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DERIVATIZED CYCLODEXTRINS AS PEPTIDOMIMETICS: INFLUENCE ON NEURITE GROWTH

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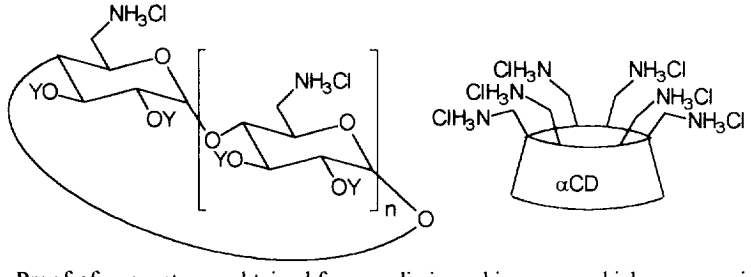
Abstract: Per-6-bromo-6-deoxy-cyclodextrins may be functionalized to yield amine-derivatized cyclodextrin mimics of glycosaminoglycan sulfate binding domains of peptides, which inhibit neurite growth in vitro.

The cyclodextrins, αCD, βCD, and γCD are naturally occurring cyclomaltooligosaccharides containing 6, 7, and 8 α-D-glucopyranosyl rings respectively.[1] The use of synthetic CD derivatives as molecular scaffolds is of some interest since the primary and secondary hydroxyl groups may be used as points of functionalization (Scheme 1).[2] By using an efficient synthesis of the per-6-deoxy-6-bromo-derivatives of αCD, βCD, per-2,3-dimethyl-2,3-dideoxy-βCD (MeβCD), and γCD,[3] seven homogeneous polyamino-substituted CD derivatives (PACDs) were synthesized as peptidomimetics for this initial study. These PACDs inhibit Nerve Growth Factor (NGF) mediated neurite growth. The mechanism of inhibition is likely associated with the ability of PACD derivatives to bind to glycosaminoglycan (GAG) sulfates. Thus PACDs may mimic the GAG sulfate-binding domains of peptides and inhibit recognition and binding of proteoglycans.

Scheme 1



αACD n = 5; Y = H
βACD n = 6; Y = H
γACD n = 7; Y = H
MeβACD n = 6; Y = Me

Proof of concept was obtained from preliminary bioassays, which were carried out using a PACD obtained by the reaction of per-6-tosyl-6-deoxy-βCD with excess tetraethylenepentamine.[4] The bioassays performed measured either (1) inhibition or (2) growth of neurites, using dorsal root ganglia (DRG) neurons.

(1) In the presence of NGF, DRG neurons extend processes on a poly-D-lysine (PDL) coated surface. When the PACD (0.5 mg/mL) was added to the growth medium, the number of neurite bearing cells was reduced significantly to 60% of control. The parent βCD and free amine had no significant effect on neurite growth under these conditions.

(2) When the surface of the incubation well was coated with the immobilized PACD in place of PDL, neurite growth was also observed. Furthermore, separate addition of two GAG sulfates, heparin and chondroitin sulfate (CS) (at 10 μg/mL), to the growth medium gave markedly different results. Addition of heparin significantly inhibited growth on the PACD, whereas CS had no effect.

These results indicated the potential for a PACD not only to inhibit neurite growth, but also to demonstrate selectivity between different GAG sulfates. The bioassays were used further and are described below. However, an improved synthesis was required and developed.

Synthesis of simple per-6-amino-CD derivatives is facile using a procedure based upon bromination of CD using the Vilsmeier-Haack reagent $[(CH_3)_2NCHBr]^+Br^-$.[3] This method is ideally suited to CD synthesis since (a) it avoids significant purification problems and (b) the isolated yields are high. Literature procedures for secondary face functionalization generally require prior protection of the primary face and ultimately a deprotection step.[5] However, the secondary face of per-6-azido-6-deoxyCD may be efficiently directly methylated without additional protective group strategies. Facile conversion to the per-6-amino-6-deoxyCD (ACD) derivatives yields hexa-, hepta-, and octa-amines, which are water soluble in their protonated forms. These ACDs may be further extended by reaction with aldehydes then reduction to provide pendant arms containing diamines or polyamines. Alternatively, direct reaction of amines with per-6-deoxy-6-bromo-CD allows synthesis of a variety of PACDs. The synthesis and activity of four ACDs and three per-6-*N,N*-dimethylaminoethylamino-6-deoxy-CD (DACD) derivatives is reported to provide prototypical data for homogeneous, water-soluble PACDs.

Heparan sulfate proteoglycans have neurite growth promoting activity associated with the GAG sulfate side chains.[6] Conversely, evidence indicates that chondroitin sulfate (CS) proteoglycans have growth inhibitory effects during development and regeneration of the nervous system.[6] The GAG-binding domains of several adhesion proteins show epitopes rich in basic amino acid residues, thus providing cationic clefts for recognition of specific sequences of the anionic GAG sulfate. For example, the heparin-binding site of Antithrombin III (AIII) contains 6 basic residues (Fig. 1).[7] The CD scaffold when regiospecifically functionalized with amine pendant groups may provide PACDs which mimic such GAG sulfate-binding sites.

The observations of (i) GAG sulfate involvement in neurite growth and (ii) neurite cell adhesion and growth in the presence of basement membrane amine templates, such as poly-D-lysine, clearly points to the potential of water-soluble amine compounds in binding of GAG sulfates and modification of neurite growth and adhesion. A large number of choices for such amine compounds exist.[8] For example, the use of phenothiazine dyes as heparin stains suggests that thiazine derivatives might provide a useful family of such amines.[9] However, we have chosen to explore the use of PACDs as a family of amines with GAG sulfate binding properties. This selection is based on features including the lack of toxicity of PACDs and the water solubility of PACDs. Moreover, variations in (a) CD scaffold, (b) amine pendant group, (c) regiochemistry, and (d) secondary face functionalization, may be used to induce GAG sulfate selectivity.

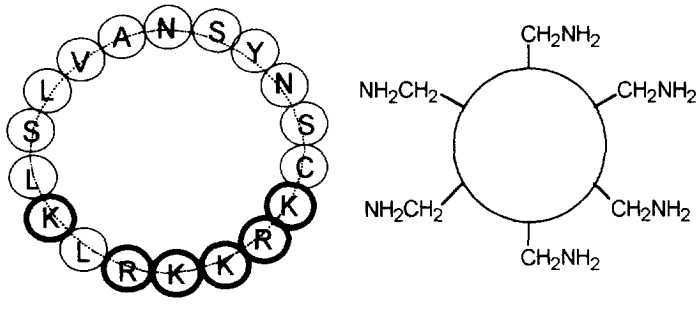


Fig. 1. Helical representation of heparin-binding site of AIII[ref 7] and αACD

The three per-6-amino-6-deoxy-CD derivatives (αACD, βACD, and γACD) were synthesized from the corresponding per-6-bromo-6-deoxy-CD, via per-6-azido-6-deoxy-CD, according to our improved procedure.[3,10] Although methods have been developed for functionalization of the secondary face of βCD, the procedure may be significantly shortened and improved by direct methylation of per-6-azido-6-deoxy-CD. The per-2,3-methoxy-per-6-amino-6-deoxy-CD (MeβACD) was prepared by addition of methyl iodide (40 equiv) to a solution of per-6-azido-6-deoxy-βCD and NaH (30 equiv) in DMF. The reaction mixture was stirred for 24 h at rt, methanol added and the mixture concentrated under vacuum. Ice-water was added and

the resulting precipitate collected and dried. MeβACD was obtained as the per-6-ammonium chloride salt by reduction of the azide with triphenylphosphine in dioxane followed by workup with ammonium hydroxide solution and isolation as previously described.[3] The product was obtained as a white solid in 92% isolated yield, calculated from the azide. These synthetic strategies yield CD derivatives, homogeneous by ^{1}H and ^{13}C NMR spectroscopy (Fig. 2) without recourse to chromatography.[11]

Each per-6-*N,N*-dimethylaminoethylamino-6-deoxyCD (αDACD, βDACD, γDACD) was synthesized from the corresponding per-6-bromo-6-deoxy-CD directly by reaction at 80 °C overnight in an excess of *N,N*-dimethylaminoethylamine as solvent. Excess diamine was removed under vacuum and the resulting product triturated with acetone, filtered and washed exhaustively with acetone. The resulting products, as white powders contained traces of diamine, presumably as the HBr salt. Careful acidification to pH approx. 3.5 with HCl and concentration gave a white powder, which was washed thoroughly with refluxing EtOH to remove all traces of diamine. The highly hygroscopic products were obtained as the HCl salts, in 80-90% isolated yield, calculated from the bromide, and were homogeneous by ^{1}H and ^{13}C NMR.[11] DACD derivatives were also amenable to analysis by electrospray (ES) mass spectroscopy (MS) as their aqueous solutions. Distributions of ions were observed, the most abundant being quadrupely protonated for βDACD and γDACD and triply protonated for αACD.[11]

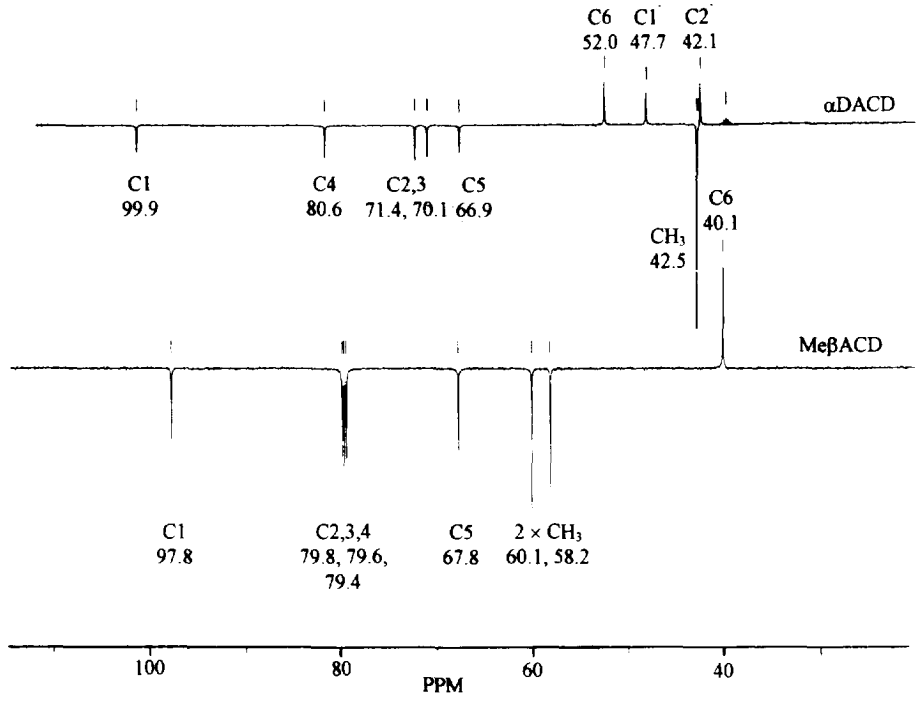


Fig. 2 100 MHz JMOD ^{13}C NMR spectra for αDACD (in DMSOd_6) and MeβACD (in D_2O).

The ability of PACD to bind to GAG sulfate in vitro can easily be demonstrated using the cationic phenothiazine dye Azure A. On colorimetric titration of Azure A with either CS or the GAG sulfate mimic, sodium polyvinylsulfonate (PVS), similar binding curves are obtained. Addition of PACD to the Azure A solution prior to titration results in binding curves displaced significantly to the right (Fig. 3), indicating competition between Azure A and PACD for binding sites on the GAG sulfate or mimic. Unfunctionalized CD has no effect. In a series of control titrations, a polyamine PAMAM Starburst™ Dendrimer was used in

place of PACD, at varying concentrations. The EC_{50}, obtained using the nonlinear regression method of Lew and Angus, was linearly dependent on [PAMAM] (Fig. 4).[12] Thus the relative efficiencies of binding for PACD were estimated from calculated EC_{50} values at given PACD concentration (Table 1). Binding of PACD to PVS may be to some extent determined by the number of amine/ammonium sites on the PACD, with an observed ordering of αACD≤βACD<γACD. However, binding to CS does not show this trend: βACD shows relative selectivity for binding CS over PVS. Furthermore, the methylated secondary face in MeβACD strongly influences the nature of binding to the GAG sulfate, as shown by the titration curve (Fig. 3).

Two separate bioassays of synthetic PACDs were performed: (1) inhibition of neurite growth, as outlined above, on a PDL-coated surface or "basement membrane", using DRG neurons and (2) support of DRG neurite growth on a PACD-coated surface. These assays would be anticipated to be complementary — a good inhibitor of growth on a PDL surface is likely itself to provide a good surface for growth.

(1) Bioassay of neurite growth used dissociated cells enriched for sensory DRG neurons from ED8 chick.[13,14] Neurite growth on plastic wells coated with PDL was assayed by counting neurite bearing cells using an optical microscope, in separate quadruplicate experiments. In the presence of NGF, DRG neurons extend processes on a PDL surface. CDs and PACDs were added in solution and incubated overnight. Each parent CD had no significant effect on neurite growth, whereas four of the five PACDs inhibited growth at 500 μg/mL (Table 2)

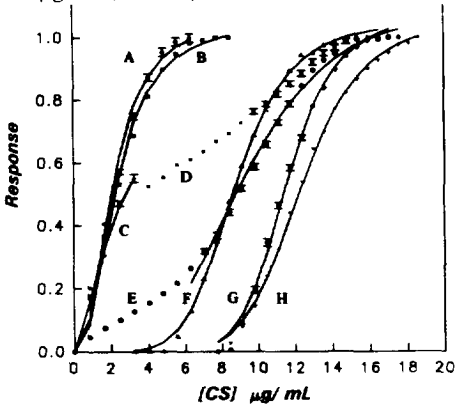


Fig. 3. Concentration-response curves for titration of Azure A by CS in the absence or presence of PACD: (A) No drug (B) MeβCD (C) + (D) MeβACD (E) αACD (F) βDACD (G) γ ACD (H) βACD. Response is δ (A(515 nm)\-A(565 nm)), normalized to a maximal response of 1.0. See Table 1 for EC_{50} values derived from these data. Curve fits from ref 12.

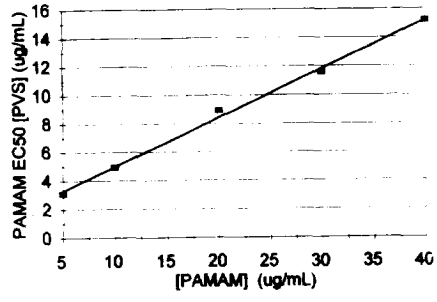


Fig. 4. EC_{50} values obtained for addition of PAMAM to PVS/Azure A colorimetric titration (expressed in units of μg/ml of PVS) plotted against concentration of PAMAM Starburst dendrimer.

(2) The ability of PACDs to act as substrates to support neurite growth was examined, again using ED8 chick DRG neurons in the presence of NGF. In this case, the plastic wells of Terasaki plates were coated overnight at 37 °C, with CD, PACD or with PDL, and flushed prior to seeding. Again parent CDs had no significant effect on neurite growth. However, PACDs which showed inhibitory properties in bioassay (1), as would be expected, provided good substrates for neurite growth.

PDL provides a good substrate for neurite growth. Several PACDs inhibit neurite growth to PDL and, furthermore, mimic PDL in providing an alternative substrate for neurite growth. Thus these PACDs are peptidomimetic, providing GAG sulfate-binding sites and acting as substitutes for in vivo basement membranes. The potential therapeutic utility of these PACDs is based upon binding selectivity between different GAG sulfates, as indicated in the initial experiments with the tetraethylenepentamine-βCD derivative.

Furthermore, PACDs showed no toxicity, whereas addition of PAMAM polyamines in the neurite growth assays led to cell death.

Table 1. Azure A colorimetric binding assay data

	conc'n mg/mL	EC_{50}(PVS), ng/mL	EC_{50}(CS), ng/ml
αACD	10	8130 ± 39	9550 ± 70
βACD	10	8130 ± 24	12300 ± 37
γACD	10	9120 ± 38	11500 ± 50
MeβACD	10	6310 ± 20	2240 ± 67
βDACD	10	4680 ± 48	8710 ± 32
none	-	2190 ± 100	2190 ± 190

At 22 °C in TRIS.HCl (50 mM, pH 7.8), from triplicate runs, measured by fitting of titration curves. CS obtained from Sigma.

Table 2. Neurite growth in the presence and absence of PACD on poly-D-lysine and PACD surfaces.

adjuvant	% neurite bearing[a,b]	% neurite bearing[a,c]
αACD	72.0 ± 4.0*	360 ± 80*
βACD	63.0 ± 6.0*	200 ± 40*
γACD	52.0 ± 7.0*	310 ± 80*
MeβACD	83.0 ± 6.9*	110 ± 10
αDACD	104 ± 11	77 ± 18
βDACD	91.6 ± 7.0	85 ± 9
γDACD	101 ± 10	92 ± 11
none	100 ± 2.8	100 ± 11
PDL	-	640 ± 110*

(a) Percentage neurite bearing relative to control. (b) At 0.5 mg/mL PACD on PDL surface. (c) On surfaces coated with PACD or PDL (10 mg/mL). * Indicates significantly different from control; errors are ± SEM.

The preliminary data from the colorimetric dye binding assay is suggestive of the potential for GAG sulfate-binding selectivity, in the differences observed between PACD binding to PVS and to CS. Simplistically, an increase in the number of amino nitrogens per molecule would increase the concentration of basic sites and hence the concentration of positive charge. This may well control binding of the simple ACDs to PVS. However, the same trend is not observed for binding to CS. Moreover, increasing the number of nitrogen sites in DACD actually leads to poorer binding to PVS and CS and more remarkably, a complete loss of biological activity towards neurite growth. It is possible that the steric bulk of the less basic, tertiary amine groups hinders binding to the annulus of secondary ammonium sites. Methylation of the secondary face in Me βACD leads to diminished inhibition of neurite growth, but more demonstrably a significant change in binding characteristics to PVS in the colorimetric assay, proving that secondary face modification can have a significant influence on binding properties.

To the best of our knowledge, this is the first study using homogeneous cyclodextrin derivatives as peptidomimetics for GAG sulfate-binding sites.[15] These preliminary results suggest PACDs may be developed with good binding and selectivity between the various GAG sulfates.

1. PACDs are observed to inhibit neurite growth to PDL.
2. Immobilized PACDs are observed to provide a substrate for neurite growth.
3. Differences observed in binding of CS and PVS in the colorimetric assay imply the potential for binding selectivity between various GAG sulfates.
4. Bioassay and binding assay data do not correlate simply with the number of PACD nitrogen sites.
5. Modification of the secondary face of the PACD may profoundly affect binding and activity.
6. The simple cationic-dye colorimetric assay has potential for measuring GAG sulfate binding selectivity.

Given the ease of synthesis of homogeneous PACD derivatives and the possibility of extended functionalization at the primary and secondary faces, the biological activity and binding data on this first generation of homogeneous PACD derivatives clearly indicates great potential for future use of PACD derivatives as peptidomimetics.

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10. Complete removal of Ph_3PO by-products from CD derivatives requires exhaustive extraction procedures, thus azide reduction by H_2/PtO_2/1 atm may be employed successfully in place of the reduction procedure employing Ph_3P/NH_2OH.
11. ^{13}C NMR data. βDACD (DMSOd_6) C1 δ 99.7 ppm; C4 δ 79.9; C2,3 δ 71.0, 70.5; C5 δ 66.7; C6 δ 52.0; C1' δ 47.7; C2' δ 42.1; CH_3 43.5ppm. γDACD (DMSOd_6) C1 δ 101.0 ppm; C4 δ 80.7; C2,3 δ 73.1, 72.6; C5 δ 70.7; C6 δ 56.6; C1' δ 49.0; C2' δ 44.3; CH_3 δ 43.7 ppm. ES-MS data: αDACD (ES+): $M+H^+$ (3%) 1393; $M+2H^+$ (25%) 697; $M+3H^+$ (100%) 465; $M+4H^+$ (65%) 349; $M+6H^+$ (45%) 233. βDACD (ES+): $M+H^+$ (<1%) 1625; $M+2H^+$ (20%) 813; $M+3H^+$ (40%) 542; $M+4H^+$ (100%) 407; $M+7^+$ (55%) 233. γDACD (ES^+): $M+2H^+$ (5%) 929; $M+3H^+$ (18%) 620; $M+4H^+$ (100%) 465; $M+8H^+$ (50%) 233.
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