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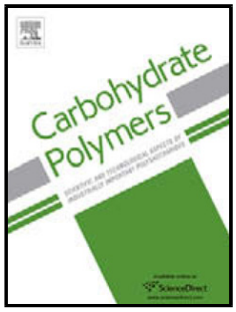

Title: The effect of urea moiety in amino acid binding by β-cyclodextrin derivatives: A 1000-fold increase in efficacy comparing to native β-cyclodextrin

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<AT>The effect of urea moiety in amino acid binding by β-cyclodextrin derivatives: a 1000-fold increase in efficacy comparing to native β-cyclodextrin

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<ABS-HEAD>Highlights► Amphiphilic Urea-cyclodextrin derivatives bind amino acids ► Amino acid binding is 1000-fold stronger in comparison to unsubstituted cyclodextrins ► Side chains attached to the cyclodextrins cause formation of nano-sized aggregates

<ABS-HEAD>Abstract

<ABS-P>Water soluble amphiphilic anion receptors based on urea-substituted β-cyclodextrin were synthesized via a copper(I) mediated azide-alkyne coupling reaction. The synthetic route was designed to minimize the number of operations of cyclodextrins. Stable products were obtained in 90% yield. They were successfully tested as amino acid receptors, showing excellent affinity constants (10^3-10^4 M^{-1}) in a highly competitive environment (pH 8 phosphate-buffered water solution). Isothermal titration calorimetry indicated that complex formation strongly depends on the hydrophobic nature of the guest and that the urea moiety of the receptor is necessary to efficiently bind amino acids.
<KWD>Keywords: cyclodextrin; azide-alkin coupling; host-guest chemistry; calorimetry; amino acids; complexes

<H1>1. Introduction

Molecular recognition of biological molecules is a broadly studied and important area of scientific interest. In particular, investigating selective interactions of amino acids or peptides is very important in order to understand and mimic the mechanisms of biological processes (Schneider, 2009). The utilization of hydrogen bond donors in the field of molecular recognition is a commonly developed area of scientific interest (Amendola, Fabbrizzi, & Mosca, 2010; Busschaert, Caltagirone, Van Rossom, & Gale, 2015; Choi & Hamilton, 2003). Such donors provide short-contact, relatively strong and spatially oriented interactions with negatively charged or polarized guests, allowing for selective and structurally-related binding.

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However, this approach is often limited by the competing environment, in which the role of the solvent cannot be neglected (Castronuovo, Elia, Pierro, & Velleca, 1999). As such, the recognition employing hydrogen bond donating groups is executed mainly in mixed solvents, where water's hydrogen bond network is shared with other polar cosolvents (e.g. acetone, acetonitrile, DMSO) (Caltagirone et al., 2008; Cametti & Rissanen, 2009; Dutta, Bose, & Ghosh, 2013).

In this study we sought to provide a water soluble compartment in which hydrogen bond donors are isolated from the solvent, so their interactions with hydrophobic L-amino acids (as model amphiphilic carboxylates) should be enhanced. For this purpose we chose β-cyclodextrin (β-CD) as a well-defined, water-soluble, amphiphilic vessel, that could be selectively modified. β-CD has a concave structure, presenting its polar hydroxy groups to the outside, while the cavity remains hydrophobic. Although several types of similar cavities have been previously tested (Dziemidowicz, Witt, & Rachoń, 2008; Mutihac, Lee, Kim, & Vicens, 2011; Richard et al., 2008; Stone, Franz, & Lebrilla, 2002) only cucurbiturils gave satisfactory (>>1000 M^{-1}) affinities with amino acids (Logsdon, Schardon, Ramalingam, Kwee, & Urbach, 2011; Rajgariah & Urbach, 2008). On the other hand, underivatized CDs bind amino acids rather poorly, with K_a's below 100 M^{-1} (Cooper & MacNicol, 1978; Matsuyama, El-Gizawy, & Perrin, 1987; Rekharsky, Schwarz, Tewari, & Goldberg, 1994). Therefore, we decided to incorporate additional hydrogen bond donors in the proximity of the cavity in order to form an isolated amphiphilic environment for the recognition of amino acids. We anticipated that a urea group, having two points of interaction, would serve as a binding site efficient enough that the modification of just one of the 21 hydroxy groups of β-CD would be necessary to provide well defined receptors.

We hypothesized that a decorating β-CD with a side-chain having both hydrophilic (urea) and lipophilic (phenyl and isobutyl) moieties will give an appropriate environment for efficient binding of amino acids, providing a receptor far more effective than underivatized β-CD. Having such a tool in hand the recognition or separation of amino acids would not have to be based on repetitive processes (as in chromatography or electrophoresis) but a single interaction would be efficient enough for molecular discrimination of particular pairs of substances.

<H1>2. Experimental

<H2>2.1. General

All solvents were used as received, unless stated otherwise. Purification of products was performed using chromatography on silica gel (Merck Kieselgel 60, 230-400 mesh) with mixtures of hexane/ethyl acetate, methanol/dichloromethane, acetonitrile/water/ammonia or using gel filtration on styrene resin (Diaion® HP-20) with water/methanol gradient. Thin-layer chromatography (TLC) was performed on silica gel plates (Merck Kieselgel 60 F_{254}). Visualization of the developed chromatogram was accomplished using UV light or ninhydrin and cerium molybdate stains. Reported NMR spectra were recorded in $CDCl_3$ or $(CD_3)_2SO$ using a Varian Unity Plus 200 MHz and Agilent 300 MHz and 500 MHz spectrometers. Chemical shifts of ^1H NMR and ^{13}C NMR are reported as δ values relative to TMS (δ = 0.00) and $CDCl_3$ (δ = 77.0) or $(CD_3)_2SO$ (δ = 39.5), respectively. The following abbreviations are used to indicate the multiplicity: s - singlet; d - doublet; t - triplet; q - quartet; m – multiplet; dm - doublet of multiplets. Mass spectra were measured on a Shimadzu LCMS-IT-TOF using ESI technique.

Distilled water (>18 MΩ/cm grade) was supplied by Mili-Q water system. Phosphate buffers were prepared by mixing specified amounts of $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot H_2O$ and subsequent titration with NaOH, to desired pH, using Elmetron CP-401 pH-meter.

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Dialysis was performed using Spectrum Labs Biotech Grade Cellulose Ester Dialysis Membrane, MWCO: 100-500 Da, preserved with 0.05% sodium azide.

<H2>2.2. Synthesis

Mono-2-propargyl-β-CD (1)
To a solution of dry β-CD (10 g, 10 mmol) in DMSO (100 mL) LiH (1 equiv.) was added and stirred overnight, until the solution became clear. Next, propargyl bromide (1 equiv.) and LiI (a few mg) were added and the mixture was stirred at 55°C for 5 h, avoiding light exposure. The solvent was evaporated under reduced pressure, resulting slurry was dissolved in water (20 mL) and precipitated with acetone (500 mL). Solid residue was filtered, air-dried and then the resulting powder was dissolved in water and passed through styrene resin (Diaion® HP-20) using gradient of water/methanol (0 to 10%) as solvent. Prior to use, the resin was conditioned in methanol for 1h and rinsed with water. Fractions containing pure product were evaporated under reduced pressure giving 4.1g of **1** (3.5 mmol, 35%). COSY, TOCSY, HSQC and HMBC NMR spectra of **1** are appended in the ESI.
^{1}HNMR (500MHz, DMSO) δ 5.80 (br s, 11H), 4.99 (d, *J* = 3.7 Hz, 1H), 4.82 (br s, 6H), 4.47 (dd, *J* = 15.8 Hz, *J* = 2.4 Hz, 1H), 4.46 (br s, 9H), 4.39 (dd, *J* = 15.8 Hz, *J* = 2.4 Hz, 1H), 3.79 (t, *J* = 9.2 Hz, 1H), 3.65–3.55 (m, 27H), 3.52 (t, *J* = 2.4 Hz, 1H), 3.43–3.40 (m, 2H), 3.37–3.27 (m).
^{13}C NMR (75 MHz, DMSO): δ 102.0–101.0, 100.1, 82.2–81.5, 79.9, 79.1, 77.8, 73.2–71.7, 72.6, 60.0–59.7, 58.7.

HRMS ESI+ m/z: 1195.3756 (MNa^+, $C_{45}H_{72}N_{35}O_3Na^+$ requires 1195.3752).

(S)-O-Tosyl-2-(Boc-amino)-4-methylpentanol
N-Boc-L-Leucinol (2.5 g, 11.5 mmol) was dissolved in dichloromethane (100 mL) and cooled to 0°C. Triethylamine (1.1 equiv.) and p-tosyl chloride (1.05 equiv.) were added and the resulting mixture was stirred overnight (allowing the mixture to warm up to rt). It was next washed with saturated sodium bicarbonate (100 mL) and brine (20 mL). Organic layer was then dried with $MgSO_4$ and evaporated under reduced pressure. The product was purified on silica gel using a 10:90 ethyl acetate:hexane mixture resulting with 2.91g of **2** (7.8 mmol, 68%).
Careful purification of the aminoalcohol **2** before the tosylation process is crucial, as mixed anhydride (formed prior to the reduction step) acts as an inhibitor.

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.4 Hz, 2H), 7.35 (d, *J* = 8.5 Hz, 2H), 4.70 – 4.34 (m, 1H), 3.97 (dd, *J* = 14.4, 11.0 Hz, 2H), 2.43 (d, *J* = 9.6 Hz, 3H), 1.64 (s, 2H), 1.50 – 1.35 (m, 9H), 1.14 (dd, *J* = 8.6, 5.9 Hz, 1H), 1.00 – 0.70 (m, 6H).
(S)-2-(Boc-amino)-1-azido-4-methylpentane (3)
2 (2.35 g, 5.9 mmol) was dissolved in DMF (20 mL) and NaN_3 (3 equiv.) was added. The addition of more than 3 equivs of NaN_3 caused the elimination of the tosylated aminoalcohol due to the basicity of azide ions in DMF. The resulting mixture was stirred at 60°C for 24 h. It was then poured into water (300 mL) and extracted with diethyl ether. The combined organic layers were then evaporated under reduced pressure. The oily residue was dried under vacuum until it solidified resulting with 2.26 g (4.7 mmol, 80%) of **3**.
^{1}H NMR (300 MHz, $CDCl_3$) δ 4.56 – 4.39 (m, 1H), 3.80 (d, *J* = 4.0 Hz, 2H), 3.54 – 3.10 (m, 2H), 1.71 – 1.51 (m, 1H), 1.49 – 1.40 (m, 9H), 0.92 (dd, *J* = 6.6, 2.2 Hz, 6H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 155.40, 79.75, 70.81, 55.35, 51.03, 48.69, 44.53, 41.48, 28.49, 25.25, 24.88, 23.08, 22.26.

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Synthesis of urea 4

(S)-2-(Boc-amino)-1-azido-4-methylpentane (600 mg, 2.5 mmol) was dissolved in 1:1 mixture of TFA and dichloromethane (5 mL) and the resulting mixture was stirred for 1 h, after which all volatiles were evaporated. In order to neutralize residual TFA and deprotonate the amine, the solid residue was treated with triethylamine (1 ml), which excess was subsequently evaporated. Dichloromethane (20 mL) was added and the solution was treated with the respective isocyanate (1 equiv., 2.5 mmol). After 24 h of stirring at rt the mixture was washed with saturated sodium bicarbonate (50 mL) and brine (50 mL). The organic layer was dried with $MgSO_4$ and the product was purified on silica gel using a 3:97 mixture of methanol:dichloromethane, yielding, after evaporation and vacuum drying, urea **4**.

(S)-1-(1-Azido-4-methylpentan-2-yl)-3-phenylurea (4a)

Yield: 590 mg (2.25 mmol, 90%)

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.49 – 7.19 (m, 5H), 7.06 (d, *J* = 6.9 Hz, 2H), 4.15 – 3.93 (m, 1H), 3.42 (ddd, *J* = 37.1, 12.1, 4.0 Hz, 2H), 2.14 (s, 2H), 1.64 (dt, *J* = 13.0, 7.5 Hz, 1H), 0.93 (d, *J* = 6.7 Hz, 6H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 187.87, 138.38, 129.51, 124.06, 121.33, 55.67, 48.48, 41.51, 25.03, 22.35.

HRMS ESI+ m/z: 262.1609 (MH^+, $C_{13}H_{19}N_5OH^+$ requires 262.1662).

(S)-1-(1-Azido-4-methylpentan-2-yl)-3-(4-nitrophenyl)urea (4b)

Yield: 705 mg (2.3 mmol, 92%)

^{1}H NMR (200 MHz, $CDCl_3$) δ 8.14 (d, *J* = 9.1 Hz, 1H), 7.92 (s, *J* = 9.6 Hz, 1H), 7.54 (d, *J* = 9.1 Hz, 1H), 5.59 (d, *J* = 8.4 Hz, 1H), 4.23 – 3.96 (m, 1H), 3.45 (ddd, *J* = 34.1, 12.3, 4.3 Hz, 1H), 1.67 (tt, *J* = 13.3, 6.5 Hz, 1H), 1.41 (dd, *J* = 9.5, 5.9 Hz, 2H), 0.93 (dd, *J* = 6.5, 2.0 Hz, 6H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 154.47, 145.90, 142.02, 125.51, 117.97, 55.56, 48.28, 41.43, 29.83, 24.98, 23.01, 22.23.

HRMS ESI+ m/z: 307.1548 (MH^+, $C_{13}H_{18}N_6O_3H^+$ requires 307.1513).

(S)-1-(1-Azido-4-methylpentan-2-yl)-3-(3,5-di(trifluoromethyl)-phenyl)urea (4c)

Yield: 894 mg (2.25 mmol, 90%)

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.76 (s, 2H), 7.54 (s, 1H), 7.45 (s, 1H), 5.30 (d, J = 8.6 Hz, 1H), 4.06 (s, 1H), 3.55 (dd, J = 12.4, 3.7 Hz, 1H), 3.37 (dd, J = 12.2, 4.6 Hz, 1H), 1.82 (bs, 1H), 1.75 – 1.53 (m, 1H), 1.53 – 1.31 (m, 2H), 0.93 (dd, J = 6.4, 1.8 Hz, 6H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 154.77, 140.24, 132.73, 120.46, 118.81, 116.33, 55.60, 48.35, 41.44, 25.01, 22.90.

Synthesis of 1-(3,5-Bis(trifluoromethyl)phenyl)-3-(2-bromoethyl)urea (6)

2-Bromoethylamine hydrobromide (1 g, 4.88 mmol), 3,5-bis(trifluoromethyl)phenyl isocyanate (0.93 mL, 5.37 mmol) and triethylamine (0.748 mL, 5.37 mmol) were dissolved in dichloromethane (20 mL) at 0°C and the resulting mixture was stirred for 4h, after which all volatiles were evaporated. The product was purified on silica gel using a 30:70 mixture of ethyl acetate:hexanes, yielding, after evaporation and vacuum drying, 0.9 g (2.34 mmol, 48%).

^{1}H NMR (400 MHz, DMSO) δ 9.42 (s, 1H), 8.08 (s, 2H), 7.56 (s, 1H), 6.73 (t, J = 5.2 Hz, 1H), 3.61 – 3.45 (m, 4H).

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^{13}C NMR (101 MHz, DMSO) δ 155.11, 142.83, 131.26, 130.94, 117.81, 114.17, 41.76, 33.47.

Synthesis of 1-(3,5-Bis(trifluoromethyl)phenyl)-3-(2-azidoethyl)urea (7)

1-(3,5-Bis(trifluoromethyl)phenyl)-3-(2-bromoethyl)urea (900 mg, 2.34 mmol) was dissolved in DMF (40 mL) at rt and sodium azide (385 mg, 5.93 mmol), and a few mg of KI were added. The resulting mixture was stirred for 24 h at 80°C. It was then poured into water (300 mL) and extracted with diethyl ether. The combined organic layers were then evaporated under reduced pressure and purified on silica gel using a 30:70 mixture of ethyl acetate:hexanes, affording 450 mg (1.3 mmol, 55%) of azide.
^{1}H NMR (400 MHz, DMSO) δ 9.34 (s, 1H), 8.09 (s, 2H), 7.54 (s, 1H), 6.67 (t, J = 5.6 Hz, 1H), 3.42 (t, J = 5.8 Hz, 2H), 3.32 (q, J = 5.4 Hz, 2H).

^{13}C NMR 13C NMR (101 MHz, DMSO) δ 154.79, 142.41, 130.75, 130.43, 117.34, 113.58, 50.38, 38.99.

Synthesis of receptors 8a-d

Mono-2-propargyl-β-CD **1** (1 g, 0.85 mmol) and the selected azide (1.1 equiv.) were dissolved in a 4:1 mixture of DMSO and water (30 mL). Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, *ca*. 20 mg) was added. The mixture was deaerated by passing argon through the solution. $CuSO_4$ (0.1 equiv., aqueous solution) and sodium ascorbate (0.2 equiv., aqueous solution) were added dropwise into the stirred reaction mixture, which was further stirred for 24 h at 50°C. After the removal of the catalyst (via the addition of ammonia to the reaction mixture and its passage through a thin pad of silica gel) the solvents were evaporated under reduced pressure and the crude product was dissolved in water (5 mL) and precipitated with acetone (500 mL). The precipitate was filtered and air dried.
(S)-2-*O*-((1-(2-(3-Phenylureido)-4-methylpentyl)-1*H*-1,2,3-triazol-4-yl)methyl)-β-CD (**8a**)

^{1}H NMR (500 MHz, DMSO) δ 8.40 (s, 1H), 8.07 (s, 1H), 7.36 (d, J = 7.6 Hz, 2H), 7.25 – 7.15 (m, 2H), 6.89 (t, J = 7.3 Hz, 2H), 6.08 (s, 3H), 5.90 (d, J = 6.5 Hz, 3H), 5.80 – 5.59 (m, 7H), 4.94 – 4.74 (m, 7H), 4.66 – 4.54 (m, 2H), 4.53 – 4.36 (m, 7H), 4.09 (dd, J = 16.5, 4.2 Hz, 2H), 3.74 – 3.48 (m, 21H), 3.32 (s, 22H), 1.73 – 1.59 (m, 2H), 1.32 – 1.11 (m, 1H), 0.86 (dd, J = 16.2, 6.6 Hz, 6H).
^{13}C NMR (126 MHz, DMSO) δ 128.64, 124.93, 121.14, 117.70, 101.93, 101.53, 100.27, 82.07, 81.59, 81.52, 81.21, 79.43, 73.25, 73.04, 72.95, 72.67, 72.48, 72.38, 72.22, 72.02, 71.75, 71.69, 64.42, 60.02, 59.91, 53.52, 47.41, 42.88, 36.67, 29.02, 28.70, 24.12, 23.18, 21.64.
HRMS ESI+ m/z: 1434.5519 (MH^+, $C_{58}H_{91}N_5O_{36}H^+$ requires 1434.5517); 1456.5321 (MNa^+, $C_{58}H_{91}N_5O_{36}Na^+$ requires 1456.5336).

(S)-2-*O*-((1-(2-(3-(4-Nitrophenyl)-ureido)-4-methylpentyl)-1*H*-1,2,3-triazol-4-yl)methyl)-β-CD (8b)
^{1}H NMR (500 MHz, DMSO) δ 9.25 (s, 1H), 8.11 (t, *J* = 13.8 Hz, 3H), 7.53 (dd, *J* = 53.5, 7.4 Hz, 2H), 6.48 (s, 1H), 6.09 – 5.47 (m, 11H), 4.99 – 4.66 (m, 7H), 4.66 – 4.30 (m, 7H), 4.09 (d, *J* = 43.9 Hz, 1H), 3.81 (t, *J* = 9.3 Hz, 1H), 3.75 – 3.46 (m, 22H), 3.46 – 3.09 (m, 22H), 1.64 (s, 2H), 1.27 (dd, *J* = 53.3, 40.5 Hz, 3H), 0.87 (dd, *J* = 17.3, 5.6 Hz, 6H).
^{13}C NMR (126 MHz, DMSO) δ 128.76, 127.76, 125.14, 124.97, 116.98, 101.96, 101.54, 100.32, 84.40, 82.12, 81.63, 81.57, 81.46, 81.17, 79.46, 73.30, 73.07, 72.97, 72.71, 72.52,

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72.40, 72.26, 72.04, 71.79, 71.70, 64.43, 60.06, 59.91, 59.79, 53.35, 47.72, 24.16, 23.18, 21.62.
HRMS ESI+ m/z: 1479.5350 (MH^+, $C_{58}H_{90}N_6O_{38}H^+$ requires 1479.5367); 1501.5163 (MNa^+, $C_{58}H_{90}N_6O_{38}Na^+$ requires 1501.5187).

(S)-2-*O*-((1-(2-(3-(3,5-Di(trifluoromethyl))-phenyl)-ureido)-4-methylpentyl)-1*H*-1,2,3-triazol-4-yl)methyl)-β-CD (**8c**)

^{1}H NMR (500 MHz, DMSO) δ 9.21 (s, 1H), 8.23 – 7.80 (m, 3H), 7.52 (s, 1H), 6.49 (s, 2H), 6.19 – 5.37 (m, 11H), 5.01 – 4.67 (m, 7H), 4.66 – 4.31 (m, 7H), 4.06 (s, 1H), 3.81 (t, J = 9.4 Hz, 1H), 3.75 – 3.46 (m, 23H), 3.46 – 3.09 (m, 25H), 1.57 (d, J = 68.8 Hz, 2H), 1.43 – 1.09 (m, 3H), 0.86 (dd, J = 19.4, 6.4 Hz, 6H).
^{13}C NMR (126 MHz, DMSO) δ 154.36, 143.41, 142.37, 130.73, 130.48, 125.02, 124.45, 122.28, 117.37, 101.96, 101.56, 100.26, 82.09, 81.58, 81.22, 79.35, 73.23, 73.06, 72.95, 72.68, 72.51, 72.40, 72.24, 72.04, 71.70, 64.32, 59.93, 53.28, 47.81, 24.14, 23.15, 21.64, 0.14.
HRMS ESI+ m/z: 1570.5255 (MH^+, $C_{60}H_{89}N_5O_{36}F_6H^+$ requires 1570.5264).

2-*O*-((1-(2-(3-(3,5-Di(trifluoromethyl))-phenylureido)-ethyl)-1*H*-1,2,3-triazol-4-yl)methyl)-β-CD (**8d**)

^{1}H NMR (500 MHz, DMSO) δ 9.53 (s, 1H), 8.15 (d, J = 7.7 Hz, 1H), 8.08 (s, 2H), 7.55 (s, 1H), 6.72 (t, J = 5.6 Hz, 1H), 5.99 – 5.83 (m, 2H), 5.83 – 5.60 (m, 11H), 4.94 – 4.76 (m, 6H), 4.60 – 4.33 (m, 14H), 3.82 (t, J = 9.5 Hz, 1H), 3.76 – 3.49 (m, 20H), 3.48 – 3.15 (m, 22H).
^{13}C NMR (126 MHz, DMSO) δ 155.32, 144.50, 144.12, 142.84, 131.43, 131.17, 130.91, 130.65, 127.04, 124.87, 124.82, 122.70, 120.53, 117.73, 114.08, 109.99, 102.37, 102.03, 100.60, 82.43, 82.01, 81.92, 81.69, 79.87, 73.61, 73.49, 73.38, 73.14, 72.92, 72.81, 72.65, 72.46, 72.22, 72.12, 64.88, 60.35, 49.70.
HRMS ESI+ m/z: 1514.4606 (MH^+, $C_{56}H_{82}N_5O_{36}F_6H^+$ requires 1514.4644).

<H2>2.3. Isothermal Titration Calorimetry

Prior to the binding measurements, compounds **8** were purified via dialysis. 500 mg of compound **8** was dissolved in 10 mL of water and placed in a dialysis tube. The sealed tube was placed in 3 L of water, which was stirred during the purification process. Water was replaced after 2, 12 and 24 hours. Finally the content of the bag was lyophilized.
Isothermal titration calorimetry (ITC) was performed using TA Instruments NanoITC calorimeter. The solutions of all hosts and guests were prepared in 50 mM phosphate buffer (pH = 8). In a typical experiment the calorimeter cell (0.949 mL) was filled with 2 mM solution of host **8**. The cell solution was stirred at 300 rpm. 10-16 mM amino acid solution (250 µl) was titrated into the calorimeter cell in 25 injections, with 300 s intervals between injections, at 25^{o}C. Measured raw heats were corrected with the heat of dilution of amino acid titrated into phosphate buffer. Heats were fitted to n-independent-sites binding model (H + nG = HG_n), which allowed to determine the K_a (affinity constant), ΔH (effective molar enthalpy of the reaction) and n (stoichiometry of guest:host interaction). Mean parameters and their uncertainties were calculated as the average of at least 3 independent experiments.
Model:

ITC measurements were conducted by the titration of amino acids into solutions of receptors. The concentrations were restricted by the heat response upon titration and solubility of the receptors. We have applied a model where one of the interacting species has n identical and independent binding sites (n-independent-sites binding model), which allowed to compare the strength of the interaction between the receptor and particular guests (amino acids).

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Interaction parameter $\bar{\nu}$ can be defined as a number of moles of the guest (G) bound to one mole of the host (H), or in terms of concentration:

$$\bar{\nu} = \frac{[G]_b}{[H]_T} = \frac{\sum_{i=1}^{n} i \cdot \beta_i \cdot [G]^i}{\sum_{i=0}^{n} \beta_i \cdot [G]^i}$$

Transformation of this relation allows to link $\bar{\nu}$ with the number of active sites of the host (n), the microscopic affinity constant K (defined for elementary reaction G + H = GH) and the unbounded guest concentration:

$$\bar{\nu} = \frac{[G]_B}{[H]_T} = \frac{nK[L]}{1+K[L]}$$

Therefore, after transformations the injection heat exchange can be infered by estimating the derivative:

$$\frac{1}{V_C} \cdot \frac{dQ}{d[G]_T} \approx \frac{1}{V_C} \cdot \frac{\Delta Q}{\Delta [G]_T} = \frac{\Delta H_{app}}{2} \left[1 - \frac{1 + [G]_T - nK[H]_T}{\sqrt{\left(1 + K[G]_T + nK[H]_T\right)^2 - 4nK^2[G]_T[H]_T}} \right]$$

Values of K, ΔH_{app} and n were calculated using nonlinear regression program NanoAnalyzer supplied by TA Instruments. Errors in estimation were calculated using Monte Carlo analysis and included in the analysis of measurement uncertainties.

<H2>2.4. Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were conducted with a Zetasizer nanoseries Nano-ZS-ZEN3600 instrument equipped with a laser of 633 nm using a ZEN-112 cuvette at 25 °C. For each measurement the number of scans was 10, the run duration was 35 s, the equilibration time was 140 s, and the time delay was 4 s. Before measurements, the samples were incubated for 2 h, then filtered through a 0.45 μm PTFE filter, sonicated for 1 min, and finally filtered again.

<H1>3. Results and Discussion

<H2>3.1. Synthesis

Our aim in this study was to synthesize a class of compounds that can be easily obtained from a simple and readily available β-CD derivative. We intended to incorporate moieties containing both polar and hydrophobic fragments that would efficiently interact with model amino acid guests. We previously reported that C-6 modified compounds are more likely to undergo intramolecular self-association, which may hamper guest binding inside the cavity (Chmurski, Stepniak, & Jurczak, 2016). In consequence, we decided to derivatize one of the secondary OH groups of β-CD. Previous studies have reported that secondary-site derivatization towards the synthesis of carbamates, esters, and ethers, results in moderate yields (Krois, Brecker, Werner, & Brinker, 2004; Martina et al., 2010; Suzuki & Nozoe, 2002). Derivatives containing aromatic substituents have been obtained via amidation or esterification reactions (Fukudome, Oiwane, Mori, Yuan, & Fujita, 2004; Hanessian, Benalil, & Viet, 1995; Hao, Tong, Zhang, & Gao, 1995; McAlpine & Garcia-Garibay, 1996; Mortellaro, Hartmann, & Nocera, 1996). Again, yields below 25% have been reported, suggesting that it is not the character of the substituent residue, but the reaction mechanism

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that is responsible for the low efficiency of monosubstitution processes. Some β-CDs substituted with charged species (such as anionic sulfonates, EDTA derivatives, protonated amines (Laferriere, Benalil, & Hanessian, 1995; Michels, Huskens, & Reinhoudt, 2002; Wenz & Höfler, 1999; Yan, Atsumi, Yuan, & Fujita, 2002)) have also been synthesized. Usually, in these systems, the β-CD moiety is responsible for interaction with the hydrophobic part of the guest, while the side-chain bearing the polar group attracts ionized parts of the interacting molecule. However, some residues attached to the upper rim of β-CD can also form self-inclusion complexes. All the derivatizations described above are characterized by low yields due to the many resulting side-products and the necessity of low efficiency purification. Usually, the synthesis is limited by the presence of reactive groups in side-arms introduced to the β-CD structure. An alternative, indirect way of derivatization is a perbenzylation-debenzylation protocol (Wang et al., 2014). In view of these facts, a Sharpless click chemistry approach (copper(I)-mediated azide-alkyne coupling – CuAAC) suggests itself as a versatile and efficient method for β-CD derivatization (Aime et al., 2009; Faugeras et al., 2012; Martos-Maldonado, Quesada-Soriano, Casas-Solvas, García-Fuentes, & Vargas-Berenguel, 2012; Rostovtsev, Green, Fokin, & Sharpless, 2002). It would allow us to minimize the number of reaction steps performed on cyclodextrins as the more demanding synthetic substrate, eliminating low yield procedures.

Due to the relatively higher acidity of the 2-OH groups (as compared to the others) we deprotonated β-CD with lithium hydride; further treatment with propargyl bromide, according to the known procedure (Casas-Solvas et al., 2009), gave mono-2-propargyl-β-CD (**1**) (Scheme 1), which was then purified by gel-filtration on styrene resin. The procedure allowed to obtain monosubstituted derivative (DS = 1). The propargyl substituent was positioned solely at C-2 position of glucose subunit (for structural elucidation see: Appendix A.)

Next we prepared azide compounds of type **4** (Scheme 2a), which were to be partners for **1** in the CuAAC method. Due to the anion binding properties of the urea group, we decided to apply it as a pH-independent donor of hydrogen bonds, thus making it possible to synthesize a class of receptors providing a metal-free binding of anions.

In order to introduce two additional attachment points into the target receptor, we modified hydrophobic amino acid so it contained a urea moiety and a nonpolar fragment. The replacement of the carboxyl group by the azide gave the intermediate for the conjugation reaction. For this purpose we used N-Boc-L-leucine which was treated with *iso*-butyl chloroformate, the resulting mixed anhydride being reduced with $NaBH_4$ to give aminoalcohol **2**. Next, aminoalcohol **2** was tosylated and subsequently treated with sodium azide, giving compound **3**. Careful purification of aminoalcohol **2** before the tosylation process is crucial, as mixed anhydride (formed prior to the reduction step) acts as an inhibitor. The addition of more than **3** equivs of NaN_3 caused elimination of tosylated aminoalcohol due to the basicity of azide ions in DMF. The cleavage of the Boc group was carried out in a 1:1 TFA:CH_2Cl_2 mixture. The resulting crude aminium trifluoroacetate was reacted with aromatic isocyanate under basic conditions, to yield urea-azide compound **4**.

The conjugation reaction of **1** with **4** was carried out under conditions we have described previously (Scheme 3) (Chmurski, Stepniak, & Jurczak, 2015). Using a 1:5 (v/v) water:DMSO mixture allowed us to apply equimolar amounts of **1** and **4**. Addition of 5 mol% of copper catalyst provided reaction completion after 24 h. After removal of the catalyst (via the addition of ammonia to the reaction mixture and its passage through a thin pad of silica gel) and addition of acetone, the final product precipitated.

We synthesized three compounds of type **8**, all of their side-arms containing a bulky substituent (*sec*-butyl) and a urea grouping of varying acidity – due to the presence of phenyl (**8a**), p-NO_2-phenyl (**8b**), or 3,5-di-CF_3-phenyl (**8c**) moieties.

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We also prepared an analog **8d**, bearing an achiral arm, in order to assess the role of the isobutyl group in complexation process and the influence of the side-chain chiral center on the chiral recognition of amino acids (Scheme 2b). Bromoethylamine (**5**) was reacted with a selected isocyanate and the resulting bromourea **6** was transformed into azidourea **7**, then subsequently subjected to CuAAC reaction with **1**.

<H2>3.2. Binding measurments

Next, we investigated the complexing properties of compounds **8a-d** towards representative hydrophobic L-amino acids. As we stressed above, β-CD derivatives would be the most promising since a cavity of this size allows the formation of inclusion complexes with a vast majority of substances possessing aromatic side-chains or hydrophobic moieties (Rekharsky & Inoue, 1998). However, β-CD:L-phenylalanine complex has low stability in water (K_a is *ca*. 18 M^{-1} in distilled water and 107 M^{-1} in a buffered solution (pH 11.3)). Several attempts have been made to modify the β-CD structure in order to enhance these affinities. For example, Tabushi, Kuroda & Mizutani (1986) regioselectively incorporated two ionic residues that provided ammonium and carboxylate binding sites. Although this β-CD derivative exhibits moderate chiral recognition of L-tryptophan, the affinity constant still does not exceed 100 M^{-1}. Because of both the high polarity and good solubility of amino acids in water, multiple intermolecular interactions have to be formed by the receptor in order to compete with the hydrogen bonding network and hydration shell stability (Whitesides & Krishnamurthy, 2005). Usually this is achieved by the synthesis of receptors based on CD, calixarene, and cucurbituril moieties or the addition of a metallic center able to introduce metal-carboxylate or metal-amine interactions. Coordination complexes with affinity constants up to 7.4×10^7 M^{-1} for L-phenylalanine:Cu^{2+}:amino-β-CD-derivative ternary complexes were reported as the most effective organometallic systems (Brown, Coates, Easton, & Lincoln, 1994; Hoof, Russell, Mcnamara, & Darcy, 2000). Another way of making complexes more stable is via the derivatization of the amino acid to increase its hydrophobicity. However, this approach cannot be considered when the receptor is meant to function in an environment that limits chemical modifications of substrates.

Receptors **8** were designed to operate in buffered aqueous solutions, so we decided to use isothermal titration calorimetry (ITC) as the tool useful for simultaneous assessment of the thermodynamic parameters, affinity constants and stoichiometry of the interacting species (Czekalla et al., 1998; Schmidtchen, 2002). We were unable to conduct NMR titrations due to the very small changes in shifts upon addition of the guest into the host solution. UV-VIS spectrometry could also not be used as a general method for all the pairs of guests and hosts. The overlapping of absorption bands of guests and hosts in some experiments caused problems with fitting to the appropriate model. ITC, on the other hand, provided very reliable data for the whole assay. Within the receptor solubility range, the detected heats were high enough and the heat data showed a sigmoidal curvature (Figure 1).

Titration experiments carried out at pH 8 (50 mM phosphate-buffered solution) showed that binding constants vary for amino acids with different hydrophobic side-chains (Figure 2). For all amino acids, binding to receptors **8a-c** was much stronger than binding to underivatized β-CD. L-Alanine formed such complexes with moderate strength (2 300 to 5 000 M^{-1}), while the more hydrophobic L-phenylalanine exhibited much greater affinity to all receptors (up to 22 400 M^{-1}). The synthesized CDs seemed to interact more strongly with an even larger hydrophobic amino acid – L-tryptophan. Interestingly, more acidic urea moieties (in **8b** and **8c**) contributed to the formation of stronger complexes, indicating that hydrogen bonding of this group plays a crucial role in the presented system. Reference compound **8d** showed no interaction with any of the amino acids investigated (Ka < 10 M^{-1}), showing that the bulky substituent is also a necessary part of the complexing system.

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The ITC method also provided information about the thermodynamics and stoichiometry of the complexation reaction (Table 1).

The enthalpies of all the reactions studied were slightly positive. The complex formation is entropy-driven and this thermodynamic parameter mostly determines the equilibrium, since the enthalpy values are similar for each amino acid (around 4.4 kJ/mol). In most cases in the literature, the formation of CD inclusion complexes exhibits both negative enthalpy and entropy. In the present study, however, the signs of both parameters were positive, as should be typical of a classic hydrophobic effect. Therefore, we suspected a complexation mechanism different from the inclusion process. The much stronger complexation of amino acids by receptors **8a-c**, in comparison to underivatized β-CD and compound **8d**, showed that the side-chain plays an important role and suggested that both its elements (the hydrogen bond donor and the bulky hydrophobic substituent) are responsible for the complexation.
<H2>3.3. Structural studies

Amphiphilic derivatives of CDs are known to form aggregates which can influence their properties and mode of interactions (Chmurski, Stępniak & Jurczak 2016, Tran et al., 2014). In the case of similar CD derivatives we have also observed formation of intra- and intermolecular self-inclusion complexes, thus affecting the stoichiometry of the guest binding. Therefore, we carried out DLS measurements to assess the degree of aggregation of compounds **8a-d** in a 50 mM phosphate buffer (pH 8) as an imitation of cell physiological conditions. This allowed us to identify polydispersed solutions of particles with average diameter of 190, 142, and 122 nm for **8a**, **8b**, and **8c**, respectively. The aggregation process occurred also in diluted solutions (0.5 mM). Interestingly, underivatized β-CD and compound **8d** did not form any aggregates. This provided us with evidence that both urea moiety and the hydrophobic side-chain are important for interactions between obtained molecules and that both affect self-association of these compounds. Provided that diameter of β-CD is about 1.5 nm, several dozens of molecules have to be involved in formation of the observed aggregates. To elucidate the mode of self-interaction between molecules of compounds **8** we have conducted ROESY experiments of **8c** in D_2O (Figure 3). We have found crosspeaks between aromatic protons and CD cavity protons (7.47 with 3.97, 3.82 and 7.80 with 3.91, 3.75 ppm, respectively). Moreover, we identified interactions between the isobutyl group and C-3 and C-5 protons of CD (3.72-3.92 ppm). This clearly indicates that both substituents of the side-chain participate in inclusion complex formation. We did not observe crosspeaks corresponding to interaction between the triazole ring and CD protons that would suggest intramolecular inclusion process. This is understandable considering the results of DLS measurements. Most probably compounds **8a-c** form aggregates via intermolecular binding of amphiphilic side-chains by CD cavities. Presented data do not determine what higher ordered structures are formed during the self-association process. Similar results were obtained for alkaline aqueous solution of compound **8c**. This shows that the hydrophobic side-chain is crucial in the self-association process.
This hypothesis is supported by the stoichiometry of the reaction measured with ITC. It can be inferred that approximately two molecules of a receptor of type **8** interact with one molecule of an amino acid, although the *n* parameter varies between 0.35 and 0.92.
For such a stoichiometry we suppose that receptors of type **8** form shallow intermolecular inclusion complexes, leading to aggregates owing to the presence of the hydrophobic fragment of the side-chain. The thermodynamic data suggest that a binding pocket is created in such a dimeric species, which behaves like a lipophilic environment isolated from the bulk solvent (Figure 4). In such a construct, urea moieties provide undisturbed hydrogen bonding allowing efficient interactions with the anionic guests, which may be the reason for such high affinities in comparison to underivatized β-CD.

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The ROESY experiments performed after addition of amino acid to the receptor solution showed no signs of interaction between the protons of the CD cavity and either the receptor's side-chain or the amino acid. This seems to confirm the non-inclusion nature of the complex formed.

<H1>4. Summary

In conclusion, we have synthesized amphiphilic monoderivatives of β-CD which are equipped with a bifunctional side-chain responsible for the formation of active aggregates and tight guest binding, due to presence of isopropyl and urea moieties, respectively. The concept of the synthesis was to limit the number of reactions performed on CD, which lead to high efficiency of the process. Receptors obtained in such a manner appeared to form aggregates. The amphiphilic binding site is formed in the at least dimeric construct allowing the urea moieties to act effectively, isolated from the bulk solvent. Typical entropy-driven complexation is observed for the interaction with L-amino acids, proving that a classic hydrophobic effect is responsible, rather than inclusion complex formation.

We can confirm that the designed β-CD decorated with a amphiphilic side-chain provide an environment for the efficient binding of amino acids, however, the mode of interaction was different than expected. Nevertheless, this approach allows hydrophobic L-amino acids to be bound with thousand-fold greater strength than for the previously reported native β-CD.

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Appendix A.

Supplementary data Supplementary data associated with this article can be found, in the online version.

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<Figure>Figure 1. Representative thermogram of ITC experiment L-Phe was titrated to **8c**.

<Figure>Figure 2. Affinity constants of receptors **8a-c** and L-amino acid complexes (estimation uncertainties are marked).

<Figure>Figure 3. ROESY spectrum of **8c** in D_2O.

<Figure>Figure 4. Illustration of the proposed binding mechanism.

<Figure>Scheme 1. Preparation of mono-2-propargyl-β-CD (**1**)

<Figure>Scheme 2. Synthesis of receptor side-chains.

<Figure>Scheme 3. Synthesis of receptors **8**

<Table>Table 1. Thermodynamic data for L-amino acid and receptor **8a-c** complexes[a]

	K (10^3 M^{-1})[b]	*n*[c]	*ΔH* (kJ/mol)	*ΔS* (kJ/mol)
	8a			
Ala	2.3±0.4	0.509±0.039	2.99±0.32	74.4
Phe	7.0±1.6	0.380±0.050	4.18±0.79	87.6
Trp	9.8±5.6	0.349±0.092	4.02±2.51	89.8
	8b			
Ala	2.1±0.2	0.773±0.030	4.89±0.27	79.8
Phe	20.2±1.9	0.481±0.006	4.9±0.09	98.8
Trp	34.4±2.0	0.393±0.003	5.42±0.07	105.0
	8c			
Ala	5.0±0.9	0.922±0.026	3.72±0.28	83.3
Phe	22.4±1.4	0.520±0.002	5.51±0.07	101.7
Trp	52.4±3.0	0.457±0.002	4.46±0.05	105.3

[a] Aa (250 μL, 10-16 mM) was titrated to **8** (949 μL, 2 mM), stirred at 300 rpm. [b] Results fitted to *n*-independent-sites binding model (where *K* is a microscopic affinity constant). [c] Stoichiometry of the interaction.

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