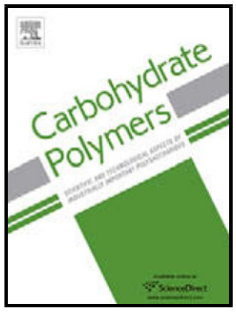

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Cyclodextrins: A promising drug delivery vehicle for bisphosphonate.

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Highlights

- Successfull cyclodextrin/bisphosphonates complex formation
- Cyclodextrins carriers for bisphosphates
- New systems for drug formulation

Abstract

Bisphosphonates are well established pharmaceutical drugs with wide applications in medicine. Nevertheless, the side chain and the nature of phosphorous groups could induce a poor aqueous solubility and act on their bioavailability. At the same time, cyclodextrins are cage molecules that facilitate transport of hydrophobic molecules to enhance the intestinal drug absorption of these molecules by forming inclusion complexes.

Here we demonstrate that cyclodextrins could be used as a bisphosphonate carrier. The formation of cyclodextrins- bisphosphonate complexes was characterized by 1D and 2D NMR spectroscopy, Isothermal Titration Calorimetry and UV-Vis spectroscopy. The results revealed that only the side chain of bisphosphonate was involved in the inclusion phenomenon and its length was a crucial parameter in the control of affinity. Findings from this study suggest that cyclodextrin will be a useful carrier for bisphosphonates.

Keywords: Bisphosphonates; cyclodextrins; host-guest complex; formation constant.

1. Introduction

Bisphosphonates (BPs) are stable analogs of pyrophosphates. Their P-C-P backbone instead of P-O-P bond in pyrophosphate are designed to resist to enzymatic hydrolysis. Due to their high affinity to Ca^{2+} ions, they exhibit a powerful binding affinity to bones (Rogers, Crockett, Coxon, & Mönkkönen, 2011; Russel, 2011) and especially hydroxyapatite (HAP) (Rogers, 2003). Bisphosphonates containing nitrogen have antiresorptive activity and inhibit the mevalonate pathway (Russel, 2011; Kavanagh et al., 2006; Merrell, Wakchoure, Lehenkari, Harris, & Selander, 2007) and the non-nitrogen bisphosphonate mimic pyrophosphate (Russel, 2011). So, bisphosphonates are used for the treatment of metabolic bone disease such as osteoporosis (Eastell, Walsh, Watts, & Siris, 2011; Lin, 1996) and Paget's disease (Reid & Hosking, 2011; Russel, 2011). As a consequence of the effect of solid tumors to develop bone diseases, they are widely used in anticancer treatment particularly for breast cancer (Muller, Migianu, Lecouvey, Kraemer, & Oudar, 2005), prostate cancer (Lee, Fong, Singer, & Guenette, 2001) and multiple myeloma (Shipman, Rogers, Apperley, Russel, & Croucher, 1997). Unfortunately, all the oral forms are poorly absorbed and their biodisponibility oscillates between 1 to 5%. Their intestinal absorption is low and mainly depends on the calcium content in food due to their high affinity to metal cations (Lin, 1996). Moreover, the charged nature of bisphosphonates and their strong hydrophilicity limit their diffusion through the lipophilic membrane and lead to a fast elimination from the blood.

To circumvent these problems, different carriers were investigated such as liposomes (Chebbi, Migianu-Griffoni, Sainte Catherine, Lecouvey, & Seksek, 2010), micelles (Seow, Xue, & Yang, 2007) and nanoparticles (Beneyettou et al., 2011). The chosen carrier should be suitable for time-controlled delivery, reduce side effects during absorption and decrease the toxicity of the drugs. We focused our attention on cyclodextrins. Cyclodextrins (CD) are α-1,4-linked cyclic oligosaccharides of D-glucopyranose with a hydrophilic exterior and a hydrophobic cavity (Szejtli, 1998) that allow the formation of inclusion complexes with hydrophobic compounds. Cyclodextrins are non-toxic and biocompatible and are widely used in soil remediation (Cho et al., 2015) and food formulation (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal Gandara, 2009; Astray, Mejuto, Morales, Rial-Otero, & Simal Gandara, 2010). Their ability to form inclusion complex was and are intensively investigated for drug delivery (Ijaz et al., 2015; Strickley, 2004; Uekama, Hirayama, & Arima, 2006) in biopharmaceuticals. To this end, the use of cyclodextrin in pharmaceutic formulation is an

effective method to enhance the solubility, chemical stability and bioavailability of drugs (Loftsson, Brewster, & Masson, 2004; Stella & Rajewski, 1997).

In the present work, we have hypothesized that cyclodextrins could be successfully employed as a carrier for bisphosphonate. To the best of our knowledge, only a few reports concerning the characterization of cyclodextrin/ bisphosphonate complexes (CD/BP) or the formation of bisphosphonate functionalized cyclodextrin derivatives were published. A bisphosphonate called alendronate was linked to cyclodextrin by click chemistry (Hein, Liu, Chen, Cullen, & Wang, 2010; Liu, Lee, Reinhardt, Marky, & Wang, 2007) for drug delivery. In addition, the formation of cyclodextrin/bisphosphonate complexes was identified by mass spectrometry (Biernacka, Betlejewska-Kielak, Witowska-Jarosz, Klosinska-Szmurlo, & Mazurek, 2014) or Raman microscopy (Daubiné et al., 2009).

This research reports the complete characterization of CD/BP complexes besides zoledronate (Daubiné et al., 2009) and alendronate (Biernacka et al., 2014). The goal of this study was to examine if cyclodextrins could be used as molecular carriers for bisphosphonates, and to determine the influence of the side chain and of the P-C-P backbone on the complexation phenomenon. Bisphosphonates with long alkyl chain were chosen because of their utilization against parasitic diseases in particular against Chagas disease (Hudock et al., 2006; Szajnman et al., 2012), a form of sleeping sickness. This disease is widespread in the world and it is considered by the World Health Organization to be one of the major parasitic disease.

The inclusion complex formation between bisphosphonate and cyclodextrin was investigated by 1D and 2D NMR spectroscopy. In addition, isothermal titration calorimetry and UV-Visible spectroscopy allowed us to determine values of formation constant.

2. Materials and methods

2.1. Materials

α-CD, β-CD, 2-hydroxypropyl-β-CD (HP-β-CD) (degree of substitution=DS=5.6) and randomly methylated-β-CD (RAMEB) (DS= 12.6) were purchased from Wacker-Chemie (Lyon, France). Methyl orange, sodium hydroxide and potassium dihydrogenophosphate were all of analytical grade and purchased from Acros Organics.

Pentadecanoyl chloride, dodecanoyl chloride, myristoyl chloride, undecanoic acid, tridecanoic acid, dimethylphosphite, trimethylphosphite, methanol, diethylether, tristrimethylsilylphosphite were purchased from Sigma Aldrich. All products were of analytical grade.

2.2. Synthesis

All chemical structures of bisphosphonate used in this study are displayed in Fig.1. In the following, the alkyl chain attached to the P-C-P backbone will be designated as the bisphosphonate side chain (thus excluding the carbon atom directly linked to phosphorus atoms). The two digits after M indicate the number of methoxy groups on either of the two P atoms.

General procedure for synthesis of bisphosphonate tetra acid (BP1, BP2, BP3, BP4, BP5, BP6).

In a 50 mL round-bottom three-neck flask equipped with a thermometer, acid chloride (2.5 mmol) was added dropwise, under argon, at -5°C, to tris trimethyl silyl phosphite (5 mmol). When addition was completed, reaction mixture was allowed to stand at room temperature for 2 h. The evolution of the reaction was monitored by ^{31}P {^{1}H} NMR (^{31}P NMR with proton decoupling). Then, volatile fractions were evaporated under reduced pressure (0.1 Torr) before being hydrolyzed with methanol. After evaporation, crude products were precipitated in diethylether.

(1-hydroxyundecane-1, 1-diyl)diphosphonic acid **BP10**

^{1}H NMR (400 MHz, DMSO): δ 1.89 – 1.70 (m, 2H, CH_2), 1.59 – 1.46 (m, 2H, CH_2), 1.33 – 1.11 (m, 14H, CH_2), 0.86 (t, 3H, ^{3}J= 8.0 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO): δ 72.82 (t, $^{1}J_{P\text{-}C}$= 154 Hz, P-*C*-P), 33.74, 31.76, 30.53, 29.22, 23.41, 22.59, 14.37. ^{31}P NMR (162 MHz, DMSO): δ 20.38. Yield: 84%

HRMS (ESI): Calcd for $C_{11}H_{25}O_7P_2$ [M - H] : 331.1079.

(1-hydroxydodecane-1, 1-diyl)diphosphonic acid **BP11**

^{1}H NMR (400 MHz, DMSO): δ 1.91 – 1.73 (m, 2H, CH_2), 1.60 – 1.43 (m, 2H, CH_2), 1.11 – 1.37 (m, 16H, CH_2), 0.85 (t, 3H, ^{3}J= 8.0 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO): δ 72.93 (t,

$^{1}J_{P\text{-}C}$= 144 Hz, P-*C*-P), 31.79, 30.49, 29.71 – 29.36, 29.22, 23.46 (t, $^{3}J_{P\text{-}C}$= 6 Hz), 22.58, 14.43. ^{31}P NMR (162 MHz, DMSO): δ 20.33. Yield 82%

HRMS (ESI): Calcd for $C_{12}H_{27}O_7P_2$ [M - H]: 345.1231.

(1-hydroxytridecane-1, 1-diyl)diphosphonic acid **BP12**

^{1}H NMR (400 MHz, DMSO): δ 1.90 – 1.70 (m, 2H, C*H*$_2$), 1.59 – 1.42 (m, 2H, C*H*$_2$), 1.37 – 1.96 (m, 18H, C*H*$_2$), 0.85 (t, 3H, ^{3}J= 8.0 Hz, C*H*$_3$). ^{13}C NMR (100 MHz, DMSO): δ 72.92 (t, $^{1}J_{P\text{-}C}$= 144 Hz, P-*C*-P), 31.78, 30.49, 29.70 – 29.37, 23.46 (t, $^{3}J_{P\text{-}C}$= 6 Hz), 22.57, 14.42. ^{31}P NMR (162 MHz, DMSO) δ 20.16. Yield 83%

HRMS (ESI): Calcd for $C_{13}H_{29}O_7P_2$ [M - H]: 359.1389

(1-hydroxytetradecane-1,1-diyl)diphosphonic acid **BP13**

^{1}H NMR (400 MHz, DMSO): δ 1.92 – 1.72 (m, 2H, C*H*$_2$), 1.61 – 1.43 (m, 2H, C*H*$_2$), 1.35 – 0.99 (m, 22H, C*H*$_2$), 0.85 (t, 3H, ^{3}J= 8.0 Hz, C*H*$_3$). ^{13}C NMR (100 MHz, DMSO): δ 72.91 (t, $^{1}J_{P\text{-}C}$= 144 Hz, P-*C*-P), 31.78, 30.51, 29.74 – 29.43, 23.42 (t, $^{3}J_{P\text{-}C}$= 6 Hz), 22.57, 14.42. ^{31}P NMR (162 MHz, DMSO): δ 20.12. Yield 80%

HRMS (ESI): Calcd for $C_{14}H_{31}O_7P_2$ [M - H] 373.1545

1-hydroxypentadecane-1,1-diyl)diphosphonic acid **BP14**

^{1}H NMR (400 MHz, DMSO): δ 1.93 – 1.73 (m, 2H, C*H*$_2$), 1.61 – 1.44 (m, 2H, C*H*$_2$), 1.35 – 1.11 (m, 22H, C*H*$_2$), 0.85 (t, 3H, ^{3}J= 8.0 Hz, C*H*$_3$). ^{13}C NMR (100 MHz, DMSO): δ 72.92 (t, $^{1}J_{P\text{-}C}$= 144 Hz, P-*C*-P), 33.97, 31.78, 30.51, 29.72 – 29.38, 29.20, 22.58, 23.47 (t, $^{3}J_{P\text{-}C}$= 6 Hz), 14.42 (CH$_3$). ^{31}P NMR (162 MHz, DMSO): δ 20.15. Yield 77%

HRMS (ESI): Calcd for $C_{15}H_{33}O_7P_2$ [M - H] : 387.1705.

(1-hydroxyhexadecane-1,1-diyl)diphosphonic acid (Monteil, Guénin, Migianu, Lutomsky, & Lecouvey, 2005) **BP15**

These data are in accordance with those reported in the literature (Monteil et al., 2005)

General procedure for synthesis of bisphosphonate monomethyl esters.

(1-hydroxy-1-(hydroxy(methoxy)phosphoryl)hexadecyl)phosphonic acid **BP15M01** was synthesized according the procedure described by Migianu (Migianu, Guénin, & Lecouvey, 2005) and these data are in accordance with those reported in the literature (Migianu et al., 2005).

General procedure for synthesis of bisphosphonate dialkyl esters.

Dimethyl (1-hydroxyhexadecane-1,1-diyl)bis(hydrogen phosphonate)(Monteil et al., 2005) **BP15M11** was synthesized according the procedure described by (Monteil et al., 2005), and these data are in accordance with those reported in the literature.

General procedure for synthesis of bisphosphonate trimethyl esters.

Methyl hydrogen (1-(dimethoxyphosphoryl)-1-hydroxyhexadecyl)phosphonate **BP15M21**

The adequate acid chloride (50 mmol) was added dropwise at -10°C under argon to trimethylphosphite (5.9 mL, 50 mL). The reaction mixture was then stirred at r.t for 2h (the end of reaction was ascertained by ^{31}P $\{^{1}H\}$ NMR or IR spectroscopy). The crude product was purified as indicated in Table 1 to furnish the corresponding α-keto-phosphonate dimethyl ester. These data are in accordance with those reported in the literature (Migianu et al., 2005).

In a 50 mL round-bottom three-neck flask equipped with a thermometer, α-keto-phosphonate dimethyl ester (5 mmol) was added dropwise, under argon, at -5°C, to dialkyl phosphite (5 mmol). When addition was completed, reaction mixture was allowed to stand at room temperature. The disappearance of tris(trimethylsilyl) phosphite during the reaction was monitored by ^{31}P $\{^{1}H\}$ NMR. The flask containing the solution was placed under an argon stream. A Pasteur pipette purged with argon was used to take 500 ul of mixture. Sample was introduced into a NMR tube purged with argon, without deuteriated solvent. If the peak of $P(OSiMe_3)_3$ doesn't appear on the spectrum, the reaction is over and the volatile fractions were evaporated under reduced pressure (0.1 Torr) before being hydrolyzed with methanol. After evaporation, crude products were precipitated in diethyl ether (Migianu, Mallard, Bouchemal, & Lecouvey, 2004).

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.67, 3.64, 3.60 (d, 9H, $^{3}J_{P\text{-}H}$= 12Hz, 3 OCH_3), 1.94 – 1.82 (m, 2H, CH_2), 1.65 – 1.51 (m, 2H, CH_2), 1.38 – 1.19 (m, 24H, CH_2), 0.92 (t, 3H, ^{3}J = 8.0 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO): δ 75.05 (t, $^{1}J_{P\text{-}C}$= 150 Hz, P-*C*-P), 53.78, 53.58, 35.10,

31.77, 30.31, 29.68 – 29.27, 29.19, 23.22, 22.57, 14.42 (CH_3). ^{31}P NMR (162 MHz, $CDCl_3$): δ 23.05 (d, $^2J_{P\text{-}P}$= 42.12 Hz), 21.85 (d, $^2J_{P\text{-}P}$= 42.12 Hz). Yield: 85%

HRMS (ESI): Calcd for $C_{19}H_{41}O_7P_2$ [M - H]: 443.2330

2.3. NMR analysis

2D ROESY spectra were recorded at 25°C on a Brucker Advance III 400 spectrometer equipped with a BBFO probe. The parameters were a mixing time of 500 ms during spin lock with 64 scans using the states-TPPI method with a 2048 time domain points (2K time domain) in F2 and 256 time increments in F1. ^1H NMR was run with FID resolution of 0.25 Hz/ point, 4s of acquisition time, 1s of recycle delay and 16 scans, for ^{31}P NMR, FID resolution of 0.97 Hz/ point, 0.5 s of acquisition time, 2s of recycle delay and 16 scans and for ^{13}C NMR, FID resolution of 0.36 Hz/ point, 1.4 s of acquisition time, 2s of recycle delay and 1500 scans.

Solutions of β-CD and bisphosphonates were prepared in 99.98% D_2O with a concentration of 5 mM and then was mixed to 1:1 ratio.

2.4. ITC analysis

An isothermal calorimeter (ITC200, MicroCal Inc., USA) was used in order to determine thermodynamic data of inclusion, for each studied complexes, at 298 K. Titration experiments consisted in 11 additions of the syringe solution (degassed phosphate buffer, 50 mM, pH=6.5, total volume 40 µL) on the cell content (same buffer, volume equal to 202.8 µL), with a first small aliquot of 1 µL followed by 10 aliquots of 3.7 µL (injection duration 7.4 s, time interval 75 s, agitation speed 1000 rpm). Heat flow was recorded as a function of time, the first injection being discarded to eliminate any error induced by material diffusion effects between the two compartments or backlash in the drive screw mechanism of the syringe. The heat produced per injection was obtained by measuring each peak area, by means of ORIGIN software. Titration experiments involved guest solution in the cell (0.25 or 0.5 mM) and host in the syringe (5 mM). Blank titrations were obtained by injecting host, guest or free buffer within a buffer solution, respectively. Effective binding signal was then obtained by subtracting to the titration experiment each host and guest blank experiment and adding the

buffer blank experiment (since this latter signal was already taken into account in each of the two other blank experiments). Complex formation constants (Kf in M^{-1}), binding stoichiometry (n) and inclusion enthalpies ($\Delta H°$ in $cal.mol^{-1}$) were deduced from binding isotherms (assuming a 1:1 stoichiometry), by nonlinear analyses of complexation heats as a function of total concentrations. Inclusion entropy and free enthalpy were deduced from the values of K and $\Delta H°$. Binding isotherms treatment were realized by means of a dedicated homemade program (Bertaut & Landy, 2014).

2.5. UV analysis

Complex formation constant (Kf) between bisphosphonates and cyclodextrins were characterized by a competition method called also spectral displacement method (Landy, Fourmentin, Salome, & Surpateanu, 2000). Methyl orange (MO) was used in its basic form (10^{-4}M in KH_2PO_4/NaOH buffer pH= 6.2). If a given concentration of bisphosphonate is added to a solution containing the CD and MO species, it causes a disturbance of the CD-MO complexation, leading to a difference between the spectra recorded in the absence and presence of the bisphosphonate. K values of CD/BP can be deduced from this absorbance difference. Stock solution of methyl orange was set to 0.1 mM. Then solutions of cyclodextrins were prepared with stock solution of methyl orange with a concentration of 0.5 mM for the α-CD and β-CD and 0.2 mM for HP-β-CD and RAMEB respectively. The concentration of bisphosphonate solution was fixed to 0.5 mM by adding the guest inside CD/ MO solution. Data were treated by the use of a dedicated algorithmic treatment (Landy, Fourmentin, Salome, & Surpateanu, 2000).

3. Results and discussions

3.1. Synthesis of bisphosphonates

Regarding the synthesis of lipophilic bisphosphonates (BPs), Merck synthesis using PCl_3 /H_3PO_3 is well documented (Kieczykowski, Jobson, Melillo, Reinhold, Grenda, & Shinkai, 1995 ; Lecouvey & Leroux, 2000), but uses harsh acidic conditions combined with heavy heating and long reaction time. These drastic conditions enable the use of fragile substrates

and the preparation of functionalized BPs. As an alternative to this pathway our group proposed a very mild and one-pot synthesis of BPs from tris(trimethylsilyl)phosphite and acyl chlorides (Lecouvey, Mallard, Bailly, Burgada, & Leroux, 2001). The interest of this procedure is that it could be indifferently used for aliphatic and aromatic substrates without any restriction concerning the nature of the functional group on the side chain.

This method enables the synthesis of various substituted BPs in a one-pot procedure under mild conditions. Moreover, reaction times were shortened and purifications were easier. This reaction was successfully extended to anhydride, aliphatic and aromatic substrates in excellent yields (Guénin, Degache, Liquier, & Lecouvey, 2004). Synthesis of esterified bisphosphonates is not possible using standard procedure used for non-esterified BPs. Most of the synthesis previously described usually goes through the making of the bisphosphonate tetraester which is then dealkylated (Turhanen, Ahlgren, Jarvinen, & Vepsalainen, 2001). The main drawbacks of these protocols are the thermal and basic instability of bisphosphonate tetraesters leading to phosphonate-phosphate isomerisation (Fitch & Moedritzer, 1962). Moreover the regioselective dealkylation to prepare partial esters is hard and does not occur in good yields (Vepsalainen, 2002). Our group has proposed a very mild and one-pot synthesis to obtain BP P,P' and P,P diesters , BP triesters and BP monoesters (scheme 2) (Migianu et al., 2005; Migianu et al., 2004). We have further exemplified this synthesis to the preparation of numerous bisphosphonate diesters varying both on the lateral chain and ester functions (Guénin, Monteil, Bouchemal, Prangé, & Lecouvey, 2007; Monteil et al., 2005)

3.2. NMR spectroscopy

Nuclear magnetic resonance spectroscopy is a major research tool useful for providing information concerning the complexation between bisphosphonates and cyclodextrins (Salvatierra, Jaime, Virgili, & Sánchez-Ferrando, 1996).

The structure of β-CD represented in Fig. 2., clearly shows that H3 and H5 hydrogens located in the inner cavity can most readily be linked to effect of guest inclusion (Djedaïni, Zhao Lin, Perly, & Wouessidjewe, 1990). H3 is located near the wider rim while H5 is on the opposite side closed to the narrower rim. Usually the guest enters through the wider rim, involving a most important shift for H3. H1, H2, H4 are set on the outside front of the cavity and should

exhibit negligible changes. For H6, the change in chemical shift will be partly dependent on the guest entry.

As a result, with reference to the proton chemical shift values of the cyclodextrin alone, the guest and the complex between these two latter, it is possible to get information on the nature of the inclusion guest orientation in the cyclodextrin and the stability of the complex thus formed. ^{1}H NMR spectra of the free bisphosphonate guest was not shown on the figure 3 due to their low solubility in deuterated water. ^{1}H NMR spectra of β-CD alone and bisphosphonate/ CD complex here BP1 were recorded and were depicted in Fig. 3.

The values reported in table 1 represent the ^{1}H chemical shift of β-CD in the free and complex state.

Assessment of the β-CD chemical shift in the absence (Schneider, Hacket, Rudiger, & Ikeda, 1998) or presence of bisphosphonate by ^{1}H NMR showed the complex formation between bisphosphonate and β-cyclodextrin. The results demonstrated that for all complexes H3 and H5 have the largest up field shifts as expected (Schneider et al., 1998), while the complex formation with bisphosphonate has little effect on H1, H2, H4 and H6 protons of β-cyclodextrin. According to their location outside the cavity, the chemical shift of H1, H2 and H4 are less affected by complexation. Moreover, the chemical shift of H6 differs slightly showing that inclusion is not deep enough for the bisphosphonate to strongly interact with H6.

2D ROESY experiment (Forgo & D'Souza, 1998; Veiga, Fernandes, Carvalho, & Geraldes, 2001) was performed to provide informations in order to understand the inclusion complexation mode and inter and intra molecular interactions between guest atoms and cyclodextrin. Fig. 4. represents the 2D NMR ROESY spectrum of β-cyclodextrin/ BP10 complex. For each studied bisphosphonate/ β-cyclodextrin complex, the ROESY spectrum displays intermolecular cross peaks between H3 and H5 protons of β-cyclodextrin and CH_3 group and methylene chain of bisphosphonate demonstrating the inclusion of these groups in the hydrophobic cavity. The same molecular correlation was observed for each complex. More specifically, the ROESY spectrum indicated two cross peaks between the H3 of the β-cyclodextrin and CH_3 group and methylene chain of bisphosphonate and showed significant correlation between H5 of the β-cyclodextrin and methylene chain of bisphosphonate. An expansion of the ROESY spectrum is displayed in Fig. 5.

The presence of cross peak between H3 and methylene and CH_3 group of bisphosphonate side chain demonstrated that the inclusion might take place though the wider rim. The overlap between H3 and H6 was so important that a cross peak between H6 and CH_2 cannot be excluded and consequently an inclusion though the narrower rim.

The flexibility of the side chain of bisphosphonate allowed the contortion of this last one inside the cavity allowing the interactions between H3 and H5 and the bisphosphonate. The end of the alkyl chain may come up along the cavity. For BP11 and BP13, low cross peak appeared on the bidimensional spectrum involving H3 of cyclodextrin and the two methylene anchored to P-C-P backbone of bisphosphonate leading to the conclusion that the P-C-P backbone may be located outside the cavity but very close to the wider rim. The P-C-P backbone may interact with the surrounding water or with some of the hydroxyl groups of β-cyclodextrin.

3.3. ITC analysis

In order to obtain a quantitative evaluation of the interactions previously highlighted by NMR, we first investigated the inclusion of BP10 to BP15 with β-CD by isothermal titration calorimetry. A representative ITC experiment is presented in Fig. 6., in the case of β-CD/BP10 complex. Injections induced negative signals, with decreasing intensities as the experiment proceeded. These results assess that a significant interaction take place between the two partners and that such formation of complexes is an exothermic reaction. As can be seen, a good fit was obtained between experimental data and theoretical simulation (for theoretical background see Bertaut & Landy, 2014), on the basis of a 1:1 stoichiometry. The same trend was observed for each studied host/guest combination. The use of theoretical models considering the presence of both 1:1 and 2:1 (or 1:2) complexes lead to uncertainties superior to the obtained values of formation constants for the highest stoichiometry, suggesting that only 1:1 inclusion compounds are present. Given the important length of such guest molecules, the fact that no 2:1 complex was observed confirmed that only the bisphosphonate side chain was available for inclusion, the bisphosphonate part being too bulky and/or hydrophilic to mobilize a second cyclodextrin.

ITC experiments afforded a complete picture of inclusion thermodynamics. Values of formation constants, inclusion free enthalpy (ΔG), enthalpy (ΔH) and entropy (-TΔS) are listed in Table 3. BP10 to BP15 complexes were characterized by a significant stability, with free enthalpy ranging from -4954 to -6482 cal/mol. Inclusion was both enthalpy and entropy

driven, the entropic contribution being roughly 2 to 3 times higher when compared to the enthalpic part. The enthalpic stabilization slightly increased with the number of methylene of the bisphosphonate side chain (i.e. on the P-C-P backbone), from -1263 to -2323 cal/mol when moving from 10 to 15 carbons. In addition, if we consider free energy of complexation as a function of the length of the alkyl part, it seems that each added methylene first improved the β-CD-BP affinity, until a total carbon content equal to 14, after what one can note a slight decrease of the complex stability. As the chain length increased, the molecular surface buried within the cyclodextrin cavity became wider, thus enhancing van der Waals interaction and thus affinity, without significant reduction of the alkyl chain flexibility. For the longest side chain (i.e. for BP15), a loss of inclusion entropy appeared and was stronger than the rise of enthalpy. Such behavior may be attributed to a more constrained conformation of the alkyl chain. It should be also kept in mind that above a certain length, free space is expected to be no longer available inside the cavity.

3.4. UV-Vis analysis

Formation constants were also evaluated by means of a spectral displacement method. Introducing a known quantity of BP within a cyclodextrin-methylorange solution indeed lead to a lower concentration of cyclodextrin-methyl orange complex, and thus to a modification of the electronic absorption of this solution. As a consequence, affinity between BPs and cyclodextrins was evaluated from the absorbance difference in the absence and the presence of BP, assuming a 1:1 stoichiometry in all cases. Corresponding formation constants are presented in Table 3. These values are in strong agreement with ITC results, which confirms that only 1:1 complexes were formed and that such spectral displacement method is well suited to the characterization of cyclodextrins/BP complexes. As this method is easy to handle in the case of numerous complexes, these competition experiments were applied to all studied inclusion compounds.

In particular, we evaluated the influence of methyl introduction on the bisphosphonate part, by considering the inclusion of BP15 to BP15M21 (Table 3), for which 0, 1, 2 and 3 methyl were introduced respectively. Similar formation constants were observed, demonstrating again that the bisphosphonate part does not seem to be involved in the inclusion phenomenon.

In addition, we investigated the inclusion behavior of modified cyclodextrins, by characterizing the interaction of RAMEB and HP-β-CD with BP10 to BP14, in comparison to the native β-CD. Corresponding stability are presented in Table 3 and expressed in terms of free energy of inclusion as a function of the number of carbons of the bisphosphonate side chain, in Fig. 7. Linear trends between free energy and chain length were observed for those three β-CDs. Slopes of the linear regressions enable the evaluation of added free energy per methylene (-0.3 to -0.4 kcal/mol). Additionally, the intercept is representative of the inclusion free energy in the absence of alkyl chain. They are ranging from 0 to 2.2 kcal/mol, which corresponds to non significant formation constants (inferior to $40M^{-1}$), confirming the quasi-absence of interactions with the bisphosphonate part, stability being essentially provided by inclusion of the alkyl side chain. If complexation behavior, in terms of order of magnitude, was similar for the three studied β-CDs, one can note that formation constants were slightly weaker for RAMEB and HP-β-CD if compared to the native β-CD. This slight decrease in affinity might be ascribed to steric hindrance induced by the chains introduced on modified β-CDs.

Finally, the influence of the cavity size on the inclusion ability was taken into account, by characterizing the stability of complexes formed between α-CD and BP11 to BP14. As can be concluded from the results listed in table 3, it seems that the inclusion ability of α-CD and β-CD are analogous (no significant differences between formation constants, when calculating the 95% confidence interval by multiplying standard deviations by a Student t value equal to 3.18).

4. Conclusion

The complex formation between bisphosphonates and various cyclodextrins was demonstrated by complementary methods such as 1D and 2D NMR spectroscopy, UV-visible and ITC analysis. Results indicated the formation of 1:1 complex of cyclodextrin/ bisphosphonates and the contribution of bisphosphonate side chain to the inclusion phenomenon. While bisphosphonates bind to various cyclodextrins with a weak influence of the cavity size and of the studied cyclodextrin derivatives, the bisphosphonate side chain

length was the main parameter controlling the complex stability. The high values of formation constant showed that cyclodextrin could improve the oral bioavailability of bisphosphonates and could be easily used for novel promising formulation.

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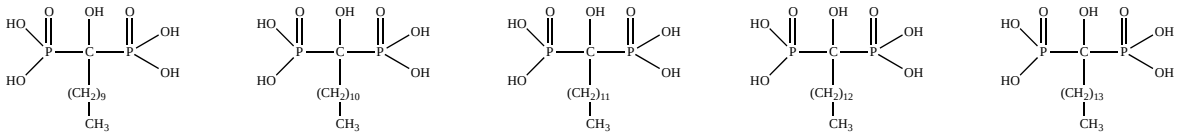
BP10 BP11 BP12 BP13 BP14



BP15 BP15M01 BP15M11 BP15M21

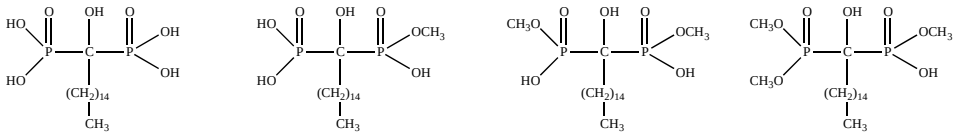


Fig.1. Chemical structure of studied bisphosphonates.

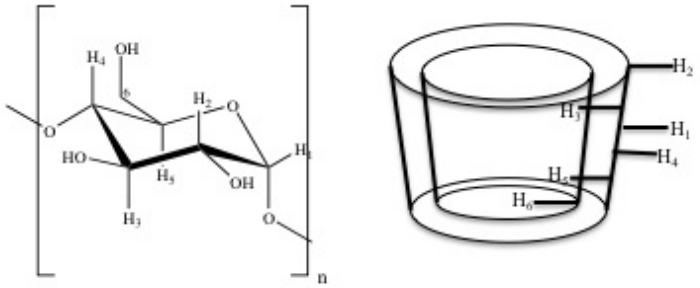


Fig. 2. Structures of the glucopyranose repeating unit and the truncated structure of β-cyclodextrin emphasizing the position of all hydrogens.

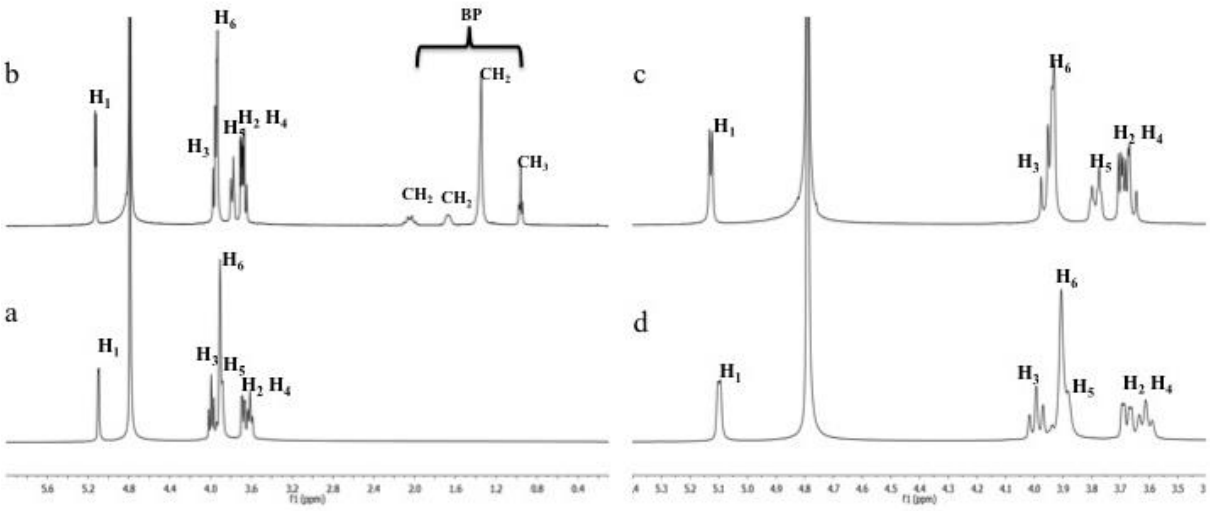


Fig. 3. ^{1}H NMR spectra of the a) β-CD alone, b) β-CD/ BP10 complex in D_2O, c) spectral zooming of (b) and d) spectral zooming of (a) between 3.4 and 5.4 ppm.

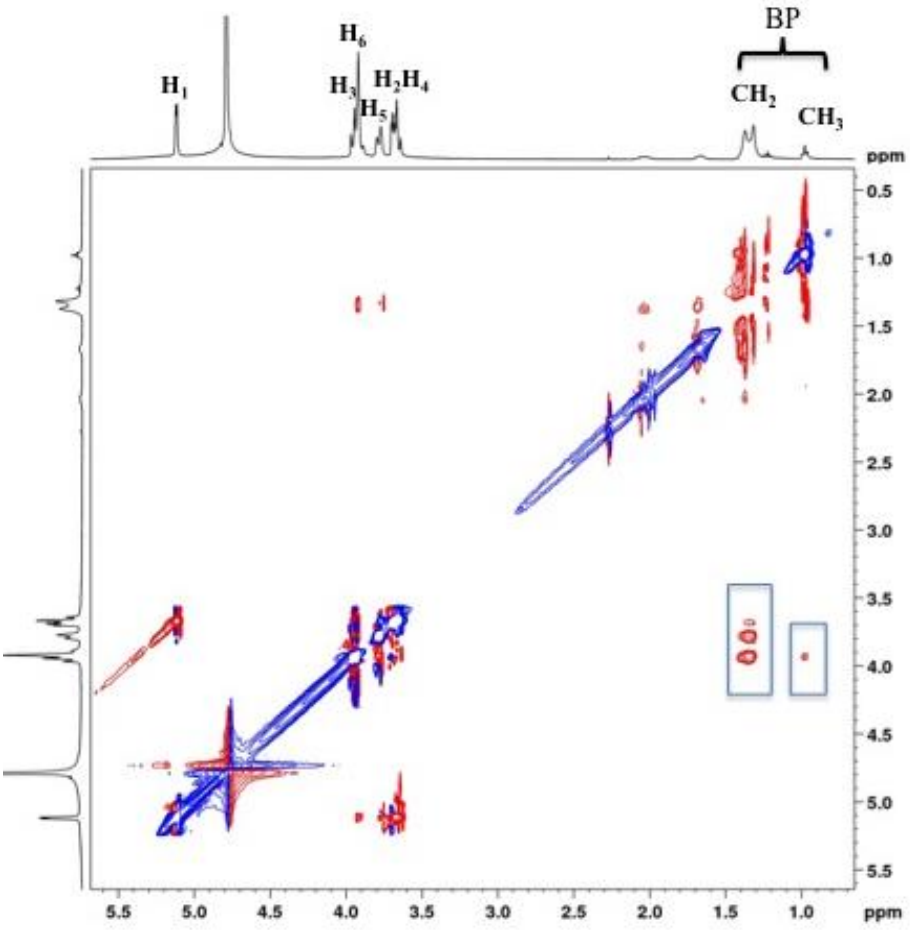


Fig. 4. ROESY spectrum of bisphosphonate β-cyclodextrin / BP10 complex in D_2O.

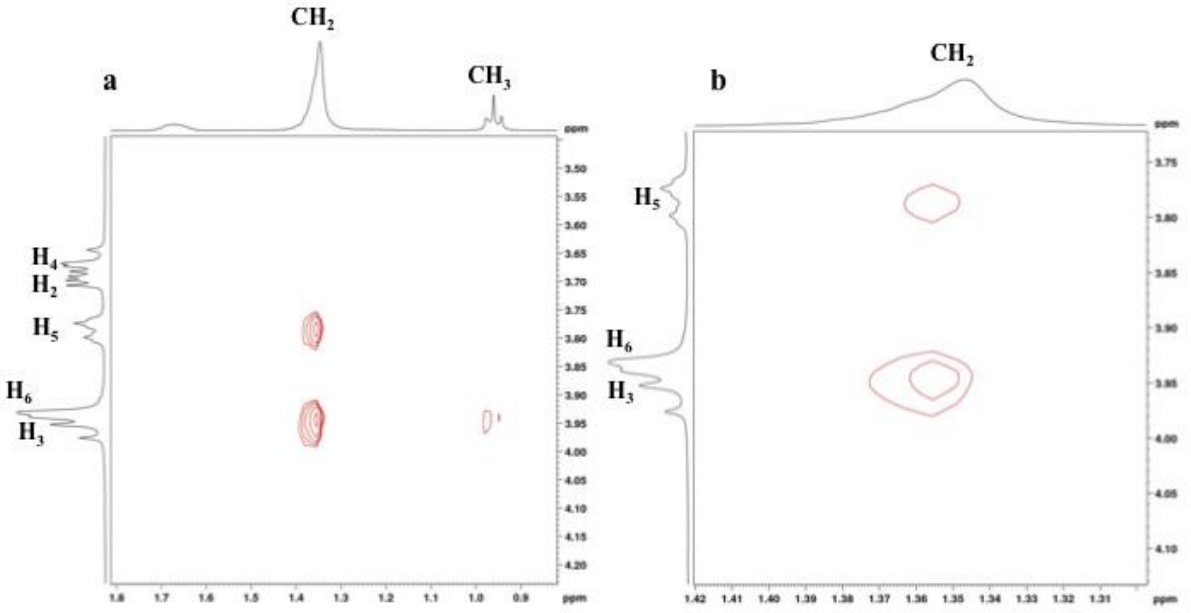


Fig. 5. (a) Expansion of ROESY spectrum of β-CD/ BP10 complex (1:1) in D_20 and (b) spectral resolution of ROESY spectrum.

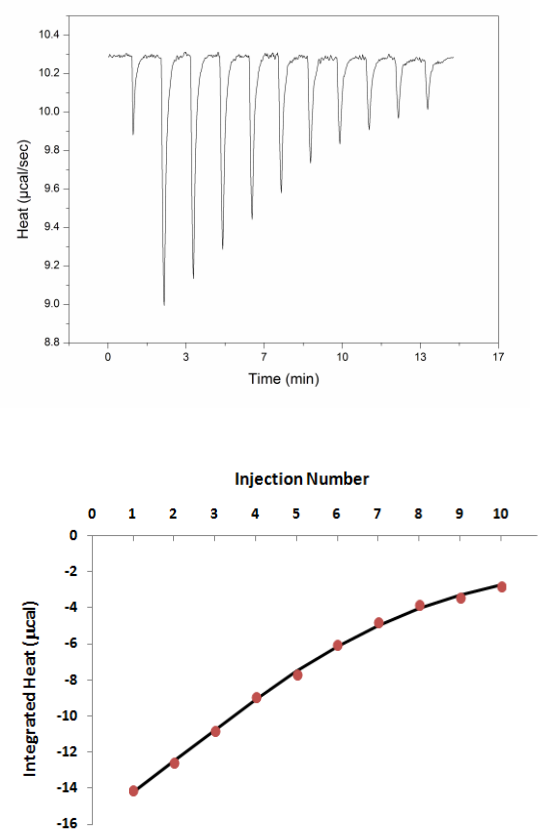


Fig. 6. ITC titration of BP10 by β-CD at 25°C. Upper and lower panels display raw thermogram and binding isotherm (dots: experimental values; solid line: best least square fit), respectively.

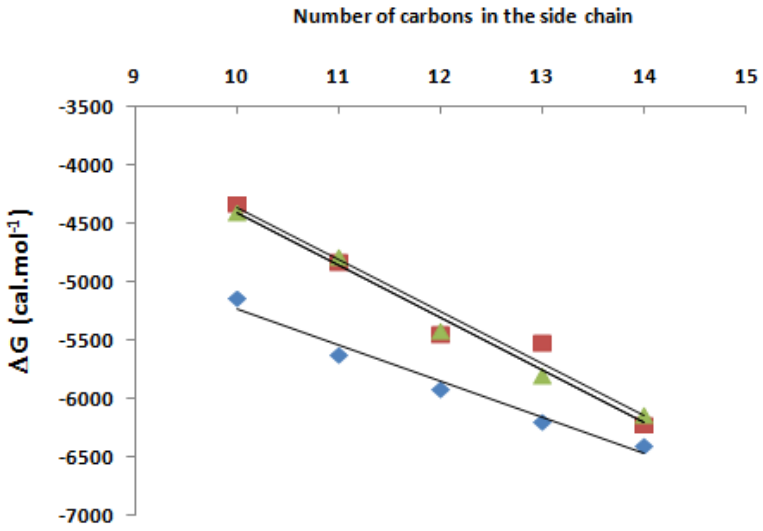
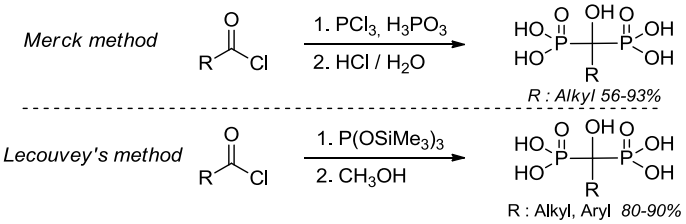
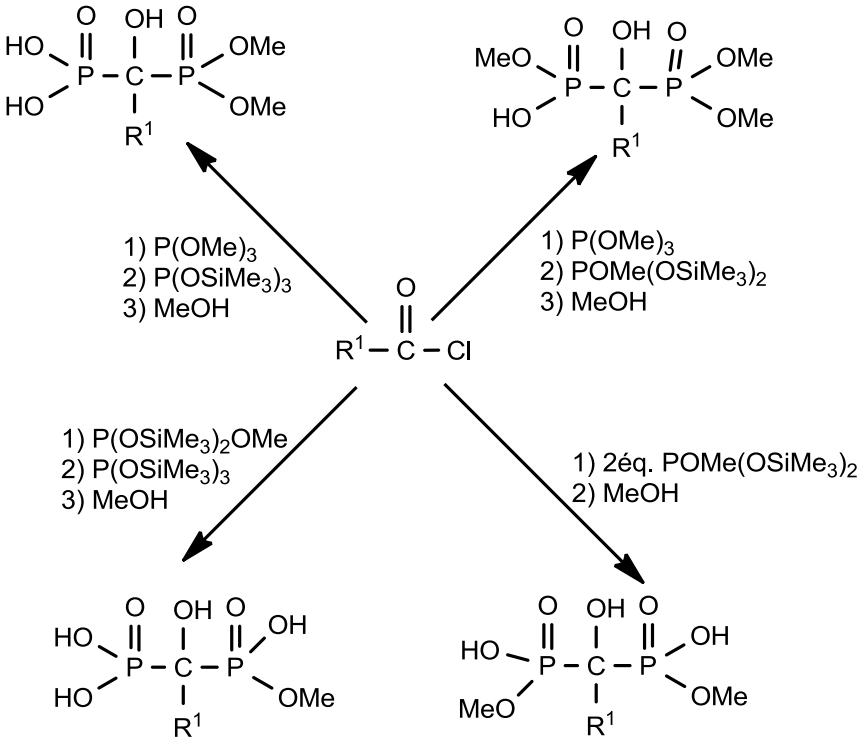


Fig.7. Free energy of inclusion (as derived from K in Table 3) as a function of alkyl length, for the complex formed between BP10 to BP14 with β-CD (blue diamond), RAMEB (red square) and HP-β-CD (green triangle).



Scheme1 Direct methods for the 1-hydroxyalkylidenebisphosphonic acid synthesis.



Scheme 2 General methods for the partial ester bisphosphonates synthesis.

Table 1: ^{1}H chemical shifts (ppm) for β-CD in the free and complex state with BP.

	β-CD	+BP10	+BP11	+BP12	+BP13	+BP14	+BP15	+BP15 M01	+BP15 M11	+BP15 M21
H1	5.10	5.12	5.10	5.11	5.11	5.11	5.09	5.09	5.09	5.13
H3	3.99	3.95	3.92	3.94	3.95	3.94	3.93	3.92	3.91	3.94
H6	3.91	3.93	3.90	3.91	3.91	3.91	3.90	3.90	3.89	3.93
H5	3.88	3.78	3.75	3.73	3.76	3.78	3.78	3.74	3.75	3.78
H2	3.68	3.69	3.66	3.68	3.67	3.69	3.67	3.66	3.66	3.69
H4	3.61	3.66	3.64	3.64	3.63	3.64	3.62	3.62	3.62	3.66

Table 2. Thermodynamic data of β-CD/BP inclusion complexes obtained by ITC at 25 °C.

	K (M^{-1})	**ΔG** ($cal.mol^{-1}$)	**ΔH** ($cal.mol^{-1}$)	**-TΔS** ($cal.mol^{-1}$)
BP10	4051 ± 468	-4901 ± 69	-1263 ± 49	-3638 ± 118
BP11	15891 ± 1184	-5708 ± 44	-1525 ± 22	-4183 ± 66
BP12	28917 ± 2138	-6061 ± 44	-1854 ± 25	-4207 ± 69
BP13	44355 ± 3573	-6313 ± 48	-1886 ± 24	-4427 ± 71
BP14	59923 ± 4749	-6491 ± 47	-2095 ± 22	-4396 ± 69
BP15	37862 ± 2377	-6220 ± 37	-2323 ± 24	-3897 ± 61

Table 3. Formation constants (K in M^{-1}) values for CD/BP inclusion complexes obtained by UV-Visible competition experiments at 25 °C. N.D.: not determined.

	β-CD	**RAMEB**	**HP-β-CD**	**α-CD**
BP10	6027 ± 478	1516 ± 151	1748 ± 174	N.D.
BP11	13600 ± 1007	3533 ± 274	3325 ± 258	8221 ± 964
BP12	22500 ± 1974	9869 ± 674	9627 ± 658	18048 ± 1884
BP13	35558 ± 3356	11300 ± 847	18168 ± 1361	24755 ± 2568
BP14	50014 ± 5219	37004 ± 3740	32738 ± 3309	55499 ± 5917
BP15	24145 ± 2183	N.D.	N.D.	N.D.
BP15M01	21876 ± 1799	N.D.	N.D.	N.D.
BP15M11	25903 ± 2289	N.D.	N.D.	N.D.
BP15M21	26618 ± 2415	N.D.	N.D.	N.D.