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SYNTHESIS OF 2-NBDLG, THE ANTIPODE OF FLUORESCENT D-GLUCOSE TRACER 2-NBDG

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Abstract D-Glucose is one of the most important energy sources for the survival of various organisms, from *E. coli* to mammals. For live-cell monitoring of glucose uptake at the single-cell level, a fluorescent D-glucose derivative 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4- yl)amino]-2-deoxy-D-glucose [2-NBDG], which we developed, has been widely used in various research fields. For the last ten years, however, researchers have awaited an optical control substance for evaluating the extent of non-specific adsorption of 2-NBDG upon plasma membrane and/or the rate of unhealthy 2-NBDG uptake through partially (or transiently) damaged membrane.

Here we introduce a fluorescent L-glucose derivative, 2-[*N*-(7-Nitrobenz-2-oxa- 1,3-diazol-4-yl)amino]- 2-deoxy-L-glucose [2-NBDLG]. L-Glucosamine is a key intermediate toward the synthesis of 2-NBDLG, but not commercially available. Although a few papers on the synthesis of L-glucosamine have been reported, a new synthetic method of L-glucosamine should be absolutely required in practical view of optical purity and preparative scale. We converted commercially available L-mannose into desired L-glucosamine by 10 steps in 14% of overall yield. The ^{1}H-NMR data of synthetic L-glucosamine were completely identical with those of commercially available D-glucosamine. On the other hand, optical purity of L-glucosamine was confirmed by comparison of specific rotation with that of D-glucosamine. L-Glucosamine thus obtained was coupled with NBD-halide to give 2-NBDLG. Use of transporter-recognizable (D-isomer) and unrecognizable (L-isomer) fluorescent analogues combined with real-time confocal microscopy, should provide valuable information on dynamism of glucose transport.

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Key words: L-Glucosamine; 2-NBDG; 2-NBDLG; GLUTs; Glucose Uptake

An essential sugar, D-glucose is one of the most important energy sources for the survival of various organisms, from *E. coli* to mammals. Recent molecular techniques have revealed increasing numbers of glucose transporters such as GLUTs (glucose transporters) and SGLTs (sodium/glucose cotransporters) that may be located in particular sites of the plasma membrane[1]. In addition, translocation of some transporters in response to insulin stimulation has been documented[2]. Historically, glucose transport activity has been monitored by radiolabeled tracers such as [^{14}C] 2-deoxy-D-glucose[3]. However, they cannot be used for time-lapse monitoring of glucose uptake at the single-cell level due to their poor spatial and temporal resolution.

In 1996, we developed a fluorescent D-glucose derivative, 2-[*N*-(7-nitrobenz-2-oxa- 1,3-diazol-4-yl)-amino]-2-deoxy-D-glucose [2-NBDG] (1)

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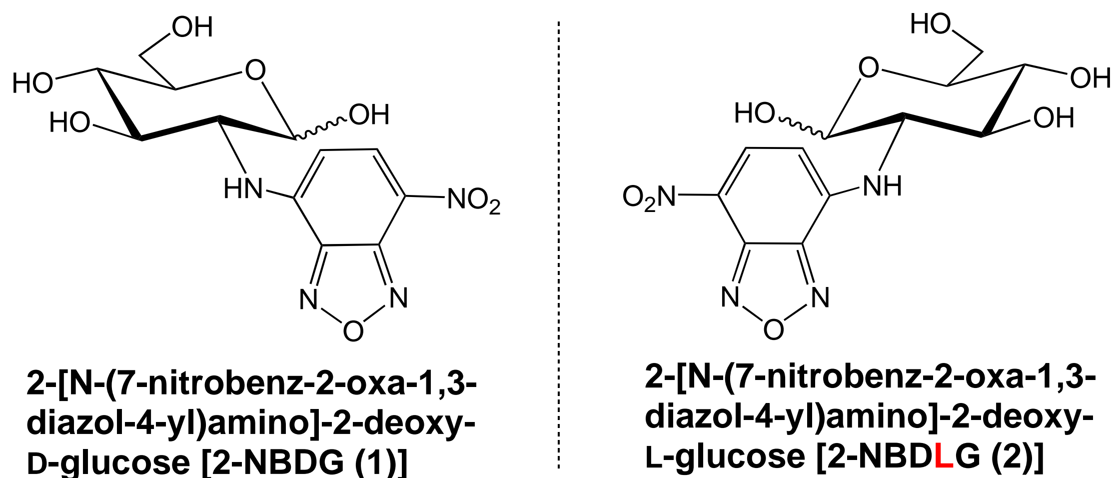

Figure 1 Structures of 2-NBDG (1) and 2-NBDLG (2)

as shown in Fig.1., that allows a more sensitive measurement of glucose uptake in single-cell of living *E. coli*[4]. Then we proved that 2-NBDG (1) is incorporated into mammalian cells through glucose transporters in a time, concentration, and temperature-dependent manner[5].

So far 2-NBDG (1) has been successfully applied in various organisms by different groups[6]. Of particular interest is its application to the brain[7], which utilizes glucose as a sole energy source, and to malignant tumor cells[8]. However, care should be taken in that the fluorescence intensity is an arbitrary measure and that previous 2-NBDG methods had no control fluorescent substrate. This is important particularly when applied to tissues consisting of heterogeneous cells showing divergent activity[6]. To overcome the difficulties, we selected 2-NBDLG (2) as shown in Fig.1., an enantiomer of 2-NBDG (1), as a control substrate for 2-NBDG (1)[9]. It is known that mammalian cells predominantly incorporate D-isomer of glucose[10]. Thus, measurement of the difference in the fluorescence derived from 2-NBDG (1) and 2-NBDLG (2) would provide critical information on the net stereospecific uptake of D-glucose into single, living cells, setting it apart from other factors such as non-specific uptake and/or transporter-unrelated binding to the cellular surface that can be serious problems in some application[11].

As shown in Fig.2., L-glucosamine (3) is an essential key intermediate for 2-NBDLG (2). Although there are a few papers[12] on synthesis of L-glucosamine (3), a new synthetic method of L-glucosamine (3) should be absolutely required in practical view of optical purity and preparative scale. Here we describe the first synthesis of 2-NBDLG (2) as well as optically pure L-glucosamine in practical scale.

L-Glucosamine (3) was synthesized from L-mannose (4) in 10 steps as shown in Fig.2. By the applications of Montgomery's method[13], transformations of a starting material, L-mannose (4) into the compound 9[14] with one free hydroxyl group at C-2 position was carried out, namely peracetylation, bromination at C-1 position, orthoester-formation, deacetylation, benzylation and acidic methanolysis. The free 2-hydroxyl group in methyl glycoside 9 was sulfonylated with trifluoromethanesulfonic anhydride in the presence of pyridine to give the compound 10[15]. By use of tetrabutylammonium azide[16] in benzene, the triflate 10 was converted to azide 11, being accompanied by inversion of

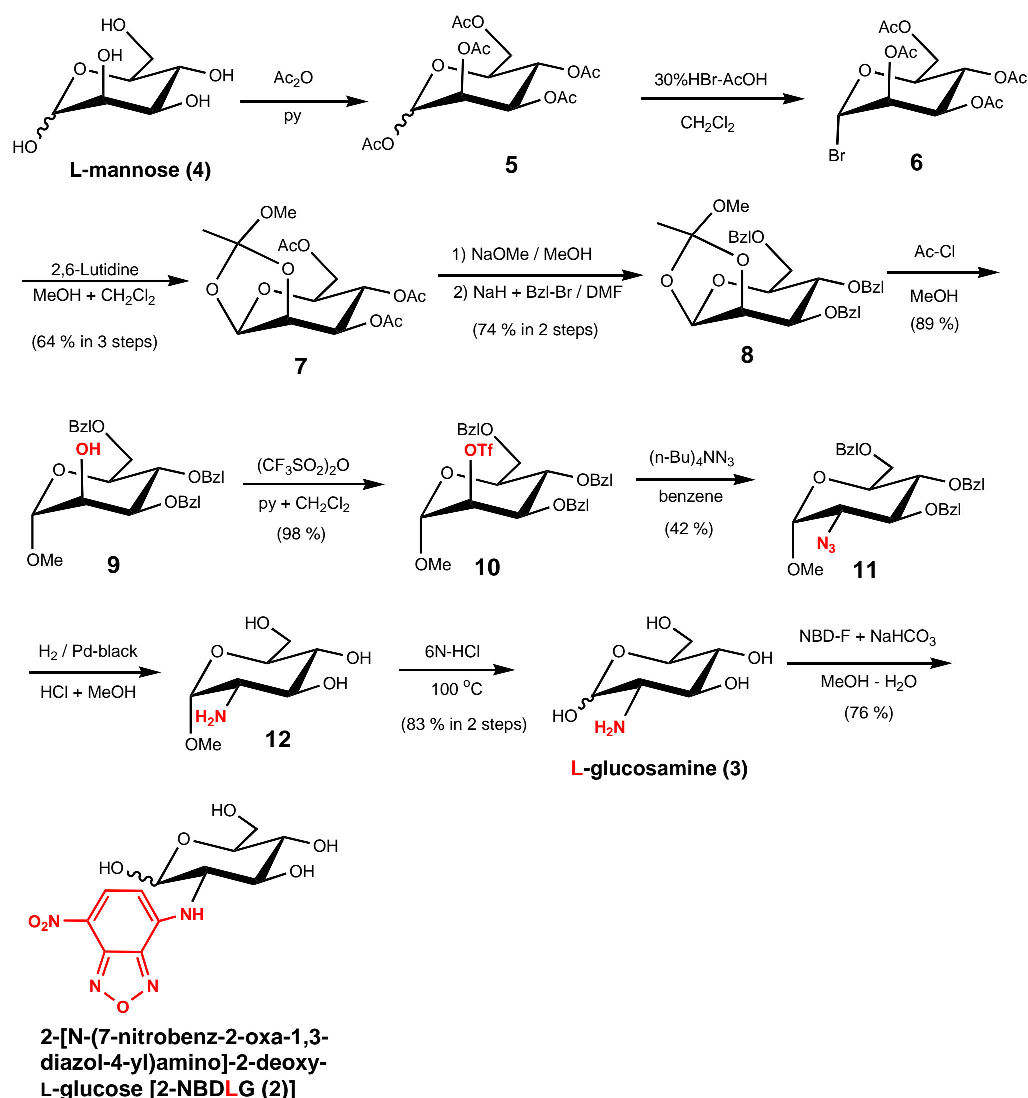

Figure 2 Synthesis of L-glucosamine (3) and 2-NBDLG (2).

the configuration at C-2 position[17]. Catalytic hydrogenation of the azide 11 gave the primary amine 12. Finally, the methyl glycoside was hydrolyzed with 6N-HCl at 100 °C[18] to form the target compound. The ^{1}H-NMR data[19] of synthetic L-glucosamine (3) thus obtained, was completely identical with that of commercially available D-glucosamine. On the other hand, optical purity of L-glucosamine was confirmed by the comparison of specific rotation with that of D-glucosamine[20]. Optically pure L-glucosamine thus obtained, was coupled with 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) to give 2-NBDLG (2) in 76% yield[21]. It was more than three times as compared with the use of 4-chloro-7-nitrobenz-2-oxa-1,3- diazole (NBD-Cl).

Use of 2-NBDG (1) has brought exciting implications including such as metabolic wave[7a] and intercellular transport of D-glucose and/or its phosphorylated form through gap junction[22]. By using of transporter-recognizable (D-isomer) and unrecognizable (L-isomer) fluorescent analogues combined with modern live-cell imaging techniques, such as real-time confocal microscopy,

should provide valuable information on dynamism of glucose transport.

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14) ^{1}H-NMR data of the compound 9 (in $CDCl_3$, 400 MHz): δ7.24-7.36 (m, 15H, Ph), δ4.80 (d, 1H, J = 1.6 Hz, H-1), δ4.69 (ABq, 2H, J = 11.9 Hz, CH_2-Ph), δ4.67 (ABq, 2H, J = 11.3 Hz, CH_2-Ph), δ4.60 (ABq, 2H, J = 12.4 Hz, CH_2-Ph), δ4.03 (m, 1H, H-2), δ3.70-3.88 (m, 5H, H-3, H-4, H-5, H-6a and H-6b), δ3.37 (s, 3H, OMe), δ2.49 (br.d, 1H, J = 2.5 Hz, C2-OH).

15) ^{1}H-NMR data of the compound 10 (in $CDCl_3$, 400 MHz): δ7.10-7.38 (m, 15H, Ph), δ5.11 (m, 1H, H-2), δ4.90 (d, 1H, J = 1.9 Hz, H-1), δ4.69 (ABq, 2H, J = 12.0 Hz, CH_2-Ph), δ4.64 (ABq, 2H, J = 10.7 Hz,

CH_2-Ph), δ4.61 (ABq, 2H, J = 11.7 Hz, CH_2-Ph), δ4.00 (dd, 1H, J = 2.9 and 8.9 Hz, H-3), δ3.69-3.84 (m, 4H, H-4, H-5, H-6a and H-6b), δ3.40 (s, 3H, OMe).

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17) ^{1}H-NMR data of the compound 11 (in $CDCl_3$, 400 MHz): δ7.15-7.38 (m, 15H, Ph), δ4.87 (ABq, 2H, J = 12.4 Hz, CH_2-Ph), δ4.83 (d, 1H, J = 3.5 Hz, H-1), δ4.66 (ABq, 2H, J = 10.7 Hz, CH_2-Ph), δ4.57 (ABq, 2H, J = 12.4 Hz, CH_2-Ph), δ3.98 (dd, 1H, J = 8.9 and 10.2 Hz, H-3), δ3.66-3.80 (m, 4H, H-4, H-5, H-6a, and H-6b), δ3.45(dd, 1H, J = 3.5, 10.4 Hz, H-2), δ3.43 (s, 3H, OMe).
Because of E2 elimination from the triflate 10 as a side reaction, the desired compound, azide 11 was obtained in low yield.

18) Under usual condition such as at 60 °C in 1N-HCl compound 12 was very stable and no hydrolysis occurred probably due to the amino group at C-2 position.

19) ^{1}H-NMR data of L-glucosamine (3) (in D_2O, 400 MHz): δ5.36 (d, 0.6H, J = 3.5 Hz, H-1α), δ4.85 (d, 0.4H, J = 8.3 Hz, H-1β), δ3.36-3.84 (m, 5H, H-3α and 3β, H-4α and 4β, H-5α and 5β, H-6a α and β, and H-6b α and β), δ3.21 (dd, 0.6H, J = 3.5, 10.6 Hz, H-2α), δ2.92 (dd, 0.4H, J = 8.3, 10.6 Hz, H-2β).
Anal. Calcd for $C_6H_{14}ClNO_5$: C, 33.42; H, 6.54; N, 6.50.
Found: C, 33.31; H, 6.46; N, 6.36.

20) $[\alpha]_D$ at 20 °C (24 h after dissolving in water)
synthetic L-glucosamine (3): –72.05 (c1.0, H_2O)
commercially available D-glucosamine: +72.20 (c1.0, H_2O)

21) ^{1}H-NMR data of 2-NBDLG (2) (in D_2O, 400 MHz): δ8.52 (d, 1H, J = 9.1 Hz, H6'), δ6.56 and δ6.54 (d x 2, 0.5H x 2, J = 9.1 Hz and J = 9.1 Hz, H5'), δ5.38 (d, 0.5H, J = 2.8 Hz, H-1α), δ4.89 (d, 0.5H, J = 8.1 Hz, H-1β), δ3.50-4.02 (m, 6H, H-2α and 2β, H-3α and 3β, H-4α and 4β, H-5α and 5β, H-6a α and β, and H-6b α and β).

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