

Supporting Information

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

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Chemicals and instrumentation

Chemicals

EDANS (*N*-(2-aminoethyl)-5-naphthylamine-1-sulfonic acid) and *N*-*tert*-butoxycarbonyl-L-phenylalanine (Boc-Phe) were purchased from Anaspec, *N*-*tert*-butoxycarbonyl-*O*-*tert*-butyl-L-serine (Boc-Ser(*t*Bu)-OH) was obtained from Bachem (Bubendorf, Switzerland). T₃P-coupling reagent (propanephosphonic acid anhydride, 2,4,6-tripropyl-1,3,5,2,4,6-trioxatriphosphorinane-2,4,6-trioxide, 50 % in THF) was a free sample from Euticals SpA, Italy. Thermolysin from *Geobacillus stearo*, papain from *Papaya latex*, DMAP (4-(dimethylamino)-pyridine) and all other chemicals were purchased from Sigma-Aldrich and used without further purification, unless indicated otherwise. The aqueous solution used in this work was a 50 mM sodium phosphate buffer (NaP) pH 8.0.

NMR spectroscopy

The ¹H and ¹³C NMR spectra were taken on a Bruker Avance 300 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) referenced with respect to residual solvent (CHCl₃ = 7.26/77.16 ppm, DMSO = 2.50/39.52 ppm)¹.

Melting point

Melting points were measured with a Melting Point B-565 (Buchi) in melting point tubes.

Mass spectrometry

High resolution mass spectra were obtained with a UPLC/Orbitrap MS system in ESI mode.

IR spectroscopy

IR spectra were recorded with a FT-IR spectrometer PerkinElmer S100 equipped with an Attenuated Total Reflection (ATR) unit.

Chromatography stationary phases

For column chromatography silica gel (Macherey-Nagel 60, particle size 0.040 - 0.063 mm) was used. The retention factor (R_f) values were determined by thin layer chromatography on pre-coated silica plates (Merck TLC Silica gel 60 F254) and spots were visualized by UV light and stained with ceric ammonium molybdate (CAM) solution, followed by treatment with a heat gun.

HPLC

HPLC was performed on Phenomenex RP Kinetex 5 μ C18 100 Å, 250 \times 4.6 mm (analytical) and 250 \times 21.2 mm (preparative) column. 0.1 % HCOOH/H₂O and methanol (MeOH) were used as eluents for HPLC experiments with flow rate of 0.7 mL/min (analytical) and 14.9 mL/min (preparative).

UV/Vis spectroscopy

A double beam Perkin Elmer Lambda 750 UV/Vis spectrophotometer equipped with a thermostated cuvette holder was used to record the absorption spectra over a wavelength range of 270 - 650 nm at 20 °C in quartz cuvettes with 1 cm light path (Hellma). Fluorescence was measured with a Cary Eclipse fluorimeter (VARIAN) equipped with a thermostated cuvette holder. Origin 8 (Microcal) was used for data analysis.

pH measurements

The pH was measured with the Mettler-Toledo SevenMulti pH-meter equipped with a micro-electrode (InLab Micro, Mettler-Toledo).

Ultrasound bath

When required, solutions were degassed in an ultrasonic bath Bandelin SONODEX Super RX 106 (240 W) *in vacuo*.

Chemical Synthesis

Hydrodabcyl (2)

The compound 4-(2',6'-dihydroxy-4'-dimethylaminophenylazo)-2-hydroxybenzoic acid (hydrobadcyl (2)) was synthesized in two steps as shown in Scheme 1 in the main text.

In the first step 5-dimethylamino-resorcinol (4) was prepared by an optimized variant of a protocol published by Petržilka and Lusuardi². The optimization implies the addition of a purification over silica gel yielding colorless crystals, rather than pink ones as described in the literature². We attributed the reported pink color to a contamination by a degradation product. This assumption is supported by the observation that the product turns pink when it is stored for several weeks in the refrigerator. If not removed, this contamination leads to a fluorescent by-product in the following step, which cannot be separated even by HPLC. Our optimized method avoids the formation of this by-product. We did not identify the impurity. However, our results suggest that the compound that was responsible of the pink discoloration was also responsible for the fluorescence of the final product. Although minimal, this modification of the protocol is requisite for obtaining 5-dimethylamino-resorcinol (4) in a pure form, and it also proves that the reaction between 4 and 4-diazosalicylic acid (5) does not lead to a fluorescent product, hence opening the way to obtain a water soluble dark quencher.

a)



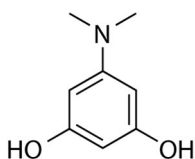
b)



Figure S1. Crystals of 5-dimethylamino-resorcinol (4). The pink crystals in a) were obtained following the protocol of Petržilka and Lusuardi² and the colorless crystals in b) were obtained following our improved protocol.

5-Dimethylamino-resorcinol² (4)

Phloroglucinol (**3**, 9.23 g, 73.2 mmol) was dissolved in a degassed mixture of DMF (128 mL) and water (95 mL) under argon. Dimethylamine hydrochloride (8 g, 91.5 mmol) was added and then pellets of solid sodium hydroxide (3.66 g, 91.5 mmol) were added one by one during 5 h. The resulting mixture was stirred overnight at room temperature. Over the next 48 h, the addition of dimethylamine hydrochloride and sodium hydroxide was repeated three times (3×9.15 mmol each). The resulting solution was concentrated *in vacuo* and the residue was purified by column chromatography (cyclohexane / ethyl acetate 1/1, R_f 0.38). The product was crystallized from dichloromethane to obtain colorless crystals (8.2 g, 73 %) which were used immediately for the next step.



Melting point: 151 °C (142 - 143 °C)²

¹H-NMR (300 MHz; DMSO-*d*₆; Me₄Si): δ = 8.81 (s, 2 H), 5.60 (s, 3 H), 2.78 (s, 6 H) ppm.

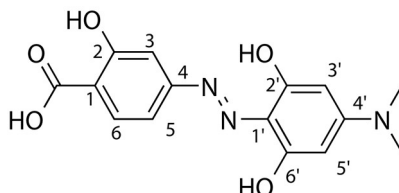
¹³C-NMR (75 MHz; DMSO-*d*₆; Me₄Si): δ = 158.8 ($\times 2$), 152.3, 92.0 ($\times 2$), 91.5, 40.1 ($\times 2$) ppm.

IR (ATR): $\tilde{\nu}$ = 3276, 2972, 2884, 2512, 1604, 1512, 1462, 1435, 1377, 1347, 1312, 1276, 1247, 1128, 1042, 1005, 987, 853, 831, 809, 686, 634, 576 cm⁻¹.

4-(2',6'-Dihydroxy-4'-dimethylaminophenylazo)-2-hydroxybenzoic acid (**2**) from azo-coupling³ of **4** with 4-diazosalicylic acid (**5**)

An ice-cold freshly prepared aqueous 2.5 M solution of sodium nitrite (9 mL, 22.5 mmol) was added dropwise to a solution of 4-aminosalicylic acid (**6**, 3.46 g, 22.5 mmol) in 6 M solution of hydrochloric acid (6 mL) at 0 - 5 °C. The resulting suspension was then stirred for another 15 min and added dropwise to a mixture of **4** (3.45 g, 22.5 mmol) and 1 M aqueous sodium hydroxide solution (23.5 mL) at 0 - 5 °C. The mixture was heated at 70 °C for 15 min and then stirred for 1 h at room temperature. Methanol was added to the resulting red suspension. This mixture was then put in an ultrasonic bath for some minutes. The impurities were filtered and the filtrate was evaporated. The remainder was diluted with 0.1 M sodium hydroxide solution, filtered, and the filtrate was treated with formic acid and ethanol adjusting a pH < 4.0. This mixture was placed in the fridge for about 15 h, and then centrifuged for 10 min at 4 °C (Heraeus Multifuge X3 FR Thermo Scientific Centrifuge operating at 4283 rpm). The pellet obtained was suspended in 0.1 % formic acid and again centrifuged

as before; this procedure was repeated three times. Then the residue was re-suspended in double-distilled water and centrifuged. This procedure was repeated twice. Finally, the product was dispersed in double-distilled water by means of an ultrasonic bath and then frozen in liquid nitrogen and dried by lyophilisation. Pure final product **2** was obtained as a red powder (5.7 g, 80 %), which was stored in a dark fridge at 4 - 8 °C. HPLC: T = 16.5 min (0.1 % HCOOH/H₂O, 55 - 45 v/v in MeOH, λ_{max} = 455 nm);



Melting point: 253 °C

¹H-NMR (300 MHz; DMSO-*d*₆; Me₄Si): δ = 7.80 (d, *J* = 8.5 Hz, 1 H, 6-H), 7.40 (d, *J* = 1.6 Hz, 1 H, 3-H), 7.18 (dd, *J* = 8.5, 1.6 Hz, 1 H, 5-H), 5.71 (s, 2 H, 3'-H, 5'-H), 3.08 (s, 6 H, NMe), ppm.

¹³C-NMR (75 MHz; DMSO-*d*₆; Me₄Si): δ = 171.6 (COOH), 162.9 (C2', C6'), 157.7 (C2, C4), 151.6 (C4'), 131.2 (C6), 124.4 (arC^q), 110.1 (arC^q), 109.7 (C5), 105.1 (C3), 91.4 (C3', C5'), 40.0 (N(CH₃)₂) ppm.

IR (ATR): $\tilde{\nu}$ = 3352, 3083, 2908, 2737, 2475, 1874, 1656, 1620, 1502, 1473, 1426, 1387, 1337, 1291, 1226, 1191, 1133, 1087, 1018, 983, 965, 886, 847, 811, 776, 729, 675 cm⁻¹.

HRMS-ESI: calcd for [M + H]⁺ C₁₅H₁₆N₃O₅ 318.1085, found 318.1081.

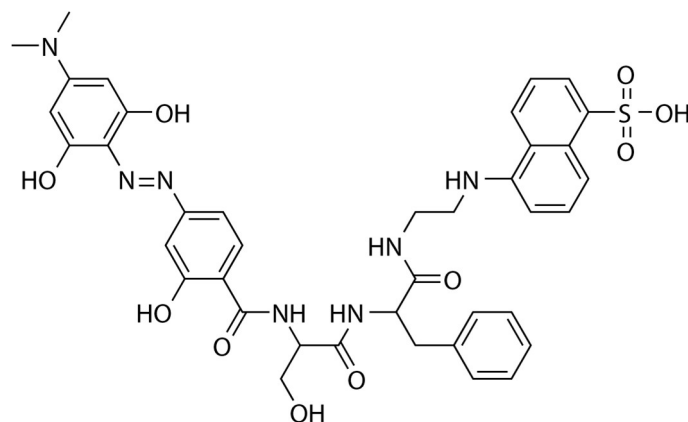
Synthesis of hydrodabcyl-Ser-Phe-EDANS (7)

The synthesis of the substrate hydrodabcyl-Ser-Phe-EDANS was performed according to the method described by S. Weimer *et al.*⁴, with the difference that in the last step triacetylated hydrodabcyl **8** was employed instead of dabcyI (**1**). For the synthesis of **8** see the dedicated paragraph below.

2-*N*-(4-((4-(*N,N*-dimethylamino)-2,6-dihydroxyphenyl)azo)-2-hydroxybenzoyl-L-serinyl-L-phenylalanylamido)-*N*-ethylaminonaphthalene-5-sulfonic acid (7)

A stirred solution of **8** (0.151 g, 0.34 mmol) in 10 mL anhydrous THF was cooled to 0 °C and treated with triethylamine (0.155 mL, 1.113 mmol). A solution of T₃P, 50 % in THF (0.32 mL, 0.534 mmol), was added dropwise. Ser-Phe-EDANS⁴ (0.17 g, 0.34 mmol) was dissolved in 6 mL anhydrous DMF and added to the reaction mixture. The resulting mixture was stirred for 1.5 h at 0 °C and then concentrated *in vacuo*. The crude product was deacetylated by addition of a solution of

piperidine (1 mL) in methanol (30 mL). The reaction mixture was stirred for 1 h at room temperature and the product was purified by HPLC after acidification with formic acid (0.1 % HCOOH/H₂O, 55 - 45 v/v in MeOH).



¹H-NMR (300 MHz; DMSO-*d*₆; Me₄Si): δ = 8.79 (d, *J* = 7.1 Hz, 1 H), 7.80 - 8.39 (m, 7 H), 7.25 - 7.37 (m, 3 H), 7.10 - 7.22 (m, 5 H), 6.55 (dd, *J* = 14.0, 7.4 Hz, 1 H), 5.75 (s, 2 H), 4.76 (dd, *J* = 12.3, 7.4 Hz, 1 H), 4.45 - 4.59 (m, 2 H), 4.39 (br s, 1 H), 3.75 - 3.64 (m, 2 H), 3.15 - 3.21 (m, 2 H), 3.06 - 3.13 (m, 8 H), 2.82 - 2.95 (m, 2 H), 1.89 (s, 1 H) ppm.

¹³C-NMR (75 MHz; DMSO-*d*₆; Me₄Si): δ = 171.2, 169.6, 167.5, 160.1, 158.6, 158.0, 154.2, 150.6, 146.2, 143.4, 137.7, 130.2, 130.1, 129.1 (× 2), 128.0 (× 2), 126.1 (× 2), 124.4, 123.7, 122.7, 122.5, 116.3, 116.2, 113.8, 110.0, 105.8, 104.9, 103.2, 91.6 (× 2), 61.5, 55.5, 54.9, 54.3, 45.8, 43.3, 40.2 (× 2), 37.8 ppm.

IR (ATR): $\tilde{\nu}$ = 3264, 2934, 1635, 1528, 1489, 1418, 1228, 1162, 1049, 1021, 1003, 887, 820, 779, 699 cm⁻¹.

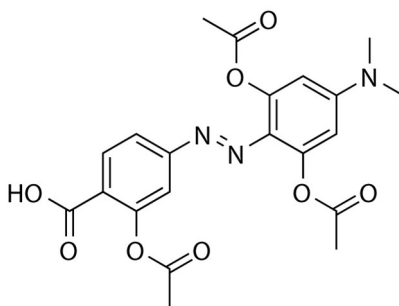
HRMS-ESI: calcd for [M + H]⁺ C₃₉H₄₂N₇O₁₀S 800.2708, found 800.2697.

Synthesis of triacetylated hydrodabcyl

Acetylation of hydrodabcyl was performed based on a literature protocol⁵.

4-(2',6'-Diacetoxy-4'-dimethylaminophenylazo)-2-acetoxybenzoic acid (8)

A mixture of **2** (3.5 g, 11 mmol), dry DMSO (10 mL), and THF (50 mL) was treated with triethylamine (9.2 mL, 66 mmol), DMAP (0.123 mg, 1.1 mmol) and acetic anhydride (4.4 mL, 46.2 mmol), and the resulting mixture was stirred at room temperature overnight. The solvent was evaporated and the remainder was partitioned between ethyl acetate and a 1 M aqueous solution of sodium hydrogen sulfate. The phases were separated and the water layer was extracted with ethyl acetate three times. The combined organic phases were dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residue was purified by column chromatography (ethyl acetate + 0.1 % HCOOH, *R_f* 0.1) and then recrystallized from cyclohexane / diethyl ether, 1:1 to obtain dark red crystals (3.9 g, 80 %).



Melting point: 130 - 131 °C

¹H-NMR (300 MHz; DMSO-*d*₆; Me₄Si): δ = 8.06 (d, *J* = 8.3 Hz, 1 H), 7.56 (dd, *J* = 8.3, 1.8 Hz, 1 H), 7.29 (d, *J* = 1.8 Hz, 1 H), 6.53 (s, 2 H), 3.09 (s, 6 H), 2.30 (s, 6 H), 2.28 (s, 3 H) ppm.

The unprocessed residual peaks in the aromatic region may be sign of partial de-acetylation of the compound after long storage in DMSO and do not represent a major problem. In fact, **8** is an analogue of hydrodabcyl (**2**) that was designed only to make the benzoic acid more susceptible to nucleophilic attack and thus facilitating the coupling of **2** through amide bond formation, as mentioned in the main text. After coupling, the acetyl groups have to be removed, preferably with mild condition. Therefore lability of these groups, as indicated by the partial de-acetylation, is not only tolerated but also solicited.

¹³C-NMR (75 MHz; DMSO-*d*₆; Me₄Si): δ = 169.4, 169.0 (× 3), 165.4, 156.7, 163.4, 151.4, 147.9, 132.9, 125.6, 124.4, 119.2, 116.0, 104.0 (× 2), 40.1 (× 2), 21.1, 20.9 (× 2) ppm.

IR (ATR): $\tilde{\nu}$ = 2925, 2849, 2644, 2536, 1772, 1758, 1684, 1617, 1603, 1538, 1484, 1419, 1355, 1281, 1201, 1184, 1117, 1085, 1044, 1020, 979, 921, 892, 881, 845, 819, 785, 744, 690, 670 cm⁻¹.

HRMS-ESI: calcd for [M + H]⁺ C₂₁H₂₂N₃O₈ 444.1401, found 444.1394.

Solubility tests

To measure the solubility, saturated solutions of the studied compounds were prepared in pure water (resistivity 18 MΩ cm, Milli Q system, Millipore) and buffered aqueous solution pH 8.0 (50 mM sodium phosphate). The solution were centrifuged at 5000 rpm for one hour (HERAEUS SEPATECH Labofuge A). The concentration of the supernatant was determined by absorption spectroscopy at 20 °C after appropriate dilution to avoid instrumental saturation.

To test the solubility of hydrodabcyl at different pH in a practical range of concentrations, buffered solutions (50 mM sodium phosphate) have been prepared at four different pH: 8.0, 7.0, 6.0 and 5.5, respectively. The amount of hydrodabcyl required to reach a concentration of 5 mM was immersed in each buffer-solution. Since we observed that keeping the solution 1 h at 30 °C in an ultrasonic bath was unnecessary for obtaining the solubility, this additional procedure was abandoned for the preparation reported here.

The solubility of hydrodabcyl was confirmed by monitoring the absorbance in function of its concentration (Figure 2 in the main text and Figure S2). Since the stock solution was too concentrated to be measured directly, three diluted solutions with different concentrations (10 μM, 20 μM and 30 μM) were prepared from the stock solution. The aliquots were taken from different part of the volume of the stock solution to test the homogeneity. The linear increase of the absorbance with the concentration of the solute according to the Lambert-Beer law indicates that the stock solution is homogeneous (Figure 2b in the main text, black line). The same experiment was successfully repeated at pH 6 to confirm the solubility also at this slightly acidic pH (Figure S2). Instead, to reach complete solubility at pH < 6.0, the pH of the stock solution (5 mM range) at pH 7.0 was decreased at the desired value with few drops of 5 M HCl solution. Following gradual acidification of the medium the mM solution of hydrodabcyl remained clear till pH 4.3. With the same procedure described above, we could prove the solubility of hydrodabcyl till pH 4.3 (Figure 2 in the main text, red line). At pH < 4.3 hydrodabcyl precipitates, therefore pH 4.3 has to be considered the lowest pH at which the solubility of hydrodabcyl in the mM range can be warranted.

The experimental data in Figure 2 in the main text and Figure S2 were analyzed according to Beer–Lambert law with the equation:

$$A_{445} = \varepsilon_{445} \cdot c \cdot l \quad (1)$$

where A_{445} is the absorbance at 445 nm, l is the path length of the light beam through the sample (in our case 1 cm), c is the molar concentration of the sample, ε_{445} is the molar extinction coefficient of the sample at 445 nm. The slope of the straight line is $\varepsilon_{445} \cdot l$. Since the path length l is known, this

procedure allows also to determine the extinction coefficient or molar absorbance of the dye at the indicated wavelength

Table S1. Summary of the results of the solubility-tests (NaP buffer = Sodium-phosphate buffer)

Solvent	Substance	Solubility	Comments
Water, 20 °C	hydrodabcyl	5.71×10^{-4} M	pH = 4.5
	dabcyl	n.d.	not determined due to precipitation
50 mM NaP buffer, pH = 8.0	hydrodabcyl	2.54×10^{-2} M	
	dabcyl	5.41×10^{-4} M	
50 mM NaP buffer, pH = 8.0	Lys-hydrodabcyl	$> 6.61 \times 10^{-3}$ M	The solution was not saturated ^a
	Lys-dabcyl	7.62×10^{-6} M	

^a In the preparation of the solution of Lys-hydrodabcyl we were limited by the product availability and not by its solubility.

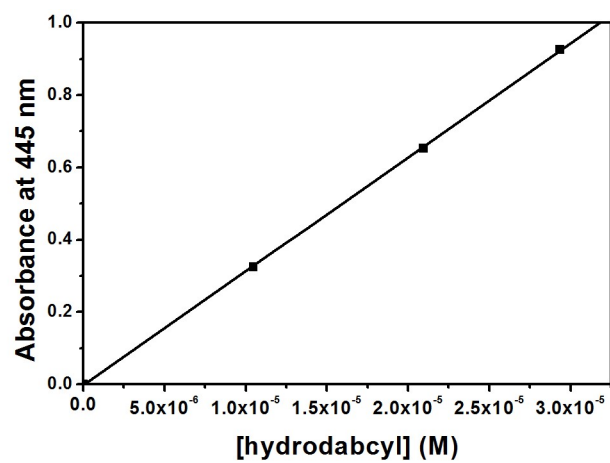


Figure S2. Concentration dependence of the absorbance at 445 nm of hydrodabcyll in buffered solution pH 6.0 at $T = 20\text{ }^{\circ}\text{C}$.

Fluorimetric and TLC assays

Fluorimetric assay

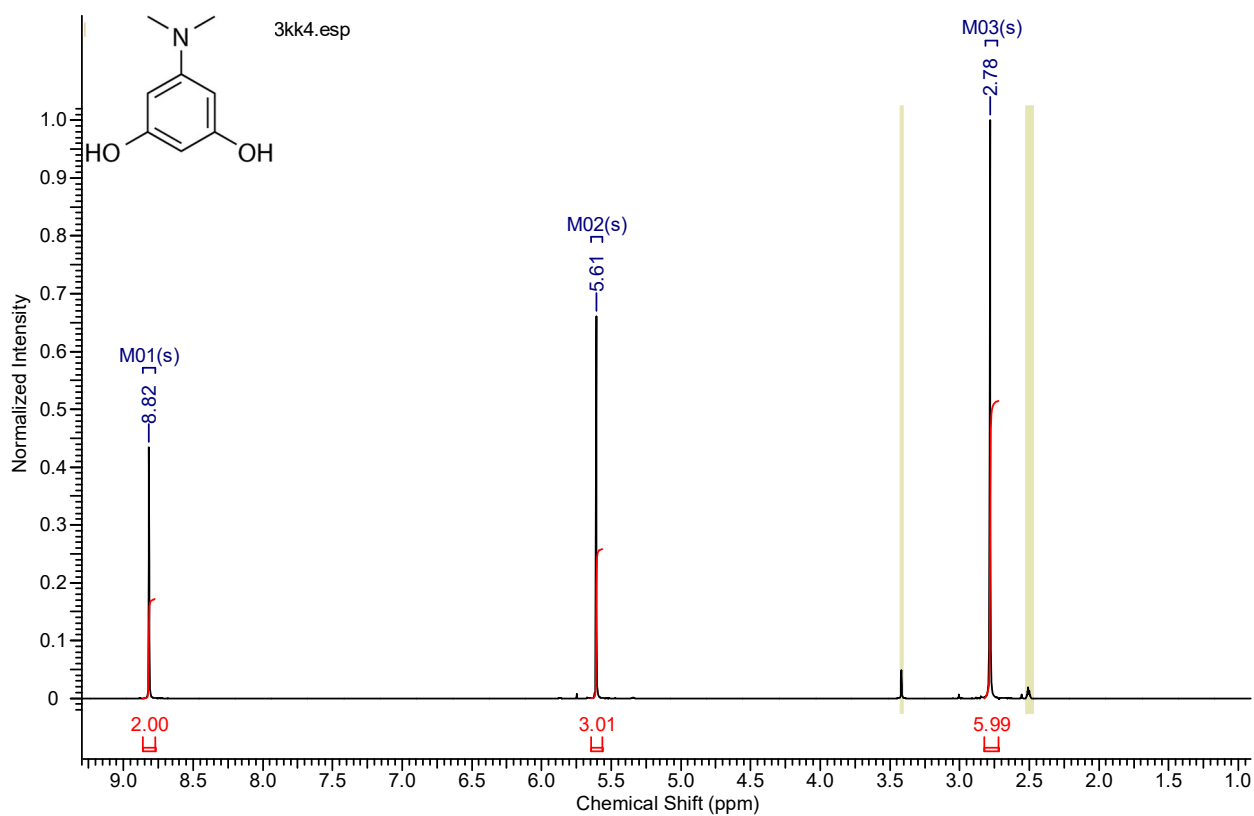
The fluorimetric assay with hydrodabcyI-Ser-Phe-EDANS was performed according to the literature⁴. Stock solutions of the substrate with a concentration of 1 mM were made in 100 mM Tris–HCl (pH 7.5) containing 2 mM CaCl₂ (thermolysin) or in 100 mM citrate pH 6.5 (papain). The concentration of the stock solutions was 6.6 mg/mL for thermolysin 4 mg/ml for papain. In typical experiments, 20 μM of the double-labelled dipeptide was incubated in 100 mM Tris–HCl/2 mM CaCl₂ (pH 7.5) or 100 mM citrate (pH 6.5) at 37 °C for 10min before hydrolysis was initiated by adding an aliquot of the protease stock solution (final concentrations of 20 μM, 100 nM and 10 nM for thermolysin and 6.0 μM for papain). Emission (λ_{ex} 336 nm, λ_{em} 515 nm) was monitored using 1-cm-pathlength cuvettes. All data given in the figures represent the means of at least three measurements. In all the experiments, the same amount (20 μM) of hydrodabcyI-Ser-Phe-EDANS (7) was used to allow the quantitative comparison of the fluorescence levels.

Thin-layer chromatography experiment

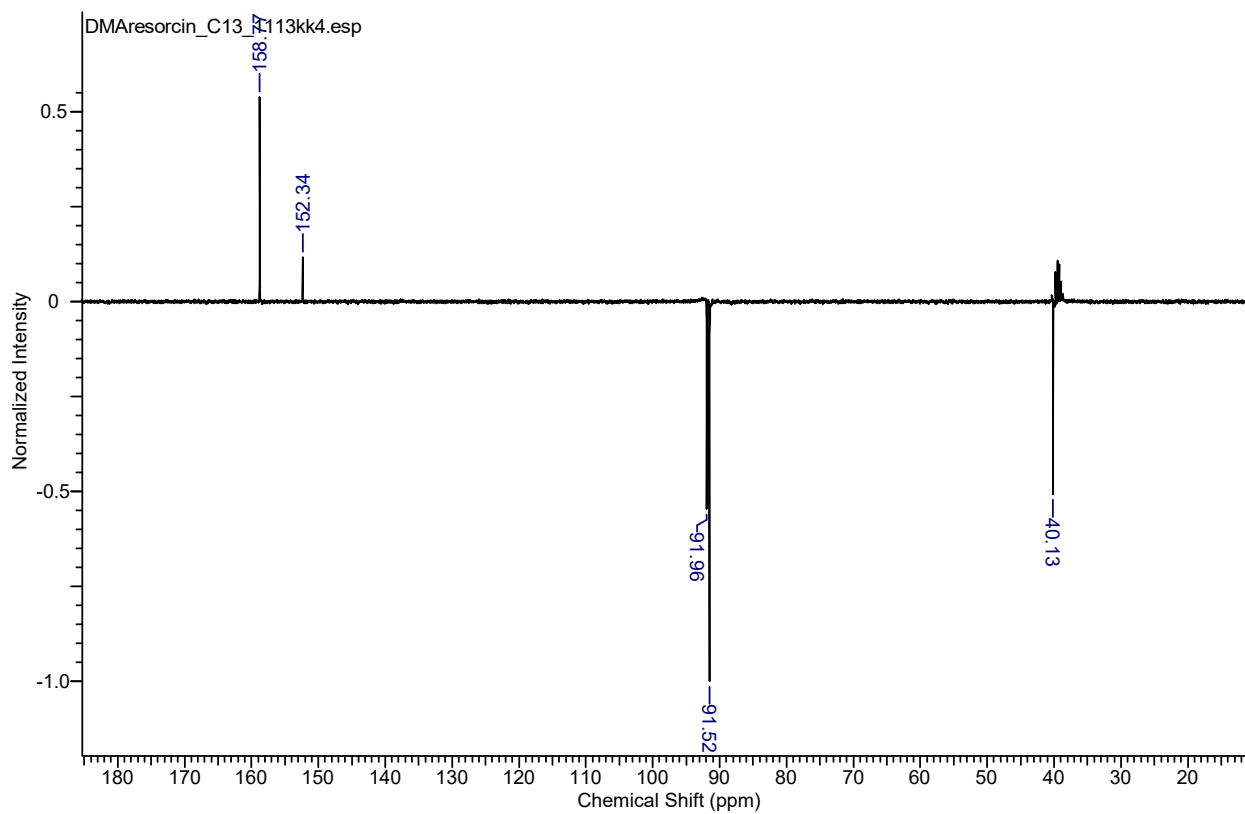
Thin-layer chromatography (TLC) was used to determine the degradation products of the proteolytic cleavage of hydrodabcyI-Ser-Phe-EDANS (7, Figure 5b in the main text). The analyzed reaction mixtures were incubated overnight, applied onto silica 60 aluminum sheets, separated by trichloromethane/methanol/water (65:25:4), and irradiated with an UV lamp at 340 nm

NMR spectra

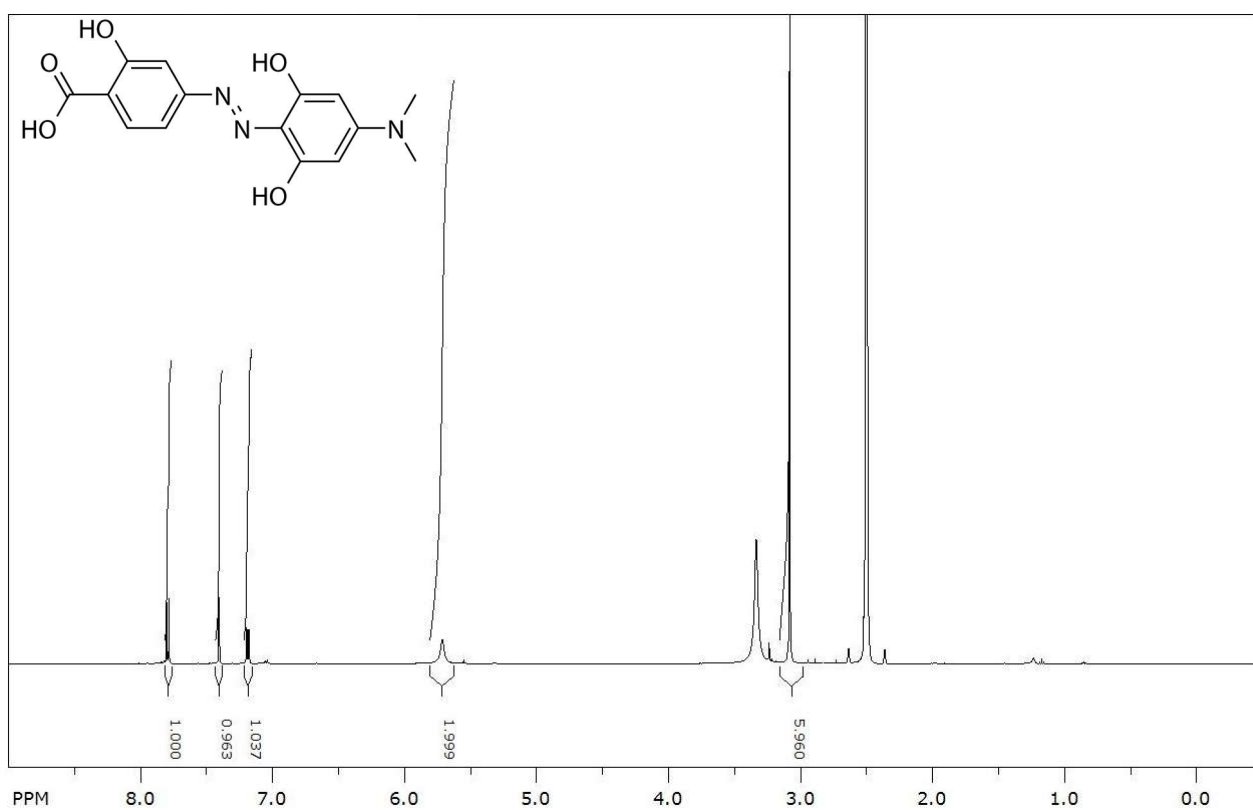
^1H NMR of compound **4** in $\text{DMSO-}d_6$



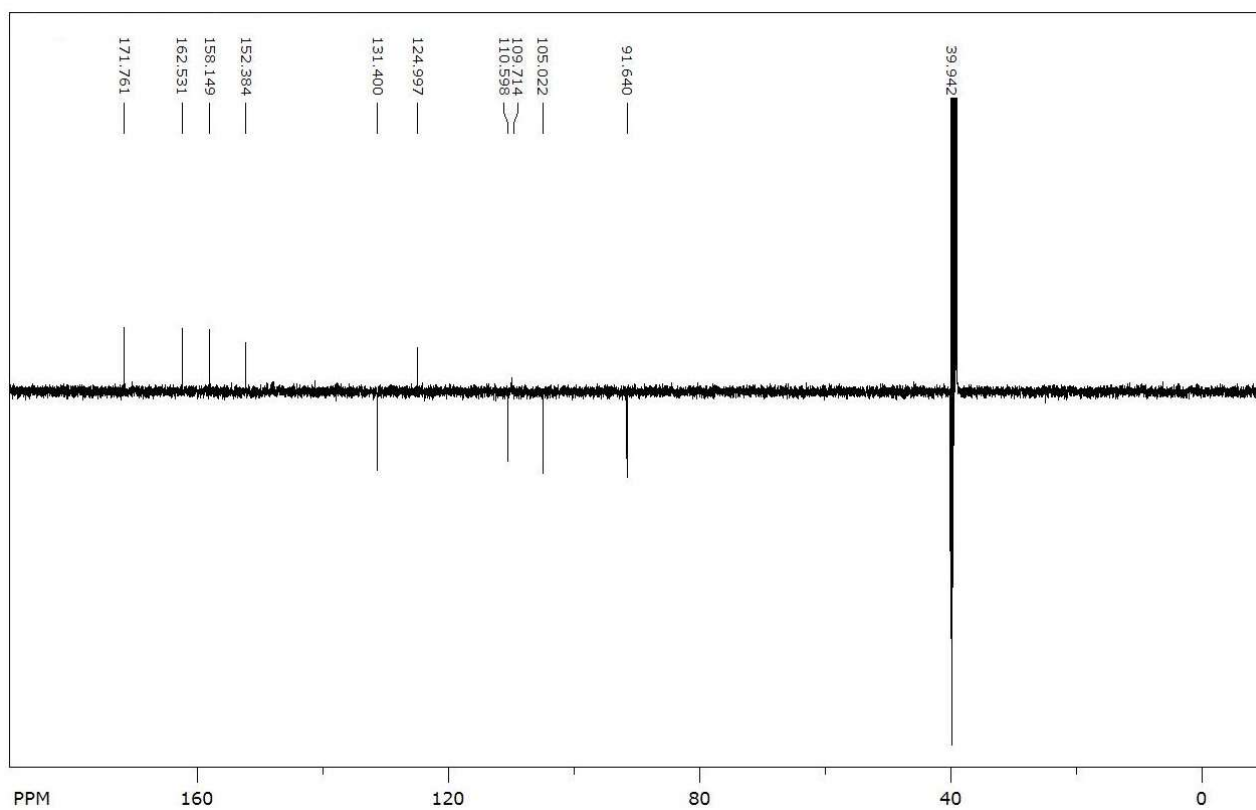
^{13}C NMR of compound **4** in $\text{DMSO-}d_6$



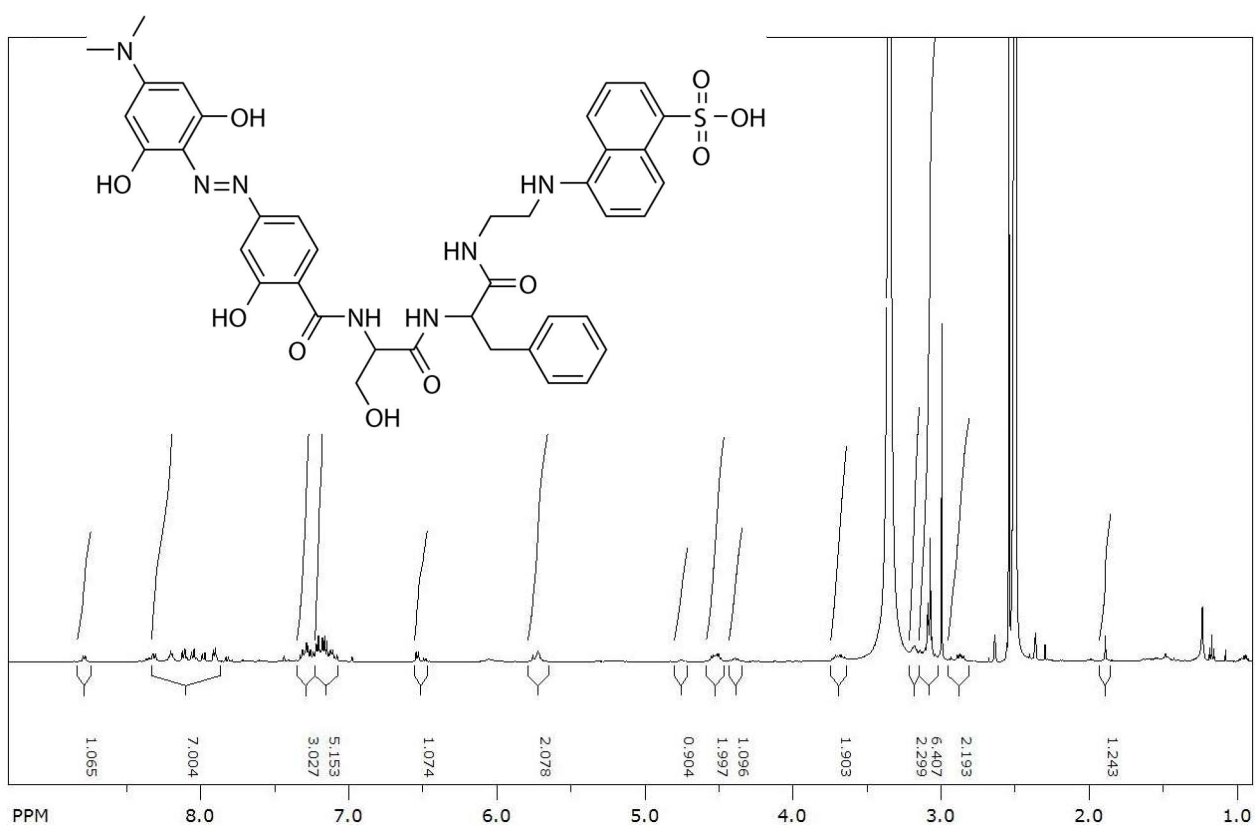
^1H NMR of compound **2** in $\text{DMSO}-d_6$



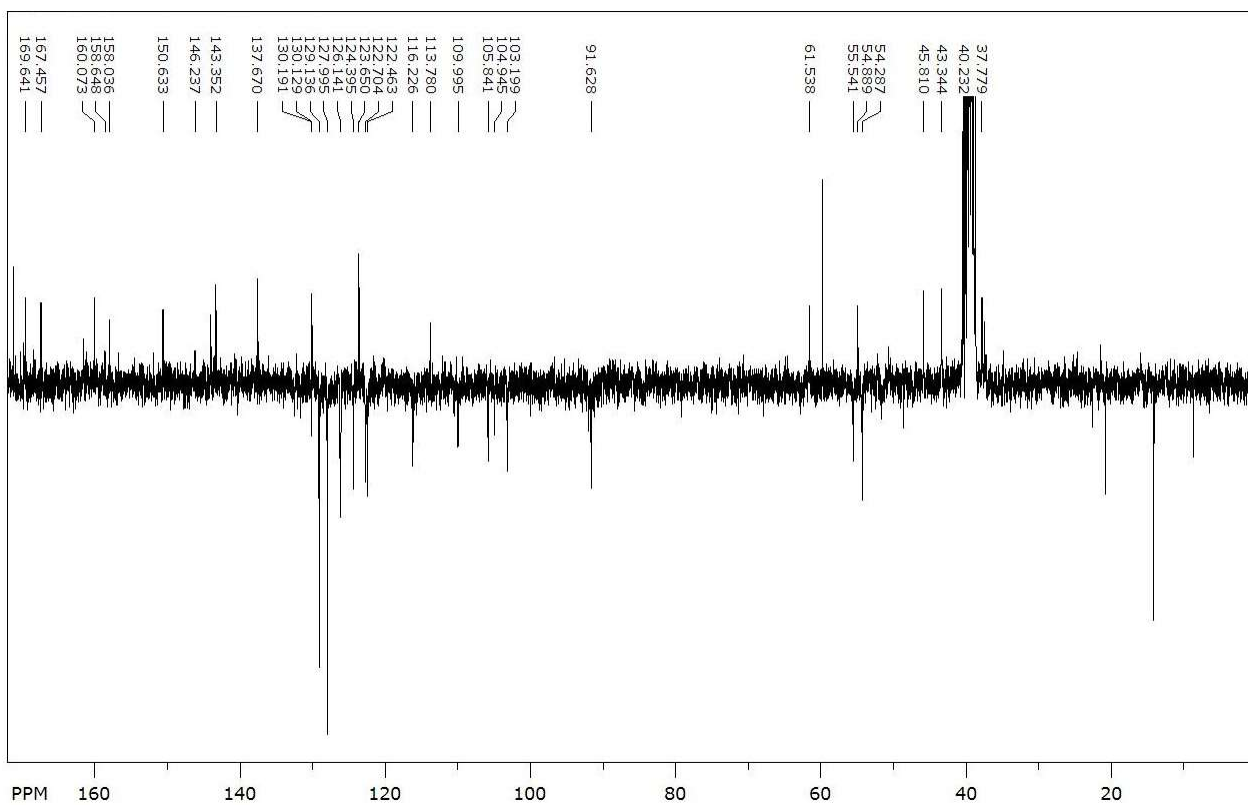
^{13}C NMR of compound **2** in $\text{DMSO}-d_6$



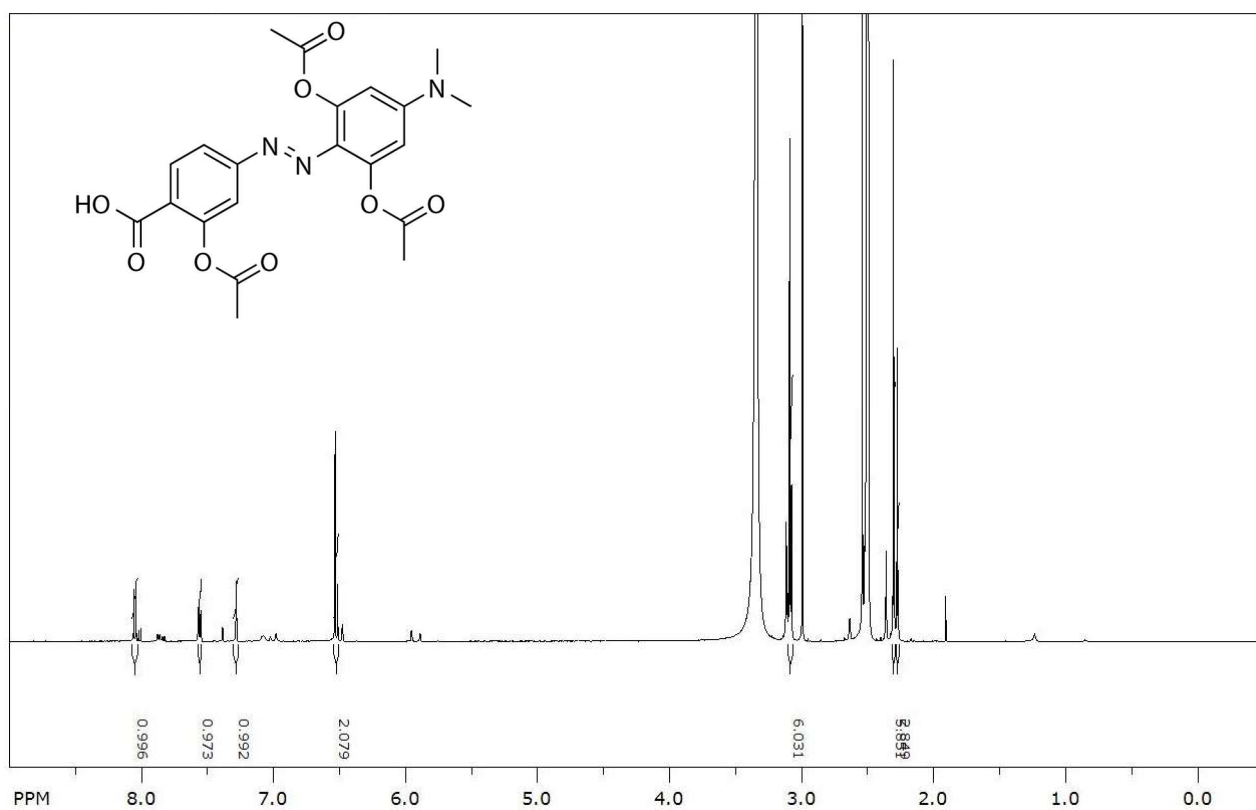
¹H NMR spectrum of compound 7



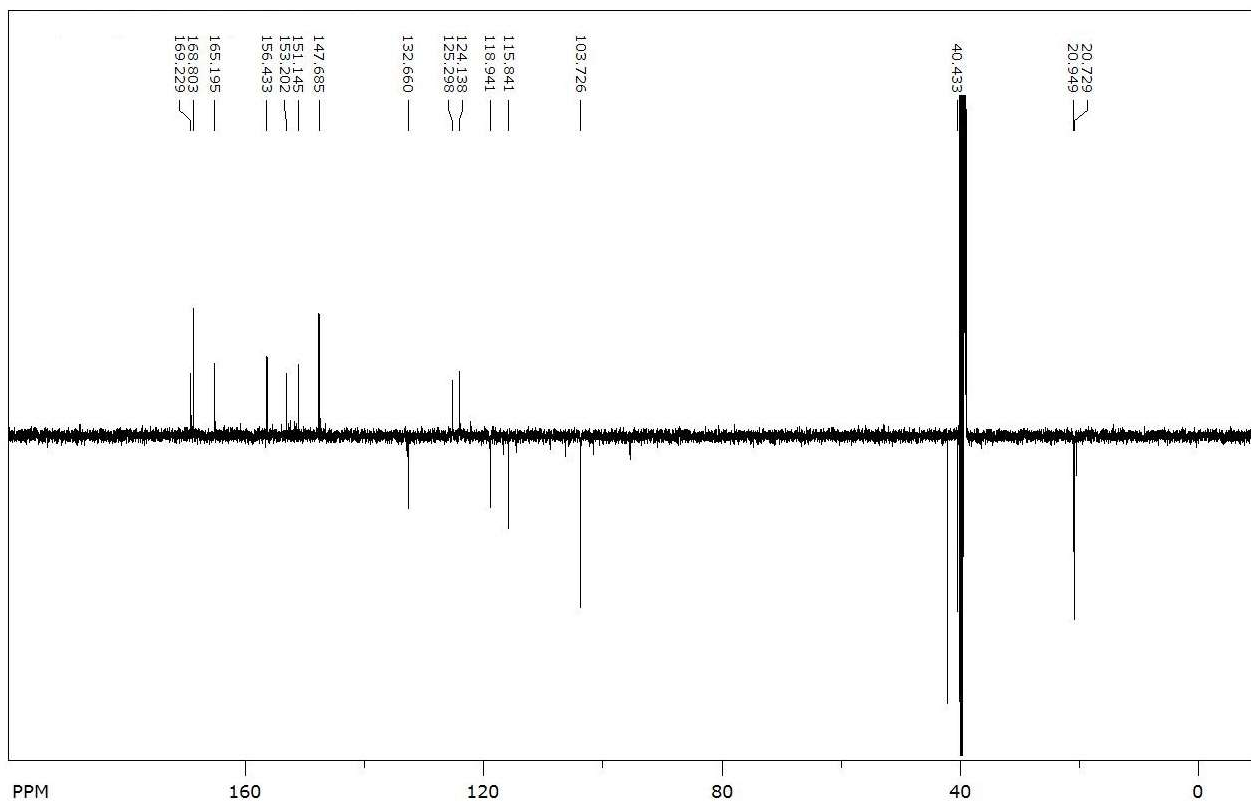
¹³C NMR spectrum of compound 7



^1H NMR spectrum of compound **8**



^{13}C NMR spectrum of compound **8**



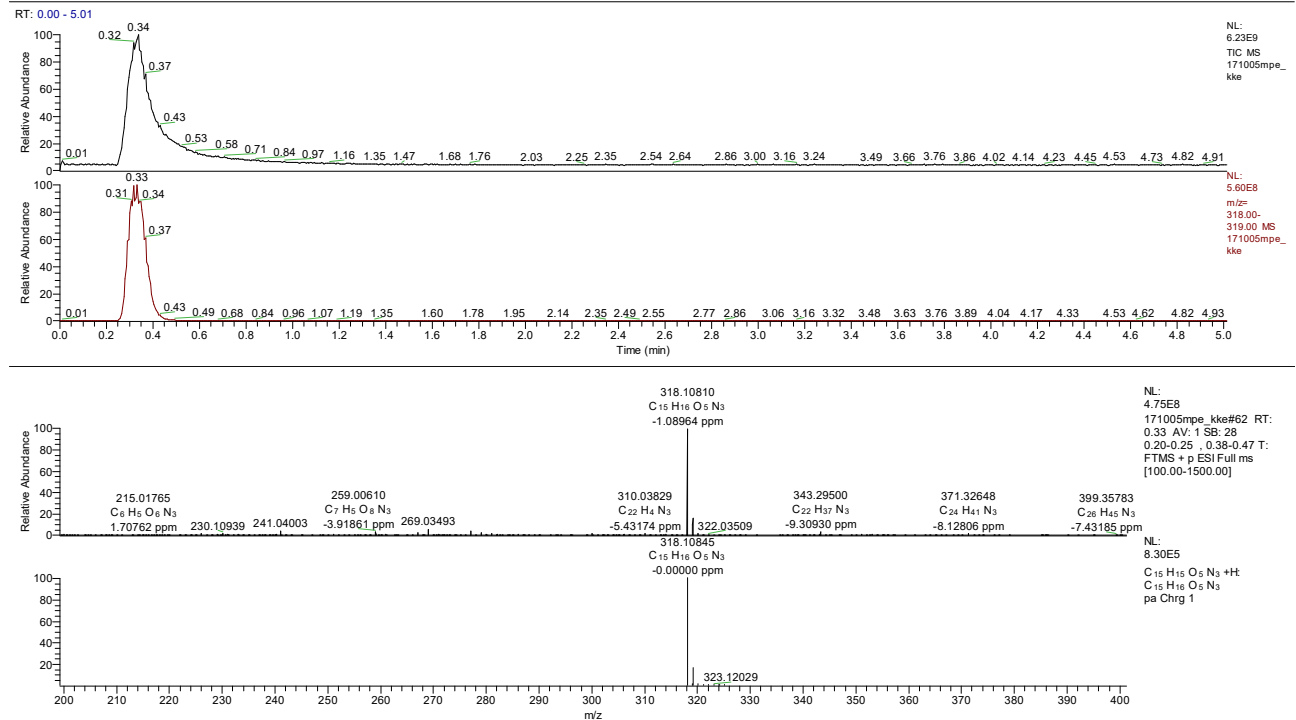
Mass Analysis

Hydrodabcyl (2)

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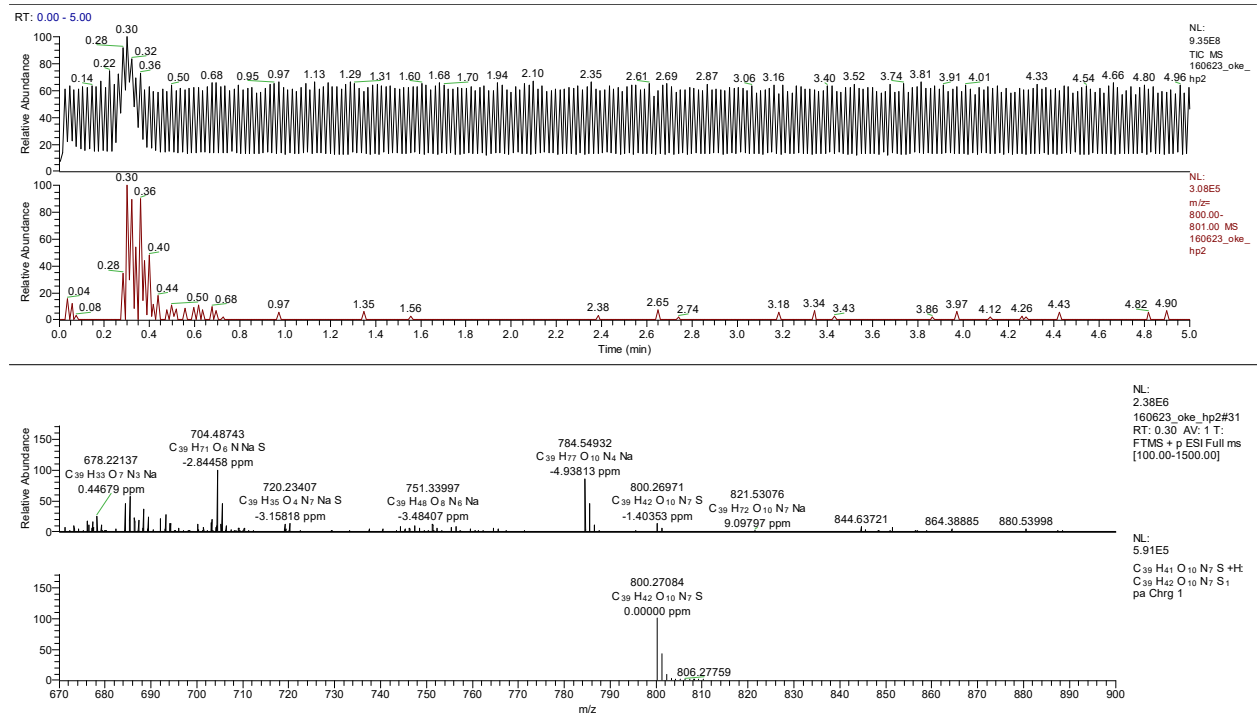
DAB(OH)3 KKe MPe in DMSO



Hydrodabcyl-Ser-Phe-EDANS (7)

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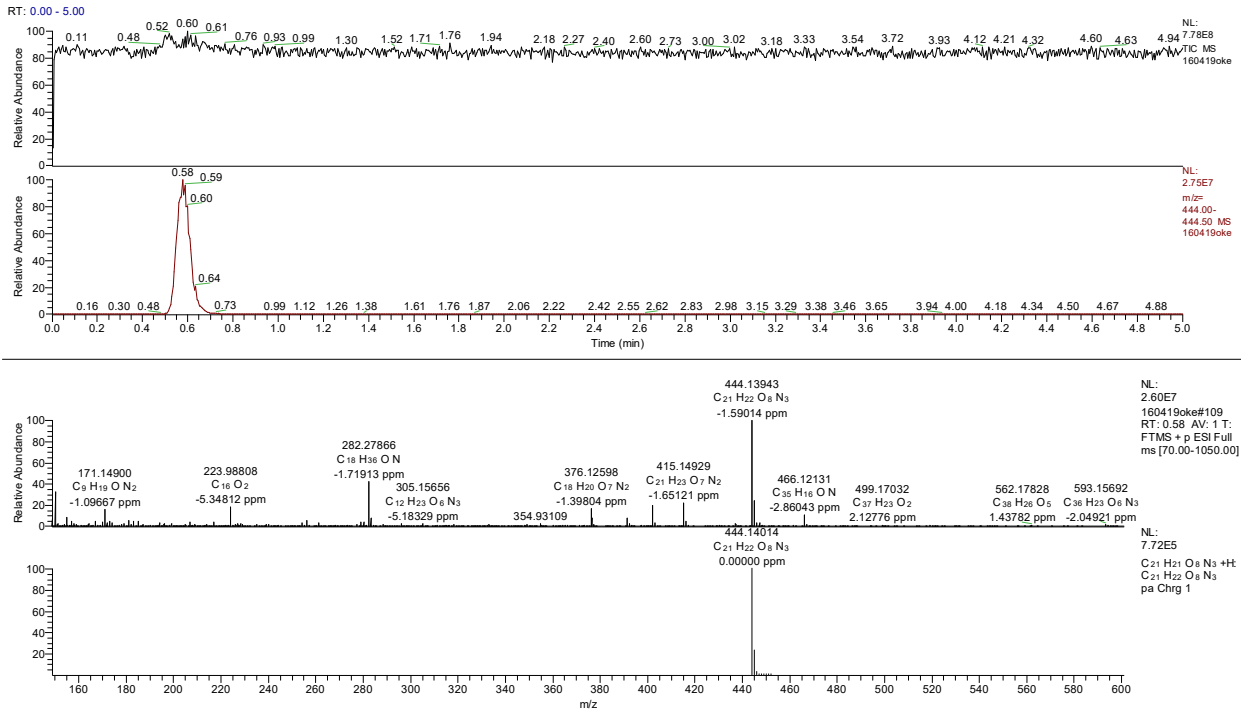
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Hydrodabcyl-triacetylated (8)

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04/19/16 09:27:58



References

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