J. Enzyme Inhibition, 2000, Vol. 15, pp. 297–310
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CARBONIC ANHYDRASE INHIBITORS; PHOSPHORYL-SULFONAMIDES – A NEW CLASS OF HIGH AFFINITY INHIBITORS OF ISOZYMES I AND II*

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(Received 1 June 1999)

A series of phosphorylated aromatic/heterocyclic sulfonamides with the general formula $ArSO_2NHPO_3H_2$ have been prepared by condensing $ArSO_2NH_2$ with phosphorus pentachloride, followed by controlled hydrolysis in the presence of formic acid. The new derivatives generally act as stronger inhibitors of two carbonic anhydrase (CA) isozymes, CA I and CA II, as compared to the parent unsubstituted sulfonamides from which they were obtained. The inhibition mechanism by this new class of CA inhibitors, as well as structure activity correlations for the series of investigated derivatives, are also discussed.

Keywords: Carbonic anhydrase; Isozymes I, II; Phosphoryl sulfonamides; pK_a

INTRODUCTION

The field of biologically active compounds saw phosphorus derivatives extensively used in recent years in the design of enzyme inhibitors,[2]

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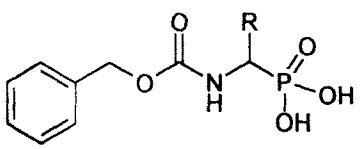
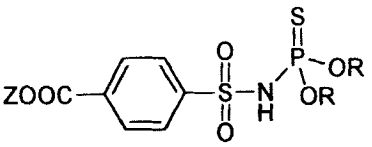
antiviral[3] or anticancer drugs.[4] Phosphorus-based ligands act as transition-state mimics for a variety of hydrolytic reactions, many such compounds being thus potent inhibitors of serine proteases[2,5] (thrombin,[2] trypsin,[2,5] etc); metallo-proteases[6] (such as collagenases,[6] angiotensin-converting enzyme[2] (ACE) or aspartic (HIV) proteases amongst others.[2,7]

Although carbonic anhydrase (CA, EC 4.2.1.1) is an extensively investigated enzyme as a target for drug design,[8-10] few phosphorus-based CA inhibitors have been investigated up to now. Thus, some phosphonic amino acid derivatives of type **1** were reported to act as weak inhibitors of red cell isozyme CA II by Osapay and Csiba,[11] and this group reported the thiophosphorylic derivatives of type **2**, possessing affinities in the range of 2–5 μM for hCA II (hCA = human CA) and 55–100 μM for hCA I, respectively.[12] In this latter study it was observed that derivatives of type **2** with R = Et were at least 100 times more active than the corresponding compounds **2** with R = Ph.[12] It appeared thus of interest to extend the previous work, in the search for stronger phosphorus-based CA inhibitors that possess even more compact groups in the neighborhood of the sulfonamide moiety.



1: R = H, Me, i-Pr



2: Z = H, Me; R = Et, Ph

In this paper we report the synthesis and CA inhibition studies on two isozymes (hCA I, and hCA II) of a series of phosphorylic sulfonamides possessing the general formula Ar-$SO_2NHPO_3H_2$. Changing the bulky substituents of the lead molecule **2** from sulfur to oxygen and from OEt to OH resulted in a drastic enhancement of affinity for the enzyme. Depending on the nature of the Ar group (aromatic or heterocyclic) both moderately active as well as tight-binding inhibitors of isozymes I and II were obtained. Thus, for the first time phosphorus-based CA inhibitors with affinities in the nanomolar range for the isozyme hCA II are reported.

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 Specord 75IR spectrometer, whereas ^{1}H-NMR spectra were obtained with a Varian

Gemmini 300 apparatus in solvents specified in each case. Chemical shifts are expressed as δ values relative to Me_4Si as standard. Mass spectroscopy was done with a MAT-311 instrument operating at 70 eV, with the electron emission of 100 μA and the ion source temperature of 150°C. Elemental analyses were done by combustion for C, H, N with an automated Carlo Erba analyzer, and were ±0.4% of the theoretical values.

Sulfonamides **3a–3k** used in synthesis were obtained from the corresponding commercially available sulfonyl chlorides (from Sigma, Acros or Aldrich) by reaction with ammonia. Dichlorophenamide **3l** was from Aldrich, whereas the benzothiazole sulfonamides **3m** and **3n** were prepared as described in the literature[13,14] from the corresponding mercaptans (from Aldrich). Acetonitrile, benzene, carbon tetrachloride, formic acid, phosphorus pentachloride or other solvents or inorganic reagents used in the synthesis were analytical grade and were used without further purification.

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Forsman *et al.*[15] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group,[16] and enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[17] 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.[18,19] Cobalt(II)–CA II was prepared by the method of Hunt *et al.*,[20] by removing zinc from the native enzyme in the presence of 50 mM pyridine-2,6-dicarboxylic acid, followed by dialysis against metal-free Tris-H_2SO_4 buffer, and addition of the stoichiometric amount of Co(II) salt.[20]

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC.[21] Solutions of substrate were prepared in anhydrous acetonitrile. The substrate concentrations used varied between $2\cdot10^{-2}$ and $1\cdot10^{-6}$ M, working at 25°C. A molar absorption coefficient ε of $18{,}400\,M^{-1}\cdot cm^{-1}$ was used for the 4-nitrophenoxide formed by hydrolysis, under the conditions of the experiments (pH 7.40), as reported in Ref. [21]. Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were made thereafter

with distilled-deionized water. Inhibitor and enzyme solutions were pre-incubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constant K_I was determined as described in Ref. [21]. The enzyme concentrations were 3.6 nM for hCA II, and 11 nM for hCA I.

General Procedure for the Preparation of Derivatives 4a–4n

An amount of 0.1 mol of sulfonamide **3a–3n** and 20.8 g (0.1 mol) of PCl_5 were stirred at 100–140°C for 40 min on an oil bath. A strong evolution of HCl was observed, leading to the formation of the title derivatives in quantitative yields. The excess phosphorus pentachloride was distilled *in vacuo*, when the trichloroderivatives **4a–4n** were obtained as crystals which were sufficiently pure to be used in the subsequent syntheses.

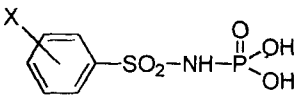
General Procedure for the Preparation of Derivatives 5a–5n

An amount of 30 mmol of trichloroderivative **4a–4n** was dissolved in 20 mL of anhydrous benzene to which 1.4 g (30 mmol) of anhydrous formic acid was added. The reaction mixture was magnetically stirred for 20 h at room temperature. After this time the compounds **5a–5n** precipitated from the reaction mixture and were filtered and used in the next synthetic step. Care should be taken not to use an excess of formic acid, which can lead to the scission of the P–N bond, with generation of free sulfonamide and phosphoric acid.[22]

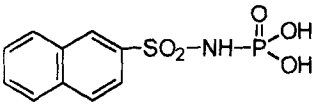
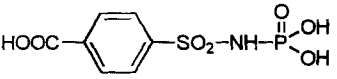
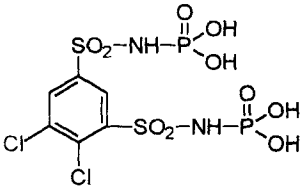
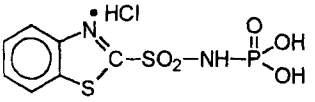
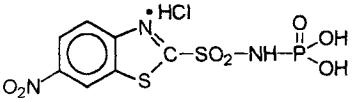
General Procedure for the Preparation of Derivatives 7a–7n

An amount of 20 mmol of compound **5a–5n** was dissolved in 15 mL of anhydrous benzene, to which 0.9 g (20 mmol) of anhydrous formic acid was added. The reaction mixture was magnetically stirred at 80°C for 1.5 h. The two layers formed were separated: the lower one contained the partially hydrolyzed derivatives, of type **6a–6n**, whereas the upper one the reaction solvent. The lower layer (which solidified on cooling) was retaken up in 15 mL of anhydrous benzene plus 0.9 g of HCOOH and heated at reflux for 3 h. On leaving the above reaction mixture overnight at 4°C, the desired phosphorylated sulfonamides **7a–7n** were obtained as crystalline powders which were filtered and dried *in vacuo*. Some of the derivatives investigated here have been previously reported in the literature[23–31] (see Tables I and II), whereas four compounds are new, being described here for the first time. A complication of the above procedure was encountered in the preparation of

TABLE I Compounds **7a–7n** synthesized in the present work, and their inhibition data against two CA isozymes. The reference where some compounds have been previously reported for the first time is also given



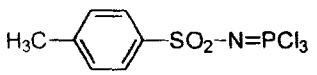
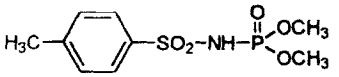
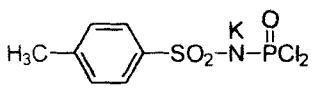
7a-7i

No.	*Compound*	*m.p.* (°C)	K_I (nM)		*Ref.*
			hCA I[a]	*hCA* II[a]	
7a	X = H	148–149	560	280	[22]
7b	X = 4-F	148–149	485	190	[23]
7c	X = 4-Cl	146–148	470	145	[23]
7d	X = 4-Br	158–162	310	60	[23]
7e	X = 4-I	180–182	42	8	[23]
7f	X = 4-CH_3	150–151	215	54	[22,24]
7g	X = 4-OCH_3	102–104	200	50	[24]
7h	X = 4-NO_2	173–175	51	15	[25]
7i	X = 2-NO_2	188–190	420	210	[25]
7j		174–175	450	200	[24]
7k		217–220	125	45	
7l		118–122	52	15	
7m		115–120	10	3	
7n		160	7	2	

[a] Human cloned isozyme.

the new derivative **7k**, due to the presence of the free carboxyl group in its molecule, which was converted to COCl during the synthesis. Still, during the usual work-up, the acyl chloride moiety was hydrolyzed to the free carboxyl one.

TABLE II CA inhibition data for compounds **4f**, **8** and **9**, and the references regarding their preparation

No.	*Compound*	*m.p.* (°C)	K_I (nM)		*Ref.*
			hCA I[a]	*hCA* II[a]	
4f		104–106	> 1000	> 1000	[26]
8		110–111	> 1000	650	[27]
9		210–213	> 1000	> 1000	[28]

[a]Human cloned isozyme.

N-Phosphoryl-4-carboxy-benzenesulfonamide **7k** IR (KBr) cm^{-1} 3410 (free NH), 3180 (assoc. NH), 2785–2700 (P–OH), 1685 (COOH), 1600 (C=C), 1318 (SO_2^{as}), 1315–1290 (P=O, C–O), 1171 (SO_2^{sym}). ^{31}P-RMN (H_3PO_4 85%), δ_{ppm}: 7.74. ^{1}H-NMR (DMSO-d_6) δ_{ppm}: 7.80–8.20 (m, AA′BB′, 4H, ArH). ^{13}C-NMR (DMSO-d_6) δ_{ppm}: 127.4 (C_2; C_6), 130.0 (C_3; C_5), 133.3 (C_4), 146.77 (C_1), 165.8 (COOH). Found: C, 29.99; H, 2.98; N, 5.30; P, 10.85. $C_7H_8NO_7PS$ (280.97) requires: C, 29.90; H, 2.87; N, 4.98; P, 11.02%. Methylation of **7k** with CH_2N_2 afforded the 4-CH_3OOC–C_6H_4–$SO_2N(CH_3)$–$P(O)(OCH_3)_2$. MS, *m/z*: 337 (M, 2.16%), 338 (M + 1, 9.7%), 273 (M – SO_2, 77.6%), 306 (M – OCH_3, 13.9%), 242 (M – SO_2 – OCH_3, 52.2%), 109 ($PO(OCH_3)_2$; 64.8%). IR (CCl_4), cm^{-1}: 1020, 1080, (P–O–(CH_3)), 1162 (SO_2^{sym}), 1273 (P=O), 1256 (C–O–(CH_3)), 1340 (SO_2^{as}), 1729 (C=O(OR)), 2854, 2926, 2965 (CH_3).

N, N′-Bisphosphoryl-4,5-dichloro-1,3-benzenedisulfonamide **7l** IR (KBr) cm^{-1} 3411 (free NH), 3280 (assoc. NH), 2780–2712 (P–OH), 1566 (C=C), 1332 (SO_2^{as}), 1233 (P=O), 1186 (SO_2^{sym}), 928 (P–O), 707 (C–Cl), 638 (C–Cl), 605 (C–Cl). ^{1}H-NMR (DMSO-d_6), δ_{ppm}: 8.18–8.28 (d, 2H, ArH). Found: C, 15.63; H, 1.9; N, 6.42; P, 13.01. $C_6H_8N_2Cl_2O_{10}P_2S_2$ (463.84) requires: C, 15.52; H, 1.74; N, 6.04; P, 13.36%. Methylation of **7l** with CH_2N_2 afforded 1,2-Cl-3,5(SO_2–$NMeP(O)(OMe)_2$)$_2$–C_6H_2. MS, *m/z*: 549 (M; 1.7%), 485 (M – SO_2; 1%), 109 ($PO(OMe)_2$; 95%), 513 (M – Cl; 15%), 449 (M – SO_2 – Cl; 15%), 349 (M – $2SO_2$ – 2Cl; 55%).

N-Phosphoryl-benzothiazole-2-sulfonamide **7m** IR (KBr), cm^{-1}: 3400–3200 (NH), 2780–2700 (P–OH), 1600 (C=C arom.), 1351 (SO_2^{as}), 1290 (P=O), 1159 (SO_2^{sym}). ^{1}H-NMR (DMSO-d_6), δ_{ppm}: 7.85–8.43 (m, 4H, ArH).

Found: C, 25.63; H, 2.58; N, 8.75; P, 9.15; Cl, 10.90. $C_7H_8N_2O_5PS_2Cl$ (330.698) requires: C, 25.42; H, 2.43; N, 8.47; P, 9.36; Cl, 10.72. Methylation of **7m** with CH_2N_2 afforded benzothiazole-2-yl-SO_2–NMe–P(O)$(OMe)_2$. MS, *m/z*: 336 (M; 1.25%), 272 (M – SO_2; 8.32%), 243 (M – SO_2 – NCH_3; 16.40%), 109 ($PO(OCH_3)_2$; 58%), 227 (M – $PO(OCH_3)_2$; 3.36%). IR (neat) cm^{-1}: 1020 (P–O–(CH_3)), 1160 (SO_2^{sym}), 1275 (P=O), 1352 (SO_2^{as}), 2850–2950 (CH_3).

N-Phosphoryl-6-nitro-benzothiazole-2-sulfonamide **7n** IR (KBr), cm^{-1}: 3400–3200 (NH), 2780–2700 (P–OH), 1600 (C=C arom.), 1352 (SO_2^{as}) 1340 (NO_2), 1290 (P=O), 1160 (SO_2^{sym}). ^{1}H-NMR (DMSO-d_6), δ_{ppm}: 7.45–8.20 (m, 3H, ArH). Found: C, 22.52; H, 1.99; N, 11.55; P, 8.03; Cl, 9.61. $C_7H_7N_3O_7PS_2Cl$ (375.695) requires: C, 22.37; H, 1.87; N, 11.18; P, 8.24; Cl, 9.43%. Methylation of **7n** with CH_2N_2 afforded 6-NO_2-benzothiazole-2-yl–SO_2–NMe-P(O)$(OMe)_2$. MS, *m/z*: 337 (M – SO_2; 0.6%), 303 (M – SO_2 – NCH_3; 0.87%), 109 ($PO(OCH_3)_2$; 28%), 273 (M – $PO(OCH_3)_2$; 0.74%). IR (neat), cm^{-1}: 1025–1150 [(P–O–CH_3), and SO_2^{sym}], 1275 (P=O), 1390–1350 (SO_2^{as} and NO_2), 2850–2950 (CH_3).

Synthesis of Derivative 8

A suspension of 0.01 mol of **9** in methanol was treated with 20 mmol of sodium methoxide dissolved in the same solvent. After 30 min stirring at room temperature, the alcohol was evaporated *in vacuo*, the residue obtained dissolved in 30 mL of water and brought to pH 2 with AcOH. The title derivative precipitated, was filtered and air dried; m.p. 110–1°C (Refs. [27,28] m.p. 110°C).

Synthesis of Derivative 9

An amount of 3 g (10 mmol) of **3f** was introduced slowly into 5 g of a saturated aqueous solution of Na_2CO_3 with vigorous stirring. The precipitated compound was filtered, washed with a small amount of ice-water and dried *in vacuo*; m.p. 210–3°C (Ref. [28] m.p. 210–3°C).

pK_a-Determination

The half neutralization point was measured by titrating the organic acids with $5.58 \cdot 10^{-2}$ M KOH in EtOH–water (30%, v/v), using a glass electrode, as described by Bell and Roblin[32] for the antibacterial sulfonamides.

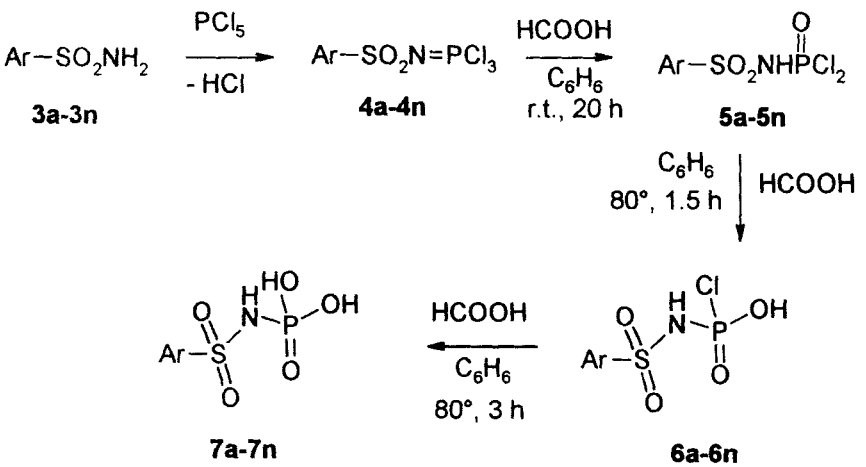
RESULTS AND DISCUSSION

The phosphorylic sulfonamides synthesized in the present work (Table I) were prepared as outlined in Scheme 1 by the Kirsanov procedure.[22–31]

Treatment of aromatic/heterocyclic sulfonamides **3a–3n** with phosphorus pentachloride afforded the trichlorophosphazasulfonaryl derivatives **4a–4n** in quantitative yield. These were then selectively hydrolyzed with the loss of one, two or three chlorine atoms, respectively, with formation of the dichloroderivatives **5a–5n**, the monochloro derivatives **6a–6n**, and finally, the desired phosphoryl sulfonamides **7a–7n**, respectively. The hydrolytic steps mentioned above were all performed with formic acid in anhydrous benzene, working at different times/temperatures, as shown in Scheme 1.

Inhibition data against two CA isozymes for compounds of type **3–7** prepared in the present work are shown in Tables I–III, whereas pK_a values for some of the derivatives **7** as well as the parent sulfonamides **3** from which they were obtained are presented in Tables III and IV.

From the above data it can be observed that with one exception (compound **7l**), the phosphorylic sulfonamides of type **7** reported here are as potent hCA I and hCA II inhibitors, as the corresponding parent, unsubstituted sulfonamides **3**. This is a remarkable finding, since generally the introduction of substituents at the SO_2NH_2 group leads to a drastic reduction in affinity for CA, as shown in the classical study of Krebs.[33] In fact, only recently the *N*-modified sulfonamide CA inhibitors were reinvestigated



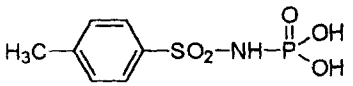
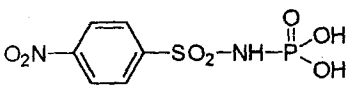
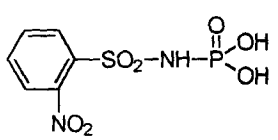
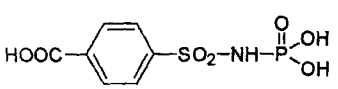
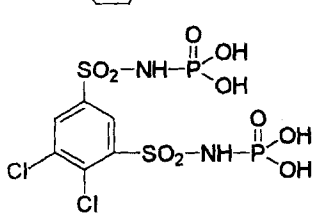
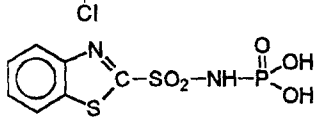
SCHEME 1

TABLE III CA inhibition data for the parent sulfonamides **3a**–**3n** from which the phosphorylated derivatives **7** were obtained

Compound	K_I (nM)[a]		pK_a[b]
	hCA I	*hCA* II	
3a	650	320	10.1
3b	520	230	9.7
3c	500	190	9.8
3d	330	100	9.9
3e	56	14	9.9
3f	350	120	10.2
3g	350	110	10.0
3h	210	60	9.3
3i	600	250	9.2
3j	630	300	10.0
3k	580	260	9.8
3l	47	10	8.3
3m	12	4	7.9
3n	10	2	7.4

[a]Human cloned isozyme. [b]In EtOH–water, 30% (v/v), at 25°C.

TABLE IV pK_a values for some of the derivatives **7** prepared in the present study

No.	*Compound*	pK_a[a]			
		pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
7f		2.79	6.36	10.25	—
7h		3.10	6.13	8.93	—
7i		2.92	5.72	9.63	—
7k		2.82	3.75	7.24	10.21
7l		2.93	5.19	9.11	—
7m		2.59	5.79	10.21	—

[a]In water, at 25°C.

by Blackburn's[34] group and by our group,[10c,35] and it was shown that efficient inhibitors can be obtained from this class too (for instance, derivatives of type $ArSO_2NHX$, with X = Cl, OH, NH_2, CN, etc., possessed the same potency as the unsubstituted sulfonamides (X = H) against hCA II). Thus, the substitution pattern reported in the present work for derivatives **7** enlarges the list of the moieties X mentioned above with which the sulfonamide moiety can be modified without loss of CA inhibitory properties. The relatively compact nature of the phosphorylic moiety in compounds **7** is critical for tight binding to the enzyme, as the bulkier derivatives **4f**, **8** or **9** (Table II) are practically devoid of CA inhibitory properties. Even the dimethyl ester **8** is 11 times less active as a hCA II inhibitor compared to the corresponding unmethylated derivative **7f**. Another factor controlling the enzyme inhibitory properties of the obtained compounds is the nature of the aromatic/heterocyclic moiety Ar. Thus, heterocyclic derivatives such as **7m**, **n** were around 10^2 times more active than the majority of the aromatic compounds (such as **7a**, **7j**, etc). In the series of aromatic derivatives, the disulfonamide **7l** behaves as a strong CA inhibitor, but its potency is slightly reduced when compared to that of dichlorophenamide **3l**, from which it was prepared. Good inhibitors were also those containing 4-nitro-, 4-carboxy- or 4-halogeno-phenyl moieties, with potencies increasing from the fluoro-derivative **7b** (a moderately active hCA II inhibitor) to the iodo-derivative **7e** (a strong hCA II inhibitor). Isozyme hCA II possessed a higher affinity for this type of *N*-modified sulfonamide inhibitor, as compared to isozyme hCA I.

In order to get more information regarding the inhibition mechanism for this new class of CA inhibitors, the interaction of compounds of types **3** and **7** with Co(II)-substituted hCA II was studied.

The Co(II) ion is a good spectroscopic probe,[10c,36-38] since Co(II)hCA has very characteristic electronic, NMR and EPR spectra which are highly sensitive to the environment around the metal ion, thus constituting an easy approach for studying the interaction of this enzyme with inhibitors, activators or substrates.[10c,36-38]

In Table V the electronic spectral data for some adducts of Co(II)hCA II with inhibitor derivatives of P(V) of type **7** and the sulfonamide **3k** for comparison are shown.

As shown by earlier studies by Bertini's group,[36-38] four bands are seen in the electronic spectrum of Co(II)hCA II in the region 400–750 nm, which are highly pH-dependent and sensitive to the environment around the metal ion. Unsubstituted sulfonamides, of the type $ArSO_2NH_2$ such as acetazolamide (5-acetylamino-1,3,4-thiadiazole-2-sulfonamide, a clinically used CA inhibitor)[8] which directly bind in ionized form to the metal ion,[10-12]

TABLE V Electronic spectral data (in the range 400–750 nm) for adducts of Co(II)hCA II with sulfonamide and phosphorylated sulfonamide inhibitors. Enzyme concentrations were in the range 0.5–1.2 mM and pH values of the media are specified. Inhibitor concentrations were in the range 1.5–3.5 mM

Adduct	*pH*	*Band position*, nm (*molar absorptivity* [$M^{-1} \cdot cm^{-1}$])
Pure enzyme	6.0	520 (180); 550 (250); 616.5 (135); 640 (100)
Pure enzyme	7.6	520 (280); 550 (380); 616.5 (280); 640 (260)
+**3k**	7.6	519 (350); 545 (210); 575 (520); 595 sh (500)
+**7e**	7.6	520 (310); 545 (200); 576 (500); 595 sh (440)
+**7h**	7.6	520 (300); 545 (205); 570 (520); 595 sh (450)
+**7j**	7.6	518 (280); 547 (210); 570 (490); 596 sh (450)
+**7k**	7.6	520 (330); 545 (210); 570 (510); 595 sh (450)
+**7n**	7.6	520 (310); 545 (200); 570 (500); 596 sh (450)

lead to intense electronic spectra, characterized primarily by molar absorptivities over 300 $M^{-1} \cdot cm^{-1}$ and the shift of the two bands from 616.5 and 640 nm in the spectrum of the pure enzyme, to wavelengths under 600 nm.[36–38] This is just the type of spectrum seen for the adduct of Co(II)-hCA II with 4-carboxy-benzenesulfonamide **3k**, shown in Table V. It was in fact envisaged that this simple aromatic sulfonamide would bind in a similar way to heterocyclic derivatives such as acetazolamide or other of its congeners. Thus, the P(V) derivatives reported here, of type **7**, seem to bind in a similar manner to the unsubstituted sulfonamide **3k**, as judged from the similarities of the electronic spectra of these adducts (Table V). Practically the first two bands in these electronic spectra (those around 520 and 545 nm) underwent slight modifications in the spectra of the P(V) adducts as compared to the spectra of the pure enzyme (except for the fact that they are much more intense), whereas the last two bands underwent major changes showing shift under 600 nm and even greater intensification.

Aromatic/heterocyclic sulfonamides bind to the metal ion within the CA active site in ionized form, as $ArSO_2NH^-$ anions.[8–10] Since heterocyclic derivatives possessing this general formula are generally more acidic than the aromatic ones (pK_a values in the range of 7.2–7.9 units for heterocyclic, versus 9.3–10.2 pK_a units for the aromatic derivatives),[8–10] it was considered that the enhanced activity observed with the heterocyclic compounds is in fact due to facilitated ionization and binding to the enzyme of the obtained anions. Thus, sulfonamides with lower pK_a values were needed in order to test this hypothesis. Compounds **7** reported here possess in fact several (three or four) dissociations over the whole pH range, being tri- or tetrabasic acids (Table IV). Three of these ionizations are due to protons of the sulfonyl–phosphoryl moiety, whereas for one compound (**7k**) a fourth ionization occurs due to the presence of the carboxyl moiety (Table IV).

As shown from the data in Tables III and IV, the pK_a of the SO_2NH moiety for compounds of type **7** is only marginally influenced by the presence of the phosphoryl moiety, being in the same range as that of the corresponding unsubstituted derivatives **3** (compare for instance the pairs **3f** and **7f**; **3h** and **7h**; **3i** and **7i**; **3k** and **7k**; etc). Generally a slight acidification of 0.3–0.4 pK_a units was observed for the phosphorylated derivatives **7** as compared to the corresponding unsubstituted sulfonamides **3** (but some exceptions from this rule were also seen). At this point, the strong inhibition observed with some of the phosphorylated derivatives **7** might indeed be due to the facilitated formation of the sulfonamido conjugate bases, but the binding to the enzyme might be realized in several ways (Figure 1).

As proposed in Figure 1, binding to the enzyme might be realized in the classical way (A), monodentately, with a $Zn-N^-$ bond, as for the unsubstituted sulfonamides.[8-10] Alternatively, a bidentate binding (B) is also possible, in which the inhibitor coordinates the Zn(II) ion by means of the sulfonamido nitrogen and one of the oxygen atoms of the phosphoryl moiety. This hypothesized trigonal bipyramidal geometry of Zn(II) might also lead to a binding of type (C), in which the inhibitor is coordinated by one of the of the oxygen atoms of the phosphoryl moiety, with Zn(II) again in its preferred tetrahedral geometry. Alternatively equilibrium between these three binding modes would not be improbable. Such a multitude of binding modes was previously found by means of paramagnetic NMR for the adduct of Co(II)hCA II with $C_6H_5SO_2NHOMe$, another inhibitor with a modified sulfonamido moiety, which binds to the metal ions both monodentately (by means of the nitrogen or oxygen atoms), as well as bidentately.[10c] The spectral data presented here for the adducts of Co(II)hCA II with compounds **7**, do not allow us at the present time to establish which of the three

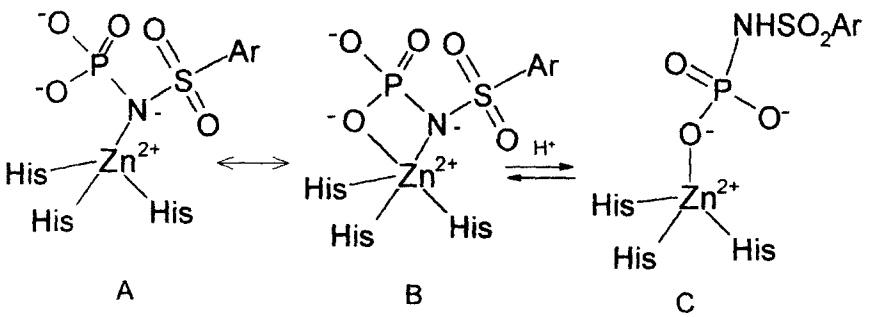


FIGURE 1 Proposed schematic binding of inhibitors of type **7** within the active site of carbonic anhydrase.

binding modes presented above best describes the interaction of this new class of inhibitors with the enzyme.

Another aspect which is important for the new derivatives **7** is that due to the presence of the very acidic protons of the phosphoryl moiety they can easily form sodium or potassium salts, which are highly soluble in water (data not shown). This property might be important for some putative applications of such inhibitors, such as the design of an anti-osteoporosis agent from the class of CA inhibitors. It is in fact known that relatively high doses of acetazolamide, a clinically used sulfonamide CA inhibitor, are useful for the treatment of osteoporosis.[39] Inhibition of CA isozymes present within osteoclasts[40] by phosphorus-based CA inhibitors of the type described here might be an interesting strategy in the design of anti-osteoporotic drugs,[41] taking also into account the clinical success of some P(V) derivatives, such as pamidronate ($H_2NCH_2CH_2C(OH)(PO_3HNa)_2$) or other bisphosphonates used as antimetastatic agents in bone cancer and related diseases.[4]

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