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A Photolabile Linker for the Mild and Selective Cleavage of Enriched Biomolecules from Solid Support

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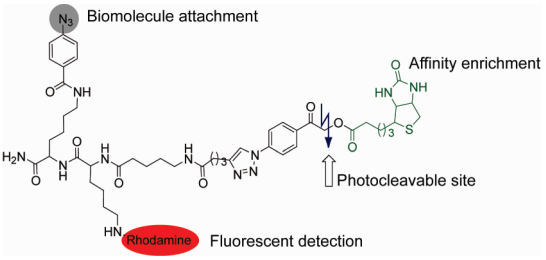

Selective release of enriched biomolecules from solid support is a desirable goal in proteomic and metabolomic studies. Here we demonstrate that photocleavage of a light-sensitive phenacyl ester bond is a suitable alternative cleavage strategy for the selective release of enriched biomolecules form avidin beads circumventing the disadvantages of conventional heat denaturation procedures.

In the era of postgenomic research, characterization of the full cellular complement such as proteins and metabolites has become a major goal to understand the molecular principles of human diseases. Rising up to this challenge several customized technologies in the field of chemistry, biology, and analytical sciences have been developed and implemented. Especially mass spectrometry (MS) has evolved as a key technology in the identification and characterization of proteins and metabolites in prokaryotic and eukaryotic cells.[1−3] Although MS is a very sensitive method for the detection of proteins and metabolites, in many cases metabolites and proteins especially those of low abundance need to be enriched on solid support in order to be detected. Several methods such as isotope coded affinity tagging (ICAT)[4] and activity based protein profiling (ABPP)[5] use biotinylated reactive groups that attach covalently onto proteins and allow their capture and enrichment via incubation with avidin or streptavidin beads. While this technology has become a standard procedure for proteomics applications, several disadvantages have limited the scope and applicability of this method including harsh, denaturing conditions to disrupt the strong biotin−avidin interaction, unspecific binding of high abundance proteins and endogenously biotinylated proteins to the beads, as well as contamination of the MS-probe with avidin monomers during the release procedure. Previously, several cleavable linkers have been applied in order to circumvent the limitations of heat-induced avidin denaturation.[6] Among those are acid and disulfide cleavable linker systems which also encountered problems due to nonselective release and premature cleavage, respectively.[6,7] A better degree of selectivity has been achieved with either protease cleavable linkers or diazobenzene derivatives that are cleaved by mild treatment with sodium dithionite.[7,8] However, although these methods represent a huge improvement compared to the heat-induced release, chemical compounds or enzymes still must be added for a successful cleavage.

Here we introduce a novel strategy to approach the goal of a selective cleavage procedure requiring light to induce a chemical bond breakage. We synthesized a photolabile phenacyl ester group and incorporated it into a multifunctionalized linker system for the enrichment and photocleavage of small molecule metabolites (Figure 1).[9,10] The first functional element of this linker is an azide group that enables the attachment of small biomolecules labeled with an alkyne-containing probe via the copper-catalyzed Huisgen cycloaddition.[11−13] Once the linker is attached to the labeled biomolecules, a biotin moiety facilitates the binding and enrichment on avidin beads. After enrichment, cleavage of biomolecules is achieved by UV-irradiation breaking the corresponding photolabile phenacyl ester group. Once released, a linker bound fluorophore provides a very sensitive visualization of labeled metabolites on HPLC. Since irradiation at 254 nm is not suitable for proteins due to expected photodamage, we decided to demonstrate the general value of our method with small molecule metabolites as an initial model system. Recently, Cravatt and Carlson introduced a method named "metabolite enrichment by tagging and proteolytic release" (METPR) in which metabolites were captured by reactive groups that are immobilized on solid support, released by proteolysis, and identified

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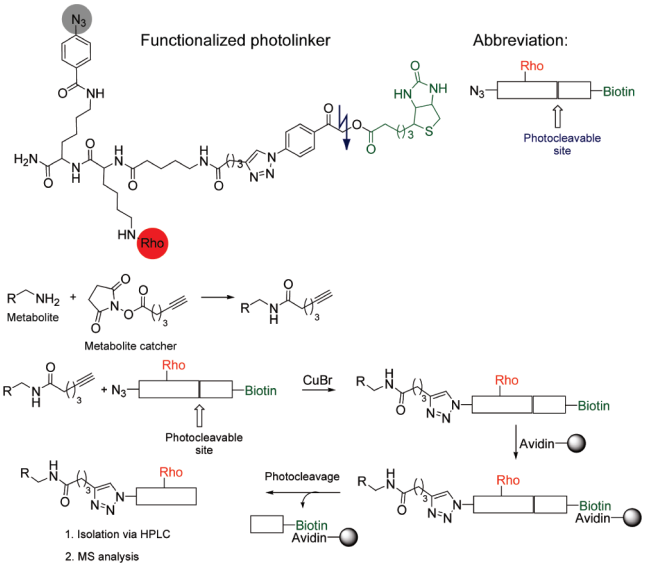
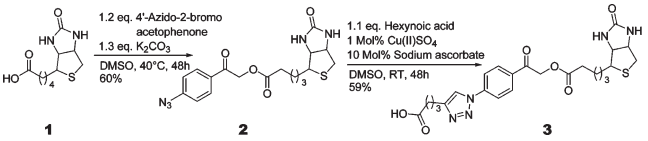


FIGURE 1. Structure of the photocleavable linker and principal workflow of the metabolite capture, enrichment, and cleavage procedure.

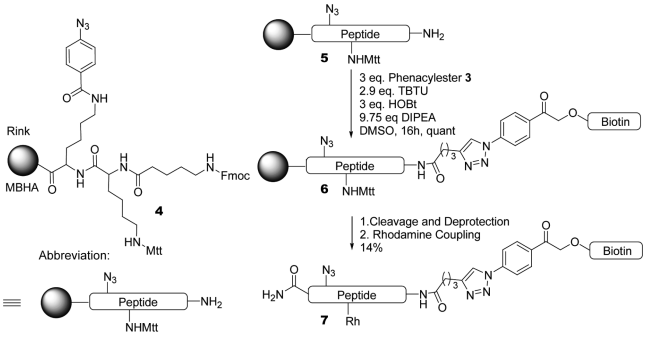
by MS out of complex sample mixtures.[14,15] On the basis of this approach, we synthesized a small *N*-hydroxy succinimide (NHS) reactive probe with an alkyne tag that can be added to metabolomic extracts and that selectively reacts with amine-containing compounds. Here we show that probe-captured model metabolites can be attached to the azide moiety of the photocleavable linker via click chemistry, immobilized on avidin beads, and selectively released via UV light.

The structure of the photocleavable linker is based on a modular assembly starting with the synthesis of a photolabile phenacyl ester group. Phenacyl esters are commonly used as photolabile protecting groups in organic synthesis.[16−19] Usually they function as terminal protecting groups, whereas our approach compromises a 4,2′-disubstituted phenacyl ester moiety linking the different functional elements of the photocleavable linker. This group is easily formed by a nucleophilic reaction between biotin and *p*-azido-2-bromoacetophenone (Scheme 1). Subsequent click chemistry between the azide moiety on the phenacyl ester and hexynoic acid introduces a free acid moiety that was coupled onto the free amine of a peptidic linker backbone that is immobilized on a rink amide resin. This immobilized peptide was built up by standard solid phase peptide synthesis (SPPS) coupling procedures and contains an azide-modified lysine and a MTT-protected lysine (Scheme 2). Removal of the peptide and the MTT group was achieved in one step via TFA treatment after successful coupling of the phenacyl ester building block **3**. The resulting free amine was coupled to *N*-hydroxy succinimide (NHS)-activated tetramethyl rhodamine (TAMRA). HPLC purification yielded the pure multifunctional photolabile linker.

SCHEME 1. Synthesis of the Biotinylated Phenacyl Ester Group



SCHEME 2. Synthesis of Functionalized Peptide Backbone



To evaluate the optimal conditions of photocleavage, we first tested the solubility of the photolinker and realized that up to 60% (v/v) DMSO in PBS buffer was required in order to dissolve the compound. For all subsequent irradiation experiments 0.8 mM DTT was added as a photoscavanger neutralizing free radicals formed during UV-irradiation.[20] Although photocleavage of the photolabile linker **7** was already induced by room light, the efficiency of this process was too low in order to be of preparative value. We therefore used a 15 W UV-hand lamp placed 20 cm above the sample and irradiated compound **7** for 5 min at 254 nm, which led to 75(±11)% of the expected cleavage product as determined by HPLC analysis (Figure 2).

The identity of the cleaved product was confirmed by mass spectrometry and revealed that the bond breakage occurred at the C−O bond and thereby released biotin as previously reported (Experimental Section).[21] Cleavage conditions could be further improved by reducing the distance of the lamp to about 1 cm above the sample. This significantly accelerated the duration of cleavage to 1 min and increased the yield (89(±2)%) (Figure 2). Compared to other cleavage procedures mentioned above, our initial experiments appeared to be advantageous due to the rapid reaction time and easy handling. We therefore evaluated the potential value of this approach in the release of immobilized small biomolecules from avidin beads. To test whether UV-induced cleavage works on solid support, the photocleavable linker (5 nmol) was incubated with avidin beads, washed, and irradiated with UV light (15 W hand lamp) for 1 min. This reaction yielded 69(±4)% fluorescent product relative to a control linker (Figure 1 in the Supporting Information) that is already used in established protocols and cleaved by conventional heat denaturation (Figure 2 in the Supporting Information).[22,23] This result

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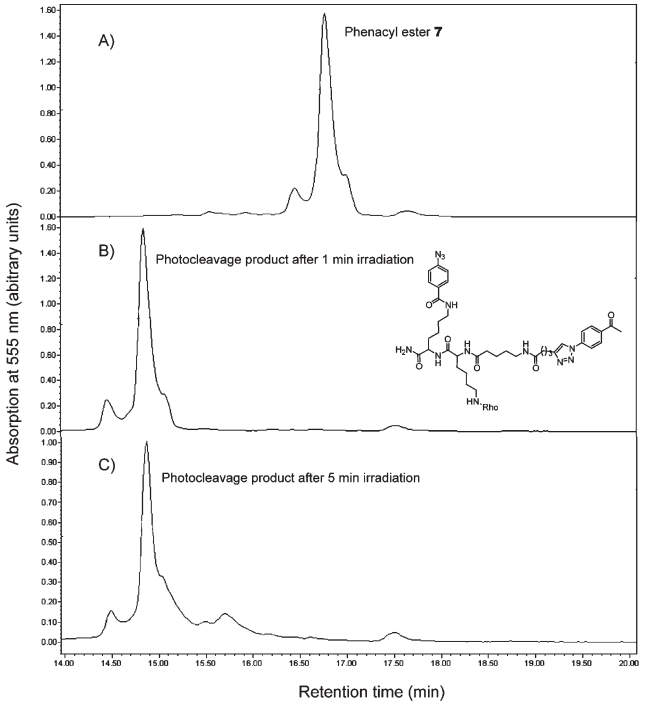

FIGURE 2. HPLC traces of the photolinker before (A) and after 1 (B) and 5 min (C) of irraditation.

indicates that the procedure is still preparative useful, although the irradiation on solid support slightly reduces the cleavage efficiency. In the next step, the efficiency of click chemistry for the probe attachment was evaluated. Unexpectedly, the standard procedure for the cycloaddition via $CuSO_4$ and TCEP did not work since it destroyed the photolinker probably by a reduction of the light-sensitive C−O bond. We therefore avoided the use of TCEP and used CuBr, which yielded a quantitative reaction. With these optimized conditions, the NHS probe was reacted with lysine and benzylamine as a representative metabolite mimic. The resulting amides were then attached to the linker via click chemistry. The reaction mixtures were each incubated with avidin beads, washed with PBS buffer, and irradiated for 7 min with a 350 W UV lamp at 254 nm, which yielded 68(±6)% lysine (Figure 3) and 70(±16)% benzylamine (Figure 3 in the Supporting Information) product relative to the heat denaturation with the control linker (Figure 4 in the Supporting Information). For these experiments, higher irradiation energy was required for bond-breaking, which is due to the presence of an additional photoactive triazole moiety. This higher irradiation energy caused the formation of two products which are a result of the quenching reaction of the radical intermediate with water to give the methyl ketone and the α-hydroxyl ketone as confirmed by mass spectrometric analysis (Supporting Information).[24]

In general, these results show that our model system validates the use of photocleavable linkers as an attractive strategy for the enrichment and release of biomolecules from solid support. Advantages of our method include rapid reaction times, easy handling, and reduced contamination as compared to established methods. Due to these advantages

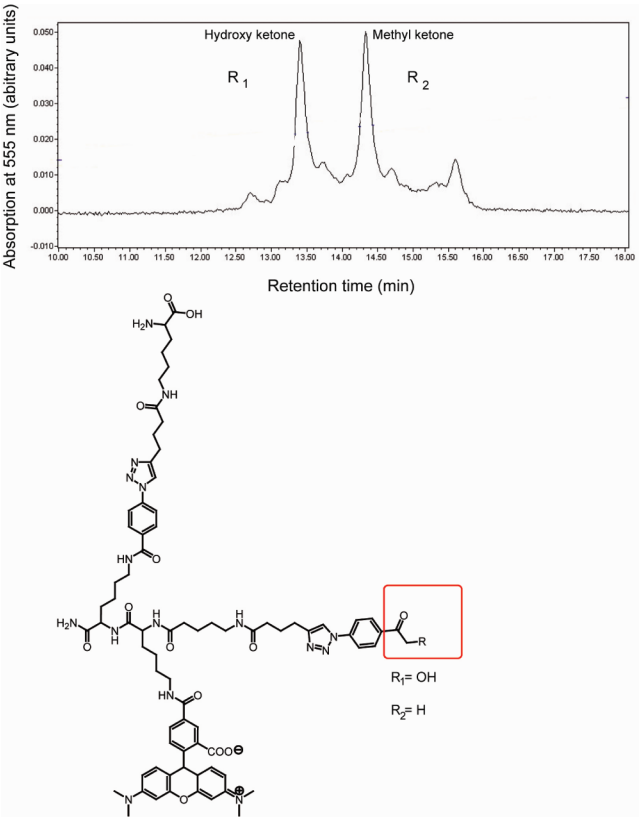

FIGURE 3. HPLC trace and structure of the photolinker after lysine capture via the metabolite reactive group and UV irradiation.

our photocleavable linker could be used in automated metabolite/metabolom screening. In future studies, the photoactivating group will be adjusted for the needs of protein chemistry and detachment from the resin should take place at wavelengths higher than 300 nm in order to avoid photodamage.

Experimental Section

Synthesis of Phenacyl Ester (2). To a solution of 4′-azido-2-bromoacetophenone (500 mg, 2.08 mmol) and Biotin (422.5 mg, 1.74 mmol) in 2.6 mL of dried DMSO was added freshly triturated K_2CO_3 (312 mg, 2.26 mmol). The reaction mixture was strirred for 48 h at 40 °C under argon atmosphere. The phenacyl ester (**2**) (424.3 mg, 60%) was obtained as a yellow solid by silica gel chromatography eluted with TCM/MeOH (4/1). MS (MALDI) *m*/*z* calcd for $C_{18}H_{21}N_5O_4S$ $(M + H)^+$ 404.13, found:404.27 $(M + H)^+$; ^{1}H NMR ($CDCl_3/CD_3OD$ (7/1), 600 MHz) δ 7.89 (d, J = 8.4 Hz, 2 H), 7.07 (d, J = 8.4 Hz, 2 H), 5.31 (d, J = 16.4, 1 H), 5.24 (d, J = 16.2, 1H), 4.50−4.46 (m, 1 H), 4.33−4.28 (m, 1 H), 3.16−3.11 (m, 1 H), 2.91−2.86 (m, J = 4.8, 1 H), 2.69 (d, J = 12.7 Hz, 1H), 2.52−2.41 (m, 2H), 1.76−1.69 (m, 3H), 1.67−1.60 (m, 1H), 1.55−1.44 (m, 2H); ^{13}C NMR ($CDCl_3/CD_3OD$ (7/1), 150 MHz) δ 191.1 (C), 173.1 (C), 163.9 (C), 145.9 (C), 130.4 (C), 129.7 (CH), 119.2 (CH), 65.7 (CH_2), 61.6 (CH), 60.1 (CH), 55.2 (CH), 40.43 (CH_2), 40.35 (CH_2), 33.4 (CH_2), 28.0 (CH_2), 24.7 (CH_2).

Synthesis of the Triazole (3). To a solution of phenacyl ester (**2**) (424.3 mg, 1.05 mmol) and hexynoic acid (127.29 μL, 1.27 mmol) in 9.1 mL of dried DMSO were added 10.50 μmol of Cu(II)SO_4 (2.62 mg dissolved in 1 mL of H_2O) and 105.00 μmol of freshly prepared sodium ascorbate (20.80 mg dissolved in 105.2 μL of H_2O). The reaction mixture was stirred for 48 h at room

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temperature under argon atmosphere. The triazole (**3**) (320 mg, 59%) was obtained as a yellow solid by silica gel chromatography eluted with TCM/MeOH (4/1). MS (ESI) m/z calcd for $C_{24}H_{29}N_5O_6S$ $(M - H)^-$ 514.174, found 514.176 $(M - H)^-$; ^{1}H NMR (DMSO, 400 MHz) δ 12.04 (s, 1H), 8.73 (s, 1H), 8.13 (d, J = 8.9 Hz, 2H), 8.06 (d, J = 8.9 Hz, 2H), 6.36 (d, J = 29.2 Hz, 2H), 5.48 (s, 2H), 4.30−4.25 (m, 1H), 4.14−4.09 (m, 1H), 3.11−3.05 (m, 1H), 2.72 (t, J = 7.6 Hz, 2H), 2.42 (t, J = 7.4 Hz, 2H), 2.30 (t, J = 7.2 Hz, 2H), 1.92−1.84 (m, 2H), 1.65−1.55 (m, 4H), 1.51−1.32 (m, 4H); ^{13}C NMR (DMSO, 100 MHz) δ 192.4 (C), 174.6 (C), 172.8 (C), 163.1 (C), 148.4 (CH), 140.6 (C), 133.6 (CH), 130.2 (CH), 120.8 (CH), 120.0 (CH), 66.8 (CH_2), 61.4 (CH), 59.6 (CH), 55.7 (CH), 40.8 (CH_2), 33.4 (CH_2), 33.4 (CH_2), 28.4 (CH_2), 28.3 (CH_2), 24.9 (CH_2), 24.8 (CH_2), 24.5 (CH_2).

Synthesis of Phenacyl Linker (7): Coupling to Resin Bound Peptide, Purification, and TAMRA Coupling. A solution of triazole (**3**) (11.1 mg, 21.6 μmol), TBTU (6.7 mg, 20.88 μmol), HOBt (2.9 mg, 21,6 μmol), and DIPEA (12.2 μL, 70.2 μmol) was stirred for 10 min at room temperature prior to addition of the deprotected resin bound peptide (**4**) (10 mg resin, 5.3 μmol, B_H = 0.53 mmol/g). The suspension was shaken for 16 h at room temperature. This coupling procedure was repeated three times to give a negative Kaiser assay. After washing with DMF (3×) and DCM (2×), the resin was treated with 100 μL of TFA/H_2O (95/5) for 2 h at room temperature. Ice cold Et_2O (1 mL) was added and the suspension was incubated for 45 min at −20 °C. After centrifugation (2 min, 17 000 g) the resulting pellet was washed with Et_2O (2×) and dried for 1 h at room temperature. The pellet was dissolved in 100 μL of dried DMF. DIPEA (3.8 μL, 21.6 μL) and a mixture of (5)- and (6)-carboxy-TAMRA-NHS isomers (3.80 mg, 7.2 μmol) were added. The reaction mixture was strirred for 16 h at room temperature. The crude product was purified via HPLC (column: XBridge BEH130 C18 5 μm (10 × 150 mm), linear gradient 0 to 100% CH_3CN/H_2O with 0.1% TFA in 60 min) to give phenacyl linker (**7**) as a purple powder (1.4 mg, 18.52%). It was possible to separate the two isomers and all studies were performed with a single isomer. t_R = 25.4 min (42.1% CH_3CN/H_2O); MS (ESI) m/z calcd for $C_{73}H_{87}N_{16}O_{13}S$ $(M)^+$ 1427.635, found 1427.632 $(M)^+$.

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Supporting Information Available: General experimental methods, additional experimental procedures, compound characterization data, copies of spectra, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.