$(Me_3Si)_2CH$) the differences between the Sb–Sb bond lengths (282.2–286.6 pm) are considerably smaller. The bond angles at the antimony atoms of **1** range between 84.66(9)° and 113.2(7)°. A similar wide range of Sb–Sb bond lengths and bond angles has also been observed in other antimony clusters, such as the anions Sb_7^{3-} [2d] or $[Sb_7M(CO)_3]^{3-}$ (M = Cr[2b], Mo[2a]). The differences in the distances and angles of **1** cause some deviations from the geometry of the realgar structure. This is apparent for the antimony atoms Sb(1), Sb(4), Sb(8), and Sb(6) bearing alkyl substiuents. They are not exactly situated in a plane; the deviations from the best plane are between −39.1 and 38.6 pm. The nonbonding distances between the antimony atoms without alkyl substituents [Sb(2)–Sb(5), Sb(2)–Sb(7), Sb(3)–Sb(5), Sb(3)–Sb(7)] vary only little between 429.0 and 432.3 pm. This corresponds quite well to the idealized geometry of the realgar type. A detailed examination of the structure of the five-membered rings reveals some deviations from an ideal envelope conformation. The atoms Sb(2), Sb(3), Sb(4), and Sb(5), for example, are not exactly in a plane either; the deviations from the best plane vary from −21.4 to 20.9 pm.

Apart from crystallographic analysis, mass spectrometry is also suitable for detecting the novel polycycles. In the chemical ionization mass spectrum, in addition to **1**, Sb_7R_5 (**2**, $R = (Me_3Si)_2CH$) was also detected through the intensive group of signals of the molecular ion. Attempts to determine the structure of **2** by X-ray structure analysis or NMR spectroscopy have not yet been successful. Analogous phosphorus compounds of the type P_7R_5 have the bicyclic norbornane structure.[3]

Experimental Section

All operations were carried out with strict exclusion of air in an argon atmosphere. Al_2O_3 was heated under reduced pressure and loaded under argon.

A solution of $RSbCl_2$ [R = $(Me_3Si)_2CH$] [6] (1.58 g, 4.5 mmol) in THF (20 mL) was added dropwise within 30 min with stirring to magnesium filings (0.15 g, 6 mmol) in THF (5 mL) activated with $BrCH_2CH_2Br$. The dark brown mixture was stirred for 1 h, the solvent was removed at reduced pressure, and the residue was washed three times with portions of petroleum ether (100 mL). The extracts were combined, concentrated to a volume of 10 mL, and separated by chromatography with petroleum ether on Al_2O_3 (Al_2O_3 neutral according to Brockmann, activity Super I, particle size 0.063–0.200 mm; column 8 × 1.5 cm). The first, intensely orange fraction contained $(SbR)_4$ (yield 0.76 g, 60%). The second, less intensely colored fraction was concentrated to a volume of 10 mL under reduced pressure. It contained $(SbR)_5$ as main component as well as SbR_3 and the polycycles **1** and **2**. Small yellow crystals of **1** were obtained when the concentrated solution was stored at −27 °C for two months (MS (CI, positive-ion mode, NH_3): **1**: *m/z* :1608–1612 [M^+]; **2**: *m/z*: 1643–1658 [M^+H]).

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[4] Crystal dimensions 0.5 × 0.4 × 0.15 mm, crystal system triclinic, space group $P\bar{1}$, cell parameters $a = 915.6(7)$, $b = 1781.5(7)$, $c = 1794.7(10)$ pm, $\alpha = 93.54(3)$, $\beta = 92.82(7)$, $\gamma = 104.56(6)°$, $V = 2.822(3)$ nm^3, $Z = 2$, $\rho_{calcd} = 1.897$ Mg m^{-3}, $2\theta_{max.} = 45.4°$, Siemens-P4 four-circle diffractometer, $Mo_{K\alpha}$ radiation, $\lambda = 71.073$ pm, scan mode 2θ-ω, $T = 173(2)$ K, 9170 measured reflections, 7365 independent reflections ($R_{int} = 0.090$) 3630 independent reflections with $(I > 2\sigma I)$, absorption coefficient 3.956 mm^{-1}, absorption correction Difabs, method of structure solutions: direct methods, program used for structure solution SHELXS-86, method of refinement full-matrix least squares at F^2, refinement program SHELXL-93, 424 free parameters, hydrogen atoms geometrically positioned and refined with a riding model, final $R(I > 2\sigma(I))$, $R1 = 0.0957$, $wR2 = 0.2507$. Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-100169. Copies of the data can be obtained free of charge on application to The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: int. code +(1223) 336-033; e-mail deposit@chemcrys.cam.ac.uk).

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Chemoenzymatic Synthesis of Fluorescent N-Ras Lipopeptides and Their Use in Membrane Localization Studies In Vivo**

Herbert Waldmann,* Michael Schelhaas, Edgar Nägele, Jürgen Kuhlmann, Alfred Wittinghofer,* Hans Schroeder, and John R. Silvius*

Dedicated to Professor Heribert Offermanns on the occasion of his 60th birthday

The lipidation of proteins, specifically the covalent attachment of a myristoyl group to an N-terminal glycine, and the S-palmitoylation and S-alkylation of cysteines by farnesyl or geranylgeranyl moieties, is among the most important modifications of proteins in nature.[1] Lipoproteins play decisive roles in numerous biological processes; in particular they are critically involved in the transduction of hormonal and mitogenic signals across the plasma membrane and from there towards the nucleus. For instance, the transmembrane G-protein-coupled receptors are S-palmitoylated, the heterotrimeric G-proteins are N-myristoylated, S-palmitoylated, and S-farnesylated, nonreceptor protein tyrosine kinases are N-myristoylated and S-palmitoylated, and the Ras proteins carry S-palmitoyl- and S-farnesyl groups. In addition, numerous further membrane-associated proteins are lipidated, for example the enzyme NO_x-synthetase,[2] and various viral envelope proteins[3] are S-palmitoylated.

Due to the important biological roles of lipid-modified proteins, the study of protein lipidation and its biological significance is at the forefront of biological research.[1] Correct lipidation of G-protein-coupled receptors[4] and the Ras proteins (vide infra) is known to be important for the proper execution

[*] Prof. Dr. H. Waldmann, Dr. M. Schelhaas, Dr. E. Nägele
Institut für Organische Chemie der Universität
Richard-Willstätter-Allee 2, D-76128 Karlsruhe (Germany)
Fax: Int. code +(721)608-4825
e-mail: waldmann@ochhades.chemie.uni-karlsruhe.de
Prof. Dr. F. Wittinghofer, Dr. J. Kuhlmann
Max-Planck-Institut für Molekulare Physiologie
Rheinlanddamm 201, D-44026 Dortmund (Germany)
Prof. J. R. Silvius, H. Schroeder
Department of Biochemistry, McGill University
Montréal, Quebec H3G1Y6 (Canada)

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of their biological functions. However, the general biological significance of different lipid groups, in particular their possible roles in biological signal transduction processes, is largely unclear and the subject of numerous hypotheses.[1] Furthermore, the detailed knowledge of the requirements for either transient or stable insertion of lipopeptides into biological bilayers may help in the design of drugs that influence pathological signal transduction processes such as those employing oncogenic Ras.

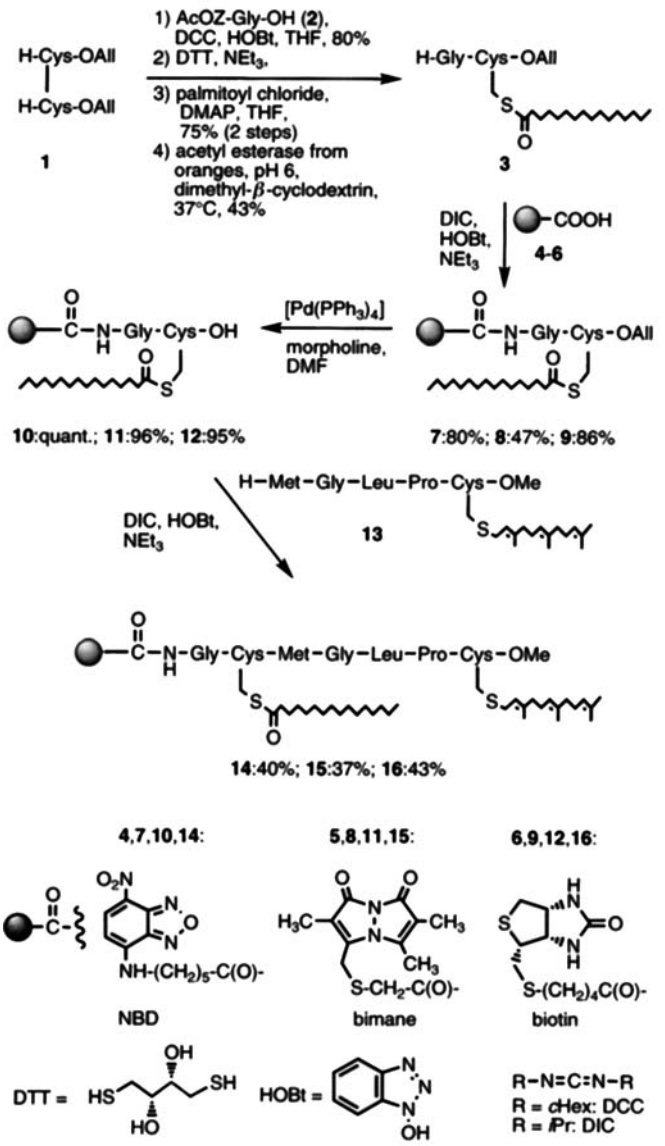
For the study of such phenomena by a combination of biophysical[5] and cell-biological[6] methods[7] useful reagents are lipidated peptides that contain the lipid groups and amino acid sequences of their parent lipoproteins, and also carry labels by which they can be traced in biological systems (i.e. fluorescent labels that can be detected by fluorescence microscopy).

We report here on an efficient method for the synthesis of fluorescent-labeled lipopeptides and on their application in the study of the specific membrane localization of lipopeptides and lipoproteins by means of membrane fusion/fluorescence microscopy and microinjection/confocal laser fluorescence microscopy.

For the development of a new synthetic, flexible method for the construction of differently lipidated peptides we have drawn from our previous experience in the construction of acid- and base-labile S-palmitoylated and S-farnesylated Ras lipopeptides.[8] The Ras proteins, a class of membrane-bound lipidated proteins that convey signals sent by growth factors further towards the nucleus, are often involved in malignant transformation.[9] They can fulfill their biological roles in the normal and the transformed state only if they are lipidated.[9]

The differently labeled N-Ras heptapeptides **14**–**16**, **17**, and **18** were chosen as target compounds. In retrosynthetic analysis they were divided into differently labeled N-terminal dipeptides and a constant C-terminal pentapeptide **13**, which carries an acid-labile farnesyl sulfanyl group (Scheme 1). Peptide **13** can be constructed in high yield by enzymatic removal of the N-terminal *p*-acetoxybenzyloxycarbonyl (AcOZ) group[8a] or base-mediated cleavage of the fluorenylmethoxycarbonyl (Fmoc) group[8c] as key steps. The synthesis of the selectively deprotected, S-palmitoylated peptides **14**–**16** is complicated by the pronounced base-lability of the sulfanylcarbonyl group. Thus, we found that in S-palmitoylated peptides these groups hydrolyze spontaneously even at pH 7 in aqueous solution.[8b] We now report that S-palmitoylated peptides can be synthesized efficiently by the combination of Pd^0-mediated cleavage of an allyl ester[10] for deprotection of the peptide chain at the C-terminus and enzymatic removal of an AcOZ group[8a] for unmasking at the N-terminus.

Thus, cystine (bis)allyl ester **1** was condensed with AcOZ-glycine **2** to give the corresponding peptide in high yield. After reductive cleavage of the disulfide bond by means of dithiothreitol (DTT), the sulfanyl groups were palmitoylated and then the N-terminal AcOZ residue was cleaved by means of the enzyme acetyl esterase from oranges to deliver the selectively unmasked, S-palmitoylated dipeptide allyl ester **3** in a straightforward manner. Dipeptide **3** was then condensed with different labels. The fluorescent 7-nitrobenz-2-oxa-1,3-diazolyl (NBD) and bimanyl groups were introduced with reagents **4** and **5**, respectively, and **3** was also coupled to the biotin derivative **6**. The fully masked peptides **7**–**9** were then treated with $[Pd(PPh_3)_4]$ in the presence of morpholine as an allyl-accepting nucleophile to give the selectively deprotected, fluorescent-labeled dipeptides **10**–**12** in high yields. In the N- and the C-terminal deprotections no undesired side reactions occurred. Thus, the conditions for the enzymatic cleavage of the AcOZ residue and the allyl ester are so mild that neither an attack on the base-sensitive thioester group nor a base-induced β-elimination[8] occurs. Finally, **10**–**12** were condensed with the N-terminally deprotected pentapeptide **13** to give the desired, differently labeled, S-farnesylated and S-palmitoylated N-Ras heptapeptides **14**–**16**. Similarly, the NBD-tagged heptapeptides **17** and **18** were prepared. In addition, the farnesylated pentapeptide **13** was coupled with NBD-aminocaproic acid **4** to give the labeled peptide **19** (Scheme 2).



Scheme 1. Chemoenzymatic synthesis of the labeled, farnesylated and palmitoylated N-Ras lipopeptides **14**–**16**.

The NBD- and the bimanyl-labeled peptides can be detected directly in biological systems and in vitro model systems by fluorescence microscopy and fluorescence spectroscopy. The biotin label can be traced with the protein streptavidin, which is also available in a fluorescent-labeled form or can be modified with colloidal gold, thus facilitating the study of model lipoproteins by fluorescence microscopy and electron microscopy.[11]

One of the prevailing questions in the study of the biological roles of the lipidation of proteins is whether particular lipidation motifs target lipoproteins to specific membranes.[5–8] Thus, the observation that several proteins in the plasma membrane carry farnesyl residues, whereas proteins in intracellular membranes

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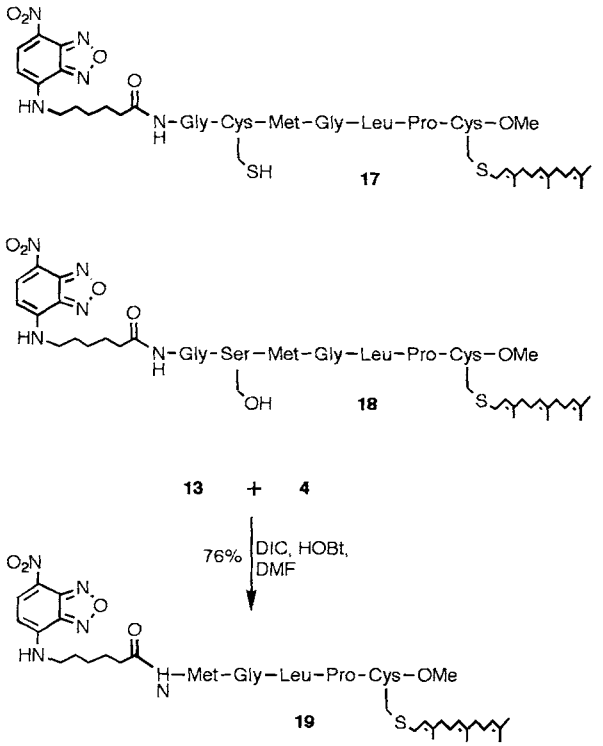

Scheme 2. Synthesis of the labeled farnesylated N-Ras peptides **17**–**19**.

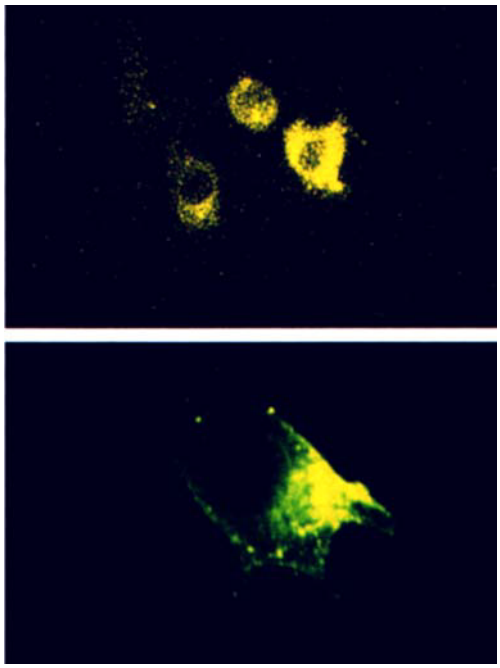

Figure 1. Fluorescence micrographs of NIH-3T3 fibroblast cells. Top: After microinjection of pentapeptide **19**; bottom: after microinjection of heptapeptide **14**. The top micrograph shows that **19** is not localized to the plasma membrane; the bottom micrograph indicates that **14** is localized in the plasma membrane.

are often geranylgeranylated (e.g. the Rab proteins[12]) has raised the notion that S-farnesylation might serve to target lipidated proteins specifically to the plasma membrane, for example by interactions with respective receptors.[1, 13]

To address this question and to determine whether the different lipidation motifs and amino acid sequences present in the Ras model peptides **14**, **17**, **18** and **19** determine a specific localization of the probes in a particular subcellular membrane, in particular the plasma membrane, the labeled peptides were subjected to in vivo studies. In one set of experiments the exclusively farnesylated and non-palmitoylable NBD-labeled pentapeptide **19** and the palmitoylated and farnesylated NBD-labeled lipoheptapeptide **14** were microinjected into NIH-3T3 fibroblast cells. After 30 min the distribution of the peptides in the cells was investigated by means of confocal laser fluorescence microscopy. The image obtained for **19**, shown at the top of Figure 1, demonstrates that the peptide is not localized in the plasma membrane. At the bottom of Figure 1 the corresponding result for **14** indicates clearly that the doubly modified peptide is specifically located in the plasma membrane. In the micrograph the fluorescence intensity is highest in the membrane around the site of injection (large yellow spot) and extends from there into the membrane of the dendrites of the cell. Several notable "hot spots" seem to represent compartments of the plasma membrane that are particularly enriched with the lipidated peptide.

In a second set of experiments the palmitoylation of the lipopeptides **17** and **18** was studied, and the subcellular locus at which this reaction occurs was determined. To this end, cultured CV-1 fibroblasts were incubated with [^{3}H]palmitic acid and sonicated vesicles (composed of phosphatidyl choline extracted from egg yolk and 1-palmitoyl-2-oleoyl phosphatidylethanolamine in a ratio of 90:10) loaded with lipopeptides.[6] The palmitoylation of the fluorescent peptides after fusion of the vesicles with the cell membrane was determined by extraction of the fibroblast cells and analysis by two-dimensional thin-layer chromatography and scintillation counting.[6] Visualization of the S-acylated peptide spot by fluorescence employing peptide **14** as reference compound revealed that the N-Ras peptide **17**, which contains a farnesylated and an unacylated cysteine, was readily S-acylated in the fibroblasts and thereby converted into the palmitoylated and farnesylated lipopeptide **14**. In contrast, the serinyl lipopeptide **18** was not acylated under these conditions at all.

The subcellular locus of S-acylation of **17** was examined by fluorescence microscopy.[6] After incubation of CV-1 fibroblasts with lipid-loaded vesicles and selective extraction of unacylated peptide, inspection of the cells revealed a preferential accumulation of fluorescence, that is, the S-acylated lipopeptide **14**, in the plasma membrane. To determine whether the peptide might have been acylated at an intracellular membrane, for example in a compartment of the endoplasmatic reticulum or of the Golgi apparatus, and then rapidly transferred in the S-acylated form to the plasma membrane, presumably by vesicular membrane transport,[14] the experiments were carried out at different temperatures. However, neither at 37 °C (at which vesicular transport is regular) nor at 15 °C (at which vesicular transport of materials from the Golgi to the plasma membrane is strongly suppressed[15]) was fluorescence detected in the Golgi apparatus or trans Golgi compartments. At both temperatures fluorescence accumulated in the plasma membrane. Given the additional fact that doubly lipid-modified peptides like **14** exhibit negligible rates of spontaneous transfer between distinct membranes[5, 6] (as opposed to lipopeptides like **19** carrying only a farnesyl group, which rapidly exchange between different membranes), these observations suggest that the plasma membrane is itself a major site of cellular S-acylation. In contrast, cells incubated with the serinyl-peptide **18** showed little residual fluorescence.

These in vivo experiments with the labeled N-Ras peptides support the mechanism for the selective subcellular localization

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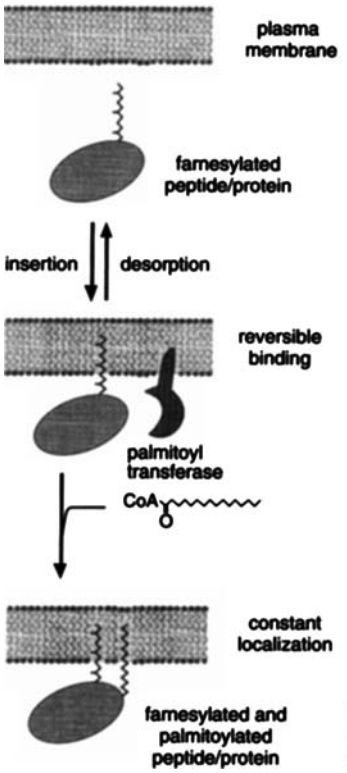
of lipoproteins recently proposed by Silvius et al.[5] (Scheme 3). According to this mechanism, the farnesylated but unacylated N-Ras C-terminal heptapeptide **17** (as well as the analogous pentapeptide **19**) can diffuse freely between different membranes. Upon S-acylation of **17** (which is not possible for **19**) in



Scheme 3. Proposed model for the targeting of lipoproteins to the plasma membrane by S-palmitoylation.

a given cellular membrane compartment the now doubly lipidated peptide can no longer be transferred between different membranes. It is thus localized to the membrane where the S-acylation reaction takes place, which in the case of the N-Ras peptide is the plasma membrane. The injected, doubly modified conjugate **14** might be rapidly incorporated into the plasma membrane near the injection site and remain there due to the anchoring influence of two lipid groups. In addition, it could undergo enzymatic[16] or nonenzymatic (vide supra) deacylation on sulfur and subsequent repalmitoylation at the plasma membrane during the time of assay.

This model should also be valid for the co- and posttranslational modification of membrane-bound lipid-modified proteins by S-farnesylation and S-palmitoylation, in particular of the Ras proteins themselves. According to this model the specific localization of lipoproteins in particular membranes would not only be determined by the first lipid group introduced in the course of their biosynthesis, but even more by the attachment of the second lipid residue at the site at which the protein remains localized. The recent identification of a plasma membrane bound protein S-acyltransferase,[17] which acylates the free cysteine in the farnesylated C-terminal hexadecapeptide of N-Ras, and similarly positioned cysteine residues in full length H-Ras strongly supports this notion.

This model of "kinetic targeting" does not address the participation of transport proteins that might be involved in the membrane targeting of lipoproteins. However, it explains how very small regions of proteins such as Lck, Fyn, and H- and N-Ras containing S-acylation sites are both necessary and sufficient for targeting to the plasma membrane when they are introduced artificially into chimeric or mutated proteins.[18] Also, a recent study has concluded that the protein Fyn is targeted to the plasma membrane by a kinetic-targeting mechanism based on acylation at that membrane[19] just as was observed for simple lipopeptides that are S-acylation substrates and that resemble the termini of proteins such as Fyn and Lck.[6]

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[20] NIH-3T3 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) in a humidified CO_2 (7.5%) incubator at 37 °C. Microinjections were performed in culture medium buffered with 20 mM Na-HEPES, pH 7.4. For injections a Zeiss Microinjection Workstation (AIS) was used along with thin borosilicate glass capillaries with filaments (Hilgenberg) having a diameter of ≤ 0.5 μm at the tip. Distribution of the fluorophore was observed 30 min after injection with a confocal laser scanning microscope (CLSM) based on the MRC 500 system (BioRad) with an excitation wavelength at 488 nm.

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