Protein Labeling

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Peptide-Templated Acyl Transfer: A Chemical Method for the Labeling of Membrane Proteins on Live Cells**

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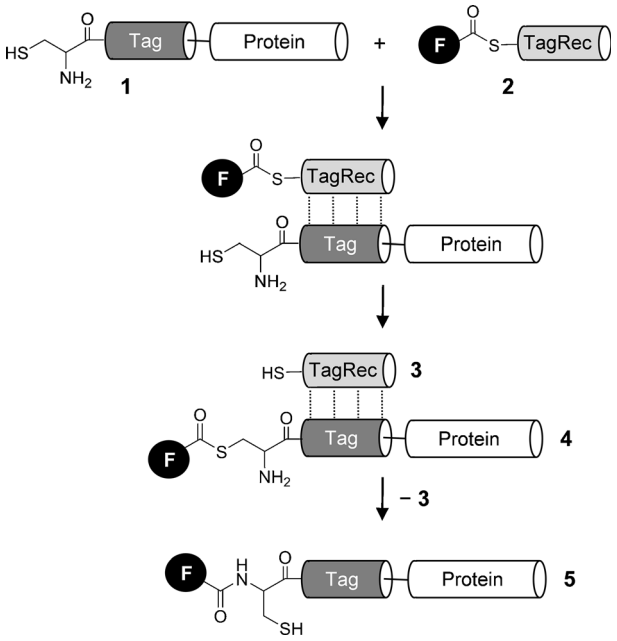
Abstract: *The development of a method is described for the chemical labeling of proteins which occurs with high target specificity, proceeds within seconds to minutes, and offers a free choice of the reporter group. The method relies upon the use of peptide templates, which align a thioester and an N-terminal cysteinyl residue such that an acyl transfer reaction is facilitated at nanomolar concentrations. The protein of interest is N-terminally tagged with a 22 aa long Cys-E3 peptide (acceptor), which is capable of forming a coiled-coil with a reporter-armed K3 peptide (donor). This triggers the transfer of the reporter to the acceptor on the target protein. Because ligation of the two interacting peptides is avoided, the mass increase at the protein of interest is minimal. The method is exemplified by the rapid fluorescent labeling and fluorescence microscopic imaging of the human Y_2 receptor on living cells.*

Methods that allow the in vivo covalent attachment of functional units to membrane proteins, for example spectroscopic labels and spin probes, are a key enabling technology in cell biological research.[1] The ideal labeling method would offer a) high selectivity for the protein of interest, b) rapid labeling for the investigation of fast processes, and c) modularity to allow the attachment of various labels. However, these demands are difficult to meet with the repertoire of naturally occurring functional groups.

Amber (STOP) and expanded size codon technologies, pyrrolysine techniques and metabolic labeling are used to introduce artificial amino acids that can be targeted by means of biocompatible conjugation reactions.[2] A more frequently explored approach involves genetically encoded tag sequences, which are fused to the protein of interest and fashioned to provide a unique microenvironment selectively addressable by a reactive probe. Here the size of the tag is of concern. Small tags are desirable to prevent interference with protein function. One way to achieve high specificity relies on proteins that undergo self-modification reactions such as the Snap,[3] Clip,[4] and Halo[5] tags. These tags are larger than 180 amino acids. Another approach is to use enzymes that promote the reaction between reactive probes and small-sized peptide tags. Relatively high substrate concentrations (> 100 μM for FGE[7] and sortase)[8] or long incubation times (1 h for lactamase)[6] are required. Only a few peptide tags are capable of forming covalent bonds without the help of additional enzymes or reagents. For example, organobisarsenic acid thioesters[9] and bisboronic acid[10] probes react with tetracysteine and tetraserine motifs, respectively. With reaction times of 30 min or more and μM concentration of reactive probes, other cysteine- or serine-rich proteins may react as well.

We sought a method that allows very rapid (< 5 min) and highly specific labeling of cell surface proteins in which minimal cargo is added to the protein of interest. We considered templated reactions that occur at high effective molarity of the aligned functional groups and, therefore, enable conjugation at dilution conditions when conventional bimolecular reactions cease to proceed. Our design involves a cysteine-mediated transfer of an arbitrary reporter group (Scheme 1). The reaction system relies on two peptides with high mutual affinity, that is, the accepting peptide tag in **1** and the tag recognition module (TagRec) in **2**. The acceptor



Scheme 1. Principle of peptide-templated acyl transfer reaction. F = reporter group, Tag = genetically encoded peptide tag, TagRec = Tag recognition module.

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peptide is fused to the protein of interest and offers an N-terminal cysteine which can be addressed by a native-chemical-ligation-like reaction.[11] The TagRec peptide in **2** recognizes the peptide tag and serves as the donor carrying a thioester-linked reporter group. Mutual recognition of the two peptides triggers a thiol-exchange reaction to form **3·4**. The subsequent S–N acyl shift leads to the irreversible transfer of the acyl group from the donor **2** to the acceptor peptide (**1**→**5**). Of note, the templated reaction proceeds through binary complexes. This and the use of a logic based on acyl transfer minimizes the mass increase at the target protein which would occur in a more conventional ligation approach.[12]

Templated acyl transfer reactions have been used in nucleic acid chemistry.[13] However, the design of a small-sized peptide that has high affinity for reactive probes is more challenging. Previous work by Ghadiri and Chmielewski on templated peptide ligation in "early molecules of life" scenarios suggested that the two strands of a coiled-coil should have the high mutual affinity required to drive fluorescence labeling at submicromolar concentration.[12a–d] Furthermore, imaging studies by Matsuzaki et al. confirmed that artificial coiled-coils form on live cells.[14] The de novo designed coiled-coil sequences K3 $(KIAALKE)_3$ and E3 $(EIAALEK)_3$ form highly stable, parallel heterodimers ($K_D = 70$ nM),[15] and therefore appeared suitable to prove the feasibility of the peptide-templated acyl transfer.[16]

The K3 peptide was equipped with a mercaptophenylacetyl (MPAA) unit at the N-terminal end and fluorescence labels such as tetramethylrhodamine (TAMRA) and Alexa Fluor 350 (AF350) were attached through thioester linkages (Figure 1a).[17] The E3 peptide served as acceptor tag and contained, for this purpose, an N-terminal cysteine residue. To assess the feasibility of the peptide-templated acyl transfer, the TAMRA-loaded donor probe **6a** was allowed to react with the acceptor **7**, both at a concentration of 5 μM. HPLC analysis with fluorescence detection showed nearly complete consumption of donor peptide **6a** and the appearance of two new peaks after a reaction time of 30 min (Figure 1b). Based on MS analysis the peaks were assigned to the transfer product **8a** and the hydrolysis product **10a**. After a reaction time of 30 min labeled product was obtained in 57% yield. To assess the specificity of the coiled-coil-directed transfer reaction, the thioester was also incubated with the cysteinyl peptide CRAESYK (**11**; Figure 1b, background). The absence of product suggests that the reaction requires the proximity induced by the coiled-coil formation.

Next we evaluated the versatility of the labeling reaction. The Cys-E3 acceptor peptide **6** was reacted with the donor conjugate **6b** armed with a sulfonated Alexa Fluor dye (AF350, **6b**). At donor and acceptor concentrations of 0.5 μM each, the transfer product (**8b**) was formed in 68% yield (Figure 1c). In this case, a doubly N- and S-linked labeling product was also formed. This is of no harm because the S-linked dye hydrolyzes to form the monolabeled **8b** (Figure S1). To put the chemoselectivity to a more stringent test, the peptide-templated acyl transfer reaction was performed in cell culture medium (Figure S2). In the absence of external reducing agents the reaction between AF350-K3

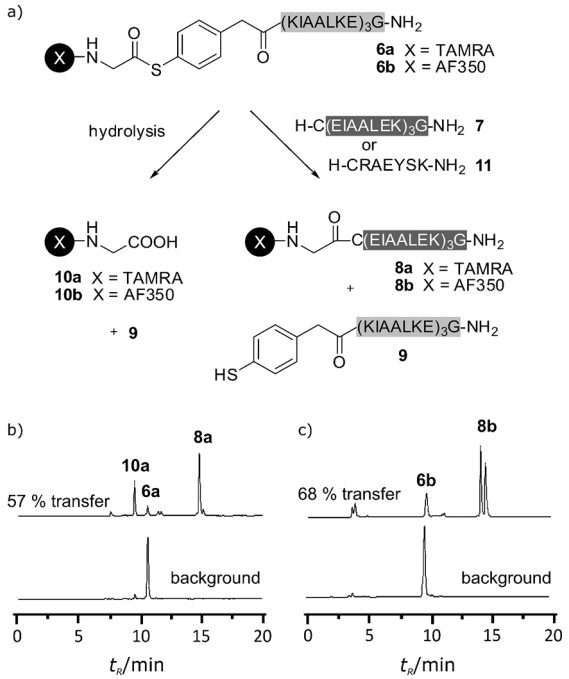

Figure 1. a) Peptide-templated acyl transfer reactions involving the donating thioester peptides **6a** or **6b** and the acceptor peptide tag **7** or the cysteinyl peptide **11** (= specificity control). HPLC analysis (fluorescence detection) after 30 min reaction between b) thioester **6a** and cysteinyl peptide **7** (top) or **11** (bottom) (5 μM peptide, 100 mM NaH_2PO_4, 1 mM TCEP, pH 7.0; $\lambda_{ex} = 555$ nm, $\lambda_{em} = 584$ nm) and c) thioester **6b** and cysteinyl peptide **7** (top) or **11** (bottom) (0.5 μM peptide, 100 mM NaH_2PO_4, 1 mM TCEP, pH 7.0; $\lambda_{ex} = 346$ nm, $\lambda_{em} = 442$ nm). Ligation product **8b** appears as a double peak due to simultaneous N- and S-acylation.

donor conjugate **6b** and cysteinyl peptide **7** afforded the labeled product in 43% yield despite the presence of fetal calf serum and cystine (200 μM).

We investigated the reactivity of thioester probe **6a** in the labeling reaction of a small-sized protein domain, the 5.8 kDa Cys-E3-tagged WW2 domain **12** (Figure 2a). SDS-PAGE and fluorescence gel imaging showed the appearance of the TAMRA band at the expected molecular weight region after only 1–2 min reaction time (Figure 2b, lanes 4 and 5). This is in line with the results of kinetic analyses of the **6a** + **7** reaction system (Figure S3). The TAMRA-labeled WW2 domain was obtained in 58% yield after 10 min. In a control experiment, acceptor peptide **12** was exposed to an unreactive K3 probe (**13a**), in which TAMRA was linked through an amide bond rather than a thioester bond. This did not lead to a fluorescent protein band in the gel (Figure 2b, lanes 10–14).

The next step included the labeling reaction of a membrane protein model. We selected the human neuropeptide Y_2 receptor (hY_2R) for N-terminal labeling. This receptor belongs to the family of G-protein-coupled heptahelix proteins and plays an important role in both epilepsy and the regulation of food intake.[18] An extracellular segment end of the hY_2R(1–46) was fused to an enhanced yellow fluorescent protein and equipped with the N-terminal Cys-E3 tag and a hemagglutinin epitope (HA). The resulting construct Cys-E3-HA-hY_2R(1-46)-eYFP-His_6 (**14**) was

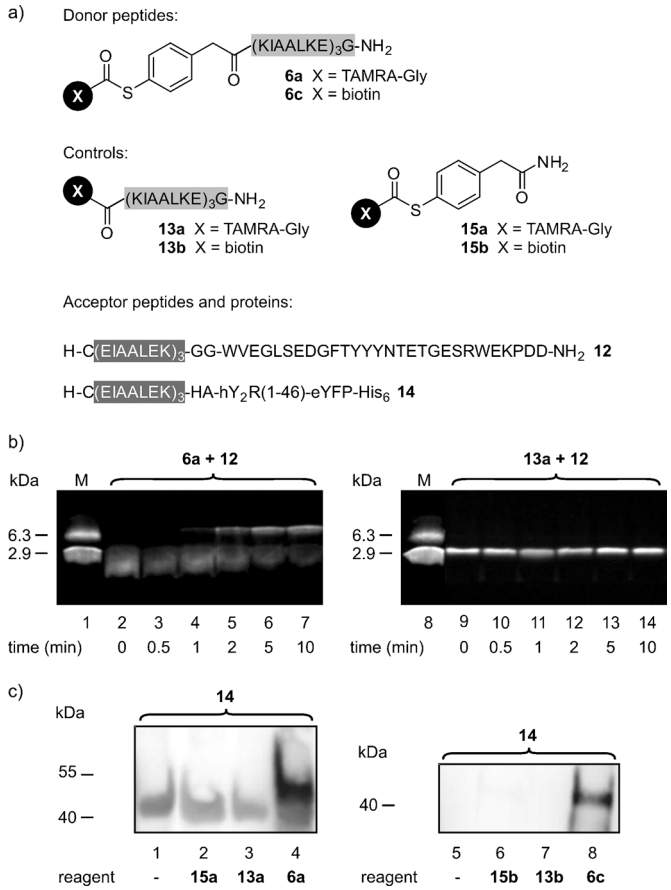

Figure 2. a) Structures of compounds employed. b) SDS-PAGE analysis (fluorescence detection) of the reaction between the Cys-E3-tagged WW2 domain **12** and thioester-linked TAMRA-K3 conjugate **6a** or the amide-linked control compound **13a**. Conditions: 0.5 μM Cys-E3-WW2 (**11**), 1.0 μM K3-peptide (**6a** or **13a**), 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM TCEP, pH 7.2; aliquots quenched by addition of 2% TFA. c) Western blot analysis of the reaction between Cys-E3-tagged hY_2R(1-46)-eYFP-His_6 with 37.5 kDa (**14**) and TAMRA-linked (**6a**, **13a**, **15a**) or biotin-linked (**6c**, **13b**, **15b**) compounds. Conditions for TAMRA labeling: 20 μM Cys-E3-hY_2R(1-46)-eYFP-His_6 (**14**), 20 μM TAMRA compound (**6a**, **13a**, **15a**), 25 mM Tris, 500 mM NaCl, pH 7.4. Conditions for biotin labeling: 10 μM Cys-E3-hY_2R(1-46)-eYFP-His (**14**), 20 μM biotin compound (**6c**, **13b**, **15b**), 25 mM Tris, 500 mM NaCl, pH 7.4; aliquots quenched by addition of 5 μL 1 M NaOH. Tris = 2-amino-2-hydroxymethyl-propane-1,3-diol.

allowed to react with the donor conjugate **6a** (Figure 2c). As negative controls, the amide-linked **13a** and a TAMRA thioester without peptide (**15a**) were tested. The donor or control peptides were incubated with the hY_2R construct **14** for 2 min. SDS-PAGE followed by Western blot analysis of reactions that involved the reactive donor **6a** showed a strong band at the molecular weight expected for the labeled product (37.5 kDa; Figure 2c, lane 4). The control experiment, in which the acceptor **14** was incubated in the absence of labeling reagent (Figure 2c, lane 1) exposed the unspecific binding of the anti-TAMRA antibody. We therefore investigated biotinylation, which can be evaluated by means of the highly specific interaction with streptavidin. A biotinylated product was formed after 2 min reaction between the E3-containing acceptor **14** and the reactive K3 conjugate **6c** (Figure 2c, lane 8). Biotinylation did not occur with control compounds **13b** and **15b** which lack either the thioester or the K3 recognition module, respectively (Figure 2c, lanes 6 and 7). This shows that the native chemical ligation **14** + **15b** is not sufficient to induce fast and efficient labeling of Cys-E3-HA-hY_2R(1-46)-eYFP-His_6 (**14**). Rather, the simultaneous presence of both the thioester structure and the K3 recognition module in reactive probes such as **6a** and **6c** is required to drive the reaction.

The coiled-coil-mediated acyl transfer reaction was subsequently applied to living cells. It was our aim to selectively label the complete hY_2R. The membrane protein was N-terminally fused with two versions of the E3 tag. In **17**, the E3 tag was equipped with the N-terminal cysteine. Construct **18** also contained the E3 tag but the N-terminal cysteine was replaced by alanine. This construct is able to form the coiled-coil yet the lack of cysteine will prevent the formation of a covalent bond. To compare the localization of the receptors, the autofluorescent eYFP was fused to the C-terminus. The hY_2R-eYFP construct (**16**) without the E3 tag served as the control. The hY_2R mutants were transiently expressed in HEK293 cells. Measurements of the eYFP emission revealed that the addition of the E3 tag did not change the localization in the membrane (Figure 3). In addition, the N-terminally modified hY_2R variants remained active, as demonstrated by an IP_3 accumulation assay after treatment with the native ligand neuropeptide Y (Table S1). Cells expressing **16**, **17**, or **18** were treated with 100 nM TAMRA-armed peptide **6a** in Hank's balanced salt solution (HBSS, pH 7.8) and 1% bovine serum albumin for only 2 min. After incubation at room temperature, the labeling solution was exchanged for HBSS. Fluorescence microscopy revealed that transfected cells expressing E3-tagged receptors were labeled with TAMRA, whereas cells which lacked the E3 tag did not emit TAMRA signals (Figure 3, compare B′, D′, G′, and I′ with A′ and F′). As a control for covalent labeling the cells were washed with a pH 9 buffer (Figure 3; C′, E′, H′, J′), which leads to dissociation of coiled-coils and thioester hydrolysis. The TAMRA emission remained high when cells expressing Cys-E3-tagged hY_2R (**17**) were subjected to the pH 9 wash (Figure 3, compare C′ with B′). In contrast, cells expressing the Ala-E3-tagged hY_2R (**18**) demonstrated a marked decrease of the TAMRA signal (Figure 3, compare E′ with D′). An additional control experiment involved the use of the unreactive TAMRA-K3 conjugate, in which the TAMRA dye was linked through an amide bond. Therefore, the coiled-coil may form but the TAMRA cannot be transferred to the E3-tagged hY_2R. Indeed, cells transfected with Cys-E3-hY_2R (**17**) and Ala-Cys-hY_2R (**18**) provided TAMRA emission after incubation with unreactive **13a** (Figure 3 G′ and I′, respectively), but the TAMRA signals vanished when the cells were subjected to the pH 9 wash (Figure 3, compare H′ with G′ and J′ with I′), indicating the dissociation of the coiled-coil. The experiments showed that robust TAMRA labeling can be achieved only when a reactive donor (**6a**) is allowed to react with a reactive acceptor (**17**). This is a strong indication for the formation of covalent bonds.

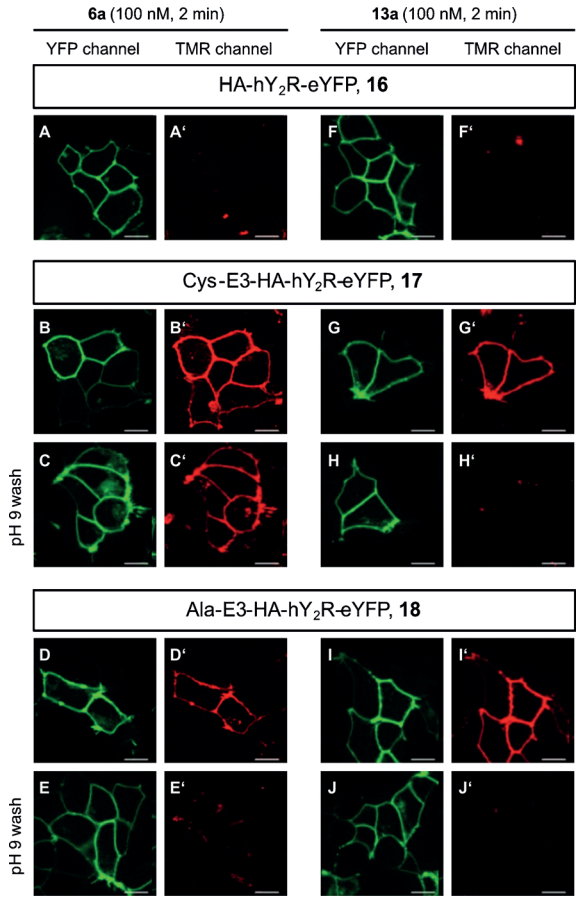

Figure 3. Fluorescence microscopy images of HEK293 cells transiently transfected with HA-hY$_2$R-eYFP (A, A′, F, F′), Cys-E3-HA-hY$_2$R-eYFP (B, B′, C, C′, G, G′, H, H′), or Ala-E3-HA-hY$_2$R-eYFP (D, D′, E, E′, I, I′, J, J′) after incubation with labeling probe **6a** (left two panels) or unreactive conjugate **13a** (right two panels). Images C, C′, E. E′, H, H′, J, and J' were obtained after an additional basic washing step. Labeling: 100 nM peptide **6a** or **13a** in HBSS containing 1% BSA, after 2 min reaction time the labeling solution was replaced with HBSS. Basic washing: 200 mM $NaHCO_3$ pH 9 for 3 min, fluorescence microscopy: 0.4–1.3 s exposure for eYFP filter, 4 s exposure for TAMRA filter, the scale bar is 10 μm.

Our data demonstrate the versatility of the peptide-templated acyl transfer reaction. The reaction proceeded in various buffers (pH 7.0–7.8) as well as on the surface of living cells. The thioaryl-based thioesters in labeling agents **6a–c** have high reactivity (**6a**: $t_{1/2}$ (HBSS) = 10 min, Figure S4). Given the high rate of the templated reaction, this helps to provide high specificity because undesired labeling by excess thioester is prevented by the competing hydrolysis. If the hydrolysis is too fast, the thioester structure can offer a convenient means for reactivity adjustments, as previous work on nucleic acid templated reactions has shown (see also Figure S5).[13a,b,19]

The labeling method requires an N-terminal cysteine residue which may perturb the folding of the extracellular domain of the targeted GPCR. To assess the generality we applied the E3/K3-templated acyl transfer to the human Y5 receptor (hY$_5$R). Both hY$_2$R and hY$_5$R contain extracellular disulfide bridges. Cys-E3 tagging did not interfere with GPCR function and live-cell labeling occurred rapidly also for hY$_5$R (Figure S5).

We evaluated the transfer of TAMRA, AF350, and biotin and we expect that the reaction is applicable to a wide range of other reporter groups. The use of the E3K3 coiled-coil motif conferred remarkable chemoselectivity on the labeling reaction. The K3 conjugates are probably not able to pass the plasma membrane. While this limits applications to extracellular targets at this stage, we consider this to be an advantage for the envisioned GPCR trafficking studies relying on ultrafast and highly specific labeling reactions. Moreover, we wish to add that the conjugation with cell-penetrating peptides may extend the scope to intracellular protein labeling. In addition, labeling by templated acyl transfer should not be restricted to coiled-coils but may be transferred to other interaction motifs involving cell-permeable proteins and peptides.

In conclusion, the rapid labeling (2 min), low concentration of labeling agent (100 nM), and small mass increases (22 amino acids + reporter) are the unique hallmarks of the coiled-coil-mediated reporter group transfer developed here. The reaction is, to the best of our knowledge, among the fastest labeling methods reported. We expect this to be useful in pulse-chase labeling experiments.

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[1] a) I. Böhme, A. G. Beck-Sickinger, *Cell Commun. Signaling* **2009**, *7*, 16; b) Y. Takaoka, A. Ojida, I. Hamachi, *Angew. Chem.* **2013**, *125*, 4182; *Angew. Chem. Int. Ed.* **2013**, *52*, 4088.
[2] a) V. W. Cornish, K. M. Hahn, P. G. Schultz, *J. Am. Chem. Soc.* **1996**, *118*, 8150; b) Y. Zeng, T. N. C. Ramya, A. Dirksen, P. E. Dawson, J. C. Paulson, *Nat. Methods* **2009**, *6*, 207; c) A. Banerjee, T. D. Panosian, K. Mukherjee, R. Ravindra, S. Gal, D. L. Sackett, S. Bane, *ACS Chem. Biol.* **2010**, *5*, 777; d) L. Wang, Z. W. Zhang, A. Brock, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 56; e) J. A. Codelli, J. M. Baskin, N. J. Agard, C. R. Berozzi, *J. Am. Chem. Soc.* **2008**, *130*, 11486; f) Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* **2003**, *125*, 3192; g) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057; h) J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16793; i) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046; j) M. Wiessler, C. Kliem, P. Lorenz, E. Müller, H. Fleischhacker, EP2006012414, **2006**; k) M. L. Blackman, M. Royzen, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 13518; l) N. K. Devaraj, R. Weissleder, S. A. Hilderbrand, *Bioconjugate Chem.* **2008**, *19*, 2297.
[3] A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, H. Vogel, K. Johnsson, *Nat. Biotechnol.* **2002**, *21*, 86.
[4] A. Gautier, A. Juillerat, C. Heinis, I. R. Correa, M. Kindermann, F. Beaufils, K. Johnsson, *Chem. Biol.* **2008**, *15*, 128.
[5] G. V. Los, L. P. Encell, M. G. McDougall, D. D. Hartzell, N. Karassina, C. Zimprich, M. G. Wood, R. Learish, R. F. Ohane, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J. Zhu, A. Darzins, D. H. Klaubert, R. F. Bulleit, K. V. Wood, *ACS Chem. Biol.* **2008**, *3*, 373.

[6] S. Mizukami, S. Watanabe, Y. Hori, K. Kikuchi, *J. Am. Chem. Soc.* **2009**, *131*, 5016.
[7] P. Wu, W. Q. Shui, B. L. Carlson, N. Hu, D. Rabuka, J. Lee, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 3000.
[8] M. W. Popp, J. M. Antos, G. M. Grotenbreg, E. Spooner, H. L. Ploegh, *Nat. Chem. Biol.* **2007**, *3*, 707.
[9] B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **1998**, *281*, 269.
[10] T. L. Halo, J. Appelbaum, E. M. Hobert, D. M. Balkin, A. Schepartz, *J. Am. Chem. Soc.* **2009**, *131*, 438.
[11] P. E. Dawson, T. W. Muir, I. Clarklewis, S. B. H. Kent, *Science* **1994**, *266*, 776.
[12] a) D. H. Lee, J. R. Granja, J. A. Martinez, K. Severin, M. R. Ghadiri, *Nature* **1996**, *382*, 525; b) K. Severin, D. H. Lee, A. J. Kennan, M. R. Ghadiri, *Nature* **1997**, *389*, 706; c) K. Severin, D. H. Lee, J. A. Martinez, M. R. Ghadiri, *Chem. Eur. J.* **1997**, *3*, 1017; d) S. Yao, I. Ghosh, R. Zutshi, J. Chmielewski, *Angew. Chem.* **1998**, *110*, 489; *Angew. Chem. Int. Ed.* **1998**, *37*, 478; e) N. Brauckhoff, G. Hahne, J. T. H. Yeh, T. N. Grossmann, *Angew. Chem.* **2014**, *126*, 4425; *Angew. Chem. Int. Ed.* **2014**, *53*, 4337.
[13] a) T. N. Grossmann, O. Seitz, *Chem. Eur. J.* **2009**, *15*, 6723; b) T. N. Grossmann, O. Seitz, *J. Am. Chem. Soc.* **2006**, *128*, 15596; c) T. N. Grossmann, L. Roeglin, O. Seitz, *Angew. Chem.* **2008**, *120*, 7228; *Angew. Chem. Int. Ed.* **2008**, *47*, 7119; d) J. Michaelis, A. Maruyama, O. Seitz, *Chem. Commun.* **2013**, *49*, 618; e) J. Michaelis, G. J. v. d. H. van Noort, O. Seitz, *Bioconjugate Chem.* **2014**, *25*, 18.
[14] Y. Yano, A. Yano, S. Oishi, Y. Sugimoto, G. Tsujimoto, N. Fujii, K. Matsuzaki, *ACS Chem. Biol.* **2008**, *3*, 341.
[15] J. R. Litowski, R. S. Hodges, *J. Biol. Chem.* **2002**, *277*, 37272.
[16] This coiled-coil motif was also used by Ball et al. to achieve rhodium-catalyzed biotinylation of proteins in cell lysate at high concentration (100 μM) of labeling agent: a) Z. Chen, B. V. Popp, C. L. Bovet, Z. T. Ball, *ACS Chem. Biol.* **2011**, *6*, 920; b) Z. Chen, F. Vohidov, J. M. Coughlin, L. J. Stagg, S. T. Arold, J. E. Ladbury, Z. T. Ball, *J. Am. Chem. Soc.* **2012**, *134*, 10138.
[17] See the Supporting Information for details on the synthesis.
[18] a) C. Walther, K. Morl, A. G. Beck-Sickinger, *J. Pept. Sci.* **2011**, *17*, 233; b) R. E. Mercer, M. J. S. Chee, W. F. Colmers, *Front. Neuroendocrinol.* **2011**, *32*, 398.
[19] a) A. Roloff, O. Seitz, *Bioorg. Med. Chem.* **2013**, *21*, 3458; b) A. Roloff, O. Seitz, *Chem. Sci.* **2013**, *4*, 432.

