Phosphotyrosine Binding by Ammonium- and Guanidinium-Modified Cyclodextrins

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Introduction

An important challenge in host−guest chemistry is the preparation of host molecules that are capable of affecting important biochemical processes in cellular settings. This requires the identification of biological targets (or guests) for which small synthetically accessible complexing agents can be rationally designed; moreover, there should be a reasonable expectation that complexation will bring about some desirable physiological outcome. One such target is phosphotyrosine. The phosphorylation of tyrosine residues on the cytosolic surfaces of receptor tyrosine kinases represents the quintessential early step in growth factor-induced mitogenesis.[1] Sites of modification are recognized and bound by specific secondary signaling proteins; these as well as subsequent protein−protein binding events provide a means for transmission of the signal from one component in the pathway to the next, ultimately leading to the activation of transcription factors that initiate cell division.[2]

In a number of human cancers, including breast and ovarian cancers, there is a strong correlation between abnormally high levels of tyrosine-phosphorylated proteins and tumor cell growth, which appears to result from the overexpression or constitutive activity of cellular tyrosine kinases.[3] One approach to inhibiting inappropriate mitogenic signaling is to inhibit the binding of secondary cytosolic proteins to tyrosine-phosphorylated receptors. We envision that this can be accomplished using small molecules that form strong specific complexes with phosphotyrosine residues on protein surfaces. Accordingly, we have initiated a program to identify host molecules with high affinity and selectivity for phosphotyrosine, employing cyclodextrins (CDs) as starting architectures. The parent compounds possess reasonably good water solubility and their ability to form inclusion complexes with aromatic guests in aqueous solution is well documented.[4] In addition, a variety of approaches to regiospecific chemical modification have been identified, allowing for the introduction of desired functionality to enhance binding affinity and selectivity.[5] Initially, we have chosen to examine derivatives of β-CD with pendant ammonium or guanidinium groups on the primary rim

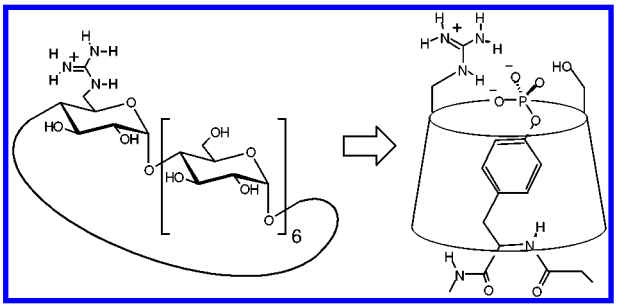
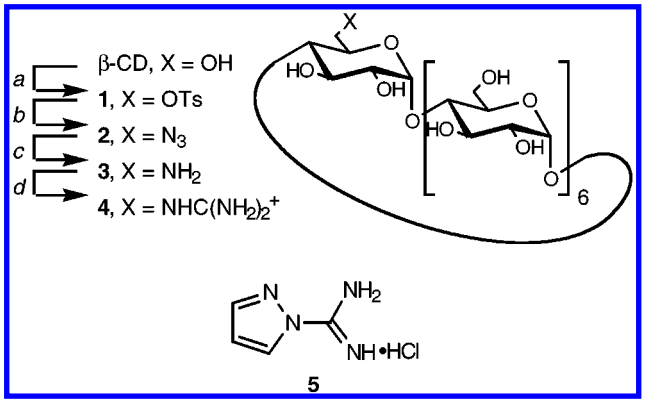


Figure 1. Structure of a guanidinium-substituted β-CD derivative and a schematic representation of an inclusion complex with phosphotyrosine on a protein surface (showing only one of the unmodified primary hydroxyl groups for clarity).

Scheme 1[a]



[a] Reagents: (a) TsCl, pyridine (30%); (b) NaN_3, KI, DMF (80%); (c) H_2, Pd/C, H_2O (90%); (d) **5**, $(i\text{-Pr})_2$NEt, DMF (33%).

of the cavity, with the expectation that these groups will take part in favorable electrostatic interactions with the phosphate group of phosphotyrosine within a complex (Figure 1).[6−8]

Results and Discussion

Guanidinium-containing β-CD derivative **4** has been prepared via known amino derivative **3**[9] using the guanylating agent 1*H*-pyrazolecarboxamidine hydrochloride (**5**)[10] (Scheme 1). A bis-guanidinium derivative of β-CD was also synthesized (Scheme 2). Here, the seven small circles represent the seven glucose units in β-CD

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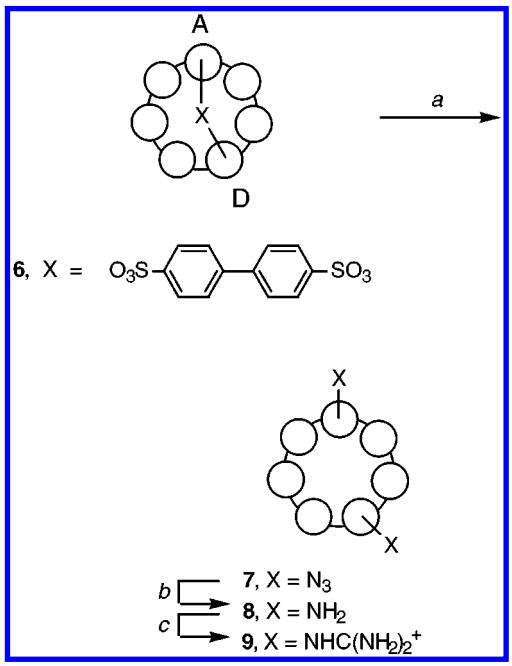
Scheme 2[a]



[a] Reagents: (a) NaN_3, KI, DMF (50%, crude); (b) H_2, Pd/C, H_2O (90%); (c) **5**, $(i\text{-Pr})_2NEt$, H_2O (74%).

and X is a functional group covalently bonded to the primary (6) carbon of the A and D sugars in the cyclodextrin ring. The required starting material **6** was prepared by the reaction of β-CD with 4,4′-biphenyldisulfonyl chloride in pyridine as reported by Tabushi and co-workers.[11] This method also produces a minor amount of the A,C isomer (ca. 10% based on isolated bis-sulfonate esters). After reaction with sodium azide and reduction, the regioisomeric amines were separated on carboxymethyl cellulose ion-exchange resin using a linear gradient of aqueous ammonium bicarbonate; pure **8** was then converted to **9** with **5**.[12] Compounds **4**, **8**, and **9** were isolated as bicarbonate salts after purification and were converted to chloride salts prior to use in binding experiments; pure **3** was isolated as the free base.

Competitive binding experiments were carried out using β-CD and derivatives with tyrosine and phosphotyrosine, using the indicator dye *m*-cresol purple. Initially, 1:1 association constants for the cyclodextrins and the indicator were determined from titration data using an iterative linear least-squares approach.[13] Subsequent competition experiments involved repeating the 1:1 experiments in the presence of a fixed concentration of amino acid, and the resulting data were analyzed as described by Pendergast and Connors.[14] Results are summarized in Table 1.

No binding was detected between β-CD and either tyrosine or phosphotyrosine.[15] The addition of a single ammonium group provides a host (**3**) that binds both amino acids with similar (low) affinity while monoguanidinium derivative **4** shows no detectable binding with either amino acid. The addition of a second positively charged group provides hosts (**8** and **9**) that display

Table 1. Association Constants (M^{-1}) for β-CD and Derivatives[a,b]

host	*m*-cresol purple	tyrosine	phosphotyrosine
β-CD	1130	n.b.d.	n.b.d.
3	1300	40 ± 10	30 ± 10
4	1590	n.b.d.	n.b.d.
8	850	30 ± 3	350 ± 8
9	1410	n.b.d.	310 ± 8

[a] Conditions: 25.0 °C, 100 mM sodium phosphate, pH 7.00. [b] n.b.d. indicates that no binding was detected.

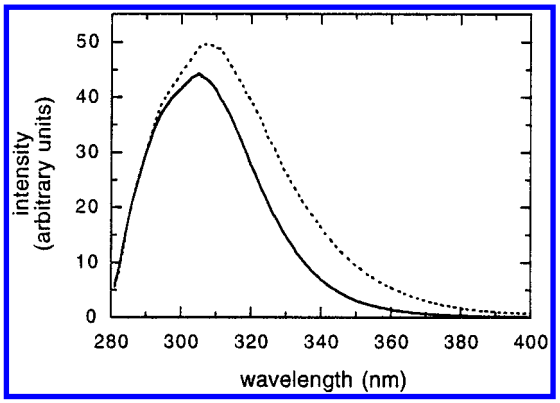


Figure 2. Fluorescence emission spectrum of phosphotyrosine in the absence (solid line) and presence (broken line) of **8**: 205 μM phosphotyrosine, 2.9 mM **8**, 25.0 °C, 100 mM sodium phosphate, pH 7.00. Excitation wavelength 274.5 nm.

significantly enhanced binding of phosphotyrosine relative to **3** and **4**. Importantly, both **8** and **9** also show a significant preference for the phosphorylated amino acid ($-\Delta\Delta G \geq 1.4$ kcal/mol), indicating that they would exhibit selectivity for phosphorylated vs nonphosphorylated tyrosine residues on a protein surface. Our results suggest that this preference results from an increase in favorable electrostatic interactions between the pendant cationic groups and the phosphate of phosphotyrosine, as no similar increase is seen for tyrosine binding. In the case of **8**, the energetic benefit of the second ammonium group (1.4 kcal/mol) is similar to that observed for the formation of a salt bridge in a wide variety of host−guest systems (1.2 ± 0.2 kcal/mol).[16]

To investigate whether binding of phosphotyrosine involves inclusion within the CD cavity, preliminary fluorescence experiments were carried out with **8** and **9** (Figure 2). Compound **8** produces a 74% increase in the quantum yield of phosphotyrosine (extrapolated to 100% bound), which is similar in magnitude to that previously observed for phenol with unmodified β-CD (71%) and is consistent with the formation of an inclusion complex.[17,18] A much smaller increase was seen with **9** (12% extrapo-

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lated to 100% bound). Increases in fluorescence intensity resulting from CD binding are thought to result primarily from decreased collisional quenching of the excited state by solvent molecules.[17] The reduced enhancement with **9** relative to that with **8** then suggests that phosphotyrosine is more solvent exposed in the former complex, although the fact that binding solutions contain 100 mM phosphate argues against purely electrostatically driven association.

The bis-ammonium and -guanidinium cyclodextrins described represent promising lead compounds in the development of phosphotyrosine-specific binding compounds. However, hosts exhibiting dissociation constants in the low micromolar to nanomolar range will be required to effectively compete for sites on tyrosine-phosphorylated receptors in vivo. More conformationally restricted analogues of **8** and **9** are currently being synthesized with the expectation that these will display higher affinities for phosphotyrosine. Experiments with phosphotyrosine-containing peptides and proteins are also presently underway.[19]

Experimental Section

General Methods. TLCs were run on precoated EM Science silica gel 60 plates and developed in 7:7:5:4 EtOAc/2-propanol/NH_4OH/water. Whatman Express-Ion Exchanger C was used for purification of **3**, **4**, **8**, and **9**. ^{1}H chemical shifts are reported downfield from external sodium 3-(trimethylsilyl)propionate-d_4 in D_2O; acetone in D_2O was used as an external standard for ^{13}C NMR spectra (δ = 31.07 ppm[20]). β-CD was obtained from Cerestar USA and used without further purification; elemental analysis indicated that the sample used in binding experiments contained 9.5 waters per CD molecule (Anal. Calcd for $C_{42}H_{70}O_{35}\cdot 9.5H_2O$: C, 38.62; H, 6.87. Found: C, 38.54; H, 6.58). *m*-Cresol purple and tyrosine were obtained from Aldrich and used without further purification. Phosphotyrosine was obtained from Sigma and was converted to its ammonium salt by lyophilization from aqueous ammonium bicarbonate followed by repeated lyophilization from deionized water; elemental analysis was consistent with the bis-ammonium salt containing 1.5 waters (Anal. Calcd for $C_9H_{18}N_3O_6P\cdot 1.5H_2O$: C, 33.52; H, 6.57; N, 13.04. Found: C, 33.48; H, 6.58; N, 12.77).

Mono-6-deoxy-6-guanidinocycloheptaamylose (4). Crude **3** (909 mg, 0.382 mmol) was suspended in 1.5 mL of dry DMF and **5** (235 mg, 1.60 mmol) and *N*,*N*-diisopropylethylamine (224 μL, 1.6 mmol) were added. The reaction was stirred for 22 h under N_2, transferred to a 40 mL beaker, and 20 mL of ether was added dropwise. The resulting suspension was stirred for 2 h, and the precipitate was collected by filtration, washed with 40 mL of ether, and dried in vacuo, providing crude **4** as a fine white powder (1.15 g). A portion of the product (810 mg) was dissolved in 250 mL of water and applied to a (carboxymethyl)-cellulose column that was eluted with a linear gradient of aqueous NH_4HCO_3 (10−200 mM). Fractions containing **4** were combined, concentrated under reduced pressure, and repeatedly lyophilized from deionized water to provide the bicarbonate salt of **4** as a fluffy white solid (308 mg, 33% yield). Data for **4** (HCO_3^- salt): R_f = 0.04; FAB-MS *m*/*z* calcd for $C_{43}H_{75}N_3O_{34}$ (M^+) 1177.4, measured 1177.0. Anal. Calcd for $C_{44}H_{75}N_3O_{37}\cdot 4H_2O$: C, 40.34; H, 6.39; N, 3.21. Found: C, 40.33; H, 6.49; N, 3.57.

The bicarbonate salt of **4** was dissolved in water, loaded on a column of Dowex 1 × 2−100 (chloride form), and eluted with water. Fractions containing **4** were combined, concentrated under reduced pressure, and lyophilized, providing the chloride salt of **4** as a clear solid. Data for **4** (Cl^- salt): mp 230 °C dec; R_f = 0.04; ^{1}H NMR (D_2O, 500 MHz) δ 5.13−5.03 (m, 7 H), 4.04−3.78 (m, 26 H), 3.70−3.50 (m, 16 H); ^{13}C NMR (D_2O, 125 MHz) δ 158.61, 102.77, 102.64, 102.35, 83.20, 82.16, 81.96, 81.90, 73.86, 73.84, 73.79, 73.73, 73.53, 72.82, 72.68, 72.63, 72.56, 71.31, 61.34, 61.13, 60.92, 42.98. Anal. Calcd for $C_{43}H_{84}ClN_3O_{39}\cdot 5H_2O$: C, 39.65; H, 6.50; Cl, 2.72; N, 3.23. Found: C, 39.48; H, 6.40; Cl, 3.07; N, 3.31.

6^A,6^D-Dideoxy-6^A,6^D-diguanidinocycloheptaamylose (9). **8** (304 mg, 0.268 mmol), **5** (162 mg, 1.11 mmol), and *N*,*N*-diisopropylethylamine (187 μL, 1.07 mmol) were stirred in 2 mL of water for 17 h at room temperature and then frozen and lyophilized. The resulting solid was dissolved in 3 mL of water, additional **5** (163 mg, 1.11 mmol) and *N*,*N*-diisopropylethylamine (93 μL, 0.54 mmol) were added, and the reaction was stirred at room temperature for 17 h. The solution was frozen and lyophilized, and the resulting solid was stirred with 40 mL of ether, collected by filtration, and washed with 60 mL of ether to provide crude **9** as a fine white powder (707 mg). This was dissolved in 250 mL of 50 mM NH_4HCO_3 and applied to a (carboxymethyl)ellulose column that was eluted with a linear gradient of aqueous NH_4HCO_3 (50−400 mM) followed by 400 mM NH_4HCO_3. Fractions containing **9** were combined, concentrated under reduced pressure, and repeatedly lyophilized from deionized water to provide the bicarbonate salt of **9** as a fluffy white solid (264 mg, 74% yield). Data for **9** (HCO_3^- salt): mp 210 °C dec; R_f = 0.0; FAB-MS *m*/*z* calcd for $C_{44}H_{78}N_6O_{33}$ ($M - H^+$) 1217.8, measured 1218.4; ^{1}H NMR (D_2O, 500 MHz) δ 5.12−5.04 (m, 7 H), 4.03−3.78 (m, 24 H), 3.69−3.50 (m, 18 H); ^{13}C NMR (D_2O, 125 MHz) δ 164.12, 158.69, 102.79, 102.66, 102.42, 83.28, 82.28, 82.25, 82.04, 82.02, 73.85, 73.82, 73.75, 73.54, 72.90, 72.85, 72.69, 72.61, 71.41, 61.39, 61.20, 60.97, 42.99. Anal. Calcd for $C_{46}H_{80}N_6O_{39}\cdot 5H_2O$: C, 38.12; H, 6.40; N, 5.80. Found: C, 37.93; H, 6.11; N, 5.71.

The bicarbonate salt of **9** was dissolved in water, loaded on a column of Dowex 1 × 2−100 (chloride form), and eluted with water. Fractions containing **9** were combined, concentrated under reduced pressure, and lyophilized, providing the chloride salt of **9** as a clear solid. Data for **9** (Cl^- salt): ^{1}H NMR (D_2O, 500 MHz) δ 5.13−5.03 (m, 7 H), 4.04−3.78 (m, 24 H), 3.70−3.50 (m, 18 H). Anal. Calcd for $C_{44}H_{88}Cl_2N_6O_{38}\cdot 5H_2O$: C, 38.29; H, 6.43; Cl, 5.14; N, 6.09. Found: C, 38.17; H, 6.37; Cl, 5.22; N, 6.03.

Binding Experiments. A solution of *m*-cresol purple (ca. 60 μM) and the desired amino acid (2−3 mM) was titrated with a solution of the cyclodextrin (ca. 8 mM) that also contained *m*-cresol purple and the amino acid at the same concentrations present in the cuvette. Absorbance at 432 nm was used for quantitation. Duplicate experiments were performed, and uncertainties are based on the error in the slopes of the resulting linear plots using least-squares analysis (95% confidence interval). In all titrations, a distinct isosbestic point was observed, consistent with 1:1 binding between the dye and the cyclodextrins examined. The pH's of solutions were checked before and after experiments and were found to change less than 0.01 during the course of an experiment; pH changes of this magnitude were found to produce changes in dye absorbance on the order of 0.002, which is within the instrumental error for individual measurements. Dye absorbance was found to be unaffected by the presence of tyrosine or phosphotyrosine at the concentrations used in competitive experiments.[21]

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