Positive effect of natural and negatively charged cyclodextrins on the stabilization of penicillins towards β-lactamase degradation due to inclusion and external guest–host association. An NMR and MS study

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Received 6th December 2005, Accepted 1st February 2006
First published as an Advance Article on the web 24th February 2006
DOI: 10.1039/b517275f

The complexation of penicillin (**1a**–**c**) and cephalosporin (**2a**,**b**) antibiotics with cyclodextrins (CDs), both natural [β-CD (**3b**) and γ-CD (**3c**)] and carboxylated [*heptakis*(6-oxycarbonylethylthio-6-deoxy)-β-CD sodium salt (**4b**) and *octakis*(6-oxycarbonylethylthio-6-deoxy)-γ-CD (**4c**) sodium salt], has been studied at neutral pH. Penicillins [ampicillin (**1a**), amoxicillin (**1b**) and dicloxacillin (**1c**) form inclusion complexes with the above CDs, as was shown by extensive NMR spectroscopic studies, whereas cephalosporins (cephalexin, cefadroxil) do not. Inclusion of the penicillins into either **3b** or **4b** was not accompanied by significant chemical shift changes in the ^{1}H NMR spectra. On the contrary, with the wider **3c** and its derivative **4c** inclusion was evidenced by both chemical shift displacements of the cavity protons and intermolecular interactions, indicating the formation of primarily 1 : 1 guest–host inclusion complexes. The binding constants for **1a/3c**, **1a/4c** and **1c/3c** were calculated as 19 ± 4, 17 ± 0.9 and 622 ± 200 M^{-1}, respectively. With **4c**, a 1 : 2 stoichiometry was also found. In addition, simultaneous formation of aggregates by external association takes place in solution, as shown by the ESI-mass spectrometric data. Studies on the hydrolysis of ampicillin under pseudo-first order conditions using an excess of **3c**, **4c** and of linear maltoheptaose at pH 7 showed that the drug hydrolysed at a similar rate in all cases. In the presence, however, of β-lactamase enzyme *and* the carboxylated host **4c**, ampicillin degraded twice as slowly (0.008 h^{-1}) as in the presence of β-lactamase alone (0.017 h^{-1}). This was explained by the effective protection provided by both inclusion and external association of the host. The interaction, therefore, of penicillins with carboxylated CDs may present a means to lessen the chemical instability of these drugs in the presence of β-lactamase enzymes.

Introduction

Penicillins (**1**) and cephalosporins (**2**) (Scheme 1) are chemically related compounds, widely used as classical antibiotics over many years.[1] The structural feature they share is a β-lactam ring fused to a five-membered ring (in **1**) or six-membered ring (in **2**) that creates a strained bicyclic system, which possesses limited stability towards hydrolysis and leading to the formation of different degradation products.[1b,2] The notion that strain in bicyclic β-lactams is responsible for exceptional reactivity and accompanying antibacterial activity, although intuitively attractive, has been seriously challenged.[1b] Indeed, there is little physicochemical evidence to suggest that the kinetic reactivity of these systems is due to an unusually strained bicycle.[1b,3] The hydrolysis of the antibiotics[1–4] is provoked by nucleophilic attack of various nucleophiles on the β-lactam ring and is catalysed by acids/bases, metal ions and oxidising agents. Self-hydrolysis of the β-lactams in water is therefore a non-negligible process, which provides **1** and **2** with a self-destructing profile. *In vivo*, a particular class of enzymes – the β-lactamases[1–5] – hydrolyse the β-lactam ring. This process constitutes the major physiological route of degradation of penicillins and, to a lesser extent, cephalosporins. β-Lactamases are produced by bacteria in order to destroy the β-lactam ring of the antibiotic and effectively annihilate the drug's activity. This enables bacteria to survive in the presence of the drug, and this the major mechanism of resistance to β-lactam antibiotics in gram-negative bacteria, a most important clinical problem.[5] Efforts to synthesise β-lactamase inhibitors, *i.e.* small molecules that act against bacterial β-lactamases of all classes, A–D,[4b] continue to be of interest,[5b,6] since such inhibitors prolong the effectiveness of β-lactam antibiotics by maintaining bacterial sensitivity to them.

Cyclodextrins (CDs, **3**) are cyclic oligosaccharides of increasing cavity size (**3a** < **3b** < **3c**) that form inclusion host–guest complexes with a variety of molecular species.[7,8] CDs increase the aqueous solubility, stability and bioavailability of many lipophilic drugs by taking up a whole or part of the drug molecule into their hydrophobic cavity, thus acting as drug carriers. Therefore, CDs have been successfully used in many pharmaceutical applications, and several review papers have been published on the subject.[9] Inclusion of the vulnerable β-lactam ring inside a CD cavity would, in principle, reduce exposure of **1** and **2** to external attack, resulting in longer-lived antibiotics in an aqueous environment. In addition, CD inclusion could possibly reduce the development of resistance to β-lactams by bacterial strains. In fact, a recent study has shown[10] that in the presence of β-CD, 2-hydroxypropyl-β-CD and methyl-β-CD, the antimicrobial activity of several antibiotics

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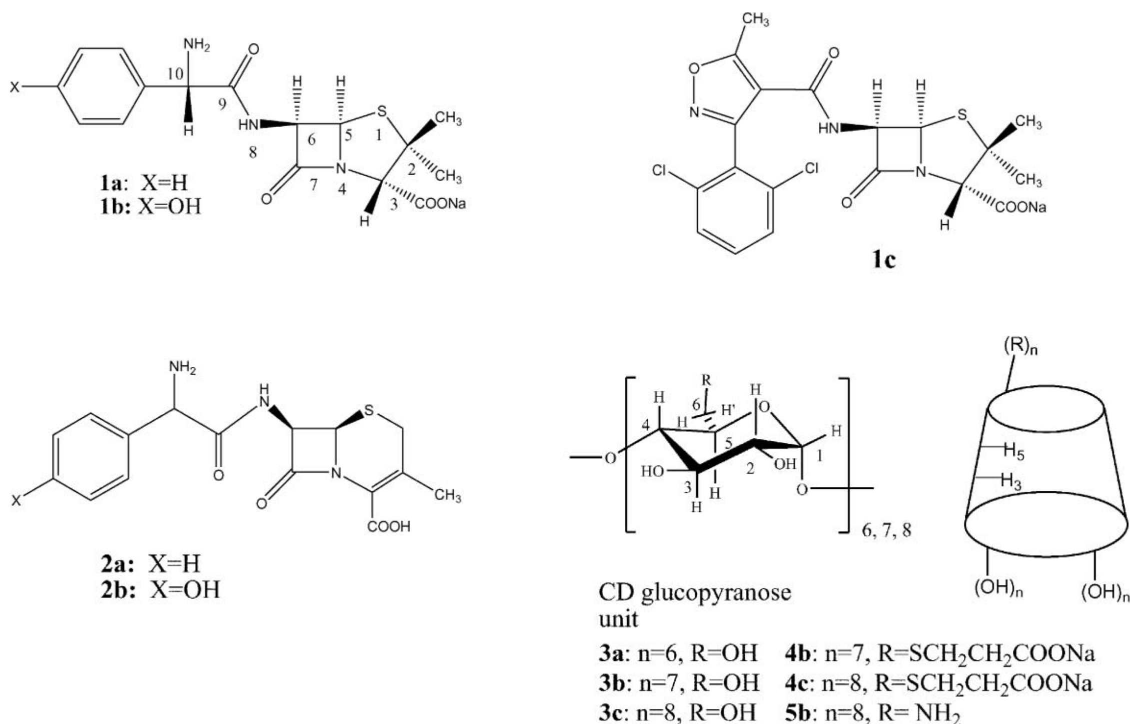

Scheme 1 Ampicillin (**1a**), amoxicillin (**1b**), dicloxacillin (**1c**), cephalexin, (**2a**) cefadroxil (**2b**) and the cyclodextrins **3**–**5** used.

is increased, particularly against gram-negative clinical strains, this action being connected to the nature and degree of substitution on the CD rim. Of the numerous CD-drug articles in the literature,[8,9b] only a few address the effect of CDs on penicillin or cephalosporin antibiotics. Aki *et al.*[11] concluded, using mainly microcalorimetry, that ampicillin formed two types of 1 : 1 and also 2 : 1 complexes with β-CD (**3b**) and with 2-hydroxypropyl-β-CD, at low pH, while in the presence of the latter, significant stabilization of ampicillin was observed. At weakly acidic or neutral pH, one 1 : 1 complex was found, but no stabilization was observed. These results disagree with a former report[12] in which the complex between ampicillin and β-CD, identified as 2 : 1, provided a higher dissolution rate and increased bioavailability of the drug in human subjects. Earlier, NMR and other spectroscopic studies of α-CD (**3a**) with penicillin V had shown the presence of one 1 : 1 complex[13] where the phenyl ring of the drug entered the secondary side of the host CD leaving the β-lactam ring exposed. Regarding the stabilization of the β-lactams in the presence of natural CDs, contradicting results indicating either decrease or acceleration of the degradation rate, have been reported.[7]

The present work aims to investigate with NMR spectroscopy the solution structures of **1** and **2** with γ-CD, a host wide enough to accommodate the bicyclic penem or cephem part of the antibiotics, as well as with CDs specifically substituted with anionic or cationic groups on their narrow rim. The NMR data are complemented with mass spectrometry measurements in order to better define the precise host–guest interactions. In addition, evaluation of the adverse or positive effects of the above CDs on the hydrolysis rate of a typical penicillin – ampicillin – is also presented, both under conditions of controlled pH and in the presence of a β-lactamase enzyme, in aqueous solution.

Results and discussion

The complexes of β-CD (**3b**), γ-CD (**3c**), *heptakis*[6-(2-carboxyethyl)thio-6-deoxy]-β-CD (**4b**),[14] *octakis*[6-(2-carboxyethyl)thio-6-deoxy]-γ-CD (**4c**)[14] and *octakis*(6-amino-6-deoxy)-β-CD (**5b**)[15] (Scheme 1) with typical penicillins and cephalosporins, namely ampicillin (**1a**), amoxicillin (**1b**), dicloxacillin (**1c**), cephalexin (**2a**) and cefadroxil (**2b**) (Scheme 1) were studied by 1D and 2D NMR spectroscopy and mass spectrometry.

NMR spectroscopic studies in aqueous solution

1D NMR experiments at pH = 7.0 (phosphate buffer) and 25 °C were carried out by monitoring the shift of the protons located inside the host cavity, H3 and H5 (Scheme 1), in the presence of increasing amounts of the guest in order to investigate the formation of the inclusion complexes in solution. Addition of each of the five antibiotics **1a**–**c** and **2a**,**b** to aqueous solutions of β-CD (**3b**) and its carboxylated derivative (**4b**) resulted in little or no shift of the protons located in the CD cavity. Surprisingly, the 2D ROESY spectrum of an ampicillin–β-CD solution clearly indicated inclusion of the guest inside the cavity, and actually in two ways, one with the phenyl ring inside and the other with the penem ring inside. The coexistence of different complexes, which are actually orientational isomers,[16] is in agreement with the results of the previously reported calorimetric studies,[11] and apparently results in the net zero chemical shift displacement of the signals of the β-CD cavity protons. This is possibly due to nearly equal shielding caused by the phenyl ring inclusion and deshielding caused by the penem ring inclusion. Therefore, consideration of chemical shift displacements alone is not safe to document

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inclusion. Also, no chemical shift changes were observed when the cephalosporins **2a** and **2b** were added to solutions of either γ-CD (**3c**) or of the derivative **4c**. However, the ROESY spectra of **3c** or **4c** with the cephalosporins **2a** and **2b** did not show any intermolecular dipolar interactions, although these CDs are wide enough to include part of the cephalosporin molecules. Therefore, the cephalosporins were not further investigated. In contrast, the penicillins induced significant shifts in the signals of H3 and H5 of the wider γ-CD (**3c**) and of **4c** having a longer cavity, indicating complexation through inclusion in solution; therefore, these systems, as amenable to detailed NMR studies, were considered further.

Molar titrations (Fig. 1) and continuous variation (or Job) plots (Fig. 2) were used in order to determine the stoichiometry of the complexes and to evaluate the strength of binding. In the plots of Fig. 1(a–c), the induced chemical shift changes ($\Delta\delta$) of the cavity protons H3 are considerably lower than those of H5, justified by the fact that in the wide γ-CD cavity the narrow-side protons (H5) are closer to the included guest, and thus more affected. The penicillins **1a** and **1b** behave similarly. The shape and curvature of the titration plot of **1a**/**4c** [Fig. 1(b)], **1c**/**3c** [Fig. 1(c)] indicate the formation of a 1 : 1 inclusion complex. For **1a**/**3c** [Fig. 1(a)] the $\Delta\delta$ values do not reach a plateau. Finally, the $\Delta\delta$ values for **1c**/**4c** [Fig. 1(d)], while small, display a turning point close to a guest : host ratio of 1 : 2. The stoichiometry of the inclusion complexes was cross-checked with continuous variation plots. As shown in Fig. 2 the plots for **1a**/**3c** [Fig. 2(a)], **1a**/**4c** [Fig. 2(b)], and **1c**/**3c** [Fig. 2(c)] indicate a stoichiometry

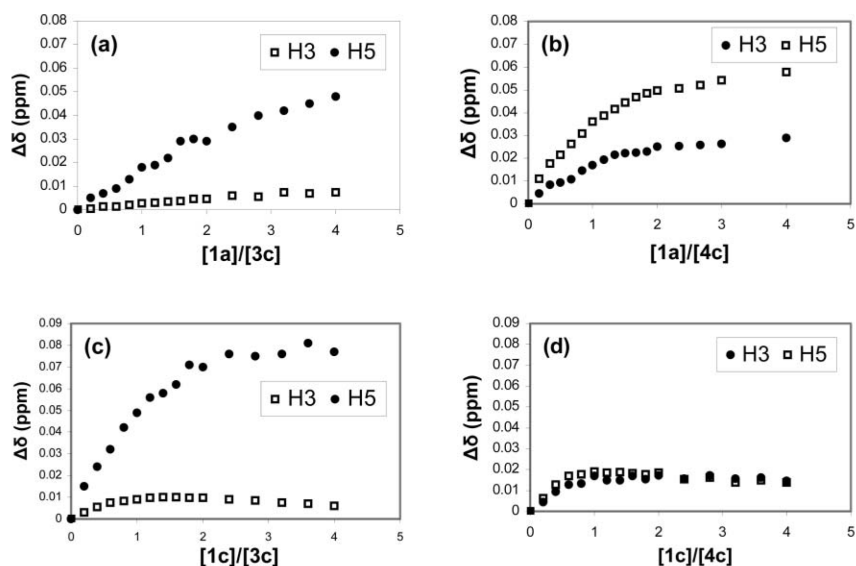

Fig. 1 Molar titration plots at pH = 7.0, 298 K of: (a) γ-CD (**3c**) with ampicillin (**1a**), [**3c**] = 13.45 mM; (b) **4c** with **1a**, [**4c**] = 6.7 mM; (c) **3c** with dicloxacillin (**1c**), [**3c**] = 9.8 mM; and (d) **4c** and **1c**, [**4c**] = 9.8 mM.

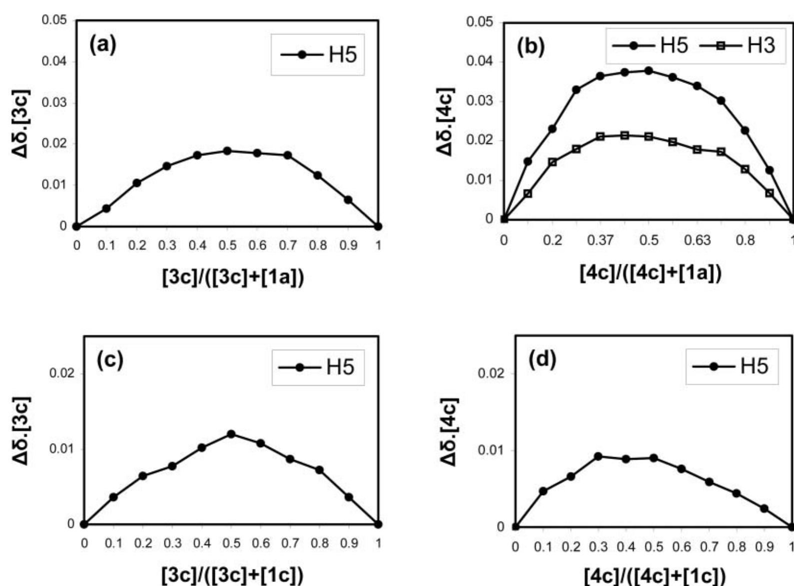

Fig. 2 Continuous variation (Job) plots at pH = 7.0, 298 K of: (a) γ-CD (**3c**) and ampicillin (**1a**) (total concentration 6.0 mM); (b) **4c** and **1a** (total concentration 4.34 mM); (c) **3c** and dicloxacillin (**1c**) (total concentration 1.5 mM); and (d) **4c** and **1c** (total concentration 3.0 mM).

of 1 : 1, whereas the corresponding plot for **1c/4c** [Fig. 2(d)] indicates a 1 : 2 guest : host stoichiometry, in agreement with molar ratio data. The presence of more than one complex in the solution of **1a/3c** could be the reason for the shallow inflection point observed. The chemical shifts of the antibiotics change upon varying their concentration in buffered D_2O (pH 7), indicating self-association phenomena, as previously observed for other water-soluble drugs.[17] As a consequence, continuous variation plots with respect to antibiotics' signals would not be reliable. In summary, data from both the molar ratio and continuous variation plots showed a 1 : 1 stoichiometry for γ-CD complexes, whereas for the carboxylate host **4c** both 1 : 1 and 1 : 2 inclusion complexes are indicated. An interesting observation in the systems studied is that the *external* protons H2 and H4 were continually affected by a gradual increase of the guest concentration, a situation not typically observed. This may arise either from inclusion of only part of the guest molecule, leaving the rest outside the cavity so as to affect the chemical shifts of the external hydrogen atoms of the host, or from externally bound molecules. The differentiation between the two cases was attempted with 2D ROESY and MS experiments, as is shown below.

The 1 : 1 binding constants for **1a/3c**, **1a/4c** and **1c/3c** were calculated by curve fitting of the titration data to the suitable equation derived for NMR data under fast exchange of host–guest complexation.[18] The corresponding values were $19 \pm 4\ M^{-1}$ ($R^2 = 0.991$), $17 \pm 0.9\ M^{-1}$ ($R^2 = 0.992$) and $622 \pm 200\ M^{-1}$ ($R^2 = 0.945$), respectively. Thus, association is weak with ampicillin but much stronger with dicloxacillin, possibly as a consequence of the large cavity of both **3c** and **4c**.

Rotating frame 2D NOESY NMR spectra (ROESY) were used to provide structural information regarding intermolecular interactions. The NMR spectra of **1a**–**c** in D_2O at pH 7.0 were similar and readily assigned with the aid of standard 2D spectra and previous literature.[19] The 2D ROESY spectra of the complexes formed by **3c** with **1a**–**c** showed some common patterns. Intermolecular correlation between the β-lactam protons H_5 and H_6 of antibiotics **1a**–**c** with H3 and H5 of cyclodextrins **3c** and **4c** were observed throughout, showing inclusion of the β-lactam ring inside the cavity. In addition, the aromatic protons [Fig. 3(a)] as well the methyl groups [Fig. 3(b)] of guests **1a**–**c** both correlated with H3 and H5 of γ-CD. Considering the crystal structure of, for example, ampicillin[20] it is evident that the bicyclic system is seriously puckered and the presence of methyl and carboxy substituents makes this part of the antibiotic large enough to occupy the γ-CD cavity, with no space for the simultaneous presence of the phenyl ring. The interaction between aromatic and cavity protons must therefore arise from an alternative mode of inclusion involving the phenyl of **1a**–**c**, *i.e.* in solution there are two 1 : 1 complexes observed, in agreement with titration results. With the longer host **4c**, intermolecular peaks of the β-lactam ring protons as well as the methyl groups [Fig. 4(b)] with H3 and H5 of **4c** were observed, as previously. The aromatic protons, however, showed correlation not only with the cavity proton H5 but also with the aliphatic arms of **4c** [Fig. 4(a)], which indicates a spatial arrangement of the complex in which the aromatic part of the antibiotic is in the primary side amongst the aliphatic substituents of the CD and the thiazolidine ring is close to the secondary side, that is, **4c** practically engulfs ampicillin (**1a**) to form a 1 : 1 complex. Dicloxacillin (**1c**), however, shows a strong

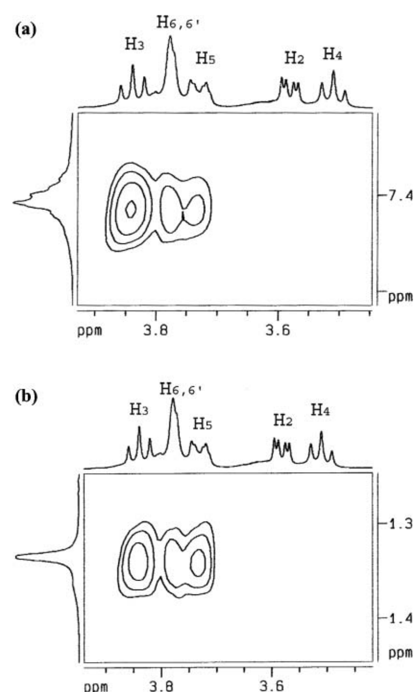

Fig. 3 Partial 2D ROESY maps of **1a/3c** (16 mM/4 mM) in D_2O, pH = 7 and at 298 K: (a) aromatic region of **1a**; (b) methyl groups of **1a**.

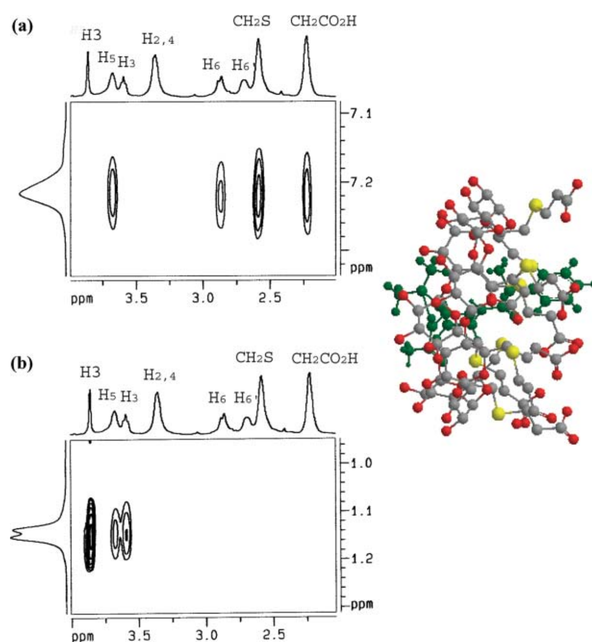

Fig. 4 Partial 2D ROESY maps of **1a/4c** (16 mM/4 mM) in D_2O, at pH = 7 (buffer phosphate) and 298 K: (a) aromatic region of **1a**; (b) methyl groups of **1a**. The singlet at *ca.* δ 3.8 is due to **H3** of **1a** and its strong correlation peak in (b) is intramolecular. A molecular representation of the proposed structure is also illustrated (ampicillin in yellow, sulfur atoms in green).

interaction of the aromatic moiety with the cavity of **4c**, showing the presence of a second host, as previous data indicated, or even a different orientation of the guest. Both **4c** and the antibiotics **1a**–**c** have carboxy groups that are partially charged at neutral

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pH (typically the pK_a of carboxylic acids is about 4.8), therefore electrostatic repulsion between the identically charged groups might lead to a head-to-tail geometry found in the complexes. No interactions were detected between antibiotic protons and external CD protons (H1, H2, H4) in the ROESY spectra.

Mass spectrometry studies

Electrospray mass spectrometry using aqueous solutions is widely used for the study of cyclodextrin complexes,[21] as is thought to realistically reproduce the solution state conditions and consequently to identify the kinds of complexes found in solution. Non-specific host–guest aggregation due to electrostatic interactions, however, has been found to significantly influence the observed signals in ESI-mass spectra of α-CD/aliphatic dicarboxylic acid solutions.[21,22] Such effects are thought to be frequently encountered when investigating hydrophobic complexes by mass spectrometry. In order to examine external interaction *vs.* inclusion complexation, several of the complexes studied previously by NMR have also been examined using negative ion mode ESI-MS at various host : guest ratios. As blank experiments, the ESI-MS of **1a**–**c** were studied in the presence of maltoheptaose, the linear analog of β-CD, which does not possess a cavity, but which can form weak complexes through aggregation or hydrogen-bonding. ESI-MS spectra of solutions of **1a**, **1b** and **1c** and the host **3c**, at a range of ratios, gave similar results. The base peak (Fig. 5) appeared at 207 *m*/*z* (lactam ring-opened fragment, also observed in the pure **1a**–**c**). The ions $[M - H]^-$ of plain **1a**, **1b** and **1c** were found at *m*/*z* 348.4, 364.4 and 468.1, respectively, and the corresponding dimers $[2M - H]^-$ at *m*/*z* 697.6, 729.8 and 939.1, respectively. The molecular ion peak of the host **3c** was located at *m*/*z* 1297. For each kind of complex **1a**–**c**/**3c** a clear peak at *m*/*z* 1471 (Fig. 5), 1479 and 1531, respectively, corresponding to a doubly charged 1 : 2 guest–host complex was observed. Only in the case of amoxicillin (**1b**) was a very weak peak present at *m*/*z* 830, corresponding to the doubly charged 1 : 1 complex. Under the same conditions, the ESI-MS of maltoheptaose with either **1a** or **1b** showed peaks at *m*/*z* 1500 and 1517, respectively (corresponding to a 1 : 1 complex formation) in addition to the above-mentioned peaks due to **1a** or **1b** and maltoheptaose alone ($[M - H]^-$ at *m*/*z* 1151). No evidence of a 1 : 2 complex was found. As the linear heptasaccharide cannot form inclusion complexes the observed 1 : 1 entity must be due to a complex formed through external association. These observations indicate that in solutions of **1a**–**c**/γ-CD cavity inclusion of the drug molecule leads to 1 : 1 complexes, whereas an additional γ-CD molecule forms the 1 : 2 complex through external association. These results are consistent with the stoichiometries obtained by the NMR experiments for the γ-CD inclusion complexes, and also explain the observation of chemical shift displacements observed for the outer protons of the host. Detection of peaks with **4c** was not possible under various conditions, and only in the presence of **1a** was a weak ion (*ca.* 15%) at guest : host 1 : 2 identified, in line with the above. The observed formation of supramolecular complexes or larger 'aggregates' involving both cavity-included and externally associated molecules has been invoked previously[23,24] as being responsible for the increased solubility or bioavailability of drugs in the presence of cyclodextrins. Apart from inclusion, mechanisms such as aggregation, surfactant-like effects or even small variation of the pH, and solvation effects (especially when charged species are involved) may often be in operation when cyclodextrins are considered.[23] This suggestion is supported by the present NMR and MS data. The above findings indicated that cyclodextrins may offer stabilization to the penicillins towards external nucleophilic attack, and this was examined next.

Hydrolysis of ampicillin

The integrity of ampicillin **1a** was monitored by NMR spectroscopy[2b,25] [Fig. 6(a)] over several days under five different

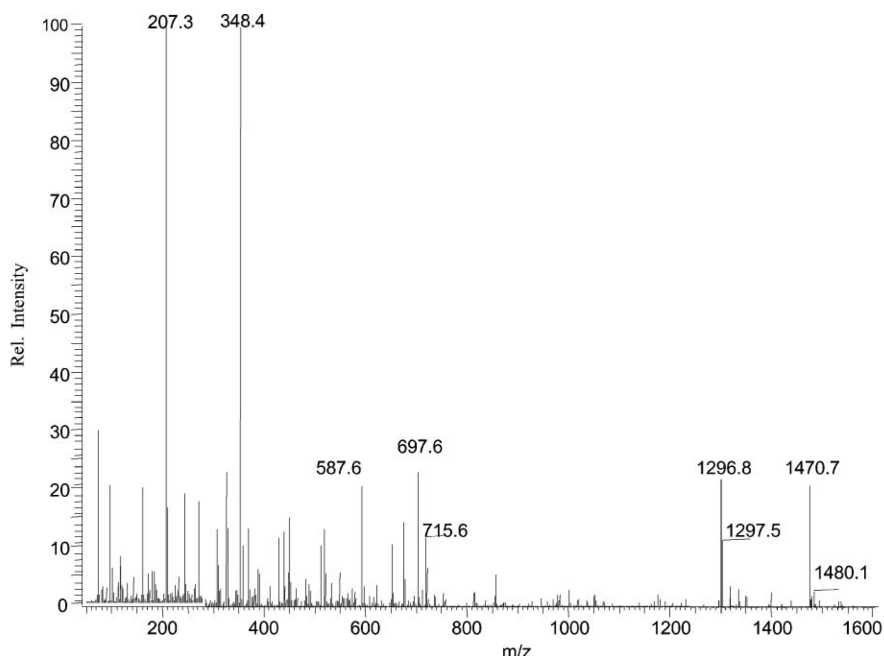

Fig. 5 ESI-MS spectra of **1a**/**3c** (0.5 : 1) in H_2O–MeOH (1 : 1, v/v).

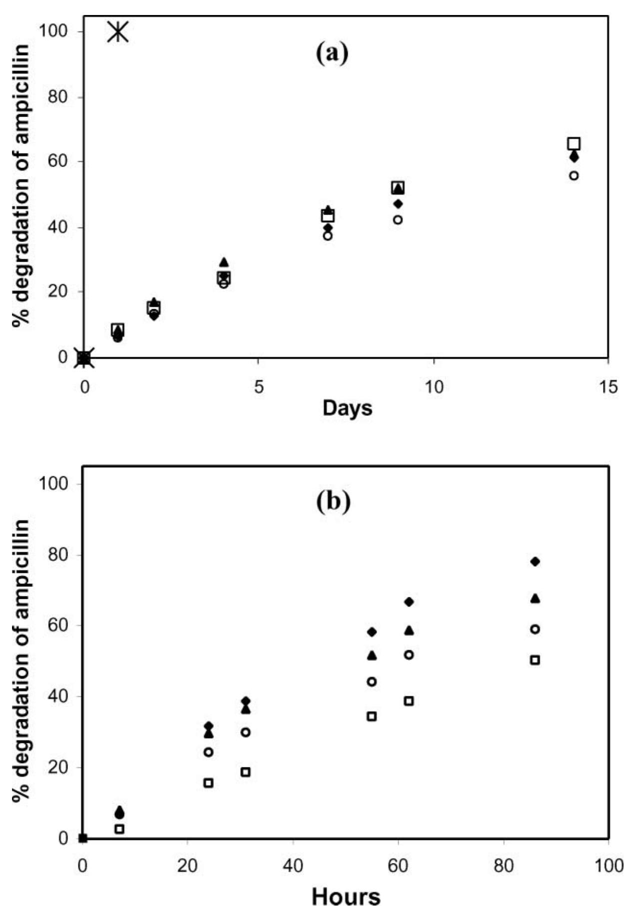

Fig. 6 (a) Degradation of **1a** on its own (◆) and in the presence of an excess of **3c** (○), maltoheptaose (▲), **4c** (□) and **5c** (×); (b) enzymatic (β-lactamase enzyme) degradation of **1a** (◆) and in the presence of an excess of maltoheptaose (▲), **3c** (○) and **4c** (□).

conditions (solutions at pH 7 and 30 °C): **1a** alone, **1a/3c** (1 : 10), **1a**/maltoheptaose (1 : 10), **1a/4c** (1 : 10) and **1a/5c** (1 : 10), where **5c** is a positively charged CD derivative synthesized as reported previously.[15] The host concentrations were high (5–10 fold) to both maximize complex formation and simulate pseudo-first order hydrolysis conditions. The ratio of **1a** to its hydrolyzed derivatives was determined by integration of the ^{1}H NMR signals of either the methyl groups or the protons on the β-lactam ring using both external and internal references. The results are summarized in Fig. 6(a). In the presence of **3c**, the rate of degradation of **1a** was slightly decreased whereas in the presence of **4c** it was slightly increased. The data were fit to a first order rate equation providing reasonably good correlation, namely rate constants of 0.068 d^{-1} (◆, **1a** alone, R^2 = 0.998), 0.058 d^{-1} (○, **1a/3c**, R^2 = 0.995), 0.070 d^{-1} (▲, **1a**/maltoheptaose, R^2 = 0.984) and 0.077 d^{-1} (□, **1a/4c**, R^2 = 0.9572), respectively. The error in the fit was ±0.002–0.008 d^{-1} and the error in the NMR peak integration was estimated to be 5–10%. The β-lactam ring of **1a** should be sheltered in the cavity of **3c** or **4c**, but the data show basically no effect on the hydrolysis rate due to inclusion. The observed slight acceleration with **4c** may be explained by the presence the carboxy groups that may catalyse the hydrolysis of the **1a** over a 15 day period. Maltoheptaose also did not affect the rate of degradation of **1a**, while the amino-γ-CD **5c** [×, Fig. 6(a)] caused complete hydrolysis of **1a** within 24 h, presumably due to the activity of its numerous amino groups. In this latter case there is no inclusion since examination of the corresponding ROESY spectrum of a **1a/5c** solution did not reveal interaction of the drug with the cavity protons. These results demonstrate that the hydrolysis of the β-lactam ring in neutral aqueous solution is not affected much by complexation with the cyclodextrins, a fact that is not surprising if one considers that in solution the most active nucleophile (except **5c**) is water itself and given the small binding constants. It was therefore decided to expose **1a** to a β-lactamase enzyme (serine-type) in the presence of the CDs (**5c** was excluded) in order to assess the true ability of the CDs to decrease the rate or to prevent the hydrolysis of **1a**, either *via* inclusion or by external association, and obstruction of the enzyme. The integrity of **1a** over time was thus followed by ^{1}H NMR for five solutions at pH 7 and 25 °C: **1a** alone, **1a**/β-lactamase, **1a**/maltoheptaose/β-lactamase (1 : 10 : cat), **1a/3c**/β-lactamase (1 : 10 : cat) and **1a/4c**/β-lactamase (1 : 5 : cat) [Fig. 6(b)]. In the presence of β-lactamase, **1a** degraded much faster than in the previous experiment, and within 3.5 d less than 40% was left. In the presence of maltoheptaose and β-lactamase, the rate of degradation of **1a** was slightly decreased [*ca.* 10%, after 84 h (3.5 d)], and therefore the external association does have an adverse effect on the action of the enzyme. In the presence of γ-CD (**3c**) the rate of degradation of **1a** was significantly decreased (*ca.* 20%, after 84 h), as expected; therefore, formation of the inclusion *and* external complex is more effective in protecting the β-lactam ring from enzymatic attack. In the presence of **4c**, the rate of degradation of **1a** is further decreased (*ca.* 30%, after 84 h); therefore, the elongated cavity of **4c**, which was found to form 1 : 2 guest : host complexes, resulted in superior resistance to enzymatic hydrolysis. In a quantitative treatment, the data fitted poorly to an exponential growth equation, but well to a first order rate equation (see Experimental section), supporting pseudo-first order rate conditions. The rate constants found were 0.017 h^{-1} (**1a** + enzyme, ◆, r^2 = 0.998), 0.011 h^{-1} (**1a** + enzyme + **3c**, ○, r^2 = 0.996), 0.014 h^{-1} (**1a** + enzyme + maltoheptaose, ▲, r^2 = 0.984) and 0.008 h^{-1} (**1a** + enzyme + **4c**, □, r^2 = 0.9572), respectively. The error of the fit was less than ±0.0004 h^{-1}. This accuracy in rate constants is unrealistically high, but what is important to note is that all samples (NMR tubes) were incubated under identical conditions, the signals were measured in an identical order each time, and the error in the hydrolysis rate should be the same in all samples; therefore, the observed differences in rates among them can be considered reliable.

From the above it is concluded that the formation of both inclusion complexes and aggregates is, to different degrees, effective in protecting ampicillin **1a** from enzymatic degradation, as the results of **3c**/maltoheptaose show. In particular, ampicillin in the presence of β-lactamase and the carboxylated host **4c** degrades twice as slowly as in the presence of β-lactamase alone.

Conclusions

NMR studies have revealed the inclusion (without change of chemical shifts) of the penicillin antibiotics **1a**–**c** in β-CD (**3b**) and its carboxylated (and elongated) derivative **4b**, whereas no inclusion was observed between **3b** or **4b** and the antibiotics belonging to the cephalosporin family **2**. Surprisingly, compounds

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2 were not encapsulated in the wider cavities of γ-CD (**3c**) and **4c** either. In contrast, all three antibiotics belonging to the penicillin family, **1a**–**c**, formed complexes with both **3c** and **4c**. The opposite behavior of the two antibiotic families may be explained by their different sizes and geometries. Penicillins contain a β-lactam ring fused to a thiazolidine ring, affording a compact butterfly shape able to enter the CDs. Cephalosporins, on the other hand, being of larger dimensions, do not enter the cavities of the CDs (even the wider γ-CDs). The penicillins enter the γ-CD host in two modes, forming primarily 1 : 1 inclusion complexes (with **4c** 1 : 2 complexes also were found) with the β-lactam ring residing inside the CD. Besides inclusion, formation of aggregates between antibiotics and CDs was found to take place in solution, as proved by the ESI-MS data. The documented inclusion of ampicillin in the CD cavity as well as the non-specific interactions with the host molecules do not practically affect the rate of self-hydrolysis of ampicillin; however, they do provide resistance to the β-lactamase enzyme. More specifically, ampicillin degrades in the presence of the enzyme at a rate two-fold higher than in the presence of the enzyme and **4c**. In the latter case the inclusion process was proven to be more effective than that of external association toward stabilization of β-lactam ring of antibiotics, since the cavity effect on the macrocyclic host γ-CD was found to be beneficial *vs.* linear maltoheptaose. The current study may present a means to improve the chemical stability of penicillins, and even reduce their allergenicity and ability to induce bacterial resistance.[10]

Experimental

All reagents used were obtained from Sigma-Aldrich and were not purified further before use. β-Lactamase from *Enterobacter cloacae* ≥97% pure powder was also obtained from Sigma-Aldrich. All solvents used were of reagent grade. When required, anhydrous dimethylformamide (DMF) was distilled over molecular sieves. All moisture-sensitive reactions were carried out under a nitrogen atmosphere. Derivatives **4b** and **4c**[14] and **5c**[15] were prepared by literature procedures.

Buffered solutions were prepared from sodium dihydrogen phosphate–sodium hydrogen phosphate in H_2O or D_2O. The pH was measured using an MP200 Mettler Toledo pH meter. Before each measurement, the electrode system was calibrated in hydrogen ion concentration units ($p[H] = -\log[H^+]$) using Buffer Reference Standard solutions (Scharlau) at 25 °C. The temperature of the solutions during the experiments was maintained to ±1°C.

^1H and ^{13}C NMR one- and two-dimensional spectra were acquired at 500 MHz. ^1H NMR spectra recorded in D_2O were referenced to HOD at δ 4.79, unless otherwise stated. Continuous variation (Job) plots were constructed by mixing varying volumes of equimolar solutions of host and guest molecules to a constant final volume, so that the resulting mixtures ranged from a 90 to 10% ratio of host : guest. All experiments were performed at 25 °C and at buffered pH 7.0.

Titrations were carried out by adding solid guest to a solution of host (around 8 mM, as indicated in each case) in buffered D_2O (pH 7.0). The additions stopped upon the formation of a precipitate. The shifts of the protons of the host molecules were internally referenced with the cyclodextrin H1 signal or HOD. For the 1 : 1 binding constant K_{11}, non-linear least squares fitting was performed with GraphPad PRISM, using the equation[18]

$$1/\Delta\delta_{obs} = 1/K_{11}(\Delta\delta_0[\text{Guest}] - \Delta\delta[\text{CD}]) + \{1/\Delta\delta_0\},$$

which applies when fast exchange is observed between the free constituents and the complex in the NMR.

Hydrolysis of ampicillin (**1a**) was followed by NMR measurements, keeping the samples at buffered pH 7.0 and at 30 °C in a regulated water bath and using a 10-fold molar excess of the cyclodextrins and of maltoheptaose. Hydrolysis of **1a** in the presence of β-lactamase enzyme was followed by NMR, keeping the samples at buffered pH 7.0 and at 25 °C in a regulated water bath and using a 10-fold excess of the cyclodextrins (5-fold of **4c**) and of maltoheptaose. All solutions were incubated 12 h in the water bath before the addition of the enzyme in order to ensure the formation of the complex between **1a** and the CDs. The hydrolyses were run in duplicate. The data obtained were fit to the (pseudo)-first order rate equation $\{\ln[\mathbf{1a}]_{init}/[\mathbf{1a}]_{left}\}$ *vs.* time elapsed, and the slope provided the calculated rate constant.

Mass spectra by ESI were recorded at the 'Mass Spectrometry and Dioxin Analysis Lab', NCSR "Demokritos". Solutions in 50% aqueous methanol were infused into an electrospray interface mass spectrometer (AQA Navigator, Finnigan) at a flow rate of 0.1 ml min^{-1}, using a Harvant Syringe pump. Negative or positive ion ESI spectra were acquired by adjusting the needle and cone voltages accordingly, best results being obtained when using the negative mode at cone voltage −70 V. Hot nitrogen gas (Dominic-Hunter UHPLCMS-10) was used for desolvation at 170 °C.

Acknowledgements

GSRT of Greece and EU, program 'Excellence in the Research Institutes' in the frame of articles 4 & 6 of N.2860/00 and EU regulations 1260/99and 438/01 (for support to D. M.) is gratefully acknowledged.

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