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Influence of Monosubstitution of Hexakis(3,6-anhydro)cyclomaltohexaose on Its Complexation Properties with Ions, with Special Attention to Heavy Metals

Lauriane Rambaud,[a] Jean-Pierre Dalbiez,[a] Badia Amekraz,[b] Christophe Moulin,[b] Bruno Perly,[a] and Cécile Baudin*[a]

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We report on the synthesis, characterisation and ionic complexation properties of mono-2-*O*-carboxymethyl-hexakis(3,6-anhydro)cyclomaltohexaose sodium salt **1**, as investigated by TLC and NMR. We demonstrate that the grafting of a carboxylate group not only modifies the complexation properties of the parent derivative in relation to heavy metals but also improves them. In this respect, we provide evidence that the gain in affinity is due to the cumulative effects of the molecular cage and the carboxylate group. Coupling of **1** to insoluble supports (bio-polymers, liposomes, …) might be expected to afford new materials for the elimination of toxic metals in biological fluids or organs.
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Introduction

In a previous paper[1] we illustrated the influence of the chemical modification of per(3,6-anhydro)cyclodextrins [per(3,6-anhydro)CDs][2–4] on their abilities to complex ions.[5–15] It has been demonstrated that chemical modification, when it involves all secondary hydroxy groups of per(3,6-anhydro)CDs, results in the formation of persubstituted derivatives in which the selectivity towards ions can be oriented and enhanced. Such properties make these per(3,6-anhydro)CDs good candidates for the elimination of toxic metals in instances of biological contamination or for the transport of radioactive metals for diagnostic or therapeutic purposes.

Both potential applications imply modification of per(3,6-anhydro)CDs. The first application requires the per(3,6-anhydro)CD to be grafted onto a solid support in order to decontaminate the biological fluids (plasma or whole blood). The second application requires a recognition signal of biological receptors or "antenna" to be grafted onto the per(3,6-anhydro)CD, as is well documented[16] for the natural cyclodextrins (CDs). This latter application combines the ionic specificity of the per(3,6-anhydro)CD and the targeting and opens up the field of new vectors dedicated to elimination or concentration of ions at the level of the organ or tissue to be decontaminated or treated.

In both cases, the grafting involves one single secondary hydroxy group, leaving the other hydroxy functions free for ulterior chemical modifications. In this respect, it is important to note that the grafting can either modify the ionic properties or impair the specificity of the cage molecule. Yamamura et al.[17] synthesised mono-2-*O*-*p*-phenylazobenzoate-heptakis(3,6-anhydro)cyclomaltoheptaose and demonstrated that this monosubstituted compound exhibits behaviour towards alkali ions different from its parent derivative. These results prompted us to investigate the properties of new monosubstituted derivatives.

We now report on the synthesis of mono-2-*O*-carboxymethyl-hexakis(3,6-anhydro)cyclomaltohexaose sodium salt **1** (Scheme 1), a new monosubstituted chelating agent derived from per(3,6-anhydro)-α-CD, which is well documented[11–13] to bind heavy metals, particularly lead. After the characterisation of this new derivative, we demonstrate the influence of the monosubstitution on its heavy metal complexation properties relative to essential physiological ions such as sodium or potassium, with the aid of NMR spectroscopy and TLC.

Results and Discussion

Before grafting the CD moiety onto the solid support or the "antenna", we elected to graft a spacer onto the CD to preclude potentially strong steric hindrance between the support or the vectorization-dedicated moiety and the per(3,6-anhydro)-α-CD during the coupling reaction.[16]

Standard coupling reactions usually involve the preparation of 6-amino-6-deoxy-CDs, synthesized in three steps from their respective parent CDs as already described elsewhere.[18,19] The three steps involved in the preparation of the monoamine require the formation of a CD monoactivated by a tosyl group, the substitution of a leaving group

[a] CEA, DRECAM/SCM, CEA Saclay,
91191 Gif-sur-Yvette, France
Fax: +33-1-69086462
E-mail: cecile.baudin@cea.fr
[b] CEA, DEN/DPC/SECR/LSRM, CEA Saclay,
91191 Gif-sur-Yvette, France
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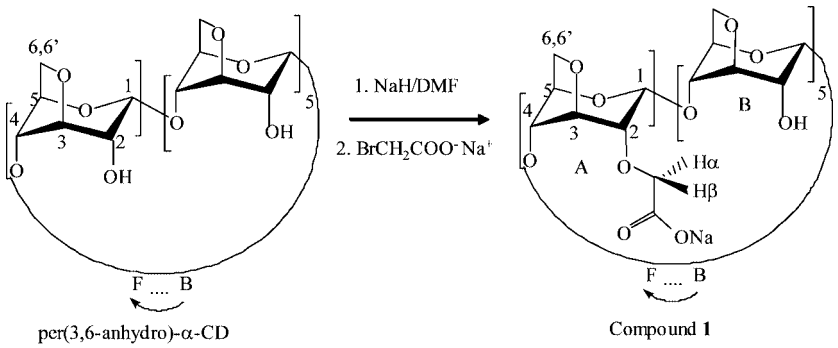

Scheme 1. Schematic representation of the synthesis of **1**.

by an azide group and the conversion of the azide group into an amine. Finally the coupling of the spacer is performed by nucleophilic addition of succinic anhydride.[16]

The same steps cannot be achieved with the per(3,6-anhydro)-α-CD because of difficultly involved in the elimination of the tosyl group.[20,21] Such behaviour prompted us to select a standard nucleophilic substitution reaction requiring the formation of the corresponding monoalkoxide through the use of sodium hydride as a nucleophilic reagent. The nucleophilic substitution reaction was carried out in the presence of sodium bromoacetate, a reagent that appears to be convenient in terms of electron-withdrawing power due to the presence of the halogen and the carboxyl function. The combined inductive effects provide an electron-deficient site, which should exhibit good reactivity during the nucleophilic substitution. In addition, the introduction of a carboxyl group is of considerable interest for further graftings.

Synthesis

The synthesis of **1** (Scheme 1) was performed in anhydrous DMF and in a basic medium, giving a mixture of five compounds: the pure and grafted per(3,6-anhydro)-α-CD and the three disubstituted derivatives.

After purification by HPLC, the chemical structure of **1** was assessed by mass spectrometry and NMR spectroscopy. Figure 1 displays the ^{1}H spectrum of a 13.2 mM solution of **1**. As **1** lacks symmetry because of its monosubstitution, the corresponding NMR spectrum appears quite complex and does not allow the shifts of protons to be measured directly. The complete assignment of **1** was therefore achieved by 2D experiments [COSY (COrrelation SpectroscopY), RELAY (RELAYed coherence transfer), HMQC (Hetero Multiple Quantum Correlation), ROESY (Rotating frame Overhauser Effect Spectroscopy), …] as described elsewhere.[22,23] Convincing evidence of the reality of the grafting of the carboxymethyl group onto the CD is to be expected in the form of the direct observation of spatial proximities between the geminal protons of the carboxymethyl group and the protons of the CD moiety. This type of information can be obtained by showing dipolar interactions (nuclear Overhauser effects) between relevant protons owing to T-ROESY (Transverse ROESY) sequence,[24,25] an improvement of the basic ROESY sequence allowing the effects due to the contribution of residual scalar transfers (HOHAHA effect) to be reduced.

Figure 2 displays a partial contour plot of a T-ROESY experiment performed on the above solution of **1**. One correlation peak is observed between H-2 of the substituted A unit and the geminal protons of the carboxymethyl group.

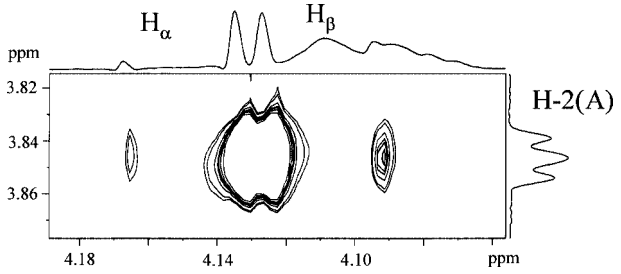

Figure 2. Partial contour plot of a T-ROESY experiment (spin lock 300 ms, 256 scans per time increment) performed at 500.13 MHz on a 13.2 mM solution of **1** at 298 K.

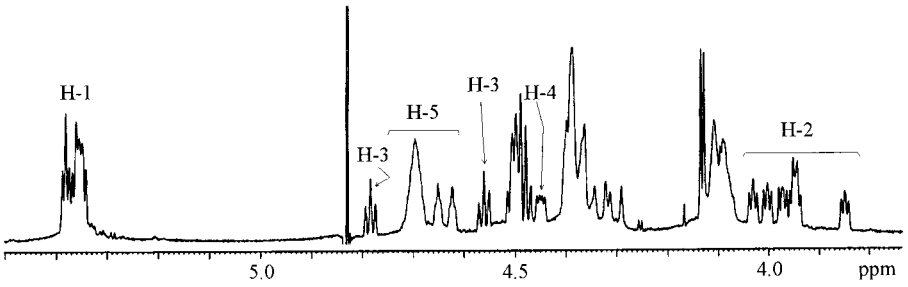

Figure 1. ^{1}H NMR spectrum (500.13 MHz, 298 K, D_2O) of a solution (13.2 mM) of **1**.

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This dipolar interaction indicates one single spatial proximity between two different moieties of **1** (i.e., the carboxymethyl group and the CD parts). This observation supports the reality of the grafting and shows unequivocally that the CH_2COONa group is grafted on unit A.

Complexation Properties

It has been shown[13] that per(3,6-anhydro)-α-CD scavenges lead with an association constant value of 2500 M^{-1} and that the hydroxy groups play a key role in the ionic complexation.[1] In this respect, the grafting of a spacer onto one anhydroglucose unit of per(3,6-anhydro)-α-CD may modify the behaviour of the host molecule and its binding properties, particularly towards lead. A NMR spectroscopic investigation was therefore performed to allow the stoichiometry to be derived and to estimate the association constant (K_a) value of **1**/Pb complex.

Figure 3 displays the influence of the concentration of $Pb(NO_3)_2$ on the ^1H spectrum of **1** in D_2O. From this Figure, two remarks can be made. Firstly, upon addition of lead the signals of **1** broaden and chemical shift variations are observed, especially in the anomeric region where the signals are not superimposed. In this case, the electronic environments of the nuclei of cyclodextrin are modified by the presence of lead. As described elsewhere, the chemical shift variations are large enough to establish the inclusion of Pb in the cavity. In the absence of crystallographic and molecular modelling data, the NMR results are convincing evidence of complexation. The broadening of the signals shows that the complexation is a dynamic process (i.e., the cation is in intermediary exchange on the NMR timescale) between the free and bound states. Secondly, at and above 0.7 equiv. of $Pb(NO_3)_2$, the shape of the spectrum line remains unmodified. Under these conditions, although the complexity of the 1D spectrum of **1** precludes the use of continuous variation techniques or the Job method[23] to ascertain the stoichiometry, it appears that the CD becomes complexed entirely when the number of equivalents x (= $[Pb(NO_3)_2]/[\mathbf{1}]$) is in the 0.6–0.7 range, with saturation occurring between 0.5 and 1 equivalent of lead. A similar experiment (data not shown) performed at 323 K shows the ^1H spectrum of **1** remaining unmodified when x is varied from 0.5 to 1.0. Within experimental uncertainties, it seems reasonable to postulate that the most likely stoichiometry of **1**/Pb complex is 2:1. The Pb cation is probably located the between cavities of two cyclodextrins, which must play the same role with regard to the lead since the chemical shift variations of H-1 are the same for two cyclodextrins. At this stage, it is reasonable to postulate that a part of the cation is hidden in the cavities. The charge of the carboxylate group allows the cation to approach the cyclodextrin and then the cyclodextrin plays its role as the complexing cavity. These results not only suggest that the natural affinity of the CD for lead is involved, but also indicate stabilisation of the complex by the charges provided by two carboxylate groups and one Pb^{2+} cation. In this context, the estimation of K_a is also of primary importance.

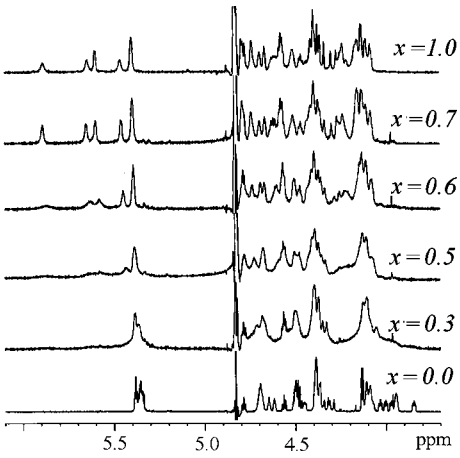

Figure 3. Influence of molar ratio (x) on ^1H NMR spectra (500.13 MHz, 298 K, D_2O) of **1**. The total concentration of **1** is 4.5 mM and the molar ratios $x = [Pb(NO_3)_2]/[\mathbf{1}]$.

Because of the reduction in the original sixfold symmetry, the determination of the variations of the chemical shifts of **1** upon complexation – and hence the K_a value – is not feasible. However, competitive experiments can be performed with compounds for which the association constants are known in order to evaluate the strength of the complex **1**/Pb.

The first competition experiment was carried out with per(3,6-anhydro)-α-CD. In Figure 4, no variation in the ^1H NMR spectrum of per(3,6-anhydro)-α-CD is observed in spite of the addition of $Pb(NO_3)_2$. Conversely, under the same conditions, modifications of the spectrum of **1** are clearly observed. As the addition of $Pb(NO_3)_2$ proceeds, the signals of **1** (↓) enlarge while those of per(3,6-anhydro)-α-CD remain unmodified. This fully supports the proposition that the K_a(**1**) affinity constant of the **1**/Pb complex is higher than the $K_{a[per(3,6\text{-anhydro})\text{-}\alpha\text{-CD}]}$ affinity constant of the per(3,6-anhydro)-α-CD/Pb complex.

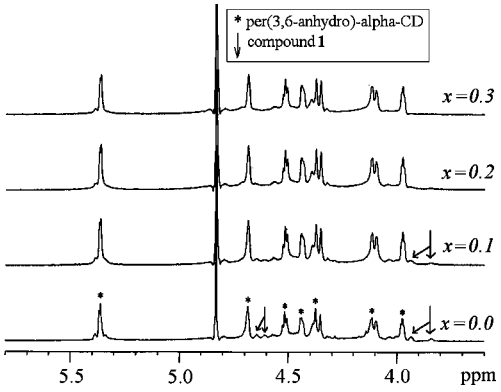

Figure 4. Influence of the concentration of $Pb(NO_3)_2$ on the ^1H NMR spectra (500.13 MHz, 298 K, D_2O) of an equimolecular mixture of **1** and per(3,6-anhydro)-α-CD. The molar ratios are given as $x = [Pb(NO_3)_2]/[\mathbf{1}]$ with [**1**] = [per(3,6-anhydro)-α-CD] = 2.9 mM. The signals of **1** are indicated by (↓) and those of per(3,6-anhydro)-α-CD by (*).

The second competition experiment, carried out with ethylenediaminetetraacetic acid disodium salt (EDTA), is

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displayed in Figure 5. With one equiv. of EDTA (x = [EDTA]/[$Pb(NO_3)_2$] = 1), weak complexation of **1** is observed, but with 1.8 equiv. (x = 1.8) of EDTA, the spectrum of free **1** is completely restored. In this case it can be concluded that the association constant K_a(**1**) value is lower than the association constant value of the EDTA/Pb complex.

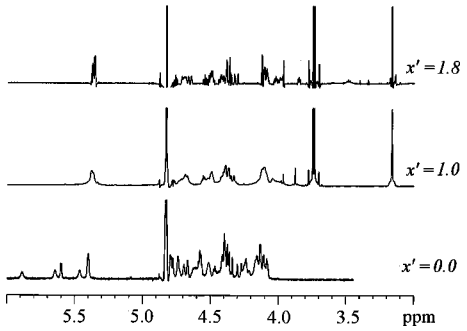

Figure 5. Influence of EDTA concentration on the ^1H NMR spectra (500.13 MHz, 298 K, D_2O) of an equimolecular mixture of **1** and $Pb(NO_3)_2$. The molar ratios are given as x' = [EDTA]/[$Pb(NO_3)_2$] with [$Pb(NO_3)_2$] = [**1**] = 4.5 mM.

In order to obtain more information on the formation of the **1**/Pb complex, a complementary mass spectrometry experiment was carried out on a mixture of **1** and $Pb(NO_3)_2$. In this experiment, the concentration of $Pb(NO_3)_2$ is twice that of **1**. The mass spectrum (data not shown) of the above mixture exhibits two main peaks, at *m*/*z* 565 and 667. These values may be ascribed to species [M – Na^+ + H^+ + Pb^{2+}]/2 and [M – Na^+ + Pb^{2+} + Pb^+]/2. The minor peak at *m*/*z* 769 may relate to the species [M – Na^+ + Pb^{2+} + Pb^+ + Pb]/2. These results provide evidence of strong interaction between **1** and lead and fully support the complexation of lead by **1**. The peak at *m*/*z* 565 probably implies a 1:1 complex, whilst the two other peaks at *m*/*z* 667 and 769 reflect a distribution rather than well determined stoichiometries. Under these conditions it appears that the stoichiometry of the complex depends on the experimental conditions, since in solution the NMR study provides a 2:1 CD/Pb complex. Keeping in mind that ESI-MS generally ionises the compounds and gives high concentrations of ionised final products, the complexes detected in ESI-MS may not reflect those observed in solution but are of considerable interest as they provide convincing evidence of complexation of lead by **1**.

After the detailed NMR investigation of lead complexation by **1**, we now extend our investigation to further toxic (Hg^{2+}, Cd^{2+}, Ba^{2+}, …) and also biological ions (Na^+, K^+, …). In this respect, the strengths of complexes are estimated by TLC on ion-loaded plates as described elsewhere.[1] In this qualitative approach, strong complexes are retained on the plate and yield high complexation indexes expressed as $1/R_f$ values.

Figure 6 displays $1/R_f$ values of **1** determined for several cations. A high $1/R_f$ value is observed for lead, which fully supports the above NMR spectroscopic data. A very high $1/R_f$ value is also found for Hg^{2+}. Although the absence of NMR spectroscopic data do not allow the presence of a 2:1 **1**/Hg^{2+} complex to be established, the TLC results suggest that the strength of the **1**/Hg^{2+} complex is greater than that of the per(3,6-anhydro)-α-cyclodextrin/Hg^{2+} complex and similar to that of the **1**/Pb^{2+} complex. The $1/R_f$ value found for the per(3,6-anhydro)-α-cyclodextrin/Hg^{2+} complex is a relative value that depends on the scale used for the high values and which, although low, does not mean the absence of complex. It can be concluded that in the case of Hg^{2+} the monosubstitution results in an enhancement of the

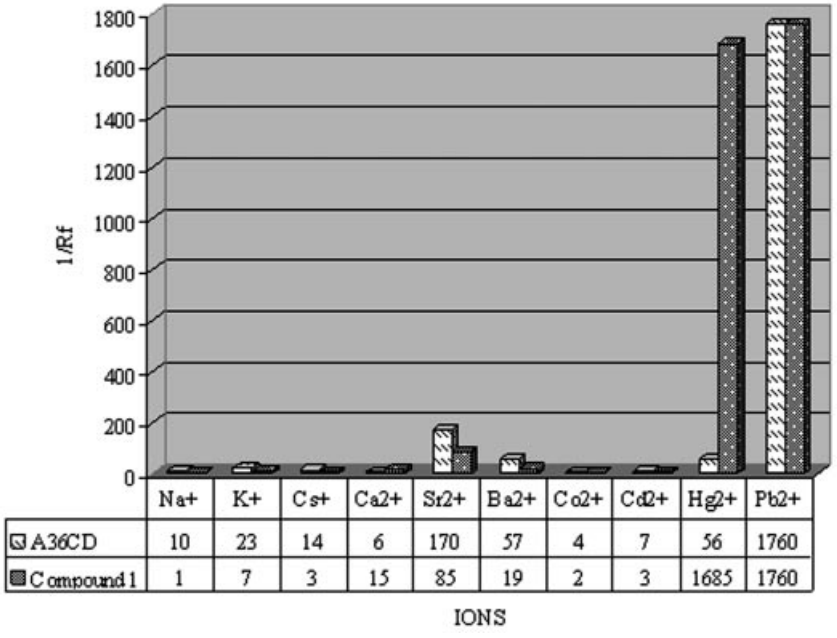

Figure 6. Ionic complexation indexes $1/R_f$ of **1** and per(3,6-anhydro)-α-CD (represented in the Table by "A36CD").

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strength of complex owing to the synergic effect of the carboxylate group. Conversely, the low $1/R_f$ values found for Na^+ and K^+ provide evidence of the lack of affinity of **1** towards biological cations, as also shown by the absence of modifications of the NMR spectrum of **1** upon addition of these cations. This result is of primary importance for the potential pharmaceutical applications of **1** (i.e., the elimination of heavy metals in living systems).

Conclusion

The above data show that the grafting of a single carboxymethyl group onto per(3,6-anhydro)-α-cyclodextrin not only modifies the affinities of the CD towards the heavy metals but also improves them. The gain in affinity achieved for **1** provides evidence that it is due to the cumulative effects of the molecular cage and the carboxyl group.

Ulterior coupling of **1** on insoluble supports (biopolymers or organized systems such as liposomes) should allow **1** to be made into a potential decontaminating agent for biological systems as far as pharmaceutical applications are concerned.[26]

Further chemical modifications of other hydroxy groups of **1** are in progress and are expected to increase the association constants of complexes with heavy metals, especially in the case of methylation, or to provide new versatile classes of chelating molecules for decontamination or targeting purposes.

Experimental Section

TLC was performed on silica gel 60 plates (E. Merck) followed by charring with 10% H_2SO_4. TLC on ion-loaded plates was performed on Polygram Ionex 25-SA-Na plates according to a procedure described elsewhere.[1]

Mass spectrometry experiments were performed on a electrospray infusion mode spectrometer (ESI-MS). Semipreparative HPLC was carried out with a Delta Pack 3000 chromatograph (Water Associates) equipped with an Evaporative Light Scattering Detector (ELSD) and a μBondapak C18-bonded silica column, with use of a linear gradient of MeOH in water from 0 to 100% in 30 min at 10 mL·min^{-1}.

^{1}H NMR experiments were performed with Bruker DRX 500 spectrometers operating at 500.13 MHz for proton. One-dimensional (1D) NMR spectra were collected using 16 K data points. Chemical shifts were measured in ppm downfield from external tetramethylsilane (TMS). D_2O was obtained from Euriso-Top (France). 2D experiments were obtained with the pulse programs available from the Bruker library. T-ROESY experiments were run with 2 K data points and 256 time increments. The phase-sensitive (TPPI) sequence was used, and processing resulted in a 1 K × 1 K (real-real) matrix. Details concerning experimental conditions are given in the Figure captions. All NMR spectroscopic data were processed and plotted by use of the UXNMR program (Bruker Analytische Messtechnik) on a Indy Silicon Graphics workstation.

Synthesis of Mono-2-*O*-carboxymethyl-hexakis(3,6-anhydro)cyclomaltohexaose Sodium Salt (1): Anhydrous conditions were required in order to make sure of the stability of the alcoholate during the reaction. Before use, the glassware and the magnetic stirring bar were oven-dried at 120 °C, and during the reaction, a stream of dry nitrogen was flushed through the reaction flask.

Sodium hydride (17 mg, 60% dispersion in mineral oil, 0.425 mmol), per(3,6-anhydro)-α-cyclodextrin (16.1 mg, 0.019 mmol, dried under vacuum for 24 hours at 100 °C), and dry DMF (3 mL) were introduced into the reaction flask and stirred for 35 minutes. Sodium bromoacetate (4.5 mg, 0.028 mmol) was added and the mixture was further stirred for 3 hours. The solvent was removed under reduced pressure and the crude product was dissolved in water (20 mL) and washed with chloroform (2 × 10 mL). The aqueous phase was taken to dryness and lyophilised. HPLC (t_R = 7.6 min) afforded chromatographically pure **1** (6 mg, 34%). R_f (DMF/BuOH/H_2O, 1:1:1) = 0.38. ^{1}H NMR (500.13 MHz, D_2O, 13.2 mm): δ = 5.382 [d, $^3J_{H-1,H-2}$ = 3.6 Hz, 1 H, H-1(F)], 5.376 [d, 1 H, H-1(C)], 5.361 [d, 1 H, H-1(A)], 5.355 [d, 1 H, H-1(D)], 5.355 [d, 1 H, H-1(E)], 5.341 [d, 1 H, H-1(B)], 4.784 [t, $^3J_{H-2,H-3}$ = 4.0 Hz, $^3J_{H-3,H-4}$ = 5.7 Hz,1 H, H-3(A)], 4.700 [t, $^3J_{H-4,H-5}$ = 2.7 Hz, $^3J_{H-5,H-6}$ = 2.0 Hz, $^3J_{H-5,H-6} \approx$ 0.0 Hz, 1 H, H-5(F)], 4.690 [t, 1 H, H-5(D)], 4.690 [t, 1 H, H-(E)], 4.685 [t, 1 H, H-5(B)], 4.648 [t, 1 H, H-5(C)], 4.618 [t, 1 H, H-5(A)], 4.560 [t, 1 H, H-3(A)], 4.500 [t, 1 H, H-3(B)], 4.498 [dd, 1 H, H-4(C)], 4.495 [t, 1 H, H-3(E)], 4.485 [t, 1 H, H-3(D)], 4.480 [t, 1 H, H-3(F)], 4.447 [dd, 1 H, H-4-(A)], 4.395 [dd, 1 H, H-4(B)], 4.395 [dd, 1 H, H-4(F)], 4.380 [dd, 1 H, H-4(E)], 4.380 [dd, 1 H, H-4(D)], 4.380 [d, $^3J_{H-5,H-6} \approx$ 0.0 Hz, $^2J_{H-6,H-6'}$ = –11.2 Hz, 1 H, H-6′(F)], 4.375 [d, 1 H, H-6′(B)], 4.375 [d, 1 H, H-6′(D)], 4.375 [d, 1 H, H-6′(E)], 4.332 [d, 1 H, H-6′(C)], 4.312 [d, 1 H, H-6′(A)], 4.152 [m, $^2J_{H\alpha,H\beta}$ = –16.2 Hz, 1 H, H_α(A)], 4.115 [dd, 1 H, H-6(C)], 4.111 [m, 1 H, H_β(A)], 4.098 [dd, 1 H, H-6(F)], 4.096 [dd, 1 H, H-6(A)], 4.090 [dd, 1 H, H-6(D)], 4.090 [dd, 1 H, H-6(E)], 4.080 [dd, 1 H, H-6(B)], 4.030 [t, 1 H, H-2(C)], 4.001 [t, 1 H, H-2(F)], 3.972 [t, 1 H, H-2(B)], 3.945 [t, 1 H, H-2(D)], 3.945 [t, 1 H, H-2(E)], 3.844 [t, 1 H, H-2(A)] ppm. ^{13}C NMR (125.77 MHz, D_2O): δ = 178.5 [C=O], 104.2 [C-1(A)], 103.4 [C-1(C)], 103.0 or 103.4[C-1 (D and E)], 102.5 [C-1 (F and B)], 83.7 [C-4 (D and E)], 82.9 [C-4 (B and F)], 81.3 [C-4 (C)], 80.8 [C-2 (A) and C-4(A)], 79.9[C-5 (A and C)], 78.6 [C-5 (B, D, E and F)], 77.5 [C-3(E)], 77.2 [C-3(D)], 76.6 [C-3(B and F)], 76.4 [C-$H_\alpha H_\beta$(A)], 75.8 [C-3(C)], 75.7 [C-3(A)], 74.2 [C-2 (B and F)], 74.1 [C-6 (B, D, E and F)], 73.7 [C-2(C)], 73.6 [C-6 (A and C)], 73.4 [C-2 (D and E)] ppm. ESI-MS (negative mode): *m/z* = 921 [M – H$^+$]$^-$, 967 [M – H$^+$ + HCOOH]$^-$.

Supporting Information (see also footnote on the first page of this article): ^{13}C, DEPT, COSY, RELAY, HMQC, ROESY and ESI-MS spectra of compound **1**.

Acknowledgments

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