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Synthesis and Per-Functionalization of Heptakis(6-O-carboxymethyl-2,3-di-O-methyl)cyclomaltoheptaose

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Abstract. *The synthesis and isolation of heptakis(6-O- carboxymethyl-2,3-di-O-methyl)cyclomalto-heptaose is described. The method provides a homogeneous product that can be used as a scaffold for the covalent attachment of a defined number of peptides, lipids, or other compounds in preparing supramolecular assemblies. In this report we describe the addition of tryptophan methyl ester and a 7-residue peptide, H_2N-WSLSLSL-$CONH_2$, to the percarboxymethylated cyclodextrin carrier.*

The attachment of peptides to a template to encourage the formation of a tertiary fold is a promising strategy in protein *de novo* design.[1] In an extension of our work on porphyrin-tethered four-helix ion channels,[1c] we sought a class of compounds that would act as suitable platforms of higher order peptide assemblies than tetramers. A suitable template should contain functional groups that directly or through simple modifications can be ligated to the peptides. In addition, we specifically looked for symmetrical and relatively rigid cyclic compounds that would promote the formation of well-defined three-dimensional protein structures containing a central ion-conducting pore. Cyclodextrins (CDs), a family of homologous cyclic oligosaccharides, meet our required criteria. Several cyclodextrin structures have been determined by X-ray crystallography. Furthermore, they contain hydroxyl groups that could be derivatized to suit our purposes. Attaching one peptide per sugar residue, α-, β -, and γ - CDs, which consist of six, seven, and eight (1-4) - linked α-glucose residues, respectively, should allow the preparation of hexa-, hepta-, and octameric peptide assemblies.

CDs have previously been derivatized with lipophilic groups to create ion channels[2] and other hydrophobic assemblies;[3] however, their purities have not been rigorously established. Numerous procedures exist for per-functionalizing CD derivatives,[4] but we have found that few provide a homogeneous product as assessed by HPLC. Further, purification of the final product is difficult due to the presence of incompletely functionalized side products with very similar properties.[5,7] The problems of identification and separation of side products are, however, diminishing with improved spectroscopic and chromatographic techniques.

We chose to protect the secondary hydroxyl groups of β-cyclodextrin as methyl ethers.[6] (2,3-Di-*O*-methyl)cyclomaltoheptaose **1** has good solubility properties in water as well as in organic solvents.[7] By converting the primary hydroxyl groups of this derivative to carboxylic acids, the peptides could be attached via their N-termini in an amide coupling reaction. Rather than oxidizing the primary hydroxyl groups directly, the carboxylic acid groups were incorporated by alkylation of CD **1** with *tert*-butyl bromoacetate to yield **2**, followed by ester hydrolysis. In this way, an additional methylene group was introduced to allow a limited

degree of flexibility between the carrier and the peptide. Removal of the *tert*-butyl groups of ester **2** under acidic conditions furnished the desired product **3** as a mixture of penta-, hexa-, and heptacarboxymethylated material, as determined by mass spectrometry. At this stage (or before ester deprotection) material unrelated to the cyclodextrins could not be removed by purification by column chromatography or by HPLC due to their similar retention times. This problem was overcome by converting the cyclodextrin carboxylic acids to their corresponding phenacyl ester derivatives **4**.[8] After flash column chromatography, followed by saponification of phenacyl esters **4**, homogeneous heptakis(6-*O*-carboxymethyl-2,3-di-*O*-methyl)cyclomaltoheptaose **3C** was isolated by HPLC purification.

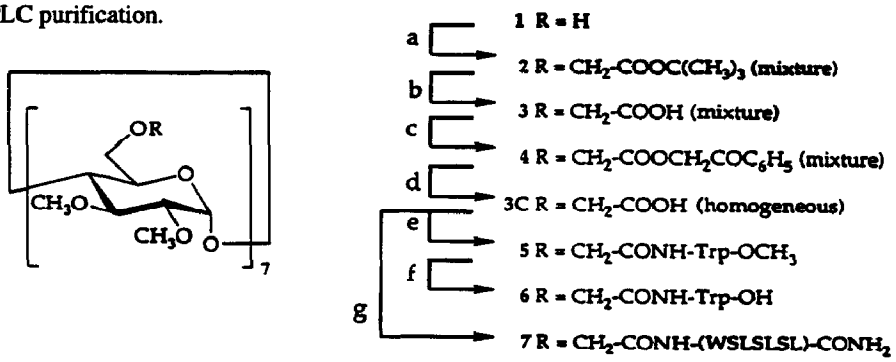

Fig. 1.[11] **a.) NaH, $BrCH_2COOC(CH_3)_3$, DMF; b.) TFA, CH_2Cl_2; c.) KF, $BrCH_2COC_6H_5$, DMF; d.) 1. LiOH, MeOH, H_2O, 2. HPLC; e.) H_2N-Trp-$COOCH_3$, HBTU, NMM, DMF; f.) LiOH, MeOH, H_2O; g.) H_2N-(WSLSLSL)-$CONH_2$, HBTU, NMM, DMSO.**

Next, with the aid of HBTU,[9] tryptophan methyl ester was coupled to heptacarboxylic acid **3C** to provide **5**. The product was virtually homogeneous, containing less than 5% of the hexasubstituted derivative, as determined by HPLC analysis. The product was saponified to give **6** and identified by FAB mass spectrometry. The same procedure was used in the addition of the β–sheet-forming 7-residue peptide H_2N-(WSLSLSL)-$CONH_2$ to carboxylic acid **3C**. The hepta-functionalized product **7** was obtained as the major product, isolated by HPLC purification, and identified by laser desorption mass spectroscopy. To our knowledge, compounds **5** and **7** represent the first examples of amino acid and peptide per-functionalized cyclodextrin derivatives, respectively.[10] The addition of α-helix-forming peptides is in progress.

In addition to its utility as a synthetic intermediate in the preparation of template-assisted proteins, per-carboxymethylated β- cyclodextrin **3C** has potential use in other areas of cyclodextrin chemistry, e. g. amphiphilic CDs, CD inclusion complexes, and CD receptor or enzyme model systems.

Experimental Procedure.[11] **3:** A solution of **1** (1.0 g, 0.75 mmol) in 30. 0 mL of DMF was rapidly added to NaH (0.63 g, 15.8 mmol) at 0 °C under inert atmosphere. After completion of addition the ice-bath was removed and the reaction mixture stirred at 21 °C. After 30 min of reaction time the gas evolution had subsided and the reaction mixture was cooled to 0 °C. *tert*-Butyl bromoacetate (4.24 mL, 26.3 mmol)) was rapidly added and the reaction mixture stirred at 21 °C. After an additional 20 h the reaction mixture was quenched with MeOH and concentrated *in vacuo*. The residue was partitioned between CH_2Cl_2 and water (200 mL each).

The organic layer was washed with an additional 200 mL of water, dried (Na_2SO_4), concentrated *in vacuo*, and purified by column chromatography (CH_2Cl_2, followed by 4% MeOH in CH_2Cl_2) to give 0.75 g (47% yield) of a thick amorphous off-white solid.

Ester deprotection. *tert*-Butyl ester **2** (0.385 g, 0.18 mmol) was dissolved in CH_2Cl_2 (10 mL) and TFA (10 mL) was added. After 2 h 15 min of reaction time at 21 °C the reaction mixture was concentrated *in vacuo* and the resulting oil co-evaporated with several portions of CH_2Cl_2 and MeOH to remove residual TFA.

Phenacylation. KF (0.73 g, 12.6 mmol) and phenacyl bromide (2.50 g, 12.6 mmol) were added to a 0.04 M solution of carboxylic acid **3** (≤ 0.18 mmol) in DMF. The reaction mixture was placed in a preheated oil bath at 55 °C and stirred under N_2. After 20 h the reaction mixture was concentrated in vacuo and the residue partitioned between diethyl ether and water (100 mL each). The aqueous layer was extracted with a portion of diethyl ether (25 mL), and the combined organic layers were washed with water (75 mL), dried (Na_2SO_4), and concentrated *in vacuo* to a yellow oil which was purified by chromatography (CH_2Cl_2, 1% MeOH, 2% MeOH, 3% MeOH, 4% MeOH, successively, 500 mL each) to provide an off-white amorphous solid (275 mg, 60% yield).

Saponification. To a solution of phenacyl ester **4** (275 mg, 0.107 mmol) in MeOH (12.0 mL) was added water (3.0 mL), followed by pulverized LiOH (315 mg, 7.51 mmol) at 21 °C. After 20 h Dowex-H^+ resin was added to the reaction mixture until pH 4. After the resin had been removed by filtration the filtrate was concentrated *in vacuo*, redissolved in MeOH, and passed through a column of LH-20 resin (4.0 x 10.5 cm) in MeOH. The product, consisting of a mixture of penta-, hexa-, and heptacarboxymethylated β-cyclodextrins, was eluted with MeOH and isolated as a colorless glass after concentration under reduced pressure. The product was dissolved in water and lyophilized to provide a white fluffy powder (160 mg, 86% yield). The penta- (**3A**), hexa- (**3B**), and heptacarboxymethylated (**3C**) cyclodextrin derivatives were then separated by HPLC chromatography on a Vydac C_{18} reversed phase support employing a gradient of 32-42 % solvent B (0.25%/min), followed by 42 % B, and obtained in 13.7% (21. 9 mg), 26.9% (43.0 mg), and 18.1% yield (29.0 mg), respectively (59% mass recovery):

3A: (with unknown distribution of positional isomers): Positive ion FAB-MS *m/z* 1621.6, 1643.6, and 1659.5 for [M + H], [M + Na] and [M + K], respectively.

3B: Positive ion FAB-MS *m/z* 1679.5, 1701.5, and 1717.5 for [M + H], [M + Na] and [M + K], respectively; ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.36 (br s, 6/7 H), 5.29 (d, 1/7 H), 4.26-4.12 (m, 2H), 3.99-3.68 (m, 5H), 3.60 (s, 3H), 3.51 (s, 3H), 3.37 (dd, 2H).

3C: Positive ion FAB-MS *m/z* 1737.4, 1759.5, and 1775.4 for [M + H], [M + Na], and [M + K], respectively; ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.36 (br s, 1 H), 4.26-4.12 (m, 2 H), 4.02-3.90 (m, 2 H), 3.90-3.78 (m, 2H), 3.72 (t, 1H), 3.60 (s, 3 H), 3.51 (s, 3 H), 3.37 (br d, 2 H).

Addition of amino acid esters and peptides to cyclodextrin **3C**.

6: N-Methylmorpholine (73 mL, diluted 1:100 with DMF, 7.17 mmole), HBTU (1.81 mg, 4.78 mmole), and tryptophan methyl ester (1.22 mg, 4.78 mmole) were added in sequence to cyclodextrin **3C** (0.593 mg, 0.341 mmole) to a total of 198 mL of DMF in an Eppendorf tube, equipped with a micro magnetic stir bar. The solution was vortexed and stirred at 21 °C. After 19 h of reaction time the reaction was concentrated *in vacuo*, and treated with LiOH in methanol and water for 24 h, as described for the saponification of phenacyl ester **4**. An aliquot was purified by HPLC chromatography on a Vydac C_{18} reversed phase support employing a gradient of 62-72% B (1%/min): Positive ion FAB-MS *m/z* 3040.59 and 3062.16 for [M + H] and [M + Na], respectively.

7: The synthesis followed the same protocol as described for **6** except DMSO was used as solvent. The product was purified by HPLC on a polystyrene reversed phase support (Hamilton, PRP-1), and eluted with a gradient of 50-100% solvent C (2%/min), followed by 100% solvent C. The major component was isolated: Laser desorption-MS *m/z* 7,365 ± 63 [M+ K], 14,718 ± 31 [2M + K][13].

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11. General. Unless otherwise noted, reagents and solvents were purchased from commercial suppliers and used without further purification. Benzotriazole tetramethyluronium hexafluorophosphate (HBTU) was purchased from Advanced ChemTech, and Sure/Seal™ DMF from Aldrich. The HPLC solvent gradient was made by mixing solvent A (0.1 % trifluoroacetic acid (TFA) in water) with solvent B (0.1% TFA in 100% CH_3CN), or C (0.1% TFA in trifluoroethanol (TFE)/iPrOH 1/1 : H_2O 9:1), as indicated in the text. Compound **1** [6a] was purified as described in the preparation of (2,3-di-*O*-methyl)cyclomaltohexaose[6b]. H_2N-WSLSLSL-$CONH_2$ (W = Trp, S = Ser, L = Leu) was prepared manually by a standard stepwise solid-phase protocol employing BOC-chemistry, [12] and purified by HPLC chromatography on a reversed phase support (Hamilton PRP-1, preparative column). Column chromatography was performed according to the method of Still, Kahn, and Mitra (*J. Org. Chem.* **1978**, *43*, 2923-26) using Silica Gel 60 (230-400 mesh).

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13. Obtaining precise mass spectra for these hydrophobic peptides was not possible due to the broadness of the peaks. The numbers represent the mean and standard deviation of three runs.

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