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Synthesis of bifunctional peptide derivatives based on a β-cyclodextrin core with drug delivery potential

Rachel J. White, Paul G. Plieger, David R. K. Harding *

Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand

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ABSTRACT

A selective, versatile, robust methodology for bifunctionalization of β-cyclodextrin is achieved allowing the attachment of peptides in varying C- and/or N-terminal combinations on resin using Fmoc SPPS. Two linkers are attached to cyclodextrin enabling selective binding to the resin (or a peptide attached to the resin). Continuation of peptide growth and/or cleavage from the resin follows, thus various combinations of peptide–cyclodextrin species are achieved. A model peptide (Gly-Ala) is used in this study to illustrate the potential of this system for attaching one or more bioactive peptides for drug transport and release purposes.

Comprised of six, seven or eight α-1,4-linked D-(+)-glucopyranose units, the cyclodextrins (α, β, and γ, respectively) are cyclic oligosaccharides with an overall shape reminiscent of a truncated cone. The narrow rim of this cone bears seven primary hydroxy groups and the wider rim bears 14 secondary hydroxy groups.[1,2] Selective modification of one or more hydroxy groups has, in the past, allowed for the grafting of various molecules with new recognition functions, yet bifunctionalization still remains a challenge.[3,4] Despite the difficulties associated with bifunctionalization, a number of groups have recently made substantial progress in selective bifunctionalization via the utilization of benzylation for protection.[3–5]

Examples of peptide-functionalized cyclodextrins are numerous, so it is somewhat surprising that bifunctionalized cyclodextrins bearing two different peptidyl units have yet to be reported. Our pursuit of new drug carriers incorporating the recognition signalling capabilities of bioactive molecules, for example, peptides, has led us to develop a versatile route to cyclodextrin functionalization. There are a large number of cellular receptors for which peptides appear to be the most versatile targeting agents. There are many publications on the grafting of single amino acids onto cyclodextrins, but little has been reported on multi-peptidyl-cyclodextrin compounds.[6–8] Of the few known examples, only a small number of these employ solid-phase peptide synthesis techniques for grafting peptides onto cyclodextrin off resin, and these result in an all or no functionalization of the β-cyclodextrin (β-CD) hydroxy groups.[7,9,10] We now report, for the first time, bifunctionalization of β-CD with a series of model peptides using solid-phase peptide synthesis. This synthetic approach allows the functionalization of cyclodextrin to one or two peptides in various combinations; for example, the attachment of β-CD to the C- and/or N-terminus of peptides as well as the functionalization of β-CD with differing peptidyl chains (Scheme 1).

Scheme 2 outlines a six-step process to bifunctionalize cyclodextrin **1** (an example of step (i) in Scheme 1) to allow for its use in Fmoc SPPS for the attachment of one or more peptides. The end product **7**, containing both amino and carboxylic functional groups, is set up for peptide synthesis. All compounds were characterized by high- or low-resolution mass spectrometry and/or NMR studies. Selective mono-derivatization of the primary hydroxy group of β-CD (**1**) was reported by Byun et al.[11]

This reaction allows the selective replacement of one primary hydroxy group by a tosyl group. Thus, treatment of β-CD (**1**) with tosyl chloride and sodium hydroxide in water at 0 °C afforded **2** (mono-6^A-(4-methylbenzenesulfonyl)-β-cyclodextrin).[11] Next, **2** was treated with 35% aqueous ammonia, refreshed daily, for seven days. Recrystallization from water gave a 90% yield of **3** (mono-6^A-amino-β-cyclodextrin) which was found to be a stable, easy to handle compound.[2] Protecting the mono-6^A-amino-β-cyclodextrin (**3**) with a Cbz group using Cbz-OSu and sodium hydrogen carbonate in dioxane/water at room temperature afforded mono-6^A-carbobenzyloxyamino-β-cyclodextrin, **4**.[12] Scale-up to 5 g of purified material was readily achieved by recrystallization from water. RP-HPLC purification using a stepwise methanol/water/TFA gradient allowed for confirmation via complete NMR characterization (see Supplementary data). Succinylation of **4**[13] resulted in the isolation of mono-6^A-carbobenzyloxyamino-succinyl-β-cyclodextrin (**5**) in a reasonable yield (40%). Dissolution in water followed by filtration of any insolubles gave the product in the filtrate suitable for use in the next step. A mixture of regioisomers of **5** was used for the remainder of the experiment. The ester linkage is stable under all

* Corresponding author. Tel.: +64 63569099; fax: +64 63505682.
E-mail address: D.R.Harding@massey.ac.nz (D.R.K. Harding).

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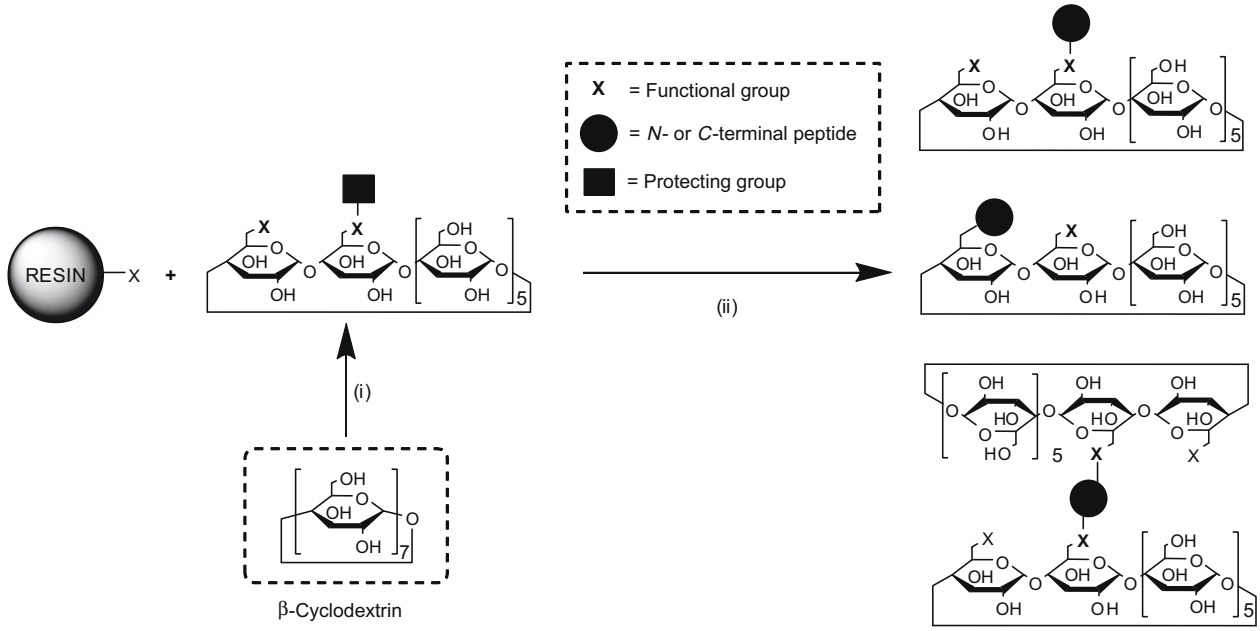
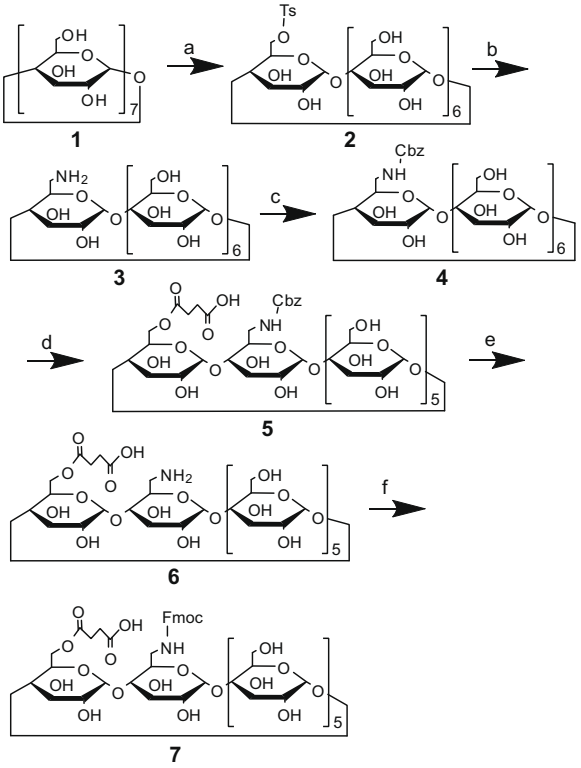


Scheme 1. Derivatization of β-cyclodextrin using solid-phase peptide synthesis. (i) Synthesis of bifunctional β-cyclodextrin to enable selective peptidyl attachment in SPPS. (ii) Attachment of peptides to β-cyclodextrin using SPPS to synthesise a number of C- and/or N-terminus-derivatized products.



Scheme 2. Bifunctional synthesis of β-CD. Reagents and conditions: (a) tosyl chloride, NaOH, H_2O, 0–5 °C, 5 h,[11] 38%; (b) 35% aq NH_3, 7 days,[2] 90%; (c) Cbz-OSu, $NaHCO_3$, dioxane/water, RT, overnight, 51%; (d) succinic anhydride, py, rt overnight, 40%; (e) H_2, Pd/C, DMF, rt, 90%; (f) Fmoc-OSu, $NaHCO_3$, dioxane/water, rt, overnight, 40%.

Fmoc SPPS reaction conditions (see Supplementary data). Isomeric purification was achieved by RP-HPLC using a stepwise methanol/water/TFA gradient followed by an acetonitrile/water/TFA gradient. This afforded a 1:2:2 ratio of the (AB/AG):(AC/AF):(AD/AE) regioisomers with the elution pattern of (AD/AE):(AC/AF):(AB/AG) as confirmed by ESI-MS studies as per Sforza et al.[14] Currently, it is not possible to differentiate between the (AB/AG), (AC/AF) and (AD/AE) pairs using mass spectrometry.

In the presence of a twofold excess of sodium chloride, un- and disubstituted β-CDs randomly break at the acetal junctions, giving rise to different polyglucose sodium-containing fragments. Therefore, the relative abundances of the di-, mono- and unsubstituted fragments generated from the three different regioisomeric pairs (AB/AG), (AC/AF) and (AD/AE) by ESI-MS can be compared to those determined by statistical analysis (see Supplementary data).[14] Figure 1 shows the identification of the three regioisomers achieved by ESI-MS studies showing three glucose units and one or no dehydrated groups (*m/z* 642 and 742, respectively). The ratio values for regioisomer peaks were found to be in agreement with literature values[14] and consistent with the expected pattern of fragmentation (inset, Fig. 1). The 642 peak is higher for the AD isomer, the 742 peak is higher for the AB isomer, and the two peaks are of similar intensity for the AC isomer (left, Fig. 1). Hydrogenolysis of the Cbz-protecting group gave mono-6^A-amino-succinyl-β-cyclodextrin, **6** as a regioisomeric mixture. Fmoc protection[12] of **6** produced mono-6^A-fluorenylmethyloxycarbonylamino-succinyl-β-cyclodextrin **7**, as a regioisomeric mixture which could be purified via recrystallization from water. For complete NMR analysis, RP-HPLC was first performed using a stepwise methanol/water/TFA gradient. This gave the elution pattern of (AC/AF):(AD/AE):(AB/AG) isomers as confirmed by ESI-MS studies (see Supplementary data). Compound **7** has been shown to be stable under all standard Fmoc SPPS reaction conditions (see Supplementary data) with the expected loss of the Fmoc group using 20% piperidine in DMF (2 × 10 min). This robust method enables the attachment of one peptide to **7** using SPPS by the selective removal of the Fmoc-protecting group (Scheme 3).

Although this work does not report new chemistry,[15] the synthetic approach and versatility allows for the addition and growth of C- and/or N-terminal peptides with the same and/or different functional properties on a solid-phase support. Peptide attachment off resin in solution using stepwise or ligation synthetic protocols is also possible (data not shown). Direct coupling to unfunctionalized β-CD was unsuccessful under all Fmoc procedures used in this study. In addition, the succinyl linker is not removed at the end of

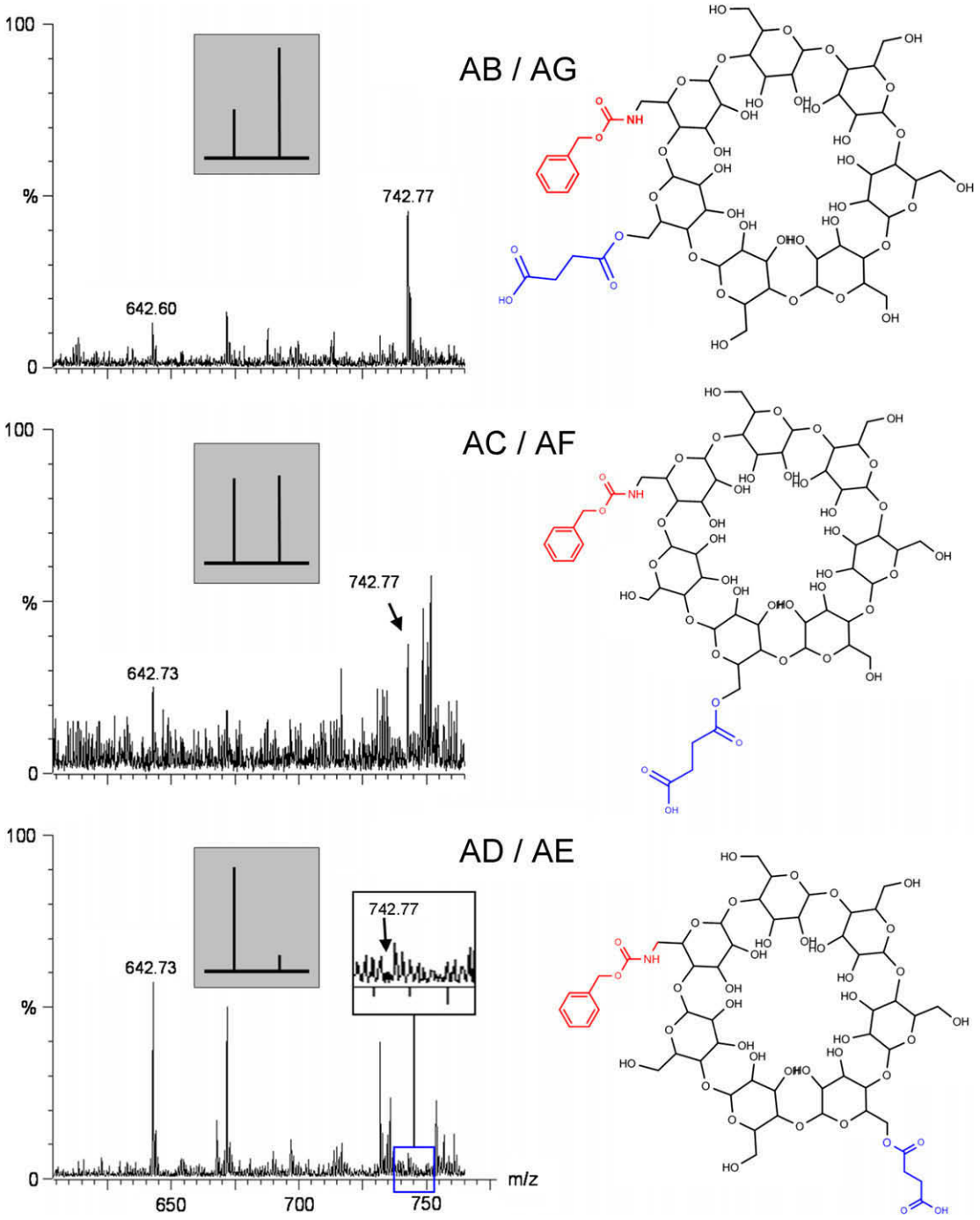
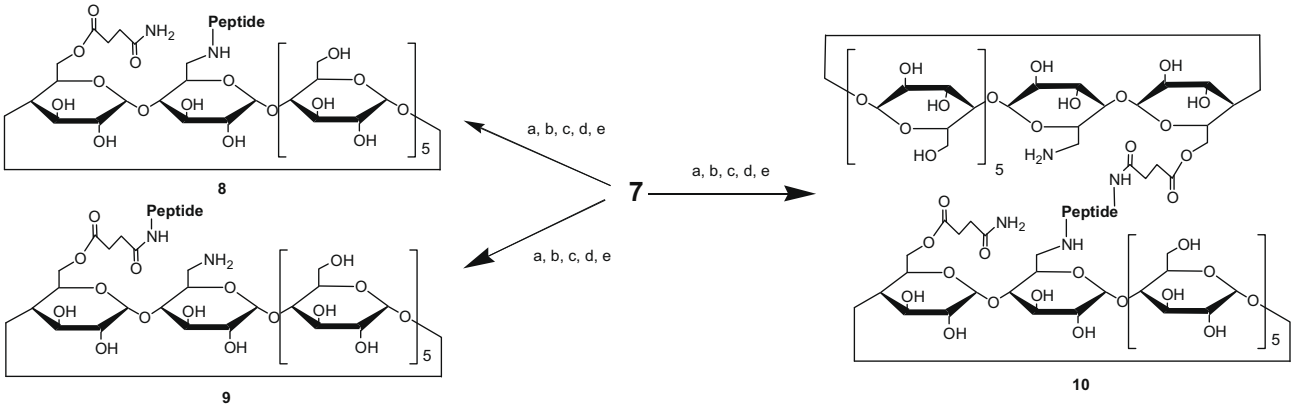


Figure 1. Experimental ESI-isomer spectra (left) with the expected pattern of fragmentation (inset); structures of mono-6^{A}-carbobenzyloxyamino-succinyl-β-cyclodextrin, **5** isomers. AB/AG isomer (top), AC/AF isomer (middle), AD/AE isomer (bottom).

this multi-step synthesis. Potentially, by using a resin that generates a peptide with acid functionality, the succinyl group is available for further functionalization.

This system has great developmental potential for the transportation of drugs and/or other molecules. Owing to the large variety of cellular receptors, peptides appear to be amongst the most versatile compounds for such targeting purposes. The grafting of peptides onto cyclodextrin also adds future therapeutic dimensions.[7] To establish proof-of-concept, the attachment of a short model peptide to **7** at both the N- and C-terminus was achieved using Fmoc SPPS to give **8** and **9**, respectively (Scheme 3). The coupling conditions for the attachment of **7** to resin and/or amino acids were studied under various amino acid coupling conditions using the Fmoc UV–vis coupling method[9] for the detection of the loss of the Fmoc group (see Supplementary data). It was found that the best method of coupling was with EDC/DIEA in excess in a 50% pyridine/DMF solvent system overnight followed by one recoupling. This gave a coupling percentage for **7** of 25% after a second recoupling. Even with a third consecutive recoupling, the coupling percentage did not improve. It is thought that the length of the spacer arm (the succinyl group) between **7** and the resin might not be long enough to enable efficient coupling. Standard Fmoc-coupling conditions were used for the addition of Fmoc amino acids [TBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium



Scheme 3. Peptide synthesis using bifunctionalized β-cyclodextrin, **7**. Reagents and conditions: (a) Rink resin (0.073 mmol/g), DMF, 3 h; (b) 20% piperidine in DMF, 2 × 10 min; (c) amino acid (4 equiv), TBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF, rt, overnight or **7** (4 equiv), EDC (4 equiv), DIEA (8 equiv), py/DMF, rt overnight; (d) DMF; (e) TFA (30%), CH_2Cl_2, rt, 30–60 min, 21% (for **8**), 15% (for **9**), 3% (for **10**) where the peptide is Ala-Gly.

tetrafluoroborate) and DIEA (diisopropylethylamine) in DMF in excess], resulting in pure 21% (10.4 mg) and 15% (7.4 mg) yields for **8** and **9**, respectively (Scheme 3). The pure products were characterized by HR-MS. The attachment of **7** at both the C- and N-termini of a peptide was also achieved (Scheme 3) using the same reaction conditions producing **10** in a pure 3% (2.4 mg) yield as confirmed by LR-MS (MALDI-TOF).

We have demonstrated a unique, versatile and robust method for the bifunctionalization of β-CD. It was interesting to note that a 1:2:2 ratio for the (AB/AG):(AC/AF):(AD/AE) isomers for the bifunctional cyclodextrin complex **5** was obtained indicating a steric preference in the overall complex. Isomer separation was achieved using RP-HPLC with characterization by ESI-MS studies. Furthermore, the attachment of peptides selectively to either the C- and/or N-terminus using Fmoc SPPS has been demonstrated. This system has great potential for the transportation of drugs and/or other molecules, especially as CD is reported as being biocompatible.[1,2,6,7] Studies are continuing into the attachment and analysis of potential bioactive peptidyl-cyclodextrin complexes for their potential use in drug transportation and delivery.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.11.118.

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