Total Synthesis of Microcystin-LF and Derivatives Thereof

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Supporting Information

ABSTRACT: Microcystins (MCs) are highly toxic natural products which are produced by cyanobacteria and, according to the WHO, are among the most dangerous water pollutants. Widespread harmful algal blooms in densely populated areas, which are favored by global warming along with eutrophication of surface waters, resulted in a shutdown of the public water supply for millions of people, for example in Toledo, Ohio, USA, (August 2014) and around lake Taihu in China (July 2007). The sole analytical method for congener-specific MC detection enabling the identification and quantification of MCs is liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS). However, the lack of certified reference compounds limits the applicability of this method. Although research on MCs has been conducted since 1878, their physiological function in cyanobacteria is still under scientific debate. Consequently, the total synthesis of MCs would provide access to a yet missing variety of reference compounds as well as microcystin derivatives for structure–activity relationship studies enabling future studies into the biological roles of MCs. Furthermore, the high cytotoxicity of MCs makes this scaffold an attractive lead structure for the development of novel potent anticancer drugs.

As shown in Figure 1, MCs consist of three D-amino acids in positions 2 and 4. Adda = (2S,3S,4E,6E,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, d-Ala = D-alanine, γ-D-Glu = γ-D-glutamic acid, Mdha = N-methyldehydroalanine, β-D-MeAsp = erythro-β-D-methylaspartic acid.

Figure 1. Microcystin-LF (MC-LF) and some amino acid variations (gray) of naturally occurring congeners. The two amino acids indicated in the name (LF in the current example) denote the natural ω-amino acids in positions 2 and 4. Adda = (2S,3S,4E,6E,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, d-Ala = D-alanine, γ-D-Glu = γ-D-glutamic acid, Mdha = N-methyldehydroalanine, β-D-MeAsp = erythro-β-D-methylaspartic acid.

INTRODUCTION

Microcystins (MCs) are highly toxic natural products which are produced by cyanobacteria and, according to the WHO, are among the most dangerous water pollutants. Widespread harmful algal blooms in densely populated areas, which are favored by global warming along with eutrophication of surface waters, resulted in a shutdown of the public water supply for millions of people, for example in Toledo, Ohio, USA, (August 2014) and around lake Taihu in China (July 2007). The sole analytical method for congener-specific MC detection enabling the identification and quantification of MCs is liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS). However, the lack of certified reference compounds limits the applicability of this method. Although research on MCs has been conducted since 1878, their physiological function in cyanobacteria is still under scientific debate. Consequently, the total synthesis of MCs would provide access to a yet missing variety of reference compounds as well as microcystin derivatives for structure–activity relationship studies enabling future studies into the biological roles of MCs. Furthermore, the high cytotoxicity of MCs makes this scaffold an attractive lead structure for the development of novel potent anticancer drugs.

As shown in Figure 1, MCs consist of three D-amino acids in positions 1, 3, and 6, the ω-amino acid Adda, and N-methyldehydroalanine (Mdha) which is involved in covalent binding of MCs to ser/thr protein phosphatases by Michael addition of a proximate cysteine residue. Two ω-amino acids in positions 2 and 4 are variable and account for the major differences amongst the more than 100 individual congeners, that have been reported so far. Despite numerous attempts the total synthesis of a natural microcystin (MC-LA) was accomplished only once. This landmark achievement featured protection of β-D-MeAsp and γ-D-Glu6 by methyl esters that were cleaved under basic conditions in the last synthetic step. Unfortunately, this treatment led to the formation of several...
uncharacterized isomers of the final product. In our interpretation the isomerization can be attributed to a cyclization involving β-D-MeAsp3 (I, Scheme 1) leading to aspartimide II which is prone to isomerization at the chiral centers in the α- and β-positions. Ring opening by basic hydrolysis can lead to the stereoisomers III of desired MC-LA as well as constitutional isomers IV. Despite extensive investigation of different methyl ester cleavage conditions, the isomerization could not be avoided and resulted in significant loss of material. To identify natural MC-LA, the isomeric mixture was separated by HPLC and the isomers were compared to an authentic sample of the cyanotoxin. The identification of the desired isomers was particularly intricate during the synthesis of unnatural derivatives for which no standard was available.

Scheme 1. Proposed Formation of Aspartimide II and Subsequent Isomerization during the Saponification of Methyl Ester Protected MC-LA I

![Scheme 1](image)

Here, we present an isomerization-free synthetic strategy and its application to the first total synthesis of MC-LF (1a) and its deuterated derivative 1b (Scheme 2) which is of interest as an internal standard during MC quantification by mass spectrometry. Since this approach delivers only one isomer, the synthesis of unnatural MC derivatives becomes feasible which we demonstrate by the preparation of derivative 1c. The alkyne-labeled 1c can be employed for toxin derivatization by click chemistry with an azide-containing reporter molecule, e.g., biotin or a fluorescent dye, and, furthermore, be used as an activity-based probe to identify interaction partners. According to crystal structures of MCs in complex with ser/thr protein phosphatases the side chain at position 4 of the MC backbone is not involved in binding and thus represents an optimal position for synthetic modifications.

## RESULTS AND DISCUSSION

### Retrosynthetic Analysis.

For the synthesis of 1a–c we followed a fragment-based strategy using tert-butyl esters as protecting groups for β-D-MeAsp3 and γ-D-Glu6 in order to suppress aspartimide formation. In contrast to the previous approach, in which N-methylphosphonylsarcosine was incorporated and subsequently converted into Mdha7 by a Horner–Wadsworth–Emmons reaction, we incorporated N-methylphenylselenocysteine (NMeSecPh) as Mdha precursor that was transformed into Mdha in the final step of the synthesis. For the macrocyclization, we decided to use the peptide bond between residues 4 and 5 which is also the cyclization site during biosynthesis of MCs. In addition, this disconnection has proven reliable in the former synthetic approach. The linear precursors 2a–c were synthesized in a convergent manner from fragments 3, 4, and 5a–c. Several syntheses of Boc-Adda−OH have been reported. We followed the route developed by Pearson et al. that delivers 3 in 13 steps with the best overall yield. Fmoc groups served as N-terminal protection of fragments 4 and 5a–c. The presence of Fmoc,

Scheme 2. Retrosynthetic Analysis of MC Derivatives 1a–c

![Scheme 2](image)
tert-butyl ester and the phenylselenocysteine moiety in fragments 4 and 5a–c required a C-terminal protection which can be cleaved in the presence of these three groups and withstands the conditions of Fmoc deprotection. In our approach we used the phenacyl (Pac) protecting group which is removed under mild reductive conditions. Tetrapeptide 4 and dipeptides 5a–c were obtained from NMeSecPh derivative 6, β-d-MeAsp derivative 7 and the building blocks 8a, 8b, and 8c.

Synthesis of Tetrapeptide 4. We started our synthesis with the preparation of tetrapeptide fragment 4 (Scheme 3).

**Scheme 3.** Synthesis of Tetrapeptide Fragment 4

**Scheme 4.** Synthesis of Fmoc-d-MeAsp-OpBu 7

**Scheme 5.** (A) Synthesis of Alkyne Labeled Building Block 8c and (B) Synthesis of Dipeptide Fragments 5a–c

Double propargylation of 17 followed by saponification of the propargyl ester gave 18 that was alkylated with phenacly bromide yielding 8c in a high yield. 8c as well as literature known 8a 17 and analogously prepared, isotopically labeled 8b were N-terminally deprotected and coupled with d-MeAsp derivative 7 to give dipeptides 5a–c (Scheme 5B).

Synthesis of Linear Heptapeptides. With all fragments in hand, heptapeptides 2a–c were assembled (Scheme 6). The C-terminal phenacyl ester of tetrapeptide 4 was reductively be explained by preferred formation of the (Z)-lithium enolate which adopts a hydrogen-in-plane conformation that is attacked opposite to the bulky nitrogen protecting groups. The isomers erythro-16 and threo-16 were readily separated by column chromatography and the configuration of erythro-16 was verified by complete deprotection and subsequent NMR analysis22 (see Supporting Information) as well as optical rotation measurement.20 Hydrogenolysis of erythro-16 followed by Fmoc protection of the free amino group gave building block 7.

The phenacyl protected propargyl tyrosine building block Boc-Tyr(Prg)-OFac 8c (Prg = propargyl) was synthesized in three steps starting from Boc-Tyr-OH 17 (Scheme 5A).
cleaved to give 19. Fmoc deprotection of dipeptides 5a–c was performed with diluted (5%) piperidine in DMF to avoid possible cleavage of the Pac ester giving 20a–c. The subsequent fragment coupling of 19 and a slight excess of 20a–c was performed with HATU/collidine leading to 21a–c. Under these conditions isomerization at the C-terminal Leu residue of fragment 19 is minimized as it was also reported by others.10,23 The hexapeptides 21a–c were N-terminally deprotected and Boc-Adda−OH 3 was coupled to give heptapeptides 22a–c. Removal of the Pac group led to macrocyclization precursors 22a–c. We found that it was essential to purify compounds 22a–c by RP-HPLC to prevent byproduct formation during the subsequent macrocyclization step.

**Macrocyclization and Final Steps.** The C-termini of 22a–c were activated as pentafluorophenyl (Pfp) ester and the t-butyl esters along with the Boc group were removed by TFA treatment (Scheme 7). Macrocyclization was induced under basic conditions applying a two-phase system of chloroform and phosphate buffer (pH = 9.5) to give cyclopeptides 23a–c which were purified by RP-HPLC. According to HPLC approximately 20% (in case of 23a and 23b) and 10% (in case of 23c), respectively, of an isomeric product, possibly due to epimerization at C-terminal amino acid during macrocyclization, were observed. Subsequent selenoxide elimination under mild oxidative conditions gave the desired MC derivatives 1a–c. All final products were fully characterized by one- and two-dimensional NMR spectroscopy and HRMS. The analytical data of synthetic MC-LF (1a) were identical to those of a commercial MC-LF sample confirming the structure of the synthetic material. In addition, coinjection of both a mixture of natural and synthetic 1a as well as a mixture of 1a and 1b led to single peaks in the LC-MS chromatograms (Supporting Information).

**Inhibition of Protein Phosphatase-1.** In order to confirm that the biological activities of synthetic and natural MC-LF are identical, we determined the potency of both compounds to inhibit the hydrolysis of p-nitrophenyl phosphate catalyzed by protein phosphatase-1 (PP1, Figure 2). As expected, the IC50 values obtained with this robust assay24 are very similar within the accuracy of this assay (synthetic 1a: IC50 = 870 pM, natural 1a: IC50 = 1.2 nM) further verifying the authenticity of the synthetic material. We also evaluated the inhibitory potency of the propargylated derivative 1c and its precursor 23c lacking the Michael acceptor Mdha. The similar IC50 value of 1c (IC50 = 1.7 nM) shows that the modification at position 4 has only a minor influence on phosphatase binding and inhibition as

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**Scheme 6. Synthesis of Linear Heptapeptides 22a–c by Fragment Couplings**

![Image of Scheme 6]

**Scheme 7. Deprotection and Macrocyclization**

![Image of Scheme 7]

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**Figure 2.** PP1 inhibition assay with natural MC-LF and synthetic compounds 1a, 1c, and 23c (values from three independent experiments). App1 = activity of PP1.
In summary, we developed a novel strategy for the synthesis of MCs. Application of the Fmoc/t-Bu protecting group strategy enabled the isomerization-free synthesis of MC-LF as well as a deuterated and an alkyne-labeled derivative thereof. The incorporation of a phenylethylcysteine moiety as precursor for the Mdha residue gave access to a novel potent and reversible phosphatase inhibitor that cannot undergo covalent binding to the protein. The synthesized MC derivatives can be broadly applied for improved MC detection and quantification and pave the way for future approaches to understanding the biological roles of MCs in various organisms.

## EXPERIMENTAL SECTION

### General Experimental Methods.
Technical solvents (petroleum ether and EtOAc) were distilled prior to use. THF and CH₂Cl₂ were distilled from Na₂O/K or CaH₂ under inert atmosphere immediately prior to use. Peptide grade DMF was used for peptide couplings. A sample of natural microcystin-LF was obtained from Enzo Life Sciences (ALX-350–081–C100, 100 μg) and used as received. The compounds 3, 10, 18, 8a, 17 and 10⁶ were synthesized according to the literature. For the reactions performed under inert gas conditions (nitrogen) Schlenk technique and oven-dried glassware were used. Analytical thin layer chromatography (TLC) was performed using TLC silica gel 60 F254 coated aluminum sheets (Merck). Spots were visualized either by UV light (λ = 254 nm) or by dipping and heating using ethanolic ninhydrin solution (3% w/v), aqueous potassium permanganate (1% w/v), anisaldehyde solution (135 mL EtOH, 5 mL conc. H₂SO₄, 15 mL glacial acetic acid, and 3.7 mL p-anisaldehyde), or Searbach’s stain (25 g phosphomolybdic acid, 10 g Ce(SO₄)₂·4H₂O, and 60 mL conc. H₂SO₄ in 1 L H₂O). Preparative flash column chromatography (FC) was performed using Gudran 60 silica gel (40–60 μm, Merck). NMR spectra were recorded on Bruker Avance III 400 or Bruker Avance III 600 instruments. Chemical shifts δ are reported in ppm relative to solvent signals (CDCl₃, δ = 7.26 ppm, CDCl₃–D₂O δ = 3.34 ppm, δ = 77.2 ppm; CDCl₃ δ = 3.34 ppm, δ = 49.0 ppm; DMSO–D₂O δ = 2.50 ppm, δ = 39.5 ppm). For all new compounds two-dimensional NMR experiments (COSY, HMQC, and HMBC) were used for signal assignments. For numbering of carbons see Supporting Information.

### Preparative synthesis

**GP1: Phenacyl Protection.** Our sample of natural microcystin-LF was obtained from the laboratory of the University of Konstanz using an Elementar vario EL elemental analyzer. Microanalytical separations. Elemental analyses were performed by the microanalytical laboratory of the University of Konstanz using an Elementar vario EL instrument. HRMS measurements were performed on a Bruker micrOTOF II (ESI-TOF) or a Thermo LTQ Orbitrap Discovery (ESI-Orbitrap) instrument.

**General Procedures.**

**GP2: Phenacyl Deprotection.** The protected peptide is dissolved in MeOH/DMF (8/2, μL mg⁻¹ peptide). Then Mg-turnings (0.16 mg/μL peptide) and acetic acid (0.8 μL mg⁻¹ peptide) are added and the reaction mixture is stirred at rt for 4 h. During the reaction, the formation of a white precipitate can be observed. After 4 h the mixture is diluted with EtOAc and washed with brine (1 ×), sat. NaHCO₃ solution (1 ×), and brine (2 ×). The organic phase is dried over Na₂SO₄, filtered, and concentrated under reduced pressure.

**GP3: Fmoc Deprotection.** The peptide is dissolved in 5% piperidine in DMF (v/v). After 3 min, the solution is diluted with DMF and the volatiles are removed under reduced pressure. The residue is co-evaporated with toluene (3 ×) and used without further purification.

**GP4: Boc/t-Bu Deprotection.** After 3 min, the solution is diluted with DMF and the volatiles are removed under reduced pressure. The residue is co-evaporated with toluene (3 ×) and used without further purification.

**GP5: Fmoc Protection.** The amino acid (1.0 equiv) is suspended in H₂O (10% w/v, 1.25 mL mmol⁻¹ amino acid). Then Fmoc-OSu (1.3 equiv) in dioxane (1.875 mL mmol⁻¹ amino acid) is added, and the mixture is stirred at rt overnight. The reaction mixture is diluted with H₂O and EtOAc, acidified with aqueous NaH₂SO₄ (1M) to pH < 3, and extracted with EtOAc (3 ×). The combined organic phases are washed with brine (1 ×), dried over Na₂SO₄, filtered, and the volatiles removed under reduced pressure. The crude product is purified with FC.

**GP6: Peptide Coupling.** The amine and carboxylic acid are dissolved in DMF. The resulting solution is cooled to 0 °C. Then base (DIPEA or collidine) and after 5 min the coupling reagent (HATU or HBTU) are added. The resulting yellowish solution is stirred at rt for 45 min and at rt overnight. The reaction mixture is diluted with EtOAc and washed subsequently with H₂O, 1:1 diluted saturated citric acid solution, 1:1 diluted saturated NaHCO₃ solution, and brine (each 1×). The organic phase is dried over Na₂SO₄, filtered, and purified with FC.

**GP7: Macrocylation.** The HPLC-purified C-terminally deprotected heptapeptide (1.0 equiv) is placed in a 4 mL glass vial. Then pentafluorophenol (1.95 equiv), dissolved in freshly distilled EtOAc (24.2 mL mmol⁻¹ peptide) and solid DCC are added. The reaction mixture is cooled to 0 °C, stirred for 90 min and then at rt for 8–10 h. Afterward, the solution is removed and the activated peptide is treated with TFA according to GP4. The resulting C-terminally activated unprotected heptapeptide is dissolved in CHCl₃ (515 mL mmol⁻¹ peptide). The resulting solution is added dropwise over a period of 10 min to a vigorously mechanically stirred mixture of CHCl₃, H₂O (1875 mL mmol⁻¹ peptide) and pH = 9.5 phosphate buffer (1M, 875 mL mmol⁻¹ peptide). After 30 min, the reaction mixture is diluted with a small amount of CHCl₃ and H₂O. The phases are separated, the aqueous phase is acidified with NaH₂SO₄ (1M) to pH < 3 and extracted with EtOAc (3 ×). The organic phases are combined, washed...
with brine, and dried over Na₂SO₄. The crude macrocyclic heptapeptide is lyophilized and purified using HPLC.

**GP8: Selenoxide Elimination.** The macrocyclic peptide is dissolved in MeCN/H₂O (3:2, 1 mL) and 30% aqueous H₂O₂ (4 μL) is added. After 1 h at rt, the reaction mixture is quenched with Me₂S (50 μL) and purified using HPLC.

**Synthesized Compounds (Sorted According to Compound Number).** 

**Microcystin-LF (1a).** The C-terminally unprotected heptapeptide 22a (15 mg, 10.9 μmol) was macrocyclized using pentadentanol (4.0 mg, 21.3 μmol) and DCC (2.8 mg, 13.3 μmol) according to GP7. The crude macrocycle 23a was purified by semipreparative RP-HPLC (gradient: 60–90% B in 30 min, tₖ = 13.3 min). The cyclic peptide 23a was transformed to 1a according to GP8 and the reaction mixture was separated by semipreparative RP-HPLC (gradient: 50–70% B in 20 min, tₖ = 13.2 min). MC-LF 1a was obtained as a white amorphous solid (2.0 mg, 19%). LC-MS analysis of synthetic 1a co-injected with natural MC-LF resulted in a single peak with the expected mass (Figure S3).

**NMR (600 MHz, CD₃OD, 300 K) δ 8.89 (d, J = 9.6 Hz, 1H, NH MeAsp), 8.17–8.08 (m, 2H, NH Leu, NH Phe), 7.46 (d, J = 8.2 Hz, 1H, NH Ala), 7.31 (d, J = 9.2 Hz, 1H, NH Adda), 7.27–7.25 (m, 2H, Ar), 7.23–7.14 (m, 6H, Ar), 6.35 (d, J = 15.5 Hz, 1H, H-5 Adda), 5.89 (s, 1H, C-CH₃), 5.51 (d, J = 9.8 Hz, 1H, H-7 Adda), 5.45 (s, 1H, C-C₅H₅), 5.34 (dd, J = 15.6, 8.6 Hz, 1H, H-4 Adda), 4.74–4.69 (m, 9H, H-3 Adda), 4.25–4.19 (m, 1H, H-α MeAsp, H-α Phe, H-α Ala), 4.45 (t, J = 7.4 Hz, 1H, H-γ Ala), 4.22–4.18 (m, 1H, H-β MeAsp, H-β Phe, H-β Ala), 3.80 (dd, J = 14.1, 3.2 Hz, 1H, H-2 Adda), 2.63 (dq, J = 12.9, 4.8 Hz, 1H, H-3 Adda), 2.30 (t, J = 8.4 Hz, 2H, -OCH₂), 1.79–1.73 (m, 2H, H-10 Adda), 1.26–2.25 (m, 4H, H-8 Adda, 2H, H-9 Adda, H-10 Adda), 2.74–2.68 (m, 2H, H-7 Adda), 2.66–2.55 (m, 4H, H-8 Adda, 2H, H-9 Adda), 2.19–2.11 (m, 1H, H-β Glu), 1.91–1.83 (m, 1H, H-7 Leu), 1.80–1.68 (m, 2H, H-2 Glu, H-γ Leu), 1.65 (s, 3H, 3xH-6' Adda), 1.53 (dd, J = 13.9, 9.8 Hz, 2H, H-1 Leu), 1.11 (d, J = 6.9 Hz, 3H, 3xH-2' Adda), 1.04 (d, J = 6.7 Hz, 3H, 3xH-1' Adda), 0.99 (s, J = 7.4 Hz, 3H, CH₃ Ala), 0.88 (d, J = 6.6 Hz, 3H, 3xH-6' Leu), 0.86 (d, J = 6.5 Hz, 3H, 3xH-5' Leu), 0.76 (d, J = 7.8 Hz, 2H, CH₂ Leu), 0.55 (s, 3H, C-CH₃), 0.33–0.27 (m, 1H, H-9 Adda), 0.32 (s, 3H, -OCH₃). The cyclic peptide 23a was transformed to 1a according to GP8 and the reaction mixture was separated by semipreparative RP-HPLC (gradient: 50–70% B in 20 min, tₖ = 13.2 min). The cyclic peptide 23a was obtained as a white amorphous solid (2.0 mg, 19%). LC-MS analysis of synthetic 1a co-injected with natural MC-LF resulted in a single peak with the expected mass (Figure S3).

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**Microcystin-LF (1b).** The macrocyclic derivative 23b (5 mg, 4.35 μmol) was transformed to 1b according to GP8, and the reaction mixture was separated by semipreparative RP-HPLC (gradient: 50–70% B in 20 min, tₖ = 13.6 min). The macrocyclic 23b was obtained as a white amorphous solid (3.3 mg, 76%). LC-MS analysis of synthetic 1b co-injected with MC-LF resulted in a single peak with the expected masses of both deuterated 1b and unidentified MC-LF (Figure S4).

**NMR (600 MHz, CD₃OD, 284 K) δ 8.16 (d, J = 6.8 Hz, 1H, NH Leu), 0.07 (d, J = 9.1 Hz, 1H, NH Adda), 7.56 (d, J = 8.4 Hz, 1H, NH Ala), 7.27–7.25 (m, 2H, Ar), 7.23–7.15 (m, 3H, Ar), 6.35 (d, J = 15.5 Hz, 1H, H-5 Adda), 5.89 (s, 1H, C-CH₃), 5.51 (d, J = 9.9 Hz, 1H, H-7 Adda), 5.46 (s, 1H, C-C₅H₅), 5.45 (dd, J = 15.6, 8.6 Hz, 1H, H-4 Adda), 4.69 (dd, J = 11.0, 8.7 Hz, 1H, H-3 Adda), 4.58–4.49 (m, 3H, H-3 MeAsp, H-3α Phe, H-3α Ala), 4.24–4.17 (m, 1H, H-β MeAsp, H-β Phe, H-β Ala), 3.49 (d, J = 14.1, 3.4 Hz, 1H, H-β H-β Phe, H-β H-β Phe, H-β Phe, H-β H-β Phe, H-β H-β Phe), 3.37 (s, 3H, -NCH₃), 3.30–3.28 (m, 1H, H-9 Adda), 3.26 (s, 3H, -OCH₃), 2.95 (dq, J = 7.2, 3.5 Hz, 1H, H-β MeAsp), 2.85 (dd, J = 14.0, 4.7 Hz, 1H, H-10 Adda), 2.88 (dd, J = 10.9, 6.9 Hz, 1H, H-2 Adda), 2.69 (dd, J = 13.9, 7.3 Hz, 1H, H-10 Adda), 2.65–2.52 (m, 4H, H-8 Adda, H-β

**Phed-δ-γ-Glu(Glu-OrBu)-N-Me cyclopentadentanol-δ-Leu-OEt (4).** Tripeptide 27 (297 mg, 449.5 μmol) was deprotected according to GP4. The trinitrophenyl-protected tripeptide 27c (157.5 μmol) and Fmoc-Glu-OrBu (249 mg, 584 μmol) were dissolved in DMF (2 mL). Peptide coupling was performed according to GP6 using HATU (222 mg, 584 μmol) and DIPEA (313 μL, 1.8 mmol). The crude product was purified by FC to give tetrapeptide 4 (390 mg, 90%) as a white

**HRMS (ESI-Orbitrap) m/z:** [M+H]⁺ Calcd for C₂H₂N₃O₂ 986.52335; Found 986.52418.
amorphous solid: \( R_f = 0.77 \) (EtOAc); 1H NMR (400 MHz, CDCl\textsubscript{3}, 300 K) \( \delta \) 7.80–7.70 (m, 4H, 4x H-\textit{Ar}), 7.68–7.54 (m, 3H, 3x H-\textit{Ar}), 7.53–7.47 (m, 1H, 2x H-\textit{Ar}), 7.45–7.35 (m, 4H, 4x H-\textit{Ar}), 7.33–7.25 (m, 2H, 2x H-\textit{Ar}), 7.24–7.19 (m, 4H, 4x H-\textit{Ar}, Na\textsubscript{A}), 6.68 (d, \( J = 8.3 \) Hz, 1H, NH Leu), 5.57 (d, \( J = 8.0 \) Hz, 1H, NH Glu), 5.35 (dd, \( J = 10.2, 5.3 \) Hz, 1H, H-\textit{NtMeSecPh}), 5.19 (d, \( J = 16.5 \) Hz, 1H, -CH\textsubscript{2}Fmoc), 4.43 (t, \( J = 6.3 \) Hz, 1H, -OCH\textsubscript{2}Fmoc), 4.41 (dd, \( J = 10.2, 7.2 \) Hz, 1H, -OCH\textsubscript{2}CH- Fmoc), 4.36–4.28 (m, 2H, -OCH\textsubscript{2}CH-Fmoc, \( H-\text{tMeAsp} \)), 4.24 (t, \( J = 7.3 \) Hz, 1H, -OCH\textsubscript{2}CH-Fmoc), 3.42 (dd, \( J = 14.2, 5.6 \) Hz, 1H, H-\textit{Phme}), 3.19 (dd, \( J = 14.2, 7.1 \) Hz, 1H, H-\textit{Phme}), 3.04 (qd, \( J = 7.1, 3.9 \) Hz, 1H, H-\textit{Phme}), 1.41 (s, 9H, tBu), 1.15 (d, \( J = 7.3 \) Hz, 3H, \( \text{CH}_{3}\text{-MeAsp} \)), 1.01 (d, \( J = 6.9 \) Hz, 3H, \( \text{CH}_{3}\text{-MeAsp} \)).

Fmoc-\( \beta\text{-MeAsp(OrBu-Phe-OMe)} \) (5a). Boc-Phe-\textsubscript{OPh}\textsubscript{17} 8a (120 mg, 313 \( \mu \)mol) was deprotected according to GP4. The obtained H-Phe\textsubscript{OPh} and Fmoc-\( \beta\text{-MeAsp-OrBu-Ph-Phe-OMe} \) 8b (140 mg, 329 \( \mu \)mol) were dissolved in DMF (3 mL). Peptide coupling was performed according to GP6 using HATU (125 mg, 329 \( \mu \)mol) and DIPEA (165 \( \mu \)L, 329 \( \mu \)mol). The crude product was purified by LCMS/MS using ES; \( m / z \) 851.3750; Found 851.3708; Anal. Calcd for C\textsubscript{24}H\textsubscript{27}NO\textsubscript{6}: C, 67.75; H, 5.37; N, 9.50; Found 67.69; 5.30; 9.55. The amino acid \( \beta\text{-MeAsp(OBz)} \) (34 \( \mu \)mol) was prepared from \( \beta\text{-MeAsp(OBz)} \) (120 \( \mu \)mol) as described in Ref. 8.

**References**


The crude product was purified by FC to give 15 (1.870 g, 72%) as a white solid. The analytical data for 15 were in agreement with the data published for its enantiomer.17,18 [α]D +17.5 (c 1.0, MeCN); 1H NMR (400 MHz, CDCl3, 300 K) δ 7.38–7.21 (m, 10H, Ar), 5.15 (d, J = 12.3 Hz, 1H, −COOCH−), 5.11 (d, J = 12.3 Hz, 1H, −COOCH−), 3.87 (d, J = 12.9 Hz, 1H, N−CH2−), 3.71 (d, J = 7.1 Hz, 5H, Ar−CH), 2.75 (dd, J = 15.6, 5.9 Hz, 1H, H−β), 2.67 (dd, J = 15.6, 7.2 Hz, 1H, H−β), 1.45 (s, 9H, t-Bu); 13C NMR (101 MHz, CDCl3, 300 K) δ 172.8 (C=O), 171.0 (C=O), 139.9 (C Ar), 135.9 (C Ar), 128.7 (C Ar), 128.5 (2C Ar), 128.4 (2C Ar), 128.4 (C Ar), 127.2 (C Ar), 118.1 (−(C(Ph)), 66.6 (−COOCH−), 58.0 (Cα), 52.2 (N−CH2−), 38.6 (C−β), 28.2 (3C, −C(Ph))).

(2R)-4-Benzyl-1-tert-butyl-N-benzyl-N-(9-phenylfluorenyl-9-y1)aspartate (15). To the solution of Bn-α-Asp(OBn)-OtBu (1.587 g, 4.274 mmol) in dry acetonitrile (47 mL) anhydrous K2PO3 (1.088 g, 5.192 mmol) and phenylfluorenyl bromide (1.324 g, 4.122 mmol) were added. The resulting heterogeneous mixture was mechanically stirred for 24 h at rt, filtered, and the solvent was removed. The crude product was purified by FC to give 15 (1.870 g, 72%) as a white solid. The analytical data for 15 were in agreement with the data published for its enantiomer.17 [α]D +25.0 (c 1.0, MeCN); 1H NMR (400 MHz, CDCl3, 300 K) δ 7.82 (d, J = 7.1 Hz, 2H, Ar), 7.74 (d, J = 7.5 Hz, 1H, Ar), 7.66 (d, J = 7.4 Hz, 1H, Ar), 7.60 (d, J = 7.5 Hz, 1H, Ar), 7.56 (d, J = 7.5 Hz, 1H, Ar), 7.47 (d, J = 7.0 Hz, 2H, Ar), 7.37 (d, J = 7.5, 1.0 Hz, H−α), 7.34–7.16 (m, 11H, Ar), 7.11–7.05 (m, 2H, Ar), 4.87 (d, J = 12.5 Hz, 1H, −COOCH−), 4.80 (d, J = 12.5 Hz, 1H, −COOCH−), 4.22 (d, J = 13.8 Hz, 1H, N−CH2−), 3.93 (dd, J = 10.9, 2.6 Hz, 1H, H−α), 3.85 (s, J = 13.9 Hz, N−CH2−), 2.61 (dd, J = 15.8, 10.9 Hz, 1H, H−β), 1.98 (dd, J = 15.9, 2.7 Hz, 1H, H−β), 1.08 (s, 9H, t-Bu); 13C NMR (101 MHz, CDCl3, 300 K) δ 171.3 (C=O), 171.2 (C=O), 147.7 (C Ar), 146.4 (C Ar), 143.8 (C Ar), 141.0 (C Ar), 140.3 (C Ar), 139.0 (C Ar), 136.0 (C Ar), 129.7 (2C Ar), 128.7 (2C Ar), 128.5 (2C Ar), 128.5 (2C Ar), 128.2 (2C Ar), 128.1 (C Ar), 128.0 (2C Ar), 127.7 (C Ar), 127.7 (C Ar), 127.4 (2C Ar), 127.3 (C Ar), 126.7 (C Ar), 126.0 (C Ar), 120.0 (2C Ar), 80.8 (−(C(Ph)), 79.7 (CPh), 66.0 (−COOCH−), 57.6 (Cα), 51.8 (N−CH2−), 34.4 (C−β), 27.8 (3C, −C(Ph))).

Anal. Calcld for C38H42N2O4: C, 80.76; H, 6.45; N, 2.30. Found: C, 80.58; H, 6.53; N, 2.46.

(2R,3S)-4-Benzyl-1-tert-butyl-N-benzyl-N-(9-phenylfluoren-9-yl)-3-methylaspartate (erythro-16). A solution of LHMDMS in THF (1.0 M, 8.25 mL, 8.25 mmol) was placed in a Schlenk flask under nitrogen atmosphere and cooled to −20 °C. First a solution of aspartic acid derivative 15 (2.0 g, 3.28 mmol in dry THF (15 mL) and then a solution of methyl iodide (707 µL, 11.35 mmol in dry THF (11 mL)) was added slowly. The mixture was stirred for 3 h at −20 °C and 30 min at room temperature and quenched with sat. aqueous NH4Cl (10 mL) and water (10 mL). The phases were separated and the aqueous phase was extracted with EtOAc (100 mL). The combined organic phases were washed with brine (1x), dried over Na2SO4, filtered, and the solvents were removed. The crude product was purified by FC to give erythro-16 (1.17 g, 57%) as a white solid: Rf = 0.4 (petroleum ether/ethyl acetate 1:3); [α]D +236.5° (c 0.8, CH2Cl2); 1H NMR (400 MHz, CDCl3, 300) δ 7.83–7.77 (m, 15H, Ar), 7.73 (d, J = 7.4 Hz, 1H, Ar), 7.62 (d, J = 7.5 Hz, 1H, Ar), 7.56 (d, J = 7.7 Hz, 1H, Ar), 7.47 (d, J = 7.0 Hz, 2H, Ar), 7.44–7.40 (m, 12H, Ar), 5.14 (d, J = 12.4 Hz, 1H, −COOCH−), 5.02 (d, J = 12.4 Hz, 1H, −COOCH−), 4.67 (d, J = 14.3 Hz, 1H, CH, N−Bn), 4.26 (d, J = 14.3 Hz, 1H, CH, N−Bn), 3.97 (d, J = 9.9 Hz, 1H, H−α), 2.75 (dq, J = 9.9, 7.0 Hz, 1H, H−β), 1.06 (s, 9H, t-Bu), 0.79 (d, J = 7.1 Hz, 3H, CH3).
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13C NMR (100 MHz, CDCl3, 300 K) δ 174.2 (−COOBn), 170.3 (−COOEtBu), 147.1 (C Ar), 146.0 (C Ar), 145.3 (C Ar), 142.2 (C Ar), 142.0 (2C Ar), 139.6 (C Ar), 136.2 (C Ar), 129.2 (2C Ar), 128.7 (C Ar), 128.6 (2C Ar), 128.4 (2C Ar), 128.3 (C Ar), 128.3 (C Ar), 128.2 (2C Ar), 128.2 (2C Ar), 128.0 (2C Ar), 127.7 (C Ar), 127.6 (C Ar), 127.5 (2C Ar), 127.4 (C Ar), 127.0 (C Ar), 126.7 (C Ar), 80.9 (−CH2(CH3)2), 80.5 (C Ph), 66.1 (CH2 Br), 64.0 (C α), 51.7 (CH3 Br), 42.6 (β), 21.7 (C CH3C); Anal. Calc'd for C14H14O N, Se: C 72.6, H 6.54, Se 20.87; Found: C 72.6, H 6.54, Se 20.87.

H-β-δ-MeAsp-OH, (3S)-β-methylaspartic Acid. The amino acid ethyl-16 (200 mg, 1.6 mmol) was suspended in methanol (2.84 mL) and 10% Pd/C catalyst (82 mg, wet, 53.76% water) was added. The reaction mixture was hydrogenated overnight at a slightly positive hydrogen pressure and filtered through a syringe filter. The filtrate was diluted 1:1 with aqueous HCl (0.1 M) and filtered twice through a syringe filter. The volatiles were removed and the residue was co-evaporated with EtOAc (1x) and toluene (2x). The resulting solid was treated with TFA (0.7 mL) according to GP4. The crude product was purified by HPLC (1% B isocratic over 10 min, tR = 4.0 min) to give the formic acid salt of H-β-δ-MeAsp-OH (40 mg, 85%). The optical rotation ([α]D was in agreement with published values for H-β-δ-MeAsp-OH ([α]D = 3.10 (C 0.05, 2 mL HCl)). A constant coupling constant of 9.0 Hz determined by 1H NMR (400 MHz, CDCl3, 300 K, 1H NMR, 1.48 (d, J = 7.2 Hz, 2H, Ar), 7.47 (7.5 Hz, 2H, H-β-δ-MeAsp-OH) as a white amorphous solid: 1H NMR (400 MHz, D2O, 300 K, pH > 14) δ 1.80 (m, 2H, H2 Ar), 4.88 (dd, J = 8.9, 7.2 Hz, 1H, H-γ-δ-MeAsp), 2.54 (s, 3H, CH3 Ala), 0.97 (d, J = 7.1 Hz, 3H, -CH3).

N-(tert-Butylocarbonyl)-O-prop-2-enyl-yl-tyrosine (Boc-Tyr(Pro)-OH) (18). 2-tert-Butylocarbonylamin-3-[(prop-2-enyloxy)-phenyl]-propionic acid propargyl ester (3633 g, 10.17 mmol) was dissolved in 1 M KOH solution in methanol (20.34 mL, 20.34 mmol) and sonicated at 2 h at 35 °C. The methanol was removed under reduced pressure. The residue was dissolved in H2O (30 mL) and the aqueous phase was washed with Et2O (2 × 30 mL). The Et2O extracts were combined, washed with brine, dried over MgSO4 and the volatiles were removed under reduced pressure. Product 18 was obtained as a white amorphous solid (31.9% yield) and used without further purification. The analytical data for 18 were in agreement with the published ones. 1H NMR (400 MHz, CDCl3) δ 7.12 (d, J = 8.5 Hz, 2H, Ar), 6.92 (d, J = 8.6 Hz, 2H, Ar), 4.92 (d, J = 7.0 Hz, 1H, NH), 4.92 (d, J = 2.4 Hz, 2H, -OCH2), 4.21 (t, J = 13.5 Hz, 3H, CH3 Ala), 3.14 (d, δ = 13.5, 5.2 Hz, 1H, H-β-Glu), 3.04 (d, δ = 13.8, 5.5 Hz, 1H, H-β-Glu); 2.51 (t, δ = 2.3 Hz, 3H, CH3-COMe), 1.42 (s, 9H, t-Bu).

Fmoc-γ-Glu(Ot-Bu)-N-MeSecPh-O-Ala-Leu-β-δ-MeAsp(Ot-Bu)-Phe-OH (21a). Tetrapeptide 4 (61 mg, 63 μmol) and dipetide 5b (50 mg, 72 μmol) were deprotected according to GP2 and GP3, respectively. The products 19 and 20a were dissolved in DMF (1 mL) and coupled according to GP6 using HATU (56 mg, 146 μmol) and collidine (25 μL, 188 μmol). The crude product was purified by FC (CH2Cl2/i-proH 99:1 to 95:5) to give 21a (49 mg, 60% as a white amorphous solid: Rf = 0.65 (CH2Cl2/i-proH 95:5), 0.65 NMR (600 MHz, DMSO-d6, 360 K) δ 8.22 (d, J = 8.0 Hz, 1H, NH), 7.94 (d, J = 7.3 Hz, 2H, Ar), 7.86 (d, J = 7.5 Hz, 2H, Ar), 7.69–7.66 (m, 4H, 3x H-α-Glu, 1H, H-β-Glu); 7.31 (d, J = 7.0 Hz, 1H, H-γ-Glu), 7.29–7.22 (m, 7H, Ar), 7.21–7.17 (m, 1H, Ar), 5.45 (d, J = 16.4 Hz, 1H, CH2 Pac), 5.42 (d, J = 16.4 Hz, 1H, CH2 Pac), 5.08–5.00 (b, 1H, H-α-NeMeSecPh), 4.72 (t, δ = 8.8, 5.0 Hz, 1H, H-α-Phe), 4.36–4.26 (m, 4H, H-δ-MeAsp, H-α-Ala, -OCH2-C-H-Fmoc), 4.32–4.12 (m, 2H, H-β-δ-MeAsp, H-γ-Glu-COMe), 4.03–3.98 (m, 1H, H-β-Glu), 3.52–3.45 (m, 1H, H-δ-MeAsp), 2.35 (d, δ = 14.1, 10.0 Hz, 1H, H-β-Phe), 1.68–1.58 (m, 1H, H-γ-MeSecPh), 1.37–1.35 (m, 1H, H-δ-MeSecPh), 0.99 (d, δ = 14.2, 9.4 Hz, 1H, H-β-Phe), 2.96–2.93 (m, 1H, H-β-MeAsp), 2.87 (bs, 3H, -NCH3), 2.42–2.34 (m, 2H, 2x H2 Y-Glu), 2.06–1.99 (m, 1H, H-β-Glu), 1.93–1.83 (m, 1H, H-β-Glu), 1.63–1.55 (m, H-γ-Leu), 1.53–1.48 (m, 2H, 2x H-β-Leu), 1.41 (s, 9H, t-Bu), 1.34 (s, 9H, t-Bu), 1.22 (d, δ = 6.8 Hz, 3H, CH3 Ala), 0.88 (d, δ = 7.1 Hz, 3H, CH3 MeAsp), 0.85 (d, δ = 6.6 Hz, 3H, 3xH-δ-Leu), 0.80 (d, δ = 6.5 Hz, 3H, 3xH-δ-Leu); HRMS (ESI-TOF) m/z: [M-H]⁺ Calc'd for C26H25N3O8Se 395.1354; Found: 395.1354.

Boc-Adda-γ-Glu(Ot-Bu)-N-MeSecPh-O-Ala-Leu-β-δ-MeAsp(Ot-Bu)-Phe-OH (22a). The hexapeptide 21a (100 mg, 77.0 μmol) was N-terminally deprotected according to GP3. The deprotected product was coupled with Boc-Adda-Oh 31e (31 mg, 70.0 μmol) according to GP6 using HATU (29 mg, 70.0 μmol) and collidine (28 μL, 210
μmol in DMF (1 mL). The crude product was purified by FC (95:5 CH₂Cl₂/1%PrOH, Rₑ = 0.55) to give phenyl glycosyl protected peptide 2a as a white amorphous solid. The phenyl glycosyl group of 2a (50 mg, 33.5 μmol) was removed according to GP2 and the crude product was purified by semipreparative RP-HPLC (gradient: 75–100% B in 20 min, tₑ = 18.4 min) to give 22b as a white amorphous solid (34 mg, 54% from 21c).

The 1H NMR spectrum (600 MHz) recorded at 300 K showed two sets of signals (ratio approximately 2:1) and peak broadening due to the presence of two rotamers of the N-methylated glu residue. The 1H NMR (600 MHz, CD₃OD, 300 K) δ 7.91 (d, J = 6.6 Hz, 1H, NH), 7.68 (b, 1H, NH), 7.50–7.49 (m, 2H, Ar), 7.30–7.21 (m, 7H, Ar), 7.21–7.14 (m, 6H, Ar), 6.27 (s, 1H, NH), 6.09 (d, J = 15.7 Hz, 1H, H-5 Adda), 5.48 (dd, J = 15.7, 6.6 Hz, 1H, H-4 Adda), 5.38 (d, J = 9.6 Hz, 1H, H-7 Adda), 5.12–5.05 (m, 1H, H-α NmesPh), 4.50–4.44 (m, 1H, H-α Phe), 4.32–4.28 (m, 1H, H-α Ala), 4.26–4.20 (m, 2H, H-β Phe, H-β NmesPh), 3.94–3.91 (m, 1H, H-β Meap), 2.88 (dd, J = 13.9, 8.8 Hz, 1H, H-β Phe), 2.83 (s, 3H, -NCH₃), 2.74 (dd, J = 14.0, 4.8 Hz, 1H, H-10 Adda), 2.66 (dd, J = 14.0, 7.3 Hz, 1H, H-10 Adda), 2.61–2.54 (m, 2H, H-2 Adda-H-8 Adda), 2.38–2.29 (m, 2H, 2xH-Glu), 2.03–1.97 (m, 1H, H-β Glu), 1.87–1.80 (m, 1H, H-10 Glu), 1.62–1.58 (m, 1H, H-β Leu), 1.56–1.52 (m, 3H, 3xH-6 Adda), 1.52–1.48 (m, 2H, H-β Leu), 1.40 (s, 9H, t-Bu), 1.38 (s, 9H, t-Bu), 1.23 (d, J = 7.1 Hz, 3H, CH₂, Ala), 1.05 (s, J = 7.0 Hz, 3H, 3xH-2 Adda), 0.97 (J = 6.8 Hz, 3H, 3xH-8 Adda), 0.90 (d, J = 7.1 Hz, 3H, CH₂, Meap), 0.86 (s, J = 6.6 Hz, 3H, 3xH-6 Leu), 0.82 (d, J = 6.5 Hz, 3H, 3xH-5 Leu), 0.77 (d, J = 10.2 Hz, 3H, 3xH-2 Leu), 0.55 (s, J = 5.5 Hz, 3H, 3xH-6 Leu), 0.46 (s, J = 7.0 Hz, 3H, 3xH-2 Leu), 0.45 (s, J = 10.2 Hz, 3H, 3xH-2 Leu), 0.43–0.42 (m, 2H, H-α Ala, H-α Mdha), 0.42–0.41 (m, 1H, H-3 Adda major rotamer), 0.40–0.40 (m, 1H, H-3 Adda major rotamer), 0.37 (d, J = 12.7 Hz, 1H, H-β NmesPh), 0.34–0.33 (m, 1H, H-β NmesPh), 0.32 (s, 3H, -NCH₃), 0.32–0.31 (m, 2H, H-β Phe-d₉-H-9 Adda), 0.31–0.29 (m, 2H, H-β Meap), 0.30 (s, 3H, H-β NmesPh), 0.29 (s, 3H, -NCH₃), 0.29 (d, J = 13.8, 9.2 Hz, 1H, H-β Phe-d₉), 0.28 (dd, J = 13.9, 7.3 Hz, 1H, H-10 Adda), 0.26 (dd, J = 13.9, 7.4 Hz, 2H, H-10 Adda-H-2 Adda), 0.25 (dq, J = 16.7, 6.7 Hz, 1H, H-8 Adda), 2.45–2.39 (m, 2H, H-γ Glu, H-γ Glu minor rotamer), 2.06–1.98 (m, 1H, H-β Glu minor rotamer) 1.95–1.87 (m, 1H, H-β Leu) 1.78–1.68 (m, 2H, H-γ Leu, H-γ Glu, H-γ Glu H₂O), 1.62 (s, 3H, 3xH-6 Adda), 1.61–1.51 (m, 1H, H-β Leu), 1.46 (s, 9H, t-Bu), 1.45 (s, 18H, 2xH-Bu), 1.42 (d, J = 7.7 Hz, 3H, CH₂, Ala), 1.11 (s, 3H, 3xH-2 Adda), 1.02 (d, J = 6.6 Hz, 3H, 3xH-6 Leu), 0.93 (d, J = 7.2 Hz, 3H, CH₂ Meap), 0.88 (d, J = 6.5 Hz, 3H, 3xH-Δ Leu) 13C NMR (151 MHz, CD₃OD, 300 K) δ = 171.1 (C, O=O), 175.2 (C, O=O), 172.4 (C, O=O), 172.5 (C, O=O), 172.6 (C, O=O), 171.8 (C, O=O). The 13C NMR spectrum (151 MHz, CD₃OD, 300 K) expressed the nucleoside core as follows: C₂⁻Glucose 1.53 (C, O=O), 1.52 (C, O=O) Glc, 3.68 (C, O=O), 3.43 (C, O=O), 2.75 (C, O=O), 2.02 (C, O=O), 1.48 (C, O=O), 1.23 (C, O=O). The 13C NMR spectrum of 21c was recorded as 23.4 (C, O=O), 23.0 & 13.7 (C, O=O). The 13C NMR spectrum of 21c was recorded as 23.4 (C, O=O), 23.0 & 13.7 (C, O=O). The 13C NMR spectrum of 21c was recorded as 23.4 (C, O=O), 23.0 & 13.7 (C, O=O). The 13C NMR spectrum of 21c was recorded as 23.4 (C, O=O), 23.0 & 13.7 (C, O=O). The 13C NMR spectrum of 21c was recorded as 23.4 (C, O=O), 23.0 & 13.7 (C, O=O).
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