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Electrospray Ionization Mass Spectrometry of Vitamin D Derivatives

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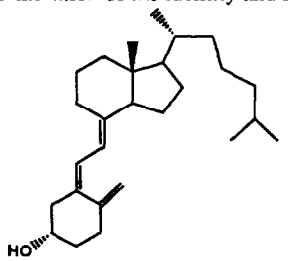
Abstract: Selective "electrospray active" tagging reagents allow the introduction of a chargeable group into neutral vitamin D molecules so they may be detected by electrospray mass spectrometry. An amino group or a crown ether are introduced via the Diels-Alder reaction of the corresponding substituted maleimides with vitamin D.

Vitamin D **1** is synthesized in the skin and is metabolized to a number of bioactive derivatives as well as degraded to inactive forms.[1] Understanding the biochemical role of vitamin D and its active metabolites requires effective and reliable methodology for determination of the identity and levels of the compounds in serum. [2]



1

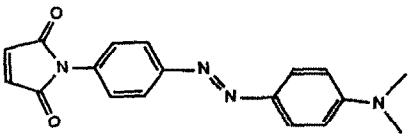
There is no general method at present to identify vitamin D metabolites *in solution* under near-physiological conditions. Radioimmunoassay methods lack specificity, so that the best current protocols involve time consuming concentration and isolation by HPLC.[3] Recent advances in the production of a number of clinically important vitamin D analogs, such as the extraordinarily potent KH1060[4] reinforces the need for more sensitive techniques for identification of such compounds in solution.

A new solution mass spectrometric technique called electrospray ionization mass spectrometry (ESI-MS) has recently emerged. Its application for analysis of biopolymers such as peptides, proteins and oligonucleotides is expanding rapidly.[5] In particular, ESI-MS uniquely generates multiply charged ions (e.g. protonated lysines of proteins) and produces intact molecular ions with no fragmentation for compounds >100,000 MW! ESI-MS involves injection of a solution of preformed ions into the instrument so that no energy is ever applied to the sample as in other ionization techniques such as electron impact (EI), chemical ionization (CI), fast atom bombardment (FAB), laser desorption (LD), etc.

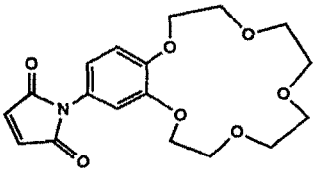
On the other hand, very few applications of ESI-MS to small molecules have appeared so far.[6] First, small molecules often may readily be determined using GC-MS. Second, neutral molecules do not appear directly in ESI-MS since they are not charged. We have recently been examining applications of ESI-MS to

organic chemistry.[7] This report describes our recent efforts to exploit specific tagging reagents[8] to make neutral molecules such as vitamin D more readily charged during the ESI-MS process. These "ESI-active" tagged molecules may then be identified in dilute solution by ESI-MS.

The well known Diels-Alder reaction[9] was selected for use as a specific derivatization process and the use of two maleimide dienophiles **2** and **3** were explored.



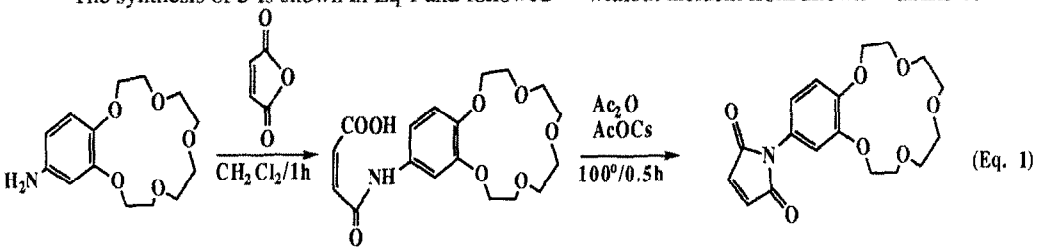
2



3

Commercially available compound **2**[10] would introduce a basic group which can be protonated and therefore be detected by ESI-MS. A new compound **3** would provide a crown ether binding site for attachment of a Na+ cation for ESI-MS detection.

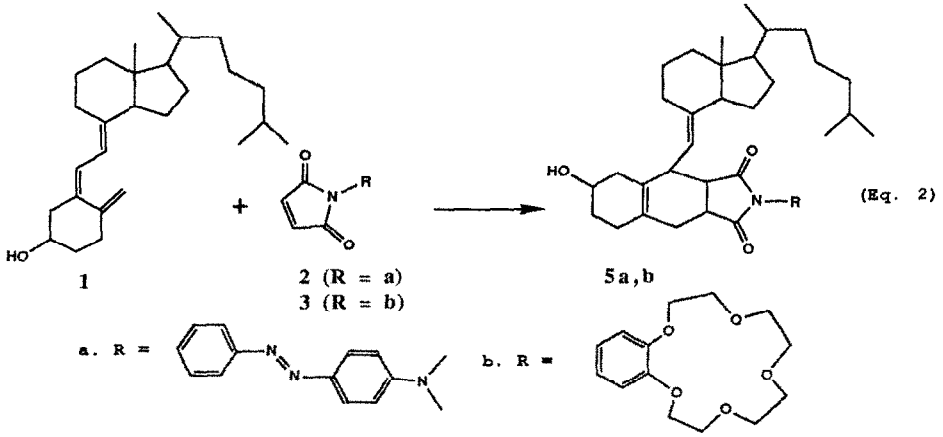
The synthesis of **3** is shown in Eq 1 and followed[11] without incident from known[12] amine **4**.



(Eq. 1)

4 **3**

The Diels-Alder reaction of vitamin D with maleimides **2** or **3** (Eq 2) proceeded smoothly over 10-20 hr in refluxing $CHCl_3$ to produce adducts **5a** and **5b**, respectively.



(Eq. 2)

1 **2** (R = a) **3** (R = b) **5a,b**

a. R = b. R =

In the case of adduct **5a**, three stereoisomeric products were isolated and their ESI-MS responses were determined to be identical. When a solution of $<10^{-4}$ M **5a** in methanol in the presence of 2.5% acetic acid was injected into the ESI-MS[13] a single strong MH^+ion was observed at m/z = 706 (Figure 1.) This result is typical of ESI-MS, i.e.fragmentation is usually not observed.

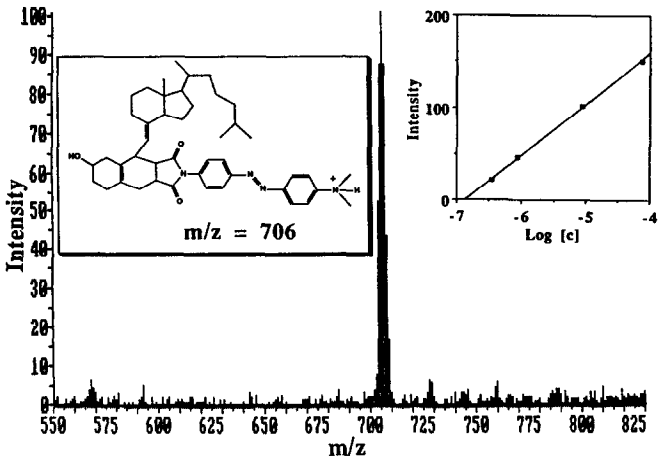


Figure 1. ESI-MS spectrum of compound **5a** in methanol containing 2.5% acetic acid. Inset shows intensity of m/z = 706 vs log [**5a**] from 10^{-4} to 10^{-7} M.

Full scan detection may be observed down to almost 10^{-7} M concentration of **5a**. Since only 2-5 μL of this solution was needed for the spectrum, less than ~1 pg of vitamin D derivative **5a** was used for the measurement. Linearity of the response is shown in the figure insets.

Electrospray MS analysis of vitamin D crown ether adduct **5b** requires the presence of 1% NaOAc to produce the spectrum shown in Figure 2. Again no fragmentation was observed, only the molecular ion $[M+Na]^+$ at m/z = 771. In contrast, the normal electron impact MS spectrum of **4b** shows only a very small molecular ion (5%) and many fragments including the expected retro Diels-Alder cleavage (e.g.base peak m/z = 384) and side-chain fragmentations.

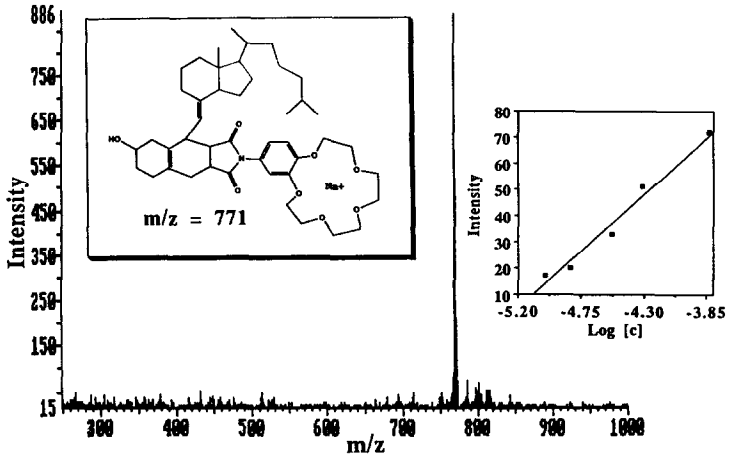


Figure 2. ESI-MS spectrum of compound **5b** in methanol containing sodium acetate. Inset shows intensity of m/z = 771 vs log [**5b**] from 10^{-4} to 10^{-5} M.

Thus we have established that the principle of ESI tagging and detection may be applied to solution characterization of vitamin D derivatives. Since it has also been reported that ESI is the most sensitive method for producing ions and detection at attomolar (10^{-18}) levels[15] are possible, we believe that ESI holds an exciting promise for investigation of dilute solutions of vitamin D derivatives and analogs. Using suitable tagging strategies, applications of ESI-MS to detection of physiological concentrations should facilitate studies of biochemical mechanisms and drug metabolism.

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