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New Tripeptidic Thrombin Inhibitors. Influence of P2 and P3 Residues on Activity and Selectivity

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Abstract—Structural variations of P2 and P3 residues in tripeptidic boroarginine thrombin inhibitors led to compounds with similar potency than reference compound DuP 714, but with enhanced selectivity for thrombin compared to plasmin.

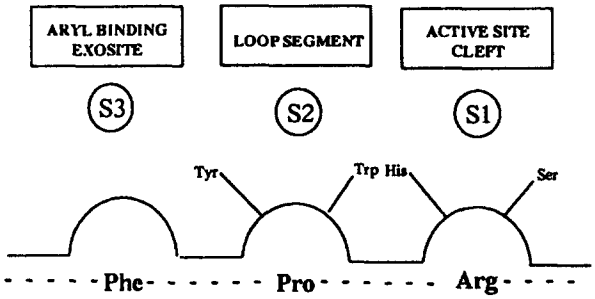
Introduction

The crucial step at the end of the coagulation cascade is the cleavage of soluble fibrinogen to fibrin by the trypsin-like serine protease thrombin; this enzyme is also responsible for the crosslinking of fibrin monomers after the activation of factor XIII. Moreover, thrombin acts on its own formation by a positive feedback mechanism, through activation of factors V and VIII. Finally, binding of thrombin to a specific membrane receptor on platelets initiates both the release reaction and aggregation.

Beside this central role in the process of coagulation, thrombin presents a vast array of cell-activating functions, among which mitogenesis, adhesion and vascular contraction are important examples; thus, a potential role for thrombin in the development of atherosclerosis has recently been proposed.[1] Current anticoagulant therapy is mainly composed of three classes of compounds: 1) heparin; 2) coumarin derivatives; 3) low molecular weight heparins. All these compounds inhibit thrombin by an indirect mechanism (activation of antithrombin III, inhibition of hepatic synthesis of vitamin K-dependent proteins, etc). Only the coumarin analogs demonstrate oral activity, but careful monitoring of the treated patients is necessary in order to avoid side effects with these substances. It is thus worthwhile to search for compounds which act as direct and selective inhibitors of thrombin, and which possess the property of being orally active.

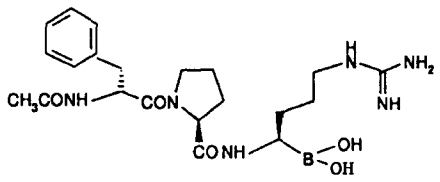
The proteolytic action of thrombin is directed toward a basic residue, with a preference for arginine; for example, fibrinogen is cleaved after the Gly-Val-Arg sequence. It has been shown that, in order to obtain a substrate analog type inhibitor, the optimal sequence to be used was D-Phe-Pro-Arg[2] (Scheme 1).

Recently, DuP 714 (**1**), a tripeptidic derivative in which the above mentioned sequence is appended to a



Scheme 1.

boronic acid, has been shown to be a potent and selective inhibitor of thrombin;[3] moreover, this compound has proven to be orally active as an anticoagulant in rats.[4]



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The goal of our studies was to improve the activity of the modified synthetic tripeptidic inhibitors for thrombin as well as their selectivity toward other proteases of the coagulation cascade and the fibrinolytic system such as plasmin. The main action of plasmin is the digestion of fibrin clots; it is thus important to avoid inhibiting its enzymatic activity.

Crystallographic studies have shown that the specificity of thrombin for its natural substrates is mainly determined by its Tyr 60A–Trp 60D characteristic loop segment. The hydrophobic cavity created by this sequence accomodates very well medium-sized hydrophobic P2 residues such as proline or valine as found in most physiological thrombin substrates and in the most efficient inhibitors.[5] One way to increase selectivity is

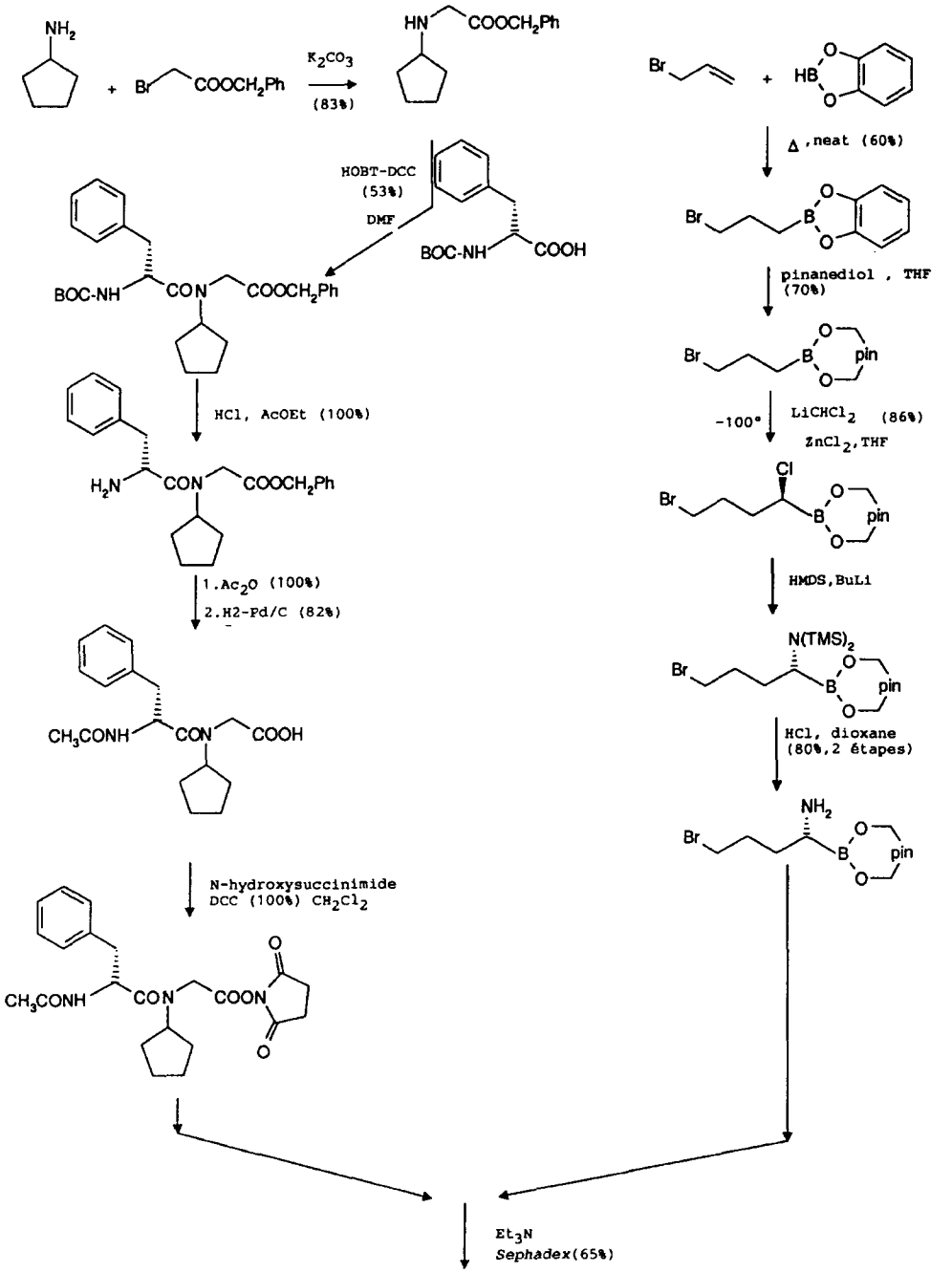
to optimize the filling of this P2 pocket in order to improve hydrophobic interactions between inhibitor and enzyme. Improving the parallel aryl–aryl interactions at P3 'aryl binding site' appeared to be another possibility for modulation of activity and selectivity. Several classical modifications of D-Phe have thus been tested.

Chemistry

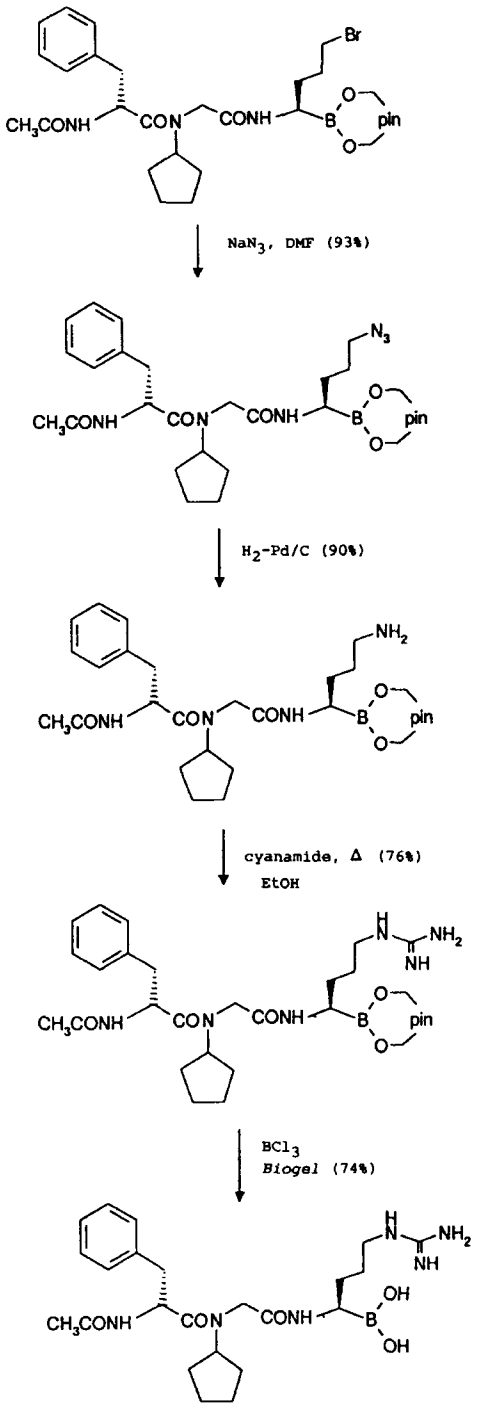
Our compounds were prepared according to the synthetic Scheme 2 described by Kettner,[3] with proline replaced by our original amino acids (see Experimental).

Biochemical Studies

Activity of the compounds was evaluated by determining the concentrations that inhibit the proteasic action of thrombin against its natural substrate, fibrinogen (Enzyme Research Laboratories) by 50% (IC_{50} values expressed in nM). Selectivity was evaluated by comparing this IC_{50} value with that obtained against plasmin, in the presence of the chromogenic substrate, S 2403 (Glu-Gly-Arg-*p*NA; Chromogenix).



Scheme 2.



Scheme 2. *Continued*

Results and Discussion

P2 Variations (Table 1)

Introduction at P2 of cyclic non-natural amino acids afforded derivatives **2** and **3** where proline is respectively replaced by perhydroindole [2*S*,3a*S*,7a*S*] (PHI) and azabicyclo[2.2.2]octane [2*S*] (ABO). In both cases, we obtained potent inhibitors, with subnanomolar IC_{50} values similar to that of DuP 714. Proline was then replaced by *N*-alkyl or *N*-cycloalkyl glycines **3** to **14**. Similar potency in the inhibition of thrombin than with DuP 714 was observed with *N*-cyclopropyl to *N*-cyclopentyl derivatives **4**–**6**. *N*-Cyclohexyl derivative **7** was twice less potent, and activity dropped sharply when proline was replaced by the bulky *N*-indan-1-yl glycine **8**. Mono or dimethylation of the cyclopentyl ring had no effect on thrombin inhibition (compounds **9** to **11**), nor did the introduction of an unsaturation within the ring (compound **12**) as well as bridging to *N*-norbornyl glycine (**13**). No gain in activity was observed when the five-membered ring was opened to yield *N*-isopentyl glycine (**14**), but homologation to *N*-cyclopentyl β-alanine (**15**) resulted in a dramatic loss in activity.

Finally, substitution on the nitrogen of glycine is necessary to obtain a strong activity, since compound **16** (proline replaced by glycine itself) is four times less potent than its best counterparts.

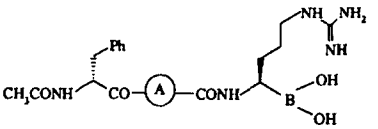
In terms of selectivity thrombin versus plasmin, the PHI and ABO derivatives **2** and **3** behaved comparably to DuP 714. However, replacement of proline by an *N*-cycloalkyl glycine decreased the plasmin inhibition potency at least ten times compared to DuP 714. This gain in selectivity culminated with *N*-cyclohexyl glycine derivative **7** which presents an IC_{50} value for plasmin inhibition approaching 2 μM. Increasing the size of the substituent to indan-1-yl or norbornyl gave compounds **8** and **13** with reduced selectivity. Curiously, compound **16**, without any substituent on nitrogen, was fairly selective for thrombin versus plasmin.

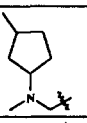
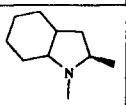
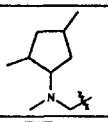
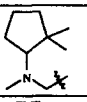
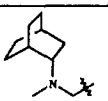
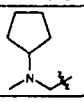
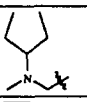
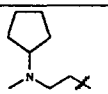
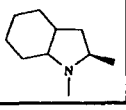
P3 Variations (Table 2)

Classical modifications were made to validate the hypothesis of the implication of the 'aryl binding site' in the selectivity profile of the inhibitors. With P2 residue as an *N*-cyclopentyl glycine, inversion of configuration (compound **17**) was deleterious for inhibition potency. Replacement of the phenyl ring of D-Phe by cyclohexyl (**19**), thienyl (**20**) or naphthyl (**21** and **22**) was without effect on the activity. Boc-protected N terminal amino group gave compound **23** with slightly diminished activity, and free amino group containing compound **24** was unstable in the biological experimental conditions, probably because of intramolecular cyclisation. No improvement in selectivity for thrombin versus plasmin was observed with this latter class of compounds as compared to the reference Ac-D-Phe derivatives described in the first part.

In summary, a potent activity is conserved when proline is replaced by different *N*-cycloalkyl glycines; however increasing too much the size of the substituent has a deleterious effect on activity. It is more interesting to note that chemical modulation of the interaction at the P2 site allows a direct approach to the optimization of selectivity for thrombin versus plasmin.

Table 1. P2 variations



S	(A)	THR IC50 (nM)	PLA IC50 (nM)	S	(A)	THR IC50 (nM)	PLA IC50 (nM)
1		0.43 ± 0.01 n = 5	28 ± 6 n = 5	9		0.62 ± 0.06 n = 5	198 ± 7 n = 3
2		0.51 ± 0.03 n = 3	31.3 ± 12.8 n = 3	10		0.72 ± 0.04 n = 4	266 ± 15 n = 3
3		0.53 ± 0.04 n = 3	20.3 ± 4.5 n = 3	11		0.56 ± 0.03 n = 5	259 ± 8 n = 2
4		0.50 ± 0.04 n = 4	398 ± 9 n = 3	12		0.86 n = 1	n.t.*
5		0.53 ± 0.01 n = 5	577 ± 12 n = 3	13		0.76 ± 0.1 n = 3	210 n = 1
6		0.38 ± 0.01 n = 5	277 ± 38 n = 5	14		0.72 ± 0.08 n = 4	329 ± 39 n = 2
7		1.03 ± 0.11 n = 4	1089 ± 38 n = 3	15		30.1 ± 2.6 n = 3	1101 n = 1
8		1.71 ± 0.05 n = 2	214 ± 14 n = 3	16		1.92 n = 1	753 ± 32 n = 2

n.t.*: not tested
THR: Thrombin, PLA: Plasmin

It would be interesting to confirm by molecular modeling studies that the P2 residue plays a critical role in the interaction of these inhibitors with plasmin. Studies on the selectivity of our compounds for thrombin compared to other enzymes, as well as evaluation of their potential as orally active thrombin inhibitors *in vivo* are currently in progress.

Experimental

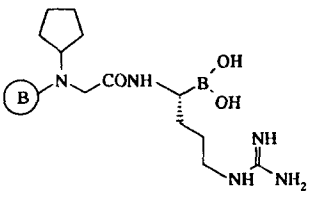
Elemental analyses were carried out by the Analytical Department of the Institut de Recherche Servier. ^{1}H NMR spectra of deuteriochloroform or DMSO-d_6 solutions (chemical shifts are given in ppm with TMS as the internal standard) were recorded on Bruker AC200 or AM300 spectrometers: *s* = singlet, *d* = doublet, *t* = triplet, *q* = quadruplet, *m* = multiplet. The preparation of [(+)-1,2-pinanediyl]-1(*R*)-amino-4-bromo-butylboronate (boroamine) is described in Ref. 3.

*Benzyl-(*N*-tert-butyloxycarbonyl-*D*-phenylalanyl)-(*N*-cyclopentyl) glycinate*

N-(*t*-Butyloxycarbonyl-D-phenylalanine) hydrochloride (25 g, 0.094 mol) is dissolved in anhydrous DMF (100 mL) and the solution is cooled to 0 °C. Triethylamine (13.1 mL), is added, followed by *N*-cyclopentylglycine benzylester (25.4 g, 0.094 mol), HOBT (14.4 g, 0.094 mol) and finally DCC (19.4 g, 0.094 mol). After 48 h at room temperature, DCU is filtered, and the solvent is evaporated. The residue is taken up by ethyl acetate, filtered, washed with water, 10% citric acid and brine. Drying on $CaSO_4$ and evaporation of the solvent gives 45 g of a crude dark orange semi solid.

Purification on silica gel (Merck, 7734, CH_2Cl_2:AcOEt 98:2) afforded 25 g (55%) of pure title compound as a thick pale yellow oil. ^{1}H NMR δ: 1.1–1.9 (8H, *m*), 1.35 (9H, *s*), 2.7 (2H, *t*), 3.95 (2H, *m*), 4.2–4.5 (1H, *m*) 4.5–4.75 (1H, *m*), 5.1 (2H, *s*), 7.05 (1H, *d*), 7.1–7.5 (10H, *m*).

Table 2. P3 variations



S	B	THR	PLA
		IC_{50} (nM)	
6	Ac-D-Phe	0.38 ± 0.01 n = 5	277 ± 38 n = 5
17	Ac-L-Phe	0.95 ± 0.05 n = 5	1623 ± 20 n = 2
18	Ac-D,L-Phe	0.70 ± 0.11 n = 3	738 ± 87 n = 3
19	Ac-D-Cha	0.70 ± 0.02 n = 3	184 n = 1
20	Ac-D-3-Thi	0.76 ± 0.06 n = 3	522 n = 1
21	Ac-D-1-Npa	0.63 ± 0.09 n = 5	348 ± 27 n = 2
22	Ac-D-2-Npa	0.70 ± 0.07 n = 3	263 ± 11 n = 2
23	Boc-D-Phe	0.87 ± 0.00 n = 2	146 n = 1
24	H-D-Phe	-	-

Benzyl-(D-phenylalanyl)-(N-cyclopentyl) glycinate

Deprotection is carried out by treating the compound obtained at the previous step (15 g, 0.031 mol) in 150 mL of anhydrous ethyl acetate by gaseous HCl during 1 h at 0–5 °C. After warming at room temperature and stirring during 1 h, the solvent is evaporated and the solid residue is dried to give 11.8 g (100%) of a white amorphous solid. ^{1}H NMR δ: 1.0–1.9 (8H, *m*), 3.0 (2H, *m*), 3.8–4.15 (3H, *m*), 4.75 (1H, *t*), 5.15 (2H, *m*), 7.3–7.4 (10H, *m*), 8.4 (3H, *brs*).

Benzyl-(N-acetyl-D-phenylalanyl)-(N-cyclopentyl) glycinate

The dipeptide obtained in step 2 (2.1 g, 0.005 mol) is dissolved in dioxane–water (10 + 5 mL), and acetic anhydride (2.4 mL, 0.0025 mol) is added. After 3 h at room temperature, the solvent is evaporated. The residue is taken up by ethyl acetate, washed with water and brine. After drying ($CaSO_4$) and evaporation of the solvent, 2.15 g (92%) of a pale yellow oil is obtained. ^{1}H NMR δ: 1.15–1.7 (8H, *m*), 1.75 (3H, *s*), 2.8 (2H, *m*), 3.95 (2H, *dd*), 4.4 (1H, *m*), 5.05 (1H, *m*), 5.15 (2H, *s*), 7.2 (5H, *m*), 7.35 (5H, *m*). Mass spectrometry (e.i.): m/z = 422.

(N-Acetyl-D-phenylalanyl)-N-cyclopentyl glycine

The benzyl ester (42 g, 0.099 mol) is hydrogenated at room temperature and pressure in anhydrous ethanol (500 mL) with 5 g of 10% Pd/C (Engelhard, ref. 5026) as a catalyst during 4 h. Filtration and evaporation affords 27 g (82%) of a white amorphous solid. ^{1}H NMR δ: 1.2–1.7 (8H, *m*), 1.7 (3H, *s*), 2.8 (2H, *dd*), 3.75 (2H, *dd*), 4.3 (1H, *m*), 5.05 (1H, *m*), 7.2 (5H, *m*), 8.2 (1H, *d*).

Succinimidyl-(N-acetyl-D-phenylalanyl)-N-cyclopentyl glycinate

To a solution of 280 mg (0.0025 mol) of *N*-hydroxysuccinimide in 30 mL of dichloromethane at 0 °C, the dipeptide (830 mg, 0.0025 mol) is added neat. DCC (515 mg, 0.0025 mol) is then added in 20 mL of dichloromethane. The solution is stirred for 18 h at room temperature, then DCU is filtered, rinced with dichloromethane and the filtrate is evaporated. A white solid (1.1 g, 100%) is obtained. ^{1}H NMR δ: 1.3–1.8 (8H, *m*), 1.75 (3H, *s*), 2.8 (4H, *s*), 2.85 (2H, *dd*), 4.25 (2H, *dd*), 4.3 (1H, *m*), 5.05 (1H, *q*), 7.2 (5H, *m*).

[(+)-1,2-Pinanediyl]-1-(R)-[N-acetyl-D-phenylalanyl]-[N-cyclopentylglycylamino]-4-bromobutylboronate

The 'boroamine' as hydrochloride (2.52 g, 0.0069 mol) is dissolved in 55 mL of dichloromethane. The succinimidyl ester (2.68 g, 0.0062 mol) in 55 mL of dichloromethane is added at room temperature. The resulting solution is then cooled at –20 °C and triethylamine (0.96 mL, 0.0068 mol) is added dropwise. The reaction is then stirred at room temperature overnight.

After evaporation of dichloromethane, the residue is taken up by ethyl acetate, washed with water, a saturated sodium bicarbonate solution, 0.2 N HCl, water and brine. Drying on $CaSO_4$, filtration and evaporation afforded 3.8 g of a crude white powder. It is purified on a 5 × 100 cm LH-20 Sephadex column, (elution: methanol). After evaporation and drying 2.5 g, (62%) of the desired compound is obtained. ^{1}H NMR δ: 0.8 (3H, *s*), 1.2 (6H, *s*), 1.2–2.1 (17H, *m*), 1.75 (3H, *s*), 2.2 (1H, *m*), 2.45 (1H, *m*), 2.9 (2H, *dd*), 3.5 (2H, *t*), 3.95 (2H, *dd*), 4.05 (1H, *d*), 4.6 (1H, *m*), 4.95 (1H, *t*), 7.25 (5H, *m*).

[(+)-1,2-Pinanediyl]-1-(R)[N-acetyl-D-phenylalanyl]-[N-cyclopentylglycylamino]-4-azidobutylboronate

The bromo-derivative (2.5 g, 0.0039 mol) in 5 mL of anhydrous DMF is treated with 0.5 g (0.0078 mol) of sodium azide. After warming at 100 °C for 4 h, the DMF is evaporated. The resulting residue is taken up by ethyl acetate, washed with water, brine and dried over calcium sulfate. After filtration and evaporation, the azido derivative is obtained as a brownish solid (2.19 g, 93%). ^{1}H NMR δ: 0.8 (3H, *s*), 1.2 (6H, *s*), 1.2–2.1 (17H, *m*), 1.75 (3H, *s*), 2.2 (1H, *m*), 2.45 (1H, *m*), 2.9 (2H, *dd*), 3.3 (2H, *t*), 3.95 (2H, *dd*), 4.05 (1H, *d*), 4.6 (1H, *m*), 4.95 (1H, *t*), 7.25 (5H, *m*).

[(+)-1,2-Pinanediyl]-1-(R)[N-acetyl-D-phenylalanyl]-[N-cyclopentylglycylamino]-4-aminobutylboronate benzenesulfonate

The azido derivative (2.19 g, 0.0036 mol) is hydro-

genated with benzene sulfonic acid (0.57 g, 0.0036 mol) and 10% Pd/C (0.5 g, Engelhard, ref. 5026) in anhydrous methanol (70 mL), at room temperature and pressure. After 7 h, the catalyst is filtered and the solvent evaporated. The desired amino derivative (2.4 g, 90%), as a benzenesulfonate salt is obtained. ^{1}H NMR δ: 0.8 (3H, *s*), 1.2–1.9 (17H, *m*), 1.25 (6H, 2*s*), 1.8 (3H, *s*), 2.05 (1H, *m*), 2.4 (1H, *m*), 2.6–3.0 (4H, *m*), 3.9 (2H, *m*), 4.2 (1H, *m*), 4.4 (1H, *m*), 4.8 (1H, *m*), 7.25 (8H, *m*), 7.65 (2H, *m*).

*[(+)-1,2-Pinanediyl]-1-(*R*)[N(acetyl-*D*-phenylalanyl)-N-cyclopentylglycylamino]-4-guanidinobutylboronate benzene sulfonate*

The amino derivative (2.4 g, 0.0032 mol) and cyanamide (0.55 g, 0.013 mol) are heated at 100 °C during 48 h in 4.5 mL of anhydrous ethanol. After evaporation of ethanol, the crude mixture is chromatographed on LH-20 Sephadex with methanol as eluent to give 1.91 g (75%) of the desired boroarginine. ^{1}H NMR δ: 0.8 (3H, *s*), 1.2–2.0 (17H, *m*), 1.2 (6H, *s*), 1.5–2.2 (2H, *m*), 1.8 (3H, *s*), 2.5 (1H, *m*), 2.8–3.0 (2H, *m*), 3.1 (2H, *m*), 3.9 (2H, *m*), 4.15 (1H, *m*), 4.3 (1H, *m*), 4.8 (1H, *m*), 7.3 (8H, *m*), 7.65 (2H, *m*).

*1-(*R*)-[(N-Acetyl-*D*-phenylalanyl)-N-cyclopentylglycyl-amino]-4-guanidino butylboronic acid monoacetate*

Deprotection of the boronic acid is carried out by treating dropwise the pinanediyl-boroester (1.91 g, 0.0024 mol) in dichloromethane (27 mL) with 9.6 mL (0.0096 mol) of a 1 M solution of BCl_3 in CH_2Cl_2 at –65 °C. The temperature is then allowed to rise to 0 °C and stirring is continued for 1 h at this temperature. Quenching is then performed with 9 mL of cold water. The organic layer is extracted with water. Combined aqueous phases are washed with ether and evaporated. The resulting residue is purified on a 5 × 100 cm P2 BioRad™ column with H_2O:CH_3COOH 9:1 as eluent.

After evaporation, the pure compound is dried over KOH, then lyophilized (0.96 g, 72%). Preparative HPLC (Hyperprep, H_2O:CH_3CN:CH_3SO_3H 825:175:2) was performed on this purified material to eliminate the trace amounts of the undesired diastereoisomer. Elemental analysis: %C calcd 54.75, found 55.37; %H calcd 7.54, found 7.08; %N calcd 15.32, found 14.88. Mass spectrometry: FAB$^+$ (glycerol): M^+ (m/z) = M – $2H_2O$ + H^+ + glycerol = 545. ^{1}H NMR δ: 1.0–1.7 (12H, *m*), 1.9 (6H, 2*s*), 2.6 (1H, *m*), 3.05 (2H, *m*), 3.2 (2H, *m*), 4.0 (2H, *dd*), 4.3 (1H, *m*), 5.15 (1H, *dd*), 7.3 (5H, *m*).

Acknowledgments

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