Synthesis, characterization and applicability of three isotope labeled azobenzene photoswitches†

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We describe a short, efficient approach for the synthesis of three novel isotope labeled azobenzene photoswitches. The synthesis is based on commercially available fully isotope labeled precursors. The target molecules have been obtained in good yields, checked for purity, and identified by NMR and IR spectroscopy and a variety of standard analytical methods (UV–vis, mp, ESI-MS, elemental analysis). Using conventional coupling techniques the three isotope labeled photoswitches can be incorporated very easily in biomacromolecules.

Introduction

Some of the main objectives in the fast growing, interdisciplinary fields of chemistry and biology are the elucidation of binding affinities and the structural dynamics of biomacromolecules. Mass-spectrometry and a variety of spectroscopic (NMR, IR, Raman and CD) techniques are used to understand these processes. An important and still not fully understood process is peptide- and protein folding. In the last years a variety of sensitive and powerful spectroscopic techniques have been developed, allowing one to follow structural dynamics in real time.[1–7]

To study protein- or peptide folding, a triggering event is required to initiate the folding or unfolding process. In ultrafast spectroscopy, in general, the triggering event is a short laser pulse. Three concepts are used in time-resolved spectroscopy to initiate the reaction: T-jumps,[8,9] phototriggers[10–12] and photoswitches.[13] A phototrigger is either a "predetermined breaking point"[14,15] or a photolabile protection group.[10–12] This means that on irradiation an irreversible reaction is initiated. In contrast, photoswitches undergo a reversible reaction, *e.g.* the photoswitch can be converted by light from state A to state B. The reverse process (B→A) can be achieved by light of a different wavelength or temperature. A variety of phototriggers and photoswitches have been successfully integrated and used in time-resolved spectroscopy.[15–20] Among the photoswitches azobenzene is the one most frequently used, since it exhibits almost perfect photophysical properties ($\lambda_{cis\rightarrow trans}$ = 430 nm *vs.* $\lambda_{trans\rightarrow cis}$ = 366 nm). It has been demonstrated,[17,21–23] that azobenzene that is crosslinked to a polypeptide allows the optical control of peptide and protein conformation. The induced conformational changes have been monitored by time-resolved spectroscopies in real time.[21,22,24,25]

The amide I band (1610–1695 cm^{-1}) of polypeptides in the mid-IR region, arising primarily from the backbone $\nu_{C=O}$ stretch mode, is particularly sensitive to peptide secondary structure, due to transition-dipole coupling effects and hydrogen bonding. Therefore the dynamics of a peptide backbone are associated with the spectral evolution of the structure sensitive amide I band and can be monitored by time-resolved infrared spectroscopy.

Recent studies using IR spectroscopy have shown that more structural information can be achieved if the amide I band becomes site selective upon isotope labeling. This results from the fact that the labeled bands are shifted slightly to lower wavenumbers.[26] Unfortunately, the $\nu_{C=C}$ stretching vibrations of the aromatic rings of the azobenzene switch also give rise to weak to medium absorptions between 1450 and 1650 cm^{-1}.[27] Moreover in such experiments one typically measures the differences between folded and unfolded states, and these differences in the amide I region are usually very small. In contrast, big changes in electronic structure take place on the azobenzene switch. Hence, although the $\nu_{C=C}$ vibrations are of weak to medium strength, in the measured difference spectrum they can be quite strong in a relative sense. Thus, the amide I bands of the peptide units are often overlapping or hidden underneath the $\nu_{C=C}$ stretching vibrations of the aromatic rings, making an unambiguous band assignment of the peptide backbone difficult.

Motivated by this fact, we conducted a study aimed at circumventing this problem. We decided to use isotopic labeling on the azobenzene photoswitch to shift the perturbing $\nu_{C=C}$ stretching vibrations well below the amide I region.

Applying density functional theory (DFT) allowed us to estimate to what extent the bands will be shifted upon isotopic labeling. These calculations (B3LYP/6-311+g*) predicted that the interfering aromatic bands will be shifted to lower wavenumbers by approximately 30–38 cm^{-1} for D–H exchange and approximately 55–60 cm^{-1} for ^{13}C–^{12}C exchange.

We describe in this paper the design, synthesis and characterization of three isotope labeled (fully deuterated or ^{13}C labeled) photoswitches that are based on an azobenzene moiety (13C-DIAA (**1**), DAMPB (**2**), DPZCl (**3**), Fig. 1). These photoswitches can be incorporated very easily in biomacromolecules by conventional coupling techniques.

Results and discussion

The three azobenzene photoswitches presented in this paper are: 4,4′-diiodoacetamide-[$^{13}C_{12}$]-azobenzene (13C-DIAA) (**1**),

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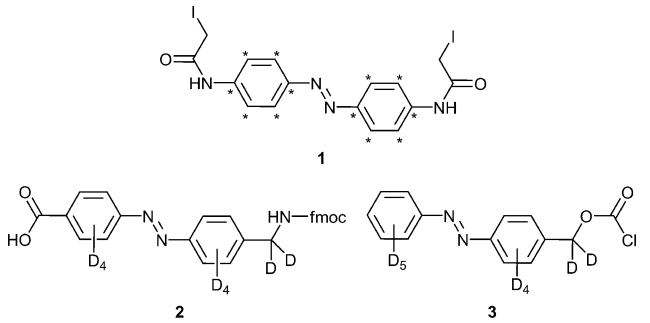


Fig. 1 Synthesized and characterized isotope photoswitches based on the azobenzene moiety (13C-DIAA (**1**), DAMPB (**2**), DPZCl (**3**)). The asterisks indicate ^{13}C atoms. Fmoc refers to the N-terminal protecting group 9*H*-fluoren-9-ylmethoxycarbonyl.

4-(4-[(9*H*-fluoren-9-ylmethoxycarbonylamino)-dideutero-methyl]-tetradeutero-phenylazo)-tetradeutero-benzoic acid (DAMPB) (**2**) and [D11]-4-phenylazo benzyl chloroformate (DPZCl) (**3**). They were assembled by an azo coupling based on a nitroso–amine reaction in glacial acetic acid as described by Schwyzer *et al.*[28] and Ulysse *et al.*[29]

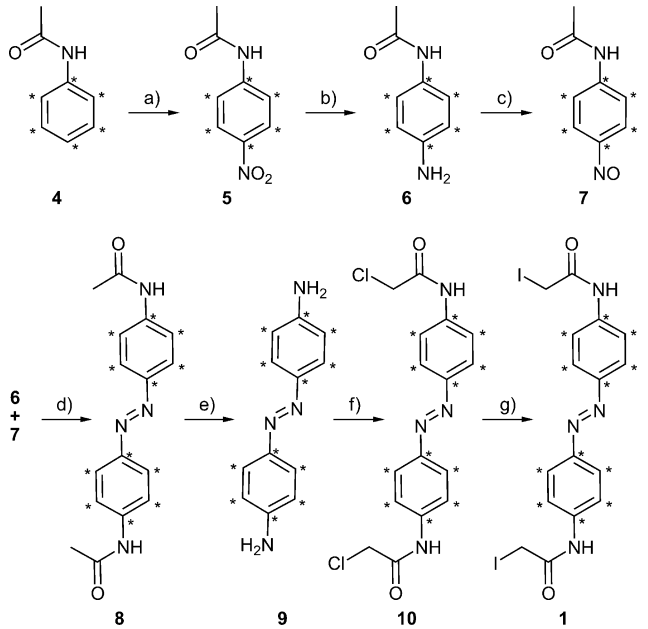
Synthesis and characterization of 13C-DIAA (1)

First, we focus on the synthesis of 13C-DIAA (**1**), which is the isotope labeled derivative of the azobenzene linker originally designed by Woolley *et al.*[30] 13C-DIAA (**1**) is a rigid switch, which can be directly cross-linked to two cysteine residues of a polypeptide chain,[25,30] while the other two switches **2** and **3** described in this work can be integrated directly into a polypeptide chain *via* an amide or urethane bond.

The synthetic route for 13C-DIAA (**1**) is outlined in Scheme 1. Starting from commercially available acetanilide-$^{13}C_6$ (**4**), we obtained 4-nitro acetanilide-$^{13}C_6$ (**5**) by treatment with guanidium nitrate[31] in an excellent 84% yield. The next step was reduction of **5** to 4-amino acetanilide-$^{13}C_6$ (**6**), which was accomplished in 85% yield. Azobenzene derivative **8** was obtained by partial oxidation of **6** in the presence of hydrogen peroxide and simultaneous coupling of the *in situ* formed 4-nitroso acetanilide-$^{13}C_6$ (**7**) to unreacted 4-aminoacetanilide-$^{13}C_6$ (**6**) in acetic acid. The acetyl capping was removed in boiling hydrogen chloride, yielding 4,4′-diaminoazobenzene-$^{13}C_{12}$ (**9**) in moderate yield (58%). Addition of chloroacetyl chloride afforded 4,4′-dichloroacetamido-azobenzene-$^{13}C_{12}$ (**10**), which was not isolated but directly converted to the final product 13C-DIAA (**1**) under Finkelstein conditions (NaI, acetone). The yield of this two-step reaction was 68%.

The final product **1** was characterized by mass spectrometry and ^1H and ^{13}C NMR spectroscopy. As 13C-DIAA exhibits aromatic rings which are ^{13}C labeled, we predicted a redshift of the interfering bands by approximately 60 cm^{-1} in the mid IR region according to our DFT calculations (B3LYP/6-311+g*). In principle one expects for the molecule three types of band in the region between 1450–1750 cm^{-1}. One band arising from the $\nu_{C=O}$ stretch modes of the 4,4′-diiodoacetamide groups and two additional sets from the $\nu_{C=C}$ stretching vibrations of the aromatic rings and the amide II modes of the NHCO moieties.

To prove that our synthesized switch **1** is spectroscopically clean and to confirm our assignment, we recorded the FTIR spectra (Fig. 2) of labeled and unlabeled **1** in a mixture of DMSO : D_2O (7 : 1). By using this mixture, the hydrogen atoms of the two diiodoacetamide groups undergo H–D exchange, a common effect during sample preparation in the field of biomolecule spectroscopy. Thus the set of bands that arises from the amide II modes vanishes. Fig. 2 shows a strong band centered at ~1690 cm^{-1}, which belongs to the $\nu_{C=O}$ stretch modes of the 4,4′-diiodoacetamide groups. Hence, the two remaining bands in the region between 1450–1610 cm^{-1} arise from the $\nu_{C=C}$ stretching vibrations of the aromatic rings and undergo the strongest spectral shift upon isotope labeling, as predicted by our DFT calculations (exp. 52 cm^{-1} and 36 cm^{-1}; calc. 60 cm^{-1} and 29 cm^{-1}).



Scheme 1 Synthetic route to 13C-DIAA (**1**). The asterisks indicate ^{13}C atoms. *Reagents and conditions:* (a) H_2SO_4, guanidium nitrate, 0 °C, 2 h (84%). (b) MeOH, Pd/C, H_2, rt, 4 h (85%). (c) + (d) AcOH, H_2O_2, rt, 6 h (16%); **7** not isolated. (e) Conc. HCl, reflux, 3 h (58%). (f) THF, TEA, chloroacetyl chloride. (g) Acetone, THF, NaI, rt, 18 h (68%); **10** not isolated.

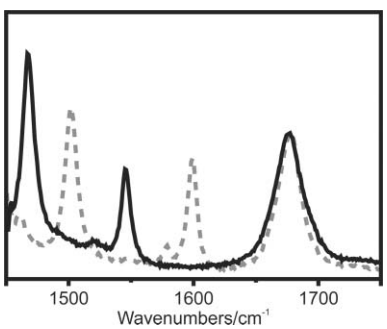


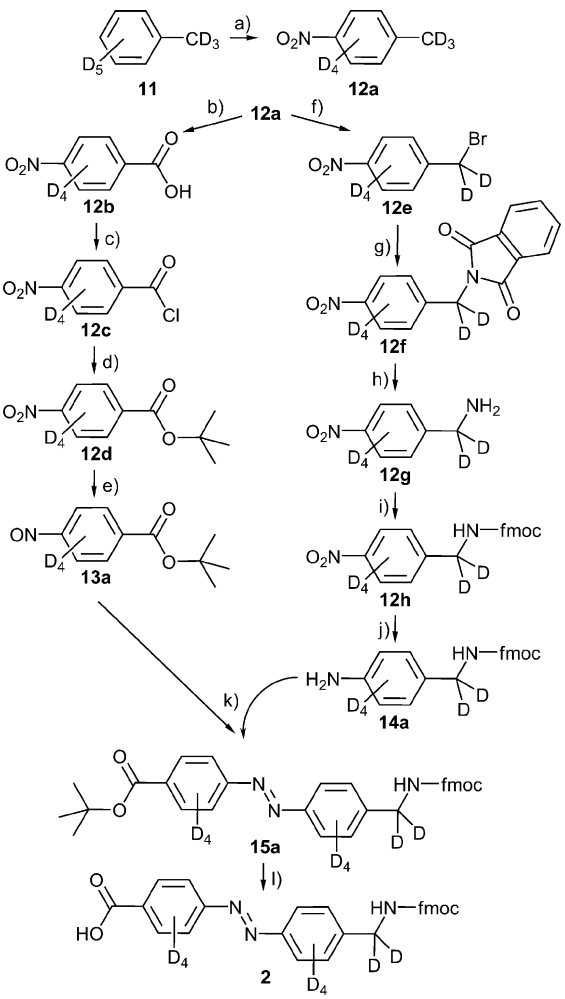
Fig. 2 FTIR spectrum of the $\nu_{C=C}$ stretching and amide I and II regions of **1** in DMSO : D_2O (7 : 1) (5–6 mM, spectral resolution 2 cm^{-1}) in comparison with the unlabeled sample. Black spectrum: ^{13}C labeled molecule 13C-DIAA (**1**). Dotted spectrum: unlabeled molecule DIAA.

Synthesis and characterization of DAMPB (2)

Compared to 4-(4-amino-phenylazo)-benzoic acid (APB), DAMPB (**2**) is fully deuterated and contains an additional

methylene group. The group is localized between the amino group and the phenyl ring of the azobenzene and prevents delocalization of the free electron pair of the amino group over the completely delocalized π-system of the azobenzene. This leads to an increased nucleophilicity and reactivity of the amino group, which offers the possibility of using DAMPB (**2**) in the form of Fmoc-DAMPB in standard peptide synthesis, both solution and SPPS. In addition, the additional methylene group offers increased flexibility, especially valuable when **2** is used for ring closing reactions in small cyclic peptides. It has been shown that the increased flexibility has no effect on the efficiency of the *cis*↔*trans* isomerization.[32,33]

The synthetic route for **2** is shown in Scheme 2. Starting from commercially available fully deuterated toluene (**11**), we obtained [D7]-4-nitro toluene (**12a**) by treatment with nitrosulfuric acid in nearly quantitative yield. One part of **12a** was oxidized without



Scheme 2 Synthetic route to DAMPB (**2**). *Reagents and conditions:* (a) HNO_3, H_2SO_4, 14 °C, 2 h, rt, 1 h (100%). (b) $Na_2Cr_2O_7$, H_2SO_4, 95 °C, ~15 h (22%). (c) $SOCl_2$, reflux, 6 h (55%). (d) THF, *tert*-BuOLi, 0 °C, 1 h, rt, 12 h (90%). (e) 2-methoxy ethanol, Zn, NH_4Cl, rt, ~3 h, $FeCl_3$, H_2O–EtOH, 0 °C, 3 h (77%). (f) CCl_4, NBS, AIBN, reflux, 3 h (17%). (g) Phthalimide potassium salt, DMF, 50 °C, 12 h (82%). (h) EtOH, THF, hydrazine hydrate, reflux, 24 h (95%). (i) DCM, DIEA, Fmoc-Cl, rt, 2 h (99%). (j) EtOH, dioxane, Pd/C, H_2, rt, 2 h (100%). (k) AcOH, 0 °C, 1 h, rt, 48 h (67%). (l) DCM, TFA, rt, 36 h (81%).

further purification, yielding pure [D4]-4-nitro benzoic acid (**12b**) in acceptable yield (22%). After that **12b** was converted to the corresponding benzoyl chloride derivative **12c** by treatment with thionyl chloride, followed by treatment with lithium *tert*-butoxide. This yielded the corresponding *tert*-butyl ester **12d** in nearly quantitative yield (90%).

Reduction of **12d** in the presence of zinc–NH_4Cl yielded the [D4]-4-nitroso benzoic acid *tert*-butyl ester (**13a**) in good yield (77%). The other part of **12a** was purified by distillation to obtain pure **12a**. Pure **12a** was subjected to Wohl–Ziegler bromination[34,35] yielding [D6]-4-nitro benzylbromide (**12e**) in acceptable yields. **12e** was subjected to Gabriel synthesis,[36] followed by treatment with hydrazine hydrate. This yielded [D6]-4-nitro benzylamine (**12g**) in very good yield. Since **12g** proved to be not very stable, it was converted directly to the Fmoc protected species **12h** by treatment with Fmoc-Cl in a near quantitative amount. After that **12h** was reduced with H_2–Pd/C to ([D6]-4-amino-benzyl)-amine-Fmoc (**14a**) in quantitative yield. Azobenzene derivative **15a** was obtained by treatment of fresh **13a** and **14a** in the presence of acetic acid. The final product **2** was obtained in good yields (81%) by removal of the *tert*-butyl ester in the presence of TFA. The final product **2** was characterized by mass spectrometry and ^1H and ^{13}C NMR spectroscopy.

Fig. 3 shows the FTIR spectra of labeled and unlabeled **2**. In both cases we observe three absorption bands. The strong band at ~1700 cm^{-1} is caused by the carbonyl groups of the carboxylic acid and the Fmoc urethane. In the case of the unlabeled **2** we observe a band centered at ~1603 cm^{-1}, which causes signal overlapping with the red part of the amide I region. This band is shifted to 1576 cm^{-1} upon isotope labeling. The observed shift of 26 cm^{-1} nicely matches our expected value of 30 cm^{-1} (B3LYP/6-311+g*).

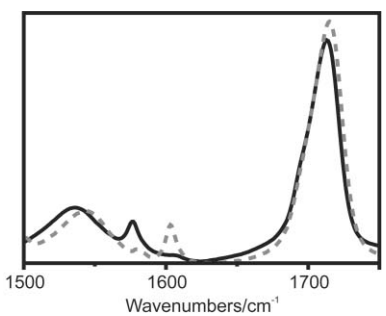
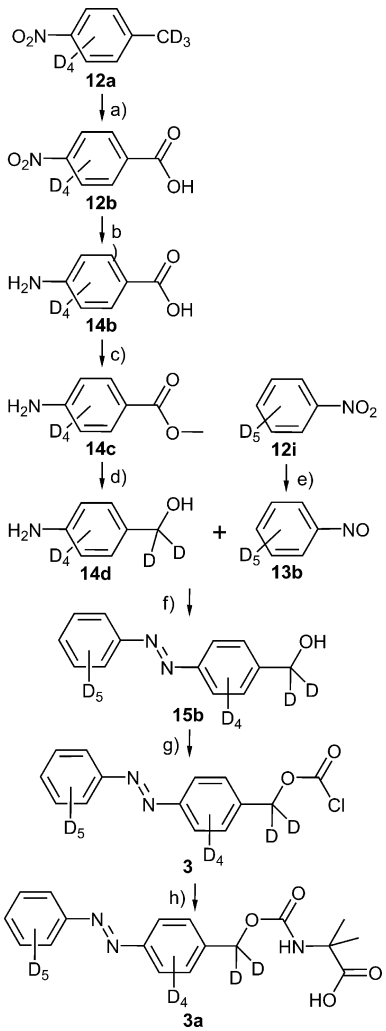


Fig. 3 FTIR spectrum of the $\nu_{C=C}$ stretching and amide I and II regions of **2** in DMSO (spectral resolution 2 cm^{-1}) in comparison with the unlabeled sample. Black spectrum: fully deuterated molecule DAMPB (**2**). Dotted spectrum: unlabeled molecule.

Synthesis and characterization of DPZCl (3)

The third photoswitch DPZCl (**3**) is aimed at being attached single-sided to a biomacromolecule. The use of this type of switch allows for the tracking of energy transport in a peptide helix upon photochemical excitation.[37]

The synthetic route for **3** is shown in Scheme 3. Starting from crude [D7]-4-nitro toluene (**12a**), we obtained [D4]-4-nitro benzoic acid (**12b**) by oxidation in acceptable yield after separation from unwanted byproducts. **12b** was converted to the corresponding amino derivative **14b** in the presence of Na_2CO_3 and zinc dust



Scheme 3 Synthetic route to DPZCl (**3**). *Reagents and conditions:* (a) $Na_2Cr_2O_7$, H_2SO_4, 95 °C, ~15 h (22%). (b) Na_2CO_3, Zn–HCl, 30 °C, 20 min (80%). (c) $SOCl_2$, MeOH, 0 °C, 1 h, rt, 12 h (~80%). (d) $LiAlD_4$, ether, reflux, 4 h (47%). (e) Zn, NH_4Cl, H_2O, rt, 20 min, H_2SO_4, 0 °C, $Na_2Cr_2O_7$, water (30%). (f) AcOH, 0 °C, 1 h, rt, 1 h (56%). (g) Triphosgene, dioxane, rt, 1 h, 40 °C, 12 h (63%). (h) Aib, 1 N NaOH, dioxane, rt, 1 h, 50 °C, 4 h, 1 N HCl (40%).

in good yield (80%). Esterification with thionyl chloride and methanol yielded the methyl ester **14c** (~80%), which was reduced with $LiAlD_4$ to [D6]-4-Amino benzyl alcohol (**14d**) in moderate yield. [D5]-Nitroso benzene (**13b**) was prepared according to the synthesis published by Shine *et al.*[38] from commercially available **12i**. Again azobenzene derivative **15b** was obtained by treatment of fresh **13b** and **14d** in the presence of acetic acid. Finally, DPZCl (**3**) was obtained in good yields by treatment of **15b** with triphosgene. The final product **3** was characterized by mass spectrometry and ^1H and ^{13}C NMR spectroscopy.

The FTIR spectra of labeled and unlabeled **3a** are shown in Fig. 4. We can see two very broad and strong absorptions (amide II and $\nu_{C=C}$; amide I and $\nu_{C=O}$) dominating the 1500–1700 cm^{-1} region. The amide II and aromatic valence vibrations are overlapping and can be seen much better in the magnification. Upon isotope labeling the band at 1606 cm^{-1} shifts to approx. 1576 cm^{-1}, where it becomes a shoulder of the strong broad band. The observed shift

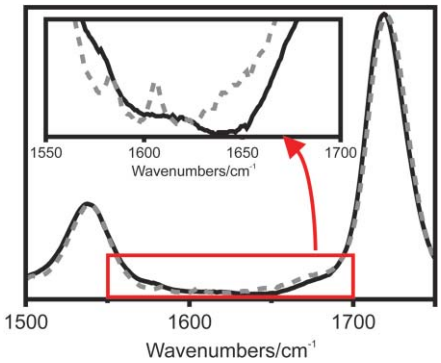


Fig. 4 FTIR spectrum of the $\nu_{C=C}$ stretching and amide I and II regions of **3a** in DMSO (spectral resolution 2 cm^{-1}) in comparison with the unlabeled sample. Black spectrum: fully deuterated molecule **3a**. Dotted spectrum: unlabeled molecule. Inset: magnification of the 1550–1700 cm^{-1} region.

of 30 cm^{-1} is in agreement with our calculated value (B3LYP/6-311+g*).

Examples of applications

Fig. 5 shows three examples for which azobenzene photoswitches have been integrated sucessfully. In the following we will explain the aim of these photoswitches in the individual model systems. In addition, we will discuss in detail for example (b) how isotope labeling of the photoswitch cleans a spectral window between 1550–1600 cm^{-1}, which in turn allows the isotope-labeling of certain amide groups, letting one study peptide folding in a site-selective manner.

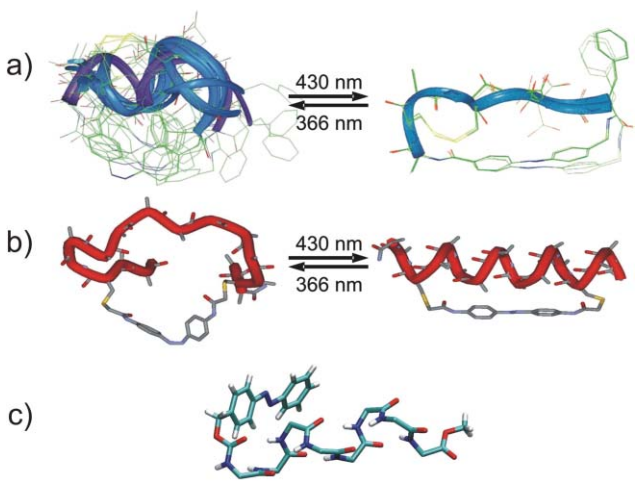


Fig. 5 Three examples for which azobenzene photoswitches have been integrated successfully. (a) cycAMPB adapted from ref. 21, (b) schematic models of FK11X adapted from ref. 25 and (c) PAZ-Aib-Ala-$(Aib)_6$-OMe adapted from ref. 37.

The folding and unfolding processes of biomolecules can be monitored by IR spectroscopic techniques with picosecond time-resolution. The aim of the azobenzene photoswitches in examples (a) and (b) is to trigger a conformational change of the peptide upon *cis–trans* isomerization of the azo-moiety. In example (a) the photoswitch has been directly integrated into the peptide

backbone (C- and N-terminal),[21] whereas it is attached *via* the cysteine side chains in example (b).[25,39] The molecule of example (c), in contrast, was designed to study heat transport phenomena through peptide helices.[37] To this end, an azobenzene moiety, which isomerizes on a 200 fs timescale and thereby becomes vibrationally hot, is attached to one side of the helix and isotope labeled amide groups are used as local thermometers at various distances from the heater along the helix.

Fig. 6 exemplifies in detail the advantages of isotope labeling the azobenzene photoswitch **1**, which was integrated in the helical system L96, a 16-residue peptide Ac-AACAKA*AAA**KAAACKA-NH_2 (where * denotes $^{13}C^{16}O$ labeling and ** denotes $^{13}C^{18}O$ labeling). The polypeptide was prepared by SPPS according to standard protocols, and the photoswitch **1** was attached to L96 as described by Woolley *et al.*[30]

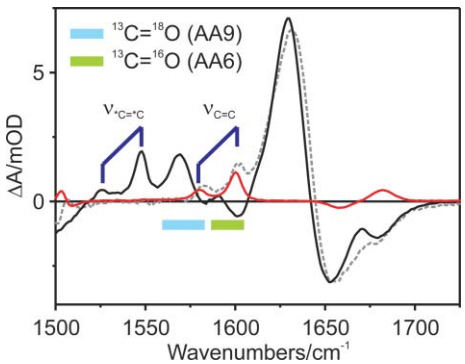


Fig. 6 FTIR difference spectra (spectral resolution 2 cm^{-1}) upon *cis–trans* isomerization of the azobenzene moiety of the molecule shown in Fig. 5(b). For comparison, a spectrum of the unlabeled photoswitch **1** is shown as well (red spectrum, without peptide). Bands appearing on irradiation are pointing upward; bands disappearing are pointing downward. Black spectrum: fully labeled L96 (*i.e.* ^{13}C labeled azobenzene moiety **1** and $^{13}C^{16}O$ -$^{13}C^{18}O$ double labeled polypeptide). Dotted spectrum: unlabeled L96 (*i.e.* neither the azobenzene moiety nor the polypeptide are isotope labeled).

Fig. 6, red curve, shows the IR spectrum of the unlabeled photoswitch **1** upon *cis-trans* isomerization. We can see two weak to medium $\nu_{C=C}$ stretching vibrations in the region between 1580 and 1600 cm^{-1} which originate from the aromatic rings of the azobenzene moiety. Fig. 6, dotted curve, shows the IR response of the unlabeled photoswitch **1** integrated in the helical system L96. The strong difference bands at 1625 and 1650 cm^{-1} report on the strengthening of the intramolecular hydrogen bonds upon folding of the helix. Comparison with the difference spectrum from the unlabeled photoswitch **1** (red spectrum) shows that the two weaker bands in the wings (1580 and 1600 cm^{-1}) originate from the photoswitch. These bands appear in exactly the spectral region where $^{13}C^{16}O$ or $^{13}C^{18}O$ labeled amide I vibrations are expected. Fig. 6, black curve, shows the response of the fully labeled system. The result demonstrates that ^{13}C labeling of the azobenzene moiety cleans a spectral range large enough to allow for $^{13}C^{16}O$ - $^{13}C^{18}O$ double labeling of L96. The hydrogen bond strengths of each of the isotope labeled amide units can now be investigated in a site-selective manner. Furthermore, since there is room for two different isotope labels, 2D-IR studies become feasible, allowing one to observe local contacts between any pair of two amino acids.[40] This will enable studies of the complex folding pathways of small peptides and proteins in unprecedented detail.

Conclusions

In summary, we have developed a short, efficient approach to the synthesis of three novel isotope labeled azobenzene photoswitches based on commercially available fully isotope labeled precursors. Azobenzene is often used in the biophysical community to initiate conformational changes and to locally deposit energy. The $\nu_{C=C}$ stretching vibrations of the unlabeled azobenzene photoswitches interfere with the structure sensitive amide I vibrations of the peptide/protein backbone. Consequently, it was the aim of this work to shift the perturbing $\nu_{C=C}$ stretching vibrations out of the amide I region by isotopic labeling. We characterized the synthesized molecules by a variety of standard analytical methods (UV–vis, mp, ESI-MS, elemental analysis). During the synthesis we did not encounter any difficulties. Fortunately, the molecules behaved like the unlabeled compounds, which made the implementation of the labeled photoswitches in biomacromolecules very easy. Consequently, we think that these universal switches are spectroscopically clean and therefore allow us to gain new insights into folding processes, which up until now have been hidden under the $\nu_{C=C}$ stretching vibrations.

Experimental

General. Melting points: Dr Tottoli apparatus, uncorrected. IR spectra: Biorad FTS-175C spectrometer. MS: ESI Esquire-LC 00028. Elementary analyses: Vario EL. TLC: Merck Silica gel 60 F/254. Column chromatography: Silica gel 60, 0.063–0.200 mm, Merck. ^1H NMR: ARX 300 (300.13 MHz), rel. to Me_4Si. ^{13}C NMR: ARX 300 (75.468 MHz). The chemical shifts given for the ^{13}C enriched products are just the enhanced signals due to isotope labeling. Chemicals were purchased from Sigma-Aldrich, if not mentioned otherwise. Deuterated compounds were purchased from Armar Chemicals (Döttingen, Switzerland).

For irradiation, we used a high-pressure mercury lamp (Oriel Corp.) with a monochromator (SPEX Minimate) operating at either $\lambda = 425$ nm or $\lambda = 366$ nm.

The abbreviations used are as follows: Aib, aminoisobutyric acid; Boc, *tert.* butoxycarbonyl; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; TFA, trifluoroacetic acid; PZ, 4-phenylazo-benzyloxycarbonyl; DPZ, deuterated 4-phenylazo-benzyloxycarbonyl; AMPB, (4-aminomethyl)-phenylazobenzoic acid; AIBN, azo-isobutyronitrile; DIEA, diisopropyl ethyl amine; DMSO, dimethylsulfoxide; EE, ethyl acetate; ether, diethyl ether; Fmoc, 9*H*-fluoren-9-ylmethoxycarbonyl; $LiAlD_4$, lithium aluminium deuteride; NBS, *N*-bromo succinimide; Pd/C, palladium 10% on charcoal; PE, petroleum ether (60–80), if not otherwise stated.

General procedure for catalytic reduction (GPA)

The nitro compound was placed in a flame-dried flask under a N_2 atmosphere. To this was added freshly distilled MeOH or dioxane–ethanol. Pd/C was added to the solution. Hydrogen was passed through the vigorously stirred solution for 2–4 h. For removal of

the hydrogen the flask was purged with N_2. The Pd/C was filtered off by using celite, and the solvent was removed *in vacuo.* Yields 80–100%.

4,4′-Diiodoacetamide-[$^{13}C_{12}$]-azobenzene (1)

4,4′-Diiodoacetamide-[$^{13}C_{12}$]-azobenzene (**1**) was synthesized according to the procedure published by Kumita *et al.*[30] Yield: 0.081 g (68%). $C_4{}^{13}C_{12}H_{14}N_4O_2I_2$, ν_{max}(DMSO)/cm^{-1}: 3278 m, 3226 m, 3120 m, 3050 m, 2965 m, 1688 s, 1585 s, 1544 s, 1508 s, 1464 s, 1300 s, 1145 s. ^{1}H-NMR (300.13 MHz, [D6]-DMSO): 10.63 (2H, s, NH), 8.10 (4H, d, arom.), 7.55 (4H, d, arom.), 3.87 (4H, s, CH_2). ^{13}C-NMR (75.468 MHz, [D6]-DMSO): 147.7, 141.4, 123.4, 119.2. MS (ESI): *m/z* 583.0 (90%, [M + Na]).

[D10]-4-(4-Fmoc-aminomethyl-phenylazo)-benzoic acid (2)

[D10]-4-(4-Fmoc-aminomethyl-phenylazo)-benzoic acid *tert*-butyl ester (**15a**) (0.544 g, 1 mmol) and TFA (4 mL) were dissolved, whilst stirring, in DCM (40 mL). Stirring was continued for about 36 h at room temperature. The reaction was quenched by the addition of water (20 mL). The precipitate formed was filtered off, and successively washed with DCM (10 mL), 5% $NaHCO_3$ (20 mL), water (20 mL) and twice with DCM (10 mL). Finally the product was stored in a desiccator over P_2O_5. This yielded 0.394 g (81%). Degree of deuteration >99%. Mp 240–242 °C. Found: C, 69.82%; D, 6.94%; N, 8.39%. $C_{29}D_{10}H_{13}N_3O_4$ requires : C, 71.44%; D, 6.82%; N, 8.62%. ν_{max}(KBr)/cm^{-1}: 3422 s, 3308 s, 3020–3070 m, 2950 m, 2207 w, 2109 w, 1688 s, 1576 m, 1532 s, 1271 s, 740 s. λ_{max}(THF)/nm 266.5 (ε/dm^3 mol^{-1} cm^{-1} 23 000), 301 (21 000), 333 (28 500) and 452.5 (675).^{1}H-NMR (300.13 MHz, [D6]-DMSO): 13.18 (1H, s, COOH), 7.25–7.95 (9H, m, Fmoc-aromatic and NH), 4.40 (2H, d, Fmoc-CH_2), 4.25 (1H, t, Fmoc-CH). ^{13}C-NMR (75.468 MHz, [D6]-DMSO): 166.8, 156.5, 154.2, 150.9, 144.1, 143.9, 140.8, 132.8, 130.4, 127.7, 127.1, 125.2, 122.5, 122.2, 120.2, 65.4, 46.9. MS (ESI): *m/z* 510.4 (95%, [M + Na]).

[D11]-4-Phenylazo benzyl chloroformate (DPZCl) (3)

Triphosgene (2.3 g, 7.75 mmol) was dissolved in dry dioxane (9 mL) and cooled to approx. 15 °C. Whilst stirring, **15b** (1.12 g, 5 mmol) was added. Stirring was continued for 1 h at room temperature. The temperature was then raised to 40 °C, and the mixture was stirred overnight. The solvent was evaporated, the compound recrystallized from hexane, and finally dried under high vacuum conditions. Yield 0.903 g (63%). Mp 73–77 °C. $C_{14}D_{11}ClN_2O_2$, ν_{max}(KBr)/cm^{-1}: 2940 w, 2274 w, 2257 w, 2168 w, 1780 s, 1371 w, 1335 w, 1298 m, 1186 s, 1064 m, 1034 m, 910 m, 812 m, 778 m, 736 m, 684 m, 608 w, 552 m, 504 w, 483 m. ^{13}C-NMR (75.468 MHz, $CDCl_3$): 153.1, 152.6, 150.9, 135.6, 130.9, 129.2, 128.8, 122.8, 72.1. MS (ESI): *m/z* 286.1 (43%, [M + H]), 206.1 (17%, [M − ClCOO]).

General procedure for DPZCl–Amino acid/Peptide coupling (e.g. [D11]-4-Phenylazo-benzyloxycarbonyl)-aminoisobutyric acid (DPZ-Aib) (3a)). Aminoisobutyric acid (0.304 g, 2.95 mmol) was dissolved in 1 N NaOH (6 mL). A solution of **3** (0.841 g, 2.95 mmol) in dioxane (7.5 mL) was added dropwise to the stirred solution. The mixture was stirred for 30 min at room temperature and for a further 2 h at 50 °C before adding 1 N NaOH (3 mL). Stirring was continued for an additional 2 h at 50 °C. After cooling to room temperature, water (9 mL) was added. Afterwards the mixture was extracted three times with ether (15 mL) to remove byproducts. The aqueous layer was acidified with 1 N HCl (~12 mL) and subsequently extracted three times with ether (30 mL). The organic layer was dried (Na_2SO_4), and the solvent evaporated. This yielded the crude product, which was further purified. The crude was dissolved in MeOH (15 mL), and insoluble parts were filtered off. The filter cake was washed twice with MeOH (7.5 mL). The combined organic phases were concentrated to ~10 ml. Addition of water (5 mL) and storage of the solution for 1 h at room temperature, followed by an additional hour in the refrigerator yielded a crystalline product, which was filtered off and dried under vacuum. This yielded 0.413 g (40%). Mp 124–126 °C. $C_{18}D_{11}H_8N_3O_4$, ν_{max}(KBr)/cm^{-1}: 3429 w, 3001 w, 1715 vs, 1581 w, 1494 s, 1310 m, 1271 m, 1224 m, 1159 m, 1089 m, 552 w, 474 w. λ_{max}(EtOH)/nm 229 (ε/dm^3 mol^{-1} cm^{-1} 15 000), 320 (26 000) and 442 (600). ^{1}H-NMR (300.13 MHz, $CDCl_3$): 9.8 (1H, bs, OH), 5.42 (1H, bs, NH), 1.60 (6H, s, Aib CH_3). ^{13}C-NMR (75.468 MHz, $CDCl_3$): 179.4, 155.1, 152.4, 152.2, 138.7, 130.2, 128.1, 122.5, 65.7, 56.3, 25.0. MS (ESI): *m/z* 375.2 (95%, [M + Na]).

4-Nitro acetanilide-$^{13}C_6$ (5)

Acetanilide-$^{13}C_6$ (**4**) (1.0 g, 7.09 mmol) was dissolved in 85% H_2SO_4 (11 mL) and cooled in an ice bath. Whilst stirring, guanidium nitrate (0.87 g, 7.1 mmol) was added slowly to the solution. The mixture was stirred for an additional 2 h at 0 °C. Afterwards the mixture was poured onto ice–water (70 mL). The precipitated product was filtered off and washed with water. Drying under vacuum yielded 1.13 g (84%). $C_2{}^{13}C_6H_8N_2O_3$, ^{1}H-NMR (300.13 MHz, [D6]-DMSO): 10.53 (1H, s, NH), 7.91–8.49 (2H, d, arom.), 7.54–8.10 (2H, d, arom.), 2.12 (3H, s, CH_3).

4-Amino acetanilide-$^{13}C_6$ (6)

Compound **6** was synthesized as described above (GPA method). **5** (1.13 g, 6.07 mmol) was dissolved in methanol (90 mL), and Pd/C (0.104 g, 0.1 mmol) was added. Reaction time 3–4 h. This yielded 0.805 g (85%). $C_2{}^{13}C_6H_{10}N_2O$, ^{1}H-NMR (300.13 MHz, [D6]-DMSO): 9.45 (1H, s, NH), 6.20–7.50 (4H, m, arom.), 4.91 (2H, s, NH_2), 1.94 (3H, s, CH_3). ^{13}C-NMR (75.468 MHz, [D6]-DMSO): 144.3, 128.6, 120.7, 113.7.

4,4′-Di(acetylamino)-[$^{13}C_{12}$]-azobenzene (8)

4-Amino acetanilide-$^{13}C_6$ (**6**) (0.312 g, 2.0 mmol) was dissolved in acetic acid (6 mL). Whilst stirring, 33% H_2O_2 (0.55 mL, 6 mmol) was added. Stirring was continued for 6 h at room temperature. Water (7 mL) was added to the mixture and stirring was continued for 5 min. The precipitated brown product was filtered off, and washed with water (5 mL). This yielded 0.05 g (16%). $C_4{}^{13}C_{12}H_{16}N_4O_2$, ^{1}H-NMR (300.13 MHz, [D6]-acetone): 9.42 (2H, s, NH), 7.58–8.12 (8H, d, arom.), 2.13 (6H, s, acetyl). ^{13}C-NMR (75.468 MHz, [D6]-acetone): 149.3, 143.1, 124.3, 120.0.

4,4′-Diamino-[$^{13}C_{12}$]-azobenzene (9)

8 (0.0617 g, 0.2 mmol) was suspended in water (10 mL). Whilst stirring, conc. HCl (5 mL) was added and the mixture was heated under reflux for 3 h. The solvent was removed *in vacuo*, and the residue was taken up in water (10 mL). The solution was basified to pH 8–9 by the addition of 1 N NaOH (approx. 1 mL). The precipitated brown product was filtered off, and was washed twice with water (5 mL). The filter cake was taken up in acetone. Removal of the solvent yielded 0.0259 g (58%) of the product. $^{13}C_{12}H_{12}N_4$, ^{1}H-NMR (300.13 MHz, [D6]-acetone): 6.48–7.90 (8H, m, arom.), 5.15 (4H, s, NH_2). ^{13}C-NMR (75.468 MHz, [D6]-acetone): 152.7, 124.8, 121.0, 114.6.

[D7]-4-Nitro toluene (12a)

[D8]-toluene (**11**) (10.75 mL, 100 mmol) was cooled by an ice bath. A precooled mixture of 10 mL 65% HNO_3 and 12 ml 98% H_2SO_4 was added dropwise over 40 min to the vigorously stirred mixture at 5–10 °C. The mixture was stirred for 2 h at 14 °C. Stirring was continued for an additional hour at 25 °C. Afterwards the mixture was poured onto ice–water (300 mL), and extracted three times with ether (50–80 mL). The combined organic layers were successively washed with 5% $NaHCO_3$ (50 mL) and twice with water (50 mL). Drying of the combined organic layers ($CaCl_2$), and removal of the solvent yielded 14.4 g (100%) crude product.

Remark: for the synthesis of **12e** it is necessary to separate the pure *para* compound **12a** from the raw product by distillation (bp 84–85 °C at 8 mbar; *ortho*: bp 80–89 °C at 9 mbar). However for the synthesis of **12b** it is much easier to separate the *para* compound from the byproduct after oxidation (*cf.* [D4]-4-nitro benzoic acid (**12b**)). Mp 49–52 °C. $C_7D_7NO_2$, ν_{max}(KBr)/cm^{-1}: 2835 w, 2314 w, 2278 w, 2126 w, 1581 s, 1510 s, 1346 s, 854 s, 639 s, 1073 m, 832 m, 821 m, 582 m. ^{13}C-NMR (75.468 MHz, [D6]-acetone): 146.8 (2 signals), 130.4, 123.7, 20.6.

[D4]-4-Nitro benzoic acid (12b)

Sodium dichromate (6 g, 20 mmol) was dissolved in water–H_2SO_4 (30 mL, 2 : 1). Crude **12a** (1.44 g, 10 mmol) was added to the solution, and the mixture was stirred overnight at 95 °C in a stopper closed apparatus. (CAUTION!!! We obtained a lower yield when the reaction was performed under reflux in an open apparatus). The mixture was allowed to cool down for 10 min before pouring in water (30 mL). After chilling the mixture for 15 min by an ice bath, the precipitate formed was filtered off. The crude was heated for 5 min at 100 °C in 5% H_2SO_4 (15 mL). Afterwards the mixture was again cooled by an ice bath and filtered off. The residue was taken up in 5% NaOH (15 mL). Insoluble parts were filtered off and the filtrate was mixed with 10% H_2SO_4 (20 mL). Once again the filter cake was washed with 5% NaOH (4mL) and the obtained filtrate was added to the H_2SO_4 mixture as well. Cooling of the mixture by an ice bath for about 5 min yielded a white precipitate, which was filtered off, washed twice with water (5 mL), and dried in a desiccator. The product already consists of *para* compound exclusively, as determined by NMR, IR and mp. No *ortho*- or *meta* byproducts were found. Further purification can be achieved by recrystallization from methanol–water. The crystalline compound (0.484 g, 2.83 mmol) was dissolved in water (10 mL), heated to 60 °C, and filtered hot. Subsequently the filter cake was washed twice with hot methanol (5 mL). Water (10 mL) was added to the filtrate, which was stored afterwards in a refrigerator for ∼15 h. The precipitate formed was filtered off, and washed twice with cold methanol–water (8 mL, 1 : 1), before placing in a desiccator. Concentration of the mother liquor yielded further product. The combined fractions afforded 0.428 g (22%). Mp 229–231 °C, (lit.[41] 239–240 °C). $C_7D_4HNO_4$, ν_{max}(KBr)/cm^{-1}: 3450 m, 2314 m, 1696 vs, 1592 s, 1538 s, 1441 s, 1349 s, 1283 vs, 938 m, 861 m, 822 m, 773 m, 640 m, 541 w. ^{13}C-NMR (75.468 MHz, [D6]-acetone): 166.0, 151.5, 136.8, 131.4, 124.1.

[D4]-4-Nitro benzoyl chloride (12c)

12b (1.71 g, 10 mmol) and $SOCl_2$ (6.3 ml, 87 mmol) were placed in the reaction flask. Whilst stirring the mixture was heated under reflux for 6 h. The clear solution was evaporated and the residue was taken up in hot hexane (20 mL, ∼60 °C). Insoluble parts were filtered off, and the filter cake was washed three times with boiling hexane (10 mL). The combined organic phases were concentrated to 20 mL. Storage in the refrigerator yielded a crystalline product, 1.05 g (55%). Mp 69–71 °C. $C_7D_4ClNO_3$, ν_{max}(KBr)/cm^{-1}: 2307 m, 2286 w, 1777 s, 1751 s, 1696 m, 1583 s, 1524 s, 1381 m, 1352 s, 1301 s, 1162 m, 1150 s, 1063 m, 1017 m, 883 s, 866 s, 821 m, 796 s, 684 m, 619 m, 581 m, 538 m, 451 m. ^{13}C-NMR (75.468 MHz, $CDCl_3$): 167.1, 151.5, 137.9, 131.9, 123.8.

[D4]-4-Nitro benzoic acid *tert*-butyl ester (12d)

In a flame-dried flask, **12c** (1.9 g, 10 mmol) was placed under a N_2 atmosphere. To this was added freshly distilled and dried THF (20 mL). Lithium *tert*-butoxide (1.3 g, 16 mmol) was added portionwise to the ice-cooled and vigorously stirred solution in such a manner that the reaction temperature did not increase beyond 10 °C. Stirring was continued for 1 h at 0 °C, and overnight at room temperature. The reaction was quenched by the portionwise addition of water (∼28 mL). The mixture was extracted three times with ether (14 mL). The combined organic layers were dried (Na_2SO_4), and the solvent was evaporated. Yield: 2.06 g (90%). Degree of deuteration >99.5%. Mp 112–113 °C. $C_{11}D_4H_9NO_4$, ν_{max}(KBr)/cm^{-1}: 2981 m, 2316 w, 2284 w, 1714 s, 1588 s, 1521 s, 1462 m, 1397 s, 1373 m, 1366 m, 1345 s, 1307 m, 1284 s, 1167 s, 1109 s, 849 m, 814 m, 635 s. ^{1}H-NMR (300.13 MHz, $CDCl_3$): 1.61 (9H, s, *tert*-Bu). ^{13}C-NMR (75.468 MHz, $CDCl_3$): 163.8, 150.3, 137.4, 130.2, 123.1, 82.7, 28.2.

[D6]-4-Nitro benzylbromide (12e)

Pure **12a** (1.44 g, 10 mmol) was dissolved in CCl_4 (10 mL). Whilst stirring NBS (1.78 g, 10 mmol) and AIBN (0.02 g, 0.12 mmol) were added successively to the solution. The mixture was heated under reflux for 3 h. Afterwards the precipitated succinimide was filtered off, and was washed twice with CCl_4 (5 mL). The solvent was removed *in vacuo*, and recrystallized from PE. This yielded 0.378 g (17%) of pure compound. Mp 94–96 °C. $C_7D_6BrNO_2$, ν_{max}(KBr)/cm^{-1}: 3428 s, 2305 s, 2288 w, 2187 w, 2149 w, 1593 s, 1534 s, 1347 s. ^{13}C-NMR (75.468 MHz, $CDCl_3$: THF (3 : 1)): 147.3, 144.4, 129.2, 123.3, 30.1. MS (ESI): *m*/*z* 221.0 (6%, [M]),

205.0 (0.4%, [M − O]), 175.0 (1.5%, [M − NO_2]), 142.1 (62%, [M − Br]), 96.1 (10%, [M − Br − NO_2]).

[D6]-4-Nitro benzylamine phthalimide (12f)

12e (2.22 g, 10 mmol) and phthalimide potassium salt (1.85 g, 10 mmol) were dissolved, whilst stirring, in DMF (30 mL) under a N_2 atmosphere. The mixture was stirred and heated at approx. 50 °C overnight. The addition of water (100 mL) afforded a white precipitate, which was filtered off, washed with water (100 mL), and twice with ethanol (100 mL). The obtained product was dried in a desiccator. This yielded 2.36 g (82%) of pure compound. Mp 167–168 °C. $C_{15}D_6H_4N_2O_4$, ν_{max}(KBr)/cm^{-1}: 2360 w, 2296 w, 1769 m, 1711 s, 1583 m, 1572 w, 1508 s, 1467 m, 1392 s, 1344 s, 1306 w, 1177 m, 1078 w, 917 s, 856 m, 826 w, 795 w, 723 s, 708 m, 667 w, 625 w, 529 w. ^{1}H-NMR (300.13 MHz, $CDCl_3$): 7.87 (2H, d, arom.), 7.76 (2H, d, arom.). ^{13}C-NMR (75.468 MHz, $CDCl_3$): 167.9, 147.6, 143.2, 134.4, 132.0, 129.1, 123.7, 123.7, 40.4. MS (ESI): *m*/*z* 343.1 (50%, [M + Na + O_2]), 311.1 (100%, [M + Na]), 242.3 (12%, [M − NO_2]).

[D6]-4-Nitro benzylamine (12g)

[D6]-4-Nitro benzylamine phthalimide (**12f**) (0.89 g, 3.09 mmol) was suspended in a mixture of ethanol (108 mL) and THF (11 mL). Whilst stirring, hydrazine hydrate (80%) (1.1 mL, 18 mmol) was added under a N_2 atmosphere. Afterwards the mixture was heated under reflux for 24 h. The resulting mixture was cooled to 0 °C. 1M oxalic acid (68 mL) was added and once again the mixture was heated under reflux for 30 min. The solution was basified with 2 N NaOH (68 mL), filtered, and the filter cake washed with water (43 mL). The filtrate was extracted five times each with DCM (46 mL). Drying of the combined organic layers (K_2CO_3), and removal of the solvent yielded the title compound, which was directly subjected to the next reaction step (**12h**) without further characterization, because the compound proved to be not very stable.

[D6]-4-Nitro benzylamine-Fmoc (12h)

Compound **12g** (0.467 g, 2.95 mmol) and DIEA (1 mL, 5.9 mmol) were dissolved whilst stirring in DCM (59 mL) under a N_2 atmosphere. Fmoc-Cl (0.768 g, 2.95 mmol) was added, and stirring was continued for 2 h at room temperature. The mixture was successively washed with 1 N HCl (15 mL), and twice with water (30 mL). The organic layer was dried (Na_2SO_4) and the solvent removed. This yielded 1.111 g (99%). Mp 149–151 °C. $C_{22}D_6H_{12}N_2O_4$, ν_{max}(KBr)/cm^{-1}: 3366 s, 3325 s, 2305 w, 2126 w, 1698 s, 1588 m, 1524 s, 1347 s, 1270 s, 1133 m, 1089 m, 758 m, 741 m. ^{1}H-NMR (300.13 MHz, $CDCl_3$): 7.27–7.80 (8H, m, Fmoc-aromatic), 5.14 (1H, s, NH), 4.54 (2H, d, Fmoc-CH_2), 4.12 (1H, t, Fmoc-CH).

[D4]-4-Nitroso benzoic acid *tert*-butyl ester (13a)

[D4]-4-Nitroso benzoic acid *tert*-butyl ester (**13a**) was synthesized according to the procedure published by Park and Standaert.[42] Yield: 1.3 g (77%). Mp 55–62 °C. R_f = 0.53 in hexane : EE (5 : 1). $C_{11}D_4H_9NO_3$, ν_{max}(KBr)/cm^{-1}: 2980 m, 2934 w, 2359 w, 2286 w, 1712 s, 1575 m, 1522 w, 1476 w, 1457 w, 1428 m, 1395 m, 1369 m, 1335 m, 1282 s, 1258 w, 1158 s, 1102 m, 1080 m, 1033 m, 940 m, 904 w, 848 m, 752 w, 709 m, 686 w, 613 m, 528 w, 497 w. ^{1}H-NMR (300.13 MHz, $CDCl_3$): 1.63 (9H, s, *tert*-Bu). ^{13}C-NMR (75.468 MHz, [D6]-acetone): 164.7, 164.4, 137.1, 130.5, 119.9, 82.5, 28.2.

[D5]-Nitroso benzene (13b)

Nitroso benzene (**13b**) was synthesized according to the procedure published by Shine *et al.*[38] A change was made during the workup procedure. Crude **13b** (2 g, 17.8 mmol) was added to a flask containing water (29 mL). Distillation of the mixture at atmospheric pressure afforded a green liquid (bp 93–98 °C at ~1000 mbar) and a white solid, which got stuck in the Liebig condenser. The white solid was washed away from the condenser with ethanol (12 mL). The combined ethanolic solution was concentrated *in vacuo* to approx. 5–6 mL. Storage overnight in the fridge yielded a white solid. Since the vapor pressure of the product is very high, the green solvent in the receiver contained further product. Upon careful concentration further product could be obtained. The product was placed in a desiccator. The combined fractions afforded 0.84 g (30%). Mp 65–67 °C. C_6D_5NO, ν_{max}(KBr)/cm^{-1}: 2287 m, 1401 s, 1360 s, 1327 m, 1290 m, 1228 w, 1150 s, 1037 w, 959 w, 934 s, 859 m, 838 w, 820 w, 809 w, 779 m, 768 m, 680 w, 664 m, 639 m, 599 w, 592 w, 559 m, 543 m, 521 s, 470 m. ^{13}C-NMR (75.468 MHz, $CDCl_3$): 165.9, 135.2, 128.9, 120.6.

([D6]-4-Amino-benzyl)-amine-Fmoc (14a)

Compound **14a** was synthesized as described above (GPA method). **12h** (1.1 g, 2.9 mmol) was dissolved in dioxane–ethanol (25 mL : 40 mL), and Pd/C (0.16 g, 0.16 mmol) was added. Reaction time 2 h. This yielded 1.022 g (100%). Mp 118–121 °C. $C_{22}D_6H_{14}N_2O_2$, ν_{max}(KBr)/cm^{-1}: 3432 s, 3321 s, 2268 w, 2247 w, 2108 w, 1689 s, 1622 m, 1590 m, 1534 s, 1465 m, 1446 s, 1269 s, 1245 s, 1125 m, 1081 m, 1066 m, 1031 m, 757 m, 741 w. ^{1}H-NMR (300.13 MHz, [D6]-DMSO): 7.85 (1H, s, NH), 7.28–7.80 (8H, m, Fmoc-aromatic), 4.35 (4H, m, Fmoc-CH_2 and NH_2), 4.20 (1H, t, Fmoc-CH).

[D4]-4-Amino benzoic acid (14b)

[D4]-4-Nitrobenzoic acid (**12b**) (0.856 g, 5 mmol) was suspended in water (20 mL). Whilst vigorously stirring, Na_2CO_3 (0.3 g, 2.8 mmol) and zinc dust (2 g, 30 mmol) were added to the solution. The mixture was heated to approx. 30 °C, and conc. HCl (10 mL) was added dropwise over 20 min. Thereafter the solution was filtered, and the filter cake was washed twice with water (7 mL). To remove unreacted **12b**, the filtrate was extracted twice with ethyl acetate (40 mL). The aqueous layer was adjusted to pH 9–10 by the addition of sat. Na_2CO_3 solution (approx. 32 mL), and the precipitated ZnOH was filtered off. Once more the filter cake was washed twice with 5% $NaHCO_3$ (10 mL). To remove byproducts, the filtrate was extracted twice with ethyl acetate (80 mL). Subsequently, the aqueous layer was adjusted to pH 3–4 by the addition of 1 N HCl (approx. 30 mL). Finally, the solution was extracted four times with ethyl acetate (80 mL). Drying of the combined organic layers (Na_2SO_4), and removal of the solvent *in vacuo* yielded 0.562 g (80%) of an off white crystalline compound. Mp 179–180 °C. $C_7D_4H_3NO_2$, ν_{max}(KBr)/cm^{-1}: 3461 s, 3364 s,

2287 vw, 1662 vs, 1575 vs, 1410 s, 1343 m, 1306 s, 1235 m, 705 m, 603 m, 542 m, 424 m. ^{13}C-NMR (75.468 MHz, D_2O): 175.5, 149.5, 130.4, 126.0, 114.7.

[D4]-4-Amino benzoic acid methyl ester (14c)

Methanol (200 mL) was placed in the reaction flask at approx. 0 °C. Whilst stirring, $SOCl_2$ (15 mL, 206 mmol) was added dropwise to the methanol in such a manner that the reaction temperature did not increase beyond 10 °C (approx. 30 min). **14b** (7.06 g, 50 mmol) was added slowly (approx. 30 min) to the solution. Stirring was continued for 1 h at 0 °C. The temperature was raised to room temperature and stirring was continued overnight. The solvent was removed *in vacuo* and the residue was taken up in water (100 mL). Neutralization with pyridine (10 mL) gave a white precipitate, which was filtered off, and washed three times with water (15 mL). Drying in a desiccator over P_2O_5 yielded 3.53 g (79%) of a crystalline compound. Mp 107–109 °C. $C_8D_4H_5NO_2$, ν_{max}(KBr)/cm^{-1}: 3411 s, 3341 s, 3329 s, 2947 m, 2285 m, 2261 m, 1683 s, 1638 m, 1575 s, 1428 s, 1374 m, 1284 s, 1221 s, 1089 s, 824 m, 708 s, 599 m, 479 m, 431 m. ^{13}C-NMR (75.468 MHz, $CDCl_3$): 167.3, 151.1, 131.2, 119.2, 113.4, 51.6.

[D6]-4-Amino benzyl alcohol (14d)

A flame dried 250 mL three-necked flask equipped with a thermometer, dropping funnel, and reflux condenser was N_2 purged. Dry ether (60 mL) was placed in the reaction flask. Whilst stirring, $LiAlD_4$ (0.84 g, 20 mmol) was added. Subsequently, a solution of **14c** (3.1 g, 20 mmol) in dry ether (100 mL) was added dropwise over a period of approx. 30 min. The mixture was heated under reflux for 3–4 h, after which it was cooled by an ice bath. Afterwards the reaction was quenched by the dropwise addition of water (7 mL). The mixture was filtered off, and the filter cake was washed three times with ether (40 mL). Drying of the combined organic phases (NaOH), and removal of the solvent yielded the crude product (100%), which was further recrystallized from benzene and placed in a desiccator. This yielded 1.22 g (47%) of a crystalline compound. Mp 60–62.5 °C, (lit.[29] 64.5 °C). $C_7D_6H_3NO$, ν_{max}(KBr)/cm^{-1}: 3377 s, 3240 s, 2273 m, 2251 w, 2203 m, 2108 m, 2069 m, 1580 s, 1439 s, 1374 s, 1236 s, 1083 s, 1045 s, 952 s, 517 s. ^{13}C-NMR (75.468 MHz, [D6]-acetone): 148.1, 131.1, 128.6, 114.6, 64.1.

[D10]-4-(4-Fmoc-aminomethyl-phenylazo)-benzoic acid *tert*-butyl ester (15a)

Fresh **13a** (0.701 g, 2 mmol) and acetic acid (20 mL) were placed in the reaction flask at approx. 0 °C. Whilst stirring, **14a** (0.634 g, 3 mmol) was added immediately. The mixture was stirred at 0 °C for 1 h and then at room temperature for an additional 48 h. The solvent was removed *in vacuo* and the residue was further purified by flash chromatography. Elution with EE–hexane (1 : 3) afforded 0.725 g (67%) of an orange crystalline compound. Mp 90–97 °C. $C_{33}D_{10}H_{21}N_3O_4$, ν_{max}(KBr)/cm^{-1}: 3457 s, 2980 m, 1712 s, 1534 s, 1369 m, 1337 m, 1272 s, 1161 s, 1078 s, 759 m, 742 m. λ_{max}(THF)/nm 266 (ε/dm^3 mol^{-1} cm^{-1} 26 000), 301 (21 000), 333 (28 000) and 449 (885). ^{13}C-NMR (75.468 MHz, $CDCl_3$): 165.3, 156.6, 154.8, 151.9, 143.9, 142.1, 141.4, 133.7, 130.1, 127.8, 127.1, 125.1, 123.1, 122.2, 120.1, 81.6, 66.8, 47.4, 28.3. MS (ESI): *m*/*z* 566.4 (99%, [M + Na]).

[D11]-4-Phenylazo benzyl alcohol (15b)

Nitroso benzene (**13b**) (1.24 g, 11 mmol) and acetic acid (9 mL) were placed in the reaction flask at approx. 0 °C. Whilst stirring, [D6]-4-amino benzyl alcohol (**14d**) (1.29 g, 10 mmol) was added immediately. The mixture was stirred at 0 °C for 1 h and then at room temperature for an additional hour. The precipitated product was filtered off. A second fraction of product was obtained upon the addition of water (38 mL) to the filtrate. After 30 min the second fraction of precipitated product was filtered and afterwards recrystallized from CCl_4. This yielded 1.25 g (56%) of a light red crystalline compound. Degree of deuteration 99.5%. Mp 135–138 °C. $C_{13}D_{11}HN_2O$, ν_{max}(KBr)/cm^{-1}: 3330 s, 2280 m, 2110 m, 1578 w, 1385 w, 1332 m, 1299 m, 1211 m, 1156 m, 1092 m, 1047 m, 970 m, 545 m, 477 m. λ_{max}(EtOH)/nm 229 (ε/dm^3 mol^{-1} cm^{-1} 13 000), 323 (23 000) and 442 (620). ^{13}C-NMR (75.468 MHz, [D6]-acetone): 153.4, 152.4, 146.8, 131.5, 129.6, 127.6, 123.1. MS (ESI): *m*/*z* 246.1 (90%, [M + Na]).

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