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RECEIVED for review July 15, 1991. Accepted January 16, 1992.

Collisionally Induced Dissociation in the Study of A-Ring Hydroxylated Vitamin D Type Compounds

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Collisionally induced dissociation (CID) is often used to determine the structure of ions based on comparison with the CID spectra of known ions. The latter are generated from judiciously selected compounds taking into account basic principles of ion chemistry. We report here on the use of this approach toward determination of the site of A-ring hydroxylation of vitamin D. Although not intrinsically an aromatic compound, vitamin D gives rise in its mass spectrum to an aromatic methylstyryl cation at *m/z* 118. A-ring hydroxylated metabolites of vitamin D would thus incorporate the extra OH group on the ion at *m/z* 118, shifting it to *m/z* 134. The position of substitution of the extra OH group on a metabolite could then be ascertained by comparing the CID spectrum of its *m/z* 134 fragment to those of the four possible (hydroxymethyl)styryl cations generated from synthesized authentic compounds. Because of their propensity to polymerize, these cations were generated in situ via the McLafferty rearrangement of the corresponding (hydroxyphenyl)ethanols. For optimum differentiation of isomeric ions, preparation of permethylated derivatives of vitamin D was necessary. The validity of the hypothesis was verified using 1,25-dihydroxyvitamin D_3 as a test compound. This method provides a viable approach for the characterization of A-ring hydroxylated metabolites of vitamin D as well as for related aromatic compounds.

INTRODUCTION

Techniques of varying sophistication are available for performing what have come to be known as MS/MS experiments. The basic goal of these methods is to gain additional information about ions in the primary mass spectrum with which most chemists are familiar. This is accomplished by observing, in one way or another, the subsequent decompositions of primary ions of interest. Applications of MS/MS include (i) the derivation of detailed information about the structure of ions which are formed in the normal EI mass spectrum; (ii) the acquisition of structural data from molecular ions generated by so-called "soft" ionization techniques—e.g., fast atom bombardment (FAB), field desorption (FD), chemical ionization (CI)—which tend to give very simple spectra that furnish molecular weight but little structural information;[1] and (iii) fast analysis of complex mixtures by utilizing the first stage of the mass spectrometric analysis as a separation technique and the second stage for identification/quantification purposes.

The usefulness of collisionally induced dissociation MS/MS methods in the study of ion structure has been well demonstrated in a number of studies by McLafferty et al.[2-4] and others.[5] One example, in particular, demonstrates its potential utility in deducing the structure of large molecules by first determining the structure of their smaller fragments based on their CID spectra. For example, in a paper by McLafferty et al.,[6] the structure of the drug "China White" ($C_{22}H_{30}N_2O$) was determined by comparing CID spectra of its mass spectral ions at *m/z* 57, 58, 91, 110, and 146 with reference CID spectra derived from known ions. Richter et al.,[7] used low-energy CID to assign the stereochemistry of fragment ions comprising intact sugar subunits of larger glycosides without resorting to chemical degradation or chromatographic separation. The CID spectra were compared to corresponding reference spectra of precursors of known stereochemistry. The same authors have extended the applicability of this approach to the study of ions containing two sugar units linked by an interglycosidic bond.[8,9] We report here on a related use of CID toward the assignment of the position of A-ring hydroxylation in vitamin D metabolites. While the example discussed here focuses on vitamin D, the approaches pursued should be more generally applicable to the characterization of hydroxylated aromatic compounds by MS/MS.

EXPERIMENTAL SECTION

Instrumentation. Normal mass spectra in this study were run on a Nuclide 12-90G single-focusing magnetic instrument or on a JEOL HX303HF instrument. Since the standards were all

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quite volatile, they were introduced from a heated reservoir through a needle valve into the ion source. MS/MS data were acquired exclusively on the JEOL HX303HF spectrometer which could be operated in either the B/E or the B^2/E linked-scan mode.

The ion accelerating energy was 3 kV, and the primary ion beam intensity was attenuated at a set value of 50% for CID experiments. All linked-scan data were acquired using the accumulation mode which saved all data points and did not convert them to a bar graph, thus enabling one to examine the true quality of the data. The collision experiments were carried out using helium as the target gas.

Proton NMR spectra were run on a Varian VXR 300-MHz instrument. Infrared spectra were run on a Perkin-Elmer 1310 spectrophotometer.

Materials. The chemicals and compounds used in this study were purchased from Aldrich Chemical Co. (Milwaukee, WI). In many cases they could be used as received. Certain compounds had to be synthesized in order to produce the desired precursor to the styryl ions of interest. The procedures involved in these syntheses are outlined below.

Permethylated 1,25-Dihydroxyvitamin D_3. The permethylation was carried out using the general procedure of Hakomori et al.[10] The dimsyl anion was formed using potassium hydride instead of sodium hydride due to its greater speed of reaction with dimethyl sulfoxide, particularly at room temperature.[11] First, a solution of dimsyl anion was produced by mixing 40 mg of potassium hydride with 5.0 mL of dimethyl sulfoxide in a flame-dried culture tube, purging the tube gently with a stream of nitrogen until the evolution of gas was complete. Three drops of this solution were added to approximately 0.5 mg of 1,25-dihydroxyvitamin D_3, and the solution was swirled for 10 min. Three drops of iodomethane were then added, and the swirling was continued for a further 20 min. Subsequently, 1 mL of chloroform was added and the solution was extracted exhaustively with water until the aqueous layer became clear. The chloroform layer was taken for mass spectral analysis, and it was revealed that the permethylation was approximately 50% complete, since a peak could be seen 14 amu lower than the expected molecular ion. The procedure was repeated after evaporating the chloroform to give a spectrum indicating less than 100% permethylation at the sterically hindered 25-position. However, permethylation of the relevant A-ring hydroxylic groups was complete judging from the 14-Da shift of the ions derived from that part of the molecule (see Results and Discussion).

2-(2-Methylphenyl)ethyl Trifluoroacetate. A 10-mg sample of 2-(2-methylphenyl)ethanol (Aldrich) was dissolved in 0.4 mL of dichloromethane followed by the addition of 0.4 mL of trifluoroacetic anhydride. The mixture was then swirled thoroughly in a reacti-vial and evaporated with a stream of nitrogen at approximately 60 °C, leaving a clear liquid which was used directly for mass spectrometry via the batch inlet.

Synthesis of Methoxylated Isomers of 2-(2-Methylphenyl)ethanol. The series of compounds 2-(3-methoxy-2-methylphenyl)ethanol, 2-(4-methoxy-2-methylphenyl)ethanol, 2-(5-methoxy-2-methylphenyl)ethanol, and 2-(6-methoxy-2-methylphenyl)ethanol was synthesized by the same method, starting with the appropriate nitromethylanisole. The general procedure involved a reduction of the nitromethylanisole to the corresponding aniline followed by replacement of the aromatic amino group with a bromine. The bromomethylanisole was reacted with *n*-butyllithium to produce the lithiated methylanisole which was then converted to the (methylphenyl)ethanol by reacting with ethylene oxide. A detailed description is given in this section for the conversion of 4-nitro-3-methylanisole to 2-(4-methoxy-2-methylphenyl)ethanol. The same details apply to the synthesis of the other isomers.

(a) 4-Methoxy-2-methylaniline. 4-Nitro-3-methylanisole (Aldrich, 10 g) was combined with 100 mL of isopropyl alcohol in a 250-mL pressure reaction bottle. Then, 57 mg of PtO_2 was added cautiously (the catalyst has been known to cause the ignition of heated vapors), the solution was connected to the Parr reaction apparatus, and vacuum was applied from a water aspirator. After gas bubbles ceased evolving, the vacuum valve was closed and sufficient H_2 was introduced to establish a modest (approximately 10 psi) pressure. Vacuum was again applied until the pressure decreased and was maintained for an additional 1 min. H_2 was then admitted until the pressure reached 45 psi and the bottle mechanically agitated while the pressure continued to be monitored. When the pressure dropped to 2 atm (29.4 psi), more H_2 was admitted until a reading of 45 psi was reached again. The progress of the reaction could be monitored by following the initial dissolution of the nitro compound, the intermediate development of dark coloration, and the subsequent clearing to a light straw-colored solution slightly clouded by the catalyst. The total pressure drop agreed well with initial calculations based on a stoichiometry of 3:1 H_2:nitro compound. While the reaction was in its intermediate stage, the rate of pressure loss was as great as 18 psi/min. At the end it slowed to 0.25 psi/min and this was taken as the signal to end the reaction by applying vacuum to the system. After the solution was filtered a 2-μm Nylon 66 filter (the filter material should be kept moist during this process or the air sweeping through can cause its slow ignition) and then solvent was removed with a rotary evaporator, 8.09 g of yellow oil were obtained (98.5% yield). Yields were generally of that order of magnitude although on a couple of occasions the catalyst apparently was poisoned. In those cases, the yield dropped as low as 75% after the vacuum distillation step which was undertaken to clear up the crude dark material. This distillation was done using a mechanical vacuum pump without a gauge.

(b) 4-Bromo-3-methylanisole. The conversion of 4-methoxy-2-methylaniline to 4-bromo-3-methylanisole was conducted as follows. First, cuprous bromide was prepared by dissolving 98 g of cupric sulfate pentahydrate and 42 g of sodium bromide with sufficient distilled water with stirring and heating. After dissolution (in one case filtration was necessary to remove a small amount of solid material that would not dissolve), 28.8 g of solid sodium bisulfite were added slowly with stirring to the hot solution. This operation was carried out under the hood. The mixture of precipitated Cu_2Br_2 and supernatant solution was cooled by placing the beaker in ice water. The solid material was washed by decanting away the supernatant solution, mixing with more distilled water, repeating once more, and finally isolating the cuprous bromide by vacuum filtration. The solid material was pressed down to remove water and was left in the filter funnel with the water aspirator running for another 2 h to dry. The total solid cuprous bromide was then combined with 60 mL of 48% HBr in a 500-mL three-necked round-bottomed flask, which was set up for simple distillation and equipped with a 250-mL dropping funnel.

The diazotization was carried out next by combining 8.84 g (0.0645 mol) of 4-methoxy-2-methylaniline with 40 mL of distilled water. Concentrated sulfuric acid (20 mL) was then added cautiously. The resulting pinkish slurry was placed in a salt–ice bath and the temperature lowered to less than 0 °C. This mixture was gently stirred, as the greater part of 4.45 g of sodium nitrite (dissolved in the minimum volume of H_2O) was added at a slow enough rate, so that the temperature was maintained below 10 °C. Starch iodide paper was used to test for excess nitrous acid as the remaining sodium nitrite was added. A little more sodium nitrite solution was carefully added until a positive starch iodide test was observed. The test was often easier to read if a drop of the diazonium solution had been diluted first in a small volume of water. No more sodium iodide was added after the first indication of an immediate blue-black color on the starch iodide paper.

The Cu_2Br_2/HBr mixture was brought to a gentle boil with a Bunsen burner, and distilled water (100 mL) was added to the 500-mL receiving flask to dilute the HBr which codistilled with the product. The cold diazonium solution was added slowly enough through the dropping funnel so that the mixture did not foam over. This took about 0.5 h. The mixture was stirred with an egg-shaped magnetic stirring bar. Boiling was maintained so that a distillate was slowly collected which contained most of the product. After the last of the diazonium solution was added and an approximately equal amount of distillate had been collected, distilled water was added through the dropping funnel and the steam distillation continued until the distillate ran quite clear into the collecting flask.

The distillate was extracted with an equal volume of pentane followed by two more 100-mL portions of pentane. The pentane solution was washed alternately with water and dilute NaOH until the aqueous phase showed no sign of red color. (This color,

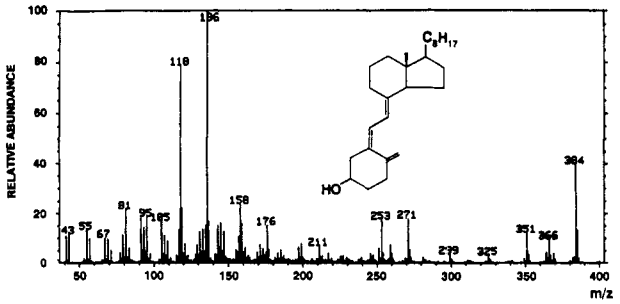


Figure 1. Electron impact (70-eV) mass spectrum of vitamin D.

presumably originating from a phenol-based dye, was very intense at the beginning of this process.) The resulting pentane solution was stirred first over NaCl and then over Na_2SO_4. It was then passed through a Pasteur pipet containing 1 in. of silica gel over a tightly packed plug of glass wool. The end of a piece of tubing with 20 psi of nitrogen helped to speed this process. If the discolored region extended to the end of the silica gel in the pipet, the process was repeated until all the polar colored material was removed, leaving a clear, colorless pentane solution which upon rotary evaporation yielded 7.55 g of 4-bromo-3-methylanisole (60% of theoretical yield).

(c) 2-(4-Methoxy-2-methylphenyl)ethanol. The formation of aryllithium reagents via the metal–halide exchange and subsequent reaction with ethylene oxide[12-15] appears to be the method of choice for the conversion of aromatic halide to phenylethanol type compound. Accordingly, (4-methoxy-2-methylphenyl)lithium was prepared by combining 1.01 g of 4-bromo-3-methylanisole, 5 mL of pentane (dried over potassium and filtered through glass wool), and 2.60 mL of 2.0 M *n*-butyllithium in pentane (Aldrich) in a flamed 50-mL round-bottomed flask equipped with a rubber septum. The flask was purged with nitrogen and then left with a 5 psi positive pressure of nitrogen and stirred with an egg-shaped magnetic stirring bar. After about 1 h, a greenish yellow precipitate began to appear. Stirring was continued for another 5 h, after which the precipitate was allowed to settle and the supernatant solution was removed with a Pasteur pipet. Care was taken to make sure that the yellow solid was not disturbed. Three 3-mL portions of pentane were added and removed in this fashion for washing the aryllithium compound. The solid was then resolvated with tetrahydrofuran.

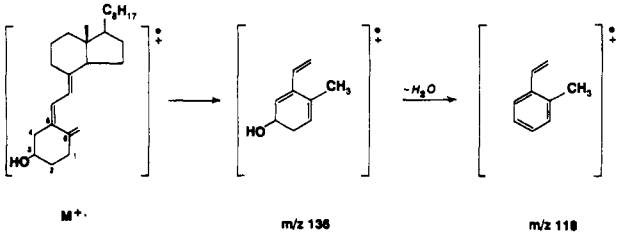
The aryllithium compound was converted to the phenylethanol by reacting with ethylene oxide. A 1.21-mL aliquot of 20% ethylene oxide in THF (density = 0.90 g/mL) was added immediately and the solution stirred 30 min. It was then quenched with water, extracted with ether, and dried over NaCl followed by $NaSO_4$. This yielded 0.56 g of crude liquid (67% of theoretical) which was purified by a simple vacuum distillation in a small Hickman still. The final step, conversion to the TFA derivative, was carried out by reaction with neat trifluoroacetic anhydride.

This procedure was used to synthesize 2-(3-methoxy-2-methylphenyl)ethanol from 3-bromo-2-methylanisole, 2-(5-methoxy-2-methylphenyl)ethanol from 3-bromo-4-methylanisole, and 2-(6-methoxy-2-methylphenyl)ethanol from 2-bromo-3-methylanisole.

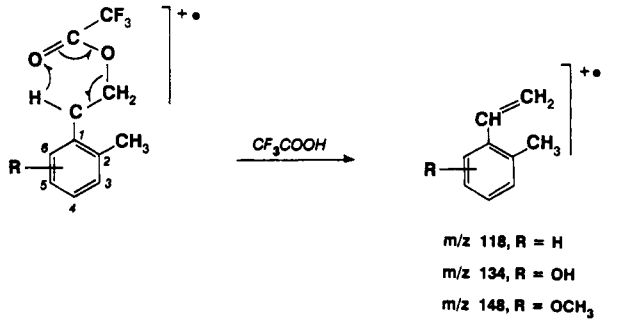
RESULTS AND DISCUSSION

Hydroxylated metabolites play an important role in vitamin D biochemistry,[16] as they do in the biochemistry of a large number of biologically active compounds. New metabolites of vitamin D are still being found and often in very small amounts which makes trace analytical techniques, such as mass spectrometry, critically important in their structural elucidation. In the study of metabolites of vitamin D one can encounter compounds where the position of the extra hydroxy group on the A-ring may be in doubt and where there is an insufficient amount available for analysis by NMR techniques. In one example, the exact location of an extra hydroxy group on the A-ring of a rat kidney metabolite could not be determined since the small amount of material, which was isolated at great effort, precluded the use of NMR techniques.

Scheme I. Fragmentation of Vitamin D Leading to *m/z* 136 and to the Methylstyryl Ion at *m/z* 118



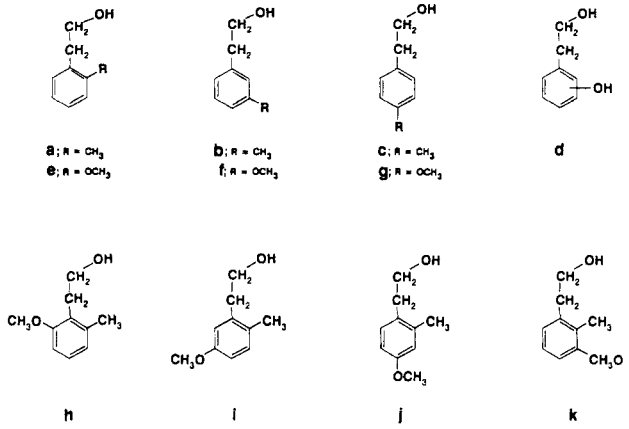
Scheme II. Generation of the Styryl Cations in the Mass Spectra of the TFA Derivatives of Ring-Substituted 2-Phenylethanols



Chemical techniques (periodate cleavage) were only sufficient to demonstrate that a vicinal diol was involved.[17]

The electron impact (EI) mass spectrum of vitamin D (Figure 1) contains ions at *m/z* 136 which lose water to give rise to the highly stable 2-methylstyryl ion at *m/z* 118. The fragmentation reactions leading to this ion in the mass spectra of vitamin D and related compounds) have been extensively studied and well characterized by Zaretskii et al.[18-20] and are summarized in Scheme I. Our approach to developing a general MS/MS method for determination of the hydroxylation site in the A-ring of vitamin D metabolites was based on this fragmentation and on the premise that the *m/z* 118 ion would be shifted by 16 Da to *m/z* 134 in the spectrum of the metabolite. The comparison of the CID spectra of the *m/z* 134 ions arising from these unknown compounds with the CID spectra of the same ions from standard aromatic compounds might thus enable one to deduce the structure of the original hydroxylated vitamin D. Experiments were conducted to test this hypothesis using the readily available 1,25-dihydroxyvitamin D_3 as a test substance and a series of compounds purchased or synthesized to provide reference ions with the basic methylstyryl structure. Different approaches to the generation of these ions were pursued.

Generation of Styryl Ions. Efforts to synthesize the neutral molecules needed to generate the hydroxy-2-methylstyryl ions were unsuccessful because of the polymerization of the compounds. As a result, alternative approaches had to be considered. It was reasoned that styryl radical ions could be generated in situ in the ion source via the McLafferty rearrangement of the trifluoroacetyl (TFA) derivatives of ring-substituted phenylethyl alcohols, as shown in Scheme II. The feasibility of this approach was tested initially with three commercially available 2-(methylphenyl)ethanols, a–c. For example, the EI mass spectra at 70 and 12 eV of the TFA derivative of the para isomer, c, are shown in Figure 2. As expected, the McLafferty rearrangement ion at *m/z* 118 dominates the spectrum, especially at the lower ionization energy. Isotopic labeling of the hydrogens α to the aromatic ring confirmed better than 99% hydrogen transfer from the α position. The feasibility of the approach was further investigated by subjecting the *m/z* 118 ions produced from the three isomers a–c to CID. The CID spectra,



generated from ions produced at 70 eV, are shown in Figure 3. Fragments corresponding to $C_6H_5^+$, $C_7H_7^+$, loss of a methyl, and cleavage through the aromatic ring (e.g., m/z 65, 51, and 39) characterize the spectra. In fact, the three spectra are also quantitatively identical, which precludes the possibility for any structural assignment even on the basis of fingerprint comparisons. It is important to point out that CID of the m/z 118 ion (Scheme I) from the spectrum of vitamin D (Figure 1) gave a spectrum essentially identical to the ones in Figure 3, thus supporting the formation of a common styryl product before CID.

Hydroxylated Compounds. The generation of styryl ions from ring-hydroxylated phenylethanols (structure d) using the TFA derivatives and the McLafferty rearrangement reaction indicated in Scheme II was considered next. Several complications arose, beginning with the difficulty to acylate selectively the aliphatic as opposed to the aromatic hydroxylic group. Moreover, even when samples were isolated from TLC after partial reaction, the CID spectra of the different positional isomers failed to exhibit any discernible variations. Accordingly, we opted to examine instead the TFA derivatives of the corresponding anisoles. Selection of the latter was further justified by the fact that the EI mass spectra of isomeric substituted anisoles are known to exhibit distinct differences due to the influence of resonance on the stabilization of positively charged ions.[21] Applicability of this approach, i.e., use of anisole ions, to the study of vitamin D would thus require the permethylation of the hydroxy metabolites as discussed later.

Methylated Derivatives. Three simple isomeric phenylethanols bearing methoxy substituents on different positions of the aromatic ring, e–g, were considered first in order to establish the applicability of this rationale. CID spectra were obtained for the corresponding methoxystyryl ions at m/z 134 generated from the McLafferty rearrangement, and these are compared in Figure 4. The spectrum of the meta isomer is distinctly different, specifically in terms of the presence of the intense peak at m/z 104 (loss of CH_2O from m/z 134). This is a fragmentation frequently encountered in the EI spectra of aromatic methoxy compounds.[22] Furthermore, while the spectra of the ortho and para isomers are qualitatively the same, distinct quantitative differences may be noted, e.g., in the relative intensity of the m/z 103 peak.

In view of the promising results obtained from the CID spectra of the methoxy derivatives, it was reasoned that the problem of determining the position of A-ring hydroxylation of vitamin D could be addressed via the use of permethylated derivatives. In line with this reasoning, the four possible 2-(methoxy-2-methylphenyl)ethanols h–k were synthesized and their TFA derivatives utilized to generate the corresponding methoxy-2-methylstyryl ions at m/z 148 via the McLafferty rearrangement. (Scheme II). The CID spectra of the four isomeric ions are shown in Figure 5. Remarkably,

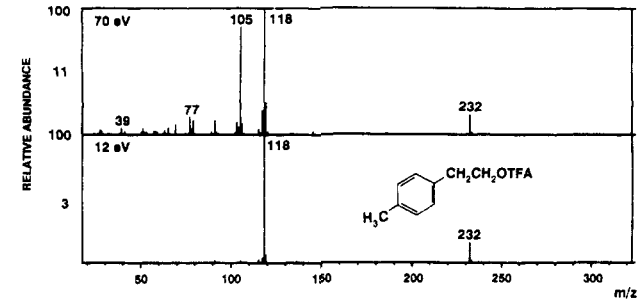


Figure 2. Electron impact mass spectra of the TFA derivative of 2-(*p*-methylphenyl)ethanol: (a) 70 eV; (b) 12 eV.

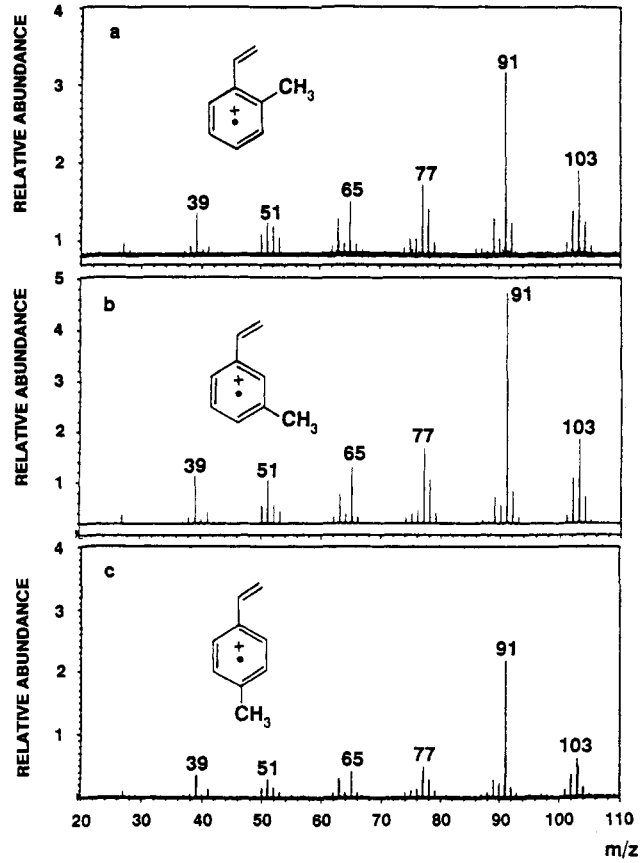


Figure 3. CID spectra of the m/z 118 ion generated from the McLafferty rearrangement product of the TFA derivative of isomeric 2-(methylphenyl)ethanols a–c.

the four mass spectra are quantitatively unique. Significant variations may be noted in the relative ratios of the ions at m/z 133 (148 – CH_3), m/z 117 (148 – CH_3O), m/z 115 (148 – CH_3OH – H), m/z 105, and m/z 103. The last two ions are probably formed by losses of CH_3 + CO and CH_3CO + 2H, respectively, from m/z 148. The CID spectra of the styryl isomeric ions from i and k (Figures 5b,d) exhibit perhaps the closest similarity. However, in addition to a variation in the ratios of m/z 117 to m/z 115 they also shows a marked difference in the relative intensity of the m/z 133 fragment. Thus the data in Figures 4 and 5 clearly demonstrate that methylation of the hydroxylic groups provides the conditions for differentiating between such isomeric compounds by CID. Significantly, the types of fragmentations observed are also typical of the EI spectra of related aromatic ions.

Application to Vitamin D Hydroxylation. 1,25-Dihydroxyvitamin D_3 (structure shown in Figure 6) was used to establish the validity and applicability of this MS/MS approach toward determining the pattern of substitution of vitamin D metabolites which have been hydroxylated on the A-ring. This would clearly hinge on the occurrence of the

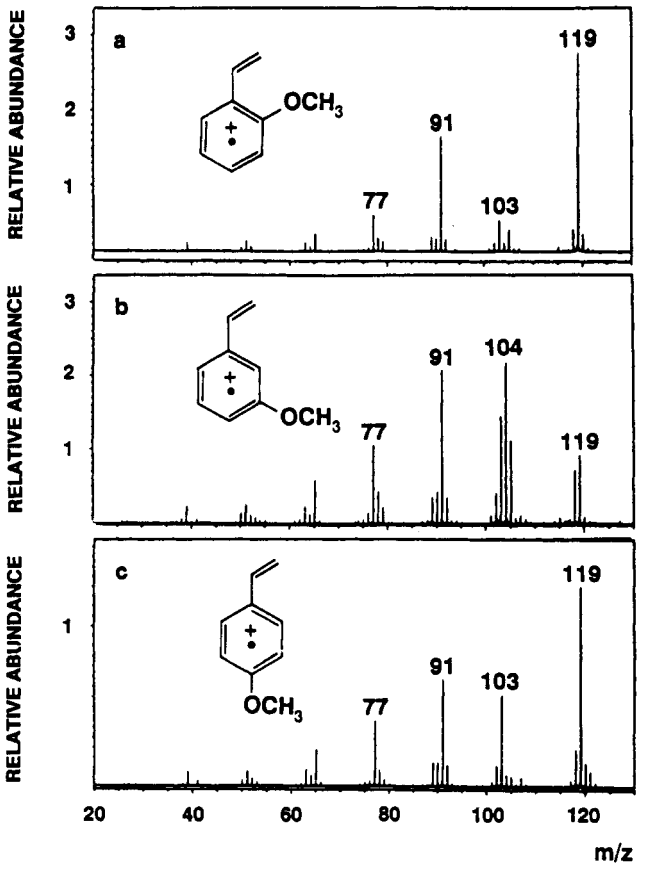


Figure 4. CID spectra of the m/z 134 ions generated from the McLafferty rearrangement of the TFA derivatives of isomeric 2-(methoxyphenyl)ethanols e–g.

process shown in Scheme I in the spectrum of permethylated 1,25-dihydroxyvitamin D_3. Indeed, the spectrum of 1,25-dihydroxyvitamin D_3 (Figure 6) shows an analogous ion at m/z 152 which dehydrates to give rise to an aromatic ion at m/z 134. Permethylation of the compound resulted in a clean shift of this set of ions to m/z 180 and m/z 148 (Figure 6b). Thus, despite the incomplete methylation of the sterically hindered 25-hydroxy group, the styryl ions in the derivative can still be used for the proposed CID studies.

The CID spectrum of m/z 148 from the 70-eV EI spectrum of methylated 1,25-dihydroxyvitamin D_3 was obtained and compared to those of the four isomeric analogues of Figure 5. It should be noted that the CID experiments were conducted under high-energy conditions which generally provide for reproducible fragmentation patterns. Despite this feature, all spectra were obtained within minutes from each other in order to further ensure retention of the same conditions of gas pressure, ion collision energy, and other relevant instrumental parameters. An excellent match with the CID spectrum of the styryl ion produced from the McLafferty rearrangement of the TFA derivative of 2-(3-methoxy-2-methylphenyl)ethanol is indicated. The two spectra are displayed on the same scale in Figure 7 for better comparison. The data confirm the soundness of the original hypothesis regarding the use of MS/MS and the comparison of CID data for the structural characterization of A-ring hydroxylated metabolites of vitamin D. Moreover, they further substantiate the assignment of a styryl structure to the m/z 118 ion in the mass spectrum of 1,25-dihydroxyvitamin D_3 and that the transitions m/z 136 → m/z 118 (loss of H_2O) and m/z 180 → m/z 148 (loss of methanol) occur apparently exclusively by elimination of the hydroxy (methoxy) substituent in the 3-position. This is not surprising since elimination from the 1-position (or the 4-position) is not favored because it would

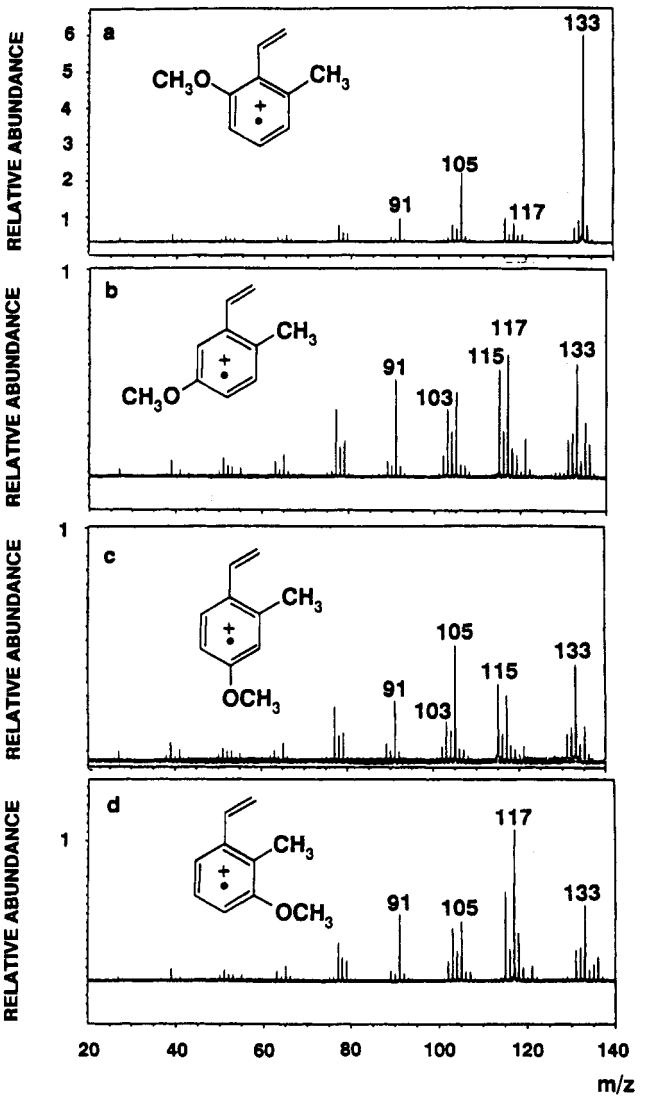


Figure 5. Comparison of the CID spectra of m/z 148 ions generated from the McLafferty rearrangement of the TFA derivatives of the four isomeric 2-(methoxy-2-methylphenyl)ethanols h–k.

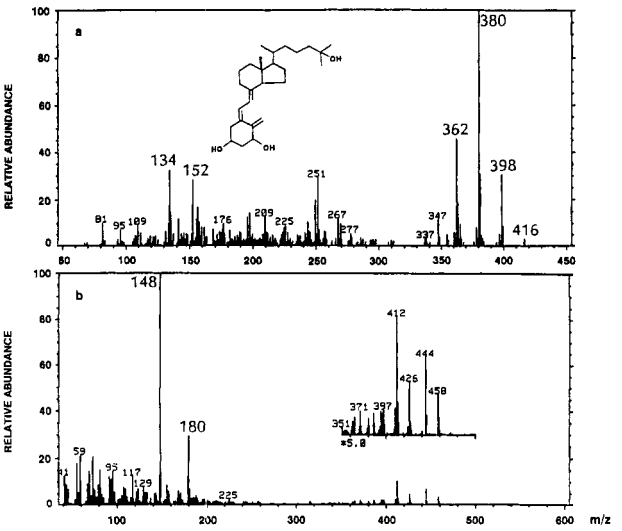


Figure 6. Electron impact mass spectra: (a) 1,25-dihydroxyvitamin D_3 (note the presence of the m/z 152 ion and its dehydration product at m/z 134); (b) permethylated 1,25-dihydroxyvitamin D_3 (note the clean shift of the m/z 152 and m/z 134 ions to m/z 180 and m/z 148, respectively).

require cleavage of a C–C bond α to an sp^2 carbon. This feature therefore permits differentiation of unknowns hy-

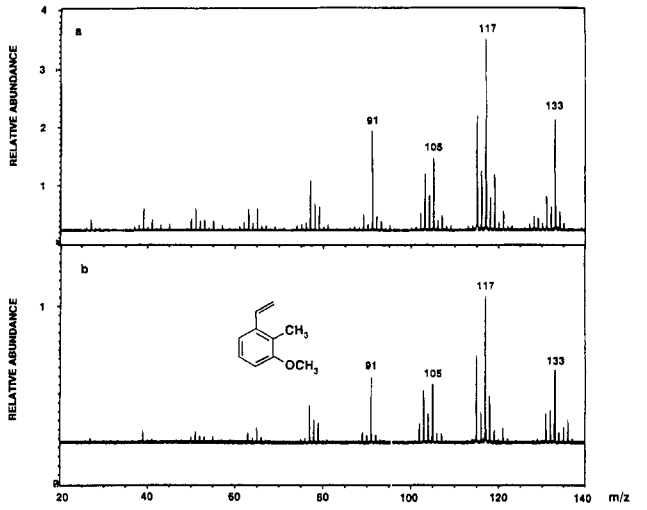


Figure 7. Comparison of the CID spectra of the (a) m/z 148 ion from the mass spectrum of permethylated 1,25-dihydroxyvitamin D_3, Figure 6b, and (b) m/z 148 ion produced from the McLafferty rearrangement in the mass spectrum of the TFA derivative of 2-(3-methoxy-2-methylphenyl)ethanol, k, Figure 5d.

droxylated in the 1- or 4-positions. A mixed spectrum would occur in the event of an unknown metabolite hydroxylated in the 2-position, but such an occurrence should be discernible from the spectral pattern and further help define this position of hydroxylation.

CONCLUSION

Tandem MS/MS with collisionally induced dissociation has been shown to be an excellent approach for elucidation of the structure of A-ring hydroxylated metabolites of vitamin D. The approach proposed here combines a sophisticated instrumental technique with classical organic synthesis as well as "ion synthesis" via the judicious selection of ionic fragmentations. The method takes advantage of the fact that key ions in the mass spectrum of vitamin D are already known to have a simple methylstyryl structure which can also be generated in the mass spectra of related organic compounds that can be prepared by conventional synthetic methods. As long as these styryl ions are produced in adequate yield, the proposed methodology should in principle be applicable to the determination of A-ring modification, irrespective of structural changes in the remaining molecule. Using radical cations, it was possible to make reasonable predictions about their CID spectra on the basis of the fragmentations observed in normal mass spectra generated by electron impact. The methoxy derivatives represent an optimal approach both from the point of view that the spectra of the different isomers can be readily distinguished from each other and also because the synthetic route for generating standards is simple. Moreover, permethylation of hydroxylic compounds can be readily carried out at low-microgram levels (see, for example, refs 23 and 24) which further enhances the utility of mass spectrometry over other spectroscopic methods for addressing structural problems of this type. Since many biologically important compounds, e.g., drug metabolites, arise from hydroxylation of an aromatic ring, the approach proposed here should be more generally applicable beyond the study of the metabolism of vitamin D.

ACKNOWLEDGMENT

Financial support for this work from the National Science Foundation, Grant No. DCB8545666 (M.F.H.), and the USPHS, Biomedical Support Grant RRO7143 (P.V.), is gratefully acknowledged. This is contribution No. 504 from the Barnett Institute.

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RECEIVED for review August 1, 1991. Accepted Janurary 30, 1992.