Synthesis of a New Water Soluble 2,2-Bifunctionalized Spin Label and Its Application to Troponin C

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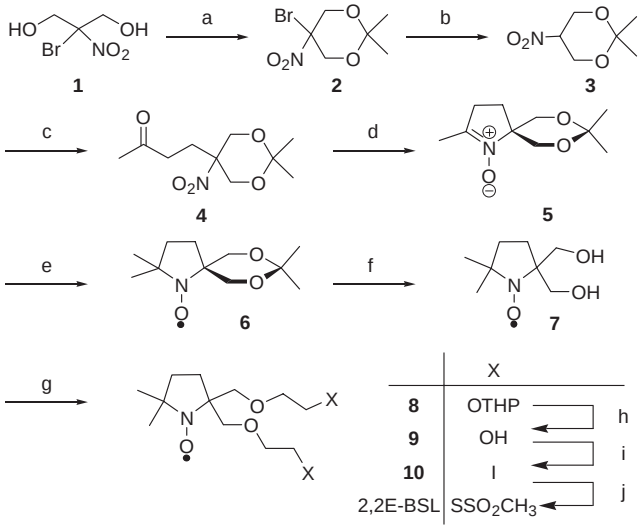
A new water soluble 2,2-bifunctionalized spin label (2,2E-BSL) having pyrrolidine nitroxide moiety was synthesized starting from a nitro compound with two hydroxymethyl group converted to the linkers of the 2,2E-BSL, and was applied to label troponin C (TnC). Labeled TnC through two linkages were successfully isolated, and 2,2E-BSL was proved to be immobilized on TnC by EPR measurement.

For clarifying a physiological protein motion, spin labels can be employed as an orientational probe.[1,2] Most of spin labels today are monofunctionalized spin labels[3] (MSL) by which protein can be modified through only one linkage. In this case, their free mobility on the protein interferes with accurate investigation of the conformational change of a protein. On the other hand, bifunctionalized spin labels (BSL) can elucidate the protein motion more exactly because of their rigid binding through two linkages. Some types of BSL which are composed of pyrrolidine nitroxide bearing 3,4-substituents[4,5] (3,4-BSL) or 2,2-substituents (2,2-BSL) have been synthesized and utilized to EPR measurement of protein. Recently, we have reported the synthesis of C_2-chiral 3,4-BSL,[5] (*R*,*R*)-BSL and (*S*,*S*)-BSL, and the labeling of troponin C with them. EPR spectra of the protein labeled with the C_2-chiral 3,4-BSL revealed that these spin labels were definitely immobilized on the α-helix. However, EPR signals would not reflect the small motion of the protein because an unpaired electron orbital on nitrogen atom of the 3,4-BSL locates almost perpendicularly to the α-helix. On the other hand, 2,2-BSLs can bind to a protein with its unpaired electron orbital parallel to α-helix and should be more sensitive for structural changes of the protein. Hideg et al. have reported a probe 2,2-BSL (2,2H-BSL) containing two aromatic rings between active thiol group and pyrrolidine nitroxide.[6] However, when we applied it for labeling TnC, most of it precipitated in the aqueous solution of the protein and the labeling was unsuccesful.[7] In the course of investigating dynamics of TnC, which is the key protein regulating muscle contraction and relaxation,[8] BSL which can bind to TnC at two cysteine residues is essential to determination of its orientation on actin filament. In this context, we designed a new 2,2-BSL having 2,2-substituents on pyrrolidine nitroxide and high water solubility for more accurate investigation of TnC dynamics. This is composed of 2,2-substituted pyrrolidine nitroxide having ether bonds in their two linkers. Although 2,2-disubstitued nitroxides are generally prepared by the addition of the Grignard reagent to nitrones, several reagents bearing protected functional groups were examined and found to yield the desired products in poor yields. Now, we designed a synthetic route starting from a nitro compound having two hydroxymethyl groups which were converted to the linkers of 2,2-BSL. The distance between each reaction center was preliminarily examined to fit that of two cysteine residues on TnC by molecular modeling.

In Scheme 1, 2-bromo-2-nitro-1,3-propanediol (**1**) was treated with 2,2-dimethoxypropane to yield bromonitrodioxane **2** in 82% yield. Reduction of **2** to remove bromine atom by $NaBH_4$ provided nitrodioxane **3** in 90% yield. By Michael addition with methyl vinyl ketone, nitroketone **4** was obtained in quantitative yield. Nitrone **5** was obtained as a white powder in 56% yield by treating **4** with zinc dust and acetic acid in ethanol. The high purity of **5** is critical to the next reaction, that is, the Grignard reaction was unsuccessful which nitrone **5** contained a small amount of impurities. Reaction of nitrone **5** with MeMgI followed by oxidation with MnO_2 provided nitroxide radical **6** in 53% yield. In an acidic deprotection of nitroxide **6**, decomposition of **6** was observed. After several unsuccessful attempts, we finally obtained diol **7** in 72% yield when **6** was treated with 80% AcOH. Diol **7** was converted to **8** by treating with NaH and THP ether of 2-bromoethanol. After deprotection of THP by oxalic



Scheme 1. Reagents and Conditions; (a) 2,2-dimethoxypropane, CSA, room temp, 82%; (b) $NaBH_4$, MeOH, rt, 90%; (c) methyl vinyl ketone, tetramethylguanidine, MeOH, rt, quant; (d) Zn, AcOH, EtOH, rt, 56%; (e) MeMgI, ether, rt, and then O_2, MnO_2, $CHCl_3$, rt, 53%; (f) 80% AcOH, 60 °C, 72%; (g) NaH, $Br(CH_2)_2OTHP$, DMF, 80 °C, 17%; (h) oxalic acid, dioxane, water, rt, quant; (i) MsCl, pyridine, rt, and then NaI, acetone, reflux, 90%; (j) $NaSSO_2CH_3$, DMSO, rt.

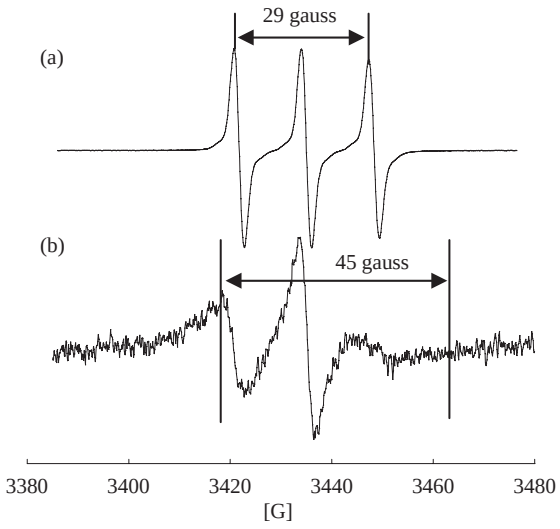


Figure 1. EPR spectra of (a) 2,2E-BSL and (b) 2,2E-BSL-labeled TnC.

acid, successive mesylation and substitution gave diiodide **10** in an overall yield of 90%. Finally, diiodide **10** was treated with $NaSSO_2CH_3$ to yield 2,2E-BSL which was directly employed for the labeling experiment.

A chicken skeletal TnC mutant (S94C) having two cysteine residues (94cys, 101cys) was expressed in *E. coli* cells. This TnC mutant was employed for the label experiment with 2,2E-BSL. The two cysteine residues were located on central E-helix of TnC and the distance between the two S atoms is calculated to be 11 Å.

The TnC mutant was purified with Sepharose Q anion exchange chromatography (2.0 × 10 cm, Amersham Biosciences). The purified TnC was treated with 5 mM of DTT in order to reduce disulfide bonds and oxidized thiol, and then, DTT was removed by Sephadex G-25 desalting column (1.5 × 20 cm, Amersham Biosciences). To the TnC solution, 2,2E-BSL was added (final concentration of 100 μM). In contrast with labeling with 2,2H-BSL, no precipitation appeared with 2,2E-BSL, owing to high water solubility. After the incubation at 4 °C for 48 h, the reaction was stopped, and then unlabeled spin label was removed by using Sephadex G-25. It is important for accurate measurement to remove the mono-labeled TnC from the mixture of labeled TnCs. For this purpose, Activated Thiol Sepharose 4B (Amersham Bioscience) was employed in our previous work.[5] In the present experiment, labeled TnC through two linkages was isolated successfully by applying anion exchange HPLC (UNO-Q, Bio-Rad). For EPR measurement, the samples were concentrated to 50 μM and stored at 4 °C.

The EPR spectrum of 2,2E-BSL itself showed a typical sharp triplet signal for nitroxide radical (Figure 1a) from which $2A_{zz}$ value was calculated to be 29 G. On the other hand, three peaks of EPR spectrum for 2,2E-BSL labeled TnC was broader than that of 2,2E-BSL, as shown in Figure 1b, indicating that the free rotation of 2,2E-BSL axis restricted by two point binding. From the broadened spectrum, the $2A_{zz}$ value was calculated to be 45 G that was much greater than that of commercially available MSL-labeled TnC (34 G). Unfortunately, the $2A_{zz}$ value of 2,2E-BSL labeled TnC was slightly smaller than that of 3,4-BSL (ca. 50 G).[5] The decrease of $2A_{zz}$ value was attributed to flexibility of sp^3 atoms of which the linkers of 2,2E-BSL consist. Additionally, the distance between two reactive groups is a little longer than that of two cysteine residues of TnC. These two factors increase the flexibility of spin lobe of 2,2E-BSL on TnC. After more than a few hours storage, the signal intensity of the labeled TnC became weak and the signals derived from the free BSL were observed although excess BSL and incomplete labeled TnC were removed by gel filtration and HPLC. Methanethiosulfonyl groups bind to cysteine residue through disulfide bond, and therefore, it is possible that a dissociation of the disulfide bonds between 2,2E-BSL and TnC might occur during storage. BSL which binds through more stable bond is necessary for more sensitive measurement and under investigation. For example, iodoacetamide group and maleimide group are preferable.

In conclusion, we synthesized a new 2,2-substituted bifunctionalized spin label 2,2E-BSL in reasonable overall yield and applied it for spin labeling of TnC. In our synthetic route, two hydroxymethyl groups of the commercially available starting material were converted to the 2,2-substituents of pyrrolidine nitroxide. Moreover, BSLs with variable linker length can be synthesized by using other alkyl halides for diol **7**. In labeling experiment, 2,2E-BSL was found to have suitable water solubility. In EPR measurement, it was confirmed that 2,2E-BSL bound to TnC with two linkages, and that completely labeled TnC was isolated by anion exchange HPLC.

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