Mechanism for the elimination of aromatic molecules from polyenes in tandem mass spectrometry

Thais Guaratini,[ab] Norberto P. Lopes,[ac] Ernani Pinto,[d] Pio Colepicolo[b] and Paul J. Gates*[a]

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We report here a general mechanism for the elimination of aromatic molecules from polyene containing natural products of several compound classes in tandem mass spectrometry.

The ongoing search for new derivatives and co-metabolites of bioactive compounds and their quantification from complex biological matrices for pharmacological and pharmacokinetic studies, often employs hyphenated techniques, for example LC-MS and HPLC-MS/MS.[1] These methods require analysis of mechanistically and structurally dependent fragmentation that is independent of the ionisation and fragmentation methods used.[2] Polyene chains commonly occur as a key structural feature in natural and synthetic compounds relating to several important classes of bioactive compounds, including: carotenoids, vitamins, antibiotics and toxins.[3–5]

Previous studies of mycolactone, a highly potent toxin that is thought to be the causative agent of Buruli fever,[6,7] showed a major product ion at *m*/*z* 659 (a loss of C_8H_{10}).[8] A further study of a new mycolactone strain showed the postulated loss of 1,3,5-trimethylbenzene (*m*/*z* 737 to 612).[9] These unusual losses could be an important common feature of polyene containing molecules and a thorough investigation of the literature reveals several reported examples of the possible loss of both toluene and benzene with no further rationalisation. For example, in a detailed systematic FAB study of carotenoids,[10] the elimination of toluene was observed. In a later independent study of the MALDI-MS/MS of carotenoids,[11] the loss of both toluene and benzene is reported. In both of these cases, no attempt at an explanation is entered into. Also, during the thermolysis of extended conjugated aliphatic chains (produced through the dehydration of poly-alcohols) elimination of aromatic molecules has been proposed to occur.[12] Supporting use of this hypothesis here, previous studies with five-membered lactones showed a good correlation between thermolysis and mass spectral induced fragmentations.[13] Together, these findings stimulated the present systematic MS/MS study of a range of polyene containing compounds (Fig. 1) to try to develop a gas-phase mechanistic rationale which correlates the observed loss of the aromatic molecule with the analyte structure.

The carotenoid pigments† (Fig. 1, **1–3**) (precursors in the biosynthesis of vitamin A and its co-metabolites[14]) play an important role in the protection of fungi, algae and plants against UV radiation as well as providing efficient suppression of reactive oxygen.[15,16] They also have very low *trans–cis* isomerisation energies. For bixin (Fig. 1, **3**) it has been reported that the all-*trans* isomer is prevalent in nature, but semi-empirical calculations show that there is little difference in the *trans–cis* stability.[17] During the ionisation and subsequent collision induced fragmentation, the internal energy of the analyte increases sufficiently to easily induce isomerisation of the polyene chain. Detailed analysis of the three carotenoid pigments by various mass spectral techniques‡ showed the ability to lose a molecule of toluene from various precursor ions (Table 1). The MS/MS analysis (see Fig. 2) of the $M^{+\bullet}$ of **2** and **3** gave the loss of toluene in the positive ion mode, whilst only the $[M - H]^-$ of **4** was observed to eliminate toluene in the negative ion mode. In the positive ion mode, bixin loses CO_2 as the dominant fragment. Low-resolution triple quadrupole ESI-MS/MS and Q-Tof nanospray-MS/MS gave the same results (results not shown). High-resolution accurate-mass ESI-FTICR-MS/MS allowed the confirmation of the formulae of the aromatic neutrals lost (Table 1).

Retinoids† are oxidised biosynthetic products from β-carotene, and are important in human nutrition and vision.[18,19] They also have low *trans–cis* isomerisation energies.[20] Our previous ESI-MS studies of the retinoids showed different ionisation processes were

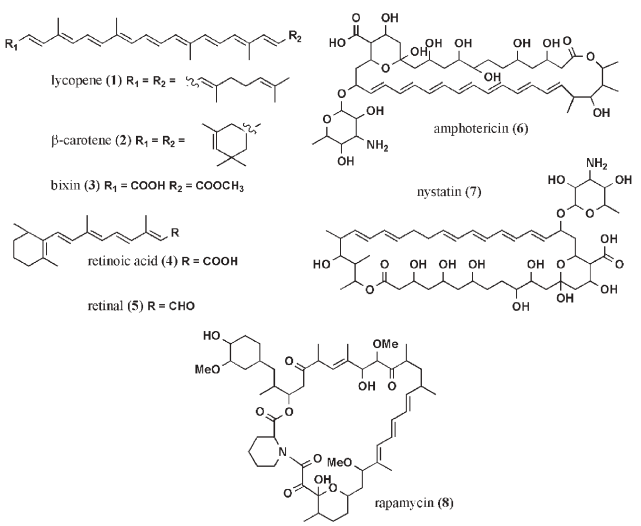


Fig. 1 Structures of the polyene containing molecules analysed.

[a]*School of Chemistry, University of Bristol, Cantock's Close, Bristol, UK BS8 1TS. E-mail: paul.gates@bristol.ac.uk; Fax: +44 117 9298611; Tel: +44 117 3317358*
[b]*Departamento de Bioquímica, Instituto de Química de São Paulo, Universidade de São Paulo, São Paulo, Brazil*
[c]*Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto-SP, Brazil. E-mail: npelopes@fcfrp.usp.br; Fax: +55 16 36024243; Tel: +55 16 36024707*
[d]*Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas de São Paulo, Universidade de São Paulo, São Paulo, Brazil*

Table 1 Precursor and product ion accurate-masses and formulae of the aromatic loss for the polyene compounds analysed. Data obtained by FTICR-MS/MS (see Fig. 2)

Compound	Precursor ion	Precursor mass	Product ion mass	Aromatic loss
lycopene	$M^{+\bullet}$	536.4326 (−2.8)[a]	444.3755 (+0.9)	C_7H_8
β-carotene	$M^{+\bullet}$	536.4350 (−3.7)	444.3743 (−1.8)	C_7H_8
bixin	$[M + Na]^+$	417.2039 (+0.5)	311.1255 (−1.3)	C_8H_{10}
retinal	$[M + H]^+$	285.2214 (+0.3)	193.1587 (0)	C_7H_8
amphotericin	$[M + Na]^+$	946.4746 (−0.4)	868.4298 (−0.3)	C_6H_6
rapamycin	*m/z* 564 − methanol	532.3028 (+0.9)	440.2413 (+1.3)	C_7H_8

[a] The mass measurement error (ppm) is given in parentheses.

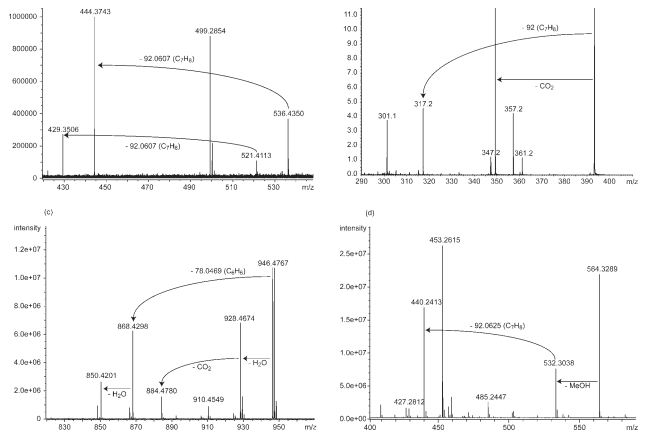
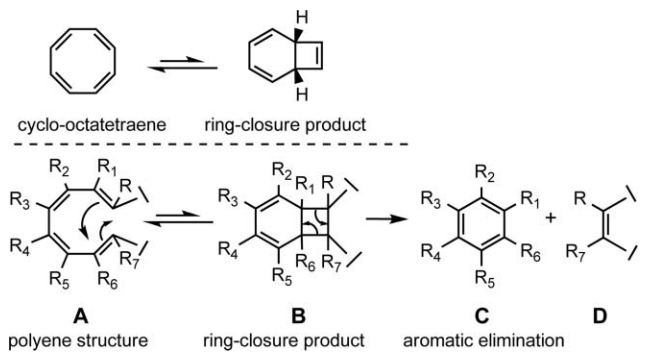


Fig. 2 Selected product ion mass spectra to demonstrate the elimination of aromatic molecules from the polyene compounds analysed. Spectrum (a) ESI-FTICR-MS/MS of β-carotene, (precursor ion, M^+, *m/z* = 536), (b) nanospray-MS/MS of bixin (precursor ion $[M - H]^-$, *m/z* = 393), (c) ESI-FTICR-MS/MS of amphotericin (precursor ion $[M + Na]^+$, *m/z* = 946) and (d) ESI-FTICR-MS^3 of rapamycin (precursor ion is *m/z* = 564 from the MS/MS of $[M + Na]^+$).

occurring when compared to the carotenoids, possibly due to differences in conjugated chain length.[21–23] In the ESI-MS/MS‡ of retinoic acid (Fig. 1, **4**) ($[M - H]^-$ negative mode) only the loss of CO_2 was observed, whereas for retinal (Fig. 1, **5**, Table 1) ($M^{+\bullet}$ or $[M + H]^+$ positive mode) the loss of the aromatic molecule from both precursors was observed.

If we consider that four conjugated double bonds might be the minimum required for this loss of an aromatic ring to occur, then structure **A** (Scheme 1) is similar to the nonaromatic compound cyclo-octatetraene. Conjugated cyclic compounds that do not have



Scheme 1 Proposed general mechanism for the elimination of aromatic molecules from polyene compounds.

a continuous overlap of *p*-orbitals are considered nonaromatic and consequently their stabilities are very similar to those of their acyclic counterparts. Based on this classical principle of aromaticity,[24] cyclo-octatetraene has an ambient temperature electrocyclic equilibrium giving rise to the phenomenon of *valence tautomerism*. Thus, a symmetry-allowed disrotatory ring-closure is highly possible (see Scheme 1).[25] If we consider the same pericyclic reaction from structure **A**, the orbital overlap will produce the same 6-membered ring as observed for cyclo-octatetraene. However, in this case, the electrons of the final double bond close the 4-membered ring resulting in structure **B**. It is well known from the literature that structures such as **B** result in two planar rings, then in the CID step in the MS/MS analysis, **C** is easily formed by a retro-pericyclic reaction eliminating the 6-membered aromatic molecule, resulting in ion **D**.

The macrocyclic compounds† (see Fig. 1): amphotericin (**6**); nystatin (**7**); and rapamycin (**8**) (produced by strains of *Streptomyces*)[26,27] are important natural products widely used in human therapy as: immunosuppressants,[28] antibiotics[27] and antifungals.[26] They are studied here‡ to confirm the above hypothesis and to demonstrate the effect of cyclisation and limited polyene chain length on the ability to eliminate an aromatic molecule. Amphotericin was observed to lose benzene in all studies performed (Fig. 2, Table 1) in contrast to nystatin, which was never observed to undergo loss of an aromatic molecule. The main difference between these two molecules is the length of the polyene chain. Amphotericin has seven conjugated double bonds with enough possibility to allow low-energy *trans–cis* isomerisation and thus elimination of benzene. Nystatin has four conjugated double bonds, but there are strong steric restrictions that probably increase the activation barrier for the *trans–cis* isomerisation. Rapamycin has three conjugated double bonds, and as expected, it was not observed to lose an aromatic molecule in ESI-MS/MS. However, in the MS^3 of the major structural fragment,[29] *m/z* 564, (Fig. 2, Table 1) it readily loses methanol to produce the four conjugated double bonds required to lose the aromatic molecule. The molecule is then observed to lose a molecule of toluene as expected.

In conclusion, we have demonstrated that four conjugated double bonds is the minimum condition (but not enough alone) required for the loss of an aromatic molecule from long-chain unsaturated compounds. For nystatin (which has four conjugated bonds) steric hindrance prevents the *trans–cis* isomerisation and no loss is observed. Rapamycin was shown to lose a molecule of toluene only after loss of a major part of the molecule and loss of methanol (producing the four conjugated double bonds required). Also, in the case of the acids, loss of CO_2 is often dominant, sometimes to the extent that the elimination of the aromatic

molecule is totally suppressed. A range of mass spectrometers were used throughout the study to demonstrate the independence of the fragmentation from instrument design and manufacturer. The mechanism of this elimination is proposed based on classical theories of aromaticity and the *valence tautomerism* of cyclo-octatetraene. The understanding of the mechanism involved in this reaction will be very helpful for all researchers involved in the quantification, analysis or elucidation of such compounds and can also be used to explain previous results in the scientific literature. Finally, this loss of an aromatic molecule is demonstrated to occur on a wide range of different instruments with a range of polyene compounds.

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Notes and references

† The carotenoid and retinoid samples were obtained in solution in either methanol or ethanol. Prior to analysis they were diluted in acetonitrile–water (50% v/v) with 0.05% formic acid to arrive at the final concentration of approximately 0.5 mg mL^{-1}. The macrolide antibiotics were all available as pure samples (Sigma/Aldrich standard products). They were dissolved in methanol–dichloromethane (50% v/v) to a concentration of approximately 0.05 mg mL^{-1}.

‡ Low-resolution ESI analyses were performed on a Quattro-LC triple quadrupole instrument (Micromass, Manchester, UK) at the Ribeirão Preto Faculty of Pharmaceutical Sciences, University of São Paulo. Nanospray ionisation analyses were performed on a QStar-XL quadrupole time-of-flight hybrid instrument (Applied Biosystems, Warrington, UK) using a Nanomate 100 automatic chip based nanospray system (Advion Biosciences, Norwich, UK) at the School of Chemistry Mass Spectrometry Facility, University of Bristol. High-resolution ESI analyses were performed on an UltrOTOF-Q quadrupole time-of-flight hybrid instrument (Bruker Daltonics, Billerica, MA, USA) at the Ribeirão Preto Faculty of Pharmaceutical Sciences, University of São Paulo. High-resolution accurate-mass ESI analyses were performed on a 7 Tesla Apex IV Fourier-transform ion cyclotron resonance instrument (Bruker Daltonics, Billerica, MA, USA) using an Apollo off-axis ESI source, at the School of Chemistry Mass Spectrometry Facility, University of Bristol. In all cases, CID-MS/MS fragmentation analyses were performed on the isolated parent ions using either N_2 or CO_2 collision gas. The MS^3 analysis of rapamycin was performed on the optimised precursor ion from the MS/MS analysis step.

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