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# A generic building block for C- and N-terminal protein-labeling and protein-immobilization

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**Abstract**—Expressed protein ligation (EPL) and bioconjugation based on the maleimide group (MIC-conjugation) provide powerful tools for protein modification. In the light of the importance of site-selectively modified proteins for the study of protein function, a flexible method for the introduction of tags and reporter groups into the C-terminus of proteins employing EPL and MIC-conjugation was developed. We describe the solid-phase synthesis of a generic building block, equipped with fluorescence markers or different functional groups. This generic building block allows for a flexible incorporation of different tags into proteins and was used for the introduction of fluorescence markers into the C-terminus of Rab and Ras GTPases by EPL or MIC-conjugation techniques. In addition, a building block appropriately modified for the incorporation of an azide into proteins was synthesized. Azide-functionalized Ras protein was immobilized on a phosphane-modified surface by means of Staudinger ligation providing a highly chemoselective ligation method for the immobilization of proteins.


## 1. Introduction

Proteins equipped with moieties such as fluorophores are efficient probes for the study of protein function. In vitro ligation methods[1] provide powerful tools for the introduction of such small organic molecules into proteins. Expressed protein ligation[2] (EPL) is a broadly applicable method for the site-specific modification of proteins. Small organic molecules made accessible by organic synthesis can be ligated by EPL to the C-terminus of proteins. Most frequently, modified peptides corresponding to the C-terminus of proteins have been used in this semi-synthetic approach to yield functionalized proteins.[3] In the light of the increasing interest in the use of precisely modified proteins as probes for the study of protein function, we have developed a flexible and general method for the synthesis of functional building blocks to be used for protein-modification.

As proteins of interest Rab GTPases, which play an important role in regulating vesicular transport processes,[4] and Ras GTPases, which are membrane-bound signal transducers occupying a central position in the MAP-kinase signal transduction pathway,[5] were chosen. Chemical labeling of Rab and Ras GTPases is a invaluable tool for the elucidation of the biological function of these small GTPases.

Because of the central role of Ras proteins in signal transduction the identification of Ras interaction

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partners or small molecules interfering with Ras-protein interactions is of utmost importance.[6] For the study of protein–protein interactions protein-immobilization is a powerful technique, reflected by an increasing interest in protein-microarray technology.[7]

For the application of protein arrays in proteomics research[7–10] site- and chemoselective immobilization of proteins via regions of the macromolecules that are not involved in interactions with other molecules is preferable or even required. Recently, chemo- and regioselective expressed protein ligation was employed for this purpose.[11]

We describe the use of the generic building block for the site-selective immobilization of Ras. Our approach to a generic building block for protein-modification and immobilization includes the introduction of an azide into the C-terminus of the Ras-protein by means of EPL allowing for the immobilization of azide-modified Ras on phosphane-functionalized surfaces by means of Staudinger ligation. The Staudinger ligation has been applied to protein immobilization by Raines and co-workers[12] and Bertozzi and co-workers[13] by non-covalent binding of a protein to an immobilized peptide sequence. The use of the Staudinger ligation in living cells by Bertozzi and co-workers[14] and for the preparation of small molecule microarrays[15] demonstrates its potential as powerful coupling strategy and its tolerance of a diverse array of functionalities. Here, we report that the Staudinger ligation provides a new chemoselective method for immobilization of proteins equipped with an azide to a phosphane-modified surface.[16]

## 2. Design of a triply substituted building block

A benzene derivative equipped with a triply orthogonal set of functional groups was chosen as core structure. It should contain a linker group for its introduction into proteins, so that the remaining two functional groups can be equipped with tags, labels or reporter groups of interest. Consequently, the desired building block was equipped with a cysteine or a maleimidocaproyl (MIC)-group which is required for introduction into proteins by expressed protein ligation or MIC-conjugation, respectively. Substituted benzene derivatives as shown in Scheme 1 represent suitable building blocks bearing one amino group for the attachment of the required cysteine or a MIC-group and a second amino group for the introduction of reporter groups. An additional carboxylic acid group may be further transformed into a thioester which would allow an N-terminal attachment to proteins containing an N-terminal cysteine by means of EPL. Such a functionalized building block would provide the possibility for protein-modification at well-defined sites.

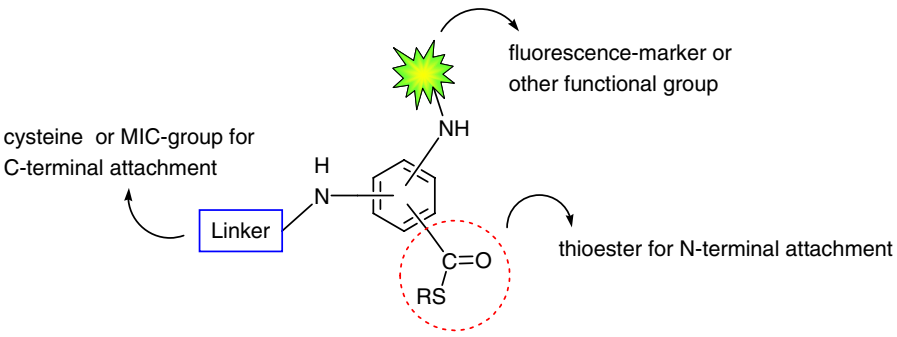
We note that a suitably functionalized amino acid, for example, lysine or glutamic acid equipped with three orthogonally stable protecting groups, would be an alternative to the aromatic building block developed by us. Since the chemistry for the functionalization of amino acids with appropriate protecting groups is well established and since arene chemistry might offer alternatives in terms of reactivity, stability and applicable reaction types and conditions, we decided to explore an aromatic building block instead of a more established approach based on a trifunctional amino acid.

## 3. Synthesis of building blocks used for protein-labeling

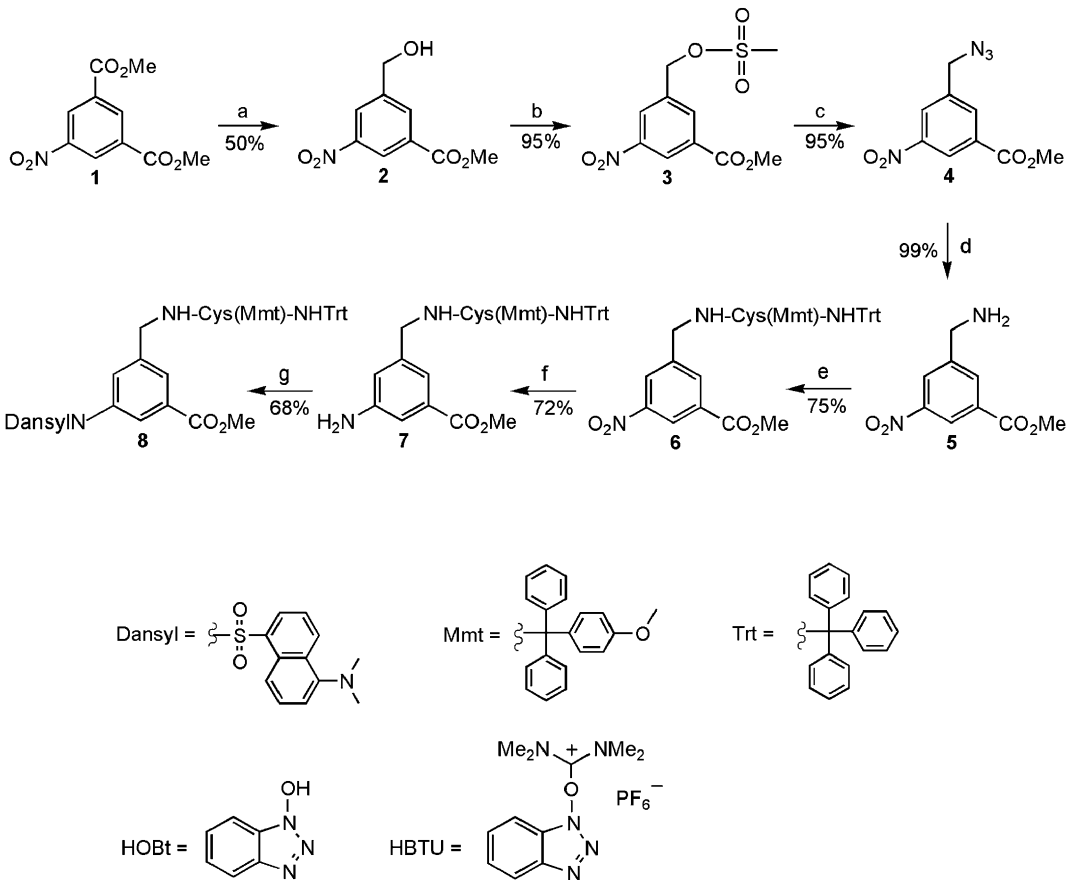
For the development of a flexible synthesis of differently modified core molecules we aimed at a solid-phase synthesis. In order to establish a suitable protocol for the synthesis of the desired building block, we decided to explore first a solution-phase strategy.

Starting from the commercially available dimethyl-5-nitro-isophthalate **1** one methyl ester was reduced to the corresponding alcohol **2** which was subsequently protected and converted to azide **4** (Scheme 2). The azide was reduced to amine **5**, which was then coupled to an Mmt- and Trt-protected cysteine derivative. The obtained intermediate **6** could be further modified. Reduction of the nitro group with palladium on charcoal under a hydrogen atmosphere, followed by attachment of the fluorescent 5-*N*,*N*-(dimethylamino)-naphthalene-1-sulfonyl(dansyl)-group, yielded fluorescently labeled benzene derivative **8** with good to excellent yield.

For the development of a solid-phase synthesis of the building block based on these reaction conditions the choice of the linker for connecting the core molecule to the solid phase had to be considered. To make this approach more broadly applicable, cleavage of the desired compounds from the resin should be possible




**Scheme 1.** Design of a triply orthogonally substituted benzene derivative as generic building block for protein labeling.




**Scheme 2.** Synthesis of fluorescently labeled building block **8** in solution. Reagents and conditions: (a) 2.5 equiv DIBAL-H, THF, −70 °C; (b) 1.5 equiv MsCl,10 equiv $Et_3N$, $CH_2Cl_2$, 30 min, 0 °C; (c) 5 equiv $NaN_3$, DMF, 50 °C, 6 h; (d) 6 equiv HS–$(CH_2)_3$–SH, 4 equiv $Et_3N$, MeOH, 12 h, rt; (e) Trt-Cys(Mmt)-OH, 1.2 equiv HOBt, 1.1 equiv HBTU, 2 equiv $Et_3N$, $CH_2Cl_2$, 12 h, rt; (f) Pd/C, $H_2$, MeOH; (g) 1.5 equiv dansylCl, 0.5 equiv DMAP, 3 equiv Py, $CH_2Cl_2$, 12 h, rt.

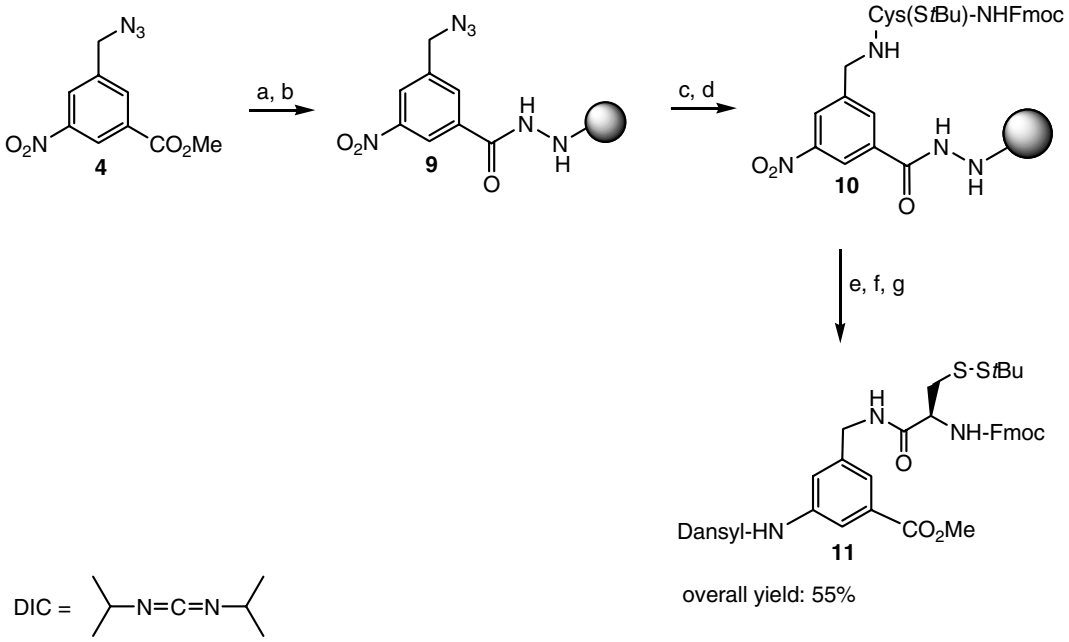
under very mild conditions giving the opportunity for further modification. These requirements are fulfilled by the oxidation-sensitive hydrazide linker.[17] Therefore, 4-Fmoc-hydrazinobenzoyl AM NovaGel (Novabiochem) was employed for the synthesis, drawing from previous experience on the synthesis of Ras- and Rab-peptides.[4] This linker allows for release of the immobilized compounds as carboxylic acids, methyl esters or amides making this approach desireably flexible.

The solid-phase synthesis started with the immobilization of the azide to the solid support (Scheme 3). The 4-Fmoc-hydrazinobenzoyl AM NovaGel resin was deprotected by agitating it twice with a solution of 30% piperidine in DMF for 10 min. After saponification of the methyl ester building block, **4** was attached to the resin using DIC/HOBt as coupling reagents in dichloromethane, yielding resin **9** after 12 h of agitation. The introduction of the protected cysteine derivative was achieved by activating the amino acid with DIC/HOBt for 20 min and treating resin **9** with the activated cysteine in the presence of tributylphosphine for amide bond formation. The resin was further treated with a 1 M solution of $SnCl_2{\cdot}2H_2O$ in DMF for the reduction of the nitro group. Afterwards, the dansyl group was attached to the resin by using dansyl chloride in the presence of DMAP and pyridine. The desired compound **11** was released from the resin by oxidation of the hydrazide to the acyldiazene with copper acetate and oxygen followed by treatment with methanol. After cleavage, the copper salts could be removed by column chromatography to give compound **11** in 55% overall yield.

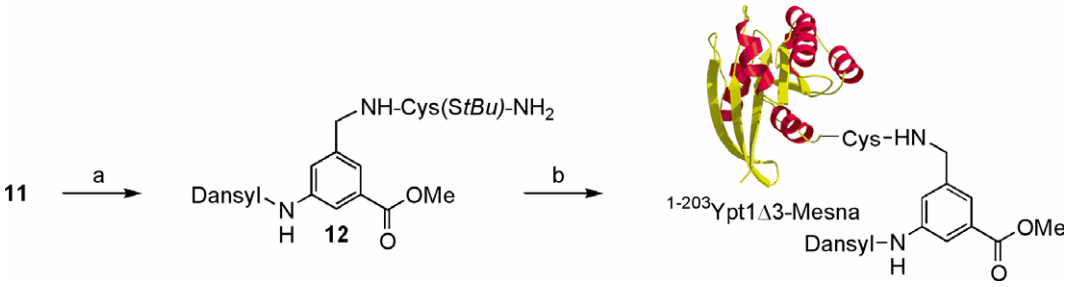
After removal of the Fmoc-group from compound **11,** fluorescently labeled compound **12** was ligated to truncated Ypt1Δ3-thioester[18] (the yeast analogue of human Rab7) by expressed protein ligation (Scheme 4). For the ligation reaction thioester-tagged Ypt1Δ3 was mixed with 5-fold molar excess of compound **12** and incubated for 12 h at room temperature. The ligation reaction did not depend on the presence of a detergent due to the good solubility of the building block in the buffer solution.

The reaction mixtures were resolved on an SDS–PAGE gel and the obtained gel was examined under UV light. Figure 1A shows that a fluorescent product could be detected at the position corresponding to the semi-synthetic Ypt1 protein. Figure 1B shows the mass spectrum of the semi-synthetic and fluorescently-labeled Ypt1-protein.

Additionally, the solid-phase synthesis of a fluorescently labeled MIC-modified building block allowing chemical




**Scheme 3.** Synthesis of the fluorescently labeled building block **11** on the solid phase. Reagents and conditions: (a) 1.5 equiv NaOH, $H_2O$/dioxane; (b) 3 equiv DIC, HOBt, $Et_3N$, $CH_2Cl_2$, hydrazide resin, 12 h, rt; (c) 3 equiv Fmoc-Cys(S-*t*-Bu)-OH, DIC, HOBt, THF, 20 min, rt; (d) 3 equiv $Bu_3P$, 12 h, rt, (e) $SnCl_2{\cdot}2H_2O$, DMF, 2 h, rt; (f) 3 equiv dansylCl, 3 equiv pyridine, 0.5 equiv DMAP, 2 h, rt; (g) 0.005 M $Cu(OAc)_2$, pyridine, MeOH, $CH_2Cl_2$, $O_2$, 3 h, rt.




**Scheme 4.** Ligation of the fluorescently labeled building blocks **11** after Fmoc-deprotection to Ypt1Δ3-thioester. Reagents and conditions: (a) $EtNH_2/CH_2Cl_2$ (1:4); (b) a—50 mM $Na_2HPO_4/NaH_2PO_4$, pH 7.5, 0.1 mM $MgCl_2$, 2 μM GDP, 150 mM 2-mercaptoethanesulfonic acid (MESNA), 12 h, rt.

labeling of proteins via MIC-conjugation and of a fluorescently labeled thioester allowing an N-terminal introduction of a fluorescence marker into the protein was carried out. To this end, amine **4** was protected with the Boc-group and after saponification compound **14** was immobilized on the solid support (Scheme 5). The immobilized building block **15** could be further modified on the solid phase. After removal of the Boc group, the amine was coupled to the dansyl group. The second amino group was generated by reduction of the nitro group and used for the introduction of the MIC-group. The desired compound **17** was cleaved from the resin and after purification by column chromatography obtained with a yield of 63%. The analogous solution-phase synthesis of desired fluorescent-labeled compound **17** provided a lower overall yield of 23%.

Apart from the higher yield of the solid-phase synthesis, a direct comparison of the solution phase and solid-phase routes revealed that in terms of efficiency, speed and flexibility the solid-phase technique is superior. Thioester **18** was generated in a similar way (Scheme 5). Due to problems encountered using thiols as nucleophiles for the final cleavage step we decided to apply a strategy used for the synthesis of peptide thioesters on the hydrazine support.[19] After oxidation of the hydrazide linker with NBS, glycine-*S*-ethyl-thioester was used as a nucleophile for the final cleavage of thioester **18** from the solid phase. The nitro group was reduced to the amine before cleavage from the resin to increase the polarity of the compound required for the ligation reaction in ligation buffer. Ligation of **18** to Rab6A-protein was achieved with a yield of ca. 10–20% of fluorescently labeled, semi-synthetic protein. Non-ligated Rab6A-protein was found to be difficult to seperate from the ligation product.

MIC-conjugation of **17** to truncated $^{1–181}$N-Ras[20] was carried out successfully using only a very small excess of compound **17** (Fig. 2A). The ligation was complete

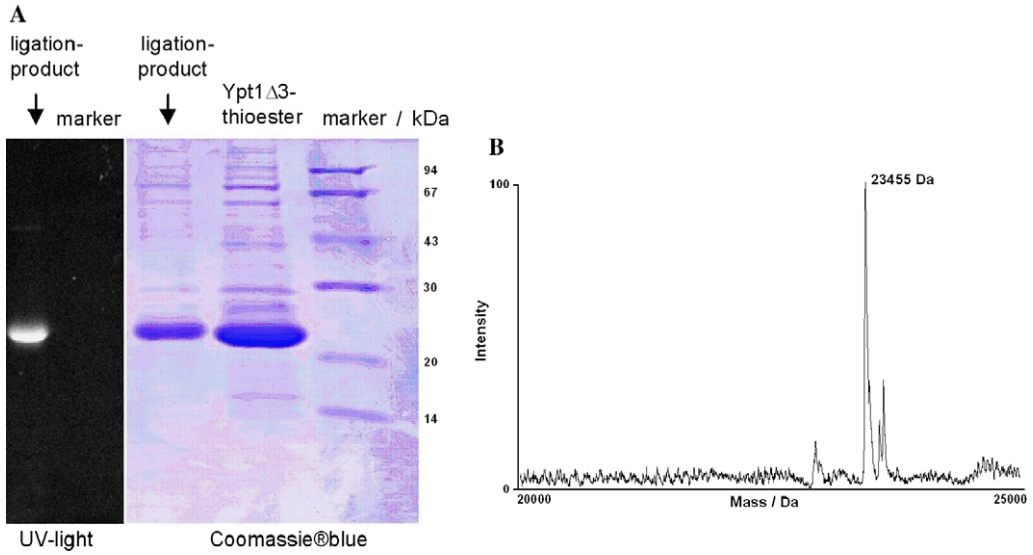
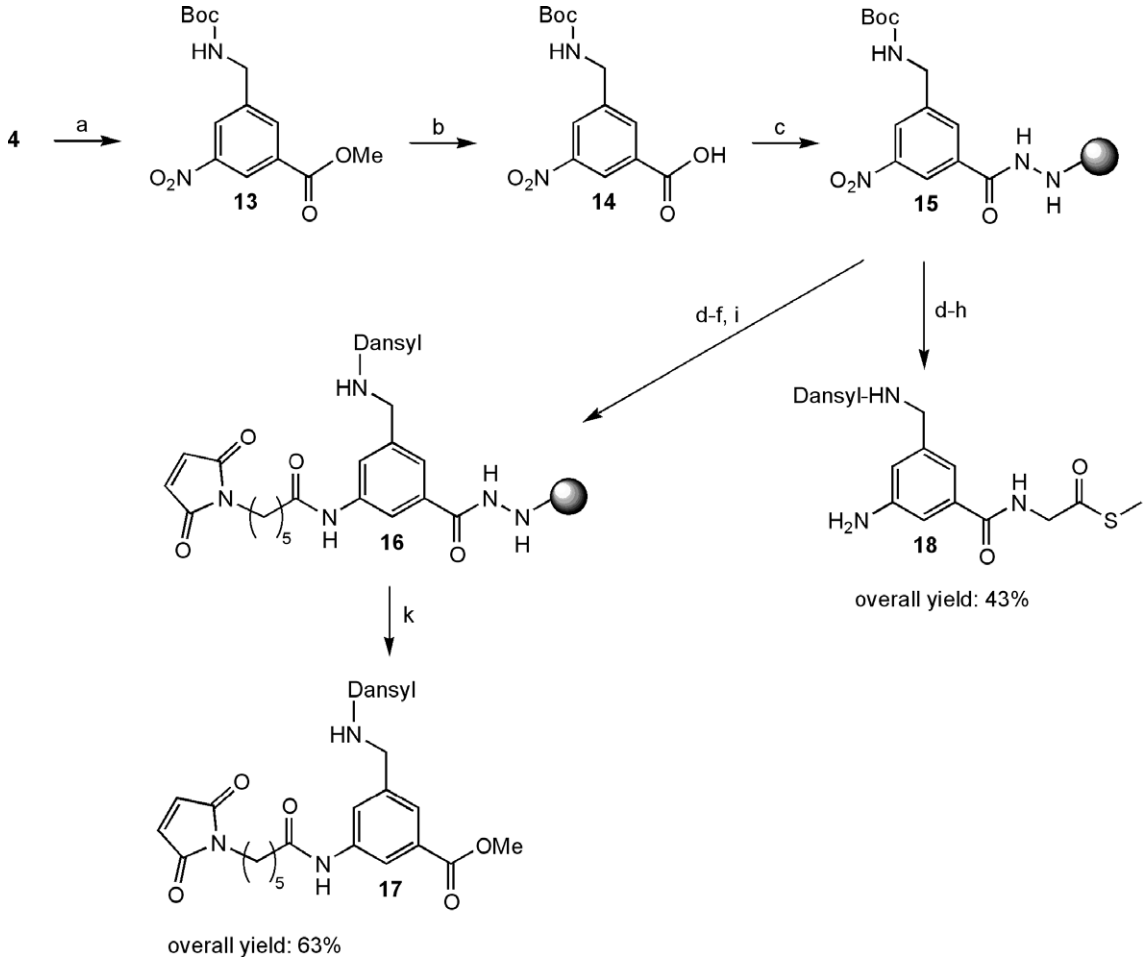



**Figure 1.** (A) SDS–PAGE gel of the fluorescently labeled semi-synthetic $^{1-203}$Ypt1-protein. The gel was photographed either in UV-light or visible light after Coomassie blue staining. (B) Deconvoluted MS-spectrum of the semi-synthetic protein. Calculated mass: 23,454 Da $[M+H]^+$.




**Scheme 5.** Synthesis of the fluorescently labeled building block **17** for MIC-conjugation and thioester **18** for N-terminal EPL. Reagents and conditions: (a) $Boc_2O$, $Et_3N$, THF, 0 °C; (b) 1.5 equiv NaOH, $H_2O$/dioxane; (c) hydrazide resin, 3 equiv HOBt, DIC, $Et_3N$, $CH_2Cl_2$, 12 h, rt; (d) 50% TFA/$CH_2Cl_2$; (e) 3 equiv dansyl-Cl, pyridine, 0.5 equiv DMAP, 3 h, rt; (f) 1 M $SnCl_2{\cdot}2H_2O$, DMF, 2 h, rt; (g) NBS, pyridine; (h) 10 equiv $H_2N$-Gly-SEt, $Et_3N$, DMF; (i) 2 equiv MIC, HOBt, HBTU, DIPEA, DMF, 12 h, rt; (k) 0.005 M $Cu(OAc)_2$, MeOH, pyridine, $O_2$.

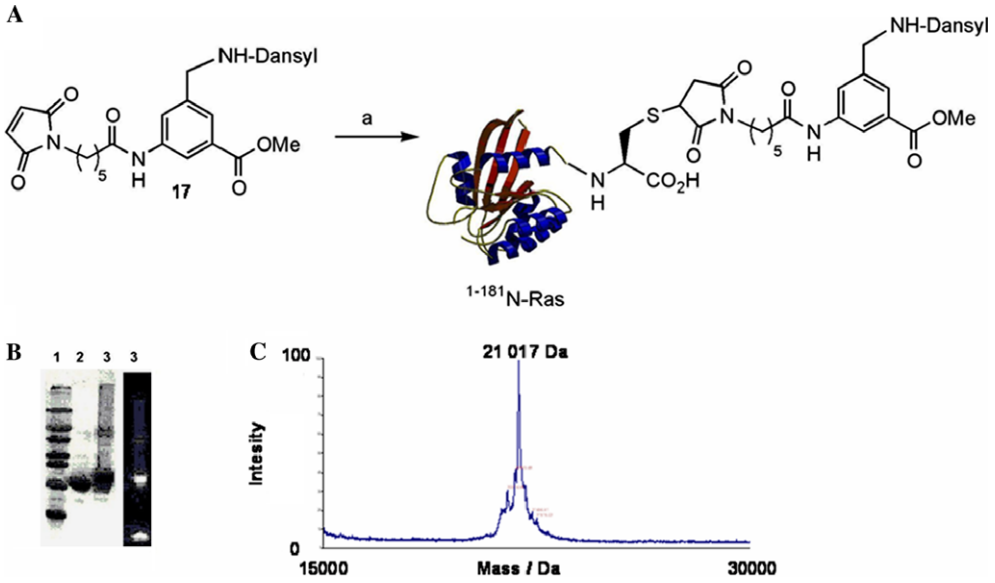



**Figure 2.** (A) Ligation of the fluorescently labeled derivative **17** with truncated $^{1-181}$N-Ras. Reagents and conditions: (a) 50 mM Tris-buffer, pH 7.4, 0.1 mM NaCl, 2 μM GDP, 12 h, rt. (B) SDS–PAGE gel of the crude ligation mixture. (C) MALDI-TOF spectra of the purified semi-synthetic protein. Calculated mass: 21,005 Da $[M+H]^+$. The deviation of the observed molecular mass from the calculated values is within the error range of the mass spectrometer. Matrix: sinapinic acid.

after 12 h and the semi-synthetic product was purified by gel filtration. Figure 2C shows the MALDI-TOF spectrum of the fluorescently labeled N-Ras protein.

## 4. Immobilization of N-Ras-protein

Encouraged by the successful ligation of the fluorescently labeled building block to the C-terminus of truncated Rab- and Ras-proteins, we aimed at the synthesis of an azide-tagged Ras-protein suitable for immobilization on a phosphane-functionalized surface by means of Staudinger ligation (Fig. 3). The hypervariable C-terminal region of the signal-transducing Ras oncoproteins is believed to primarily mediate protein-membrane interactions and to be less important for Ras-effector interactions than other regions of the protein.[5c] Therefore, immobilization of Ras via the C-terminus is desirable.

For visualization of proteins on the surface Cy5 was also coupled to the building block as suitable fluorescence marker.

For immobilisation experiments, benzene derivative **15** was appropriately functionalized (Scheme 6). The modification of the building block with Cy5 was achieved on the solid support. Due to its high polarity, Cy5 adhered to the resin and could hardly be removed from the solid

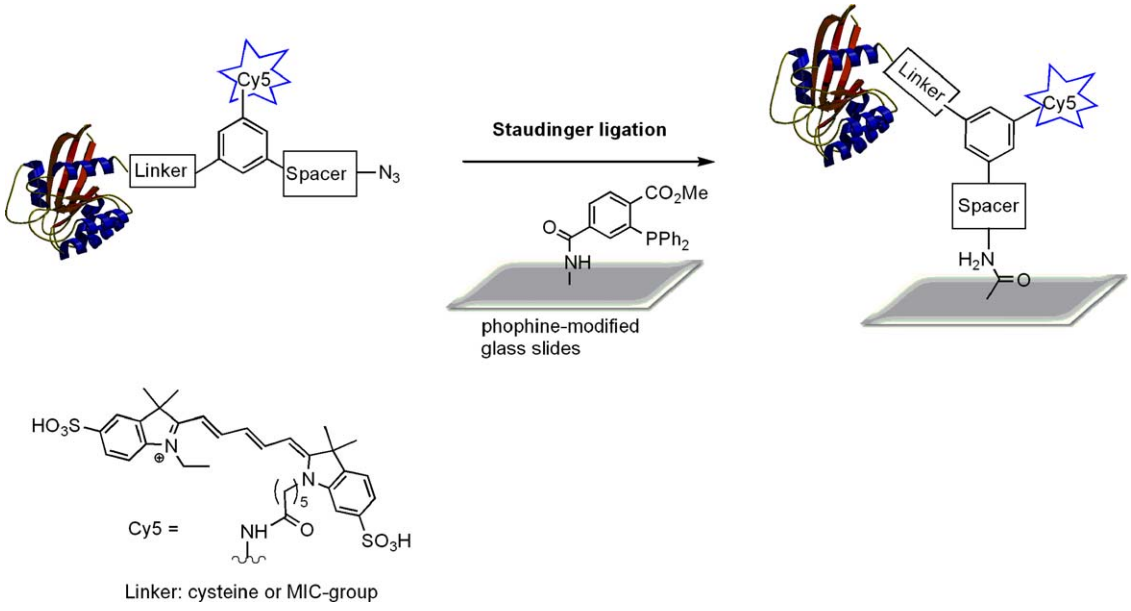
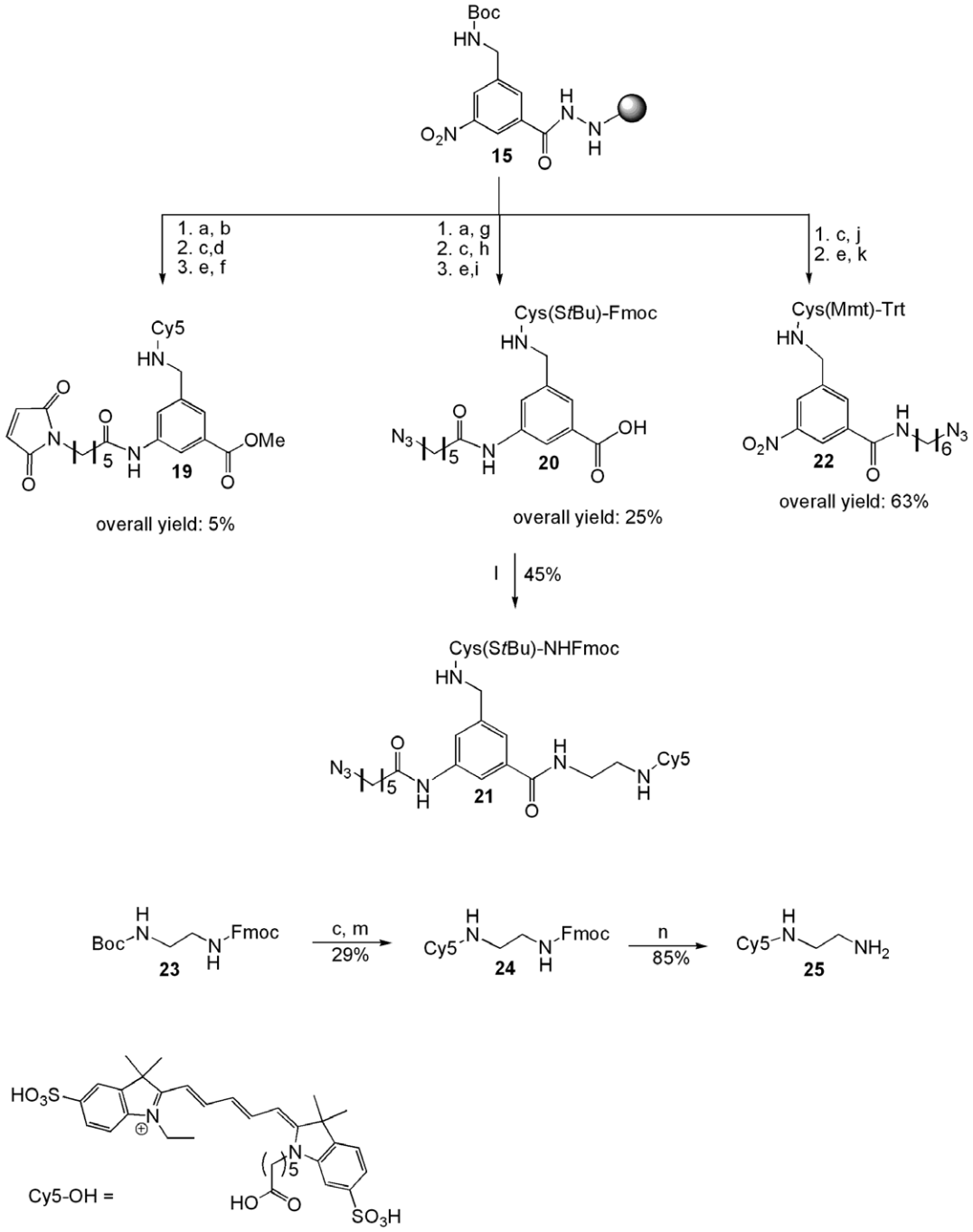



**Figure 3.** Concept for immobilization of functionalized proteins on phosphine-modified glass-slides via Staudinger ligation.




**Scheme 6.** Synthesis of different functionalized benzene derivatives on the solid phase. Reagents and conditions: (a) $SnCl_2{\cdot}2H_2O$, DMF, 2 h, rt; (b) 3 equiv MIC, HOBt, DIC, 12 h, rt; (c) 50% TFA/$CH_2Cl_2$, 30 min; (d) 6 equiv Cy5, HOBt, HBTU, $Et_3N$, 12 h, rt; (e) NBS, pyridine; (f) MeOH/$CH_2Cl_2$; (g) 3 equiv $N_3$–$(CH_2)_5$–$CO_2H$, HOBt, DIC, DIPEA, $CH_2Cl_2$, 12 h, rt; (h) 3 equiv Fmoc-Cys(S-*t*-Bu)-OH, 3 equiv HOBT, HBTU, DIPEA, 4 h, rt; (i) $H_2O$/THF; (m) 4 equiv Cy5-Et-$NH_2$, 4 equiv HOBt, HBTU, DIPEA, 12 h, rt; (j) 4 equiv Trt-Cys(Mmt)-OH, 4 equiv HOBT, HBTU, DIPEA, 4 h, rt; (k) 4 equiv $H_2N$–$(CH_2)_6$–$N_3$, THF; (l) 4 equiv Cy5-Et-$NH_2$, 4 equiv HOBt, HBTU, DIPEA, 12 h, rt; (m) 3 equiv Cy5, 8 equiv HOBt, HBTU, 4 equiv DIPEA; (n) 10% piperidine/DMF, 5 min.

support. To solubilize the fluorophore, methanol had to be used as solvent which, however, leads to insufficient swelling of the resin. Therefore, compound **19** was obtained with only 5% yield. To overcome this problem, we decided to synthesize triply substituted building block **21.** To this end, azide- and cysteine-functionalized carboxylic acid **20** was cleaved from the solid support by using a mixture of water and THF. Cy5 could then be coupled to **20** as amide to yield the desired triply functionalized compound **21**, which was purified by preparative HPLC. The amino derivative of Cy5 employed in this sequence was synthesized as shown in Scheme 6. Ethylenediamine derivative **23** was deprotected and then coupled to Cy5 yielding **24** in moderate yield. In the final step the Fmoc-group was removed under basic conditions, which are in general tolerated by Cy5.

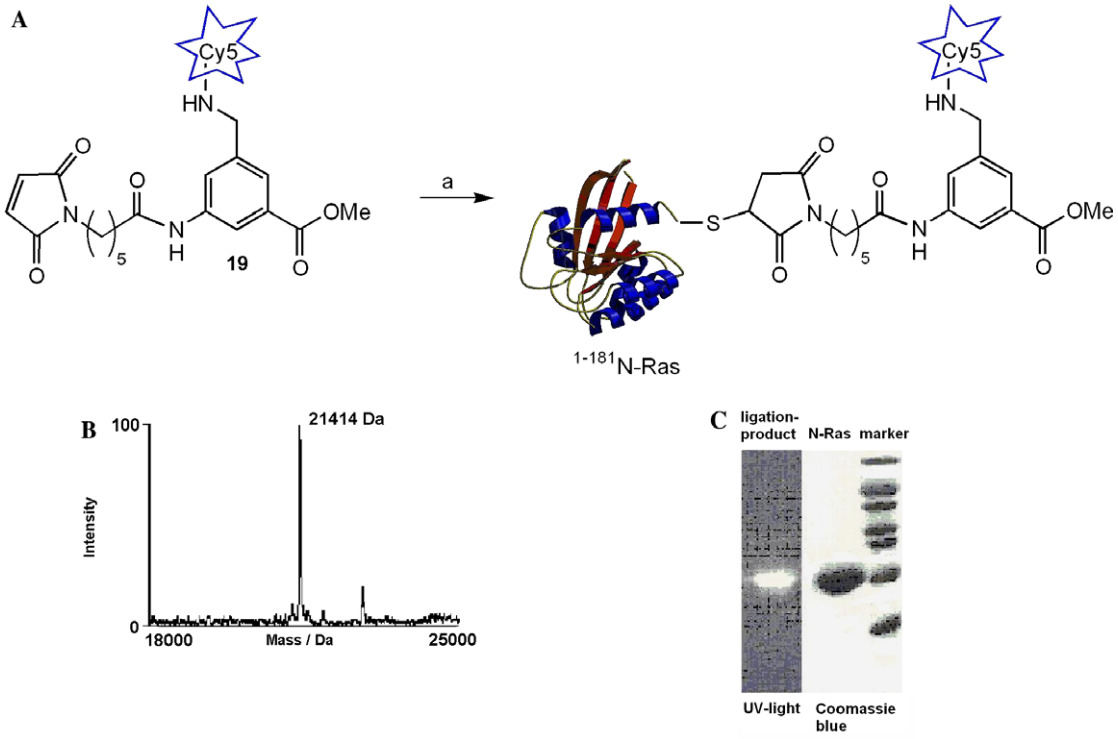



**Figure 4.** (A) Ligation of **19** to truncated $^{1-181}$N-Ras-protein. Reagents and conditions: (a) 50 mM Tris-buffer, pH 7.4, 0.1 mM NaCl, 2 μM GDP, 12 h, rt. (B) Deconvoluted MS-spectrum of the semi-synthetic protein (calculated mass: 21,411 Da $[M+H]^+$) and SDS–PAGE gel of the purified semi-synthetic protein. The gel was photographed either in UV-light or visible light after Coomassie blue staining.

The Cy5-labeled compound **19** was ligated to truncated $^{1-181}$N-Ras by MIC-conjugation (Fig. 4A). Only a slight excess of compound **19** was required for a successful ligation reaction yielding the semi-synthetic and Cy5-labeled Ras-protein quantitatively. Figure 4B shows the mass spectrum of the labeled N-Ras-protein after purification and the SDS–PAGE gel of the purified ligation product.

The azide-modified building block **22** was generated on solid phase by using one amino group of the building block **15** for the introduction of the cysteine, whereas the desired azide was introduced in the final cleavage step (Scheme 6). To avoid the formation of undesired complexes formed by copper-salts and azides, NBS was used to oxidize the hydrazide linker. By proper selection of the cleavage nucleophile, a further functionalization could be achieved. For synthesis of compound **22**, 6-azido-hexylamine was used as nucleophile. The cleavage proceeded smoothly, although in contrast to methanol, the excess of amine could not be removed by straightforward evaporation or extraction. However, simple column chromatography sufficed to purify the desired compound **22**.

To introduce an azide into the C-terminus of N-Ras, intermediate **22** was deprotected and afterwards ligated to truncated $^{1-180}$N-Ras-thioester by expressed protein ligation (Fig. 5A). After incubation of the thioester-tagged N-Ras-protein with the azide-modified building block for 12 h at room temperature, the obtained semi-synthetic N-Ras-protein was purified by gel filtration using DEAE-column chromatography. Figure 5B shows the LC/MS trace of the semi-synthetic N-Ras. It is important to note that the ligation reaction with azide-carrying building block is efficient in the presence of excess thiol, that is, the azide is not reduced to the amine under these conditions.

The reduction of alkyl azides by mono- and dithiols has been investigated before. Thus, the monothiol mercaptoethanol reduces alkyl azides much slower than, for example, 1,4-dithiothreitol.[20] Accordingly, azide **4** is efficiently reduced by propane-1,3-dithiol (Scheme 4) whereas the monothiol mercaptoethanesulfonic acid does not reduce the alkyl azide under the conditions described above.

For the immobilization of the azide-functionalized Ras-protein glass-slides initially modified with an intermediate layer of generation 4 PAMAM dendrimers were chosen as carriers to achieve maximum surface coverage.[15] For covalent attachment of the protein by means of Staudinger ligation the surface of the slides had to be decorated with an appropriately functionalized phosphine.[14a] This phosphane has been used for in vivo studies applying the Staudinger ligation.[14c] Phosphane groups on surfaces in general are prone to oxidation, the phosphine used here was developed by Bertozzi et al. to limit air oxidation.[21] For the spotting experiments, the azide-modified protein (positive control; PC) as well as the non-modified Ras-protein as a negative control (NC) were dissolved in phosphate buffer at 200–100 μM by means of a piezo-driven spotting robot.[22] Subsequent to the spotting process the slides were washed with buffer and blocked with bovine serum

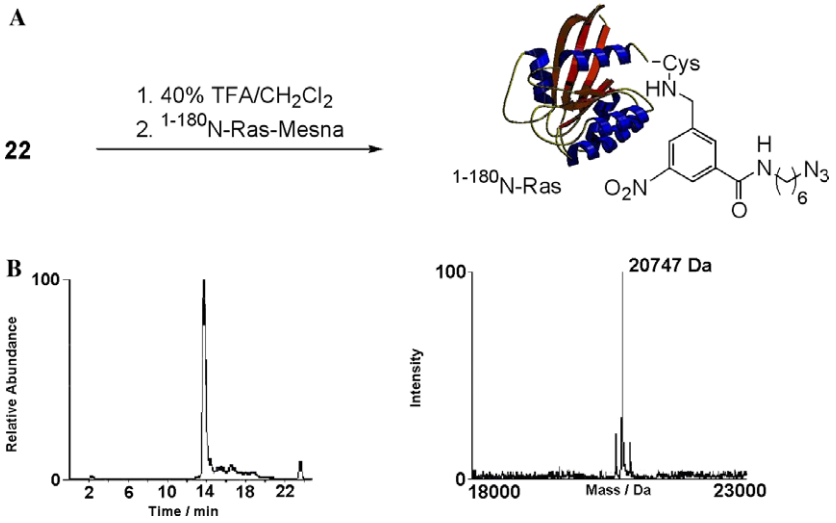



**Figure 5.** (A) Ligation of compound **22** after removal of all protection groups to $^{1-180}$N-Ras-thioester. (B) Deconvoluted LC–MS spectra of the purified semi-synthetic protein. Calculated mass: 20,747 Da $[M+H]^+$.

albumin (BSA)-containing blocking solution to prevent non-specific binding of protein reagents. The slides were then treated with Cy5-labeled Ras-antibody (50 nM, 1 h at room temperature). The fluorescent signals were recorded and quantified after removal of excess reagent. This preliminary result for the immobilization of

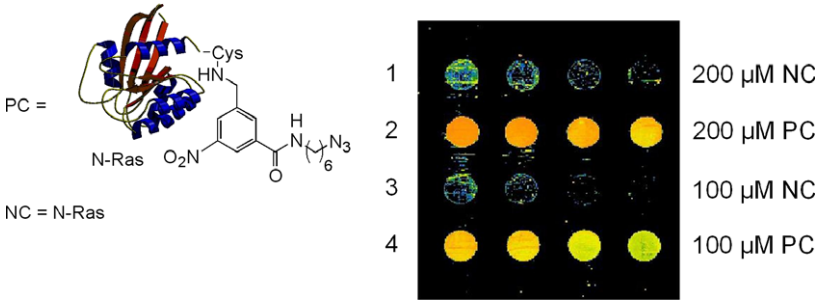



**Figure 6.** Preliminary results of immobilization of azide-functionalized N-Ras-protein (2, 4 and 6) as positive control (PC) and non-modified N-Ras-protein (1, 3 and 5) as negative control (NC) with various concentrations.

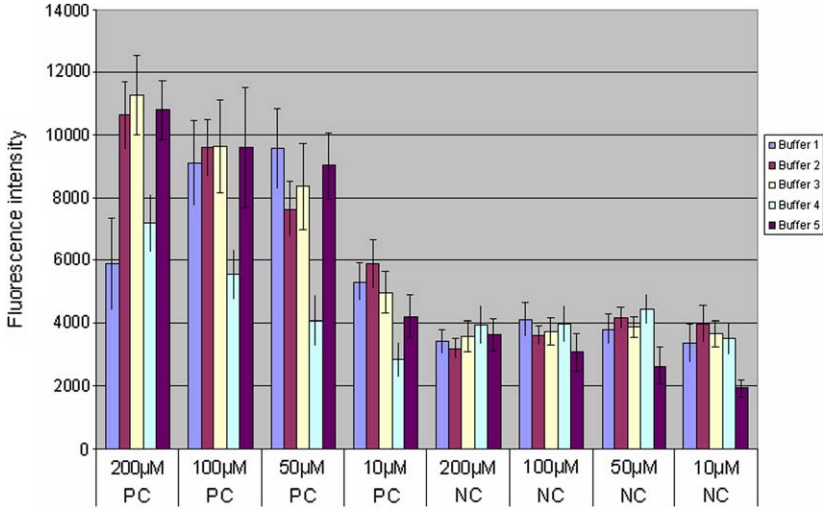



**Figure 7.** Influence of different buffers on the immobilization of azide-modified N-Ras-protein (PC). Reagents and conditions: Buffer 1: 20 mM Tris/Cl, 5 mM $MgCl_2$, 150 mM NaCl, pH 7.4 + 0.05% Tween 20. Buffer 2: 20 mM Tris, 5 mM $MgCl_2$, pH 7.4 + 0.05% Tween 20. Buffer 3: 10 mM Hepes, 150 mM NaCl, 5 mM $MgCl_2$, pH 7.4 + 0.05% Tween 20. Buffer 4: 20 mM Tris, 5 mM $MgCl_2$, 2 mM DTE, pH 7.6 + 0.05% Tween 20. Buffer 5: 50 mM $Na_2HPO_4$, 2 mM NaCl, 2 mM $MgCl_2$, pH 7.5 + 0.05% Tween 20. NC: non-modified N-Ras-protein.

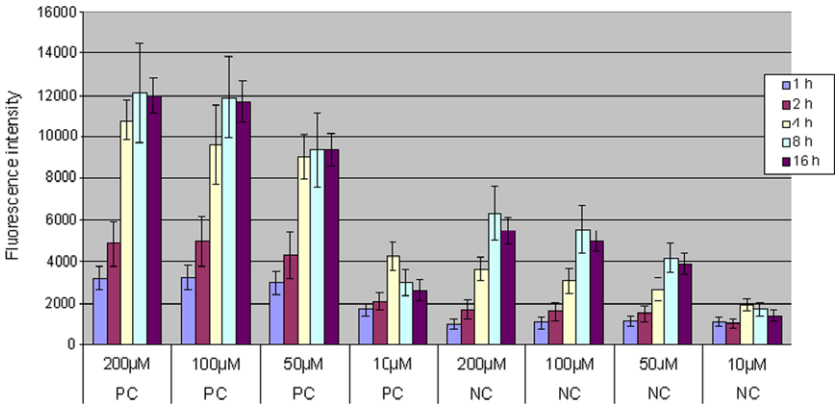



**Figure 8.** Time course and concentration dependence of immobilization by Staudinger ligation.

azide-modified Ras-protein is shown in Figure 6. The immobilization of azide-modified protein (PC) gave clear fluorescent signals, whereas no significant signals were obtained at any concentration for the negative control (NC). Encouraged by these findings we aimed at the study of the influence of different buffers, reaction time and concentration dependence of immobilization of azide-modified Ras-protein by Staudinger Ligation. The influence of different buffers on the immobilization of azide-modified N-Ras-protein (PC) is shown in Figure 7. No significant differences were observed between buffer 1, 2, 3 and 5. Buffer 4 gave inferior results, that is, the use of DTE leads to a decrease of immobilized protein. The immobilization was carried out at pH 7.4–7.6 due to the pH-dependent stability of the Ras-protein. We note, however, that the Staudinger ligation proceeds well over a relatively wide pH range (5–8.5).[23]

The time course and concentration dependence of immobilization by Staudinger ligation was studied using different concentrations of azide-modified N-Ras-protein (PC) and non-modified N-Ras-protein (NC) in phosphate buffer (Buffer 5: 50 mM $Na_2HPO_4$, 2 mM NaCl, 2 mM $MgCl_2$, pH 7.5 + 0.05% Tween 20) for 1–16 h as shown in Figure 8. After incubation with Cy5-labeled Ras-antibody (50 nM, 1 h), the fluorescence signals were recorded and quantified. The minimum concentration for acceptable levels of immobilization was found to be ca. 50 µM. An immobilization time of 4 h was sufficient for obtaining clear and reproducible signals.

These results demonstrate that the immobilization of the azide-modified Ras-protein onto the phosphane-modified glass-slides was successful.

## 5. Conclusion

In summary, a generic building block for protein modification was developed. It provides a flexible and generally applicable tool for the introduction of different functional groups of interest (e.g., fluorescent groups) into proteins. The benzene derivative equipped with a triply orthogonal set of functional groups could be easily modified on the solid phase in a controlled way and was successfully applied to the introduction of fluorescence markers into Rab and Ras GTPases.

Additionally, the building block was used for the covalent attachment of an azide into the C-terminus of the Ras-protein yielding azide-functionalized Ras, which could be further immobilized on phosphane-modified glass-slides via highly chemoselective Staudinger ligation. This new strategy for the site-specific immobilization of proteins onto a glass surface may be used for the creation of protein microarrays.

## 6. Experimental

### 6.1. General

Chemicals were obtained from Acros, Advanced Chemtech, Aldrich, Biosolve, Fluka or Novabiochem and used without further purification. Analytical chromatography was performed on Merck silica gel $60F_{254}$ aluminium sheets and Merck aluminium oxide 60 $F_{254}$ aluminium sheets. Chromatography was performed using Merck Silica gel 60 and Fluka aluminium oxide. Dichloromethane, triethylamine, diisopropylethylamine and piperidine were refluxed under argon over $CaH_2$, THF over Na/K, methanol over magnesium and freshly distilled prior to usage. $^1H$ and $^{13}C$ NMR data were recorded on a Bruker DRX 500 spectrometer at room temperature. NMR spectra were calibrated to the solvent signals of $CDCl_3$ (7.26 and 77.00 ppm). ESI-MS was performed on a Agilent 1100 series binary pump together with a reversed-phase HPLC column (Macherey-Nagel) and a Finnigan Thermoquest LCQ. Preparative HPLC was preformed on Agilent 1100 series with a VP125/21 Nucleosil 120-7C4 column (Macherey-Nagel) run at a flowrate of 20.0 mL/min, eluant was monitored at 215 nm. FAB measurements were taken with a Jeol SX 102A spectrometer using 3-nitrobenzyl alcohol (3-NBA) as matrix. MALDI-TOF spectra were recorded with a Voyager-DE Pro BioSpectrometer from PerSeptive Biosystems using 2,5-dihydroxybenzoic acid (DHB) as matrix. Optical rotations were measured using a Schmidt+Haensch Polartronic HH8. Yield and scale

of the solid-phase reactions are given with respect to the Fmoc loading as supplied by Novabiochem. All reactions were carried out under an argon atmosphere in dry solvents unless otherwise noted.

### 6.2. General procedures for the solid-phase synthesis of building blocks using the phenylhydrazide linker

For all building blocks commercially available Fmoc-4-hydrazinobenzoyl AM NovaGel resin from Novabiochem was used, (Fmoc loading of 0.56 mM/g). All reactions were carried out under an argon atmosphere in polyethylene syringe reactors equipped with a fritted disc. Agitation was achieved using an orbital shaker.

**6.2.1. Loading of the resin (general procedure GP1).** Swelling of the Fmoc-4-hydrazinobenzoyl NovaGel resin was carried out in dichloromethane. For Fmoc cleavage the resin was treated with a degassed solution of 35% piperidine in DMF for 7 min twice. The resin was subsequently washed six times with DMF and three times with dichloromethane. Afterwards 3 equiv of the acid to be coupled to the resin was preactivated for 5 min with 3 equiv HOBt, 3 equiv DIC and 3 equiv of $Et_3N$ in dichloromethane. The solution was added to the resin and the mixture was agitated for 12 h at room temperature. The resin was subsequently washed six times with dichloromethane and three times with DMF.

**6.2.2. Cleavage of the Boc-group (GP2).** The Boc-protecting group was cleaved by agitating the resin once for 5 min with a solution of 50% TFA in dichloromethane. The resin was subsequently washed five times with dichloromethane and five times with DMF.

**6.2.3. Reduction of the nitro group (GP3).** The resin was treated for 2 h at room temperature with a solution of 1M $SnCl_2{\cdot}2H_2O$ in DMF and subsequently washed five times with dichloromethane, DMF and ethyl acetate.

**6.2.4. Couplings (GP4).** *Method A.* Amino acids were coupled using HBTU/HOBt as activating reagents. Three equivalents of amino acid was preactivated for 5 min with 3 equiv HBTU, 3 equiv HOBt and 3 equiv *i*-PrNEt$_2$ in dichloromethane. The solution was added to the resin and agitated for 4 h at room temperature. In order to suppress racemisation, cysteine derivatives were coupled using DIC/HOBt for activation. The resin was subsequently washed six times with dichloromethane and three times with DMF.

*Method B.* A solution of 3 equiv dansylchloride, 3 equiv pyridine and 0.5 equiv DMAP in DMF was added to the resin, and the mixture was left standing for 3 h at room temperature. The resin was afterwards subsequently washed six times with dichloromethane and three times with DMF.

**6.2.5. Cleavage from the solid support (GP5).** *Method A.* The resin was treated with a solution of $Cu(OAc)_2$ (5.4 mg, 29 μmol), pyridine (48 μL, 588 μmol) and methanol (240 μL, 5.9 mmol) in dichloromethane (7 mL) under an oxygen atmosphere for 3 h at room temperature. The resin was filtered off and the solvent was evaporated under reduced pressure.

*Method B.* The resin was treated with a solution of 0.02 M NBS (5 equiv), and 0.02 M pyridine (5 equiv) in dichloromethane for 45 min. The resin was filtered off and washed five times with dichloromethane. Cleavage was achieved by agitating the resin with a solution of methanol/dichloromethane (1:100) for 2 h. The resin was filtered off and the solvent was evaporated under reduced pressure.

### 6.3. 3-Hydroxymethyl-5-nitro-benzoic acid methyl ester (2)

To a solution of 7 g (0.029 mmol) 5-nitro-isophthalic acid dimethyl ester (**1**) in 200 mL THF, 2.5 equiv DIBAL-H (72.5 mL of 1 M-solution in toluene) was added slowly under an argon-atmosphere at −78 °C. The reaction mixture was allowed to warm to room temperature and 50 mL methanol were added. The solvent was evaporated under reduced pressure and the remaining residue was dissolved in ethyl acetate. The organic phase was washed once with each 1 N HCl and a saturated solution of NaCl and was dried over $Na_2SO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (silica gel, cyclohexane/ethyl acetate 9:1, 7:3 (v/v)) yielding **2** as an off-white solid (3.06 g, 1.45 mmol, 50%).

TLC: 0.36 (cyclohexane/ethyl acetate 1:1 (v/v)); mp = 75–76 °C.

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.74 (s, 1H, arom CH-4), 8.43 (s, 1H, arom CH-6), 8.34 (s, 1H, arom CH-2), 4.88 (s, 2H, C$H_2$OH), 3.88 (s, 3H, $CO_2CH_3$), 2.14 (s, 1H, O*H*).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 164.99 ($CO_2CH_3$), 148.60 (C-5), 143.60 (C-3), 133.05 (C-2), 131.88 (C-1), 125.24 (C-4), 123.44 (C-6), 63.49 (*C*$H_2$OH), 52.83 ($CO_2CH_3$).

HR-FAB-MS (*m*/*z*): calcd for $[C_9H_{10}NO_5]^+$: 212.0559 $[M+H]^+$, found: 212.0576.

### 6.4. 3-Methanesulfonyloxymethyl-5-nitro-benzoic acid methyl ester (3)

To a solution of 1.50 g (7.11 mmol) 3-hydroxymethyl-5-nitro-benzoic acid methyl ester (**2**) in 20 mL dichloromethane 10 equiv $Et_3N$ (9.85 mL, 71.1 mmol) and 2 equiv mesyl chloride (1.10 mL, 14.22 mmol) was added slowly under argon at 0 °C. The reaction mixture was stirred for 15 min at 0 °C and subsequently treated with 10 mL of a saturated solution of NaCl. The organic phase was washed with 1 N $KHSO_4$ and a sat. solution of $Na_2CO_3$ and dried over $Na_2SO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (silica gel, cyclohexane/ethyl acetate 9:1, 8:2 (v/v)) yielding **3** as a pale yellow solid (1.95 g, 6.75 mmol, 95%).

TLC: 0.32 (cyclohexane/ethyl acetate 1:1 (v/v)); mp = 82 °C.

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.84 (s, 1H, arom CH-6), 8.44 (s, 1H, arom CH-4), 8.37 (s, 1H, arom CH-2), 5.33 (s, 2H, C$H_2$O), 3.98 (s, 3H, $CO_2CH_3$), 3.08 (s, 3H, $O_2SCH_3$).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 164.35 ($CO_2CH_3$), 136.38 (C-3), 134.61 (C-2), 132.59 (C-1), 126.84 (C-4), 125.01 (C-6), 68.17 ($CH_2$O), 53.04 ($CO_2CH_3$), 38.19 ($O_2SCH_3$).

HR-FAB-MS (*m*/*z*): calcd for $[C_{10}H_{11}NO_7SNa]^+$: 312.0154 $[M+Na]^+$, found: 312.0142.

### 6.5. 3-Azidomethyl-5-nitro-benzoic acid methyl ester (4)

To a solution of 1.30 g (4.50 mmol) 3-methanesulfonyloxymethyl-5-nitro-benzoic acid methyl ester (**3**) in 20 mL DMF 5 equiv of sodium azide (1.46 g, 22.49 mmol) were added. The reaction mixture was stirred for 2 h at 50 °C. The solvent was evaporated under reduced pressure and the remaining residue was dissolved in ethyl acetate. The organic phase was washed once with water and a saturated solution of NaCl and was dried over $Na_2SO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (silica gel, cyclohexane/ethyl acetate 9:1, 7:3 (v/v)) yielding **4** as a pale yellow solid (1.06 g, 4.5 mmol, quant.).

TLC: 0.83 (cyclohexane/ethyl acetate 1:1 (v/v)); mp = 69–72 °C.

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.75 (s, 1H, arom CH-6), 8.35 (s, 1H, arom CH-4), 8.29 (s, 1H, arom CH-2), 4.57 (s, 2H, C$H_2N_3$), 3.97 (s, 3H, $CO_2CH_3$).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 164.47 ($CO_2CH_3$), 148.47 (C-5), 138.38 (C-3), 134.23 (C-2), 132.31 (C-1), 126.37 (C-4), 124.00 (C-6), 53.25 ($CH_2N_3$), 52.83 ($CO_2CH_3$).

FAB/LR-MS (*m*/*z*): calcd for $[C_9H_8NO_4]^+$: 194.05 $[M-N_3]^+$, found: 194.00.

### 6.6. 3-Aminomethyl-5-nitro-benzoic acid methyl ester (5)

To a solution of 200 mg (0.85 mmol) 3-azidomethyl-5-nitro-benzoic acid methyl ester (**4**) in 5 mL methanol 4 equiv $Et_3N$ (470 μL, 3.39 mmol) and 6 equiv of propanedithiol (511 μL, 5.08 mmol) were added under an argon atmosphere. The reaction mixture was stirred for 12 h at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (silica gel, dichloromethane/methanol 10:1 (v/v)) yielding **5** as a pale yellow solid (151.7 mg, 0.723 mmol, 85%).

TLC: 0.3 (dichloromethane/methanol 10:1 (v/v)); mp = 84 °C.

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.61 (s, 1H, arom CH-6), 8.30 (s, 1H, arom CH-4), 8.21 (s, 1H, arom CH-2), 3.91 (s, 2H, C$H_2NH_2$), 3.86 (s, 3H, $CO_2CH_3$).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 164.97 ($CO_2CH_3$), 148.31 (C-5), 144.60 (C-3), 133.80 (C-2), 131.72 (C-1), 125.87 (C-4), 122.88 (C-6), 52.55 ($CH_2NH_2$), 44.57 ($CO_2CH_3$).

HR-FAB-MS (*m*/*z*): calcd for $[C_9H_{11}N_2O_4]^+$: 311.0719 $[M+H]^+$, found: 211.0730 (in 3-NBA-Matrix).

### 6.7. 3-{Amino-[L-cysteine-(*S*- monomethoxytrityl)-*N*-trityl]-methyl}-5-nitro-benzoic acid methyl ester (6)

To a solution of 1.57 g (2.54 mmol) Trt-Cys(Mmt)-OH in 5 mL dichloromethane 389 mg (2.54 mmol) HOBt and 320 μL (2.54 mmol) DIC were added. Activation of the amino acid to the active ester was controlled by TLC. Afterwards, a solution of 500 mg (2.12 mmol) 3-azidomethyl-5-nitro-benzoic acid methyl ester (**5**) in 1 mL dichloromethane was added to the reaction mixture. The reaction mixture was stirred for 12 h at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (silica gel, cyclohexane/ethyl acetate 8:2, 7:3 (v/v)) yielding **6** as a white solid (1.67 g, 2.01 mmol, 95%).

TLC: 0.5 (cyclohexane/ethyl acetate 7:3 (v/v)); mp = 118–119 °C; $[\alpha]_D^{20}$ +55.7 (*c* 1.0, $CHCl_3$).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.83 (s, 1H, arom CH-6), 8.39 (s, 1H, arom CH-4), 8.33 (s, 1H, arom CH-2), 7.98 (m, 1H, NH), 7.45–7.51 (m, 6H, 6× arom CH Trt, Mmt), 7.36–7.21 (m, 21H, 21× arom CH Trt, Mmt), 6.81 (d, *J* = 5.0 Hz, 2H, 2× arom CH Mmt), 4.57 (s, 2H, C$H_2$NH), 4.01 (s, 3H, $CO_2CH_3$), 3.90 (m, 1H, α-CH), 3.78 (s, 3H, $OCH_3$ Mmt), 2.81–2.85 (m, 2H, β-$CH_2$), 2.65 (m, 1H, NH).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 173.75 (CONH), 158.16, 148.43, 144.95, 144.59, 141.22, 136.41, 134.34, 131.76, 130.94, 129.49, 129.44, 128.83, 128.70, 128.01, 127.99, 127.92, 127.81, 127.78, 126.93, 126.77, 126.43, 123.3 (22× arom CH), 113.23 (2× arom CH Mmt), 71.44 (quart C Trt), 66.75 (quart C Mmt), 56.24 ($OCH_3$ Mmt), 55.12 (α-CH), 52.75 ($CO_2CH_3$), 42.26 ($CH_2$NH), 36.79 (β-$CH_2$).

ESI-MS+ *m*/*z*: calcd for $C_{51}H_{45}N_3O_6SNa$ $[M+Na]^+$: 850.3, found: 850.4.

MALDI-TOF (DHB) calcd for $C_{51}H_{45}N_3O_6SNa$ $[M+Na]^+$: 850.3, found: 850.4.

FAB/LR-MS (*m*/*z*): calcd 828.30 $[M+H]^+$, found: 828.00.

### 6.8. 3-{Amino-[L-cysteine-(*S*-monomethoxytrityl)-*N*-trityl]-methyl}-5-amino-benzoic acid methyl ester (7)

To a solution of 50 mg (0.06 mmol) 3-{amino-[L-cysteine-(*S*Mmt)-*N*-trityl]-methyl}-5-nitro-benzoic acid methyl ester (**6**) in 3 mL methanol 10 mg palladium on charcoal was added. The reaction mixture was stirred for 4 h under an hydrogen atmosphere at room temperature. The catalyst was filtered off and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (silica, cyclo-

hexane/ethyl acetate 1:1 (v/v)) yielding **7** as a white solid (47.8 mg, 0.06 mmol, quant.).

TLC: 0.44 (cyclohexane/ethyl acetate 1:1 (v/v)); mp = 124–126 °C; $[\alpha]_D^{20}$ +18.0 (*c* 1.0, $CHCl_3$).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 7.95 (s, 1H, arom CH), 7.42–7.35 (m, 5H, 5× arom CH), 7.27–7.24 (m, 3H, 3× arom CH), 7.23–7.10 (m, 20H, 20× arom CH), 7.03 (s, 1H, arom CH), 6.65 (d, $^3J$ = 10.8 Hz, 2H, 2× arom CH Mmt), 4.00–3.98 (dd, $^3J_1$ = 15.0 Hz, $^3J_2$ = 7.1 Hz, 2H, C$H_2$NH), 3.82 (s, 3H, $CO_2CH_3$), 3.68 (s, 3H, O$CH_3$ Mmt), 3.66 (m, 1H, α-CH), 3.08 (m, 1H, β-$CH_{2a}$), 2.99 (m, 1H, β-$CH_{2b}$), 2.86 (br s, 2H, $NH_2$), 2.41–2.36 (m, 1H, NH).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 173.24 (CONH), 158.15, 145.31, 144.92, 144.87, 136.58, 130.95, 129.49, 129.46, 128.86, 127.98, 127.92, 127.86, 126.93, 126.78, 126.74 (15× arom CH), 113.44 (2× arom CH Mmt), 71.67 (quart C Trt), 66.58 (quart C Mmt), 56.79 (O$CH_3$ Mmt), 55.27 (α-CH), 52.29 ($CO_2CH_3$), 43.21 ($CH_2$NH), 36.97 (β-$CH_2$).

MALDI-TOF (DHB) calcd for $C_{51}H_{47}N_3O_4SNa$ $[M+Na]^+$: 820.3, found: 820.6; calcd for $C_{51}H_{47}N_3O_4SK$ $[M+K]^+$: 836.3, found: 836.6.

FAB/LR-MS (*m*/*z*): calcd: 798.33 $[M+H]^+$, found: 799.15.

### 6.9. 3-{Amino-[L-cysteine-(*S*-monomethoxytrityl)-*N*-trityl]-methyl}-5-(5-dimethylamino-naphthalen-1-sulfonylamino)-benzoic acid methylester (8)

To a solution of 770 mg (0.966 mmol) 3-{amino-[L-cysteine-(*S*Mmt)-*N*-trityl]-methyl}-5-amino-benzoic acid methyl ester (**7**) in 3 mL dichloromethane 1.5 equiv dansyl chloride (7.6 mg, 0.028 mmol), 3 equiv pyridine (4.46 mg, 0.056 mmol) and 0.5 equiv dimethylaminopyridine (1.7 mg, 0.015 mmol) were added. The reaction mixture was stirred for 4 h at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (silica, cyclohexane/ethyl acetate 8:2, 1:1 (v/v)) yielding **8** as a pale yellow solid (786.0 mg, 0. 763 mmol, 79%).

TLC: 0.58 (cyclohexane/ethyl acetate 1:1 (v/v)); mp = 120–121 °C; $[\alpha]_D^{20}$ −46.1 (*c* 1.0, $CHCl_3$).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.46 (d, $^3J$ = 8.5 Hz, 1H, H-2 dansyl), 8.28 (d, $^3J$ = 9.2 Hz, 1H, H-4 dansyl), 8.20 (d, $^3J$ = 6.8 Hz, 1H, H-8 dansyl), 7.55–7.44 (m, 4H, 4× arom CH), 7.40–7.35 (m, 7H, 7× arom CH), 7.26–7.04 (m, 22H, 22× arom CH), 6.65 (d, $^3J$ = 8.0 Hz, 2H, 2× arom CH Mmt), 6.30 (br s, 1H, NH), 3.88–3.92 (dd, $^3J_1$ = 14.9 Hz, $^3J_2$ = 6.0 Hz, 2H, C$H_2$NH), 3.72 (s, 3H, $CO_2CH_3$), 3.66 (s, 3H, O$CH_3$ Mmt), 3.64 (m, 1H, α-CH), 3.12–3.05 (m, 2H, β-$CH_2$), 2.84 (s, 6H, N($CH_3$)$_2$), 2.32 (dd, $^3J_1$ = 12.3 Hz, $^3J_2$ = 6.3 Hz, 1H, NH).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 172.98 (CONH), 165.85 ($CO_2CH_3$), 151.72, 144.89, 144.50, 139.42, 137.10, 136.27, 133.87, 130.98, 130.66, 130.61, 129.93, 129.54, 129.25, 129.16, 128.55, 128.36, 127.70, 127.55, 126.51, 126.69, 124.49, 123.94, 122.76, 119.69, 118.17, 115.02 (26× arom CH), 112.94 (2× arom CH Mmt), 69.63 (quart C Trt), 66.13 (quart C Mmt), 56.12 (O$CH_3$ Mmt), 54.85 (α-CH), 51.94 ($CO_2CH_3$), 45.07 ($CH_2$NH), 42.47 (N($CH_3$)$_2$), 36.45 (β-$CH_2$).

ESI-MS+ *m*/*z*: calcd for $C_{63}H_{58}N_4O_6S_2$ [M]: 1030.3, found: 850.4 [M−Mmt+2Na+H].

MALDI-TOF (DHB) calcd for $C_{63}H_{58}N_4O_6S_2$ [M]: 1030.3, found: 811.31 [M−Mmt+Na+H].

HR-FAB-MS (*m*/*z*): calcd: 1031.3876 $[M+H]^+$, found: 1031.3936.

### 6.10. 3-{Amino-[L-cysteine-(*S*-*tert*-butyl)-*N*-fluorenylmethoxy-carbonyl]-methyl}-5-(5-dimethylamino-naphthalene-1-sulfonyl-amino)-benzoic acid methyl ester (11)

The loaded resin (**9**) (50 mg, 0.05 mmol) was agitated in 2 mL dichloromethane. To a solution of 126.2 mg (0.2 mmol) Fmoc-Cys(S-*t*-Bu)-OH in 2 mL THF 30.6 mg (0.2 mmol) HOBt and 25.2 μL DIC (0.2 mmol) were added. Activation of the amino acid was controlled by TLC. The reaction mixture was stirred for 10 min and then added to the resin. To the resin 6 equiv $Bu_3P$ (74 μL, 0.3 mmol) was added subsequently. After 12 h the resin was filtered off and washed five times with dichloromethane and five times with DMF. The reduction of the nitro group was carried out according to GP3. Afterwards, the resin was treated with 27 mg (0.1 mmol) dansyl chloride, 3 mg ($2.5 \times 10^{-5}$ mol) DMAP and 5 μL (0.06 mmol) pyridine as described in GP4 (method B). The cleavage from the resin was carried out according to GP5 (method A). The crude product was purified by column chromatography (silica gel, cyclohexane/ethyl acetate 6:4 (v/v)) yielding **11** as a yellow solid (22.7 mg, 0.027 mmol, 55%).

TLC: 0.64 (cyclohexane/ethyl acetate 1:1 (v/v)); mp = 112–113 °C; $[\alpha]_D^{20}$ −85.1 (*c* 1.0, $CHCl_3$).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.46 (d, $^3J$ = 8.5 Hz, 1H, H-2 dansyl), 8.20 (d, $^3J$ = 8.8 Hz, 1H, H-4 dansyl), 7.75–7.72 (m, 4H, H-8 dansyl, H-1, H-8 Fmoc, arom CH-4), 7.58–7.50 (m, 4H, H-4, H-5 Fmoc, H-3 dansyl, arom CH-6), 7.40–7.26 (m, H, H-6, H-7 dansyl, H-2, H-3, H-6, H-7 Fmoc), 7.12 (s, 1H, arom CH-2), 4.42–4.18 (m, 5H, H-9, H-10 Fmoc, C$H_2$NH), 4.01 (t, $^3J$ = 8.8 Hz, 1H, α-CH); 3.78 (s, 3H, O$CH_3$), 3.15–3.03 (m, 8H, β-$CH_2$ Cys, N($CH_3$)$_3$), 1.33 (s, 9H, S-*t*-Bu).

ESI-MS+ *m*/*z*: calcd for $C_{43}H_{47}N_4O_5S_3$ $[M+H]^+$: 827.2, found: 827.5, calcd for $C_{43}H_{46}N_4O_5S_3Na$ $[M+Na]^+$: 859.2, found: 859.5.

HR-FAB-MS (*m*/*z*): calcd: 827.2606 $[M+H]^+$, found: 827.2552.

### 6.11. 3-[Amino-(*tert*-butyloxycarbonyl)-methyl]-5-nitro-benzoic acid methyl ester (13)

To a solution of 340 mg (1.61 mmol) 3-aminomethyl-5-nitro-benzoic acid methyl ester (**4**) in 15 mL THF a solu-

tion of 2 equiv $Et_3N$ (446 μL, 3.22 mmol) and 1.5 equiv $Boc_2O$ (457 mg, 2.09 mmol) in 5 mL THF was added at 0 °C. The reaction mixture was stirred for 12 h and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (silica, cyclohexane/ethyl acetate 7:3 (v/v)) yielding **13** as a colourless oil (499 mg, 1.61 mmol, quant.).

TLC: 0.67 (cyclohexane/ethyl acetate 1:1 (v/v)).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.73 (s, 1H, arom CH-6), 8.32 (s, 1H, arom CH-4), 8.27 (s, 1H, arom CH-2), 5.13 (br s, 1H, NH), 4.45 (s, 2H, C$H_2$NH), 3.97 (s, 3H, $CO_2CH_3$), 1.46 (s, 9H, O-*t*-Bu).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 164.87 ($CO_2CH_3$), 148.55 (C-5), 142.18 (C-3), 133.77 (C-2), 132.03 (C-1), 125.81 (C-4), 123.33 (C-6), 52.78 ($CO_2CH_3$), 43.67 ($CH_2$NH), 28.29 (O-*t*-Bu).

HR-FAB/MS (*m*/*z*): calcd for $[C_{14}H_{18}N_2O_6]^+$: 311.1243 $[M+H]^+$, found: 311.1241.

### 6.12. 3-[Amino-(*tert*-butyloxycarbonyl)-methyl]-5-nitro-benzoic acid (14)

To a solution of 140 mg (0.45 mmol) 3-[amino-(*tert*-butyloxycarbonyl)-methyl]-5-nitro-benzoic acid methyl ester (**13**) in 2 mL water/dioxane (1:1) 1.5 equiv NaOH (675 μL of a 1 N solution, 0.675 mmol) was added over 1 h. The pH of the solution was adjusted to pH 2 with 1 N HCl. The reaction mixture was extracted three times with ethyl acetate. The combined organic phases were dried over $Na_2SO_4$. The solvent was removed under reduced pressure yielding pure **14** (133 mg, 0.45 mmol, quant.) as a colourless oil.

TLC: 0.2 (cyclohexane/ethyl acetate 1:1 (v/v)).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.56 (s, 1H, arom CH-6), 8.25 (s, 1H, arom CH-4), 8.20 (s, 1H, arom CH-2), 4.29 (s, 2H, C$H_2$NH), 3.21 (s, 3H, $CO_2H$), 1.37 (s, 9H, O-*t*-Bu).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 164.18 ($CO_2CH_3$), 148.68 (C-5), 143.18 (C-3), 133.71 (C-2), 132.82 (C-1), 125.46 (C-4), 122.37 (C-6), 43.05 ($CH_2$NH), 27.61 (O-*t*-Bu).

HR-FAB/MS (*m*/*z*): calcd for $[C_{13}H_{16}N_2O_5Na]^+$: 319.0906 $[M+Na]^+$, found: 319.0901.

### 6.13. 3-[Amino-(5-dimethylaminonaphthalene-1-sulfonyl)-methyl]-5- (maleimidocaproyl-amino)-benzoic acid methyl ester (17)

The loading of 150 mg Fmoc-4-hydrazinobenzoyl resin (0.56 mmol/g, 0.147 mmol) with compound **14** was carried out according to GP1. Removal of the Boc-group was carried out according to GP2. The resin was treated with 94 mg (0.348 mmol) dansyl chloride, 10.4 mg (0.085 mmol) DMAP and 17.4 μL (0.348 mmol) pyridine as described in GP4 (method B). The nitro group was reduced according to GP3 and the resin was treated with 61.3 mg (0.294 mol) maleimidocaproic acid, 91 mg (0.588 mol) HOBt, 336 mg (0.882 mol) HBTU and 52 μL (0.294 mol) *i*-Pr$NEt_2$ according to GP4 (method A). Cleavage from the resin was carried out according to GP5 (method A). The crude product was purified by column chromatography (silica gel, cyclohexane/ethyl acetate 1:1, 4:6 (v/v)) yielding **17** as a colourless oil (49 mg, 0.081 mol, 63%).

TLC: 0.22 (cyclohexane/ethyl acetate 1:1 (v/v)).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.40 (d, $^3J$ = 10.5 Hz, 1H, H-2 dansyl), 8.22 (d, $^3J$ = 10.8 Hz, 1H, H-4 dansyl), 8.13 (d, $^3J$ = 9.1 Hz, 1H, H-8 dansyl), 7.80 (s, 1H, arom CH-6), 7.53 (s, 1H, arom CH-4), 7.45 (t, $^3J$ = 9.5 Hz, 1H, H-3 dansyl), 7.40 (t, $^3J$ = 10.3 Hz, 1H, H-7 dansyl), 7.30 (s, 1H, arom CH-2), 7.09 (d, $^3J$ = 10.0 Hz, 1H, H-6 dansyl), 6.62 (s, 2H, C$H$=C$H$ Mic), 5.27 (s, 1H, NH), 4.00 (s, 2H, C$H_2$NH), 3.76 (s, 3H, $CO_2CH_3$), 3.44 (t, $^3J$ = 5.0 Hz, 2H, ε-$CH_2$), 2.82 (s, 6H, N($CH_3$)$_2$), 2.25 (t, $^3J$ = 9.5 Hz, 2H, α-$CH_2$), 1.64 (m, 2H, β-$CH_2$), 1.55 (m, 2H, δ-$CH_2$), 1.28 (m, 2H, γ-$CH_2$).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 172.02 (CONH), 170.91 ($CO_2CH_3$), 166.55 (OCNCO), 157.70, 138.39, 137.61, 134.88 (4× arom CH), 133.97 (C=C), 130.15, 130.01, 129.47, 129.39, 129.34, 128.01, 123.88, 123.49, 123.12, 119.77, 119.66, 115.09 (12× arom CH), 51.98 ($CO_2CH_3$), 46.37 ($CH_2$NH), 45.26 (N($CH_3$)$_2$), 41.57 (ε-$CH_2$), 37.40 (α-$CH_2$), 28.50 (δ-$CH_2$), 26.07 (β-$CH_2$), 24.71 (γ-$CH_2$).

ESI-MS+ *m*/*z*: calcd for $C_{13}H_{35}N_4O_7S$ $[M+H]^+$: 607.2, found: 607.5.

MALDI-TOF (DHB) calcd for $C_{13}H_{34}N_4O_7SNa$ $[M+Na]^+$: 629.2, found: 629.6.

FAB/LR-MS (*m*/*z*): calcd: 607.21 $[M+H]^+$, found: 607.84.

### 6.14. 3-[Amino-(5-dimethylaminonaphthalene-1-sulfonyl)-methyl]-5-amino-benzoic acid-(glycine-*S*-ethylthio ester) amide (18)

The loading of 50 mg Fmoc-4-hydrazinobenzoyl resin (0.56 mmol/g, 0.05 mmol) with compound **14** was carried out according to GP1. Removal of the Boc-group was carried out according to GP2. The resin was treated with 40 mg (0.15 mmol) dansyl chloride, 3.4 mg (0.03 mmol) DMAP and 7.5 μL (0.15 mmol) pyridine according to GP4 (method B). The reduction of the nitro group was carried out according to GP3. The cleavage from the resin was carried out according to GP5 (method B). In the final cleavage step the resin was treated with a solution of 10 equiv glycine ethyl thioester (0.5 mmol, 250 mg) and 10 equiv *i*-Pr$NEt_2$ (0.5 mmol, 86 μL) in 2 mL DMF for 3 h. The resin was filtered off and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (silica gel, dichloromethane/methanol 50:1, 20:1 (v/v)) yielding **18** as a yellow solid (10.8 mg, 0.021 mol, 43%).

TLC: 0.50 (dichloromethane/methanol 20:1 (v/v)).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.43 (d, $^3J$ = 10.3 Hz, 1H, H-2 dansyl), 8.21 (d, $^3J$ = 11.5 Hz, 1H, H-4 dansyl), 8.14 (d, $^3J$ = 9.0 Hz, 1H, H-8 dansyl), 7.48 (t, $^3J$ = 10.0 Hz, 1H, H-3, dansyl), 7.43 (t, $^3J$ = 10.5 Hz, 1H, H-3, dansyl), 7.10 (d, $^3J$ = 8.8 Hz, 1H, H-6 dansyl), 6.80 (s, 2H, arom CH-2), 6.72 (s, 2H, arom CH-6), 6.41 (s, 1H, arom CH-4), 6.30 (br s, 1H, NH), 4.17 (br s, 1H, NH), 3.91-3.87 (m, 2H, C$H_2$NH), 3.62–3.61 (m, 2H, $CH_2$ Gly), 3.41 (s, 2H, $NH_2$), 3.09–3.08 (m, 2H, C$H_2$CH$_3$), 2.81 (s, 6H, N(C$H_3$)$_2$), 1.18 (s, 3H, CH$_2$C$H_3$).

ESI-MS+ *m*/*z*: calcd for $C_{24}H_{28}N_4O_4S_2$ $[M+H]^+$: 501.1, found: 501.4.

HR-FAB-MS (*m*/*z*): calcd: 501.1630 $[M+H]^+$, found: 501.1644.

### 6.15. 3-(Cy5-aminomethyl)-5-(maleimidocaproyl-amino)-benzoic acid methyl ester (19)

Resin **15** (150 mg, 0.147 mmol) was agitated in 3 mL dichloromethane. Reduction of the nitro group was carried out according to GP3. The resin was afterwards treated with 93.0 mg (0.441 mmol) maleimidocaproic acid (MIC), 68.3 mg (0.441 mmol) HOBt and 69.1 μL (0.441 mmol) DIC according to PG4 (method A). Removal of the Boc-group was carried out according to GP2. Afterwards, the resin was treated with 576.8 mg (0.882 mmol) Cy5, 134.9 mg (0.882 mmol) HOBt, 330.8 mg (0.882 mmol) HBTU and 132.0 μL (0.882 mmol) $Et_3N$ in 2 mL DMF for 12 h. Cleavage from the resin was carried out according to GP5 (method B). The crude product was purified by HPLC (linear gradient of water (solvent A) and $CH_3CN$ (solvent B); 20–100 B% 15 min) yielding **19** as a blue solid (7.4 mg, $7.35 \times 10^{-5}$ mol, 5%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.32–8.26 (m, 2H, arom. CH Cy5), 8.05 (s, 1H, arom CH-6), 7.98–7.84 (m, 3H, H-4, 2× arom CH Cy5), 7.67 (s, 1H, arom CH-2), 7.34–7.29 (dd, $^3J_1$ = 10.3 Hz, $^3J_2$ = 6.4 Hz, 2H, 2× arom CH Cy5), 6.75 (s, 2H, *H*C=C*H* Mic), 6.67–6.60 (m, 1H, C=C*H*Cy5), 6.34–6.27 (t, $^3J$ = 16.6 Hz, 2H, *H*C=C*H*Cy5), 4.38 (s, 2H, C$H_2$NH), 4.14–4.12 (m, 4H, ε-$CH_2$, $N^+$=C–C*H*=, $C_{quart}$=C*H* Cy5), 3.86 (s, 3H, $CO_2CH_3$), 3.47 (t, $^3J$ = 8.3 Hz, 2H, ε-$CH_2$ Mic), 2.35 (t, $^3J$ = 8.5 Hz, 2H, α-$CH_2$ Cy5), 2.29 (t, $^3J$ = 8.2 Hz, 2H, α-$CH_2$ Mic), 1.85–1.60 (m, 6H, β-$CH_2$, δ-$CH_2$ Mic, N–C$H_2$–$CH_3$ Cy5), 1.70 (s, 12H, 4× $CH_3$ Cy5), 1.60 (t, $^3J$ = 8.5 Hz, 2H, δ-$CH_2$ Cy5), 1.50 (m, 2H, γ-$CH_2$ Cy5), 1.37 (t, $^3J$ = 9.0 Hz, 2H, γ-$CH_2$ Mic), 1.33–1.28 (m, 3H, $NCH_2$–C$H_3$ Cy5).

ESI-MS+ *m*/*z*: calcd for $C_{52}H_{62}N_5O_{12}S^+$ $[M]^+$ 1012.4, found: 1012.4.

FAB/LR-MS (*m*/*z*): calcd: 1013.38 $[M+H]^+$, found: 1013.52.

### 6.16. 3-{Amino-[L-cysteine-(*S*-*tert*-butyl)-*N*-fluorenyl-methoxy-carbonyl]-methyl}-5-(6-azido-hexoyl-amino)-benzoic acid (20)

Resin **15** (50 mg, 0.05 mmol) was agitated in 2 mL dichloromethane. The reduction of the nitro group was carried out according to GP3. According to GP4 (method A) a solution of 21.3 mg (0.15 mmol) 6-azido-hexanoic acid, 22.9 mg (0.15 mmol) HOBt, 56.3 mg (0.15 mmol) HBTU and 20.2 μL (0.15 mmol) $Et_3N$ in 2 mL dichloromethane was added to the resin. Removal of the Boc-protecting group was carried out according to GP2. According to PG4 (method A) the resin was treated with 126.2 mg (0.2 mmol) Fmoc-Cys(S-*t*-Bu)-OH, 30.6 mg (0.2 mmol) HOBt, 74.4 mg (0.2 mmol) HBTU and 34.5 μL (0.2 mmol) *i*-PrN$Et_2$ in 2 mL dichloromethane. The cleavage from the resin was carried out according to GP5 (method B). In the final cleavage step the resin was treated with 100 μL $H_2O$ in 900 μL THF for 3 h. The resin was filtered off and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (silica gel, dichloromethane/methanol 50:1, 20:1 (v/v)) yielding **20** as a white solid (8.9 mg, 0.021 mol, 25%).

TLC: 0.30 (dichloromethane/methanol 20:1 (v/v)); mp: 92–93 °C; $[\alpha]_D^{20}$ −54.9 (*c* 1.0, $CHCl_3$).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 7.91 (br s, 1H, NH), 7.73 (d, $^3J$ = 9.6 Hz, 2H, H-1, H-8 Fmoc), 7.69–7.63 (m, 3H, H-4, H-5 Fmoc, arom CH-6), 7.52 (m, 2H, H-2, H-7 Fmoc), 7.39–7.30 (m, 4H, H-3, H-6 Fmoc, 2× arom CH-4, CH-2), 4.49 (m, 1H, H-9 Fmoc), 4.39–4.29 (m, 4H, H-10 Fmoc, C$H_2$NH), 4.12 (m, 1H, α-CH Cys), 3.17 (t, $^3J$ = 8.0 Hz, 2H, C$H_2N_3$), 3.11–3.00 (m, 2H, β-$CH_2$ Cys), 2.26 (m, 2H, α-$CH_2$), 1.61 (m, 2H, β-$CH_2$), 1.51 (t, $^3J$ = 8.8 Hz, 2H, δ-$CH_2$), 1.33–1.28 (m, 2H, γ-$CH_2$), 1.24 (s, 9H, S-*t*-Bu).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 172.29 (CONH), 143.54, 141.08, 140.71, 128.16, 127.58, 126.96, 126.51, 126.01, 124.96, 119.78, 117.12, 110.78 (12× arom C), 66.34 (O*C*H$_2$ Fmoc), 51.07 (α-CH Cys), 48.09 (*C*H$_2$NH), 46.98 (*C*H$_2$N$_3$), 43.45 (β-$CH_2$ Cys), 42.64 (CH Fmoc), 36.71 (α-*C*H$_2$), 29.61 (S-*t*-Bu), 28.41(δ-*C*H$_2$), 26.15 (γ-*C*H$_2$), 24.82 (β-*C*H$_2$).

ESI-MS+ *m*/*z*: calcd for $C_{36}H_{43}N_6O_6S_2$ $[M+H]^+$: 719.3, found: 719.2, calcd for $C_{36}H_{42}N_6O_6S_2Na$ $[M+Na]^+$: 741.3, found: 741.3.

HR-FAB-MS (*m*/*z*): calcd: 719.2675 $[M+H]^+$, found: 719.2669.

### 6.17. 3-{Amino-[L-cysteine-(*S*-*tert*-butyl)-*N*-fluorenyl-methoxy-carbonyl]-methyl}-5-(6-azido-hexoyl-amino)-benzoic acid-(Cy5-ethyl) amide (21)

To a solution of 8.2 mg ($1.15 \times 10^{-5}$ mol) 3-{amino-[L-cysteine-(*S*-*tert*-butyl)-*N*-fluorenylmethoxycarbonyl]-methyl}-5-(6-azido-hexoyl-amino)-benzoic acid (**25**) in 1 mL DMF a solution of 8 mg ($1.15 \times 10^{-5}$ mol) Cy5-

ethylamine (**29**), 7.0 mg ($4.58 \times 10^{-5}$ mol) HOBt, 17.4 mg ($4.58 \times 10^{-5}$ mol) HBTU and 4 μL ($2.29 \times 10^{-5}$ mol) *i*-PrNEt$_2$ was added. The reaction mixture was stirred for 12 h at room temperature. the solvent was removed under reduced pressure and the crude product was purified by HPLC (linear gradient of water (solvent A) and $CH_3CN$ (solvent B); 20–100 B% 15 min) yielding **21** as a blue solid (7.2 mg, $5.18 \times 10^{-6}$ mol, 45%).

$[\alpha]_D^{20}$ +32.6 (*c* 1.0, $CHCl_3$).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.97 (s, 1H, arom CH-4), 8.29–8.27 (m, 2H, 2× arom. CH Cy5), 7.87 (d, $^3J$ = 6.8 Hz, 2H, H-1, H-8 Fmoc), 7.82 (d, $^3J$ = 10.4 Hz, 1H, NH), 7.75 (d, $^3J$ = 9.3 Hz, 2H, H-4, H-5 Fmoc), 7.65–7.60 (m, 4H, 4× arom. CH Cy5), 7.42 (s, 1H arom. CH-2), 7.34–7.20 (m, 5H, H-2, H-3, H-6, H-7 Fmoc, arom CH-6), 6.63 (t, $^3J$ = 15.4 Hz, 1H, C=C*H*Cy5), 6.27 (t, $^3J$ = 21.0 Hz, 2H, *H*C=C*H*Cy5), 4.46–4.43 (m, 3H, H-9, H-10 Fmoc), 4.22 (t, $^3J$ = 5.8 Hz, 1H, α-CH Cys), 4.13–4.10 (m, 2H, C$H_2$NH), 3.26–3.19 (m, 12H, β-$CH_2$ Cys, N$^+$=C–C*H*= Cy5, $C_{quart}$=C*H*Cy5, HN–C$H_2$–C$H_2$–NH, ε-$CH_2$Cy5, $CH_2N_3$), 2.31–2.27 (m, 2H, δ-$CH_2$ Cy5), 2.17 (t, $^3J$ = 19.5 Hz, 2H, α-$CH_2$Cy5), 1.70 (s, 12H, 4× $CH_3$Cy5), 1.67–1.59 (m, 6H, β-$CH_2$Cy5, 2× $CH_2$), 1.40–1.28 (m, 9H, NCH$_2$–C$H_3$Cy5, γ-$CH_2$Cy5, 2× $CH_2$), 1.32 (s, 9H, S-*t*-Bu).

ESI-MS+ *m*/*z*: calcd for $C_{71}H_{87}N_{10}O_{12}S_4$ [M]$^+$: 1399.5, found: 1399.5 [M]$^+$.

MALDI-TOF (DHB) calcd for $C_{71}H_{87}N_{10}O_{12}S_4$ [M]$^+$: 1399.5, found: 1399.3 [M]$^+$.

### 6.18. 3-{Amino-[L-cysteine-(*S*-monomethoxytrityl)-*N*-trityl]-methyl}-5-nitro-benzoic acid-(6-azido-hexyl) amide (22)

The loaded resin (**15**) (150 mg, 0. 147 mmol) was agitated in 2 mL dichloromethane. Removal of the Boc-protecting group was carried out according to GP2. According to GP4 (method A) the resin was treated with a solution of 283.5 mg (0.441 mmol) Trt-Cys(Mmt)-OH, 68.3 mg (0.441 mmol) HOBt, 69.1 μL (0.441 mmol) DIC and 66.0 μL (0.441 mmol) $Et_3N$ in 2 mL dichloromethane. Cleavage from the resin was carried out according to GP5 (method B). In the final cleavage step the resin was treated with a solution of 4 equiv 6-azido-hexylamine (0.588 mmol, 83.5 mg) in 3 mL THF for 3 h. The resin was filtered off and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (silica gel, cylohexane/ethyl acetate 6:4 (v/v)) yielding **22** as a white solid (79.5 mg, 0.085 mol, 58%).

TLC: 0.60 (cylohexane/ethyl acetate 1:1 (v/v)); mp: 115–116 °C; $[\alpha]_D^{20}$ +47.9 (*c* 1.0, $CHCl_3$).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.47 (s, 1H, arom CH-6), 8.15 (s, 1H, arom CH-4), 7.94 (s, 1H, arom CH-2), 7.35–7.10 (m, 27H, 27× arom CH Trt, Mmt), 6.45 (br s, 1H, NH), 4.14–4.11 (m, 2H, C$H_2$NH), 3.79–3.68 (m, 1H, α-CH Cys), 3.66 (s, 3H, OC$H_3$), 3.34–3.22 (m, 6H, C$H_2N_3$, β-$CH_2$ Cys, C$H_2$NH), 2.33–2.22 (m, 2H, $CH_2$), 1.55 (t, $^3J$ = 9.2 Hz, 2H, $CH_2$), 1.33–1.26 (m, 4H, 2× $CH_2$).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 174.85 (*C*ONH), 164.93 (*C*ONH), 158.24, 148.45, 146.78, 144.93, 145.81, 144.61, 141.25, 136.39, 136.36, 131.92, 130.84, 129.43, 129.40, 129.19, 128.80, 128.73, 128.65, 128.50, 128.38, 128.13, 128.03, 128.00, 127.98, 127.90, 127.84, 127.82, 127.71, 127.55, 127.41, 127.26, 127.16, 127.08, 126.94, 126.85, 126.82, 124.75, 120.83, 113.28, 113.14 (39× arom C), 71.47 (quart C Trt), 66.80 (quart C Mmt), 56.30 (O*C*H$_3$), 55.12 (α-CH Cys), 51.28 (*C*H$_2$NH), 42.35 (*C*H$_2$N$_3$), 40.14 (*C*H$_2$NH), 36.79 (β-CH$_2$ Cys), 28.66 (*C*H$_2$), 26.88 (*C*H$_2$), 26.41 (*C*H$_2$), 26.32 (*C*H$_2$).

ESI-MS+ *m*/*z*: calcd for $C_{56}H_{55}N_7O_5SNa$ [M+Na]$^+$: 960.4, found: 960.3.

MALDI-TOF (DHB) calcd for $C_{56}H_{55}N_7O_5S$ [M]: 937.40, found: 704.1 [M−Mmt+H+K]$^+$.

FAB/LR-MS (*m*/*z*): calcd: 937.40 [M+H]$^+$, found: 937.26.

### 6.19. 1-*N*-Fluorenylmethoxycarbonyl-2-*N*-(*tert*-butyloxycarbonyl)-ethane-1,2-diamine (23)

To a solution of *N*-(*tert*-butyloxycarbonyl)-ethane-1,2-diamine (100 mg, 0.625 mmol) in 5 mL dichloromethane/methanol (1:1) 316.2 mg (0.938 mmol) Fmoc-OSu and 173.2 μL (1.25 mmol) $Et_3N$ were added at 0 °C. The reaction mixture was stirred for 12 h at room temperature and the organic phase was washed with a solution of 1 N $NaHSO_4$ and $NaHCO_3$. The combined organic phases were dried over $MgSO_4$. The solvent was removed under reduced pressure yielding pure **23** (186 mg, 0.487 mmol, 78%) as a white solid.

TLC: 0.50 (cyclohexane/ethyl acetate 1:1 (v/v)); mp: 158–159 °C.

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 7.76 (d, $^3$J = 9.5 Hz, 2H, H-1, H-8 Fmoc), 7.58 (d, $^3J$ = 9.0 Hz, 2H, H-4, H-5 Fmoc), 7.40 (t, $^3J$ = 9.5 Hz, 2H, H-2, H-7 Fmoc), 7.31 (t, $^3J$ = 9.0 Hz, 2H, H-3, H-6 Fmoc), 4.40 (d, $^3J$ = 8.0 Hz, 2H, OC$H_2$ Fmoc), 4.21 (t, $^3J$ = 8.0 Hz, 1H, C*H* Fmoc), 3.27 (m, 4H, 2× NHC$H_2$), 1.45 (s, 9H, C(C$H_3$)$_3$).

$^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 156.71 ($CO_2$NH), 143.90, 141.31, 127.76, 127.02, 119.94 (4× *C*H Fmoc), 66.68 (O*C*H$_2$ Fmoc), 47.26 (*C*H Fmoc), 41.60 (N*C*H$_2$), 40.56 (N*C*H$_2$), 28.35 (C(*C*H$_3$)$_2$).

ESI-MS+ *m*/*z*: calcd for $C_{22}H_{26}N_2O_4Na$ [M+Na]$^+$: 405.2, found: 405.2.

MALDI-TOF (DHB) calcd for $C_{22}H_{26}N_2O_4Na$ [M+Na]$^+$: 405.2, found: 405.5.

HR-FAB-MS (*m*/*z*): calcd: 383.1893 $[M+H]^+$, found: 383.1998.

### 6.20. 1-*N*-Fluorenylmethoxycarbonyl-2-*N*-(Cy5)-ethane-1,2-diamine (24)

For removal of the Boc-group 43.5 mg (0.114 mmol) 1-*N*-fluorenylmethoxycarbonyl-2-*N*-(*tert*-butyloxycarbonyl)-ethane-1,2-diamine (**23**) was dissolved in 1 mL of a solution of 50% TFA in dichloromethane. The reaction mixture was stirred for 10 min at room temperature and the solvent was coevaporated with toluene (10 mL). The crude product was used without further purification. To a solution of 50 mg ($7.615 \times 10^{-5}$ mol) Cy5 in 2 mL DMF 8 equiv HOBt (93.2 mg, 0.5 mmol), 8 equiv DIC (94.4 μL, 0.610 mmol) and 4 equiv $Et_3N$ (42.0 μL, 0.304 mmol) were added. The reaction mixture was stirred for 20 min at room temperature. The crude 1-*N*-fluorenylmethoxycarbonyl-ethane-1,2-diamine (32 mg, 0.114 mmol) was added and the reaction mixture was stirred for 48 h. The solvent was evaporated under reduced pressure and the crude product purified by HPLC (linear gradient of water (solvent A) and $CH_3CN$ (solvent B); 20–100 B% 15 min) yielding **24** as a blue solid (20 mg, 0.217 mol, 29%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.30–8.23 (m, 2H, 2× arom. CH), 7.97 (m, 4H, 4× arom. CH), 7.74 (d, $^3J$ = 9.1 Hz, 2H, H-1, H-8 Fmoc), 7.60 (d, $^3J$ = 9.3 Hz, 2H, H-4, H-5 Fmoc), 7.36–7.26 (m, 4H, H-2, 3, 6, 9 Fmoc), 6.63 (m, 1H, C=C*H*), 6.28 (t, $^3J$ = 17.0 Hz, 2H, *H*C=C*H*), 4.30 (d, $^3J$ = 8.5 Hz, 2H, $N^+$=C–C*H*=, $C_{quart}$=C*H*), 4.19–4.12 (m, 2H, H-10 Fmoc), 4.02 (t, $^3J$ = 6.8 Hz, 1H, H-9 Fmoc), 3.28–3.20 (m, 6H, HN–C$H_2$–C$H_2$–NH, ε-$CH_2$), 2.18 (t, $^3J$ = 9.0 Hz, 2H, α-$CH_2$), 1.73 (m, 2H, δ-$CH_2$), 1.71 (s, 12H, 4× $CH_3$), 1.65 (t, $^3J$ = 9.5 Hz, 2H, δ-$CH_2$), 1.42–1.39 (m, 2H, γ-$CH_2$), 1.35 (t, $^3J$ = 8.8 Hz, 2H, $NCH_2$–C$H_3$).

ESI-MS+ *m*/*z*: calcd for $C_{50}H_{57}N_4O_9S_2$ $[M]^+$: 921.36, found: 921.7.

MALDI-TOF (DHB) calcd for $C_{50}H_{57}N_4O_9S_2$ $[M]^+$: 921.36, found: 921.7; calcd for $C_{50}H_{57}N_4O_9S_2Na$ $[M+Na]^+$: 943.4, found: 943.7.

### 6.21. Cy5-ethylamine (25)

A solution of 12 mg ($1.346 \times 10^{-5}$ mol) 1-*N*-fluorenylmethoxycarbonyl-2-*N*-(Cy5)-ethane-1,2-diamine (**24**) in 700 μL DMF was cooled to 0 °C with an ice-bath. To the solution 300 μL piperidine was added at 0 °C and the reaction mixture was allowed to warm up to room temperature. The reaction mixture was stirred for 1 h and the solvent was evaporated under reduced pressure. The crude product was purified by HPLC yielding (linear gradient of water (solvent A) and $CH_3CN$ (solvent B); 20–100 B% 15 min) **25** as a blue solid (8 mg, $1.144 \times 10^{-5}$ mol, 85%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.29–8.27 (m, 2H, 2× arom. CH), 7.89–7.85 (m, 4H, 4× arom. CH), 7.33 (t, $^3J$ = 12.5 Hz, 1H, NH), 6.69 (t, $^3J$ = 15.1 Hz, 1H, C=C*H*), 6.34 (t, $^3J$ = 18.8 Hz, 2H, *H*C=C*H*), 4.58 (br s, 1H, NH), 4.18–4.14 (m, 2H, $N^+$=C–C*H*=, $C_{quart}$=C*H*), 3.35 (m, 4H, HN–C$H_2$–C$H_2$–NH), 2.90 (m, 2H, ε-$CH_2$), 2.21 (t, $^3J$ = 8.5 Hz, 2H, α-$CH_2$), 1.83 (m, 2H, δ-$CH_2$), 1.73 (s, 12H, 4× $CH_3$), 1.65 (t, $^3J$ = 9.0 Hz, 2H, β-$CH_2$), 1.39–1.36 (m, 5H, $NCH_2$–C$H_3$, γ-$CH_2$).

ESI-MS+ *m*/*z*: calcd for $C_{35}H_{47}N_4O_7S_2$ $[M]^+$: 699.3, found: 699.7.

MALDI-TOF (DHB) calcd for $C_{35}H_{47}N_4O_7S_2$ $[M]^+$: 699.3, found: 699.7; calcd for $C_{35}H_{47}N_4O_7S_2Na$ $[M+Na]^+$: 721.3, found: 721.6.

FAB/LR-MS (*m*/*z*): calcd: 700.29 $[M+H]^+$, found: 700.07.

### 6.22. Protein expressions and ligation reactions

**6.22.1. Cloning, expression and purification of $^{1–203}$Ypt1Δ3-MESNA.** Ypt1 truncated by three-amino-acid residues was C-terminally fused to an intein/chitin-binding domain assembly as implemented in the pTWIN-1 vector (New England Biolabs). Protein expression in *Escherichia coli* and purification of thioester-tagged protein were performed as described earlier.[3c] The resulting Ypt1Δ3-MESNA thioester protein was desalted on a PD-10 column (Amersham) equilibrated with 10 mM Na-phosphate, pH 7.5, 0.1 mM $MgCl_2$, 2 mM GDP and concentrated.

**6.22.1.1. Ligation of compound 12 to $^{1–203}$Ypt1Δ3-MESNA.** In the ligation reaction 10 μL $^{1–203}$Ypt1-MESNA (12 mg/mL) was mixed with 5 equiv of 30 mM compound **12** (dissolved in $H_2O$/acetonitrile 1:4) in 50 μL of 50 mM $Na_2HPO_4/NaH_2PO_4$, pH 7.5, 0.1 mM $MgCl_2$, 2 μM GDP and 10 μL of 150 mM 2-mercaptoethanesulfonic acid (MESNA) and allowed to react overnight at room temperature with vigorous agitation. The reaction mixture was centrifuged (1 min at 13,000 rpm) to remove protein and peptide precipitate. The pellet was washed with methanol, methylene chloride (4 times), methanol (4 times) and distilled water (4 times). Semisynthetic protein obtained was quantitatively determined by HPLC–MS (calculated mass: 23454 $[M+H]^+$, found: 23455).

**6.22.2. Cloning, expression and purification of $^{1–181}$N-Ras.** Truncation of the full-length N-Ras cDNA (Accession No. X02751) was achieved using high-fidelity *Pfu* DNA polymerase (Stratagene). For the truncated version of N-Ras two stop codons were introduced at positions 182 and 183 of the N-Ras cDNA. The resulting fragment N-RasΔ181 was purified and digested with EcoRI and SmaI, and was subcloned into the ptac expression vector. The plasmid was sequenced to prove PCR fidelity and correct mutagenesis, and transformed into the *E. coli* strain CK600K (Stratagene).

*Escherichia coli* carrying the expression plasmid for N-RasΔ181 was cultivated in LB-medium containing 100 μg/mL ampicillin and 25 μg/mL canamycin at 37 °C. Expression was induced at a cell density absor-

bance of $A_{600}$ = 0.6 with 500 μM of isopropyl β-D-thiogalactoside and incubation was continued at 30 °C overnight. Pellets were resuspended in 20 mM Tris (pH 7.4), 5 mM $MgCl_2$, and 2 mM DTE (buffer A). Phenylmethylsulfonyl fluoride (PMSF) to 0.1 mM and 0.5 mg DNase I were added and the cells were lysed using a microfluidizer (Microfluidics). Following centrifugation, the clear supernatant was applied to a DEAE–Sepharose column equilibrated with buffer A. After washing with 2 column volumes, the protein was eluted over 5 column volumes using a linear gradient of 0–1 M NaCl in buffer A. Fractions containing N-Ras were determined by SDS–PAGE, pooled and concentrated by precipitation with 3 M ammonium sulfate (final concentration) for 30 min. After centrifugation, the precipitate was resuspended in gel filtration buffer (buffer A containing 200 mM NaCl, 10 μM GDP) and applied to a gel filtration column (HiLoad Superdex 200, Amersham Pharmacia Biotech). Fractions containing N-Ras were pooled and again concentrated by ammonium sulfate precipitation. All purification steps were carried out at 4 °C. Purified protein was stored at −80 °C.

**6.22.2.1. Conjugation of MIC-labeled compound 17 to $^{1–181}$N-Ras.** In the conjugation reaction 10 μL $^{1–181}$N-Ras (50 mg/mL) was mixed with 1.2 equiv of 40 mM compound **17** (dissolved in $H_2O$/acetonitrile 1:4) in 50 μL of 50 mM Tris-buffer, pH 7.4, 0.1 mM NaCl and 2 μM GDP and allowed to react overnight at room temperature with vigorous agitation. The reaction mixture was centrifuged (1 min 13,000 at rpm) to remove protein and peptide precipitate.

The ligation mixture was purified afterwards on a DEAE-gel filtration column (FPLC-system), eluted with a linear gradient of 20 mM Tris/HCl, pH 7.4, and 5 mM $MgCl_2$. The solution of purified, semi-synthetic protein was concentrated to 40 g/L (yield: 98%, calculated mass: 23,454 $[M+H]^+$, found: 23,455 by MALDI-TOF).

**6.22.2.2. Conjugation of MIC-labeled compound 19 to $^{1–181}$N-Ras.** In the conjugation reaction 10 μL $^{1–181}$N-Ras (40 mg/mL) was mixed with 1.2 equiv of 30 mM compound **19** (dissolved in $H_2O$) in 50 μL of 50 mM Tris-buffer, pH 7.4, 0.1 mM NaCl and 2 μM GDP and allowed to react overnight at room temperature with vigorous agitation. The reaction mixture was centrifuged (1 min at 13,000 rpm) to remove protein and peptide precipitate.

The ligation mixture was purified afterwards on a DEAE-gelfiltration column (FPLC-system), eluted with a linear gradient of 20 mM Tris/HCl, pH 7.4 and 5 mM $MgCl_2$. The solution of purified, semi-synthetic protein was concentrated to 30 g/L (yield: 98%, calculated mass: 21411 $[M+H]^+$, found: 21414 by HPLC–MS).

**6.22.3. Cloning and expression of $^{1–180}$H-Ras and generation of the corresponding MESNA-thioester.** To produce the protein core for native chemical ligation, the DNA sequence coding for truncated H-Ras (amino residues 1–180) was cloned into the IMPACT (Intein-Mediated Purification with an Affinity Chitin-binding Tag) vector pTYB2 (New England Biolabs). E. coli Bl21(DE3) were transformed with the Ras-intein plasmid and expression of the protein was performed at 20 °C due to strong aggregation of the fusion protein at higher temperatures. After harvesting of the bacteria, the cells were lysed by ultrasonification and the soluble fraction was applied to a chitin-modified affinity column. The Ras-MESNA thioester was formed on column by application of 200 mM 2-mercaptoethanesulfonic acid for 30 min at 4 °C and concentrated by size-exclusion filtration. After aliquotation, the protein was shock-frozen and stored at −80 °C.

The synthesized building block **22** was treated with 40% TFA/$CH_2Cl_2$. After complete deprotection (followed by TLC), the solvent was removed under reduced pressure and deprotected compound **22** was ligated to $^{1–180}$N-Ras-MESNA.

In the ligation reaction 10 μL N-Ras-thioester (77.2 mg/mL) was mixed with 5 equiv of 40 mM deprotected compound **22** (dissolved in $H_2O$/acetonitrile 1:5) in 50 μL of 50 mM $Na_2HPO_4/NaH_2PO_4$, pH 7.5, 0.1 mM $MgCl_2$, 2 μM GDP and 10 μL of 150 mM 2-mercaptoethanesulfonic acid and allowed to react overnight at room temperature with vigorous agitation. The reaction mixture was centrifuged (1 min at 13,000 rpm) to remove protein and peptide precipitate.

The ligation mixture was purified on a DEAE-gel filtration column (FPLC-system), eluted with a linear gradient of 20 mM Tris/HCl, pH 7.4 and 5 mM $MgCl_2$. The solution of purified, semi-synthetic protein was concentrated to 13 g/L (yield: 95%, calculated mass: 20747 $[M+H]^+$, found: 20747 by HPLC–MS).

### 6.23. Preparation of the phosphine-functionalized slides

Dry *N*,*N*-dimethylformamide (60 mL) (DMF), *N*,*N*-diisopropylethyl amine (4.8 mM) (DIPEA), 1-hydroxybenzotriazole (4.8 mM) (HOBt) and *N*,*N*′-diisopropylcarbodiimide (4.8 mM) (DIC) were added to *N*-Fmoc-protected aminocaproic acid (4.8 mM) and stirred for 10 min under argon to activate the acid. Then, the solution was transferred into a Schlenk flask containing four $NH_2$-terminated polyamidoamine-dendrimer-coated glass slides (Chimera Biotec, Dortmund) mounted on a Teflon rack. The mixture was stirred overnight under argon, the supernatant solution was removed and the slides were rinsed twice with DMF, then stirred twice for 20 min in DMF for washing and rinsed again with DMF. Afterwards, they were treated twice for 20 min with 60 mL of a 20% piperidine solution in DMF for deprotection. The washing procedure was repeated adding a 15 min stirring step in dichloromethane (DCM) as well as rinsing with DCM. During the whole procedure the slides were kept in the Schlenk flask under argon. The slides were then dried in vacuo. In the following step, the phosphane reagent carrying the free acid function (5.2 mM), dry DMF (65 mL), HOBt (5.2 mM), DIPEA (10.4 mM) and DIC (5.2 mM) were added subsequently under argon to the slides. The mixture was stirred overnight and the supernatant solution was again

removed followed by a washing procedure consisting of rinsing twice with dry DMF, stirring in dry DMF/DCM (1:1) twice for 20 min and rinsing again twice with dry DMF. While stirring, the solution was degassed three times. Afterwards, the slides were treated twice for 10 min with a solution of acetic anhydride (4.8 mM) and pyridine (43.2 mM) in dry DMF (60 mL). After removing the supernatant, the slides were rinsed with dry DMF and dry DCM, stirred twice for 20 min in dry DCM, rinsed again with dry DCM, dried in vacuo and stored under argon until usage. Again, the solution was degassed three times while stirring as mentioned above.

### 6.24. Spotting process

The spotting process of 10–200 μM protein solution in different buffers (50 mM $Na_2HPO_4$, 2 mM NaCl and 2 mM $MgCl_2$, pH 7.5) was carried out using a GeSiM Nanoplotter (Gesim, Dresden, Germany) by spotting 0.25 nL droplets of protein solutions (spot size 400 μm diameter) followed by incubation overnight.

The slides were incubated with bovine serum albumin (BSA) blocking-buffer (Chimera Biotec, Dortmund) for 30 min and subsequently washed twice with phosphate buffer (50 mM $Na_2HPO_4$, 2 mM NaCl, 2 mM $MgCl_2$ and 0.05 vol % Tween 20) for 30 min. The slides were incubated with 50 nM Ras-antibody (50 μM TETBS, Tween, EDTA and Tris-buffer, pH 7.4) for 45 min and afterwards washed with phosphate buffer (50 mM $Na_2HPO_4$, 2 mM NaCl and 2 mM $MgCl_2$) for 30 min.

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