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Synthesis of Thioether-Linked Analogues of the 2,3-Sialyl-TF and MECA-79 Antigens: Mucin-Type Glycopeptides Associated with Cancer and Inflammation

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antigens · carbohydrates · chemoselective ligation · glycopeptides · sulfation

Interest in the synthesis of sulfated and sialylated oligosaccharides is motivated in part by their unique expression in tissues of certain disease states, including chronic inflammation and many types of cancer. In nature, sulfated and sialylated carbohydrates are abundantly expressed on mucins, such as GlyCAM-1, MAdCAM-1, CD34, and MUC-1.[1] To date, the synthesis of an *O*-linked glycopeptide containing a sulfated oligosaccharide has not been reported. This is most likely due to the acid-sensitive nature of carbohydrate sulfate esters[2] and a lack of suitable methods for their protection during peptide synthesis.[3] While syntheses of glycopeptides containing sialic acid have been described, the preparation of these compounds is still a difficult task.[4] Here we present an efficient method for the convergent assembly of *O*-linked glycopeptides bearing sulfated and sialylated oligosaccharides in a chemoselective fashion.

Previously, we reported the use of glycosyl amino acid **1** (Scheme 1) for the synthesis of glycopeptide analogues by thiol alkylation.[5] Building block **1** contains an unnatural 3-thio-GalNAc residue that can undergo condensation with *N*-bromoacetamido sugars, following its incorporation into a peptide by Fmoc-based solid-phase peptide synthesis (SPPS). The thiol

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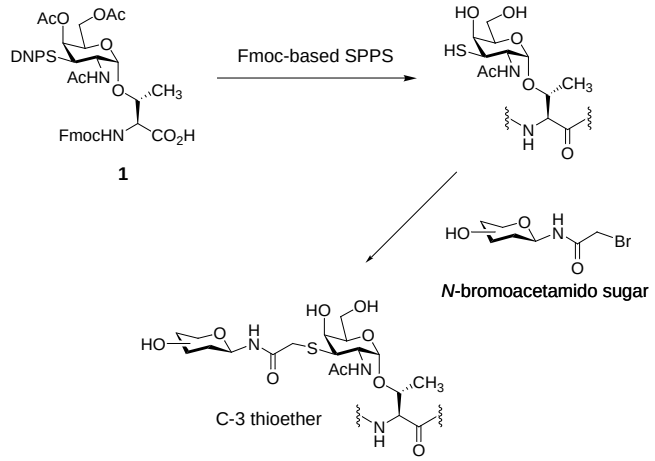
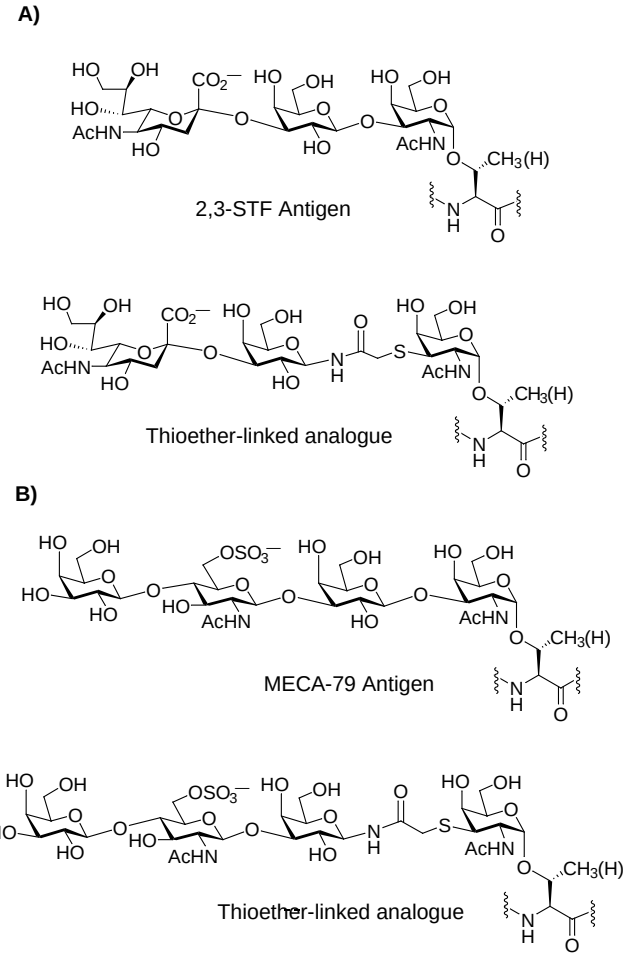


Scheme 1. *Synthesis of glycopeptide analogues by alkylation of 3-ThioGalNAc. Fmoc = 9-fluorenylmethoxycarbonyl, DNP = 2,4-dinitrophenyl.*

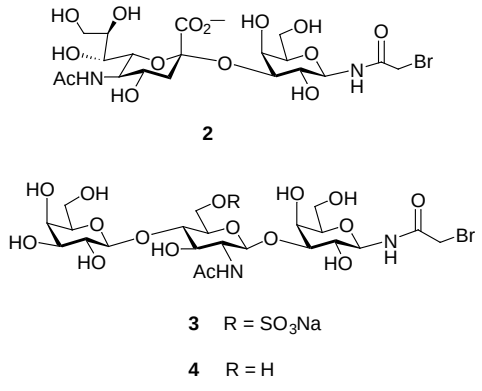
group can be alkylated selectively over all natural amino acids except cysteine. The resulting product contains an unnatural thioether linkage at the C-3 position, a common branch point of mucin-type oligosaccharides, but retains the native sugar peptide linkage. Conservation of the native core GalNAc residue is a key feature of this approach, given the importance of this moiety in establishing proper organization of the underlying peptide backbone.[6] In this manner, carbohydrate ligands can be appended to native mucin-like scaffolds in a chemoselective fashion. Since the oligosaccharide portion of the glycopeptide is introduced following peptide synthesis, exposure to harsh acid, such as trifluoroacetic acid (TFA), is avoided. Hence, this thiol alkylation strategy is ideally suited for the synthesis of glycopeptides containing acid-sensitive groups, such as sulfate esters and sialic acid residues.

The structures synthesized in the present study correspond to thioether-linked analogues of the mucin-related 2,3-sialyl-TF (STF) and MECA-79 antigens (Scheme 2). The presence of the STF antigen on breast tumors has been demonstrated.[1d] Thus, glycopeptide fragments containing this structure are of interest in the preparation of anticancer vaccines.[7] The MECA-79 antigen, which was recently identified by Fukuda and coworkers,[8] is of interest due to its unique expression at sites of chronic inflammation. Binding of the rat monoclonal antibody MECA-79 to cell-surface mucins containing this epitope inhibits L-selectin-dependent adhesion.[1b, 9] Since the interaction between L-selectin and cell-surface mucins is a key step in the process of inflammation, antibodies with specificities similar to MECA-79 may serve as antiinflammatory agents. Glycopeptide mimics bearing MECA-79 antigen analogues may be used as synthetic antigens to elicit such antibodies.

In order to generate glycopeptide analogues bearing the STF and MECA-79 antigens, bromoacetamide derivatives **2** and **3** were prepared (Scheme 3). For the purpose of future biological investigations, the unsulfated version (**4**) of the MECA-79 antigen was also synthesized. In general, *N*-bromoactemido sugars can be readily prepared from the corresponding glycosyl amines.[10] Glycosyl azides are stable to a variety of reaction conditions and



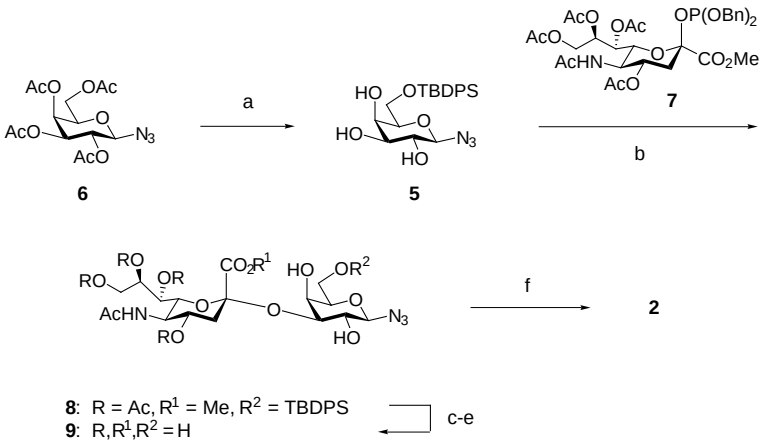
Scheme 2. *Structures of the 2,3-STF and the MECA-79 antigens and their thioether-linked derivatives.*



Scheme 3. *Target* N*-bromoacetamide derivatives **2**, **3**, and **4**.*

were therefore selected as intermediates in the synthesis of compounds **2** – **4**. The reduction of anomeric azides to glycosyl amines can be achieved under mild conditions, compatible with the presence of sialic acid residues and sulfate esters.

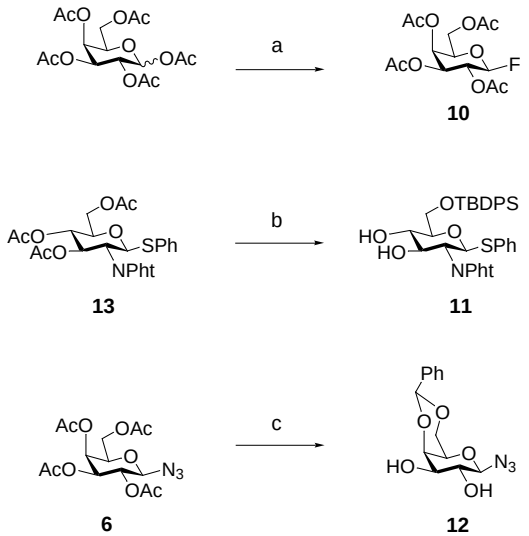
The synthesis of disaccharide **2** is depicted in Scheme 4. Glycosylation of 6-*O*-TBDPS β-galactosyl azide **5**, prepared in two steps from the known glycosyl azide **6**,[11] was achieved with high stereo- and regioselectivity by using sialyl phosphite **7**[12] as a



Scheme 4. *a) 1. NaOMe, MeOH; 2. TBDPSCl, imidazole, DMF, 89%; b) TMSOTf, 3 Å molecular sieves, CH_3CN, −40 °C, 2 h, 44%; c) TBAF, AcOH, THF, RT, overnight; d) NaOMe, MeOH, RT, 24 h; e) NaOH, THF, RT, 24 h, 92% (3 steps); f) 1. H_2, Pd/C, MeOH; 2. bromoacetic anhydride, 1 M $NaHCO_3$, RT, 2 h, 66%. TBDPS = tert-butyldiphenylsilyl, DMF = N,N-dimethylformamide, TMS = trimethylsilyl, Tf = trifluoromethanesulfonyl, TBAF = tetrabutylammonium fluoride, THF = tetrahydrofuran.*

donor. The resulting α-linked disaccharide **8** was deprotected by treatment with TBAF/AcOH, followed by deacetylation and saponification. The free glycosyl azide **9** was then reduced by catalytic hydrogenation to give the corresponding glycosyl amine, which was immediately treated with bromoacetic anhydride in the presence of sodium bicarbonate. The resulting *N*-bromoacetamido sugar **2** was obtained in good yield (66%) following purification by size-exclusion chromatography.

The synthesis of the MECA-79 antigen derivative **3** was achieved through the use of intermediates **10**, **11**, and **12** (Scheme 5), which were prepared as shown, from peracetylated D-galactose, thioglycoside **13**, and glycosyl azide **6**, respectively.

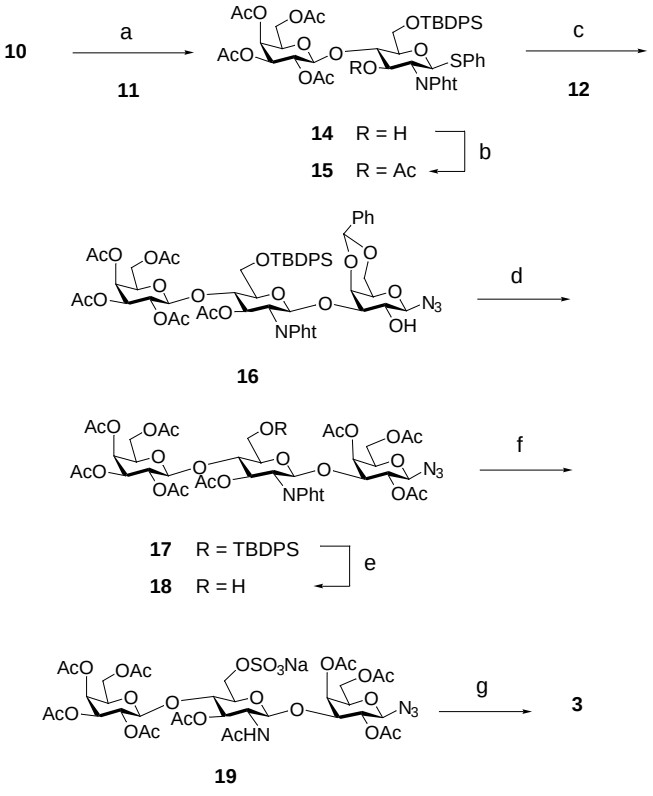


Scheme 5. *a) 1. $N_2H_4 \cdot HOAc$, CH_3CN, 45%; 2. DAST, CH_2Cl_2, 0 °C, 66%; b) 1. NaOMe, MeOH; 2. TBDPSCl, imidazole, DMF, 83%; c) 1. NaOMe, MeOH; 2. $PhCH(OMe)_2$, CSA, CH_3CN, 64%. Pht = N-phthalimido, DAST = diethylaminosulfur trifluoride, CSA = (±)-camphorsulfonic acid.*

As depicted in Scheme 6, regioselective glycosylation of the 4-OH group of diol **11** with donor **10**[13] was accomplished by using $SnCl_2$/AgOTf[14] as a catalyst, to provide disaccharide **14** in good yield (69%). Following acetylation, disaccharide **15** was obtained.

Glycosylation of diol **12** with thioglycoside **15**, promoted by NIS/TfOH, furnished the desired trisaccharide **16** in good yield (75%). Cleavage of the benzylidene acetal followed by acetylation of the free hydroxy groups gave trisaccharide **17**. Removal of the 6-*O*-TBDPS group with TBAF/AcOH gave compound **18**, which was then treated with $SO_3 \cdot$pyridine to install the 6-*O*-sulfate ester. Subsequent removal of the *N*-phthalimido group, followed by acetylation, furnished trisaccharide **19** in 51% yield. Deprotection of the sulfated trisaccharide **19** with NaOMe, followed by reduction of the glycosyl azide yielded the intermediate glycosyl amine, which was immediately treated with bromoacetic anhydride to give the target *N*-bromoacetamide **3**. Purification of **3** was accomplished by size-exclusion chromatography. The unsulfated trisaccharide **4** was generated in a similar fashion starting from intermediate **18** as outlined in Scheme 7.

The glycopeptide scaffolds (**21**[5] and **22**, Scheme 8) prepared in this study correspond to a 17-amino acid fragment of the



Scheme 6. *a) AgOTf, $SnCl_2$, CH_2Cl_2, toluene, 69%; b) Ac_2O, pyridine, DMAP, 86%; c) NIS, TfOH, CH_2Cl_2, 75%; d) 1. 80% AcOH, 80 °C; 2. Ac_2O, pyridine, 72%; e) TBAF, AcOH, THF, 98%; f) 1. $SO_3 \cdot$pyridine; 2. MeOH/$N_2H_4 \cdot H_2O$ (5:1), 70–75 °C; 3. Ac_2O, pyridine; 4. IRC-50 (Na^+) resin, 51%; g) 1. NaOMe, MeOH, H_2O; 2. H_2, Pd/C, H_2O; 3. bromoacetic anhydride, 1 M $NaHCO_3$, 25%. DMAP = 4-dimethylaminopyridine, NIS = N-iodosuccinimide.*

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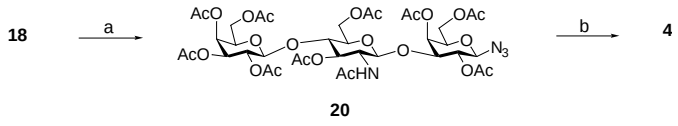
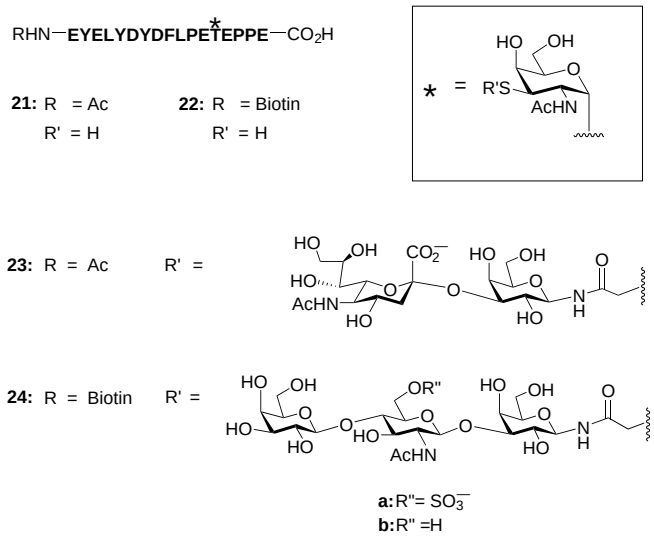


Scheme 7. a) 1. MeOH/$N_2H_4 \cdot H_2O$ (5:1), 70 – 75 °C; 2. Ac_2O, pyridine, 67%; b) 1. NaOMe, MeOH, H_2O; 2. H_2, Pd/C, H_2O; 3. bromoacetic anhydride, 1 M $NaHCO_3$, 45%.



*Scheme 8. Thioether-linked glycopeptides containing no extension (**21**, **22**), STF (**23**), and the MECA-79 antigens (**24a**, **b**).*

P-selectin glycoprotein ligand-1 (PSGL-1).[15] An *O*-linked glycan was incorporated at Thr12 of the peptide fragment by using building block **1** as previously described.[5] Peptide synthesis was carried out on 4-methylbenzhydrylamine (MBHA) rink amide resin employing *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate/1-hydroxy-1*H*-benzotriazole (HBTU/HOBt) ester activation in the presence of *N*,*N*-diisopropylethylamine (DIEA). The N terminus of glycopeptide **22** was biotinylated for use in future biological studies. Cleavage of the DNP thioether and reduction of the azide was carried out on resin by treatment with 1,4-dithiothreitol (DTT) and 1,8-diazabicyclo[5.4.0]undec-7-ane (DBU).[5] Acetylation of the resulting 3-thiol and 2-amino groups was achieved by reaction with Ac_2O in pyridine. Cleavage and deprotection of the glycopeptide was accomplished by treatment with Reagent K.[16] Following precipitation from Et_2O, the crude glycopeptide was deacetylated with 10% aqueous hydrazine hydrate (in the presence of DTT) and purified by reversed-phase HPLC. The identities of the target glycopeptide fragments (**21** and **22**) were confirmed by ESI-MS.

Ligation of glycopeptides **21** and **22** with derivatives **2** – **4**, was carried out by incubating each glycopeptide at 37 °C in the presence of an excess of *N*-bromoacetamido sugar for 12 h in sodium phosphate buffer at pH 7.2. The thioether-linked products (**23**, **24a** and **24b**; Scheme 8) were isolated by reversed-phase HPLC and their identities confirmed by ESI-MS.

Given the difficulty in synthesizing glycopeptides containing acid-sensitive sulfated and sialylated oligosaccharides, the syntheses of the glycopeptide analogues described here highlight the utility of the thiol alkylation approach. To date, no glycopeptides containing sulfated oligosaccharides have been synthesized by traditional methods. Moreover, the need to synthesize complex glycosyl amino acids is also circumvented by the thiol alklyation approach. We are now in the process of investigating the binding of these analogues to their corresponding antibodies (that is, MECA-79 and anti-STF). The results of these experiments will be reported elsewhere.

Experimental Section

Glycopeptide 22: The synthesis of glycopeptide **22** was carried out on MBHA rink amide resin (0.05 mmol scale) with N^{α}-Fmoc-protected amino acids and *N*,*N*′-dicyclohexylcarbodiimide (DCC)-mediated HOBt ester activation in *N*-methylpyrrolidine (NMP; Perkin-Elmer ABI 431A synthesizer, user-devised cycles). Glycosyl amino acid **1** (5.0 equiv) and D-biotin (5.0 equiv) were coupled manually, by using HBTU/HOBt/DIEA in DMF. Azide reduction/DNP cleavage was achieved by treatment of the resin-bound glycopeptide with DTT (4.0 equiv) and DBU/DIEA (1.0 equiv) in DMF for 30 min (2 ×). Following acetylation with Ac_2O/pyridine (1:2), the resin was washed thoroughly with DMF and CH_2Cl_2. Peptide cleavage/deprotection was accomplished by treatment with Reagent K (94.5% TFA, 2.5% H_2O, 2.5% ethanedithiol, and 1% triethylsilane)[16] at RT for 5 h. The crude peptide was precipitated with Et_2O, dissolved in 50% aqueous CH_3CN, and lyophilized. The crude glycopeptide was deacetylated with 10% aq. $N_2H_4 \cdot H_2O$ in the presence of DTT (excess) at RT for 30 min and then purified by preparative reversed-phase HPLC with a gradient of 10 – 60% CH_3CN in water (0.1% TFA) over 50 min to give 42 mg (32%) of **22**: ESI-MS (neg) calcd 2594, found 2594.

Thiol alkylation: Bromoacetamide **2**, **3**, or **4** (5 mg, 7 – 8 μmol) was added to glycopeptide **21**[5] or **22** (5 mg, 2.0 μmol) in sodium phosphate buffer (500 μL, 0.1 M, pH 7.2). The reaction mixture was incubated at 37 °C overnight and the thioether-linked product was isolated by semipreparative reversed-phase HPLC with a gradient of 10 – 60% CH_3CN in water (0.1% TFA) over 50 min. The products were lyophilized and analyzed by ESI-MS (neg): calcd for **23** 2920, found 2921; calcd for **24a** 3259, found 3258; calcd for **24b** 3179, found 3178.

Full experimental details and spectral characterization for all synthetic intermediates and HPLC data for all glycopeptides are provided in the Supporting Information.

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Modelling of Photointermediates Suggests a Mechanism of the Flip of the β-Ionone Moiety of the Retinylidene Chromophore in the Rhodopsin Photocascade

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isomerization · molecular dynamics · molecular modeling · photochemistry · rhodopsin

Light causes an extremely rapid 11-*cis*-to-all-*trans* isomerization of the retinylidene chromophore of rhodopsin.[1] This isomerization leads to bleaching intermediates in the photoactivation cascade. An early photointermediate, bathorhodopsin (Batho), which already contains a photoisomerized all-*trans* retinylidene chromophore, slowly ($\approx$1 μsec) decays by conformational changes to metarhodopsin I (Meta I) through lumirhodopsin (Lumi). The *cis-trans* photoisomerization of the retinylidene chromophore of rhodopsin occurs within the limited space of opsin, which results in a highly strained conformation of the chromophore. In a photoaffinity labeling experiment, Nakanishi et al. showed that a modified β-ionone moiety cross-linked Trp265 on transmembrane segment 6 (TM6) both in the rhodopsin and Batho states, which suggests that the cyclohexenyl moiety remains unchanged in the rhodopsin-to-Batho transition. In the subsequent Batho-to-Lumi transition, the moiety flipped from TM6 towards TM4.[2]

The flip of the modified β-ionone moiety suggests that TM3 and TM4 rearrange to accommodate the modified β-ionone moiety, as schematically shown in Figure 1, while the helix arrangement of Batho remains unchanged. Spectroscopic analyses such as UV/Vis, resonance Raman, and FTIR spectra of Batho and Lumi have revealed that Lumi has an almost relaxed all-*trans* chromophore with the protonated Schiff base (PSB), whereas Batho has a twisted double bond at C11=C12.[3] However, little is known about the structural origins of the twisted structure of the Batho chromophore and the flip of the β-ionone moiety in the Batho-to-Lumi transition.

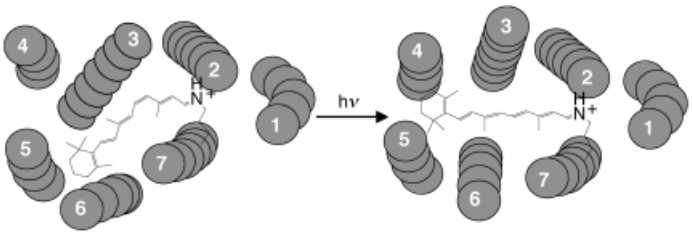

Figure 1. *The flip of the β-ionone moiety and the rearrangement of TM3 and TM4 in the photoactivation. A view from an intradiscal side.*

We previously formulated a structural model of the Batho chromophore[4] by using models of the transmembrane helices constructed from a projection map of rhodopsin.[5] The crystal structure of rhodopsin has recently been solved at a resolution of 2.8 Å[6] and this could greatly aid structural analysis of the process of formation of the photoactivated intermediates in the photocascade.

In the study reported herein, we applied restrained molecular dynamics simulation to the isomerization of the chromophore. Based on the present results, we propose structural models for Batho and Lumi that provide a structural explanation for the flip of the chromophore and the motion of TM3 and TM4.

The extraordinarily rapid photoisomerization ($\approx$200 fs) at a low temperature (77 K) leaves most of the opsin structure unaffected.[7] We generated candidate structures of the Batho chromophore in the crystal structure of opsin by using molecular dynamics and subsequent structure minimization and freezing

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