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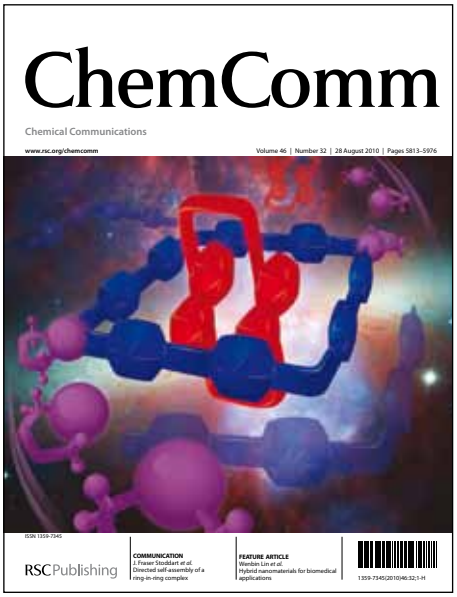

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ARTICLE TYPE

# 1,3,5-Trisubstituted Benzenes as Fluorescent Photoaffinity Probes for Human Carbonic Anhydrase II Capture

*Partha Sarathi Addy,[a] Baisakhee Saha,[b] N. D. Pradeep Singh,[a] Amit K. Das,[b*] Jacob T. Bush,[c] Clarisse Lejeune,[c] Christopher J. Schofield [c]* and Amit Basak[a*+]*

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**The synthesis of small molecule based 1,3,5-trisubstituted benzenes for photo-mediated capture of human carbonic anhydrase II with visualisation by fluorescence is described.**

The 'capture' of proteins by small molecules via irreversible cross-linking mediated by photo-irradiation is of interest in the field of proteomics (for reviews see ref. 1). The technique has the potential for profiling protein-binding by small molecules, an objective of importance both for basic cell biology and in pharmaceutical science. Capture compounds, or photoaffinity probes, are typically endowed with three functions comprising (i) a selectivity function, such as an enzyme inhibitor, (ii) a photo-cross linking group (capture function) and (iii) a sorting group to enable separation of the captured protein from biological mixtures, such as biotin or an alkyne for subsequent modification. The captured protein(s) can be isolated using streptavadin beads and identified by mass spectrometry or western blotting (for examples see ref. 2). We are interested in developing methods for protein capture and visualisation without necessitating an isolation or chemical conjugation step (**Figure 1**).[3]

Various 'tripodal' molecules have been employed for protein capture by small molecules, for example amino acids, peptides and trisubstituted aromatics including 1,2,4-trisubstituted benzene derivatives.[4] However, there are few examples of the use of 1,3,5-trisubstituted benzenes for this purpose.[5] Here we report that 5-amino dimethylisophthalate can be readily modified to produce compounds (**1**, **2**) suitable for fluorescence based monitoring of protein capture (**Figure 2**) as exemplified by work on human carbonic anhydrases (HCA), increases in the level of some isoforms of which are indicative of disease especially those related to hypoxia (CA IX).

We employed two sulphonamide derivatives that are known inhibitors for human carbonic anhydrase II (HCA II) as the selectivity function.[6] An aryl azide was chosen for photo-mediated cross-linking due to its ability to react irreversibly via nitrene formation.[7, 8] A propargylated pyrene derivative was selected as a fluorescent visualisation function.

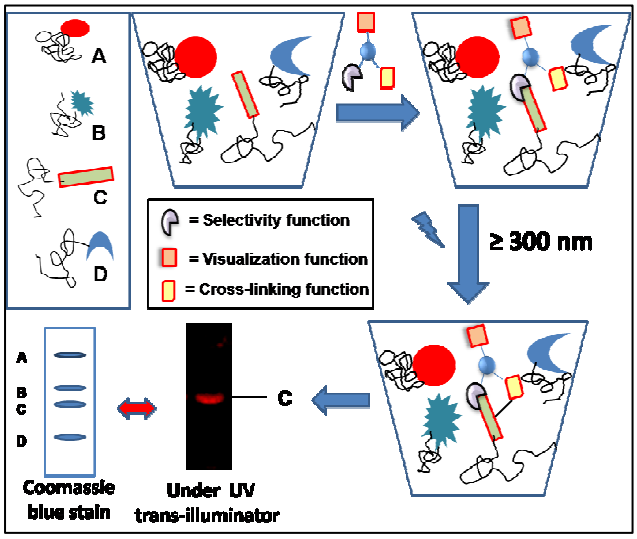



**Figure 1:** Working principle of fluorescence based visualisation of capture.

It was envisioned that the three groups could be attached to 5-amino dimethyl isophthalate acting as a tripodal template. A polar linker (diamino ethylene) was introduced between the visualization group and the template in order to distance the former from the other two groups and to increase the hydrophilicity (Figure 2).

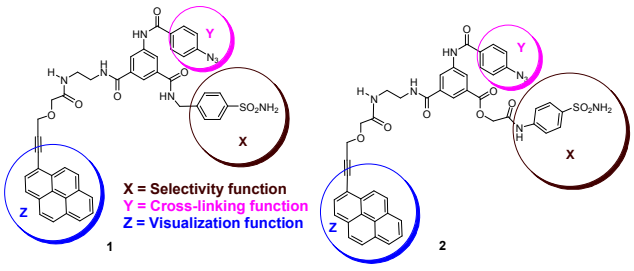
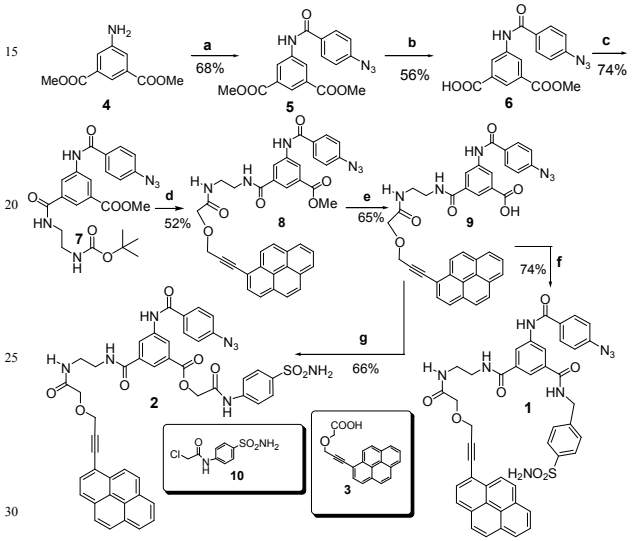



**Figure 2**: Design of capture compounds

The synthesis started with BOP- mediated amide coupling with 4-azidobenzoic acid to link the cross-linking function to amino dimethyl isophthalate **4**. The product, **5,** was hydrolysed (NaOH) to give acid **6**. Subsequent coupling of **6** with mono-Boc protected ethylenediamine afforded amide **7**. Deprotection ($CF_3CO_2H$) followed by coupling of the resultant amine with acid **3** gave amide **8** the ester of which was hydrolysed (NaOH). The resultant free acid **9** was coupled with 4-aminomethyl-benzenesulfonamide to furnish the capture compound, **1**. Reaction of **8** with chloro sulphonamide **10** using potassium carbonate in DMF produced the other capture compound **2** (**Scheme 1**).[9] The fluorescent tag **3** was prepared from 1-bromopyrene via a 3-step protocol (see ESI).




**Scheme 1:** Synthesis of capture compounds **1** and **2**. *Reagents and conditions*: a) *p*-Azidobenzoic acid, BOP-reagent, diisopropylethylamine (DIPEA), $CH_2Cl_2$ reflux, 24 h; b) NaOH, MeOH, 50 °C, 3 h; c) MonoBOC protected ethylenediamine, BOP-reagent, DIPEA, $CH_2Cl_2$, 40 °C, 48 h; d) (i) $CF_3CO_2H$, $CH_2Cl_2$, 0 °C - rt, 20 min; (ii) Acid **3**, BOP-reagent, DIPEA, $CH_2Cl_2$:DMF (5:1), rt, 20 h; e) NaOH, MeOH, 50 °C, 6 h; f) 4-Aminomethyl-benzenesulfonamide, BOP-reagent, DIPEA, $CH_2Cl_2$:DMF (5:1), 40 °C, 48 h; g) $K_2CO_3$, 2-Chloro-N-(4-sulfamoylphenyl)acetamide **10**, dry DMF, rt, 20 h.

We then investigated the inhibition of HCA II by **1** and **2**. Both were found to be reversible (competitive) inhibitors with compound **2** showing stronger inhibition ($IC_{50}$: **1** 16.7 μM, **2** = 1.2 μM, for kinetic plots see ESI). The issue of suitability of the template for the proposed capture compounds and the efficacy of the fluorescence based technique for visualising protein capture was then addressed. Thus, **1** and **2** were incubated with HCA II at various concentrations (15 min), irradiated (UV λ ≥ 300 nm, 7 min) and then directly subjected to polyacrylamide gel electrophoresis (PAGE).[10] Exposure of the gel to UV-transillumination showed clear fluorescence bands at the expected region, which was further confirmed by Coomassie blue staining (**Figure 3**).

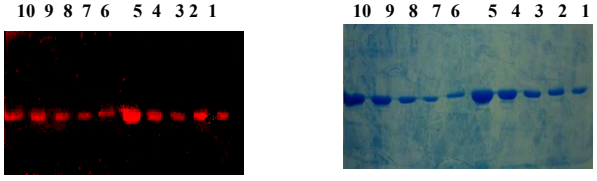



**Figure 3**: Result of gel electrophoresis analysis of capture of HCA II by **1** and **2** at different protein concentrations, as visualised by UV (left) and Coomassie blue (right). Lanes 1-5 represent incubation with **1** (100 μM), lanes 6-10 represent incubation with **2** (100 μM). The final concentration of protein in lanes 1-5 was 4, 6, 8, 20 and 40 μM, respectively. HEPES buffer (pH 7.2) was used.

The results validate compounds **1** and **2** as fluorescent capture compounds for HCA II. Capture is visible by in gel analysis under a UV- transilluminator down to HCA II concentrations of 4 μM, with the concentration of the capture compounds at 50 μM.

We then investigated the selectivity of the probes. The process of incubation and photolysis was carried with a mixture of proteins *i.e.* HCA II, bovine serum albumin (BSA) and Lysozyme. Under UV-transillumination, only the fluorescence band corresponding to HCA II was visible, whereas Coomassie blue staining of the same gel showed bands corresponding to the three proteins (**Figure 4**).

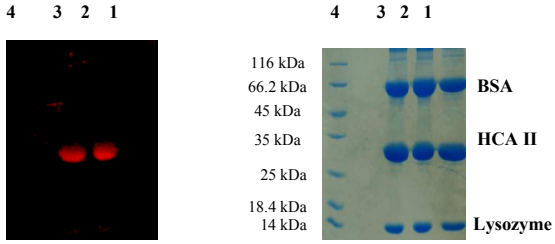



**Figure 4**: Results of gel electrophoresis analysis of capture of HCA II in a mixture by **1** and **2** visualised using UV (left) and Coomassie blue (right). Lane 1: mixture of BSA, HCA II and Lysozyme (all at 21 μM), lane 2: protein mixture (21 μM) + **1** (50 μM) with irradiation, lane 3: protein mixture (21 μM) + **2** (50 μM) with irradiation, lane 4: molecular weight marker. HEPES buffer (pH 7.2) was used.

The experiment was repeated with cell lysates of *E. coli* where the selective capturing of HCA II was clearly apparent by PAGE (**Figure 5**). Thus, both **1** and **2** showed high selectivity towards HCA II demonstrating the suitability of our 1,3,5-trisubstituted benzene template for capture and subsequent fluorescence-based visualisation.

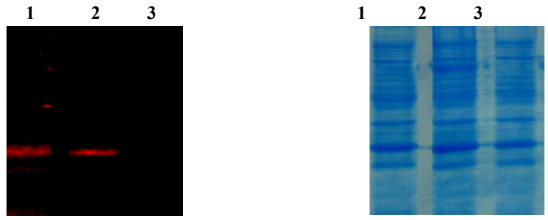



**Figure 5**: Gel electrophoresis of capture of HCA II from a cell lysate by **1** and **2** visualised using UV (left) and Coomassie blue (right). **Lanes 1**: 0.5 mg cell + **1** (10 μl, 1 mM) + 90 μL buffer (HEPES); **2**: 0.5 mg cell + **2** (10 μl, 1mM) + 90 μL buffer; **3**: 0.5 mg cell + 100 μL buffer.

The capture of HCA II by both compounds **1** and **2** was validated by MALDI mass spectrometric analyses. Thus, the incubated photo-reacted mixture was directly analysed using a MALDI-TOF mass spectrometer which revealed a new peak at m/z 29919.24 (**1**) or 29964.05 (**2**) corresponding to [$M^+$ (HCA II) + (**1** or **2**) $-N_2 + H^+$)] (see ESI). The mass spectrum of the incubated photo reacted mixture containing three proteins showed the peak corresponding to capture of HCA II only (**Figure 5** for capture by **2**; see ESI for **1**).[11]

In conclusion, we have described two readily accessible small molecule probes, **1** and **2,** for selective capture of HCA II via photo-irradiation allowing fluorescent visualization. The ability of 5-amino dimethyl isophthalate to act as a tripodal template for capture molecule design was demonstrated. We believe that 1,3,5-trisubstituted benzene based photo-reactive probes coupled with fluorescence based visualisation offers a simple and effective method for protein capture, which should be of utility in evaluating drug toxicity by studying their off-target interactions, inhibitor design and early disease diagnosis.

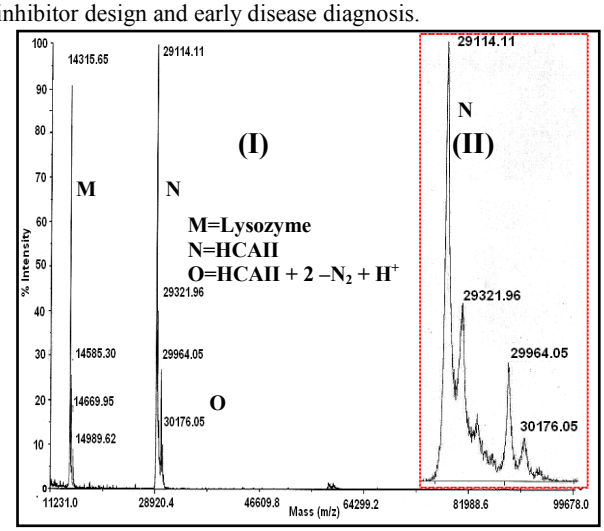



**Figure 5:** MALDI spectra: **(I)** Mixture of HCA II, BSA and Lysozyme + **2**, incubated and photo-reacted; **(II)** expanded spectrum.

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[a]*Department of Chemistry,* [b]*Department of Biotechnology, Indian Institute of Technology Kharagpur 721302 India*
[c]*Department of Chemistry, Chemistry Research Laboratory, University of Oxford, 12 Mansfield Road, Oxford, UK OX1 3TA*
*E-mail: absk@chem.iitkgp.ernet.in*
*Fax: +91 3222 282552; Tel: +91 3222 283300*

†Electronic Supplementary Information (ESI) available: Experimental procedure, compound characterization, copies of NMR, Inhibition Kinetics, ESI and MALDI-TOF MS.

## Notes and references

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[9] Spectroscopic data: **For 1:** $\delta_H$ ($d_6$-DMSO): 10.52 (1H, s), 9.21 (1H, t, $J$ = 5.5 Hz), 8.66 (1H, bs), 8.42 (1H, d, $J$ = 9.0 Hz), 8.39-8.27 (4H, m), 8.26-8.07 (5H, m), 8.02 (2H, d, $J$ = 8.5 Hz), 7.77 (2H, d, $J$ = 8.5 Hz), 7.49 (2H, d, $J$ = 8.5 Hz), 7.22 (2H, d, $J$ = 8.5 Hz), 4.72 (2H, s), 4.53 (2H, d, $J$ = 5.5 Hz), 4.12 (3H, s), 3.16 (2H, s), 3.14 (3H, s). $\delta_C$ ($CDCl_3$): 169.6, 166.8, 166.7, 166.2, 144.2, 143.6, 143.2, 139.9, 135.9, 135.6, 131.9, 131.6, 131.3, 131.2, 131.0, 131.4, 129.5, 129.0, 128.3, 127.8, 127.4, 126.7, 126.6, 126.3, 125.5, 125.2, 124.2, 123.9, 122.9, 122.7, 121.7, 119.7, 116.8, 91.9, 85.5, 69.4, 59.5, 43.1, 38.5, 31.9; $\nu_{max}$ ( KBr, $cm^{-1}$): 3752, 3413, 2923, 2857, 2364, 2122, 1651, 1599, 1547, 1452, 1333, 1284, 1155, 1099, 1028; $\lambda_{max}$ (DMSO): 365 nm (ε 30,985 $M^{-1}cm^{-1}$), 347 nm (ε 24.630 $M^{-1}cm^{-1}$), 285 nm (ε 35,384 $M^{-1}cm^{-1}$); HRMS: Calcd for $C_{45}H_{36}N_8O_7S+Na^+$ 855.2325, found 855.2346. **For 2:** $\delta_H$ ($d_6$-DMSO): 10.60 (2H, s), 8.83 (1H, s), 8.65 (1H, s), 8.57 (1H, s), 8.45 (1H, d, $J$ = 8.5 Hz), 8.34 (1H, d, $J$ = 7.0 Hz), 8.29 (2H, d, $J$ = 9.5 Hz), 8.23-8.04 (8H, m), 7.76 (4H, bs), 7.25 (4H, bs), 4.99 (2H, s), 4.72 (2H, s), 4.14 (2H, s), 3.32 (4H, s); $\delta_C$ ($CDCl_3$): 169.4, 166.2, 165.4, 146.5, 143.9, 141.7, 139.1, 136.4, 136.1, 135.6, 134.9, 133.9, 133.6, 132.3, 131.9, 131.6, 131.4, 130.8, 130.1, 129.7, 129.2, 128.8, 128.5, 127.6, 127.2, 126.4, 126.3, 125.2, 125.0, 124.2, 123.7, 119.4, 119.3, 116.5, 91.7, 85.3, 69.2, 59.3, 56.2, 38.3, 34.7; $\nu_{max}$ ( KBr, $cm^{-1}$): 3855, 3751, 3398, 2924, 2853, 2357, 2127, 1745, 1696, 1606, 1542, 1399, 1308, 1243, 1155, 1102, 1021; $\lambda_{max}$ (DMSO): 365 nm (ε 27,369 $M^{-1}cm^{-1}$), 347 nm (ε 21,200 $M^{-1}cm^{-1}$), 285 nm (ε 34,615 $M^{-1}cm^{-1}$); HRMS: Calcd for $C_{46}H_{36}N_8O_9S+Na^+$ 899.2224, found 899.2259.

[10] **Capture experiment protocol**: For capture experiments, the HCA II concentration was kept at 0.5 µM and total volume was made up to 100 µL with buffer (20 mM HEPES; pH 7.2). HCA II and capture compounds (10-100 µM) were mixed by vortexing followed by centrifugation. For cell lysate preparation, 5 mL of induced, lag phase BL21(DE3)pLysS cells carrying plasmid pACA/HCA II were resuspended in 0.5 mL of buffer (50 m*M* Tris; pH 8.0 , 50 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 1 mM phenyl methanesulfonyl fluoride, 0.2 mM $ZnSO_4$), sonicated and centrifuged at 10,000 rpm for 10 minutes. The cell lysate thus obtained was mixed with the 50 µM capture compounds. To probe the selectivity of the capture compounds towards HCA II, BSA and lysozyme were used as controls. BSA and lysozyme were mixed at equimolar concentrations with HCA II. Exactly same method was followed as used with HCA II. The compounds were incubated with proteins for 15 min, then photo-irradiated with medium pressure mercury lamp at ≥ 300 nm for 7 minutes followed by SDS-PAGE and mass spectrometric analysis.

[11] The mass spectra didn't show any peak for BSA probably due to its high MW (66.5 kDa).