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Synthesis and characterization of glycosylated nitrogen mustard derivatives and their interaction with bovine serum albumin

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Abstract To improve the specificity of nitrogen mustards towards tumor cells, glucose-nitrogen mustard, fructose-nitrogen mustard, and lactose-nitrogen mustard were prepared as three novel glycosylated nitrogen mustard derivatives by esterification of bis(2-chloroethyl)carbamic chloride (BCC) with glucose, fructose, and lactose, respectively. BCC was synthesized from bis(2-chloroethyl)amine hydrochloride and triphosgene. The topic products were characterized by infrared (IR) and mass spectrometry (MS), and their interaction with bovine serum albumin was investigated by measuring fluorescence spectra in tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) buffer solution at physiological conditions.

Keywords Glycosylated ester · Nitrogen mustards · Synthesis · BSA

Introduction

The nitrogen mustards are an important group of alkylating agents that have been commonly used in clinical cancer chemotherapy for more than 30 years [1]. Like almost all other anticancer drugs, their clinical efficacy has been limited by their toxicity to normal tissues [2]. Thus, it is important for pharmacochemists to reduce their side-effects and prolong their duration of activity [3, 4]. Many works have reported that it is possible to improve their targeting of tumor cells by linking them with different drug carriers such as lectins, glycoproteins, and lipids.

Carbohydrates are the most ubiquitous chemicals in living systems, being present in most cell surfaces, and have been traditionally associated with energy production and as building blocks [5]. However, it has been observed that they

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play a more pervasive and sophisticated role in drug delivery to specific sites. The chemical structures of carbohydrates contain many hydroxyl groups. In some cases, it has been indicated that linking of antitumor drugs to carbohydrates can simultaneously improve the hydrophilic nature of the drugs. So, significant effort has been invested in the development of carbohydrate-based cell-specific drugs. In recent years, various glycoside anticancer agents, such as bleomycins, epipodophyllotoxin, and cardioactive glycosides, containing carbohydrate moieties have been reported [6].

To improve the specificity of nitrogen mustards towards tumor cells, three glycosylated nitrogen mustard derivatives were synthesized, and their interaction with BSA was studied spectroscopically.

Experimental

Apparatus and reagents

Fourier-transform IR (FTIR) spectra were recorded from KBr pellets using a Nicolet Avatar FT/IR-360 spectrophotometer. MS spectra were recorded using a HP6890/5973 Agilent 1,100 liquid mass spectrometer or Agilent 5973–6890 gas chromatography (GC)/MS system. Fluorescence spectra were measured using a PerkinElmer LS/45 fluorophotometer. Thin-layer chromatography (TLC) analysis was performed on glass sheets coated with Merck silica gel GF_{254}, and compounds were visualized by I_2 vapor.

Chemicals used included bis(2-chloroethyl)amine hydrochloride (98 %; Aladdin Reagent), triphosgene (99 %; Aladdin Reagent), glucose (AR; National Pharmaceutical Group Chemical Reagent), fructose (BR; National Pharmaceutical Group Chemical Reagent), and lactose (AR; National Pharmaceutical Group Chemical Reagent). All other chemical reagents were of analytical grade and were distilled before use.

Synthesis of bis(2-chloroethyl)carbamic chloride (BCC)

BCC was prepared according to literature [7]. The main procedure was as follows: To a solution of 0.3625 g (1.2 mmol) triphosgene in CH_2Cl_2 (20 mL), triethylamine (1.0 mL) and bis(2-chloroethyl)amine hydrochloride 0.5194 g (3 mmol) in CH_2Cl_2 (10 mL) was added dropwise during 40 min and stirred for an additional 1.5 h in an ice-water bath. Methanol was used as quencher, and formation of reaction products was monitored by TLC at R_f 0.42 (1:2 petroleum ether:ethyl acetate). Further purification was not necessary, and the product was used directly in the next step.

Synthesis of glucose-nitrogen mustard (GNM)

To a mixture containing 0.2970 g (1.5 mmol) glucose in water (10 mL) and 0.6120 g (3 mmol) BCC in CH_2Cl_2 (20 mL) was added triethylamine to keep pH in

the range 8–10. The reaction was stirred at room temperature for 8 h while being monitored by TLC. After reaction completion, the mixture was separated. The organic layer was washed with HCl (0.1 mmol L^{-1}) and saturated brine, dried over Na_2SO_4, and evaporated. The product was further purified by flash chromatography on silica gel as needed, and a slightly yellowish, fragrant syrup was obtained. MS *m/z*: 500.9 $[M\text{-}18]^+$. IR (KBr)/cm^{-1}: 3,600–3,400 (ν_{O-H}), 2,966 (ν_{C-H}), 1,739 ($\nu_{C=O}$), 1,441 (ν_{C-N}), 1,300–1,041 (ν_{C-O-C}), 763 and 670 (ν_{C-Cl}).

Synthesis of fructose-nitrogen mustard (FNM)

Fructose (0.5402 g, 3.0 mmol) in water (10 mL) and 0.6120 g (3 mmol) BCC in CH_2Cl_2 (20 mL) were mixed in a flask, and triethylamine was added to keep pH in the range 8–10. The reaction was stirred at room temperature overnight. After reaction completion, the aqueous solution was removed. The organic layer was washed with HCl (0.1 mmol L^{-1}) and saturated brine, dried over Na_2SO_4, and evaporated. The terminal product was purified by flash chromatography on silica gel, and a yellowish, fragrant syrup was obtained. MS *m/z*: 317 $[M\text{-}CH_2OH]^+$. IR (KBr)/cm^{-1}: 3,660–3,485 (ν_{O-H}), 2,966 (ν_{C-H}), 1,731 ($\nu_{C=O}$), 1,466 (ν_{C-N}), 1,300–1,041 (ν_{C-O-C}), 767 and 671 (ν_{C-Cl}).

Synthesis of lactose-nitrogen mustard (LNM)

A mixture containing 0.5400 g (1.5 mmol) lactose in water (10 mL) and 0.6120 g (3 mmol) BCC in CH_2Cl_2 (20 mL) was adjusted to alkaline (pH in the range 8–10) using triethylamine. After stirring for 24 h at room temperature, the mixture was separated. The organic layer was washed with HCl (0.1 mmol L^{-1}) and saturated brine, dried over Na_2SO_4, and evaporated. The result was purified like GNM, and a yellow oil with unpleasant smell was obtained. MS *m/z*: 680.7 $[M]^+$. IR (KBr)/cm^{-1}: 3,485 (ν_{O-H}), 2,962 (ν_{C-H}), 1,748 ($\nu_{C=O}$), 1,433 (ν_{C-C}, ν_{C-N}), 1,300–1,200 (ν_{C-O-C}), 767, 661 (ν_{C-Cl}).

Interaction of BSA with GNM, FNM, and LNM

Stock solution of BSA (6 μM) in aqueous Tris-HCl buffer solution (100 mL) of pH 7.4 was prepared, while stock solutions of the products were prepared in dehydrated alcohol because of their lower solubility in water. For interaction studies with protein, 3.0 mL aqueous solution of BSA (6 μM) was titrated with various concentrations of the compounds ranging from 0 to 70 μL; the total volume of alcohol did not exceed 100 μL. The presence of 100 μL alcohol did not induce major structural changes in BSA. Each solution was mixed thoroughly before spectral measurements at room temperature.

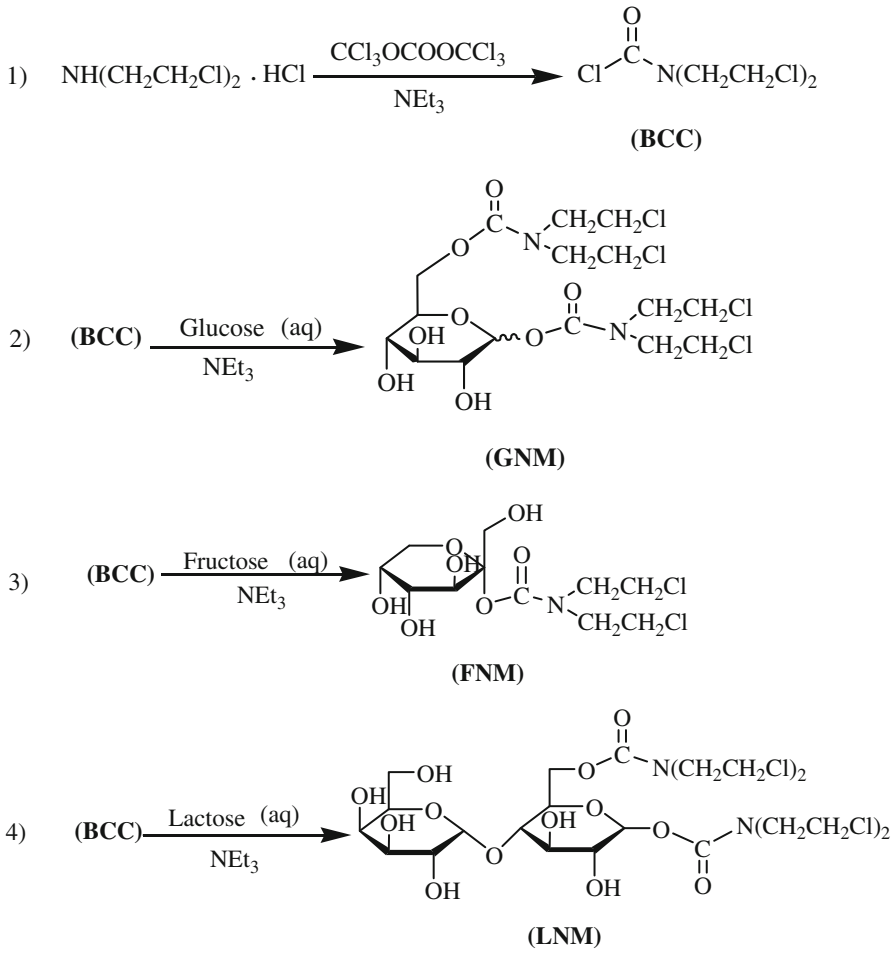
Fluorescence measurements were taken by exciting the protein solution at 288 nm. An excitation wavelength of 288 nm was applied to selectively excite tryptophan residues in protein.

Results and discussion

Synthesis of GNM, FNM, and LNM

To improve the specificity of nitrogen mustards towards tumor cells and research their biological activity further, the present article focused on synthesis and characterization of three glycosylated nitrogen mustard derivatives. The synthetic route is shown in Scheme 1.

In the process of this research, we found that the sugars had poor solubility in water, dimethylformamide, and ethanol. However, water was chosen as the best solution for its low boiling point, nontoxicity, easy separation with dichloromethane compared with dimethylformamide, and easy reaction with BCC (unlike ethanol).



Scheme 1 Synthesis of GNM, FNM, and LNM

Moreover, when dimethylformamide was applied in this reaction, the whole system was dark-colored at the end of this step. Tetrabutylammonium bromide was used as phase-transfer catalyst to promote the reaction, but we found that it was difficult to remove it completely. In this step, HCl was prepared as byproduct. To make the reaction reach completion and to improve productivity, the reaction should be carried out under alkaline conditions.

Characterization of GNM

GNM was prepared by means of esterifying reaction of glucose and BCC. Whether the reaction occurs can be determined from the characteristic bands of ester absorption peaks in IR spectra. The glucose spectrum presents the following bands: O–H stretching vibration at 3,264 cm^{-1}, C–H stretching vibration at 2,941 and 2,884 cm^{-1}, C–C stretching vibration at 1,437–1,323 cm^{-1}, and C–O at 1,160–1,017 cm^{-1}, while the GNM spectra display new absorption peaks at 1739, 1300–1158, 763, and 670 cm^{-1}, respectively, corresponding to C=O, C–O–C (characteristic band of ester), and C–Cl stretching vibration. The molecular weight of GNM is 518, whereas we measured $[M\text{-}18]^+ = 500.9$; this difference may be caused by loss of molecular H_2O. Moreover, the CH_2OH fragment was not found by MS, further illustrating that the reaction happened as desired. From the IR spectra and MS it can be concluded that synthesis of GNM was successful.

Characterization of FNM

Comparison of the IR spectra between FNM and fructose reveals that the reaction occurred. The fructose spectrum presents the following bands: O–H stretching vibration at 3,393 cm^{-1}, C–H stretching vibration at 2,938 cm^{-1}, and C–C stretching vibration at 1,407 cm^{-1}, while the FNM spectra display new absorption peaks at 1731 cm^{-1}, 1300–1161 cm^{-1}, 767, and 671 cm^{-1}, corresponding to C=O, C–O–C, and C–Cl stretching vibration. The molecular weight of FNM is 348, whereas we measured $[M\text{-}CH_2OH]^+ = 317$ and $[CH_2OH + 1]^+ = 32$ by MS; this may be because of instability of the glycosylated derivative under high temperature and additionally proves that BCC was grafted onto C1–OH of fructose. From the IR spectra and MS it can be concluded that the synthesis of FNM was successful.

Characterization of LNM

The lactose IR spectrum presented O–H stretching vibration at 3,528–3,379 cm^{-1}, C–H stretching vibration at 2,895 cm^{-1}, C–C stretching vibration at 1,407 cm^{-1}, and C–O at 1,160–1,062 cm^{-1}. In comparison with the lactose IR spectrum, the LNM spectra display new absorption peaks at 1,748 cm^{-1}, 1,300–1,155 cm^{-1}, 767, and 671 cm^{-1}, corresponding to C=O, C–O–C, and C–Cl stretching vibration. The molecular weight of LNM is 680, and we measured $[M]^+ = 680.7$ by MS. From all these results, we conclude that the product is obtained as desired.

Synthesis of BCC

BCC is an important synthetic intermediate, being crucial for the subsequent steps in the whole process. It was prepared from bis(2-chloroethyl)amine hydrochloride and triphosgene in methylene dichloride at 0–5 °C for 2 h in presence of triethylamine. The nitrogen in BCC acts as a nucleophile with triphosgene to form a N–C bond. The triphosgene-mediated reaction of BCC outlined here has advantages over existing methods: the reaction can be carried out without handling hazardous phosgene, the starting material is commercially available, formation of SO_2 or $POCl_3$ (as occurs with chlorinating agents such as thionyl chloride and phosphorus pentachloride) is avoided, and a nitrogen atmosphere is not necessary [8]. The initiator and organic base for this step can be dimethylformamide, triethylamine, or pyridine; however, triethylamine was chosen because of the easy isolation of its hydrochloride salt from organic solvents, the lack of dark-colored impurities, and its suitability for further use. Progress of the reaction was monitored conveniently by TLC.

Interaction of BSA with GNM, FNM, and LNM

Serum albumins are the most abundant proteins in plasma [9, 10]. It is well known that BSA is often used as a target protein molecule because of its low cost, ready availability, and unusual ligand-binding properties [11, 12]. Moreover, the whole structure of the BSA molecule is similar to that of the human serum albumin (HSA) molecule, meaning that many studies of BSA can be considered to be consistent with HSA [13]. To research the interaction of the three glycosylated nitrogen mustard derivatives with BSA, the quenching intrinsic fluorescence of protein was used.

The fluorescence spectrum of BSA presents strong emission with maximum at 356 nm when excited at 288 nm. The results showed that, with addition of GNM, FNM, and LNM, respectively, the intrinsic fluorescence of BSA was gradually quenched compared with pure BSA solution. It can be concluded that the environment of tryptophan is highly affected by the interaction with the derivatives.

As many studies have reported on the interaction of small molecules with BSA, it is possible to estimate the quenching mode by using the Stern–Volmer Eq. (1) [14].

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q], \quad (1)$$

where F_0 is the fluorescence intensity of BSA solution without quencher at 356 nm, F is the fluorescence intensity of BSA solution in the presence of quencher at 356 nm, K_q is the biomolecular quenching rate constant, τ_0 is the fluorophore lifetime in the absence of quencher, K_{SV} is the Stern–Volmer quenching constant, and $[Q]$ is the quencher concentration [15, 16]. Figure 1 shows Stern–Volmer plots of BSA-GNM, BSA-FNM, and BSA-LNM, respectively.

Figure 1 shows that the Stern–Volmer plots were linear and did not change from linearity with increasing concentration of the three derivatives, to some extent revealing the occurrence of a single type of quenching, either static or dynamic. The corresponding plot expressions are listed in Table 1.

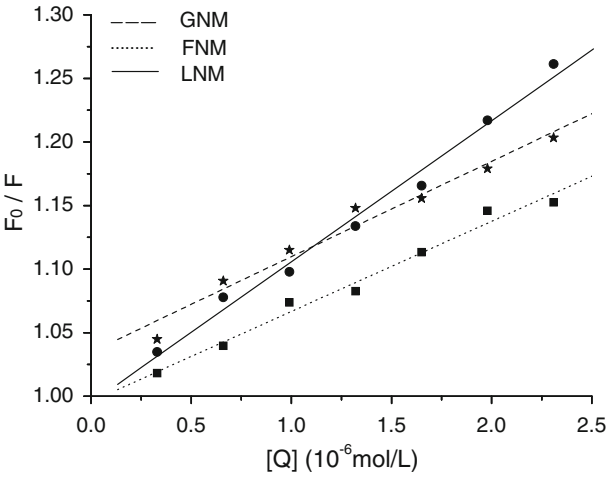


Fig. 1 Stern–Volmer plot of BSA + GNM, BSA + FNM, and BSA + LNM solutions with different GNM, FNM, and LNM concentrations

Table 1 Quenching constants of BSA + GNM, BSA + FNM, and BSA + LNM systems calculated according to Stern–Volmer plots

System	K_{SV} (L/mol)	K_q (L/mol s)	R
BSA + GNM	7.499×10^4	7.499×10^{12}	0.98461
BSA + FNM	7.088×10^4	7.088×10^{12}	0.99163
BSA + LNM	1.1098×10^5	1.1098×10^{13}	0.9938

According to the Stern–Volmer equation, K_{SV} values for the three systems were obtained. Generally, for most protein molecules, τ_0 is $\sim 10^{-8}$ s. Therefore, K_q could be calculated from the relation $K_{SV} = K_q \tau_0$. Since the maximum value of Kq for a diffusion-controlled quenching process with a biopolymer is about 2.0×10^{10} L/mol s [17], the high value of the quenching rate constants imply that the quenching is static. Table 1 shows that the K_q values of the three system were all $>2.0 \times 10^{10}$. Thus, it can be concluded that the quenching model of these novel glycosylated nitrogen mustard derivatives with BSA is static quenching.

Conclusions

BCC was successfully grafted to glucose, fructose, and lactose, respectively, to obtain GNM, FNM, and LNM. The products were characterized by IR and MS, and their biological activity was measured in Tris-HCl buffer solution at pH 7.4. The quenching model for these novel glycosylated nitrogen mustard derivatives with BSA is static quenching.

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