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Protease Inhibitors, Part 13: Specific, Weakly Basic Thrombin Inhibitors Incorporating Sulfonyl Dicyandiamide Moieties in their Structure

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A series of compounds has been prepared by reaction of dicyandiamide with alkyl/arylsulfonyl halides as well as arylsulfonylisocyanates to locate a lead for obtaining weakly basic thrombin inhibitors with sulfonyldicyandiamide moieties as the S1 anchoring group. The detected lead was sulfanilyl-dicyandiamide (K_I of 3 μM against thrombin, and 15 μM against trypsin), which has been further derivatized at the 4-amino group by incorporating arylsulfonylureido as well as amino acyl/dipeptidyl groups protected at the amino terminal moiety with benzyloxycarbonyl or tosylureido moieties. The best compound obtained (ts-D-Phe-Pro-sulfanilyl-dicyandiamide) showed inhibition constants of 9 nM against thrombin and 1400 nM against trypsin. pKa measurements showed that the new derivatives reported here do indeed possess a reduced basicity, with the pKa of the modified guanidine moieties in the range 7.9–8.3 pKa units. Molecular mechanics calculations showed that the preferred tautomeric form of these compounds is of the type $ArSO_2N=C(NH_2)$ NH-CN, probably allowing for the formation of favorable interaction between this new anchoring group and the active site amino acid residue Asp 189, critical for substrate/inhibitor binding to this type of serine protease. Thus, the main finding of the present paper is that the sulfonyldicyandiamide group may constitute an interesting alternative for obtaining weakly basic, potent thrombin inhibitors, which bind with less affinity to trypsin.

Keywords: Thrombin, trypsin, inhibitor, dicyandiamide, sulfonyl, reduced basicity, tautomerism

INTRODUCTION

Thrombin (EC 3.4.21.5) plays a key role in the control of thrombus formation, and has become a highly investigated target for the design of orally active, low molecular weight inhibitors, with potential use as antithrombotic drugs.[1–5] Much progress has been registered ultimately in the design and synthesis of highly effective, active site directed inhibitors, with affinity for the enzyme in the low nanomolar range, as well as specificity against other related serine proteases (such as trypsin),[1–5] but the "ideal" thrombin inhibitor has not yet been obtained.[2,3] The majority of the available potent inhibitors

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generally show very poor oral bioavailability, strongly bind to plasma proteins, and/or do not have an appropriate plasma half-life after oral dosing.[2,3] Many of these pharmacokinetic problems are due to the high basicity of the majority of these first generation thrombin inhibitors, which contain arginine or amidine-based S1 anchoring moieties. These problems seem to be circumvented to some degree by the recent report of a large number of weakly basic inhibitors which do not contain the above mentioned classical S1 anchoring moieties, but instead, incorporate isosteres of these moieties, as well as other structural modifications that alter the highly basic pKa of the arginine/amidine-based inhibitors (which is in the range of 11–13).[3] Among the most promising new S1 anchoring groups recently reported must be mentioned the following moieties: oxoguanidine A (pKa 7);[6] hydroxyguanidine B (pKa 9)[7]; acylguanidine C (pKa 7.6);[8] aminohydrazone D (pKa 8.7);[9] benzamidrazone E (pKa 8.9);[10] sulfonylguanidine F (pKa 8.3);[11,12] sulfonylaminoguanidine G (pKa 8.4);[13,14] imidazole H (pKa around 7);[15] 1-aminoisoquinoline I (pKa 7.5);[16] 2-aminopyridine J (pKa c. 7);[17] benzylamine K (pKa 9.4);[18] aniline L (pKa not provided, but stated as "neutral")[19,20a] and benzonitrile M[20b] among others (Figure 1). Compounds incorporating some of these groups, such as LB-30057 **1**, CGH 1668 **2**,

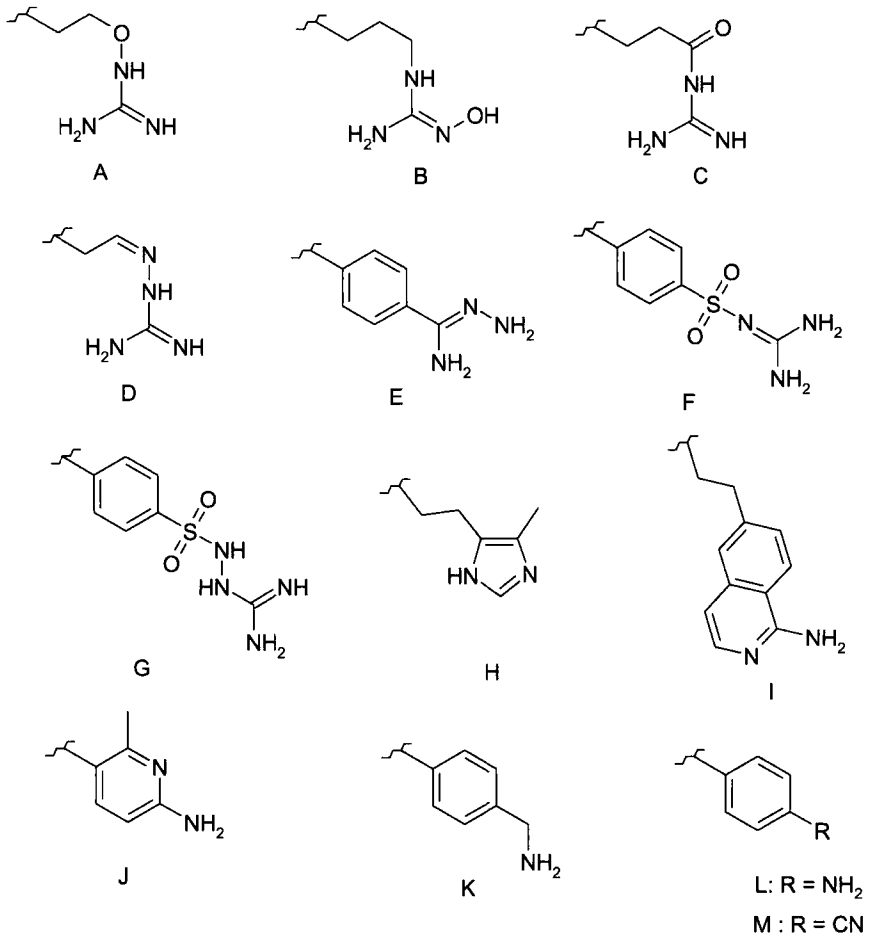
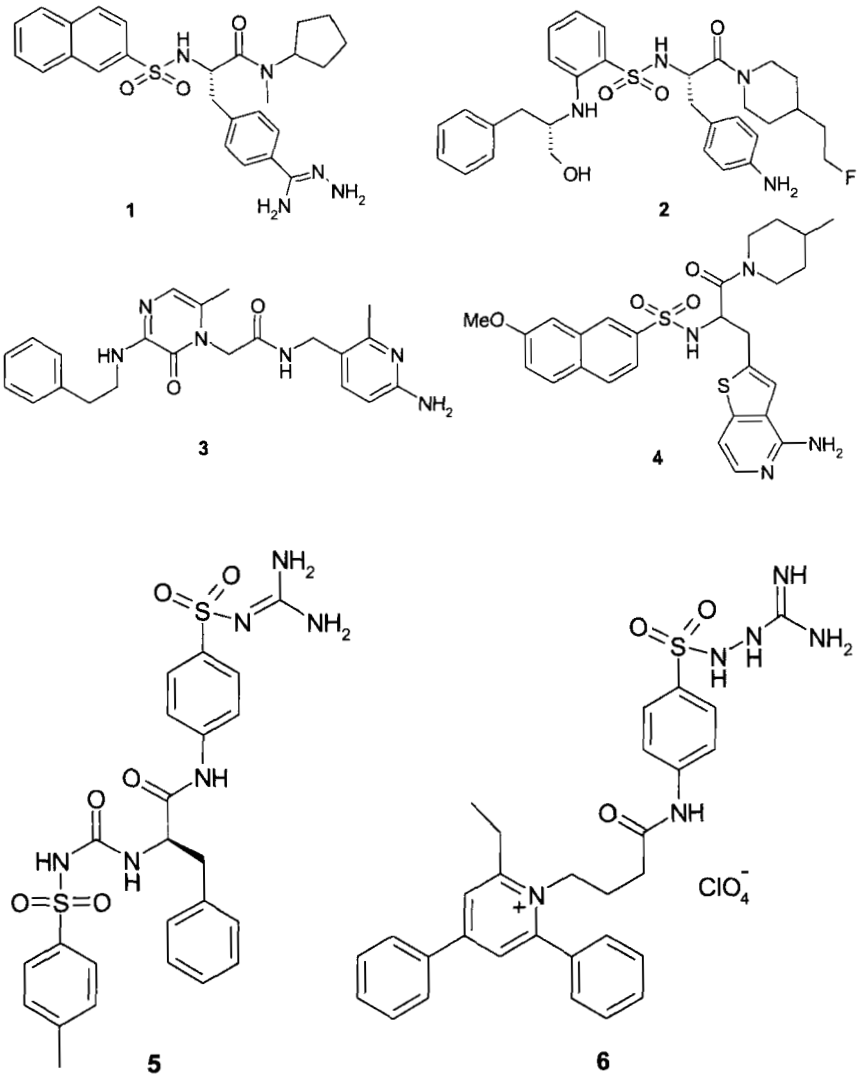


FIGURE 1 S1 anchoring groups of types A-M with reduced basicity incorporated in thrombin inhibitors.



L-375378 **3** or the Organon derivative **4**, are currently in clinical investigations as antithrombotic drugs.[4,5]

In previous reports from this group,[11–14] it was shown that derivatives incorporating benzenesulfonyl-guanidine/aminoguanidine moieties as S1 anchoring groups, of types **5** and **6** among others, not only possessed good thrombin inhibitory properties and specificity for thrombin over trypsin, but also showed pKa values in the range of 8.2–8.4, making them interesting candidates for further investigations. In this paper we extend our investigations for the search of weakly basic serine protease inhibitors, reporting that sulfonyl-dicyandiamide derivatives acts as inhibitors of two serine proteases, human thrombin and human trypsin, with good specificity for the first enzyme over the second one. The compounds reported here were obtained by reaction of dicyandiamide with alkyl/arylsulfonyl halides or arylsulfonyl isocyanates. An interesting lead compound obtained in this manner, i.e., sulfanilyl-dicyandiamide, was then further derivatized in order to enhance thrombin inhibitory properties, by attaching moieties that would assure favorable interactions with different binding

sites of the enzyme, such as for instance the P2 pocket.[4] Since dicyandiamide and its sulfonylated derivatives possess many possible tautomeric structures, calculations have been performed in order to establish the preferred tautomeric form(s) that probably interacts with the enzyme.

MATERIALS AND METHODS

Calculations

Calculations were done with Gaussian 94[21] and MOPAC 93.[22] For dicyanamide two tautomeric structures and for benzenesulfonyldicyanamide three tautomers were generated using Hyperchem 5.1[23] and optimized using Hyperchem's MM + molecular mechanics optimizer. These geometries were further optimized with MOPAC 93 using the AM1 Hamiltonian[24], and with Gaussian 94 using the density functional B3LYP/6–31G**. For the semiempirical calculations the vacuum geometries were further optimized using COSMO,[25] while for the density functional method a one-point calculation was run using SCIPCM[26] at the vacuum density functional geometry. The structures thus obtained were used in one point calculations to obtain the heats of formation in vacuo and in a medium of dielectric constant 72.7 using the COSMO[27] approximation (by MOPAC), and with the B3LYP method[27,28] and in the latter medium using SCIPCM[26] (by Gaussian).

Chemistry

Melting points were detained on a heating plate microscope (not corrected), IR spectra as KBr pellets on a 400–4000 cm^{-1} Perkin-Elmer 16PC FTIR spectrometer, ^{1}H-NMR spectra on a Varian Gemini 200 apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard) Elemental analysis ($\pm$ 0.4% of the theoretical values, calculated for the proposed formulas – data not shown) was done on a Carlo Erba Instrument CHNS Elemental Analyser, Model 1106. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Preparative HPLC was performed on a Dynamax-60A column (25 × 250 mm), with a Beckman EM-1760 instrument. The detection wavelength was 254 nm. Sulfonyl halides, dicyandiamide, triethylamine, carbodiimides, amino acids, Cbz-amino acids, dipeptides, and arylsulfonyl isocyanates used in the syntheses were commercially available compounds (from Sigma-Aldrich, Fluka or Acros). Acetonitrile, acetone, dioxane, ethyl acetate (E. Merck, Darmstadt, Germany) or other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions. Inogatran was from Astra Hassle (Molndal, Sweden). Benzamidine, NAPAP, human thrombin, human trypsin and Chromozym TH were from Sigma-Aldrich (Milan, Italy).

General procedure for the preparation of compounds A1–A36

Methods A and C: An amount of 10 mM sulfonyl halide (chloride for method A and fluoride for method C) was dissolved in 50 mL of acetone and the stoichiometric amount of dicyandiamide (0.84 g = 10 mM) dissolved in 5 mL of water was added dropwise, together with the stoichiometric amount of solid $NaHCO_3$ or NaOH required for the neutralization of the acid formed in reaction.[11,13] The mixture was magnetically stirred at 25 °C for 5 h and then the reaction mixture was acidified with 0.1 N HCl solution to pH 4.5. The obtained sulfonyl derivatives generally precipitated by leaving the above mentioned reaction mixture at 4 °C overnight and were then filtered and recrystallized from ethanol.

Method B: An amount of 0.42 g = 5 mmol of dicyandiamide and 0.84 mL (5 mmol) of triflic anhydride were suspended in 10 mL of acetone and 0.35 mL (5 mmol) of triethylamine was added dropwise.[11,13] The mixture was magnetically

stirred at 4 °C for 5 h. The solvent was then evaporated *in vacuo,* and the tan residue treated with 5 mL of cold water and acidified with 0.1 N HCl solution. The triflate salt of triethylamine being water soluble was thus separated from the triflated dicyandiamide (much less water soluble) by a simple filtration. The latter compound was recrystallized from ethanol.

Method D: An amount of 0.84 g = 10 mmol of dicyandiamide, 0.70 mL (10 mmol) of triethylamine and 10 mmol of sulfobenzoic cyclic anhydride or tetrabromo-*O*-sulfobenzoic cyclic anhydride were heated at reflux in 50 mL of anhydrous acetonitrile for 2 h.[11,13] After evaporation of the solvent, the products were treated with 10 mL of water, acidified with 0.1 N HCl solution till pH 4, and the precipitated derivatives filtered, dried and recrystallized from methanol.

General procedure for the preparation of N-arylsulfonylureido derivatives

An amount of 10 mM of amino acid/dipeptide/dicyandiamide/**A18** was suspended/dissolved in 50 mL of anhydrous acetone and the corresponding amount (10 mM) of arylsulfonyl isocyanate was added dropwise.[29,30] The reaction mixture was stirred at 4 °C for 4–6 h, when TLC showed that it was complete. Evaporation of the solvent *in vacuo* afforded white foams of *N*-arylsulfonylureido protected derivatives, as well as the arylsulfonylureido dicyandiamides **A37–A41**, **B1–B5**, which were recrystallized from ethanol-water (1:1, v/v).

General procedure for the preparation of compounds B6–B13

An amount of 1 mM of *N*-Cbz- or *N*-arylsulfonylureido-protected amino acid/dipeptide was dissolved/suspended in 25 mL of anhydrous acetonitrile, and treated with 190 mg (1 mM) of EDCI. HCl and 15 μL (1 mM) of triethylamine.[31] The mixture was stirred at 4 °C for 1 h in order to allow the formation of the mixed-anhydride type activated amino acyl compound, then 240 mg (1 mM) of **A18** was added over a period of 1 h. The reaction mixture was magnetically stirred at 4 °C for 16–20 h (TLC control) untill all the amino acid/dipeptide was converted. The solvent was evaporated *in vacuo* and the residue taken up in ethyl acetate (5 mL), poured into a 5% solution of sodium bicarbonate (5 mL) and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate, filtered and the solvent removed *in vacuo*. Preparative HPLC (Dynamax-60A column (25 × 250 mm); 90% acetonitrile/6% methanol/4% water; flow rate of 30 mL/min) afforded the pure compounds **B6–B13** as colorless solids.

Full characterization data for some representative compounds of each subseries is shown below.

4-Fluorophenylsulfonyl-dicyandiamide, **A8** As colorless crystals, mp 203–4 °C. IR (KBr), cm^{-1}: 1170 (SO_2^{sym}), 1182 (imide III), 1365 (SO_2^{as}), 1580 (imide II), 1678 (imide I), 2180 (CN), 3360 br (NH, NH_2): ^{1}H-NMR (DMSO-d_6), δ, ppm: 6.83 (br s, 2H, NH_2), 7.05–7.52 (m, AA′BB′, $J_{AB} = 7.7$ Hz, 4H, ArH, *p*-F-phenylene), 8.14 (br s, 1H, NH). Found: C, 39.85; H, 3.16; N, 23.03; S, 13.51. $C_8H_7FN_4O_2S$, requires. C, 39.67; H, 2.91; N, 23.13; S, 13.24%.

4-Toluenesulfonylureido-dicyandiamide, **A40** As colorless crystals, mp 310–3 °C. IR (KBr), cm^{-1}: 1114 (SO_2^{sym}), 1185 (imide III), 1357 (SO_2^{as}), 1540 (amide II), 1657 (amide I), 2180 (CN), 3360 br (NH, NH_2): ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (s, 3H, Me), 6.81 (br s, 2H, NH_2), 7.12–7.84 (m, AA′BB′, $J_{AB} = 7.5$ Hz, 4H, ArH, *p*-Me-phenylene), 8.10 (br s, 1H, NH). Found: C, 42.61; H, 3.74, N, 24.69, S, 11.34. $C_{10}H_{11}N_5O_3S$ requires: C, 42.70; H, 3.94, N, 24.90, S, 11.40%.

4-(N-Benzyloxycarbonyl-D-phenylalanylamido)-benzenesulfonyl-dicyandiamide, **B6** Its m.p. 211–2 °C: IR (KBr), cm^{-1}: 1143 (SO_2^{sym}), 1188 (imide III), 1371 (SO_2^{as}), 1540 (amide II), 1645 (amide I), 2180 (CN), 3360 br (NH, NH_2). ^{1}H-NMR (DMSO- D_2O), δ, ppm: 3.10–3.56 (m, 2H, CH_2CH of Phe), 4.13 (dd, $^3J_{HH} = 5.0$ Hz, $^3J_{HH} = 7.8$ Hz,

1H, CH_2CH of Phe), 5.04 (s, 2H, $PhCH_2O$), 6.81 (br s, 2H, NH_2) – disappears immediately due to deuteration, 7.20–7.45 (m, 10H, H_{arom} of Phe and $PhCH_2O$), 7.61 (d, $^3J_{HH} = 7.9$ Hz, 2H, H_{ortho} of $XNHC_6H_4SO_2$), 7.90 (d, $^3J_{HH} = 7.9$ Hz, 2H, H_{meta} of $XNHC_6H_4SO_2$), 8.20 (br s, 1H, N*H* from the anchoring group); ^{13}C-NMR (DMSO- D_2O), δ, ppm: 41.1 (s, CH_2CH of Phe), 59.6 (s, *C*HCH2 of Phe), 74.2 (s, $PhCH_2O$), 130.1 (s, C_{para} of Phe), 133.4 (s, C_{meta} of $XNHC_6H_4SO_2$), 133.9 (s, C_{meta} of Phe), 134.7 (s, C_{ortho} of Phe), 135.5 (s, C_{ortho} of $XNHC_6H_4SO_2$), 141.0 (s, C_{ipso} of Phe), 143.7 (s, C_{para} of $XNHC_6H_4SO_2$), 148.9 (s, C_{ipso} of $XNHC_6H_4SO_2$), 173.5 (s, Phe-*C*ONH), 179.5 (s, $PhCH_2OCO$), 181.3 (s, $SO_2N{=}C$). Found: C, 57.42; H, 4.83; N, 16.08, S, 6.33. $C_{25}H_{24}N_6O_5S$ requires: C, 57.68; H, 4.65; N, 16.14, S, 6.16%.

4-(4-Toluenesulfonylureido-D-phenylalanyl-L-prolylamido)-benzenesulfonyl-dicyandiamide, **B9** Its m.p. 255–7 °C (dec.): IR (KBr), cm^{-1}: 1140 and 1158 (SO_2^{sym}), 1180 (imide III), 1357 (SO_2^{as}), 1540 (amide II), 1650 (amide I), 2180 (CN), 3360 br (NH, NH_2): ^{1}H-NMR (DMSO- D_2O), δ, ppm: 1.19–1.44 (m, 1H, *H*CH of Pro), 1.55–1.71 (m, 1H, HC*H*), 1.75–1.89 (m, 2H, CH_2 of Pro), 2.60 (s, 3H, $CH_3C_6H_4$), 3.10–3.53 (m, 2H, CH_2CH of Phe), 3.20–3.42 (m, 2H, CH_2N of Pro), 3.75–3.80 (m, 1H, *C*HCO of Pro), 4.10 (dd, $^3J_{HH} = 5.0$ Hz, $^3J_{HH} = 7.4$ Hz, 1H, CH_2CH of Phe), 6.83 (br s, 2H, NH_2) – disappears immediately due to deuteration, 7.16–7.58 (m, 7H, H_{ortho} of tosyl and H_{arom} of Phe), 7.65 (d, $^3J_{HH} = 7.8$ Hz, 2H, H_{ortho} of $XNHC_6H_4SO_2$), 7.90 (d, $^3J_{HH} = 7.8$ Hz 2H, H_{meta} of $XNHC_6H_4SO_2$), 7.91 (d, $^3J_{HH} = 8.3$ Hz, 2H, H_{meta} of tosyl), 8.22 (br s, 1H, N*H* from the anchoring group); ^{13}C-NMR (DMSO- D_2O), δ, ppm: 15.2 (s, CH_2 of Pro), 21.4 (s, CH_2 of Pro), 26.3 (s, $CH_3C_6H_4$), 41.8 (s, CH_2CH of Phe), 46.9 (s, CH_2N of Pro), 59.5 (s, $CHCH_2$ of Phe), 64.5 (s, CHCO of Pro), 130.2 (s, C_{para} of Phe), 132.5 (s, C_{meta} of $CH_3C_6H_4$), 132.8 (s, C_{meta} of $XNHC_6H_4SO_2$), 133.7 (s, C_{meta} of Phe), 134.0 (s, C_{ortho} of Phe), 135.1 (s, C_{ortho} of $CH_3C_6H_4$), 135.9 (s, C_{ortho} of $XNHC_6H_4SO_2$), 141.8 (s, C_{ipso} of Phe), 1424 (s, C_{para} of $XNHC_6H_4SO_2$), 145.6 (s, C_{para} of $CH_3C_6H_4$), 148.1 (s, C_{ipso} of $CH_3C_6H_4$), 148.9 (s, C_{ipso} of $XNHC_6H_4SO_2$), 149.5 (s, NHCONH), 172.0 (s, Phe-CONH), 174.7 (Pro-CONH), 182.1 (s, *C*(=NH)). Found: C, 53.12; H, 4.61; N, 16.35; S, 9.58. $C_{30}H_{32}N_8O_7S_2$ requires: C, 52.93; H, 4.74; N, 16.46; S, 9.42%.

4-(N-Benzyloxycarbonyl-D-phenylalanylprolyl-amido)-benzenesulfonyl-dicyandiamide, **B10** Its m.p. 229–30 °C. IR (KBr), cm^{-1}: 1143 and 1159 (SO_2^{sym}), 1180 (imide III), 1363 (SO_2^{as}), 1540 (amide II), 1648 (amide I), 2180 (CN), 3365 br (NH, NH_2). ^{1}H-NMR (DMSO- D_2O), δ, ppm: 1.12–1.36 (m, 1H, *H*CH of Pro), 1.51–1.64 (m, 1H, HC*H*), 1.75–1.87 (m, 2H, CH_2 of Pro), 3.12–3.54 (m, 2H, *C*H2CH of Phe), 3.20–3.35 (m, 2H, CH_2N of Pro), 3.75–3.83 (m, 1H, *C*HCO of Pro), 4.15 (dd, $^3J_{HH} = 5.0$ Hz, $^3J_{HH} = 7.8$ Hz, 1H, CH_2CH of Phe), 5.10 (s, 2H, $PhCH_2O$), 6.85 (br s, 2H, NH_2) – disappears immediately due to deuteration, 7.04–7.46 (m, 10H, H_{arom} of Phe and $PhCH_2O$), 7.69 (d, $^3J_{HH} = 7.9$ Hz, 2H, H_{ortho} of $XNHC_6H_4SO_2$), 7.90 (d, $^3J_{HH} = 7.9$ Hz, 2H, H_{meta} of $XNHC_6H_4SO_2$), 8.24 (br s, 1H, N*H* from the anchoring group); ^{13}C-NMR (DMSO- D_2O), δ, ppm: 15.4 (s, CH_2 of Pro), 21.3 (s, CH_2 of Pro), 41.7 (s, *C*H2CH of Phe), 46.4 (s, CH_2N of Pro), 59.9 (s, $CHCH_2$ of Phe), 64.5 (s, CHCO of Pro), 75.7 (s, $PhCH_2O$), 130.8 (s, C_{para} of Phe), 133.6 (s, C_{meta} of $XNHC_6H_4SO_2$), 133.9 (s, C_{meta} of Phe), 134.6 (s, C_{ortho} of Phe), 135.2 (s, C_{ortho} of $XNHC_6H_4SO_2$), 141.0 (s, C_{ipso} of Phe), 142.5 (s, C_{para} of $XNHC_6H_4SO_2$), 148.9 (s, C_{ipso} of $XNHC_6H_4SO_2$), 173.2 (s, Phe-*C*ONH), 174.8 (Pro-*C*ONH), 179.5 (s, $PhCH_2OCO$), 182.0 (s, *C*(=NH)). Found: C, 58.22; H, 5.35; N, 15.60; S, 5.47. $C_{30}H_{31}N_7O_6S$ requires: C, 58.34; H, 5.06; N, 15.87; S, 5.19%.

Biochemistry

Enzyme assays; K_I determinations

Human thrombin and human trypsin were purchased from Sigma-Aldrich (Milan, Italy); their concentrations were determined from the absorbance at 280 nm and the extinction coefficients

furnished by the supplier. The activity of such preparations was in the range of 2500–3000 NIH units/mg. The potency of standard and the novel inhibitors was determined from the inhibition of the enzymatic (amidolytic) activity of these serine proteases, at 21 °C, using Ts-Gly-Pro-Arg-pNA (Chromozym TH) from Sigma as substrate, by the method of Lottenberg *et al.*[33] The substrate was reconstituted as 4 mM stock in ultrapure water and brought to pH 4 with hydrochloric acid. Substrate concentrations were determined from absorbance at the isosbestic wavelength for the peptide-*p*-nitroanilide – *p*-nitroaniline mixtures. Extinction coefficients of 8270 $L \cdot mol^{-1} \cdot cm^{-1}$ in the buffer used (0.01 M Hepes – 0.01 M Tris – 0.1 M NaCl – 0.1 % polyethylene glycol 6000) were employed. The rate of *p*-nitroanilide hydrolysis was determined from the change in absorbance at 405 nm using an extinction coefficient for *p*-nitroaniline of 9920 $L \cdot mol^{-1} \cdot cm^{-1}$ for the above-mentioned reaction buffer. Measurements were made using a Perkin-Elmer spectrophotometer interfaced with a PC. Initial velocities were thus estimated using the direct linear plot-based procedure as reported by Lottenberg *et al.*[32] K_I-s were then determined according to Dixon, using a linear regression program.[33] The K_I values determined are the means of at least three determinations.

pKa Determination

The half neutralization point was measured by titrating the organic acids/bases with 0.05 N NaOH or 0.05 N HCl in EtOH – water (30%, v/v), using a glass electrode, as described by Bell and Roblin[34] for the structurally-related antibacterial sulfonamides and adapted by us for previously reported serine protease inhibitors.[12]

RESULTS AND DISCUSSION

The new compounds reported in this paper, of types **A1–A36**,were obtained by reaction of alkyl/arylsulfonyl halides or their derivatives with dicyandiamide, similar to the procedure previously reported[11,13] for the alkyl/arylsulfonylation of guanidine/aminoguanidine (Table I). The reaction was performed under Schotten-Baumann conditions and led to the desired derivatives in high yields (65–90%, data not shown). The sulfonylurea derivatives **A37–A41** on the other hand, were prepared by reaction of dicyandiamide with arylsulfonyl isocyanates[35]. Compounds **B1–B5** were obtained by reaction of sulfanilyl-dicyandiamide **A18** with arylsulfonyl isocyanates, by the method previously reported for the synthesis of aromatic/heterocyclic sulfonamides incorporating arylsulfonylureido moieties (Scheme 1).[35] Similarly were prepared some amino acid/dipeptide derivatives which were then used for derivatization of the lead compound **A18** (see later in the text). Such tosylureido-containing amino acids/dipeptides, or the N-benzyloxycarbonyl protected analogues were then coupled with sulfanilyl-dicyandiamide **A18** in the presence of carbodiimides (such as EDCI),[31] leading to the new derivatives of type **B6–B13**, by the procedure previously reported for the preparation of thrombin inhibitors containing sulfonylguanidine/sulfonylaminoguanidine moieties as S1 anchoring group (Scheme 1 and Table - II).[11,13] It should be noted that under the conditions used for the syntheses (carbodiimide in acetonitrile or acetone at 4 °C) only the aminophenyl group of **A18** was acylated, whereas the cyanoguanidine moiety (-NH-C(=NH)-NH-CN) was unaffected thus allowing preparation of these new derivatives without the need to protect the latter moiety by diverse protecting groups such as butyloxycarbonyl (Boc), trityl, tritylsulfenyl, etc.[36]

The new compounds reported here were characterized by standard procedures (elemental analysis, IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy) which confirmed their structure (see Experimental for details).

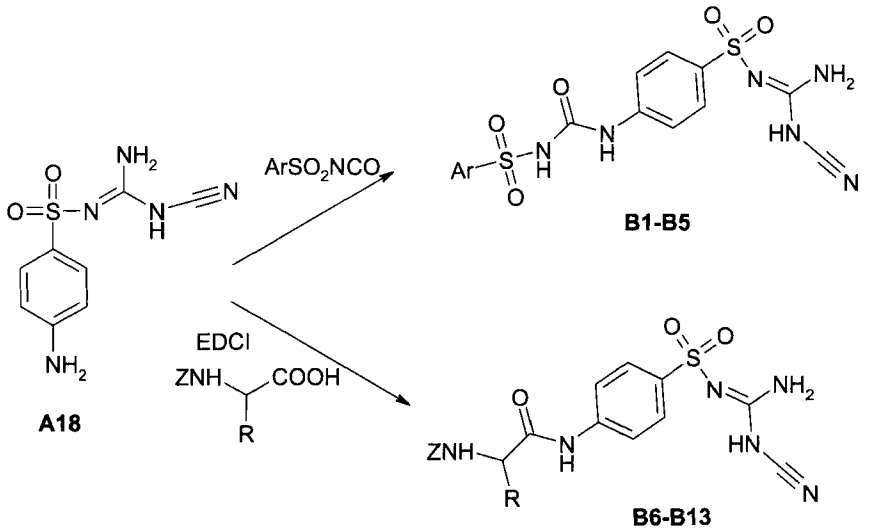
An important factor that might influence the binding of these derivatives to the enzyme, is the

TABLE I Sulfonyl dicyandiamide derivatives **A1–A41** prepared in the present study, with their inhibition data against human thrombin and human trypsin, and the synthetic method used. R-SO_2N=C(NH_2)NHCN (**A1–A36**), R-N=C(NH_2)NHCN (**A37–A41**)

Compound **A**	R	K_I(μM)[a]		Synthetic method*
		Thrombin	Trypsin	
1	Me_2N–	12	35	A
2	CH_3–	21	40	A
3	CH_3CH_2–	13	27	A
4	$CH_3CH_2CH_2$–	12	25	A
5	CF_3–	7	36	B
6	$PhCH_2$–	8	19	C
7	Ph	12	21	A
8	*p*-F-C_6H_4–	10	15	A
9	*p*-Cl-C_6H_4–	12	16	A
10	*p*-Br-C_6H_4–	8	15	A
11	*p*-I-C_6H_4–	7	13	A
12	*p*-CH_3-C_6H_4–	7	15	A
13	*p*-O_2N-C_6H_4–	6	10	A
14	*m*-O_2N-C_6H_4–	7	12	A
15	*o*-O_2N-C_6H_4–	5	10	A
16	3-Cl-4-O_2N-C_6H_3–	4	10	A
17	*p*-AcNH-C_6H_4–	7	12	A
18	*p*-H_2N-C_6H_4–	3	15	C
19	C_6F_5–	5	13	A
20	*o*-HOOC-C_6H_4–	9	14	D
21	*m*-HOOC-C_6H_4–	7	15	A
22	*p*-HOOC-C_6H_4–	7	13	A
23	o-HOOC-C_6Br_4–	3	12	D
24	*p*-CH_3O-C_6H_4–	3	12	A
25	2,4,6-$(CH_3)_3$-C_6H_2–	4	13	A
26	4-CH_3O-3-H_2N-C_6H_3–	4	11	C
27	2-HO-3,5-Cl_2-C $_6H_2$–	3	13	A
28	4-Me_2N-C_6H_4-N = N-C_6H_4–	1	12	A
29	*p*-Ac-C_6H_4–	7	10	A
30	1-Naphthyl	5	12	A
31	2-Naphthyl	6	15	A
32	5-Dimethylamino-1-naphthyl-	4	13	A
33	*n*-C_4F_9–	5	15	A
34	*n*-C_8F_{17}	3	12	A
35	2-thienyl	7	15	A
36	Camphor-10-yl	4	9	A
37	$PhSO_2NHCO$	2	13	E
38	4-F-$C_6H_4SO_2NHCO$	2	14	E
39	4-Cl-$C_6H_4SO_2NHCO$	1.5	11	E
40	4-Me-$C_6H_4SO_2NHCO$	1.6	10	E
41	2-Me-$C_6H_4SO_2NHCO$	1.8	12	E
–	Benzamidine	3	5	–

*A: RSO_2Cl + dicyandiamide; B: triflic anhydride + dicyandiamide; C: RSO_2F + dicyandiamide; D – sulfobenzoic cyclic anhydride + dicyandiamide; E – RSO_2NCO + dicyandiamide. [a]K_I-s values were obtained from Dixon plots using a linear regression program, from at least three different assays. Spreads around the mean (data not shown) were ± 10–20% of the shown values.

tautomeric form in which the dicyandiamide moiety exists. A literature search showed that the tautomerism of dicyandiamide or its derivatives has not been investigated previously. Thus, we have performed some mechanical quantum calculations in order to establish which is the



SCHEME 1 Synthesis of derivatives **B1-B13**.

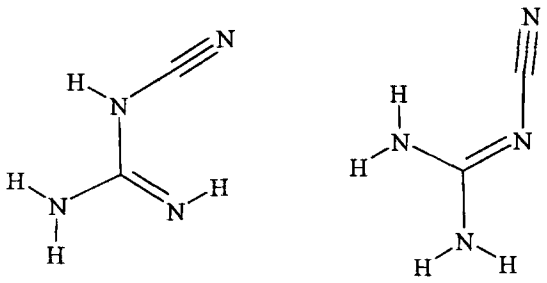
TABLE II Derivatives **B1–B13** obtained from sulfanilyl-dicyandiamide **A18** as lead, with their inhibition data against human thrombin and human trypsin. Inhibition data of standard inhibitors are also included. *p*-XNH-C_6H_4-SO_2N=C(NH_2)NHCN (**B1–B13**)

Compound	X*	K_I (nM)[a]	
		Thrombin	Trypsin
B1	$PhSO_2NHCO$	86	1300
B2	4-F-$C_6H_4SO_2NHCO$	42	1100
B3	4-Cl-$C_6H_4SO_2NHCO$	40	1150
B4	4-Me-$C_6H_4SO_2NHCO$	36	1550
B5	2-Me-$C_6H_4SO_2NHCO$	39	1300
B6	Cbz-*D*-Phe	31	950
B7	ts-*D*-Phe	28	1340
B8	ts-*L*-Pro	34	1500
B9	ts-*D*-PhePro	9	1400
B10	Cbz-*D*-PhePro	12	1400
B11	ts-GlyHis	19	1600
B12	ts-β-AlaHis	13	1250
B13	ts-*L*-ProGly	17	1500
–	Inogatran	15	540
–	NAPAP	6.5	690

[a]K_I-s values were obtained from Dixon plots using a linear regression program, from at least three different assays. Spreads around the mean (data not shown) were ± 5–10% of the shown values. *Cbz = $PhCH_2OCO$; ts = *p*-MeC_6H_4-SO_2NHCO-; these groups acylate the amino-terminal H_2N moiety. When configuration is not specified, *L*-amino acid moieties were employed. The usual polypeptide format is used: the amino-terminal residue is written first (and it is always protected either by a Cbz or ts moiety), whereas the carboxyterminal residue is acylating the sulfanilyl-dicyandiamide N-4 amino group.

favored tautomer of these compounds, both in vacuum as well as in solution.

Both AM1 and the density functional method predicted structure 2 for dicyandiamide to be more stable than structure 1 (Scheme 2 and Table III). In both cases, solvation enhanced the difference. Because the differences are quite large, we are confident that these results are valid. Three structures were then calculated for benzenesulfonyl dicyandiamide **A7** (Scheme 3 and Table IV). The resulting energies are shown in Table IV. It will be seen that structure 4 is of lowest energy by AM1. Structure 3 has considerably higher energy both in vacuum and in



Structure 1 Structure 2

SCHEME 2 Tautomeric structures of dicyandiamide.

TABLE III Energies of the two tautomers of dicyandiamide, calculated in vacuum and in solution ($\varepsilon = 72.7$) by the semi-empirical AM1 method and COSMO, and by the density functional B3LYP method and SCIPCM model

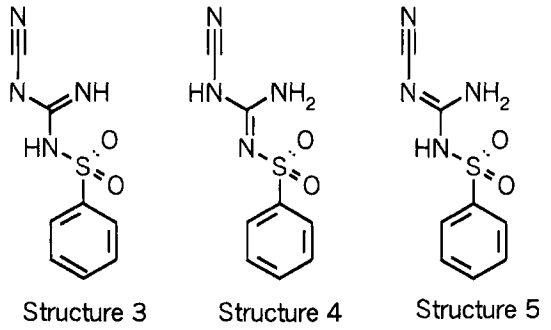
Method	Structure 1	Structure 2	Difference
AM1[a]	64.807 kcal	54.381 kcal	10.426 kcal
AM1 COSMO[b]	45.381 kcal	30.081 kcal	15.300 kcal
B3LYP/6–31G**[a]	−297.599756 au	−297.624842 au	15.742 kcal
B3LYP/6–31G** SCIPCM[b]	−297.619631 au	−297.648051 au	17.834 kcal

[a]In vacuum. [b]In solution.

TABLE IV Energies of the three tautomers of benzenesulfonyldicyandiamide **A7**, calculated in vacuum and in solution ($\varepsilon = 72.7$) by the semi-empirical AM1 method and COSMO, and by the density functional B3LYP method and SCIPCM model

Method	Structure 3	Difference	Structure 4	Structure 5	Difference
AM1[a]	28.938[1]	9.400[1]	19.538[1]	24.791[1]	5.253[1]
AM1 COSMO[b]	−2.991[1]	14.097[1]	−17.088[1]	−14.233[1]	2.855[1]
B3LYP/6–31G**[a]	−1077.227407[2]	12.099[1]	−1077.246688[2]	−1077.243619[2]	1.926[1]
B3LYP/6–31G** SCIPCM[b]	−1077.244073[2]	13.940[1]	−1077.266288[2]	−1077.262272[2]	2.520[1]

[1]kcal. [2]au. [a]In vacuum. [b]In solution.



SCHEME 3 Tautomeric structures of benzenesulfonyl dicyandiamide **A7**.

solution, but structure 5, though higher in energy than structure 4, is not so high that it can be ruled out, especially in solution. Structure 4 is also of lowest energy by the density functional method *in vacuo*, but only by approximately 2 kcal. In solution, structure 4 is a little more stabilised relative to structure 5 by the SCIPCM model. An appreciable fraction of the compound will thus exist in equilibrium in the structure 5 form, but from all of the models considered, structure 4 will predominate and consequently, we have formulated the new compounds reported here in this tautomeric form. The agreement between the semiempirical and *ab initio* results is satisfactory, but some doubt is introduced by the fact that neither solvation model takes into account the specific hydrogen bonding capability of water. Similar calculations on such well studied systems as the glycine zwitter ion however do give results in accord with experiment. Obviously, this tautomeric form mentioned above may participate in favorable interactions (hydrogen bonds and/or electrostatic interactions) with the carboxylate moiety of Asp 189 in the S1 pocket of the enzyme.

From the calculations reported above, and also by taking into considerations some novel type of low basicity S1 anchoring groups such as B, E-G or M (Figure 1), it is expected that the sulfonyldicyandiamide moiety will confer serine protease binding affinity to the new derivatives reported here. Indeed, biological activity data of the simple derivatives **A1–A41**, against two serine proteases, human thrombin and human trypsin, showed that these compounds act as medium potency inhibitors, with affinities in the micromolar range for both enzymes (Table I).

In the series of sulfonylated derivatives **A1–A36**, the following structure-activity correlations have been observed: (i) the weakest thrombin/trypsin inhibitors were those incorporating aliphatic or simple aromatic (phenyl 4-fluoro/chloro-phenyl) moieties; these inhibitors possessed inhibition constants in the range 10–21 μM against thrombin, and 15–40 μM against trypsin; (ii) more potent inhibition was observed with compounds incorporating other substituted-arylsulfonyl moieties, such as 4-bromo/iodo-phenyl-, 2-/3- or 4-nitrophenyl-, 4-acetyl/acetamido-phenyl-, 2-/3- or 4-carboxyphenyl-, 1- or 2-naphthyl- or thienylsulfonyl among others. These compounds possessed inhibition constants in the range 4–9 μM against thrombin, and 10–15 μM against trypsin; (iii) the most active compounds in this subseries were those containing 4-aminophenylsulfonyl-, 2-carboxy-tetrabromophenylsulfonyl-4-methoxyphenylsulfonyl-, 2-hydroxy-3,5-dichlorophenylsulfonyl-, perfluorooctylsulfonyl- and dabsyl moieties in their molecule, with inhibition constants of 1–3 μM against thrombin, and 12–15 μM against trypsin; (iv) even more active were the derivatives of type **A37–A41**, incorporating arylsulfonylureido-dicyandiamide moieties, which all possessed inhibition constants of c. 1.5–2 μM against thrombin, and 10–14 μM against trypsin. Best inhibitors of this type reported here had the same order of thrombin inhibitory potency as the simple (and very basic) inhibitor benzamidine, which does not discriminate between thrombin and trypsin, whereas the dicyandiamide derivatives showed some degree of specificity for the first *versus* the latter serine protease.

The relatively good thrombin inhibitory activity (as well as specificity over trypsin) of the sulfanilyl derivative **A18**, correlated with the intensification of these inhibitory properties in derivatives incorporating arylsulfonyluerido moieties (such as in **A37–A41**) (Table I) prompted us to use **A18** as lead molecule for obtaining even more potent thrombin inhibitors incorporating sulfonyl-dicyandiamide moieties as S1 anchoring moiety. Thus, in a first subseries of such derivatives, the 4-aminophenyl moiety of the lead was derivatized by means of the arylsulfonylureido moieties mentioned above, leading to the new compounds **B1–B5**. These compounds did indeed show increased thrombin inhibitory properties (K_I-s in the range of 39–86 nM against thrombin and 1100–1500 nM against trypsin) as compared to the parent compound **A18**. Furthermore, the classical D-Phe-Pro-(Arg) scaffold (or fragments of it) have been incorporated in the molecules of the new inhibitors **B6-B13**. The first type of derivatives investigated incorporated Cbz-Pro, tosylureido-Pro or tosylureido-Phe moieties only (as proline is known to be the P2 residue in many potent thrombin inhibitors, whereas D-Phe is generally the P3 residue of such peptidomimetic inhibitors).[1–5] Indeed, such derivatives (**B6–B8**) showed increased affinity for thrombin (28–34 nM), maintaining also the selectivity over trypsin. Even better inhibitors were those incorporating both amino acids (Phe and Pro) of the above mentioned scaffold, with the benzyloxycarbonyl- or tosylureido-protected aminoterminal derivatives **B9** and **B10** being the best inhibitors in this entire series of dicyandiamide derivatives. These compounds showed thrombin inhibition levels of the same order of magnitude as the clinically used inhibitor inogatran, being only slightly less active than the very potent inhibitor NAPAP described by Stürzebecher.[1,3] The activity of compounds **B11–B13** incorporating Gly-His, Ala-His and Pro-Gly scaffolds respectively (with the aminoterminal moiety derivatized again by means of tosylureido isocyanate)[11,13] was slightly diminished as compared to the previously mentioned compounds, but they were still relatively potent and selective thrombin inhibitors with inhibition constants of 13–19 nM against thrombin and 1250–1600 nM against trypsin.

pKa values have been measured for several simple dicyandiamide derivatives investigated here, in order to prove that they possess a

TABLE V pK_a data for several serine protease inhibitors

Compound	pK_a[a] Guanidino/amidino moiety	SO_2NH moiety
Inogatran	12.3	–
NAPAP	12.6	–
Sulfaguanidine	8.4	7.0
A7	8.2	7.0
A19	7.9	6.7
A37	8.3	7.5

[a] pK_a values were determined in 30% Et-OH – water (v/v) as described in the Experimental section.

reduced basicity (Table V). Indeed, such values were in the range of 7.9–8.3 pKa units, proving that this anchoring group may constitute a valid alternative for obtaining serine protease inhibitors with weak basicity. It is probable that these compounds bind in a neutral state to the enzyme, which we expect to further facilitate the favorable interaction between the carboxylate moiety of Asp 89 and this new anchoring group.

In conclusion, we report here the finding that the sulfonyldicyandiamide group may constitute an interesting alternative for obtaining weakly basic thrombin inhibitors. The simple aromatic sulfonyldicyandiamides prepared in the present study, as well as the corresponding arylsulfonylureido-dicyandiamides, showed affinities in the low micromolar ramge for thrombin and c. 10–20 μM for trypsin. Further derivatization of the detected lead sulfanilyldicyandiamide ($K_I = 3$ μM against thrombin) by means of arylsulfonyl isocyanates or amino acids/dipeptides incorporated/incorporating the classical scaffold D-Phe-Pro(Arg), led to derivatives active in the low nanomolar range against thrombin and maintaining the specificity for this serine protease over the related enzyme trypsin.

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