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ORIGINAL ARTICLE

A new non-natural arginine-like amino acid derivative with a sulfamoyl group in the side-chain

Rosaria De Marco · Maria L. Di Gioia · Antonella Leggio · Angelo Liguori · Francesca Perri · Carlo Siciliano · Maria C. Viscomi

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Abstract Sulfamoylation of the L-ornithine methyl ester side-chain generates a non-natural arginine isostere which can be coupled with *N*-Fmoc-L-proline to synthesize analogues which maintain the structural characteristics of the biologically important Pro-Arg dipeptide sequence. As a probe of its biological importance, the sulfamoylated amino acid derivative was also incorporated as P1 residue in tripeptide structures matching the *C*-terminal subsequence of fibrinogen. The reported results demonstrate that the functionalization of L-ornithine side-chain with a neutral sulfamoyl group can generate an arginine bioisostere which can be used for the synthesis of prototypes of a new class of human thrombin inhibitors.

Keywords Ornithine · Sulfamoyl group · Arginine bioisosteres · Tripeptides · Thrombin inhibitors · Anticoagulants

Abbreviations

DMF	*N*,*N*-Dimethylformamide
MTBE	Methyl-*tert*-butyl-ether
DMAP	4-(*N*,*N*-Dimethylamino)pyridine
DIEA	*N*,*N*-Diisopropylethylamine
HOBt	1-Hydroxybenzotriazole
EDCI	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
TFA	Trifluoroacetic acid
α-CHCA	α-Cyano-4-hydroxy-*trans*-cynnamic acid
DEPT	Distorsionless enhancement by polarization transfer
TOCSY	Total correlation spectroscopy
DQF-COSY	Double quantum filtered correlation spectroscopy
TLC	Thin layer chromatography
FCC	Flash column chromatography

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R. De Marco · M. L. Di Gioia · A. Leggio · A. Liguori (✉) · F. Perri · C. Siciliano · M. C. Viscomi
Dipartimento di Scienze Farmaceutiche,
Università della Calabria, Via P. Bucci, Cubo 15/C,
87036 Arcavacata di Rende (CS), Italy
e-mail: A.Liguori@unical.it

Introduction

Arginine is a natural amino acid containing a strongly basic guanidine residue which is protonated at physiological pH values. Either alone or as a constituent of peptide structures, arginine plays a leading role in establishing non-covalent interactions with the active sites of a large number of enzymes involved in biologically important processes (Schug and Lindner, 2005; Tyndall et al. 2005; Hadden et al. 2005; Maryanoff 2004; Sugase et al. 2004; Reddy et al. 2004; Tung and Weissleder 2003; Kim et al. 2003; Rockwell et al. 2002; Pellegrini et al. 2002; James et al. 1999; Shearer et al. 1997) and of the eukaryotic 26S proteasome (Groll et al. 2006). Arginine-rich peptide motifs bound to RNA and DNA strands are also involved in the natural fate of nucleic acids (Austin et al. 2002). However, the requirement for arginine as the major determinant for selective recognition in biological systems is not rigorous. In fact, protonation of the guanidine residue determines lack of oral bioavailability and selectivity, and increases the toxicity of many arginine containing substrates and

inhibitors. Replacement of arginine by any other amino acid in homologous peptide sequences, modification of its side-chain or guanidine moiety (Kokko et al. 2001) by introducing non-polar and/or neutral groups of reduced basicity, and elimination of the cationic site from the side-chain (Zega et al. 2004; Tapparelli et al. 1993) represent valuable approaches to afford a series of pharmaceutically relevant synthetic peptides (Schmuck and Geiger 2005; Isaacs et al. 2006; Peterlin-Mašič et al. 2003; Powers et al. 2002; Lee et al. 2000) of greater protease specificity, bioavailability, inhibition potency, and stability against proteolysis together with improved binding affinities for many biologically relevant receptors (Balbo et al. 2003; Lee et al. 2003; St-Denis et al. 2002; Fischer et al. 2000). The growing demand for an ideal protease inhibitor has increased the need for assorted libraries of synthetic compounds. In this context, non-proteinogenic amino acids are of considerable interest.

Motivated by pharmacological interest, and by the plethora of biological aspects related to the role of Arg and of its modified isosteres, we have exploited a facile synthetic access to a new L-ornithine derivative with the aim to develop a new class of human thrombin inhibitors. We report the synthesis of the polar and uncharged arginine-like derivative **6** (Fig. 1), a compound featuring a masked sulfamoyl group on the δ carbon atom of the side-chain in substitution of the highly basic guanidine residue of natural arginine. The selected group is a constituent of biologically active sulfonamides (Schaal et al. 2001; Langenhan et al. 2001; Supuran et al. 2000) and sulfamoyl carbamates (Dougherty et al. 2005), but among the different moieties proposed for the chemical functionalization of the L-ornithine side-chain (Salemme et al. 1997) no mention can be found in the literature about the employment of the sulfamoyl group. The $NHSO_2NH_2$ moiety in the L-ornithine skeleton could define a particular tetrahedral pharmacophore, surrogate of the guanidine group of arginine, because it is an efficient H-bonding acceptor (Radkiewicz et al. 1998) and should confer to compound **6** a weakly acidic character, due to the possible loss of the SO_2NHR proton (Quan et al. 2003).

Materials and methods

All reagents were used as purchased and without further purification. All solvents were purified and dried by standard procedures and distilled prior to use. Melting points were determined with a Kofler hot-stage apparatus at atmospheric pressure and are uncorrected. Mono-dimensional (1D) ^{1}H-NMR spectra were recorded at 300 MHz, by using a dilute solution of each compound in $CDCl_3$ or DMSO-d_6. Chemical shift values (δ) are

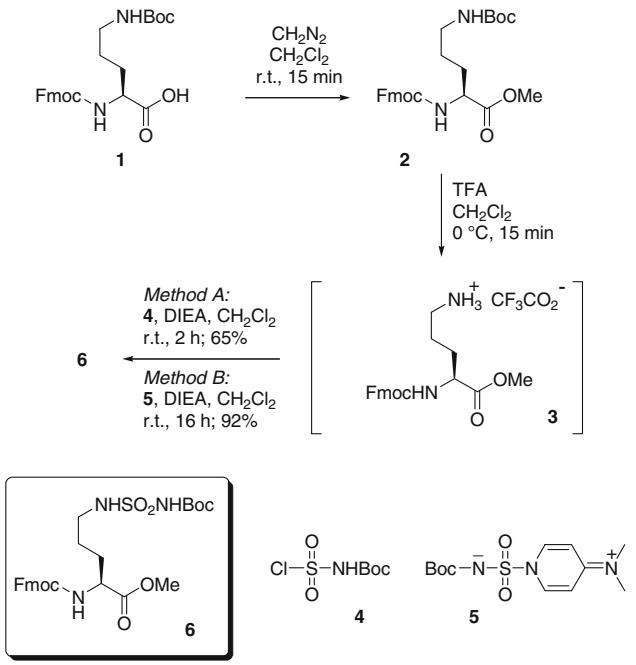

Fig. 1 Synthesis of the amino acid derivative **6**

expressed in ppm and calibrated on the resonance frequency of the central line of the solvent signal ($CDCl_3$: ^{1}H, singlet, 7.24 ppm; ^{13}C, triplet, 77.0 ppm. DMSO-d_6: ^{1}H, quintet, 2.54 ppm; ^{13}C, septet, 39.5 ppm). Coupling constants (J) are reported in Hz. ^{13}C-NMR spectra were recorded at 75 MHz, and ^{13}C-DEPT analyses were obtained with the WALTZ-16 pulse cpd sequence for the proton decoupling resolution. Two-dimensional (2D) homonuclear ^{1}H,^{1}H-TOCSY spectra were obtained using the MLEV pulse sequence for the isotropic mixing (Bax and Davis 1985; Braunschweiler and Ernst 1983) with an 80-ms spin lock period. Coupling constant values were extracted (Rence et al. 1983) from the two-dimensional homonuclear ^{1}H,^{1}H-DQF-COSY (Derome and Williamson 1990) and the mono-dimensional proton analysis. All mono- and two-dimensional spectra were recorded at 298 K, and the spectral results were consistent with the proposed structures. For MALDI MS analysis a 1-μL portion of a premixed solution of each sample and α-CHCA (0.3% in TFA) was spotted on the matrix target, dried at room temperature, and analyzed in the positive mode. The progress of all reactions was monitored by TLC using silica gel 60-F_{254} precoated glass plates, and UV light (254 nm) or 0.2% ninhydrin in ethanol and charring as visualizing agent. Kieselgel 60H without gypsum was used for FCC. The methylene chloride solution of diazomethane was prepared from *N*-methyl-*N*-nitrosourea using a classical procedure (Leggio 1997). The concentration of the diazomethane solution (0.66 M) was obtained by back-titration performed with a standard

benzoic acid solution. (CAUTION: diazomethane is highly toxic; hence, this reagent must be handled carefully). Methylene chloride solutions of diazomethane are stable for long periods if stored on KOH pellets at −20°C. All reactions were performed using flame-dried glassware and under an inert atmosphere (dry N_2). For in vitro clotting assays an automatic coagulometer was used with a maximum coagulation time limit of 500 s for TT and APTT tests. TT and APTT determinations were performed with the kits Dade BC Thrombin Reagent and Dade Actin FSL, respectively.

Synthesis of N^{α}-Fmoc-N^{δ}-Boc-L-ornithine methyl ester (2)

A 0.66 M solution of diazomethane in CH_2Cl_2 (8.5 mL, 5.6 mmol) was added dropwise to a suspension of **1** (0.73 g, 1.6 mmol) in CH_2Cl_2 (5 mL). The resulting mixture was stirred at r.t. for 15 min, and the conversion of the precursor was monitored by TLC ($CHCl_3$:CH_3OH = 95:5). The solvent was removed under reduced pressure condition and the residue was solubilised in AcOEt (5 mL), and washed with a 5% aqueous solution of $NaHCO_3$ (2 × 5 mL) and once with brine (5 mL). The organic extracts were dried over Na_2SO_4, filtered and evaporated to dryness under vacuum. The solid residue was co-evaporated with a 1:1 Et_2O/*n*-hexane mixture and compound **2** was recovered as a white crystalline solid. Yield: 0.72 g, 96%. Mp 149–151°C. $R_f = 0.69$. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.75 (d, J = 7.4 Hz, 2 H), 7.58 (d, J = 7.4 Hz, 2 H), 7.38 (t, J = 7.4 Hz, 2 H), 7.30 (t, J = 7.4 Hz, 2 H), 5.43 (d, J = 8.0 Hz, 1 H), 4.55 (t, J = 6.9 Hz, 1 H), 4.31-4-42 (m, 3 H), 3.74 (s, 3 H), 3.12 (m, 2 H), 1.79-1.94 (m, 1 H), 1.46-1.74 (m; 3 H), 1.42 (s, 9 H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.8, 156.0, 143.8, 141.3, 128.9, 127.7, 125.1, 119.9, 79.3, 77.5, 67.0, 53.6, 52.5, 47.1, 40.0, 29.8, 28.4, 26.1 ppm. Anal. Calcd for $C_{26}H_{32}N_2O_6$: C, 66.65; H, 6.88; N, 5.98. Found: C, 66.86; H, 6.89; N, 5.96.

Synthesis of N^{α}-Fmoc-L-ornithine methyl ester trifluoroacetate (3)

A solution of **2** (0.72 g, 1.54 mmol) in CH_2Cl_2 (3 mL) was treated with a solution of TFA in CH_2Cl_2 (9:1, 5 mL). The resulting mixture was stirred at 0°C for 15 min. After this time the consumption of **3** was complete as checked by TLC ($CHCl_3$:CH_3OH = 95:5; compound **3** furnished a yellow spot to the ninhydrin test), and the volatile components of the mixture were removed under vacuum. The oily residue was co-evaporated with toluene (3 × 5 mL) and finally with a 1:1 MTBE/*n*-hexane mixture. The obtained glassy solid was dried under vacuum and immediately subjected to the next reaction step. $R_f = 0.11$. MS (MALDI) *m/z* Calcd for $C_{21}H_{25}N_2O_4^+$ 369.1810; found: 369.1847 (Δm = + 10 ppm).

N^{α}-Fmoc-N^{δ}-sulfamoyl(Boc)-L-ornithine methyl ester (6)

Method A

A solution of the sulfamoyl chloride **4** (0.95 g, 4.62 mmol), freshly prepared as reported in the literature (Spillane et al. 1998; Kloek and Leschinsky 1976), in dry ethanol-free CH_2Cl_2 (3 mL), was added dropwise to a solution of **3** (0.57 g, 1.54 mmol; amount estimated on the basis of a quantitative transformation of **2** into **3** by acidolysis) in dry ethanol-free CH_2Cl_2 (3 mL) containing DIEA (1.18 mL, 6.78 mmol). The resulting mixture was stirred at r.t. and the progress of the reaction was monitored by TLC ($CHCl_3$:CH_3OH = 95:5). After 2 h, the solvent was removed under vacuum and the oily residue was solubilised in AcOEt (5 mL). The organic solution was washed with a 5% aqueous solution of $KHSO_4$ (3 × 5 mL), a 5% aqueous $NaHCO_3$ (3 × 5 mL) and once with brine (5 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated to dryness under vacuum. The pale yellow solid residue was co-evaporated three times with a 1:1 Et_2O/*n*-hexane mixture to give **6** as a pale yellow glassy solid. FCC showed pure **6** as a pale yellow powder. Yield: 0.55 g, 65%.

Method B

Compound **3**, obtained from the unblocking of **2** (0.46 g, 1 mmol), and DIEA (0.33 ml, 2 mmol) was added to a solution of the azanide **6** (0.3 g, 1 mmol) in dry ethanol-free CH_2Cl_2 (5 mL). The mixture was stirred at r.t. for 16 h, monitoring the conversion of **3** by TLC ($CHCl_3$:CH_3OH = 95:5). The solvent was removed under vacuum and the solid residue was solubilised in EtOAc (10 mL). The organic solution was washed with a 5% aqueous solution of $KHSO_4$ (3 × 5 mL), a 5% aqueous solution of $NaHCO_3$ (3 × 5 mL), and once with brine (5 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated to dryness under vacuum to give pure **6** as pale yellow powder. Yield: 0.49 g, 92%. Mp 119–121°C. $R_f = 0.56$. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.01 (s broad, 1 H), 7.74 (d, J = 7.4 Hz, 2 H), 7.58 (d, J = 7.4 Hz, 2 H), 7.38 (t, J = 7.4, 2 H), 7.30 (t, J = 7.4 Hz, 2 H), 5.44 (d, J = 8.3 Hz, 1 H), 5.35 (t, J = 6.16 Hz, 1 H), 4.20-4.43 (m, 3 H), 4.19 (t, J = 6.7 Hz, 1 H), 3.73 (s, 3 H), 3.08

(m, 2 H), 1.83-2.00 (m, 1 H), 1.54-1.78 (m, 3 H), 1.46 (s, 9 H) ppm. ^{13}C-NMR DEPT (75 MHz, $CDCl_3$) δ 172.8 (CO), 156.1 (CO), 155.9 (CO), 143.8 (C), 141.2 (C), 128.0 (CH), 127.6 (CH), 125.1 (CH), 119.9 (CH), 79.3 (C), 67.0 (CH_2), 53.6 (CH), 52.5 (CH_3), 47.1 (CH), 40.0 (CH_2), 29.7 (CH_2), 28.4 (CH_3), 26.1 (CH_2) ppm. Anal. Calcd for $C_{26}H_{33}N_3O_8S$: C, 57.02; H, 6.07; N, 7.67. Found: C, 57.21; H, 6.09; N, 7.68.

N^{α}-Acetyl-N^{δ}-sulfamoyl-L-ornithine methyl ester (8)

Removal of Fmoc group from **6** by the mercaptoacetic acid/sodium methoxide reagent system (molar ratio **6**/$HSCH_2CO_2H$/MeONa 1:3:5)

Mercaptoacetic acid (0.09 mL, 1.26 mmol) was added to a solution of sodium methoxide (0.11 g, 2.1 mmol) in CH_3OH (1 mL) at 0°C. A solution of **6** (0.23 g, 0.42 mmol) in CH_3CN (10 mL) was then added and the resulting mixture was maintained under magnetic stirring at 50°C. After 3.5 h, TLC ($CHCl_3$:CH_3OH = 95:5) showed the complete conversion of **6**. The mixture was made acidic (pH 5) by adding 0.1 N aqueous solution of HCl, and extracted with Et_2O (3 × 10 mL). The ethereal extracts were discarded off and the aqueous phase was made basic (pH 8) with a 10% aqueous solution of $NaHCO_3$, and then extracted with AcOEt (3 × 10 mL). The collected organic layers were dried over Na_2SO_4, filtered and evaporated to dryness under vacuum to give an oily residue which was immediately treated with an excess of freshly distilled acetic anhydride, in a 1:1 mixture of CH_2Cl_2 (5 mL) and a 10% aqueous solution of $NaHCO_3$ (5 mL). The biphasic system was vigorously stirred at r.t., and the reaction was monitored by TLC ($CHCl_3$:CH_3OH = 95:5). After 1 h, the mixture was concentrated under vacuum and the aqueous residue was extracted with AcOEt (3 × 5 mL). The organic phase was washed with a 5% aqueous solution of $NaHCO_3$ (3 × 5 mL) and once with brine (5 mL), then dried over Na_2SO_4, filtered, and evaporated to dryness under vacuum. The solid residue was purified by FCC to give the acetylated compound **8** as pale yellow foam with the following yields: 0.03 g, 21%. R_f = 0.34. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.19 (d, J = 8.0 Hz, 1 H), 6.44 (m, 3 H), 4.19 (m, 1 H), 3.62 (s, 3 H), 2.85 (m, 2 H), 1.84 (s, 3 H), 1.37-1.81 (m, 4 H) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6) δ 172.7, 169.4, 51.7, 41.9, 39.8, 28.3, 25.4, 22.1 ppm. Anal. Calcd for $C_8H_{17}N_3O_5S$: C, 35.95; H, 6.41; N, 15.72. Found: C, 36.05; H, 6.40; N, 15.72.

FCC of the crude material recovered from the reaction furnished compound **10** as the principal product. Yield: 0.11 g, 68%.

N^{α}-Acetyl-N^{δ}-sulfamoyl(Boc)-L-ornithine methyl ester (10)

Removal of Fmoc group from **6** by the mercaptoacetic acid/sodium methoxide reagent system (molar ratio **6**/$HSCH_2CO_2H$/MeONa 1:5:8)

A solution of **6** (0.23 g, 0.42 mmol) in CH_3CN (10 mL) was reacted with mercaptoacetic acid (0.15 mL, 2.1 mmol) and sodium methoxide (0.18 g, 3.36 mmol) in CH_3OH (2 mL). TLC monitoring of the reaction mixture showed complete conversion of **6** after 5.5 h at 50°C. The oily residue recovered from the work-up of the reaction mixture (see preparation of compound **8**) was immediately acetylated with an excess of freshly distilled acetic anhydride, in a 1:1 mixture of CH_2Cl_2 (5 mL) and a 10% aqueous solution of $NaHCO_3$ (5 mL). The biphasic system was vigorously stirred at r.t., and the reaction was monitored by TLC ($CHCl_3$:CH_3OH = 95:5). After 1 h, the mixture was concentrated under vacuum and the aqueous residue was extracted with AcOEt (3 × 5 mL). The organic phase was washed with a 5% aqueous solution of $NaHCO_3$ (3 × 5 mL) and once with brine (5 mL), then dried over Na_2SO_4, filtered, and evaporated to dryness under vacuum. The oily residue was chromatographed to give **10** as the only reaction product, as pale yellow foam, with the following yields: 0.14 g, 90%. R_f = 0.65. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.28 (s broad, 1 H), 6.54 (d, J = 7.9 Hz, 1 H), 5.74 (t, J = 6.8 Hz, 1 H), 4.56 (m, 1 H), 3.71 (s, 3 H), 3.05 (m, 2 H), 2.0 (s, 3 H), 1.52-1.96 (m, 4 H), 1.43 (s, 9 H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.9, 170.6, 150.5, 83.5, 52.5, 51.6, 42.9, 29.5, 28.0, 25.1, 22.9 ppm. Anal. Calcd for $C_{13}H_{25}N_3O_7S$: C, 42.50; H, 6.86; N, 11.44. Found: C, 42.59; H, 6.88; N, 11.46.

Synthesis of dipeptide 12

A solution of Fmoc-Pro-OH (0.14 g; 0.42 mmol) in dry ethanol-free CH_2Cl_2 (2 mL) was treated with HOBt monohydrate (0.07 g, 0.46 mmol), EDCI (0.1 g, 0.52 mmol), DIEA (0.18 mL, 1.03 mmol), and the resulting mixture was stirred at r.t. for 12 h. Compound **9** (0.14 g, 0.42 mmol; amount estimated on the basis of a complete conversion of **6** in the unblocking step performed as described for the preparation of **10**) solubilised in dry ethanol-free CH_2Cl_2 (2 mL) was then added, and the stirring was maintained for further 12 h. After this time, TLC ($CHCl_3$:CH_3OH = 95:5) showed the complete consumption of **9**. The mixture was then evaporated to dryness under vacuum and the residue was solubilised with AcOEt (5 mL), washed with a 5% aqueous solution of $KHSO_4$ (3 × 5 mL), 5% aqueous solution of $NaHCO_3$

(3 × 5 mL), and once with brine (5 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness under vacuum to give a solid residue which was triturated with a 1:4 MTBE/*n*-hexane mixture. The precipitate was collected and dried under vacuum. Dipeptide **12** was recovered pure as a yellowish powder. Yield: 0.24 g, 88%. ^{1}H-NMR (300 MHz, $CDCl_3$) 75:25 mixture of rotamers A and B δ 8.43 (s broad, 0.75 H, A), 7.74 (d, $J = 7.4$ Hz, 2 H), 7.60 (m, 2 H), 7.52 (s broad, 0.25 H, B), 7.38 (t, $J = 7.4$ Hz, 2 H), 7.29 (t, $J = 7.4$ Hz, 2 H), 6.75 (d, $J = 8.1$ Hz, 0.75 H, A), 6.57 (s broad, 0.25 H, B), 5.46 (t, $J = 6.9$ Hz, 0.75 H, A), 5.33 (s broad, 0.25 H, B), 4.58 (m, 1 H), 4.41 (m, 3 H), 4.13-4.30 (m, 2 H), 3.71 (s, 3 H), 3.31-3.65 (m, 2 H), 2.90-3.29 (m, 2 H), 2.11 (m, 2 H), 1.91 (m, 2 H), 1.56-1.78 (m, 4 H), 1.43 (s, 2.25 H, A + B), 1.39 (s, 6.75 H, A + B) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) 75:25 mixture of the two rotamers A and B δ 172.3, 171.9, 155.7, 150.7, 144.0, 143.7, 141.3, 127.7, 127.1, 125.2, 120.0, 83.5, 67.9, 60.5, 52.5, 51.7, 47.1, 43.1, 31.6, 29.7, 29.5, 28.9, 27.9, 24.8, 24.7 ppm. MS (MALDI) *m/z* Calcd for $C_{31}H_{41}N_4O_9S^+$ 644.2523; found: 644,2452 (Δm = -11 ppm). Anal. Calcd for $C_{31}H_{40}N_4O_9S$: C, 57.75; H, 6.25; N, 8.69. Found: C, 57.94; H, 6.27; N, 8.67.

Synthesis of tripeptide 13

Unblocking of 12

A solution of **12** (0.32 g, 0.5 mmol) in CH_3CN (10 mL) was reacted with mercaptoacetic acid (0.18 mL, 2.5 mmol) and sodium methoxide (0.21 g, 4.0 mmol) in CH_3OH (2 mL). The progress of the reaction was monitored by TLC ($CHCl_3$:CH_3OH = 95:5), showing the complete conversion of **6** after 5.5 h at 50°C. The mixture was then made acidic (pH 5) by adding a 0.1 N aqueous solution of HCl, and extracted with Et_2O (3 × 10 mL). The ethereal extracts were discarded off and the aqueous phase was made basic (pH 8) with a 10% aqueous solution of $NaHCO_3$, and then extracted with AcOEt (3 × 10 mL). The collected organic layers were dried over Na_2SO_4, filtered and evaporated to dryness under vacuum to give a glassy pale yellow solid which was used in the next step of coupling without further purification.

Coupling

A solution of Boc-D-Phe-OH (0.13 g; 0.5 mmol) in freshly distilled DMF (2 mL) was treated with EDCI (0.31 g, 0.52 mmol), HOBt monohydrate (0.08 g, 0.55 mmol), DIEA (0.27 mL, 1.6 mmol), and the resulting mixture was stirred at r.t. for 2 h. The glassy solid obtained from the unblocking of **12** (0.21 g, 0.5 mmol; amount estimated on the basis of a complete conversion of **12** in the unblocking step performed as described) solubilised in freshly distilled DMF (2 mL) was then added dropwise to the mixture, and the stirring was maintained for further 24 h. After this time, TLC ($CHCl_3$:CH_3OH = 95:5) showed the complete consumption of Boc-D-Phe-OH. The mixture was evaporated to dryness under vacuum and the solid residue was solubilised with AcOEt (5 mL), washed with a 5% aqueous solution of $KHSO_4$ (3 × 5 mL), 5% aqueous solution of $NaHCO_3$ (3 × 5 mL), and once with brine (5 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness under vacuum to give a solid residue which was triturated with a 1:4 MTBE/*n*-pentane mixture. The precipitate was collected and dried under vacuum. Tripeptide **13** was recovered pure as yellowish powder. Yield: 0.30 g, 89%. ^{1}H-NMR (300 MHz, $CDCl_3$) 70:30 mixture of rotamers A and B δ 7.18-7.41 (m, 6 H), 7.06 (d, $J = 7.1$ Hz, 1 H), 6.24 (m, 0.3 H, B), 6.02 (m, 1 H), 5.89 (d, $J = 6.9$ Hz, 0.7 H, A), 4.52-4.74 (m, 0.3 H, B), 4.50 (m, 0.7 H, A), 4.30-4.48 (m, 0.3 H, B), 4.28 (m, 0.7 H, A), 4.17 (m, 1 H), 3.70-3.90 (m, 1 H), 3.76 (s, 3 H), 3.52 (m, 0.3 H, B), 3.49 (m, 1 H), 3.28 (m, 0.7 H, A), 2.90-3.16 (m, 0.3 H, B), 2.67 (m, 0.7 H, A), 1.10-2.15 (m, 8 H), 1.52 (s, 3 H), 1.41 (s, 3 H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) 70:30 mixture of the two rotamers A and B δ 172.2, 172.0, 171.7, 170.9, 157.03, 155.3, 136.22, 134.7, 129.8, 129.6, 129.4, 129.1, 128.5, 128.0, 127.1, 126.8, 124.3, 120.4, 60.2, 55.7, 53.8, 52.44, 51.9, 51.4, 49.1, 47.2, 43.0, 41.7, 40.4, 39.4, 33.9, 33.3, 33.1, 32.7, 29.6, 28.3, 28.1, 25.6, 25.3, 25.0, 24.7. MS (MALDI) *m/z* Calcd for $C_{30}H_{48}N_5O_{10}S^+$ 669.3041; found: 669.3135 (Δm = + 14 ppm). Anal. Calcd for $C_{30}H_{47}N_5O_{10}S$: C, 53.80; H, 7.07; N, 10.46. Found: C, 52.7; H, 7.33; N, 10.04.

Synthesis of tripeptide trifluoroacetate 14

A solution of **13** (0.30 g; 0.45 mmol) in CH_2Cl_2 (3 mL) was treated with a solution of TFA in CH_2Cl_2 (9:1, 5 mL). The resulting mixture was stirred at 0°C for 30 min. After this time the consumption of **13** was complete as checked by TLC ($CHCl_3$:CH_3OH = 95:5; compound **14** furnished a yellow spot to the ninhydrin test), and the volatile components of the mixture were removed under vacuum. The oily residue was co-evaporated with toluene (3 × 5 mL), absolute EtOH (3 × 5 mL), and triturated with a 1:2 MTBE/*n*-pentane mixture. The obtained precipitate was collected, dried under vacuum and finally lyophilized to give **14** as a pale yellow glassy solid. Yield: 0.25 g, 90%. $R_f = 0.10$. ^{1}H-NMR (300 MHz, DMSO-d_6/D_2O) δ 7.11-7.40 (m, 5 H), 6.50 (s broad, 2 H), 4.02-4-41 (m, 3 H), 3.61 (s, 3 H), 3.4-3.6 (m, 2 H, partially overlapped by the water signal), 2.68-3.14 (m, 4 H), 1.34-1.90

(m, 8 H) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6/D_2O) δ 172.9, 171.8, 166.8, 134, 94, 130.0, 129.0, 127.9, 59.7, 52.3, 52.1, 49.2, 47.2, 42.5, 37.2, 29.6, 28.6, 26.0, 24.2. MS (MALDI) *m/z* Calcd for $C_{20}H_{32}N_5O_6S^+$ 470.2070; found: 470.2014 ($\Delta m = -12$ ppm). Anal. Calcd for $C_{22}H_{31}N_5O_5S \cdot CF_3CO_2H$: C, 45.28; H, 5.53; N, 12.00. Found: C, 44.02; H, 5.68; N, 11.65.

Clotting assays for tripeptides 13 and 14

Preparation of stock solution

Tripeptides **13** and **14** to be tested were dissolved in DMSO just prior to use and diluted with PBS buffer (pH 8) to yield a final 152 mM stock solution for tripeptide **13**, and a 337 mM stock solution for tripeptide trifluoroacetate **14**.

Clotting assays

In vitro coagulation assays were performed with pooled human plasma. Clotting time was measured using an automatic coagulometer according to the manufacturer's instructions. In order to exclude any influence of DMSO in clotting assays, a blank prepared by mixing the pooled human plasma (800 µL) and DMSO (200 µL) was subjected to TT and APTT tests. Control values of both the parameters were not modified with respect to those registered for a blank prepared by mixing the pooled human plasma (800 µL) and PBS (200 µL).

Determination of thrombin time (TT)

To each of the tubes containing plasma (800 µL), increasing amounts (25, 50, 75, 100, 125, and 150 µL; corresponding to 8.4, 16.8, 25.3, 33.7, 42.1, and 50.5 µmol, respectively) of the stock solutions of tripeptide **14** were added. The same protocol was applied to prepare samples of tripeptide **13**. The resulting samples were diluted to a total volume of 1 mL by adding PBS, vortexed, and incubated at 37°C for 1 minute, and then placed on the instrument sample wheel. The coagulometer automatically dispensed thrombin time reagent in the sample of each tube. The time for the appearance of a fibrin clot was measured and each value was obtained from a set of three separate experiments. Control value of clotting time for TT tests was determined for three experiments using a blank prepared by mixing the pooled human plasma (800 µL) and PBS (200 µL), and was 15.5 s (mean value; SD = ±0.6; $n = 3$). Clotting time values exceeding the coagulometer time limit (500 s) were not determined.

Determination of activated partial thromboplastin time (APTT)

A second series of samples prepared as described for TT determinations was treated with the activated partial thromboplastin time reagent. The coagulometer automatically dispensed the appropriate reagent in the sample of each tube. The time for the appearance of a fibrin clot was measured and each value was obtained from a set of three separate experiments. The same blank used for the control value of clotting time for TT tests was used to determine a control value of 31.2 sec (mean value; SD = ±1.2; $n = 3$) for clotting time in APTT tests. Clotting time values exceeding the coagulometer limit (500 s) were not determined.

Results and discussion

The preparation of **6** started from N^{α}-Fmoc-N^{δ}-Boc-L-ornithine (**1**), and involved a three-step procedure (Fig. 1).

Methyl ester **2** was obtained by treatment of the starting precursor **1** with a dichloromethane solution of diazomethane (Di Gioia et al. 2003), at room temperature. Methylation afforded **2** in yields of 96%, and pure enough to be subjected to the next synthetic step without need for chromatography. Removal of the Boc group from **2** was performed by acidolysis at 0°C, using TFA. Conversion of **2** was complete and the resulting salt **3**, not isolated, was immediately subjected to sulfamoylation. The α-amino function in the side-chain of **3** was finally functionalized using the sulfamoylating reagent **4** (Fig. 1, Method A), in the presence of DIEA. Reagent **4** was efficiently prepared from commercially available chlorosulfonyl isocyanate (CSI) and *tert*-butanol in dichloromethane, under the experimental conditions already described for conventional routes to this reagent (Spillane et al. 1998; Kloek and Leschinsky 1976). Although **4** is rapidly formed in a quantitative yield by this approach, its use in the derivatization of the salt **3** proved to be troublesome. The chloride **4** is very unstable, and it cannot be stored for a long period. This aspect limits the possibility of an exact stoichiometric dosage of **4** imposing that the reagent must be prepared immediately before any sulfamoylation step. Moreover, under the experimental conditions adopted for the experiment in which **4** is used, the methyl ester **6** can be recovered as a pure product only after chromatography, and in yields not exceeding 65%. We found the zwitterionic azanide **5** to be the optimal reagent for the sulfamoylation of the trifluoroacetate **3** (Fig. 1, Method B). It can be obtained in very high yields and purity by a known procedure (Winum et al. 2001). Since **5** is a stable crystalline solid, it can also be stored for prolonged periods and easily

dosed for any purpose. The reaction of trifluoroacetate **3** with **5** was performed in the presence of DIEA to generate the free δ-amino group; and a simple work-up of the reaction mixture allowed the recovery of the methyl ester **6** in yields of 92%, with no need for chromatography. The use of a base, e.g. DIEA, is strongly recommended: without the base, compound **6** can be recovered pure only after column chromatography and in yields not higher than 65%. DMAP produced during the sulfamoylation did not provoke removal of the base-labile protecting group Fmoc. The methyl ester **6** was fully characterized by NMR analysis, and both 1D- and 2D-homonuclear techniques confirmed the structure proposed for the new arginine-like derivative **6**. The Fmoc group was removed from the α-amino function of **6** avoiding the use of nitrogenated species. This was done in order to facilitate the separation of the unprotected methyl ester from the crude reaction mixture. As reported elsewhere (Di Gioia et al. 2004a, b; Leggio et al. 2000), the Fmoc group can alternatively be removed from the corresponding methyl esters by reagent systems composed of $AlCl_3$ and toluene or *N*,*N*-dimethylaminoaniline, but acid-labile protecting groups, e.g. Boc, are not compatible with the experimental conditions adopted for the Lewis acid-assisted treatment. Fmoc group was straightforwardly removed by treating **6** with the reagent system composed of mercaptoacetic acid and sodium methoxide, upon conditions similar to those adopted for the removal of the nosyl group from *N*-methylated amino acid derivatives (Di Gioia et al. 2003). The appropriate stoichiometric ratio between the two components of the reagent system, referred to one equivalent of **6**, was found to be 5:8. Upon these conditions, treatment of **6** at 50°C for 5.5 h afforded **9** (Fig. 2), which was not separated and immediately transformed into the corresponding acetyl derivative **10** by treating the crude material obtained from the unblocking step with an excess of acetic anhydride. Compound **10** was obtained in yields of 90%, which was reliable with the complete conversion of the precursor **6** into **9**. Removal of the Fmoc masking group was chemospecific, since both the Boc-protected sulfamoyl moiety and the methyl ester functionality were not affected by the treatment. A different composition of the reagent system mercaptoacetic acid/sodium methoxide, e.g. a 3:5 stoichiometric ratio, gave **10** in lower yields (68%) together with modest amounts (21%) of **8**. The latter compound was probably formed through the unprotected intermediate species **7**, which could be generated by a concomitant removal of both the Fmoc and Boc masking groups (Fig. 2).

Since proline occupies a special place among the natural amino acids, we coupled **9** with *N*-Fmoc-L-proline. The sequence Pro–Arg, in fact, is often selected as an ideal chemical probe in conformational studies, and in

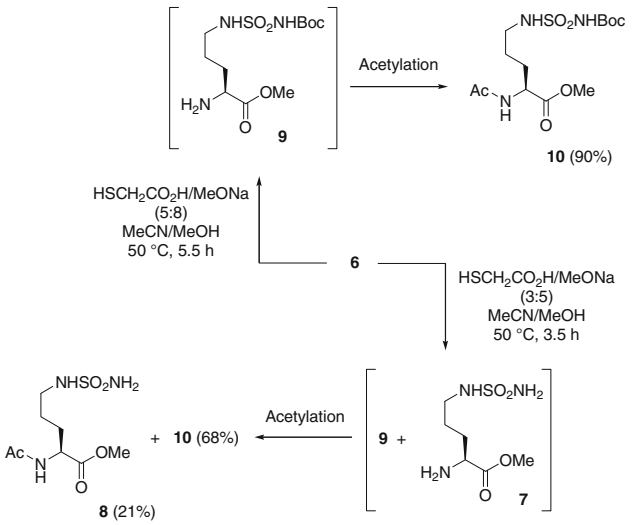

Fig. 2 Removal of the Fmoc group from **6**

determining the absolute specificity and bioavailability of peptide inhibitors of proteolytic enzymes (Koskinen et al. 2005; Groll et al. 2002; De Tar De Los and Luthra 1977). In order to evaluate the structural and conformational role of **6** in short peptide sequences, we prepared the dipeptide *N*-Fmoc-L-Pro-L-Orn-(N^{δ}-SO_2NHBoc)-OMe (**12**) by adopting a Fmoc-chemistry protocol usually used for the solution synthesis of peptides, upon conditions that can avoid the racemisation of the reaction partners (Romoff 2003). *N*-Fmoc-L-proline (**11**) was activated with the system HOBt/EDCI/DIEA and allowed to react with **9**, obtained from **6** after removal of the Fmoc group by treatment with a 5:8 mixture of mercaptoacetic acid and sodium methoxide as previously described. The only product obtained from the reaction was dipeptide **12**, which was recovered in yields of 88% after flash column chromatography (FCC). 1D- and 2D-homonuclear NMR analysis of **12** showed resonances which were consistent with the proposed structure. The 1D ^{1}H spectrum, recorded in $CDCl_3$ at 298 K, showed the presence of two sets of rotamers arising from the slow *trans*/*cis* isomerization around the CO–N linkage between the Fmoc group and the proline ring. Based on the integration of non-overlapping signals, e.g. those appearing at 6.75 and 6.57 ppm, attributable to the α-NH proton of the ornithine skeleton included in the *trans* form and the same proton in the *cis* rotamer, respectively, we calculated a *trans*/*cis* ratio of 75:25 for the rotamers. The precise ratio was determined by the integration of the signals of both the α-NH protons which were assigned to the respective rotamer in analogy with the results reported in the literature for small dipeptides containing proline as the *N*-terminal residue (Sugawara et al. 2001). 2D-homonuclear NMR showed resonances clearly attributable to the protons belonging to

the proline and the ornithine skeletons, respectively. In particular, the cross-signals appearing in the contour plot of the TOCSY analysis performed in $CDCl_3$ at 298 K confirmed the presence of the *trans*/*cis* rotamer mixture and showed correlation patterns typical of the peptide sequence Pro-Orn. No other signals relative to protons attributable to any possible diastereomer of **12** appeared in the 1D and 2D proton spectra. On the other hand, the 1D- and 2D-NMR analysis of a sample of the crude dipeptide **12**, revealed resonances attributable only to the given structure, confirming that, limited to the sensitivity of NMR techniques, no racemisation of the α-carbon atom present in **6** occurred during the coupling. Thus, the stereochemistry of the protected arginine-like derivative **6** is retained both during its synthetic process and the Fmoc removal, as well as in the final coupling step.

In an effort to evaluate the biological role of the sulfamoyl group placed in the L-ornithine side-chain, **6** was finally used as building block in the synthesis of serine-protease inhibitors. In particular, compound **6** was coupled with the dipeptide Boc-D-Phe-Pro-OH to prepare tripeptide analogues of the *C*-terminal subsequence of fibrinogen, the natural thrombin substrate. Thrombin, the blood-clotting enzyme, is a serine protease with trypsin-like specificity and is able to cleave Arg-Xaa peptide bonds but only in a very limited number of substrates (Das and Kimball 1995; Bode et al. 1992). This enzyme has a critical position in the blood coagulation cascade and thus a central role in the regulation of haemostasis (Weitz and Crowther 2002; Hauptmann and Stürzebecher 1999). Moreover, for the prevention and treatment of thrombosis the control of thrombin activity is a key target (McDonald 2005) and the discovery of new classes of inhibitors of this enzyme could lead to useful drugs for treating thrombotic disorders, which constitute a serious source of mortality and morbidity in patients worldwide.

Removal of the Fmoc group from **12** was performed as previously described for **6** using the system composed by mercaptoacetic acid and sodium methoxide, in the 5:8 stoichiometric ratio. Boc-D-Phe-OH was then coupled to dipeptide **12**, after activation of the free carboxylic group by the system HOBt/EDCI/DIEA in the presence of DIEA. The fully protected tripeptide *N*-Boc-D-Phe-L-Pro-L-Orn-(N^{δ}-SO_2NHBoc)-OMe (**13**) was recovered pure by FCC in yields of 88%, and ^{1}H and ^{13}C NMR analysis confirmed the expected structure. In particular, the proton spectrum registered in $CDCl_3$ at 298 K showed the presence of a mixture of rotamers with a *trans/cis* ratio of 70:30, due to the different geometries of the peptide bond between the D-Phe and Pro residues. Acidolysis of **13** by using a solution of TFA in CH_2Cl_2 afforded D-Phe-L-Pro-L-Orn-(N^{δ}-SO_2NH_2)-OMe·CF_3COOH (**14**) which was obtained by precipitation of the crude product from a 1:2 MTBE/*n*-pentane mixture and further lyophilisation in order to remove all the residues of *tert*-butanol and any trace of possible co-products generated during the unblocking step. The ^{1}H NMR spectrum of the trifluoroacetate salt **14** registered at 298 K in DMSO-d_6/D_2O showed exchange of all protons on the N atoms, except for those of the SO_2NH_2 moiety, but did not display well resolved resonances for the other protons. Structure of **14** was definitively attributed by correlation with the corresponding resonances observed in the proton spectrum obtained for **13** and the molecular weight of the salt was confirmed by MALDI mass spectrometry.

Both tripeptides **13** and **14** were then subjected to in vitro coagulation assays in order to evaluate, in a set of preliminary experiments, their inhibitory effects against human thrombin. The TT (Thrombin Time), a measure of the thrombin-fibrinogen reaction in vitro (Jim 1957), and the activated partial thromboplastin time (APTT), the parameter used to evaluate the anticoagulant effect on the thrombin produced by the intrinsic pathway of the coagulation cascade (Proctor and Rappaport 1961), were determined using pooled human plasma treated with samples of **13** and **14** at different concentrations. Among the two tripeptides, **13** did not show appreciable inhibitory potency in all the performed experiments, while **14** exhibited a good and dose-dependent activity as determined by the clotting time of human plasma in TT and APTT tests. In particular, a sample containing 8.4 μmol of trifluoroacetate **14** prolonged the clotting time of pooled human plasma to 217.2 s (mean value, SD = ±10.2; $n = 3$), about three times the control value fibrin clot time in TT determination, and to 65.4 s (mean value, SD = ±2.5; $n = 3$), twice the corresponding control value in APTT measurements. With a more concentrated sample of **14** (25.3 μmol) a TT value of 493.7 s was recorded, while APTT was 123.8 s. Another sample containing 33.7 μmol of **14** showed a not detectable TT, with a value exceeding the coagulometer time limit (500 s), and an APTT value of 296.8 s was recorded. A not detectable (>500 s) APTT value was also obtained when a sample of the potential thrombin inhibitor was prepared using 42.1 μmol of tripeptide **14**.

Conclusion

In conclusion, we have synthesized the non-natural arginine-like α-amino acid derivative **6** which displays on the side-chain a masked sulfamoyl moiety. Compound **6** can easily be coupled with the dipeptide motif D-Phe-Pro to produce tripeptides of high biological interest. Since the neutral polar function in the side-chain of **6**, at physiological pH values, is certainly capable of H-bonding through its heteroatoms (Garrett et al. 1964), it could be

regarded as an efficient anchoring moiety for a wide series of substrates and inhibitors in their binding with the recognition subsites of biological receptors. In particular, rational modification of the *C*-terminal tripeptide lead structure found in fibrinogen by inserting **6** in substitution of the natural arginine at P1 position generates compounds which are active as thrombin inhibitors. Thus, **6** can really regarded as an arginine mimic and could be strategic for studies on the importance of the physiological role of different kinds of proteases, especially in the discovery of promising candidates for future development of clinically useful agents.

The synthesis of the target peptide and the evaluation of its anticoagulant activity constitute only the initial phase of a more complex process that involves more experiments that could subsequently enhance or reduce the interest for the same product with regard its use in real situations.

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