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Studies on the development of capture compounds for selective detection of HCA II†

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Abstract : The work carried out in our laboratory on the development of protein capture/labeling agent over the last few years has been discussed. The chronological improvement of the design technology of such agents and the demonstration of enediyne acting as photo cross-linker are also elaborated. The research demonstrated the power of this technique in various applications including designing of new inhibitors and development of diagnostic tools.

Keywords : Protein profiling, template, HCA II, fluorescent probe, enediyne, azidinaphthalimide.

Introduction

Photo-affinity based protein profiling[1] is a rapidly developing technique in the field of chemical biology. It employs a small organic molecule called the protein capture or labeling compound (depending upon the final objective) and is useful in studying complex biological interactions. The decipheration of human genome[2] has witnessed the emergence of the field of proteomics[3] which involves exploration of the structure-function and characterization of proteins expressed by various genes present in the entire genome. 2D gel electrophoresis coupled with MALDI MS and use of protein capture agents are at the forefront of proteomics research. A protein capture compound consists of three functions, also called hands. They are : (a) the reactivity hand; (b) the selectivity hand and (c) the reporting or sorting hand. These three hands are tethered to a particular template. A protein capture compound as represented in Fig. 1 can be used for various purposes which include : (a) identification of lead compounds for drug targets, (b) rapid detection of proteins of interest and (c) off-target interactions which are the main causes of side-effects of drugs. The template around which the hands are incorporated, plays an important role to ensure highest possible interaction between the targeted enzyme and the capture molecule. A template free tethering system using linkers may be too flexible and thus adopting a particular conformation required to interact with the target protein and proper positioning of the cross-linking moiety may not be entropy-favourable. The working principle of capture compound is very interesting. Using its selectivity hand, the capture compound first interacts with the targeted enzyme. By doing so, it selectively comes closer to the enzyme. Upon suitable photo-perturbation, the reactivity hand gets activated and forms covalent linkage with a nearby reactive residue in the interacted protein. The interaction is then confirmed by mass spectroscopy or gel assay by the use

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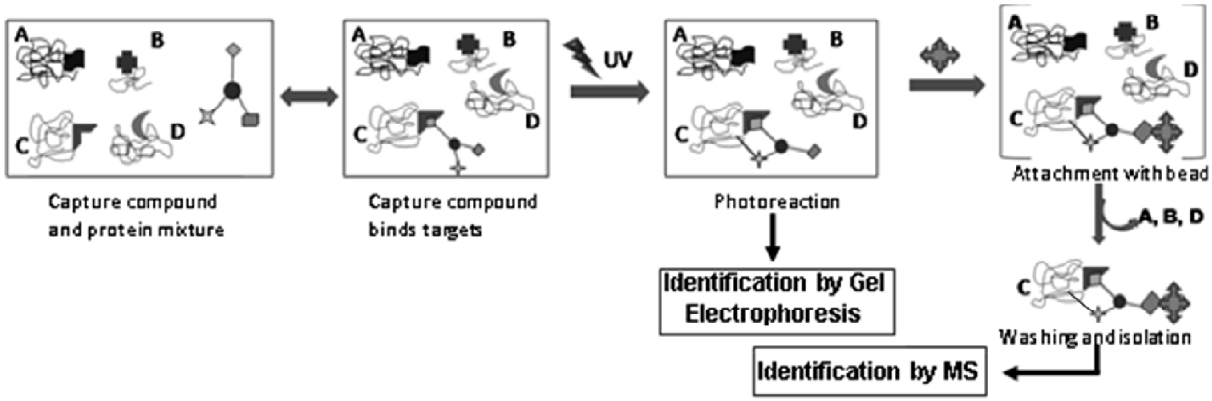


Fig. 1. Schematic representation of Principle and Design of a capture agent.

of reporting hand. Generally inhibitor of an enzyme is considered as the selecti-vity hand. Consequently this part can be varied according to the target enzyme. The other two constituents (reactivity and sorting/reporter hands) are varied to provide better efficiency of capturing and also better sensitivity of detection. There are some specific photo cross-linking groups and reporter groups that are available in the literature. Most of the capture molecules synthesized are based on these groups[1,4]. In this manuscript, we present the progress made in the field of template-based protein capture research that include design, synthesis and profiling of biomolecules in our laboratory over the past few years.

Structural features of 'Capture' compounds

Selectivity hand

As have been mentioned, a protein capture compound is endowed with three functional hands which are often crafted on a template. Regarding the selectivity hand, a prior knowledge of the active site of the target protein forms the basis of design of this functionality. A structural entity capable of interacting in a reversible manner with the active site/receptor site of the protein of interest serves the purpose. Weak forces like H-bonding, electrostatic, hydrophobic and π-stacking form the basis of such reversible binding.

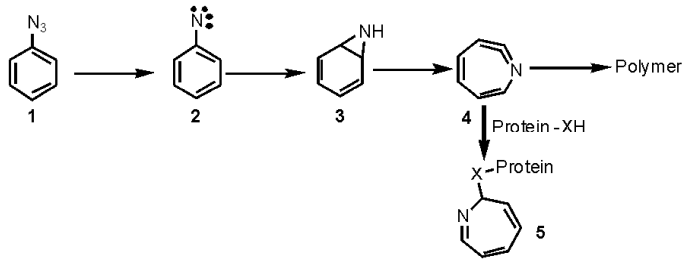
Reactivity hand

This is the functionality which the capture compound employs to form irreversible cross-link with the protein. For the cross-linking to happen, the reactivity hand should be in close proximity with protein residues which is ensured by the reversible binding of the selectivity hand. Generally, photo-reactive groups are used as reactivity hand which upon irradiation form cross-links with the interacted protein (target) or group of proteins (off-targets). Photo-irradiation generally results in the formation of highly reactive intermediates like carbene or nitrene, which are capable of forming the covalent linkage with the reactive amino acid residues in the vicinity of the active site. Aromatic azides[5], aliphatic or aromatic diazirines[6] and benzophenones[7] are the widely used photo-reactive groups in capture compounds for photo-affinity labelling purpose. Recently, enediyne moiety has been demonstrated to show photo-cross linking ability. Since our work is mostly devoted to using aryl azide and enediyne as photo-reactive probes, the photo chemistry of these two moieties are described here.

Use of aryl azide

The mechanistic details of the decomposition of aryl azides have been extensively studied by means of matrix isolation spectroscopy, laser flash photolysis and various theoretical studies. Till today it has

been established that an aryl azide upon UV photolysis generates singlet phenyl nitrene and molecular nitrogen. In the liquid phase this singlet nitrene isomerizes to form 1,2-azacycloheptatetraene. Later on, it was revealed that it is basically a two step process involving bicyclic benzazirine. This benzazirine intermediate was trapped by ethane thiol. In proteomic analysis this 1,2-azacycloheptatetraene intermediate basically is attacked by the necleophilic sites of the interacted protein which results in the covalent bond formation between the protein and the capture compound[8] (Scheme 1). Aryl azides are very often used in capture compounds because of their small size. Moreover these derivatives can be synthesized very easily from readily accessible starting materials, stable in dark and very reactive upon photo-excitation.



Scheme 1. Fate of aryl azide upon photo-irradiation in presence of protein.

A major disadvantage of using aryl azides as photo-cross linker is that their maximum absorption wavelength is below 300 nm, which could induce damage to the biological system upon photo-irradiation.

Use of enediyne

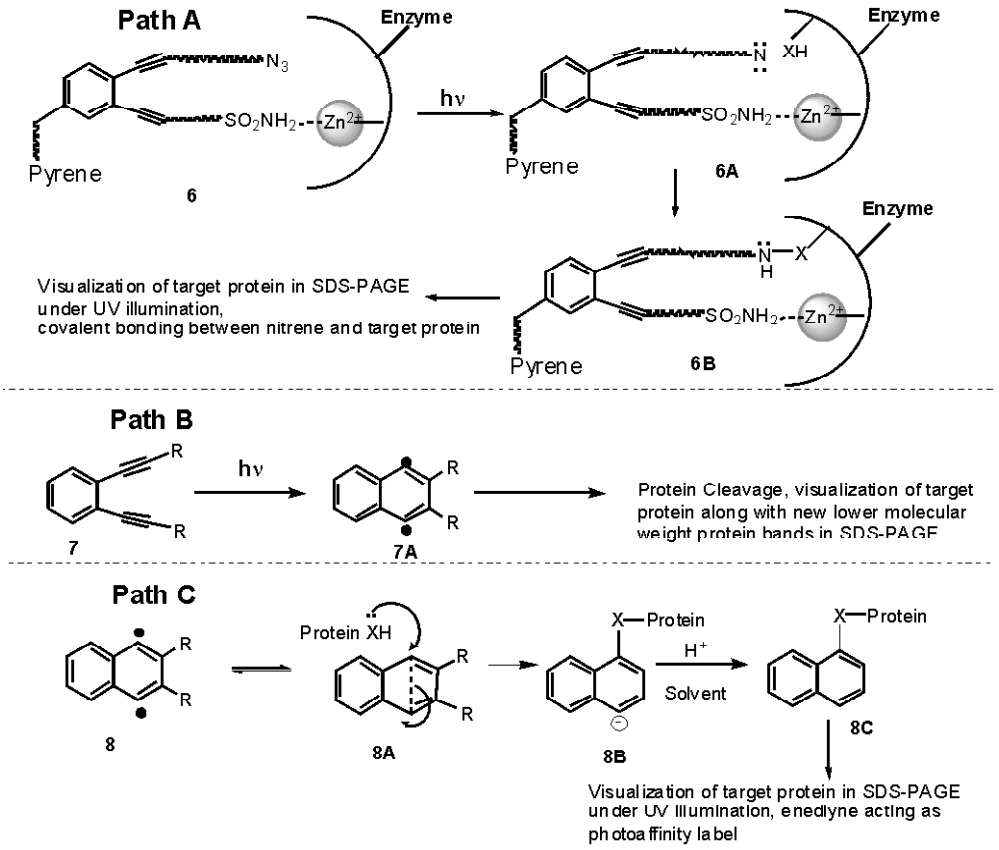
The enediyne moiety itself may undergo photo-Bergman cyclization[9] under UV irradiation and may give rise to a radical mediated cleavage of protein molecule by the 1,4-diradical generated through Bergman cyclization of the enediyne. In 2007 Perrin *et al*.[10] reported addition of H-X (X = Cl, Br, I) to cyclodeca-1,5-diyn-3-ene to form 1-halotetrahydronaphthalene upon slight heating. The authors proposed a zwitterionic form of the 1,4-diradical generated after Bergman cyclization of the enediyne that underwent H-X addition. Thus, considering the zwitter-ionic form of the diradical, a second possibility may be the protein itself cross-links to the capture compound by the nuclophilic amino acid residue present in it. It is to be mentioned here that the zwitterionic form may be considered as the major contributor to the excited state during photochemical cycloaromatization. All these possibilities are shown in Scheme 2.

Present work

Type 1 : Tripodal template-based design

(A) 1,3,5-Trisubstituted benzene based systems :

Two fluorescence based capture compounds 8 and 9 targeted against human carbonic anhydrase II (HCA II) were synthesized. Basic design of the molecule is very simple and was based on the principle as stated earlier. 5-Amino isophthalate was selected as the template with three linking moieties (two esters and one amine functionalities) for attaching the three hands. To the template, reactive hand (4-azido benzoic acid) was first attached. This was followed by the pyrene based fluorophore that was tethered *via* an ethylene diamine linker to keep this moiety far from the main interactive hands. Finally the selectivity hand (an amino benzene sulphonamide) was attached (Fig. 2). The selection of sequential attachment of the three hands was decided by considering the solubility problems encountered during synthesis which involved a series of amide coupling and esterification reactions[8].



Scheme 2. Photo-cross linking mechanism of enediynes.

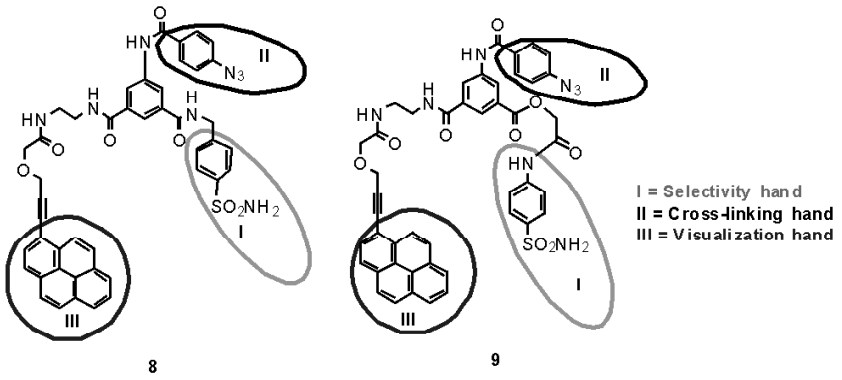


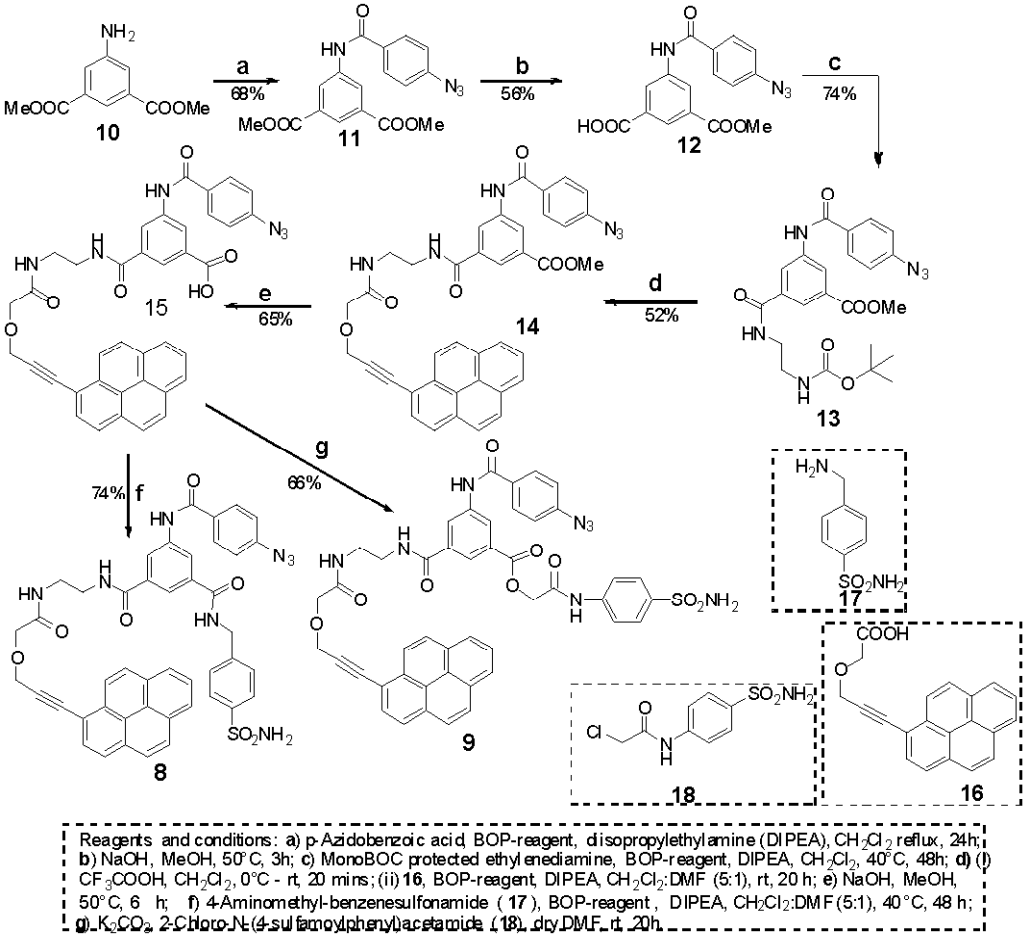
Fig. 2. Target capture molecules 8 and 9.

The synthesis started with BOP-mediated amide coupling with 4-azidobenzoic acid to introduce the cross-linking hand into the template. The resulting compound 11 was hydrolysed with one equivalent of sodium hydroxide to furnish the acid 12. Subsequent coupling of 12 with mono-BOC protected ethylenediamine afforded compound 13. The latter was deprotected with TFA and the free amine was reacted with the pyrene-based acid 16 using BOP-reagent. The resulting coupling product 14 was hydrolysed with sodium hydroxide to produce the free acid 15. Another BOP-mediated amide coupling be-

tween the acid 15 and 4-aminomethyl-benzenesulfonamide (17) afforded the capture compound 8. On the other hand, reaction of compound 15 with sulphonamide 18 using potassium carbonate in DMF produced the other capture compound 9. The yields of individual steps and the reaction conditions are mentioned in Scheme 3.

Initial studies were aimed towards finding the suitability of compounds 8 and 9 in forming cross-links with the protein and also whether the cross-linked protein is visible in an SDS PAGE gel under UV-illumination. The compounds at different concentrations were incubated with HCA II for 15 min. The incubated mixtures were then irradiated with UV light of λ 365 nm for 5 min and then directly run on a polyacrylamide gel. Exposure of the gel to UV-transilluminator showed clear fluorescence band for the protein at the expected region which was further confirmed by staining the same gel with Coomassie blue (Fig. 3). The result validated our concept of fluorescence based protein capture technology. We have also inferred that with the current fluorescence probe, one can go down up to a HCA II concentration of 4 μM for the capture to be visible under fluorescence in SDS-PAGE analysis keeping the concentration of the capture compounds at 50 μM.

Once the ability of the compounds 8 and 9 to capture HCA II was established, the next issue was to check their selectivity. To address this issue, the entire process of incubation and photolysis was car-



Scheme 3. Synthesis of capture compounds 8 and 9.

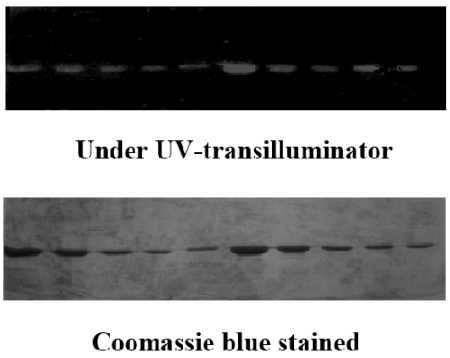


Fig. 3. Result of gel electrophoresis showing capture of HCA II by 8 and 9.

ried with a mixture of three proteins namely HCA II, BSA and Lysozyme. The result was very encouraging; only the fluorescence band corresponding to HCA II could be visible under UV-transilluminator, whereas the Coomassie blue staining of the same gel showed all three protein bands (Fig. 4). The contents in different lanes are shown (the protein concentration in all the experiments was kept at 21 μM). The mass spectrum of the incubated photo-reacted mixture containing three proteins showed the peak at M^+ + capture Compound – N_2 only for HCA-II (Fig. 5).

The experiment was repeated with cell lysate of *E. coli* where the selective capturing of HCA II could be apparent from the gel electrophoresis study (Fig. 6). Thus, both compounds 8 and 9 showed high selectivity towards HCA II which could be nicely demonstrated by our fluorescence based capture (FBC).

(B) 1,2,4-Trisubstituted benzene based systems :

Our next attention was drawn to the design of enediyne based protein capture agents using 1,2,4-trisubstituted benzene as the template. Towards that objective, two enediyne based protein-capture compounds 19 and 20 were synthesized[11]. Like the earlier described systems 8 and 9, both these molecules have an aryl sulfonamide for reversible binding with Human Carbonic Anhydrase II (HCA II) and a pyrene moiety for the visualization of a capture event. While compound 19 has an aryl azide as a photo crosslinking

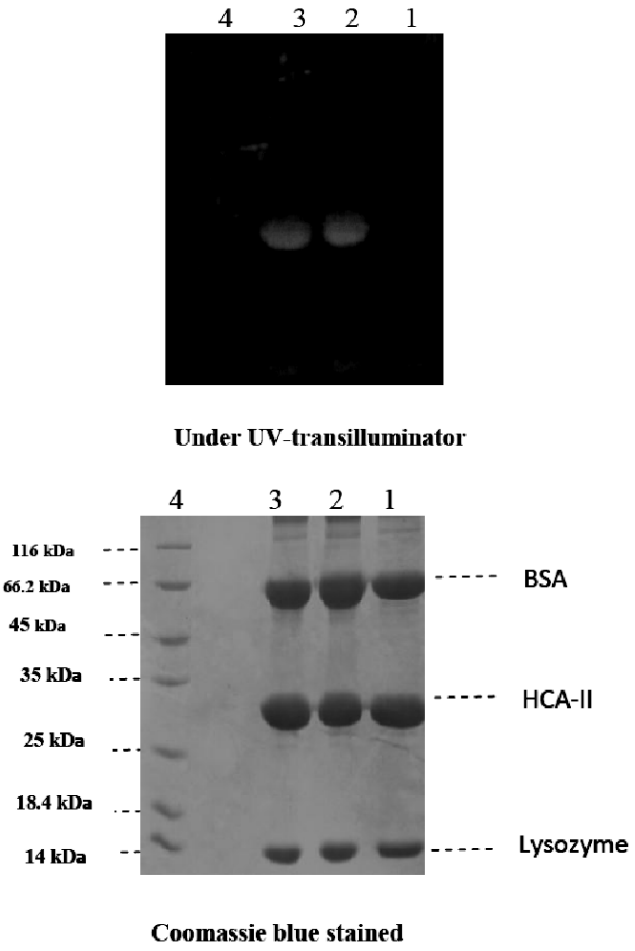


Fig. 4. Result of gel electrophoresis showing capture of HCA II in a mixture by 8 and 9.

agent, compound 20 lacks the azide moiety. The initial basis of our design was to find out whether an enediyne moiety can be used as a template for protein capture. It was hoped that the enediyne framework would allow the photo crosslinking moiety, namely the azide to be in close proximity with the protein surface after the sulfonamide was anchored to the active site of HCA II. Photo-irradiation should yield nitrene which is expected to form cross-links with the protein (Path I). As mentioned, another interesting point worthy of consideration was the possibility of radical mediated protein cleavage once the diradical was generated via photo BC of the enediynes (Path II). A third point to take note of was the reported halide addition to the zwitterionic form of the

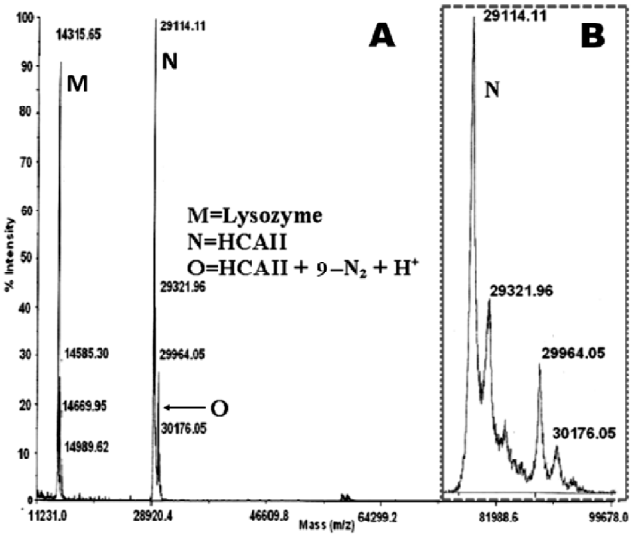


Fig. 5. MALDI spectra : (A) mixture of HCA II, BSA and lysozyme + compound 9, incubated and photo-reacted; (B) expanded spectrum.

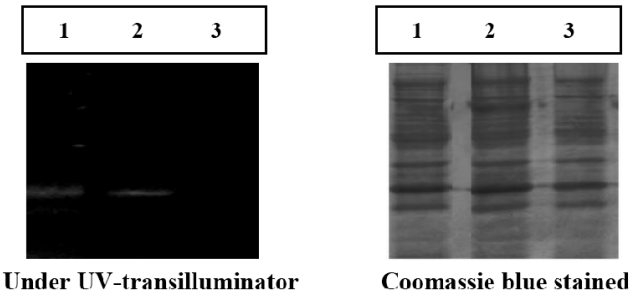
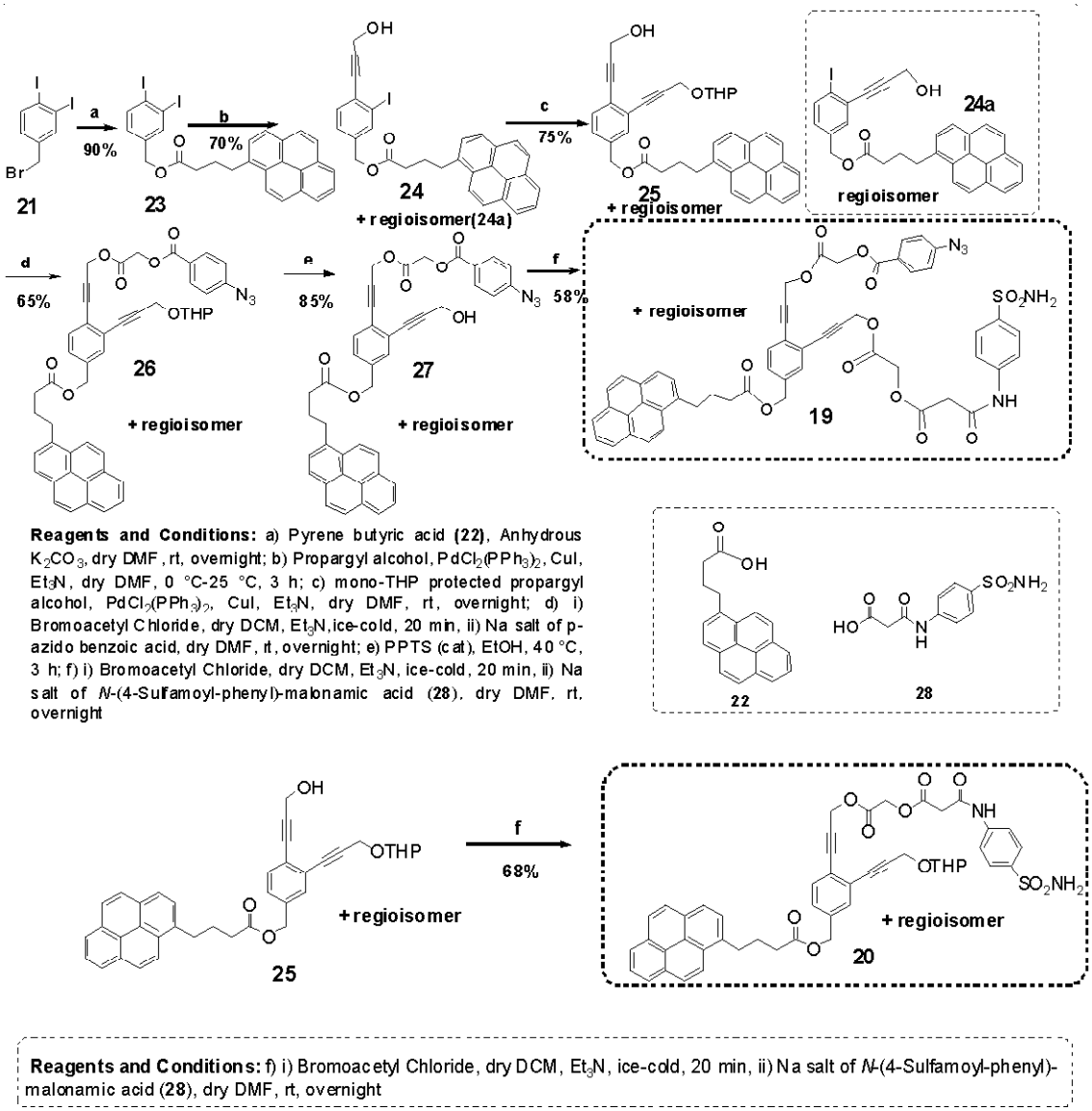


Fig. 6. Gel electrophoresis of capture of HCA II from cell lysate by 8 and 9. Lane 1 : 0.5 mg cell + compound 8 (10 μL, 1 mM) + 90 μL buffer (HEPES); Lane 2 : 0.5 mg cell + compound 9 (10 μL, 1 mM) + 90 μL buffer (HEPES); Lane 3 : 0.5 mg cell + 100 μL buffer.

diradical reported first by Perrin and O'Connor *et al*.[10]. A similar addition of a nucleophilic amino acid might allow the crosslinking of the protein (Path III).

The synthesis of the tripodal compound 19 started from 3,4-diiodo benzyl bromide 21 which was first reacted with the K-salt of pyrene butyric acid 22. The benzyl bromide 21 was synthesized by following a protocol reported earlier. The resulting ester 23 upon Sonogashira coupling[12] with propargyl alcohol maintaining the temperature from initial 0°C to ultimately 25°C over a period of 3 h produced the alcohol 24. At this stage the product was a mixture of inseparable regioisomers which was carried forward to the next sequence of reactions. Compound 24 was again made to undergo Sonogashira coupling with mono-THP protected propargyl alcohol to produce 25. The reaction with bromoacetyl chloride followed by esterification with the Na-salt of 4-azidobenzoic acid incorporated the photoaffinity label (compound 26). The THP group was then removed and the resulting alcohol 27 was similarly esterified via bromoacetyl chloride followed by the Na-salt of malonylsulfanilamide 28 to afford the target molecule 19. The dipodal molecule 20 without the azide handle was synthesized directly from the enediyne mono-protected alcohol 25 via a similar esterification protocol (Scheme 4). The linker groups with the three hands were chosen meticulously to maintain a distance between them and also for proper balancing of hydrophilicity and hydrophobicity. Before embarking upon the actual capture experiment, we evaluated the inhibition potential of the sulfonamides. It was found that compounds 19 and 20 both inhibited HCA II with compound 19 having lower IC50 value of 1.89 μM as against 10.76 μM for 20. The enediyne 25 without any sulfonamide moiety expectedly failed to inhibit the enzyme.

Backed by the inhibition result, we proceeded with the capture experiment. Compound 19 was first evaluated. In a typical experiment, compound 19 and the protein in HEPES buffer and DMSO were incubated for 20 min and then irradiated for 60 min with light of wavelength ≥300 nm. The mixture was then analysed by gel electrophoresis. The gel was visualized under UV illumination and then stained with Coomassie blue. Comparison of the gel pictures gave indication of protein capture. The experiment was then repeated with compounds 20 and 25. Analysis of the gel pictures showed that both compounds 19 and 20 were capable of protein capture (Figs. 7P and 7Q respectively) and compound 20 appeared to have better efficiencies (Fig. 7R). Compound 25 lacking



Scheme 4. Synthesis of enediyne based capture compounds.

the selectivity hand (sulfonamide) expectedly did not show any cross-linking (Lane 1, Fig. 7R). The results demonstrate that the presence of the azide functionality is not mandatory in order to generate cross links as revealed by the capturing of HCA II by compound 20. The enediyne moiety can perform the role of photoaffinity labeling under UV irradiation. Thus pathway C involving photo BC followed by nucleophilic addition as shown in Scheme 2 seemed to be the process occurring under UV-light which was necessary to induce cross-links. This was demonstrated by carrying out the incubation of HCA II with compound 20 without any photoirradiation and expectedly no protein capture was observed in the gel (Lane 1, Fig. 7S), which further confirms that the capture process requires photoirradiation.

Type 2 : Template with built-in photo-affinity and linker hands

In spite of significant progress, the present technology of design of template-based protein labeling probes faces the challenge of assembling of individual units (selectivity, reactivity and visualizing units) on to the template via linkers. In addition, the

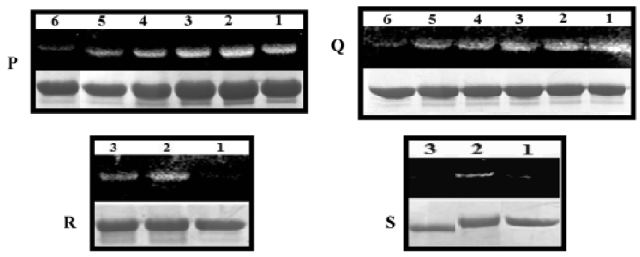


Fig. 7. Cross linking experiments with enediynes : results of gel electrophoresis analysis of HCA II capture as visualized by UV (upper panel) and Coomassie blue (lower panel). (P) Irradiation of reaction mixture containing Lanes 1-5 : HCA II (40 μM) + compound 19 (20, 10, 5, 2.5 and 1.75 μM respectively) and Lane 6 : HCA II (40 μM) + DMSO (2%) as the control. (Q) Lanes 1-5 : HCA II (40 μM) + compound 20 (40, 20, 10, 5, 2.5 μM respectively), Lane 6 : HCA II (40 μM) + DMSO (2%). (R) Lanes 1-3 : HCA II (20 μM) + compounds 3, 20, 19 respectively (each 10 μM). (S) Lanes 1 and 2 : Incubation of 50 μL mixture of HCA II (20 μM) + compound 20 (100 μM) without and with irradiation respectively, Lane 3 : irradiation of mixture HCA II (20 μM) + DMSO (2%). For all experiments HEPES buffer (pH 7.2) was used and total volume of each reaction mixture was kept at 50 μL.

usual practice of employing sterically demanding fluorescent labels like pyrene/fluorescein may seriously hamper the binding to the active site of the target enzyme/receptor and also may create solubility problems. Recently, Schofield *et al*.[13] has simplified the design via a nicely executed 4-component Ugi reaction to make the photo-affinity scaffold. In a departure from Schofield's approach, we felt the complexity involved in the design of the protein capture agents can be simplified if the template itself is inherently fluorescent or becomes fluorescent after cross-linking. The task can be made even simpler, if the template has an in-built photo reactive group along with a linker to attach the selectivity hand. This newly designed sterically less-demanding template will act as a 3-in-1 unit bypassing the present challenge of joining of individual functional hands. The principle of the proposed design of labeling molecule and the earlier adapted ones is shown in Fig. 8.

Based upon the above principle, we have synthesized a series of azidonaphthalimides 30A-I attached with carboxylic acid-based linkers of different lengths and substitution. The azidonaphthalimides are known to undergo high degree of increment of fluorescence intensity upon conversion to an amine (effected chemically or via UV-irradiation[14]). With this expectation, these carboxylic acids were attached to a sulfonamide moiety (well-known reversible inhibitors for CA[15]) to generate compounds 29A-I (Fig. 8). These have shown to label and visualize HCA II with high efficiency. The dependence of cross linking efficiency with the linker length and the side chain (derived from α-amino acids) has also been shown.

A 3-step protocol as shown in Scheme 5 was followed to synthesize of the labeling agents 29A-J starting from the commercially available 4-bromo-1,8-naphthalic anhydride 31. In the first step, the bromide 31 was treated with NaN_3 in moist DMF. The resulting azido anhydride 32 was converted to the naphthalic imide templates with terminal carboxylic acid functionality 30A-I by treatment with various amino acids 34A-I in refluxing dry ethanol[16]. The product, obtained after cooling, was collected by filtration. For the synthesis of HCA II labeling agents 29A-I, the templates 30A-I were treated with bromoacetyl sulfanilamide 33 in presence of K_2CO_3 in dry DMF at room temperature for 8 h. Filtration followed by removal of DMF by lyophilization gave crude mixture from which the products were purified by repeated precipitation from acetone-hexane (for 29A-G) or by column chromatography (for 29H-I). All new compounds were characterized by NMR and HRMS data.

We next proceeded with the labeling experiment. The sulphonamides 29A-I were separately incubated with HCA II for 15 min and then irradiated (> 254 nm) in short pulses for 30 min. The mixture was then analysed by SDS PAGE. The gel was visualised under UV illumination followed by staining with Coomassie blue[17]. Comparison of the gel pictures gave indication of protein labeling. Though all the compounds were able to show efficient cross-linking, the extent varied with the spacer length and nature of α-substituent. Image analysis pattern of the gel pictures using Image J revealed a ~2-fold increase of cross-linking efficiency while going from

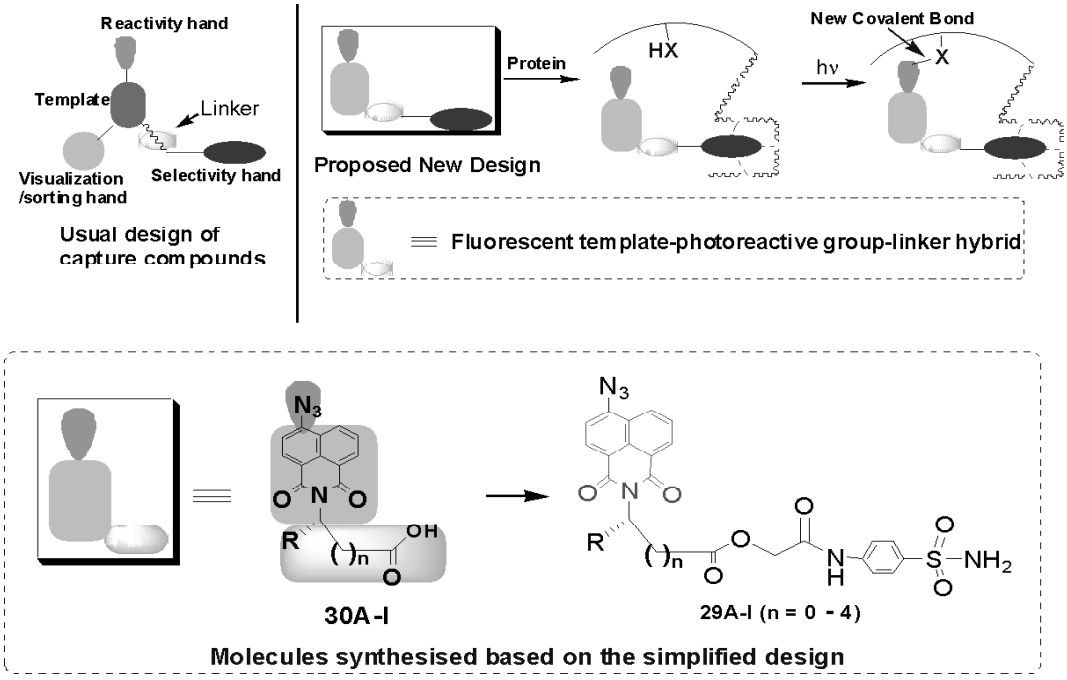
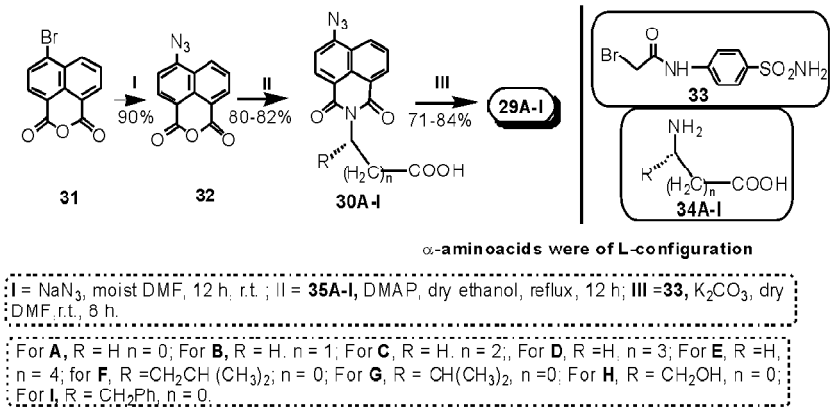


Fig. 8. Improved design of protein labeling agents.



Scheme 5. Synthesis of newly designed capture compounds 29A-I.

one carbon spacer (glycine) to a two carbon β-alanine-based spacer. The cross-linking efficiency (further increased by ~2.5-fold (as compared to 29A) a three carbon spacer (γ-amino butyric acid) which remained more or less the same upon further increase of spacer length. The final order of dependency of cross-linking on the type of spacer was : glycine < β-alanine < γ-amino butyric acid ≈ valeric acid ≈ caproic acid. The relevant gel pictures and the bar diagram showing the cross linking efficiencies are shown in Fig. 9.

Regarding the effect of side chains derived from L-α-amino acids, the cross-linking efficiency is not significantly affected except for phenyl alanine based sulfonamide 29I for which poor cross-linking was observed. The final order of efficiency of cross-linking of amino acid-based linkers was : phenyl alanine << leucine < serine < valine. The evaluated IC_{50} values of the sulfonamides (except 29I) for HCA II were found to be in low micromolar ranges, which in general, is in accordance with high efficiency of cross-linking. The fluctuation of cross-linking effi-

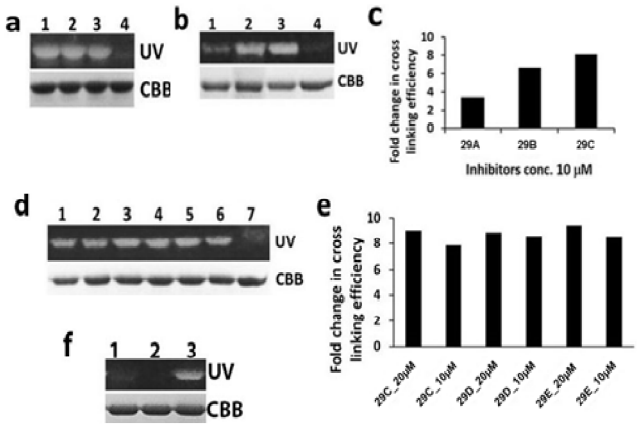


Fig. 9. Results of gel electrophoresis analysis of HCA II-labelling by compounds 29A-E. (a) Lanes 1–3 : Irradiation of a mixture containing HCA II (40 μM) + compound 1B (40, 20, 10 μM respectively) and Lane 4 : HCA II + DMSO (2%) control. (b) Lanes 1–3 : Irradiation of mixture HCA II (20 μM) + compound 29A, 29B, 29C respectively (all at 10 μM) and Lane 4 : HCA II + DMSO (2%) control. (c) Image analysis of Fig. 2b. (d) Lanes 1–6 : Irradiation of a mixture of HCA II (20 μM) + compounds 29C, 29D and 29E (each at 20 and 10 μM concentrations respectively); Lane 7 : HCA II + DMSO (2%) control. (e) Image analysis of Fig. 2d. (f) Irradiation of a mixture containing HCA II (10 μM) + compound 4c (20 μM, Lane 1), HCA II-DMSO control (Lane 2) and 1c (20 μM, Lane 3).

ciency on the nature of linker is probably due to the relative proximity of the azide group with a nearby amino acid side chain capable of cross-linking with a nitrene. To know the site of cross-linking, MALDI MS of tryptic digestion of the protein cross-linked with 29C was compared with that for the native protein digestion after denaturation to find out the site of cross-linking.

Apart from the expected digestion fragments as reported in the literature[18], the cross-linked protein showed additional bands at *m/z* 2914 and 2966 which were absent in the MALDI MS of the tryptic digested fragments of native HCA II. The observed results can be explained if the cross-linking has taken place with the fragment "LIQFHFHWGSLDGQGSEHTVDK" (containing amino acids 90-111) (Mol. wt. 2538). MS/MS analysis on fragment at *m/z* 2966 showed new peaks at *m/z* 1142, 1010, 812 and 651 which indicated that cross-linking has most likely taken place with a glutamine residue Q[19]. Preliminary docking studies (not included) also indicated close proximity of the azide group and the HCA II residue 90-111.

The sulfonamides are also selective against HCA II. In presence of other proteins, these can cross-link and detect the presence of HCA II. This was demonstrated by carrying the cross-linking experiment with a mixture of HCA II, BSA and lysozyme (Fig. 10a) which showed most prominent fluorescent band for HCA II. The experiment was repeated with cell lysates of *E. coli* with over expressed HCA II where the selective capturing of HCA II was clearly apparent by PAGE (Fig. 10b). Thus, the GABA sulphonamide 29C showed high selectivity towards HCA II in the presence of other enzymes demonstrating the suitability of our template for labeling and visualization of over-expressed protein[20].

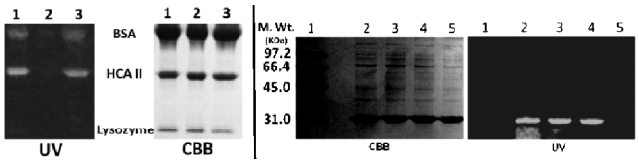


Fig. 10. (a) Capture experiment with a mixture of proteins, namely lysozyme, HCA II and BSA. Lanes 1, 3 : HCA II (20 μM) + compounds 29B and 29A (10 μM) respectively; Lane 2 : HCA II (20 μM) + DMSO (2%) control. (b) Lanes 2–4 : Irradiation of a 50 μL mixture containing cell lysate (10 μL) and compound 29C (final concentrations were 100, 50, 25 μM respectively); Lane 5 : Control without compound; Lane 1 : MW marker.

Conclusion and future perspectives

In conclusion, the design of protein capture compounds has been improved are made simpler over the years. Initially we started with a tripodal template, using a 1,3,5-trisubstituted benzene ring in which the three functionalities, essential for protein capturing were attached. These molecules were able to capture HCA II efficiently. This was followed by the design and synthesis of a enediyne linker based 1,2,4-benzene based capture system. During performing of the capture experiment, it was shown that the enediyne itself can undergo Photo-Bergman Cyclization which leads to cross-linking of an amino acid residue via an anionic addition to *p*-benzyne. How-

ever, the complexity of synthesizing both the systems prohibited us to explore the capture of other proteins. A breakthrough in this direction was achieved by using a turn-on fluorescent template with a built in photoaffinity functionality and a linker to serve as protein capture agent. Various selectivity hand depending on the target protein can be attached via a simple esterification, hydroxamate etc. In future this template will be used to screen possible inhibitors again some disease causing enzymes.

References

1. (a) D. J. Lapinsky, *Bioorg. Med. Chem.,* 2012, 20, 6237; (b) J. Sumranjit, and J. C. Sang, *Molecules*, 2013, 18, 10425.
2. (a) "Concepts of genetics", 10th ed., Pearson Education, San Francisco, 2012; (b) B. M. Kadakkuzha and S. V. Puthanveettil, *Molecular BioSystems,* 2013, 9, 1807.
3. (a) P. P. Geurink, M. M. Prely, G. A. van der Marel, R. Bischoff and H. S. Overkleeft, *Top. Curr. Chem.,* 2012, 324, 85; (b) L. I. Willems, W. A. van der Linden, N. Li, K. Y. Li, N. Liu, S. Hoogendoorn, G. A. van der Marel, B. I. Florea and H. S. Overkleeft, *Acc. Chem. Res.,* 2011, 44, 718 and references therein; (c) W. P. Heal, T. H. T. Dang and E. W. Tate, *Chem. Soc. Rev.,* 2011, 40, 246.
4. (a) G. Dorman and G. D. Prestwich, *Trends Biotechnol*., 2000, 18, 64; (b) Y. Hatanaka and Y. Sadakane, *Curr. Top. Med. Chem.,* 2002, 2, 271.
5. (a) E. Leyva, D. de Loera and S. Leyva, *Tetrahedron Lett.,* 2008, 49, 6759; (b) Q. Peng, Y. Xia, F. Qu, X. Wu, D. Campese and L. Peng, *Tetrahedron Lett.,* 2005, 46, 5893; (c) M. S. Platz, *Acc. Chem. Res.,* 1995, 28, 487.
6. (a) L. Dubinsky, B. P. Krom and M. M. Meijler, *Bioorg. Med. Chem.,* 2012, 20, 554; (b) M. Hashimoto and Y. Hatanaka, *Eur. J. Org. Chem.,* 2008, 2008, 2513.
7. R. E. Galardy, L. C. Craig and M. P. Printz, *Nature New Biol*., 1973, 242, 127.
8. P. S. Addy, B. Saha, N. D. P. Singh, A. K. Das, J. T. Bush, C. Lejeune, C. J. Schofield and A. Basak, *Chem. Commun*., 2013, 49, 1930.
9. (a) A. Evenzahav and N. J. Turro, *J. Am. Chem. Soc.,* 1998, 120, 1835; (b) N. Choy, B. Blanco, J. Wen, A. Krishnan and K. C. Russell, *Org. Lett*., 2000, 2, 3761; (c) G. V. Karpov and V. V. Popik, *J. Am. Chem. Soc*., 2007, 129, 3792; (d) A. Poloukhtine and V. V. Popik, *J. Org. Chem*., 2006, 71, 7417; (e) D. R. Pandithavidana, A. Poloukhtine and V. V. Popik, *J. Am. Chem. Soc.,* 2009, 131, 351; (f) R. K. Mohamed, P. W. Peterson and I. V. Alabugin, *Chem. Rev*., 2013, 113, 7089.
10. (a) B. L. Rodgers, J. M. O'Connor and C. L. Perrin, *J. Am. Chem. Soc.,* 2007, 129, 4795; (b) P. W. Peterson, R. K. Mohamed and I. V. Alabugin, *Eur. J. Org. Chem*., 2013, 2505.
11. J. Das, S. Roy, S. Halnor, A. K. Das and A. Basak, *Org. Biomol. Chem*., 2017, 15, 1122.
12. (a) R. Chinchilla and C. Nájera, *Chem. Soc. Rev.,* 2011, 40, 5084; (b) R. Chinchilla and C. Nájera, *Chem. Rev.,* 2007, 107, 874; (c) S. Mandal, S. S. Bag and A. Basak, *Chem. Rev.,* 2003, 103, 4077; (d) M. Kar and A. Basak, *Chem. Rev.,* 2007, 107, 2890.
13. J. T. Bush, L. J. Walport, J. F. McGouran, I. K. H. Leung, G. Berridge, S. S. van Berkel, A. Basak, B. M. Kessler and C. J. Schofield, *Chem. Sci*., 2013, 4, 4115
14. The azidonaphthalimides are known to undergo a high degree of fluorescence intensity increment upon conversion to amines (done chemically or via UV-irradiation). For reference, see : (a) L. Zhang, S. Li, M. Hong, Y. Xu, S. Wang, Y. Liu, Y. Qian and J. Zhao, *Org. Biomol. Chem*., 2014, 12, 5115; (b) Y. Guo, T. Zeng, G. Shi, Y. Cai and R. Xiec, *RSC Adv*., 2014, 4, 33626; (c) Q. Xu, L. He, H. Wei and W. Lin, *J. Fluoresc.,* 2015, DOI: 10.1007/s10895-015-1582-5.
15. (a) A. Scozzafava, A. Casini and C. T. Supuran, *Med. Res. Rev*., 2003, 23, 146; (b) D. Vullo, G. Manole, A. Casini, A. Scozzafava and C. T. Supuran, *Curr. Med. Chem.,* 2004, 2, 49; (c) C. T. Supuran, *Nat. Rev. Drug Discovery*, 2008, 7, 168; (d) J. Y. Winum, A. Scozzafava, J. L. Montero and C. T. Supuran, *Mini Rev. Med. Chem.,* 2006, 6, 921; (e) J. Y. Winum, A. Scozzafava, J. L. Montero and C. T. Supuran, *Med. Res. Rev*., 2005, 25, 186; (f) A. Maresca, C. Temperini, H. Vu, N. B. Pham, S. A. Poulsen, A. Scozzafava, R. J. Quinn and C. T. Supuran, *J. Am. Chem. Soc*., 2009, 131, 3057.
16. Y. Cai, J. Zhan, H. Shen, D. Mao, S. Ji, R. Liu, B. Yang, D. Kong, L. Wang and Z. Yang, *Anal. Chem*., 2016, 88, 740.
17. It may be mentioned that no cross-linking was observed without any UV-exposure.
18. Web.expasy.org/peptide_mass/. The entered protein was CAH2_HUMAN (P00918) from Uni Prot KB/Swiss-Prot and the selected enzyme was trypsin.
19. Nitrene insertion to an amide NH has been reported : L. M. Gornostaev and T. I. Lavrikova, *Zh. Org. Khim*., 1984, 20, 874.
20. M. Singha, S. Roy, S. D. Pandey, S. S. Bag, P. Bhattacharya, M. Das, A. S. Ghosh, D. Ray and A. Basak, *Chem. Commun.*, 2017, 53, 13015.