Macrocyclic Modalities Combining Peptide Epitopes and Natural Product Fragments

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Supplementary Figure S1 | Independent stepwise fragment synthesis of fused-di-pyrrolidine NP unit to establish absolute stereochemistry of PepNats.

15 \[ \xrightarrow{\text{Cu(MeCN)}_2\text{BF}_4, \text{Rg-Teosphere, } \text{Et}_2\text{N, DCM, rt, 3 h}} \] 18a, R = Me, 95% (\[\alpha\]_D = 108.6)

18b, R = Ph, 40% (\[\alpha\]_D = 107.2)

16 \[ \xrightarrow{\text{Allyl bromide, rt, 16 h}} \] 17

19a \[ \xrightarrow{\text{TFA/DCM (50:50), 0 °C to rt, 8 h}} \] 19a, R = Me, quantitative (\[\alpha\]_D = +60.7)

19b, R = Ph, quantitative (\[\alpha\]_D = +85.3)

19a: MeHN-GLGFK(MYF)-PyBOB, HOAt, DMF

20a: minor diastereomer of imine/cycloaddition on resin

20b: MeHN-RINMK(MYF)-PyBOB, HOAt, DMF

20b: minor diastereomer of imine/cycloaddition on resin

H1-minor

H1-major

K1-minor

K1-major

On resin imine/cycloaddition strategy gave:
Supplementary Figure S2 | RP-HPLC-MS and NMR comparison of the imine/cycloaddition strategy to form PepNats H1-major, the independent stepwise fragment enantiopure synthesis of H1-minor and the racemic independent synthesis of both endo products.

a HPLC comparison using analytical RP-HPLC-MS (Method I-C), HPLC profile are shown at 210 nm

Imine/cycloaddition of PepNat H1-major, crude

d.r. 90:10

Independent enantiopure synthesis of H1-minor, crude

d.r. 65:35

Mixture of PepNat H1-major and H1-minor, crude

d.r. 50:50

Independent racemic synthesis of the two endo products H1-racemic

major diastereomer (2R, 3S, 4R, 5S)

minor diastereomer (2S, 3R, 4S, 5R)
b $^1$H NMR comparison for PepNat H1-major (top) and H1-minor (bottom)

$^1$H NMRs were acquired using 700 MHz in CD$_3$OH at room temperature. The comparison between the major diastereomer H1-major obtained using imine/cycloaddition strategy (top) showed a clear difference compared to the diastereomer synthesized via the independent enantiopure fragment synthesis H1-minor (bottom). A strong shift of chemical displacement is observed for the methyl group (yellow, H$^{112,113,114}$) substituent of the fused di-pyrrolidine and the N-methyl (blue, H$^{104,105,106}$) substituted phenylalanine in $\alpha$ of the NP moiety. These observations are in line with the strong conformational change observed between the major and the minor diastereomers using NMR conformational analysis (Supplementary Figure S5b and S6b; for details see Section 6).
Supplementary Figure S3 | RP-HPLC-MS and NMR comparisons of the imine/cycloaddition strategy to form major PepNats K1-major, the independent stepwise fragment enantiopure synthesis of K1-minor and the racemic independent synthesis of both endo products.

a) HPLC comparison using optimized RP-HPLC-MS (Method II-C), HPLC profile are shown at 210 nm.

It can be noted that the independent enantiopure synthesis of K1-minor was challenging and a small amount of the desired PepNat was formed (middle HPLC profile). This reveals the robustness of the imine/cycloaddition strategy on resin allowing to isolated mg amount of both diastereomers.
The NMRs were acquired using 700 MHz in CD$_3$OD at room temperature. The comparison demonstrates that the major (top) and minor (bottom) diastereomers of PepNat K1 have different chemical shifts. Therefore, the two diastereomers adopt two distinct conformations in solution, as demonstrated for PepNat H1 (for details see Section 6).
Supplementary Figure S4 | Proposed favored mechanism and stereoselection for the 1,3-dipolar cycloaddition between maleimide dipolarophile and cyclic peptidic ylide intermediate on solid support.
Supplementary Figure S5 | Conformational analysis of the major diastereomer (2R, 3S, 4R, 5S) PepNat H1-major using 2D NMR analysis and NMR derived solution conformation.

a Slice from the ROESY spectrum (right) for H1-major corresponding to the chemical shift of the N-methyl group of the fused di-pyrrolidine moiety (protons 112,113,114). Long-range NOEs are labelled with green dots in the structure (left).

b NMR solution structure of H1-major macrocycle. PepNat conformations were sampled using Maestro’s Macrocycle Sampling algorithm (OPLS3 force field; Low-mode MD) using a 25 Kcal/mol energy window. The 1704 conformers generated in the conformational sampling were filtered using four long-range constraints (112,113,114) – (139,140), (112,113,114) – (133,137), (112,113,114) – (148) and (116,117) – (139,140), with an upper limit of 5.5 Å. The 21 conformers that complied with those distance restraints were subjected to solvent explicit 10 ns MD simulations, their trajectories clustered and the most populated clusters for each conformation selected. MSpin NOE Fitter algorithm was utilized to select which cluster agreed best with the NMR experimental data.
**Supplementary Figure S6 | Conformational analysis of the minor diastereomer (2S, 3R, 4S, 5R) PepNat H1-minor using 2D NMR analysis and NMR derived solution conformation.**

**a** Slice from the ROESY spectrum (right) for H1-minor corresponding to the chemical shift of the N-methyl group of the fused di-pyrrolidine moiety (protons 150,151,152). Long-range NOEs are labelled with green dots in the structure (left).

**b** NMR derived solution conformation for H1-minor. PepNat conformations were sampled using Maestro’s Macrocycle Sampling algorithm (OPLS3 force field; Low-mode MD) using a 25 Kcal/mol Energy window. The 1611 conformers generated in the conformational sampling were filtered using the three long-range constraints (75,78) – (30,31), (150,151,152) – (119,120,121) and (119,120,121) – (50,51) with an upper limit of 5.5 Å. The 35 conformers that complied with those distance restraints were subjected to solvent explicit 10 ns MD simulations, their trajectories clustered and the most populated clusters for each conformation selected. MSpin NOE Fitter algorithm was utilized to select which clusters agreed best with the NMR experimental data.
Supplementary Figure S7 | NMR analysis of the minor diastereomer (2S, 3R, 4S, 5R) of PepNat K1-minor.

Slice from the NOESY spectrum for PepNat K1-minor corresponding to the chemical shift of the fused di-pyrrolidine phenyl ortho protons (protons 97,105). Long-range NOEs are labelled with green dots in the structure (for details see Section 6).
Supplementary Figure S8 | SPR sensorgrams for selected examples of DINNN containing PepNats.
Example sensorgrams for selected PepNats D1–D4, D7, D8, D10, D12 and D18 interacting with immobilized hSPSB2 using SPR (red). Each PepNat was injected at 0.020 µM, 0.078 µM, 0.313 µM, 1.25 µM and 5 µM concentration in quick succession followed by a longer dissociation time. A binding kinetic model was fitted to each experimental trace (black). All experiments were conducted in triplicates.
Supplementary Figure S9 | Binding and functional curves for selected examples of Rff/RFF containing PepNats.
Example concentration response curves for selected PepNats in MC1R, MC3R, MC4R, MC5R radioligand binding assays and MC1R, MC3R, MC4R cAMP assays. Responses were normalized to reference agonist NDP-α-MSH and presented as % effect of control, with full competition in binding defined as -100% and full agonism in cAMP assays defined as 100%. Data were fitted using a four-parameter logistic fit and derived IC₅₀ and EC₅₀ values are reported in table S5. Curve fits were removed for compounds not reaching an upper plateau in a cAMP assay or if considered inactive in a binding assay. Threshold to define a compound as active was set to -30% in binding assays and 20% in cAMP assays respectively. Data are from at least three independent experiments and error bars represent ± Standard Error of the Mean (SEM).
Table S1 | Imine macrocyclization of linear peptide on solid support.

![Diagram](attachment:image.png)

| entry | epitope | imine conditions | temperature | time | conversion (%) |
|-------|---------|-----------------|-------------|------|----------------|
| 1     | ALFPGF  | 1% AcOH in DMF  | rt          | 1.5 h| 91             |
| 2     | ALFPGF  | 5% AcOH in DMF  | rt          | 1.5 h| ≥ 99           |
| 3     | ALFPGF  | Et₃N (3 eq.), 4Å MS, DMF | rt          | 1.5 h| 4              |
| 4     | ALFPGF  | Et₃N (3 eq.), CH(OCH₃)₃ (196 eq.), DMF | rt          | 1.5 h| ≥ 99           |

The ALFPGF sequence was bound to solid support using Rink Amide low loading resin (loading 0.36 mmol/g).

* The conversion was obtained by integration of the product and remaining starting material using analytical RP-HPLC-MS, after reduction of the imine using NaBH₃CN (20 eq.) and subsequent TFA cleavage from the solid support.

To date, the literature is lacking examples of intramolecular imine macrocyclization on solid support. Only few examples are available either intramolecular imine formation in solution or intermolecular imine formation on solid support. According to the screening conditions depicted in the table above, trimethyl orthoformate afforded good compatibility with the resin and the subsequent cycloaddition conditions (entry 4). The full conversion to the desired imine was assessed by selective reduction of the Schiff base using sodium cyanoborohydride and subsequent cleavage from the resin followed by analytical RP-HPLC-MS.
**Table S2 | Screening condition for the 1,3-dipolar cycloaddition on solid support.**

| entry | epitope | dipolarophile | conditions | solvent | time | conv (%)* | d.r. ** |
|-------|---------|---------------|------------|---------|------|-----------|---------|
| 1     | ALFPGF  | 6             | AgOAc, PPh₃, Et₃N | DCM     | 16 h | 88        | 75:25   |
| 2     | ALFPGF  | 10            | AgOAc, PPh₃, Et₃N | DCM     | 60 h | 50        | n.d.    |
| 3     | ALFPGF  | 11            | Cu(CH₃CN)₂BF₄, Fe(Cp)₂PCy₂PPh₃, Et₃N | DCM | 16 h | 49        | n.d.† |
| 4     | ALFPGF  | 11            | Cu(CH₃CN)₂BF₄, R-Fesulphos, Et₃N | DCM     | 16 h | 100       | n.d.† |
| 5     | ALFPGF  | 11            | Cu(CH₃CN)₂BF₄, R-BINAP, Et₃N | DCM     | 16 h | 67        | n.d.† |
| 6     | ALFPGF  | 11            | diphenylphosphate, Et₃N | DCM     | 48 h | ≤ 5       | n.d.    |
| 7     | ALFPGF  | 6             | Cu(CH₃CN)₂BF₄, R-Fesulphos, Et₃N | DCM     | 2 h | ≥ 99      | n.d.‡ |
| 8     | ALFPGF  | 6             | LiBr, Et₃N | THF     | 16 h | ≥ 99      | 92:8    |
| 9     | ALFPGF  | 7             | LiBr, Et₃N | THF     | 16 h | ≥ 99      | 89:11   |
| 10    | ALFPGF  | 10            | LiBr, Et₃N | THF     | 16 h | ≥ 99      | 70:30   |
| 11    | ALFPGF  | 11            | LiBr, Et₃N | THF     | 42 h | ≥ 99      | 37:22:21:20 |
| 12    | ALFPGF  | 6             | Et₃N | THF     | 16 h | 35        | 3 dia.  |
| 13    | DINNN   | 6             | LiBr, Et₃N | THF     | 16 h | ≥ 99      | 96:4    |
| 14    | DINNN   | 6             | Et₃N | THF     | 16 h | ≥ 99      | 44:57   |

The peptide epitope sequence is bound to solid support using Rink Amide LL Resin (loading 0.28 to 0.40 mmol·g⁻¹). The epitope is represented using single capital letter code amino acid. *The conversion was determined by integration of the product and SM using analytical RP-HPLC profile at 210 nm. **The diastereomeric ratio was determined by integration of the HPLC profile at 210 nm. n.d. not determined, a complex mixture of diastereomer was observed using optimized analytical RP-HPLC-MS. †complex LC-MS profile was observed with various diastereomers and consequent amount of side products. ‡complex mixture of diastereomers (≥ 4).

After screening catalyst systems (see table above), lithium bromide led to cleaner crude profile, demonstrated good reliability with the different dipolarophiles and gave ≥ 99% conversion to the desired cyclic ALFPGF and DINNN PepNats on solid support (entries 8-11 and 13). Lithium bromide proved to be essential for the cycloaddition on resin, as in its absence the cycloaddition could not reach completion (entries 12 and 14). Moreover, avoiding the use of heavy metal containing catalyst is of major advantages to develop a robust method to create PepNats collection readily available for biological evaluation.
Table S3 | Synthesis of macrocyclic PepNats.

| entry | PepNat  | epitope | aa | dipolarophile | crude d.r** | mass (mg)† | yield (%)† | purity (%)‡ |
|-------|---------|---------|----|--------------|-------------|------------|-----------|------------|
| 1     | A1-major| ALFPGF  | G  | 6            | 92:8        | 2.7        | 8         | 99         |
| 2     | A2-major| ALFPGF  | G  | 7            | 89:11       | 3.4        | 10        | 9        |
| 3     | A3-major| ALFPGF  | G  | 10           | 70:30       | 4.5        | 14        | 98         |
| 4     | A4-mixt.| ALFPGF  | G  | 11           | 37:22:21:20 | 3.1        | 8         | 92         |
| 5     | B1-major| RFFNAF  | Y  | 6            | 53:47       | 1.2        | 2         | 95         |
| 6     | B2-minor| RFFNAF  | G  | 6            | 78:22       | 1.4        | 2         | 99         |
| 7     | B3-major*| RFFNAF | G  | 7            | 61:39       | 0.7        | 1         | 95         |
| 8     | C1-major| RFFNAF  | Y  | 6            | 84:16       | 2.4        | 2         | 84         |
| 9     | C2-major| RFFNAF  | Y  | 11           | 30:31:28:11 | 2.2        | 1         | 96         |
| 10    | C3-major| RFFNAF  | Y  | 12           | 26:39:24:11 | 1.8        | 1         | 92         |
| 11    | C4-major| RFFNAF  | G  | 6            | 71:29       | 1.8        | 4         | 98         |
| 12    | D1-major| DINNN   | G  | 6            | 90:4        | 13.1       | 11        | 96         |
| 13    | D2-major| DINNN   | G  | 7            | 83:17       | 4.3        | 5         | 83         |
| 14    | D3-major| DINNN   | G  | 8            | 81:19       | 13.5       | 10        | 89         |
| 15    | D4-major| DINNN   | G  | 10           | 59:41       | 2.7        | 6         | 85         |
| 16    | D5-mixt.| DINNN   | G  | 12           | 11:10:17:62 | 1.1        | 3         | 96         |
| 17    | D6-major| DINNN   | G  | 13           | 9:36:7:48   | 2.2        | 6         | 89         |
| 18    | D7-major| DINNN   | G  | 14           | 71:29       | 1.1        | 4         | 88         |
| 19    | D19-major| EEKDINNNVK | T | 6            | 70:30       | 1.1        | 2         | 91         |
| 20    | D20-major| DINNNV  | G  | 6            | 84:16       | 8.3        | 5         | 99         |
| 21    | D21-major| INNN    | G  | 6            | 66:34       | 3.1        | 3         | 74         |
| 22    | D23-major| NNN     | G  | 6            | 75:25       | 5.7        | 12        | 98         |
| 23    | E1-major| AHASN   | G  | 6            | 74:26       | 1.1        | 3         | 81         |
| 24    | F1-major*| RFFNA   | Y  | 6            | 64:36       | 2.2        | 3         | 97         |
| 25    | F2-minor| RFFNA   | Y  | 7            | 53:47       | 1.7        | 2         | 90         |
| 26    | G1-major| RFFNA   | Y  | 6            | 75:25       | 1.1        | 2         | 92         |
| 27    | G2-mixt.| RFFNA   | G  | 11           | 12:32:18:38 | 2.4        | 5         | 82         |
| 28    | H1-major| GLGF    | F  | 6            | 90:10       | 4.9        | 5         | 98         |
| 29    | H2-major| GLGF    | F  | 7            | 91:9        | 1.7        | 5         | 99         |
| 30    | H3-major| GLGF    | 11 | 8:68:5:19    | 1.1        | 4         | 99         |
| 31    | I1-major| LKPI    | G  | 6            | 80:20       | 2.9        | 8         | 83         |
| 32    | I2-mixt.| LKPI    | G  | 7            | 60:40       | 2.6        | 5         | 86         |
| 33    | I3-mixt.| LKPI    | G  | 13           | 15:44:14:27 | 1.5        | 4         | 92         |
The peptide epitope sequence is bound to solid support using Rink Amide LL Resin (loading 0.28 to 0.40 mmol.g⁻¹).

The epitope is represented as single capital letter code amino acid with o-amino acid indicated by lower case letter.
mixt.: mixture of diastereomers. * reverse diastereoselectivity observed with minor* = (2R, 3S, 4R, 5S) diastereomer, major* = (2S, 3R, 4S, 5R) diastereomer (for details see Experimental Section). ** The cycloaddition diastereomeric ratio (d.r.) is depicted as major:minor diastereomers unless more than two diastereomers were obtained. The d.r. was determined from the crude product by integration of the HPLC profile at 210 nm recorded using either the analytical RP-HPLC-MS (I) or the Optimized Analytical RP-HPLC-MS (II) (for details see Experimental Section). † Isolated mass and overall isolated yield of the major diastereomer (2R, 3S, 4R, 5S) PepN at from the starting unfunctionalized resin, unless otherwise noted (for details see Experimental Section). ‡ Purity was determined by integration of the product peak on the HPLC profile at 210 nm (for details see Experimental Section). § PepNat isolated as mixture of diastereomers (for the d.r. of the isolated product see Experimental Section). ¶ The isolated diastereomer contains the (2S, 3R, 4S, 5R) stereocenters due to reverse selectivity of the cycloaddition due to steric hinderance.
Table S4 | Impact of the NMe amino acid and the amine linker length on the diastereoselectivity of the cycloaddition with maleimide dipolarophile on solid support using the DINNN epitope.

| entry | PepNat | nMe-aa | n | dipolarophile | crude d.r. | mass (mg) | yield (%) | purity (%) |
|-------|--------|--------|---|---------------|------------|-----------|-----------|------------|
| 1     | D1-major | NMeF   | 4 | 6             | 96:4       | 5.1       | 11        | 96         |
| 2     | D8-major | NMeF   | 3 | 6             | 96:4       | 5.3       | 13        | 97         |
| 3     | D9-major | NMeF   | 2 | 6             | 95:5       | 1.3       | 3         | 97         |
| 4     | D10-major | NMeF | 1 | 6             | 84:16      | 7.0       | 4         | 98         |
| 5     | D11-minor | NMeG   | 4 | 6             | 54:46      | 12.7      | 8         | 90         |
| 6     | D12-major | NMeL   | 4 | 6             | 91:9       | 12.4      | 10        | 95         |
| 7     | D13-major | NMeV   | 4 | 6             | 85:15      | 12.9      | 11        | 98         |
| 8     | D14-minor | P      | 4 | 6             | 57:43      | 1.1       | 3         | 94         |
| 9     | D15-minor* | NMeF  | 4 | 6             | 64:36*     | 2.6       | 5         | 97         |
| 10    | D16-mixt. | F      | 4 | 6             | 28:35:37   | 2.0#      | 2         | 81         |
| 11    | D2-major | NMeF   | 4 | 7             | 83:17      | 4.3       | 5         | 83         |
| 12    | D17-major | NMeF  | 1 | 7             | 58:42      | 2.3       | 2         | 79         |
| 13    | D18-major | NMeV  | 4 | 7             | 62:38      | 1.6       | 1         | 95         |

The DINNN epitope sequence is bound to solid support using Rink Amide LL Resin (loading 0.28 to 0.40 mmol.g\(^{-1}\)). The epitope is represented as single capital letter code amino acid with o-amino acid indicated by lower case letter. * reverse diastereoselectivity observed with minor* = {2R, 3S, 4R, 5S} diastereomer (for details see Experimental Section). ** The cycloaddition diastereomeric ratio is depicted as major:minor diastereomers unless more than two diastereomers were obtained. The d.r. was determined from the crude product by integration of the HPLC profile at 210 nm recorded using either the analytical RP-HPLC-MS (I) or the Optimized Analytical RP-HPLC-MS (II) (for details see Experimental Section). † Isolated mass of the major diastereomer unless otherwise noted, after purification by RP-HPLC-MS (for details see Experimental Section). ‡ Overall isolated yield of the major diastereomer from the starting unfunctionalized resin. § The purity was determined by integration of the product peak on the 210 nm HPLC profile. ¶ The PepNats product was isolated as mixture of diastereomers, the diastereomeric ratio of the product can be found in the Experimental Section.
| entry | compound | structure |
|-------|----------|-----------|
| 28 | YCRFNACG | YCRFNACG |
| 29 | YCRFNASG | YCRFNASG |
| 30 | B1-major | YFFNAPK |
| 31 | B2-minor | YFFNAPK |
| 32 | B3-major* | YFFNAPK |
| 33 | C1-major | YFFNAPK |
| 34 | C2-minor* | YFFNAPK |
| 35 | C3-major | YFFNAPK |
| 36 | C5-minor | YFFNAPK |
| 37 | C4-major | YFFNAPK |
| 38 | F1-major* | YFFNAPK |
| 39 | F1-minor* | YFFNAPK |
| 40 | F1-major* | YFFNAPK |
| 41 | F2-minor | YFFNAPK |
| 42 | F2-minor | YFFNAPK |
| 43 | G1-major | YFRNAY |
| 44 | J1-major | YFFNFK |
| 45 | J2-major | YFFNFK |
| 46 | J3-major | YFFNFK |
| 47 | J3-major* | YFFNFK |
| 48 | K1-major | YFFNFK |
| 49 | K2-major | YFFNFK |
| 50 | K3-major | YFFNFK |
| 51 | K4-major | YFFNFK |
| 52 | K4-major* | YFFNFK |
| 53 | K5-major | YFFNFK |
| 54 | N1-minor* | YFFFK |
| 55 | N1-major | YFFFK |
| 56 | N2-minor* | YFFFK |
| 57 | N2-minor* | YFFFK |
| 58 | O1-major | YFFFK |
| 59 | O1-minor | YFFFK |
| 60 | O2-major | YFFFK |

The disulfide bridge decapetide 28 showed differences in binding on the human receptors compared to the mouse receptors reported in the literature.  The affinity differences between the two species is likely due to the low similarity (~76%) between the human and the mouse melanocortin receptors. In the functional assay, the cyclic decapetide showed weak partial agonistic activity at hMC1R. Agonist effect could not be observed on hMC3R and hMC4R. Additionally, modification of Phe$^{112}$ and Phe$^{113}$ to their $\alpha$-analogues reduced the selectivity profile of the disulfide bridge decapetide. However, selective and almost full agonistic activity at hMC1R was observed (29, EC$^{50}$ = 0.14 $\mu$M ± 0.01 $\mu$M; % effect = 84). This result confirms the hypothesis that the conformation adopted by the two phenylalanine hot spots can increase the agonistic activity at the MC1R.
2) Materials and Methods

2) A) Chemical Synthesis Materials and Methods

All reactants, reagents and commercially available compounds 6, 7, 9 and 10 were purchased from commercial sources (ABCR, Alfa Aesar, Acros Organics, Novabiochem (Merck), Sigma-Aldrich, Roth, trc Canada, Strem, Iris Biotechnology) and were used without further purification unless otherwise noted. Dry solvents (DMF, THF, Et₂O, NMP, DCM) and bases (DIPEA, Et₃N) were used as commercially provided without additional treatment.

Reactions were monitored on Merck pre-coated silica gel 60 F254 TLC plates and visualized using 254 nm UV light. They were then revealed with a KMnO₄ developing bath if required.

Flash chromatography was performed using Flash Master on a Grace Revaleris® HP Silica.

NMR data were collected on Bruker AVANCE 500 (500 MHz ¹H, 125 MHz ¹³C), AVANCE 600 (600 MHz ¹H, 150 MHz ¹³C) and AVANCE 700 (700 MHz ¹H, 175 MHz ¹³C). ¹H, ¹³C, COSY, NOESY, ROESY and e-HSQC spectra were obtained in CDCl₃, CD₃OD, CD₃OH or DMSO-d₆. ¹H, COSY and e-HSQC spectra were recorded with proton decoupling. Chemical shifts are reported in ppm. Data are reported in the following order: chemical shift (δ) values are reported in ppm with the solvent resonance as internal standard; multiplicities are indicated s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet); coupling constants (J) are given in Hertz (Hz).

Analytical RP-HPLC-MS (I) was performed using a Thermo Scientific Dionex® UltiMate 3000 equipped with a DAD-3000 detector (210, 254, 280 and 350 nm) coupled to a LCQ Fleet ion trap mass spectrometer. Heated electrospray ionization mass spectra were recorded in 110-2000 Da range using an EC Nucleodur C₁₈ gravity column (150 mm x 3 mm, particle size 1.8 μm) from Macherey-Nagel. The diastereomeric ratio was determined by integration of the chromatogram at 210 nm.

Method I-A: A step gradient of 10 to 10% for 0.5 min then 10 to 95% for 7.0 min of MeCN + 0.1% TFA in H₂O + 0.1% TFA was applied at a flow rate of 0.4 mL/min.

Method I-B: A step gradient of 10 to 10% for 0.5 min then 10 to 55% for 15.0 min followed by 55 to 95% in 0.5 min of MeCN + 0.1% TFA in H₂O + 0.1% TFA was applied at a flow rate of 0.4 mL/min.

Method I-C: A step gradient of 10 to 10% for 0.5 min then 10 to 70% for 15.0 min followed by 70 to 95% in 0.5 min of MeCN + 0.1% TFA in H₂O + 0.1% TFA was applied at a flow rate of 0.4 mL/min.

Method I-D: A step gradient of 5 to 5% for 0.5 min then 5 to 45% for 15.0 min followed by 45 to 95% in 0.5 min of MeCN + 0.1% TFA in H₂O + 0.1% TFA was applied at a flow rate of 0.4 mL/min.

Method I-E: A step gradient of 20 to 30% for 20.0 min followed by 45 to 95% in 0.5 min of MeCN + 0.1% TFA in H₂O + 0.1% TFA was applied at a flow rate of 1 mL/min.
Optimized Analytical RP-HPLC-MS (II) was performed using a Thermo Scientific Dionex® UltiMate 3000 equipped with a DAD-3000 detector (210, 230, 254 and 280 nm) coupled to a Velos Pro Dual-Pressure Linear Ion Trap Mass Spectrometer. Heated electrospray ionization mass spectra were recorded in 110-2000 Da range using an EC Nucleodur C18 gravity column (150 mm x 2 mm, particle size 1.8 μm) from Macherey-Nagel. A flow rate of 0.75 mL/min with a column temperature of 60 °C was applied. The gradient system was adjusted according to the elution profile and is indicated for the single compounds. The diastereomeric ratio was determined by integration of the chromatogram at 210 nm.

**Method II-A** A step gradient of 11.1 to 11.1% for 1 min then 11.1 to 49.5% for 23 min followed by 49.5 to 95.0% for 1 min of 11 mM aqueous HCO$_2$NH$_4$ / MeCN (10:90) solution + 0.03% TFA in 11 mM aqueous HCO$_2$NH$_4$ + 0.03% TFA.

**Method II-B** A step gradient of 5.5 to 5.5% for 1 min then 5.5 to 27.5% for 16 min followed by 27.5 to 95.0% for 3 min of 11 mM aqueous HCO$_2$NH$_4$ / MeCN (10:90) solution + 0.03% TFA in 11 mM aqueous HCO$_2$NH$_4$ + 0.03% TFA.

**Method II-C** A step gradient of 11.1 to 11.1% for 1 min then 11.1 to 49.5% for 34 min followed by 49.5 to 95.0% for 1 min of 11 mM aqueous HCO$_2$NH$_4$ / MeCN (10:90) solution + 0.03% TFA in 11 mM aqueous HCO$_2$NH$_4$ + 0.03% TFA.

**Method II-D** A step gradient of 5.5 to 5.5% for 1 min then 5.5 to 27.5% for 11 min followed by 27.5 to 95.0% for 1 min of 11 mM aqueous HCO$_2$NH$_4$ / MeCN (10:90) solution + 0.03% TFA in 11 mM aqueous HCO$_2$NH$_4$ + 0.03% TFA.

**Method II-E** A step gradient of 11.1 to 11.1% for 1 min then 11.1 to 49.5% for 18 min followed by 49.5 to 95.0% for 1 min of 11 mM aqueous HCO$_2$NH$_4$ / MeCN (10:90) solution + 0.03% TFA in 11 mM aqueous HCO$_2$NH$_4$ + 0.03% TFA.

**Method II-F** A step gradient of 27.5 to 27.5% for 1 min then 27.5 to 72.5% for 18 min followed by 72.5 to 95.0% for 1 min of 11 mM aqueous HCO$_2$NH$_4$ / MeCN (10:90) solution + 0.03% TFA in 11 mM aqueous HCO$_2$NH$_4$ + 0.03% TFA.

Analytical Purity-RP-HPLC was performed on an Agilent Technologies 1260 Infinity series chromatograph equipped with a 1260 DAD UV detector (G4212B) detector. A step gradient of 5 to 5% for 2 min then 5 to 65% in 30 min followed by 65 to 95% for 1 min of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA was applied to an EC Nucleodur C$_{18}$ gravity column (50 mm x 2 mm, 1.8 μm) from Macherey-Nagel, at a flow rate of 1 mL/min. The purity was determined by integration of the chromatogram at 210 nm.

Semi-Preparative RP-HPLC (I) was performed on an Agilent Technologies 1260 Infinity series chromatograph coupled with a 1260 DAD VL UV detector (G1315D) and an Agilent Technology 6120 Quadrupole LC/MS (ESI“). The chromatograph was equipped with a Nucleodur C$_{18}$ Gravity column (125 mm x 10 mm, 5 μm) from Macherey-Nagel. A step gradient from H$_2$O + 0.1% TFA to MeCN + 0.1% TFA was used at a flow rate of 6 mL/min. The gradient system was adjusted according
to the elution profile of the crude product. The relevant fractions containing the desired product were collected, analyzed by RP-HPLC-MS and accordingly pooled and lyophilized.

**HRMS** were obtained on a Thermo Scientific LTQ Fleet Orbitrap mass spectrometer coupled to an *Accela HPLC*-System (HPLC column: Hypersyl GOLD, 50 mm x 1 mm, particle size 1.9 μm) equipped with an electron spray ionization source (ESI). The mass detection was recorded in the range of 150 to 2000 Da.

### 2) B) Biochemical Assays Materials and Methods

**Surface Plasmon Resonance (SPR)**

All SPR experiments were conducted on a Biacore T200, S200 or 8K instrument (GE Healthcare) at 20 °C using HBS-P+ as running buffer (GE Healthcare). A CMS sensor chip (GE Healthcare) was conditioned three times at 60 s using 50 mM NaOH, 1 M NaCl prior to standard NHS/EDC activation (e.g. amine coupling in the Biacore instruction manual) of the sensor surface for 420 s. Recombinant human SPSB2 (product ID: ab134592, Abcam, Cambridge, UK) was diluted to 200 nM using 10 mM Sodium acetate pH 5.5 and injected over the activated surface for 300 s. This surface and a surface subjected to only NHS/EDC activation were deactivated using 100 mM Tris-HCl, pH 8.0. The empty surface was used as a reference surface.

All PepNat and peptide reference interaction experiments were conducted by diluting 10 mM DMSO stocks of PepNats and peptide references using digital dispensing (Tecan) to 10 μM, 2.5 μM, 625 nM, 156 nM and 40 nM or 1 μM, 250 nM, 63 nM, 16 nM and 4 nM. All concentrations were serially injected over both the SPSB2 containing- and empty surfaces using single-cycle kinetics (GE Healthcare). The resulting sensorgrams were reference- and blank subtracted prior to fitting of the data using a binding kinetic model (Biacore evaluation software).

**DNA constructs, cell culturing, transfections and generation of stable cell lines**

DNA constructs and cell lines were employed as previously described by Durek *et al.*⁷ “cDNAs encoding human MC1 (Genbank accession number NM_002386), MC3 (NM_019888), and MC4 (NM_005912) and MC5 (NM_005913) were cloned into the pcDNA4/TO vector (ThermoFisher). In the constructs, a consensus Kozak sequence (GCCACC) was incorporated immediately before the start ATG. DNA was amplified, isolated and sequenced using standard techniques.

T-Rex™-293 cells (ThermoFisher) were cultured in DMEM medium, supplemented with 10% foetal bovine serum and 5 μg/ml blasticidin at 37°C in a humidified atmosphere with 10% CO2. Lipofectamine 2000 (ThermoFisher) was used for transfections according to the manufacturer’s recommendations. Resistant polyclonal populations were generated by selection with Zeocin (0.3 mg/ml, ThermoFisher) for about two weeks after transfection. Individual clones were isolated using flow cytometry, subsequently expanded, tested for responsiveness to [Nle⁴, dPhe⁷]-α-Melanocyte Stimulating Hormone (NDP-α-MSH) and cryopreserved.” To induce receptor
expression, MC1R and MC4R transfected cells were treated with 0.1 ng/mL and MC3R transfected cells with 10 ng/mL doxycyclin, for 24 h before assay.

**Melanocortin receptor radioligand binding and cAMP assays.**

Binding and cAMP assays were performed as described by Durek et al. “A 384-well format scintillation proximity assay (SPA) was used to identify compounds that show competitive binding to the human Melanocortin receptor 1,3,4 or 5 ligand binding domain (LBD). Membranes expressing the Melanocortin receptor binds to the wheat germ agglutinin (WGA) coated polyvinyltoluene (PVT) SPA beads. The inhibition of the scintillation signal by displacement of $^{[125]}$-NDP-α-MSH by test compounds were detected by a 1450 MicroBeta Trilux scintillation counter (PerkinElmer).

Binding assays were performed in a 384 format PS-microplate (Greiner 781095). Membranes were prepared from HEK293-6E suspension cells transiently expressing human MC1R, MC3R, MC4R or MC5R. The assays were done as follows: 20 µl WGA coated PVT SPA bead (Perkin Elmer RPNQ0001) resuspended in Assay buffer (25mM Hepes pH 7, 1.5 mM CaCl$_2$, 1 mM MgSO$_4$, 0.2 % BSA (Sigma A3059), 1 mM 1,10-Phenanthroline monohydrate (Sigma P9375)) at a final concentration of 0.5 µg/µl (MC1R and MC4R) or 2 µg/µl (MC3R and MC5R) was mixed with 0.5 µl of compounds at various concentrations or DMSO. 20 µl membrane homogenate to a final concentration of 5 µg/µl (MC1R), 50 µg/µl (MC3R) or 10 µg/µl (MC4R, MC5R) were added together with 10 µl $^{[125]}$-NDP-α-MSH (2200 Ci/mmol specific activity, PerkinElmer NEX352) to a final concentration of 0.1 nM. Plates were incubated for 16-23 h in the dark at room temperature before reading on a 1450 MicroBeta Trilux.”

Measurements of cAMP production were performed using LANCE® cAMP kits (PerkinElmer) in a total assay volume of 12 µl/well. In brief, ligands were dispensed into small-volume 384-well plates (Greiner) before addition of 1000 cells/well in buffer (Hank’s balanced salt solution with Ca$^{2+}$ and Mg$^{2+}$ (HBSS) (Thermo Fisher), 20 mM HEPES pH7.4 (Thermo Fisher), 0.1% BSA (Sigma)). cAMP production was stimulated for 45 min at 37°C in the presence of 1 mM IBMX (Sigma) and 250 µM Alexa Fluor® 647-anti cAMP antibody (LANCE cAMP kit). The reaction was stopped with detection buffer containing 10 µM Biotin-cAMP and 15 µM LANCE Eu-W8044 labelled streptavidin (PerkinElmer LANCE cAMP kit). cAMP production was detected by homogenous time resolved fluorescence (HTRF) ($\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 665 and 615 nm) using a Pherastar (BMG Labtech).

PepNats, literature compounds and references were tested in ten-point concentration response (½ log serial dilution) with 100, 10 or 1 µM as final start concentrations. Raw data output was analyzed in Screener software (Genedata AG) and pEC$_{50}$ and pIC$_{50}$ values calculated using a four-parameter logistic fit.
2) C) Representative Procedures

Automated Solid Phase Peptide Synthesis (RP_01)

Linear peptide precursors were synthesized with a SyroXP solid-phase peptide synthesizer using Rink Amide AM resin Low Loading 1 (100-200 mesh, loading: 0.16–0.40 mmol/g). The peptide was first covalently bound to the required resin after Fmoc deprotection. A 3-step cycle (Fmoc deprotection, chain elongation via double coupling procedure and capping) was used as described in the table below with extensive DMF washing in between.

Conditions for the SPPS with a Syro I peptide synthesizer

| Cycle† | Reagents | Protocol |
|--------|----------|----------|
| Fmoc deprotection | 20% piperidine in DMF | 3 min reaction, 10 s vortex, 1 min break, 20 s emptying then 5 min reaction, 10 s vortex, 1 min break, 20 s emptying |
| Double coupling of amino acid | Fmoc-aa-OH (3 eq.) in Oxyma Pure (0.5 M) HATU (3 eq.) in DMF (0.5 M) DIPEA (10 eq.) in NMP (2 M) | 40 min reaction, 15 s vortex, 2 min break, 20 s emptying 40 min reaction, 15 s vortex, 2 min break, 20 s emptying |
| Capping | Ac2O in NMP (10%) DIPEA in NMP (2 M) | 10 min reaction, 10 s vortex, 1 min break, 20 s emptying |

† All the cycles were performed at room temperature

Finally, the linear functionalized peptide resin 2 was collected and washed with DCM (3 × 6 mL), DMF (3 × 6 mL) and DCM (3 × 6 mL) and quickly dried with vacuum filtration under a stream of argon. The resin was stored at –20 °C until further use in the next reaction step.

Test cleavage of the linear functionalized peptide resin 2:

The test cleavage was performed on a small portion of the functionalized resin (ca. 2 mg) using a solution of TFA/H₂O/TIS (600 μL, 95:2.5:2.5, v/v/v). After shaking for 1.5 to 3 h at room temperature, the filtrate was transferred to a 1.5 mL conical Eppendorf tube and cooled Et₂O was added. The resulting white precipitate was collected by centrifugation (4000 rpm, 4 min). The supernatant was removed. The white residue was quickly dried under a stream of argon and dissolved in H₂O + 0.1% TFA and MeCN + 0.1% TFA (80 μL, 50:50, v/v) and analyzed by Analytical RP-HPLC-MS (I).

If no precipitation occurred in Et₂O, the sample was dried under a flow of argon and the remaining residue was dissolved in H₂O + 0.1% TFA and MeCN + 0.1% TFA (80 μL, 50:50, v/v) and analyzed by Analytical RP-HPLC-MS (I).

Mtt Cleavage and 4-carboxybenzaldehyde coupling (RP_02)

Step 1:

The functionalized peptide resin 2 (250.0 mg, 85.0 μmol, unfunctionalized starting loading: 0.34 mmol/g) was treated with a solution of DCM/TFA/TIS (8 mL, 95:2:3, v/v/v) for 2 min at room temperature. The solution was removed under vacuum filtration and the resin was washed with
DCM (3 x 6 mL). The procedure was repeated seven to eight additional times. Finally, the resin was washed with DCM (3 x 6 mL), 5% DIPEA in DCM (3 x 6 mL), DMF (3 x 6 mL) and DCM (3 x 6 mL).

**Step 2:** To a solution of 4-carboxybenzaldehyde (38.3 mg, 255.0 μmol, 3 eq.) in DMF (3 mL) was added HATU (97.0 mg, 255.0 μmol, 3 eq.) followed by DIPEA (118 μL, 0.68 mmol, 8 eq.). The resulting mixture was stirred for 5 min before adding it to a suspension of Mtt deprotected peptide resin from step 1 in DMF (2 mL). The suspension was shaken at room temperature for 2.5 h. The solution was removed by vacuum filtration and the resin was washed with DCM (3 x 6 mL), DMF (3 x 6 mL) and DCM (3 x 6 mL). The desired aldehyde containing peptide resin was quickly dried under vacuum filtration with a flow of argon.

**Test cleavage for the aldehyde functionalized peptide 3:**

To assess the completion of the aldehyde coupling, a small portion of the functionalized resin (ca. 2 mg) was treated with a solution of TFA/H₂O (600 μL, 95:5, v/v). After shaking for 20 min, the filtrate was transferred to a 1.5 mL conical Eppendorf tube and cooled Et₂O was added. The white precipitate was collected by centrifugation (4000 rpm, 4 min) and quickly dried under a stream of argon. The obtained solid was dissolved in H₂O + 0.1% TFA and MeCN + 0.1% TFA (80 μL, 50:50, v/v) and analyzed by Analytical RP-HPLC-MS (I).

If no precipitation occurred in Et₂O, the sample was dried under a flow of argon and the remaining residue was dissolved in H₂O + 0.1% TFA and MeCN + 0.1% TFA (80 μL, 50:50, v/v) and analyzed by Analytical RP-HPLC-MS (I).

**Fmoc deprotection (RP_03)**

The Fmoc protecting group was removed by treating the aldehyde containing resin 3 (250.0 mg, 85.0 μmol, loading 0.34 mmol/g) with a solution of 20% piperidine in DMF (6 mL) for 4 min. The solution was removed by vacuum filtration and the resin was washed with DMF (3 x 6 mL). The deprotection was repeated two additional times for 4 min each. The resin was finally washed with DMF (4 x 6 mL) and DCM (4 x 6 mL) and directly used into the next step without further modification.

**Intramolecular imine cyclization followed by 1,3-dipolar cycloaddition (RP_04)**

**Step 1:**

The above Fmoc deprotected peptide resin (250.0 mg, 85.0 μmol, starting unfunctionalized loading: 0.34 mmol/g) was treated with Et₃N (36 μL, 255.0 μmol, 3 eq.) in DMF (910 μL) and trimethyl orthoformate (1.8 mL, 16.7 mmol, 196 eq.) at room temperature for 2.5 h to form the intermediate cyclic imine 4.
Step 2:
To the resin suspension from step 1 was added a solution of dipolarophile (6−14) (215.5 μmol, 2.5 eq.), LiBr (18.5 mg, 212.5 μmol, 2.5 eq.) and Et₃N (36 μL, 255.0 μmol, 3 eq.) in THF (2.7 mL). The resulting suspension was shaken from 16 to 48 h at room temperature. The solution was removed by vacuum filtration and the resin was washed with DCM (3 × 6 mL), DMF (3 × 6 mL) and DCM (3 × 6 mL). The desired PepNat cycloadduct resin 5 then quickly dried under vacuum filtration with a flow of argon.

Test cleavage for the PepNat cycloadduct resin 5:
The test cleavage was performed with a small portion of the resin from step 2 (ca. 2 mg) using a solution of TFA/H₂O/TIS (600 μL, 95:2.5:2.5, v/v/v). After shaking for 1.5 to 3.5 h at room temperature, the filtrate was collected by vacuum filtration in a 1.5 mL conical Eppendorf tube and cooled Et₂O was added. The resulting white precipitate was collected by centrifugation (4000 rpm, 4 min). The supernatant was removed and the white residue was quickly dried under air and dissolved in H₂O + 0.1% TFA and MeCN + 0.1% TFA (80 μL, 50:50, v/v) and analyzed by RP-HPLC-MS (I or II).
If no precipitation occurred in Et₂O, the sample was dried under a flow of argon and the remaining residue was dissolved in H₂O+0.1%TFA and MeCN+0.1%TFA (80 μL, 50:50, v/v) and analyzed by RP-HPLC-MS (I or II).
The diastereomeric ratio of the cycloaddition was determined using RP-HPLC-MS (I or II) and integration of the chromatogram at 210 nm.

Full cleavage from the Rink Amide Low Loading resin:
The resin 5 from step 2 was shaken with a solution of TFA/H₂O/TIS (8 mL, 95:2.5:2.5, v/v/v) at room temperature for 1.5 to 3.5 h. The solution was then transferred to a falcon tube and ice cooled Et₂O (40 mL) was added. After 20 min at 0 °C, the resulting precipitate was collected by centrifugation (4000 rpm, 4 °C, 10 min) and dissolved in MeCN/H₂O (34 mL, 5:95, v/v) and freeze dried overnight to afford the crude PepNat.

Purification by semi-preparative RP-HPLC-MS:
The crude PepNat was dissolved in DMSO (ca. 1 mL) and purified by semi-preparative RP-HPLC-MS. Selected pure fractions were combined and lyophilized to afford the desired PepNat (A−P). The reported yield corresponds to the overall isolated yield after purification by semi-preparative RP-HPLC from the starting unfunctionalized Rink Amide resin, through a total of six steps after the SPPS linear precursor synthesis on resin, unless otherwise noted.
3) Experimental Section

3) A) Synthesis of Lower Loaded Rink Amide Resin and Loading Quantification

Representative Procedure for the Synthesis of Lower Loaded Rink Amide Resin

**Step 1:** Commercially available Rink Amide Low Loading resin (602 mg, 0.22 mmol, loading 0.36 mmol/g) was treated with 20% piperidine in DMF (6 mL) for 4 min. The treatment was repeated 2 additional times. The resin was finally washed with DMF (3 × 8 mL) and DCM (3 × 8 mL) then quickly dry under a flow of argon.

**Step 2:** To a solution of Fmoc-Gly-OH (97 mg, 0.33 mmol) and N-acetylglycine (38 mg, 0.33 mmol) in DMF (6 mL) was added HCTU (259 mg, 0.65 mmol) followed by DIPEA (0.3 mL, 1.73 mmol). The mixture was stirred for few minutes and added to the resin from step 1 in suspension in DMF (4 mL). The suspension was shaken for 2 h at room temperature. The solution was removed by vacuum filtration and the resin was washed DMF (3 × 8 mL) and DCM (3 × 8 mL) then quickly dry under a flow of argon.

**Step 3:** To a solution of Fmoc-Gly-OH (97 mg, 0.33 mmol) and N-acetylglycine (38 mg, 0.33 mmol) in DMF (6 mL) was added HATU (247 mg, 0.65 mmol) followed by DIPEA (0.3 mL, 1.73 mmol). The mixture was stirred for few minutes and added to the resin from step 2 in suspension in DMF (4 mL). The suspension was shaken for 16 h at room temperature. The resin was washed with DMF (3 × 8 mL) and DCM (3 × 8 mL) then Et₂O (4 × 8 mL) and finally dried under vacuum to afford the lower loaded Rink Amide resin (0.11 mmol of Fmoc-Gly available for functionalization, calculated loading 0.18 mmol/g, 51% loading reduction).

Resin Loading Quantification by UV Absorbance

A sample of the desired dry resin (ca. 10 mg) was treated with 20% piperidine in DMF (10 mL) for 30 min. The filtrate was collected by filtration. The mean absorbance of the dibenzofulvene–piperidine adduct contained in the filtrate was determined at 301 nm with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Nanodrop 2000c, Quartz cuvette with optical path length = 1 cm) as an average of three independent measurements. The initial concentration was calculated using the Lambert–Beer’s law equation with the molar extinction coefficient of 8021 l.mol⁻¹.cm⁻¹. The final loading of the resin was deduced from the calculated initial concentration and reported in Figure 3a.
3) B) Synthesis and Characterization of Linear Peptide Precursors 2 on Solid Support

The following linear peptides 2 were synthesized according to the representative procedure (RP_01). The successful formation of the desired linear peptide was confirmed by test cleavage as described above followed by analytical RP-HPLC-MS (I) analysis using method (I-A). The table below summarizes the obtained linear peptides 2 with the observed retention time and [M+H]^+ ion.

| peptide | sequence       | MW (g.mol⁻¹) | t_R (min) | [M+H]^+ found |
|----------|----------------|--------------|-----------|---------------|
| 2a       | GFALFPFGKG-Resin | 1275.50      | 4.5       | 1275.7        |
| 2b-1     | GFRFFNAFKY-Resin | 1531.76      | 3.9       | 1531.7        |
| 2b-2     | GFRFFNAFKG-Resin | 1425.63      | 3.6       | 1424.8        |
| 2c-1     | GFRffNAFKY-Resin | 1531.76      | 3.9       | 1531.8        |
| 2c-2     | GFRffNAFKG-Resin | 1425.63      | 3.8       | 1426.7        |
| 2d-1     | GFDDNNNKG-Resin  | 1213.30      | 3.9       | 1213.7        |
| 2d-2     | GFDDNNNOrnG-Resin| 1199.27      | 3.9       | 1200.4        |
| 2d-3     | GFDDNNNDAbG-Resin| 1185.25      | 3.9       | 1185.6        |
| 2d-4     | GFDDNNNDapG-Resin| 1171.22      | 4.0       | 1171.5        |
| 2d-5     | GFDDNNNKG-Resin  | 1123.77      | 3.2       | 1123.7        |
| 2d-6     | GLDINNNKG-Resin  | 1179.28      | 3.8       | 1180.1        |
| 2d-7     | GPDDNNNKG-Resin  | 1149.21      | 3.3       | 1149.8        |
| 2d-8     | GfDDNNNKG-Resin  | 1213.30      | 3.9       | 1213.7        |
| 2d-9     | GFDDNNNKG-Resin  | 1199.27      | 3.7       | 1199.8        |
| 2d-10    | GVDINNNKG-Resin  | 1165.27      | 3.6       | 1165.6        |
| 2d-11    | GFEEKDINNNVKKT-Resin | 1871.08 | 2.8       | 936.3*        |
| 2d-12    | GFDDNNNKVKG-Resin| 1313.43      | 3.7       | 1313.3        |
| 2d-13    | GfINNNKG-Resin   | 1098.23      | 3.8       | 1098.6        |
| 2d-14    | GVINNNKG-Resin   | 1050.24      | 3.4       | 1050.5        |
| 2d-15    | GFNNNK-Resin     | 985.07       | 6.4       | 985.4         |
| 2e       | GFHAASNKG-Resin  | 1123.24      | 3.0       | 1123.4        |
| 2f       | GFRFFNAKY-Resin  | 1384.66      | 3.7       | 1384.7        |
| 2g-1     | GFRffNAKY-Resin  | 1384.66      | 3.7       | 1384.5        |
| 2g-2     | GFRffNAKG-Resin  | 1278.46      | 3.6       | 1278.6        |
| 2h-1     | GFGLGFKF-Resin   | 1107.30      | 4.6       | 1107.5        |
| 2h-2     | GFGLGFK-Resin    | 1065.24      | 4.5       | 1065.5        |
| 2i       | GFLKPIKG-Resin   | 1094.35      | 3.6       | 1094.5        |
| 2j-1     | GFRFFNKY-Resin   | 1313.50      | 3.6       | 1313.6        |
| 2j-2     | GFRFFNKG-Resin   | 1207.38      | 3.6       | 1207.5        |
| 32k-1    | GFRffNKY-Resin   | 1313.50      | 3.7       | 1313.6        |
| 32k-2    | GFRffNKG-Resin   | 1207.38      | 3.6       | 1207.8        |
| 2l       | GFRANK-Resin     | 984.11       | 3.2       | 984.7         |
| 2m       | GFRGDK-Resin     | 914.02       | 3.2       | 914.5         |
| 2n-1     | GFRFFKY-Resin    | 1199.40      | 3.7       | 1199.6        |
| 2n-2     | GFRFFKG-Resin    | 1093.28      | 3.6       | 1093.6        |
| 2n-3     | GFRFFK-Resin     | 1036.23      | 3.7       | 1036.6        |
| 2o-1     | GFRffKY-Resin    | 1199.40      | 3.7       | 1199.7        |
| 2o-2     | GFRffKG-Resin    | 1093.28      | 3.7       | 1093.7        |
| 2o-3     | GFRffK-Resin     | 1036.23      | 3.8       | 1036.7        |
| 2p       | GFFLGK-Resin     | 903.08       | 4.4       | 903.6         |

Bold residues are N-methylated. Dap = Diaminopropanoic acid. Dab = Diaminobutanoic acid. Orn = Ornithine.
Lower case letters indicate α-amino acids. * indicate that the observed mass corresponds to [M+H]^+.
3) C) Synthesis and Characterization of Aldehyde Containing Linear Peptides 3 on Solid Support

The aldehyde functionalized peptides 3 were prepared from the respective linear peptide precursors 2 using Mtt deprotection followed by amide coupling of the 4-carboxybenzaldehyde as described in RP_02. The completion was confirmed by test cleavage with a solution of TFA/H₂O (95:5) as specify in the representative procedure followed by analytical RP-HPLC-MS (i) analysis using method (I-A). The table below summarizes the observed tᵣ and [M+H]⁺ ion of peptide 3.

| peptide | sequence | MW (g.mol⁻¹) | tᵣ (min) | [M+H]⁺ found |
|---------|----------|--------------|----------|--------------|
| 3a      | GFALFPFK(Ald)G-Resin | 1407.61 | 6.2 | 1407.4 |
| 3b-1    | GFRFFNAFK(Ald)Y-Resin | 1664.89 | 4.9 | 1664.7 |
| 3b-2    | GFRFFNAFK(Ald)G-Resin | 1557.75 | 4.7 | 1557.9 |
| 3c-1    | GFRFFNAFK(Ald)Y-Resin | 1633.87 | 4.7 | 1644.8 |
| 3c-2    | GFRFFNAFK(Ald)G-Resin | 1557.75 | 4.7 | 1558.8 |
| 3d-1    | GFDDINNK(Ald)G-Resin | 1345.41 | 4.7 | 1345.4 |
| 3d-2    | GFDDINNOrn(Ald)G-Resin | 1331.39 | 5.0 | 1332.3 |
| 3d-3    | GFDDINNNDab(Ald)G-Resin | 1317.36 | 5.0 | 1317.3 |
| 3d-4    | GFDDINNNDap(Ald)G-Resin | 1303.34 | 4.9 | 1303.3 |
| 3d-5    | GGDINNNK(Ald)G-Resin | 1256.29 | 4.1 | 1255.5 |
| 3d-6    | GGDINNNK(Ald)G-Resin | 1311.42 | 4.7 | 1311.6 |
| 3d-7    | GPDINNNK(Ald)G-Resin | 1281.34 | 4.3 | 1281.4 |
| 3d-8    | GPDINNNK(Ald)G-Resin | 1345.41 | 4.7 | 1347.2 |
| 3d-9    | GPDINNNK(Ald)G-Resin | 1331.39 | 4.6 | 1331.4 |
| 3d-10   | GVDINNNK(Ald)G-Resin | 1297.39 | 4.6 | 1297.6 |
| 3d-11   | GFEEDINNNVKK(Ald)T-Resin | 2003.20 | 3.28 | 1002.3* |
| 3d-12   | GFDDINNNVK(Ald)G-Resin | 1444.57 | 4.9 | 1444.5 |
| 3d-13   | GFDDINNNK(Ald)G-Resin | 1230.35 | 4.9 | 1230.2 |
| 3d-14   | GVDINNNK(Ald)G-Resin | 1182.11 | 4.6 | 1182.3 |
| 3d-15   | GDNINNK(Ald)G-Resin | 1117.19 | 4.5 | 1117.1 |
| 3e      | GFAPASKN(Ald)G-Resin | 1255.35 | 7.7 | 1255.4 |
| 3f      | GFRFFNAK(Ald)Y-Resin | 1517.71 | 4.6 | 1516.8 |
| 3g-1    | GFRFFNAK(Ald)Y-Resin | 1517.71 | 4.5 | 1516.8 |
| 3g-2    | GFRFFNAK(Ald)G-Resin | 1410.58 | 4.4 | 1410.8 |
| 3h-1    | GFGGFK(Ald)F-Resin | 1239.42 | 6.4 | 1239.5 |
| 3h-2    | GFGGFK(Ald)-Resin | 1197.36 | 6.4 | 1197.3 |
| 3i      | GFLKPK(Ald)G-Resin | 1226.49 | 4.2 | 1226.6 |
| 3i-1    | GFRFFNKN(Ald)Y-Resin | 1445.62 | 4.6 | 1445.6 |
| 3i-2    | GFRFFNKN(Ald)G-Resin | 1339.60 | 4.6 | 1339.7 |
| 3k-1    | GFRFFNKN(Ald)Y-Resin | 1445.62 | 4.5 | 1445.8 |
| 3k-2    | GFRFFNKN(Ald)G-Resin | 1339.50 | 4.4 | 1339.9 |
| 3l      | GFRANK(Ald)G-Resin | 1116.23 | 4.0 | 1116.8 |
| 3m      | GFRGDK(Ald)-Resin | 1046.13 | 4.0 | 1046.6 |
| 3n-1    | GFRFFK(Ald)Y-Resin | 1331.52 | 4.6 | 1331.6 |
| 3n-2    | GFRFFK(Ald)G-Resin | 1225.40 | 4.4 | 1225.7 |
| 3n-3    | GFRFFK(Ald)-Resin | 1168.34 | 4.7 | 1168.8 |
| 3o-1    | GFRFFK(Ald)Y-Resin | 1331.52 | 4.7 | 1331.7 |
| 3o-2    | GFRFFK(Ald)G-Resin | 1225.40 | 4.6 | 1225.7 |
| 3o-3    | GFRFFK(Ald)-Resin | 1168.34 | 4.7 | 1168.8 |
| 3p      | GFFLK(Ald)G-Resin | 1035.21 | 6.2 | 1035.3 |

(Ald) indicate coupling of 4-carboxybenzaldehyde on the free amine side chain residue. Bold residues are n-methylated. Dap = Diaminopropanoic acid. Dab = Diaminobutyanoic acid. Orn = Ornithine. Lower case letters indicate d-amino acids.
3) D) Synthesis of Dipolarophiles 8, 11–14

1-(4-Methoxyphenyl)-1H-pyrrole-2,5-dione (8)

Step 1: Maleic anhydride (1.50 g, 15.30 mmol) was dissolved in Et₂O (25 mL) then 4-methoxyaniline (1.88 g, 15.30 mmol) in Et₂O (13 mL) was added dropwise using a dropping funnel at room temperature. The resulting cloudy yellow reaction mixture was stirred at room temperature for 1.5 h. The reaction mixture was then cooled to 0 °C. The precipitate was collected by filtration and dried under vacuum to afford the intermediate N-substituted maleanilic acid as a yellow orange solid.

Step 2: To a solution of sodium acetate (0.50 g, 6.12 mmol) in acetic anhydride (8 mL, 15.30 mmol) was added the intermediate maleanilic acid solid from step 1 at room temperature under inert atmosphere. The reaction mixture was refluxed for 45 min. The reaction mixture was cooled to room temperature and then poured into a water-ice mixture (75 mL). The precipitate was recovered by filtration, washed ice-cold water (3 × 30 mL) and dried under high vacuum to afford the crude product (2.61 g) as a green yellow solid. The crude product was recrystallized from EtOH/H₂O (15 mL, 2:1, v/v) to afford the desired product 8 (1.92 g, 9.47 mmol, 62% yield) as a green crystalline solid.

¹H NMR (500 MHz, CDCl₃) δ 7.23 (d, J = 8.92 Hz, 2H), 6.98 (d, J = 8.93 Hz, 2H), 6.83 (s, 2H), 3.82 (s, 3H); ¹³C NMR (126 MHz, CDCl₃) δ 170.0, 159.3, 134.3 (× 2), 127.7 (× 2), 123.9, 114.6 (× 2), 55.2. The NMR data are in agreement with the data reported in the literature.⁸

(E)-3-benzylideneindolin-2-one (11)

To indolin-2-one oxindole (1.00 g, 7.51 mmol) in dry EtOH (11.0 mL) was added benzaldehyde (915 µL, 9.01 mmol) followed by piperidine (74.2 µL, 0.75 mmol). After refluxing the resulting mixture at 70°C for 6.5 h, the reaction was cooled to room temperature which triggered a spontaneous precipitation. The residue was dissolved in absolute EtOH (10 mL) and stored at −20 °C overnight. The yellow precipitate was filtered off, dried under reduce pressure. The resulting solid was...
purified by recrystallization from EtOH (11 mL) to afford the title product (1.10 g, 66% yield) as a yellow crystalline solid and E-isomer (E/Z ≥ 95:5).

$^1$H NMR (500 MHz, CDCl$_3$) δ 8.54 (s, 1H), 7.85 (s, 1H), 7.67 (d, $J$ = 7.3 Hz, 2H), 7.65 (d, $J$ = 7.8 Hz, 1H), 7.49 (dd, $J$ = 11.4, 4.5 Hz, 2H), 7.44 (dd, $J$ = 6.3, 3.7 Hz, 1H), 7.22 (t, $J$ = 7.3 Hz, 1H), 7.21 (d, $J$ = 7.8 Hz, 1H), 6.87 (t, $J$ = 7.7, 0.8 Hz, 1H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 170.3, 141.6, 137.8, 135.0, 130.1, 129.8, 129.5 (× 2), 128.8 (× 2), 127.6, 123.2, 122.0, 121.9, 110.3. The NMR data are in agreement with the one reported in the literature.$^9$

$^{(E)}$-3-(4-methoxybenzylidene)indolin-2-one (12)

![Image of structure 12]

To a solution of indolin-2-one (500 mg, 3.76 mmol) in EtOH (10 mL) was added $p$-anisaldehyde (548 µl, 4.51 mmol) followed by piperidine (37.1 µl, 0.38 mmol). The reaction mixture was refluxed for 6 h then cooled to room temperature which triggered a spontaneous precipitation. To the suspension was added additional EtOH (15 mL) before storing overnight at -20°C. The yellow solid was filtered off and washed with cold EtOH. The crude solid was recrystallized using EtOH (20 mL) to afford the title product (2.02 g, 66% yield) as yellow solid and E isomer (E/Z ≥ 99:1).

$R_f$ 0.47 (AcOEt – cHex 50:50); $^1$H NMR (500 MHz, CDCl$_3$) δ 8.46 (s, 1H), 7.81 (s, 1H), 7.78 (d, $J$ = 7.7 Hz, 1H), 7.69 (d, $J$ = 8.7 Hz, 2H), 7.22 (m, 1H), 7.00 (m, 3H), 6.93 (d, $J$ = 7.6 Hz, 1H), 3.89 (s, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$) δ 170.5, 161.2, 141.1, 138.6, 134.8, 131.8 (2C), 129.6, 127.2, 122.8, 122.1, 122.1, 114.3, 114.0 (2C), 110.4, 55.6; HRMS (ESI) calculated for C$_{16}$H$_{14}$NO$_2$ [M+H]$^+$ = 252.10191, found 252.10163.

$^{(E)}$-3-(4-fluorobenzylidene)indolin-2-one (13)

![Image of structure 13]

To a solution of indolin-2-one (2.00 g, 14.02 mmol) in EtOH (40 mL) was added 4-fluorobenzaldehyde (1.90 mL, 18.04 mmol) followed by piperidine (1.40 mL, 16.5 mmol). The reaction mixture was refluxed for 1.5 h then cooled to room temperature which triggered a spontaneous precipitation. To the suspension was added additional EtOH (15 mL) before storing
overnight at -20°C. The yellow solid was filtered off and washed with cold EtOH. The crude solid was recrystallized using EtOH (10 mL) to afford the title product (291.6 mg, 31% yield) as yellow solid and E isomer \((E/Z \geq 99:1)\).

\[ R_f 0.55 \text{ (AcOEt – cHex 50:50); } ^1H \text{ NMR (500 MHz, CDCl}_3\text{) } \delta 8.89 \text{ (s, 1H), 7.79 \ (s, 1H), 7.64 - 7.7 \ (m, 2H), 7.61 \ (d, J=7.7 \text{ Hz, 1H), 7.21} - 7.26 \text{ (m, 1H), 7.17 (t, J=8.6 \text{ Hz, 2H), 6.94 (d, J=7.8 \text{ Hz, 1H), 6.89 (t, J=7.7 Hz, 1H); } ^13C \text{ NMR (126 MHz, CDCl}_3\text{) } \delta 170.4, 163.4 \text{ (}^{1}J(CF) = 248 \text{ Hz), 141.7, 136.6 \text{ (}^{5}J(CF) = 2.3 \text{ Hz), 134.5 \text{ (}^{4}J(CF) = 8.5 \text{ Hz), 131.5 \ (× 2, } ^{3}J(CF) = 8.2 \text{ Hz), 130.2, 127.6, 123.0, 122.1, 121.6, 116.1 \ (× 2, } ^{2}J(CF) = 22.6 \text{ Hz), 110.6; HRMS (ESI) calculated for C}_{15}H_{11}NOF \ [M+H]^+ = 240.08192, \text{ found 240.08164. }}\]

**5-Benzylidene-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione (14)**

![](image)

To a clear solution of 1,3-dimethylbarbituric acid (300 mg, 1.92 mmol) in H\(_2\)O (13 mL) was added in one portion benzaldehyde (0.195 mL, 1.92 mmol) at room temperature. The reaction mixture was stirred at rt for 4 h. The white precipitate was collected by filtration and wash with cold water (20 mL) and cold ether (5 mL). The solid was dried under reduce pressure overnight to afford the desired product 9 (390 mg, 83 %) as a white solid.

\[^1H \text{ NMR (400 MHz, CDCl}_3\text{) } \delta 8.58 \text{ (s, 1H), 8.06 (d, J = 7.3 \text{ Hz, 2H), 7.53 (m, 1H), 7.48 (d, J = 7.8 \text{ Hz, 2H), 3.43 (s, 3H), 3.38 (s, 3H); } ^13C \text{ NMR (101 MHz, CDCl}_3\text{) } \delta 162.6, 159.5 \ (× 2), 151.4, 133.6 \ (× 2), 133.1, 132.8, 128.4 \ (× 2), 117.7, 29.3, 28.6. \text{ The NMR data are in agreement with the data reported in the literature.}^{10}}\]
3) E) Synthesis on Solid Support and Characterization of PepNats

[N-Me-fused-di-pyrrolidine-nMeFALFPGFK]G, A1

The titled fused di-pyrrolidine-peptide PepNat was prepared from supported aldehyde peptide resin 3a (80 mg, 9.6 µmol, calculated loading 0.12 mmol/g, starting unfunctionalized loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (2.7 mg, 24.0 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-B showed that the cycloaddition proceeded with a 8:92 diastereomeric ratio (tR, minor = 11.7 min, tR, major = 11.8 min with [M+H]+ = 1278.6 and [M+2H]2+ = 640.0). The crude product (13.7 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 25% to 85% of MeCN + 0.1% TFA in H2O + 0.1% TFA in 25 min at a flow rate of 6 mL/min to afford the major PepNat A1-major (2.7 mg, 2.11 µmol, 22% cycloaddition yield and 8% overall yield) as a white fluffy solid and a single diastereomer (d.e. = 91%).

Major diastereomer A1-major: RP-HPLC-MS (I): Method I-B, tR, minor = 11.7 min; tR, major = 11.9 min, [M+H]+ = 1278.7, [M+2H]2+ = 640.0, d.r. = 7:98; Analytical Purity-RP-HPLC: 99%; HRMS (ESI) calculated for C67H84O13N13 [M+H]+ = 1278.63061, found 1278.63135.

[N-Ph-fused-di-pyrrolidine-nMeFALFPGFK]G, A2

The titled PepNat was prepared from supported aldehyde peptide resin 3a (70 mg, 24.5 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide
(7) (10.6 mg, 61.3 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 89:11 diastereomeric ratio ($t_{R,\text{major}} = 4.9 \text{ min}, t_{R,\text{minor}} = 5.3 \text{ min}$ with $[\text{M+H}]^+ = 1340.8$ and $[\text{M+2H}]^{2+} = 671.3$) according to the RP-HPLC-MS (I) chromatogram (Method I-A). The crude product (14.9 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 25% to 85% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 25 min at a flow rate of 6 mL/min to yield the major diastereomer A2-major (3.4 mg, 2.50 µmol, 10%) as a white fluffy solid and a single diastereomer (d.e. = 90%).

**Major diastereomer A2-major:** RP-HPLC-MS (I): Method I-A, $t_{R,\text{major}} = 4.9 \text{ min}, t_{R,\text{minor}} = 5.3 \text{ min}$, $[\text{M+H}]^+ = 1341.4$, $[\text{M+2H}]^{2+} = 671.1$; RP-HPLC-MS (I): Method I-B, $t_{R,\text{major}} = 13.2 \text{ min}; t_{R,\text{minor}} = 13.6 \text{ min}$, $[\text{M+H}]^+ = 1341.4$, $[\text{M+2H}]^{2+} = 671.1$, d.r. = 90:10; Analytical Purity-RP-HPLC: 94%; HRMS (ESI) calculated for C$_{72}$H$_{86}$O$_{13}$N$_{13}$ $[\text{M+H}]^+ = 1340.64626$, found 1340.64692.

[pyrrolidine-κMeFALFPGFK]G, A3

![A3-major](image)

The *titled PepNat* was prepared from supported aldehyde peptide resin 3a (70 mg, 24.5 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with dimethyl maleate (10) (8.8 mg, 61.3 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-A showed that the cycloaddition proceeded with a 70:30 diastereomeric ratio ($t_{R,\text{major}} = 4.9 \text{ min}, t_{R,\text{minor}} = 5.1 \text{ min}$ with $[\text{M+H}]^+ = 1311.7$ and $[\text{M+2H}]^{2+} = 656.7$). The crude product (13.3 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 25% to 85% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 25 min at a flow rate of 6 mL/min to afford the major diastereomer A3-major (4.5 mg, 3.43 µmol, 14%) as a white fluffy solid and a single diastereomer (d.e. = 66%).

**Major diastereomer A3-major:** RP-HPLC-MS (I): Method I-A, $t_{R,\text{major}} = 4.8 \text{ min}; t_{R,\text{minor}} = 5.0 \text{ min}$, $[\text{M+H}]^+ = 1311.6$, $[\text{M+2H}]^{2+} = 656.5$; RP-HPLC-MS (I): Method I-B, $t_{R,\text{major}} = 12.9 \text{ min}; t_{R,\text{minor}} = 13.4 \text{ min}$, $[\text{M+H}]^+ = 1311.6$, $[\text{M+2H}]^{2+} = 656.6$, d.r. = 83:17; Analytical Purity-RP-HPLC: 98%; HRMS (ESI) calculated for C$_{68}$H$_{87}$O$_{15}$N$_{12}$ $[\text{M+H}]^+ = 1311.64084$, found 1311.64165.
The titled 3,3′-pyrrolidinyl-spirooxindole PepNat was prepared from supported aldehyde peptide resin 3a (80 mg, 28.0 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with (E)-3-benzylidene-indolin-2-one 11 (15.5 mg, 84.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 37:22:21:20 diastereomer ratio (t\text{R,dia1} = 30.2 min, t\text{R,dia2} = 30.4 min, t\text{R,dia3} = 31.0 min, t\text{R,dia4} = 31.5 min with [M+H]^+ = 1389.6 and [M+2H]^{2+} = 695.1) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). Purification by semi-preparative-RP-HPLC of the crude product (13.9 mg), using a step gradient of 25% to 85% of MeCN + 0.1% TFA in H\textsubscript{2}O + 0.1% TFA in 25 min at a flow rate of 6 mL/min afforded the product A4-mixt. (3.1 mg, 2.23 µmol, 8%) as a white fluffy solid and an inseparable mixture of four diastereomers (d.r. = 63:5:21:11).

Isolated mixture of diastereomers A4-mixt.: Optimized RP-HPLC-MS (II): Method II-C, t\text{R,dia1} = 30.2 min, t\text{R,dia2} = 30.4 min, t\text{R,dia3} = 31.0 min, t\text{R,dia4} = 31.5 min, [M+H]^+ = 1388.7 and [M+2H]^{2+} = 695.1, d.r. 63:5:21:11; Analytical Purity-RP-HPLC: 92%; HRMS (ESI) calculated for C\textsubscript{77}H\textsubscript{90}O\textsubscript{12}N\textsubscript{13} [M+H]^+ = 1388.68264, found 1388.68560.

The titled fused di-pyrrolidine-peptide macrocycle was prepared from supported aldehyde peptide resin 3b-1 (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar...
cycloaddition on resin with N-methylmaleimide (6) (14.2 mg, 127.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 47:53 diastereomeric ratio (t<sub>R,minor</sub> = 19.0 min, t<sub>R,major</sub> = 19.6 min, with [M+H]<sup>+</sup> = 1535.7, [M+2H]<sup>2+</sup> = 768.3) according to the RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (10.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 15% to 55% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to give the major diastereomer B1-major (1.2 mg, 0.78 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. = 82%).

**Major diastereomer B1-major:** Optimized RP-HPLC-MS (II): Method II-C, t<sub>R,minor</sub> = 19.1 min, t<sub>R,major</sub> = 19.4 min, [M+H]<sup>+</sup> = 1535.8, [M+2H]<sup>2+</sup> = 768.2, d.r. = 9:91; Analytical Purity-RP-HPLC (210 nm): 95%; HRMS (ESI) calculated for C<sub>80</sub>H<sub>96</sub>O<sub>15</sub>N<sub>17</sub> [M+H]<sup>+</sup> = 1534.72663, found 1534.72833.

\[
\text{[N-Me-fused-di-pyrrolidine-\text{NMeFRFFNAFK}]G, B2}
\]

![Diagram of peptide B2-minor](image)

The **titled fused di-pyrrolidine-peptide PepNat** was prepared from supported aldehyde peptide resin 3b-2 (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (14.2 mg, 127.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 22:78 diastereomeric ratio (t<sub>R,minor</sub> = 17.0 min, t<sub>R,major</sub> = 25.8 min, with [M+H]<sup>+</sup> = 1429.9) according to the RP-HPLC-MS (II) chromatogram (Method II-C). The cycloaddition proceeded with a 22:78 diastereomeric ratio (t<sub>R,minor</sub> = 17.0 min, t<sub>R,major</sub> = 25.8 min, with [M+H]<sup>+</sup> = 1429.9) according to the RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (29.9 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 45% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to give the minor diastereomer B2-minor (1.4 mg, 0.98 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Minor diastereomer B2-minor:** Optimized RP-HPLC-MS (II): Method II-C, t<sub>R</sub> = 17.6, [M+H]<sup>+</sup> = 1428.7, [M+2H]<sup>2+</sup> = 715.2; Analytical Purity-RP-HPLC (210 nm): 99%; HRMS (ESI) calculated for C<sub>73</sub>H<sub>90</sub>O<sub>14</sub>N<sub>17</sub> [M+H]<sup>+</sup> = 1428.68477, found 1428.67921.
The *titled fused di-pyrrolidine-peptide hybrid macrocycle* was prepared from supported aldehyde peptide resin 3b-2 (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (22.1 mg, 127.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 61:39 diastereomeric ratio ($t_R,_{\text{minor}} = 19.2$ min, $t_R,_{\text{major}} = 25.7$ min, with [M+H]$^+$ = 1491.7 and [M+2H]$^{2+}$ = 746.2) according to the RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (29.9 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 45% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to yield the minor diastereomer **B3-major** (0.7 mg, 0.47 µmol, 1%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Minor diastereomer B3-major**: Optimized RP-HPLC-MS (II): Method II-C, $t_R = 19.3$, [M+H]$^+$ = 1491.7, [M+2H]$^{2+}$ = 746.2; Analytical Purity-RP-HPLC (210 nm): 95%; HRMS (ESI) calculated for C$_{78}$H$_{92}$O$_{14}$N$_{17}$ [M+H]$^+$ = 1490.70042, found 1490.70131.

The *titled PepNat* was prepared from supported aldehyde peptide resin 3c-1 (250 mg, 100.0 µmol, loading 0.40 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (27.8 mg, 250.0 µmol) according to the representative procedure (RP-04).
MS (I), Method I-B showed that the cycloaddition proceeded with a 16:84 diastereomeric ratio ($t_{R,\text{minor}} = 8.7$ min, $t_{R,\text{major}} = 9.2$ min with $[M+H]^+ = 1535.6$ and $[M+2H]^{2+} = 768.1$). The crude product (46.2 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 50% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 40 min at a flow rate of 6 mL/min to afford the major diastereomer **C1-major** (2.4 mg, 1.56 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer C1-major**: RP-HPLC-MS (I): Method I-B, $t_R = 9.2$ min, $[M+H]^+ = 1535.6$, $[M+2H]^{2+} = 768.2$; Analytical Purity-RP-HPLC (210 nm): 84%; HRMS (ESI) calculated for C$_{80}$H$_{96}$O$_{15}$N$_{17}$ $[M+H]^+ = 1534.72663$, found 1534.72751.

**[Ph-pyrrolidinyl-spirooxindole-nMeFRffNAFK]Y, C2**

![Diagram of C2-major and C2-minor diastereomers]

The **titled 3,3'-pyrrolidinyl-spirooxindole-peptide hybrid macrocycle** was prepared from supported aldehyde peptide resin 3c-1 (300 mg, 120.0 µmol, loading 0.40 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with (E)-3-benzylidene-indolin-2-one (11) (66.4 mg, 300.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 30:31:28:11 diastereomeric ratio ($t_{R,\text{dia1,minor}} = 8.5$ min, $t_{R,\text{dia2, major}} = 8.8$ min, $t_{R,\text{dia3}} = 9.1$ min, $t_{R,\text{dia4}} = 9.2$ min with $[M+H]^+ = 1645.7$ and $[M+2H]^{2+} = 823.2$) according to the RP-HPLC-MS (I) chromatogram (Method I-C). The crude product (63.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 5% to 37% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 18 min at a flow rate of 30 mL/min to give the minor diastereomer **C2-minor** (1.7 mg, 1.03 µmol, 0.9%) as white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the major diastereomer **C2-major** (2.2 mg, 1.34 µmol, 1%) as white fluffy solid and single diastereomer (d.e. ≥ 99%).

**1ˢᵗ diastereomer C2-minor**: RP-HPLC-MS (I): Method I-C, $t_R = 8.6$ min, $[M+H]^+ = 1645.6$, $[M+2H]^{2+} = 823.3$; Analytical Purity-RP-HPLC (210 nm): 98%; HRMS (ESI) calculated for C$_{90}$H$_{102}$O$_{14}$N$_{17}$ $[M+H]^+ = 1644.77867$, found 1644.76639.

**2ⁿᵈ diastereomer C2-major**: RP-HPLC-MS (I): Method I-C, $t_R = 8.8$ min, $[M+H]^+ = 1645.6$, $[M+2H]^{2+} = 823.3$; Analytical Purity-RP-HPLC (210 nm): 96%; HRMS (ESI) calculated for C$_{90}$H$_{102}$O$_{14}$N$_{17}$ $[M+H]^+ = 1644.77867$, found 1644.78069.
The titled PepNat was prepared from supported aldehyde peptide resin 3c-1 (250 mg, 100.0 µmol, loading 0.40 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with (E)-3-(4-methoxybenzylidene)indolin-2-one (12) (62.8 mg, 250.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 26:39:24:11 diastereomeric ratio ($t_{R,\text{dia1, minor}} = 8.4$ min, $t_{R,\text{dia2, major}} = 8.7$ min, $t_{R,\text{dia3}} = 9.1$ min, $t_{R,\text{dia4}} = 9.5$ min with [M+H]$^+$ = 1675.7 and [M+2H]$^{2+}$ = 838.3) according to the RP-HPLC-MS (I) chromatogram (Method I-C). Purification by semi-preparative-RP-HPLC of the crude product (26.2 mg), using a step gradient of 5% to 37% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 18 min at a flow rate of 30 mL/min afforded the minor diastereomer C3-minor (1.4 mg, 0.84 µmol, 1%) as white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the major diastereomer C3-major (1.8 mg, 1.08 µmol, 1%) as white fluffy solid and a single diastereomer (d.e. ≥ 99%).

1$^{\text{st}}$ diastereomer C3-minor: RP-HPLC-MS (I): Method I-C, $t_R = 8.4$ min, [M+H]$^+$ = 1675.6, [M+2H]$^{2+}$ = 838.3; Analytical Purity-RP-HPLC (210 nm): 96%; HRMS (ESI) calculated for C$_{91}$H$_{104}$O$_{15}$N$_{17}$ [M+H]$^+$ = 1674.78923, found 1674.79102.

2$^{\text{nd}}$ diastereomer C3-major: RP-HPLC-MS (I): Method I-C, $t_R = 8.6$ min, [M+H]$^+$ = 1675.6, [M+2H]$^{2+}$ = 838.3; Analytical Purity-RP-HPLC (210 nm): 92%; HRMS (ESI) calculated for C$_{91}$H$_{104}$O$_{15}$N$_{17}$ [M+H]$^+$ = 1674.78923, found 1644.78107.
[N-Me-fused-di-pyrrolidine-nMeFRffNAFK]G, C4

C4-major

The titled fused di-pyrrolidine-peptide hybrid macrocycle was prepared from supported aldehyde peptide resin 3c-2 (120 mg, 33.6 µmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (9.3 mg, 84.0 µmol) according to the representative procedure (RP-04). Optimized Analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 29:71 diastereomeric ratio (tR,minor = 16.3 min, tR,major = 17.3 min with [M+H]⁺ = 1429.2 and [M+2H]²⁺ = 715.5). The crude product (16.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 15% to 45% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 30 min at a flow rate of 6 mL/min to afford the major diastereomer C4-major (1.8 mg, 1.26 µmol, 4%) as a white fluffy solid and single diastereomer (d.e. ≥ 99%).

Major diastereomer C4-major: Optimized RP-HPLC-MS (II): Method II-C, tR = 17.4 min, [M+H]⁺ = 1429.0, [M+2H]²⁺ = 715.3; Analytical Purity-RP-HPLC (210 nm): 98%; HRMS (ESI) calculated for C₇₃H₉₀O₁₄N₁₇[M+H]⁺ = 1428.68477, found 1428.68501.

[N-Me-fused-di-pyrrolidine-nMeFDINNNK]G, D1

D1-major

The titled DINNN PepNat was prepared from supported aldehyde peptide resin 3d-1 (135 mg, 35.0 µmol, calculated loading 0.16 mmol/g, starting unfunctionalized loading 0.26 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (9.7 mg, 87.5 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-A showed that the cycloaddition proceeded with a 96:4 diastereomeric ratio (tR, major = 3.3 min, tR, minor = 3.5
min with [M+H]+ = 1216.6 and [M+2H]2+= 609.2. The crude product (16.2 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 35% of MeCN + 0.1% TFA in H2O + 0.1% TFA in 25 min at a flow rate of 6 mL/min to yield the major PepNat **D1-major** (5.1 mg, 3.8 µmol, 24% cycloaddition yield and 11% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer D1-major**: RP-HPLC-MS (I): Method I-A, tR = 3.3 min, [M+H]+ = 1216.9, [M+2H]2+= 609.0; Optimized RP-HPLC-MS (II): Method II-A, tR = 7.7 min, [M+H]+ = 1216.7, [M+2H]2+ = 609.1; Analytical Purity-RP-HPLC (210 nm): 96%; HRMS (ESI) calculated for C55H74O17N15 [M+H]+ = 1216.53816, found 1216.53975.

**[N-Ph-fused-di-pyrrolidine-nMeFDINNNK]G, D2**

![D2-major](image)

The **titled fused di-pyrrolidine-peptide hybrid macrocycle** was prepared from supported aldehyde peptide resin 3d-1 (200 mg, 70.0 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (30.5 mg, 175.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 83:17 diastereomeric ratio (tR,major = 6.9 min, tR,minor = 7.8 min with [M+H]+ = 1279.0 and [M+2H]2+ = 640.1) according to the analytical RP-HPLC-MS (I) chromatogram (Method I-C). The crude product (78.8 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 100% of MeCN + 0.1% TFA in H2O + 0.1% TFA in 55 min at a flow rate of 6 mL/min to yield the major diastereomer **D2-major** (4.3 mg, 3.36 µmol, 5%) as a white fluffy solid and single diastereomer (d.e. ≥ 99%).

**Major diastereomer D2-major**: RP-HPLC-MS (I): Method I-C, tR = 6.9 min, [M+H]+ = 1279.2, [M+2H]2+ = 640.1; Analytical Purity-RP-HPLC (210 nm): 83%; HRMS (ESI) calculated for C60H76O17N15 [M+H]+ = 1278.55381, found 1278.55629.
The titled DINNN PepNat was prepared from supported aldehyde peptide resin 3d-1 (340 mg, 51 µmol, calculated loading 0.15 mmol/g, starting unfunctionalized loading 0.26 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-(4-methoxyphenyl)maleimide (8) (24.0 mg, 127.5 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a 81:19 diastereomeric ratio ($t_{R,\text{major}}$ = 7.0 min, $t_{R,\text{minor}}$ = 8.0 min with $[M+H]^+ = 1309.0$ and $[M+2H]^{2+} = 655.2$). The crude product (58.2 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 30 min at a flow rate of 6 mL/min to give the product D3-major (13.5 mg, 10.3 µmol, 20% cycloaddition yield and 10% overall yield) as a white fluffy solid and single diastereomer (d.e. ≥ 99%).

Major diastereomer D3-major: RP-HPLC-MS (I): Method I-C, $t_{R} = 7.0$ min, $[M+H]^+ = 1309.3$, $[M+2H]^{2+} = 655.2$; Analytical Purity-RP-HPLC (210 nm): 89% HRMS (ESI) calculated for C$_{61}$H$_{79}$O$_{18}$N$_{15}$ $[M+H]^+ = 1309.57220$, found 1309.55915.

The titled pyrrolidine-peptide macrocycle was prepared from supported aldehyde peptide resin 3d-1 (100 mg, 35.0 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on
resin with dimethyl maleate (10) (12.6 mg, 87.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 41:59 diastereomeric ratio \((t_{R,\text{minor}} = 12.4 \text{ min}, t_{R,\text{major}} = 12.8 \text{ min})\) with \([M+H]^+ = 1249.7 \text{ and } [M+2H]^{2+} = 625.5\) according to the optimized analytical RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (33.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 45 min at a flow rate of 6 mL/min to yield the minor diastereomer D4-minor (2.9 mg, 2.32 µmol, 7%) as a white fluffy solid and single diastereomer (d.e. = 30%) and the major diastereomer D4-major (2.7 mg, 2.16 µmol, 6%) as a white fluffy solid and single diastereomer (d.e. ≥ 99%).

Minor diastereomer D4-minor: Optimized RP-HPLC-MS (II): Method II-C, \(t_R = 12.4 \text{ min} \) and \(t_R = 12.9 \text{ min}, [M+H]^+ = 1249.6, [M+2H]^{2+} = 637.1, \text{d.r.} = 65:35; \) Analytical Purity-RP-HPLC (210 nm): 71%; HRMS (ESI) calculated for \(C_{56}H_{77}O_{19}N_{14}[M+H]^+ = 1249.54839, \) found 1249.54976.

Major diastereomer D4-major: Optimized RP-HPLC-MS (II): Method II-C, \(t_R = 12.7 \text{ min}, [M+H]^+ = 1249.7, [M+2H]^{2+} = 636.5; \) Analytical Purity-RP-HPLC (210 nm): 85%; HRMS (ESI) calculated for \(C_{56}H_{77}O_{19}N_{14}[M+H]^+ = 1249.54839, \) found 1249.54704.

\[
[4\text{-OMePh-pyrrolidinyl-spirooxindole-NMeFDINNNK}]G, D5
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The titled PepNat was prepared from supported aldehyde peptide resin 3d-1 (80 mg, 28.0 µmol, loading 0.35 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with \((E)-4\text{-methoxybenzylidene-indolin-2-one} (12) (17.6 mg, 70.0 µmol)\) according to the representative procedure (RP-04). The cycloaddition proceeded with a 11:10:17:62 diastereomeric ratio \((t_{R,\text{dia1}} = 16.5 \text{ min}, t_{R,\text{dia2}} = 17.8 \text{ min}, t_{R,\text{dia3}} = 18.3 \text{ min}, t_{R,\text{dia4}} = 18.8 \text{ min with } [M+H]^+ = 1356.7 \text{ and } [M+2H]^{2+} = 679.1\) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (30.6 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 48 min at a flow rate of 6 mL/min to yield the product D5-mixt. (1.1 mg, 0.81 µmol, 3%) as a white fluffy solid and an inseparable mixture of diastereomers (d.r. = 15:10:75).

Mixture of diastereomers D5-mixt.: Optimized RP-HPLC-MS (II): Method II-C, \(t_{R,\text{dia2}} = 17.8 \text{ min, } t_{R,\text{dia3}} = 18.4 \text{ min, } t_{R,\text{dia4}} = 18.9 \text{ min with } [M+H]^+ = 1356.8 \text{ and } [M+2H]^{2+} = 679.2, \text{d.r.} = 15:10:75; \) Analytical
Purity-RP-HPLC (254 nm): 96%; HRMS (ESI) calculated for C_{66}H_{82}O_{17}N_{15} [M+H]^+ = 1356.60076, found 1356.59802.

\[ \text{[4-F-Ph-pyrrolidinyl-spirooxindole-nMeFDINNNK]}G, D6 \]

![Diagram of D6-major](image)

The titled 3,3'-pyrrolidinyl-spirooxindole-peptide hybrid macrocycle was prepared from supported aldehyde peptide resin 3d-1 (80 mg, 28.0 µmol, loading 0.35 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with (E)-4-fluorobenzylidene-indolin-2-one (13) (16.7 mg, 70.0 µmol) according to the representative procedure (RP-04, Cu/BINAP). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a diastereomeric ratio 9:36:7:48 (t_{R,dia1} = 7.4 min; t_{R,dia2} = 8.0 min; t_{R,dia3} = 8.2 min; t_{R,dia4} = 8.7 min; with [M+H]^+ = 1345.4 and [M+2H]^{2+} = 673.1). The crude product (25.5 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H_{2}O + 0.1% TFA in 45 min at a flow rate of 6 mL/min to afford the major diastereomer D6-major (2.2 mg, 1.64 µmol, 6%) as a white fluffy solid a single diastereomers (d.e. = 66%).

**Major diastereomer D6-major:** RP-HPLC-MS (I): Method I-C, t_{R,dia1} = 7.4 min; t_{R,dia2} = 8.0 min; t_{R,dia3} = 8.2 min; t_{R,dia4} = 8.8 min, [M+H]^+ = 1345.0, [M+2H]^{2+} = 673.1, d.r. = 6:3:8:83; Analytical Purity-RP-HPLC (210 nm): 89%; HRMS (ESI) calculated for C_{65}H_{79}O_{16}N_{15}F [M+H]^+ = 1344.58078, found 1344.58143.

\[ \text{[pyrrolidinyl-spirobarbiturate-nMeFDINNNK]}G, D7 \]

![Diagram of D7-major](image)
The titled pyrrolidinyl-spirobarbiturate PepNat was prepared from supported aldehyde peptide resin 3d-1 (80 mg, 7.2 µmol, calculated loading 0.09 mmol/g, starting unfunctionalized loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with 5-benzylidene-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione (14) (17.1 mg, 70.0 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a 29:71 diastereomeric ratio (t<sub>R</sub>,minor = 8.3 min; t<sub>R</sub>,major = 8.7 min, with [M+H]<sup>+</sup> = 1350.2 and [M+2H]<sup>2+</sup> = 675.5). The crude product (20.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 45 min at a flow rate of 6 mL/min to give the product D7-major (1.1 mg, 0.82 µmol, 11% cycloaddition yield and 4% overall yield) as a white fluffy solid and a single diastereomer (d.e. = 71%).

Major diastereomer D7-major: RP-HPLC-MS (I): Method I-C, t<sub>R</sub>,minor = 8.3 min, t<sub>R</sub>,major = 8.7 min, [M+H]<sup>+</sup> = 1350.4, [M+2H]<sup>2+</sup> = 675.4, d.r. = 14:85; Analytical Purity-RP-HPLC (210 nm): 88%; HRMS (ESI) calculated for C<sub>63</sub>H<sub>81</sub>O<sub>18</sub>N<sub>16</sub>[M+H]<sup>+</sup> = 1349.59093, found 1349.59237.

[N-Me-fused-di-pyrrolidine-NMeFDINNOrN]G, D8

The three-carbon length DINNN PepNat was prepared from supported aldehyde peptide resin 3d-2 (100 mg, 35.0 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (9.7 mg, 87.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 96:4 diastereomeric ratio (t<sub>R</sub>,major = 7.5 min, t<sub>R</sub>,minor = 7.7 min with [M+H]<sup>+</sup> = 1203.1 and [M+2H]<sup>2+</sup> = 602.0) according to the RP-HPLC-MS (I) chromatogram (Method I-B). The crude product (16.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 45% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to afford the product D8-major (5.3 mg, 4.41 µmol, 13%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Major diastereomer D8-major: RP-HPLC-MS (I): Method I-B, t<sub>R</sub> = 7.5 min, [M+H]<sup>+</sup> = 1203.4, [M+2H]<sup>2+</sup> = 602.2; Analytical Purity-RP-HPLC: 97%; HRMS (ESI) calculated for C<sub>54</sub>H<sub>72</sub>O<sub>17</sub>N<sub>15</sub>[M+H]<sup>+</sup> = 1202.52251, found 1202.52395.
The *titled two carbon length DINNN PepNat* was prepared from supported aldehyde peptide resin 3d-3 (100 mg, 35.0 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with *N*-methymaleimide (6) (9.7 mg, 87.5 µmol) according to the representative procedure (RP-04). Optimized analytical RP-HPLC-MS (II), Method II-A showed that the cycloaddition proceeded with a 95:5 diastereomeric ratio (t<sub>R,major</sub> = 6.6 min; t<sub>R,minor</sub> = 7.7 min with [M+H]⁺ = 1189.0 and [M+2H]²⁺ = 595.0). The crude product (13.4 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 45% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to yield the major diastereomer **D9-major** (1.3 mg, 1.01 µmol, 3%) as a white fluffy solid and single diastereomer (d.e. ≥ 99%).

**Major diastereomer D9-major:** Optimized RP-HPLC-MS (II): Method II-A, t<sub>R</sub> = 6.6 min, [M+H]⁺ = 1188.6, [M+2H]²⁺ = 595.0; Analytical Purity-RP-HPLC: 97%; HRMS (ESI) calculated for C<sub>53</sub>H<sub>70</sub>O<sub>17</sub>N<sub>15</sub> [M+H]⁺ = 1188.50686, found 1188.50638.

The *titled one carbon length DINNN PepNat* was prepared from supported aldehyde peptide resin 3d-4 (380 mg, 152.0 µmol, loading 0.40 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with *N*-methymaleimide (6) (43.0 mg, 380 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 84:16 diastereomeric ratio (t<sub>R,major</sub> = 15.1 min; t<sub>R,minor</sub> = 15.5 min with [M+H]⁺ = 1174.7 and [M+2H]²⁺ = 588.0) according to the optimized analytical RP-
HPLC-MS (II) chromatogram (Method II-C). The crude product (18.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 45% of MeCN + 0.1% TFA in H2O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to give the major diastereomer D10-major (7.0 mg, 5.96 µmol, 4%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer D10-major:** RP-HPLC-MS (I): Method I-C, \( t_R = 5.9 \text{ min} \), \([\text{M}+\text{H}]^+ = 1174.8 \) and \([\text{M}+2\text{H}]^{2+} = 588.0 \); Optimized RP-HPLC-MS (II): Method II-C, \( t_R = 15.2 \) min, \([\text{M}+\text{H}]^+ = 1174.7 \) and \([\text{M}+2\text{H}]^{2+} = 588.0 \); Analytical Purity-RP-HPLC: 98%; \(^1\)H NMR (700 MHz, CD₃OD, 25 °C) \( \delta 8.11 (d, J = 7.1 \text{ Hz}, 1H), 7.98 (d, \ J = 8.4 \text{ Hz}, 2H), 7.63 (d, \ J = 8.2 \text{ Hz}, 2H), 7.34 (t, \ J = 7.6 \text{ Hz}, 1H), 7.28 – 7.24 (m, 3H), 4.96 – 4.91 (m, 2H), 4.61 – 4.57 (m, 2H), 4.45 (dd, \ J = 7.7, 5.5 \text{ Hz}, 1H), 4.38 (dd, \ J = 9.4, 5.2 \text{ Hz}, 1H), 4.31 (d, \ J = 9.0 \text{ Hz}, 1H), 4.28 – 4.24 (m, 1H), 4.17 (dd, \ J = 11.4, 4.4 \text{ Hz}, 1H), 4.07 (t, \ J = 8.2 \text{ Hz}, 1H), 4.02 – 3.96 (m, 2H), 3.94 – 3.84 (m, 2H), 3.80 (dd, \ J = 14.2, 2.9 \text{ Hz}, 1H), 3.43 – 3.39 (m, 1H), 3.35 – 3.32 (m, 1H), 3.26 – 3.22 (m, 1H), 3.18 – 3.15 (m, 1H), 3.07 (dd, \ J = 15.6, 5.4 \text{ Hz}, 1H), 2.93 (dd, \ J = 11.6, 6.1 \text{ Hz}, 1H), 2.87 (dd, \ J = 15.6, 7.8 \text{ Hz}, 1H), 2.79 (s, 3H), 2.70 (s, 3H), 2.55 (dd, \ J = 14.5, 9.8 \text{ Hz}, 1H), 2.49 (dd, \ J = 14.5, 5.2 \text{ Hz}, 1H), 2.12 – 2.04 (m, 1H), 1.76 – 1.69 (m, 1H), 1.23 – 1.16 (m, 1H), 0.96 (d, \ J = 6.8 \text{ Hz}, 3H), 0.92 (t, \ J = 7.4 \text{ Hz}, 3H), the NH and OH protons were excluded due to exchange with the deuterated methanol solvent; \(^{13}\)C NMR (176 MHz, CD₃OD, 25 °C) \( \delta 178.4, 176.1, 175.8, 175.7, 175.5, 174.9, 174.4, 173.1, 173.1, 172.9, 172.8, 172.2, 171.8, 170.9, 169.7, 139.5, 134.8, 130.5 (×2), 130.0 (×2), 128.7 (×2), 128.5 (×2), 127.9, 101.4, 69.2, 63.9, 63.0, 59.1, 56.5, 54.1, 53.9, 53.2, 52.1, 51.9, 51.4, 48.6, 43.4, 42.2, 39.5, 39.2, 38.7, 37.2, 36.0, 35.5, 34.4, 25.9, 25.9, 16.2, 11.5; HRMS (ESI) calculated for C₅₂H₆₈O₁₇N₁₅ \([\text{M}+\text{H}]^+ = 1174.49121\), found 1174.49062.

\[ \text{N-Me-fused-di-pyrrolidine-\textit{N}MeGDINNNK\textit{G}, D11} \]

The *titled PepNat* was prepared from supported aldehyde peptide resin 3d-5 (400 mg, 136.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (37.6 mg, 340.0 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a 54:46 diastereomeric ratio (\( t_{R,\text{major}} = 4.2 \text{ min} \); \( t_{R,\text{minor}} = 5.1 \text{ min} \) with \([\text{M}+\text{H}]^+ = 1126.6 \) and \([\text{M}+2\text{H}]^{2+} = 563.8 \)). The crude product (130.2 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 5% to 50% of MeCN.
+ 0.1% TFA in H₂O + 0.1% TFA in 40 min at a flow rate of 6 mL/min to afford the minor diastereomer D11-minor (12.7 mg, 11.3 µmol, 8%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Minor diastereomer D11-minor: RP-HPLC-MS (I): Method I-C, tᵣ = 5.1 min, [M+H]⁺ = 1126.6, [M+2H]²⁺ = 564.1; Analytical Purity-RP-HPLC (210 nm): 90%; HRMS (ESI) calculated for C₄₈H₆₇O₁₈N₁₄ [M+H]⁺ = 1127.47523, found 1127.48935.

[N-Me-fused-di-pyrrolidine-nMeLDINNNK]G, D12

The titled fused di-pyrrolidine PepNat was prepared from supported aldehyde peptide resin 3d-6 (361 mg, 48.0 µmol, calculated loading 0.13 mmol/g, starting unfuctionalized loading 0.26 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide 6 (13.0 mg, 120 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 91:9 diastereomeric ratio (tᵣ,major = 8.2 min; tᵣ,minor = 10.1 min with [M+H]⁺ = 1182.6 and [M+2H]²⁺ = 592.0) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (79.8 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to afford the major diastereomer D12-major (12.4 mg, 9.57 µmol, 20% cycloaddition yield and 10% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the minor diastereomer D12-minor (1.9 mg, 1.47 µmol, 3% cycloaddition yield and 1% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Major diastereomer D12-major: Optimized RP-HPLC-MS (II): Method II-C, tᵣ = 8.2 min, [M+H]⁺ = 1182.6 and [M+2H]²⁺ = 592.0; Analytical Purity-RP-HPLC: 95%; HRMS (ESI) calculated for C₅₂H₇₆O₁₇N₁₅ [M+H]⁺ = 1182.55381, found 1182.55411.

Minor diastereomer D12-minor: Optimized RP-HPLC-MS (II): Method II-C, tᵣ = 10.1 min, [M+H]⁺ = 1182.6 and [M+2H]²⁺ = 592.0; Analytical Purity-RP-HPLC: 96%; HRMS (ESI) calculated for C₅₂H₇₆O₁₇N₁₅ [M+H]⁺ = 1182.55381, found 1182.55415.
The titled fused di-pyrrolidine PepNat was prepared from supported aldehyde peptide resin 3d-10 (385 mg, 38.0 µmol, calculated loading 0.13 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide 6 (10.6 mg, 100.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 85:15 diastereomeric ratio ($t_R,\text{major} = 5.8 \text{ min}; t_R,\text{minor} = 7.9 \text{ min}$ with $[\text{M+H}]^+ = 1168.59$ and $[\text{M+2H}]^{2+} = 584.97$) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (78.9 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 5% to 50% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to afford the major diastereomer D13-major (12.9 mg, 11.1 µmol, 27% cycloaddition yield and 11% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the minor diastereomer D13-minor (5.8 mg, 4.5 µmol, 12% cycloaddition yield and 6% overall yield) as a white solid and a single diastereomer (d.e. ≥ 99%).

Major diastereomer D13-major: Optimized RP-HPLC-MS (II): Method II-C, $t_R = 5.8 \text{ min}$, $[\text{M+H}]^+ = 1168.6$ and $[\text{M+2H}]^{2+} = 585.0$; Analytical Purity-RP-HPLC: 98%; HRMS (ESI) calculated for C$_{51}$H$_{73}$O$_{15}$N$_{15}$ $[\text{M+H}]^+ = 1168.53871$, found 1168.54024.

Minor diastereomer D13-minor: Optimized RP-HPLC-MS (II): Method II-B, $t_R = 7.8 \text{ min}$, $[\text{M+H}]^+ = 1168.6$, $[\text{M+2H}]^{2+} = 585.0$; Analytical Purity-RP-HPLC: 99%; HRMS (ESI) calculated for C$_{51}$H$_{73}$O$_{15}$N$_{15}$ $[\text{M+H}]^+ = 1168.53871$, found 1168.53890.

[N-Me-fused-di-pyrrolidine-PDINNNK]G, D14
The titled proline containing PepNat was prepared from supported aldehyde peptide resin 3d-7 (100 mg, 35.0 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (9.7 mg, 87.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 57:43 diastereomeric ratio ($t_{R,\text{major}} = 5.0$ min; $t_{R,\text{minor}} = 5.4$ min with $[\text{M+H}]^+ = 1153.7$ and $[\text{M+2H}]^{2+} = 577.3$) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-B). The crude product (16.4 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 35% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 25 min at a flow rate of 6 mL/min to afford the product D14-minor (1.1 mg, 1.00 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Minor diastereomer D14-minor**: Optimized RP-HPLC-MS (II): Method II-B, $t_R = 5.4$ min, $[\text{M+H}]^+ = 1152.9$, $[\text{M+2H}]^{2+} = 576.9$; Analytical Purity-RP-HPLC: 94%; HRMS (ESI) calculated for C$_{50}$H$_{70}$O$_{17}$N$_{15}$ [M+H]$^+$ = 1152.50686, found 1152.50732.

\[ [\text{N-Me-fused-di-pyrrolidine-NMefDINNNK}]_G, D15 \]

The fused di-pyrrolidine-DINNN PepNat was prepared from supported aldehyde peptide resin 3d-8 (150 mg, 42.0 µmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (9.7 mg, 87.5 µmol) according to the representative procedure (RP-04). Optimized RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 36:64 diastereomeric ratio ($t_{R,\text{minor}}^* = 7.2$ min; $t_{R,\text{major}}^* = 7.7$ min with $[\text{M+H}]^+ = 1216.8$ and $[\text{M+2H}]^{2+} = 609.1$). The crude product (25.2 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 40% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 30 min at a flow rate of 6 mL/min to yield the product D15-minor* (2.6 mg, 2.14 µmol, 5%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Minor* diastereomer D15-minor**: Optimized RP-HPLC-MS (II): Method II-C, $t_R = 7.3$ min, $[\text{M+H}]^+ = 1216.9$, $[\text{M+2H}]^{2+} = 609.0$; Analytical Purity-RP-HPLC: 97%; HRMS (ESI) calculated for C$_{55}$H$_{74}$O$_{17}$N$_{15}$ [M+H]$^+$ = 1216.53816, found 1216.53877.

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The *titled PepNat* was prepared from supported aldehyde peptide resin 3d-9 (300 mg, 102.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with *N*-methylmaleimide (6) (28.6 mg, 255.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 28:35:37 diastereomeric ratio (t<sub>R,dia1</sub> = 5.0 min; t<sub>R,dia2</sub> = 5.4 min; t<sub>R,dia3</sub> = 5.9 min with [M+H]<sup>+</sup> = 1202.7 and [M+2H]<sup>2+</sup> = 602.1) according to the RP-HPLC-MS (I) chromatogram (Method I-C). The crude product (48.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to afford the product D16-mixt. (2.0 mg, 1.66 µmol, 2%) as a white fluffy solid and an inseparable mixture of diastereomers (d.r. = 47:53).

**Mixture of diastereomers D16-mixt.:** RP-HPLC-MS (I): Method I-C, t<sub>R,major</sub> = 5.4 min, t<sub>R,minor</sub> = 5.8 min, [M+H]<sup>+</sup> = 1202.6, [M+2H]<sup>2+</sup> = 602.1, d.r. = 47:53; Analytical Purity-RP-HPLC: 81%; HRMS (ESI) calculated for C<sub>65</sub>H<sub>76</sub>O<sub>12</sub>N<sub>11</sub> [M+H]<sup>+</sup> = 1202.56694, found 1202.56882.

The *titled N-phenyl fused di-pyrrolidine one carbon linker PepNat* was prepared from supported aldehyde peptide resin 3d-4 (300 mg, 102.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with *N*-phenylmaleimide (7) (44.6 mg, 255.0 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a 58:42 diastereomeric ratio (t<sub>R,major</sub> = 6.5 min; t<sub>R,minor</sub> = 7.9 min with
[M+H]^+ = 1236.9 and [M+2H]^{2+} = 619.1). The crude product (55.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H_2O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to give the major diastereomer D17-major (2.3 mg, 1.86 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer D17-major:** RP-HPLC-MS (I): Method I-C, t_R = 6.5 min, [M+H]^+ = 1237.2, [M+2H]^{2+} = 619.0; Analytical Purity-RP-HPLC: 79%; HRMS (ESI) calculated for C_{57}H_{70}O_{17}N_{15} [M+H]^+ = 1236.50686, found 1236.50923.

The **titiled PepNat** was prepared from supported aldehyde peptide resin 3d-10 (243 mg, 31.6 µmol, calculated loading 0.13 mmol/g, starting unfunctionalized loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (13.7 mg, 80.0 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 62:38 diastereomeric ratio (t_{R,major} = 11.1 min; t_{R,minor} = 13.3 min with [M+H]^+ = 1230.6 and [M+2H]^{2+} = 616.1). Purification of crude product (55.2 mg) by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H_2O + 0.1% TFA in 37 min at a flow rate of 6 mL/min afforded the major diastereomer D18-major (1.6 mg, 1.19 µmol, 4% cycloaddition yield and 1% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the minor diastereomer D18-minor (0.9 mg, 0.67 µmol, 2% cycloaddition yield and 1% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer D18-major:** Optimized RP-HPLC-MS (II): Method II-C, t_R = 11.0 min, [M+H]^+ = 1230.6, [M+2H]^{2+} = 616.0; Analytical Purity-RP-HPLC: 95%; HRMS (ESI) calculated for C_{56}H_{76}O_{17}N_{15} [M+H]^+ = 1230.55381, found 1230.55451.

**Minor diastereomer D18-minor:** Optimized RP-HPLC-MS (II): Method II-C, t_R = 13.3 min, [M+H]^+ = 1230.7, [M+2H]^{2+} = 616.0; purity = 88%; HRMS (ESI) calculated for C_{56}H_{76}O_{17}N_{15} [M+H]^+ = 1230.55381, found 1230.56052.
The titled *PepNat* was prepared from supported aldehyde peptide resin 3d-11 (100 mg, 34.0 µmol, initial unfunctionalized loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with *N*-methylmaleimide (6) (9.1 mg, 89.0 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a 70:30 diastereomeric ratio (t<sub>R, major</sub> = 3.5 min, t<sub>R, minor</sub> = 3.8 min with [M+H]<sup>+</sup> = 1873.9 and [M+2H]<sup>2+</sup> = 937.7). The crude product (35.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 5% to 35% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to yield the major *PepNat* D19-major (1.1 mg, 0.59 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer D19-major:** RP-HPLC-MS (I): Method I-C, t<sub>R</sub> = 3.5 min, [M+H]<sup>+</sup> = 1874.5, [M+2H]<sup>2+</sup> = 937.7; Analytical Purity-RP-HPLC (210 nm): 91%; HRMS (ESI) calculated for C<sub>8</sub>H<sub>12</sub>O<sub>2</sub>N<sub>2</sub> [M+H]<sup>+</sup> = 1873.9006, found 1873.91383.

The titled *macrocycle* was prepared from supported aldehyde peptide resin 3d-12 (370 mg, 130 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with *N*-methylmaleimide (6) (36.0 mg, 330.0 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 84:16 diastereomeric ratio (t<sub>R, major</sub> = 11.3 min, t<sub>R, minor</sub> = 12.2 min with [M+H]<sup>+</sup> = 1315.7 and [M+2H]<sup>2+</sup> =
The crude product (78.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 45% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to yield the major PepNat **D20-major** (8.3 mg, 6.3 µmol, 5%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer D20-major**: RP-HPLC-MS (II): Method II-C, tᵣ = 11.4 min, [M+H]⁺ = 1315.8, [M+2H]²⁺ = 658.6; Analytical Purity-RP-HPLC (210 nm): 73%; HRMS (ESI) calculated for C₆₀H₮₂O₁₈N₁₆ [M+H]⁺ = 1315.60713, found 1315.60930.

\[
[N\text{-Me-fused-di-pyrrolidine-nMeFINNNK}]G, \text{ D21}
\]

![D21-major](image)

The **titled macrocycle** was prepared from supported aldehyde peptide resin **3d-13** (294 mg, 100.0 µmol, starting unfunctionalized loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (28.0 mg, 250 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a 66:34 diastereomeric ratio (tᵣ, major = 6.4 min, tᵣ, minor = 6.7 min with [M+H]⁺ = 1101.5 and [M+2H]²⁺ = 551.3). The crude product (67.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 2% to 55% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to yield the major product **D21-major** (3.1 mg, 2.8 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer D21-major**: RP-HPLC-MS (I): Method I-C, tᵣ = 6.4 min, [M+H]⁺ = 1001.6, [M+2H]²⁺ = 551.4; Analytical Purity-RP-HPLC (210 nm): 74%; HRMS (ESI) calculated for C₅₁H₆₈O₁₄N₁₄ [M+H]⁺ = 1101.51176, found 1101.51389.
The titled macrocycle was prepared from supported aldehyde peptide resin 3d-14 (694 mg, 100 µmol, starting unfunctionalized loading 0.36 mmol/g, calculated loading 0.14 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (46.6 mg, 288 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a 58:42 diastereomeric ratio (t_R, major = 6.5 min, t_R, minor = 6.9 min with [M+H]^+ = 1115.73). The crude product (270.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 50% of MeCN + 0.1% TFA in H_2O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to yield the major product D22-major (7.6 mg, 6.8 µmol, 7% cycloaddition yield and 3% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Major diastereomer D22-major: RP-HPLC-MS (I): Method I-C, t_R = 6.5 min, [M+H]^+ = 1115.7 [M+2H]^{2+} = 558.3; Analytical Purity-RP-HPLC (210 nm): 85%; HRMS (ESI) calculated for C_{53}H_{70}O_{15}N_{12} [M+H]^+ = 1115.52742, found 1115.52987.

The titled truncated NNN PepNat was prepared from supported aldehyde peptide resin 3d-15 (250 mg, 27.5 µmol, starting unfunctionalized loading 0.19 mmol/g, calculated loading 0.11 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (13.3 mg, 120 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-A
showed that the cycloaddition proceeded with a 75:25 diastereomeric ratio ($t_R,_{\text{major}} = 4.5 \text{ min}, \ t_R,_{\text{minor}} = 5.3 \text{ min}$ with $[M+H]^+ = 988.5$ and $[M+2H]^{2+} = 494.8$). The crude product (36.8 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to yield the major PepNat D23-major (5.7 mg, 5.8 µmol, 21% cycloaddition yield and 12% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer D23-major:** RP-HPLC-MS (I): Method I-A, $t_R = 4.5 \text{ min}$, $[M+H]^+ = 988.7$, Analytical Purity-RP-HPLC (210 nm): 98%; HRMS (ESI) calculated for C$_{45}$H$_{57}$O$_{13}$N$_3$ $[M+H]^+ = 988.42771$, found 988.42999.

$$[N\text{-Me-fused-di-pyrrolidine-NMeFAHASNK}]G, \text{ E1}$$

The **titled fused di-pyrrolidine-peptide** PepNat was prepared from supported aldehyde peptide resin 3e (100 mg, 40.0 µmol, loading 0.40 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (11.0 mg, 100.0 µmol) according to the representative procedure (RP-04). Optimized analytical RP-HPLC-MS (II), Method II-B showed that the cycloaddition proceeded with around 26:74 diastereomeric ratio ($t_R,_{\text{minor}} = 8.8 \text{ min}; \ t_R,_{\text{major}} = 8.9 \text{ min}$ (peak overlap) with $[M+H]^+ = 1126.7$ and $[M+2H]^{2+} = 564.0$). The crude product (30.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 5% to 45% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to afford the major diastereomer E1-major (1.1 mg, 0.98 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer E1-major:** Optimized RP-HPLC-MS (II): Method II-B, $t_R = 8.7 \text{ min}$, $[M+H]^+ = 1126.7$, $[M+2H]^{2+} = 564.0$; Analytical Purity-RP-HPLC: 81%; HRMS (ESI) calculated for C$_{52}$H$_{68}$O$_{14}$N$_{15}$ $[M+H]^+ = 1126.50647$, found 1126.50735.
The titled fused di-pyrrolidine-peptide hybrid macrocycle was prepared from supported aldehyde peptide resin 3f (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (14.2 mg, 127.5 µmol) according to the representative procedure (RP-04). Optimized analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 64:36 diastereomeric ratio ($t_{R,\text{major}^*} = 15.5$ min, $t_{R,\text{minor}^*} = 18.1$ min with [M+H]$^+$ = 1387.7 and [M+2H]$^{2+}$ = 694.6). The crude product (51.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 15% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to afford the major diastereomer F1-major$^*$ (2.2 mg, 1.59 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the minor diastereomer F1-minor$^*$ (1.4 mg, 1.01 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer F1-major$^*$:** Optimized RP-HPLC-MS (II): Method II-C, $t_R = 15.3$, [M+H]$^+$ = 1387.8, [M+2H]$^{2+}$ = 694.6; Analytical Purity-RP-HPLC (210 nm): 97%; HRMS (ESI) calculated for C$_{71}$H$_{87}$O$_{14}$N$_{16}$ [M+H]$^+$ = 1387.65822, found 1387.65295.

**Minor diastereomer F1-minor$^*$:** Optimized RP-HPLC-MS (II): Method II-C, $t_R = 18.1$, [M+H]$^+$ = 1387.8, [M+2H]$^{2+}$ = 694.6; Analytical Purity-RP-HPLC (210 nm): 75%; HRMS (ESI) calculated for C$_{71}$H$_{87}$O$_{14}$N$_{16}$ [M+H]$^+ = 1387.65822$, found 1387.66040.
The titled fused di-pyrrolidine-peptide macrocycle was prepared from supported aldehyde peptide resin 3f (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (22.1 mg, 127.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 47:53 diastereomeric ratio ($t_{R,\text{minor}} = 17.8$ min, $t_{R,\text{major}} = 22.4$ min, with [M+H]$^+$ = 1449.7 and [M+2H]$^{2+}$ = 725.7) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (33.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 15% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to give the minor diastereomer F2-minor (1.7 mg, 1.17 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Minor diastereomer F2-minor: Optimized RP-HPLC-MS (II): Method II-C, $t_{R} = 17.7$, [M+H]$^+$ = 1450.7, [M+2H]$^{2+}$ = 725.7; Analytical Purity-RP-HPLC (210 nm): 90%; HRMS (ESI) calculated for C$_{76}$H$_{89}$O$_{14}$N$_{16}$ [M+H]$^+$ = 1449.67387, found 1449.67564.

The titled fused di-pyrrolidine-peptide hybrid macrocycle was prepared from supported aldehyde peptide resin 3g-1 (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (22.1 mg, 127.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 47:53 diastereomeric ratio ($t_{R,\text{minor}} = 17.8$ min, $t_{R,\text{major}} = 22.4$ min, with [M+H]$^+$ = 1449.7 and [M+2H]$^{2+}$ = 725.7) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (33.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 15% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to give the minor diastereomer F2-minor (1.7 mg, 1.17 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Minor diastereomer F2-minor: Optimized RP-HPLC-MS (II): Method II-C, $t_{R} = 17.7$, [M+H]$^+$ = 1450.7, [M+2H]$^{2+}$ = 725.7; Analytical Purity-RP-HPLC (210 nm): 90%; HRMS (ESI) calculated for C$_{76}$H$_{89}$O$_{14}$N$_{16}$ [M+H]$^+$ = 1449.67387, found 1449.67564.
representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (14.2 mg, 127.5 µmol) according to the representative procedure (RP-04). Optimized Analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 25:75 diastereomeric ratio ($t_{R,\text{minor}} = 12.0$ min, $t_{R,\text{major}} = 14.6$ min with $[\text{M+H}]^+ = 1387.6$ and $[\text{M+2H}]^{2+} = 694.7$). The crude product (46.2 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 15% to 45% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 30 min at a flow rate of 6 mL/min to give the major diastereomer product **G1-major** (1.1 mg, 0.79 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer G1-major**: Optimized RP-HPLC-MS (II): Method II-C, $t_R = 14.8$ min, $[\text{M+H}]^+ = 1387.8$, $[\text{M+2H}]^{2+} = 694.7$; Analytical Purity-RP-HPLC (210 nm): 92%; HRMS (ESI) calculated for C$_{71}$H$_{86}$N$_{16}$O$_{14}$ $[\text{M+H}]^+ = 1387.65822$, found 1387.66758.

[Ph-\text{pyrrolidinyl-spirooxindole-\text{NMeFRffNAK}]G, G2}]

The **titled 3,3’-pyrrolidinyl-spirooxindole-peptide PepNat** was prepared from supported aldehyde peptide resin **3g-2** (120 mg, 33.6 µmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with [E]-3-benzylidene-indolin-2-one (11) (18.6 mg, 84.0 µmol) according to the representative procedure (RP-04). Optimized Analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 12:32:18:38 diastereomeric ratio ($t_{R,\text{dia1}} = 21.6$ min, $t_{R,\text{dia2}} = 22.0$ min, $t_{R,\text{dia3}} = 23.6$ min, $t_{R,\text{dia4}} = 23.9$ min with $[\text{M+H}]^+ = 1392.1$ and $[\text{M+2H}]^{2+} = 696.8$). The crude product (14.2 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 20% to 50% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 30 min at a flow rate of 6 mL/min to yield the product **G2-mixt.** (2.4 mg, 1.73 µmol, 5%) as a white fluffy solid and an inseparable mixture of diastereomers (d.r. = 11:36:25:28).

**Mixture of diastereomers G2-mixt.**: Optimized RP-HPLC-MS (II): Method II-C, $t_{R,\text{dia1}} = 21.8$ min, $t_{R,\text{dia2}} = 22.2$ min, $t_{R,\text{dia3}} = 23.7$ min, $t_{R,\text{dia4}} = 24.1$ min, $[\text{M+H}]^+ = 1391.8$, $[\text{M+2H}]^{2+} = 696.7$, d.r. = 11:36:25:28; Analytical Purity-RP-HPLC (210 nm): 82%; HRMS (ESI) calculated for C$_{74}$H$_{87}$O$_{12}$N$_{16}$ $[\text{M+H}]^+ = 1391.66839$, found 1391.66903.
The titled fused di-pyrrolidine-GLGF PepNat was prepared from supported aldehyde peptide resin 3h-1 (220 mg, 88.0 µmol, loading 0.40 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (24.4 mg, 220.0 µmol) according to the representative procedure (RP-04). Optimized Analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 90:10 diastereomeric ratio (tR,major = 22.8 min, tR,minor = 23.3 min with [M+H]+ = 1110.7). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a 90:10 diastereomeric ratio (tR,major = 9.7 min, tR,minor = 10.0 min with [M+H]+ = 1111.3 and [M+2H]2+ = 555.9). Purification by semi-preparative-RP-HPLC of the crude product (24.4 mg), using a step gradient of 25% to 55% of MeCN + 0.1% TFA in H2O + 0.1% TFA in 30 min at a flow rate of 6 mL/min yielded the major diastereomer H1-major (4.9 mg, 4.1 µmol, 5%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer H1-major:** RP-HPLC-MS (I): Method I-C, tR = 9.7 min, [M+H]+ = 1110.7, [M+2H]2+ = 556.1; Optimized RP-HPLC-MS (II): Method II-C, tR = 22.9 min, [M+H]+ = 1110.8, [M+2H]2+ = 556.1; Analytical Purity-RP-HPLC: 98%; 1H NMR (700 MHz, CD3OH, 25 ºC) 0.98 (d, J = 6.7 Hz, 3H), 0.98 (d, J = 6.5 Hz, 3H), 1.39 (m, 1H), 1.50 (m, 1H), 1.51 (m, 1H), 1.53 (m, 1H), 1.58 (m, 1H), 1.62 (m, 1H), 1.78 (m, 1H), 1.80 (m, 1H), 1.88 (dd, J = 13.7, 11.0, 4.0 Hz, 1H), 2.32 (s, 3H), 2.79 (s, 3H), 2.89 (dd, J = 8.9, 10.8 Hz, 1H), 2.91 (dd, J = 9.2, 11.1 Hz, 1H), 3.03 (dd, J = 14.0, 5.9 Hz, 1H), 3.13 (dd, J = 13.9, 5.6 Hz, 1H), 3.31 (m, 1H), 3.33 (m, 1H), 3.48 (dd, J = 14.0, 4.4 Hz, 1H), 3.58 (m, 1H), 3.61 (m, 1H), 3.63 (m, 1H), 3.66 (m, 1H), 3.82 (t, J = 8.3 Hz, 1H), 4.00 (t, J = 7.9 Hz, 1H), 4.19 (dd, J = 11.4, 4.4 Hz, 1H), 4.29 (dd, J = 17.0, 7.8 Hz, 1H), 4.31 (m, 1H), 4.33 (m, 1H), 4.49 (dd, J = 8.1 Hz, 1H), 4.56 (m, 1H), 4.65 (ddd, J = 11.0, 9.3, 3.8 Hz, 1H), 4.86 (s, 1H), 7.04 (s, 1H), 7.16 (t, J = 7.1 Hz, 2H), 7.18 – 7.28 (m, 11H), 7.34 (m, 2H), 7.45 (d, J = 5.2 Hz, 1H), 7.48 (s, 1H), 7.57 (d, J = 8.1 Hz, 2H), 7.71 (d, J = 9.2 Hz, 1H), 7.78 (d, J = 7.9 Hz, 1H), 7.85 (m, 2H), 8.22 (d, J = 7.6 Hz, 1H), 8.30 (m, 2H), 8.34 (dd, J = 7.7, 5.0 Hz, 1H), the NH proton of the fused di-pyrrolidine NP was not reported; 13C NMR (176 MHz, CD3OH, 25 ºC) 21.8, 23.6, 23.8, 25.1, 25.2, 29.6, 32.3, 34.5, 38.2, 38.7, 38.9, 40.2, 43.9, 44.1 (x2), 48.7, 51.8, 52.3, 54.6, 55.5, 57.1, 63.3, 64.2, 69.3, 127.7, 127.8, 127.9, 128.18 (x2), 128.23 (x2), 129.4 (x2), 129.7 (x2), 129.8 (x2), 130.1 (x2), 130.2 (x2), 130.3 (x2), 135.8, 138.0,
138.2 (x2), 139.4, 169.4, 170.4, 171.2, 171.3, 171.4, 173.7, 174.0, 174.7, 175.2, 175.8, 178.3; HRMS (ESI) calculated for C_{59}H_{72}O_{11}N_{11} [M+H]^+ = 1110.54073, found 1110.54122;

\[ \text{[N-Ph-fused-di-pyrrolidine-\text{\textit{N}}MeFGLGFK]} \text{F, H2} \]

![Diagram of H2-major]

The **titled PepNat** was prepared from supported aldehyde peptide resin 3h-1 (100 mg, 28.0 µmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (12.2 mg, 70.0 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-C showed that the cycloaddition proceeded with a 91:9 diastereomeric ratio (t_{R,major} = 10.8 min, t_{R,minor} = 13.2 min with [M+H]^+ = 1173.1 and [M+2H]^{2+} = 782.4). The crude product (14.5 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to give the major diastereomer **H2-major** (1.7 mg, 1.45 µmol, 5%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer H2-major:** RP-HPLC-MS (I): Method I-C, t_{R} = 10.9 min, [M+H]^+ = 1172.5, [M+2H]^{2+} = 587.1; Analytical Purity-RP-HPLC: 99%; HRMS (ESI) calculated for C$_{64}$H$_{74}$O$_{11}$N$_{11}$ [M+H]^+ = 1172.55638, found 1172.55793.

\[ \text{[Ph-pyrrolidinyl-spirooxindole-\text{\textit{N}}MeFGLGFK]} \text{, H3} \]

![Diagram of H3-major]

**H3-major**
The titled 3,3’-pyrrolidinyl-GLGF PepNat was prepared from supported aldehyde peptide resin 3h-2 (100 mg, 28.0 µmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with (E)-3-benzylidene-indolin-2-one (11) (15.5 mg, 84.0 µmol) according to the representative procedure (RP-04). Optimized analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 8:68:5:19 diastereomer ratio ($t_{R,\text{dia}1} = 26.5$ min, $t_{R,\text{dia}2} = 27.1$ min, $t_{R,\text{dia}3} = 27.8$ min, $t_{R,\text{dia}4} = 29.0$ min with [M+H]$^+$ = 1073.8 and [M+2H]$^{2+}$ = 537.6). The crude product (18.2 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 55 min at a flow rate of 6 mL/min to afford the major PepNat H3-major (1.1 mg, 1.03 µmol, 4%) as a white fluffy solid and a single diastereomer (d.e. = 74%).

Major diastereomer H3-major: Optimized RP-HPLC-MS (II): Method II-C, $t_{R,\text{dia}1} = 26.5$ min, $t_{R,\text{dia}2} = 27.1$ min, [M+H]$^+$ = 1073.9, [M+2H]$^{2+}$ = 537.6 with d.r. = 13:87; Analytical Purity-RP-HPLC: 99%; HRMS (ESI) calculated for C$_{60}$H$_{69}$O$_9$N$_{10}$ [M+H]$^+$ = 1073.52435, found 1073.52564.

[N-Me-fused-di-pyrroline-NMeFLKPIK]G, I1

The titled fused di-pyrroline-peptide macrocycle was prepared from supported aldehyde peptide resin 3i (120 mg, 33.6 µmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (9.4 mg, 84.0 µmol) according to the representative procedure (RP-04). Optimized Analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 20:80 diastereomeric ratio ($t_{R,\text{minor}} = 14.2$ min, $t_{R,\text{major}} = 14.7$ min with [M+H]$^+$ = 1098.8 and [M+2H]$^{2+}$ = 549.8). The crude product (19.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min afforded the major diastereomer I1-major (2.9 mg, 2.65 µmol, 8%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Major diastereomer I1-major: Optimized RP-HPLC-MS (II): Method II-C, $t_R = 14.8$ min, [M+H]$^+$ = 1097.9, [M+2H]$^{2+}$ = 549.7; Analytical Purity-RP-HPLC: 83%; HRMS (ESI) calculated for C$_{56}$H$_{80}$O$_{12}$N$_{11}$ [M+H]$^+$ = 1098.59824, found 1098.60092.
The *titled PepNat* was prepared from supported aldehyde peptide resin 3i (120 mg, 33.6 μmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (14.7 mg, 84.0 μmol) according to the representative procedure (RP-04). Optimized RP-HPLC-MS (II), Method II-C showed the desired product at $t_R = 18.4$ and 18.6 min ($[M+H]^+ = 1159.7$ and $[M+2H]^{2+} = 580.6$) as an inseparable mixture of diastereomers (d.r. ~ 40:60). The crude product (36.6 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 40 min at a flow rate of 6 mL/min to give the desired product I$_2$-mixt. (2.6 mg, 2.24 μmol, 5%) as a white fluffy solid and an inseparable mixture of diastereomer (d.r. ~ 55:45).

**Mixture of diastereomers I$_2$-mixt.:** Optimized RP-HPLC-MS (II): Method II-C, $t_R = 18.3$ and 18.6 min, $[M+H]^+ = 1159.8$, $[M+2H]^{2+} = 580.6$ (d.r. ~ 55:45); Analytical Purity-RP-HPLC: 86%; HRMS (ESI) calculated for C$_{61}$H$_{83}$O$_{11}$N$_{12}$ [M+H]$^+$ = 1159.62988, found 1159.63120.

The *titled 3,3'-pyrrolidinyl-spirooxindole PepNat* was prepared from supported aldehyde peptide resin 3i (120 mg, 36.6 μmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with (E)-3-(4-fluorobenzylidene)indolin-2-one (13) (20.3 mg, 91.5 μmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 15:44:14:27 diastereomeric ratio ($t_{R, dia1} = 8.3$ min, $t_{R, dia2} = 8.9$ min, $t_{R, dia3} = 10.0$ min, $t_{R, dia4} = 10.8$ min with $[M+H]^+ = 1226.9$ and
\([\text{M+2H}]^{2+} = 613.8\) according to the RP-HPLC-MS (II) chromatogram (Method II-F). The crude product (24.6 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H\(_2\)O + 0.1% TFA in 40 min at a flow rate of 6 mL/min afforded the product \(I3\)-mxt. (1.5 mg, 1.23 µmol, 4%) as a white fluffy solid and a mixture of two diastereomers (d.r. = 85:15).

**Mixture of diastereomers \(I3\)-mxt.:** Optimized RP-HPLC-MS (II): Method II-F, \(t_{R,\text{dia3}} = 10.1\) min; \(t_{R,\text{dia4}} = 10.9\) min, [M+H]\(^+\) = 1226.0, [M+2H]\(^{2+}\) = 613.8, d.r. 15:85; Analytical Purity-RP-HPLC: 92%; HRMS (ESI) calculated for C\(_{67}\)H\(_{86}\)O\(_{10}\)N\(_{12}\)F [M+H]\(^+\) = 1225.65684, found 1225.65957.

**[N-Me-fused-di-pyrrolidine-NMeFRFFNK]Y, J1**

The *titled PepNat* was prepared from supported aldehyde peptide resin 3j-1 (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with \(N\)-methylmaleimide (6) (14.2 mg, 127.5 µmol) according to the representative procedure (RP-04). Optimized analytical RP-HPLC-MS (II), Method II-C showed that the cycloaddition proceeded with a 17:83 diastereomeric ratio (\(t_{R,\text{minor}} = 15.1\) min, \(t_{R,\text{major}} = 15.4\) min with [M+H]\(^+\) = 1317.6 and [M+2H]\(^{2+}\) = 659.2). The crude product (26.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 5% to 55% of MeCN + 0.1% TFA in H\(_2\)O + 0.1% TFA in 38 min at a flow rate of 6 mL/min to yield the major diastereomer \(J1\)-major (2.1 mg, 1.60 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer \(J1\)-major:** Optimized RP-HPLC-MS (II): Method II-C, \(t_R = 15.5\), [M+H]\(^+\) = 1316.7, [M+2H]\(^{2+}\) = 659.1; Analytical Purity-RP-HPLC (210 nm): 77%; HRMS (ESI) calculated for C\(_{68}\)H\(_{82}\)O\(_{13}\)N\(_{15}\) [M+H]\(^+\) = 1316.62110, found 1316.62269.
The titled fused di-pyrrolidine-peptide macrocycle was prepared from supported aldehyde peptide resin 3j-1 (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (22.1 mg, 127.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 73:27 diastereomeric ratio ($t_R$ major = 17.8 min, $t_R$ minor = 22.4 min, with [M+H]$^+$ = 1378.7 and [M+2H]$^{2+}$ = 690.1) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (26.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 15% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 35 min at a flow rate of 6 mL/min afforded the major diastereomer J2-major* (1.8 mg, 1.31 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Major diastereomer J2-major*: Optimized RP-HPLC-MS (II): Method II-C, $t_R$ = 17.3, [M+H]$^+$ = 1378.8, [M+2H]$^{2+}$ = 690.1; Analytical Purity-RP-HPLC (210 nm): 84%; HRMS (ESI) calculated for C$_{73}$H$_{84}$O$_{13}$N$_{15}$ [M+H]$^+$ = 1378.63675, found 1378.63781.
The titled fused di-pyrrolidine-peptide PepNat was prepared from supported aldehyde peptide resin 3j-2 (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (22.1 mg, 127.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 78:22 diastereomeric ratio (tr,major* = 15.8 min, tr,minor* = 22.0 min, with [M+H]+ = 1273.6 and [M+2H]2+= 637.1) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (16.2 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 20% to 60% of MeCN + 0.1% TFA in H2O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to give the major* diastereomer J3-major* (4.1 mg, 3.22 µmol, 6%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the minor* diastereomer J3-minor* (2.4 mg, 1.89 µmol, 4%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer J3-major**: Optimized RP-HPLC-MS (II): Method II-C, tr = 15.6, [M+H]+ = 1272.7, [M+2H]2+= 637.1; Analytical Purity-RP-HPLC (210 nm): 82%; HRMS (ESI) calculated for C₆₆H₇₈O₁₂N₁₅ [M+H]+ = 1272.59489, found 1272.59649.

**Minor diastereomer J3-minor**: Optimized RP-HPLC-MS (II): Method II-C, tr = 21.6, [M+H]+ = 1272.7, [M+2H]2+= 637.1; Analytical Purity-RP-HPLC (210 nm): 82%; HRMS (ESI) calculated for C₆₆H₇₈O₁₂N₁₅ [M+H]+ = 1272.59489, found 1272.59654.
The titled phenyl substituted fused di-pyrrolidine-RffN PepNat was prepared from supported aldehyde peptide resin 3k-1 (150 mg, 42.0 µmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (18.2 mg, 105.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 44:56 diastereomeric ratio \( t_{R,\text{minor}} = 14.9 \text{ min} \), \( t_{R,\text{major}} = 17.0 \text{ min} \) with \([M+H]^+ = 1379.9\) and \([M+2H]^{2+} = 690.3\) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (15.4 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 20% to 50% of MeCN + 0.1% TFA in \( \text{H}_2\text{O} + 0.1\% \text{TFA} \) in 30 min at a flow rate of 6 mL/min to afford the minor diastereomer K1-minor (1.7 mg, 1.23 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the major diastereomer K1-major (1.7 mg, 1.23 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Minor diastereomer K1-minor:** Optimized RP-HPLC-MS (II): Method II-C, \( t_R = 14.9 \text{ min} \), \([M+H]^+ = 1379.0, [M+2H]^{2+} = 690.2\); Analytical Purity-RP-HPLC (210 nm): 99%; \(^1\)H NMR (700 MHz, CD\(_3\)OD, 25 °C) 1.21 (m, 1H), 1.25 (m, 1H), 1.31 (m, 1H), 1.35 (m, 1H), 1.39 (m, 1H), 1.51 (m, 1H), 1.55 (m, 1H), 1.58 (m, 1H), 1.72 (m, 1H), 1.88 (m, 1H), 2.07 (m, 1H), 2.37 (dd, \( J = 24.5, 12.0 \text{ Hz} \)), 2.42 (m, 1H), 2.66 (s, 2H), 2.72 (dd, \( J = 15.2, 9.5 \text{ Hz} \)), 2.92 (m, 2H), 3.01 (t, \( J = 7.1 \text{ Hz} \)), 3.07 (dd, \( J = 13.5, 8.2 \text{ Hz} \)), 3.16 (dd, \( J = 14.1, 4.7 \text{ Hz} \)), 3.33 (s, 3H), 3.35 (m, 2H), 3.36 (m, 1H), 3.70 (dd, \( J = 16.2, 4.2 \text{ Hz} \)), 4.02 (m, 1H), 4.06 (t, \( J = 8.6 \text{ Hz} \)), 4.32 (dt, \( J = 9.5, 4.4 \text{ Hz} \)), 4.32 (m, 1H), 4.34 (m, 1H), 4.35 (m, 1H), 4.36 (m, 1H), 4.50 (dd, \( J = 10.3, 8.4, 4.6 \text{ Hz} \)), 4.64 (d, \( J = 8.2 \text{ Hz} \)), 4.82 (m, 1H), 5.22 (dd, \( J = 11.7, 4.0 \text{ Hz} \)), 6.68 (m, 4H), 6.96 (t, \( J = 7.4 \text{ Hz} \)), 7.03 (t, \( J = 7.3 \text{ Hz} \)), 7.11 (d, \( J = 8.2 \text{ Hz} \)), 7.17 (m, 3H), 7.23 (t, \( J = 6.9 \text{ Hz} \)), 7.25 – 7.33 (m, 7H), 7.37 (d, \( J = 4.3 \text{ Hz} \)), 7.45 (m, 1H), 7.71 (d, \( J = 8.0 \text{ Hz} \)), 7.81 (d, \( J = 8.1 \text{ Hz} \)), 8.03 (d, \( J = 9.2 \text{ Hz} \)), 8.07 (d, \( J = 8.9 \text{ Hz} \)), 8.10 (d, \( J = 8.5 \text{ Hz} \)), 8.19 (d, \( J = 9.1 \text{ Hz} \)), 8.41 (d, \( J = 6.3 \text{ Hz} \)); \(^{13}\)C NMR (176 MHz, CD\(_3\)OD, 25 °C) 25.3, 26.6, 28.1, 28.5, 32.2, 31.7, 34.5, 37.0, 37.5, 40.0, 41.0, 41.1, 41.6, 49.5, 50.3, 51.9, 52.9, 54.6, 55.8, 56.2, 56.4, 57.0, 63.6, 64.0, 64.7, 116.1 (x2), 127.4, 127.6 (x2), 127.9, 128.1, 128.4 (x2), 128.5 (x2), 129.2 (x4), 129.7 (x3), 129.8 (x2), 130.1 (x2), 130.6 (x2), 130.8
(x2), 131.3 (x2), 133.1, 135.0, 137.3, 138.1, 138.7, 139.6, 157.2, 158.5, 169.8, 171.8, 172.2, 173.1, 173.2 (x2), 174.2, 174.3, 174.6, 174.8, 176.6, 179.1; HRMS (ESI) calculated for \( \text{C}_{73}\text{H}_{84}\text{O}_{13}\text{N}_{15}[\text{M+H}]^+ \) = 1378.63675, found 1378.63692;

**Major diastereomer K1-major:** Optimized RP-HPLC-MS (II): Method II-C, \( t_R = 17.2 \) min, \([\text{M+H}]^+ = 1378.7, [\text{M+2H}]^{2+} = 690.1\); Analytical Purity-RP-HPLC (210 nm): 92%; HRMS (ESI) calculated for \( \text{C}_{73}\text{H}_{84}\text{O}_{13}\text{N}_{15}[\text{M+H}]^+ \) = 1378.63675, found 1378.63692.

\[ \text{[N-Me-fused-di-pyrrolidine-NMeFRffNK]} \text{G, K2} \]

![Diagram of K2-minor](image)

The *titled fused di-pyrrolidine-peptide macrocycle* was prepared from supported aldehyde peptide resin **3k-2** (190 mg, 17.1 µmol, starting unfunctionalized loading 0.16 mmol/g, calculated loading 0.09 mmol/g)) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with \( N \)-methylmaleimide (6) (5.0 mg, 40.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 50:50 diastereomeric ratio (\( t_{R,\text{minor}} = 4.9 \) min, \( t_{R,\text{major}} = 6.8 \) min with \([\text{M+H}]^+ = 1211.6 \) and \([\text{M+2H}]^{2+} = 606.3\) according to the RP-HPLC-MS (I) chromatogram (Method I-C). The crude product (28.0 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 45% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to give the minor diastereomer **K2-minor** (0.6 mg, 0.50 µmol, 3% cycloaddition yield and 2% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Minor diastereomer K2-minor:** RP-HPLC-MS (I): Method I-C, \( t_R = 4.9 \) min, \([\text{M+H}]^+ = 1210.6, [\text{M+2H}]^{2+} = 606.1\); Analytical Purity-RP-HPLC (210 nm): 98%; HRMS (ESI) calculated for \( \text{C}_{61}\text{H}_{76}\text{O}_{12}\text{N}_{15}[\text{M+H}]^+ \) = 1210.57924, found 1210.58074.
The **titiled fused di-pyrroldine PepNat** was prepared from supported aldehyde peptide resin **3k-2** (245 mg, 83.3 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with **N-phenylmaleimide (7)** (72.1 mg, 208.3 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 48:52 diastereomeric ratio (t_R,minor = 5.5 min, t_R,major = 6.3 min, with [M+H]^+ = 1272.5 and [M+2H]^2+ = 637.0) according to the analytical RP-HPLC-MS (I) chromatogram (Method I-C). Purification by semi-preparative-RP-HPLC of the crude product (79.3 mg), using a step gradient of 10% to 45% of MeCN + 0.1% TFA in H_2O + 0.1% TFA in 37 min at a flow rate of 6 mL/min afforded the minor diastereomer **K3-minor** (0.6 mg, 0.47 µmol, 1%) and the major diastereomer **K3-major** (0.3 mg, 0.79 µmol, 1%) as white fluffy solids and a single diastereomers (d.e. ≥ 99%).

**Minor diastereomer K3-minor:** Analytical RP-HPLC-MS (I): Method I-C, t_R = 5.6 min, [M+H]^+ = 1272.6, [M+2H]^2+ = 637.1; Analytical Purity-RP-HPLC (210 nm): 90%; HRMS (ESI) calculated for C_{66}H_{78}O_{12}N_{15} [M+H]^+ = 1272.59489, found 1272.59637.

**Major diastereomer K3-major:** Analytical RP-HPLC-MS (I): Method I-C, t_R = 6.4 min, [M+H]^+ = 1272.6, [M+2H]^2+ = 637.1; Analytical Purity-RP-HPLC (210 nm): 85%; HRMS (ESI) calculated for C_{66}H_{78}O_{12}N_{15} [M+H]^+ = 1272.59489, found 1272.59580.

**[N-4-OMePh-fused-di-pyrroldine-nMeFffNK]G, K4**
The titled PepNat was prepared from supported aldehyde peptide resin 3k-2 (300 mg, 102.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (8) (57.1 mg, 330.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 45:55 diastereomeric ratio ($t_{R,\text{minor}} = 12.2$ min, $t_{R,\text{major}} = 14.3$ min with $[M+H]^+ = 1302.7$ and $[M+2H]^{2+} = 652.1$) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (35.5 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 50% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to yield the minor diastereomer K4-minor (5.8 mg, 4.45 µmol, 4%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the major diastereomer K4-major (5.9 mg, 4.53 µmol, 4%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Minor diastereomer K4-minor: Optimized RP-HPLC-MS (II): Method II-C, $t_R = 12.4$, $[M+H]^+ = 1302.7$, $[M+2H]^{2+} = 652.1$; Analytical Purity-RP-HPLC (210 nm): 97%; HRMS (ESI) calculated for C$_{67}$H$_{80}$O$_{13}$N$_{15}$ [M+H]$^+$ = 1302.60545, found 1302.60733.

Major diastereomer K4-major: Optimized RP-HPLC-MS (II): Method II-C, $t_R = 14.2$, $[M+H]^+ = 1302.7$, $[M+2H]^{2+} = 652.1$; Analytical Purity-RP-HPLC (210 nm): 99%; HRMS (ESI) calculated for C$_{67}$H$_{80}$O$_{13}$N$_{15}$ [M+H]$^+$ = 1302.60545, found 1302.60726.

\[N-3,4,5-\text{OMePh-fused-di-pyrrolidine-\text{nMeFRffNK}}]G, K5

The titled PepNat was prepared from supported aldehyde peptide resin 3k-2 (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-(3,4,5-trimethoxyphenyl)maleimide (9) (33.6 mg, 127.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 33:67 diastereomeric ratio ($t_{R,\text{minor}} = 4.8$ min, $t_{R,\text{major}} = 6.1$ min with $[M+H]^+ = 1362.6$ and $[M+2H]^{2+} = 682.1$) according to the analytical RP-HPLC-MS (I) chromatogram (Method I-C). The crude product (40.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 50% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 33 min at a
flow rate of 6 mL/min to give the major diastereomer **K5-major** (1.6 mg, 1.17 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer K5-major**: RP-HPLC-MS (I): Method I-C, $t_R = 6.1$ min, [M+H]$^+$ = 1362.7, [M+2H]$^{2+}$ = 682.2; Analytical Purity-RP-HPLC (210 nm): 97%; HRMS (ESI) calculated for C$_{69}$H$_{84}$O$_{15}$N$_{15}$ [M+H]$^+$ = 1362.62658, found 1362.62842.

![Image of L1 major](attachment:image.png)

**[N-Me-fused-di-pyrrolidine-nMeFRANK]G, L1**

The *titled fused di-pyrrolidine RAN PepNat* was prepared from supported aldehyde peptide resin **3l** (65 mg, 22.8 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide (6) (6.3 mg, 56.9 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 11:89 diastereomeric ratio ($t_{R, minor} = 4.4$ min, $t_{R, major} = 4.7$ min with [M+H]$^+$ = 987.6 and [M+2H]$^{2+}$ = 494.5) according to the RP-HPLC-MS (I) chromatogram (Method I-B). The crude product (12.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 5% to 65% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 30 min a flow rate of 6 mL/min to afford the major diastereomer **L1-major** (3.1 mg, 3.14 µmol, 14%) as a white fluffy solid and a single diastereomer (d.e. = 72%).

**Major diastereomer L1-major**: RP-HPLC-MS (I): Method I-B, $t_{R, minor} = 4.3$ min; $t_{R, major} = 5.0$ min, [M+H]$^+$ = 987.6, [M+2H]$^{2+}$ = 494.5, d.r. = 14:86; Analytical Purity-RP-HPLC: 96%; HRMS (ESI) calculated for C$_{46}$H$_{63}$O$_{11}$N$_{14}$ [M+H]$^+$ = 987.47953, found 987.47992.
[N-Ph-fused-di-pyrrolidine-nMeFRANK]G, L2

The *titled* PepNat was prepared from supported aldehyde peptide resin 3l (120 mg, 42.0 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with *N*-phenylmaleimide (7) (18.2 mg, 105.0 µmol) according to the representative procedure (RP-04). Analytical RP-HPLC-MS (I), Method I-A showed that the cycloaddition proceeded with a 78:22 diastereomeric ratio (tR,major = 2.8 min, tR,minor = 3.2 min with [M+H]+ = 1049.8 and [M+2H]2+ = 525.7). The crude product (14.8 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 5% to 65% of MeCN + 0.1% TFA in H2O + 0.1% TFA in 30 min at a flow rate of 6 mL/min to yield the major diastereomer **L2-major** (1.5 mg, 1.43 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer L2-major:** RP-HPLC-MS (I): Method I-A, tR = 2.8 min, [M+H]+ = 1049.6, [M+2H]2+ = 525.5; Analytical Purity-RP-HPLC: 70%; HRMS (ESI) calculated for C51H65O11N14 [M+H]+ = 1049.49518, found 1049.49402.

[N-Me-fused-di-pyrrolidine-nMeFRGDK], M1

The *titled* fused *di-pyrrolidine-tripeptide macrocycle* was prepared from supported aldehyde peptide resin 3m (100 mg, 36.0 µmol, loading 0.36 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with *N*-methylmaleimide (6) (9.7 mg, 87.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 82:18 diastereomeric ratio (tR,major = 4.1 min, tR,minor = 5.4 min with [M+H]+ = 917.6 and [M+2H]2+ = 459.5) according to the RP-HPLC-MS (I) chromatogram (Method I-E). The crude product (13.4 mg) was purified by semi-
preparative-RP-HPLC using a step gradient of 10% to 35% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 25 min at a flow rate of 6 mL/min to afford the major diastereomer **M1-major** (2.9 mg, 3.16 µmol, 9%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer M1-major**: Analytical RP-HPLC-MS (I): Method I-E, tᵣ = 4.1 min, [M+H]⁺ = 917.6, [M+2H]²⁺ = 459.5; Analytical Purity-RP-HPLC: 95%; HRMS (ESI) calculated for C₄₃H₅₇O₁₁N₁₂ [M+H]⁺ = 917.42643, found 917.42697.

[N-Ph-fused-di-pyrrolidine-NMeFRFFK]Y, N1

![N1-major*](image)

The **titled fused di-pyrrolidine-peptide PepNat** was prepared from supported aldehyde peptide resin 3n-1 (385 mg, 7.7 µmol, starting unfunctionalized loading 0.26 mmol/g, calculated loading 0.02 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (4.0 mg, 23.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 66:34 diastereomeric ratio (tᵣ,major* = 17.4 min, tᵣ,minor* = 24.8 min, with [M+H]⁺ = 1264.7 and [M+2H]²⁺ = 633.1) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). Purification by semi-preparative-RP-HPLC of the crude product (16.2 mg), using a step gradient of 20% to 70% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 40 min at a flow rate of 6 mL/min afforded the major* diastereomer **N1-major** (1.6 mg, 1.26 µmol, 16% cycloaddition yield and 1% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the minor* diastereomer **N1-minor** (1.0 mg, 0.79 µmol, 10% cycloaddition yield and 1% overall yield) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer N1-major**: Optimized RP-HPLC-MS (II): Method II-C, tᵣ = 17.6, [M+H]⁺ = 1264.7, [M+2H]²⁺ = 633.1; Analytical Purity-RP-HPLC (210 nm): 99%; HRMS (ESI) calculated for C₆₉H₇₈O₁₁N₁₃ [M+H]⁺ = 1264.59383, found 1264.59513.

**Minor diastereomer N1-minor**: Optimized RP-HPLC-MS (II): Method II-C, tᵣ = 24.8, [M+H]⁺ = 1264.7, [M+2H]²⁺ = 633.1; Analytical Purity-RP-HPLC (210 nm): 86%; HRMS (ESI) calculated for C₆₉H₇₈O₁₁N₁₃ [M+H]⁺ = 1264.59383, found 1264.59580.
The *titled fused di-pyrrolidine-peptide PepNat* was prepared from supported aldehyde peptide resin *3n-2* (150 mg, 51.0 µmol, loading 0.34 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with *N*-phenylmaleimide (7) (22.1 mg, 127.5 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 64:36 diastereomeric ratio (*t_R,major* = 15.3 min, *t_R,minor* = 22.7 min, with [M+H]^+ = 1158.6 and [M+2H]^2+ = 580.1) according to the RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (36.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 15% to 55% of MeCN + 0.1% TFA in H_2O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to yield the major* diastereomer N2-major* (1.5 mg, 1.30 µmol, 3%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%) and the minor* diastereomer N2-minor* (0.8 mg, 0.69 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

**Major diastereomer N2-major*: Optimized RP-HPLC-MS (II): Method II-C, *t_R* = 15.4, [M+H]^+ = 1158.6, [M+2H]^2+ = 580.0; Analytical Purity-RP-HPLC (210 nm): 99%; HRMS (ESI) calculated for C_{62}H_{72}O_{10}N_{13} [M+H]^+ = 1158.55196, found 1158.55331.

**Minor diastereomer N2-minor*: Optimized RP-HPLC-MS (II): Method II-C, *t_R* = 22.7, [M+H]^+ = 1158.7, [M+2H]^2+ = 580.1; Analytical Purity-RP-HPLC (210 nm): 95%; HRMS (ESI) calculated for C_{62}H_{72}O_{10}N_{13} [M+H]^+ = 1158.55196, found 1158.55331.
The titled PepNat was prepared from supported aldehyde peptide resin 3n-3 (300 mg, 120.0 µmol, loading 0.40 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (52.0 mg, 300.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 72:28 diastereomeric ratio (t<sub>R,major*</sub> = 16.3 min, t<sub>R,minor*</sub> = 23.9 min, with [M+H]<sup>+</sup> = 1101.7 and [M+2H]<sup>2+</sup> = 551.6) according to the RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (21.1 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 50% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 40 min at a flow rate of 6 mL/min to give the major* diastereomer N3-major* (2.7 mg, 2.45 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).

Major diastereomer N3-major*: Optimized RP-HPLC-MS (II): Method II-C, t<sub>R</sub> = 16.0 min, [M+H]<sup>+</sup> = 1101.8 and [M+2H]<sup>2+</sup> = 551.6; Analytical Purity-RP-HPLC (210 nm): 98%; HRMS (ESI) calculated for C<sub>60</sub>H<sub>69</sub>O<sub>9</sub>N<sub>12</sub>[M+H]<sup>+</sup> = 1101.53050, found 1101.53094.

The titled fused di-pyrrolidine-Rff PepNat was prepared from supported aldehyde peptide resin 3o-1 (190 mg, 8.0 µmol, starting unfunctionalized loading 0.26 mmol/g, calculated loading 0.03 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-methylmaleimide.
(6) (2.7 mg, 24 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 39:61 diastereomeric ratio (t<sub>R,minor</sub> = 13.9 min, t<sub>R,major</sub> = 14.6 min with [M+H]<sup>+</sup> = 1202.7 and [M+2H]<sup>2+</sup> = 602.1) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (12.4 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 15% to 55% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 35 min at a flow rate of 6 mL/min to afford the minor diastereomer O1-<i>minor</i> (0.6 mg, 0.50 µmol, 6% cycloaddition yield and 1% overall yield) as a white fluffy solid and single diastereomer (d.e. ≥ 99%) and the major diastereomer O1-<i>major</i> (0.7 mg, 0.58 µmol, 7% cycloaddition yield and 1% overall yield) as a white fluffy solid and single diastereomer (d.e. ≥ 99%).

Minor diastereomer O1-<i>minor</i>: Optimized RP-HPLC-MS (II): Method II-C, t<sub>R</sub> = 14.1 min, [M+H]<sup>+</sup> = 1202.17, [M+2H]<sup>2+</sup> = 602.1; Analytical Purity-RP-HPLC (210 nm): 99%; HRMS (ESI) calculated for C<sub>64</sub>H<sub>76</sub>O<sub>11</sub>N<sub>13</sub>[M+H]<sup>+</sup> = 1202.57818, found 1202.57936.

Major diastereomer O1-<i>major</i>: Optimized RP-HPLC-MS (II): Method II-C, t<sub>R</sub> = 14.6 min, [M+H]<sup>+</sup> = 1202.7, [M+2H]<sup>2+</sup> = 602.1; Analytical Purity-RP-HPLC (210 nm): 99%; HRMS (ESI) calculated for C<sub>64</sub>H<sub>76</sub>O<sub>11</sub>N<sub>13</sub>[M+H]<sup>+</sup> = 1202.57818, found 1202.57936.

[N-Ph-fused-di-pyrrolidine-nMeFRffK]Y, O2

The titled PepNat was prepared from supported aldehyde peptide resin 3o-1 (150 mg, 42.0 µmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with N-phenylmaleimide (7) (18.2 mg, 105.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 24:76 diastereomeric ratio (t<sub>R,minor</sub> = 17.5 min, t<sub>R,major</sub> = 18.3 min with [M+H]<sup>+</sup> = 1265.0 and [M+2H]<sup>2+</sup> = 633.2) according to the RP-HPLC-MS (II) chromatogram (Method II-C). Purification by semi-preparative-RP-HPLC of the crude product (18.2 mg), a step gradient of 20% to 50% of MeCN + 0.1% TFA in H<sub>2</sub>O + 0.1% TFA in 30 min at a flow rate of 6 mL/min afforded the major diastereomer O2-<i>major</i> (1.1 mg, 0.87 µmol, 2%) as a white fluffy solid and a single diastereomer (d.e. ≥ 99%).
**Major diastereomer O2-major:** RP-HPLC-MS (II): Method II-C, $t_R = 18.0$ min, $[M+H]^+ = 1265.2$, $[M+2H]^{2+} = 633.3$; Analytical Purity-RP-HPLC (210 nm): 98%; HRMS (ESI) calculated for $C_{69}H_{78}O_{11}N_{13}$ $[M+H]^+ = 1264.59383$, found 1264.59401.

$[N$-Me-$fused$-$di$-$pyrrolidine$-N$Me$FFLK\textsc{G}]\textsc{G}$, P1

The titled fused di-pyrrolidine-dipeptide macrocycle was prepared from supported aldehyde peptide resin 3p (120 mg, 33.6 µmol, loading 0.28 mmol/g) using first the Fmoc deprotection representative procedure (RP-03) followed by intramolecular imine cyclization and 1,3-dipolar cycloaddition on resin with $N$-methylmaleimide (6) (9.4 mg, 84.0 µmol) according to the representative procedure (RP-04). The cycloaddition proceeded with a 57:43 diastereomeric ratio ($t_{R, major} = 28.4$ min, $t_{R, minor} = 28.7$ min with $[M+H]^+ = 906.8$) according to the optimized RP-HPLC-MS (II) chromatogram (Method II-C). The crude product (10.3 mg) was purified by semi-preparative-RP-HPLC using a step gradient of 10% to 55% of MeCN + 0.1% TFA in H$_2$O + 0.1% TFA in 37 min at a flow rate of 6 mL/min to afford the major diastereomer PepNat P1-major (1.1 mg, 1.19 µmol, 4%) as a white fluffy solid and a single diastereomer (d.e. = 80%).

**Major diastereomers P1-major:** Optimized RP-HPLC-MS (II): Method II-C, $t_{R, major} = 28.4$ min, $t_{R, minor} = 28.6$, $[M+H]^+ = 906.8$, d.r. 90:10; Analytical Purity-RP-HPLC: 98%; HRMS (ESI) calculated for $C_{48}H_{50}O_{9}N_{9}$ $[M+H]^+ = 906.45085$, found 906.45085.
4) Independent Synthesis in Solution to Establish the Absolute Configuration of PepNats

4) A) Independent Enantiopure Synthesis in Solution of the (2S, 3R, 4S, 5R)-Configured Cycloadduct 19a and 19b

**Allyl 4-formyl benzoate (16)**

![Allyl 4-formyl benzoate (16)]

To a solution of 4-formylbenzoic acid (15) (1.00 g, 6.66 mmol) in DMF (13.3 mL) was added cesium carbonate (2.60 g, 7.99 mmol). After 10 min, allyl bromide (1.44 ml, 16.65 mmol) was added dropwise and the resulting reaction mixture was stirred for 16 h at room temperature. H₂O (15 mL) was then added and the resulting mixture was extracted with Et₂O (5 × 30 mL). The combined organic layers were dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product (1.21 g) was purified by flash chromatography (cHex – Et₂O 100:0 to 80:20) to afford the desired allyl ester product 16 (0.95 g, 75% yield) as a colorless oil.

Rᵣ 0.40 (cHex – Et₂O 80:20); ¹H NMR (400 MHz, CDCl₃) δ 10.10 (s, 1H), 8.21 (d, J = 8.58 Hz, 2H), 7.95 (d, J = 8.58 Hz, 2H), 6.04 (ddt, J = 5.74, 10.43, 17.18 Hz, 1H), 5.43 (dq, J = 1.50, 17.20 Hz, 1H), 5.32 (dq, J = 1.25, 10.43 Hz, 1H), 4.85 (dt, J = 1.39, 5.74 Hz, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 191.7, 165.4, 139.4, 135.3, 131.9, 130.4 (2C), 129.7 (2C), 119.0, 66.3. The NMR data are in agreement with the one reported in the literature.¹¹

**Allyl (E)-4-(((2-methoxy-2-oxoethyl)imino)methyl)benzoate (17)**

![Allyl (E)-4-(((2-methoxy-2-oxoethyl)imino)methyl)benzoate (17)]

A suspension of tert-butyl glycinate HCl salt (1.00 g, 5.99 mmol), anhydrous magnesium sulfate (0.75 g, 6.24 mmol) and triethylamine (0.84 mL, 5.99 mmol) in DCM (23 mL) was stirred at room temperature for 1.25 h under inert atmosphere. A solution of allyl ester 16 (0.95 g, 4.99 mmol) in DCM (17 mL) was then added to the suspension. The resulting reaction mixture was stirred for 20 h at room temperature. The mixture was filtered off to remove the magnesium sulfate. The filtrate was quickly washed with water (50 mL). The organic layer was separated and the aqueous layer was extracted with DCM (5 × 50 mL). The combined organic layers were dried over magnesium sulfate anhydrous, filtered, evaporated, and dried under reduced pressure to afford the desired unstable imine 17 (1.70 g, quantitative yield) as a pale-yellow oil, which was directly used into the next step without further purification.
\[
^{1}H \text{ NMR (400 MHz, DMSO-} d_6) \delta 8.43 \text{ (s, 1H), 8.06 (d, } J = 8.30 \text{ Hz, 2H), 7.91 (d, } J = 8.29 \text{ Hz, 2H), 5.98 – 6.12 \text{ (m, 1H), 5.42 (dd, } J = 1.61, 17.25 \text{ Hz, 1H), 5.29 (dd, } J = 1.40, 10.48 \text{ Hz, 1H), 4.82 (d, } J = 5.43 \text{ Hz, 2H), 4.36 (s, 2H), 1.44 (s, 9H).}
\]

tert-Butyl (1S,3R,3aS,6aR)-3-[(allyloxy)carbonyl]phenyl]-5-methyl-4,6-dioxooctahydropyrrolo[3,4-c]pyrrole-1-carboxylate (18a)

To a solution of (Rp)-2-(tert-butylthio)-1-(diphenylphosphino)ferrocene (0.69 g, 0.15 mmol) and tetrakis(acetonitrile)copper(I) tetrafluoroborate (0.05 g, 0.15 mmol) in DCM (13.7 mL) at room temperature was added a solution of imine 17 (1.51 g, 4.99 mmol) in DCM (25 mL) followed by triethylamine (0.70 mL, 4.99 mmol) and 1-methyl-1H-pyrrole-2,5-dione (6) (0.61 g, 5.49 mmol). The resulting yellow clear reaction mixture was stirred at room temperature for 3 h. The resulting reaction mixture was concentrated under reduce pressure. The crude orange solid was purified by flash chromatography (AcOEt + 1% 7N NH₃ in MeOH – cHex 0:100 to 60:40) to afford the desired title cycloadduct 18a (1.97 g, 95% yield) as a white creamy solid.

Rf 0.42 (AcOEt + 1% 7N NH₃ in MeOH – cHex 50:50); [α]_D^{20} +108.6 (c 0.14, CH₂Cl₂); \(^{1}H \text{ NMR (500 MHz, CDCl}_3) \delta 8.03 \text{ (d, } J = 8.27 \text{ Hz, 2H), 7.43 (d, } J = 8.23 \text{ Hz, 2H), 6.03 (ddt, } J = 5.68, 10.82, 16.20 \text{ Hz, 1H), 5.36–5.48 (m, 1H), 5.23–5.32 (m, 1H), 4.81 (d, } J = 5.68 \text{ Hz, 2H), 4.50 (dd, } J = 4.99, 8.38 \text{ Hz, 1H), 3.89–4 (m, 1H), 3.55 (t, } J = 7.16 \text{ Hz, 1H), 3.45 (t, } J = 8.14 \text{ Hz, 1H), 2.85 (s, 3H), 2.40 (s, 1H), 1.58 (s, 9H); 13C \text{ NMR (126 MHz, CDCl}_3) \delta 175.7, 174.7, 174.7, 168.5, 166.0, 142.3, 132.3, 130.1, 129.9 (× 2), 127.2 (× 2), 118.5, 82.7, 65.7, 63.4, 62.5, 49.7, 48.0, 28.2 (× 3), 25.1; \text{ HRMS (ESI) calculated for } C_{22}H_{27}N_{2}O_{6} [M+H]^+ = 415.18636, \text{ found 415.18545}.)

tert-Butyl (1S,3R,3aS,6aR)-3-[(allyloxy)carbonyl]phenyl]-4,6-dioxo-5-phenyloctahydropyrrolo[3,4-c]pyrrole-1-carboxylate (18b)

To a solution of (Rp)-2-(tert-butylthio)-1-(diphenylphosphino)ferrocene (14.46 mg, 0.03 mmol) and tetrakis(acetonitrile)copper(I) tetrafluoroborate (9.92 mg, 0.03 mmol) in DCM (2.2 mL) at room
temperature was added a solution of imine 17 (319.00 mg, 1.05 mmol) in DCM (4 mL) followed by triethylamine (0.027 mL, 0.19 mmol) and 1-phenyl-1H-pyrrole-2,5-dione (7) (200.00 mg, 1.16 mmol). The resulting yellow clear reaction mixture was stirred at room temperature for 3 h. The mixture was directly concentrated under reduced pressure. The residual green solid was purified by flash chromatography (AcOEt – cHex 0:100 to 60:40) to afford the phenyl substituted cycloadduct 18b (202.3 mg, 40% yield) as a white creamy solid.

Rf 0.32 (AcOEt – cHex 40:60); [α]D 20°C +107.2 (c 0.14, CH2Cl2); 1H NMR (500 MHz, DMSO-d6) δ 7.97 – 7.91 (m, 2H), 7.61 – 7.55 (m, 2H), 7.44 (dd, J = 8.3, 6.9 Hz, 2H), 7.42 – 7.32 (m, 1H), 7.11 – 7.05 (m, 2H), 6.05 (ddt, J = 17.2, 10.7, 5.4 Hz, 1H), 5.40 (dd, J = 17.2, 1.7 Hz, 1H), 5.27 (dd, J = 10.4, 1.5 Hz, 1H), 4.82 – 4.77 (m, 2H), 4.50 (dd, J = 8.8, 4.6 Hz, 1H), 3.91 (dd, J = 6.8, 4.3 Hz, 1H), 3.70 (ddd, J = 7.9, 6.7, 1.3 Hz, 1H), 3.61 (d, J = 8.3 Hz, 1H), 3.58 (dd, J = 7.4, 3.3 Hz, 1H), 1.48 (s, 9H); 13C NMR (126 MHz, CDCl3) δ 174.7, 173.7, 168.4, 166.0, 142.3, 132.3, 131.7, 130.2, 130.0 (× 2), 129.2 (× 2), 128.7, 127.3 (× 2), 126.3 (× 2), 118.5, 82.8, 65.7, 63.7, 62.8, 49.7, 48.1, 28.3 (× 3); HRMS (ESI) calculated for C27H29N2O6 [M+H]+ = 477.20201, found 477.20147.

(15,3R,3aS,6aR)-3-[(allyloxy)carbonyl]phenyl]-5-methyl-4,6-dioxooctahydropyrrolo[3,4-c]pyrrole-1-carboxylic acid (19a)

Cycloadduct 18a (0.98 g, 2.36 mmol) was dissolved in DCM (25 mL). The solution was cooled to 0 °C, then TFA (25 mL, 326.47 mmol) was added dropwise. The reaction mixture was allowed to slowly warm to room temperature and stirred for 8 h at room temperature. Toluene (25 mL) was added and the resulting mixture was concentrated under reduced pressure to give the desired acid 19a (1.17 g, quantitative yield) as a yellow brown solid.

RP-HPLC-MS (I): Method I-A tR = 3.38 min, [M+H]+ = 359.1; [α]D 20°C +60.7 (c 0.14, CH2Cl2); 1H NMR (600 MHz, DMSO-d6) δ 7.91 (d, J = 8.3 Hz, 2H), 7.47 (d, J = 8.2 Hz, 2H), 6.05 (ddt, J = 15.9, 10.7, 5.4 Hz, 1H), 5.41 (dd, J = 17.2, 1.5 Hz, 1H), 5.25–5.31 (m, 1H), 4.80 (d, J = 5.4 Hz, 2H), 4.47 (d, J = 8.4 Hz, 1H), 3.97–3.90 (m, 1H), 3.61–3.57 (m, 1H), 3.53 (m, 1H), 3.41 (s, 1H), 3.33 (s, 1H), 2.67 (s, 3H); 13C NMR (151 MHz, DMSO) δ 206.6, 176.1, 175.0, 165.4, 137.4, 132.7, 128.8 (× 2), 127.7 (× 2), 125.3, 118.0, 65.1, 62.3, 61.0, 48.9, 47.2, 24.5; HRMS (ESI) calculated for C18H19N2O6 [M+H]+ = 359.12376, found 359.12347.
(1S,3R,3aS,6aR)-3-{4-[allyloxy]carbonyl]phenyl}-4,6-dioxo-5-phenyloctahydropyrrolo[3,4-c]pyrrole-1-carboxylic acid (19b)

The phenyl substituted cycloadduct 18b (0.19 g, 0.40 mmol) was dissolved in DCM (4.7 mL). The solution was cooled to 0 °C then TFA (4.7 mL) was added dropwise. The reaction mixture was allowed to slowly warm to room temperature and stirred for 8 h. Toluene (10.0 mL) was added and the resulting mixture was concentrated under reduce pressure to afford the desired acid 19b (0.21 g, quantitative yield) as a brown orange solid.

RP-HPLC-MS (I): Method I: t_R = 4.51 min, [M+H]^+ = 421.2; [α]_D^{20°C} +85.3 (c 0.14, CH₂Cl₂); ^1H NMR (700 MHz, DMSO-d₆) δ 7.97 (d, J = 8.23 Hz, 2H), 7.64 (d, J = 8.12 Hz, 2H), 7.45 (t, J = 7.81 Hz, 2H), 7.38 (t, J = 7.45 Hz, 1H), 7.13 (d, J = 7.69 Hz, 2H), 6.05 (ddt, J = 5.44, 10.78, 15.92 Hz, 1H), 5.35 – 5.49 (m, 1H), 5.23 – 5.31 (m, 1H), 4.80 (d, J = 5.35 Hz, 2H), 4.73 (s, 1H), 4.21 (s, 1H), 4.18 (s, 1H), 3.87 (d, J = 6.07 Hz, 2H), 3.75 – 3.8 (m, 2H); ^13C NMR (176 MHz, DMSO-d₆) δ 206.5, 165.3, 132.6, 132.2, 128.9, 128.9, 128.8 (× 2), 128.3 (× 2), 128.2, 127.9 (× 2), 126.8 (× 2), 118.0 (× 2), 65.1, 62.6, 61.3, 48.5, 47.0, 30.7; HRMS (ESI) calculated for C₂₃H₂₁N₂O₆ [M+H]^+ = 421.13941, found 421.13808.

4) B) Independent Synthesis in Solution of the (2S, 3R, 4S, 5R)-Configured PepNats H1-minor and K1-minor

AllylO₂C-[19a]-nMeFGLGFK(Mtt)F-resin (20a)

Step 1: The Fmoc protecting group was removed by treating the linear peptide precursor FmocHN-nMeF-GLGFK(Mtt)F-resin (180.0 mg, 0.07 mmol, loading 0.40 mmol/g), obtained using automated solid phase peptide synthesis according to the representative procedure (RP_01), with a solution of 20% piperidine in DMF (3 mL) for 4 min. The solution was removed by filtration and the resin was washed with DMF (2 × 4 mL) and DCM (2 × 4 mL). The deprotection was repeated 2
additional times for 4 min each. Finally, the resin was washed with DMF (3 × 4 mL) and DCM (3 × 4 mL) and quickly dried under a stream of argon.

**Step 2:** A solution of cycloadduct 19a (67.9 mg, 0.14 mmol), PyBOP (74.9 mg, 0.14 mmol) and HOAt (19.6 mg, 0.14 mmol) in DMF (4 mL) was added to the unprotected N-terminal amine resin from step 1, followed by DIPEA (0.10 mL, 0.58 mmol). The resulting suspension was shaken at room temperature for 3.5 h. The filtrate was removed then the resin was washed with DMF (3 × 4 mL) and DCM (3 × 4 mL), and quickly dried under a steam of argon. The coupling procedure from step 2 was repeated 2 additional times for 20 h each at room temperature.

**Test cleavage of the functionalized resin 20a from step 2:**

The test cleavage was performed on a small portion of resin (ca. 4 mg) using a solution of TFA/H₂O/TIS (800 μL, 95:2.5:2.5; v/v/v/v). After shaking for 1.5 h, the filtrate was collected by filtration and dried under a flow of argon. The remaining residue was dissolved in MeCN + 0.1% TFA and H₂O + 0.1% TFA (80 μL, 1:1, v/v) and analyzed by RP-HPLC-MS to confirm the formation of the desired unbound and unprotected peptide product (MW = 1168.36 g.mol⁻¹) in 100% conversion and as a single diastereomer.

RP-HPLC-MS (I): Method I-A tᵣ = 3.97 min, [M+H]⁺ = 1169.6, [M+2H]²⁺ = 585.2.

**AllylO₂C-[19b]-nMeFRffNK(Mtt)Y-resin (20b)**

![image](image.png)

**Step 1:** The Fmoc protecting group was removed by treating the linear peptide precursor FmocHN-nMeF-RffNK(Mtt)F-resin (250.0 mg, 0.09 mmol, loading 0.34 mmol/g), obtained using automated solid phase peptide synthesis according to the representative procedure (RP_01), with a solution of 20% piperidine in DMF (3 mL) for 4 min. The solution was removed by filtration and the resin was washed with DMF (2 × 4 mL) and DCM (2 × 4 mL). The deprotection was repeated 2 additional times for 4 min each. Finally, the resin was washed with DMF (3 × 4 mL) and DCM (3 × 4 mL) and quickly dried under a stream of argon.

**Step 2:** A solution of cycloadduct 19b (91.0 mg, 0.17 mmol), PyBOP (88.0 mg, 0.17 mmol) and HOAt (23.1 mg, 0.17 mmol) in DMF (4.8 mL) was added to the unprotected N-terminal amine resin from step 1, followed by DIPEA (0.12 mL, 0.68 mmol). The resulting suspension was shaken at room temperature for 16 h. The filtrate was removed then the resin was washed with DMF (3 × 4 mL)
and DCM (3 × 4 mL), and quickly dried under a steam of argon. The coupling procedure from step 2 was repeated 2 additional times for 16 h each at room temperature.

**Test cleavage of the functionalized resin 20b from step 2:**

The test cleavage was performed on a small portion of resin (ca. 4 mg) using a solution of TFA/H₂O/TIS (800 μL, 95:2.5:2.5; v/v/v/v). After shaking for 1.5 h, the filtrate was collected by filtration and dried under a flow of argon. The remaining residue was dissolved in MeCN + 0.1% TFA and H₂O + 0.1% TFA (100 μL, 1:1, v/v) and analyzed by RP-HPLC-MS to confirm the formation of the desired unbound and unprotected peptide product (MW = 1436.64 g.mol⁻¹) in 77% conversion and as a single diastereomer.

RP-HPLC-MS (I): Method I-A tᵣ = 6.85 min, [M+H]⁺ = 1436.6, [M+2H]²⁺ = 719.3.

**HO₂C-[19a]-NMeFGLGFK(Mtt)F-resin (21a)**

![Resin 21a](image)

Functionalized resin 20a (180.0 mg, 0.07 mmol, loading 0.40 mmol/g) was suspended in previously degazed 1,2-dichloroethane (2.0 mL) at room temperature under argon. To the resin suspension was added phenylsilane (0.173 mL, 1.40 mmol) followed by a solution of tetrakis(triphenylphosphine)-palladium(0) (40.0 mg, 0.04 mmol) in degazed 1,2-dichloroethane (4.2 mL). The resulting yellow suspension was shaken for 3 h at room temperature under argon. The resin was washed with DMF (2 × 4 mL) then consecutively with a solution of 0.5 % DIPEA in DMF (5 × 4 mL) and a solution of 0.5 % sodium diethylthiocarbamate trihydrate in DMF (5 × 4 mL) and extensively with DMF (4 × 4 mL) and DCM (4 × 4 mL) to be finally dried under a flow of argon.

**Test cleavage of free acid resin 21a:**

A small portion (ca. 4 mg) of the resin was used to performed a test cleavage using a solution of TFA/H₂O/TIS (800 μL, 95:2.5:2.5; v/v/v/v). After shaking for 2.5 h, the filtrate was collected by filtration and cooled Et₂O was added. The white precipitate was centrifuged. The residual white solid was dissolved in H₂O + 0.1% TFA and MeCN + 0.1% TFA (100 μL, 50:50) and analyzed by RP-HPLC-MS to confirm the formation of the desired unprotected and unbound peptide product (MW = 1128.93 g.mol⁻¹) in 100% conversion and as a single diastereomer.

RP-HPLC-MS (I): Method I-A tᵣ = 3.48 min, [M+H]⁺ = 1128.6 and [M+2H]²⁺ = 565.1.
HO₂C-[19b]-NMeFGLGFK(Mtt)F-resin (21b)

Functionalized resin 20b (250.0 mg, 0.09 mmol, loading 0.34 mmol/g) was suspended in previously degazed 1,2-dichloroethane (2.4 mL) at room temperature under argon. To the resin suspension was added phenylsilane (0.21 mL, 1.70 mmol) followed by a solution of tetrakis(triphenylphosphine)-palladium(0) (49.0 mg, 0.04 mmol) in degazed 1,2-dichloroethane (5.0 mL). The resulting yellow suspension was shaken for 4.5 h at room temperature under argon. The resin was washed with DMF (2 × 4 mL) then consecutively with a solution of 0.5 % DIPEA in DMF (5 × 4 mL) and a solution of 0.5 % sodium diethylthiocarbamate trihydrate in DMF (5 × 4 mL) and extensively with DMF (4 × 4 mL) and DCM (4 × 4 mL) to be finally dried under a flow of argon.

Test cleavage of free acid resin 21b:

A small portion (ca. 4 mg) of the resin was used to perform a test cleavage using a solution of TFA/H₂O/TIS (800 μL, 95:2.5:2.5; v/v/v/v). After shaking for 2.5 h, the filtrate was collected by filtration and cooled Et₂O was added. The white precipitate was centrifuged. The residual white solid was dissolved in H₂O + 0.1% TFA and MeCN + 0.1% TFA (100 μL, 50:50) and analyzed by RP-HPLC-MS to confirm the formation of the desired unprotected and unbound peptide product (MW = 1396.57 g.mol⁻¹) in 100% conversion and as a single diastereomer.

RP-HPLC-MS (I): Method I-A tᵣ = 2.97 min, [M+H]⁺ = 1396.6 and [M+2H]²⁺ = 699.3.

[N-Me-fused-di-pyrrolidine-NMeFGLGFK]F, H₁-minor

Step 1: Functionalized resin 21a (180.0 mg, 0.07 mmol, loading 0.40 mmol/g) was shaken with a solution of DCM/TFA/TIS (4 mL, 95:2:3) for 2 min at room temperature. The solution was removed by filtration and the resin was washed with DCM (3 × 6 mL). The Mtt deprotection was repeated 8
additional times for 2 min each. Finally, the resin was washed with DCM (3 × 6 mL), 5% DIPEA in DCM (3 × 6 mL) and DMF (3 × 6 mL).

Step 2: To a suspension of the Mtt unprotected resin from step 1 in DMF (1.8 mL) was added a solution of PyBOP (14.6 mg, 0.28 mmol) and HOAt (38.0 mg, 0.28 mmol) in DMF (1.8 mL) and DCM (3.5 mL) followed by DIPEA (0.073 mL, 0.42 mmol). The suspension was shaken at room temperature for 16 h. The resin was washed with DCM (3 × 6 mL), DMF (3 × 6 mL) and DCM (3 × 6 mL).

Step 3: The resin from step 2 was shaken with a solution of TFA/H₂O/TIS (6 mL, 95:3:2; v/v/v) for 2.5 h at room temperature. The desired product was precipitated by adding cooled diethyl ether (35 mL) to the TFA filtrate. The precipitate was collected by centrifugation (4000 rpm, 4 °C, 10 min) and dissolved in MeCN/H₂O (10 mL, 10:90, v/v) and freeze dried overnight. The crude product (23.3 mg) was purified by semi-preparative RP-HPLC-MS using a step gradient of 10% to 50% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 33 min at a flow rate of 6 mL/min to afford the (2S, 3R, 4S, 5R)-configured PepNat, H1-minor (1.7 mg, 2% yield) as a white fluffy solid and single diastereomer (d.e. ≥ 99%).

(2S, 3R, 4S, 5R)-Configured GLGF PepNat obtained through independent synthesis in solution = Minor diastereomer of imine/cycloaddition on resin strategy H1-minor: RP-HPLC-MS (I): Method I-C, tᵣ = 10.0 min, [M+H]⁺ = 1111.2, [M+2H]²⁺ = 556.0; ¹H NMR (700 MHz, CD₃OH, 25 °C) 0.96 (d, J = 6.5 Hz, 3H), 1.00 (d, J = 6.7 Hz, 3H), 1.38 (m, 1H), 1.46 (m, 1H), 1.53 (m, 2H), 1.67 (m, 3H), 1.82 (m, 1H), 1.94 (ddd, J = 13.6, 11.2, 4.4 Hz, 1H), 2.78 (dd, J = 14.3, 9.6 Hz, 1H), 2.84 (s, 3H), 2.92 (dd, J = 13.8, 8.7 Hz, 1H), 2.99 (dd, J = 14.5, 4.9 Hz, 1H), 3.13 (m, 1H), 3.16 (s, 3H), 3.21 (m, 1H), 3.23 (dd, J = 14.5, 7.6 Hz, 1H), 3.36 (dd, J = 16.9, 5.9 Hz, 1H), 3.45 (dd, J = 16.8, 5.9 Hz, 1H), 3.50 (dd, J = 17.3, 5.1 Hz, 1H), 3.66 (dd, J = 14.3, 4.5 Hz, 1H), 3.74 (m, 1H), 3.77 (t, J = 8.3 Hz, 1H), 3.91 (t, J = 7.8 Hz, 1H), 4.03 (dd, J = 7.5, 4.5 Hz, 1H), 4.13 (m, 3.9 Hz, 1H), 4.15 (m, 1H), 4.20 (dd, J = 17.3, 6.8 Hz, 1H), 4.49 (ddd, J = 11.9, 8.5, 4.0 Hz, 1H), 4.57 (d, J = 8.1 Hz 1H), 4.59 (dd, J = 7.6, 6.1 Hz, 1H), 4.69 (d, J = 8.4 Hz, 1H), 7.03 (s, 1H), 7.15 – 7.27 (m, 11H), 7.29 (t, J = 7.6 Hz, 2H), 7.38 (d, J = 8.0 Hz, 2H), 7.48 (s, 1H), 7.55 (d, J = 7.5 Hz, 2H), 7.58 (d, J = 6.0 Hz, 1H), 7.67 (d, J = 8.4 Hz, 1H), 7.74 (d, J = 8.4 Hz, 1H), 7.75 (d, J = 8.1 Hz, 2H), 7.87 (d, J = 6.9 Hz, 1H), 8.27 (t, J = 6.1 Hz, 1H), 8.31 (t, J = 6.0 Hz, 1H), 8.44 (dd, J = 7.3, 4.6 Hz, 1H), the NH of the fused di-pyrrolidine NP unit was excluded; ¹³C NMR (176 MHz, CD₃OH, 25 °C) 21.6, 23.1, 23.5, 25.2, 25.6, 29.6, 31.6, 35.0, 38.0, 38.4, 38.7, 39.1, 41.8, 43.0, 44.0, 48.0, 50.6, 52.7, 55.0, 55.4, 56.3, 62.2, 64.2, 69.2, 127.2, 127.7, 127.7, 128.2 (x2), 128.6 (x2), 129.4 (x2), 129.4 (x2), 129.4 (x2), 130.2 (x2), 130.3 (x2), 130.5 (x2), 135.5, 138.2, 138.2, 139.7, 141.4, 170.7, 170.8, 170.9, 171.9, 172.0, 173.9, 174.4, 175.5, 175.8, 176.0, 176.5; HRMS (ESI) calculated for C₅₉H₇₂N₁₁O₁₁ [M+H]⁺ = 1110.54073, found 1110.54287.
**[N-Ph-fused-di-pyrrolidine-nMeFRffNK]Y, K1-minor**

![K1-minor](image)

**Step 1:** Functionalized resin **21b** (250.0 mg, 0.09 mmol, loading 0.34 mmol/g) was shaken with a solution of DCM/TFA/TIS (3 mL, 95:2:3) for 2 min at room temperature. The solution was removed by filtration and the resin was washed with DCM (3 × 4 mL). The Mtt deprotection was repeated 7 times for 2 min each. Finally, the resin was washed with DCM (3 × 4 mL), 5% DIPEA in DCM (3 × 4 mL), DMF (3 × 4 mL) and DCM (3 × 4 mL).

**Step 2:** To a suspension of the partially unprotected resin from step 1 in DMF (2 mL) was added a solution of PyBOP (177.0 mg, 0.34 mmol) and HOAt (46.3 mg, 0.34 mmol) in DMF (2.5 mL) and DCM (4.5 mL) followed by DIPEA (0.089 mL, 0.51 mmol). The suspension was shaken at room temperature for 16 h. The resin was washed with DCM (3 × 6 mL), DMF (3 × 6 mL) and DCM (3 × 6 mL).

**Step 3:** The resin from step 2 was shaken with a solution of TFA/H₂O/TIS (8 mL, 95:3:2; v/v/v) for 3 h at room temperature. The desired product was precipitated by adding cooled diethyl ether (35 mL) to the TFA filtrate. The precipitate was collected by centrifugation (4000 rpm, 4 °C, 8 min) and dissolved in MeCN/H₂O (15 mL, 10:90, v/v) and freeze dried overnight. The crude product (50.4 mg) was purified by semi-preparative RP-HPLC-MS using a step gradient of 20% to 50% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 30 min at a flow rate of 6 mL/min to afford the (2S, 3R, 4S, 5R)-configured PepNat, **K1-minor** (2.1 mg, 2% yield) as a white fluffy solid and single diastereomer (d.e. ≥ 99%).

*(2S, 3R, 4S, 5R)-Configured RffN PepNat obtained through independent synthesis in solution = Minor diastereomer of imine/cycloaddition on resin strategy **K1-minor:** Optimized RP-HPLC-MS (II): Method II-C, tᵣ = 14.8 min, [M+H]⁺ = 1378.6, [M+2H]²⁺ = 690.0; Analytical Purity-RP-HPLC: 88%; HRMS (ESI) calculated for C₇₃H₆₄O₁₅N₁₅ [M+H]⁺ = 1378.63675, found 1378.64060.*
4) C) Independent Racemic Synthesis in Solution Used for the LC-MS Comparison Depicted in Supplementary Figure S2 and S3

The independent racemic synthesis of the endo cycloadduct products 19a-racemic and 19b-racemic was performed using the procedure described in Section 4)A) for the cycloadducts 19a and 19b. However, the 1,3-dipolar cycloaddition step was performed using two different achiral conditions (Method A: AgOAc, Et3N, DCM, MeCN and Method B: LiBr, Et3N, THF) to afford racemic cycloadduct 18a-racemic with $[\alpha]_{D}^{20}{ }^\circ C +2.5$ ($c$ 0.14, CH2Cl2) and 18b-racemic with $[\alpha]_{D}^{20}{ }^\circ C +3.2$ ($c$ 0.14, CH2Cl2).

The racemic cycloadducts 19a-racemic and 19b-racemic were then introduced in the required linear peptides, using the procedure described in Section 4)B) to afford the racemic PepNat H1-racemic and K1-racemic, containing both the (2S, 3R, 4S, 5R)- and (2R, 3S, 4R, 5S)-stereocenters for the fused di-pyrrolidine NP moiety. Supplementary Figure S2a and S3a showed the crude HPLC profile after test cleavage for the racemic independent synthesis of PepNats H1-racemic and K1-racemic respectively.
5) Synthesis and Characterization of Reference Peptides and Peptidomimetics

5) A) Synthesis of Literature Reference Linear and Cyclic Peptides 22–25

Linear peptides 22, 23 and disulfide bridged peptide 24

The acetylated peptide 22 and 23 were synthesized according to the representative procedure (RP_01) followed by an additional final N-acylation step using 10% Ac₂O in DMF / 2 M DIPEA in DMF (1:3, v/v) for 5 × 15 min. Then the linear peptides were unprotected and cleaved from the resin using TFA/H₂O/TIPS (95:2.5:2.5, v/v/v). After cleavage, the peptide was precipitated with cold diethyl ether and dissolved in H₂O/MeCN (90:10, v/v) followed by lyophilization. The crude product was purified by semi-preparative RP-HPLC (Atlantis rep T3 OBD column, 10µ silica, 19 mm diameter, 250 mm length), using decreasingly polar mixtures of water (containing 0.1% formic acid or 0.1% TFA) and MeCN as eluents. Fractions containing the desired compound were evaporated to dryness to afford the desired linear peptides 22 (7.5 mg, 4.7 µmol) as a white solid with 88% purity and 23 (12.1 mg, 19.2 µmol) as a white solid with 86% purity.

The disulfide bridged peptide 24 (10 mg, purity 95%) was purchased from the Chinese peptide company.

The table below summarizes the peptide sequence, related molecular weight, observed retention time and [M+H]⁺ ion found.

| peptide sequence | MW (g.mol⁻¹) | t_R (min) [method] | [M+H]⁺ found |
|------------------|-------------|-------------------|--------------|
| 22 Ac-KEKDIINNNVKKT-NH₂ | 1600.77 | 0.8 [method I-D] | 1601.9 |
| 23 Ac-DINNN-NH₂ | 629.63 | 2.6 [method I-D] | 630.2 |
| 24 [CVDINNNC] | 933.02 | 11.1 [method I-E] | 933.3 |

[CWDINNNβA], 25

Cyclic peptide 25 was synthesized by adapting the procedure described by Sadek et al.16

2-Chlorotrityl chloride resin (118 mg, 0.2 mmol) was pre-swelled in DCM (10 mL) for 1 h. To the resin was loaded a solution of Fmoc-βAla-OH (31.3 mg, 0.1 mmol) and DIPEA (6 µL, 0.5 mmol) in DMF (5 mL). The suspension was shaken for 1.5 h before capping for 30 min. The resin was then washed with DMF (3 × 5 mL), DCM (3 × 5 mL) and Et₂O (4 × 5 mL) and quickly dried under vacuum filtration. The pre-loaded Fmoc-βAla-2-chlorotrityl resin (calculated loading 0.67 mmol/g) was used for the peptide elongation via SPPS using the representative procedure (RP_01) to afford Fmoc-WDINNNβA-2-chlorotrityl resin. After Fmoc deprotection, the peptide was cleaved from the resin using a solution of 30% HFIP in DCM for 2 times 30 min. The peptide was precipitated in Et₂O, dried and lyophilized to afford the crude linear sequence WDINNNβA (155 mg).

A portion of the crude linear peptide (30 mg, 2.8 µmol) was cyclized with HCTU (3 eq.) and DIPEA (6 eq.) in DMF (40 mL) for 16 h at room temperature. The resin was washed with DMF (3 × 5 mL) and DCM (3 × 5 mL) and quickly dried under reduce pressure. The side chain protecting group were
finally removed with a solution of TFA/TIS/H₂O (8 mL, 95:2.5:2.5, v/v/v) for 3 h. The filtrated was recovered and Et₂O (40 mL) was added. The resulting precipitate was collected by centrifugation and purified by semi-preparative RP-HPLC to afford the desired product 25 (2.4 mg) as a white fluffy solid with 96% purity. LC-MS using method I-C showed the desired product at t<sub>R</sub> = 3.0 min with [M+H]<sup>+</sup> = 828.4.

5) B) Synthesis of Literature Reference Cyclic Peptidomimetic 26

2-((2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzyl)oxy)-4-(tert-butoxy)-4-oxobutanoic acid (A)

![Chemical Structure of A](image)

The titled fragment A was synthesized following the six steps procedure described by Harjani, J. R. et al. starting from commercially available L-malic acid to yield 2-((2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzyl)oxy)-4-(tert-butoxy)-4-oxobutanoic acid (A) (3.00 g) with 97% purity. Analytical HPLC-MS (method I-A) showed the desired product at t<sub>R</sub> = 8.04 min with [M+H]<sup>+</sup> = 874.4. ¹H NMR (300 MHz, DMSO-d6) δ 11.35 (s, 1H), 9.80 (s, 1H), 8.24 (d, J = 8.2 Hz, 1H), 7.95 – 7.74 (m, 5H), 7.50 – 6.97 (m, 22H), 4.99 – 4.85 (m, 1H), 4.56 (d, J = 9.5 Hz, 1H), 4.48 – 4.34 (m, 1H), 4.32 – 4.19 (m, 2H), 4.16 (t, J = 8.3 Hz, 1H), 3.91 (dd, J = 10.6, 2.9 Hz, 1H), 2.99 (dd, J = 13.4, 3.6 Hz, 1H), 2.58 (dd, J = 15.5, 2.9 Hz, 1H), 2.40 (t, J = 12.4 Hz, 1H), 2.23 (dd, J = 15.4, 10.7 Hz, 1H), 1.35 (s, 9H). The analytical data are in agreement with the one reported in the literature.¹⁷

[ VIII-DINNN ], 26

![Chemical Structure of VIII-DINNN](image)

The above building block A (296.8 mg, 0.34 mmol) was first manually loaded on 2-chlorotrityl chloride resin (200 mg, 0.34 mmol, loading 1.7 mmol/g) using DIPEA (0.28 mL, 1.7 mol) in DMF
(5 mL). The coupling was repeated two additional times. The resin was washed with DMF (3 × 8 mL) and DCM (3 × 8 mL). The functionalized resin was further functionalized using SPPS according to the representative procedure (RP_01) to afford the required linear peptide: Fmoc-INNN-A-resin. The fully protected linear peptide was then cleaved from the 2-chlorotrityl resin using 30% HFIP in DCM (4 mL) for 40 min at room temperature. To a solution of the obtained crude linear peptide (406 mg, 0.22 mmol) in DMF/DCM 1:1 (440 mL) was treated with a solution of HCTU (273.9 mg, 0.66 mmol) and DIPEA (0.22 mL, 1.32 mmol) in DMF (10 mL). The reaction mixture was stirred for 48 h, concentrated under reduce pressure and the obtained residue was stirred with a solution of TFA:TIS:DODT (20 mL, 95:2.5:2.5, v/v/v) for 2 h at room temperature. Then, Et₂O (60 mL) was added to the reaction mixture and the precipitate was collected by filtration to afford the crude product (208 mg). Purification by semi-preparative-RP-HPLC using a step gradient of 5% to 50% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 40 min at a flow rate of 20 mL/min gave the desired cyclic peptidomimetic 26 (22.7 mg, 0.03 mmol, 13.2% yield) with 98% purity, as a white fluffy solid and a single observed diastereoisomer.

Analytical RP-HPLC-MS (I): Method I-A, tᵣ = 2.71 min, [M+H]⁺ = 677.42; HRMS (ESI) calculated for C₉₇H₄₀O₁₁N₈ [M+H]⁺ = 677.28948, found 677.28166. The analytical data are in agreement with the one previously reported in the literature.¹⁷

5) C) Synthesis of Cyclic Phenylmethanamine DINNN Peptidomimetic, 27

[Phenylmethanamine-nMeFDINNNK]G, 27

![Diagram of peptidomimetic 27]

Step 1: The Fmoc protecting group was removed by treating the aldehyde containing resin 3d-1 (300.0 mg, 35.0 µmol, calculated loading 0.12 mmol/g, starting unfunctionalized loading 0.26 mmol/g) with a solution of 20% piperidine in DMF (8 mL) for 4 min. The solution was removed by vacuum filtration and the resin was washed with DMF (3 × 8 mL). The deprotection was repeated two additional times for 4 min each. The resin was finally washed with DMF (4 × 8 mL) and DCM (4 × 8 mL) and directly used into the next step without further modification.

Step 2: A solution of 5% AcOH in DMF (5 mL) was added to the free amine peptide resin form step 1 in DMF (4 mL). The suspension was shaken for 1 h at room temperature then NaBH₃CN (43.4 mg, 20 eq.) was added in one lump to the suspension. The suspension was shaken for 16 h at room
temperature. The resin was washed with DMF (3 × 8 mL) and DCM (3 × 8 mL) and quickly dried under vacuum filtration. The protecting group and the peptide were cleaved from the solid support using a solution of TFA/TIS/H2O (10 mL, 95:2.5:2.5, v/v/v) for 3 h at room temperature. The filtrate was then collected, and the peptide was precipitated by addition of Et2O (40 mL) and collected by centrifugation. The crude product (110 mg) was purified by semi-preparative RP-HPLC to afford the desired cyclic peptidomimetic 27 (11.1 mg, 0.01 mmol, 29% imine/reductive amination yield, 13% overall yield) as a white fluffy solid with ≥ 99% purity. LC-MS using method I-C showed the desired product at tR = 4.4 min with [M+H]⁺ = 1107.5 and [M+2H]²⁺ = 554.4.

5) D) Synthesis of Literature Reference Cyclic Disulfide Bridged RFF/Rff Peptides 28 and 29

Synthesis and Characterization of Linear Peptide Precursors on Solid Support

The following linear peptides were synthesized according to the representative procedure (RP_01). The successful formation of the desired linear peptide was confirmed by test cleavage as described in the representative procedure section followed by analytical RP-HPLC-MS (I) analysis using method (I-B). The table below summarizes the obtained linear peptides with the observed retention time and [M+H]⁺ ion.

| peptide | sequence                        | MW (g mol⁻¹) | tR (min) | [M+H]⁺ found |
|---------|---------------------------------|--------------|----------|--------------|
| B       | Fmoc- YCRFFNAFCY-resin          | 1553.6       | 4.3      | 1554.8       |
| C       | Fmoc- YCRffNFAFCY-resin         | 1553.6       | 4.2      | 1554.8       |

Lower case letters indicate β-amino acids. The cysteine amino acids are protected with Trityl protecting group.

Y[CRFFNAFC]Y, 28

Step 1: The resin B (90 mg, 0.03 mmol) was suspended in 20% piperidine in DMF (4 mL) and shaken for 4 min. The deprotection was repeated 2 additional time for 4 min. The resin was then washed with DMF (3 × 2 mL), DCM (3 × 2 mL) then DMF (3 × 2 mL) and quickly dried.

Step 2: The free amine resin from step 1 was cleaved from the Rink Amide Resin LL using a solution of TFA/TIS/H₂O (4 mL, 95:2.5:2.5, v/v/v) by shaking for 1.5 h at room temperature. The filtrate was collected and cooled Et₂O (40 mL) was added on ice. The slurry was kept at 0 °C for 30 min. The resulting white precipitate was then centrifuged (4000 rpm, 10 min, 4 °C). The supernatant was removed, and the residual white solid was dissolved in H₂O (40 mL) and freeze-dried to afford the unbound and unprotected peptide (37.6 mg) as a white fluffy solid.

Step 3: The crude solid from step 2 was dissolved in DMSO (1.72 mL) and acetic acid (0.86 mL) then added dropwise to a solution of DMSO (0.86 mL) and water (14.7 mL). The resulting colorless reaction mixture was stirred for 48 h at room temperature. The reaction mixture was then diluted with additional water and freeze dried overnight to afford the crude disulfide bridge product. The crude product was purified by semi-preparative RP-HPLC using a step gradient of 10% to 70% of MeCN + 0.1% TFA in H₂O + 0.1% TFA in 30 min at a flow rate of 6 mL/min to give the desired disulfide bridge product (12.3 mg, 25% yield) as a white fluffy solid with ≥ 99% purity. The analytical
RP-HPLC-MS (I) using method (I-A) showed the desired cyclic disulfide bridge peptide 28 at \( T_R = 2.8 \) min, \([\text{M+H}]^+ = 1330.6\) and \([\text{M+H}]^{2+} = 666.2\).

**Y[CRffNAFC]Y, 29**

**Step 1:** The resin C (90 mg, 0.03 mmol) was suspended in 20% piperidine in DMF (4 mL) and shaken for 4 min. The deprotection was repeated 2 additional time for 4 min. The resin was then washed with DMF (3 \( \times \) 2 mL), DCM (3 \( \times \) 2 mL) then DMF (3 \( \times \) 2 mL) and quickly dried.

**Step 2:** The resin from step 1 was cleaved from the Rink Amide Resin LL using a solution of TFA/TIS/H\(_2\)O (4 mL, 95:2.5:2.5, v/v/v) by shaking for 1.5 h at room temperature. The filtrate was then collected by filtration and cooled Et\(_2\)O (40 mL) was added on ice. The slurry was kept at 0 °C for 30 min. The resulting white precipitate was then centrifuged. The supernatant was removed, and the residual white solid was dissolved in H\(_2\)O (40 mL) and freeze-dried overnight to afford the unbound and unprotected peptide (32.4 mg) as a white fluffy solid.

**Step 3:** The crude solid from step 2 was dissolved in DMSO (1.48 mL) and acetic acid (0.74 mL) then added dropwise to a solution of DMSO (0.74 mL) and water (12.7 mL). The resulting colorless reaction mixture was stirred for 48 h at room temperature. The reaction mixture was then diluted with additional water and freeze dried overnight to afford the crude disulfide bridge product. The crude product was purified by preparative RP-HPLC using step gradient of 5 to 65% for 30 min of MeCN + 0.1% TFA in H\(_2\)O + 0.1% TFA at a flow rate of 6 mL/min to afford the desired disulfide bridge product 29 (6.3 mg, 17% yield) as a white fluffy solid with \( \geq 99\% \) purity. The analytical RP-HPLC-MS (I) using method (I-A) showed the desired cyclic disulfide bridge peptide 29 at \( T_R = 2.73 \) min, \([\text{M+H}]^+ = 1330.7\) and \([\text{M+H}]^{2+} = 666.2\).
6) NMR Conformational Analysis and Spectra of Selected PepNats

6) A) Conformational Analysis by NMR of PepNats H1 and K1

All NMR spectra were recorded on a Bruker 700 MHz. NMR data was acquired in CD$_3$OH and chemical shifts ($\delta$ values), given in parts per million (ppm), referenced to the CD$_3$OH residual solvent signal (3.31 ppm). To identify which NH groups were buried or forming intramolecular hydrogen bonds, NMR spectra were also run in fully deuterated CD$_3$OD (single endpoint, no rates were calculated). For the structural assignment of the peptides 1D $^1$H, 2D COSY, $^1$H-$^{13}$C HSQC/HMBC spectra were acquired using the standard pulse sequences available in TopSpin 3.5 and 4.0 (Bruker GmbH). Conformational analysis was carried out using the 2D $^1$H, $^1$H ROESY with 1048 and 512 data points in F2 and F1 respectively, a spectral width of 14 ppm, mixing time of 300 ms and a relaxation delay of 3 s. To determine the relative nOe intensities for pairs of spins, the extraction of F2-slices in the 2D ROESY at the F1-chemical shift of each resonance was carried out and the signals then integrated. To improve accuracy, the PANIC method (Peak Amplitude Normalisation for improved cross-relaxation) was applied, where the nOe intensities were normalized relative to the diagonal for each slice. Correction factors were then applied to compensate for the number of spins in each environment (corrected integral). For each molecule the integral for two protons at a known distance was used as reference to calibrate the other interproton distances in the molecule using the equation below:

$$\frac{\eta_{1S}}{\eta_{2S}} = \frac{r_{1S}^{-6}}{r_{2S}^{-6}}$$

where $\eta_{1S}$ is the intensity of the NOE between I and S (S being the inverted spin) and $r_{1S}^{-6}$ is the internuclear distance between I and S.

The sampling of the conformational space for the peptides was carried out with Maestro Macrocycle Sampling algorithm using the OPLS3 force field (version 11.6.013, Schrödinger) with an energy threshold of 25 Kcal/mol to allow a full exploration of the rotation around the peptidic bonds. The NMR restraints were collected in the form of NOEs and $J_{NH-H\alpha}$ couplings. The resulting conformers were filtered to a reduced set of conformations that fulfilled key long-range NOEs in the macrocycle. To avoid being too restrictive and missing possible conformers complying with the NMR data, the filter for those distances was set to an upper limit of 5.5 Å. Each of the conformers from the reduced set was subjected to solvent explicit MD simulations (Desmond Molecular Dynamics software module, D.E. Shaw, v4.4, running inside Maestro) using 10 ns in length runs with energy value recording every 1.2 ps and trajectory recording every 4.8 ps. The trajectories were then RMSD clustered using the Clustering tool in Maestro and the most populated cluster was taken as the conformation which the molecule spent most time in the dynamics run. The average structure for each of the most populated clusters was extracted. A new set of
conformations comprising each of those averaged structures was used together with the NMR restraints (NOEs and $J$ couplings) as input for MSpin NOE Fitter algorithm$^5$ (version 2.4.0-713; MestReLab Research). No-NOE constraints were also used and set to a default distance of 5.0 Å. The Fitter algorithm fits NMR data to ensembles of conformers rather than a priori assuming the presence of a single, low energy conformation in solution. The H-bond information was used for further refinement at the end of the analysis and it was introduced as distance restraints (equivalent to NOEs) between the NH and the H-bond acceptor in the peptide. For each NH potentially forming a H-bond according to the NMR data in CD$_3$OD (ie, not exchanging with the solvent) all the possible interactions with nearby acceptors were considered in the calculation, and only those that complied with the rest of the NMR data were kept as distance restraints.

**Conformational analysis of PepNat H1-major:**

![Structure diagram]

**NMR assignments of H1-major in CD$_3$OH at 25 °C**

| Atom | Chemical Shift (δ ppm) | Atom | Chemical Shift (δ ppm) |
|------|------------------------|------|------------------------|
| 1 C  | 23.8                   | 82  H | 0.95                   |
| 2 C  | 25.2                   | 83  H | 0.95                   |
| 3 C  | 21.8                   | 84  H | 0.95                   |
| 4 C  | 43.9                   | 85  H | 1.79                   |
| 5 C  | 52.3                   | 86  H | 0.98                   |
| 7 C  | 174.7                  | 87  H | 0.98                   |
| 9 C  | 44.1                   | 88  H | 0.98                   |
| 11 C | 171.4                  | 89  H | 1.88                   |
| 13 C | 69.3                   | 90  H | 1.50                   |
| 14 C | 34.5                   | 91  H | 4.65                   |
| 15 C | 139.4                  | 92  H | 7.71, d, $J$ = 9.2 Hz  |
| 16 C | 130.3                  | 93  H | 3.62                   |
| 17 C | 129.8                  | 94  H | 4.29                   |
| 18 C | 127.8                  | 95  H | 8.34, dd, $J$ = 5.0, 7.7 Hz |
| 19 C | 129.8                  | 96  H | 4.19                   |
|   C   |     H     |         |     |
|------|-----------|---------|-----|
|  20 C |    130.3  |    97 H | 3.33|
|  22 C |     38.9  |    98 H | 3.48|
|  23 C |    169.4  |    99 H | 7.26|
|  25 C |     63.3  |   100 H | 7.34|
|  27 C |     64.2  |   101 H | 7.26|
|  28 C |     51.8  |   102 H | 7.34|
|  29 C |     48.7  |   103 H | 7.26|
|  30 C |    175.2  |  104 H  | 2.79|
|  33 C |     25.1  |  105 H  | 2.79|
|  34 C |    178.3  |  106 H  | 2.79|
|  36 C |    138.2  |  107 H  | 4.49|
|  37 C |    128.2  |  109 H  | 4.86|
|  38 C |    128.2  |  110 H  | 3.82|
|  39 C |    135.8  |  111 H  | 4.00|
|  40 C |    128.2  |  112 H  | 2.32|
|  41 C |    128.2  |  113 H  | 2.32|
|  42 C |    170.4  |  114 H  | 2.32|
|  45 C |     40.2  |  115 H  | 7.57|
|  46 C |     29.6  |  116 H  | 7.85|
|  47 C |     23.6  |  117 H  | 7.85|
|  48 C |     32.3  |  118 H  | 7.57|
|  49 C |    54.6   |  119 H  | 8.30, J= 5.4 Hz|
|  51 C |    174.0  |  120 H  | 3.33|
|  53 C |     57.1  |  121 H  | 3.58|
|  54 C |     38.2  |  122 H  | 1.53|
|  55 C |    138.0  |  123 H  | 1.62|
|  56 C |    130.1  |  124 H  | 1.39|
|  57 C |    129.7  |  125 H  | 1.50|
|  58 C |    127.9  |  126 H  | 1.79|
|  59 C |    129.7  |  127 H  | 1.58|
|  60 C |    130.1  |  128 H  | 4.34|
|  62 C |    171.3  |  129 H  | 8.22, d, J= 7.6 Hz|
|  64 C |     44.1  |  130 H  | 4.31|
|  66 C |    171.3  |  131 H  | 2.91|
|  68 C |    173.7  |  132 H  | 3.03|
|  71 C |     55.5  |  133 H  | 7.19|
|  72 C |     38.7  |  134 H  | 7.22|
|  73 C |    138.2  |  135 H  | 7.16|
|  74 C |    130.2  |  136 H  | 7.22|
|  75 C |    129.4  |  137 H  | 7.19|
|  76 C |    127.7  |  138 H  | 7.45, d, J= 5.2 Hz|
|  77 C |    129.4  |  139 H  | 3.67|
|  78 C |    130.2  |  140 H  | 3.67|
|  79 C |    175.8  |  141 H  | 8.31, t, J= 5.7 Hz|
|  142 H |  7.78, d, J= 7.9 Hz|
|  143 H |          |        | 4.56|
|  144 H |          |        | 2.89|
|  145 H |          |        | 3.13|
|  146 H |          |        | 7.22|
|  147 H |          |        | 7.23|
|  148 H |          |        | 7.16|
|  149 H |          |        | 7.23|
|  150 H |          |        | 7.22|
|  151 H |          |        | 7.48|
|  152 H |          |        | 7.04|
**NH protons H92, H95, H129, H138 and H142 were not fully exchanging when the NMR experiments were run in CD$_3$OD, indicating either the formation of intramolecular hydrogen bonds or being buried within the structure. Protons H119, H141 and H151/152 did fully exchange, indicating that they were solvent exposed.**

The 1704 conformers generated in the conformational sampling were filtered using four long-range constraints: (112,113,114) – (139,140), (112,113,114) – (133,137), (112,113,114) – (148) and (116,117) – (139,140), with an upper limit of 5.5 Å. 21 conformers that

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| Atom 1 | Atom 2 | Normalized integral | Corrected integral | Calculated distance (Å) | Average distance cluster (Å) |
|--------|--------|---------------------|-------------------|-------------------------|-----------------------------|
| 145    | 144    | 106.0               | 106.0             | 1.8*                    | 1.8                         |
| 95     | 112,113,114 | 1.5               | 0.5               | 4.3                     | 4.0                         |
| 95     | 104,105,106 | 6.9               | 2.3               | 3.4                     | 3.0                         |
| 95     | 111    | 1.6                 | 1.6               | 3.6                     | 4.1                         |
| 95     | 96     | 9.5                 | 9.5               | 2.7                     | 3.0                         |
| 95     | 92     | 16.2                | 16.2              | 2.4                     | 2.5                         |
| 141    | 91     | 21.5                | 21.5              | 2.3                     | 2.3                         |
| 141    | 138    | 12.8                | 12.8              | 2.5                     | 4.1                         |
| 141    | 112,113,114 | 0.4               | 0.4               | 4.5                     | 4.6                         |
| 119    | 124,125 | 5.6                | 5.6               | 2.9                     | 3.6                         |
| 119    | 122,123 | 5.0                 | 5.0               | 3.0                     | 3.3                         |
| 129    | 124,125 | 10.3                | 10.3              | 2.6                     | 3.1                         |
| 129    | 131,132 | 6.0                | 6.0               | 2.9                     | 3.1                         |
| 129    | 130    | 73.0                | 73.0              | 1.9                     | 3.5                         |
| 129    | 133,137 | 3.0                | 1.5               | 3.6                     | 4.4                         |
| 129    | 138    | 3.3                 | 3.3               | 3.2                     | 3.4                         |
| 129    | 142    | 9.0                 | 9.0               | 2.7                     | 3.0                         |
| 129    | 116,117 | 0.8                | 0.4               | 4.5                     | 5.6                         |
| 116,117| 139,140 | 4.9                | 2.5               | 3.3                     | 3.6                         |
| 116,117| 130    | 1.1                 | 1.1               | 3.8                     | 6.4                         |
| 116,117| 133,137 | 0.4                | 0.2               | 5.1                     | 9.0                         |
| 116,117| 138    | 1.7                 | 1.7               | 3.5                     | 4.8                         |
| 142    | 126,127 | 3.6                | 3.6               | 3.1                     | 4.1                         |
| 142    | 131,132 | 1.4                | 0.7               | 4.1                     | 3.3                         |
| 92     | 112,113,114 | 2.5               | 0.8               | 4.0                     | 4.0                         |
| 92     | 97,98  | 2.9                 | 1.5               | 3.6                     | 3.9                         |
| 115,118| 89,90  | 1.2                 | 0.6               | 4.2                     | 4.4                         |
| 115,118| 139,140 | 3.9                | 1.9               | 3.5                     | 3.6                         |
| 138    | 112,113,114 | 2.4               | 0.8               | 4.0                     | 3.3                         |
| 100,102| 104,105,106 | 0.8              | 0.3               | 4.8                     | 5.0                         |
| 99,103 | 107    | 7.7                 | 3.8               | 3.1                     | 4.1                         |
| 147,149| 112,113,114 | 0.7               | 0.4               | 4.6                     | 6.2                         |
| 133,137| 112,113,114 | 1.0               | 0.5               | 4.4                     | 4.2                         |
| 107    | 104,105,106 | 48.6              | 16.2              | 2.4                     | 2.8                         |
| 130    | 112,113,114 | 1.0               | 1.0               | 3.9                     | 4.3                         |
| 96     | 104,105,106 | 13.0              | 13.3              | 2.5                     | 2.4                         |
| 111    | 104,105,106 | 10.5              | 10.5              | 2.6                     | 3.0                         |
| 112,113,114| 139,140 | 2.5                | 1.2               | 3.7                     | 3.6                         |
| **92** | 24     |                     |                   | 1.8                     | 2.1                         |
| **95** | 31     |                     |                   | 1.8                     | 1.9                         |
| 82,83,84| 115    | No NOE              |                   | 6.8                     | 10.9                        |
| 82,83,84| 118    | No NOE              |                   | 8.1                     | 6.3                         |
| 82,83,84| 112,113,114 | No NOE            |                   | 6.7                     | 7.1                         |
| 89,90  | 104,105,106 | No NOE         |                   | 6.7                     | 7.1                         |
| 115,118| 104,105,106 | No NOE         |                   | 6.7                     | 7.1                         |

* Reference distance; ** IHB constraints
complied with those distance restraints were subjected to 10 ns MD simulations, and the most populated cluster from each of them selected for further analysis with MSpin. The cluster below showed the best fit with the experimental NMR data (NOEs, $J$ couplings and H-bonds).

Solution conformation of compound H1-major derived using NMR and computational data

Conformational analysis of PepNat H1-minor:

NMR assignments of H1-minor in CD$_3$OH at 25 °C

| Atom | Chemical Shift ($\delta$ ppm) | Atom | Chemical Shift ($\delta$ ppm) |
|------|-----------------------------|------|-----------------------------|
| 3 C  | 173.9                       | 2 H  | 7.74, d, $J$= 7.8 Hz        |
| 5 C  | 55.0                        | 6 H  | 4.15                        |
| 9 C  | 174.4                       | 8 H  | 7.87, d, $J$= 6.9 Hz        |
| 10 C | 56.3                        | 11 H |                             |
| 12 C | 38.0                        | 13 H | 2.99                        |
| 15 C | 138.2                       | 14 H | 2.78                        |
| 16 C | 130.2                       | 17 H | 7.20                        |
| 18 C | 129.3                       | 19 H | 7.25                        |
| 20 C | 127.7                       | 21 H | 7.25                        |
|   | C   |   | H   |   |
|---|-----|---|-----|---|
| 22 | 129.3 | 23 | H   | 7.25 |
| 24 | 130.2 | 25 | H   | 7.20 |
| 28 | 170.8 | 27 | H   | 7.58, d, J= 6.0 Hz |
| 29 | 43.0  | 30 | H   | 3.36 |
| 34 | 175.5 | 31 | H   | 3.45 |
| 36 | 52.7  | 33 | H   | 8.31, t, J= 6.0 Hz |
| 40 | 171.9 | 37 | H   | 4.49 |
| 41 | 44.0  | 39 | H   | 7.67, d, J= 8.4 Hz |
| 46 | 172.0 | 42 | H   | 3.50 |
| 47 | 69.2  | 43 | H   | 4.20 |
| 49 | 35.0  | 45 | H   | 8.27, d, J= 6.1 Hz |
| 52 | 141.4 | 48 | H   | 4.03 |
| 53 | 130.5 | 50 | H   | 3.66 |
| 55 | 129.4 | 51 | H   | 3.23 |
| 57 | 127.2 | 54 | H   | 7.55 |
| 59 | 129.4 | 56 | H   | 7.29 |
| 61 | 130.5 | 58 | H   | 7.20 |
| 64 | 170.7 | 60 | H   | 7.29 |
| 65 | 55.4  | 62 | H   | 7.55 |
| 66 | 48.0  | 73 | H   | 7.38 |
| 67 | 50.6  | 75 | H   | 7.76 |
| 68 | 64.2  | 78 | H   | 7.76 |
| 71 | 139.7 | 80 | H   | 7.38 |
| 72 | 128.6 | 84 | H   | 8.44, dd, J= 4.9, 6.2 Hz |
| 74 | 128.2 | 86 | H   | 3.74 |
| 76 | 135.5 | 87 | H   | 3.20 |
| 77 | 128.2 | 89 | H   | 1.53 |
| 79 | 128.6 | 90 | H   | 1.67 |
| 81 | 170.9 | 92 | H   | 1.38 |
| 85 | 39.1  | 93 | H   | 1.46 |
| 88 | 29.6  | 95 | H   | 1.67 |
| 91 | 23.1  | 96 | H   | 1.67 |
| 94 | 31.6  | 97 | H   | 4.69 |
| 98 | 176.0 | 103| H   | 3.77 |
| 100| 176.5 | 104| H   | 3.91 |
| 109| 41.8  | 105| H   | 4.60 |
| 112| 25.6  | 110| H   | 1.94 |
| 114| 21.6  | 111| H   | 1.53 |
| 118| 23.5  | 113| H   | 1.82 |
| 124| 62.2  | 115| H   | 0.96 |
| 126| 175.8 | 116| H   | 0.96 |
| 131| 38.7  | 117| H   | 0.96 |
| 134| 138.2 | 119| H   | 1.00 |
| 135| 130.3 | 120| H   | 1.00 |
| 137| 129.4 | 121| H   | 1.00 |
| 139| 127.7 | 125| H   | 4.57 |
| 141| 129.4 | 128| H   | 7.03 |
| 143| 130.3 | 129| H   | 7.48 |
| 145| 38.4  | 132| H   | 2.92 |
| 149| 25.2  | 133| H   | 3.13 |
|   | 136. H | 7.25 |
|   | 138. H | 7.25 |
|   | 140. H | 7.20 |
|   | 142. H | 7.25 |
|   | 144. H | 7.25 |
|   | 146. H | 3.16 |
|   | 147. H | 3.16 |
|   | 148. H | 3.16 |
|   | 150. H | 2.84 |
|   | 151. H | 2.84 |
|   | 152. H | 2.84 |
The 1611 conformers generated in the conformational sampling were filtered using three long-range constraints: (75,78) – (30,31), (150,151,152) – (119,120,121) and (119,120,121) – (50,51) with an upper limit of 5.5 Å. 35 conformers that complied with those distance restraints were subjected to 10 ns MD simulations, and the most populated cluster from each of them selected for further analysis with MSpin. The cluster below showed the best fit with the experimental NMR data (NOEs, J couplings and H-bonds).

| Atom 1 | Atom 2 | Normalized integral | Corrected integral | Calculated distance (Å) | Average distance cluster (Å) |
|--------|--------|---------------------|--------------------|------------------------|-----------------------------|
| 132    | 133    | 109.0               | 109.0              | 1.8*                   | 1.8                         |
| 33     | 111    | 16.0                | 16.0               | 2.5                    | 2.9                         |
| 33     | 37     | 88.0                | 88.0               | 1.8                    | 2.3                         |
| 33     | 27     | 14.0                | 14.0               | 2.5                    | 4.6                         |
| 45     | 146,147,148 | 5.6              | 1.9               | 3.5                    | 3.3                         |
| 45     | 48     | 11.6                | 11.6               | 2.6                    | 2.9                         |
| 45     | 39     | 12.7                | 12.7               | 2.5                    | 2.6                         |
| 45     | 119,120,121 | 0.9               | 0.3               | 4.8                    | 4.8                         |
| 8      | 13,14  | 8.2                 | 4.1                | 3.1                    | 2.8                         |
| 8      | 11     | 47.0                | 23.5               | 2.3                    | 2.6                         |
| 8      | 17,25  | 2.8                 | 1.4                | 3.7                    | 4.0                         |
| 8      | 2      | 6.0                 | 6.0                | 2.9                    | 4.4                         |
| 75.78  | 115,116,117 | 0.4               | 0.4               | 4.6                    | 4.9                         |
| 75.78  | 30,31  | 5.0                 | 2.5                | 3.3                    | 4.3                         |
| 2      | 13     | 2.4                 | 2.4                | 3.4                    | 5.6                         |
| 39     | 42,43  | 5.3                 | 5.3                | 2.9                    | 3.4                         |
| 27     | 11     | 10.0                | 10.0               | 2.7                    | 2.9                         |
| 27     | 8      | 2.9                 | 2.9                | 3.3                    | 4.6                         |
| 54,62  | 146,147,148 | 1.8              | 0.6               | 4.2                    | 4.2                         |
| 73,80  | 115,116,117 | 0.2               | 0.1               | 6.0                    | 5.3                         |
| 73,80  | 119,120,121 | 0.7               | 0.2               | 5.0                    | 3.8                         |
| 73,80  | 110,111 | 3.0                 | 1.5               | 3.6                    | 3.8                         |
| 73,80  | 150,151,152 | 0.7               | 0.2               | 5.0                    | 4.8                         |
| 73,80  | 30,31  | 0.6                 | 0.3                | 4.7                    | 4.3                         |
| 146,147,148 | 104            | 6.2                | 6.2                | 2.9                    | 3.3                         |
| 146,147,148 | 105            | 8.6                | 8.6                | 2.7                    | 2.8                         |
| 146,147,148 | 48             | 12.4               | 12.4               | 2.6                    | 2.5                         |
| 150,151,152 | 119,120,121 | 1.0                 | 0.3               | 4.7                    | 5.1                         |
| 150,151,152 | 110,111       | 0.5                 | 0.3                | 4.9                    | 6.8                         |
| 119,120,121 | 50,51         | 0.4                 | 0.2                | 5.1                    | 5.3                         |
| 115,116,117 | 37            | 5.8                 | 5.8                | 2.9                    | 3.9                         |

* Reference distance
Solution conformation of compound H1-minor derived using NMR and computational data

Conformational analysis of PepNat K1-minor:

NMR assignments of K1-minor in CD$_3$OD at 25 °C

| Atom | Chemical Shift (δ ppm) | Atom | Chemical Shift (δ ppm) |
|------|------------------------|------|------------------------|
| 3 C  | 174.6                  | 2 H  | 8.10, d, $J$= 8.5 Hz   |
| 5 C  | 55.8                   | 6 H  | 4.02                   |
| 9 C  | 174.3                  | 8 H  | 8.41, d, $J$= 6.3 Hz   |
| 10 C | 51.9                   | 11 H | 4.32                   |
| 12 C | 37.0                   | 13 H | 2.42                   |
| 15 C | 174.2                  | 14 H | 2.72                   |
| 22 C | 172.2                  | 21 H | 7.45, br               |
| 24 C | 57.0                   | 25 H | 4.35                   |
| 28 C | 171.8                  | 27 H | 8.19, d, $J$= 9.1 Hz   |
| 30 C | 56.4                   | 31 H | 4.78                   |
| 34 C | 173.1                  | 33 H | 8.03, d, $J$= 9.2 Hz   |
| 35 C | 54.6                   | 36 H | 4.36                   |
| 39 C | 173.1 | 38 H | 8.07, d, J= 8.9 Hz |
|-----|-------|------|-------------------|
| 40 C | 63.6  | 41 H | 5.22              |
| 42 C | 34.5  | 43 H | 3.70              |
| 45 C | 138.7 | 44 H | 3.35              |
| 46 C | 129.2 | 47 H | 7.37              |
| 48 C | 130.1 | 49 H | 7.37              |
| 50 C | 128.1 | 51 H | 7.27              |
| 52 C | 130.1 | 53 H | 7.37              |
| 54 C | 129.2 | 55 H | 7.37              |
| 57 C | 173.1 | 66 H | 7.68              |
| 58 C | 64.0  | 68 H | 7.77              |
| 59 C | 50.3  | 71 H | 7.77              |
| 60 C | 52.9  | 73 H | 7.68              |
| 61 C | 64.7  | 77 H | 7.03, overlapped  |
| 64 C | 139.6 | 79 H | 2.92              |
| 65 C | 128.4 | 80 H | 3.35              |
| 67 C | 128.5 | 82 H | 1.35              |
| 69 C | 135.0 | 83 H | 1.25              |
| 70 C | 128.5 | 85 H | 1.31              |
| 72 C | 128.4 | 86 H | 1.22              |
| 74 C | 169.8 | 88 H | 1.72              |
| 78 C | 41.0  | 89 H | 1.39              |
| 81 C | 28.5  | 90 H | 4.85              |
| 84 C | 25.3  | 97 H | 7.17              |
| 87 C | 31.7  | 99 H | 7.26              |
| 91 C | 174.8 | 103 H| 7.26              |
| 93 C | 179.1 | 105 H| 7.17              |
| 95 C | 133.1 | 107 H| 4.06              |
| 96 C | 127.6 | 108 H| 4.34              |
| 98 C | 129.8 | 109 H| 4.64              |
| 102 C| 129.8 | 113 H| 1.89              |
| 104 C| 127.6 | 114 H| 1.55              |
| 112 C| 28.1  | 116 H| 1.58              |
| 115 C| 26.6  | 117 H| 1.51              |
| 118 C| 41.6  | 119 H| 3.01              |
| 123 C| 158.5 | 120 H| 3.01              |
| 130 C| 41.1  | 131 H| 3.37              |
| 133 C| 138.1 | 132 H| 3.07              |
| 134 C| 130.8 | 135 H| 7.28              |
| 136 C| 129.7 | 137 H| 7.31              |
| 138 C| 127.9 | 139 H| 7.23              |
| 140 C| 129.7 | 141 H| 7.31              |
| 142 C| 130.8 | 143 H| 7.28              |
| 144 C| 40.0  | 145 H| 2.07              |
| 147 C| 137.3 | 146 H| 2.37              |
| 148 C| 130.6 | 149 H| 6.68              |
| 150 C| 129.2 | 151 H| 6.96              |
| 152 C| 127.4 | 153 H| 7.03              |
| 154 C| 129.2 | 155 H| 6.96              |
| 156 C| 130.6 | 157 H| 6.68              |
| 159 C| 56.2  | 160 H| 4.50              |
| 161 C| 176.6 | 167 H| 3.16              |
| 166 C| 37.5  | 168 H| 2.92              |
| 169 C| 129.6 | 171 H| 7.11              |
| 170 C| 131.3 | 173 H| 6.69              |
| 172 C| 116.1 | 176 H| 6.69              |
| 174 C| 157.2 | 178 H| 7.11              |
| 175 C| 116.1 | 182 H| 3.33              |
| 177 C| 131.3 | 183 H| 3.33              |
| 181 C| 32.2  | 184 H| 3.33              |
nOe Correlations and inter-proton distances (Å) calculated from analysing F2-slices of 2D ROESY.

| Atom 1 | Atom 2 | Normalized integral | Corrected integral | Calculated distance (Å) |
|--------|--------|---------------------|--------------------|-------------------------|
| 14     | 13     | 222.7               | 222.7              | 1.8*                    |
| 8      | 13     | 4.7                 | 4.7                | 3.4                     |
| 8      | 14     | 3.9                 | 3.9                | 3.5                     |
| 8      | 6      | 15.3                | 15.3               | 2.8                     |
| 8      | 11     | 114.9               | 114.9              | 2.0                     |
| 8      | 2      | 15.1                | 15.1               | 2.8                     |
| 2      | 13,14  | 5.0                 | 2.5                | 3.8                     |
| 2      | 6      | 11.7                | 11.7               | 2.9                     |
| 2      | 11     | 6.3                 | 6.3                | 3.2                     |
| 38     | 182,183,184 | 8.1              | 2.7                | 3.7                     |
| 38     | 41     | 5.5                 | 5.5                | 3.3                     |
| 33     | 36     | 12.7                | 12.7               | 2.9                     |
| 33     | 97,105 | 8.2                 | 4.1                | 3.5                     |
| 41     | 135,143 | 1.8               | 0.9                | 4.5                     |
| 68,71  | 145,146 | 1.3               | 0.7                | 4.7                     |
| 68,71  | 149,157 | 2.0               | 1.0                | 4.4                     |
| 68,71  | 151,155 | 0.9               | 0.5                | 5.0                     |
| 68,71  | 153    | 2.2                 | 2.2                | 3.9                     |
| 66,73  | 145,146 | 1.0               | 0.5                | 4.9                     |
| 68,71  | 135,143 | 1.8               | 0.9                | 4.5                     |
| 66,73  | 25     | 11.6                | 11.6               | 2.9                     |
| 66,73  | 149,157 | 1.2               | 0.6                | 4.8                     |
| 66,73  | 151,155 | 0.6               | 0.3                | 5.3                     |
| 66,73  | 21     | 0.8                 | 0.8                | 4.6                     |
| 66,73  | 135,143 | 2.5               | 1.3                | 4.2                     |
| 99,103 | 113    | 4.3                 | 2.2                | 3.9                     |
| 99,103 | 145,146 | 9.4              | 4.7                | 3.4                     |
| 99,103 | 25     | Overlapped          | Overlapped         | 5.0                     |
| 99,103 | 31     | Overlapped          | Overlapped         | 5.0                     |
| 99,103 | 149,157 | Overlapped         | Overlapped         | 5.0                     |
| 97,105 | 116,117 | 4.4              | 2.2                | 3.8                     |
| 97,105 | 113    | 1.1                 | 1.1                | 4.3                     |
| 97,105 | 145,146 | 2.8              | 1.4                | 4.2                     |
| 97,105 | 25     | 2.4                 | 2.4                | 3.8                     |
| 97,105 | 31     | Overlapped          | Overlapped         | 5.0                     |
| 97,105 | 149,157 | 1.5              | 0.7                | 4.6                     |
| 151,155 | 85,86  | 2.4                | 1.2                | 4.3                     |
| 151,155 | 79 or 168 | 1.9              | 1.9                | 3.9                     |
| 151,155 | 6      | 2.3                 | 2.3                | 3.8                     |
| 173,176 | 88,89  | Overlapped          | Overlapped         | 5.0                     |
| 149,157 | 6      | Overlapped          | Overlapped         | 5.0                     |
| 109    | 182,183,184 | 68.2         | 22.7              | 2.6                     |

* Reference distance
6) B) NMR Spectra of Selected PepNats

D10-major

$^1$H NMR (700 MHz, CD$_3$OD, 25 °C)

$^{13}$C NMR (176 MHz, CD$_3$OD, 25 °C)
H1-major

$^1$H NMR (700 MHz, CD$_3$OH, 25 °C)

$^{13}$C NMR (176 MHz, CD$_3$OH, 25 °C)
H1-minor

$^1$H NMR (700 MHz, CD$_3$OH, 25 °C)

$^{13}$C NMR (176 MHz, CD$_3$OH, 25 °C)
PepNat **K1-major** showed two species in solution with a 80:20 ratio (measured by $^1$H integration). nOe peaks (blue, different color of diagonal) and exchange peaks (red, same color of diagonal) are visible in the ROESY spectrum. The exchange peaks connect the NMR signals from the major and the minor species. Therefore, these two species are not chemically different, but the same chemical entity present in solution in two conformations in slow exchange.

$^1$H NMR (700 MHz, CD$_3$OH, 25 °C)
$^{13}$C NMR (176 MHz, CD$_3$OH, 25 °C)

$^1$H $^1$H ROESY
**K1-minor**

$^1$H NMR (700 MHz, CD$_3$OD, 25 °C)

$^{13}$C NMR (176 MHz, CD$_3$OD, 25 °C)
7) RP-HPLC-MS Spectra of PepNats

PepNat A1 Crude HPLC Profile

Analytical RP-HPLC-MS (I); Method I-B

| Apex RT | Area    | %Area |
|---------|---------|-------|
| 11.66   | 868232.6| 8.33  |
| 11.83   | 9549841 | 91.67 |

PepNat A1-major

Analytical RP-HPLC-MS (I); Method I-B

| Apex RT | Area    | %Area |
|---------|---------|-------|
| 11.72   | 354657.2| 7.28  |
| 11.88   | 4516926 | 92.72 |
PepNat A2-major
Analytical RP-HPLC-MS (I); Method I-B

| Apex RT | Area   | %Area |
|---------|--------|-------|
| 13,18   | 15951087 | 89,96 |
| 13,63   | 1781026  | 10,04 |

PepNat A3-major
Analytical RP-HPLC-MS (I); Method I-B

| Apex RT | Area   | %Area |
|---------|--------|-------|
| 12,9    | 1267051 | 82,8  |
| 13,38   | 263215,5 | 17,2  |
**PepNat A4-crude**

Optimized RP-HPLC-MS (II); Method II-C

| Apex RT | Area   | %Area |
|---------|--------|-------|
| 30,15   | 3049047| 36,6  |
| 30,43   | 1855346| 22,3  |
| 30,95   | 1758960| 21,1  |
| 31,46   | 1666472| 31,7  |

**PepNat A4-mixt.**

Optimized RP-HPLC-MS (II); Method II-C

| Apex RT | Area | %Area |
|---------|------|-------|
| 30,15   | 8400931 | 63,2  |
| 30,44   | 664283,6 | 5     |
| 30,95   | 2834636 | 21,3  |
| 31,45   | 1394246 | 10,5  |
PepNat B1-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

| Apex RT | Area   | %Area |
|---------|--------|-------|
| 19,1    | 2171552| 9.18  |
| 19,44   | 21475663| 90.82 |

PepNat B2-minor
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat B3-major*

Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat C1-major

Optimized Analytical RP-HPLC-MS (I); Method I-B
PepNat C2-major
Optimized Analytical RP-HPLC-MS (I); Method I-C

PepNat C2-minor
Optimized Analytical RP-HPLC-MS (I); Method I-C
PepNat C3-major
Optimized Analytical RP-HPLC-MS (I); Method I-C

PepNat C3-minor
Optimized Analytical RP-HPLC-MS (I); Method I-C
PepNat C4-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat D1-major
Optimized Analytical RP-HPLC-MS (I); Method I-A
PepNat D2-major
Analytical RP-HPLC-MS (I); Method I-C

PepNat D3-major
Analytical RP-HPLC-MS (I); Method I-C
PepNat D4-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat D4-minor
Optimized Analytical RP-HPLC-MS (II); Method II-C

| Apex RT | Area   | %Area |
|---------|--------|-------|
| 12,36   | 1034041| 64,99 |
| 12,89   | 557105,9| 35,01 |
**PepNat D5-mixt.**
Optimized Analytical RP-HPLC-MS (II); Method II-C

| Apex RT | Area   | %Area |
|---------|--------|-------|
| 17,8    | 51087,53 | 15,25 |
| 18,35   | 33013,027 | 9,85  |
| 18,86   | 250889,776 | 74,89 |

**PepNat D6-major**
Analytical RP-HPLC-MS (I); Method I-C

| Apex RT | Area   | %Area |
|---------|--------|-------|
| 7,43    | 193396,3 | 6,32  |
| 7,98    | 81013,12 | 2,65  |
| 8,22    | 254624,3  | 8,32  |
| 8,77    | 2530523  | 82,71 |
PepNat D7-major
Analytical RP-HPLC-MS (I); Method I-C

| Apex RT | Area  | %Area |
|---------|-------|-------|
| 8.33    | 233671.5 | 14.73 |
| 8.71    | 1352465   | 85.27 |

PepNat D8-major
Analytical RP-HPLC-MS (I); Method I-B
**PepNat D9-major**

Optimized Analytical RP-HPLC-MS (II); Method II-A

**PepNat D10-major**

Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat D11-minor
Analytical RP-HPLC-MS (I); Method I-C

PepNat D12-major
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat D14-minor
Optimized Analytical RP-HPLC-MS (II); Method II-B

PepNat D15-minor*
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat D16-mixt.
Analytical RP-HPLC-MS (I); Method I-C

| Apex RT | Area   | %Area |
|---------|--------|-------|
| 5.35    | 1043636| 46.72 |
| 5.84    | 1189967| 53.28 |

PepNat D17-major
Optimized Analytical RP-HPLC-MS (I); Method I-C
**PepNat D18-major**

Optimized Analytical RP-HPLC-MS (II); Method II-C

**PepNat D18-minor**

Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat E1-major
Analytical RP-HPLC-MS (II); Method II-B

PepNat F1-major*
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat F1-minor*  
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat F2-minor  
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat G1-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat G2-mixt.
Optimized Analytical RP-HPLC-MS (II); Method II-C

| Apex RT | Area     | %Area |
|---------|----------|-------|
| 21,76   | 1273827  | 10,6  |
| 22,15   | 4364676  | 36,32 |
| 23,72   | 2997108  | 24,94 |
| 24,09   | 3380121  | 28,13 |
PepNat H1-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat H2-major
Optimized Analytical RP-HPLC-MS (I); Method I-C
PepNat H3-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

| Apex RT | Area     | %Area |
|---------|----------|-------|
| 26.47   | 2294396.09 | 12.61 |
| 27.09   | 15897278.5 | 87.39 |

PepNat I1-major
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat I2-mixt.
Optimized Analytical RP-HPLC-MS (II); Method II-C

| Apex RT | Area      | %Area |
|---------|-----------|-------|
| 18,33   | 15451150  | 55,19 |
| 18,56   | 12542871  | 44,81 |

PepNat I3-mixt.
Optimized Analytical RP-HPLC-MS (II); Method II-F

| Apex RT | Area      | %Area |
|---------|-----------|-------|
| 10,12   | 261828,238| 15,41 |
| 10,85   | 1437602,86| 84,59 |
**PepNat J1-major**
Optimized Analytical RP-HPLC-MS (II); Method II-C

![Chromatogram](image1)

**PepNat J2-major**
Optimized Analytical RP-HPLC-MS (II); Method II-C

![Chromatogram](image2)
PepNat J3-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat J3-minor
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat K1-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat K1-minor
Optimized Analytical RP-HPLC-MS (II); Method II-C
**PepNat K2-minor**

Optimized Analytical RP-HPLC-MS (I); Method I-C

![Graph 1](image1.png)

RT: 4.91

**PepNat K3-major**

Optimized Analytical RP-HPLC-MS (I); Method I-C

![Graph 2](image2.png)

RT: 6.39
PepNat K3-minor
Optimized Analytical RP-HPLC-MS (I); Method I-C

PepNat K4-major
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat K4-minor
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat K5-major
Optimized Analytical RP-HPLC-MS (I); Method I-C
PepNat L1-major
Optimized Analytical RP-HPLC-MS (I); Method I-B

| Apex RT | Area   | %Area |
|---------|--------|-------|
| 4.32    | 1539631| 14.03 |
| 5.01    | 9431898| 85.97 |

PepNat L2-major
Analytical RP-HPLC-MS (I); Method I-A
PepNat M1-major
Optimized Analytical RP-HPLC-MS (II); Method II-E

PepNat N1-major*
Optimized Analytical RP-HPLC-MS (II); Method II-C
**PepNat N1-minor**

Optimized Analytical RP-HPLC-MS (II); Method II-C

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**PepNat N2-major**

Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat N2-minor*  
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat N3-major*  
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat O1-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat O1-minor
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat O2-major
Optimized Analytical RP-HPLC-MS (II); Method II-C

PepNat P1-major
Optimized Analytical RP-HPLC-MS (II); Method II-C
PepNat H1-minor obtained through independent synthesis in solution
Optimized Analytical RP-HPLC-MS (I); Method I-C

PepNat K1-minor obtained through independent synthesis in solution
Optimized Analytical RP-HPLC-MS (II); Method II-C
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