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Cyclodextrin-drug conjugates – synthesis and study of their properties
Konjugáty cyclodextrin-léčivo – syntéza a studium jejich vlastností

Diploma thesis

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Prague, 2024

DECLARATION

Herewith I declare that I have written my master's thesis by myself and that all sources are listed in the bibliography. Neither this work nor its significant part was used to obtain other academic titles.

Prague, 22.08.2024 Anna-Marie Dušková

ABSTRACT

The diploma thesis deals with the synthesis of conjugates of the antitumor drug 5-fluorouracil with cyclodextrins. Cyclodextrins are connected to the drug by linkers of different lengths, which are unstable in an acidic environment and therefore expected to release the drug in the vicinity of tumor cells. Cyclodextrins serve as a delivery carrier of the mentioned cancerostatic, which complexes the drug and increases its solubility, stability, and bioavailability.

Several synthetic procedures were proposed to obtain suitable conjugates for this purpose. As a part of this work, a total of 12 conjugates of fluorouracil with α-, β- and γ-cyclodextrins were prepared and characterized.

Keywords: cyclodextrin, drug, conjugate

ABSTRAKT

Diplomová práce se zabývá syntézou konjugátů protinádorového léčiva 5-fluoruracilu s cyklodextriny. Cyklodextriny jsou s léčivem spojeny pomocí linkerů různé délky, které jsou v kyselém prostředí nestabilní a proto je očekáváno uvolnění léčiva v okolí nádorových buněk. Cyklodextriny slouží jako nosič zmíněného kancerostatika, které komplexací zvyšují jeho rozpustnost, stabilitu a biologickou dostupnost.

Bylo navrženo několik syntetických postupů k získání konjugátů, které byly uznány vhodnými pro tento účel. V rámci této práce bylo připraveno a charakterizováno celkem 12 konjugátů fluoruracilu s α-, β- a γ-cyclodextriny.

Klíčová slova: cyklodextrin, léčivo, konjugát

LIST OF ABBREVIATIONS

Ac	acetyl
Bn	benzyl
Boc	tert-butyloxycarbonyl
Boc_2O	di-tert-butyl dicarbonate
Boc-Gly	N-tert-butyloxycarbonyl glycine
Bu	butyl
CD	cyclodextrin
Cl-AcOH	chloroacetic acid
d	dublet
dd	doublet of dublets
DCC	*N,N*'-dicyklohexylkarbodiimid
DCM	dichloromethane
DIBAL-H	diisobutylaluminiumhydrid
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMP	Dess-Martin periodinane
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Et	ethyl
EtOAc	ethyl acetate
FA	folic acid

FdUDP fluorodeoxyuridine diphosphate

FR folate receptor

FU 5-fluorouracil

FUA 5'-fluorouracil-1-acetic acid

FUDP 5-fluorouridine diphosphate

FUMP 5-fluorouridine monophosphate

FUTP 5-fluorouridine triphosphate

HP CD hydroxypropylated cyclodextrin

HP-β-CD hydroxypropylated β-cyclodextrin

HRMS high-resolution mass spectrometry

IBX iodoxybenzoic acid

IR infra-red spectroscopy

IUPAC International Union of Pure and Applied Chemistry

LC-MS (ESI) liquid chromatography-electrospray ionization-mass spectrometry

m multiplet

M-β-CD methylated β-cyclodextrin

MALDI-TOF matrix-assisted laser desorption/ionization

Me methyl

MeCN acetonitrile

MS mass spectrometry

NHS N-hydroxysuccinimide

NMR nuclear magnetic resonance

PGE1 prostaglandin E1

Ph	phenyl
RNA	ribonucleic acid
rpm	rounds per minute
rt	room temperature
s	singlet
SBE-CD	sulfobutylether-cyclodextrin
t	triplet
TBDMS	tert-butyldimethyl silyl
TBDMSCl	tert-butyldimethylsilyl chloride
tBu	tert-butyl
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxyl
THF	tetrahydrofuran
TLC	thin-layer chromatography
TS	thymidylate synthase
Ts	tosyl, toluenesulfonyl
UV	ultraviolet
UV-Vis	ultraviolet–visible

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1. INTRODUCTION

Cyclodextrins are cyclic oligosaccharides that contain a different number of α-D-glucose units connected with 1,4-glycosidic bonds arranged in a circle. Due to their low prices and unique structure, they are used in the pharmacy, cosmetic, and food industries.[1] Molecules of cyclodextrins have the shape of a hollow cone. The outer, hydrophilic part of the molecule, is responsible for their ability to dissolve in polar solvents. The inner part of the molecule that forms a cavity is hydrophobic, so it can make inclusion complexes with lipophilic substances, which can then be easily dissolved in polar solvents, such as water. This property of cyclodextrins is probably the most important and allows to use them in pharmaceutical formulations as solubilizers or stabilizers.[2]

One of the disadvantages of using cyclodextrins in drug formulations is that the complexes can disintegrate when conditions (temperature, concentration, pH) change. When the complex was created only based on non-bonding interactions, the drug can be removed from the cavity of the cyclodextrin molecule and lost before delivery to the target structure. This problem can be solved using a covalent linkage between cyclodextrin and drug moiety.[3]

5-Fluorouracil is a drug which is widely used in cancer treatment.[4] However, the use of fluorouracil as an anticancer agent still has several limitations, including, for example, high cytotoxicity to healthy cells, low bioavailability, the need to use high doses of the drug, and poor solubility.[5] These problems can be overcome using a delivery system – cyclodextrins can be applied for this purpose.

2. OBJECTIVES

This work aims to synthesize several conjugates of different types of cyclodextrins with an anticancer drug - fluorouracil. To connect these two parts of the target molecule, linkers of different lengths, rigidity, and different stabilities should be used – the linker must be unstable in an acidic environment, which will allow the drug to be released in the target tissue.

Based on this knowledge, several conjugates were proposed – including conjugates provided with amide, ester, imine, and hydrazide bonds which connect both key parts of the target conjugate molecule.

3. THEORETICAL OVERVIEW

3.1. Cyclodextrins

Cyclodextrins (CD) are cyclic oligosaccharides consisting of α-D-glucopyranose units connected with 1,4-glycosidic bonds. These non-reducing sugars are products of the enzymatic degradation of starch, which is also the reason why they are very cheap[6]. They were first described in the 19th century by Villiers[7], and now they are produced in the amount of several thousand tons per year.[8]

3.1.1. Structure and properties

The molecules of cyclodextrins contain α-D-glucopyranose units in the chair conformation and the resulting molecules have the shape of a hollow cylinder. Primary hydroxyl groups (6-OH) are located at the narrower edge of the molecule, so this side is called the primary side. Secondary hydroxyl groups (2-OH and 3-OH) are situated in the wider part of the cyclodextrin molecule, called the secondary side (Figure 1).

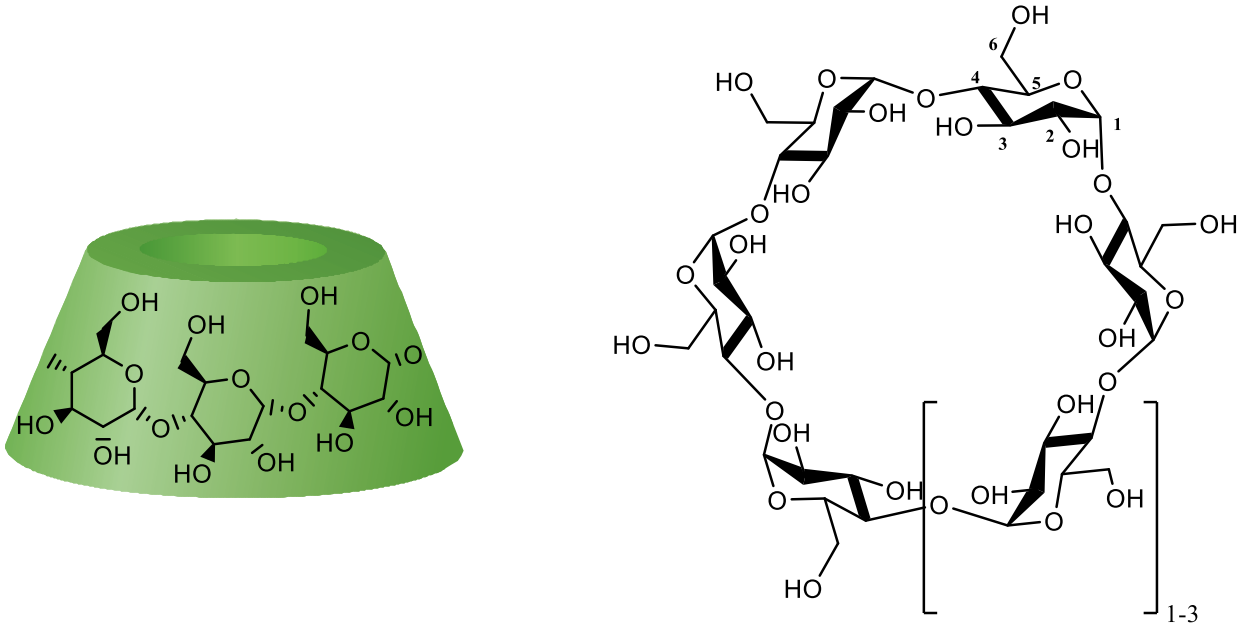


Figure 1: Different ways to depict cyclodextrin molecules.

Depending on the number of glucose units in a molecule, we distinguish three basic types of cyclodextrins, which differ in some properties (Table 1) – the count of sugar units in these compounds is 6-8, and we refer to them as α-, β- and γ-cyclodextrins. In addition, cyclodextrins with a different number of glucose subunits were also prepared[9–11], but their use in practice is limited because of their higher price, complicated synthesis, and worse properties in comparison to α-, β- and γ-cyclodextrins – cyclodextrins with smaller rings have a too small cavity to complex molecules, on the other hand, cyclodextrins with larger rings are deformed. [6]

Table 1: Properties of cyclodextrins.[6,12,13]

	α-cyclodextrin	β-cyclodextrin	γ-cyclodextrin
Number of glucose units	6	7	8
Molecular weight (g/mol)	972	1035	1297
Diameter of cavity (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Outer diameter (Å)	14.6	15.4	17.5
Solubility (25 °C; g/100 ml)	12.8	1.8	25.6
p*K*a (25 °C)	12.33	12.20	12.08

Cyclodextrins are white crystalline compounds soluble in water and some other polar solvents (DMF, DMSO, pyridine). They are stable under basic conditions, but they undergo hydrolysis in acidic environment (under harsher conditions than acyclic oligosaccharides).[14] The rate of hydrolysis differs from the type of cyclodextrin but information published in different articles is contradictory – Kimura *et al*.[15] published study in which γ-CD was found as the most stable due to its flexibility. Compared to this article, Li *et al*. states that α-cyclodextrin is more resistant to acid hydrolysis in comparison to β- and γ-CD.[16]

3.2. Substituted cyclodextrins

Substituted cyclodextrins have one or more hydroxyl groups modified with other functional groups. Currently, more than 11,000 different cyclodextrin derivatives are known[17,18] – it includes at least 2000 derivatives of α-, 8000 derivatives of β- and 1000 derivatives of γ-cyclodextrin. Cyclodextrins are modified because of the improvement of some properties of natural cyclodextrins, but also to improve the stability of cyclodextrin complexes[19]. Some of these derivatives are commercially available.

Depending on reaction conditions, cyclodextrins can be modified at 6-OH, 2-OH, or 3-OH. The most common method for modification of cyclodextrins is electrophilic substitution. Two general approaches can be used to achieve substitution on cyclodextrin moiety[20] – the first approach is called the direct method – it includes work with unprotected cyclodextrin, and the second one uses some protected cyclodextrin derivatives (Scheme 1). The exclusive modification of cyclodextrins is quite complex, caused by a similar reactivity of all hydroxyl groups. Another factor is the possible complexation of the reagent into the cyclodextrin cavity, which may lead to undesirable products.

Without the usage of protecting groups, hydroxyl groups of cyclodextrins can be selectively substituted due to the different properties of particular -OH groups. 6-OH's groups have the highest reactivity caused by their steric accessibility and flexibility compared to C-2 and C-3 –OH groups, which are connected with hydrogen bonds. If an excess weak base is combined with a non-complexing electrophile, C-6 hydroxyls are preferentially attacked, and we get cyclodextrin derivatives substituted at the primary rim.[21] 2-OH's are the most acidic, so substitution in this position can take place using the exact amount of a strong base and non-complexing electrophile.[22] Selective modification at the 3-OH position is usually achieved by complexation.[23,24] This kind of reaction strongly depends on used solvents and reaction time and requires further purification (the side product of this reaction tends to be C-2 substituted cyclodextrins).[25]

Another option to get selectively modified cyclodextrin is to use protecting groups. The most used strategy for modifying cyclodextrins on the primary rim is silylation with tert-butyldimethylsilyl chloride (TBDMSCl). The yield of this reaction is usually about 90%.[26,27] Cyclodextrins protected on the primary rim can then be modified at C-2 and C-3 –OH groups and subsequently deprotected on the primary side.

Selective modification on the secondary side is much more complicated due to the higher reactivity of primary hydroxyl groups. If 6-OH's are protected, 2-OH's react preferentially due to their better accessibility and higher acidity, so products are usually modified at 2-positions. To achieve a product selectively modified at 3-position, several strategies were invented – it can be carried out, for example, through 2,3-anhydro-cyclodextrin intermediate, which is treated with some nucleophile. The ring-opening reaction gives preferentially products substituted at the 3-position because this position is easily accessible for nucleophilic attack.[28]

3.2.1. Monosubstituted cyclodextrins

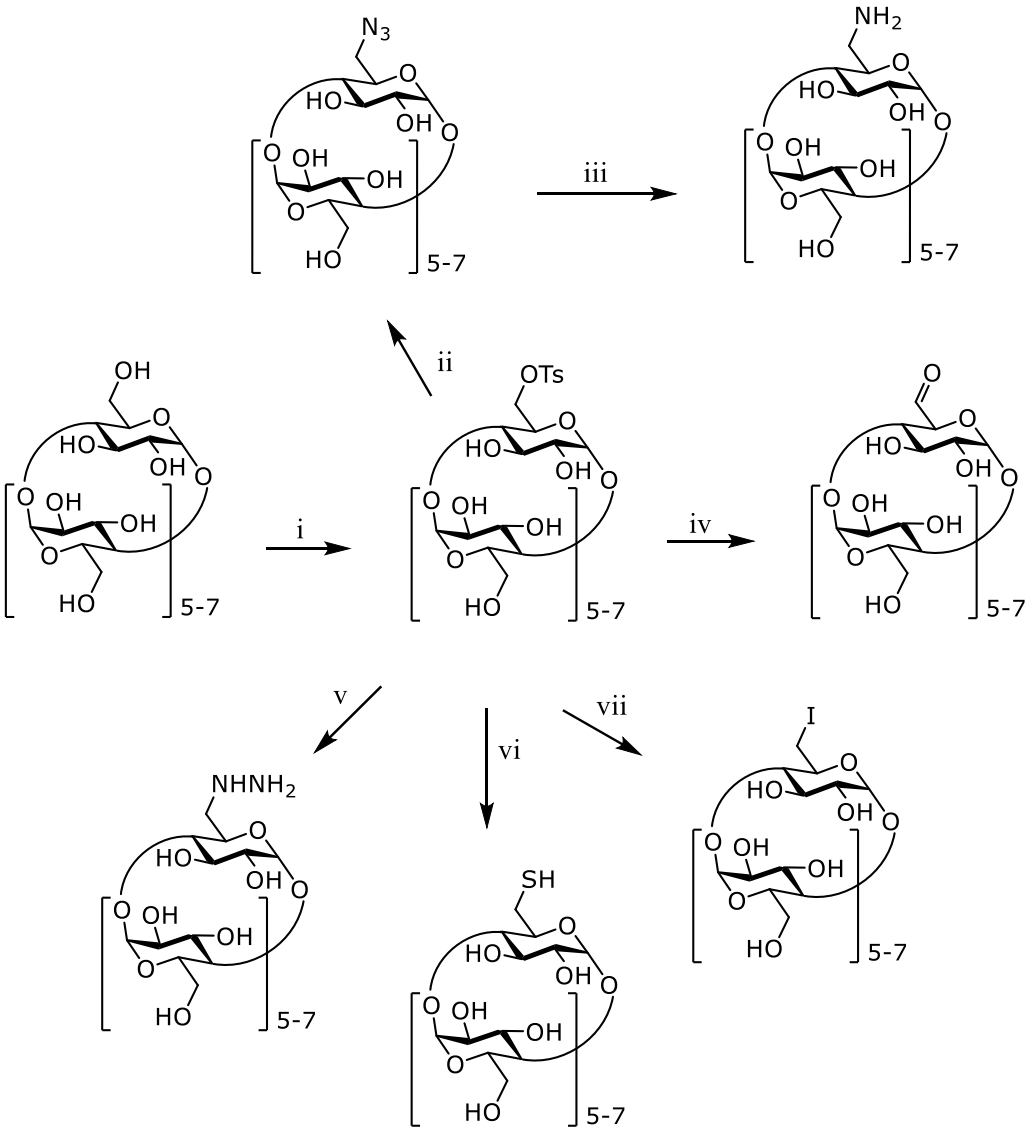
During monosubstitution on cyclodextrin moiety, exact amounts of reactants are used, but multiple substitutions cannot be completely preserved. In these cases, further purification is required, so the yield of monosubstituted derivative is usually about 15-35 %.[29–31]

3.2.1.1 Monosubstitution at 6-position

Monosubstitution at the 6-position is usually achieved by substitution at one of the hydroxyl groups, where is the hydrogen replaced with sulfonyl moiety, which is a better leaving group. The products of these reactions are used for further modifications, such as oxidation reactions,[32] azidation and amine formation,[33] synthesis of monothio derivatives,[34] reaction with hydrazine and hydroxylamine,[35] iodination[36], and many others. Alkaline bases cannot be used for these kinds of reactions because of the intramolecular reaction resulting in the formation of 3,6-anhydro cyclodextrin.[37] Monosubstitution can be done with various agents, including *p*-toluenesulfonyl (Ts) chloride and *p*-toluenesulfonyl anhydride. This reaction proceeds usually in pyridine or water containing a base and requires further purification because of the presence of unreacted cyclodextrins and highly substituted derivatives. The total yield of the sulfonylation reaction is about 20-30%.[38]

Another strategy to achieve derivatives monosubstituted at the 6-position is to use an indirect method and perbenzylated precursor. Cyclodextrin is first perbenzylated and then selectively debenzylated with diisobutylaluminiumhydrid (DIBAL).[39] These reactions proceed in better

overall yield but are tedious compared to the direct method. Different ways to selectively modify a product at the 6-position are depicted below (Scheme 1).



Scheme 1: Synthesis of various types of 6-substituted cyclodextrin derivatives. i) TsCl, pyridine, rt; ii) NaN_3, DMF, 80 °C; iii) PPh_3, then H_2O, DMF, 90 °C; iv) DMSO, collidine, 130 °C; v) NH_2NH_2, H_2O, rt; vi) NaOH, thiourea, rt; vii) *N*-iodosuccinimide, PPh_3, DMF, 50 °C

3.2.1.2 Monosubstitution at 2-position

Monosubstitution at 2-position can be achieved by direct or indirect method as in the case of substitution at 6-position. An example of a direct method is the synthesis of 2-*O*-tosyl-β-cyclodextrin, which can be done by the reaction of cyclodextrin with *m*-nitrophenyl tosylate.[40] In this case, selectivity is driven by the formation of an inclusion complex between cyclodextrin and substrate. An example of selectivity based on the different basicity of each type of hydroxyl group is the synthesis of 2-hydroxypropyl-cyclodextrin (HP CD).[41] Amount of the used base is critical for regioselectivity of the reaction, which in the case of the exact amount of a strong base leads exclusively to the formation of cyclodextrins substituted at 2-position. As with C-6 modified cyclodextrins, the C-2 modified cyclodextrin derivative can also be synthesized by an indirect method. Described cases are selective de-*O*-methylation at position 2 with DIBAL[42] or the synthesis starting from the perbenzylated derivative[43], in which the hydrazine in pyridine is used as an agent for deprotection at position 2.

3.2.1.3 Monosubstitution at 3-position

Synthesis of cyclodextrin derivative selectively modified at the 3-position is complicated due to the higher reactivity of two other types of hydroxyl groups. The most used methods for selective modification at the 3-position of cyclodextrins are based on the complexation of the reagent into the cyclodextrin cavity. An example of this approach is the synthesis of C-3 sulfonate using β-naphthalenesulfonyl chloride in 30 % aqueous MeCN as solvent.[44] Complexation is responsible for the higher yield of C-3 substituted cyclodextrin, but it's still required to purify products using chromatography techniques. Another disadvantage is that the product of this reaction is not a good precursor for further modifications. Another approach is to use the previously mentioned 2,3-anhydro cyclodextrin, which can undergo nucleophilic ring-opening reaction to get preferentially derivative modified at 3-position.[28] Only a few procedures giving preferentially 3-substituted derivative in really high yield were previously reported - one of them is the synthesis of 3-*O*-cinnamyl cyclodextrin derivative.[31] In this reaction, β-cyclodextrin is reacted with cinnamyl bromide to get the target product with a regioselectivity higher than 90 % and a yield of about 30%, which is comparable to C-6 modified cyclodextrins. The reaction of α-cyclodextrin under the same condition does not lead to the same result and affords the mixture of products due to the

smaller cavity of α-cyclodextrin and the impossibility to create an inclusion complex in the desired orientation.[45] 3-*O*-Cinnamyl-α-cyclodextrin can be synthesized by reaction of the appropriate alkylation reagent in the presence of LiH in DMSO, as well as 3-*O*-allyl derivative[45,46], which can be reacted to get carboxymethyl- or formylmethyl-cyclodextrin.

3.2.2. Di- and multiply-substituted cyclodextrins

In the case of cyclodextrins with two or more substitutions, the appropriate nomenclature is used, which indicates the position of each of the substituents on cyclodextrin moiety. There are two options for naming substituted cyclodextrins (Figure 2) – the first follows the IUPAC recommendations, and if we look from the primary side, we count clockwise[47]. For the labeling of the position, Roman numerals are used (I-VIII). The second option is used more often, and we count in the opposite direction.[18,48] Instead of Roman numerals, we use uppercase letters (A-H). This approach is called Tabushi numbering.

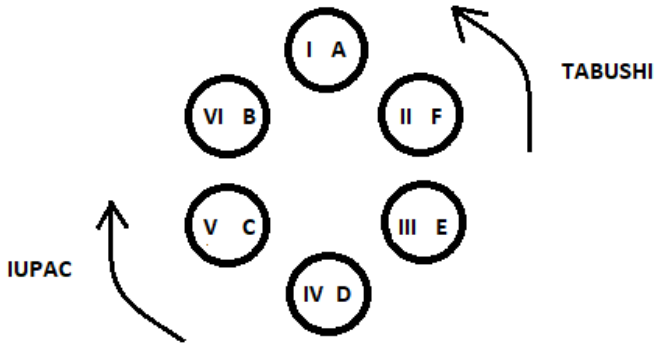


Figure 2: Numbering of α-cyclodextrin units – view from the primary side. IUPAC = I-VI, TABUSHI = A-F.

Synthesis of selectively di- and multiply-substituted cyclodextrins is usually more difficult in comparison to monosubstituted derivatives. As in the case of monosubstituted cyclodextrins, two different approaches are used – the direct method, which starts from an unmodified cyclodextrin, and the indirect method, which uses persubstituted intermediates and is usually more selective than the direct method.

In the case of the direct method, the classical approach is that cyclodextrin is reacted with a so-called capping reagent (Figure 3). There are several compounds, that were recently successfully used for this purpose – 1,4-dibenzoylbenzene-3′,3″-disulfonyl imidazole was used to synthesize 2^A,2^C-disulfonyl substituted β-cyclodextrin[49], later the work was extended to the synthesis of 2^A,2^D-disulfonyl cyclodextrins, which were carried out with sulfonyl reagent obtained by Wolff–Kishner reduction of 4,4′-dibenzoylbiphenyl followed by reaction with imidazole.[50] Mesitylensulfonyl chloride in pyridine was used to synthesize primary mesitylensulfonates of α-cyclodextrin, which could then be selectively transformed to di- or tri-substituted cyclodextrins.[51] Selective tritylation of α-cyclodextrin by trityl chloride leads to the formation of 6^A,6^C,6^E-tri(triphenylmethyl)-α-cyclodextrin, which can be then protected at remaining positions and after hydrolysis of trityl groups provides the useful precursor for further synthesis.[52]

Figure 3: Synthesis of selectively disubstituted cyclodextrins by the capping method.

There are many more examples of the synthesis of selectively substituted cyclodextrin, which were carried out by the indirect method. One of the most favorite approaches is the usage of perbenzylated precursor, which can be then selectively debenzylated by diisopropylaluminium hydride (DIBAL-H).[42] This method is very useful for synthesis of 6^A,6^D-substituted α- and β-cyclodextrins derivatives, but when is applied to γ-cyclodextrin, leads to a mixture of products. This reaction is very sensitive to reaction conditions, such as solvents, temperature and reaction time.[53–55] DIBAL-H was recently applied in the selective demethylation of permethylated derivatives, which takes place at secondary rim.[42] Selectively demethylated derivative was also obtained from the derivative methylated on the primary rim and benzylated on the secondary side.[56]

3.2.3. Persubstituted cyclodextrins

Persubstituted cyclodextrins are used as substrates for selective modifications as described before, but they are also widely used in several applications. We can distinguish cyclodextrins persubstituted in all positions and cyclodextrins persubstituted in selected positions.

In the case of cyclodextrins substituted in all positions, there are plenty of derivatives that were successfully prepared - perbenzylated cyclodextrins were already mentioned, as well as permethylated derivatives[57]; both of these derivatives can be prepared by a reaction of cyclodextrin with an excess of appropriate reagent (benzyl bromide or methyl iodide together with NaH). Other very popular derivatives are peracetylated cyclodextrins, prepared by a reaction of cyclodextrins with acetanhydride.[58] Partially and persilylated cyclodextrins were prepared by a reaction with trimethylsilylimidazole. This reaction strongly depends on the solvent used.[59] When the reaction was carried out in pyridine, only partially substituted products were obtained. High degree of substitution was achieved with the mixture DMF/$CHCl_3$ – chloroform was used to solubilize partially silylated cyclodextrin molecules and homogenize the reaction mixture.

Cyclodextrins persubstituted at selected positions are prepared for many reasons, for example, to get compounds selectively modified on the secondary side (usually at 2-position) or to get compounds substituted with different protecting groups on the primary and secondary rim. In this case, 6-OH hydroxyls can be protected, for example, by tert-butyldimethylsilyl (TBDMS) groups – this is achieved by the reaction of cyclodextrin with TBDMSCl in the presence of imidazole.[60] Protected cyclodextrin can be, in the next step, persubstituted on the secondary side and then deprotected on the primary rim.[27]

Another approach uses per-6-halo-cyclodextrins, which are very useful precursors for further synthesis. Per-6-iodo-cyclodextrins were prepared directly from cyclodextrins reacted with I_2 and PPh_3 in DMF under heating to 70 °C.[61] Per-6-bromo-cyclodextrins are usually prepared by a direct bromination[62] or by an indirect method, which includes protection of the primary side with TBDMS groups and subsequent methylation of the secondary rim. Reaction with bromine and PPh_3 in dichloromethane gives per-6-bromo-cyclodextrin.[27] Halogen atoms can be replaced by a large number of other functional groups, such as azido group and amino groups[63], thiol or alkylthiol groups[64], and carboxylate moieties.[65]

3.3. Cyclodextrins inclusion complexes

The ability to create inclusion complexes is the most important property of cyclodextrins. Inclusion complexes are aggregates based on non-covalent interactions, in which one molecule (host) includes the other (guest).[66] Thanks to their structure, cyclodextrins are a type of compounds, which are good hosts for wide spectra of molecules of different sizes – these compounds are for example hydrocarbons[67], steroids[68], amino acids[69], aromatic compounds[70], drugs[71], and many others. Usually, the type of included compound depends on the size of the cyclodextrin cavity; α-CD includes small molecules, such as linear hydrocarbons or benzene, β-CD includes bigger aromatic compounds or substituted benzene, and larger molecules (steroids) are complexed by γ-CD. As always, there are exceptions - it was confirmed, for example, that α-CD forms a complex with stigmasterol[72], where we would expect complexation with γ-CD.

Inclusion complexes are based mainly on two types of interactions – hydrophobic effect and Van der Waals forces. Therefore, the formation of complexes is favored especially in an aqueous environment, where the presence of water molecules in the cavity is energetically disadvantageous; the replacement of water by guest molecules is controlled thermodynamically.[73]

The strength of the resulting complex is indicated by the stability constant *K* (also called the binding constant or the association constant), which is practically an expression of chemical equilibrium; for the easiest case, where the ratio of host and guest in the resulting complex is 1:1, we get:

$$\text{CD} + \text{guest} \rightleftharpoons \text{complex} \qquad K = [\text{complex}] / [\text{CD}][\text{guest}] \qquad (1)$$

The value of the constant for CD complexes is typically between 10^2-10^5 depending on the guest molecule and conditions.[74–76] Several methods exist to determine its size, including ultraviolet–visible spectroscopy (UV-Vis)[77], nuclear magnetic resonance spectroscopy (NMR)[78], microcalorimetry[79], and potentiometric titration.[80]

Besides the mentioned ratio 1:1, cyclodextrins also form complexes in other ratios, for example, 2:2[81]. The host-guest ratio can be non-stoichiometric when a molecule of appropriate guest occupies more than one cavity (this is typical for polyrotaxanes) or when is the cavity occupied by more than one guest. For example, the host-guest ratio can be determined by NMR titration or by UV-Vis

spectroscopy. When the guest molecule is covalently linked to the cyclodextrin moiety, NMR titration can be used to prove, if the resulting complex is intra- or intermolecular (in intramolecular complex, the guest is complexed by the cavity of its „own" cyclodextrin; in the case of intermolecular complexation, the guest is complexed by the cyclodextrin cavity, with which it is not covalently linked to). Different types of cyclodextrin complexes are depicted in Figure 4.

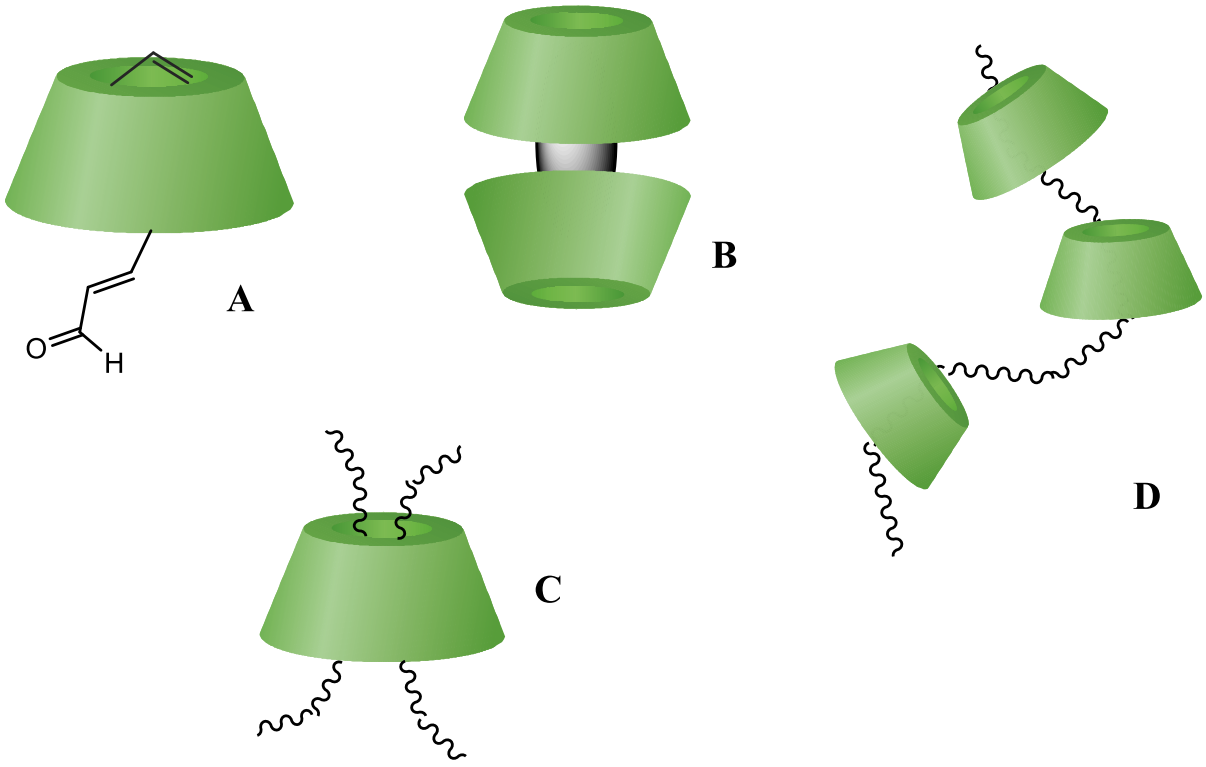


Figure 4: Different types of cyclodextrin inclusion complexes – A) host-guest ratio 1:1, B) host-guest ratio 2:1, C) host-guest ratio 1:2, D) host-guest ratio 3:1.

3.4. Pharmaceutical applications of cyclodextrins

Cyclodextrins were first used as a part of a medicinal product in 1976.[82] This drug was Prostarmon E™, which was sold in Japan in the form of sublingual tablets and which contained prostaglandin E_2/β-cyclodextrin. From then until now, there are several dozens of pharmaceuticals, which contain cyclodextrins.[83] Previously, cyclodextrins have been used mainly as a part of drug formulation in the form of non-covalent complexes. Nowadays, there are also several drugs in which cyclodextrin is covalently bonded to an active substance.[84]

The usage of cyclodextrins in drug formulations has several advantages, such as increasing the solubility of active substance, decreasing its volatility, modulation of taste or odour, increasing bioavailability, protecting against the action of oxidants and other adverse reactions before delivery to the target structure. In some cases, native cyclodextrins are used; in others, some cyclodextrin derivatives were chosen instead of native ones because of better properties important for the specific case. The derivatives are, for example, hydroxypropylated β-cyclodextrin (HP-β-CD), which has higher solubility than β-cyclodextrin[85], methylated β-cyclodextrin (M-β-CD), which also shows anticancer activity by itself[86], sulfobutylated derivatives (SBE-CD), which are not only good solubilizing agents but also improve stability of many commercially available drugs[83] (Table 2). Nowadays, one parenteral drug is administered as a vaccine, which uses sulfolipo-cyclodextrin as a drug delivery carrier.[83,87,88]

Table 2: Some commercially available drugs containing cyclodextrins.[83]

Drug	CD	Trade name	Dosage form
Benzoyl peroxide	γ-CD	Nujevi Acne	cream
Estradiol	M-β-CD	Aerodiol	nasal spray
Nicotine	β-CD	Nicorex	tablet
PGE1	α- and β-CD	Limaprost	tablet
Progesterone	HP-β-CD	Lubion	injection
Remdesivir	SBE-CD	Veklury	i.v. injection

3.5. Fluorouracil as a drug

Fluorouracil (FU) is a drug widely used in the therapy of various types of cancer since the 1960s.[89] Recently, it was used in the treatment of colorectal cancer, but also to heal other types of gastrointestinal cancers, breast cancer, and many others.[90] In the case of breast, head, and neck cancers, fluorouracil is used mainly in combination with other anticancer drugs.[91] In the treatment of colorectal cancer, the influence of fluorouracil is still irreplaceable.[92]

3.5.1. Mechanism of action

After entering an organism, the fluorouracil molecule is transformed into several active compounds. Fluorouracil does not show cytotoxicity by itself, but through its active metabolites[93,94] – anabolic route leads to the formation of 5-fluorouridine monophosphate (FUMP), which after phosphorylation gives 5-fluorouridine diphosphate (FUDP). FUDP is converted into fluorodeoxyuridine triphosphate (FUTP) or fluorodeoxyuridine diphosphate (FdUDP).

Fluorouracil is structurally similar to uracil, a naturally occurring component of RNA. Due to the similarity of structures of these substances, fluorouracil can be transported to the cell by the same system[95] and then further metabolized inside the cell into the mentioned active substances. The actual mechanism of action is twofold (Figure 5). First, fluorouracil is an enzyme inhibitor, which inhibits enzyme thymidylate synthase (TS) through one of its metabolites, the second is incorporation into RNA or DNA, which in the case of RNA leads to changes in function, in the case of DNA to the damage of DNA chain.[96]

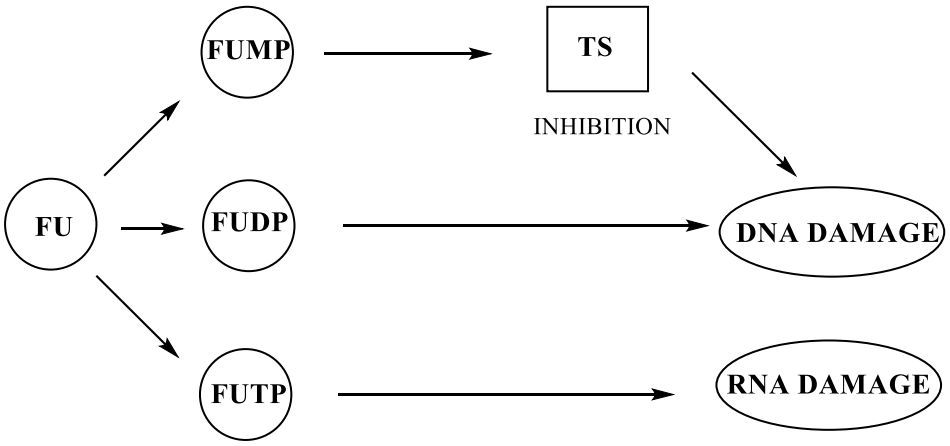


Figure 5: Fluorouracil – mechanism of action (simplified by Longley *et al.*).[90]

3.5.2. Use in therapy

Fluorouracil is used mostly in cancer therapy. This compound is quite effective, but on the other hand shows a high level of cardiotoxicity[97], continuous infusions lead to neurological problems, stomatitis and Hand-foot syndrome.[98] As usual in the case of drugs, there is also a significant influence of resistance to the treatment.[99] Fluorouracil is administered in native form, in the form of prodrugs or analogues. There are also new nucleobases, which were synthesized to replace fluorouracil.[100] Despite all the limitations, fluorouracil is still the third most used chemotherapeutic agent.[101]

Fluorouracil is administered in the form of infusions. In the case of gastrointestinal cancers, continuous intravenous administration is usually used; in the case of hematologic cancers, intravenous bolus infusions are applied. The treatment can also combine both approaches for better modulation of the treatment effect; it always depends on the particular case.[89]

4. RESULTS AND DISCUSSION

This work aimed to synthesize conjugates of an anticancer drug, fluorouracil, with different types of cyclodextrins. Both components of the target molecule should be linked by a linker that breaks down under physiological conditions – this would provide the free drug molecule and cyclodextrin, which is not harmful to the organism.

The plan was to use linkers, which are not stable under acidic pH. This idea was inspired by the fact that the pH is lower in tumour tissue than around healthy cells.[102,103] Based on this knowledge, cyclodextrin-drug conjugate should be delivered even to tumour cells, where it would be broken down into a drug and cyclodextrin.

When the target conjugate was planned, amide, ester, imine, oxime, and hydrazide linkages were discussed (Figure 6). Because of their stability, amide bonds were selected as controls for possible complexation measurements. Based on the structure of fluorouracil, the oxime linker was found to be unsuitable for this particular case.

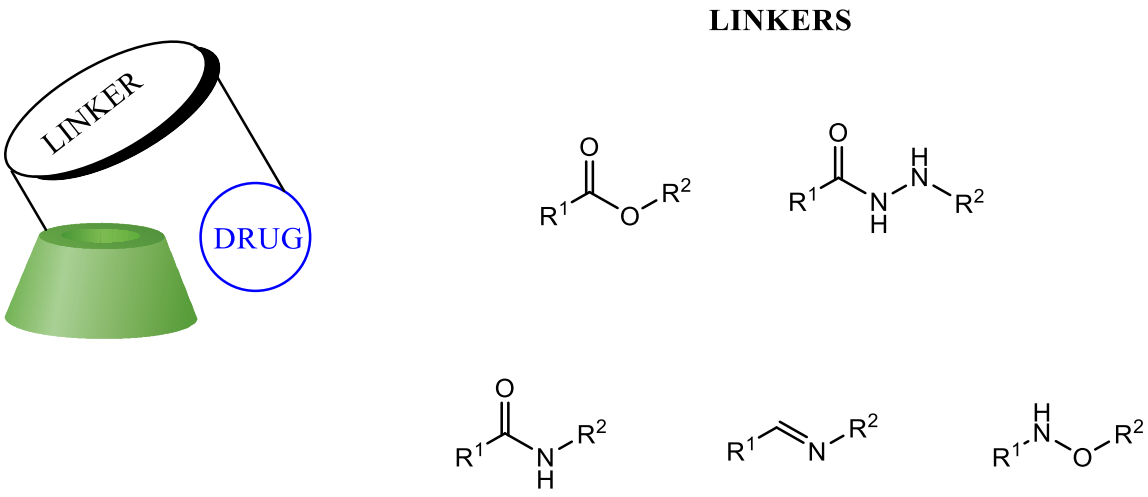


Figure 6: CD-FU conjugate and its possible linkers.

Several factors were considered during the planning of synthesis; the drug should be connected with the type of bond, which allows it to decompose on the target place (the low stability

connection between cyclodextrin and linker was not required), the products of decomposition should not be toxic (except the fluorouracil native molecule), and the linker should have a length and flexibility that allows complexation.

The synthesis started from some well-known cyclodextrin derivatives. These first conjugates were planned to be prepared easily from cyclodextrins with an amino functional group and fluorouracil acetic acid (amide bond) or from non-modified cyclodextrins with the same reagent (ester bond). Synthesis was described previously with β-cyclodextrin[104] (amide linkage only with amino-CD) and has been planned to be extended to α- and γ-cyclodextrins (Figure 7).

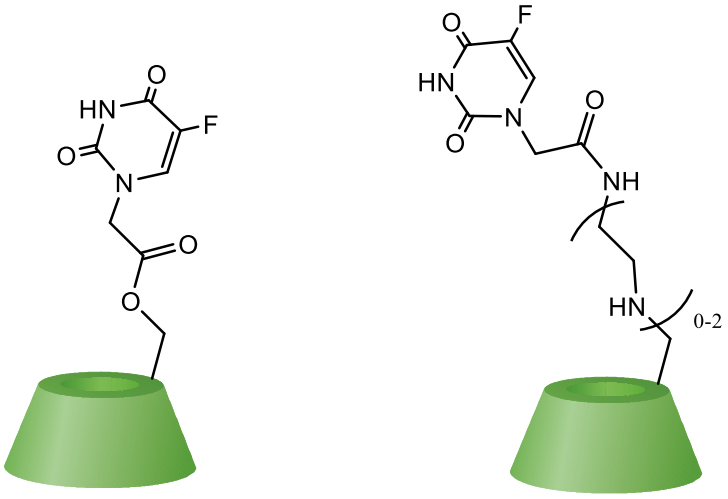


Figure 7: Structures of the designed conjugates with ester and amide linkage.

The second idea was to use an ester bond between fluorouracil and a linker and an amide linkage between CD and a linker molecule. For this purpose, we chose dicarboxylic acids of a different length. This concept was based on the knowledge, that the ester bond between the fluorouracil molecule and dicarboxylic acid chain should undergo hydrolysis; the product of hydrolysis tends to be 5-fluoro-1-hydroxymethyluracil, which quickly breaks into free drug and formaldehyde.[105]

This kind of connection between fluorouracil and a linker was also planned to be used to synthesize cyclodextrin conjugates with imine bond linkage. In this case, the glycine molecule would be connected to the fluorouracil derivative with its carboxylic group and amino group to formyl-cyclodextrins.

Conjugates with hydrazide linkage should have been synthesized from cyclodextrin carboxylic acids, fluorouracil hydrazide, and/or hydrazinyl-CDs and fluorouracil carboxylic acid (Figure 8).

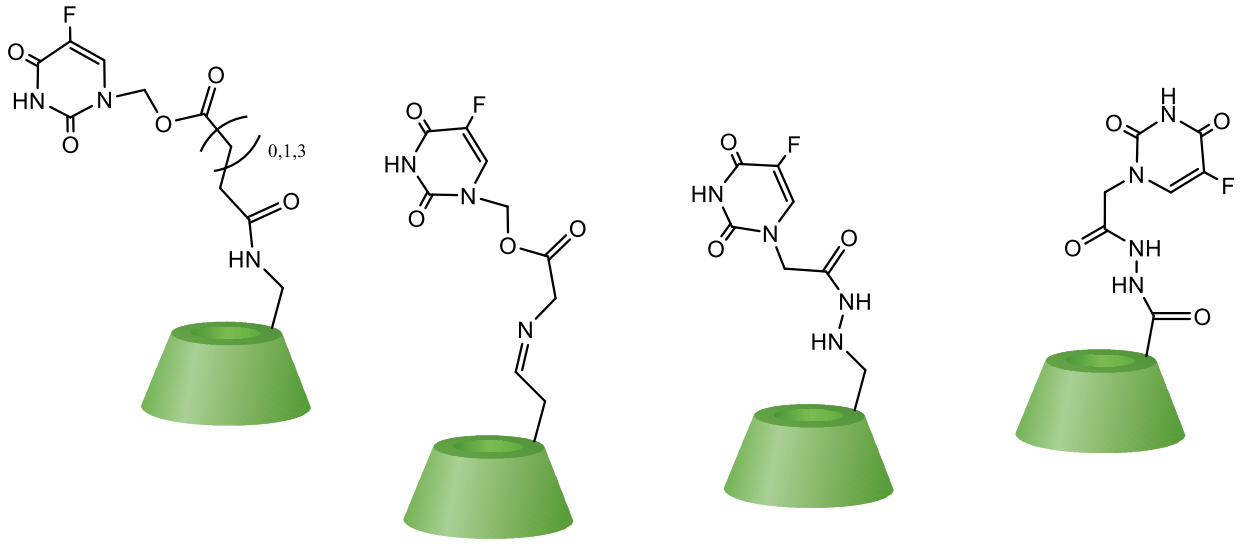


Figure 8: Structures of the designed conjugates with ester, hydrazide, and imine linkage.

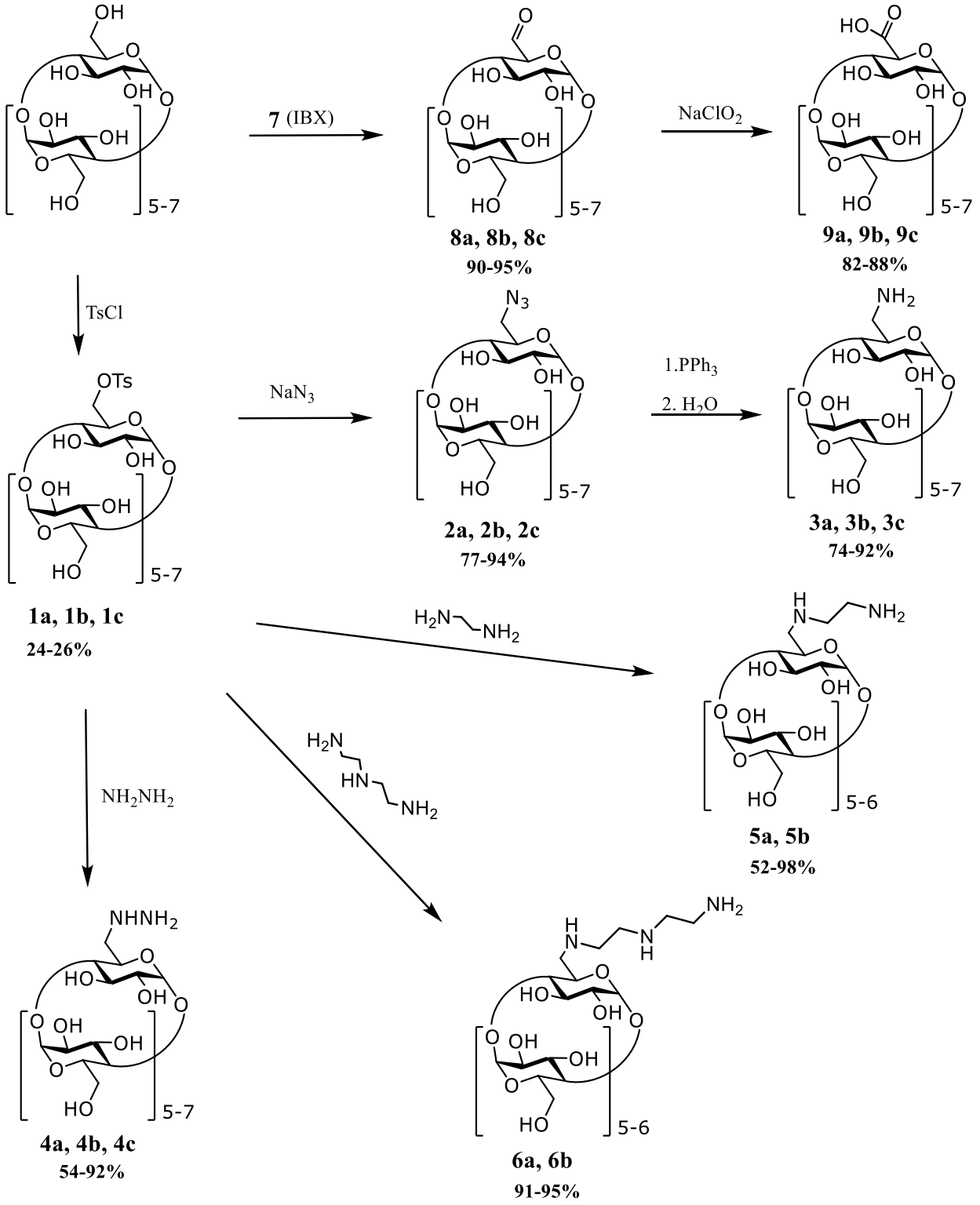
Except for the conjugates described previously, it was planned to synthesize some conjugates provided with a targeting group. Cancer cells are known to overexpress folate receptors (FR).[106,107] Therefore, folic acid can be connected to some of these FU-CD conjugates. In our lab, amino-azido CD was previously successfully prepared, which was ideal for this purpose – the amino group would be connected to FU *via* a succinic acid linker and the azido group was a perfect substrate for click reaction between CD and folic acid modified by reaction with propargylamine.

4.1. Synthesis of monosubstituted cyclodextrin derivatives

The synthesis started with modifications of cyclodextrins (Scheme 2) – the first reaction was the formation of 6^A-*O*-*p*-toluenesulfonyl-β-cyclodextrin **(1a)**. Several approaches were previously described, but not all contain a purification step necessary to obtain a pure monosubstituted derivative. Another described approach is based on reaction followed by chromatography; these methods are suitable for obtaining pure products, but their big disadvantage is the time requirement. In our case, the procedure published by Popr *et al.*[38] was followed. In this case, the reaction proceeds with tosyl chloride as a tosylation agent and does not require purification by chromatography; the pure product is obtained by refluxing the crude product in the mixture of H_2O/MeOH in a ratio 1:1 and subsequent crystallization from the resulting solution. Another advantage of this method is that the reaction is carried out in water containing NaOH, which is more user-friendly than pyridine or DMF.

Synthesis of 6^A-*O*-*p*-toluenesulfonyl-α-cyclodextrin **(1b)** and 6^A-*O*-*p*-toluenesulfonyl-γ-cyclodextrin **(1c)** required different approaches, which included mentioned chromatography. Cyclodextrins were reacted with 0.9 eq of tosyl chloride, the reaction was carried out in dry pyridine. The dryness of used reagents is necessary to achieve high yields. When not dried solvents are used, the yield decreases rapidly independently to used tosyl chloride equivalents, caused by the fact, that in a non-dried solvent, tosyl chloride reacts firstly with the water present in the mixture.[108] Therefore, the dryness of used pyridine was checked by Karl-Fischer titration, and CD's were dried under reduced pressure at 70 °C using an oil pump. Thanks to this approach, yields of both reactions were higher than 20% which is a very good result in the field of preparation of monosubstituted cyclodextrins.

Monotosylated cyclodextrins were used to synthesize azide derivatives **(2a-c)** using modified well-known procedures.[33,109] Briefly, dried cyclodextrins were diluted in dry DMF and reacted with 20 eq of sodium azide. The yields of this reaction were between 80-90%. The work-up of this reaction was modified to remove the remaining sodium azide completely; the product was precipitated with acetone, which contained about 10 % water, this process was repeated three times. The purity of the product has been proven by infrared spectroscopy (IR), which did not show any signal related to free azide. Water usage in the purification step probably caused a lower yield compared to published articles.



Scheme 2: Synthesis of monosubstituted cyclodextrins – a) β, b) α, c) γ.

Azido-cyclodextrins were precursors in the synthesis of amino-CD derivatives **(3a-c)**. This procedure is also described many times in different articles, experienced procedure was followed.[33] Reaction proceeded in DMF and the product was isolated by precipitation with acetone. Residual DMF present in dried products was removed by dissolving the product in water and its evaporation – this procedure was repeated several times.

Monotosylated cyclodextrins were used as substrates for reaction with hydrazine hydrate resulting in the formation of cyclodextrin hydrazines **(4a-c)**. This reaction was previously described on β-cyclodextrin[35]; in this work, it was extended to get the remaining cyclodextrin derivatives.

For the synthesis of α- and β-cyclodextrin modified with diamine molecules, tosyl CD's were used once more – CD was reacted with appropriate diamine (ethylenediamine – **5a-b**; diethylenetriamine – **6a-b**) to get CD with longer linkage to an amino-functional group. Diamines were heated with CD's overnight; products were obtained by diluting the reaction mixture in water and precipitation with excess acetone. The procedure was repeated until an amine was completely removed (detected by thin-layer chromatography (TLC) using ninhydrin). The yields were usually higher than 90%.

Another synthesis that started from non-modified cyclodextrin was the synthesis of cyclodextrin carboxylic acid. The first step of this sequence was to get monoformyl-cyclodextrins, which should then be selectively oxidized to get carboxylic acid. Previously, several approaches to get formyl-CD's were published, including synthesis using tosylated cyclodextrin[32,110], oxidation of native cyclodextrin with Dess-Martin periodinane (DMP)[111,112] or indirect method, which included perbenzylation and subsequent selective deprotection.[113,114]

First, the method described by Yoon *et al.* was tried. The authors used Kornblum oxidations; synthesis started from tosylated derivatives, which were dissolved in DMSO. The reaction required heating to 130 °C in the presence of collidine. The formation of monoformyl-CD was confirmed by NMR (chemical shift of formyl proton was 9.69 for β-CD; this peak disappeared after the addition of water which was caused by the formation of a hydrate) but apart from the desired product, the presence of a side product in the reaction mixture was also observed (proven by NMR – the peak with chemical shift 9.21, signal did not disappear even after adding water). The spectra of the Kornblum oxidation are depicted in Figures 9 and 10.

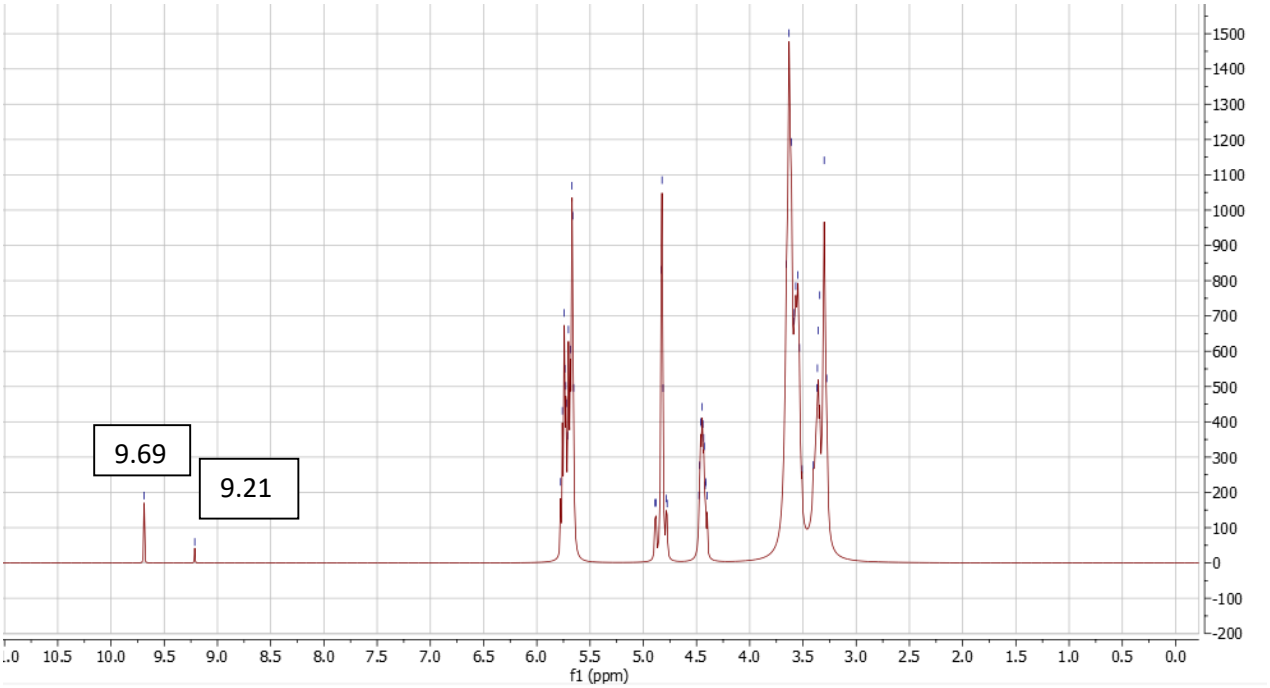


Figure 9: Kornblum oxidation – the spectrum of products in DMSO.

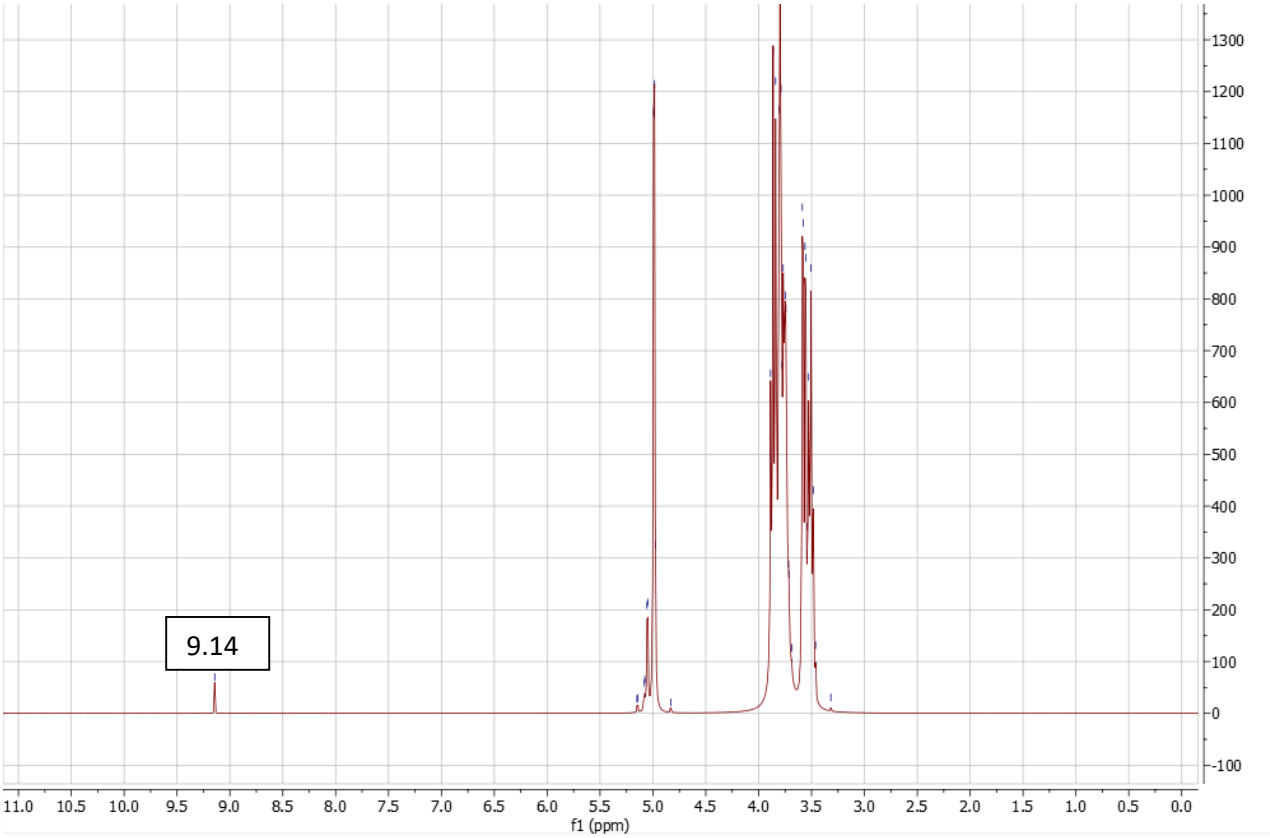


Figure 10: Kornblum oxidation – the spectrum of products in D_2O.

According to the published article, the second compound was identified as a product of β-elimination.[115] This α,β-unsaturated aldehyde did not form a hydrate even after the water addition. According to the proposed reaction mechanism, several different bases were used to compare the result of the mentioned reaction (collidine, lutidine, Hunig base, triethylamine, triisopropylamine) but the side product was still present in lower or higher amounts (higher amount of the side product was observed in cases when non-sterically hindered base was used; this fact corresponds to the proposed reaction mechanism). The side product was also present when a reaction was carried out at a lower temperature and prolonged reaction time. According to the published procedure[116], the reaction was repeated under microwave irradiation at 135 °C, which shortened the reaction time to 15 minutes. This approach did not lead to the desired result; the side product was still present in the reaction mixture.

Due to the mentioned facts, the synthetic strategy was switched to the oxidation of non-modified CD by DMP. Compared to Kornblum oxidation, the transformation requires only one reaction step; the disadvantage is possible over-oxidation, which occurs if the reaction is allowed to proceed too long. There are several articles, which were published in connection with oxidation of cyclodextrins by DMP. Articles differ in reaction times and equivalents of an oxidation agent; the result of the reaction strongly depends on its quality. It was tried to use old DMP prepared in our lab, commercially available DMP (Aldrich), DMP prepared just before use, and 2-iodoxybenzoic acid (IBX), an intermediate of the DMP synthesis. The best results were achieved by IBX prepared just before use – in comparison to DMP, it was easier to remove it from the reaction mixture (IBX is soluble only in DMSO).

IBX was prepared following the published procedure.[117] The ideal time for the synthesis of monosubstituted derivative was determined by kinetic measurements – 50 mg of appropriate CD was reacted with 1.1 eq of synthesized IBX and the reaction was monitored by NMR. The ideal reaction time was determined to be 100 min for all CD's (Figure 11). After prolonged reaction times, cyclodextrins were over-oxidized – over-oxidation was the fastest in the case of α-CD; over oxidation of γ-CD was the slowest. Because of a little amount of water in all mixtures, the integration of H1 signals was chosen to determine the amount of the oxidized product. Because non-modified CD's have identical retardation factor (R_F) with oxidized products, determined time was used as a standard for reactions in larger scales **(8a-8c)**.

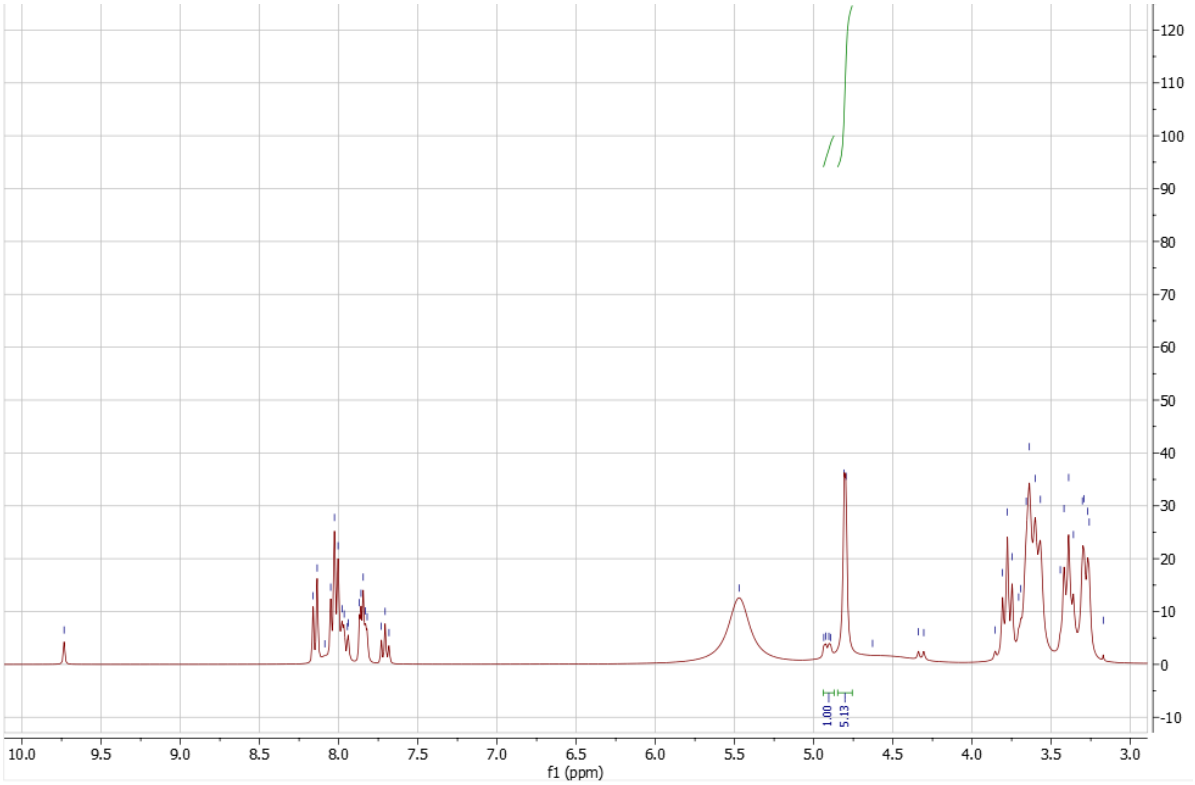


Figure 11: Oxidation of α-CD by DMP – reaction time 100 min.

Cyclodextrins monoaldehydes were used as substrates for the following oxidation to carboxylic acids. Cyclodextrin monocarboxylic acid was previously successfully prepared by Yoon *et al.*[32]; in this case, authors used bromine in phosphonate buffer as an oxidation agent. The yield of this transformation was only 24% and the reaction was carried out for 5 d, therefore, we tried to find a better way. Other approaches, such as oxidation with 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO), were also described.[118] This method is unsuitable for getting selectively monooxidized CD's and leads to a mixture of products.

First, oxidation by hydrogen peroxide was carried out; this idea was inspired by the synthesis of oxidized trehalose.[119] In the case of cyclodextrins, the method did not work even if we applied different concentrations of peroxide and various temperatures. At low concentrations and temperatures, the reaction did not proceed; under higher temperatures or with higher concentrations of hydrogen peroxide, the mixture of products was obtained.

The strategy was finally switched to the Pinnick oxidation.[120] This approach was successful and the oxidation proceeded in a yield higher than 80% and in a short reaction time (up to 24 h). Because of a high product polarity, it was impossible to use reverse-phase chromatography for purification; gel chromatography was chosen to obtain pure desalted compounds **(9a-9c)**.

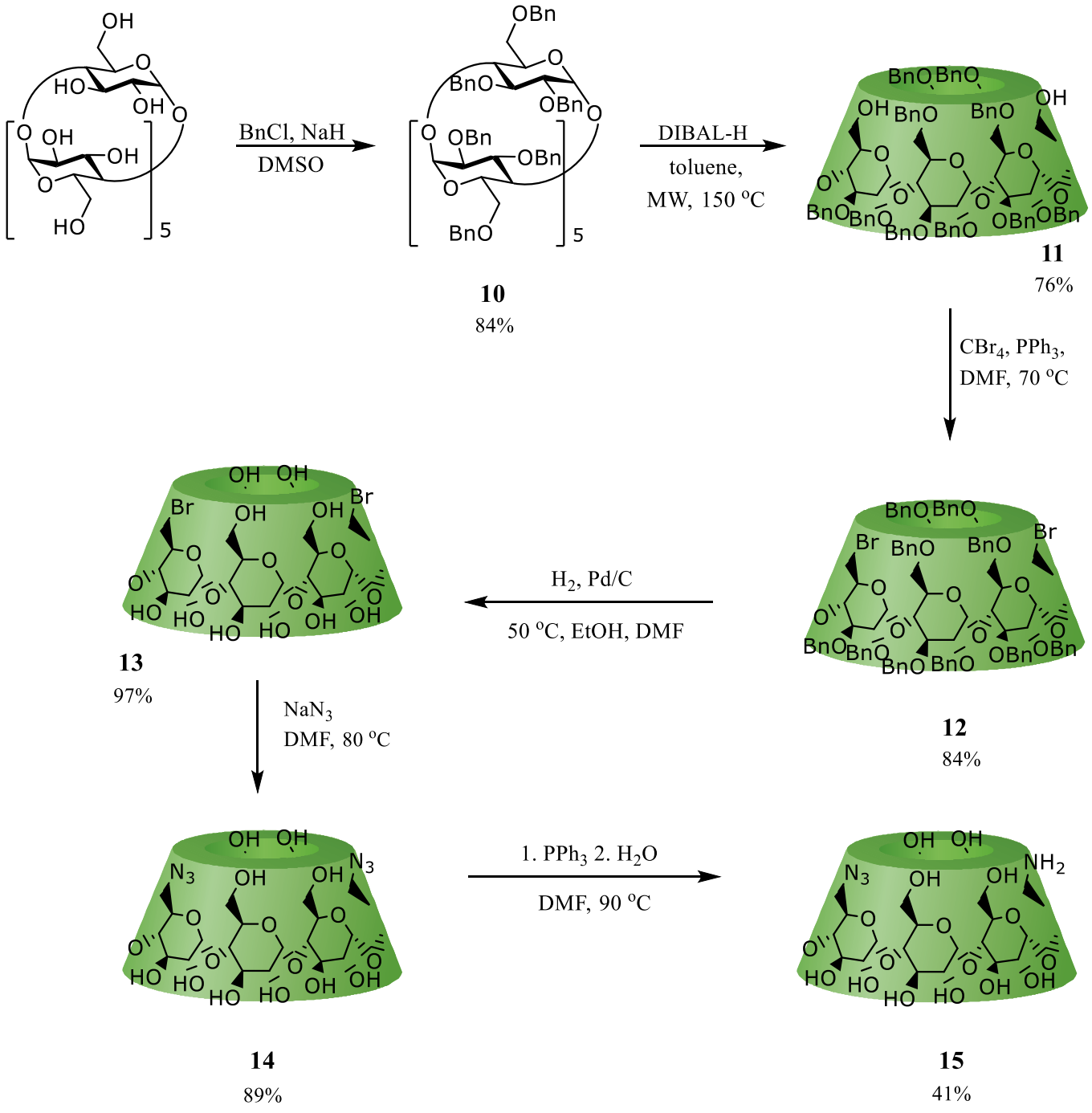
4.2. Synthesis of disubstituted cyclodextrin derivatives

To synthesize selectively disubstituted cyclodextrin derivative, α-CD was selected as a starting material. Synthesis of an amino-azido α-CD (Scheme 3) started with perbenzylation of non-modified α-CD, which was then selectively deprotected by DIBAL, brominated, deprotected, reacted with sodium azide, and finally semi-reduced to get a target compound.

Perbenzylated product **(10)** was obtained in a high yield following the method described previously[121]; to get the pure product from the amount used, it was necessary to lyophilize the residue for 5 d (yellow oil was obtained after evaporation under reduced pressure).

Selective debenzylation was achieved by reacting the perbenzylated derivative with an excess DIBAL-H (10 eq) under microwave irradiation.[122] Complete conversion was observed after 1 h under heating to 150 °C.

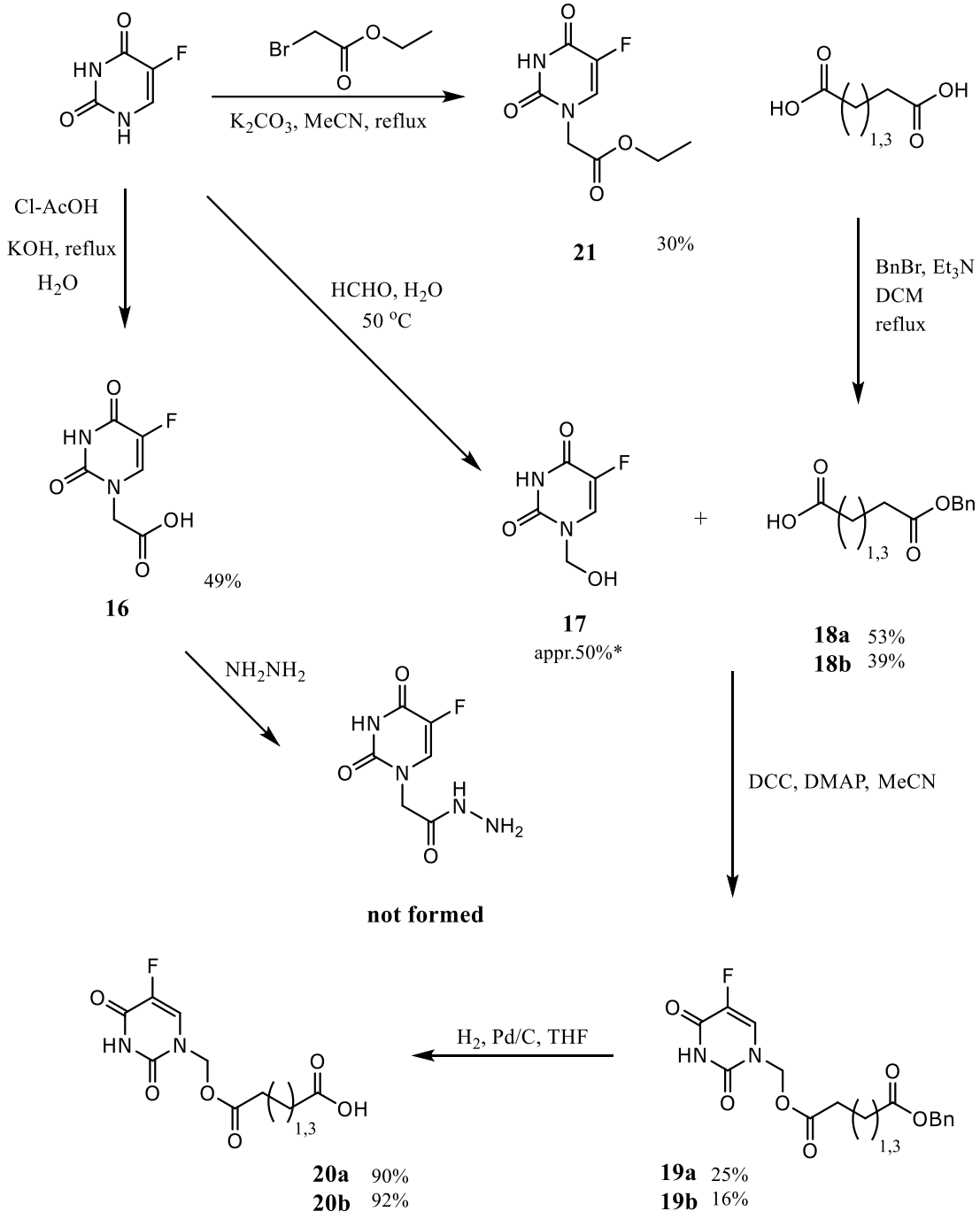
The di-debenzylated derivative **(11)** was converted to the di-bromo derivative **(12)** and then deprotected following the procedure published in the literature.[123] Azidation of the resulting compound **(13)** was performed using the method already described for the synthesis of monosubstituted derivatives.[33] The product **(14)** was purified by precipitation with acetone/water mixture. Amino-azido α-cyclodextrin **(15)** was then obtained by the partial reduction with 0.7 eq of PPh_3.



Scheme 3: The synthesis of disubstituted α-cyclodextrin derivatives.

4.3. Modifications of 5-fluorouracil

Several molecules based on 5-FU have been proposed and found to be suitable candidates for the synthesis of conjugates. Some syntheses were successful and the resulting molecules were the basis for conjugate synthesis, some molecules could not be prepared (Scheme 4).



Scheme 4: Modifications of 5-fluorouracil. *Yield estimated from the NMR spectrum.

The modification of 5-fluorouracil started with a reaction with halogenated acetic acid. Two approaches were tried – the first was carried out with bromoacetic acid at 40 °C[124], and the second was performed with chloroacetic acid at 100 °C.[125] Unlike both published procedures, the yield of the reaction was around 50% (in published articles, the yield was around 90%); the final crystallization of the resulting fluorouracil acetic acid (FUA) **(16)** from the acidic solution was carried out in the fridge and took several days.

Another modification tried was the reaction of 5-FU with an aqueous solution of formaldehyde. This reaction is mentioned in several different articles.[126–129] It was necessary to modify the procedure to get a compound that could be used for further reactions. The main problem was that heating a reaction mixture to a temperature higher than 60 °C led to the decomposition of a product back to FU or polymerization. Therefore, the temperature at which the reaction was carried out was set up to 50 °C and monitored during the reaction as well as the evaporation of the solvents and drying of the product. The reaction was performed in 15% formaldehyde instead of reaction in 37% formaldehyde solution. The resulting mixture of products **(17)** (containing 5-FU, FU substituted at N1, FU substituted at N3, and disubstituted FU) was immediately used to further reactions without purification.

It was tried to oxidize the product of the reaction to a formyl derivative according to the published patent.[130] Several approaches were used to get a 5-fluorouracil-1-carboxaldehyde but none of them was successful; first of all, hydroxymethyl FU was reacted with MnO_2 in MeCN, $CHCl_3$, acetone, or their mixtures under reflux but the reaction was quenched due to the polymerization of starting material (only small amount of a product was obtained). Another used method was based on the reaction of FU with HCOOH in the presence of DCC but this reaction was unsuccessful either. The reaction of FU and ammonium formate at 80 °C with different molar ratios of reactants did not lead to the formation of a desired product even after the addition of zinc chloride as a Lewis acid. The synthetic strategy was switched to the synthesis of some derivative that can be prepared in a reasonable yield.

The mixture of products from the reaction of FU with formaldehyde was finally used to synthesize FU connected with the dicarboxylic acid moiety threw an ester linkage, which can be reacted with amino-CD to get a final conjugate. First of all, appropriate dicarboxylic acid (succinic or adipic acid) was partially benzylated to get esters **18a-b**.[131]

Semi-benzylated succinic acid was then reacted with the mixture **(17)** in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC), and 4-dimethylaminopyridine (DMAP) to get a protected succinylFU **(19a)**. The yield of the reaction over two steps was approximately 25% which did not correspond to the published literature[132], probably because of the lower content of 1-hydroxymethyl FU in the starting mixture.

Protected adipoylFU **(19b)** was prepared from the same mixture **(17)**, but the reaction was performed with ethyl(dimethylaminopropyl) carbodiimide (EDCI) and N-hydroxysuccinimide (NHS) as coupling agents.[133] The overall yield was not higher than 16%; that was probably caused by the same reason described in the previous case.

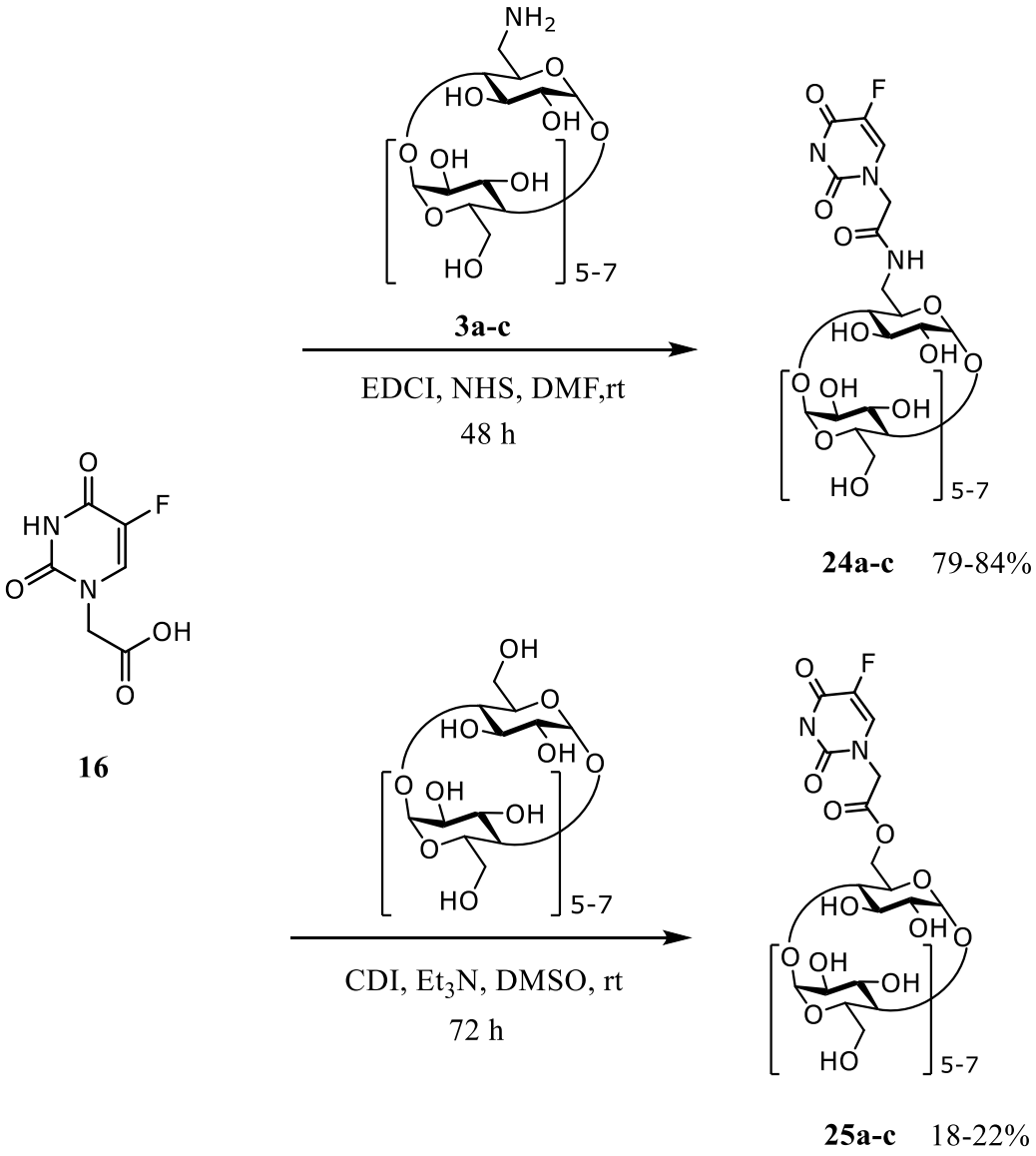
Both compounds were deprotected by the palladium-catalyzed reduction under a slight overpressure of hydrogen at rt to get **(20a)** and **(20b)**.[134]

As the next compound synthesized, ethyl 5-fluorouracil-1-acetate **(21)** was selected. This compound was prepared from FU by the reaction with ethyl bromoacetate following the method described for the synthesis starting from uracil.[135] The resulting compound was used for the reaction with hydrazine hydrate to the corresponding hydrazide. This compound was already isolated from the marine sponge and characterized[136] but it has not yet been synthesized. The hydrazinolysis of an ester was reported on natural nucleobases.[137] In this particular case, the reaction led to the decomposition of a starting compound.

Another synthesis of FU derivative was its conjugation with glycine molecule which is a perfect candidate to create a longer linkage, not harmful after decomposition of the conjugate. Glycine was planned to be connected with FU as described before by the reaction of protected amino acid with the mixture **(17)** or by a hydrazide linkage coming from the reaction of 1-carbethoxymethyl-5-fluorouracil with protected glycine hydrazide. Protected glycine was prepared according to the published procedure[138] from an unprotected glycine with di-tert-butyl dicarbonate (Boc_2O) giving protected glycine (Boc-Gly) **(22)**. The synthesis of hydrazide **(23)** started from commercially available N-Boc-Gly methyl ester which was reacted with a slight excess of hydrazine hydrate overnight. The reaction mixture was evaporated to get a pure target compound. Both compounds were not further used due to lack of time.

4.4. Synthesis of FU-CD conjugates

The synthesis started from two conjugates of β-CD and FUA (Scheme 5) which were previously successfully prepared. Compared to the published literature[104], the synthesis was extended to α- and γ-CD.



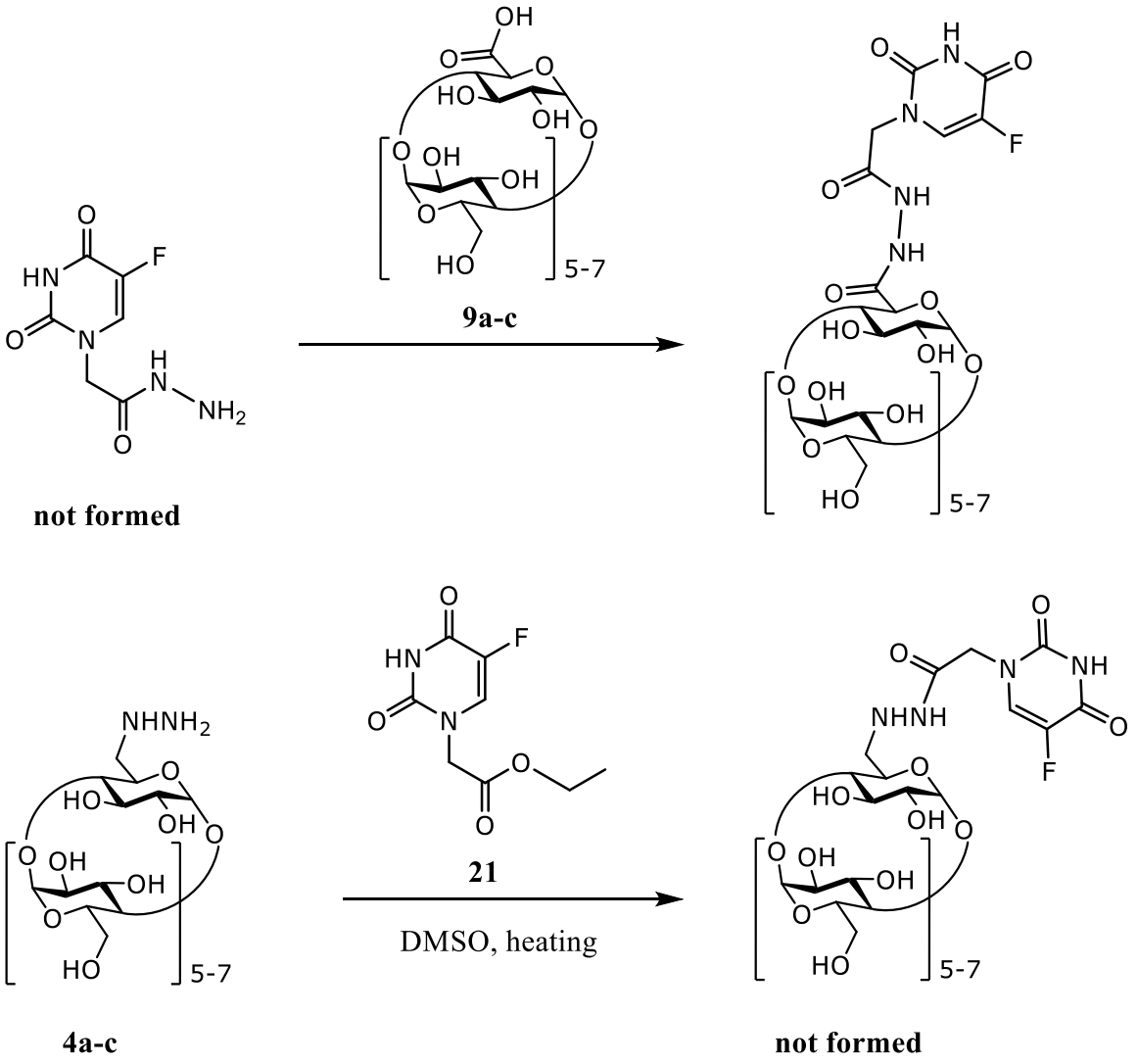
Scheme 5: Synthesis of FUA/CD conjugates.

The synthesis of the first prepared conjugates **(24a-24c)** started from amino-CD's **(3a-b)** and FUA **(16)**. Reactions were performed with DCC as a coupling reagent. Because of its complicated separation from the reaction mixture, DCC was replaced by EDCI which was combined with NHS and used in a slight excess (2.4 eq). Synthesized conjugates were not soluble enough in water to measure their complexation.

For the synthesis of FUA/CD conjugates connected with an ester linkage **(25a-25c)**, non-modified cyclodextrins were used. Reactions were carried out in DMSO and with carbonyldiimidazole (CDI) in combination with triethylamine (Et_3N). The yield of these reactions was usually around 20%, which was consistent with the fact that it is a modification of native CD. The mixture of products contained monosubstituted CD as well as a starting material and di- and tri-substituted derivatives. This mixture was separated by chromatography. The ideal reaction time was set up to 72 h. Another approach for the synthesis which was recently described by Wei *et al*.[139] promised to be applicable for the preparation of conjugates modified with fluorouracil on the secondary rim. This approach did not lead to the formation of the desired product.

The synthesis of conjugates of FU and cyclodextrins modified by diamines **(5a-b, 6a-6b)** was also tested. The yield of such kind of reaction did not exceed 50%, which was caused by the formation of side products coming from reactions on secondary nitrogen; these conjugates are not a part of this work.

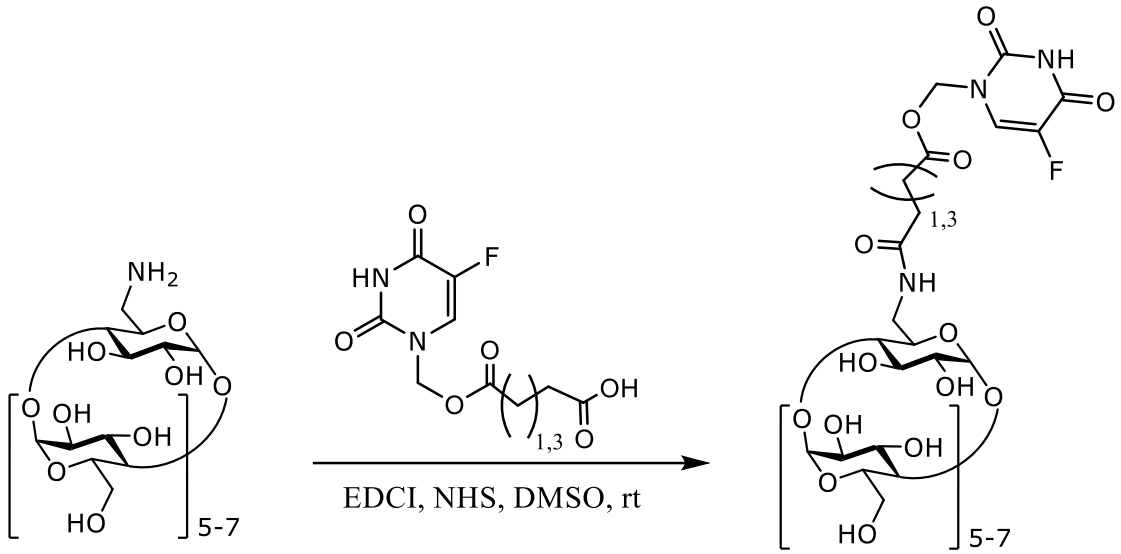
The synthesis of hydrazide conjugates turned out to be very complicated. Used approaches for its synthesis are depicted below (Scheme 6). The first idea was to connect the hydrazide derivative of FUA with formyl-CD's **(8a-8c)**. Because this hydrazide was not prepared, the strategy was changed to the synthesis starting from hydrazino-CD's **(4a-c)** and 1-carbethoxymethyl-5-fluorouracil **(21)**. This approach was not successful; even after heating the reaction mixture to 90 °C, only a small amount of a target compound was detected by LC-MS (after 24 h from the start of the reaction). Another planned approach started from hydrazide of Gly/FU **(23)** and carboxy-CD's **(9a-c)**, was not completed due to lack of time.



Scheme 6: Synthetic routes to get conjugates provided with a hydrazide linkage.

The synthesis of conjugates between FU and amino-CD's provided with dicarboxylic acid linkage **(26a-c, 27a-c)** (Scheme 7) was on the beginning planned to start from hydroxymethyl-FU **(17)** and dicarboxylic acids connected to CD moiety. These dicarboxylic acid-CD derivatives were prepared with succinic and adipic acid (reaction with malonic acid led to the decarboxylation reaction). Due to changes in our strategy, these compounds are not a part of this work. Finally, the synthesis was carried out with EDCI and NHS as same as in the case of first prepared conjugates. Starting compounds were amino-CD's **(3a-c)** and FU connected to appropriate dicarboxylic acid

(20a) and **(20b)**. This change occurred because of the number of reaction steps compared to the original idea. In comparison to a shorter linkage in the case of FUA/amino-CD's conjugates, reactions were quicker and proceeded in better yields (around 85%).



3a-c **26a-c, 27a-c**

84-91%

Scheme 7: Conjugates of FU/amino-CD's with dicarboxylic acid linkage.

The conjugate provided with a targeting group (Figure 12) was not prepared due to the fact that it was not possible to obtain pure folic acid (FA) functionalized with alkyne moiety (in a reasonable number of steps). The reaction was carried out with propargylamine as it was described before[140] but led to a formation of a mixture of products.

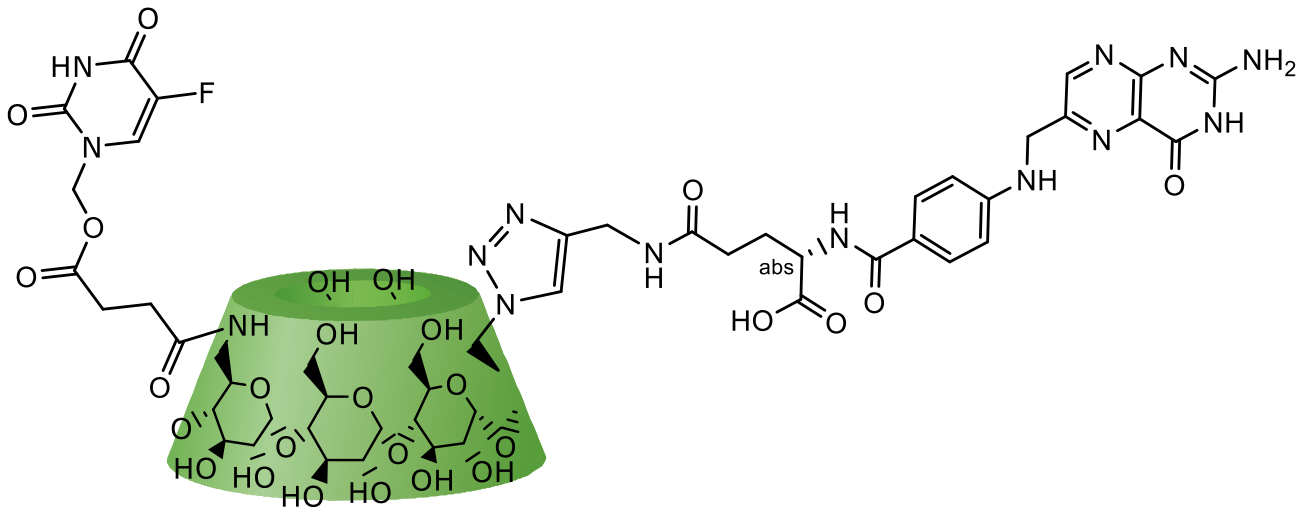


Figure 12: The designed conjugate provided with a targeting group.

5. CONCLUSION

During the work on this diploma thesis, several conjugates of cyclodextrins and fluorouracil were proposed. First of all, cyclodextrins were modified with appropriate functional groups – a total of 22 differently modified cyclodextrins were prepared. As a part of the synthetic process, the approach to the synthesis of cyclodextrin carboxylic acids was changed to the better strategy, which allowed to shorten the reaction time and rapidly increase the yield.

Several modifications of 5-fluorouracil were performed to achieve a molecule that could be connected to the appropriate cyclodextrin derivative. Some of the proposed molecules could not be prepared which led to changes in our synthetic strategy. In the end, some reasonable molecules containing 5-fluorouracil were obtained and used for conjugation with cyclodextrins.

A total of 12 conjugates of 5-fluorouracil and α-, β- and γ-cyclodextrins were prepared and characterized. These conjugates can be further studied, for example, in terms of ability to create complexes, stability and biological activity.

6. EXPERIMENTAL SECTION

6.1. General methods, chemicals, and instruments

For thin-layer chromatography, silica gel plates 60 F_{254} (Merck) were used. Used mobile phases are the following:

S1: $CHCl_3$/MeOH/H_2O 5/4/1
S2: nPrOH/H_2O/EtOAc/NH_3 6/3/1/1
S3: $CHCl_3$/MeOH 9/1
S4: hexane/EtOAc 1/1
S5: hexane/EtOAc 3/1
S6: nBuOH/H_2O/AcOH 3/1/1
S7: EtOH/NH_3/H_2O 20/5/3
S8: nPrOH/H_2O/EtOAc/NH_3 5/5/3/3
S9: hexane/EtOAc 2/1

For visualization of compounds on TLC plates were used 50% H_2SO_4, ninhydrin (200 mg of ninhydrin in 95 ml of butanol with 5 ml AcOH), bromocresol green (100 mg of bromocresol green in 75 ml of ethanol), and basic solution of $KMnO_4$ (1.5 g of $KMnO_4$, 10 g of K_2CO_3 and 1.25 ml of 10% NaOH dissolved in 200 ml of water). All of these methods included heating with a heat gun. Some compounds were detectable by UV light (wavelength 254 and 366 nm). Ammonia used in mobile phases was in the form of a 24% solution.

Chemicals and solvents were purchased from Merck, Wako, Fluorochem, Sigma Aldrich, Penta, Lach-ner, and P-lab. Chemicals were used without further purification, all solvents were distilled before usage, and the water used was deionized. Dry solvents were obtained by drying over molecular sieves (3 Å, Carl Roth). Chromatography was performed with LiChroprep RP-18, 40-63 μm (Merck) or Silia*Flash* P60, 40-63 μm (SiliCycle). Flash column chromatography was performed on COMPACT PREPARATIVE SYSTEM (50 ml/min, TOY18DAD800; ECOM). Different ion exchangers purchased from Fluka were used to separate charged compounds. Sephadex G-10 and Sephadex G-25, which were used for gel chromatography, were obtained from Lachema and Pharmacia.

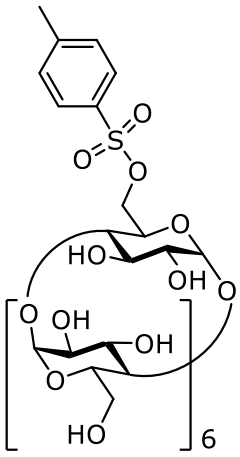
Solvents were evaporated by rotary vacuum evaporators from Büchi at temperatures up to 60°C and products were dried using an oil pump from LAVAT at 50-80 °C.

NMR spectra were measured on Bruker Avance III 400 MHz, Varian NMR System 300 MHz, and Bruker Avance III 600 MHz. Deuterated solvents were purchased from ARMAR, Sigma Aldrich, and Deutero. The mass spectra (MS) were measured by LCMS-2020 Shimadzu or by Bruker Esquire 3000. HRMS spectra were obtained by Agilent 6530 Q-TOF LC/MS. Thermo Nicolet - Avatar 370 5T-IR device was used to measure IR spectra.

Freeze dryer Labconco FreeZone 2.5 was used for lyophilizations, reactions under microwave irradiation were carried out in Microwave Synthesizer Discover purchased from CEM.

6.2. Synthetic procedures

6^{A}-*O*-*p*-Toluenesulfonyl-β-cyclodextrin (1a)



β-Cyclodextrin (50.0 g, 44.0 mmol, 1.0 eq) was dissolved in water (1100 ml), and then tosyl chloride (12.6 g, 66.0 mmol, 1.5 eq) was added. The reaction mixture was left to react at rt for 3 h under stirring, and then NaOH was added dropwise (22 g in 200 ml of water, 550 mmol). After 10 min, the reaction mixture was filtered, and the filtrate was neutralized by HCl and left overnight at 4 °C. The resulting precipitate was filtered, and dissolved at boiling in 50% methanol solution in water (100 ml). The mixture was left to cool down and then filtered. The resulting crystals were filtered off, and the crystallization was repeated twice in the same way. The product was dried using an oil pump at 60 °C. Pure product was obtained in the form of white crystals, and the amount of 14.53 g, the yield was 26%. The NMR spectra were in agreement with the literature.[38,141]

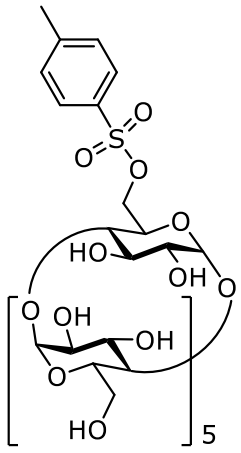
R_F = 0.45 (S1, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.75 (d, J = 8.1 Hz, 2 H, H2′), 7.43 (d, J = 8.1 Hz, 2 H, H3′), 5.83-5.62 (m, 14 H, OH-2, OH-3), 4.86-4.74 (m, 7 H, H1), 4.53-4.33 (m, 6 H, OH-6), 4.31 (s, 1 H, H6), 4.19 (dd, J^1 = 11.0 Hz, J^2 = 6.3 Hz, 1 H, H6), 3.75-3.15 (m, 49 H, H2, H3, H4, H5, H6, overlapped with H_2O), 2.43 (s, 3 H, H5′) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 145.28, 133.14, 130.36, 128.06, 102.40, 81.98, 73.64-72.27, 70.19, 69.38, 60.38, 21.68 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{49}H_{76}O_{37}S$: 1311.4 $[M+Na]^+$, found 1311.4. $[M+Na]^+$.

6^A-*O*-*p*-Toluenesulfonyl-α-cyclodextrin (1b)



Dried α-cyclodextrin (51.6 g, 53.0 mmol, 1.0 eq) was dissolved in pyridine (750 ml; water content 51.2 ppm) at 50 °C under a nitrogen atmosphere. After complete dissolving, tosyl chloride (9.1 g, 48.0 mmol, 0.9 eq) in pyridine (80 ml) was added during 20 min (the mixture was cooled down in an ice bath). The resulting mixture was left to react at rt. After one hour, the pyridine was evaporated using an oil pump at 60 °C. The residue was precipitated with acetone (1580 ml) and then filtered. Precipitate was dissolved in water and separated by flash column chromatography (H_2O/MeOH gradient; product eluted in 20 % MeOH). Solvents were evaporated under reduced pressure and the product was dried using an oil pump at 60 °C. The pure product was obtained in the form of white crystals and the amount of 15.10 g, the yield was 25%. The NMR spectra were in agreement with the literature.[38]

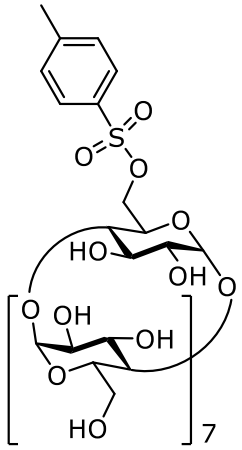
R_F = 0.42 (S1, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.77 (d, *J* = 8.4 Hz, 2 H, H2´), 7.45 (d, *J* = 8.2 Hz, 2 H, H3´), 5.56-5.38 (m, 12 H, OH-2, OH-3) 4.81-4.77 (m, 4 H, H1), 4.72 (d, *J* = 3.2 Hz, 1 H, H1), 4.66 (d, *J* = 3.2 Hz, 1 H, H1), 4.52-4.44 (m, 5 H, OH-6) 4.33-4.23 (m, 2 H, H6), 3.89-3.13 (m, 34 H, H2, H3, H4, H5, H6), 2.41 (s, 3 H, H5´) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 145.26, 132.91, 130.41, 128.17, 102.60-102.34, 102.00, 82.78-82.02, 73.79-71.88, 70.13, 69.35, 60.45-60.11, 21.62 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{43}H_{66}O_{32}S$: 1149.3 $[M+Na]^+$, found 1149.3 $[M+Na]^+$.

6^{A}-*O*-*p*-Toluenesulfonyl-γ-cyclodextrin (1c)



Dried γ-cyclodextrin (3000 mg, 2.313 mmol, 1.0 eq) was dissolved in pyridine (50 ml; water content 61.9 ppm) at 50 °C under a nitrogen atmosphere. After complete dissolving, tosyl chloride (402.6 mg, 2.111 mmol, 0.9 eq) in pyridine (3.6 ml) was added during 10 minutes (the mixture was cooled down in an ice bath). The resulting mixture was left to react at rt overnight, then pyridine was evaporated using an oil pump at 60 °C. The residue was dissolved in water (10 ml), precipitated with acetone (25 ml), and filtered. Precipitate was dissolved in water and separated by flash column chromatography (H_2O/MeOH gradient; product eluted in 20 % MeOH). Solvents were evaporated under reduced pressure and the product was dried using an oil pump at 60 °C. The pure product was obtained in the form of white crystals and the amount of 812.6 mg, the yield was 24%. The NMR spectra were in agreement with the literature.[38]

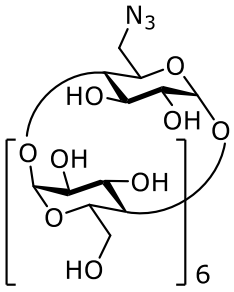
R_F = 0.43 (S1, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.77 (d, J = 7.5 Hz, 2 H, H2′), 7.45 (d, J = 7.7 Hz, 2 H, H3′), 5.91-5.66 (m, 16 H, OH-2, OH-3), 4.93-4.87 (m, 6 H, H1), 4.83-4.78 (m, 2 H, H1), 4.60-4.38 (m, 7 H, OH-6), 4.30 (d, J = 9.5 Hz, 1 H, H6), 4.20 (m, 1 H, H6), 3.77-3.20 (m, 38 H, H2, H3, H4, H5, H6, overlapped with H_2O), 2.42 (s, 3 H, H5′) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 145.34, 132.99, 130.47, 128.56, 128.03, 125.97, 102.69, 102.15, 101.58, 81.69-81.02, 80.59, 73.54-72.44, 69.96, 69.47, 60.43, 60.17, 59.83, 21.60 ppm.

LC-MS (ESI) m/z calculated for $C_{55}H_{86}O_{42}S$: 1474.3 $[M+Na]^+$, found 1473.5 $[M+Na]^+$.

6^A-Azido-6^A-deoxy-β-cyclodextrin (2a)



6^A-*O*-*p*-Toluenesulfonyl-β-cyclodextrin (31.08 g, 24.06 mmol, 1.0 eq) was dissolved in DMF (300 ml), then sodium azide (31.59 g, 481.17 mmol, 20.0 eq) was added under stirring. The reaction mixture was heated to 80 °C and left to react overnight. After 16 h, the reaction mixture was cooled down to rt and then precipitated with acetone (900 ml). The crude product was redissolved in water and precipitated with the acetone/water mixture (10% water, 200 ml); this procedure was repeated three times. The product was dried using an oil pump at 60 °C. Pure product was obtained in the form of white powder and the amount of 26.25 g, the yield was 94%. NMR spectra were in agreement with the literature.[109]

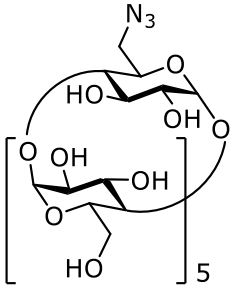
R_F = 0.30 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 5.03-4.97 (m, 7 H, H1), 3.96-3.70 (m, 28 H, H3, H5, H6), 3.62-3.45 (m, 14 H, H2, H4) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.81, 81.08, 73.02, 72.00, 71.74, 60.24, 51.01 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{42}H_{69}O_{34}N_3$: 1183.0 $[M+Na]^+$, found 1182.9 $[M+Na]^+$.

6^A-Azido-6^A-deoxy-α-cyclodextrin (2b)



Dried 6^A-*O*-*p*-Toluenesulfonyl-α-cyclodextrin (486.8 mg, 0.432 mmol, 1.0 eq) was dissolved in DMF (5 ml), then sodium azide (564.4 g, 8.682 mmol; 20.0 eq) was added. The reaction mixture was heated to 80 °C and left to react overnight. After 20 h, the reaction mixture was cooled down to rt a then precipitated with acetone/water mixture (67 ml of acetone and 8 ml of water). The precipitate was filtered and washed with acetone. The product was dried using an oil pump at 65 °C. The pure product was obtained in the form of white powder and the amount of 379.6 mg, the yield was 88%. The NMR spectra were in agreement with the literature.[142]

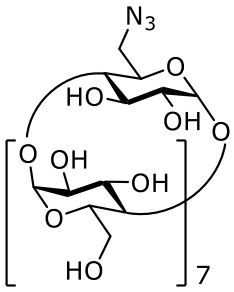
R_F = 0.29 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.66-5.34 (m, 12 H, OH-2, OH-3), 4.89-4.73 (m, 6 H, H1), 4.62-4.44 (m, 5 H, OH-6) 3.89-3.50 (m, 24 H, H3, H5, H6), 3.50-3.16 (m, 12 H, H2, H4, overlapped with H_2O) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.39, 82.15, 81.35, 81.21, 73.29, 72.16, 71.97, 71.68, 71.60, 70.73, 60.40, 31.39 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{36}H_{59}O_{29}N_3$: 1021.0 $[M+Na]^+$, found 1020.4 $[M+Na]^+$.

6^A-Azido-6^A-deoxy-γ-cyclodextrin (2c)



6^A-*O*-*p*-Toluenesulfonyl-γ-cyclodextrin (192.9 mg, 0.133 mmol, 1.0 eq) was dissolved in DMF (2 ml), then sodium azide (179.7 mg, 2.764 mmol, 20.1 eq) was added. The reaction mixture was heated to 80 °C and left to react overnight under stirring. After 24 h, the reaction mixture was left to cool down and then was precipitated with acetone/water mixture (27 ml of acetone, 3 ml of water), the precipitate was filtered and washed. The product was dried using an oil pump at 60 °C. 6^A-Azido-6^A-deoxy-γ-cyclodextrin was obtained in the form of white powder and the amount of 136.1 mg, the yield was 77%.

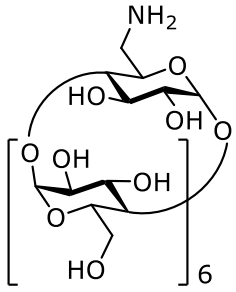
R_F = 0.29 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.93-5.67 (m, 16 H, OH-2, OH-3), 4.96-4.84 (m, 8 H, H1), 4.65-4.51 (m, 7 H, OH-6), 3.75-3.49 (m, 32 H, H3, H5, H6), 3.43-3.26 (m, 16 H, H2, H4, overlapped with H_2O) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 102.15, 81.13, 73.48-72.52, 60.40, 51.05 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{48}H_{79}O_{39}N_3$: 1345.1 $[M+Na]^+$, found 1345.2 $[M+Na]^+$.

6^AAmino-6^A-deoxy-β-cyclodextrin (3a)



6^A-Azido-6^A-deoxy-β-cyclodextrin (1160.0 mg, 1.0 mmol, 1.0 eq) was dissolved in DMF (23 ml), then triphenylphosphine (289.0 mg, 1.1 mmol, 1.1 eq) was added under stirring. The reaction mixture was left to react at rt for 2 h, then water (2.5 ml) was added dropwise and the reaction mixture was heated to 90 °C and left to react overnight under the same conditions. After 18 h, the reaction mixture was left to cool down at rt. The product was then precipitated with acetone (420 ml), and the precipitate was filtered and washed with acetone; this procedure was repeated twice. The product was dried using an oil pump at 70 °C. 6^A-Amino-6^A-deoxy-β-cyclodextrin was obtained as a white powder and in the amount of 838.9 mg, the yield was 74%. The NMR spectra were in agreement with the literature.[33]

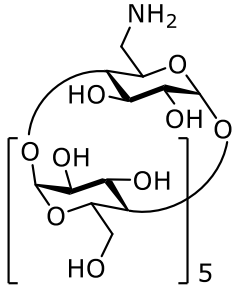
R_F = 0.17 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.83-5.61 (m, 14 H, OH-2, OH-3), 4.94-4.78 (m, 7 H, H1), 4.52-4.40 (m, 6 H, OH-6), 3.73-3.52 (m, 28 H, H3, H5, H6), 3.41-3.26 (m, 14 H, H2, H4, overlapped with H_2O) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 102.38, 82.08, 81.95, 81.89, 73.52, 72.88, 72.51, 60.40 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{42}H_{71}O_{34}N$: 1135.0 $[M+H]^+$, found 1134.9 $[M+H]^+$.

6^A-Amino-6^A-deoxy-α-cyclodextrin (3b)



6^A-Azido-6^A-deoxy-α-cyclodextrin (230.5 mg, 0.231 mmol, 1.0 eq) was dissolved in DMF (4 ml), then triphenylphosphine (70.4 mg, 0.268 mmol, 1.2 eq) was added under stirring. The reaction mixture was left to react at rt for 2 h, then water was added dropwise (0.5 ml) and the reaction mixture was heated to 90 °C and left to react overnight under the same conditions. After 21 h, the reaction mixture was left to cool down at rt and precipitated with acetone (50 ml). The product was dried using an oil pump at 60 °C. 6^A-Amino-6^A-deoxy-β-cyclodextrin was obtained in the form of white powder and the amount of 207.3 mg, the yield was 92%. The NMR spectra were in agreement with the literature.[33]

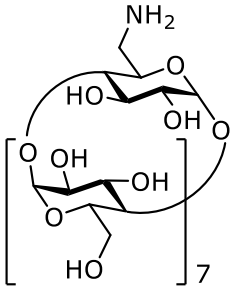
R_F = 0.21 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.61-5.40 (m, 12 H, OH-2, OH-3), 4.91-4.75 (m, 6 H, H1), 4.59-4.44 (m, 5H, OH-6), 3.83-3.52 (m, 24 H, H3, H4, H6), 3.48-3.20 (m, 12 H, H2, H4, overlapped with H_2O) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 102.44, 82.58, 73.73, 72.58, 60.45 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{36}H_{61}O_{29}N$: 973.0 $[M+H]^+$, found 972.4 $[M+H]^+$.

6^A-Amino-6^A-deoxy-γ-cyclodextrin (3c)



6^A-Azido-6^A-deoxy-γ-cyclodextrin (42.4 mg, 0.032 mmol, 1.0 eq) was dissolved in DMF (1 ml), then triphenylphosphine (10.0 mg, 0.038 mmol, 1.2 eq) was added under stirring. The reaction mixture was left to react at rt for 2 h, then water was added dropwise (0.1 ml) and the reaction mixture was heated to 90 °C and left to react under the same conditions. After 5 h, the reaction mixture was left to cool down at rt. The reaction mixture was precipitated with acetone (9 ml), and the precipitate was filtered and washed. The product was dried using an oil pump at 60 °C. 6^A-Amino-6^A-deoxy-γ-cyclodextrin was obtained in the form of white powder and the amount of 33.8 mg, the yield was 81%. The NMR spectra were in agreement with the literature.[143]

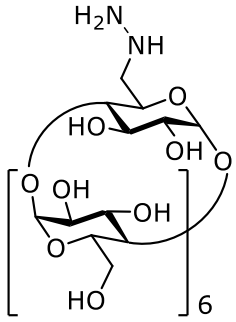
R_F = 0.24 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 5.04 (s, 8 H, H1), 3.91-3.73 (m, 30 H, H3, H5, H6), 3.62-3.48 (m, 16 H, H2, H4), 3.41 (t, *J* = 4.4 Hz, 1 H, H6) 3.00 (d, *J* = 9.5 Hz, 1 H, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.62, 80.38, 72.89, 72.29, 71.72, 60.18 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{48}H_{81}O_{39}N$: 1297.1 $[M+H]^+$, found 1297.1 $[M+H]^+$.

6^A-Hydrazino-6^A-deoxy-β-cyclodextrin (4a)



6^A-*O*-*p*-Toluenesulfonyl-β-cyclodextrin (377.5 mg, 0.293 mmol, 1.0 eq) was dissolved in hydrazine hydrate solution (1.5 ml; 78-82 %). The reaction mixture was left to react at rt under stirring. After 24 h, the reaction mixture was precipitated with ethanol (20 ml) and centrifuged at 6000 rpm (3 × 20 min). The supernatant was removed and the residue was diluted with water (2 ml). Water was removed from the product using an oil pump at 60 °C and the resulting product was dried at the same temperature. 6^A-Hydrazino-6^A-deoxy-α-cyclodextrin was obtained in the form of white crystals and the amount of 308.0 mg, the yield was 92%. The NMR spectra were in agreement with the literature.[35]

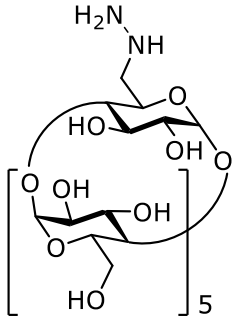
R_F = 0.09 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 4.99 (s, 7 H, H1), 3.95-3.72 (m, 26 H, H3, H5, H6), 3.63-3.44 (m, 13 H, H2, H4), 3.39 (t, *J* = 3.4 Hz, 1 H, H4) 3.14 (m, 1 H, H6), 2.91 (m, 1 H, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.81, 101.47, 83.28, 81.07, 80.79, 73.08-72.80, 72.10-71.63, 69.62, 60.22 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{42}H_{72}O_{34}N_2$: 1150.2 $[M+H]^+$, found 1149.4 $[M+H]^+$.

6^A-Hydrazino-6^A-deoxy-α-cyclodextrin (4b)



6^A-*O*-*p*-Toluenesulfonyl-α-cyclodextrin (251.7 mg, 0.223 mmol, 1.0 eq) was dissolved in 1.0 ml of hydrazine hydrate solution (78-82 %). The reaction was left to proceed at rt under stirring. After 22 h, the reaction mixture was diluted with water (1 ml) and precipitated with ethanol (25 ml). The precipitate was separated by centrifugation at 6000 rpm (3 × 20 min), then the supernatant was removed and the residue was diluted with water (2 ml). Water was removed from the product using an oil pump at 60 °C and the resulting product was dried at the same temperature. 6^A-Hydrazino-6^A-deoxy-α-cyclodextrin was obtained in the form of white crystals and the amount of 119.8 mg, the yield was 54%.

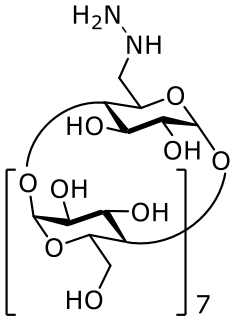
R_F = 0.05 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 5.02-4.93 (m, 6 H, H1), 3.96-3.68 (m, 22 H, H3, H5, H6), 3.61-3.44 (m, 11 H, H2, H4), 3.39 (t, *J* = 3.4 Hz, 1 H, H4), 3.19 (m, 1 H, H6), 2.90 (m, 1 H, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.35, 101.19, 83.38, 81.32, 81.16, 73.25, 73.06, 71.97, 71.64, 69.95, 60.38 ppm.

HRMS (ESI) *m*/*z* calculated for $C_{36}H_{62}O_{29}N_2$: 987.3511 $[M+H]^+$, found 987.3517 $[M+H]^+$.

6^A-Hydrazino-6^A-deoxy-γ-cyclodextrin (4c)



6^A-*O*-*p*-Toluenesulfonyl-γ-cyclodextrin (111.0 mg, 0.076 mmol, 1.0 eq) was dissolved in hydrazine hydrate solution (0.5 ml; 78-82%) and water (0.5 ml). The reaction was left to proceed at rt under stirring. After 25 h, the reaction mixture was precipitated with ethanol (10 ml). The precipitate was separated by centrifugation at 6000 rpm (3 × 20 min), then the supernatant was removed and the residue was diluted with water (2 ml). Water was removed from the product using an oil pump at 60 °C and the resulting product was dried at the same temperature. 6^A-Hydrazino-6^A-deoxy-γ-cyclodextrin was obtained in the form of white crystals and the amount of 76.0 mg, the yield was 76%.

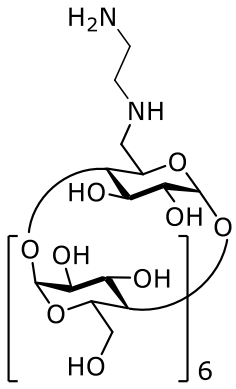
R_F = 0.10 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 5.03 (m, 8 H, H1), 3.93-3.72 (m, 30 H, H3, H5, H6), 3.62-3.46 (m, 15 H, H2, H4), 3.40 (t, *J* = 3.1 Hz, 1 H, H4), 3.12 (m, 1 H, H6), 2.91 (m, 1 H, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.62, 80.39, 72.88, 72.27, 71.73, 60.17 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{48}H_{82}O_{39}N_2$: 1312.3 $[M+H]^+$, found 1311.5 $[M+H]^+$.

6^A-((2-aminoethyl)amino)-6^A-deoxy-β-cyclodextrin (5a)



Dried 6^A-*O*-*p*-Toluenesulfonyl-β-cyclodextrin (150.4 mg, 0.232 mmol, 1.0 eq) was dissolved in ethylendiamine (1.0 ml) under a nitrogen atmosphere. The reaction mixture was heated to 80 °C and left to react at this temperature under stirring for 4 h. The product was precipitated with acetone (17.5 ml) and filtered. Precipitation was repeated three times to remove ethylenediamine (detected by TLC). The crude product was dried using an oil pump at 60 °C. 6^A-((2-aminoethyl)amino)-6^A-deoxy-β-cyclodextrin was obtained in the form of white powder and the amount of 134.0 mg, the yield was 98%. The NMR spectra were in agreement with the literature.[57,144,145]

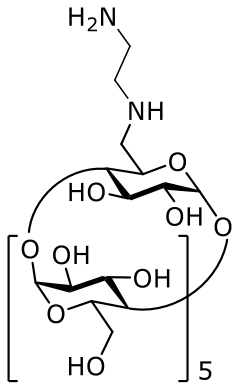
R_F = 0.11 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 4.99 (s, 7 H, H1), 3.95-3.72 (m, 26 H, H3, H5, H6), 3.57 (m, 7 H, H2) 3.50 (t, *J* = 3.4 Hz, 6 H, H4), 3.38 (t, *J* = 3.4 Hz, 1 H, H4), 2.98 (d, *J* = 9.2 Hz, 1 H, H6), 2.83-2.58 (m, 5 H, NHCH_2C$H_2$$NH_2$, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.79, 101.50, 83.44, 81.07, 80.87, 73.04, 72.96, 72.01, 71.79, 70.40, 60.22, 49.52, 49.13, 39.41 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{44}H_{76}O_{34}N_2$: 1200.2 $[M+Na]^+$, found 1199.9 $[M+Na]^+$.

6^A-((2-aminoethyl)amino)-6^A-deoxy-α-cyclodextrin (5b)



Dried 6^A-*O*-*p*-toluenesulfonyl-α-cyclodextrin (150.4 mg, 0.133 mmol, 1.0 eq) was dissolved in ethylenediamine (0.8 ml) under a nitrogen atmosphere. The reaction mixture was heated to 80 °C and left to react under stirring for 3.5 h. After cooling down the reaction mixture was diluted with water (1 ml) and precipitated with acetone (150 ml). Precipitation was repeated three times to remove unreacted ethylenediamine (detected by TLC). 6^A-((2-aminoethyl)amino)-6^A-deoxy-α-cyclodextrin was obtained in the form of light orange powder and the amount of 69.8 mg, the yield was 52%.

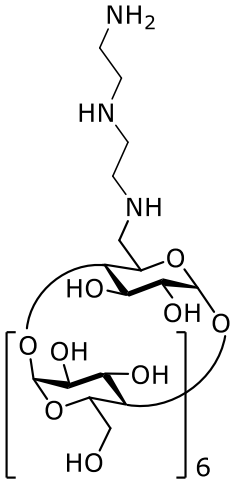
R_F = 0.14 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 4.97 (s, 6 H, H1), 3.97-3.67 (m, 22 H, H3, H5, H6), 3.61-3.41 (m, 11 H, H2, H4), 3.37 (t, *J* = 3.2 Hz, 1 H, H4), 3.00 (d, *J* = 3.5 Hz, 1 H, H6), 2.85 (d, *J* = 9.5 Hz, 1 H, H6), 2.65-2.50 (m, 4 H, C*H*$_2$) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.41, 81.21, 73.28, 72.02, 71.63, 60.41, 41.53, 30.25 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{38}H_{66}O_{29}N_2$: 1038.1 $[M+Na]^+$, found 1037.8 $[M+Na]^+$.

6^A-((2-((2-aminoethyl)amino)ethyl)amino)-6^A-deoxy-β-cyclodextrin (6a)



Dried 6^A-*O*-*p*-toluenesulfonyl-β-cyclodextrin (150.9 mg, 0.117 mmol, 1.0 eq) was dissolved in ethylendiamine (1.0 ml) under a nitrogen atmosphere. The reaction mixture was heated to 80 °C and allowed to react under stirring for 1 h. After cooling down the reaction mixture was precipitated with acetone (17.5 ml) and filtered. Precipitation was repeated three times to remove unreacted diethylenetriamine (detected by TLC). The crude product was washed with acetone and dried using an oil pump at 60 °C. 6^A-((2-((2-aminoethyl)amino)ethyl)amino)-6^A-deoxy-β-cyclodextrin was obtained in the form of yellow powder and the amount of 136.0 mg, the yield was 95%. The NMR spectra were in agreement with the literature.[146]

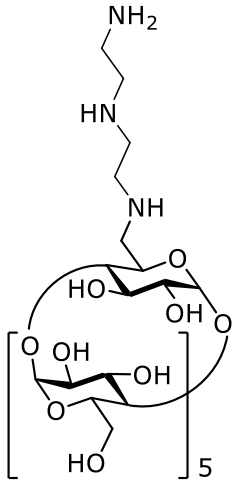
R_F = 0.14 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 4.98 (s, 7 H, H1), 3.89-3.70 (m, 26 H, H3, H5, H6), 3.56 (m, 7 H, H2) 3.49 (t, *J* = 3.5 Hz, 6 H, H4), 3.35 (t, *J* = 3.5 Hz, 1 H, H4), 2.98 (d, *J* = 9.6 Hz, 1 H, H6), 2.69-2.53 (m, 9 H, C*H*$_2$, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.86, 81.10, 73.09, 73.02, 72.01, 71.84, 60.20, 50.20, 48.82, 39.66, 30.21 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{46}H_{81}O_{34}N_3$: 1243.3 $[M+Na]^+$, found 1243.1 $[M+Na]^+$.

6^A-((2-((2-aminoethyl)amino)ethyl)amino)-6^A-deoxy-α-cyclodextrin (6b)



Dried 6^A-*O*-*p*-toluenesulfonyl-α-cyclodextrin (150.6 mg, 0.134 mmol, 1.0 eq) was dissolved in diethylenetriamine (1.4 ml) under a nitrogen atmosphere. The reaction mixture was heated to 80 °C and allowed to react under stirring for 1 h. After cooling down the reaction mixture was diluted in water (1 ml) and precipitated with acetone (150 ml). Precipitation was repeated three times to remove unreacted diethylenetriamine (detected by TLC). The crude product was washed with acetone and dried using an oil pump at 60 °C. 6^A-((2-((2-aminoethyl)amino)ethyl)amino)-6^A-deoxy-α-cyclodextrin was obtained in the form of yellow powder and the amount of 128.5 mg, the yield was 91%.

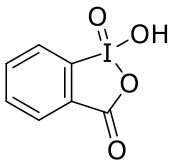
R_F = 0.18 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 4.99-4.94 (m, 6 H, H1), 3.93-3.70 (m, 22 H, H3, H5, H6), 3.54 (m, 6 H, H2) 3.49 (t, *J* = 3.5 Hz, 5 H, H4), 3.35 (t, *J* = 3.2 Hz, 1 H, H4), 3.04 (d, *J* = 9.1 Hz, 1 H, H6), 2.70-2.55 (m, 9 H, CH_2, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 101.32, 81.19, 73.21, 73.14, 71.98, 71.61, 60.38, 50.00, 48.83, 39.58, 30.22 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{40}H_{71}O_{29}N_3$: 1081.1 $[M+Na]^+$, found 1080.9 $[M+Na]^+$.

2-Iodoxybenzoic acid (7)

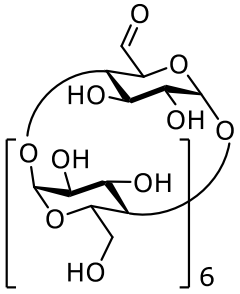


To a stirred solution of oxone (37.2 g, 0.060 mol, 3.0 eq) in water (200 ml), 2-iodobenzoic acid (5.0 g, 0.020 mol, 1.0 eq) was added in three portions. The mixture was left to react at 70 °C under stirring. After 1 h, the mixture was placed on ice and stirred for an additional 30 min. The resulting crystals were filtered and washed with acetone (6 × 10 ml) and water (3 × 10 ml). The product was obtained in the form of white crystals, which were dried by air; the amount was 3.960 g, and the yield was 71%. The NMR spectra were in agreement with the literature.[147]

^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.20-7.76 (m, aromatic H, 4 H) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 167.96, 147.02, 133.87, 133.42, 131.91, 130.56, 125.47 ppm.

6^{A}-Formyl-6^{A}-deoxy-β-cyclodextrin (8a)



To the flask with IBX (121.0 mg, 0.432 mmol, 1.1 eq) in DMSO (10 ml), β-cyclodextrin (501.1 mg, 0.441 mmol, 1.0 eq) in DMSO (5 ml) was added under stirring. The reaction was left to proceed at rt for 95 min. The product was precipitated with cold acetone (450 ml), and the resulting mixture was given to the freezer for 1 h and then filtered. Precipitate was washed with acetone, dissolved in water, and purified by flash column chromatography (H_2O/MeOH gradient, product eluted in 10 % MeOH). Solvents were evaporated under reduced pressure and the product was dried using an oil pump at 60 °C. 6^{A}-Formyl-6^{A}-deoxy-β-cyclodextrin was obtained in the form of white powder and the amount of 438.6 mg, the yield was 90%. The NMR spectra were in agreement with the literature.[32]

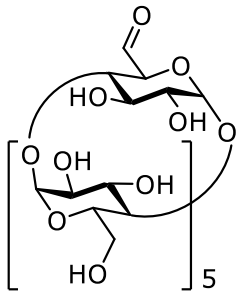
R_F = 0.27 (S2, 50% H_2SO_4)

^{1}H NMR (300 MHz, DMSO-d_6): δ = 9.69 (s, 1 H, C*H*O), 5.70 (br s, 14 H, OH-2, OH-3), 4.94 (s, 1 H, H1), 4.83 (s, 6 H, H1), 4.45 (br s, 6 H, OH-6), 4.20 (d, J = 4.1 Hz, 1 H, H5), 3.73-3.20 (m, 39 H, H2, H3, H4, H5, H6) ppm.

^{13}C NMR (75 MHz, DMSO-d_6): δ = 198.8, 102.4, 87.9, 82.0, 73.5, 72.9, 72.5, 60.4 ppm.

LC-MS (ESI) m/z calculated for $C_{42}H_{68}O_{35}$: 1132.0 $[M-H]^-$, found 1132.3 $[M-H]^-$.

6^A-Formyl-6^A-deoxy-α-cyclodextrin (8b)



α-Cyclodextrin (1002.0 mg, 1.030 mmol, 1.0 eq) was dissolved in DMSO (10 ml), then was added to the flask with IBX (319.6 mg, 1.141 mmol, 1.1 eq) in DMSO (20 ml). The reaction was left to proceed at rt for 90 min, then the product was precipitated with cold acetone (900 ml). The resulting mixture was given to the freezer for 1 h and then filtered. The precipitate was washed with acetone, dissolved in water (15 ml), and purified by flash column chromatography (H_2O/MeOH gradient; product eluted in 10 % MeOH). Solvents were evaporated under vacuum and the product was dried using an oil pump at 60 °C. 6^A-Formyl-6^A-deoxy-α-cyclodextrin was obtained in the form of white powder and the amount of 909.4 mg, the yield was 91%.

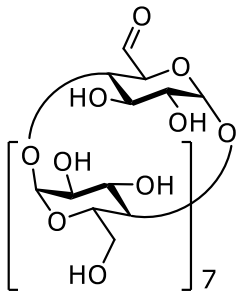
R_F = 0.25 (S2, 50% H_2SO_4)

^{1}H NMR (300 MHz, DMSO-d_6): δ = 9.73 (s, 1 H, CHO), 5.47 (br s, 12 H, OH-2, OH-3), 4.91 (m, 1 H, H1), 4.80 (m, 5 H, H1), 4.48 (br s, 5 H, OH-6), 4.32 (d, J = 4.5 Hz, 1 H, H5), 3.87-3.48 (m, 21 H, H3, H5, H6), 3.47-3.18 (m, 12 H, H2, H4) ppm.

^{13}C NMR (75 MHz, DMSO-d_6): δ = 199.1, 102.6, 82.6, 75.0-68.8, 63.1, 58.9 ppm.

LC-MS (ESI) m/z calculated for $C_{36}H_{58}O_{30}$: 969.8 $[M-H]^-$, found 970.3 $[M-H]^-$.

6^A-Formyl-6^A-deoxy-γ-cyclodextrin (8c)



γ-Cyclodextrin (505.9 mg, 0.390 mmol, 1.0 eq) was dissolved in DMSO (5 ml), then was added to the flask with IBX (121.0 mg, 0.432 mmol, 1.1 eq) in DMSO (10 ml). The reaction was left to proceed at rt for 95 min, then the product was precipitated with cold acetone (450 ml). The resulting mixture was given to the freezer for 1 h and then filtered. Precipitate was washed with acetone, dissolved in water (10 ml), and purified by flash column chromatography (H_2O/MeOH gradient; product eluted in 10% MeOH). Solvents were evaporated under vacuum and the product was dried using an oil pump at 60 °C. 6^A-Formyl-6^A-deoxy-γ-

cyclodextrin was obtained in the form of white powder and the amount of 479.8 mg, the yield was 95%.

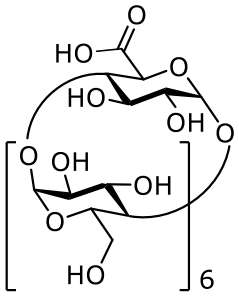
R_F = 0.29 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.67 (s, 1 H, CHO), 5.92-5.60 (m, 16 H, OH-2, OH-3), 4.99 (s, 1 H, H1), 4.89 (m, 7 H, H1), 4.60-4.40 (m, 7 H, OH-6), 4.14 (d, J = 4.5 Hz, 1 H, H5), 3.75-3.45 (m, 29 H, H3, H5, H6), 3.42-3.30 (m, 16 H, H2, H4, overlapped with H_2O) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 102.15, 81.39, 73.37, 73.05, 72.64, 60.44 ppm.

HRMS (ESI) m/z calculated for $C_{48}H_{78}O_{40}$: 1293.3997 $[M-H]^-$, found 1293.3991 $[M-H]^-$.

6^A-Carboxy-6^A-deoxy-β-cyclodextrin (9a)



6^A-Formyl-6^A-deoxy-β-cyclodextrin (50.1 mg, 0.044 mmol, 1.0 eq) was dissolved in H_2O (1.5 ml) with tBuOH (0.5 ml). 0.2 ml of 2-methyl-2-butene was dropped into the reaction mixture, then $NaClO_2$ (13.6 mg, 0.150 mmol, 3.4 eq) and NaH_2PO_4 (22.2 mg, 0.185 mmol, 4.2 eq) were added and the reaction was left to proceed overnight. After 12 h, no traces of starting material were detectable by TLC, solvents were evaporated under reduced pressure and the residue was desalted by gel chromatography. 6^A-Carboxy-6^A-deoxy-β-cyclodextrin was obtained as a white solid the amount of 44.6 mg, yielding 88%. The NMR spectra were in agreement with the literature.[32]

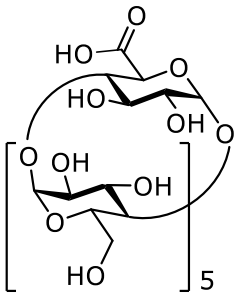
R_F = 0.14 (S2, 50% H_2SO_4)

^{1}H NMR (300 MHz, D_2O): δ = 4.94 (s, 1 H, H1), 4.89 (s, 6 H, H1), 4.14 (d, J = 4.5 Hz, 1 H, H5), 3.85-3.52 (m, 25 H, H3, H5, H6), 3.50-3.39 (m, 14 H, H2, H4) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 175.5, 104.6, 84.0, 75.5, 74.8, 74.3, 62.5 ppm.

LC-MS (ESI) m/z calculated for $C_{42}H_{68}O_{36}$: 1148.1 $[M-H]^-$, found 1148.7 $[M-H]^-$.

6^A-Carboxy-6^A-deoxy-α-cyclodextrin (9b)



6^A-Formyl-6^A-deoxy-α-cyclodextrin (50.0 mg, 0.052 mmol, 1.0 eq) was dissolved in H_2O (1.5 ml) with tBuOH (0.5 ml). 0.2 ml of 2-methyl-2-butene was dropped into the reaction mixture, then $NaClO_2$ (16.4 mg, 0.182 mmol, 3.5 eq) and NaH_2PO_4 (25.1 mg, 0.209 mmol, 4.0 eq) were added and the reaction was left to proceed at rt for 10 h. Solvents were evaporated under reduced pressure, the crude product was desalted by gel chromatography. 6^A-Carboxy-6^A-deoxy-α-cyclodextrin was obtained in the form of white powder and the amount of 42.0 mg, the yield was 82%.

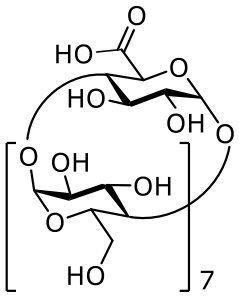
R_F = 0.12 (S2, 50% H_2SO_4)

^{1}H NMR (300 MHz, D_2O): δ = 4.91 (s, 1 H, H1), 4.87 (s, 5 H, H1), 4.25 (d, *J* = 4.9 Hz, 1 H, H5), 3.97-3.30 (m, 33 H, H2, H3, H4, H5, H6) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 172.4, 105.2, 104.2, 76.2-74.0, 62.1 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{36}H_{58}O_{31}$: 985.9 $[M-H]^-$, found 985.9 $[M-H]^-$.

6^A-Carboxy-6^A-deoxy-γ-cyclodextrin (9c)



6^A-Formyl-6^A-deoxy-γ-cyclodextrin (49.6 mg, 0.038 mmol, 1.0 eq) was dissolved in H_2O (1.5 ml) with tBuOH (0.5 ml). 0.2 ml of 2-methyl-2-butene was dropped into the reaction mixture, then $NaClO_2$ (10.1 mg, 0.112 mmol, 2.9 eq) and NaH_2PO_4 (18.1 mg, 0.151 mmol, 4.0 eq) were added and the reaction was left to proceed at rt overnight. After 18 h, solvents were evaporated under reduced pressure and the crude product was desalted by gel chromatography. 6^A-Carboxy-6^A-deoxy-γ-cyclodextrin was obtained in the form of white powder and the amount of 43.8 mg, the yield was 88%.

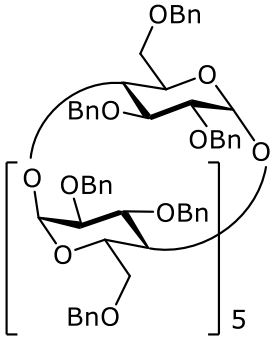
R_F = 0.16 (S2, 50% H_2SO_4)

^{1}H NMR (300 MHz, D_2O): δ = 4.99 (s, 1 H, H1), 4.94 (s, 7 H, H1), 4.08 (d, *J* = 3.9 Hz, 1 H, H5), 3.84-3.25 (m, 37 H, H2, H3, H4, H5, H6) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 175.0, 104.6, 76.4-74.0, 61.5 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{48}H_{78}O_{41}$: 1310.3 $[M-H]^-$, found 1311.0 $[M-H]^-$.

2^{A-F},3^{A-F},6^{A-F}-Octadeca-*O*-benzyl-α-cyclodextrin (10)



Dried α-cyclodextrin (10.0 g, 10.3 mmol, 1.0 eq) was dissolved in DMSO (240 ml). 60 % NaH in mineral oil (13.6 g, 339 mmol, 33.0 eq) was added in 10 min and the reaction mixture was left to stir at rt for 15 min and then was cooled down in an ice bath. After 10 min, benzyl chloride (32 ml, 278 mmol, 27.0 eq) was added under stirring for 10 min. The reaction was left to proceed at rt. After 22 h, water (250 ml) was added to the reaction mixture in 30 min and the product was extracted with diethyl ether (3 × 600 ml). Organic phases were combined and dried with $MgSO_4$. After 24 h, $MgSO_4$ was removed by filtration, and volatiles were evaporated under reduced pressure. The crude product (yellow oil) was dissolved in DCM (200 ml) and the product was separated by flash column chromatography (gradient hexane/ethyl acetate; product eluted with 20% EtOAc). Solvents were evaporated under reduced pressure; pure product was obtained by lyophilization in the form of white crystals and the amount of 22.4 g, the yield was 84%. The NMR spectra were in agreement with the literature.[148,149]

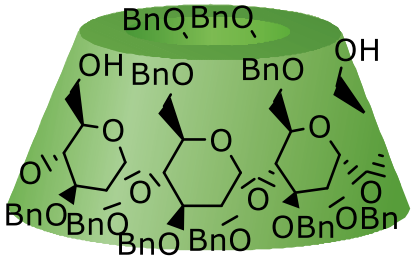
R_F = 0.50 (S5, 50% H_2SO_4)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.30-7.10 (m, 90 H, aromatic H), 5.20 (d, *J* = 10.9 Hz, 6 H, C3-O-CH_2Ph), 5.10 (d, *J* = 3.4 Hz, 6H, H1), 4.89 (d, *J* = 10.6 Hz, 6 H, C3-O-CH_2Ph), 4.54-4.30 (m, 24 H, C6-O-CH_2Ph, C2-O-CH_2Ph), 4.19-3.88 (m, 24 H, H3, H4, H5, H6), 3.54-3.45 (m, 12 H, H2, H6) ppm.

^{13}C NMR (100 MHz, $CDCl_3$): δ = 139.39, 138.40, 138.20, 128.45-126.85, 98.66, 81.03, 79.16, 77.38, 77.06, 76.74, 75.61, 73.42, 72.78, 71.57, 69.12 ppm.

MS (MALDI-TOF) *m*/*z* calculated for $C_{162}H_{168}O_{30}$: 2618.3 $[M+Na]^+$, found 2618.5 $[M+Na]^+$.

$2^{A-F},3^{A-F},6^{B-C},6^{D-F}$-Hexadeca-*O*-benzyl-α-cyclodextrin (11)



Perbenzylated α-cyclodextrin (1.0 g, 0.386 mmol, 1.0 eq) was dissolved in 1 M DIBAL-H in toluene (4 ml, 10 eq), the mixture was cooled in an ice bath. After 10 min, the reaction mixture was heated to 150 °C under microwave irradiation for 60 min. The mixture was cooled in an ice bath, and the water (15 ml) was added. The crude product was extracted with EtOAc (60 ml) and the organic phase was filtered and dried over $MgSO_4$. After filtration, volatiles were distilled off and the product was purified by flash column chromatography (hexan/EtOAc 3:1). Fractions with a product were combined and evaporated under the reduced pressure at 40 °C. The product was dried using an oil pump at 70 °C. $2^{A-F},3^{A-F},6^{B-C},6^{D-F}$-Hexadeka-O-benzyl-α-cyclodextrin was obtained as a yellowish solid the amount of 710.4 mg, the yield was 76%. The NMR spectra were in agreement with the literature.[150]

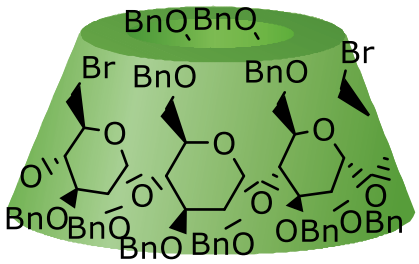
R_F = 0.46 (S9, 50% H_2SO_4)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.32-7.01 (m, 80 H, aromatic H), 5.75 (d, *J* = 3.9 Hz, 2 H, H1), 5.43 (d, *J* = 10.2 Hz, 2 H, C*H*$_2$Ph), 5.17 (d, *J* = 10.4 Hz, 2 H, C*H*$_2$Ph), 4.92-4.84 (m, 4H, C*H*$_2$Ph), 4.83-4.70 (m, 10 H, H1, C*H*$_2$Ph), 4.58-3.55 (m, 50 H, H2, H3, H4, H5, H6, C*H*$_2$Ph), 3.45-3.41 (m, 4 H, H2), 3.54-3.45 (br s, 2 H, OH) ppm.

^{13}C NMR (100 MHz, $CDCl_3$): δ = 139.40-137.83, 128.64-126.30, 98.41, 97.95, 97.80, 81.88, 81.74, 81.15, 80.75, 79.20, 76.56, 74.40-72.10, 71.40, 69.82, 61.90 ppm.

MS (MALDI-TOF) *m/z* calculated for $C_{148}H_{156}O_{30}$: 2438.1 $[M+Na]^+$, found 2438.8 $[M+Na]^+$.

$2^{A-F},3^{A-F},6^{B-C},6^{E-F}$-Hexadeca-*O*-benzyl-$6^{A,D}$-dibromo-$6^{A,D}$-dideoxy-α-cyclodextrin (12)



To the CBr_4 (1197.1 mg, 3.609 mmol, 14.6 eq) in a three-necked flask cooled in an ice bath, PPh_3 (947.0 mg, 3.611 mmol, 14.6 eq) in DMF (1.5 ml) was added. After the formation of a precipitate, $2^{A-F},3^{A-F},6^{B-C},6^{D-F}$-Hexadeka-O-benzyl-α-cyclodextrin (600 mg, 0.248 mmol, 1.0 eq) in DMF (1.5 ml) was dropped to the reaction mixture and the reaction was let to proceed at 70 °C under stirring. After 6 h, MeOH (1.0 ml) was added and the mixture was cool down at rt. Toluene (30 ml) was used to extract the crude product, it was washed with water (3 × 15 ml) and brine (1 × 15 ml). Organic layers were combined, dried over Na_2SO_4 and evaporated under reduced pressure. The pure product was obtained by column chromatography (hexane/EtOAc 3:1), lyophilization yielded brownish amourphous solid the amount of 530.0 g, the yield was 84%. The NMR spectra were in agreement with the literature.[123]

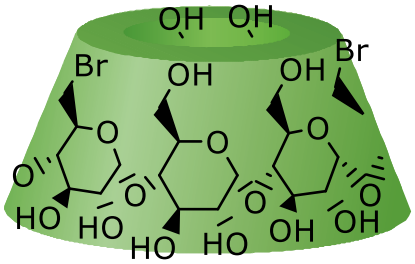
R_F = 0.54 (S5, 50% H_2SO_4)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.32-7.05 (m, 80 H, aromatic H), 5.28-3.32 (m, 74 H, H1, H2, H3, H4, H5, H6, C*H*$_2$Ph) ppm.

^{13}C NMR (100 MHz, $CDCl_3$): δ = 139.20-138.01, 128.35-126.84, 98.89, 80.87-79.81, 78.91, 71.82-68.89, 34.95 ppm.

MS (MALDI-TOF) *m*/*z* calculated for $C_{148}H_{154}O_{28}Br_2$: 2563.9 $[M+Na]^+$, found 2564.2 $[M+Na]^+$.

$6^{A,D}$-Dibromo-$6^{A,D}$-dideoxy-α-cyclodextrin (13)



$2^{A-F},3^{A-F},6^{B-C},6^{E-F}$-Hexadeka-O-benzyl-$6^{A,D}$-dibromo-$6^{A,D}$-dideoxy-α-cyclodextrin (500.6 mg, 0.197 mmol, 1.0 eq) was dissolved in DMF (8 ml) and EtOH (8 ml) in a three-necked flask filled with argon. Pd/C (150 mg) was added and the apparature was evacuated with argon and the mixture was degassed by ultrasound. The reaction was left to proceed under

a slight overpressure of hydrogen (balloon) at 50 °C overnight. After 11 h, the mixture was filtered through Celite pad and volatiles were distilled off under reduced presure. The product was dried using an oil pump at 70 °C. $6^{A,D}$-Dibromo-$6^{A,D}$-dideoxy-α-cyclodextrin was obtained as white crystals the amount of 209.4 mg, the yield was 97%. The NMR spectra were in agreement with the literature.[123]

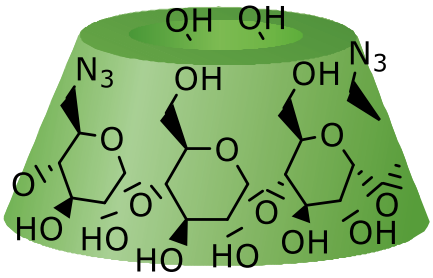
R_F = 0.43 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.78-5.35 (m, 12 H, OH-2, OH-3), 4.86-4.81 (m, 6 H, H1), 4.65-4.55 (m, 4 H, OH-6), 4.00-3.25 (m, 36 H, H2, H3, H4, H5, H6, overlapped with H_2O) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 102.30, 84.40-82.60, 72.60-72.08, 69.51, 60.35, 36.29 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{36}H_{58}O_{28}Br_2$: 1121.1 $[M+Na]^+$, found 1120.4 $[M+Na]^+$.

$6^{A,D}$-Diazido-$6^{A,D}$-dideoxy-α-cyclodextrin (14)



To the closed vial with dibromo-α-CD **(13)** (150.0 mg, 0.137 mmol, 1.0 eq) in DMF (2.0 ml), sodium azide was added (178.4 mg, 2.744 mmol, 20.0 eq) and the mixture was heated to 80 °C overnight. After 16 h, the product was precipitated with acetone contained water (10%; 25 ml). The precipitate was filtered and washed with acetone; the precipitation was repeated three times to remove impurities. The pure product was dried using an oil pump at 70 °C. The amount of obtained $6^{A,D}$-Diazido-$6^{A,D}$-dideoxy-α-cyclodextrin was 124.6 mg, yield 89%. The NMR spectra were in agreement with the literature.[142]

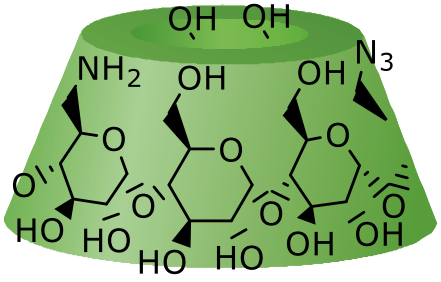
R_F = 0.40 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 5.04-4.95 (m, 6 H, H1), 3.95-3.70 (m, 24 H, H3, H5, H6), 3.64-3.45 (m, 12 H, H2, H4) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 101.38, 101.32, 101.19, 82.16, 81.35, 73.24, 73.02, 72.16, 71.96, 71.66, 71.59, 71.52, 70.71, 60.50, 60.46, 51.24 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{36}H_{58}O_{28}N_6$: 1045.3 $[M+Na]^+$, found 1045.4 $[M+Na]^+$.

6^{A}-Amino-6^{D}-azido-$6^{A,D}$-dideoxy-α-cyclodextrin (15)



To the closed vial with diazido-α-CD **(14)** (90.0 mg, 0.088 mmol, 1.0 eq) in DMF (2.0 ml), PPh_3 was added (16.2 mg, 0.062 mmol, 0.7 eq) and the reaction mixture was left to react at rt. After 2 h, water (0.2 ml) was added and the reaction mixture was heated to 90 °C and left to react overnight under stirring. After 18 h, the product was precipitated with acetone (15 ml) and the precipitate was purified by reverse-phase chromatography (water/MeOH gradient; the product eluted in 10% MeOH). Fractions with the product were combined and volatiles were evaporated under reduced pressure at 60 °C, the product was dried using an oil pump at the same temperature. The product was obtained as a white solid of amount 36.3 mg, the yield was 41%.

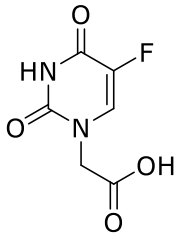
R_F = 0.37 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.79-5.25 (m, 12 H, OH-2, OH-3), 5.00-4.69 (m, 6 H, H1), 4.69-4.40 (br s, 4 H, OH-6), 4.00-3.39 (m, 34 H, H2, H3, H4, H5, H6), 3.00-2.75 (br s, 2 H, ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 102.38,102.28, 102.17, 83.40-81.80, 73.60-70.00, 60.35, 50.84, 50.25, 41.80 ppm.

LC-MS (ESI) *m/z* calculated for $C_{36}H_{60}O_{28}N_4$: 997.3 $[M+H]^+$, found 997.5 $[M+H]^+$.

5-Fluorouracil-1-acetic acid (16)



5-fluorouracil (520.5 mg, 4.000 mmol, 1.0 eq) was dissolved in water (8 ml) with KOH (716.3 mg). The resulting mixture was refluxed for 1 h, and then chloroacetic acid (579.1 mg, 6.128 mmol, 1.6 eq) in water (4 ml) was dropped into the flask in three portions. pH was adjusted to ~ 10 by 1 M NaOH solution and the reaction was left to proceed under reflux. After 3 h, the pH was decreased to 5 by 35% HCl, the solution was filtered and the filtrate was acidified to pH 2 and left to crystalize in the fridge. The resulting crystals were filtered and dried by air. The product was obtained in the form of colorless needles and the amount of 368.7 mg, the yield was 49%. The NMR spectra were in agreement with the literature.[151]

R_F = 0.38 (S2, UV light)

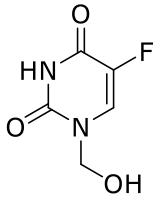
^{1}H NMR (400 MHz, DMSO-d_6): δ = 13.22 (s, 1 H, $CH_2COO\mathit{H}$), 11.91 (d, J = 11.9 Hz, 1 H, H3), 8.08 (d, J = 8.9 Hz, 1H, H6), 4.36 (s, 2H, $C\mathit{H}_2COOH$) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 169.74, 157.90, 150.11, 139.67, 130.96, 49.08 ppm.

^{19}F NMR (376 Hz, DMSO-d_6): δ = -170.10 ppm.

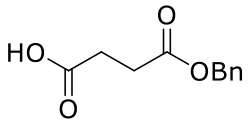
HRMS (ESI) m/z calculated for $C_6H_5O_4N_2F$: 187.0161 $[M-H]^-$, found 187.0159 $[M-H]^-$.

5-Fluoro-1-hydroxymethyluracil (17)



5-fluorouracil (262.2 mg, 2.016 mmol, 1.0 eq) was suspended in water (2 ml), then 38 % formaldehyde solution (0.3 ml, 3.796 mol, 1.9 eq) was added. The resulting mixture was left to react under stirring at 50 °C for 2.5 h. Volatiles were evaporated under reduced pressure at 50 °C and the product was dried using an oil pump at the same temperature (the temperature never crossed 52 °C). The resulting product (colorless oil) was used for appropriate reactions without further purification.

4-(Benzyloxy)-4-oxobutanoic Acid (18a)



Succinic acid (404.1 mg, 3.422 mol, 1.0 eq) was dissolved in DCM (10 ml), then triethylamine (0.5 ml, 3.587 mmol, 1.0 eq) was added and the resulting mixture was stirred for 10 min. After that, benzyl bromide (0.45 ml, 3.789 mmol, 1.1 eq) was added in one portion and the reaction mixture was left to reflux overnight. After 23 h, the mixture was left to cool down and volatiles were evaporated under reduced pressure. The crude product was redissolved in THF (20 ml) and ethyl acetate (20 ml). The organic layer was extracted with 15 % Na_2CO_3 (3 × 30 ml). The water phase was washed with diethyl ether (30 ml) and then acidified by concentrated HCl to pH 1. The product was extracted with ethyl acetate (3 × 40 ml) and then washed with saturated NaCl solution. Organic phases were dried over Na_2SO_4 overnight, then filtered and evaporated at 40 °C to dryness. The product was obtained in the form of white powder and the amount of 380.6 mg, the yield was 53%. The NMR spectra were in agreement with the literature.[131]

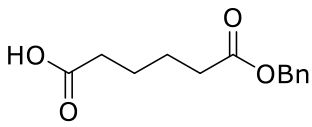
R_F = 0.74 (S7, bromocresol green)

1H NMR (300 MHz, $CDCl_3$): δ = 7.36 (s, 5 H, aromatic H), 5.17 (s, 2 H, PhCH_2O), 2.71 (s, 4 H, CO(CH_2)$_2$COOH) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 177.87, 171.98, 135.70, 128.60, 128.32, 128.23, 66.68, 28.91, 28.87 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{11}H_{12}O_4$: 207.2 $[M-H]^-$, found 207.1 $[M-H]^-$.

6-(Benzyloxy)-6-oxohexanoic Acid (18b)



Adipic acid (1615.8 mg, 11.057 mmol, 1.0 eq) was dissolved in DCM (40 ml), then triethylamine (1.8 ml, 12.914 mmol, 1.2 eq) was added and the resulting mixture was stirred for 10 min. After that, benzyl bromide (1.4 ml, 11.787 mmol, 1.1 eq) was added in one portion and the reaction mixture was left to reflux overnight. After 24 h, volatiles were evaporated under reduced pressure, and the crude product was redissolved in THF (20 ml) and ethyl acetate (20 ml). The organic layer was extracted with 15 % Na_2CO_3 (3 × 30 ml). The water phase was washed with diethyl ether (30 ml) and then acidified by concentrated HCl to pH 1. The product was extracted with ethyl acetate (3 × 40 ml) and then washed with saturated NaCl solution. Organic phases were dried over Na_2SO_4 overnight, then filtered and evaporated at 40 °C to dryness. The product was obtained in the form of a white sticky solid and the amount of 1006.8 mg, the yield was 38%. The NMR spectra were in agreement with the literature.[152]

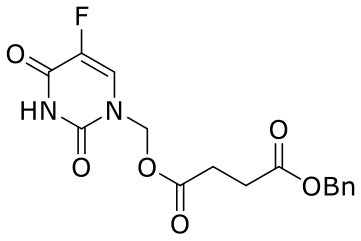
R_F = 0.66 (S7, bromocresol green)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.42-7.32 (m, 5 H, aromatic H), 5.14 (s, 2 H, PhCH_2O), 2.41 (m, 4 H, adipic acid), 1.71 (m, 4 H, adipic acid) ppm.

^{13}C NMR (100 MHz, $CDCl_3$): δ = 179.37, 173.16, 135.96, 128.59, 128.26, 128.24, 66.30, 33.89, 33.60, 24.27, 24.06 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{13}H_{16}O_4$: 235.3 $[M-H]^-$, found 235.1 $[M-H]^-$.

1-[(5-Fluoro-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidinyl)methyl]-4-(phenylmethyl) butanedioate (19a)



Crude 5-fluoro-1-hydroxymethyluracil (2.016 mmol, 1.0 eq) was dissolved in MeCN (6 ml), then DMAP (21.0 mg, 0.172 mmol, 0.1 eq) and 4-(benzyloxy)-4-oxobutanoic acid (338.6 mg, 1.626 mmol, 0.8 eq) were added under stirring. The reaction mixture was moved to the ice bath, and then DCC (561.7 mg, 2.722 mmol, 1.4 eq) was added in four portions in 10 minutes. The reaction mixture was left to react at room temperature under stirring. After 23 h, the mixture was left to cool down, then filtered, and volatiles were evaporated under reduced pressure. The crude product (yellow oil) was dissolved in ethyl acetate (15 ml) and washed with water (2 × 15 ml). The organic layer was dried over Na_2SO_4 overnight, then filtered and evaporated at 40 °C to dryness. The product (white powder) was isolated by flash column chromatography (hexane/acetone 2:1). Pure product was obtained in the form of white powder and the amount of 140.0 mg, the total yield after two steps was 25%. The NMR spectra were in agreement with the literature.[133]

R_F = 0.46 (S3, UV light and bromocresol green)

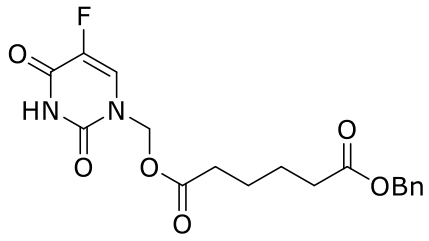
^{1}H NMR (400 MHz, DMSO-d_6): δ = 11.99 (s, 1 H, H3), 8.11 (d, J = 8.1 Hz, 1H, H6), 7.37 (s, 5 H, aromatic H), 5.59 (s, 2 H, NCH_2O), 5.09 (s, 2H, OCH_2Ph) 2.65 (s, 4 H, COCH_2CH_2CO) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.22, 172.16, 157.88, 149.69, 139.76, 136.48, 129.98, 128.90, 128.51, 128.38, 71.05, 66.16, 28.93, 28.90 ppm.

^{19}F NMR (376 Hz, DMSO-d_6): δ = -168.70 ppm.

LC-MS (ESI) m/z calculated for $C_{16}H_{15}FN_2O_6$: 373.3 $[M+Na]^+$, found 373.1 $[M+Na]^+$.

1-[(5-Fluoro-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidinyl)methyl]-6-(phenylmethyl) hexanedioate (19b)



Crude 5-fluoro-1-hydroxymethyluracil (2.013 mmol, 1.4 eq) was dissolved in MeCN (6 ml) with potassium carbonate (208.8 mg, 1.511 mmol, 1.0 eq); 6-(Benzyloxy)-6-oxohexanoic acid (346.3 mg, 1.466 mmol, 1.0 eq) was added and the reaction mixture was cooled in an ice bath. NHS (171.6 mg, 1.491 mmol, 1.0 eq) and EDCI (352.9 mg, 1.841 mmol, 1.3 eq) were added under stirring. The reaction was left to proceed at rt. After 22 h, the mixture was left to cool down and after filtration, volatiles were evaporated under reduced pressure. The crude product (yellow oil) was dissolved in ethyl acetate (15 ml) and then washed with water (15 ml), 1 M HCl (15 ml), and brine (15 ml). The organic layer was dried over Na_2SO_4 overnight, then was filtered and evaporated at 40 °C to dryness. The product (oil) was isolated by flash column chromatography (hexane/acetone 2:1). Pure product was obtained in the form of a white sticky solid and the amount of 90.6 mg, the total yield after two steps was 16%. The NMR spectra were in agreement with the literature.[127]

R_F = 0.43 (S3, UV light and bromocresol green)

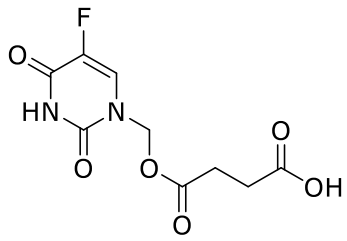
^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.64 (s, 1 H, H6), 7.38 (s, 5 H, aromatic H), 5.65 (s, 2 H, NCH_2O), 5.14 (s, 2 H, OCH_2Ph), 2.42 (s, 4 H, adipic acid), 1.71 (s, 4 H, adipic acid) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 178.44, 173.18, 157.10, 149.39, 141.40, 139.00, 128.78-128.12, 69.67, 66.32, 34.00-33.33, 24.33-23.88 ppm.

^{19}F NMR (376 Hz, DMSO-d_6): δ = -164.22 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{18}H_{19}FN_2O_6$: 401.4 $[M+Na]^+$, found 401.1 $[M+Na]^+$.

1-[(5-Fluoro-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidinyl)methyl] butanedioate (20a)



Three-necked flask was filled with argone, then 1-[(5-Fluoro-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidinyl)methyl] 4-(phenylmethyl) butanedioate (115.0 mg, 0.328 mmol, 1.0 eq) was added and dissolved in anhydrous THF (6.5 ml). To the resulting solution, 10 % Pd/C (40.0 mg) was added, the flask was evacuated with argon, and the reaction proceeded at rt under stirring and a slight hydrogen overpressure. After 3 h, the reaction mixture was filtered through a Celite® pad. Volatiles were evaporated under reduced pressure at 40 °C. The pure product was obtained in the form of a white solid and the amount of 76.4 mg, the yield was 90%. The NMR spectra were in agreement with the literature.[133]

R_F = 0.24 (S3, UV light and bromocresol green)

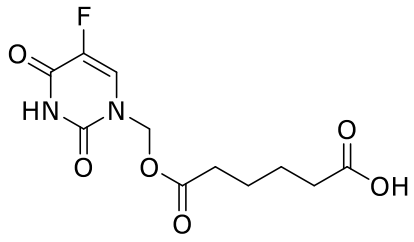
^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.04 (br s, 1 H, H3), 8.10 (d, J = 8.1 Hz, 1H, H6), 5.58 (s, 2 H, NCH_2O), 2.59-2.53 (m, 2 H, COCH_2CH_2CO), 2.51-2.46 (m, 2H, COCH_2CH_2CO) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 173.66, 172.45, 157.97, 149.65, 140.96, 129.80, 70.90, 29.25, 28.98 ppm.

^{19}F NMR (376 Hz, DMSO-d_6): δ = -168.73 ppm.

LC-MS (ESI) m/z calculated for $C_9H_9FN_2O_6$: 259.2 $[M-H]^-$, found 259.0 $[M-H]^-$.

1-[(5-Fluoro-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidinyl)methyl] hexanedioate (20b)



The three-necked flask was filled with argon, then 1-[(5-Fluoro-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidinyl)methyl] 4-(phenylmethyl) butanedioate (115.0 mg, 0.328 mmol, 1.0 eq) was added and dissolved in anhydrous THF (6.5 ml). To the resulting solution, 10% Pd/C (40.0 mg) was added, the flask was evacuated with argon, and the reaction proceeded at rt under stirring and a slight hydrogen overpressure. After 3 h, the reaction mixture was filtered through a Celite® pad. Volatiles were evaporated under reduced pressure at 40 °C. The pure product was obtained in the form of a white

solid and the amount of 76.4 mg, the yield was 90%. The NMR spectra were in agreement with the literature.[127]

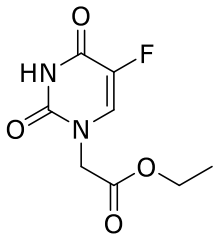
R_F = 0.21 (S3, UV light and bromocresol green)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.40 (br s, 1 H, H3), 7.58 (d, *J* = 8.1 Hz, 1 H, H6), 5.60 (d, *J* = 6.8 Hz, 2 H), 2.40-2.25 (m, 4 H, adipic acid), 1.65-1.39 (m, 4 H, adipic acid) ppm.

^{19}F NMR (376 Hz, $CDCl_3$): δ = -165.02 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{11}H_{13}FN_2O_6$: 287.2 $[M-H]^-$, found 287.0 $[M-H]^-$.

Ethyl 5-fluorouracil-1-acetate (21)



5-fluorouracil (606.6 mg, 4.663 mmol, 1.0 eq) and K_2CO_3 (955.4 mg, 6.913 mmol, 1.5 eq) were dissolved in MeCN (20 ml). The resulting mixture was stirred for 10 min, then 600 µl of ethyl bromoacetate was dropped into the reaction mixture in three portions (908.4 mg, 5.439 mmol, 1.2 eq). The reaction mixture was refluxed for 2 h. The carbonate was filtered off and volatiles were evaporated under reduced pressure at 40 °C. The crude product was dissolved in 10 ml DCM (10 ml) and separated by flash column chromatography (hexane/ethyl acetate 1:1). Fractions with the product were evaporated under reduced pressure at 40 °C. The product was obtained as a white powder in the amount of 306.0 mg, the yield was 30%.

R_F = 0.41 (S3, UV light)

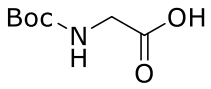
^{1}H NMR (400 MHz, DMSO-d_6): δ = 11.97 (s, 1 H, H3), 8.09 (d, *J* = 8.1 Hz, 1 H, H6), 4.47 (s, 2H, CH_2COOCH_2CH_3), 4.17 (q, 2 H, CH_2COOCH_2CH_3), 1.22 (t, *J* = 1.2 Hz, 3 H, CH_2COOCH_2CH_3) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 168.34, 157.89, 150.09, 139.81, 130.78, 61.78, 49.10, 14.47 ppm.

^{19}F NMR (376 Hz, DMSO-d_6): δ = -169.74 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_8H_9O_4N_2F$: 239.2 $[M+Na]^+$, found 239.2 $[M+Na]^+$.

N-Boc-glycine (22)



Glycine (1048.2 mg, 13.963 mmol, 1.0 eq) was dissolved in water (50 ml) with dioxane (50 ml). NaOH (834.0 mg, 20.850 mmol, 1.5 eq) was added to the resulting solution and the reaction mixture was placed into an ice bath. Di-tert-butyl-dicarbonate (3343.1 mg, 15.318 mmol, 1.1 eq) was dissolved in dioxane (25 ml) and then added to the reaction (reaction flask was in an ice bath). The reaction was left to proceed at rt. After 22 h, the mixture was concentrated under reduced pressure at 40 °C, the residue was acidified with hydrochloric acid to adjust pH = 6 and extracted with ethyl acetate (2 × 50 ml). Organic phases were combined and washed with saturated $NaHCO_3$ and saturated NaCl. The organic phase was dried over Na_2SO_4 overnight. Solvents were evaporated under reduced pressure at 40 °C. The product was obtained in the form of white crystals and the amount of 2202.0 mg, the yield was 90%. The NMR spectra were in agreement with the literature.[153]

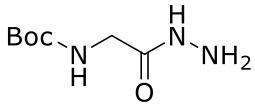
R_F = 0.81 (S6, UV light and ninhydrine).

^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.44 (br s, 1 H, NHCH$_2$COO*H*), 7.06 (t, *J* = 7.1 Hz, 1 H, N*H*CH$_2$COOH), 3.58 (d, *J* = 3.6 Hz, 2 H, NHC*H*$_2$COOH), 1.38 (s, 9 H, (C*H*$_3$)$_3$CO) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.24, 156.29, 78.48, 42.25, 28.65 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_7H_{13}O_4N$: 198.2 $[M+Na]^+$, found 198.1 $[M+Na]^+$.

N-Boc-glycine hydrazide (23)



N-boc-glycine methyl ester (125.3 mg, 0.662 mmol, 1.0 eq) was dissolved in 1.85 ml of ethanol. Hydrazine hydrate (150 µl, 2.996 mmol, 4.5 eq) was added and the resulting mixture was left to react under stirring at rt. After 24 h, the mixture was evaporated at 50 °C and dried using an oil pump at the same temperature. The resulting product (white powder) was synthesized in the amount of 125.0 mg, the yield was 100%.

R_F = 0.50 (S6, UV light and ninhydrine)

^{1}H NMR (400 MHz, D_2O): δ = 3.67 (s, 2 H, NHC*H*$_2$CO), 1.36 (s, 9 H, (C*H*$_3$)$_3$CO) ppm.

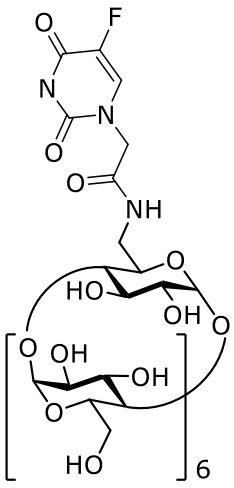
^{13}C NMR (100 MHz, D_2O): δ = 171.40, 158.10, 81.70, 42.16, 27.55 ppm.

HRMS (ESI) *m*/*z* calculated for $C_6H_4O_4N_2F$: 187.0161 $[M-H]^-$, found 187.0159 $[M-H]^-$.

General procedure for the synthesis of 5FUA/NH_2-CD conjugates (GP1)

5-fluorouracil acetic acid (1.3 eq) and NHS (2.4 eq) were dissolved in DMF (1 ml per 50.0 mg of starting amino-CD). The reaction mixture was cooled in an ice bath, and EDCI (2.4 eq) was added under stirring. After 1 h, the reaction mixture was left to warm at rt, appropriate amino-CD (1.0 eq) was added and the reaction was left to proceed for 48 h. The product was precipitated with EtOH (10 ml per 50.0 mg of starting amino-CD), the precipitate was filtered off and purified by flash column chromatography (RP-C18 and water/MeOH gradient; the product was eluted in 10% MeOH). Appropriate fractions were concentrated under reduced pressure at 60 °C, the product was dried using an oil pump at the same temperature.

5FUA/NH_2-β-CD conjugate (24a)



GP1 was followed; the product was obtained as a white powder of 106.1 mg, yielding 80%. The NMR spectra were in agreement with the literature.[104]

R_F = 0.17 (S2, 50% H_2SO_4)

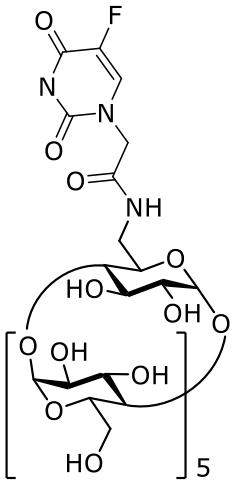
^{1}H NMR (400 MHz, D_2O): δ = 7.72 (d, *J* = 8.1 Hz, 1 H, H6‘), 5.03-4.95 (m, 7 H, H1), 4.44 (d, *J* = 4.4 Hz, 2 H, C*H*$_2$), 3.95-3.70 (m, 28 H, H3, H5, H6), 3.64-3.45 (m, 14 H, H2, H4) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 168.87, 150.88, 131.47, 131.14, 101.94, 101.76, 83.07, 81.21, 81.08, 80.86, 73.01, 72.75, 72.02, 71.89, 71.73, 70.25, 60.22, 50.75, 40.41 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -167.75 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{48}H_{74}O_{37}N_3F$: 1327.3 $[M+Na]^+$, found 1326.4 $[M+Na]^+$.

5FUA/NH_2-α-CD conjugate (24b)



GP1 was followed; the product was obtained as a white powder of 50.6 mg, yielding 79%.

R_F = 0.15 (S2, 50% H_2SO_4)

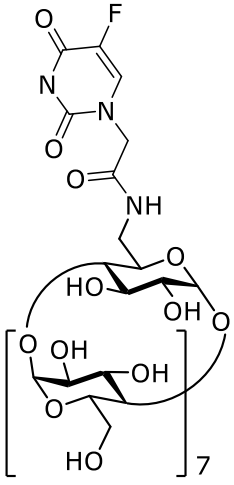
^{1}H NMR (300 MHz, D_2O): δ = 7.71 (d, *J* = 8.0 Hz, 1 H, H6‘), 5.00-4.92 (m, 6 H, H1), 4.44 (s, 2 H, CH_2), 3.90-3.62 (m, 24 H, H3, H5, H6), 3.59-3.41 (m, 12 H, H2, H4) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 168.4, 150.6, 131.6, 100.9, 81.5, 73.2-70.6, 60.1, 49.8, 40.0 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -167.94 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{42}H_{64}O_{32}N_3F$: 1143.1 $[M+H]^+$, found 1142.9 $[M+H]^+$.

5FUA/NH_2-γ-CD conjugate (24c)



GP1 was followed; the product was obtained as a white powder in the amount of 39.3 mg, yielding 84%.

R_F = 0.17 (S2, 50% H_2SO_4)

^{1}H NMR (300 MHz, D_2O): δ = 7.74 (d, *J* = 8.0 Hz, 1 H, H6‘), 5.11-4.97 (m, 8 H, H1), 4.43 (s, 2 H, CH_2), 3.92-3.64 (m, 32 H, H3, H5, H6), 3.67-3.49 (m, 16 H, H2, H4) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 169.4, 152.0, 132.3, 101.8, 82.4, 74.4-71.2, 60.9, 50.8, 41.2 ppm.

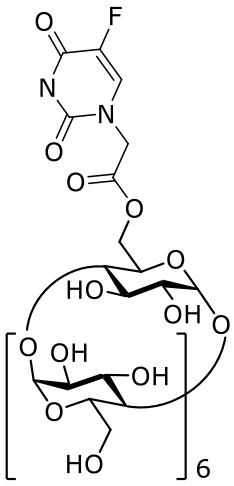
^{19}F NMR (376 MHz, D_2O): δ = -166.10 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{54}H_{84}O_{42}N_3F$: 1489.4 $[M+Na]^+$, found 1490.0 $[M+Na]^+$.

General procedure for the synthesis of 5FUA/CD conjugates (GP2)

5-fluorouracil acetic acid (1.0 eq) and CDI (3.0 eq) were dissolved in DMSO (3 ml per 63.0 mg of FUA). The reaction was left to proceed at rt for 3 h and then was dropped into a stirred solution of appropriate CD (1.0 eq) in DMSO (1 ml per 100 mg of CD) with Et_3N (1.2 ml per 100 mg of CD). After 3 d, the reaction mixture was precipitated with acetone (30 ml per 100.0 mg of starting amino-CD), the precipitate was filtered off and purified by flash column chromatography (RP-C18 and water/MeOH gradient; the product was eluted in 10% MeOH). Appropriate fractions were concentrated under reduced pressure at 60 °C, the product was dried using an oil pump at the same temperature.

5FUA/β-CD conjugate (25a)



GP2 was followed; the product was obtained as a white powder the amount of 156.4 mg, yielding 22%. The NMR spectra were in agreement with the literature.[104]

R_F = 0.35 (S2, 50% H_2SO_4)

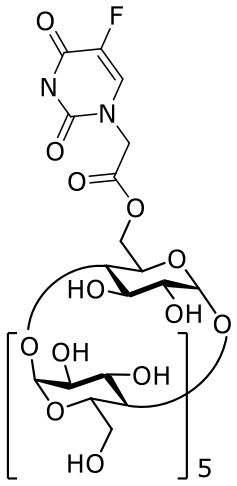
^{1}H NMR (400 MHz, D_2O): δ = 7.90 (d, *J* = 8.1 Hz, 1 H, H6′), 5.08-5.03 (m, 6 H, H1), 5.00 (s, 1 H, H1), 4.45 (d, *J* = 4.1 Hz, 2 H, CH_2), 4.30-3.58 (m, 42 H, H2, H3, H4, H5, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 170.3, 152.1, 132.4, 131.9, 103.1, 82.9-82.6, 74.6-72.5, 61.6, 51.2, 41.8 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -165.63 ppm.

LC-MS (ESI) *m*/*z* calculated for $C_{48}H_{74}O_{38}N_2F$: 1329.3 $[M+Na]^+$, found 1328.9 $[M+Na]^+$.

5FUA/α-CD conjugate (25b)



GP2 was followed; the product was obtained as a white powder the amount of 113.0 mg, yielding 18%.

R_F = 0.32 (S2, 50% H_2SO_4)

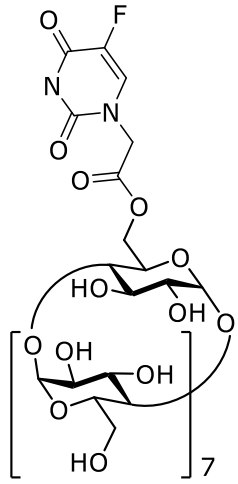
^{1}H NMR (400 MHz, D_2O): δ = 7.87 (d, *J* = 8.1 Hz, 1 H, H6′), 5.05-4.95 (m, 5 H, H1), 4.90 (s, 1 H, H1), 4.45 (d, *J* = 4.1 Hz, 2 H, C*H*$_2$), 4.24-3.52 (m, 36 H, H2, H3, H4, H5, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 169.8, 152.0-131.9, 103.6, 82.5-82.0, 74.0-72.1, 61.9, 50.6, 41.1 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -164.84

LC-MS (ESI) *m*/*z* calculated for $C_{42}H_{64}O_{33}N_2F$: 1145.1 $[M+H]^+$, found 1144.8 $[M+H]^+$.

5FUA/γ-CD conjugate (25c)



GP2 was followed; the product was obtained as a white powder the amount of 136.0 mg, yielding 20%.

R_F = 0.39 (S2, 50% H_2SO_4)

^{1}H NMR (400 MHz, D_2O): δ = 7.94 (d, *J* = 8.1 Hz, 1 H, H6′), 5.14-5.08 (m, 6 H, H1), 5.04 (s, 1 H, H1), 4.45 (d, *J* = 4.1 Hz, 2 H, C*H*$_2$), 4.40-3.63 (m, 48 H, H2, H3, H4, H5, H6) ppm.

^{13}C NMR (100 MHz, D_2O): δ = 170.9, 153.0-131.4, 102.1, 82.6-81.8, 74.0-71.9, 62.0, 52.2, 43.4 ppm.

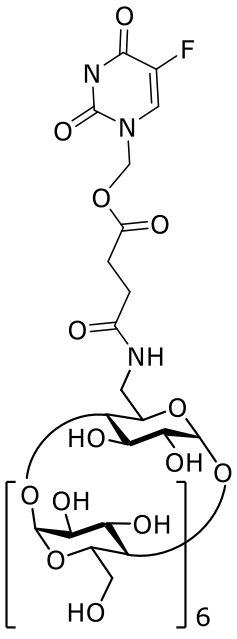
^{19}F NMR (376 MHz, D_2O): δ = -166.25

LC-MS (ESI) *m*/*z* calculated for $C_{54}H_{84}O_{43}N_2F$: 1469.4 $[M+H]^+$, found 1470.0 $[M+H]^+$.

General procedure for the synthesis of succinylFU/NH_2-CD conjugates and adipoylFU/NH_2-CD conjugates (GP3)

1-[(5-Fluoro-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidinyl)methyl] butanedioate / 1-[(5-Fluoro-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidinyl)methyl] hexanedioate (1.0 eq) was dissolved in DMSO (1 ml per 10.0 mg of acid) with NHS (1.6 eq). The reaction was cooled in an ice bath, then EDCI (1.4 eq) was added. The reaction was left to proceed at rt. After 3 h, appropriate amino-CD (1.0 eq) was added, and the reaction continued under the same conditions. After 24 h, the mixture was diluted with water (1 ml per 1 ml of DMSO) and precipitated with acetone (10 ml per 10.0 mg of acid). The precipitate was separated by centrifugation (6000 rpm, 10 min) and redissolved in water. The product was purified by flash column chromatography (water/MeOH gradient, product eluted in 10% MeOH).

succinylFU/NH_2-β-CD conjugate (26a)



GP3 was followed; the product was obtained as a white powder in the amount of 36.0 mg, yielding 84%.

R_F = 0.32 (S2, 50% H_2SO_4)

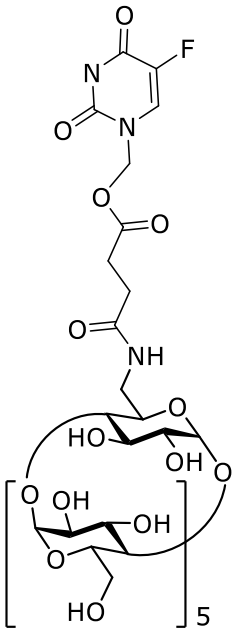
^{1}H NMR (300 MHz, D_2O): δ = 7.66 (d, *J* = 7.7 Hz, 1 H, H6′), 5.58 (s, 2 H, CH_2), 5.04 – 4.95 (m, 7 H, H1), 4.08-3.48 (m, 42 H, H2, H3, H4, H5, H6), 2.61-2.41 (m, 4 H, COCH_2CH_2CO) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 173.9, 172.8, 165.7, 151.8, 131.5, 131.1, 101.2, 83.2-80.0, 73.0-69.9, 60.5, 50.7, 42.0-39.8 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -169.39

LC-MS (ESI) *m/z* calculated for $C_{51}H_{78}O_{39}N_3F$: 1399.2 $[M+Na]^+$, found 1400.0 $[M+Na]^+$.

succinylFU/NH_2-α-CD conjugate (26b)



GP3 was followed; the product was obtained as a white powder in the amount of 29.2 mg, yielding 88%.

R_F = 0.30 (S2, 50% H_2SO_4)

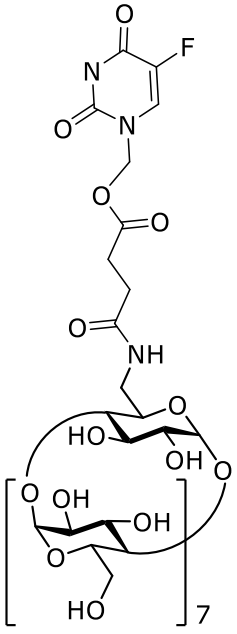
^{1}H NMR (300 MHz, D_2O): δ = 7.89 (d, J = 7.7 Hz, 1 H, H6‘), 5.63 (s, 2 H, CH_2), 5.04-4.94 (m, 6 H, H1), 3.97-3.31 (m, 36 H, H2, H3, H4, H5, H6), 2.59-2.37 (m, 4 H, COCH_2CH_2CO) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 173.5, 172.4, 165.0, 152.0, 131.5, 131.1, 101.6, 83.0-79.9, 72.8-70.6, 60.0, 50.2, 41.0-39.9 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -166.98

LC-MS (ESI) *m*/*z* calculated for $C_{45}H_{68}O_{34}N_3F$: 1237.0 $[M+Na]^+$, found 1237.2 $[M+Na]^+$.

succinylFU/NH_2-γ-CD conjugate (26c)



GP3 was followed; the product was obtained as a white powder in the amount of 20.0 mg, yielding 90%.

R_F = 0.34 (S2, 50% H_2SO_4)

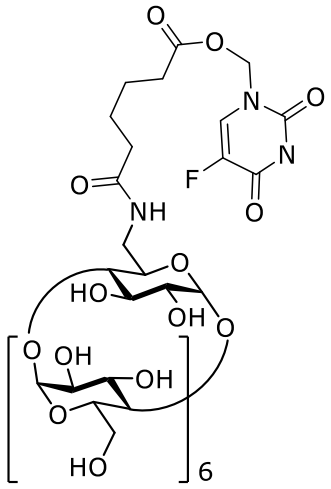
^{1}H NMR (300 MHz, D_2O): δ = 7.78 (d, *J* = 7.8 Hz, 1 H, H6‘), 5.61 (s, 2 H, CH_2), 5.13-4.96 (m, 8 H, H1), 3.98-3.45 (m, 48 H, H2, H3, H4, H5, H6), 2.63-2.48 (m, 4 H, COCH_2CH_2CO) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 170.9, 170.0, 164.2, 151.9, 131.0, 130.1, 101.9, 83.2-79.3, 73.0-68.6, 60.0, 50.3, 42.9-39.4 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -166.76

LC-MS (ESI) *m*/*z* calculated for $C_{57}H_{88}O_{44}N_3F$: 1561.3 $[M+Na]^+$, found 1562.0 $[M+Na]^+$.

adipoylFU/NH_2-β-CD conjugate (27a)



GP3 was followed; the product was obtained as a white powder in the amount of 49.8 mg, yielding 91%.

R_F = 0.40 (S2, 50% H_2SO_4)

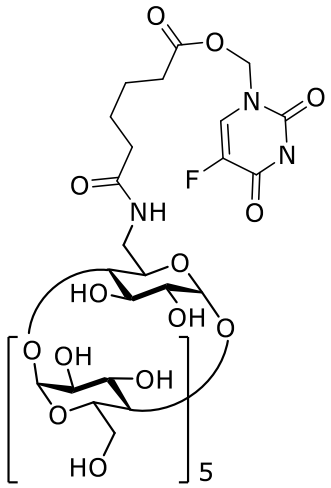
^{1}H NMR (300 MHz, D_2O): δ = 7.60 (d, *J* = 7.7 Hz, 1 H, H6′), 5.54 (s, 2 H, CH_2), 5.06– 4.95 (m, 7 H, H1), 4.00-3.44 (m, 42 H, H2, H3, H4, H5, H6), 2.55-2.41 (m, 4 H, adipic acid), 1.92-1.80 (m, 4 H, adipic acid) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 172.7, 172.2, 165.0, 151.4, 131.4, 131.0, 100.8, 83.2-80.4, 73.2-70.8, 60.1, 50.2, 44.0-42.8, 34.5-33.6 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -167.22

LC-MS (ESI) *m*/*z* calculated for $C_{53}H_{82}O_{39}N_3F$: 1427.2 $[M+Na]^+$, found 1427.4 $[M+Na]^+$.

adipoylFU/NH_2-α-CD conjugate (27b)



GP3 was followed; the product was obtained as a white powder in the amount of 36.0 mg, yielding 88%.

R_F = 0.39 (S2, 50% H_2SO_4)

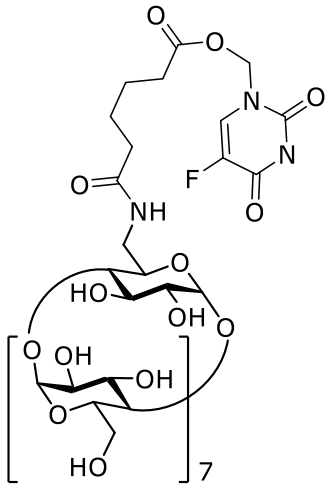
^{1}H NMR (300 MHz, D_2O): δ = 7.82 (d, J = 7.7 Hz, 1 H, H6‘), 5.58 (s, 2 H, CH_2), 5.03-4.97 (m, 6 H, H1), 3.93-3.24 (m, 36 H, H2, H3, H4, H5, H6), 2.53-2.37 (m, 4 H, adipic acid), 1.77-1.65 (m, 4 H, adipic acid) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 173.2, 172.0, 164.9, 152.4, 131.9, 131.0, 101.0, 82.1-80.0, 72.8-69.5, 59.8, 50.6, 43.0-41.5, 33.0-31.5 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -168.06

LC-MS (ESI) *m*/*z* calculated for $C_{47}H_{72}O_{34}N_3F$: 1265.1 $[M+Na]^+$, found 1265.1 $[M+Na]^+$.

adipoylFU/NH_2-γ-CD conjugate (27c)



GP3 was followed; the product was obtained as a white powder in the amount of 16.4 mg, yielding 85%.

R_F = 0.40 (S2, 50% H_2SO_4)

^{1}H NMR (300 MHz, D_2O): δ = 7.76 (d, *J* = 7.8 Hz, 1 H, H6‘), 5.60 (s, 2 H, C*H*$_2$), 5.10-4.98 (m, 8 H, H1), 3.96-3.35 (m, 48 H, H2, H3, H4, H5, H6), 2.59-2.30 (m, 4 H, adipic acid), 1.80-1.62 (m, 4 H, adipic acid) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 171.9, 170.5, 166.2, 153.6, 131.0, 130.1, 100.2, 83.0-80.1, 73.4-71.1, 59.8, 49.6, 41.9-39.0, 34.5-33.2 ppm.

^{19}F NMR (376 MHz, D_2O): δ = -167.76

LC-MS (ESI) *m*/*z* calculated for $C_{59}H_{92}O_{44}N_3F$: 1589.4 $[M+Na]^+$, found 1590.1 $[M+Na]^+$.

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