	P	F	F	Q	D	C	G	CONH ₂	172
30	NH ₂	A	C	E	M	G	Y	F	Q	D	C	G	CONH ₂	173
31	NH ₂	A	C	E	M	G	W	F	Q	D	C	G	CONH ₂	174
32	NH ₂	A	C	E	M	G	F(4 - Cl)	F	Q	D	C	G	CONH ₂	175
33	NH ₂	A	C	E	M	G	L	F	Q	D	C	G	CONH ₂	176
34	NH ₂	A	C	E	M	G	F	L	Q	D	C	G	CONH ₂	177
35	NH ₂	A	C	E	M	G	F	Y	Q	D	C	G	CONH ₂	178
36	NH ₂	A	C	E	M	G	F	W	Q	D	C	G	CONH ₂	179
37	NH ₂	A	C	E	M	G	Y	Y	Q	D	C	G	CONH ₂	180
38	NH ₂	A	C	E	M	G	L	L	Q	D	C	G	CONH ₂	181
39	NH ₂	A	C	E	M	G	F	F	K	D	C	G	CONH ₂	182
40	NH ₂	A	C	E	M	G	F	F	G	D	C	G	CONH ₂	183
41	NH ₂	A	C	E	M	G	F	F	L	D	C	G	CONH ₂	184
42	NH ₂	A	C	E	M	G	F	F	Q	E	C	G	CONH ₂	185
43	NH ₂	A	C	E	M	G	F	F	Q	L	C	G	CONH ₂	186
44	NH ₂		C	E	M	G	F	F	A	D	C		CONH ₂	230
45	NH ₂		hC	E	M	G	F	F	Q	D	C	G	CONH ₂	187
46	NH ₂		C	E	M	G	F	F	Q	D	hC	G	CONH ₂	188
47	NH ₂		hC	E	M	G	F	F	Q	D	hC	G	CONH ₂	189
49	NH ₂		C	E	dM	G	F	F	A	D	C		CONH ₂	190
50	NH ₂		C	E	α- Me M	G	F	F	A	D	C		CONH ₂	191

51	NH ₂		C	E	M	G	4- CN -F	F	A	D	C		CONH ₂	192
53	NH ₂		C	E	M	G	hF	F	A	D	C		CONH ₂	193
54	NH ₂		C	E	M	G	F	hF	A	D	C		CONH ₂	194
55	NH ₂		C	E	M	G	F	F5- F	A	D	C		CONH ₂	195
56	NH ₂		C	E	M	G	F	Tic	A	D	C		CONH ₂	196
57	NH ₂		C	E	M	G	F	F	P	D	C		CONH ₂	197
58	NH ₂		C	E	M	G	F	F	R	D	C		CONH ₂	198
59	NH ₂		C	E	M	G	F	F	R	L	C		CONH ₂	199
60	NH ₂		dC	E	M	G	F	F	A	C			CONH ₂	200
61	NH ₂		dC	E	M	G	F	F	A	hC			CONH ₂	201
62	NH ₂		dC	E	M	G	F	F	A	dC			CONH ₂	202
63	NH ₂		dC	E	M	G	F	F	A	dhC			CONH ₂	203
64	NH ₂		dC	E	M	G	F	F	C				CONH ₂	204
65	NH ₂		dC	E	M	G	F	F	hC				CONH ₂	205
66	NH ₂		dC	E	M	G	F	F	dC				CONH ₂	206
67	NH ₂		dC	E	M	G	F	F	dhC				CONH ₂	207
68	NH ₂		dhC	E	M	G	F	F	A	C			CONH ₂	208
69	NH ₂		dhC	E	M	G	F	F	A	hC			CONH ₂	209
70	NH ₂		dhC	E	M	G	F	F	A	dC			CONH ₂	210
71	NH ₂		dhC	E	M	G	F	F	A	dhC			CONH ₂	211
72	NH ₂		dhC	E	M	G	F	F	C				CONH ₂	212
73	NH ₂		dhC	E	M	G	F	F	hC				CONH ₂	213
74	NH ₂		dhC	E	M	G	F	F	dC				CONH ₂	214
75	NH ₂		dhC	E	M	G	F	F	dhC				CONH ₂	215
80	NH ₂	A	C	A	M	G	F	F	Q	D	C	G	CONH ₂	216
81	NH ₂	A	C	E	A	G	F	F	Q	D	C	G	CONH ₂	217
82	NH ₂	A	C	E	M	A	F	F	Q	D	C	G	CONH ₂	218
83	NH ₂	A	C	E	M	G	A	F	Q	D	C	G	CONH ₂	219
84	NH ₂	A	C	E	M	G	F	A	Q	D	C	G	CONH ₂	220
85	NH ₂	A	C	E	M	G	F	F	A	D	C	G	CONH ₂	221
86	5- FAM	A	C	E	M	G	F	F	Q	D	C	G	CONH ₂	222

88	NH ₂	A	C	E	M	G	F	F	Q	A	C	G	CONH ₂	223
89	Ac		C	E	M	G	F	F	Q	D	C		CONH ₂	224
Ac = acetyl; β A = beta-alanine; F5(F) = Phe with 5 fluoride atoms F; Nle = norleucine; dhC = D-homo-cysteine; dC = D-cysteine; hC = homo-cysteine; Tic = amino-1,2,3,4-tetrahydroisoquinoline-3-carboxyl; hF = homo-phenylalanine; α -MeM = alpha-methyl-methionine; dM = D-methionine; Cba = 2-amino-4-cyanobutyric acid; 4-CN-F = Phe with CN on C4; F(4-Cl) = Phe with Cl on C4; 5-FAM = flourescin														

Table 6, Part B			
Results for Fluorescence Polarisation (FP) competition assay – using EE-86 (5-FAM-EE-02) as ligand – and ThermoFluor (TF) assay with melting curve shift values (ΔT).			
EE	K _D -FP (nM)	K _D -TF (nM)	ΔT -TF (K)
2	51.02	5.1	12.7
3	2154	115.2	8.4
4	NI	N.A.	N.A.
5	NI	54305.9	0.1
6	24430	1248	5.6
7	26410	1994.9	5
8	5849	1209.7	5.7
8	1022	111.6	8.4
10	NI	61245.6	-0.1
11	153.9	10.3	10.9
12	7.463	0.3	14
13	72.44	4.1	11.7
14	39.72	1.5	12.6
15	54.78	5.7	11.4
16	9667	1965.9	5.1
17	NI	50119.3	0.2
18	NI	52891.3	0.1
19	NI	5362.5	3.8
20	783.3	34	10.1
21	1037	47.4	9.8
22	140.6	4.2	12.3

23	NI	5272	3.9
24	NI	47073.5	0.3
25	NI	99193.8	-1.1
26	NI	12010.1	2.7
27	NI	41256.4	0.6
28	1885	1120	7.8
29	NI	57102.3	0.1
30	3.897	11.6	14.5
31	1831	580.4	8
32	54.57	20.1	12.3
33	NI	13085.6	3
34	66250	6932.2	4.1
35	40.13	8.6	13.3
36	770.5	227	9.3
37	165	27.5	12
38	NI	56323.6	0.1
39	80.71	33.1	11.7
40	147.7	51.6	11.2
41	51.55	8.8	13.4
42	43.06	16.7	12.8
43	2963	175.2	9.7
44	29.7	10.4	13.4
45	328.3	554.4	9.8
46	770.6	1743.1	8.5
47	1898	3977.4	7.5
49	NI	9455.3	3.6
50	100.6	9.9	13.3
51	NI	8506.6	3.8
53	3418	1027.1	7.3
54	1818	586.7	8
55	726.4	49.3	11.4
56	NI	371.7	8.6
57	NI	5805.2	4.4
58	29.88	6.5	13.8
59	142	45.3	11.6

60	NI	11262	2.8
61	NI	28424.6	1.3
62	2820	482.2	7.1
63	8299	2004	5.2
64	NI	22445.9	1.7
65	NI	15291.7	2.3
66	62110	5461.6	3.9
67	6929	5017.4	4
68	22460	4684.9	4.1
69	NI	23800.6	1.6
70	NI	8820.2	3.3
71	9111	1829.7	5.3
72	NI	16609.4	2.1
73	NI	20260.4	1.8
74	17150	5493.1	3.8
75	NI	44793.6	0.5
80	NI	8763.1	3.3
81	NI	23459.9	1.6
82	NI	10609.9	3
83	NI	44685.4	0.5
84	NI	24747.2	1.6
85	81.07	8.9	12.1
86	63.1	-	-
88	386.3	101.7	9.3
89	235.8	41.7	10.4
NI = no interaction. EE-44 was chosen as the optimized sequence. Data has been updated using new thermodynamic data for EE-02 and EE-44 from ITC assay.			

Using the above values of FP and TF, some conclusions about the SAR can be derived. This will be broken down into interacting (EMGFF (SEQ ID NO: 139)) and non-interacting (ACXXXXXADCG: SEQ ID NO: 43) residues of EE-02. The residues are numbered as they appear on EE-02; i.e. A¹ is found in the first position, C² in the second, E³ in the third, and so on, counting from the N-terminus up until G¹¹ in the C-terminus.

EE-03 to EE-10: None of these sequences bind better than EE-02.

EE-11: Acetylation of the N-terminus of EE-02 caused a drop in binding affinity, but only slightly – by a factor of 2, from K_D of 5.1 to 10.3 nM and an accompanying drop of ΔT from 12.7 to 10.9K, as studied by thermofluor.

EE-12 to EE-15: Deletion of residues found outside the macrocycle (i.e., before C² and after C¹⁰) had a positive effect on binding (EE-12, K_D of 5.1 to 0.3nM with an increase in ΔT from 12.7 to 14K, as studied by thermofluor). In general, however, the effect was small. On the other hand, adding residues on the C-terminus had little effect on binding (EE-14/15). This indicated a huge tolerance for any residues found outside the macrocycle.

EE-16 to EE-18: Deletion of residues found inside the macrocycle had detrimental effects. Even though A⁸ and D⁹ were non-interacting, deleting them sequentially (EE-16, then EE-17) lowered binding significantly in the first instance (A⁸ deletion, EE-16), and completely obliterated binding in the second instance (A⁸ and D⁹ deletion, EE-17). This implied that although these two residues (A⁸ and D⁹) were not interacting directly with eIF4E, they play a vital role in binding – perhaps indirectly. Homo-cysteine (hC¹⁰) in EE-18 were used to attempt to recover binding.

EE-19: Substituting Glu (E³) for Asp (D³) caused a huge decrease in binding, possibly due to lower interaction with R¹¹² of eIF4E, as Asp is one methylene unit shorter than Glu.

EE-20 to EE-26: Substituting Met (M⁴) with any other hydrophobic residues, except norleucine (Nle), caused a depreciation of binding affinity. Leu (L), for example, caused a depreciation of binding constant (K_D) from 5.1 to 47.4nM, with an accompanying change in ΔT from 12.7 to 9.8K, as studied by thermofluor. Nle was tolerated and could be a useful alternative to methionine in the M⁴ position, although this was disproved in later series of the peptide. Any other residues, such as Phe (EE-26), Asp (EE-25) or Glu (EE-24), in this position completely obliterated binding (K_D of 12 to 100 μ M).

EE-27 to EE-29: Gly (G⁵) was found to be intolerant to changes. Substituting Gly for sarcosine (Sar) – an N-methyl-glycine residue – caused a dramatic drop in binding affinity, from K_D of 5.1 to 1120 nM and an accompanying change in ΔT from 12.7 to 7.8K, as studied by thermofluor. Any other residues substituted in this position obliterated binding (between K_D of 41 to 57 μ M).

EE-30 to EE-33: Substituting the Phe (F⁶) by any other aromatic groups reduced binding affinity; *para*-chloro-Phe was, however, slightly tolerated (K_D of 20.1nM and ΔT of

12.3K) – indicating an ability to add substituents on the Phe (F⁶) group to influence and probe effects of electronics. Substituting with a non-aromatic residue, Leu (L), obliterated binding.

EE-34 to EE-36: Substitution of Phe (F⁷) for other aromatic groups in this position seem to affect bind affinity differently. While Trp reduces binding (K_D of 227 nM and ΔT of 9.3K, as studied by thermofluor), Tyr (Y) improves binding slightly (K_D of 8.6 nM and ΔT of 13.3K, as studied by thermofluor). Substituting with a non-aromatic residue, Leu (L), obliterated binding.

EE-37 to EE-38: Substituting both F⁶ and F⁷ with Tyr (EE-37) were tolerated (K_D of 27.5nM and ΔT of 12K, as studied by thermofluor). Substituting both F⁶ and F⁷ with Leu (EE-38), however, completely obliterated binding. It can be deduced that F⁶ and F⁷ are both bound to the aromatic pockets of eIF4E cap-binding site. This will be further inspected later.

EE-39 to EE-41: Replacing the Gln (Q⁸) with any other residues seem to have minimal impact – even though these residues vary wildly in functional groups. For example, Lys (K), Gly (G) and Leu (L) gave somewhat similar binding affinity (K_D between 8.8 to 51.6 nM and ΔT between 11.2 to 13.4K). This might indicate a high tolerance of functional groups in this position.

EE-42 to EE-43: The Asp (D⁹) position was observed to be tolerant only for Glu (E) substitution – where both residues have the same carboxylic acid functional group – only slightly lowering binding affinity to a K_D of 16.7nM and ΔT of 12.8K, as studied by thermofluor. Placing Leu (L) on this position significantly reduced binding to a K_D of 175.2nM and ΔT of 9.7K.

EE-44 to EE-47: Truncated sequences were then probed in this set of sequences. As mentioned at the start of Section 3, EE-44, with Gln (Q⁸) substituted for Ala (A⁸) and A¹ and G¹¹ removed from the sequence, gave better binding affinity than EE-02 (with EE-44 demonstrating a K_D of 10.4 nM and ΔT of 13.4 K, as studied by thermofluor). Varying combinations of homo-Cys (hC) analogues on C² and C¹⁰ positions were attempted in EE-45 to EE-47 with only A¹ removed from the sequence. These peptides, however, had their binding affinities obliterated – highlighting the importance that the native disulphide bond from Cys (C) played.

EE-49 to EE-50: Reversing the stereochemistry of Met (M⁴) to *D*-Met in EE-49 obliterated binding – indicating that the binding is stereo-specific. Substituting M⁴ for unnatural α-methyl-methionine (α-met-M) in EE-50, on the other hand, was well tolerated (with a K_D of 9.9 nM and ΔT of 13.3K, as studied by thermofluor).

EE-51 to EE-53: While Tyr (Y) and *para*-chloro-Phe were both shown to be tolerated, previously (in EE-30 and EE-32, respectively), larger and more electron-withdrawing groups such as *p*-CN-Phe in EE-51 and homo-Phe in EE-43 were found to have obliterated binding in EE-52 (K_D of 8.5 μ M and ΔT of 3.8K) and drastically reduced binding in EE-53 (K_D of 1027nM and ΔT of 7.3K).

EE-54 to EE-56: larger groups on Phe in the F⁷ position were not tolerated, with the exception of pentafluoro-phenylalanine (F₅-F) in EE-55, which has a reasonable K_D of 49.3 nM and ΔT of 11.4K, as studied by thermofluor, for a large change in the aromatic ring. Homo-Phe substitution on the F⁷ position in EE-54 caused a huge drop in binding affinity (K_D of 587nM and ΔT of 8K, as studied by thermofluor). Similarly, Tic (a 1,2,3,4-tetrahydroisoquinoline-3-carboxyl-containing residue) substitution on this F⁷ position impaired binding affinity with a K_D of 372nM and ΔT of 8.6K, as studied by thermofluor.

EE-57 to EE-59: Substituting Arg (R) groups on the A⁸ position of EE-44 had little impact on binding affinity – again highlighting the ability for this position to tolerate different functional groups. Placing Pro (P) on this position, however, caused an obliteration of binding (K_D of 5.8 μ M and ΔT of 4.4K, as studied by thermofluor) – which might have suggested that an important role of this A⁸ position is conformation.

EE-60 to EE-75: Varying combination of truncations (by removing A⁸ and D⁹ residues) and replacement of Cys (C² and C¹⁰ positions) with either homo-Cys (hC) or *D*-Cys (*d*C) or *D*-homo-Cys (*dh*C) were done on this set of peptides. While all the peptides tested gave poor to no binding, EE-62 (with D⁹ removed and with both C² and C¹⁰ substituted for *d*C) gave the highest binding affinity, with a K_D of 482nM and ΔT of 7.1K, as studied by thermofluor. Binding affinities for this set varied from K_D between 0.5 and 45 μ M, and ΔT between 0.5 and 7.1K, as studied by thermofluor.

EE-80 to EE-85 and EE-88: Alanine (A) scanning was performed on the EE-02 peptide scaffold. As suspected, all positions except the Q-8 position had their binding affinities obliterated. Substituting Ala (A⁸) into the Q⁸ position, on the other hand, improved binding marginally (with a K_D of 8.9nM and ΔT of 12.1 K, as studied by thermofluor).

EE-86: N-terminal 2-FAM labelled EE-02, for use in FP assay.

EE-89: Acetylated peptide of EE-44 with Q⁸ instead of A⁸. Acetylation of the N-terminus was shown to reduce binding affinity for this analogue of EE-44 (K_D of 42nM and ΔT of 10.4K, as studied by thermofluor). It is possible that the N-terminal amine plays a role in

forming a temporary salt-bridge with Asp (D⁹) that facilitates binding – as observed also in effects of substituting D⁹.

In summary, the initial SAR study using native disulphide bond constraints is shown (**Fig. 13**).

5

EXAMPLE 4

Biophysical assays: Results for EE-48 – a methylene bridge linchpin

4.1 SPR results for EE-48:

EE-48 was studied first by Surface Plasmon Resonance (SPR). Results from SPR studies indicated binding with K_D of 1600nM – a huge drop from EE-44 which displayed a K_D of 69 nM (**Fig. 14**, a 23-fold drop in binding affinity).

10

4.2 ITC results for EE-48:

Isothermal calorimetry (ITC) experiment was performed on EE-48 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (15 µM) with the peptide ligand (200 µM), at 25°C (20 titrations of 2.5 µL per injection with 300 seconds intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 1421 (± 267) nM, with an enthalpy change, ΔH, of –23.6 (± 2.8)kJ/mol and entropy change, ΔS, of 32.8 (± 10.6) J/mol·K (**Fig. 15**).

20

4.3 Comparison of biophysical data and X-ray crystal structures: EE-48 vs EE-44 with eIF4E:

EE-48 analyzed gave us a glimpse of the effects of replacing the disulphide bond with a methylene bridge. The binding affinity was drastically reduced from a K_D of 53 nM as studied on ITC for EE-44, to a K_D of 1350nM, as studied on ITC – a 200-fold decrease in binding affinity (**Fig. 16**). Such a drastic effect over a small change in linker length and properties were previously observed in EE-45 to EE-47, where homo-Cys were used in place of Cys on the C² and C¹⁰ positions to vary linker length. The implication of this is important, as it might indicate that simply trying to modify the disulphide bonds with linchpins might not achieve the desired effect. Co-crystals with eIF4E were obtained (**Fig. 17**).

30

EXAMPLE 5

Biophysical assays: Results for HTC peptides EE-94

EE-91 to EE-94 were ordered from Chinese Peptide Company (CPC) for studies on head-to-tail cyclized (HTC) peptides (Table 7). Both EE-91 and EE-94 were eventually re-synthesized in-house for further analysis. A moderately acceptable hit was found using EE-94 (K_D of about 1.1 μ M and ΔT of 6.3K, as studied by thermofluor – comparable to EE-48). This peptide was eventually co-crystallized with eIF4E.

Table 7																	
Results for HTC peptides EE-91 to 94																	
		Peptide Amino Acid Positions															
EE	N-Ter	1	2	3	4	5	6	7	8	9	10	11	C-Ter	K_D -FP (nM)	K_D -TF (nM)	ΔT -TF (K)	SEQ ID NO:
91	cyclo		β A	E	M	G	F	F	A	D	β A			99790	3867.4	4.4	4
92	cyclo		G	E	M	G	F	F	A	D	G			NI	18827.9	2.0	225
93	cyclo		G	E	M	G	F	F	A	D	β A			25690	4246.8	4.3	140
94	cyclo		β A	E	M	G	F	F	A	D	G			2980	1133.6	6.3	5
β A = beta-alanine																	

5.1 ITC results for EE-94:

Isothermal calorimetry (ITC) experiment was performed on EE-94 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (15 μ M) with the peptide ligand (200 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 2305 ($\pm$ 264) nM, with an enthalpy change, ΔH , of $-21.8 (\pm 1.5)$ kJ/mol and entropy change, ΔS , of 34.7 (± 4.2) J/mol·K (**Fig. 18**).

5.2 Comparison of biophysical assay results and X-ray crystal structure for EE-94 with eIF4E:

An X-ray crystal structure of EE-94 binding to eIF4E was also attained (**Fig. 18**, right, and **Fig. 19**). Summary of the results and SAR from different ring sizes and position of amide linkage in HTC peptides EE-91 to EE-94 are shown in **Fig. 19**. The full X-ray crystal structure is shown below (**Fig. 20**).

EXAMPLE 6

Evolution of HTC Peptide EE-94 to Side-to-Tail Cyclized (STC) peptide EE-108

EE-108 was evolved from EE-94 by replacing the amide bond with an all-carbon bond, with the introduction of an N-terminus to capture additional interaction with eIF4E's N⁵⁰ (**Fig. 21**).

5 The first set of peptides we studied on this system was EE-106 to EE-108 (Table 8).

Table 8																	
Results for STC peptides EE-106 to EE-108																	
		Peptide Amino Acid Position															
EE	N-	1	2	3	4	5	6	7	8	9	10	11	C-	K _D -FP (nM)	K _D -TF (nM)	ΔT-TF (K)	SEQ ID NO:
106	cyclo		bA	E	M	G	F	F	A	A	G			-	6431.8	3.8	6
107	cyclo		ε-K	E	M	G	F	F	A	A				9247	1797.3	5.7	7
108	cyclo		ε-K	E	M	G	F	F	A	D				1115.9	888.6	8	8
In this set, the best binding affinities were achieved by EE-108. βA = beta-alanine; εK = epsilon-lysine (lysine linked via its side-chain amino group to C-terminal amino acid in an amide linkage)																	

6.1 ITC results for EE-108:

10 Isothermal calorimetry (ITC) experiment was performed on EE-108 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (15 μM) with the peptide ligand (200 μM), at 25°C (20 titrations of 2.5 μL per injection with 300 seconds intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 1130 (± 153) nM, with an enthalpy change, ΔH, of -25.8 (± 0.8) kJ/mol and entropy change, ΔS, of 27.5 (± 2.7) J/mol K (**Fig. 22**).

6.2 X-ray crystal structure for EE-108 with eIF4E:

EE-108 was co-crystallized with eIF4E. The crystals obtained were analyzed by X-ray crystallography to obtain the structure shown in **Fig. 23**.

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EXAMPLE 7

Biophysical assays and Met4 evolution to EE-124

The oxygen variant in EE-122, i.e., O-methyl-L-homoserine (Hse(OMe)) in place of M⁴, did not perform well – giving a binding affinity K_D value of 10,381 nM and ΔT of 4.1K. The alkyl chain variant (EE-123) using Nle in place of M⁴ from EE-108 also had a drastic drop in binding affinity (K_D of 7,587 nM and ΔT of 4.7K, as studied by thermofluor), which was unexpected since the same replacement in EE-22 from EE-02 saw only a slight drop in binding affinity. From this study, we have discovered a new leading peptide with the best binding data so far for unnatural disulphide-replaced peptides – EE-124, the selenomethionine (Semet) analogue of M⁴ in EE-108, with a K_D of 584 nM and ΔT of 9.1K, as studied by thermofluor. These results are summarized in Table 9.

10

Table 9																	
Results from fourth set of HTC peptides prepared to study effects of different groups on Phe-6/7 and Met-4.																	
		Peptide Amino Acids															
EE	N-Ter	1	2	3	4	5	6	7	8	9	10	11	C-Ter	K _D -FP (nM)	K _D -TF (nM)	ΔT-TF (K)	SEQ ID NO:
12	cyclo		ε-K	E	Hse (OMe)	G	F	F	A	D				51420	10380.8	4.1	9
12	cyclo		ε-K	E	Nle	G	F	F	A	D				17340	7587.2	4.7	10
12	cyclo		ε-K	E	Semet	G	F	F	A	D				803.7	584	9.1	11
εK = epsilon-lysine (lysine linked via its side-chain amino group to C-terminal amino acid in an amide linkage); Semet = selenomethionine; Hse(OMe) = O-methyl-L-homoserine; Nle = norleucine;																	

7.1 ITC results for EE-124:

Isothermal calorimetry (ITC) experiment was performed on EE-124 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (15 μM) with the peptide ligand (200 μM), at 25°C (20 titrations of 2.5 μL per injection with 300 seconds intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 622.1 (± 109.7) nM, with an enthalpy change, ΔH, of −27.7 (± 1.3)kJ/mol and entropy change, ΔS, of 26.0 (± 5.9) J/mol·K (**Fig. 24**).

20

7.2 Comparison of biophysical assay results and X-ray crystal structure for EE-124 with eIF4E

Results from the biophysical assays of EE-124 were compared with EE-108 in **Fig. 25**. EE-124 was co-crystallized with eIF4E. The crystals obtained were analyzed by X-ray crystallography to obtain the structure shown in **Fig. 26**.

5

EXAMPLE 8

Biophysical assays and Phe6/Phe7 evolution to EE-129:

Employing perfluorinated phenylalanine (F₅-Phe) on the F⁶ position as its binding site on eIF4E is able to accommodate such changes to the phenyl ring (**Fig. 27**, top, middle).

10 *ortho*-Fluoro-Phe (*o*-F-Phe) on the F⁷ position would also be introduced since partial fluorination on the phenyl ring would enhance the polarity of the aromatic ring – favoring a buildup of positive charges (δ^+) especially on the proton *para*- to the fluorine. This would enhance the edge-to-face pi-pi stacking interactions with F⁷; this interaction is shown in **Fig. 27** (top right). This generated the lead STC peptide **EE-129**.

15 EE-130 has a K_D of 331.5 nM by ITC, a two-fold improvement from EE-124, as predicted. To confirm that the effects of perfluorinated phenyl ring is specific for the F⁶ position, the residue was introduced to the F⁷ position (EE-132) and both the F⁶ and F⁷ positions (EE-131). As predicted, perfluorinations on the phenyl groups in these positions caused a decrease in binding affinities; EE-132 saw a decrease in K_D to 1082.5 nM, while EE-131 saw a larger
20 decrease of K_D to 1913nM. Building from EE-130, EE-129 with the same perfluorinated phenyl on F⁶ position, but with an additional 2-fluoro-phenyl group in the F⁷ position, gave an optimum K_D of 169.7 nM, as studied by ITC. To show that these effects were specific for the *ortho*-position of the phenyl group on F⁶, EE-133 was tested – possessing fluorine substituents on both the *para*- and *meta*-positions. This new peptide gave a lower binding affinity K_D of 1121.5 nM,
25 highlighting the success of our rationalization approach in EE-129 (**Fig. 28**).

8.1 ITC results for EE-129:

Isothermal calorimetry (ITC) experiment was performed on EE-129 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a
30 buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (17 μ M) with the peptide ligand (110 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 169.7

(± 13.6) nM, with an enthalpy change, ΔH , of $-47.9 (\pm 0.92)$ kJ/mol and entropy change, ΔS , of $-30.9 (\pm 3.6)$ J/mol·K (**Fig. 29**).

8.2 ITC results for EE-130:

5 Isothermal calorimetry (ITC) experiment was performed on EE-130 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (22 μ M) with the peptide ligand (170 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated two more times for a total of three times. The K_D obtained was 360.8 (± 69.7) nM, with an enthalpy change, ΔH , of $-38.8 (\pm 3.7)$ kJ/mol and entropy change, ΔS , of $-6.7 (\pm 10.8)$ J/mol·K (**Fig. 30**).

8.3 ITC results for EE-131:

15 Isothermal calorimetry (ITC) experiment was performed on EE-131 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (15 μ M) with the peptide ligand (150 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated one more time for a total of two times. The K_D obtained was 828.7 (± 106.0) nM, with an enthalpy change, ΔH , of $-35.4 (\pm 3.2)$ kJ/mol and entropy change, ΔS , of $-2.1 (\pm 11.9)$ J/mol·K (**Fig. 31**).

8.4 ITC results for EE-133:

20 Isothermal calorimetry (ITC) experiment was performed on EE-133 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (15 μ M) with the peptide ligand (150 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated one more time for a total of two times. The K_D obtained was 1093.5 (± 123.7) nM, with an enthalpy change, ΔH , of $-52.7 (\pm 1.2)$ kJ/mol and entropy change, ΔS , of $-62.6 (\pm 3.1)$ J/mol·K (**Fig. 32**).

EXAMPLE 9

30 Detailed *in silico* Crystal structure (CS) analysis:

We used crystal structure coordinates for four cyclic peptide-protein complexes, EE-44, EE-108, EE-48, and EE-94 with the resolution of 2.35 Å, 2.70 Å, 2.70 Å and 2.10 Å, respectively. All these cyclic peptides interact with the protein in a very similar manner, keeping

the inter protein-peptide interactions very similar. The main difference is found in EE-108 peptide with significant loop movement near residue Asn-50 caused by its interaction with the Linker. From the crystal structures analyzed (**Fig. 33**), it is apparent that the majority of the interactions are retained among them and the main difference in the peptide conformations are in its non-interacting region. The conformational changes reflecting peptides' flexibility and rigidity are mainly due to the changes in their linker-types. E³ is a critical residue involved in salt bridge formations with R¹¹² of eIF4E. M⁴ interacts with D⁹⁰ and also has a weaker polar interaction with S⁹² of eIF4E. M⁴ also makes hydrophobic interactions with W⁴⁶, F⁴⁷, F⁴⁸ and Y⁹¹. G⁵ makes a hydrogen bond with W¹⁰² and is also important for turn conformation of the peptide. Aromatic rings of F⁶ and F⁷ have significant stacking interactions with residues W¹⁰² and W⁵⁶ respectively. A⁸ and D⁹ residues do not make any specific interactions in the crystal structures. The details are summarized in Table 10. The main intra-peptide interaction is i, i+3 hydrogen bond between the backbone atoms of E³ (carbonyl O) and F⁶ (amide N, Table 11). This is important for maintaining the β -turn adopted by the peptide. This turn is classified as 'Type-II β turn' based on the dihedral angles of residues M⁴ (-55, 121) and G⁵ (110, -24). The crystal waters in the vicinity of E³-R¹¹² eIF4E are conserved in these four structures.

Table 10

	EE44	EE108	EE94	EE48
Linker(O): Arg_157(NH)	3.2	3.9	5.0	3.4
Glu_3(OE1,2) : Arg_112(NH1,2)	2.9, 4.7	4, 5.1	4.1, 6.7	2.8, 4.9
Met_4 (N):Asp_90(OD)	2.8	2.7	2.9	2.7
Gly_5 (O): Trp_102(N)	2.9	3.1	2.9	3
Glu_5(N) : Pro_100(O)	3.3	3.2	3.1	3.2
Linker(N): Asn_50(OD)	9.3	2.3	8.8	7.3
Distances in Å of interactions between protein and the peptide are shown here for the available structures.				

Table 11

	EE02	EE108	EE94	EE48
Glu_3(O):Phe_6(N)	3.1	2.7	2.8	3.1
Phe_6(O):Glu_3(N)	4.3	3.6	3	4.4

Phe_6(O):Linker(N)	5.1	5.6	4.8	4.8
Distances in Å of intra-peptide interactions are shown here for the available structures.				

EXAMPLE 10

¹³C, ¹⁵N eIF4E NMR: Chemical Shift Perturbation (CSP) study:

Protein binding studies were performed to assess changes to apo eIF4E (**Fig. 34**) upon ligand binding; eIF4E's interactions with peptides EE-124 (**Fig. 35**) and EE-129 (Fig. S32) were studied on ¹³C, ¹⁵N protein NMR (600/800MHz). Chemical shift perturbations (CSP) were imaged (**Fig. 36, 37 and 39**).

Interactions found for eIF4E/EE-124 complex (**Fig. 36**): ¹H-¹⁵N HSQC perturbations (i.e. changes in chemical shifts) were observed for W⁴⁶, F⁴⁸, K⁴⁹, N⁵⁰, W⁵⁶, G⁸⁸, C⁸⁹, S⁹², E⁹⁹, M¹⁰¹, W¹⁰², R¹⁰⁹, G¹¹⁰, R¹¹², G¹⁵¹, C¹⁷⁰, E¹⁷¹, S¹⁹⁹, D²⁰², A²⁰⁴ and K²¹². Two contiguous regions proximal to the peptide EE-124 binding site show chemical shift changes upon peptide binding: (1) W⁴⁶, F⁴⁸, K⁴⁹ and N⁵⁰, and (2) R¹⁰⁹, G¹¹⁰, S¹⁹⁹, D²⁰², A²⁰⁴ and K²¹². Interestingly, in our protein NMR study (shown in yellow), classical m⁷GTP phosphate binding region (R¹⁵⁷, K¹⁵⁹ and K¹⁶²) is relatively unaffected, by EE-124 binding to eIF4E. Direct interacting regions between EE-124 and eIF4E shown as W⁵⁶, G⁸⁸, C⁸⁹, S⁹², E⁹⁹, M¹⁰¹, W¹⁰², R¹¹² and G¹⁵¹. Furthermore, although X-ray co-crystal structures suggested EE-124 has competing interactions between R¹¹² and R¹⁵⁷, protein NMR study showed otherwise; Only R¹¹² is affected (**Fig. 37**).

Similar to EE-124, interaction mapping for EE-129 shows extensive perturbations in the cap-binding site & surrounding contiguous regions (**Fig. 38**). However, EE-129 was not completely bound in these protein NMR studies, perhaps due lower solubility relative to EE-124. Binding differences are most noticeable for W¹⁰², G¹¹⁰ and E¹⁷¹ and could be related to differences in the macrocyclic peptide's F⁶ aromatic pocket interactions with eIF4E (**Fig. 39**); i.e. differences in effects of perfluorinated phenylalanine in EE-129's F⁶ position versus non-fluorinated phenylalanine in EE-124's F⁶ position.

10.1 Methods for 1H-15N TROSY-HSQC NMR study of ¹³C, ¹⁵N eIF4E and its peptide complexes:

Solution conditions used for structure determination were 0.2mM ¹³C-¹⁵N-eIF4E (human) with or without 0.2mM macrocyclic peptides (EE-124 or EE-129) in 50 mM phosphate buffer (pH 7.4), 100 mM NaCl, 1 mM dithiothreitol (DTT), 0.02% NaN₃ (Volpon et al., EMBO J 25: 5138-5149 (2006)). All NMR spectra were recorded at 298K on a Varian Inova 600 MHz

spectrometer or a Bruker 800 MHz spectrometer, both equipped with cryogenic probes. The data was processed and analyzed using NMRPipe (Delaglio et. al., J. Biomolec. NMR 6: 277-293(1995)) and CARA (Keller, The CARA/Lua Programmers Manual.

<http://cara.nmrsoftware.org/downloads/NMR.014-0.5x.PDF> (2004)), respectively.

5 We assigned the chemical shifts of Apo-eIF4E and eIF4E/EE-124 or eIF4E/EE-129 according to previous works done by Miura et al., J. Biomolec. NMR 27: 279-280 (2003) and Volpon et. al. op. cit. All NMR samples were deuterated and Non Uniform Sampling (NUS) was used in the two indirect dimensions to collect triple resonance data, and with Poisson Gap Sampling to sample 12–15% of the indirect grid (Hyberts et al., J. Amer. Chem. Soc. 132: 2145-2147 (2010)). The hmsIST program was employed to reconstruct and process the data (Hyberts
10 et al., J. Biomolecular NMR 52: 315-327 (2012)).

10.2 Recombinant ^{13}C , ^{15}N eIF4E expression:

The plasmid containing the full-length cDNA for human eIF4E (Chugai
15 pharmaceutical Co., Ltd) was expressed in Escherichia coli BL21 (DE3), using minimal media containing $^{13}\text{C}_6$ glucose as the sole carbon source and $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source (Miura et al. op. cit.). The protein was purified according to the procedures by Volpon et. al. op. cit.

10.3 ^1H , ^{13}C and ^{19}F NMR spectra and assignments for EE-124 and EE-129 peptides:

20 ^1H , ^{13}C and ^{19}F NMR data of EE-124 and EE-129 peptides alone were collected in d_6 -DMSO and d_6 -DMSO with 20% D_2O .

EE-124: ^1H NMR (400MHz, d_6 -DMSO) δ_{H} 12.30 (bs, 4H), 8.51 (bs, 1H), 8.35 (s, 1H), 8.24 (s, 1H), 8.18-8.13 (m, 4H), 7.89 (d, $J = 8.0\text{Hz}$, 2H), 7.54 (t, $J = 6.0\text{Hz}$, 1H), 7.30-7.26 (m, 2H), 7.23-7.13 (m, 5H), 7.09-7.06 (m, 2H), 6.60 (bs, 1H), 4.56 (dd, $J = 12.0, 4.0\text{Hz}$, 1H),
25 4.38-4.3 (m, 2H), 4.26-4.15 (m, 3H), 3.92 (dd, $J = 16.0, 4.0\text{Hz}$, 1H), 3.76 (t, $J = 6.0\text{Hz}$, 1H), 3.61 (dd, $J = 16.0, 4.0\text{Hz}$, 1H), 3.16 (dd, $J = 12.0, 4.0\text{Hz}$, 1H), 3.07-3.05 (m, 2H), 2.86 (dd, $J = 16.0, 12.0\text{Hz}$, 1H), 2.77-2.53 (m, 4H), 2.47-2.25 (m, 4H), 2.08-1.66 (m, 8H), 1.38-1.2 (m, 6H) see **Fig. 40**; ^1H NMR (400MHz, d_6 -DMSO with 20% D_2O) δ_{H} 8.41-8.05 (m, 2H), 7.51 (t, $J = 4.0\text{Hz}$, 1H), 7.28-6.99 (m, 7H), 4.51 (t, $J = 6.0\text{Hz}$, 1H), 4.36-4.32 (m, 1H), 4.23-4.08 (m, 6H),
30 3.99-3.92 (m, 5H), 3.87-3.82 (m, 1H), 3.72 (t, $J = 6.0\text{Hz}$, 1H), 3.57 (d, $J = 16.0\text{Hz}$, 1H), 3.14-3.04 (m, 2H), 3.85-2.55 (m, 5H), 2.46-2.24 (m, 3H), 1.99-1.66 (m, 6H), 1.37-1.19 (m, 5H) see **Fig. 42**; ^{13}C NMR (100MHz, d_6 -DMSO) δ_{C} 173.9, 172.2, 171.9, 171.25, 171.2, 171.11, 171.1, 169.9, 169.1, 168.8, 157.79 (q, $^2J_{\text{CF}_3} = 44.0\text{Hz}$, 1C_{TFA}), 138.4, 138.3, 129.7, 129.5, 128.7, 128.6,

126.9, 126.8, 117.8 (q, $^1J_{CF3} = 283.0\text{Hz}$, $1C_{TFA}$), 55.8, 55.3, 53.8, 52.9, 52.5, 50.1, 49.7, 42.4, 38.5, 37.3, 36.9, 36.7, 33.2, 30.8, 28.4, 27.5, 21.2, 20.6, 18.5, 3.9 see **Fig. 44**; ^{19}F NMR (376.5MHz, d_6 -DMSO) δ_F -73.82 (TFA) see **Fig. 46**.

EE-129: ^1H NMR (400MHz, d_6 -DMSO) δ_H 12.22 (bs, 4H), 8.41-7.05 (m, 16H),
 5 4.55-4.53 (m, 1H), 4.38 (dd, $J = 12.0, 8.0\text{Hz}$, 1H), 4.28-4.19 (m, 3H), 3.87-3.70 (m, 3H), 3.64-
 3.43 (m, 4H), 3.19 (dd, $J = 12.0, 4.0\text{Hz}$, 1H), 3.10 (bs, 1H), 3.03-2.87 (m, 3H), 2.66-2.60 (m,
 1H), 2.54-2.52 (m, 1H), 2.47-2.21 (m, 3H), 1.99-1.85 (m, 4H), 1.82-1.72 (m, 1H), 1.69-1.65 (m,
 1H), 1.39-1.19 (m, 5H) see **Fig. 41**; ^1H NMR (400MHz, d_6 -DMSO with 20% D_2O) δ_H 8.39-7.98
 10 4.14-4.10 (m, 1H), 4.05-4.03 (m, 2H), 4.00-3.97 (m, 3H), 3.93-3.92 (m, 3H), 3.80-3.73 (m, 2H),
 3.67-3.63 (m, 1H), 3.15 (dd, $J = 16.0, 6.0\text{Hz}$, 1H), 3.06-2.95 (m, 2H), 2.90-2.83 (m, 1H), 2.66
 (dd, $J = 16.0, 4.0\text{Hz}$, 1H), 2.56-2.54 (m, 1H), 2.45-2.35 (m, 2H), 2.32-2.22 (m, 2H), 2.0-1.65 (m,
 6H), 1.41-1.19 (m, 5H) see **Fig. 43**; ^{13}C NMR (100MHz, d_6 -DMSO) δ_C 174.0, 171.8, 171.1,
 170.2, 170.0, 169.5, 169.3, 168.8, 162.1, 159.7, 157.7₉ (q, $^2J_{CF3} = 44.0\text{Hz}$, $1C_{TFA}$), 145.1 (d, $^1J_{CF}$
 15 = 244.0 Hz, $1C_{PhF}$), 140.7, 136.9 (d, $^1J_{CF} = 244.0\text{Hz}$, $1C_{PhF}$), 135.6 (d, $^2J_{CF} = 23.0\text{Hz}$, $1C_{PhF}$),
 130.0 (d, $^1J_{CF} = 244.0\text{Hz}$, $1C_{PhF}$), 129.9₆ (d, $^1J_{CF} = 244.0\text{Hz}$, $1C_{PhF}$), 124.8, 124.7, 124.2, 117.8
 (q, $^1J_{CF3} = 283.0\text{Hz}$, $1C_{TFA}$), 115.2, 115.0, 111.6, 111.4, 53.7, 53.1, 52.6, 52.4, 52.1, 49.6, 49.0,
 42.1, 38.0, 36.5, 33.0, 30.4, 29.4, 28.0, 27.3, 23.9, 20.6, 20.2, 18.2, 3.5 see **Fig. 45**; ^{19}F NMR
 (376.5MHz, d_6 -DMSO) δ_F -73.82 (TFA), -117.84 (2-fluoro-phenylalanine), -142.2 (d, $J = 26.3$
 20 Hz, $2F_{ortho}$, pentafluorophenylalanine), -156.8 (t, $J = 22.6\text{Hz}$, $2F_{para}$, pentafluorophenylalanine), -
 163.0 (td, $J = 22.6, 7.5\text{Hz}$, $2F_{meta}$, pentafluorophenylalanine) see **Fig. 47** and **Fig. 48**.

EXAMPLE 11

NanoCLICK assay description:

25 The NanoCLICK assay is a high-throughput, target-agnostic cell permeability
 assay, developed in MSD, that combines NanoBRET technology with intracellular Click
 chemistry.

The NanoCLICK assay essentially measures the cumulative cytosolic exposure of
 a peptide in a concentration-dependent manner. It has been named NanoClick as it combines in-
 30 cell copper-free Click chemistry and monitoring of a NanoBRET signal in cells. The assay is
 based on cellular expression of the NanoLuc-HaloTag system and relies on the Click reaction of
 azide-containing peptides with DiBac-chloroalkane (CA) anchored to the HaloTag. The
 subsequent introduction of an azido-dye followed by the NanoLuc substrate allows the detection

of a BRET signal that is reduced by the presence of Click-reactive peptides in the cytosol. The readout can be expressed as a permeability ratio of EC₅₀s when compared to the response of a low permeability control. We validated the assay using known cell-penetrating peptides for the p53/MDM2 model system. The assay has been applied across multiple programs and has been used to guide and establish structure-permeability relationships in the optimization of macrocyclic peptides for cellular potency across intracellular PPI target programs.

In this Example, the assay was performed using 384 well white assay plates (Perkin Elmer CUSG03874) at a density of 6000 cells/well and incubated at 37°C 5% CO₂ overnight. DIBAC-CA was diluted in assay buffer (OptiMem without phenol red + 1% FBS) and added to cells at a final concentration of 3 µM and incubated at 37°C 5% CO₂ for 1 hour.

Cells were subsequently centrifuged using a BlueWasher (Blue Cat Bio) to remove the DIBAC-CA solution and washed two times with HBSS (Ca⁺⁺, Mg⁺⁺). Then, 30 µL of assay buffer was added back to cell plates. Peptides were serially diluted four-fold in DMSO with a Hamilton Star, and then delivered into assay plates with an acoustic liquid handler Labcyte ECHO (300 nL, 1% DMSO in-well concentration). After incubating cells with peptide for the desired time (4 hours or 18 hours), the HaloTag ligand, NanoBret618-azide, was added to each well at a final concentration of 10 µM. After 1 hour incubation at 37°C 5% CO₂, NanoBRET NanoGlo Substrate and Extracellular NanoLuc Inhibitor (Promega) were added according to the manufacturer's recommended protocol and immediately read on the enVISION™. For lytic assays, 50 µg/mL of digitonin was added to the assay buffer during the DIBAC, peptide and NB618-AZ incubation steps. When the assay was run at 4°C, the assay buffer was pre-chilled to the assay temperature and added to cell plates after DiBAC-CA treatment. Assays run at 4°C were incubated in a refrigerator. The steps following peptide incubation were also run at the same temperature prior to reading the plate on the enVISION™. The readout consists of a ratio of the donor and acceptor wavelengths.

The positive permeable control peptide was azide-ATSP-7041 (Ac-K(N₃)-betaAla-LTF-R8-EYWAQ-Cba-S5-SAA-NH₂ (SEQ ID NO: 44), where R8 and S5 refer to the i,i+7 stapling positions using (R)-2-(7-octenyl)Ala and (S)-2-(4'-pentenyl)Ala, respectively, which gave EC₅₀ 4 hour ratio of 0.03 and EC₅₀ 18 hour ratio of 0.01.

The negative impermeable control peptide was EEE-azide-ATSP-7041 (Ac-EEE-K(N₃)-betaAla-LTF-R8-EYWAQ-Cba-S5-SAA-NH₂ (SEQ ID NO: 45), where R8 and S5 refer to the i,i+7 stapling positions using (R)-2-(7-octenyl)Ala and (S)-2-(4'-pentenyl)Ala, respectively, which gave EC₅₀ 4 hour ratio of >1 and EC₅₀ 18 hour ratio of >1 (Table 12).

NanoCLICK EC₅₀_RATIO was determined using the EC₅₀ obtained for the peptide-of-interest against the EC₅₀ obtained from the negative impermeable control peptide:

$$EC_{50_RATIO} = EC_{50_}[peptide] / EC_{50_}[impermeable\ EEE\text{-}azide\text{-}ATSP\text{-}7041].$$

Table 12									
NanoCLICK assays performed on positive permeable control and negative impermeable control peptides									
		4 hr, n=1		4 hr, n=2		18 hr, n=1		18 hr, n=2	
Name	Target	EC ₅₀	EC ₅₀ _R ATIO	EC ₅₀	EC ₅₀ _R ATIO	EC ₅₀	EC ₅₀ _R ATIO	EC ₅₀	EC ₅₀ _R ATIO
ATSP-7041	Positive control	152.36	0.03	96.9	0.01	74.36	0.01	57.35	0.01
Impermeable ATSP-7041	Negative control	5318.58	1	>10000	>1	>10000	>1	>10000	>1

5

EXAMPLE 12

NanoBIT assay description:

NanoBIT assays were performed by HD Biosciences (HDBio, Shanghai, China); Cellularly expressed NanoBIT complex used in the assay has a binding K_D of 5.9nM.

10

Objective: (1) Test the validation set compounds in NanoBIT assay with 20 µg/mL digitonin to permeabilize the cells, (2) without digitonin, and (3) Test the effect of protease inhibitor on the compounds dose response.

Reagents and Consumable List:

Item	Supplier	Catalog No.
Plasmid LgBIT-eIF4E	Merck	
Plasmid SmBIT-VHEE26	Merck	
Plasmid SmBIT-Halo	Merck	
Plasmid pCDNA3	Merck	
L-006404568-000T (M7 GTP)	Merck	

DMSO	Sigma	472301-1L
Digitonin	Sigma	D141
Protease inhibitor (cOmplete™, EDTA-free)	Roche	4693132001
DMEM	Gibco	11965092
Opti-MEM I	Invitrogen	11058021
Lipofectamine 3000	Life	L3000015
Nano-Glo Live Cell Assay System	Promega	N2013
384 LDV compound plate	Labcyte	LP-0200
MultidropCombi small tube metal tip dispensing cassette	ThermoFisher	24073295
white 384w assay plate	Corning	3570

Equipment List:

Item	Supplier
Echo 550	Labcyte
MultidropCombi	Thermo Scientific
enVISION™	Perkin Elmer
ViewLux	Perkin Elmer
Plate Centrifuge	
Tissue Culture Incubator (37°C/5% CO ₂)	Thermo Scientific
Single and Multi-channel pipette	Rainin
Countess II	Thermo

Validation compounds: EE-111, EE-124, EE-129, EE-130, EE-131, EE-132, EE-133, and

5 M⁷GTP.

Cells used: HEK293F

Growing and transfecting cells:

1. Cells may be grown and passed 1:3-1:5 ratio in 150mm dishes with 10% FBS DMEM and passes with 0.25 % trypsin;
- 10 2. Expand cells to 10 dishes and culture to 90% confluence before transfecting the cells; *On the day to transfecting cells:*

a. Prepare DNA-Lipofectamine 3000 mixture. Ratio Lipo3000/DNA is 1.5 (every 1 µg of DNA with 1.5 µL of Lipo3000), ratio P3000/DNA is 2 (every 1 µg of DNA with 2 µL of P3000). Per single 150mm dish, prepare Mix 1 and Mix 2 as following.

Mix 1: 60µL Lipo3000 in 1250µL of Opti-MEM I (0% FBS, no red phenol);

5 *Mix 2:* 20µg plasmid 1 (e.g. LgBIT-eIF4E) + 20 µg plasmid 2 (e.g. SmBIT-VHEE26) in 1250µL of Opti-MEM I (0% FBS, no red phenol); Add 80µL P3000 in Mix 2, re-suspend and then add Mix 2 into Mix 1, resuspend and incubate at room temperature for 15 minutes.

b. Remove DMEM media from each 150 mm dish, add in 18 mL 0% FBS Opti-MEM (no red phenol).

10 c. Add the DNA transfection mix to each dish, rock to mix and incubate at 37°C, 5% CO₂ overnight.

Harvesting and freezing transfected cells:

1. Remove media, wash cells with 5 mL of PBS, remove PBS and detach the cells from the surface of the plate using 2 mL trypsin per dish.

2. Re-suspend cells with 8 mL of media and collect into 50 mL tubes. Centrifuge at 1200 rpm for 5 minutes at room temperature.

3. Discard supernatant and resuspend cells again with freezing media (90% FBS + 10% DMSO) to achieve a final concentration of 3 M/mL.

20 4. Dispense 1ml aliquots into sterile cryovials and freeze down the cells at a cooling rate of about 1 °C per minute (1 °C/min).

5. Transfer cells to vapor phase Liquid N₂ within 3 days.

Assay Procedure

25 1. Prepare the doses of validation set compounds on LDV plate. Performing 3.162-folds, 12 points serial dilution for all compounds from a top concentration of 10 mM. Transfer 200 nL dose to the assay plate.

2. Thaw assay ready frozen vial of cells (SmBIT-VH-EE26/LgBIT-eIF4E transfected cells) in 37°C water bath, then transfer the cells to 5 mL Opti-MEM I® no red phenol in 15 mL tube.

30 Centrifuge at 1200 rpm for 5 minutes at room temperature.

3. Discard supernatant and re-suspend cells again in 0% FBS Opti-MEM I no red phenol. Count cell number and adjust cell density to 1 x 10⁶ cells per mL (2X working cell suspension) using 0% FBS Opti-MEM I® no red phenol.

4. Prepare 2X working solution of protease inhibitor. Dissolve one tablet of Protease inhibitor (Roche, cOmplete™, EDTA-free) in 20 mL 0% FBS Opti-MEM I no red phenol.

5. *Conditional:* Prepare 2X working digitonin solution without protease inhibitor by dilute the digitonin stock (1 mg/mL) with 0% FBS Opti-MEM I. Prepare 2X working digitonin solution with protease inhibitor by dilute the digitonin stock with 2X working solution of protease inhibitor prepared in step 4. The working concentrations of digitonin is 40 µg/mL for the assay (20 µg/mL final concentration).

6. *Conditional:* Mix 2X working digitonin solutions (with and without protease inhibitor) with 2X working cell suspension of equal volume, respectively. Permeabilize the cells at room temperature for 20 minutes.

7. Add 20 µL of the cell lysates to designated well containing 200nL compound dose (final top concentration is 100 µM). Incubate at room temperature for 60 minutes.

8. Equilibrate NanoGlo® buffer to room temperature and use it to dilute the NanoGlo® substrate at a ratio of 1:19 buffer to substrate.

9. Add 5µL of the diluted substrate to each well.

10. Read plate on ViewLux® at room temperature after 30 minutes.

Data Analysis: Data was analyzed by GraphPad Prism®. The compound IC₅₀ values are calculated by using following equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}$, X: log of dose or concentration, Y: Response, decreasing as X increases, Top and Bottom: Plateaus in same units as Y, logIC₅₀: same log units as X, HillSlope: Slope factor or Hill slope, unitless.

12.1 LDH assay procedure:

Objective: Test the dose response of validation set compounds (including iDNA79) in LDH assay using cells transfected by LgBIT-eIF4E and SmBIT-VHEE26 plasmids.

Reagents and Consumable List

Item	Supplier	Catalog No.
Cell transfected by LgBIT-eIF4E and SmBIT-VHEE26 plasmids		
iDNA79	Merck	
DMSO	Sigma	472301-1L

Digitonin	Sigma	D141
DMEM	Gibco	11965092
Opti-MEM I	Invitrogen	11058021
CytoTox™ 96 Non-Radioactive Cytotoxicity Assay Kit	Promega	G1780
384 LDV compound plate	Labcyte	LP-0200
Multidrop Combi™ small tube metal tip dispensing cassette	ThermoFisher	24073295
white 384w assay plate	Corning	3570
Clear 384w assay plate	Perkin Elmer	6007658

Equipment List

Item	Supplier
Echo 550	Labcyte
Multidrop Combi™	Thermo Scientific
enVISION™	Perkin Elmer
Plate Centrifuge	
Tissue Culture Incubator (37°C/5% CO ₂)	Thermo Scientific
Single and Multi-channel pipette	Rainin
Countess II™	Thermo

Validation compounds: EE-111, EE-124, EE-129, EE-130, EE-131, EE-132, EE-133, M⁷GTP, and iDNA79.

Cells used: HEK293F

Assay procedure

1. Prepare the doses of validation compounds on LDV plate. Performing 3.162-folds, 12 points serial dilution for all compounds (except for iDNA79) from a top concentration of 10 mM. Performing 3.162-folds, 12 points serial dilution for iDNA79 from a top concentration of 5 mM. Transfer 200 nL dose to assay plate 1 (Corning #3570) by Echo 550®.
2. Thaw assay ready frozen vial of cells (SmBIT-VH-EE26/LgBIT-eIF4E transfected cells) by 37°C water bath, then transfer the cells to 5mL Opti-MEM I® no red phenol in 15 mL tube. Centrifuge at 1200 rpm for 5 minutes at room temperature.

3. Discard supernatant and re-suspend cells again in 0% FBS Opti-MEM I[®] no red phenol. Count cell number and adjust cell density to 5×10^5 cells per mL using 0% FBS Opti-MEM I[®] no red phenol.
4. Add 20 μ L of the cell suspension to designated well of assay plate 1 containing 200nL compound dose. Incubate at room temperature for 2 hours.
5. Transfer 10 μ L of supernatant from assay plate 1 to assay plate 2 (PE #6007658). Then add in 10 μ L CytoTox[™] Reagent. Incubate at room temperature for 30 minutes.
6. Add 10 μ L Stop Solution to each well of assay plate 2.
7. Read 492 nM absorbance on enVISION[™] at room temperature within 60 minutes.

Data Analysis: Data was analyzed by GraphPad[™] (Prism). The compound EC₅₀ values were calculated by using following equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))}$ X: log of dose or concentration, Y: Response, increasing as X increases, Top and Bottom: Plateaus in same units as Y, logEC₅₀: same log units as X, HillSlope: Slope factor or Hill slope, unitless

EXAMPLE 13

Biophysical/Cellular assays for EE-155 EE-169 N₃, EE-171, EE-233, EE-246 and EE-249:

N-methylation scan was done on the EE-129 molecule, and the D9NMeD mutation was found to bind marginally better (EE-155 K_D = 151.1nM and ΔT of 12.7K, as studied on Thermofluor). This gave us EE-155. However, N-methylation on this same position after swapping for other residues obliterated binding affinities. In an effort to improve hydrophobicity, we also made the D9L mutation with an azidolysine on the A⁸ position, to give EE-169 N₃. Although it has worse binding affinities [EE-169 N₃ K_D = 401.7 ($\pm$ 179.0)nM, as studied on FP] than the parent compound EE-129/EE-129 N₃, it is cell-permeable (EC₅₀ 4 hour ratio of 0.375 and EC₅₀ 18 hour ratio of 0.06, as studied on target-agnostic NanoCLICK assay, Table 13), and gave cellular activities on NanoBIT assay (with 8 μ M Endo-Porter EC₅₀18h = 9696.1nM, Table 14) when used together with Endo-Porter delivery formulation (without 8 μ M Endo-Porter EC₅₀18h = >100000 nM).

Table 13

NanoCLICK assay for EE-169 N₃.

		4 hr, n=1		4 hr, n=2		18 hr, n=1		18 hr, n=2	
Name	Target	EC ₅₀	EC _{50_R} ATIO	EC ₅₀	EC _{50_R} ATIO	EC ₅₀	EC _{50_R} ATIO	EC ₅₀	EC _{50_R} ATIO
ATSP-7041	Protein control	152.36	0.03	96.9	0.01	74.36	0.01	57.35	0.01
Impermeable ATSP-7041	Negative control	5318.58	1	>10000	>1	>10000	>1	>10000	>1
EE-169 N3	eIF4E	2730.72	0.51	2432.78	0.24	480.92	0.05	689.31	0.07

Table 14								
NanoBIT assays with Endo-Porter peptide-based delivery agent (formulation)								
Name	NanoBIT with 8uM Endoporters EC50 (N=1) nM	NanoBIT with 8uM Endoporters EC50 (n=2) nM	NanoBIT EC50 (N=1) nM	NanoBIT EC50 (n=2) nM	LDH (with 8uM endoporters) EC50 (N=1) nM	LDH (with 8uM Endoporters) EC50 (n=2) nM	LDH EC50 (N=1) nM	LDH EC50 (n=2) nM
EE-168 N3	>100000	>100000	>100000	>100000	>100000	>100000	>100000	>100000
EE-169 N3	9025.542	10366.686	>100000	>100000	>100000	>100000	>100000	>100000

EE-169 gave appreciable activities.

D-Propargylglycine-PEG₂-Poly-*D*-Arg₁₀ (SEQ ID NO: 37) CPP (*d*Pra-PEG₂-*d*R₁₀ (SEQ ID NO: 37)) was conjugated onto parent peptide EE-129 N₃ by employing Copper-catalyzed azide-alkyne cycloaddition (CuAAC) of both peptides in solution, to generate EE-171 – our biological tool compound and the positive control for our NanoBIT assays (**Fig. 49**). EE-171 has excellent binding affinities with eIF4E [$K_D = 20.0 (\pm 0.6)$ nM, as studied on FP]. As positive control for our NanoBIT assay, EE-171 also has excellent cellular activity [EC₅₀ 4 hours = 7011.0 ($\pm$ 1187.0) nM and EC₅₀ 18 hours = 2765.5 ($\pm$ 694.3) nM, with low LDH leakage EC₅₀ 18 hours = 19220.0 nM]. For the negative control, we have employed All-*D*-EE-171, by conjugating *D*-Propargylglycine-PEG₂-Poly-*D*-Arg₁₀ (SEQ ID NO: 37) CPP (*d*Pra-PEG₂-*d*R₁₀ (SEQ ID NO: 37)) onto All-*D*-EE-129 N₃. This negative control peptide and its parent peptide,

All-D-EE-129 N₃, gave no binding affinities ($K_D = >300000$ nM for both peptides, as studied on FP), and has little cellular activity [EC_{50} 4 hours = >100000 nM and EC_{50} 18 hours = 7968.2 nM, with similar LDH leakage EC_{50} 18 hours = 16852. 8 nM].

Passively permeable and cell-active compound EE-233 was obtained from D9dH mutation of EE-129 N₃. This compound had good binding affinity ($K_D = 124.3$ nM, as studied on FP) and modest cell activity (EC_{50} 18 hours = 4512.9 nM). Further iteration of D9X and A8X mutants did not produce better cell-active compounds but did give peptides that bind much better than the parent peptide EE-129/EE-129 N₃. Specifically, EE-242 ($K_D = 37.1$ nM, as studied on FP) and EE-246 ($K_D = 25.3$ nM, as studied on FP). Replacing the azidolysine on EE-233 with Ala [i.e., K(N₃)8A mutation] in EE-249 gave better results for binding affinity ($K_D = 43.9$ nM, as studied on FP) but not cell activity (EC_{50} 18 hours = >100000 nM). In fact, cell-activity was obliterated in EE-249, indicating a role that lipophilic azidolysine may play in passive permeability for EE-233.

13.1 NanoBIT with permeabilized cells:

To demonstrate that the low or absent NanoBIT activities for some of the peptides, we performed the same with digitonin-induced permeabilized cells (Table 15). The following values were obtained for compounds EE-171 (positive control tool compound), EE-168 N₃ (D9H mutant of EE-129 N₃), EE-169 N₃ (D9L mutant of EE-129 N₃), EE-206 N₃ (D9Phe(*p*-guanidino) mutant of EE-129 N₃), EE-209 N₃ [D9K(N₃), and K(N₃)8Phe(*p*-guanidino) mutant of EE-129 N₃], and EE-233 N₃ (D9dH mutant of EE-129 N₃).

Table 15		
NanoBIT assay with permeabilized cells (by employing digitonin) to demonstrate permeabilization as the problem		
Name	4 hr NanoBIT w/ digitonin EC_{50} (N=1) nM	4 hr NanoBIT w/ digitonin EC_{50} (n=2) nM
EE-171	652.101	3952.548
EE-168 N ₃	1209.81	18816.534
EE-169 N ₃	1871.901	7092.337
EE-206 N ₃	5647.619	
EE-209 N ₃	3736.677	4621.202

EE-233 N ₃	1188.212	214.742
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13.2 ITC result for EE-155:

Isothermal calorimetry (ITC) experiment was performed on EE-155 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (10 μ M) with the peptide ligand (100 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 150.23 ($\pm$ 30.5) nM, with an enthalpy change, ΔH , of $-37.7 (\pm 1.0)$ kJ/mol and entropy change, ΔS , of $4.3 (\pm 5.0)$ J/mol K (**Fig. 50**).

13.2 ITC result for EE-169 N₃:

Isothermal calorimetry (ITC) experiment was performed on EE-169 N₃ to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (10 μ M) with the peptide ligand (100 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 second intervals). The experiment was repeated two more times for a total of three times. The K_D obtained was 384.1 ($\pm$ 189.9) nM, with an enthalpy change, ΔH , of $-4.05 (\pm 1.30)$ kJ/mol and entropy change, ΔS , of $110.0 (\pm 4.4)$ J/mol K (**Fig. 51**). Interestingly, this indicated a shift in binding mechanism, where entropy increase contributed to most of the binding.

13.3 ITC result for EE-171:

Isothermal calorimetry (ITC) experiment was performed on EE-171 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (10 μ M) with the peptide ligand (100 μ M), at 25°C (20 or 30 titrations of 2.5 μ L or 1.0 μ L per injection, respectively, with 300 seconds intervals). The experiment was repeated two more times for a total of three times. The K_D obtained was 70.1 ($\pm$ 15.6) nM, with an enthalpy change, ΔH , of $-51.7 (\pm 7.9)$ kJ/mol and entropy change, ΔS , of $-36.4 (\pm 24.5)$ J/mol K (**Fig. 53**).

13.4 ITC result for EE-233 N₃:

Isothermal calorimetry (ITC) experiment was performed on EE-233 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a

buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (10 μ M) with the peptide ligand (100 μ M), at 25°C (22 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated two more times for a total of three times. The K_D obtained was 86.5 ($\pm$ 37.9) nM, with an enthalpy change, ΔH , of $-16.7 (\pm 1.4)$ kJ/mol and entropy change, ΔS , of 80.0 (± 8.0) J/mol·K (**Fig. 54**).

13.5 ITC result for EE-246:

Isothermal calorimetry (ITC) experiment was performed on EE-246 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (10 μ M) with the peptide ligand (100 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 50.1 ($\pm$ 14.3) nM, with an enthalpy change, ΔH , of $-31.1 (\pm 3.0)$ kJ/mol and entropy change, ΔS , of 35.6 (± 7.8) J/mol·K (**Fig. 55**).

13.6 ITC result for EE-249:

Isothermal calorimetry (ITC) experiment was performed on EE-249 to determine the thermodynamic data and binding affinity. The experiment was performed by titrating a buffered solution (PBS, 2% DMSO, 0.05% Tween 20) of eIF4E (10 μ M) with the peptide ligand (100 μ M), at 25°C (20 titrations of 2.5 μ L per injection with 300 seconds intervals). The experiment was repeated three more times for a total of four times. The K_D obtained was 104.0 ($\pm$ 53.5) nM, with an enthalpy change, ΔH , of $-36.9 (\pm 4.5)$ kJ/mol and entropy change, ΔS , of 10.8 ($\pm$ 13.5) J/mol·K (**Fig. 56**).

EXAMPLE 14

Peptide Synthesis and Purifications:

Solid Phase Peptide Synthesis (SPPS): Peptides were synthesized on a Liberty Blue Automated Microwave Peptide synthesizer from CEM Corporation, using standard solid phase synthesis employing Fmoc chemistry. N,N'-Diisopropylcarbodiimide (DIC) with ethyl (hydroxyimino)cyanoacetate (Oxyma Pure) as coupling reagents for amide bond formations between amino acids. H-Ramage resins (100-200 mesh, 0.6 mmol/g loading, 100% PEG-based ChemMatrix® resins, PCAS BioMatrix Inc.) were employed for disulfide-constrained peptide synthesis, while 2-Chlorotrityl chloride (CTC) resins (200-400 mesh, 1.1 mmol/g loading, 1% divinylbenzene, Iris Biotech GmbH), pre-loaded with either Fmoc-Asp(OH)-Oallyl or Fmoc-

Glu(OH)-Oallyl, were used for synthesis of the head-to-tail cyclized (HTC) and side-to-tail cyclized (STC) peptides (**Fig. 57**). All amino acids were dissolved to 0.2 M concentration in anhydrous DMF (N,N dimethylformamide). The amino acids were activated with equimolar amounts of Oxyma Pure solution (0.5 M in anhydrous DMF), and a 2-fold molar excess of DIC solution (1.0 M in anhydrous DMF). Reactions were typically performed at 0.1 mmol scale. Every synthesis cycle included: (1) Fmoc amino acid deprotection by 20% piperidine in anhydrous DMF (90°C microwave assisted heating, 2 minutes), and (2) coupling (double couplings were performed for difficult couplings) with Fmoc-protected amino acid/DIC/Oxyma (at 5:5:10 equiv.; 90°C microwave assisted heating, 3 minutes). Cycles of deprotections and couplings were repeated with the desired monomers/residues until the full linear peptide sequence was completed (**Fig. 57**).

Cleavage and Deprotection: The resin-bound peptides were deprotected and cleaved from the solid support by treatment with 0.1 M HCl in trifluoroethanol or hexafluoroisopropanol, according to reports by Palladino & Stetsenko, Organic Letters 14: 6346-6349 (2012), or with TFA/H₂O/TIS/DTT (95:1.5:2.5:1.0, v/v; 10 mL) at room temperature (RT) for 3-4 hours, with shaking. After filtration of the resin, crude peptides were concentrated by rotary evaporation and precipitated from the cleavage solution using cold diethyl ether (40 mL) and collected by centrifugation (4000 rpm). The crude peptide was washed with cold diethyl ether (20 mL) and dried *in vacuo*. The crude peptide was dissolved in acetonitrile/DI water (1:3, v/v, 15 mL). The solution was frozen and lyophilized before purification.

Synthesis of HTC and STC peptides: Initial activated CTC resins (1.1 mmol/g) used were stirred with Fmoc-Asp(OH)-Oallyl or Fmoc-Glu(OH)-Oallyl (5.0 equiv.) and 20.0 equivalent of diisopropylamine (DIPEA) in dichloromethane (CH₂Cl₂) to pre-load the CTC resins with the appropriate residue for Head-to-Tail Cyclized (HTC) peptides (e.g. EE-94) and Side-to-Tail Cyclized (STC) peptides (e.g. EE-108). The linear peptide sequence was grown using the Liberty Blue MW synthesizer, and the final resin-bound peptides were collected for the final steps; Deprotection of the N-terminal Fmoc-group with 20% piperidine in DMF, followed by the deprotection of the C-terminal allyl ester group on the initial Asp or Glu residue with two rounds of reactions with 1.0 equiv. Pd(PPh₃)₄ and 4.0 equivalents phenylsilane in CH₂Cl₂, with multiple DMF/CH₂Cl₂ washes (4x) in between reactions. The free C-terminus and free amine on the N-terminus used for the final macrocyclization step (repeated two to three times) using a solution of HATU (4.0 equivalents), HOAt (8.0 equivalents) and DIPEA (8.0 equivalents) for 4 hours each time. HATU refers to (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-

b]pyridinium 3-oxide hexafluorophosphate and HOAt refers to 1-Hydroxy-7-azabenzotriazole. The reaction was monitored on HPLC-MS with mini-cleavage. Upon completion, the peptides were deprotected and cleaved, concentrated *in vacuo*, precipitated in cold diethyl ether, and purified on HPLC to generate white powders (20-70% yield).

5 *Synthesis of Methylene bridged peptide EE-48*: EE-48 was synthesized by employing solution-based alkylation using diiodomethane (**Fig. 58**). To a solution of reduced EE-44 (0.5mM, free cysteines) in a round-bottom flask was added ACN:H₂O (1:1), 2.0 equivalents of diiodomethane and 4.0 equivalents of triethylamine. The solution was stirred overnight and the reaction analyzed by LC-MS to ensure completion. The solution was
10 concentrated *in vacuo*, frozen and lyophilized before it was purified on RP-HPLC (67% yield).

Synthesis of CPP-conjugated peptides EE-171 and All-D-EE-171: EE-129 N₃ and its mirror image, All-D-EE-129 N₃ were conjugated separately to the CPP: D-Propargylglycine-PEG₂-Poly-D-Arg₁₀ (SEQ ID NO: 37) CPP (*d*Pra-PEG₂-*d*R₁₀ (SEQ ID NO: 37)) via Copper-catalyzed azide-alkyne cycloaddition (CuAAC) of both peptides in solution, to generate EE-171
15 and All-D-EE-171. *Procedure*: To a solution of EE-129 N₃ or All-D-EE-129 N₃ in a round-bottom flask (0.5 mM) was dissolved in 20 mL ACN:H₂O (1:1) before adding a solution of copper(II) sulfate pentahydrate (20.0 equiv.), sodium ascorbate (100.0 equivalents) and THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine, 10.0 equiv.) pre-dissolved in 5 mL ACN:H₂O (1:1). The reaction was monitored on HPLC-MS. Upon completion, the solution is concentrated
20 *in vacuo*, and filtered through a reverse-phase column at 5% ACN in H₂O to remove most of the copper salts. The filtrate is then frozen and lyophilized to a white powder (50-60% yield).

HPLC Purification: Purification was performed by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on Shimadzu Prominence using C-12 Jupiter 4u Proteo 90A, AXIA (250 x 21.2mm) or Gemini 5u C18 110A (250 x 10.0mm) *Mobile*
25 *phase*: (A) 0.1 % TFA in HPLC water and (B) 0.1 % TFA in HPLC acetonitrile; Or *Mobile phase*: (A) 5 mM HCl in HPLC water and (B) 5 mM HCl in HPLC acetonitrile; flow rate: 15 mL/min; UV wavelength λ = 220 and 280 nm; gradient: 5-40% B over 45 minutes. UV absorbing fractions containing the target m/z ions were collected and the fractions containing product were confirmed by LC/MS. Purity of fractions was confirmed by UPLC-MS and HPLC-MS.

Lyophilization of combined fractions containing pure peptide resulted in the final product as a white powder.

14.1 Analytical methods (HPLC-MS, UPLC-MS and HRMS):

5 *HPLC-MS*: Shimadzu LC-30AD instrument integrated with a LCMS2020 mass spectrometer; Column: Phenomenex Jupiter Proteos 110A, 150 × 2.1 mm, 5 μm. Samples were prepared in 0.1% TFA solution, with sample tray and column temperature kept at 15°C and 25°C, respectively. *Mobile phase*: (A) 0.1 % formic acid in HPLC water and (B) 0.1 % formic acid in HPLC acetonitrile; gradient: 60% B in 25 minutes; flow rate: 1.0 mL/minutes; UV wavelength λ = 190 nm and 220 nm. Mass spectrum (m/z) was recorded under ESI conditions.

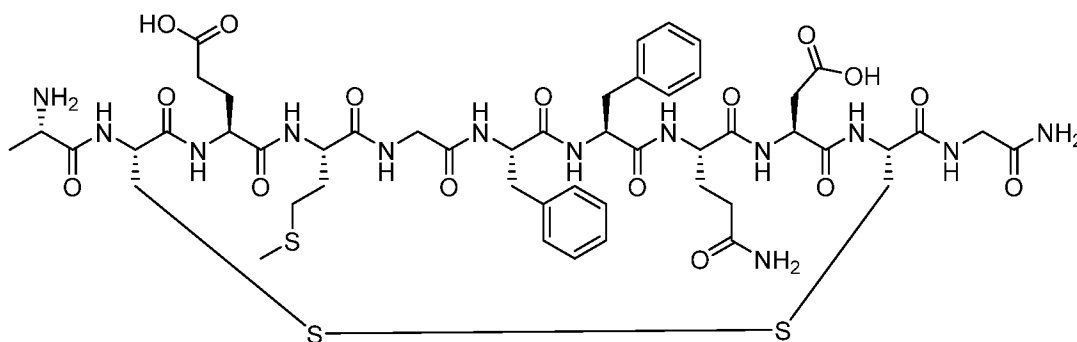
10 *UPLC-MS*: Agilent Technologies 1290 Infinity 2 UHPLC with DAD; Column: Aeris 1.7 μm PEPTIDE XB-C18 100 LC Column 150 x 2.1 mm. Samples were prepared in 5 mM HCl solution, pH 2.3, with sample tray and column temperature kept at 30°C. *Mobile phase*: (A) 0.1 % formic acid in HPLC water and (B) 0.1 % formic acid in HPLC acetonitrile; gradient: 60% B in 25 min; flow rate: 0.2 mL/minutes; UV wavelength λ = 220 nm. Mass spectrum (m/z) was recorded under ESI conditions.

20 *High-resolution mass spectrometry (HRMS)*: HRMS were sent to the Institute of Chemical and Engineering Sciences (ICES), Agency for Science, Technology and Research (A*STAR), and recorded on an Agilent 6200 Series TOF and 6500 Series Q-TOF LC/MS System, using ESI-TOF.

EXAMPLE 15

Exemplary peptides of the present invention.

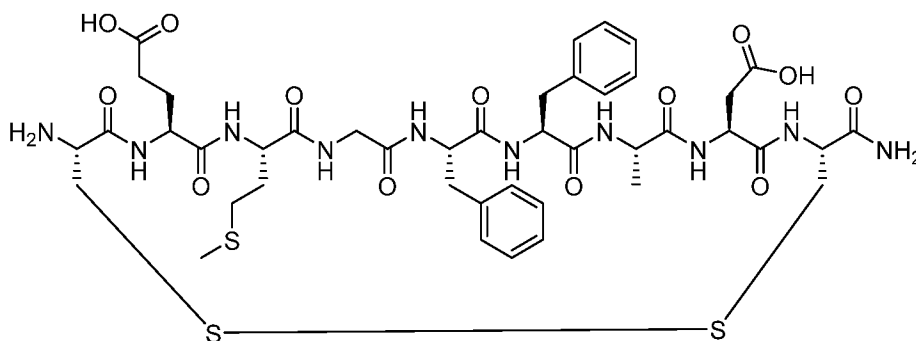
EE-02 peptide: ACEMGFFQDCG-NH₂, disulfide bond, SEQ ID NO: 1.



Chemical Formula: $C_{50}H_{69}N_{13}O_{16}S_3$; Exact Mass [M]: 1203.41474; Mass found $[M+H]^+$: **1204.2**; Mass found $[M+2H]^{+2}$: **602.8**; Mass found $[M-H]^-$: **1202.2**; Mass found $[M-2H]^{-2}$: **600.8**
HRMS (ESI-TOF) found $[M+H]^+$ 1204.4199 $C_{50}H_{70}N_{13}O_{16}S_3$ requires 1204.42257; error: 2.22 ppm

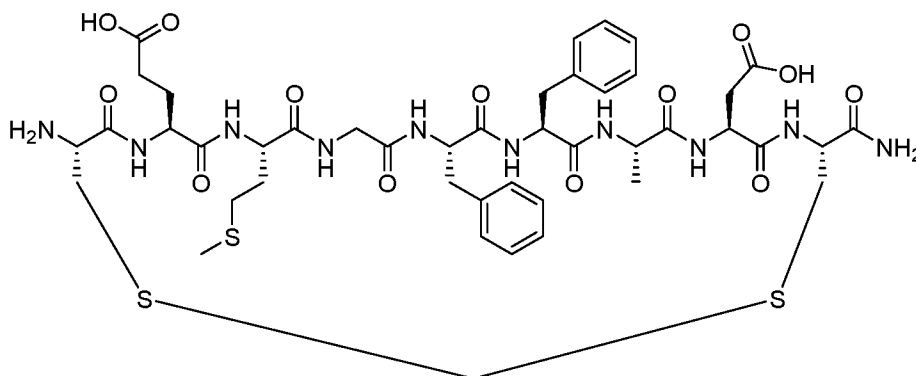
5

EE-44 peptide: CEMGFFADC-NH₂, disulfide bond, SEQ ID NO: 2.



- 10 Chemical Formula: $C_{43}H_{58}N_{10}O_{13}S_3$; Exact Mass [M]: 1018.33469; Mass found $[M+H]^+$: **1019.1**; Mass found $[M-H]^-$: **1017.1**
HRMS (ESI-TOF) found $[M+H]^+$ 1019.3399 $C_{43}H_{59}N_{10}O_{13}S_3$ requires 1019.3425; error: 2.55 ppm

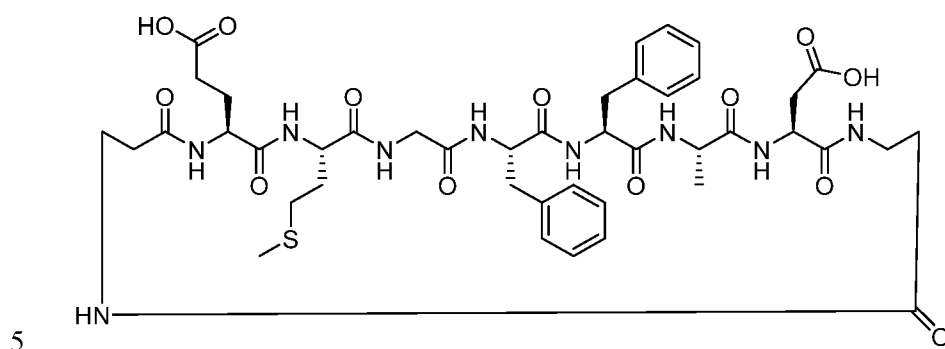
- 15 *EE-48 peptide*: C*EMGFFADC*-NH₂, Cys*-Cys* methylene bridge, SEQ ID NO:3.



Chemical Formula: $C_{44}H_{60}N_{10}O_{13}S_3$; Exact Mass [M]: 1032.35034; Mass found $[M+H]^+$: **1033.1**; Mass found $[M+2H]^{+2}$: **517.1**; Mass found $[M-H]^-$: **1031.1**
HRMS (ESI-TOF) found $[M+H]^+$ 1033.3558 $C_{44}H_{61}N_{10}O_{13}S_3$ requires 1033.3582; error:

- 20 2.32 ppm

EE-91 peptide: cyclo[βA-EMGFFAD-βA], Head-to-Tail Cyclized (HTC), SEQ ID NO: 4 (βA: beta-L-alanine (βA))

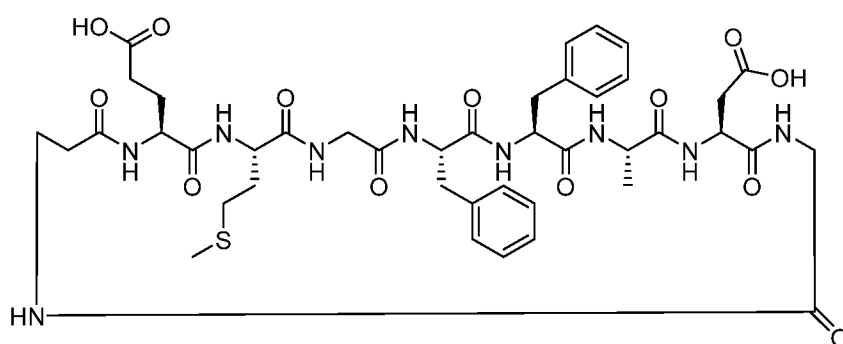


Chemical Formula: $C_{43}H_{57}N_9O_{13}S$; Exact Mass [M]: 939.37965; Mass found $[M+H]^+$: **940.3**;
Mass found $[M-H]^-$: **938.3**

HRMS (ESI-TOF) found $[M+H]^+$ 940.3855 $C_{43}H_{58}N_9O_{13}S$ requires 940.3875; error: 2.13

10 ppm

EE-94 peptide: cyclo[βA-EMGFFAD-G], Head-to-Tail Cyclized (HTC), SEQ ID NO: 5.

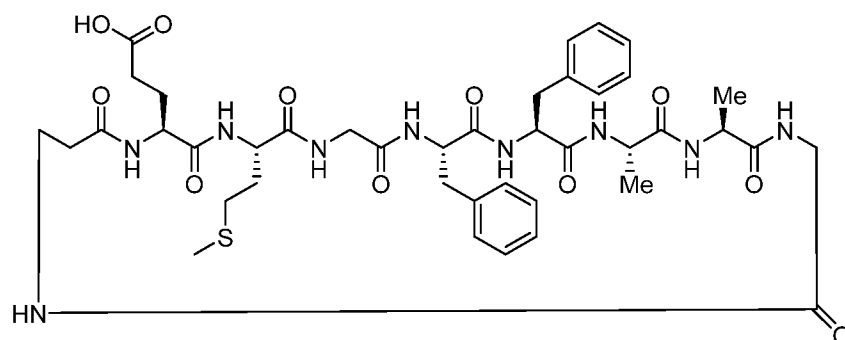


Chemical Formula: $C_{42}H_{55}N_9O_{13}S$; Exact Mass [M]: 925.36400; Mass found $[M+H]^+$: **926.2**;
Mass found $[M-H]^-$: **924.2**

HRMS (ESI-TOF) found $[M+H]^+$ 926.3694 $C_{42}H_{56}N_9O_{13}S$ requires 926.3718; error: 2.59
ppm

20

EE-106 peptide: cyclo[βA-EMGFFAA-G], Head-to-Tail Cyclized (HTC), SEQ ID NO: 6.



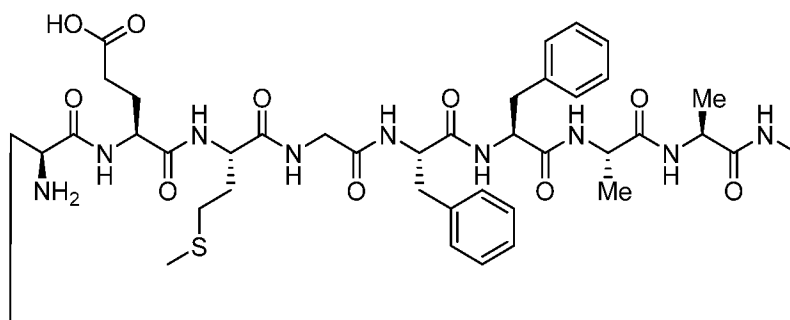
Chemical Formula: $C_{41}H_{55}N_9O_{11}S$; Exact Mass [M]: 881.37417; Mass found $[M+H]^+$: **882.2**;

Mass found $[M-H]^-$: **880.2**

HRMS (ESI-TOF) found $[M+H]^+$ 882.3801 $C_{41}H_{56}N_9O_{11}S$ requires 882.3820; error: 2.15

5 ppm

EE-107 peptide: cyclo[εK*-EMGFFAA], Side-to-Tail Cyclized (STC), SEQ ID NO: 7.



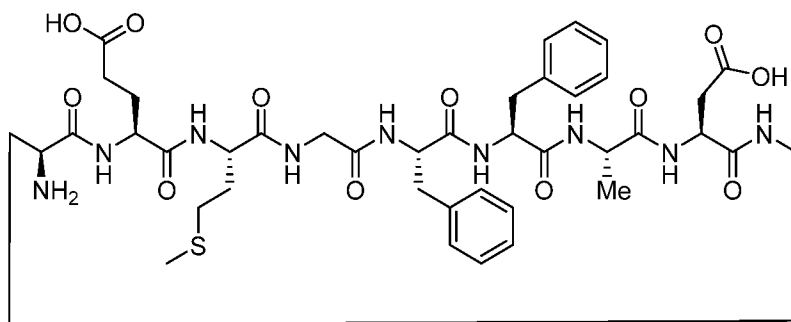
10 Chemical Formula: $C_{42}H_{59}N_9O_{10}S$; Exact Mass [M]: 881.41056; Mass found $[M+H]^+$: **882.5**;

Mass found $[M-H]^-$: **880.5**

HRMS (ESI-TOF) found $[M+H]^+$ 882.4172 $C_{42}H_{60}N_9O_{10}S$ requires 882.4184; error: 1.36

ppm

15 *EE-108 peptide*: cyclo[εK*-EMGFFAD], Side-to-Tail Cyclized (STC), SEQ ID NO: 8.

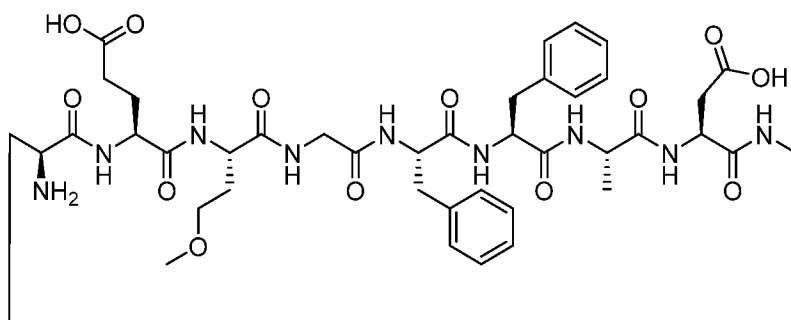


Chemical Formula: C₄₃H₅₉N₉O₁₂S; Exact Mass [M]: 925.40039; Mass found [M+H]⁺: **926.2**;
Mass found [M-H]⁻: **924.2**

- 5 **HRMS (ESI-TOF)** found [M+H]⁺ 926.4063 C₄₃H₆₀N₉O₁₂S requires 926.4082; error: 2.05 ppm

EE-122 peptide: cyclo[εK*-E-Hse(OMe)-GFFAD], Side-to-Tail Cyclized (STC), SEQ ID NO: 9.
Hse(OMe): O-methyl-L-homoserine.

10

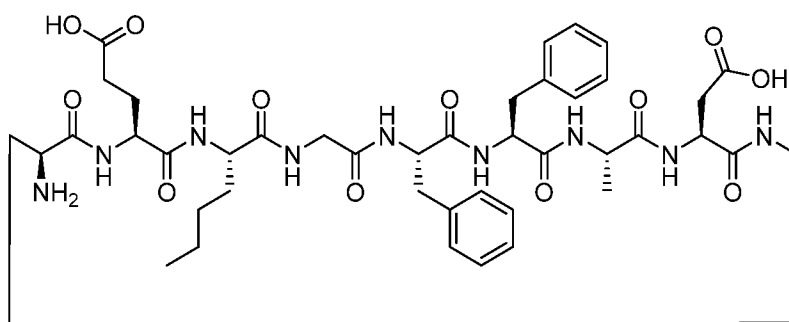


Chemical Formula: C₄₃H₅₉N₉O₁₃; Exact Mass [M]: 909.42323; Mass found [M+H]⁺: **910.2**;
Mass found [M-H]⁻: **908.2**

HRMS (ESI-TOF) found [M+H]⁺ 910.4292 C₄₃H₆₀N₉O₁₃ requires 910.4311; error: 2.09 ppm

15

EE-123 peptide: cyclo[εK*-E-Nle-GFFAD], Side-to-Tail Cyclized (STC), SEQ ID NO: 10. Nle: L-norleucine.



Chemical Formula: C₄₄H₆₁N₉O₁₂; Exact Mass [M]: 907.44397; Mass found [M+H]⁺: **908.3**;

Mass found [M-H]⁻: **906.2**

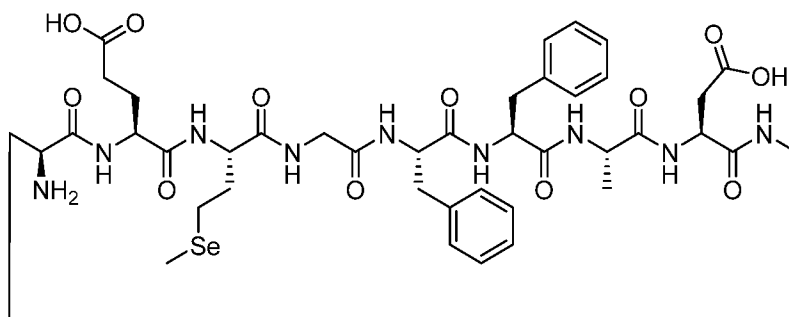
HRMS (ESI-TOF) found [M+H]⁺ 908.4498 C₄₄H₆₂N₉O₁₂ requires 908.4518; error: 2.20 ppm

5

EE-124 peptide: cyclo[εK*-E-Semet-GFFAD], Side-to-Tail Cyclized (STC), SEQ ID NO: 11.

Semet: *L*-selenomethionine.

UPLC-MS: EE-124 UV



10

Chemical Formula: C₄₃H₅₉N₉O₁₂Se; Exact Mass [M]: 973.34484; Mass found [M+H]⁺: **974.1**;

Mass found [M-H]⁻: **972.2**

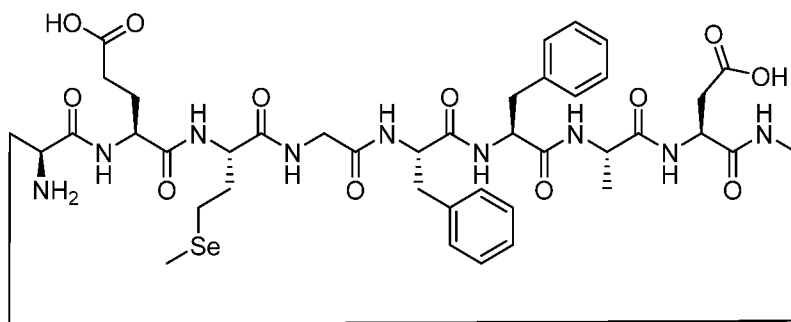
HRMS (ESI-TOF) found [M+H]⁺ 974.3511 C₄₃H₆₀N₉O₁₂Se requires 974.3527; error: 1.64

15

ppm *EE-129 peptide*: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-AD], Side-to-Tail Cyclized

(STC), SEQ ID NO: 12. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine;

Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine.



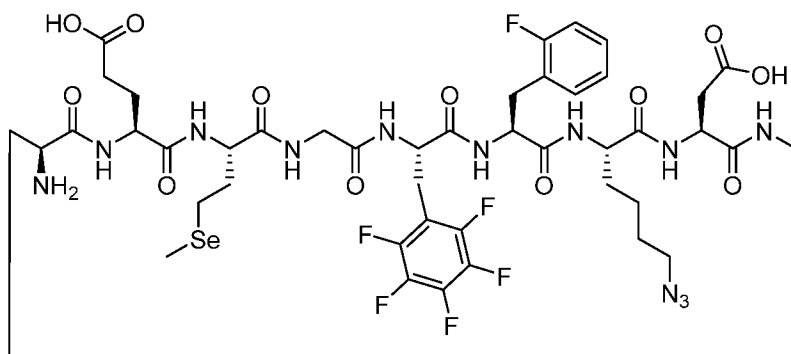
Chemical Formula: C₄₃H₅₃F₆N₉O₁₂Se; Exact Mass [M]: 1081.28831; Mass found [M+H]⁺: 1082.1; Mass found [M-H]⁻: 1080.1

HRMS (ESI-TOF) found [M+H]⁺ 1082.2939 C₄₃H₅₄F₆N₉O₁₂Se requires 1082.2961; error:

5 2.03 ppm

EE-129 N₃ peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-D], Side-to-Tail Cyclized (STC), SEQ ID NO: 13. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; K(N₃): *L*-azidolysine.

10

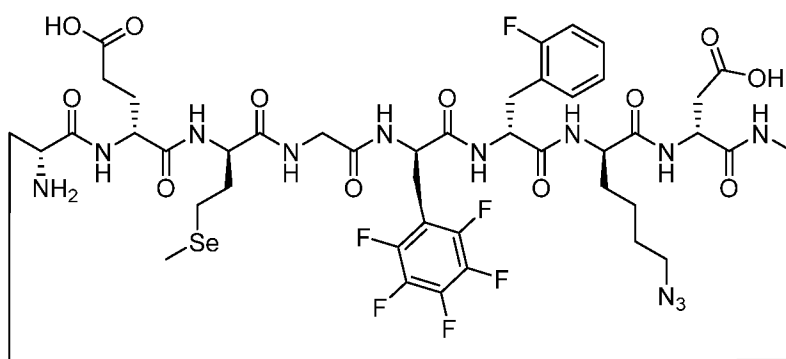


Chemical Formula: C₄₆H₅₈F₆N₁₂O₁₂Se; Exact Mass [M]: 1164.33666; Mass found [M+H]⁺: 1165.1; Mass found [M-H]⁻: 1163.1

15 HRMS (ESI-TOF) found [M+H]⁺ 1165.3422 C₄₆H₅₉F₆N₁₂O₁₂Se requires 1165.3445; error: 1.97 ppm

All-D-EE-129 N₃ peptide: cyclo[dεK*-dE-dSemet-dG-dPhe(F₅)-dPhe(2-F)-dK(N₃)-dD], Side-to-Tail Cyclized (STC) and CuAAC-conjugated CPP, SEQ ID NO: 14. dSemet: *D*-

selenomethionine; *d*Phe(F₅): *D*-pentafluorophenylalanine; *d*Phe(2-F): 2-fluoro-*D*-phenylalanine or *ortho*-fluoro-*D*-phenylalanine; *d*K(N₃): *D*-azidolysine.



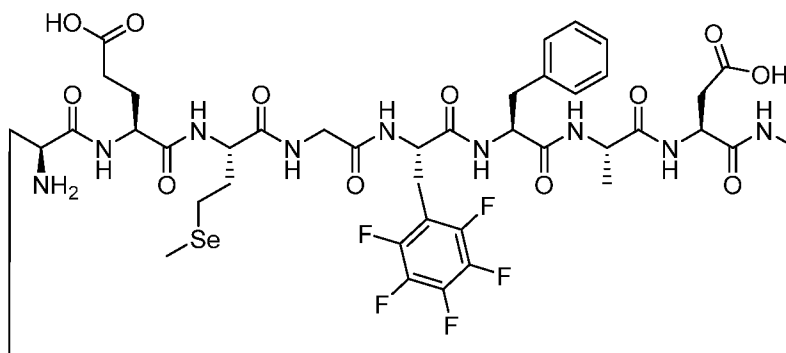
5

Chemical Formula: C₄₆H₅₈F₆N₁₂O₁₂Se; Exact Mass [M]: 1164.33666; Mass found [M+H]⁺: 1165.1; [M-H]⁻: 1163.1

HRMS (ESI-TOF) found [M+H]⁺ 1165.3426 C₄₆H₅₉F₆N₁₂O₁₂Se requires 1165.3445; error: 1.63 ppm

10

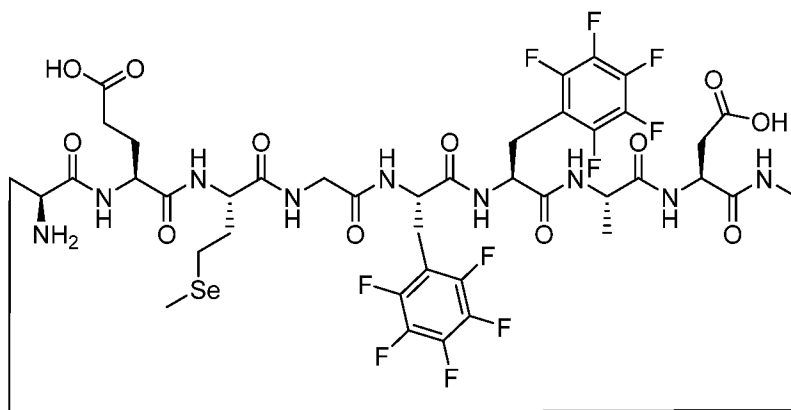
EE-130 peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-FAD], Side-to-Tail Cyclized (STC), SEQ ID NO: 15. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine.



15 Chemical Formula: C₄₃H₅₄F₅N₉O₁₂Se; Exact Mass [M]: 1063.29773; Mass found [M+H]⁺: 1064.1; Mass found [M-H]⁻: 1062.1

HRMS (ESI-TOF) found [M+H]⁺ 1064.3037 C₄₃H₅₅F₅N₉O₁₂Se requires 1064.3055; error: 1.75 ppm

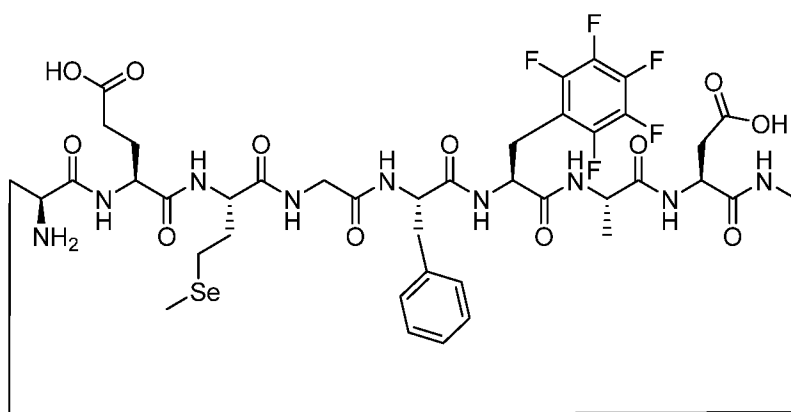
EE-131 peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(F₅)-AD], Side-to-Tail Cyclized (STC), SEQ ID NO: 16. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine.



- 5 Chemical Formula: C₄₃H₄₉F₁₀N₉O₁₂Se; Exact Mass [M]: 1153.25062; Mass found [M+H]⁺: **1154.4**; Mass found [M-H]⁻: **1152.4**

HRMS (ESI-TOF) found [M+H]⁺ 1154.2562 C₄₃H₅₀F₁₀N₉O₁₂Se requires 1154.25845; error: 1.95 ppm

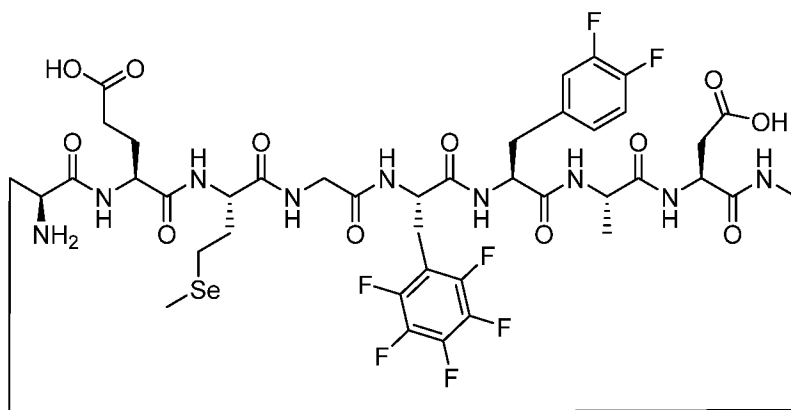
- 10 *EE-132 peptide*: cyclo[εK*-E-Semet-GF-Phe(F₅)-AD], Side-to-Tail Cyclized (STC), SEQ ID NO: 17. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine.



- 15 Chemical Formula: C₄₃H₅₄F₅N₉O₁₂Se; Exact Mass [M]: 1063.29773; Mass found [M+H]⁺: **1064.1**; Mass found [M-H]⁻: **1062.1**

HRMS (ESI-TOF) found [M+H]⁺ 1064.3038 C₄₃H₅₅F₅N₉O₁₂Se requires 1064.30556; error: 1.65 ppm

EE-133 peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(3,4-F₂)-AD], Side-to-Tail Cyclized (STC), SEQ ID NO: 18. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(3,4-F₂): 3,4-difluoro-*L*-phenylalanine or *meta-para*-difluoro-*L*-phenylalanine.



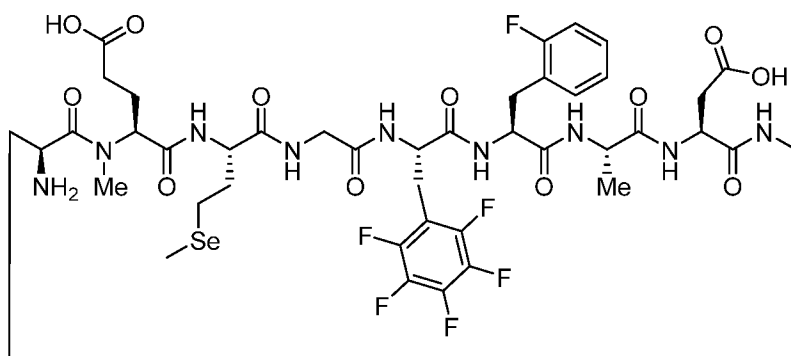
5

Chemical Formula: C₄₃H₅₂F₇N₉O₁₂Se; Exact Mass [M]: 1099.27889; Mass found [M+H]⁺: **1100.1**; Mass found [M-H]⁻: **1098.1**

HRMS (ESI-TOF) found [M+H]⁺ 1100.2848 C₄₃H₅₃F₇N₉O₁₂Se requires 1100.2867; error: 1.73 ppm

10

EE-154 peptide: cyclo[εK*-NMeE-Semet-G-Phe(F₅)-Phe(2-F)-AD], Side-to-Tail Cyclized (STC), SEQ ID NO: 19. NMeE: *N*-methyl-*L*-glutamic acid; Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine.

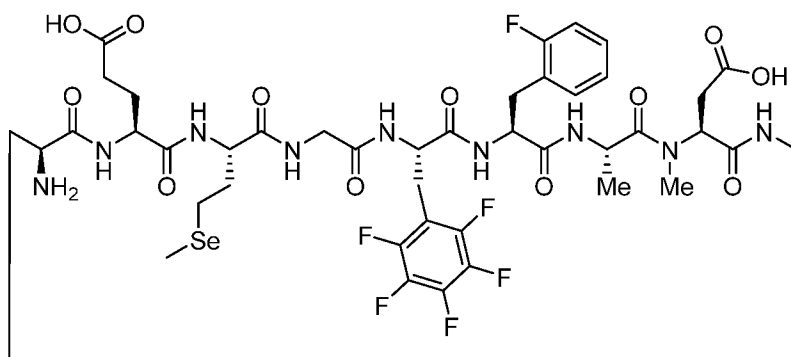


15

Chemical Formula: C₄₄H₅₅F₆N₉O₁₂Se; Exact Mass [M]: 1095.30396; Mass found [M+H]⁺: **1096.1**; Mass found [M-H]⁻: **1094.2**

HRMS (ESI-TOF) found $[M+H]^+$ 1096.3098 $C_{44}H_{56}F_6N_9O_{12}Se$ requires 1096.3118; error: 1.82 ppm

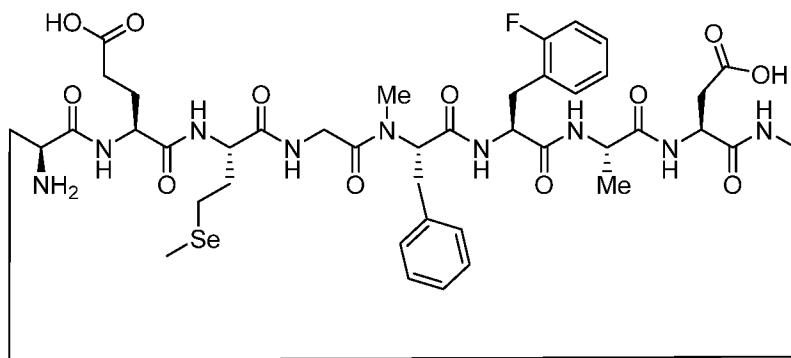
EE-155 peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-A-NMeD], Side-to-Tail Cyclized (STC), SEQ ID NO: 20. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; NMeD: *N*-methyl-*L*-aspartic acid.



Chemical Formula: $C_{44}H_{55}F_6N_9O_{12}Se$; Exact Mass $[M]$: 1095.30396; Mass found $[M+H]^+$: 1096.1; Mass found $[M-H]^-$: 1094.1

HRMS (ESI-TOF) found $[M+H]^+$ 1096.3101 $C_{44}H_{56}F_6N_9O_{12}Se$ requires 1096.3118; error: 1.55 ppm

EE-159 peptide: cyclo[εK*-E-Semet-G-NMeF-Phe(2-F)-AD], Side-to-Tail Cyclized (STC), SEQ ID NO: 21. Semet: *L*-selenomethionine; NMeF: *N*-methyl-*L*-phenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine.



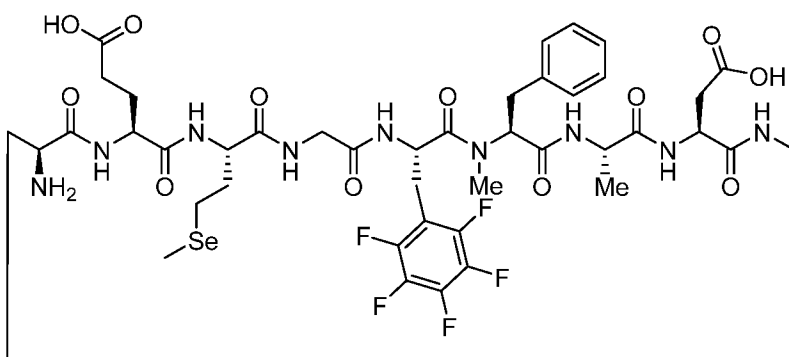
Chemical Formula: $C_{44}H_{60}FN_9O_{12}Se$; Exact Mass [M]: 1005.35107; Mass found $[M+H]^+$:

1006.2; Mass found $[M-H]^-$: **1004.1**

HRMS (ESI-TOF) found $[M+H]^+$ 1006.3586 $C_{44}H_{61}FN_9O_{12}Se$ requires 1006.3589; error: 0.30 ppm

5

EE-160 peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-NMeF-AD], Side-to-Tail Cyclized (STC), SEQ ID NO: 22. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; NMeF: *N*-methyl-*L*-phenylalanine.

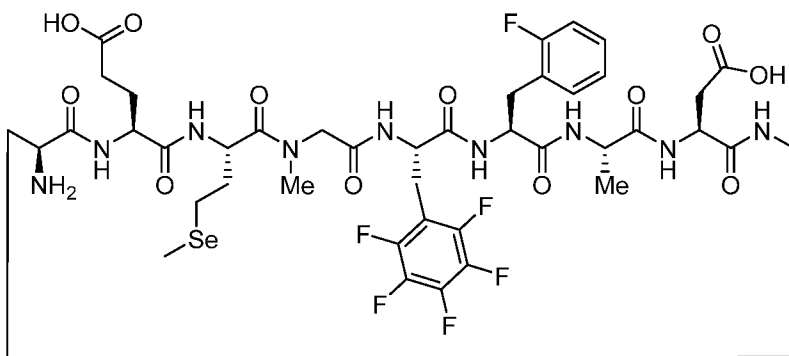


10 Chemical Formula: $C_{44}H_{56}F_5N_9O_{12}Se$; Exact Mass [M]: 1077.31338; Mass found $[M+H]^+$:

1078.4; Mass found $[M-H]^-$: **1075.8**

HRMS (ESI-TOF) found $[M+H]^+$ 1078.3190 $C_{44}H_{57}F_5N_9O_{12}Se$ requires 1078.3212; error: 2.04 ppm

15 *EE-162 peptide*: cyclo[εK*-E-Semet-Sar-Phe(F₅)-Phe(2-F)-AD], Side-to-Tail Cyclized (STC), SEQ ID NO: 23. Semet: *L*-selenomethionine; Sar: *N*-methyl-glycine or sarcosine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine.

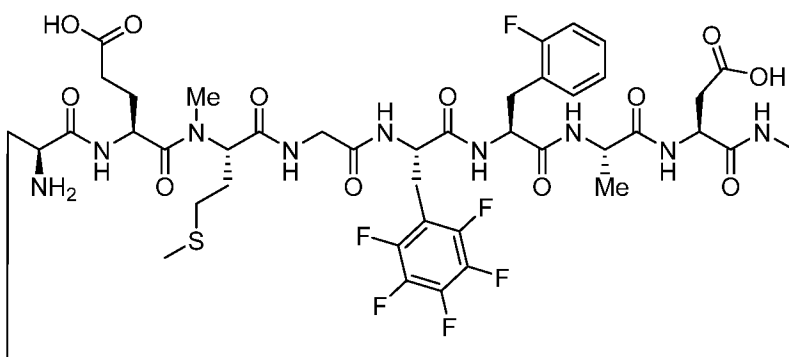


Chemical Formula: $C_{44}H_{55}F_6N_9O_{12}Se$; Exact Mass [M]: 1095.30396; Mass found $[M+H]^+$: **1096.1**; Mass found $[M-H]^-$: **1094.1**

HRMS (ESI-TOF) found $[M+H]^+$ 1096.3098 $C_{44}H_{56}F_6N_9O_{12}Se$ requires 1096.3118; error: 1.82 ppm

5

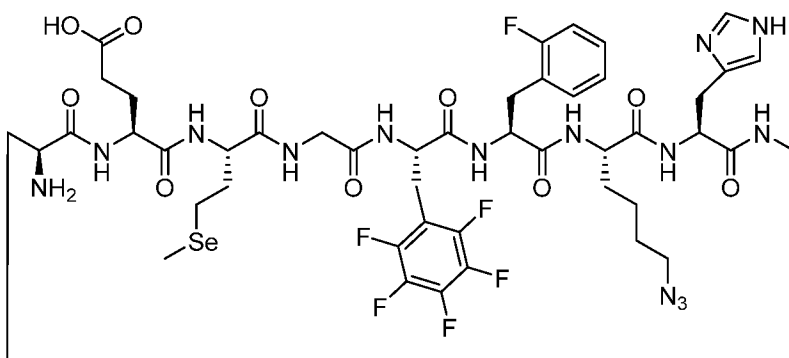
EE-164 peptide: cyclo[εK*-E-NMeS-G-Phe(F₅)-Phe(2-F)-AD], Side-to-Tail Cyclized (STC), SEQ ID NO: 24. NMeS: N-methyl-L-methionine; Phe(F₅): L-pentafluorophenylalanine; Phe(2-F): 2-fluoro-L-phenylalanine or *ortho*-fluoro-L-phenylalanine.



10 Chemical Formula: $C_{44}H_{55}F_6N_9O_{12}S$; Exact Mass [M]: 1047.35951; Mass found $[M+H]^+$: **1048.2**; Mass found $[M-H]^-$: **1046.2**

HRMS (ESI-TOF) found $[M+H]^+$ 1048.3660 $C_{44}H_{56}F_6N_9O_{12}S$ requires 1048.3673; error: 1.24 ppm

15 *EE-168 N₃ peptide*: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-H], Side-to-Tail Cyclized (STC), SEQ ID NO: 25. Semet: L-selenomethionine; Phe(F₅): L-pentafluorophenylalanine; Phe(2-F): 2-fluoro-L-phenylalanine or *ortho*-fluoro-L-phenylalanine; K(N₃): L-azidolysine.

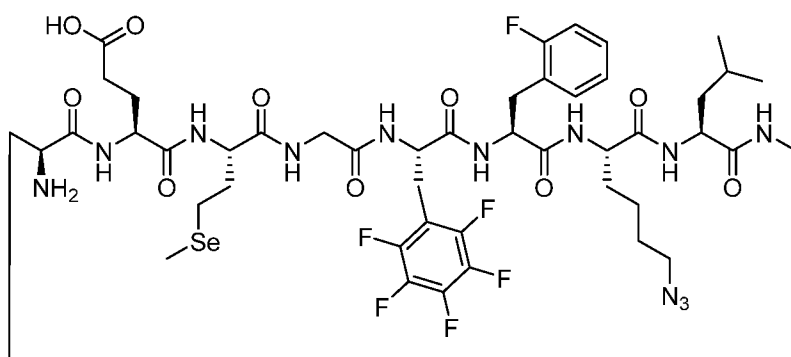


Chemical Formula: $C_{48}H_{60}F_6N_{14}O_{10}Se$; Exact Mass [M]: 1186.36863; Mass found $[M+H]^+$: **1187.1**; $[M+2H]^{+2}$: **594.0**; Mass found $[M-H]^-$: **1185.1**

HRMS (ESI-TOF) found $[M+H]^+$ 1187.3749 $C_{48}H_{61}F_6N_{14}O_{10}Se$ requires 1187.3764s; error: 1.31 ppm

5

EE-169 N₃ peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-L], Side-to-Tail Cyclized (STC), SEQ ID NO: 26. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; K(N₃): *L*-azidolysine.



10

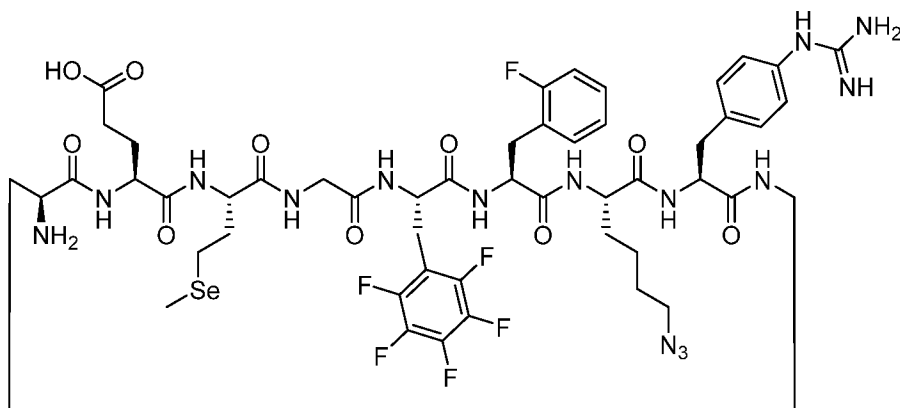
Chemical Formula: $C_{48}H_{64}F_6N_{12}O_{10}Se$; Exact Mass [M]: 1162.39378; Mass found $[M+H]^+$: **1163.2**; $[M+2H]^{+2}$: **582.4**; Mass found $[M-H]^-$: **1161.2**

HRMS (ESI-TOF) found $[M+H]^+$ 1163.3996 $C_{48}H_{65}F_6N_{12}O_{10}Se$ requires 1163.4016; error: 1.72 ppm

15

EE-206 N₃ peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-Phe(*p*-guanidino)], Side-to-Tail Cyclized (STC) SEQ ID NO: 27. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; K(N₃): *L*-azidolysine; Phe(*p*-guanidino): 4-(guanidino)-*L*-phenylalanine or *para*-(guanidino)-*L*-phenylalanine.

20

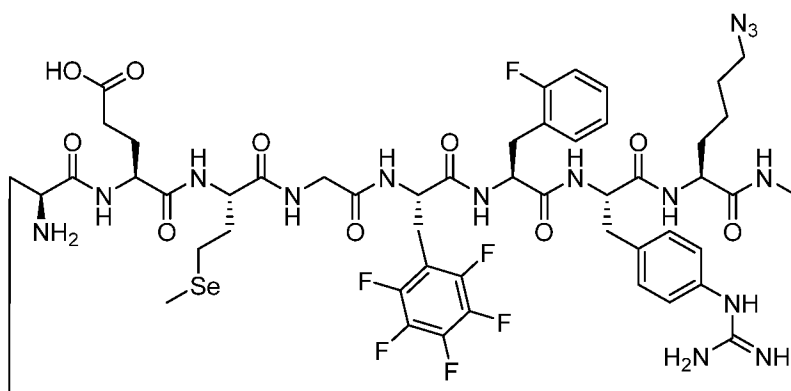


Chemical Formula: $C_{52}H_{65}F_6N_{15}O_{10}Se$; Exact Mass [M]: 1253.41082; 1162.39378; Mass found $[M+H]^+$: **1254.2**; $[M+2H]^{+2}$: **627.7**; Mass found $[M-H]^-$: **1252.2**

HRMS (ESI-TOF) found $[M+H]^+$ 1254.4167 $C_{52}H_{66}F_6N_{15}O_{10}Se$ requires 1254.41865; error:

5 1.55 ppm

EE-209 N₃ peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-Phe(*p*-guanidino)-K(N₃)], Side-to-Tail Cyclized (STC), SEQ ID NO: 28. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine;
 10 Phe(*p*-guanidinium): 4-(guanidino)-*L*-phenylalanine or *para*-(guanidino)-*L*-phenylalanine;
 K(N₃): *L*-azidolysine.



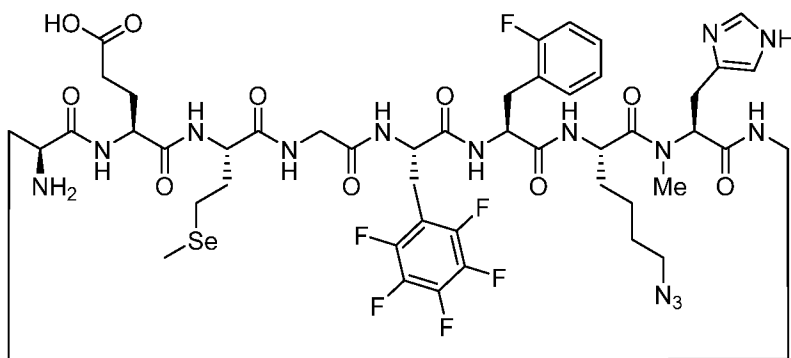
Chemical Formula: $C_{52}H_{65}F_6N_{15}O_{10}Se$; Exact Mass [M]: 1253.41082; 1162.39378; Mass found $[M+H]^+$: **1254.2**; $[M+2H]^{+2}$: **627.7**; Mass found $[M-H]^-$: **1252.2**

HRMS (ESI-TOF) found $[M+H]^+$ 1254.4159 $C_{52}H_{66}F_6N_{15}O_{10}Se$ requires 1254.41865; error:

2.19 ppm

EE-231 N₃ peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-NmeH], Side-to-Tail Cyclized (STC) (SEQ ID NO: 29). Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; K(N₃): *L*-azidolysine; NMeH: N-methyl-*L*-histidine.

5



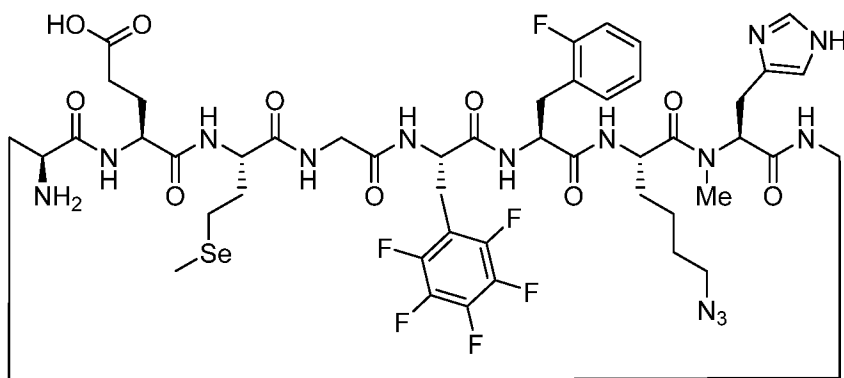
Chemical Formula: C₄₉H₆₂F₆N₁₄O₁₀Se; Exact Mass [M]: 1200.38428; Mass found [M+H]⁺: **1201.1**; [M+2H]²⁺: **601.2**; Mass found [M-H]⁻: **1199.1**

HRMS (ESI-TOF) found [M+H]⁺ 1201.3900 C₄₉H₆₃F₆N₁₄O₁₀Se requires 1201.3921; error:

10 1.75 ppm

EE-233 N₃ peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-dH], Side-to-Tail Cyclized (STC), SEQ ID NO: 30. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; K(N₃): *L*-azidolysine; dH:

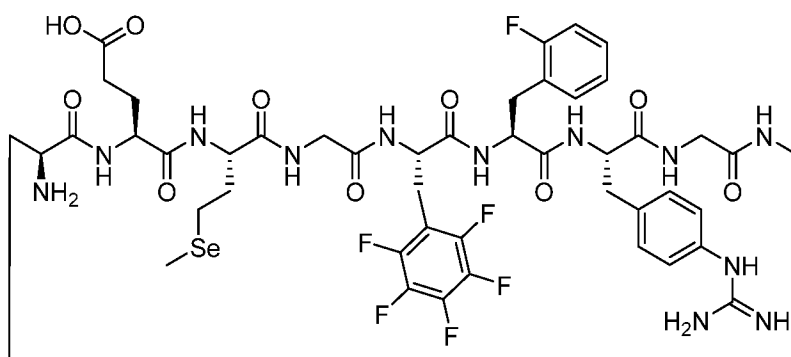
15 *D*-histidine.



Chemical Formula: C₄₈H₆₀F₆N₁₄O₁₀Se; Exact Mass [M]: 1186.36863; Mass found [M+H]⁺: **1187.1**; [M+2H]²⁺: **594.2**; Mass found [M-H]⁻: **1185.1**

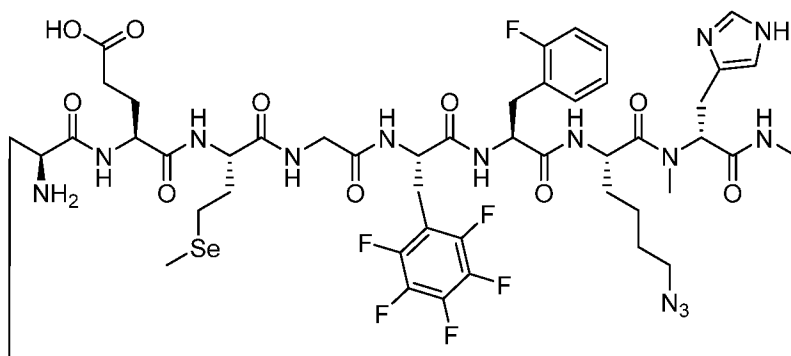
HRMS (ESI-TOF) found $[M+H]^+$ 1187.3745 $C_{48}H_{61}F_6N_{14}O_{10}Se$ requires 1187.37645; error: 1.64ppm

- 5 *EE-242 peptide*: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-Phe(*p*-guanidino)-G], Side-to-Tail Cyclized (STC), SEQ ID NO: 31. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; Phe(*p*-guanidinium): 4-(guanidino)-*L*-phenylalanine or *para*-(guanidino)-*L*-phenylalanine.



- 10 Chemical Formula: $C_{48}H_{58}F_6N_{12}O_{10}Se$; Exact Mass $[M]$: 1156.34683; 1162.39378; Mass found $[M+2H]^{+2}$: 1157.1 ; $[M+2H]^{+2}$: 579.2; Mass found $[M-H]^-$: 1155.1
HRMS (ESI-TOF) found $[M+H]^+$ 1157.3530 $C_{48}H_{59}F_6N_{12}O_{10}Se$ requires 1157.35465; error: 1.425 ppm

- 15 *EE-244 N₃ peptide*: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-NMedH], Side-to-Tail Cyclized (STC), SEQ ID NO: 32. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; K(N₃): *L*-azidolysine; NMedH: N-methyl-*D*-histidine.

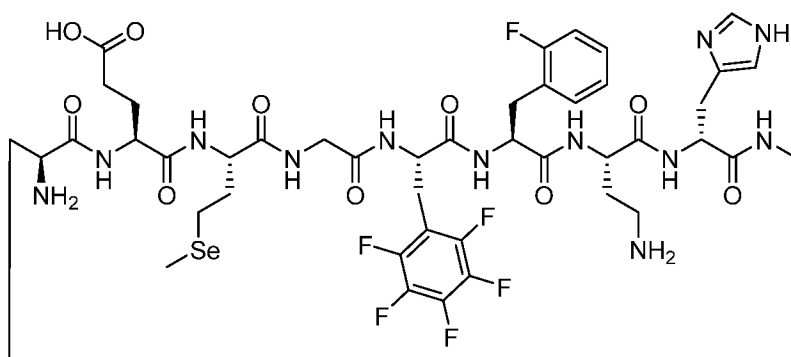


Chemical Formula: $C_{49}H_{62}F_6N_{14}O_{10}Se$; Exact Mass [M]: 1200.38428; Mass found $[M+H]^+$: **1201.1**; $[M+2H]^{+2}$: **601.2**; Mass found $[M-H]^-$: **1199.1**

HRMS (ESI-TOF) found $[M+H]^+$ 1201.3900 $C_{49}H_{63}F_6N_{14}O_{10}Se$ requires 1201.3921; error: 1.75 ppm

5

EE-246 peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-Dab-*d*H], Side-to-Tail Cyclized (STC), SEQ ID NO: 33. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; Dab: *L*-2,4-diaminobutyric acid; *d*H: *D*-histidine.



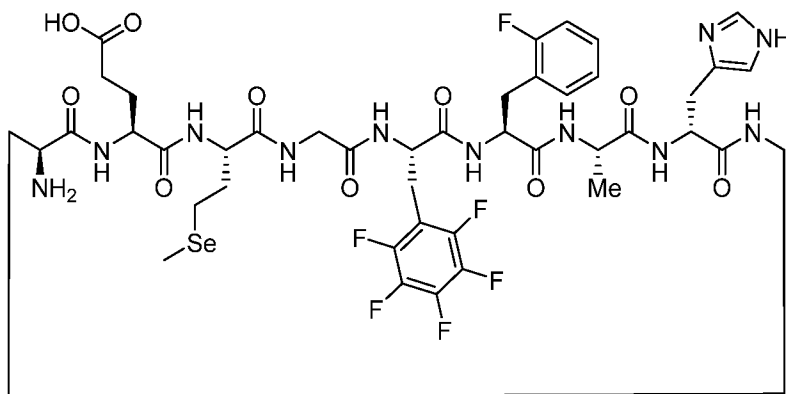
10

Chemical Formula: $C_{46}H_{58}F_6N_{12}O_{10}Se$; Exact Mass [M]: 1132.34683; Mass found $[M+H]^+$: **1133.1**; $[M+2H]^{+2}$: **567.1**; $[M+3H]^{+3}$: **378.4**; Mass found $[M-H]^-$: **1131.1**

HRMS (ESI-TOF) found $[M+H]^+$ 1133.3535 $C_{46}H_{59}F_6N_{12}O_{10}Se$ requires 1133.3546; error: 1.015 ppm

15

EE-249 peptide: cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-A-*d*H], Side-to-Tail Cyclized (STC), SEQ ID NO: 34. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; *d*H: *D*-histidine.



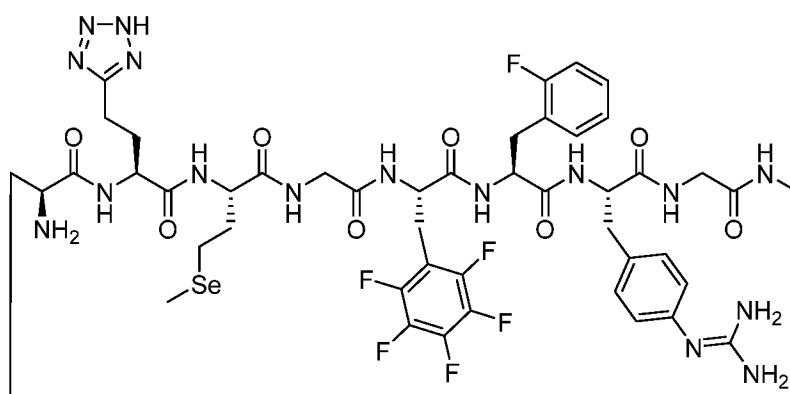
Chemical Formula: $C_{45}H_{55}F_6N_{11}O_{10}Se$; Exact Mass [M]: 1103.32028; Mass found $[M+H]^+$: **1104.1**; $[M+2H]^{+2}$: **552.7**; Mass found $[M-H]^-$: **1102.1**

HRMS (ESI-TOF) found $[M+H]^+$ 1104.3267 $C_{45}H_{56}F_6N_{11}O_{10}Se$ requires 1104.3281; error: 1.27 ppm

5

EE-260 peptide: cyclo[ϵK^* -E(T)-Semet-G-Phe(F₅)-Phe(2-F)-Phe(*p*-guanidino)-G], Side-to-Tail Cyclized (STC), SEQ ID NO: 35. E(T): (*S*)-2-amino-4-(1H-tetrazole-5-yl)butanoic acid; Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; Phe(*p*-guanidinium): 4-(guanidino)-*L*-phenylalanine or *para*-

10



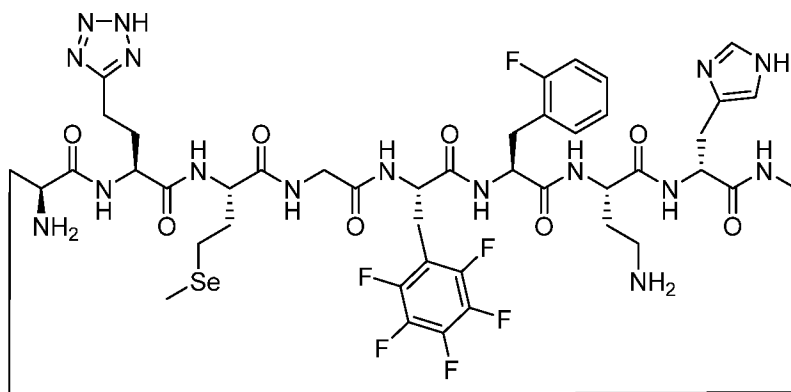
Chemical Formula: $C_{48}H_{58}F_6N_{16}O_8Se$; Exact Mass [M]: 1180.36929; Mass found $[M+H]^+$: **1181.1**; $[M+2H]^{+2}$: **591.2**; Mass found $[M-H]^-$: **1179.1**

HRMS (ESI-TOF) found $[M+H]^+$ 1181.3752 $C_{48}H_{59}F_6N_{16}O_8Se$ requires 1181.3771; error:

15 1.61 ppm

EE-261 peptide: cyclo[ϵK^* -E(T)-Semet-G-Phe(F₅)-Phe(2-F)-Dab-*d*H], Side-to-Tail Cyclized (STC), SEQ ID NO: 36. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or *ortho*-fluoro-*L*-phenylalanine; Phe(*p*-guanidinium): 4-(guanidino)-*L*-phenylalanine or *para*-(guanidino)-*L*-phenylalanine; E(T): (*S*)-2-amino-4-(1H-tetrazole-5-yl)butanoic acid; Dab: *L*-2,4-diaminobutyric acid; *d*H: *D*-histidine.

20



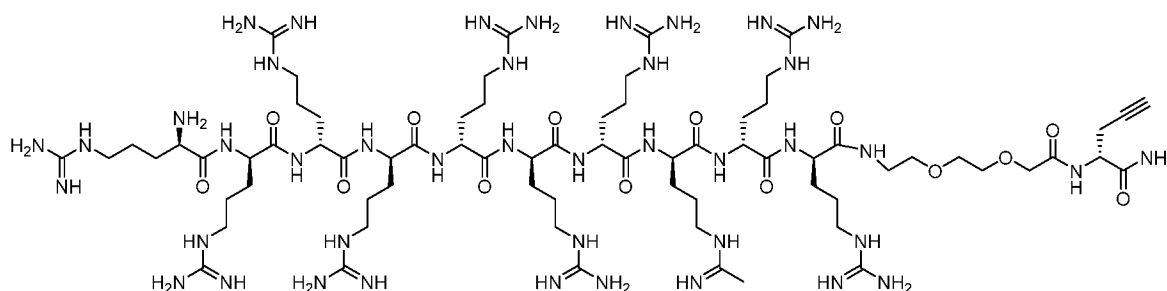
Chemical Formula: $C_{46}H_{58}F_6N_{16}O_8Se$; Exact Mass [M]: 1156.36929; Mass found $[M+H]^+$:

1157.1; $[M+2H]^{+2}$: **579.2**; $[M+3H]^{+3}$: **386.5**; Mass found $[M-H]^-$: **1155.1**

HRMS (ESI-TOF) found $[M+H]^+$ 1157.3748 $C_{46}H_{59}F_6N_{16}O_8Se$ requires 1157.3771; error:

5 1.99 ppm

dPra-PEG₂-dR₁₀ peptide: *D*-Propargylglycine-PEG₂-Poly-*D*-Arg₁₀ CPP, SEQ ID NO: 37. PEG₂: 12-amino-4,7,10-trioxadodecanoic acid; *dR*: *D*-arginine



10

Chemical Formula: $C_{71}H_{139}N_{43}O_{14}$; Exact Mass [M]: 1818.14867; Mass found $[M+3H]^{+3}$:

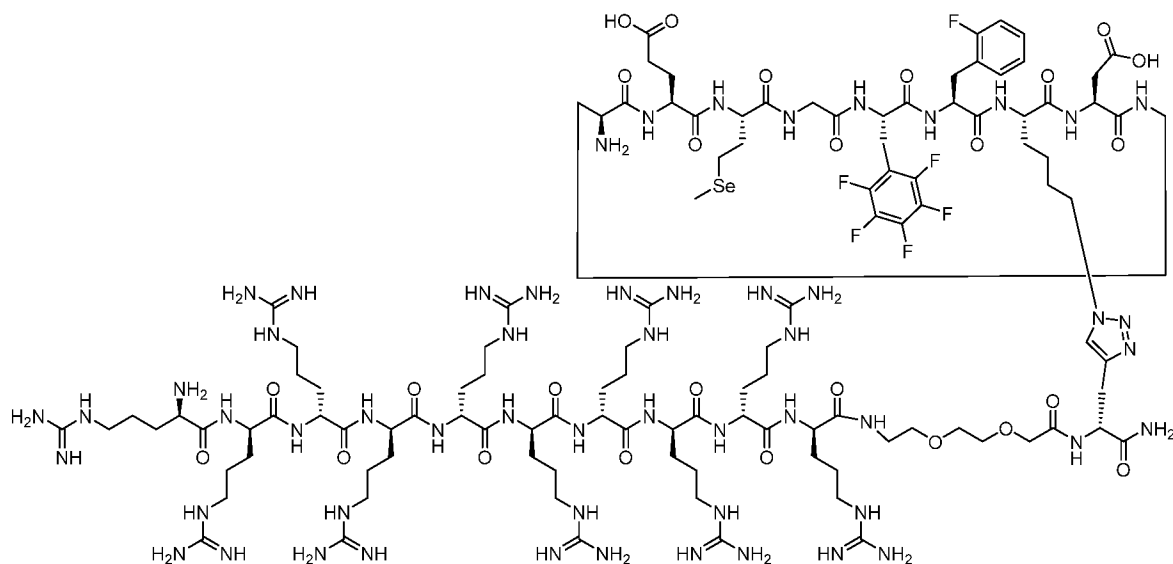
607.2; $[M+4H]^{+4}$: **455.5**; $[M+5H]^{+5}$: **364.7**; $[M+6H]^{+6}$: **304.1**

HRMS (ESI-TOF) found $[M+5H]^{+5}$ 364.6391 $C_{71}H_{144}N_{43}O_{14}$ requires 364.6375; error: 4.39

15 ppm

EE-171 peptide: cyclo[ϵ K*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(triazole-*d*Pra-PEG₂-*d*R₁₀)-D], Side-to-Tail Cyclized (STC) and CuAAC-conjugated CPP, SEQ ID NO: 38. Semet: *L*-selenomethionine; Phe(F₅): *L*-pentafluorophenylalanine; Phe(2-F): 2-fluoro-*L*-phenylalanine or

ortho-fluoro-*L*-phenylalanine; K(N₃): *L*-azidolysine; PEG₂: 12-amino-4,7,10-trioxadodecanoic acid; *dR*: *D*-arginine.

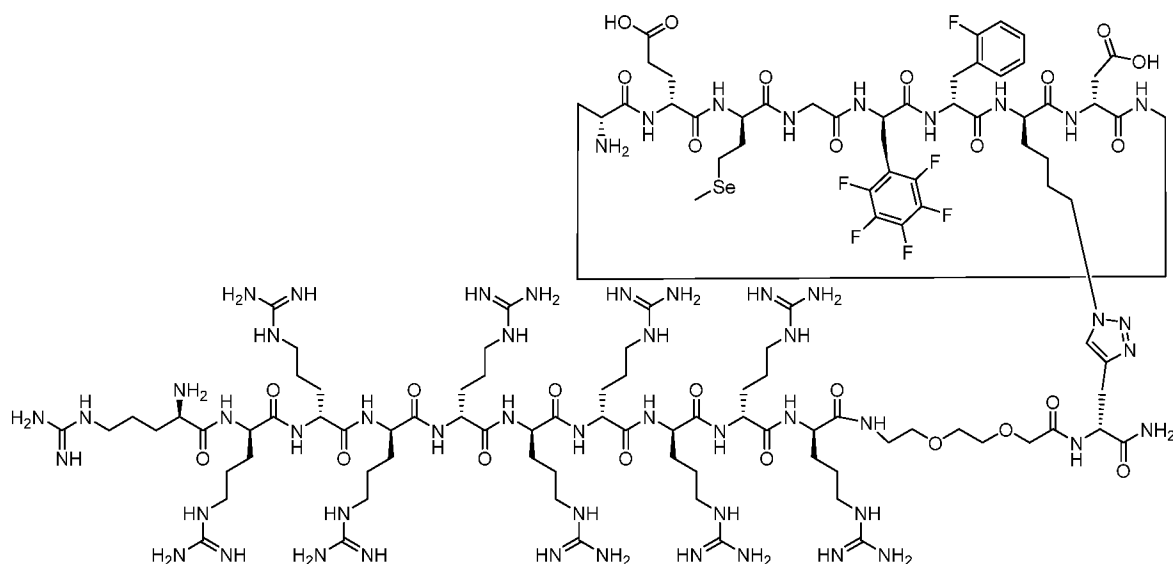


Chemical Formula: C₁₁₇H₁₉₇F₆N₅₅O₂₆Se; Exact Mass [M]: 2982.48532; Mass found

5 [M+3H]⁺³: 995.4; [M+4H]⁺⁴: 746.8; [M+5H]⁺⁵: 597.5; [M+6H]⁺⁶: 498.1; [M+7H]⁺⁷: 427.1

HRMS (ESI-TOF) found [M+5H]⁺⁵ 597.5075 : C₁₁₇H₂₀₂F₆N₅₅O₂₆Se requires 597.5049; error: 4.35 ppm

10 *All-D-EE-171 peptide*: cyclo[*dεK**-*dE*-*dSemet*-*dG*-*dPhe*(F₅)-*dPhe*(2-F)-*dK*(triazole-*dPra*-PEG₂-*dR*₁₀)-*dD*], Side-to-Tail Cyclized (STC) and CuAAC-conjugated CPP, SEQ ID NO: 39. *dSemet*: *D*-selenomethionine; *dPhe*(F₅): *D*-pentafluorophenylalanine; *dPhe*(2-F): 2-fluoro-*D*-phenylalanine or *ortho*-fluoro-*D*-phenylalanine; *dK*(N₃): *D*-azidolysine; PEG₂: 12-amino-4,7,10-trioxadodecanoic acid.



Chemical Formula: $C_{117}H_{197}F_6N_{55}O_{26}Se$; Exact Mass [M]: 2982.48532; Mass found [M+2H]⁺₂: 1492.4; [M+3H]⁺₃: 995.1; [M+4H]⁺₄: 746.8; [M+5H]⁺₅: 597.7; [M+6H]⁺₆: 498.2; [M+7H]⁺₇: 427.1

- 5 **HRMS (ESI-TOF)** found [M+5H]⁺₅ 597.5076 : $C_{117}H_{202}F_6N_{55}O_{26}Se$ requires 597.5049; error: 4.52 ppm

EXAMPLE 16

Fluorescence Based Thermal Shift Assay (Thermofluor) Measurements:

- 10 A fluorescence based thermal shift assay was used to screen and rank the rationally designed eIF4E binding derivative peptides. The fluorescent dye SYPRO orange (Invitrogen) was used to monitor thermal denaturation of eIF4E. Binding of the dye molecule to eIF4E, as it unfolds due to thermal denaturation, results in a sharp increase in the fluorescence intensity. The midpoint of this transition is termed the T_m . The thermal shift assay was
- 15 conducted in a LightCyclerTM (Roche). Protein samples studied were made up to a total volume of 50 μ l in PBS (Phosphate Buffered Saline) with SYPRO Orange, (Invitrogen, 5000 $\times$ DMSO stock) at a 3.125 $\times$ concentration. The final protein concentration was 10 μ M. Protein samples were incubated with derivative peptides at a concentration of 100 μ M. The plate was heated from 20 to 90°C with a heating rate of 1°C/minute. The fluorescence intensity was measured
- 20 with Ex/Em: 533/640 nm.

The fluorescence data against temperature derived from the LightCyclerTM were fitted to Eq. (1) to obtain ΔH_u , ΔC_{pu} , and T_m by nonlinear regression using the program Prism 4.0, Graphpad:

$$(1) F_t = F_{post} + \frac{(F_{pre} - F_{post})}{1 + \exp \left\{ \frac{-\Delta H_u}{R} \left(\frac{1}{T} - \frac{1}{T_m} \right) + \frac{\Delta C_{pu}}{R} \left[\ln \left(\frac{T}{T_m} \right) + \frac{T_m}{T} - 1 \right] \right\}}$$

where F_t is the fluorescence intensity at temperature T ; T_m is the midpoint temperature of the protein-unfolding transition, F_{pre} and F_{post} are the pretransitional and posttransitional fluorescence intensities, respectively, R is the gas constant, ΔH_u is the enthalpy of protein unfolding, and ΔC_{pu} is the heat capacity change on protein unfolding. In the absence of ligand, $T_m = T_0$, $\Delta C_{pu} = \Delta C_{T_0pu}$, and $\Delta H_u = \Delta H_{T_0u}$ (Zhou et al., PloS one 7: e47235-e47235 (2012)).

To calculate the ligand-binding affinity at T_m for the derivative eIF4E binding peptides, use Eq. (2):

$$(2) K_{L(T_m)} = \frac{\exp \left(\frac{-\Delta H_u^{T_0}}{R} \left(\frac{1}{T_m} - \frac{1}{T_0} \right) + \Delta C_{pu}^{T_0} \left[\ln \left(\frac{T_m}{T_0} \right) + \frac{T_0}{T_m} - 1 \right] \right)}{L_{T_m}}$$

To compare binding affinities for the derivative peptides to eIF4E calculated from the thermal shift data, the binding affinity at temperature T [$K_L(T)$] must be calculated. $K_L(T)$ can be calculated from $K_L(T_m)$ using Eq. (3):

$$(3) K_{L(T)} = K_{L(T_m)} \exp \left(\frac{-\Delta H_{L(T)}}{R} \left(\frac{1}{T} - \frac{1}{T_m} \right) \right)$$

where $K_L(T)$ is the ligand association constant at temperature T , and $\Delta H_{L(T)}$ is the van't Hoff enthalpy of binding at temperature T . The value of $\Delta H_{L(T)}$ was taken to be -5 kcal/mol (Zhou et al. op. cit.).

EXAMPLE 17

96 well eIF4E Cap-Binding Competition Fluorescence Polarization (FP) Assays:

The 96-well high-throughput Competition Fluorescence Polarization (FP) assays were performed by HD Biosciences (HDBio, Shanghai, China). The EE-02^{FAM} probe used to develop the fluorescence polarization (FP) technique were. Reagents used include (1) Full length eIF4E (wild type, 500 μ M stock); (2) FAM labelled tracer peptide [EE-02^{FAM}, sequence (read N-to-C): (5-FAM)-ACEMGFFQDCG-NH₂ (SEQ ID NO: 46)] in 1 mM DMSO stock, purchased from Chinese Peptide Company (CPC, Hangzhou, China); (3) competitive peptide-of-interest in 10 mM DMSO stock. The equipment used include (1) Bioshaker (500 rpm, 2 minutes); (2) 96 well black plate (PROPYLENE, COSTAR); (3) enVISIONTM Multi-plate Reader. 5-FAM: 5-Carboxyfluorescein.

Micro-Plate Setup (Volume = 50 μ l).

Final Microplate Assay concentrations: (1) 0.625 μ M eIF4E; (2) 50 nM EE-02^{FAM}; (3) X nM Titrant Competitor Peptide (5nM-20 μ M concentration range), 12 titration points; (4) Final Buffer concentrations (3% v/v DMSO, PBS, 0.1% Tween 20).

5 *EE-02^{FAM} only control:* (1) 50nM EE-02^{FAM}, Final Buffer concentrations (3% v/v DMSO, PBS, 0.1% Tween 20).

Positive Control: 0.625 μ M eIF4E, 50 nM EE-02^{FAM}, 10 μ M EE-44, Final Buffer concentrations (3% v/v DMSO, PBS, 0.1% Tween 20).

Negative Control: 0.625 μ M eIF4E, 50 nM EE-02^{FAM} Final Buffer concentrations (3% v/v DMSO, PBS, 0.1% Tween 20).

10

Typical Plate Setup:

Replicate 1 (Cpd X Titration, 12 points)											
Replicate 2 (Cpd X Titration, 12 points)											
Replicate 3 (Cpd X Titration, 12 points)											
Replicate 1 (Cpd Y Titration, 12 points)											
Replicate 2 (Cpd Y Titration, 12 points)											
Replicate 3 (Cpd Y Titration, 12 points)											
EE-02 ^{FAM} Only			Positive Control			Negative Control					

K_d Determination for FP:

Purified eIF4E protein was titrated against 50nM FAM-labelled EE-02.

15 Dissociation constant for EE-02^{FAM} was determined by fitting the experimental data to a 1:1 binding model equation shown below:

$$r = r_o + (r_b - r_o) \times \frac{(K_d + [L]_t + [P]_t) - \sqrt{K_d + [L]_t + [P]_t)^2 - 4[L]_t[P]_t}}{2[L]_t}$$

where [P] is the protein concentration (eIF4E), [L] is the labelled peptide concentration, r is the anisotropy measured, r_o is the anisotropy of the free peptide, r_b is the anisotropy of the
 20 eIF4E:FAM-labeled peptide complex, K_d is the dissociation constant, [L]_t is the total FAM labelled peptide concentration, and [P]_t is the total eIF4E concentration. The determined apparent K_d value for EE-02^{FAM} was 60.1 $\pm$ 2.3nM.

Apparent K_d values were then determined for unlabelled compounds via competitive fluorescence anisotropy experiments. Titrations were carried out with the

concentration of eIF4E held constant at 625 nM and the EE-02^{FAM} peptide at 50 nM. The competing molecules were then titrated against the complex of the FAM labeled peptide and protein. Apparent K_d values were determined by fitting the experimental data to the equations shown below:

$$\begin{aligned}
 r &= r_o + (r_b + r_o) \times \frac{2\sqrt{(d^2 - 3e)} \cos(\theta/3) - 9}{3K_{d1} + 2\sqrt{(d^2 - 3e)} \cos(\theta/3) - d} \\
 d &= K_{d1} + K_{d2} + [L]_{st} + [L]_t - [P]_t \\
 e &= ([L]_t - [P]_t)K_{d1} + ([L]_{st} - [P]_t)K_{d2} + K_{d1}K_{d2} \\
 f &= -K_{d1}K_{d2}[P]_t \\
 \theta &= \arccos \left[\frac{-2d^3 + 9de - 27f}{2\sqrt{(d^2 - 3e)^3}} \right]
 \end{aligned}$$

- [L]_{st} and [L]_t denote labelled ligand and total unlabeled ligand input concentrations, respectively. K_{d2} is the dissociation constant of the interaction between the unlabelled ligand and the protein. In all competitive types of experiments, it is assumed that $[P]_t > [L]_{st}$, otherwise considerable amounts of free labeled ligand would always be present and would interfere with measurements. K_{d1} is the apparent K_d for the labeled peptide used in the respective experiment, which has been experimentally determined as described in the previous paragraph. Readings were carried out with an enVISIONTM Multilabel Reader (PerkinElmer). Curve-fitting was carried out using Prism, (GraphPadTM).

EXAMPLE 18

- Surface Plasmon Resonance (SPR) experiment for EE-48:

EE-48 stock peptide solution was dissolved in 100% DMSO to a concentration of 10 mM; Running buffer: 10mM HEPES 0.15M NaCl, 1mM DTT, 0.1% Tween 20, pH 7.6. EE-48 stock solution was diluted into 1.03x running buffer to make a peptide solution with 3% DMSO final concentration. Working concentrations were reached until 3% DMSO was reached.

- Surface Plasmon Resonance (SPR) experiments on EE-48 were performed on a Biacore T100 machine.

Immobilization of eIF4E on CM5 sensor chip: The CM5 chip was conditioned with a 6 second injections of (1) 100 mM HCL, (2) 0.1% SDS and (3) 50 mM NaOH, at a flow rate of 100 μ L/minute. The sensor chip surface was activated via a mixture of NHS (115 mg/ml) and EDC (750mg/ml) for 7 minutes at 10 μ L/minutes. Purified eIF4E was diluted with 10 mM

sodium acetate (NaOAc) buffer (pH 5.0) to a final concentration of 0.5 μ M, with m⁷GTP present in a 2:1 ratio in order to stabilize eIF4E. The amount of eIF4E immobilized on the activated surface was controlled by altering the contact time of the protein solution and was approximately 1000RU. After the immobilization of the protein, excess active succinimide ester groups were
5 quenched by 1.0 M ethanolamine (pH 8.5, 7-minute injection at 10 μ L/minutes).

SPR run: Six buffer blanks were injected to first equilibrate the instrument, followed by a solvent correction curve, and a further two blank injections. The solvent correction curve was obtained by varying the amounts of pure DMSO to 1.03x running buffer to generate a range of DMSO concentrations (3.8%, 3.6%, 3.4%, 3.2%, 3%, 2.85%, 2.7% and
10 2.5%) for the solvent correction curve. The compounds were injected for 60 seconds and dissociation was monitored for 180 seconds (flow rate of 50 μ L/minute). The data collection rate was 10Hz. K_D was determined using the BiaEvaluation software (Biacore), by calculating from the responses of the eIF4E coated CM5 chips at equilibrium and during dissociation/association phases. Both these equilibrium and kinetic data were fitted to 1:1 binding models. EE-48 K_D
15 was determined from three separate titrations. Within each titration at least two concentration points were duplicated to ensure stability and robustness of the chip surface.

EXAMPLE 19

Isothermal Titration Calorimetry (ITC):

20 Overnight dialysis of protein and peptides were carried out in buffer containing 1x phosphate buffered saline (PBS) pH 7.2, 1% DMSO, and 0.01% Tween-20. Approximately 100–200 μ M of peptide was titrated into 10–15 μ M of purified recombinant human eIF4E protein, over 20–30 injections of either 1.0 or 2.5 μ L each. All experiments were performed in duplicates using the Affinity ITC from TA Instruments. Data analysis was carried out using the associated
25 NanoAnalyze software.

EXAMPLE 20

20.1 Automated Ligand Identification System (ALIS) for discovery of EE-233 N3:

30 Automated Ligand Identification System (ALIS) with Affinity selection coupled to mass spectrometry (AS-MS) allows the identification of protein–ligand interactions without the need for labels in free solution. During the affinity selection phase, the protein of interest is incubated with a mixture of macrocyclic peptide ligands (comfortable library sizes of 25). Molecules that do not bind eIF4E are then washed away via size-exclusion chromatography

before the protein–ligand complex is analyzed by MS for ligand identification directly, following a decomplexation step (Reverse-phase HPLC dissociates and separates the bound ligands from target using low pH and high temperature of 40 °C). These experiments were repeated with varying amounts of library concentrations to rank order the results. (**Fig. 59** and **60**).

Two ALIS libraries of 25 peptides each were constructed using EE-129 N₃ as the parent peptide. The first library consists of all *L*-residues on the D⁹ position (**Fig. 59**: Library 1 (D9X mutations of EE-129 N₃) consisting of L-residues. EE-129 N₃ parent peptide was present (red), along with internal standard D9G (green).), whilst the second consisted of mostly *D*-residues on the same D⁹ position (**Fig. 60**: Library 2 (D9X mutations of EE-129 N₃) consisting of mostly D-residues. D9G mutation serves as internal standard (green). Top hit was identified to be D9dH mutant, EE-233 N₃. BT = breakthrough – this means that effects of non-specific hydrophobic interactions may contribute to binding.). Control peptide used included parent peptide EE-129 N₃ and D9G peptides present in both libraries (acting as internal standards). D9dH was immediately identified as the strongest binder in the second library, which eventually led us to EE-233 N₃.

On the other hand, the same libraries for A8X mutations of EE-129 N₃ gave too many hits, indicating the promiscuity of the 8th-position.

Proteins used: eIF4E (470µM) and Invertase from baker's yeast (*S. cerevisiae*) from Sigma-Aldrich.

eIF4E protein buffer: 54 mM HEPES pH 7.5, 156 mM NaCl.

Binding conditions: Final buffer conditions: 2.5% DMSO, 50 mM HEPES, 150 mM NaCl.

Libraries preparation: Library dilution buffer: 50 mM HEPES pH 7.5, 150 mM NaCl. Solution made in DMSO from powder at about 3 mM per peptides (75 mM total of material).

Library 1: Mixtures diluted in DMSO 1:1500, then in HEPES buffer, 1:20 (about 0.05 µM per peptide).

Library 2: Mixtures diluted in DMSO 1:1875, then in HEPES buffer, 1:20 (about 0.04 µM per peptide). The final dilution is 1:1 with the protein at different concentrations

20.2 Protein titration protocol:

For each protein titration experiment, seven levels of protein concentration were prepared. These corresponded to the following screening concentrations of eIF4E protein: 5 µM, 2.5 µM, 1.25 µM, 0.625 µM, 0.31 µM, 0.16 µM and 0.08 µM. Since the ALIS system required a

substantial protein peak in the UV detection window to trigger the valve that shuttles the complex onto the LC-MS portion of the instrument, a non-binding carrier protein (Invertase) was added at a screening concentration of 2.5 μ M to all protein screening concentrations. To make the 1 mM Invertase stock, 5 mg of Invertase was reconstituted in 59 μ L of protein dilution buffer. Then, 5 μ L of this solution was diluted in 245 μ L protein dilution buffer to create a working stock of 20 μ M solution of Invertase. A portion of this stock was further diluted to create a 10 μ M Invertase working stock solution.

The protein titration curve was created by mixing the protein and the library solutions. The protein levels for each solution were created at 2x the screening concentration for both eIF4E and Invertase. For example, the highest level of the titration curve was created by mixing the 20 μ M eIF4E solution and the 20 μ M Invertase solution at a ratio of 1:1 to create a solution that was 10 μ M eIF4E and 10 μ M Invertase. This allowed for a screening at the maximum concentration of 5 μ M eIF4E and 2.5 μ M Invertase. Then, 40 μ L of the highest concentration eIF4E/Invertase solution was reserved to be used in a reference control, and enough volume was reserved for the highest sample in the titration curve. The remaining volume of the solution was diluted 1:1 with 10 μ M Invertase. This created a new solution that has 2.5 μ M eIF4E and 2.5 μ M Invertase. This process of serial dilution was repeated until all seven target protein concentrations were created, with each containing 5 μ M Invertase, which translates to 2.5 μ M screening concentration of Invertase in each well.

20.3 Combination of Protein Curve and Compound Mixtures for Protein Titration Experiment:

For each screening sample, 5 μ L of library was mixed with 5 μ L of appropriate concentration protein solution. For counter-screening samples, 5 μ L of library was mixed with 5 μ L of 10 μ M Invertase alone. These samples allowed for the investigation of the propensity of each compound to bind to the carrier protein used in these experiments.

After mixing the library solutions with the protein solutions, the plate was incubated at room temperature for 30 minutes. After incubation, the plate was transferred to the autosampler and chilled to 4°C.

ALIS system used for this experiment: Agilent 1260 Cap pump (Model G1376A), Agilent IsoPump™ 1260 binary LC pump (Model G1310A) (RPC wash), Agilent IsoPump™ 1260 binary LC pump (Model G1310B) (SEC wash), Agilent Quat Pump (Model G1311A) (running buffer), Agilent 1260 autosampler (Model G1377A), Agilent 1200 VWD Detector (Model G1314A), Agilent 1200 VWD Detector (Model G1314A).

LC/MS system: 10 minute method, Dual column system used. UV detection 62.5 attenuation. Line 17 orbitrap (Thermo Fisher Orbitrap™ Exactive plus), RPC column: Targa C18 column, 0.5mm I.D. x 50mM length, 5µm packing material (Higgins Analytical, Mountain View, CA).

5 *Reverse Phase Column:* Higgins Analytical, Proto 300 C4 5µM, Particle Size: 5µM, Pore Size: 300Å, Dimensions: 50 x 0.5mM, Part Number: 189554.

EXAMPLE 21

21.1 Computer Simulations:

10 ACE and NME caps were added to both eIF4E and the macrocyclic peptides through the use of t-leap in AMBER11. The MD simulations were performed using the TIP3P water model, and a minimum distance of 12Å was set between the solute and solvation box boundary. The forcefield ff99SB was chosen for all simulated systems. Each system underwent the following 3-phase minimization protocol: (1) Steepest descent method for 1000 cycles, with
15 the solutes frozen with a force constant of 500 kcal mol⁻¹ angstrom⁻², (2) Steepest descent method for 1000 cycles, with the solvent frozen with a force constant of 500 kcal mol⁻¹ angstrom⁻² and (3) Steepest descent method for 1000 cycles, followed by 1000 cycles of conjugate gradient method for another 1000 cycles. This was done on the whole system. The system was then heated from 1F to 300F over 30 ps. The MD simulations were run using both
20 the SANDER and CUDA module of the AMBER11 package. A step size of 2fs with the constraint algorithm SHAKE was used. Two replicates with different random seed numbers were carried out for each system, each for a length of 50ns, for a total of 100ns per system.

EXAMPLE 22

25 22.1 Crystallizations:

 The eIF4E:macrocyclic peptide complexes were crystallized by vapor diffusion using the sitting drop method. Crystallization drops were setup with eIF4E and macrocyclic peptides at concentrations of 75 µM and 150 µM respectively. Sitting drops were set up in 48 well Intelli-Plates™ (Hampton Research) with 1 µL of the protein sample mixed with 1 µL of the
30 mother-well solution. Crystals grew over a period of one week in 10–20% of Polyethylene glycol monomethyl ether 5,000 and 100 mM Hepes or Bis-Tris at pHs of 6.5, 7.0, and 7.5. For X-ray data collection at 100 K, crystals were transferred to an equivalent mother liquor solution containing 20% (v/v) glycerol and then flash frozen in liquid nitrogen.

EXAMPLE 23

23.1 X-ray Crystal Data Collection and Refinement:

The data was collected on a X8 Proteum rotating anode source (Bruker) using a CCD detector. The crystal diffracted to a resolution of 2.2Å and was integrated and scaled using PROTEUM2 (Bruker). The initial phases of the ternary complexed crystals of eIF4E were solved by molecular replacement with the program PHASER using the human eIF4E structure complexed with the eIF4G1 peptide (PDB accession code: 2W97) as a search model. The starting models were subjected to rigid body refinement and followed by iterative cycles of manual model building in Coot and restrained refinement in Refmac 6.0TM. The macrocyclic peptides were added into clearly visible electron density. REFMAC library files for the ligand molecule were generated using PRODRG. Models were validated using PROCHECK and the MOLPROBITY webserver. Final models were analyzed using PYMOL (Schrödinger).

The sequences disclosed herein are presented in Table 16.

Table 16 Sequences		
SEQ ID NO:	Description	Sequence
1	EE-02 peptide (ACEMGFFQDCG-NH ₂): C2-C10 disulfide bond C-terminal amide	ACEMGFFQDCG
2	EE-44 peptide (CEMGFFADC-NH ₂): C1-C9 disulfide bond C-terminal amide	CEMGFFADC
3	EE-48 peptide (C*EMGFFADC*-NH ₂): C1-C9 methylene bridge C-terminal amide	CEMGFFADC
4	EE-91 peptide (cyclo[βA-EMGFFAD-βA]): Head-to-Tail Cyclized (HTC) X1 = beta-L-alanine X9 = beta-L-alanine	XEMGFFADX

	1-10 amide bond linkage between X1 amino group and X9 carboxyl group	
5	EE-94 peptide (cyclo[bA-EMGFFAD-G]): Head-to-Tail Cyclized (HTC) X1 = beta-L-alanine 1-10 amide bond linkage between X1 amino group and G9 carboxyl group	XEMGFFADG
6	EE-106 peptide (cyclo[βA-EMGFFAA-G]): Head-to-Tail Cyclized (HTC) X1 = beta-L-alanine 1-10 amide bond linkage between X1 amino group and G9 carboxyl group	XEMGFFAAG
7	EE-107 peptide (cyclo[εK*-EMGFFAA]): Side-to-Tail Cyclized (STC) 1-8 amide bond linkage between K1 epsilon amino group and A8 C-terminus carboxyl group	KEMGFFAA
8	EE-108 peptide (cyclo[εK*-EMGFFAD]): Side-to-Tail Cyclized (STC) 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEMGFFAD
9	EE-122 peptide (cyclo[εK*-E-Hse(OMe)-GFFAD]): Side-to-Tail Cyclized (STC). X3 = Hse(OMe): O-methyl-L-homoserine. 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGFFAD
10	EE-123 peptide (Cyclo[εK*-E-Nle-GFFAD]): Side-to-Tail Cyclized (STC). X3 = Nle: L-norleucine. 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGFFAD
11	EE-124 peptide (Cyclo[εK*-E-Semet-GFFAD]): Side-to-Tail Cyclized (STC).	KEXGFFAD

	X3 = Semet: L-selenomethionine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	
12	EE-129 peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-AD]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KXGXXAD
13	EE-129 N₃ peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-D]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = K(N₃): L-azidolysine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGXXXD]
14	All-D-EE-129 N₃ peptide (cyclo[dεK*-dE-dSemet-dG-dPhe(F₅)-dPhe(2-F)-dK(N₃)-dD]): Side-to-Tail Cyclized (STC). X3 = dSemet: D-selenomethionine X5 = dPhe(F₅): D-pentafluorophenylalanine X6 = dPhe(2-F): 2-fluoro-D-phenylalanine or ortho-fluoro-D-phenylalanine X7 = dK(N₃): D-azidolysine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGXXXD
15	EE-130 peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-FAD]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine	KEXGXFAD

	X5 = Phe(F ₅): L-pentafluorophenylalanine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	
16	EE-131 peptide (cyclo[εK*-E-Semet-G-Phe(F ₅)-Phe(F ₅)-AD]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F ₅): L-pentafluorophenylalanine X6 = Phe(F ₅): L-pentafluorophenylalanine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGXXAD
17	EE-132 peptide (cyclo[εK*-E-Semet-GF-Phe(F ₅)-AD]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X6 = Phe(F ₅): L-pentafluorophenylalanine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGFXAD
18	EE-133 peptide (cyclo[εK*-E-Semet-G-Phe(F ₅)-Phe(3,4-F ₂)-AD]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F ₅): L-pentafluorophenylalanine X6 = Phe(3,4-F ₂): 3,4-difluoro-L-phenylalanine or meta-para-difluoro-L-phenylalanine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGXXAD
19	EE-154 peptide (cyclo[εK*-NMeE-Semet-G-Phe(F ₅)-Phe(2-F)-AD]): Side-to-Tail Cyclized (STC). X2 = NMeE: N-methyl-L-glutamic acid X3 = Semet: L-selenomethionine X5 = Phe(F ₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KXXGXXAD

20	EE-155 peptide (cyclo[εK*-E-Semet-G-Phe(F ₅)-Phe(2-F)-A-NMeD]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X8 = NMeD: N-methyl-L-aspartic acid 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGXXAX
21	EE-159 peptide (cyclo[εK*-E-Semet-G-NMeF-Phe(2-F)-AD]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = NMeF: N-methyl-L-phenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine; 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGXXAD
22	EE-160 peptide (cyclo[εK*-E-Semet-G-Phe(F ₅)-NMeF-AD]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F ₅): L-pentafluorophenylalanine X6 = 6NMeF: N-methyl-L-phenylalanine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXGXXAD
23	EE-162 peptide (cyclo[εK*-E-Semet-Sar-Phe(F ₅)-Phe(2-F)-AD]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X4 = Sar: N-methyl-glycine or sarcosine X5 = Phe(F ₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	KEXXXXAD
24	EE-164 peptide (cyclo[εK*-E-NMeS-G-Phe(F ₅)-Phe(2-F)-AD]): Side-to-Tail Cyclized (STC). X3 = NMeS: N-methyl-L-methionine	KEXGXXAD

	X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine 1-8 amide bond linkage between K1 epsilon amino group and D8 C-terminus carboxyl group	
25	EE-168 N₃ peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-H]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = K(N₃): L-azidolysine 1-8 amide bond linkage between K1 epsilon amino group and H8 C-terminus carboxyl group	KEXGXXXH
26	EE-169 N₃ peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-L]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = K(N₃): L-azidolysine 1-8 amide bond linkage between K1 epsilon amino group and L8 C-terminus carboxyl group	KEXGXXXL
27	EE-206 N₃ peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-Phe(<i>p</i>-guanidino)) Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = K(N₃): L-azidolysine X8 = Phe(<i>p</i>-guanidinium): 4-(guanidino)-L-phenylalanine or para-(guanidino)-L-phenylalanine	KEXGXXXX

	1-8 amide bond linkage between K1 epsilon amino group and X8 C-terminus carboxyl group	
28	EE-209 N₃ peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-Phe(<i>p</i>-guanidino)-K(N₃)]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = Phe(<i>p</i>-guanidinium): 4-(guanidino)-L-phenylalanine or para-(guanidino)-L-phenylalanine X8 = K(N₃): L-azidolysine 1-8 amide bond linkage between K1 epsilon amino group and X8 C-terminus carboxyl group	KEXGXXXX
29	EE-231 N₃ peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-NmeH]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = K(N₃): L-azidolysine X8 = NMeH: N-methyl-L-histidine 1-8 amide bond linkage between K1 epsilon amino group and X8 C-terminus carboxyl group	KEXGXXXX
30	EE-233 N₃ peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-dH]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine; X5 = Phe(F₅): L-pentafluorophenylalanine; X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine; X7 = K(N₃): L-azidolysine; H8 = dH: D-histidine	KEXGXXXH

	1-8 amide bond linkage between K1 epsilon amino group and H8 C-terminus carboxyl group	
31	EE-242 peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-Phe(<i>p</i>-guanidino)-G]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine; X5 = Phe(F₅): L-pentafluorophenylalanine; X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine; X7 = Phe(<i>p</i>-guanidinium): 4-(guanidino)-L-phenylalanine or para-(guanidino)-L-phenylalanine 1-8 amide bond linkage between K1 epsilon amino group and X8 C-terminus carboxyl group	KEXGXXXG
32	EE-244 N₃ peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-K(N₃)-NMedH]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine; X5 = Phe(F₅): L-pentafluorophenylalanine; X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine; K(N₃): X7 = L-azidolysine; X8 = NMedH: N-methyl-D-histidine 1-8 amide bond linkage between K1 epsilon amino group and X8 C-terminus carboxyl group	KEXGXXXX
33	EE-246 peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-Dab-dH]): Side-to-Tail Cyclized (STC) X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = Dab: L-2,4-diaminobutyric acid; H8 = dH: D-histidine 1-8 amide bond linkage between K1 epsilon amino group and H8 C-terminus carboxyl group	KEXGXXXH

34	EE-249 peptide (cyclo[εK*-E-Semet-G-Phe(F₅)-Phe(2-F)-A-dH]): Side-to-Tail Cyclized (STC). X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine H8 = dH: D-histidine 1-8 amide bond linkage between K1 epsilon amino group and H8 C-terminus carboxyl group	KEXGXXAH
35	EE-260 peptide (cyclo[εK*-E(T)-Semet-G-Phe(F₅)-Phe(2-F)-Phe(<i>p</i>-guanidino)-G]): Side-to-Tail Cyclized (STC). X2 = E(T): (S)-2-amino-4-(1H-tetrazole-5-yl)butanoic acid X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = Phe(<i>p</i>-guanidinium): 4-(guanidino)-L-phenylalanine or para-(guanidino)-L-phenylalanine 1-8 amide bond linkage between K1 epsilon amino group and G8 C-terminus carboxyl group	KXXGXXXG
36	EE-261 peptide (cyclo[εK*-E(T)-Semet-G-Phe(F₅)-Phe(2-F)-Dab-dH]): Side-to-Tail Cyclized (STC). X2 = E(T): (S)-2-amino-4-(1H-tetrazole-5-yl)butanoic acid X3 = Semet: L-selenomethionine X5 = Phe(F₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = Dab: L-2,4-diaminobutyric acid H8 = dH: D-histidine	KXXGXXXH

	1-8 amide bond linkage between K1 epsilon amino group and H8 C-terminus carboxyl group	
37	dPra-PEG ₂ -dR ₁₀ peptide (<i>D</i> -Propargylglycine-PEG ₂ -Poly- <i>D</i> -Arg ₁₀ CPP). PEG ₂ : 12-amino-4,7,10-trioxadodecanoic acid) R1-10 = dR: D-Arginine R10 = C-terminus carboxy group linked to PEG ₂ -D-propargylglycine by an amide bond	RRRRRRRRRR
38	EE-171 peptide (cyclo[εK*-E-Semet-G-Phe(F ₅)-Phe(2-F)-K(triazole- <i>d</i> Pra-PEG ₂ - <i>d</i> R ₁₀)-D]): Side-to-Tail Cyclized (STC) and CuAAC-conjugated CPP. X3 = Semet: L-selenomethionine X5 = Phe(F ₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = K(N ₃): L-azidolysine X7 azide group linked to dPra-PEG ₂ -dR ₁₀ peptide (<i>D</i> -Propargylglycine-PEG ₂ -Poly- <i>D</i> -Arg ₁₀ (SEQ ID NO: 37) CPP in a triazole linkage	KEXGXXXD
39	All-D-EE-171 peptide (cyclo[<i>d</i> εK*- <i>d</i> E- <i>d</i> Semet- <i>d</i> G- <i>d</i> Phe(F ₅)- <i>d</i> Phe(2-F)- <i>d</i> K(triazole- <i>d</i> Pra-PEG ₂ - <i>d</i> R ₁₀)- <i>d</i> D]): Side-to-Tail Cyclized (STC) and CuAAC-conjugated CPP. X3 = Semet: L-selenomethionine X5 = Phe(F ₅): L-pentafluorophenylalanine X6 = Phe(2-F): 2-fluoro-L-phenylalanine or ortho-fluoro-L-phenylalanine X7 = K(N ₃): L-azidolysine X7 azide group linked to dPra-PEG ₂ -dR ₁₀ peptide (<i>D</i> -Propargylglycine-PEG ₂ -Poly- <i>D</i> -Arg ₁₀ (SEQ ID NO: 37) CPP in a triazole linkage	KEXGXXXD
40	Head-to-Tail Cyclized (HTC) peptide X1 = beta-L-alanine	XEMGFFADX

	X9 = beta-L-alanine or glycine 1-10 amide bond linkage between X1 amino group and X9 carboxyl group	
41	Peptide comprising EE-02	ACEMGFFQDCG
42	Peptide comprising EE-44	CEMGFFADC
43	Peptide 43	ACXXXXXADCG
44	azide-ATSP-7041 (Ac-K(N ₃)-betaAla-LTF-R8-EYWAQ-Cba-S5-SAA-NH ₂ , where R8 and S5 refer to the i,i+7 stapling positions using (R)-2-(7-octenyl)Ala and (S)-2-(4'-pentenyl)Ala, respectively. X1 = K(N ₃): L-azidolysine X2 = beta-L-alanine X6 = (R)-2-(7-octenyl)Ala X12 = Cba X13 = (S)-2-(4'-pentenyl)Ala 1-16 = N-terminal azide; C-terminal amide; X6, X13 staple	XXLTFXEYWAQ-XXSAA
45	EEE-azide-ATSP-7041 (Ac-EEE-K(N ₃)-betaAla-LTF-R8-EYWAQ-Cba-S5-SAA-NH ₂ , where R8 and S5 refer to the i,i+7 stapling positions using (R)-2-(7-octenyl)Ala and (S)-2-(4'-pentenyl)Ala, respectively. X4 = K(N ₃): L-azidolysine X5 = beta-L-alanine X9 = (R)-2-(7-octenyl)Ala X15 = Cba X16 = (S)-2-(4'-pentenyl)Ala 1-16 = N-terminal azide; C-terminal amide; X9, X16 staple	EEEXXLTFXEYW AQXXSAA
46	EE-02 ^{FAM} A1- conjugated to 5-FAM (Flourescin) G11 – C-terminal amide	ACEMGFFQDCG
	Macrocyclic peptide	XXXGXXXX

	X1 comprises a Lys residue in which the epsilon amino group is in amide linkage with the carboxyl group of amino acid X8; X2 comprises Glu, N-methyl-Glu, or (S)-2-amino-4-(1H-tetrazole-5-yl)butanoic acid; X3 comprises Met, O-methyl-homoserine, Norleucine (Nle), N-methyl-Met, or selenomethionine; X5 comprises Phe, N-methyl-Phe, or pentafluorophenylalanine; X6 comprises Phe, N-methyl-Phe, or 2-fluorophenylalanine; X7 comprises Ala, azidolysine, 4-(guanidino)-phenylalanine, or 2,4-diaminobutyric acid; and, X8 comprises Asp, N-methyl-Asp, D-His, N-methyl-His, azidolysine, or 4-(guanidino)-phenylalanine	
48-138	See Table 5	
139	Interacting peptide	EMGFF

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having
5 ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the present invention is limited only by the claims attached herein.

WHAT IS CLAIMED:

1. A macrocyclic peptide that can inhibit eIF4E by binding to eIF4E in its “apo” form, comprising:

5 8 to 12 amino acids of which two adjacent amino acids thereof are aromatic amino acids,

wherein from an N-terminal to C-terminal direction, a first aromatic amino acid comprising a first aromatic group that can interact with a first aromatic pocket of the eIF4E comprising amino acid residues W¹⁰², W¹⁶⁶, and H²⁰⁰ and a second aromatic amino acid
10 comprising a second aromatic amino acid group that can interact with a second aromatic pocket of the eIF4E bounded by amino acid residues W⁵⁶ and F⁴⁸, and

wherein the macrocyclic peptide is optionally linked to a cell penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane.

15 2. The macrocyclic peptide of claim 1, wherein the aromatic amino acid is phenylalanine or an analog thereof.

3. The macrocyclic peptide of claim 1, wherein the first aromatic amino acid is a phenylalanine analog and the second aromatic amino acid is a phenylalanine analog, wherein
20 the phenylalanine analogs may be the same or different.

4. The macrocyclic peptide of claim 1, wherein the first aromatic amino acid is pentafluorophenylalanine and the second aromatic amino acid is 2-fluorophenylalanine.

25 5. The macrocyclic peptide of claim 1, wherein each of the 8 to 12 amino acids comprises a D-amino acid.

6. The macrocyclic peptide of claim 1, wherein the 8 to 12 amino acids of which two adjacent amino acids thereof are aromatic comprise from an N-terminal to C-terminal
30 direction the amino acid sequence GX¹X²,

wherein G is glycine, X¹ is a phenylalanine or phenylalanine analog, and X² is a phenylalanine or phenylalanine analog, and

wherein the macrocyclic peptide is optionally linked to a cell penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane.

7. The macrocyclic peptide of claim 6, wherein X^1 is a phenylalanine analog,
5 and X^2 is a phenylalanine analog.

8. The macrocyclic peptide of claim 7, wherein the phenylalanine analog is N-methyl-phenylalanine, pentafluorophenylalanine, or 2-fluorophenylalanine.

10 9. The macrocyclic peptide of claim 6, wherein X^1 is pentafluorophenylalanine and X^2 is 2-fluorophenylalanine.

10. A macrocyclic peptide comprising the formula

15
$$X^1X^2X^3G^4X^5X^6X^7X^8$$

wherein X^1 comprises a lysine in which the epsilon amino group thereof and the carboxyl group at the C-terminus of X^8 are linked by an amide bond; X^2 comprises glutamic acid, N-methyl-glutamic acid, or (S)-2-amino-4-(1H-tetrazole-5-yl)butanoic acid; X^3 comprises
20 methionine, N-methyl-methionine, or selenomethionine; X^5 comprises phenylalanine, N-methyl-phenylalanine, or pentafluorophenylalanine; X^6 comprises phenylalanine, N-methyl-phenylalanine, or 2-fluorophenylalanine; X^7 comprises azidolysine or azidolysine linked to a cell penetrating moiety that is capable of transporting the macrocyclic compound across the cell membrane; and, X^8 comprises aspartic acid or glutamic acid.

25 11. The macrocyclic peptide of claim 10, wherein X^5 comprises a pentafluorophenylalanine.

12. The macrocyclic peptide of claim 10, wherein X^5 comprises a
30 pentafluorophenylalanine and X^6 comprises a 2-fluorophenylalanine.

13. The macrocyclic peptide of claim 10, wherein X^3 comprises selenomethionine.

14. The macrocyclic peptide of claim 10, wherein X³ comprises selenomethionine, X⁵ comprises a pentafluorophenylalanine, X⁶ comprises a 2-fluorophenylalanine, and X⁷ comprises azidolysine.

5

15. The macrocyclic peptide of claim 10, wherein X² comprises glutamic acid, X³ comprises selenomethionine, X⁵ comprises a pentafluorophenylalanine, X⁶ comprises a 2-fluorophenylalanine, X⁷ comprises azidolysine, and X⁸ comprises aspartic acid.

10

16. The macrocyclic peptide of claim 10, wherein X² comprises glutamic acid, X³ comprises selenomethionine, X⁵ comprises a pentafluorophenylalanine, X⁶ comprises a 2-fluorophenylalanine, X⁷ comprises azidolysine linked to a cell penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane, and X⁸ comprises aspartic acid.

15

17. The macrocyclic peptide of claim 10, wherein one or more of X¹-X⁸ comprises a D-amino acid.

20

18. The macrocyclic peptide of claim 10, wherein each of X¹-X⁸ comprises a D-amino acid.

25

19. The macrocyclic peptide of claim 10, wherein the macrocyclic peptide comprises an amino acid sequence set forth in the group of amino acid sequences consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 38, and SEQ ID NO: 39.

30

20. The macrocyclic peptide of claim 10, wherein the macrocyclic peptide comprises an amino acid sequence set forth in the group of amino acid sequences consisting of

SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, and SEQ ID NO: 32.

21. The macrocyclic peptide of any one of claims 1 to 20, wherein the cell-
5 penetrating moiety comprises a cell-penetrating peptide (CPP).

22. The macrocyclic peptide of claim 21, wherein the CPP comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID
10 NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82,
15 SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109,
20 SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 123, SEQ ID NO: 124, SEQ ID NO: 125, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ
25 ID NO: 136, SEQ ID NO: 137, and SEQ ID NO: 138.

23. The macrocyclic peptide of claim 21, wherein the CPP is a poly(Arg) polymer comprising 5 to 15 arginine residues (SEQ ID NO: 141).

30 24. The macrocyclic peptide of claim 23, wherein the poly(Arg) is a poly-L-arginine or poly-D-arginine polymer comprising 5 to 15 L-Arg (SEQ ID NO: 141) or D-Arg residues (SEQ ID NO: 142).

25. The macrocyclic peptide of claim 23, wherein the poly(Arg) polymer comprises 10 L-Arg residues (SEQ ID NO: 113) or 10 D-Arg residues (SEQ ID NO: 37).

26. The macrocyclic peptide of claim 23, wherein the CPP comprises an
5 alkyne group, optionally linked to the CPP by a linking moiety.

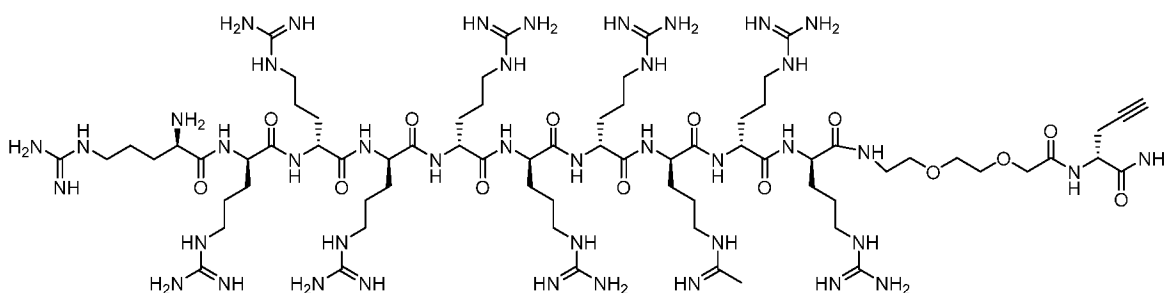
27. The macrocyclic peptide of claim 26, wherein the alkyne group is directly linked to the C-terminus of the CPP or indirectly linked to the C-terminus of the CPP by a linking moiety.

28. The macrocyclic peptide of claim 26, wherein the C-terminus carboxy group of the poly-D-arginine polymer is covalently linked to the alkyne group by a linking moiety comprising a polyethylene glycol (PEG) linker.

29. The macrocyclic peptide of claim 28, wherein the PEG linker comprises one to 10 PEG units.

30. The macrocyclic peptide of claim 28, wherein the alkyne group comprises proargylglycine.

31. The macrocyclic peptide of claim 30, wherein the cell penetrating moiety comprises the formula:



(SEQ ID NO: 37).

32. A composition comprising a macrocyclic peptide of any one of claims 1-31 and a pharmaceutically acceptable carrier.

33. A method for the treatment of a cancer comprising administering to an individual having a cancer a therapeutically effective amount of a macrocyclic peptide of any one of claims 1-31 or composition of claim 32 to treat the cancer.

5

34. The method of claim 33, wherein the cancer is selected from the group cancers that overexpress eIF4E.

35. The method of claim 34, wherein the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

10

36. Use of a macrocyclic peptide of any one of claims 1-31 or composition of claim 32 for the manufacture of a medicament for the treatment of cancer.

15

37. The use of claim 36, wherein the cancer is selected from the group cancers that overexpress eIF4E.

20

38. The use of claim 37, wherein the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

25

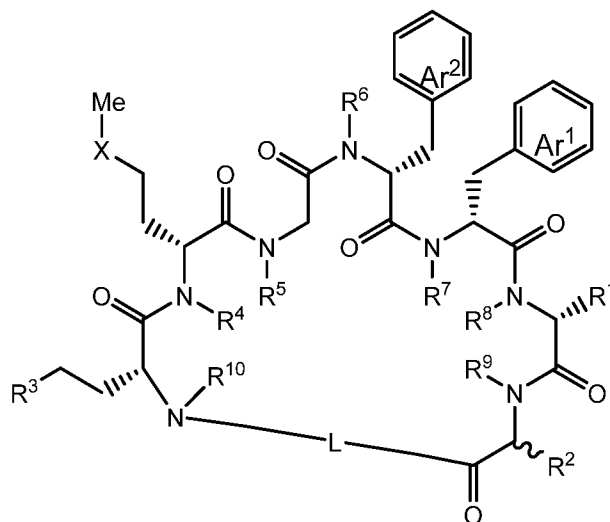
39. The macrocyclic peptide of any one of claims 1-31 or composition of claim 32 for use in the treatment of a cancer.

40. The method of claim 39, wherein the cancer is selected from the group cancers that overexpress eIF4E.

30

41. The method of claim 40, wherein the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

42. A compound comprising Formula I



wherein R^1 comprises a C_1 - C_6 alkylene- R^{11} ; R^2 comprises a CH_2COOH ,

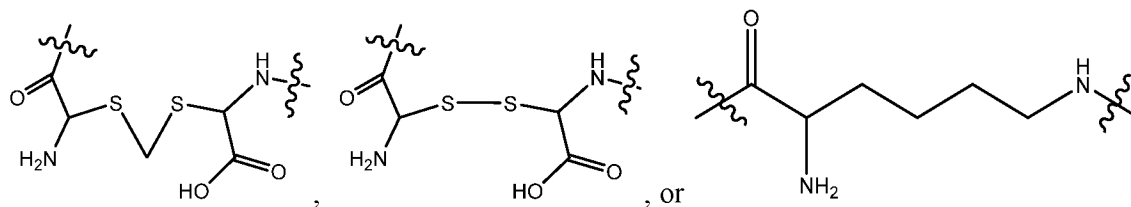
- 5 CH_2CH_2COOH , or $CH_2(5\text{-imidazole})$; R^3 is CH_2COOH ; $R^4 - R^{10}$ each independently comprises a CH_3 or H ; R^{11} comprises H , N_3 , alkyne, succinimide, maleimide, aryl-p-guanidino, 5-imidazole, or NH_2 ; X comprises sulfur (S) or selenium (Se); Ar^1 comprises a benzene, pentafluorobenzene, 2-fluoro-benzene, hydroxybenzene, p-guanidino-benzene, or 3,4-fluoro-benzene; and Ar^2 comprises a benzene, pentafluorobenzene group, 2-fluoro-benzene, p-guanidino-benzene, or
- 10 3,4-fluoro-benzene; and, L comprises a linker;

wherein the compound binds eIF43 in its open form; and

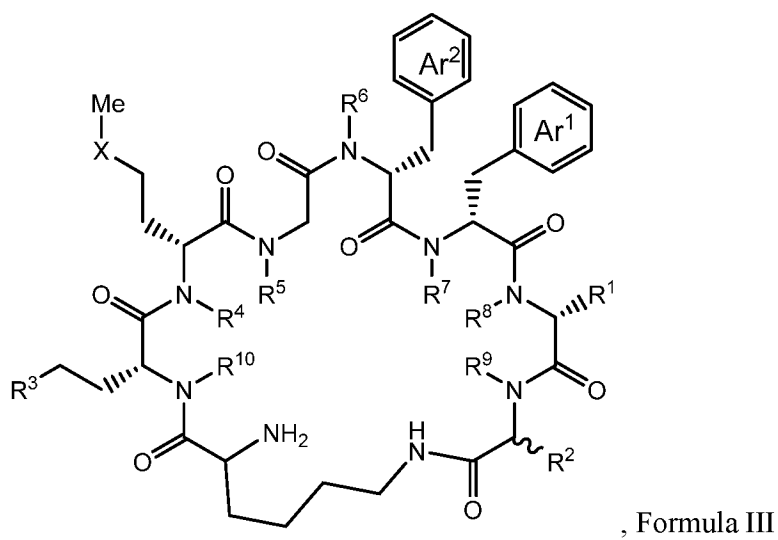
wherein the compound is optionally covalently linked to a cell-penetrating moiety capable of being transported across a cell membrane.

- 15 43. The compound of claim 42, wherein R^1 is a C_1 - C_6 alkylene- R^{11} ; R^2 is a CH_2COOH , CH_2CH_2COOH , or $CH_2(5\text{-imidazole})$; R^3 is CH_2COOH ; $R^4 - R^{10}$ each independently is a CH_3 or H ; R^{11} is H , N_3 , alkyne, succinimide, maleimide, aryl-p-guanidino, 5-imidazole, or NH_2 ; X is sulfur (S) or selenium (Se); Ar^1 is a benzene, hydroxybenzene, pentafluorobenzene, 2-fluoro-benzene, p-guanidino-benzene, or 3,4-fluoro-benzene; and Ar^2 is a benzene,
- 20 pentafluorobenzene group, 2-fluoro-benzene, p-guanidino-benzene, or 3,4-fluoro-benzene.

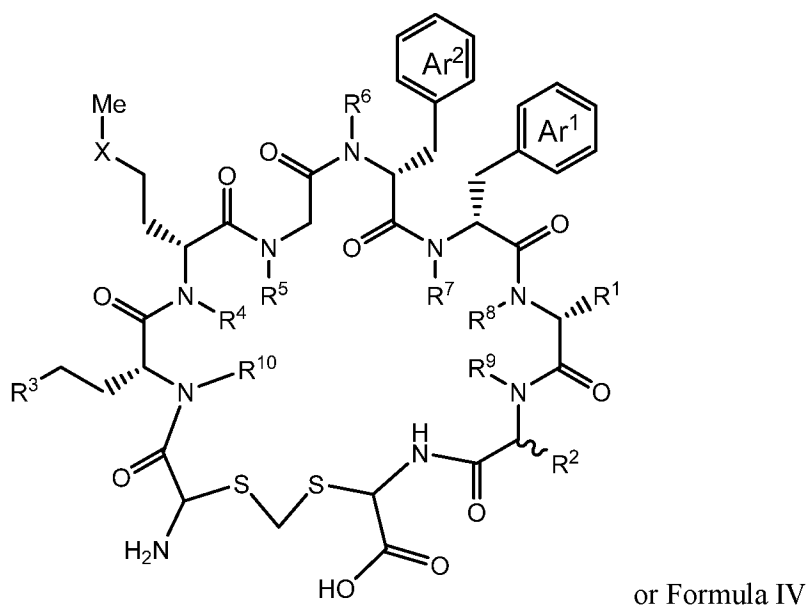
44. The compound of claim 42, wherein L has the formula



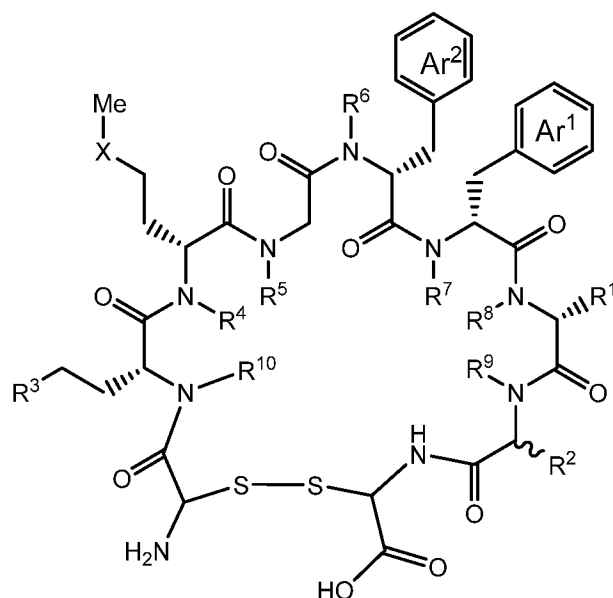
45. A compound comprising Formula II



5



or Formula IV



wherein R^1 comprises a C_1 - C_6 alkylene- R^{11} ; R^2 comprises a CH_2COOH , CH_2CH_2COOH , or $CH_2(5\text{-imidazole})$; R^3 is CH_2COOH ; $R^4 - R^{10}$ each independently comprises a CH_3 or H ; R^{11} comprises H , N_3 , alkyne, succinimide, maleimide, aryl- p -guanidino, 5-imidazole, or NH_2 ; X comprises sulfur (S) or selenium (Se); Ar^1 comprises a benzene, hydroxybenzene, pentafluorobenzene, 2-fluoro-benzene, p -guanidino-benzene, or 3,4-fluoro-benzene; and Ar^2 comprises a benzene, pentafluorobenzene group, 2-fluoro-benzene, p -guanidino-benzene, or 3,4-fluoro-benzene;

wherein the compound binds eIF43 in its open form; and

wherein the compound is optionally covalently linked to a cell-penetrating moiety capable of facilitating the transport of the compound across the cell membrane.

46. The compound of claim 45, wherein R^1 is a C_1 - C_6 alkylene- R^{11} ; R^2 is a CH_2COOH , CH_2CH_2COOH , or $CH_2(5\text{-imidazole})$; R^3 is CH_2COOH ; $R^4 - R^{10}$ each independently is a CH_3 or H ; R^{11} is H , N_3 , alkyne, succinimide, maleimide, aryl- p -guanidino, 5-imidazole, or NH_2 ; X is sulfur (S) or selenium (Se); Ar^1 is a benzene, hydroxybenzene, pentafluorobenzene, 2-fluoro-benzene, p -guanidino-benzene, or 3,4-fluoro-benzene; and Ar^2 is a benzene, pentafluorobenzene group, 2-fluoro-benzene, p -guanidino-benzene, or 3,4-fluoro-benzene.

47. The compound of claim 45, wherein

(a) R^1 comprises 1-azidobutane ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}_3$); R^2 comprises CH_2COOH or $\text{CH}_2(5\text{-imidazole})$; R^3 comprises $\text{CH}_2\text{CH}_2\text{COOH}$; $R^4 - R^{10}$ each independently comprises CH_3 or H ; X comprises sulfur (S) or selenium (Se); Ar^1 comprises pentafluorobenzene; and Ar^2 comprises 2-fluoro-benzene;

5 (b) R^1 is 1-azidobutane ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}_3$); R^2 is CH_2COOH or $\text{CH}_2(5\text{-imidazole})$; R^3 is $\text{CH}_2\text{CH}_2\text{COOH}$; $R^4 - R^{10}$ each independently is CH_3 or H ; X is sulfur (S) or selenium (Se); Ar^1 is pentafluorobenzene; and Ar^2 comprises 2-fluoro-benzene; or

(c) R^1 is 1-azidobutane ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}_3$); R^2 is CH_2COOH ; R^3 is $\text{CH}_2\text{CH}_2\text{COOH}$; $R^4 - R^{10}$ each is H ; X is selenium (Se); Ar^1 is pentafluorobenzene; and Ar^2 comprises 2-fluoro-benzene;

wherein the 1-azidobutane is optionally linked to a cell-penetrating moiety.

48. The compound of any one of claims 45-47, the N_3 or 1-azidobutane is linked to a cell-penetrating moiety comprising an alkyne group in a triazole linkage.

15

49. The compound of claim 45-47, wherein the cell-penetrating moiety comprises a cell-penetrating peptide (CPP).

50. The compound of claim 49, wherein the CPP comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID

30

NO: 121, SEQ ID NO: 122, SEQ ID NO: 123, SEQ ID NO: 124, SEQ ID NO: 125, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, and SEQ ID NO: 138.

5

51. The compound of claim 49, wherein the CPP is a poly(Arg) polymer comprising 5 to 15 arginine residues (SEQ ID NO: 141).

10 52. The compound of claim 51, wherein the poly(Arg) polymer is a poly-L-arginine or poly-D-arginine polymer comprising 5 to 15 L-Arg (SEQ ID NO: 141) or D-Arg residues (SEQ ID NO: 142).

15 53. The compound of claim 51, wherein the poly(Arg) polymer is a poly-L-arginine (SEQ ID NO: 113) or poly-D-arginine polymer comprising 10 arginine residues (SEQ ID NO: 37).

54. The compound of claim 51, wherein the CPP further comprises an alkyne group.

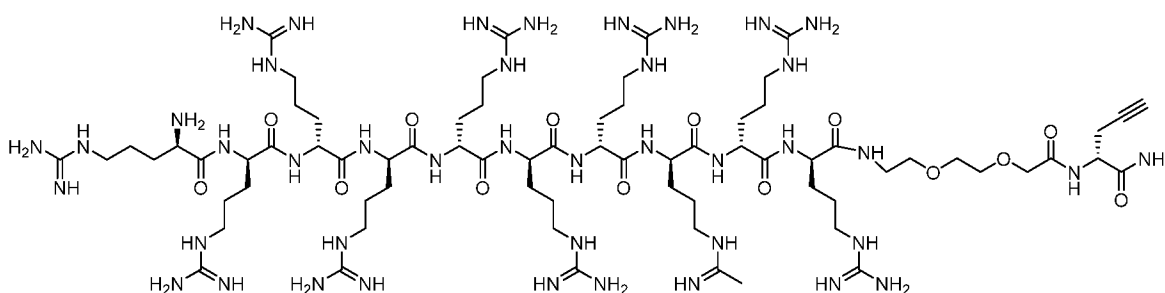
20 55. The compound of claim 54, wherein the alkyne group is directly linked to the C-terminus of the CPP or indirectly linked to the C-terminus of the CPP by a linking moiety.

25 56. The compound of claim 55, wherein the C-terminus carboxy group of the poly-D-arginine polymer is covalently linked to the alkyne group by a linking moiety comprising a polyethylene glycol (PEG) linker.

57. The compound of claim 56, wherein the PEG linker comprises one to 10 PEG units.

30 58. The compound of claim 54, wherein the alkyne group comprises proargylglycine.

59. The compound of claim 49, wherein the cell penetrating moiety comprises the formula:



5 (SEQ ID NO: 37).

60. A composition comprising any one of the compounds of claims 45-59 and a pharmaceutically acceptable carrier.

10 61. A method for the treatment of a cancer comprising administering to an individual having a cancer a therapeutically effective amount of a macrocyclic peptide of any one of claims 45-59 or composition of claim 60 to treat the cancer.

15 62. The method of claim 61, wherein the cancer is selected from the group cancers that overexpress eIF4E.

20 63. The method of claim 62 wherein the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

64. Use of a macrocyclic peptide of any one of claims 45-59 or composition of claim 60 for the manufacture of a medicament for the treatment of cancer.

25 65. The use of claim 64, wherein the cancer is selected from the group cancers that overexpress eIF4E.

66. The use of claim 65, wherein the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

5 67. The macrocyclic peptide of any one of claims 45-59 or composition of claim 60 for use in the treatment of a cancer.

68. The method of claim 67, wherein the cancer is selected from the group cancers that overexpress eIF4E.

10

69. The method of claim 68, wherein the cancer that overexpress eIF4E is selected from the group consisting of breast, bladder, colorectum, uterine cervix, non-Hodgkin lymphoma, and head and neck cancers.

15

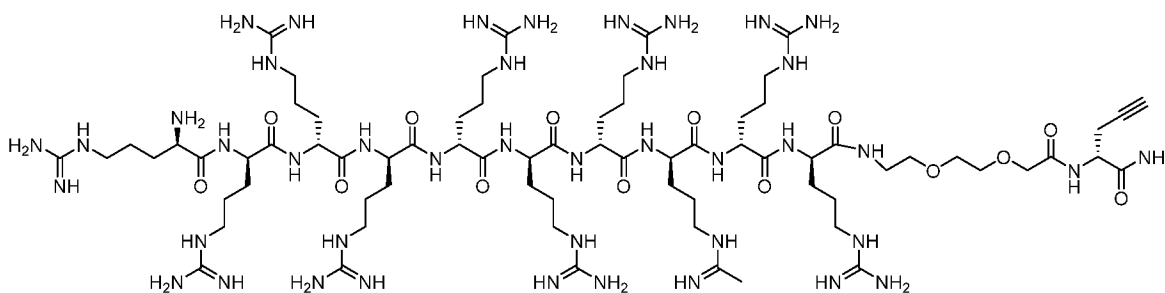
70. A compound comprising a macrocyclic peptide covalently linked to a cell-penetrating moiety comprising a poly-D-arginine polymer directly or indirectly covalently linked at the C-terminus carboxy group to an alkyne group.

71. The compound of claim 70, wherein the poly-D-arginine polymer
20 comprises five to 15 D-arginine residues (SEQ ID NO: 142).

72. The compound of claim 71, wherein the poly-D-arginine polymer comprises 10 D-arginine residues (SEQ ID NO: 37).

25

73. The compound of claim 70, wherein poly-D-arginine polymer comprises the formula



(SEQ ID NO: 37).

74. A method for producing a compound that is cell permeable comprising:

(a) providing a macrocyclic peptide comprising an azido group and a cell-penetrating moiety comprising a poly-D-arginine polymer directly or indirectly covalently linked at the C-terminus carboxy group to an alkyne group; and

(b) conjugating the azido group of the macrocyclic peptide to the alkyne group to produce the cell permeable macrocyclic compound.

75. The method of claim 74, wherein the poly-D-arginine polymer comprises

5 to 15 D-Arg residues (SEQ ID NO: 142).

76. The method of claim 75, wherein the poly-D-arginine polymer comprises

10 D-Arg residues (SEQ ID NO: 37).

77. The method of claim 74, wherein the alkyne group comprises

proargylglycine.

78. The method of claim 74, wherein the alkyne group is directly to the C-

terminus of the poly-D-arginine polymer or indirectly linked to the C-terminus of the poly-D-arginine polymer by a linking moiety.

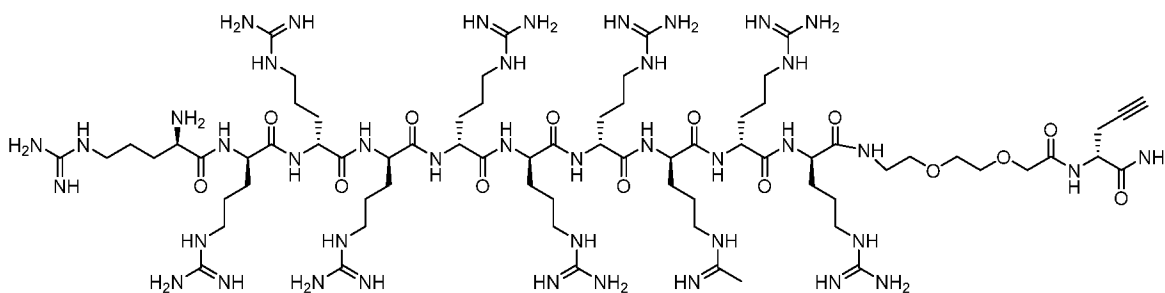
79. The method of claim 74, wherein the C-terminus carboxy group of the

poly-D-arginine polymer is covalently linked to the alkyne group by a linking moiety comprising a polyethylene glycol (PEG) linker.

80. The method of claim 79, wherein the PEG linker comprises one to 10 PEG

units.

81. The compound of claim 74, wherein the cell penetrating moiety comprises:



(SEQ ID NO: 37).

82. The present invention provides a cell-penetrating moiety comprising a
5 poly-D-arginine polymer directly or indirectly covalently linked at the C-terminus carboxy group to an alkyne group.

83. The compound of claim 82, wherein the poly-D-arginine polymer
comprising 5 to 15 arginine residues (SEQ ID NO: 141).

10

84. The compound of claim 83, wherein the poly-D-arginine polymer
comprises 10 D-Arg residues (SEQ ID NO: 37).

85. The compound of claim 82, wherein the poly-D-arginine polymer is
15 indirectly covalently linked to the poly-D-arginine polymer by a linking moiety.

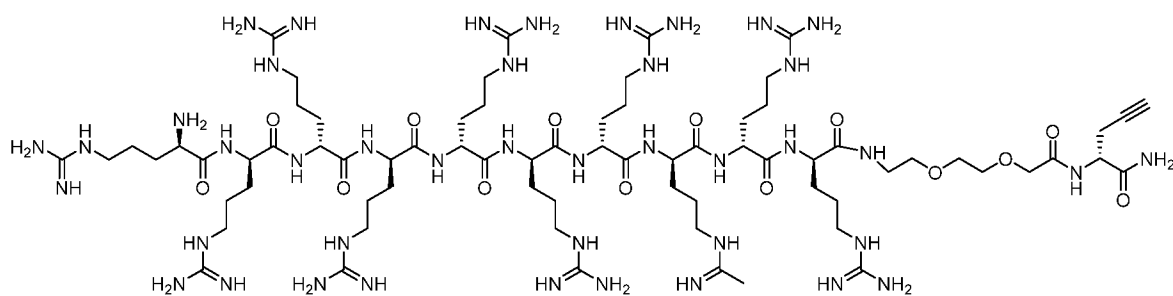
86. The compound of claim 82, wherein the C-terminus carboxy group of the
poly-D-arginine polymer is indirectly covalently linked to the alkyne group by a linking moiety
comprising a polyethylene glycol (PEG) linker.

20

87. The compound of claim 86, wherein the PEG linker comprises one to 10
PEG units.

88. The compound of claim 82, wherein the alkyne group comprises
25 proargylglycine.

89. The compound of claim 82, wherein the cell penetrating moiety comprises the formula



5 (SEQ ID NO: 37).

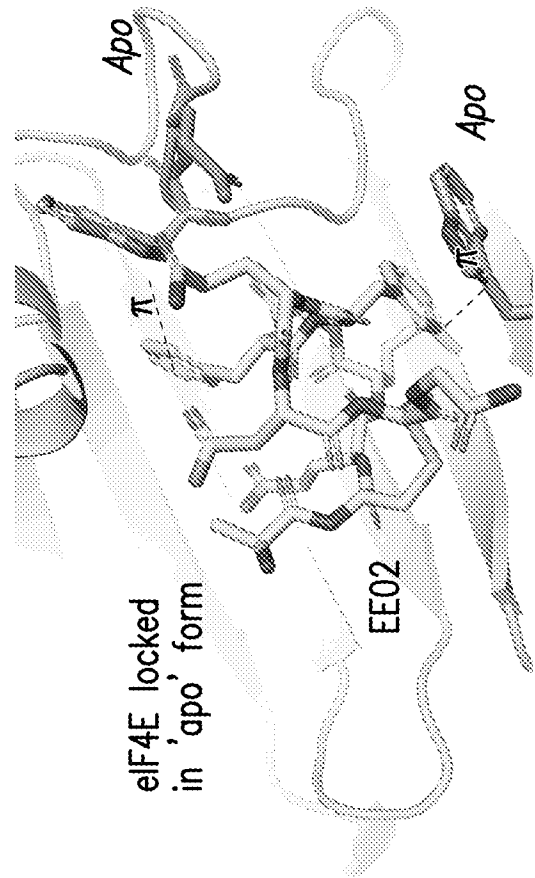


FIG.1B

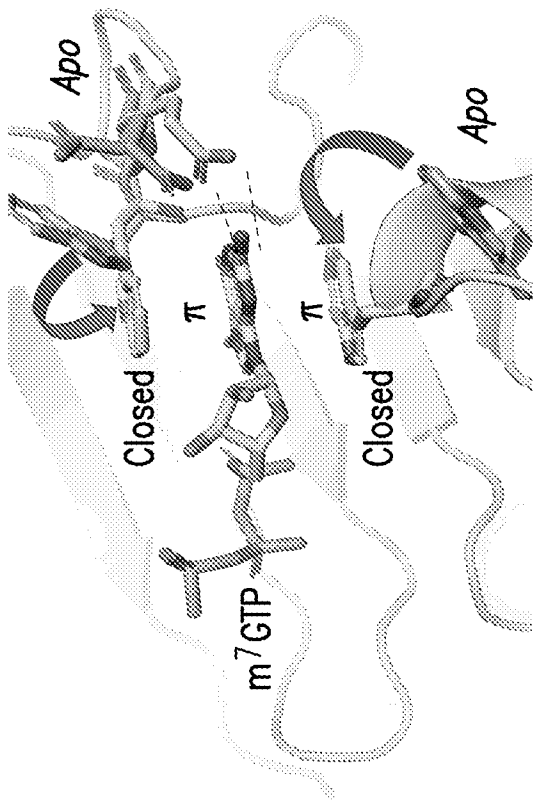


FIG.1A

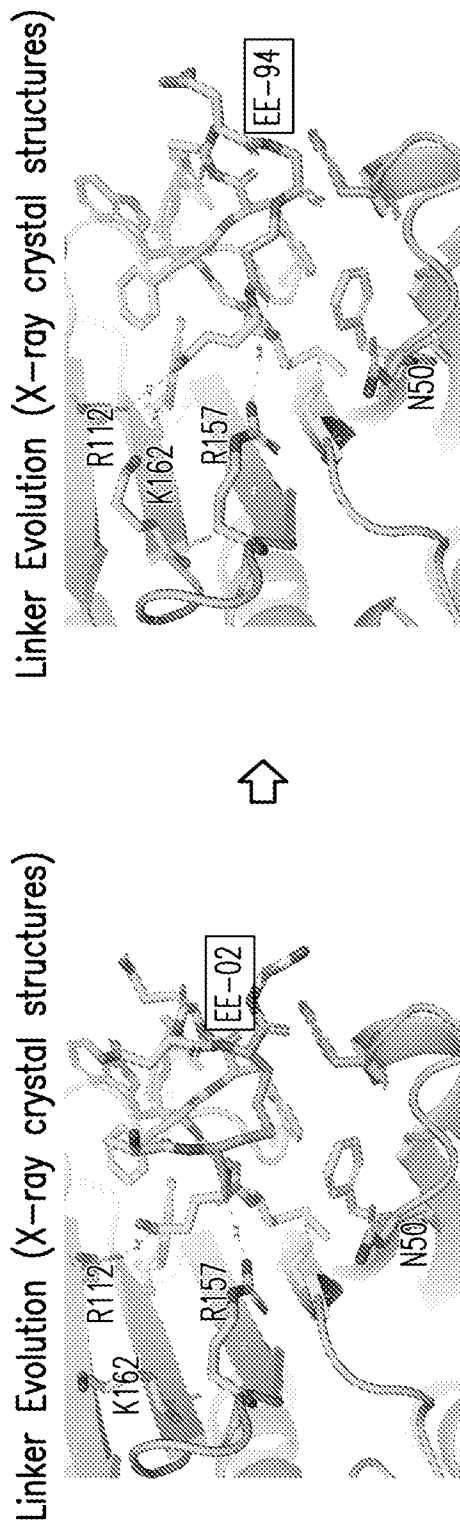


FIG. 2A

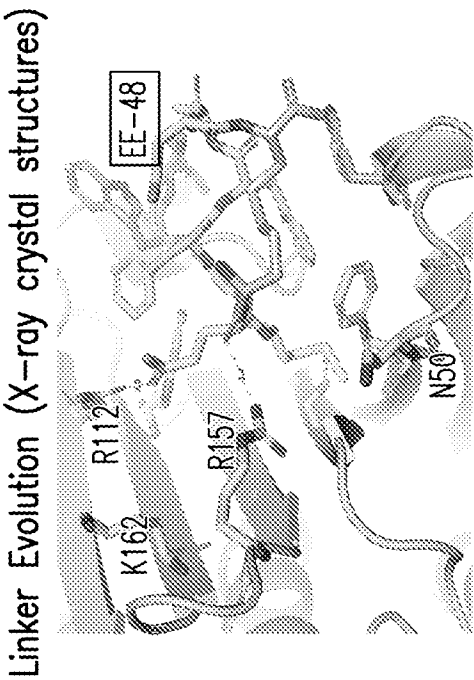


FIG. 2C

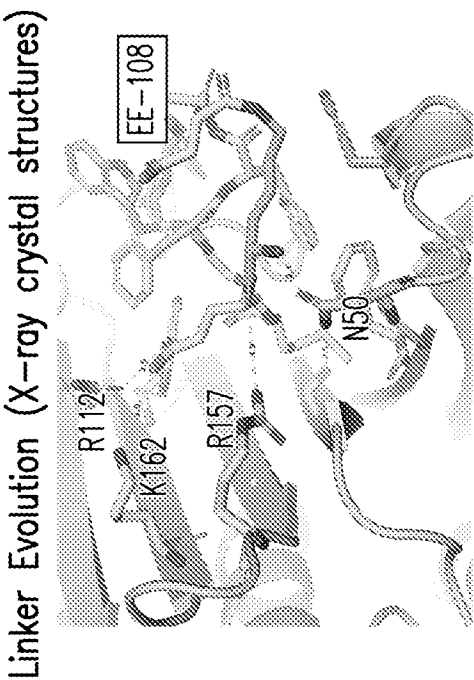


FIG. 2D

FIG. 2B

Structure-Activity-Relationship
(SAR) Study from EE-108 ^{3/65}

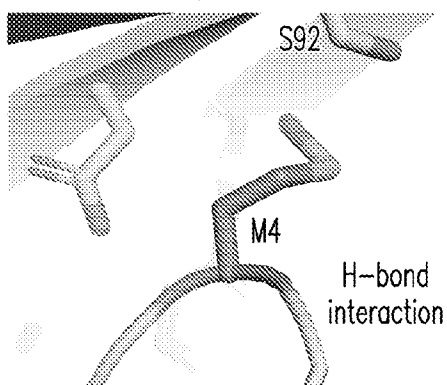


FIG.3A

Structure-Activity-Relationship
(SAR) Study from EE-108

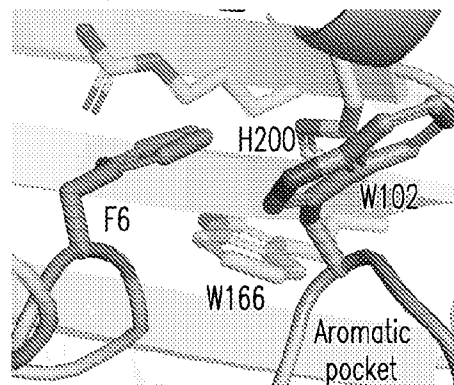


FIG.3B

Structure-Activity-Relationship
(SAR) Study from EE-108

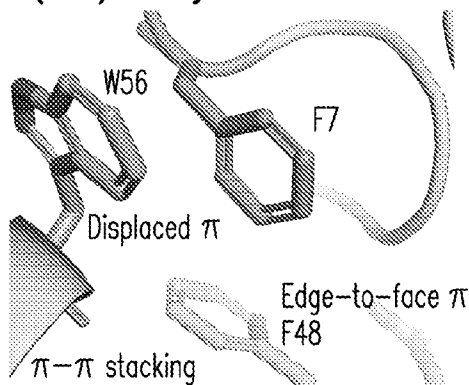


FIG.3C

Structure-Activity-Relationship
(SAR) Study from EE-108

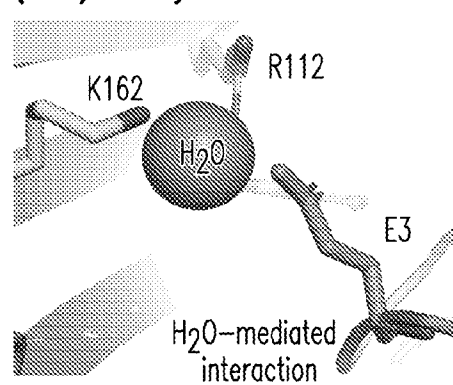
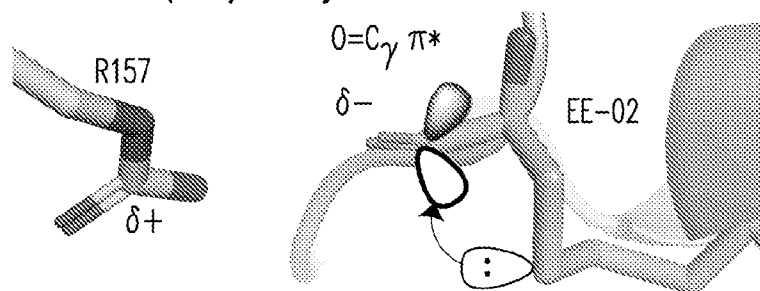


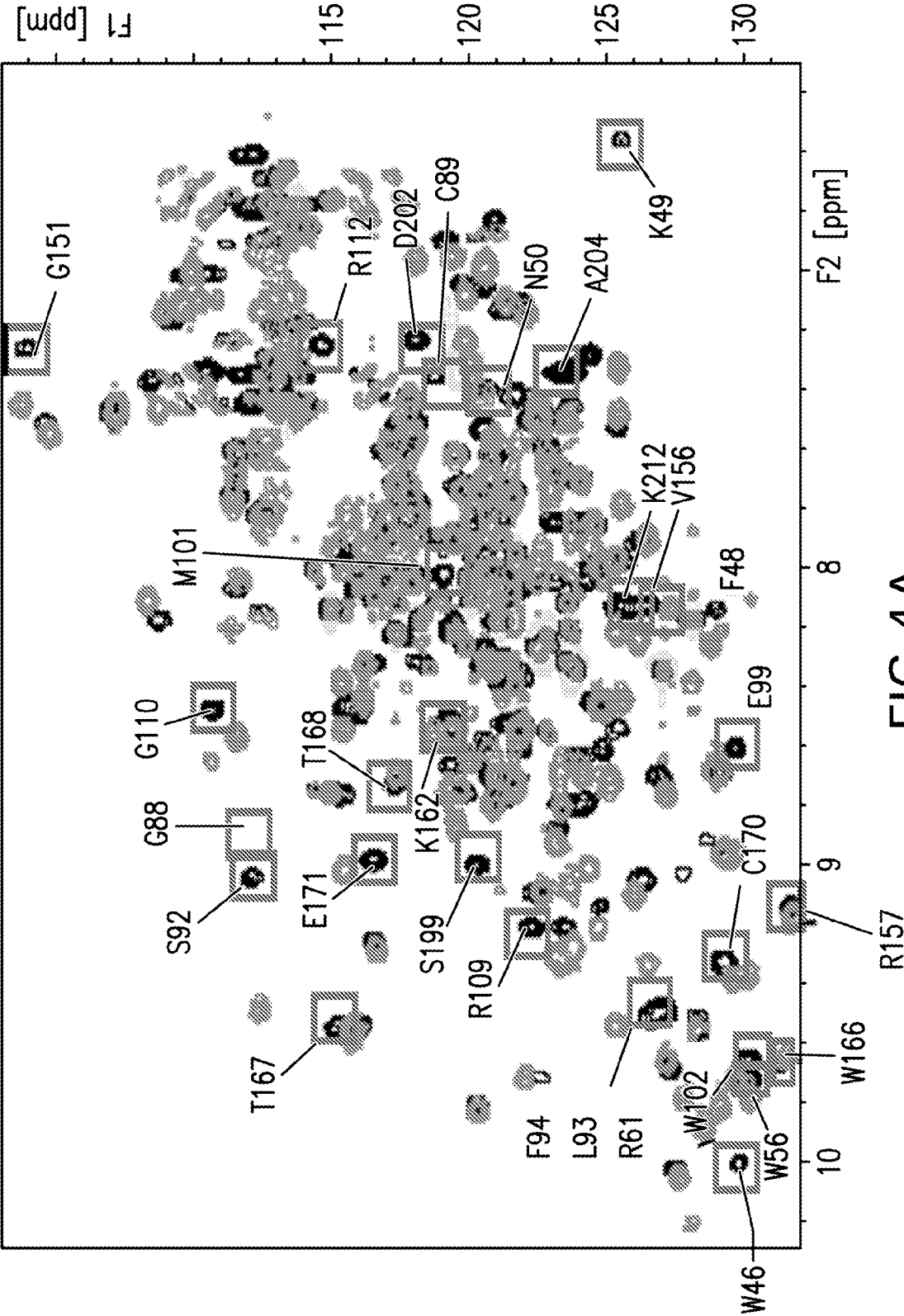
FIG.3D

Structure-Activity-Relationship
(SAR) Study from EE-108



Disulfide (S-S) bond donation into carbonyl induces stronger polar interaction with R157. This, in turn, stabilized S-S bonds in EE-02/44.

FIG.3E



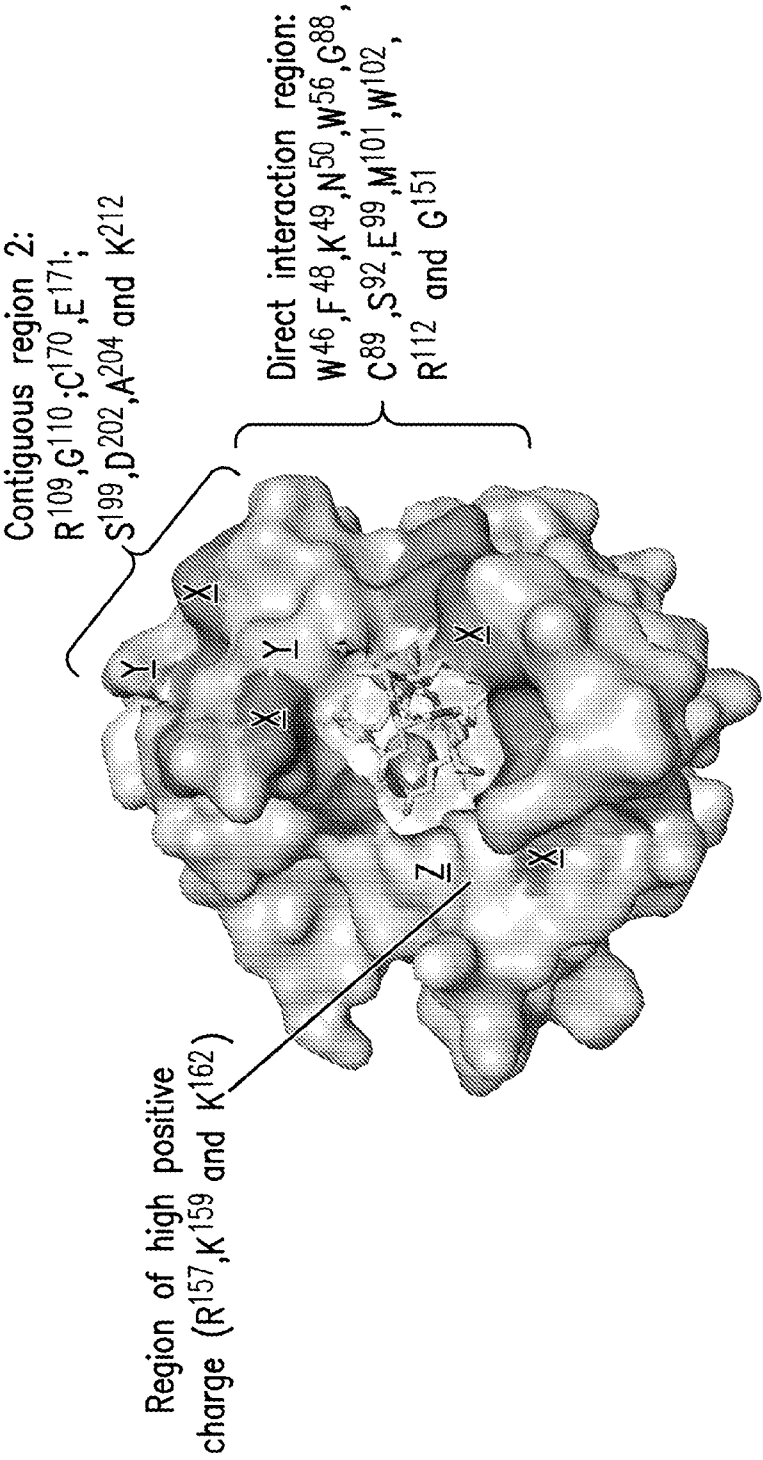
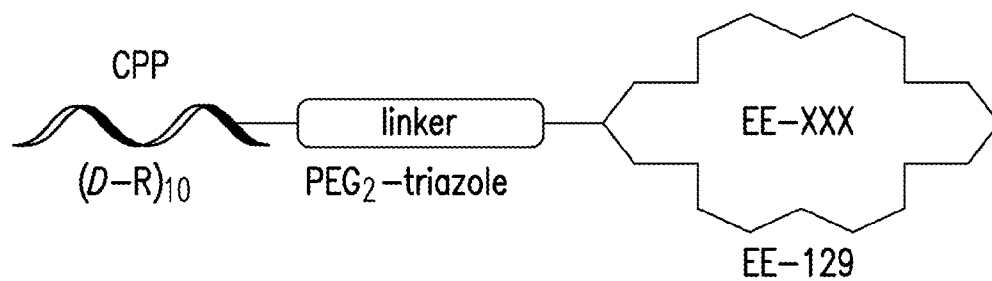


FIG.4B

6/65

Passive Permeable Development
Cell-Permeable Peptide (CPP) Conjugate



EE-171: Biological cell assays / target validation

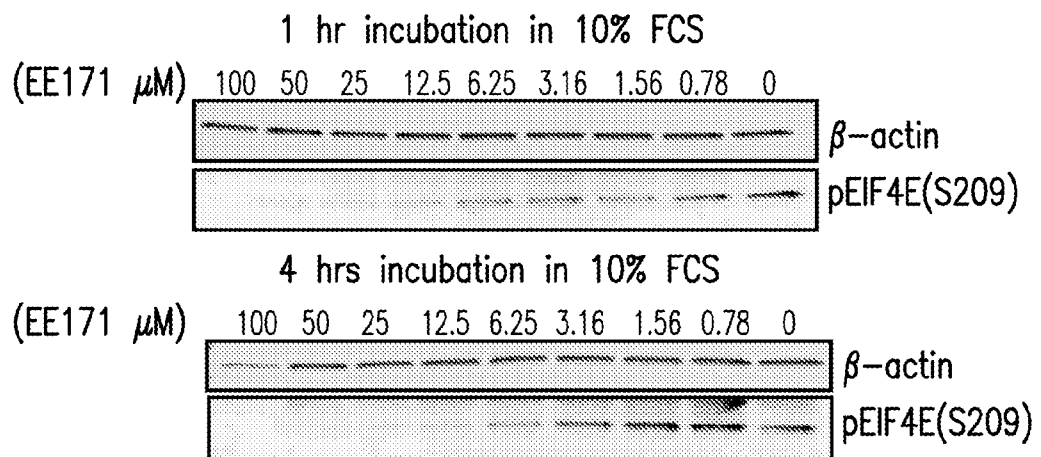


FIG.5A

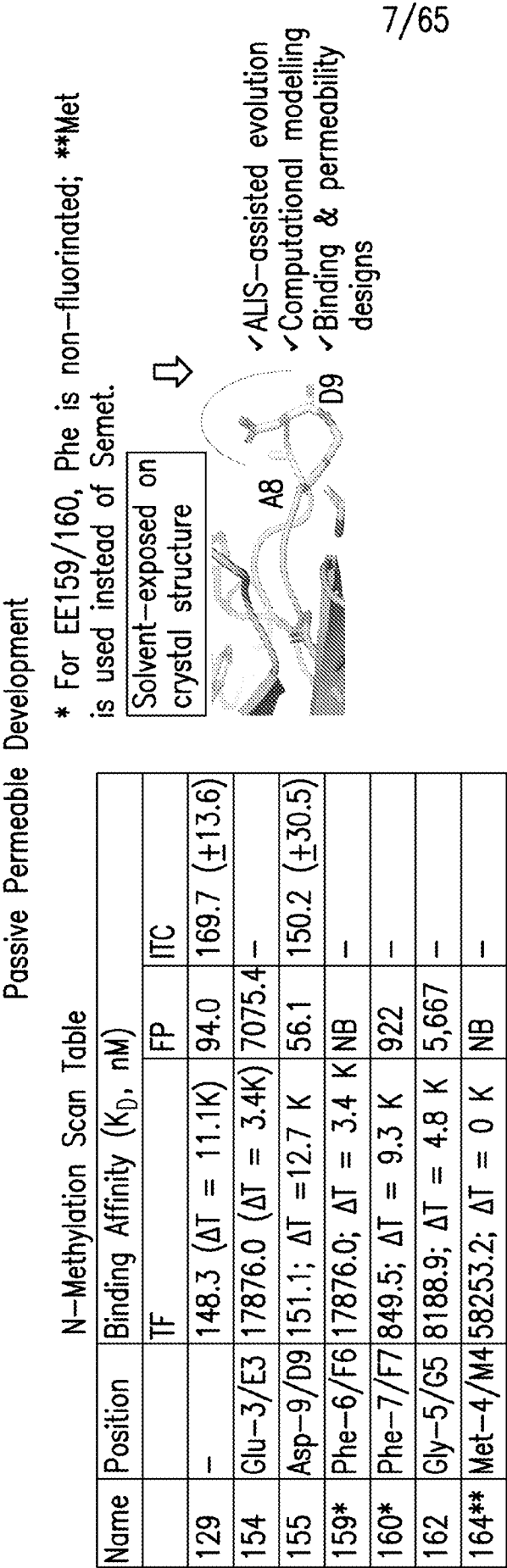


FIG.5B

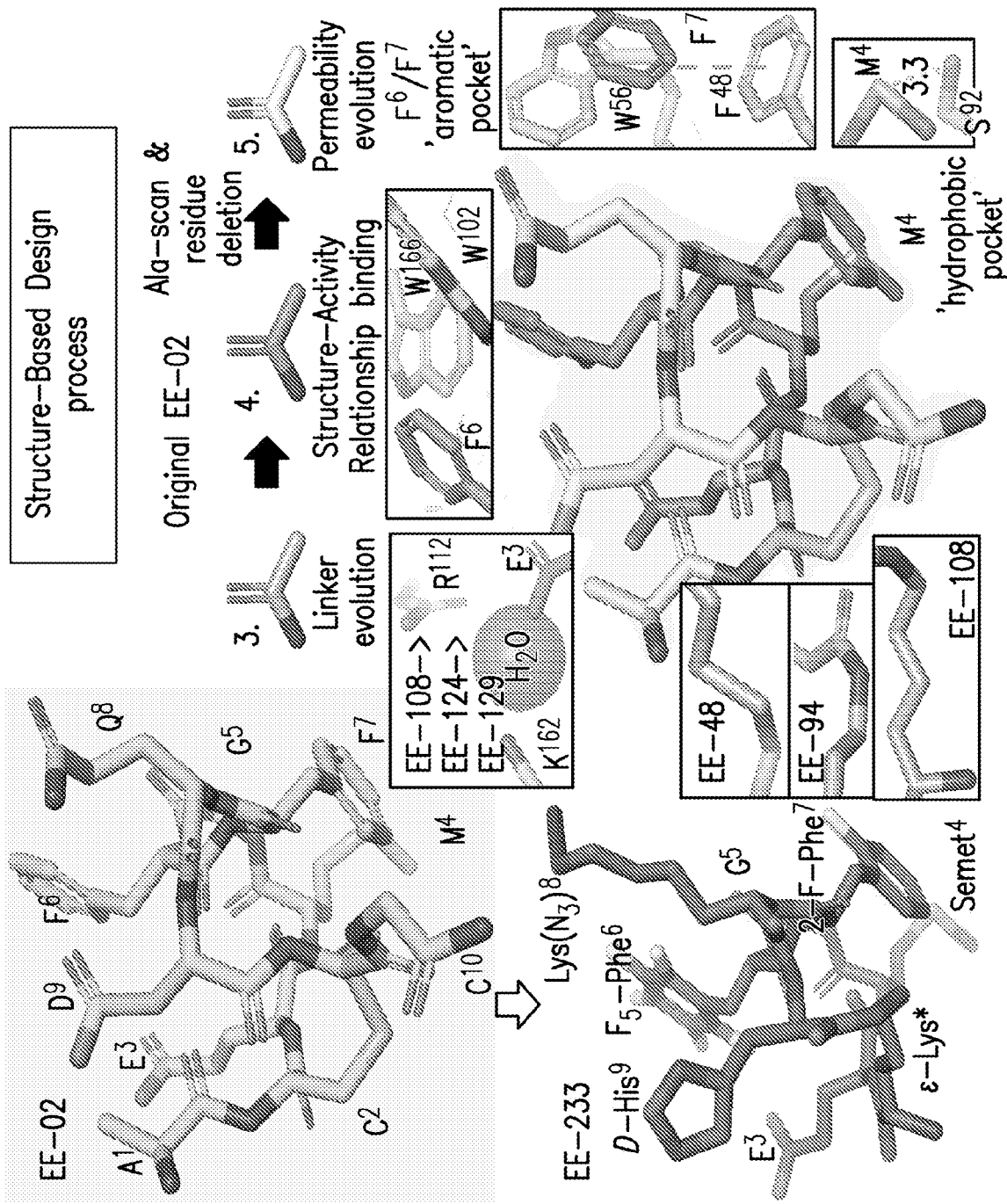


FIG. 6

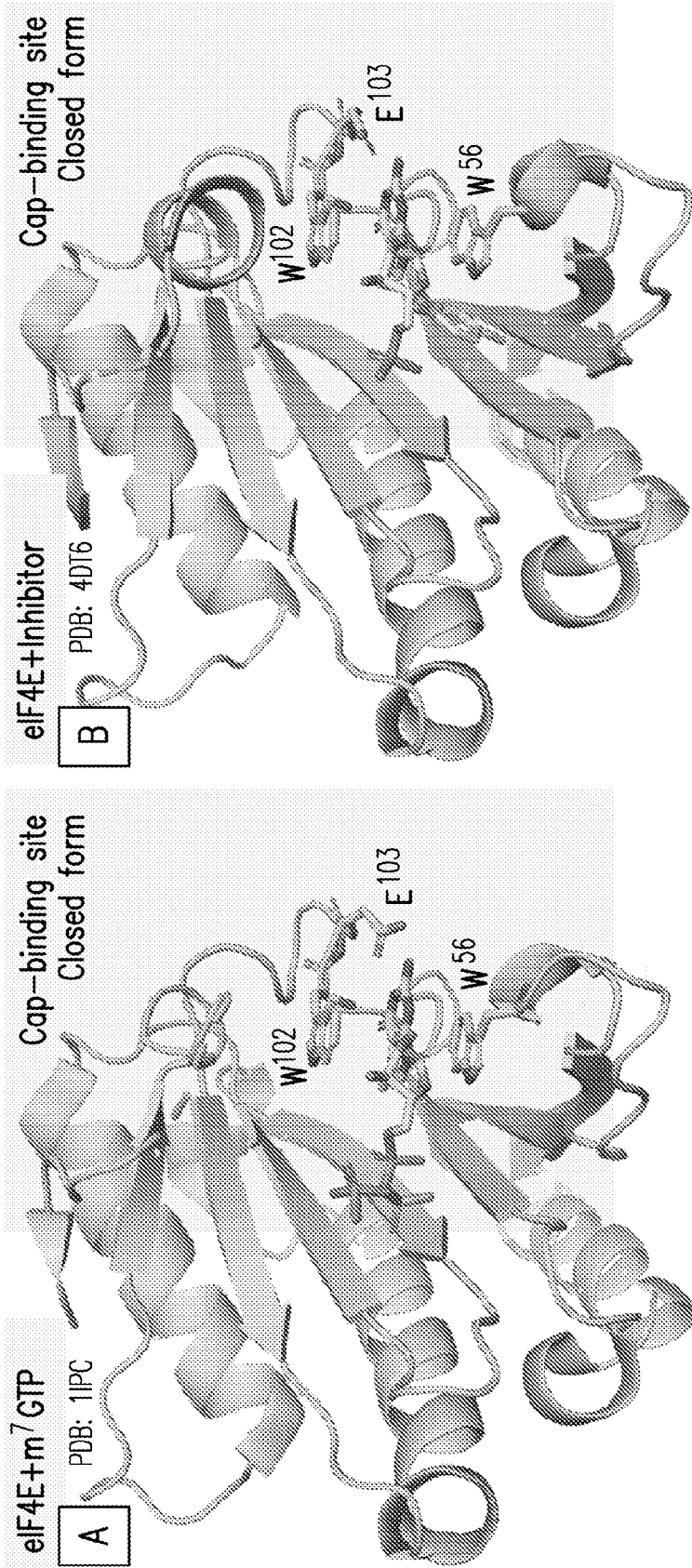


FIG.7A

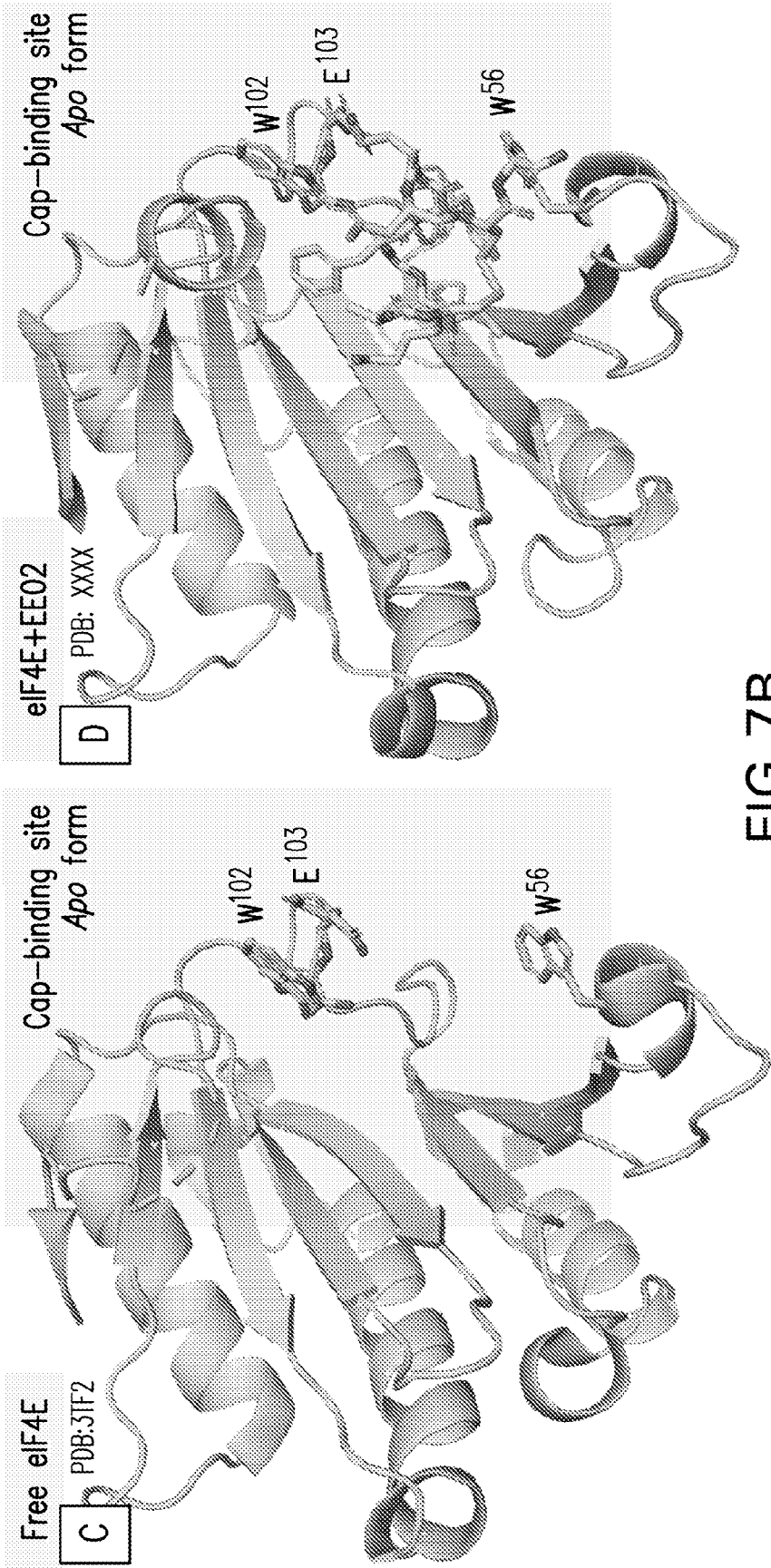


FIG. 7B

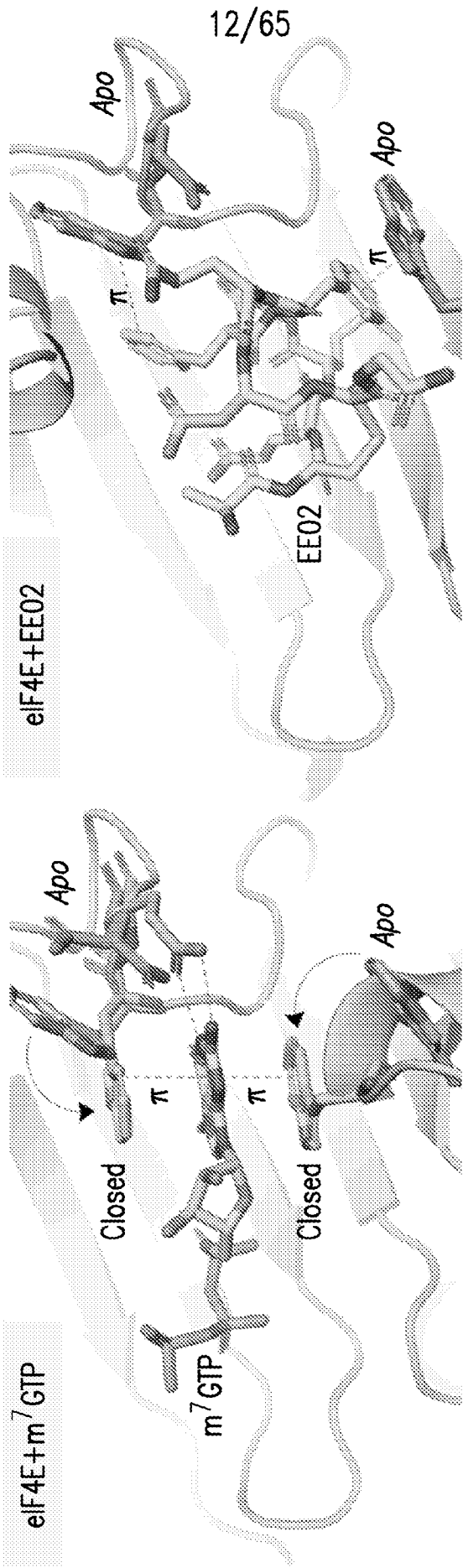


FIG.7C

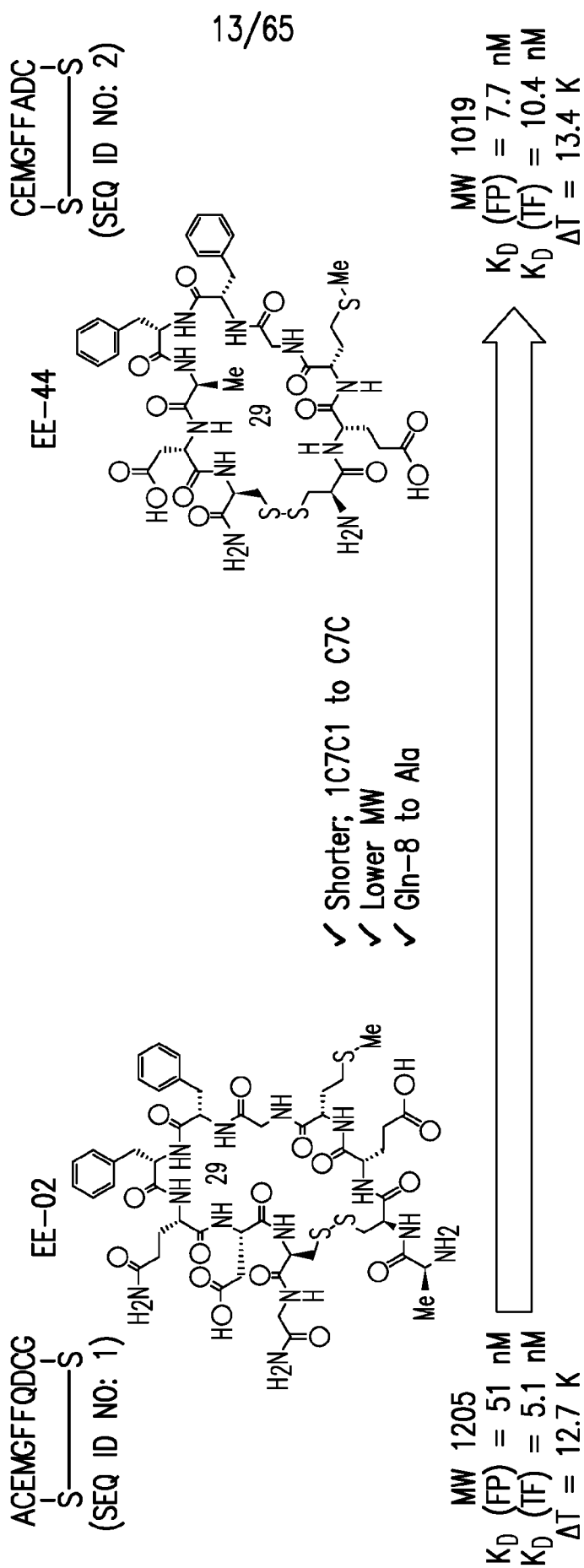


FIG.8

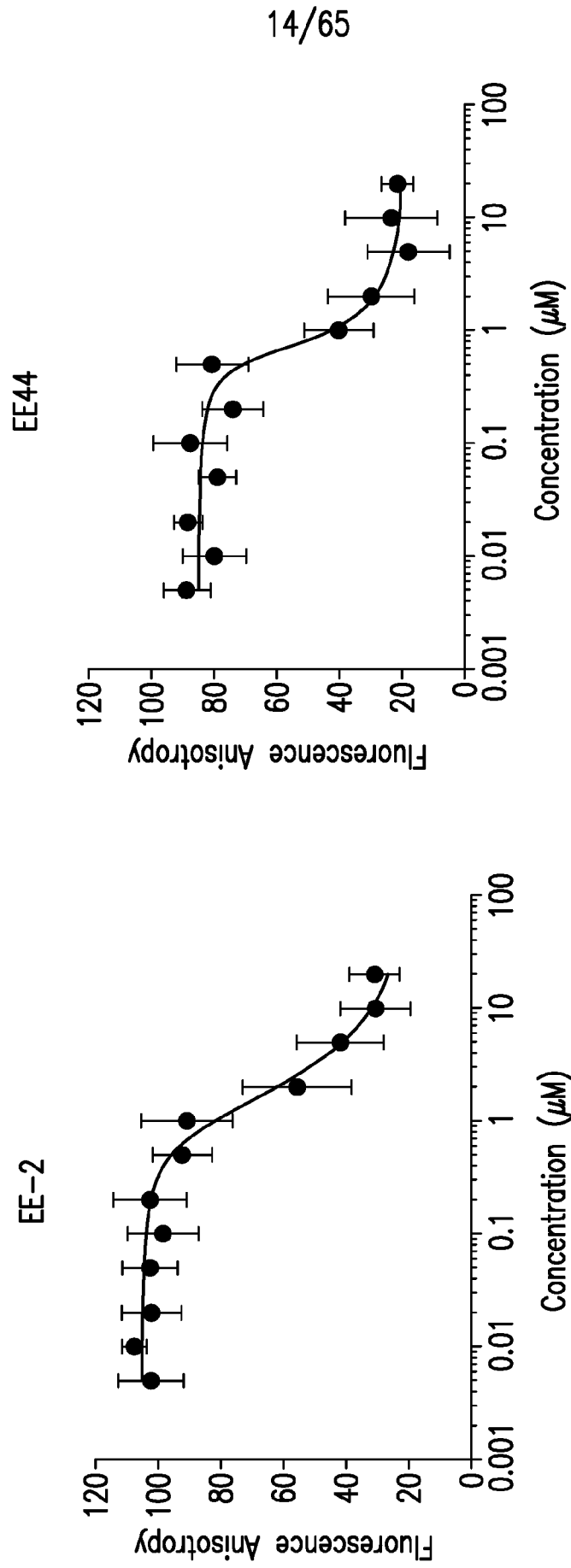


FIG.9

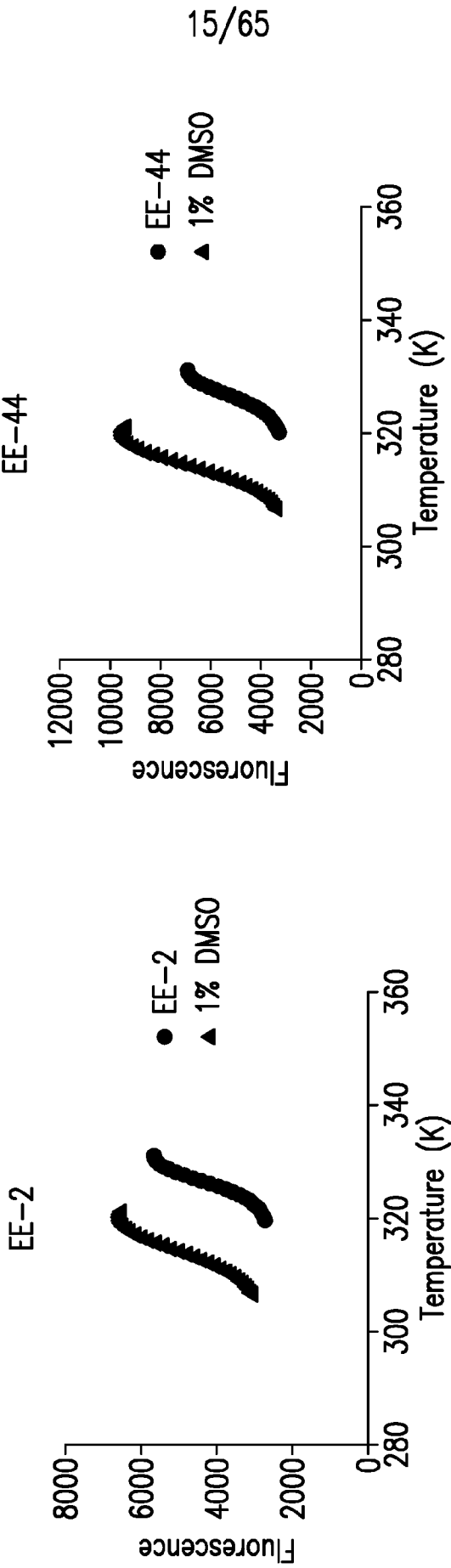
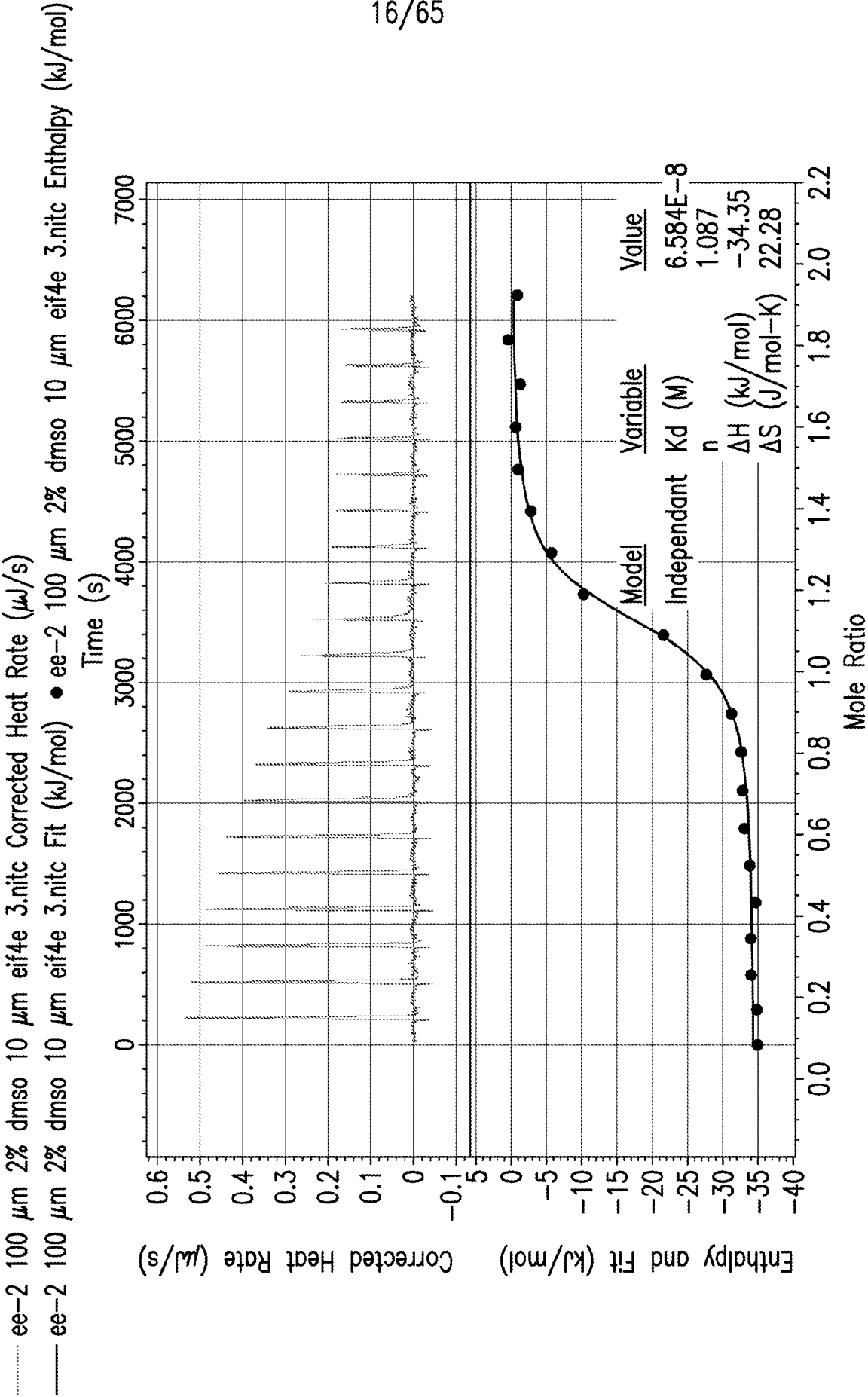


FIG.10



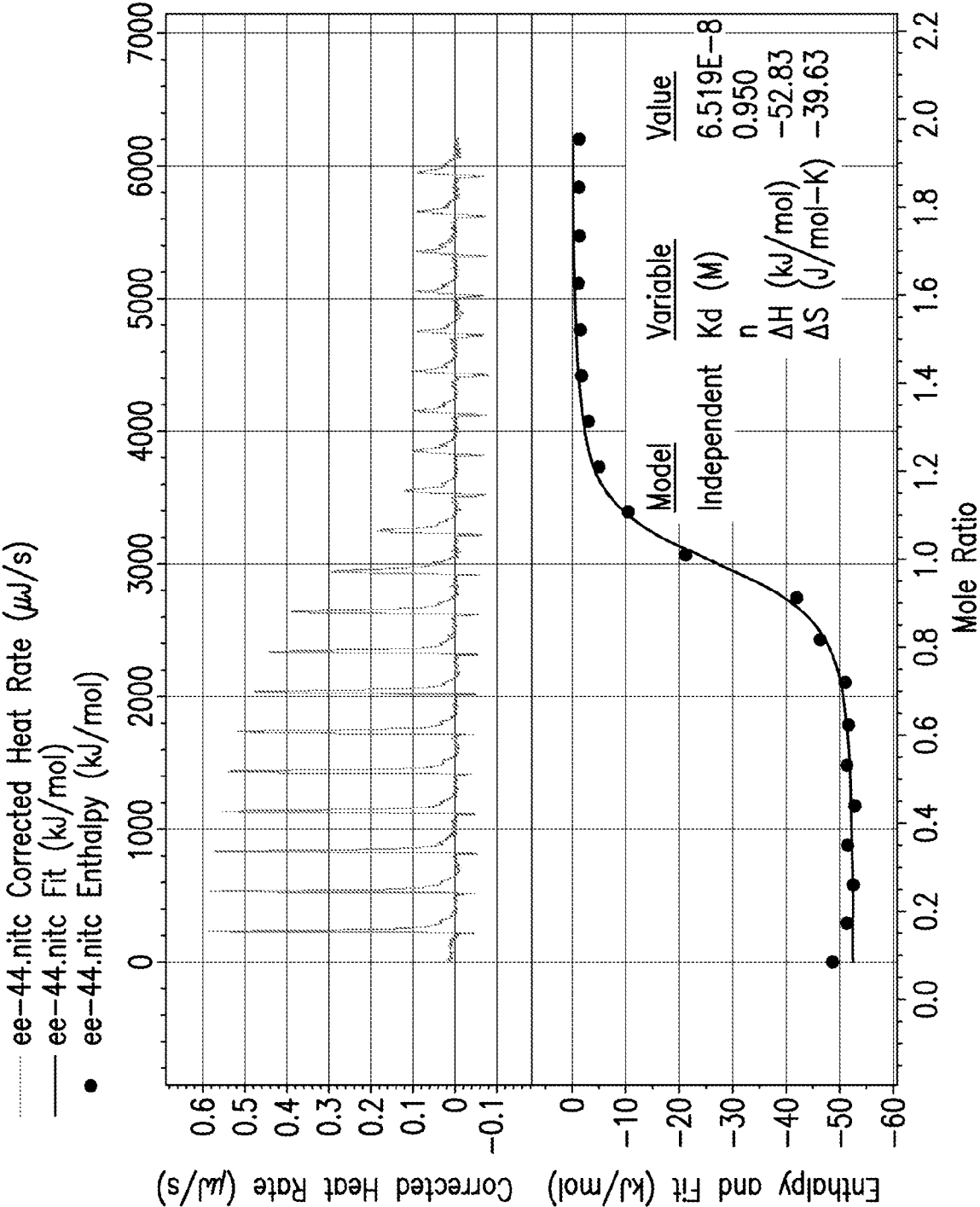


FIG.12

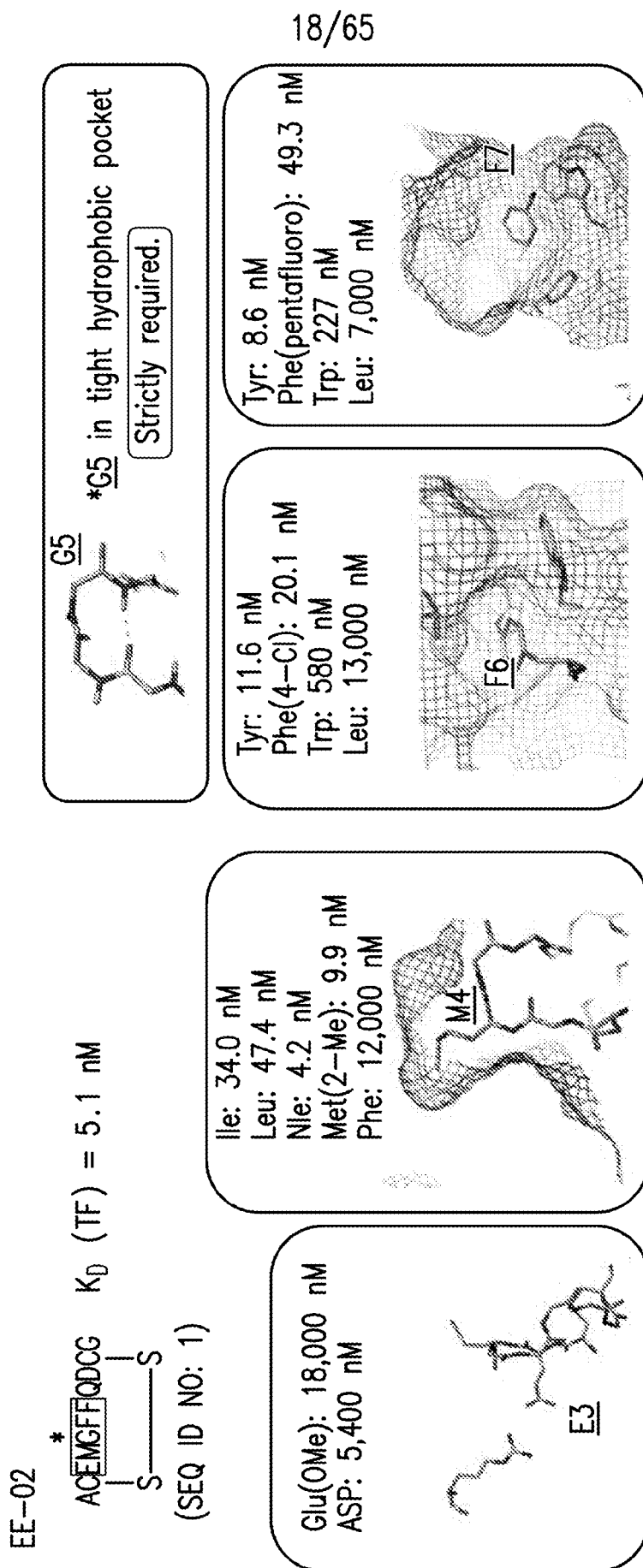


FIG. 13

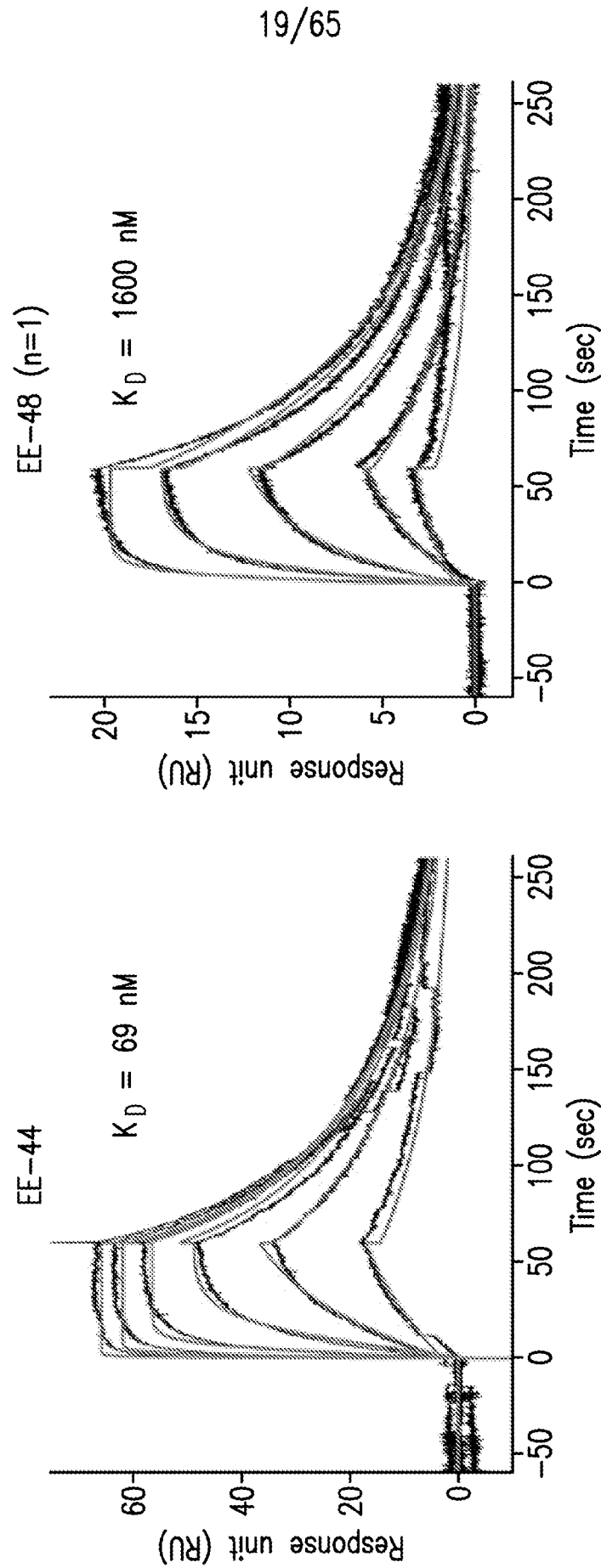


FIG.14

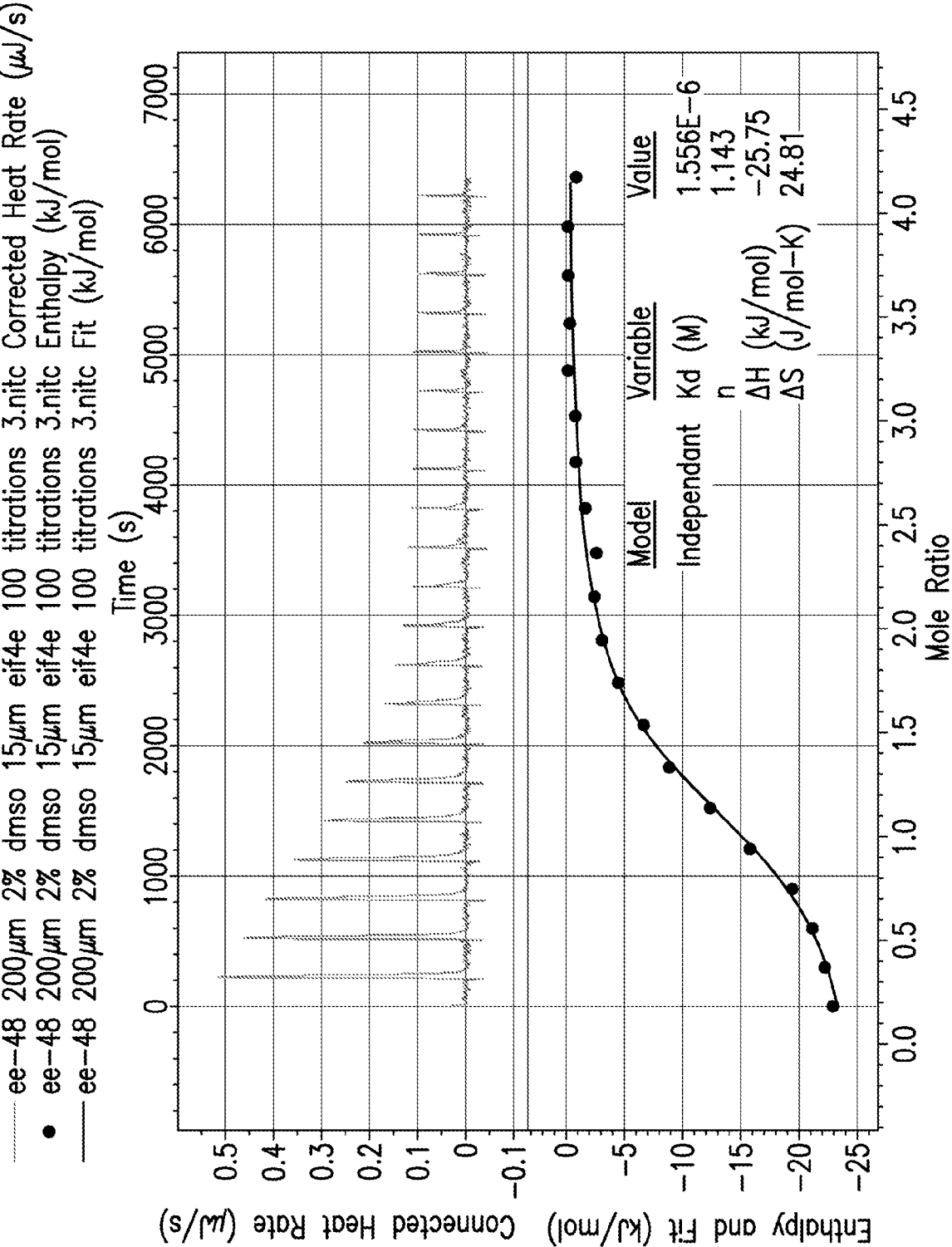


FIG.15

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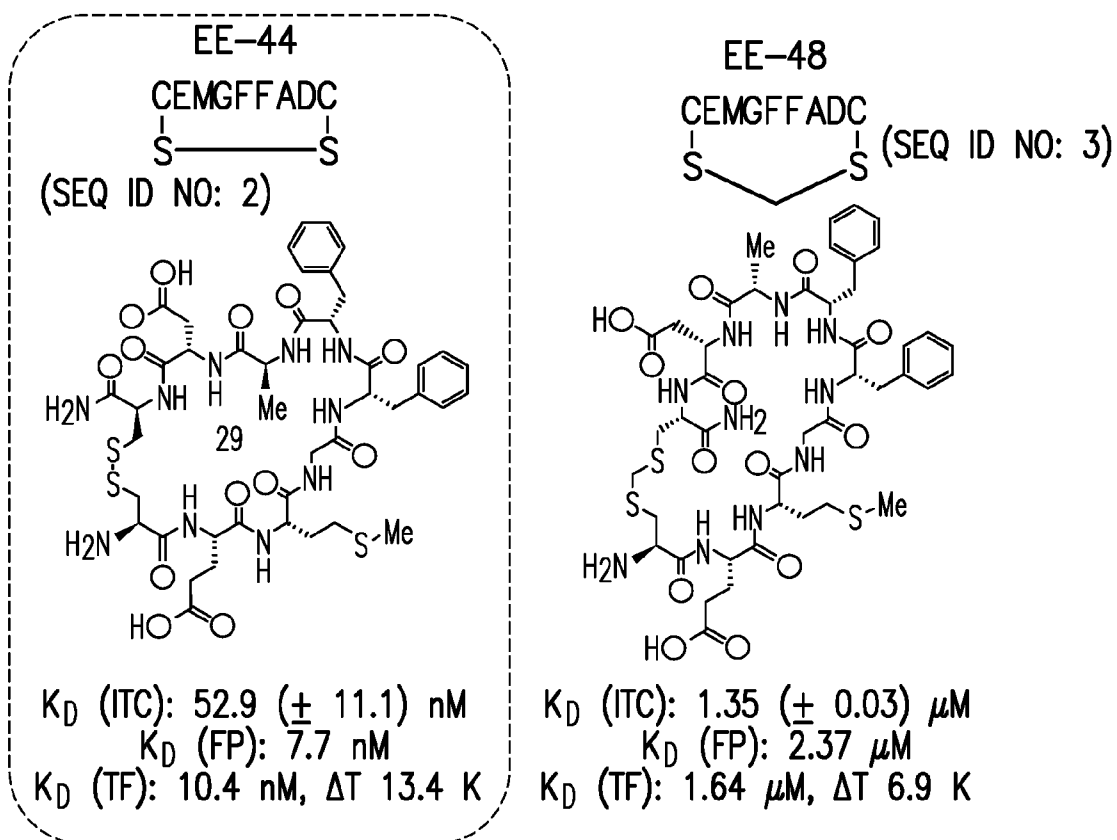


FIG.16

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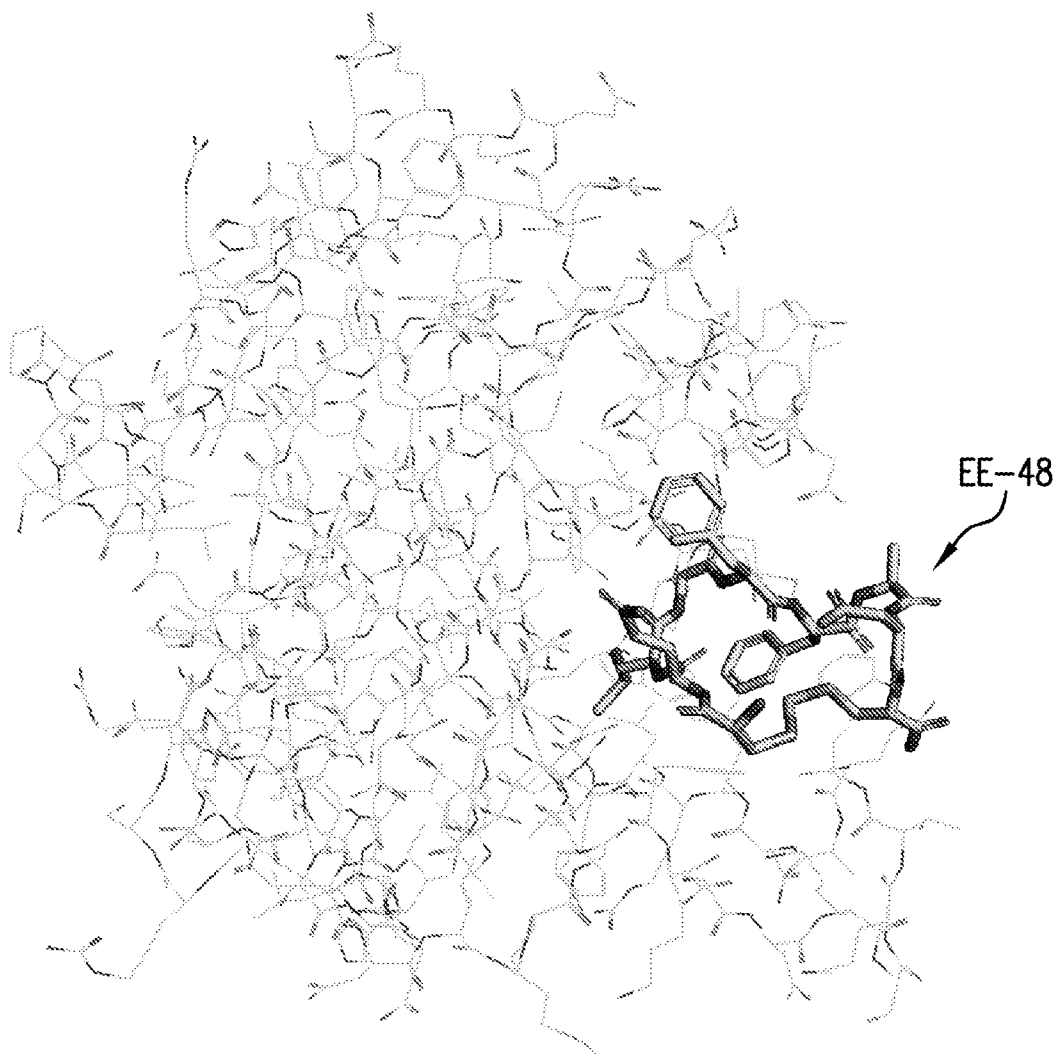


FIG.17

ee-94a 200 μm 2% dmso 15 μm eif4e 4.nitc Corrected Heat Rate (μJ/s) ee-94a 200 μm 2% dmso 15 μm eif4e 4.nitc Enthalpy (kJ/mol)

ee-94a 200 μm 2% dmso 15 μm eif4e 4.nitc Fit (kJ/mol) ee-94a 200 μm 2% dmso 15 μm eif4e 4.nitc Enthalpy (kJ/mol)

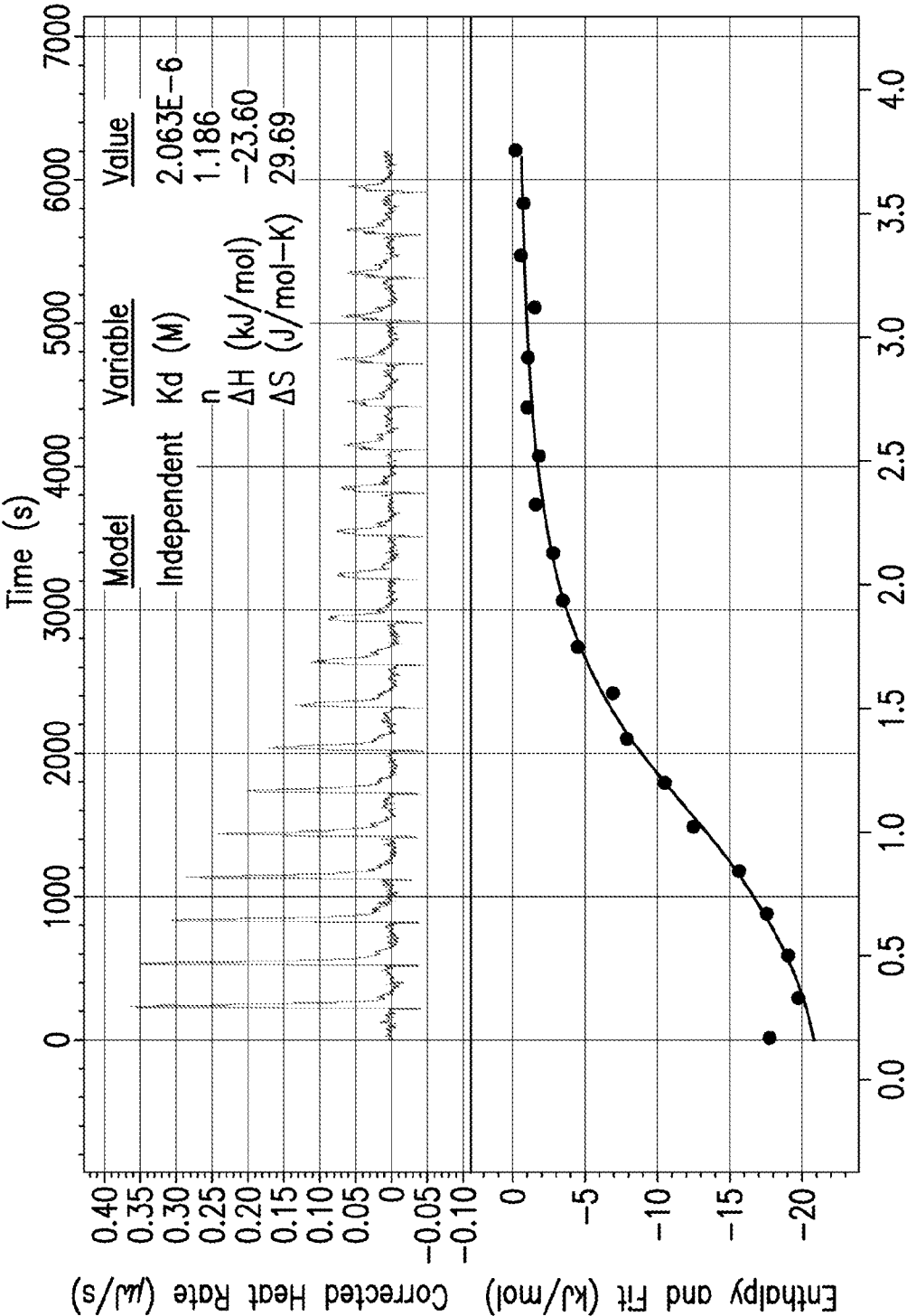


FIG.18

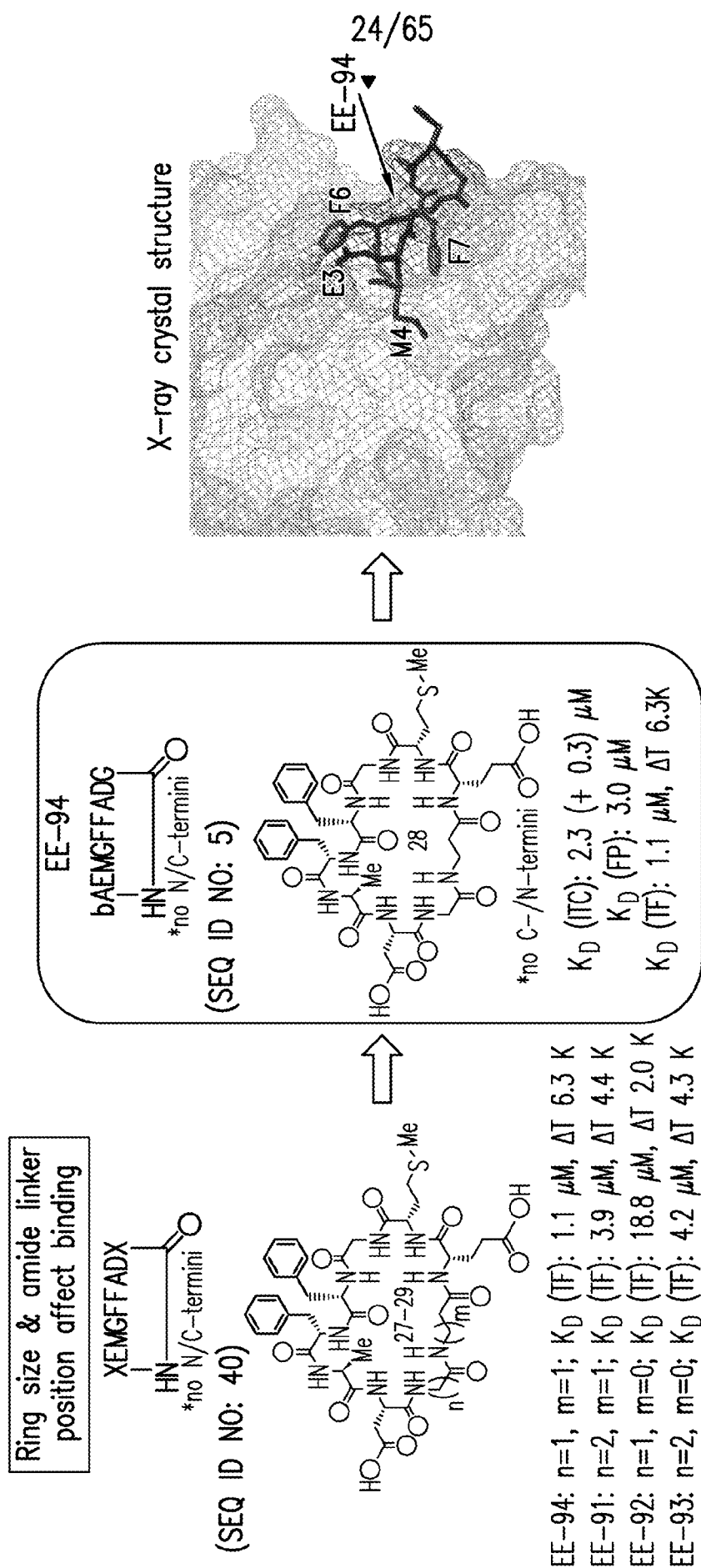


FIG. 19

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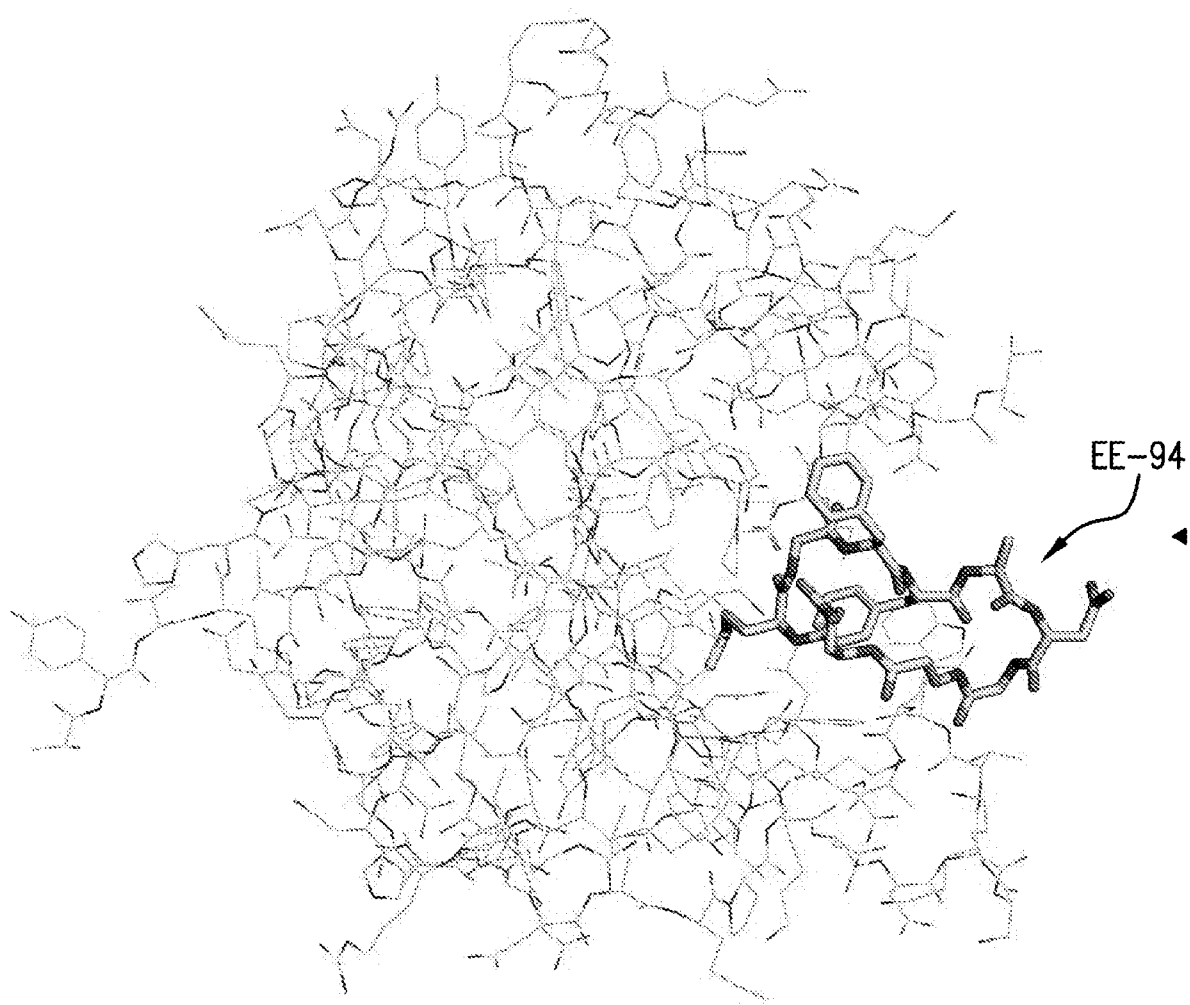


FIG.20

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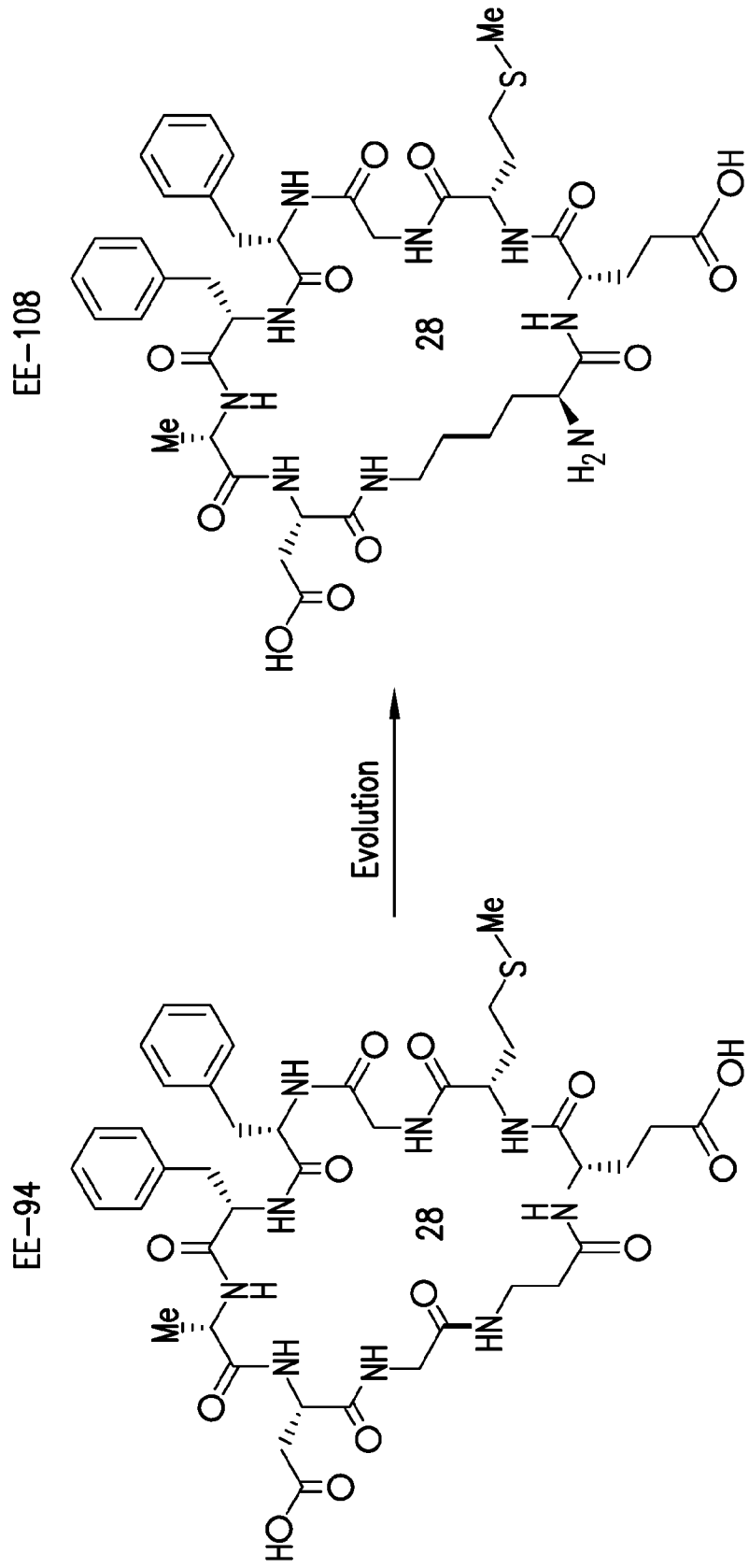


FIG.21

..... ee-108 200 μm 2% dmso 15 μm eif4e 3.nitc Corrected Heat Rate (μJ/s)
—— ee-108 200 μm 2% dmso 15 μm eif4e 3.nitc Fit (kJ/mol) • ee-108 200 μm 2% dmso 15 μm eif4e 3.nitc Enthalpy (kJ/mol)

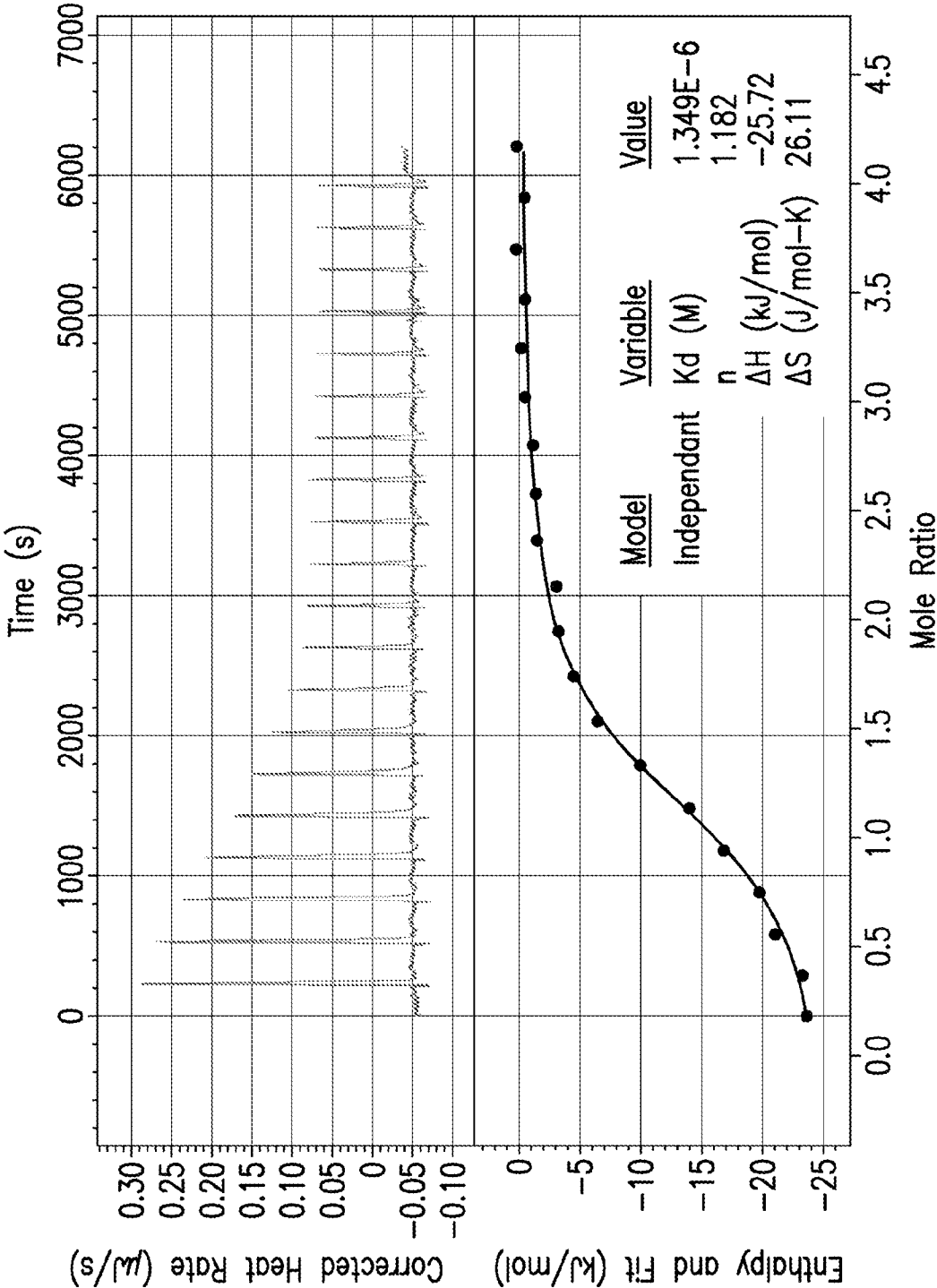


FIG.22

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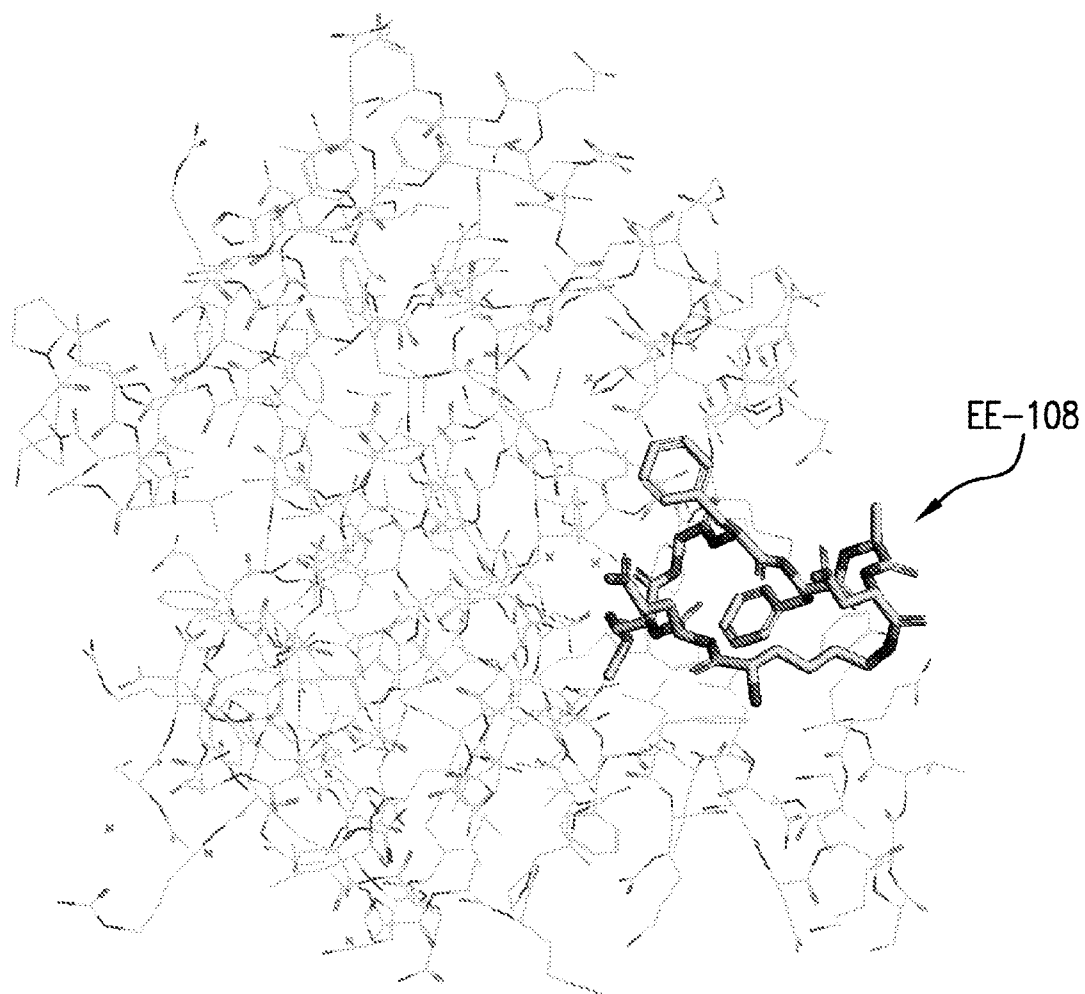


FIG.23

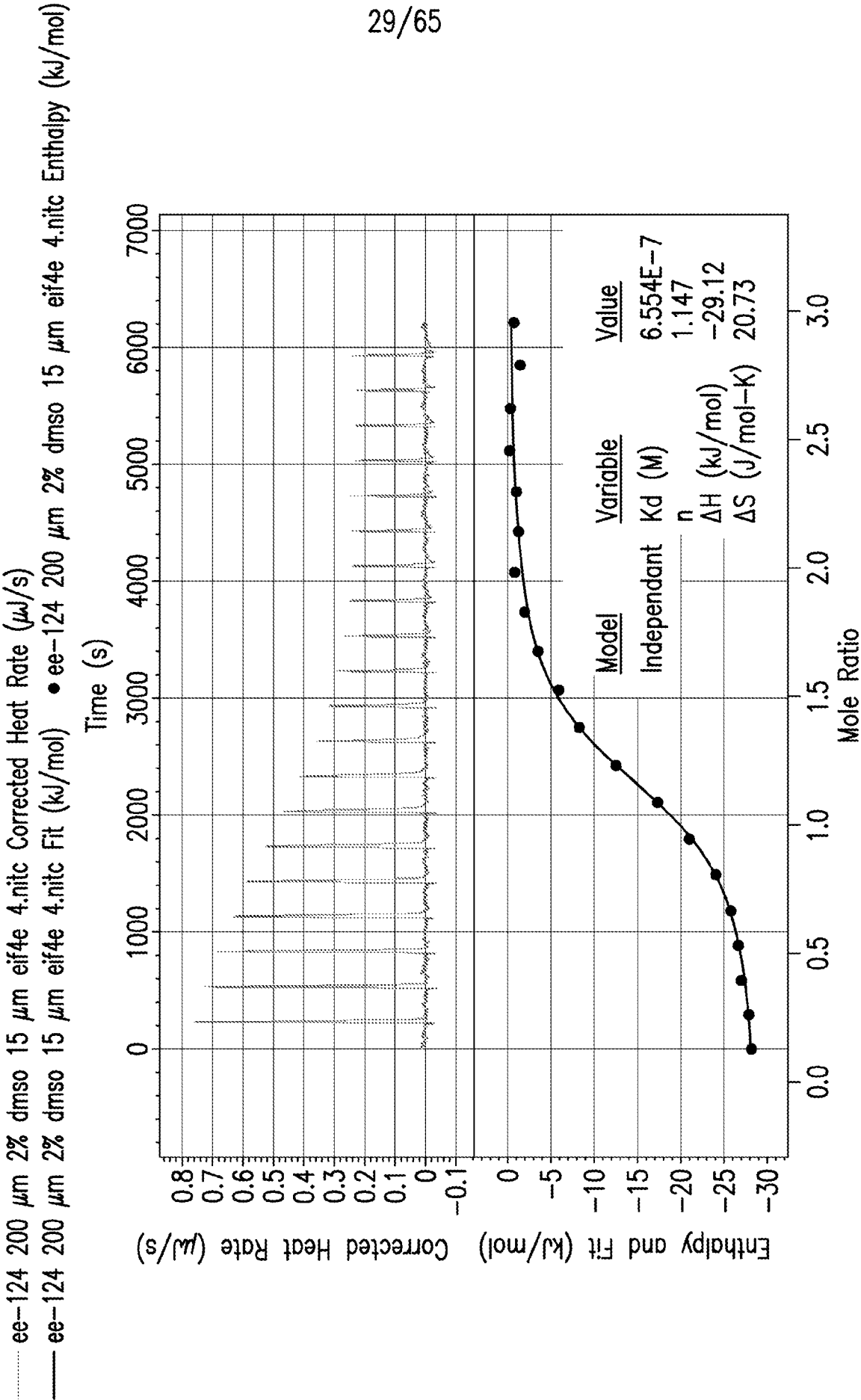


FIG.24

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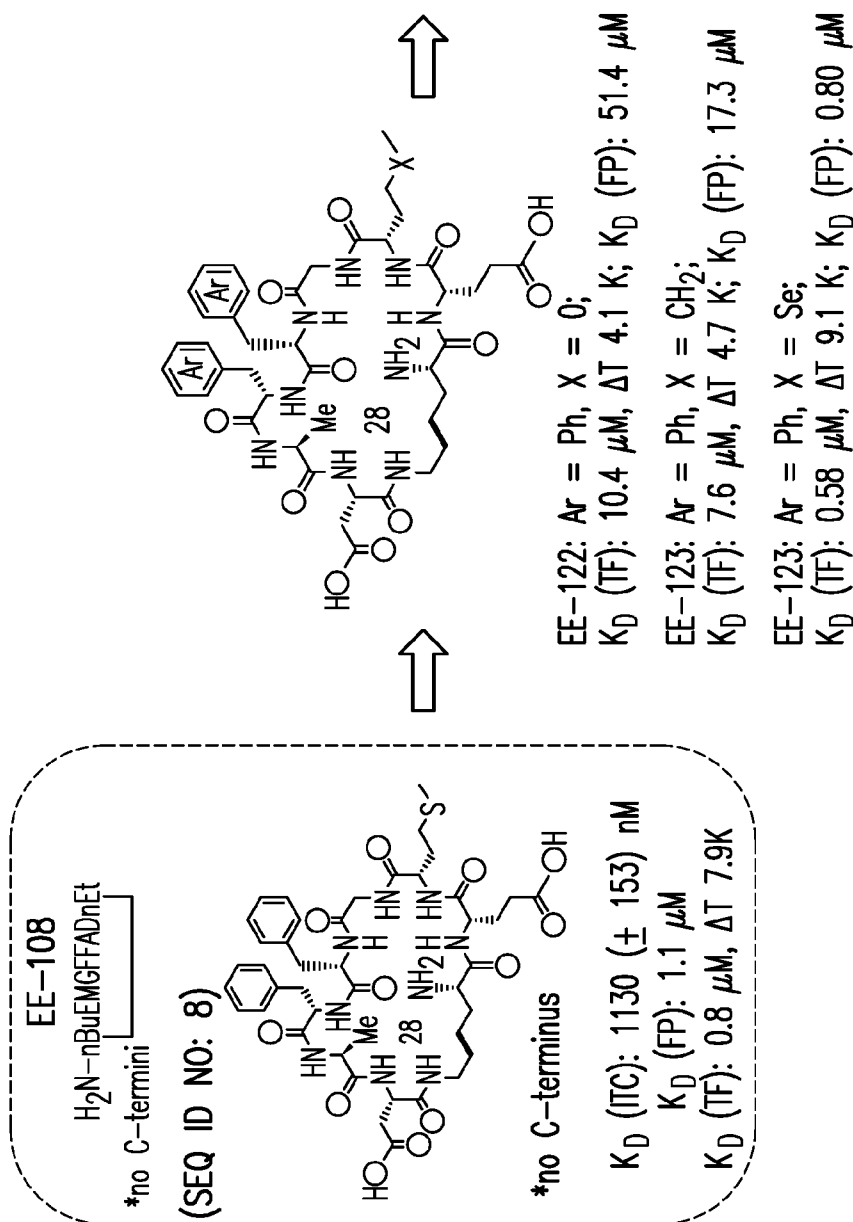
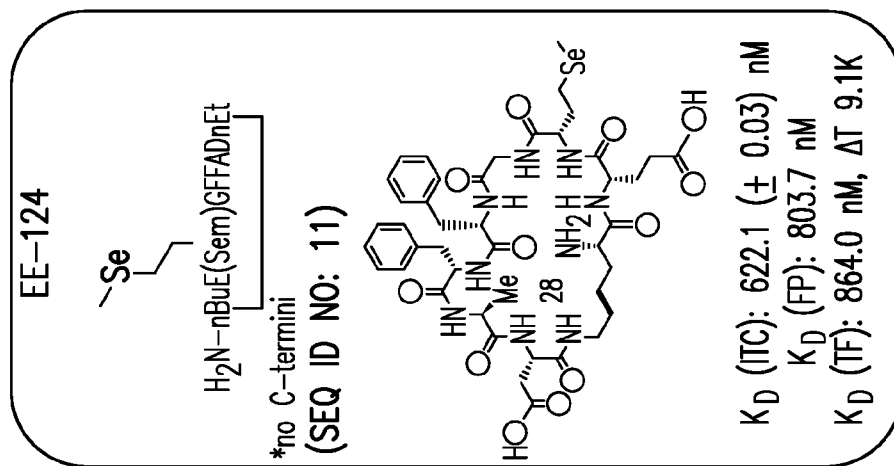


FIG.25

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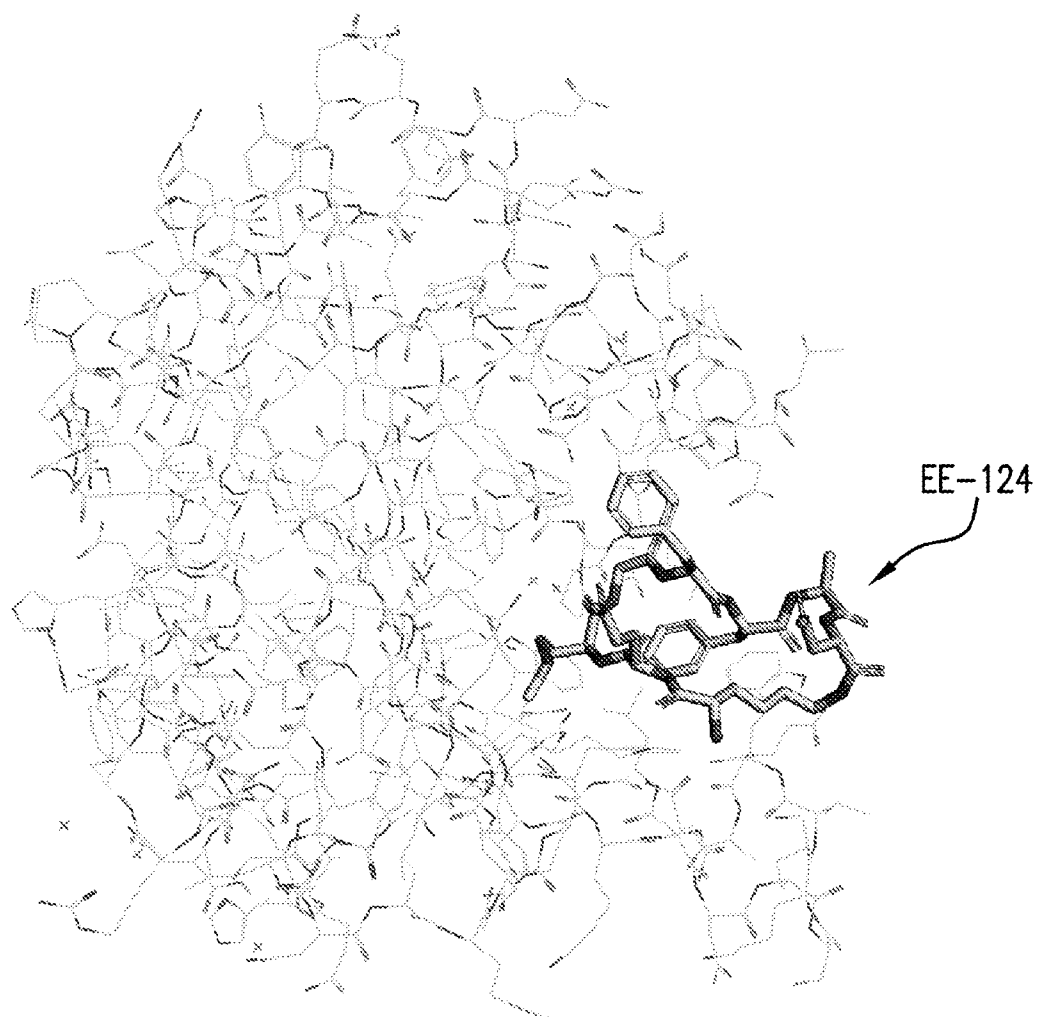


FIG.26

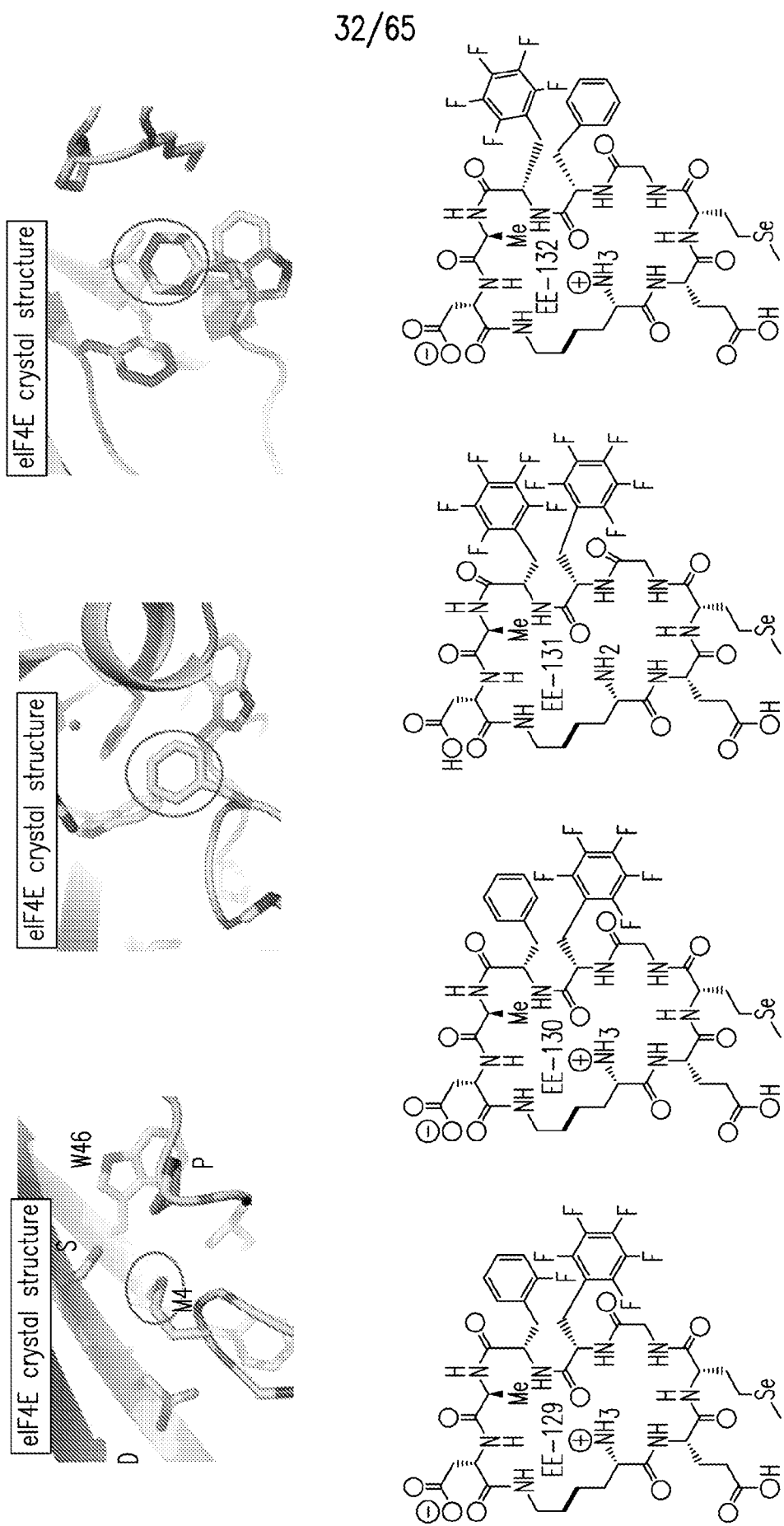


FIG.27

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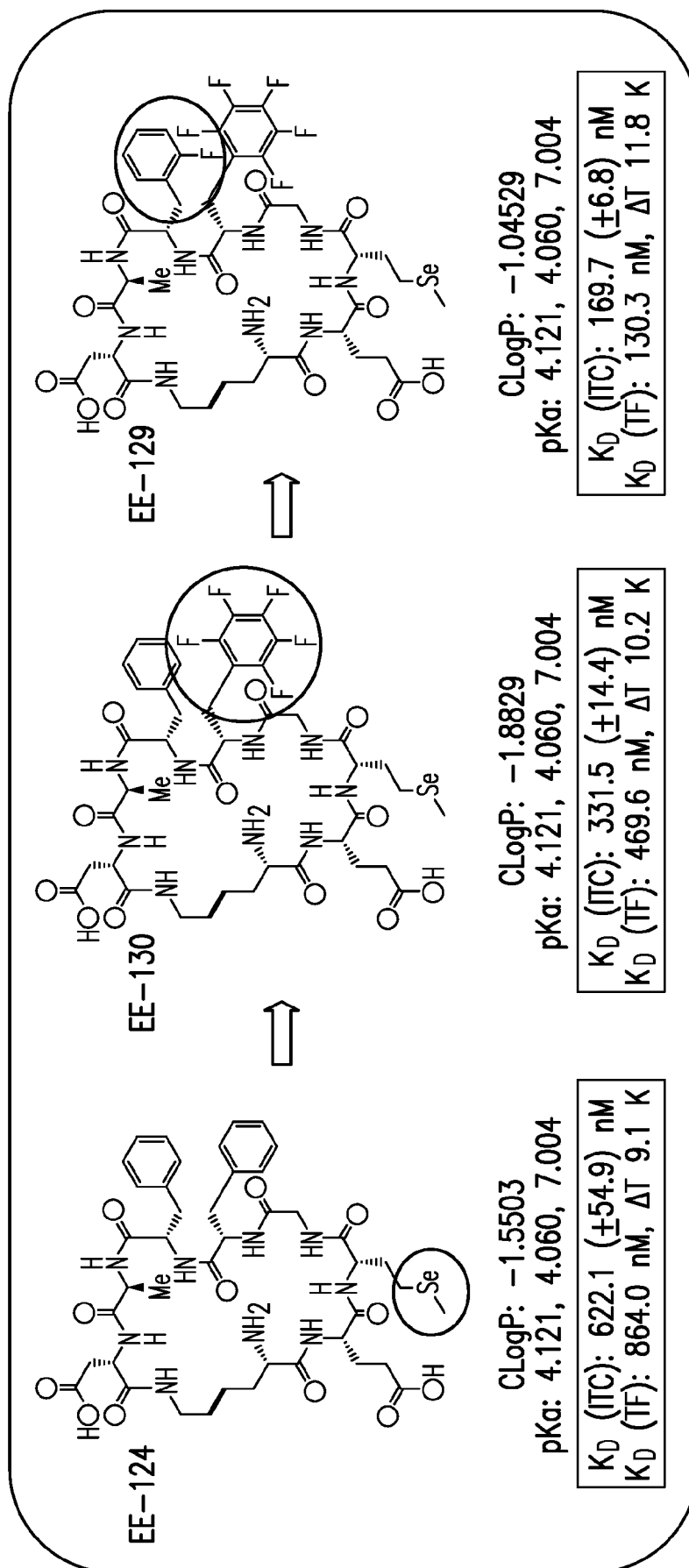
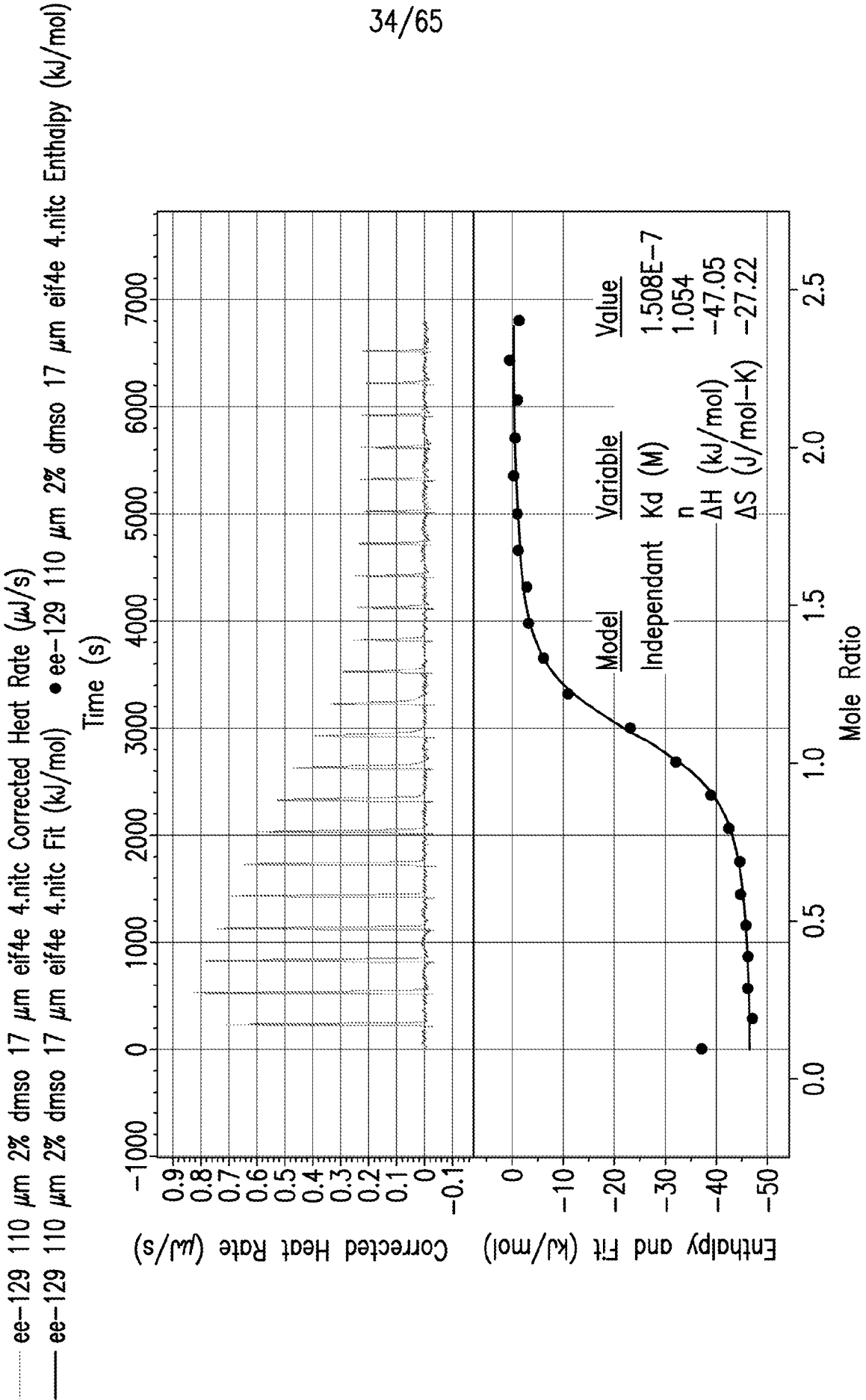


FIG. 28



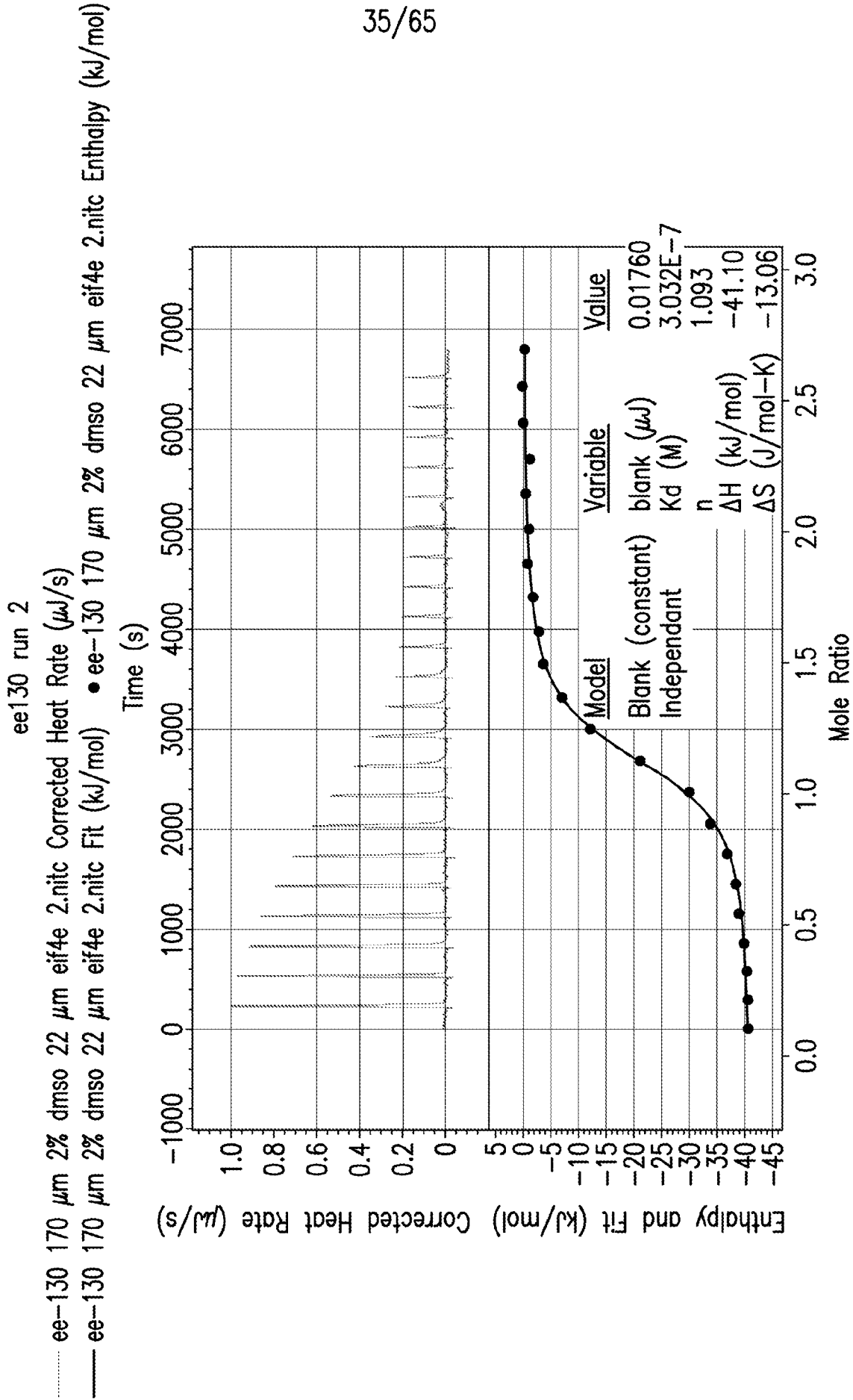


FIG.30

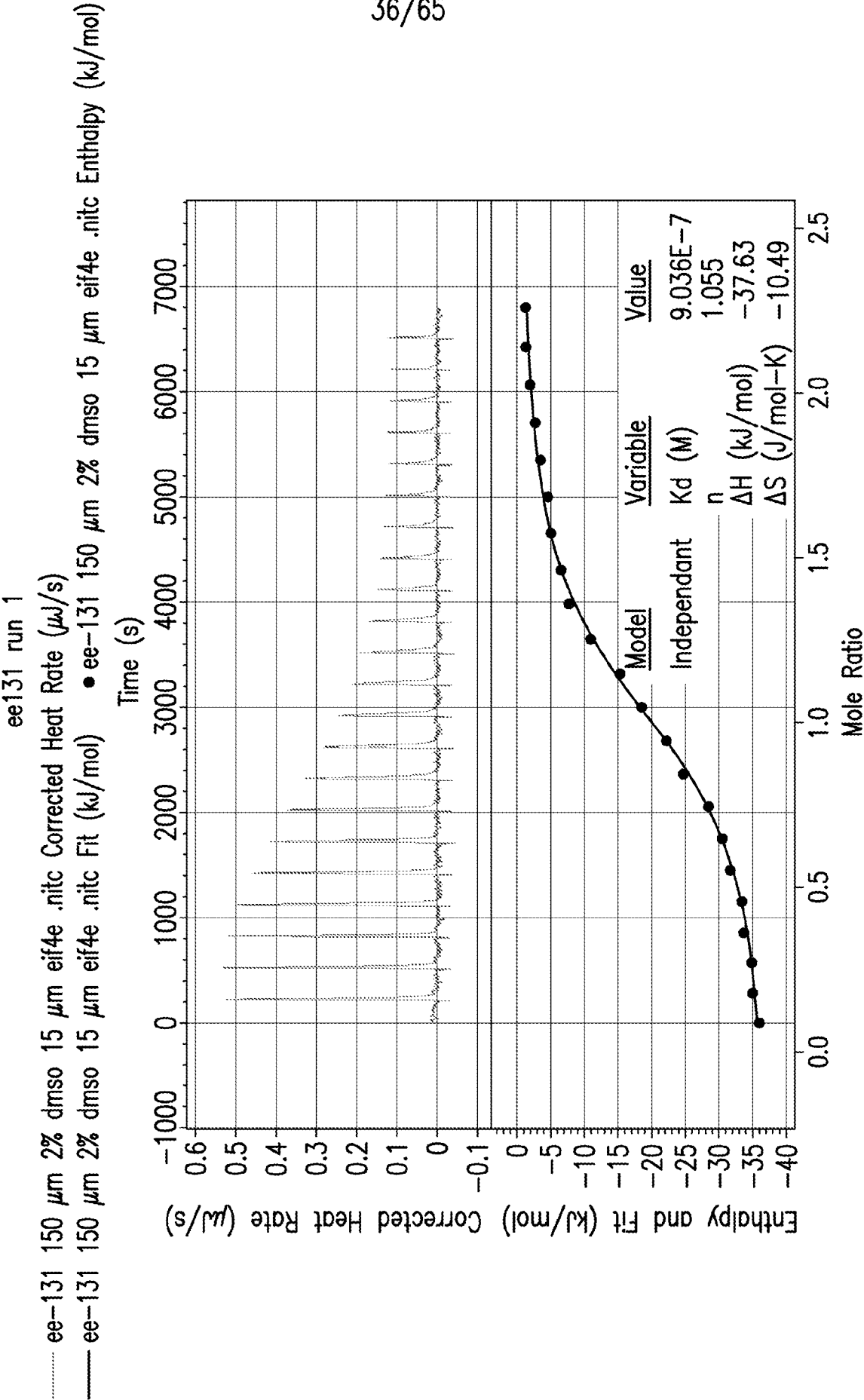


FIG.31

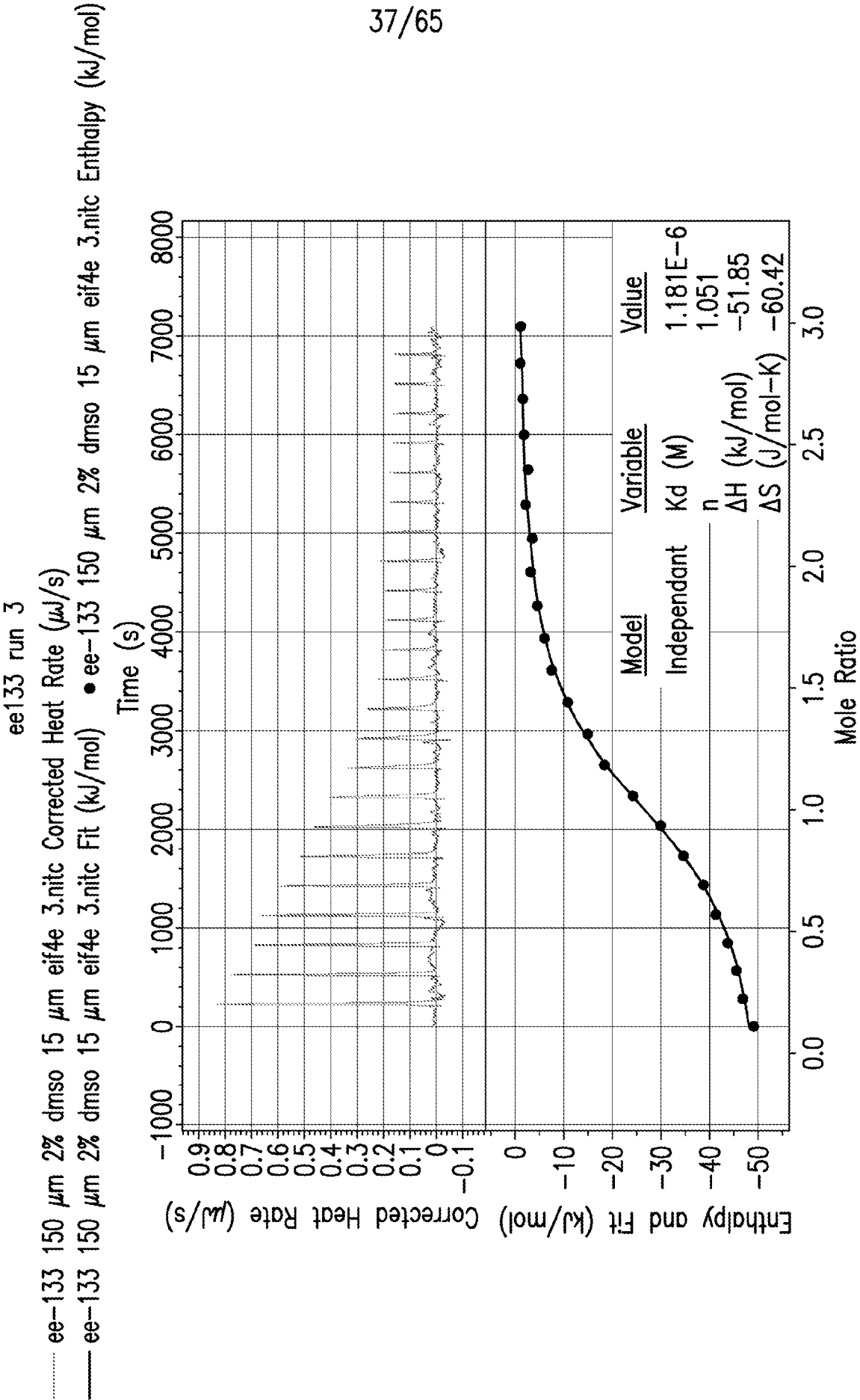


FIG.32

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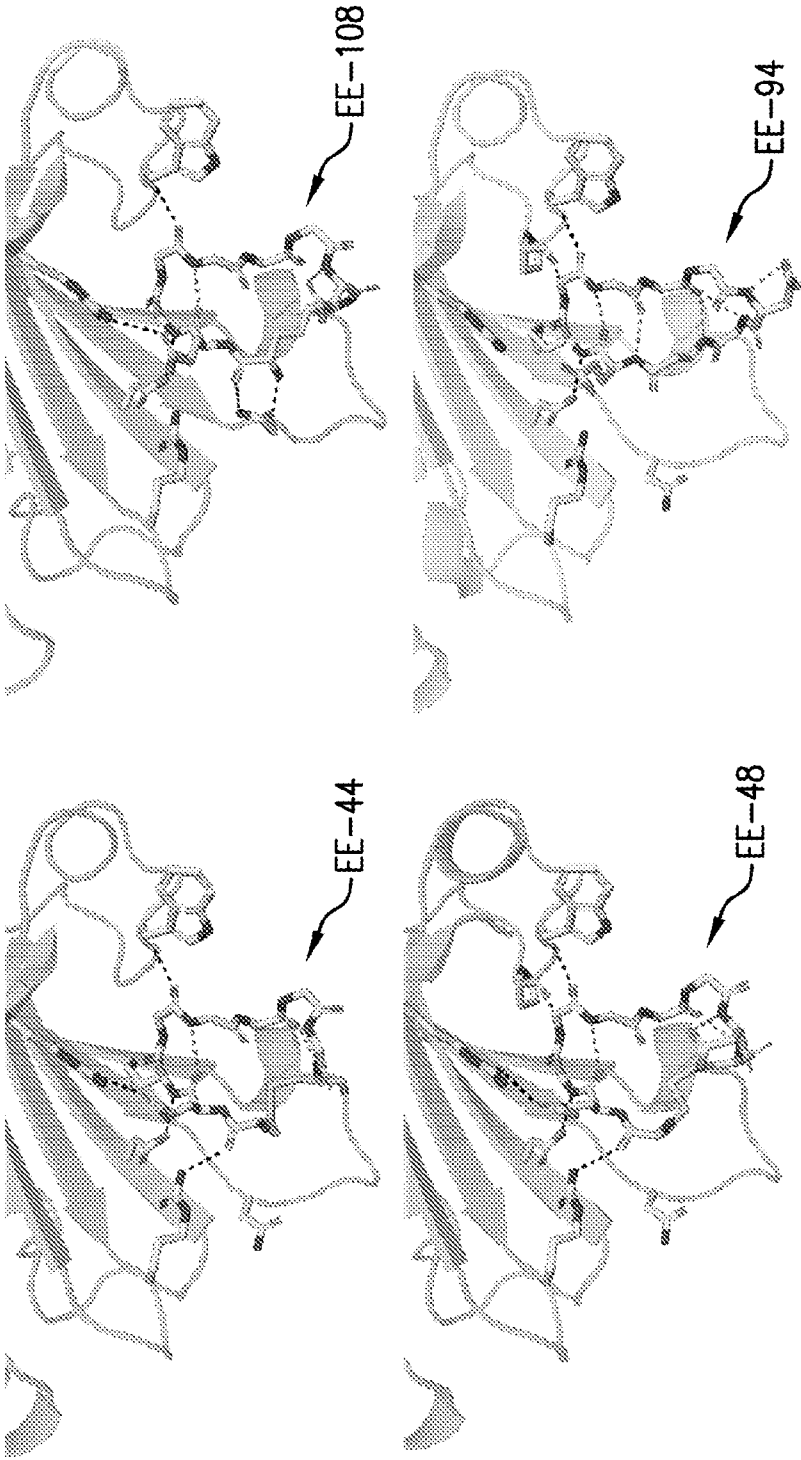
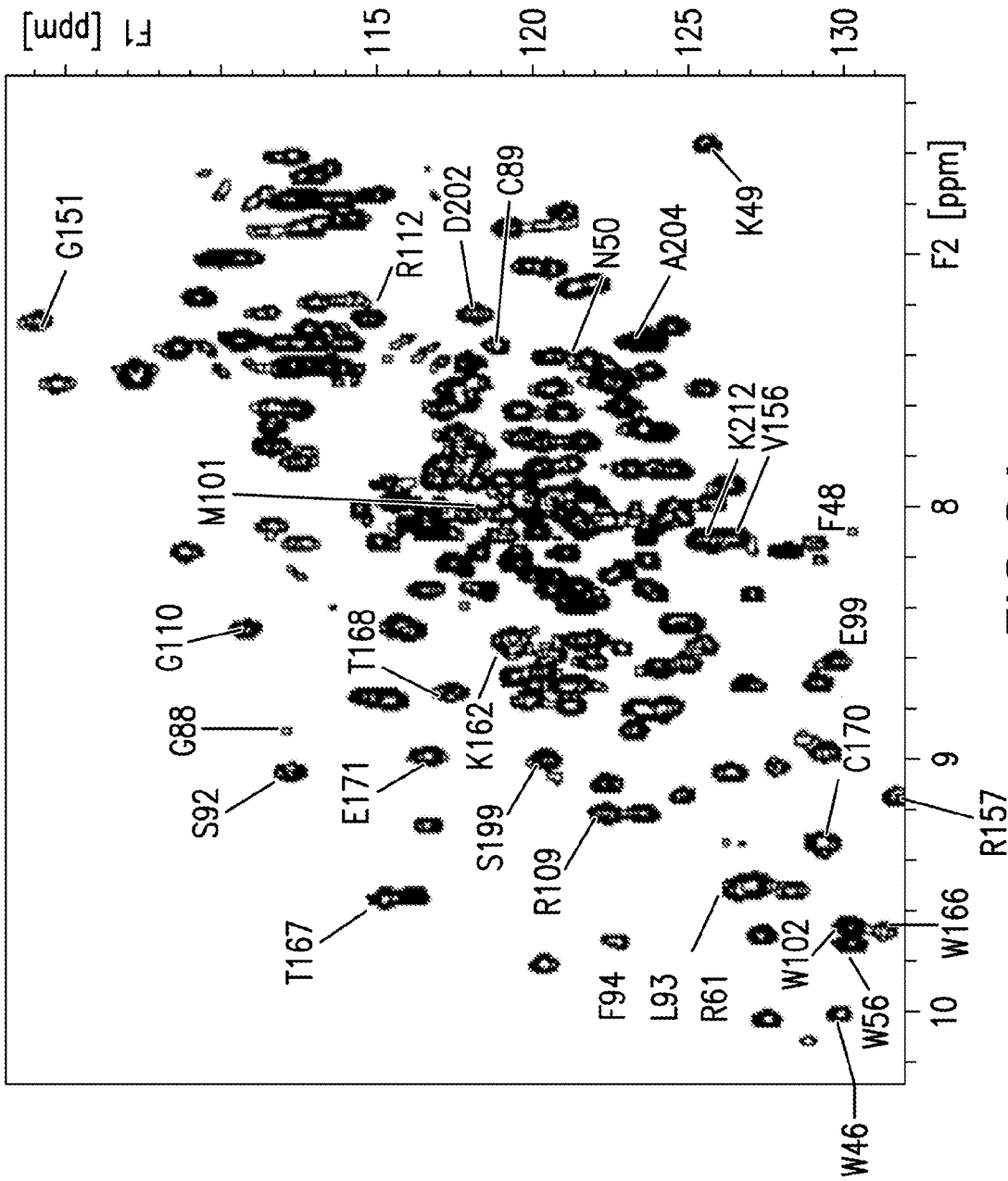


FIG.33

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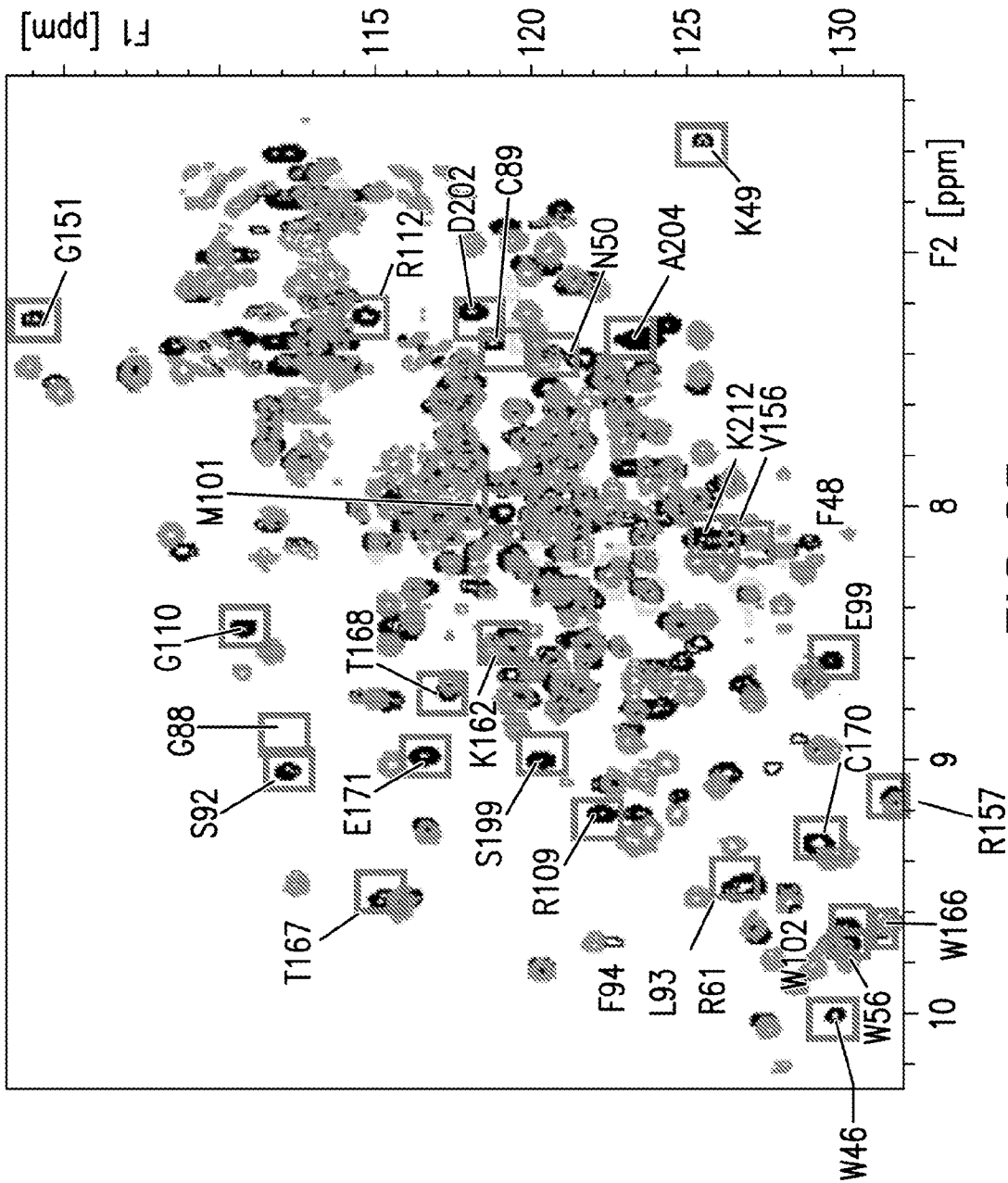


FIG. 35

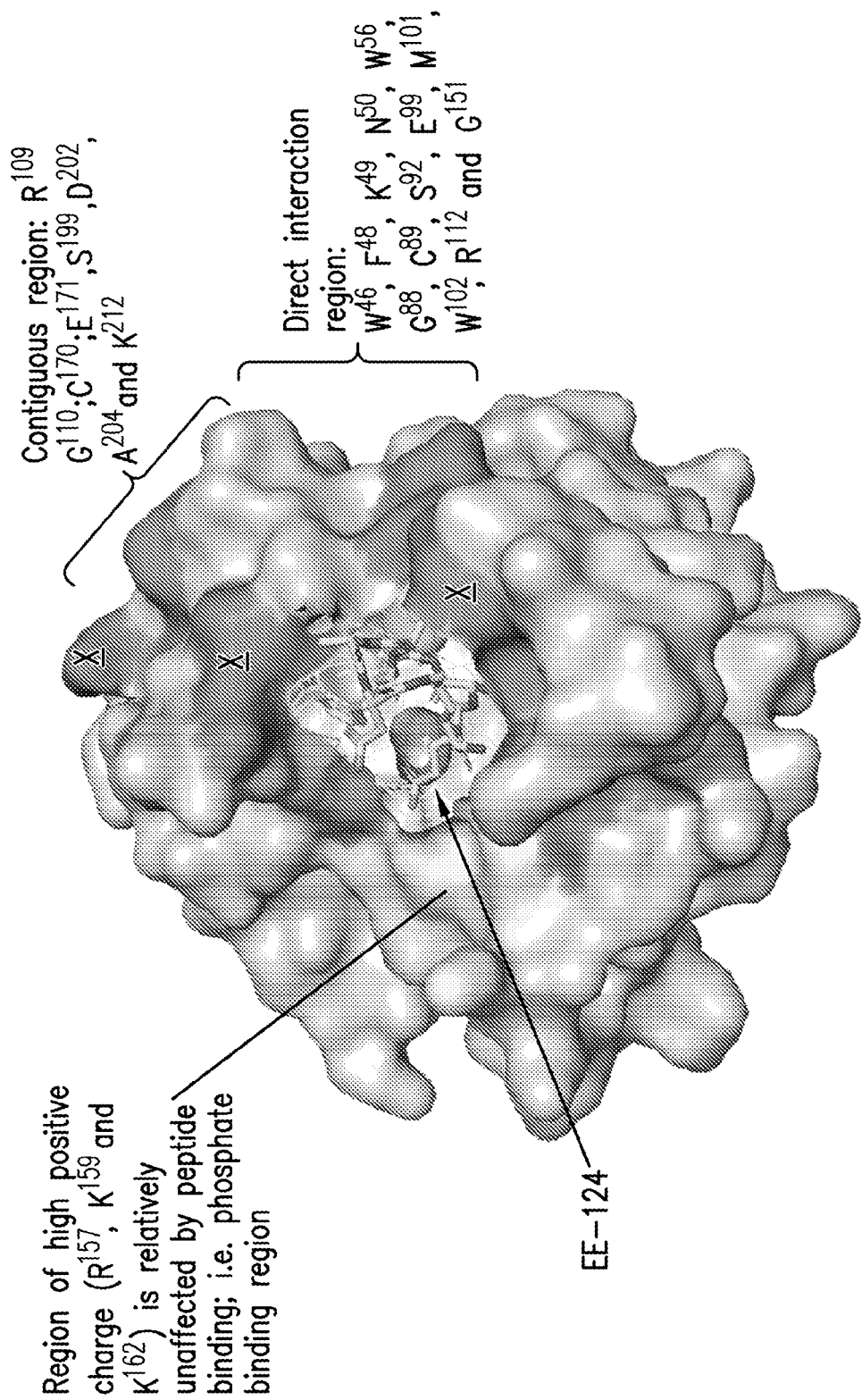
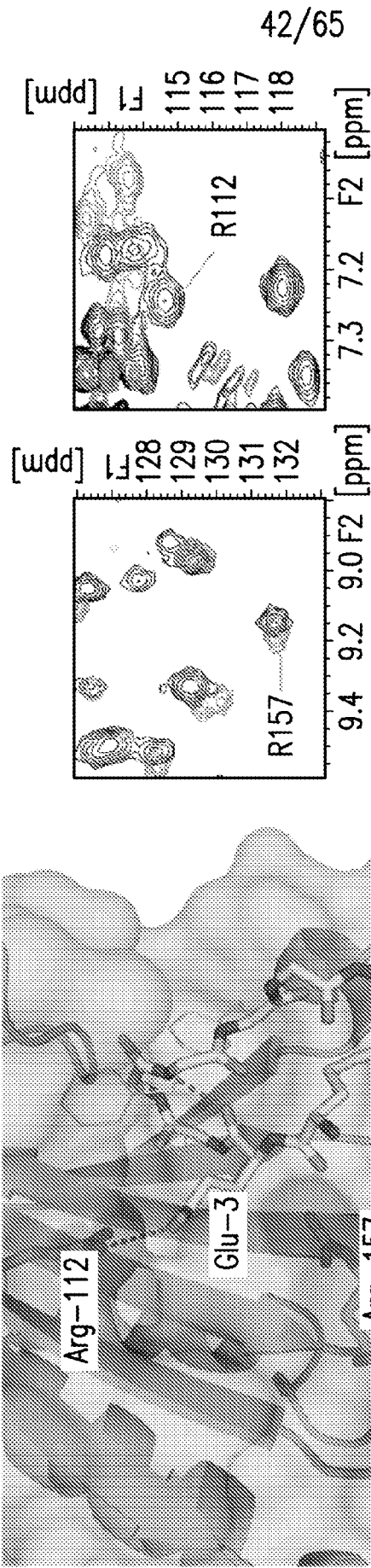


FIG.36



Crystal structures suggest competition between two hydrogen bonds (E³ on EE-124 with R¹¹² or R¹⁵⁷)

¹³C, ¹⁵N protein NMR:
R¹¹² is affected by EE-124 binding;
R¹⁵⁷ is relatively unaffected

FIG.37

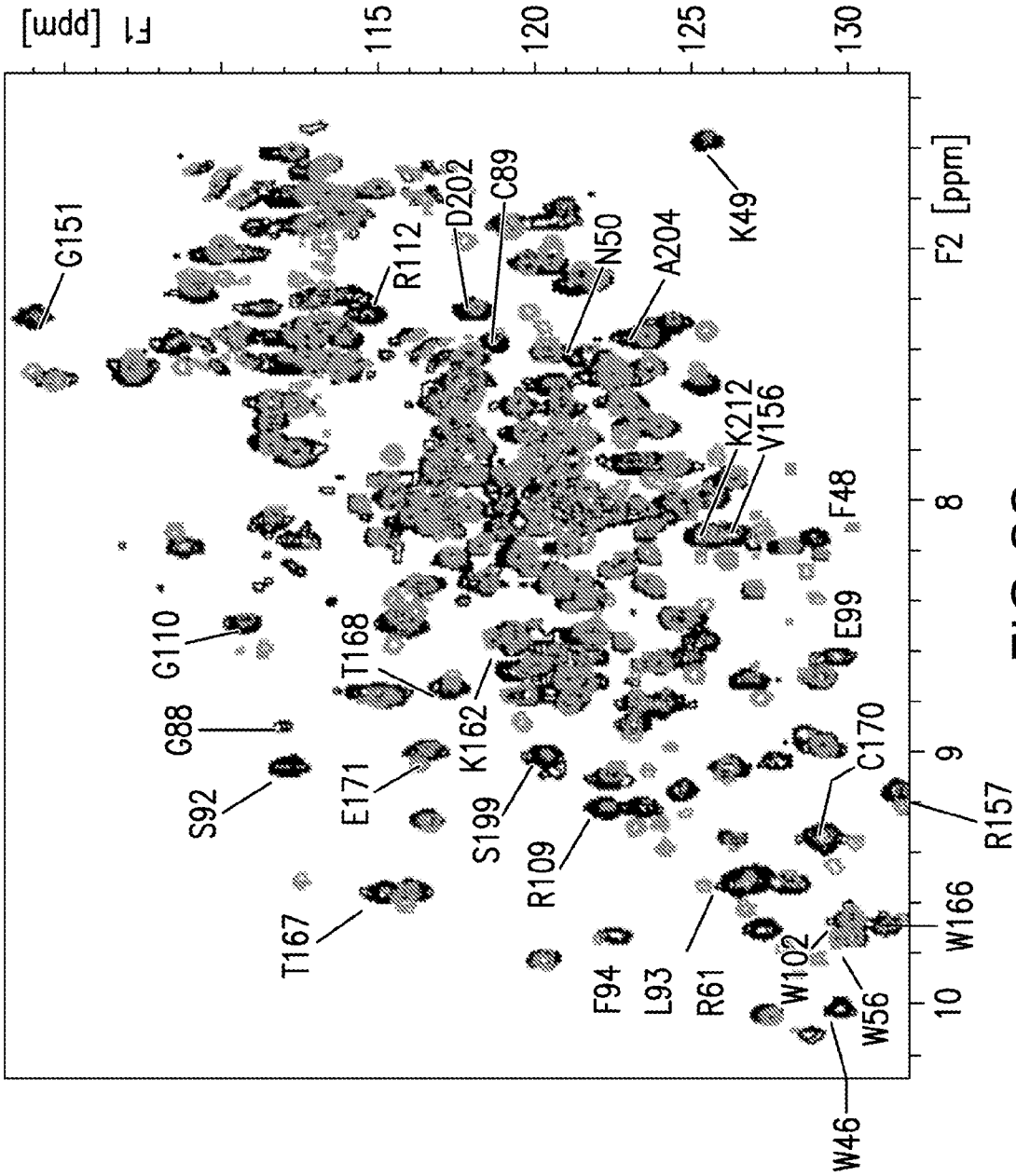


FIG.38

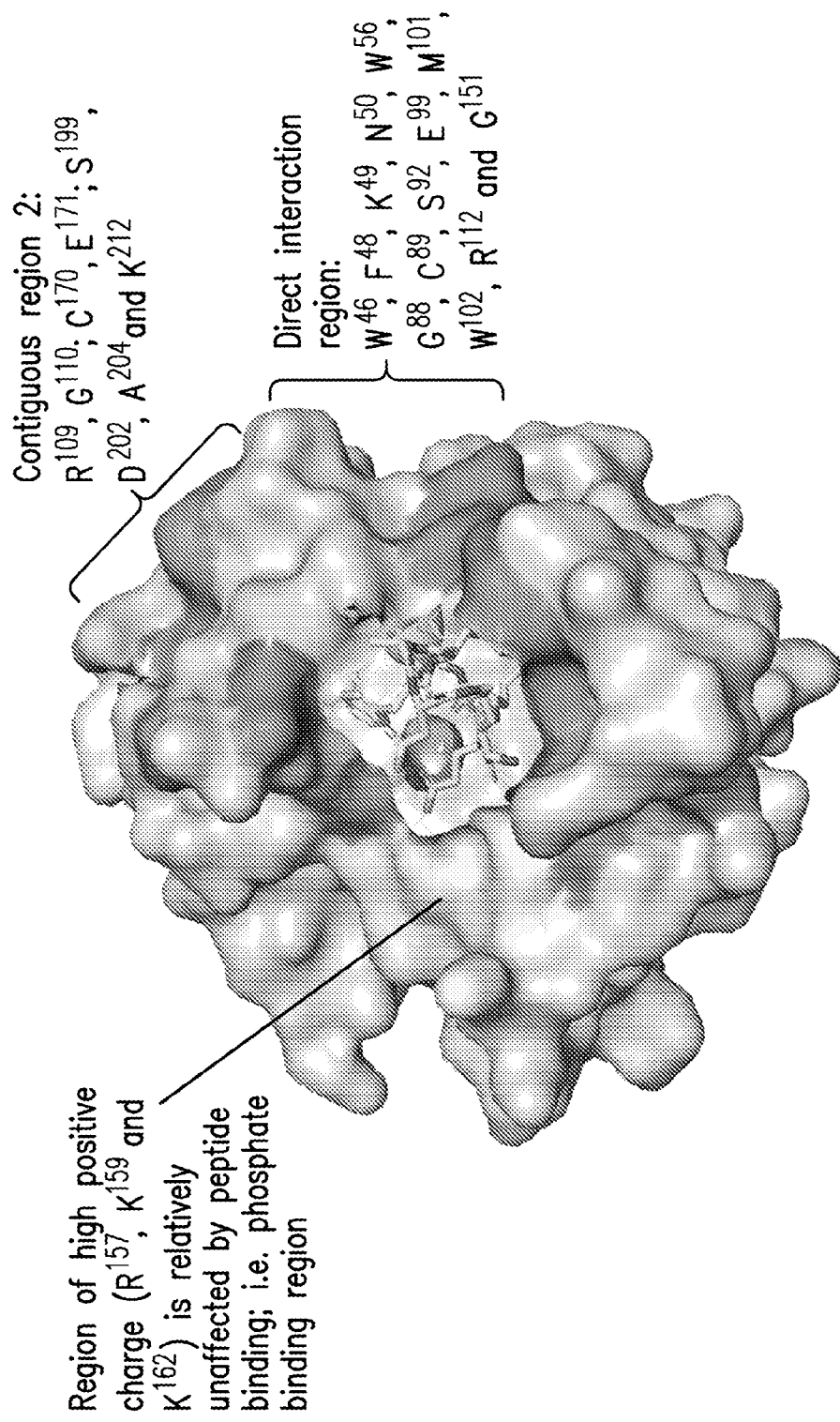


FIG.39

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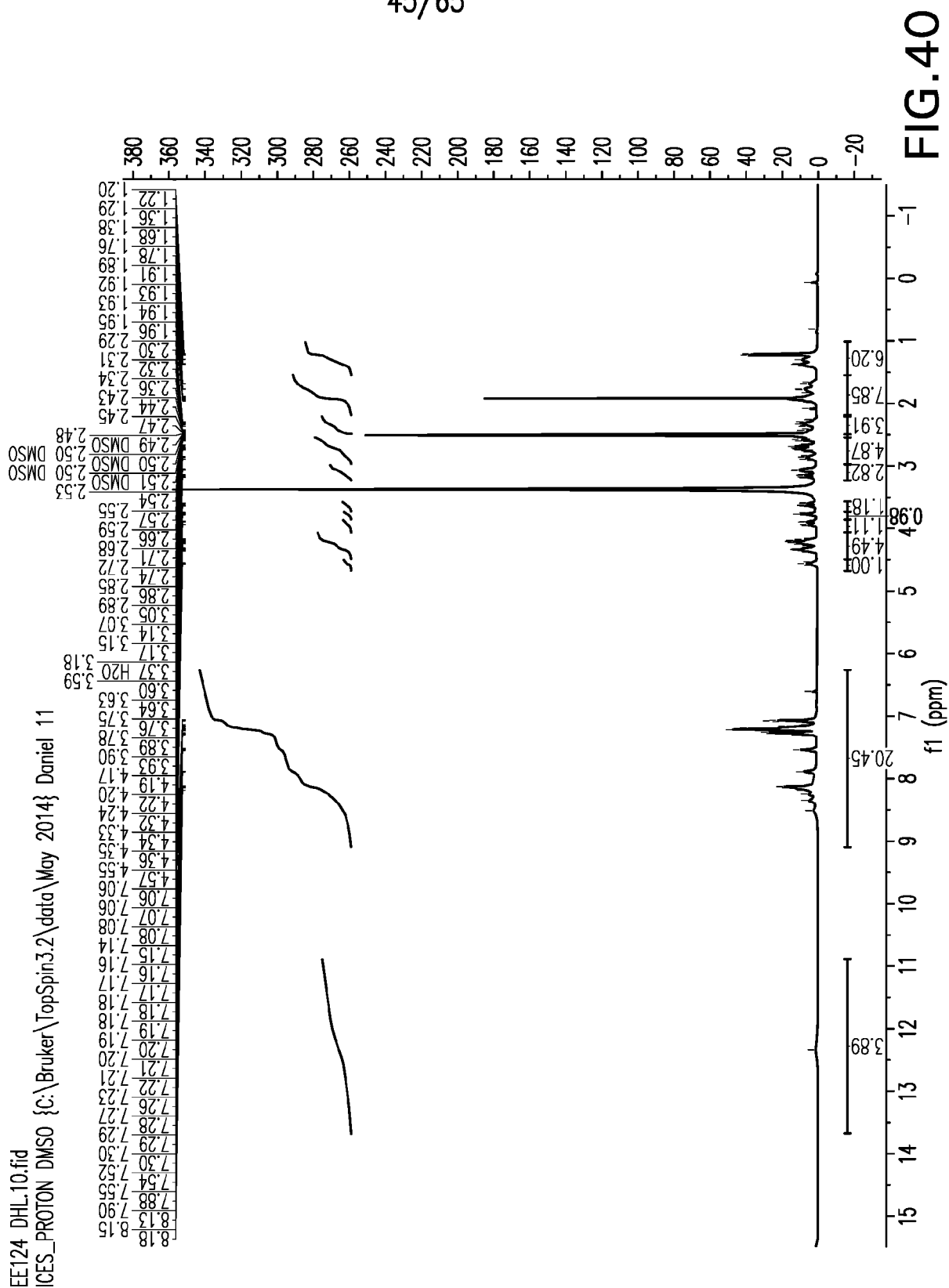


FIG.40

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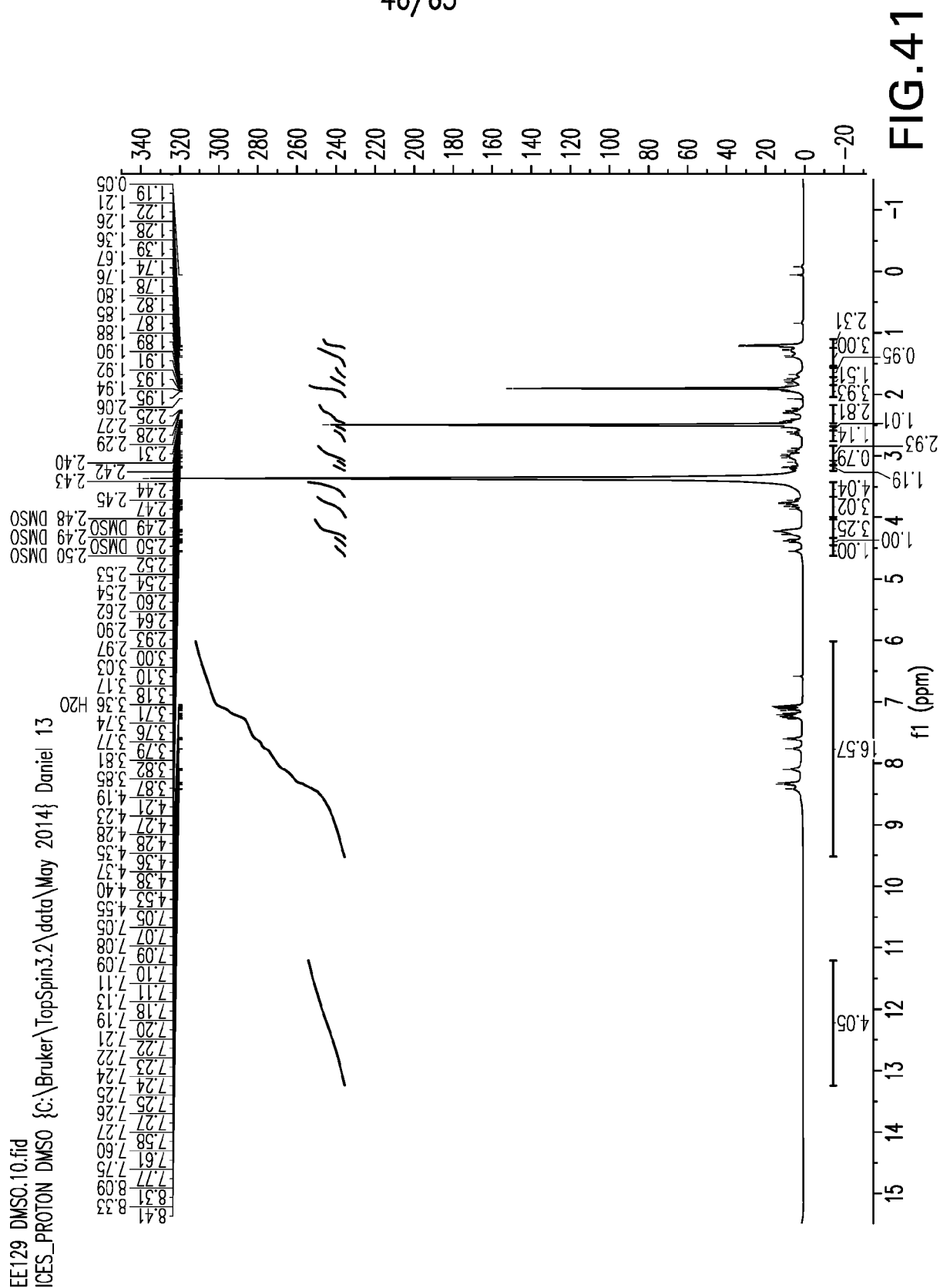
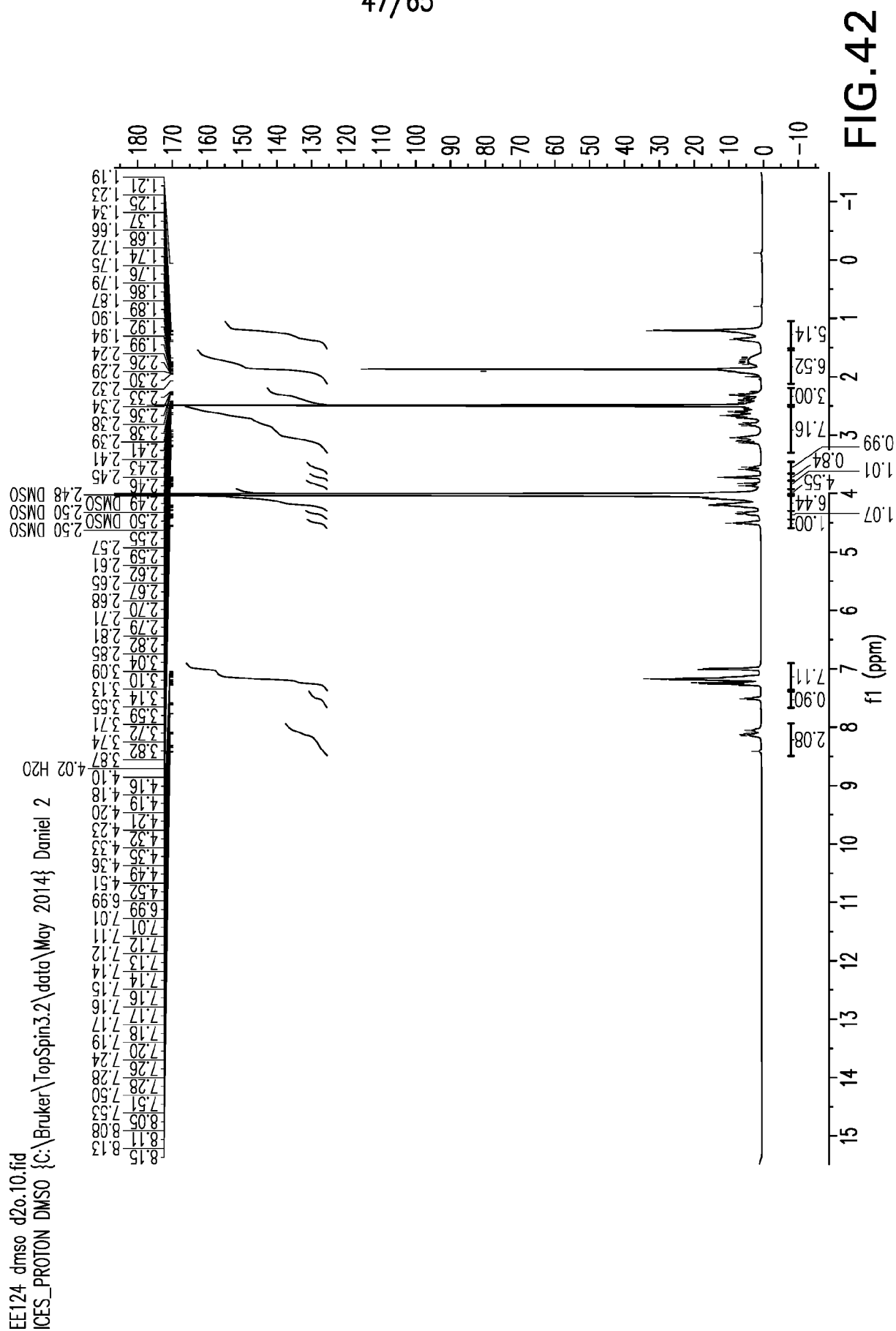
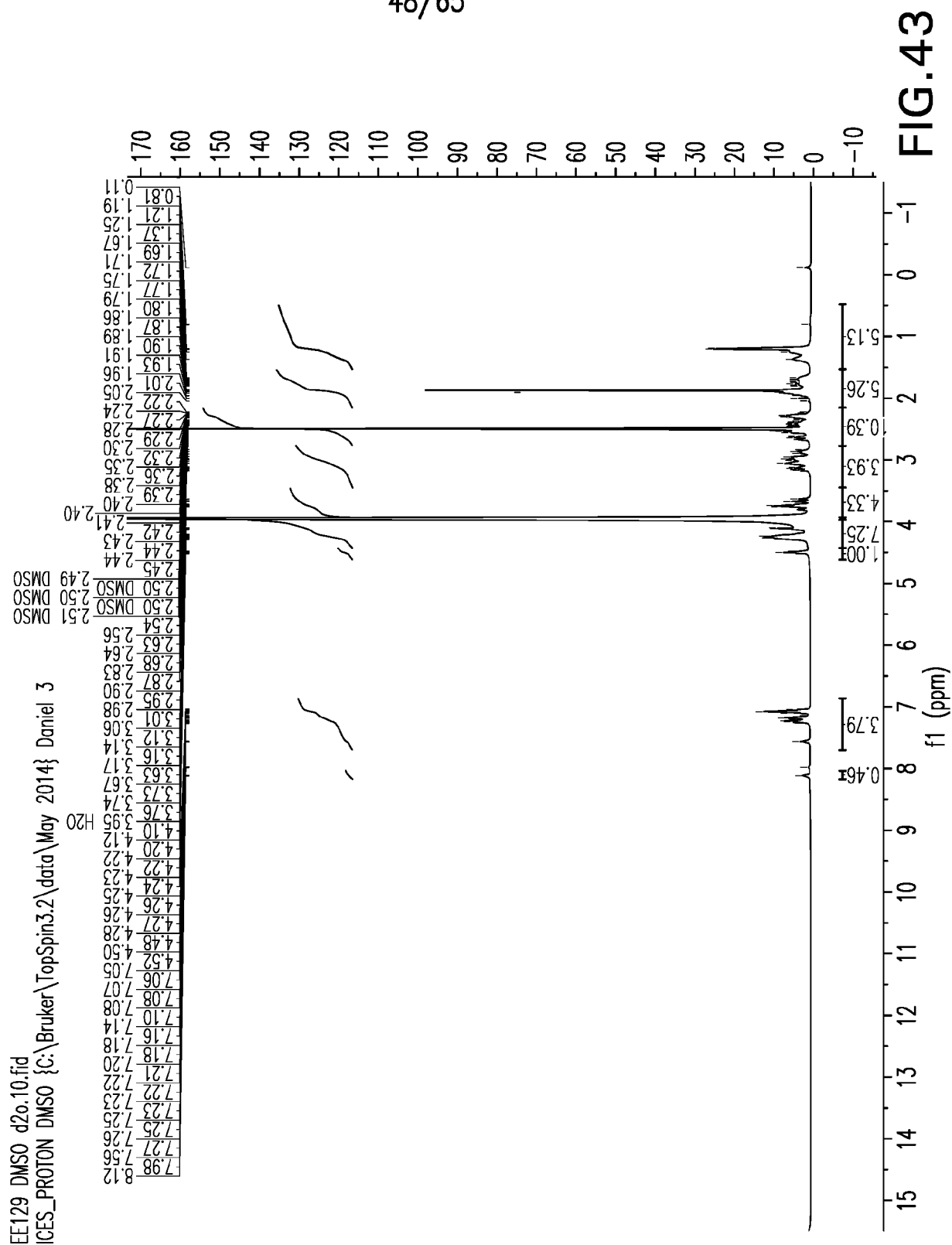


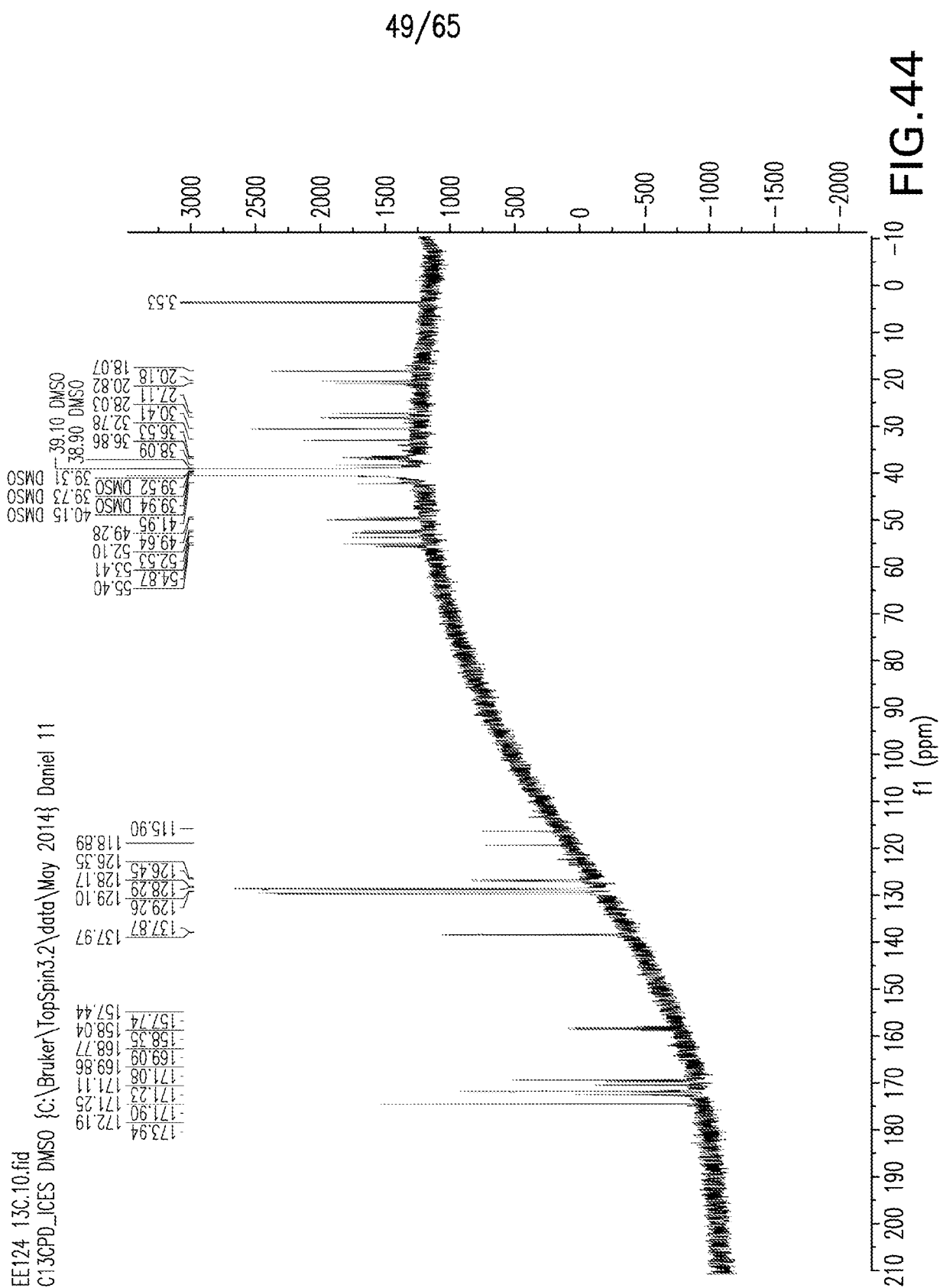
FIG. 41

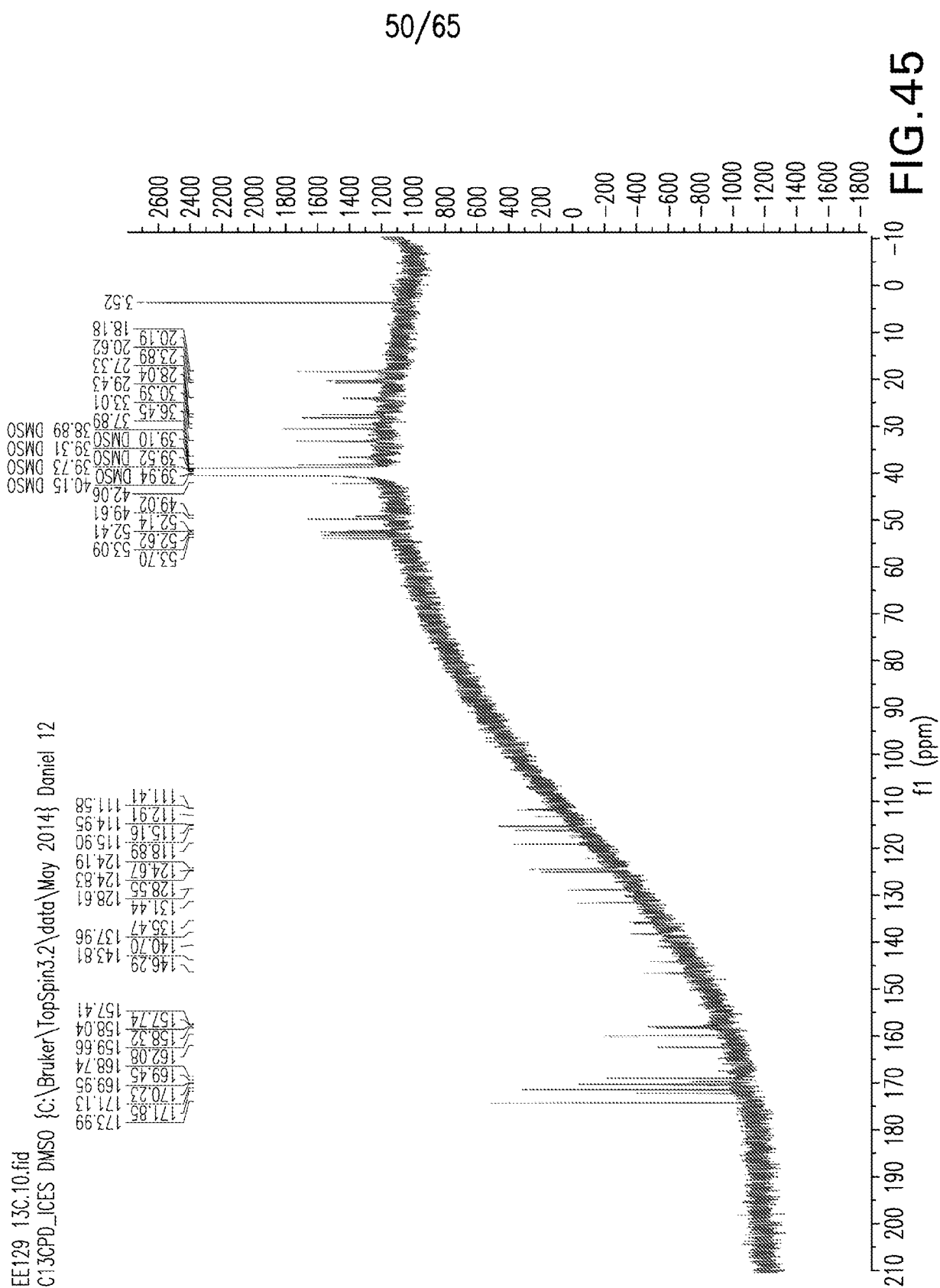
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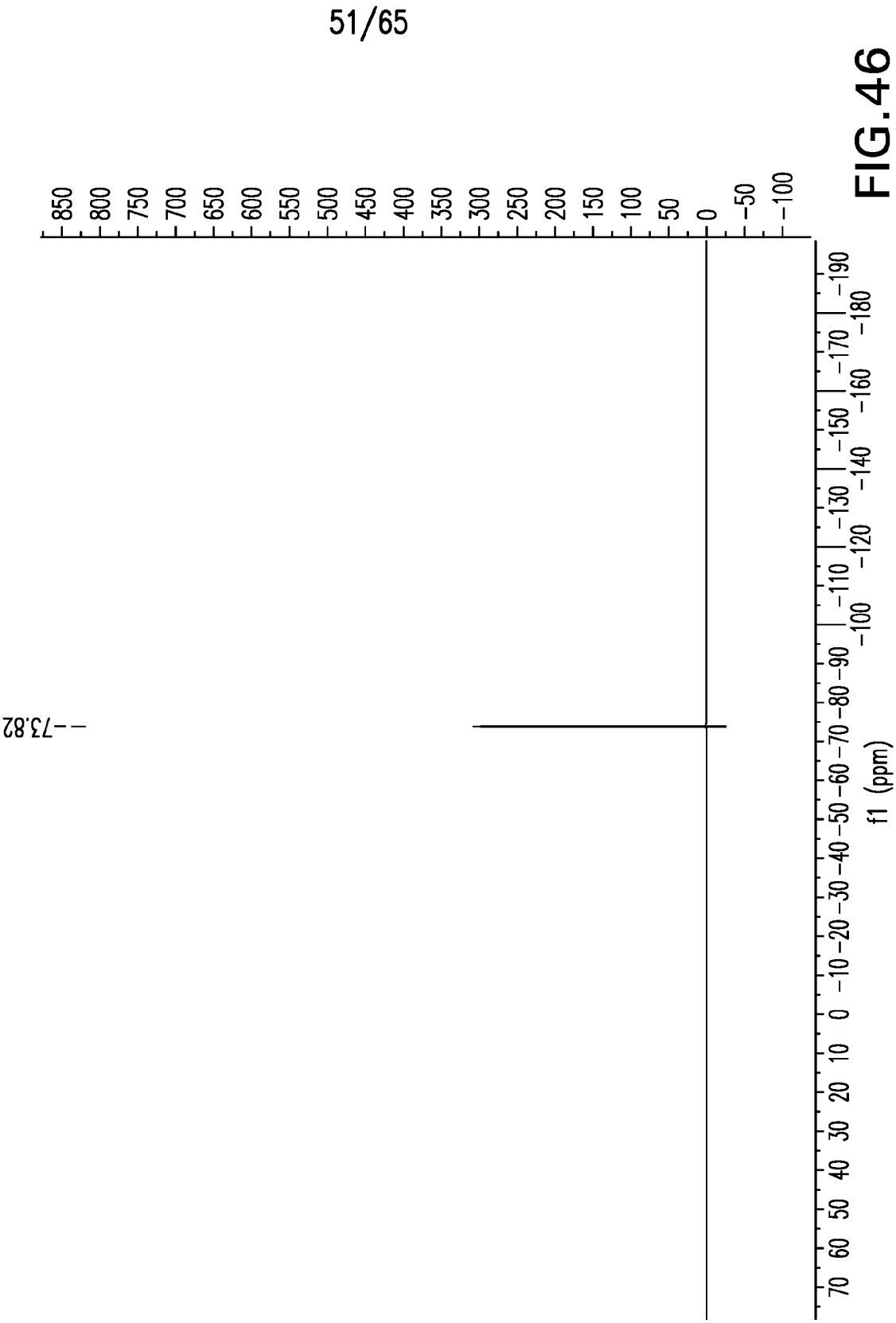
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ee124.1.fid
bruker_19F_1D_1Hdecoupled DMSO {C:\Bruker\TOPSPIN.2\data\medchem\Yong Yao\ medchem 43



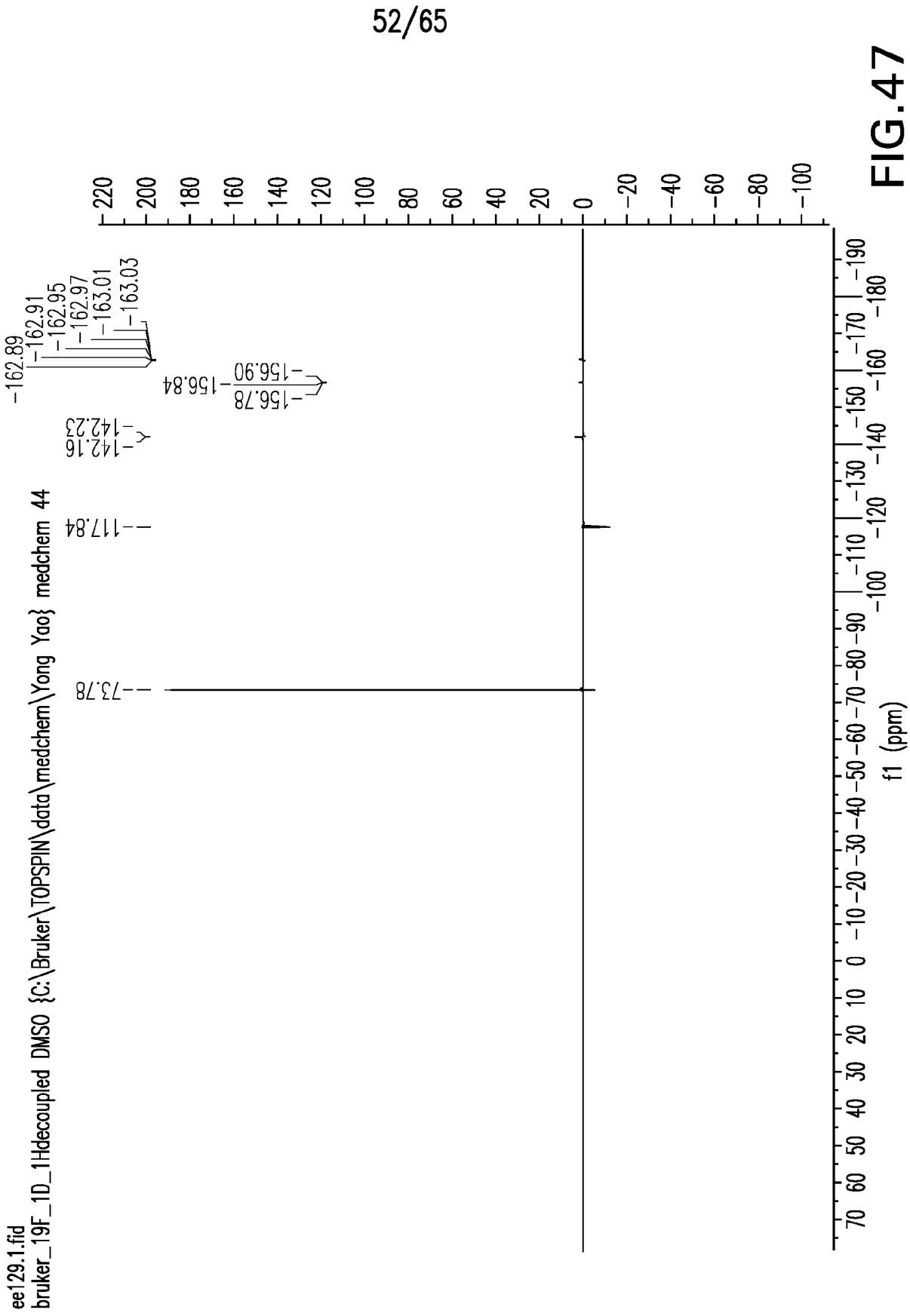


FIG.47

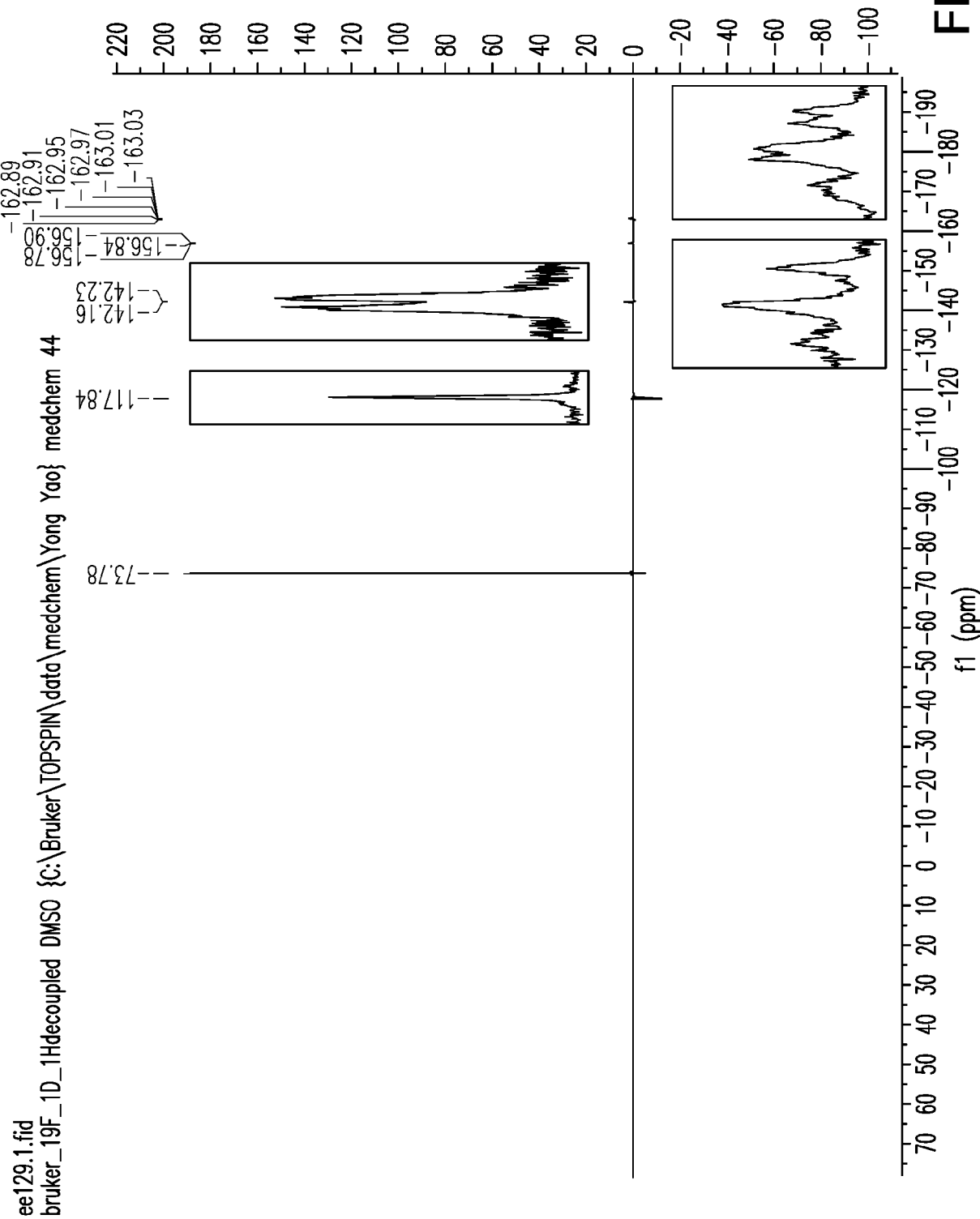
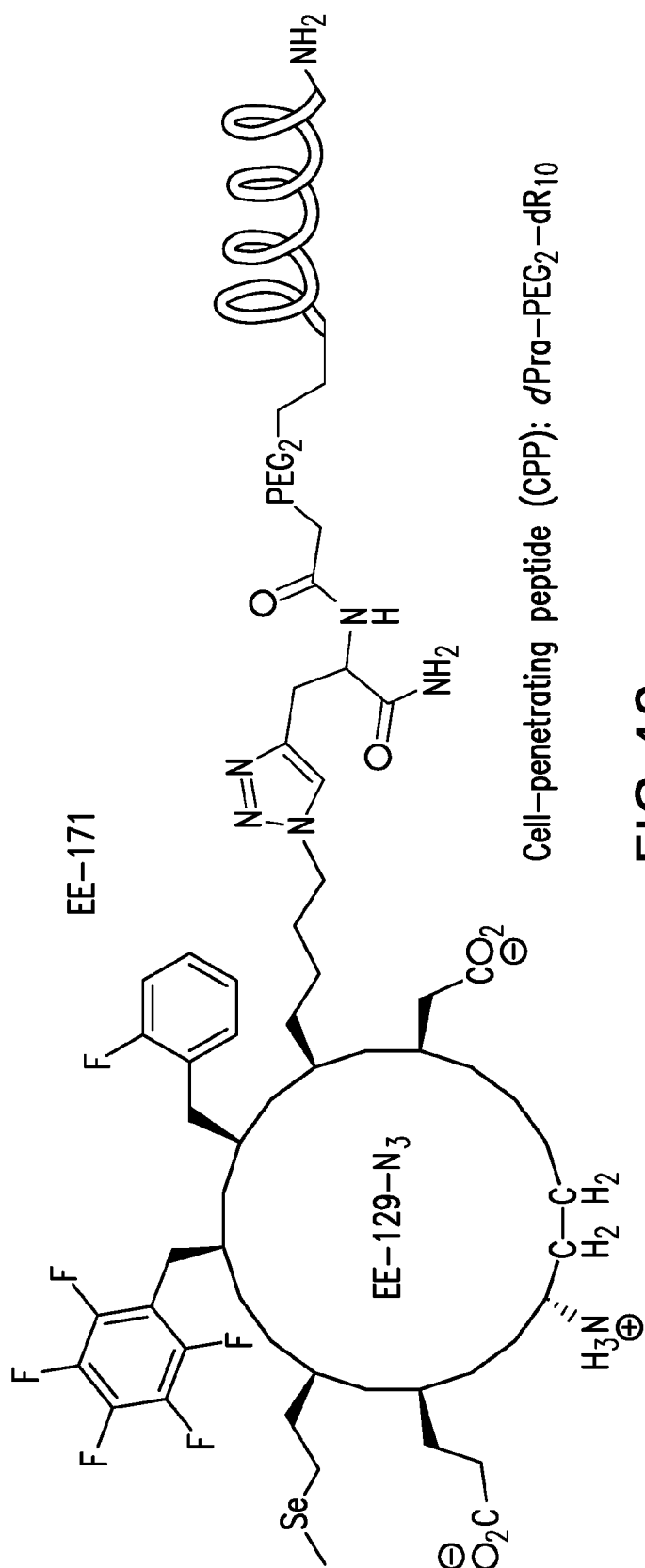


FIG.48

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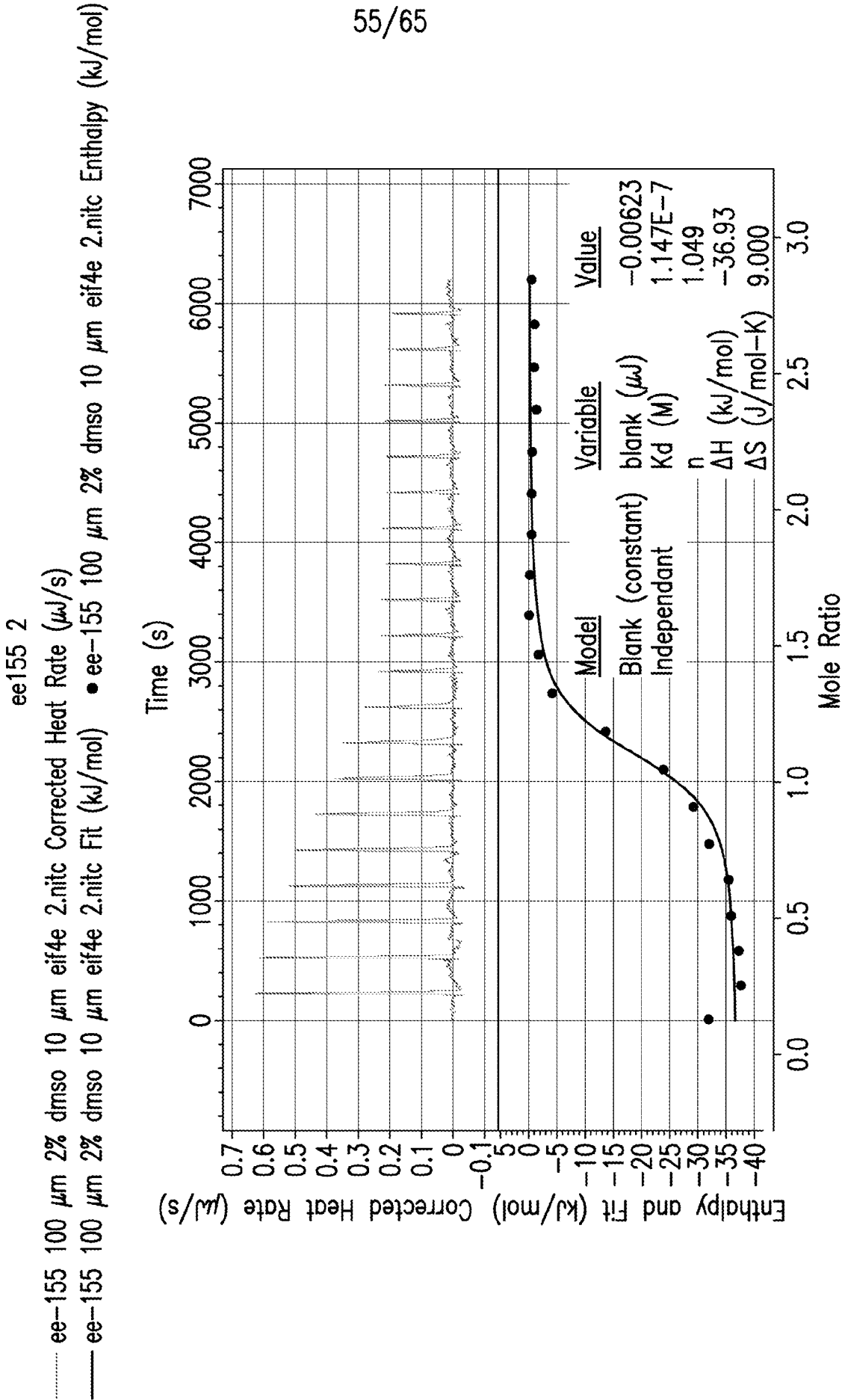


FIG.50

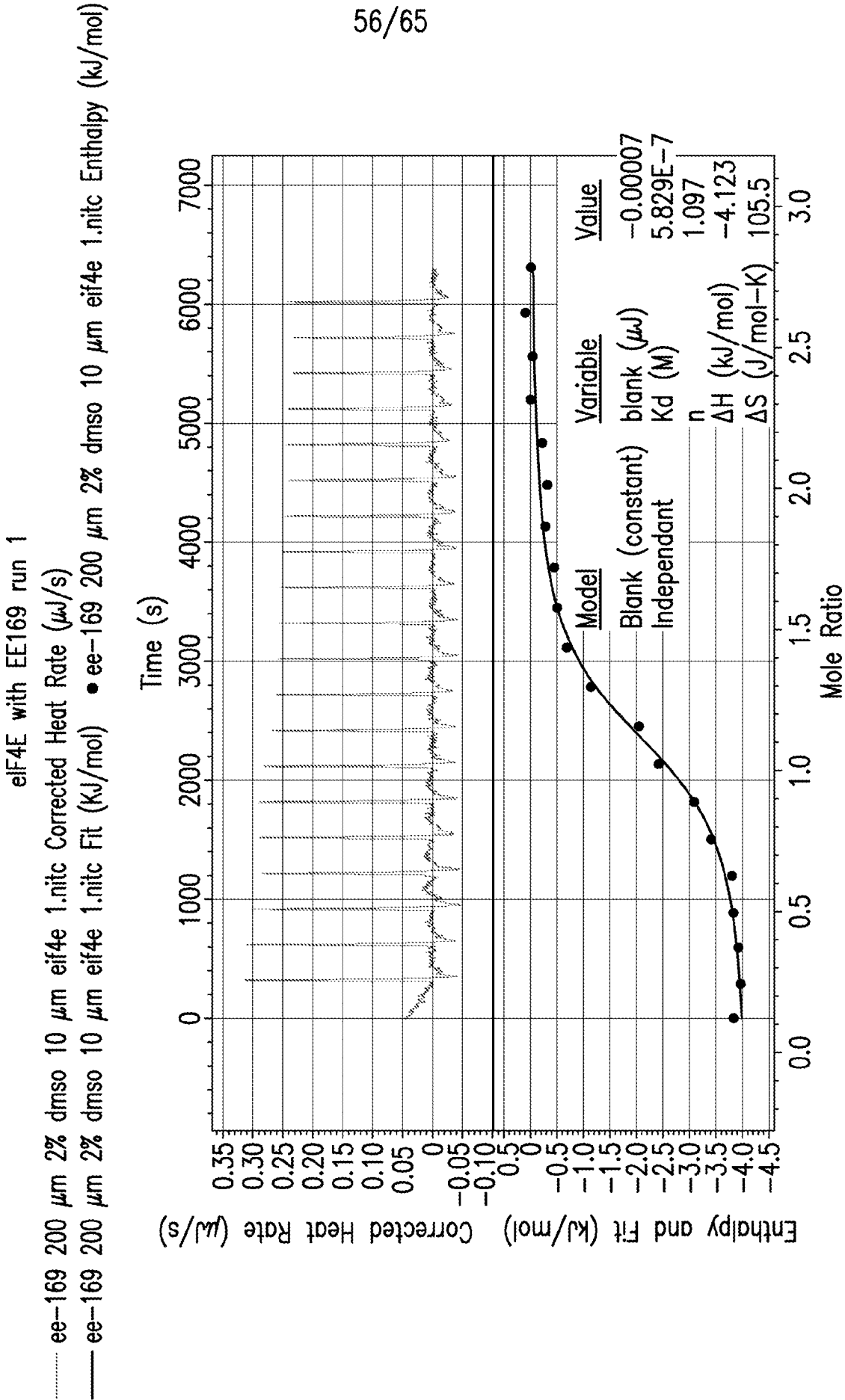


FIG.51

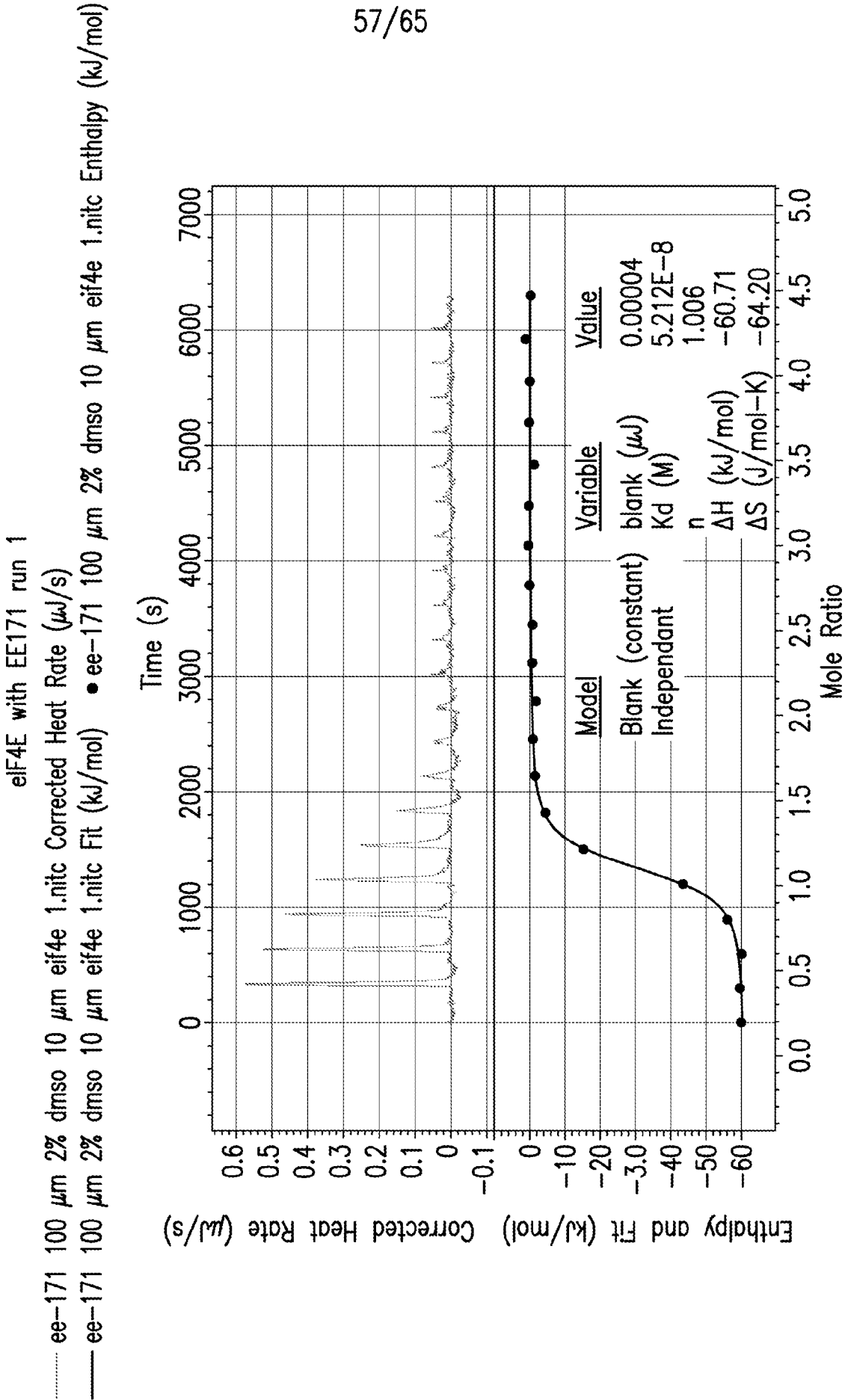


FIG.52

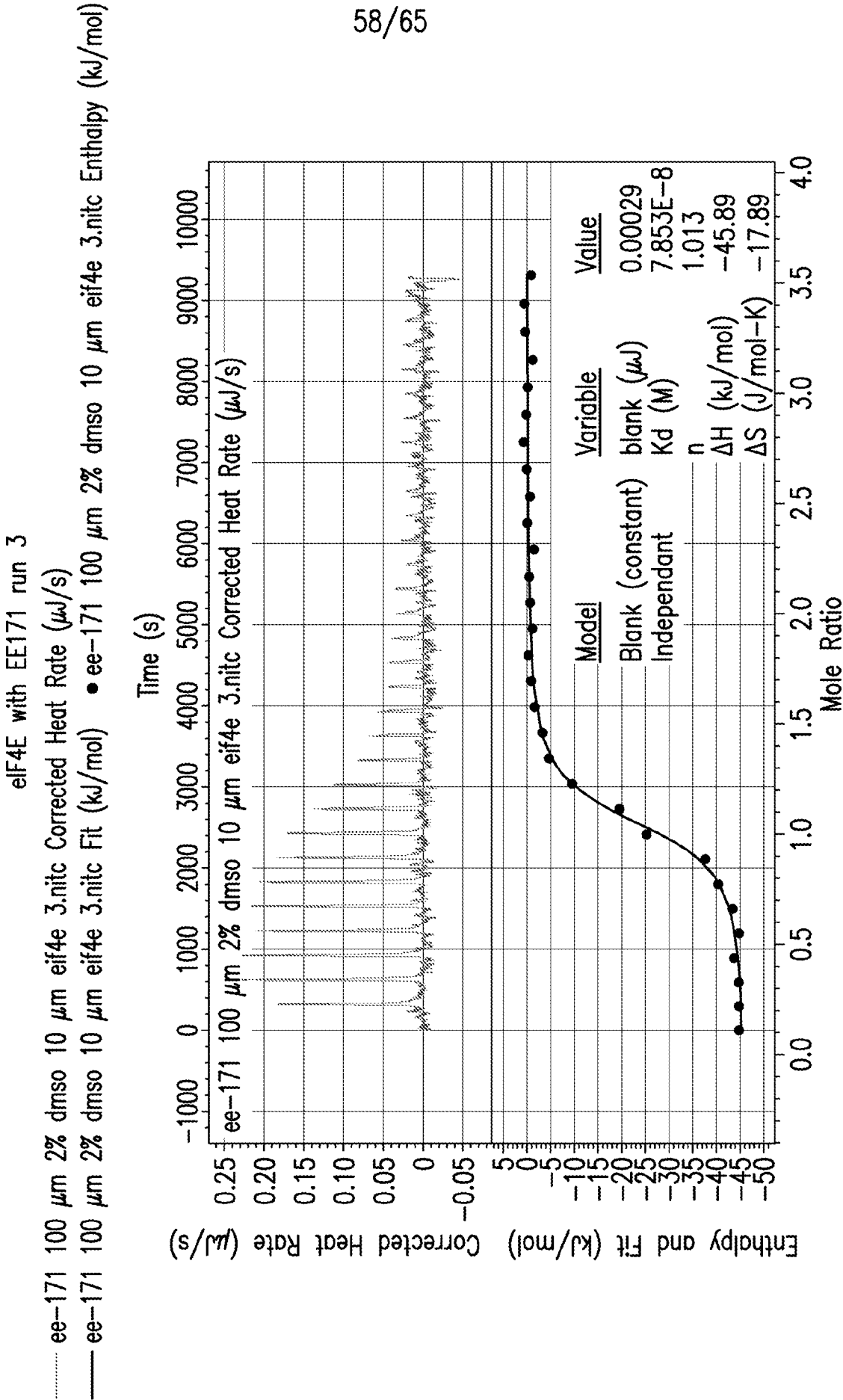


FIG.53

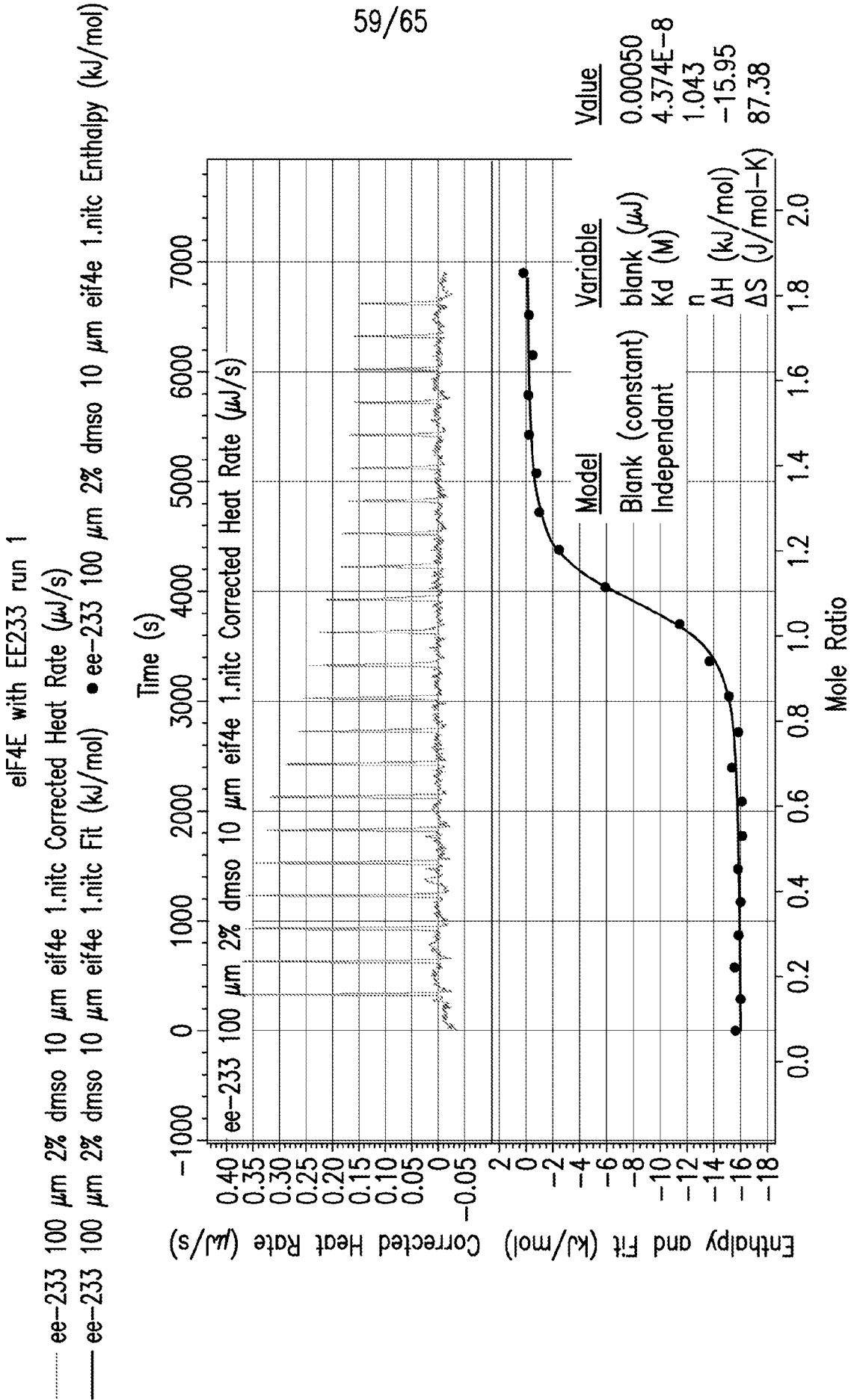


FIG.54

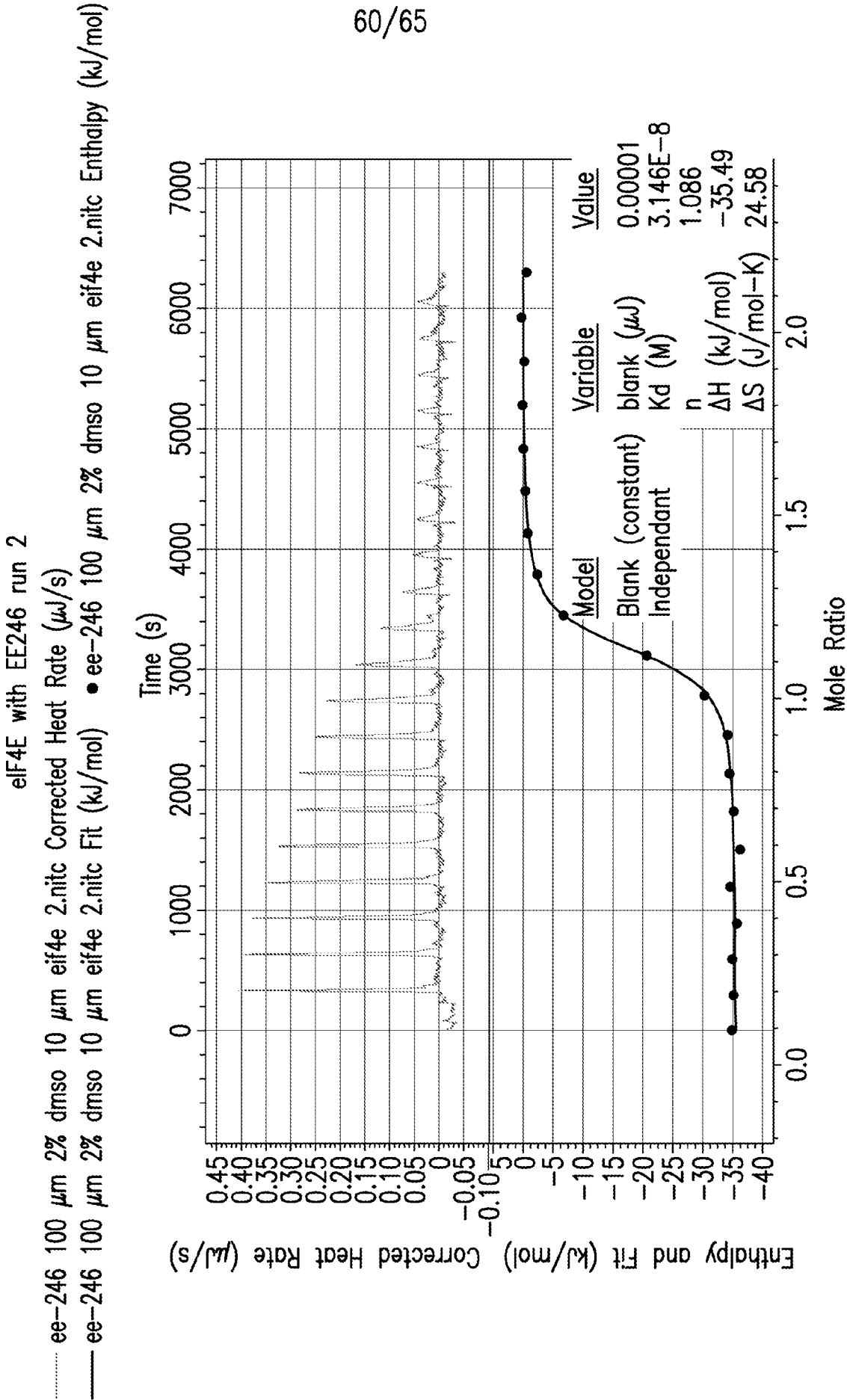


FIG.55

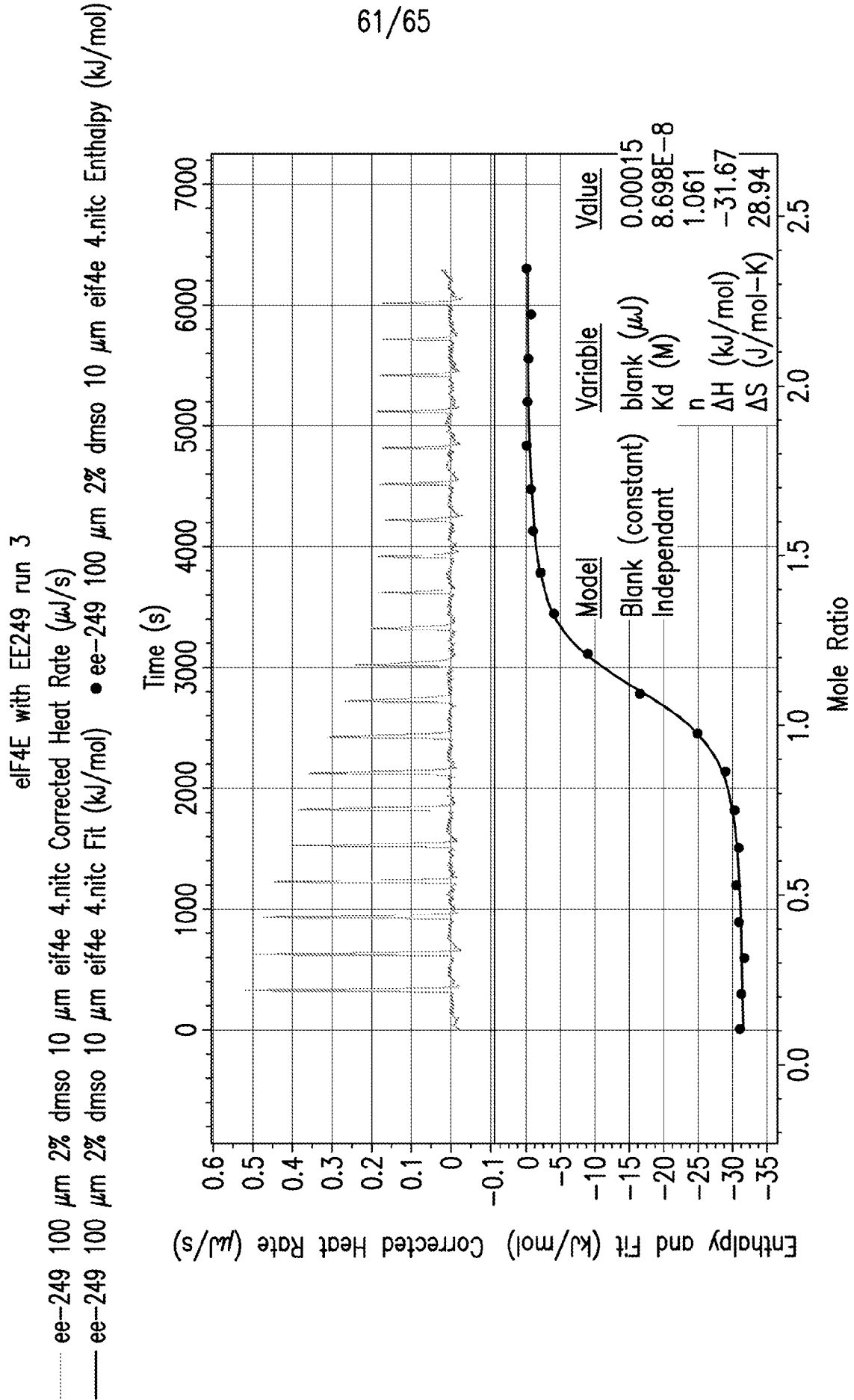


FIG.56

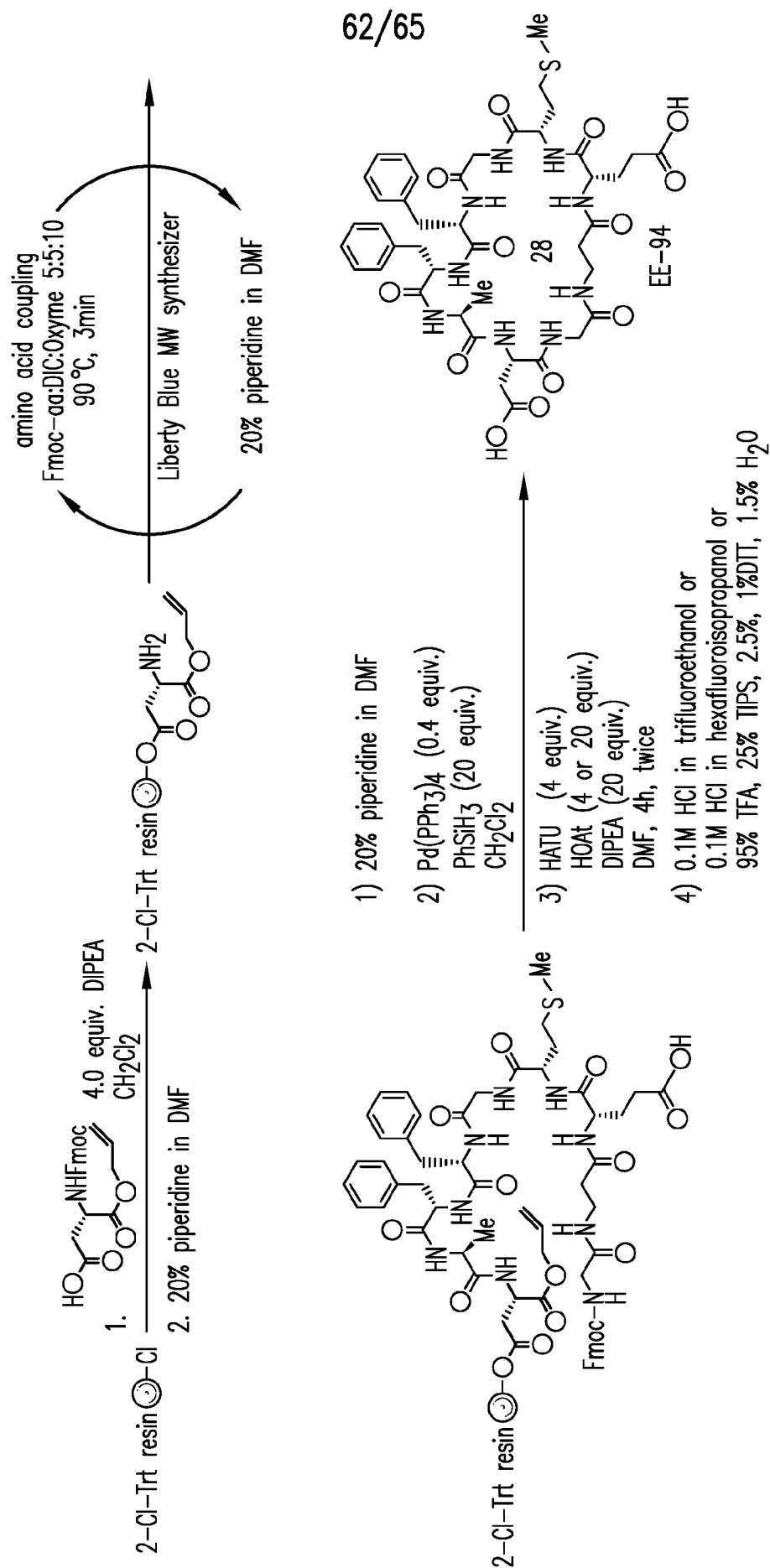


FIG.57

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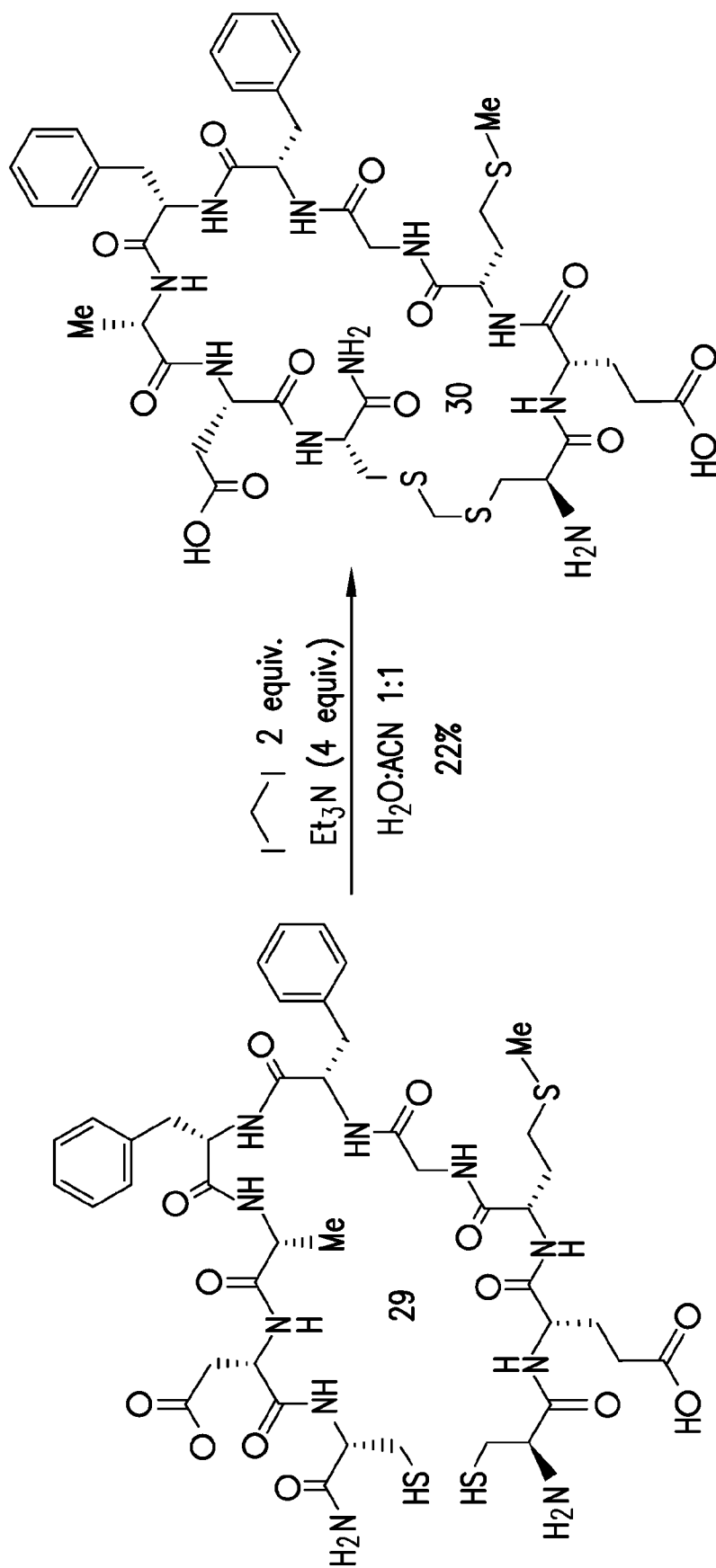


FIG. 58

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					[peptide] = 0.04 μ M per peptide, mixture of 25						
					[eIF4E] titration						
				Library 2							
	X =			peptides	0.08 μ M	0.16 μ M	0.31 μ M	0.62 μ M	1.25 μ M	2.5 μ M	5 μ M
C45H58F6N12O10Se	D-A			1				y			No Binding
C48H65F6N15O10Se	D-R			2		y BT					
C46H59F6N13O11Se	D-N			3				y			
C46H58F6N12O12Se	D-D			4			y				
C45H58F6N12O10SSe	D-C			5							y
C47H61F6N13O11Se	D-Q			6				y			
C47H60F6N12O12Se	D-E			7				y			
C44H56F6N12O10Se	D-G			8					y		
C48H60F6N14O10Se	D-H			9	y BT						
C48H64F6N12O10Se	D-I			10					y		
C48H65F6N13O10Se	D-K			11		y BT					
C47H62F6N12O10SSe	D-M			12				y			
C51H62F6N12O10Se	D-F			13					y		
C47H60F6N12O10Se	D-P			14				y			
C45H58F6N12O11Se	D-S			15					y		
C46H60F6N12O11Se	D-T			16				y			
C53H63F6N13O10Se	D-W			17					y		
C51H62F6N12O11Se	D-Y			18					y		
C47H62F6N12O10Se	D-V			19					y		
C46H61F6N13O10Se	L-Dab			20						y	
C45H59F6N13O10Se	L-Dap			21						y	
C47H63F6N13O10Se	L-Orn			22						y	
C46H60F6N12O11Se	L-hSer			23					y		
C52H64F6N12O11Se	L-Ser(OBn)			24							y
C47H60F6N12O12Se	L-Ser(OAc)			25				y			

Internal
standard D9G

FIG.60

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2022/046814

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - INV. - C07K 7/50; A61P 35/00 (2023.01) ADD. - A61K 38/12 (2023.01) CPC - INV. - C07K 7/50; A61K 38/12 (2023.01) ADD. - A61P 35/00 (2023.01) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) See Search History document Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched See Search History document Electronic database consulted during the international search (name of database and, where practicable, search terms used) See Search History document		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2020/204828 A1 (AGENCY FOR SCIENCE TECHNOLOGY AND RESEARCH) 08 October 2020 (08.10.2020) entire document	1, 2, 5, 6, 10, 17-19, 21, 22
Y	US 2020/0270305 A1 (PIVARIS BIOSCIENCE GMBH) 27 August 2020 (27.08.2020) entire document	1, 2, 5, 6, 10, 17-19, 21, 22
A	US 2019/0071469 A1 (AILERON THERAPEUTICS INC.) 07 March 2019 (07.03.2019) entire document	1, 2, 5, 6, 10, 17-19, 21, 22
A	WO 2021/123047 A1 (HERANTIS PHARMA OYJ) 24 June 2021 (24.06.2021) entire document	1, 2, 5, 6, 10, 17-19, 21, 22
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "D" document cited by the applicant in the international application "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 27 January 2023		Date of mailing of the international search report MAR 08 2023
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300		Authorized officer Taina Matos Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2022/046814

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2022/046814

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 82-89
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 82-89 are held as omnibus claims as they refer to an invention "present invention provides."

3. ☒ Claims Nos.: 32-41, 60-69
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 2, 5, 6, 10, 17-19, 21, 22

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2022/046814

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-31, 42-59, and 70-73 are drawn to macrocyclic peptides that can inhibit eIF4E by binding to eIF4E in its "apo" form, compounds comprising Formula I thereof, compounds comprising Formula II thereof, and compounds comprising a macrocyclic peptide covalently linked to a cell-penetrating moiety thereof.

Group II: claims 74-81 are drawn to methods for producing a compound that is cell permeable.

The first invention of Group I+ is restricted to a macrocyclic peptide compound selected to be SEQ ID NO: 1, and a cell-penetrating peptide (CPP) selected to be SEQ ID NO: 48. It is believed that claims 1, 2, 5, 6, 10, 17-19, 21, and 22 read on this first named invention and thus these claims will be searched without fee to the extent that they read on SEQ ID NOS: 1 and 48.

Applicant is invited to elect additional compounds and CPPs, and their respective, corresponding, SEQ ID NOS to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be a macrocyclic peptide compound selected to be SEQ ID NO: 2, and a cell-penetrating peptide (CPP) selected to be SEQ ID NO: 49. Additional antibody compounds and CPPs, and their respective, corresponding, SEQ ID NOS will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I+, macrocyclic peptides that can inhibit eIF4E by binding to eIF4E in its "apo" form, compounds comprising Formula I thereof, compounds comprising Formula II thereof, and compounds comprising a macrocyclic peptide covalently linked to a cell-penetrating moiety thereof, are not present in Group II; and the special technical features of Group II, methods for producing a compound that is cell permeable, are not present in Group I+.

The Groups I+ formulas do not share a significant structural element responsible for binding to eIF4E in its "apo" form, requiring the selection of alternative compounds and CPPs where "macrocyclic peptide comprising the formula X1X2X3G4X5X6X7X8," where "the macrocyclic peptide comprises an amino acid sequence set forth in the group of amino acid sequences consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 38, and SEQ ID NO: 39," and "the CPP comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 123, SEQ ID NO: 124, SEQ ID NO: 125, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, and SEQ ID NO: 138."

Additionally, even if Groups I+ and II were considered to share the technical features of a macrocyclic peptide that can inhibit eIF4E by binding to eIF4E in its "apo" form, comprising: 8 to 12 amino acids of which two adjacent amino acids thereof are aromatic amino acids, wherein from an N-terminal to C-terminal direction, a first aromatic amino acid comprising a first aromatic group that can interact with a first aromatic pocket of the eIF4E comprising amino acid residues W102, W166, and H200 and a second aromatic amino acid comprising a second aromatic amino acid group that can interact with a second aromatic pocket of the eIF4E bounded by amino acid residues W56 and F48, and wherein the macrocyclic peptide is optionally linked to a cell penetrating moiety that is capable of transporting the macrocyclic peptide across the cell membrane; a macrocyclic peptide having the core structure of the formula X1X2X3G4X5X6X7X8; a compound having the core structure of Formula I; a compound having the core structure of Formula II; and a compound comprising a macrocyclic peptide covalently linked to a cell-penetrating moiety, these shared technical features do not represent a contribution over the prior art.

Specifically, WO 2020/204828 to Agency for Science, Technology and Research (hereinafter, "Agency") teaches a macrocyclic peptide, comprising: 8 to 12 amino acids of which two adjacent amino acids thereof are aromatic amino acids (Para. [00126], [a] novel macrocyclic peptide that binds eIF4E at the cap-binding site; Paras. [0007], [0061], present invention relates to a peptide that binds to elongation initiation factor 4E (eIF4E) comprising the amino acid sequence CEX-1GX2X3X4X5C (SEQ ID NO: 1), specifically wherein the peptide comprises the amino acid sequence selected from the group consisting of ACEMGFFQDCG (SEQ ID NO: 2)), wherein from an N-terminal to C-terminal direction, a first aromatic amino acid comprising a first aromatic group and a second aromatic amino acid comprising a second aromatic amino acid group (Para. [00126], [a] novel macrocyclic peptide that binds eIF4E at the cap-binding site; Paras. [0007], [0061], present invention relates to a peptide that binds to elongation initiation factor 4E (eIF4E) comprising the amino acid sequence CEX-1GX2X3X4X5C (SEQ ID NO: 1), specifically wherein the peptide comprises the amino acid sequence selected from the group consisting of ACEMGFFQDCG (SEQ ID NO: 2)); a macrocyclic peptide having the core structure of the formula X1X2X3G4X5X6X7X8 (Para. [00126], [a] novel macrocyclic peptide that binds eIF4E at the cap-binding site; Paras. [0007], [0061], present invention relates to a peptide that binds to elongation initiation factor 4E (eIF4E) comprising the amino acid sequence

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CEX-1GX2X3X4X5C (SEQ ID NO: 1), specifically wherein the peptide comprises the amino acid sequence selected from the group consisting of ACEMGFFQDCG (SEQ ID NO: 2)); a compound having the core structure of Formula I (Para. [00126], [a] novel macrocyclic peptide that binds eIF4E at the cap-binding site; Paras. [0007], [0061], present invention relates to a peptide that binds to elongation initiation factor 4E (eIF4E) comprising the amino acid sequence CEX-1GX2X3X4X5C (SEQ ID NO: 1), specifically wherein the peptide comprises the amino acid sequence selected from the group consisting of ACEMGFFQDCG (SEQ ID NO: 2)); a compound having the core structure of Formula II (Para. [00126], [a] novel macrocyclic peptide that binds eIF4E at the cap-binding site; Paras. [0007], [0061], present invention relates to a peptide that binds to elongation initiation factor 4E (eIF4E) comprising the amino acid sequence CEX-1GX2X3X4X5C (SEQ ID NO: 1), specifically wherein the peptide comprises the amino acid sequence selected from the group consisting of ACEMGFFQDCG (SEQ ID NO: 2)); and a compound comprising a macrocyclic peptide covalently linked to a cell-penetrating moiety (Para. [0064], peptides of the present invention may be conjugated to one or more additional compounds. In one embodiment, the peptide is conjugated to one or more additional peptides; Para. [0065], the one or more additional peptides is a cell penetrating peptide).

In regards to "that can inhibit eIF4E by binding to eIF4E in its "apo" form," "that can interact with a first aromatic pocket of the eIF4E comprising amino acid residues W102, W166, and H200," and "that can interact with a second aromatic pocket of the eIF4E bounded by amino acid residues W56 and F48," a recitation of the intended use of the claimed invention must result in a structural difference between the claimed invention and the prior art in order to distinguish the claimed invention from the prior art. If the prior art is capable of performing the intended use, it meets the claim. In this case, SEQ ID NO: 2 of Agency appears to be capable of providing functionality for inhibiting eIF4E by binding to eIF4E in its "apo" form, interacting with a first aromatic pocket of the eIF4E comprising amino acid residues W102, W166, and H200, and interacting with a second aromatic pocket of the eIF4E bounded by amino acid residues W56 and F48.

The inventions listed in Groups I+ and II therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.