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Differentiation of cyclosporin A from isocyclosporin A by liquid chromatography/electrospray ionization mass spectrometry with post-column addition of divalent metal salt

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RATIONALE: Cyclosporin A (CsA) rearranges to its isomer isocyclosporin A (isoCsA) upon acid hydrolysis and also during ionization in the ion source of the mass spectrometer. It has been reported that both compounds could not be differentiated by tandem mass spectrometry (MS/MS) using atmospheric pressure ionization (API) sources and ambiguously differentiated by using other sources. In order to analyze these compounds which are common fungal metabolites, it is relevant to develop a simple method for their differentiation.
METHODS: CsA and isoCsA were analyzed by liquid chromatography/mass spectrometry (LC/MS) with post-column addition of metal ion solutions in a quadrupole time-of-flight instrument equipped with an electrospray ionization (ESI) source.
RESULTS: Mass spectra of CsA obtained upon post-column addition of solutions of Ca(II), Cu(II) and Zn(II) showed complexes between cyclosporin and the metal, including $[2CsA + Me]^{2+}$ and $[CsA–H + Me]^{+}$. These complexes were not observed in the spectra of isoCsA. The same results were observed at different metal concentrations.
CONCLUSIONS: Differentiation via metal complexation in positive ion mode LC/ESI-MS was performed to simultaneously distinguish CsA and its isomer isoCsA. Copyright © 2014 John Wiley & Sons, Ltd.

Cyclosporin A (CsA), cyclo[−MeBmt^{1}-Abu^{2}-Sar^{3}-MeLeu^{4}-Val^{5}-MeLeu^{6}-Ala^{7}-D-Ala^{8}-MeLeu^{9}-MeLeu^{10}-MeVal^{11}-], where MeBmt stands for (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-methylamino-6-octenoic acid, is a secondary metabolite produced by various fungal species including *Tolypocladium inflatum* (Fig. 1). It is a well-known commercial cyclic undecapeptide with immunosuppressing activity, and is used in bone marrow and organ transplantation treatment.[1–4] This compound has also a variety of other biological properties, which include anti-inflammatory, antifungal and antiparasitic activities.[1] The isomerization of CsA to isocyclosporin A (isoCsA) has been reported, via a N,O-acyl migration upon acidic treatment of CsA in aqueous solution or in organic solvents.[5] This intramolecular rearrangement was reported to take place also *in situ* in an ion trap.[6]

Ion rearrangements usually lead to an increase in the complexity of mass spectra and ambiguity in their interpretation, but the N-O peptidyl shift in $[M + H]^{+}$ ions of cyclosporins provides a preferential 1–11 splitting of the CsA backbone, which leads to a simpler interpretation.[6] Although the analysis of the amino acid sequence is not compromised, both isomers still need to be distinguished. Havlíček and coworkers studied in detail the fragmentation of cyclosporins,[6–8] employing different ionization techniques such as fast-atom bombardment (FAB), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption/ionization (MALDI), and electrospray ionization (ESI), and also different analyzers like double focusing BE, triple quadrupole, time-of-flight (TOF) and quadrupole ion trap. These authors found that the collision-induced dissociation (CID) mass spectrum of the $[M + H]^{+}$ ion of isoCsA was identical to that of CsA.[6] Although the differentiation of CsA and isoCsA by high-energy CID has been discussed, and a b_n-17$^{1–11}$ series has been observed in the spectra of isocyclosporins, the differences were subtle and ambiguous. In the same work, CsA and isoCsA could be distinguished by liquid chromatography (LC) since both compounds have different retention times.[9]

Differentiation of isomers by the use of metal complexation and electrospray was previously achieved, for example in hydroxypyridine *N*-oxides[10] and polyaromatic hydrocarbons.[11] The same strategy was explored for the differentiation of CsA and isoCsA by metal complexation, based on the ability of cyclosporins to form stable adducts with Na^{+}, Mg^{2+}, Ca^{2+}, Cu^{2+} and Zn^{2+} in solution.[12–15] On the other hand, post-column addition of metal solutions in LC/MS has been successfully employed for the improvement of signal response[16] and in isomer differentiation of flavonoids[17] and carbohydrates.[18] Since CsA and isoCsA are usually analyzed by LC in complex natural matrices, the differentiation of both metabolites was investigated by the use of post-column addition of metal ion solutions.

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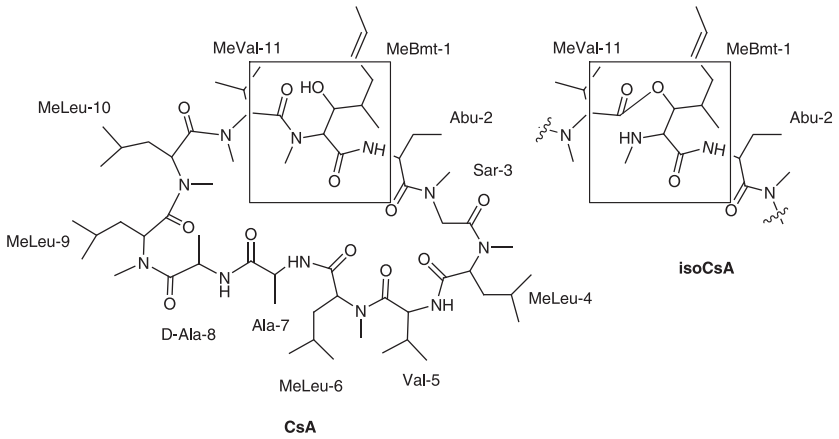

Figure 1. Structures of CsA and isoCsA.

EXPERIMENTAL

Materials and samples

Cyclosporin A (CsA) was isolated from a commercial sample of Sandimmun® (Novartis) and the structure was confirmed by nuclear magnetic resonance (NMR).[19] Isocyclosporin A was prepared from CsA as previously reported.[5] LC/MS-grade methanol and water were purchased from Carlo Erba (Milan, Italy) and Tedia (OH, USA) respectively. Formic acid (p.a., ACS) was purchased from Merck KGaA (Darmstadt, Germany). CsA (0.017 mM) and isoCsA (0.011 mg/mL) solutions were prepared using methanol.

Post-column addition of metal ion solutions

Aqueous metal ion stock solutions (10 mM) of $CaCl_2$, $CuSO_4.5H_2O$ and $ZnCl_2$ were prepared. Appropriate dilutions in water were performed to produce the working stock solutions, with the following final concentrations: 5, 1, 0.5 and 0.1 mM. The metal ion solutions were

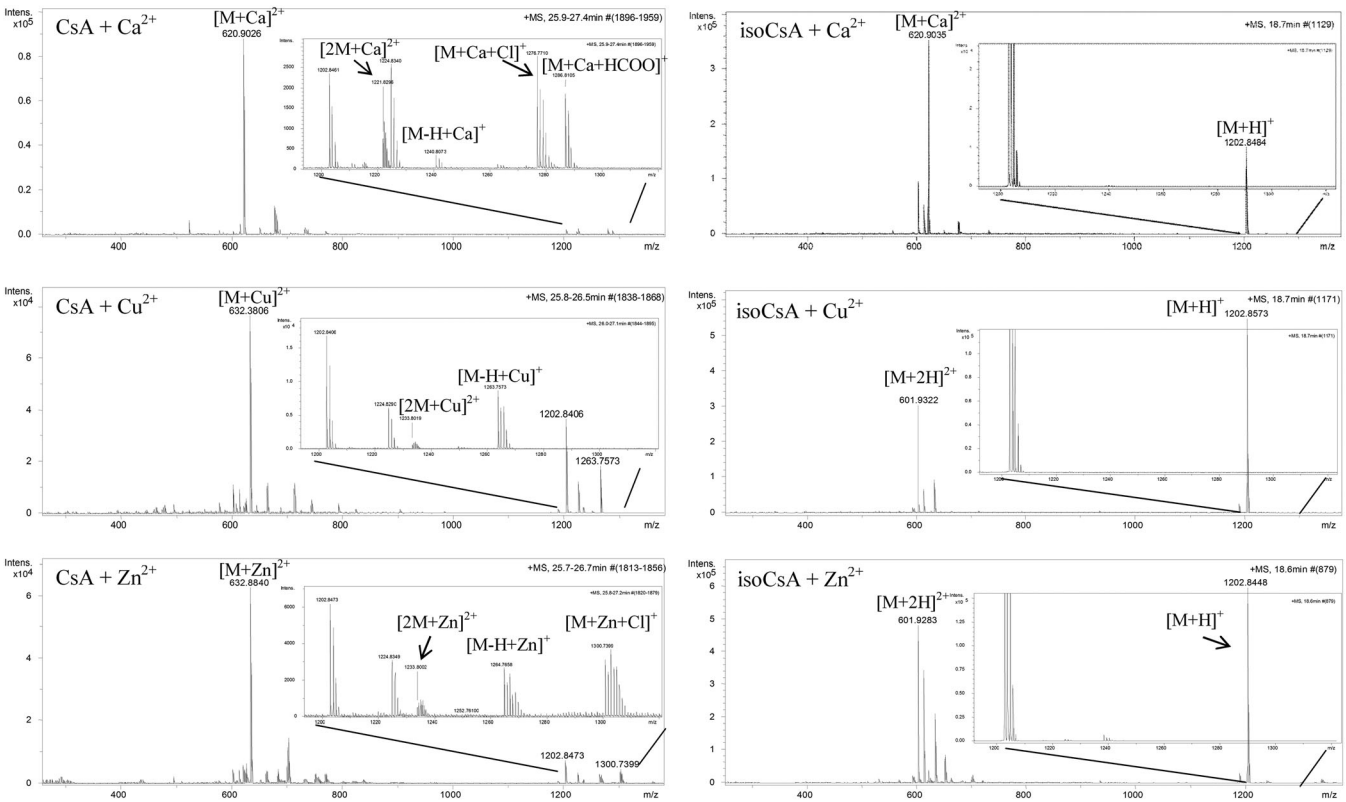

Figure 2. Mass spectra of CsA and isoCsA obtained by LC/MS with post-column addition of Ca(II), Cu(II), and Zn(II). Magnified images of the regions at m/z 1200–1300 are also shown.

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introduced by means of a syringe pump at a flow rate of 3 μL/min, via a T-junction before entrance into the ion source.

HPLC/MS analysis

Samples were analyzed on an Agilent 1200 liquid chromatograph (Agilent Technologies, Wilmington, DE, USA) coupled to a Bruker micrOTOF-Q II mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with an ESI source. The instrument was operated in positive ion mode at a capillary voltage of 4.5 kV, an end plate offset of −500 V, a drying temperature of 160 °C, and N_2 as drying gas at 6.0 L/min and as nebulizer gas at a pressure of 3.0 bar, respectively. Multi-point mass calibration from m/z 100 to 1500 in positive ion mode was carried out by using a sodium formate solution. Data acquisition and processing were carried out with Bruker Compass Data Analysis software (version 4.0) supplied with the instrument.

The samples were chromatographed on a Luna C18 column (3 μm, 2.0 × 100 mm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of water containing 0.1% formic acid (A) and methanol (B). The flow rate was 0.3 mL/min and the column temperature was set at 30 °C. Linear gradient elution was performed as follows: 55% B (0–2 min), 55–100% B (2–30 min), 100% B (30–40 min). Retention times: 18.7 min isoCsA, 26.1 min CsA. Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

Methanol solutions of CsA and isoCsA were subjected to LC/MS with post-column addition of $CaCl_2$, $CuSO_4$ or $ZnCl_2$ solutions. Control experiments were also performed without post-column addition. Among the different metal ions, Ca^{2+}, Cu^{2+}, and Zn^{2+} were chosen because they are endogenous metals that are known to form complexes with pure CsA. $CuSO_4$ was used instead of $CuCl_2$ because it has the same capacity to produce metal adducts, but it does not form adducts with the counterion Cl.[10] The chromatographic conditions were chosen based on previous analysis of complex mixtures of natural fungal extracts containing cyclosporins.

Table 1. m/z relationships (with error) and relative intensities of metal complexes and doubly protonated CsA and isoCsA compared with that of singly protonated CsA[a]

M		CsA				IsoCsA		
ME		Ca^{2+}	Cu^{2+}	Zn^{2+}		Ca^{2+}	Cu^{2+}	Zn^{2+}
$[M+H]^+$ $C_{62}H_{112}N_{11}O_{12}$	1202,8472 (1.2) 100	1202,8461 (2.1) 100	1202,8406 (6) 100	1202,8473 (1.1) 100	1202,8484 (0.2) 100	1202,8486 (0.1) 100	1202,8573 (7.2) 100	1202,8448 (1.5) 100
$[M+2H]^{2+}$	601,9234 (6.0) 15	601,9296 (2.7) 58	601,9236 (6) 43	601.92973 (3.0) 62	601,9293 (2.2) 77	601,9275 (0.8) 83	601,93220 (7.1) 47	601,9283 (3) 79
$[M+Na]^+$	1224,8311 (0.4) 646	1224,8340 (2.8) 107	1224,8290 (1.3) 31	1224,8349 (3.5) 54	1224,8302 (0.3) 48	n.o.	n.o.	n.o.
$[M+H+Na]^{2+}$	612,9150 (6.3) 81	612,9166 (3.7) 56	612,9142 (7.7) 30	612,9171 (3.0) 64	612,9212 (3.7) 92	612,9219 (4.8) 43	612,9229 (6.5) 13	612,9186 (0.5) 53
$[M+2Na]^{2+}$	623.9037 (4.2) 229	n.o.	623,8901 (7.4) 14	n.o.	623.9102 (0.5) 99	n.o.	n.o.	n.o.
$[M\text{-}H+ME]^+$		1240.8073 (9.0) 12	1263,7573 (6.8) 53	1264,7658 (2.9) 44		n.o.	n.o.	n.o.
$[M+ME+Cl]^+$		1276,7710 (1.0) 124	n.o.	1300,7399 (0.8) 75		1276,7688 (2.7) 0.2	n.o.	n.o.
$[M+ME+HCOO]^+$		1286,8105 (7.3) 68	n.o.	n.o.		n.o.	n.o.	n.o.
$[M+ME]^{2+}$		620,9026 (1.2) 5322	632,3806 (6.8) 255	632,8840 (1.2) 902		620,9035 (2.3) 233	632,3886 (5.9) 18	632,8814 (5.3) 34
$[2M+ME]^{2+}$		1221,8296 (5.9) 68	1233.2928 (10.0) 4	1233.8002 (4.2) 8		n.o.	n.o.	n.o.

[a]All values in a column represent m/z relationship with error and relative intensities of the specified complex resulting from post-column addition of 10 mM metal solutions. Relative intensity values are scaled to the intensity of the protonated peptide (intensity = 100) and are an average of at least 3 experiments.
ME: Ca(II), Cu(II) or Zn(II); n.o. not observed.

The ESI mass spectra of CsA and isoCsA typically showed $[M+H]^+$, $[M+Na]^+$, $[M+2H]^{2+}$, $[M+H+Na]^{2+}$ and $[M+2Na]^{2+}$ ions. However, the relative abundances of these ions were different for both isomers, with $[M+Na]^+$ being the most abundant ion for CsA and $[M+H]^+$ and $[M+H+Na]^{2+}$ for isoCsA. Although these results may imply a differentiation, it is based in the relative abundance of the same ions, which may also vary under different chromatographic conditions.

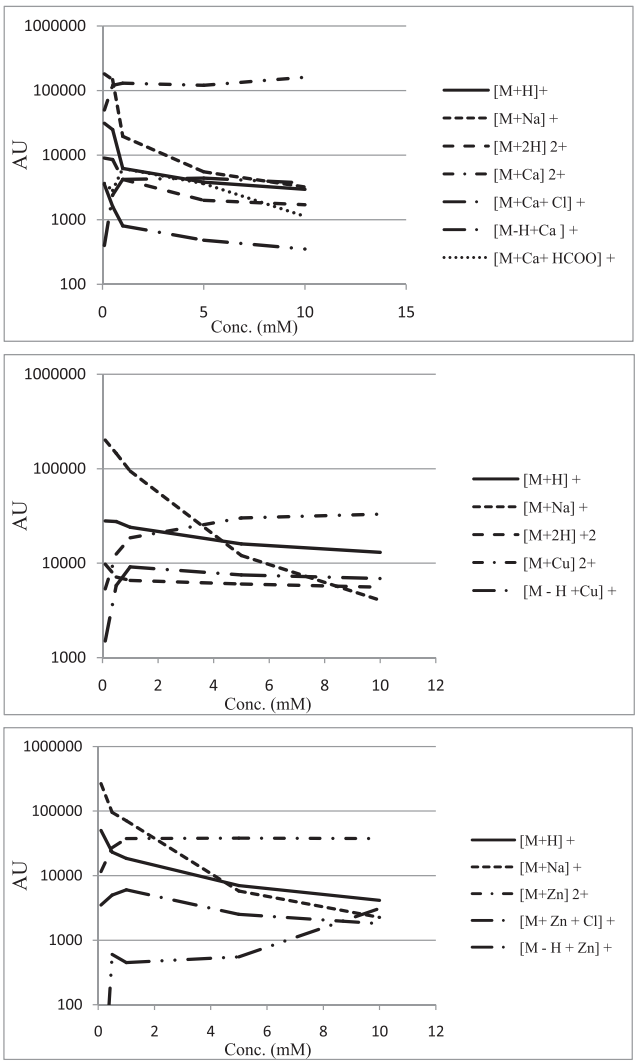
The ESI mass spectra of CsA resulting from post-column addition of 10 mM solutions of Ca(II), Cu(II) and Zn(II) show ions at m/z 620,9014 ($[M+Ca]^{2+}$), 632,3849 ($[M+Cu]^{2+}$) and 632,8847 ($[M+Zn]^{2+}$), respectively, as the base peaks (Fig. 2). These results agree with those previously reported for direct infusion spectra of Ca(II), although these ions were not the most intense ions for Cu(II) and Zn(II) in their infusion spectra.[15,20] Other ions, observed in the mass spectra at m/z 1200–1300, were assigned to $[2CsA+Ca]^{2+}$ and $[CsA+Ca+X]^+$, where X is Cl or HCOO in the case of Ca(II) addition, $[2CsA+Cu]^{2+}$ and $[CsA\text{-}H+Cu]^+$ in the case of Cu(II) addition and $[2CsA+Zn]^{2+}$, $[CsA+Zn+Cl]^+$ and $[CsA–H+Zn]^+$ in the case of Zn(II) addition (Table 1).

The presence of adducts with deprotonated CsA in the case of Cu^{2+} and Zn^{2+} addition is consistent with the known fact that deprotonation occurs on an amide nitrogen, and deprotonated ions can be stabilized by these metals due to their ability to coordinate nitrogen and oxygen atoms. This stabilization is less pronounced for Ca^{2+} which binds more likely to an amide carbonyl oxygen atom in CsA.[13] In contrast, the ions $[M+H]^+$ and $[M+ME]^{2+}$, where ME is either Ca^{2+}, Cu^{2+} or Zn^{2+}, were predominant in the mass spectra of isoCsA. None of the aforementioned ions at m/z 1200–1300 were observed (Fig. 2 and Table 1), except for the ion $[isoCsA+Ca+Cl]^+$, which was observed at 0.2% relative abundance.

In order to determine the effect of metal concentration on the observed species, different concentrations were infused post-column. As expected in all cases for CsA, a decrease in metal concentration produced an increase in the singly and doubly charged protonated and sodiated species. The species $[CsA+ME]^{2+}$ continued to be the base peak in all the spectra up to a concentration of 1 mM for Ca^{2+} and 5 mM for Cu^{2+} and Zn^{2+}. Although at low metal ion concentrations the $[M+Na]^+$ ion had a higher intensity, the distinctive ions at m/z 1200–1300 were observed even at 0.1 mM of metal solution (Fig. 3). The relative intensities of the adduct ions suggest the following order of affinity of CsA for metal ions: $Ca^{2+} > Cu^{2+} > Zn^{2+}$, which is the same order reported previously.[13,15] These findings indicate that the tested metals, particularly Cu^{2+} and Zn^{2+}, are well suited for the unambiguous differentiation of CsA and isoCsA at all the tested concentrations, since none of the diagnostic ions at m/z 1200–1300 were observed for isoCsA.

In order to obtain a deeper understanding of this differentiation, MS/MS experiments of $[CsA\text{-}H+ME]^+$ or $[CsA+ME+X]^+$ ions were performed. The MS/MS spectrum of $[CsA+Ca+Cl]^+$ shows mainly a single fragment ion at m/z 1128.7055 corresponding to the loss of HCl and the side chain of the amino acid MeBmt (Fig. 4). This fragment was observed previously in an ion trap, although among many other fragment ions.[20]

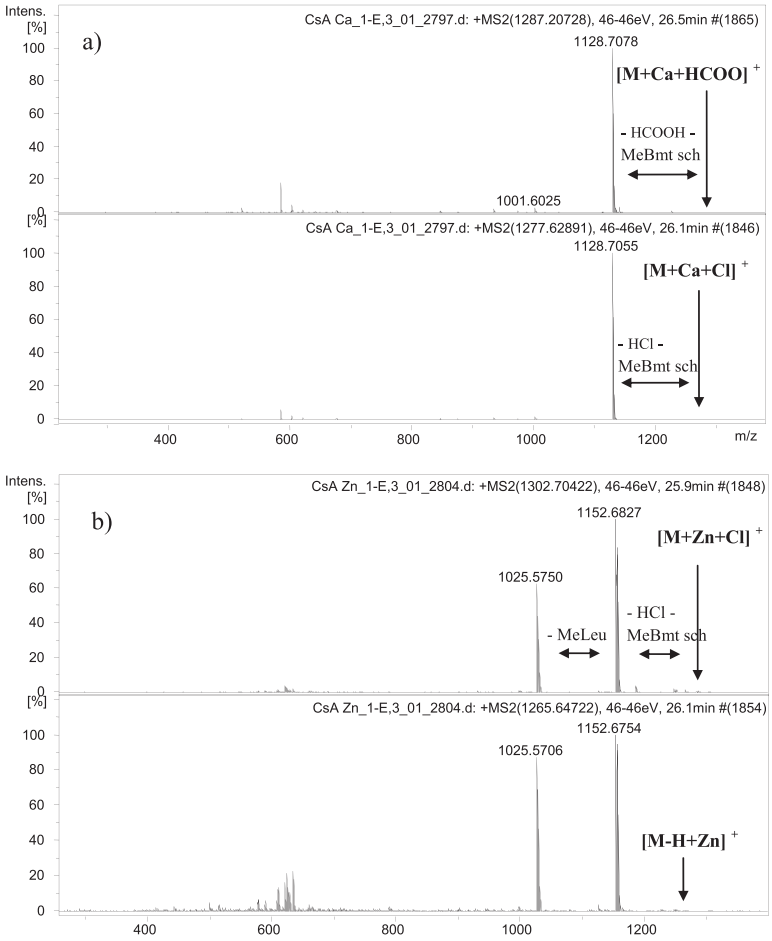
The MS/MS spectra of $[CsA\text{-}H+Zn]^+$ and $[CsA+Zn+Cl]^+$ are nearly identical with two fragment ions at m/z 1152.6727 (loss of the MeBmt side chain and loss of the MeBmt side chain and HCl, respectively) and m/z 1025.5750, which originates from the loss of 127 u (MeLeu) from the former fragment (Fig. 4). The MS/MS spectrum of $[CsA+H]^+$ did not show fragment ions with the loss of the MeBmt side chain; therefore, the cationized adduct formation facilitated the loss of the side chain, thereby weakening the C_α–C_β bond of MeBmt. For example, deprotonation of CsA in the amide nitrogen of Abu (amino acid 2) to form the amidate leads to a proton transfer from the hydroxyl of MeBmt to the nitrogen of Abu with loss of the MeBmt side chain (Fig. 5).



(AU: arbitrary units)

Figure 3. Relationship between the observed species and the concentration of metal solution.

It is noteworthy that CsA has only four amide protons (at amino acids 2, 5, 7, and 8); NMR studies confirmed that the intramolecular hydrogen bonds present in CsA are missing in the metal-bound conformation[15] and that a stronger coordination of Ca(II) occurs via the amide carbonyl oxygens especially in the region of the antiparallel β-pleated sheet (residues 11 to 5; Fig. 1).[13] The calculated structure of the CsA-Mg complex in water shows an octahedral coordination of the Mg^{2+} ion with the carbonyl oxygens of residues 1, 4, 5, and 6 and water molecules.[14] These facts



MeBmt sch: MeBmt side-chain
m/z 1128.7078 $C_{55}H_{98}CaN_{11}O_{11}$ (error: -0.9 ppm), m/z 1152.6727, $C_{55}H_{98}N_{11}O_{11}Zn$ (error: 0.5 ppm);
m/z 1025.5750, $C_{48}H_{85}N_{10}O_{10}Zn$ (error: 1.3 ppm)

Figure 4. MS/MS spectra of (a) $[CsA + Ca + HCOO]^+$ and $[CsA + Ca + Cl]^+$ and (b) $[CsA + Zn + Cl]^+$ and $[CsA\text{-}H + Zn]^+$.

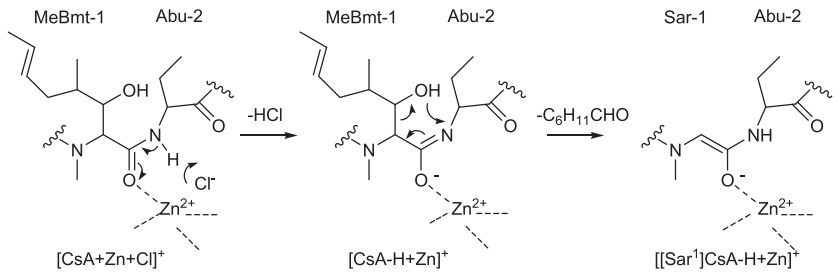


Figure 5. Proposed mechanism for the fragmentation of $[CsA + Zn + Cl]^+$.

agree with the proposed mechanism of fragmentation, and they also explain the loss of MeLeu subsequent to the loss of the MeBmt side chain. The loss of MeLeu 6 is reasonable after an initial ring opening between residues 5 and 6 (or 6 and 7), especially in the case of metal binding to carbonyl oxygens 1, 4, 5, and 6 (as was proven in the case of Mg(II)), or at least in the region between amino acids 11 to 5.

The loss of the MeBmt side chain is relevant, as it can explain the differences in the mass spectra of CsA and isoCsA. Since the metal cation, the counterion attachment, or the H atom detachment in CsA should all be in the MeBmt region, this loss gives rise to the only difference observed between CsA and isoCsA. Although the MS/MS spectrum of the $[CsA\text{-}H + Cu]^+$ ion shows the fragment ion due to the loss of the MeBmt side chain, the excessive number of fragments make it of little analytical use. To the best of our knowledge, the structure of isoCsA and its capability to complex cations have not been previously studied, although isoCsA is known to exist naturally in a conformation which is different from that of CsA.[5]

CONCLUSIONS

The present results indicate that CsA and isoCsA are easily differentiated by the observation of the following species: $[2CsA + ME]^{2+}$, $[CsA–H + ME]^{+}$ and/or $[CsA + ME + X]^{+}$ upon post-column addition of Cu^{2+} and Zn^{2+}. Although these ions are observable for CsA, none of these species are detected in the case of isoCsA. This difference in behavior may be explained on the basis of their structural variation, namely the replacement of an amide functionality in CsA by an ester in isoCsA. These structural changes take place precisely in the region of the molecule where the metal cation is attached, giving rise to the observed differences.

Acknowledgements

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