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CARBONIC ANHYDRASE INHIBITORS: *N*-CYANOSULFONAMIDES, A NEW CLASS OF HIGH AFFINITY ISOZYME II AND IV INHIBITORS*

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A series of *N*-cyanosulfonamides has been prepared by reaction of alkyl-, arylalkyl- and arylsulfonyl halides or sulfonic acid anhydrides with cyanamide, or by reaction of cyanogen bromide with sulfamide/sulfamic acid. Other compounds have been obtained from sulfenyl chlorides, acyl chlorides, or tosyl isocyanate and cyanamide. Inhibition of three carbonic anhydrase (CA) isozymes, hCA I, hCA II and bCA IV (h = human; b = bovine) with the prepared compounds has been investigated. Very good inhibitors, as well as compounds with moderate activity against these isozymes were found, depending on the R group at which the metal-coordinating moiety of the inhibitor molecule was attached. Compounds of the types RSNHCN and RCONHCN were much less active in inhibiting all the investigated isozymes as compared to the strong inhibitors possessing the general formula RSO_2NHCN. Susceptibility to inhibition with the *N*-cyanosulfonamides was generally: hCA II > bCA IV ≫ hCA I. Spectroscopic studies on Co(II)-substituted hCA II proved that the new inhibitors directly bind to the metal ion within the enzyme active site, similarly to the classical inhibitors of the unsubstituted sulfonamide type.

Keywords: *N*-cyanosulfonamides; Cyanamides; Carbonic anhydrase; Isozyme I, II, IV; Co(II)-substitution; Inhibition mechanism

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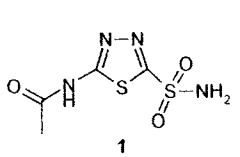
INTRODUCTION

In connection with our X-ray crystallographic work on the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1),[2] it was observed that cyanamide, a linear molecule isoelectronic to the physiological CA substrate, CO_2, was initially acting as a weak inhibitor of this enzyme ($K_I = 61 \pm 3$ mM for hCA II (h = human isozyme), and 238 ± 9 mM for hCA I, respectively) being subsequently hydrolyzed within the active site to urea.[3] Although many analogies can be formally found between the physiological reaction catalyzed by the CA-s (the reversible CO_2 hydration to bicarbonate – reaction 1) and that of cyanamide hydration to urea (reaction 2), the kinetic and presumably mechanistic differences between them are tremendous. Thus, reaction 1 is one of the fastest catalyzed by an enzyme, with a turnover number (TON = k_3) approaching $10^6\ s^{-1}$ for hCA II,[4] whereas the second reaction is a very slow process, with a TON of $10^{-5}\ s^{-1}$ (and the same isozyme).[3] More than that, urea formed in the reaction (unlike bicarbonate formed in the analogous CO_2 hydration) is so strongly bound within the enzyme active site that it cannot be displaced even by very high affinity CA inhibitors added in solution, such as the heterocyclic sulfonamides of type **1–3**. Only by denaturing the enzyme, is the CA–urea adduct destroyed and urea liberated in solution.[3] This has been explained after solving the X-ray structure of hCA II soaked in cyanamide solutions, by the observation that a urea molecule coordinated to the Zn(II) ion of the enzyme also participates in an extended network of eight hydrogen bonds with water molecules and amino acid residues present at the bottom of the active site.[3] Presumably, cyanamide is also directly bound to the metal ion of the enzyme, a water molecule/hydroxide ion still being coordinated to this, as shown by spectroscopic data on the Co(II)-hCA II adduct of cyanamide.[3] This water molecule/hydroxide ion is the nucleophile subsequently attacking the coordinated cyanamide molecule, which is converted into urea.[3] The differences between the catalytic mechanism of CO_2 hydration are indeed remarkable.[2–4]



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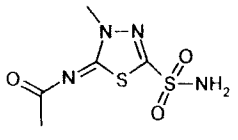

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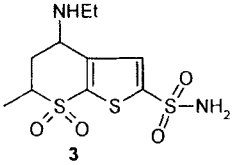

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$$O=C=O + H_2O \Longleftrightarrow HCO_3^- + H^+ \qquad (1)$$

$$HN{=}C{=}NH + H_2O \Longleftrightarrow H_2NCONH_2 \quad (2)$$

$$\underset{\mathbf{4}}{H_2N{-}C{\equiv}N} \Longleftrightarrow \underset{5}{HN{=}C{=}NH} \quad (3)$$

As seen from Figure 1, the urea molecule formed by cyanamide hydration within the CA active site displaces the metal bound water molecule/hydroxide ion of the native enzyme, and is directly bound to the metal ion by means of a nitrogen atom, which furthermore is engaged in hydrogen bonds with the side chain of Thr 199 and with Wat 123, the so-called "deep water" molecule.[3,4] The second nitrogen atom of urea is engaged in hydrogen bonds with the side chain of Thr 200, and with two water molecules, Wat 99 and Wat 122, whereas the urea oxygen atom is hydrogen bonded to Thr 200, Wat 122 and Wat 123.

The behaviour of cyanamide as a suicide substrate of CA-s, and its transformation to urea which remains blocked within the active site, as shown by the above mentioned X-ray crystallographic work,[3] prompted us to design

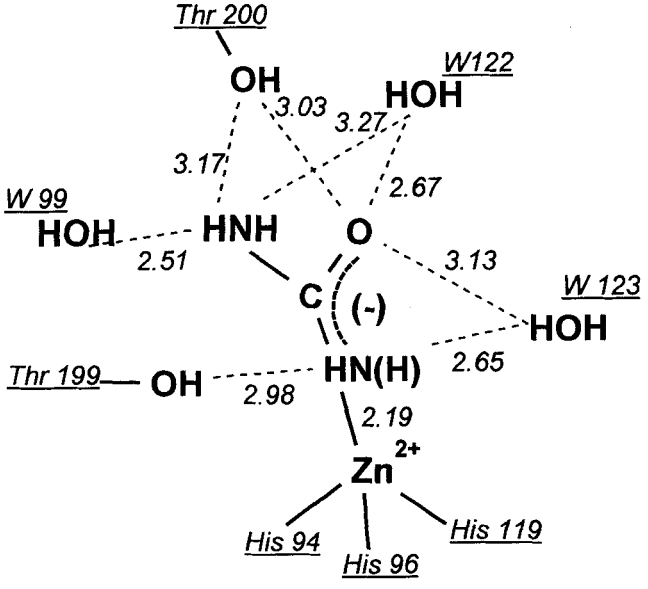

FIGURE 1 Schematic binding of urea to the Zn(II) ion and amino acid side chains and hydrogen bonds involved in anchoring the inhibitor at the bottom of the active site cavity for the urea–hCA II adduct, obtained by active site hydrolysis of cyanamide. The figure has been generated from the X-ray coordinates of the urea–hCA II adduct (file code 1BAV) as reported by this group previously.[3]

structurally related inhibitors and investigate their behaviour against the major CA isozymes, hCA I, hCA II and bCA IV (b = bovine isozyme). Mention should be made that cyanamide exists in two tautomeric forms, the amide form **4** (H_2NCN) and the carbodiimide one **5** (HN=C=NH), as shown in equation (3). Many of its derivatives obviously display, the same type of tautomerism.[5] In this paper we report the preparation of *N*-cyanosulfonamides, *N*-cyanosulfenamides and acyl cyanamides, by reaction of cyanamide with sulfonyl halides/sulfonic acid anhydrides, sulfenyl halides or acyl chlorides. Other structurally related compounds were prepared from cyanogen bromide and sulfamide/sulfamic acid. All the obtained derivatives were assayed for their interaction with the above-mentioned three CA isozymes. None of the prepared derivatives behaved as a suicide substrate of the CA isozymes, but some of them acted as very potent inhibitors for the esterase activity of these enzymes (with 4-nitrophenyl acetate as substrate). Spectroscopic work on Co(II)-substituted hCA II proved that the new inhibitors directly bind to the metal ion within the active site, similarly to the prototypical inhibitors of the unsubstituted sulfonamide type.

MATERIALS AND METHODS

Melting points were determined with a heating plate microscope and are not corrected. IR spectra were obtained in KBr pellets with a Perkin-Elmer 16PC FTIR spectrometer and ^{1}H-NMR spectra with a Varian 300CXP apparatus in DMSO-d_6 as solvent. Chemical shifts are expressed as δ values relative to Me_4Si as standard. Elemental analyses were done by combustion for C, H, N with an automated Carlo Erba analyzer, and were $\pm 0.4\%$ of the theoretical values.

Sulfonyl halides, sulfonic acid anhydrides, triethylamine, sulfamic acid, sulfamide and cyanamide used in synthesis were commercially available (from Sigma, Acros or Aldrich). Tosyl isocyanate was from Acros. Acetonitrile, acetone (Merck) and other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions. Sulfonamides **1**–**3** used as standards in the enzymatic assay were commercially available from Sigma, Aldrich or Merck, Sharp and Dohme.

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL2l (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Forsman *et al.*[6] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group,[7] and enzymes were purified by affinity chromatography

according to the method of Khalifah *et al.*[8] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1} \cdot cm^{-1}$ for CA I and 54 $mM^{-1} \cdot cm^{-1}$ for CA II, respectively, based on $M_r = 28.85$ kDa for CA I, and 29.30 kDa for CA II, respectively.[9,10] bCA IV was isolated from bovine lung microsomes, and its concentration was determined by titration with ethoxzolamide.[11,12]

Co(II)-hCA II was prepared as described in the literature by removing zinc from the native enzyme with pyridine-2,6-dicarboxylic acid, followed by dialysis against metal-free Tris-H_2SO_4 buffer, and by addition of the stoichiometric amount of Co(II) salt.[13]

Initial rates of 4-nitrophenyl acetate (Sigma) hydrolysis were monitored spectrophotometrically, at 400 nm and 25°C, with a Cary 3 instrument interfaced to a personal computer.[14] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between $2 \cdot 10^{-2}$ and $1 \cdot 10^{-6}$ M. A molar absorption coefficient $\epsilon = 18{,}400\ M^{-1} \cdot cm^{-1}$ was used for the 4-nitropenolate formed by hydrolysis, under the conditions of the experiments (pH 7.80), as previously reported.[14] Non-enzymic hydrolysis rates were always subtracted from the observed rates. At least duplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the average of such results (standard error of ±5–10% of the reported values). The enzymatic rates (without inhibitor) were obtained as the average of at least three experiments.

General Procedure for the Preparation of Compounds 6–45

The *N*-cyanosulfonamides **6–34** were prepared as previously described by our[15] and Roblin's[16] groups, by reaction of arylsulfonyl halides or sulfonic acid anhydrides, with cyanamide in aqueous acetone.

Methods A and B An amount of 10 mM sulfonyl halide (chloride for method A, and fluoride for method B) was dissolved in 50 mL of acetone and the stoichiometric amount (10 mM, 420 mg) of cyanamide dissolved in 5 mL of water was added dropwise, together with a solution of NaOH 1 M. The mixture was magnetically stirred at 25°C for 5 h, then the reaction mixture was acidified with 0.1 N HCl solution to pH 3. The *N*-cyanosulfonamides obtained generally precipitated by leaving the reaction mixture at 4°C overnight and were then filtered and recrystallized from ethanol.

Method C 210 mg (5 mmol) of cyanamide and 0.84 mL (5 mmol) of triflic anhydride were suspended in 10 mL of acetone and 0.35 mL (5 mmol) of triethylamine was added dropwise. The mixture was magnetically stirred

at 4°C for 5 h. The solvent was then evaporated *in vacuo*, and the tan residue treated with 5 mL of cold water. The triflate salt of triethylamine being water soluble was thus separated from CF_3SO_2NHCN **8** (much less water soluble) by simple filtration. The latter compound was recrystallized from *iso*-butanol.

Method D 420 mg (10 mmol) of cyanamide, 0.70 mL (10 mmol) of triethylamine and 10 mmol of sulfobenzoic cyclic anhydride or tetrabromo-*O*-sulfobenzoic cyclic anhydride were heated at reflux in 50 mL of anhydrous acetonitrile for 2 h, with a small amount of *p*-toluenesulfonic acid added as catalyst. After evaporation of the solvent, the products were treated with 10 mL of water and the precipitated derivatives **22** or **23** filtered and recrystallized from ethanol.

Method E As above for Method A, but using arylsulfenyl chlorides instead of arylsulfonyl halides.

Method F An amount of 0.76 mL (5 mmol) tosyl isocyanate dissolved in 10 mL of anhydrous acetonitrile was treated with 210 mg (5 mmol) of cyanamide dissolved in 20 mL of the same solvent. The mixture was stirred at room temperature for 1 h, then the solvent was evaporated and the precipitated foam (**37**) washed with 10 mL of water, filtered off and recrystallized from ethanol–water (3 : 2, v/v).

Method G An amount of 10 mmol of acyl chloride in 20 mL of anhydrous tetrahydrofuran was treated with the stoichiometric amount of cyanamide dissolved in 5 mL of 1 M NaOH solution. The reaction mixture has been magnetically stirred at 25°C for 6 h, then acidified with 0.1 N HCl to pH 3. The acyl-cyanamides precipitated after several hours at 4°C, and were recrystallized from ethanol.

Method H and I A general procedure for the derivatization of amines with cyanogen bromide was used.[17] An amount of 10 mmol of sulfamide or sulfamic acid was dissolved in 5 mL of 1 N NaOH solution, and the stoichiometric amount of BrCN was added. The mixture was magnetically stirred at 4°C for 4 h, adjusted to pH 5 with concentrated HCl solution and the mixture left at 4°C overnight, when the reaction products **44** and **45**, respectively, crystallized as prisms.

N,N-Dimethyl-N'-cyano-sulfamide, **6** As colorless crystals, mp 189–90°C. IR (KBr), cm^{-1}: 1140 (SO_2^{sym}), 1334 (SO_2^{as}), 2180 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 4.80 (s, 6H, Me_2N); 8.79 (s, 1H, SO_2NH). Found, C, 24.09; H, 4.61; N, 28.12. $C_3H_7N_3O_2S$ requires: C, 24.16; H, 4.73; N, 28.17%.

N-Cyano-phenylmethylsulfonamide, **7** As colorless crystals, mp 185–6°C. IR (KBr), cm^{-1}: 1176 (SO_2^{sym}), 1365 (SO_2^{as}), 2180 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.23 (s, 2H, PhCH_2); 7.15–7.59 (m, 5H, ArH from Ph); 9.21 (s, 1H, SO_2NH). Found, C, 48.59; H, 4.03; N, 14.20. $C_8H_8N_2O_2S$ requires: C, 48.97; H, 4.11; N, 14.28%.

N-Cyano-trifluoromethylsulfonamide, **8** As colorless crystals, mp 236–8°C (dec.). IR (KBr), cm^{-1}: 1169 (SO_2^{sym}), 1345 (SO_2^{as}), 2180 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 9.85 (s, 1H, SO_2NH). Found, C, 13.95; H, 0.91; N, 16.03. $C_2HF_3N_2O_2S$ requires: C, 13.80; H, 0.58; N, 16.09%.

N-Cyano-4-fluorophenylsulfonamide, **9** As colorless crystals, mp 208–9°C. IR (KBr), cm^{-1}: 1171 (SO_2^{sym}), 1360 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.11–7.49 (m, AA′BB′, $J_{AB}=7.4$ Hz, 4H, ArH, *p*-F-phenylene); 9.30 (s, 1H, SO_2NH). Found, C, 41.62; H, 2.19; N, 13.87. $C_7H_5FN_2O_2S$ requires: C, 42.00; H, 2.52; N, 13.99%.

N-Cyano-4-chlorophenylsulfonamide, **10** As colorless crystals, mp 212–4°C. IR (KBr), cm^{-1}: 1175 (SO_2^{sym}), 1366 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.10–7.56 (m, AA′BB′, $J_{AB}=7.4$ Hz, 4H, ArH, *p*-Cl-phenylene); 9.30 (s, 1H, SO_2NH). Found, C, 38.89; H, 2.60; N, 12.80. $C_7H_5ClN_2O_2S$ requires: C, 38.81; H, 2.33; N, 12.93%.

N-cyano-4-bromophenylsulfonamide, **11** As colorless crystals, mp 210–2°C. IR (KBr), cm^{-1}: 1179 (SO_2^{sym}), 1370 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.15–7.47 (m, AA′BB′, $J_{AB}=7.4$ Hz, 4H, ArH, *p*-Br-phenylene); 9.25 (s, 1H, SO_2NH). Found, C, 32.25; H, 2.11; N, 10.50. $C_7H_5BrN_2O_2S$ requires: C, 32.20; H, 1.93; N, 10.73%.

N-Cyano-4-iodophenylsulfonamide, **12** As colorless crystals, mp 218–20°C. IR (KBr), cm^{-1}: 1185 (SO_2^{sym}), 1377 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.10–7.50 (m, AA′BB′, $J_{AB}=7.5$ Hz, 4H, ArH, *p*-I-phenylene); 9.26 (s, 1H, SO_2NH). Found, C, 27.30; H, 1.95; N, 8.90. $C_7H_5IN_2O_2S$ requires: C, 27.29; H, 1.64; N, 9.09%.

N-Cyano-4-toluenesulfonamide, **13** As colorless crystals, mp 249°C, Ref. 15 mp 247–9°C. IR (KBr), cm^{-1}: 1115 (SO_2^{sym}), 1350 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (s, 3H, Me); 7.30–8.10 (m, AA′BB′, $J_{AB}=7.4$ Hz, 4H, ArH, *p*-Me-phenylene); 10.21 (s, 1H, SO_2NH). Found, C, 48.66; H, 4.25; N, 14.03. $C_8H_8N_2O_2S$ requires: C, 48.97; H, 4.11; N, 14.28%.

N-Cyano-4-nitrophenylsulfonamide, **14** As yellow crystals, mp 236–8°C. IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1340 (NO_2), 1365 (SO_2^{as}), 1510 (NO_2), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.08–7.89 (m, AA′BB′,

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(m, 4H, ArH, *m*-H_2N-phenylene); 9.89 (s, 1H, SO_2NH). Found, C, 42.39; H, 3.58; N, 21.24. $C_7H_7N_3O_2S$ requires: C, 42.63; H, 3.58; N, 21.31%.

N-Cyano-pentafluorophenylsulfonamide, **21** As colorless crystals, mp 175–7°C (dec). IR (KBr), cm^{-1}: 1148 (SO_2^{sym}), 1336 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 10.70 (s, 1H, SO_2NH). Found, C, 30.02; H, 0.45; N, 10.14. $C_7HF_5N_2O_2S$ requires: C, 30.89; H, 0.37; N, 10.29%.

N-Cyano-2-carboxyphenylsulfonamide, **22** As colorless crystals, mp 293–6°C (dec.). IR (KBr), cm^{-1}: 1153 (SO_2^{sym}), 1354 (SO_2^{as}), 1720 (COOH), 2190 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.15–7.67 (m, 4H, ArH, *o*-HOOC-phenylene); 9.89 (s, 1H, SO_2NH); 10.35 (br s, 1H, COOH). Found, C, 42.54; H, 2.50; N, 12.28. $C_8H_6N_2O_4S$ requires: C, 42.48; H, 2.67; N, 12.38%.

N-Cyano-2-carboxytetrabromophenylsulfonamide, **23** As colorless crystals, mp 219–21°C. IR (KBr), cm^{-1}: 1158 (SO_2^{sym}), 1370 (SO_2^{as}), 1720 (COOH), 2190 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 9.97 (s, 1H, SO_2NH); 10.42 (br s, 1H, COOH). Found, C, 17.50; H, 0.43; N, 5.06. $C_8H_2Br_4N_2O_4S$ requires: C, 17.74; H, 0.37; N, 5.17%.

N-Cyano-p-methoxyphenylsulfonamide, **24** As colorless crystals, mp 241–4°C. IR (KBr), cm^{-1}: 1170 (SO_2^{sym}), 1315 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.50 (s, 3H, MeO); 7.10–7.83 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-MeO-phenylene); 10.06 (s, 1H, SO_2NH). Found, C, 45.56; H, 4.10; N, 13.05. $C_8H_8N_2O_3S$ requires: C, 45.28; H, 3.80; N, 13.20%.

N-Cyano-2,4,6-trimethylphenylsulfonamide, **25** As colorless crystals, mp 219–20°C. IR (KBr), cm^{-1}: 1170 (SO_2^{sym}), 1319 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (s, 3H, 4-Me); 2.75 (s, 6H, 2,6-Me_2); 7.10–7.85 (m, 2H, ArH); 9.90 (s, 1H, SO_2NH). Found, C, 53.32; H, 5.17; N, 12.28. $C_{10}H_{12}N_2O_2S$ requires: C, 53.55; H, 5.39; N, 12.49%.

N-Cyano-4-methoxy-3-aminophenylsulfonamide, **26** As colorless crystals, mp 213–4°C. IR (KBr), cm^{-1}: 1160 (SO_2^{sym}), 1353 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.50 (s, 3H, MeO); 5.22 (s, 2H, H_2N-phenylene); 7.20–7.64 (m, 3H, ArH, trisubstituted-phenyl); 10.12 (s, 1H, SO_2NH). Found, C, 41.98; H, 4.13; N, 18.35. $C_8H_9N_3O_3S$ requires: C, 42.28; H, 3.99; N, 18.49%.

N-Cyano-2-hydroxy-3,5-dichlorophenylsulfonamide, **27** As tan crystals, mp 173–4°C. IR (KBr), cm^{-1}: 1140 (SO_2^{sym}), 1339 (SO_2^{as}), 2185 (CN), 3060 (NH + OH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 6.22 (br s, 1H, HO); 7.35 (s, 1H,

ArH, 4H); 7.80 (s, 1H, ArH, 6H) 10.24 (s, 1H, SO_2NH). Found, C, 31.56; H, 1.33; N, 10.24. $C_7H_4Cl_2N_2O_3S$ requires: C, 31.48; H, 1.51; N, 10.49%.

N-Cyano-p-(4-dimethylaminophenylazo)-benzenesulfonamide, **28** As yellow crystals, mp 213–4°C. IR (KBr), cm^{-1}: 1160 (SO_2^{sym}), 1310 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.00 (s, 6H, Me_2N); 7.08–8.10 (m, AA′BB′, $J_{AB}=7.4$ Hz, 8H, ArH); 10.15 (s, 1H, SO_2NH). Found, C, 54.56; H, 4.50; N, 21.05. $C_{15}H_{15}N_5O_2S$ requires: C, 54.70; H, 4.59; N, 21.26%.

N-Cyano-5-dimethylamino-1-napthalenesulfonamide, **29** As yellow crystals, mp 219–21°C. IR (KBr), cm^{-1}: 1169 (SO_2^{sym}), 1328 (SO_2^{as}), 2180 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.00 (s, 6H, Me_2N); 7.66–7.80 (m, 4H, H^2, H^3, H^6, H^7 from naphthalene); 7.96 (d, 1H, H^4 from the substituted naphthyl); 8.15 (m, 1H, H^8 from the substituted naphthyl); 10.20 (S, 1H, SO_2NH). Found, C, 56.60; H, 4.80; N, 15.12. $C_{13}H_{13}N_3O_2S$ requires: C, 56.71; H, 4.76; N, 15.26%.

N-Cyano-1-naphthalenesulfonamide, **30** As white crystals, mp 271–3°C. IR (KBr), cm^{-1}: 1164 (SO_2^{sym}), 1361 (SO_2^{as}), 2180 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.66–7.79 (m, 4H, H^2, H^3, H^6, H^7 from naphthalene); 7.94 (m, 1H, H^5 or H^8 from the substituted naphthyl); 7.98 (d, 1H, H^4 from the substituted naphthyl); 8.16 (m, 1H, H^5 or H^8 from the substituted naphthyl); 10.20 (s, 1H, SO_2NH). Found, C, 56.69; H, 3.30; N, 11.79. $C_{11}H_8N_2O_2S$ requires: C, 56.89; H, 3.47; N, 12.06%.

N-Cyano-2-naphthalenesulfonamide, **31** As white crystals, mp 265–6°C. IR (KBr), cm^{-1}: 1160 (SO_2^{sym}), 1380 (SO_2^{as}), 2180 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.66–7.74 (m, 2H, H^6, H^7 from naphthalene); 7.94 (m, 1H, H^5 or H^8 from the substituted naphthyl); 7.96 (d, 1H, H^3 or H^4 from the substituted naphthyl); 8.20 (d, 1H, H^4 or H^3 from the substituted naphthyl); 8.33 (s, 1H, H^1 of the substituted naphthyl); 8.35 (dd, 9.7 Hz, 2.3 Hz, 1H, H^8 or H^5 from the substituted naphthyl); 10.21 (s, 1H, SO_2NH). Found, C, 56.64; H, 3.55; N, 11.98. $C_{11}H_8N_2O_2S$ requires: C, 56.89; H, 3.47; N, 12.06%.

N-Cyano-perfluoro-n-butylsulfonamide, **32** As colorless crystals, mp 105–7°C. IR (KBr), cm^{-1}: 1180 (SO_2^{sym}), 1365 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 10.50 (s, 1H, SO_2NH). Found, C, 18.29; H, 0.45; N, 8.46. $C_5HF_9N_2O_2S$ requires: C, 18.53; H, 0.31; N, 8.69%.

N-Cyano-perfluoro-n-octylsulfonamide, **33** As colorless crystals, mp 96–7°C. IR (KBr), cm^{-1}: 1176 (SO_2^{sym}), 1362 (SO_2^{as}), 2175 (CN), 3060 (NH).

^{1}H-NMR (DMSO-d_6), δ, ppm: 10.40 (s, 1H, SO_2NH). Found, C, 20.78; H, 0.40; N, 5.25. $C_9HF_{17}N_2O_2S$ requires: C, 20.62; H, 0.19; N, 5.34%.

N-Cyano-2-thienylsulfonamide, **34** As colorless crystals, mp 242–3°C. IR (KBr), cm^{-1}: 1154 (SO_2^{sym}), 1350 (SO_2^{as}), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 6.28 (dd, 3.8 Hz, 2.5 Hz, 1H, from thienyl); 6.88 (dd, 3.8 Hz, 1.8 Hz, 1H, from thienyl); 7.16 (dd, 2.5 Hz, 1H, from thienyl); 10.20 (s, 1H, SO_2NH). Found, C, 32.09; H, 2.40; N, 14.66. $C_5H_4N_2O_2S_2$ requires: C, 31.91; H, 2.14; N, 14.88%.

N-Cyano-4-nitrobenzenesulfenamide, **35** As yellow crystals, mp 129–13°C. IR (KBr), cm^{-1}: 1075 and 1250 (NO_2), 1340 (NO_2), 2175 (CN), 3080 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 5.10 (br s, 1H, NH); 7.08–7.56 (m, AA′BB′, $J_{AB} = 7.5$ Hz, 4H, ArH). Found, C, 43.21; H, 2.40; N, 21.39. $C_7H_5N_3O_2S$ requires: C, 43.07; H, 2.58; N, 21.53%.

N-Cyano-2-nitrobenzenesulfenamide, **36** As pale yellow crystals, mp 165–8°C. IR (KBr), cm^{-1}: 1075 and 1254 (NO_2), 1330 (NO_2), 2180 (CN), 3080 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 5.20 (br s, 1H, NH); 7.15–7.88 (m, 4H, ArH). Found, C: 42.99; H, 2.45; N, 21.12. $C_7H_5N_3O_2S$ requires: C, 43.07; H, 2.58; N, 21.53%.

N-(4-Tosylamidocarbonyl)-cyanamide, **37** As colorless crystals, mp 250–2°C (dec.). IR (KBr), cm^{-1}: 1125 (SO_2^{sym}), 1290 (amide III), 1355 (SO_2^{as}), 1540 (amide II), 1680 (amide I), 2180 (CN), 3065 and 3190 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (s, 3H, Me from tosyl); 7.05–7.59 (m, AA′BB′, $J_{AB} = 7.2$ Hz, 4H, ArH phenylene from tosyl); 7.91 (s, 1H, SO_2NH); 8.42 (s, 1H, CONHCN). Found, C, 45.50; H, 3.70; N, 17.25. $C_9H_9N_3O_3S$ requires: C, 45.18; H, 3.79; N, 17.56%.

Pentafluorobenzoylcyanamide, **38** As colorless crystals, mp 179–81°C. IR (KBr), cm^{-1}: 1280 (amide III), 1545 (amide II), 1680 (amide I), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 8.54 (s, 1H, CONH). Found, C, 45.45; H, 0.45; N, 11.69. $C_8HF_5N_2O$ requires: C, 40.70; H, 0.43; N, 11.86%.

Nicotinoylcyanamide, **39** As colorless crystals, mp 137–8°C. IR (KBr), cm^{-1}: 1280 (amide III), 1540 (amide II), 1620 (C=N), 1670 (amide I), 2180 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.15–7.98 (m, 4H, ArH); 7.70 (s, 1H, CONH). Found, C, 57.23; H, 3.43; N, 28.55. $C_7H_5N_3O$ requires: C, 57.14; H, 3.43; N, 28.56%.

Isonicotinoylcyanamide, **40** As colorless crystals, mp 140–1°C. IR (KBr), cm^{-1}: 1280 (amide III), 1560 (amide II), 1620 (C=N), 1675 (amide I),

2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.15–7.72 (m, AA′BB′, $J_{AB} = 7.9$ Hz, 4H, ArH); 7.75 (s, 1H, CONH). Found, C, 57.25; H, 3.09; N, 28.19. $C_7H_5N_3O$ requires: C, 57.14; H, 3.43; N, 28.56%.

2,4-Dichlorobenzoylcyanamide, **41** As white crystals, mp 150–1°C. IR (KBr), cm^{-1}: 1285 (amide III), 1550 (amide II), 1680 (amide I), 2175 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.05–7.64 (m, 3H, ArH); 7.70 (s, 1H, CONH). Found, C, 44.70; H, 2.05; N, 13.01. $C_8H_4Cl_2N_2O$ requires: C, 44.68; H, 1.87; N, 13.03%.

2-Thienylcarbonylcyanamide, **42** As colorless crystals, mp 177–9°C. IR (KBr), cm^{-1}: 1280 (amide III), 1540 (amide II), 1675 (amide I), 2180 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 6.20 (dd, 3.7, 2.4, 1H, from thienyl); 6.85 (dd, 3.7, 1.8, 1H, from thienyl); 7.14 (dd, 2.4, 1.8, 1H, from thienyl); 8.04 (s, 1H, CONH). Found, C, 47.45; H, 2.71; N, 18.13. $C_6H_4N_2OS$ requires: C, 47.36; H, 2.65; N, 18.41%.

N,N-Diphenylaminocarbamoylcyanamide, **43** As colorless crystals, mp 123–5°C. IR (KBr), cm^{-1}: 1274 (amide III), 1540 (amide II), 1670 (amide I), 2180 (CN), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.20–7.85 (m, 10H, 2 Ph); 7.87 (s, 1H, CONH). Found, C, 70.56; H, 4.39; N, 17.50. $C_{14}H_{11}N_3O$ requires: C, 70.87; H, 4.67; N, 17.71%.

N-Cyano-sulfamide, **44** As white crystals, mp 275–6°C (dec.). IR (KBr), cm^{-1}: 1125 (SO_2^{sym}), 1350 (SO_2^{as}), 2180 (CN), 3190 (NH + NH_2). Found, C, 9.55; H, 2.70; N, 34.30, S, 26.51. $CH_3N_3O_2S$ requires: C, 9.92; H, 2.50; N, 34.69; S, 26.46%.

N-Cyano-sulfamic acid (sodium salt), **45** As white crystals, mp > 300°C. IR (KBr), cm^{-1}: 1120 (SO_2^{sym}), 1340 (SO_2^{as}), 2180 (CN), 3190 (NH + OH). Found, C, 8.70; H, 1.05; N, 19.29, S, 22.53. CHN_2NaO_3S requires: C, 8.34; H, 0.70; N, 19.44; S, 22.25%.

RESULTS AND DISCUSSION

It was recently reported by this group[15] that in addition to the unsubstituted sulfonamides of the type RSO_2NH_2, certain derivatives possessing the general formula RSO_2NHX (where X may be a group such as OH, OCH_3, NH_2, NHMe, Cl, etc) act as powerful inhibitors of CA isozymes I and II, sometimes their potency being greater than that of the corresponding unsubstituted sulfonamide. It thus appeared of interest to test whether some compounds from the recently described[15] classes might lead to new types of high affinity inhibitors and to study in detail their mechanisms of action at

the molecular level. The *N*-cyano-sulfonamides and some of their derivatives appeared of particular interest, since cyanamide has recently been shown to act both as an inhibitor as well as a substrate of several CA isozymes.[3]

N-cyanosulfonamides **6**–**34** (Table I) were obtained by reaction of alkyl-, arylalkyl- and arylsulfonyl halides or sulfonic acid anhydrides with cyanamide. Some other structurally related derivatives have also been prepared by reaction of cyanamide with arylsulfenyl halides, aroyl halides as well as tosyl isocyanate. Reaction of cyanogen bromide with sulfamic acid or sulfamide afforded two derivatives (**44** and **45**) which contain in their molecule both the sulfonamido as well as NHCN moieties, both of which interact with the CA active site, as mentioned in the introductory section (Table I).

Inhibition data with compounds **6**–**45**, as well as standard CA inhibitors **1**–**5**, against three isozymes CA I, II and IV, are shown in Table II.

The following remarks can be made regarding the inhibition data shown in Table II. *N*-cyanosulfonamides **6**–**34**, and other structurally related derivatized cyanamides of the types **35**–**45**, generally act as efficient inhibitors of the three investigated CA isozymes. *N*-cyanosulfonamides acted as more potent inhibitors when compared to the aroyl cyanamides, which in turn were more inhibitory than the *N*-cyanosulfenamides. All these compounds were however much more inhibitory than the underivatized cyanamide **4**. In the series of derivatives reported here, the nature of the groups to which the SO_2NHCN (or SNHCN, CONHCN) moiety is linked, was critical for the biological activity of the inhibitors. Thus, among the *N*-cyanosulfonamides **6**–**34**, high affinity inhibitors were associated with the presence of perfluoroalkyl/aryl-moieties such as those present in the derivatives **8**, **21**, **32** and **33**. Some of these compounds (**21**, **32** or **33**) possess stronger inhibitory properties than the classical sulfonamide inhibitors **1**–**3**, such as acetazolamide, methazolamide or dorzolamide, against isozymes II and IV, being at the same time much less effective isozyme I inhibitors than the above-mentioned sulfonamides.[18,19] Less effective inhibitors in this subseries (such as **6**, **7**, **12**, **13**, **18**, **19**, **24**–**26**, **30**, **31**, etc) were those containing moieties such as nitrophenyl-, 3-chloro-4-nitrophenyl-, 2-hydroxy-3,5-dichlorophenyl-, 4-dimethylaminophenylazo-phenyl- or 2-thienyl, but all these compounds were generally slightly less effective inhibitors than acetazolamide, methazolamide or dorzolamide. The relatively ineffective inhibitors contained groups such as tosyl-, 4-acetamidophenylsulfonyl-, aminophenylsulfonyl-, 4-methoxyphenylsulfonylamido-, mesityl-, etc. It is remarkable that the two *N*-sulfenyl cyanamides **35** and **36** had very weak inhibitory power and were only slightly more active than cyanamide **4**. The aroyl cyanamides **38**–**43** possessed an intermediate behaviour between the

TABLE I *N*-Cyanosulfonamides **6–34**, *N*-cyanosulfenamides **35**, **36**, aroyl-cyanamides **37–43** and sulfamic acid/sulfamide derivatives **44**, **45** prepared in the present study, with their synthetic methods

RSO_2NHCN	RSNHCN	RCONHCN
6–34	**35,36**	**37–43**

Compound	*R*	*Synthesis method*	*Yield*
6	Me_2N-	A	50
7	$PhCH_2-$	B	64
8	CF_3-	C	28
9	*p*-F–C_6H_4-	A	49
10	*p*-Cl–C_6H_4-	A	70
11	*p*-Br–C_6H_4-	A	67
12	*p*-I–C_6H_4-	A	81
13	*p*-CH_3–C_6H_4-	A	82
14	*p*-O_2N–C_6H_4-	A	57
15	*m*-O_2N–C_6H_4-	A	69
16	*o*-O_2N–C_6H_4-	A	48
17	3-Cl–4-O_2N–C_6H_3-	A	60
18	*p*-AcNH–C_6H_4-	A	74
19	*p*-H_2N–C_6H_4-	B	38
20	*m*-H_2N–C_6H_4-	B	45
21	C_6F_5-	A	63
22	*o*-HOOC–C_6H_4-	D	95
23	*o*-HOOC–C_6Br_4-	D	96
24	*p*-CH_3O–C_6H_4-	A	55
25	2,4,6-$(CH_3)_3$–C_6H_2-	A	50
26	4-CH_3O–3-H_2N–C_6H_3-	A	46
27	2-HO–3,5-Cl_2–C_6H_2-	A	38
28	4-Me_2N–C_6H_4–N=N–C_6H_4-	A	71
29	5-Dimethylamino–1-naphthyl–	A	65
30	1-Naphthyl	A	87
31	2-Naphthyl	A	90
32	*n*-C_4F_9-	A	18
33	*n*-C_8F_{17}	A	37
34	2-thienyl	A	75
35	*p*-O_2N–C_6H_4-	E	76
36	*o*-O_2N–C_6H_4-	E	57
37	TsNH	F	92
38	C_6F_5-	G	51
39	Nicotinoyl	G	73
40	Isonicotinoyl–	G	75
41	2,4-$Cl_2C_6H_3-$	G	69
42	2-thienyl	G	79
43	Ph_2N	G	87
44	— H_2NSO_2NHCN	H	57
45	— $HOSO_2NHCN$	I	32

A – cyanamide + RSO_2Cl; B – cyanamide + RSO_2F; C – cyanamide + triflic anhydride; D – cyanamide + sulfobenzoic cyclic anhydride; E – cyanamide + RSCl; F – cyanamide + tosyl isocyanate; G – cyanamide + RCOCl; H – sulfamide + BrCN; I – sulfamic acid + BrCN.

TABLE II CA inhibition data for compounds **6–45** and standard CA inhibitors **1–3** (IC_{50}, the mean of two different assays, represents the molarity of inhibitor producing a 50% decrease of enzyme specific activity for the 4-nitrophenylacetate hydrolysis reaction[14])

Compound	IC_{50}(nM)[a]		
	hCA I[b]	hCA II[b]	bCA IV[c]
1 (acetazolamide)	200 ± 4	7 ± 0.2	120 ± 9
2 (methazolamide)	10 ± 1	9 ± 0.5	145 ± 6
3 (dorzolamide)	30,000 ± 500	2 ± 0.1	3 ± 0.1
4 (cyanamide)	(238 ± 9) · 10^6	(61 ± 3) · 10^6	(145 ± 7) · 10^6
6	125,000 ± 300	210 ± 5	290 ± 10
7	180,000 ± 200	270 ± 9	330 ± 10
8	30,000 ± 500	12 ± 0.5	19 ± 0.4
9	150,500 ± 450	69 ± 4	110 ± 5
10	170,000 ± 200	81 ± 8	120 ± 6
11	180,000 ± 500	80 ± 5	110 ± 5
12	190,000 ± 300	103 ± 8	125 ± 9
13	350,000 ± 600	450 ± 10	650 ± 15
14	245,000 ± 500	35 ± 3	70 ± 5
15	260,000 ± 700	39 ± 5	78 ± 7
16	220,000 ± 500	64 ± 4	130 ± 9
17	170,000 ± 600	33 ± 2	62 ± 5
18	400,000 ± 500	420 ± 13	540 ± 20
19	620,000 ± 800	370 ± 10	505 ± 12
20	650,000 ± 500	450 ± 15	480 ± 10
21	30,000 ± 200	4 ± 0.1	9 ± 1
22	250,000 ± 400	90 ± 7	125 ± 8
23	180,000 ± 700	80 ± 10	150 ± 12
24	239,000 ± 900	400 ± 6	610 ± 13
25	320,000 ± 1000	400 ± 14	585 ± 10
26	580,000 ± 1000	420 ± 17	630 ± 20
27	85,000 ± 200	31 ± 2	49 ± 4
28	125,000 ± 300	32 ± 3	59 ± 6
29	340,000 ± 800	41 ± 8	75 ± 5
30	430,000 ± 200	112 ± 11	230 ± 9
31	400,000 ± 500	105 ± 5	240 ± 10
32	35,000 ± 200	5 ± 0.2	12 ± 0.7
33	27,000 ± 300	3 ± 0.1	10 ± 0.5
34	95,000 ± 500	25 ± 3	40 ± 2
35	> 10^6	> 1000	> 1000
36	> 10^6	> 1000	> 1000
37	300,000 ± 200	80 ± 12	120 ± 10
38	75,000 ± 100	45 ± 5	106 ± 8
39	550,000 ± 1000	800 ± 25	960 ± 17
40	680,000 ± 3000	880 ± 16	970 ± 18
41	> 10^6	> 1000	> 1000
42	700,000 ± 1000	400 ± 7	530 ± 14
43	510,000 ± 200	450 ± 20	690 ± 21
44	5,500 ± 100	19 ± 0.3	19 ± 1
45	7,000 ± 150	20 ± 0.8	25 ± 3

[a]Mean ± average spread (from two determinations).
[b]Human (cloned) isozyme.
[c]Isolated from bovine lung microsomes.

N-cyanosulfonamides and the *N*-cyano sulfenamides mentioned above. In this small subseries, again the perfluorophenyl derivative **38** was the most effective inhibitor, whereas 2,4-dichlorobenzoylcyanamide **41** was only slightly more efficient than cyanamide **4** itself. A special mention should be made regarding the two sulfamic acid derivatives **44** and **45**. These two compounds, unlike others in this series, appreciably inhibited hCA I, acting also as good hCA II and bCA IV inhibitors. The sulfamide derivative **44** was slightly more active than the sulfamic acid **45**.

In order to test our hypothesis that *N*-cyanosulfonamides might act as suicide substrates of CA-s, similarly to the unsubstituted cyanamide **4**,[3] tosylcyanamide **13** was incubated for different periods of time (2–72 h) with variable concentrations (in the millimolar–micromolar range) of hCA II and hCA I (data not shown). The expected reaction where the inhibitor would act as suicide substrate is shown below (equation (4)):

$$\underset{\mathbf{13}}{\text{TsNH-CN}} + H_2O \rightarrow \underset{\mathbf{46}}{\text{TsNH-CO-NH}_2} \qquad (4)$$

Tosyl-urea, **46**, possibly formed in the above reaction has previously been prepared by our group and shown to act as a weak CA inhibitor, binding monodentately to the metal ion within the enzyme active site.[20] Thus, the above mentioned reaction mixtures were assayed by a HPLC method for the presence of tosyl-urea. Under all the conditions in which the experiments were performed, with or without denaturation of the enzyme after the incubation, tosyl-urea **46** could not be detected, whereas tosyl-cyanamide **13** was found to be unhydrolyzed (data not shown). Thus, it can be concluded that unlike cyanamide **4**, its arylsulfonylated derivatives of the type reported here do not act as suicide substrates of CA-s.

A final remark should be made regarding the mechanism of action of this new class of CA inhibitors. In order to have more insights regarding the binding mode of these inhibitors within the active site, the electronic spectra of Co(II)-substituted hCA II and of its adducts with the classical and the new type of inhibitors studied here were recorded (Table III)

It is well documented that the Co(II) ion is a good spectroscopic probe for many metallo-enzymes,[21] and Co(II)CA-s possess very characteristic electronic, NMR and EPR spectra, these being highly sensitive to the environment around the metal ion, so constituting an easy approach for studying the interaction of this enzyme with inhibitors or substrates.[2,3,15,20–22]

The electronic spectral data (Table III) indicate that the new derivatives reported here bind to the Co(II) ion in the enzyme active site giving rise

TABLE III Electronic spectroscopic data for Co(II)-hCA II and its adducts with different inhibitors

Adduct	pH	*Absorption maximum*, nm (ϵ, $M^{-1} \cdot cm^{-1}$)			
Co(II)-hCA II (pure)	7.20	520 (240)	540 (310)	616.5 (220)	640 (200)
+cyanamide[a]	7.20	525 (45)	573 (50)	605 sh (30)	
+urea[b]	7.20	520 (240)	540 (300)	616 (220)	642 (200)
+**1**[c] (acetazolamide)	7.20	518 (380)	549 (210)	574 (520)	595 (500)
+**13**[c]	7.20	520 (300)	550 (210)	575 (340)	595 (350)
+**21**[c]	7.20	520 (305)	549 (210)	575 (400)	595 (450)
+**38**[c]	7.20	520 (300)	552 (200)	578 (350)	597 (420)
+**44**[c]	7.20	518 (205)	550 (270)	—	600 (250)

[a][cyanamide] = 240 nM, no incubation.
[b][urea] = 500 mM, incubation of enzyme and inhibitor solutions for 10 h at 4°C; [a,b] from Ref. 3.
[c][inhibitor] = 0.05–0.10 mM; enzyme concentrations in all cases were in the range of 0.1–0.4 mM.

to a pseudotetrahedral geometry, similarly to the unsubstituted sulfonamides, of which acetazolamide **1** is a well-known example.[21] Such adducts are characterized by intense spectra with molar absorbances above $300\,M^{-1}\,cm^{-1}$,[20–22] and this pseudotetrahedral geometry has been confirmed by X-ray crystallographic data for some of these complexes.[23] Binding is presumably achieved in all cases through the ionized nitrogen atom of the SO_2NHCN (CONHCN) moiety.

Derivative **44** possesses an electronic spectrum in the complex which is indicative of the existence of equilibria between tetra- and penta-coordinated species, as pointed out by Bertini *et al.*[21,22] for anions such as chloride, azide. Mention should be made that sulfamide, from which this compound is a structurally related derivative, has also been shown by us to bind in a similar manner to the metal ion within the CA II active site.[15]

Thus it can be concluded that the *N*-cyanosulfonamides and their derivatives reported here, behave as potent CA I, II and IV inhibitors, their mechanism of action closely resembling that of the unsubstituted sulfonamides, the prototypical CA inhibitors. Unlike the unsubstituted cyanamide from which these compounds have generally been prepared, they do not behave as suicide substrates of the CA-s, and are not hydrolyzed within the active site to the corresponding aryl/alkyl-sulfonyl ureas.

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