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Mass spectrometric fragmentation behaviour of cAMP analogues

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ABSTRACT

The mass spectrometric fragmentation of a number of analogues of the biological second messenger, adenosine 3′,5′-cyclic monophosphate (cAMP), was undertaken in order to study the effects of modifications of the naturally-occuring compound on the tandem mass spectrometry data obtained. Such analogues are being investigated as biomedical treatments and also as experimental research tools; the data reported here will assist the structural elucidation of novel cAMP analogues produced in the future by acting as a reference guide as to the effect of different modifications on the mass spectrometric fragmentation patterns.

1. Introduction

The application of tandem mass spectrometry (MS/MS) for the elucidation of the structure of nucleobases, nucleosides, nucleotides and cyclic nucleotides has been well documented [1–8]. Recent advances in the field of mass spectrometry now allow both the ability to trap and fragment initial MS/MS derived product ions (and continue this trend in MS^n analyses) and to perform accurate mass analysis to the sub-ppm level. The use of modern MS/MS experiments enables the comparison of fragmentation pathways of cyclic nucleotides adding to the available information regarding their mass spectrometric behaviour. In nucleoside/nucleotide research, MS^n experiments have been utilised to study the fragmentation pathways and gas phase reaction of many compounds in great detail [9,10]. Mass spectrometric analysis has previously led to the identification of nucleosides, such as 3′-deoxyadenosine as an antibiotic – cordycepin [11], and the identification of potential tumour cell markers such as 5′-deoxycytidine from urine samples of patients suffering with cancer of the head and neck [12]. To date, less information has been gathered regarding the fragmentation behaviour of modifications of the cyclic nucleotides. Novel adenosine cyclic nucleotide derivatives have been previously investigated by fast-atom bombardment MS (FAB-MS) for molecular weight information and fast-atom bombardment mass-analysed ion kinetic energy spectrometry (FAB-MIKES) for structural information [7]. Chemically synthesised analogues (especially of adenosine cyclic monophosphate (cAMP)) have numerous roles both as potential therapeutics and as experimental research tools. Recent studies have shown that 8-Cl-cAMP causes the down-regulation of the Type I regulatory unit and up-regulation of Type II regulatory unit of cAMP-dependent protein kinase, and is recognised as an anti-proliferative agent [13–15]. Such derivatives have potential applications in treating brain tumours, as it has been shown that dibutyryl cAMP decreases the growth of semiconfluent cultures in rat brain tumour cells [16,17]. The activity of some protein kinases that can be either stimulated or inhibited by a selection of cyclic nucleotide derivatives, has also been reported with some having therapeutic activity [18]. Further derivatives, such as N^6-benzyl cAMP, and its chlorinated derivatives, have demonstrated that different chemical modification of the second messenger causes the cAMP analogue to alter the cell cycle kinetics of leukemia cells at different points [19]. As experimental tools, cAMP analogues can be used as membrane permeable agents (in the case of chlorophenylthio-cAMP) that affect cAMP activity. These inhibit adenylate cyclase activity which causes the abolition of native cAMP formation, enabling the study of the effects of varying exogenously added cAMP levels in many processes including neuron potentiation [20]. Furthermore, other structural analogues of cAMP have found applications as affinity materials for selectively binding proteins which bind cAMP in different cells and metabolic conditions, for example 2-(6-aminohexylamino) cAMP has been used in “cAMP capture compound mass spectrometry” from cell lysates of *Escherichia coli* [21]. This current study examines the mass spectrometric fragmentation behaviour of synthetic derivatives of cAMP with a view to obtaining a better understanding of their fragmentation processes. In addition to the intrinsic interest in these processes *per se*, such

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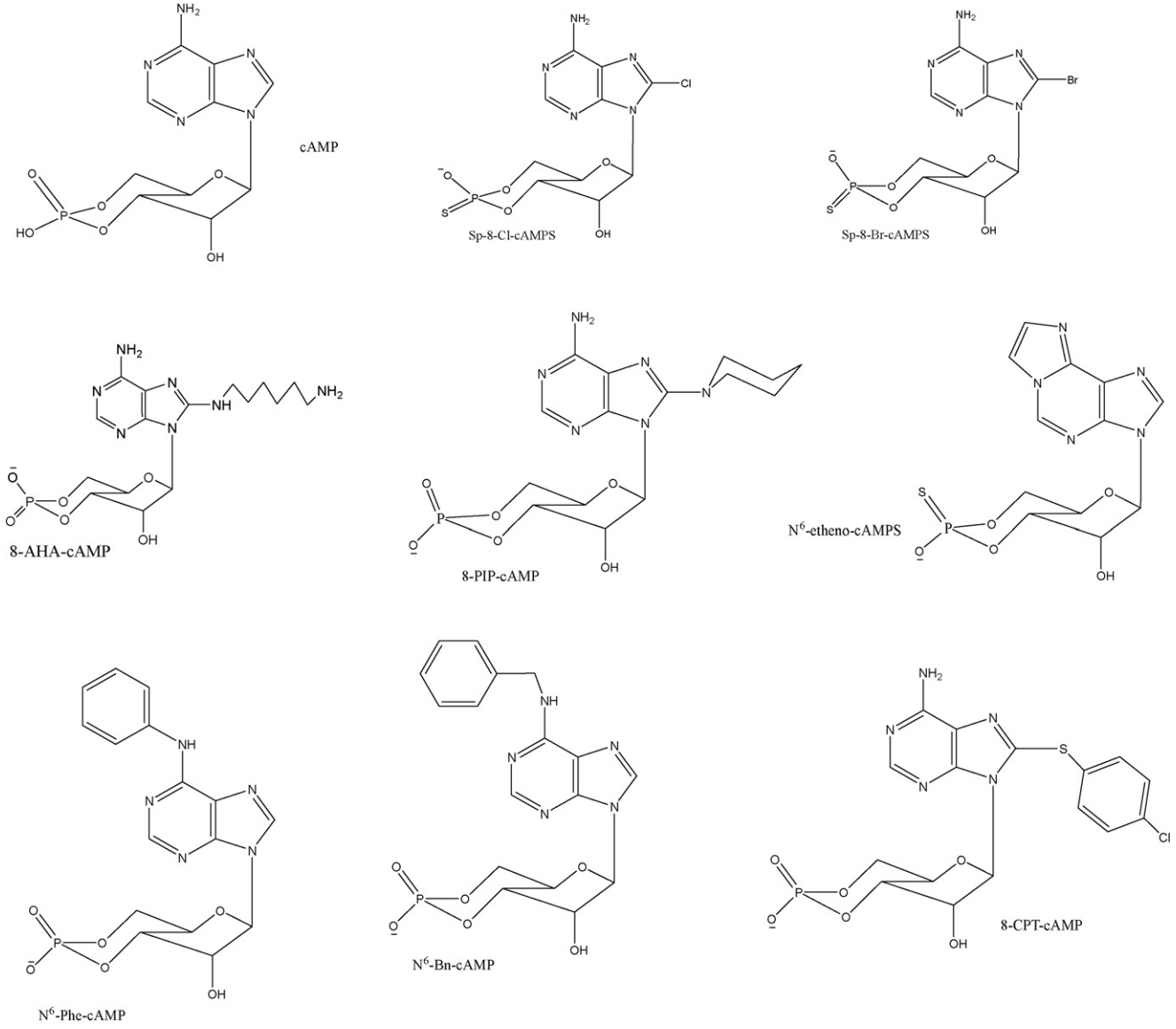

Fig. 1. Structure of cAMP and the derivatives studied.

information is of value as it allows data from other newly synthesised analogues to be compared to this dataset for structural validation.

2. Material and methods

2.1. Materials

Adenosine-3′,5′-cyclic monophosphate (cAMP) was obtained from Sigma (Poole, Dorset, U.K.) and 8-chloroadenosine-3′,5′-cyclic monophosphorothioate (Rp/Sp-8-Cl-cAMPS), 8-bromoadenosine-3′,5′-cyclic monophosphorothioate (Rp/Sp-8-Br-cAMPS), 8-(2-aminohexyl)aminoadenosine-3′,5′-cyclic monophosphate (8-AHA-cAMP), 8-piperidinoadenosine-3′,5′-cyclic monophosphate (8-PIP-cAMP), N^6-benzyladenosine-3′,5′-cyclic monophosphate (N^6-Bn-cAMP), 8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphate (8-CPT-cAMP), N^6-ethenoadenosine-3′,5′-cyclic monophosphate (N^6-etheno-cAMP) and N^6-phenylyladenosine-3′,5′-cyclic monophosphate (N^6-Phe-cAMP) that were obtained from Biolog Life Sciences Institute (Bremen, Germany). Methanol (MeOH) and acetonitrile (ACN) were obtained from Fisher Scientific (Loughborough, Leic., U.K.) with formic acid (HCOOH) being purchased from Analar (Dorset, U.K.). The oxygen-free nitrogen was supplied by BOC Ltd. (Guildford, Surrey, U.K.) and the Milli-Q purified water was prepared 'in-house' using the Elix® and Milli-Q® ultrapure water purification system obtained from Millipore U.K. Ltd. (Watford, U.K.). 1 mg of the each of the cAMP analogues was dissolved in water and then further diluted to concentrations ranging from 2 to 10 pmol/μL with 50/50 methanol/water prior to mass spectrometric analysis.

2.2. Mass spectrometry

The LCQ – Deca XP ion trap mass spectrometer was equipped with a nano-electrospray source (ThermoFinnigan, U.K.). The electrospray source was operated in negative ion mode and full scan mass spectra were recorded using a maximum injection time of 200 ms and 3 microscans with a spray voltage of 3.0 kV, capillary voltage of 15 V and capillary temperature of 160 °C. During the MS/MS experiments the precursor ions were selected and isolated from a full scan mass spectrum. Collision energy sufficient to fragment all of the precursor ions or the selected ions was applied to

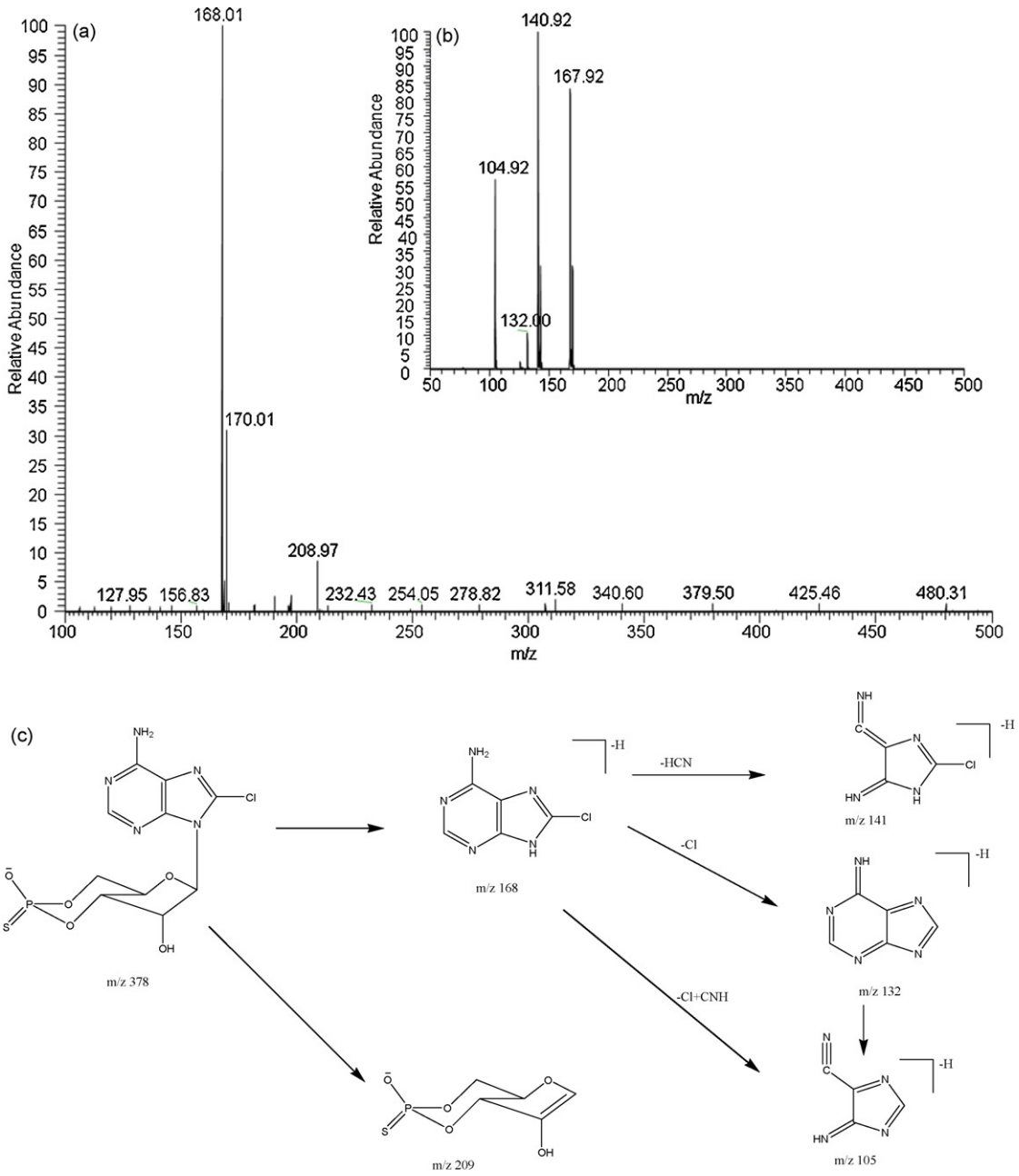

Fig. 2. (a) MS/MS spectrum of 8-Cl-cAMPS (*m*/*z* 377). (b) MS^3 spectrum of 8-Cl-cAMPS (*m*/*z* 168 via *m*/*z* 377). (c) Fragmentation scheme for 8-Cl-cAMPS

produce fragmentation spectra. LCQ – Deca XP control and data acquisition were performed by the Xcalibur software. For accurate mass analysis, an Advion nanosprayer (Advion, U.K.) was utilised, which applied the spray voltage of +1.55 kV and infused the samples directly into an Orbitrap mass spectrometer (ThermoFinnigan, U.K.) operated in negative ionisation mode. Following calibration with a sheath gas flow of 2 (arbitary units) and a capillary temperature of 200 °C, combinations of CID, HCD and in source fragmentation were used in order to generate the product ions formed with different derivatives requiring different conditions for optimal product ion formation.

3. Results

The application of tandem mass spectrometry for the elucidation of the structure of cAMP has been previously documented [7], with negative ionisation mass spectrometry commonly being utilised due to the increased ion current produced compared to positive ionisation analysis. The fragmentation of cAMP in negative ionisation mode in initial MS/MS analysis exhibits the loss of the sugar and phosphate group producing the deprotonated base moiety as the only product ion formed. The cleavage of the glycosidic bond being common to nucleotides, except C-nucleotides and nucleosides in which the bond is more stable [9]. This ion can be further fragmented and loses HCN (represented by a mass loss of 27 Da) and NH_2CN (represented by a loss of 42 Da). These losses are characteristic of the adenosine base and occur after ring opening of the six-member ring of the base as shown in Supplementary Fig. 1(a) and (b). The fragmentation behaviour of various derivates of cAMP (molecular structures shown in Fig. 1) was therefore studied and compared to the data acquired during the analysis of cAMP. The first set of analogues studied were cAMP which had been modified to a small degree on both the sugar and the base. In particular, cAMP with a halogen substitution on the base and a thiophos-

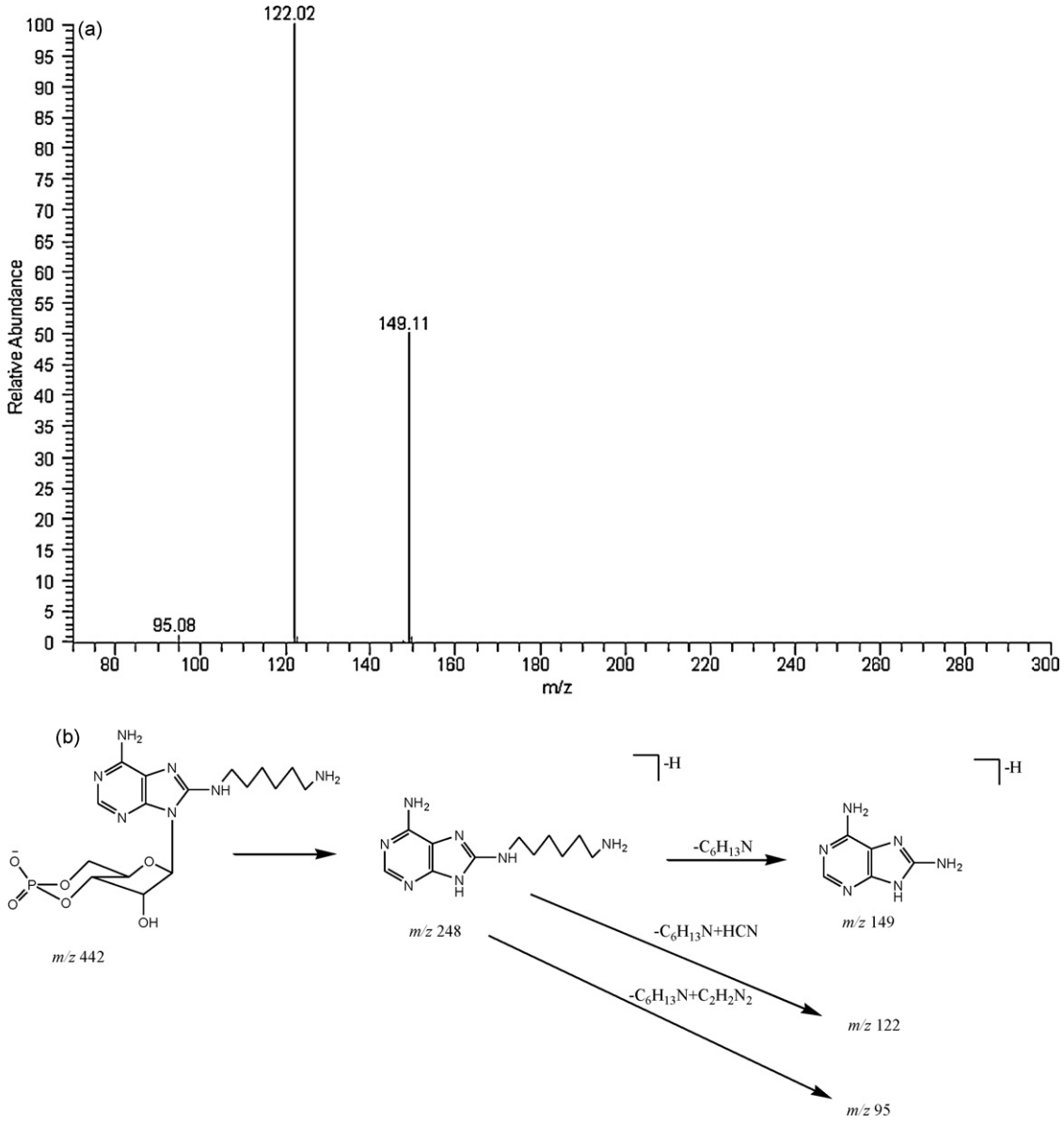

Fig. 3. (a) MS^3 spectrum of 8-AHA-cAMP (m/z 248 via m/z 442). (b) Fragmentation scheme for 8-AHA-cAMP

phate substitution on the sugar was examined (8-Cl-cAMPS and 8-Br-cAMPS). These sulphur-containing groups offer two possible isomers depending upon the orientation of the sulphur; both isomers (Rp and Sp) produced identical mass spectrometric fragmentation data in our analysis.

When 8-Cl-cAMPS was studied under MS/MS conditions, the first difference between the fragmentation spectra obtained and that of unmodified cAMP was the presence of an additional product ion (other than the deprotonated base) at m/z 209. Fig. 2(a) shows the MS/MS spectrum of the chlorinated nucleotide, using an isolation window wide enough to fragment both the C^{35} and C^{37} isotope containing nucleotides. It can be seen that the deprotonated base ion exhibits the expected chloride isotope pattern whilst the m/z 209 does not, demonstrating that the m/z 209 product ion does not contain the chloride. Accurate mass analysis (see Supplementary Table 1 for all accurate mass data throughout the manuscript) identified the ion as the ionised sugar moiety derived from the breakage of the glycosidic bond. This suggests that the presence of the chloride allows for the more stable formation of the sugar derived ion compared to the unmodified cAMP. Further fragmentation of the m/z 209 generates product ions arising from loss of the phosphate group with the charge maintained on the sugar and the same process with the charge retained on the phosphate (data not shown), however this data does not aid in the characterisation of the modified nucleotide. Further fragmentation of the deprotonated nucleobase (again isolating both isotope ions, Fig. 2(b)) gives rise to product ions at m/z 141 (which exhibits the characteristic chloride isotope pattern), m/z 132 and m/z 105 (the latter two not having the same chloride derived isotope pattern). Hence the m/z 141 ion arises from the loss of sections of the purine ring, whilst the other two ions arise from the loss of the chloride and the chloride along with CNH (see Fig. 2(c)).

Fragmentation of 8-Br-cAMPS was also undertaken in a similar fashion (isolating all bromine derived isotope ions at each stage where applicable) and MS/MS generated the same expected ions – the deprotonated nucleobase and the sugar derived m/z 209. This again highlights the impact on a halogen on the nucleotide, increasing the stability and/or ability to form the sugar derived product ion during MS/MS of the nucleotide. Therefore, the presence of this ion in any MS/MS spectra has the potential to be used as a character-

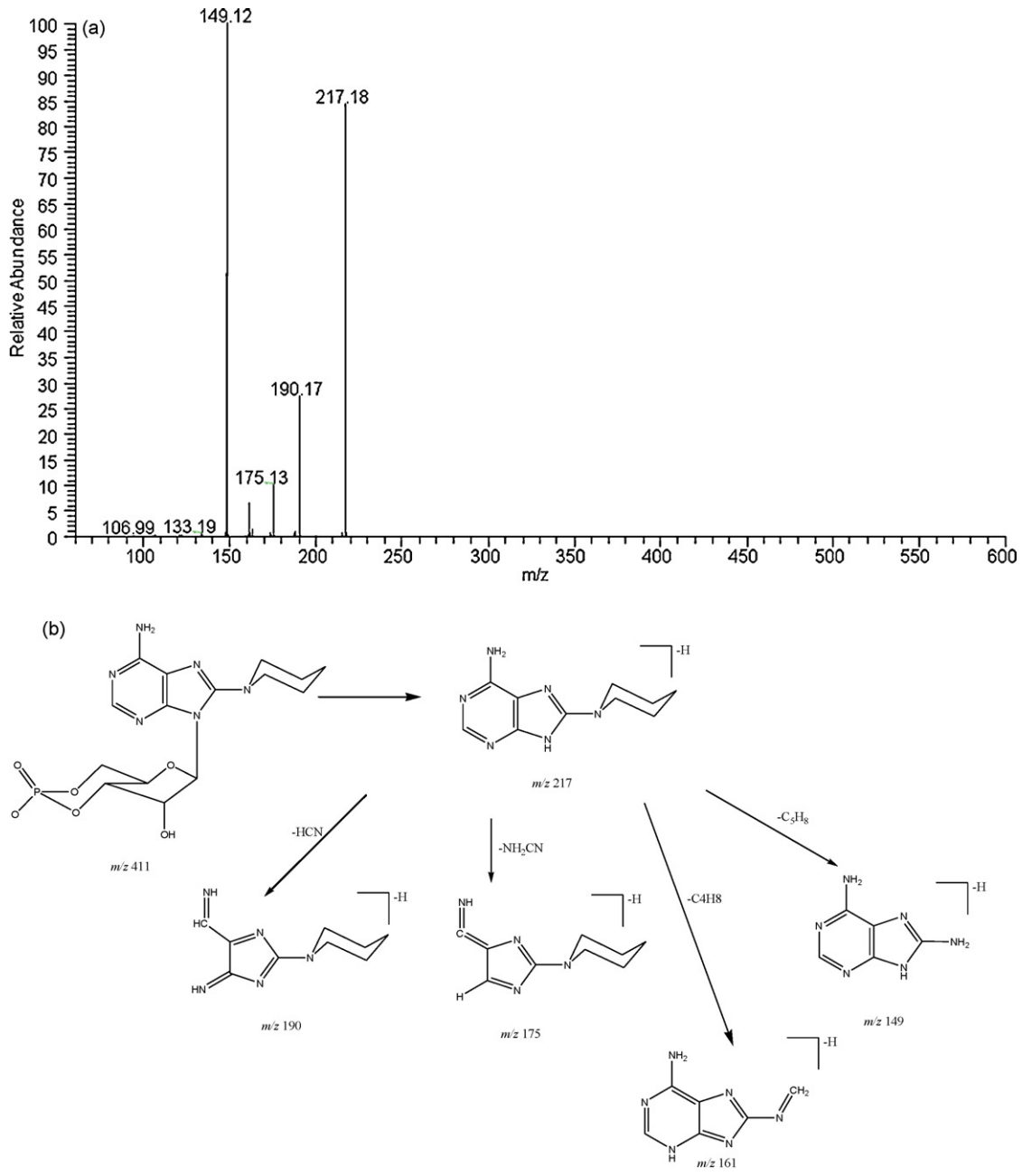

Fig. 4. (a) MS^3 spectrum of 8-PIP-cAMP (m/z 217 via m/z 411). (b) Fragmentation scheme for 8-PIP-cAMP

istic product ion of a halogenated nucleotide and the mass of the ion can indicate whether the phosphate has addition atoms, such as the sulphur in cAMPS derivatives, within its structure. Similar to the chlorinated derivative, MS^3 of the bromine derivative of cAMPS (via the deprotonated nucleobase) generated ions derived from the loss of HCN, the halogen (as HBr) and the two lost together (see Supplementary Fig. 2(a–c)) with again the isotope pattern clearly distinguishing fragment ions which have lost the halogen during their formation.

The second set of modified cyclic AMP compounds studied had different modifications present on the base, bound to the base via nitrogen in five cases and sulphur in the last. The first modification studied was the addition of an aminohexyl group attached to the C8 position on the imidazole ring of the nucleobase via an additional amino group (8-AHA-cAMP). MS/MS analysis resulted in the complete loss of the sugar and phosphate group leaving the deprotonated base (see Supplementary Fig. 3). However, a more abundant product ion is also present in the MS/MS spectrum which represents the further fragmentation of the deprotonated base (during MS/MS analysis) resulting in the additional loss of the aminohexyl chain (Fig. 3(a)) leaving only the additional amino group attached at position 8 on the purine (m/z 149). This ion is also seen in the MS^3 spectrum fragmenting the deprotonated nucleobase alongside ions representing the further loss of a single HCN (m/z 122) and, less abundantly, the loss of two HCN (m/z 95) (see Fig. 3(b)) in addition to the cleavage of the aminohexyl chain. Whilst, without stable isotope labelling of the compound, it is not possible to determine which atoms constitute the lost HCN moieties, it is thought likely that the losses arise from the six-member ring which – as demonstrated for cAMP [22] – undergoes ring opening during base fragmentation. It is therefore possible that the additional amino group is retained throughout the fragmentation process.

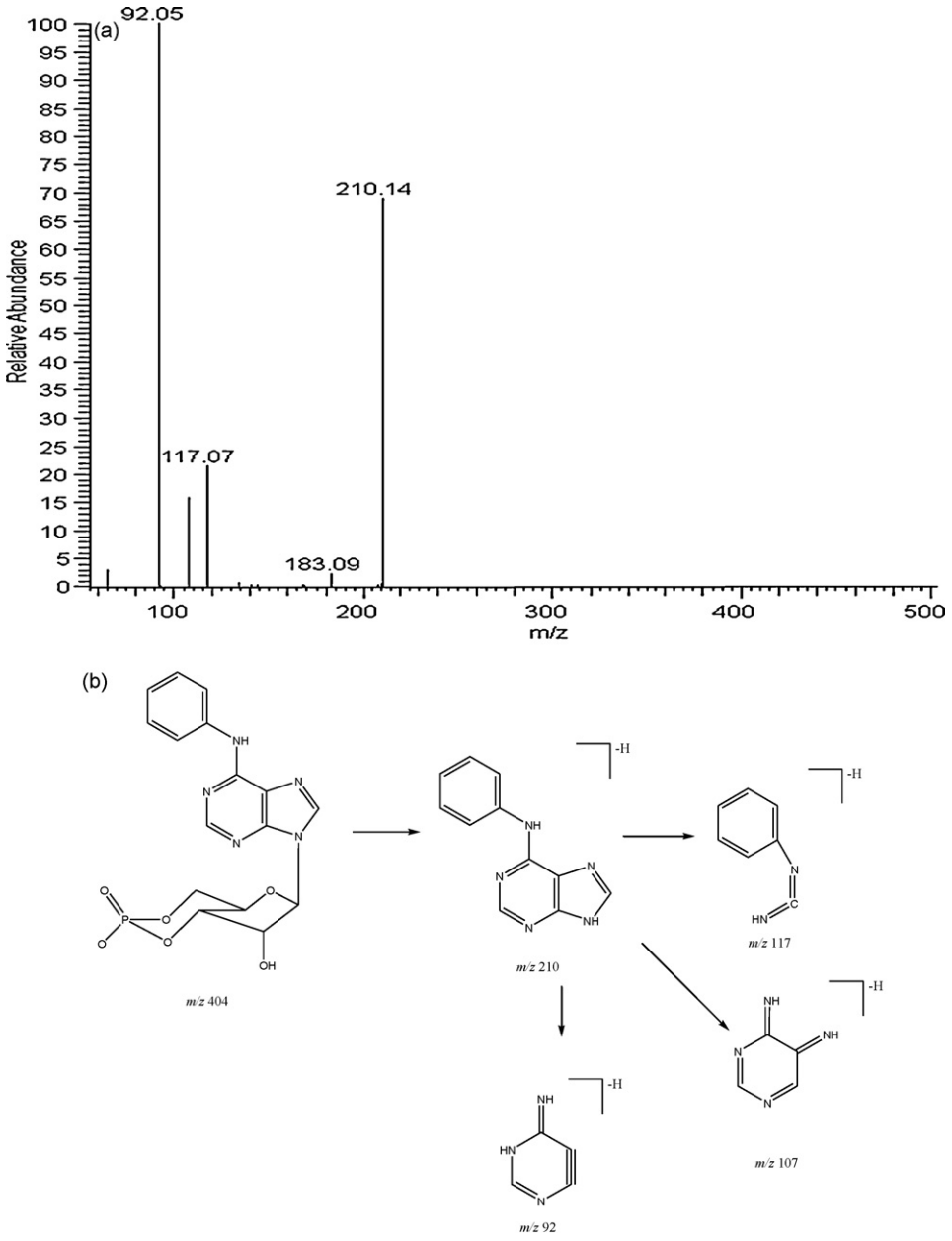

Fig. 5. (a) MS^3 spectrum of N^6-Phe-cAMP (*m*/*z* 210 via *m*/*z* 404). (b) Fragmentation scheme for N^6-Phe-cAMP

Similarly, 8-PIP-cAMP represents the modification of cAMP at the carbon 8 position by the addition of a nitrogen-linked modification. In the case of 8-PIP-cAMP however, the modification is a piperidine ring. Again, during MS/MS the sugar and phosphate are lost (Supplementary Fig. 4), unlike the fragmentation of 8-AHA-cAMP, using the same energy input no fragmentation of the piperidine substituent was found. Further fragmentation results predominantly in the loss of the majority of the modification with the nitrogen retained giving rise to the same *m*/*z* 149 product ion detected in further 8-AHA-cAMP fragmentation (Fig. 4(a)). However, other product ions in which the modification is retained intact and losses occur via ring opening and loss of HCN and NH_2CN (as seen for unmodified cAMP) are also detected (Fig. 4(b)). The presence of these ions suggests that the cyclic nature of the modification provides a partially more stable modification compared to the linear AHA-type modification. However, an ion derived from the fragmentation of the modification group is detected at *m*/*z* 161 (accurate mass analysis suggesting an empirical formula of $C_6H_5N_6$, 1.089 mmu mass accuracy). This ion is believed to arise via the fragmentation of the cyclic modification and loss of all but the nitrogen and one carbon atom as seen in Fig. 4(b).

N^6-Phe-cAMP comprises of a phenyl ring attached to the adenosine base via an existing nitrogen which is not a part of the central purine ring structure (attached to carbon 6). Whilst the initial MS/MS fragmentation follows the predicted route of the production of the deprotonated base (analogous to unmodified cAMP – Supplementary Fig. 5), the further fragmentation is more divergent from that seen for previous modifications (Fig. 5(a)). A product ion at *m*/*z* 117 derived an empirical formula of $C_7H_5N_2$ during accurate mass analysis (1.335 mmu mass difference) and this potentially represents the retention of the charge on the modification and the retention of a carbon and nitrogen from the purine base as suggested in Fig. 5(b). Further product ions are detected which are believed to have arisen from the loss of the phenyl ring and parts

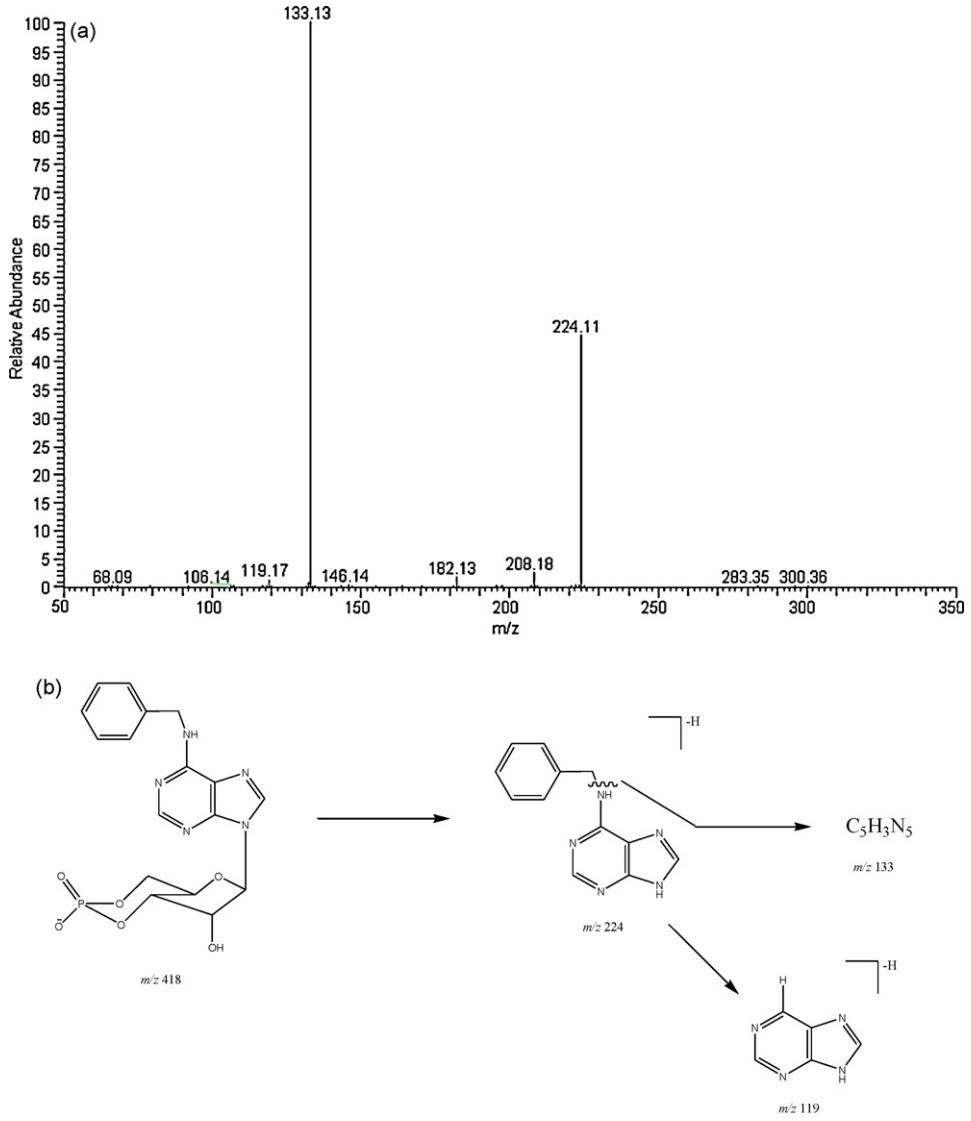

Fig. 6. (a) MS^3 spectrum of N^6-Bn-cAMP (m/z 224 via m/z 418). (b) Fragmentation scheme for N^6-Bn-cAMP

of the imidazole ring of the purine base. An ion at m/z 107 gave an empirical formula of $C_4H_3N_4$ during accurate mass analysis. The only mechanism by which the requisite number of carbons and nitrogens are available from the precursor ion for such a formula is via the retention of the six-member ring and the two nitrogens as shown in Fig. 5(b). Furthermore, by far the most abundant product ion is detected at m/z 92 and this ion is thought to represent a rearrangement of the pyrimidine ring (minus the modification and the three atoms of the five-member ring (empirical formula $C_4H_2N_3$, mass accuracy 1.526 mmu).

Following the analysis of the N^6-phenyl derivative of cAMP, the study of the N^6-benzyl derivative was undertaken. The two are very similar, with the benzyl derivative having an additional carbon group between the unsaturated ring of the modification and the nitrogen attached to the adenine base. As expected, the loss of the sugar and phosphate predominates during MS/MS analysis (Supplementary Fig. 6), however during further fragmentation of the deprotonated base, distinct losses compared to those exhibited by the similar phenyl derivative were noted. The most abundant product ion is that at m/z 133. This product ion has an empirical formula of $C_5H_3N_5$ therefore it must arise from the nucleobase and hence represent the complete loss of the modification from the cAMP derivative. However, the benzyl derivatives deprotonated base retained the nitrogen of the adenine base, unlike the phenyl derivative. The additional carbon between the six-carbon ring of the modification must therefore weaken the bond to the adenine nitrogen and hence the retention of this nitrogen by the base is preferred (Fig. 6(a) and (b)). Interestingly, the deprotonated base product ion has an m/z of 133, indicating a different rearrangement of atoms during fragmentation compared to previous modifications. The loss of the modification along with the adenine nitrogen is detected, producing a product ion at m/z 119, although with markedly less relative abundance.

The next derivative studied was N^6-etheno-cAMP which exhibits cyclisation between N1 and N^6 of the adenine base via two additional carbons (Fig. 1). After the usual loss of the sugar and phosphate (Supplementary Fig. 7), the commonly determined loss of HCN was observed (at m/z 131) – presumably via the usual ring opening mechanism – alongside the production of the product ion at m/z 92 as noted in the further fragmentation of N^6-phe-cAMP (see Fig. 7(a)). As the etheno derivative does not contain an additional six-member ring besides that within the usual purine

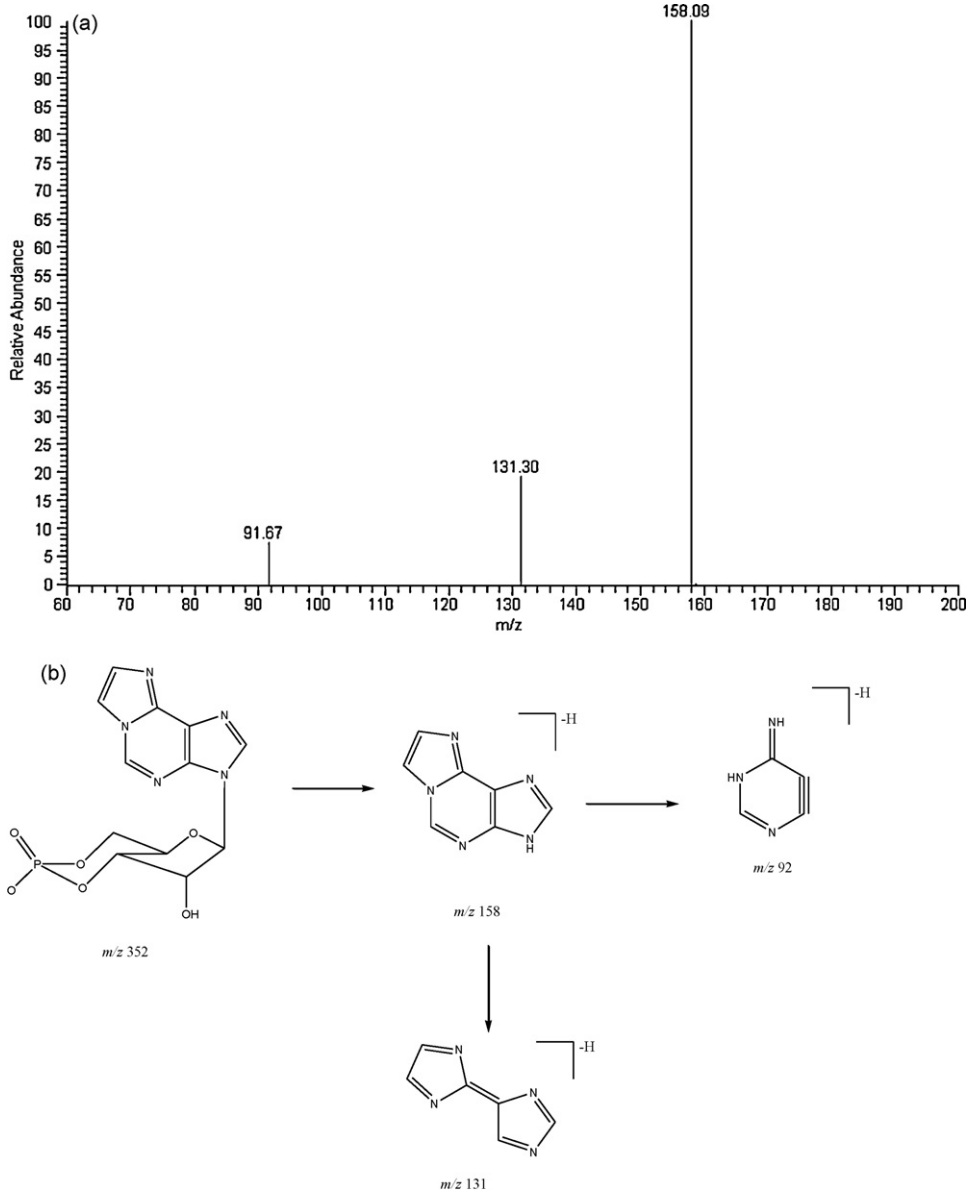

Fig. 7. (a) MS^3 spectrum of N^6-etheno-cAMP (m/z 158 via m/z 352). (b) Fragmentation scheme for N^6-etheno-cAMP

structure, this finding further substantiates the proposal that the loss of two distinct sections of the derivative under fragmentation conditions is possible leaving the central section of the purine (the pyrimidine ring) intact. Therefore, the m/z 92 product ion may also have been formed in this manner rather than arising from the release of the etheno itself (Fig. 7(b)). However, it is still possible that the m/z 92 ion consists of the imadazole ring containing the etheno bridge, N^1, C^6 and N^6 of the purine system and stable isotope labelling of the compound at specific atoms would be required to confirm which arrangement forms the ion.

Finally, we examined the data obtained when fragmenting a derivative with an additional halogenated ring structure attached to the adenine base via a sulphur atom rather than a nitrogen or carbon (CPT-cAMP – see Fig. 1). After the initial production of the deprotonated base as the lone product ion in MS/MS analysis (m/z 276, see Supplementary Fig. 8), the main product ion formed via further fragmentation represents the loss of HCN from the modified base (m/z 249 – Fig. 8(a)). There are a number of other product ions detected and accurate mass analysis was required to identify the species lost during fragmentation to generate these ions (Fig. 8(b)). The product ion at m/z 166 represents an empirical formula of $C_5H_4N_5S$ and hence represents the loss of the entire modification except the sulphur atom retained at carbon 8 (see Supplementary Table 1 for accurate mass values). The fragmentation of the phenyl ring attached to the sulphur with only two carbons and the sulphur retained by the deprotonated base ion is also detected at m/z 190 and the fragmentation at the other end of the compound – releasing two carbons and two nitrogens from the purine six-member ring – is also observed (m/z 222). Interestingly, there are a series of ions at m/z 244, 243 and 242 which represent exact mass differences between them of a single proton (see Supplementary Fig. 9). The accurate mass data gained, strongly suggest that these ions arise via the loss of the sulphur which joins the adenine base to chlorinated ring with the two lower m/z ions representing additional losses of one or two hydrogens as well as the sulphur. Such ion formation would require the breakage of the sulphur bridge between the two moieties and their reconnection in the gas phase and the mechanism by which this would occur (or alternatively whether the two rings sit one above the other spatially and join prior to loss of the sulphur) is at present unclear.

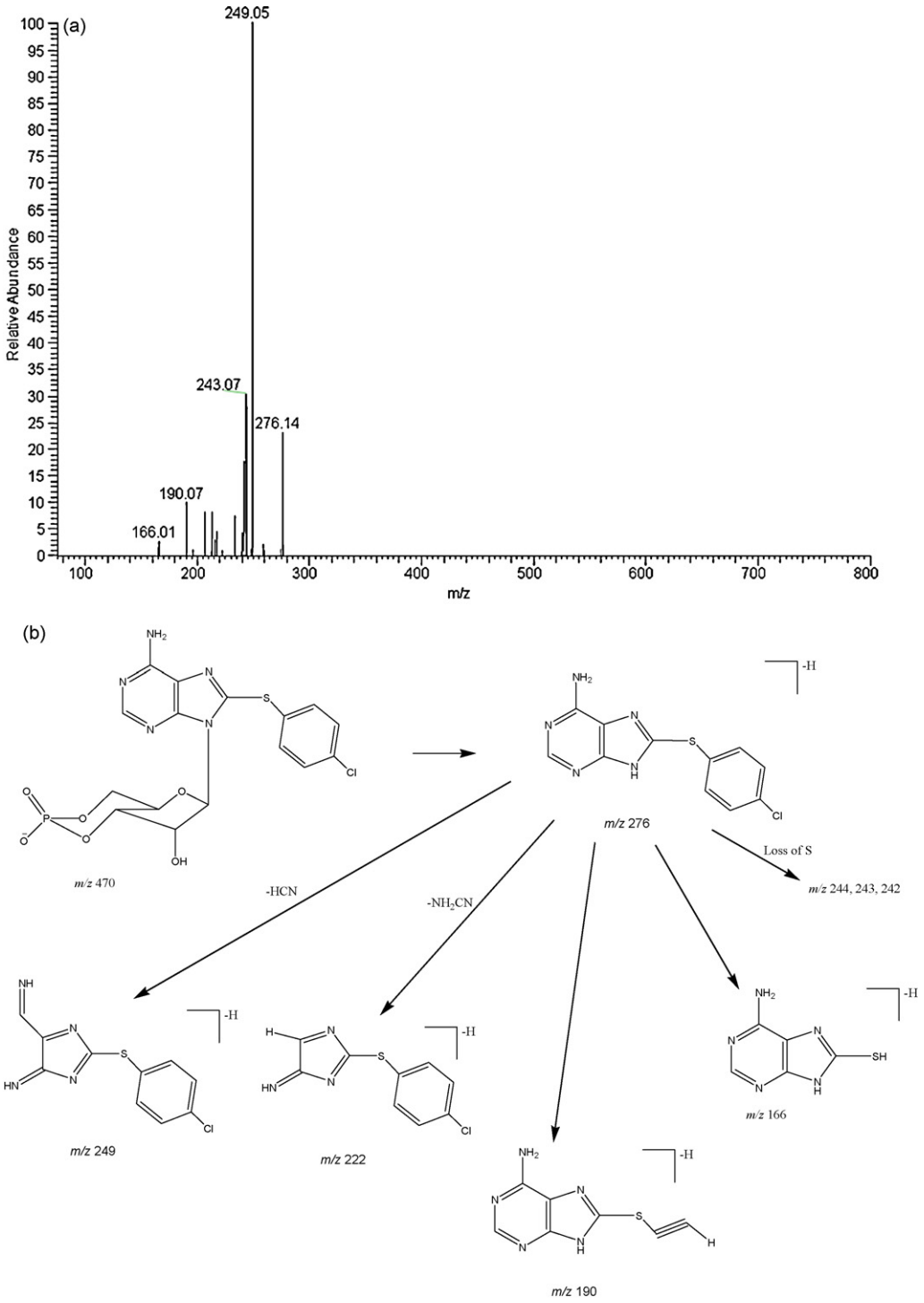

Fig. 8. (a) MS^3 spectrum of 8-CPT-cAMP (m/z 276 via m/z 470). (b) Fragmentation scheme for 8-CPT-cAMP

4. Conclusion

The aim of the study was to determine whether the derivatisation of cAMP caused a change in the tandem mass spectrometric behaviour of the compounds synthesised and whether the study of this behaviour was of value in studying novel synthesised analogues of cAMP which may have potential value as medicinal treatments or experimental research tools. The results obtained indicate a number of interesting features exhibited in the fragmentation spectra of cAMP derivatives that are of potential value. The first note is that, with the exception of the halogenated cAMPS, MS/MS purely indicates the loss of the sugar and the phosphate providing little information regarding the nature of any modification. Under further fragmentation halogenated derivatives produce a deprotonated base ion at m/z 132 (rather than 134) and a further ion at m/z 105 representing the further loss of CHN from this de-halogenated ion. The incorporation of halogens is also clearly evident in the isotope ratios for the intact deprotonated molecule as would be expected. Derivatives with nitrogen-linked modification groups at the carbon 8 position differ in their fragmentation behaviour depending upon whether the modification is cyclic or linear in nature. Linear modifications are lost comparatively easily

compared to cyclic modifications with their loss exhibited during MS/MS fragmentation of the intact cyclic nucleotide. Furthermore, the data clearly indicate that the addition of a carbon between modification groups attached to the carbon-6 linked nitrogen of cAMP leads to distinctly different fragmentation behaviour. More extensive modifications – such as CPT-cAMP- have been shown to lead to more complex fragmentation spectra which exhibit more diverse fragment ions (including product ion series one proton apart). The combined dataset and understanding of the characteristic behaviour of these cAMP derivatives during mass spectrometric analysis is hoped to aid the further elucidation of novel cAMP derivatives synthesised for both medical and experimental purposes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.06.015.

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