Polymerization reactions: A 1-L glass autoclave was charged with toluene (200 mL) and triisobutylaluminum (0.5 mL). The mixture was stirred (700 rpm), thermostated at 25 °C, and saturated with ethylene (2 bar). The polymerization reaction was started by the injection of a solution of the respective zwitterionic complex in toluene, generated in situ by treatment of **8a** or **8b** (ca. 35 μmol) with an equimolar amount of $B(C_6F_5)_3$ in toluene (8 mL). After 1 h the reaction was quenched by adding aqueous HCl in methanol (15 mL). The polymer was precipitated with additional methanol (100 mL), collected by filtration, washed with 6 N aqueous HCl (100 mL), water (200 mL), acetone (50 mL), and then dried in vacuo.

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Photoactivatable Synthetic Ras Proteins: "Baits" for the Identification of Plasma-Membrane-Bound Binding Partners of Ras**

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The regulation of cell growth and differentiation by proteins of the Ras superfamily[1] requires the correct subcellular distribution of the small GTP-binding proteins (GTP = guanosine triphosphate).[2] The biological function of Ras is strictly dependent on its correct translocation to the plasma membrane, and this localization is directly linked to posttranslational S-farnesylation and S-palmitoylation of Ras.[3]

The real mechanism of translocation is still the subject of debate. On the one hand for K-Ras^B, which embodies a polycationic hexalysine stretch and a farnesyl thioether at the C terminus, a model was proposed in which unspecific but highly anionic "sites" (formed at least in part by the lipid bilayer) at the plasma membrane instead of a classical specific proteinaceous receptor are responsible for association of this Ras isoform with the plasma membrane.[4] On the other hand for H- and N-Ras, which have S-farnesyl and S-palmitoyl substituents at the C terminus, a membrane-trapping model[5] was postulated in which a prenyl protein specific palmitoyl-

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transferase (PTase) is located in the plasma membrane. However, these proposals have been challenged by recent findings that suggest that K-Ras and H-/N-Ras traffic to the cell surface through different routes and that palmitoyl substitution of the H- and N-isoform occurs in the endoplasmatic reticulum.[6]

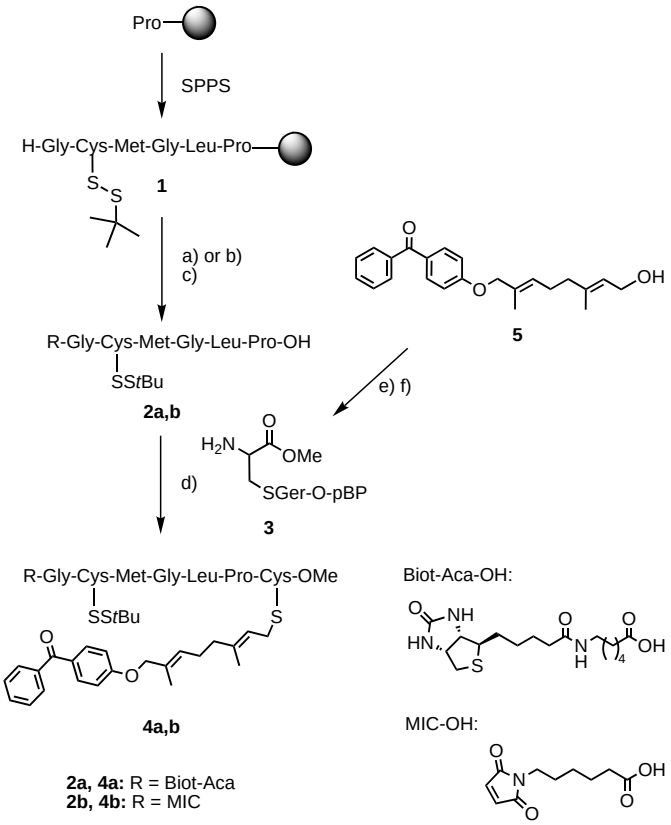
In addition, the correlation of subcompartmentation of the plasma membrane[7] with signal transduction events has received increasing attention. Thus, H-Ras is enriched in membrane domains with high levels of sphingolipids and cholesterol (rafts),[8] whereas K-Ras shows a random distribution in the plasma membrane.[6] To unravel the mechanisms that direct Ras localization and to identify putative binding partners of Ras in the plasma membrane or other intracellular compartments, new tools that go beyond the techniques available from regular biological methodology alone are urgently needed.

In addressing this challenge, we have synthesized Ras lipopeptides and a Ras protein that incorporate a photoactivatable isoprenoid thioether. Our evaluations demonstrated that the lipopeptides will be suitable "baits" for covalent coupling of proteins, whereas the protein construct demonstrated biological activity required to trap Ras binding partners.

As suitable chemical tools for covalent trapping and identification of possible membrane-embedded binding partners of the Ras-proteins, lipopeptides were considered that meet the following requirements:

1. They should embody the characteristic C-terminal amino acid sequence of the Ras protein that is in contact with the membrane and most likely also with a putative binding partner. To fulfill this criterion, the C-terminal heptapeptide sequence of N-Ras was chosen which terminates in a S-farnesyl-substituted cysteine methyl ester.[9]
2. The peptides should be selectively localized to and stably inserted into the plasma membrane of cells, in our case by insertion of a palmitoylatable cysteine residue that is located at the C terminus of N-Ras. This cysteine was masked as a *tert*-butyl disulfide, which can be cleaved readily by treatment with dithiothreitol. The cysteine thiol functional group liberated is readily derivatized with a palmitoyl group, and the resulting S-palmitoyl- and S-farnesyl-substituted Ras peptide is selectively localized at the plasma membrane.[10, 11]
3. The conjugate should contain a group that can be photoactivated,[12] preferably incorporated into the membrane-embedded isoprenoid residue. For this purpose the benzophenone group (BP) was chosen as an established photophore.[13, 14]
4. They should carry a group that allows the isolation and detection of the covalently crosslinked products by established methods. For this purpose the biotin label was chosen because it can serve both as a tag for product enrichment and as a detection label.
5. The synthesis strategy should give access to photoactivatable lipopeptides for the labeling experiments and to protein–lipopeptide conjugates, which can be used to determine the activity of the lipopeptides. The maleimidocaproic acid (MIC) group was chosen to couple the lipopeptide to a Ras mutant with a C-terminal cysteine residue.[15, 16] Such peptides were required to demonstrate that the conjugate is selectively directed to the plasma membrane and that the photophore does not significantly influence the binding to a putative Ras binding protein, if at all.

These criteria are met by the peptides **4a** and **4b**, which were synthesized as shown in Scheme 1. The N-terminal hexapeptide of the Ras sequence was synthesized efficiently



Scheme 1. Solid-phase synthesis of the hexapeptides **2a/b** and fragment coupling with the BP-labeled isoprenyl-derivatized cysteine **3**. a) Biot-Aca-OH, HBTU, HOBt, DIPEA, DMF; b) MIC-OH, HBTU, HOBt, DIPEA, DMF; c) TFA in CH_2Cl_2 (10%), **2a**: 62%, **2b**: 33%; d) **3**, EDCI, HOBt, CH_2Cl_2, **4a**: 43%, **4b**: 55%; e) NCS, DMS, CH_2Cl_2; f) CysOMe · HCl, NH_3, MeOH, 66% over two steps. BP = benzophenone, SPPS = solid-phase peptide synthesis, DEAD = diethylazodicarboxylate, HBTU = 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, DMS = dimethyl sulfide, EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, NCS = *N*-chlorosuccinimide.

on a solid support. The peptide was linked to the polymeric carrier through the chlorotrityl (chlorotriphenylmethyl) linker, and Fmoc-protected amino acids were employed for chain elongation. After assembly of the hexapeptide and N-terminal deprotection, either biotinylaminocaproic acid (Biot-Aca-OH) or maleimidocaproic acid (MIC-OH) were introduced followed by release of the Biot-Aca- and MIC-labeled peptides **2a** and **2b** from the solid support. Both compounds were isolated in high yields and then coupled with the S-alkylated cysteine methyl ester **3** to yield the target peptides **4a** and **4b**. In **3** the terminal isoprene unit of the farnesyl

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group has been replaced by the benzophenone photophore, which approximates it in size and lipophilicity. The lipid-modified and benzophenone-labeled cysteine methyl ester **3** was synthesized by conversion of allyl alcohol **5**[17] into the corresponding allyl chloride and subsequent nucleophilic substitution with cysteine methyl ester (Scheme 1).

The synthesis strategy is both efficient and flexible. The chosen combination of solid-phase and solution synthesis and the assembly of the target peptides from building blocks that can be varied readily opens up the opportunity to generate a variety of further analogues rapidly if required. Furthermore, variation of the photophore is straightforward. Thus, a variety of suitable tools for further biological investigations can be generated efficiently by means of this approach. To investigate if the benzophenone isoprenoid groups could be applied to the photoaffinity labeling of Ras interaction partners, we performed exposure experiments with solutions of lipopeptide **4a** (1.6 μM) and bovine serum albumin (BSA; 8 μM). The reaction mixture was exposed to UV light and samples were taken before exposure to UV light, and after 5, 15, 30, and 60 min of exposure. The time course of the reaction was analyzed by SDS-PAGE and Western Blot. Biotinylated BSA was detected by incubation with a streptavidine–alkaline phosphatase conjugate and subsequent enzymatic reaction. The benzophenone moiety was stable in the absence of UV irradiation, whereas exposure to 300—400-nm light led to rapid photoactivation and coupling of the lipopeptide to BSA (Figure 1). Five minutes of exposure to UV light were sufficient for maximum biotinylation of the BSA sample.

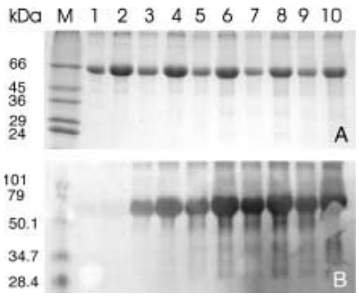

Figure 1. Coupling of biotinylated lipopeptide with BSA. A solution of lipopeptide **4a** (10 μM) and a fivefold excess of BSA were exposed to UV light, and samples were taken before (1, 2) and 5 min (3, 4), 15 min (5, 6), 30 min (7, 8), and 60 min (9, 10) after start of irradiation. Aliquots corresponding to 3 μg (1, 3, 5, 7, 9) and 5 μg (2, 4, 6, 8, 10) of BSA were applied in SDS-PAGE (A) and Western Blot (B).

Utilization of lipopeptides with a benzophenone isoprenoid group for the affinity labeling of Ras-interaction proteins requires that the replacement of the farnesyl group by a geranyl benzophenone moiety does not prevent the recognition of the lipopeptides by receptors or modifying enzymes for Ras. We generated a lipoprotein by coupling oncogenic N-Ras mutant N-RasG12VΔ181 (which has a truncated C terminus) with lipopeptide **4b** (which corresponds to the native C-terminus of N-Ras with a free palmitoylation site and a geranyl benzophenone instead of the farnesyl thioether at the last amino acid residue) as shown in Scheme 1. The reaction of the MIC group of the lipopeptide with the free cysteine residue at position 181 of the N-Ras mutant proceeded almost quantitatively.

N-RasG12VΔ181 terminates in a free cysteine residue, which selectively reacts with the MIC group. The coupling product was extracted by treatment with Triton-X114 and purified by ion-exchange chromatography. Gel electrophoresis gave a single band for the hydrophobic protein (Figure 2). Mass spectrometric analysis (Figure 3) gave rise to signals for the coupling product without the S-*tert*-butyl protecting group at the cysteine group, which can be substituted with a palmitoyl group (N-RasG12V^{CysGerBP}, 21659 Da), and a smaller signal for the coupling product with the protecting group (21747 Da). These data verify a 1:1 stoichiometry for the protein–lipopeptide coupling product. Specific reaction of MIC lipopeptide **4b** with the C-terminal cysteine (Cys181) of N-RasG12VΔ181 was validated by tryptic digestion of the chimeric protein with trypsin and chymotrypsin. ESI-MS and MALDI-TOF analysis of the proteolytic products excluded significant formation of lipopeptide thioethers with Cys51, Cys80, or Cys118 of N-RasG12VΔ181 (data not shown).

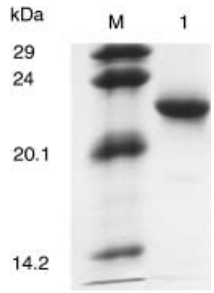

Figure 2. Characterization of the coupling product of N-RasG12VΔ181 with lipopeptide **4a** by SDS-PAGE. SDS-PAGE of the coupling product shows a protein band with an apparent mass of 22 kDa and a purity >90%.

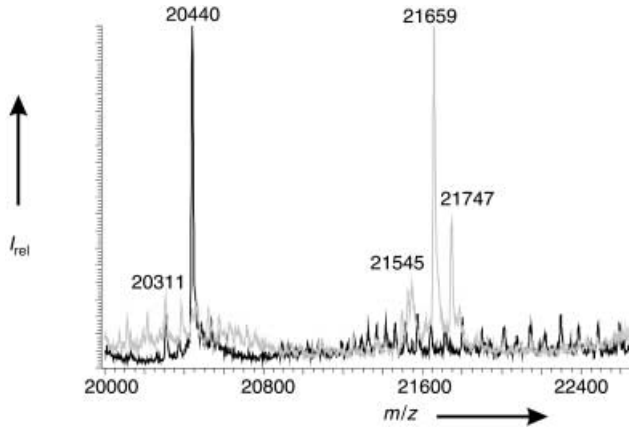

Figure 3. Characterization of the coupling product of N-RasG12VΔ181 with lipopeptide **4a** by ESI MS. Mass spectra of N-RasG12VΔ181 (black) and the coupling product of N-RasG12VΔ181 with lipopeptide **4a** (gray). The base peak in the spectra corresponds to the theoretical mass of the N-RasG12VΔ181 that has been truncated at the C terminus (20440 Da). The minor peak at 20311 Da can be assigned to N-RasG12VΔ181 without the N-terminal Met1 (theoretical mass: 20309 Da). The MS spectrum of the coupling product shows a major peak at 21659 Da and a minor peak at 21747 Da. These signals match the masses of the coupling product N-RasG12VΔ181–**4a** after and before removal of the S-*tert*-butyl protecting group, respectively.

The rat pheochromocytoma cell line PC12 can be induced to differentiate by oncogenic Ras mutants.[18] This effect can be correlated to the transforming potential of Ras mutants in a microinjection assay.[19] To ensure that benzophenone lipopeptides are applicable as photoaffinity probes for the identification of Ras receptors and modifiers, we analyzed the

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differentiating potential of the oncogenic coupling product of N-RasG12VΔ181 and the benzophenone lipopeptide **4b** and compared it with the efficiency of N-RasG12V full-length protein and the "natural" chimera with a C-terminal farnesyl thioether (N-RasG12V^{CysFar}) to induce the corresponding morphological pattern in PC12 cells. Gratifyingly, the lipoprotein with the benzophenone isoprenoid moiety induced neurite-like outgrowths in PC12 cells (Figure 4), thus indicating biological activity of the protein. This result implies that the construct with a geranyl benzophenone thioether and a carboxymethylation at the C terminus is sufficient to address the oncogenic chimera to its functional location, which includes palmitoylation of the free cysteine of the lipopeptide moiety.

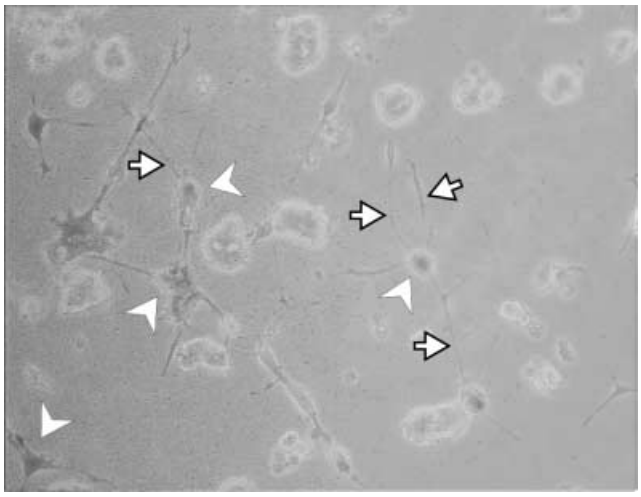

Figure 4. Differentiation of PC12 cells by N-RasG12V^{CysGerBP}. Overlay of a phase contrast and fluorescence microscopy image of PC12 cells after coinjection of 100 μM oncogenic Ras construct and FITC-dextran. Only cells with a fluorescent marker (dark cell body, white arrowheads) have formed neurite-like outgrowths (black-bordered white arrows). FITC = fluorescein isothiocyanate.

The data show that the potential of the benzophenone derivative to induce neurite-like outgrowths is approximately one third of the values recorded for N-RasG12V (full length) or N-RasG12V^{CysFar} (Figure 5). This activity is sufficient to allow the successful execution of biological experiments, including photoactivation studies.

Our results demonstrate that introduction of a benzophenone group as a photoactivatable ligand in the isoprenoid moiety of Ras-lipopeptides does not obstruct the ability of a corresponding oncogenic Ras-lipoprotein chimera to differentiate PC12 cells. Furthermore, photochemical activation of the benzophenone can be initiated by exposure to near-UV light (350 – 360 nm), thus limiting photodamage of the protein targets. Therefore, probes such as peptide **4a** with the C-terminal peptide sequence of Ras-proteins, a benzophenone isoprenoid substitution of the farnesyl group, and a suitable affinity tag (e.g. biotin) are promising tools for the isolation of Ras-modifying enzymes and Ras-interaction partners, in particular of plasma-membrane-localized proteins.

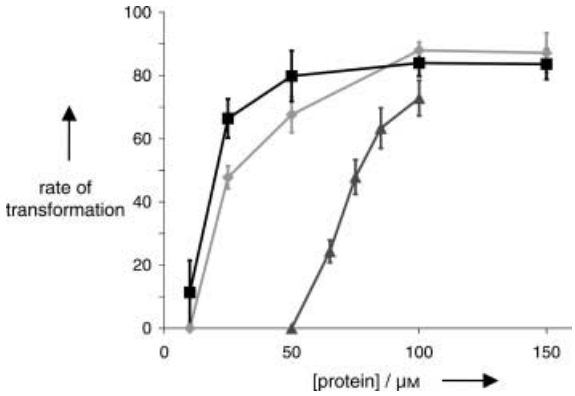

Figure 5. Differentiation efficiency of oncogenic N-Ras constructs. PC12 cells were microinjected with a protein solution that contained N-RasG12V^{CysGerBP} (▲), N-RasG12V^{CysFar} (◆), and full-length N-RasG12V (■) in the given concentrations.

Studies with green fluorescent protein constructs of Ras proteins have promoted the idea that it is the hydrophobically modified C terminus of the Ras isoforms that determines targeting and localization,[6, 20] whereas the G domain is less important for targeting the protein. The Ras C terminus is flexible and was not resolved in the crystal structures of the protein[21, 22] and its complexes[23] nor in the NMR spectroscopic analysis of H-Ras.[24] Therefore, it is most probable that affinity tags presenting the Ras C terminus are sufficient for recognition by and binding to putative receptors and modifiers.

By combining the power of organic synthesis with cell biology numerous synthetic lipopeptide and lipoprotein "baits" are accessible. For instance different natural or non-natural hydrophic modifications, alternative affinity tags, or radiolabels may be introduced or the entire peptide backbone may be replaced by a nonpeptide scaffold, thereby opening up a multitude of possible applications. The combination of chemical synthesis, cell biology, and elaborated biochemical and mass spectrometric approaches delineated herein should provide a new powerful approach to solve some of the most prevailing problems and answer open questions concerning signal transduction through Ras proteins.

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Bismuthine BiH_3: Fact or Fiction? High-Resolution Infrared, Millimeter-Wave, and Ab Initio Studies**

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Standard Inorganic Chemistry textbooks[1] report that bismuthine, BiH_3, is a common although unstable Group 15 hydride. Typically only the boiling point[2] of $+16.8\,^{\circ}C$ is mentioned for characterization. Apart from the early observation of a "volatile bismuth hydride" by Paneth in 1918,[3] its relevance in analytical chemistry,[4] and a mass spectrometric study,[5] all experimental information on BiH_3 (synthesis, isolation, and vapor pressure) originates from the report by Amberger in 1961.[2] To the best of our knowledge nobody has to date been able to repeat Amberger's (admittedly presumptuous) synthesis of BiH_3 by decomposition of CH_3BiH_2 at -55 to $-45\,^{\circ}C$, or to develop an alternative route to this compound. Hence, unlike the case of the short-lived monohydride BiH,[6] neither the structure nor the vibrational spectrum of BiH_3 have been determined experimentally.

We report herein the successful repetition of Amberger's synthesis of BiH_3 and its unambiguous characterization by independent, modern spectroscopic methods, which are supported by ab initio caclulations. Our particular interest in BiH_3 concerns those structural and spectroscopic properties that are expected to be unique for BiH_3. First, BiH_3 should have the smallest H-X-H bond angle (ca. 90.0°) of any of the hydrides of the composition XH_2 and XH_3 (Figure 1). In the vibrational ground state, BiH_3 should thus be an oblate symmetric top ($I_a = I_b < I_c$) extremely close to the spherical top ($I_a = I_b = I_c$) limit, even more so than SbH_3.[7] In vibrationally excited states it may switch to a prolate symmetric top with $I_a < I_b = I_c$. Therefore it should reveal, with unprecedented distinction, particular ground and excited state rotational energy patterns that require appropriate reductions of the rotational – vibrational Hamiltonian.[8] Owing to the near-rectangular H-Bi-H angle, the heavy central atom, the expected small HBi/BiH′ coupling, and the large anharmonicity of the BiH stretching motion, BiH_3 would also be a prototype molecule for local mode behavior.[9]

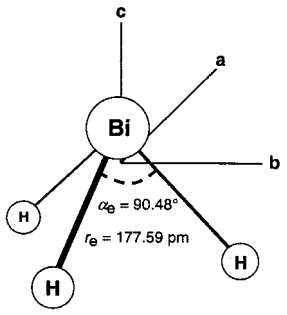

Figure 1. Molecular structure of BiH_3 with principal axes of inertia indicated; α_e, r_e = bond angle and length in the equilibrium structure.

After numerous failures we were able to repeat the reported synthesis[2] and eventually obtained apparently pure BiH_3 in quantities sufficient to enable us to carry out gas-phase infrared (IR) and millimeter-wave (MMW) measurements over periods of minutes to hours. In brief, we first prepared $Bi(CH_3)_3$ from $BiCl_3$ and CH_3MgI, and then reacted this with $BiCl_3$ to give CH_3BiCl_2 by using standard procedures.[10] CH_3BiCl_2 was then reduced with $LiAlH_4$ in di-*n*-butyl ether at $-78\,^{\circ}C$ to give CH_3BiH_2 whose disproportionation at -55 to $-45\,^{\circ}C$ yielded BiH_3 along with methylbismuthanes. Some hydrogen, which formed by decomposition of the hydrides, was pumped off while the reaction mixture was cooled by using liquid nitrogen. Thereafter this mixture was allowed to warm to about $-50\,^{\circ}C$ and volatile material expanded into cooled absorption cells until a total vapor pressure of between 10 and 100 Pa was reached.

IR spectra were recorded in the region for stretching fundamentals $\nu_1(A_1)/\nu_3(E)$ at about $1700\,cm^{-1}$ with a resolution of $4.4 \times 10^{-3}\,cm^{-1}$ (Figure 2), and in the region for bending modes $\nu_2(A_1)/\nu_4(E)$ at about $750\,cm^{-1}$ with a resolution of $6.6 \times 10^{-3}\,cm^{-1}$ (Figure 3).[11] An external dou-

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