

Diels–Alder Ligation of Peptides and Proteins

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Abstract: The development of the Diels–Alder cycloaddition as a new method for the site-specific chemoselective ligation of peptides and proteins under mild conditions is reported. Peptides equipped with a 2,4-hexadienyl ester and an N-terminal maleimide react in aqueous media to give cycloadducts in high yields and depending on the amino acid sequence with high stereoselectivity. Except for the cysteine SH group the transformation is compatible with all amino acid side chain functional groups. For ligation to proteins the hexadienyl group was attached to avidin and streptavidin noncova-

lently by means of complex formation with a biotinylated peptide or by covalent attachment of a hexadienyl ester-containing label to lysine side chains incorporated into the proteins. Site-specific attachment of the hexadienyl unit into a Rab protein was achieved by means of expressed protein ligation followed by protection of the generated cysteine SH by means of Ellman's

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reagent. The protein reacted with different maleimido-modified peptides under mild conditions to give the fully functional cycloadducts in high yield. The results demonstrate that the Diels–Alder ligation offers an advantageous and technically straightforward new opportunity for the site-specific equipment of peptides and proteins with further functional groups and labels. It proceeds under very mild conditions and is compatible with most functional groups found in proteins. Its combination with other ligation methods, in particular expressed protein ligation is feasible.

Introduction

The site-specific equipment of proteins and other biomolecules with additional functional groups for subsequent biochemical and biological investigations, for example, fluorophores, spin probes, photoactivatable groups and affinity

tags is among the most frequently employed and important methods of research in the life sciences.^[1] Recently, in particular, aldehyde assisted ligations,^[2] native chemical ligation,^[3] expressed protein ligation,^[4] expressed enzymatic ligation,^[5] Staudinger ligation^[6] and the application of the Huisgen azide cycloaddition^[7] have been developed as powerful new methods advancing the field significantly. However, due to the multifunctionality of biomacromolecules in general and proteins in particular and the manifold applications of such techniques there is a major and continuing demand for the development of new technology providing alternatives to the methods mentioned above. The chemistry required must be compatible with the functional groups found in proteins and proceed chemoselectively under mild conditions and in aqueous solution, preferably in the absence of any potentially denaturing cosolvent. The Diels–Alder reaction is a highly selective transformation and can proceed in water with a higher velocity and selectivity than in organic solvents.^[8] Its compatibility with biomolecules has been explored elegantly in the bioconjugation and/or immobilization of oligonucleotides and other biomolecules^[9,10] as well as microarray development.^[11,12] Here we report the development of the Diels–Alder cycloaddition as a method for

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the chemoselective ligation of peptides and proteins under mild conditions.^[13]

Results and Discussion

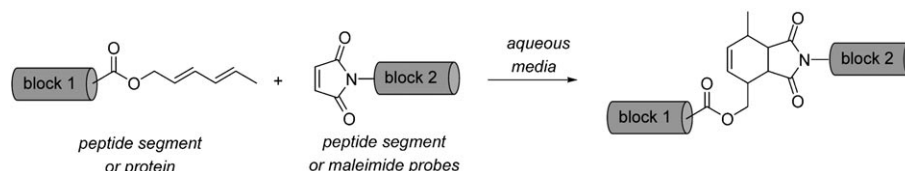
The scope of the ligation approach was initially investigated using model peptides equipped with a *trans,trans*-2,4-hexadienyl group at the C-terminus or with a maleimido dienophile at the N-terminus (Scheme 1). The ligation method was further employed for the covalent modification of protein dienyl derivatives with maleimido-labeled probes.

Diels–Alder ligation of peptides

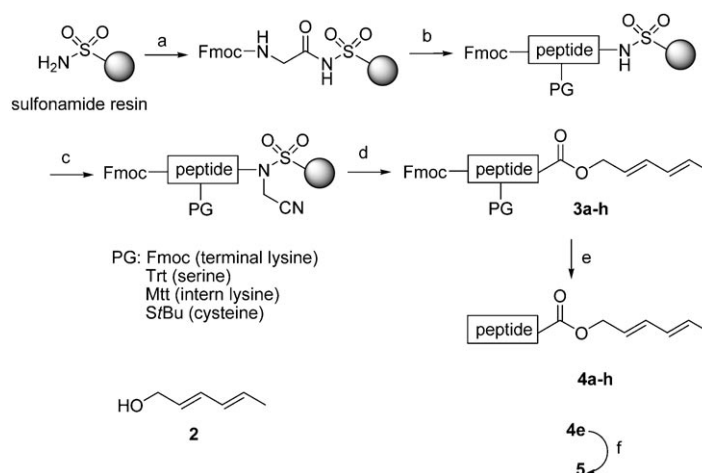
Synthesis of peptide dienyl esters: Due to the acid-sensitivity of the diene, for the synthesis of the peptide hexadienyl esters in solution or on the solid phase, base- or mildly acid-labile protecting groups were applied.

Initially a tripeptide hexadienyl ester, H-Val-Ala-Gly-OH **1**, was synthesized in solution by preparing a 9-fluorenylmethoxycarbonyl (Fmoc) tripeptide *tert*-butyl ester, cleavage of the *tert*-butyl ester and re-esterification with *trans,trans*-2,4-hexadienol (**2**; see Scheme S1 in the Supporting Information). For longer peptides the re-esterification proceeded only with unsatisfactory yields, and, therefore, these peptides were synthesized by means of solid-phase synthesis using the sulfamylbutyryl linker resin developed by Backes and Ellman^[14] and Fmoc, trityl (Trt), 4-methyltrityl (Mtt) or *tert*-butylthiol (S_tBu) groups for protecting N-terminal and reactive side-chain groups of the amino acids (Scheme 2). Due to the poor nucleophilicity of the sulfonamide function, attachment of the first amino acid may result in low loading and possible racemization.^[15] For this reason, glycine was selected as the C-terminal amino acid for all diene–peptide sequences. Still quantitative loading of Fmoc-glycine was only achieved using a large excess of amino acid and coupling reagents over extended time (Scheme 2).

After peptide chain elongation on resin employing a 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/*N*-hydroxybenzotriazole (HOBt)/*N,N*-diisopropylethylamine (DIPEA) coupling protocol, the sulfonamide linker was activated by alkylation with iodoacetonitrile/DIPEA. Treatment of the resin with hexadienol **2**/4-(dimethylamino)pyridine (DMAP) resulted in simultaneous release and esterification of the protected peptides **3** (Scheme 2). The crude products were purified by reversed-phase HPLC to remove excess hexadienol and DMAP (Figure S1,



Scheme 1. Schematic representation of the Diels–Alder ligation of peptides and proteins.



Scheme 2. Solid-phase synthesis of peptide hexadienyl esters. a) Fmoc-Gly-OH (8 equiv), DIC (8 equiv), *N*-methylimidazole (8 equiv), DCM/DMF 4:3, 2 × 18 h, quantitative loading; b) SPPS: i) 20% piperidine in DMF, 10 min (2 ×), ii) Fmoc-AA-OH (4 equiv), HBTU (4 equiv), HOBt (4 equiv), DIPEA (8 equiv), DMF; c) ICH₂CN (25 equiv), DIPEA (10 equiv), NMP, 24 h; d) i) hexadienol **2** (20 equiv), DMAP (0.5 equiv), THF, 24 h; ii) (only for **3b**) 1% TFA/5% TIS in DCM, 2 h; e) 20% piperidine in DCM or DMF, 40 min; f) DTT, 0.1 M NH₄CO₃/DMF 5:3, 2.5 h. DCM = dichloromethane, DMF = dimethylformamide, SPPS = solid-phase peptide synthesis, NMP = *N*-methylpyrrolidinone, THF = tetrahydrofuran.

Supporting Information). When necessary, the trityl protecting group was removed by treatment of the cleaved peptide with 1% trifluoroacetic acid (TFA)/5% triisopropylsilane (TIS) in dichloromethane for 2 h at room temperature. Peptide hexadienyl esters **3a–h** were isolated in 43–16% overall yield after HPLC purification (Table 1). The Fmoc protecting group was removed with 20% piperidine in dichloromethane (DCM) or DMF to give peptides **4a–h** after HPLC

Table 1. Results for the synthesis of peptide hexadienyl esters **3** and **4**.

After cleavage from resin			After removal of protecting groups		
Diene	Peptide sequence	Yield [%] ^[a]	Diene	Peptide sequence	Yield [%] ^[a]
3a	Fmoc-K ^{Fmoc} PFLG	43	4a	KPFLG	72
3b	Fmoc-PC ^{S<sub>t</sub>Bu} SMG	20	4b	PC ^{S<sub>t</sub>Bu} SMG	51
3c	Fmoc-K ^{Fmoc} LGFAG	33	4c	KLGFAG	83
3d	Fmoc-K ^{Fmoc} LGK ^{Mtt} AG	32	4d	KLGK ^{Mtt} AG	73
3e	Fmoc-K ^{Fmoc} C ^{S<sub>t</sub>Bu} GVFG	22	4e	KC ^{S<sub>t</sub>Bu} GVFG	80
3f	Fmoc-K ^{Fmoc} FPIGLFG	16 ^[b]	4f	KFPIGLFG	91
3g	Fmoc-K ^{Fmoc} FPIGLGFG		4g	KFPIGLGFG	92
3h	Fmoc-C ^{S<sub>t</sub>Bu} GPAG	37	4h	C ^{S<sub>t</sub>Bu} GPAG	35
			5	KCGVFG	72

[a] Yields are reported after HPLC purification. [b] During assembly of **3g** one glycine residue coupling was not complete resulting in the formation of two products **3f** and **3g** in 12 and 4% yield, respectively.

purification and lyophilization. Deprotection of the *S*tBu group was accomplished by reduction of the disulfide bond of peptide **4e** with dithiothreitol (DTT) in ammonium bicarbonate medium for 2 h, affording deprotected diene **5**. Surprisingly, in the case of compound **4d**, the Mtt group, which is known to be a very acid sensitive protecting group for amines,^[16] could not be removed by treatment with 1% TFA/5% triethylsilane (or TIS) in DCM. If the concentration of TFA was increased to 5% partial removal of the Mtt group was observed, however, also considerable decomposition of the diene occurred. Therefore peptide **4d** was subsequently employed in Mtt-protected form.

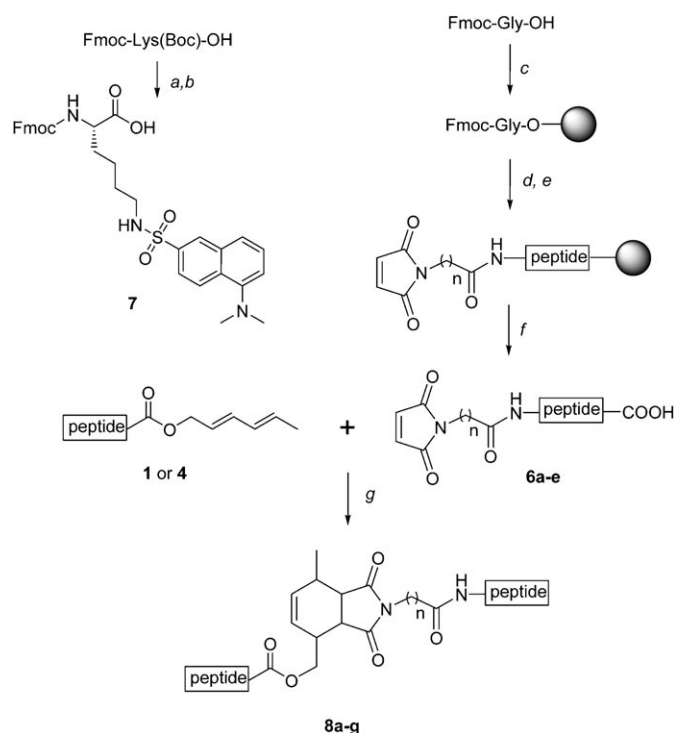
Synthesis of peptide derived dienophiles: The synthesis of the N-terminal maleimide functionalized peptides **6** was achieved by means of the Fmoc/*t*Bu solid-phase strategy on a Wang resin and the HBTU/HOBt/DIPEA coupling protocol (Scheme 3). In order to investigate a possible influence of linker length on the course of the ligation reaction N-maleimido-glycine^[17] or N-maleimido- β -alanine were utilized for the incorporation of the maleimide group in the last step of the peptide synthesis. Since the maleimide moiety is stable under acidic conditions,^[17] side chain deprotection and cleavage were achieved by treatment of the resin with TFA and scavengers, affording N-maleimido peptides **6a–e** in high purity after lyophilization (Table 2). Fmoc-Lys(dansyl)-OH (**7**) was applied as building block for the synthesis of fluorescently labeled peptide **6e**.

Table 2. Results for the synthesis of N-maleimido-peptides **6**.

Maleimide	Linker (<i>n</i>)	Peptide sequence	Overall yield [%]
6a	1	YTG	62
6b	1	TQFHG	60
6c	1	SEWIG	53
6d	1	AKTSAESYSG	59
6e	2	SKTK(dansyl)G	57

Peptide ligation by means of Diels–Alder reaction: The Diels–Alder ligation of the peptide hexadienyl esters **1** or **4** and maleimides **6** to give cycloadducts **8** (Scheme 3) was performed in aqueous solution at room temperature (Table 3). The diene and dienophile were mixed in equal amounts in most cases and usually at 10 mM concentration and allowed to react for 24 h. If required, methanol or DMF was added for peptide solubilization in the aqueous solution.

A typical time course diagram for the Diels–Alder ligation of diene and dienophile modified peptides is given in Figure 1. After overnight reaction the ligation products were



Scheme 3. Synthesis of the N-terminal maleimido-peptides **6** and Diels–Alder ligation of the dienyl and maleoyl peptides. *a*) TFA/DCM 1:1, 80 min; *b*) dansyl-Cl, NaHCO₃, MeOH/H₂O 5:2, overnight, 52%; *c*) Wang resin, DIC, DMAP, DMF, overnight, quantitative loading; *d*) SPPS: i) 20% piperidine in DMF, 10 min (2×); ii) Fmoc-AA-OH (4 equiv), HBTU (4 equiv), HOBt (4 equiv), DIPEA (8 equiv), DMF; *e*) N-maleoyl-glycine (*n*=1) or N-maleoyl- β -alanine (*n*=2), DIC, HOBt, DCM/DMF 1:1; *f*) TFA/TIS/H₂O 95:2.5:2.5, 2–3 h. *g*) aqueous media, room temperature. Dansyl = 5-dimethylaminonaphthalene-1-sulfonyl.

formed in 70% to quantitative yield as revealed by HPLC analysis. In some cases, consumption of the starting materials was complete only after longer reaction times (Table 3, entries 4, 6, 7). The use of DMF as a co-solvent seems to slow down the rate of cycloaddition (Table 3, entry 3 vs 4). Application of an excess of dienophile over diene considerably shortened the coupling time and led to complete conversion of the hexadienyl peptide to the new cycloadduct (Table 3, entries 1 and 5).

Table 3. Results for the Diels–Alder ligation of hexadiene- and maleimido-peptides.

Entry	Cycloadduct	Diene peptide	Maleimido peptide	Solvent	<i>t</i> [h]	Conversion [%] ^[a]	Isolated after HPLC [%]
1	8a	1	6a ^[b]	H ₂ O/MeOH 10:3	20	quant.	87
2	8b	4a	6a	H ₂ O/MeOH 4:1	24	93	60
3	8c	4d	6b	H ₂ O/MeOH 20:1	24	95	74
4	8d	4d	6c	H ₂ O/DMF 4:1	47	84	64
5	8e	4b	6a ^[c]	H ₂ O/MeOH 3:2	24	quant.	32
6	8f	4f	6d	H ₂ O	48	93	69
7	8g	4g	6d	H ₂ O	48	92	67

[a] Based on the consumption of diene–peptide determined by analytical HPLC. [b] Dienophile was added in excess (1.2 equiv). [c] Dienophile was added in excess (2.4 equiv).

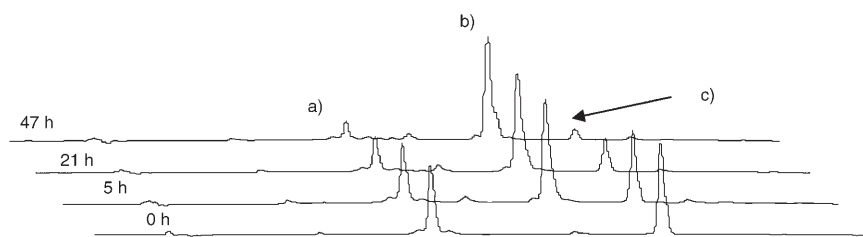


Figure 1. Time course for the ligation of peptides **4f** (c) and **6d** (a) to give cycloadduct **8f** (b) followed by HPLC analysis (entry 6, Table 3).

All ligation products were isolated by HPLC purification and identified by mass spectroscopy and NMR (see Section S3 in the Supporting Information). These results revealed that the Diels–Alder ligation is chemoselective and compatible with reactive amino acids such as Lys, His and Trp. Also, no nucleophilic addition of the N-terminal amino group to the α,β -unsaturated double bond of the dienophile was observed under these conditions. However, we recognized the potentially troublesome Michael addition reaction that can take place between the side chain of cysteine residues and the maleimide function.^[1] Indeed, when hexadiene **5**, which possesses a free cysteine residue, was treated with maleimide **6b**, the formation of a doubly labeled product resulting from both nucleophilic addition and cycloaddition reaction was observed (68 % conversion, MALDI-TOF: m/z : calcd for: 2142, found: 2143). To avoid this side reaction, protection of the cysteine side chain during Diels–Alder ligation is required, as illustrated for the reaction of peptides **4b** and **6a** (Table 3, entry 5), where the sulfhydryl moiety is masked as a disulfide by the *S*tBu protecting group.

In additional experiments, we investigated the Diels–Alder peptide ligation using different diene and dienophile building blocks. However, peptides incorporating a cyclopentadiene moiety rapidly dimerized in aqueous media^[18] and peptides having a furan group at the C-terminus or an acrylamide group at the N-terminus, were not reactive enough.

Stereoselectivity of the Diels–Alder ligation: The Diels–Alder ligation investigated in this study potentially results in the formation of four diastereomers. Only if *endo/exo*- and face-selectivity are complete will one stereoisomer be formed. In order to investigate this possibility the stereose-

lectivity was scrutinized employing model compounds amenable to spectroscopic analysis. The *endo/exo* selectivity of the cycloaddition reaction involving the peptidyl hexadiene and -maleimide was first investigated by using simple achiral cycloadducts as model molecules (see Section S4, Supporting Information). NOE investigations revealed that only the *endo* isomers were formed (Scheme S2, Supporting Information).

The Diels–Alder ligation process to give peptides **8a** and **8b** led to the formation of the expected *endo* products (Figure 2) as confirmed by comparison of their NMR values

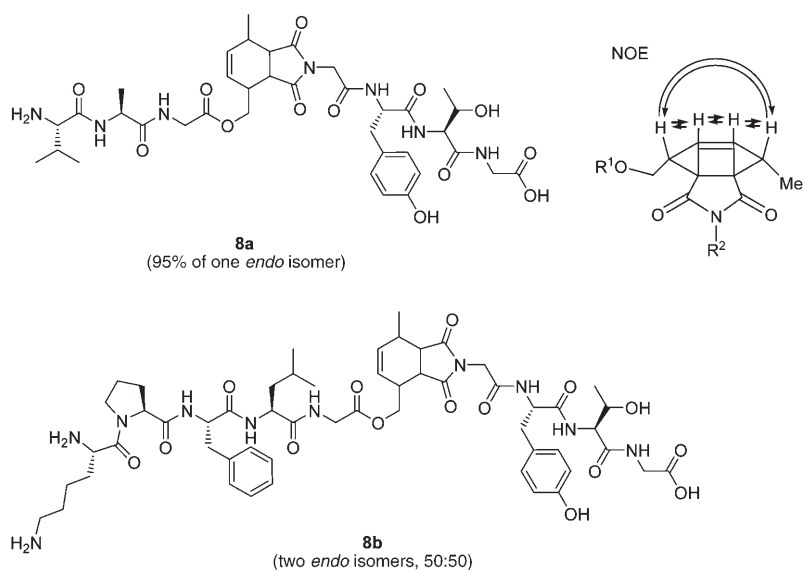


Figure 2. Structures of cycloadducts **8a** and **8b**.

with the data found for the model cycloadducts (Table S1, Supporting Information). Surprisingly, cycloadduct **8a** was obtained as a single *endo* isomer (95 %) whereas the cycloadduct **8b** was obtained as a mixture of the two *endo* isomers (50:50). These results suggest that a favourable hydrogen-bond pattern between the two reacting peptide chains may fix the diene and dienophile groups in a specific position that favors a face-selective *endo* attack. Spectroscopic and chromatographic analysis of the other ligation products **8c–g** did not permit a conclusive determination of the stereoselectivity of the cycloaddition processes.

Diels–Alder ligation of proteins

In order to generate tailor-made proteins we sought to combine the Diels–Alder ligation method with other conjugation techniques. In such a combined strategy the protein of interest is initially functionalized with a diene unit and then the

resulting protein-derived diene can be further functionalized by Diels–Alder reactions with different dienophiles under very mild conditions. Initially the Diels–Alder ligation of proteins was investigated employing a dienyl peptide equipped with a biotin unit that was employed for non-covalent attachment to streptavidin and avidin. These experiments revealed that the cycloaddition proceeded smoothly giving rise to the desired protein cycloadducts (Section S5, Supporting Information).

Conjugation of proteins by means of Diels–Alder cycloaddition:

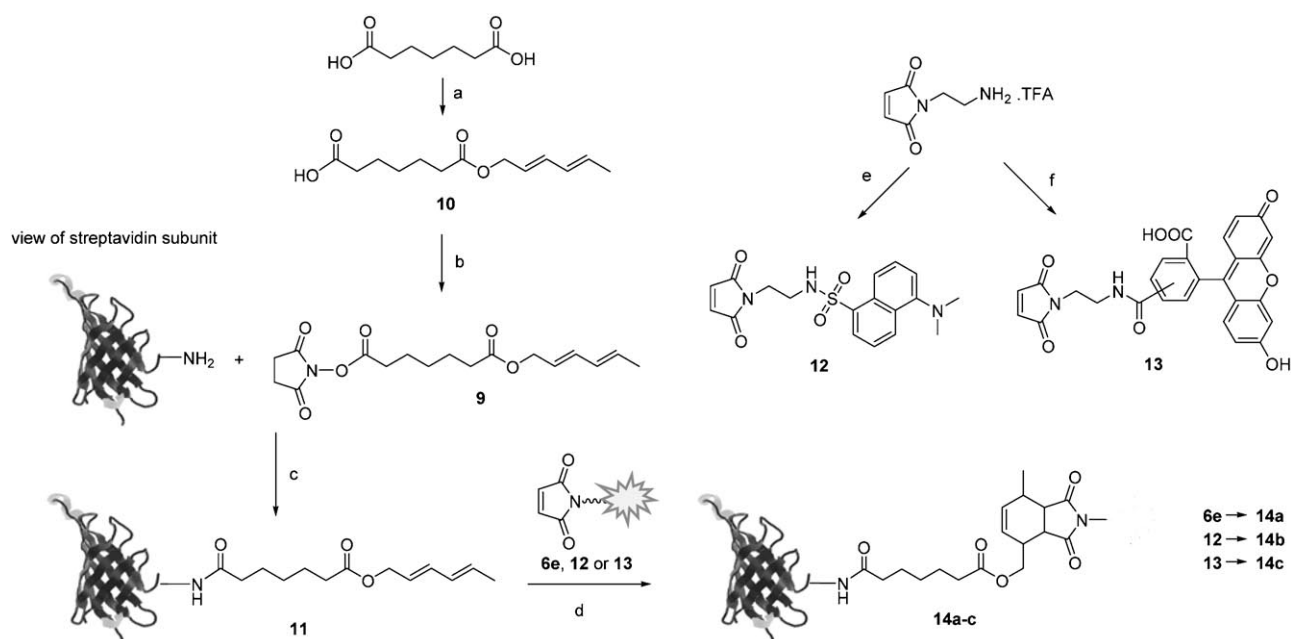
For the development of the Diels–Alder bioconjugation method, we devised the heterobifunctional cross-linker **9**, which embodies a 2,4-hexadiene functionality and the *N*-hydroxysuccinimidoyl (NHS) group that can react with amines (Scheme 4).

Cross-linker **9** was prepared in two steps by mono-esterification of 2 equiv pimelic acid with 1 equiv hexadienol **2** followed by transformation of the diene **10** into the *N*-hydroxysuccinimidyl ester **9** (Scheme 4). Linker **9** was first attached to streptavidin molecules by acylation of lysine residues through the NHS moiety. The molar ratio between the cross-linker and streptavidin was kept relatively low (6:1) to prevent multiple labeling of the streptavidin molecules. Analysis of the MALDI-TOF spectra of modified streptavidin revealed that on average each streptavidin subunit was equipped with one diene-linker (Figure S4B, Supporting Information).

The diene-modified protein **11** was subjected to Diels–Alder ligation with three different fluorescent labeled maleimide compounds **6e**, **12** and **13**, respectively in water.

Dansyl- (**12**) and fluorescein-maleimide (**13**) were synthesized from the precursor 2-maleimido-ethanamine^[19] as shown in Scheme 4. The cycloaddition reaction was carried out at 100 μ M protein concentration using 30-fold excess of dienophile at 25 °C for 24 h (Scheme 4). After removal of unligated dienophile, the formation of the new fluorescent protein **14** was verified by SDS-PAGE for all three conjugation reactions (Figure 3a). In addition, the mass of the expected ligation cycloadduct was confirmed by MALDI-TOF analysis (Supporting Information Figure 4). Incubation of unmodified streptavidin and dienophile molecules **6e**, **12** and **13** resulted in no detectable product (lanes 3, 5 and 7 in Figure 3a), thus confirming that the observed conjugation of maleimide probes proceeded via Diels–Alder ligation.

Next we analyzed the efficiency and selectivity of the cycloaddition at different pH values. We chose not to carry out the reactions at pH above 7 since under these conditions a nucleophilic addition of amino groups to the dienophile double bond may take place.^[1] The ligation between diene conjugate **11** and about 100-fold excess of dansylated maleimido-peptide **6e** was investigated in sodium phosphate buffer in the pH range of 5.5 to 7.0 for 24 h at 25 °C. To monitor possible side reactions, control reactions were performed in which the streptavidin-conjugate was substituted by unmodified streptavidin. As shown in Figure 3b the Diels–Alder cycloaddition is selective at pH 5.5 to 6.5 but loses its selectivity at higher pH. The appearance of a fluorescent band for the reaction of streptavidin with **6e** at pH 7 (lane 9 in Figure 3b) indicated that the maleimido-compound also reacts unspecifically with the protein molecule under these conditions.



Scheme 4. Diels–Alder conjugation of streptavidin. a) Hexadienol **2**, DIC, DMAP, THF, overnight, 43%; b) *N*-hydroxysuccinimide, DIC, DMAP, THF, overnight, 82%; c) 6 equiv cross linker **9**, H₂O, 2 h, 25 °C, purified by membrane ultracentrifugation (Microcon, 10 kDa cut-off); d) 30 equiv dienophile, H₂O, 24 h, 25 °C, purified by gel filtration (DyeEx spin columns); e) dansyl chloride, DIPEA, DMF, 1 h, 80%; f) fluorescein succinimidyl ester, DIPEA, DMF, 2.5 h, 46%.

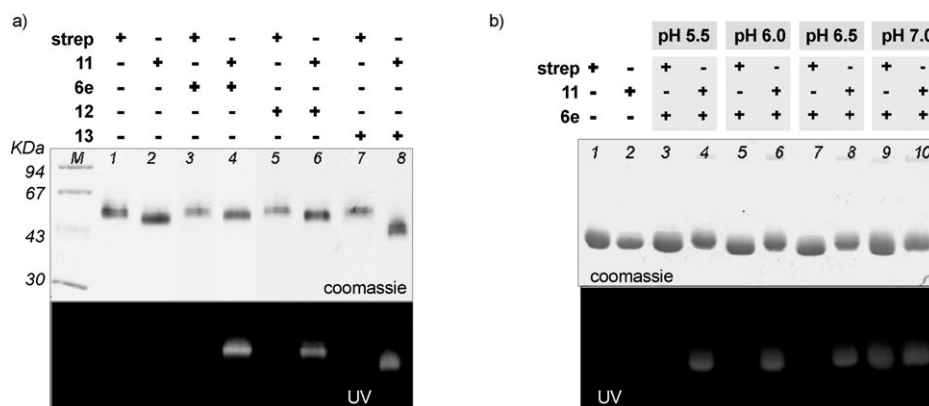


Figure 3. a) SDS-PAGE analysis of the streptavidin conjugates **14a**, **14b** and **14c** obtained by Diels–Alder ligation of streptavidin–diene **11** (lane 2) and maleimides **6e** (lane 4), **12** (lane 6) and **13** (lane 8), respectively. Control experiments showed that no binding occurs when wild type streptavidin (lane 1) is combined with maleimides **6e** (lane 3), **12** (lane 5) or **13** (lane 7) in water. b) SDS-PAGE analysis for the Diels–Alder conjugation of diene **11** and maleimide **6e** at pH 5.5 to 7.0. The observed protein bands represent streptavidin subunits as the tetrameric protein complex was denatured upon heating of the sample at 80°C for 3 min with denaturing loading buffer prior to gel loading. Strep: streptavidin, M: molecular weight marker.

Site-specific labeling of a Rab protein: The Expressed Protein Ligation (EPL) method^[4] is a suitable tool to equip a given protein with the diene functionality at a specific position. By means of this method, the hexadiene group can be specifically attached to the C-terminus of the protein by reaction of a recombinant thioester tagged protein with a peptide or amino acid carrying for example a cysteine at the N-terminus and a hexadienyl unit at the C-terminus. The EPL process generates a nucleophilic cysteine residue at the ligation site which should be temporary protected (along with other accessible cysteine side chains present in the protein) to avoid undesired modification of the mercapto group in the subsequent reaction with maleimido probes. After the Diels–Alder ligation process, the masked cysteines are transformed back into the free thiol form.

This approach was successfully implemented using a Rab protein. The small Rab GTPases are key regulators of vesicular traffic, which act in vesicle budding, docking and fusion of intracellular peptides.^[20] Many processes in which the Rab proteins are involved are only partially understood, making differently and site-specifically labeled Rab proteins versatile probes to address open questions.^[21–23]

As part of this proposed methodology for the labeling of Rab proteins, Ellmann's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)^[24] was employed as an appropriate cysteine blocking agent that efficiently reacts with the mercapto groups of the protein to form stable disulfide bonds and renders them inert toward further reactions with maleimides. To prove the efficiency of Ellmann's reagent to mask the cysteine residues of Rab proteins, several control experiments were performed in which wild-type Rab7 was treated with dansylated maleimide **6e** with or without prior DTNB protection (Section S7, Supporting Information).

With a suitable cysteine masking procedure in hands, functionalization of Rab7 by means of the expressed protein ligation method to generate the Rab hexadienyl ester was

attempted. The method uses a genetically engineered intein and a chitin binding domain (CBD) as fusion partners to express and purify the desired Rab thioester.^[21,23] In this study, we employed a Rab protein from *Canis familiaris* (Rab7ΔC3-MESNA thioester **15**, see Scheme 5) truncated by three amino acids.

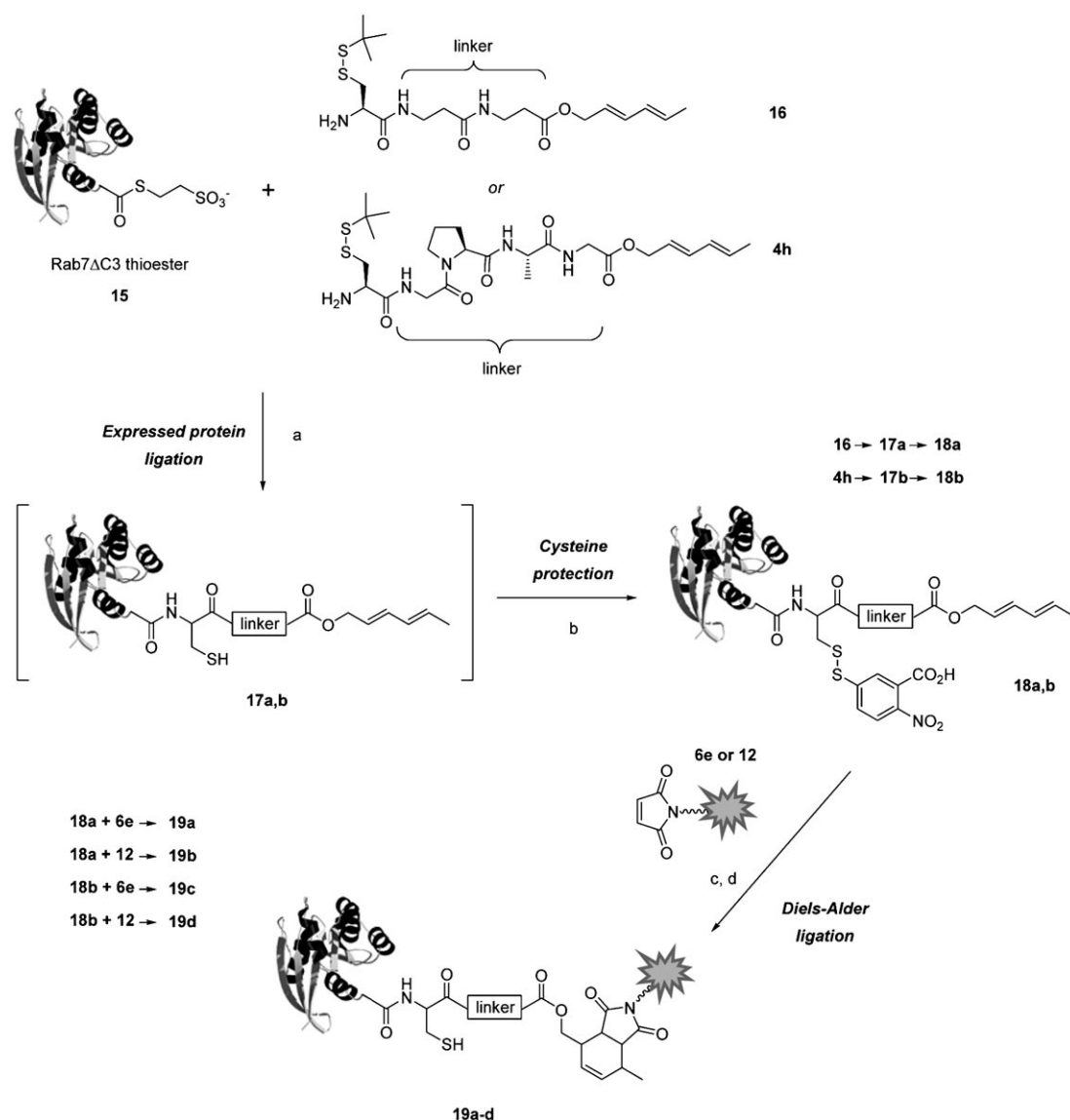
For the dienyl linker construction, two different cysteinyl hexadiene linkers **16** and **4h** were synthesized (Section S8, Supporting Information).

For the construction of the C-terminally esterified Rab7 protein, Rab7 thioester **15** was ligated with peptide hexadienyl esters **16** or **4h** under reducing

conditions overnight at 16°C and in the presence of GDP and MgCl₂ for stabilization (Scheme 5).^[21] An excess of 20 equivalents of the dienyl compound was employed to ensure complete conversion. Small amounts of the detergent CHAPS were also included to increase protein and peptide solubilization under the ligation conditions. The identity of the ligated products **17a,b** was confirmed by ESI-MS (Figure S6, Supporting Information).

Without intermediate purification, the ligated hexadienyl protein **17** was directly submitted to cysteine masking with excess Ellmann's reagent yielding masked protein hexadienyl esters **18a** and **18b** (Scheme 5). These proteins were then dialyzed against DA buffer (5 mM sodium phosphate pH 6.0, 20 mM NaCl, 0.2 mM MgCl₂, 20 μM GDP) to remove all small molecules (MESNA, Ellmann's reagent, dienyl peptide). As determined by mass spectrometry (Figure S6, Supporting Information), the two accessible cysteine residues of the truncated Rab7 protein **18** were in fact masked by the disulfide groups. In the case of hexadiene **18b**, the reagent MESNA itself formed a stable disulfide bond with a cysteine residue of the protein, acting also as a masking agent.

Next the protected hexadienyl Rab7 proteins **18a,b** were subjected to Diels–Alder reaction with peptide-derived dienophile **6e** and dansyl derivative **12** (Scheme 5). The cycloaddition ligation was carried out in buffer at pH 6.0 and at a protein concentration of approximately 40 μM for 24 h at room temperature affording fluorescent labeled Rab7 proteins **19a–d**. The ligation efficiency varied between 50 and 90% depending on the excess of dienophile employed (Table S2, Supporting Information). The coupling reactions were terminated by addition of excess dithiothreitol which traps the dienophile and simultaneously converts the disulfides into unmasked thiols. Because of the release of the chromogenic thionitrobenzoic acid (TNB) group into solution, the ligation solution became yellowish at this point.



Scheme 5. Combination of expressed protein ligation and Diels–Alder ligation for site-specific modification of the Rab7 protein. a) 0.25 mM thioester tagged Rab7ΔC3 **15** in 5 mM sodium phosphate buffer pH 7.5, 20 mM NaCl, 20 mM MESNA, 0.4% CHAPS, 10 μM GDP, 0.2 mM MgCl₂ and 5 mM peptide **16** or **4h**, overnight, 16 °C; b) DTNB, 4 h, 25 °C, then dialysis against DA buffer (5 mM sodium phosphate buffer pH 6.0, 20 mM NaCl, 0.2 mM MgCl₂, 20 μM GDP); c) 1 equiv 40 μM Rab7 **18** in DA buffer, 100 equiv maleimide **6e** or **12**, 24 h, 25 °C. d) DTT, 2 h, 25 °C. MESNA: sodium 2-mercaptoethane sulfonate, CHAPS: 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate, GDP: guanosine 5'-diphosphate, DA: Diels–Alder.

The three-step ligation process was followed by SDS-PAGE (Figure 4) and all intermediates and final products were identified by ESI-MS mass spectrometry (Figure S6, Supporting Information). In the presence of 100-fold excess of dienophile the hexadienyl protein is converted into the expected fluorescent conjugates with high efficiency (90%). Undesired multiple labeling of Rab7 was not detected at significant levels.

The Diels–Alder conjugation of the masked hexadienyl Rab protein with the dansylated labeled dienophile rendered this protein insoluble in the reaction solution. The formation of aggregates could not be avoided even when the ligation was carried out in the presence of cosolvent (glycerol) or detergents (CHAPS, Triton X-100 or Tween-20), or

at lower protein concentration (10–20 μM). This behavior was also formerly observed during the semisynthesis of lipitated Rab proteins.^[21–22]

The purification of the Rab protein **19a** was performed similarly to the procedure described for the isolation of semisynthetic prenylated Rab proteins.^[21a] After incubation of the Rab hexadienyl ester **18a** with maleimide **6e**, the protein precipitate was separated by centrifugation and washed with methanol in order to remove unligated dienophile. The resulting pellet was dissolved in denaturation buffer containing 6 M guanidinium chloride and DTT (unmasking of the cysteine at this point) and then refolded by a 25-fold dilution with renaturation buffer. Subsequently, the folded protein was dialyzed and concentrated.

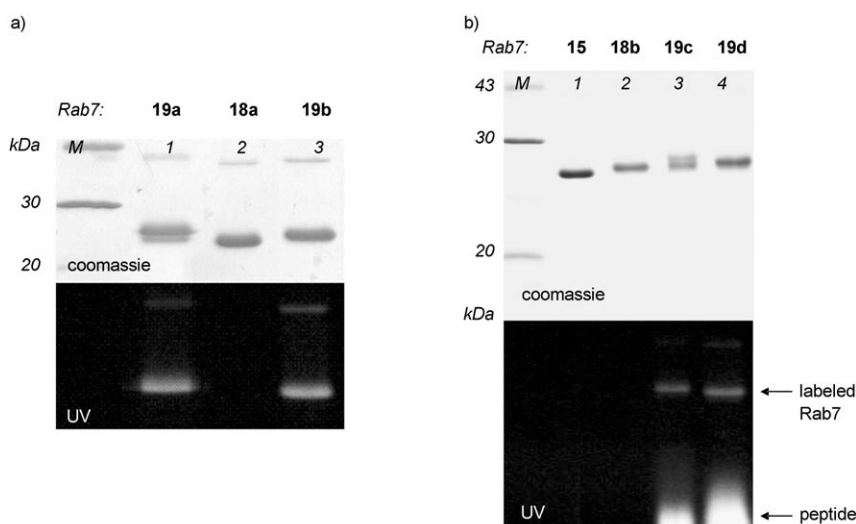


Figure 4. SDS-PAGE analysis of the Diels–Alder ligation between masked Rab7 hexadienyl ester **18** and male-imide probes **6e** and **12**. Diels–Alder labeled Rab7 **19a–d** appeared as a unique fluorescent protein band at ca 24 kDa. Ligation conditions: **18a** and 100-fold **6e** (lane 1 in a), **18a** and 100-fold **12** (lane 3 in a), **18b** and 50-fold **6e** (lane 3 in b), **18b** and 100-fold **12** (lane 4 in b).

In order to establish whether the generated protein was natively folded and functionally active we analyzed its interaction with the Rab Escort Protein 1 (REP-1). REP-1 is an accessory factor that facilitates prenylation of Rab GTPases by presenting them to Rab geranylgeranyltransferase and subsequently by delivering them to the target membranes. Recognition of Rab proteins by REP-1 occurs via a large protein/protein interface and requires integrity of the Rab GTPase ternary structure.^[25] To analyze the interaction of Rab7 with REP-1 we took advantage of the dansyl label attached to the flexible C-terminus of Rab7 that enables the use of fluorescence spectroscopy to monitor the interaction between both proteins. When the solution of semisynthetic Rab7 **19a** excited at 333 nm was titrated with increasing concentrations of REP-1 a dose dependent and saturable increase of fluorescence emission at 440 nm was observed (Figure 5). Titration data could be fitted using a quadratic equation describing the binding curve and were consistent with a 1:1 stoichiometry and a K_d value of 2 nM. This K_d value is very close to the one obtained earlier with fluorescent GDP-bound Rab7.^[26] The obtained data show that the developed procedure yields fully active functionalized Rab7 protein.

The use of the two-step ligation strategy delineated above is particularly indicated if reporter groups or tags need to be introduced into proteins that are not stable under the conditions of expressed protein ligation. For instance, in the course of a different research program we observed that the fluorescence of the BODIPY label, one of the most advantageous and very often used fluorophores, is lost during attempted ligation of BODIPY-functionalized peptides to proteins by means of EPL. However, fluorescent-labeled Rab proteins are formed if diene-modified Rab protein **18a** is

subjected to Diels–Alder ligation with BODIPY-labeled peptides.^[13]

Thus, upon treatment of protein dienyl ester **18a** with male-imide-tagged doubly lipidated peptide **20** the desired lipid-modified protein **21** was formed and isolated (Scheme 6 and Figure 6). The ligated protein was identified by ESI-MS (m/z : calcd for: 25 496 Da; found: 25 495 Da [M^+]). Lipidated peptide **20** represents a fluorescent-analogue of the S-palmitoylated and S-farnesylated C-terminus of the human Ras protein and was synthesized as described previously.^[27] Notably the synthesis of chimeric lipoprotein **21** also dem-

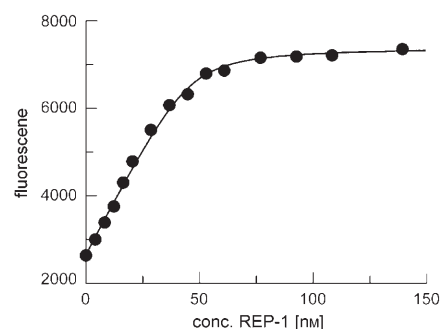
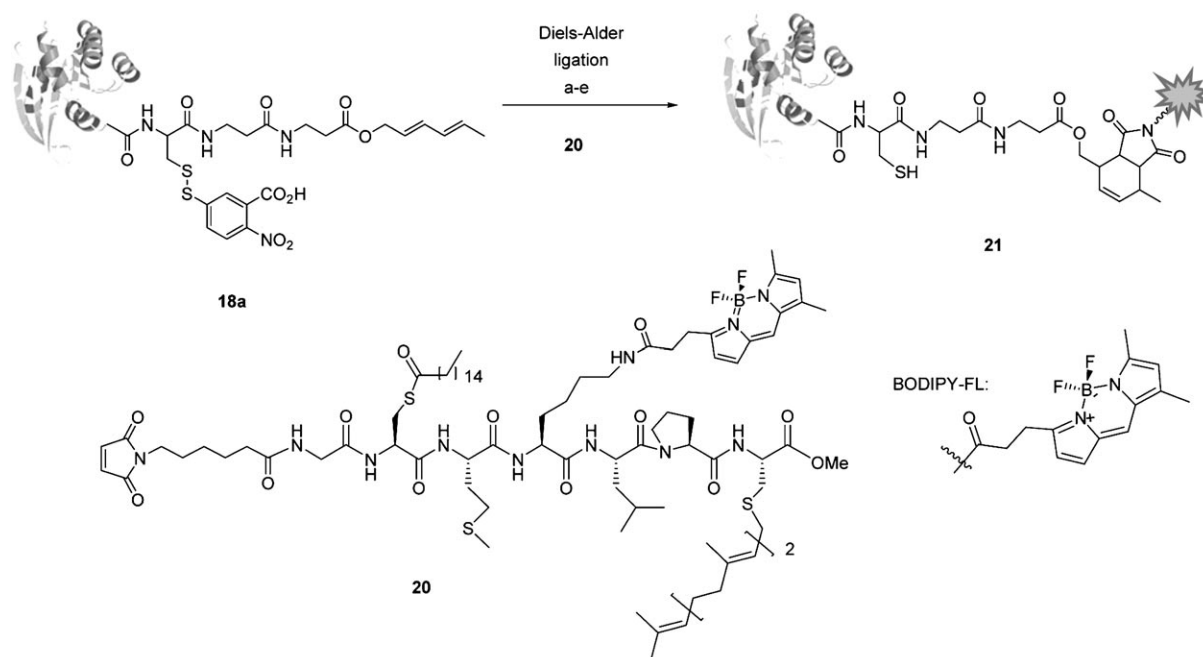


Figure 5. Spectrofluorometric titration of **19a** with REP-1. Conditions: 25°C, 25 mM HEPES pH 7.2, 40 mM NaCl, 2 mM $MgCl_2$, 2 mM DTE and 20 μ M GDP. The concentrations of **19a** complexes were 50 nM. The excitation/emission wavelengths were fixed at 333/440 nm. Data were fitted to a quadratic equation as implemented in the program Grafit 5.1 (Eritacus software) leading to K_d value of 1.9 ± 0.5 nM and consistent with 1:1 stoichiometry. DTE: dithioerythritol, HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid.

onstrates that the Diels–Alder ligation is applicable to the synthesis of sensitive protein conjugates such acid- and base-sensitive lipoproteins.^[28]

Conclusion

We have shown that the Diels–Alder ligation offers a new opportunity for the site-selective functionalization of proteins and peptides. It proceeds under very mild conditions with high selectivity and is compatible with most functional groups found in proteins. Its combination with other ligation methods, in particular expressed protein ligation, is feasible and allows for the rapid equipment of a given protein with different functional groups in a site-specific manner, that is



Scheme 6. Combination of expressed protein ligation and Diels–Alder ligation for the synthesis of a lipidated protein. a) 1 equiv 40 μM Rab7 **18a** in DA buffer, 100 equiv maleimide **20** (in MeOH/CH₂Cl₂ 3:1), 24 h, 25 °C (ligated product **21** precipitates); b) centrifugation and washing of the pellet with MeOH (3 $\times$) and to remove excess peptide; c) the precipitate was dissolved in denaturation buffer (100 mM Tris-HCl, pH 8.0, 6 M guanidinium-HCl, 100 mM DTE, 1% CHAPS, 1 mM EDTA) over night at 4 °C, d) protein renaturated by diluting 25-fold dropwise into refolding buffer (50 mM HEPES, pH 7.5, 2.5 mM DTE, 2 mM MgCl₂, 10 μM GDP, 1% CHAPS, 400 mM arginine-HCl, 400 mM trehalose, 0.5 mM PMSF, 1 mM EDTA) 20-fold stirring at 25 °C 3 h, e) protein was concentrated up to ca. 40 μM by ultracentrifugation (Amicon 10 kDa cut-off). Tris: tris(hydroxymethyl)aminomethane, EDTA: ethylenediaminetetraacetate, PMSF: phenylmethylsulfonyl fluoride.

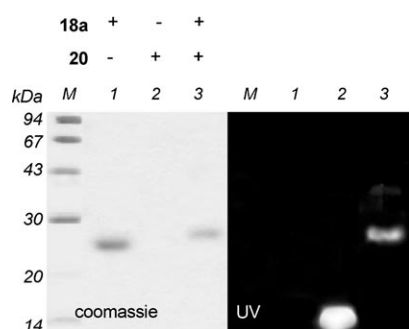


Figure 6. SDS-PAGE analysis of the Diels–Alder ligation product **21** (lane 3) obtained from masked Rab7 hexadienyl ester **18a** (lane 1) and maleimide BODIPY-peptide **20** (lane 2).

at the C-terminus. The Diels–Alder coupling involving the maleimide segment should be carried out at slightly acidic conditions and in the absence of reactive thiol groups or other groups of similar nucleophilicity. If the protein possesses reactive cysteine residues in its structure, the ligation conditions must be adjusted to prevent unspecific reactions by temporary blocking of the sulfhydryl groups. The Diels–Alder reaction leads to a non-traceless ligation site, but the final cycloadduct skeleton is relatively small and should not significantly influence the protein structure. The 2,4-hexadiene moiety is stable under physiological conditions and can be easily incorporated chemically into biomolecules from the commercially available precursor *trans,trans*-2,4-

hexadienol. Maleimide derivatives and probes are available from commercial suppliers.

Taken together the results described herein and the previous results for the bioconjugation of oligonucleotides and saccharides demonstrates that the Diels–Alder [4+2] cycloaddition is an advantageous method for covalent biomolecule modification and a real alternative for the in vitro assembly of semisynthetic proteins and biopolymers.

Experimental Section

General methods: Peptide synthesis grade solvents and deionized water (Millipore Q-Plus System) were used for all experiments. Peptides and reagents, unless otherwise noted, were purchased from commercial suppliers. Purifications were performed by reversed-phase HPLC on Agilent preparative HPLC 1100 Series system using a Nucleodur C18 Gravity column (Macherey–Nagel) and detection at 215 nm. Linear gradients of solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in water) were applied at 25 mL min⁻¹ flow rate. Optical rotations were measured in a Schmidt and Haensch Polartronic HH8 polarimeter at 589 nm and concentrations are given in g per 100 mL solvent. NMR spectra were recorded using a Varian Mercury 400 MHz spectrometer and calibrated according with solvent standard peaks. Analytical reversed-phase HPLC was carried out in a Hewlett Packard HPLC 1100 system using a Nucleosyl 100-5 C18 Nautilus column, detection at 215 and 254 nm and linear gradients of solvent B in solvent A at 1 mL min⁻¹ flow rate. Electrospray mass spectrometric analyses (ESI-MS) were performed on a Finnigan LCQ spectrometer. Mass spectra from proteins were deconvoluted using the Biomass program included in the Xcalibur software (ThermoFinnigan). Fast atom bombardment (FAB) mass spectra were recorded on a

Finnigan MAT MS 70 spectrometer, using *m*-nitrobenzylalcohol as matrix. MALDI-TOF measurements were carried out with Voyager-DE Pro Biospectrometry Workstation from PerSeptive Biosystems utilizing α -cyano-hydroxycinnamic acid as matrix. Calculated masses were obtained using the program ChemDraw Ultra (CambridgeSoft Corporation). Streptavidin and avidin proteins were obtained from Sigma. Tris/glycine 15% gels were applied for denaturing SDS-PAGE, with fluorescence detection at 302 nm (Reprostar II UV illuminator, Camag) and subsequent staining with Coomassie Blue. Rab7 thioester,^[21] and REP-1^[25] were expressed and purified as described in the respective references.

General procedure for solid-phase peptide synthesis (SPPS)

Peptides were assembled by manual solid-phase synthesis using the HBTU/HOBt/DIPEA activation method. Typically couplings were carried out with 4 equiv Fmoc-amino acid, 4 equiv HBTU, 4 equiv HOBt and 8 equiv DIPEA in DMF, for a minimum of 1 h and monitored by the Kaiser test. Fmoc-deprotection was accomplished by treating the resin with 20% piperidine in DMF for 10 minutes twice. N-Maleimidoyl glycine^[17] or N-maleimido- β -alanine were coupled using 4 equiv of the respective maleimide, 4 equiv DIC and 4 equiv HOBt in DCM/DMF 1:1 for 2 h or allowed to react overnight.

General procedure for the solid-phase synthesis of the fully protected hexadienyl peptide esters 3 using the sulfonamide linker resin

First amino acid loading and peptide assembly: The resin loaded with Fmoc-glycine was obtained by treatment of 4-sulfamylbutyryl AM resin (Novabiochem) with a mixture of Fmoc-Gly-OH (7 equiv), *N*-methylimidazole (7 equiv) and DIC (7 equiv) in DCM/DMF 4:3 overnight (quantitative loading as determined by the UV-Fmoc method). The peptide chain was assembled as indicated in the general procedure above.

Activation/cleavage from resin: Iodoacetone (25 equiv) and DIPEA (10 equiv) were dissolved in NMP (4 mL mmol⁻¹ ICH₂CN), filtered through basic alumina, and added to the fully protected peptidyl resin (pre-swollen in DCM and NMP). The resulting mixture was shielded from light and shaken at room temperature for 18–24 h. The resin was washed with NMP (5 $\times$) and THF (3 $\times$). The activated resin was directly transferred to a round bottom flask and treated with a solution of *trans-trans*-2,4-hexadienyl-1-ol (**2**; 20 equiv) and DMAP (0.5 equiv) in dry THF (10 mL g⁻¹ resin) for 24 h. The resin was filtered and washed several times with THF. The filtrates were combined and THF was removed under reduced pressure. The crude product was purified by reversed-phase HPLC. Fractions containing the product (analyzed by MALDI-TOF) were combined and dried by lyophilization.

Fmoc-Lys(Fmoc)-Pro-Phe-Leu-Gly-OHxd (3a**):** Starting from sulfonamide resin (122 mg) loaded with Fmoc-Gly (0.075 mmol), a colorless solid (35 mg, 0.032 mmol, 43%) was obtained. ¹H NMR (CDCl₃, 400 MHz): δ = 0.62–0.80 (m, 6H, 2 $\times$ CH₃ Leu), 1.10–2.10 (m, 16H, δ -CH₂ Lys, γ -CH₂ Lys, β -CH₂ Leu, γ -CH Leu, CH₃ Hxd, β -CH₂ Lys, β -CH₂ Pro, γ -CH₂ Pro), 2.9–3.2 (m, 4H, β -CH₂ Phe, ϵ -CH₂ Lys), 3.45–3.70 (m, 2H, ϵ -CH₂ Pro), 3.75–3.90 (m, 2H, α -CH₂ Gly), 4.00–4.55 (m, 12H, α -CH Lys, α -CH Pro, α -CH Leu, α -CH Phe, CH₂ Hxd, 2 $\times$ CH₂ Fmoc, 2 $\times$ CH Fmoc), 5.36–5.49 (m, 1H, CH=CHCH₃), 5.60–5.68 (m, 1H, CH₂CH=CH), 5.82–5.94 (m, 1H, CH=CHCH₃), 6.06–6.13 (m, 1H, CH₂CH=CH), 7.00–7.10 (m, 5H, Ar Phe), 7.13–7.23 (m, 4H, 2 $\times$ Ar Fmoc), 7.25–7.35 (m, 4H, 2 $\times$ Ar Fmoc), 7.40–7.55 (m, 4H, 2 $\times$ Ar Fmoc), 7.60–7.70 ppm (m, 4H, 2 $\times$ Ar Fmoc); ESI-MS: *m/z*: calcd for C₆₄H₇₂N₇O₁₀: 1085.5; found: 1085.3 [M+H]⁺.

Fmoc-Pro-Cys(SrBu)-Ser-Met-Gly-OHxd (3b**):** Starting from sulfonamide resin (151 mg) loaded with Fmoc-Gly (0.10 mmol) the product was cleaved from the resin as Fmoc-Pro-Cys(SrBu)-Ser(Trt)-Met-Gly-OHxd and purified by RP-HPLC. The resulting fractions containing product were combined and concentrated in vacuo. After evaporation of the solvent, partial Trt deprotection was detected (MALDI and LC-MS). In order to achieve complete removal of the Trt group, the crude product (58 mg) was further treated with DCM/TFA/TIS 100:1:5 (1 mL) for 2 h at room temperature. Solvents were removed in vacuo and the product was purified by RP-HPLC, affording colorless solid (18 mg, 0.02 mmol, 20%); ¹H NMR (CD₃OD, 400 MHz): δ = 1.20/1.32 (s, 9H, *t*Bu), 1.73 (d, 3H, *J* = 6.6 Hz, CH₃ Hxd), 1.86–2.10 (m, 4H, γ -CH₂ Pro, β -CH₂ Pro), 2.06

(s, 3H, CH₃ Met), 2.10–2.38 (m, 2H, β -CH₂ Met), 2.44–2.65 (m, 2H, γ -CH₂ Met), 2.92–3.20 (m, 2H, β -CH₂ Cys), 3.41–3.70 (m, 2H, ϵ -CH₂ Pro), 3.78–3.88 (m, 2H, β -CH₂ Ser), 3.92 (m, 2H, α -CH₂ Gly), 4.18–4.56 (m, 6H, CH Fmoc, CH₂ Fmoc, 3 $\times$ α -CH), 4.59 (d, 2H, *J* = 6.5 Hz, CH₂ Hxd), 4.62–4.66 (m, 1H, α -CH), 5.57–5.63 (m, 1H, CH=CHCH₃), 5.70–5.79 (m, 1H, CH₂CH=CH), 6.01–6.08 (dd, 1H, *J* = 15.0, 10.4 Hz, CH=CHCH₃), 6.21–6.28 (dd, 1H, *J* = 15.0, 10.3 Hz, CH₂CH=CH), 7.32 (t, 2H, *J* = 7.4 Hz, Ar Fmoc), 7.40 (t, 2H, *J* = 7.4 Hz, Ar Fmoc), 7.65 (t, 2H, *J* = 7.4 Hz, Ar Fmoc), 7.80 ppm (d, 2H, *J* = 7.4 Hz, Ar Fmoc) and weak amide signals; ESI-MS: *m/z* for C₄₃H₅₇N₅O₃S₃: 884.3; found: 884.4 [M+H]⁺; MALDI-TOF: *m/z*: 906.8 [M+Na]⁺, 922.8 [M+K]⁺.

Fmoc-Lys(Fmoc)-Leu-Gly-Phe-Ala-Gly-OHxd (3c**):** Starting from sulfonamide resin (51 mg) loaded with Fmoc-Gly (0.043 mmol) a colorless solid (16 mg, 0.014 mmol, 33%) was obtained. ¹H NMR ([D₆]DMSO, 400 MHz): δ = 0.81 (d, 3H, *J* = 6.5 Hz, CH₃ Leu), 0.82 (d, 3H, *J* = 6.5 Hz, CH₃ Leu), 1.23 (d, 3H, *J* = 7.1 Hz, CH₃ Ala), 1.22–1.65 (m, 9H, 3 $\times$ CH₂ Lys, β -CH₂ Leu, γ -CH Leu), 1.70 (d, 3H, *J* = 6.6 Hz, CH₃ Hxd), 2.71–2.77 (m, 1H, β -CH₂ Phe), 2.92–2.99 (m, 2H, CH₂ Lys), 2.99–3.05 (m, 2H, β -CH₂ Phe), 3.55–3.71 (m, 2H, α -CH₂ Gly), 3.77–3.91 (m, 2H, α -CH₂ Gly), 3.94–4.00 (m, 1H, α -CH), 4.14–4.35 (m, 8H, 2 $\times$ CH Fmoc, 2 $\times$ α -CH, 2 $\times$ CH₂ Fmoc), 4.51–4.55 (m, 1H, α -CH), 4.56 (d, 2H, *J* = 6.2 Hz, CH₂ Hxd), 5.60 (ddd, 1H, *J* = 12.8, 6.4, 6.4 Hz, CH=CHCH₃), 5.69–5.78 (m, 1H, CH₂CH=CH), 6.04 (dd, 1H, *J* = 14.9, 10.2 Hz, CH=CHCH₃), 6.27 (dd, 1H, *J* = 15.0, 10.3 Hz, CH₂CH=CH), 7.13–7.26 (m, 5H, Ar Phe), 7.31 (t, 4H, *J* = 7.4 Hz, Ar Fmoc), 7.40 (t, 4H, *J* = 7.4 Hz, Ar Fmoc), 7.66–7.71 (m, 4H, Ar Fmoc), 7.84–7.86 (m, 1H, NH), 7.87 (d, 4H, *J* = 7.4 Hz, Ar Fmoc), 7.98 (d, 1H, *J* = 8.2 Hz, NH), 8.05 (t, 1H, *J* = 5.3 Hz, NH), 8.13 (t, 1H, *J* = 5.7 Hz, NH), 8.19 ppm (d, 1H, *J* = 7.6 Hz, NH); ESI-MS: *m/z*: calcd for C₆₄H₇₃N₇O₁₁: 1116.5; found: 1116.2 [M+H]⁺; MALDI-TOF: *m/z*: 1155.1 [M+K]⁺.

Fmoc-Lys(Fmoc)-Leu-Gly-Lys(Mtt)-Ala-Gly-OHxd (3d**):** Starting from sulfonamide resin (59 mg) loaded with Fmoc-Gly (0.05 mmol) a colorless solid (21 mg, 0.016 mmol, 32%) was obtained. ESI-MS: *m/z*: calcd for C₈₁H₉₂N₈O₁₁: 1353.7; found: 1353.4 [M+H]⁺; FAB-LRMS: *m/z*: 1352.79 [M]⁺. Compound **3d** was not further characterized and directly converted into compound **4d**.

Fmoc-Lys(Fmoc)-Cys(SrBu)-Gly-Val-Phe-Gly-OHxd (3e**):** Starting from Fmoc-Gly sulfonamide resin (164 mg) loaded with Fmoc-Gly (0.056 mmol), a colorless solid (14 mg, 0.012 mmol, 22%) was obtained. [α]_D²⁰ = –13.5 (*c* = 0.2, MeOH); ¹H NMR ([D₇]DMF, 400 MHz): δ = 0.92 (d, 3H, *J* = 6.8 Hz, CH₃ Val), 0.94 (d, 3H, *J* = 6.8 Hz, CH₃ Val), 1.46 (s, 9H, *t*Bu), 1.63–1.72 (m, 4H, γ -CH₂ Lys, δ -CH₂ Lys), 1.88 (d, 3H, *J* = 6.6 Hz, CH₃ Hxd), 1.90–2.10 (m, 2H, β -CH₂ Lys), 2.16–2.25 (m, 1H, β -CH Val), 3.28–3.50 (m, 6H, ϵ -CH₂ Lys, β -CH₂ Phe, β -CH₂ Cys), 4.00–4.21 (m, 4H, 2 $\times$ α -CH₂ Gly), 4.34–4.48 (m, 8H, 2 $\times$ CH Fmoc, 2 $\times$ α -CH, 2 $\times$ CH₂ Fmoc), 4.78 (d, 2H, *J* = 6.4 Hz, CH₂ Hxd), 4.85–4.90 (m, 2H, 2 $\times$ α -CH), 5.78–5.85 (m, 1H, CH=CHCH₃), 5.90–5.98 (m, 1H, CH₂CH=CH), 6.22–6.28 (dd, 1H, *J* = 15.0, 11.0 Hz, CH=CHCH₃), 6.45–6.52 (dd, 1H, *J* = 15.0, 10.4 Hz, CH₂CH=CH), 7.34 (t, 1H, *J* = 7.2 Hz, Ar Phe), 7.41 (t, 2H, *J* = 7.2 Hz, Ar Phe), 7.47 (d, 2H, *J* = 7.0 Hz, Ar Phe), 7.50 (t, 4H, *J* = 7.4 Hz, Ar Fmoc), 7.60 (t, 4H, *J* = 7.4 Hz, Ar Fmoc), 7.84–7.97 (m, 8H, Ar Fmoc, 4 $\times$ CONH), 8.05 (d, 4H, *J* = 7.4 Hz, Ar Fmoc), 8.35 (t, 1H, *J* = 6.0 Hz, CONH), 8.49 (t, 1H, *J* = 5.8 Hz, CONH), 8.66 ppm (d, 1H, *J* = 7.5 Hz, CONH); ESI-MS: *m/z*: calcd for C₆₇H₇₉N₇O₁₁S₂: 1222.5; found: 1222.2 [M+H]⁺; FAB-LRMS: *m/z*: 1221.7 [M]⁺.

Fmoc-Lys(Fmoc)-Phe-Pro-Ile-Gly-Leu-Phe-Gly-OHxd (3f**) and Fmoc-Lys(Fmoc)-Phe-Pro-Ile-Gly-Leu-Gly-Phe-Gly-OHxd (**3g**):** Sulfonamide resin (106 mg) loaded with Fmoc-Gly (0.09 mmol) was used. The coupling of the second glycine residue was incomplete, yielding a mixture of two peptides **3f** and **3g** which were separated by means of HPLC: 16% overall yield: **3f** (14.5 mg, 0.010 mmol, 12%) and **3g** (5.4 mg, 0.0037 mmol, 4%). **3f**: MALDI-TOF: *m/z*: calcd for C₈₁H₉₅NaN₉O₁₃: 1424.7; found: 1424.7 [M+Na]⁺, 1440.7 [M+K]⁺; **3g**: MALDI-TOF: *m/z*: calcd for C₈₃H₉₈N₁₀O₁₄: 1481.7; found: 1481.7 [M+Na]⁺, 1497.7 [M+K]⁺. Compounds **3f** and **3g** were not further characterized and immediately converted to compounds **4f** and **4g**.

Fmoc-Cys(SrBu)-Gly-Pro-Ala-Gly-O-hexadienylester (3h**):** Sulfonamide resin (291 mg) loaded with Fmoc-Gly (0.24 mmol) was used. The product

was purified by flash chromatography, eluting with DCM and DCM/MeOH 100:1–20:1 to give a colorless solid (70 mg, 0.09 mmol, 37%). R_f = 0.31 (DCM/MeOH 10:1); MALDI-TOF: m/z : calcd for $C_{40}H_{51}NaN_5O_8S_2$: 816.3; found: 816.6 $[M+Na]^+$, 833.5 $[M+K]^+$. Compound **3h** was not further characterized and immediately converted to compound **4h**.

General procedure for Fmoc-protecting group removal to give hexadienyl esters 4: A solution of 5–30 mg Fmoc-protected peptide **3** was treated with 0.5–2.0 mL 20% piperidine in DMF or DCM for 40 minutes at room temperature. The reaction mixture was coevaporated with methanol to remove excess of piperidine. The product was purified by reversed-phase-HPLC, fractions containing the product (evaluated by MALDI-TOF) were combined and dried by lyophilization.

H-Lys-Pro-Phe-Leu-Gly-OHxd (4a): Starting from **3a** (33 mg, 0.032 mmol) a colorless solid (15 mg, 0.023 mmol, 72%) was obtained. $[\alpha]_D^{20}$ = -27.9° (c = 0.3, MeOH); 1H NMR (CD_3OD , 400 MHz): δ = 0.91 (d, 3H, J = 6.1 Hz, CH_3 Leu), 0.94 (d, 3H, J = 6.2 Hz, CH_3 Leu), 1.46–1.82 (m, 7H, δ - CH_2 Lys, γ - CH_2 Lys, β - CH_2 Leu, γ -CH Leu), 1.74 (d, 3H, J = 6.7 Hz, CH_3 Hxd), 1.84–2.23 (m, 6H, β - CH_2 Pro, γ - CH_2 Pro, β - CH_2 Lys), 2.90–3.00 (m, 2H, ϵ - CH_2 Lys), 3.02 (dd, 1H, J = 13.9, 7.7 Hz, β - CH_2 Phe), 3.13 (dd, 1H, J = 13.9, 6.5 Hz, β - CH_2 Phe), 3.56–3.63 (m, 1H, ϵ - CH_2 Pro), 3.67–3.73 (m, 1H, ϵ - CH_2 Pro), 3.81–3.96 (m, 2H, α - CH_2 Gly), 4.25 (t, 1H, J = 6.1 Hz, α -CH), 4.42 (dd, 1H, J = 9.5, 5.5 Hz, α -CH), 4.50 (t, 1H, J = 5.2 Hz, α -CH), 4.57 (t, 1H, J = 6.6 Hz, α -CH), 4.61 (d, 2H, J = 6.6 Hz, CH_2 Hxd), 5.61 (ddd, 1H, J = 13.5, 6.5, 6.5 Hz, $CH=CHCH_3$), 5.72–5.81 (m, 1H, $CH_2CH=CH$), 6.06 (dd, 1H, J = 15.0, 10.4 Hz, $CH=CHCH_3$), 6.28 (dd, 1H, J = 15.0, 10.4 Hz, $CH_2CH=CH$), 7.17–7.30 ppm (m, 5H, Ar Phe); ESI-MS: m/z : calcd for $C_{34}H_{52}N_6O_6$: 641.4; found: 641.4 $[M+H]^+$; FAB-HRMS: m/z : 640.3945 $[M]^+$.

H-Pro-Cys(SrBu)-Ser-Met-Gly-OHxd (4b): Starting from **3b** (6 mg, 6.8 μ mol) a colorless solid (2.3 mg, 3.5 μ mol, 51%) was obtained. $[\alpha]_D^{20}$ = -17.1° (c = 0.1, MeOH); 1H NMR (CD_3OD , 400 MHz): δ = 1.35 (s, 9H, tBu), 1.75 (d, 3H, J = 6.6 Hz, CH_3 Hxd), 1.90–2.21 (m, 6H, γ - CH_2 Pro, β - CH_2 Pro, β - CH_2 Met), 2.09 (s, 3H, CH_3 Met), 2.38–2.64 (m, 2H, γ - CH_2 Met), 3.02 (dd, 1H, J = 13.7, 9.1 Hz, β - CH_2 Cys), 3.23 (dd, 1H, J = 13.6, 5.0 Hz, β - CH_2 Cys), 3.30–3.45 (m, 2H, ϵ - CH_2 Pro), 3.75 (dd, 1H, J = 10.9, 5.9 Hz, β - CH_2 Ser), 3.85 (dd, 1H, J = 10.9, 5.4 Hz, β - CH_2 Ser), 3.94 (m, 2H, α - CH_2 Gly), 4.31 (dd, 1H, J = 8.4, 6.3 Hz, α -CH Pro), 4.40 (t, 1H, J = 5.7 Hz, α -CH Met), 4.56 (dd, 1H, J = 9.0, 4.9 Hz, α -CH Ser), 4.62 (d, 2H, J = 6.7 Hz, CH_2 Hxd), 4.69 (dd, 1H, J = 9.0, 5.1 Hz, α -CH Cys), 5.61 (ddd, 1H, J = 13.2, 6.5, 6.5 Hz, $CH=CHCH_3$), 5.73–5.81 (m, 1H, $CH_2CH=CH$), 6.06 (dd, 1H, J = 15.0, 10.4 Hz, $CH=CHCH_3$), 6.27 ppm (dd, 1H, J = 15.1, 10.3 Hz, $CH_2CH=CH$); ESI-MS: m/z : calcd for $C_{28}H_{47}N_5O_7S_3$: 662.3; found: 662.2 $[M+H]^+$, 684.3 $[M+Na]^+$; MALDI-TOF: m/z : 662.8 $[M+H]^+$, 684.8 $[M+Na]^+$, 700.8 $[M+K]^+$.

H-Lys-Leu-Gly-Phe-Ala-Gly-OHxd (4c): Starting from **3c** (7 mg, 0.0063 mmol) a colorless solid (3.5 mg, 0.0052 mmol, 83%) was obtained. $[\alpha]_D^{20}$ = -7.2° (c = 0.3, MeOH); 1H NMR (CD_3OD , 400 MHz): δ = 0.95 (d, 3H, J = 6.5 Hz, CH_3 Leu), 0.97 (d, 3H, J = 6.5 Hz, CH_3 Leu), 1.38 (d, 3H, J = 7.2 Hz, CH_3 Ala), 1.45–1.91 (m, 9H, $3 \times CH_2$ Lys, β - CH_2 Leu, γ -CH Leu), 1.74 (d, 3H, J = 6.4 Hz, CH_3 Hxd), 2.90–3.20 (m, 4H, β - CH_2 Phe, CH_2 Lys), 3.88–3.97 (m, 4H, $2 \times \alpha$ - CH_2 Gly), 4.34–4.42 (m, 2H, $2 \times \alpha$ -CH), 4.58–4.61 (m, 2H, $2 \times \alpha$ -CH), 4.62 (d, 2H, J = 6.2 Hz, CH_2 Hxd), 5.62 (ddd, 1H, J = 13.6, 6.6, 6.6 Hz, $CH=CHCH_3$), 5.72–5.81 (m, 1H, $CH_2CH=CH$), 6.06 (dd, 1H, J = 15.0, 10.5 Hz, $CH=CHCH_3$), 6.28 (dd, 1H, J = 15.1, 10.3 Hz, $CH_2CH=CH$), 7.18–7.29 ppm (m, 5H, Ar Phe); ESI-MS: m/z : calcd for $C_{34}H_{53}N_7O_7$: 671.4; found 671.4 $[M]^+$; MALDI-TOF: m/z : 672.9 $[M+H]^+$, 694.9 $[M+Na]^+$, 710.9 $[M+K]^+$.

H-Lys-Leu-Gly-Lys(Mtt)-Ala-Gly-OHxd (4d): Starting from **3d** (21 mg, 15.5 μ mol) a colorless solid (10.2 mg, 11.2 μ mol, 73%) was obtained. 1H NMR (CD_3OD , 400 MHz): δ = 0.94 (d, 3H, J = 6.4 Hz, CH_3 Leu), 0.96 (d, 3H, J = 6.4 Hz, CH_3 Leu), 1.29–1.40 (m, 2H, γ - CH_2 Lys), 1.39 (d, 3H, J = 7.2 Hz, CH_3 Ala), 1.44–1.55 (m, 2H, γ - CH_2 Lys), 1.58–1.93 (m, 11H, $2 \times \delta$ - CH_2 Lys, β - CH_2 Leu, $2 \times \beta$ - CH_2 Lys, γ -CH Leu), 1.74 (d, 3H, J = 6.6 Hz, CH_3 Hxd), 2.38 (s, 3H, CH_3 Mtt), 2.85–2.99 (m, 4H, $2 \times \epsilon$ - CH_2 Lys), 3.77–3.96 (m, 5H, $2 \times \alpha$ - CH_2 Gly, α -CH), 4.26–4.43 (m, 3H, $3 \times \alpha$ -CH), 4.58 (d, 2H, J = 6.6 Hz, CH_2 Hxd), 5.59 (ddd, 1H, J = 13.3, 6.6, 6.6 Hz, $CH=CHCH_3$), 5.71–5.80 (m, 1H, $CH_2CH=CH$), 6.05 (dd, 1H, J =

15.0, 10.4 Hz, $CH=CHCH_3$), 6.28 (dd, 1H, J = 15.0, 10.6 Hz, $CH_2CH=CH$), 7.18 (d, 2H, J = 8.4 Hz, Ar Mtt), 7.27–7.33 (m, 6H, Ar Mtt), 7.43–7.49 ppm (m, 6H, Ar Mtt); FAB-HRMS: m/z : calcd for $C_{34}H_{52}N_6O_6$: 909.5602; found 909.5684 $[M+H]^+$.

H-Lys-Cys(SrBu)-Gly-Val-Phe-Gly-OHxd (4e): Starting from **3e** (11 mg, 9.0 μ mol) a colorless solid (5.6 mg, 7.2 μ mol, 80%) was obtained. $[\alpha]_D^{20}$ = -9.0° (c = 0.1, MeOH); 1H NMR (CD_3OD , 400 MHz): δ = 0.73 (d, 3H, J = 6.8 Hz, CH_3 Val), 0.82 (d, 3H, J = 6.8 Hz, CH_3 Val), 1.34 (s, 9H, tBu), 1.45–1.55 (m, 2H, γ - CH_2 Lys), 1.63–1.70 (m, 2H, δ - CH_2 Lys), 1.74 (d, 3H, J = 6.6 Hz, CH_3 Hxd), 1.84–1.95 (m, 2H, β - CH_2 Lys), 2.01–2.10 (m, 1H, β -CH Val), 2.94 (t, 2H, J = 7.6 Hz, ϵ - CH_2 Lys), 2.98–3.07 (m, 2H, β - CH_2 Phe), 3.20 (dt, 2H, J = 13.9, 6.4 Hz, β - CH_2 Cys), 3.68 (d, 1H, J = 16.1 Hz, α -CH Val), 3.94 (s, 2H, α - CH_2 Gly), 3.98 (t, 1H, J = 6.4 Hz, α -CH Lys), 4.08 (t, 2H, J = 8.2 Hz, α - CH_2 Gly), 4.63 (d, 2H, J = 6.6 Hz, CH_2 Hxd), 4.63–4.66 (m, 2H, α -CH Cys, α -CH Phe), 5.58–5.65 (m, 1H, $CH=CHCH_3$), 5.72–5.82 (m, 1H, $CH_2CH=CH$), 6.03–6.10 (dd, 1H, J = 15.0, 10.4 Hz, $CH=CHCH_3$), 6.25–6.32 (dd, 1H, J = 15.1, 10.5 Hz, $CH_2CH=CH$), 7.18–7.30 ppm (m, 5H, Ar Phe); MALDI-TOF: m/z : calcd for $C_{37}H_{59}N_7O_8S_2$: 778.4; found 778.8 $[M+H]^+$, 800.8 $[M+Na]^+$, 816.7 $[M+K]^+$.

H-Lys-Phe-Pro-Ile-Gly-Leu-Phe-Gly-OHxd (4f): Starting from **3f** (14.5 mg, 10.3 μ mol) a colorless solid (9.0 mg, 9.4 μ mol, 91%) was obtained. $[\alpha]_D^{20}$ = -28.6° (c = 0.3, MeOH); 1H NMR (CD_3OD , 400 MHz): δ = 0.82 (dd, 3H, J = 6.3, 4.3 Hz, CH_3 Ile), 0.87 (d, 3H, J = 6.3 Hz, CH_3 Ile), 0.91–1.00 (m, 6H, $6 \times CH_2$ Leu), 1.21–1.71 (m, 10H, CH_2 Ile, δ - CH_2 Lys, γ - CH_2 Lys, β - CH_2 Leu, β - CH_2 Lys), 1.74 (d, 3H, J = 6.6 Hz, CH_3 Hxd), 1.83–2.14 (m, 6H, β - CH_2 Pro, γ - CH_2 Pro, γ -CH Leu, β -CH Ile), 2.90–3.04 (m, 4H, $2 \times 1H$ β - CH_2 Phe, ϵ - CH_2 Lys), 3.17–3.26 (m, 2H, $2 \times 1H$ β - CH_2 Phe), 3.32–3.52 (m, 1H, ϵ - CH_2 Pro), 3.76–3.95 (m, 6H, ϵ - CH_2 Pro, $2 \times \alpha$ - CH_2 Gly), 4.08 (t, 1H, J = 7.0 Hz, α -CH Pro), 4.22–4.28 (m, 2H, $2 \times \alpha$ -CH), 4.53–4.67 (m, 2H, $2 \times \alpha$ -CH), 4.62 (d, 2H, J = 6.5 Hz, CH_2 Hxd), 5.61 (ddd, 1H, J = 13.6, 6.4, 6.4 Hz, $CH=CHCH_3$), 5.72–5.81 (m, 1H, $CH_2CH=CH$), 6.06 (dd, 1H, J = 15.0, 10.5 Hz, $CH=CHCH_3$), 6.28 (dd, 1H, J = 15.2, 10.5 Hz, $CH_2CH=CH$), 7.17–7.34 ppm (m, 10H, Ar Phe); ESI-MS: m/z : calcd for $C_{51}H_{73}N_9O_9$: 958.6; found: 958.7 $[M+H]^+$; MALDI-TOF: m/z : 958.9 $[M+H]^+$, 980.9 $[M+Na]^+$, 996.8 $[M+K]^+$; FAB-LRMS: m/z : 958.75 $[M+H]^+$.

H-Lys-Phe-Pro-Ile-Gly-Leu-Gly-Phe-Gly-OHxd (4g): Starting from **3g** (5.4 mg, 3.7 μ mol) a colorless solid (3.5 mg, 3.4 μ mol, 92%) was obtained. $[\alpha]_D^{20}$ = -25.7° (c = 0.2, MeOH); 1H NMR (CD_3OD , 400 MHz): δ = 0.82 (dd, 3H, J = 6.3, 3.1 Hz, CH_3 Ile), 0.87 (d, 3H, J = 6.5 Hz, CH_3 Ile), 0.90–1.00 (m, 6H, $2 \times CH_3$ Leu), 1.17–1.72 (m, 10H, CH_2 Ile, δ - CH_2 Lys, γ - CH_2 Lys, β - CH_2 Leu, β - CH_2 Lys), 1.73 (d, 3H, J = 6.6 Hz, CH_3 Hxd), 1.83–2.04 (m, 5H, β - CH_2 Pro, γ - CH_2 Pro, γ -CH Leu), 2.04–2.13 (m, 1H, β -CH Ile), 2.89–3.04 (m, 4H, $2 \times 1H$ β - CH_2 Phe, ϵ - CH_2 Lys), 3.17–3.26 (m, 2H, $2 \times 1H$ β - CH_2 Phe), 3.32–3.52 (m, 1H, ϵ - CH_2 Pro), 3.72–4.02 (m, 8H, ϵ - CH_2 Pro, $3 \times \alpha$ - CH_2 Gly), 4.10 (m, 1H, α -CH), 4.21–4.28 (m, 2H, $2 \times \alpha$ -CH), 4.49–4.56 (m, 2H, $2 \times \alpha$ -CH), 4.62 (d, 2H, J = 6.5 Hz, CH_2 Hxd), 5.62 (ddd, 1H, J = 13.2, 6.7, 6.7 Hz, $CH=CHCH_3$), 5.71–5.82 (m, 1H, $CH_2CH=CH$), 6.06 (dd, 1H, J = 14.9, 10.5 Hz, $CH=CHCH_3$), 6.27 (dd, 1H, J = 14.6, 10.0 Hz, $CH_2CH=CH$), 7.19–7.35 ppm (m, 10H, Ar Phe); ESI-MS: m/z : calcd for $C_{53}H_{78}N_{10}O_{10}$: 1015.6; found: 1015.7 $[M+H]^+$; MALDI-TOF: m/z : 1015.9 $[M+H]^+$, 1037.8 $[M+Na]^+$, 1053.8 $[M+K]^+$.

H-Cys(SrBu)-Gly-Pro-Ala-Gly-OHxd (4h): Starting from **3h** (70 mg, 0.09 mmol) a colorless solid (18 mg, 0.05 mmol, 35%) was obtained. $[\alpha]_D^{20}$ = -18.5° (c = 0.2, MeOH); 1H NMR (CD_3OD , 400 MHz): δ = 1.37 (s, 9H, tBu), 1.38 (d, 3H, J = 7.3 Hz, CH_3 Ala), 1.75 (d, 2H, J = 6.7 Hz, CH_3 Hxd), 1.86–2.38 (m, 4H, β - CH_2 , γ - CH_2 Pro), 3.07 (dd, 1H, J = 8.5, 14.3 Hz, β - CH_2 Cys), 3.26 (dd, 1H, J = 5.2, 14.1 Hz, β - CH_2 Cys), 3.52–3.74 (m, 2H, δ - CH_2 Pro), 3.89–4.01 (m, 2H, α - CH_2 Gly), 4.05–4.17 (m, 3H, α - CH_2 Gly, α -CH Cys), 4.36–4.46 (m, 2H, $2 \times \alpha$ -CH), 4.62 (d, 2H, J = 6.6 Hz, CH_2 Hxd), 5.61 (ddd, 1H, J = 13.8, 7.1, 7.1 Hz, $CH=CHCH_3$), 5.72–5.81 (m, 1H, $CH_2CH=CH$), 6.06 (dd, 1H, J = 15.1, 10.5 Hz, $CH=CHCH_3$), 6.28 (dd, 1H, J = 15.2, 10.4 Hz, $CH_2CH=CH$); ESI-MS: m/z : calcd for $C_{25}H_{41}N_5O_6S_2$: 572.3; found: 572.3 $[M+H]^+$, 594.3 $[M+Na]^+$; FAB-HRMS: m/z : 571.2521 $[M]^+$.

H-Lys-Cys-Gly-Val-Phe-Gly-OHxd (5): Peptide **4d** (4.2 mg, 5.4 μ mol) was dissolved in a degassed solution (800 μ L) of 0.1 M ammonium bicar-

bonate/DMF 5:3 under an argon atmosphere. DTT (12 μ L, 0.14 mmol) dissolved in degassed DMF (12 μ L) was added to the peptide solution and the mixture was stirred under argon at room temperature for 2.5 h. The crude product was dried by lyophilization, redissolved in DMF, filtered and purified by reversed-phase HPLC, to afford a colorless solid (2.7 mg, 3.9 μ mol, 72%); ^1H NMR (CD_3OD , 400 MHz): δ =0.73 (d, 3H, J =6.8 Hz, CH_3 Val), 0.82 (d, 3H, J =6.8 Hz, CH_3 Val), 1.28–1.33 (m, 1H, SH), 1.47–1.54 (m, 2H, γ - CH_2 Lys), 1.63–1.72 (m, 2H, δ - CH_2 Lys), 1.74 (d, 3H, J =6.6 Hz, CH_3 Hxd), 1.86–1.92 (m, 2H, β - CH_2 Lys), 2.00–2.07 (m, 1H, β -CH Val), 2.81–3.03 (m, 4H, ϵ - CH_2 Lys, β - CH_2 Phe), 3.20 (dt, 2H, J =8.8, 5.0 Hz, β - CH_2 Cys), 3.77 (d, 1H, J =16.1 Hz, α -CH Val), 3.94 (s, 2H, α - CH_2 Gly), 3.98 (t, 1H, J =6.4 Hz, α -CH Lys), 4.07 (t, 2H, J =6.4 Hz, α - CH_2 Gly), 4.53 (t, 2H, α -CH), 4.62 (d, 2H, J =6.6 Hz, CH_2 Hxd), 4.66 (t, 2H, J =5.0 Hz, α -CH), 5.58–5.65 (m, 1H, $\text{CH}=\text{CHCH}_3$), 5.72–5.81 (m, 1H, $\text{CH}_2\text{CH}=\text{CH}$), 6.03–6.10 (ddd, 1H, J =15.0, 10.4 Hz, $\text{CH}=\text{CHCH}_3$), 6.25–6.32 (dd, 1H, J =15.1, 10.5 Hz, $\text{CH}_2\text{CH}=\text{CH}$), 7.18–7.30 ppm (m, 5H, Ar Phe); MALDI-TOF: m/z : calcd for $\text{C}_{33}\text{H}_{41}\text{N}_7\text{O}_7\text{S}$: 690.4; found: 691.0 [$M+\text{H}$] $^+$, 713.0 [$M+\text{Na}$] $^+$, 729.0 [$M+\text{K}$] $^+$.

General procedure for the solid-phase synthesis of the maleimido-peptides 6 using Wang resin: Wang resin (Novabiochem) was loaded with Fmoc-Gly-OH using 4 equiv amino acid, 4 equiv DIC and 0.1 equiv DMAP in DMF (overnight, quantitative loading by UV-Fmoc determination). After peptide chain assembly was complete (SPPS), the peptidyl resin was treated with TFA containing 2.5% TIS and 2.5% H_2O as scavengers for 2–3 h. After partial evaporation of the resulting solution, the crude peptide was precipitated by adding diethyl ether, filtered and washed with diethyl ether. Finally the products were redissolved in a mixture of MeOH/water and lyophilized.

N-Maleimido-Gly-Tyr-Thr-Gly-OH (6a): Starting from Wang resin (301 mg) loaded with Fmoc-glycine (0.31 mmol) a colorless solid (91 mg, 0.19 mmol, 62%) was obtained. $[\alpha]_{\text{D}}^{20}$ =+16.2° (c =0.4, DMF); ^1H NMR (D_2O , 400 MHz): δ =0.99 (d, 3H, J =6.4 Hz, CH_3 Thr), 2.81–2.90 (m, 2H, β - CH_2 Tyr), 3.77 (s, 2H, α - CH_2 Gly), 4.02–4.07 (m, 1H, α -CH Thr), 4.10 (d, 2H, J =2.8 Hz, α - CH_2 Gly), 4.17 (d, 1H, J =4.0 Hz, β -CH Thr), 4.50 (t, 1H, J =7.8 Hz, α -CH Tyr), 6.66 (d, 2H, J =8.4 Hz, Ar Tyr), 6.75 (s, 2H, $\text{CH}=\text{CH}$), 6.97 ppm (d, 2H, J =8.4 Hz, Ar Tyr); ^{13}C NMR (D_2O , 100 MHz): δ =18.8, 36.4, 39.9, 41.4, 55.7, 58.9, 67.2, 115.7, 127.8, 130.7, 134.7, 154.5, 169.0, 171.7, 171.9, 172.7, 173.2 ppm; ESI-MS: m/z : calcd for $\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_9$: 477.1; found: 477.0 [$M+\text{H}$] $^+$, 499.0 [$M+\text{Na}$] $^+$; FAB-HRMS: m/z : 499.1458 [$M+\text{Na}$] $^+$.

N-Maleimido-Gly-Thr-Gln-Phe-His-Gly-OH (6b): Starting from Wang resin (276 mg) loaded with Fmoc-glycine (0.32 mmol), a colorless solid (140 mg, 0.19 mmol, 60%) was obtained. $[\alpha]_{\text{D}}^{20}$ =−20.2° (c =1.0, DMF); ^1H NMR ($[\text{D}_6]\text{DMSO}$, 400 MHz): δ =1.00 (d, 3H, J =6.2 Hz, CH_3 Thr), 1.61–1.68 (m, 1H, β - CH_2 Gln), 1.80–1.88 (m, 1H, β - CH_2 Gln), 1.99–2.11 (m, 2H, γ - CH_2 Gln), 2.78 (dd, 1H, J =13.6, 9.4 Hz, β - CH_2), 2.83–3.01 (m, 2H, $2\times 1\text{H}$ β - CH_2), 3.11 (dd, 1H, J =15.2, 5.4 Hz, β - CH_2), 3.68–3.84 (m, 2H, α - CH_2 Gly), 3.91–3.98 (m, 1H, β -CH Thr), 4.14–4.30 (m, 4H, α - CH_2 Gly, $2\times\alpha$ -CH), 4.43 (dd, 1H, J =8.8, 5.4 Hz, α -CH), 4.62 (dd, 1H, J =7.7, 6.0 Hz, α -CH), 6.82 (brs, 1H, CH His), 7.08 (s, 2H, $\text{CH}=\text{CH}$), 7.16–7.26 (m, 5H, Ar Phe), 7.35 (brs, 1H, CH His), 8.02 (d, 1H, J =7.6 Hz, CONH), 8.07 (d, 1H, J =7.5 Hz, CONH), 8.14 (t, 1H, J =5.8 Hz, CONH), 8.20 (d, 1H, J =8.1 Hz, CONH), 8.35 (d, 1H, J =8.5 Hz, CONH), 8.95 ppm (s, 1H, NH); ESI-MS: m/z : calcd for $\text{C}_{32}\text{H}_{39}\text{N}_5\text{O}_{11}$: 726.3; found: 726.3 [$M+\text{H}$] $^+$; MALDI-TOF: m/z : 726.9 [$M+\text{H}$] $^+$, 748.9 [$M+\text{Na}$] $^+$, 764.9 [$M+\text{K}$] $^+$.

N-Maleimido-Gly-Ser-Glu-Trp-Ile-Gly-OH (6c): Starting from Wang resin (350 mg) loaded with Fmoc-glycine (0.40 mmol) a colorless solid (152 mg, 0.21 mmol, 53%) was obtained. $[\alpha]_{\text{D}}^{20}$ =−24.5 (c =0.7, DMF); ^1H NMR ($[\text{D}_6]\text{DMSO}$, 400 MHz): δ =0.80 (t, 3H, J =7.4 Hz, CH_3 Ile), 0.84 (d, 3H, J =6.8 Hz, CH_3 Ile), 1.00–1.13 (m, 1H, CH_2 Ile), 1.38–1.47 (m, 1H, CH_2 Ile), 1.65–1.75 (m, 2H, β - CH_2 Glu), 1.83–1.92 (m, 1H, β -CH Ile), 2.19 (t, 2H, J =8.0 Hz, γ - CH_2 Glu), 2.89 (dd, 1H, J =14.8, 8.6 Hz, β - CH_2 Trp), 3.11 (dd, 1H, J =14.8, 5.4 Hz, β - CH_2 Trp), 3.49–3.59 (m, 2H, β - CH_2 Ser), 3.65–3.80 (m, 2H, α - CH_2 Gly), 4.12 (d, 2H, J =5.5 Hz, α - CH_2 Gly), 4.17–4.28 (m, 2H, $2\times\alpha$ -CH), 4.34 (dd, 1H, J =7.7, 5.9 Hz, α -CH), 4.59 (dd, 1H, J =8.1, 5.7 Hz, α -CH), 6.96 (t, 1H, J =8.0 Hz, Ar Trp), 7.04 (t, 1H, J =8.0 Hz, Ar Trp), 7.07 (s, 2H, $\text{CH}=\text{CH}$),

7.11 (d, 1H, J =2.3 Hz, Ar Trp), 7.30 (d, 1H, J =8.0 Hz, Ar Trp), 7.56 (d, 1H, J =7.8 Hz, Ar Trp), 7.86 (d, 1H, J =8.9 Hz, CONH), 8.02 (d, 1H, J =8.0 Hz, CONH), 8.10–8.12 (m, 2H, $2\times\text{CONH}$), 8.33 (d, 1H, J =7.8 Hz, CONH), 10.76 ppm (d, 1H, J =2.0 Hz, NH Trp); ESI-MS: m/z : calcd for $\text{C}_{33}\text{H}_{41}\text{N}_7\text{O}_{12}$: 728.3; found: 728.1 [$M+\text{H}$] $^+$; MALDI-TOF: m/z : 750.8 [$M+\text{Na}$] $^+$, 766.8 [$M+\text{K}$] $^+$.

N-Maleimido-Gly-Ala-Lys-Thr-Ser-Ala-Glu-Ser-Tyr-Ser-Gly-OH (6d): Starting from Wang resin (285 mg) loaded with Fmoc-glycine (0.29 mmol), a colorless solid (196 mg, 0.17 mmol, 59%) was obtained. $[\alpha]_{\text{D}}^{20}$ =−7.7° (c =0.3, DMF); ^1H NMR (D_2O , 400 MHz): δ =1.05 (d, 3H, J =6.4 Hz, CH_3 Thr), 1.20–1.37 (m, 2H, γ - CH_2 Lys), 1.26 (d, 6H, J =7.2 Hz, $2\times\text{CH}_3$ Ala), 1.47–1.57 (m, 2H, δ - CH_2 Lys), 1.58–1.78 (m, 2H, β - CH_2 Lys), 1.79–1.97 (m, 2H, β - CH_2 Glu), 2.31 (t, 2H, J =7.5 Hz, γ - CH_2 Glu), 2.82–2.92 (m, 4H, ϵ - CH_2 Lys, β - CH_2 Tyr), 3.63–3.79 (m, 6H, $3\times\beta$ - CH_2 Ser), 3.83 (s, 2H, α - CH_2 Gly), 4.10–4.33 (m, 11H, $8\times\alpha$ -CH, α - CH_2 , β -CH Thr), 4.50 (t, 1H, J =7.5 Hz, α -CH), 6.67 (d, 2H, J =8.5 Hz, Ar Tyr), 6.80 (s, 2H, $\text{CH}=\text{CH}$), 7.00 ppm (d, 2H, J =8.5 Hz, Ar Tyr); ESI-MS: m/z : calcd for $\text{C}_{47}\text{H}_{68}\text{N}_{12}\text{O}_{21}$: 1137.5; found: 1137.5 [$M+\text{H}$] $^+$; MALDI-TOF: m/z : 1137.7 [$M+\text{H}$] $^+$, 1159.7 [$M+\text{Na}$] $^+$, 1175.6 [$M+\text{K}$] $^+$; FAB-LRMS: m/z : 1136.45 [M] $^+$.

N-Maleimido- β -Ala-Ser-Lys-Thr-Lys(dansyl)-Gly-OH (6e): Starting from Wang resin (147 mg) loaded with Fmoc-glycine (0.13 mmol) a colorless solid (67 mg, 0.074 mmol, 57%) was obtained. $[\alpha]_{\text{D}}^{20}$ =−19.0° (c =0.4, MeOH); ^1H NMR (CD_3OD , 400 MHz): δ =1.16 (d, 3H, J =6.4 Hz, CH_3 Thr), 1.22–1.43 (m, 4H, $2\times\gamma$ - CH_2 Lys), 1.47–1.73 (m, 6H, $2\times\alpha$ - CH_2 Lys, β - CH_2 Lys), 1.73–1.81 (m, 1H, β - CH_2 Lys), 1.94–2.03 (m, 1H, β - CH_2 Lys), 2.54 (t, 2H, J =6.7 Hz, CH_2 β Ala), 2.84 (t, 2H, J =6.3 Hz, ϵ - CH_2 Lys), 2.94–3.00 (m, 8H, ϵ - CH_2 Lys, $2\times\text{CH}_3$ Dan), 3.68–3.83 (m, 4H, CH_2 β Ala, β - CH_2 Ser), 3.84–3.93 (m, 2H, α - CH_2 Gly), 4.13–4.19 (m, 1H, β -CH Thr), 4.25 (dd, 1H, J =9.1, 4.8 Hz, α -CH), 4.29–4.34 (m, 2H, $2\times\alpha$ -CH), 4.43 (dd, 1H, J =9.7, 4.7 Hz, α -CH), 6.79 (s, 2H, $\text{CH}=\text{CH}$), 7.39 (d, 1H, J =7.6 Hz, Ar Dan), 7.59–7.64 (m, 2H, Ar Dan), 8.20 (d, 1H, J =7.3 Hz, Ar Dan), 8.42 (d, 1H, J =8.7 Hz, Ar Dan), 8.53 ppm (d, 1H, J =8.6 Hz, Ar Dan); ESI-MS: m/z : calcd for $\text{C}_{40}\text{H}_{57}\text{N}_9\text{O}_{13}$: 904.4; found: 904.7 [$M+\text{H}$] $^+$; MALDI-TOF: m/z : 904.9 [$M+\text{H}$] $^+$, 926.9 [$M+\text{Na}$] $^+$, 942.9 [$M+\text{K}$] $^+$; FAB-HRMS: m/z : 904.3904 [$M+\text{H}$] $^+$.

Fmoc-Lys(dansyl)-OH (7): Fmoc-Lys(Boc)-OH (500 mg, 1.1 mmol) was treated with TFA/DCM 1:1 (10 mL) for 80 min at room temperature. Excess of TFA was removed by coevaporation with toluene and the peptide was dried under reduced pressure (colorless oil, Fmoc-Lys-OH-TFA). The side-chain deprotected peptide was dissolved in MeOH/ H_2O 5:2 (35 mL), followed by addition of NaHCO_3 (270 mg, 3.2 mmol) and dansyl chloride (432 mg, 1.6 mmol). The reaction mixture was stirred for 19 h at room temperature. The pH was adjusted to 2 by adding HCl 1 M and the product was extracted $3\times$ with DCM, washed with brine, dried over Na_2SO_4 and concentrated in vacuo. Purification was performed by silica gel flash chromatography, eluting first with DCM, followed by DCM/MeOH 10:1, affording (336 mg, 0.56 mol, 52%) a light yellow oil (fluorescent). R_f =0.10 (DCM/MeOH 10:1); $[\alpha]_{\text{D}}^{20}$ =−5.5° (c =0.4, CHCl_3); ^1H NMR (CD_3OD , 400 MHz): δ =1.07–1.34 (m, 4H, γ - CH_2 , δ - CH_2), 1.35–1.70 (m, 2H, β - CH_2), 2.77–2.84 (m, 2H, ϵ - CH_2), 2.79 (s, 6H, $2\times\text{CH}_3$ Dan), 3.95 (dd, 1H, J =4.5, 9.2 Hz, α -CH), 4.20 (t, 1H, J =6.7 Hz, CH Fmoc), 4.33 (d, 2H, J =6.0 Hz, CH_2 Fmoc), 7.06 (d, 1H, J =7.1 Hz, Ar Dan), 7.10–7.21 (m, 2H, Ar Dan), 7.27 (t, 2H, J =7.4 Hz, Ar Fmoc), 7.40 (t, 2H, J =7.4 Hz, Ar Fmoc), 7.50 (d, 2H, J =7.0 Hz, Ar Fmoc), 7.64 (d, 2H, J =7.5 Hz, Ar Fmoc), 8.17 (d, 1H, J =7.2 Hz, Ar Dan), 8.34 (d, 1H, J =8.7 Hz, Ar Dan), 8.51 ppm (d, 1H, J =8.5 Hz, Ar Dan); ^{13}C NMR (CD_3OD , 100 MHz): δ =22.4, 29.1, 31.8, 42.8, 45.6, 47.3, 54.0, 67.2, 115.5, 119.3, 120.0, 123.5, 125.4, 127.1, 127.8, 128.5, 129.6, 129.8, 130.0, 130.5, 135.1, 141.4, 143.9, 144.1, 152.0, 156.8, 174.8 ppm; MALDI-TOF: m/z : calcd for $\text{C}_{33}\text{H}_{35}\text{N}_3\text{O}_6\text{S}$: 600.2; found: 600.4 [$M-\text{H}$] $^+$, 624.4 [$M+\text{Na}$] $^+$.

General procedure for the Diels–Alder ligation: Diene- and dienophile-peptides were dissolved in water at room temperature to a concentration of 10 mM. If required, methanol or DMF was added in minimum amounts to support peptide solubilization (Table 3). The ligation was monitored by HPLC analysis. After appropriate reaction time (20–48 h), the ligation

product was directly purified by reversed phase HPLC and finally lyophilized.

H-Val-Ala-Gly-O-cyclo-N-Gly-Tyr-Thr-Gly-OH (8a): Stirring of Val-Ala-Gly-OHxd (**1**; 11 mg, 0.033 mmol) and **6a** (19 mg, 0.039 mmol) in H₂O/MeOH 10:3 (1 mL) for 20 h gave a colorless solid (23 mg, 0.029 mmol, 87%). $[\alpha]_D^{20} = +11.7^\circ$ ($c = 1.0$, DMF); ¹H NMR (CD₃OD, 400 MHz): $\delta = 1.03$ (d, 3H, $J = 6.8$ Hz, CH₃ Val), 1.06 (d, 3H, $J = 6.9$ Hz, CH₃ Val), 1.14 (d, 3H, $J = 6.4$ Hz, CH₃ Thr), 1.39 (d, 3H, $J = 7.2$ Hz, CH₃ Ala), 1.40 (d, 3H, $J = 7.0$ Hz, CH₃ cyclo), 2.14–2.24 (m, 1H, β -CH Val), 2.44–2.52 (m, 1H, H₆), 2.65–2.72 (m, 1H, H₃), 2.88 (dd, 1H, $J = 13.7$, 7.8 Hz, β -CH₂ Tyr), 3.02 (dd, 1H, $J = 13.9$, 6.3 Hz, β -CH₂ Tyr), 3.18 (dd, 1H, $J = 8.4$, 7.2 Hz, H₅), 3.39 (dd, 1H, $J = 8.5$, 6.1 Hz, H₄), 3.67 (d, 1H, $J = 5.6$ Hz, α -CH Val), 3.84–4.09 (m, 6H, $3 \times \alpha$ -CH₂ Gly), 4.13–4.20 (m, 1H, β -CH Thr), 4.31 (t, 1H, $J = 3.5$ Hz, α -CH), 4.47 (ddd, 1H, $J = 14.3$, 7.1, 3.2 Hz, CH₂ cyclo), 4.51–4.63 (m, 3H, CH₂ cyclo, $2 \times \alpha$ -CH), 5.71–5.79 (m, 2H, CH=CH), 6.69 (d, 2H, $J = 7.4$ Hz, Ar Tyr), 7.04 ppm (d, 2H, $J = 8.5$ Hz, Ar Tyr); ESI-MS: m/z : calcd for C₃₇H₅₁N₇O₁₃: 802.4; found: 802.4 $[M+H]^+$; MALDI-TOF: m/z : 802.9 $[M+H]^+$, 824.9 $[M+Na]^+$, 840.9 $[M+K]^+$.

H-Lys-Pro-Phe-Leu-Gly-O-cyclo-N-Gly-Tyr-Thr-Gly-OH (8b): Stirring of **4a** (10 mg, 0.015 mmol) and **6a** (7 mg, 0.015 mmol) at room temperature in H₂O/MeOH 4:1 (1 mL) for 24 h gave a colorless solid (10 mg, 0.009 mmol, 60%). $[\alpha]_D^{20} = -23.2^\circ$ ($c = 0.3$, MeOH); ¹H NMR (CD₃OD, 400 MHz): $\delta = 0.90$ (d, 3H, $J = 6.0$ Hz, CH₃ Leu), 0.94 (d, 3H, $J = 6.0$ Hz, CH₃ Leu), 1.14 (d, 3H, $J = 6.4$ Hz, CH₃ Thr), 1.39/1.40 (ratio 1:1, d, 3H, $J = 7.3$ Hz, CH₃ cyclo), 1.51–1.74 (m, 7H, γ -CH₂ Lys, δ -CH₂ Lys, β -CH₂ Leu, γ -CH Leu), 1.87–2.09 (m, 5H, γ -CH₂ Pro, β -CH₂ Lys, β -CH₂ Pro), 2.16–2.26 (m, 1H, β -CH₂ Pro), 2.45–2.51 (m, 1H, H₆), 2.64–2.73 (m, 1H, H₃), 2.87 (dd, 1H, $J = 13.9$, 7.8 Hz, β -CH₂ Tyr), 2.92–3.15 (m, 5H, β -CH₂ Tyr, ϵ -CH₂ Lys, β -CH₂ Phe), 3.13/3.20 (ratio 1:1, dd, 1H, $J = 8.5$, 7.2/8.4, 7.2 Hz, H₅), 3.38/3.46 (ratio 1:1, dd, 1H, $J = 8.4$, 6.2/8.5, 6.3 Hz, H₄), 3.55–3.64 (m, 1H, δ -CH₂ Pro), 3.67–3.74 (m, 1H, δ -CH₂ Pro), 3.83–4.11 (m, 6H, $3 \times \alpha$ -CH₂ Gly), 4.13–4.19 (m, 1H, β -CH Thr), 4.23–4.28 (m, 1H, α -CH), 4.32 (t, 1H, $J = 3.7$ Hz, α -CH), 4.39–4.46 (m, 1H, CH₂ cyclo), 4.48–4.52 (m, 1H, CH₂ cyclo), 4.54–4.65 (m, 4H, $4 \times \alpha$ -CH), 5.70–5.78 (m, 2H, CH=CH), 6.69 (d, 2H, $J = 8.4$ Hz, Ar Tyr), 7.03 (d, 2H, $J = 8.5$ Hz, Ar Tyr), 7.18–7.31 ppm (m, 5H, Ar Phe); ESI-MS: m/z : calcd for C₅₅H₇₆N₁₀O₁₅: 1117.6; found: 1117.6 $[M+H]^+$; MALDI-TOF: m/z : 1118.0 $[M+H]^+$, 1140.0 $[M+Na]^+$, 1156.0 $[M+K]^+$.

H-Lys-Leu-Gly-Lys(Mtt)-Ala-Gly-O-cyclo-N-Gly-Thr-Gln-Phe-His-Gly-OH (8c): Compounds **4d** (2.0 mg, 2.2 μ mol) and **6b** (1.6 mg, 2.2 μ mol) were stirred in H₂O/MeOH 10:1 (200 μ L) for 24 h. Partial removal of Mtt protection groups from lysine side chain took place during the reaction, giving two cycloadducts **8c** (with Mtt) and **8c'** (without Mtt). These compounds were isolated by reversed-phase HPLC in 74% overall yield. **8c**: colorless solid (1.3 mg, 0.80 μ mol, 43%), compound with Mtt group. ¹H NMR (CD₃OD, 400 MHz): $\delta = 0.94$ (d, 3H, $J = 6.7$ Hz, CH₃ Leu), 0.96 (d, 3H, $J = 6.7$ Hz, CH₃ Leu), 1.20 (d, 3H, $J = 6.2$ Hz, CH₃ Thr), 1.39 (d, 6H, $J = 7.1$ Hz, CH₃ Ala, CH₃ cyclo), 1.3–2.3 (m, CH₂ Lys, CH₂ Gln, CH₂CH Leu), 2.37 (s, 3H, CH₃ Mtt), 2.44–2.51 (m, 1H, H₆), 2.63–2.72 (m, 1H, H₃), 2.9–3.4 (m, β -CH₂ Phe, β -CH₂ His, β -CH₂ Lys, H₅, H₄), 3.7–4.7 (m, 5H, α -CH₂ Gly, α -CH, CH₂ cyclo), 5.72–5.76 (m, 2H, CH=CH), 7.17–7.49 (m, 20H, Ar Phe, Ar Mtt, CH His), 8.73 ppm (brs, 1H, NH His); MALDI-TOF: m/z : calcd for C₈₃H₁₁₁N₁₇O₁₈: 1378.7; found: 1379.1 $[(M-Mtt)+H]^+$, 1417.1 $[(M-Mtt)+K]^+$, 257.3 $[Mtt]^+$. **8c'**: colorless solid (0.8 mg, 0.58 μ mol, 31%), compound without Mtt group. ¹H NMR (CD₃OD, 400 MHz): $\delta = 0.95$ (d, 3H, $J = 6.6$ Hz, CH₃ Leu), 0.98 (d, 3H, $J = 6.6$ Hz, CH₃ Leu), 1.20 (d, 3H, $J = 6.4$ Hz, CH₃ Thr), 1.40 (d, 6H, $J = 7.5$ Hz, CH₃ Ala, CH₃ cyclo), 1.4–2.3 (m, CH₂ Lys, CH₂ Gln, CH₂CH Leu), 2.47–2.53 (m, 1H, H₆), 2.67–2.74 (m, 1H, H₃), 2.9–3.4 (m, β -CH₂ Phe, β -CH₂ His, β -CH₂ Lys, H₅, H₄), 3.7–4.7 (m, α -CH₂ Gly, α -CH, CH₂ cyclo), 5.73–5.77 (m, 2H, CH=CH), 7.35 (brs, 1H, CH His), 7.18–7.29 (m, 5H, Ar Phe), 8.71 ppm (brs, 1H, NH His); MALDI-TOF: m/z : calcd for C₆₃H₉₅N₁₇O₁₈: 1378.7; found: 1379.5 $[M+H]^+$, 1401.4 $[M+Na]^+$, 1417.4 $[M+K]^+$.

H-Lys-Leu-Gly-Lys(Mtt)-Ala-Gly-O-cyclo-N-Gly-Ser-Glu-Trp-Ile-Gly-OH (8d): Compounds **4d** (2.0 mg, 2.2 μ mol) and **6c** (1.6 mg, 2.2 μ mol) were stirred in H₂O/DMF 4:1 (200 μ L) for 48 h. Partial removal of Mtt

protection groups from lysine side chain took place during the reaction, giving two cycloadducts **8d** (with Mtt) and **8d'** (without Mtt). These compounds were isolated by reversed-phase HPLC in 64% overall yield. **8d**: colorless solid (1.2 mg, 0.73 μ mol, 40%), compound with Mtt group. ¹H NMR (CD₃OD, 400 MHz): $\delta = 1.40$ (d, 3H, CH₃ Hxd), 2.38 (s, 3H, Mtt), 5.67–5.75 (m, 2H, CH=CH), 7.1–7.5 ppm (Mtt); MALDI-TOF: m/z : calcd for C₈₄H₁₁₃N₁₅O₁₉: 1380.7; found: 1381.5 $[(M-Mtt)+H]^+$, 1403.4 $[(M-Mtt)+Na]^+$, 1419.4 $[(M-Mtt)+K]^+$, 257.3 $[Mtt]^+$. **8d'**: colorless solid (0.6 mg, 0.43 μ mol, 24%), compound without Mtt group. ¹H NMR ([D₆]DMSO, 400 MHz): $\delta = 1.2$ (d, 3H, CH₃ Hxd), 5.02–5.75 ppm (m, 2H, CH=CH); MALDI-TOF: m/z : calcd for C₆₄H₉₇N₁₅O₁₉: 1380.7; found: 1381.3 $[M+H]^+$, 1403.2 $[M+Na]^+$, 1419.2 $[M+K]^+$.

H-Pro-Cys(SiBu)-Ser-Met-Gly-O-cyclo-N-Gly-Tyr-Thr-Gly-OH (8e): Stirring of **4b** (2.0 mg, 3.0 μ mol) and **6a** (3.5 mg, 7.3 μ mol) in H₂O/MeOH 3:2 (500 μ L) for 24 h gave a colorless solid (1.1 mg, 0.97 μ mol, 32%). $[\alpha]_D^{20} = -31.5^\circ$ ($c = 0.1$, MeOH); ¹H NMR (CD₃OD, 400 MHz): $\delta = 1.14$ (d, 3H, $J = 6.5$ Hz, CH₃ Thr), 1.35 (brs, 9H, *t*Bu Cys), 1.40/1.41 (ratio 1:1, d, 3H, $J = 7.0$ Hz, CH₃ cyclo), 1.89–2.21 (m, 5H, γ -CH₂ Pro, β -CH₂ Pro, β -CH₂ Met), 2.08 (s, 3H, CH₃ Met), 2.42–2.65 (m, 4H, β -CH₂ Pro, H₆, γ -CH₂ Met), 2.66–2.72 (m, 1H, H₃), 2.85–2.92 (m, 1H, β -CH₂ Tyr), 2.97–3.05 (m, 2H, β -CH₂ Tyr, β -CH₂ Cys), 3.2–3.5 (m, β -CH₂ Cys, H₅, H₄), 3.56–4.19 (m, 11H, δ -CH₂ Pro, β -CH₂ Ser, $3 \times \alpha$ -CH₂ Gly, β -CH Thr), 4.29–4.45 (m, 3H, $3 \times \alpha$ -CH), 4.53–4.66 (m, 4H, $2 \times \alpha$ -CH, CH₂ cyclo), 4.69–4.73 (m, 1H, α -CH), 5.68–5.80 (m, 2H, CH=CH), 6.68/6.69 (ratio 1:1, d, 2H, $J = 8.5$ Hz, Ar Tyr), 7.03/7.04 ppm (ratio 1:1, d, 2H, $J = 8.3$ Hz, Ar Tyr); ESI-MS: m/z : calcd for C₄₉H₇₁N₉O₁₆S₃: 1138.4; found: 1138.4 $[M+H]^+$; MALDI-TOF: m/z : 139.1 $[M+H]^+$, 1161.0 $[M+Na]^+$, 1177.0 $[M+K]^+$.

H-Lys-Phe-Pro-Ile-Gly-Leu-Phe-Gly-O-cyclo-N-Gly-Ala-Lys-Thr-Ser-Ala-Glu-Ser-Tyr-Ser-Gly-OH (8f): Stirring of **4f** (2.8 mg, 2.9 μ mol) and **6d** (3.3 mg, 2.9 μ mol) in H₂O (300 μ L) for 48 h gave a colorless solid (3.5 mg, 1.7 μ mol, 69%). $[\alpha]_D^{20} = -22.9^\circ$ ($c = 0.1$, MeOH); ¹H NMR (CD₃OD, 400 MHz): $\delta = 0.81$ –1.00 (m, 12H, $2 \times$ CH₃ Ile, $2 \times$ CH₃ Leu), 1.19 (d, 3H, $J = 6.3$ Hz, CH₃ Thr), 1.19–1.77 (m, 22H, $2 \times \beta$ - γ - δ -CH₂ Lys, $2 \times$ CH₃ Ala, CH₂ Ile, β -CH₂ Leu), 1.43 (d, 3H, $J = 7.3$ Hz, CH₃ cyclo), 1.81–2.20 (m, 8H, β -CH₂ Glu, β -CH₂ Pro, γ -CH₂ Pro, γ -CH Leu, β -CH Ile), 2.41–2.46 (m, 2H, γ -CH₂ Glu), 2.47–2.53 (m, 1H, H₆), 2.66–2.74 (m, 1H, H₃), 2.89–3.26 (m, 11H, $2 \times \epsilon$ -CH₂ Lys, β -CH₂ Tyr, $2 \times \beta$ -CH₂ Phe, H₅), 3.34–3.57 (m, 3H, H₄, ϵ -CH₂ Pro), 3.71–4.00 (m, 14H, $3 \times \beta$ -CH₂ Ser, $4 \times \alpha$ -CH₂ Gly), 4.06–4.68 (m, 15H, $13 \times \alpha$ -CH, CH₂ cyclo), 5.71–5.77 (m, 2H, CH=CH), 6.68 (d, 2H, $J = 8.4$ Hz, Ar Tyr), 7.08 (d, 2H, $J = 8.5$ Hz, Ar Tyr), 7.17–7.34 ppm (m, 10H, Ar Phe); ESI-MS: m/z : calcd for C₉₈H₁₄₃N₂₁O₃₀: 1048; found: 1048 $[M+H]^+$; MALDI-TOF: m/z : 2098 $[M+H]^+$, 2120 $[M+Na]^+$, 2135 $[M+K]^+$.

H-Lys-Phe-Pro-Ile-Gly-Leu-Gly-Phe-Gly-O-cyclo-N-Gly-Ala-Lys-Thr-Ser-Ala-Glu-Ser-Tyr-Ser-Gly-OH (8g): Stirring of **4g** (2.8 mg, 2.7 μ mol) and **6d** (3.1 mg, 2.7 μ mol) in H₂O (270 μ L) for 48 h gave a colorless solid (3.2 mg, 1.5 μ mol, 67%). $[\alpha]_D^{20} = -22.2^\circ$ ($c = 0.1$, MeOH); ¹H NMR (CD₃OD, 400 MHz): $\delta = 0.82$ –1.00 (m, 12H, $2 \times$ CH₃ Ile, $2 \times$ CH₃ Leu), 1.19 (d, 3H, $J = 6.3$ Hz, CH₃ Thr), 1.20–1.74 (m, 22H, $2 \times \beta$ - γ - δ -CH₂ Lys, $2 \times$ CH₃ Ala, CH₂ Ile, β -CH₂ Leu), 1.43 (d, 3H, $J = 7.3$ Hz, CH₃ cyclo), 1.83–2.19 (m, 8H, β -CH₂ Glu, β - γ -CH₂ Pro, γ -CH Leu, β -CH Ile), 2.41–2.47 (m, 2H, γ -CH₂ Glu), 2.47–2.53 (m, 1H, H₆), 2.66–2.74 (m, 1H, H₃), 2.89–3.26 (m, 11H, $2 \times \epsilon$ -CH₂ Lys, β -CH₂ Tyr, $2 \times \beta$ -CH₂ Phe, H₅), 3.32–3.57 (m, 3H, H₄, ϵ -CH₂ Pro), 3.70–4.02 (m, 16H, $3 \times \beta$ -CH₂ Ser, $5 \times \alpha$ -CH₂ Gly), 4.06–4.68 (m, 16H, $14 \times \alpha$ -CH, CH₂ cyclo), 5.73–5.77 (m, 2H, CH=CH), 6.68 (d, 2H, $J = 8.6$ Hz, Ar Tyr), 7.06–7.09 (m, 2H, Ar Tyr), 7.17–7.33 ppm (m, 10H, Ar Phe); ESI-MS: m/z : calcd for C₁₀₀H₁₄₆N₂₂O₃₁: 1076; found: 1076 $[M+H]^+$; MALDI-TOF: m/z : 2155 $[M+H]^+$, 2177 $[M+Na]^+$, 2193 $[M+K]^+$.

Pimeloyl succinimidylyl hexadienyl ester (9): Pimeloyl diene ester **10** (160 mg, 0.66 mmol), *N*-hydroxysuccinimide (96 mg, 0.83 mmol) and DMAP (8.6 mg, 0.07 mmol) were dissolved in dry THF (6 mL). Then DIC (118 μ L, 0.76 mmol) was added dropwise at room temperature and the reaction mixture was allowed to react overnight. The solvent was removed under reduced pressure and the urea was precipitated by adding EtOAc/cyclohexane and separated by filtration. The crude product was purified by silica gel flash chromatography cyclohexane/EtOAc 3:1 $\rightarrow$

1:1, to give a colorless oil (184 mg, 0.55 mmol, 82 %). R_f = 0.31 (cyclohexane/EtOAc 1:1); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): δ = 1.38–1.46 (m, 2H, CH_2), 1.60–1.68 (m, 2H, CH_2), 1.69–1.77 (m, 2H, CH_2), 1.73 (d, 3H, J = 6.6 Hz, CH_3 Hxd), 2.30 (t, 2H, J = 7.5 Hz, CH_2), 2.58 (t, 2H, J = 7.5 Hz, CH_2), 2.80 (brs, 4H, $2 \times \text{CH}_2$ NHS), 4.54 (d, 2H, J = 6.6 Hz, CH_2 Hxd), 5.59 (ddd, 1H, J = 14.0, 6.6, 6.6 Hz, $\text{CH}=\text{CHCH}_3$), 5.68–5.77 (m, 1H, $\text{CH}_2\text{CH}=\text{CH}$), 6.02 (dd, 1H, J = 15.0, 10.7 Hz, $\text{CH}=\text{CHCH}_3$), 6.21 ppm (dd, 1H, J = 15.1, 10.5 Hz, $\text{CH}_2\text{CH}=\text{CH}$); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): δ = 18.3, 24.4, 24.5, 25.8, 28.4, 30.9, 34.1, 65.0, 123.9, 130.6, 131.4, 135.0, 168.7, 169.4, 173.4 ppm; ESI-MS: m/z : calcd for $\text{C}_{17}\text{H}_{23}\text{NaNO}_6$: 360.1; found: 360.1 $[\text{M}+\text{Na}]^+$; FAB-HRMS: m/z : 360.1448 $[\text{M}+\text{Na}]^+$.

Pimeloyl hexadiene ester (10): A solution of DIC (619 μL , 4 mmol) in THF (5 mL) was added dropwise over a 30-minute period at room temperature to a solution of pimelic acid (3.2 g, 20 mmol), *trans,trans*-2,4-hexadien-1-ol (393 mg, 4 mmol) and DMAP (49 mg, 0.4 mmol) in THF (30 mL). The reaction mixture was stirred overnight. The solvent was then evaporated and the urea was removed by precipitation with EtOAc/cyclohexane. The filtrate was concentrated under reduced pressure and the product was purified by silica gel flash chromatography cyclohexane/EtOAc 10:3, affording a colorless oil (401 mg, 1.7 mmol, 43 %). R_f = 0.16 (cHex/EtOAc 10:3); $^1\text{H NMR}$ (CDCl_3 , 400 MHz): δ = 1.33–1.41 (m, 2H, CH_2), 1.60–1.68 (m, 4H, $2 \times \text{CH}_2$), 1.75 (d, 3H, J = 6.6 Hz, CH_3 Hxd), 2.32 (t, 2H, J = 7.4 Hz, CH_2), 2.35 (t, 2H, J = 7.4 Hz, CH_2), 4.56 (d, 2H, J = 6.6 Hz, CH_2 Hxd), 5.61 (ddd, 1H, J = 13.6, 6.4, 6.4 Hz, $\text{CH}=\text{CHCH}_3$), 5.70–5.79 (m, 1H, $\text{CH}_2\text{CH}=\text{CH}$), 6.04 (dd, 1H, J = 15.1, 10.5 Hz, $\text{CH}=\text{CHCH}_3$), 6.24 ppm (dd, 1H, J = 15.2, 10.4 Hz, $\text{CH}_2\text{CH}=\text{CH}$); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): δ = 18.3, 24.5, 24.8, 28.7, 34.0, 34.3, 65.1, 123.9, 130.6, 131.5, 135.1, 173.6, 179.9 ppm; ESI-MS: m/z : calcd for $\text{C}_{13}\text{H}_{20}\text{NaO}_4$: 263.1; found: 263.1 $[\text{M}+\text{Na}]^+$; MALDI-TOF: m/z : 263.2 $[\text{M}+\text{Na}]^+$, 279.2 $[\text{M}+\text{K}]^+$.

Preparation of the streptavidin–diene conjugate (11)

A solution of streptavidin (1.09 mg, 21 nmol) in water (450 μL) was combined with of a freshly prepared 50 mM solution (2.5 μL) of the diene cross-linker **9** in DMF (125 nmol) for 30 min at 25 °C. The reaction mixture was transferred to a Microcon centrifugal filtration device (10 kDa cut-off), diafiltered with four changes of water and concentrated to a final volume protein concentration of 19 mg mL^{-1} .

N-Dansyl-2-maleimido-ethylamine (12): 2-Maleimido-ethylamine^[19] (38 mg, 0.15 mmol), dansyl chloride (54 mg, 0.20 mmol) and DIPEA (78 μL , 0.45 mmol) were dissolved in 2.0 mL dry DMF and the solution was stirred at room temperature for 1 h. Acetic acid (120 μL) was added and the solution was concentrated under high vacuum. The residue was redissolved in DCM and the organic solution was washed with HCl 0.1 M (3 $\times$), brine (1 $\times$), dried over Na_2SO_4 and concentrated. The product was purified by reversed-phase HPLC and freeze-dried to give a light yellow solid (45 mg, 0.12 mmol, 80 %). $^1\text{H NMR}$ (CD_3OD , 400 MHz): δ = 3.09–3.13 (m, 2H, CH_2), 3.11 (s, 6H, $\text{N}(\text{CH}_3)_2$), 3.45 (t, 2H, J = 5.7 Hz, CH_2), 6.45 (s, 2H, $\text{CH}=\text{CH}$ maleoyl), 7.56 (d, 1H, J = 7.7 Hz, Ar), 7.62–7.68 (m, 2H, Ar), 8.19 (d, 1H, J = 7.3 Hz, Ar), 8.44 (d, 1H, J = 8.6 Hz, Ar), 8.48 ppm (d, 1H, J = 8.6 Hz, Ar); $^{13}\text{C NMR}$ (CD_3OD , 100 MHz): δ = 37.2 (CH_2), 40.3 (CH_2), 45.3 ($\text{N}(\text{CH}_3)_2$), 116.8 (Ar), 122.5 (Ar), 124.5 (Ar), 127.8 (Ar), 128.1 (Ar), 128.5 (Ar), 129.4 (Ar), 129.5 (Ar), 133.5 ($\text{CH}=\text{CH}$), 170.9 ppm ($\text{C}=\text{O}$); MALDI-TOF: m/z : calcd for $\text{C}_{18}\text{H}_{19}\text{N}_3\text{O}_3\text{S}$: 374.1; found: 374.5 $[\text{M}+\text{H}]^+$, 396.5 $[\text{M}+\text{Na}]^+$, 412.5 $[\text{M}+\text{K}]^+$; FAB-HRMS: m/z : 373.1098 $[\text{M}]^+$.

N-Fluoresceinoyl-2-maleimido-ethylamine (13): 2-Maleimido-ethylamine^[19] (9 mg, 0.035 mmol), fluorescein succinimidyl ester (15 mg, 0.028 mmol) and DIPEA (16 μL , 0.106 mmol) were dissolved in dry DMF (1.0 mL) and the solution was stirred at room temperature for 2.5 h. Acetic acid (15 μL) was added and the product was directly purified by reversed-phase HPLC, affording a yellow solid (6.5 mg, 0.013 mmol, 46 %). $^1\text{H NMR}$ (CD_3OD , 400 MHz): δ (I/II ratio 1:0.65) = 3.49/3.61 (t, 2H, J = 6.1 Hz, CH_2), 3.67/3.78 (t, 2H, J = 5.0 Hz, CH_2), 6.22–6.67 (m, 2H, Xan), 6.71/6.82 (s, 2H, $\text{CH}=\text{CH}$ maleoyl), 6.71–6.82 (m, 2H, Xan), 6.78–6.80 (m, 2H, Xan), 7.31 (d, 1H, J = 8.0 Hz, Ar [II]), 7.54 (s, 1H, Ar [I]), 8.03 (d, 1H, J = 8.0 Hz, Ar [I]), 8.11 (d, 1H, J = 8.0 Hz, Ar [I+II]), 8.34 ppm (s, 1H, Ar [II]); ESI-MS: m/z : calcd for $\text{C}_{27}\text{H}_{18}\text{N}_2\text{O}_4$:

499.1; found: 499.5 $[\text{M}+\text{H}]^+$; MALDI-TOF: m/z : 499.5 $[\text{M}+\text{H}]^+$, 521.5 $[\text{M}+\text{Na}]^+$, 537.5 $[\text{M}+\text{K}]^+$.

Diels–Alder ligation of the streptavidin–diene conjugate and fluorescently labeled dienophiles (14): Hexadiene-conjugate **11** at 3–5 mg mL^{-1} concentration in water was incubated with 30-fold excess of maleimides **6e**, **12** or **13** and kept at 25 °C for 24 h while shaking. After this time, the excess dienophile was removed by passing the reaction mixture through a spin gel filtration column (DyeEx columns from Qiagen). The ligated protein was analyzed by SDS-PAGE and MALDI-TOF experiments. The same procedure was performed by carrying out the ligation of **11** with 100-fold excess of **6e** in 20 mM sodium phosphate buffer pH 5.5, 6.0, 6.5 or 7.0.

Ligation of the Rab7AC3 thioester and peptides 16 or 4h (17): An aliquot of a stock solution (100 μL) of Rab7AC3 thioester **15** (6.8 mg mL^{-1} in buffer 25 mM sodium phosphate pH 7.5, 25 mM NaCl, 0.5 M MESNA, 10 μM GDP, 2 mM MgCl_2 , 30 nmol) was combined with ligation buffer (13 μL ; 25 mM sodium phosphate pH 7.5, 100 mM NaCl, 100 mM MESNA, 2 % CHAPS, 50 μM GDP, 1 mM MgCl_2) and 10 μL of a solution of peptide **16** or **4h** (60 mM in methanol, 600 nmol) was added. The final concentrations were 250 μM for Rab7 thioester and 5 mM for the peptide (ca. 20 equivalents). The ligation reaction mixture was incubated overnight at 16 °C with slight shaking. Small samples were removed for analysis by ESI-MS and SDS-PAGE. The resulting solution of ligated protein **17** was directly submitted to the next step without further purification.

Masking of cysteine residues with Ellmann's reagent (18): An aliquot of dienylyl Rab7 **17** (ca. 5.7 mg mL^{-1}) solution (85 μL) was mixed with 30 mM DTNB (115 μL ; in 60 mM sodium phosphate pH 8) at 25 °C for 4 h. Subsequently the yellowish reaction solution was dialyzed against DA buffer (5 mM sodium phosphate buffer pH 6.0, 20 mM NaCl, 0.2 mM MgCl_2 and 20 μM GDP). The final concentration was approximately 1.2–1.5 mg mL^{-1} of a colorless solution. Small samples were removed for analysis by ESI-MS and SDS-PAGE. The protein solution was shock frozen and stored at –80 °C.

Diels–Alder ligation of Rab7 hexadienyl ester 30 and maleimide compounds (19): In different scale experiments, 10–300 μL of Rab7 hexadienyl ester **18** solution in DA buffer at concentration of approximately 1 mg mL^{-1} (ca. 40 μM protein) were combined with 30 to 100 equivalents of maleimide compounds **6e** or **12**. The ligation mixture was incubated at 25 °C for 24 h. Under these conditions, the ligated protein usually precipitated gradually during the reaction course. The reaction was quenched by adding 200 mM DTT (50 equiv relative to the amount of dienophile added). The deprotection of the cystein residues was visually noticed by the development of a yellow color upon addition of DTT resulted from the release of the TNB groups into solution. After 2 h, the reaction mixture was analyzed by SDS-PAGE and ESI-MS.

Purification of the Rab7 cycloadduct 19a: After incubation of Rab7 hexadienyl ester **18a** with maleimide **6e**, the reaction mixture was centrifuged and the supernatant was removed. The pellet was washed with methanol (2 $\times$) to remove excess dienophile and then redissolved in denaturing buffer (100 mM Tris-HCl pH 8.0, 6 M guanidinium-HCl, 100 mM DTE, 1 % CHAPS and 1 mM EDTA) to a concentration of about 1 mg mL^{-1} (the solution became yellowish because of the TNB group release) and incubated overnight at 4 °C. The protein was refolded by diluting it 25-fold dropwise with folding buffer (100 mM HEPES pH 7.5, 5 mM DTE, 2 mM MgCl_2 , 100 μM GDP, 1 % CHAPS) and incubated at room temperature for 3 h with slight stirring. The folded protein was submitted to two different procedures respectively: 1) it was dialyzed against buffer 25 mM HEPES pH 7.5, 40 mM NaCl, 3 mM DTE, 2 mM MgCl_2 and 20 μM GDP, concentrated by ultracentrifugation (Microcon 10 kDa cut-off) and stored at –80 °C for subsequently use in spectrofluorometric assays; or 2) an equimolar amount of the REP-1 protein was added and the solution was incubated overnight at 4 °C, and the resulting complex was dialyzed against 25 mM HEPES pH 7.5, 40 mM NaCl, 3 mM DTE, 2 mM MgCl_2 and 20 μM GDP, concentrated by ultracentrifugation (Amicon 10 kDa cut-off) and stored at –80 °C.

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