New fluorescent probes for carbonic anhydrases†

Jayati Banerjee,[a] Manas K. Haldar,[a] Sumathra Manokaran,[b] Sanku Mallik*[a] and D. K. Srivastava*[b]

Received (in Austin, TX, USA) 30th January 2007, Accepted 1st April 2007
First published as an Advance Article on the web 20th April 2007
DOI: 10.1039/b701421j

We report the synthesis and fluorescence properties of naphthalenesulfonamide derivatives as active site probes for carbonic anhydrases.

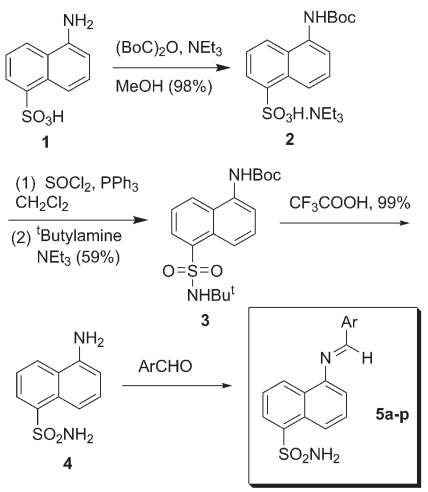
Due to the marked sensitivity of fluorescence spectroscopy, fluorescent probes have found a wide range of applications as diagnostics and imaging tools in biomedical sciences. Since the number of protein targets of human diseases have increased tremendously in the recent genomics and proteomics era,[1] there is an increasing need for the development of newer fluorescent probes. Fluorogenic probes are frequently used for designing enzyme substrates, as well as for the detection of target proteins and enzymes under both *in vivo* and *in vitro* conditions.[2]

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitously-distributed zinc-containing metalloenzymes.[3] These enzymes catalyze the reversible hydration of CO_2 to form HCO_3^-, which is involved in a variety of biosynthetic reactions, such as gluconeogenesis, the synthesis of certain amino acids, lipogenesis, ureagenesis and pyrimidine nucleotide biosynthesis.[4] Besides, these enzymes are involved in pH homeostasis, ion transport, water and electrolyte balance, bone resorption, calcification and tumorogenicity.[3,4] Amongst these CA isozymes, CA II has been studied most extensively.[5] Systemic and topical inhibitors of CA II are employed to lower the elevated intraocular pressure in glaucoma.[5] In recent years, inhibitors for the tumorogenic, hypoxia-induced CA XII have been synthesized and screened for potential anticancer activity.[5,6]

In pursuit of developing inhibitors against carbonic anhydrases, we resorted to determining binding affinities of inhibitors, both by performing steady-state kinetic experiments (K_i) as well as by the ligand displacement method (K_d).[7] While attempting to measure the binding affinities of carbonic anhydrase inhibitors, we noted that dansylamide has been widely utilized as a fluorescent probe for carbonic anhydrases.[8] Aside from dansylamide, there are a few reports of other fluorescent probes for carbonic anhydrases in the literature.[9] However, those probes have not been extensively utilized due to the high costs of their starting materials or challenging synthetic protocols.[9] Another major drawback of all fluorescent probes has been their high quantum yields in the free forms, and such quantum yields are not significantly altered upon binding of the probes to the CA sites.[9] This feature, aside from exhibiting lower sensitivity, also provides a high background fluorescence while performing both equilibrium ligand displacement as well as transient kinetic experiments. In view of the above limitations, we proceeded to synthesize a variety of naphthalenesulfonamide derivatives as fluorescent probes for carbonic anhydrases and determined their fluorescence properties in the absence and presence of recombinant human carbonic anhydrase isozymes I and II.

In the effort of synthesizing naphthalenesulfonamide derivatives, we decided to first prepare aminonaphthalenesulfonamide and then derivatize the amino group with a variety of substituted benzaldehyde moieties. The synthesis of 4-aminonaphthalene-1-sulfonamide was reported in the literature.[10] However, in our hands, this procedure gave very low yields for the intermediates and the final product, and we were unsuccessful in scaling up the reactions. Consequently, we developed an efficient synthesis of this compound (**4**) starting from inexpensive, commercially-available 4-aminonaphthalene-1-sulfonic acid (**1**) (Scheme 1, synthetic details are provided in the ESI†).

Firstly, the amino group of **1** was protected with the Boc-group employing the standard protocol. This protection also improved the solubility of compound **2** in organic solvents. The sulfonic acid was converted to the *tert*-butyl protected sulfonamide by the reaction of *in situ* generated sulfonyl chloride with *tert*-butylamine. In the final step, both the Boc and *tert*-butyl protecting groups were removed using trifluoroacetic acid to produce **4** in 57% overall yield. We have performed these reactions on a multigram

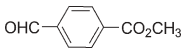
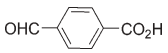
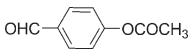
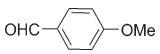
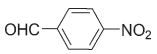
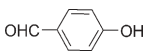
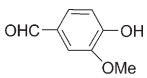
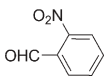
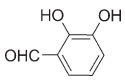
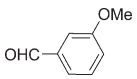
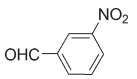
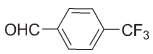
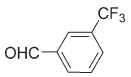
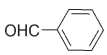
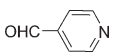


Scheme 1 Synthesis of the fluorogenic probes.

[a]*Department of Pharmaceutical Sciences, North Dakota State University, Fargo, North Dakota 58105, USA. E-mail: sanku.mallik@ndsu.edu; Fax: +1 701-231-8666; Tel: +1 701-231-7888*
[b]*Department of Chemistry, Biochemistry and Molecular Biology, North Dakota State University, Fargo, North Dakota 58105, USA. E-mail: dk.srivastava@ndsu.edu; Fax: +1 701-231-8831; Tel: +1 701-231-7831*

† Electronic supplementary information (ESI) available: Syntheses, probe integrity, kinetics and fluorescence data, spectrophotometric and spectrofluorometric studies, determinations of dissociation constants and quantum yields and NMR data. See DOI: 10.1039/b701421j

Table 1 Structures of the substituted benzaldehydes used, yields and dissociation constants of the probes with recombinant human CA I and CA II

Product	Aldehyde	Yield (%)	K_d(CA I)/ nM	K_d(CA II)/ nM
5a		60	>5000	>5000
5b		47	1460	3300
5c		47	40	97
5d		30	30	30
5e		54	20	300
5f		39	1000	3000
5g		62	25	47
5h		56	107	720
5i		76	30	60
5j		59	150	130
5k		75	70	100
5l		28	26	80
5m		45	34	960
5n		61	121	100
5o		48	235	130
5p		60	130	1500

scale and the final product is easily purified by recrystallization. The amine **4** was then reacted with a variety of substituted benzaldehydes to produce the fluorogenic probes **5a–p** (Scheme 1). The structures of the aldehydes used in the imine formation, as well as the yields of the probes, are shown in Table 1.

Due to the presence of aryl sulfonamide groups, several of these probes were found to serve as potent inhibitors for both CA I and CA II (Table 1, for details, see ESI†). All of our synthesized probes showed strong absorption bands in the UV-Vis region with absorption maxima in the 330–370 nm range (Table 2). We observed that under our experimental conditions, the probes are not in equilibrium with the precursor aldehydes, both in the absence and presence of CA (see ESI†). When the free forms of these probes (5 μM in 25 mM HEPES buffer, pH = 7.0, containing 10% DMSO) were excited at the wavelengths of their absorption maxima, most of the probes (except for compound **5a**) showed very weak fluorescence emission intensity. However, the fluorescence emission intensities of these probes were markedly enhanced in the presence of recombinant human CA I or CA II (Fig. 1A). We determined the quantum yields of free fluorophores as well as those bound to the active sites of CA I and CA II (Table 2, details are provided in ESI†).

As shown in Table 2, several of our synthesized probes showed significant enhancements in quantum yields when bound to the enzymes. Many of these quantum yield enhancements (*e.g.*, for **5c**, **5f**, **5i**, **5j**, **5k**, **5o** and **5p**) upon binding to the enzymes were significantly higher than that observed with the parent compound, dansylamide.[8] In fact, the emission intensity of compound **5h** was so weak in solution that we could not determine the quantum yield. In contrast, the compound turned highly fluorescent in the presence of recombinant human CA I or CA II. Two of our synthesized probes showed higher enhancements in quantum yield upon binding with CA I as compared to that of CA II (*e.g.*, **5b** and **5n**), while the others showed either a reverse trend (*e.g.*, **5c**, **5d**, **5i**, **5l** and **5o**) or nearly equal enhancements in the quantum yields with both of these isozymes (*e.g.*, **5e**, **5f**, **5g**, **5j**, **5k** and **5p**).

A comparison of the fluorescence profiles of free *versus* enzyme bound probes reveals that the emission intensities of the free probes are quenched in aqueous solution. However, this is not a

Table 2 Photophysical properties of the synthesized fluorophores: maximum absorption wavelength (λ_{abs}, nm), emission maxima (λ_{em}, nm) when excited at λ_{abs} in 25 mM HEPES buffer containing 10% DMSO (pH = 7.0, 5 μM probe solution) in the presence of recombinant human CA I (10 μM) and CA II (10 μM), quantum yields (Φ) in the presence of CA I and CA II and the enhancements in quantum yields in the presence of CA I and CA II

Probes	λ_{abs}	λ_{em}(free)	λ_{em}(CA I)	λ_{em}(CA II)	Φ(CA I)	Φ(CA II)	Φ(CA I)/Φ(free)	Φ(CA II)/Φ(free)
5a	342	522	516	520	0.022	0.011	1.4	1.0
5b	342	516	468	467	0.026	0.013	22.4	13.3
5c	333	522	467	465	0.044	0.062	47.2	66.9
5d	330	519	470	468	0.014	0.024	30.7	55.2
5e	358	528	471	468	0.015	0.011	12.1	7.9
5f	336	528	470	466	0.139	0.144	40.9	42.3
5g	342	520	473	465	0.020	0.017	28.4	23.9
5h	330	—	470	464	0.012	0.017	—	—
5i	330	526	470	464	0.036	0.045	39.5	49.2
5j	330	525	470	465	0.073	0.064	77.7	70.0
5k	330	528	470	469	0.073	0.077	53.1	55.8
5l	336	526	475	463	0.043	0.063	20.4	30.2
5m	330	—	470	462	0.018	0.015	—	—
5n	330	525	468	466	0.073	0.041	31.1	17.6
5o	330	—	470	465	0.012	0.015	47.7	60.6
5p	330	518	475	465	0.116	0.107	45.6	41.9

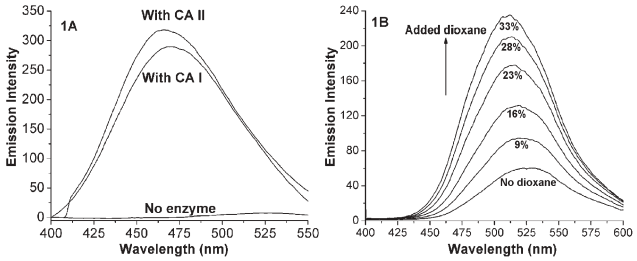


Fig. 1 **(A)** The emission spectrum of **5f** (5 μM) and in the presence of recombinant CA I and CA II (10 μM, λ_{ex} = 336 nm) in 25 mM HEPES buffer (pH = 7.0) containing 10% DMSO. **(B)** The emission intensities of **5f** (5 μM, λ_{ex} = 336 nm) in 25 mM aqueous HEPES buffer (pH = 7.0) containing varying amounts of added dioxane are shown. The volume percentages of added dioxane are indicated on the plot.

Table 3 The absorption maxima of Reichardt's dye in aqueous buffer with various amounts of dioxane, the $E_T(30)$ values and the corresponding quantum yields of **5f**

Dioxane (%)	λ_{abs}	$E_T(30)$	Φ
0	452	63.2	0.007
9	462	61.9	0.012
16	470	60.8	0.019
23	480	59.5	0.028
28	495	57.7	0.036
33	505	56.6	0.043

self-quenching process, since increasing the concentration of the probes in buffer solution resulted in regular increases in the emission intensity (data not shown). Since the active site pockets of carbonic anhydrases are predominantly hydrophobic,[11] the question arose whether the enhancements in the fluorescence quantum yields of these probes upon binding to CA I and CA II were due to changes in polarity of the solvent media. To probe this, we determined the fluorescence emission spectra and quantum yields of **5f** in 25 mM HEPES buffer (pH = 7.0) as a function of increasing concentration of added dioxane (Fig. 1B, Table 3). Note that as the hydrophobicity of the solvent medium increases (as determined by the $E_T(30)$ values[12]), the fluorescence emission intensity as well as the quantum yield keep on increasing. Clearly, the hydrophobicity of the active site pockets of CA I and CA II is responsible, at least in part, for the enhanced quantum yields of the fluorescent probes in Table 1. However, we could not ascertain the contribution of active site resident Zn^{2+} ion on the enhancement of the emission intensity due to precipitation of Zn^{2+}-free (apo) CA II in the presence of the parent compound dansylamide. Sulfonamides are known[13] to be weak binders of Zn^{2+} ions in solution and we did not observe any changes in the fluorescence emission spectra of the probes in the presence of up to 50 mM of $ZnSO_4$ in aqueous solution (see ESI†).

In conclusion, we have synthesized several new naphthalene-sulfonamide derivatives as fluorescent probes for CAs. Due to their low quantum yields in their free forms in aqueous buffer and significantly enhanced quantum yields when bound to the enzymes, they will find applications in a variety of thermodynamic and kinetic experiments for the binding of enzyme inhibitors to various isoforms of carbonic anhydrases.

This research was supported by the NIH grants 1R01 CA113746 to DKS and SM, and 1R01 GM 63204 to SM.

Notes and references

1 P. Imming, C. Sinning and A. Meyer, *Nat. Rev. Drug Discovery*, 2006, **5**, 821; R. M. Hoffman, *Nat. Rev. Cancer*, 2005, **5**, 796.
2 K. E. Sapsford, L. Berti and I. L. Medinta, *Angew. Chem., Int. Ed.*, 2006, **45**, 4562; N. S. Finney, *Curr. Opin. Chem. Biol.*, 2006, **10**, 238.
3 A. Scozzafava, T. Owa, A. Mastrolorenzo and C. T. Supuran, *Curr. Med. Chem.*, 2003, **10**, 925.
4 S. Pastorekova, S. Parkkila, J. Pastorek and C. T. Supuran, *J. Enzyme Inhib. Med. Chem.*, 2004, **19**, 199.
5 J. Y. Winum, A. Scozzafava, J. L. Montero and C. T. Supuran, *Expert Opin. Ther. Pat.*, 2006, **16**, 27.
6 C. T. Supuran, D. Vullo, G. Manole, A. Casini and A. Scozzafava, *Curr. Med. Chem.: Cardiovasc. Hematol. Agents*, 2004, **2**, 49.
7 B. C. Roy, A. L. Banerjee, D. L. Kloche, M. Swanson, X. Jia, M. K. Haldar, S. Mallik and D. K. Srivastava, *J. Am. Chem. Soc.*, 2004, **126**, 13206.
8 A. L. Banerjee, S. Tobwala, B. Ganguli, S. Mallik and D. K. Srivastava, *Biochemistry*, 2005, **44**, 3673; C. A. Lesburg, C. Huang, D. W. Christianson and C. A. Fierke, *Biochemistry*, 1997, **36**, 15780; B. W. Clare and C. T. Supuran, *Eur. J. Med. Chem.*, 1997, **32**, 311.
9 Y. Takaoka, H. Tsutsumi, N. Kasagi, E. Nakata and I. J. Hamachi, *J. Am. Chem. Soc.*, 2006, **128**, 3273; C. A. Fierke and R. B. Thompson, *BioMetals*, 2001, **14**, 205; R. B. Thompson, W. O. Whetsell, Jr., B. P. Maliwal, C. A. Fierke and C. J. Frederickson, *J. Neurosci. Methods*, 2000, **96**, 35.
10 A. M. Trushkin, A. G. Kazantsev, N. V. Kuznetsov, A. D. Yakhimovich, G. Y. Moguchaya, E. V. Rodina, N. A. Gridneva, I. G. Sharina and A. A. Nedospasov, *Biokhimiya (Moscow)*, 1994, **59**, 1521.
11 J. D. Cox, J. A. Hunt, K. M. Compher, C. A. Fierke and D. W. Christianson, *Biochemistry*, 2000, **39**, 13687; A. Jain, G. M. Whitesides, R. S. Alexander and D. W. Christianson, *J. Med. Chem.*, 1994, **37**, 2100.
12 M. T. Morgan, M. A. Carnahan, C. E. Immoos, A. A. Ribeiro, S. Finkelstein, S. J. Lee and M. W. Grinstaff, *J. Am. Chem. Soc.*, 2003, **125**, 15485.
13 S. Chaves, S. M. Marques, A. Cachudo, M. A. Esteves and M. A. Santos, *Eur. J. Inorg. Chem.*, 2006, 3853.