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ORIGINAL ARTICLE

Calcium and zinc complexes of pyrroglutamate analogs detected by electrospray ionization mass spectrometry

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Abstract The complexation of calcium and zinc cations by pyrroglutamate analogs has been studied in the gas phase by means of electrospray ionization mass spectrometry (ESI–MS). Complexes were obtained from the solutions of calcium perchlorate and zinc perchlorate in acetonitrile. The complexes with calcium are singly and doubly charged with various stoichiometries while zinc complexes are singly charged except for one ligand. Solvation with acetonitrile and presence of perchlorate counter-ions are observed when the complexes are in the gas phase. The complexes formed with both metals are mainly L_2M and LM species. All tested compounds are better complexing agents for calcium than for zinc.

Keywords Non natural aminoacids · Aminophosphonates · Aminobisphosphonates · Zinc complexes · Calcium complexes · Electrospray ionization mass spectrometry

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Introduction

Non-natural aminoacids and their aminophosphonic analogs have numerous biological activities useful in medicinal chemistry, such as inhibitors of various proteolytic enzymes, agonists or antagonists of CNS (central nervous system) receptors, and metal complexing agents (Kafarski and Lejczak 2001; Braeuner-Osborne et al. 2000; Kiss et al. 1994; Bessis et al. 2003; Naydenove et al. 2007; Kudzin et al. 2008). Geminal bisphosphonic acids are metabolically stable analogs of naturally occurring inorganic pyrophosphates (Masschelein and Stevens 2007; Haelters et al. 2008). Fleisch et al. (1969) showed that such compounds are able to impair the formation and dissolution of calcium phosphate in vitro, due to their capacity of chelating divalent cations. Several bisphosphonic acids are currently used for the treatment of various diseases of bone mineral metabolism like osteoporosis, Paget's disease, tumor osteolysis or hypercalcemia of malignancy (Fleisch et al. 1966, 1969; Francis et al. 1969; Widler et al. 2002). In the course of discovery programs dedicated to the search of penicillin binding protein (PBP) inhibitors, we have previously prepared several aminoacid, aminophosphonic and aminobisphosphonic derivatives related to glutamate, pyrroglutamate and aspartate, and evaluated their activities against several β-lactamases (bacterial defence enzymes) and R39 enzyme (soluble enzyme used as a model of membrane-bound bacterial DD-transpeptidase-carboxypeptidases) (Beck et al. 2008, 2009a, b). Selected compounds behaved as modest to good inhibitors of class A and class D β-lactamases, and R39 enzyme, which are all serine proteases. Unfortunately, none of these compounds were active against zinc β-lactamases (class B). In order to understand this absence of activity, the complexing ability of our molecules toward zinc cation has been studied by

electrospray ionization mass spectrometry (ESI–MS). Because of the potential application in the treatment of various bone diseases of calcium complexing agents, the chelation potential of our molecules toward calcium cation has been parallely investigated using ESI–MS. Among the series of molecules available (Beck et al. 2009a, b), five representatives were chosen for this study, which possess structural similarities (Fig. 1). Four of them are pyrrolidinones, whereas one is a non-cyclic amide. Compounds **1** and **2** are α-aminoacid derivatives; **3**, **4** and **5** are α-aminophosphonic derivatives. All compounds feature a quaternary α carbon atom-bearing ester chains. In particular, **5** displays a geminal bisphosphonate structure.

The correct attribution of supramolecular structures which are present in relatively small amounts, through the use of traditional tools such as UV/CD or NMR is a very difficult task. ESI–MS, an accurate method generally coupled to liquid chromatography for the analysis of aminoacids (Langrock et al. 2006) has been shown to be well suited for the study of singly and multiply charged metal-coordinated species. This technique permits the supramolecular species to be transferred from the solution to the gas phase (Schalley 2001). Due to the high sensitivity of this technique, only very little quantity of sample is required. This soft ionization process allows detecting their formed species and determining their stoichiometry as illustrated through numerous complexation studies with Ca, Mg, Zn or Pt (Sudhir et al. 2005; Mineo et al. 2002; Frański and Gierczyk 2005; Frańska 2007; Konaklieva et al. 2007; Maton et al. 2005).

The purpose of this work is to investigate the calcium and zinc complexations with pyrroglutamate analogs **1**–**5** by means of the mild and readily accessible ESI–MS technique.

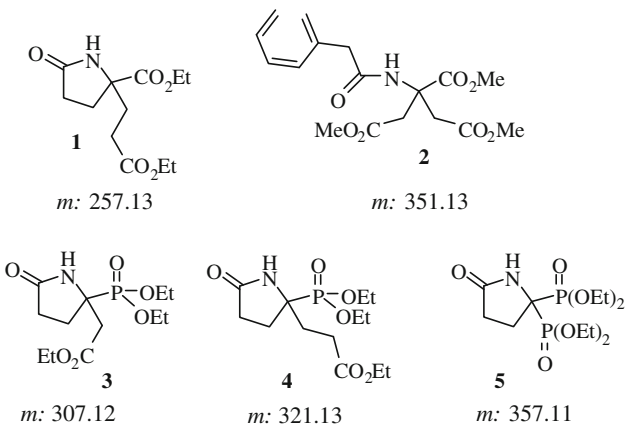


Fig. 1 Structures of the analyzed compounds **1**–**5** and their corresponding monoisotopic masses

Materials and methods

Materials

Molecules **1**–**5** have been synthesized as previously described (Beck et al. 2009a, b). In Brief, the Schiff's base prepared by condensation of glycine ethyl ester with *m*-chlorobenzaldehyde is treated with potassium carbonate and an excess of ethyl acrylate (EtOH, 20°C, 16 h); imine hydrolysis of the resulting bis-Michael adduct (1 N HCl, CH_3CN, 20°C, 30 min then basic workup) leads directly to **1** in 45% overall yield after chromatography on silica gel (Scheme 1).

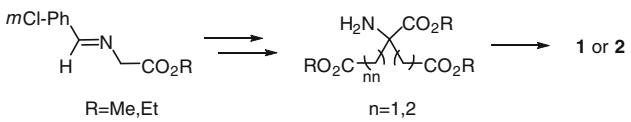
Similarly, the Schiff's base prepared by condensation of glycine methyl ester with *m*-chlorobenzaldehyde is treated with methyl bromoacetate in excess (Bu_4NBr_{catal}, K_2CO_3, CH_3CN, 50°C, 27 h); imine hydrolysis as above and acylation with phenylacetylchloride (pyridine, CH_2Cl_2, 0–20°C, 5 h) gives **2** in 27% overall yield after chromatography on silica gel (Scheme 1).

The Schiff base obtained by condensation of diethyl (aminomethyl)phosphonate with *m*-chlorobenzaldehyde is first monoalkylated with ethyl bromoacetate (LDA, THF, −78°C) and then alkylated again with ethyl acrylate in the presence of sodium ethoxide (EtOH, reflux, 2 h). Imine hydrolysis (1 N HCl, CH_3CN, 20°C, 30 min, then aqueous NaOH work-up) and spontaneous cyclization affords **3** in 23% overall yield after chromatography on silica gel (Scheme 2).

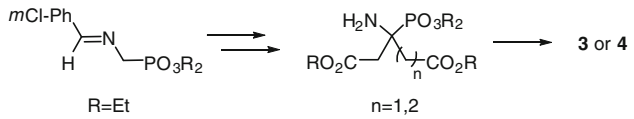
The previous Schiff's base can be symmetrically bis-alkylated by treatment with an excess of ethyl acrylate (EtOH, EtONa, 80°C, 24 h). Imine hydrolysis as above leads to **4** in 25% overall yield after chromatography on silica gel (Scheme 2).

Lastly, the Schiff's base resulting from the condensation of tetraethyl (aminomethylene) diphosphonate with *m*-chlorobenzaldehyde is reacted under Michael addition conditions (Ethyl acrylate, EtOAc, EtOH, 80°C, 2 h); further imine hydrolysis furnishes **5** in 34% overall yield after chromatography on silica gel (Scheme 3).

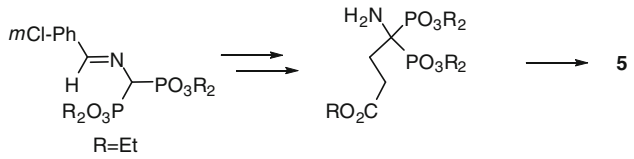
*5-Carbethoxy-5-(2-carbethoxyethyl)-2-pyrrolidinone (±**1**)* ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.23 (t, $^3J_{(H,H)}$ = 7.2 Hz, 3H, CH_3), 1.30 (t, $^3J_{(H,H)}$ = 7.2 Hz, 3H, CH_3), 2.05–2.11 (m, 2H, CH_2), 2.19–2.25 (m, 1H, CH_2), 2.31–2.35 (m, 2H, CH_2), 2.37–2.41 (m, 2H, CH_2), 2.45–2.51 (m, 1H, CH_2), 4.13 (q, $^3J_{(H,H)}$ = 7.2 Hz, 2H,



Scheme 1 Synthesis of molecules **1** and **2**



Scheme 2 Synthesis of molecules **3** and **4**



Scheme 3 Synthesis of molecule **5**

OCH_2), 4.22 (q, $^3J_{(H,H)} = 7.2$ Hz, 2H, OCH_2), 6.41 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.3 (CH_3), 29.3 (CH_2), 29.8 (CH_2), 30.6 (CH_2), 33.8 (CH_2), 61.1 (CH_2), 62.2 (CH_2), 64.9 (C), 172.6 (CO ester), 173.2 (CO ester), 177.1 (CO lactam).

*Trimethyl 2-(2-phenylacetamido)aminopropane-1,2,3-tricarboxylate (**2**)* ^1H NMR ($CDCl_3$, 300 MHz) δ 2.85 (d, $^2J_{(H,H)} = 15.5$ Hz, 2× 1H, CH_2), 3.53 (s, 2H, CH_2), 3.56 (s, 6H, 2× OCH_3), 3.63 (d, $^2J_{(H,H)} = 15.5$ Hz, 2× 1H, CH_2), 3.79 (s, 3H, OCH_3), 6.87 (br s, 1H, NH), 7.23–7.40 (m, 5H, CH_{aryl}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 39.7 (CH_2), 44.5 (CH_2), 52.0 (CH_3), 53.6 (CH_3), 59.2 (C), 127.5 (CH_{Ar}), 129.0 (CH_{Ar}), 129.4 (CH_{Ar}), 134.7 (CH_{Ar}), 170.0 (CO ester), 171.0 (CO ester), 171.8 (CO amide) ppm.

*5-Diethoxyphosphoryl-5-(ethoxycarbonyl)methyl-pyrrolidin-2-one (±**3**)* ^1H NMR ($CDCl_3$, 300 MHz) δ 1.28 (t, $^3J_{(H,H)} = 7.1$ Hz, 3H, CH_3), 1.35 (td, $^3J_{(H,H)} = 7.1$ Hz, $^4J_{(H,P)} = 2.7$ Hz, 6H, 2× CH_3), 2.22 (m, 1H, CH_2), 2.36 (m, 1H, CH_2), 2.57 (m, 3H, CH_2), 2.90 (dd, $^2J_{(H,H)} = 15.1$ Hz, $^3J_{(H,P)} = 7.3$ Hz, 1H, CH_2), 4.18 (m, 6H, 3× OCH_2), 6.21 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2 (CH_3), 16.7 (d, $^3J_{(C,P)} = 5.6$ Hz, CH_3), 28.5 (CH_2), 29.7 (CH_2), 40.7 (d, $^3J_{(C,P)} = 9.4$ Hz, CH_2), 57.8 (d, $^1J_{(C,P)} = 164.7$ Hz, C), 61.3 (CH_2), 63.3 (d, $^2J_{(C,P)} = 7.6$ Hz, CH_2), 63.8 (d, $^2J_{(C,P)} = 7.4$ Hz, CH_2), 169.6 (d, $J_{(C,P)} = 11$ Hz, CO ester), 177.3 (d, $^3J_{(C,P)} = 3.7$ Hz, CO lactam) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz) δ 24.6 ppm.

*5-(Diethoxyphosphoryl)-5-[2-(ethoxycarbonyl)ethyl]-pyrrolidin-2-one (±**4**)* ^1H NMR ($CDCl_3$, 300 MHz) δ 1.25 (t, $^3J_{(H,H)} = 7.1$ Hz, 3H, CH_3), 1.34 (td, $^3J_{(H,H)} = 7.1$ Hz, $^4J_{(H,P)} = 2.3$ Hz, 6H, 2× CH_3), 1.97 (m, 2H, CH_2), 2.36 (m, 6H, 3× CH_2), 4.16 (m, 6H, 3× OCH_2), 6.24 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.3 (CH_3), 16.7 (d, $^3J_{(C,P)} = 5.6$ Hz, CH_3), 27.3 (CH_2), 28.5 (d, $^3J_{(C,P)} = 7.5$ Hz, CH_2), 30.0 (CH_2), 30.7 (d, $^3J_{(C,P)} = 9.2$ Hz, CH_2), 59.3 (d, $^1J_{(C,P)} = 162.2$ Hz, C), 61.0 (CH_2), 63.1 (d, $^2J_{(C,P)} = 7.8$ Hz, CH_2), 63.7 (d, $^2J_{(C,P)} = 7.4$ Hz, CH_2), 173.0 (CO ester), 178.0 (d, $^3J_{(C,P)} = 7.4$ Hz, CO lactam) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz) δ 26.2 ppm.

*2,2-Bis-(diethoxyphosphoryl)-pyrrolidin-5-one (**5**)* ^1H NMR ($CDCl_3$, 300 MHz) δ 1.36 (t, $^3J_{(H,H)} = 7.1$ Hz, 12H, 4× CH_3), 2.55 (m, 4H, 2× CH_2), 4.24 (m, 8H, 4× OCH_2), 5.84 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) δ 16.5 (CH_3), 24.8 (CH_2), 29.2 (CH_2), 58.7 (t, $^1J_{(C,P)} = 152.5$ Hz, C), 177.7 (CO lactam) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz) δ 19.8, 19.9 ppm.

Mass spectrometry

All measurements were carried out in acetonitrile (HPLC grade, Fisher Scientific). The salts used for the complexation studies were calcium perchlorate and zinc perchlorate. They were purchased from Alpha (highest quality available) and were vacuum-dried over P_2O_5.

Mass spectra were obtained on a ESI–MS triple quadrupole, TSQ Quantum (Thermo FINNIGAN, San Jose, CA, USA). The spectra were recorded in the positive ion mode. The ESI source was operated at 3.3 kV, and the heated capillary set at 260°C. Different temperatures and tube lens voltages and CID sources have been tested to set the optimal conditions for detecting complexes and not adducts statistically formed in the ESI source. For instance, capillary temperature was varied between 150 and 320°C.

The perchlorate salts were added to 10 mL of acetonitrile solution containing one of the ligands **1–5** at a concentration of 10^{-4} M. Then four solutions were prepared by mixing the solution of salt previously prepared and solutions of ligand at a concentration of 10^{-4} M in order to study the formation of complexes according to the number of salt equivalents (0.5, 1, 2, 5 equiv.). The solution obtained was then directly infused into the ESI source, the flow rate was 3–7 μL min^{-1}. Nitrogen was used as the nebulizing and desolvating gas at flow rates of 100 and 300 L h^{-1}, respectively. Every scan was recorded from 200 to 1500 *m/z* in no less than 5 s and at least 20 scans were averaged to yield the final spectrum. Every spectrum was reproduced three times. The spectra were recorded in profile mode, for three different SCID (Source Collision Induced Dissociation) values: 0, −20, −40 V but only results at −20 V are presented here except for compound **5** (−40 V). These SCIDs have been chosen because they permit to maximize the desolvation and to minimize the fragmentation. To check the attribution of the observed peaks, their isotopic patterns were compared with the simulated pattern of their suggested molecular formula.

In order to represent the evolution of each species in function with the metal concentration, the surface of the peaks has been integrated and reported to the integral sum of all peaks in each spectrum. The isotopic clusters with a

relative intensity less than 5% were not taken into account. This representation is not a real quantification but rather a visualization of different species present at each cation concentration. Mass spectrometry measurements were performed on pure compounds **1**–**5** before complexation experiments. For all compounds, the most intense signal in the spectrum corresponded to singly charged ligand (L) cationized by sodium ion $[LNa]^+$.

Biochemical assays

The enzymes were produced and purified at the University of Liège (CIP-ULg, Belgium). BcII (Carfi et al. 1998) and VIM-4 (Pournaras et al. 2002) are zinc β-lactamases of class B, and OXA-10 (Paetzel et al. 2000) is a serine β-lactamase of class D. The catalytic parameters of the tested enzymes are as follows: k_{cat} (s^{-1}) = 40 (BcII), 40 (VIM-4) and 400 (OXA-10); K_m (μM) = 10 (BcII), 10 (VIM-4) and 20 (OXA-10); k_{cat}/K_m ($M^{-1}s^{-1}$) = 4×10^6 (BcII), 4×10^6 (VIM-4) and 2×10^7 (OXA-10).

The enzymes (1–100 nM) were incubated with the tested compounds (100 μM) in phosphate (50 mM, pH 7) or acetate buffer (50 mM, pH 5). In the case of metallo-enzymes (class B), $ZnCl_2$ (50 μM) added to HEPES buffer (10 mM, pH 7) was used. The tested compounds were dissolved in DMSO at 10–100 mM and then diluted with the buffer (final concentration of DMSO in the test solution ≤2%); ≤2% DSMO had no effect on the enzyme activity. The hydrolysis rate of nitrocefine was followed by spectrophotometry at 482 nm with UVIKON 860, 940 and XL instruments connected to a computer through an RS232 line. The residual activity was determined by comparison with the variation of the absorbance of the reference (sample without inhibitor). The results are expressed as a percentage of the initial activities; variations of results are within an error of ±3%. All experiments were performed at least three times.

Results and discussion

Four solutions of ligands mixed with different quantities of calcium perchlorate or zinc perchlorate solutions were prepared and analyzed by ESI–MS. The solution 1 contains 1 equiv. of ligand and 0.5 equiv. of salt, the solution 2 contains 1 equiv. of ligand and 1 equiv. of salt, the solution 3 contains 1 equiv. of ligand and 2 equiv. of salt and the solution 4 contains 1 equiv. of ligand and 5 equiv. of salt.

Complexes between calcium and ligands

In solutions 1–3, uncomplexed ligand is still remaining as indicated by $[LH]^+$ and $[LNa]^+$ signals. Even if LM species could be observed in the solution 4 (5 equiv. of Ca^{2+}), the signals arising from the salts totally dominated the spectrum and it was the case for all the ligands. Singly and doubly charged complexes have been observed; as typical examples, all the species formed when ligands were mixed with 1 equiv. of calcium salt (solution 2), are summarized in Table 1.

When ligand **1** was treated with 0.5 equiv. of calcium salt (solution 1), the complexes observed are L_2Ca and L_3Ca species as indicated by signals at *m/z* 276.7 ($[L_2Ca]^{2+}$), 405.1 ($[L_3Ca]^{2+}$) and 652.8 ($[L_2CaClO_4]^+$). Suspected doubly charged ions were clearly confirmed using the possible enhanced resolution of the Quantum instrument. In solutions 2 and 3, the quantities of L_2Ca complexes diminished and LCa complexes were visible on the spectrum as recorded by signals at *m/z* 395.6 ($[LCaClO_4]^+$) and 436.6 ($[LCaClO_4CH_3CN]^+$). These ions were confirmed as singly charged complexes from their isotopic peaks, which were clearly separated by 1 mass unit. Only traces of complexes L_2Ca_2 have been recorded: $[L_2Ca_2(ClO_4)_3]^+$ at 892.6 *m/z*. The spectra of ligand **1** in solution 1–4 are presented in Fig. 2.

The complexes recorded for ligand **2** in solution 1 are L_2Ca and LCa species at *m/z* 370.7 ($[L_2Ca]^{2+}$), 489.7 ($[LCaClO_4]^+$) and 840.6 ($[L_2CaClO_4]^+$). When the concentration of Ca^{2+} increased (solutions 2 and 3), the proportion of LCa complex increased compared to those of L_2Ca complexes, and L_2Ca_2 complexes appeared.

The structures of ligands **3** and **4** differ just by an additional methylene group on the lateral chain and their behavior in the presence of calcium is very similar. The most intense signals in the spectrum of solution 1 corresponded to L_2Ca complexes with traces of L_3Ca complex and LCa complex in the case of ligand **4**. When the ligands **3**–**4** were treated with 1 equiv. of calcium perchlorate (solution 2), L_2Ca and LCa species have been observed (Table 1). In the case of ligand **4**, the complexes $[L'CaClO_4]^+$ at *m/z* 321.6 and $[L'CaClO_4CH_3CN]^+$ at *m/z* 362.7 were formed, respectively, from the complexes $[LCaClO_4]^+$ at *m/z* 459.7 and $[LCaClO_4CH_3CN]^+$ at *m/z* 500.7 by loss of diethyl phosphite molecule. Fang and co-workers have already observed the loss of alkyl phosphite during the study of [(4-substituted benzoylamino)phenylmethyl] phosphonic acid diisopropyl esters under ESI–MS (Fang et al. 2007). Only traces of $[L_2Ca_2(ClO_4)_3]^+$ complexes have been detected at *m/z* 1300.0. In solution 3, the main complex observed is LCa species although small amount of L_2Ca complexes were still visible.

Ligand **5** has been studied at higher SCID of −40 V because this condition permits to maximize the desolvation and to minimize the fragmentation. When SCID = 0 and −20 V, the quantities of complexes LM and L_2M formed are lower. No complex was observed when mixing **5** and

Table 1 Positive ESI–MS data for compounds **1–5** (L) mixed with 1 equiv. of calcium perchlorate (solution 2) in acetonitrile

Complexes[a]	**1**	**2**	**3**	**4**	**5**
$[LH\text{-}HP(O)(OEt)_2]^+$	/	/	/	183.7 (4)	/
$[LH]^+$	257.7 (10)	/	/	/	/
$[L_2Ca\text{-}HP(O)(OEt)_2]^{2+}$	/	/	257.6 (7)	/	/
$[L'CaClO_4]^+$	/	/	/	321.6 (8)	/
$[L_2Ca]^{2+}$	276.7 (8)	370.7 (16)	326.7 (11)	/	376.4 (9)
$[L'CaClO_4CH_3CN]^+$	/	/	/	362.7 (8)	/
$[LCaClO_4]^+$	395.6 (12)	489.7 (26)	445.6 (14)	459.7 (13)	495.2 (6)
$[LCaClO_4CH_3CN]^+$	436.6 (8)	/	486.6 (9)	500.7 (9)	/
$[L_2CaClO_4]^+$	652.8 (11)	840.6 (17)	752.7 (22)	780.8 (16)	852.2 (73)
$[L_2Ca_2(ClO_4)_3]^+$	892.6 (5)	1080.5 (14)	990.6 (8)	1018.6 (6)	/
$[L_3Ca_2(ClO_4)_3]^+$	/	/	1300.0 (7)	/	/

Number in parentheses indicates the percentage of each species calculated as explained in Materials and methods

[a] L′ = L-diethylphosphite

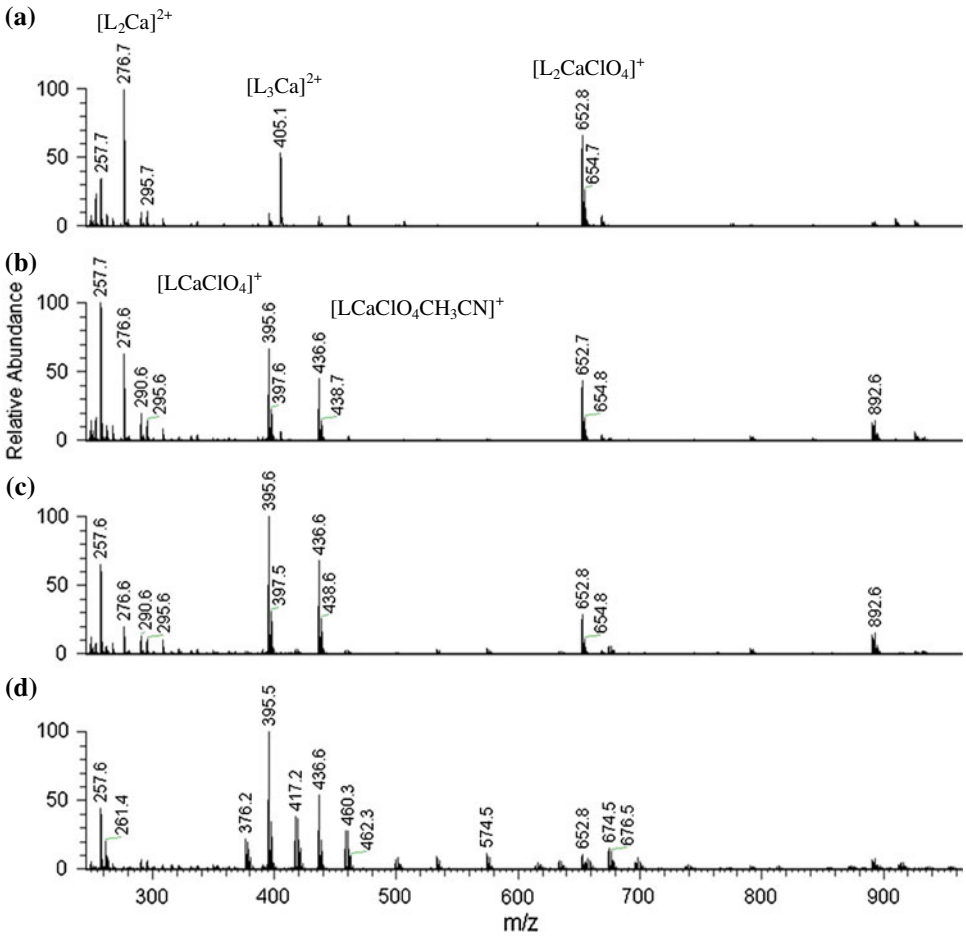


Fig. 2 ESI mass spectra obtained from acetronitrile solutions containing ligand **1** and $Ca(ClO_4)_2$ at different concentrations at SCID = −20 V: **a** 1 equiv. ligand **1** and 0.5 equiv. $Ca(ClO_4)_2$; **b** 1 equiv. ligand **1** and 1 equiv. $Ca(ClO_4)_2$; **c** 1 equiv. ligand **1** and 2 equiv. $Ca(ClO_4)_2$; **d** 1 equiv. ligand **1** and 5 equiv. $Ca(ClO_4)_2$

0.5 equiv. of calcium salt; only signals belonging to **5** were recorded. The most intense signals on the spectrum of solution 2 corresponded to L_2Ca complexes (84% of signals of the spectrum) with trace of LCa complex as revealed at *m/z* 376.4 ($[L_2Ca]^{2+}$), 495.2 ($[LCaClO_4]^+$) and 852.2 ($[L_2CaClO_4]^+$) (Table 1). In solution 3, LCa and L_2Ca were observed approximately in the same proportion.

The presence of acetonitrile and perchlorate in the complexes indicates that the ligands cannot fulfill the coordination sphere of the cation. The complexes more often observed are $[L_2CaClO_4]^+$, $[LCaClO_4]^+$ and $[LCaClO_4CH_3CN]^+$. The small amounts of complexes featuring two Ca^{2+} cations could be real binuclear complexes or aggregates formed during the ESI process.

Complexes between zinc and ligands

In solutions 1–3, a lot of signals of ligands **1** and **2**, which are not chelated by zinc, were recorded. Even if several complexes could be observed in solution 4, the signals arising from the salts dominated totally the spectrum for all the ligands, as before. Only singly charged complexes were observed, except for ligand **2**. All the species formed when ligands **1–5** were mixed with 1 equiv. of zinc salt (solution 2) are summarized in Table 2.

When ligand **1** was treated with 0.5 equiv. of zinc salt (solution 1), the signals observed corresponded to $[LH]^+$, L_2Zn and L_3Zn species (Fig. 3). Only one L_2Zn complex has been identified: $[L_2ZnClO_4]^+$ at *m/z* 676.7.

Fragmentation experiment allowed us to identify other L_2Zn complexes at *m/z* 577.1 and 531.0 as shown in Fig. 4. There is a loss of 100 Da between $[L_2ZnClO_4]^+$ at *m/z* 677.2 and the fragment at *m/z* 577.1. Two possibilities could explain this fragmentation. The first hypothesis is the elimination of ethyl acrylate. This very common fragmentation process corresponds to a retro-Michael reaction and has already been observed by Shen et al. (2004). The second hypothesis is the loss of $HClO_4$, and then the complex at *m/z* 577.1 should be attributed to $[L_2Zn\text{-}H]^+$. Mineo et al. (2002) have already observed the substitution of labile proton by zinc during the study of L-carnosine/zinc complexes by ESI–MS. Simulation of the isotopic pattern revealed two patterns too similar for allowing unambiguous distinction between these two losses. The complex at *m/z* 531.0 could be explained by loss of EtOH, giving the formula $[LL''Zn\text{-}H]^+$. In solution 2, the observed complexes were L_2Zn, L_3Zn and LZn species. The main formed complexes were $[LZnClO_4]^+$ at *m/z* 419.4 in the solution 3 while the proportion of L_2Zn and L_3Zn complexes diminished. Only LZn species were observed in the solution 4.

There is still free ligand **2** in solutions 1–3 as revealed by signals at *m/z* 351.7 ($[LH]^+$) and 373.7 ($[LNa]^+$. The complexes observed in solutions 1 and 2 were L_2Zn and LZn species (Table 2). The proportion of L_2Zn complexes decreased whereas the proportion of LZn increased in solution 3. LZn and trace of L_2Zn complexes have been detected in solution 4.

Ligands **3** and **4** behaved similarly toward zinc. In solution 1, most of the signals (62% for **3** and 77% for **4**) on the spectrum belonged to free ligands. Only one complex has been observed and attributed to $[L_2Zn\text{–}H]^+$ at *m/z* 676.8 (for **3**) and 704.8 (for **4**). The loss of 100 Da toward $[L_2ZnClO_4]^+$ complex could result only from the loss of perchloric acid since the elimination of ethyl acrylate is not possible for ligand **3**. This result suggests that the same fragmentation process already observed with ligand **1**, is most probably caused by the loss of perchloric acid rather than by the elimination of ethyl acrylate. When the ligands were mixed with 1 equiv. of zinc salt (solution 2), the proportion of non chelated ligands decreased whereas the proportion of L_2Zn complexes increased and trace of $[LZnClO_4]^+$ was detected at *m/z* 469.6 (for **3**) and 483.7 (for **4**). The peak at *m/z* 538.7, only observed for ligand **3**, was attributed to $[LL'Zn\text{-}H]^+$ fragment formed by loss of perchloric acid and phosphonate group. In solution 3, the complexes observed were L_2Zn, LZn and L_2Zn_2 species. Finally, the same species were detected in solution 4.

Ligand **5** has been studied at SCID = −40 V. No complex was observed when mixing **5** and 0.5 or 1 equiv. of zinc salt (solutions 1 and 2); only signals belonging to **5** were recorded. In solution 3, LZn and L_2Zn species were observed approximately in the same proportion as shown in Table 2. LZn observed complexes are $[LZnClO_4]^+$ and $[LZnClO_4CH_3CN]^+$. It is surprising to observe a complex containing a molecule of acetonitrile at this SCID. Traces of L_2Zn_2 complexes have also been recorded. Only one

Table 2 Positive ESI–MS data for compounds **1–5** (L) mixed with 1 equiv. of zinc perchlorate (solution 2) in acetonitrile

Complexes[a]	**1**	**2**	**3**	**4**	**5**[b]
$[LH\text{-}HP(O)(OEt)_2]+$	/	/	169.5 (19)	183.7 (22)	/
$[LH]^+$	257.5 (16)	351.7 (8)	/	/	/
$[LNa]^+$	/	373.6 (9)	329.7 (11)	343.7 (5)	379.3 (3)
$[L_2Zn]^{2+}$	/	382.5 (8)	/	/	/
$[LZnClO_4]^+$	419.4 (11)	531.6 (23)	469.7 (7)	483.7 (6)	519.1 (30)
$[LZnClO_4CH_3CN]^+$	/	/	/	/	557.6 (3)
$[LL''Zn\text{-}H]^+$	530.7 (9)	/	/	/	/
$[LL'Zn\text{-}H]^+$	/	/	538.7 (9)	/	/
$[L_2Zn\text{-}H]^+$	576.7 (14)	/	676.8 (30)	704.8 (26)	776.3 (22)
$[L_2ZnClO_4]^+$	676.7 (7)	866.8 (21)	776.8 (7)	804.8 (17)	876.3 (12)
$[L_2Zn_2(ClO_4)_3]^+$	/	/	/	/	1138.5 (6)
$[L_3Zn(ClO_4)_3]^+$	934.0 (13)	/	/	/	/

Number in parentheses indicates the percentage of each species calculated as explained in Materials and methods

[a] L″ = L-EtOH

[b] No complexes were detected in solution 2; complexes presented were recorded in solution 3

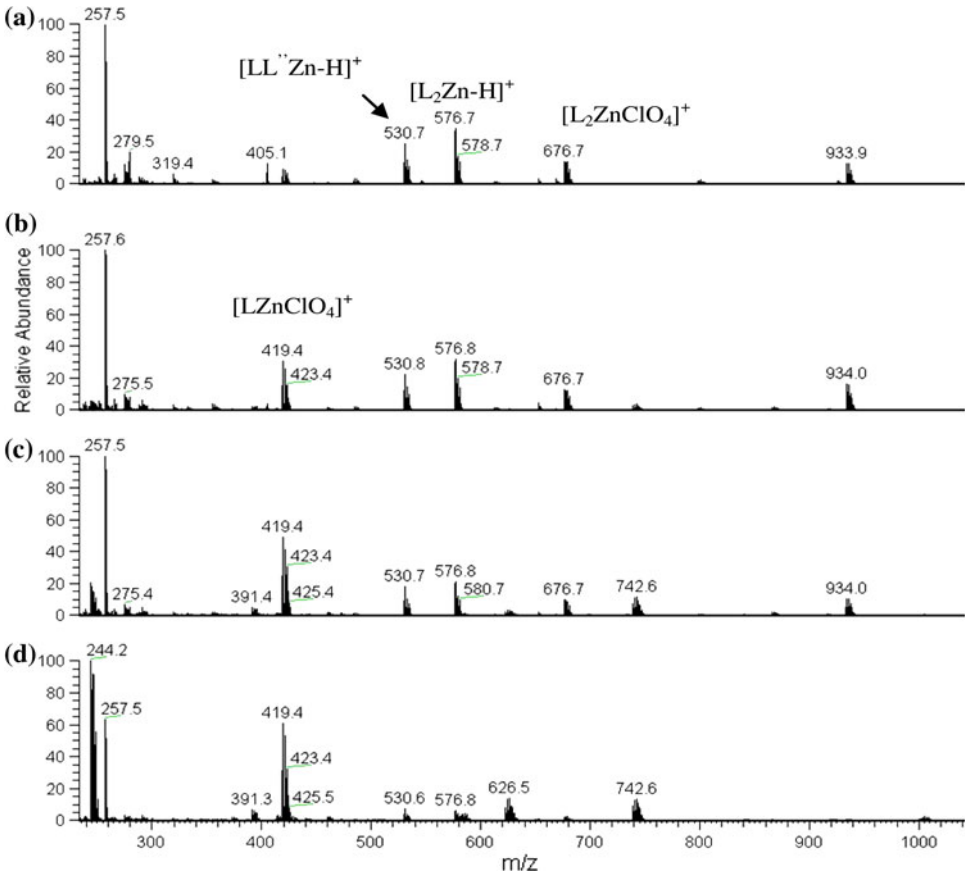


Fig. 3 ESI mass spectra obtained from acetronitrile solutions containing ligand **1** and $Zn(ClO_4)_2$ at different concentrations at SCID = −20 V: **a** 1 equiv. ligand **1** and 0.5 equiv. $Zn(ClO_4)_2$; **b** 1 equiv. ligand **1** and 1 equiv. $Zn(ClO_4)_2$; **c** 1 equiv. ligand **1** and 2 equiv. $Zn(ClO_4)_2$; **d** 1 equiv. ligand **1** and 5 equiv. $Zn(ClO_4)_2$

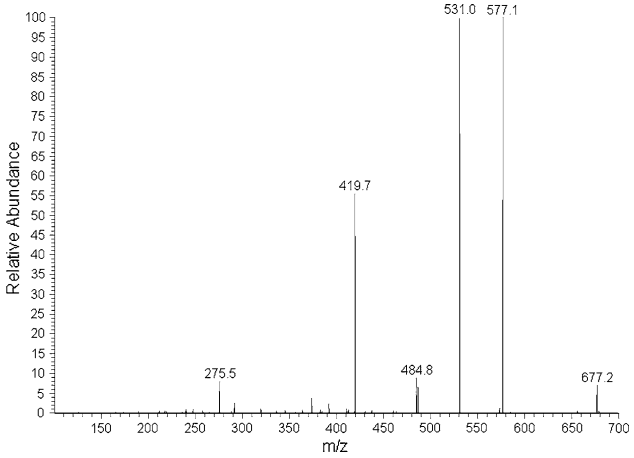


Fig. 4 Positive ion ESI–MS/MS spectrum of the isolated ion at *m/z* 677, $[L_2ZnClO_4]^+$

complex has been observed on the spectrum of the solution 4: $[LZnClO_4]^+$ at *m/z* 557.6.

In the series of ligands **1**–**5**, the zinc complex formed with loss of perchloric acid, due to the substitution of a labile proton by zinc ($[L_2Zn\text{-}H]^+$), appears mainly for the 5-diethoxyphosphoryl-2-pyrrolidinones **3**–**5** regarding the 5-ethoxycarbonyl-2-pyrrolidinone **1**, and is not detected in the case of phenylacetamide derivative **2**. This observation is consistent with the NH acidity range of the respective amide functions.

Inhibition of β-lactamases

The production of β-lactamases is the most common mechanism of bacterial resistance to the β-lactam antibiotics, i.e., penicillins, cephalosporins and now carbapenems (Macheboeuf et al. 2006). The currently marketed β-lactamase inhibitors in therapeutical use are efficient mainly against the serine β-lactamases of class A (Bryskier et al. 2005). Their chemical structures involve a β-lactam ring always.

In order to discover novel "hits" for the inhibition of the most problematical β-lactamases, namely the proteases from classes B and D, the screening of non β-lactamic compounds could be a valuable strategy (Sandanayaka and Prashad 2002). We have tested our ligands **1**–**5** against clinically representative β-lactamases: two zinc proteases (class B: BcII and VIM-4) and one serine protease (class D: OXA-10) particularly sensitive to lipophilic inhibitors (Beck et al. 2009b).

Tested compounds and enzymes were incubated (37°C, 30 min), and then a chromogenic substrate (nitrocefine) was added and its hydrolysis rate was monitored at 482 nm for determining the enzymes residual activity. The results

Table 3 Evaluation of ligands **1–5** as potential inhibitors of β-lactamases

Compound (100 μM)	BcII (pH 5) (%)	VIM-4 (pH 7) (%)	OXA-10 (pH 7) (%)
1	100	96	97
2	100	100	n.d.
3	92	100	93
4	100	100	85
5	100	100	84

Results expressed in percentages (%) of enzymatic initial activity

in Table 3 are expressed in percentages (%) of initial activity; it means that low % values indicate active inhibitors. In such screening, the limit for considering a compound as potential "hit" is fixed at 80%.

Clearly the ligands **1–5** are devoid of activity against the zinc β-lactamases and two of them (**4**, **5**) are weakly active against the serine β-lactamase.

Conclusion

Solutions of Ca(II) and pyrroglutamate analogues **1–5** and Zn(II) and pyrroglutamate analogues **1–5** at different concentrations of cations were studied by ESI–MS. To ensure the detection of real complexes and not adducts formed in the source, different ESI parameters have been tested. Doubly and singly charged complexes were observed containing different ligands and various stoichiometries. The complexes containing perchlorate counter ion have single positive charge. The most abundant complex ions in the case of calcium had general formula $[L_2CaClO_4]^+$ and $[LCaClO_4]^+$. There is no consensus for zinc complexes because several fragmentation processes were involved. The loss of perchloric acid, ethanol or diethyl phosphite has been observed and confirmed by fragmentation experiment. The most abundant complex ions in the case of ligands **1** and **5** had general formula $[L_2Zn\text{-}H]^+$ and $[LZnClO_4]^+$. In the case of ligand **2**, $[L_2ZnClO_4]^+$ and $[LZnClO_4]^+$ were the major complexes. Species $[L_2Zn\text{-}H]^+$ was the most abundant for ligands **3** and **4**. Ester and amide functions, acetonitrile, perchlorate and phosphonate group are complexing motifs toward calcium and zinc. Reasonable structures for the major complexes may be proposed based on bibliographic researches in the database of X-ray structures (see supplementary material). All the tested molecules appeared to be better complexing agents for calcium than for zinc. A lot of signals of ligands not chelated by zinc were detected. Hence, the absence of activity of our molecules against the class B β-lactamases (zinc peptidases) could be due, at least in part, to their weak ability of chelating zinc.

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