Journal of Peptide Science
J. Peptide Sci. **8**: 418–430 (2002)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.397

Studies on the Synthesis of Cyclic Pentapeptides as LHRH Antagonists and the Factors that Influence Cyclization Yield

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Received 2 February 2002
Accepted 18 March 2002

Abstract: Six cyclic pentapeptides containing two or three non-protein amino acids have been synthesized by cyclization of linear precursors in dilute solution and characterized by TLC, HPLC, NMR, melting point, specific rotation etc. A total of 72 cyclization reactions were carried out to study the factors that influence head-to-tail cyclization: linear precursor sequence, coupling reagent, residue configuration, the proportion of DMAP additive, concentration, reaction temperature and reaction time. The cyclic pentapeptides will be modified by active moieties and evaluated as LHRH antagonists. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: LHRH antagonist; cyclic pentapeptide; cyclization; coupling reagent; 2-chlorotrityl chloride resin

INTRODUCTION

LHRH was isolated and identified from the hypothalamic neurons of sheep and pigs in 1971 [1,2]. Since then more than 5000 LHRH analogues have been synthesized. Most of these analogues are antagonists. The search among them has been for highly active, and long-lasting LHRH analogues with low side effects which could be used for the therapy of hormone-dependent cancer and birth control, and some have been found that showed high antagonistic activity [3,4]. Until now, most of the LHRH antagonists investigated have been linear peptide analogues with poor water solubility, instability in the body and have the disadvantage of causing histamine release [5–7]. Moreover, the study of the relationship between structure and bioactivity is complicated by the flexibility of such linear peptides [8–10]. Small cyclic peptides (cyclic pentapeptides and cyclic hexapeptides) have better stability to enzymatic degradation and it is easy to study the structure–bioactivity relationship because of their restricted conformations [11]. We believe that the study of small cyclic peptide LHRH analogues will be of future importance.

In the synthesis of homodetic cyclic peptides, the readiness of a linear peptide precursor to cyclize depends on the size of the ring to be closed. Usually, no difficulties arise from the cyclization of free linear peptides containing seven or more amino acid residues [12]. However, the cyclization of pentapeptides is often difficult because cyclodimerization to a

Abbreviations: Aph, β-(4-aminophenyl)-alanine; BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate; DEPBT, 3-(diethoxyphosphoryloxy)-1,2,3- benzotriazin-4-(3*H*)-one; EDCI·HCl, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride; Nal, β-(2-naphthyl)-alanine; Pal, β-(3-pyridyl)-alanine; Phg, phenylglycine;

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cyclodecapeptide can occur easily [13,14]. An effective method to prevent cyclodimerization is to close the linear peptide precursor in highly dilute solutions (10^{-3}–10^{-4} M) [15,16]. The cyclization reaction may take several hours or several days to complete unless a highly effective coupling reagent, such as HATU, HBTU etc, is used. Prolonged existence of the activated carboxyl component increases the possibility of racemization of the *C*-terminal residue. The development of reliable and convenient methods for the synthesis of cyclic pentapeptides has therefore been a continuing concern.

Head-to-tail cyclizations are carried out in highly dilute solution [17]. In principle, two different approaches are employed for the cyclization of linear precursors. In one approach the activation step and ring formation are separated by applying active ester activation [18–21]. The alternative approach causes the free linear peptide to be cyclized with the help of coupling reagents in a 'one-pot' method [17]. For our investigation, we chose the second approach in order to simplify the synthesis protocol.

In this paper, we report and discuss the synthesis of cyclic pentapeptides as LHRH antagonists (Table 1) and the factors that influenced the cyclization yield.

RESULTS AND DISCUSSION

Synthesis and Physical Properties of Cyclic Pentapeptides

Several problems occurred while cyclizing the linear pentapeptides in our programme. The target cyclic pentapeptides contained two or three unnatural amino acid residues, and their large side chains inhibited ring formation. Further, the crude products often contained numerous by-products and could only be purified by HPLC.

A series of cyclic pentapeptides were prepared by various methods (Table 1). The structures and purity of the cyclic pentapeptides were characterized by FAB-MS (or/and ESI-MS), NMR, TLC, HPLC, melting point, specific optical rotation [α] etc.

The TLC R_f values of cyclic pentapeptides containing Aph(Boc) residues (**1, 3, 5, 6**) were obviously different from the cyclic pentapeptides containing D-Aph(Boc) residues (**2**, **4**). It was suggested that the desired cyclic pentapeptide could be separated from the epimerization product via TLC. For example, cyclo-(Trp(Boc)-Phg-Arg(Tos)-Aph(Boc)-D-Ala) could be separated from cyclo-(Trp(Boc)-Phg-Arg(Tos)-Aph(Boc)-Ala). Therefore, it can be inferred that the by-products of epimerization have been removed from the desired cyclic pentapeptides.

Table 1 The Cyclic Pentapeptides Synthesized in this Work

No.	Cyclic pentapeptide
1	Cyclo-(Trp(Boc)-Phg-Arg(Tos)-Aph(Boc)-D-Ala)
2	Cyclo-(Trp(Boc)-Phg-Arg(Tos)-D-Aph(Boc)-D-Ala)
3	Cyclo-(D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala)
4	Cyclo-(D-Nal-Phg-Arg(Tos)-D-Aph(Boc)-D-Ala)
5	Cyclo-(D-Pal-Phg-Arg(Tos)-Aph(Boc)-D-Ala)
6	Cyclo-(Cit-Arg(Tos)-Aph(Boc)-D-Ala-Trp(Boc))

The Factors that Influence Cyclization Results

A total of 72 cyclization reactions were carried out in order to study the factors that influence head-to-tail cyclization results. The factors included sequence (i.e. using different linear precursors of the same cyclic pentapeptides), coupling reagents, configuration, DMAP additive proportion, concentration, temperature and reaction time.

Influence of linear peptide precursors. The linear precursors sequence and the coupling reagent choice are two of the most important considerations that govern the success or failure of a cyclization reaction [22,23]. The cyclic pentapeptide **3** was chosen as a model for an initial investigation of the significance of the linear precursor sequence. All five linear precursors of cyclic pentapeptide **3** were cyclized by the EDCI/DMAP method, our initially preferred cyclization method, because it combined strong activating power with good solubility of the resultant urea and the products were generally easy to purify.

After the coupling reaction, the mixture was washed with aqueous citric acid and Na_2CO_3 as described in the experimental section. The residue was first purified by preparative TLC (eluant: $CHCl_3$/MeOH = 9 : 1) and then by HPLC on a semi-preparative silica column (eluant: $CHCl_3$/MeOH = 20 : 1) to yield pure cyclic pentapeptide. The cyclization yields were based on the product isolated by HPLC (Table 2).

The best cyclization yields were obtained when the linear precursor H-D-Nal-Phg-Arg(Toc)-Aph(Boc)-D-Ala-OH (**3-a**) was cyclized. The cyclization yield

Table 2 Synthesis of **3** from Different Linear Precursors with EDCI/DMAP

No.	Linear pentapeptide precursor	Yield (%)[a]
3-a	H-D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	9
3-b	H-D-Ala-D-Nal-Phg-Arg(Tos)-Aph(Boc)-OH	4
3-c	H-Aph(Boc)-D-Ala-D-Nal-Phg-Arg(Tos)-OH	5
3-d	H-Arg(Tos)-Aph(Boc)-D-Ala-D-Nal-Phg-OH	8
3-e	H-Phg-Arg(Tos)-Aph(Boc)-D-Ala-D-Nal-OH	<1

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

from H-Arg(Tos)-Aph(Boc)-D-Ala-D-Nal-Phg-OH (**3-d**) was almost the same as that from **3-a**. The lowest cyclization yield was obtained from the linear precursor **3-e**. The yields from linear precursors **3-b** and **3-c** were moderate.

The steric bulk of the amino acid residue at the *C*-terminal apparently played a more important role in the cyclization yield than that of the *N*-terminal. The side chains of Aph(Boc), Arg(Tos) and D-Nal are much larger than those of Phg and D-Ala. In precursor **3-a**, a small D-Ala residue was placed at the *C*-terminal and large D-Nal residue was at *N*-terminal, while in precursor **3-b**, a large Aph(Boc) residue was placed at the *C*-terminal and a small D-Ala residue was placed at the *N*-terminal. The cyclization yield of precursor **3-a** was higher than that of **3-b**. Similar results could be obtained after comparison of cyclization yield of **3-d** with that of **3-e**.

It can be concluded that amino acid residues with bulky side chains should not be placed at the *C*-terminal in the sequence chosen for the linear precursor peptide in the planning of cyclopentapeptide synthesis in this class.

Three linear precursors (**5-a, 5-b, 5-d**) of cyclic pentapeptide **5** were cyclized in DCM/DMF (9 : 1) solution (10^{-4} M) (Table 3). The solubility of the linear precursors of **5** was much lower than that of **3**, and the solvent for the cyclization reaction therefore had to contain more than 10% DMF. Coupling reagents and other reaction conditions for the synthesis of **5** were similar to those of cyclic pentapeptide **3**.

The structure of cyclic peptide **5** was similar to that of **3** except that the D-Nal residue in **3** was replaced by D-Pal in **5**. It could be predicted that the precursors of **3** and **5** would have similar cyclization results. Precursors **5-a** and **5-d**, in which D-Ala and Phg were placed at the *C*-terminal respectively, were

Table 3 Synthesis of **5** from Different Linear Precursors

No.	Linear pentapeptide precursor	Reagent	Yield (%)[a]
5-a	H-D-Pal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	EDCI/DMAP	11
5-b	H-D-Ala -D-Pal-Phg-Arg(Tos)-Aph(Boc)-OH	EDCI/DMAP	3
5-d	H-Arg(Tos)-Aph(Boc)-D-Ala-D-Pal-Phg-OH	HBTU/DMAP	3
5-a	H-D-Pal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	HBTU/DMAP	5

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

cyclized in order to determine the best precursor for cyclization. The cyclization yield of **5-a** was a little higher than that of **5-d** when HBTU/DMAP was used as the coupling reagent. Further, for linear precursor **5-a**, EDCI/DMAP (yield 11%) was a better coupling reagent than HBTU/DMAP (yield 5%).

Knowing that the precursors that contained large amino acid residues at the *C*-terminal inhibited ring formation, it was not necessary to investigate all the possible precursors with large amino acid residues (Aph(Boc), D-Aph(Boc), D-Nal, Arg(Tos)) at the *C*-terminal. We chose precursor **5-b** with Aph(Boc) at the *C*-terminal as a model to confirm the influence the amino acid residue side-chain bulk at the *C*-terminal on ring formation.

It was found that **5-a** was a better linear precursor than **5-b** for ring closure when EDCI/DMAP was used as a coupling reagent. Precursor **5-b** was not suitable for cyclization because of side-chain steric bulk at the *C*-terminal position. The low cyclization yield of precursor **5-b** provided more evidence that large amino acid residues at the *C*-terminal inhibited ring closure. A large amount of precipitate, which proved to be a by-product was also formed in the cyclization of **5-b**.

There is a significant structural difference when comparing the cyclic pentapeptides **1** and **6** with **3** and **5**. Only one D-amino acid residue (D-Ala) is present in **1** and **6**. The configuration sequence of the amino acid residues of **3** and **5** is D-L-L-L-D, while the configuration sequence of **1** and **6** is L-L-L-L-D.

Three precursors (**1-a**, **1-b**, **1-e**) of cyclic pentapeptide **1** and two precursors (**6-a**, **6-e**) of cyclic pentapeptide **6** were cyclized under the same conditions as those used for the synthesis of cyclic

Table 4 Synthesis **1** and **6** from Different Linear Precursors with EDCI/DMAP

No.	Linear pentapeptide precursor	Yield (%)[a]
1-a	H-Trp(Boc)-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	26
1-b	H-D-Ala -Trp(Boc)-Phg-Arg(Tos)-Aph(Boc)-OH	12
1-e	H-Phg-Arg(Tos)-Aph(Boc)-D-Ala-Trp(Boc)-OH	7
6-a	H-Trp(Boc)-Cit-Arg(Tos)-Aph(Boc)-D-Ala-OH	22
6-e	H-Cit-Arg(Tos)-Aph(Boc)-D-Ala-Trp(Boc)-OH	5

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

pentapeptide **3**. The results are listed in Table 4. The highest yields obtained for precursor **1-a** and **6-a** were 26% and 22%, respectively. Moreover, the purity of the crude products **1** and **6** from precursors **1-a** and **6-a** respectively was much higher than that from other precursors. This result was consistent with the results in the synthesis of cyclic peptides **3** and **5**. In short, the precursor with a small amino acid residue at the *C*-terminal position facilitated ring formation.

Five different precursors of **1** or **6** with configuration sequences as indicated gave yields as follows:

1-a (26%) or **6-a** (22%) : L-L-L-L-D

1-b (12%) : D-L-L-L-L

1-e (7%) or **6-e** (5%) : L-L-L-D-L

The amino acid residue Aph(Boc) at the *C*-terminal position had very large steric bulk which inhibited ring formation. As a result, the cyclization yield from precursor **1-b** was much lower than that from **1-a**. The lower cyclization yield from precursor **6-e** could be explained in view of its configurational sequence and steric bulk. The large side chain of Trp(Boc) inhibited ring formation. In the linear pentapeptide containing only one D-amino acid residue, the precursor with a D-amino acid residue at the *C*-terminal position underwent ring formation much more easily than the precursor with a D-amino acid residue at the middle position.

In conclusion, the optimal precursors for synthesis of cyclic pentapeptides **1**, **3**, **5** and **6** had similar primary structure properties with respect to the *C*-terminal residue. The D-Ala residue, which was placed at the *C*-terminal position, facilitated ring formation, and resulted in the highest yield.

Further proof of these principles was obtained with the synthesis of cyclic pentapeptide **4**. Precursors of **4** were cyclized with coupling reagent HBTU/DMAP. The cyclization reactions were carried out in DCM/DMF (10 : 1) solution with the concentration of linear peptide at 3×10^{-4} M. After the cyclization reaction, the mixture was washed and purified by preparative TLC and then by HPLC. The cyclization results are summarized in Table 5.

The steric bulk of the Phg side chain is greater than that of D-Ala, but the cyclization of precursor **4-d** in which Phg was at the *C*-terminal position nevertheless provided a better yield than that of **4-a** in which D-Ala was at the *C*-terminal position. The different results were presumably caused by the different conformations of the linear precursors.

Table 5 Synthesis of **4** from Different Linear Precursors with HBTU/DMAP

No.	Linear pentapeptide precursor	Yield (%)[a]
4-a	H-D-Nal-Phg-Arg(Tos)-D-Aph(Boc)-D-Ala-OH	2
4-d	H-Arg(Tos)-D-Aph(Boc)-D-Ala-D-Nal-Phg-OH	19
4-e	H-Phg-Arg(Tos)-D-Aph(Boc)-D-Ala-D-Nal-OH	trace

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

Table 6 Cyclization Results with Linear Pentapeptides having Different Configurational Sequences with EDCI/DMAP

No	Linear pentapeptide precursor	Configurational sequence	Yield (%)[a]
1-a	H-Trp(Boc)-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	L-L-L-L-D	26
3-a	H-D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	D-L-L-L-D	9
5-a	H-D-Pal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	D-L-L-L-D	11

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

Further studies of the influence of amino acid configurations and their sequences. As indicated above, a higher yield was obtained from the cyclization of precursor **1-a** than from the cyclization of precursor **3-a** (or **5-a**) (Table 6). The only configurational difference between **1-a** and **3-a** (or **5-a**) is in their *N*-terminal residues. Precursor **1-a** contained an L-amino acid residue, Trp(Boc), at the *N*-terminal, but precursor **3-a** or **5-a** contained a D-amino acid residue, D-Nal or D-Pal, at the *N*-terminal. Even though the side chain (Trp(Boc)) of **1-a** is much larger than that of D-Nal (**3-a**) or D-Pal (**5-a**), it was much easier to cyclize.

It was the orientation of the *N*-terminal side chain, not the size, that led to the different cyclization results in this case. The orientation of the *N*-terminal side chain (i.e. the configuration of the *N*-terminal residue) is a significant factor governing the success or failure of a cyclization.

A linear precursor with the configurational sequence L-L-L-L-D was an easier substrate for head-to-tail cyclization than the linear precursor with the configurational sequence D-L-L-L-D if the *C*-terminal residues of the two precursors were the same. Precursors of cyclic pentapeptide **3** or **5** contained three L-amino acid residues and two D-amino acid residues; while precursors of cyclic pentapeptide **1** contained four L-amino acid residues and one D-amino acid residue.

Cyclization yields : L-L-L-L-D(**1-a** 26%)

> D-L-L-L-D(**3-a** 9%, **5-a** 11%)

For the linear pentapeptide containing three D-amino acid residues, precursor L-D-D-D-L was easier to cyclize than precursor D-L-L-D-D if the amino acid residue at the *C*-terminal position was not very large. In precursor **4-d**, two *L*-amino acid residues were at the *N*- and *C*-terminal and the three D-amino acid residues were in the middle position. The cyclization yield of **4-d** was much better than that of **4-a** (Table 5).

L-D-D-D-L (**4-d** 19%) > D-L-L-D-D (**4-a** ¡ 2%)

The influence of different coupling reagents. Many reagents have been reported for the synthesis of cyclic peptides [24–29]. EDCI, BOP, HBTU and DEPBT were used in our work with additives such as HOSu, HOBt or DMAP.

The cyclic pentapeptide first successfully obtained was **3**, by cyclizing precursor H-Arg(Tos)-Aph(Boc)-D-Ala-D-Nal-Phg-OH (**3-d**) with EDCI/ HOSu. In order to search for better coupling reagents, EDCI/HOBt, EDCI/DMAP and BOP/HOBt were used respectively in the synthesis (Table 7). Precursor **3-d** was cyclized in DCM/DMF (100 : 1) solution (10^{-4} M) with different coupling reagents. The attempt to cyclize precursor **3-d** failed when BOP/HOBt was used. The best result among the reagents explored was obtained when EDCI/ DMAP was used. Moreover, the crude residue in that case, after washing with aqueous citric acid and Na_2CO_3, contained the smallest amount of by-products.

Table 7 Cyclization Yields from **3-a**, **3-b**, **3-d**, **6-a** with Different Coupling Reagents

No.	Linear pentapeptide precursor	Reagent	Yield (%)[a]
3-a	H-D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	EDCI/DMAP	9
3-a	H-D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	EDCI/HOSu	5
3-b	H-D-Ala-D-Nal-Phg-Arg(Tos)-Aph(Boc)-OH	EDCI/DMAP	5
3-b	H-D-Ala-D-Nal-Phg-Arg(Tos)-Aph(Boc)-OH	EDCI/HOBt	4
3-d	H-Arg(Tos)-Aph(Boc)-D-Ala-D-Nal-Phg-OH	EDCI/DMAP	8
3-d	H-Arg(Tos)-Aph(Boc)-D-Ala-D-Nal-Phg-OH	EDCI/HOBt	6
3-d	H-Arg(Tos)-Aph(Boc)-D-Ala-D-Nal-Phg-OH	EDCI/HOSu	5
3-d	H-Arg(Tos)-Aph(Boc)-D-Ala-D-Nal-Phg-OH	BOP/HOBt	trace
6-a	H-Trp(Boc)-Cit-Arg(Tos)-Aph(Boc)-D-Ala-OH	EDCI/DMAP	22
6-a	H-Trp(Boc)-Cit-Arg(Tos)-Aph(Boc)-D-Ala-OH	EDCI/HOSu	19

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

The yield of pure product from the EDCI/HOBt cyclization was higher than from the EDCI/HOSu cyclization. However, the crude residue contained more by-products when EDCI/HOBt was used than when EDCI/HOSu was used even after washing. The by-products may arise from side reactions between HOBt and DCM under basic conditions; they could not be completely removed by washing with aqueous citric acid and Na_2CO_3 [30]. The cyclization yield from precursor **3-d** with different coupling reagents

was found to be in the following order:

EDCI/DMAP > EDCI/HOBT
> EDCI/HOSu > BOP/HOBt.

Similar results were obtained with precursors **3-a** (H-D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH) and **6-a** (H-Trp(Boc)-Cit-Arg(Tos)-Aph(Boc)-D-Ala-OH). Moreover, a better yield was obtained with EDCI/DMAP than with EDCI/HOBt when the cyclization of precursor **3-b** was carried out. EDCI/DMAP has proved to be a better cyclization reagent than EDCI/HOSu, BOP/HOBt, EDCI/HOBt and BOP/HOBt for ring formation of **3-a**, **3-b**, **3-d** and **6-a**.

Linear pentapeptide **2-a** was cyclized by DEPBT which gave the best results and HBTU/DMAP (Table 8). DEPBT was developed by our research group [31,32]. It has been successfully used for the synthesis of linear peptides by solid-phase or solution methods with a remarkable resistance to racemization [33]. In addition, DEPBT has been proved to be a very good reagent for the synthesis of cyclic peptides [34,35].

It is interesting to compare the reaction progress and the results of cyclization of precursor **5-a** and **3-a** when applying EDCI/DMAP and HBTU/DMAP reagents (Tables 9 and 10). In the cyclization of **5-a** using the strongly activating reagent HBTU/DMAP, only traces of linear precursor were detectable by TLC after a reaction time of 1 h. However, the amount of the desired cyclic pentapeptide changed only slightly after that time and a substantial amount of by-product formation was evident. The by-product may be a polymer, judging by its much lower solubility than the cyclic pentapeptide. Ring closure of precursor **5-a** with EDCI/DMAP afforded a better purity of crude product and a higher yield of pure cyclic pentapeptide.

Table 8 Cyclization Yields from **2-a** (H-Trp(Boc)-Phg-Arg(Tos)-D-Aph(Boc)-D-Ala-OH) with Different Coupling Reagents

Reagent	Yield (%)[a]
DEPBT	22
HBTU/DMAP	12

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

Table 9 Cyclization Yields from **5-a** (H-D-Pal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH) with Different Coupling Reagents

Reagent	Yield (%)[a]
EDCI/DMAP	11
HBTU/DMAP	5

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

Table 10 Cyclization Yields from **3-a** (H-D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH) with Different Coupling Reagents

Reagent	Yield (%)[a]
HBTU/DMAP	12
EDCI/DMAP	9

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

However, the cyclization yield from precursor **3-a** with HBTU/DMAP was better than with EDCI/DMAP (Table 10). The influence of different coupling reagents on the cyclization yields was dependent on the structure of the *N*-terminal amino acid residue.

The configurational sequences of the two precursors (**5-a** and **3-a**) are the same: D-L-L-L-D. The structures of **5-a** and **3-a** are the same except for the *N*-terminal residues. The main differences between the *N*-terminal residues are in their side chain bulk and polarity. The side chain of the D-Nal residue in precursor **3-a** has larger bulk but lower polarity than that of the D-Pal residue. The side chain of the D-Nal residue is hydrophobic, while that of D-Pal is hydrophilic. It could be inferred that HBTU/DMAP is a more suitable reagent than EDCI/DMAP for the cyclization of a linear D-L-L-L-D precursor in which the side chain of the *N*-terminal residue is a large hydrophobic moiety. However, EDCI/DMAP is a more suitable reagent than HBTU/DMAP for the cyclization of a linear D-L-L-L-D precursor in which the side chain of the *N*-terminal residue is a hydrophilic moiety.

The influence of added DMAP. The cyclization of precursor **6-a** was carried out using EDCI/DMAP with different amounts of DMAP additive. The best cyclization yield (30%) was obtained when the molar ratio between EDCI and DMAP was 5 : 2 with a pH

Table 11 Cyclization Yields from **6-a** (H-Trp(Boc)-Cit-Arg(Tos)-Aph(Boc)-D-Ala-OH) with Different between EDCI : DMAP Ratios

EDCI : DMAP	Yield (%)[a]
5 : 2	30
1 : 1	22

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

of 7.5 (Table 11). A lower yield (22%) was obtained when the molar ratio between EDCI and DMAP was 1 : 1 (pH 8.5). Adding more DMAP led to racemization of the *C*-terminal residue. In addition, excessive DMAP caused the crude product to become yellow. Therefore, the molar ratio between EDCI and DMAP should not be lower than 5 : 2.

HBTU/DMAP was used for cyclization of precursor **4-a** with different amounts of DMAP additive. In contrast to experience with EDCI/DMAP, the HBTU/DMAP method needed more DMAP (HBTU/DMAP = 2 : 3), the optimal pH value (8.5) for the HBTU/DMAP method was higher than the optimal value (7.5) for the EDCI/DMAP method.

The influence of concentration. Precursor **6-a** (H-Trp(Boc)-Cit-Arg(Tos)-Aph(Boc)-D-Ala-OH) was cyclized by EDCI/DMAP at different concentrations of linear peptide. The best cyclization results were obtained when the concentration of **6-a** was 10^{-4} M (Table 12). Similar results were obtained when other linear precursors were cyclized.

Highly dilute conditions are usually employed in the cyclization of linear peptide precursors in solution. However, there has been no report about the exact optimal concentration. According to the literature, the concentrations of linear precursors are usually about 10^{-3} M with concentrations of coupling reagents at $1–5 \times 10^{-3}$ M. In our synthesis, the linear precursor concentrations were usually in the range of $1–3 \times 10^{-4}$ M. A decreased concentration of linear precursors would diminish the cyclodimerization. On the other hand, higher concentrations of a coupling reagent would increase the chance of the linear peptide and coupling reagent coming together. This increased chance would favour the cyclization reaction. Procedures with lower concentrations of linear precursors (10^{-4} M) and higher concentrations of coupling reagents ($3–6 \times 10^{-3}$ M) were therefore used in our work.

A stepped concentration protocol was also adopted. The linear precursor concentration was usually 10^{-4} M and the concentration of the coupling reagent was in the range of 6×10^{-4} M–3×10^{-3} M, but after a while, the solution was gradually concentrated.

Table 12 Cyclization Yields from **6-a** with EDCI/DMAP at Different Concentrations

Concentration of linear pentapeptide (M)	Yield (%)[a]
1×10^{-4}	30
3×10^{-4}	16

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

The influence of reaction temperature. The cyclization of precursor **3-a** (H-D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH) was carried out with HBTU/DMAP at varied temperatures. It was found that a decreased reaction temperature could improve the product purity and increase the cyclization yield. A yield of 8% was obtained when the reaction was carried out at room temperature for 20 h, while a yield of 12% was obtained when the reaction solution was stirred at −15 °C for 2 h, and then stirred at 0 °C for 2 h and at room temperature for 20 h (Table 13).

A protocol of gradually increasing the reaction temperature was developed for our cyclization reactions: it proved effective and resulted in improved yields. At the outset, the mixture was stirred at −15 °C for 2 h, and then allowed to

Table 13 Reaction Times for EDCI/DMAP and HBTU/DMAP Cyclizations of **3-a** at Different Reaction Temperatures

Reagent	Time at −15 °C	Time at 0 °C	Time at r.t.	Yield (%)[a]
EDCI/DMAP	4 days	2 days	2 days	9
HBTU/DMAP	2 h	2 h	20 h	12
HBTU/DMAP	—	—	20 h	8

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

attain room temperature gradually. A decreased reaction temperature could prevent racemization of the *C*-terminal residue. A lower reaction temperature in the beginning is especially important in order to increase the cyclization yield for the HBTU/DMAP method since HBTU is a strongly activating reagent.

This protocol was also effective when EDCI/DMAP was used for cyclization. The reaction was allowed to run at −15 °C for about 4 days and then at 0 °C for 2 days. Finally the temperature was increased to room temperature and the reaction solution was stirred for another 2 days.

The influence of reaction time at low temperature. The reaction time in the synthesis of cyclic pentapeptide varied according to different linear precursors and coupling reagents. Usually, the reaction was stopped when no linear precursor was detectable by TLC.

It was very interesting to analyse the crude product of cyclic pentapeptide **1** from precursor **1-a** (H-Trp(Boc)-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH) when two reactions by EDCI/DMAP method were carried out for different reaction times at −12 °C.

In the first reaction (Reaction A), the reaction mixture was kept at −12 °C for 4 days, and then continued at room temperature as usual. In the second reaction (Reaction B), the mixture was kept at −12 °C for 30 days, and then continued at room temperature as usual. It was found that the crude product from Reaction B was purer than that from Reaction A. When the crude product from Reaction B was analysed by TLC, only one by-product spot near the desired cyclic pentapentide spot was observed. This by-product could be removed by purification on preparative TLC. More by-product spots were observed in the crude product from Reaction A. Moreover, the isolated yield by HPLC from Reaction B (32%) was higher than that from Reaction A (26%). In short, an increased time at low temperature improved the purity of the crude product and enhanced the yield.

The Best Synthetic Results for each Cyclic Pentapeptide

The best results for the synthesis of each cyclic pentapeptide are summarized in Table 14. The best cyclization yield was obtained by cyclization of linear precursors with D-Ala or Phg at the *C*-terminal. Three of them were obtained by the method of EDCI/DMAP, two of them were obtained by HBTU/DMAP, and one was obtained by DEPBT. Of all the cyclic pentapeptides, the yield of **1** was the highest and the yield of **5** was the lowest.

CONCLUSION

Six cyclic pentapeptides, which will be modified in later work with active groups, have been synthesized, purified by HPLC and characterized by TLC, HPLC, NMR, melting point and specific rotation.

The cyclic pentapeptide by-product, which was formed as a result of racemization of the *C*-terminal residue, could be completely removed by preparative TLC.

Although each cyclic pentapeptide has its most suitable linear precursor, some regularities were found. For the *C*-terminal residue of the linear precursor, steric bulk plays an important role. Of all the linear precursors, those with the smaller D-Ala or Phg as the *C*-terminal residue were usually the easiest to cyclize. Lower yields were obtained when the precursors had the larger D-Nal, Trp(Boc), Aph(Boc) or Arg(Tos) residues at the *C*-terminal

Table 14 The Best Results for Each Cyclic Pentapeptide

Cyclic pentapeptide	Linear precursor	Reagent	Yield (%)[a]
1	H-Trp(Boc)-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	EDCI/DMAP	32
2	H-Trp(Boc)-Phg-Arg(Tos)-D-Aph(Boc)-D-Ala-OH	DEPBT	22
3	H-D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	HBTU/DMAP	12
4	H-Arg(Tos)-D-Aph(Boc)-D-Ala-D-Nal-Phg-OH	HBTU/DMAP	19
5	H-D-Pal-Phg-Arg(Tos)-Aph(Boc)-D-Ala-OH	EDCI/DMAP	11
6	H-Trp(Boc)-Cit-Arg(Tos)-Aph(Boc)-D-Ala-OH	EDCI/DMAP	30

[a] The yield of cyclic pentapeptide was based on the amount of product isolated by HPLC.

position. However, for the *N*-terminal residue; the orientation of the side chain of the *N*-terminal amino acid residue (i.e. residue configuration) is a dominant factor governing the success or failure of a cyclization, not its bulk.

HBTU/DMAP was found to be a suitable reagent for cyclization of the linear pentapeptide in which the side chain of the *N*-terminal residue is large and hydrophobic. DEPBT was a suitable reagent for cyclization when the configurational sequence was L-L-L-D-D. EDCI/DMAP has a wider range of applicability for the synthesis of cyclic pentapeptides and good cyclization results were obtained by EDCI/DMAP in all except a few cases. Moreover, the following order of cyclization efficiency for EDCI was found to be:

$$\text{EDCI/DMAP} > \text{EDCI/HOBt} > \text{EDCI/HOSu}.$$

The use of concentration and reaction temperature gradients was found to be effective for preventing racemization, decreasing by-products, and increasing cyclization yields. Running the reaction at a lower temperature for a long period of time was advantageous.

After comparison of all the cyclization results, it was found that the cyclic pentapeptides containing only one D-amino acid residue were easier to synthesize than those containing two or three adjacent D-amino acid residues. The linear precursor with the configurational sequence of L-L-L-D-D was easier to cyclize than that of D-L-L-D-D.

EXPERIMENTAL

General Data

Free linear pentapeptides were synthesized by SPPS on a 2-chlorotrityl resin using an Fmoc protecting strategy [36–39]. DCM was dried over K_2CO_3, and distilled over CaH_2. DMF was distilled in vacuum over ninhydrin to remove the free amino residues, and then dried over 4 Å Zeolite. Other reagents were used as purchased from commercial suppliers, without further purification. ^{1}H NMR spectra were obtained using a 500 MHz IR-ATS-MO/ Kernresonanz. FAB-MS were determined on a VG-ZAB-HS or a Bruker APEX™II. ESI-MS used a NanoESI. Specific rotation was measured with a Perkin Elmer MC-241. Melting points were obtained with a Yanaco micro melting point apparatus.

Analytical TLC was performed on precoated GF_{254} silica gel plates from Merck using UV detection at 365 nm. Preparative TLC plates were prepared in our own laboratory using GF_{254} silica gel.

HPLC purification of cyclic pentapeptides was performed on a silica column (6 μm HR silica column, 300 × 7.8 mm) with a Waters 600 E HPLC apparatus. UV detection wavelength on a Water 486 monitor was selected at 280 nm for cyclic pentapeptides **1**, **2**, **3**, **4**, **6** and 254 nm for cyclic pentapeptide **5**. Eluent: A ($CHCl_3$/MeOH = 25 : 1) for **2**; B ($CHCl_3$/MeOH = 22.5 : 1) for **4**; C ($CHCl_3$/MeOH = 20 : 1) for **1** and **3**; D ($CHCl_3$/MeOH = 9 : 1) for **6**; E ($CHCl_3$/MeOH/i – PrOH = 12.5 : 1 : 0.6) for **5**.

General Cyclization Procedure

With EDCI/DMAP. DCM/DMF (100 : 1, 500 ml) containing 0.05 mmol (1 eq) linear pentapeptide was cooled to −15 °C. EDCI (1.22 mmol, 240 mg, 25 eq) and DMAP (0.5 mmol, 61 mg, 10 eq) were added to the solution. The mixture was stirred at −15 °C for 2 h and then kept at a −15 °C for 2 days. EDCI (0.5 mmol, 96 mg, 10 eq) and DMAP (0.2 mmol, 24 mg, 4 eq) were added, and the mixture was kept at −15 °C for another 2 days, and kept at 0 °C for 2 days. After that, the mixture was stirred at room temperature for 2 days and then concentrated to 50 ml. After stirring at room temperature for 2 days, 50 ml DCM was added. The mixture was washed with 2% citric acid (2 × 50 ml), 2% Na_2CO_3(2 × 50 ml) and brine (5 × 50 ml), dried over Na_2SO_4, and evaporated. The residue was purified by TLC plate to obtain the crude product. Further purification by HPLC was carried out on a silica column.

With EDCI/HOSu. DCM/DMF (100 : 1, 300 ml) containing 0.03 mmol (1 eq) linear pentapeptide was cooled to −15 °C. EDCI (0.90 mmol, 173 mg, 30 eq), HOSu (1.2 mmol, 138 mg, 40 eq) and DIEA (1.8 mmol, 310 μl, 60 eq) were added. After stirring at −15 °C for 2 h, the mixture was kept at −15 °C for 2 days. Then, the mixture was kept at 0 °C for 2 days and stirred at room temperature for 2 days. After that, the mixture was concentrated to 30 ml and stirred at room temperature for 2 days. 30 ml DCM was added. The solution was washed and purified with the same method as the EDCI/DMAP method to give pure product.

With EDCI/HOBt. DCM/DMF (100 : 1, 300 ml) containing 0.03 mmol (1 eq) linear precursor, HOBt (0.3 mmol, 40 mg, 10 eq) and DIEA (0.6 mmol,

100 μl) were cooled at −15 °C and then EDCI (0.3 mmol, 58 mg, 10 eq) was added. The mixture was stirred at −15 °C for 2 h and then kept at −15 °C for 2 days. The solution was stirred at 0 °C for 2 h and then stirred at room temperature. Every 24 h, additional EDCI (5 eq) and HOBt (5 eq) were added. The pH was kept at 8–9 by addition of DIEA. After 7 days, the solvent was concentrated to 30 ml and stirring was continued at room temperature for 2 days. 30 ml DCM was added. The solution was washed and purified with the same method as the EDCI/DMAP method to give pure product.

With HBTU/DMAP. DCM/DMF (9 : 1, 100 ml) containing 0.03 mmol (1 eq) linear pentapeptide was cooled to −15 °C. HBTU (0.12 mmol, 45 mg, 4 eq) and DMAP (0.18 mmol, 22 mg, 6 eq) were added. The solution was stirred at −15 °C for 2 h and at room temperature for 8 h. Additional HBTU (0.06 mmol, 23 mg, 2 eq) and DMAP (0.09 mmol, 11 mg, 3 eq) were added to the mixture and stirred at room temperature for 12 h. The solution was concentrated to about 30 ml and stirred at room temperature for 8 h. After that, 30 ml DCM was added. The solution was washed and purified with the same method as the EDCI/DMAP method to give pure product.

With BOP/HOBt. DCM/DMF (100 : 1, 300 ml) containing 0.03 mmol (1 eq) linear precursor, HOBt (0.3 mmol, 40 mg, 10 eq) and DIEA (0.6 mmol, 100 μl) was cooled to −15 °C and treated with BOP (0.3 mmol, 133 mg, 10 eq). The mixture was stirred at −15 °C for 2 h and then kept at −15 °C for 24 h. After that, the solution was stirred at 0 °C for 5 h and then stirred at room temperature. Every 24 h, additional BOP (5 eq) and HOBt (5 eq) were added. The pH was kept at 8–9 by addition of DIEA. After 7 days, the solvent was concentrated to 30 ml and stirring was continued at room temperature for 2 days. 30 ml DCM was added. The solution was washed and purified with the same method as the EDCI/DMAP method to give pure product.

With DEPBT. DCM/DMF (100 : 1, 204 ml) containing 0.06 mmol (1 eq) linear pentapeptide (3×10^{-4} M) was cooled to −15 °C. DEPBT (0.3 mmol, 90 mg, 5 eq) and DIEA (0.48 mmol, 84 μl) were added to the solution. After stirring at −15 °C for 2 h and at 0 °C for another 2 h, the solution was stirred at room temperature. Every 2 days, 5 eq DEPBT was added. The pH was kept at 8 by addition of DIEA. After 6 days, the mixture was concentrated to 60 ml and stirring was continued at room temperature for 2 days. The solution was washed and purified with the same method as the EDCI/DMAP method to give pure product.

Characterization data

cyclo-(Trp(Boc)-Phg-Arg(Tos)-Aph(Boc)-D-Ala) (1). m.p. 246°–248 °C; $[\alpha]_D^{20} = -46°$ (c 0.1, $CHCl_3$/MeOH = 1 : 1); FAB-MS: $m/z = 1063(M+H)^+$, ESI-MS: $m/z = 1063.3$ $(M+H)^+$, 1085.3 $(M+Na)^+$, 1101.2 $(M+K)^+$; TLC R_f: 0.43 ($CHCl_3$/MeOH = 9 : 1); Retention time on HPLC: 8.52 min (eluent: $CHCl_3$/MeOH = 20 : 1, flow: 2 ml/min); ^{1}H NMR (DMSO, 500 MHz) δ 1.06 (d, $J = 6.3$ Hz, 3H, CH_2-β (D-Ala)), 1.19 (m, 2H, CH_2-γ (Arg)), 1.45 (s, 9H, $3 \times CH_3$ (Boc)), 1.61 (s, 9H, $3 \times CH_3$(Boc)), 1.64 (m, 2H, CH_2-β (Arg)), 2.33 (s, 3H, CH_3(Tos)), 2.71 and 2.89 (2d, $J = 10.0$ Hz, 2H, CH_2-β (Aph)), 2.96 (m, 2H, CH_2-δ (Arg)), 3.00 and 3.23 (2d, $J = 11.6$ Hz, 2H, CH_2-β (Trp)), 3.70 (m, 1H, CH-α (Arg)), 4.24 (t, $J = 7.2$ Hz, 1H, CH-α (D-Ala)), 4.40 (m, 1H, CH-α (Aph)), 4.48 (t, $J = 8.0$ Hz, 1H, CH-α (Trp)), 5.44 (d, 1H, CH-α (Phg)), 6.40–7.30 (broad, 3H, $3 \times$ NH (Arg)), 7.01–8.03 (t, d, s, 18H, arom CH(Trp, Tos, Phg, Aph)), 7.86 (d, $J = 9.2$ Hz, 1H, NH-α (Aph)), 8.06 (d, $J = 9.2$ Hz, 1H, NH-α (Arg)), 8.20 (d, $J = 8.0$ Hz, 1H, NH-α (Phg)), 8.53 (d, $J = 9.2$ Hz, 1H, NH-α (D-Ala)), 8.69 (d, $J = 9.2$ Hz, 1H, NH-α (Trp)), 9.17 (s, 1H, Ar-NH-CO (Aph)).

cyclo-(Trp(Boc)-Phg-Arg(Tos)-D-Aph(Boc)-D-Ala) (2). m.p. 265°–270 °C (dec); $[\alpha]_D^{20} = -17°$ (c 0.1, $CHCl_3$/MeOH = 1 : 1); FAB-MS: $m/z = 1063$ $(M+H)^+$, 1085 $(M+Na)^+$, ESI-MS: $m/z = 1063.2$ $(M+H)^+$; TLC R_f: 0.53 ($CHCl_3$/MeOH = 9 : 1); Retention time on HPLC: 7.85 min (eluent: $CHCl_3$/MeOH = 22.5 : 1, flow: 2 ml/min); ^{1}H NMR (DMSO, 500 MHz) δ 0.88 (d, $J = 6.4$ Hz, 3H, CH_2-β(D-Ala)), 1.01 (m, 2H, CH_2-γ (Arg)), 1.18 (s, 9H, $3 \times CH_3$(Boc)), 1.34 (m, 2H, CH_2-β (Arg)), 1.36 (s, 9H, $3 \times CH_3$(Boc)), 2.12 (s, 3H, CH_3(Tos)), 2.58 (d, $J = 8.6$ Hz, 2H, CH_2-β (D-Aph)), 2.72 (m, 2H, CH_2-δ (Arg)), 2.87 (d, 2H, CH_2-β (Trp)), 3.65 (m, 1H, CH-α (Arg)), 3.96 (m, 1H, CH-α (Ala)), 4.09 (m, 1H, CH-α (D-Aph)), 4.26 (m, 1H, CH-α (Trp)), 5.05 (d, $J = 8.2$ Hz, 1H, CH-α (Phg)), 6.40–7.30 (broad, 3H, $3 \times$ NH (Arg)), 6.80–7.38 (t, d, s, 17H, arom CH(Trp, Tos, Phg, D-Aph)), 8.10 (d, $J = 9.0$ Hz, 1, arom (Trp)), 7.51 (d, $J = 9.0$ Hz, 1H, NH-α (D-Aph)), 7.81 (d, 1H, NH-α (Arg)), 8.08 (d, $J = 8.2$ Hz, 1H, NH-α (Phg)), 8.17 (d, $J = 8.0$ Hz, 1H, NH-α (D-Ala)), 8.22 (d, $J = 9.2$ Hz, 1H, NH-α (Trp)), 8.98 (s, 1H, Ar-NH-CO (D-Aph)).

cyclo-(D-Nal-Phg-Arg(Tos)-Aph(Boc)-D-Ala) (3). m.p. 251°–254 °C; $[\alpha]_D^{20} = -24°$ (c 0.1, $CHCl_3$/MeOH = 1 : 1); FAB-MS $m/z = 996$ $(M + Na)^+$, ESI-MS: $m/z = 974.2$ $(M + H)^+$, 996.3 $(M + Na)^+$; TLC R_f: 0.50 ($CHCl_3$/MeOH = 9 : 1); Retention time on HPLC: 5.75 min (eluent: $CHCl_3$/MeOH = 30 : 1, flow: 3 ml/min); ^{1}H NMR (DMSO, 500 MHz) δ 0.92 (d, J = 6.2 Hz, 3H, CH_3-β (D-Ala)), 1.36 (m, 2H, CH_2-γ (Arg)), 1.45 (s, 9H, 3 × CH_3(Boc)), 1.57 (m, 2H, CH_2-β (Arg)), 2.30 (s, 3H, CH_3(Tos)), 2.76 and 2.85 (2d, J = 9.6 Hz, 2H, CH_2-β (Aph)), 3.06 (m, 2H, CH_2-δ (Arg)), 3.13 and 3.24 (2d, J = 10.2 Hz, 2H, CH_2-β (D-Nal)), 3.70 (m, 1H, CH-α (D-Ala)), 3.84 (d, J = 5.8 Hz, t, 1H, CH-α (Arg)), 4.35 (d, J = 8.6 Hz, t, 1H, CH-α (Aph)), 4.43 (m, 1H, CH-α (D-Nal)), 5.31 (d, J = 8.2 Hz, 1H, CH-α (Phg)), 6.40–7.30 (broad, 3H, 3 × NH (Arg)), 6.94–7.84 (t, d, s, 20H, arom CH(Aph, Phg, Tos, D-Nal)), 7.81 (d, J = 9.2 Hz, 1H, NH-α (Aph)), 8.17 (d, J = 10.2 Hz, 1H, NH-α (D-Nal)), 8.29 (d, J = 6.0 Hz, 1H, NH-α (D-Ala)), 8.76 (d, J = 8.2 Hz, 1H, NH-α (Phg)), 8.91 (d, J = 9.2 Hz, 1H, NH-α (Arg)), 9.17 (s, 1H, Ar-NH-CO (Aph)).

cyclo-(D-Nal-Phg-Arg(Tos)-D-Aph(Boc)-D-Ala) (4). m.p. 216°–218 °C; $[\alpha]_D^{20} = +18°$ (c 0.1, $CHCl_3$/MeOH = 1 : 1); FAB-MS $m/z = 996$ $(M + Na)^+$, ESI-MS: $m/z = 974.2$ $(M + H)^+$, TLC R_f: 0.40 ($CHCl_3$/MeOH = 9 : 1); Retention time on HPLC: 10.12 min (eluent: $CHCl_3$/MeOH = 20 : 1, flow: 2 ml/min); ^{1}H NMR (DMSO, 500 MHz) δ 1.10 (m, 2H, CH_2-γ (Arg)), 1.28 (m, 3H, CH_3-β (D-Ala)), 1.37 (m, 2H, CH_2-β (Arg)), 1.41 (s, 9H, 3 × CH_3(Boc)), 2.26 (s, 3H, CH_3(Tos)), 2.52 and 2.72 (2m, 2H, CH_2-β (D-Aph)), 2.91 (m, 2H, CH_2-δ (Arg)), 3.06 and 3.25 (2d, J = 10.6 Hz, 2H, CH_2-β (D-Nal)), 3.97 (m, 1H, CH-α (D-Ala)), 4.08 (m, 1H, CH-α (Arg)), 4.26 (m, 2H, CH-α (Aph, D-Nal)), 5.50 (d, J = 8.4 Hz, 1H, CH-α (Phg)), 6.40–7.30 (broad, 3H, 3 × NH (Arg)), 7.06–7.82 (t, d, s, 20 H, arom CH(D-Aph, Phg, Tos, D-Nal)), 7.85 (d, J = 8.0 Hz, 1H, NH-α (D-Aph)), 8.32 (d, J = 8.4 Hz, 1H, NH-α (D-Nal)), 8.39 (d, J = 7.9 Hz, 1H, NH-α (D-Ala)), 8.58 (d, J = 8.4 Hz, 1H, NH-α (Phg)), 9.10 (s, 1H, Ar-NH-CO (D-Aph)), 9.16 (d, J = 6.8 Hz, 1H, NH-α (Arg)).

cyclo-(D-Pal-Phg-Arg(Tos)-Aph(Boc)-D-Ala) (5). m.p. 196°–199 °C; $[\alpha]_D^{20} = -27°$ (c 0.05, $CHCl_3$/MeOH = 1 : 1); FAB-MS: $m/z = 925$ $(M + H)^+$, ESI-MS: $m/z = 925.2$ $(M + H)^+$; TLC R_f: 0.49 ($CHCl_3$/MeOH = 5 : 1); Retention time on HPLC: 6.58 min (eluent: $CHCl_3$/MeOH = 9 : 1, flow: 3 ml/min); ^{1}H NMR (DMSO, 500 MHz) δ 1.11 (d, J = 6.5 Hz, 3H, CH_3-β (D-Ala)), 1.17 (m, 2H, CH_2-γ (Arg)), 1.45 (s, 9H, 3 × CH_3(Boc)), 1.59 (m, 2H, CH_2-β (Arg)), 2.32 (s, 3H, CH_3(Tos)), 2.83 and 2.98 (2d, J = 7.0 Hz, 2H, CH_2-β (Aph)), 2.95 (m, 2H, CH_2-β (D-Pal), 2.98 (m, 2H, CH_2-δ (Arg)), 4.01 (m, 1H, CH-α (Arg)), 4.14 (m, 1H, CH-α (Pal)), 4.15 (m, 1H, CH-α (Ala)), 4.58 (m, 2H, CH-α (Aph)), 5.16 (d, J = 7.0 Hz, 1H, CH-α (Phg)), 6.40–7.30 (broad, 3H, 3 × NH (Arg)), 7.04–8.39 (t, d, s, 17H, arom CH(Aph, Phg, Tos, Pal)), 7.96 (d, J = 7.2 Hz, 1H, NH-α (D-Ala)), 8.05 (broad, 1H, NH-α (D-Pal)), 8.09 (broad, 1H, NH-α (Arg)), 8.35 (d, J = 7.5 Hz, 1H, NH-α (Aph)), 8.53 (d, J = 7.0 Hz, 1H, NH-α (Phg)), 9.21 (s, 1H, Ar-NH-CO (Aph)).

cyclo-(Cit-Arg(Tos)-Aph(Boc)-D-Ala)-Trp(Boc)) (6). m.p. 212°–214 °C; $[\alpha]_D^{20} = -32°$ (c 0.1, $CHCl_3$/MeOH = 1 : 1); FAB-MS $m/z = 1087$ $(M + H)^+$, 1109 $(M + Na)^+$, ESI-MS: $m/z = 1087.3$ $(M + H)^+$, 1109.2 $(M + Na)^+$; TLC R_f: 0.44 ($CHCl_3$/MeOH = 5 : 1); Retention time on HPLC: 8.33 min (eluent: $CHCl_3$/MeOH = 9 : 1, flow: 2 ml/min); ^{1}H NMR (DMSO, 500 MHz) δ 1.00 (m, 3H, CH_3-β(D-Ala)), 1.28 (m, 2H, CH_2-γ (Cit)), 1.35 (m, 2H, CH_2-γ (Arg)), 1.43 (s, 9H, 3 × CH_3(Boc)), 1.58 (s, 9H, 3 × CH_3(Boc)), 2.33 (s, 3H, CH_3(Tos)), 2.73 and 2.88 (2d, 2H, CH_2-β (Aph)), 2.96 (m, 2H, CH_2-δ (Cit)), 3.06 (m, 2H, CH_2-δ (Arg)), 3.00 and 3.23 (2d, J = 12.0 Hz, 2H, CH_2-β (D-Trp)), 3.75 (m, 1H, CH-α (Cit)), 4.10 (m, 1H, CH-α (Arg)), 4.18 (m, 1H, CH-α (Ala)), 4.38 (m, 2H, CH-α (Aph)), 4.42 (m, 1H, CH-α (Trp)), 5.38 (s, 2H, CO-NH_2(Cit)), 5.96 (t, 1H, NH-CO (Cit)), 6.40–7.30 (broad, 3H, 3 × NH (Arg)), 7.00–7.66 (t, d, s, 13H, arom CH(Trp, Aph, Tos)), 7.76 (d, J = 8.6 Hz, 1H, NH-α (Aph)), 7.80 (d, J = 8.6 Hz, 1H, NH-α (Cit)), 8.15 (d, J = 9.2 Hz, 1H, NH-α (Arg)), 8.39 (d, J = 9.4 Hz, 1H, NH-α (Ala)), 8.56 (d, J = 6.4 Hz, 1H, NH-α (Trp)), 9.18 (s, 1H, Ar-NH-CO (Aph)).

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