Highly regioselective glucosylation of 2′-deoxynucleosides by using the crude β-glycosidase from bovine liver

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ARTICLE INFO

ABSTRACT

An enzymatic regioselective approach for the glucosylation of a series of 2′-deoxynucleosides was described by using the crude β-glycosidase from bovine liver that is less expensive and can be simply prepared in a standard organic laboratory. With the glucosylation of 2′-deoxyuridine as a model reaction, the effects of several key factors on the enzymatic reaction were examined. The optimum enzyme dosage, buffer pH and temperature were 0.05 U/ml, 9.5 and 42 °C, respectively. The presence of alkali β-glycosidase as the main active component in the crude enzyme extract might account for the high glucosylation activity at pH 9.5. In addition, the desired 5′-O-glucosylated derivatives of 2′-deoxynucleosides were synthesized with the yields of 22–72% and exclusive 5′-regioselectivities (>99%).

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1. Introduction

Nucleoside analogs constitute a vital class of anticancer and antiviral agents (Sanghvi and Cook, 1993; Yoo and Jones, 2006). For example, floxuridine (5-fluoro-2′-deoxyuridine, FUdR) has been used extensively in the clinical treatment of colon carcinoma and hepatic metastases for many years (Kemeny et al., 1999); and idoxuridine has proven to be a useful antiviral agent in the treatment of herpes simplex virus and varicella-zoster virus infections (De Clercq, 2004). However, these nucleoside agents generally suffer from poor bioavailability, low selectivity and various side effects, etc. in the clinical practice; thus, the chemical modified nucleosides without such drawbacks represent a promising direction for their pharmaceutical applications (De Clercq and Field, 2006; Landowski et al., 2005). It was reported that glycosylated derivatives of many active compounds could mask their toxicity and/or improve their pharmacokinetic properties. For instance, the toxicity of 5′-O-β-D-galactosyl-5-fluorouridine was more than 100-fold less than the parent compound to bone marrow cells in Balb/c mice (Abraham et al., 1994). In addition, it has been demonstrated that the cellular uptake of many active components could be significantly enhanced after glycosylation modification, due to the active absorption mediated by glucose transport system (Mizuma et al., 1992). D-Glucose

conjugates of 7-chlorokynurenic acid, a potential neuroprotective agent, were able to facilitate the transport of this active compound across the blood–brain barrier (Battaglia et al., 2000).

Additionally, disaccharide nucleosides are an important group of natural compounds, which consist of a disaccharide that is linked to a base moiety through an N-glycosidic bond. Such compounds were often found as minor structural elements in a variety of biopolymers such as tRNA and poly(ADP-ribose) (Efimtseva et al., 2009), although their biological roles were still obscure. Moreover, a number of biologically active disaccharide nucleosides have been isolated and characterized (Efimtseva and Mikhailov, 2004). For example, the amicetin group antibiotics possess a broad spectrum of biological activities such as antibacterial, fungicidal, herbicidal, antitumor and antiviral activities; and adenophostins A and B, two disaccharide nucleosides, are the most powerful agonists of inositol 1,4,5-triphosphate receptors, which play an important role in Ca^{2+} release (Efimtseva and Mikhailov, 2002). Hence, the synthesis of such compounds is attracting increasing interest in the field of structural biology of biopolymer as well as in the pharmaceutical industry.

Enzymatic modification of the parent drugs, by virtue of simplicity, mild reaction conditions, exquisite selectivity and being environmentally friendly, is a promising alternative to chemical methods (Diaz-Rodriguez et al., 2005; Li et al., 2008, 2009a). Biocatalytic modification of nucleosides was reviewed comprehensively by our group (Li et al., 2010) and Ferrero et al. (Ferrero and Gotor, 2000). It was found that there were only few reports on enzymatic glycosylation of nucleosides in the two decades. Binder et al.,

for example, described the synthesis of four O-β-D-galactosyl-nucleosides using β-galactosidase from *Aspergillus oryzae* through transgalactosylation, but the yields (3–7%) were pretty low (Binder et al., 1995). Andreotti et al. (2007) successfully synthesized a group of 5′-O-β-D-galactosylated nucleoside derivatives using the marine β-galactosidase from *Aplysia fasciata*. The yields generally ranged from low to good (12–60%), with the exception of 5′-O-β-D-galactosyl-uridine with a yield of 80%.

Recently, our group reported the regioselective galactosylation of FUdR catalyzed by a commercial β-galactosidase from bovine liver with a high yield (75%) and an excellent 5′-regioselectivity (>99%) (Zeng et al., 2010). Herein, we continued to extend our interest to the glucosylation modification of a group of 2′-deoxynucleosides by using the crude β-glycosidase from bovine liver, a less expensive biocatalyst (Scheme 1). To the best of our knowledge, the enzymatic synthesis of β-D-glucosyl-containing disaccharide nucleosides was reported for the first time.

2. Materials and methods

2.1. Materials

p-Nitrophenyl β-D-glucopyranoside (pNPGlc) was supplied by Alfa Aesar (USA). o-Nitrophenyl β-D-galactoside (oNPGal) was from Genebase Bioscience (Guangzhou, China). FUdR was purchased from Shanghai Hanhong (China). 2′-Deoxyuridine, thymidine and idoxuridine were bought from Tuoxin Biotechnology & Science (China). 5-Bromo-2′-deoxyuridine and the commercial β-galactosidase were obtained from Sigma–Aldrich (USA). All other chemicals were obtained from commercial sources and with the highest purity available.

2.2. Preparation of the crude β-glycosidase

Fresh bovine liver was obtained from a local market, diced into approximately 1 cm cubes and stored at −20 °C prior to use. All procedures were performed at 4 °C or on ice unless otherwise stated. To 100 ml phosphate buffer (10 mM, pH 6.0) containing protease inhibitors [1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% NaN_3, 17 mM PMSF, 1% (v/v) isopropanol], 100 g bovine liver was added, and homogenized. The homogenate was centrifuged at $10\,000 \times g$ for 30 min at 4 °C, and the pellet was discarded. The supernatant was re-centrifuged at $30\,000 \times g$ for 60 min at 4 °C. The lipid layer on the surface of the supernatant was carefully removed with a pipette, and then the extract was incubated at 50 °C with gentle stir for 20 min, followed by treatment on ice for another 20 min. Then the extract was centrifuged at $15\,000 \times g$ for 20 min at 4 °C, and ammonium sulfate was added to the supernatant to 75% saturation. After incubation for 20 h, the precipitate was collected by centrifugation for 20 min at $8\,000 \times g$ and dissolved in Na_2HPO_4–citric acid buffer (50 mM, pH 6.0). The solution was dialyzed against this buffer overnight and the crude extract was collected by centrifugation at $15\,000 \times g$ for 10 min at 4 °C.

2.3. Enzyme activity assay of the crude β-glycosidase

To 0.5 ml phosphate buffer (100 mM, pH 7.0), 20 μl the crude enzyme solution and 0.5 ml pNPGlc solution (50 mM) were added. The reaction was conducted for 10 min at 37 °C and 200 rpm, and then stopped by adding 8.98 ml Na_2CO_3 (1 M). The released p-nitrophenol was assayed spectrophotometrically at 400 nm. One unit of glycosidase activity was defined as the amount of enzyme required to catalyze the release of 1 μmol p-nitrophenol per min under the conditions given. The specific activity of the crude β-glycosidase was 1.50 U/ml.

2.4. General procedure for enzymatic glucosylation of 2′-deoxynucleosides

In a typical experiment, 2 ml glycine–NaOH buffer (100 mM, pH 9.5) containing 0.04 mmol 2′-deoxynucleoside, 0.02 mmol pNPGlc and 0.1 U enzyme was incubated in a 15 ml Erlenmeyer shaking flask capped with a septum at 200 rpm and 42 °C. Aliquots were withdrawn at specified time intervals from the reaction mixture. The samples were treated at 100 °C for 5 min to denature the enzyme, and then diluted by 25 times with the corresponding mobile phase prior to HPLC analysis. Yields were calculated by the ratio of the actual product concentration to the theoretic product concentration based on the pNPGlc. The regioselectivity was defined as the percentage of the HPLC peak area of the desirable product in all the glucosylated products. All the enzymatic glucosylation experiments were carried out duplicate.

2.5. Synthesis, purification and structure determination of disaccharide nucleosides

The product structure was determined by ^{1}H and ^{13}C NMR (Bruker AVANCE Digital 400 MHz NMR spectrometer, Germany) at 400 and 100 MHz, respectively.

5′-O-β-D-glucosyl-2′-deoxyuridine **3a** (13% isolated yield). To 20 ml phosphate buffer (100 mM, pH 9.5) containing pNPGlc (0.2 mmol, 60 mg) and 2′-deoxyuridine (0.4 mmol, 91.2 mg), 1.0 U the crude β-glycosidase was added. The reaction was conducted at 42 °C and 200 rpm for 11 h (monitored by HPLC). The mixture was treated at 100 °C for 10 min to denature the enzyme, filtered and then evaporated under reduced pressure leading to a residue, which was purified by column chromatography on silica gel. ^{1}H NMR (D_2O): δ 2.45–2.48 (m, 2H, H2′), 3.32–3.55 (m, 4H, H2″ + H3″ + H4″ + H5″), 3.74–3.79 (m, 1H, H4′), 3.87–3.99 (m, 2H, H6″), 4.21–4.26 (m, 2H, H5′), 4.55 (d, J = 7.6 Hz, 1H, H1″), 4.58–4.62 (m, 1H, H3′), 5.93 (d, J = 8.0 Hz, 1H, H6), 6.33 (t, J = 6.4 Hz, H1′), 7.96 (d, J = 8.0 Hz, 1H, H5). ^{13}C NMR (D_2O): δ 38.96 (C2′), 61.07 (C6″), 69.30 (C5′), 69.95 (C4″), 71.09 (C3′), 73.44 (C2″), 75.98 (C5″), 76.13 (C3″), 85.67 (C4′), 86.13 (C1′), 102.18 (C5), 102.51 (C1″), 142.36 (C6), 151.71 (C2), 166.41 (C4).

5′-O-β-D-glucosyl-FUdR **3b** (34% isolated yield). The reaction conditions were same as those for the synthesis of the compound **3a** except for FUdR (0.4 mmol, 98.4 mg). The reaction time was 11 h. ^{1}H NMR (D_2O): δ 2.37–2.49 (m, 2H, H2′), 3.33–3.57 (m, 4H, H2″ + H3″ + H4″ + H5″), 3.72–3.76 (m, 1H, H4′), 3.90–3.99 (m, 2H, H6″), 4.26 (apparent d, 2H, H5′), 4.56 (d, J = 8.0 Hz, 1H, H1″), 4.62 (br s, 1H, H3′), 6.31 (t, J = 6.4 Hz, 1H, H1′), 8.12 (d, J = 6.4 Hz, 1H, H6). ^{13}C NMR (D_2O): δ 38.84 (C2′), 60.82 (C6″), 68.46 (C5′), 69.66 (C4″), 70.55 (C3′), 73.11 (C2″), 75.59 (C5″), 75.79 (C3″), 85.46 (C4′), 85.84 (C1′), 101.89 (C1″), 125.59, 125.93 (C6), 139.22, 141.53 (C5), 149.73 (C2), 158.97, 159.23 (C4).

5′-O-β-D-glucosyl-thymidine **3c** (15% isolated yield). The reaction conditions were same as those for the synthesis of the compound **3a** except for thymidine (0.4 mmol, 96.8 mg). The reaction time was 22 h. ^{1}H NMR (D_2O): δ 1.95 (s, 3H, H7), 2.39–2.50 (m, 2H, H2′), 3.34–3.45 (m, 2H, H2″ + H4″), 3.50–3.58 (m, 2H, H3″ + H5″), 3.74–3.78 (m, 1H, H4′), 3.88–4.00 (m, 2H, H6″), 4.24 (t, 2H, H5′), 4.56 (d, J = 8.0 Hz, 1H, H1″), 4.61–4.64 (m, 1H, H3′), 6.35 (t, J = 6.8 Hz, 1H, H1′), 7.73 (s, 1H, H6); ^{13}C NMR (D_2O): δ 11.84 (C7), 38.76 (C2′), 61.09 (C6″), 69.44 (5′), 70.03 (C4″), 71.19 (C3′), 73.51 (C2″), 76.01 (C5″), 76.18 (C3″), 85.51 (C4′), 85.59 (C1′), 102.57 (C5), 111.41 (C1″), 137.68 (C6), 151.76 (C2), 166.53 (C4).

5′-O-β-D-glucosyl-5-bromo-2′-deoxyuridine **3d** (10% isolated yield). The reaction conditions were same as those for the synthesis of the compound **3a** except for 5-bromo-2′-deoxyuridine (0.4 mmol, 112.8 mg). The reaction time was 22 h. ^{1}H NMR (D_2O): δ 2.45–2.48 (m, 2H, H2′), 3.44–3.59 (m, 4H, H2″ + H3″ + H4″ + H5″),