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Identification of the products of reaction between pyridoxal phosphate and amiclenomycin and other related 1-amino-cyclohexa-2,5-dienes

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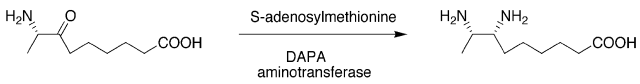
Abstract—Amiclenomycin, a natural product containing the 1-amino cyclohexa-2,5-diene moiety is an inhibitor of 7;8-diaminopelargonic acid aminotransferase, a pyridoxal phosphate (PLP) dependent enzyme involved in biotin biosynthesis. The postulated mechanism implies the aromatisation of the Schiff base formed between PLP and amiclenomycin. Aromatic adducts have been obtained by heating PLP with amiclenomycin and other related 1-amino cyclohexa-2,5-dienes. They were fully characterized by UV–visible and ESI mass spectrometry and provide standards for identification of the enzyme-derived products.

1. Introduction

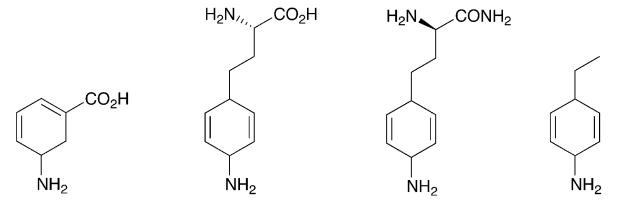
Amiclenomycin **1a**, isolated from different *streptomyces* strains, either free[1] or included in peptides,[2–5] is an inhibitor of biotin biosynthesis.

It has been established by Hotta et al.[6] that the target was 7,8-diaminopelargonic (DAPA) aminotransferase (EC 2.6.1.62), a pyridoxal phosphate (PLP) dependent enzyme which catalyses the following reaction (Scheme 1).



Scheme 1. Reaction catalysed by DAPA aminotransferase.

These authors observed that during preincubation with the inhibitor, inactivation of the enzyme was taking place. However, 50% of the activity was restored after extensive dialysis, showing that no covalent bond was present, at least to this extent, in the enzyme–inhibitor complex. This was tentatively interpreted by a tight-binding of amiclenomycin to the enzyme, but not demonstrated and we decided to reinvestigate the origin of the inhibition.



Scheme 2. The different aminocyclohexadienes discussed in this study.

From the structural analogy between amiclenomycin and gabaculine (Scheme 2), namely the presence of an amino-cyclohexadienyl moiety, we postulated that the mechanism of action of both compounds could be similar. Gabaculine is a well-known inhibitor of several PLP enzymes[7–12] and in that case, the mechanism of inhibition has been well established:[7,13] the enzyme catalyses the formation of an aromatic adduct with PLP, whose affinity for the protein is such that the inhibition appears irreversible and the complex can be dissociated only after denaturation of the enzyme.

The formation of such an aromatic adduct between amiclenomycin and PLP, non-covalently bound to the protein, would be consistent with the reversibility (at least partial) of the inhibition described in that case, assuming that here its affinity for the enzyme is lower. The likely mechanism of formation of the postulated adduct **2**, derived from the one proposed for gabaculine,[7] is depicted on Scheme 3.

Keywords: pyridoxal phosphate; amiclenomycin; 1-amino-cyclohexa-2,5-dienes; *syn* 1,4-elimination; ESI mass spectrometry.

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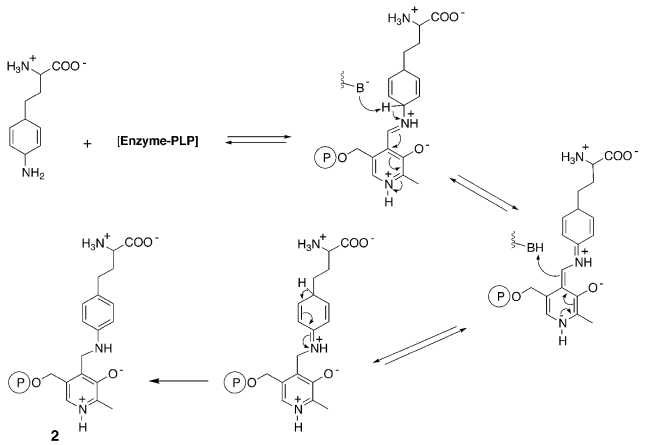


Scheme 3. Postulated mechanism for the inhibition of DAPA aminotransferase by amiclenomycin.

This hypothesis had of course to be evidenced and mass spectrometry was obviously the suitable technique to confirm the structure of the product derived from amiclenomycin and the non-covalent nature of its interaction with the protein.

For that purpose, it was useful to run the mass spectrum of a reference compound, a priori accessible by heating a mixture of amiclenomycin and PLP.[14] This is reported in this paper. We have also analysed the products of reaction of PLP with several other amino-cyclohexadienes. Indeed, there was a doubt about the stereochemistry of amiclenomycin, which was tentatively postulated to be *trans*.[6] To remove this ambiguity, we achieved the synthesis of both isomers[15] and we could conclude that the natural product was in fact the *cis* one. As we obtained during this synthetic approach two other pairs of *cis* and *trans* amino-cyclohexadienes, **3** and **4** (Scheme 2)[15,16] we tested their inhibitory power of DAPA aminotransferase. All of them inhibit the enzyme, but their potency depends on the nature of the side-chain and also on the stereochemistry, the *cis* isomers being always more potent that the *trans* ones.[17] Thus it was important to examine if all these compounds gave the same kind of aromatic adducts.

2. Results and discussion

2.1. Preparation of the PLP-aminocyclohexadienes adducts: general procedure

Each aminocyclohexadiene was heated in water at 95 °C with a stoichiometric amount of PLP in a closed quartz cuvette and the course of the reaction was followed by UV–visible spectroscopy. When the evolution of the spectrum has stopped, an aliquot of the mixture was withdrawn and, after a tenfold dilution in a 1/1 acetonitrile/water mixture, analysed by using ESI external source combined with ion-trap mass spectrometry (IT/MS).

2.2. Structural analysis

2.2.1. Adducts of PLP with *cis* and *trans* 1-amino-4-ethyl-cyclohexadienes-2,5 4a and 4b. The evolution of the UV spectrum during the reaction of the *cis* compound **4a** is shown on Fig. 1.

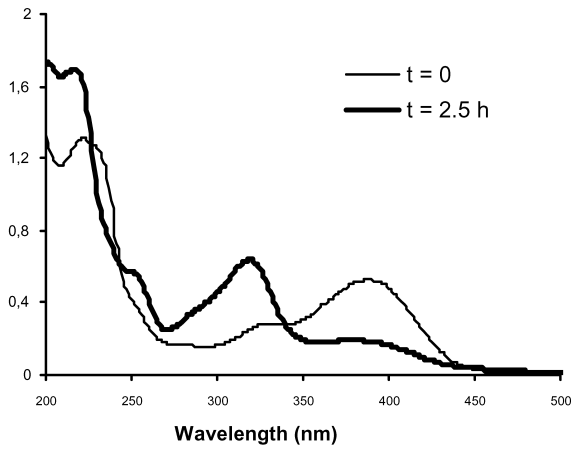


Figure 1. Evolution of the UV–visible spectrum of the PLP/**4a** mixture.

After heating, the PLP absorptions at 388 and 330 nm disappeared and new bands at 318 and 250 nm, with a shoulder at 290 nm were appearing, which can be attributed to the pyridoxamine structure[18] of **6** (Scheme 8). This structure was confirmed by the ESI mass spectrum which shows a quasi-molecular peak at *m/z* 353 corresponding to protonated molecule (Fig. 2(a)).

The CID product spectrum of this ion (Fig. 2(b)) displays fragment ions at *m/z* 255, 232 and 134, the very likely structure of which is represented in Scheme 4.

The *trans* isomer **4b** shows a quite different behaviour. The

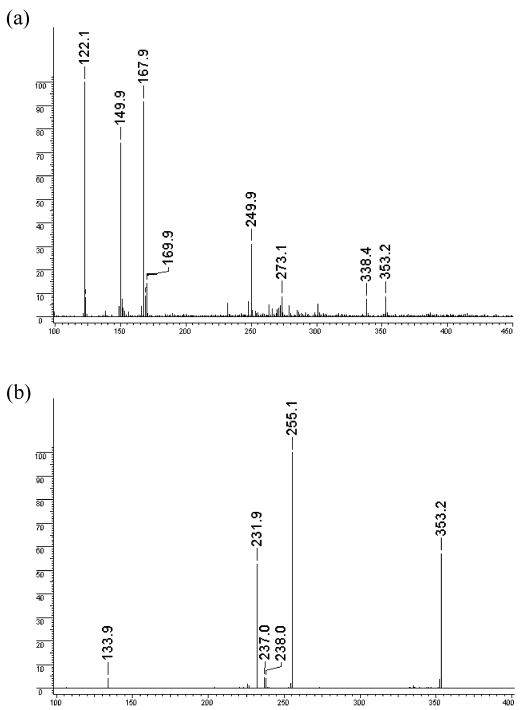
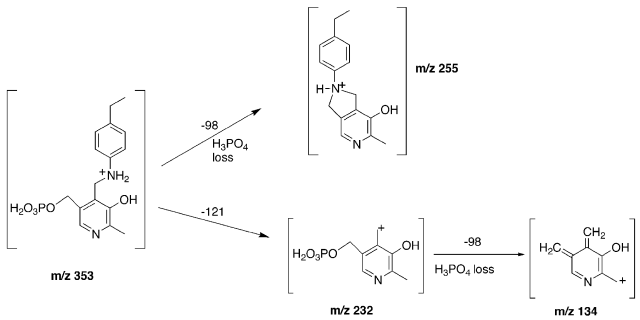


Figure 2. (a) ESI mass spectrum of the reaction product of PLP and **4a** (after 2.5 h heating). (b) CID product spectrum of the *m/z* 353 ions from (a).



Scheme 4. Fragment ions observed on the CID product spectrum of the *m/z* 353 ions.

expected quasi-molecular peak at *m/z* 353 does not appear significantly in the ESI mass spectrum. The major peaks are found at *m/z* 150 and 168 (Fig. 3(a)).

However, a CID analysis at *m/z* 353, the expected quasi-molecular peak value, gives fragment ions at *m/z* 255 and 232, identical to those observed with the *cis* isomer **4a** (Fig. 3(b)), which prove the presence of the aromatic adduct (but clearly in a much lower amount than in the *cis* case).

Tandem mass spectrometry allows investigation of minor peaks present in the noise. The use of an ion-trap mass spectrometer instead of a triple quadrupole instrument increases the sensitivity, because ions can be stored in the trap.

On the UV spectrum, which remains unchanged only after 14 h, the band at 317 nm attributed to the pyridoxamine moiety is however present (data not shown). In a control

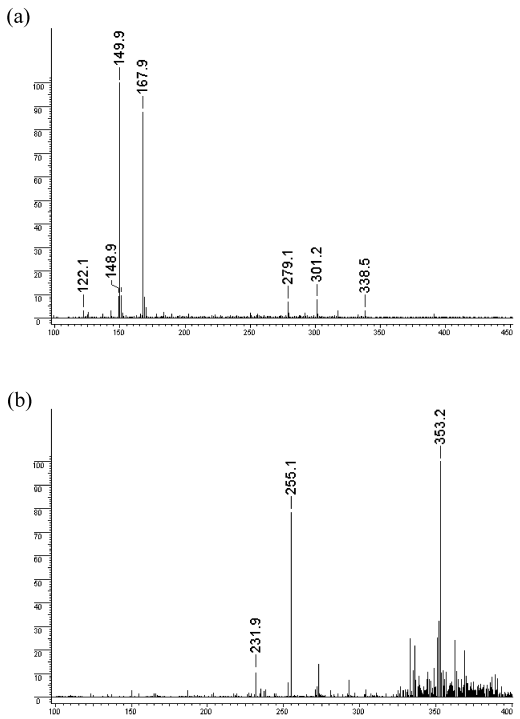


Figure 3. (a) ESI mass spectrum of the reaction product of PLP and **4b** (after14 h heating). (b) CID product spectrum of the *m/z* 353 ions from (a).

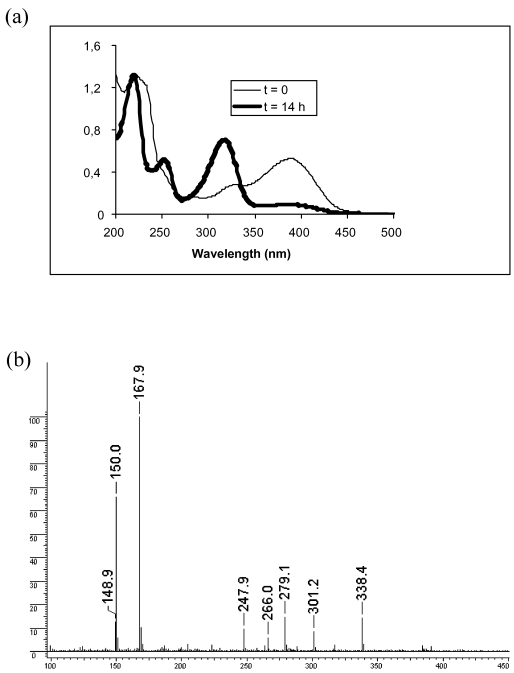


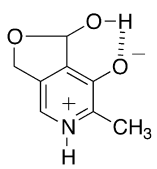
Figure 4. (a) Evolution of the UV–visible spectrum of a PLP solution heated at 95°C. (b) ESI mass spectrum of a PLP solution heated at 95°C for 14 h.

experiment, an aqueous solution of PLP was heated under the conditions used for the mixture. The same spectral changes were observed (Fig. 4(a)).

When analysed by ESI mass spectrometry, this solution presented only two important peaks at *m/z* 168 and 150 (Fig. 4(b)). The peak at *m/z* 168 corresponds to the hemiacetal form of pyridoxal (Scheme 5), as confirmed by NMR, and the peak at *m/z* 150 corresponds to a water elimination from the hemiacetal. The observed UV–visible spectrum is in agreement with the one published by Matsushima and Martell.[18]

The presence of PLP hemiacetal can be explained if one assumes an elimination of ammonia from **4b**, leading to ethylbenzene (not detected). This process, competitive with the formation of the aromatic adduct, leaves unreacted PLP, which looses its phosphate group, albeit more slowly than the formation of the aromatic adduct.

The presence of both peaks at *m/z* 168 and 150 in the ESI mass spectrum of Fig. 2(a) indicates that the same competition can occur in the reaction of the *cis* isomer **4a**. Due to the fact that the UV–visible spectra of the aromatic adduct and of PLP hemiacetal are interfering, it is difficult to measure precisely the proportions of the two compounds.



Scheme 5. The hemiacetal form of pyridoxal.

However, it is clear that the formation of the aromatic adduct is favoured with the *cis* isomer.

This different behaviour can be rationalised by a stereochemically favoured elimination of ammonia in the case of the *trans* isomer, assuming a *syn* elimination leading to ethyl benzene.[19–22] The easier elimination of ammonia in the *trans* aminocyclohexadienes has already been observed: in the ESI mass spectra of *cis* amiclenomycin and *cis* amiclenomycin amide, the quasi-molecular peaks were always present, whereas they were not observed with the *trans* isomers. In that case, only the aromatic products ions, resulting from ammonia elimination were detected.

2.2.2. Adducts of PLP with the *cis* and *trans* amides 3a and 3b. The adducts were prepared as described above and analysed in the same way. The results are quite consistent with those obtained with **4a** and **4b**. Similar UV–visible spectra were obtained after reaction, with maxima at 323 and 250 nm and a shoulder at 289 nm.

The expected quasi-molecular ions of the aromatic adduct are observed at *m/z* 425 on the ESI mass spectrum of the reaction product with the *cis* amide **3a** (Fig. 5(a)).

The fragmentations (Fig. 5(b)) generated under CID conditions are the same as those of the adduct prepared from **4a**, leading to product ions at *m/z* 327 and 232 ions, with supplementary ions at *m/z* 239, 194 and 149 due to the presence of a second protonation site on the amide function, as proposed on Scheme 6. Ions at *m/z* 310, 232, 134 and 106 not detectable on Fig 5(b) were however clearly observed on

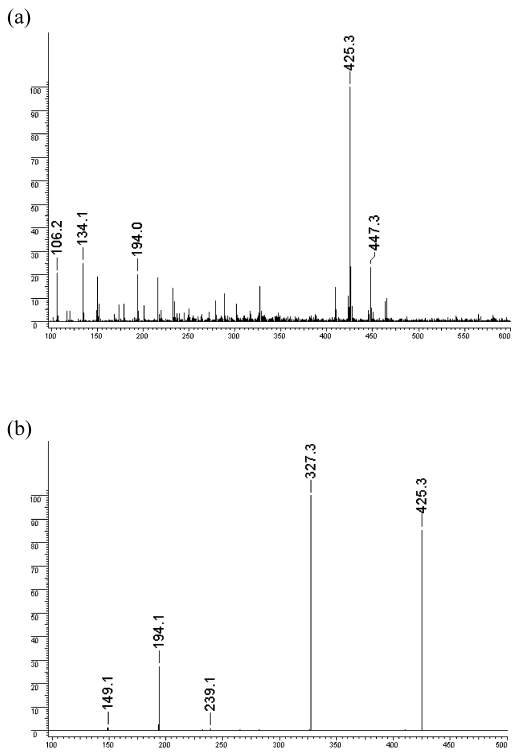
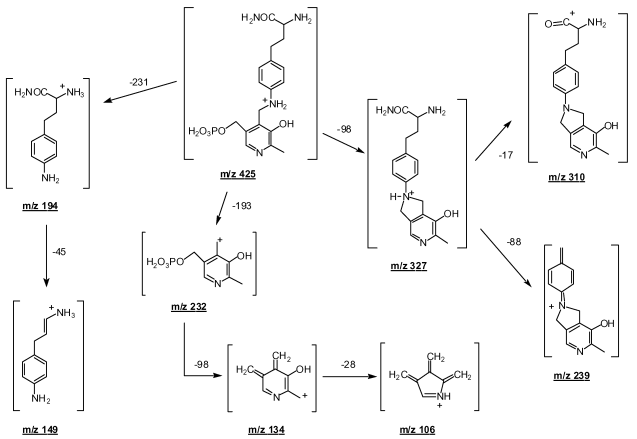


Figure 5. (a) ESI mass spectrum of the reaction product of PLP and **3a** (after 1.5 h. heating). (b) CID product spectrum of the *m/z* 425 ions from (a).



Scheme 6. Fragment ions observed on the CID product spectrum of the *m/z* 425 ions.

the CID product spectrum of the *m/z* 425 ions obtained with a Quattro 1 instrument (Micromass) with ESI source (data not shown). The difference between the two spectrometers is due to the residence time in the collision cell (ar 100 μs) and ion-trap (ar 100 ms).

With the *trans* isomer **3b**, as already observed for **4b**, the quasi-molecular ions of the adduct at *m/z* 425 is hardly visible on the ESI mass spectrum. The main peaks are again at *m/z* 150 and 168, revealing the presence of pyridoxal hemiacetal. The UV–visible spectrum, with bands at 317 and 254 nm is also consistent. However, the CID product spectrum of the selected *m/z* 425 ions shows only two peaks, at *m/z* 327 and 194, expected for the quasi-molecular ion of the adduct, confirming their presence in small amounts (data not shown).

2.2.3. Adducts of PLP with amiclenomycin 1a. The study was limited to the case of the *cis* isomer, amiclenomycin **1a**. The UV–visible spectrum obtained after heating the mixture was identical to the preceding ones, with bands at 323 and 288 nm and a shoulder at 254 nm.

The ESI mass spectrum shows the quasi-molecular peak at *m/z* 426, but only after addition of formic acid (final concentration: 0.5%) to the sample before injection. The CID product spectrum of these ions (Fig. 6) shows fragment ions at *m/z* 328 and 195, corresponding to those of the amide (*m/z* 327 and 194). The fragments at *m/z* 239, 232, 149 and

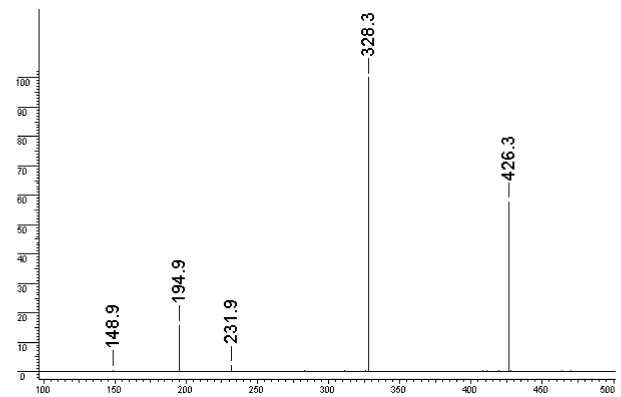
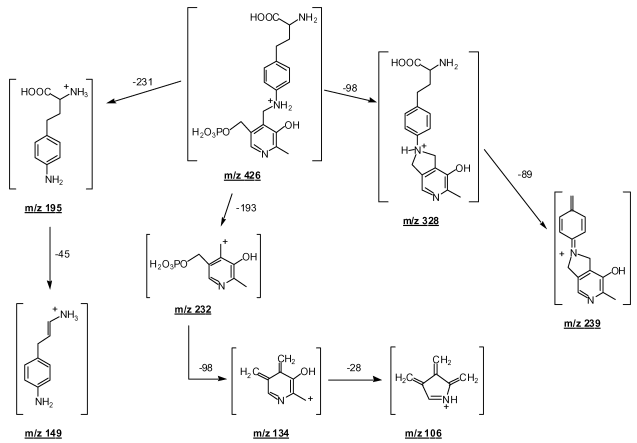


Figure 6. CID product spectrum of the *m/z*426 ions.

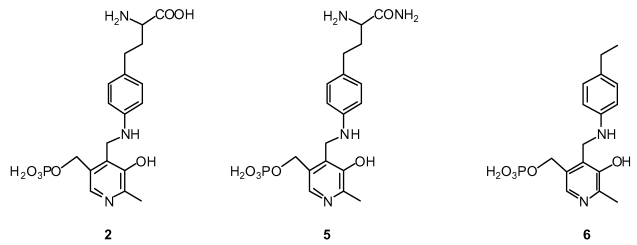


Scheme 7. Fragments observed on the CID product spectrum of the *m/z* 426 ions.

106 are conserved, confirming that they have lost the α-amino acid moiety (Scheme 7). Ions at *m/z* 239 and 106 were observed only on the CID product spectrum of the *m/z* 426 ions obtained with a Quattro 1 instrument on positive mode (data not shown).

3. Conclusion

By heating the mixture of PLP and the 1-amino-cyclohexa-2,5-dienes **1a**, **3a**, **3b**, **4a**, **4b**, we could synthesise the aromatic adducts **2**, **5** and **6** (Scheme 8). Their structure was established without ambiguity by mass spectrometry.



Scheme 8. Aromatic adducts characterized by mass spectrometry.

According to the ESI mass spectra, they are formed with a better yield with the *cis* isomers than with the *trans* ones. This behaviour can be rationalised by assuming an elimination of ammonia, stereochemically favoured with the *trans* compounds, competitive with the reaction with PLP.

This characterisation of the adducts issued from amino cyclohexadienes **1**, **3** and **4**, was a necessary step, which will allow their identification, if they are present, in the inactivated enzyme.

4. Experimental

4.1. Preparation of the adducts

An aqueous solution of PLP (125 μL, 250 nmol) and an aqueous solution of amino cyclohexadiene (125 μL, 250 nmol) were introduced in a quartz cuvette (250 μL, l=0.1 cm). The cuvette was heated at 95°C in an oil bath and the reaction was followed by UV–visible spectroscopy (Uvikon 930).

4.1.1. PLP hemiacetal. An aqueous solution of PLP (0.01 mmol, 10 mL) was stirred and heated at 95°C for 15 h under argon atmosphere. The mixture was then lyophilised and the residue solubilised in D_2O. The hemiacetal form of PLP was identified by NMR.

^{1}H NMR (400 MHz, D_2O) δ 2.18 (s, 3H, CH_3), 4.76 (d, ^2J=13 Hz, 1H, CH_2), 4.94 (d, ^2J=13 Hz, 1H, CH_2), 6.27 (s, 1H, O–CH–O), 7.28 (s, 1H, $CH_{aromatic}$).

4.2. Mass spectrometry

ESI mass spectra (positive mode) and the collision induced dissociation (CID) spectra of selected ions, under low energy conditions, were generally performed in an ion-trap mass spectrometer IT/MS (Esquire 3000, Bruker, Bremen, Germany). The sample were prepared in H_2O/CH_3CN (100 μM) and were introduced into the ESI source by infusion using a syringe pump (120 μL/h).

Conditions for the ESI source were as follows: source temperature 250°C, drying gas (N_2) flow: 7 L/min, nebulising gas 7 psi, capillary voltage 4000 V, capillary offset 70 V, skimmer 27 V, octopole RF 120Vpp.

For MS/MS experiments, the quasi-molecular ions displayed in the ESI mass spectrum are selected by using broad-band isolation method. These selected ions, in a second step, can be excited by collision with helium in the trap cell and then decompose. Resonant excitation conditions were chosen (0.77 V, isolation width 1 Th). All mass spectra are averages of 2 min accumulation.

A triple quadrupole mass spectrometer (Quattro, Micromass) equipped with a ESI source was also used for complementary MS/MS experiments.

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