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High-resolution nuclear magnetic resonance spectroscopy studies of polysaccharides crosslinked by sodium trimetaphosphate: a proposal for the reaction mechanism

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Abstract—An NMR spectroscopy study (^{31}P, ^{1}H, ^{13}C) of the postulated crosslinking mechanism of sodium trimetaphosphate (STMP) on polysaccharides is reported using methyl α-D-glucopyranoside as a model. In a first step, reaction of STMP with Glc-OMe gives grafted sodium tripolyphosphate (STPP$_g$). On the one hand, STTP$_g$ can react with a second alcohol functionality to give a crosslinked monophosphate. On the other hand, a monophosphate (grafted phosphate) could be obtained by alkaline degradation of STPP$_g$. NMR spectroscopy allows to detect the various species formed and to obtain the crosslinking density of STMP–polysaccharides hydrogels.
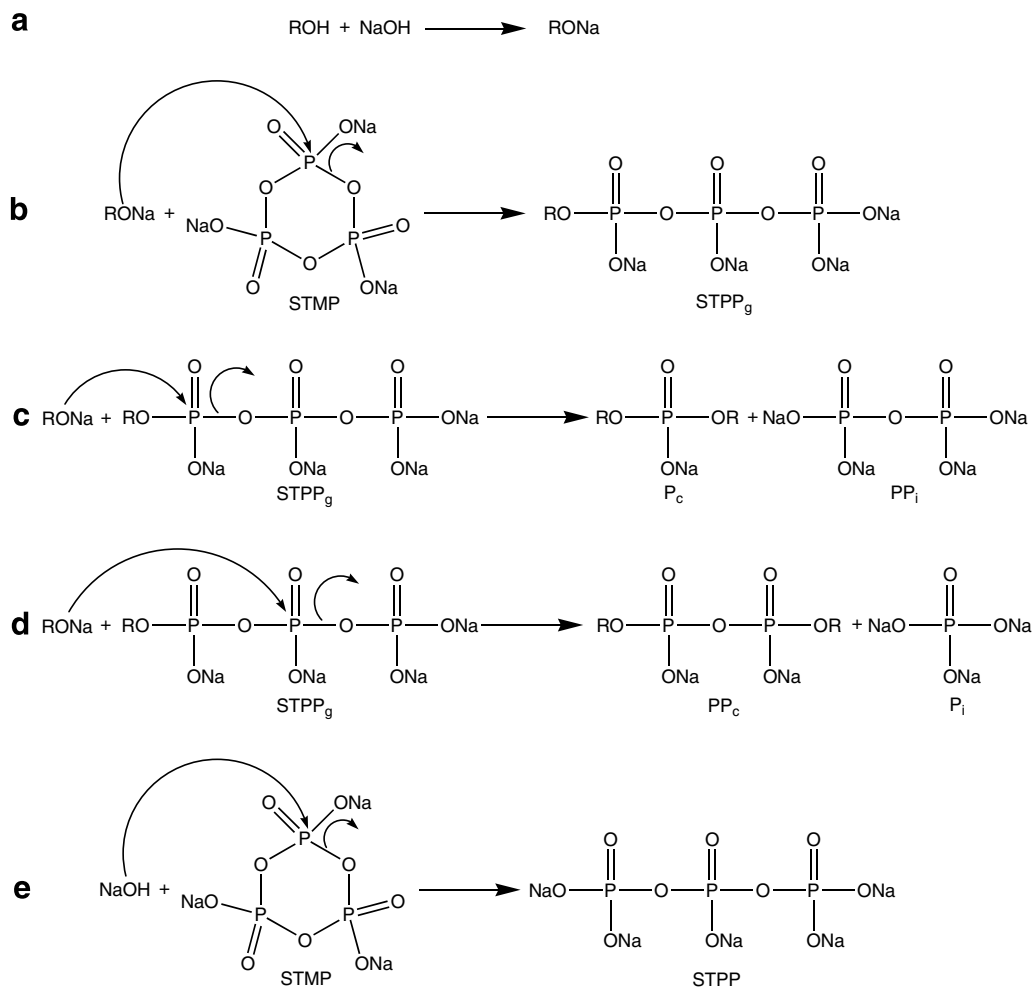© 2007 Elsevier Ltd. All rights reserved.

1. Introduction

Sodium trimetaphosphate (STMP, Scheme 1b) is a cyclic triphosphate, obtained by condensation of phosphoric acid (P$_i$) and pyrophosphate (PP$_i$) at high temperature. Being nontoxic,[1] STMP is often used for the preparation of starch phosphates.[2] These starch phosphates form very consistent colorless pastes, with a good resistance to freezing and defrosting. Widely used in food industry, they are classified into two main categories: monostarch phosphated and crosslinked starch or distarch. In spite of its uses in various domains (for instance, for starch modification,[1–3] synthesis of hydrogels for pharmaceutical purposes),[4,5] the crosslinking mechanism of polysaccharides by STMP is not clearly established. The most reliable mechanism was proposed by Lim and Seib in a paper[2] focused on the preparation of wheat and corn starch phosphates. Therein, they assumed a crosslinking in two steps. The first step consists of the opening of the STMP cycle by starch in the presence of sodium hydroxide (Scheme 1b). The second step is the addition of a new polymer chain, thus affording the crosslinking with the release of PP$_i$ (Scheme 1c). This mechanism was proposed by analogy with the STMP degradation at high temperature and the hydrolysis of adenosine triphosphate in

Scheme 1. STMP reaction with polysaccharide (ROH) in alkaline medium. Alcoholate formation (a), STMP opening (b), monophosphate crosslink formation (c), pyrophosphate crosslink formation (d) and alkaline degradation of STMP (e).

the presence of sodium hydroxide. More recently this mechanism was completed by Seib and Woo[1] who described the formation of pyrophosphate links at alkaline pH (Scheme 1d).

In this paper, we wish to propose a general NMR method (with a classical high resolution probe) for the assignment of species formed during the reaction of polysaccharides with STMP and thus to clarify the crosslinking mechanism. The reaction of pullulan, chosen as exopolysaccharide model, with STMP led to the formation of hydrogels. However, the main problem of NMR on polymers and hydrogels lies in the very long correlation time (τ_c) and the short relaxation times (T_2 has a low value for polysaccharides), which have direct effects on the resolution (broad NMR line, Fig. 1). Consequently, its seem difficult to isolate and assign the molecular species generated during the crosslinking of pullulan by STMP, using classical NMR probe (without using HR-MAS NMR). As a result, the study was performed with methyl α-D-glucopyranoside (Glc-OMe), which is stable at alkaline pH and gives spectra better resolved than pullulan.

2. Results and discussion

2.1. Preliminary studies

First, STMP was dissolved in water without any pH correction (9:1 water–D_2O; pH = 5.4). Three ^{31}P NMR peaks could be observed (data not shown) at −21.25 ppm (s, Int. = 1), −9.49 ppm (s, Int. = 0.006) and 0.32 ppm (s, Int. = 0.002), which correspond, respectively, to STMP, PP_i and P_i. However, integration of these signals showed that P_i and PP_i are present at very low concentrations. These data suggest that these components are derived from the STMP synthesis. In addition, kinetic study indicated that STMP is hardly hydrolyzed after several days under these conditions (data not shown).

After dissolution of STMP in a strongly alkaline medium (pH = 13.5), two new ^{31}P NMR peaks appeared instantaneously at −18.3 ppm (t, 19.1 Hz) and −4.48 ppm (d, 19.1 Hz), respectively. Both signals were attributed to tripolyphosphate (STPP) by comparison with a commercially available sample. STPP could result from the cycle opening of STMP by sodium hydroxide
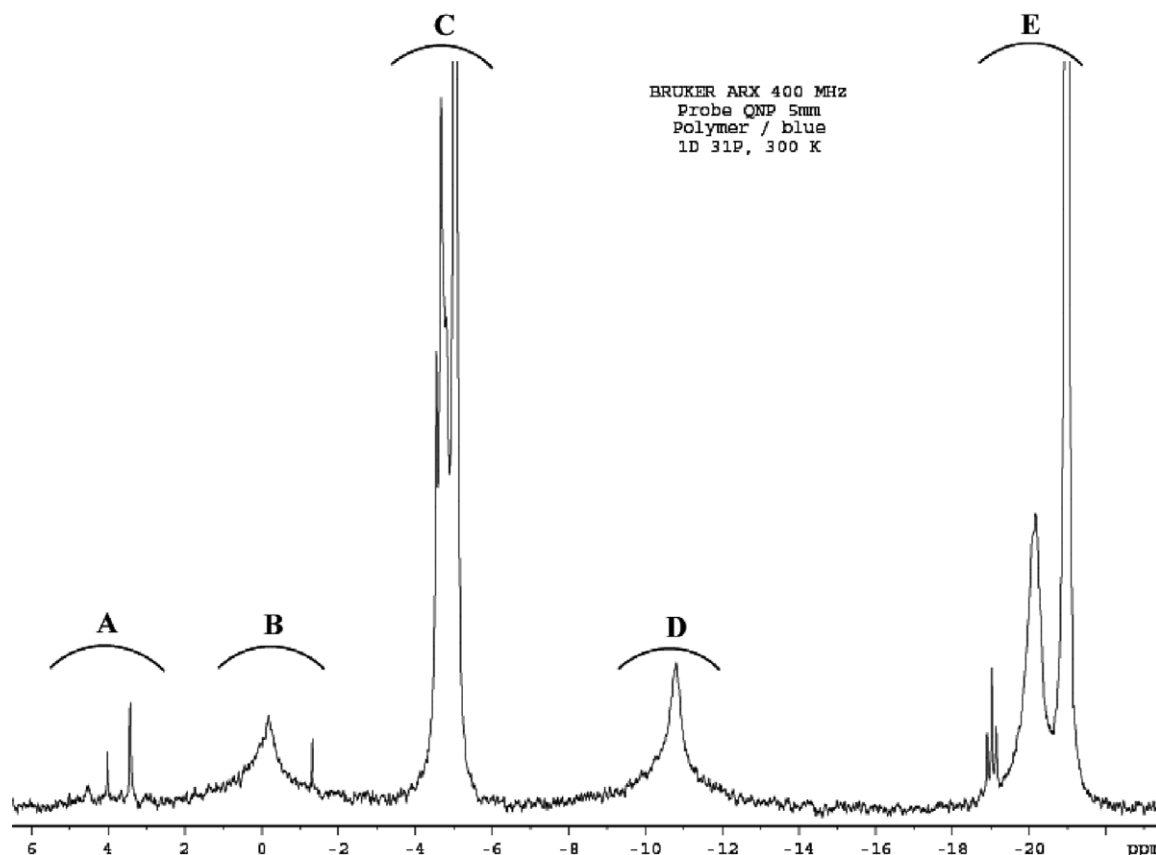
Figure 1. ^{31}P NMR spectrum of a pullulan hydrogel crosslinked with STMP ([pullulan] = 1.23 M, [STMP] = 0.1 M, pH at the beginning of the reaction = 13.5, 9:1 water–D$_2$O, 300 K, 161.97 MHz).

(Scheme 1e), a phenomenon which was highlighted at 100 °C by Bell in 1949.[6] The kinetic study suggested that the STMP hydrolysis is immediate, is temperature-dependent (two times faster at 323 K than at 298 K) and is strongly correlated to the base concentration with a degradation inhibition starting from pH 11.[7] Since STPP appeared during the reaction, its stability at pH = 13.5 and its reactivity with sugar were studied. STPP was stable at this pH and did not react with sugar under alkaline conditions during 72 h (data not shown). Moreover, the PP$_i$ concentration was unchanged during all the kinetic studies in alkaline medium.

2.2. Reaction between STMP and Glc-OMe

Comparatively to the ^{31}P NMR spectrum of STMP in alkaline medium, the NMR spectrum resulting from the reaction between STMP and Glc-OMe at pH 13.5 revealed numerous peaks (Fig. 2). These signals correspond to those observed in the pullulan hydrogel crosslinked with STMP spectrum (Fig. 1). This result means that Glc-OMe could be an appropriate model for the study of the polysaccharides crosslinking reactions with STMP, exactly as pullulan.

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded regularly during the reaction between Glc-OMe and STMP. Since phosphorylated glycosides formed during the reaction were present in lower quantities than Glc-OMe, ^{31}P spectra gave the most interesting informations (Fig. 2). Before going any further, an appropriate internal standard, stable at high pH values, was necessary for the kinetic studies. Likewise, the formation of unknown species during the reaction (associated with pH and ionic strength dependency on ^{31}P chemical shifts)[8] involved the assignment of the ^{31}P signals appearing in alkaline medium.

Classical ^{31}P concentration standard, such as PO(OMe)$_3$, was not suitable because of its rapid degradation at high pH. Then, NaPF$_6$, which is stable at pH 13.5 and did not interfere with the reaction, was used as standard. Concerning the assignments, the resonances corresponding to the impurities present in the internal standard and to the STMP hydrolysis products were easily detected. For example, the signals at 3.7 ppm (s), −1.6 ppm (s), −14.2 ppm (d, 16.5 Hz) ppm and −20.0 ppm (d, 16.5 Hz) corresponded to impurities introduced with NaPF$_6$, whereas those at −21.2 ppm (s), −5.4 ppm (s) and 3.5 ppm (s) corresponded, respectively, to STMP, PP$_i$ and P$_i$ (Fig. 2). In the same manner, the signals of STPP were easily detected at −4.9 ppm (d, 19.1 Hz) and −19.3 ppm (t, 19.1 Hz).

In order to assign the uncharacterized ^{31}P signals, 2D NMR experiments ^{31}P–^{31}P COSY (Fig. 3) and ^{1}H–^{31}P
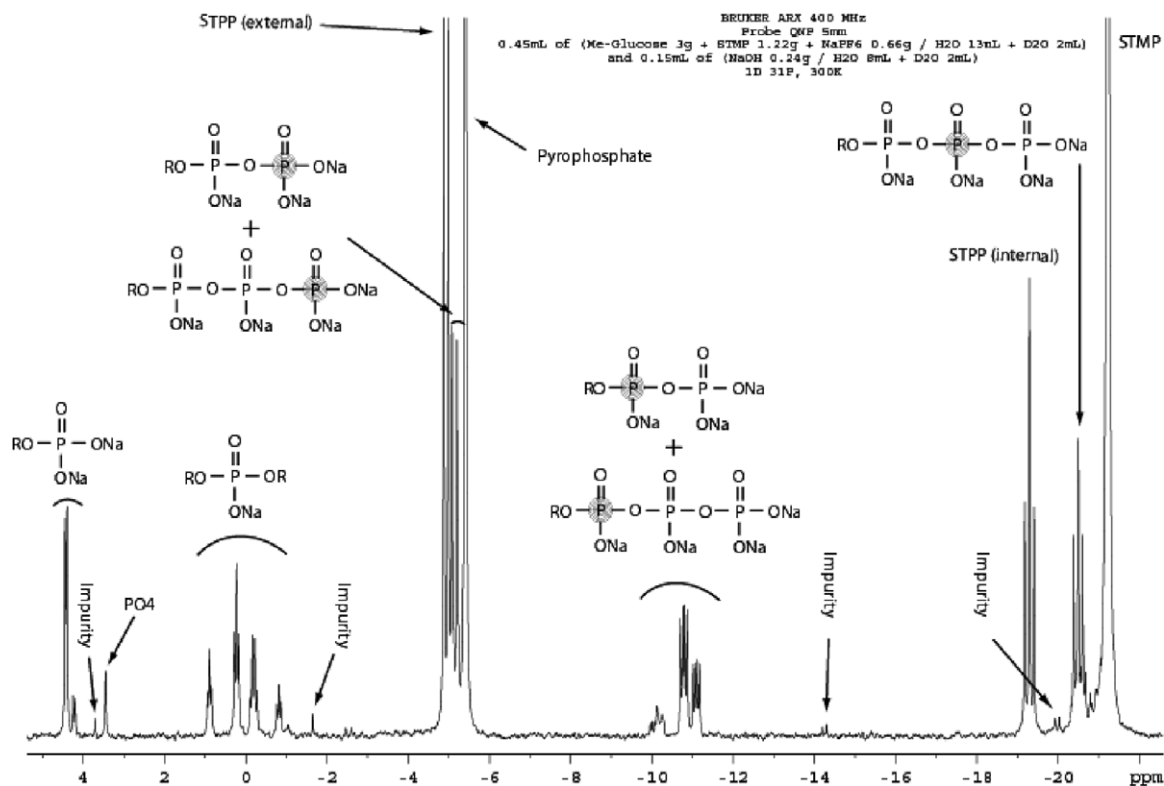
Figure 2. ^{31}P NMR spectrum obtained during the reaction of STMP with Glc-OMe in alkaline medium ([Glc-OMe] = 0.77 M, [STMP] = 0.2 M, pH at the beginning of the reaction = 13.5, 9:1 water–D$_2$O, 300 K, 161.97 MHz).

heteroCOSY (Fig. 4) were carried out. COSY experiments were used to determine which phosphorus atom is connected to another by only a few chemical bonds and the heteroCOSY indicated which phosphorus is directly linked to Glc-OMe. Furthermore, the hetero-COSY allowed to reveal the correlations between ^{31}P and ^{1}H, even for the resonances not easily visible on the 1D ^{1}H spectrum (comparatively to Glc-OMe, phosphorylated glycosides never exceed 5%; Fig. 4: see the ^{1}H projection). The COSY experiment confirms the assignment of the STPP (only one correlation between the internal and external ^{31}P, Fig. 3) and using concomitantly COSY, heteroCOSY and ^{1}H–^{31}P or ^{31}P–^{31}P coupling constants values, the following assignments were suggested (Fig. 2):

- δ = 4.43 ppm (d, $^{3}J_{PH}$ = 7.3 Hz) and 4.23 ppm (d, $^{3}J_{PH}$ = 8.0 Hz), for grafted monophosphate on a sugar (phosphate monoesters, noted P$_g$, zone A).
- δ = 0.89 ppm (at, $^{3}J_{PH}$ = 8.3 Hz), 0.23 ppm (at $^{3}J_{PH}$ = 8.0 Hz), −0.19 ppm (aq, $^{3}J_{PH}$ = 8.5 Hz) and −0.82 ppm (at $^{3}J_{PH}$ = 8.9 Hz), for crosslinks with one phosphorus between two sugars (phosphate diesters, noted P$_c$, zone B).
- δ = −5.1 ppm (d, $^{2}J_{PP}$ = 19.7 Hz), −10.13 ppm (m), −10.79 ppm (dd, $^{3}J_{PH}$ = 9.5 Hz, $^{2}J_{PP}$ = 17.2 Hz), −11.10 ppm (dd, $^{3}J_{PH}$ = 8.9 Hz, $^{2}J_{PP}$ = 17.2 Hz),

−20.46 ppm (t, $^{2}J_{PP}$ = 18 Hz) for grafted pyrophosphate or tripolyphosphate on a sugar (noted PP$_g$ and STPP$_g$, zones C, D and E). In fact, the integration comparisons of the signal at −20.46 ppm (only STPP$_g$) and the signals between −9.5 and −11.5 ppm (STPP$_g$ and/or PP$_g$) indicated clearly that STPP$_g$ is the major product comparatively to PP$_g$ (data not shown). Indeed, the sum of integrations of the signals between −9.5 and −11.5 ppm was always almost equal to the integration value of the signal at −20.46 ppm. Thus the formation of PP$_g$ could be considered as negligible and STPP$_g$ was the only product of the reaction.

Due to the four possible active sites on Glc-OMe, numerous combinations could be obtained during the formation of the link between the sugar and phosphate (e.g., the phosphate diesters zone B, Figs. 1 and 2). However, the final goal of this work being the study of hydrogels and these polymers giving too broad NMR signals to define exactly the positions of crosslinks, the characterization of these positions on Glc-OMe was not attempted.

Finally, our assignments were found to be in agreement with previous published chemical shifts of polyphosphate carbohydrates, cell phosphorus metabolites or polynucleotide analogues.[9–12]
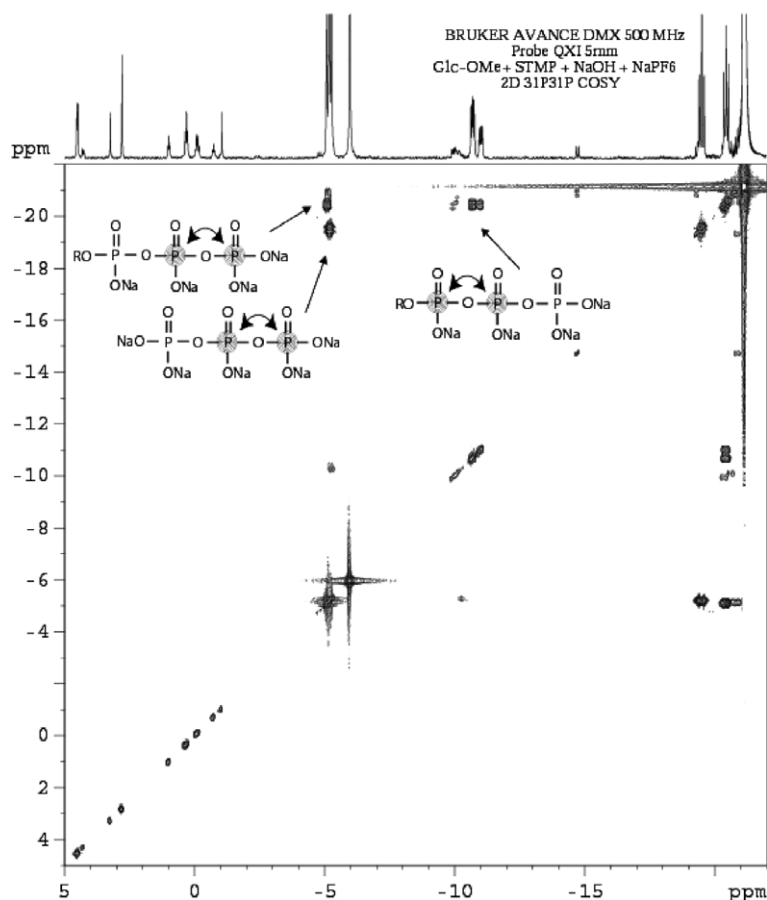
Figure 3. ^{31}P–^{31}P COSY spectrum obtained during the reaction of STMP with Glc-OMe in alkaline medium ([Glc-OMe] = 0.77 M, [STMP] = 0.2 M, pH at the beginning of the reaction = 13.5, 9:1 water–D_2O, 300 K, 202.46 MHz).

These assignments highlight three interesting regions. The zone B corresponding to phosphated link between two sugars (P_c), the zone A corresponding to one phosphorus grafted to one sugar (P_g) and zone C, D or E corresponding to tripolyphosphate grafted to one sugar ($STPP_g$). These areas being also present on the ^{31}P MNR spectra of the crosslinked pullulan (Fig. 1), it will be possible, if the reaction is carried out in the presence of reference (such as sodium hexafluorophosphate), to estimate the percentage of monografted phosphorus (P_g, $STPP_g$) and the amount of phosphorus taking part in the crosslinking reaction.

To determine precisely which reaction occurred during the crosslinking reaction, NMR kinetic study was also performed throughout the reaction of STMP with Glc-OMe in alkaline medium. For this purpose, the evolution of the pH, the consumption of the STMP and the formation of the other species were examined. During the first 150 min of reaction, the pH decrease was rather slow (Fig. 5a). Then, the pH fell very quickly and was stabilized around a value of 10. The STMP opening by NaOH or by the sugar was immediate (Fig. 5b); the concentration decreased quickly to reach a plateau after approximately 1500 min (25 h). This time corresponds

to the stabilization of the pH near 10. The pH was therefore, most probably, not sufficient to ensure on the one hand the reaction of the sugar with STMP and on the other hand the STMP degradation. STPP formation was immediate (Fig. 5c), with an increase of the concentration during the first 750 min (12.5 h), and then a plateau was reached. STPP formation is due to alkaline degradation of STMP. Thus, STMP degradation is instantaneous and stops after nearly 13 h of reaction. The pH corresponding to the end of STMP degradation was around 12. For this pH, it is possible that STMP opening by a sugar is preferential to its degradation. Like for STPP, the formation of $STPP_g$ was immediate (Fig. 5d) and a plateau was also reached after about 750 min. $STPP_g$ corresponds to the opening of the STMP cycle by a sugar and is an intermediate in the crosslinking reaction. Indeed, $STPP_g$ is consumed during the reaction (Scheme 2), which means that the formation of $STPP_g$ can be masked by its consumption. Appearance and disappearance kinetics of $STPP_g$ would be comparable and consequently its concentration would seem constant. We have seen that the degradation of the STMP in STPP reaches a plateau after nearly 13 h, then after this time, the disappearance of the
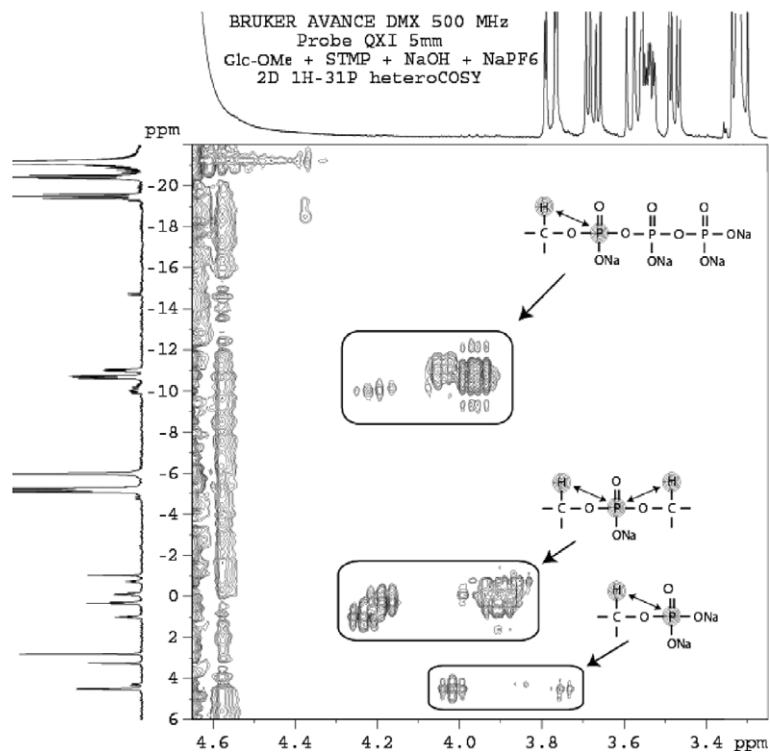
Figure 4. $^1H–^{31}P$ heteronuclear COSY spectrum obtained during the reaction of STMP with Glc-OMe in alkaline medium ([Glc-OMe] = 0.77 M, [STMP] = 0.2 M, pH at the beginning of the reaction = 13.5, 9:1 water–D_2O, 300 K, 500.13–202.46 MHz).

Figure 5. Evolution of pH (a), of STMP concentration (b), of STPP concentration (c) and of $STPP_g$ concentration (d), during the reaction of STMP with Glc-OMe in alkaline medium.

STMP should correspond to the opening of the cycle by an alcohol moiety. Interestingly, during the whole reaction, the P_i concentration remained constant (data not shown).
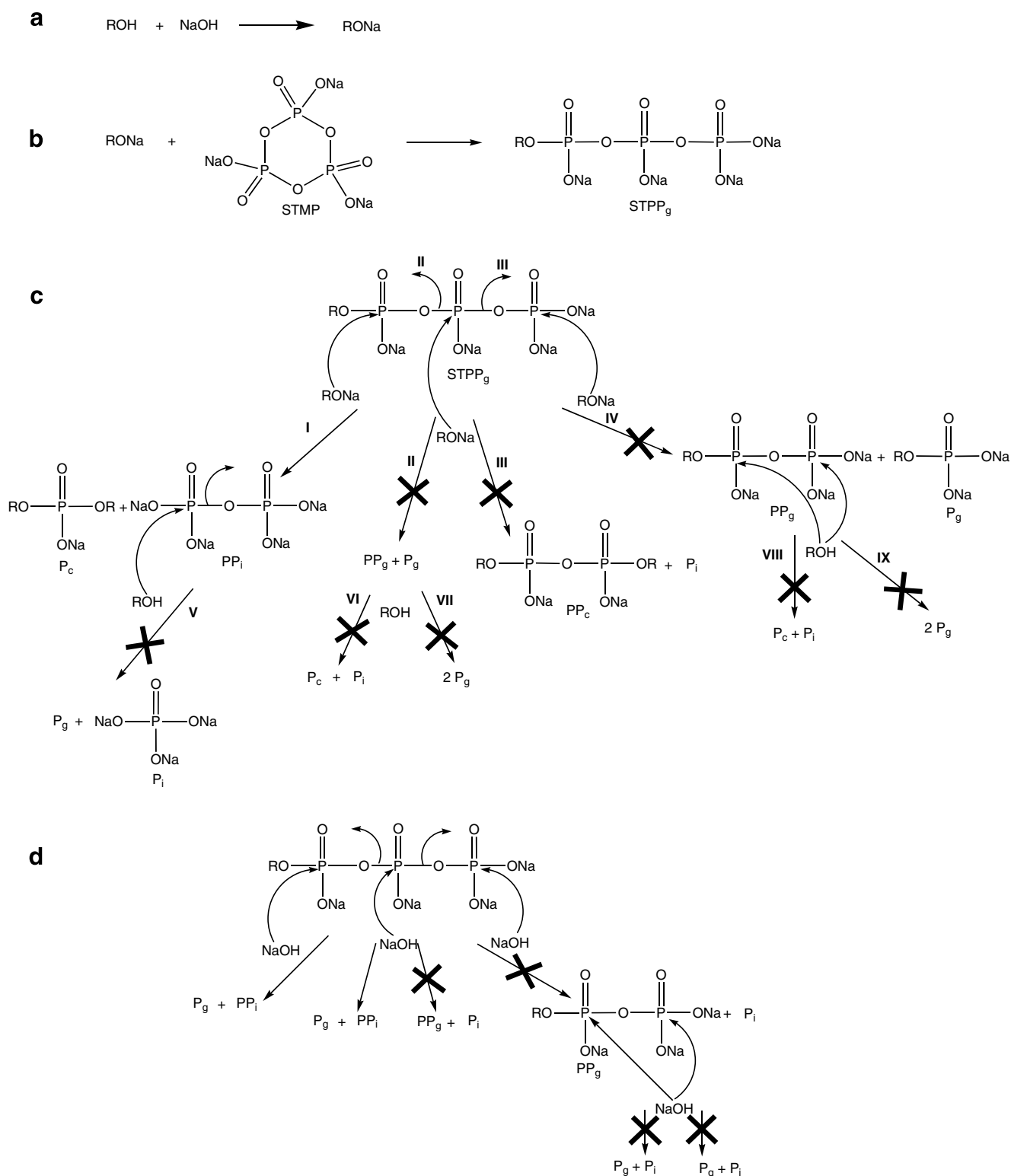
Scheme 2. Schematic representation of all the reactions being able to occur during the reticulation of Glc-OMe (ROH) by STMP. Alcoholate formation (a), 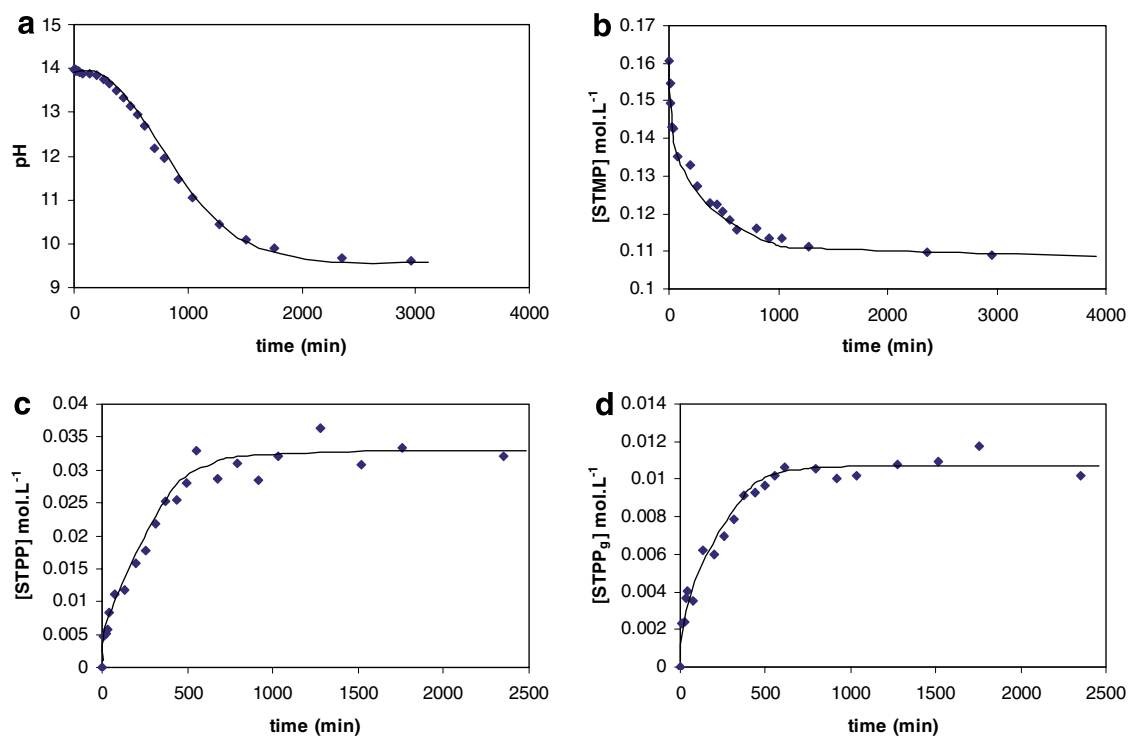STMP opening (b), all the possible reactions between Glc-OMe and $STPP_g$ (c) and all the possible ways of degradation of $STPP_g$ (d).

As the reaction seemed to stop when the pH became too low (pH < 10), the experiment was repeated but one NaOH pellet was added to the reaction medium when the plateau was reached (Table 1). Before the introduction of the NaOH pellet, the results obtained corroborate our previous conclusions. Indeed, a concomitant decrease of the STMP concentration with an increase of the other components was clearly observed

Table 1. Concentration of the various compounds present in the reaction medium according to time and pH, before and after the addition of one NaOH pellet, during the reaction of STMP with Glc-OMe in alkaline medium

Time (min)	STMP (mM)	$STPP_g$ (mM)	STPP (mM)	PP_i (mM)	P_c (mM)	P_g (mM)	P_i (mM)	pH
0	208	—	—	3	—	—	1	13.5
30	195	7	4	5	ND	ND	2	13.2
210	148	20	16	26	15	7	2	13.0
1130	116	20	23	39	23	12	1	10.1
6960	117	20	23	41	24	12	2	9.6
Addition of one NaOH pellet								
30	68	12	41	72	41	26	2	>13.9
86,000	0	3	62	70	5	104	2	>13.9

(except for P_i). After 210 min (at the most) it was observed that the $STPP_g$ concentration becomes constant, while the concentrations of the other compounds still increase (Table 1). This last observation again exhibited a consumption of $STPP_g$ as fast as its formation. Following the introduction of one NaOH pellet in the NMR tube, P_c concentration increased while $STPP_g$ concentration decreased. This result pointed out, under these very aggressive conditions, that $STPP_g$ consumption, in favour of P_c, becomes more important than $STPP_g$ production. Moreover, STMP concentration strongly decreased while concentration of the other components increased (except for $STPP_g$ and P_i). After 1 h, PP_i precipitates and thus its concentration will remain unchanged. Finally, when all the STMP was consumed, the very aggressive character of the reaction medium induced a degradation of P_c in favour of P_g. The results obtained indicate that under these extreme conditions, STMP degradation products (STPP, PP_i) and P_g are mainly obtained.

It should be noticed that during the reactions, no P_i was formed (P_i signal integration remained constant, Table 1). This fact is of great importance to clarify the crosslinking mechanism by STMP.

2.3. Mechanism

Using all the data obtained during this study, it becomes possible to propose a mechanism for the polysaccharides crosslinking by STMP. The formation of a crosslink is accomplished in two steps; the opening of the STMP cycle by an alcohol moiety followed by the reaction with a second alcohol moiety. However, it is possible, a priori, to consider numerous reaction combinations. Indeed, after formation of the $STPP_g$, attacks by another alcohol moiety on different sites are possible (Scheme 2). Obviously in a symmetrical way, the degradation of $STPP_g$ (hydroxide attacks) should also be considered. The results presented in this paper indicate that under the conditions of the study, the STPP and PP_i were stable, the formation of PP_g could be neglected and that there was no P_i formation. Thus it is possible to remove, from the possible theoretical mechanisms, all the reac-

tions producing orthophosphate (reactions **III**, **V**, **VI** and **VIII**, Scheme 2). Pathways **II** and **IV** (Scheme 2) are also in disagreement with our results. Indeed, because formation of PP_g could be negelected, these two reactions should continue until the formation of 3 P_g (reactions **VII** and **IX**, Scheme 2). However, the results obtained when the first plateau is reached (before the introduction of one NaOH pellet, e.g., after 1130 min, Table 1) indicate that comparatively to the others compounds, P_g is not produced in very large quantity. Furthermore, the only two possibilities of producing PP_i are the formation of P_c (reaction **I**, Scheme 2) and the degradation of $STPP_g$ by hydroxide attack, giving P_g. Thus, because the sum of the concentrations of P_c and P_g is always almost equal to the concentration of PP_i (Table 1), the reaction ways producing 3 P_g (reactions **II** + **VII** and **IV** + **IX**, Scheme 2) could also be excluded, P_g being produced only during the degradation of $STPP_g$. Finally, in alkaline medium, we propose the reaction mechanism summarized in Figure 6. This mechanism is valid only under the classical conditions of reaction (pH < 13.5) and in the presence of STMP. Indeed in the other cases (addition of one pellet of NaOH), $STPP_g$ and P_c are degraded and at the end of the reaction, only STPP, PP_i and P_g are obtained (Table 1).

It could be concluded that, in a first step, Glc-OMe in alcoholate form and hydroxide compete for STMP as the most reactive species, giving STPP or $STPP_g$ formation (Fig. 6). Further attack of $STPP_g$ by Glc-OMe in alcoholate form preferably gives P_c and PP_i. Obviously, PP_i is the better leaving group than P_i, which is not observed. Thus no PP_c is generated. The hydroxide also preferably attacks the $STPP_g$ forming P_g and PP_i. These observations are in agreement with the fact that phosphate monoesters as P_g are better more acidic than P_i and thus should be the better leaving groups. Obviously, STPP is a less reactive species, since it will in any case form P_i and PP_i.

$STPP_g$ has a structure similar to adenosine 5′-triphosphate (ATP). Thus it may be interesting to compare our results with those concerning the ATP hydrolysis. The extracellular degradation of ATP is catalyzed by a
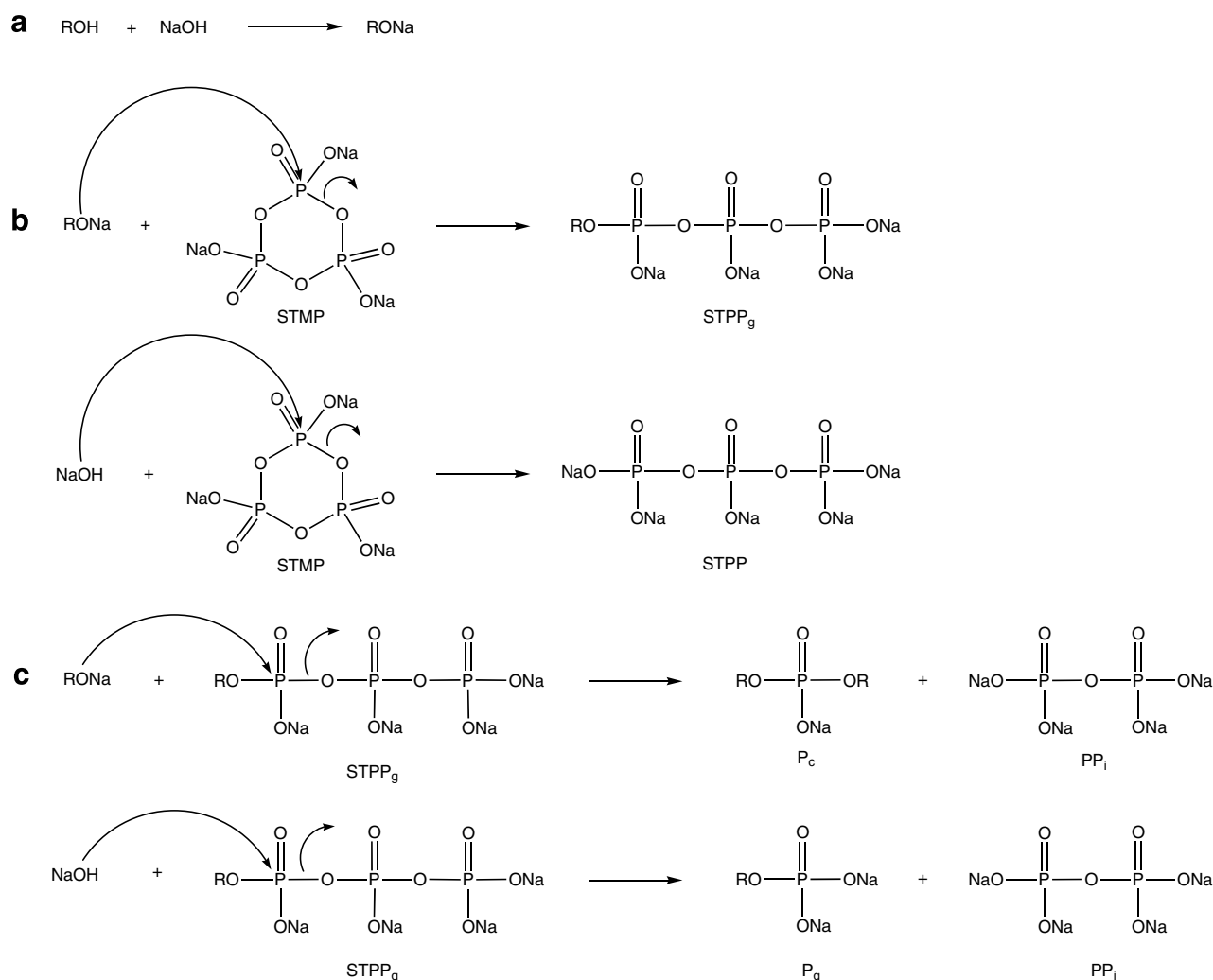
Figure 6. Proposed mechanism for the crosslinking reaction of polysaccharides by STMP in alkaline medium. Alcoholate formation (a), STMP opening (b) and crosslinking or degradation of $STPP_g$ (c).

family of enzymes called nucleoside triphosphate diphosphohydrolases (NTPDases).[13] In the biological conditions, these enzymes hydrolyze the γ and β phosphate of ATP, giving ADP and/or AMP.[14,15] Our reaction conditions are too distant of the enzymatic conditions in the living world to make a clear comparison. However, the results obtained in this study in very basic medium can be explained by the fact that attack of nucleophile on a phosphate ester is strongly suppressed by negative charge.[16,17] Indeed, in very basic medium it is on α phosphate that the repulsion by the negative charges is less strong. It is thus natural to obtain mainly P_c and P_g (Fig. 6). The medium giving the results closest to the biological conditions is certainly obtained at acid pH. Indeed, the acid-catalyzed hydrolysis of ATP has been found to give rise to both ADP and AMP.[18] In this pH condition, phosphate esters are protonated, allowing a more easy attack of nucleophiles, and the γ phosphate may undergo unimolecular elimination to form monometaphosphate.[17]

3. Conclusions

By NMR spectroscopy we were able to propose a mechanism for the reaction between Glc-OMe and STMP. This mechanism could be transposed to the reaction of pullulan with STMP in the same conditions, which leads to the formation of hydrogels. The reaction of polysaccharides with STMP, giving $STPP_g$ which is the first stage of the crosslinking reaction, and the STMP degradation by NaOH start immediately. Among all the possibilities of reactions on formed $STTP_g$, it seems that only the attack on α phosphate is privileged. The results obtained indicate that in the presence of STMP and between pH 10 and 13.5, in addition to species stable like PP_i or STPP, the reaction produces mainly P_c and P_g. With the studied conditions, the ratio between P_c and P_g seems to be, respectively, 2:1. Under very aggressive conditions (pH > 13.9 and/or absence of STMP), the degradation reactions prevail and produce mainly P_g, PP_i and STPP. PP_i, which in this case is formed in very

large quantity, precipitates when its concentration in solution is close to 70 mM. Conversely, the crosslinking reaction is stopped when the pH solution reaches 10. This pH is not high enough to ensure the reaction with STMP or with $STPP_g$. The pH seems to be a crucial parameter for the reaction.

Moreover, owing to NMR, it is now possible to quantify easily the phosphated species present in hydrogels and thus to obtain the degree of crosslinking. Having clarified the reticulation mechanism and having a reliable method to quantify the various species produced, it is now interesting to compare the properties of hydrogels obtained as a function of the reaction conditions (pH, ratio of reagents, temperature, etc.). Indeed, the crosslinking rate, the charges or the quantity of PP_i and STPP are believed to have an impact on the properties of hydrogels. We are currently studying hydrogels obtained by crosslinking of pullulan under various conditions in order to supplement our knowledge on this type of reaction.

4. Materials and methods

4.1. Materials

Pullulan PI20 (M_n = 160,000 g mol^{-1}, determined from SEC/MALLS measurements)[19] was purchased from Hayashibara Biochemical Laboratory (Okayama, Japan). STMP, STPP, sodium hexafluorophosphate, PP_i and methyl α-D-glucopyranoside were purchased from Sigma Chemical Company (Lyon, France). Sodium hydroxide, sodium chloride and hydrogen chloride were purchased from VWR (Fontenay-sous-bois, France). Deuterium oxide was purchased from SDS (Peypin, France).

4.2. Hydrogel synthesis

The synthesis of hydrogels based on pullulan and crosslinked with STMP was described previously.[20] For NMR study, pullulan (1 g, 6 mmol) was dissolved in water (13 mL at a fixed pH of 13.5) and gently stirred for 2 h. Then, an aqueous solution of STMP (1.22 g in 2 mL, 3.9 mmol) was added under vigorous stirring and 0.6 mL of the mixture was quickly poured in a 5-mm NMR tube. The reaction was studied at 300 K and for every sample the final proportion of H_2O and D_2O was fixed to 90:10, respectively. NMR study of the reaction between Glc-OMe and STMP was carried out with the same procedure as described above.

4.3. NMR spectroscopy

NMR experiments were performed either on a BRUKER ARX 400 spectrometer (for 1D spectra), equipped with a 5 mm QNP ^{1}H/^{13}C/^{31}P/^{19}F probe, interfaced to a Silicon Graphics INDIGO2 workstation or on a BRUKER AVANCE DMX 500 spectrometer (for 2D spectra), equipped with a 5 mm QXI ^{1}H/^{13}C/^{31}P/^{15}N probe, interfaced to Silicon Graphics O2 workstation. All data were processed on a Silicon Graphics O2 workstation using XWINNMR and AURELIA software (BRUKER S.A., Wissembourg, France). ^{31}P spin-lattice relaxation times (T_1) of few representative phosphorus containing compounds used in this study (P_i, PP_i, STPP) were measured by using inversion recovery sequence.[21] To ensure full relaxation between each scan and obtain an accurate integration, a relaxation delay of 30 s was used in all 1D ^{31}P NMR experiments. After data integration, the concentrations were calculated by comparison to $NaPF_6$, at an accurately known concentration (as an internal standard). All chemical shifts are expressed in parts per million (ppm), from 85% phosphoric acid as an external reference (0.0 ppm) for ^{31}P and from DSS as an external reference (0.0 ppm) for ^{1}H. All spectra were recorded at a temperature of 298 or 300 K, except for the temperature dependence study of the STMP hydrolysis in alkaline medium. For ^{31}P 1D NMR spectra, 16 transients were typically collected at a frequency of 161.97 MHz with a flip angle of 90° (9.5 μs), a spectral width of 41,667 Hz, an acquisition time of 0.79 s and 65,536 data points. Data were processed with multiplication prior to Fourier transformation by an exponential function (line broadening was 3.0 Hz) and without zero filling. The 2D gradient-enhanced ^{31}P–^{31}P COSY[22,23] without ^{1}H decoupling and proton detected ^{1}H–^{31}P heteronuclear COSY (heteroCOSY)[24] without ^{31}P decoupling were recorded in the absolute value mode. Usually, 512 (COSY), 256 (heteroCOSY) equally spaced evolution time period (τ_1 = 3 μs) values were acquired, averaging 32 (COSY), 64 (heteroCOSY) transients of 2048 points with 10,162 Hz of spectral width in both dimensions (COSY) or with 4251 and 10,122 Hz of spectral width in ω_2 and ω_1, respectively (heteroCOSY). Time domain data matrices were zero filled in ω_1 dimension to 1 K (COSY) or 512 (heteroCOSY), thus yielding a digital resolution of 4.96 and 9.92 Hz/point (COSY) or 2.08 and 19.77 Hz/point (heteroCOSY) in ω_2 and ω_1, respectively. A $\pi/8$ (COSY) or $\pi/2$ (heteroCOSY) shifted sine-bell in both dimensions was used before transformation. Gradient values were set to ratio 20:20 (COSY) or 30:12.14 (heteroCOSY). Relaxation delay was 3.5 and 2 s for COSY and heteroCOSY, respectively.

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