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Carbonic anhydrase activators
Part 24. High affinity isozymes I, II and IV activators, derivatives of 4-(4-chlorophenylsulfonylureido-amino acyl)ethyl-1*H*-imidazole

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Abstract

N-1-Tritylsulfenyl histamine was synthesized by reaction of histamine (Hst) with tetrabromophthalic anhydride followed by protection of its imidazole moiety with tritylsulfenyl chloride. After hydrazinolysis, it afforded a key intermediate which was derivatized at the aminoethyl group in order to obtain new types of activators of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1). Reaction of the key intermediate with 4-chlorophenylsulfonylureido amino acids (cpu-AA) in the presence of carbodiimides afforded, after deprotection of the imidazolic nitrogen atom, a series of compounds with the general formula cpu-AA-Hst (cpu, 4-$ClC_6H_4SO_2NHCO$). Some structurally related dipeptide derivatives with the general formula cpu-AA1-AA2-Hst (AA, AA1 and AA2 represent amino acyl moieties) were also prepared by a strategy similar to that applied for the amino acyl compounds mentioned above. The new derivatives proved to be efficient activators of three CA isozymes. Best activity was detected against hCA I and bCA IV, for which some of the new compounds showed affinities in the 1–10 nM range (h, human; b, bovine isozymes). hCA II, on the other hand, was less prone to activation by the new derivatives, which possessed affinities around 20–50 nM for this isozyme. This new class of CA activators might lead to the development of drugs/diagnostic agents for CA deficiency syndrome, a genetic disease of bone, brain and kidneys.

Keywords: Carbonic anhydrase; Histamine; Amino acid; 4-Chlorophenylsulfonyl isocyanate; Sulfonylureas; Enzyme activators

1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) deficiency syndrome is a genetic disease of bone, brain and kidney affecting a large number of people (Sly, 1991; Sly and Hu, 1995). In this condition, a certain CA isozyme gene (generally CA II, I or IV) is either not expressed, or its protein product is unstable due to deleterious mutations, and the corresponding CA isozyme is absent in the blood, kidney or lung of such people (Sly, 1991; Sly and Hu, 1995). No pharmacologically specific treatment for this condition is available as yet, although the possibility of activating certain CA isozymes has been investigated thoroughly in the last few years (Clare and Supuran, 1994; Supuran and Puscas, 1994; Briganti et al., 1997, 1998, 1999). Furthermore, in many CA deficiency syndrome cases, only one CA isozyme is absent, the other isozymes generally being present in normal concentrations (Sly, 1991). Thus, their activation by exogenous modulators of activity might lead to restoration of the normal CA activity in patients affected by this life-threatening genetic disorder (Supuran et al., 1993, 1996b,c).

Indeed, carbonic anhydrase inhibitors of the unsubstituted sulfonamide type, RSO_2NH_2, are widely used drugs for the treatment or prevention of a variety of diseases, such as glaucoma (Supuran, 1994; Supuran et al., 1998a,b, 1999; Scozzafava et al., 1999a,b), epilepsy (Reiss and Oles, 1996), gastric and duodenal ulcers (Supuran, 1994), osteoporosis (Raisz et al., 1988) and acid–base disequilibria (Supuran et al., 1996a), among others. In contrast to inhibitors, activators of this enzyme, for which at least 14 different isozymes have so far been isolated in higher vertebrates (Hewett-Emmett, 1999), have been much less investigated. Only recently have the X-ray crystallographic structures of the first adducts of the physiologically relevant isozyme II (hCA II) with the activators histamine

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(Briganti et al., 1997) and phenylalanine (in this case a tertiary complex, in which azide is also bound to the Zn(II) ion) been reported. Furthermore, a few other QSAR (Clare and Supuran, 1994) or synthetic chemistry (Supuran et al., 1993, 1996b,c; Ilies et al., 1997; Briganti et al., 1999) studies have been reported in the field of CA activators.

The lead molecule considered by us for obtaining tighter binding CA activators was histamine (**1**) itself. As seen from the X-ray coordinates with which Fig. 1 was generated, the activator molecule is bound at the entrance of the hCA II active site cavity, where it is anchored by hydrogen bonds to amino acid side-chains and to water molecules (Briganti et al., 1997). Such hydrogen bonds involve only the nitrogen atoms of the imidazole moiety, whereas the terminal aliphatic amino group does not make any contact with the enzyme, but extends away from the cavity into the solvent. On the other hand, the Nδ1 and Nε2 atoms of the histamine imidazole ring are engaged in hydrogen bonds with the side-chains of Asn 62, His 64, Gln 92 and Wat152. Thus, it is of interest to derivatize the lead at its aliphatic NH_2 moiety in order to exploit the binding energy of such modified groups with polar amino acid residues at the edge of the active site, such as His 3, His 4, His 10, His 15, His 17 and Asp 19 (Fig. 1). This approach has been used successfully both by Whiteside's (Avila et al., 1993; Gao et al., 1995, 1996) and our groups (Supuran et al., 1998a,b, 1999; Scozzafava et al., 1999a,b) for the design of tight-binding, isozyme-specific sulfonamide CA

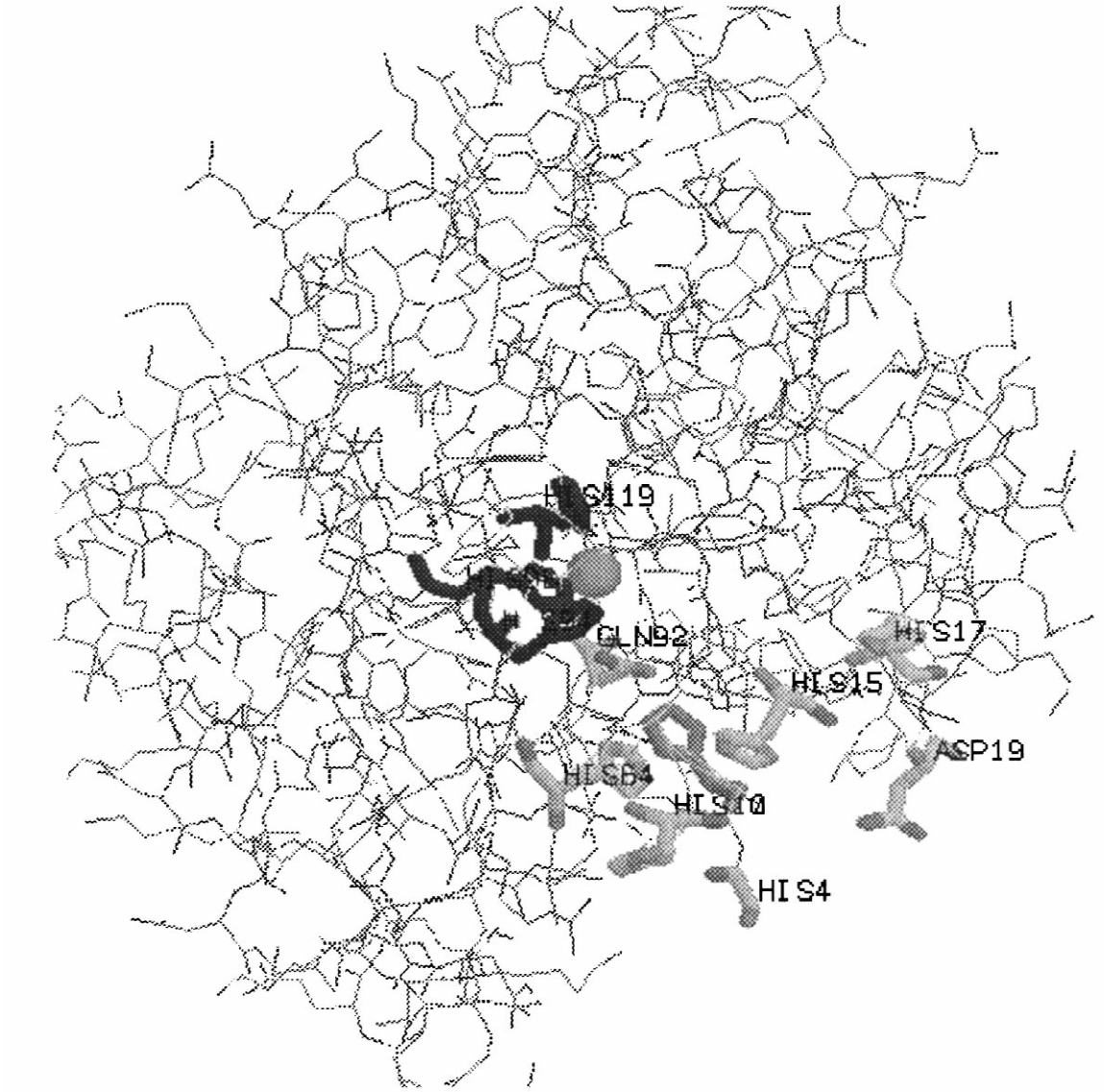


Fig. 1. hCA II–histamine adduct: the Zn(II) ion (central sphere) and its three histidine ligands (His 94, His 96 and His 119) are shown at the center of the active site, whereas histamine is situated at the entrance, between residues His 64 (left) and Gln 92 (above). Amino acid residues at the rim of the active site, such as His 4, His 10, His 15, His 17 and Asp 19, thought to be important for the binding of the activators, are also evidenced. The figure was generated from the X-ray coordinates of the hCA II–histamine adduct reported by this group (Briganti et al., 1997) using the program RasMol for Windows 2.6. The coordinates of this structure are deposited in the Brookhaven Protein Database (PDB entry 4TST).

inhibitors. Furthermore, we recently (Scozzafava and Supuran, 1999a) reported some carboxamido and ureido derivatives of histamine (derivatized at the aliphatic amino group) possessing high affinities for the three CA isozymes mentioned above. Thus, it is of interest to explore other types of moieties that might be attached at the aliphatic end of the molecule, and chlorophenylsulfonylureido-amino acyl groups (chlorophenylsulfonylureido groups are abbreviated as 'cpu') are of interest due to the possible interactions of these highly polarized groups with amino acid residues at the edge of the active site. Such interactions would presumably lead to increased stabilities of the enzyme–activator (E–A) adducts.

In this paper we report the synthesis of a series of 4-chlorophenylsulfonylureido-amino acyl/dipeptidyl histamine derivatives possessing the general formula cpu-AA-Hst and cpu-AA1-AA2-Hst (AA, AA1, AA2, amino acyl moieties; Hst, histamine) obtained by reaction of appropriately protected histamine (Briganti et al., 1999) with chlorophenylsulfonylureido amino acids/dipeptides in the presence of carbodiimide derivatives. The new compounds were assayed as activators of three CA isozymes, hCA I, hCA II and bCA IV (h, human; b, bovine isozyme), and generally showed very good activities. SAR in this series of derivatives is also discussed. Ex vivo data showed that some of the new compounds strongly activate red cell CA isozymes.

2. Materials and methods

2.1. Chemistry

Melting points were determined with a heating plate microscope and are not corrected. IR spectra were obtained using KBr pellets with a Perkin-Elmer 16PC FTIR spectrometer, while ^{1}H-NMR spectra were recorded with a Varian 300CXP apparatus using solvents specified in each case. ^{13}C-NMR spectra were registered at 75 MHz using the same apparatus. Chemical shifts are expressed as δ values relative to Me_4Si as standard. Elemental analyses were performed by combustion for C, H, N with an automated Carlo Erba analyzer, and were $\pm$0.4% of the theoretical values. Preparative HPLC was carried out on C_{18} reversed-phase Bondapack or Dynamax-60A (25×250 mm) columns, with a mobile phase of 90% acetonitrile/8% methanol/2% water (30 ml/min).

Compounds used in synthesis (histamine, natural and non-natural amino acids, tritylsulfenyl chloride, tetrabromophthalic anhydride, hydrazine, etc.) were commercially available (from Sigma, Acros or Aldrich). The 4-chlorophenylsulfonylureido-amino acid/dipeptide derivatives were prepared as described previously (Scozzafava and Supuran, 1999b) by the reaction of 4-chlorophenylsulfonyl isocyanate (Aldrich) with amino acids/dipeptides (from Sigma or Aldrich). Acetonitrile, acetone, dioxane (Merck) and other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions.

2.1.1. Preparation of N-1-tritylsulfenyl-histamine (3)

An amount of 5.55 g (50 mM) of histamine and 23.15 g (50 mM) of tetrabromophthalic anhydride were suspended in 300 ml of dry toluene and refluxed under Dean–Stark conditions until water was separated (generally 2–3 h). The solvent was evaporated in vacuo, the crude product dissolved in 150 ml of anhydrous acetonitrile and treated with 15.5 g (50 mM) of tritylsulfenyl chloride and 6.95 ml (50 mM) of triethylamine. The mixture was stirred at room temperature for 3 h (TLC control), the solvent was then evaporated and the crude product (**2**) stirred with 100 ml of water and ice. The tan precipitate obtained was filtered, dried and used directly in the deprotection step. Hydrazinolysis was effected by dissolving the above-mentioned precipitate in 200 ml of ethanol, adding 15 ml hydrazinium hydroxide, followed by stirring for 5 h at room temperature. The solvent was then evaporated, a small excess of 2 N HCl solution added, and the precipitated tetrabromophthalhydrazide was filtered and discarded, whereas the solution containing **3** was brought to pH 7 with solid $NaHCO_3$, brought to a small volume by in vacuo evaporation of the solvent, and precipitated **3** was then recrystallized from ethanol (yield 80%, based on histamine, after the three steps described above). Tan crystals, m.p. 177–178°C, ^{1}H-NMR (300 MHz, DMSO-d_6), δ, ppm: 2.47 (t, 2H, J = 7.0 Hz, CH_2); 2.96 (q, 2H, J = 6.2, 12.5 Hz, $H_2NC\underline{H}_2$); 4.23 (m, 2H, NH_2); 7.10–7.30 (m, 15H, trityl); 7.34 (m, 1H, imidazole CH); 8.35 (s, 1H, imidazole CH); Anal., found: C, 75.12; H, 5.83; N, 10.88%; $C_{24}H_{23}N_3S$ requires: C, 74.77; H, 6.01; N, 10.90%.

2.1.2. General procedure for the preparation of 4-chlorophenylsulfonylureido amino acids/dipeptides cpu-AA and cpu-AA1-AA2

An amount of 20 mM of amino acid/dipeptide was suspended/dissolved in 50 ml of anhydrous acetone or acetonitrile, and a stoichiometric amount of 4-chlorophenylsulfonyl isocyanate was added in one portion with energetic stirring and eventual cooling of the reaction mixture. The mixture was then stirred for 1–2 h at 4°C, the solvent was evaporated in vacuo and the product purified either by recrystallization from water–ethanol (1:1, v/v), or by preparative HPLC (in the case of cpu-GlyGly, cpu-His, cpu-Val, cpu-Trp and cpu-Phe, when the chlorophenylsulfonylureido-amino acid/dipeptide contained variable amounts of unreacted amino acid and 4-chlorobenzenesulfonamide). Conditions were: C_{18} reversed-phase Bondapack or Dynamax-60A (25×250 mm) columns; 90% acetonitrile/8% ethanol/2% water, 30 ml/min. Remarkably, the reaction of L-Lys monohydrochloride or L-Arg monohydrochloride with 4-chlorophenylsulfonyl

isocyanate under the conditions mentioned above led to the formation of only one very pure product, i.e. the α-derivatized compound, without derivatization of the ε-amino moiety in the case of Lys, or the guanidino moiety in the case of Arg. This is probably due to the fact that H^+ acts, in this case, as a very good side-chain protecting group for these two amino acids. This has been further confirmed by the synthesis of α-cpu-Lys and α-cpu-Arg from the appropriately protected amino acid derivatives (*N*-ε-acetyl-L-Lys and ω-*N*-tritylsulfenyl-L-Arg) and 4-chlorophenylsulfonyl isocyanate, followed by deprotection of the side-chain under standard conditions (data not shown).

*2.1.3. General procedure for the preparation of compounds **A1–A24***

An amount of 10 mM *N*-1-tritylsulfenyl-histamine (**3**) was dissolved in 50 ml of anhydrous acetonitrile and then treated with a solution obtained from 10 mM 4-chlorophenylsulfonylureido amino acid/dipeptide (10 mM) dissolved in 10 ml of the same solvent, followed by 10 mM diisopropyl-carbodiimide (or EDCI·HCl + Et_3N) and 10 mM 1-hydroxybenzotriazole in anhydrous acetonitrile as solvent. The reaction mixture was stirred for 3–9 h at 4°C (TLC control). The solvent was then evaporated in vacuo and the residue taken up in ethyl acetate (50 ml), poured into a 5% solution of sodium bicarbonate (50 ml) and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate and filtered, and the solvent removed in vacuo. In many cases the compounds of type **4** precipitated, were filtered, dried and deprotected at the N-1 imidazolic moiety in the following way. Crude **4** was dissolved in 20 ml of dioxane and treated with 25 ml of a 4 M HCl solution in dioxane, followed by heating at 40°C for 6–8 h (TLC control). The solvent was then evaporated under reduced pressure, the residue taken up in 50 ml of a 5% solution of sodium bicarbonate and the tritylsulfenyl chloride formed during the deprotection step extracted in 2×50 ml of Et_2O. The water phase was evaporated in vacuo to a small volume, when generally compounds **A1**–**A24** precipitated by allowing the mixture to stand overnight at 4°C. The pure compounds were obtained after recrystallization from ethanol–water (1:1, v/v). In some cases, preparative HPLC was performed (C_{18} reversed-phase Bondapack or Dynamax-60A (25×250 mm) columns; 90% acetonitrile/8% methanol/2% water, 30 ml/min) in order to obtain the pure title derivatives.

*2.1.3.1. 4-[β-(4-Chlorophenylsulfonylureido-glycylamido)-ethyl)]-1H-imidazole (**A1**)*

White crystals, m.p. 278–280°C (dec.); IR (KBr), cm^{-1}: 1152 (SO_2^{sym}), 1289 (amide III), 1371 (SO_2^{as}), 1579 (amide II), 1710 (amide I), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.51 (t, 2H, $J = 7.0$, Hst CH_2); 2.97 (t, 2H, $J = 7.0$, Hst CONHC$\underline{H}_2$); 3.66 (s, 2H, C$\underline{H}_2$ of Gly); 7.34 (m, 1H, imidazole CH); 7.62 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 7.94 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.22 (br s, 3H, CONH + NHCONH); 8.35 (s, 1H, imidazole CH); 8.80 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.3 (s, $\underline{C}H_2$ of Hst); 37.6 (s, $\underline{C}H_2$ of Hst); 40.4 (s, $\underline{C}H_2$ of Gly); 122.4 (s, C-4 of Hst); 130.9 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.9 (s, NH$\underline{C}$ONH); 134.8 (s, C-5 of Hst); 135.2 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.6 (s, C-2 of Hst); 145.9 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.1 (s, $\underline{C}_{para}$ of ClC_6H_4); 167.1 (CONH); Anal., found: C, 43.39; H, 4.35; N, 17.96%; $C_{14}H_{16}ClN_5O_4S$ requires C, 43.58; H, 4.18; N, 18.15%.

*2.1.3.2. 4-[β-(4-Chlorophenylsulfonylureido-alanylamido)-ethyl)]-1H-imidazole (**A2**)*

White crystals, m.p. 255–257°C; IR (KBr), cm^{-1}: 1152 (SO_2^{sym}), 1285 (amide III), 1370 (SO_2^{as}), 1574 (amide II), 1710 (amide I), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.84 (d, $^3J_{HH} = 6.5$, 3H, CHC$\underline{H}_3$ of Ala); 2.47 (t, 2H, $J = 7.0$, Hst CH_2); 3.00 (t, 2H, $J = 7.0$, Hst CONHC$\underline{H}_2$); 3.99 (q, 1H, C$\underline{H}$ of Ala); 7.34 (m, 1H, imidazole CH); 7.60 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 7.99 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.25 (br s, 3H, CONH + NHCONH); 8.37 (s, 1H, imidazole CH); 8.84 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.3 (s, CH$\underline{C}H_3$ of Ala); 33.0 (s, $\underline{C}H_2$ of Hst); 37.7 (s, $\underline{C}H_2$ of Hst); 34.6 (s, $\underline{C}HCH_3$ of Ala); 122.5 (s, C-4 of Hst); 131.5 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.7 (s, NH$\underline{C}$ONH); 134.5 (s, C-5 of Hst); 135.7 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.7 (s, C-2 of Hst); 145.2 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.4 (s, $\underline{C}_{para}$ of ClC_6H_4); 167.8 (CONH); Anal., found: C, 45.19; H, 4.46; N, 17.50%; $C_{15}H_{18}ClN_5O_4S$ requires C, 45.06; H, 4.54; N, 17.51%.

*2.1.3.3. 4-[β-(4-Chlorophenylsulfonylureido-β-alanylamido)-ethyl)]-1H-imidazole (**A3**)*

White crystals, m.p. 248–249°C (dec.); IR (KBr), cm^{-1}: 1153 (SO_2^{sym}), 1286 (amide III), 1370 (SO_2^{as}), 1575 (amide II), 1710 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.48 (t, 2H, $J = 7.0$, Hst CH_2); 2.78 (t, $^3J_{HH} = 6.6$, 1H, $CH_2C\underline{H}_2CO$ of β-Ala); 3.02 (t, 2H, $J = 7.0$, Hst CONHC$\underline{H}_2$); 3.29 (t, $^3J_{HH} = 6.7$, 1H, $CH_2C\underline{H}_2CO$ of β-Ala); 3.47 (t, $^3J_{HH} = 6.3$, 2H, C$\underline{H}_2CH_2CO$ of β-Ala); 7.30 (m, 1H, imidazole CH); 7.60 (d, $^3J_{HH} = 8.2$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 8.03 (d, $^3J_{HH} = 8.2$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.28 (br s, 3H, CONH + NHCONH); 8.37 (s, 1H, imidazole CH); 8.85 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.3 (s, $\underline{C}H_2$ of Hst); 37.2 (s, NH$\underline{C}H_2CH_2$ of β-Ala); 38.4 (s, $\underline{C}H_2$ of Hst); 40.5 (s, $CH_2\underline{C}H_2CO$ of β-Ala); 122.6 (s, C-4 of Hst); 130.5 (s, $\underline{C}_{meta}$ of $CH_3C_6H_4$); 132.8 (s, NH$\underline{C}$ONH); 134.9 (s, C-5 of Hst); 135.6 (s, $\underline{C}_{ortho}$ of $CH_3C_6H_4$); 137.1 (s, C-2 of Hst); 144.8 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.9 (s, $\underline{C}_{para}$ of ClC_6H_4); 167.9 (CONH); Anal., found: C, 44.92; H, 4.55; N, 17.32%; $C_{15}H_{18}ClN_5O_4S$ requires C, 45.06; H, 4.54; N, 17.51%.

*2.1.3.4. 4-[β-(4-Chlorophenylsulfonylureido-n-butyramido)-ethyl)]-1H-imidazole (**A4**)*

White crystals, m.p. 252–253°C; IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1284 (amide III), 1370 (SO_2^{as}), 1580 (amide II), 1712 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.49 (t, 2H, $J = 7.0$ Hz, Hst CH_2); 2.77 (t, $^3J_{HH}$ = 6.5, 1H, $(CH_2)_2C\underline{H}_2CO$ of GABA); 2.98 (t, 2H, $J = 7.0$ Hz, Hst $CONHC\underline{H}_2$); 3.31 (t, $^3J_{HH} = 6.7$, 1H, $(CH_2)_2C\underline{H}_2CO$ of GABA); 3.35–3.59 (m, 4H, $(C\underline{H}_2)_2CH_2CO$ of GABA); 7.36 (m, 1H, imidazole CH); 7.60 (d, $^3J_{HH} = 8.2$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 8.03 (d, $^3J_{HH}$ = 8.2, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.28 (br s, 3H, CONH+NHCONH); 8.37 (s, 1H, imidazole CH); 8.82 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.2 (s, $\underline{C}H_2$ of Hst); 37.4 (s, $NH\underline{C}H_2CH_2$ of GABA); 37.9 (s, $\underline{C}H_2$ of Hst); 40.5 (s, $CH_2CH_2\underline{C}H_2CO$ of GABA); 41.4 (s, $CH_2\underline{C}H_2CH_2CO$ of GABA); 122.6 (s, C-4 of Hst); 130.8 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.7 (s, NH$\underline{C}$ONH); 134.6 (s, C-5 of Hst); 135.3 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.5 (s, C-2 of Hst); 144.9 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.5 (s, $\underline{C}_{para}$ of ClC_6H_4); 167.7 (CONH); Anal., found: C, 46.55; H, 4.70; N, 16.81%; $C_{16}H_{20}ClN_5O_4S$ requires C, 46.43; H, 4.87; N, 16.92%.

*2.1.3.5. 4-[[β-(4-Chlorophenylsulfonylureido-glycyl)glycylamido]-ethyl)]-1H-imidazole (**A5**)*

White crystals, m.p. 219–221°C; IR (KBr), cm^{-1}: 1154 (SO_2^{sym}), 1288 (amide III), 1375 (SO_2^{as}), 1580 (amide II), 1715 (amide I), 3063 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.52 (m, 6H, Hst $CH_2 + 2C\underline{H}_2$ of GlyGly); 2.99 (t, 2H, $J = 7.0$, Hst $CONHC\underline{H}_2$); 7.33 (m, 1H, imidazole CH); 7.69 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 8.05 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.28 (br s, 3H, CONH+NHCONH); 8.37 (s, 1H, imidazole CH); 8.80 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.3 (s, $\underline{C}H_2$ of Hst); 35.6 (s, $\underline{C}H_2$ of GlyGly); 37.8 (s, $\underline{C}H_2$ of Hst); 122.7 (s, C-4 of Hst); 130.8 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.9 (s, NH$\underline{C}$ONH); 134.6 (s, C-5 of Hst); 135.6 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.8 (s, C-2 of Hst); 145.3 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.9 (s, $\underline{C}_{para}$ of ClC_6H_4); 167.6 (CONH); 172.0 (CONH between the two glycyl moieties); Anal., found: C, 43.50; H, 4.21; N, 18.75%; $C_{16}H_{19}ClN_6O_5S$ requires C, 43.39; H, 4.32; N, 18.98%.

*2.1.3.6. 4-[β-(4-Chlorophenylsulfonylureido-valylamido)-ethyl)]-1H-imidazole (**A6**)*

White crystals, m.p. 208–210°C; IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1283 (amide III), 1375 (SO_2^{as}), 1576 (amide II), 1710 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.10 (d, $^3J_{HH} = 6.7$, 6H, $CH(C\underline{H}_3)_2$ of Val); 2.29–2.50 (m, 1H, $C\underline{H}(CH_3)_2$ of Val); 2.57 (t, 2H, $J = 7.0$, Hst CH_2); 3.04 (t, 2H, $J = 7.0$, Hst $CONHC\underline{H}_2$); 3.79 (d, $^3J_{HH} = 4.3$, 1H, NHC$\underline{H}$CH of Val); 7.36 (m, 1H, imidazole CH); 7.70 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 8.06 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.24 (br s, 3H, CONH+NHCONH); 8.39 (s, 1H, imidazole CH); 8.84 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.3 (s, $CH(\underline{C}H_3)_2$ of Val); 33.5 (s, $\underline{C}H_2$ of Hst); 34.2 (s, $\underline{C}H(CH_3)_2$ of Val); 37.9 (s, $\underline{C}H_2$ of Hst); 64.0 (s, NH$\underline{C}$HCH of Val); 122.6 (s, C-4 of Hst); 130.7 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.7 (s, NH$\underline{C}$ONH); 134.4 (s, C-5 of Hst); 134.8 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.5 (s, C-2 of Hst); 145.3 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.8 (s, $\underline{C}_{para}$ of ClC_6H_4); 168.1 (CONH); Anal., found: C, 47.66; H, 5.09; N, 16.24%; $C_{17}H_{22}ClN_5O_4S$ requires C, 47.72; H, 5.18; N, 16.37%.

*2.1.3.7. 4-[β-(4-Chlorophenylsulfonylureido-leucylamido)-ethyl)]-1H-imidazole (**A7**)*

White crystals, m.p. 211–212°C; IR (KBr), cm^{-1}: 1154 (SO_2^{sym}), 1280 (amide III), 1376 (SO_2^{as}), 1575 (amide II), 1713 (amide I), 3064 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.45 (d, $^3J_{HH} = 6.7$, 6H, $CH(C\underline{H}_3)_2$ of Leu); 1.53 (m, 2H, CH_2 of Leu); 2.55 (t, 2H, $J = 7.0$, Hst CH_2); 3.05 (t, 2H, $J = 7.0$, Hst $CONHC\underline{H}_2$); 3.70 (m, 1H, NHC$\underline{H}$CH$_2$ of Leu); 4.24 (m, 1H, $CHCH_2C\underline{H}(CH_3)_2$ of Leu); 7.30 (m, 1H, imidazole CH); 7.65 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 8.02 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.25 (br s, 3H, CONH+NHCONH); 8.37 (s, 1H, imidazole CH); 8.83 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 21.5 (s, $CH(\underline{C}H_3)_2$ of Leu); 31.8 (s, $\underline{C}H_2$ of Leu); 33.4 (s, $\underline{C}H_2$ of Hst); 34.2 (s, $\underline{C}H(CH_3)_2$ of Leu); 37.6 (s, $\underline{C}H_2$ of Hst); 56.0 (s, NH$\underline{C}$HCH$_2$ of Leu); 122.5 (s, C-4 of Hst); 130.8 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.6 (s, NH$\underline{C}$ONH); 134.9 (s, C-5 of Hst); 135.5 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.8 (s, C-2 of Hst); 145.0 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.8 (s, $\underline{C}_{para}$ of ClC_6H_4); 167.8 (CONH); Anal., found: C, 48.99; H, 5.51; N, 15.67%; $C_{18}H_{24}ClN_5O_4S$ requires C, 48.92; H, 5.47; N, 15.85%.

*2.1.3.8. 4-[β-(4-Chlorophenylsulfonylureido-iso-leucylamido)-ethyl)]-1H-imidazole (**A8**)*

White crystals, m.p. 194–196°C; IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1282 (amide III), 1373 (SO_2^{as}), 1575 (amide II), 1715 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.15 (d, $^3J_{HH} = 6.5$, 3H, $C\underline{H}_3$ of Ile); 1.23 (t, 3H, $^3J_{HH} = 6.7$, CH_3 of Et moiety of Ile); 1.56 (m, 2H, CH_2 of Ile); 2.54 (t, 2H, $J = 7.0$, Hst CH_2); 3.04 (t, 2H, $J = 7.0$, Hst $CONHC\underline{H}_2$); 3.20 (m, 1H, EtC$\underline{H}$(Me)- of Ile); 3.78 (m, 1H, NHC$\underline{H}$CH of Ile); 7.36 (m, 1H, imidazole CH); 7.70 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 8.00 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.25 (br s, 3H, CONH+NHCONH); 8.38 (s, 1H, imidazole CH); 8.84 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 21.7 (s, $CH\underline{C}H_3$ of Ile); 22.9 (s, $\underline{C}H_3CH_2$ of Ile); 31.4 (s, $\underline{C}H_2$ of Ile); 33.4 (s, $\underline{C}H_2$ of Hst); 34.1 (s, $\underline{C}H(CH_3)_2$ of Leu); 37.7 (s, $\underline{C}H_2$ of Hst); 46.5 (s, Et$\underline{C}$H(Me)- of Ile); 55.2 (s, NH$\underline{C}$HCH$_2$ of Ile); 122.7 (s, C-4 of Hst); 130.7 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.8 (s, NH$\underline{C}$ONH); 134.7 (s, C-5 of Hst); 134.5 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.5 (s, C-2 of Hst); 145.6 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.7 (s, $\underline{C}_{para}$ of ClC_6H_4); 167.9

(CONH); Anal., found: C, 48.64; H, 5.56; N, 15.79%; $C_{18}H_{24}ClN_5O_4S$ requires C, 48.92; H, 5.47; N, 15.85%.

*2.1.3.9. 4-[β-(4-Chlorophenylsulfonylureido-asparaginylamido)-ethyl)]-1H-imidazole (**A9**)*

White crystals, m.p. 245–246°C; IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1286 and 1295 (amide III), 1370 (SO_2^{as}), 1575 (amide II), 1710 and 1724 (amide I), 3066 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.55 (t, 2H, $J = 7.0$, Hst CH_2); 2.62 (d, 2H, $^3J_{HH} = 6.3$, $C\underline{H}_2$ of Asn); 3.03 (t, 2H, $J = 7.0$, Hst $CONHC\underline{H}_2$); 4.54 (m, 1H, $NHC\underline{H}CH_2$ of Asn); 7.33 (m, 1H, imidazole CH); 7.61 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 7.97 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.10–8.32 (br s, 5H, $CONH_2 + CONH + NHCONH$); 8.37 (s, 1H, imidazole CH); 8.84 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 30.0 (s, $CH\underline{C}H_2$ of Asn); 33.3 (s, $\underline{C}H_2$ of Hst); 37.9 (s, $\underline{C}H_2$ of Hst); 70.6 (s, $\underline{C}HCH_2$ of Asn); 122.6 (s, C-4 of Hst); 130.9 (s, $\underline{C}_{meta}$ of ClC_6H_4); 134.5 (s, $NH\underline{C}ONH$); 134.9 (s, C-5 of Hst); 135.8 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.1 (s, C-2 of Hst); 145.6 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.0 (s, $\underline{C}_{para}$ of ClC_6H_4); 177.0 (CONH); 178.5 (s, $\underline{C}ONH_2$ of Asn); Anal., found: C, 43.47; H, 4.19; N, 19.00%; $C_{16}H_{19}ClN_6O_5S$ requires C, 43.39; H, 4.32; N, 18.98%.

*2.1.3.10. 4-[β-(4-Chlorophenylsulfonylureido-glutaminylamido)-ethyl)]-1H-imidazole (**A10**)*

White crystals, m.p. 233–235°C; IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1285 and 1294 (amide III), 1372 (SO_2^{as}), 1575 (amide II), 1710 and 1726 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.53 (t, 2H, $J = 7.0$, Hst CH_2); 2.58–2.69 (m, 2H, $C\underline{H}_2$ of Gln); 3.03 (t, 2H, $J = 7.0$, Hst $CONHC\underline{H}_2$); 3.07–3.38 (m, 2H, $C\underline{H}_2$ of Gln); 4.645 (m, 1H, $NHC\underline{H}CH_2$ of Gln); 7.34 (m, 1H, imidazole CH); 7.66 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 7.97 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.10–8.34 (br s, 5H, $CONH_2 + CONH + NHCONH$); 8.39 (s, 1H, imidazole CH); 8.85 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 30.5 (s, $CH\underline{C}H_2CH_2$ of Gln); 33.4 (s, $\underline{C}H_2$ of Hst); 37.9 (s, $\underline{C}H_2$ of Hst); 44.5 (s, $CHCH_2\underline{C}H_2$ of Gln); 72.5 (s, $\underline{C}HCH_2CH_2$ of Gln); 122.6 (s, C-4 of Hst); 130.7 (s, $\underline{C}_{meta}$ of ClC_6H_4); 133.6 (s, $NH\underline{C}ONH$); 134.8 (s, C-5 of Hst); 135.3 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.9 (s, C-2 of Hst); 145.2 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.0 (s, $\underline{C}_{para}$ of ClC_6H_4); 177.4 (CONH); 179.1 (s, $CH_2\underline{C}ONH_2$ of Gln); Anal., found: C, 44.56; H, 4.55; N, 18.21%; $C_{17}H_{21}ClN_6O_5S$ requires C, 44.69; H, 4.63; N, 18.39%.

*2.1.3.11. 4-[β-(4-Chlorophenylsulfonylureido-arginylamido)-ethyl)]-1H-imidazole (**A11**)*

White crystals, m.p. 274–275°C (dec.); IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1280 (amide III), 1375 (SO_2^{as}), 1582 (amide II), 1713 (amide I), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.70–2.05 (m, 2H, $CHCH_2C\underline{H}_2$ of Arg); 2.49 (t, 2H, $J = 7.0$ Hz, Hst CH_2); 2.51–2.62 (m, 2H, $CHC\underline{H}_2CH_2$ of Arg); 2.75 (t, $^3J_{HH} = 6.5$, 1H, $(CH_2)_2C\underline{H}_2CO$ of Arg); 3.04 (t, 2H, $J = 7.0$ Hz, Hst CONH $C\underline{H}_2$); 3.30–3.46 (m, 2H, $CH_2C\underline{H}_2NH$ of Arg); 3.51–3.67 (m, 1H, $CH_2C\underline{H}(NH)CO$ of Arg); 7.35 (m, 1H, imidazole CH); 7.67 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 7.98 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.25 (br s, 3H, $CONH + NHCONH$); 8.35 (s, 1H, imidazole CH); 8.83 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 29.6 (s, $CH_2\underline{C}H_2CH_2$ of Arg); 33.5 (s, $\underline{C}H_2$ of Hst); 35.4 (s, $CHCH_2\underline{C}H_2$ of Arg); 37.8 (s, $\underline{C}H_2$ of Hst); 45.9 (s, $CH_2\underline{C}H_2NH$ of Arg); 59.8 (s, $CH_2\underline{C}H(NH)CO_2H$ of Arg); 122.5 (s, C-4 of Hst); 130.8 (s, $\underline{C}_{meta}$ of ClC_6H_4); 131.8 (s, $NH\underline{C}ONH$); 134.6 (s, C-5 of Hst); 134.9 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.4 (s, C-2 of Hst); 144.8 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.9 (s, $\underline{C}_{para}$ of ClC_6H_4); 161.6 (s, $NH\underline{C}(=NH)NH_2$ of Arg); 170.8 (CONH); Anal., found: C, 44.66; H, 5.31; N, 23.08%; $C_{18}H_{25}ClN_8O_4S$ requires C, 44.58; H, 5.20; N, 23.11%.

*2.1.3.12. 4-[β-[α-(4-Chlorophenylsulfonylureido-lysylamido)]-ethyl)]-1H-imidazole (**A12**)*

White crystals, m.p. 223–224°C; IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1286 (amide III), 1375 (SO_2^{as}), 1579 (amide II), 1711 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.66–2.23 (m, 6H, $CH(C\underline{H}_2)_3CH_2$ of Lys); 2.49 (t, 2H, $J = 7.0$ Hz, Hst CH_2); 2.99 (t, 2H, $J = 7.0$ Hz, Hst $CONHC\underline{H}_2$); 3.15 (t, $^3J_{HH} = 6.7$, 2H, $CH_2C\underline{H}_2NH_2$ of Lys); 3.88 (t, $^3J_{HH} = 6.7$, 1H, $CH_2C\underline{H}(NH)CO$ of Lys); 7.33 (m, 1H, imidazole CH); 7.66 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 7.99 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.29 (br s, 3H, $CONH + NHCONH$); 8.36 (s, 1H, imidazole CH); 8.83 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 26.8 (s, $H_2NCH_2(\underline{C}H_2)_3$ of Lys); 31.5 (s, $H_2NCH_2(\underline{C}H_2)_3$ of Lys); 33.2 (s, $\underline{C}H_2$ of Hst); 34.8 (s, $H_2NCH_2(\underline{C}H_2)_3$ of Lys); 37.5 (s, $\underline{C}H_2$ of Hst); 43.8 (s, $H_2N\underline{C}H_2(CH_2)_3$ of Lys), 58.9 (s, $CH_2\underline{C}H(NH)CO$ of Lys); 122.3 (s, C-4 of Hst); 130.8 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.5 (s, $NH\underline{C}ONH$); 134.8 (s, C-5 of Hst); 135.7 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.6 (s, C-2 of Hst); 145.1 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 148.8 (s, $\underline{C}_{para}$ of ClC_6H_4); 171.5 (CONH); Anal., found: C, 47.50; H, 5.74; N, 18.24%; $C_{18}H_{25}ClN_6O_4S$ requires C, 47.31; H, 5.51; N, 18.39%.

*2.1.3.13. 4-[β-(4-Chlorophenylsulfonylureido-histidylamido)-ethyl)]-1H-imidazole (**A13**)*

White crystals, m.p. 269–270°C (dec.); IR (KBr), cm^{-1}: 1154 (SO_2^{sym}), 1283 (amide III), 1377 (SO_2^{as}), 1585 (amide II), 1713 (amide I), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.49 (t, 2H, $J = 7.0$, Hst CH_2); 2.99 (t, 2H, $J = 7.0$, Hst $CONHC\underline{H}_2$); 3.32–3.52 (m, 2H, $CHC\underline{H}_2$ of His); 4.01–4.09 (m, 1H, $C\underline{H}CH_2$ of His); 7.33 (m, 1H, imidazole CH of Hst); 7.44 (s, 1H, $C\underline{H}$-5 of His); 7.59 (d, $^3J_{HH} = 8.2$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 7.95 (d, $^3J_{HH} = 8.2$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.24 (br s, 3H, $CONH + NHCONH$); 8.36 (s, 1H,

imidazole CH); 8.51 (s, 1H, CH-2 of His); 8.83 (s, 2H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.0 (s, CH_2 of Hst); 37.9 (s, CH_2 of Hst); 40.5 (s, CH_2 of His); 58.7 (s, CH_2CH of His); 122.2 (s, C-4 of His); 122.6 (s, C-4 of Hst); 131.1 (s, C_{meta} of ClC_6H_4); 132.5 (s, NHCONH); 134.3 (s, C-5 of His); 134.7 (s, C-5 of Hst); 135.3 (s, C_{ortho} of ClC_6H_4); 137.0 (s, C-2 of Hst); 137.5 (s, C-2 of His); 145.4 (s, C_{ipso} of ClC_6H_4); 148.6 (s, C_{para} of ClC_6H_4); 167.4 (CONH); Anal., found: C, 46.51; H, 4.32; N, 21.03%; $C_{18}H_{20}ClN_7O_4S$ requires C, 46.40; H, 4.33; N, 21.04%.

*2.1.3.14. 4-[β-(4-Chlorophenylsulfonylureido-phenylglycylamido)-ethyl)]-1H-imidazole (**A14**)*

White crystals, m.p. 212–213°C (dec.); IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1285 (amide III), 1375 (SO_2^{as}), 1580 (amide II), 1710 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.52 (t, 2H, $J = 7.0$, Hst CH_2); 2.99 (t, 2H, $J = 7.0$, Hst CONHCH_2); 4.05 (m, 1H, PhCH of Phg); 7.29–7.58 (m, 8H, H_{ortho} of ClC_6H_4 + H of Phg + imidazole CH of Hst); 7.98 (d, $^3J_{HH} = 8.2$, 2H, H_{meta} of ClC_6H_4); 8.23 (br s, 3H, CONH + NHCONH); 8.35 (s, 1H, imidazole CH); 8.85 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.3 (s, CH_2 of Hst); 37.9 (s, CH_2 of Hst); 60.4 (s, PhCH of Phg); 122.5 (s, C-4 of Hst); 132.8 (s, C_{meta} of ClC_6H_4); 132.7 (s, NHCONH); 133.6 (s, C_{meta} of Phg); 134.5 (s, C_{ortho} of Phg); 134.9 (s, C-5 of Hst); 135.6 (s, C_{ortho} of ClC_6H_4); 137.3 (s, C-2 of Hst); 141.5 (s, C_{ipso} of Phg); 145.2 (s, C_{ipso} of ClC_6H_4); 148.9 (s, C_{para} of ClC_6H_4); 166.5 (CONH); Anal., found: C, 52.15; H, 4.29; N, 15.11%; $C_{20}H_{20}ClN_5O_4S$ requires C, 52.00; H, 4.36; N, 15.16%.

*2.1.3.15. 4-[β-(4-Chlorophenylsulfonylureido-phenylalanylamido)-ethyl)]-1H-imidazole (**A15**)*

White crystals, m.p. 199–200°C; IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1286 (amide III), 1371 (SO_2^{as}), 1583 (amide II), 1710 (amide I), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.51 (t, 2H, $J = 7.0$, Hst CH_2); 3.01 (t, 2H, $J = 7.0$, Hst CONHCH_2); 3.10–3.54 (m, 2H, CH_2CH of Phe); 4.08 (dd, $^3J_{HH} = 5.0$, $^3J_{HH} = 7.8$, 1H, CH_2CH of Phe); 7.32–7.64 (m, 8H, H_{ortho} of ClC_6H_4 + H_{arom} of Phe + imidazole CH of Hst); 7.97 (d, $^3J_{HH} = 8.2$, 2H, H_{meta} of ClC_6H_4); 8.24 (br s, 3H, CONH + NHCONH); 8.35 (s, 1H, imidazole CH); 8.82 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.1 (s, CH_2 of Hst); 37.9 (s, CH_2 of Hst); 41.6 (s, CH_2CH of Phe); 59.2 (s, CH_2CH of Phe); 122.5 (s, C-4 of Hst); 132.3 (s, C_{meta} of ClC_6H_4); 132.6 (s, NHCONH); 133.9 (s, C_{meta} of Phe); 134.5 (s, C_{ortho} of Phe); 134.8 (s, C-5 of Hst); 135.2 (s, C_{ortho} of ClC_6H_4); 137.3 (s, C-2 of Hst); 141.9 (s, C_{ipso} of Phe); 145.4 (s, C_{ipso} of ClC_6H_4); 148.6 (s, C_{para} of ClC_6H_4); 166.9 (CONH); Anal., found: C, 53.24; H, 4.66; N, 14.62%; $C_{21}H_{22}ClN_5O_4S$ requires C, 53.00; H, 4.66; N, 14.71%.

*2.1.3.16. 4-[β-(4-Chlorophenylsulfonylureido-tryptophanylamido)-ethyl)]-1H-imidazole (**A16**)*

White crystals, m.p. 216–218°C (dec.); IR (KBr), cm^{-1}: 1152 (SO_2^{sym}), 1280 (amide III), 1375 (SO_2^{as}), 1581 (amide II), 1710 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.48 (t, 2H, $J = 7.0$, Hst CH_2); 2.99 (t, 2H, $J = 7.0$, Hst CONHCH_2); 3.40 (dd, $^3J_{HH} = 9.0$, $^2J_{HH} = 14.6$, 1H, CH_2CH of Trp); 3.65 (dd, $^3J_{HH} = 4.1$, $^2J_{HH} = 15.0$, 1H, CH_2CH of Trp); 4.17 (dd, $^3J_{HH} = 4.3$, $^3J_{HH} = 8.0$, 1H, CH_2CH of Trp); 7.22–7.86 (m, 8H, H_{ortho} of ClC_6H_4 + H_{arom} of Trp + 1H, imidazole CH of Hst); 7.95 (d, $^3J_{HH} = 8.1$, 2H, H_{meta} of ClC_6H_4); 8.24 (br s, 3H, CONH + NHCONH); 8.35 (s, 1H, imidazole CH); 8.85 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 31.6 (s, CH_2CH of Trp); 33.2 (s, CH_2 of Hst); 37.9 (s, CH_2 of Hst); 58.5 (s, CH_2CH of Trp); 113.6 (s, C-2 of Trp); 116.9 (s, C-7 of Trp); 122.5 (s, C-4 of Hst); 123.4 (s, C-5 of Trp); 124.8 (s, C-6 of Trp); 126.8 (s, C-4 of Trp); 129.3 (s, C-1 of Trp); 130.9 (s, C_{meta} of ClC_6H_4); 132.1 (s, C-8 of Trp); 132.7 (s, NHCONH); 134.6 (s, C-5 of Hst); 135.5 (s, C_{ortho} of ClC_6H_4); 137.3 (s, C-2 of Hst); 141.8 (s, C-3 of Trp); 145.6 (s, C_{ipso} of ClC_6H_4); 148.0 (s, C_{para} of ClC_6H_4); 168.7 (CONH); Anal., found: C, 53.75; H, 4.48; N, 16.12%; $C_{23}H_{23}ClN_6O_4S$ requires C, 53.64; H, 4.50; N, 16.32%.

*2.1.3.17. 4-[β-(4-Chlorophenylsulfonylureido-prolylamido)-ethyl)]-1H-imidazole (**A17**)*

M.p. 255–256°C; IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1288 (amide III), 1375 (SO_2^{as}), 1590 (amide II), 1721 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.18–1.39 (m, 1H, HCH of Pro); 1.55–1.65 (m, 1H, HCH of Pro); 1.70–1.85 (m, 2H, CH_2 of Pro); 2.52 (t, 2H, $J = 7.0$, Hst CH_2); 2.99 (t, 2H, $J = 7.0$, Hst CONHCH_2); 3.16–3.35 (m, 2H, CH_2N of Pro); 3.75–3.83 (m, 1H, CHCO of Pro); 7.34 (m, 1H, H-5 of Hst); 7.51 (d, $^3J_{HH} = 8.3$, 2H, H_{ortho} of ClC_6H_4); 7.95 (d, $^3J_{HH} = 8.3$, 2H, H_{meta} of ClC_6H_4); 8.21 (br s, 2H, CONH + Pro-NCONH); 8.39 (s, 1H, H-2 of Hst); 8.80 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 15.6 (s, CH_2 of Pro); 21.3 (s, CH_2 of Pro); 33.1 (s, CH_2 of Hst); 37.9 (s, CH_2 of Hst); 46.6 (s, CH_2N of Pro); 64.6 (s, CHCO of Pro); 122.5 (s, C-4 of Hst); 132.5 (s, NHCON); 132.4 (s, C_{meta} of ClC_6H_4); 134.8 (s, C-5 of Hst); 135.4 (s, C_{ortho} of ClC_6H_4); 137.8 (s, C-2 of Hst); 145.6 (s, C_{para} of ClC_6H_4); 148.7 (s, C_{ipso} of ClC_6H_4); 170.7 (s, Pro-CONH); Anal., found: C, 47.60; H, 4.69; N, 16.31%; $C_{17}H_{20}ClN_5O_4S$ requires: C, 47.94; H, 4.73; N, 16.44%.

*2.1.3.18. 4-[β-(4-Chlorophenylsulfonylureido-pipecolylamido)-ethyl)]-1H-imidazole (**A18**)*

M.p. 236–238°C; IR (KBr), cm^{-1}: 1148 (SO_2^{sym}), 1282 (amide III), 1375 (SO_2^{as}), 1589 (amide II), 1710 (amide I), 3066 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.19–1.41 (m, 1H, HCH of Pip); 1.50–1.66 (m, 1H, HCH); 1.70–1.92 (m,

4H, 2CH_2 of Pip); 2.52 (t, 2H, $J = 7.0$, Hst CH_2); 2.98 (t, 2H, $J = 7.0$, Hst CONHC$\underline{H}_2$); 3.16–3.39 (m, 2H, CH_2N of Pip); 3.70–3.86 (m, 1H, C$\underline{H}$CO of Pip); 7.30 (m, 1H, H-5 of Hst); 7.46 (d, $^3J_{HH} = 8.3$, 2H, H_{ortho} of ClC_6H_4); 7.97 (d, $^3J_{HH} = 8.3$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.23 (br s, 2H, CONH + Pip-NCONH); 8.34 (s, 1H, H-2 of Hst); 8.83 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 15.6 (s, CH_2 of Pip); 18.3 (s, CH_2 of Pip); 21.6 (s, CH_2 of Pip); 33.0 (s, $\underline{C}H_2$ of Hst); 37.9 (s, $\underline{C}H_2$ of Hst); 46.5 (s, CH_2N of Pip); 64.6 (s, $\underline{C}$HCO of Pip); 122.4 (s, C-4 of Hst); 132.2 (s, NH$\underline{C}$ON); 132.9 (s, $\underline{C}_{meta}$ of ClC_6H_4); 134.5 (s, C-5 of Hst); 135.4 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.3 (s, C-2 of Hst); 145.5 (s, $\underline{C}_{para}$ of ClC_6H_4); 148.9 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 170.6 (s, Pip-$\underline{C}$ONH); Anal., found: C, 49.23; H, 5.15; N, 15.67%; $C_{18}H_{22}ClN_5O_4S$ requires: C, 49.14; H, 5.04; N, 15.92%.

*2.1.3.19. 4-[β-(4-Chlorophenylsulfonylureido-nipecotylamido)-ethyl)]-1H-imidazole (**A19**)*

M.p. 232–233°C; IR (KBr), cm^{-1}: 1147 (SO_2^{sym}), 1280 (amide III), 1375 (SO_2^{as}), 1590 (amide II), 1711 (amide I), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.24–1.65 (m, 4H, CH_2CH_2 of Nip); 2.51 (t, 2H, $J = 7.0$, Hst CH_2); 2.99 (t, 2H, $J = 7.0$, Hst CONHC$\underline{H}_2$); 3.16–3.37 (m, 4H, CH_2NCH_2 of Nip); 3.70–3.88 (m, 1H, C$\underline{H}$CO of Nip); 7.31 (m, 1H, H-5 of Hst); 7.53 (d, $^3J_{HH} = 8.1$, 2H, H_{ortho} of ClC_6H_4); 7.94 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.23 (br s, 2H, CONH + Nip-NCONH); 8.35 (s, 1H, H-2 of Hst); 8.82 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 16.3 (s, CH_2 of Nip); 21.2 (s, CH_2 of Nip); 33.3 (s, $\underline{C}H_2$ of Hst); 37.9 (s, $\underline{C}H_2$ of Hst); 46.6 (s, CH_2N of Nip); 47.2 (s, NCH_2 of Nip); 53.6 (s, $\underline{C}$HCO of Nip); 122.4 (s, C-4 of Hst); 132.2 (s, NH$\underline{C}$ON); 132.8 (s, $\underline{C}_{meta}$ of ClC_6H_4); 134.8 (s, C-5 of Hst); 135.5 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.3 (s, C-2 of Hst); 145.3 (s, $\underline{C}_{para}$ of ClC_6H_4); 148.9 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 170.6 (s, Nip-$\underline{C}$ONH); Anal., found: C, 49.08; H, 5.09; N, 15.84%; $C_{18}H_{22}ClN_5O_4S$ requires: C, 49.14; H, 5.04; N, 15.92%.

*2.1.3.20. 4-[β-(4-Chlorophenylsulfonylureido-iso-nipecotylamido)-ethyl)]-1H-imidazole (**A20**)*

M.p. 264–265°C (dec.); IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1279 (amide III), 1373 (SO_2^{as}), 1591 (amide II), 1715 (amide I), 3063 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.86–2.30 (m, 8H, 2 CH_2CH_2 of Inp); 2.48 (t, 2H, $J = 7.0$, Hst CH_2); 2.99 (t, 2H, $J = 7.0$, Hst CONHC$\underline{H}_2$); 3.21–3.54 (m, 1H, C$\underline{H}$CO of Inp); 7.33 (m, 1H, H-5 of Hst); 7.50 (d, $^3J_{HH} = 8.3$, 2H, H_{ortho} of ClC_6H_4); 7.93 (d, $^3J_{HH} = 8.3$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.24 (br s, 2H, CONH + Inp-NCONH); 8.35 (s, 1H, H-2 of Hst); 8.81 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 21.3 (s, CH_2 of Inp); 33.3 (s, $\underline{C}H_2$ of Hst); 37.9 (s, $\underline{C}H_2$ of Hst); 47.1 (s, NCH_2 of Inp); 53.2 (s, $\underline{C}$HCO of Inp); 122.3 (s, C-4 of Hst); 132.1 (s, NH$\underline{C}$ON); 132.5 (s, $\underline{C}_{meta}$ of ClC_6H_4); 134.9 (s, C-5 of Hst); 135.3 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.5 (s, C-2 of Hst); 145.3 (s, $\underline{C}_{para}$ of ClC_6H_4); 148.4 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 170.9 (s, Inp-$\underline{C}$ONH); Anal., found: C, 48.90; H, 4.95; N, 15.78%; $C_{18}H_{22}ClN_5O_4S$ requires: C, 49.14; H, 5.04; N, 15.92%.

*2.1.3.21. 4-[β-(4-Chlorophenylsulfonylureido-glycyl-histidylamido)-ethyl]-1H-imidazole (**A21**)*

M.p. 221–223°C; IR (KBr), cm^{-1}: 1153 (SO_2^{sym}), 1287 (amide III), 1375 (SO_2^{as}), 1594 (amide II), 1715 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.52 (t, 2H, $J = 7.0$, Hst CH_2); 2.99 (t, 2H, $J = 7.0$, Hst CONHC$\underline{H}_2$); 3.35–3.46 (m, 2H, CHC$\underline{H}_2$ of His); 3.65 (s, 2H, C$\underline{H}_2$ of Gly); 4.52–4.66 (m, 1H, C$\underline{H}$CH_2 of His); 7.34 (s, 2H, C$\underline{H}$-5 of His + Hst); 7.58 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 7.89 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.30 (br s, 4H, 2CONH + NHCONH); 8.36 (s, 2H, C$\underline{H}$-2 of His + Hst); 8.84 (s, 2H, imidazole NH from His and Hst); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.2 (s, $\underline{C}H_2$ of Hst); 37.9 (s, $\underline{C}H_2$ of Hst); 40.6 (s, $\underline{C}H_2$ of Gly); 59.6 (s, $\underline{C}$HCH_2 of His); 122.4 (s, $\underline{C}$-4 of His); 122.8 (s, C-4 of Hst); 130.7 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.0 (s, $\underline{C}$-5 of His); 132.3 (s, NH$\underline{C}$ONH); 134.5 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 134.9 (s, C-5 of Hst); 137.3 (s, $\underline{C}$-2 of His); 137.7 (s, C-2 of Hst); 139.8 (s, $\underline{C}_{para}$ of ClC_6H_4); 145.4 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 175.5 (s, $CH_2\underline{C}$O of Gly); 176.8 (s, $\underline{C}$ONH of His); Anal., found: C, 45.88; H, 4.32; N, 21.24%; $C_{20}H_{23}ClN_8O_5S$ requires: C, 45.93; H, 4.43; N, 21.43%.

*2.1.3.22. 4-[β-(4-Chlorophenylsulfonylureido-β-alanyl-histidylamido)-ethyl]-1H-imidazole (**A22**)*

M.p. 225–227°C; IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1289 (amide III), 1375 (SO_2^{as}), 1592 (amide II), 1715 (amide I), 3061 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (t, 2H, $J = 7.0$, Hst CH_2); 2.79–2.90 (m, 2H, C$\underline{H}_2$ of β-Ala); 2.99 (t, 2H, $J = 7.0$, Hst CONHC$\underline{H}_2$); 3.12–3.26 (m, 2H, C$\underline{H}_2$ of β-Ala); 3.34–3.45 (m, 2H, CHC$\underline{H}_2$ of His); 4.57–4.65 (m, 1H, C$\underline{H}$CH_2 of His); 7.33 (s, 2H, C$\underline{H}$-5 of His + Hst); 7.60 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{ortho}$ of ClC_6H_4); 7.90 (d, $^3J_{HH} = 8.1$, 2H, $\underline{H}_{meta}$ of ClC_6H_4); 8.29 (br s, 4H, 2CONH + NHCONH); 8.34 (s, 2H, C$\underline{H}$-2 of His + Hst); 8.85 (s, 2H, imidazole NH from His and Hst); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.3 (s, $\underline{C}H_2$ of His); 33.5 (s, $\underline{C}H_2$ of Hst); 37.4 (s, NH$\underline{C}H_2CH_2$ of β-Ala); 37.9 (s, $\underline{C}H_2$ of Hst); 40.5 (s, $CH_2\underline{C}H_2$CO of β-Ala); 59.6 (s, $\underline{C}$HCH_2 of His); 122.2 (s, $\underline{C}$-4 of His); 122.6 (s, C-4 of Hst); 130.7 (s, $\underline{C}_{meta}$ of ClC_6H_4); 132.8 (s, NH$\underline{C}$ONH); 134.2 (s, $\underline{C}$-5 of His); 134.7 (s, C-5 of Hst); 135.6 (s, $\underline{C}_{ortho}$ of ClC_6H_4); 137.4 (s, $\underline{C}$-2 of His); 137.7 (s, C-2 of Hst); 145.9 (s, $\underline{C}_{para}$ of ClC_6H_4); 149.2 (s, $\underline{C}_{ipso}$ of ClC_6H_4); 175.3 (s, $CH_2\underline{C}$O of β-Ala); 176.4 (s, $\underline{C}$ONH of His); Anal., found: C, 47.13; H, 4.70; N, 20.76%; $C_{21}H_{25}ClN_8O_5S$ requires: C, 46.97; H, 4.69; N, 20.87%.

*2.1.3.23. 4-[β-(4-Chlorophenylsulfonylureido-phenylalanyl-prolylamido)-ethyl]-1H-imidazole (**A23**)*

M.p. 223–224°C (dec.); IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1289 (amide III), 1377 (SO_2^{as}), 1590 (amide II), 1714 (amide I), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.19–1.42 (m, 1H, HCH of Pro); 1.55–1.66 (m, 1H, HCH); 1.75–1.87 (m, 2H, CH_2 of Pro); 2.50 (t, 2H, $J = 7.0$, Hst CH_2); 2.99 (t, 2H, $J = 7.0$, Hst CONHCH_2); 3.12–3.56 (m, 2H, CH_2CH of Phe); 3.20–3.39 (m, 2H, CH_2N of Pro); 3.75–3.88 (m, 1H, CHCO of Pro); 4.14 (dd, $^3J_{HH} = 5.0$, $^3J_{HH} = 7.8$, 1H, CH_2CH of Phe); 7.29–7.59 (m, 8H, H_{ortho} of $ClC_6H_4 + H_{arom}$ of Phe + H-5 of Hst); 7.98 (d, $^3J_{HH} = 8.3$, 2H, H_{meta} of ClC_6H_4); 8.29 (br s, 4H, 2CONH + NHCONH); 8.37 (s, 1H, CH-2 of Hst); 8.84 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 15.2 (s, CH_2 of Pro); 21.5 (s, CH_2 of Pro); 33.4 (s, CH_2 of Hst); 37.9 (s, CH_2 of Hst); 41.4 (s, CH_2CH of Phe); 47.1 (s, CH_2N of Pro); 59.5 (s, CHCH_2 of Phe); 64.8 (s, CHCO of Pro); 122.4 (s, C-4 of Hst); 130.3 (s, C_{para} of Phe); 132.6 (s, NHCONH); 132.8 (s, C_{meta} of ClC_6H_4); 133.9 (s, C_{meta} of Phe); 134.6 (s, C_{ortho} of Phe); 134.9 (s, C-5 of Hst); 135.5 (s, C_{ortho} of ClC_6H_4); 137.7 (s, C-2 of Hst); 141.0 (s, C_{ipso} of Phe); 145.4 (s, C_{para} of ClC_6H_4); 148.4 (s, C_{ipso} of ClC_6H_4); 172.4 (s, Phe-CONH); 174.2 (Pro-CONH); Anal., found: C, 54.56; H, 5.13; N, 14.48%; $C_{26}H_{29}ClN_6O_5S$ requires: C, 54.49; H, 5.10; N, 14.66%.

*2.1.3.24. 4-[β-(4-Chlorophenylsulfonylureido-prolyl-glycylamido)-ethyl]-1H-imidazole (**A24**)*

M.p. 233–235°C; IR (KBr), cm^{-1}: 1147 (SO_2^{sym}), 1290 (amide III), 1375 (SO_2^{as}), 1592 (amide II), 1717 (amide I), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.18–1.42 (m, 1H, HCH of Pro); 1.55–1.68 (m, 1H, HCH); 1.73–1.91 (m, 2H, CH_2 of Pro); 2.51 (t, 2H, $J = 7.0$, Hst CH_2); 2.99 (t, 2H, $J = 7.0$, Hst CONHCH_2); 3.16–3.35 (m, 2H, CH_2N of Pro); 3.69 (s, 2H, CH_2 of Gly); 3.75–3.84 (m, 1H, CHCO of Pro); 7.36 (m, 1H, H-5 of Hst); 7.65 (d, $^3J_{HH} = 8.1$, 2H, H_{ortho} of ClC_6H_4); 7.97 (d, $^3J_{HH} = 8.1$, 2H, H_{meta} of ClC_6H_4); 8.25 (br s, 3H, 2CONH + Pro-NCONH); 8.35 (s, 1H, H-2 of Hst); 8.83 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 15.4 (s, CH_2 of Pro); 21.2 (s, CH_2 of Pro); 33.3 (s, CH_2 of Hst); 37.8 (s, CH_2 of Hst); 40.5 (s, CH_2 of Gly); 46.7 (s, CH_2N of Pro); 64.8 (s, CHCO of Pro); 122.3 (s, C-4 of Hst); 132.5 (s, NHCONH); 132.7 (s, C_{meta} of ClC_6H_4); 134.8 (s, C-5 of Hst); 135.3 (s, C_{ortho} of ClC_6H_4); 137.3 (s, C-2 of Hst); 145.5 (s, C_{para} of ClC_6H_4); 148.4 (s, C_{ipso} of ClC_6H_4); 175.6 (s, Pro-CONH); 176.6 (s, CONH of Gly); Anal., found: C, 47.51; H, 4.75; N, 17.21%; $C_{19}H_{23}ClN_6O_5S$ requires: C, 47.25; H, 4.80; N, 17.40%.

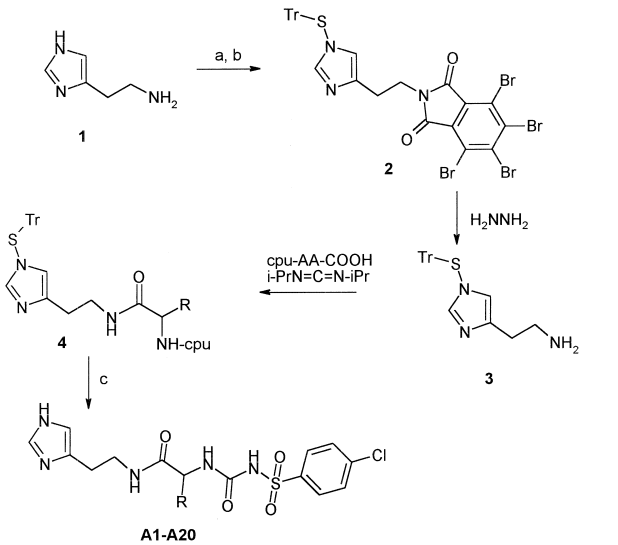
2.1.4. Enzyme assay

hCA I and hCA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog et al. (1991) (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group (Behravan et al., 1990) and enzymes were purified by affinity chromatography according to the method of Khalifah et al. (1977). Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM^{-1} cm^{-1} for CA I and 54 mM^{-1} cm^{-1} for CA II, based on $M_r = 28.85$ kDa for CA I and 29.30 kDa for CA II (Lindskog and Coleman, 1964; Steiner et al., 1975). CA IV was isolated from bovine lung microsomes and its concentration was determined by titration with ethoxzolamide (Maren et al., 1993).

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced to an IBM-compatible PC (Pocker and Stone, 1967). Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25°C. A molar absorption coefficient ε of 18,400 M^{-1} cm^{-1} was used for the 4-nitrophenolate formed by hydrolysis, under the conditions of the experiments (pH 7.40, 10 mM Tris buffer), as reported in the literature (Pocker and Stone, 1967). Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were performed for each activator concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of activator (1 mM) were prepared in distilled-deionized water with 10–15% (v/v) DMSO (which is not inhibitory/activatory at these concentrations) and dilutions up to 0.1 nM were prepared thereafter with distilled-deionized water. Activator and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–A (enzyme–activator) complex. The activation constant K_A was determined as described by Briganti et al. (1997). Enzyme concentrations were 3.1 nM for hCA II, 9.5 nM for hCA I and 31 nM for bCA IV (this isozyme has a decreased esterase activity (Baird et al., 1997) and higher concentrations had to be used for the measurements).

2.1.5. Ex vivo CA activation

An amount of 2 ml of freshly isolated human blood was thoroughly washed several times with 5 ml of Tris buffer (pH 7.40, 5 mM) and centrifuged for 10 min. The obtained erythrocytes were then treated with 2 ml of a 5 μM solution of CA activator. Incubation was carried out at 37°C with gentle stirring for periods of 30–60 min. After that time, the red cells were centrifuged again for 10 min, the supernatant discarded, and the cells washed three times with 5 ml of the above-mentioned buffer, in order to eliminate all unbound compound. The cells were then lysed in 5 ml of distilled water, centrifuged to eliminate



Scheme 1. Synthesis of compounds **A1**–**A24**. Reagents: a – tetrabromophthalic anhydride; b – tritylsulfenyl chloride; c – dioxane–HCl.

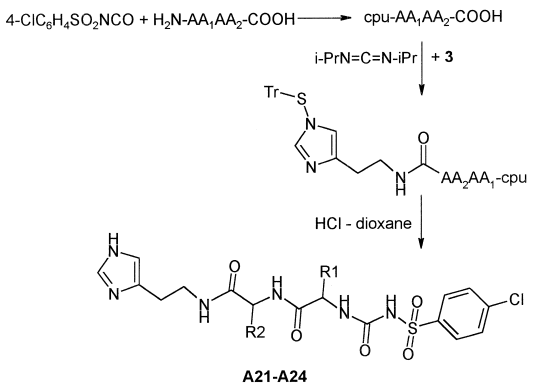
membranes and other insoluble materials, and CA activity assayed as described above. Blank experiments were performed in which no activator was added to the blood red cells treated as described above, and CA activity determined under such conditions was recorded as 100%.

3. Results

The synthesis of compounds **A1**–**A24** is shown in Schemes 1 and 2.

Activation data of isozymes hCA I, hCA II and bCA IV with the new derivatives (**A1**–**A24**) and the standard activator histamine (**1**) are presented in Table 1.

Ex vivo activation data with some of the new compounds reported here are shown in Table 2.



Scheme 2. Synthesis of compounds **A1**–**A24**.

4. Discussion

The study of CA activators has only recently registered some progress, by the report of the first X-ray crystallographic data (Briganti et al., 1997, 1998) of adducts of histamine and phenylalanine with the rapid isozyme hCA II. Taking into account the binding mode of this compound to the enzyme, we then designed tighter-binding activators by derivatization of the aminoalkyl group of histamine by means of alkyl/arylsulfonyl, carboxamido or ureido moieties, among others (Briganti et al., 1999; Scozzafava and Supuran, 1999a). Here we extend the above-mentioned studies (Briganti et al., 1999; Scozzafava and Supuran, 1999a) and report synthesis and activation data for a new class of tight-binding activators obtained by chemical modification of the aliphatic amino group of the lead compound, histamine (**1**).

The key intermediate for obtaining novel types of activators reported in this paper, *N*-1-tritylsulfenyl histamine (**3**), was obtained by non-exceptional procedures, involving the initial protection of the primary amine moiety by means of phthalimide derivatives, followed by protection of the imidazolic NH moiety with tritylsulfenyl chloride, and hydrazinolysis of the phthalimido moiety under mild conditions (Scheme 1). The overall yield of the three steps was good (around 80%) and the purification procedures quite simple. The approach shown here would thus predict that the tritylsulfenyl (TrS) moiety might be a good protecting group for the side-chains of 'difficult' amino acids such as histidine and arginine for (solid-phase) peptide synthesis (Briganti et al., 1999). Subsequent reaction of the key intermediate **3** with 4-chlorophenylsulfonylureido-amino acid/dipeptide derivatives in the presence of carbodiimides afforded a series of *N*-tritylsulfenylated compounds (**4**), which were deprotected under standard conditions (dioxane–HCl) leading to the desired derivatives **A1**–**A24**. All the new compounds reported here have been characterized by IR, ^{1}H- and ^{13}C-NMR spectroscopy, as well as elemental analysis ($\pm$0.4% of the theoretical data, calculated for the proposed formulas).

The CA activation data of Table 1 show significant differences between the investigated isozymes in their behavior towards both 'classical' activators, such as histamine (**1**), as well as the new class of derivatives described in the present work. Thus, histamine (**1**) is a potent hCA I activator, and a relatively weak hCA II activator, whereas isozyme bCA IV possesses intermediate behavior. The most interesting finding of the present study is represented by the high susceptibility of the cytosolic rapid isozyme hCA II to be activated by some of the histamine derivatives of types **A1**–**A24** as compared to the lead molecule (some compounds with activation constants in the range 0.02–0.04 μM were obtained). Moreover, the highly abundant and most prone to activation (by histamine) isozyme hCA I was also susceptible to activation

Table 1
CA isozymes I, II and IV activation with histamine (**1**) (Hst) the 4-chlorophenylsulfonylureido-aminoacyl derivatives cpu-AA-Hst (**A1**–**A20**), and the 4-chlorophenyl-sulfonylureido-dipeptide derivatives (**A21**–**A24**)[a]

No.	**A1**–**A20**: cpu-AA-Hst		**A21**–**A24**: cpu-AA_1-AA_2-Hst			
	X	AA/AA_1AA_2	K_A[b] (μM)			Yield[c]
			hCA I[d]	hCA II[d]	bCA IV[e]	
1	Histamine	–	2	125	41	–
A1	–	Gly	0.21	10	1.9	51
A2	–	L-Ala	0.20	14	3.2	59
A3	–	β-Ala	0.20	15	1.6	68
A4	–	GABA	0.19	13	1.2	62
A5	–	GlyGly	0.14	8	1.1	55
A6	–	L-Val	0.17	7	4.4	41
A7	–	L-Leu	0.18	8	3.2	56
A8	–	L-Ile	0.19	8	4.0	53
A9	–	L-Asn	0.16	7	4.1	77
A10	–	L-Gln	0.13	6	2.3	80
A11	–	L-Arg	0.03	1.1	0.4	75
A12	–	L-Lys	0.04	1.2	0.4	68
A13	–	L-His	0.03	0.9	0.6	81
A14	–	L-Phg	0.10	11	3.3	75
A15	–	L-Phe	0.09	8	2.1	80
A16	–	L-Trp	0.20	10	5.3	91
A17	–	L-Pro	0.17	9	3.1	60
A18	–	L-Pip	0.18	9	3.2	56
A19	–	D,L-Nip	0.16	8	3.1	71
A20	–	D,L-Inp	0.15	7	3.0	70
A21		L-GlyHis	0.002	0.03	0.03	41
A22	–	L-β-AlaHis	0.001	0.02	0.007	52
A23		L-PhePro	0.004	0.05	0.008	38
A24		L-ProGly	0.006	0.04	0.011	36

[a] Phg, phenylglycine; Pip, pipecolic acid (piperidine-2-carboxylic acid); Nip, nipecotic acid (piperidine-3-carboxylic acid); Inp, isonipecotic acid (piperidine-4-carboxylic acid); cpu, 4-Cl-$C_6H_4SO_2NHCO$-.
[b] Mean from at least three determinations by the esterase method (Pocker and Stone, 1967). Standard error was in the range 5–10%.
[c] Based on histamine.
[d] Human cloned isozyme.
[e] Purified from bovine lung microsomes.

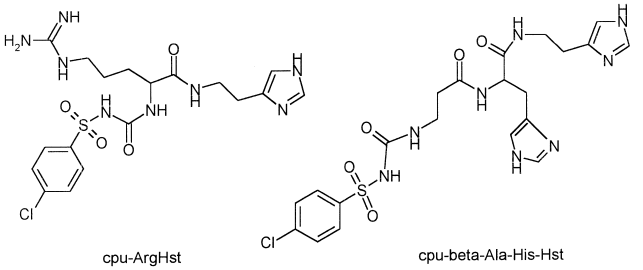
by the new derivatives reported here (with constants in the nanomolar range for the most active derivatives), but differences in activity are not so pronounced as compared to the situation for the rapid isozyme hCA II. bCA IV, on the other hand, exhibits intermediate behavior towards the new class of activators, with activation constants in the 0.007–0.020 μM range for the most active compounds. Efficient CA activators were: (i) derivatives of basic amino acids (Arg, Lys, His), such as **A11**–**A13**, as well as the phenylglycine and phenylalanine derivatives **A14** and **A15**; (ii) slightly less active were the compounds derived from Pro, Pip, Nip, Inp, Asn and Gln as well as the hydrophobic amino acid derivatives (Val, Leu, Ile, Trp); (iii) GlyGly (**A5**) and GABA (**A4**) derivatives were more active than the β-Ala derivative (**A3**), which in turn was more active than the Ala or Gly derivatives (**A2** and **A1**); (iv) the best activators in this series were those derived from dipeptides such as Gly–His, β-Ala–His (carnosine), Phe–Pro or Pro–Gly. These compounds possessed activation constants in the 1–30 nM range against hCA I and bCA IV, and in the 20–50 nM range against hCA II. Probably, the many heteroatoms present in the arylsulfonylureido-dipeptidyl moieties confer to the obtained compounds 'sticky' properties, i.e. they are able to participate in many interactions

Table 2
Ex vivo CA activation data after 30 and 60 min incubation of human erythrocytes with solutions containing 5 μM activators **1**, **A11**, **A13**, **A22** and **A23**

Activator	% CA activity[a]	
	30 min	60 min
1 (histamine)	121±3	130±5
A11	152±5	197±9
A13	175±7	215±10
A22	190±6	245±8
A23	194±5	253±9

[a] Mean±standard error ($n = 3$); erythrocyte CA activity (hCA I+hCA II) in the absence of activator is taken as 100%.

with amino acid residues from the active site, thus assuring the formation of very stable E–A (enzyme–activator) adducts.



A special mention should also be made regarding compounds such as **A11**, **A12**, **A13**, **A21** and **A22**, which due to the fact that they possess secondary moieties able to shuttle protons, in addition to the parent histamine moeity (the guanidino, ε-amino or imidazolic ring of histidine, respectively), behave as very effective CA activators against all three isozymes investigated here.

The rate-limiting step of reactions catalyzed by CA is a proton transfer process between the active site and the reaction medium (Steiner et al., 1975; Briganti et al., 1997). CA activators of the type reported in the present work presumably intervene in the catalytic cycle of the enzyme, leading to the formation of CA–activator complexes (similarly to enzyme–inhibitor adducts, but without substitution of the metal bound solvent molecule), in which the activator bound within the active site facilitates the rate-limiting proton transfer steps (Supuran et al., 1993, 1996b,c; Clare and Supuran, 1994; Briganti et al., 1997, 1998; Ilies et al., 1997; Lindskog, 1997). The driving force of this effect might be the fact that intramolecular reactions are more rapid than intermolecular reactions (Clare and Supuran, 1994; Supuran and Puscas, 1994). Thus, in the presence of activators (A), the rate-limiting step is described by (Supuran et al., 1993, 1996a,b; Clare and Supuran, 1994; Briganti et al., 1997, 1998; Ilies et al., 1997; Lindskog, 1997)

$$EZn^{2+}\text{–}OH_2 + A \Leftrightarrow [EZn^{2+}\text{–}OH_2\text{-}A] \Leftrightarrow [EZn^{2+}\text{–}HO^{-}\text{-}AH^{+}] \Leftrightarrow EZn^{2+}\text{–}OH^{-} + AH^{+}$$

enzyme–activator complexes (1)

In addition to the imidazolic moiety which can participate in the proton transfer processes between the active site and the environment (similarly to histamine (**1**)), the new compounds reported here also possess arylsulfonylureido amino acyl/dipeptidyl tails, which probably provide increased stability to the enzyme–activator adduct, thus allowing for more efficient activation processes as compared to **1**. Indeed, the active site edge of all three CA isozymes investigated here contain a high proportion of polar amino acid residues (such as the histidine cluster composed of His 3, His 4, His 10, His 15 and His 17, as well as Asp 19) which might interfere with polar groups such as Cl-C_6H_4-SO_2NHCO-amino acyl. In fact, such amino acid residues might explain the different catalytic properties of the diverse isozymes, as well as their diverse susceptibility to inhibition/activation by modulators of activity (Lindskog, 1997; Briganti et al., 1997, 1998, 1999). Such residues could easily participate in the formation of hydrogen bonds (as well as other types of interactions) with the histamine derivatives reported here. This might in fact explain the greater efficiency of the compounds reported in the present work in activating this isozyme as compared to histamine, which is a relatively weak hCA II activator.

The data of Table 2 show that, after incubation of normal blood red cells (containing approximately 150 μM hCA I and 20 μM hCA II, Maren et al., 1993) with micromolar concentrations of histamine (**1**) or histamine derivatives synthesized in the present work (such as **A11**, **A13**, **A22**, **A23**, etc.), the total CA activity in the treated cells is enhanced as compared to that of cells treated only with buffer in a blank experiment. Thus, histamine produces only a weak activation of around 120% after 0.5 h incubation, and of around 130% of the basal CA activity after 1 h incubation with the red cells. Some of the new histamine derivatives tested ex vivo (which showed strong in vitro CA activity enhancements) produced activations of 150–195% after 0.5 h incubation, and of 195–250% after 1 h incubation. These are clear-cut experiments demonstrating that some of the compounds reported in this paper might act as effective in vivo CA activators, with potential use in the treatment of CA deficiency syndrome.

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