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Racemization-Free Synthesis of S-Alkylated Cysteines via Thiol-ene Reaction

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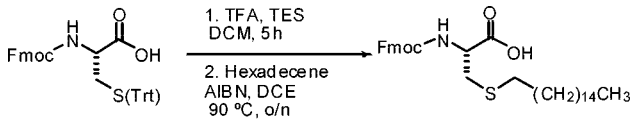
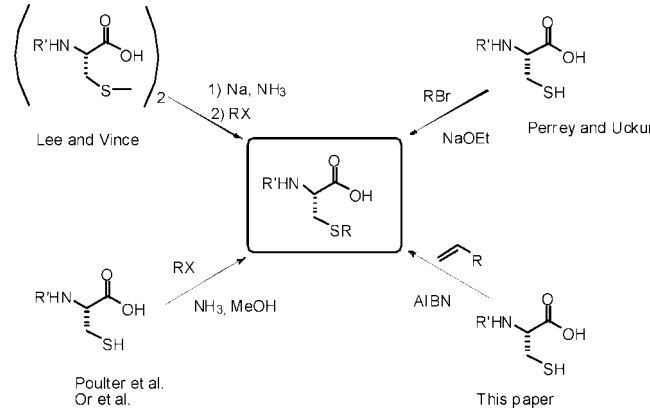


Treatment of differently protected and unprotected cysteines with a variety of alkenes in the presence of a radical initiator (AIBN) afforded the corresponding S-alkylated cysteines in high yields and without racemization.

Post-translational modifications of cysteines play crucial roles in the localization and functioning of many proteins. An illustrative example is their influence on the signal transduction pathways regulated by proteins of the Ras superfamily.[1–3] The small GTPases H-, K-, and N-Ras control, for example, cell proliferation and differentiation. Mutations in this class of proteins lead to uncontrolled cell growth and cancer.[4] The Ras proteins are post-translationally modified via lipidation of their C-termini. This creates a hydrophobic peptide stretch which confers membrane affinity to the proteins and is essential for their localization and functioning. In the three Ras proteins, the lipidation pattern includes S-isoprenylation (either farnesyl or geranylgeranyl moieties, for the three Ras proteins, only farnesyl) and S-palmitoylation. Farnesylation is a stable, nonreversible, modification, but palmitoylation is a dynamic process due to the lability of the thioester moiety and corresponding palmitoylating and depalmitoylating enzymes.[5–7] The palmitoylation process, its regulation, and its influence on protein localization and functioning still poses open questions.[8–11] In order to investigate these phenomena in detail, we have previously prepared Ras proteins, both native and modified, via a combination of peptide synthesis, molecular biology, and protein ligations.[9,12,13] This has given important insights in the above listed processes. These studies have involved the synthesis of lipidated peptides featuring several differently lipidated cysteine building blocks.[11] These included cysteines featuring the natural farnesyl and palmitoyl modifications but also cysteines with non-natural modifications such as the hexadecylated cysteine **4**, which bears a hydrolysis-resistant hexadecyl thioether. This building block has been used for the synthesis of lipidated Ras proteins whose palmitoyl functionality cannot be hydrolyzed anymore.[9,14]

SCHEME 1. Different Strategies for the Synthesis of Alkylated Cysteines



The typical methods used up to now by us and others for the preparation of these alkylated cysteine building blocks are based on the selective alkylation of the cysteine thiol functionality under basic conditions (Scheme 1).[15–17] Vince et al. prepared a propylated cysteine by reduction of cystine with sodium in liquid

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ammonia followed by addition of the alkyl halide.[18] This method has been applied by other groups as well for the preparation of decylated cysteine.[17] Poulter et al. prepared farnesylated cysteine and cysteine methyl ester using farnesyl chloride in an ammonia/methanol solution.[19] This method has, for example, also been used by Or et al. to alkylate a model tetrapeptide with different alkyl substituents.[20] More recently, Uckun et al. used sodium ethoxide for the S-alkylation of *N*-acetyl cysteine with different alkyl bromides.[16]

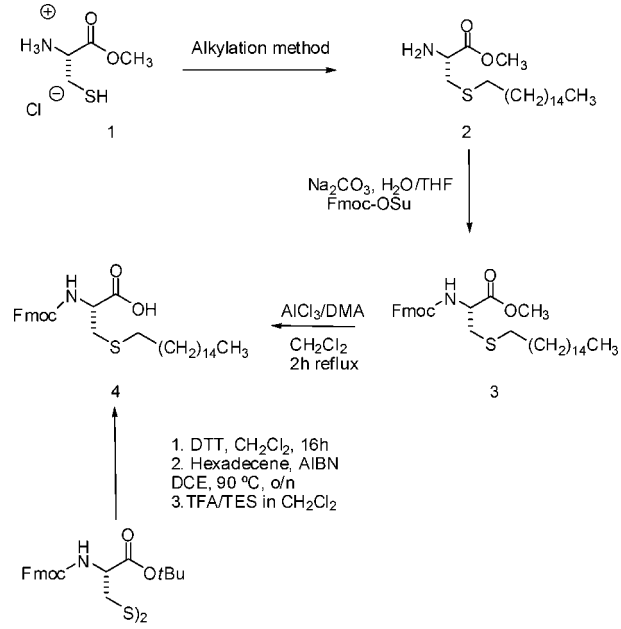
The preparation of alkylated cysteines and the hexadecylated cysteine **4** specifically has, however, typically been characterized by low conversion under these nucleophilic conditions and difficult purification procedures. This is in part due to the poor solubility and amphiphilic character of the product, resulting in nonoptimal yields.[15] Taking into account the basic conditions of the alkylation, attempts to obtain higher yields using more drastic conditions usually result in cysteine racemization especially when cysteine methyl ester is used.[16] Here, the results of a systematic search for high yielding racemization-free reaction conditions for **4** and other alkylated cysteines are reported, with a special focus on the thiol-ene reaction.

The typical protocol for the synthesis of hexadecylated cysteine **4** has been sulfur deprotection of Fmoc-cysteine(Trt)-OH under acid conditions followed by S-alkylation with hexadecyl iodide in DMF and triethylamine as a base.[15] This protocol yields **4** in yields up to 30%, after extensive column chromatography. Poulter′s conditions (which employ a solution of ammonia in methanol) can be successfully applied for the synthesis of farnesylated and geranylgeranylated cysteines as has previously been shown.[15,19,21] In the case of prenylbromides or chlorides, the reactivity of the allyl halide electrophile is increased due to π conjugation in the transition state. For normal aliphatic halides, such as hexadecyl bromide, this activation does not exist. Concomitantly, only traces of product could be detected when we applied these conditions for the alkylation of cysteine with hexadecyl bromide. When this alkylation reaction was performed under the same conditions using cysteine methyl ester instead of free cysteine, the hexadecylated cysteine methyl ester could be obtained in 20% yield. This could be increased to 52% with longer reaction times at room temperature (Table 1). With all this information in hand and taking into account that these methods proceed in poor yields in the case of long aliphatic halides, we turned our attention to examine other conditions for the cysteine alkylation. We decided to study the applicability of the thiol-ene reaction for the synthesis of alkylated cysteines.

The addition of a thiyl radical to a multiple bond, also known as thiol-ene reaction, allows the formation of thioethers through radical-induced addition of thiols to alkenes.[22–24] This reaction follows a mechanism in which a thiyl radical formed by a radical source adds to an alkene double bond, producing a carbon radical, which in turn can abstract a new hydrogen from another thiol and thus propagating the cycle. The thiol-ene reaction can be promoted by UV light radiation or by radical initiators. The main drawback of the addition of thiyl radicals to olefins is the strong reversibility of this step.[25] Therefore, this reaction is usually coupled to an irreversible process in order to drain the equilibrium to the desired product. This methodology has been successfully applied for the synthesis of several natural products. Miyata and co-workers used a sulfanyl radical addition coupled to cyclization and elimination reactions for the synthesis of kainic acid.[22,26] Nevertheless, the number of applications of this reaction for the synthesis of alkylated cysteines is very limited. The few examples known can be found mainly in the polymer field and usually focus on generating functionalized materials, with the aim of obtaining cysteine-based polymers starting from monomers having olefin and mercapto groups derived from cysteine.[27,28] A photochemically induced addition of thiols to alkenes has recently been successfully used by Kunz and co-workers for the coupling of glycopeptides to thiol groups of carrier proteins as synthetic vaccines.[29] The results of our studies as well as the applicability of this reaction for the synthesis of differently substituted cysteines are discussed below.

We first tried the thiol-ene reaction with 1-hexadecene and cysteine methyl ester as substrates and UV light irradiation (254 nm) as the radical initiator (Table 1, entry 3). Unfortunately,

TABLE 1. Yield of Alkylation Step and Enantiomeric Excess of the Hexadecylated Cysteine 4



entry	alkylation method	yield of **3** (%)	ee of **4** (%)
1	hexadecyl bromide, NH_3/MeOH, 3 h at 0 °C then 1 h at rt	20	80
2	hexadecyl bromide, NH_3/MeOH, 3 h at 0 °C then 3 h at rt	52	68
3	hexadecene/DMF, 40 °C, 254 nm	15	86
4	hexadecene, AIBN, DCE, 90 °C, 3 h	74	86
5	hexadecene, AIBN, DCE, 90 °C, o/n (from Fmoc-cystine-O*t* Bu)		99

(18) Lee, C. K.; Vince, R. *J. Med. Chem.* **1978**, *21*, 176–179.
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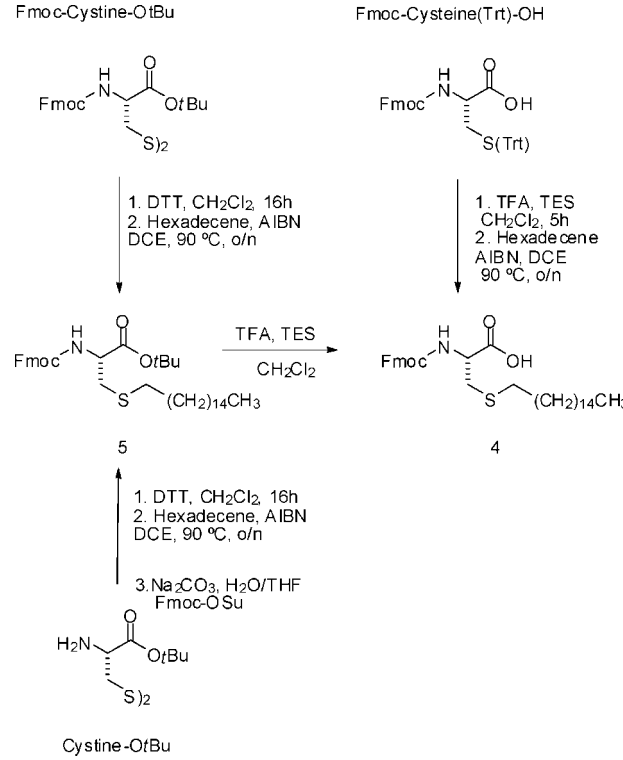
low yields were obtained (15–30%) despite long reaction times (12–72 h). The cysteine disulfide was the main product. However, when the same reaction was performed using AIBN as the radical initiator (Table 1, entry 4), the hexadecylated cysteine **2** was successfully obtained in 91% yield. This compound could be easily transformed to the Fmoc-protected analogue **3**, with an overall yield of 74%. In order to obtain the desired cysteine derivative **4**, a cleavage of the methyl ester of **3** was required. This was performed using $AlCl_3$/dimethylaniline (DMA), which was reported to selectively remove the methyl ester over the Fmoc functionality and not to affect the enantiopurity of the product.[30,31] However, in our hands, for the cysteine substrates, this method resulted in partial racemization of the amino acid. After cleavage of the methyl ester moiety, the enantiomeric excess of the desired hexadecylated Fmoc-protected cysteine **4** was studied by chiral normal phase HPLC. When the alkylation was performed using Poulter's conditions and after methyl ester hydrolysis, cysteine **4** was obtained with 80% ee, which decreased to 68% when longer reactions times at room temperature were used for the alkylation. A lowered ee of 86% was also observed when the thiol-ene reaction was used for the alkylation of the cysteine. Both the alkylation promoted by UV radiation and by AIBN as chemical initiator featured this lowered ee of 86%. Considering that the thiol-ene reaction is not affecting the enantiopurity[29] (vide infra), it can be concluded that the racemization results from the methyl ester hydrolysis using the $AlCl_3$/DMA method.[30,31] To avoid racemization, differently protected cysteine derivatives were explored for the thiol-ene reaction to synthesize hexadecylated cysteine **4**.

Thiol functionalities adjacent to an ester group have been described to be more reactive in the thiol-ene reaction than those with a free carboxylic acid.[32] Therefore, instead of using a methyl ester, we used the conveniently protected cysteine derivative *N*-Fmoc, *O*-*tert*-butyl-protected cystine (Fmoc-cystine-O*t* Bu). After reduction of the disulfide bond with dithiothreitol (DTT), the reaction of the resulting free thiol with hexadecene using AIBN as the radical initiator followed by removal of the *tert*-butyl group under acidic conditions afforded the hexadecylated cysteine **4** in an overall yield of 42% after three steps (Scheme 2 and Table 2, entry 1). The yield of the reaction sequence increased to 57% (in four steps) when first the radical alkylation was performed on reduced cystine-O*t* Bu, followed by Fmoc protection of the amino group (Table 2, entry 2). The analysis of these hexadecylated cysteines after the *tert*-butyl deprotection by chiral HPLC showed >99% ee, hereby proving the suitability of this method with respect to racemization free synthesis (Table 1, entry 5). These findings additionally show that for hexadecylated cysteine the methyl ester deprotection method using $AlCl_3$/DMA[30,31] does not proceed racemization-free.

The thiol-ene reaction was also studied using Fmoc-cysteine (Trt)-OH to determine the effects of the unprotected acid functionality on the reaction. Although, in this case, the yield obtained was lower than that in the case of the cysteine methyl ester, the hexadecylated cysteine **4** could be isolated after two reaction steps in a 55% yield from Fmoc-cysteine(Trt)-OH (Table 2, entry 4). Lower yields but in a similar range were

SCHEME 2. Different Synthesis Approaches to Alkylated Cysteine 4, Based on the Thiol-Ene Reaction[a]



[a] DTT: dithiothreitol; AIBN: azobisisobutyronitrile; TES: triethylsilane; DCE: dichloroethane.

TABLE 2. Cysteine Reagents and Conditions for the Synthesis of Hexadecylated Cysteine 4

entry	thiol	solvent	overall yield of **4** (%)
1	Fmoc-cystine-O*t* Bu	DCE	42
2	cystine-O*t* Bu	DCE	57
3	Fmoc-cysteine(Trt)-OH	hexane/*i*-PrOH	47
4	Fmoc-cysteine(Trt)-OH	DCE	55
5	Fmoc-cysteine(Trt)-OH	DCE (1 equiv of alkene)	43

obtained when dichloroethane (DCE) was replaced by hexane/isopropanol as a solvent system (Table 2, entry 3). The yield decreased to 43% when instead of 3 equiv only 1 equiv of the hexadecene was used, showing that an excess of one of the two reagents is beneficial.

In order to demonstrate the general applicability of the method, a selected number of other alkenes were also reacted with the mercapto group of the cysteine under the established conditions (Table 3). The thiol-ene reaction with Fmoc-cysteine(Trt)-OH proceeded in high yields both with the shorter 1-octene (Table 3, entry 1) and the branched 2-methyl-1-hexene (Table 3, entry 2). The internal alkene *trans*-2-octene also reacted under the same conditions although in lower yields (Table 3, entry 3). This reaction was not regioselective and yielded the two regioisomers **8a** and **8b**. The reaction is tolerant to a range of biologically relevant functional elements. It could be successfully applied for the attachment of the fluorescent dansyl derivative (**10**) (Table 3, entry 4) and a biotin marker (**12**) (Table 3, entry 5). These reactions proceeded in satisfying yields, taking into account that these were performed with equimolar amounts of the cysteine and the allyl-functionalized dansyl or biotin derivative.

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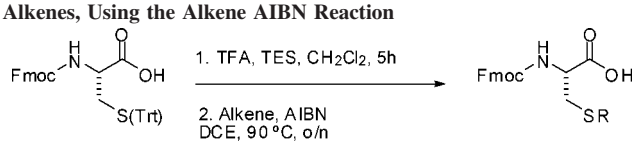
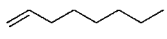
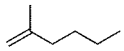
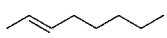
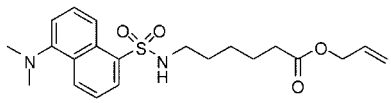
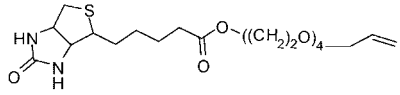
TABLE 3. Alkylation of Fmoc-Cysteine(Trt)-OH with a Variety of Alkenes, Using the Alkene AIBN Reaction



TES:triethylsilane; AIBN: Azobisisobutyronitrile; DCE: dichloroethane

Entry	Alkene	Cp	Yield
1		**6**	57
2		**7**	91
3		**8a, 8b**	28
4	 **9**	**10**	35
5	 **11**	**12**	43

Concluding, herein we described a convenient and practical methodology for the synthesis of alkylated cysteines under nonracemizing conditions. The approach is widely applicable to various alkyl groups and additional functional substituents. The use of the thiol-ene reaction has allowed the generation of several alkylated cysteines in satisfying yields. Complex substituents can be introduced onto the sulfur such as for compounds **10** and **12**, which would be difficult to perform using classic methodologies. Moreover, the experimental procedure is simple and easy to perform, uses inexpensive starting materials, and avoids toxic reagents.

Experimental Section

L-9*H*-Fluoren-9-ylmethoxycarbonylamino)-3-hexadecylsulfanyl propionic acid (4). Fmoc-cysteine(Trt)-OH (4 g, 6.8 mmol) was deprotected following reported conditions.[15] The residue was dissolved in 30 mL of DCE (solvent was degassed under an argon stream in a sonicator bath for 15 min prior to use). To this solution of cysteine in solvent (0.26 M) were added 3 equiv of alkene and 0.5 equiv of AIBN, and the mixture was brought to reflux at 90 °C until TLC analysis of the crude reaction mixture indicated that the cysteine had been completely consumed. The solution was allowed to cool to room temperature, and the solvent was removed under reduced pressure. The resulting residue was purified by flash chromatography using CH_2Cl_2/MeOH (95:5) to provide the desired product **4** (2.0 g, 55%) (0.1 equiv of AIBN has been also used, obtaining similar reaction yields).

Analytical data: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87 (t, 3H, J = 6.8 Hz), 1.24 (s, 26H), 1.51 (m, 2H), 2.52 (m, 2H), 3.02 (m, 2H), 4.20 (t, 1H, J = 6.8 Hz), 4.38 (s, 2H), 4.37 (s, 1H), 5.76 (br s, 1H), 7.27 (t, 2H, J = 7.2 Hz), 7.36 (t, 2H, J = 7.3 Hz), 7.55 (s, 2H), 7.73 (d, 2H, J = 7.59 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.0, 22.6, 28.7, 29.1, 29.2, 29.47, 29.49, 29.55, 29.59, 29.60, 29.63, 31.8, 32.8, 34.1, 47.0, 53,4, 67.3, 119.9, 125.0, 127.0, 127.6, 130.3, 141.2, 143.5, 143.7, 155.8, 175.0; $[\alpha]^{20}_D$ –8.9 (*c* 0.22, CH_2Cl_2); LC-MS (ESI) calcd for $C_{34}H_{50}NO_4{}^{32}S$ 568.35 $[M + H]^+$, found 568.23 $[M + H]^+$, R_t = 9.76 min; HR-MS calcd for $C_{34}H_{50}O_4N_1{}^{32}S_1$ 568.3455 $[M + H]^+$, 590.3275 $[M + Na]^+$, found 568.3451 $[M + H]^+$, 590.3270 $[M + Na]^+$.

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Supporting Information Available: Experimental procedures, characterization data of all new and known compounds, and copies of NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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