PDF hosted at the Radboud Repository of the Radboud University
Nijmegen

The following full text is a publisher’s version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/177868

Please be advised that this information was generated on 2018-11-04 and may be subject to change.
Peptide-Appended Permethylated β-Cyclodextrins with Hydrophilic and Hydrophobic Spacers

Abbas H. K. Al Temimi, ‡ Thomas J. Boltje, ‡ Daniel Zollinger, §§ Floris P. J. T. Rutjes, ‡ and Martin C. Feiters†,‡

†Institute for Molecules and Materials, Radboud University, Heijendaalseweg 135, 6525 AJ Nijmegen, The Netherlands
‡Okklo Life Sciences BV, Pivot Park, Molenstraat 110, 5349 TD Oss, The Netherlands
§QareFree, Blauwoogvlinder 2, 4814 ST Breda, The Netherlands

ABSTRACT: A novel synthetic methodology, employing a combination of the strain-promoted azide–alkyne cycloaddition and maleimide–thiol reactions, for the preparation of permethylated β-cyclodextrin-linker-peptidyl conjugates is reported. Two different bifunctional maleimide cross-linking probes, the polyethylene glycol containing hydrophilic linker bicyclo[6.1.0] nonyne-maleimide and the hydrophobic 5'-dibenzoazacyclooctyne-maleimide, were attached to azide-appended permethylated β-cyclodextrin. The successfully introduced maleimide function was exploited to covalently graft a cysteine-containing peptide (Ac-Tyr-Arg-Cys-Amide) to produce the target conjugates. The final target compounds were isolated in high purity after purification by isocratic preparative reverse-phase high-performance liquid chromatography. This novel synthetic approach is expected to give access to many different cyclodextrin–linker peptides.

INTRODUCTION

Cyclodextrins (CDs) are cyclic oligosaccharides containing six, seven, or eight (α-1,4)-linked D-glucopyranoside units, named α-, β-, and γ-cyclodextrin, respectively. These macromolecules have a hydrophilic exterior which makes them water-soluble and a hydrophobic interior that can accommodate small lipophilic molecules. As such, CDs are used as pharmaceutical excipients that can solubilize various poorly soluble drugs and a hydrophobic interior that can accommodate small lipophilic molecules. As such, CDs are used as pharmaceutical excipients that can solubilize various poorly soluble drugs through the formation of water-soluble drug–CD complexes. Hence, CDs are applied in the pharmaceutical industry to promote the bioavailability, safety, stability, and solubility of drug molecules. Moreover, CDs (mainly β-CDs as depicted in Scheme 1) have been used for many years in biochemical studies to extract cholesterol from plasma membranes. The functionality and applications of the cyclodextrins can be further expanded by the addition of functional groups that allow for cell and tissue specific targeting. The conjugation of small peptides is especially interesting in this respect. However, the synthesis of cyclodextrin–peptide conjugates with different spacers/linkers presents a nontrivial scientific challenge, due to the presence of multiple reactive groups. Despite several previous reports on the attachment of small peptides directly to the CD core, there is as yet no flexible synthetic route that allows for insertion of linkers of choice between the peptide and CD. In this study, we have exploited the −SH of a cysteine-based model peptide to successfully accomplish its covalent grafting to an azide-appended permethylated β-cyclodextrin (PMβCD) via hydrophilic and hydrophobic linkers, utilizing the strain-promoted azide–alkyne cycloaddition (SPAAC) reaction followed by the maleimide–thiol coupling.

As proof of concept, we applied a multistep strategy (Scheme 1, cartoon representation of β-CD reproduced from ref 13) to use the polyethylene glycol (PEG)-containing hydrophilic linker bicyclo[6.1.0] nonyne-maleimide (BCN-Mal, derived from BCN-PEG-maleimide) and the hydrophobic linker 5'-dibenzoazacyclooctyne-maleimide (DIBAC-Mal, derived from DIBAC-maleimide)11), with a model cysteine containing peptide (Ac-Tyr-Arg-Cys-Amide) to provide the desired conjugates by the final maleimide-cysteine coupling reaction step. This model peptide is of interest because it contains the phenolic hydroxyl and guanidine groups in addition to the reactive thiol of cysteine. The reason for selecting PMβCD-N3 instead of βCD-N3 as the starting point for the conjugation to access the target conjugates 10 and 13 (Scheme 1) is that both the latter and model peptide 9 contain multiple reactive (nucleophilic) groups which could give rise to side products. Previous studies by Shi et al. have shown that permethylated CDs are soluble both in water and in organic solvents and do not undergo side reactions during coupling. Thus, we envisaged that the use of permethylated azide-
appended β-CD instead of its hydroxyl analogue will simplify the synthetic approach. The mono-6-azido-permethylated β-CD 4 was obtained from precursor β-CD 1 by a selective tosylation of the primary hydroxyl group on the outer face to give mono 6-OTs-β-CD 2,18 followed by azide displacement resulting in hydroxyl azide-appended β-CD 3,19 and permethylation of the remaining hydroxyl groups to afford the key intermediate 4,14,20,21 which is the starting point for access to a wide range of compounds of the β-CD family. As method for the conjugation of the permethylated azide-appended β-CD 4 with the cross-linking reagent [either the hydrophilic BCN-Mal 7 (path A in Scheme 1) or the hydrophobic DIBAC-Mal 11 (path B in Scheme 1)] we chose a click reaction, viz., the SPAAC reaction between permethylated azide-appended β-CD 4 and alkyne-appended BCN (or DIBAC), because it is metal-free and the resultant product is therefore suitable for application in living systems.22 The coupling of 4 with the commercially available bifunctional hydrophilic cross-linking reagent (BCN-Mal) 7 resulted in maleimide-functionalized PMβCD 8 (path A). The model peptide 9 (Ac-Tyr-Arg-Cys-Amide) was synthesized by standard Fmoc solid-phase peptide synthesis (see Supporting Information for details) and reacted with its terminal cysteine thiol to the maleimide in 8 to give the target conjugate with hydrophilic linker PMβCD-Mal-BCN-Peptide 10. Similarly, the introduction of the hydrophobic linker DIBAC-Mal 11 was carried out using the same synthetic approach (SPAAC reaction followed by maleimide−thiol coupling to peptide 9) to afford the final target conjugate 13 (path B).

## RESULTS AND DISCUSSION

**Synthesis of mono-6-azido PMβCD 4.** The synthesis of key intermediate 4 has been reported22 but was modified to improve the yield of the desired product. Regioselective tosylation of a single hydroxyl group at the 6-position was achieved according to a reported method18 by reacting β-CD 1
with 1.2 equiv of tosyl chloride in the presence of 8 N NaOH and water/acetonitrile as a solvent to afford the compound 2 in a yield of 26%. 1H and 13C NMR corroborated the identity of 6-β-CD-OTs 2 (Figures S2 and S3). ESI-MS analysis confirmed the formation of target compound 2, but also revealed the presence of traces of impurities (unreacted and ditosylated β-CD, Figure S1). It is not straightforward to remove these impurities due to the high polarity of these compounds. Since TLC, 1H NMR, ESI-MS, and LC-MS had proven the structure and acceptable purity of the product, it was used in the next step without further purification. The azidation step was performed with excess sodium azide in water. After precipitation in acetone followed by centrifugation, the mono-6-azido β-CD (Nα-βCD) 3 was obtained in a yield of 85%. ESI-MS showed the mass of the compound 3 (Figure S4), and 1H and 13C NMR identified its chemical structure (Figures S5 and S6). As in the case of 2, the presence of some β-CD, remaining from the previous step, was apparent from the 1H NMR spectrum of the target azide- appended CD, as confirmed by LC-MS. After recrystallization from acetone, the azido-β-CD intermediate was used subsequently without further purification. The next step, the capping of all remaining hydroxyl groups, was performed with excess methyl iodide (25.0 equiv) in the presence of the strong base NaH (25.0 equiv) and anhydrous DMF as a solvent compatible with this reaction. The purity and the chemical structure of 4 were determined by LC-MS, ESI-MS, 1H NMR, and 13C NMR (Figures S7, S8, S9, and S10, respectively). Since the crude product showed a single spot in TLC, it could only be purified by a prep-HPLC system provided with two UV detectors at 254 and 215 nm. It eluted at 17.0 min (Figure S11) and the yield after this step was 34%. Analytical HPLC (Figure S12) showed that compound 4 was pure, and the impurities remaining from previous steps had been effectively removed.

**Synthesis of Maleimide-Functionalized Hydrophilic Linker-Attached PM/CD 8.** The linker BCN-Mal 7 possesses two functional ends, dibenzocyclooctyne moiety for click reaction with azide functionalized compounds and maleimide end for cysteine-maleimide conjugation reaction. The starting materials for the preparation of 7, BCN-(PEO)₃- NH₂, S and Mal-NHS 6, were obtained from SynAffix B.V. (Oss, The Netherlands). The bifunctional reagent was obtained using PBS (pH 7.2) with DMSO or DMF as solvent (1:1 v/v). The LC-MS and ESI-MS data showed the formation of the product as a main peak (Figures S13 and S14), with only traces of a side product in LC-MS. Without the buffer, only a small amount of the target BCN-Mal is formed, and the unwanted BCN-Mal-BCN (Figure S15) represents the dominant product; this is formed due to a high pH value which causes the amine group from BCN-(PEO)₃-NH₂ to attack both electrophilic sites, the NHS and the maleimide moieties, in Mal-NHS. The product 8 was obtained by SPAAC reaction in 42% yield after purification by flash column chromatography on a Biotage system. TLC analysis clearly indicated the yellow spot for the double bond maleimide moiety after dipping in KMnO₄ stain. The formation of 8 was indicated by LC-MS and MALDI-TOF MS analyses (Figures S16 and S17) and confirmed by 1H NMR (Figure S18), showing a singlet around 6.7 ppm for the triazole. IR analysis showed that the azide peak of 4 at 2100–2200 cm⁻¹ completely disappeared after the reaction with BCN-Mal 7.

**Synthesis, prep-HPLC Purification, and Characterization of the Peptide 9.** The model peptide Ac-YRC-NH₂ (9) was synthesized with a semiautomated peptide synthesizer (Laboretç SP 640, Bubendorf, Switzerland) with standard Fmoc α-protection [23] [see experimental protocol S6 and Scheme 1 in the Supporting Information] from the protected amino acids Fmoc-Cys(Trt)−OH, Fmoc-Arg(Pbf)−OH, and Fmoc-Tyr(Bu)−OH. Coupling reactions of Fmoc amino acids were achieved in DMF applying amino acid/HOBT/DIPCIDI/ Breipohl resin in the molar ratio 3.0:3.6:3.3:1.0. Acetylation of the N-terminus was achieved with Ac₂O/DIPEA in DMF using a molar ratio 1:1 (equiv). The coupling reactions and Fmoc deprotections were monitored at intervals with the color Kaiser tests [24,25] until completed. Upon prep-HPLC purification, the product 9 gave a chromatogram with a major peak in addition to a small peak representing its associated dimer form in MS as determined by LC-MS and MALDI-TOF MS (Figures S19 and S20), {calc. [M + H]+ 482.2; obsd. 482.3}. The model peptide was characterized and checked for purity by various analytical techniques including 1H NMR, 13C NMR, prep-HPLC, and analytical HPLC (Figures S21, S22, S23, S24, respectively).

**Preparation, prep-HPLC Purification and Characterization of the PM/CD-Hydrophilic Linker-Peptide Conjugate 10.** One of the most widespread specific covalent coupling reactions is the conjugation of a protein or peptide bearing a thiol moiety which can react with maleimide reagents. The cysteine thiol can be efficiently and site-specifically labeled with maleimides, disulfides, or halocetyl compounds. Maleimide can be coupled to sulphydryl groups when the pH of the reaction is between 6.5 and 7.5. The formed thioether linkage is stable but can be cleaved with reducing agents, such as TCEP, dithiothreitol (DTT), and β-mercaptoethanol (βME).

Based on the previously published procedures from our group [27,28] it was our goal to access the target conjugates by reacting PM/CD-BN-Mal 8 with model peptide 9 at suitable conditions (Scheme 1A). The conjugation of maleimide attached to hydrophobic linker appended-PM/CD 8 with thiol cysteine-based model peptide 9 was accomplished in PBS buffer at pH 7.2 for 4 h at room temperature employing TCEP.HCl as reducing agent and a small amount of DMF. It is essential to degas the buffer for 1 h by allowing a flow of nitrogen, since a cysteine-containing peptide is prone to oxidation. Also, a freshly prepared stock solution of the odorless tris(2-carboxyethyl)-phosphine reducing agent (TCEP-HCl) at pH 7 was used for the reaction. This circumvents the problems of adding TCEP.HCl in solid form which potentially results in a sharp drop of pH.

However, as confirmed by LC-MS and ESI-MS analyses, the conjugate 10 was not produced efficiently due to an impurity which appeared at a retention time very close to that of the main conjugate. It is probably from the starting material peptide 9 which is added in excess relative to the maleimide-attached PM/CD 8 in the final reaction. It was decided to systematically optimize the reaction conditions by variation of the buffer and its pH values, reaction time, temperature, and the stoichiometry of the reactants. Varying the pH in the range of 6–7.8 did not result in reduction of the amount of impurity in the reaction product, nor did raising the temperature to 37 °C at pH 7.2, or adding the maleimide-attached PM/CD 8 in steps.

When we explored the effect of the molar ratio of 9 and 10 on the reaction, we found that besides the stoichiometry other factors were also important, viz., the PBS buffer (100 μM, pH 7.2), using a fresh solution (100 μM) of TCEP-HCl, and incubation for 4 h at room temperature. The best results were obtained when using 1.3 equiv of the model peptide relative to
the Mal-appended PMβ/CD. The presence of the target conjugate 10 in the product was supported by the observation of a significant doubly charged peak [M+2H]²⁺ at 1206.3 in ESI-MS (Figure S25) and the observation of the [M + H]⁺ and [M + Na]⁺ adducts in MALDI-TOF analysis (Figure S26), and further confirmed by its ¹H NMR spectrum, in which the absence of maleimide protons at 6.7 ppm indicates that the addition of thiol peptide has taken place (Figure S27). LC-MS for the crude product of the conjugation reaction gave a strong peak at 1206.2 corresponding to the calculated mass of the target compound 10, but also an unidentified impurity at 1090.6 (20% of strongest peak, Figure 1a). Attempted separation by gradient prep-HPLC was not successful, but the final conjugate could be purified by prep-RP-HPLC in isocratic mode. Notably, the compound was successfully separated from the impurity by this method as confirmed by LC-MS analysis before and after purification (Figure 1b, Figure S28).

Even after separation, it was not possible to establish the identity of the impurity, as there was not enough material for NMR analysis and the LC-MS and ESI-MS results were insufficient to hypothesize a relevant structure. It could not be correlated to any known structure originating from the reactants, or to possible side products.

Preparation, RP-HPLC Purification, and Characterization of the PMβ/CD-Hydrophobic Linker-Peptide Conjugate 13. In a parallel approach we explored cyclo-dextrin–peptide conjugates with hydrophobic spacers. The synthesis of maleimide attached to hydrophobic linker appended PMβ/CD 12 was carried out identically to that of 8, except for using the hydrophobic linker DIBAC-Mal 11, which was used directly as obtained commercially. Intermediate 12 was synthesized and purified by a Biotage chromatographic system. The mass of 12 was confirmed by MALDI-TOF MS (Figure S30) and the formation of the triazole ring by ¹H NMR which showed a singlet at approximately 6.7 ppm (Figure S31). Analysis by LC-MS revealed two very close chromatographic peaks in a 1:1 ratio which obviously result from regioisomerism (Figure S29). These regioisomers were not separated and used as a mixture for the rest of the synthesis, since they exhibit identical reactivity toward azides. The triazole isomers were combined for the conjugation with peptide 9 to PMβ/CD-hydrophobic linker-peptide 13. An excess (1.3 equiv) of the model peptide 9 relative to the Mal-appended hydrophobic linker PMβ/CD was used under the same conditions that afforded 10 from 8 and 9. LC-MS analysis of the product of the reaction of 12 and 9 showed the mass of the target conjugate 13 at 1174.8 [M+2H]²⁺ (Figure 2b) as well as an unidentified impurity at 1059.2 (Figure 2a), which is comparable to that obtained for the side product of the reaction of 8 and 9 to give 10, although there were slight differences in the retention times and the final yields. Likewise, the final target conjugate 13 with the hydrophobic linker could be obtained in pure form after isocratic prep-HPLC purification (Figure S32) in a yield of 14%. The final product was further confirmed by the disappearance of the maleimide protons at 6.7 ppm in the ¹H NMR spectrum (Figure S33). The LC-MS analysis of the final conjugate before and after prep-HPLC isocratic purification is shown in Figure 2a and b, respectively.

CONCLUSIONS

The synthesis and characterization of the conjugates PMβ/CD-BCN-Mal-peptide 10 and PMβ/CD-DIBAC-Mal-peptide 13 with hydrophilic and hydrophobic linkers have been successfully accomplished. We developed a robust and reliable
The best of our knowledge, the only synthetic route toward a combination of an azide-appended cyclodextrin and a peptide via suitable hydrophilic and hydrophobic linkers. We consider this novel methodology for the preparation of PM/βCD-linker-peptide derivatives to be a promising valuable research tool for the development of interesting therapeutics.

**EXPERIMENTAL PROCEDURES**

**General Methods.** $^1$H NMR spectra were recorded on a Varian Inova-400 (400 MHz) spectrometer at 300 K and chemical shifts are given in parts per million ($\delta$) relative to tetramethylsilane as an internal reference ($\delta = 0.00$ ppm). Coupling constants are reported as $J$-values in Hz. The following abbreviations are used to designate the multiplicities: $s =$ singlet, $d =$ doublet, $t =$ triplet, $q =$ quartet, $m =$ multiplet.

$^{13}$C NMR spectra were recorded on a Bruker Avance III 500 MHz NMR and the chemical shifts are internally referenced to the residuals selected solvent. Mass spectra were confirmed by ESI-MS (Thermo Finnigan LCQ Advantage Max). Liquid chromatography electrospray ionization mass spectrometry was measured on a Shimadzu LCMS-QP8000 (Duisburg, Germany) single quadrupole benchtop mass spectrometer operating in a positive ionization mode. Infrared (IR) spectra were recorded on a IR-ATR Bruker TENSOR 27 spectrometer; only the absorption frequencies ($\text{cm}^{-1}$) of major peaks are reported. Optical rotations of samples were measured on a Perkin & Elmer Polarimeter 241 using a 10 cm, 1 mL cell. All reactions were magnetically stirred and monitored by TLC on Kieselgel 60 F254 (Merck, Darmstadt, Germany); Spots were visualized under UV light (254 nm) and were stained with ninhydrin, 2,4-dinitrophenylhydrazine (DNP), or aqueous KMnO$_4$ (depending on the reaction), followed by heating on a hot plate. $R_f$ values were obtained with solvent mixtures indicated. Unreacted TCEP reagent and degassed PBS buffer (pH 7.2) were removed by size exclusion chromatography using a Sephadex G10 column. The model peptide was synthesized on a semiautomated peptide synthesizer (Labortec SP 640, Labortec AG) by applying the Fmoc-protocol. Accurate molecular weights of the model peptide and compounds were confirmed by ESI-MS technique using a Micromass Platform II (Altrincham, United Kingdom) single quadrupole benchtop mass spectrometer operating in a positive ionization mode. The mass of each compound was measured and the observed monoisotopic $[M + H]^+$ values were correlated with the calculated $[M + H]^+$ values by use of (ChemBioDraw Ultra 14) Cambridge software program. Other adducts $[M + Na]^+$ and $[M + K]^+$ have also been detected and identified. The room temperature in the reactions is in the range $20 \pm 25^\circ$C.

Compounds 8 and 11 were purified by flash column chromatography on a Biotage Isolera One chromatography system (silicyle 4g cartridge, size 230–400 mesh 40–63 $\mu$m, Quebec, Canada) using a 15% methanol/dichloromethane gradient. Analytical HPLC was performed on a Shimadzu LC-20A Prominence system (Shimadzu, Quebec, Canada) using a 15% methanol/dichloromethane gradient. Analytical HPLC was performed on a Shimadzu LC-20A Prominence system (Shimadzu, Quebec, Canada) using a 15% methanol/dichloromethane gradient. Analytical HPLC was performed on a Shimadzu LC-20A Prominence system (Shimadzu, Quebec, Canada) using a 15% methanol/dichloromethane gradient.

**Compound Characterization.** The identity of the compounds was confirmed and further characterized by TLC, $^1$H NMR, $^{13}$C NMR, LC-MS, Maldi-Tof MS, ESI-MS, IR, and optical rotation ($\text{[a]}\_D$).

**Bioconjugate Chemistry**
Reverse-Phase HPLC Characterization of the Final Conjugates 10 and 13. Both final conjugates were purified on a Phenomenex Gemini-NX 3u C18 110A reversed-phase column (150 × 2.12 mm) with HPLC system using an isocratic elution at a constant flow rate of 10 mL/min at 30 °C. The analytical procedure for both conjugates was as follows: in the isocratic phase, the acetonitrile concentration was kept at 40% for 25 min, followed by a gradient from 40% to 100% over 3 min. Conjugates 10 and 13 elute at 15.5 and 20.4 min, respectively. Buffer A: 0.1% (v/v) TFA in water; Buffer B: CH₃CN containing 0.1% (v/v) TFA. All the samples were filtered through a 0.22-μm syringe filter before injection.

LC-MS Characterization and Analytical HPLC Analysis. LS-MS analysis for all the compounds was performed on a Thermo Finnigan LCQ-Fleet ESI-ion trap (Thermo Fisher, Breda, The Netherlands) equipped with a Phenomenex Gemini-NX C18 column, 50 × 2.0 mm, particle size 3 μM (Phenomenex, Utrecht, The Netherlands). An acetonitrile/water gradient containing 0.1% formic acid was used for elution (5−100%, 1−20 min, flow 0.2 mL min⁻¹).

Analysis of Purified Compounds and the Model Peptide by MALDI-TOF Mass Spectroscopy. The purified compounds and lyophilized fractions of model peptide were analyzed by matrix assisted laser desorption/ionization time-of-flight mass spectrometer (Bruker Biflex III Maldi-Tof MS, Germany). 10 μL of the required compound was mixed with 10 μL of the supernatant (1:1) Maldi matrix solution (α-cyano-4-hydroxy-cinnamic acid; 5.0 mg weighed in an Eppendorf vial, mainly by A. H. K. Al Temimi and M. C. Feiters.

Details of synthetic procedures and characterization (LC-MS, MALDI-TOF MS, ESI-MS, ¹H NMR, ¹³C NMR, HPLC) for compounds 2, 3, 4, 7, 8, 9, 10, 11, 12, and 13 (PDF)

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
This work was supported by the Dutch Organization for Scientific Research (NWO) within the framework of FONDS NCI-KIEM project 731.014.104. Dr. Stephan Peters, Dr. Alex Zwiers (Okklo Life Sciences), Dr. Marco Felici, Jan Dommerholt, Dr. Roel Hammink, Dr. Vijayendar Reddy Yedulla, Dr. Dennis W. P. M. Löwik, and Dr. Rajat K. Das (Radboud University) are gratefully acknowledged for their insightful discussions.

ABBREVIATIONS LIST
Ac₂O, acetic anhydride; β-CD, beta-cyclodextrin; BCD-Mal, bicyclo[6.1.0] nonyne-maleimide; DIPCDI, N,N′-disopropylcarbodiimide; DIPEA, N,N-disopropylethylamine; DMS, dimethylsulfoxide; DTT, dithiothreitol; ESI-MS, electrospray ionization-mass spectrometry; HOBT, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography mass spectrometry; MALDI-TOF, matrix-assisted laser-induced desorption-time-of-flight; NHS, N-hydroxysuccinimide; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PEO, poly(ethylene oxide); SPAAC, strain-promoted azide–alkyne cycloaddition; TCEP.HCl, tris(2-carboxyethyl)phosphine hydrochloride; TLC, thin layer chromatography

REFERENCES
(1) Kurkov, S. V., and Lofthson, T. (2013) Cyclodextrins. Int. J. Pharm. 453, 167−180.
(2) de Miranda, J. C., Martins, T. E. A., Veiga, F., and Ferraz, H. G. (2011) Cyclodextrins and ternary complexes: technology to improve solubility of poorly soluble drugs. Braz. J. Pharm. Sci. 47, 4.
(3) Lofthson, T., and Brewster, M. E. (1997) Cyclodextrins as pharmaceutical excipients. Pharm. Technol. S, 26−34.
(4) Tsai, Y., Tsai, H., Wu, C., and Tsai, F. (2010) Preparation, characterisation and activity of the inclusion complex of paeonol with β-cyclodextrin. Food Chem. 120, 837−841.
(5) Yannakopoulou, K. (2012) Cationic cyclodextrins: cell penetrating agents and other diverse applications. J. Drug Delivery Sci. Technol. 22, 243−249.
(6) Arun, R. (2008) Cyclodextrins as Drug Carrier Molecule: A Review. Sci. Pharm. 76, 567−598.
(7) Zidovetzki, R., and Revitan, I. (2007) Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. Biochim. Biophys. Acta, Biomembr. 1768 (6), 1311−1324.
(8) Irie, T., Fukanaga, K., and Pitha, J. (1992) Hydroxypropylocyclodextrins in parenteral use. I: Lipid dissolution and effects on lipid transfers in vitro. J. Pharm. Sci. 81, 521−53.
(9) Ohtani, Y., Irie, T., Uekama, K., Fukanaga, K., and Pitha, J. (1989) Differential effects of alpha-, beta- and gammacyclocyclodextrins on human erythrocytes. Eur. J. Biochem. 186, 17−22.
(10) Ohno, H., and Slote, J. P. (1996) Cyclodextrin-mediated removal of steroids from monolayers: effects of sterol structure and phospholipids on desorption rate. Biochemistry 35, 8018−8024.
(11) White, R. J., Pleger, P. G., and Harding, D. R. K. (2010) Synthesis of bi-functionalised peptide derivatives based on a β-cyclodextrin core with drug delivery potential. Tetrahedron Lett. 51 (5), 800−803.
(12) Ruiz Garcia, Y., Zelenka, J., Rabon, V., Iyer, A., Budilinskiš, M., Kraus, T., Smith, C. I. E., and Madder, A. (2015) Cyclodextrin-peptide...
conjugates for sequence specific DNA binding. Org. Biomol. Chem. 13, 5273–5278.

(13) http://www.itncyclon.eu/files/Newsletter/Newsletter_30-9-13.pdf.

(14) Loukou, C., Changeret-Barret, P., Rager, M. N., Plaza, P., Martin, M. M., and Mallet, J. M. (2011) The design, synthesis and photochemical study of a biomimetic cyclodextrin model of Photoactive Yellow Protein (PYP). Org. Biomol. Chem. 9, 2209.

(15) Shi, J., Guo, D.-S., Ding, F., and Liu, Y. (2009) Unique Regioselective Binding of Permethylated α-Cyclodextrin with Azobenzene Derivatives. Eur. J. Org. Chem. 2009, 923–931.

(16) Liu, Y., Shi, J., and Guo, D.-S. (2007) Novel Permethylated α-Cyclodextrin Derivatives Appended with Chromophores as Efficient Fluorescent Sensors for the Molecular Recognition of Bile Salts. J. Org. Chem. 72, 8227–8234.

(17) Diaz, E., Jankowski, C. K., Hocquelet, C., del Rio, F., and Barrios, H. (2008) The unambiguous assignment of NMR spectra of per-O-methylated 6-mono and 6,6-diamino-Beta-cyclodextrines. Can. J. Chem. 86, 726–736.

(18) Miyake, K., Irie, T., Hirayama, F., and Uekama, K. Improved solubility and oral bioavailability of cyclosporin A by hydrophilic cyclodextrin complexation. In Proceedings of the Ninth International Symposium on Cyclodextrins. Torres Labandeira, J. J., and Vila-Jato, J. L., eds. Kluwer Academic Publishers: Dordrecht, 1999, 293–296.

(19) Lovrinovic, M., and Niemeyer, C. M. (2007) Microtiter plate-based screening for the optimization of DNA-protein conjugate synthesis by means of expressed protein ligation. ChemBioChem 8 (1), 61–67.

(20) Molina, A. G., Kungurtsev, V., Virta, P., and Lönnberg, H. (2012) Acetylated and Methylated α-Cyclodextrins as Viable Soluble Supports for the Synthesis of Short 2’-Oligodeoxyribonucleotides in Solution. Molecules 17, 12102–12120.

(21) Felici, M., Marzé-Pérez, M., Hatzakis, N. S., Nolte, R. J. M., and Feiters, M. C. (2008) α-Cyclodextrin-Appended Giant Amphiphile: Aggregation to Vesicle Polymersomes and Immobilisation of Enzymes. Chem. - Eur. J. 14, 9914–9920.

(22) Van Hest, J. C. M., and van Delft, F. L. (2011) Protein modification by strain-promoted alkyne-azole cycloaddition. ChemBioChem 12, 1309–1312.

(23) Merrifield, R. B. (1963) Solid Phase Peptide Synthesis. The Synthesis of a Tetrapeptide. J. Am. Chem. Soc. 85, 2149–2154.

(24) Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal. Biochem. 34 (2), 595–598.

(25) Sarin, V. K., Kent, S. B., Tam, J. P., and Merrifield, R. B. (1981) Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. Anal. Biochem. 117, 147–157.

(26) Kim, Y., Hu, S. O., Gassman, N. R., Korlann, Y., Landorf, E. V., Collart, F. R., and Weiss, S. (2008) Efficient site-specific labeling of proteins via cysteines. Bioconjugate Chem. 19 (3), 786–791.

(27) Georgieva, J. V., Brinkhuis, R. P., Stojanov, K., Weijers, C. A. G. M., Zuilhof, H., Rutjes, F. P. J. T., Hoekstra, D., van Hest, J. C. M., and Zuhorn, I. S. (2012) Peptide-mediated blood-brain barrier transport of polymersomes. Angew. Chem., Int. Ed. 51, 8339–8342.

(28) Stojanov, K., Georgieva, J. V., Brinkhuis, R. P., van Hest, J. C. M., Rutjes, F. P. J. T., Dierckx, R. A. J. O., de Vries, E. F. J., and Zuhorn, I. S. (2012) In vivo biodistribution of prion- and GM1-targeted polymersomes following intravenous administration in mice. Mol. Pharmaceutics 9, 1620–1627.