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Danièle Pro, Samuel Huguet, Mustapha Arkoun, Caroline Nugier-Chauvin, José Maria Garcia-Mina, et al.. From algal polysaccharides to cyclodextrins to stabilize a urease inhibitor.. Carbohydrate Polymers, 2014, 112, pp.145-51. 10.1016/j.carbpol.2014.05.075 . hal-01063896v1

HAL Id: hal-01063896

<https://hal.science/hal-01063896v1>

Submitted on 7 Oct 2014 (v1), last revised 14 Oct 2014 (v2)

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1 **Highlights**

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- A new formulation of NBPT, a known urease inhibitor, with cyclodextrin was prepared
- The complex CD/NBPT was fully characterized
- The stability of NBPT and that of CD/NBPT in acidic media were studied by NMR
- IC_{50} of the CD/NBPT complex was determined

Accepted Manuscript

9 **From algal polysaccharides to cyclodextrins to stabilize a urease inhibitor**

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32 **From algal polysaccharides to cyclodextrins to stabilize a urease inhibitor**

33

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48 **Abstract:** *N*-Butyl-phosphorotriamide (NBPT) is a fertilizer widely used for its urease

49 inhibiting properties. Nevertheless, formulations currently commercialized are complex and

50 do not avoid severe decrease of activity due to the low stability of the bioactive compound

51 under acidic conditions. According to its structure, NPBT was thought to be able to interact

52 with both polar additives, by its phosphoramidate function, and hydrophobic ones, through its

53 alkyl chain. In this context, and in order to simplify formulations of this bioactive compound,

54 a panel of natural polysaccharides was studied, including starch, β -(1,3)-glucans,

55 carraghenans and alginates. We also used cyclodextrins, characterized the most stable

56 inclusion complex with α -cyclodextrin and evaluated the stability of NBPT thus protected
57 against hydrolysis under acidic conditions.

58

59 **Keywords:** NBPT; Carraghenans; Alginates; (1,3)-Glucans; Starch; Cyclodextrins

60

61 **1. Introduction**

62 The small molecule of urea is the source of two major scientific discoveries. Not only it is the
63 first molecule chemically synthesized by Wöhler in 1828, but the study of its biodegradation
64 allowed the first crystallization of an enzyme, the Jack Bean urease, in 1926 by Sumner
65 (Sumner, 1926), who was subsequently awarded by the Nobel Prize for this breakthrough in
66 1946. Meantime, urea has become an important component of many fertilizers. However, as
67 only 30% of the available nitrogen is absorbed by plants, improving the efficiency of the
68 nitrogen use in agriculture generally requires adding inhibitors acting on nitrification and/or
69 ureases. The application of the latter significantly reduces ammonia volatilization, as well as
70 damages to seedlings. Plants are also able to assimilate nitrogen as urea, and urease inhibitors
71 promote this type of nutrition, which is also beneficial for plant growth (Trenkel, 2010).
72 Finally, the use of such compounds able to increase the lifetime of urea in soils is not
73 exclusive to agriculture, but find applications in dairy farming where they can significantly
74 decrease nitrogen emissions due to urine and manure (Leinker, Reinhardt-Hanisch, Hartung &
75 von Borell, 2005; Zaman, Nguyen, Blennerhassett & Quin, 2008).

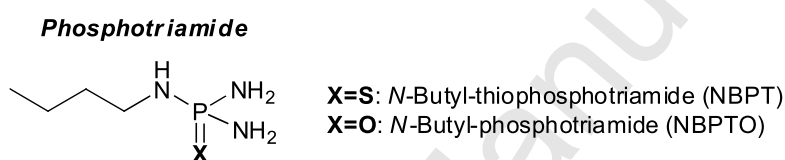
76 Numerous studies have led to the marketing of a large panel of molecules having anti-ureasic
77 activities [for instance hydroquinone, phosphotriamide compounds (Domínguez et al., 2008;
78 Font et al., 2008; Trenkel, 2010)], or able to influence nitrification phenomena [ammonium
79 thiosulfate, thiourea, or more complex products, i.e. pyrimidinic, triazolic, thiadiazolic
80 derivatives, dicyandiamide (DCD) (Nelson & Huber, 1992; Trenkel, 2010)], both contributing

81 to improve nitrogen absorption by plants. In addition to the range of such bioactive molecules,
82 many formulations are proposed. Inhibitors may be available as pure compounds or directly
83 incorporated into fertilizers. They can also be formulated in liquid or solid phases. This allows
84 matching a wide range of treatments to soil characteristics and infrastructure of the users. For
85 instance, commercial forms of DCD are an excellent example of adaptability, since it is
86 possible to obtain this single molecule or in combination with other inhibitors of nitrification
87 possibly mixed with urea, or even incorporated into fertilizers containing ammonium salts and
88 many other nutrients. Similarly, *N*-butyl-thiophosphotriamide (NBPT) can be purchased in a
89 liquid formulation (Agrotain), but also in a solid one containing DCD, or in solid granules
90 with urea and DCD. Consequently, commercially available inhibitors stabilize nitrogen for
91 several days to several weeks.

92

93 The development of new compounds and/or formulations is part of an integrated approach to
94 farming, keeping in mind that control of the global impact on environment is a priority.
95 However, decades of use of urease inhibitors, and of basic researches, highlighted the
96 complexity of using them efficiently. Among influencing parameters are the quality of soils
97 (pH, nature of the organic matter and possibility to find colloids, porosity, biodiversity linked
98 with numerous microorganisms), abiotic factors (temperature, water quality), and own
99 properties of the inhibitors (volatility, hydrophilic/hydrophobic balance, stability and mobility
100 in soils) (Subbarao et al., 2006). In this context, we have initiated a program and first
101 proposed to covalently connect known nitrification inhibitors to mono- and disaccharides,
102 especially in order to increase their ecocompatibility (Pro et al., 2012). Herein, we propose
103 another approach lying on the opportunity to formulate the known urease inhibitor NBPT
104 (Fig. 1) with natural polysaccharides and derivatives thereof. It is recognized that NBPT has
105 already inhibition properties ($IC_{50} = 100$ nM), but this thiophosphoramidate is readily converted

106 into its *O*-counterpart NBPTO that is the real inhibitor in soils ($IC_{50} = 1 \text{ nM}$). However, two
 107 major limitations are generally encountered with this inhibitor patented in 1985 (Kole,
 108 Swerdloff, Rogic, Hendrickson & Van Der Puy, 1985): the first one is its hydrophobicity that
 109 requires more or less complex formulations, and the second but more limiting one relies on its
 110 low stability, and so loss of activity, in acidic media (Domínguez et al., 2008). The ability of
 111 carbohydrates to adopt many conformations, and/or to interact with their aqueous
 112 environment (basic vs. acidic, presence of salts) (Cui, 2005), offers great opportunities to
 113 develop new formulations.



114

115 **Fig.1.** Structure of the studied urease inhibitor NBPT.

116

117 2. Material and methods

118 2.1. Materials

119 Polysaccharides used in this study are commercially available. NBPT was provided by Timac
 120 Agro International (Dinard) and was further purified by recrystallisation from water. ^1H , ^{13}C ,
 121 ^{31}P NMR spectra were recorded with a Brüker ARX 400 spectrometer at 400 MHz, 100 MHz,
 122 and 162 MHz for ^1H , ^{13}C and ^{31}P , respectively. Chemical shifts are given in δ units (ppm).
 123 Coupling constants J were calculated in Hertz (Hz). Abbreviations were used to precise signal
 124 multiplicity: s (singlet), d (doublet), t (triplet), m (multiplet), dd (double doublet)... The
 125 HRMS were measured at the Centre Regional de Mesures Physiques de l'Ouest (CRMPO,
 126 Université de Rennes 1) with a MS/MS ZabSpec TOF Micromass using *m*-nitrobenzyllic
 127 alcohol as a matrix and accelerated caesium ions for ionization.

128

129 2.2. Degradation tests in acidic media by ^{31}P NMR

130 The necessary amount of formulation for having 10 mg of NBPT was mixed with 250 μL
 131 water in NMR tubes (blank solution consisted in 40 mg Agrotain in 250 μL of water). The
 132 solution was heated until complete dissolution of NBPT. 250 μL of acidic buffer solution
 133 ($\text{CH}_3\text{COOH}/\text{CH}_3\text{COONa}$, 500 mM, pH 5.5) was added. Then, a fused capillary containing a
 134 invariant H_3PO_4 solution in D_2O was inserted, and the degradation of NBPT was monitored
 135 by ^{31}P NMR. The degradation of NBPT was deduced from the decrease of the signal at 60.5
 136 ppm (corresponding to the NBPT) compared to the constant -0.3 ppm peak's area
 137 (corresponding to the H_3PO_4).

138

139 2.3. Formulations with cyclodextrin $\text{CD}\alpha$ and determination of the inclusion complex thanks
140 to the Job's method

141 A solution of α -cyclodextrin ($\text{CD}\alpha$) in water (10 mM) and a solution of NBPT in methanol
 142 (10 mM) were prepared. Different ratio of both solutions were mixed together in test tubes
 143 (from 0:10 to 10:0), for a total volume of 1 mL, before freeze-drying. The resulting solid was
 144 dissolved into 1 mL of D_2O , and analyzed by ^1H NMR. For each ratio of NBPT (r , Eq. 1) and
 145 for each proton (i) of NBPT, the variation of chemical shift ($\Delta\delta_r^i$) was calculated as (Eq. 1):

146
$$r = [\text{NBPT}]/([\text{NBPT}] + [\text{CD}\alpha]) \text{ (Eq. 1)}$$

147
$$\Delta\delta_r^i = \delta_r^i - \delta_1^i \text{ (Eq. 2)}$$

148 For each ratio of $\text{CD}\alpha$ ($1-r$) and for each proton (j) of $\text{CD}\alpha$, the variation of chemical shift
 149 $\Delta\delta_{1-r}^j$ was calculated as (Eq. 3):

150
$$\Delta\delta_{1-r}^j = \delta_{1-r}^j - \delta_0^j \text{ (Eq. 3)}$$

151 Then $r \cdot \Delta\delta_r^i$ and $(1-r) \cdot \Delta\delta_{1-r}^j$ were plotted against r . Resulting curves went through a maximum,
 152 at the r value corresponding to the ratio of the inclusion complex.

153

154 2.4. Determination of the association constant

155 The association constant was obtained according to the method of Benesi-Hildebrand (Benesi
156 & Hildebrand, 1949). A solution of NBPT in methanol (5 mM) and a solution of CD- α in
157 water (125 mM) were prepared. Different amounts of these two solutions were mixed with
158 water for a total volume of 500 μ L, a constant concentration of NBPT (1 mM), and a varying
159 concentration of CD- α (from 50 to 100 mM).

160 Then, mixtures were freeze-dried and the resulting solid dissolved into 500 μ L of D₂O, before
161 ¹H NMR analysis. For each concentration of CD- α ([CD- α]), and for each proton (*i*) of
162 NBPT, the variation of chemical shift $\Delta\delta_{[\text{CD-}\alpha]}^i$ was calculated thanks to equation 4:

$$163 \quad \Delta\delta_{[\text{CD-}\alpha]}^i = \delta_{[\text{CD-}\alpha]}^i - \delta_0^i \text{ (Eq. 4)}$$

164 Then, $1/\Delta\delta_{[\text{CD-}\alpha]}^i$ was plotted against $1/[\text{CD-}\alpha]$. Resulting curves obey to a linear equation.

165 The association constant of the complex (K_a) and the variations of chemical shifts of NBPT in
166 the pure complex ($\Delta\delta_{\text{Cpx}}^i$) were deduced from equations of the intercept and the slope of the
167 curves:

$$168 \quad \text{intercept} = 1/\Delta\delta_{\text{Cpx}}^i \text{ (Eq. 5) and slope} = 1/K_a \cdot \Delta\delta_{\text{Cpx}}^i \text{ (Eq. 6)}$$

169 This method was applied on the four signals corresponding to the butyl chain of the NBPT.

170 The four estimations of the association constant were averaged.

171

172

173 2.5. Evaluation of the inhibitory activity

174 One milliliter of buffer solution (KH₂PO₄/K₂HPO₄, 70 mM, pH 7), 1 mL of inhibitor solution
175 (at different concentrations), and 10 μ L of jack bean urease solution (2.8 mg/mL, Sigma U-
176 1500 35.500) were mixed in test tubes and incubated for 30 min at 37 °C (blank solution
177 consisted of the same mixture without inhibitor). Afterward, 1 mL of urea solution (6 mg/mL)
178 was added, with incubation again for 30 min at 37 °C. The reaction was stopped by the

179 addition of 2 mL of 0.033 N HCl. Each inhibitor dose was tested in triplicate. The level of
180 ammonium released was determined photometrically, using the ammonium Test from Merck
181 (Ref 1.14752.0001). The inhibition percentage $Inh(\%)$ was calculated as:

$$182 \quad Inh(\%) = 100 - 100(A_{Inh}/A_B) \text{ (Eq. 7)}$$

183 where A_{Inh} and A_B are the absorbance at 692 nm in the test tubes, with and without inhibitor,
184 respectively.

185

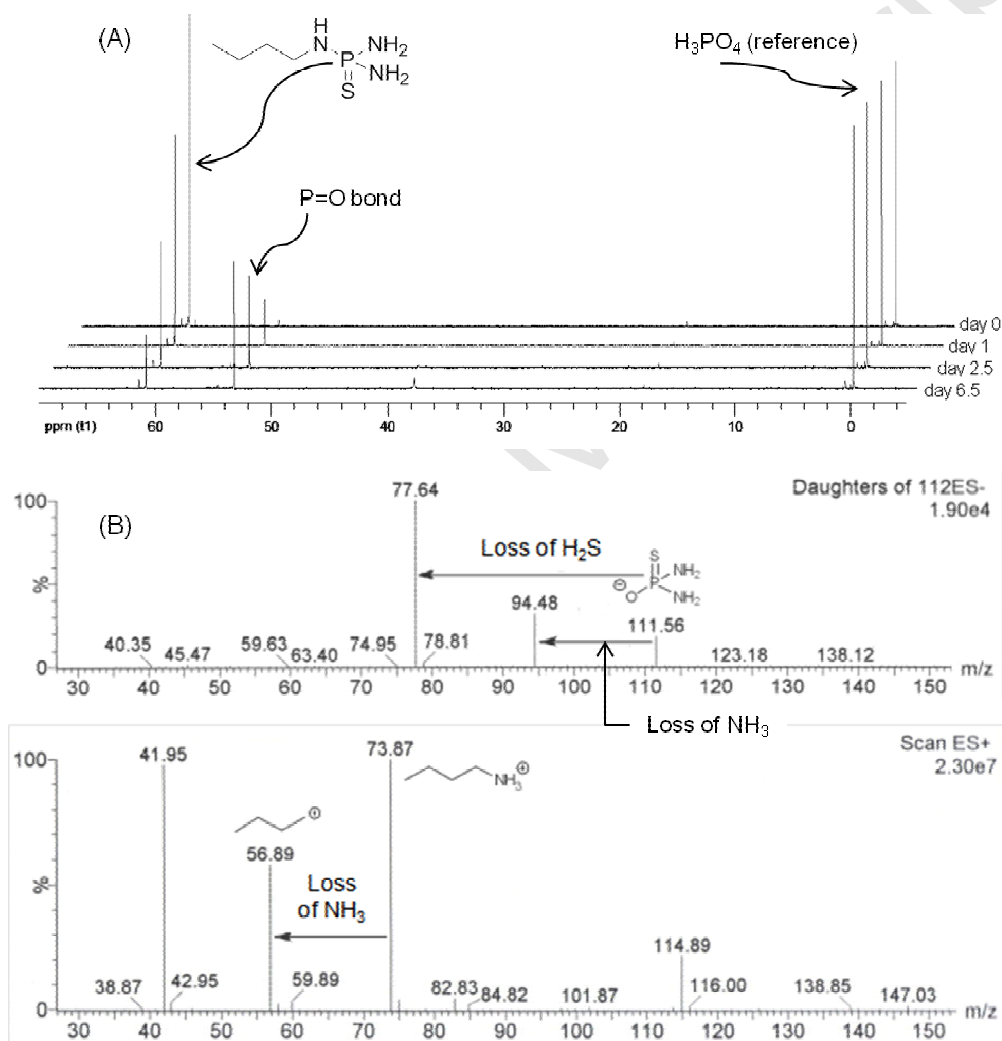
186 **3. Results and discussion**

187 Before managing formulations with polysaccharides, a simple method for monitoring
188 degradation of NBPT, solubilized in a solvent, under acidic conditions was developed. While
189 some analysis were already proposed (Abraham, Benson & Jardine, 1983; Douglass &
190 Hendrickson, 1991), NMR data, and more especially ^{31}P NMR in the case of phosphorous-
191 containing compounds, offers the opportunity to also give structural data of resulting products
192 (Fig. 2). In order to mimic acidic soils, this study was therefore performed in an acetate buffer
193 at pH 5.5 over one week. It was firstly observed that the intensity of the initial signal at nearly
194 60 ppm disappeared in favor of another one at 53 ppm. Secondly, no more NBPT could be
195 detected after one week in this medium. Moreover, the high field chemical shift
196 corresponding to the newly formed compound indicated a break of at least one P-N linkage,
197 substituted by a P-O bond. This result was subsequently complemented and corroborated by a
198 high resolution mass spectrometry (HRMS) analysis (Fig. 2). Indeed, two peaks at m/z 111.56
199 and 73.87 appeared, thus demonstrating the apparition of diamoniphosphoric acid (DATP)
200 and *n*-butylamine, respectively. This showed the favored cleavage between the P atom and the
201 N one connected to the butyl chain, both P-NH₂ linkages remaining stable. Moreover, under
202 the conditions used, no NBPTO was observed. That means that the P=S bond is stable under
203 conditions used and that S/O exchange occurs only in soils, probably thanks to bacterial

204 activity. Finally, the monitoring of the degradation of NBPT over one week allowed us to
 205 show that it followed a first order kinetics at pH 5.5 ($k = 2.2 \times 10^{-6} \pm 0.8 \times 10^{-6} \text{ s}^{-1}$, $n = 15$; See
 206 Supplementary materials).

207

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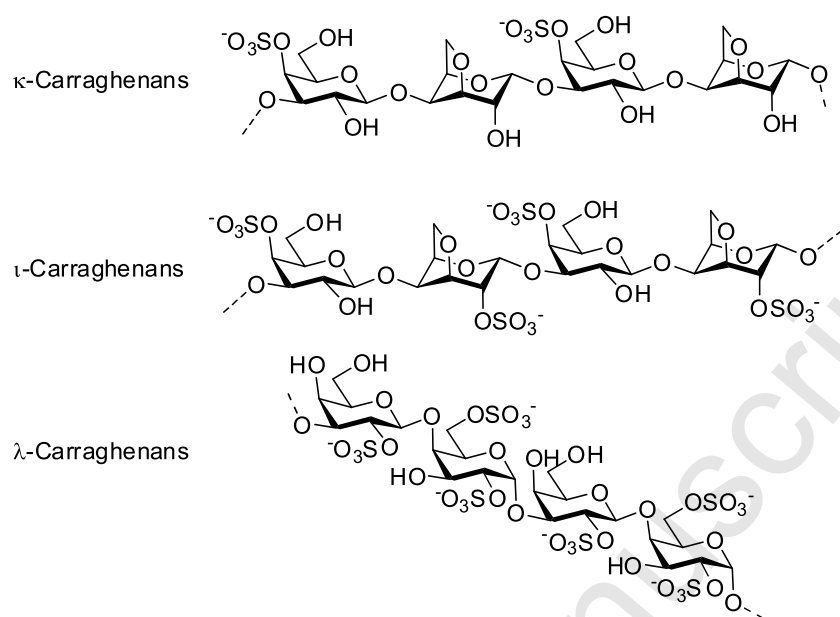
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210 **Fig. 2.** (A) ^{31}P NMR monitoring and (B) HRMS analysis of the degradation of NBPT at pH

211 5.5.

212

213 With this analytical method in hand, it was further planned to build the inhibitor around an
214 environment conducive to its preservation in acidic media. However, NBPT must remain free
215 in order to emerge this background, and be gradually released to exert the desired biological
216 function on urease. On the basis of conformational and physico-chemical adaptability of
217 polysaccharides, we first studied carraghenans (Fig. 3), well known carbohydrates extracted
218 from red seaweeds *Rhodophyceae*. κ - and ι -Carraghenans are characterized by sequence of D-
219 galactopyranosyl (D-Galp) residues having alternatively 4C_1 and 1C_4 conformations, the latter
220 resulting from a 3,6-anhydro-bridge, and they can adopt double strand conformations. λ -
221 Carraghenans are also built of sulfated D-Galp entities, but differ from the latter families by
222 the absence of anhydro-bridge and by the number of anionic groups, thus resulting in tertiary
223 ribbon structure (Cui, 2005). Despite their charges, carraghenans are able to interact with
224 small volatile compounds (Hambleton, Fabra, Debeaufort, Dury-Brun & Voilley, 2009). We
225 thus hypothesized that it could possibly interact either with the butyl chain of NBPT, even
226 weakly, or also with the more polar phosphoramidate part of the inhibitor, or both functions. On
227 this basis, the studied inhibitor was dissolved in saturated solution of κ -, ι - or λ -carraghenans
228 buffered at pH 5.5. Its degradation was monitored by ${}^{31}P$ NMR and compared to that of NBPT
229 formulated as Agrotain. Unfortunately, no protecting effect was observed and the
230 disappearance of NBPT in carbohydrate solutions over a period of 6 days followed a kinetic
231 comparable with that of commercially available formulation (See Supplementary Materials).



232

233 **Fig. 3.** Primary structures of κ -, ι -, and λ -carraghenans.

234

235 In a second stage, alginates were also tried to potentially carry the target thiophosphoramidate.

236 As carraghenans, they present anionic groups, i.e. carboxylates, but not sulfates. We thus

237 anticipated that carboxylate groups in alginates could quench some protons from the media,

238 thus preventing NBPT hydrolysis. Moreover, L-guluronic entities, but not D-mannuronic

239 residues, may form cavities which can accommodate divalent cations such as calcium (Fig. 4).

240 Thus, in the presence of calcium salts, the polyguluronic chains gather around cations,

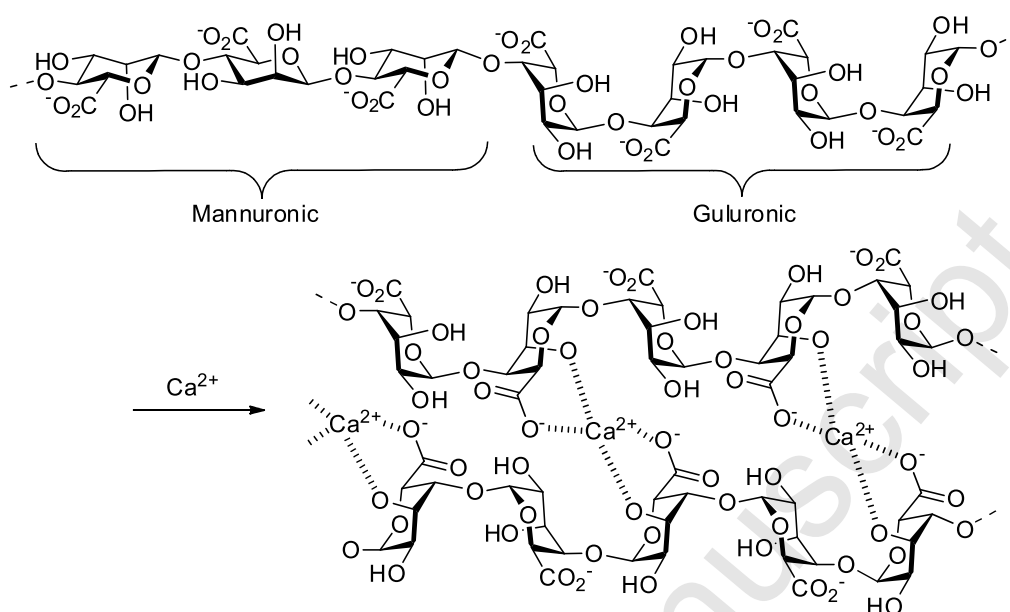
241 creating a network of channels with some facial amphiphilicity. We have however observed

242 no significant improvement of the formulations, using alginates alone or these

243 polysaccharides in the presence of calcium salts to provide beads. The latter cannot retain

244 efficiently the NBPT molecules since they diffuse into the aqueous solution within only a few

245 minutes.

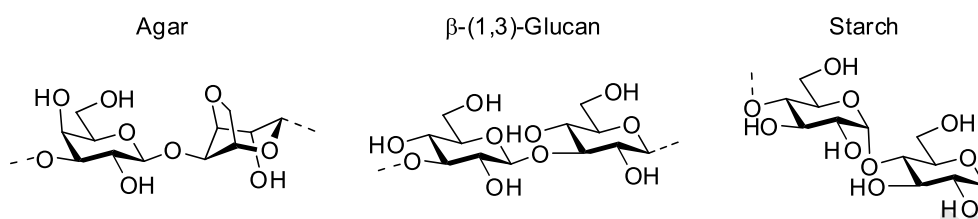


246

247 **Fig. 4.** A conformational model for alginates.

248

249 These results led us to turn to neutral polysaccharides. Agar present tertiary conformations
 250 close to that of κ - and ι -carraghenans; laminarines, also called β -(1,3)-glucans, can organize
 251 in triple helix, and was already used to guide the synthesis of hydrophobic nanomaterials
 252 (Lehtovaara & Gu, 2011; Young, Dong & Jacobs, 2000); starch was also selected since it is
 253 well established that it can encapsulate iodine, lipids, surfactants by formation of inclusion
 254 complexes (Heinemann, Escher & Conde-Petit, 2003) (Fig. 5). Once again, no stabilization
 255 was obtained using both agar and laminarines. Nevertheless, a slight improvement was
 256 observed using starch previously unstructured in a boiling aqueous solution. Indeed, 60% of
 257 NBPT remained unchanged after nine days while Agrotain was decomposed at 60% over the
 258 same period. We hypothesize that this positive effect can be assigned to the presence of
 259 helices that prefigure maltocyclodextrins.

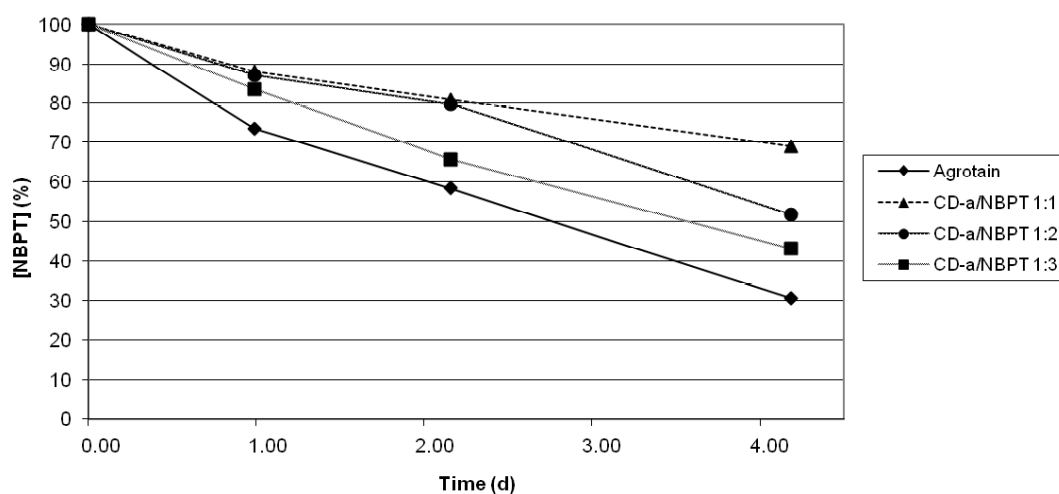


260

261 **Fig. 5.** Main skeleton for agar, β -(1,3)-glucans and starch.

262

263 Therefore, this result encouraged us to use pure cyclodextrins instead of polysaccharides,
 264 since they are commonly used in the pharmaceutical industry but also in the food and plant
 265 health fields (Shen, Yang, Wang, Zhou & Chen, 2012). We expected that the lipophilic cavity
 266 of a cyclodextrin interact with the NBPT butyl chain to form a complex able to protect the
 267 inhibitor from surrounding acidic medium. It was firstly easily established that the more
 268 suitable cyclodextrin likely to favorably interact with NBPT was the smaller one containing
 269 six glucosyl residues (α -cyclodextrin, CD- α) (Fig. given in Supplementary Material).
 270 Secondly, the optimal molar ratio CD- α /NBPT was experimentally determined as 1:1, and
 271 Fig. 6 shows that at least 70% of NBPT remained stable after four days at pH 5.5, while 60%
 272 of NBPT from Agrotain formulation was hydrolyzed under the same conditions. This
 273 corresponds to an increase of 75% stability

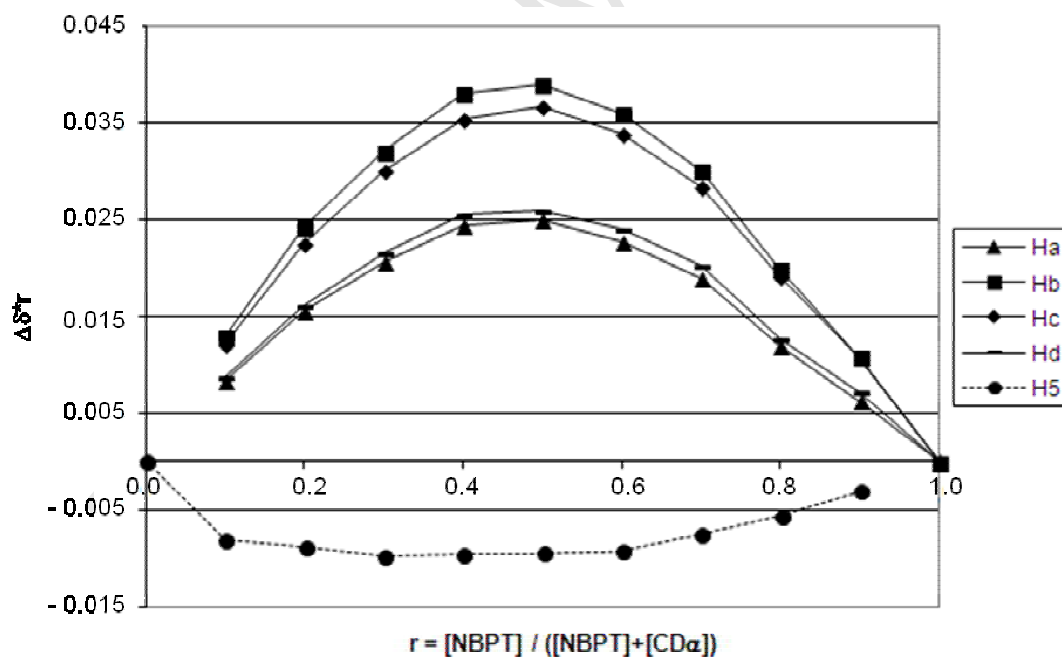


274

275 **Fig. 6.** Stabilization of NBPT according to the molar ratio CD- α /NBPT.

276

277 Finally, the precise nature of the suspected complex was determined by ^1H NMR thanks to the
 278 Job's plot method (Marçon, Mathiron, Pilard, Lemaire-Hurtel, Dubaele & Djedaini-Pilard,
 279 2009). This approach is based on the correlation between the complex concentration in
 280 solution and the chemical shift variations of protons in both the host (CD- α) and the guest
 281 (NBPT; see Supplementary Material). More particularly, the complex concentration is
 282 proportional to the $\Delta\delta_X \cdot r_X$ where $\Delta\delta_X$ corresponds to the chemical shift variation for proton X
 283 and r_X is the ratio $[\text{NBPT}]/([\text{NBPT}]+[\text{CD-}\alpha])$. Because of low variations observed from
 284 protons of CD- α , the Job graphic was drawn from signals corresponding to the butyl chain
 285 (Fig. 7). A 1:1 complex was thus unambiguously established.



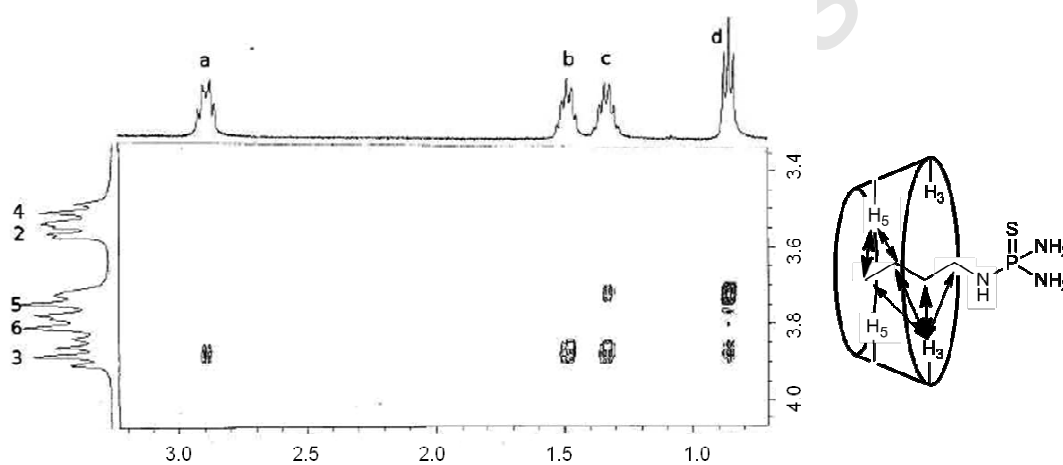
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287 **Fig. 7.** Determination of the CD α /NBPT according to the Job's plot method (Marçon,
 288 Mathiron, Pilard, Lemaire-Hurtel, Dubaele & Djedaini-Pilard, 2009). Ha-Hd are for the alkyl
 289 chain of NBPT and H5 for the CD α .

290

291 Moreover, this study was complemented by a T-ROESY NMR analysis (Fig. 8) in order to
 292 identify precise molecular interactions between the inhibitor and its ligand. The resulting
 293 spectrum clearly established that H-5 of the maltocyclodextrin are close to the ethyl terminal
 294 group of the alkyl chain of NBPT, and that H-3 of the former, exposed from the bigger crown,
 295 could interact with all protons of the butyl chain, but more particularly with the core CH₂.

296



297

298 **Fig. 8.** Focus on NMR T-ROESY spectrum of the inclusion complex CD α /NBPT.

299

300 Finally, in order to characterize the dynamic of the equilibrium, the association constant K_a
 301 was determined according to the Benesi-Hildebrand method, applied on ¹H NMR signals
 302 corresponding to the butyl chain of the NBPT (Benesi & Hildebrand, 1949). Using a large
 303 excess of CD- α and a constant concentration in NBPT, all resulting straights corresponding to
 304 the protons of NBPT gave a value of $94 \pm 4 \text{ M}^{-1}$, slightly in favor of the formation of the
 305 complex.

306

307 Having these physicochemical data in hands, the inhibitory activity of the established more
308 stable complex was evaluated *in vitro* at pH 7, under optimal conditions for the activity of
309 Jack bean urease. A value of 1.5 μM was obtained, i.e. 15 times higher than the IC_{50} of free
310 NBPT (100 nM). As expected, ligation of NBPT by the α -cyclodextrin was accompanied by a
311 significant decrease of inhibiting property, but this lower activity has to be put into
312 perspective with increased stability over time at pH 5.5 and with the significant simplicity of
313 the formulation. To conclude this study, the 1:1 inclusion complex as well as solutions of CD-
314 α /NBPT with excess cyclodextrin (ratio: 2:1 and 5:1) were deposited on an acidic soil.
315 Monitoring of the urea concentration showed that this simple new formulation had similar
316 efficiency than that of Agrotain (See Supplementary Material). Since activity *in vitro* was
317 more interesting for the complex, limitations could be attributed to the bacterial activity from
318 the soil.

319

320 4. Conclusion

321 Our study is part of the current trend to formulate bioactive compounds, especially in the field
322 of agriculture, as simple as possible using a limiting number of additives, obviously
323 environmentally friendly. In this context, a new formulation of NBPT, a urease inhibitor
324 widely used as fertilizer, was searched with the main goal of increasing its stability over time
325 under acidic conditions. Our approach was based on molecular characteristics of this
326 thiophosphotriamide since it presents a polar phosphoramidate function and a lipophilic butyl
327 chain. Therefore, a panel of polysaccharides, neutral or charged, with different
328 physicochemical properties, was tested. Nevertheless, the best results were obtained with the
329 α -cyclodextrin used as additive. The 1:1 inclusion complex was fully characterized and
330 presented moderate inhibition activity (1.5 μM) but a significant increased stability at pH 5.5
331 compared to that of commercial Agrotain (75% after 4 days). The resulting inclusion complex

332 is a stable solid which is highly hydrosoluble. Application of solutions in an acidic soil finally
333 showed that inclusion complex of NBPT in cyclodextrin- α had similar inhibition efficiency
334 than Agrotain.

335

336 **Aknowledgements**

337 DP is really grateful to the Région Bretagne, Ille et Vilaine, and Rennes Métropole for PhD
338 fellowship. The present results are part of the AZOSTIMER project, awarded by the Pôle de
339 Compétitivité Mer Bretagne and supported by the TIMAC Agro International (Groupe
340 Roullier). The authors thank Jean-Paul Guégan (ENSCR) for NMR recording.

341

342 **References**

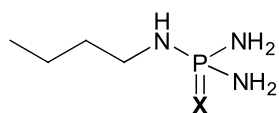
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Captions

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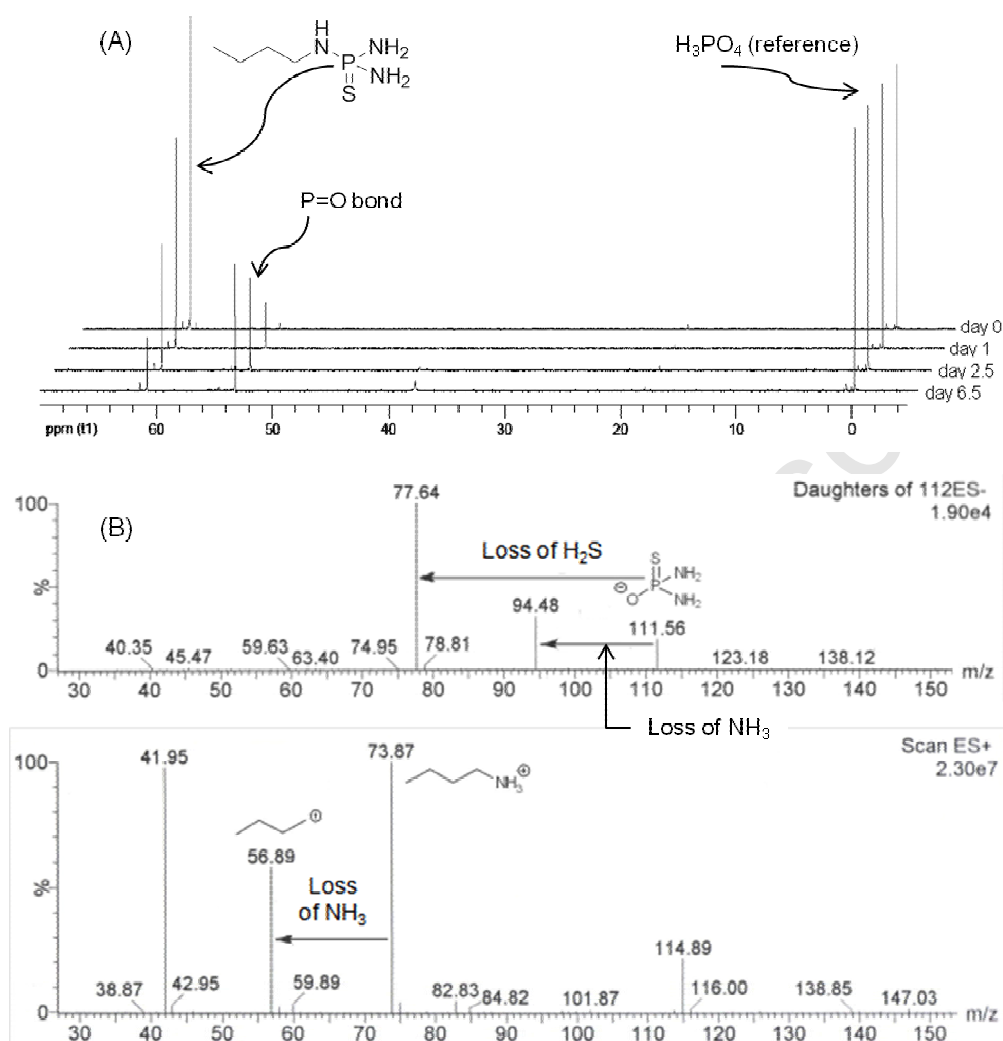
Phosphotriamide

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X=S: *N*-Butyl-thiophosphotriamide (NBPT)**X=O:** *N*-Butyl-phosphotriamide (NBPTO)397 **Fig.1.** Structure of the studied urease inhibitor NBPT.

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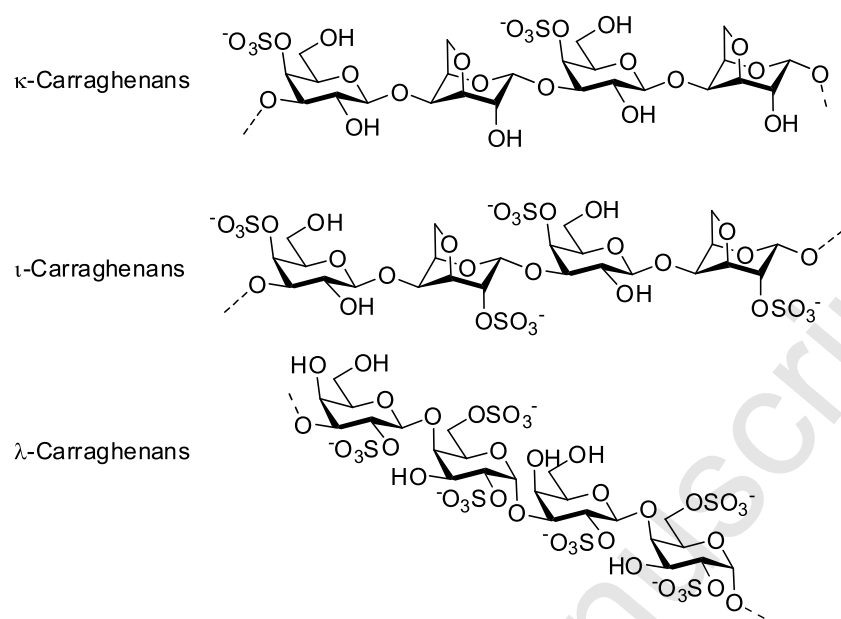
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399 **Fig. 2.** (A) ^{31}P NMR monitoring and (B) High Resolution Mass Spectrometry analysis of the
 400 degradation of NBPT at pH 5.5.

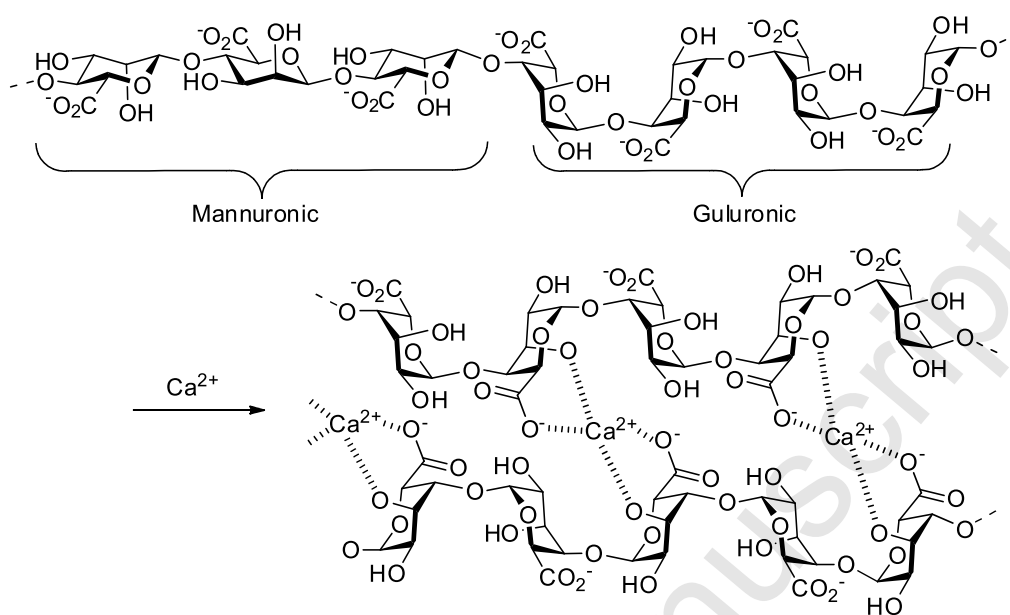
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402 **Fig. 3.** Primary structures of κ -, ι -, and λ -carraghenans.

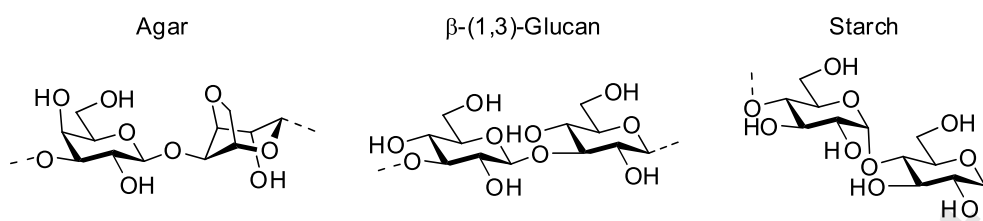
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404 **Fig. 4.** A conformational model for alginates.

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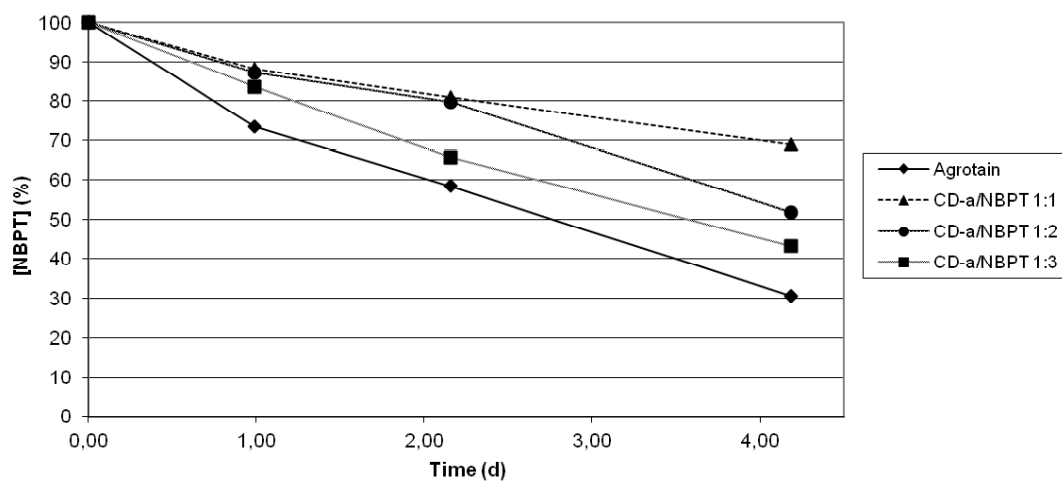


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406 **Fig. 5.** Main skeleton for agar, β -(1,3)-glucans and starch.

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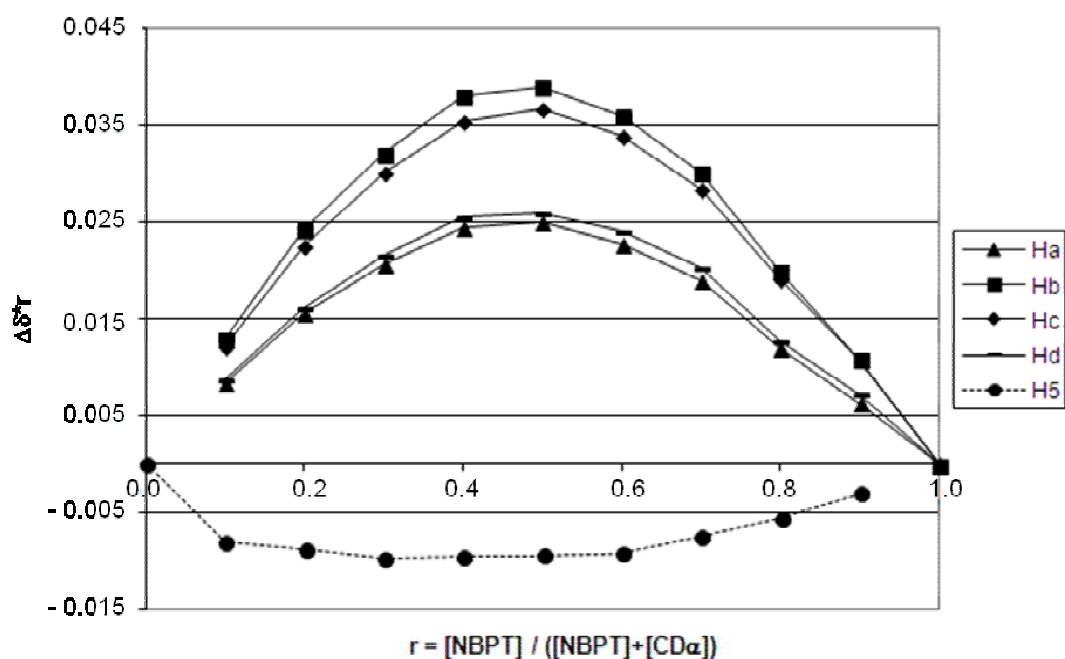
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408 **Fig. 6.** Stabilization of NBPT according to the molar ratio CD- α /NBPT.

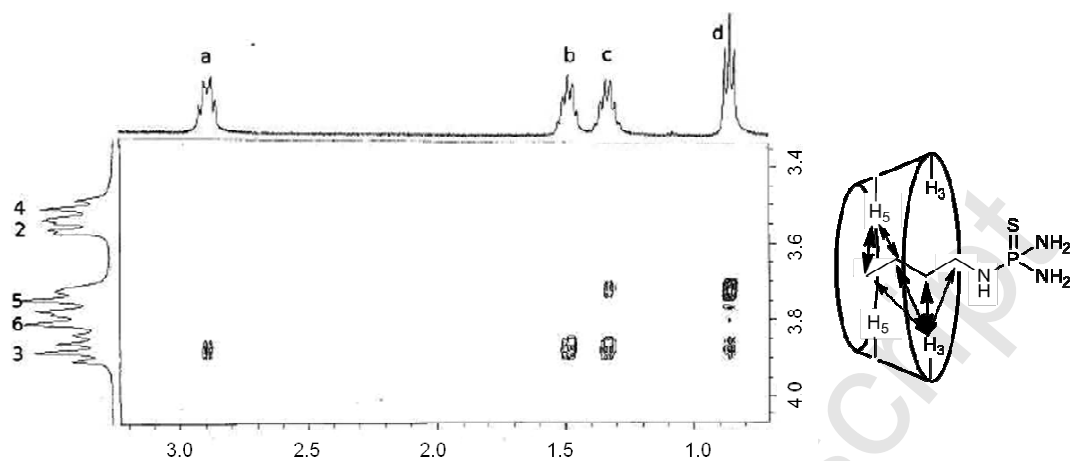
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410 **Fig. 7.** Determination of the $CD\alpha/NBPT$ according to the Job's plot method (Marçon,
 411 Mathiron, Pilard, Lemaire-Hurtel, Dubaele & Djedaini-Pilard, 2009). Ha-Hd are for the alkyl
 412 chain of NBPT and H5 for the $CD\alpha$.

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413

414 **Fig. 8.** Focus on NRM T-ROESY spectrum of the inclusion complex CD α /Nzz

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