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NMR spectroscopic study of cyclodextrin inclusion complexes with A-007 prodrugs

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Abstract— One- and two-dimensional NMR spectroscopy was used to demonstrate the formation of inclusion cyclodextrin complexes with several A-007 prodrugs. These complexes are comprised from the encapsulation of the two phenol moieties of the A-007 prodrugs within the cyclodextrin cavity. Considering the size of the two phenol moieties of the A-007 prodrugs compared to the sizes of α-, β-, and γ-cyclodextrin cavities, we observed complementary binding of the A-007 prodrug with only β-cyclodextrin, which was also demonstrated spectroscopically. The β-cyclodextrin inclusion complexes increased the prodrug solubility and modified the prodrug half-life in water. Therefore, β-cyclodextrin inclusion complexes can be used as an essential form of A-007 prodrug delivery.

Keywords: Cyclodextrin; Inclusion complexes; A-007 prodrugs; Molecular aggregation; NOESY

1. Introduction

4,4′-Dihydroxybenzophenone-2,4-dinitrophenylhydrazone (A-007, Fig. 1) has recently completed a phase I clinical trial, where it was used as a therapeutic with the targeted treatment of advanced cancer, with minimal toxicity.[1] It was speculated that A-007 activity comes from complementary binding to lymphocyte receptors. This speculation was supported by experimental findings that indicate that A-007 interacts, presumably through the electron-rich (phenol) and the electron-poor (dinitrophenyl) moieties, with the CD45 receptor through complementary interactions.[2]

Despite promising results in the clinical trials, there is a major disadvantage to using A-007 as a broad scale therapeutic. A-007 has low water solubility and in its current formulation, is used only as a topically applied 0.25% gel.[3] To make use of this promising anticancer drug orally or intravenously, the short-term obstacle must be to overcome the limited solubility of A-007 in water. Two possibilities exist to overcome the water solubility problem: the first is to make a hydrolyzable prodrug; and the second is to make an A-007 complex with a water soluble host, such as cyclodextrin.[4] Considering the complex structure of A-007, we hypothesized using a combination of these two previously described methods, which would utilize the chemical transformation of A-007 into a more water soluble prodrug and then further increase the water solubility of this newly formed prodrug through the formation of cyclodextrin inclusion complexes. Recently, we explored the influence

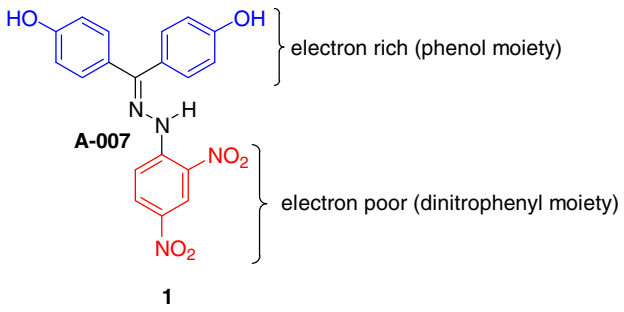

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Figure 1. Chemical structure of the anticancer compound A-007.

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of cyclodextrins on the formation of chiral molecular associates using amino acid derivatives.[5] The application of cyclodextrins in drug delivery is well documented in the literature.[6] For example, the toxicity and compatibility of using cyclodextrins in drug delivery has been previously explored and utilized for several commercialized drugs on the market today.[6,7] Here we spectroscopically explore both the stability of our recently synthesized A-007 prodrug in aqueous solution, as well as the ability of our A-007 prodrug to form cyclodextrin inclusion complexes.

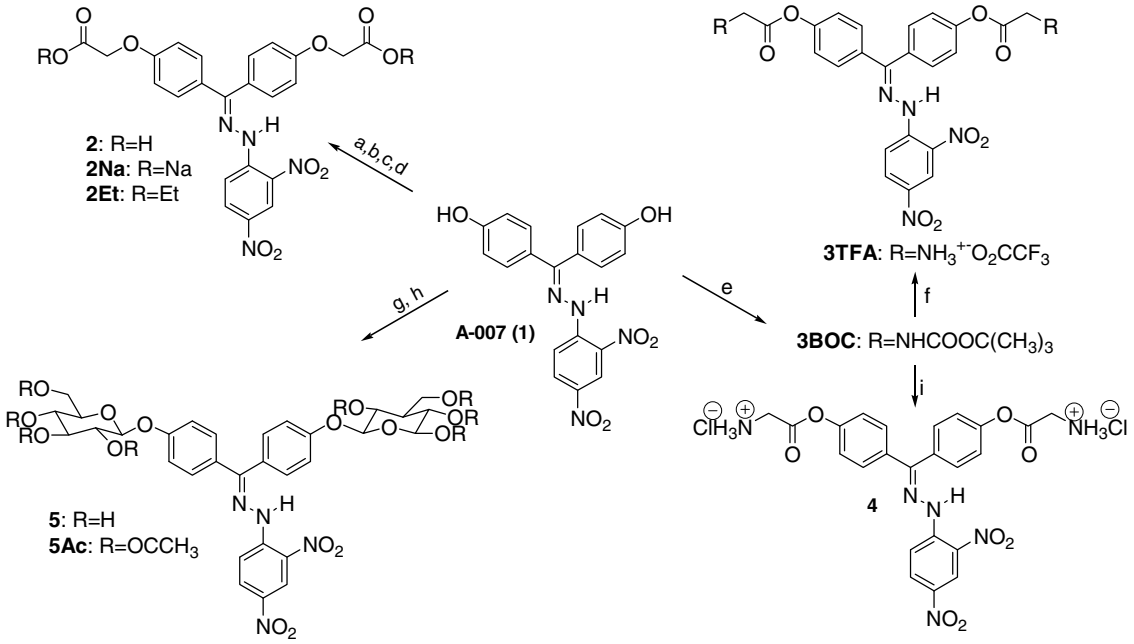
2. Results and discussion

To explore cyclodextrin inclusion complexes with A-007 prodrugs, we prepared neutral, anionic, and cationic prodrugs (Scheme 1). In all of these preparations, the starting material used was the previously synthesized compound, A-007, and was prepared from commercially available starting materials following our previously described method.[1]

Acetic acid derivatives of salt **2** were prepared from A-007 with ethyl 2-bromoacetate, followed by hydrolysis and acidification according to previously reported methods of preparation for similar phenol derivatives.[8] The trifluoroacetic acid salt **3TFA** was prepared by the acylation of A-007 with Boc-glycine [$(CH_3)_3COCONHCH_2CO_2H$] in the presence of dicyclohexylcarbodiimide (DCC), followed by trifluoroacetic acid (CF_3CO_2H) deprotection in CH_2Cl_2.[9] The hydrochloric acid salt **4** was prepared in a similar manner as the trifluoroacetic acid salt, except that the Boc-deprotection was carried out with methanolic HCl instead of CF_3CO_2H–CH_2Cl_2.

Glucosylation of A-007 was performed in two steps. The first step involved Schmidt's glucosylation[10] of A-007 with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-trichloroacetimidate,[11] followed by sodium hydroxide hydrolysis in CH_2Cl_2–MeOH (Scheme 1). The structural properties of these A-007 prodrugs are ideal for testing the nature of their biological activity as well as the administration to patients in combination with cyclodextrins.[12]

The simplest possible prodrug of A-007 is its sodium salt (**1Na**). This compound will be converted into free A-007 under physiological conditions. Unfortunately, even this double sodium salt is not soluble in water in high concentrations. While at an elevated temperature (50 °C) a 10 mM water solution can be formed, upon cooling, a red precipitate forms at room temperature. It is reasonable to speculate that because A-007 is a surfactant by nature, molecular aggregates could form in aqueous media. This should be reflected in chemical shift of the aromatic protons of **1Na** versus its concentration. However, our spectroscopic studies revealed no noticeable difference in chemical shift of 1 mM and 10 mM concentration of **1Na** in water media (Fig. 2), suggesting that A-007 does not form molecular aggregates, such as micelles.[13] The formation of micelles in aqueous media is a desirable property for these prodrugs, because micellar stability can be a function of concentration. Ideally, a



Scheme 1. Synthetic routes of preparation of A-007 into the prodrug formulation. Reagents and conditions: (a) NaOH/CH3OH; (b) $BrCH_2CO_2C_2H_5$; (c) $NaOH/H_2O$–CH_3OH; (d) HCl/H_2O; (e) $(CH_3)_3COCONHCH_2COOH/DCC/CH_2Cl_2$; (f) CF_3CO_2H/CH_2Cl_2; (g) 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate/BF_3–THF/CH_2Cl_2; (h) $NaOH/CH_2Cl_2$–CH_3OH; (i) $(COCl)_2/CH_3OH/CH_2Cl_2$.

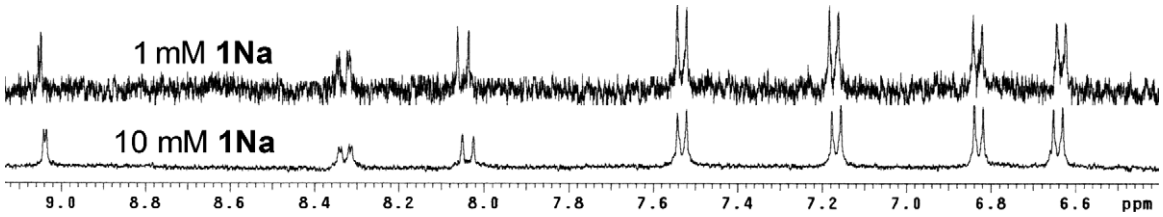


Figure 2. [1]H NMR spectra of aqueous **1Na.**

prodrug should be stable at higher aqueous concentrations, above the critical micellar concentration (CMC) and it hydrolyzes easily at lower or below CMC concentrations. In this way, the prodrug can be stored at higher concentrations and upon administration it slowly hydrolyzes in the blood stream.

Ideally, compounds to be used as prodrugs should be water soluble or have the ability to form cyclodextrin inclusion complexes. To test the capability of **1Na** to be used as a prodrug of A-007, we analyzed the capacity of **1Na** to form a cyclodextrin inclusion complex with α-, β-, and γ-cyclodextrin. We prepared a 10 mM solution of **1Na** with β-cyclodextrin, and observed that the solution stayed clear for several days, a dramatic improvement considering that the precipitate forms in approximately 30 min from 10 mM **1Na** without β-cyclodextrin. (A 10 mM solution was made by mixing together A-007 with sodium hydroxide in D_2O and sonicated at 50 °C for 5 min. A solution that is 0.1 mM is the highest water concentration of **1Na** that remains stable for several days.)

To determine whether a strong cyclodextrin inclusion complex is formed, it is necessary to observe changes in the spectroscopic pattern of the compound/complex, where these changes should be reflected by changes in the NMR chemical shifts. In our analyses, we found no noticeable change in chemical shifts of aromatic protons of **1Na** in combination with α- or γ-cyclodextrins. This can be explained by the fact that the α-cyclodextrin cavity is too small to encapsulate the phenol moieties of **1Na**, while the γ-cyclodextrin cavity is too large to forms strong inclusion complexes with **1Na**. On the other hand it is well documented that β-cyclodextrin forms a strong inclusion complex with phenol groups,[14] and this observation corresponds to our spectroscopic data of **1Na** and β-cyclodextrin. We observed a significant chemical shift in the NMR spectra of free **1Na** and **1Na** in aqueous β-CD (Fig. 3). The major chemical shift changes for **1Na** were observed with a 2:1 ratio between β-CD and **1Na**. This implies the possibility of a ternary cyclodextrin complex formation between one molecule of **1Na** and two molecules of β-CD.[24]

Because our one-dimensional NMR spectroscopic study of β-CD complexation with **1Na** is not conclusive, additional spectroscopic investigation is essential. There are two additional spectroscopic methods that can shed more light on the nature of the complex, electron spray mass spectroscopy (ES-MS) and the two-dimensional nuclear Overhauser spectroscopy (NOESY). Electron spray mass spectroscopy (ES-MS)[15] is an important tool used for the characterization of non-covalent binding and using this spectroscopic technique, the ternary complex signal at 1331.1 *m/z* was observed in the β-cyclodextrin and **1Na** water solution. This signal corresponds to the molecular mass of two cyclodextrins and one **1Na** (Fig. 4).

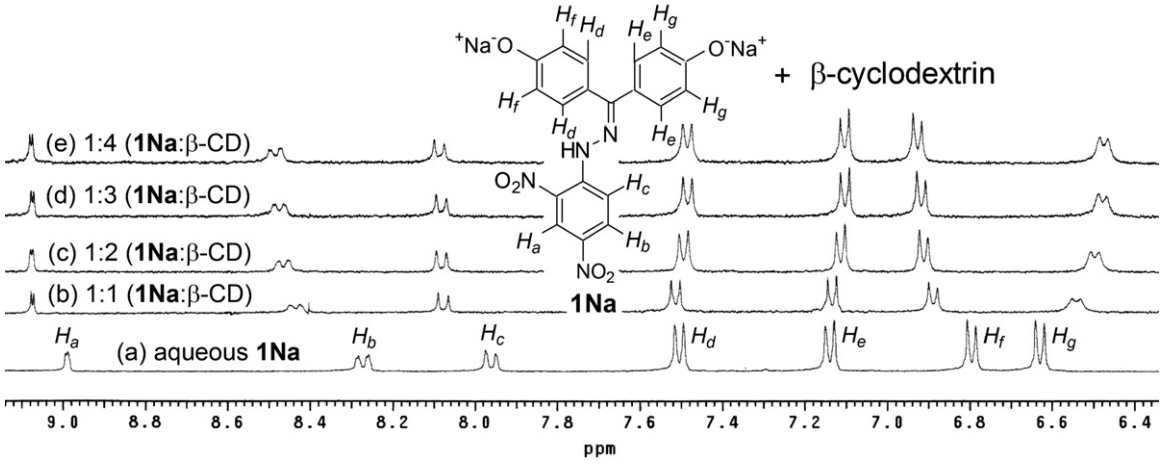


Figure 3. [1]H NMR spectra of aqueous β-cyclodextrin of **1Na** (10 mM).

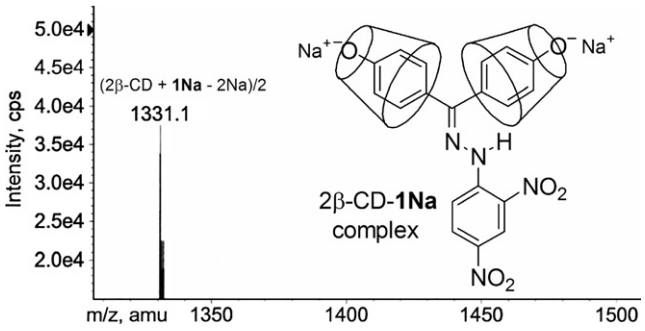


Figure 4. Negative ES-MS spectra of **1Na** (0.01 mM) and β-cyclodextrin (0.03 mM).

Two-dimensional NOESY (nuclear Overhauser spectroscopy) can be used to demonstrate that two protons or groups of protons are in proximity, as the protons must be within 3.5 Å of each other.[16] This spectroscopic method is a valuable tool to study β-cyclodextrin inclusion complexes with A-007 prodrugs. Using NOESY, we found that the 2D NOESY spectra of our β-cyclodextrin complex with **1Na** showed intense cross peaks indicating interactions between β-cyclodextrin and phenol moiety of **1Na** (Fig. 5). These two cross peaks indicate the presence of these groups in the vicinity of 2–5 Å. However there are no crossing signals between β-cyclodextrin hydrogens and the hydrogens of the 2,4-dinitrophenyl moiety of **1Na**, suggesting that this part of the **1Na** molecule is not encapsulated in the β-cyclodextrin cavity. In summary, all spectroscopic studies, including the 1D NMR, 2D NOESY, and ES-MS, indicate that there is the formation of a ternary inclusion complex that substantially increases the water solubility of the simple A-007 prodrug, **1Na**.

Our second compound, the sodium salt **2Na**, does not strictly belong to the group of A-007 prodrugs because it cannot be directly converted into A-007 itself. Nevertheless, we chose to include this compound in our study due to the fact that phenol ethers metabolize via oxidative O-dealkylation, resulting in the formation of a free phenol group, or in our case to A-007.[23] In addition, the corresponding acid **2** also has anticancer activity that is similar to the original A-007. Unfortunately, the low solubility of this acid also hampers its oral or intravenous administration. Using carboxylic acid salts as a polar group of **2Na**, we expected this compound to possess physical properties similar to surfactants, lending it capable of forming molecular assemblies similar to taxol prodrugs reported by Nicolaou and coworkers.[17] First, we explored the change of the NMR chemical shift of **2Na** versus its concentration in pure water. There was a substantial downfield chemical shift observed in the spectra when the concentration of **2Na** increased from 1 mM to 10 mM, while the saturation was achieved after the 10 mM concentration (after CMC).

In the aqueous cyclodextrin solution of **2Na** we expected to observe the appropriate NMR chemical shift changes when the cyclodextrin inclusion complex

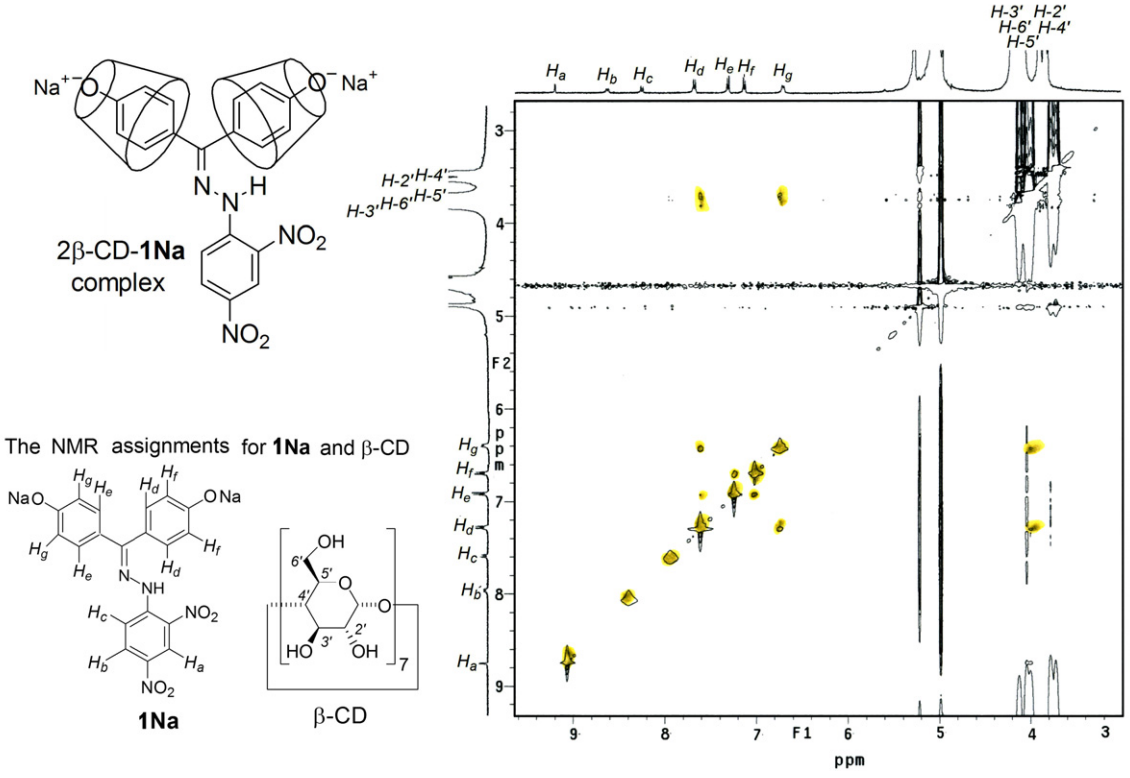


Figure 5. 2D NOESY spectra of **1Na** (10 mM) in aqueous β-cyclodextrin (30 mM).

is formed. There were no noticeable changes when either α-cyclodextrin or γ-cyclodextrin was added into aqueous **2Na** (10 mM). This observation did not come as a surprise as the α-cyclodextrin cavity is too small (4.7–5.3 Å) while γ-cyclodextrin cavity is too big (7.5–8.3 Å) to form strong inclusion complexes with **2Na**.[18] However, there was a substantial difference with β-cyclodextrin (Fig. 6). This difference is profound on the chemical shift of the phenol moiety of the prodrug, indicating that this part of the molecule interacts with β-cyclodextrin. As was the case with **1Na**, the β-cyclodextrin titration of 10 mM solution of **2Na** suggests that a ternary (one molecule of **2Na** and two molecules of β-cyclodextrin) complex is formed. This was based on the fact that after a ratio of 1:2 was reached, further increases of β-cyclodextrin did not produce noticeable chemical shift changes in the NMR spectra of **2Na**.

Two-dimensional NOESY spectra clearly support our assumption that the ternary β-cyclodextrin inclusion complex with **2Na** is formed (Fig. 7). There are several NOE cross couplings between the two phenol moieties of prodrug **2Na** and the cyclodextrin hydrogens. However, we observed no cross coupling between hydrogens from the 2,4-dinitrophenyl moiety of **2Na** and the β-cyclodextrin hydrogens. Furthermore, cross coupling between the methylene hydrogens of the acetic acid moieties and β-cylcodextrin hydrogens was observed as well. All of these cross couplings strongly suggest that the formation of a ternary β-cyclodextrin inclusion complex with **2Na** is formed in water media as demonstrated in Figure 7.

Both of the previously described prodrugs (**1Na** and **2Na**) are sodium salts and therefore are negatively charged organic molecules that revert to the corresponding

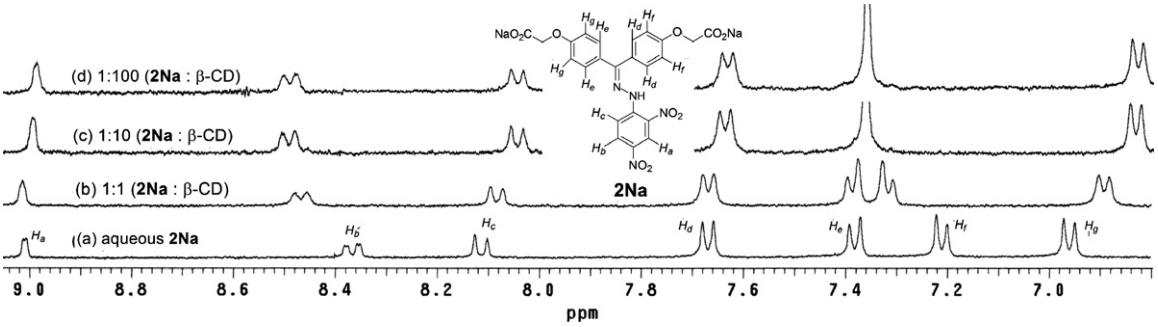


Figure 6. ^{1}H NMR spectra of **2Na** (10 mM) in aqueous β-cyclodextrin.

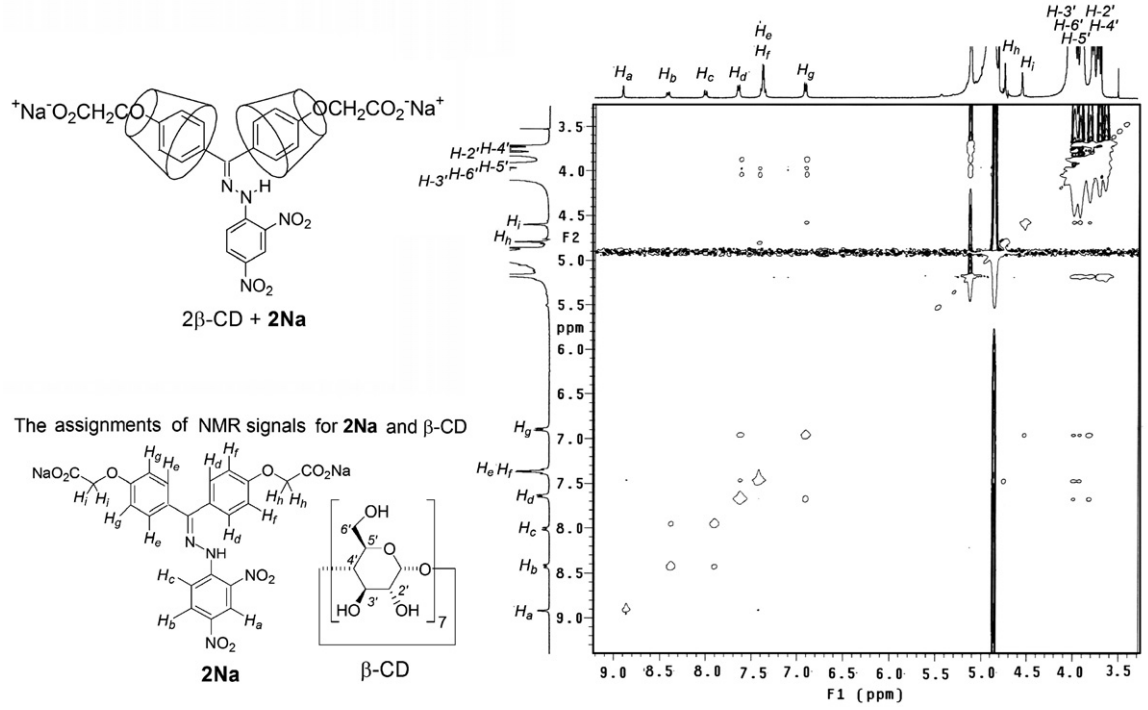


Figure 7. 2D NOESY of **2Na** (10 mM) in aqueous β-cyclodextrin (30 mM).

target low water soluble drugs upon acidification (**1** and **2**, respectively). At physiological pH (7.4) both of these prodrugs must be used in combination with β-cyclodextrin. To ensure a proper drug delivery using prodrugs, it is of crucial importance to explore 'neutral' (less pH sensitive) A-007 prodrugs. A second very important point in the design of the A-007 prodrug is its timely release to the targeted area. Therefore, the half-life of drug released from the prodrug is an important factor in drug design.[19] The ideal prodrug is the one that is instantly transformed into the target drug at the site in which biological activity is desired.

Glucosides of active biological compounds are very often used in ADEPT (antibody-directed enzyme prodrug therapy) strategy.[20] This strategy provides the means to selectively deliver an active drug to its intended target from an inactive prodrug precursor. In the first step, the enzyme (β-glucuronidase) is delivered to the tumor site by monoclonal antibody–antigen recognition, and in the second step, enzyme cleavage of the prodrug liberates the active drug to the tumor cell surface. β-Glucuronidase is an appropriate enzyme for glycosylated anticancer drugs. In normal tissue, glucosidase is located in the liposome of the cell and it is found in very low concentrations in the blood. High concentrations of this enzyme are present extracellularly in necrotic areas.

Considering this, our glucoside **5** should be an ideal prodrug for A-007. After delivery of the A-007 derivative, glucoside **5**, to the vicinity of the cancer cell, the free drug, A-007, should be delivered by enzymatic glycoside bond cleavage. To further support this hypothesis, it was also determined in vitro that prodrug **5** has slightly higher anticancer activity than the original A-007 (the results of this study will be published elsewhere). However, the solubility of **5** is still relatively low (~2 mg/1 mL of water). The solubility of this compound is fivefold higher in the presence of β-cyclodextrin (from ~2 mg/1mL of water to 10 mg/1 mL of 30 mM β-CD). Even so, we could not observe substantial change of aromatic NMR chemical shift of **5** with any of the three cyclodextrins. However, in the ES-MS spectra of **5** in aqueous β-cyclodextrin, there is a weak signal that corresponds to the complex between one β-CD and one molecule of **5** (Fig. 8). The only plausible explanation is that inter-molecular interactions between **5** and β-cyclodextrin occurred between glucose moieties of **5** and the outside wall of β-cyclodextrin. That will increase the solubility of **5** in β-CD, show the molecular associate signal in ES-MS, but not have influence in the chemical shift of aromatic signals that are relatively far away from the molecular interactions.

To explore the influence of cationic A-007 prodrugs on delivery (half-life), solubility, and anticancer activity, we prepared and studied the physical properties of glycine derived prodrugs **3TFA** and **4**. These compounds are hydrolyzable prodrugs sensitive to the moisture and their stability in aqueous media might be questionable, based on the shelf-life of the compounds.[21] Considering that these compounds have surfactant-like properties, it is reasonable to expect that they might be able to form molecular aggregates similar to micelles in aqueous media. Aggregation was nicely demonstrated by following the NMR chemical shift for aromatic protons versus concentration of **4** in water media (Fig. 9). We observed a substantial downfield chemical shift with increasing concentration, with the maximal effect observed at 10 mM. At this concentration, aqueous **4** is stable at room temperature for long periods (at least three months). We assume that at this concentration, **4** forms molecular

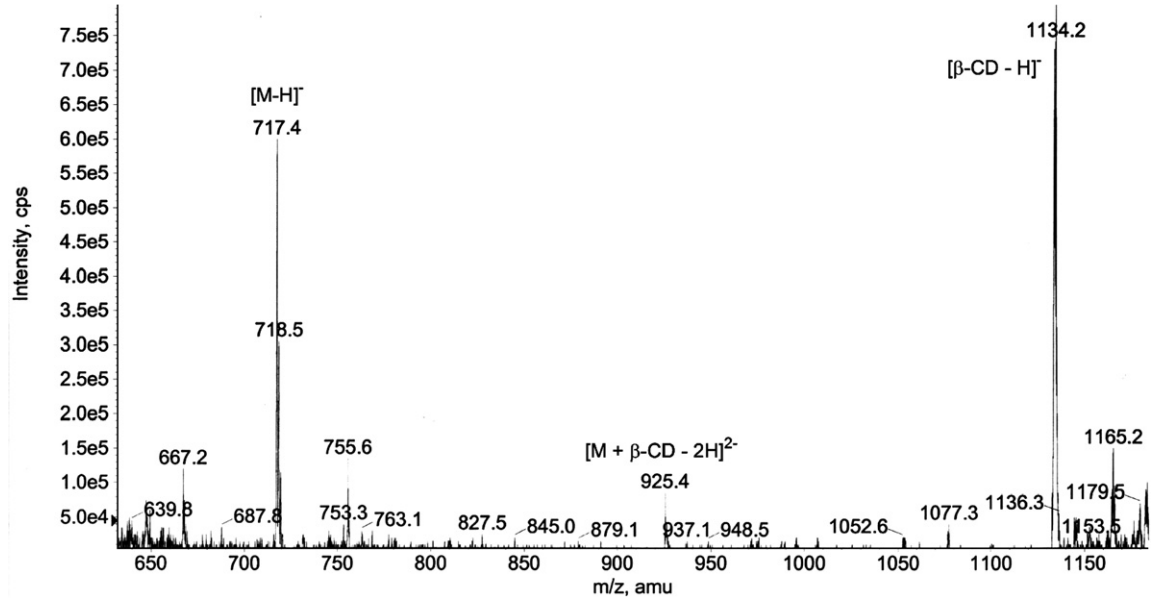


Figure 8. Negative ES-MS of diglucoside **5** (0.01 mM) and aqueous β-CD (0.03 mM).

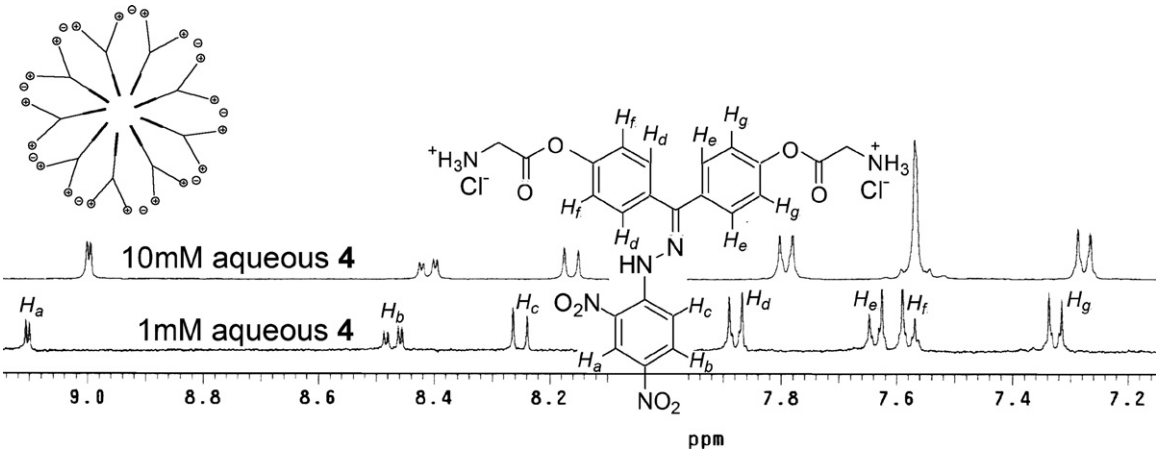


Figure 9. Dependence of concentration of **4** on the chemical shift.

aggregates similar to micelles (Fig. 9), which slow down the hydrolysis of the ester group.

To confirm this assumption, we also explored the water stability of **4** at 1 mM concentration (Fig. 10). Almost instantly, hydrolysis of the two ester groups of **4** started and the precipitation of A-007 could be observed. Due to the exceptionally low water solubility of A-007, we were not able to obtain the NMR spectra of pure A-007 in water, and the additional signals in the NMR spectra of **4** after one and two days correspond to the mono ester of A-007 (partially hydrolyzed product of **4**). Neither the NMR spectra of **4** after one day nor the NMR spectra of **4** in water after two days can be used for computing the half-life of this drug because both of the NMR spectra contain substantial amounts of precipitate and their NMR acquisition time is progressively longer. The half-life was determined by evaluating the amount of precipitate versus time. It was determined that the half-life of **4** as a 1 mM concentration at room temperature was around 140 min. As mentioned above, at a 10 mM concentration the formation of the precipitate was not observed even after a few months at 5 °C.

There are some interesting observations regarding cyclodextrin inclusion complexes with **3TFA**. Experimental evidence indicates that α-cyclodextrin forms complexes with trifluoroacetic acid.[22] If this is true, then the trifluoroacetate anion of **3** should be complexed by α-cyclodextrin, resulting in a slight downfield chemical shift for all aromatic protons. We did not observe any evidence that supports the formation of the α-cyclodextrin inclusion complex with the aromatic moieties of **3TFA** (Fig. 11).

On the other hand, there is a noticeable difference in the chemical shift of the **3TFA** protons in aqueous β-cyclodextrin, indicating strong interactions between β-cyclodextrin and **3TFA**. Unfortunately, we were not able to record a reliable two-dimensional NOESY NMR spectra due to fact that **3TFA** hydrolyzed in water media. In the NMR sample there are three components (**3TFA**, monoester of A-007, and A-007) in complex with β-cyclodextrin. The molar ratio of these three components varies with the progression of the NMR recording time. This is clearly demonstrated on the one-dimensional spectrum of **3TFA** in water and β-cyclodextrin (Fig. 11). Even for the freshly prepared sample of **3TFA** in water, the NMR spectra (acquired after 10 min) shows the presence of partially hydrolyzed **3TFA**. Full hydrolysis of **3TFA** generates A-007 that immediately precipitates from the solution. Aqueous

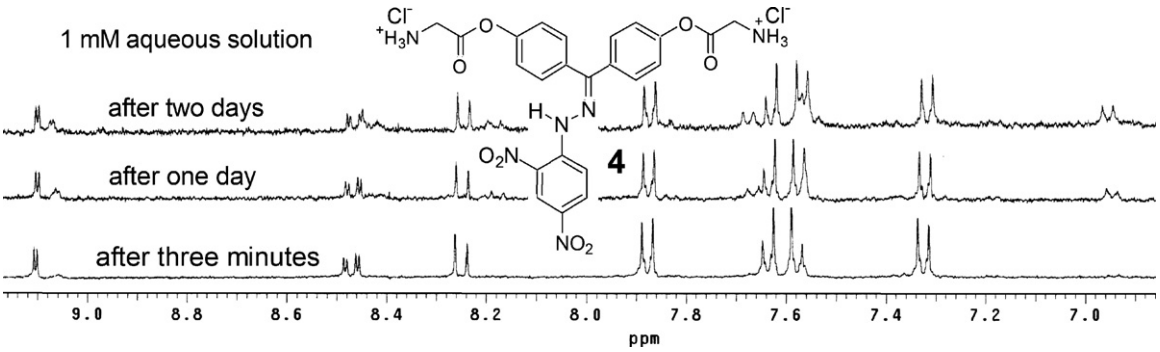


Figure 10. ^{1}H NMR spectra of **4** at 1 mM at different time points.

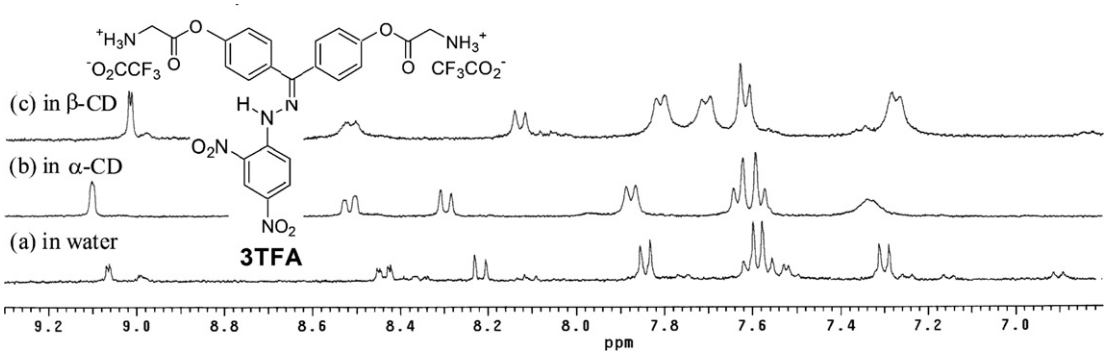


Figure 11. ^{1}H NMR spectra of **3TFA** (10 mM) in α-CD (100 mM) and β-CD (100 mM).

solution of **3TFA** and β-cyclodextrin (with 1:10 molar ratio) stays clear for a few hours, but the NMR spectrum indicates the presence of all three molecular species.

Regardless of the fact that the characterization of the β-cyclodextrin inclusion complex with **3TFA** is hard to evaluate by two-dimensional NMR spectroscopy, we believe that the ^{1}H NMR spectra supports the formation of the β-cyclodextrin inclusion complex. Furthermore, this prodrug half-life (~80 min in aqueous β-cyclodextrin) seems to be ideal for pharmacological study of this prodrug.

3. Conclusion

Several interesting A-007 prodrugs were prepared through ester, ether, and acetal linkages of polar molecules to A-007. All the prodrugs have significantly higher water solubility (10–50 mM) as compared to original A-007. To further increase their water solubility, alter their water half-life, and biodelivery, cyclodextrin inclusion complexes were studied. Through one-dimensional NMR, two-dimensional NOESY, and negative ES-MS spectroscopy studies, it was demonstrated that inclusion complexes with β-cyclodextrin were formed for all but A-007 glucoside **5**. It seems that the α-cyclodextrin cavity is too small and the γ-cyclodextrin cavity is too large to form stable inclusion complexes with these studied A-007 prodrugs. It was postulated that β-cyclodextrin forms a 2:1 inclusion complex with the studied A-007 prodrugs. None of the three studied cyclodextrins form inclusion complexes with the diglucoside of A-007, although they slightly increase the prodrug water solubility. This prodrug was prepared for targeted cancer therapy (ADEPT).

4. Experimental

Melting points were taken on an Electrothermal IA 9000 Digital Melting Point Apparatus and are uncorrected. The ^{1}H and ^{13}C NMR spectra were run on Varian 300 MHz Gemini2000 and on Varian 400 MHz Unity in $CDCl_3$, DMSO-d_6, or D_2O as solvent and internal standards. Two-dimensional NOESY spectra were recorded on Varian INOVA 500 MHz spectrophotometer with D_2O as a solvent and internal standard (4.80 ppm). The mass spectra were recorded on a Micromass Quattro 2 Triple Quadropole Mass Spectrometer. The prepared ammonium A-007 prodrugs are hydroscopic and decompose by ester hydrolysis. However under dry conditions they can be stored for at least several months. All compounds have melting points higher than 200 °C and decompose before melting. For ammonium compounds **3TFA** and **4** carbon NMR spectra were not reported because they decompose in solution during NMR acquisition time. For these compounds ^{1}H NMR samples were prepared immediately before recording the spectrum.

4.1. Preparation of ethyl {4-[[(2,4-dinitrophenyl)hydrazono]-(4-ethoxycarbonyl-methoxyphenyl)-methyl]-phenoxy}-ethanoate (2Et)

An acetone (500 mL) suspension of A-007 (3.94 g, 0.01 mol), potassium carbonate monohydrate (3.12 g, 0.01 mol), and ethyl 2-bromoacetate (3.34 g, 0.02 mol) was sonicated at room temperature for 2 h and heated at reflux overnight. The solvent from dark red suspension was evaporated to the solid residue. The solid residue was mixed with CH_2Cl_2 (200 mL), stirred at room temperature for 2 h, and the insoluble material was separated by filtration. The filtrate was washed with 10% potassium carbonate, dried over anhydrous magnesium sulfate and evaporated to deep red solid residue (5.3 g, 94%). According to the NMR spectroscopy product was more than 96% pure and was used in next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.30 (1H, NH), 9.09 (1H, d, $J = 2.4$ Hz), 8.35 (1H, dd, $J_1 = 9.3$, $J_2 = 2.4$ Hz), 8.18 (1H, d, $J = 9.3$ Hz), 7,64 (2H, d, $J = 9$ Hz), 7.32 (2H, d, $J = 8.4$ Hz), 7.30 (2H, d, $J = 8.7$ Hz); 7.17 (2H, d, J = 8.4 Hz), 6.94 (2H, d, $J = 9.0$ Hz), 4.76 (2H, s), 4.69 (2H, s), 4.37 (2H, q,

$J = 6.9$ Hz), 4.31 (2H, q, $J = 6.9$ Hz), 1.36 (3H, t, $J = 6.9$ Hz), 1.34 (3H, t, $J = 6.9$ Hz); ^{13}C NMR (300 MHz, $CDCl_3$) δ 168.7, 159.8, 159.4, 155.2, 144.7, 138.0, 130.6, 130.1, 129.9, 129.6, 124.9, 123.7, 116.8, 116.3, 114.8, 65.6, 65.5, 14.4. Anal. Calcd for $C_{27}H_{26}N_4O_{10}$ (MW 566.52): C, 57.24; H, 4.63; N, 9.89. Found: C, 57.08; H 4.77, N 9.72.

4.2. Preparation of (4-{(4-carboxymethoxy-phenyl)-[(2,4-dinitro-phenyl)-hydrazono]-methyl}-phenoxy)-acetic acid (2)

Water (150 mL), sodium hydroxide (1.6 g, 0.04 mol), and MeOH (150 mL) were mixed with CH_2Cl_2 (150 mL) solution of crude A-007 ester (5.3 g, 9.3 mmol). This solution was stirred at room temperature for 4 h. The reaction mixture was concentrated to 1/3 of its original volume at 40 °C and reduced pressure, diluted with water (100 mL), and neutralized with concentrated hydrochloric acid (10 mL). The solid material was separated by filtration, washed with water (3×100 mL), and dried at 110 °C for a few hours to afford a product that was pure by NMR spectroscopy (4.5 g, 95%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 11.12 (1H, s), 8.79 (1H, d, $J = 2.4$ Hz), 8.34 (1H, dd, $J_1 = 9.6$ Hz, $J_2 = 2.4$ Hz), 8.17 (1H, d, $J = 9.6$ Hz), 7.54 (2H, d, $J = 8.8$ Hz), 7.38 (2H, d, $J = 8.4$ Hz), 7.20 (2H, d, $J = 8.4$ Hz), 6.99 (2H, d, $J = 8.4$ Hz), 4.81 (2H, s), 4.73 (2H, s); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 169.93, 169.90, 159.6, 159.0, 154.6, 144.1, 137.1, 130.1, 130.0, 129.6, 129.3, 123.8, 122.9, 116.6, 115.8, 114.7, 64.7, 64.6. Anal. Calcd for $C_{23}H_{18}N_4O_{10}$ (MW 510.41): C, 54.12; H, 3.55; N, 10.98. Found: C, 53.98; H, 3.63; N, 10.87.

4.3. Preparation of *tert*-butoxycarbonylamino-acetic acid 4-{[4-(2-*tert*-butoxycarbonylamino-acetoxy)-phenyl]-[(2,4-dinitro-phenyl)-hydrazono]-methyl}-phenyl ester (3BOC)

tert-Butoxycarbonylamino-acetic acid (175 mg, 1 mmol), A007 (197 mg, 0.5 mmol), and dicyclohexylcarbodiimide (248 mg, 1.2 mmol) were taken in 20 mL of dry CH_2Cl_2. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. The progress of the reaction was monitored by proton NMR spectroscopy. The reaction mixture was concentrated to half of the original volume and filtered. The white residue was discarded and the filtrate was further concentrated and subjected to column chromatography on a silica gel column with CH_2Cl_2 and EtOAc (10:1) producing pure product (71 mg; 20%). ^{1}H NMR ($CDCl_3$, 400 MHz) 11.20 (1H, s), 9.01 (1H, d, $J = 2.8$ Hz), 8.33 (1H, dd, $J_1 = 9.6$ Hz, $J_2 = 2.0$ Hz), 8.14 (2H, d, $J = 9.6$ Hz), 7.43 (2H, d, $J = 8.8$ Hz), 7.38 (2H, d, $J = 8.4$ Hz), 7.15 (2H, d, $J = 8.8$ Hz), 5.28 (2H, m), 4.22 (2H, d, $J = 4.8$ Hz), 4.18 (2H, d, $J = 4.4$ Hz), 1.48 (9H, s), and 1.47 (9H, s); ^{13}C NMR ($CDCl_3$, 400 MHz) 169.1, 168.9, 155.9, 153.6, 152.2, 152.0, 144.5, 136.4, 134.3, 130.1, 129.9, 129.8, 129.3, 123.5, 121.8, 116.7, 80.5, 42.8, 28.4. Anal. Calcd for $C_{33}H_{36}N_6O_{12}$ (MW 708.67): C, 55.93; H, 5.12; N, 11.86. Found: C, 55.73; H, 5.12; N, 11.77.

4.4. Preparation of 3,3′-(4,4′-((2-(2,4-dinitrophenyl)-hydrazono)methylene)bis(4,1-phenylene)bis(oxy))bis-(2-oxoethylammonium trifluoroacetate) (3TFA)

A mixture of CH_2Cl_2–trifluoroacetic acid (9 mL:1 mL) wa cooled to 0 °C and **3BOC** was added (354 mg, 0.5 mmol). The reaction mixture was stirred at 0 °C for 1 h and solvent was evaporated under a stream of nitrogen resulting in red crystalline product (361 mg; 98%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 11.04 (1H, s), 8.83 (1H, d, $J = 2.4$ Hz), 8.48 (3H, m), 8.83 (3H, m), 8.26 (2H, d, $J = 9.3$ Hz), 7.78 (2H, d, $J = 8.8$ Hz), 7.66 (2H, d, $J = 8.4$ Hz), 7.55 (2H, d, $J = 8.4$ Hz), 7.32 (2H, d, $J = 8.8$ Hz), 4.21 (2H, m), 4.16 (2H, m). Anal. Calcd for $C_{27}H_{22}F_6N_6O_{12}\cdot 2H_2O$ (MW 772.52): C, 41.98; H, 3.39; N, 10.88. Found: C, 42.02; H, 3.37; N, 10.75.

4.5. Preparation of 3,3′-(4,4′-((2-(2,4-dinitrophenyl)-hydrazono)methylene)bis(4,1-phenylene)bis(oxy))bis-(2-oxoethylammonium chloride) (4)

A CH_2Cl_2 (0.2 mL) solution of **3BOC** (45 mg; 0.06 mmol) was injected into an ice cold MeOH (2 mL) solution of hydrochloric acid (made by the addition of 0.2 mL of oxalyl chloride). A red hydroscopic precipitate was immediately formed. The product was separated by filtration, washed with CH_2Cl_2 (3×0.5 mL), and dried under nitrogen to produce a product that was pure by NMR spectroscopy (33 mg, 95%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 11.04 (1H, s), 8.33 (1H, d, $J = 2$ Hz), 8.45 (1H, dd, $J_1 = 9.6$ Hz, $J_2 = 2.0$ Hz), 8.26 (1H, d, $J = 9.6$ Hz), 7.77 (2H, d, $J = 8.4$ Hz), 7.64 (2H, d, $J = 8.0$ Hz), 7.56 (2H, d, $J = 8.4$ Hz), 7.32 (2H, d, $J = 8.4$ Hz), 4.18 (2H, s), 4.13 (2H, s). Negative ES-MS m/z 507.4 ($M-2HCl-H^+$), 450.3 ($M-3HCl-COCH_2NH_2^+$), 393.4 ($M-2HCl-2COCH_2NH_2-H^+$); Anal. Calcd for $C_{23}H_{22}Cl_2N_6O_8\cdot 2H_2O$ (MW 617.39): C, 44.74; H, 4.24; N, 13.61. Found: C, 44.56; H, 4.31; N, 13.45.

4.6. Preparation of 4,4′-dihydroxybenzophenone-2,4-dinitrophenylhydrazone bis(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside) (5Ac)

2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-trichloroacetimidate (370 mg, 0.75 mmol) and A-007 (118 mg, 0.3 mmol) were stirred under an atmosphere of nitrogen in anhydrous CH_2Cl_2 (20 mL) with 3 Å molecular sieves for 1 h. The solution was cooled in an ice-bath for

30 min before $BF_3{\cdot}OEt_2$ (2 mL) was added and stirred for 1 min the ice-bath followed by 1 h at room temperature. The solution was added to an ice cold satd aq $NaHCO_3$ solution (100 mL) with vigorous stirring and then extracted with ether (2 × 75 mL). The organic layers were combined and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography (CH_2Cl_2–CH_3CO_2Et 10:1) to give **5Ac** as red crystalline solid (43 mg; 20%). ^1H NMR ($CDCl_3$, 400 MHz) δ 11.28 (1H, s), 9.09 (1H, d, $J = 2.8$ Hz), 8.37 (1H, dd, $J_1 = 2.8$ Hz, $J_2 = 9.6$ Hz), 8.17 (1H, d, $J = 9.2$ Hz), 7.61 (2H, d, $J = 8.8$ Hz), 7.31 (2H, d, $J = 8.8$ Hz), 7.25 (2H, d, $J = 8.8$ Hz), 5.40–5.10 (6H, m), 4.30 (2H, m), 4.20 (2H, t), 3.9 (2H, m), 2.13 (3H, s), 2.09 (3H, s), 2.08 (3H, s), 2.075 (6H, s), 2,071 (3H, s), and 2.065 (3H, s). Negative ES-MS 1011.8 $[M-H]^-$; positive ES-MS 1035.8 $[M+Na]^+$. Anal. Calcd for $C_{45}H_{48}N_2O_{23}$ (MW 1012.88): C, 53.36; H, 4.78; N, 5.53. Found: C, 53.25; H, 4.88; N, 5.42

4.7. Preparation of 4,4′-dihydroxybenzophenone-2,4-dinitrophenylhydrazone bis(β-D-glucopyranoside) (5)

Octaacetate **5Ac** (101 mg, 0.1 mol) was dissolved in 2:1 MeOH–CH_2Cl_2 (30 mL). Sodium hydroxide (1 M) was added to a pH ~ 9–10. The solution was stirred at room temperature overnight, neutralized with acidic Dowex resin, and evaporated. The solid residue was dried under reduced pressure at room temperature to give 68 mg (95%) of red crystalline product. ^1H NMR (DMSO-d_6, 400 MHz) δ 11.13 (1H, s), 8.80 (2H, d, $J = 2.4$ Hz), 8.40 (2H, dd, $J_1 = 9.6$ Hz, $J_2 = 2.4$ Hz), 8.19 (2H, d, $J = 9.6$ Hz), 5.54 (1H, d, $J = 4.8$ Hz), 5.48 (1H, d, $J = 4.8$ Hz), 5.18 (4H, m), 4.99 (1H, d, $J = 7.2$ Hz), 4.92 (1H, D, $J = 7.4$ Hz), 4.74 (1H, t, $J = 5.6$ Hz), 4.67 (1H, t, $J = 5.6$ Hz), 3.2 (4H, m); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 159.4, 159.0, 154.9, 144.5, 137.6, 130.6, 130.3, 129.7, 129.6, 125.0, 123.4, 117.8, 117.0, 116.6, 100.6, 100.3, 77.3, 76.8, 73.6, 73.5, 70.0, 61.0. Positive ES-MS 731.5 $[M+Na]^+$; Negative ES-MS 717.4 [M−H]. Anal. Calcd for $C_{31}H_{34}N_4O_{16}$ (MW 718.62) C, 51.81; H, 4.77; N, 7.80. Found: C, 51.65; H, 4.88; N, 7.68.

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