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PAPER

Fast and efficient MCR-based synthesis of clickable rhodamine tags for protein profiling†

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Protein profiling probes are important tools for studying the composition of the proteome and as such have contributed greatly to the understanding of various complex biological processes in higher organisms. For this purpose the application of fluorescently labeled activity or affinity probes is highly desirable. Especially for *in vivo* detection of low abundant target proteins, otherwise difficult to analyse by standard blotting techniques, fluorescently labeled profiling probes are of high value. Here, a one-pot protocol for the synthesis of activated fluorescent labels (*i.e.* azide, alkynyl or NHS), based on the Ugi-4-component reaction (Ugi-4CR), is presented. As a result of the peptoidic structure formed, the fluorescent properties of the products are pH insensitive. Moreover, the applicability of these probes, as exemplified by the labeling of model protein BSA, will be discussed.

Introduction

The term *proteome*, comprising the complete set of expressed proteins of a prokaryotic and eukaryotic genome origin, was first introduced in the mid 1990s.[1] Since then the investigation of the composition of the proteome has become a fast developing scientific field at the interface of bioinformatics, chemical and biological sciences. Based on the obtained information of these combined fields, a better understanding of complex biological processes in cells and tissues, such as protein–protein interactions and altering protein activity by post-translational modifications (*e.g.* glycosylation, phosphorylation and prenylation), has been achieved. Moreover, the combination of fields has strongly propelled the development of new biomarkers for early diagnoses of diseases and the discovery of new drug target proteins associated with specific physiological states.[2–4]

For the analysis of the proteome different approaches have been developed. The classical approach involves the separation of proteins of whole cell lysate by two-dimensional gel electrophoresis (2D-PAGE), followed by the analysis of the separated protein spots by mass spectrometric techniques.[5] This approach, however, suffers some significant drawbacks, such as the inability of analysing low-abundant or membrane-bound proteins.[6] Consequently, Cravatt and co-workers, among others, proposed a more chemical approach termed *activity-based protein profiling* (ABPP). In general the probes applied in ABPP consist of two (or three) functionalities, depending on the type of proteins studied. The primary element is the *selectivity group* for specific target recognition (*e.g.* reversible/irreversible inhibitor, substrate or cofactor) see Fig. 1. A *reactive group* is installed for the covalent attachment of the (reversible) probe to the target protein (generally comprised of electrophilic or photoreactive groups). Finally, the ABPP probe is decorated with a *reporter-tag* for target detection or purification purpose (*e.g.* fluorophores or biotin).[7]

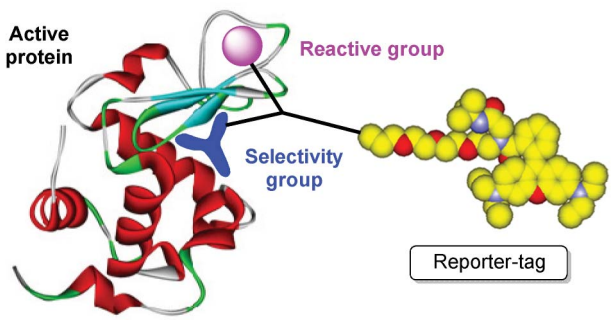

Fig. 1 General build-up of an ABPP probe.

As a result of the high molecular mass of these chemical probes, the cellular uptake and distribution is often hampered, thus limiting the *in vivo* applications. To overcome these limitations a "tag-free" activity-based probe (ABP) was developed. By providing a probe-head, consisting of a selectivity and a reactive group, and a reporter-tag, both carrying a bioorthogonal functional handle, conjugation of both the probe-head and the reporter tag can be

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achieved. This then allows for the detection of the covalently attached probe-head to the protein. Two commonly used post-target conjugation strategies are the copper-catalyzed [3 + 2] azide–alkyne cycloaddition (CuAAC)[8] and the Staudinger ligation.[9]

The multifaceted demands for a reporter tag include: high reactivity, easily obtainable, highly flexible in terms of linker-design, and compatibility under various biological conditions (*e.g.* different pH-values).

In this paper we describe the synthesis of rhodamine containing reporter-tags, carrying different bioorthogonal functional handles, such as linear alkynes, strained alkynes and azides. In addition, the broad applicability of the protocol presented here is extrapolated to the synthesis of other activated reporter tags, *i.e.* NHS-esters (*N*-hydroxysuccinimide). The selection of rhodamine was based on the advantages over other fluorescent dyes, *e.g.* high photostability, good membrane permeability, and good commercial availability. However, the major drawback of working with rhodamine and its derivatives is the pH-sensitivity. Secondary amides of rhodamine tend to undergo intramolecular cyclization leading to the leuko-form (Fig. 2). To avoid the loss of fluorescence, tertiary amides, produced *via* multi-step approaches,[10] are commonly applied. Examples of such modifications of rhodamine and their applications in the labelling of biomolecules have been reviewed by Beija *et al.*[11] and Gonçalves.[12]

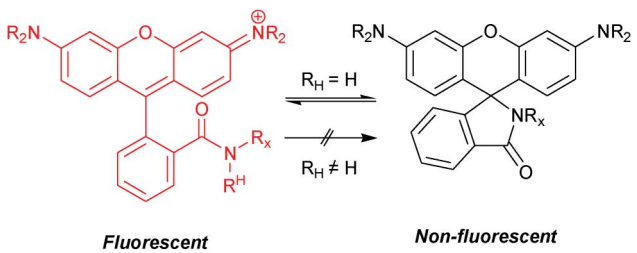


Fig. 2 Cyclization leading to a non-fluorescent leuko-form of rhodamine.

For a fast, high yielding and structurally versatile synthesis of tertiary amide rhodamine probes we envisioned the application of one step, one pot, multicomponent reactions (MCR), more specifically the Ugi-4-component reaction (Ugi-4CR).[13] To the best of our knowledge this approach was considered only once before (in this particular case rhodamine B was reacted with amine functionalities of proteins *via* a direct multicomponent reaction).[14]

The reporter tags obtained by the herein reported MCR approach can be synthesised *via* an efficient protocol using no additional reagents and producing water as the sole by-product. Moreover, the formation of a tertiary amide bond, inherent to all Ugi reactions, is expected to reduce the pH-sensitivity of the rhodamine-based reported tags.

Results and discussion

The introduction of the Ugi-4CR as the key step in the synthesis of functional rhodamine probes has significant advantages over other synthetic approaches: (1) simple reaction protocols; (2) by varying the individual components a library of structurally diverse reporter tags can be synthesised giving fast access to the most suitable tags; (3) no use of additional toxic and expensive coupling reagents is required and; (4) high flexibility of the Ugi-4CR concerning availability, or synthetic accessibility, of different starting materials.

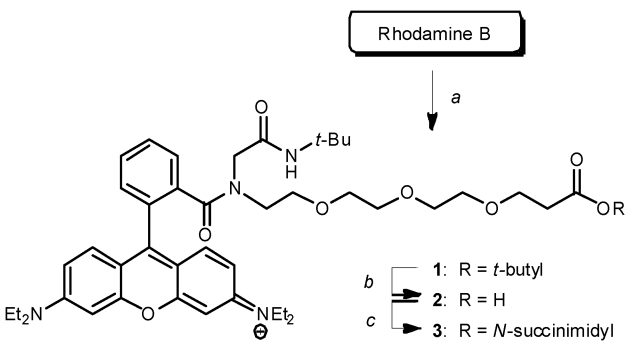
Most of the Ugi-reactive building blocks used in this project were obtained from commercial sources. The PEGylated azide building blocks, on the other hand were synthesised starting from ethylene glycols possessing different chain lengths in order to affect the water solubility of the resulting compounds.[15]

The design of specific rhodamine probes was based on our interest in constructing probes having either reactive groups for direct coupling to biomolecules or having bioorthogonal functional handles allowing fast post-functionalisation using either CuAAC or SPAAC.

Synthesis of rhodamine-based reporter-tags

NHS-reactive rhodamine probe. To covalently modify proteins in biological samples, side chain selective modification reagents are necessary. These reagents should react under specific conditions with a pre-targeted single, or limited number of, side chains. The most commonly used reactive groups in the chemical modification of proteins are amine selective reagents (*e.g. N*-hydroxysuccinimide esters, imidoesters and activated aryl fluorides) and compounds which show a high reactivity towards thiol groups (maleimides and α-halocarbonyl compounds).[16]

Synthesis of an NHS-containing rhodamine chemical probe (*e.g.* **3**), can be accessed *via* a three step procedure starting from commercially available starting materials (Scheme 1).



Scheme 1 Ugi-derived NHS-rhodamine probe. *Reagents and conditions*: (a) paraformaldehyde, *tert*-butyl 12-amino-4,7,10-trioxadodecanoate, *tert*-butyl isocyanide, rhodamine B, MeOH, μW (100 °C, 1 h), (63%); (b) H_3PO_4 (85 v/v%), toluene, RT, 3 h, (65%); (c) *N*-hydroxysuccinimide, DMAP, EDCI, CH_2Cl_2, RT, 24 h, (75%).

The combination of paraformaldehyde, *tert*-butyl 12-amino-4,7,10-trioxadodecanoate, *tert*-butyl isocyanide and rhodamine B in the Ugi-4CR led to the *tert*-butyl ester protected precursor **1** in 63% yield. The reaction was performed in methanol under microwave-heating conditions (during our studies we observed that employing microwave heating speeds up the reaction tremendously while leaving the yield of the reaction unaffected). After subsequent deprotection with phosphoric acid in toluene,[17] carboxylic acid **2** was transformed into the activated NHS-ester **3** by treatment with *N*-hydroxysuccinimide, under standard coupling conditions. After aqueous workup the product was isolated in 75% yield.

To test the applicability of the obtained rhodamine probe **3** in the functionalisation of proteins, purified BSA (bovine serum

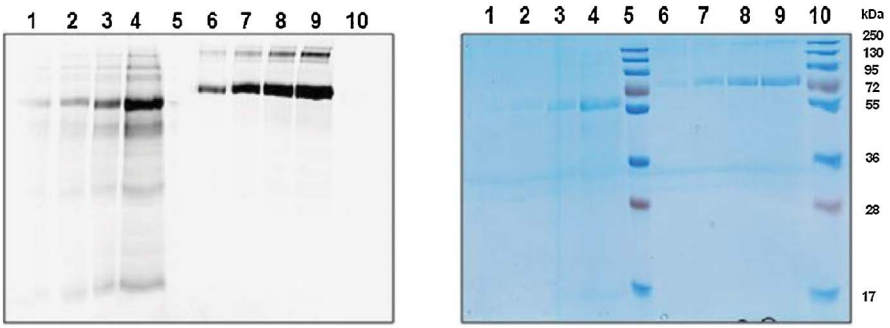
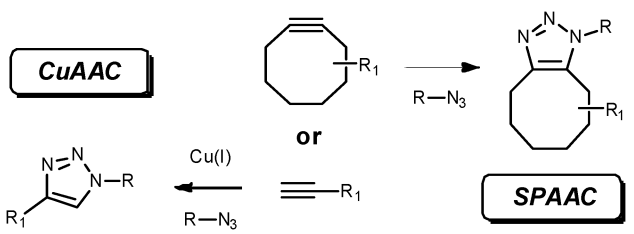


Fig. 3 SDS-PAGE gel of labelled BSA and a crude protein mixture (*A. thaliana* (Col-0)), visualised by in-gel fluorescence-scanning (left) or *via* Coomassie-staining (right).

albumin, ~66.8 kDa) and a crude extract of *A. thaliana* (Col-0) were incubated with NHS-ester **3**. The samples were run on SDS-PAGE gels and subsequently analysed by means of fluorescence scanning and Coomassie-blue staining (Fig. 3). As expected compound **3** covalently attached to purified BSA. Moreover, in comparison with the Coomassie-blue stained gel, the application of this fluorescence probe is more sensitive for detecting labelled proteins than the commonly used staining technique. This is additionally confirmed by labelling a crude extract with compound **3**. In this case protein bands are only visible in the Coomassie-blue stained gel at the highest protein concentration applied, whereas in the fluorescence scan clearly several protein bands became visible at the lowest concentration tested (presumably mostly RuBisCO (~53 kDa)).

However, for selective modification of biomolecules this approach is rather unsuitable, due to the multitude of competing nucleophiles in complex biological samples. This lack of selectivity could be overcome by the introduction of an azide as a bioorthogonal handle. In the past decade several reactions and suitable reagents were developed to react with azides under physiological conditions. Examples of such reactions are (i) the Staudinger ligation,[18] using substituted phosphine reagents, (ii) the copper-catalyzed [3 + 2] azide–alkyne cycloaddition reaction (CuAAC),[19] and (iii) the strain-promoted azide–alkyne cycloaddition reaction (SPAAC) making use of cyclooctyne reagents.[20,21]

"Clickable" rhodamine derivatives. The copper catalysed regioselective cycloaddition of an azide and alkyne leading to 1,4-substituted 1,2,3-triazoles, independently developed by Sharpless[22] and Meldal[23] (Scheme 2), has frequently been applied as a tool for the modification of proteins and other biomolecules.



Scheme 2 Formation of 1,2,3-triazoles using CuAAC and SPAAC.

However this coupling method is strongly dependent on the presence of a copper catalyst, which is a significant drawback in its application *in vivo*, due to the toxicity of copper(I). Consequently, Bertozzi and co-workers developed a class of highly strained cyclooctynes, which react rapidly with azides without any additional promoter species.[24] This reaction (*i.e.* SPAAC, Scheme 2) has found widespread application in the field of chemical biology.[25]

Applying the Ugi-4CR, rhodamine derivatives were synthesised in a one-step procedure, carrying an azide or alkyne functionality (Scheme 3). The azide bearing compounds **4a** and **4b**, which differ only in the number of ethylene glycol units, were synthesised in the same manner as compound **1** using the Ugi-4CR. Paraformaldehyde, isopropylamine, rhodamine B and 1-azido-11-isocyano-3,6,9-trioxaundecane[15] or 1-azido-17-isocyano-3,6,9,12,15-pentaoxaheptadecane[15] were combined to give azido-rhodamines **4a** and **4b** in 74% and 70% yield respectively. Alkyne-modified rhodamine **5** was generated by varying the starting materials of the Ugi-4CR. To this end acetylene-PEG_4-amine served as the amine instead of isopropylamine and *tert*-butyl isocyanide was used as the isocyanide component to generate alkyne **5** in a satisfying yield of 61%. Using a dibenzoazacyclooctyne[26] derivative, a strained clickable rhodamine-derivative was prepared in 73% yield (**6**). Currently, potential applications of these "clickable" rhodamine tags (*e.g.* capturing phosphorylated proteins) are under investigation in our laboratories.

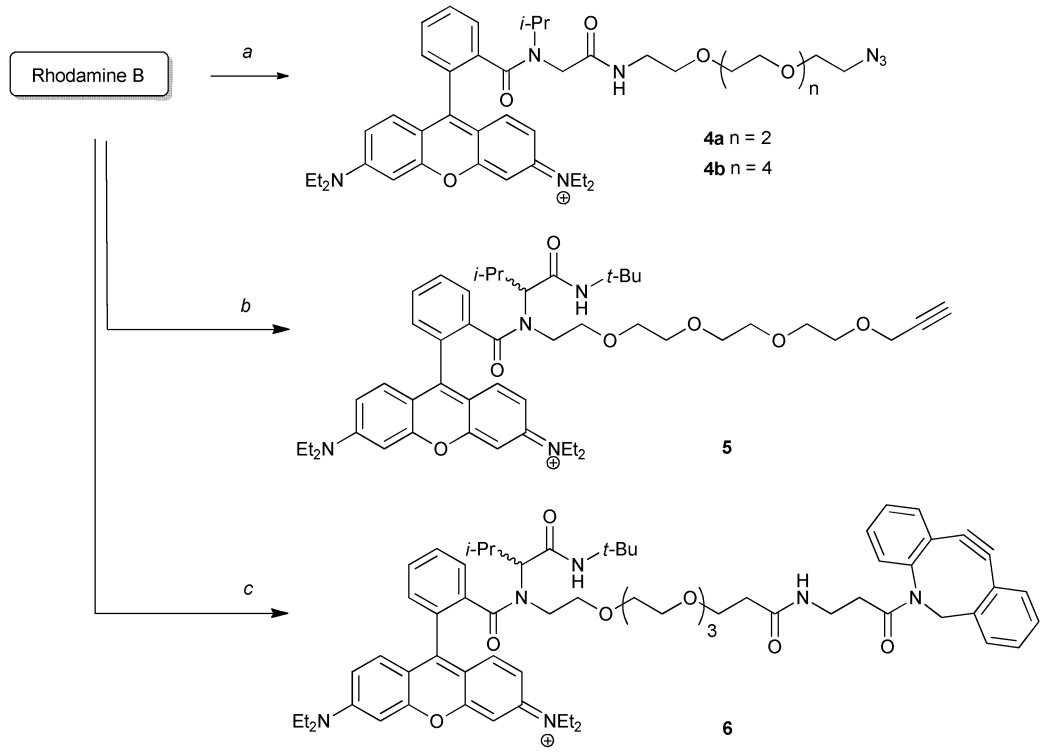
Fluorescent properties of Ugi-modified rhodamine dyes

To prove the proposed pH-independence of the Ugi-rhodamine dyes, compound **4b** as a model compound was dissolved in Britton–Robinson buffer and the pH was adjusted with NaOH solution (0.2 M), see Fig. 4. During the experiment no disappearance of fluorescence was observed at the different pH-values tested. This was previously observed for compounds having a secondary amide bond under basic conditions.[27]

Moreover, we observed an increase in fluorescence intensity under acidic conditions due to the degree of protonation and hence alteration of the electron-donating properties of the tertiary amines.[28] Consequently, this change in fluorescence intensity disappears at neutral respectively basic conditions.

Versatility of the Ugi-4CR in modifying rhodamine dyes

To test the versatility of the presented Ugi-protocol other rhodamine dyes were modified *via* the Ugi-4CR (Scheme 4). For this purpose we chose the most commonly used rhodamine dyes,



Scheme 3 Ugi-derived rhodamine clickable probes. *Reagents and conditions*: (a) paraformaldehyde, isopropylamine, 1-azido-11-isocyano-3,6,9-trioxaundecane (or 1-azido-17-isocyano-3,6,9,12,15-pentaoxaheptadecane), rhodamine B, MeOH, μW (100 °C, 1 h), for **4a** (74%) and **4b** (70%); (b) isobutyraldehyde, acetylene-PEG_4-amine, *tert*-butyl isocyanide, rhodamine B, μW (100 °C, 1 h), (61%); (c) isobutyraldehyde, dibenzylcyclooctyne-PEG_4-amine, *tert*-butyl isocyanide, rhodamine B, MeOH, RT, 24 h, (73%).

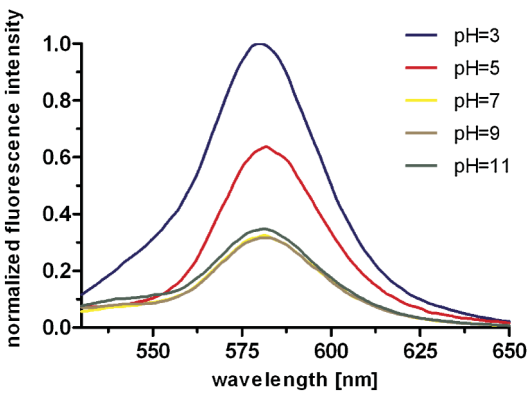
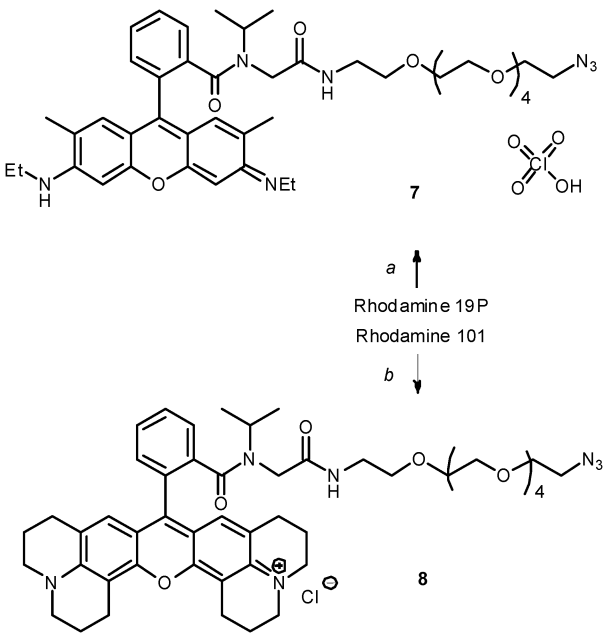


Fig. 4 Fluorescent intensity of **4b** as a function of pH. Spectra were taken in a BR-buffer solution (10 mM) at an excitation wavelength of 510 nm (sample concentration of **4b**: 0.1 μM).

i.e. rhodamine 19P and rhodamine 101. Each individual dye was reacted with paraformaldehyde, isopropylamine and 1-azido-17-isocyano-3,6,9,12,15-pentaoxaheptadecane similar to the protocol applied for the synthesis of **4b**. The yields of the reactions varied from good (81% for rhodamine 19P) to unsatisfying (24% for rhodamine 101).

This dramatic decrease in yield may be attributed to the inner salt form of rhodamine 101, which makes an additional protonation step necessary. Nevertheless, with these results in hand it becomes apparent that the Ugi-4CR is a suitable protocol for the modification of a wide range of rhodamine dyes bearing a free carboxylic acid moiety (*e.g.* rhodamine 110, rhodamine 116, *etc.*).



Scheme 4 Modification of related rhodamine dyes. *Reagents and conditions*: (a) paraformaldehyde, isopropylamine, 1-azido-17-isocyano-3,6,9,12,15-pentaoxaheptadecane, rhodamine 19P, MeOH, (81%); (b) paraformaldehyde, isopropylamine, 1-azido-17-isocyano-3,6,9,12,15-pentaoxaheptadecane, rhodamine 101, MeOH, μW (100 °C, 1 h), (24%).

The corresponding fluorescence spectra of compounds **7** and **8** were recorded under physiological conditions (Fig. 5). From

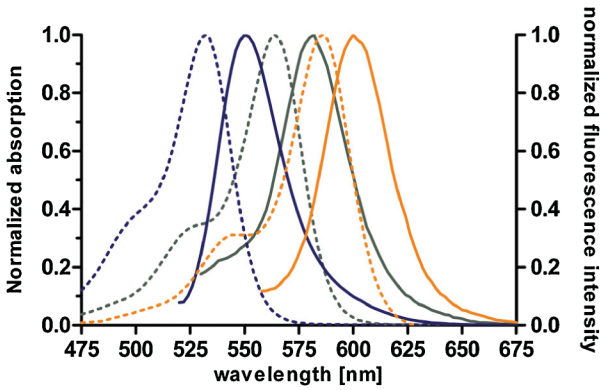


Fig. 5 Normalized absorption (dashed line) and fluorescence emission (solid line) spectra of a 0.1 μM solution containing compound **4b** (green), **7** (blue) and **8** (orange) in BR-buffer solution (10 mM, pH = 7).

Table 1 Spectral properties of rhodamine B, **4b**, **7** and **8**[a]

Compound	λ_{abs}/nm	λ_{em}/nm	ε/M cm^{-1}
Rhodamine B	554	576	87 013
4b	564	582	67 093
7	532	550	71 751
8	586	600	66 823

[a] Sample concentration 0.1 μM in BR-buffer solution (10 mM).

these spectra the spectral properties corresponding to compounds **4b**, **7** and **8** were determined (Table 1). Compared to unmodified rhodamine B the absorption and fluorescence maxima of **4b** were almost unaffected by chemical modification of the rhodamine scaffold (bathochromic shift). The same is true for compounds **7** and **8** compared to already published data.[29]

Conclusions

The facile modification of rhodamine-based fluorophores, introducing multiple functionalities, is of significant importance in the field of chemical biology. Therefore, easy, fast and reliable techniques to synthesise such compounds are highly appreciated. Different approaches have been developed using multi-step procedures,[11] whereas applying multicomponent reactions have not been applied for this purpose. Using the well-documented Ugi-4CR for the modification of rhodamine B we were able to produce a reactive fluorescent probe carrying *N*-hydroxysuccinimide ester suitable for subsequent amine coupling reactions. The reactivity of this probe was demonstrated by the efficient coupling of purified protein BSA under physiological conditions.

In addition, rhodamine B was efficiently modified *via* the Ugi multicomponent reaction, introducing azides (**4a**, **4b**) and alkynes (**5**, **6**). These ready-to-use fluorescent tags can be applied in various *in vivo* applications, such as activity-based protein profiling or labelling of azide respectively alkyne modified biomolecules either *via* CuAAC or SPAAC.[21]

In this paper we focused on the modification of rhodamine dyes by means of the Ugi-4CR, however, other affinity labels such as biotin have been demonstrated to be swiftly incorporated by this method as well (results not included).

In general, we demonstrated the successful application of the Ugi-4CR as a powerful tool for the preparation of functional fluorescent probes. This fast, efficient, facile and diverse method for producing probes for protein profiling can be performed by inexperienced chemists or biologists, thus allowing a broad scientific audience to make use of this methodology. In our opinion the application of multicomponent reactions will become increasingly important within the field of chemical biology. Investigations regarding the synthesis of chemical probes for proteomic studies are currently underway in our laboratories.

Experimental section

General experimental information

Unless otherwise stated all chemicals and solvents were obtained commercially and were used without further purification. All ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on either a 300 MHz Varian MERCURY-VX 300 apparatus (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR, respectively) or on a 400 MHz Varian MERCURY-VX 400 apparatus (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, respectively). Chemical shifts are reported in δ values (ppm) with tetramethylsilane (TMS) as internal standard. ESI-MS spectra were obtained from an API-150EX spectrometer. HRMS spectra were recorded on an FT-ICR Bruker Apex III 70e mass spectrometer. Absorption spectra were recorded on a Jasco V-560 UV/VIS spectrophotometer. Fluorescence was measured on a Perkin Elmer Luminescence Spectrometer LS50B. Reactions under microwave heating were performed in an Emrys Microwave reactor (Biotage). Purification of the crude products by column chromatography was performed on silica gel 60 (230–400 mesh, 0.040–0.063 mm), Merck, Germany. TLC identification of products and reactants were performed on silica gel coated aluminium foil (silica gel 60 F_{254} with fluorescence indicator), Merck, Germany. Acetylene-PEG_4-amine was purchased from Jena Bioscience and dibenzylcyclooctyne-PEG_4-amine from Click Chemistry Tools.

Protein labeling and analysis. For *in vitro* protein labeling 50 μg protein were dissolved in a 10 μL buffer solution, containing aqueous urea buffer (8 M) and Tris-buffer (30 mM), adjusted to pH = 8.5 to ensure effective labelling. After addition of 1 μL of NHS ester **3** (400 pmol μL^{-1}, DMF) samples were incubated for 30 min on ice in a dark environment. The labelling reaction was stopped by addition of 1 μL lysine solution (10 mM, pH 9.0) and further incubation for 10 min on ice in the dark. Separation of the proteins was carried out by SDS-PAGE. Therefore Laemmli-buffer was added and the samples were denatured for 5 min at 95 °C. The SDS-PAGE was performed according to the Biorad instruction manual using a Protean 3 cell, a 12% gel and a continuous buffer system. After the subsequent washing of the gel (three times in water for 1–2 min) the labelled proteins were visualised by in-gel fluorescence scanning using a Typhoon 9410 Imager (GE Healthcare). The gel was stained with Coomassie afterwards.

General procedure A: Ugi-4CR

A solution of aldehyde (1 equiv.) and primary amine (1 equiv.) in methanol was stirred at room temperature for 2 h to preform the

imine. After the addition of isocyanide (1 equiv.) and carboxylic acid (1 equiv.) the resulting mixture was allowed to stir at room temperature overnight. The solvent was evaporated and the residue was purified by column chromatography (CC).

General procedure B: microwave-assisted Ugi-4CR

Imine formation was performed as described in the general procedure above. Subsequently isocyanide (1 equiv.) and carboxylic acid (1 equiv.) were added and the resulting mixture was heated in the microwave (1 h, 100 °C). After evaporation of all volatile material the residue was purified by column chromatography.

***N*-[9-(2-{[2-(*tert*-Butylamino)-2-oxoethyl](14,14-dimethyl-12-oxo-3,6,9,13-tetraoxapentadec-1-yl)carbamoyl}phenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene]-*N*-ethylethanaminium chloride (1).** According to general procedure B: paraformaldehyde (30.0 mg, 1.00 mmol), *tert*-butyl 12-amino-4,7,10-trioxadodecanoate (357 mg, 1.03 mmol), *tert*-butyl isocyanide (0.12 mL, 1.06 mmol), rhodamine B (481.2 mg, 1.00 mmol) in methanol (3 mL) were reacted. CC purification using a gradient of CH_2Cl_2/MeOH afforded **1** as a dark purple semi-solid (540 mg, 63%). R_f 0.28 (CH_2Cl_2/MeOH 9 : 1); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.33 (t, J = 7.0 Hz, 12H), 1.41 (s, 9H), 1.44 (s, 9H), 2.49 (t, J = 6.7 Hz, 2H), 3.07 (t, J = 6.9 Hz, 2H), 3.28 (t, J = 5.1 Hz, 2H), 3.31–3.40 (m, 2H), 3.46 (t, J = 5.0 Hz, 2H), 3.50–3.64 (m, 10H), 3.65–3.73 (m, 4H), 4.36 (s, 2H), 6.67 (s, 2H), 7.27–7.31 (m, 1H), 7.34–7.45 (m, 3H), 7.55–7.60 (m, 2H), 7.87 (dd, J = 5.7, 3.1 Hz, 1H), 8.64 (s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 12.6, 28.0, 28.6, 36.2, 45.1, 46.0, 51.2, 54.0, 66.8, 67.0, 69.9, 70.1, 70.2, 70.3, 95.5, 113.9, 127.8, 129.1, 129.2, 129.8, 130.7, 132.4, 136.9, 155.6, 156.7, 157.6, 167.6, 169.2, 170.8; ESI-MS m/z: 815.6 $[M]^+$; HRMS: Calcd for $C_{47}H_{67}O_8N_4$ 815.4953, found 815.4939.

***N*-[9-(2-{[2-(*tert*-Butylamino)-2-oxoethyl](2-{2-[2-(2-carboxyethoxy)ethoxy]ethoxy}ethyl)carbamoyl}phenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene]-*N*-ethylethanaminium chloride (2).** To a solution of **1** (101 mg, 0.12 mmol) in toluene (2 mL) phosphoric acid (85% v/v, 0.04 mL, 0.59 mmol) was added and the reaction mixture was stirred at room temperature for 3 h. After ESI-MS indicated completion of the reaction, distilled water (20 mL) was added and the aqueous phase was washed with ethyl acetate (3 × 20 mL). The aqueous phase was subsequently saturated with solid NaCl and extracted with isopropanol/dichloromethane (2 : 1 v/v). The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was taken up in a small volume of $CHCl_3$ and filtered to remove residual inorganic salts. Evaporation of the filtrate afforded the free carboxylic acid as a dark purple oil, which was used directly in the next step without further purification (62.3 mg, 65%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.33 (t, J = 6.9 Hz, 12H), 1.40 (s, 9H), 2.66 (t, J = 6.0 Hz, 2H), 3.11 (t, J = 5.9 Hz, 2H), 3.18–3.24 (m, 2H), 3.37–3.50 (m, 4H), 3.52–3.81 (m, 14 H), 4.28 (s, 2H), 6.68 (s, 2H), 7.26–7.31 (m, 2H), 7.38 (d, J = 9.4 Hz, 2H), 7.59 (dd, J = 5.3, 3.5 Hz, 2H), 7.86 (dd, J = 5.7 3.1 Hz, 1H), 7.95 (br s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 12.7, 28.7, 35.6, 45.5, 46.0, 51.3, 53.8, 66.8, 68.4, 70.2, 70.3, 70.4, 70.7, 95.6, 114.0, 127.8, 129.4, 129.9, 130.7, 132.5, 136.4, 155.6, 156.9, 157.6, 167.9, 169.3, 173.4; ESI-MS m/z: 759.6 $[M]^+$; HRMS: Calcd for $C_{43}H_{59}O_8N_4$ 759.4327, found 759.4314.

***N*-{9-[2-([2-(*tert*-Butylamino)-2-oxoethyl]{2-[2-(2-{3-[(2,5-dioxopyrrolidin-1-yl)oxy]-3-oxopropoxy}ethoxy)ethoxy]ethyl}carbamoyl)phenyl]-6-(diethylamino)-3*H*-xanthen-3-ylidene}-*N*-ethylethanaminium chloride (3).** Compound **2** (50.5 mg, 63.5 µmol), *N*-hydroxysuccinimide (11.2 mg, 97.3 µmol) and a catalytic amount of DMAP were dissolved in dry CH_2Cl_2 (5 mL) and cooled to 0 °C. Afterwards EDCI·HCl (19.4 mg, 101.2 µmol) was added and the ice bath was removed. Stirring was continued at room temperature overnight. The organic solvent was washed consecutively with 1 mL aqueous HCl (5%) and 2 mL distilled water. Finally the organic phase was dried over Na_2SO_4 and evaporated to yield **3** as a dark purple semi-solid, which was stored under an inert-gas atmosphere (42.2 mg, 75%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.30–1.37 (m, 12H), 1.41 (s, 9H), 2.81–2.85 (m, 4H), 2.88 (t, J = 6.6 Hz, 2H), 3.08 (t, J = 6.9 Hz, 2H), 3.28 (t, J = 5.1 Hz, 2H), 3.35 (br s, 2H), 3.43–3.48 (m, 2H), 3.49–3.70 (m, 14H), 3.82 (t, J = 6.4 Hz, 2H), 4.35 (s, 2H), 6.67 (s, 2H), 7.26–7.30 (m, 2H), 7.35–7.40 (m, 2H), 7.57 (dd, J = 5.7, 3.4 Hz, 2H), 7.87 (dd, J = 5.9, 2.9 Hz, 1H), 8.58 (s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 12.7, 25.4, 25.6, 28.7, 32.1, 45.2, 46.1, 51.3, 54.0, 65.7, 67.0, 69.9, 70.3, 70.4, 70.6, 95.5, 114.0, 127.9, 129.2, 129.9, 130.7, 132.5, 137.1, 155.7, 156.9, 157.7, 166.7, 167.7, 168.9, 169.3, 169.7; ESI-MS m/z: 856.5 $[M]^+$; HRMS Calcd for $C_{47}H_{62}O_{10}N_5$ 856.4491, found 856.4498.

***N*-[9-{2-[(14-Azido-2-oxo-6,9,12-trioxa-3-azatetradec-1-yl)(1-methylethyl)carbamoyl]phenyl}-6-(diethylamino)-3*H*-xanthen-3-ylidene]-*N*-ethylethanaminium chloride (4a).** According to general procedure B: paraformaldehyde (12 mg; 0.40 mmol), isopropylamine (30 µL 0.40 mmol), rhodamine B (192 mg; 0.40 mmol) and 1-azido-11-isocyano-3,6,9-trioxaundecane (91.3 mg; 0.40 mmol) in MeOH (2 mL) were reacted. CC purification using a gradient of CH_2Cl_2/MeOH afforded **4a** as a dark purple oil (230 mg; 74%). R_f 0.26 (CH_2Cl_2/MeOH 9 : 1); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.66–0.99 (m, 6H), 1.33 (t, J = 7.0 Hz, 12H), 3.40 (t, J = 5.1 Hz, 2H), 3.44–3.81 (m, 22H), 4.11–4.24 (m, 1H), 4.29 (s, 2H), 6.65 (s, 2H), 7.29–7.36 (m, 4H), 7.53–7.58 (m, 2H), 8.03–8.05 (m, 1H), 9.05 (t, J = 5.6 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 12.7, 18.6, 38.9, 46.1, 47.0, 49.5, 50.7, 53.4, 69.4, 69.9, 70.2, 70.6, 95.6, 114.0, 128.1, 128.9, 129.0, 130.0, 132.3, 137.9, 155.6, 157.4, 157.6, 169.3, 170.1; ESI-MS m/z: 742.6 $[M]^+$; HRMS Calcd for $C_{41}H_{56}O_6N_7$ 742.4287, found 742.4287.

***N*-[9-{2-[(20-Azido-2-oxo-6,9,12,15,18-pentaoxa-3-azaicos-1-yl)(1-methylethyl)carbamoyl]phenyl}-6-(diethylamino)-3*H*-xanthen-3-ylidene]-*N*-ethylethanaminium chloride (4b).** According to general procedure B: paraformaldehyde (30 mg, 1.00 mmol), isopropylamine (86 µL, 1.00 mmol), 1-azido-17-isocyano-3,6,9,12,15-pentaoxaheptadecane (325.5 mg, 1.03 mmol) and rhodamine B (480.8 mg, 1.00 mmol) in methanol (5 mL) were reacted. CC purification using a gradient of CH_2Cl_2/MeOH afforded **4b** as a dark purple oil (607 mg, 70%). R_f 0.45 (CH_2Cl_2/MeOH 90 : 10); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.74–0.95 (m, 6H), 1.33 (t, J = 7.0 Hz, 12H), 3.40 (m, 2H), 3.53–3.69 (m, 30H), 4.19 (m, 1H), 4.26 (s, 2H), 6.65 (s, 2H), 7.25–7.31 (m, 1H), 7.31–7.38 (m, 3H), 7.54–7.60 (m, 2H), 8.01–8.06 (m, 1H), 8.99 (m, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 12.6, 18.6, 30.9, 38.9, 46.1, 50.0, 50.6, 69.3, 69.9, 70.1, 70.5, 70.6, 95.6, 113.9, 128.1, 128.9, 129.0, 129.9, 130.7, 132.3, 155.6, 157.3, 157.6, 169.3, 170.1; ESI-MS m/z: 830.7 $[M]^+$; HRMS Calcd for $C_{45}H_{64}O_8N_7$ 830.4811, found 830.4806.

***N*-[9-(2-{[1-(*tert*-Butylcarbamoyl)-2-methylpropyl](prop-2-yn-1-yl)carbamoyl}phenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene]-*N*-ethylethanaminium chloride (5).** According to general procedure B: acetylene-PEG$_4$-amine (100 mg, 0.43 mmol), isobutyraldehyde (39.6 µL, 0.43 mmol), rhodamine B (208 mg, 0.43 mmol) and *tert*-butyl isocyanide (49.8 µL, 0.43 mmol) in methanol (3 mL) were reacted. CC purification using a gradient of CH_2Cl_2/MeOH afforded **5** as a purple oil (222 mg, 61%). R_f 0.21 (CH_2Cl_2/MeOH 9 : 1); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.25–0.35 (m, 3H), 0.82 (d, J = 6.4 Hz, 3H), 1.17 (s, 9H), 1.28–1.41 (m, 12H), 2.17–2.35 (m, 1H), 2.45 (t, J = 2.3 Hz, 1H), 3.15–3.41 (m, 2H), 3.47–3.76 (m, 23H), 4.20 (d, J = 2.3 Hz, 2H), 6.75 (br s, 1H), 6.79–6.83 (m, 2H), 6.90–7.01 (m, 2H), 7.26 (dd, J = 9.7, 7.3 Hz, 2H), 7.31–7.36 (m, 1 H), 7.63–7.77 (m, 3 H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 12.6, 18.0, 19.7, 25.7, 28.3, 46.2, 50.6, 53.4, 58.3, 67.5, 69.0, 70.3, 70.4, 70.5, 74.5, 79.5, 96.1, 96.3, 113.4, 113.7, 114.2, 128.9, 129.7, 130.1, 132.5, 135.7, 155.1, 155.5, 155.6, 157.5, 157.6, 169.1, 171.0; ESI-MS m/z: 811.4 [M]$^+$; HRMS Calcd for $C_{48}H_{67}N_4O_7$ 811.5004, found 811.4983.

***N*-[9-(2-{[1-(*tert*-Butylcarbamoyl)-2-methylpropyl][19-(11,12-didehydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)-15,19-dioxo-3,6,9,12-tetraoxa-16-azanonadec-1-yl]carbamoyl}phenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene]-*N*-ethylethanaminium chloride (6).** According to general procedure A: rhodamine B (25.0 mg, 0.05 mmol), isobutyraldehyde (3.65 mg, 0.05 mmol), *tert*-butyl isocyanide (4.20 mg, 0.05 mmol) and dibenzylcyclooctyne-PEG$_4$-amine (25.0 mg, 0.05 mmol) in methanol (10 mL) were reacted. CC purification using a gradient of CH_2Cl_2/MeOH afforded **6** as a purple oil (42 mg, 73%). R_f 0.30 (CH_2Cl_2/MeOH 10 : 1); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.25–0.96 (m, 6H), 1.03–1.22 (m, 9H), 1.24–1.52 (m, 12H), 1.84–2.64 (m, 5H), 3.10–3.79 (m, 28H), 4.59–4.69 (m, 1H), 5.05–5.19 (m, 2H), 6.60–7.90 (m, 18H), 8.31 (br s, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 12.6, 17.4, 18.5, 19.7, 25.7, 28.3, 28.4, 34.7, 35.1, 36.7, 45.9, 46.0, 46.1, 51.2, 53.4, 55.4, 67.0, 67.6, 70.1, 70.3, 70.4, 80.1, 96.1, 96.2, 96.3, 96.3, 113.5, 113.8, 114.1, 114.6, 125.4, 127.1, 127.7, 128.1, 128.2, 128.6, 129.0, 129.8, 130.1, 131.1, 131.2, 131.3, 132.0, 133.2, 133.7, 135.7, 148.0, 151.0, 155.3, 155.5, 155.7, 157.6, 157.7, 164.2, 167.5, 169.1, 170.8, 171.8; ESI-MS m/z: 1103.6 [M]$^+$; HRMS Calcd for $C_{66}H_{83}N_6O_9$ 1103.6216, found 1103.6187.

***N*-[(3*E*)-9-{2-[(20-Azido-2-oxo-6,9,12,15,18-pentaoxa-3-azaicos-1-yl)(1-methylethyl)carbamoyl]phenyl}-6-(ethyl-amino)-2,7-dimethyl-3*H*-xanthen-3-ylidene]ethanaminium perchlorate (7).** According to general procedure B: rhodamine 19P (98.1 mg, 0.19 mmol), paraformaldehyde (8.1 mg, 0.26 mmol), isopropylamine (21.9 µL, 0.27 mmol), 1-azido-17-isocyano-3,6,9,12,15-pentaoxaheptadecane (64.5 mg, 0.20 mmol) in methanol (2 mL) were reacted. CC purification using a gradient of CH_2Cl_2/MeOH afforded **7** as a dark purple oil (140 mg, 81%). R_f 0.25 (CH_2Cl_2/MeOH 90 : 10); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.67–1.04 (m, 6H), 1.39 (t, J = 7.2 Hz, 6H), 2.14–2.27 (m, 6H), 3.11–3.22 (m, 2H), 3.30–3.43 (m, 4H), 3.43–3.82 (m, 24H), 3.84–4.02 (m, 1H), 5.99 (br s, 1H), 6.28 (t, J = 5.4 Hz, 1H), 6.35 (br s, 1H), 6.66–6.75 (m, 2H), 6.91–7.01 (m, 2H), 7.35 (dd, J = 5.7, 2.8 Hz, 1H), 7.59–7.78 (m, 3H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 13.7, 17.4, 20.7, 38.7, 39.1, 43.9, 50.6, 69.5, 69.9, 70.2, 70.4, 70.5, 70.6, 93.9, 114.0, 125.4, 127.1, 129.3, 129.7, 130.0, 130.7, 135.6, 154.5, 156.1, 157.3, 168.5, 169.0; ESI-MS m/z: 801.3 [M]$^+$; HRMS Calcd for $C_{43}H_{60}O_8N_7$ 802.4498, found 802.4478.

***N*-(17-Azido-3,6,9,12,15-pentaoxaheptadec-1-yl)-*N*^{2}-(1-methylethyl)-*N*^{2}-{[2-(2,3,6,7,12,13,16,17-octahydro-1*H*,5*H*,11*H*,15*H*-pyrido[3,2,1-*ij*]quinolizino[1′,9′:6,7,8]chromeno[2,3-*f*]quinolin-4-ium-9-yl)phenyl]carbonyl}glycinamide chloride (8).** Paraformaldehyde (13.2 mg, 0.42 mmol) and isopropylamine (36 µL, 0.42 mmol) were dissolved in methanol (2 mL) and stirred at room temperature for 2 h to preform the imine. To this solution a second solution containing 1-azido-17-isocyano-3,6,9,12,15-pentaoxaheptadecane (134 mg, 0.425 mmol) and rhodamine 101 inner salt (206 mg, 0.42 mmol) dissolved in methanol (1 mL) and acidified with conc. HCl (34.6 µL) were added. The resulting reaction mixture was heated in the microwave (1 h at 100 °C). After evaporation of all volatile material the crude residue was purified by column chromatography using a gradient of CH_2Cl_2/MeOH to yield **8** as a purple oil (91 mg, 24%). R_f 0.36 (CH_2Cl_2/MeOH 9 : 1); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.76–1.09 (m, 6H), 1.89–2.20 (m, 8H), 2.38–2.83 (m, 4H), 2.94–3.10 (m, 4H), 3.21–3.72 (m, 32H), 4.18 (s, 2H), 4.21–4.36 (m, 1H), 6.65–6.84 (m, 2H), 7.21–7.30 (m, 1H), 7.51–7.61 (m, 1H), 7.60–7.72 (m, 1H), 8.16–8.25 (m, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 18.7, 19.5, 19.7, 20.4, 20.7, 38.8, 46.7, 48.3, 50.2, 50.5, 50.8, 51.0, 69.2, 69.8, 70.0, 70.4, 70.5, 104.7, 113.2, 123.2, 126.5, 126.9, 128.2, 128.8, 129.3, 129.4, 129.5, 131.0, 131.6, 135.8, 137.1, 150.9, 151.1, 151.8, 151.9, 152.8, 155.0, 168.6, 169.1, 169.9; ESI-MS m/z: 878.6 [M]$^+$; HRMS Calcd for $C_{49}H_{64}N_7O_8$ 878.4811, found 878.4812.

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