A small azide-modified thiazole-based reporter molecule for fluorescence and mass spectrometric detection

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Full Research Paper

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Abstract

Molecular probes are widely used tools in chemical biology that allow tracing of bioactive metabolites and selective labeling of proteins and other biomacromolecules. A common structural motif for such probes consists of a reporter that can be attached by copper(I)-catalyzed 1,2,3-triazole formation between terminal alkynes and azides to a reactive headgroup. Here we introduce the synthesis and application of the new thiazole-based, azide-tagged reporter 4-(3-azidopropoxy)-5-(4-bromophenyl)-2-(pyridin-2-yl)thiazole for fluorescence, UV and mass spectrometry (MS) detection. This small fluorescent reporter bears a bromine functionalization facilitating the automated data mining of electrospray ionization MS runs by monitoring for its characteristic isotope signature. We demonstrate the universal utility of the reporter for the detection of an alkyne-modified small molecule by LC–MS and for the visualization of a model protein by in-gel fluorescence. The novel probe advantageously compares with commercially available azide-modified fluorophores and a brominated one. The ease of synthesis, small size, stability, and the universal detection possibilities make it an ideal reporter for activity-based protein profiling and functional metabolic profiling.

Introduction

Fluorescent dyes are widely used for detection and monitoring in the fields of chemistry, biochemistry, molecular biology, medicine and material sciences. Due to sensitive and selective detection methods and unproblematic toxicology they have almost completely replaced radioactive tags. Widely used representatives include dansyl chloride, fluoresceins, rhodamines and boron-dipyrromethenes (BODIPYs) [1]. Dansyl chloride, with a maximum UV–vis absorption at 369 nm, is one of the first extrinsic fluorescent dyes introduced in this field and is still widely used in protein labeling [2]. Later, fluoresceins and
rhodamines found applications in this area as well because of
advantageous UV–vis absorption maxima (480–600 nm) and
more bathochromic emission wavelengths (510–615 nm) [3].

A successful class of fluorophores also used for probing in life
science comprises the heterocyclic thiazoles. This structural
element can be found in commercial products, such as thiazole
orange, SYBR® Green I or TOTO®, which are, e.g., used for
DNA labeling. In these compounds the thiazole ring is part of a
benzothiazole. We set out to minimize the structural complexity
of the fluorophores to achieve higher atom economy and reduce
the interaction with biomacromolecules. In this context it was
critical to realize that the thiazole moiety itself can also act as a
fluorophore, especially the class of 4-hydroxythiazoles [4,5].
4-Hydroxythiazoles are now becoming commercially available
but are also easily accessible by synthesis with a broad range of
substitution patterns. Substantial manipulations of the UV–vis
excitation and emission wavelengths of these compounds are
thus possible [6].

The design of molecular probes based on fluorophores requires
the attachment of the fluorescent reporter to bio(macro)mole-
cules or synthetic probes. Especially “click chemistry”, intro-
duced by Sharpless and coworkers in 2001 [7], is a widely used
strategy to attach fluorophores covalently to other molecules.
Among “click” reactions the Cu(I)-catalyzed azide–alkyne
cycloaddition (CuAAC) is often considered as the prototypical
transformation [7-9]. Due to the mild conditions and the use of
aqueous solvents it is an efficient tool for bioorthogonal chem-
istry even inside of living systems [10]. One application of this
concept for functional analysis of proteins is the activity-based
protein profiling (ABPP) [11,12]. This proteomic strategy uses
small probes designed to target active members of enzyme
families [13]. These are often based on natural products to
investigate their protein targets and eventually their mode of
action [14,15]. ABPP probes contain two structural units: (1) a
reactive group that reacts with the protein target and (2) a
reporter unit for detection which could be, e.g., a fluorophore, a
MS-tag, biotin or a combination of these [16,17]. For in vivo or
in situ applications the alkyn (or azide) modified reactive
group is usually applied to living organisms and after cell lysis
the reporter is introduced by CuAAC [16]. Fluorophore tagged
proteins can then be visualized by gel electrophoresis [17].

Besides fluorescence detection, mass spectrometry (MS) is
also suited for the monitoring of tagged biological samples.
Several probes have been designed for use with liquid chroma-
tography–mass spectrometry (LC–MS). The probes attach coval-
ently to target functional groups like amines, aldehydes/ketones,
carboxylic acids and enhance their detection limit in
LC–electrospray ionization (ESI) MS. This can be achieved by
introduction of charged species like ammonium or phos-
phonium for ionization in the positive mode [18-20]. Bromine
[19,21,22] or chlorine [23] containing tags were also intro-
duced as they generate a unique isotopic pattern and therefore
enhance recognition and identification of labeled small mole-
cules. These specific isotopic patterns also enable data
processing by cluster analysis [19] or other algorithms for an
automated structure mining [24,25].

Here we introduce the rational design, synthesis and applica-
tion of a small thiazole-based, azide-tagged reporter molecule
that supports universally, fluorescence, UV and MS detection.
We thoroughly characterize its reactivity and utility with
different detection methods and compare it with common
commercially available fluorophores. As proof of principle
protein and amino acid labeling with an alkyn containing reac-
tive probe according to the ABPP concept is introduced using a
new reporter molecule.

Results and Discussion
Design of the reporter
We aimed to combine high UV absorption and fluorescence
with the possibility of unambiguous mass spectrometric detect-
ion (LC–ESIMS) in one reporter molecule. An azide function-
ality guarantees compatibility with widely applicable CuAAC
approaches, that are, for instance, used in the field of ABPP
where fluorescent reporter azides act as part of protein probes.
To avoid the need for expensive detection systems for in-gel
fluorescence we adjusted the excitation and emission wave-
lengths of the reporter to basic laboratory documentation equip-
ment (365 nm UV-transilluminator, digital camera and a low
cost commercial UV filter). Introduction of at least one atom
with characteristic isotopic pattern like bromine or chlorine is
necessary for a unique mass spectrometric detection of labeled
substances. However, introduction of these heavy atom
substituents in a fluorophore is challenging since it often results
in decreased fluorescence due to intersystem crossing [26].
When working with reversed-phase LC–MS not only a specific
isotopic pattern but also balanced polarity of the reporter is
required. Addition of a nonpolar reporter shifts the retention
time of polar analytes in reversed-phase chromatography to
higher values. This is especially advantageous in the detection
of small polar analytes [23,27]. On the other hand the polarity
of the reporter needs to be high enough to work with biological
samples in aqueous solution. Ideally a pH-independent fluores-
cence should guarantee for unbiased detection in tissues or
under variable analytical conditions. In the light of our detailed
knowledge of luminescence properties of pyridylthiazoles we
considered this compound class to be ideally suited for the
above mentioned tasks [28]. Based on previous considerations
on the luminescent properties of pyridylthiazoles we decided to
synthesize 4-(3-azidopropoxy)-5-(4-bromophenyl)-2-(pyridin-2-yl)thiazole (BPT, 1, Figure 1) as target molecule fulfilling the above mentioned requirements.

Scheme 1: Synthesis of the azide-bearing 4-hydroxythiazole derivative 1.

The thioamide 2 bears an electron-withdrawing substituent in form of a 2-pyridyl moiety, which is important for an efficient fluorescence of the final product [6]. The α-bromoester 3 bears a bromine atom at the 4-position of the phenyl ring, which is introduced to facilitate MS detection.

For comparison, we also synthesized and tested a bromine modified dansyl derivative N-(3-azidopropyl)-6-bromo-5-(dimethylamino)naphthalene-1-sulfonamide (BNS, 6, Figure 2). Dansyl chloride is brominated according to the literature [30] to produce 6-bromo-5-(dimethylamino)naphthalene-1-sulfonyl chloride (7) and subsequently treated with 3-azidoprop-1-amine to provide the fluorescence/MS tag 6.

Characterization of BPT (1) and comparison with other azide modified fluorophores
We characterized the new thiazole reporter BPT (1) regarding its absorption and emission properties as well as its quantum
yield and compared it with other commercially available fluorophores of similar size (Figure 2). We chose N-(3-azidopropyl)-S-(dimethylamino)naphthalene-1-sulfonamide (DNS, 8) with a fluorophore system exhibiting a large stokes shift [1] suitable for fluorescence detection with UV filters. Derivatization with dansyl chloride is used for labeling of primary and secondary amines or phenols resulting in enhanced ESI signals and shifted retention times of labeled polar analytes in reversed-phase LC [27]. For comparison with BPT (1) we also introduced bromine into the aromatic system of DNS (8) to receive BNS (6). Furthermore, we utilized N-(3-azidopropyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (NBD, 9), a cheap fluorophore previously used for probes [1,31] or as fluorescent tag [32,33] (Figure 2).

UV–vis spectra of all substances were recorded in an aqueous solution containing 20% THF (v/v) (Figure 3) and their molar absorption coefficients ε at their absorption maxima (λabs,max) were calculated (Table 1). Notably, introduction of bromine into DNS (8) decreases the molar absorption coefficient more than twice and makes the resulting compound BNS (6) unsuitable for excitation with an UV transilluminator whereas BPT (1) offers a very good absorbance at 365 nm, a standard UV excitation wavelength.

We then recorded fluorescence spectra of all fluorophores and determined fluorescence quantum yields Φ (Table 1). To characterize BPT (1) in a non-interacting solvent we used cyclohexane resulting in an emission maximum of 444 nm and a quantum yield of Φ = 0.96. In an aqueous solution containing 20% THF (v/v) the maximum of emission (λem,max) is shifted to 455 nm (Figure 4). The quantum yield of BPT (1) in this solution is Φ = 0.87 which makes it convenient for fluorescence detection in aqueous media, e.g., for biological applications. In contrast, the quantum yield of BNS (6) in 80% water/20% THF (v/v) was below 0.03 and rendering it inappropriate for this purpose.

The UV properties allow UV detection after LC separation as could be shown by ultra-performance liquid chromatography (UPLC) coupled to a photodiode array detector using the

![Figure 3: UV–vis spectra of 20 µM solutions of the azide modified fluorophores BPT (1), DNS (8), NBD (9) and BNS (6) in THF/water (20:80; v/v).](image)

![Figure 4: Normalized absorbance and fluorescence of BPT (1) in 20% THF/80% water (v/v), excitation at 374 nm.](image)

| Table 1: Spectral properties of BPT (1), DNS (8), NBD (9) and BNS (6) in THF/water (20:80; v/v). |
|---------------------------------------------------------------|
| **λabs,max [nm]** | **ε [L/(mol cm)]** | **λem,max [nm]** | **Φ** |
|------------------|------------------|------------------|-------|
| BPT (1) | 374 (376a) | 15.5 × 10³ | 455 (444a) | 0.87 (0.96a) |
| DNS (8) | 331 | 4.3 × 10³ | 546 | n.d. |
| NBD (9) | 344/477 | 8.5 × 10³ b/28.1 × 10³ | 545 | 0.51d |
| BNS (6) | 327 | 1.9 × 10³ | 550 | <0.03 |
| **Lit. ε [L/(mol cm)]** | **Lit. λem,max [nm]** |
|------------------|------------------|
| 4.2 × 10³ e,f | 506 nm°, 520 nm° | 22.1 × 10³ d | 524 nm° |

*a In cyclohexane; b at 344 nm (which is a local maximum of NBD), since we excite fluorescently labeled biomolecules in gels with a 365 nm UV transilluminator this region is of crucial importance; c quantum yield, d in ethanol [45], e solvent not specified, f from [46]; n.d. - not determined; Lit. - values found in literature.
solvents A (water/acetonitrile/formic acid 98:2:0.1; v/v/v) and B (acetonitrile/0.1% formic acid; v/v). The peaks of equimolar amounts were integrated at their absorption maxima resulting in the highest integrated peak area for NBD (9) followed by BPT (1) (Figure 5A). BPT is the least polar substance among the tested fluorophores and elutes at 90% B (Table 2). Polar analytes are often poorly retained in reversed-phase chromatography [23]. Thus, after CuAAC BPT (1) will shift retention times of polar analytes to higher values as it is achieved with other labeling reagents like dansyl chloride [27] or p-chlorophenylalanine containing tags [23]. Nevertheless, BPT (1) shows sufficient water solubility when working in aqueous solutions with low amounts of organic co-solvent. For instance during implementation of CuAAC we use 3.5% DMSO and 4.5% t-BuOH in our protocol which ensures solubility of BPT (1).

Table 2: Solvent composition at time of elution of BPT (1), DNS (8), NBD (9) and BNS (6) measured with C18-UPLC–ESIMS using a linear gradient of solvents A (water/acetonitrile/formic acid 98:2:0.1; v/v/v) and B (acetonitrile/0.1% formic acid; v/v) and solvent composition and masses of imines 11–14 formed in a model reaction between L-lysine and DDY (10) followed by CuAAC with the different reporter molecules.

| reporter | comp. B (%) | imine | comp. B (%) | m/z* |
|----------|-------------|-------|-------------|------|
| BPT (1)  | 90          | 11    | 33          | 691.19 |
| DNS (8)  | 58          | 12    | 20          | 609.31 |
| NBD (9)  | 49          | 13    | 24          | 539.26 |
| BNS (6)  | 74          | 14    | 31          | 687.22 |

*Calculated monoisotopic masses; comp. - solvent composition.

UPLC–ESIMS measurements were employed to characterize ionization properties of the fluorophores (Figure 5B). In positive mode BPT (1) gives a slightly higher peak area than DNS (8). Derivatization with dansyl chloride has been previously introduced as an ionization enhancing procedure for LC–ESIMS. Thereby increased linear responses of tested amino acids by over two orders of magnitude compared to underderivatized samples were observed [27]. Interestingly, the novel BPT (1) is even superior to the established DNS (8) but introduced the additional benefit of a characteristic isotope pattern. Ionization of the brominated dansyl system BNS (6) resulted in a clearly lower intensity response. In negative mode ionization of BPT (1) is not adequate (data not shown).

Taken together in the comparison of all four fluorophores BPT (1) has superior properties for detection if fluorescence, UV absorption and MS properties are concerned.

Visualization of small molecules by mass spectrometric detection

To demonstrate the universal application possibilities, we next coupled reporter molecules with a synthetic reactive group as commonly used in ABPP approaches. The alkyne-modified (2E,4E)-deca-2,4-dien-9-ynal (DDY, 10) served as reactive group. DDY (10) mimics the natural product 2,4-decadienal that is produced by some diatoms as potential chemical defense metabolite against their grazers [34]. Structure-activity tests have revealed that 2,4-decadienal can be modified in the alky terminal without loss of function [35]. Thus the alkyne modified α,β,γ,δ-unsaturated aldehyde 10 can serve as a tool for the elucidation of the mode of action of the compound class of polyunsaturated aldehydes. DDY (10) was initially transformed with L-lysine to form an imine before CuAAC was performed with the four azides BPT (1), DNS (8), NBD (9) and BNS (6). After one hour of incubation with lysine the respective reporter, the ligand 1-(1-benzyltriazol-4-yl)-N,N-bis((1-benzyltriazol-4-
Procedure of the model reaction between L-lysine and DDY (10) to form an imine (only one of two possible reactions shown) followed by CuAAC with the azide-modified fluorophores. The rectangle represents the respective reporter unit. For clarity only reactions with the terminal lysine are depicted.

After addition and incubation of DDY (10) with the catalase, we applied CuAAC with the four fluorophores. The products were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by in-gel fluorescence detection (Figure 8). BPT/DDY gives the brightest signal whereas intensities of DNS/DDY and BNS/DDY-labeled catalase are clearly lower. The lowest signal was emitted by NBD/DDY/catalase, which is probably due to very low fluorescence quantum yields reported for NBD derivatives of primary amines in water [39]. Furthermore, NBD (9) is not suitable for standard SDS-PAGE (12% gels) as the dye smears and therefore potentially covers fluorescent signals of proteins of lower masses.

The novel probe has further implications since a combination of fluorescence and mass tagging might prove beneficial in proteomics studies. Mass tags containing bromine [24,37,40,41] and chlorine [24,38] have been reported in proteomics related applications. Additionally, bromine containing tags called isotope-differentiated binding energy shift tags (IDBEST™) were designed to introduce a mass-defect in peptides for better sequence coverage of proteins [25,42]. The reporter molecule BPT (1) contains bromine and tagged proteins can thus easily be identified with both, fluorescent and MS techniques.

Conclusion

We introduce the azide-modified thiazole-based reporter molecule BPT (1) with superior properties for fluorescence, UV and MS detection compared to other common reporters. BPT (1) can be easily synthesized and attached to terminal alkyne-modified molecules via CuAAC. We show model experiments that demonstrate the suitability of the molecule in labeling small...
molecules and in ABPP investigations. Fluorescence and MS offer orthogonal opportunities for detection and make this reporter a universal tool for targeting molecules of different sizes and properties.

**Experimental Synthesis**

Experimental details are available in Supporting Information File 1.

**Sample preparation and measurements**

**UV–vis and fluorescence spectroscopy**

Solutions of each fluorophore in THF/water (20 µM, 20:80; v/v) were prepared out of 5 mM stock solutions in DMSO. UV–vis spectra were recorded with a GENESYS™ 10 S spectrophotometer (Thermo Fischer Scientific Inc., Waltham, MA, USA) with 10 mm quartz cells. Quantum yields were obtained as described in [43] using quinine sulfate in 0.05 M sulfuric acid as fluorescent standard with a Varian Cary 500 spectropho-
Figure 8: Fluorescent labeling of catalase treated with DDY (10) followed by CuAAC with all four reporter molecules and in-gel fluorescence detection at 365 nm. Equivalent amounts of protein were labeled and loaded on the gel. (Protein ladder masses in kDa).

LC–ESIMS and UV–vis detection
For LC–MS measurements we used an Acquity™ Utrapreformance LC (Waters, Milford, MA, USA) coupled to a Waters 996 PDA detector and a Q-Tof microMS (Waters Micromass, Manchester, England). A Kinetex C18 reversed-phase column (2.1 mm × 50 mm, 1.7 µm particle size, Phenomenex, Torrance, CA, USA) was used. For UV detection and ionization in positive and negative mode 10 µL of 10 µM solutions of each fluorophore were injected. For model reactions between DDY (10) and lysine followed by CuAAC, 5 µL were injected.

Incubation with DDY (10) and CuAAC
**For L-lysine:** L-Lysine (30 µL 1 mM, prepared from a 50 mM stock in water) were added to 1.47 mL methanol followed by 4 µL (0.13 mM) of DDY (10) (prepared from a 50 mM stock in DMSO) and mixed on a vortex mixer. 132 µL of this solution were transferred to a 1.5 mL Eppendorf tube (Eppendorf AG, Hamburg, Germany) and the following substances were added (procedure adapted from [16]): 3 µL (0.1 mM) of BPT (1) or the other reporter molecules (5 mM stock in DMSO), 9 µL (0.1 mM) TBTBA solution (1.7 mM stock in DMSO/tert-butanol, 1:4, v/v) and 3 µL (20 mM) freshly prepared ascorbic acid solution (1.00 M in water). Samples were vortexed and 1 µL (1 mM) copper(II) sulfate solution (50 mM in water) was added. Samples were vortexed again and stored on ice for 1 hour.

**For catalase from bovine liver:** Catalase from bovine liver (2.5 mg) was dissolved in 1 mL phosphate buffer (59.0 mM Na₂HPO₄, 7.6 mM KH₂PO₄, pH 7.6) and 2 µL (0.01 mM) of DDY (10, 5 mM stock in DMSO) were added. The sample was incubated for one hour. 44 µL of this solution was transferred to an Eppendorf tube and the following substances were added (procedure adapted from [16]): 1 µL (0.1 mM) of BPT (1) or the other reporter molecules (5 mM stock in DMSO), 3 µL (0.1 mM) TBTBA solution (1.7 mM stock in DMSO/tert-butanol, 1:4, v/v) and 1 µL (20 mM) of freshly prepared ascorbic acid solution (1.00 M in water). Samples were vortexed and 1 µL (1 mM) copper(II) sulfate solution (50 mM in water) was added. Samples were vortexed again and stored on ice for 1 hour.

**SDS-PAGE and in-gel fluorescence detection**
Aliquots (10 µL) of each pre-incubated catalase (9 µL of untreated catalase and 1 µL of deionized water) were mixed with 10 µL of 2× loading buffer [44] and heated to 95 °C for 6 min. A protein ladder (PageRuler unstained protein ladder, Thermo Scientific) and 15 µL of each sample were loaded on a 12% SDS mini gel and separated in a Mini-Protean® Tetra gel cell (Bio-Rad, Hercules, CA, USA) and a commercially available UV filter (HMC Hoya Multi-Coated Filter, Hoya, Tokyo, Japan). The gel was stained with RAPIDstain™ (G-Biosciences, St. Louis, MO, USA).

**Supporting Information**
Supporting Information File 1
Synthetic procedures and characterization data of synthetic compounds.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-10-258-S1.pdf]

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