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A Photo-Cross-Linking Strategy to Map Sites of Protein–Protein Interactions

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Chemical and photochemical cross-linking are potentially powerful approaches for investigating protein–protein interactions and identification of interaction sites.[1] The approaches complement high-resolution X-ray diffraction and NMR spectroscopy methods for three-dimensional protein structure analysis, especially when proteins are only available in small amounts or are difficult to crystallize. The (photo)cross-linked products are typically analyzed by proteolytic digestion and mass spectrometry, yielding, in ideal cases, sequence and specific amino acid(s) of the interaction sites. The fast reaction of photo-cross-linking allows its application to short-lived protein complexes. To facilitate mass spectrometric analysis, enrichment of the digested photo-cross-linked products is desirable. Herein, we describe a novel photo-cross-linking strategy based on such an enrichment step and capable of mapping sites of protein–protein interactions. As a test case, we applied the strategy to a model system represented by the G-protein-coupled receptor (GPCR) rhodopsin and its G protein transducin (Gt).[2] These two proteins are key enzymes in the visual process and were chosen previously for (photo)-cross-linking studies.[3]

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GPCRs, also called seven-transmembrane receptors, are among the most important drug targets and are involved in many physiological processes by routing extracellular signals across the cell membrane to different intracellular signaling pathways.[4] Binding of extracellular ligands causes a conformational change in the GPCR to enable its cytoplasmic domain to catalyze GDP/GTP exchange in heterotrimeric G proteins ($G\alpha\beta\gamma$); the process that initiates the signaling cascade. Visual signal transduction in the retinal rod cell is a prototypical sample of G-protein-coupled signaling systems in which the GPCR rhodopsin with its covalently bound chromophore 11-*cis*-retinal acts as a photoreceptor to detect single photons.[2,5] In its photochemically functional core, rhodopsin contains the covalently bound ligand 11-*cis*-retinal, which stabilizes the inactive rhodopsin state. Photon absorption causes retinal *cis*→*trans* isomerization, leading to in situ formation of an activating ligand in the binding site and subsequent activation of rhodopsin.

The crystal structures of rhodopsin, as well as that of opsin, the ligand-free apoprotein, are known.[6] The latter features a more open conformation of the cytoplasmic domain, which represents (with respect to G protein coupling) an active GPCR state.[2] For signal transfer to the cognate G protein called transducin (Gt), the C terminus of the $G\alpha$ subunit, $Gt\alpha$(340-350) (Figure 1), binds into a central crevice in the cytoplasmic domain opened in the active receptor.[7] A second binding site on the G protein is the prenylated C terminus of the $G\gamma$ subunit. In the case of Gt, the binding site $Gt\gamma$(60-71) is farnesylated (Figure 1; $Gt\gamma$(60-71)far).[8] The binding site of $Gt\gamma$(60-71)far on rhodopsin is less clear, although there is multiple evidence that it is involved in initial docking to the receptor.[9]

Herein, we set out to determine the binding site of $Gt\gamma$-(60-71)far on light-activated rhodopsin by preparing a synthetic photoactivable peptide derived from the farnesylated $Gt\gamma$ C terminus. For this purpose, a derivative of the native $Gt\gamma$(60-71)far peptide (Scheme 1 A, peptide 1) was synthesized, which contained a *p*-ethynylbenzoyl moiety modified Phe-64, yielding the photoactivable ethynylated benzophenone probe (Scheme 1 B, peptide 2). The MALDI-TOF-MS

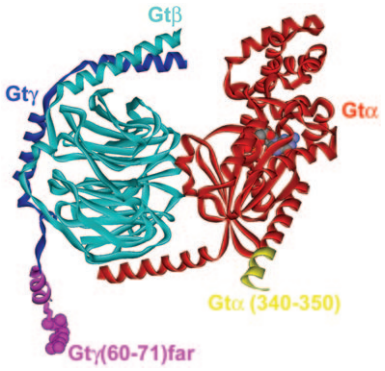
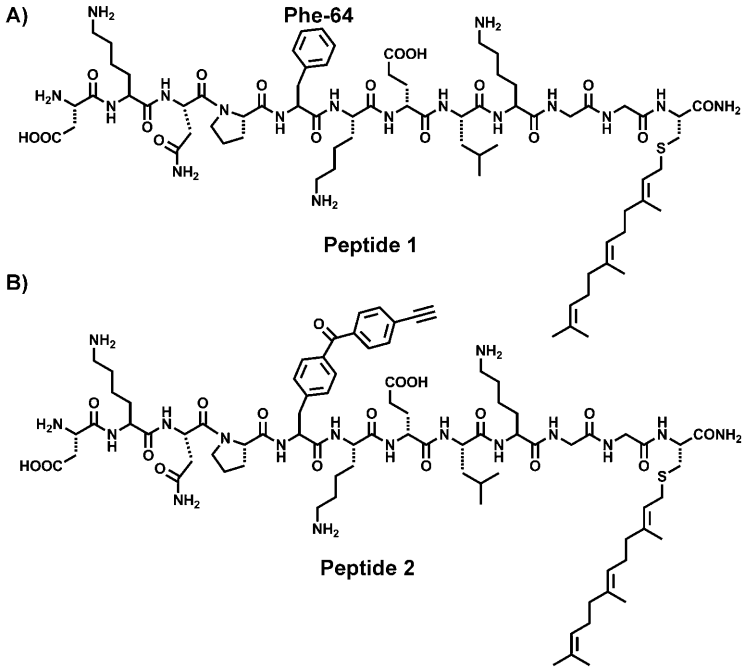


Figure 1. Crystal structure of the heterotrimeric G protein transducin, Gtαβγ (PDB accession 1GOT). Rhodopsin interacting sites are the C-terminal regions of Gtγ and Gtα (shown in purple and yellow, respectively), and modeled in their active receptor interacting conformations known from NMR spectroscopy.[10]

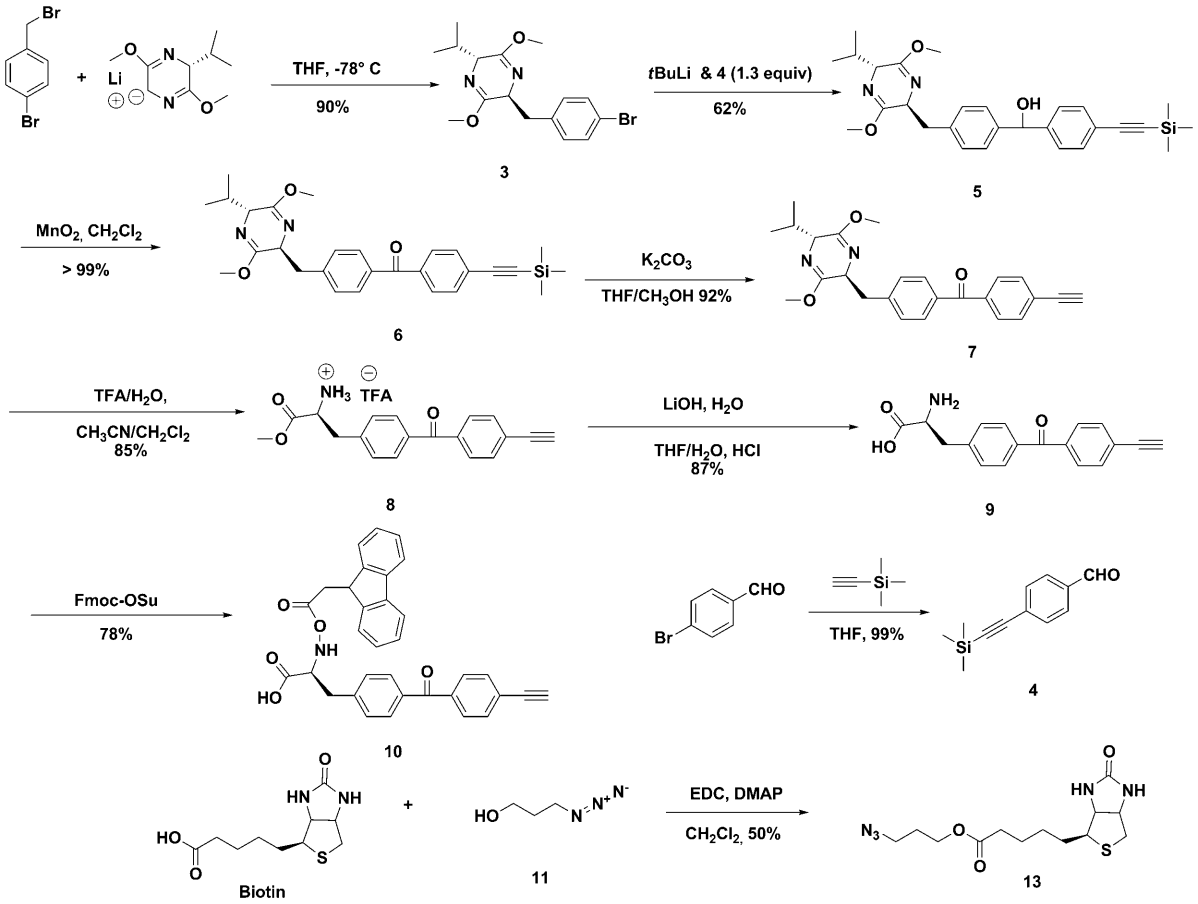


Scheme 1. Peptide 1: native Gtγ(60-71)far peptide derived from the transducin Gtγ subunit (^{60}DKNPFKELKGGC^{71}-farnesyl). Peptide 2: photoactivable Phe-64 *p*-ethynylbenzoyl-modified peptide 1.

spectrum of peptide 2 is shown in Figure S1 in the Supporting Information.

Position 64 was chosen because earlier studies suggested that Phe-64 is part of the rhodopsin interacting surface.[11] Further, a benzophenone group at this position had little effect on the affinity of the Gtγ(60-71)far-derived peptide for active rhodopsin.[12] Introduction of the alkyne group to the benzophenone allows click chemistry, which has proven to be useful in labeling biomolecules for in vitro and in vivo studies, to be performed.[13] Peptide 2 was synthesized by using the *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) strategy with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) activation chemistry and Fmoc-protected 4-ethynylated *p*-benzoyl-L-phenylalanine (Ebp), which was prepared as described in Scheme 2. The corresponding *p*-benzoyl-L-phenylalanine is known as a useful photoactivable probe.[14] The C-terminal cysteine in peptide 2 was farnesylated by using farnesyl bromide.[15] For photo-cross-linking and subsequent identification of the interaction site (Scheme 3), the respective photolabile peptide 2 was incubated with native rod cell disc membranes (rhodopsin represents 90% of the total protein in these membranes). Orange light was used to form activated rhodopsin, and its complex with peptide 2 was cross-linked by irradiation with 350 nm light. After solubilization in lauryldimethylamine oxide and reduction with dithiothreitol, the cross-linked protein–peptide complex was alkylated with iodoacetamide and precipitated with trichloroacetic acid (TCA). CNBr cleavage (that was used earlier to specifically cleave rhodopsin at methionine sites)[3d,16] then yielded a mixture of peptide fragments which were subjected to "click-chemistry" in the presence of biotin propyl azide (Schemes 2 and 3). Click chemistry, that is, Cu^{I}-catalyzed chemo-selective coupling between organic azides and terminal alkynes (like the ethynyl group of Ebp), was used to add a biotin-tag to the cross-linked peptidic fragments for further purification by biotin-avidin affinity chromatography (Scheme 3). The [3+2] cycloaddition reaction with the azido group in biotin propyl azide represents a fast and selective chemical transformation under mild conditions.

Only the biotin-tag-derivatized photo-cross-linked rhodopsin peptidic fragments bind to avidin–agarose. These fragments were eluted with 8 M guanidine-HCl, desalted, and fractionated on an Atlantis dC18 (3 μm, 4.6×150 mm, Waters) reverse-phase column by HPLC (Figure S2 in the Supporting Information). Purified peptides were mixed with α-cyano-4-hydroxycinnamic acid as the matrix and subjected to MALDI-TOF mass spectrometry. Results of the analysis of the biotin-tag-derivatized fragments are shown in Figure 2. Three signals corresponding to *m*/*z* of 3370.1 (peak 1), 3441.6 (peak 2), and 3485.0 (peak 3) were observed. The rhodopsin peptidic fragment in peak 1 had an actual *m*/*z* value of 1375.7, calculated by subtracting the *m*/*z* value of 1994.4 for peptide 2 modified with biotin propyl azide. A *m*/*z* value of 1375.7 (or 1375.0 as de-

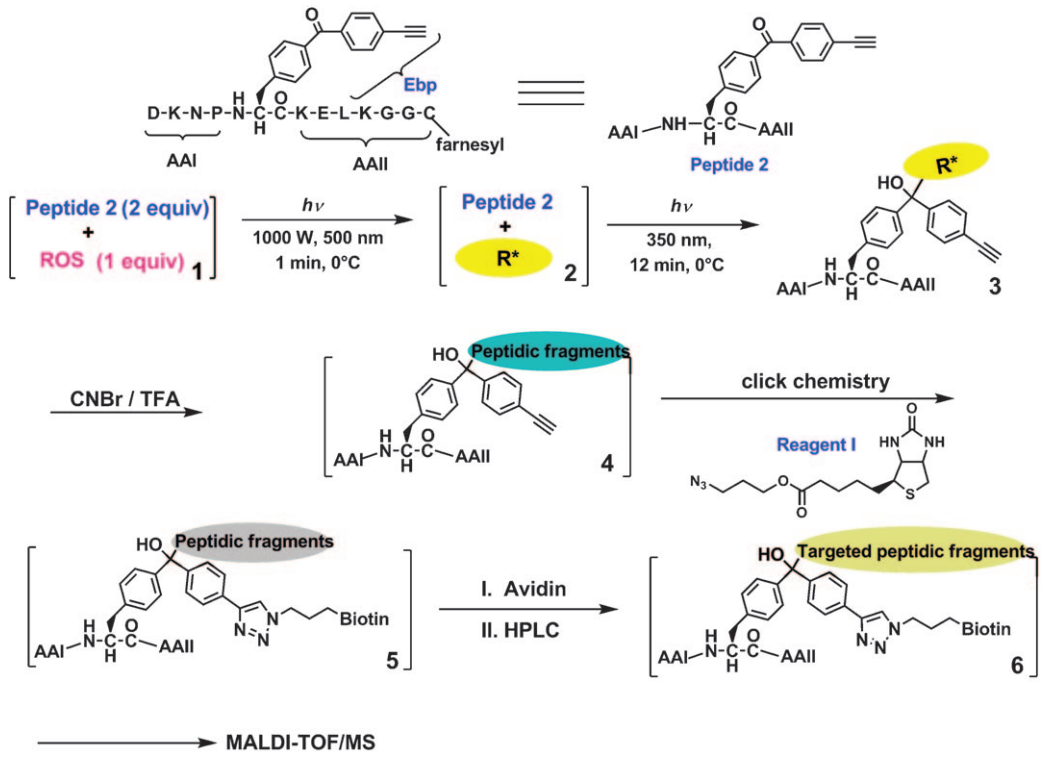


Scheme 2. Synthesis of the "clickable" amino acid and the corresponding biotinylated conjugate (see the Supporting Information). Fmoc-OSu = *N*-(9-fluorenylmethoxycarbonyloxy)succinimid, TFA = trifluoroacetic acid, DMAP = 4-(dimethylamino)pyridine, EDC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide.

termined in another experiment; Figure S3 in the Supporting Information) is consistent with the expected mass of 1374.7 $[M+H]^+$ of a peptide fragment corresponding to the sequence ^{144}SNFRFGENHAIM^{155} of rhodopsin.[16] This sequence covers the cytoplasmic end of transmembrane helix 4 and the C-terminal half of the second cytoplasmic loop connecting transmembrane helices 3 and 4 (Figure 3). The identification of the other two peaks, probably resulting from incomplete CNBr cleavage,[17] was not possible, so far, and in this specific case was hampered by the use of a highly hydrophobic peptide probe.

Our work aimed at testing a new photo-cross-linking strategy to map the interaction sites between activated rhodopsin and Gtγ(60-71)far. Our study extends previous covalent (photo)-cross-linking approaches on rhodopsin/Gt interaction using different strategies, that is, reactive groups and analysis procedure.[3a–c] In previous studies, photoactive reagents were attached to the sulfhydryl group of cytoplasmic monocysteine rhodopsin mutants to primarily map the rhodopsin contact sites on the Gtα subunit, whereas we have utilized the benzophenone moiety in Ebp/Phe-64 of Gtγ(60-71)far (Scheme 3). The combination of click chemistry in the presence of biotin propyl azide as a probe, followed by MALDI-TOF mass spectrometry analysis, resulted in the identification of the interacting peptide sequence Ser144-Met155 on rhodopsin, which contains the cytoplasmic end of helix 4 and half of the second cytoplasmic loop. This loop is likely to play a role in the two-step interaction between activated rhodopsin and Gt and is assumed to rearrange according to a current modeling study.[9b,18]

In summary, we developed a new photo-cross-linking strategy that makes use of *p*-ethynylbenzoyl-modified phenylalanine and exploits click chemistry to purify the photo-cross-linked products for subsequent mass spectrometric identification of proteolytic peptide fragments. Expression systems were developed to generate proteins with genetically encoded benzophenone by using *p*-benzoyl-L-phenylalanine and amber codon suppression methodology.[19] We envision that the ethynylated *p*-benzoyl-L-phenylalanine (compound **9** in Scheme 2) might be incorporated into proteins in vivo, which adds a high potential to the presented photo-cross-linking strategy for proteome research.



Scheme 3. Strategy for photo-cross-linking of peptide 2 to light-activated rhodopsin (R*) and subsequent analysis of cross-linked sites on rhodopsin. Peptide 2, Gtγ(60-71)far peptide with *p*-ethynylbenzoyl modified Phe-64. Reagent I, biotin propyl azide was prepared by a two-step synthesis (see the Supporting Information).

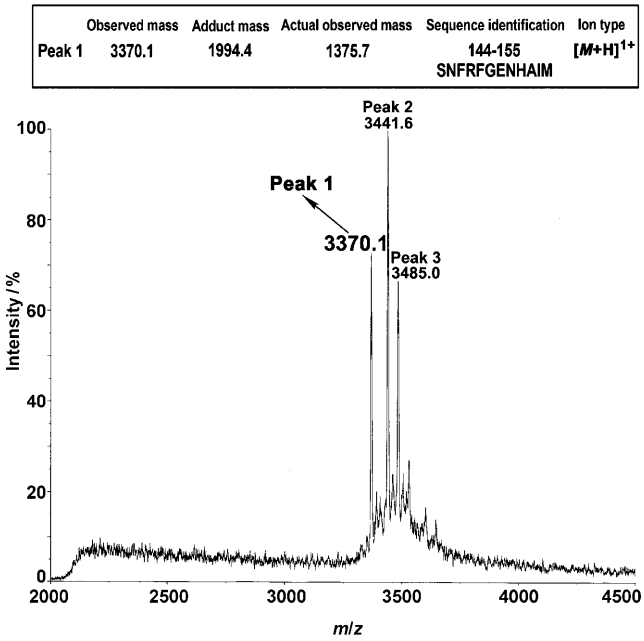


Figure 2. MALDI-TOF mass spectrum of rhodopsin fragments photo-cross-linked to peptide 2. The photo-cross-linked rhodopsin was digested with CNBr, labeled with biotinylated reagent I (Scheme 3), purified by avidin–agarose, and subjected to MS. Inset: the amino acid sequence and expected mass of the identified rhodopsin fragment (peak 1).

Experimental Section

General experimental procedure: Bovine retinal rod outer segment membranes were prepared under dim red light[20] and washed with 5 M urea to strip off surface-bound proteins.[21] Washed rod outer segment membranes for extra MII measurements were prepared according to reference [9b]. THF and diethyl ether were distilled from sodium/benzophenone ketyl. Dichloromethane was distilled over CaH_2. All other reagents and starting materials were purchased and used as received (Aldrich, St. Louis, MO). Avidin agarose resin was purchased from ThermoFisher Scientific. Reactions were monitored by analytical TLC using silica gel 60 F254 plates and spots were visualized by UV light irradiation (254 nm) or oxidative stain (PMA = phosphomolybdic acid). Flash column chromatography was performed by using silica gel (230–400 mesh). Analytical HPLC was performed on a Waters instrument with UV detector (200–700 nm) by using a C18 reverse-phase column (Waters, 4.6 mm × 150 mm) to analyze synthetic Gtγ-derived peptides. Water and acetonitrile were used as eluents in various gradient programs at a flow of 0.6 mL min^{-1}. ^{1}H NMR spectra were recorded with Bruker (300 or 400 MHz) spectrometers. The chemical shifts are expressed in ppm (δ) downfield from tetramethylsilane (in $CDCl_3$). UV/Vis spectra were recorded at 25 °C in a Perkin–Elmer Lambda 40 spectrophotometer. Mass spectra were measured on a JEOL KMS-HX110/100A HF mass spectrometer under FAB conditions or a Voyager DE MALDI-TOF mass spectrometer (Applied Biosystems). Synthetic compounds **3**–**9**, and **13** (see Scheme 2 and descriptions in the Supporting Information) were subjected to FABMS.

Peptide synthesis: This was performed on an ABI 433A automated peptide synthesizer (Applied Biosystems) on a 0.1 mM scale with 300 mg TentaGel S-RAM-Fmoc-resin (capacity 0.22 mmol g^{-1} RAPP Polymere GmbH Tübingen, Germany) by using the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)/*tert*-butyl alcohol strategy. The following side-chain protecting groups were used: *tert*-butoxycarbonyl (Lys), *tert*-butyl ester (Asp and Glu) and trityl (Asn and Cys). Couplings were performed with *O*-(7-aza-

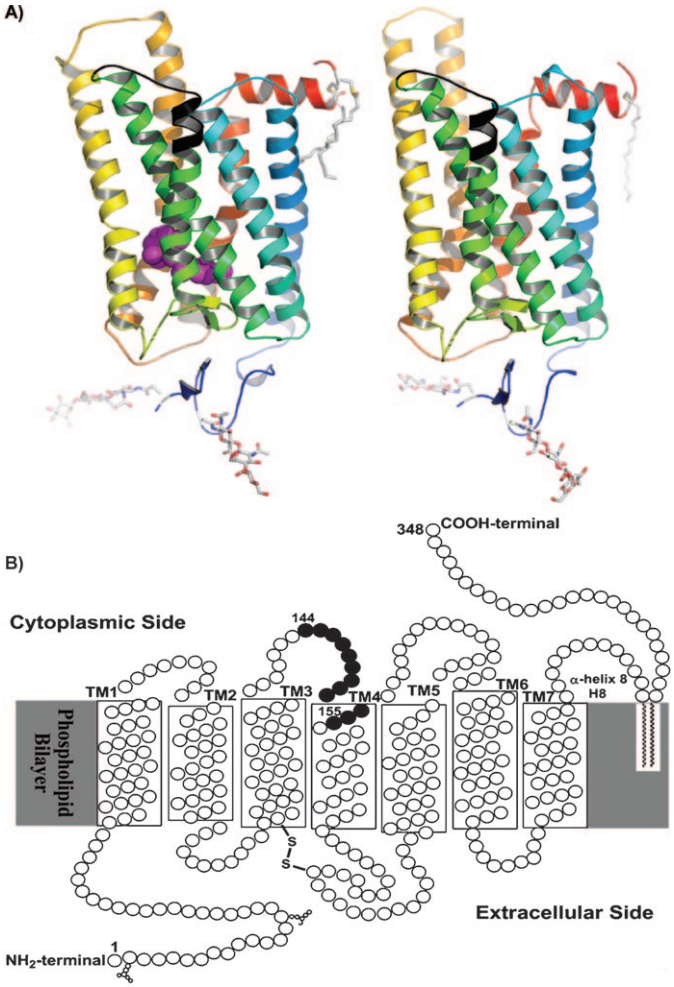


Figure 3. Crystal structures (A) of rhodopsin (left, PDB accession number 1GZM) and opsin (right, PDB accession number 3CAP), and transmembrane topological model (B) of rhodopsin. Rhodopsin and opsin contain seven transmembrane helices (blue to orange in (A) and TM1 to TM7 in (B)), followed by cytoplasmic helix 8 (red in (A) and H8 in (B)), which is terminated by palmitoylated Cys322 and Cys323 (in opsin only palmitoylation of Cys322 is shown). Oligosaccharides are attached to Asn2 and Asn15 in the N-terminal region. The transmembrane helices are linked by cytoplasmic (top) and extracellular (bottom) loops. In (A), C-terminal residues 325–348 are not shown. The rhodopsin/opsin peptide fragment (^{144}SNFRFGENHAIM^{155}), shown in black (A) and as filled circles (B), was identified by cross-linking experiments with peptide 2 (Gtγ(60-71)far peptide containing *p*-ethynylbenzoyl-modified phenylalanine. The identified sequence (Figure 2, inset) covers most of the second cytoplasmic loop (connecting transmembrane helices 3 and 4) and the cytoplasmic end of transmembrane helix 4.

benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) in *N*-methylpyrrolidone as a coupling agent. Deprotection of the Fmoc group was performed during the complete synthesis with 20% piperidine in DMF. The final deprotection from the resin was performed with 95% TFA in water containing 3% triisopropylsilane and 5% phenol. The crude protein was farnesylated with farnesyl bromide and purified by reverse-phase (RP) HPLC on a 7 μ Zorbax SB C18 column (21.2×250 mm, Prep HT) with a linear gradient of 50–60% B over 45 min (A: water (1000 mL), TFA (2 mL); B: acetonitrile (500 mL), water (100 mL), TFA (1 mL)) at a flow rate of 10 mL min^{-1} with spectrophotometric monitoring at $\lambda = 220$ nm. The fractions were checked by RP-HPLC (Shimadzu LC10) on a VYDAC C18 column (4.6×250 mm, 5 μ, 300 Å) with a linear gradient of 10 to 100% B over a period of 45 min (A: water (1000 mL), TFA (2 mL); B: acetonitrile (500 mL), water (100 mL), TFA (1 mL)) to give the final pure product. Peptide farnesylation was carried out as follows: 5 μM peptide was dissolved in 50% *n*-propyl alcohol and Na_2CO_3 was added to a final concentration of 20 mM. The solution was then treated with 30 μM farnesyl bromide in a 10% solution of *n*-propyl alcohol for 18–24 h at room temperature in the dark. Farnesylated peptides were purified by reverse-phase chromatography by using a PepRPC fast protein liquid chromatography column (GE Healthcare) using a linear (0–100%) gradient of acetonitrile in water containing 0.1% TFA. The molecular weight of purified peptides was determined by electrospray mass spectrometry by using a Vestec VT 201 mass spectrometer. Purified peptides were lyophilized and stored at −20 °C under nitrogen. Immediately before the experiments, the peptides were dissolved in appropriate buffers or deionized water and adjusted to pH 7 to obtain stock solutions of 1–10 mM.

Modified amino acid synthesis: The modified amino acid was synthesized by slight modification of a previous route.[12] For the synthesis of *p*-(4-hydroxybenzoyl)phenylalanine, *p*-bromobenzyl bromide was treated with (*R*)-Schollkopf reagent, and then incubated with 4-[(trimethylsilyl)ethynyl]benzaldehyde. Subsequent oxidation, deprotections, and hydrolysis gave a single diastereomer (100% *de*) as indicated. Biotin-tag-derivatized amino acid was obtained by treating biotin propyl azide with above compound.

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Keywords: binding sites · G-protein-coupled receptors · mass spectrometry · photochemistry · protein–protein interactions

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