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Synthesis and DNA-binding Properties of Trehalose-tethered Monomeric and Dimeric Berberines

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Trehalose-tethered monomeric and dimeric berberines were synthesized in 50% and 30% from the reaction of berberrubine with 6-tosyl-α,α'-trehalose and 6,6'-ditosyl-α,α'-trehalose, respectively, and fully characterized by MS (HR and ESI) and NMR (^{1}H, ^{13}C, COSY and HSQC). Spectrophotometric and spectrofluorimetric titrations indicated that compared with berberine, trehalose-tethered monomeric berberine had comparable DNA-binding affinity toward calf-thymus DNA, whereas trehalose-spaced dimeric berberine exhibited higher DNA-binding affinity. The potential application of these conjugates is also briefly discussed.

Key Words : Protoberberine, Trehalose, DNA binder, Non-covalent interaction

Introduction

Carbohydrates play a crucial role in the functions of biological systems and are widely present in many naturally-occurring antitumor antibiotics, such as Aclacinomycin, Pluramycin A and Olivomycin A. [1] These antibiotics feature an aromatic subunit and sugar moiety, both of which are common structural components of many DNA-binding molecules. Because the aromatic subunit plays a DNA intercalating role, whereas the carbohydrate residue binds within the DNA minor groove through multiple hydrogen bonds, carbohydrate-containing antibiotics generally exhibit high DNA-binding affinities.[2]

On the basis of this multivalent binding mode, some carbohydrate-tethered DNA-binding molecules have been reported and show promising bioactivities. For example, Toshima *et al.* designed 2,6-dideoxyaminosugar-containing anthraquinones. [3] These molecules bind selectively to a TGC sequence, and significantly their potencies are affected by the nature of the aminosugar moiety. That is, the conjugate containing a D-aminosugar is stronger in DNA binding than the one bearing a corresponding L-sugar. Thus, the sugar moiety contributes to the binding, and furthermore, the binding activity is translated into cytotoxicity. In addition, they also reported artificial hybrides containing aminosugar and a 2-phenylquinoline aromatic moiety as the intercalator as well as the photo-reactive group. These hybrides bind to and cleave DNA strands through a photo-mediated radical mechanism, which leads to cytotoxicity that increases when evaluated under ultraviolet irradiation.[4] These and other studies[5] raise the possibility that carbohydrate-aromatic conjugates are promising in developing DNA-binding and anti-tumor antibiotic agents. Construction of such conjugates in which the sugar moieties are generally linked to the aromatic subunits *via* glucosidic bonds, however, requires tedious synthetic manipulations.

Given the ready availability and structural modification of α,α'-trehalose and as part of our sustained interest in creating novel DNA binders based on berberine **1**,[6,7] herein we report the synthesis and DNA-binding properties of trehalose-tethered monomeric and dimeric berberines **2** and **3** (Figure 1). Because both berberine and trehalose are biologically active natural products having multiple physiological activities, their conjugates such as compounds **2** and **3** may integrate the activities of berberine and trehalose, and are therefore expected to find wide applications, for example as potential *anti*-HIV agents.[8] In addition, the trehalose moiety is expected to enhance the water-solubility of berberine dimers.[7]

Experimental Section

General. NMR spectra were recorded at a Bruker Avance AV 400 (or 100) spectrometer. ESI-MS and HR-ESI-MS spectra were measured on Waters UPLC/Quattro Premier

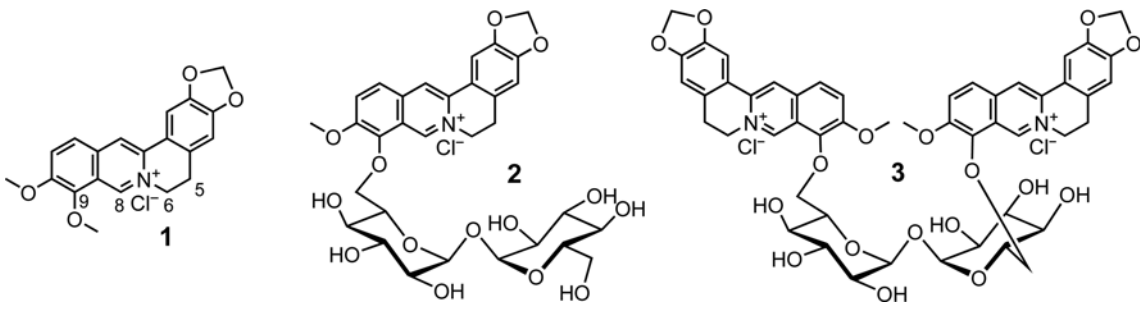


Figure 1. Structures of berberine **1**, and its trehalose-tethered derivatives **2** and **3**.

XE and Shimadzu LCMS-IT-TOF mass spectrometers, respectively. Silica gel 60 Å (reagent pure, Qingdao Haiyang Chemical Co. Ltd) was used for column chromatography. Analytical thin-layer chromatography was performed on silica gel plates 60 GF254 (chemical pure, Qingdao Haiyang Chemical Co. Ltd). Detection on TLC was made by use of iodine, UV (254 or 365 nm) and 20% aqueous H_2SO_4. UV-Vis and fluorescence spectra were measured on a TU-1901 spectrophotometer and a Shimadzu RF-5301PC spectrofluorimeter, respectively, using conventional quartz cells of 1 cm path.

Calf-thymus (CT) DNA was purchased from Sigma Chemical Co. (St Louis, USA). The concentration of CT DNA was determined spectrophotometrically using the molar extinction coefficient of 13200 $mol^{-1}\cdot cm^{-1}$/base pair at 260 nm. Berberrubine **4** was prepared according to reported procedures.[9] All the other chemicals were of analytical reagent grade and used without further purification.

Synthesis of Compounds 2 and 3.

6-Tosyl-α,α'-trehalose 5: The synthesis of compound **5** was conducted according to reported procedures.[10] Thus, to a solution of trehalose (1.9 g, 5.6 mmol) in anhydrous pyridine (80 mL) was added a solution of *p*-toluenesulfonyl chloride (1.2 g, 6.3 mmol) in anhydrous pyridine (20 mL). The resulting mixture was stirred at −35 °C for 9 h. Then, pyridine was evaporated under reduced pressure and the obtained residues were isolated by chromatography on an open reverse-phase column, eluting from H_2O to 15% aqueous MeOH, to give compound **5** (0.75 g, 27%) having ESI-MS *m/z* 496.1 ($[M]^+$).

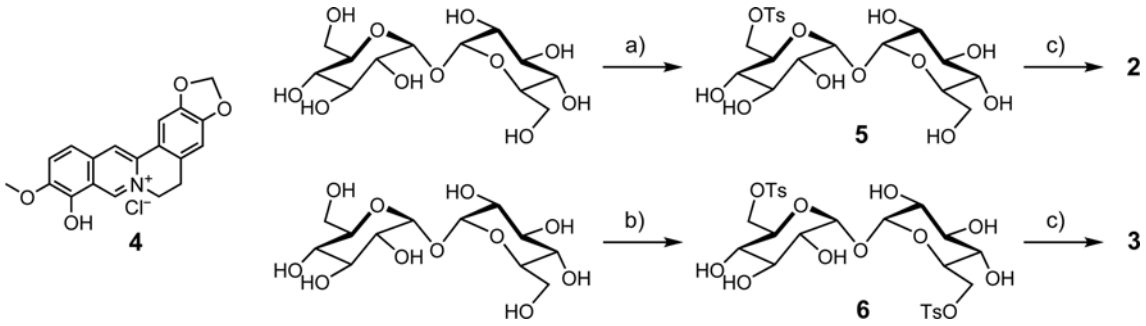
6,6'-Ditosyl-α,α'-trehalose 6: The synthesis of compound **6** was conducted according to reported procedures.[10] Thus, to a solution of trehalose (2.3 g, 6.8 mmol) in anhydrous pyridine (70 mL) was added a solution of *p*-toluenesulfonyl chloride (3.4 g, 17.9 mmol) in anhydrous pyridine (30 mL). The resulting mixture was stirred at −35 °C for 5 h. Then, pyridine was evaporated under reduced pressure and the obtained residues were isolated by chromatography on an open reverse-phase column, eluting from H_2O to 50% aqueous MeOH, to give compound **6** (0.85 g, 19%) having ESI-MS *m/z* 673.5 ($[M+Na]^+$).

Compound 2: To a solution of berberrubine **4** (275 mg, 0.86 mmol) in MeCN (20 mL) was added compound **5** (400 mg, 0.81 mmol). The resulting mixture was refluxed for 5 d. After the solvent was removed under reduced pressure, the obtained residues were subject to anion exchange into chloride form, and isolated by chromatography on an open reverse-phase column, eluting from H_2O to 10% aqueous MeOH, to give compound **2** (240 mg, 50%) as a yellow powder, having ^{1}H-NMR (400 MHz, D_2O, MeCN as an internal standard, δ 1.98 ppm) δ 9.23 (s, 1H), 7.78 (s, 1H), 7.65 (d, *J* = 9.2 Hz, 1H), 7.45 (d, *J* = 9.12 Hz, 1H), 6.80 (s, 1H), 6.62 (s, 1H), 5.84 (d, *J* = 3.44 Hz, 2H), 5.13 (d, *J* = 3.76 Hz, 1H, H1), 4.81 (d, *J* = 3.80 Hz, 1H, H1'), 4.58 (overlapped with solvent, 2H), 4.41-4.33 (m, 2H, H6), 4.02-3.98 (m, 1H, H5), 3.81 (s, 3H), 3.80 (t, *J* = 9.76 Hz, 1H, H3), 3.74-3.67 (m, 3H, H3', H5' and H6'), 3.63-3.59 (m, 2H, H2 and H6'), 3.52 (t, *J* = 9.64 Hz, 1H, H4), 3.36 (dd, *J* = 3.80 and 9.64 Hz, 1H, H2'), 3.28 (t, *J* = 9.40 Hz, 1H, H4'), 2.94 (t, *J* = 5.64 Hz, 2H); ^{13}C-NMR (100 MHz, D_2O, MeCN as internal standard, δ 1.30 ppm) δ 150.84, 150.43, 147.97, 144.23, 141.89, 137.65, 133.17, 130.28, 126.77, 123.91, 121.55, 119.73, 119.65, 108.83, 104.77, 102.87, 93.80 (C1 and C1'), 73.24 (C6), 73.14 (C2), 73.02 (C3), 72.79 (C5'), 71.65 (C5), 71.53 (C4), 70.37 (C2'), 70.16 (C4'), 61.03 (C6'), 57.04, 56.45, 26.95; ESI MS *m/z* 647.0 ($[M-Cl]^+$), and HR MS for $C_{31}H_{36}NO_{14}^+$ ($[M-Cl]^+$) calcd: 646.2136, found 646.2147.

Compound 3: To a solution of berberrubine **4** (250 mg, 0.78 mmol) in MeCN (20 mL) was added compound **6** (205 mg, 0.32 mmol). The resulting mixture was refluxed for two weeks. After the solvent was removed under reduced pressure, the obtained residues were subject to anion exchange into chloride form, and isolated by chromatography on an open reverse-phase column, eluting from H_2O to 50% aqueous MeOH, to give compound **3** (79 mg, 30%) as a yellow powder, having ^{1}H-NMR (400 MHz, DMSO-d_6 with 2 drops of D_2O) δ 9.63 (s, 2H), 8.81 (s, 2H), 8.12 (d, *J* = 11.5 Hz, 2H), 7.99 (d, *J* = 11.5 Hz, 2H), 7.72 (s, 2H), 7.03 (s, 2H), 6.14 (s, 4H), 4.87 (br, 4H), 4.82 (d, *J* = 3.5 Hz, 2H, H1), 4.50 (br, 4H, H6), 4.19-4.17 (br, 2H, H5), 4.03 (s, 6H), 3.74-3.67 (overlapped with solvent, 2H, H3), 3.38-3.34 (m, 4H, H2 and H4), 3.17 (br, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6 with 2 drops of D_2O) δ 150.37, 150.25, 148.08, 145.38, 143.36, 137.84, 133.42, 130.93, 127.16, 123.72, 121.67, 120.70, 120.52, 108.77, 105.82, 102.49, 94.52 (C1), 74.24 (C6), 73.02 (C3), 71.88 (C5), 71.79 and 70.44 (C2 and C4), 57.52, 56.03, 26.08; ESI-MS *m/z* 475.6 ($[M-2Cl]^{2+}$), and HR MS for $C_{50}H_{50}N_2O_{17}$ ($[M-2Cl]^{2+}$) calcd: 475.1555, found 475.1546.

Spectrofluorimetric Titrations. Spectrofluorimetric titrations were carried out by fixing the concentration of compound **2** or **3**, while gradually increasing the concentration of CT DNA. Specifically, to a solution of compound **3** (1.96×10^{-6} M) in 50 mM Tris-HCl buffer (pH 6.35) were added aliquots of CT DNA (7.24×10^{-4} M) solution containing compound **3** (1.96×10^{-6} M) in the same buffer. This operation ensured the gradual increase of the concentration of CT DNA, while kept the concentration of compound **3** constant. After the mixture was equilibrated, the corresponding fluorescence spectra were measured at room temperature. This operation was repeated until saturation reached. The spectrofluorimetric titration of compound **2** was conducted in a similar way. The association constants (K_a's) were derived from the analysis of the relationship between the fluorescence intensity at 522 nm and the concentration of compound **2** or **3**.

Spectrophotometric Titrations. Spectrophotometric titrations were performed by fixing the concentration of compound **2** or **3**, while gradually increasing the concentration of CT DNA. Specifically, to a solution of compound **3** (9.8×10^{-6} M) in 50 mM Tris-HCl buffer (pH 6.35) were added aliquots of CT DNA (7.24×10^{-4} M) solution containing compound **3** (9.8×10^{-6} M) in the same buffer. This operation ensured the gradual increase of the concentration of CT DNA, while kept the concentration of compound **3** constant. After the mixture was equilibrated, the corresponding absorption spectra were recorded at room temperature. This operation was



Scheme 1. Synthesis of compounds **2** and **3**. Reagents and conditions: a) TsCl (1.1 eq), anhydrous pyridine, –35 °C; b) TsCl (2.6 eq), anhydrous pyridine, –35 °C; c) **4**, CH_3CN, reflux.

repeated until saturation reached. The spectrophotometric titration of compound **2** was conducted in a similar fashion.

Results and Discussion

Synthesis of Compounds 2 and 3. The synthesis of compounds **2** and **3** is shown in Scheme 1. Thus, reaction of berberrubine **4** with compounds **5** and **6**, respectively, and subsequent anion-exchange into chloride form, afforded compounds **2** and **3** in 50% and 30% yields, respectively. The structures of compounds **2** and **3** were confirmed by MS (ESI and HR) and NMR (^{1}H, ^{13}C, COSY and HSQC). Compound **2** gave a mass spectrum with the *m/z* value corresponding to $[M\text{-}Cl]^+$, and in the ^{1}H NMR spectrum, the ratios of the integrated areas for the protons of berberine subunit to those for sugar protons were in full accord with the expected structure. Compound **3** gave a mass spectrum with the *m/z* value corresponding to $[M\text{-}2Cl]^{2+}$, and only one set of NMR signals was observed, indicative of its symmetric structure. The proton and carbon signals were assigned on the basis of COSY and HSQC.

Interaction of Compounds 2 and 3 with DNA. The binding affinities of compounds **2** and **3** toward CT DNA were evaluated by means of fluorescence spectrometry.[6a,7,11] Figure 2(a) shows the spectrofluorimetric titration spectra of compound **3** with CT DNA. It can be seen that the weak fluorescence of compound **3** was largely enhanced upon the addition of CT DNA, which is indicative of the interaction between compound **3** and CT DNA. Similar phenomenon was observed for compound **2** upon the addition of CT DNA. Analyses of the relationship between the fluorescence intensities and the concentrations of CT DNA by nonlinear curve fitting methods afforded the association constants (K_a's) of compounds **2** and **3** (Table 1). For comparison, the binding constant of berberine **1** with CT DNA was measured in a similar way. It can be seen that compared with berberine, compound **2** had comparable DNA-binding affinity, whereas compound **3** exhibited *ca* 7-fold higher affinity.

The interactions of compounds **2** and **3** with CT DNA were then monitored by absorption spectrometry.[6a-c,11] It is known that the interaction of a DNA-binding molecule with DNA usually accompanies spectral changes in which wavelength shifts, absorbance changes and/or isosbestic points provide useful information for understanding of the binding strength and mode.[12] As shown in Figure 2(b) and Table 1, upon the addition of CT DNA, both compounds **2** and **3** exhibited hypochromicities and bathochromic shifts. These spectroscopic variations convincingly suggest that compounds **2** and **3** are capable of forming stable complexes with CT DNA. The fact that compound **2** showed similar hypochromicity and bathochromic shift with berberine, whereas

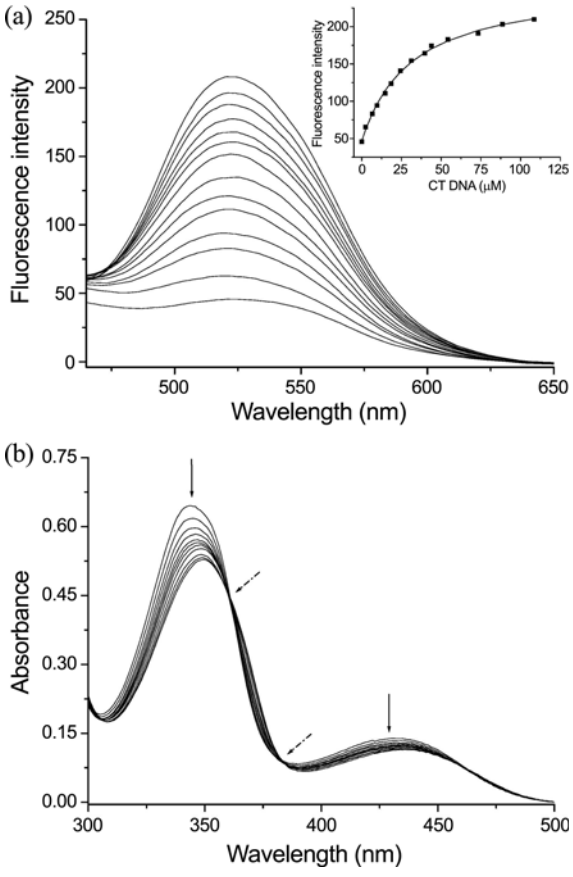


Figure 2. (a) Fluorescence spectra of compound **3** (1.96×10^{-6} M) with CT DNA of increasing concentrations ($0 \sim 2.17 \times 10^{-4}$ M in base pair) in 50 mM Tris-HCl (pH 6.35) at room temperature, ex 345 nm. The inset indicates the relationship between the fluorescence intensity at 522 nm and the concentration of CT DNA. (b) Spectrophotometric titrations of compound **3** (9.80×10^{-6} M) with CT DNA of increasing concentrations ($0 \sim 1.18 \times 10^{-4}$ M in base pair) in 50 mM Tris-HCl (pH 6.35) at room temperature. The solid arrows indicate the decreasing absorption bands during the course of titration; dash-dot arrows indicate the isosbestic points.

Table 1. Association constants (K_a's, M^{-1}) and photophysical properties of compounds **1-3** bound to CT DNA[a]

Compound	K_a	Red Shift/nm[b]	Hypochromicity/%[b]	Isosbestic Point/nm
1	$(4.91 \pm 0.42) \times 10^3$	2	9.5	354, 387
2	$(2.64 \pm 0.05) \times 10^3$	2	7.0	355, 393
3	$(3.38 \pm 0.22) \times 10^4$	6	16.3	361, 383

[a]Measured in 50 mM Tris-HCl buffer (pH 6.35) at room temperature. [b]Obtained at 345 nm. The concentrations of compounds **2** and **3** were 1.53×10^{-5} M and 9.80×10^{-6} M, respectively.

compound **3** exhibited larger hypochromicity and bathochromic shift suggests that compound **2** had comparable DNA-binding affinity with berberine, whereas compound **3** exhibited higher affinity.[11] These results are in agreement with those obtained from the spectrofluorimetric titration experiments. On the other hand, during the titrations with CT DNA, two well-resolved isosbestic points were observed for both compounds **2** and **3**, revealing the existence of one preferential, almost exclusive binding mode of compounds **2** and **3** with CT DNA.[11] According to the hypochromicities and bathochromic shifts, compounds **2** and **3** interact with CT DNA, most probably *via* an intercalation mode.

The present results may be rationalized if the structures of compounds **2** and **3** are taken into consideration. Computer-aided modeling study on the berberine-DNA complex has suggested that berberine binds to DNA from its C_5-C_6-N^+-C_8 side (Figure 1).[13] This was experimentally supported by our earlier study that introducing bulky groups at the 9-position leads to a dramatic decrease in the binding affinity.[14] According to this, the trehalose group in compound **2**, because of its bulkiness, may inhibit the intercalation of the berberine subunit into the base pairs of DNA. However, it is poly-hydroxylated and thus able to impart the binding affinity through multivalent hydrogen bonding with DNA. Thus, compound **2** exhibited comparable affinity with berberine. On the other hand, we have shown in previous studies that the affinities of dimeric berberines are dependent on the spacer length.[7] For example, the dimer linked with three-atomed propyl chain shows up to 100-fold higher DNA-binding affinity than berberine, whereas the one having seven-atomed heptyl chain has comparable affinity with berberine. According to this, compound **3** should have comparable affinity with berberine, as it can be viewed as one dimeric analog that is linked with nine atoms. However, the trehalose group enhanced the interaction of compound **3** with DNA through multiple hydrogen bonds. Thus, compound **3** exhibited higher affinity than berberine.

Conclusion

In summary, two trehalose-modified monomeric and dimeric berberines have been successfully synthesized and fully characterized on the basis of NMR (^{1}H, ^{13}C and 2D) and MS (ESI and HR). Spectrophotometric and spectrofluorimetric titrations have indicated that, compared with berberine, trehalose-tethered monomeric berberine had comparable DNA-binding affinity, whereas trehalose-spaced dimeric berberine exhibited higher DNA-binding affinity. Thus, the dimer may be exploitable as a new DNA binder. Efforts aimed at exploiting the potential applications of these conjugates as biomedical agents are currently under investigation in our laboratories.

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