CHEMBIOCHEM

DOI: 10.1002/cbic.200900349

Synthesis of *S*-Adenosyl-L-homocysteine Capture Compounds for Selective Photoinduced Isolation of Methyltransferases

Christian Dalhoff,[b] Michael Hüben,[a] Thomas Lenz,[b] Peter Poot,[a] Eckhard Nordhoff,[c] Hubert Köster,*[b] and Elmar Weinhold*[a]

Understanding the interplay of different cellular proteins and their substrates is of major interest in the postgenomic era. For this purpose, selective isolation and identification of proteins from complex biological samples is necessary and targeted isolation of enzyme families is a challenging task. Over the last years, methods like activity-based protein profiling (ABPP) and capture compound mass spectrometry (CCMS) have been developed to reduce the complexity of the proteome by means of protein function in contrast to standard approaches, which utilize differences in physical properties for protein separation. To isolate and identify the subproteome consisting of *S*-adenosyl-L-methionine (SAM or AdoMet)-dependent methyltransferases (methylome), we developed and synthesized trifunctional capture compounds containing the chemically stable cofactor product *S*-adenosyl-L-homocysteine (SAH or AdoHcy) as selectivity function. SAH analogues with amino linkers at the N6 or C8 positions were synthesized and attached to scaffolds containing different photocrosslinking groups for covalent protein modification and biotin for affinity isolation. The utility of these SAH capture compounds for selective photoinduced protein isolation is demonstrated for various methyltransferases (MTases) acting on DNA, RNA and proteins as well as with *Escherichia coli* cell lysate. In addition, they can be used to determine dissociation constants for MTase–cofactor complexes.

Introduction

The proteome, that is, the entire set of proteins expressed by a genome, cell, tissue or organism at a given time under defined conditions, is the prevalent bioanalytical challenge in the postgenomic era. The vast number of proteins usually present in cells or organisms preclude an entire direct proteomic analysis. Protein separation by physical properties like molecular weight, charge, and hydrophobicity can prefractionate the complex protein mixtures, but thereafter time consuming and extensive analyses of the single fractions might be necessary to further separate and identify the protein(s) of interest. Alternatively, functional-directed separation of proteome subsets from whole proteomes can reduce the complexity and allow for identification of the protein(s) of interest by standard bioanalytical methods. Affinity chromatography and chip technology achieve functional separations by immobilized ligands, but suffer from surface effects and steric hindrance. Additionally, the polymeric network has to be swollen in appropriate solvents, which have to be compatible with the solubility properties of the proteins. Since size and solubility of the targeted proteins are a priori unknown, many proteins can remain undetected. Thus, methods in which the critical affinity-based interaction takes place in solution are desirable.

Activity-based protein profiling (ABPP) has been used for targeted isolation of many enzyme classes, including proteases, kinases, phosphatases, glycosidases, hydrolases, histone deacetylases and oxidoreductases.[1] This approach is based on synthetic compounds containing two functional moieties: one moiety is responsible for binding (selectivity function) and covalent modification (reactivity function) of specific proteins, and the other one for detection (e.g., fluorophores) or isolation (sorting function).

However, the design of ABPP compounds is not trivial because the reactivity function has to be incorporated into the selectivity group, for example, a small molecule substrate, without affecting its binding properties. Thus, a separation of selectivity and reactivity function is often desirable.[2]

Capture compound mass spectrometry (CCMS) is based on capture compounds (CC; Figure 1), which are small molecules comprised of three different functionalities.[3] They contain: 1) a *selectivity function* for specific, reversible binding to targeted proteins, 2) a *reactivity function* for photoinduced covalent attachment to the targeted proteins, and 3) a *sorting function* for isolation of the covalently captured proteins on solid supports. The CCs offer two advantages compared to simple affini-

[a] *Dr. M. Hüben, P. Poot, Prof. Dr. E. Weinhold*
Institut für Organische Chemie, RWTH Aachen University
Landoltweg 1, 52056 Aachen (Germany)
Fax: (+49) 241-80-92528
E-mail: elmar.weinhold@oc.rwth-aachen.de

[b] *Dr. C. Dalhoff, T. Lenz, Prof. Dr. H. Köster*
Caprotec Bioanalytics, GmbH
Volmerstrasse 5, 12489 Berlin (Germany)
Fax: (+49) 30-6392-3989
E-mail: hubert.koester@caprotec.com

[c] *Dr. E. Nordhoff*
Max-Planck-Institut für Molekulare Genetik
Ihnestrasse 63-73, 14195 Berlin (Germany)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900349.

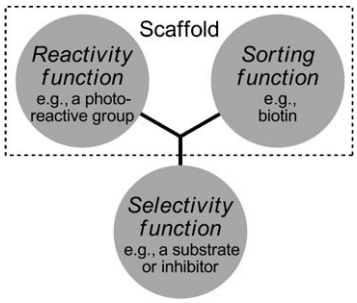
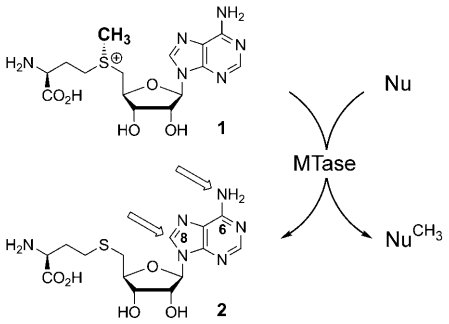


Figure 1. General composition of trifunctional capture compounds (CC). By attaching different selectivity functions to a CC scaffold, which contains both the reactivity and sorting function, CCs for a wide variety of protein classes can be obtained.



Scheme 1. Methyltransferases (MTases) transfer the activated methyl group from SAM (**1**) to nucleophilic positions (Nu) in various substrates, like DNA, RNA, proteins and small molecules; this leads to the formation of SAH (**2**). In addition, the N6 and C8 position for the attachment to capture compound scaffolds are indicated.

ty-based separations on solid supports (pull-down experiments). First, the critical selective affinity interaction takes place in solution and avoids surface-associated problems, and second, the transformation of weak noncovalent interactions into a covalent bond allows stringent washing conditions to remove unspecific (noncovalently bound) proteins.

In previous studies we used a hydrophobic CC scaffold with lysine as trifunctional core to attach azidobenzoic acid (reactivity function for photocrosslinking) and biotin amidohexanoic acid (sorting function for binding to avidin-coated beads) for the isolation of carbonic anhydrase.[3] Paying attention to the fact that most enzymes are located in the cytoplasm, we designed a more hydrophilic CC scaffold using aspartic acid as the core and a PEG linker for attaching biotin. The synthesis of the CC scaffold is a straightforward sequence of amide-forming reactions and the selectivity function can be introduced at the end of the synthesis. This convergent synthesis gives the opportunity to target different protein classes by simply changing the selectivity function.

The sulfonium compound *S*-adenosyl-L-methionine (**1**, SAM, AdoMet) is a ubiquitous cofactor and one of the most widely used enzyme substrates, second only to adenosine triphosphate (ATP).[4] Due to the activation of the adjacent carbon atoms by the pivotal sulfonium center, SAM (**1**) is involved not only in several transfer reactions, including almost all methyl transfer reactions in biological systems, but also in radical generation and other transformations.[5] SAM-dependent enzymes are widely spread in all phyla of life and represent about 2% of the total proteins in *E. coli*, making SAM-dependent enzymes an interesting target for proteomic research. After the methyl group transfer reaction catalyzed by methyltransferases (MTases), the demethylated cofactor product *S*-adenosyl-L-homocysteine (**2**, SAH, AdoHcy) is formed (Scheme 1). The resulting thioether function is chemically much more stable than the activated sulfonium center in SAM, and SAH is a general competitive product inhibitor of MTases.[6] This combination of chemical stability and high binding affinity makes SAH, in contrast to SAM, a promising selectivity function for capturing SAM-dependent MTases (the methylome).

SAH (**2**) has been previously directly attached to solid supports via the amino or carboxylic acid group of the amino acid side chain.[7] However, based on our experience with aziridine-based SAM analogues for labeling DNA using MTases,[8] X-ray structures of MTases in complex with these cofactor analogues[9] and analysis of PDB data,[10] the exocyclic N6 position or C8 position of the adenine heterocycle (Scheme 1) are much less recognized by MTases and more suitable for linker introduction and subsequent attachment of CC scaffolds. To target these positions we implemented new synthetic routes to adenine-modified SAH derivatives.

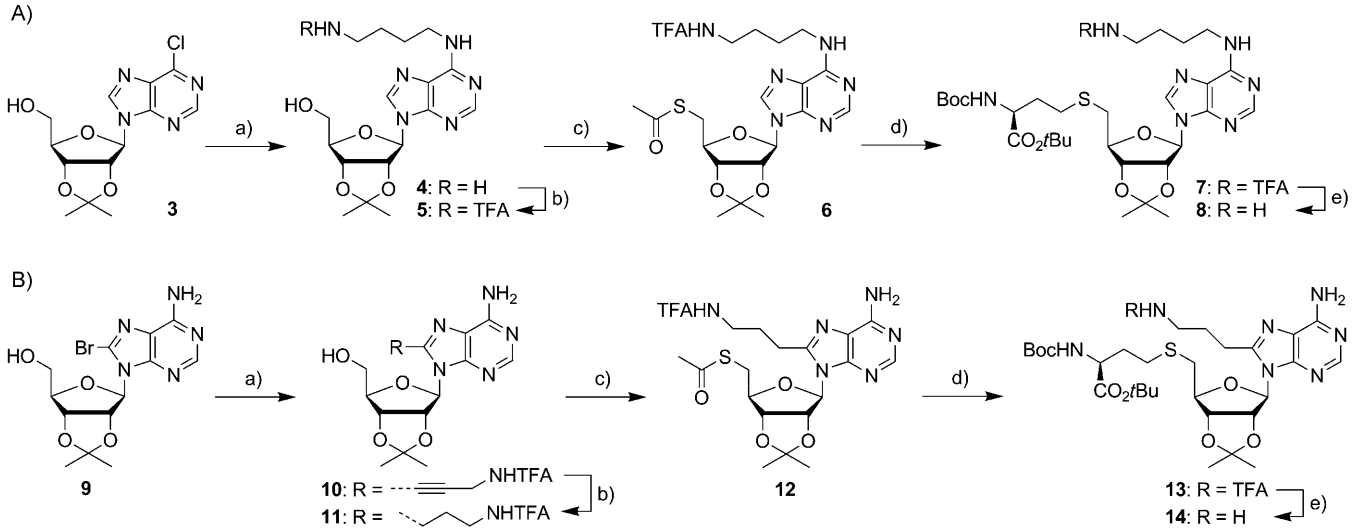
Here we report the synthesis of four SAH-based CCs with two different attachment positions for two scaffolds bearing different photocrosslinking groups. These SAH-CCs are evaluated by using purified MTases and used to determine the dissociation constant K_D for MTase–cofactor complexes. In addition, we demonstrate that they can be used to isolate SAH-binding enzymes from *E. coli* cell lysate.

Results and Discussion

Synthesis of N6 and C8 linker-functionalized SAH derivatives

Chemical synthesis of SAH (**2**) and analogues is commonly performed with C5′-activated adenosines, like 5′-*O*-tosyl or 5′-deoxy-5′-halo derivatives, or by in situ activation of the 5′ position and coupling with thiolates.[11] However, this approach suffers from a competing intramolecular attack of N3 of the adenine ring onto C5′ leading to irreversible cyclonucleoside formation.[12] This intramolecular cyclization can be avoided by reversing the reactivity as demonstrated by coupling of various alkyl bromides with protected 5′-deoxy-5′-thioadenosine.[13] This strategy was also used in the present work to incorporate the full homoserine side chain into linker-functionalized SAH derivatives.

The SAH derivative bearing an amino linker at N6 was synthesized starting from 2′,3′-*O*-isopropylidene-protected 6-chloropurine-β-D-riboside (**3**; Scheme 2 A). Substitution with 1,4-diaminobutane gave the linker-functionalized adenosine derivative **4** and treatment with ethyl trifluoroacetate yielded the amino-protected adenosine derivative **5**.[8b] Introduction of sulfur was performed with thioacetic acid under Mitsunobu



Scheme 2. Synthesis of N6 and C8 linker-functionalized SAH derivatives. A) Experimental conditions for **8**: a) 1,4-diaminobutane, NEt_3, EtOH, 60 °C, 96%; b) ethyl trifluoroacetate, NEt_3, MeOH, room temperature, 92%; c) PPh_3, DEAD, AcSH, THF, −10 °C then 0 °C, 94%; d) 1: NH_3, MeOH, 0 °C; 2: *N*-Boc-γ-tosyl-homoserine-*tert*-butylester, KOH, 18-crown-6, THF, −10 °C, 79% for both steps; e) NH_3, H_2O, MeOH, room temperature, 90%. B) Experimental conditions for **14**: a) *N*-(2-propinyl)-2,2,2-trifluoroacetamide, $(PPh_3)_2PdCl_2$, CuI, NEt_3, DMF, room temperature, 75%; b) H_2, PtO_2, MeOH, room temperature, 92%; c) PPh_3, DEAD, AcSH, THF, −10 °C then 0 °C, 87%; d) 1: NH_3, MeOH, 0 °C; 2: *N*-Boc-γ-tosyl-homoserine-*tert*-butylester, KOH, 18-crown-6, THF, −10 °C, 69% for both steps; e) NH_3, H_2O, MeOH, room temperature, 65%. Details are given in the Supporting Information.

conditions[14] and led to the thioester **6**. Selective cleavage of the thioester was obtained in dry methanol saturated with gaseous ammonia whereas ammonolysis in methanol/water led to additional removal of the TFA group. Since the resulting 5′-thiol has a strong tendency for disulfide formation under aerobe conditions the subsequent coupling step was performed without purification and under rigorous exclusion of oxygen. For introducing the amino acid side chain, commercially available Boc-Asp-*t*Bu was converted into the desired homoserine derivative according to a literature procedure.[15] During the reduction of a mixed anhydride intermediate in methanol, cooling to −10 °C proved to be crucial, because otherwise the corresponding methylether was obtained. The resulting alcohol was activated as *p*-toluenesulfonic ester and treated with freshly cleaved thioester **6** to yield the protected SAH derivative **7** in good yield. Cleavage of the TFA group led to the primary amine **8**, which was coupled to the different CC scaffolds (vide infra).

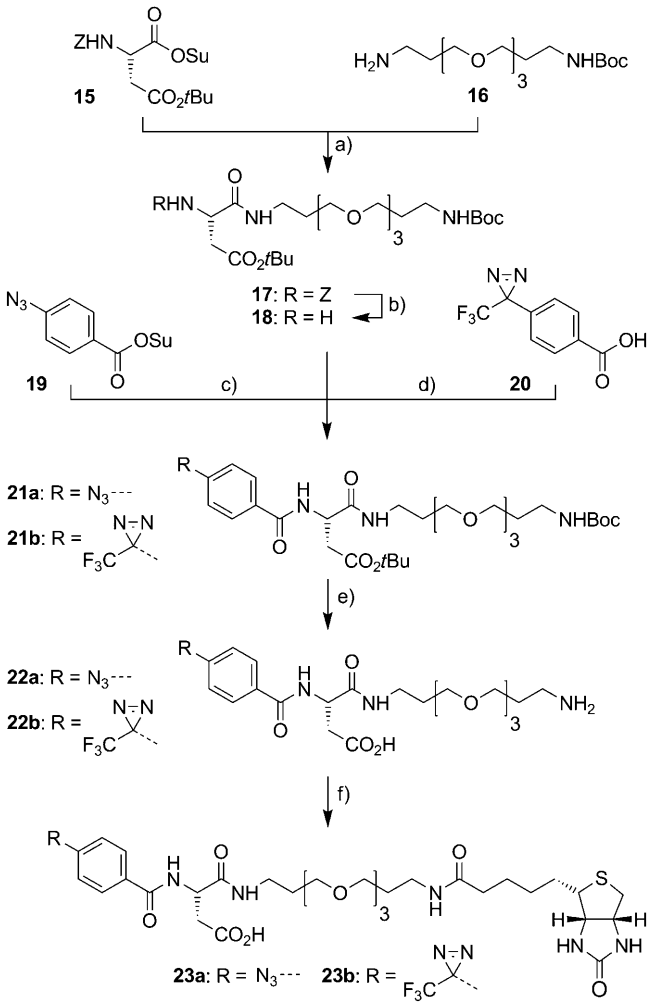
Synthesis of the SAH derivative with an amino linker at C8 started from 8-bromo-2′,3′-*O*-isopropylideneadenosine (**9**). Initially, we followed the synthetic route for the N6-modified SAH derivative **8**. Substitution with 1,4-diaminobutane and protection of the primary amine gave the corresponding adenosine derivative, but attempts to introduce thioacetic acid at the 5′ position via the Mitsunobu reaction failed. Under these condition an intramolecular cyclization occurred and led to an *N*8,5′-anhydroadenosine derivative.[16] A successful approach to C8-modified SAH was obtained by palladium-catalyzed Sonogashira cross-coupling[17] of **9** with TFA-protected propargyl amine, which avoids a secondary amine at the 8 position (Scheme 2B). The resulting alkyne **10** was reduced by catalytic hydrogenation to form **11** with a flexible linker at the 8 position. Subsequent Mitsunobu reaction gave the desired thioester **12**, which was, after liberating the 5′-thiol, coupled with the activated homoserine derivative. Finally, the resulting SAH derivative **13** was treated with ammonia in methanol/water to give the primary amine **14** for attachment to the CC scaffolds (vide infra).

Hydrophilic capture compound scaffolds

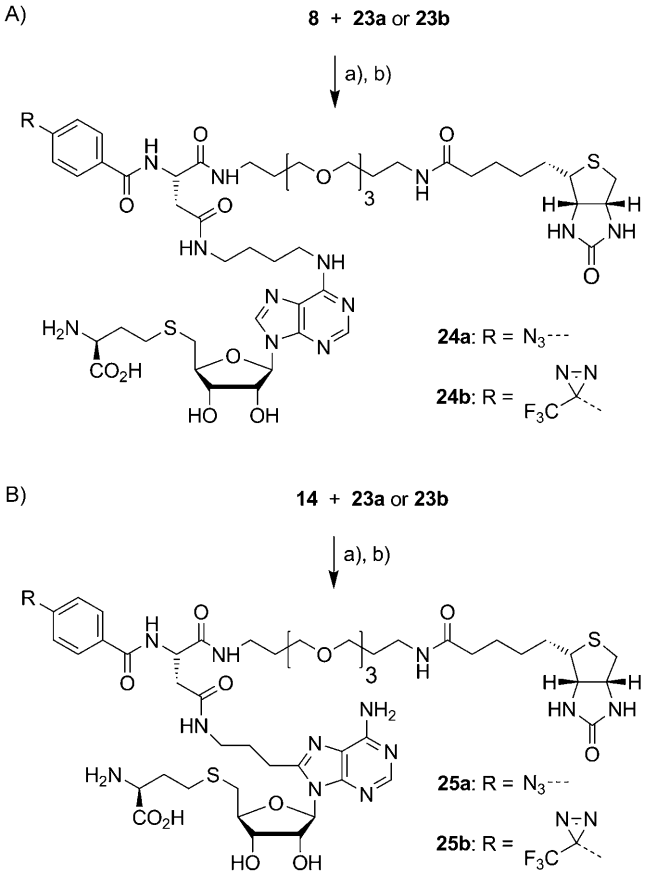
Synthesis of the new CC scaffolds (Scheme 3) is based on amide bond forming reactions, starting from commercially available trifunctional core Z-Asp(*t*Bu)OSu (**15**) and linker *N*-Boc-4,7,10-trioxa-1,13-tridecanediamine (**16**). After coupling amino acid **15** with linker **16** the benzyloxycarbonyl group (Z group) of the product **17** was removed by catalytic hydrogenation and the resulting amine **18** was used to introduce two photocrosslinking groups (reactivity functions). 4-Azidobenzoic acid was incorporated by using the corresponding *N*-hydroxysuccinimidylester **19**, whereas 4-(3-(trifluoromethyl)-3*H*-diazirine-3-yl)benzoic acid (**20**) was introduced by carbodiimide/HOBt chemistry and yielded the coupling products **21a** and **21b**, respectively. Removal of the *tert*-butyloxycarbonyl group (Boc group) and cleavage of the *tert*-butylester under acidic conditions led to the amines **22a** and **22b**, which were coupled with *N*-hydroxysuccinimidyl biotin (sorting function) to yield the hydrophilic CC scaffolds **23a** and **23b**, respectively.

Capture compounds with SAH as selectivity function

The primary amino groups of the linker-functionalized SAH derivatives **8** and **14** were coupled with the carboxylic acids of CC scaffolds **23a** and **23b** by using carbodiimide/HOBt



Scheme 3. Synthesis of capture compound scaffolds. Experimental conditions: a) NEt_3, DCM, 0 °C then room temperature, 20 h, 95%; b) H_2, Pd/C, MeOH, room temperature, 48 h, 90%; c) NEt_3, DCM, room temperature, 24 h, 94%; d) diisopropylcarbodiimide, HOBt, DMAP, NEt_3, THF, 0 °C then room temperature, 44 h, 95%; e) **21a**, TFA, MeOH, room temperature, 48 h; **21b**, HCl/dioxane, DCM, 0 °C, 2 h; f) **22a**, *N*-hydroxysuccinimidyl biotin, NEt_3, THF, room temperature, overnight, 39% for the last two steps; **22b**, *N*-hydroxysuccinimidyl biotin, NEt_3, DMSO, 20% for the last two steps. Details are given in the Supporting Information.



Scheme 4. Synthesis of SAH-based trifunctional capture compounds with different attachment positions and photocrosslinking groups. A) Experimental conditions for N6-linked CCs **24a** and **24b**: a) diispropylcarbodiimide, HOBt, DMAP, dimethylacetamide, 50 °C for 1 h then room temperature for 12 h; b) HCl in dioxan, DCM, 0 °C for 1 h, then add H_2O, 0 °C for 1 h; **24a**: 30% for both steps, **24b**: 63% for both steps. B) Experimental conditions for C8-linked CCs **25a** and **25b**: a) diispropylcarbodiimide, HOBt, DMAP, dimethylacetamide, 50 °C for 1 h then room temperature for 12 h; b) HCl in dioxan, DCM, 0 °C for 90 min, then add H_2O, 0 °C for 90 min (**25a**) or 60 min (**25b**); **25a**: 28% for both steps; **25b**: 43% for both steps. Details are given in the Supporting Information.

chemistry (Scheme 4). Afterwards all remaining protection groups of the SAH fragments (2′,3′-*O*-isopropylidene, Boc and *tert*-butyl group) were removed in one step under acidic conditions. This yielded a set of four biotinylated CCs with two different attachment positions to SAH (N6: **24a** and **24b**, C8: **25a** and **25b**) and two different photocrosslinking groups (4-azido-benzoic acid: **a** series, 4-(3-(trifluoromethyl)-3*H*-diazirine-3-yl)benzoic acid: **b** series) which were used for comparative capture studies with MTases.

Capturing various MTases

The functionality and specificity of the new SAH-CCs **24a**, **24b**, **25a** and **25b** are demonstrated and compared by isolation of MTases acting on different substrates. Purified enzymes were incubated with an excess of the respective SAH-CC, the reactivity function was activated by irradiation with UV light and covalently crosslinked enzymes were isolated by using the biotin sorting function for binding to streptavidin-coated magnetic beads. Captured MTases were then analyzed and visualized by SDS-PAGE and silver staining.

First, we tested SAH-CC **24a** carrying the aromatic azide scaffold at the N6 position of SAH with the DNA adenine N6 MTase from *Thermus aquaticus* (M.TaqI). Analysis by SDS-PAGE (Figure 2) showed that M.TaqI can be isolated by the CC technology (lane 3) and capture yields ranged between 5–10% under saturating conditions. Importantly, several control experiments established that capture is specific and requires covalent bond formation. No capture is observed in the presence of the competitor SAH (**2**; lane 4); this indicates that SAH-CC **24a** binds to the cofactor binding site of M.TaqI and prebinding to the protein is required for photocrosslinking. In addition, the CC scaffold **23a** lacking the selectivity function does not lead to the isolation of M.TaqI (lane 5), which demonstrates

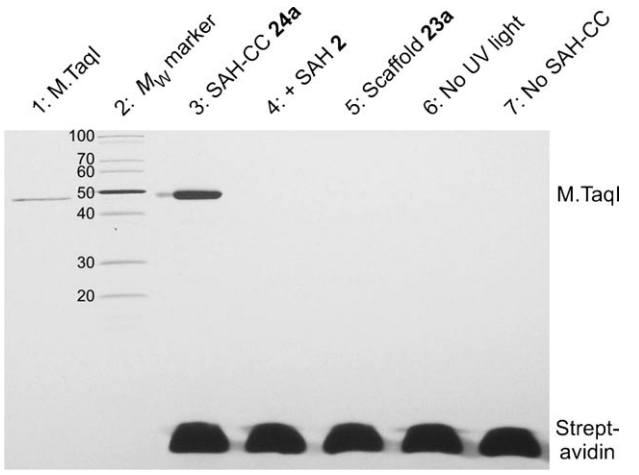


Figure 2. Capturing the DNA MTase M.TaqI (1 μM) with SAH-CC **24a** (10 μM). Samples were analyzed by SDS-PAGE and silver staining. Lane 1: M.TaqI standard; lane 2: molecular weight (M_W) marker with indicated molecular weights in kDa; lane 3: capture experiment; lane 4: control in the presence of SAH (**2**; 6.8 mM) as competitor; lane 5: control with the CC scaffold **23a** (10 μM); lane 6: control without UV irradiation; lane 7: control in the absence of CC.

that the selectivity function is essential. No capturing occurs without UV irradiation (lane 6) showing that covalent bond formation is required for protein isolation. Finally, no capturing occurs in the absence of the SAH-CC (lane 7).

We then compared all four SAH-CCs **24a**, **24b**, **25a** and **25b** containing different photocrosslinking functions (**a** = aromatic azide, **b** = aromatic diazirine) and different attachment positions (**24** = adenine-N6, **25** = adenine-C8) in their ability to capture M.TaqI (Figure 3). Slightly more intense bands were obtained with the aromatic diazirines **24b** and **25b** (lanes 5 and 6) than with the aromatic azides **24a** and **25a** (lanes 2 and 3).

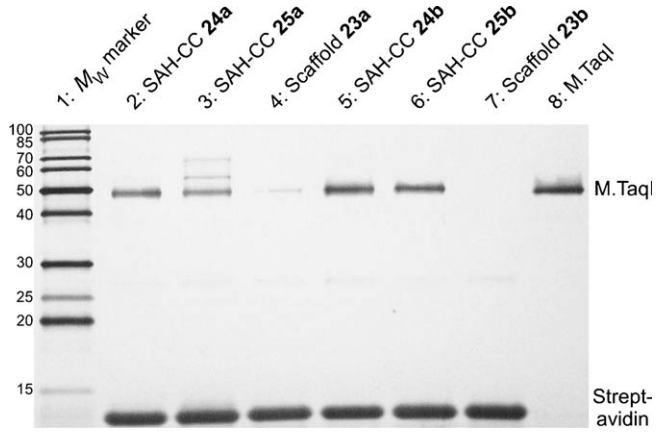


Figure 3. Capturing the DNA MTase M.TaqI (1 μM) with all four SAH-CCs **24a**, **25a**, **24b**, or **25b** (2 μM each; nonsaturating conditions). Samples were analyzed by SDS-PAGE and silver staining. Lane 1: molecular weight (M_W) marker with indicated molecular weights in kDa; lanes 2–4: capture experiments with SAH-CCs **24a**, **25a** or CC scaffold **23a** carrying an aromatic azide for photocrosslinking; lanes 5–7: capture experiments with SAH-CCs **24b**, **25b** or CC scaffold **23b** carrying an aromatic diazirine for photocrosslinking; lane 8: M.TaqI standard (100 ng).

From a practical view this small improvement in capture yield might not balance the much higher costs (or much longer synthesis) for the 4-(3-(trifluoromethyl)-3*H*-diazirine-3-yl)benzoic acid (**20**) compared to 4-azidobenzoic acid. In addition, the N6-linked SAH-CCs **24a** and **24b** (lanes 2 and 5) gave somewhat higher capture yields than the C8-linked SAH-CCs **25a** and **25b** (lanes 3 and 6). Control experiments with the CC scaffolds **23a** and **23b** lacking the SAH selectivity functions did not lead to the isolation of M.TaqI in appreciable amounts. This again demonstrates that the selectivity function is required to direct the probes to the protein and enables photocrosslinking within the protein–probe complex.

Having successfully tested the four CCs with the adenine-specific DNA MTase M.TaqI, we were interested in testing further MTases acting on various other substrates. Capture experiments were performed with the DNA cytosine-C5 MTase M.HhaI, the RNA cytosine-C5 MTase Trm4 and the protein histone H3 lysine 9 MTase Clr4. All three MTases were isolated by using SAH-CC **24a** and capture yields ranged between 2–10% (Figure 4). Two control experiments were performed to demonstrate that initial noncovalent interactions between the SAH selectivity function and proteins are required for photocrosslinking. Competition by an excess of SAH (**2**) completely (lanes 3 and 7) or strongly (lane 11) blocks photocrosslinking and no (lanes 4 and 8) or almost no (lane 12) proteins are detected when using the CC scaffold **23a**, which lacks the selectivity function.

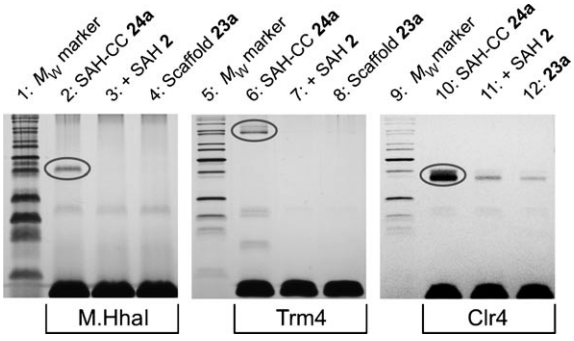


Figure 4. Capturing the DNA MTase M.HhaI (1 μM), the RNA MTase Trm4 (0.5 μM) and the protein MTase Clr4 (1 μM) with SAH-CC **24a** (5 μM for M.HhaI, 20 μM for Trm4 and 10 μM for Clr4). Samples were analyzed by SDS-PAGE and silver staining. Lanes 1, 5 and 9: molecular weight (M_W) marker ranging from 15 to 200 kDa; lanes 2, 6, and 10: capture experiments with the three MTases (bands corresponding to the isolated MTases are circled in gray); lanes 3, 7 and 11: control experiments in the presence of SAH (**2**; 7.3 mM for M.HhaI, 4.0 mM for Trm4 and 6.8 mM Clr4); lanes 4, 8 and 12: control experiments with the CC scaffold **23a** (5 μM for M.HhaI, 20 μM for Trm4 and 10 μM for Clr4).

In addition to SDS-PAGE, we analyzed M.TaqI captured with SAH-CC **24a** by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). A mixture of M.TaqI and crosslinked M.TaqI yielded two strong signals (Figure 5). Photocrosslinking by aromatic azides is initiated by loss of nitrogen (N_2) and the resulting singlet nitrene can rapidly decay into various activated species for crosslinking.[18] The experimental mass dif-

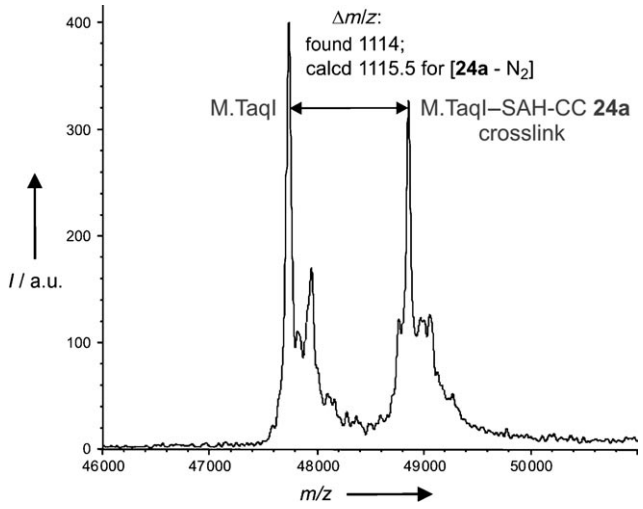


Figure 5. MALDI-MS spectrum with magnification in the 46–51 kDa range of a mixture of the DNA MTase M.TaqI and M.TaqI crosslinked with SAH-CC **24a**. The experimental (found) mass difference of the two main signals is indicated and matches the calculated (calcd) mass difference. Satellite signals mainly originate from MALDI-specific adducts with salts and matrix molecules.

ference between unmodified and modified M.TaqI was 1114, which is in very good agreement with the calculated mass increase resulting from covalent attachment of SAH-CC **24a** (*m/z* 1115.5 for $[M-N_2]$). In addition, MALDI-MS analysis of peptides obtained after tryptic fragmentation identified M.TaqI with a sequence coverage of 65%.

Determination of dissociation constants

The dissociation constant K_D for M.TaqI and SAH-CC **24b** was determined by "capture titration" by using a constant concentration of M.TaqI (250 nM) and increasing concentrations of SAH-CC **24b** (0–5 μM) as shown in Figure 6A. This approach is based on the very fast time scale of photolytic reactions (nanoseconds to milliseconds) and under ideal conditions the photolytic crosslinking reaction can take a snapshot of an enzyme–ligand complex in equilibrium. In the experiment, however, the time interval for photoactivation and subsequent crosslinking is not infinitely small. Ligand exchange can take place during the irradiation process, which could lead to deviations from the true solution equilibrium. The yield of the photolytic crosslinking reaction within the enzyme–ligand complex is assumed not to be quantitative but constant, so that the amount of isolated enzyme–ligand crosslink is proportional to the concentration of the enzyme–ligand complex in solution before irradiation. For measuring the amount of covalently crosslinked enzyme–ligand complex, the M.TaqI–SAH-CC crosslink was isolated by using streptavidin-coated magnetic beads, subjected to SDS-PAGE and subsequently silver stained. The bands were quantified by integration by using the software ImageJ,[19] and fitting of the quadratic binding equation for one binding site (Equation (9) given in the Supporting Information) to the data resulted in a K_D of 1.4 μM for the complex of M.TaqI and SAH-CC **24b**. A control experiment with a constant saturating concentration of SAH-CC **24b** (5 μM) and increasing initial M.TaqI concentrations (up to 500 nM) showed a linear increase in integrated band intensities (the coefficient of determination of a linear fit to the data points was $R^2 = 0.998$) with respect to the initial M.TaqI concentration (Figure S1 in the Supporting Information). This result verifies that the yield of isolated M.TaqI–SAH-CC crosslink and hence the crosslinking yield is constant for the applied concentration range. Additionally, from the same experiment, the detection limit for M.TaqI in the capture assay was determined to be about 5 pmol (50 nM in 100 μL). The detection limit for assayed M.TaqI by direct MS/MS analysis of peptides obtained after capturing and tryptic fragmentation was approximately 100 fmol (1 nM in 100 μL; data not shown), which demonstrates the higher sensitivity of CCMS compared to capturing and simple SDS-PAGE analysis.

In a second set of experiments (Figure 6B) we determined the dissociation constant K_D for M.TaqI and SAH (**2**) by "competitive capture titration". Constant concentrations of M.TaqI (250 nM) and SAH-CC **24b** (1 μM) were treated with increasing concentrations of the natural cofactor product SAH (**2**; 0–125 μM), which led to an almost complete inhibition of crosslinking. This result is best explained by binding of SAH to the cofactor binding site of M.TaqI and hindrance of complex formation with SAH-CC **24b** and, thus, crosslinking. Fitting Equation (16) given in the Supporting Information, which describes analytically the competitive binding of two ligands to the same binding site, to the obtained data points yielded a K_D of 2.3 μM for the complex of M.TaqI and SAH (**2**). The obtained K_D value is in excellent agreement with the published K_D of (2.4 ± 0.5) μM, which was determined in a fluorescence assay.[20] Although the accuracy of quantification by SDS-PAGE and silver staining might be lower than by spectroscopic or radioactive methods, capture titrations only require a UV lamp, a strong magnet and standard biochemical laboratory equipment, which makes them a low-cost alternative to other binding assays.

Capturing SAH-binding proteins from *E. coli* lysate

The utility of our SAH-CCs to isolate proteins from complex mixtures was tested by using a cell lysate from *E. coli*. Analysis by SDS-PAGE (Figure 7) showed clear functional enrichment of proteins compared to the control with added SAH (**2**). Most importantly, we were able to identify proteins from the gel by treating gel slices with trypsin, analyzing peptides by MALDI-MS/MS and interrogating protein sequence databases. Besides streptavidin from the magnetic beads and naturally biotinylated biotin carboxyl carrier protein of acetyl-CoA carboxylase (BCCP), we found two SAH-binding proteins from *E. coli*: trans-aconitate 2-MTase (tam)[21] and 5′-methylthioadenosine/*S*-adenosylhomocysteine (MTA/SAH) nucleosidase (MtnN).[22] SAH binds with micromolar affinity to trans-aconitate 2-MTase (72% inhibition at 0.38 mM SAH)[21] and to MTA/SAH nucleosidase (K_M for SAH is 4.3 μM).[22] This demonstrates that the SAH-CC allows isolation of MTases and other SAH-binding proteins from complex protein mixtures like cell lysates in a form that is suitable for identification by MALDI-MS/MS. Since direct

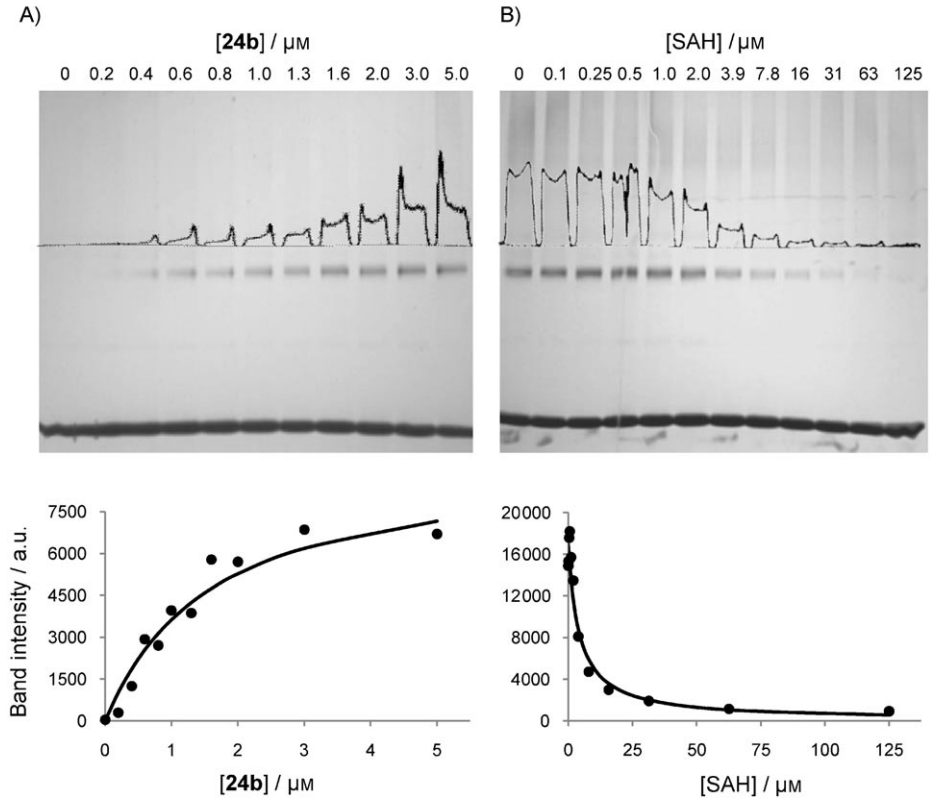


Figure 6. Determination of dissociation constants (K_D) for the complexes of M.TaqI with SAH-CC: A) **24b**, or B) SAH (**2**) by "capture titration". After irradiation, crosslinked M.TaqI was isolated by using streptavidin-coated magnetic beads, and subjected to SDS-PAGE with silver staining. Crosslinked M.TaqI corresponding to the bands in the middle of the two gels was quantified by using the software ImageJ (integrated band intensities are shown above the protein bands). Plots of the integrated M.TaqI band intensities versus the concentration of SAH-CC **24b** or SAH (●) and least-square fits (black lines) of the corresponding equation to the data points are shown below the gels. A) The K_D for the complex of SAH-CC **24b** with M.TaqI was determined by varying the initial SAH-CC **24b** concentration (detailed above the gel) at a constant initial M.TaqI concentration of 250 nM. Fitting of Equation (9) (see the Supporting Information) to the data points gave a K_D of 1.4 μM. B) The K_D for the complex of SAH with M.TaqI was determined by varying the initial SAH concentration (detailed above the gel) at constant initial M.TaqI and SAH-CC **24b** concentrations of 250 nM and 1 μM, respectively. Fitting of Equation (16) (see the Supporting Information) to the data points gave a K_D of 2.3 μM.

protein identification from the magnetic beads is much more sensitive than SDS-PAGE (see above results with M.TaqI), we expect to find many more MTases by CCMS.

Conclusions

We have established synthesis routes to previously unknown N6- and C8-modified SAH derivatives suitable for attachment to CC scaffolds. However, the protected intermediates **8** and **14** containing primary amines should also be useful for coupling to other chemical entities or solid supports leading to various applications. Our design of SAH-CC proved to be viable and all four SAH-CCs **24a**, **24b**, **25a** and **25b** were successfully used for the isolation of the DNA MTase M.TaqI. In fact, the binding affinity of SAH-CC **24b** to M.TaqI is even slightly increased compared to the binding affinity of the natural cofactor product SAH (**2**) as directly determined by capture titration. Functional studies with other purified MTases acting on different substrates confirmed the ability of our SAH-CCs to isolate MTases for follow-up analysis by SDS-PAGE and mass spectrometry (CCMS). Importantly, the SAH-CCs also allow for isolation and identification of SAH-binding proteins from complex protein mixtures like *E. coli* lysate. This paves the way for isolating functionally defined subproteomes, like the methylome. In addition, the linear relationship between protein concentration and amount of captured protein, as demonstrated with M.TaqI, should be useful for quantitative comparison of expression patterns in different cell states (functional protein expression profiling).

Experimental Section

General: All solvents and chemicals were of analytical grade unless otherwise noted. HPLC analysis and purification was performed with a Waters Breeze system and NMR spectra were recorded by using a Mercury 300 spectrometer (Varian) or an Ultrashield 400 spectrometer (Bruker). Mass spectrometric analysis was performed on a Scout MTP Ultraflex II MALDI-TOF-MS/MS equipped with a solid-state SmartBeam laser (Bruker Daltonics, Bremen, Germany).

The MTases M.TaqI,[23] M.HhaI[24] and Clr4[25] were over-expressed and purified as described before. Trm4 was kindly provided by Priv.-Doz. Dr. Mark Helm (Institut für Pharmazie und Molekulare Biotechnologie, Universität Heidelberg, Germany). Silver staining of gels was performed with the ProteoSilver Plus silver stain kit (Sigma). The peptide calibration standards angiotensin I and ACTH 18-39 (corticotropin-like intermediate lobe peptide) were purchased from Bachem and porcine trypsin was from Promega. All synthesis procedures and equations for determining dissociation constants K_D are given in detail in the Supporting Information.

Preparation of *E. coli* lysate: Four Erlenmeyer flasks (5 L) containing LB medium (2.5 L each) were inoculated with an *E. coli* DH5α culture (50 mL each) grown to exponential phase (OD_{600} of 0.8) and incubated at 37 °C and 166 rpm until an OD_{600} of 0.8 was reached. The cells were harvested by centrifugation (4200 *g*, 4 °C, 30 min) and the cell pellet (25 g) was stored at −20 °C for 14 h. The cells were resuspended in lysis buffer (100 mL, 6.7 mM MES, 6.7 mM HEPES, 6.7 mM NaOAc, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.2 mM PMSF, 10% (*v/v*) glycerol, pH 7.5), were cooled on ice and treated in aliquots with ultrasound (Brason Sonifier 250, big tip, duty cycle 10%, output control 10). Cell debris was removed by centrifugation (35000 *g*, 4 °C, 60 min) and the com-

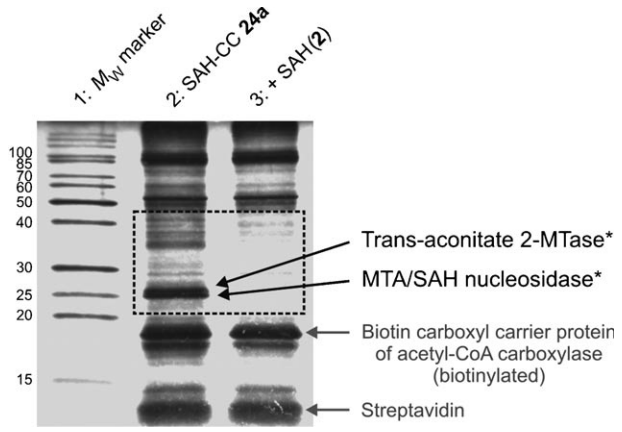


Figure 7. Capturing SAH-binding proteins with SAH-CC **24a** (5 μM) from *E. coli* lysate (3.5 mg mL^{-1}). Samples were analyzed by SDS-PAGE and silver staining. In addition, gel slices containing protein bands were excised, treated with trypsin, and resulting peptides were analyzed by MALDI-MS/MS. Lane 1: molecular weight (M_W) marker with indicated molecular weights in kDa; lane 2: capture experiment; lane 3: control in the presence of SAH (**2**) as competitor. A clear enrichment of proteins as compared to the control is observed in the molecular weight range between 20 and 50 kDa (dashed box) and two SAH-binding proteins (*) were identified.

bined supernatants were concentrated (to 17.5 mL) by ultrafiltration (Jumbosep 10 K, Pall, 500 *g*, 4 °C, overnight). Small molecules were removed by gel filtration (NAP5 columns, GE Healthcare; equilibrated with lysis buffer). Aliquots (500 μL) were applied to the columns, lysis buffer (200 μL) was added and proteins were eluted with lysis buffer (650 μL). The combined fractions (23 mL) were supplemented with lysis buffer (37 mL) and concentrated (to 11 mL) by ultrafiltration (Jumbosep 10 K, Pall, 350 *g*, 4 °C, overnight). Glycerol (9 mL) was added and the cell lysate (total protein concentration 35 mg mL^{-1}) stored at −20 °C.

General procedure for capture experiments: The capture procedure is described for M.TaqI and SAH-CC **24a**. For the other MTases or *E. coli* lysate and SAH-CCs, capturing was performed in an analogous manner and details regarding concentrations are given in the respective figure legend. M.TaqI (1 μM) was incubated with SAH-CC **24a** (10 μM) in capture buffer (100 μL, 20 mM HEPES, 10 mM $Mg(OAc)_2$, 50 mM KOAc, 10% (*v/v*) glycerol, pH 7.9) in thin-walled PCR tubes (0.5 mL, Eppendorf) at 4 °C for 5 min. The tubes were placed into a reflector bowl (8.2 cm diameter) filled with ice water and the samples were irradiated (ten times) with a studio flash lamp (Alienbees B1600 640 WS without UV filter, 2 cm distance between flashtube and samples). Controls in the presence of CC scaffold or additionally containing SAH (**2**) were treated in the same way. After exposure to UV light, all samples were supplemented with SAH (100 μM final concentration) and centrifuged (16000 *g*, 4 °C, 10 min). The samples were applied to Zeba™ gel filtration columns (0.5 mL, Pierce) previously equilibrated with wash buffer (50 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 0.05% octyl-β-D-glucopyranoside, pH 7.5) containing SAH (100 μM) and proteins were directly eluted to streptavidin-coated magnetic beads (200 μg, MyOne™ Streptavidin C1 Dynabeads®, Invitrogen, prepared according to the instruction of the manufacturer) in 10 μL wash buffer containing SAH (100 μM). Then the samples were incubated on an orbital shaker (Thermomixer 5433, Eppendorf) at 4 °C for 40 min. Beads were collected with a strong neodymium magnet for 1 min and the supernatants were removed. The beads were suspended in wash buffer containing SAH (100 μM; 180 μL for each sample), transferred into a new tube, incubated on the orbital shaker at 4 °C for 5 min and magneto-precipitated. This washing step was repeated twice. After removal of the supernatants the beads were washed twice with basic wash buffer (180 μL, 50 mM NH_4OAc, 0.05% octyl-β-D-glucopyranoside, pH 9.0). Then the beads were washed again two times with wash buffer containing SAH (100 μM; 180 μL for each sample) and two times with water (180 μL, Milli-Q grade).

SDS-PAGE analysis: For SDS-PAGE (15% gel), the supernatants were removed, the beads were suspended in SDS sample buffer (14 μL, 50 mM Tris-HCl, 2.5% SDS, 10% glycerol, 320 mM β-mercaptoethanol, 0.05% bromphenole blue, pH 6.8) and heated to 95 °C for 10 min. After electrophoresis protein bands were visualized by silver staining.

In gel trypsinolysis: Gel slices were treated twice with aqueous acetonitrile (400 μL, 50%) for 30 min while shaking, once with acetonitrile (400 μL) for 5 min and once with reducing buffer (50 μL, 10 mM DTT, 50 mM NH_4HCO_3) at 37 °C for 30 min. After an additional treatment with acetonitrile (400 μL) for 5 min, iodoacetamide solution (50 μL, 20 mM in 50 mM NH_4HCO_3) was added and the gel samples were incubated at room temperature in the dark for 30 min. After solvent removal a solution of 2,2′-thiodiethanol (50 μL, 20 mM) was added and incubation was continued at room temperature for 10 min. The liquid was removed and the gel samples were treated twice with aqueous acetonitrile (400 μL, 50%) for 5 min and once with acetonitrile (400 μL) for 10 min. After removing the liquid the gel samples were dried in a vacuum centrifuge for 30 min. A solution of trypsin (12 μL, 2.5 ng μL^{-1} in 50 mM NH_4HCO_3, pH 7.8) was added to the gel pieces, and after 10 min incubation on ice NH_4HCO_3 solution (50 mM, pH 7.8), sufficient to just cover the gel pieces, was added and followed by incubation, overnight, at 37 °C. For peptide extraction, an aqueous solution (20 μL) of trifloroacetic acid (TFA; 0.1%) and octyl-β-D-glucopyranoside (0.5 mM) was added and the gel samples were placed on a shaker for 20 min. Of each sample, two aliquots of the supernatant (0.5 μL) were aspirated and directly prepared for MALDI.

MALDI mass analysis: Thin microcrystalline layers of α-cyano-4-hydroxycinnamic acid were prepared on AnchorChips 384/600 as described before.[26] Each aliquot was deposited on a separate matrix-coated sample anchor and left to dry at ambient conditions. The dried samples were washed with TFA solution (3 μL, 0.1%) and analyzed by MALDI-MS/MS. Positively charged ions in the *m/z* range 700–4000 Da were detected and 4000 single-shot spectra were accumulated for each analysis. Optimal values for fixed laser attenuation were determined prior to analysis by evaluation of a few fractions. Spectrum processing was performed automatically with the software FlexAnalysis 3.0. Automatic detection of peptide monoisotopic signals was performed by using the algorithm SNAP with a signal-to-noise threshold of six. Internal mass correction was performed by using the signals of two reference peptides (angiotensin I, $[M+H]^+$ 1296.6853 and ACTH 18–39, $[M+H]^+$ 2465.1989) included in the MALDI matrix solution.

Protein identification was performed by using the Mascot software 2.2 (Matrixscience, London, UK) searching the UniProt/SwissProt and UniProt/Trembl sequence databases. The following settings were used for the searches. Mass error tolerance for peptide masses: 15 ppm; mass error tolerance for the fragment ions: 0.35 Da; fixed modification: carbamidomethylation; variable modification: methionine oxidation; number of missed cleavage sites: 1; type of instrument: MALDI-TOF-PSD. Identifications based on peptide mass fingerprinting were considered very likely to be correct if

the score was greater than 80. For identification by MS/MS a different score is calculated and in this case the threshold for identification was set to 50.

Determination of dissociation constants by capture titration: M.TaqI (250 nM) was incubated with varying concentrations of SAH-CC **24b** (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.3, 1.6, 2.0, 3.0, and 5.0 μM) in a slightly modified capture buffer (100 μL; 20 mM HEPES, 10 mM $Mg(OAc)_2$, 50 mM KOAc, 10% (*v/v*) glycerol, 0.02% Triton X-100, pH 7.9) placed in thin-walled tubes (0.2 mL) of a 12-tube strip (Thermo-Strip, Thermo Scientific) at 2 °C in the sample holder of a CaproBox™ (Caprotec Bioanalytics GmbH) for 10 min. The sample tubes (open lids) were irradiated with UV light (310 nm, 10 mW cm^{-1}) within the CaproBox™ at 2 °C for 10 min. Each sample was supplemented with SAH (**2**; 20 μL, 10 mM), 5× wash buffer (25 μL, 250 mM Tris-HCl, 5 M NaCl, 5 mM EDTA, 0.25% octyl-β-D-glucopyranoside, pH 7.5) and streptavidin-coated magnetic beads (50 μL, 10 mg mL^{-1}) by carefully inverting the tube with closed lid after each addition. The beads were kept in suspension by rotating the samples on a rotator wheel at 4 °C for 1 h and then collected by using a caproMag™ magnetic particle collector (Caprotec Bioanalytics, GmbH) containing a strong N52 neodymium magnet. The supernatants were discarded and the beads were washed three times with wash buffer (200 μL), three times with basic wash buffer (200 μL) and once with water (200 μL) by repeated resuspension, magneto-precipitation and removal of the supernatants. Finally, the collected beads were resuspended in SDS sample buffer (20 μL), heated to 95 °C for 10 min and analyzed by SDS-PAGE (OLS® ProPage 4–20% Tris-glycine precast gel and 25 mM Tris base, 200 mM glycine, 0.1% SDS, pH 8.3 running buffer) with subsequent silver staining. To determine the dissociation constant K_D of the M.TaqI–SAH complex, a mixture of M.TaqI (250 nM) and SAH-CC **24b** (1 μM) was incubated with varying concentrations of SAH (**2**; 0, 0.12, 0.24, 0.49, 0.98, 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, and 125 μM) and the samples were processed as described above.

Intensities of protein bands were integrated from pictures of silver-stained SDS-PAGE gels by using the software ImageJ[19] and plotted against the initial concentrations of SAH-CC **24b** (Figure 6A) or SAH (Figure 6B). The dissociation constant K_{D1} of the complex between M.TaqI and SAH-CC **24b** was determined by varying the parameters *C* and K_{D1} in a least-square fit of Equation (9) (see the Supporting Information) to the data points by using the solver module of MS Excel. Analogously, the dissociation constant K_{D2} of the complex between M.TaqI and SAH was determined by a least-square fit of Equation (16) (see the Supporting Information) to the corresponding data points by using the solver module of MS Excel. The K_{D1} from above was used as a constant and the parameters *C* and K_{D2} were varied.

Competing financial interest: C.D., T.L. and H.K. are employees of Caprotec Bioanalytics GmbH (Berlin, Germany), a company that produces and markets a SAH caproKit containing capture compound **24a**. C.D. and T.L. own stock options and H.K. and E.W. own stocks.

Acknowledgements

We thank Kerstin Glensk and Dr. Wibke Peters for the preparation of M.TaqI, M.HhaI and Clr4 and Priv.-Doz. Dr. Mark Helm for providing Trm4. This work was supported by the Human Frontier Science Program Organization (HFSP Award 2007, RGP0058/2007-C).

Keywords: CCMS · photoaffinity labeling · proteomics · SAH · transferases

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Received: June 3, 2009
Published online on January 4, 2010

