ORGANIC MASS SPECTROMETRY, VOL. 24, 679–688 (1989)

Identification of Butyryl Derivatives of Cyclic Nucleotides by Positive Ion Fast Atom Bombardment Mass Spectrometry and Mass-analysed Ion Kinetic Energy Spectrometry†

Russell P. Newton,‡ Terence J. Walton,‡ Salem A. Basaif,‡ Andrea M. Jenkins,‡ A. Gareth Brenton,§ Dipankar Ghosh§ and Frank M. Harris§
‡Biochemistry Research Group, School of Biological Sciences, and
§Mass Spectrometry Research Unit, Department of Chemistry,
University College of Swansea, Singleton Park, Swansea SA2 8PP, UK

The syntheses of the dibutyryl derivatives of the 3′,5′-cyclic monophosphates of adenosine, guanosine and cytidine are described. The fast atom bombardment mass spectra of these compounds are discussed, together with the mass-analysed ion kinetic energy spectra of their protonated molecular ions and of diagnostic fragments. A protocol for the identification of the derivatives is reported which includes criteria for confirming retention of the cyclic phosphodiester moiety, substitution of both heterocyclic base and ribose ring, and butyrylation of the 2′-O-position. The origins of significant fragments in the spectra are discussed.

INTRODUCTION

Cyclic nucleotides are a group of compounds containing a heterocyclic base, a ribofuranose ring and a phosphodiester moiety (**1**). The biochemical significance of cyclic nucleotides lies in their effects upon metabolic regulation; adenosine 3′,5′-cyclic monophosphate (cyclic AMP), for example, is the intracellular mediator of the action of a large number of extracellular mammalian hormones, its activity in this context leads to its description as a second messenger and makes it a potential pharmacological target.[1] Mass spectrometric analysis of cyclic nucleotides was relatively unsuccessful until the advent of 'soft' ionization techniques, but the use of fast atom bombardment (FAB) mass spectrometry in combination with collision-induced dissociation (CID)/mass-analysed ion kinetic energy (MIKE) spectrometry has provided a means of unambiguous identification of cyclic nucleotides,[2,3] capable of easy distinction between 2′,3′- and 3′,5′-isomers. Application of these techniques to tissue extracts has led to demonstrations of the natural occurrence of guanosine 3′,5′-cyclic monophosphate (cyclic GMP)[4] in higher plants, and of cytidine-, uridine-, inosine-, and 2′-deoxythymidine-3′,5′-cyclic monophosphates (cyclic CMP, -UMP, -IMP and -dTMP, respectively) in mammalian tissues.[5–7]

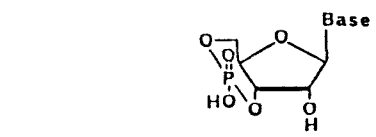
Biochemical studies of the effects of cyclic nucleotides are hampered by the fact that these molecules do not pass readily through cell membranes, thus an intravenous injection of cyclic nucleotide is ineffective for studies of intracellular effects. This problem is frequently overcome by administration of cyclic nucleotide derivatives containing lipophilic side-chains; these side-chains protect the cyclic nucleotide from hydrolysis by phosphodiesterases in the blood stream and enable rapid permeation through cell membranes.[8] Once inside the cell, esterases remove the side-chains and generate the parent cyclic nucleotide, the metabolic effects of which can then be determined experimentally.

The major difficulty in this approach is the unambiguous synthesis of the cyclic nucleotide derivative. The simplest effective lipophilic derivative is a dibutyryl cyclic nucleotide, for example N^4,2′-O-dibutyryl cyclic CMP (**2**). The synthesis of a derivative of this type is by a sequential synthesis with the ribose ring substitution proceding much more rapidly than the heterocycle substitution; routine examination of the end-product is by chromatographic means.[9] Many chromatographic procedures so used lack the resolution to determine whether an end product is solely the synthetic target, for example N^2,2′-O-dibutyryl cyclic GMP (**3**) or alternatively, if one of the phosphodiester bonds has been hydrolysed to yield a dibutyryl non-cyclic mononucleotide (**4**), or if only one of the butyryl substitutions had occurred to yield a monoester (**5**, **6**), or if a 2′,3′-cyclic nucleotide isomer has been 'artefactually generated' (**7**), or if two butyryl substituents are present but are not at the intended positions (**8**). As retention of the 3′,5′-cyclic phosphate group and substitution of one butyryl residue at 2′-O and a second on the heterocyclic ring is necessary for pharmacological experimentation, the misidentification of any of **4**–**8** as the dibutyryl 3′,5′-cyclic nucleotide, or their presence as contaminants in any preparation administered to experimental animals, would seriously compromise any conclusions drawn. In view of the successful application of FAB/MIKE spectrometry to cyclic nucleotide analysis, it appeared that this technique potentially provided a rapid and sensitive means of indicating the degree and position of substitu-

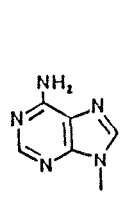
† Dedicated to Professor Allan Maccoll on the occasion of his 75th birthday.

0030–493X/89/080679–10 $05.00

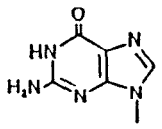
Received 14 April 1989



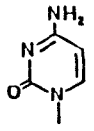
1



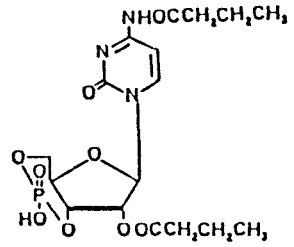
Adenine



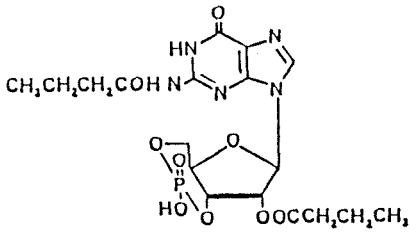
Guanine



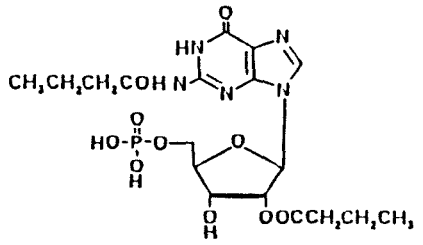
Cytosine



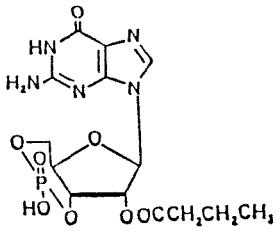
2



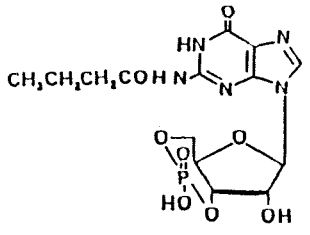
3



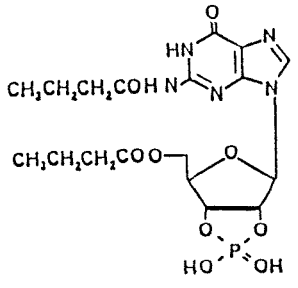
4



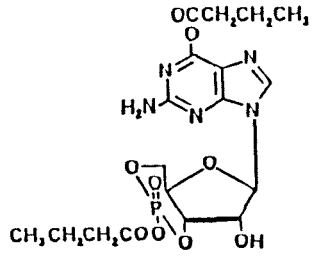
5



6



7



8

tion within a cyclic nucleotide derivative and whether the 3′,5′-cyclic phosphate group had been retained during the preparation; this paper describes the analysis of the dibutyryl derivatives of cyclic AMP, cyclic GMP and cyclic CMP by FAB/MIKE spectrometry.

RESULTS

FAB mass spectra

In the FAB mass spectrum of the monosodium salt of N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate, there was a significant peak at *m/z* 470, corresponding to the protonated molecular ion of the free acid form, $[MH]^+$, accompanied by two large peaks at *m/z* 492 and *m/z* 514, corresponding to the monosodium ($[MNa]^+$) and disodium ($[MNa_2]^+$) adducts of the free nucleotide, together with prominent peaks at *m/z* 508 and *m/z* 530 assigned to the monopotassium adduct ($[MK]^+$) and the mixed adduct ion ($[MNaK]^+$) of the free nucleotide, respectively (Fig. 1(a)). The absence of any significant peaks at *m/z* values expected for the analogous, non-cyclic dibutyryl AMP ($[MH]^+ = 488$, $[MNa]^+ = 510$ and $[MNa_2]^+ = 532$) indicated that the cyclic ribotide system was conserved. Peaks for ions derived from the butyrylated ribotide and butyrylated

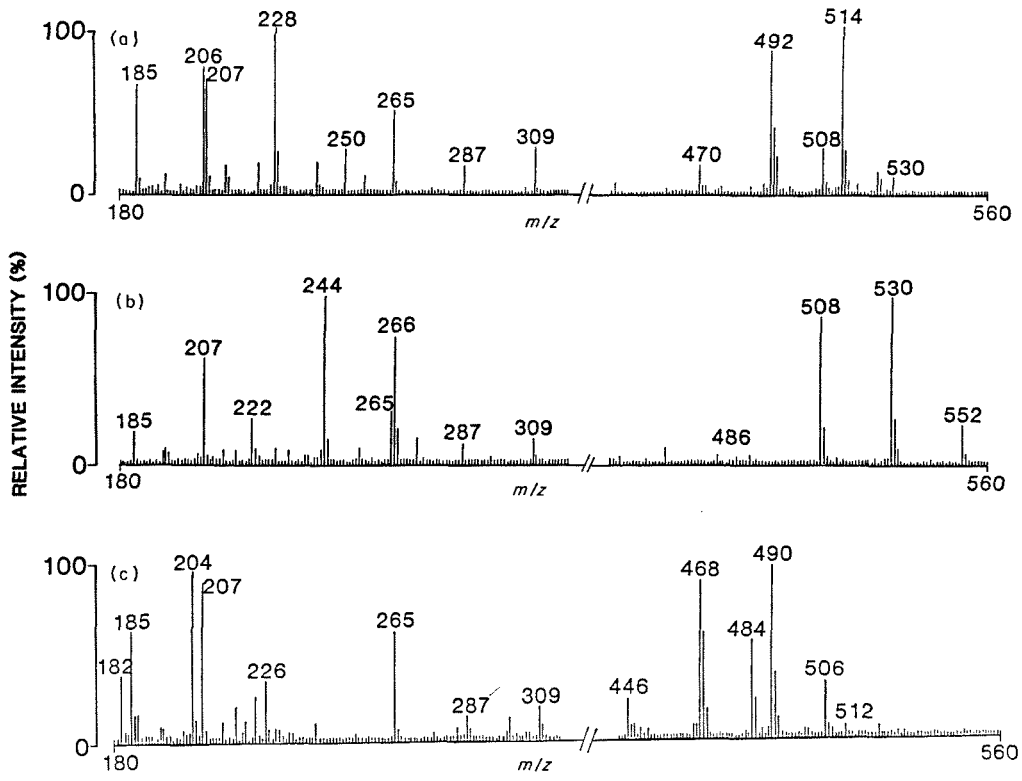


Figure 1. Positive-ion FAB mass spectra of (a) the sodium salt of N^6,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate, (b) the sodium salt of N^2,2'-O-dibutyryl guanosine 3',5'-cyclic monophosphate, and (c) the sodium salt of N^4,2'-O-dibutyryl cytidine 3',5'-cyclic monophosphate.

nucleotide base were also prominent in the spectrum. The series of ions at *m/z* 265 ([butyrylribose–cyclic PO_4]$^+$), *m/z* 287 ([Na–butyrylribose–cyclic PO_4]$^+$) and *m/z* 309 ([Na_2–butyrylribose–cyclic PO_4]$^+$) can be assigned as originating from the O-monobutyrated cyclic ribotide system. Support for these assignments was provided by the fragmentation patterns in the FAB mass spectra of the sodium salts of 2'-O-monobutyryl-adenosine-3',5'-cyclic monophosphate (O-monobutyryl-cAMP) and N^6-monobutyryl-adenosine-3',5'-cyclic monophosphate (*N*-monobutyryl-cAMP). In the spectrum of O-monobutyryl-cAMP (Fig. 2(a)), although no significant peak was present at *m/z* 265, there was a prominent peak at *m/z* 287, [Na–butyrylribose–cyclic PO_4]$^+$, whereas there was no significant peak at *m/z* 287 in the spectrum of *N*-monobutyryl-cAMP (Fig. 2(b)). Fragment ions at *m/z* 309 were, however, present in the spectra of both monobutyrylated cyclic nucleotide derivatives (Fig. 2(a) and (b)), and its presence in the *N*-monobutyryl-cAMP was tentatively assigned to the sodiated-glycerol adduct ion of the free ribotide (*m/z* 309, [gro–ribose–cyclic PO_4–Na]$^+$), isobaric with [Na_2–butyrylribose–cyclic PO_4]$^+$. The assignment of the ion at *m/z* 287 as primarily [Na–butyrylribose–cyclic PO_4]$^+$ and not the putative isobaric glycerol adduct in [gro–ribose–cyclic PO_4]$^+$ was further supported by its MIKE spectrum (Fig. 4) as described below.

In the mass spectrum of dibutyryl-cAMP (Fig. 1(a)), the large peak at *m/z* 206 ([butyryladenine]$^+$) together with the large peak at *m/z* 228 ([butyryladenine – Na]$^+$) and the prominent peak at *m/z* 250 ([butyryladenine–Na_2]$^+$) were assigned as originating from the butyrylated purine base. Species isobaric with the latter two ions at *m/z* 228 and *m/z* 250 derived from the butyrylated base might possibly arise from adducts of the free base adenine with glycerol, i.e. [adenine–gro]$^+$ and [adenine–gro–Na]$^+$, respectively. However, since significant peaks at these masses are absent from the spectrum of O-monobutyryl-cAMP (Fig. 2(a)), it appears unlikely that glycerol adduct ions make any contribution to the peaks at *m/z* 228 and *m/z* 250 in the spectrum of dibutyryl-cAMP. In the mass spectrum of N^6-monobutyryl-cAMP, the peaks at *m/z* 206 and *m/z* 250, but not *m/z* 228, were large, indicating the former two ions as useful markers for identification of butyrylated base moieties.

The FAB mass spectra of N^2,2'-O-dibutyrylguanosine-3',5'-cyclic monophosphate (dibutyryl-cGMP) and N^4,2' - O - dibutyrylcytidine - 3'5' - monophosphate (dibutyryl-cCMP) showed fragmentation patterns generally analogous to that of dibutyryl-cAMP. In the case of dibutyryl-cGMP [$MH^+ = 486$] the monosodium and disodium adduct-ion peaks were intense [*m/z* 508 and *m/z* 530] and in this case were accompanied by a prominent peak arising from the trisodium adduct [*m/z* 552] (Fig. 1(b)). The characteristic peaks indicating a butyrylated ribotide system (*m/z* 265, 287 and 309) were accompanied by three peaks arising from the butyrylated guanine base at *m/z* 222 ([butyrylguanine]$^+$), *m/z* 244 ([butyrylguanine–Na]$^+$) and *m/z* 266 ([butyrylguanine–Na_2]$^+$). In the spectrum of dibutyryl-cCMP [$MH^+ = 446$], the intense sodium adduct-ion peaks at *m/z* 468 ([MNa]$^+$) and *m/z* 490 ([MNa_2]$^+$)

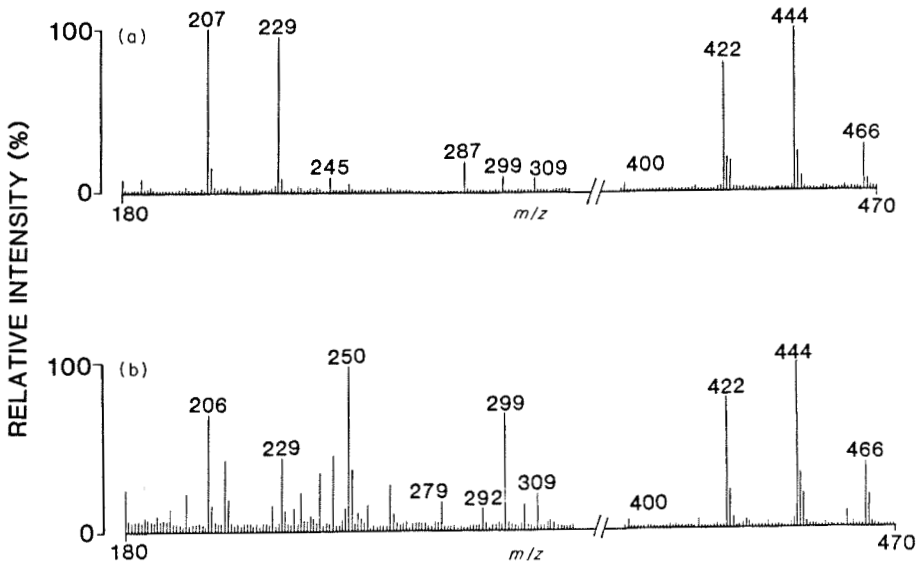


Figure 2. Positive-ion FAB mass spectra of (a) the sodium salt of 2′-O-monobutyryl adenosine 3′,5′-cyclic monophosphate, and (b) the sodium salt of N^6-monobutyryl adenosine 3′,5′-cyclic monophosphate.

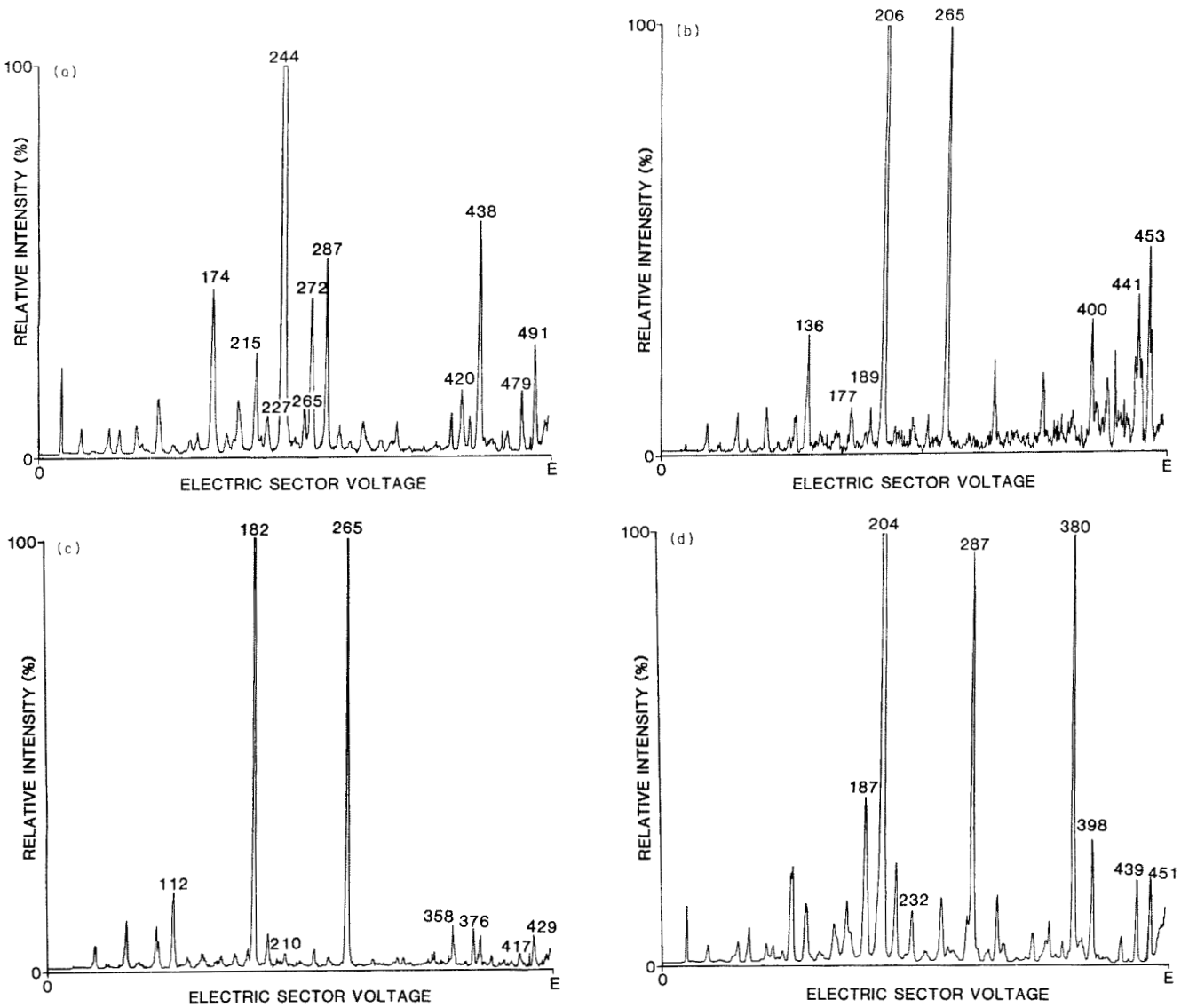


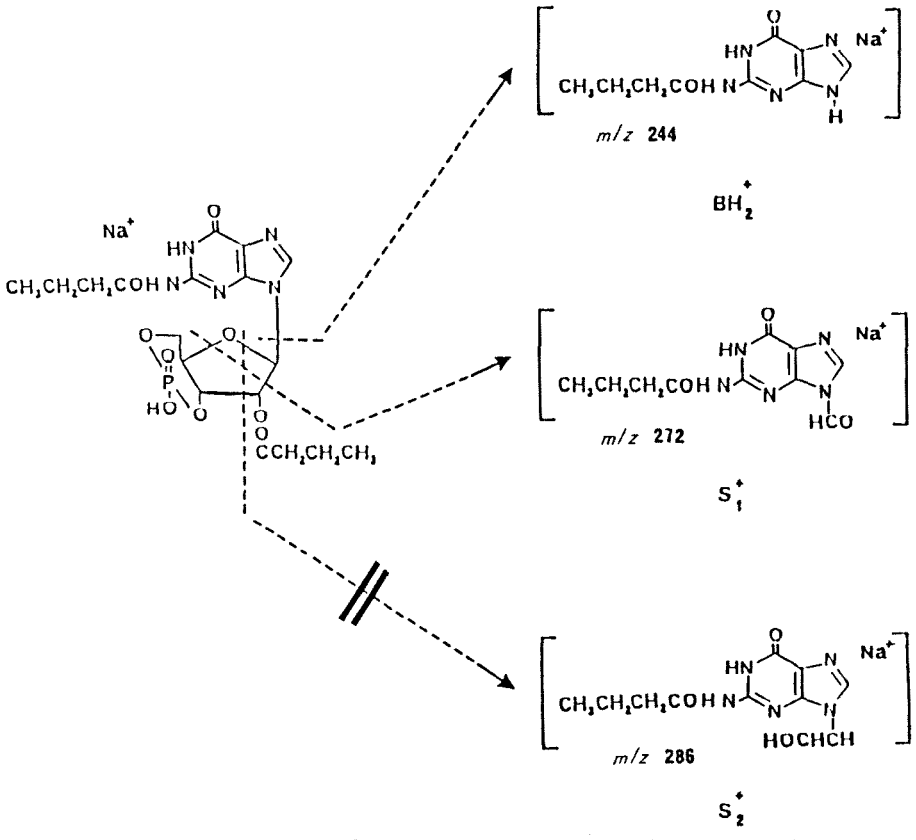
Figure 3. MIKE spectra of the protonated molecular ions of (a) the sodium salt of N^2,2′-O-dibutyryl guanosine 3′,5′-cyclic monophosphate (*m/z* 508) (b) N^6,2′-O-dibutyryl adenosine 3′,5′-cyclic monophosphate (*m/z* 470) (c) N^4,2′-O-dibutyryl cytidine 3′,5′-cyclic monophosphate (*m/z* 446) and (d) the sodium salt of N^4,2′-O-dibutyryl cytidine 3′,5′-cyclic monophosphate (*m/z* 468).

were accompanied (Fig. 1(c)) by prominent potassium adduct-ion peaks at *m/z* 484 ($[MK]^+$) and *m/z* 506 ($[MNaK]^+$) as well as a weak trisodium adduct ion at *m/z* 512 ($[MNa_3]^+$). The ions at *m/z* 265, 287 and 309 indicated the presence of the butyrylated ribotide system, while the large peak at *m/z* 182 ($[butyrylcytosine]^+$ together with the very large peak at *m/z* 204 ($[butyrylcytosine–Na]^+$) and prominent ion peak at *m/z* 226 ($[butyrylcytosine–Na_2]^+$) confirmed the presence of the butyrylated cytosine moiety. The FAB mass spectrum of each dibutyrylcyclic nucleotide thus affords series of ions derived from the intact molecule, the butyrylated ribotide system and the butyrylated nucleotide base for subsequent analysis by MIKE spectroscopy.

MIKE spectra

The MIKE spectrum obtained from the protonated molecular ion (*m/z* 508) of the sodium salt of dibutyryl cyclic GMP contained the major peak at *m/z* 244, corresponding to the sodium adduct of the base, N^2-butyryl guanine (Fig. 3(a)). Other major fragments in the MIKE spectrum include those arising from the loss of $CH_3CH_2CH_2CO$ (−70), CH_3CH_2 (−29) and either OH or NH_3 (−17), both from the parent molecular ion and from the sodium adduct of the butyryl base, occurring at *m/z* 438, 479 and 491 and at *m/z* 174, 215 and 227, respectively. (The loss of a fragment of 17 u is ambiguous in potential interpretation and will be further discussed after consideration of the full series of spectra.) Of interest is the presence of a fragment at *m/z* 272, corresponding to the S_1 fragment (Scheme 1) equivalent to the protonated base +28 u, and the absence of the S_2 fragment (Scheme 1) equivalent to the protonated base +42 u at *m/z* 286. The absence of the latter is indicative of a substituent at the 2′-O-position.[4,5] The fragment at *m/z* 287 is assigned to the sodium adduct of monobutyryl ribose cyclic phosphate, arising from the loss of the butyryl base moiety from the parent ion, whilst a smaller peak at *m/z* 265, corresponding to the same fragment but with the loss of the sodium atom, is also apparent.

The MIKE spectrum (Fig. 3(b)) obtained from the protonated molecular ion (*m/z* 470) of dibutyryl cyclic AMP, the free acid form, has a large peak at *m/z* 265 corresponding to the butyryl ribose cyclic phosphate fragment, but no sodium adduct at *m/z* 287. The MIKE spectrum of dibutyryl cyclic AMP contained an analogous fragmentation pattern to that obtained from the dibutyryl cyclic GMP salt, with the major peak corresponding to the protonated base, butyryl adenine, at *m/z* 206, and fragments at *m/z* 136, 177 and 189, and at *m/z* 400, 441 and 453 corresponding to loss of 70, 29 and 17 u from the parent ion and the protonated base, respectively. However, no evidence of a significant S_1 or S_2 fragment ion is apparent; a further difference between the spectra of the cyclic GMP and cyclic AMP derivatives is the relative abundance of $[MH - 70]^+$, which is a much larger contributor to the spectrum of the former than the latter. The different structures of the two purine nucleotides may affect the ease with which the butyryl moiety is lost, or alternatively, the presence



Scheme 1. Fragmentation of N^2,2′-O-dibutyryl guanosine 3′,5′-cyclic monophosphate.

or absence of sodium ions may be a significant factor in determining the fragmentation process. In support of the latter, it is interesting that a fragment at *m/z* 420 occurs in the dibutyryl cyclic GMP–Na-salt spectrum, corresponding to a loss of butyric acid ($[MH - 88]^+$), while no analogous fragment is apparent in the spectrum of the free acid dibutyryl cyclic AMP.

In the MIKE spectra of the dibutyryl derivative of a third cyclic nucleotide, cyclic CMP, containing a pyrimidine base, obtained from both the protonated molecular ion of the free acid, *m/z* 446 (Fig. 3(c)), and from the sodium salt, *m/z* 468 (Fig. 3(d)), intense peaks corresponding to the protonated base, *m/z* 182, or the sodium adduct of butyryl cytosine, *m/z* 204, and the butyryl ribose cyclic phosphate fragment, *m/z* 265, or its sodium salt, *m/z* 287, were observed in the relevant spectrum. In the spectrum obtained from the sodium salt of dibutyryl cyclic CMP (Fig. 3(d)) ions at *m/z* 451 and 187, (corresponding to loss of either NH_3 or OH from both the parent ion [m/z 468 − 17] and the cytosine–sodium adduct [*m/z* 204 − 17], respectively) were present, whereas the spectrum derived from the parent ion of the free acid contained a peak at *m/z* 429 representing loss of OH or NH_3 from the parent ion but did not contain an ion corresponding to the same loss from the base moiety (Fig. 3(c)). Both spectra contained fragments at $[MH - 29]^+$ and $[MH - 70]^+$, at *m/z* 439 and 398 and *m/z* 417 and 376, corresponding to a loss of CH_3CH_2 and of $CH_3CH_2CH_2CO$; a major difference between the two was the contribution of the fragment corresponding to the loss of butyric acid $[MH - 88]^+$ from the parent, it being of significantly greater intensity in the spectrum of the sodium salt at *m/z* 380 than at *m/z* 358 in the free acid spectrum, a situation similar to that observed in comparing the spectra of Fig. 3(a) and 3(b). No indication of ions at *m/z* 224 (Fig. 3(c)) or 246 (Fig. 3(d)), which would arise from an S_2 fragmentation was apparent, but peaks corresponding to an S_1 fragmentation are apparent at *m/z* 232 (Fig. 3(d)) and to a lesser extent at *m/z* 210 (Fig. 3(c)).

The MIKE spectra obtained from *m/z* 287 (Fig. 4), corresponding to the sodium adduct of butyryl ribose cyclic monophosphate and common to the FAB-MS spectra of each of the sodium salts of the dibutyryl cyclic nucleotides, having arisen from loss of the base, had the major fragment at *m/z* 216, corresponding to a loss of $CH_3CH_2CH_2CO$ together with prominent ions at *m/z* 199, corresponding to a loss of $CH_3CH_2CH_2COOH$ from the parent ion, and at *m/z* 194, corresponding to a loss of sodium from *m/z* 216. Other fragments included those arising from loss of O and H_2O from the parent ion at *m/z* 271 and 269, respectively, and minor peaks due to loss of both butyryl and phosphate moieties from the parent ion (*m/z* 287) at *m/z* 119, due to the loss of $-POH_2$ from the major fragment at *m/z* 167, and due to the presence of $[NaH_3PO_4]^+$ at *m/z* 121. This fragmentation pattern is consistent with that expected for a ribose cyclic phosphate containing a butyryl group at the 2′-O-position. Butyrylation involving esterification of an oxygen atom which was part of the cyclic phosphate group would have been anticipated to yield fragments containing a butyryl phosphate component at *m/z* 168 or *m/z* 190

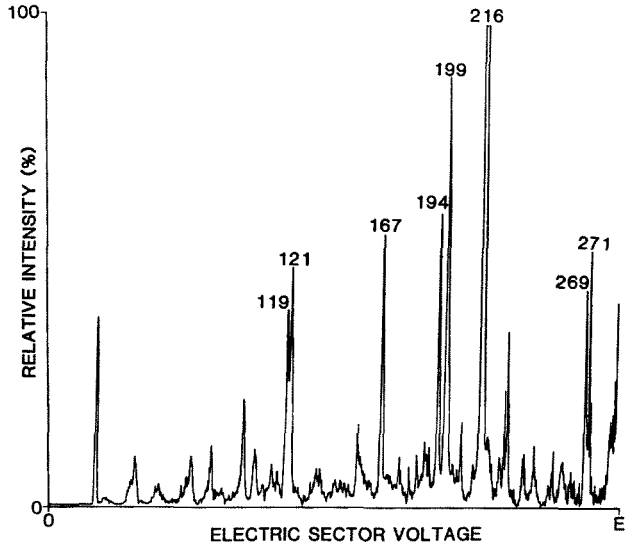


Figure 4. MIKE spectrum of the sodium adduct of butyryl ribose cyclic monophosphate (*m/z* 287).

(sodium adduct), or their dehydrated fragments (*m/z* 150 or 172), and fragments arising from S_1 and S_2 type fragmentation at *m/z* 31 ($[H_2CHO]^+$) and *m/z* 44 ($[HCHCHOH]^+$); no such fragments are evident in the spectrum (Fig. 4). MIKE spectrometry of *m/z* 265, obtained from the mass spectra of free acid forms of the dibutyryl cyclic nucleotides yielded lower fragment ion currents but indicated analogous fragmentation had taken place to that described above for the sodium salt.

In the MIKE spectrum obtained from the sodium salt of N^6-monobutyryl cyclic AMP (Fig. 5), no fragments derived from butyryl ribose cyclic phosphate were evident at *m/z* 287 and 265, as would be predicted in the absence of any migration of the butyryl group from the heterocyclic ring system to the ribose ring. The major fragment is at *m/z* 228, corresponding to the sodium adduct of the substituted base, butyryladenine; serial

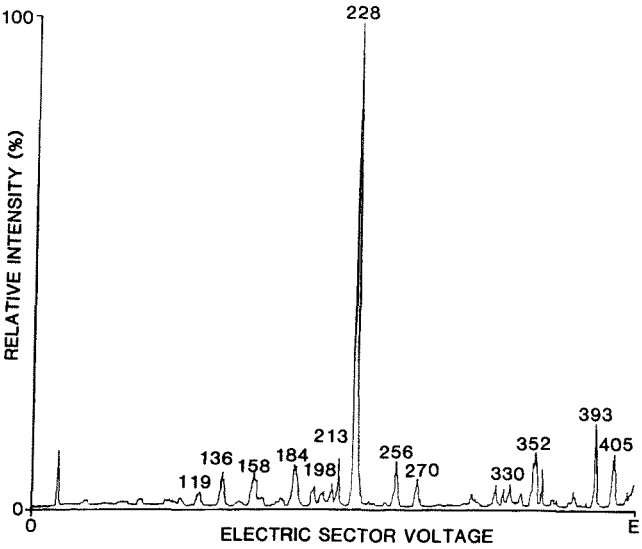


Figure 5. MIKE spectrum of the protonated molecular ion of the sodium salt of N^6-monobutyryl adenosine 3′,5′-cyclic monophosphate (*m/z* 422).

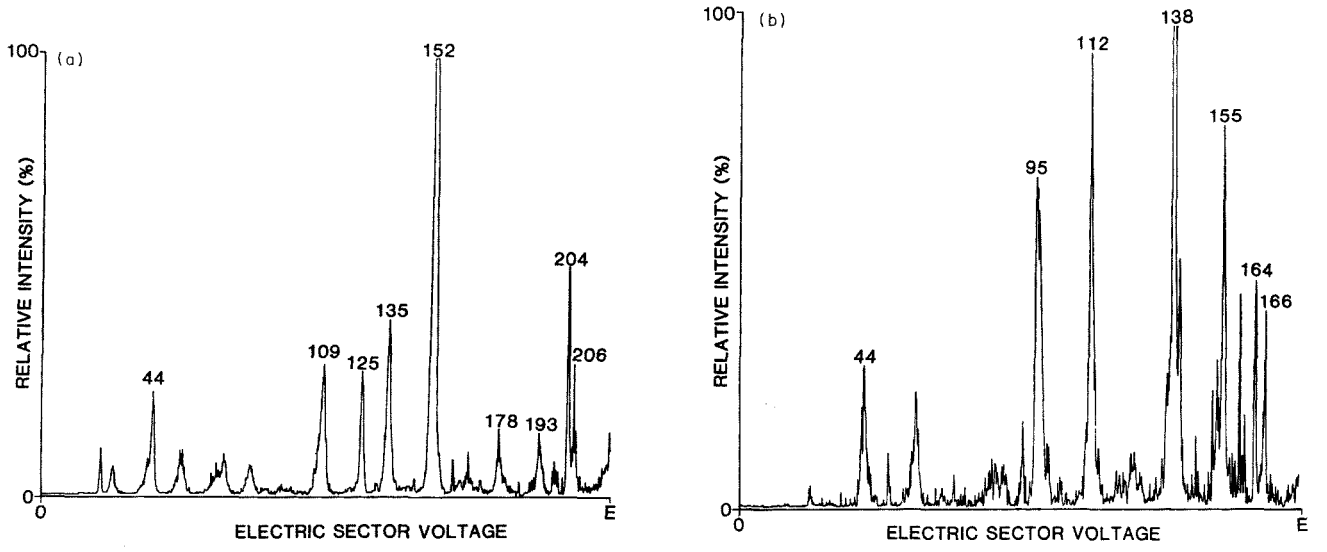


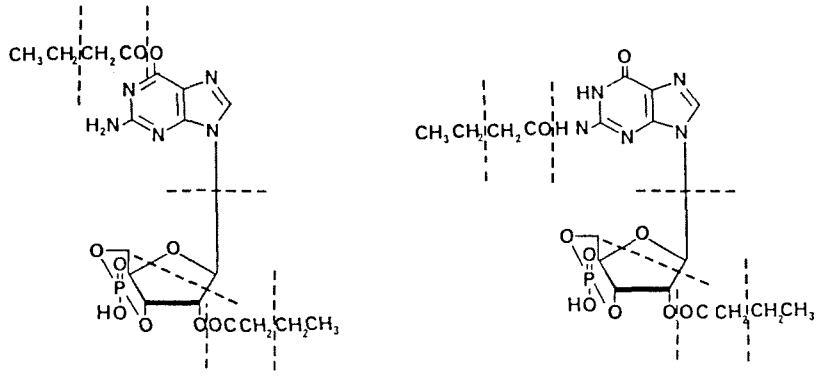
Figure 6. MIKE spectra of monobutyryl base fragment ions arising from (a) dibutyryl cyclic GMP (*m/z* 222), and (b) dibutyryl cyclic CMP (*m/z* 182).

fragmentation of the butyryl side-chain from this fragment was apparent from peaks at *m/z* 213, 198, 184 and 158, the latter ion corresponding to the sodium adduct of the base, adenine, which was itself present as a protonated fragment at *m/z* 136. The absence of a substituent at the 2′-O-position permits both the S_1 and S_2 cleavage; fragments are present at *m/z* 256 and 270 corresponding to $[BH_2 + 28]^+$ and $[BH_2 + 42]^+$ as a result of these processes. The protonated ion of cyclic AMP (*m/z* 330) and its sodium adduct (*m/z* 352) and fragments corresponding to loss of OH or NH_3 (*m/z* 405) and CH_3CH_2 (*m/z* 393) are also apparent.

In both dibutyryl cyclic CMP and dibutyryl cyclic GMP butyrylation of the heterocyclic base could occur by either amide formation or esterification, and it was hoped that the position of the butyryl substitution of the heterocyclic base could be deduced from MIKE spectrometry of the fragment corresponding to the butyryl base. In the MIKE spectrum arising from the butyryl guanine fragment (*m/z* 222) from dibutyryl cyclic GMP (Fig. 6(a)), the major fragment is the protonated base guanine at *m/z* 152, with peaks arising from this by loss of OH or NH_3 (*m/z* 135), HCN (*m/z* 125) and CONH (*m/z* 109). Other significant assigned fragment ions are at *m/z* 206, 204, 193 and 178 corresponding to losses of O, H_2O, CH_3CH_2 and $CH_3CH_2CH_2$, respectively. In the MIKE spectrum (Fig. 6(b)) obtained from the butyryl base fragment (*m/z* 182) from dibutyryl cyclic CMP, the protonated base, *m/z* 112, was relatively less intense with the major fragment occurring at *m/z* 138, corresponding to $[R{=}C{=}O]^+$ where R is the parent protonated base. The peak at *m/z* 95 represents a loss of 17 u (OH or NH_3) from the protonated base; no such loss is apparent from the parent butyryl base, which instead has fragments corresponding to the loss of H_2O and O at *m/z* 164 and 166. These processes, loss of 17 u from the protonated base, but losses of 16 and 18 u from the butyrylated form, are common to both the guanine and cytosine derivatives (Fig. 6(a) and 6(b)) and may assist in the deduction of the position of substitution; the MIKE spectrum of the butyryladenine fragment, obtained from dibutyryl cyclic AMP, which does not contain any oxygen substitution in the heterocyclic base, was very much weaker and the only reproducible fragment obtained was at *m/z* 136, corresponding to the protonated base.

DISCUSSION

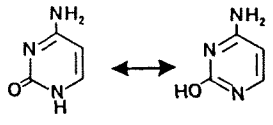
The application of FAB mass spectrometry and MIKE spectrometry provides a useful method of identifying dibutyryl cyclic nucleotide derivatives. The number of butyryl substituents can be readily determined from the FAB mass spectrum; in addition, the absence of an ion at $[MH + 18]^+$ in the spectrum can be used as an indication that no dibutyryl non-cyclic nucleotide monophosphate has been produced as a result of the hydrolysis of one or other or the phosphodiester bonds during the synthesis. The location of the butyryl residue on the ribose ring can be assigned to the 2′-O-position by virtue of the absence of a fragment ion corresponding to $[BH_2 + 42]^+$, the potential result of an S_2 cleavage, and the absence of any butyryl phosphate fragments in the MIKE spectra of both the protonated molecular ion and the protonated butyryl ribose cyclic phosphate. That butyryl substitution of the heterocyclic base has taken place is evident from the existence of the protonated butyryl base fragment ion in the MIKE spectra, but the position of this substitution is not so simply deduced (Scheme 2). For the purpose of identifying dibutyryl cyclic nucleotide derivatives suitable for administration during biochemical investigations, the procedure described above is completely satisfactory, since, although base butyrylation is essential, its position on either the NH_2 or OH side-chains is acceptable. However, the position of substitution is of interest, as is the nature of the 17 u fragment loss observed in each of the spectra. We have considered the possibility of



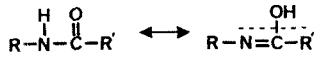
Scheme 2. Analogous fragmentation of O^4,2′-O-dibutyryl guanosine 3′,5′-cyclic monophosphate and N^2,2′-O-dibutyryl guanosine 3′.5′-cyclic monophosphate.

various artefact processes, in particular fragmentations arising in the first field-free region, giving rise to the loss of 17 u and as yet we have no evidence that these occur. The peak corresponding to the loss of 17 u occurs at, or very close to, an integer number on the mass scale which indicates it is real, as artefact peaks often show their presence by their deviation from an expected integer position.

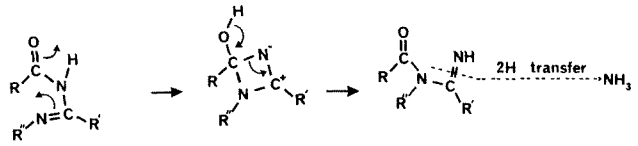
In underivatized cyclic nucleotides, the loss of a 17 u fragment from the protonated molecular ion and from the protonated base has been assigned to NH_3 loss.[2–7] In the MIKE scans of each of the protonated molecular ions of the dibutyryl cyclic nucleotides (Fig. 3(a)–(d)) the highest mass fragment ion observed corresponds to $[MH - 17]^+$; in the MIKE scans of the butyryl ribose cyclic phosphate there are two large fragment ion peaks at $[MH - 18]^+$ and $[MH - 16]^+$ deduced to have arisen from H_2O and O loss (Fig. 3), but none at $[MH - 17]^+$. If the assumption were made that a free NH_2 group is necessary for the loss of a 17 u fragment, then the loss of fragments of 18 and 16 u from the butyryl guanine and butyryl cytosine fragments (Fig. 6(a) and (b)) implies that the butyrylation of these bases is via the NH_3 substituent, a hypothesis supported by the presence of fragments corresponding to the loss of 17 u, i.e. NH_2, from the protonated bases in the same spectra. On this basis, however, it is difficult to explain the existence of $[MH - 17]^+$ in the MIKE spectra of the three dibutyryl cyclic nucleotides; it is feasible that there is a loss of OH from the cytosine and guanine rings as a result of lactim–enol tautomerism,



but such an explanation would not hold for the dibutyryl cyclic AMP nor the monobutyryl cyclic AMP (Fig. 4). If the 17 u fragment is lost from the butyryl base moiety and is the same entity in each case then it could be as a result of OH loss from the amide,



which is the common feature of each of the butyrylated bases. Alternatively, it could occur as a result of NH_3 loss from a rearrangement, for example, of the type



In order to distinguish between the different possibilities, and to ascertain the identy of the 17 u fragment in each case, tandem mass spectrometry of the free bases, butyrylated bases and butyrylated cyclic nucleotides employing high-resolution MIKE scan spectrometry, or alternatively, use of isotopic labelling, for example the use of [^{18}O]-butyric anhydride in the synthesis, will be required. In addition, a parent-ion scan of M – 17, such as a high-voltage scan, may be very informative in elucidating the fragmentation mechanism(s) involved.

EXPERIMENTAL

Preparation of butyryl cyclic nucleotide derivatives

Dibutyryl cyclic nucleotides were prepared by modifications of previously published methods.[9,10]

(a) N^6,2′-O-dibutyryl adenosine 3′,5′-cyclic monophosphate (dibutyryl cyclic AMP). cAMP (50 mg) was dissolved in the minimum quantity of cold, aqueous 0.4 M triethylamine. The solution was evaporated to dryness *in vacuo*, taken up in anhydrous pyridine, and re-evaporated to dryness. The whole operation was repeated once, then the residue dried for 5 h under high vacuum. The powder was taken up in 1.5 cm^3 of hot anhydrous pyridine; after cooling, 0.75 g of butyric acid anhydride were added and the mixture boiled for 3–4 min. The resultant solution was rapidly cooled then left at room temperature, protected from light and moisture. After 6 days, the reaction was complete, as indicated by TLC analysis on silica-gel G with isopropanol: water 3 : 2 (vol:vol) as developing solvent.

The reaction mixture was cooled in ice and 1 cm^3 of water was added in small quantities with stirring. After

5 h at 4 °C, it was evaporated (30–40 °C), by using high vacuum for 24 h. $BaI_2.2H_2O$ (30 mg) in 0.2 cm^3 of water was then added, followed by 2 cm^3 of absolute alcohol and 8 cm^3 of anhydrous ether. After standing at 4 °C for 2 h, the resultant precipitate was recovered by centrifugation. The supernatant was evaporated to dryness and taken up by a solution of 15 mg of BaI_2 in 0.1 cm^3 of water. By adding 1 cm^3 of alcohol and 5 cm^3 of dry ether, a second preparation of barium salt was obtained. This was added to the first precipitate and purified by dissolving in water and reprecipitating with alcohol and ether, then drying *in vacuo* over H_2SO_4.

The sodium salt of dibutyryl-cAMP was obtained by treating the product with sodium perchlorate. Dibutyryl-cAMP barium salt (25 mg) in 0.5 cm^3 of methanol was added to 0.5 cm^3 of 1 M sodium perchlorate in anhydrous acetone. The precipitation of the sodium salt was completed by adding 5 cm^3 of anhydrous acetone. The precipitate was collected, freeze-dried and identified as putative sodium dibutyryl cyclic AMP by TLC and UV absorbance spectrophotometry.[10]

(b) N^2,2′-O-dibutyryl guanosine 3′,5′-cyclic monophosphate (dibutyryl cyclic GMP). cGMP (1 g) was dissolved under stirring in a mixture of 60 cm^3 of distilled water and 2 cm^3 of a 40% aqueous solution of tetrabutyl ammonium hydroxide. The solution was freeze-dried and re-suspended in 150 cm^3 of dry pyridine and, after adding 4.6 cm^3 of butyric anhydride, refluxed for 6 h. The solvent was removed *in vacuo*, the residue was taken up in 30 cm^3 of ice water and re-evaporated to dryness, and the process repeated. The residue was taken up in 40 cm^3 of distilled water and extracted 10 times with ether. The organic phase was discarded, and traces of ether in the water layer were removed in a rotary evaporator. Charcoal (10 mg) was added, shaken, and removed by filtration; this process was repeated. The filtrate was freeze-dried, re-dissolved in the minimum amount of water and applied to TLC silica-gel, developed with isopropanol : water, 80 : 20 (vol : vol). The band of dibutyryl-cGMP was detected by UV absorbance and the silica-gel containing the dibutyryl-cGMP was removed and extracted twice with isopropanol : water, 80 : 20 (vol : vol) and the filtered extract freeze-dried. The resultant yellow powder was identified as putative dibutyryl cyclic GMP by TLC and UV absorbance spectrophotometry.[10]

(c) N^4,-2′-O-dibutyryl cytidine 3′,5′-cyclic monophosphate. cCMP 150 mg was taken up in 5 cm^3 of dry pyridine and concentrated to a foam. This was repeated twice and then the foam was left at room temperature for 2 h. Dry pyridine (4 cm^3) was added and heated to 120 °C momentarily under nitrogen to effect maximum dissolution. The solution was cooled, 1 cm^3 of butyric anhydride added, and the solution reheated to 120 °C for 10 min, then left at room temperature for 36 h under nitrogen and protected from light. After cooling in ice, 2 cm^3 of water was added, the solution stirred for 4 h, then the mixture concentrated under reduced pressure. A 1 : 1 (vol : vol) mixture of hexane : ether (5 cm^3) was added which was decanted from the oil after vigorous shaking. This was repeated once. The gum taken up in 1 cm^3 of isopropanol : water, 85 : 15 (vol : vol) was applied to thin layers of silica-gel and the chromatograms developed with a mixture of isopropanol : water : ammonium hydroxide, 80 : 12.5 : 2.5 (vol : vol : vol). Bands were detected by UV absorbance and silica-gel containing the derivative was removed from the plates and extracted with isopropanol : water, 80 : 20 (vol : vol). The extract was concentrated under reduced pressure then passed through a 25 cm^3 Amberlite IR-120 (Na^+ form) column followed by 100 cm^3 of water. After freeze-drying a light yellow powder was obtained, and identified as putative sodium dibutyryl cyclic CMP by TLC and UV absorbance spectrophotometry.[9]

(d) N^6-monobutyryl adenosine 3′,5′-cyclic monophosphate and 2′-O-monobutyryl adenosine 3′,5′-cyclic monophosphate were obtained commercially from the Sigma Chemical Co., Poole, UK.

Mass spectrometry

Positive-ion fast atom bombardment mass spectra were obtained on a VG ZAB 2F mass spectrometer fitted with a VG FAB source and ion gun. Xenon atoms were used to bombard the samples, the ion-gun conditions being typically 8 kV accelerating potential and 1 mA discharge current. The source accelerating voltage was 8 kV. Samples were prepared by dissolving in glycerol : water, 1 : 1 (vol : vol) at a concentration of 1–10 $\mu g\ \mu l^{-1}$ and then placing 3 µl on the FAB target. Under these conditions the sample lifetime was 4–5 min.

To generate CID spectra nitrogen was used as the collision gas in the second field-free region gas cell at a pressure of 6×10^{-6} Torr. MIKE spectra were obtained by scanning the electric sector voltage under the control of a data system and were signal averaged over at least four scans.

Acknowledgements

The authors gratefully thank SERC and the Wellcome Trust for support.

REFERENCES

1. R. H. Herman and O. D. Taunton, *Principles of Metabolic Control in Mammalian Systems*, ed. by R. H. Herman, R. M. Cohn and P. D. McNamara, pp. 424–480, Plenum Press, New York (1980).
2. E. E. Kingston, J. H. Beynon and R. P. Newton, *Biomed. Mass Spectrom.* **11**, 367 (1984).
3. E. E. Kingston, J. H. Beynon, R. P. Newton and J. G. Liehr, *Biomed. Mass Spectrom.* **12**, 522 (1985).
4. R. P. Newton, E. E. Kingston, D. E. Evans, L. M. Younis and E. G. Brown, *Phytochem.* **23**, 1367 (1984).
5. R. P. Newton, B. J. Salvage, S. G. Salih and E. E. Kingston, *Bichem. Soc. Trans.* **11**, 354 (1983).

6. R. P. Newton, S. G. Salih, B. J. Salvage and E. E. Kingston, *Biochem. J.* **221**, 665 (1984).
7. R. P. Newton, E. E. Kingston, N. A. Hakeen, S. G. Salih, J. H. Beynon and C. D. Moyse, *Biochem. J.* **236**, 431 (1986).
8. T. H. Posternak, *Cyclic Nucleotides and Therapeutic Perspectives*, ed. by G. Cehovic and G. A. Robinson, pp 4–7, Pergamon Press, Oxford (1979).
9. W. Wierenga and J. A. Wolterson, *J. Carbohydrate, Nucleosides and Nucleotides*, **4**, 335 (1977).
10. T. Posternak and G. Weinman, *Methods in Enzymology*, **38**, 399 (1974).