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PROTEASE INHIBITORS: PART 4. SYNTHESIS OF WEAKLY BASIC THROMBIN INHIBITORS INCORPORATING PYRIDINIUM-SULFANILYLAMINOGUANIDINE MOIETIES

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Three series of derivatives have been prepared by reaction of sulfanilylaminoguanidine with pyrylium salts, with the pyridinium derivatives of glycine and with the pyridinium derivatives of β-alanine, respectively. The new compounds were assayed as inhibitors of two serine proteases, thrombin and trypsin. The study showed that in contrast to the leads, possessing K_I's around 100–300 nM against thrombin, and 450–1420 nM against trypsin, respectively, the new derivatives showed inhibition constants in the range of 15–50 nM against thrombin, whereas their affinity for trypsin remained relatively low. Derivatives of β-alanine were more active than the corresponding glycine derivatives, which in turn were more inhibitory than the pyridinium derivatives of sulfanilylaminoguanidine possessing the same substitution pattern at the pyridinium ring. Thus, the present study proposes two novel approaches for the preparation of high affinity, specific thrombin inhibitors: a novel S1 anchoring moiety in the already large family of arginine/amidine-based inhibitors, i.e., the $SO_2NHNHC(=NH)NH_2$ group, and novel non-peptidomimetic scaffolds obtained by incorporating alkyl-/aryl-substituted-pyridinium moieties in the hydrophobic binding site(s). The first one is important for obtaining bioavailable thrombin inhibitors, devoid of the high basicity of the commonly used arginine/amidine-based inhibitors, whereas the second one may lead to improved water solubility of such compounds.

Keywords: Thrombin; Trypsin; Sulfanilylaminoguanidine; Pyridinium salts; Pyridinium amino acid; Non-basic thrombin inhibitor

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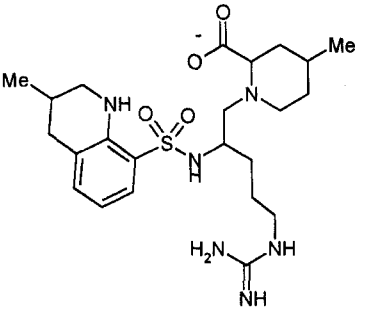
INTRODUCTION

Thrombin (EC 3.4.21.5) has become an important target for drug design in recent years, in the search for low molecular-weight, potent and selective inhibitors with applications as diagnostic and therapeutic agents for the increasingly common thrombotic diseases.[1–8] Although a large number of potent active site-directed thrombin inhibitors, such as peptide aldehydes,[9,10] boronates,[11] benzamidine-[2,3,12,13] or arginine/guanidine-derived[14] inhibitors have been reported, none of them meets all the criteria needed for an ideal antithrombotic drug.[2,15] Thus, the largest majority of the presently available low-molecular weight inhibitors, such as argatroban (MQPA) **1**,[16] inogatran **2**,[8] NAPAP **3**,[17] 4-TAPAP **4** or its 3-amidino-isomer, 3-TAPAP **5**,[2,17] are poorly bioavailable, either due to their high basicity, connected with the presence of guanidino/amidino moieties in their molecule, or are not absorbable orally, or are rapidly eliminated from the circulation, mainly due to their peptidic nature. Although recently some non-basic S1 anchoring groups have been incorporated in the molecules of some thrombin inhibitors,[3,7,18] the presence of guanidino/benzamidino moieties in such compounds is critical, since it is by means of the interaction of these highly polar groups with Asp 189, the central amino acid residue from the specificity pocket, that the enzyme–inhibitor adduct is initially formed (obviously, a lot of other secondary interactions are responsible for the formation of high affinity adducts between thrombin and its inhibitors).[3–5,12–14] Thus, a challenge for drug design would be to exploit the intrinsically high affinity of guanidino-/benzamidino-containing inhibitors for the thrombin active site, but at the same time avoiding the undesired properties connected with their too high basicity. In this paper we propose a novel approach for designing such tight-binding inhibitors, by using sulfonilaminoguanidino moieties as anchoring groups to the specificity S1 pocket. The presence of the SO_2 group in the neighborhood of the aminoguanidino moiety strongly reduces the basicity of the latter, presumably without precluding the binding of inhibitors within the enzyme active site.

In this paper we report the preparation and serine protease inhibitory properties (against human thrombin and human trypsin) of three series of compounds obtained by reaction of sulfanilylaminoguanidine with pyrylium salts, with the pyridinium derivatives of glycine (prepared from Gly and pyrylium salts) and with the pyridinium derivatives of β-alanine (obtained from β-Ala and pyrylium salts), respectively. From the point of view of their thrombin inhibitory properties, as well as that of their specificity for thrombin over trypsin, some of our compounds showed inhibition constants



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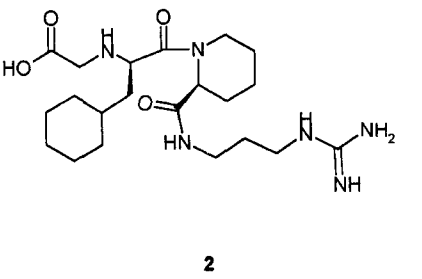

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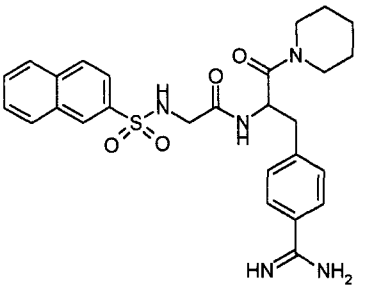

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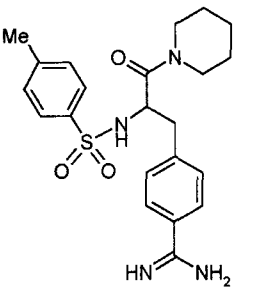

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of the same order of magnitude as those of the clinically used compounds MQPA **1**,[16] and inogatran **2**,[8] in the 15–50 nM range against thrombin, whereas maintaining a much lower trypsin affinity (inhibition constants around 1200–1500 nM) as compared to the above-mentioned clinically used derivatives.

MATERIALS AND METHODS

Melting points were determined on a heating plate microscope (not corrected), IR spectra as KBr pellets at 400–4000 cm^{-1} on a Perkin-Elmer 16PC FTIR spectrometer and ^{1}H-NMR spectra on a Varian 300CXP apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard). Elemental analysis (±0.4% of the theoretical values, calculated for the proposed formulas – data not shown) was done on a Carlo Erba Instrument CHNS Elemental Analyzer, 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. Triethylamine, carbodiimides, and amino acids

used in the syntheses were commercially available compounds (from Sigma, Acros or Aldrich). Sulfanilylaminoguanidine was prepared as previously reported.[19] Pyrylium salts were prepared as described in the literature.[20–23] 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 Chem. Co. (St Louis, MO, USA).

General Procedure for the Preparation of Compounds A(1–16)

Methods A

An amount of 0.22 g (1 mM) of sulfanilylaminoguanidine **7** and the stoichiometric amount of pyrylium salt **6** and 140 μL of triethylamine (1 mM) were dissolved/suspended in 20 mL of absolute methanol. The mixture was refluxed for 30 min, then 0.45 mL of glacial acetic acid were added and refluxation was continued for other 2 h. The cold mixture was treated with 100–200 mL of diethyl ether for the precipitation of the pyridinium salts **A1–A16** which were recrystallized from water with 2–5% perchloric acid.

Method B

An amount of 0.65 g (2.9 mM) of sulfanilylaminoguanidine **7** and 2.9 mM of pyrylium salt **6** were suspended in 5 mL of anhydrous methanol and poured into a stirred mixture of 14.5 mM of triethylamine and 5.8 mM of acetic anhydride. After 5 min stirring, another 10 mL of methanol were added to the reaction mixture, which was heated to reflux for 15 min. Then 14.5 mM of acetic acid was added and heating was continued for 2–5 h. The role of the acetic anhydride was to react with the water formed during the condensation reaction between the pyrylium salt and the aromatic amine so as to shift the equilibrium towards the formation of the pyridinium salts of type **A1–A16**. In the case of sulfanilylaminoguanidine, this procedure was the only one which gave acceptable yields of pyridinium salts possessing 2-methyl groups. The precipitated pyridinium salts obtained were then purified by treatment with concentrated ammonia solution (which also converts the eventually unreacted pyrylium salt to the corresponding pyridine which is soluble in acidic medium), reprecipitation with perchloric acid and recrystallization from water with 2–5% $HClO_4$.

General Procedure for the Preparation of Derivatives 10 and 11

An amount of 10 mM of amino acid (Gly or β-Ala) was suspended/dissolved in 50 mL of anhydrous acetonitrile and the stoichiometric amount (10 mM) of pyrylium salt **6** and triethyl amine (10 mM, 1.47 mL) were added. The reaction mixture was heated at reflux for 4 h, then 2.5 mL of glacial acetic acid was added and refluxing was continued for another 2 h. The obtained reaction mixture was treated as described above (Method A), in order to obtain the pure intermediates **10** and **11** (recrystallized from water with 2–5% perchloric acid).

General Procedure for the Preparation of Compounds B, C(1–16)

An amount of 1 mM of pyridinium-amino acid derivative **10, 11** was dissolved/suspended in 25 mL of anhydrous acetonitrile or acetone, and then treated with 224 mg (1 mM) of sulfanilylaminoguanidine **7** and 190 mg (1 mM) of EDCI · HCl or di-isopropyl-carbodiimide. The reaction mixture was magnetically stirred at room temperature for 15 min, then 30 μL (2 mM) of triethylamine were added and stirring was continued for 16 h at 4°C. 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 and filtered, and the solvent removed *in vacuo*. Preparative HPLC (Dynamax-60A column (25 × 250 mm) using 90% acetonitrile/8% methanol/2% water and a flow rate of 30 mL/min afforded the pure compounds **B, C(1–16)** as colorless solids.

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2,4,6-trimethylpyridinium perchlorate **A1** as white crystals, m.p. 278–9°C (yield of 30%). IR (KBr), cm^{-1}: 625, 740, 1100, 1175, 1290, 1345, 1580, 1675, 3040, 3245, 3335. ^{1}H-NMR (TFA), δ, ppm: 2.56 (s, 6H, 2,6-$(Me)_2$), 2.81 (s, 3H, 4-Me), 7.35–7.85 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 8.10 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{15}H_{20}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2-isopropyl-4,6-dimethylpyridinium perchlorate **A2** as pale yellow crystals, m.p. 259–61°C (yield of 45%). IR (KBr), cm^{-1}: 625, 680, 1100, 1175, 1290, 1345, 1580, 1675, 3020, 3235. ^{1}H-NMR (TFA), δ, ppm: 1.50 (d, 6H, 2Me from *i*-Pr), 2.70 (s, 3H, 6-Me), 2.83 (s, 3H, 4-Me), 3.48 (heptet, 1H, CH from *i*-Pr), 7.25–8.45 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 7.98 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{17}H_{24}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2,6-di-isopropyl-4-methylpyridinium perchlorate **A3** as tan crystals, m.p. 217–8°C (yield of 82%). IR (KBr),

cm^{-1}: 625, 685, 820, 1100, 1175, 1290, 1345, 1580, 1675, 3030, 3250. ^{1}H-NMR (TFA), δ, ppm: 1.51 (d, 12H, 4Me from 2 *i*-Pr), 2.80 (s, 3H, 4-Me), 3.42 (heptet, 2H, 2CH from 2 *i*-Pr), 7.31–8.51 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 8.05 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{19}H_{28}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2,6-dimethyl-4-phenylpyridinium perchlorate **A4** as white crystals, m.p. 283–4°C (yield of 54%). IR (KBr), cm^{-1}: 625, 690, 1100, 1175, 1290, 1345, 1580, 1675, 3030, 3260, 3330. ^{1}H-NMR (TFA), δ, ppm: 2.58 (s, 6H, 2,6-$(Me)_2$), 8.10–9.12 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal. $C_{20}H_{22}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2,6-diethyl-4-phenylpyridinium perchlorate **A5** as yellow crystals, m.p. 268–9°C (yield of 41%). IR (KBr), cm^{-1}: 625, 765, 1100, 1175, 1290, 1345, 1580, 1675, 3040, 3270, 3360. ^{1}H-NMR (TFA), δ, ppm: 1.43 (t, 6H, 2 Me from ethyl), 2.82 (q, 4H, 2 CH_2 from Et), 7.68–8.87 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal. $C_{22}H_{26}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2,6-di-n-propyl-4-phenylpyridinium perchlorate **A6** as yellowish crystals, m.p. 223–5°C (yield of 62%). IR (KBr), cm^{-1}: 625, 775, 1100, 1175, 1290, 1345, 1580, 1675, 3060, 3220, 3315. ^{1}H-NMR (TFA), δ, ppm: 1.01 (t, 6H, 2 Me from propyl), 1.70 (sextet, 4H, $2CH_2$ (β) from *n*-Pr), 2.80 (t, 4H, 2 CH_2 (α) from *n*-Pr), 7.55–8.78 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal. $C_{24}H_{30}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2,6-di-isopropyl-4-phenylpyridinium perchlorate **A7** as white crystals, m.p. 206–7°C (yield of 69%). IR (KBr), cm^{-1}: 625, 1100, 1175, 1290, 1345, 1580, 1675, 3060, 3270, 3315. ^{1}H-NMR (TFA), δ, ppm: 1.45 (d, 12H, 4 Me from *i*-Pr), 2.95 (heptet, 2H, 2 CH from *i*-Pr), 7.92–8.97 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal. $C_{24}H_{30}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2-methyl-4,6-diphenylpyridinium perchlorate **A8** as white crystals, m.p. 270–1°C (yield of 45%). IR (KBr), cm^{-1}: 625, 770, 1100, 1175, 1290, 1345, 1580, 1675, 3040, 3245, 3350. ^{1}H-NMR (TFA), δ, ppm: 2.72 (s, 3H, 2-Me), 7.55–8.73 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. $C_{25}H_{24}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2-ethyl-4,6-diphenylpyridinium perchlorate **A9** as pale-yellow crystals, m.p. 237–8°C (yield of 58%). IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1175, 1290, 1345, 1580, 1675, 3040, 3250, 3350. ^{1}H-NMR (TFA), δ, ppm: 1.50 (t, 3H, Me from ethyl), 2.97 (q, 2H, CH_2), 7.40–8.57 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. $C_{26}H_{26}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2-n-propyl-4,6-diphenylpyridinium perchlorate **A10** as white crystals, m.p. 240–1°C (yield of 75%). IR (KBr), cm^{-1}: 625, 700, 1100, 1175, 1290, 1345, 1580, 1675, 3030, 3270, 3350. ^{1}H-NMR (TFA), δ, ppm: 1.05 (t, 3H, Me from propyl), 1.93 (sextet, 2H, β-CH_2 from *n*-Pr), 2.93 (t, 2H, α-CH_2 from *n*-Pr), 7.38–8.53 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. $C_{27}H_{28}N_5O_2S^+$ ClO_4^- (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2-isopropyl-4,6-diphenylpyridinium perchlorate **A11** as white crystals, m.p. 213–4°C (yield of 49%). IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1175, 1290, 1345, 1580, 1675, 3040, 3250, 3360. ^{1}H-NMR (TFA), δ, ppm: 1.52 (d, 6H, 2 Me from *i*-propyl), 2.52–3.25 (m, 1H, CH from *i*-Pr), 7.33–8.60 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. $C_{27}H_{28}N_5O_2S^+$ ClO_4^- (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2-n-butyl-4,6-diphenylpyridinium perchlorate **A12** as white crystals, m.p. 249–51°C (yield of 70%). IR (KBr), cm^{-1}: 625, 710, 770, 1100, 1175, 1290, 1345, 1580, 1675, 3060, 3260, 3345. ^{1}H-NMR (TFA), δ, ppm: 0.90 (t, 3H, Me from butyl), 1.10–2.15 (m, 4H, CH_3-*CH_2*-*CH_2*-CH_2 from *n*-Bu), 2.97 (t, 2H, α-CH_2 from *n*-Bu), 7.25–8.52 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. $C_{28}H_{30}N_5O_2S^+$ ClO_4^- (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenylmethyl)-2-tert-butyl-4,6-diphenylpyridinium perchlorate **A13** as white crystals, m.p. 206–8°C (yield of 69%). IR (KBr), cm^{-1}: 625, 765, 1100, 1175, 1290, 1345, 1580, 1675, 3060, 3270. ^{1}H-NMR (TFA), δ, ppm: 1.90 (s, 9H, *t*-Bu), 6.83–8.83 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{28}H_{30}N_5O_2S^+$ ClO_4^- (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2,4,6-triphenylpyridinium perchlorate **A14** as yellow crystals, m.p. 248–50°C (yield of 84%). IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1175, 1290, 1345, 1580, 1675, 3030, 3260, 3350. ^{1}H-NMR (TFA), δ, ppm: 7.47–8.63 (m, 21H, ArH from 1,4-phenylene, pyridinium and 2,4,6-Ph_3). Anal. $C_{30}H_{26}N_5O_2S^+$ ClO_4^- (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2,6-diphenylpyridinium perchlorate **A15** as yellow-orange crystals, m.p. 257–8°C (yield of 34%). IR (KBr), cm^{-1}: 625, 705, 765, 1100, 1175, 1290, 1345, 1580, 1675, 3050, 3260. ^{1}H-NMR (TFA), δ, ppm: 6.71–8.40 (m, 17H, ArH from 1,4-phenylene, 2,6-Ph_2 and 3,4,5-H from pyridinium). Anal. $C_{24}H_{21}N_5O_2S^+$ ClO_4^- (C, H, N, S).

1-N-(4-Guanidinoaminosulfonyl-phenyl)-2,3,4,6-tetramethylpyridinium perchlorate **A16** as white crystals, m.p. 265–7°C (yield of 33%). IR (KBr), cm^{-1}: 625, 750, 1100, 1175, 1290, 1345, 1580, 1675, 3040, 3245, 3330. ^{1}H-NMR (TFA), δ, ppm: 2.45 (s, 3H, 3-Me), 2.50 (s, 3H, 4-Me), 2.55 (s, 3H, 6-Me),

2.75 (s, 3H, 2-Me), 8.03–9.17 (m, 5H, ArH from 1,4-phenylene and pyridinium 5-H). Anal. $C_{16}H_{22}N_5O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2,4,6-trimethylpyridinium perchlorate **B1** as white-tan crystals, m.p. 282–3°C (yield of 78%). IR (KBr), cm^{-1}: 625, 680, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3030, 3250. ^{1}H-NMR (TFA), δ, ppm: 2.70 (s, 3H, 4-Me), 2.85 (s, 6H, 2,6-$(Me)_2$), 4.12 (s, 2H, Gly CH_2), 7.13–8.41 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 8.00 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{17}H_{22}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2-isopropyl-4,6-dimethylpyridinium perchlorate **B2** as light orange crystals, m.p. 215–7°C (yield of 66%). IR (KBr), cm^{-1}: 625, 680, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3020, 3235. ^{1}H-NMR (TFA), δ, ppm: 1.50 (d, 6H, 2Me from *i*-Pr), 2.80 (s, 3H, 6-Me), 2.90 (s, 3H, 4-Me), 3.48 (heptet, 1H, CH from *i*-Pr), 4.12 (s, 2H, Gly CH_2), 7.25–8.43 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 7.98 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{19}H_{26}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2,6-di-isopropyl-4-methylpyridinium perchlorate **B3** as tan crystals, m.p. 221–3°C (yield of 71%). IR (KBr), cm^{-1}: 625, 820, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3030, 3250. ^{1}H-NMR (TFA), δ, ppm: 1.51 (d, 12H, 4Me from 2 *i*-Pr), 2.83 (s, 3H, 4-Me), 3.42 (heptet, 2H, 2CH from 2 *i*-Pr), 4.12 (s, 2H, CH_2), 7.31–8.51 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 8.03 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{21}H_{30}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2,6-dimethyl-4-phenylpyridinium perchlorate **B4** as orange-red crystals, m.p. 248–9°C (yield of 61%). IR (KBr), cm^{-1}: 625, 765, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3050, 3265. ^{1}H-NMR (TFA), δ, ppm: 3.00 (s, 6H, 2,6-$(Me)_2$), 4.12 (s, 2H, CH_2), 7.21–8.51 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{22}H_{24}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2,6-diethyl-4-phenylpyridinium perchlorate **B5** as tan crystals, m.p. 235–6°C (yield of 62%). IR (KBr), cm^{-1}: 625, 770, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3060, 3230. ^{1}H-NMR (TFA), δ, ppm: 1.55 (t, 6H, 2 Me from Et), 3.30 (q, 4H, 2 CH_2 from Et), 4.12 (s, 2H, N^+-CH_2), 7.08–8.63 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{24}H_{28}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2,6-di-n-propyl-4-phenylpyridinium perchlorate **B6** as tan crystals, m.p. 227–8°C (yield of 50%). IR (KBr), cm^{-1}: 625, 775, 1100, 1175, 1290, 1345, 1535, 1580,

1640, 1675, 3060, 3240. ^{1}H-NMR (TFA), δ, ppm: 1.15 (t, 6H, 2 Me from Pr), 1.90 (sextet, 4H, 2 CH_2 from Pr), 3.18 (t, 4H, 2 CH_2 from Pr), 4.12 (s, 2H, N^+-CH_2), 7.10–8.50 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{26}H_{32}N_5O_3S^+$ ClO_4^- (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2,6-di-iso-propyl-4-phenylpyridinium perchlorate **B7** as tan crystals, m.p. 213–5°C (yield of 74%). IR (KBr), cm^{-1}: 625, 775, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3060, 3240. ^{1}H-NMR (TFA), δ, ppm: 1.55 (d, 12H, 4 Me from *i*-Pr), 3.53 (heptet, 2H, 2 CH from *i*-Pr), 4.13 (s, 2H, N^+-CH_2), 7.23–8.65 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{26}H_{32}N_5O_3S^+$ ClO_4^- (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2-methyl-4,6-diphenylpyridinium perchlorate **B8** as yellow crystals, m.p. 259–60°C (yield of 59%). IR (KBr), cm^{-1}: 625, 770, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3050, 3250. ^{1}H-NMR (TFA), δ, ppm: 3.00 (s, 3H, 2-Me), 4.12 (s, 2H, CH_2), 7.08–8.58 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{27}H_{26}N_5O_3S^+$ ClO_4^- (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2-ethyl-4,6-diphenylpyridinium perchlorate **B9** as white crystals, m.p. 228–30°C (yield of 80%). IR (KBr), cm^{-1}: 625, 705, 770, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3050, 3250. ^{1}H-NMR (TFA), δ, ppm: 1.60 (t, 3H, Me from Et), 3.27 (q, 2H, CH_2 from Et), 4.12 (s, 2H, N^+-CH_2), 7.08–8.60 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{28}H_{28}N_5O_3S^+$ ClO_4^- (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2-n-propyl-4,6-diphenylpyridinium perchlorate **B10** as white-yellowish crystals, m.p. 210–1°C (yield of 79%). IR (KBr), cm^{-1}: 625, 685, 770, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3080, 3250. ^{1}H-NMR (TFA), δ, ppm: 1.18 (t, 3H, Me from Pr), 2.10 (sextet, 2H, CH_2 from *n*-Pr), 3.20 (t, 2H, CH_2 from *n*-Pr), 4.12 (s, 2H, N^+-CH_2), 7.08–8.63 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{29}H_{30}N_5O_3S^+$ ClO_4^- (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2-isopropyl-4,6-diphenylpyridinium perchlorate **B11** as tan crystals, m.p. 230–1°C (yield of 65%). IR (KBr), cm^{-1}: 625, 710, 770, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3070, 3250. ^{1}H-NMR (TFA), δ, ppm: 1.55 (d, 6H, 2 Me from *i*-Pr), 3.55 (heptet, 1H, CH from *i*-Pr), 4.10 (s, 2H, N^+-CH_2), 7.08–8.63 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{29}H_{30}N_5O_3S^+$ ClO_4^- (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2-n-butyl-4,6-diphenylpyridinium perchlorate **B12** as tan crystals, m.p. 217–9°C

(yield of 49%). IR (KBr), cm^{-1}: 625, 690, 770, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3080, 3250. ^{1}H-NMR (TFA), δ, ppm: 0.93 (t, 3H, Me from *n*-Bu), 1.55 (sextet, 2H, CH_2 from *n*-Bu), 2.05 (quintet, 2H, CH_2 from *n*-Bu), 3.17 (t, 2H, CH_2 from *n*-Bu), 4.12 (s, 2H, N^+-CH_2), 7.08–8.58 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{30}H_{32}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2-tert-butyl-4,6-diphenylpyridinium perchlorate **B13** as white crystals, m.p. 221–2°C (yield of 54%). IR (KBr), cm^{-1}: 625, 705, 765, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3060, 3270. ^{1}H-NMR (TFA), δ, ppm: 1.90 (s, 9H, *t*-Bu), 4.22 (s, 2H, CH_2), 6.83–8.83 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{30}H_{32}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2,4,6-triphenylpyridinium perchlorate **B14** as orange crystals, m.p. 244–5°C (yield of 75%). IR (KBr), cm^{-1}: 625, 705, 770, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3050, 3270. ^{1}H-NMR (TFA), δ, ppm: 4.09 (s, 2H, CH_2), 6.70–8.56 (m, 21H, ArH from 1,4-phenylene, 2,4,6-Ph_3 and 3,5-H from pyridinium). Anal. $C_{32}H_{28}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2,6-diphenylpyridinium perchlorate **B15** as yellow-orange crystals, m.p. 245–6°C (yield of 35%). IR (KBr), cm^{-1}: 625, 705, 765, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3050, 3260. ^{1}H-NMR (TFA), δ, ppm: 4.13 (s, 2H, CH_2), 6.71–8.40 (m, 17H, ArH from 1,4-phenylene, 2,6-Ph_2 and 3,4,5-H from pyridinium). Anal. $C_{26}H_{23}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylmethyl]-2,3,4,6-tetramethylpyridinium perchlorate **B16** as white-tan crystals, m.p. 255–7°C (yield of 60%). IR (KBr), cm^{-1}: 625, 800, 1100, 1175, 1290, 1345, 1535, 1580, 1640, 1675, 3030, 3305. ^{1}H-NMR (TFA), δ, ppm: 2.60 (s, 3H, 4-Me), 2.77 (s, 3H, 3-Me), 2.87 (s, 6H, 2,6-$(Me)_2$), 4.12 (s, 2H, CH_2), 7.21–8.50 (m, AA′BB′, 4H, ArH from 1,4-phenylene), 7.90 (s, 1H, ArH, 5-H from pyridinium). Anal. $C_{18}H_{24}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2,4,6-trimethylpyridinium perchlorate **C1** as white crystals, m.p. 269–71°C (yield of 88%). IR (KBr), cm^{-1}: 625, 680, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3060, 3250, 3330. ^{1}H-NMR (TFA), δ, ppm: 2.66 (s, 3H, 4-Me), 2.88 (s, 6H, 2,6-$(Me)_2$), 3.12 (t, 2H, CH_2), 4.05 (t, 2H, CH_2), 7.47–8.38 (m, 6H, ArH from 1,4-phenylene and 3,5-H from pyridinium). Anal. $C_{18}H_{24}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2-isopropyl-4,6-dimethylpyridinium perchlorate **C2** as white crystals, m.p. 249–50°C

(yield of 80%). IR (KBr), cm^{-1}: 625, 685, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3040, 3255, 3380. ^{1}H-NMR (TFA), δ, ppm: 1.47 (d, 6H, 2Me from *i*-Pr), 2.68 (s, 3H, 4-Me), 2.90 (s, 3H, 6-Me), 3.10–3.75 (m, 3H, CH from *i*-Pr + CH_2), 4.03 (t, 2H, CH_2), 7.33–8.35 (m, 6H, ArH from 1,4-phenylene and 3,5-H from pyridinium). Anal. $C_{20}H_{27}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2,6-di-isopropyl-4-methylpyridinium perchlorate **C3** as white crystals, m.p. 255–6°C (yield of 75%). IR (KBr), cm^{-1}: 625, 685, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3040, 3235, 3410. ^{1}H-NMR (TFA), δ, ppm: 1.48 (d, 12H, 4Me from 2 *i*-Pr), 2.70 (s, 3H, 4-Me), 3.15–3.79 (m, 4H, 2CH from 2 *i*-Pr + CH_2), 4.02 (t, 2H, CH_2), 7.33–8.27 (m, 6H, ArH from 1,4-phenylene and 3,5-H from pyridinium). Anal. $C_{22}H_{32}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2,6-dimethyl-4-phenylpyridinium perchlorate **C4** as white crystals, m.p. 227–9°C (yield of 79%). IR (KBr), cm^{-1}: 625, 690, 780, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3050, 3280. ^{1}H-NMR (TFA), δ, ppm: 3.08 (s, 6H, 2,6-$(Me)_2$), 3.15 (t, 2H, CH_2), 4.03 (t, 2H, CH_2), 7.55–8.37 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{23}H_{26}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2,6-diethyl-4-phenylpyridinium perchlorate **C5** as white crystals, m.p. 234–5°C (yield of 77%). IR (KBr), cm^{-1}: 625, 700, 780, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3060, 3240, 3335. ^{1}H-NMR (TFA), δ, ppm: 1.67 (t, 6H, 2 Me from Et), 3.15–3.80 (m, 6H, 2 CH_2 from Et + CH_2 from ethylene bridge), 4.07 (t, 2H, CH_2 from ethylene bridge), 7.57–8.50 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{25}H_{30}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2,6-di-n-propyl-4-phenylpyridinium perchlorate **C6** as white crystals, m.p. 222–4°C (yield of 60%). IR (KBr), cm^{-1}: 625, 685, 775, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3050, 3255, 3335. ^{1}H-NMR (TFA), δ, ppm: 1.23 (t, 6H, 2 Me from Pr), 2.03 (q, 4H, 2 CH_2 from Pr), 3.07–3.75 (m, 6H, 2 CH_2 from Pr + CH_2 from ethylene bridge), 4.05 (t, 2H, CH_2 from ethylene bridge), 7.55–8.43 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{27}H_{34}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2,6-di-isopropyl-4-phenylpyridinium perchlorate **C7** as white crystals, m.p. 247–9°C (yield of 69%). IR (KBr), cm^{-1}: 625, 685, 765, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3060, 3270, 3350. ^{1}H-NMR (TFA), δ, ppm: 1.60 (d, 12H, 4 Me from *i*-Pr), 3.10–3.83 (m, 4H, 2 CH from *i*-Pr + CH_2 from ethylene

bridge), 4.13 (t, 2H, CH_2 from ethylene bridge), 7.47–8.43 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{27}H_{34}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2-methyl-4,6-diphenylpyridinium perchlorate **C8** as white crystals, m.p. 230–1°C (yield of 71%). IR (KBr), cm^{-1}: 625, 675, 775, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3050, 3245, 3435. ^{1}H-NMR (TFA), δ, ppm: 3.03–3.39 (m, 5H, 2-Me + CH_2 from ethylene bridge), 4.06 (t, 2H, CH_2 from ethylene bridge), 7.05–8.45 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{28}H_{28}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2-ethyl-4,6-diphenylpyridinium perchlorate **C9** as white crystals, m.p. 236–7°C (yield of 58%). IR (KBr), cm^{-1}: 625, 685, 750, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3050, 3220, 3390. ^{1}H-NMR (TFA), δ, ppm: 1.72 (t, 3H, Me from Et), 2.90–3.78 (m, 4H, CH_2 from Et + CH_2 from ethylene bridge), 4.08 (t, 2H, CH_2 from ethylene bridge), 6.88–8.47 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{29}H_{30}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2-n-propyl-4,6-diphenylpyridinium perchlorate **C10** as white crystals, m.p. 236–8°C (yield of 62%). IR (KBr), cm^{-1}: 625, 705, 775, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3080, 3255, 3340. ^{1}H-NMR (TFA), δ, ppm: 1.32 (t, 3H, Me from Pr), 2.17 (sextet, 2H, CH_2 from *n*-Pr), 2.82–3.66 (m, 4H, CH_2 from *n*-Pr + CH_2 from ethylene bridge), 4.09 (t, 2H, CH_2 from ethylene bridge), 6.83–8.43 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{30}H_{32}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2-isopropyl-4,6-diphenylpyridinium perchlorate **C11** as white crystals, m.p. 243–5°C (yield of 75%). IR (KBr), cm^{-1}: 625, 700, 765, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3070, 3250, 3350. ^{1}H-NMR (TFA), δ, ppm: 1.70 (d, 6H, 2 Me from *i*-Pr), 3.15 (t, 2H, CH_2 from ethylenic bridge), 3.50–4.03 (m, 1H, CH from *i*-Pr), 4.11 (t, 2H, CH_2 from ethylenic bridge), 6.95–8.53 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{30}H_{32}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2-n-butyl-4,6-diphenylpyridinium perchlorate **C12** as white crystals, m.p. 227–8°C (yield of 79%). IR (KBr), cm^{-1}: 625, 685, 7650, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3080, 3255, 3330. ^{1}H-NMR (TFA), δ, ppm: 1.15 (t, 3H, Me from *n*-Bu), 1.38–2.45 (m, 4H, 2 CH_2 from *n*-Bu), 3.00–3.68 (m, 4H, CH_2 from *n*-Bu + CH_2 from ethylenic bridge), 4.10 (t, 2H, CH_2 from ethylenic

bridge), 7.02–8.43 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{31}H_{34}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2-tert-butyl-4,6-diphenylpyridinium perchlorate **C13** as white crystals, m.p. 229–31°C (yield of 62%). IR (KBr), cm^{-1}: 625, 700, 765, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3060, 3250, 3370. ^{1}H-NMR (TFA), δ, ppm: 1.92 (s, 9H, *t*-Bu), 3.14 (t, 2H, CH_2), 4.10 (t, 2H, CH_2 from ethylene bridge), 6.90–8.77 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{31}H_{34}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2,4,6-triphenylpyridinium perchlorate **C14** as yellow crystals, m.p. 233–4°C (yield of 78%). IR (KBr), cm^{-1}: 625, 680, 770, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3050, 3260, 3335. ^{1}H-NMR (TFA), δ, ppm: 3.12 (t, 2H, CH_2 from ethylene bridge), 4.05 (t, 2H, CH_2 from ethylene bridge), 6.57–8.40 (m, 21H, ArH from 1,4-phenylene, 2,4,6-Ph_3 and 3,5-H from pyridinium). Anal. $C_{33}H_{30}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2,6-diphenylpyridinium perchlorate **C15** as yellow crystals, m.p. 228–9°C (yield of 24%). IR (KBr), cm^{-1}: 625, 700, 760, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3050, 3240, 3325. ^{1}H-NMR (TFA), δ, ppm: 3.07 (t, 2H, CH_2), 4.13 (t, 2H, CH_2 from ethylene bridge), 6.55–8.50 (m, 17H, ArH from 1,4-phenylene, 2,6-Ph_2 and 3,4,5-H from pyridinium). Anal. $C_{27}H_{25}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

1-N-[(4-Guanidinoaminosulfonyl-phenyl)aminocarbonylethyl]-2,3,4,6-tetramethylpyridinium perchlorate **C16** as white crystals, m.p. 219–21°C (yield of 56%). IR (KBr), cm^{-1}: 625, 680, 1100, 1175, 1285, 1345, 1540, 1580, 1645, 1675, 3030, 3245, 3325. ^{1}H-NMR (TFA), δ, ppm: 2.52 (s, 3H, 3-Me), 2.62 (s, 3H, 4-Me), 2.83 (s, 3H, 6-Me), 2.92 (s, 3H, 2-Me), 3.13 (t, 2H, CH_2), 4.07 (t, 2H, CH_2), 7.61–8.55 (m, 5H, ArH from 1,4-phenylene + 5-H from pyridinium). Anal. $C_{19}H_{26}N_5O_3S^+ ClO_4^-$ (C, H, N, S).

Enzyme Assays; K_I Determinations

Human thrombin and human trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and their concentrations were determined from the absorbance at 280 nm and the extinction coefficients furnished by the supplier. The activity of the thrombin preparations was in the range 2500–3000 NIH units/mg. The potency of standard and novel inhibitors was determined from the inhibition of the enzymatic (amidolytic) activity of these serine proteases, at 21°C, using Ts-Gly-Pro-Arg-*p*NA (Chromozym TH)

from Sigma as substrate, by the method of Lottenberg *et al.*[24] 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 of 379 nm for the peptide-*p*-nitroanilide–*p*-nitroaniline mixtures. An extinction coefficients of 8270 $L \cdot mol^{-1} \cdot cm^{-1}$ at 379 nm in the used buffer (0.01 M Hepes – 0.01 M Tris – 0.1 M NaCl – 0.1% polyethylene glycol 6000; pH 7.80) was 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 Cary 3 spectrophotometer interfaced with a PC. Initial velocities were thus estimated using the direct linear plot-based procedure as reported by Lottenberg *et al.*[24] K_I's were then determined according to Dixon, using a linear regression program.[25] The K_I values determined are the means of at least three determinations.

pK_a Determination

The half neutralization point was measured by titrating the organic acids/bases with 0.05 N NaOH and 0.05 N HCl in EtOH–water (30%, v/v), using a glass electrode, as described by Bell and Roblin[26] for the structurally-related antibacterial sulfonamides.

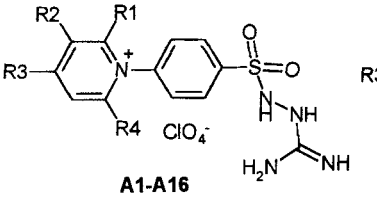
RESULTS AND DISCUSSION

Compounds prepared by reaction of di-, tri- or tetrasubstituted pyrylium salts with sulfanilylaminoguanidine, of types **A1–A16**, as well as the corresponding Gly derivatives of types **B1–B16** and β-Ala derivatives **C1–C16** are shown in Table I.

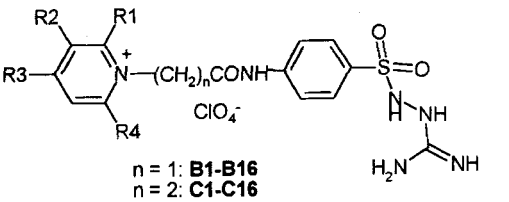
Routine synthetic procedures were used for the reactions of pyrylium salts with nucleophiles (for the preparation of compounds **A,B,C(1–16)** as well as the pyridinium amino acid intermediates **10** and **11**)[27,28] whereas for attaching the pyridinium-amino acyl moieties the condensation reactions in the presence of carbodiimide derivatives were used, as outlined in Scheme 1.[29,30]

Sulfanilylaminoguanidine **7** was reacted with di-, tri- or tetrasubstituted pyrylium salts **6** leading to the pyridinium derivatives **A1–A16**. Alternatively, reaction of pyrylium salts with Gly or β-Ala afforded the pyridinium amino acid derivatives **10** and **11** respectively, which were coupled with **7** in the presence of EDCI or diisopropylcarbodiimide as condensing agents, leading to compounds **B1–B16**, and **C1–C16**, respectively.

TABLE I Pyridinium-sulfanilylaminoguanidines **(A1–A16)**, pyridinium-methyl-carboxamido- **(B1–B16)** and pyridinium-ethylcarboxamido-sulfanilylaminoguanidines **(C1–C16)** prepared in the present study, with their inhibition data against human thrombin and human trypsin



A1-A16



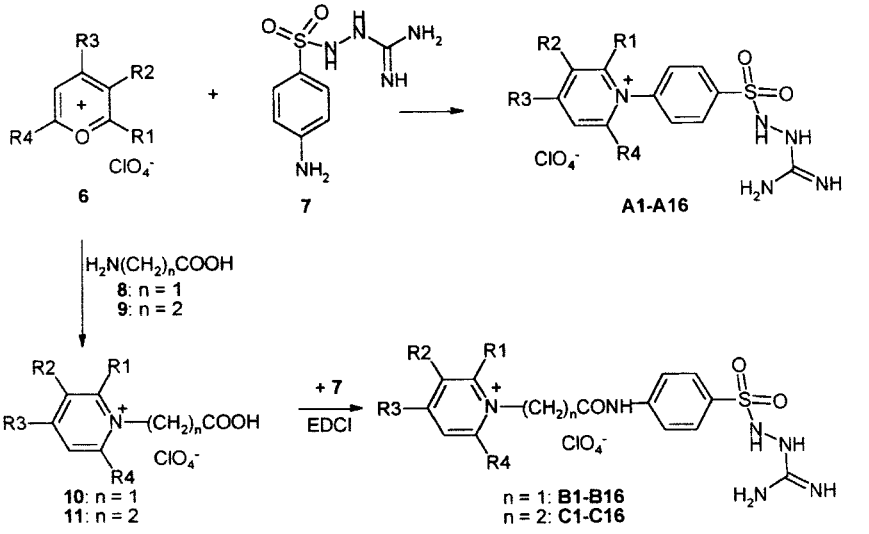
n = 1: **B1-B16**
n = 2: **C1-C16**

Compound	*n*	R^1	R^2	R^3	R^4	K_I[a] (nM)	
						Thrombin	*Trypsin*
A1	—	Me	H	Me	Me	80 ± 5	1270 ± 80
A2	—	*i*-Pr	H	Me	Me	75 ± 6	1180 ± 90
A3	—	*i*-Pr	H	Me	*i*-Pr	92 ± 5	1460 ± 105
A4	—	Me	H	Ph	Me	39 ± 2	1110 ± 75
A5	—	Et	H	Ph	Et	35 ± 3	1100 ± 65
A6	—	*n*-Pr	H	Ph	*n*-Pr	50 ± 7	1150 ± 60
A7	—	*i*-Pr	H	Ph	*i*-Pr	46 ± 5	1200 ± 85
A8	—	Me	H	Ph	Ph	30 ± 2	1250 ± 70
A9	—	Et	H	Ph	Ph	29 ± 2	1240 ± 75
A10	—	*n*-Pr	H	Ph	Ph	34 ± 3	1200 ± 80
A11	—	*i*-Pr	H	Ph	Ph	33 ± 2	1210 ± 75
A12	—	*n*-Bu	H	Ph	Ph	58 ± 5	1340 ± 120
A13	—	*t*-Bu	H	Ph	Ph	30 ± 3	1100 ± 90
A14	—	Ph	H	Ph	Ph	51 ± 4	1950 ± 130
A15	—	Ph	H	H	Ph	55 ± 6	1950 ± 100
A16	—	Me	Me	Me	Me	71 ± 6	1300 ± 90
B1	1	Me	H	Me	Me	70 ± 5	1210 ± 90
B2	1	*i*-Pr	H	Me	Me	62 ± 4	1120 ± 60
B3	1	*i*-Pr	H	Me	*i*-Pr	70 ± 7	1320 ± 95
B4	1	Me	H	Ph	Me	30 ± 3	1100 ± 70
B5	1	Et	H	Ph	Et	25 ± 3	1020 ± 60
B6	1	*n*-Pr	H	Ph	*n*-Pr	48 ± 4	1150 ± 45
B7	1	*i*-Pr	H	Ph	*i*-Pr	42 ± 5	1175 ± 75
B8	1	Me	H	Ph	Ph	20 ± 2	1250 ± 80
B9	1	Et	H	Ph	Ph	15 ± 1	1200 ± 100
B10	1	*n*-Pr	H	Ph	Ph	21 ± 2	1210 ± 65
B11	1	*i*-Pr	H	Ph	Ph	20 ± 2	1175 ± 90
B12	1	*n*-Bu	H	Ph	Ph	50 ± 5	1200 ± 60
B13	1	*t*-Bu	H	Ph	Ph	22 ± 3	1100 ± 55
B14	1	Ph	H	Ph	Ph	47 ± 4	1900 ± 60
B15	1	Ph	H	H	Ph	54 ± 5	1700 ± 65
B16	1	Me	Me	Me	Me	70 ± 6	1260 ± 90
C1	2	Me	H	Me	Me	66 ± 4	1130 ± 80
C2	2	*i*-Pr	H	Me	Me	45 ± 4	1100 ± 100
C3	2	*i*-Pr	H	Me	*i*-Pr	70 ± 5	1270 ± 45
C4	2	Me	H	Ph	Me	27 ± 2	1050 ± 75
C5	2	Et	H	Ph	Et	23 ± 2	1010 ± 55
C6	2	*n*-Pr	H	Ph	*n*-Pr	45 ± 5	1100 ± 100
C7	2	*i*-Pr	H	Ph	*i*-Pr	40 ± 4	1100 ± 80

TABLE I (*Continued*)

Compound	*n*	R^1	R^2	R^3	R^4	K_I (nM)	
						Thrombin	*Trypsin*
C8	2	Me	H	Ph	Ph	16 ± 2	1200 ± 75
C9	2	Et	H	Ph	Ph	13 ± 1	1160 ± 50
C10	2	*n*-Pr	H	Ph	Ph	18 ± 2	1150 ± 60
C11	2	*i*-Pr	H	Ph	Ph	19 ± 1	1140 ± 70
C12	2	*n*-Bu	H	Ph	Ph	40 ± 5	1220 ± 85
C13	2	*t*-Bu	H	Ph	Ph	16 ± 2	1110 ± 50
C14	2	Ph	H	Ph	Ph	40 ± 5	1320 ± 45
C15	2	Ph	H	H	Ph	46 ± 3	1500 ± 50
C16	2	Me	Me	Me	Me	69 ± 5	1200 ± 40

[a] K_I's values were obtained from Dixon plots using a linear regression program,[25] from at least three different assays.

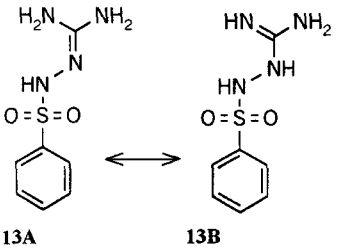


SCHEME 1 Synthesis of sulfanilylaminoguanidine derivatives.

The lead molecule considered by us for obtaining novel types of thrombin inhibitors was benzamidine **12**, one of the simplest such compounds, which possesses an inhibition constant $K_I = 300$ nM against human thrombin. Moreover, the X-ray crystallographic structure for the complex of benzamidine with the enzyme has recently been reported (PDB entry: 1DWB).[31] From the X-ray data it was observed that the amidino moiety of the inhibitor is anchored to the S1 specificity pocket of the enzyme, interacting electrostatically and by means of hydrogen bonds with Asp 189. Several other van der Waals contacts between the inhibitor molecule and the enzyme were

also seen.[31] Obviously, benzamidine is a weak thrombin inhibitor, since the binding energy is only gained due to the strong electrostatic interaction of the carboxylate of Asp 189 and the positively charged amidino moiety. On the other hand, as already mentioned in the introductory section, the amidino moiety possesses too high a basicity to be bioavailable enzyme inhibitors, and it appeared thus of great interest to elaborate non-basic variants of this attractive serine protease anchoring group. The sulfonylaminoguanidino moiety appeared as an attractive candidate for such a purpose, since the presence of the SO_2 moiety in the neighborhood of the strong base, aminoguanidine, should drastically weaken its basicity. Such modified anchoring groups should not presumably interfere with the binding of the inhibitor to the enzyme, since the hydrogen-bonding donor/acceptor properties as well as the possibility to interact electrostatically with the enzyme for the compounds incorporating them should not differ too much from those of the classical amidino-/guanidino-based inhibitors of types **1–5** or **12**. The sulfonylaminoguanidines possess a large number of possible tautomeric forms, and this factor might be a critical one for the binding of such a compound to thrombin. Thus, in a previous work[19] we have shown that arylsulfonylaminoguanidines, among which sulfanilylaminoguanidine **7** belongs, possess moderate but specific thrombin inhibitory properties. Moreover, by means of AM1 and MOPAC calculations it was demonstrated that the tautomer of type **13A** of benzenesulfonylaminoguanidine is less stable than the tautomer **13B** (Scheme 2), a situation that might be relevant for binding to the enzyme.[19] Thus, we presume that the same is true for the pyridinium-based compounds reported here, i.e., that the symmetrical tautomers of type **13A** are less stable than the corresponding non-symmetrical tautomers of type **13B**.

Three series of pyridinium containing sulfanilylaminoguanidines **A1–A16**, **B1–B16** and **C1–C16** were prepared in order to test the above-mentioned hypothesis (Table I). These compounds were obtained by



13A **13B**

SCHEME 2 Benzenesulfonylaminoguanidine tautomers.

TABLE II Inhibition data of two serine proteases with standard inhibitors **1–3** and **12**, and sulfanilylaminoguanidine **7**

Compound		K_I (nM)[a]	
		Thrombin	*Trypsin*
1	Argatroban[b]	19 ± 2	—
2	Inogatran	15 ± 1	540 ± 11
3	NAPAP	6.5 ± 0.05	690 ± 24
7	Sulfanilylaminoguanidine	91 ± 4	1425 ± 100
12	Benzamidine	300 ± 5	450 ± 6

[a]K_I's values were obtained from Dixon plots using a linear regression program,[25] from at least three different assays. [b]From Ref. [5].

reactions of pyrylium salts with sulfanilylaminoguanidine, or alternatively, by condensation of sulfanilylaminoguanidine with pyridinium derivatives of glycine or β-alanine (obtained from the two amino acids and pyrylium salts, by the original procedure of Balaban's and Neidlein's groups).[21,22,32–34]

The following should be noted regarding the serine protease inhibition data in Tables I and II for the new compounds and standard inhibitors: (i) the pyridinium derivatives **A, B, C(1–16)** reported here generally behave as stronger thrombin inhibitors compared to the lead molecules from which they were derived, i.e., benzamidine **12** and sulfanilylaminoguanidine **7**. At the same time, their affinity for trypsin is relatively low, which constitutes a positive feature for the putative clinical use of such compounds, (ii) in the three subseries of investigated compounds, thrombin inhibitory properties increased from the pyridinium derivatives of sulfanilylaminoguanidine **A(1–16)** to the corresponding pyridinium-Gly-derivatives **B(1–16)**, with the pyridinium-β-Ala derivatives **C(1–16)** behaving as the most active inhibitors in the whole series of reported compounds (obviously, this discussion takes into account the same substitution pattern at the pyridinium ring for compounds in the three investigated subseries), (iii) the nature of R1–R4 groups substituting the pyridinium ring was critical for the biological activity of the obtained compounds, similarly to the situation seen for the carbonic anhydrase sulfonamide inhibitors reported previously.[19,20] Thus, tri- or tetraalkylpyridinium as well as 2,6-di- or 2,4,6-triphenylpyridinium moieties were generally less effective than 2-alkyl-4,6-diphenylpyridinium groups in bestowing strong thrombin inhibitory properties to the compounds incorporating them. Practically, the most active derivatives in all three subseries were those containing 2-alkyl-4,6-diphenylpyridinium moieties, such as 2-methyl-, 2-ethyl-, 2-*iso*-propyl- or 2-*tert*-butyl-4,6-diphenylpyridinium groups. Replacing the 2-alkyl group mentioned above with a bulky phenyl one (such as in compounds **A14**, **B14** or **C14**) or with a longer aliphatic

chain (*n*-butyl, such as in **A12**, **B12** or **C12**) led to a drastic reduction in thrombin inhibitory effects. On the other hand, compounds possessing 2,6-dialkyl-4-phenylpyridinium moieties (such as **A,B,C(4, 5)**) possessed a behavior intermediate between the strong inhibitors of the type **A,B,C(8, 9, 11, 13)** and the relatively weak inhibitors of type **A,B,C(1–3, 14–16)**. Anyhow, the best substitution for bestowing strong thrombin inhibitory properties was that incorporating the 2-ethyl-4,6-diphenylpyridinium moiety in the molecules of the new derivatives. Some of the compounds containing this substitution pattern, such as **B9** and **C9** (but also the structurally-related compounds **B8**, **B10**, **B11**, **C8**, **C10** and **C11**) showed thrombin inhibitory properties of the same order of magnitude as the clinically used derivatives argatroban **1** and inogatran **2**, although they are less effective as compared to the very potent inhibitor NAPAP (Table II). A special mention should be made regarding the fact that the new compounds reported here possess a much lower affinity for trypsin as compared to the standard inhibitors **2** and **3**, which constitutes a highly desirable feature in a compound to be developed for clinical use.

pK_a values for the amidino/guanidino as well as sulfonamido moieties of some of the newly synthesized serine protease inhibitors and standard compounds such as inogatran, argatroban and NAPAP (Table III) show that the approach proposed here for reducing the basicity of such an enzyme inhibitor is a successful one. Thus, unlike the highly basic guanidines/amidines of type **1–3** (pK_a's around 12.3–12.6), sulfanilylaminoguanidine **7** and its derivatives reported here (such as compounds **A9**, **B9** or **C9**) have pK_a values for the guanidino moiety around of 8.1–8.5, being at least 10^4 times less basic than the previously mentioned derivatives. Furthermore, due to the presence of the sulfonyl moiety in their molecules, these compounds also

TABLE III pK_a data for serine protease inhibitors **1–3,7, A9, B9** and **C9**

Compound	pK_a[a]	
	Guanidino/amidino moiety	*SO_2NH moiety*
1 Argatroban[b]	12.5	—
2 Inogatran[b]	12.3	—
3 NAPAP	12.6	—
7 Sulfanilylaminoguanidine	8.4	7.1
A9	8.2	7.1
B9	8.3	7.2
C9	8.3	7.3

[a]pK_a values were determined in 30% Et-OH–water (v/v) as described in the Experimental section. [b]From Ref. [8].

possess a weakly acidic character, with another ionization step around pK_a 7, due to the loss of the SO_2NH proton. Thus, the $SO_2NHNHC(NH_2)=NH$ group will exist appreciably as the neutral molecule at pH 7.4, suitable for oral absorption.

The strong thrombin inhibitory properties of some compounds reported in this study might be explained by taking into account the X-ray crystallographic structure of the enzyme as well as those of some of its complexes with guanidine-/amidine-based inhibitors.[4,5,33,34] Thus, it was shown that effective binding is achieved when a proline, a pipecolic acid or a similarly non-hydrophilic moiety is present in the P2 position, which allows favorable interactions with the enzyme S2 cavity (comprising among others amino acid residues Trp 60D and Tyr 60A), as well as when hydrophobic (generally aromatic: Ph, Ts, naphthyl) groups are present at P3 which allow strong interactions with the S3 site (comprising residues Leu 99; Trp 215 and Ile 174 among others).[4,5,35,36] Some moieties present in the compounds prepared by us might thus just possess the required structural elements for the formation of high affinity adducts with thrombin, although this needs to be confirmed by molecular modeling. For example, for the strongest inhibitor reported in this paper, **C9** ($K_I = 13$ nM against thrombin), the CH_2CH_2CO moiety might interact with the S2 cavity, whereas the two phenyls substituting the pyridinium moiety probably bind within the aryl binding site (S3). Obviously, the sulfonylaminoguanidino moiety of all these inhibitors probably fills the specificity S1 pocket, interacting with Asp 189, as discussed earlier.

In conclusion, three series of weakly basic sulfanilylaminoguanidine derivatives have been prepared by reaction of sulfanilylaminoguanidine with di-, tri- or tetrasubstituted pyrylium salts (bearing alkyl, aryl or combination of the two moieties in their molecule) and with the corresponding Gly-pyridinium and β-Ala-pyridinium derivatives, respectively. Qualitative SAR proved that the most potent thrombin inhibitors bore a 2-alkyl-4,6-diarylpyridinium moietie, and that the β-Ala derivatives were more active than the corresponding Gly derivatives, which in turn were more active than the corresponding pyridinium-sulfanilylaminoguanidines. The obtained compounds generally possessed a low affinity for trypsin, which might be considered a positive feature for the putative pharmacological development of such thrombin inhibitors. Thus, our study proposes two novel approaches for the preparation of high affinity, specific thrombin inhibitors: (1) a novel S1 anchoring moiety of the arginine/amidine type i.e., the $SO_2NHNHC(=NH)NH_2$ group and, (2) novel non-peptidomimetic scaffolds obtained by incorporating alkyl-/aryl-substituted-pyridinium moieties

in the hydrophobic binding site(s). The first approach is important for obtaining bioavailable thrombin inhibitors, devoid of the high basicity of the commonly used arginine-/amidine-based inhibitors, whereas the second one may lead to improved water solubility of such compounds due to facilitated salt formation to give the required lipophilic–lipophobic ratio for intestinal absorption and reduced plasma binding, in order to increase availability of the free drug.

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References

[1] (a) A. Patel, H.J. Smith, J. Stürzebecher (1998) "Design of enzyme inhibitors as drugs", in *Introduction to the Principles of Drug Design and Action*, (Smith, H.J., Ed.), pp. 261–330. Harwood Academic Publishers, OPA; Amsterdam; (b) J.A. Shafer (1998) *Curr. Opin. Chem. Biol.*, **2**, 458–465; (c) P.E. Sanderson and A.M. Naylor-Olsen (1998) *Curr. Med. Chem.*, **5**, 289–304.

[2] (a) J. Stürzebecher and J. Meier (1995) *J. Enzyme Inhib.*, **9**, 1–2; (b) T. Steimetzer, M. Batdorsdhjin, P. Kleinwachter, L. Seyfarth, G. Greiner, S. Reissmann and J. Stürzebecher (1999) *J. Enz. Inhib.*, **14**, 203–216.

[3] J. Stürzebecher, D. Prasa, J. Hauptmann, H. Vieweg and P. Wikstrom (1997) *J. Med. Chem.*, **40**, 3091–3099.

[4] (a) V. Pavone, G. De Simone, F. Nastri, S. Galdiero, N. Staiano, A. Lombardi and C. Pedone (1998) *J. Biol. Chem.*, **379**, 987–1006; (b) S.R. Stone and B.F. Le Bonniec (1998) Thrombin. In *Handbook of Proteolytic Enzymes – CD-ROM* (Barrett, A.J., Rawlings, N.D. and Woessner, J.F., Eds.), Chapter 55. Academic Press; London.

[5] R.A. Engh, H. Brandstetter, G. Sucher, A. Eichinger, U. Baumann, W. Bode, R. Huber, T. Poll, R. Rudolph and W. von der Saal (1996) *Structure*, **4**, 1353–1362.

[6] R.E. Babine and S.L. Bender (1997) *Chem. Rev.*, **97**, 1359–1472.

[7] F.R. Salemme, J. Spurlino and R. Bone (1997) *Structure*, **5**, 319–324.

[8] U.G. Eriksson, L. Renberg, U. Bredberg, A.C. Teger-Nilsson and C.G. Regardh (1998) *Biopharm. Drug Dispos.*, **19**, 55–64.

[9] (a) S. Bajusz, E. Szell, D. Bagdy, E. Barbas, G. Horvath, M. Dioszegi, Z. Fittler, G. Szabo, A. Juhasz, E. Tomori and G. Szilagyi (1990) *J. Med. Chem.*, **33**, 1729–1735; (b) A. Wienand, C. Ehrhardt, R. Metternich and C. Tapparelli (1999) *Bioorg. Med. Chem.*, **7**, 1295–1307.

[10] R. Krishnan, E. Zhang, K. Hakansson, R.K. Arni, A. Tulinsky, M.S. Lim-Wilby, O.E. Levy, J.E. Semple and T.K. Brunck (1998) *Biochemistry*, **37**, 12094–13103.

[11] G. Claeson, M. Philipp, E. Agner, M.F. Scully, R. Metternich, V.V. Kakkar, T. DeSoyza and L.H. Niu (1993) *Biochem. J.*, **290**, 309–312.

[12] J. Stürzebecher, D. Prasa, E. Bretschneider, W. Bode, M. Bauer, H. Brandstetter, P. Wikstrom and H. Vieweg (1993). New developments in the field of benzamidine-derived thrombin inhibitors. In *DIC – Pathogenesis, Diagnosis, and Therapy of Disseminated Intravascular Fibrin Formation*, (Muller-Berghaus, G., Madlener, K., Blomback, M. and Cate, J.W., Eds.), pp. 183–190. Excerpta Medica: Amsterdam, London, New York, Tokyo.

[13] W.C. Lumma, K.M. Witherup, T.J. Tucker, S.F. Brady, J.T. Sisko, A.M. Naylor-Olsen, S.D. Lewis, B.J. Lucas and J.P. Vacca (1998) *J. Med. Chem.*, **41**, 1011–1013.

[14] J.E. Semple, D.C. Rowley, T.K. Brunck, T. Ha-Uong, N.K. Minami, T.D. Owens, S.Y. Tamura, E.A. Goldman, D.V. Siev, R.J. Ardecky, S.H. Carpenter, Y. Ge, B.M. Richard, T.G. Nolan, K. Hakanson, A. Tulinsky, R.F. Nutt and W.C. Ripka (1996) *J. Med. Chem.*, **39**, 4531–4536.
[15] J.J. Sixma and P.G. de Groot (1992) *Thromb. Res.*, **68**, 507–512.
[16] S. Okamoto, K. Kinjo, A. Hijikata, R. Kikumoto, Y. Tamao, K. Ohkubo and S. Tonomura (1980) *J. Med. Chem.*, **23**, 827–830.
[17] J. Stürzebecher, F. Markwardt, B. Voigt, G. Wagner and P. Walsmann (1983) *Thromb. Res.*, **29**, 635–642.
[18] W.C. Groutas, R. Kuang, R. Venkataraman, J.B. Epp, S. Ruan and O. Prakash (1997) *Biochemistry*, **36**, 4739–4750.
[19] (a) C.T. Supuran, A. Scozzafava, F. Briganti and B.W. Clare (2000) *J. Med. Chem.*, **43** (in press); (b) B.W. Clare, A. Scozzafava, F. Briganti and C.T. Supuran (1999) *J. Enz. Inhib.* (in press).
[20] A.T. Balaban, A. Dinculescu, G.N. Dorofeenko, G.W. Fischer, A.V. Koblik, V.V. Mezheritskii and W. Schroth (1982) "Pyrylium salts: Syntheses, reactions and physical properties". In *Advances in Heterocyclic Chemistry*, (Katritzky, A.R., Ed.), pp. 8–360. Academic Press; New York.
[21] C.T. Supuran, E. Pop and A. Dinculescu (1994) *Heterocycles*, **37**, 667–671.
[22] R. Neidlein and P. Witerzens (1975) *Monats. Chem.*, **106**, 643–648.
[23] C.T. Supuran and B.W. Clare (1995) *Eur. J. Med. Chem.*, **30**, 687–696.
[24] R. Lottenberg, U. Christensen, C.M. Jackson and P.L. Coleman (1981) *Meth. Enzymol.*, **80**, 341–361.
[25] H.C. Hemker, G.M. Willems and S.A. Beguin (1986) *Thromb. Haemostas.*, **56**, 9–17.
[26] P.H. Bell and R.O. Roblin (1942) *J. Am. Chem. Soc.*, **64**, 2905–2917.
[27] C.T. Supuran, G. Manole, A. Dinculescu, A. Schiketanz, M.D. Gheorghiu, I. Puscas and A.T. Balaban (1992) *J. Pharm. Sci.*, **81**, 716–719.
[28] C.T. Supuran, A. Scozzafava, M.A. Ilies, B. Iorga, T. Cristea, F. Chiraleu and M.D. Banciu (1998) *Eur. J. Med. Chem.*, **33**, 577–594.
[29] (a) G.W. Anderson, J.E. Zimmerman and F.M. Callahan (1963) *J. Am. Chem. Soc.*, **85**, 3039; (b) J.C. Sheehan and S.L. Ledis (1973) *J. Am. Chem. Soc.*, **95**, 875–879.
[30] (a) A. Scozzafava, L. Menabuoni, F. Mincione, F. Briganti, G. Mincione and C.T. Supuran (1999) *J. Med. Chem.*, **42**, 2641–2650; (b) C.T. Supuran, A. Scozzafava, L. Menabuoni, F. Mincione, F. Briganti and G. Mincione (1999) *Eur. J. Pharm. Sci.*, **8**, 317–328; (c) A. Scozzafava, F. Briganti, G. Mincione, L. Menabuoni, F. Mincione and C.T. Supuran (1999) *J. Med. Chem.*, **42**, 3690–3700.
[31] D.W. Banner and P. Hadvary (1991) *J. Biol. Chem.*, **266**, 20 085–20 093.
[32] A.T. Balaban and C. Toma (1966) *Tetrahedron Suppl.*, **7**, 1–7.
[33] A. Dinculescu and A.T. Balaban (1980) *Rev. Roum. Chim.*, **25**, 1505–1528.
[34] C.T. Supuran, M.D. Banciu and A.T. Balaban (1993) *Rev. Roum. Chim.*, **38**, 199–205.
[35] W. Bode, D. Turk and A. Karshikov (1992) *Protein Sci.*, **1**, 426–471.
[36] M.T. Stubbs and W. Bode (1993) *Thromb. Res.*, **69**, 1–58.