

## Synthesis and Biological Activity of Photoactivatable *N*-Ras Peptides and Proteins

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**Abstract:** A modular strategy for the assembly of farnesylated *N*-Ras heptapeptides carrying a photoactivatable benzophenone (BP) group within the lipid residue is described. This strategy is based on the fragment condensation of a *N*-terminal hexapeptide synthesized on the solid support with a cysteine methyl ester which is modified with different farnesyl analogues, incorporating the photophor. At the *N*-terminus of the peptides different functional groups can be attached, e.g., biotin for product enrichment and detection after photoactivation or a maleimido (MIC) linker, allowing for the coupling to proteins carrying a *C*-terminal free cysteine. Using this strategy, 24 peptides were synthesized, incorporating farnesyl analogues with four different chain lengths. Two of these photoactivatable conjugates were ligated to oncogenic human *N*-RasG12VΔ181. A cellular transformation assay revealed that the semisynthetic proteins retain their biological activity despite the photolabel. The first photolabeling experiments with a geranyl-BP-labeled *N*-Ras construct and the farnesyl-sensitive guanine nucleotide exchange factor hSos1 indicate that this photoaffinity labeling system can be particularly useful for studying protein–protein interactions, e.g., the participation of the farnesyl group in Ras signaling, which is still discussed with controversy.

### Introduction

Within the complex framework of cellular signaling proteins, the Ras proteins occupy a paramount position. Cycling between the GTP-bound (activated) and the GDP-bound (inactive) states, they act as molecular switches that translate growth-promoting signals into changes in gene expression.<sup>1,2</sup> Ras proteins are involved in the regulation of cellular programs as diverse as cell growth and differentiation, the cell cycle, and apoptosis. Hence, it is not surprising that point mutations of the *ras* genes which lead to improper signaling are directly linked to various diseases, in particular cancer.<sup>3</sup> In a time of changing paradigms in cancer research, it seems doubtless that ragged growth signaling is one of the cellular traits of cancer development.<sup>4</sup> Disrupting the aberrant Ras signal is found among the most promising objectives in cancer therapy: mutated Ras proteins are met in 20–30% of all human tumors, underlining the outstanding contribution of oncogenic Ras to deregulated growth signaling.<sup>2</sup>

To be functional, the Ras proteins need to be localized at the plasma membrane.<sup>5</sup> This localization is mediated through lipid modifications that are attached to the protein after its translation. Whereas the first steps of the posttranslational modification resulting in the attachment of a farnesyl group have been elucidated quite well, the molecular details of *H*- and *N*-Ras palmitoylation have not yet been revealed unambiguously.<sup>6</sup> In particular, it is not clear at which locus within the cell this palmitoylation occurs and which are the proteins involved in the thioester formation. Therefore, there are still open questions concerning the mechanism of membrane targeting. A further subject of debate is the role of the farnesyl group in protein–protein interactions.<sup>7</sup> For example, a high increase in catalytic activity and binding affinity is observed for the interaction with hSos1 upon farnesylation of unprocessed Ras.<sup>8,9</sup>

We have previously reported our concept of combining chemical and molecular biological methodologies to address biological questions that would be difficult (or impossible) to answer with either chemical or biological tools alone.<sup>1,10</sup> Such a combined approach calls for the development of new chemical methodologies for the synthesis of the required bioprobes. In

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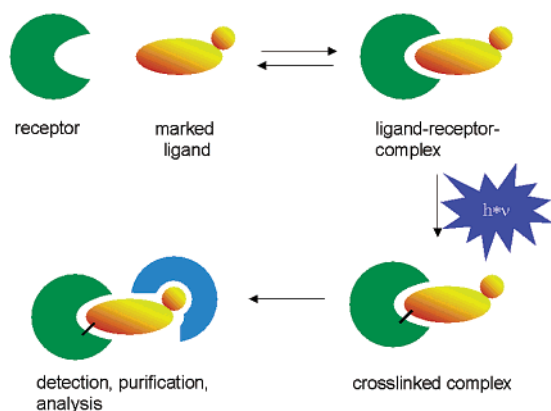
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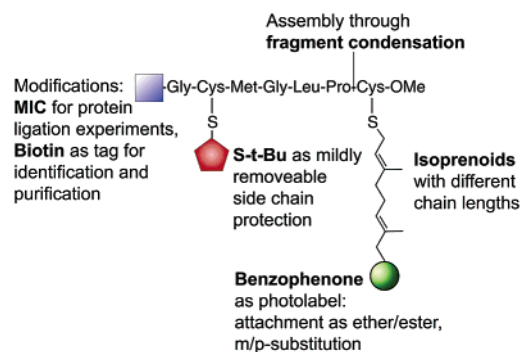
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**Figure 1.** General principle of photoaffinity labeling.



**Figure 2.** Strategy for the modular construction of photoactivatable human *N*-Ras peptides.

particular, it requires the successful synthesis and biological evaluation of differently lipidated functional Ras proteins.<sup>11</sup>

Given the fact that the mechanisms of Ras palmitoylation—and thereby the mechanisms for its localization to the plasma membrane—could not be unraveled conclusively, the employment of new techniques extending beyond existing methodology appears to be required. “Photoaffinity labeling” (PAL) has gained considerable merit in analyzing protein–protein interactions (Figure 1).<sup>12,13</sup> In the light of these findings, we reasoned that biologically active Ras proteins incorporating a photoactivatable group could serve as efficient tools.<sup>14</sup>

In this paper we describe a “construction kit” for the modular assembly of differently modified probes. Our approach is based on the combined solid- and solution-phase synthesis of peptide fragments incorporating benzophenones (BPs) as photolabels and provides a rapid and highly flexible access to peptides and proteins with “tailor-made” properties.

## Results and Discussion

**Synthetic Considerations.** In devising the overall strategy, we reasoned that the photoactivatable peptides and proteins should fulfill the following criteria (Figure 2).

(1) They should embody the characteristic *C*-terminal amino acid sequence of human Ras proteins mediating membrane binding and most likely involved in the interaction with putative binding partners in the course of proper palmitoylation. To fulfill this criterion, the *C*-terminal heptapeptide sequence of *N*-Ras was chosen, terminating in an *S*-farnesylated cysteine methyl ester.<sup>15</sup>

(2) The peptides should be capable of proper localization to the plasma membrane. For this purpose, a palmitoylatable cysteine located in the proximity of the *N*-Ras *C*-terminus had to be present. Earlier investigations had demonstrated that it is sufficient for the proper localization and biological activity in vivo to support the peptide with a palmitoylation site.<sup>16,17</sup> After microinjection into PC12 cells, palmitoylation is performed by the cellular machinery. The use of a *tert*-butyl disulfide masked cysteine allows the liberation of the thiol function under mild reductive conditions.

(3) The photolabel should be embodied within the farnesyl moiety of the membrane anchor. This should provide a high chance of interaction with possible membrane-embedded binding partners of Ras.<sup>18</sup> In vitro this positioning of the photolabel should enable the mapping of binding sites of already known, isoprenoid-sensitive Ras interaction partners. As photolabels, benzophenones were chosen, since they are chemically stable and their activation at wavelengths of 350–360 nm is not damaging to proteins.<sup>19</sup> Additionally, benzophenones have been successfully applied to map binding sites between small G-proteins and their regulators,<sup>20</sup> and BP-labeled isoprenoid pyrophosphates were used for labeling protein prenyltransferases.<sup>21</sup>

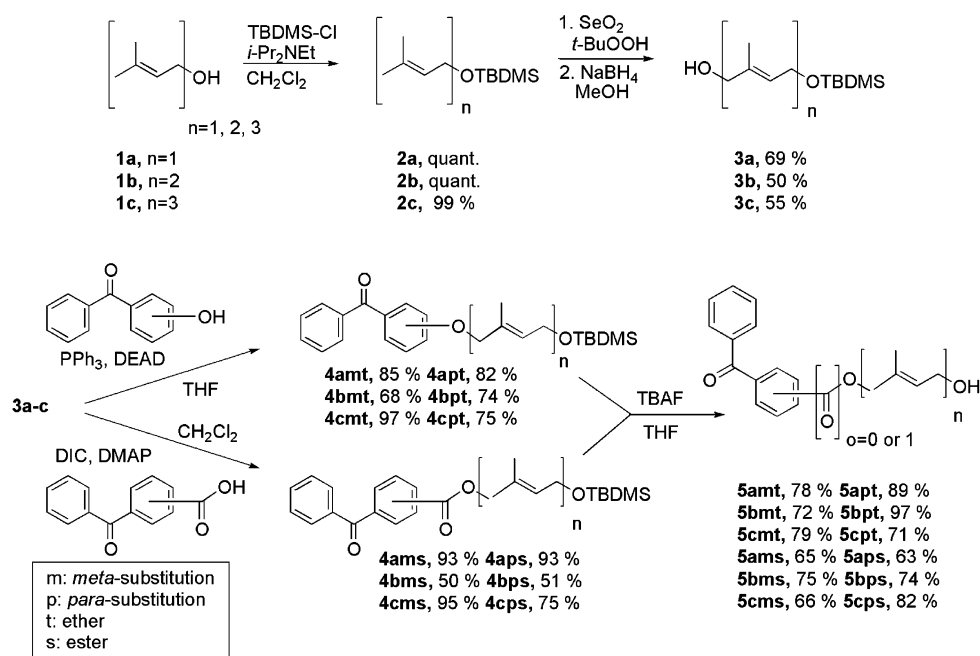
(4) At the *N*-terminus the peptides should carry groups that enable further modifications or handling. For photolabeling experiments, a marker group is needed that allows the detection—and possibly purification—of photo-cross-linked products. As such a marker biotin was employed, since it offers both possibilities. Additionally the validity of the concept had to be proven by showing that the BP label does not affect the biological processing of the peptide. For this purpose, a maleimido group (MIC) was incorporated for selective ligation of the peptides to *C*-terminally truncated oncogenic Ras mutants.<sup>11</sup>

As the key step of a modular synthesis we identified the fragment condensation of an *N*-terminal hexapeptide, incorporating the palmitoylation site and the *N*-terminally attached modifier MIC or biotin, with the *C*-terminal cysteine methyl ester modified with different BP-carrying farnesyl analogues.

The site for fragment coupling has several advantages. First, fragment condensations with glycine or proline as the *C*-terminal amino acid are particularly favorable, since racemization is not an issue. Second, this disconnection yields two fragments of

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**Scheme 1.** Synthesis of BP-Modified Prenyl Analogues **5**<sup>a</sup>

<sup>a</sup> Abbreviations: DEAD, diethylazodicarboxylate; DMAP, *N,N*-dimethylaminopyridine; DIC, diisopropylcarbodiimide.

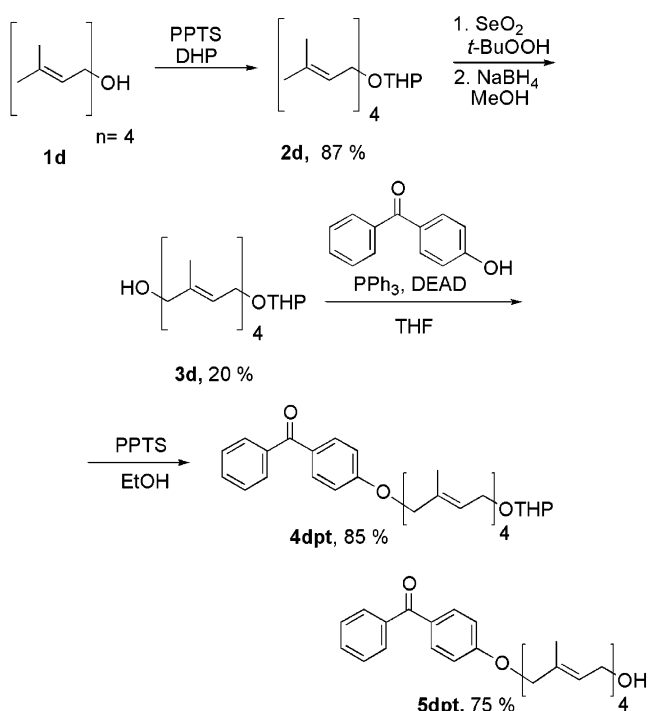
similar complexity, which is desirable for a high-yielding, convergent synthesis. Third, the *N*-terminal hexapeptide is accessible by means of a solid-phase peptide synthesis, thereby allowing for rapid assembly of different building blocks in sufficient quantities.

These features render the proposed synthetic strategy a modular and flexible construction kit, in which any of the components can be exchanged at will according to the planned biological and photolabeling experiments. If desired, also, e.g., fluorescent markers or other traceable functional groups could be incorporated at the *N*-terminus.

**Synthesis of BP-Modified Lipidated Cysteine Methyl Esters.** As photoactivatable farnesyl analogues *meta*- and *para*-substituted benzophenones were chosen to be linked to the isoprenoid via either an ester or an ether bond. Isoprenoids of three different lengths were synthesized, starting from prenyl, geraniol, or farnesol, giving rise to a total of 12 photoactivatable isoprenoid analogues (Scheme 1).

The synthesis was performed employing established methods for the synthesis of BP ethers and esters of prenyl and geraniol.<sup>22</sup> After protection with TBDMS, the isoprenoids **2** were oxidized using the Sharpless allylic oxidation protocol. Partly formed aldehyde was reduced with NaBH<sub>4</sub>. Benzoylphenols were attached to the alcohols **3** using Mitsunobu conditions with PPh<sub>3</sub> and DEAD; the esters **4** were generated under Steglich conditions with DIC and DMAP. Final TBDMS removal with fluoride delivered the isoprenoid analogues **5** in overall yields of 19–50%.

Additionally, *p*-benzoylphenyl geranylgeranyl-16-hydroxy ether (**5dpt**) was prepared in 13% overall yield using the THP protecting group instead of the TBDMS ether (Scheme 2).

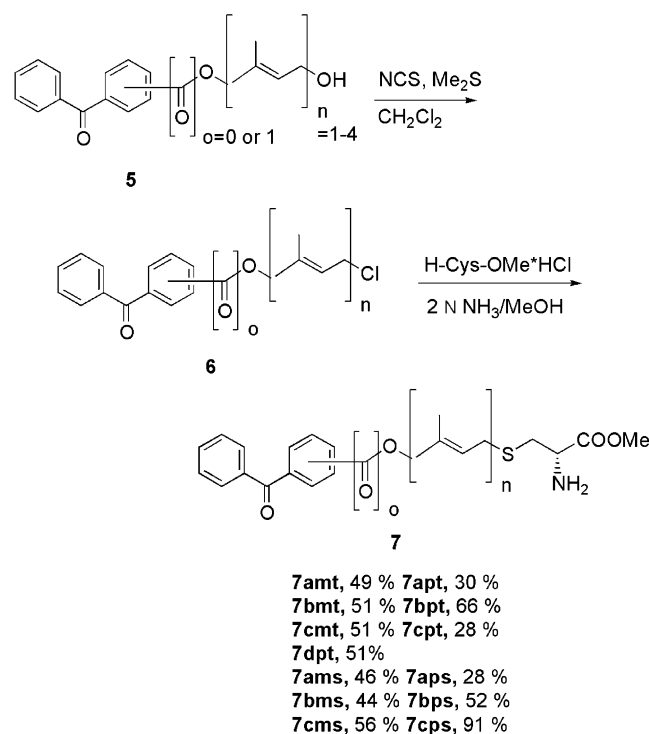
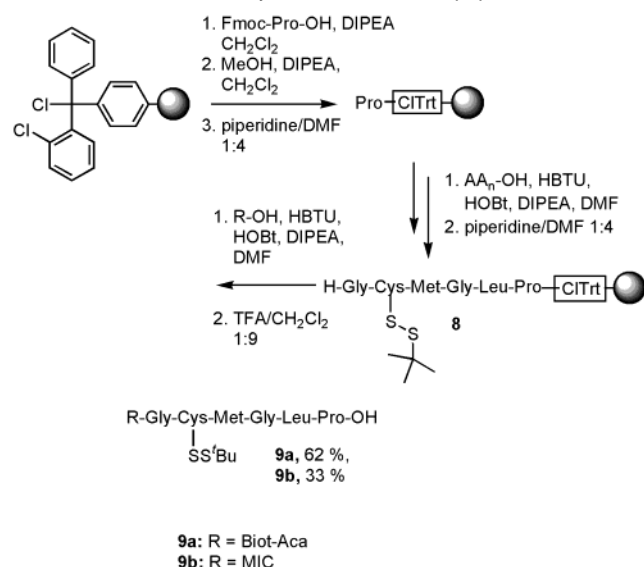
**Scheme 2.** Synthesis of the BP-Modified Geranylgeranyl Analogue **5dpt**<sup>a</sup>

<sup>a</sup> Abbreviations: PPTS, pyridinium *p*-toluenesulfonate; DHP, dihydropyran.

The 13 isoprenoid analogues **5** were converted into the corresponding chlorides **6** using the Corey–Kim protocol and employed in the alkylation of cysteine methyl ester to deliver the *S*-lipidated amino acid esters **7** in 28–91% overall yields (Scheme 3).

**Solid-Phase Peptide Synthesis and Fragment Condensation.** The synthesis of the hexapeptide fragment was achieved

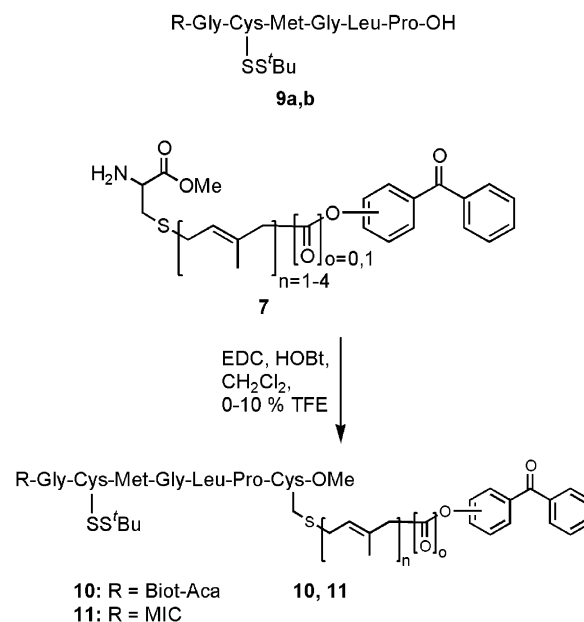
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**Scheme 3.** Coupling of the BP-Modified Prenyl Analogues **5** to Cysteine Methyl Ester<sup>a</sup><sup>a</sup> Abbreviation: NCS, *N*-chlorosuccinimide.**Scheme 4.** Solid-Phase Synthesis of the Hexapeptides **9a** and **9b**<sup>a</sup>

<sup>a</sup> Abbreviations: DIPEA, diisopropylethylamine; HBTU, 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole.

using the 2-chlorotrityl linker on a polystyrene resin and employing an Fmoc protocol for chain elongation (Scheme 4).

The first amino acid was attached to the resin under basic conditions, and subsequently unreacted chloride functions were

**Scheme 5.** Fragment Couplings of the Hexapeptides **9a** and **9b** with the BP-Modified Isoprenylated Cysteines **7**<sup>a</sup>

<sup>a</sup> Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; TFE, 2,2,2-trifluoroethanol.

**Table 1.** Fragment Condensation of the Hexapeptides **9a,b** with the BP-Labeled Prenylated Cysteines **7**

entry	peptide nr	yield (%)	entry	peptide nr	yield (%)
1	<b>10amt</b>	73	14	<b>11amt</b>	45
2	<b>10apt</b>	77	15	<b>11apt</b>	65
3	<b>10ams</b>	41	16	<b>11ams</b>	80
4	<b>10aps</b>	49	17	<b>11aps</b>	59
5	<b>10bmt</b>	55	18	<b>11bmt</b>	23
6	<b>10bpt</b>	43	19	<b>11bpt</b>	55
7	<b>10bms</b>	76	20	<b>11cmt</b>	72
8	<b>10bps</b>	44	21	<b>11cpt</b>	48
9	<b>10cmt</b>	44	22	<b>11cms</b>	83
10	<b>10cpt</b>	42	23	<b>11cps</b>	59
11	<b>10cms</b>	45	24	<b>11dpt</b>	48
12	<b>10cps</b>	44			
13	<b>10dpt</b>	65			

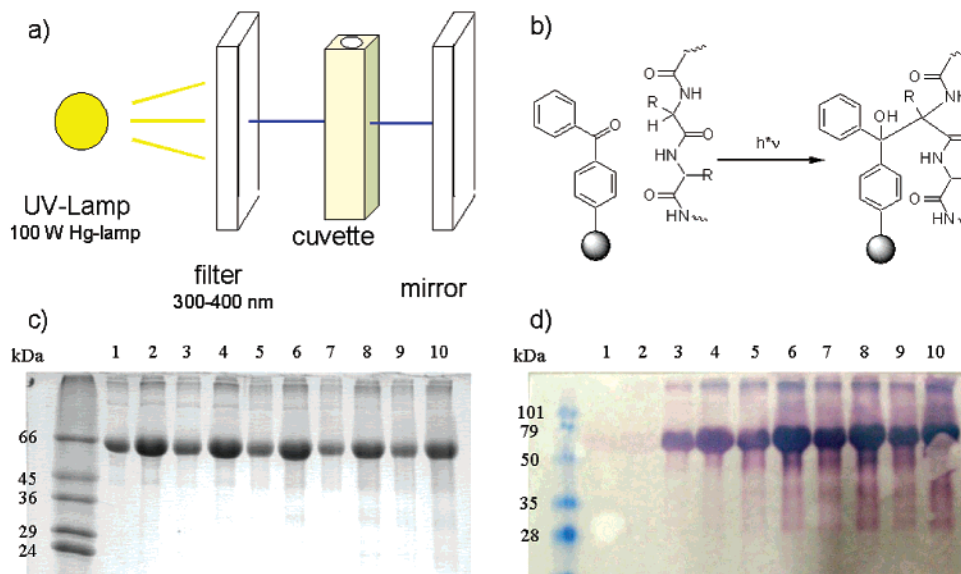
blocked by treatment with MeOH and base. After Fmoc removal with 20% piperidine in DMF, the chain was elongated using HBTU/HOBt activation. After successful synthesis of the hexapeptide, the terminal amino function was liberated and the selectively unmasked, solid-phase-bound intermediate was coupled to biotinylaminocaproic acid (Biot-Aca-OH) or maleimidocaproic acid (MIC-OH). Final detachment of the products was achieved by treating the resin with 10% TFA in CH<sub>2</sub>Cl<sub>2</sub>, furnishing the hexapeptides **9a** and **9b** in high yield.

The fragment couplings with the BP-labeled prenylated cysteine methyl esters **7** were carried out in CH<sub>2</sub>Cl<sub>2</sub> using EDC/HOBt for carboxyl activation (Scheme 5, Table 1).

Successful coupling of biotinylated peptide **9a** required the addition of 5–10% (v/v) 2,2,2-trifluoroethanol (TFE) to enhance the solubility of the peptide. Otherwise the condensation reactions proceeded uneventful and yielded the desired compounds in a reliable manner.

In total, 24 peptides were prepared, including 13 biotinylated and 11 MIC-modified compounds. Typically, the yields for the fragment condensation are in a preparatively useful range of 40–60%.





**Figure 3.** Experimental setup of the photo-cross-linked system (a). Benzophenones preferably insert into backbone C–H bonds (b). SDS–PAGE (c) and Western blot (d) of the photo-cross-linking of **10bpt** and BSA. Samples were taken prior to irradiation (lanes 1 and 2) and after 5 min (lanes 3 and 4), 15 min (lanes 5 and 6), 30 min (lanes 7 and 8), and 60 min (lanes 9 and 10). Of each sample, aliquots of 3  $\mu$ g (lanes 1, 3, 5, 7, and 9) and 5  $\mu$ g (lanes 2, 4, 6, 8, and 10) were used.

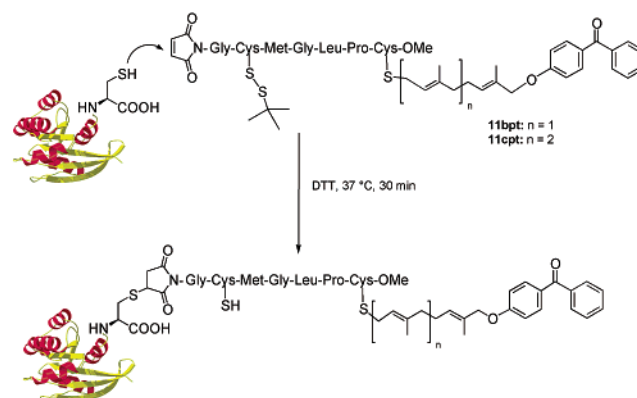
The biotinylated peptides **10** open up the opportunity to carry out photoaffinity labeling experiments, whereas the MIC peptides **11** can be ligated to C-terminally truncated oncogenic Ras proteins.

**Biological Investigations.** Prior to the application of the probes in biological photolabeling experiments it had to be assured that the photolabeling system as such is functional. Thus, the cross-link has to be achievable by UV irradiation, and the readout system with biotin–streptavidin conjugation has to be functional. Also it had to be assured that the biological activity of proteins incorporating the BP analogues would not be altered decisively by the photolabel.

The applicability of the peptides in photolabeling experiments was investigated in a model experiment by UV irradiation of a solution containing biotinylated peptide **10bpt** (10  $\mu$ M) and albumin from bovine serum (BSA; 50  $\mu$ M) (Figure 3a,b). BSA was selected as a low-specificity bait with good binding properties for hydrophobic groups<sup>23</sup> and a broad selectivity ranging from C14 to C20 fatty acid chains.<sup>24</sup> Samples were taken before treatment and after 5, 15, 30, and 60 min. In all irradiated samples the photo-cross-linked product could be detected by an enzyme-catalyzed color reaction after denaturing gel electrophoresis (SDS–PAGE), followed by Western blot and incubation with a streptavidin–alkaline phosphatase conjugate (Figure 3c,d).

The biological activity of Ras proteins incorporating a benzophenone-modified farnesyl group was examined for the geranyl-BP- and farnesyl-BP-modified MIC peptides **11bpt** and **11cpt**. These two peptides were ligated to the oncogenic *N*-Ras mutant *N*-RasG12V $\Delta$ 181. This Ras protein terminates in a C-terminal cysteine which represents the only surface-accessible SH group for conjugate addition to the MIC group (Scheme 6).

**Scheme 6.** Ligation of Peptides **11bpt** and **11cpt** to Oncogenic RasG12V $\Delta$ 181



This ligation strategy has been successfully used and validated for the synthesis of lipidated and fluorescent protein conjugates.<sup>11</sup>

The coupling products were extracted by treatment with Triton-X114 and purified via ion-exchange chromatography. By means of mass spectrometry the quality and stoichiometry of the ligated products were determined; in both cases the ligation led to products with a 1:1 stoichiometry (Figure 4).

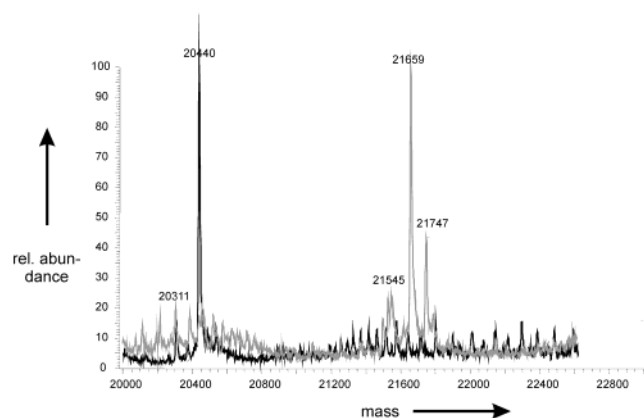
The ligated proteins were microinjected to phaeochromocytoma cells (PC12), which are known to differentiate in the presence of oncogenic Ras mutants,<sup>25</sup> forming neurite-like outgrowths. Both for geranyl-BP-labeled (Figure 5a) and for farnesyl-BP-labeled proteins (Figure 5b) the differentiation of the cells was observed.

Quantitative analysis of the biological response for the **11bpt** conjugate showed that the transforming potential of the semi-synthetic protein is ca. one-third the potential of full-length RasG12V.<sup>14</sup> This activity is high enough to conclude that the processing of the BP-labeled mutant is not essentially altered.

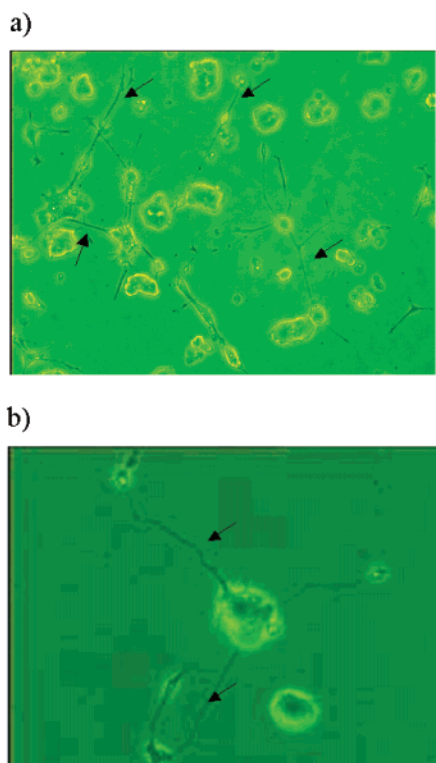
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**Figure 4.** Characterization of the coupling product (gray trace) of *N*-RasG12VΔ181 (black trace) with peptide **11bpt** by means of ESI-MS. The peak at 20440 Da corresponds to the theoretical mass of the C-terminally truncated protein; the minor peak at 20311 Da can be assigned as the protein without the *N*-terminal Met<sup>1</sup> (theoretical mass 20309 Da). The MS of the coupling product shows a major peak at 21659 Da and a minor one at 21747 Da, matching the molecular masses of the coupled products after and before removal of the thiobutyl protecting group.



**Figure 5.** Differentiation of PC12 cells after microinjection of the ligated product of *N*-RasG12VΔ181 and **11bpt** (a) or **11cpt** (b) in 100  $\mu$ M concentration. The formation of neurite-like outgrowths (black arrows) is observed.

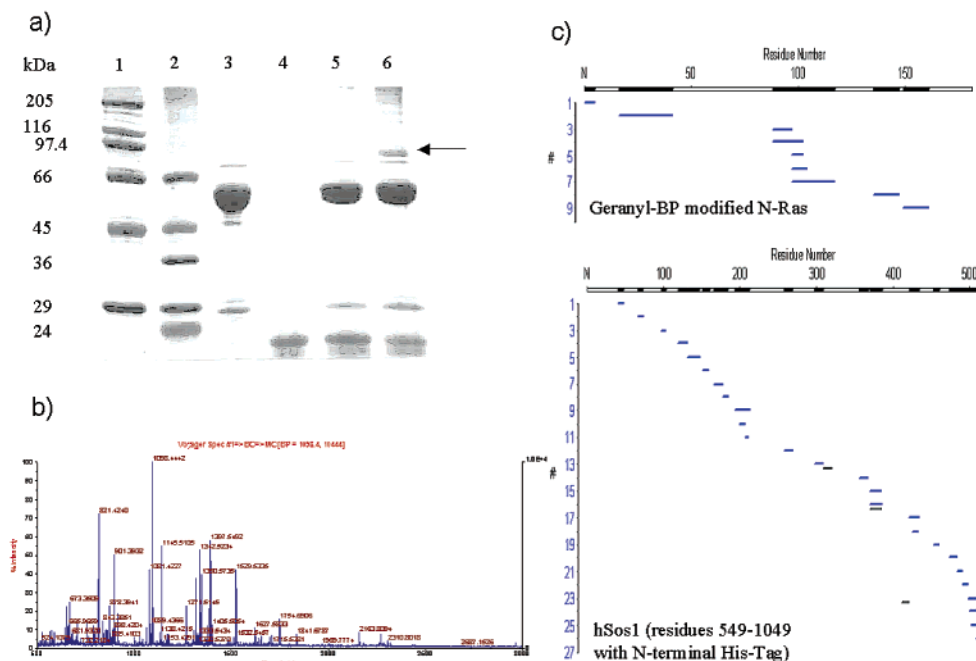
The biological activity of the MIC-coupled Ras proteins together with the observation that NBD-labeled derivatives of the farnesylated *N*-Ras C-terminal heptapeptide are taken up by cells and reversibly palmitoylated<sup>16</sup> makes the application of the BP-labeled lipopeptides in cellular photolabeling experiments very promising. With these tools it should be possible to address for instance open questions in the cascade of cellular processing events leading to the palmitoylation of Ras proteins, and to identify further cellular binding partners of Ras.

The bioactivity of the semisynthetic photoactivatable Ras proteins renders these constructs also ideal tools for the mapping of binding sites on farnesyl-sensitive Ras interaction partners. The first promising photolabeling results were obtained for the Ras guanine nucleotide exchange factor Sos1 (Figure 6). The catalytic domain of Sos is responsible for the activation of Ras proteins by exchange of GDP by GTP, thus “switching” the protein “on”. This domain consists of 500 amino acids and contains blocks of sequences that are conserved in other Ras-specific nucleotide exchange factors such as Cdc25.<sup>26</sup> While the catalytic domain of Cdc25 efficiently promotes guanine nucleotide exchange on both prenylated and unprocessed Ras, the catalytic domain of Sos shows higher activity on prenylated Ras.<sup>9</sup> In an attempt to map the putative binding site for the prenyl group on hSos1, we first ligated the geranyl-BP-modified MIC peptide **11bpt** to C-terminally truncated *N*-Ras wild-type protein (*N*-Raswt.Δ181). The *N*-Ras construct (20  $\mu$ M) was incubated at 0  $^{\circ}$ C for 1 h with the catalytic domain of hSos1 (residues 564–1049, 20  $\mu$ M). One portion of the solution was then UV-irradiated for 60 min. A photo-cross-linked product of about 80 kDa could be detected after denaturing gel electrophoresis (SDS–PAGE) (Figure 6a). With respect to the relative mass of this new band, the cross-linked product was assumed to consist of geranyl-BP-modified *N*-Ras protein covalently bound to hSos1 in a 1:1 stoichiometry. This assumption could be confirmed by in-gel digestion using trypsin and subsequent MALDI-TOF analysis. The list of peptide masses found in the mass spectrum (Figure 6b) was compared with the theoretically expected masses after trypsin digestion of the used hSos1 fragment and the *N*-Ras–MIC construct. The found matches cover a high percentage of both proteins (Figure 6c). As a test for the specificity of product formation we performed an additional cross-linking experiment with glutathione *S*-transferase (GST) as a non-Ras-binding reaction partner. Using identical protein concentrations and conditions, no GST–Ras coupling product could be identified by means of SDS–PAGE or in an anti-GST Western blot. The same result was obtained if a mixture of hSos, RasGerBP, and GST (each 20  $\mu$ M) was UV-exposed. In this experiment, only the Ras–Sos band was detected due to formation of the corresponding heterodimer (data not shown).

These results are first steps in our attempt to explain the preferential catalytic activity of hSos1 against prenylated Ras. As benzophenones have been successfully applied to map binding sites on other proteins,<sup>13,19</sup> we are looking forward to determining the region or even the exact amino acids of hSos1 that are modified by the photoinitiated covalent coupling reaction. The crystal structure of a complex of C-terminally truncated *H*-Ras (residues 1–166) with hSos1 (residues 564–1049) has already been solved.<sup>27</sup> The analysis of Ras proteins covalently linked to Sos via its C-terminal moiety should allow the role of the farnesyl group in this protein–protein interaction to be determined. Geranyl- and farnesyl-BP-labeled Ras proteins might also be used for the analysis of other protein interactions, for which a participation of the prenyl group has been reported.<sup>7</sup>

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**Figure 6.** Photoaffinity labeling of hSos1 (residues 549–1049) with geranyl-BP-modified *N*-Ras wild type. Analysis of the photo-cross-linking reaction by SDS–PAGE (a): lanes 1 and 2, molecular mass markers (Sigma); lane 3, hSos1; lane 4, geranyl-BP-modified *N*-Ras; lane 5, photoaffinity labeling sample before photoirradiation; lane 6, photoaffinity labeling sample after 60 min of photoirradiation. The arrow indicates the location of the photo-cross-linked product. MALDI-TOF-MS spectrum of the cross-linked product after in-gel digestion with trypsin (b). Representation of found sequence matches for the semisynthetic Ras construct and hSos1 after trypsin digestion of the cross-linked peptide.

## Experimental Section

**Materials and Methods.** All reactions were carried out in oven-dried glassware under an atmosphere of argon. The solid-phase reactions were performed in solid-phase shaking reactors, also under an atmosphere of argon. The solvents used were reagent grade. Tetrahydrofuran (THF) was freshly distilled over sodium under an argon atmosphere prior to use. Dichloromethane was dried by distillation over calcium hydride. All reagents used were of the highest quality available and used as received from the suppliers.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were measured on a Varian Mercury 400 MHz or a Bruker DRX 500 MHz spectrometer.  $^{13}\text{C}$ – $^1\text{H}$  substitution was determined with a DEPT-135 pulse sequence, differentiating signals of methyl and methine carbons pointing “up” (+) from methylene carbons pointing “down” (–) and quaternary carbons that are missing (o). MALDI-TOF MS spectra were acquired by averaging over 500 pulses using dihydroxybenzoic acid (DHB) as the matrix. HPLC–ESI-MS spectra were recorded on a Finnegan LCQ spectrometer connected to a Hewlett-Packard HPLC system equipped with a column changer. Analytical C4 reversed-phase columns purchased from Macherey-Nagel were used (CC250/4 Nucleosil 120-5C4; flow rate 1.0 mL/min; solvent A, 0.1% HCOOH in  $\text{H}_2\text{O}$ ; solvent B, 0.1% HCOOH in MeOH; gradient program, 20% B (0 min), 20% B (2 min), 90% B (30 min), 95% B (37 min), 20% B (38 min), 20% B (45 min)). GC–MS spectra were recorded using a Macherey&Nagel Optima 1 (0.2 mm  $\times$  25 m) capillary column and the following temperature program: injector, 150  $^\circ\text{C}$ ; oven, 50  $^\circ\text{C}$  (0 min), 50  $^\circ\text{C}$  (2 min), 250  $^\circ\text{C}$  (10 min), 250  $^\circ\text{C}$  (12 min). High-resolution FAB-MS spectra were recorded at the Department of Chemistry, University of Karlsruhe.

**General Procedure for the Introduction of the TBDMS Group (Preparation of Compounds 2a–c).** A mixture of isoprenyl alcohol (1; 1.0 equiv), (TBDMS)Cl (1.2 equiv), and *i*-PrNEt (2.0 equiv) in  $\text{CH}_2\text{Cl}_2$  (1 mL/mmol) was stirred at room temperature for 3–6 h. The reaction mixture was washed with saturated aqueous solutions of  $\text{NH}_4\text{Cl}$ ,  $\text{NaHCO}_3$ , and NaCl, dried over  $\text{MgSO}_4$ , and concentrated in vacuo. The crude compounds were purified by flash column chromatography

on silica gel. Following this procedure, compounds 2a–c were prepared in quantitative yields.

**General Procedure for the Allylic Oxidation (Preparation of Compounds 3a–d).** Compound 2 (1.0 equiv) was added under stirring to a mixture of  $\text{SeO}_2$  (0.05 equiv), salicylic acid (0.1 equiv), and *t*-BuOOH (80% in (*t*-BuO) $_2$ - $\text{H}_2\text{O}$ , 3:2 v/v, 3.5 equiv) in  $\text{CH}_2\text{Cl}_2$  (1 mL/mmol). After 12 h, toluene was added and the reaction mixture was concentrated in vacuo. The residue was diluted with  $\text{Et}_2\text{O}$  and washed with saturated aqueous  $\text{NaHCO}_3$  and brine, dried over  $\text{MgSO}_4$ , and concentrated in vacuo. The residue was dissolved in MeOH (2 mL/mmol) and cooled to  $-15$   $^\circ\text{C}$ .  $\text{NaBH}_4$  (0.2 equiv) was added in small portions. After addition of acetone, the reaction mixture was concentrated in vacuo. The residue was dissolved in EtOAc and washed with saturated aqueous solutions of  $\text{NH}_4\text{Cl}$  and brine, dried over  $\text{MgSO}_4$ , and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel. Following this procedure, compounds 3a–d were prepared; their characterization is given in the Supporting Information.

**General Procedure for Prenyl Etherification (Preparation of Compounds 4xxr).** A mixture of alcohol 3 (1.0 equiv), *m*- or *p*-hydroxybenzophenone (1.2 equiv),  $\text{PPh}_3$  (1.5 equiv), and DEAD (1.5 equiv) in THF (1–2 mL/mmol) was stirred at room temperature for 4–8 h. After addition of EtOAc, the mixture was washed with  $\text{H}_2\text{O}$  and brine, dried over  $\text{MgSO}_4$ , and concentrated in vacuo. The crude compounds were purified by flash column chromatography on silica. Following this procedure, compounds 4amt, 4apt, 4bmt, 4bpt, 4cmt, 4cpt, and 4dpt were prepared; their characterization is given in the Supporting Information.

**General Procedure for Removal of the TBDMS Group (Preparation of Compounds 5a–c).** The TBDMS ether 4 was stirred at 0  $^\circ\text{C}$  with TBAF (1.2 equiv) in THF (5 mL/mmol) for 1 h. After dilution with EtOAc, the mixture was washed with brine, dried over  $\text{MgSO}_4$ , and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel. Following this procedure, compounds 5amt, 5apt, 5bmt, 5bpt, 5cmt, and 5cpt were prepared; their characterization is given in the Supporting Information.



**General Procedure for Coupling to Cysteine Methyl Ester (Preparation of Compounds 7).** To a solution of 1.1 equiv of *N*-chlorosuccinimide in CH<sub>2</sub>Cl<sub>2</sub> (2–5 mL/mmol) was added dropwise at –40 °C dimethyl sulfide (2 equiv). After being warmed to 0 °C for 5 min, the mixture was cooled to –40 °C, and a solution of **5** in CH<sub>2</sub>Cl<sub>2</sub> was added dropwise. The resulting mixture was allowed to warm to 0 °C over 1 h and stirred at 0 °C for 1 h. The mixture was diluted with cyclohexane, washed twice with ice-cold brine, dried over MgSO<sub>4</sub>, and concentrated in vacuo. The residue was used for the alkylation step without further purification.

The residue was dissolved in MeOH or THF and added at 0 °C to a solution of l-cysteine methyl ester hydrochloride (1.1 equiv) in 2 N NH<sub>3</sub>/MeOH (5–10 mL/mmol). After 1 h, the reaction mixture was allowed to warm to room temperature for 1 h. After dilution with CH<sub>2</sub>Cl<sub>2</sub> or ether, the mixture was washed with water. After thorough back-extraction the combined organic layers were dried over MgSO<sub>4</sub> and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel. Following this procedure, 13 compounds **7xxx** were prepared. Their characterization is given in the Supporting Information.

**Solid-Phase Synthesis of H-GC(S-*t*-Bu)MGLP 8.** 2-Chlorotriyl polystyrene resin (loading 1.08 mmol/g) was loaded by shaking with 1.2 equiv of Fmoc-proline and 4.8 equiv of DIPEA in 5 mL of dry CH<sub>2</sub>Cl<sub>2</sub> for 2 h. Unreacted linker groups were blocked by subsequent treatment with MeOH and DIPEA in CH<sub>2</sub>Cl<sub>2</sub> for 0.5 h. The loading of the resin was determined to be 0.25 mmol/g. After Fmoc cleavage with 20% piperidine in DMF, the peptide **8** was synthesized using the following protocol: The Fmoc-amino acids (4 equiv) were coupled to the resin with 3.6 equiv of HBTU, 4.8 equiv of HOBt, and 8 equiv of DIPEA in DMF for 90 min after 5 min of preactivation. For capping, the resin was treated twice with 10% acetic anhydride in pyridine for 5 min. Deprotection was accomplished through repeated washing with a solution of 20% piperidine in DMF for 10 min. Between all steps the resin was washed thoroughly with DMF. For further elaboration of the *N*-terminus the Fmoc group was removed by the standard cleavage procedure. The quality of the product was assured by HPLC–ESI-MS: *t*<sub>R</sub> = 16.57 min, purity 90%; *m/z* (M) calcd for C<sub>42</sub>H<sub>58</sub>N<sub>6</sub>O<sub>5</sub>S 886.34, found 885.2 [M – H]<sup>–</sup>.

**Synthesis of Biot-Aca-GC(S-*t*-Bu)MGLP 9a.** For the biotinylation Biot-Aca-OH (4 equiv) was activated for 10 min with 3.6 equiv of HBTU, 4.8 equiv of HOBt, and 16 equiv of DIPEA in DMF and subsequently shaken for 2 h with the immobilized hexapeptide **8**. After the reaction the resin was washed thoroughly with CH<sub>2</sub>Cl<sub>2</sub>. **9a** was cleaved from the resin with 10% TFA in CH<sub>2</sub>Cl<sub>2</sub>, and the solvent was removed by evaporation in vacuo under repeated addition of toluene for azeotropic removal of TFA, yielding 393.6 mg of **9a** (87% starting from Pro-loaded resin) as a colorless oil. Further purification was not required. The assignment of the proton signals was achieved by H–H COSY. [α]<sub>D</sub><sup>20</sup> = –27.0° (*c* = 1.08, MeOH). <sup>1</sup>H NMR (500 MHz, MeOD): δ = 0.96 (dd, 6H, *J* = 10.0, 6.5 Hz, δ-Leu), 1.26 (s, 9H, *t*-Bu), 1.21–1.73 (m, 15H, 6 CH<sub>2</sub> chain, β-Leu, γ-Leu), 2.00 (s, 3H, CH<sub>3</sub> Met), 1.85–2.05 (m, 4H, γ-Pro, γ-Met), 2.07–2.20 (m, 6H, 2 CH<sub>2</sub>C=O Biot-Aca, β-Pro), 2.40–2.58 (m, 2H, δ-Pro), 2.60–2.65 (m, 2H, S-CH<sub>2</sub> Biot), 2.82–2.87 (m, 1H, S-CH Biot), 2.93 (dd, 1H, *J* = 7.7, 13.6 Hz, β-Cys), 3.04–3.14 (m, 3H, *N*-CH<sub>2</sub> Aca, β'-Cys), 3.65–3.90 (m, 4H, 2 α-Gly), 4.20–4.24 (m, 2H, *N*-CH Biot, α-Pro), 4.32–4.36 (m, 1H, α-Met), 4.39–4.43 (m, 1H, *N*-CH Biot), 4.53 (dd, 1H, *J* = 7.7, 5.7 Hz, α-Cys), 4.61 (dd, 1H, *J* = 10.3, 4.3 Hz, α-Leu). HPLC–ESI-MS: *t*<sub>R</sub> = 13.79 min, purity 85%; *m/z* 1004.4 [M + H]<sup>+</sup>, 1027.4 [M + Na]<sup>+</sup>. HR-FAB-MS: *m/z* calcd for C<sub>43</sub>H<sub>74</sub>N<sub>9</sub>O<sub>10</sub>S<sub>4</sub> 1004.444, found 1004.446.

**Synthesis of MIC-GC(S-*t*-Bu)MGLP 9b.** MIC-OH (4 equiv) was activated for 10 min with 3.6 equiv of HBTU, 4.8 equiv of HOBt, and 8 equiv of DIPEA in DMF and subsequently shaken for 90 min with the immobilized hexapeptide **8**. After the reaction the resin was washed

thoroughly with CH<sub>2</sub>Cl<sub>2</sub>. **9b** was cleaved from the resin with 10% TFA in CH<sub>2</sub>Cl<sub>2</sub>, and the solvent was removed by evaporation in vacuo under repeated addition of toluene for azeotropic removal of TFA. Purification by flash chromatography (SiO<sub>2</sub>; EtOAc/MeOH, 10:1) yields 375.8 mg of **9b** (70% starting from proline-loaded resin) as a colorless oil. The assignment of the proton signals was achieved by H–H COSY. [α]<sub>D</sub><sup>20</sup> = –43.5° (*c* = 2.67, MeOH). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ = 0.73–0.98 (m, 6H, δ-Leu), 1.12–1.37 (m, 11H, CH<sub>2</sub> MIC, *t*-Bu), 1.39–1.73 (m, 7H, 2 CH<sub>2</sub> MIC, β-Leu, γ-Leu), 1.98–2.19 (m, 7H, β'-Met, β'-Pro, γ-Pro, CH<sub>3</sub> Met), 2.20–2.41 (m, 4H, α-CH<sub>2</sub> MIC, β-Met, β-Pro), 2.47–2.74 (m, 2H, γ-Met), 2.98–3.19 (m, 2H, β-Cys), 3.42–3.57 (m, 2H, *N*-CH<sub>2</sub> MIC), 3.58–3.69 (m, 1H, δ'-Pro), 3.77–4.04 (m, 5H, 2 α-Gly, δ-Pro), 4.09–4.23 (m, 1H, α-Pro), 4.24–4.56 (m, 2H, α-Met, α-Cys), 4.62–4.79 (m, 1H, α-Leu), 6.69 (s, 2H, HC=CH). HPLC–ESI-MS: *t*<sub>R</sub> = 11.11 min, purity >90%; *m/z* 858.2 [M + H]<sup>+</sup>, 880.3 [M + Na]<sup>+</sup>. HR-FAB-MS: *m/z* calcd for C<sub>37</sub>H<sub>59</sub>N<sub>7</sub>O<sub>10</sub>S<sub>3</sub> 857.3536, found 857.3464.

**Fragment Couplings of the BP-Labeled Cysteines 7 with the Biotinylated Peptide 9a.** For fragment coupling, 1.0 equiv of the biotinylated hexapeptide **9a** was stirred with 1.0 equiv of the prenylated cysteine **7**, 1.3 equiv of EDC, and 1.5 equiv of HOBt in CH<sub>2</sub>Cl<sub>2</sub> with 5% (v/v) 2,2,2-trifluoroethanol at room temperature for 16–24 h. The reaction mixture was diluted with EtOAc, washed successively with 0.5 N aqueous HCl, saturated NaHCO<sub>3</sub>, and brine, dried over MgSO<sub>4</sub>, and concentrated in vacuo. The crude product was purified by flash chromatography.

**Synthesis of Biot-Aca-GC(S-*t*-Bu)MGLPC(8-(4-benzoylphenyloxy)geranyl)-OMe (10bpt).** The reaction was performed on a scale of 62.7 μmol. Purification using EtOAc/MeOH, 5:1 (v/v), yields **10bpt** (40 mg, 43%) as a pale brown oil. [α]<sub>D</sub><sup>20</sup> = –38.5° (*c* = 1.13, CHCl<sub>3</sub>). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ = 0.89–0.98 (m, 6H), 1.20–1.75 (m, 17H), 1.28 (s, 9H), 1.68 (s, 3H), 1.74 (s, 3H), 2.01–2.40 (m, 14H), 2.50–2.68 (m, 4H), 2.72–3.23 (m, 10H), 3.50–3.70 (m, 2H), 3.73 (s, 3H), 3.80–4.02 (m, 2H), 4.20–4.36 (m, 2H), 4.43 (s, 2H), 4.52–4.63 (m, 2H), 4.64–4.80 (m, 3H), 5.17 (t, 1H, *J* = 7.2 Hz), 5.50 (t, 1H, *J* = 7.3 Hz), 7.14 (m, 1H), 7.30–7.40 (m, 3H), 7.40–7.52 (m, 2H), 7.52–7.60 (m, 1H), 7.76–7.82 (d, 2H, *J* = 8.2 Hz). HPLC–ESI-MS: *t*<sub>R</sub> = 26.01 min, purity >90%; *m/z* 1453.4 [M + H]<sup>+</sup>, 1476.4 [M + Na]<sup>+</sup>. MALDI-MS (DHB, calibrated): *m/z* calcd for C<sub>70</sub>H<sub>104</sub>N<sub>10</sub>O<sub>13</sub>S<sub>5</sub>Na<sup>+</sup> 1475.629, found 1475.626.

**Synthesis of Biot-Aca-GC(S-*t*-Bu)MGLPC(12-(4-benzoylphenyloxy)farnesyl)-OMe (10cpt).** The reaction was performed on a scale of 35.6 μmol. Purification using EtOAc/MeOH, 9:1 (v/v), yields **10cpt** (22.9 mg, 42%) as a pale brown oil. [α]<sub>D</sub><sup>20</sup> = –11.2° (*c* = 1.17, MeOH). <sup>1</sup>H NMR (400 MHz, MeOD): δ = 0.73–1.00 (m, 6H), 1.29 (s, 9H), 1.19–1.79 (m, 17H), 1.62 (s, 3H), 1.65 (s, 3H), 1.73 (s, 3H), 1.93–2.36 (m, 14 H), 2.07 (s, 3H), 2.46–2.78 (m, 4H), 2.84–3.04 (m, 3H), 3.06–3.22 (m, 4H), 3.40–4.14 (m, 6H), 3.71 (s, 3H), 4.18–4.32 (m, 1H), 4.42–4.73 (m, 8H), 5.11 (t, 1H, *J* = 7.2 Hz), 5.19 (t, 1H, *J* = 7.6 Hz), 5.57 (t, 1H, *J* = 7.2 Hz), 7.04 (d, 2H, *J* = 8.8 Hz), 7.52 (t, 2H, *J* = 7.6 Hz), 7.62 (t, 1H, *J* = 7.6 Hz), 7.71 (d, 2H, *J* = 7.2 Hz), 7.79 (d, 2H, *J* = 8.8 Hz). HPLC–ESI-MS: *t*<sub>R</sub> = 25.75 min, purity >90%; *m/z* 1521.5 [M + H]<sup>+</sup>, 1543.6 [M + Na]<sup>+</sup>. MALDI-MS (DHB): *m/z* 1543.65 [M + Na]<sup>+</sup>, 1560.73 [M + K]<sup>+</sup>. FAB-MS (NBA): *m/z* 1543.1 (M<sup>+</sup> + Na).

**Fragment Couplings of the BP-Labeled Cysteines 7 with the MIC-Modified Peptide 9b.** For fragment coupling, 1.0 equiv of the MIC hexapeptide **9b** was stirred with 1.0 equiv of the prenylated cysteine **7**, 1.3 equiv of EDC, and 1.5 equiv of HOBt in CH<sub>2</sub>Cl<sub>2</sub> at room temperature for 16–24 h. The reaction mixture was diluted with EtOAc, washed successively with 0.5 N aqueous HCl, saturated NaHCO<sub>3</sub>, and brine, dried over MgSO<sub>4</sub>, and concentrated in vacuo. The crude product was purified by flash chromatography.

**Synthesis of MIC-GC(S-*t*-Bu)MGLPC(4-(4-benzoylphenyloxy)-prenyl)-OMe (11bpt).** The reaction was performed on a scale of 51 μmol. Purification using EtOAc/MeOH, 10:1 (v/v), yields **11bpt** (36



mg, 55%) as a pale brown oil.  $[\alpha]_D^{20} = -28.6^\circ$  ( $c = 0.21$ ,  $\text{CHCl}_3$ ).  $^1\text{H}$  NMR (400 MHz,  $\text{MeOD}$ ):  $\delta = 0.93$  (dd, 6H,  $J = 8.0, 6.4$  Hz), 1.20–1.37 (m, 11H), 1.55–1.80 (m, 7H), 1.67 (s, 3H), 1.75 (s, 3H), 1.98–2.34 (m, 12H), 2.08 (s, 3H), 2.55–2.75 (m, 2H), 2.90–3.20 (m, 4H), 3.34–3.95 (m, 8H), 3.74 (s, 3H), 4.09–4.13 (m, 1H), 4.47 (s, 2H), 4.52–4.88 (m, 4H), 5.14–5.21 (m, 1H), 5.50–5.56 (m, 1H), 6.68 (s, 2H), 6.96 (d, 2H,  $J = 9.0$  Hz), 7.47 (t, 2H,  $J = 8.2$  Hz), 7.57 (t, 1H,  $J = 7.4$  Hz), 7.75 (d, 2H,  $J = 8.2$  Hz), 7.81 (d, 2H,  $J = 8.6$  Hz). HPLC–ESI-MS:  $t_R = 27.77$  min, purity >90%;  $m/z$  1307.2  $[\text{M} + \text{H}]^+$ , 1329.3  $[\text{M} + \text{Na}]^+$ . MALDI-MS (DHB):  $m/z$  1329.60  $[\text{M} + \text{Na}]^+$ , 1345.57  $[\text{M} + \text{K}]^+$ .

**Synthesis of MIC-GC(S-*t*-Bu)MGLPC(12-(3-benzoylphenoxy)-farnesyl)-OMe (11cpt).** The reaction was performed on a scale of 33.5  $\mu\text{mol}$ . Purification using  $\text{EtOAc}/\text{MeOH}$ , 9:1 (v/v), yields **11cpt** (33.2 mg, 72%) as a pale brown oil.  $[\alpha]_D^{20} = -42.2^\circ$  ( $c = 0.25$ ,  $\text{MeOH}$ ).  $^1\text{H}$  NMR (400 MHz,  $\text{MeOD}$ ):  $\delta = 0.73$ –0.98 (m, 6H), 1.23–1.48 (m, 11H), 1.49–1.78 (m, 7H), 1.61 (s, 3H), 1.66 (s, 3H), 1.73 (s, 3H), 1.92–2.34 (m, 16H), 2.07 (s, 3H), 2.44–2.80 (m, 2H), 2.95–3.06 (m, 1H), 3.07–3.23 (m, 3H), 3.70 (s, 3H), 3.42–4.13 (m, 8H), 4.20–4.27 (m, 1H), 4.40–4.64 (m, 4H), 4.65–4.73 (m, 1H), 5.11 (t, 1H,  $J = 7.0$  Hz), 5.18 (t, 1H,  $J = 7.0$  Hz), 5.56 (t, 1H,  $J = 7.0$  Hz), 6.78 (s, 2H), 7.03 (d, 2H,  $J = 8.4$  Hz), 7.51 (t, 2H,  $J = 7.6$  Hz), 7.61 (t, 1H,  $J = 7.6$  Hz), 7.71 (d, 2H,  $J = 7.6$  Hz), 7.77 (d, 2H,  $J = 8.4$  Hz). HPLC–ESI-MS:  $t_R = 28.68$  min, purity >90%;  $m/z$  1375.4  $[\text{M} + \text{H}]^+$ , 1397.6  $[\text{M} + \text{Na}]^+$ . MALDI-MS (DHB):  $m/z$  1397.25  $[\text{M} + \text{Na}]^+$ , 1412.26  $[\text{M} + \text{K}]^+$ .

**Coupling of the Biotinylated Lipopeptide 11bpt to BSA.** A solution of 50  $\mu\text{M}$  bovine serum albumin (BSA) and 10  $\mu\text{M}$  lipopeptide **11bpt** was incubated in 20 mM HEPES, pH 7.4, 5 mM  $\text{MgCl}_2$  in a quartz cuvette at room temperature and exposed to UV light (300–400 nm). Samples were taken at different time points of exposure, and the reaction was analyzed by SDS–PAGE and Western blot with a streptavidin–AP (alkaline phosphatase) detection system (Promega).

**Plasmid Construction.** Truncation of the full-length *N*-Ras cDNA (accession no. X02751) and the introduction of the point mutation G12V were achieved using the high-fidelity *Pfu* DNA polymerase (Stratagene). For the truncated version of *N*-Ras two stop codons were introduced to positions 182 and 183 of the *N*-Ras cDNA. The resulting fragment *N*-Ras $\Delta$ 181 was purified and digested with *Eco*RI and *Sma*I and was subcloned into the pTac expression vector.<sup>28</sup> The plasmid was sequenced to prove PCR fidelity and correct mutagenesis and was transformed into the *E. coli* strain CK600K (Stratagene).

**Expression and Purification of the *N*-Ras Protein.** The *E. coli* strain CK600K carrying the expression plasmid for either *N*-Raswt. $\Delta$ 181 or *N*-RasG12V $\Delta$ 181 was cultivated in LB medium containing 100  $\mu\text{g}/\text{mL}$  ampicillin and 25  $\mu\text{g}/\text{mL}$  canamycin at 37  $^\circ\text{C}$ . Expression was induced at a cell density absorbance of  $A_{600} = 0.6$  with 500  $\mu\text{M}$  isopropyl  $\beta$ -D-thiogalactoside, and incubation was continued at 30  $^\circ\text{C}$  overnight. Pellets were resuspended in 20 mM Tris (pH 7.4), 5 mM  $\text{MgCl}_2$ , 2 mM DTE (buffer A). Phenylmethylsulfonyl fluoride (PMSF) to 0.1 mM and 0.5 mg of DNase I were added, and the cells were lysated using a microfluidizer (Microfluidics). Following centrifugation the clear supernatant was applied to a DEAE-Sepharose column equilibrated with buffer A. After being washed with two column volumes, the protein was eluted over five column volumes using a linear gradient of 0–1 M NaCl in buffer A. Fractions containing *N*-Ras were determined by SDS–PAGE, pooled, and concentrated by precipitation with 3 M ammonium sulfate (final concentration) for 30 min. After centrifugation the precipitate was resuspended in gel filtration buffer (buffer A containing 200 mM NaCl, 10  $\mu\text{M}$  GDP) and applied to a gel filtration column (HiLoad Superdex 200, Amersham Pharmacia Biotech). Fractions containing *N*-Ras were pooled and again concentrated by ammonium sulfate precipitation. All purification steps were carried out at 4  $^\circ\text{C}$ . Purified protein was stored at  $-80^\circ\text{C}$ .

**Coupling of 11bpt and 11cpt to *N*-Ras.** Prior to coupling the *N*-Ras $\Delta$ 181 protein was passed through a HiTrap gel filtration column (Amersham Pharmacia Biotech) to remove any excess salts and DTE required for storage of the protein. At all stages of the coupling reaction, all samples containing protein, unless otherwise stated, were kept at or below 4  $^\circ\text{C}$ . To a solution of the peptide dissolved in methanol (50  $\mu\text{L}$ ) in a 2 mL Eppendorf tube was added a buffer of 11% Triton X-114 (Fluka) containing 30 mM Tris/HCl, 100 mM NaCl (1 mL). The solution was sonicated for approximately 15 min until a slightly cloudy homogeneous solution was obtained. The detergent solution was cooled to 0  $^\circ\text{C}$ , and 1 mL of an aqueous solution (buffer: 20 mM Tris/HCl, 5 mM  $\text{MgCl}_2$ , pH 7.4) containing 10 mg of the Ras protein was added. The coupling reaction was performed with stoichiometric amounts of peptide and protein. The coupling mixture was covered with argon and incubated at 4  $^\circ\text{C}$  for 16 h. The solution was then centrifuged. The supernatant was transferred into a 15 mL centrifuge tube, diluted with 3 mL of buffer (containing 2 mM DTE), and heated to 37  $^\circ\text{C}$ , resulting in a phase separation of the detergent phase from the aqueous phase after centrifugation at room temperature. The aqueous phase was removed and extracted two more times with an 11% Triton X-114 detergent solution ( $2 \times 1$  mL). The detergent phases were combined and washed three times with fresh buffer ( $3 \times 7$  mL). The protein extract was diluted to 2% Triton X-114 with fresh buffer, and applied to a DEAE-Sepharose column. After being washed with 20 mM Tris/HCl, 5 mM  $\text{MgCl}_2$ , 2 mM DTE, pH 7.4, the bound protein was eluted with a sodium chloride gradient (0–1 M NaCl) and concentrated. The product was analyzed by electrospray mass spectrometry (ESI-MS; Finnigan) and SDS–PAGE.

**Differentiation Assay.** PC12 cell culture and microinjections were performed as described.<sup>23,29,30</sup> Briefly, PC12 cells were prestimulated with NGF (100 ng/mL) for 3 days and kept in NGF-free medium for another 2 days. The modified proteins were diluted in PBS containing 10  $\mu\text{M}$  fluorescein dextran as a marker for identification of injected cells. The transformed cells showed a typical differentiated phenotype with neurite-like outgrowth 40 h after injection. The transformation efficiency was calculated from at least four independent experiments.

**Expression and Purification of hSos1 (564–1049).** The human Sos1 catalytic domain construct (amino acids 564–1049) was cloned into the bacterial expression vector pET-28 (Novagen) at cloning sites *Bam*HI and *Xho*I and kindly provided by B. E. Hall.<sup>31</sup> The plasmid was transformed into the BL21(DE3) strain of *E. coli*. The bacteria were grown in LB medium containing 100  $\mu\text{g}/\text{mL}$  kanamycin at 37  $^\circ\text{C}$ . After reaching an absorbance of 0.5 unit at 600 nm, they were induced to synthesize polyhistidine-tagged Sos protein by 500  $\mu\text{M}$  isopropyl  $\beta$ -D-thiogalactoside for 16 h at 30  $^\circ\text{C}$ . Pellets were resuspended in 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM  $\beta$ -mercaptoethanol, 30 mM imidazole (buffer B). Prior to disruption of the cells using a microfluidizer (Microfluidics), PMSF to 0.1 mM and 0.5 mg of DNase I were added. After centrifugation the clear supernatant was collected and purified on a Ni-NTA column. The column was washed with buffer B. Bound polyhistidine-tagged Sos was eluted using a linear imidazole gradient (30–500 mM) in buffer B.

**Coupling of the Geranyl-BP-Modified *N*-Raswt. $\Delta$ 181 to the Catalytic Domain of hSos1.** A solution of 20  $\mu\text{M}$  geranyl-BP-modified *N*-Raswt. $\Delta$ 181 and 20  $\mu\text{M}$  hSos (564–1049) was incubated in 20 mM HEPES, pH 7.4, 5 mM  $\text{MgCl}_2$ , 2 mM  $\beta$ -mercaptoethanol, 15 mM *n*-octylglucopyranoside for 1 h at 4  $^\circ\text{C}$ , transferred into a quartz cuvette, and exposed to UV light (300–400 nm) for 1 h at 4  $^\circ\text{C}$ . The reaction was analyzed by SDS–PAGE. The obtained photo-cross-linked product was excised from the gel for trypsin digestion. To remove contaminants associated with sodium dodecyl sulfate–polyacrylamide gel electro-

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phoresis, the gel spot was first destained by several steps of dehydration and rehydration. The gel piece was dehydrated through incubation with 10–20  $\mu\text{L}$  of acetonitrile for 10 min. After removal of the fluid it was rehydrated again with 0.1 M  $\text{NH}_4\text{HCO}_3$  for 5 min. The same volume of acetonitrile was added for 15 min and the supernatant removed afterward. The gel spot was then dried in a speed vacuum for 30 min. A 20  $\mu\text{L}$  sample of 10 mM DTE in 0.1 mM  $\text{NH}_4\text{HCO}_3$  was added, and reduction was performed for 45 min at 56 °C. After the solution was cooled to room temperature, the supernatant was discarded and the gel spot was further dehydrated using 20  $\mu\text{L}$  of acetonitrile for 5 min. The fluid was again removed, and 20  $\mu\text{L}$  of 0.055 mM iodacetamide in 0.1 mM  $\text{NH}_4\text{HCO}_3$  was added. This step prevents the cysteine residues from oxidation and recombining to disulfide bonds. Stable *S*-carboxyamidomethyl derivatives were formed. After incubation in the dark for 15 min, the supernatant was removed and a further washing step by first rehydrating with 0.1 mM  $\text{NH}_4\text{HCO}_3$  and then dehydrating with acetonitrile was performed. The gel spot was dried in a speed vacuum for 1 h and then covered with 12.5 ng/ $\mu\text{L}$  trypsin (Sigma) in 50 mM  $\text{NH}_4\text{HCO}_3$ , 5 mM  $\text{CaCl}_2$  for 45 min at 4 °C. After removal of the supernatant the gel spot was covered with the same buffer but without trypsin and incubated overnight at 37 °C. The supernatant obtained in this way generated and extacted protein fragments and was used for

the subsequent MALDI-TOF analysis. A comparison of the peptide masses found in the mass spectrum with the theoretically expected masses after trypsin digestion of the used hSos1 fragment and the *N*-Ras–MIC construct was performed using PAWS.

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**Supporting Information Available:** General procedure for the preparation of BP esters **4xxs** and characterization of compounds **2a–d**, **3a–d**, **4xxx**, **5xxx**, **7xxx**, **10xxx** (except **10bpt** and **10cpt**), and **11xxx** (except **11bpt** and **11cpt**). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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