Letter

Hydrodabcyl: a superior hydrophilic alternative to the dark fluorescence quencher dabcyl

Oxana Kempf, Karl Kempf, Rainer Schobert, and Elisa Bombarda

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.7b03488 • Publication Date (Web): 26 Oct 2017

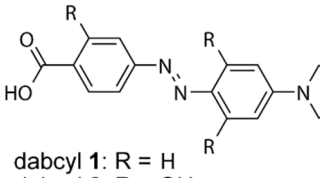
Hydrodabcyl: a superior hydrophilic alternative to the dark fluorescence quencher dabcyl

Oxana Kempf,[†] Karl Kempf, [‡] Rainer Schobert[‡] and Elisa Bombarda*,[†]

[†]Department of Biochemistry and [‡]Organic Chemistry Laboratory, University of Bayreuth, Universitaetsstr. 30, 95440, Bayreuth, Germany

ABSTRACT: Dark fluorescence quenchers are non-fluorescent dyes that can modulate the fluorescence signal of an appropriate fluorophore donor in a distance-dependent manner. Dark quenchers are extensively used in many biomolecular analytical applications, such as studies with fluorogenic protease substrates or nucleic acids probes. A very popular dark fluorescence quencher is dabcyl, which is a hydrophobic azobenzene derivative. However, its insolubility in water may constitute a major drawback, especially during the investigation of biochemical systems whose natural solvent is water. We designed and synthesized a new azobenzene-based dark quencher with excellent solubility in aqueous media, which represents a superior alternative to the much-used dabcyl. The advantage of hydrodabcyl over dabcyl is exemplarily demonstrated for the cleavage of the fluorogenic substrate hydrodabcyl-Ser-Phe-EDANS by the proteases thermolysin and papain.

Biomolecular processes are extensively studied by employing fluorescence dyes[1]. There is a broad range of fluorescent dyes the spectroscopic characteristics of which depend on several physico-chemical parameters such as their hydrophobicity and oxidation state, or the pH value and ionic strength of the medium[2]. The strategy of using such single-labeled probes was refined by the development of dual-labeled probes which combine a reporter dye with a quencher moiety[3]. Typically, these probes may exist in two conformations differing in their fluorescence properties: a 'closed' form in which the reporter and the quencher are in close proximity and an 'open' form in which these groups are spatially separated. If the quencher is a fluorescence dye itself, both the increase in the fluorescence of the quencher and the decrease in the fluorescence of the reporter can be followed. However, an overlap of quencher and reporter fluorescence spectra may cause background noise necessitating a meticulous instrumental set-up and data analysis. Dark quenchers (e.g., non-fluorescent dyes) offer a solution to this problem because they do not occupy any crucial emission bandwith. Dual-labeled probes comprising a reporter and a dark quencher are also called fluorogenic probes since the biochemical event to be observed causes their transition from a non-fluorescent to a (typically strongly) fluorescent form.



dabcyl **1**: R = H
hydrodabcyl **2**: R = OH

Figure 1. Chemical structures of 4-(4'-dimethylaminophenylazo)benzoic acid (dabcyl (**1**)) and 4-(2',6'-dihydroxy-4'-dimethylaminophenylazo)-2-hydroxybenzoic acid (hydrodabcyl (**2**)).

Dabcyl (4-(4'-dimethylaminophenylazo)benzoic acid (**1**), Figure 1) is a dark quencher in dual-labeled probes widely used for a variety of biomolecular applications such as enzymatic catalysis and nucleic acid probes[4–7]. The absorption band of dabcyl (**1**) in the range of 350-550 nm overlaps with the emission band of many common fluorescent dyes such as 5-((2'-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS) ($\lambda_{em,\ Max}$ = 490 nm), bimane ($\lambda_{em,\ Max}$ = 480 nm), and many fluorescein, coumarin and rhodamine derivatives. Dabcyl (**1**) is an azobenzene push-pull functionalized at the para-positions of the phenyl rings. Its conjugated π-system and its lack of polar substituents render it a hydrophobic compound, virtually insoluble in aqueous media. In fact, stock solutions of dabcyl (**1**) need to be prepared in DMSO. Although dabcyl (**1**) is one of the most popular acceptors for developing FRET-based probes, its insolubility in water severely limits its use in biological systems where the natural solvent is water. Even when this hydrophobicity can be partly compensated for by the hydrophilicity of the substrate to which dabcyl (**1**) is linked (e.g., long DNA segments or peptide chains), it poses a real problem in the case of comparatively small substrates. Such solubility problems were reported for various dabcyl-labeled substrates[5,8]. In particular, any incomplete dissolution leads to inaccurate estimations of the concentrations and consequently to erroneous calculations of stability and rate constants. Most attempts to overcome the problems stemming from the insolubility of dabcyl (**1**) such as performing the enzymatic assays in mixtures of water and DMSO[5] can be source of new problems due to the modification of the polar environment of the reaction. Instead of adjusting the polarity of the solvent we designed and synthesized a new azobenzene derivative, hydrodabcyl (**2**), that is spectroscopically dabcyl-like, yet far more hydrophilic when compared to dabcyl (**1**).

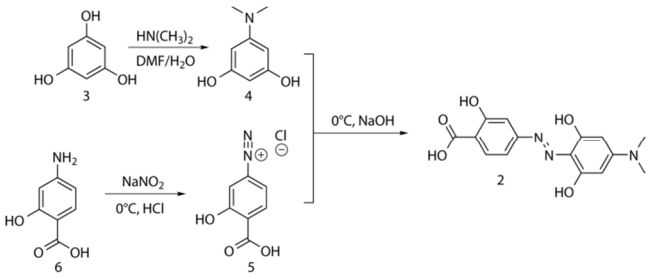
EXPERIMENTAL SECTION

All procedural information can be found in the Supporting Information.

RESULT AND DISCUSSION

Technically, hydrodabcyl (**2**) (4-(2',6'-dihydroxy-4'-dimethylaminophenylazo)-2-hydroxybenzoic acid, Figure 1) is a trishydroxy derivative of the parent dabcyl (**1**).Since phenolic OH groups were known to affect the emitting properties of a molecule, which is apparent for instance from the intensity increase and red-shift of the fluorescence when going from phenylalanine to tyrosine, it took some deliberation to ensure the darkness of the new chromophore. We found that the addition of three hydroxy groups to the azobenzene of **1** led to a non-fluorescent molecule. This finding is in line with a previous work on the coumarin chromophore that showed that the addition of an OH group at C-7 of coumarin gives rise to a distinctly fluorescent molecule while attachment of three OH groups affords non-fluorescent coumarin derivatives[9]. Accordingly, trihydroxydabcyl derivatives were not fluorescent either. Moreover, the three hydroxy groups had to be positioned in a way that precludes the formation of catechol chelate complexes with biologically relevant metal ions (e.g. Fe(III)[10]) which would possibly interfere with the system under investigation. This aspect is particularly important for the investigation of enzymatic reactions in which metals are essential cofactors and for possible applications *in vivo*.

Scheme 1. Synthesis of hydrodabcyl (2).



Hydrodabcyl (**2**) was synthesized in two steps (Scheme 1, for details see SI†). Phloroglucinol (**3**) was converted with dimethylamine to 5-dimethylamino-resorcinol (**4**), which in turn was reacted with 4-diazosalicylic acid (**5**) to give hydrodabcyl (**2**). The intermediate **4** was prepared according to a protocol by Petrzilka and Lusuardi [11] and obtained as pink crystals as described (Figure S1 in SI). However, when used as such for the azo coupling reaction with **5**, it afforded a fluorescent product that proved unsuitable as a dark quencher. A careful examination of every single step of the procedure pointed to an impurity as the cause of the pink coloration and possibly of the fluorescence of the final product (*cf.* SI). In fact, when the unspecified impurity was removed by a preceding column chromatography, **4** was obtained as colorless crystals (Figure S1 in SI). Its reaction with **5** afforded **2** as a non-fluorescent compound in 58 % yield over both steps. Though seemingly marginal, this modification to the original protocol is crucial for obtaining **2** in a quality that allows its use as a dark quencher.

Hydrodabcyl (**2**) is soluble in water, up to a concentration of 5.7×10^{-4} M at 20 °C. In contrast, it was not possible to prepare an aqueous solution of dabcyl (**1**) due to precipitation even at low concentration (Figure 2a). The attachment of hydroxy groups to the dabcyl core imparts water solubility without adding electric charges as would have the addition of ionic solubilizers such as sulfonate groups[12]. Consequently, hydrodabcyl (**2**) will not significantly modify the electrostatic profile of the molecule to which it is linked and thus the binding properties of the labeled molecule are expected not to be altered much. This aspect has important implications for investigations of biological systems in which molecular interactions are frequently driven by electrostatics (e.g. enzymatic reactions). Additionally, the carboxyl group of hydrodabcyl (**2**) remains available for the coupling to an amino group in the substrate through a standard amide bond formation. Practical advantages of the good solubility of hydrodabcyl (**2**) are the ease of preparing solutions and of cleansing used glassware.

Saturated solutions of hydrodabcyl (**2**) in water show a pH of 4.5, which is significantly lower than the pH of pure water. In fact, both dabcyl (**1**) and hydrodabcyl (**2**) bear a carboxyl group and have therefore an acidic character. The acidic environment favors the protonation of the carboxyl group, thus increasing the hydrophobic character of the molecule. While the hydroxy groups of hydrodabcyl (**2**) compensate for this effect, dabcyl (**1**) turns even less soluble in water as the pH drops. Nevertheless, experiments are normally carried out at controlled pH, therefore, we performed the same test in a buffered aqueous solution at pH 8.0 where the carboxyl group is supposed to be deprotonated and thus charged. Expectedly, in an aqueous buffer of pH 8.0 the solubility of both dabcyl (**1**) and hydrodacyl (**2**) is increased, distinctly so in the case of hydrodabcyl (**2**) (25 mM) but still unsufficiently so in the case of dabcyl (**1**) (*cf.* Table 1 in SI), confirming the superiority of hydrodabcyl.

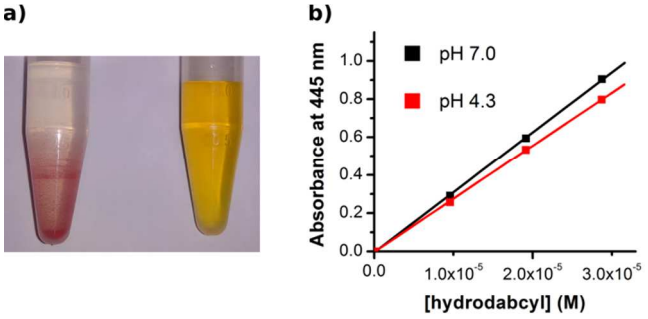


Figure 2. (a) Solubility in water: on the left, evident precipitation of dabcyl (**1**) (intended concentration *ca.* 7 µM); on the right, a clear solution of hydrodabcyl (**2**) (7 µM). (b) Concentration dependence of the absorbance at 445 nm of hydrodabcyl (**2**) in a buffered solution at pH 7.0 (black) and at pH 4.3 (red). The solution at pH 4.3 was obtained by gradual acidification of the solution at pH 7.0 (SI for details). The temperature was set at 20 °C.

In the majority of their applications, quenchers such a dabcyl (**1**) or hydrodabcyl (**2**) are linked to the actual molecules of interest, e.g. to peptide substrates in order to monitor protease activities. Both dabcyl (**1**) and hydrodabcyl (**2**) can be readily coupled to an amino group of a peptide via a standard amide bond formation. However, this linkage eliminates the free carboxylic acid on the quenchers and so the option to improve their solubility, or that of their peptide conjugates, by elevating the pH. To investigate this aspect, we linked the two dark quenchers to the amino group of a lysine to obtain the compounds Lys-dabcyl and Lys-hydrodabcyl. Interestingly, in a buffered aqueous solution at pH 8.0, the concentration of Lys-dabcyl was only 7.6×10^{-6} M whereas we could prepare a 6.6 mM solution of Lys-hydrodabcyl without reaching satura-

tion. Accordingly, the solubility of Lys-hydradabcyl is presumably much higher than 6.6 mM and therefore close to that of L-Lys which is about 40 mM or 5.8 g kg^{-1} of water[13]. In other words, amide conjugation of hydrodabcyl (**2**) to peptides does not significantly alter the solubility of the latter, whereas conjugation to dabcyl (**1**) is expected to lower their solubility in aqueous media. The modification of the solubility of a reactant in a biochemical process may hamper and distort its experimental study considerably. For instance, a distinctly reduced solubility of the product of an enzymatic reaction may hinder its release from the active site, resulting in an artifactual inhibiting effect. The solubility of hydrodabcyl was accurately measured spectroscopically at different pH by certifying the linear dependence between absorbance and concentration (Figure 2b and SI for more details). From basic pH values down to pH 6.0, solutions with concentrations in the millimolar range can be prepared directly at the desired pH. At pH < 6.0 the solubility is lower, however, solutions with millimolar concentrations can still be prepared by gradual acidification of an alkaline solution down to a pH value of 4.3. The excellent solubility of hydrodabcyl (**2**) and its conjugates with biomolecules makes it the quencher of choice in most biochemical applications.

The absorption spectra of dabcyl (**1**) and hydrodabcyl (**2**) in DMSO are quite similar with the latter showing a slight bathochromic shift of the main absorption band and a greater molar absorbance (Figure 3). Both effects enhance the quenching power of hydrodabcyl (**2**) at larger wavelengths and advocate its preferential use, even in applications where dabcyl (**1**) has been customarily employed, so far. On the other hand, in aqueous solution, the absorption maximum of hydrodabcyl (**2**) (λ_{Max} = 445 nm, ε_{445} = 43000 M^{-1} cm^{-1}) is shifted toward smaller wavelength (hypsochromic shift) in comparison to its spectrum in DMSO (λ_{Max} = 470 nm, ε_{470} = 37000 M^{-1} cm^{-1}) as shown in Figure 3. However, this hypsochromic shift is partly compensated by its greater molar absorbance in aqueous solution, thus rendering hydrodabcyl (**2**) an effective quencher at wavelengths up to 500 nm also in aqueous solution.

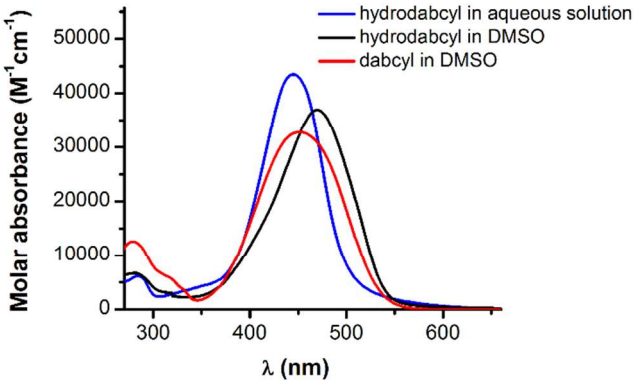


Figure 3. Comparison of molar absorbances of dabcyl (**1**) in DMSO (red curve, λ_{Max} = 451 nm, ε_{451} = 32000 M^{-1} cm^{-1}), hydrodabcyl (**2**) in DMSO (black curve, λ_{Max} = 470 nm, ε_{470} = 37000 M^{-1} cm^{-1}) and **2** in buffered aqueous solution pH 8.0 (blue curve λ_{Max} = 445 nm, ε_{445} = 43000 M^{-1} cm^{-1}); all at 20 °C.

Hydrodabcyl (**2**) has the requisite properties for serving as a useful dark quencher in fluorogenic probes. In order to observe hydrodabcyl (**2**) in action we synthesized a peptidic substrate featuring only two amino acids: serine labeled with hydrodabcyl (**2**) as quencher-acceptor and phenylalanine labeled with EDANS as fluorophore donor (Figure 4 and SI for details of the synthesis procedure). A dipeptide is the minimal substrate for protease reactions, ideal to prove the applicability or even superiority of hydrodabcyl (**2**) in labeling small molecules. The dipeptide Ser-Phe was chosen because it is a preferential cleavage site of the P1' protease thermolysin, an enzyme that is commercially available. Furthermore, the same dipeptide labeled with EDANS and dabcyl (**1**) had already been tested with several proteases[14], therefore allowing a comparison of dabcyl (**1**) and hydrodabcyl (**2**) during a typical biochemical application. It should be noted that in this case hydrodabcyl (**2**), due to its high polarity, needs to have its hydroxy groups protected by acetylation prior to the amide bond formation in order to increase yields and reactivity. Subsequent deprotection under mild conditions then restores the advantageous polarity and solubility of the conjugate (*cf.* SI).

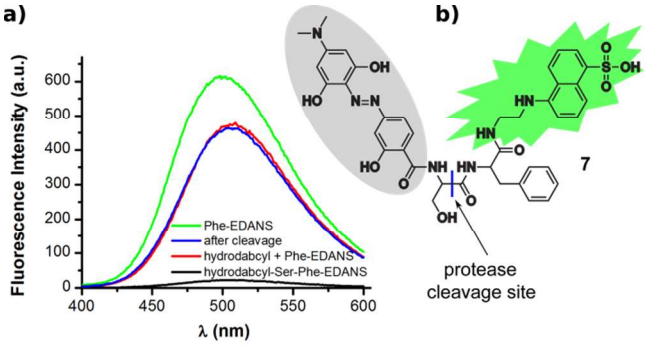


Figure 4. a) Fluorescence spectra of 20 µM hydrodabcyl-Ser-Phe-EDANS (**7**) alone (black) and after 5 h incubation with thermolysin (blue). For comparison, the fluorescence spectra of 20 µM Phe-EDANS alone (green) and an equimolar amount of hydrodabcyl (**2**) + Phe-EDANS (red). The spectra were recorded in 100 mM Tris-HCl (pH 7.5) containing 2 mM $CaCl_2$ at 37 °C; λ_{Ex}= 336 nm. b) Chemical structure of hydrodabcyl-Ser-Phe-EDANS (**7**) The quencher hydrodabcyl (**2**) and the fluorophore EDANS are marked in gray and green, respectively. The protease cleavage site is indicated by a blue bar.

In the double-labeled substrate, the fluorescence of EDANS linked to the Phe moiety is largely quenched by hydrodabcyl (**2**) linked to the Ser moiety (black curve in Figure 4). The fluorescence of EDANS decreases also when an equimolar amount of hydrodabcyl (**2**) is added to Phe-EDANS (green and red curves in Figure 4). However, when the fluorophore and the quencher are not kept close together, as in the double labeled dipeptide, the quenching efficiency is lower in line with the distance-dependence of energy-transfer-based processes. Interestingly, the fluorescence intensity curve detected when the labeled dipeptide was incubated with the enzyme thermolysine (blue curve in Figure 4) coincided with the curve of Phe-EDANS in presence of an equimolar amount of hydrodabcyl (**2**) (red curve in Figure 4) and not with the fluorescence intensity curve of Phe-EDANS alone (green curve in Figure 4). This finding indicates that the hydrodabcyl moiety has a certain quenching ability even when not covalently linked to the fluorophore and that this ability is roughly the same for hydrodabcyl (**2**) and its amide with serine. This fact has to be taken into account during the procedure to determine the reference points for calibrating the signal accurately. Several type of physical quantities can be estimated provided the appropriate calibration of the signal is made.

Figure 5a shows the restoration of the fluorescence of EDANS as a function of time upon adding different concentrations of thermolysin to a solution of the labeled dipeptide. As expected, the larger the excess of the substrate with respect to the enzyme, the slower the reaction. Interestingly, despite the different concentration ratio between labeled substrate and enzyme, the same fluorescence level is eventually reached, indicating that the same amount of substrate molecules has been cleaved. When the enzyme is added in stoichiometric amount (1:1) to the labeled substrate the reaction is so fast that it cannot be observed on the chosen time scale. Under these conditions, the initial amount of substrate is expected to be fully cleaved, defining the maximum fluorescence level corresponding to a complete cleavage of the substrate. Consequently, since all the time traces reached the same fluorescence level, we conclude that the cleavage reaction proceeded to completion, independently of the amount of enzyme added. In order to confirm the exhaustive cleavage of the substrate, fresh enzyme was added to the mixture of substrate and enzyme after reaching signal saturation. Since no appreciable fluorescence increase was observed, we excluded the presence of non-cleaved substrate and concluded that the fluorescence level truly corresponds to the fluorescence signal of the fully processed substrate.

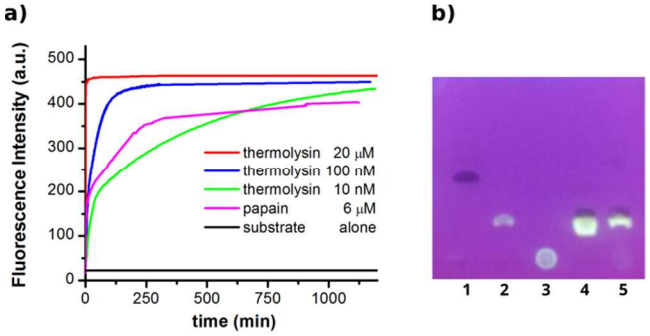


Figure 5: a) Time traces of the hydrolysis of 20 μM of hydrodabcyl-Ser-Phe-EDANS (**7**) at 37 °C, (λ_{Ex}= 336 nm, λ_{Em}=515 nm) by thermolysin (different concentrations) and papain (6 μM). Thermolysin was added in different enzyme to substrate concentration ratios: 1:2000 (green), 1:200 (blue), 1:1 (red). The magenta curve describes the hydrolytic reaction by papain. The black line corresponds to the fluorescence level of the substrate alone, before adding the enzyme. b) Products of hydrodabcyl-Ser-Phe-EDANS after (**7**) proteolytic cleavage indicated by TLC: hydrodabcyl-Ser-Phe-EDANS (**7**) (lane 1, R_f = 0.5), Phe-EDANS (lane 2, R_f = 0.3), EDANS (lane 3, R_f = 0 - 0.1), reaction mixture after proteolytic cleavage with thermolysin (lane 4, bright spot R_f = 0.3 and dark spot R_f = 0.33) and with papain (lane 5, bright spot R_f = 0.3 and dark spot R_f = 0.33).

Additional evidence for the cleavage of the substrate was provided by the determination of the degradation product with thin-layer chromatography (TLC) (Figure 5b). Hydrodabcyl (**2**) confers an orange-red color to the substrate to which it is attached making it visible under UV light as a dark spot. Accordingly, the double-labeled substrate hydrodabcyl-Ser-Phe-EDANS (**7**) appeared as a dark spot with a retention factor (R_f) of 0.5 (Figure 5b, lane 1). The references of the potential products EDANS and Phe-EDANS are shown as white spots in lanes 2 and 3 of Figure 5b. In agreement with the work of Weimer et al.[14], EDANS remained close to the starting line (R_f = 0 - 0.1), whereas Phe-EDANS had an R_f value of 0.3. The mixture resulting from the reaction of thermolysin with an excess of double-labeled substrate (ratio 1:200) was applied in lane 4. The presence of a bright spot with the same R_f as Phe-EDANS together with the absence of a dark spot with the same R_f as the intact double-labeled substrate indicates that thermolysin cleaved the Ser-Phe peptide bond in the double-labeled substrate. The dark spot with an R_f of 0.33 overlapping with the bright spot of Phe-EDANS most likely corresponds to hydrodabcyl-Ser, the other product of the cleavage. Interestingly, the R_f of the putative spot of hydrodabcyl-Ser is lower than 0.53, the R_f of the putative spot of dabcyl-Ser, as reported in the literature[14] . The same trend can be recognized when observing that the double-labeled substrate including hydrodabcyl (**2**) has a lower retention factor (R_f = 0.5) than the one including dabcyl (**1**) (R_f = 0.72) [14]. As a lower retention factor R_f is an indication of higher polarity, this result is in keeping with the higher polarity conferred by hydrodabcyl (**2**) as compared to dabcyl (**1**). Taken together our results demonstrate the retention of the activity of thermolysin in cleaving the peptide bond between serine and phenylalanine in the hydrodabcyl-Ser-Phe-EDANS (**7**) despite the presence of the chromophores. They also corroborate the usefulness and the advantages of hydrodabcyl (**2**) in fluorogenic probes.

Furthermore, we tested the proteolysis of hydrodabcyl-Ser-Phe-EDANS (**7**) by another enzyme, the cysteine protease papain (Figure 5). In the work of Weimar et al.[14], where the fluorogenic substrate was dabcyl-Ser-Phe-EDANS, only a small portion of the dipeptide was processed after an incubation of several hours with papain. In our experiment, we incubated the same amount of hydrodabcyl-Ser-Phe-EDANS (**7**) (20 μM) with 6 μM of papain. This relatively high concentration of papain was necessary to allow the detection of the reaction in a comparable time range. This shows that papain is much slower than thermolysin in cleaving the dipeptide, in agreement with the findings of Weimar et al.[14]. However, in contrast to them, papain was able to cleave the entire amount of the fluorogenic substrate, when dabcyl (**1**) was substituted with hydrodabcyl (**2**), as evidenced both by the evolution of the fluorescence signal and the analysis of the degradation products by TLC (Figure 5). This result is noteworthy, as it shows that the hydrophobicity of dabcyl (**1**) may indeed represent a handicap for fluorogenic probes when used in biochemical system (e.g. interfering with proteolysis) and points out the higher applicability of hydrodabcyl (**2**).

CONCLUSION

Hydrodabcyl (**2**) is a new dark fluorescence quencher with an optimal solubility-stability-absorption profile. Its small dimension, the absence of charged groups, and its absorption range make hydrodabcyl (**2**) the dark quencher of choice in tandem with many commercially available fluorescence donors. Hydrodabcyl (**2**) overcomes the problem of insolubility in aqueous media, doing away with the need of organic co-solvents, and it lends itself ideally to high-throughput enzymatic tests. Thus, hydrodabcyl (**2**) represents a vastly improved and superior alternative to the very popular dabcyl (**1**) in the design of fluorogenic probes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting data, chemical synthesis and characterization (PDF)

AUTHOR INFORMATION

Corresponding Author

* elisa.bombarda@uni-bayreuth.de.

Notes

The authors declare no competing financial interests. A patent for hydrodabcyl is pending (WO 2016/083611 A1, issued 02.06.2016).

ACKNOWLEDGMENT

The present work was supported by the DFG grant BO 3578/1 and by the RTG 1640 (Photophysics of Synthetic and Biological Multichromophoric Systems). We thank Prof. Dr. G. Matthias Ullmann for fruitful discussions and Markus Petermichl for helping in acquiring NMR spectra.

REFERENCES

(1) Lakowicz, J. R. Principles of fluorescence spectroscopy, 3rd ed.; Springer: New York, 2006.

(2) Hauglang, R. P. Handbook of fluorescence probes and research products, 9th ed.; Molecular Probes, Inc.: Eugene, OR (USA), 2002.

(3) Johansson, M. K.; Cook, R. M. *Chem. - Eur. J.* **2003**, *9*, 3466–3471.

(4) Bernacchi, S.; Mely, Y. *Nucleic Acids Res.* **2001**, *29*, E62-2.

(5) Matayoshi, E. D.; Wang, G. T.; Krafft, G. A.; Erickson, J. *Science* **1990**, *247*, 954–958.

(6) Tyagi, S. *Nat. Biotechnol.* **1996**, *14*, 947–948.

(7) Tyagi, S.; Marras, S. A.; Kramer, F. R. *Nat. Biotechnol.* **2000**, *18*, 1191–1196.

(8) Holskin, B. P.; Bukhtiyarova, M.; Dunn, B. M.; Baur, P.; de Chastonay, J.; Pennington, M. W. *Anal. Biochem.* **1995**, *227*, 148–155.

(9) Balaiah, V.; Seshadri, T. R.; Venkateswarlu, V. *Proc. - Indian. Acad. Sci.* **1942**, *16*, 68–82.

(10) Avdeef, A.; Sofen, S. R.; Bregante, T. L.; Raymond, K. N. *J. Am. Chem. Soc.* **1978**, *100*, 5362–5370.

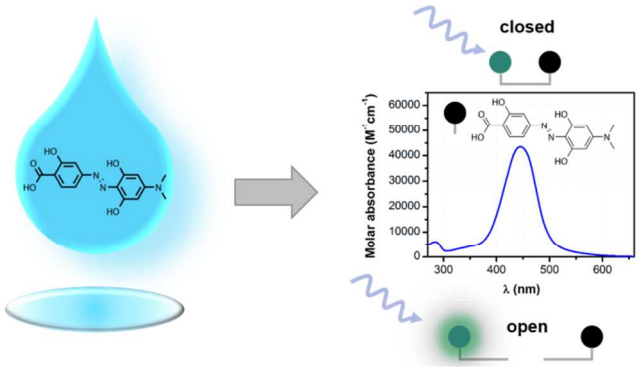
(11) Petrzilka, T.; Lusuardi, W. G. *Helv. Chim. Acta* **1973**, *56*, 510–518.

(12) Loudwig, S.; Bayley, H. *J. Am. Chem. Soc.* **2006**, *128*, 12404–12405.

(13) Lide, D. R. CRC Handbook of Chemistry and Physics, 85th Edition.

(14) Weimer, S.; Oertel, K.; Fuchsbauer, H. L. *Anal. Biochem.* **2006**, *352*, 110–119.

For TOC only


closed
Molar absorbance (M⁻¹cm⁻¹)
60000
50000
40000
30000
20000
10000
0
300
400
500
600
λ (nm)
open