

STABLE COMPOSITIONS OF FOXY-5 HEXAPEPTIDE WITH HIGH SOLUBILITY

FIELD OF THE INVENTION

The present disclosure relates to new, highly soluble compositions of the Wnt hexapeptide

- 5 Foxy-5. The present disclosure further relates to methods for the production of said compositions and stability assessments thereof.

BACKGROUND OF THE INVENTION

- 10 Foxy-5 is a formylated, WNT5A-derived hexapeptide and WNT5A mimetic with potential anti-metastatic activity currently in development as a drug candidate for the prevention of tumor spread and recurrence in several common forms of cancer. Foxy-5 has the formula **For-Met-Asp-Gly-Cys-Glu-Leu-OH** (SEQ_NO 1, [Figure 1](#)).

Upon intravenous administration, Foxy-5 binds to and activates WNT5A receptors, predominantly of the Frizzled family, which activates WNT5A-mediated signaling.

- 15 Foxy-5 is intended to compensate for the deficiency of protein WNT5A in tumor tissues noted in patients with cancer types where the WNT5A ligand acts as a cancer suppressor, in order to reduce the risk of metastasis. A sub-analysis from a recent retrospective study of patients with colorectal cancer in stage III, shows that the proportion of patients with low expression of WNT5A is significantly higher than that observed in
20 previous studies in patients with stage II colorectal cancer (CRC). Patients with CRC stage III tumors differ from stage II mainly by the presence of tumor cells in lymph nodes adjacent to the primary tumor, thereby being more aggressive and faster progressing. A low level of WNT5A has been observed in close to 70 percent of patients in stage III, compared with approximately 45 percent of patients with less advanced tumor stages. Similar findings have
25 been made in breast cancer tissues, where absence or a low level of WNT5A has been observed in 70% of aggressive triple-negative breast cancer in comparison with approximately 45% in less aggressive breast cancer types. In several types of cancer, including breast-, colon-, prostate- and hepatocellular cancer, it has been demonstrated that a lack or low level of WNT5A in the primary tumor is associated with a more rapid recurrence when compared with
30 tumor with normal or near normal expression of the WNT5A protein. This supports the hypothesis that the WNT5A level significantly influences the course of disease.

Based on a completed Phase 1b study with Foxy-5 aimed at documenting the drug candidate's safety profile, pharmacokinetics and dose determination for Phase 2, Foxy-5 is

now undergoing a Phase 2 clinical trial study, the NeoFox study, which includes patients with stage II / III colon cancer, who at diagnosis are considered to have a high risk of recurrence after the primary tumour has been surgically resected. The assessment of the risk of recurrence is performed according to established assessment criteria. The aim of the study is to demonstrate that Foxy-5 reduces the risk of relapse, primarily in patients with low expression of WNT5A. The study is currently underway at 28 hospitals in Spain (16) and Hungary (12).

Foxy-5 itself and a classical solid-phase (SPSS) method for its preparation are described in International Pat. Publication No. WO 2006/130082 (A PEPTIDE LIGAND TO IMPAIR CANCER CELL MIGRATION). The active pharmaceutical ingredient (API) for the preclinical and early clinical studies has been produced by this route. Solution phase routes for Foxy-5 developed by applicant are disclosed in International Patent Publication No. WO 2020/038878 (Solution phase routes for Wnt hexapeptides) and WO 2020/120198 (Linear solution phase synthesis for Wnt hexapeptides).

The current manufacturing process of Foxy-5 applies a product concentration for the final lyophilization of only 3 g/L due to the limited solubility of the salt free drug substance (DS) in water. This presents a bottleneck which severely limits the production throughput for the commercial manufacture of Foxy-5.

Initial attempts at overcoming this issue resulted in the development of an alternative lyophilization process using an aqueous 30% acetonitrile solution (instead of neat water) as solvent. It was demonstrated in small scale experiments and verified on a 35 g scale that the developed process is applicable for DS concentrations up to 7 g/L, *i.e.* a >100% increase in productivity. While this achievement is impressive, it nonetheless remains problematic to produce Foxy-5 on commercial scale when the final step requires such a high dilution.

Consequently, an increase of batch size by other solubilizing techniques than solvent polarity adjustments was proposed. From drug product (DP) formulation studies it was known that a change from the salt free form of Foxy-5 to the sodium salt will increase DS solubility to levels of at least 20 g/L.

It is noted in this context that Foxy-5 contains three carboxylic acid moieties and no free amino groups, as the terminal amino group is formylated ([Figure 1](#)). Therefore, it is only possible to prepare salts of Foxy-5 with basic/alkaline compounds. It is noted that some fine

chemical suppliers offer a compound marketed as the “trifluoroacetic salt of Foxy-5”, which in reality is just a lyophilizate containing free trifluoroacetic acid.

A conversion of Foxy-5 to its sodium salt was investigated by applicant. As Foxy-5 contains three carboxylic groups (Asp², Glu⁵, and the C-terminal carboxylic acids), the mono, di, and tri-sodium salts were generated by treating aqueous slurries of Foxy-5 (25 g/L) with 1 eq., 2 eq. and 3 eq. of sodium hydroxide. As starting material, a batch of Foxy-5 free acid containing very low amounts of two known impurities, the aspartimide (< 0.1%) and the dimer, (< 0.1%) was used (structures, see Figure 2).

The mono-sodium salt of Foxy-5 could not be completely dissolved, and shortly after NaOH addition (1 eq., pH = 3.84), gel formation was observed.

The di-sodium salt could be completely dissolved within 10 minutes after addition of NaOH (2 eq., pH = 4.57). HPLC analysis of the solution showed an increased content of the aspartimide impurity, while dimer formation was only detected on a negligible level. After lyophilization, 0.07% dimer and 0.79% aspartimide were detected.

The tri-sodium salt dissolved within 5 minutes after addition of NaOH (3 eq., pH = 6.76) with minimal increase of the aspartimide impurity, but increased formation of the dimer was observed. After lyophilization, 0.41% dimer and 0.17% of the aspartimide were detected.

Of the three salts, the trisodium salt was found to have the highest solubility, > 100 gr/ltr, and thus initially appeared to be a promising new drug substance candidate.

However, upon stability testing at 40 °C it was discovered by mass spectrometry that the trisodium salt of Foxy-5 quickly developed significant amounts of the above discussed impurities (Figure 3), reaching a level of between 15-20% after 4 weeks at 40 °C.

Further studies of aqueous solutions of Foxy-5 at different pH values revealed that at pH 9 the content of the aspartimide significantly decreases directly after complete dissolution. After stirring of the Foxy-5 solution for 2 hours at pH 9 (see Figure 4), the aspartimide peak in the HPLC chromatogram completely disappeared (< LOD). It is known that ring opening of aspartimides occurs at high pH, leading to the formation of either the parent compound and the β-Asp and the D-Asp derivatives. However, significant dimer formation was observed (2.5% after 2 hours at pH = 9). The identification of the dimer in the chromatogram was confirmed by co-elution with separately synthesized reference material. In addition to the two known impurities (the aspartimide and the dimer), a third impurity in the tri-sodium salt was surprisingly identified as the desulfurized Cys → **Ala** analog of Foxy-5 (see Figure 2 and 3), albeit in low amounts.

In summary, although solubility in water can be increased by converting Foxy-5 to a trisodium salt, this compound is much too unstable to qualify as a drug substance. There thus remains a need for a different DS form of Foxy-5 having satisfactory solubility and stability, both for further clinical trial supply and for future commercial purposes.

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DEFINITIONS

As used herein, the term "nitrogen base" shall mean a basic compound comprising at least one nitrogen atom.

As used herein, the term "mono salt" or "mono-salt" shall mean a composition comprising one equivalent of Foxy-5 and 0.5-1.45 equivalents of a nitrogen base, as defined herein.

As used herein, the term "di salt" or "di-salt" shall mean a composition comprising one equivalent of Foxy-5 and 1.50 - 2.49 equivalents of a nitrogen base, as defined herein.

As used herein, the term "tri salt" or "tri-salt" shall mean a composition comprising one equivalent of Foxy-5 and 2.5 – 3.1 equivalents of a nitrogen base, as defined herein.

As used herein, the term HT-XRPD shall mean High Throughput X-Ray Powder Diffraction.

As used herein, the term HPLC-CAD shall refer to HPLC with Charged Aerosol Detection.

As used herein, the term UPLC-MS shall refer to Ultra performance liquid chromatography with Mass Spectrometry detection

As used herein, the term "thermocycling" shall refer to a process where a solid composition according to the invention is suspended in a solvent or solvent mixture followed by 1 - 3 cycles where the the resulting suspension in each cycle is heated with stirring at a rate of $10^{\circ}\text{C} \pm 2^{\circ}\text{C} / \text{hr}$ until a temperature of $50^{\circ}\text{C} \pm 2^{\circ}\text{C}$ is reached, followed by cooling the suspension at a rate of:

- a. $20^{\circ}\text{C} \pm 2^{\circ}\text{C} / \text{h}$ for the 1st cycle,
- b. $10^{\circ}\text{C} \pm 2^{\circ}\text{C} / \text{hr}$ for the 2nd cycle, and
- c. $5^{\circ}\text{C} \pm 2^{\circ}\text{C} / \text{hr}$ for 3rd cycle.

until a temperature of $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$ is reached. The suspension is finally aged by stirring it for 1-3 days at $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The temperature profile is illustrated in figure 11.

SUMMARY OF THE INVENTION

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Foxy-5 is as mentioned a formylated hexapeptide of formula **For-Met-Asp-Gly-Cys-Glu-Leu-OH** containing three carboxylic acid groups: Asp², Glu⁵, and the C-terminal carboxylic acids groups, and can thus form mono-, di- and tri-salts with basic compounds. It has now been found that Foxy-5 forms highly soluble compositions with various nitrogen bases (defined herein as basic compounds containing at least one nitrogen atom) in the approximate ratios

1:1, 1:2 and 1:3 (Foxy-5 : Base). These compositions are for convenience referred to in the following as mono- di- and tri-salts of Foxy-5, even though the compositions – which are isolated as amorphous lyophilizates - have not all been properly (stoichiometrically) characterized as salts.

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Surprisingly, the stabilities of these compositions when measured at 40 °C have been found to vary considerably, which will be discussed in the following. An overview of all compositions according to the invention and their impurity profiles is given in Figure 10.

10 **In a first aspect** the present invention provides a solid composition comprising For-Met-Asp-Gly-Cys-Glu-Leu-OH (SEQ_NO 1, Foxy-5) and a nitrogen base selected from ammonia, quaternary ammonium hydroxides, alkylated guanidines, amino acids and primary and secondary amines.

In a second aspect the present invention provides a method for producing a composition
15 according to the first aspect, comprising the following steps:

a) Mixing the free acid form of For-Met-Asp-Gly-Cys-Glu-Leu-OH (SEQ_NO 1, Foxy-5),
either as

- i. a suspension in a solvent, or
- ii. as a neat powder,

20 with either

- iii. 0.5 - 1.49 equivalents of a nitrogen base to prepare a mono-salt of Foxy-5, or
- iv. 1.50 - 2.49 equivalents of a nitrogen base to prepare a di-salt of Foxy-5, or
- v. 2.5 – 3.1 equivalents of a nitrogen base to prepare a tri-salt of Foxy-5,

b) Stirring the reaction mixture until a clear solution is obtained, and

25 c) Isolating a solid composition by precipitation, or by lyophilizing or spray-drying the solution,

wherein the nitrogen base is a compound selected from ammonia, quaternary ammonium hydroxides, alkylated guanidines, amino acids, and primary and secondary amines, and wherein all steps a-c are optionally conducted in an inert atmosphere.

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In a third aspect of the present invention, the resulting solid composition of step c) of the second aspect is further subjected to a thermocycling process, comprising:

- 5 i. Providing a suspension of the lyophilized or spray-dried product in a solvent or solvent mixture,
- ii. Subjecting the suspension to a thermocycling process comprising 1 - 3 cycles between 5°C - 50°C $\pm$ 2°C, followed by aging the suspension for 1-3 days at 5°C $\pm$ 2°C,
- iii. isolating the thermocycled product, e.g. by filtration or centrifugation,
- during which thermocycling process the suspension is heated at a rate of 10°C $\pm$ 2°C /hr until a temperature of 50°C $\pm$ 2°C is reached, followed by cooling the suspension at a rate of:
- 10 a. 20°C $\pm$ 2°C /h for the 1st cycle,
- b. 10°C $\pm$ 2°C /hr for the 2nd cycle, and
- c. 5°C $\pm$ 2°C /hr for 3rd cycle,
- until a temperature of 5°C $\pm$ 2°C is reached. The suspension is finally aged by stirring it for 1-3 days at 5°C $\pm$ 2°C before isolation, e.g. by filtration or centrifugation.
- 15 The temperature profile for the thermocycling process is illustrated in figure 11.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1

- 20 The figure shows the chemical structure of Foxy-5 (SEQ_NO 1). Foxy-5 is a linear peptide consisting of six amino acids with a formylated N-terminus. All optically active amino acid residues are in the L-configuration. The molecular formula of Foxy-5 is C₂₆H₄₂N₆O₁₂S₂, and the molecular mass is 694.8 g/mol (average mass).

Figure 2

- 25 The figure shows the chemical structure of three main impurities in Foxy-5:
- A) The aspartimide derivative of Foxy-5
- B) The Cys-Cys dimer derivative of Foxy-5
- C) The Cys $\rightarrow$ Ala-analog of Foxy-5

Figure 3

- 30 The figure shows the purity (HPLC area%) values at 0, 2 and 4 weeks storage at 40 °C of three known impurities in the trisodium salt: The "Aspartimide", the "Dimer", and the "Ala-analog". For details, see the Detailed Description.

Figure 4

The figure shows the tracking of high pH treatment of Foxy-5 by HPLC: starting material (bottom trace), after dissolution (middle trace) and after 2 hrs at pH 9 (top trace). The HPLC traces have been offset in the y-direction for purposes of comparison.

5 Figure 5

The figure shows the purity (HPLC area%) values at 0, 2 and 4 weeks storage at 40 °C of the tri-salts (top figure) and the di-salts (bottom) of the invention. As can be seen, there is a greater variability in stability at 4 weeks for the tri-salts than for the di-salts.

Figure 6

- 10 The top figure compares the purity (HPLC area%) values at 0, 2 and 4 weeks storage at 40 °C of the tri-salts produced with amino acids (i.e. Lys, Arg and His) vs. other salt formers (i.e. choline, diethylamine, ammonia, N-Me-glucamine and tromethamine). The bottom figure shows a similar comparison for the di-salts. As can be seen, there is a general trend that salts produced with amino acids have a higher stability than the salts produced with other salt
15 formers.

Figure 7

The figure shows the purity (HPLC area%) values at 0, 2 and 4 weeks storage at 40 °C of a selection of salts according to the invention having a purity of at least 90% by HPLC (area%) after 4 weeks storage at 40 °C.

20 Figure 8

- The figure shows the purity (HPLC area%) values of the freshly lyophilized tri-ammonium (NH₃), tri-tromethamine (TRO) and tri-N-methyl-glucamine (NMG) salts of Foxy-5 prepared by either ¹) slow addition of base to an aqueous suspension of Foxy-5, keeping the pH <6 throughout the procedure, or by ²) adding the full amount of base in one portion. As can be
25 seen, the purity of the tri-ammonium salt is quite strongly influenced by the mode of addition, whereas the purity of the tri-TRO and tri-NMG salts is relatively unaffected.

Figure 9

The figure shows the powder X-ray diffractograms (PXRD) of lyophilizates of tri-valent salts of Foxy-5 (bottom to top): Ammonia tri-salt, N-methyl-D-glucamine tri-salt, Tromethamine tri-salt and Histidine tri-salt. The diffractograms have been offset in the y-direction for purposes
5 of comparison.

Figure 10

The figure is an overview table of all conducted experiments resulting in isolated solid compositions according to the invention, divided into “di-salts” and “tri-salts”. The table demonstrates the development in impurity profile over time (0, 2 and 4 weeks storage @
10 40°C) for each composition and for the starting material (Foxy-5 free acid).

Figure 11

The figure shows the temperature profile used for “thermocycling” the Foxy-5 compositions HIS-GEN14, HIS-GEN15 (L-Histidine 1:2 and 1:3) and NMG-GEN16 (N-Methyl-D-glucamine (1:2)). The samples were suspended in an organic solvent and stirred at a temperature
15 between 5 – 50°C ±2°C. The thermocycling profile included 3 cycles between 5 – 50°C ±2°C and aging for 3 days at 5°C±2°C. The samples were thus heated at 10°C/h until a temperature of 50°C ±2°C was reached, and next cooled at 20°C/h (1st cycle), 10°C/h (2nd cycle) and 5°C/h (3rd cycle) until a temperature of 5°C±2°C was reached. Finally the suspensions were aged by stirring at 5°C±2°C for 3 days.

Figure 12

Figure 12 contains two tables: The top table present the results of the HPLC-CAD analysis of the Foxy-5-L-Histidine salts. The bottom table present the results of the HPLC-CAD analysis of the N-Methyl-D-glucamine salts of Foxy-5. As can be seen, the ratios determined using the HPLC-CAD method were all close to the expected ratio of 1:1, 1:2 and 1:3. The slight deviation
25 that was observed is probably due to the hygroscopicity of the samples.

Figure 13

Figure 13 shows an overlay of HT-XRPD patterns of Foxy-5-L-Histidine (1:2) from bottom to top (Exp. ID GEN5, Exp. ID TCP7 (thermocycled in acetone), Exp. ID TCP9 (thermocycled in acetonitrile/water (90/10))).

Figure 14

Figure 14 shows an overlay of HT-XRPD patterns of Foxy-5-L-Histidine (1:3) from bottom to top (Exp. ID GEN8, Exp. ID TCP11 (thermocycled in ethanol), Exp. ID TCP15 (thermocycled in ethyl acetate)).

5 Figure 15

Figure 15 shows an overlay of HT-XRPD patterns of Foxy-5-N-Methyl-D-Glucamine (MEG) (1:2) from bottom to top (Exp. ID GEN7, Exp. ID TCP18 (thermocycled in tetrahydrofuran), Exp. ID TCP20 (thermocycled in ethyl acetate)).

DETAILED DESCRIPTION

As mentioned in the summary hereinabove a linear solution phase manufacturing method for the Foxy-5 hexapeptide (hereinafter referred to as "Foxy-5") has been published in applicant's international patent application WO 2020/120198 which involves a product concentration of only 3 g/L for the final lyophilization due to the limited solubility of the salt free drug substance (DS) in water. As further mentioned, an initial strategy for overcoming this issue resulted in the production of the tri-sodium salt of Foxy-5, which was found to have a good aqueous solubility but an extremely poor stability which rendered the tri-sodium salt unusable as a new drug substance candidate.

It has now been found that Foxy-5 forms highly soluble compositions with various nitrogen bases. Surprisingly, many of these compositions are significantly more stable than the tri-sodium salt of Foxy-5, which potentially allows for a substantial increase in production throughput for the commercial manufacture of Foxy-5.

This finding was made when performing a screening for salts of Foxy-5 using the following potential "salt formers": ammonium hydroxide, L-arginine, calcium hydroxide, choline hydroxide, diethylamine, L-lysine, L-histidine, magnesium hydroxide, N-methyl-D-glucamine, and tromethamine. In view of the known issues associated with the sodium salts of Foxy-5, this selection of salt formers was believed to provide a sufficient breadth of chemically different candidates which would also eventually satisfy the regulatory requirements for pharmaceutical components and excipients. The selected "salt formers" are thus selected from compounds with GRAS approval and/or Stahl class 1 or 2 designation (see P. H. Stahl and C. G. Wermuth, Handbook of Pharmaceutical Salts: Properties, Selection, and Use, Second Edition, Wiley-VCH: Zürich (2011)).

As mentioned hereinabove the compositions resulting from the salt screening study are for convenience referred to in the following as mono-, di- and tri-salts of Foxy-5, even though the compositions – which are isolated as amorphous lyophilizates - have not been properly (stoichiometrically) characterized as salts.

Tri-salts of Foxy-5 were produced by adding about three equivalents of base to an aqueous suspension of 20-30 mg/mL of the Foxy-5 free acid using two different approaches: adding the calculated amount of base in one portion to the aqueous suspension of the Foxy-5 free acid, or by titrating the relevant base slowly, keeping the pH of the suspension < 6.0 throughout the procedure.

Clear solutions were obtained for all but the calcium and magnesium salts, which remained suspensions, indicating that for all but the calcium and magnesium salts a solubility of at least 20 gr/L was obtained. The clear solutions were lyophilized and the solid lyophilizates characterized by PXRD and HPLC (see Experimental section and [Figure 10](#) for an overview of all compositions). All were found to be amorphous (see [Figure 9](#) for exemplary PXRD diagrams). For some salt formers successful attempts preparing tri-salts with a solubility of ~50 gr/L were made, and for some tri-salts a solubility of >100 gr/L was reached.

The previously studied highly soluble tri-sodium salt (see the Background section) was also generated for comparison.

Di-salts were also prepared with all of the salt formers that led to clear solutions in the trivalent salt experiments, namely, ammonium hydroxide, L-arginine, choline hydroxide, diethylamine, L-lysine, L-histidine, N-methyl-D-glucamine, and tromethamine. Again, clear solutions were obtained in all cases, indicating that also the di-salts of Foxy-5 had a solubility in water that equals or exceeds 20 mg/mL. The solutions were lyophilized and the solid lyophilizates characterized by PXRD and HPLC (see Experimental section). All were found to be amorphous. For some salt formers successful attempts preparing di-salts with a solubility of ~50 gr/L were made. Mono-salts were prepared in a similar way.

The resulting lyophilizates were all amorphous solids as evidenced by PXRD analysis. In many cases the solids were slightly hygroscopic.

In a first aspect the present invention provides a solid composition comprising For-Met-Asp-Gly-Cys-Glu-Leu-OH (SEQ_NO 1, Foxy-5) and a nitrogen base, wherein the nitrogen base is a compound selected from ammonia, quaternary ammonium hydroxides, alkylated guanidines, amino acids, and primary and secondary amines.

In an embodiment of the first aspect, the solid composition contains 0.5 - 3.1 equivalents of a nitrogen base selected from ammonia, quaternary ammonium hydroxides, alkylated guanidines, amino acids, and primary and secondary amines.

In a preferred embodiment of the first aspect, the present invention provides a "tri-salt" comprising Foxy-5 (For-Met-Asp-Gly-Cys-Glu-Leu-OH, SEQ_NO 1) and 2.5 - 3.1 equivalents of a nitrogen base selected from ammonia, quaternary ammonium hydroxides, alkylated guanidines, amino acids, and primary and secondary amines.

In another preferred embodiment of the first aspect, the present invention provides a “di-salt” comprising Foxy-5 (For-Met-Asp-Gly-Cys-Glu-Leu-OH, SEQ_NO 1) and 1.50 - 2.49 equivalents of a nitrogen base selected from ammonia, quaternary ammonium hydroxides, alkylated guanidines, amino acids, and primary and secondary amines.

5 In another embodiment of the first aspect, the present invention provides a “mono-salt” comprising Foxy-5 (For-Met-Asp-Gly-Cys-Glu-Leu-OH, SEQ_NO 1) and 0.5 – 1.49 equivalents of a nitrogen base selected from ammonia, quaternary ammonium hydroxides, alkylated guanidines, amino acids, and primary and secondary amines.

10 As mentioned in the background section, the tri-sodium salt of Foxy-5 had a high aqueous solubility but was found to be surprisingly unstable in comparison to the free acid form. The major impurities in the sodium salts were found to be (see [Figure 2](#)):

- the **Cys** disulfide (dimer) of Foxy 5,
- 15 • the **Cys** → **Ala** substitution, *i.e.* the desulfurized derivative of Foxy 5, and
- the **Asp** aspartimide derivative.

The stability analysis of the compositions according to the first aspect of the present invention have demonstrated that these compositions contain the same impurities as the known sodium salts, albeit in varying amounts. The development of said impurities over time also varies considerably between the compositions.

20

A stability study of the compositions according to the first aspect (and the previously prepared trisodium salt) at 40°C for two and four weeks was performed. Some general trends were found. As appears from [Figure 5](#), the absolute purity of the compositions when analysed right after lyophilization (0 weeks) and monitored at 40°C for two and four weeks, was found to differ substantially. The purity of the tri-salts varied more than the purity of the di-salts (HPLC purity 78-95% at 4 weeks for the tri-salts vs. 87-96% at 4 weeks for the di-salts). Furthermore, as appears from [Figure 6](#), the absolute purity of the compositions comprising amino acids was generally higher after 4 weeks at 40 °C when compared with compositions comprising other salt formers (value at 4 weeks: ~90% vs. ~85% for tri-salts and ~95% vs. ~90% for di-salts).

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In an embodiment the present invention provides a composition comprising Foxy-5 and 2.5 – 3.1 equivalents of an amino acid, such as 2.8 – 3.05 equivalents of an amino acid, such as 2.9 – 3.02 equivalents of an amino acid, such as 2.95 – 3.01 equivalents of an amino acid, such as 3.0 equivalents of an amino acid.

- 5 In another embodiment the invention provides a composition comprising Foxy-5 and 1.50 - 2.49 equivalents of an amino acid, such as 1.7 – 2.1 equivalents of an amino acid, such as 1.8 – 2.05 equivalents of an amino acid, such as 1.9 – 2.03 equivalents of an amino acid, such as 1.95 – 2.01 equivalents of an amino acid, such as 2.0 equivalents of an amino acid.

- In preferred embodiments the present invention provides a composition comprising
10 Foxy-5 and an amino acid selected from lysine, arginine, or histidine.

- Most of the compositions of the present invention are as mentioned hygroscopic to some degree and many were found to turn sticky when subjected to humid air. Assay analysis in combination with elemental analysis has however revealed that some of the compositions of
15 the present invention can be characterized as proper salts which have formed non-sticky hydrates, such as di- and tetrahydrates (see Experimental section). As the compositions of the present invention were isolated as freeze-dried amorphous solids (lyophilizates), such hydrates cannot be as precisely defined as crystalline hydrates, but many of the compositions have nevertheless been found to be quite well-defined such as salts with an approximate ratio
20 of 1:2 (“di-salts”) or 1:3 (“tri-salts”) between Foxy-5 and the salt-forming base.

Finally, Figure 7 show the compositions having an purity measured by HPLC (area%) of at least 90% after 4 weeks storage at 40 °C. As can be seen, compositions of the present invention with high stability are not limited to a particular salt form.

- 25 In another embodiment of the first aspect, the present invention thus provides a composition wherein the nitrogen base is a compound selected from amino acids, ammonia, and primary and secondary amines.

In preferred embodiments of the first aspect, the nitrogen base employed is selected from ammonia, L-lysine, L-histidine, N-methyl-D-glucamine, and tromethamine.

- 30 In other preferred embodiments, the present invention provides a composition comprising Foxy-5 and 1.50 - 2.49 equivalents, such as 1.7 – 2.1 equivalents, such as 1.9 – 2.03 equivalents, such as 1.95 – 2.01 equivalents, such as 2.0 equivalents of a nitrogen base selected from ammonia, L-lysine, L-histidine and tromethamine.

In other preferred embodiments, the present invention provides a composition comprising Foxy-5 and 2.5 – 3.1 equivalents, such as 2.9 – 3.03 equivalents, such as 2.95 – 3.01 equivalents, such as 3.0 equivalents, of a nitrogen base selected from L-histidine and N-methyl-D-glucamine.

5 In other preferred embodiments, the present invention provides a composition comprising the di-ammonium salt, the di-histidine salt, the tri-histidine salt, the di-lysine salt, the di-N-methyl-D-glucamine salt, the tri-N-methyl-D-glucamine salt and the di-tromethamine salt of Foxy-5, which salts optionally comprise between 0-5 equivalents of water calculated in relation to Foxy-5.

10 In a particularly preferred embodiment, the present invention provides a composition comprising Foxy-5 and L-histidine, such as between 1.5 – 3.1 equivalents of L-histidine, such as between 1.7 - 2.49 equivalents of L-histidine, such as 1.7 – 2.1 equivalents of L-histidine or such as between 2.5 – 3.1 equivalents of L-histidine, which compositions optionally comprise between 0-5 equivalents of water calculated in relation to Foxy-5.

15

In a second aspect the present invention provides a method for producing a composition according to the first aspect, comprising the following steps:

- a) Mixing the free acid form of For-Met-Asp-Gly-Cys-Glu-Leu-OH (SEQ_NO 1, Foxy-5), either as
- 20 i. a suspension in a solvent, or
- ii. as a neat powder,
- with either
- iii. 0.5 - 1.49 equivalents of a nitrogen base to prepare a mono-salt of Foxy-5, or
- iv. 1.50 - 2.49 equivalents of a nitrogen base to prepare a di-salt of Foxy-5, or
- 25 v. 2.5 – 3.1 equivalents of a nitrogen base to prepare a tri-salt of Foxy-5,
- b) Stirring the reaction mixture until a clear solution is obtained, and
- c) Isolating a solid composition by precipitation, or by lyophilizing or spray-drying the solution,

wherein the nitrogen base is a compound selected from ammonia, quaternary ammonium

30 hydroxides, alkylated guanidines, amino acids, and primary and secondary amines, and wherein all steps a) – c) are optionally conducted in an inert atmosphere.

In preferred embodiments of the second aspect, the nitrogen base employed for salt formation in step iii), iv) or v) is selected from ammonia, L-lysine, L-histidine, N-methyl-D-glucamine, and tromethamine.

- 5 In preferred embodiments, the concentration of Foxy-5 in step i) is about 20-30 mg/mL in water. In other preferred embodiments, the nitrogen base employed for salt formation in step vi) is 1.50 - 2.49 equivalents, such as 1.7 – 2.1 equivalents, such as 1.9 – 2.03 equivalents, such as 1.95 – 2.01 equivalents, such as 2.0 equivalents, of a nitrogen base selected from ammonia, L-lysine, L-histidine and tromethamine.
- 10 In other preferred embodiments, the nitrogen base employed for salt formation in step v) is 2.5 – 3.1 equivalents, such as 2.9 – 3.03 equivalents, such as 2.95 – 3.01 equivalents, such as 3.0 equivalents, of a nitrogen base selected from L-histidine and N-methyl-D-glucamine.

As mentioned hereinabove, two different approaches were used in the preparation of the compositions according to the first aspect: adding the calculated amount of base in one
15 portion to the aqueous suspension of the Foxy-5 free acid, or titrating the relevant base slowly, keeping the pH of the reaction mixture < 6.0 throughout the procedure.

Upon HPLC analysis of the freshly lyophilized compositions, it was found that the “slow” titration produced a composition which contains a lower level of impurities than compositions prepared using the “quick” approach, see [Figure 8](#). As can be seen, the effect is
20 largest for the “tri-salt” of ammonia where the purity drops from about 98% to 95% (slow addition → quick addition). In comparison, the purities of the “tri-salts” of tromethamine and tri-N-methyl-glucamine are only slightly lower when these salts are formed by quick addition. Without being bound by theory, the inventors believe this phenomenon may be related to the pKa value of the salt-forming base: ammonium hydroxide is the strongest of the three
25 bases with a pKa of 9.3, whereas both tromethamine and N-methyl-glucamine have a pKa of 8.0.

In embodiments of the second aspect, the nitrogen base employed for salt formation in step b) is added in one portion to the aqueous suspension of the Foxy-5 free acid. In other
embodiments of the second aspect, the nitrogen base employed for salt formation in step b)
30 is added gradually to the aqueous suspension of the Foxy-5 free acid, keeping the pH of the reaction mixture < 6.0 throughout the procedure.

In yet other embodiments of the second aspect, the final clear solution in step b is lyophilized to provide a solid composition of the present invention. In other embodiments of

the second aspect, a solid composition is obtained from the final clear solution in step b by precipitation, including by crystallization. In some embodiments, precipitation or crystallization is achieved by addition of one or more antisolvents, by evaporation, by cooling or by a combination hereof.

5

Some of the compositions of the present invention were selected for scale-up. As part hereof the initially isolated amorphous product was subjected to a thermocycling process in which a suspension of the amorphous product in an organic solvent underwent 1-3 cycles of alternating heating and cooling between $5^{\circ}\text{C} - 50^{\circ}\text{C} \pm 2^{\circ}\text{C}$ followed by an aging step where the suspension was stirred for 1-3 days at $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$. It was surprisingly found that the obtained, thermocycled material is less fluffy and static than the initially obtained material and thus easier to handle.

In a third aspect of the present invention, the resulting solid composition of step c) of the second aspect is further subjected to a thermocycling process, comprising:

15

- i. Providing a suspension of the lyophilized or spray-dried product in a solvent or solvent mixture,
- ii. Subjecting the suspension to a thermocycling process comprising 1 - 3 cycles between $5^{\circ}\text{C} - 50^{\circ}\text{C} \pm 2^{\circ}\text{C}$, followed by aging the suspension for 1-3 days at $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$,
- iii. isolating the thermocycled product, e.g. by filtration or centrifugation,

20

during which thermocycling process the suspension is heated at a rate of $10^{\circ}\text{C} \pm 2^{\circ}\text{C} / \text{hr}$ until a temperature of $50^{\circ}\text{C} \pm 2^{\circ}\text{C}$ is reached, followed by cooling the suspension at a rate of:

25

- a. $20^{\circ}\text{C} \pm 2^{\circ}\text{C} / \text{hr}$ for the 1st cycle,
- b. $10^{\circ}\text{C} \pm 2^{\circ}\text{C} / \text{hr}$ for the 2nd cycle, and
- c. $5^{\circ}\text{C} \pm 2^{\circ}\text{C} / \text{hr}$ for 3rd cycle,

until a temperature of $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$ is reached. The suspension is finally aged by stirring it for 1-3 days at $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$ before isolation, e.g. by filtration or centrifugation.

The temperature profile for the thermocycling process is illustrated in figure 11.

30

In preferred embodiments of the third aspect the organic solvent is selected from ethanol, acetone, tetrahydrofuran (THF), acetonitrile/water (90/10), and ethyl acetate.

In preferred embodiments of the third aspect the thermocycled product is the Foxy-5-L-Histidine salt in the ratio 1:2 and 1:3, or the Foxy-5-N-Methyl-D-glucamine salt in the ratio 1:2. Typical HT-XRPD diagrams can be found in Figures 13-15.

EXPERIMENTAL

Determination of the pKa Values of Foxy-5

The pKa values of the Foxy-5 free acid (lot: 1058263) were measured by potentiometric titration at 25°C using a Sirius T3 instrument equipped with SiriusT3Control
 5 Vers. 2.0.0.0 software. An automated aqueous titration method was used with initial values of 1.0 mg of solid API dissolved in 1.5 mL of ISA water and three sequential titrations on the same sample. In all three titrations, the sample solution was adjusted with 0.5 M KOH to a starting pH of 12 and slowly titrated with 0.5 M HCl to a final pH of 2.

Each titration curve was fit using the SiriusT3Refine Vers. 2.0.0.0 software, and the
 10 corresponding pKa values were calculated. Values of the pKa for all four theoretically predicted acidic sites were observed in the experimentally accessible range with an RMSD of 0.071. The results of the fit are summarized in below Table 1.

Theoretical pKa values were also calculated with ACD/Percepta 14.51.0 (Build 3382) using both the classic and GALAS modes and the ACD_Prediction Vers. 2.0.0.0 package within
 15 the Sirius T3 Vers. 2.0.0.0 software, the latter being the program used to measure the pKa. The assignments of the calculated values to specific sites on the molecule were made by the software. As Table 1 shows, the three prediction methods gave values that agreed with each other to within 0.1 to 0.5 units. The experimental values are reasonably close to the predicted ones with the measured values for the thiol of the cysteine group and the carboxylic acid of
 20 the L-aspartic acid lying essentially in the middle of the corresponding predicted ranges and the terminal carboxylic acid and the carboxylic acid of the L-glutamic acid group being just outside the upper end of the predicted range.

Table 1 Measured and calculated pKa values for Foxy-5

pKa [measured]	pKa [calculated]			Predicted site (type)
	ACD Labs ^{a)}	ACD Labs ^{b)}	Sirius T3 ^{c)}	
9.01	9.2 ± 0.1	8.7 ± 0.5	9.16	CYS thiol (acidic)
4.79	4.4 ± 0.1	4.7 ± 0.4	4.45	GLU carboxylic acid (acidic)
3.98	4.1 ± 0.1	3.9 ± 0.5	4.19	ASP carboxylic acid (acidic)
3.39	3.3 ± 0.1	3.2 ± 0.4	3.34	terminal carboxylic acid (acidic)
a) These values were calculated using the classic approach in ACD/Percepta 14.51.0 (Build 3382).				
b) These values were calculated using the GALAS approach in ACD/Percepta 14.51.0 (Build 3382).				
c) These values were calculated using the ACD_Prediction Vers. 2.0.0.0 package within the Sirius T3 Vers. 2.0.0.0 software. No error bar was provided by the software.				

In the following a detailed description is given for each produced composition according to the present invention, divided into “tri-salts”, *i.e.* Foxy-5 compositions which contain about three equivalents of a nitrogen base, “di-salts”, *i.e.* Foxy-5 compositions which contain about two equivalents of a nitrogen base, and “mono-salts”, *i.e.* Foxy-5 compositions which contain about one equivalent of a nitrogen base.

The stability test data for all formed compositions and the starting material itself (Foxy-5 free acid) were measured at 0, 2 and 4 weeks storage @ 40 °C and included in [Figure 10](#).

10 **Analytical conditions for stability measurements**

The following parameters for the substance-specific UPLC method were employed for the purity measurements of the stability samples:

Table 2 UPLC method parameters

Dionex UltiMate 3000RS UPLC instrument with Chromeleon Version 7.2 software.	
Parameter	Description
column (reversed-phase RP18)	Waters, Acquity CSH Phenyl-Hexyl, 150 mm x 2.1 mm, 1.7- μ m particles
sample dilution system	87:13 (v/v) H ₂ O/MeCN
mobile phase A	99.9:0.1 (v/v) 0.1% TFA (v/v) in H ₂ O/MeCN
mobile phase B	0.1% TFA (v/v) in MeCN
gradient	0.0 min - 88% A, 12% B 1.0 min - 88% A, 12% B 25.0 min - 74% A, 26% B 25.5 min - 10% A, 90% B 28.5 min - 10% A, 90% B 29.0 min - 88% A, 12% B 35.0 min - 88% A, 12% B
Flow	0.35 mL min ⁻¹
Concentration	approximately 0.1-0.3 mg/mL
injection volume	5 μ L
detection	220 nm
column temperature	40°C
sample temperature	r.t.

Ratio determination for Foxy-5 salts

Elemental analysis (C, H, N, O and S) was initially carried out on a number of isolated Foxy-5 salts (see the following Experimental section), i.e. the ammonia di-salt and tri-salt and the histidine di-salt and tri-salt. For these salts the found values were only in reasonable
 5 agreement with various hydrates of the salts, and it was therefore decided to investigate other analytical methods for the ratio determination in order to prove unequivocally that 1:1, 1:2 and 1:3 salts of Foxy-5 have been produced by the methods disclosed herein.

The determination of the ratio of nine different Foxy-5 salts, (L-Histidine, ammonium, and N-
 10 Methyl-D-glucamine each with three different ratios 1:1, 1:2 and 1:3) was thus attempted with HPLC-CAD and ¹H-NMR.

Solubility in water

The aqueous solubility of the nine salts was determined using a quantitative solubility method. The material was suspended in water for 24 hours, after which an aliquot of the
 15 mother liquor is analyzed by UPLC. In case the material was dissolved after 24 hours, the solubility was given as greater than (>).

An overview of the solubility results can be found in the below table 3:

20 **Table 3** solubility results Foxy-5 NMG, NH3 and HIS salts

Exp. ID	Counterion (CI)	Ratio Foxy-5 : CI	Aq.solubility (mg/ml)
NMG-GEN4	NMG	1 : 1	22.8
NH3-GEN3	NH3	1 : 1	19.5
HIS-GEN2	HIS	1 : 1	20.3
NMG-GEN7	NMG	1 : 2	>140
NH3-GEN6	NH3	1 : 2	31.2
HIS-GEN5	HIS	1 : 2	>71
NMG-GEN10	NMG	1 : 3	>140
NH3-GEN9	NH3	1 : 3	> 130
HIS-GEN8	HIS	1 : 3	>140

It can be concluded that the prepared 1:2 and 1:3 salts all had aqueous solubilities exceeding 20 mg/ml whereas the 1:1 salts had aqueous solubilities at around 20 mg/ml. For comparison, the free Foxy-5 hexapeptide has an aqueous solubility of around 3 mg/ml.

HPLC-CAD

- 5 An HPLC-CAD method was developed for the determination of the ratio of the Foxy-5-L-Histidine- and Foxy-5-N-Methyl-D-glucamine-salts. The method is however not suitable for ammonium salts of Foxy-5, as an ammonium salt is included in the mobile phase.

10 A calibration line was measured for L-Histidine and N-Methyl-D-glucamine using a stock solution with a concentration of 0.1 mg/ml and 10 different injection volumes (from 1 µl to 5.5 µl in 0.5µl steps). The amount of Foxy-5-salt used for the analysis was calculated, so that the concentration of the counterion would be about 0.1 mg/ml. The ratio was then calculated using the expected area vs. the actual measured area (see Figure 12, top table for L-Histidine and Figure 12, bottom table for N-Methyl-D-glucamine). The ratios determined using the
15 HPLC-CAD method were all close to the expected ratio of 1:1, 1:2 and 1:3. HPLC-CAD is thus well suited to determine the ratio between Foxy-5 and its respective counterion. The current specific HPLC method however prevents such ratio determination for ammonium salts of Foxy-5, but with a suitable modification of the HPLC mobile phase this problem can be obviated.

¹H-NMR

20 ¹H-NMR is the standard method to determine the ratio of an API with its co-former in a salt screening process, as long as the co-former is organic and visible in ¹H-NMR. L-Histidine and N-Methyl-D-glucamine both contain protons that are visible in ¹H-NMR. Ammonia does contain protons, but they are usually not visible in ¹H-NMR and therefore not quantifiable.

25

It was therefore only attempted to determine the ratio of Foxy-5 and L-Histidine, as well as N-Methyl-D-glucamine with ¹H-NMR. The ratio determination went well with the Foxy-5-N-Methyl-D-glucamine 1:2 salts, as the methyl group of N-Methyl-D-glucamine shifted from 2.26 ppm before salt formation to 2.48 ppm after salt formation. The integration of the
30 methyl group resulted in a ratio of 1:2.15 for GEN16, 1:2.01 for TCP18, and 1:2.02 for TCP20. ¹H-NMR is thus a good method to determine the ratio of Foxy-5-L-NMG salts.

The ratio determination of the Foxy-5-L-Histidine salts was, however, more challenging. GEN14 was determined to have the expected ratio of 1:2. Other samples, however, were determined to have a ratio of about 1:0.1 which could be explained by the poor solubility of L-Histidine in DMSO-d6. The L-Histidine probably started crystallizing in the NMR solvent after
5 some time and was therefore not detected during the measurement.

New samples were prepared in D₂O and indeed the protons of L-Histidine gave a much stronger signal, but the ratio of Foxy-5 to L-Histidine was still determined lower than expected, namely 1:1.4 for the 1:2 samples and 1:2.1 for the 1:3 samples. The integration
10 within the peptide itself deviated also from the molecular structure. It was therefore decided to test an NMR method with a longer relaxation delay, which was named "qNMR". It was tested on two samples, GEN15 and TCP11, both Foxy-5-L-Histidine salts in the ratio of 1:3. The qNMR method resulted in a proton integration that is a good fit with the molecular formula of the peptide and the ratio with L-Histidine was determined to be close to 1:3.

15 In conclusion it can be said that by the analytical methods described hereinabove for the ratio determination, it has been demonstrated that 1:1, 1:2 and 1:3 salts of Foxy-5 with good aqueous solubility can be produced by the methods disclosed herein. The solubility determination show a clear trend from the low solubility of the free Foxy-5 peptide (3 mg/ml)
20 over the increased solubility of the 1:1 salts (about 20 mg/ml) to the significantly increased solubility of the 1:2 and 1:3 ratio salts (up to 150 mg/ml).

Scale-ups and thermocycling of Foxy-5 salts

Three salts were selected for scale-up, i.e. Foxy-5-Histidine in the ratio 1:2 and 1:3 and Foxy-
25 5-N-Methyl-D-glucamine in the ratio 1:2. These salts were selected because their solubility is higher than 31 mg/ml and because a pH < 6 could be maintained during their preparation. The ammonium hydroxide 1:2 salt also fulfills these requirements, but was however not selected for scale-up at this time as there is presently no precise analytical method available to determine the ratio of this salt.

30

The scale-up was performed for all selected salts at a 300 mg scale. In all cases (Foxy-5-L-Histidine (1:2), Foxy-5-L-Histidine (1:3) and Foxy-5-N-Methyl-D-glucamine (1:2)) solids were obtained. The solids were analyzed using ^1H -NMR, UPLC-MS, HPLC-CAD and TGMS.

The obtained solids were subjected to a temperature profile in 5 different solvents (see Figure 11). The results of the thermocycling experiments are listed in below Table 4.

Table 4 results of thermocycling Foxy-5 NMG and HIS salts

Exp. ID	Description	API, mg	Solvent	Vol., μL	XRPD
TCP6	HIS 1:2	53	Ethanol	1000	Am
TCP7	HIS 1:2	53	Acetone	1000	Am
TCP8	HIS 1:2	53	THF	1000	Am
TCP9	HIS 1:2	53	MeCN/H ₂ O (90/10)	200	Am
TCP10	HIS 1:2	53	EtOAc	2000	Am
TCP11	HIS 1:3	55	Ethanol	1000	Am*
TCP12	HIS 1:3	55	Acetone	1000	Am
TCP13	HIS 1:3	55	THF	1000	Am
TCP14	HIS 1:3	55	MeCN/H ₂ O (90/10)	200	Am*
TCP15	HIS 1:3	55	EtOAc	2000	Am
TCP16	NMG 1:2	52	Ethanol	1000	Deliq.
TCP17	NMG 1:2	52	Acetone	1000	Am
TCP18	NMG 1:2	52	THF	1000	Am
TCP19	NMG 1:2	52	MeCN/H ₂ O (90/10)	200	Deliq.
TCP20	NMG 1:2	52	EtOAc	2000	Am

Am = amorphous

Am* = amorphous + some peaks from crystallized His

Deliq. = deliquescent

The XRPD diagrams of the thermocycled samples can be found in Figures 13-15. The scale up and thermocycling resulted mostly in amorphous material, although a few samples were found to be deliquescent, both before and after thermocycling. For the HIS 1:3 salts it was observed that the XRPD diagrams contained some peaks, indicating crystallinity. On closer inspection it turned out that the peaks originated from a small excess of pure L-Histidine which had precipitated as the monoclinic crystalline modification.

It was surprisingly found that the obtained, thermocycled material is easier to handle than the initially obtained freeze-dried material, as it is less fluffy and static.

5 **Formation of “tri-salts”**

Foxy-5 tri-salts are generally prepared by adding the relevant base to a suspension of Foxy-5 in water or another suitable solvent. The base may be added neat or in solution, and either in one portion or gradually, keeping the pH <6 during the procedure.

Arginine tri-salt

10 **EXPERIMENT ARG-P1**

89.7 mg (0.129 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 67.7 mg (3 equiv.) of L-arginine was added under an N₂ purge $\Rightarrow$ clear, colorless solution with pH 7.31 was obtained; let stir at r.t. for 2 h $\Rightarrow$ clear, colorless solution with pH 15 7.27 was obtained; filtered through a syringe filter (13-mm diameter, 0.2- μm PVDF) into a 10-mL round-bottomed flask; resulting solution frozen in dry ice; lyophilized for 18 h 15 min $\Rightarrow$ white solid was obtained; characterized by PXRD; stored in the freezer and characterized in duplo by HPLC 14 d later. PXRD confirms that the sample is amorphous; HPLC shows purities of 96.47 area-% and 96.40 area-% for the two injections.

20 **Calcium tri-salt**

EXPERIMENT Ca-P1

89.6 mg (0.129 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 28.8 mg (3 equiv.) of Ca(OH)₂ was added $\Rightarrow$ dilute, white suspension with pH 25 12.36 was obtained; let stir at r.t. for 2 h $\Rightarrow$ dilute, white suspension with pH 12.34 was obtained; let stir at r.t. overnight $\Rightarrow$ white suspension was obtained; experiment terminated because minimum solubility was not reached.

Choline tri-salt

EXPERIMENT CHO-P1

89.6 mg (0.129 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ white suspension was obtained; 95.4 μL (3 equiv.) of 46% choline hydroxide (aq) (dens. = 1.073 g mL^{-1}) was added under $\text{N}_2 \Rightarrow$ beige solution with pH 8.51 was obtained; let stir at r.t. for 2 h $\Rightarrow$ beige solution
5 with pH 8.43 was obtained; filtered through a $0.2 \mu\text{m}$ PVDF syringe filter into a 10-mL round-bottomed flask; solution frozen and lyophilized $\Rightarrow$ beige solid was obtained; characterized by PXRD; characterized in duplo by HPLC. PXRD confirms that the sample is amorphous; HPLC shows purities of 92.14 area-% and 92.08 area-% for the two injections.

Diethylamine tri-salt

10 EXPERIMENT DEA-P1

90.5 mg (0.130 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 40.2 μL (3 equiv.) of diethylamine (dens. = 0.707 g mL^{-1}) was added under an N_2 purge $\Rightarrow$ clear, colorless solution with pH 5.43 was obtained; let stir at r.t. for 2 h $\Rightarrow$ clear,
15 colorless solution with pH 5.45 was obtained; filtered through a syringe filter (13-mm diameter, $0.2 \mu\text{m}$ PVDF) into a 10-mL round-bottomed flask; resulting solution frozen in dry ice; lyophilized for 18 h 15 min $\Rightarrow$ white solid was obtained; characterized by PXRD; stored in the freezer and characterized in duplo by HPLC 14 d later; aliquots of this sample were stored in closed containers at 40°C for 2 weeks and 4 weeks and examined by HPLC; they were
20 observed to have a glassy appearance after both 2 and 4 weeks of storage at 40°C . PXRD confirms that the sample is amorphous; HPLC shows purities of 97.26 area-% and 97.22 area-% for the two injections.

Lysine tri-salt

EXPERIMENT LYS-P1

25 90.3 mg (0.130 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 56.8 mg (3 equiv.) of L-lysine was added under an N_2 purge $\Rightarrow$ clear, colorless solution with pH 6.39 was obtained; let stir at r.t. for 2 h $\Rightarrow$ clear, colorless solution with pH 6.46 was obtained; filtered through a syringe filter (13-mm diameter, $0.2 \mu\text{m}$ PVDF) into a 10-
30 mL round-bottomed flask; resulting solution frozen in dry ice; lyophilized for 18 h 15 min $\Rightarrow$ white solid was obtained; characterized by PXRD; stored in the freezer and characterized by

HPLC 14 d later; aliquots of this sample were stored in closed containers at 40°C for 2 weeks and 4 weeks and characterized in duplo by HPLC. PXRD confirms that the sample is amorphous; HPLC shows purities of 97.44 area-% and 97.39 area-% for the two injections.

Magnesium tri-salt

- 5 EXPERIMENT Mg-P1
- 89.6 mg (0.129 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 22.7 mg (3 equiv.) of $\text{Mg}(\text{OH})_2$ was added $\Rightarrow$ thick white suspension was obtained (too thick to measure pH); let stir at r.t. for 2 h $\Rightarrow$ white suspension with pH 9.06
- 10 was obtained; let stir at r.t. overnight $\Rightarrow$ white suspension was obtained; terminated experiment because minimum solubility not reached. Since no salt with a solubility in excess of 30 g L^{-1} was obtained, no characterization was carried out.

Sodium tri-salt

- EXPERIMENT Na-P1
- 15 90.5 mg (0.130 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; added 3 mL of degassed ultrapure water (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 388.6 μL (3 equiv.) of 1 M NaOH (aq) was added under an N_2 purge $\Rightarrow$ clear, colorless solution with pH 8.23 was obtained; let stir at r.t. for 2 h $\Rightarrow$ clear, colorless solution with pH 8.17 was obtained; filtered through a syringe filter (13-mm diameter, $0.45 \mu\text{m}$ PVDF)
- 20 into a 10-mL round-bottomed flask; resulting solution frozen in dry ice; lyophilized for 24 h 05 min $\Rightarrow$ white solid was obtained; stored in the freezer and characterized by HPLC 5 d later; the sample was difficult to weigh because it was very hygroscopic; aliquots of this sample were stored in closed containers at 40°C for 2 weeks and 4 weeks and examined in duplo by HPLC; the 4-week sample was difficult to weigh due to hygroscopicity. PXRD confirms that the
- 25 sample is amorphous; traces of NaCl are possibly present; HPLC shows purities of 95.11 area-% for both injections.

Ammonia tri-salt

EXPERIMENT NH3-P8

- 100.3 mg (0.144 mmol) of Foxy-5 hexapeptide was weighed out into a 15-mL Supelco vial; 3.333 mL of ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) under an N₂ purge $\Rightarrow$ glassy suspension. The suspension was stirred at 500 rpm and an pH electrode was inserted. 0.1 M of NH₄OH (aq) was dosed at 0.05 mL min⁻¹, pausing to measure pH periodically. At 15 min, 0.50 mL of base had been added and the pH was 3.84; at 45 min, a total of 1.34 mL of base had been added, and the pH was 4.09; at 1 h 06 min, a total of 2.37 mL of base had been added, and the pH was 4.42; at 1 h 14 min, a total of 2.74 mL of base had been added, and the pH was 4.64; a clear, colorless solution was obtained; at 1 h 45 min, a total of 3.74 mL of base had been added (equal to 2.59 equivalents), and the pH was 5.81; the dosing was paused; at 3 h 40 min, the pH was 6.00; the remaining 0.58 mL of base was not added; the experiment was terminated. The clear, colorless solution was placed into a 20-mL round-bottomed flask, frozen in a mixture of dry ice and isopropanol, and lyophilized. HPLC (in duplo) and elemental analyses were carried out. HPLC shows purities of 98.08 area-% and 98.05 area-% for the two injections and corresponding assay values of 68.76% and 68.71%, respectively.
- The ratio of the nitrogen to sulfur content does not quite comply with a tri-salt, but this is not surprising because the amount added (based on gravimetric calculations) was only 2.59 equivalents. Elemental analysis (C, H, N, O and S) of the product (below) is in good agreement with a salt comprising 2.6 equivalents of ammonia and 2 equivalents of water.

CHNOS analysis	Ammonia "tri-salt" dihydrate $\text{C}_{26}\text{H}_{42}\text{N}_6\text{O}_{12}\text{S}_2 \cdot (\text{NH}_3)_{2.6} (\text{H}_2\text{O})_2$						
	%C	%H	%N	%O	%S	N/S ratio	Stoichiometry
Theoretical	40,25	7,01	15,61	28,87	8,26	1,89	1 : 2.65
Found:	40,9	7,31	15,23	28,51	8,06	1,89	1 : 2.65

N-methyl-D-glucamine tri-salt

EXPERIMENT NMG-P5

- 90.3 mg (0.130 mmol) of Foxy-5 hexapeptide was weighed out into a 7-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$). The pH meter was inserted and pH of 2.66 was measured. A N₂ purge was applied and stirring was set up at 700 rpm. 0.78 mL of a 0.5 M aqueous solution of N-methyl-D-glucamine (4.8803 g in 50 mL of water) was slowly added with a dosimeter and the pH was measured at different time points. After the addition of 0.25 mL of 0.5 M NMG (aq), a cloudy solution was observed with some material

on the bottom of the reactor. pH was 4.01. A clear solution was obtained after the addition of 0.70 mL of the 0.5 M N-methyl-D-glucamine solution; the pH was 5.51. A total of 0.74 mL of 0.5 M N-methyl-D-glucamine solution was added (equal to 2.85 eq); pH was 5.99. After 2h of stirring, the pH of the solution was 6.01. The clear, colorless solution was placed into a 20-
5 mL round-bottomed flask, frozen in dry ice; and lyophilized overnight. A white solid was obtained and characterized by PXRD and HPLC. PXRD confirms that the sample is amorphous; HPLC shows purities of 96.90 area-% and 96.69 area-% for the two injections.

Tromethamine tri-salt

10 EXPERIMENT TRO-P3

90.0 mg (0.1295 mmol) of Foxy-5 hexapeptide was weighed out into a 7-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity ≥ 18.2 M Ω cm). The pH meter was inserted and pH of 2.69 was measured. A N₂ purge was applied and stirring was set up at 700 rpm. A suspension was observed. 0.39 mL of a 0.5 M aqueous solution of tromethamine (1.2115 g in
15 20 mL of water) was slowly added with a dosimeter over 30 minutes and the pH was measured at different time points. After the addition of 0.29 mL (1.12 eq) of 0.5 M TRO (aq), almost all the solid was dissolved and pH was 4.04. A clear solution was obtained after the addition of 0.59 mL of the 0.5 M TRO solution (2.28 eq); the pH was 4.99. A total of 0.71 mL of 0.5 M TRO solution was added (equal to 2.74 eq); pH was 5.95. After 2h of stirring, the pH of the solution
20 was 5.97. The clear, colorless solution was placed into a 20-mL round-bottomed flask; frozen in the freezer; and lyophilized overnight. A white solid was obtained and characterized by PXRD. The sample was then stored in the freezer and characterized in duplo by HPLC 5 d later. HPLC shows purities of 98.34 area-% and 98.35 area-% for the two injections.

Histidine tri-salt

25 EXPERIMENT HIS-P2

100.4 mg (0.145 mmol) of Foxy-5 hexapeptide was weighed out into a 7-mL Supelco vial; 3.33 mL of ultrapure water was added (resistivity ≥ 18.2 M Ω cm) under an N₂ purge $\Rightarrow$ suspension of white, gel-like solid observed; stirred at 500 rpm and inserted an electrode to measure a pH of 2.64; 0.0985 M of L-histidine (aq) was dosed at 0.1 mL min⁻¹, pausing to measure pH; at
30 2 min, 0.30 mL of base had been added, and the pH was 3.50; at 9 min, a total of 1.00 mL of base had been added, and the pH was 3.50; at 19 min, a total of 2.00 mL of base had been

added, and the pH was 4.23; a mostly clear solution with a few clumps of gel-like solid was observed; at 29 min, a total of 3.00 mL had been added, and the pH was 4.61; at 39 min, a total of 4.00 mL of base had been added, and the pH was 5.11; a clear, colorless solution was observed; at 43 min, a total of 4.38 mL of base had been added, and the pH was 5.29; let stir
5 for several hours; at 3 h 23 min after the start of dosage, the pH was measured to be 5.27, and the experiment was terminated; the clear, colorless solution was placed into a 20-mL round-bottomed flask, resulting solution frozen in dry ice, and lyophilized for 19 h 05 min and yielded a fluffy, white solid; HPLC (in duplo) and elemental analyses were carried out. PXRD confirms that the sample is amorphous (Figure 8). HPLC shows purities of 98.25 area-% and
10 98.21 area-% for the two injections and corresponding assay values of 62.12% and 61.95%, respectively. Elemental analysis (C, H, N, O and S) of the product (below) is in good agreement with the amorphous product being a tri-salt tetrahydrate.

CHNOS analysis	Histidine tri-salt tetrahydrate $C_{26}H_{42}N_6O_{12}S_2 \cdot (C_6H_9N_3O_2)_3 (H_2O)_4$						
	%C	%H	%N	%O	%S	N/S ratio	Stoichiometry
Theoretical	42,93	6,29	17,17	28,46	5,14	3,28	1 : 3.00
Found:	42,89	6,48	17,19	28,27	5,16	3,33	1 : 3.09

15
Solubility determination for the histidine tri-salt

12.9 mg of the above produced Histidine salt was carefully redissolved in water at a stirring rate of 500 rpm and a water dosing rate of 0.01 mL/min. Complete dissolution was observed after addition of a total of 0.08 mL of water, corresponding to a solubility of at least 161 g/L.

20
EXPERIMENT HIS-GEN8
70.1 mg (0.101 mmol) Foxy-5 hexapeptide was slurried in 5 ml water at ambient temperature and 0.1 M histidine (aq.) was added slowly with stirring until about 2.97 - 2.98 molar equivalents had been added. The pH was 5.45. The experiment was terminated and the
25 colorless solution was lyophilized as described above. HT-XRPD confirms that the sample is amorphous; UPLC shows a purity of 96.0 area-%.
HPLC-CAD confirms that the sample is a 1:3 Foxy-5 hexapeptide : L-Histidine salt.

EXPERIMENT HIS-GEN15

Experiment HIS-P2 was scaled up as follows: 199.7 mg Foxy-5 hexapeptide was combined with 134.2 mg L-histidine in 7 ml degassed water and stirred until complete dissolution of the starting materials. A pH of 5.41 was observed. The solution was subsequently lyophilized overnight. A white solid was obtained and characterized by HPLC-CAD to have a 1:3.19 ratio
5 between Foxy-5 and L-Histidine but was more accurately determined by qNMR in D₂O to have a 1:3 ratio (Foxy-5:L-histidine). UPLC analysis showed a chemical purity of 98.9 area %.

5 samples of the obtained solid from exp. HIS-GEN15 (each about 50 mg) were suspended in 1000 µL ethanol (exp.TCP11), 1000 µL acetone (exp.TCP12), 1000 µL THF (exp.TCP13), 200 µL
10 acetonitrile/water (90:10) (exp.TCP14) and 2000 µL ethyl acetate (exp.TCP15). The suspensions were subjected to thermocycling and aged for 3 days at app.5°C (see temperature profile Figure 11). Upon completion of the aging period, the solids were collected by centrifugation and analyzed by HT-XRPD, as wet and vacuum-dried samples. Material from the thermocycling experiments was amorphous, but - especially from ethyl acetate - was found
15 to have improved properties with regards to the handling of the material when compared to the initially obtained lyophilizate. Lyophilized Foxy-5, both as such and as compositions with nitrogen bases is often found to be a fine and bulky material with a pronounced tendency to be static and therefore difficult to handle (during weighing, for instance).

Formation of "di-salts"

20 Foxy-5 di-salts are generally prepared by adding the relevant base to a suspension of Foxy-5 in water or another suitable solvent. The base may be added neat or in solution, and either in one portion or gradually, keeping the pH <6 during the procedure.

Arginine di-salt

EXPERIMENT ARG-P2

25 90.5 mg (0.130 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 45.1 mg (2 equiv.) of L-arginine was added under an N₂ purge $\Rightarrow$ clear, colorless solution with pH 4.57 was obtained; let stir at r.t. for 2 h $\Rightarrow$ clear, colorless solution with pH 4.55 was obtained; filtered through a syringe filter (13-mm diameter, 0.2-µm PVDF) into a 10-
30 mL round-bottomed flask; resulting solution frozen in dry ice; lyophilized for 19 h 30 min $\Rightarrow$ white solid was obtained; characterized by PXRD; stored in the freezer and characterized

in duplo by HPLC 6 d later. PXRD confirms that the sample is amorphous; HPLC shows purities of 97.70 area-% and 97.67 area-% for the two injections.

Choline di-salt

EXPERIMENT CHO-P2

- 5 89.1 mg (0.128 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 63.6 μL (2 equiv.) of 46% choline hydroxide (aq) (dens. = 1.073 g mL^{-1}) was added under an N_2 purge $\Rightarrow$ beige solution with pH 4.78 was obtained; let stir at r.t. for 2 h $\Rightarrow$ beige solution with pH 4.78 was obtained; filtered through a syringe filter (13-mm diameter, 0.2 μm PVDF) into a 10-mL round-bottomed flask; resulting solution frozen in dry ice; lyophilized for 19 h 30 min $\Rightarrow$ white solid was obtained; characterized by PXRD; and characterized in duplo by HPLC. PXRD confirms that the sample is amorphous; HPLC shows purities of 94.42 area-% and 94.27 area-% for the two injections.

Diethylamine di-salt

15 EXPERIMENT DEA-P2

- 90.1 mg (0.130 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 26.8 μL (2 equiv.) of diethylamine (dens. = 0.707 g mL^{-1}) was added under an N_2 purge $\Rightarrow$ dilute white suspension with pH 4.68 was obtained; let stir at r.t. for 2 h $\Rightarrow$ clear, colorless solution with pH 4.54 was obtained; filtered through a syringe filter (13-mm diameter, 0.2- μm PVDF) into a 10-mL round-bottomed flask; resulting solution frozen in dry ice; lyophilized for 19 h 30 min $\Rightarrow$ white solid was obtained; characterized by PXRD; stored in the freezer and characterized by HPLC 6 d later; aliquots of this sample were stored in closed containers at 40°C for 2 weeks and 4 weeks and examined in duplo by HPLC. PXRD confirms that the sample is amorphous; HPLC shows purities of 97.30 area-% and 97.41 area-% for the two injections.

Lysine di-salt

EXPERIMENT LYS-P2

- 30 89.6 mg (0.129 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension

was obtained; 37.0 mg (2 equiv.) of L-lysine was added under an N₂ purge $\Rightarrow$ clear, colorless solution with pH 4.53 was obtained; let stir at r.t. for 2 h $\Rightarrow$ clear, colorless solution with pH 4.52 was obtained; filtered through a syringe filter (13-mm diameter, 0.2- μ m PVDF) into a 10-mL round-bottomed flask; resulting solution frozen in dry ice; lyophilized for 19 h 30 min $\Rightarrow$ white solid was obtained; characterized by PXRD; stored in the freezer and characterized by HPLC 6 d later; aliquots of this sample were stored in closed containers at 40°C for 2 weeks and 4 weeks and examined in duplo by HPLC. PXRD confirms that the sample is amorphous; HPLC shows purities of 97.69 area-% and 97.73 area-% for the two injections.

10 Ammonia di-salt

EXPERIMENT NH3-P6

100.7 mg (0.145 mmol) of Foxy-5 hexapeptide was weighed out into a 7-mL Supelco vial; 3.33 mL of ultrapure water was added (resistivity ≥ 18.2 M Ω cm) under an N₂ purge $\Rightarrow$ gel-like, white suspension observed; stirred at 500 rpm and inserted an electrode to measure a pH of 2.63; 1 M of NH₄OH (aq) was dosed at 0.05 mL min⁻¹, pausing to measure pH; at 2 min, 0.10 mL of base had been added, and the pH was 3.19; at 4 min, a total of 0.20 mL of base had been added, and the pH was 3.11; a colorless solution was observed on top of the white suspension; at 5 min, 0.29 mL of base (the di-salt stoichiometry) has been added, and the pH was 3.18; at 7 min, the pH was 5.49, and the solution was nearly clear; at 12 min, the pH was 4.60, and the solution was clear; at 2 h 12 min, the pH was measured to be 4.56, and the experiment was terminated; the clear, colorless solution was placed into a 20-mL round-bottomed flask, resulting solution frozen in dry ice, and stored in the fridge for 5 d; it was then lyophilized for 27 h 35 min and yielded a white solid; HPLC (in duplo) and elemental analyses were carried out. PXRD confirms that the sample is amorphous. HPLC shows purities of 97.62 area-% and 97.66 area-% for the two injections and corresponding assay values of 89.32% and 89.08%, respectively. Elemental analysis (C, H, N, O and S) of the product (below) is in good agreement with the amorphous product being a di-salt dihydrate.

CHNOS analysis	Ammonia di-salt dihydrate C ₂₆ H ₄₂ N ₆ O ₁₂ S ₂ * (NH ₃) ₂ (H ₂ O) ₂						
	%C	%H	%N	%O	%S	N/S ratio	Stoichiometry
Theoretical	40,74	6,88	14,80	29,22	8,27	1,75	1 : 2.00
Found:	41,41	6,97	14,21	29,35	8,06	1,76	1 : 2.07

EXPERIMENT NH3-GEN6

81.3 mg (0.117 mmol) Foxy-5 hexapeptide was slurried in 5 ml water at ambient temperature
5 and 0.1 M histidine (aq.) was added slowly with stirring until about 1.97 - 1.98 molar
equivalents had been added. The pH was 4.36. The experiment was terminated and the
colorless solution was lyophilized as described above. HT-XRPD confirms that the sample is
amorphous; UPLC shows a purity of 93.7 area-%.

Due to the presence of ammonia in the HPLC mobile phase, it was not possible to confirm that
10 *the isolated compound is a 1:2 Foxy-5 hexapeptide : NH3 salt.*

N-methyl-D-glucamine di-salt

EXPERIMENT NMG-P6

90.2 mg (0.130 mmol) of Foxy-5 hexapeptide was weighed out into a 7-mL Supelco vial; 3 mL
15 of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$). The pH meter was inserted
and pH of 2.67 was measured. A N₂ purge was applied to the headspace (N₂ atmosphere)
and stirring was set up at 700 rpm. A suspension was observed. 0.52 mL of a 0.5 M aqueous
solution of N-methyl-D-glucamine (4.8803 g in 50 mL of water) was slowly added with a
dosimeter and the pH was measured at different time points. After the addition of 0.35 mL of
20 0.5 M NMG (aq), almost all the solid was dissolved and a solution with some agglomerates
was observed. pH was 4.13. A clear solution was obtained after the addition of 0.50 mL of the
0.5 M N-methyl-D-glucamine solution; the pH was 4.57. A total of 0.52 mL of 0.5 M N methyl-
D-glucamine solution was added (correspond to 2.0 eq); pH was 4.64. After 2h of stirring, the
pH of the solution was 4.63. The clear, colorless solution was placed into a 20-mL round-
25 bottomed flask; resulting solution frozen in dry ice and lyophilized overnight. A white solid
was obtained and characterized by PXRD and HPLC. Aliquots of this sample were stored in
closed containers at 40°C for 2 weeks and 4 weeks and examined in duplo by HPLC; they were
observed to have a sticky, glassy appearance after 2 weeks of storage at 40°C. PXRD confirms
that the sample is amorphous; HPLC shows purities of 96.64 area-% and 96.58 area-% for the
30 two injections.

EXPERIMENT NMG-GEN7

79.3 mg (0.114 mmol) Foxy-5 hexapeptide was slurried in 5 ml water at ambient temperature and 0.5 M N-methyl-D-glucamine solution (aq.) was added slowly with stirring until about 1.97 - 1.98 molar equivalents had been added. The pH was 4.73. The experiment was terminated and the colorless solution was lyophilized as described above. HT-XRPD confirms that the sample is amorphous; UPLC shows a purity of 95.0 area-%. HPLC-CAD confirms that the sample is a 1:2 Foxy-5 hexapeptide:N-Methyl-D-glucamine salt.

EXPERIMENT NMG-GEN16

Experiment NMG-P6 was scaled up as follows: 200.3 mg Foxy-5 hexapeptide was combined with 113.5 mg N-methyl-D-glucamine in 7 ml degassed water and stirred until complete dissolution of the starting materials. A pH of 4.68 was observed. The solution was subsequently lyophilized overnight. A white solid was obtained and characterized by UPLC, TGMS, ¹H-NMR and HPLC-CAD. The UPLC shows a chemical purity of 99.0 % (area %). ¹H-NMR measurements and HPLC-CAD of NMG-GEN16 confirm that the sample is a 1:2 Foxy-5 hexapeptide : N-Methyl-D-glucamine salt.

The obtained solid was subjected to thermocycling, as follows:

5 samples of the obtained solid from exp. NMG-GEN16 (each about 50 mg) were suspended in 1000 µL ethanol (exp.TCP16), 1000 µL acetone (exp.TCP17), 1000 µL THF (exp.TCP18), 200 µL acetonitrile/water (90:10) (exp.TCP19) and 2000 µL ethyl acetate (exp.TCP20). The suspensions were subjected to thermocycling and aged for 3 days at app.5°C (see temperature profile Figure 11). Upon completion of the aging period, the solids were collected by centrifugation and analyzed by HT-XRPD, as wet and vacuum-dried samples. Material from the thermocycling experiments was amorphous, but - especially from ethyl acetate - was found to have improved properties with regards to the handling of the material when compared to the initially obtained lyophilizate. Lyophilized Foxy-5, both as such and as compositions with nitrogen bases is often found to be a fine and bulky material with a pronounced tendency to be static and therefore difficult to handle (during weighing, for instance).

Tromethamine di-salt

EXPERIMENT TRO-P2

90.1 mg (0.130 mmol) of Foxy-5 hexapeptide was weighed out into a 4-mL Supelco vial; 3 mL of degassed ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) $\Rightarrow$ glassy, white suspension was obtained; 31.4 mg (2 equiv.) of tromethamine was added under an N₂ purge $\Rightarrow$ clear, colorless solution with pH 4.88 was obtained; let stir at r.t. for 2 h $\Rightarrow$ clear, colorless solution
5 with pH 4.86 was obtained; filtered through a syringe filter (13-mm diameter, 0.2- μm PVDF) into a 10-mL round-bottomed flask; resulting solution frozen in dry ice; lyophilized for 19 h 30 min $\Rightarrow$ white solid was obtained; characterized by PXRD; stored in the freezer and characterized in duplo by HPLC 6 d later. PXRD confirms that the sample is amorphous; HPLC shows purities of 97.77 area-% and 97.78 area-% for the two injections.

10

Histidine di-salt

EXPERIMENT HIS-P3

99.2 mg (0.143 mmol) of Foxy-5 hexapeptide was weighed out into a 7-mL Supelco vial; 3.33 mL (i.e. 30 mg/mL) of ultrapure water was added (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) under an N₂ purge
15 $\Rightarrow$ suspension of white, gel-like solid observed; stirred at 500 rpm and inserted an electrode to measure a pH of 2.65; 0.0985 M of L-histidine (aq) was dosed at 0.1 mL min^{-1} , pausing to measure pH; at 1 min, 0.17 mL of base had been added, and the pH was 3.10; at 10 min, a total of 1.00 mL of base had been added, and the pH was 3.90; at 20 min, a total of 2.00 mL of base had been added, and the pH was 4.40; a mostly clear solution with a few clumps of
20 gel-like solid was observed; at 28 min, a total of 2.82 mL had been added, and the pH was 4.73; at 1 h 10 min, a clear, colorless solution was observed; at 2 h 28 min after the start of dosage, the pH was measured to be 4.50, and the experiment was terminated; the clear, colorless solution was placed into a 20-mL round-bottomed flask, resulting solution frozen in dry ice, and lyophilized for 19 h 05 min and yielded a fluffy, white solid; HPLC and elemental
25 analyses were carried out; aliquots of this sample were stored in closed containers at 40°C for 2 weeks and 4 weeks and examined by HPLC; the sample was observed to remain a fluffy white solid after 2 and 4 weeks of storage at 40°C. HPLC shows purities of 98.02 area-% and 98.00 area-% for the two injections and corresponding assay values of 67.88% and 67.86%, respectively. Elemental analysis (C, H, N, O and S, below) of the product is in good agreement
30 with the amorphous product being a di-salt dihydrate.

CHNOS analysis	Histidine di-salt dihydrate						
	$C_{26}H_{42}N_6O_{12}S_2 \cdot (C_6H_9N_3O_2)_2 (H_2O)_2$						
	%C	%H	%N	%O	%S	N/S ratio	Stoichiometry
Theoretical	42,37	6,36	15,60	29,71	5,95	2,62	1 : 2.00
Found:	42,98	6,67	15,15	29,43	5,77	2,63	1 : 2.01

Solubility determination for the histidine di-salt

The above experiment was repeated using half the amount of water (60 mg/ml) for the initial slurring of Foxy-5 hexapeptide. A final concentration of 31.6 g L⁻¹ was achieved for the histidine di-salt at a pH of 5.82. The experiment was also successfully repeated adding the histidine solution to neat (solid) Foxy-5 hexapeptide which resulted in a final concentration of 49.3 g L⁻¹ at a pH of 5.78. In both cases the final solutions were frozen and lyophilized for 25 hrs yielding a fluffy, white solid.

EXPERIMENT HIS-GEN5

78.4 mg (0.113 mmol) Foxy-5 hexapeptide was slurried in 5 ml water at ambient temperature and 0.1 M histidine (aq.) was added slowly with stirring until about 1.97 - 1.98 molar equivalents had been added. The pH was 4.61. The experiment was terminated and the colorless solution was lyophilized as described above. HT-XRPD confirms that the sample is amorphous; UPLC shows a purity of 93.9 area-%.

HPLC-CAD confirms that the sample is a 1:2 Foxy-5 hexapeptide : L-Histidine salt.

EXPERIMENT HIS-GEN14

Experiment HIS-P3 was scaled up as follows: 220.1 mg Foxy-5 hexapeptide was combined with 98.5 mg L-histidine in 7 ml degassed water and stirred until complete dissolution of the starting materials. A pH of 4.65 was observed. The solution was subsequently lyophilized overnight. A white solid was obtained and characterized by HPLC-CAD to have a 1:2.10 ratio between Foxy-5 and L-Histidine but the ratio was more accurately determined by ¹H NMR to be 1:2.03. UPLC analysis showed a chemical purity of 98.9 area %.

5 samples of the obtained solid from exp. HIS-GEN14 (each about 50 mg) were suspended in 1000 µL ethanol (exp.TCP6), 1000 µL acetone (exp.TCP7), 1000 µL THF (exp.TCP8), 200 µL acetonitrile/water (90:10) (exp.TCP9) and 2000 µL ethyl acetate (exp.TCP10). The suspensions were subjected to thermocycling and aged for 3 days at app.5°C (see temperature profile

Figure 11). Upon completion of the aging period, the solids were collected by centrifugation and analyzed by HT-XRPD, as wet and vacuum-dried samples. Material from the thermocycling experiments was amorphous, but - especially from ethyl acetate - was found to have improved properties with regards to the handling of the material when compared to the initially obtained lyophilizate. Lyophilized Foxy-5, both as such and as compositions with nitrogen bases is often found to be a fine and bulky material with a pronounced tendency to be static and therefore difficult to handle (during weighing, for instance).

10 Formation of "mono-salts"

Foxy-5 mono-salts are generally prepared by adding the relevant base to a suspension of Foxy-5 in water or another suitable solvent. The base may be added neat or in solution, and either in one portion or gradually, keeping the pH <6 during the procedure. The reaction mixture is then stirred until the starting material has dissolved. The final solution of the mono-salt is optionally filtered followed by being lyophilized or spray-dried.

Histidine mono-salt

EXPERIMENT HIS-GEN2

90.6 mg (0.131 mmol) Foxy-5 hexapeptide was slurried in 5 ml water at ambient temperature and 0.1 M histidine (aq.) was added slowly with stirring until about 0.97 - 0.98 molar equivalents had been added. The pH was 3.88. The experiment was terminated and the slightly opalescent, colorless solution was lyophilized as described above. HT-XRPD confirms that the sample is amorphous; UPLC shows a purity of 95.2 area-%.

HPLC-CAD confirms that the sample is a 1:1 Foxy-5 hexapeptide : L-Histidine salt.

Ammonia mono-salt

EXPERIMENT NH3-GEN3

92.6 mg (0.134 mmol) Foxy-5 hexapeptide was slurried in 5 ml water at ambient temperature and 1 M of NH₄OH (aq) was dosed at 0.05 mL min⁻¹ with stirring until about 0.99 molar equivalents had been added. The pH was 3.87. The experiment was terminated and the

slightly opalescent, colorless solution was lyophilized as described above. HT-XRPD confirms that the sample is amorphous; UPLC shows a purity of 94.4 area-%.

Due to the presence of ammonia in the HPLC mobile phase, it was not possible to confirm that the isolated compound is a 1:1 Foxy-5 hexapeptide : NH₃ salt.

5

N-methyl-D-glucamine mono-salt

EXPERIMENT NMG-GEN4

89.5 mg (0.129 mmol) Foxy-5 hexapeptide was slurried in 5 ml water at ambient temperature and 0.26 ml (1 molar equivalent) of a 0.5 M aqueous solution of N-methyl-D-glucamine (4.88 g in 50 mL of water) was slowly added with stirring. After about 2 hours an almost clear, slightly opalescent solution was obtained. The pH was 3.91 at this point. The experiment was terminated and the solution was lyophilized as described above. HT-XRPD confirms that the sample is amorphous; UPLC shows a purity of 94.3 area-%.

HPLC-CAD confirms that the sample is a 1:1 Foxy-5 hexapeptide : NMG salt.

15

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CLAIMS

1. A solid composition comprising For-Met-Asp-Gly-Cys-Glu-Leu-OH (SEQ_NO 1, Foxy-5) and
a nitrogen base, wherein the nitrogen base is a compound selected from ammonia,
5 quaternary ammonium hydroxides, alkylated guanidines, amino acids, and primary and
secondary amines, and wherein said solid composition optionally comprises water.
2. The solid composition according to claim 1 which contains 0.5 - 3.1 molar equivalents of
said nitrogen base, calculated in relation to Foxy-5.
- 10 3. The solid composition according to any one of claim 1 or 2 wherein the nitrogen base is a
compound selected from amino acids, ammonia, and primary and secondary amines.
4. The solid composition according to any one of claim 1 to 3 wherein the nitrogen base is
15 selected from ammonia, L-lysine, L-histidine, N-methyl-D-glucamine, and tromethamine.
5. The solid composition according to any one of claim 1 to 4 comprising Foxy-5 and 1.50 -
2.49 equivalents, such as 1.7 - 2.1 equivalents, such as 1.9 - 2.03 equivalents, such as
1.95 - 2.01 equivalents, such as 2.0 equivalents of a nitrogen base selected from
20 ammonia, L-lysine, L-histidine and tromethamine.
6. The solid composition according to any one of claim 1 to 4 comprising Foxy-5 and 2.5 -
3.1 equivalents, such as 2.9 - 3.03 equivalents, such as 2.95 - 3.01 equivalents, such as
3.0 equivalents, of a nitrogen base selected from L-histidine and N-methyl-D-glucamine.
- 25 7. The solid composition according to claim 1 selected from the di-ammonium salt, the di-L-
histidine salt, the tri-L-histidine salt, the di-L-lysine salt, the di-N-methyl-D-glucamine salt,
the tri-N-methyl-D-glucamine salt and the di-tromethamine salt of Foxy-5, which salts are
amorphous and optionally comprise between 0-5 equivalents of water calculated in rela-
30 tion to Foxy-5.
8. The solid composition according to claim 2 wherein the nitrogen base is L-histidine.

9. The solid composition according to claim 8 comprising Foxy-5 and between 1.50 - 2.49 equivalents of L-histidine such as 1.7 – 2.1 equivalents of L-histidine.
10. The solid composition according to claim 8 comprising Foxy-5 and between 2.5 – 3.1 equivalents of L-histidine.
11. A method for producing a solid composition according to any one of the preceding claims, comprising the following steps:
- a) Mixing the free acid form of For-Met-Asp-Gly-Cys-Glu-Leu-OH (SEQ_NO 1, Foxy-5), either as
 - i. a suspension in a solvent, or
 - ii. as a neat powder,with either
 - iii. 0.5 – 1.49 equivalents of a nitrogen base to prepare a monosalt of Foxy-5, or
 - iv. 1.50 - 2.49 equivalents of a nitrogen base to prepare a disalt of Foxy-5, or
 - v. 2.5 – 3.1 equivalents of a nitrogen base to prepare a tri-salt of Foxy-5,
 - b) Stirring the reaction mixture until a clear solution is obtained, and
 - c) Isolating a solid composition by precipitation, crystallization or by lyophilizing or spray-drying the solution,
- wherein the nitrogen base is a compound selected from ammonia, quaternary ammonium hydroxides, alkylated guanidines, amino acids, and primary and secondary amines, and wherein all steps a) – c) are optionally conducted in an inert atmosphere.
12. A method for producing a solid composition according to claim 11 wherein water is employed as solvent.
13. A method for producing a solid composition according to any one of claim 11-12, wherein the nitrogen base is added gradually to Foxy-5 whilst keeping the pH of the reaction mixture below 6.0.
14. A method for producing a solid composition according to any one of claim 11-13, wherein the resulting clear solution from step b) is lyophilized.

15. A method for producing a solid composition according to any one of claim 11-14 wherein the resulting solid composition is further subjected to a thermocycling process comprising:

- 5 i. Providing a suspension of the lyophilized or spray-dried product in a solvent or solvent mixture,
- ii. Subjecting the suspension to a thermocycling process comprising 1 - 3 cycles between 5°C - 50°C $\pm$ 2°C, followed by aging the suspension for 1-3 days at 5°C $\pm$ 2°C,
- 10 iii. isolating the thermocycled product, e.g. by filtration or centrifugation, during which thermocycling process the suspension is heated at a rate of 10°C $\pm$ 2°C /hr until a temperature of 50°C $\pm$ 2°C is reached, followed by cooling the suspension at a rate of:
- a. 20°C $\pm$ 2°C /h for the 1st cycle,
- b. 10°C $\pm$ 2°C /hr for the 2nd cycle, and
- 15 c. 5°C $\pm$ 2°C /hr for 3rd cycle,
- until a temperature of 5°C $\pm$ 2°C is reached, followed by aging the suspension by stirring it for 1-3 days at 5°C $\pm$ 2°C before isolation.

- 20 16. A method for producing a solid composition according to claim 15, wherein the organic solvent is selected from ethanol, acetone, tetrahydrofuran (THF), acetonitrile/water (90/10), and ethyl acetate.