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On-resin Head-to-tail Cyclization of Cyclotetrapeptides: Optimization of Crucial Parameters

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Abstract: Cyclotetrapeptides are constrained cyclic peptides whose synthesis is considered a difficult task. A methodology based on on-resin head-to-tail cyclization by anchoring the side chain of a trifunctional amino acid was investigated. A series of model cyclotetrapeptides containing the RGD sequence cyclo(Xaa-Arg-Gly-Asp) (Xaa = Ala, Phe, Phg, D-Ala, D-Phe, D-Phg) was synthesized with no cyclodimerization by-products. An evaluation and optimization study of all of the parameters directly involved in the ring closure was performed. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Arg-Gly-Asp sequence; cyclization; cyclotetrapeptide; solid-phase synthesis

INTRODUCTION

Cyclic peptides are important tools in medicinal chemistry because they exhibit, compared with their linear precursors, a reduced conformational flexibility resulting in improved metabolic stability and, possibly, enhanced biological activity, receptor selectivity and bioavailability. Moreover, their constrained geometry allows conformational investigations for epitope/pharmacophore studies [1], providing a predictable conformation of diverse functionalities around a core, whose flexibility depends on the ring size.

Cyclization is the key step in the synthesis of constrained head-to-tail cyclopeptides [2,3], due to the high tendency of the corresponding linear peptides to oligomerize [4]. Classical approaches to the synthesis of a cyclic peptide generally involve preparation of the partially protected linear precursor by solution or solid-phase approaches, followed by cyclization in solution under high dilution conditions [5]. However, solution-phase methodologies, even under high dilution conditions, suffer from several drawbacks, such as cyclodimerization and cyclooligomerization side reactions.

Abbreviations: AcOH, acetic acid; Al, allyl; tBu, *tert*-butyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; DCU, *N*,*N*′-dicyclohexylurea; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DKP, 2,5-diketopiperazine; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; EtOAc, ethyl acetate; Fmoc, 9*H*-fluoren-9-ylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; MeCN, acetonitrile; MeOH, methanol; NMM, *N*-methylmorpholine; Pbf, 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl; Phg, phenylglycine or α-aminophenylacetic acid; PyBop, (1-hydroxy-1*H*-benzotriazolato-*O*)tris-1-pyrrodinyl phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; Tos, tosyl or *p*-toluenesulphonyl.

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It has been previously reported that cyclization can be performed while the peptide remains anchored on a solid support, thus taking advantage from the pseudodilution phenomenon [6], which favours intrachain resin-bound reactions, minimizing inter-chain interactions. Homodetic head-to-tail cyclopeptides can also be conveniently synthesized by solid-phase peptide synthesis (SPPS) by anchoring a trifunctional amino acid to the resin through its side chain [7–17]. The combination of this strategy with an orthogonal three-dimensional protection scheme, such as the Fmoc/tBu/OAl SPPS [15], results in a powerful head-to-tail cyclization methodology [7,8,11,14–16].

Biological activity of cyclotetrapeptides, characterized by a rigid scaffold [18–21], is strongly dependent on their constrained conformation [22–28]. Solution approaches to the preparation of cyclotetrapeptides do not always avoid cyclooligomerization [29–34]. This phenomenon is indeed critical in the synthesis of cyclotetrapeptides, where the 12-member ring closure is not favoured over the intermolecular cyclization, due to the intrinsic rigidity induced by the π-character of the peptide bond.

Syntheses of cyclotetrapeptides by a solid-phase cyclization-cleavage strategy, based on intrachain aminolysis of the peptides, were described [35–38]. In particular, this approach was used by Nishino *et al.* [37,38] for the preparation of cyclo(Arg-Gly-Asp-Phg), a cyclic tetrapeptide presenting an inhibitory activity toward cell adhesion. This study showed that the extent of oligomerization strongly depends on the choice of the *C*-terminal residue (from 20% of dimer when Phg is in the *C*-terminal position, to 40% with Gly).

The on-resin head-to-tail cyclization was applied to the synthesis of His containing cyclotetrapeptides [8], by anchoring Fmoc-His-OAl through its side chain to a trityl resin. Cyclo(His-Gly-His-Gly), usually prone to cyclodimerization [39], was synthesized with no evidence of oligomer formation.

In this paper a systematic study on the synthesis of cyclotetrapeptides by on-resin head-to-tail cyclization [40], using the Fmoc/tBu/OAl three-dimensional protection scheme is described. A comparative study was undertaken to evaluate all of the parameters (resin, coupling reagent and base) that could play an important role in the ring-closure reaction. A series of Arg-Gly-Asp (RGD) containing cyclotetrapeptides cyclo(Xaa-Arg-Gly-Asp) (Xaa = Ala, Phe, Phg, D-Ala, D-Phe, D-Phg) was chosen as model peptides. In particular, the synthesis of cyclo(Phg-Arg-Gly-Asp) was previously reported as presenting cyclodimerization to some extent [37,38]. Moreover, the RGD tripeptide is a universal cell-recognition sequence of several extracellular matrix proteins bound to integrins, which are involved in different important physiologically processes like cell differentiation, platelet aggregation and tumour metastasis. Incorporation of this sequence into cyclic penta- and hexapeptides resulted in highly potent and selective inhibitors of integrins [41–43].

MATERIALS AND METHODS

Protected amino acids and resins were obtained from Calbiochem-Novabiochem AG (Laufelfingen, Switzerland). TBTU, HOBt and PyBop were purchased from Advanced Biotech Italia (Milano, Italy). Peptide grade DMF was obtained from Scharlau (Barcelona, Spain). Dichloromethane was distilled from CaH_2 before use. The trityl chloride and the Wang resin were dried under vacuum before functionalization. $Pd(PPh_3)_4$ was weighed under Ar and dissolved in dry DCM immediately before use. RP-HPLC analytical analyses were performed on a Beckmann System Gold instrument (model 125) equipped with a diode array (model 168), on a Phenomenex Jupiter C18 column (5 µm, 250 × 4.6 mm) using a flow rate of 1 ml/min and with the following solvent systems: 0.1% TFA in H_2O (A), 0.1% TFA in MeCN (B). Semipreparative RP-HPLC analyses were performed using a flow rate of 4 ml/min on a Phenomenex Jupiter C18 column (10 µm, 250 × 10.0 mm). Mass spectra were registered on the ESI LCQ Advantage mass spectrometer (ThermoFinnigan) and on the VG 70-250 FAB-MS spectrometer (Micromass). LC-ESI MS analyses were performed on a Phenomenex Aqua C18 column (5 µm, 150 × 2.0 mm) (flow rate: 200 µl/min), or a Vydac 218MS51 C18 Mass Spec column (5 µm, 250 × 1.0 mm) (flow rate: 30 µl/min), on a ThermoFinnigan Surveyor HPLC system coupled to the ESI-MS, using the solvent systems: H_2O (A), MeCN (B), 1% TFA in H_2O (C). Racemization tests were performed on the GC apparatus Fractovap 4160 (Carlo Erba) equipped with Chirasil-Val capillary columns.

SPPS was performed in the eight reaction-block of the AdvancedChemTech automatic synthesizer APEX 396. Functionalization of the resins, allyl deprotections and cyclizations, were performed in Teflon reactors of the manual synthesizer PLS 4x4 (AdvancedChemTech).

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Fmoc-Asp-OAl (1)

A solution of DCC (0.6 g, 2.9 mmol) in allyl alcohol (1.0 ml, 14.7 mmol) was slowly added at 0 °C to a stirred suspension of Fmoc-Asp(OtBu)-OH (1.0 g, 2.4 mmol) in dry DCM (20 ml). The mixture was stirred at room temperature for 16 h, and the reaction monitored by TLC (DCM/MeOH 10 : 1; $R_f = 0.90$). The white precipitate was filtered off and the excess of DCC was eliminated with AcOH (0.5 ml). The filtered solution was concentrated to dryness, and the residue recrystallized from EtOAc/hexane. The product was treated with TFA (7 ml) at 0 °C and stirred for 2 h, monitoring deprotection by TLC (DCM/MeOH 10 : 1; $R_f = 0.35$). The solution was evaporated to dryness and the residue recrystallized from Et_2O/hexane giving a white powder (0.60 g, 62% yield). ^1H NMR ($CDCl_3$, 200 MHz), δ (ppm): 2.86–3.16 (2 H, m, β-H_2), 4.20 (1 H, t, Fmoc 9-H), 4.3–4.7 (3 H, α-H and Fmoc 10-H_2), 4.65 (2 H, d, allyl O-CH_2), 5.20–5.34 (2 H, $CH_2 = CH$), 5.8–6.0 (2 H, NH and $CH_2{=}CH$), 7.32 (4 H, m, Fmoc 2-H, 3-H, 6-H and 7-H), 7.57 (2 H, d, Fmoc 1-H and 8-H), 7.73 (2 H, d, Fmoc 4-H and 5-H), 10.0 (1 H, br s, COOH). An alternative synthesis of this product is reported in the literature [15,16].

Fmoc-Asp(trityl-resin)-OAl (2)

The trityl resin (1.0 g, 0.82 mmol/g) was dried under vacuum and then swollen with dry DCM. A solution of Fmoc-Asp-OAl (**1**) (0.155 g, 0.41 mmol) and DIPEA (0.57 ml, 3.28 mmol) in dry DCM (10 ml) was added to the resin. After 2 h, the resin was washed with DMF (3 × 2 min) and DCM (2 × 2 min), and then endcapped with DCM/MeOH/DIPEA (17 : 2 : 1) (2 × 2 min). After washing with DCM (2 × 2 min), DMF (2 × 2 min) and DCM (2 × 2 min), the resin was dried under vacuum and the resin loading (0.17 mmol/g) was determined from the Fmoc release monitored by UV absorption at 301 nm.

Fmoc-Asp(Wang-resin)-OAl (2′)

DIPCDI (0.32 ml, 2.0 mmol) was added to a solution of Fmoc-Asp-OAl (**1**) (1.5 g, 4.0 mmol) in dry DCM (15 ml). The solution was stirred at 0 °C under N_2. After 20 min the solution was concentrated and the residue dissolved in DMF (10 ml). This terminally protected amino acid solution and a solution of DMAP (12 mg, 0.1 mmol) in DMF (0.5 ml) were added to the Wang resin (1.0 g, 1.2 mmol/g), pre-swollen in DMF for 30 min, and vortexed for 1 h. The resin was washed with DMF (3 × 2 min) and DCM (2 × 2 min), and then endcapped with acetic anhydride (1.9 ml, 20 equiv) and NMM (2.2 ml, 20 equiv) in DCM (10 ml) for 1.5 h. The resin, washed with DCM (2 × 2 min), DMF (2 × 2 min) and DCM (2 × 2 min), was dried under vacuum and the resin loading (0.25 mmol/g) was determined from the Fmoc release monitored by UV absorption at 301 nm.

General Procedure for the Solid-Phase Synthesis of the Linear Peptides

The linear tetrapeptides anchored to the resin, **4a–f** and **4′a–f** were synthesized on the APEX 396 automatic synthesizer following the standard Fmoc protocol. The resin was swollen for 40 min in DMF. Fmoc group removal (except for the second residue) was performed by treating the resin with 20% piperidine in DMF (1 × 10 min + 1 × 15 min) and washing with DMF (5 × 2 min). To avoid DKP formation, Fmoc deprotection on the second residue was performed with a fast protocol (20% piperidine in DMF, 3 × 5 min). Couplings were carried out with a mixture of Fmoc-Xaa-OH (3 equiv), HOBt (3 equiv), TBTU (3 equiv) and NMM (6 equiv) in DMF for 45 min, and repeated once again.

Cleavage

The peptide-resin was treated for 2.5 h with TFA/H_2O (95 : 5). The resin was filtered off and the solution was concentrated by flushing with N_2. The peptide was precipitated from cold Et_2O, centrifuged and lyophilized.

Removal of Allyl Protecting Group

Method 1. The peptide-trityl resins **3a–f** (0.65 g, 0.17 mmol/g) were dried under vacuum and swollen in dry DCM (2 × 20 min) under Ar. The resins were treated for 3 h with a solution of $Pd(PPh_3)_4$ (0.38 g, 0.33 mmol) in $CHCl_3$/AcOH/NMM (37 : 2 : 1) (12 ml). The resins were washed with a solution of 0.5% DIPEA in DMF (2 × 2 min), a solution of 0.5% sodium diethyldithiocarbamate in DMF (2 × 5 min) and DCM (3 × 2 min). The Fmoc group was then removed with 20% piperidine in DMF (1 × 10 min + 1 × 15 min) and the resins washed with DMF (5 × 2 min).

Method 2. The peptide-resins **3a–f** or **3′a–f** were dried under vacuum and swollen in dry DCM (2 × 20 min) under Ar. The resins were shaken

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for 5 min with a solution of $PhSiH_3$ (24 equiv) in dry DCM under Ar, and then a solution of $Pd(PPh_3)_4$ (0.25 equiv) in dry DCM was added. After 40 min the resins were washed with dry DCM (1×5 min). The treatment with $PhSiH_3/Pd(PPh_3)_4$ was repeated once again. The resins were washed with DCM (1×5 min), a solution of 0.5% sodium diethyldithiocarbamate in DMF (2×5 min), DMF (3×2 min) and DCM (3×2 min). The Fmoc group was then removed with 20% piperidine in DMF (1×10 min $+ 1 \times 15$ min) and the resins washed with DMF (5×2 min).

Alanyl-arginyl-glycyl-aspartic Acid (5a)

Trityl resin approach. Fmoc-Asp(trityl-resin)-OAl (**2**) (200 mg, 0.17 mmol/g) was treated as previously described. Before the allyl and Fmoc deprotections, a micro-cleavage of the peptide-resin **3a** (100 mg) was performed as a control. The protected peptide was obtained in 57% yield (6.5 mg). Allyl and Fmoc deprotections were then performed on the peptide-resin **3a** following method 1. After cleavage, the linear peptide **5a** was obtained in 11% yield (1.5 mg).

Wang resin approach. Fmoc-Asp(Wang-resin)-OAl (**2**′) (30 mg, 0.25 mmol/g) was treated as previously described. After allyl and Fmoc deprotections following method 2, cleavage from the resin **4′a** gave the linear peptide **5a** in 97% yield (3.0 mg). RP-HPLC: isocratic 5% B, $R_t = 2.3$ min. ESI MS (m/z): found 418.5 $[M+H]^+$, calcd 418.2.

Phenylalanyl-arginyl-glycyl-aspartic Acid (5b)

Trityl resin approach. Fmoc-Asp(trityl-resin)-OAl (**2**) (200 mg, 0.17 mmol/g) was treated as previously described. Before the allyl and Fmoc deprotections, a micro-cleavage of the peptide-resin **3b** (100 mg) was performed as control. The protected peptide was obtained in 62% yield (8.0 mg). Allyl and Fmoc deprotections were then performed on the peptide-resin **3b** following method 1. The product **4b** cleaved from the resin gave the linear peptide **5b** in 11% yield (1.8 mg).

Wang resin approach. Fmoc-Asp(Wang-resin)-OAl (**2**′) (45 mg, 0.25 mmol/g) was treated as previously described. After allyl and Fmoc deprotections following method 2, the product **4′b** was cleaved from the resin. After cleavage, the linear peptide **5b** was obtained in 85% yield (3.1 mg). RP-HPLC: 5%–20% B in 20 min, $R_t = 2.2$ min. ESI MS (m/z): found 494.5 $[M+H]^+$, calcd 494.2.

Cyclization Methods

The peptide-resins **4a–f** or **4′a–f** (100 mg, 0.25 mmol/g) were vortexed at room temperature for 4 h with a solution of coupling reagents (1 equiv) and base (2 equiv) in DMF (1 ml). The following coupling systems were used: TBTU/DIPEA (method A); TBTU/HOBt/DIPEA (method B); TBTU/2,6-collidine (method C); PyBop/DIPEA (method D). After cyclization, the resins **6a–f** or **6′a–f** were washed with DMF (3×2 min) and DCM (2×2 min). The reactions were checked by the Kaiser test [44].

Cyclo(alanyl-arginyl-glycyl-aspartyl) (7a)

Trityl resin approach. Fmoc-Asp(trityl-resin)-OAl (**2**) (1.5 g, 0.17 mmol/g) was treated as previously

Table 1 Data for the Cyclotetrapeptides **7a–f**

Product	Overall yield (%)	Cyclization yield (%)	R_t (min)[a]	Racemization level (%)[d]	$[M+H]^+$ found (calcd.)
7a	15	95	10.6[b]	n.d.[e]	400.2 (400.1)
7b	6	80	11.1[c]	11	476.2 (476.2)
7c	8	85	10.8[b]	40	462.2 (462.2)
7d	6	80	11.0[b]	n.d.[e]	400.2 (400.1)
7e	6	>95	11.0[c]	7	476.2 (476.2)
7f	10	>95	11.2[b]	46	462.2 (462.2)

[a] RP-HPLC: Phenomenex Jupiter C18 column using as solvents: A 0.1% TFA in H_2O; B 0.1% TFA in CH_3CN.
[b] Isocratic 5% B.
[c] 5%–15% B in 20 min.
[d] Determined by LC-MS.
[e] Not detected.

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described. Allyl deprotection was performed following method 1 and for the cyclization reaction method A was used. After cleavage from the trityl resin, the product **7a** (15 mg) was obtained with an overall yield of 15% and a cyclization yield of 95%. RP-HPLC: isocratic 5% B, $R_t = 10.6$ min. The racemization level, determined by LC-MS, is reported in Table 1. GC test: racemization not detected. FAB MS (m/z): found 400.1 $[M + H]^+$, calcd 400.2.

Comparative study. Cyclopeptide **7a** was synthesized from Fmoc-Asp(trityl-resin)-OAl (**2**) (100 mg, 0.20 mmol/g) and from Fmoc-Asp(Wang-resin)-OAl (**2**′) (100 mg, 0.25 mmol/g) following the procedures previously described. Allyl deprotection was performed following method 2. Coupling reagents were compared in the cyclization step (data are reported in Table 2).

Cyclo(phenylalanyl-arginyl-glycyl-aspartyl) (7b)

Trityl resin approach. Fmoc-Asp(trityl-resin)-OAl (**2**) (1.5 g, 0.17 mmol/g) was treated as previously described. Allyl deprotection was performed following method 1 and for the cyclization reaction method A was used. After cleavage from the trityl resin, the product **7b** (7 mg) was obtained with an overall yield of 6% and a cyclization yield of 80%. RP-HPLC: 5%–15% B in 20 min, $R_t = 11.1$ min. FAB MS (m/z): found 476.2 $[M + H]^+$, calcd 476.2. The racemization level, determined by LC-MS, is reported in Table 1.

Comparative study. Cyclopeptide **7b** was synthesized from Fmoc-Asp(trityl-resin)-OAl (**2**) (100 mg, 0.20 mmol/g) and from Fmoc-Asp(Wang-resin)-OAl (**2**′) (100 mg, 0.25 mmol/g) following the procedures previously described. Allyl deprotection was performed following method 2. The coupling reagents were compared in the cyclization step (data are reported in Table 2).

Cyclo(phenylglycyl-arginyl-glycyl-aspartyl) (7c)

Trityl resin approach. Fmoc-Asp(trityl-resin)-OAl (**2**) (1.5 g, 0.17 mmol/g) was treated as previously described. Allyl deprotection was performed following method 1, and for the cyclization reaction method A was used. After cleavage from the trityl resin, the product **7c** (9 mg) was obtained with an overall yield of 8% and a cyclization yield of 85%. RP-HPLC: isocratic 5% B, $R_t = 10.8$ min. FAB MS (m/z): found 462.2 $[M + H]^+$, calcd 462.2. Racemization level, determined by LC-MS, is reported in Table 1.

Wang resin approach. Fmoc-Asp(Wang-resin)-OAl (**2**′) (150 mg, 0.25 mmol/g) was treated as previously described. Allyl deprotection was performed following method 2 and for the cyclization reaction method A was used. After cleavage from the Wang resin, the product **7c** (13.5 mg) was obtained with an overall yield of 78% and a cyclization yield of 75%. The racemization (38%) was evaluated by LC-MS.

Cyclo(D-alanyl-arginyl-glycyl-aspartyl) (7d)

Fmoc-Asp(trityl-resin)-OAl (**2**) (1.5 g, 0.17 mmol/g) was treated as previously described. Allyl deprotection was performed following method 1 and for

Table 2 Relative Amounts[a] of Cyclotetrapeptides (**7a–b**) and Cyclotrimers (**9a–b**) in the Crude Product

Cyclization method	Trityl resin Overall yield (%)		Wang resin Overall yield (%)		Cyclization yield (%)			
	7a	**7b**	**7a**	**7b**	**7a**	**9a**	**7b**	**9b**
A	6	5	75	65	84	2	65[b]	n.d.[d]
B	4	6	90	71	83	4	62[b]	5
C	6	6	85	42	80	6	63[b,c]	8
D	6	5	77	40	77[c]	3	67[b,c]	7

[a] Calculated by HPLC from the area determined at 215 nm (conditions as reported in Table 1).
[b] Racemization level determined by LC-ESI MS: 11% for **7b** (method A); 14% for **7b** (method B); 21% for **7b** (method C); 20% for **7b** (method D).
[c] Linear peptide: 5% for **7a** (method D); 9% for **7b** (method C); 15% for **7b** (method D).
[d] Not detected.

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the cyclization reaction method A was used. After cleavage from the trityl resin, the product **7d** (5 mg) was obtained with an overall yield of 6% and a cyclization yield of 80%. RP-HPLC: isocratic 5% B, $R_t = 11.0$ min. FAB MS (m/z): found 400.1 $[M + H]^+$, calcd 400.2. The racemization level, determined by LC-MS, is reported in Table 1.

Cyclo(D-phenylalanyl-arginyl-glycyl-aspartyl) (7e)

Fmoc-Asp(trityl-resin)-OAl (**2**) (1.5 g, 0.17 mmol/g) was treated as previously described. Allyl deprotection was performed following method 1 and for the cyclization reaction method A was used. After cleavage from the trityl resin, the product **7e** (7 mg) was obtained with an overall yield of 6% and a cyclization yield >95%. RP-HPLC: 5%–15% B in 20 min, $R_t = 11.0$ min. Racemization level, determined by LC-MS, is reported in Table 1. GC racemization test: 4.5% D-Asp, 1.4% L-Phe. FAB MS (m/z): found 476.2 $[M + H]^+$, calcd 476.2.

Cyclo(D-phenylglycyl-arginyl-glycyl-aspartyl) (7f)

Fmoc-Asp(trityl-resin)-OAl (**2**) (1.5 g, 0.17 mmol/g) was treated as previously described. Allyl deprotection was performed following method 1 and for the cyclization reaction method A was used. After cleavage from the trityl resin, the product **7f** (12 mg) was obtained with an overall yield of 10% and a cyclization yield >95%. RP-HPLC: isocratic 5% B, $R_t = 11.2$ min. Racemization level, determined by LC-MS, is reported in Table 1. GC racemization test: 20.4% D-Asp, 52.2% L-Phg. FAB MS (m/z): found 462.2 $[M + H]^+$, calcd 462.2.

Cyclo(alanyl-arginyl-glycyl-aspartyl)$_2$ (8a)

Cyclopeptide **8a** was synthesized from Fmoc-Asp(Wang-resin)-OAl (**2′**) (150 mg, 0.25 mmol/g) on the APEX 396 by SPPS as previously described. After allyl and Fmoc deprotections (method 2), cyclization was performed using method A. After cleavage and lyophilization, the product **8a** (15 mg, 50% yield) was analysed by LC-MS on a Phenomenex Aqua column (0–5% B in 20 min, $R_t = 18.7$ min). ESI MS (m/z): found: 799.7 $[M + H]^+$, calcd 799.4.

Cyclo(phenylalanyl-arginyl-glycyl-aspartyl)$_2$ (8b)

Cyclopeptide **8b** was synthesized from Fmoc-Asp(Wang-resin)-OAl (**2′**) (150 mg, 0.25 mmol/g) on the APEX 396 by SPPS as previously described. After allyl and Fmoc deprotections (method 2), cyclization was performed using method A. After cleavage and lyophilization, the product **8b** (16 mg, 46%) was analyzed by LC-MS on a 218MS51 Vydac column (5%–20% B in 20 min, $R_t = 7.5$ min). ESI MS (m/z): found 951.5 $[M + H]^+$, calcd 951.4.

RESULTS AND DISCUSSION

A systematic study is reported on the synthesis of various cyclotetrapeptides usually prone to cyclooligomerization, evaluating and optimizing all parameters directly involved in the cyclization step. A series of RGD-containing cyclotetrapeptides [cyclo(Xaa-Arg-Gly-Asp); Xaa = Ala, Phe, Phg, D-Ala, D-Phe, D-Phg] were synthesized by using the three-dimensional protection scheme Fmoc/tBu/OAl and anchoring the Asp side-chain to the resin (Scheme 1). The use of a three-dimensional protection scheme allows the orthogonal deprotection of the *C*- and *N*-termini for the subsequent on-resin head-to-tail cyclization. The solid-phase cyclization, compared with the solution one, takes advantage of the pseudo-dilution phenomenon. The syntheses were performed under different conditions in order to evaluate the role of the following parameters: nature of resin, coupling reagent and base used for cyclization. Moreover, the influence of the steric hindrance of different amino acids involved in the ring closure was evaluated. The synthesis of cyclo(Phg-Arg-Gly-Asp), previously reported as presenting cyclodimerization to some extent [37], dependent on the residues directly involved in the ring closure, was chosen as a model to compare the cyclization-cleavage methodology on an oxime resin with the on-resin head-to-tail cyclization strategy proposed in this paper.

Syntheses of Cyclo(Xaa-Arg-Gly-Asp) (7a-f)

To evaluate the effect of the steric hindrance of the amino-acid side chain of the residues involved in the ring closure, the series of RGD-containing cyclotetrapeptides cyclo(Xaa-Arg-Gly-Asp) (**7a–f**), with Xaa = Ala, Phe, Phg, D-Ala, D-Phe, D-Phg, was synthesized.

The syntheses were performed by anchoring Fmoc-Asp-OAl (**1**) to the resin through its side chain. A low resin loading (0.15–0.25 mmol/g) was used to take advantage of the pseudo-dilution phenomenon. The Asp side chain was bound

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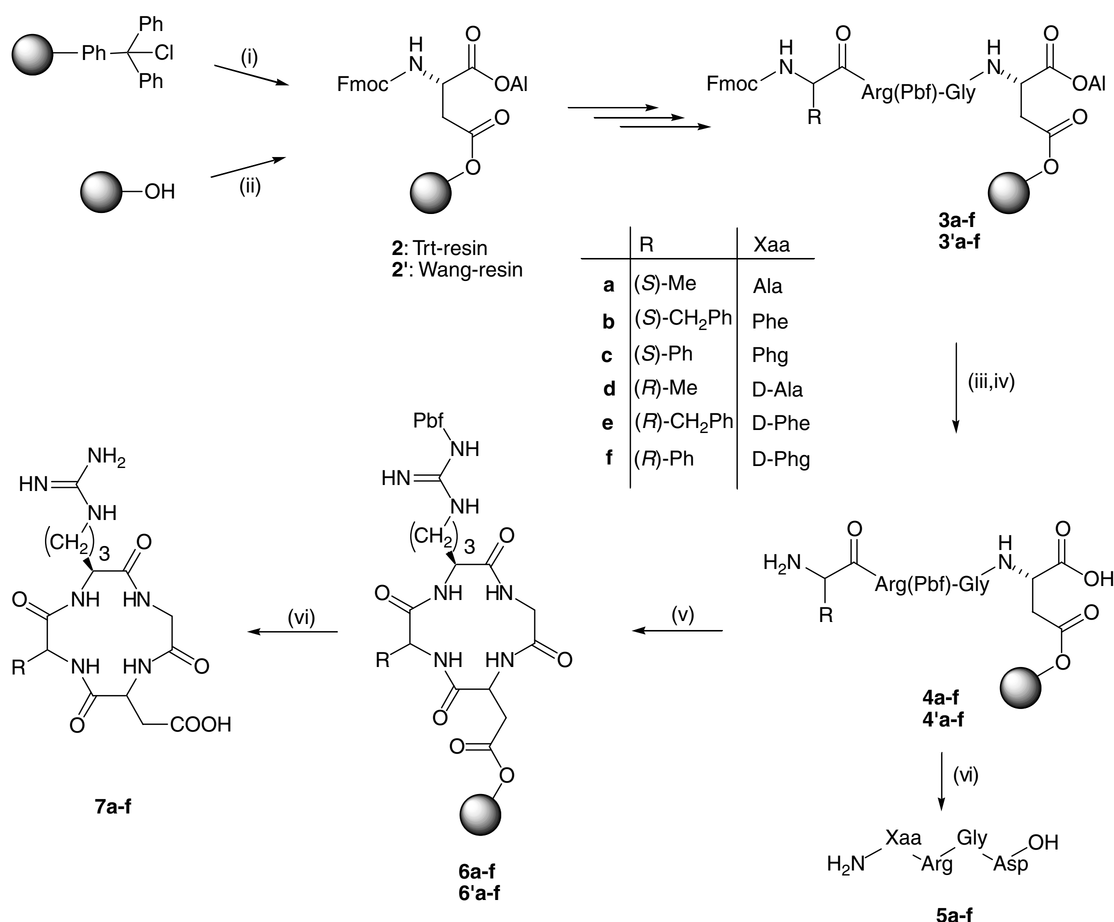

Scheme 1 Synthesis of RGD-containing cyclotetrapeptides. Reagents: (i) Fmoc-Asp-OAl (**1**), DIPEA, 2 h, r.t.; (ii) Fmoc-Asp-OAl (**1**), DIPCDI, DMAP, 1.5 h, r.t.; (iii) $PhSiH_3$, $Pd(PPh_3)_4$ (2×40 min); (iv) 20% piperidine in DMF (1×10 min + 1×15 min); (v) TBTU, DIPEA, 4 h, r.t.; (vi) TFA/H_2O (95 : 5), 3 h, r.t.

to the trityl resin, which was chosen for the versatility of its linker to further exploit cyclopeptide libraries containing different trifunctional amino acids. The linear tetrapeptides Fmoc-Xaa-Arg-Gly-Asp(trityl-resin)-OAl (**3a–f**) were synthesized using TBTU/HOBt/NMM as coupling systems by the Fmoc/tBu SPPS on an automatic synthesizer (APEX 396, AdvancedChemTech).

To avoid a nucleophilic attack of the free amino function leading to by-products, deprotection of the *C*-terminal carboxyl function must be carried out before the last Fmoc removal [45]. Allyl deprotection was then performed using $Pd(PPh_3)_4$ in $CHCl_3$/AcOH/NMM (37 : 2 : 1), under anhydrous conditions (method 1). Treatment of the resin with 20% piperidine in DMF led to the linear peptides **4a–f** anchored to the solid support and deprotected at the *C*- and *N*-termini. Cyclization was performed using TBTU/DIPEA as the coupling systems (method A). According to empirical rules [25], no difference in terms of reactivity was found during the cyclization between the peptides containing at the *N*-terminal position an amino acid with an L or D stereochemistry, all reactions being completed in 4 h. Cleavage from the resin provided the six RGD-containing cyclotetrapeptides **7a–f** in low crude yields (Table 1). Cyclotetrapeptides **7a–f** were characterized by HPLC and FAB MS, as reported in Table 1. Racemization level was evaluated by LC-ESI MS for cyclotetrapeptides **7a–f**. Cyclopeptides **7a, 7e** and **7f** were also analysed by the GC racemization test. The data were in accordance with the LC-MS results.

Racemization at the α-carbon of the amino acids involved in the ring closure was greatly influenced by the amino-acid side chain. As expected, the racemization level increased with the steric hindrance at the α-carbon from Ala to Phe, and from Phe to Phg.

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However, both for Phe and Phg, racemization cannot be only due to steric effects, but it may also be significantly influenced by an electronic effect of the phenyl moiety. Therefore, a true steric effect could have been examined only by studying hindered aliphatic residues such as Val, Ile or Leu. No racemization was detected in the case of the Ala containing cyclotetrapeptides **7a** and **7d**, while for cyclo(Phe-Arg-Gly-Asp) (**7b**) and cyclo(D-Phe-Arg-Gly-Asp) **7e** 5%–10% of racemization was found. The presence of the phenyl group directly bound to the α-carbon (D-Phg of **7f**) dramatically increases the extent of racemization (GC racemization test: 20.4% D-Asp, 52.2% L-Phg) with respect to the D-Phe containing cyclopeptide **7e** (GC racemization test: 4.5% D-Asp, 1.4% L-Phe). In the particular case of Phg, not only a steric effect can be envisaged, but also a charge stabilizing effect by the α-phenyl group. Moreover, as during the coupling reaction racemization usually occurs at the residue involved in the *C*-activation, racemization at the α-carbon of Phg can only be justified on the basis of an anion-stabilizing conjugation effect of its side-chain phenyl moiety.

Influence of the Resin on the Synthesis of the Linear Peptides

The low yields obtained for the synthesis of the cyclotetrapeptides **7a–f** with the trityl resin prompted us to investigate the cyclization step on other solid supports. Thus, it was decided to compare the trityl resin [46] with the Wang resin [47]. As references, the syntheses of Fmoc-Ala-Arg-Gly-Asp(resin)-OAl (**3a** or **3′a**) and Fmoc-Phe-Arg-Gly-Asp(resin)-OAl (**3b** or **3′b**) were carried out both from Fmoc-Asp(trityl-resin)-OAl (**2**) and Fmoc-Asp(Wang-resin)-OAl (**2′**), by using Fmoc/tBu SPPS. Moreover, it was decided to remove the allyl group under neutral conditions (method 2), using Ph_3SiH and $Pd(PPh_3)_4$ in catalytic amounts, under anhydrous conditions [48]. Treatment of the resins with a 20% piperidine solution in DMF led to the linear peptides **4a** or **4′a** and **4b** or **4′b** anchored to the solid support and deprotected at the *C*- and *N*-termini.

By cleavage-control it was observed that in the case of the trityl resin an undesired early cleavage occurred during the allyl deprotection. Before the allyl deprotection step, good yields were obtained (57% for **3a**, 62% for **3b**); while after the $Pd(PPh_3)_4$ treatment very low yields of the linear tetrapeptides **5a** (11%) and **5b** (11%) were found. No significant improvement of the yield was found by performing the allyl deprotection under neutral conditions with the trityl resin (method 2) (Table 2), in comparison with that described for method 1 (Table 1). On the contrary, cleavage from the Wang resins, **4′a** and **4′b**, gave the linear peptides in good yields (**5a**: 97%; **5b**: 85%). Then, the early cleavage, occurring with the trityl resin, does not seem related to the scavengers and to the conditions used during the allyl removal, but to the high lability of the ester bond between the Asp β-carboxyl and the trityl group. It can be hypothesized that the C^{α}-terminal Asp carboxyl function is acid enough to cleave the peptide from the trityl resin.

Influence of Different Coupling Reagents on Cyclization

To investigate the effect of different coupling reagents on cyclization step, the ring closure of the linear tetrapeptides H-Ala-Arg-Gly-Asp(Wang-resin)-OH (**4′a**) and H-Phe-Arg-Gly-Asp(Wang-resin)-OH (**4′b**) were compared using the following coupling systems: TBTU/DIPEA (Method A); TBTU/HOBt/DIPEA (Method B); TBTU/2,6-collidine (Method C); PyBop/DIPEA (Method D) (Table 2).

On-resin cyclization of **4′a** and **4′b** was performed in 4 h at room temperature with systems A and B. System C, chosen to compare DIPEA with a more hindered base to minimize racemization, showed a comparable reactivity with systems A and B in the synthesis of **6a**, but in the case of **6b** the cyclization reaction proceeded slowly (10 h). PyBop/DIPEA (D) always led to slow cyclization reactions (10 h for **6a** and **6b**). Systems C and D did not give complete conversion of the linear peptide in the ring closure of cyclo(Phe-Arg-Gly-Asp) (**7b**), a 10%–15% of the corresponding linear peptide **5b** was detected (Table 2).

As reported in Table 2, cyclotetrapeptides are the main products, and the yield of conversion of the linear peptide on the resin and the purity are strictly dependent on the activating reagents used and on the different steric hindrance of the amino acid side chain. High cyclization yield is obtained in the case of the supported Ala-containing, linear tetrapeptide **4′a**, while for cyclo(Phe-Arg-Gly-Asp) (**7b**) more by-products are present. Systems A and B gave the best results in terms of reactivity, yield and purity. Cyclotetrapeptides undergo ion/molecule reactions under the ionization conditions of the mass spectrometry technique (aside from fragmentation), such as ESI-MS, as previously reported by Schmidt and Langner [29]. In fact, ESI-MS investigations of our cyclotetrapeptides revealed oligomer cyclopeptide

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clusters. However, under high dilution conditions the $[M + H]^+$ ion of the desired cyclotetrapeptide could be detected. Cyclotetrapeptides were unequivocally characterized by FAB-MS (Table 3).

To detect possible amounts of cyclodimers in the reaction crude of **7a** and **7b**, the two cyclooctapeptides cyclo(Ala-Arg-Gly-Asp)$_2$ (**8a**) and cyclo(Phe-Arg-Gly-Asp)$_2$ (**8b**) were synthesized as reference products. HPLC and co-elution experiments of the crude cyclo(Ala-Arg-Gly-Asp) (**7a**) and the corresponding cyclodimer **8a** showed different retention times (R_t). On these bases, no cyclodimer is formed to any appreciable extent. Moreover, no racemization occurred during formation of the cyclotetrapeptide **7a**. Also in the case of **7b** no cyclodimerization occurred, but a small extent of racemization was detected by LC-ESI MS. No improvement, in terms of racemization level, was found using system C.

In conclusion, cyclization of Asp-containing tetrapeptides, whose side chain is anchored to the Wang resin, following Fmoc/tBu/OAl SPPS, under pseudo-dilution conditions and using the activating system A, prevented cyclodimerization. Surprisingly, a very small amount of cyclotrimers **9a** and **9b** was detected (Table 2). However, the use of system A allows the control of cyclotrimer formation (<2%). As expected, long acidic cleavage of cyclo(Ala-Arg-Gly-Asp) (**7a**) from the resin gave aspartimide by-products.

Cyclo(Phg-Arg-Gly-Asp) (**7c**) was previously reported by Nishino *et al.* [37] by using the cyclization-cleavage strategy of the corresponding linear peptide H-Phg-Arg(Tos)-Gly-Asp(OcHex)-resin on an oxime resin (loading 0.5 mmol/g). Using this approach, a 50% yield of the cyclotetrapeptide **7c**, along with a 20% yield of cyclodimer, were obtained after 20 h. In order to compare this strategy with our cyclization strategy, **7c** was synthesized on the Wang resin, in 4 h, using TBTU/DIPEA (method A) as the activating system. Cleavage from the resin provided the desired cyclotetrapeptide **7c** with an overall yield of 78% and a cyclization yield of 75%, together with only a 12% of the cyclotrimer by-product. It is evident that this approach is more convenient in terms of yield and reaction time, and that cyclooligomerization is more limited. Moreover, separation from the cyclotrimer is easier than from the cyclodimer (obtained by the Nishino's methodology) because of its higher chromatographic retention time.

Table 3 HPLC and Mass Analyses of the Cyclotetrapeptides in Comparison with the Corresponding Linear Tetrapeptides and Cyclooligomers

Product	R_t (min)	$[M + H]^+$ found[f] (calcd.)
H-Ala-Arg-Gly-Asp-OH (**5a**)	2.3[a,b]	418.5 (418.2)
Cyclo(Ala-Arg-Gly-Asp) (**7a**)	4.3[a,b]; 16.5[a,c]	400.1 (400.2)
Cyclo(Ala-Arg-Gly-Asp)$_2$ (**8a**)	18.7[a,c]	799.7 (799.4)
Cyclo(Ala-Arg-Gly-Asp)$_3$ (**9a**)	7.3[a,b]	1198.8 (1199.2)
H-Phe-Arg-Gly-Asp-OH (**5b**)	2.2[a,d]	494.5 (494.2)
Cyclo(Phe-Arg-Gly-Asp) (**7b**)	12.1[a,d]; 6.6[e]	476.2 (476.2)
Cyclo(Phe-Arg-Gly-Asp)$_2$ (**8b**)	7.5[e]	951.5 (951.4)
Cyclo(Phe-Arg-Gly-Asp)$_3$ (**9b**)	14.7[a,d]	1427.6 (1427.2)

[a] RP-HPLC: Phenomenex Aqua C18 column (150 × 2.0 mm), flow rate 200 µl/min, solvent system A: H_2O, B: CH_3CN, C: 1% TFA in H_2O.
[b] Isocratic 5% B (10% C) in 20 min.
[c] 0–5% B (10% C) in 20 min.
[d] 5%–40% B (10% C) in 20 min.
[e] 5%–20% B in 20 min (2% C) on a 218MS51 C18 MassSpec Vydac column (250 × 1.0 mm), flow rate 30 µl/min.
[f] Determined by ESI MS.

CONCLUSION

In this paper, the synthesis is reported of a series of RGD-containing cyclotetrapeptides cyclo(Xaa-Arg-Gly-Asp) (**7a–f**) with no cyclodimerization by-products and only a very small amount of cyclotrimers (<2%). It was therefore demonstrated that the head-to-tail cyclization through side-chain anchoring of an Asp residue to a Wang resin, by using the Fmoc/tBu/OAl protection scheme, is an efficient approach to the synthesis of constrained cyclotetrapeptides using TBTU/DIPEA as the activating system in the cyclization step.

The optimization of all of the parameters and the understanding of their role can be considered a starting point for the synthesis of libraries of constrained cyclopeptides. Moreover, even if the on-resin head-to-tail cyclization through amino acid side chain anchoring is strictly dependent on the presence of a trifunctional amino acid, the proposed synthetic approach presents the great advantage of obtaining cyclic scaffolds still bound to the resin with functionalities ready for further modifications.

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