cyclo(-arginyl-sarcosyl-aspartyl-phenylglycyl-)$_2$. Simple synthesis of an RGD-related peptide with inhibitory activity for platelet aggregation

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The dimerization–cleavage of the tetrapeptide precursor bound to the benzophenone oxime resin afforded cyclo[-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-]$_2$, a cyclic analogue of the RGD peptide. The yield and the selectivity between the tetra-/octa-peptide formed through the cyclization–cleavage depended on the sequence of the tetrapeptide. The ratio of tetra-/octa-peptide also depended on the substitution level of the oxime resin with the peptide segment. The cyclic octapeptide was also synthesized through the solution-phase dimerization–cyclization from the linear tetrapeptide precursor. The solution-phase dimerization–cyclization was very efficiently mediated with BOP/HOBt as the condensation reagent. ^{1}H NMR and CD spectra suggested that the cyclic octapeptide was of some restricted conformation, which might be involved in the preferred formation of the octapeptide in both solid- and solution-phase syntheses. The octapeptide showed potent inhibitory activity toward platelet aggregation; however, it showed no activity toward cell adhesion.

Introduction

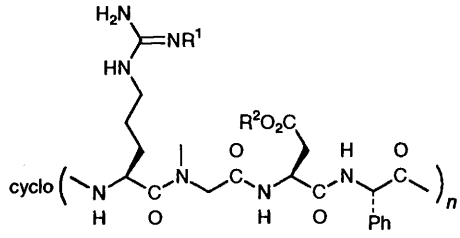
The Arg-Gly-Asp unit in fibrinogen had been shown to participate in the aggregation of human platelets.[1,2]† Synthetic peptides containing an Arg-Gly-Asp sequence often act as antagonists for the receptor protein. Hence, a number of peptides with an Arg-Gly-Asp sequence and their mimics were synthesized, in the expectation that they show some inhibitory activity toward platelet aggregation.[3] Owing to their restricted conformations, cyclic peptides often mimic protein surfaces. Therefore, cyclic peptides containing an Arg-Gly-Asp sequence have been well studied from the viewpoint of both their biological activities and their conformation.[4] We have recently reported the syntheses, conformation and biological activity of cyclo(-Arg-Gly-Asp-Phg-) as a moderate inhibitor of platelet aggregation.[5] During the syntheses of various cyclic peptides containing an -Arg-Gly-Asp-Phg- sequence and its analogues, we found that the title compound cyclo(-Arg-Sar-Asp-Phg-)$_2$ (**3**) was formed in fair yield by using the solid-phase synthesis and cyclization–cleavage method on the oxime resin. In this paper, we report not only the synthesis of this cyclic octapeptide, but also its potent inhibitory activity for platelet aggregation.

Results and discussion

Solid-phase syntheses

We first studied the dependence between the peptide sequence on the oxime resin and the cyclization efficiency in the solid-phase synthesis and cyclization–cleavage method.[5a] We examined three tetrapeptide resins, Boc-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-oxime resin **7**, Boc-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-oxime resin **8** and Boc-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-oxime resin **9** (Scheme 1). Since Arg(Tos)-oxime resin is unstable due to spontaneous cleavage with the formation of the lactam,[5a] we omitted the fourth sequence of Boc-Sar-Asp(O*c*Hex)-Phg-Arg(Tos)-oxime resin. Thus, these three tetrapeptide–resins (**7**, **8** and **9**) were assembled by the solid-phase syntheses on *p*-nitrobenzophenone oxime resin by using BOP/HOBt as the condensation reagent. The Kaiser tests[6] showed that each coupling step on the resin took place quantitatively for substrates **7** and **8**. However, compound **9** was formed in poor yield (15% based on the first amino acid). Probably, cyclo[-Arg(Tos)-Sar-] would be formed as a by-product in the synthesis of compound **9**.[7]

The N-terminal Boc groups of the tetrapeptide–resins **7**, **8** and **9** were removed with 25% TFA in CH_2Cl_2 (by volume). Then, DIEA and AcOH (2.0 mol equiv. per peptide,



	n	R^1	R^2
1	1	H	H
2	1	Tos	c-Hex
3	2	H	H
4	2	Tos	c-Hex
5	3	H	H
6	3	Tos	c-Hex

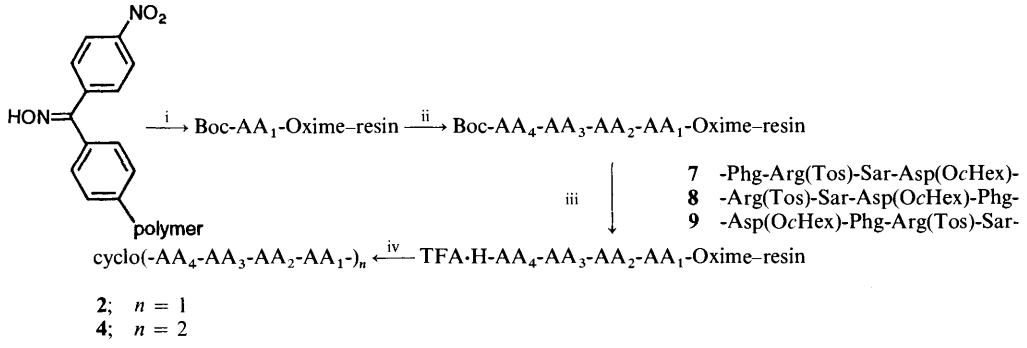
Structures of cyclo(-Arg-Sar-Asp-Phg-)$_n$

† Abbreviations used are according to ref. 2. Other abbreviations: AA, amino acid; Phg, L-phenylglycine; Sar, sarcosine; Boc, *tert*-butoxycarbonyl; Tos, *p*-tolylsulfonyl; *c*Hex, cyclohexyl; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; DIEA, *N*,*N*-diisopropylethylamine; BOP, (benzotriazol-1-yloxy)tris(diethylamino)phosphonium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate.

Table 1 Syntheses of cyclo[-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-]$_n$ (**2**, n = 1; **4**, n = 2; **6**, n = 3) by the solid-phase synthesis and cyclization–cleavage method on the oxime–resin

Entry	Precursor sequence on oxime resin (Boc-AA$_n$-oxime–resin)		Amino acid substitution[a]	Yield (%)[b]	2/4/6[c]
1	-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-	**7**	0.49	64	41:59:0
2	-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-	**8**	0.49	24	12:88:0
3	-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-	**9**	0.49	15	*d*
4	-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-	**7**	0.11	45	79:21:0
5	-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-	**7**	0.23	52	73:27:0
6	-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-	**7**	0.38	62	60:40:0
7	-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-	**7**	0.46	64	51:49:0
8	-[Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-]$_2$-	**10**	0.49	45	0:100:0
9	-[Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-]$_2$-	**11**	0.49	45	0:100:0
10	-[Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-]$_3$-	**12**	0.49	38	0:9:91

[a] Millimoles of the first amino acid per gram of resin. [b] After 24 h at 298 K. Based on the first amino acid. [c] Determined by HPLC. [d] Not determined.



Scheme 1 *Reagents:* i, Boc-AA-OH, DCC; ii, stepwise solid-phase syntheses; iii, TFA; iv, DIEA and AcOH

respectively) in DMF were added to the peptide-resins. An excess of DIEA was employed to complete the liberation of the free amino group from the TFA salt. AcOH was used to keep the reaction media in a neutral to slightly acidic condition. The catalytic role of acetate anion in the cyclization–cleavage is not yet clearly understood. The reaction mixtures were shaken at 298 K and subjected to HPLC analysis, where two reaction products were detected. The signals corresponded to the cyclic tetrapeptide cyclo[-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-] **2** and the cyclic octapeptide cyclo[-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-]$_2$ **4**.

In Table 1 (entries 1–3) are summarized the results of the cyclization–cleavage of the three tetrapeptide–resins with different sequences. The tetrapeptide tethered to the oxime resin with an Asp(O*c*Hex) residue (**7**) gave a mixture of cyclic tetrapeptide **2** and octapeptide **4** in 41:59 ratio (entry 1) in moderate total yield [64% per Boc-Asp(O*c*Hex)-oxime resin]. The peptide with Phg at C-terminus (**8**) gave a lower yield, but the cyclic octapeptide produced preferentially the tetrapeptide (12:88, entry 2). The selectivity of the products (**2**/**4**) may reflect the conformation of the tetrapeptide segment on the oxime resin (see Conformational aspects sub-section). The -Phg-Arg(Tos)-Sar-Asp(O*c*Hex)- sequence in compound **7** might be of a bending conformation. The amino group of the Phg residue of compound **7** could easily attack the oxime ester carbonyl group intramolecularly to form cyclic tetrapeptide **2**. However, the -Arg(Tos)-Sar-Asp(O*c*Hex)-Phg- sequence in structure **8** seems to be rather extended, where the amino group of Arg(Tos) could hardly attack the carbonyl group intramolecularly. Hence, the cyclization–cleavage of compound **8** was slow and much more of the intermolecular dimerization product was formed than in the case of compound **7**. The product of the cyclization–cleavage from H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-oxime resin **9** was small in quantity, because of the inefficient elongation of the peptide segment from H-Sar-oxime resin. The content of peptides **2**/**4** was not determined in this case.

Since the formation of peptide **4** includes a bimolecular process, we next examined the formation of cyclic tetra-/octa-peptides with different concentrations of the tetrapeptide segment on the oxime resin. The varieties of oxime resins with different substitution levels of Boc-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg- group were synthesized (Table 1, entries 1, 4–7). For the peptide–resins with lower substitution levels, each coupling reaction with 3 mol equiv. of Boc-amino acid was very slow. In such cases, the coupling reactions were performed with 6 mol equiv. Boc-amino acids per the peptide segments instead of the 3 mol equiv. of reagents usually employed. Fig. 1 shows the time courses of cyclization–cleavage from H-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-oxime resin **7** with different substitution levels on the oxime resin (0.11 and 0.49 mmol/gram of resin). For each resin, the formation of peptide–resins **2** and **4** took place in the initial stage of the reaction. These results indicated that the

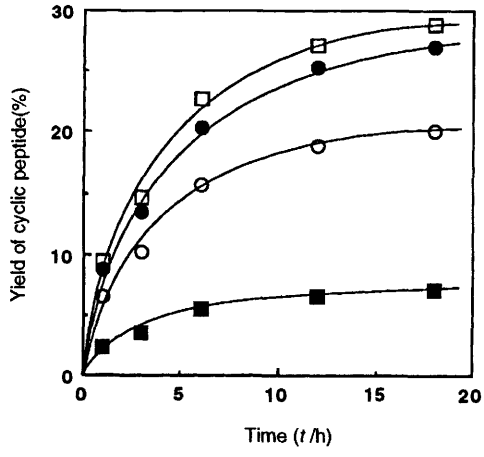


Fig. 1 Time courses of formation of cyclic tetrapeptide **2** and cyclic octapeptide **4**. **2** (●) and **4** (■) from compound **7** with low substitution level (0.11 mmol g^{-1}), and **2** (○) and **4** (□) from compound **7** with high substitution level (0.49 mmol g^{-1}).

formation of linear octapeptides on the oxime resin took place quickly and that the cyclization was the rate-determining step for the formation of cyclic octapeptide **4** (Scheme 2). In the cyclization–cleavage from compound **7**, the higher the substitution level on the oxime resin the higher is the content of the cyclic octapeptide **4** (Table 1, entry 1, 4–7). The same tendency in the cyclization–dimerization was reported by Ösapay *et al.*[8] Probably the linear tetrapeptide H-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-oxime resin is extended and rigid, and the octapeptide segment formed on the resin would be bent and flexible.

An independent experiment also showed the flexible nature of the linear octapeptide segment on the oxime resin. The cyclization–cleavage of tetra- and dodeca-peptide–oxime resins was examined (Table 1, entries 8–10). The loss of the intermediate peptides during assembly of the linear peptide–resins would explain the lower yields of cyclic peptides. For the cyclization–cleavage from the octapeptide–resin **10** and **11** no sequence dependence on the yield was observed, which was in contrast to the situation with the tetrapeptide–resin.

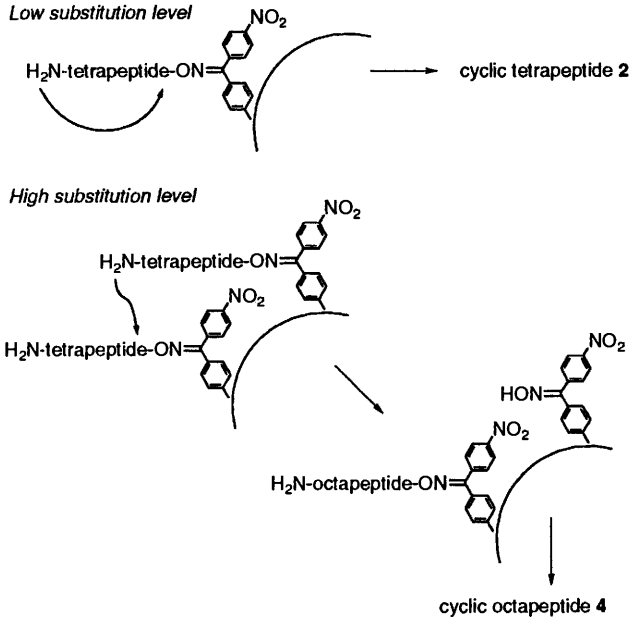
Boc-[-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-]$_2$-oxime resin **10**

Boc-[-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-]$_2$-oxime resin **11**

Boc-[-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-]$_3$-oxime resin **12**

Solution-phase syntheses

Next, we examined the solution-phase synthesis of octapeptide **4**. In general, cyclic peptides were synthesized under high-dilution conditions to avoid risk of polymerization. However, solid-phase synthesis afforded the dimerization–cyclization product **4** in fair yield as described above, which prompted us to synthesize compound **4** under less dilute conditions in the solution phase. We therefore searched for an efficient condensation reagent for use under mild dilution conditions. The cyclization was examined using linear tetrapeptide H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OH **13** as the starting material, with the expectation that the dimerization–cyclization would take place to form cyclic octapeptide **4**. The Sar residue was chosen as the C-terminus because this amino acid has no chiral centre.



Scheme 2 Illustration for the formation of the cyclic tetrapeptide **2** and cyclic octapeptide **4** from compound **7** with different substitution levels

The dimerization–cyclization of compound **13** was carried out by dissolution of substrate **13** (as an HCl salt) and HOBt (1.2 mol equiv.) in DMF (1.0×10^{-5} mol dm^{-3} concentration for **13**) with various condensation reagents at 273 K. The condensation reagents examined were BOP, PyBOP, HBTU, DCC and EDC with or without additives. The reaction mixtures were analysed by reversed-phase HPLC to quantify the starting material, intermediates and cyclic peptide after appropriate intervals. Regardless of the condensation reagents used, cyclic tetrapeptide **2** and polymeric peptides were not generated at all, but the cyclic octapeptide **4** was obtained in high yield. Since the cyclic tetrapeptide was hardly formed in the solution-phase cyclization, paradoxically, it is noteworthy that the solid-phase synthesis and cyclization–cleavage on the oxime–resin with low substitution level is the only method available for the preparation of such cyclic tetrapeptides with strictly controlled conformations.

H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OH **13**

H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OBt **14**

H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-Asp
-(O*c*Hex)-Phg-Arg(Tos)-Sar-OBt **15**

Fig. 2 shows the time courses of the dimerization–cyclization of compound **13** by using BOP/HOBt, DCC/HOBt without additive, and DCC/HOBt/Bu_4NPF_6 systems. The formation of cyclic octapeptide **4** precursor from **13** probably involved three steps; formation of the active ester (*O*-benzotriazole ester) of linear tetrapeptide **14** (▲), dimerization of this tetrapeptide to form the linear octapeptide active ester **15** (△), and cyclization of this linear octapeptide to yield compound **4** (○). Unfortunately, attempted isolation of the OBt esters of the tetra- and octa-peptide (**14** and **15**) failed because of the reactive nature of these intermediates. Surprisingly rapid cyclization took place in the BOP/HOBt system (Fig. 2a). The starting material **13** quickly disappeared and compound **4** was produced almost quantitatively within 30 min. On the other hand, cyclization took place over 12 h in the DCC/HOBt system (Fig. 2b). Cyclization of the *N*-hydroxy-succinimide ester of H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OH in pyridine (data not

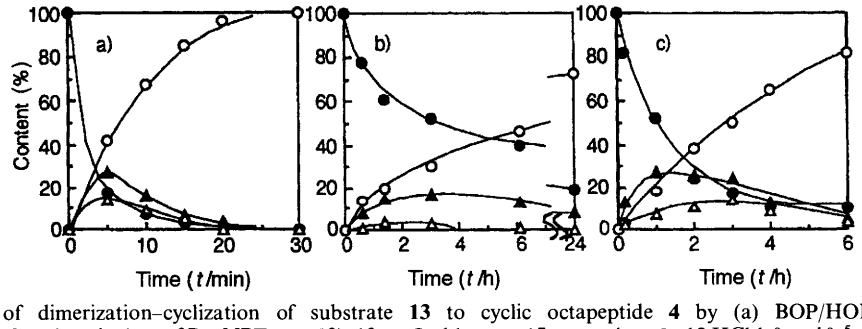


Fig. 2 Time courses of dimerization–cyclization of substrate **13** to cyclic octapeptide **4** by (a) BOP/HOBt, (b) DCC/HOBt, (c) DCC/HOBt/Bu_4NPF_6 (1.2 mol equivalent of Bu_4NPF_6 per **13**). **13** = ●; **14** = ▲; **15** = △; **4** = ○. **13**·HCl 1.0×10^{-5} mol dm^{-3} in DMF with 1.2 mol equiv. of the coupling reagents at 273 K.

Table 2 Solution-phase synthesis of cyclo[-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-]$_2$ **4** from HCl·H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OH (**13**·HCl) with various condensation reagents[a]

Entry	Condensation reagents	Additive	Mol of additive/**4**	$10^5 \times K_1/s^{-1}$ [b]
1	BOP/HOBt			190.0
2	PyBOP/HOBt			270.0
3	*HBTU/HOBt*			120.0
4	DCC/HOBt			3.3
5	DCC/HOBt	Bu_4NPF_6	0.5 equiv.	5.1
6	DCC/HOBt	Bu_4NPF_6	1.2 equiv.	6.6
7	DCC/HOBt	Bu_4NPF_6	2.0 equiv.	1.1
8	DCC/HOBt	Bu_4NBF_4	1.2 equiv.	3.5
9	DCC/HOBt	Bu_4NCl	1.2 equiv.	0.9
10	EDC/HOBt			14.8
11	EDC/HOBt	KPF_6	1.2 equiv.	38.5

[a] **13**·HCl 1.0×10^{-5} mol dm^{-3} in DMF, Et_3N (1.0 mol equiv.), condensation reagent/HOBt (1.2 mol equiv.) at 273 K. [b] Rate constant for the formation of compound **4**.

shown) was also very sluggish and resulted in a 70% yield after 24 h.

The use of PyBOP/HOBt or HBTU/HOBt instead of BOP/HOBt gave similar results (Table 2, entries 1–3). The yields of compounds **4** after 30 min were quantitative for both the PyBOP/HOBt and HBTU/HOBt systems. These reagents must mediate the cyclization reactions by the same mechanism as BOP/HOBt. The carboxy group should be activated as an acyloxyphosphonium salt [–C(=O)–O–$P^+(NMe_2)_3$] and/or OBt ester, as has been argued previously.[9] The rapid reaction mediated by BOP/HOBt has been considered to be due to the fast formation of the active ester.[10] However, the rapid disappearance of the reaction intermediates **14** and **15** suggests that the reactions of these active esters with the amino groups might be also accelerated in these systems. The nucleophilic reactivity of the amino group might be increased by the coexisting PF_6^- anion, through the interaction between the NH group and the FP moiety. Unfortunately, ^{19}F and ^{31}P NMR spectra of the reaction mixture did not indicate the existence of the N–H · · · F–P bond, suggesting that the interaction of the amino group and the PF_6^-, if it existed, is very weak. However, control experiments supported the hypothesis that PF_6^- activated the amino group. The addition of Bu_4NPF_6 (1.2 mol equiv.) to the DCC/HOBt system shortened the reaction to be complete in 6 h (Fig. 2c), probably owing to the activation of the amino groups by the PF_6^- anion.

The effects of the additive salts in the DCC/HOBt and EDC/HOBt system were further examined. Table 2 summarizes the first-order rate constants observed for the dimerization–cyclization of compound **13** in the various conditions. Addition of Bu_4NPF_6 (0.5 to 1.2 mol equiv., entries 5,6) to the DCC/HOBt system accelerated the reaction. However, the cyclization was sluggish with 2.0 mol equiv. Bu_4NPF_6 (entry 7). Under such conditions, large amounts of Bu_4N^+ formed the ammonium salt of the carboxylate and/or HOBt, which weakened the reactivity. Bu_4NBF_4 did not accelerate the cyclization and Bu_4NCl retarded it (entries 8,9). These salts were ineffective because BF_4^- and Cl^- are too weakly basic as to activate the amino group, but coexisting Bu_4N^+ deactivated the carboxylic group. The use of EDC/HOBt/KPF_6 system clearly showed the deactivation mechanism by Bu_4N^+ (entries 10,11). The addition of KPF_6 to EDC might form the PF_6^- salt {1-[3-(dimethylamino)propyl]-3-ethylcarbodiimidehydrohexafluorophosphate} *in situ* without generating Bu_4N^+. Therefore, a rapid and high-yield cyclization occurred in the EDC/HOBt/-KPF_6 system.

Since the linear peptide **13** has no asymmetric amino acid at C-terminus, there was no problem on racemization. The BOP/HOBt and related reagents, however, are known to cause

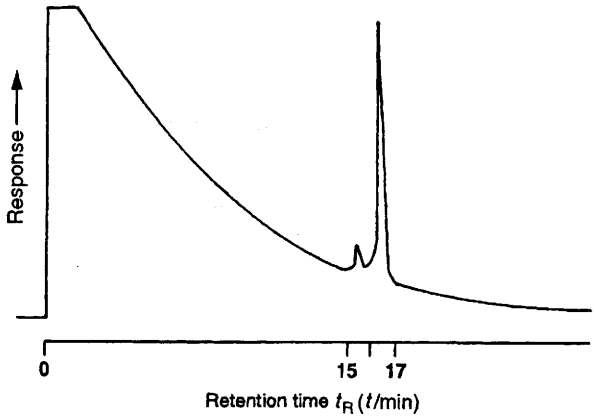


Fig. 3 HPLC profile of the reaction mixture of the dimerization–cyclization of H-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-OH by BOP/HOBt in DMF. The signals at 15.60 and 16.46 min are due to cyclo [-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-]$_2$ **4** and cyclo[-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-D-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-] (*epi*-**4**), respectively. Eluent: linear gradient of 46–78% CH_3CN/0.1% TFA for 30 min.

10–20% racemization during condensation of the fragments, probably because rather much more amine is necessary for the condensation.[11] Therefore, we prepared H-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-OH and treated it with BOP/HOBt, in order to examine the racemization caused by this reagent. Again, dimerization–cyclization occurred in high yield. Two cyclic octapeptides were found by HPLC analysis, in a 95:5 ratio, which were well separated (Fig. 3, retention times were 15.60 and 16.46 min, eluted with a linear gradient of 46–78% CH_3CN/0.1% TFA for 30 min). The latter was revealed to be the cyclic octapeptide cyclo[-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-]$_2$ **4**. The other was revealed to be cyclo[-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-D-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-] (*epi*-**4**), by comparison with authentic samples synthesized independently. About 10% of racemization may be unavoidable, but the desired cyclic peptide was easily separated from its epimer as shown by Fig. 3, due to the strict conformational difference between them.

Conformational aspects of the cyclic octapeptide in [$^2H_6$]DMSO

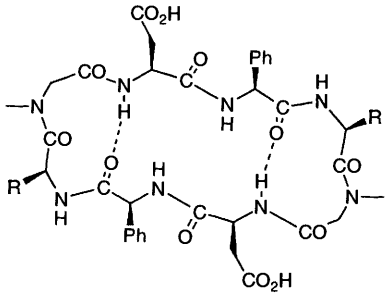
In order to investigate the mechanistic aspects of the dimerization–cyclization of the tetrapeptide precursor, the structure of the cyclic octapeptide was studied by ^1H NMR spectroscopy. The fully deprotected cyclic octapeptide **3** was employed for this study, because the Tos and O*c*Hex protecting groups in compound **4** confused the assignments of the signals. After removal of the protection with anhydrous HF, cyclic tetrapeptide **1** and octapeptide **3** were separated and purified by reversed-phase HPLC. In the ^1H NMR spectrum of compound **3** in [$^2H_6$]DMSO (at 303 K), only three signals for the amide protons were observed, indicating that compound **3** is of C_2 symmetry on the ^1H NMR time-scale. Table 3 shows the chemical shifts of the amide protons and their temperature coefficients, along with the $^3J_{HN\alpha}$-values of these amide protons and the C(carbonyl, $i-1$)–N(i)–C(α, i)–C(carbonyl, i) dihedral angles ϕ. Cyclo(-Arg-Sar-Asp-Phg-)$_3$ **5** was unsymmetrical on the ^1H NMR time-scale and assignments of the signals were not accomplished.

As is well known, the amide protons that participated in intramolecular hydrogen bonding, show little temperature dependence in their chemical shifts. The amide protons that participated in hydrogen bonding with the solvent DMSO show large temperature dependence.[4a,g] As shown in Table 3, the Asp-NH of compound **3** apparently participated in the intramolecular hydrogen bonding. The temperature dependence of the Phg-NH suggests that this amide proton also participated in intramolecular hydrogen bonding. Considering that various conformations would be allowed for the Sar residue like

Table 3 ^{1}H NMR parameters of the amide protons of compound **3**[a]

N–H	Chemical shift δ/ppm[b]	Temperature coefficients $\Delta\delta \times \Delta T^{-}$/ppb K^{-1}[c]	Coupling constant $^3J_{HN\alpha}$/Hz[b]	Dihedral angle ϕ/°
Arg	8.78	−5.06	8.8	−98 or −142
Asp	8.74	−1.74	5.5	−71
Phg	8.68	−3.46	7.9	−90 or −150

[a] Measured in [$^2H_6$]DMSO. [b] At 303 K. [c] Measured at 303, 313, 323, 333 and 343 K.



Supposed structure of compound **3** with the turn unit of the Phg-Arg-Sar-Asp sequence {R = -[CH_2]$_3$NHC(=NH)NH_2}. Only Asp-NH · · · Phg-CO intramolecular hydrogen bondings are depicted for clarity.

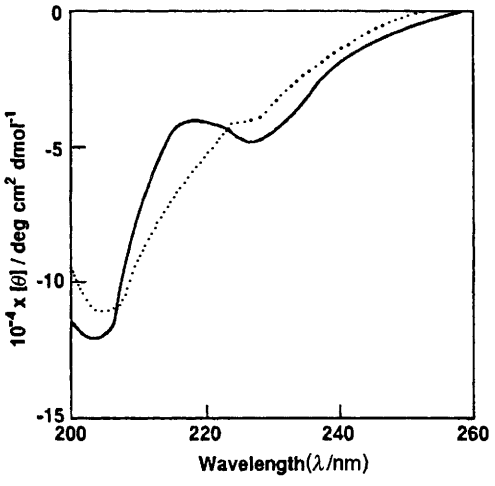


Fig. 4 CD spectra of compound **3** in MeOH (——) and in water (· · ·). [**3**] = 3.0 × 10^{-5} mol dm^{-3}. Measured at 303 K with a quartz cell of 1 mm path length.

glycine, a type II-like β-turn made with the Phg-Arg-Sar-Asp sequence might be one of the possible structures. The cyclic structure of compound **3** may be made up with two turn units and two rather extended -Asp-Phg- units. When compound **3** is of C_2 symmetrical conformation and contains two Phg-Arg-Sar-Asp turn units, strong intramolecular hydrogen bondings between the Asp-NH and Phg-CO might exist. The rather weak intramolecular hydrogen bonding between the Phg-NH and Asp-CO moieties might also be observed.

CD spectra of compound **3** in both MeOH and water (Fig. 4) supported the hypothesis described above. In both solvents, strong negative Cotton effects were observed at 205 nm. In MeOH, a clear negative Cotton effect was also observed at 228 nm, but only a shoulder peak was observed here in water. These spectra suggest the existence of a β-turn structure in compound **3**, although the CD spectra gave little quantitative information. Owing to the fact that the hydrogen bond would be weak in water, the Cotton effects were small in intensity both at 210 and 230nm.

Thus, the temperature dependence of the ^{1}H NMR chemical shifts and CD spectra both suggested the existence of the turn structure in compound **3**. Here we again speculate from the ^{1}H NMR spectra. The $^3J_{HN\alpha}$-value reflects the dihedral angle between NH and C$^{\alpha}$H bonds; therefore this value is often employed to elucidate the secondary structure of peptides. By using the equation of Wüthrich and co-workers [12] the dihedral angle ϕ (the dihedral angle between C(carbonyl, i − 1)–N(i) and C(α, i)–C(carbonyl, i) was evaluated. The $^3J_{HN\alpha}$-value for the antiparallel β-sheet structure (ϕ = 139°) is reported to be 8.9 Hz and that for the parallel β-sheet (ϕ = −119°) is 9.7 Hz.[13] The $^3J_{HN\alpha}$-value for the α-helix structure (ϕ = 57°) is 3.9 Hz. The $^3J_{HN\alpha}$-values of the Asp and Phg residues of compound **3** were in the mid range of sheets and helices, therefore these residues are supposed to be in some extended form under NMR conditions. The Phg residue might separate the two turn units and stabilize the conformation of the molecule. However, the $^3J_{HN\alpha}$-value for the Arg residue (8.8 Hz) is too large for the second amino acid residue of the type-II β-turn. The usual $^3J_{HN\alpha}$-value for the type-II β-turn (ϕ = 60°) is 4.2 Hz. The characteristics of the Sar residue might explain such a discrepancy between the too large $^3J_{HN\alpha}$-value for the Arg residue and the supposed turn sequence of Phg-Arg-Sar-Asp. The NMe group of Sar might sterically interact with the α-proton or the side-chain of the Arg residue. Hence, the Phg-Arg-Sar-Asp turn structure of compound **3** might be distorted. The bond length C(Arg-carbonyl)–N(Sar) may be different from that of usual amide bonds, and this might be one reason for the large $^3J_{HN\alpha}$-value of Arg.

Anyway, the information on the conformation of the cyclic octapeptide **3** was useful for the interpretation of both the solid-phase and the solution-phase syntheses. As for the solid-phase syntheses, the Boc-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)–resin **7** might have a bent conformation containing the -Phg-Arg-Sar-Asp- turn structure. Therefore, the amino group of the N-terminus may be close to the resin and could attack the ester carbonyl group of the Asp-oxime–resin. The intramolecular cyclization–cleavage of compound **7** might take place to form the cyclic tetrapeptide **2**. Boc-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg–resin **8** might have an extended structure consisting of Asp(O*c*Hex)-Phg residues. Hence, the amino group of the Arg residue cannot be close to the Phg carbonyl group. The amino group of the Arg residue would preferably attack the carbonyl group of the Phg of another peptide segment, resulting in the formation of the cyclic octapeptide **4**. The preferred formation of the cyclic octapeptide in the solution-phase synthesis is also explained by its conformation. The amino group of the N-terminus of the H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-active ester **14** is not close to the C-termini. However, when dimerization occurred to form H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-active ester **15** the N-termini can be close to the C-terminus active ester because this linear octapeptide has a bent moiety consisting of a -Phg-Arg(Tos)-Sar-Asp(O*c*Hex)- sequence. Therefore, after dimerization occurred to form the linear octapeptide, rapid cyclization of precursor **15** took place to form cyclic octapeptide **4**.

Biological activity of cyclic peptides

The cyclic tetrapeptide cyclo(-Arg-Sar-Asp-Phg-) **1** showed no

Table 4 Inhibition of human fibronectin platelet aggregation and cell adhesion by the cyclic and linear -Arg-Sar-Asp-peptides

Peptide	Platelet aggregation IC_{50}/μmol dm^{-3}	Cell adhesion IC_{50}/μmol dm^{-3}
cyclo(-Arg-Sar-Asp-Phg-) **1**	300	> 100
cyclo(-Arg-Sar-Asp-Phg-)$_2$ **3**	0.36	> 100
cyclo(-Arg-Sar-Asp-Phg-)$_3$ **5**	1	> 100
H-Arg-Sar-Asp-Phg-OH	200	> 100
cyclo(-Arg-Sar-Asp-DPhg-)$_2$	300	> 100
cyclo(-Arg-Sar-Asp-Phg-Ala-Sar-Ala-Phg-)	10	> 100

inhibitory activity toward human platelet aggregation nor in cell adhesion to extracellular matrix components (Table 4). The cyclic octapeptide cyclo(-Arg-Sar-Asp-Phg-)$_2$ **3** showed potent inhibitory activity toward the platelet aggregation (IC_{50}, 0.36 μmol dm^{-3}). It is very interesting that compound **3** showed no inhibitory activity in the cell adhesion test. The cyclic dodecapeptide cyclo(-Arg-Sar-Asp-Phg-)$_3$ **5** showed some inhibitory activity toward platelet aggregation (IC_{50}, 1.0 μmol dm^{-3}) and again showed no inhibitory activity in the cell adhesion test. In order to elucidate the relation between the cyclic structure and biological activity, the linear tetrapeptide H-Arg-Sar-Asp-Phg-OH, cyclic octapeptide with D-amino acids cyclo(-Arg-Sar-Asp-DPhg-)$_2$ and cyclo(-Arg-Sar-Asp-Phg-Ala-Sar-Ala-Phg-) were synthesized and subjected to the biological assay. The conformation of cyclo(-Arg-Sar-Asp-DPhg-)$_2$ must be much different from that of compound **3**. In fact, cyclo(-Arg-Sar-Asp-DPhg-)$_2$ showed no inhibitory activity in either the platelet aggregation or the cell adhesion tests. Cyclo(-Arg-Sar-Asp-Phg-Ala-Sar-Ala-Phg-) has little inhibitory activity in platelet aggregation, which might imply that the conformation of this cyclic octapeptide is somewhat similar to that of compound **3**. Anyway, compound **3** showed a characteristic compared to the many RGD-peptides studied previously, in having potent inhibitory activity in platelet aggregation but not in the cell adhesion test. This fact can be attributed to the cyclic structure of compound **3**. The linear RGD-peptides and proteins may, by varying their conformation, fit two receptor sites for platelet aggregation and cell adhesion. However, the conformation of compound **3** would be restricted and consequently this cyclic octapeptide might fit only the receptor site for platelet aggregation.

Experimental

Materials and methods

Amino acid derivatives and reagents for peptide syntheses were from Watanabe Chemical Co. (Hiroshima, Japan). *p*-Nitrobenzophenone oxime–resin (0.49 mmol of oxime group per 1.0 g resin) was prepared according to the reported method.[7,14] HPLC analyses were carried out on a Hitachi L-6200 intelligent pump equipped with a Hitachi L-4200 UV-VIS detector and a Hitachi D-2500 chromato-integrator. The analyses were performed on MS-GEL C18 120 Å (4.6 × 150 mm or 10 × 250 mm, Asahi Glass, Tokyo) columns eluted with a linear gradient of CH_3CN/water/TFA = 10:90:0.1 to 100:0:0.1 (v/v/v) over a period of 30 min, at a flow rate of 1.0 cm^3 min^{-1} and detection at 220 nm unless otherwise noted. FAB-MS were obtained by a JEOL DX-300 mass spectrometer equipped with a JEOL JMA-3100 mass data analysis system, using glycerol as matrix. CD spectra were recorded on a JASCO J-500A spectropolarimeter, using a quartz cell of 1 mm pathlength at 298 K. The concentration of the polypeptide for the CD measurements was 30 μmol dm^{-3}. ^1H NMR spectra were obtained by a JEOL GSX-400 spectrometer. The concentration of the polypeptide for NMR measurements was 2.0 mmol dm^{-3} in [$^2H_6$]DMSO. The elemental analyses were carried out with a Yanagimoto MT-3 analyser.

Solid-phase synthesis

Synthesis of the tetrapeptide-oxime–resin. Boc-Phg-Arg(Tos)-Sar-Asp(OcHex)-oxime-resin 7. To the *p*-nitrobenzophenone oxime–resin (4.0 g, corresponding to 2.0 mmol of the oxime group) pre-swelled with CH_2Cl_2 were added Boc-Asp(OcHex)-OH (1.3 g, 4.0 mmol), DCC (0.82 g, 4.0 mmol) and CH_2Cl_2 (60 cm^3) and the resultant mixture was shaken overnight. Then the resin was filtered off, washed successively with CH_2Cl_2 (60 cm^3, 2 ×), CH_2Cl_2–EtOH (1:1 v/v; 60 cm^3, 4 ×) and CH_2Cl_2 (60 cm^3, 2 ×) and dried *in vacuo* over P_2O_5. The yield was 4.5 g. The picrate assay[15] of an aliquot indicated the nearly quantitative introduction of the Boc-Asp(OcHex)- group on the resin [0.46 mmol of Asp(OcHex) group per 1.0 g resin].

Boc-Asp(OcHex)-oxime–resin [4.5 g, 2.0 mmol of Asp(OcHex) group] thus obtained was washed successively with CH_2Cl_2 (60 cm^3, 2 ×) and TFA/CH_2Cl_2 (1:3 v/v; 60 cm^3), then was treated with TFA/CH_2Cl_2 (1:3 v/v; 60 cm^3) for 30 min to deprotect the Boc group. The resin was washed with CH_2Cl_2 (60 cm^3, 2 ×), propan-2-ol (60 cm^3), CH_2Cl_2 (60 cm^3, 3 ×) and DMF (60 cm^3). To the H-Asp(OcHex)-oxime–resin (TFA salt) thus obtained was added a mixture of Boc-Sar-OH (1.1 g, 6.0 mmol), BOP (2.6 g, 6.0 mmol), HOBt·H_2O (0.92 g, 6.0 mmol), DIEA (1.3 g, 10 mmol) and DMF (60 cm^3) and the resultant mixture was shaken for 30 min. After filtration, the resin was washed successively with DMF (60 cm^3, 3 ×) and CH_2Cl_2 (60 cm^3, 2 ×), then dried in *vacuo* over P_2O_5. The Kaiser test[6] indicated the disappearance of the amino group; that is, Boc-Sar-Asp(OcHex)-oxime–resin was synthesized quantitatively. Similar elongation of peptides afforded Boc-Phg-Arg(Tos)-Sar-Asp(OcHex)-oxime–resin **7**.

Cyclization–cleavage of the tetrapeptide–resin from the oxime–resin to yield cyclic peptides. The Boc group of compound **7** (0.5 mmol) was removed in a similar manner with Boc-Asp(OcHex)-oxime–resin. To the tetrapeptide–resin was added a DMF (15 cm^3) solution of AcOH (60 mg, 1.0 mmol) and DIEA (0.13 g, 1.0 mmol) and the resultant mixture was shaken at room temperature for 12 h. Small aliquots (20 mm^3) were withdrawn at appropriate intervals and subjected to HPLC analysis. The resin was filtered and washed with DMF (15 cm^3, 3 ×). The filtrate and washings were collected, concentrated and solidified by addition of water. The yield of cyclic peptides was 0.23 g (64% based on compound **7**). The cyclic tetra-, octa- and dodeca-peptides were separated by HPLC.

cyclo[-Arg(Tos)-Sar-Asp(OcHex)-Phg-] **2**, HPLC t_R 19.64 min; FAB-MS *m/z* 712 [(M + H)$^+$].

cyclo[-Arg(Tos)-Sar-Asp(OcHex)-Phg-]$_2$ **4**, HPLC t_R 25.38 min; FAB-MS *m/z* 1424 [(M + H)$^+$] (Found: C, 53.5; H, 7.0; N, 13.3. $C_{68}H_{90}N_{14}O_{16}S_2$·$5H_2O$ requires C, 53.96; H, 6.66; N, 12.95%.)

cyclo[-Arg(Tos)-Sar-Asp(OcHex)-Phg-]$_3$ **6** HPLC retention t_R 27.64 min; FAB-MS *m/z* 2134 [(M + H)$^+$].

Syntheses of tetrapeptide–resin with low substitution level. For instance, Boc-Asp(OcHex)-oxime–resin [0.10 mmol of Asp(OcHex) group per 1.0 g resin] was prepared as follows. To the oxime–resin (2.0 g, corresponding to 1.0 mmol of oxime group) pre-swelled with CH_2Cl_2, were added Boc-Asp(OcHex)-OH

(0.13 g, 0.40 mmol), DCC (80 mg, 0.40 mmol) and CH_2Cl_2 (30 cm^3). After shaking of the resultant mixture overnight, the resin was washed as usual and then the unchanged oxime group was capped with an acetyl group. To the resin was added a mixture of Ac_2O (0.51 g, 5.0 mmol) and DIEA (0.26 g, 2.0 mmol) in CH_2Cl_2 (15 cm^3), then the resultant mixture was shaken for 30 min. The resin was filtered off, washed and dried as usual to obtain the resin with low substitution. The picrate assay indicated 0.11 mmol of Boc-Asp(O*c*Hex)- group per 1.0 g resin. In the coupling of Boc-amino acids to the oxime resin with low substitution level, excesses of reagents (6 mol equiv. for amino acids, BOP and HOBt·H_2O, 8 mol. equiv. for DIEA) were employed because otherwise the reaction was too slow.

Solution-phase synthesis

Boc-Arg(Tos)-Sar-OH. To a mixture of Boc-Arg(Tos)-OH (4.3 g, 10 mmol), H-Sar-OBzl·TosOH (4.2 g, 12 mmol), HOBt·H_2O (0.15 g, 1.0 mmol) and DMF (20 cm^3) were added DCC (2.5 g, 12 mmol) and triethylamine (1.0 g, 10 mmol) at 273 K. After 24 h, the solvent was evaporated off and the residue was taken up in EtOAc. The EtOAc solution was washed successively with aq. citric acid, aq. $NaHCO_3$ and water, then was evaporated to give Boc-Arg(Tos)-Sar-OBzl (4.4 g, 75%). This dipeptide was sufficiently pure and was used for further synthesis without any purification.

Boc-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OBzl. Boc-Arg(Tos)-Sar-OBzl (3.2 g, 5.5 mmol) was dissolved in TFA (20 cm^3) at 273 K. After 30 min, the solution was evaporated to give TFA·H-Arg(Tos)-Sar-OBzl. The coupling of Boc-Phg-OH with TFA·H-Arg(Tos)-Sar-OBzl as described above yielded Boc-Phg-Arg(Tos)-Sar-OBzl. Further deprotection by TFA and coupling with Boc-Asp(O*c*Hex)-OH gave Boc-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OBzl as a powder [1.7 g, 35% based on Boc-Arg(Tos)-Sar-OBzl]. Boc-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OBzl had HPLC t_R 27.46 min; FAB-MS *m*/*z* 920 $[(M + H)^+]$.

HCl·H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OH (13·HCl). Under H_2, Boc-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OBzl (1.0 g, 1.1 mmol) was hydrogenated in MeOH (5.0 cm^3) with 5% Pd–charcoal. After 3 h, the catalyst was filtered off and the filtrate was evaporated to give Boc-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OH as an oil. The oil was dissolved in 1,4-dioxane (3.2 cm^3) and 4 mol dm^{-3} HCl–1,4-dioxane (3.2 cm^3) was added. After 2 h, the solution was evaporated and the resultant oil was solidified by addition of diethyl ether, the yield was 0.77 g (93%). HCl·H-Asp(O*c*Hex)-Phg-Arg(Tos)-Sar-OH (**13**·HCl) had HPLC t_R 16.99 min; FAB-MS *m*/*z* 730 $[(M - Cl)^+]$.

HCl·H-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-OH. A mixture of Boc-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-oxime–resin **7** (3.0 g, corresponding to 1.3 mmol peptide) and *N*-hydroxypiperidine[16] (0.40 g, 3.9 mmol) in DMF (45 cm^3) was shaken for 24 h. Then the resin was filtered off, and washed with DMF (45 cm^3, 3 ×). The filtrate and washings were collected and evaporated to dryness. The residue was dissolved in AcOH (45 cm^3), then sodium 'hydrosulfite' ($Na_2S_2O_4$) (1.1 g, 6.5 mmol) and water (6.0 cm^3) were added. After 60 min, the solvent was evaporated off and the residues were solidified with water to afford Boc-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-OH. Treatment with HCl–1,4-dioxane gave HCl·H-Phg-Arg(Tos)-Sar-Asp(O*c*Hex)-OH (0.60 g, 61% based on **7**), FAB-MS *m*/*z* 730 $[(M - Cl)^+]$.

Dimerization–cyclization of compound 13·HCl by the DCC/HOBt/Bu_4NPF_6 system. To an ice-cooled solution of compound **13**·HCl (7.7 mg, 10 μmol), HOBt·H_2O (1.8 mg, 12 μmol) and Bu_4NPF_6 (4.6 mg, 12 μmol) in DMF (1.0 cm^3) were added Et_3N (10 mg, 10 μmol) and DCC (2.5 mg, 12 μmol) successively. The reaction mixture was maintained at 273 K and aliquots (5.0 mm^3) were withdrawn at intervals for HPLC analyses.

Dimerization–cyclization of compound 13·HCl by BOP/HOBt. To an ice-cooled solution of compound **13**·HCl (380 mg, 0.50 mmol) and HOBt·H_2O (92 mg, 0.60 mmol) in DMF (50 cm^3) were added BOP (0.27 g, 0.60 mmol) and Et_3N (0.14 g, 1.4 mmol) successively. After 1 h, the solvent was evaporated off and the residue was solidified by addition of water. The solid was reprecipitated from MeOH–diethyl ether. The yield of cyclic octapeptide **4** was 214 mg (60%).

Deprotection of the cyclic peptide

cyclo(-Arg-Sar-Asp-Phg-) 1. The protected peptide **2** (52 mg, 74 μmol) was treated with anhydrous HF (10 cm^3) in the presence of anisole (1.0 cm^3) at 273 K for 1 h.[17] After evaporation, the residue was dissolved in 10% AcOH and purified by HPLC. The yield of peptide **1** was 14 mg (40%); HPLC t_R 9.89 min; FAB-MS *m*/*z* 476 $[(M + H)^+]$. The following two peptides were similarly prepared.

cyclo(-Arg-Sar-Asp-Phg-)$_2$ 3. HPLC t_R 11.44 min; FAB-MS *m*/*z* 951 $[(M + H)^+]$ (Found: C, 44.5; H, 5.0; N, 15.2. $C_{42}H_{58}N_{14}O_{12}\cdot 2(CF_3CO_2H)\cdot 4H_2O$ requires C, 44.16; H, 5.48; N, 15.67%.)

cyclo(-Arg-Sar-Asp-Phg-)$_3$ 5. HPLC t_R 13.10 min; FAB-MS *m*/*z* 1427 $[(M + H)^+]$.

Biological activity assay of the cyclic peptides

The platelet-aggregation assay and the cell-adhesion assay of the cyclic peptides were performed as described before.[5b]

References

1 T. K. Gartner and J. S. Bennett, *J. Biol. Chem.*, 1985, **260**, 11891; E. F. Plow, M. D. Pierschbacher, E. Ruoslahti, G. A. Marguerie and M. H. Ginsberg, *Proc. Natl. Acad. Sci. USA*, 1985, **82**, 8057; M. Adler and G. Wagner, *Biochemistry*, 1992, **31**, 1031; N. S. Cook, G. Kottirsch and H.-G. Zerwes, *Drugs Fut.*, 1994, **19**, 134 and references cited therein.

2 IUPAC–IUB Joint Commission on Biochemical Nomenclature, *Eur. J. Biochem.*, 1984, **138**, 9.

3 J. Samanen, F. E. Ali, T. Romoff, R. Calvo, E. Sorenson, J. Vasko, B. Storer, D. Berry, D. Bennett, M. Strohsacker, D. Powers, J. Stadel and E. Nichols, *J. Med. Chem.*, 1991, **34**, 3114; L. Alig, A. Edenhofer, P. Hadvary, M. Hurzeler, D. Knopp, M. Muller, B. Steiner, A. Trzeciak and T. Weller, *J. Med. Chem.*, 1992, **35**, 4393; G. D. Hartman, M. S. Egbertson, W. Halczenko, W. L. Laswell, M. E. Duggam, R. L. Smith, A. M. Naylor, P. D. Manno, R. J. Lynch, G. Zhang, C. T.-C. Chang and R. J. Gould, *J. Med. Chem.*, 1992, **35**, 4640; W. C. Johnson, Jr., T. G. Pagono, C. T. Basson, J. A. Madri, P. Gooley and I. M. Armitage, *Biochemistry*, 1993, **32**, 268 and references cited therein.

4 (*a*) H. Kumagai, M. Tajima, Y. Ueno, Y. Giga-Hama and M. Ohba, *Biochem. Biophys. Res. Commun.*, 1991, **177**, 74; (*b*) M. P. Williamson, J. S. Davies and W. A. Thomas, *J. Chem. Soc., Perkin Trans. 2*, 1991, 601; (*c*) R. Mizutani, I. Shimada, Y. Ueno, M. Yoda, H. Kumagai and Y. Arata, *Biochem. Biophys. Res. Commun.*, 1992, **182**, 966; (*d*) R. S. McDowell and T. R. Gadek, *J. Am. Chem. Soc.*, 1992, **114**, 9245; (*e*) K. Kopple, P. W. Baures, J. W. Bean, C. A. D'Ambrosio, J. L. Hughes, C. E. Peishoff and D. S. Eggleston, *J. Am. Chem. Soc.*, 1992, **114**, 9615; (*f*) L.-H. Zhang and P. Ma, *Tetrahedron Lett.*, 1994, **35**, 5765; (*g*) A. C. Bach, II, C. J. Eyermann, J. D. Gross, M. J. Bower, R. L. Harlow, P. C. Weber and W. F. DeGrado, *J. Am. Chem. Soc.*, 1994, **116**, 3207 and references cited therein.

5 (*a*) N. Nishino, M. Xu, H. Mihara, T. Fujimoto, Y. Ueno and H. Kumagai, *Tetrahedron Lett.*, 1992, **33**, 1479; (*b*) A. Isoai, Y. Ueno, Y. Giga-Hama, H. Goto and H. Kumagai, *Cancer Lett.*, 1992, **65**, 259.

6 E. Kaiser, R. L. Colescott, C. D. Bossinger and P. I. Cook, *Anal. Biochem.*, 1970, **34**, 595.

7 N. Nishino, M. Xu, H. Mihara and T. Fujimoto, *Bull. Chem. Soc. Jpn.*, 1992, **65**, 991.

8 G. Ösapay, M. Bouvier and J. W. Taylor, *Techniques in Protein Chemistry II*, Academic Press, New York, 1991, p. 221.

9 D. Hudson, *J. Org. Chem.*, 1988, **53**, 617.

10 R. Schmidt and K. Neubert, *Int. J. Pept. Protein Res.*, 1991, **37**, 502; A. Ehrlich, S. Rothemund, M. Brudel, M. Beyermann, L. A. Carpino and M. Bienert, *Tetrahedron Lett.*, 1993, **34**, 4781;

J. S. McMurray, R. J. A. Budde and D. F. Dyckes, *Int. J. Pept. Protein Res.*, 1993, **42**, 209.
11 N. L. Benoiton, Y. C. Lee, R. Steinaur and F. M. F. Chen, *Int. J. Pept. Protein Res.*, 1992, **40**, 559.
12 A. Pardi, M. Bikketer and K. Wüthrich, *J. Mol. Biol.*, 1984, **180**, 741.
13 K. Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, 1986.
14 T. Sasaki, M. A. Findeis and E. T. Kaiser, *J. Org. Chem.*, 1991, **56**, 3159.
15 B. F. Gisin, *Anal. Chim. Acta*, 1972, **58**, 248.
16 B. O. Handford, J. H. Jones, G. T. Young and T. F. N. Johnson, *J. Chem. Soc.*, 1965, 6814.
17 S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, 1965, **38**, 1412.

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