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Bidirectional Macrocyclization of Peptides by Double Multicomponent Reaction

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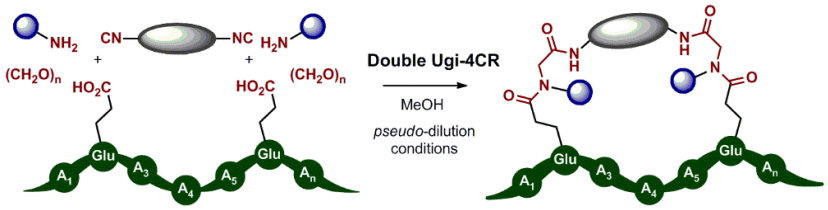
Graphical Abstract



Text: Peptide macrocyclization by multicomponent reactions

Abstract

Increasing the diversity of peptide cyclization methods is an effective way to access new types of macrocyclic chemotypes featuring a wide variety of ring sizes and topologies. Multicomponent reactions (MCRs) are processes capable to generate great levels of molecular diversity and complexity with low synthetic cost. In an endeavor to further exploit MCRs in the field of cyclopeptides, we describe a bidirectional multicomponent approach for the synthesis of *N*-alkylated macrocyclic peptides of varied sequences and cross-linking positions. The process relies on the execution of two Ugi reactions between peptide diacids and diisocyanides. Variation of the amino component enabled the installation of exocyclic elements of diversity, while skeletal diversity was created through different side chain and backbone cyclizations. This procedure shows promise for the rapid scanning of the chemical space of macrocyclic peptides for applications in chemical biology and drug discovery.

Keywords

Peptides, macrocycles, cyclic peptides, multicomponent reactions, macrocyclizations

Introduction

The introduction of conformational constraints in peptides by means of cyclization is a common strategy in the design of peptide ligands[1] and mimetics of protein epitopes.[2] Upon creating conformationally constrained peptide mimics, cyclization is among the most synthetically feasible ways to reduce the intrinsic flexibility of both the peptide backbone and the side chains.[3] This usually leads to an increased ligand binding affinity to the biological target, while improving the pharmacological properties compared to the acyclic parent.[4] The backbone preorganization derived from cyclization has also been extensively used to access peptidic templates for the appropriate arrangement of recognition elements[5]and to induce peptide secondary structures (α-helices, β-hairpins, etc.) in sequences not likely to have them.[6,7] As a result, cyclized peptides have been designed for a variety of applications ranging from the investigation of protein folding to the development of peptide-based therapeutic agents and materials.

Different synthetic procedures based on peptide coupling protocols[3,8] and chemoenzymatic[9] approaches are available for the head-to-tail cyclization of peptides. Nevertheless, these methods mostly focus on the formation of the natural amide bond, which has limited the biological/pharmacological evaluation of other types of chemical linkages. A different scenario shows up in the side chain-to-side chain cyclization, for which a variety of strategies leading to the incorporation of structurally varied linkages have been reported.[3,7,10] Thus, the formation of amide bonds between the side chains of Lys and Asp/Glu residues,[7,11] the Cu^{I}-catalyzed alkyne-azide1,3-dipolar cycloaddition,[12] the olefin metathesis with Grubbs catalysts[13] and the nucleophilic (aromatic) substitution[10,14] are among the most powerful approaches utilized by chemists to reduce the conformational space of peptides through side chain-to-side chain cyclization. In some cases, the covalent bridges derived from those processes have been introduced as surrogates of the natural disulfide linkage in the pursuit of more stable, but conformationally identical, analogues of bioactive peptide sequences.[7,12,15] These non-natural

linkages are also frequently introduced aiming at achieving by covalent means what nature does through non-covalent interactions, as it is the case of the stabilization of helical structures by linking the side chains of residues located at the *i*, *i* + 4 and *i*, *i* + 7 positions of the peptide sequence.[7,11,16]

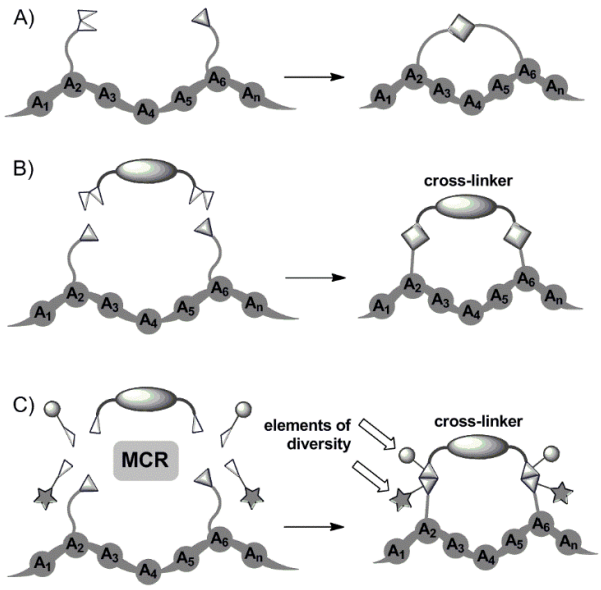


Figure 1. Schematic representation of A) unidirectional, B) bidirectional and C) bidirectional multicomponent macrocyclizations of peptide side chains (C is exemplified with a four-component reaction).

As shown in figure 1A, a common characteristic of these conventional methods is their unidirectionality, which derives from the utilization of two complementary functional groups that participate in a single reaction. A different approach is the utilization of a bidirectional macrocyclization strategy, in which a pair of amino acid side chains bearing the same functional groups (i.e, Cys, Lys and Asp/Glu) reacts doubly with a cross-linker bifunctionalized with reactive groups matching in reactivity with those of the peptide. This second strategy, exemplified in figure 1B, has been much less exploited than the unidirectional one, albeit it has rendered outstanding contributions to the field of folded peptides.[7,17] In terms of molecular design, the bidirectional strategy is perhaps more versatile, as it allows for the tunable variation of the structure and properties of the cross-linking moiety. Thus, excellent reports have described

the utilization of flexible,[18] linear rigid,[19] photoisomerizable[17,20] and near-infrared fluorescent[21] cross-linkers either to stabilize or derivatize folded peptides.

Despite of the advances in peptide cyclization methods, a key factor limiting the broader exploration of macrocyclic peptide scaffolds is their low structural diversity. During the construction of cyclic peptide libraries, the diversity elements are usually incorporated either prior or after the cyclization step. In this sense, the utilization of multicomponent reactions (MCRs) in peptide cyclization may offer a venue of opportunities, as they allow for the rapid generation of molecular diversity during the ring closing step.[22] Herein we describe a bidirectional multicomponent macrocyclization approach for the synthesis of structurally diverse *N*-alkylated macrocyclic peptides. Figure 1C illustrates the concept of a bidirectional macrocyclization based on two MCRs, which enable tethering a pair of side chains with a bifunctional cross-linker. We implement this idea not only for side chain-to-side chain, but also for the side chain-to-backbone and backbone-to-backbone macrocyclizations. In this work, the bidirectional macrocyclization relies on two Ugi four-component reactions (Ugi-4CRs) between peptide diacids and aromatic cross-linkers, eventually including exocyclic amino acid appendages as *N*-substituents, allover in one pot.

The Ugi-4CR is the condensation of a primary amine, an oxo compound (i.e., ketone or aldehyde), a carboxylic acid and an isocyanide. Despite of the fact that two of the reactive components are functional groups present in peptide termini and side chains, this reaction has been rarely utilized for peptide cyclization.[23] The reason for this may be the inherent slow kinetics of the Ugi-4CR under the high dilution typically used for macrocyclization and the frequent mismatch between the intrinsic folding of the peptide and the one required for the Mumm rearrangement to take place. Attempts to expand the substrate scope of the Ugi-4CR to the cyclization of peptoids (i.e., *N*-alkylated peptides) have demonstrated that even short peptoids can be effectively cyclized with this MCR,[24] possibly owing to the fact that the easier access to *s-cis* amide bonds facilitates the two termini to come closer. Alternatively, a modification of the

original Ugi-4CR by using amphoteric aziridine aldehydes has enabled the highly efficient and stereoselective head-to-tail cyclization of peptides of variable sizes.[25]

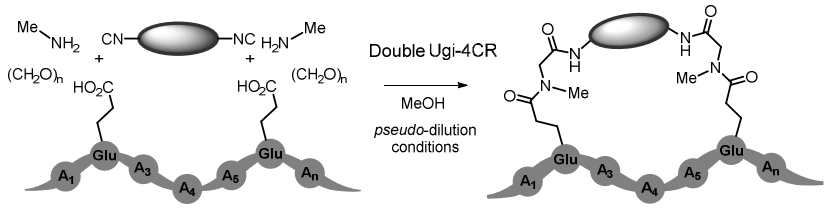
The potential of MCRs for the rapid generation of molecular complexity and diversity has been previously exploited in bidirectional macrocyclizations[22] based on double Ugi-4CRs[26] and well as Passerini,[27] Staudinger[27] and Zhu[28] three-component reactions. This strategy has been utilized for the one-pot synthesis of macrocycles incorporating natural scaffolds like steroids,[26] lipids,[29] carbohydrates[30] and nitrogenated heterocycles.[26a,30] However, to our knowledge neither the Ugi-4CR nor related MCRs have been utilized for the macrocyclization of peptide side chains. Based on our experience with a variety of substrates,[26-30] we anticipated that using flexible side chains like those of Glu and Lys would allow for the Ugi-4CR-based macrocyclization to proceed more efficiently than with the terminal amino and carboxylic groups.

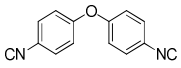
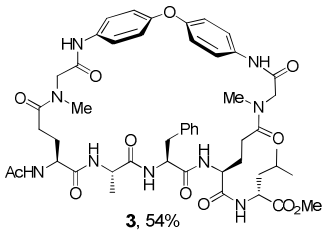
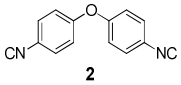
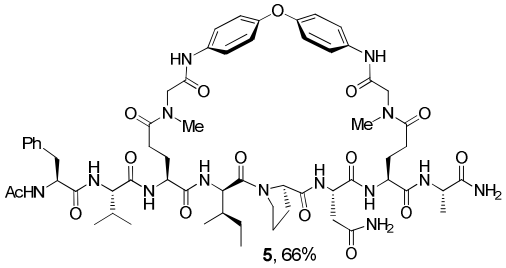
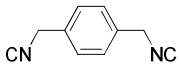
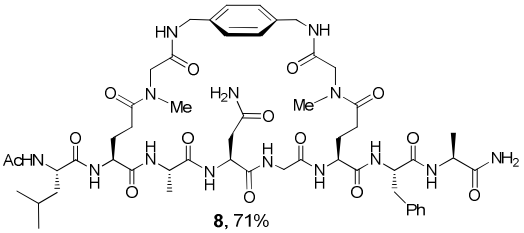
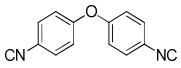
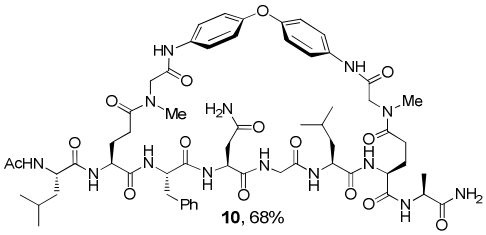
Results and Discussion

Table 1 illustrates the results of the bidirectional macrocyclization of peptide side chains by means of a double Ugi-4CR procedure, which leads to the formation of two parallel *N*-substituted dipeptide backbones as tethers of the cross-linker. The partial *N*-methylation,[31] and *N*-alkylation in general,[24] is known to be a successful way to improve the pharmacological properties of peptides. Important characteristics such as metabolic stability, membrane permeability and pharmacokinetics, are increased in *N*-alkylated peptides when compared to conventional ones.[31]Despite of the possible incorporation of two different elements of exocyclic diversity using a four component reaction (see Fig. 1C), we initially focused on the combination of methylamine and paraformaldehyde to seek the best experimental conditions. Peptides including two glutamic acids were produced either by standard solution or solid-phase protocols[32] (see Supporting Information), and then subjected to macrocyclization based on the diacid/diisocyanide combination. Aromatic cross-linkers were chosen as diisocyanide components because of their recognized capability to rigidify peptides upon macrocyclization.[10,19] Among them, we focused

on the utilization of the biarylether cross-linker, as this moiety frequently occurs in natural macrocyclic peptides like vancomycin, K-13, bouvardin, OF4949-III, and biphenomycin-A.[33]

Table 1. Side chain-to-side chain bidirectional macrocyclization of peptides by double Ugi-4CR.[a]



Peptide diacid	Diisocyanide	Macrocyclic Peptide[b]
Ac-Glu-Ala-Phe-Glu-Leu-OMe **1**	 **2**	 **3**, 54%
Ac-Phe-Val-Glu-Ile-Pro-Asn-Glu-Ala-NH_2 **4**	 **2**	 **5**, 66%
Ac-Leu-Glu-Ala-Asn-Gly-Glu-Phe-Ala-NH_2 **6**	 **7**	 **8**, 71%
Ac-Leu-Glu-Phe-Asn-Gly-Leu-Glu-Ala-NH_2 **9**	 **2**	 **10**, 68%

[a]Macrocyclizations carried out for 96 h under *pseudo*-dilution conditions comprising the slow addition of the twobifunctional components. [b]Yield of isolated pure product.

Peptides are commonly cyclized under high dilution conditions (0.01-0.1 mM), unless their intrinsic folding favors the ring closure as it is the case of peptides including reverse turns. Thus, large volume of solvents and long reactions times are required if a sufficient amount of the cyclic

peptide is wanted. A solution for this is the implementation of *pseudo*-dilution conditions in which a solution of the linear peptide is slowly added (usually by means of a syringe pump) to the reaction mixture. Nevertheless, in bidirectional macrocyclizations the synthetic design is even more elaborated, as the two different building blocks need to be under dilution to avoid formation of complex mixtures of (a)cyclic oligomers. In this sense, a proper understanding of the intrinsic reaction kinetics and the substrate characteristic (e.g., rigidity, folding, etc.) are crucial in the selection of the addition flow rate. For example, whereas cyclizations based on peptide coupling are usually completed within 24 h, those based on the Ugi-4CR require from 72 h to 96 h for ensuring a yield of isolated pure product higher than 50%.[26,30] Nevertheless, it must be noticed that eight new bonds are formed in a bidirectional Ugi-4CR-based macrocyclization, whilst either bidirectional peptide couplings[18] or nucleophilic substitutions[19] renders only two new bonds.

Based on these considerations, different macrocyclizations conditions – all based on the *pseudo*-dilution protocol – were tested aiming to reach a compromise between chemical efficiency and reaction time. The best results were obtained by the slow addition with syringe pumps of both the peptide diacid and the diisocyanide to a reaction mixture containing the preformed imine. Typically, two solutions, one of the peptide diacid and another of the diisocyanide (0.15 mmol in 10 mL of MeOH each), are simultaneously added (flow rate 0.2 $mL.h^{-1}$) to a methanolic solution of the imine (0.5 mmol, in 50 mL). Under these conditions, the addition was complete within 50 h, albeit the stirring was kept up for additional two days to ensure a conversion of at least 70%, as indicated by analytic HPLC analysis. As example, figure 2 shows the HPLC traces of the multicomponent macrocyclizations of peptides **6** and **9** with diisocyanides**7** and **2**, respectively, after 96 hours of reaction. This HPLC monitoring proved high conversion onto macrocycles **8** and **10**, while only a minor amount of the acyclic peptides **6** and **9** remained unreactive after this time. Thus, the general reaction time was fixed to 96 h to enable comparison of the macrocyclization outcomes when varying the different components.

As depicted in table 1, a variety of macrocyclic peptides featuring different sequences and cross-linking positions were obtained in good yields of isolated pure products. Although the goal of this article is not to study the peptide folding characteristics derived from macrocyclization, the synthetic design focused on locating the Glu residues at *i*, *i* + 3, *i*, *i* + 4 and *i*, *i* + 5 positions and thus addressing the influence of this variation on the macrocyclization efficiency. Interestingly, macrocycle **3** was obtained with the lowest yield among the fourside chain-to-side chain macrocylizations, while there were no great differences in the macrocyclization outcome of peptides having the Glu at *i*, *i* + 4 and *i*, *i* + 5 positions (i.e., **4**, **6** and **9**). A reason for this may be that the intrinsic folding of peptide **1** does not favor the proximity of the Ugi-reactive groups during the multicomponent ring closure. This is supported by the fact that the acyclic intermediate of compound **3** (i.e., the one derived from only one Ugi-4CR) was isolated in 13% yield, which suggest a slow final ring closure. In contrast, the intermediate of macrocycles **5, 8** and **10** were detected only in minor amounts by HPLC and ESI-MS analysis.

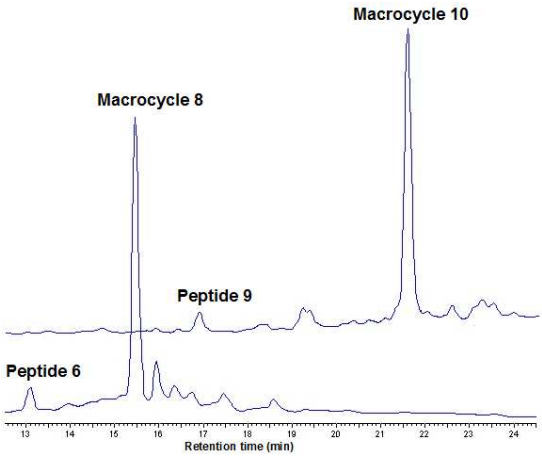


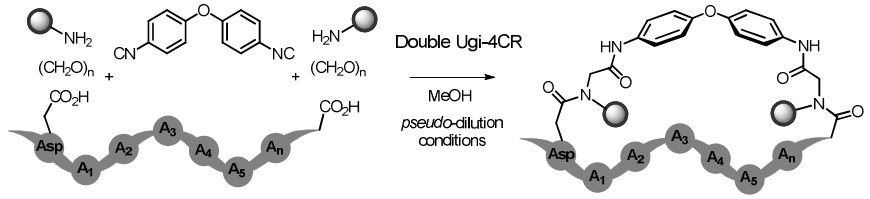
Figure 2. HPLC monitoring of the crude bidirectional macrocyclizations giving rise to macrocycles **8** and **10**. Gradient: 5%→60% of acetonitrile in 0.1% (v/v) TFA in water over 35 min at a flow rate of 0.8 mL/min.

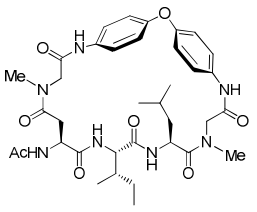
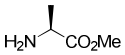
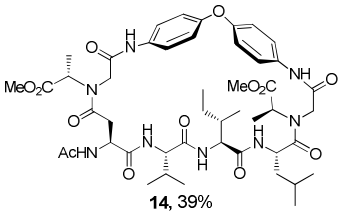
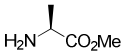
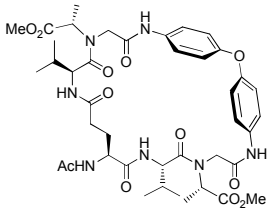
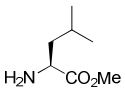
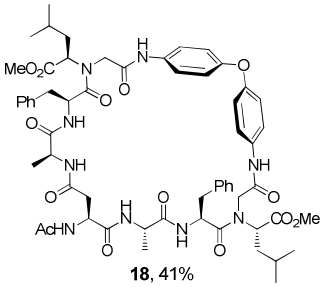
Besides of the variation of the amino acid sequence and the position for the side chain cross-linking, we were interested on exploiting the potential of MCRs for the generation of exocyclic diversity during the bidirectional macrocyclization. We also turned to address the scope of the

side chain-to-backbone and backbone-to-backbone cyclizations, once again relying on the efficient diacid/diisocyanide combination of building blocks. As shown in table 2, a variety of peptide diacids bearing the side chain and terminal carboxylic groupswere subjected to bidirectional macrocyclization with biaryl ether diisocyanide **2** and either methylamine or *C*-protected amino acids as amino components. Peptides were produced through typical solution-phase protocols,[32] relying on two different strategies: *i*) the incorporation of an Asp residue at the *N*-terminus (i.e., **11** and **13**) to enable the macrocyclization using the Asp side chain and the *C*-terminal carboxylic acids and *ii*) a bidirectional growing approach based on a double coupling of either an amino acid to *N*-protected Glu (i.e., **15**) or a peptide to *N*-protected Asp (i.e., **17**). This latter strategy enabled the assembly of bifunctional peptides bearing two *C*-terminal carboxylic acidsto be used for the bidirectional multicomponent macrocyclization. In all cases, peptides were produced according to the Boc/Bzl solution-phase protocol, then deprotected at both termini and capped by acetylation at the *N*-terminus (see Electronic Supplementary Information).

These chain-to-backbone and backbone-to-backbone macrocyclizations were carried out under the same reaction conditions that the side chain-to-side chain ones, and nonetheless their chemical efficiency was lower. The rationale of this might be found in the mechanism of the Ugi-4CR-based ring closure, which proceeds *via* a macrocyclic intermediate (i.e., the α-adduct) that evolves through an intramolecular acylation (Mumm rearrangement) to the final macrocycle. Thus, several factors may disfavor the final ring closing step, including: *i*) a high conformational constraint, *ii*) a poor proximity of the Ugi-reactive groups provoked either by a mismatch in size between the two building blocks or unfavorable peptide folding, and *iii*) a high steric hindrance at the migrating group in macrocyclic α-adduct.

Table 2. Side chain-to-backbone and backbone-to-backbone bidirectional macrocyclizations of peptides by double Ugi-4CR.[a]



Peptidediacid	Amine	MacrocyclicPeptide[b]
Ac-Asp-Ile-Leu-OH **11**	Me-NH_2	 **12**, 40%
Ac-Asp-Val-Ile-Leu-OH **13**		 **14**, 39%
Val-OH Ac-Glu-Val-OH **15**		 **16**, 44%
Ala-Phe-OH Ac-Asp-Ala-Phe-OH **17**		 **18**, 41%

[a]Macrocyclizations were carried out for 96 h under *pseudo*-dilution conditions comprising the slow addition of the two bifunctional components. [b]Yield of isolated pure product.

However, all macrocycles in table 2 were produced in about 40% yield, and in all cases the acyclic intermediates derived from one Ugi-4CR were isolated in 15-20%, regardless of the different length of the peptide diacids. Accordingly, we believe it is the low conformational flexibility of the peptide backbones – perhaps combined with an unfavorable folding – what

makes the final ring closing step difficult. This may also explain why the side chain-to-side chain multicomponent macrocyclization (Table 1) is rather more efficient, as the higher conformational freedom of the Glu side chains may enable the ring closure without a costly conformational change of the peptide backbone. To assess whether this is solely a kinetic problem or a deviation of the reaction course to different by-products, we carried out a parallel experiment with the synthesis of macrocycle **14** during 144 h. To our delight, the yield of isolated pure product **14** increased up to 67%, which proves that the macrocyclization efficiency can be improved with longer reaction times. On the other hand, the use of a higher concentration – derived from a faster flow rate addition of building blocks – is not recommended, as this leads to formation of complex mixtures of larger (a)cyclic oligomers.

Different from any other class of peptide macrocyclization method, this approach enables the double installation of exocyclic appendages as further elements of diversity during each of the Ugi-4CRs. As noticed in the structures of macrocycles **14**, **16** and **18**, two new amino acid residues were incorporated to the peptide sequence during the bidirectional macrocyclization. Thus, the approach allows not only for the introduction of conformational constraints but also for the enlargement of the peptide sequence in a one-pot procedure. In cases where the bidirectional macrocyclization is rather challenging, longer reaction times can be utilized to produce crude products of higher purity, thus enabling the rapid construction of macrocyclic peptide combinatorial libraries.

Conclusions

We have described a bidirectional multicomponent approach for the macrocyclization of peptides using the carboxylic groups either of the Glu and Asp side chains or of the *C*-terminus. The process comprises the execution of two Ugi-4CRs under *pseudo*-dilution conditions, which enabled the assembly of a variety of *N*-alkylated macrocyclic peptide featuring dissimilar sequences and cross-linking positions. The implementation of different side chain and backbone cyclizations enabled a rapid scanning of the topological space of hybrid biaryl ether-peptide

macrocycles, while provided useful information regarding the substrate scope and the optimized reaction conditions. In general, the side chain-to-side chain macrocyclizations took place with higher efficiency than the side chain-to-backbone and backbone-to-backbone macrocyclizations, whereas it was proven that yields can be improved with longer reaction times. An important difference between this method and other bidirectional approaches is the capability of installing elements of exocyclic diversity during the ring closing step. We believe this is a promising approach for the combinatorial production and screening of new chemotypes of peptidic macrocycles, as both the generation of structural diversity and macrocyclization are accomplished simultaneously, allover in a single synthetic operation.

Experimental Section

General

Fmoc-protected amino acids, MBHA resin, TBTU, EDC, and HOBt were obtained from Bachem (Switzerland). DIC was from Merck (Germany). Peptide synthesis grade DMF, dichloromethane, DIEA, TFA, and HPLC-grade acetonitrile were from Caledon (Canada). Synthesis of peptides **4**, **6** and **9** was carried out manually on MBHA resin by a stepwise solid-phase Fmoc strategy, while peptides **1**, **11**, **13**, **15**, and **17** were synthesized by a stepwise solution-phase Boc/Bzl strategy, as described in the Supplementary Information. Diisocyanides **2**[29] and **7**[30] were prepared as described previously. HPLC analysis was performed with a system AKTA 100 (GE Healthcare, USA) in a reverse-phase (RP) C18 column (Vydac, 4.6× 150 mm, 5μm). A linear gradient from 5% to 60% of solvent B in solvent A over 35 min at a flow rate of 0.8 mL/min was used. The preparative purification was performed on the HPLC system LaChrom (Merck Hitachi, Germany). Separation was achieved by RP C18 column (Vydac, 25 × 250 mm, 25 μm). A linear gradient from 15% to 45% of solvent B in solvent A over 50 min at a flow rate of 5 mL/min was used. Detection was accomplished at 226 nm. Solvent A: 0.1% (v/v) TFA in water. Solvent B: 0.05% (v/v) TFA in acetonitrile. Flash column chromatography was performed on silica gel 60

(Merck, >230 mesh). Peptidic macrocycles were purified to >95% either by column chromatography or by RP-HPLC and characterized by electrospray ionization mass spectrometry (ESI-MS) in a hybrid quadrupole-time-of-flight instrument (QTOF1,Waters, USA) fitted with a nanospray ion source. The high resolution ESI mass spectra were obtained from a Bruker Apex 70e Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with an Infinity™ cell, a 7.0 Tesla superconducting magnet. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury 400 spectrometer at 399.94 MHz and 100.57 MHz, respectively. Chemical shifts (δ) are reported in ppm relative to the TMS (^{1}H NMR) and to the solvent signal (^{13}C NMR).

General bidirectional macrocyclization by doubleUgi-4CRs: A solution of paraformaldehyde (0.5 mmol) and the amine (0.5mmol) in MeOH (20 mL) was stirred for 2 h at room temperature (when an amino acid methyl ester hydrochloride was used, 0.5 mmol of NEt_3 was added to enable formation of the corresponding imine). The reaction mixture was diluted up to 50 mL by addition of methanol (30 mL). Two solutions, one of the peptide diacid (0.15 mmol) and another of the diisocyanide (0.15 mmol) in 10 mL of MeOH each one, were simultaneously slowly added to the reaction mixture using syringe pumps (flow rate 0.2 $mL.h^{-1}$). After addition was complete, the reaction mixture was stirred for additional 48 h and then concentrated under reduced pressure. A mixture of water/acetonitrile (1:1, 10 mL) was added and the suspension is sonicated and centrifuged. After removal of the supernatant, the crude product was washed with diethyl ether (10 mL) and centrifuged twice, then suspended in water/acetonitrile (1:1, 5 mL) and lyophilized. The resulting product was further purified either by flash column chromatography (CH_2Cl_2/MeOH) or by preparative RP-HPLC to furnish the puremacrocyclic peptide.

Peptidic Macrocycle 3: Peptide **1** (98 mg, 0.15mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2{\cdot}HCl$ (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification

on silica (CH_2Cl_2/MeOH10:1) afforded the pure macrocycle **3** (78 mg, 54%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ= 9.14 (s, 1H), 9.00 (s, 1H), 7.49 (d, *J* = 8.6 Hz, 2H), 7.41 (d, *J* = 8.7 Hz, 2H), 7.33-7.18 (m, 5H), 6.89 (m, 4H), 6.75-6.69 (m, 2H), 6.52 (d, *J* = 6.2 Hz, 1H), 6.41 (m, 1H), 4.56-4.50 (m, 2H), 4.37 (m, 1H), 4.19 (m, 1H), 4.14 (m, 1H), 3.93 (d, *J* = 13.5 Hz, 1H), 3.91 (d, *J* = 13.6 Hz, 1H), 3.83 (d, *J* = 13.5 Hz, 1H), 3.79 (d, *J* = 13.6 Hz, 1H), 3.67 (s, 3H), 3.25 (dd, *J* = 13.1, 4.5 Hz, 1H), 3.03 (dd, *J* = 13.1, 5.9 Hz, 1H), 2.89 (s, 3H), 2.86 (s, 3H), 2.31-2.24 (m, 4H), 2.00 (m, 2H), 1.97 (s, 3H), 1.92 (m, 2H), 1.63 (m, 1H), 1.60 (m, 1H), 1.53 (m, 1H), 1.29 (d, *J* = 6.7 Hz, 3H), 0.91-0.58 (m, 6H).^{13}C NMR (100 MHz, $CDCl_3$) δ = 175.0, 173.4, 172.3, 171.8, 171.7, 170.9, 170.6, 169.7, 167.9 (CO), 156.9, 137.6, 133.5 (C), 129.4, 129.1, 127.3, 121.3, 120.9, 119.5,119.0, 57.7, 55.8, 55.1, 54.0 (CH), 52.6 (CH_3), 50.1 (CH), 45.8, 44.8, 41.9, 39.6 (CH_2), 38.2, 37.8 (CH_3), 34.1, 33.2, 30.3, 30.1 (CH_2), 26.0 (CH), 23.9, 23.7, 18.6 (CH_3). R_f = 0.45 (CH_2Cl_2/MeOH 15:1). HRMS (ESI-FT-ICR) *m/z*: 992.4497 $[M+Na]^+$; calcd. for $C_{49}H_{63}O_{12}N_9Na$: 992.4494.

Peptidic Macrocycle 5: Peptide **4** (144 mg, 0.15mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2$·HCl (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Purification by preparative RP-HPLC afforded the pure macrocycle **5** (125 mg, 66%) as awhite amorphous solid. R_t = 20.5 min. ^{1}H NMR (600 MHz, DMSO-d_6): δ = 10.02 (s, 1H), 9.67 (s, 1H), 8.12 (d, *J* = 7.7 Hz, 1H), 8.00 (d, *J* = 7.0 Hz, 1H), 7.93 (m, 1H), 7.83 (m, 2H), 7.53-7.44 (m, 4H), 7.40 (d, *J* = 6.7 Hz, 1H), 7.19-7.14 (m, 5H), 7.09 (d, *J* = 6.7 Hz, 1H), 6.95 (d, *J* = 7.0 Hz, 1H), 6.87 (m, 4H), 6.80 (d, *J* = 8.7 Hz, 1H), 4.54-4.44 (m, 2H), 4.38 (m, 1H), 4.27-4.19 (m, 2H), 4.05 (m, 1H), 3.91 (m, 1H), 3.58 (m, 1H), 3.06-2.95 (m, 2H), 2.86 (m, 3H), 2.76 (m, 3H), 2.70-2.56 (m, 2H), 2.32 (m, 1H), 2.20 (m, 1H), 1.88 (m, 1H), 1.79 (m, 1H), 1.74 (s, 3H), 1.39 (m, 1H), 1.19 (d, *J* = 7.1 Hz, 3H), 1.10 (t, *J* = 7.3 Hz, 2H), 0.84-0.59 (m, 12H). ^{13}C NMR (150 MHz, DMSO-d_6): δ = 174.3, 174.2, 173.9, 172.8, 172.6, 172.4, 171.7, 171.3, 170.8, 170.7, 169.2, 169.1 (CO), 158.3, 158.2, 153.1, 152.9, 138.0, 134.3, 134.2 (C), 129.1, 127.9, 126.1, 121.4, 121.0, 120.9, 119.2, 118.7, 118.5, 118.4,

54.5, 53.8, 52.9, 52.6, 52.3, 51.8, 51.7, 50.4, 49.5, 49.5, 48.4 (CH), 47.1, 45.7 (CH_2), 40.9, 40.6, 40.1 (CH_2), 37.2, 37.1 (CH_3), 36.8 (CH), 34.5, 31.3 (CH_2), 30.6 (CH), 29.7, 29.1, 28.9, 28.7, 27.5, 24.4, 24.1 (CH_2), 22.4 (CH_3), 22.1 (CH_2), 19.1, 18.1, 17.4, 14.9, 13.9, 10.7, 10.6, 8.6 (CH_3). ESI-MS *m/z*: 1287.40 $[M+Na]^+$; calcd. for $C_{62}H_{84}O_{15}N_{14}Na$: 1287.38.

Peptidic Macrocycle 8: Peptide **6** (134 mg, 0.15 mmol), diisocyanide **7** (24 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2{\cdot}HCl$ (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Purification by preparative RP-HPLC afforded the pure macrocycle **8** (121 mg, 71%) as a white amorphous solid. R_t = 15.5 min. ^{1}H NMR (600 MHz, DMSO-d_6): δ = 8.41 (m, 1H), 8.26 (m, 1H), 8.09 (d, *J* = 6.8 Hz, 1H), 8.00 (m, 1H), 7.97-7.91 (m, 2H), 7.90 (d, *J* = 6.8 Hz, 1H), 7.88 (d, *J* = 6.1 Hz, 1H), 7.78 (d, *J* = 7.8 Hz, 1H), 7.34 (d, *J* = 6.4 Hz, 1H), 7.22-7.09 (m, 9H), 7.03 (d, *J* = 5.5 Hz, 1H), 6.92 (s, 1H), 6.85 (m, 1H), 4.49-4.40 (m, 2H), 4.27-4.24 (m, 2H), 4.22-4.17 (m, 3H), 4.16 (m, 1H), 4.11 (m, 1H), 3.93 (dd, *J* = 13.2, 5.3 Hz, 1H), 3.90-3.83 (m, 2H), 3.67 (m, 1H), 3.52 (m, 1H), 3.00 (m, 1H), 2.90 (m, 3H), 2.76 (m, 3H), 2.50 (m, 1H), 2.42 (dd, *J* = 15.5, 6.4 Hz, 1H), 2.32 (m, 1H), 2.23 (m, 2H), 2.14 (m, 1H), 1.84 (m, 1H), 1.79 (s, 3H), 1.76 (m, 1H), 1.69 (m, 1H), 1.55 (m, 1H), 1.37 (m, 2H), 1.17 (m, 6H), 0.82 (m, 3H), 0.78 (m, 3H). ^{13}C NMR (150 MHz, DMSO-d_6):δ = 174.0, 172.5, 172.4, 172.4, 172.2, 172.1, 171.8, 171.8, 171.7, 171.4, 171.3, 171.2, 171.1, 170.5, 169.4, 168.8, 168.7, 168.4, 168.2, 168.1 (CO), 158.1, 157.8, 138.0, 137.9, 137.7, 137.4 (C), 129.2, 128.1, 127.3, 127.1, 126.9, 126.3, 53.9, 52.3, 51.8, 51.2, 51.1 (CH), 50.7, 50.6 (CH_2), 49.8, 48.3, 48.2 (CH), 42.5, 42.1, 41.7, 40.7, 37.1 (CH_2), 36.8, 36.4 (CH_3), 34.3, 28.7, 28.4 (CH_2), 27.6 (CH), 24.2, 22.9, 22.5, 21.6, 18.2, 18.1, 17.5 (CH_3). ESI-MS *m/z*: 1133.53 $[M+H]^+$, 567.25 $[M+2H]^{2+}$; calcd. for $C_{53}H_{77}O_{14}N_{14}$: 1133.57.

Peptidic Macrocycle 10: Peptide **9** (140mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2{\cdot}HCl$ (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Purification by preparative RP-HPLC afforded the pure macrocycle **10** (126 mg, 68%) as a white amorphous solid. R_t = 21.6 min. ^{1}H

NMR (600 MHz, DMSO-d_6): δ = 10.00 (s, 1H), 9.89 (s, 1H), 8.26 (d, *J* = 6.5 Hz, 1H), 7.99 (m, 1H), 7.91 (dd, *J* = 10.4, 6.5 Hz, 1H), 7.77 (m, 1H), 7.55 (m, 4H), 7.38 (d, *J* = 6.8 Hz, 1H), 7.26 – 7.13 (m, 5H), 6.90 (m, 4H), 4.53 (m, 1H), 4.25 (m, 1H), 4.19-4.14 (m, 2H), 4.09 (m, 1H), 4.01 (m, 1H), 3.78 (m, 1H), 3.03 (m, 3H), 2.84 (m, 3H), 2.41 (m, 1H), 2.32 (m, 1H), 1.88 (m, 1H), 1.81 (s, 1H), 1.57 (m, 2H), 1.46 (m, 1H), 1.37 (m, 2H), 1.19 (m, 3H), 0.90-0.76 (m, 12H). ^{13}C NMR (150 MHz, DMSO-d_6): δ = 174.1, 173.2, 172.7, 172.3, 172.0, 171.9, 171.1, 170.7, 169.3, 168.8, 167.3, 166.8 (CO), 157.8, 157.6, 153.2, 152.9, 137.5, 134.3, 134.1 (C), 129.2, 127.9, 126.2, 121.3, 121.0, 119.2, 119.0, 118.8, 118.7, 52.5, 51.9, 51.7, 51.1, 50.8, 50.2, 49.7, 48.0 (CH), 42.1, 40.8, 37.5, 37.3 (CH_2), 36.9, 36.8 (CH_3), 34.45, 28.8, 28.1, 27.5 (CH_2), 24.2 (CH), 23.0, 22.4, 21.5, 18.1 (CH_3). ESI-MS *m/z*: 1239.61 $[M+H]^+$, 620.28 $[M+2H]^{2+}$; calcd. for $C_{60}H_{83}O_{15}N_{14}$: 1239.60.

Peptidic Macrocycle 12: Peptide **11** (60 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2{\cdot}HCl$ (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification on silica (CH_2Cl_2/MeOH 12:1) afforded the pure macrocycle **12** (43 mg,40%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.12 (s, 1H), 8.99 (s, 1H), 7.65 (d, *J* = 7.4 Hz, 1H), 7.56 (d, *J* = 6.1 Hz, 1H), 7.48 (d, *J* = 8.5 Hz, 2H), 7.42 (d, *J* = 8.5 Hz, 2H), 6.88 (m,4H), 6.73 (d, *J* = 6.1 Hz, 1H), 4.57 (m, 1H), 4.54 (m, 1H), 4.27 (m, 1H), 3.90 (d, *J* = 13.5 Hz,2H), 3.81 (d, *J* = 13.5 Hz, 1H), 3.76 (d, *J* = 13.5 Hz,1H), 2.93 (s, 3H), 2.88 (s, 3H), 2.73 (dd, *J* = 14.2, 6.3 Hz, 1H), 2.66 (dd, *J* = 14.2, 3.3 Hz, 1H), 1.99 (s, 3H), 1.86 (m, 1H), 1.59 (m, 1H), 1.53-1.41 (m, 2H), 1.32 (m, 1H), 1.20 (m, 1H), 0.94-0.76 (m, 12H). ^{13}C NMR (40 MHz, $CDCl_3$) δ = 174.4, 173.1, 172.8, 172.1, 170.8, 169.5, 168.9 (CO), 157.5, 133.2 (C), 121.9,121.6, 120.0, 119.2, 61.2, 56.1, 53.8 (CH), 46.8, 46.5, 41.2, 40.8 (CH_2), 38.2, 37.5 (CH_3), 36.7 (CH_3), 26.4 (CH_2), 25.7 (CH), 24.2, 22.7, 16.1, 12.6 (CH_3). R_f = 0.48 (CH_2Cl_2/MeOH 15:1). HRMS (ESI-FT-ICR) *m/z*: 730.3543 $[M+Na]^+$; calcd. for $C_{36}H_{49}O_8N_7Na$: 730.3540.

Peptidic Macrocycle 14: Peptide **13** (75 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol), HCl·Ala-OMe(70 mg, 0.5 mmol) and Et_3N (70 μL, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification on silica (CH_2Cl_2/MeOH 12:1) afforded the pure macrocycle **14** (56 mg, 39%) as a white amorphous solid. In a parallel experiment comprising 144 h of reaction, macrocycle **14** was obtained in 67% yield (96 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.19 (s, 1H), 9.11 (s, 1H), 7.72 (d, *J* = 6.1 Hz, 1H), 7.53 (d, *J* = 8.6 Hz, 2H), 7.48 (d, *J* = 8.5 Hz, 2H), 7.45-7.31 (m, 2H), 6.91 (m, 4H), 6.50 (d, *J* = 5.3 Hz, 1H), 4.63 (m, 1H), 4.54 (m, 1H), 4.44 (q, *J* = 5.3 Hz, 1H), 4.37-4.29 (m, 2H), 4.18 (m, 1H), 3.94 (d, *J* = 14.0 Hz, 1H), 3.89 (d, *J* = 13.6 Hz, 1H), 3.82 (d, *J* = 14.0 Hz, 1H), 3.78 (d, *J* = 13.6 Hz, 1H), 3.69 (s, 3H), 3.67 (s, 3H), 2.72 (dd, *J* = 13.4, 6.8 Hz, 1H), 2.64 (dd, *J* = 13.4, 6.1 Hz, 1H), 2.03 (m, 1H), 1.98 (s, 3H), 1.82 (m, 1H), 1.59 (m, 1H), 1.48-1.40 (m, 3H), 1.39 (d, *J* = 7.0 Hz, 3H), 1.37 (d, *J* = 7.0 Hz, 3H), 1.28 (m, 1H), 1.15 (m, 1H), 0.98-0.89 (m, 9H), 0.82 - 0.73 (m, 9H). ^{13}C NMR (40 MHz, $CDCl_3$) δ = 174.0, 172.7, 172.5, 172.1, 171.6, 170.7, 170.5, 170.1, 169.4, 168.8 (CO), 156.6, 133.4 (C), 121.3, 120.9, 119.4, 119.2, 59.8, 57.1,54.7, 54.4 (CH), 52.6, 52.4(CH_3), 51.3, 49.8 (CH), 44.8, 44.4, 41.6, 40.9 (CH_2), 37.7, 31.5 (CH), 27.1 (CH_2), 25.8 (CH), 24.1, 22.6, 19.9, 17.1, 16.8, 15.7, 11.7 (CH_3).R_f = 0.50 (CH_2Cl_2/MeOH 15:1). HRMS (ESI-FT-ICR) *m/z*: 973.4645 $[M+Na]^+$; calcd. for $C_{47}H_{66}O_{13}N_8Na$: 973.4647.

Peptidic Macrocycle 16: Peptide **15** (58 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol), HCl·Ala-OMe (70 mg, 0.5 mmol) and Et_3N (70μL, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification on silica (CH_2Cl_2/MeOH 15:1) afforded the pure macrocycle **16** (55 mg,44%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.35 (s, 1H), 9.13 (s, 1H), 7.55 (d, *J* = 8.6 Hz, 1H), 7.49 (d, *J* = 8.6 Hz, 2H), 7.43 (d, *J* = 8.6 Hz, 2H), 6.85 (m, 4H), 6.70 (d, *J* = 7.1 Hz, 1H), 6.49 (d, *J* = 6.5 Hz, 1H), 4.37 (q, *J* = 6.0 Hz , 2H), 4.30-4.24 (m, 2H), 4.13 (m, 1H), 4.01 (m, 1H), 3.98 (d, *J* = 14.0 Hz, 1H), 3.96 (d, *J* = 13.5 Hz , 1H), 3.83 (d, *J* = 13.5 Hz ,

1H), 3.80 (d, *J* = 14.0 Hz, 1H), 3.69 (s, 6H), 2.33 (m, 2H), 2.12 (m, 2H), 1.97 (s, 3H), 1.94 (m, 2H), 1.48 (d, *J* = 6.9 Hz, 3H), 1.40 (d, *J* = 6.8 Hz, 3H), 0.96-0.88 (m, 12H).^{13}C NMR (100 MHz, $CDCl_3$)δ = 175.1, 173.1, 172.4, 171.3, 171.1, 169.1, 168.1 (CO), 156.7, 133.8 (C), 121.3, 120.7, 119.3, 119.2, 60.1, 58.6, 54.6, 52.6 (CH), 52.5(CH_3), 51.1 (CH), 45.8, 42.9, 34.2 (CH_2), 31.6, 31.2 (CH), 30.6 (CH_2), 23.4, 19.7, 19.2, 17.3, 16.9 (CH_3). R_f = 0.51 (CH_2Cl_2/MeOH 15:1). HRMS (ESI-FT-ICR) *m/z*: 860.3809 $[M+Na]^+$; calcd. for $C_{41}H_{55}O_{12}N_7Na$: 860.3806.

Peptidic Macrocycle 18: Peptide **17** (92 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol), HCl·Leu-OMe (91 mg, 0.5 mmol) and Et_3N (70μL, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification on silica (CH_2Cl_2/MeOH 10:1) afforded the pure macrocycle **18** (71 mg,41%) as a white amorphous solid.^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.23 (s, 1H), 9.16 (s, 1H), 7.95 (d, *J* = 5.8 Hz, 1H), 7.69 (d, *J* = 6.3 Hz, 1H), 7.49 (d, *J* = 8.6 Hz, 2H), 7.41 (d, *J* = 8.4 Hz, 2H), 7.34 (d, *J* = 7.10 Hz, 1H), 7.22-7.15 (m, 10H), 6.95 (m, 4H), 6.87 (d, *J* = 7.8 Hz, 1H), 6.42 (d,*J* = 6.5 Hz, 1H), 4.58 (m, 1H), 4.50 (m, 2H), 4.43 (m, 1H),4.39 (m, 1H), 4.29-4.23 (m, 2H), 3.97 (d, *J* = 13.2 Hz, 1H), 3.95 (d, *J* = 13.5 Hz, 1H), 3.84 (d, *J* = 13.2 Hz, 1H), 3.80 (d, *J* = 13.5 Hz, 1H), 3.70 (s, 3H), 3.68 (s, 3H), 3.11 (m, 2H), 2.82 (m, 2H), 2.63 (m, 2H), 2.00 (s, 3H), 1.65 (m, 2H), 1.60-1.51 (m, 2H), 1.33 (d, *J* = 7.1 Hz, 3H), 1.30 (d, *J* = 6.4 Hz, 3H), 0.95-0.89 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 174.0, 173.3, 173.2, 172.8, 170.6, 170.4, 168.7(CO), 157.3, 136.4, 133.9(C), 129.5, 128.6, 127.1, 121.9, 120.9, 120.2, 119.2, 58.1, 56.0, 55.4, 54.8, 53.7 (CH),52.5 (CH_3), 51.2, 49.6 (CH), 45.7, 45.2, 42.9, 40.5, 39.6, 39.5 (CH_2), 24.8 (CH), 22.7, 22.2, 18.0, 17.2 (CH_3).R_f = 0.47 (CH_2Cl_2/MeOH 15:1). HRMS (ESI-FT-ICR) *m/z*: 1168.5335 $[M+Na]^+$; calcd. for $C_{60}H_{75}O_{14}N_9Na$: 1168.5331.

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Electronic Supplementary Information: Experimental procedures and spectroscopic data of peptide acids. ESI-MS and HPLC chromatograms of the final peptidic macrocycles.

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