Tetrahedron Letters, Vol.31, No.43, pp 6163-6166, 1990
Printed in Great Britain

0040-4039/90 $3.00 + .00
Pergamon Press plc

INTERMEDIATES IN THE VITAMIN B_{12} BIOSYNTHETIC PATHWAY OF *Propionibacterium shermanii* BY FAST ATOM BOMBARDMENT MASS SPECTROMETRY

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ABSTRACT: Simple matrix conditions have been discovered which have given 100% success in examination of a large number of isolated intermediates or derivatives on the Vitamin B_{12} biosynthetic pathway of *Propionibacterium shermanii*, including metal-free and metal-containing porphyrins.

INTRODUCTION: No single instrumental or chemical technique can suffice to determine the complete molecular structure of a complex organic natural product molecule. Mass spectrometry, especially with the development of the fast atom bombardment (FAB) technique, can assume a much more active role in this task than was previously possible. Finding the specific matrix and sample preparation conditions for a particular group of natural products, however, remains a primary obstacle to significant progress in structure elucidation of the target molecules.

We have investigated this question in relation to determining the structures of intermediates along the biosynthetic pathway of vitamin B_{12} in the bacterium, *Propionibacterium shermanii* [1]. The pathway of this organism can be manipulated by nutritional and culture conditions to accumulate various intermediates or sideproducts. Purification of these materials, chemical reactions and modifications of the molecules, refeeding of labeled compounds to the organism, etc., permit our assembling a detailed description of the chemical events leading from uroporphyrinogen III, the last intermediate at a branch point in the general porphyrin biosynthetic pathway, after which intermediates are committed to the production of vitamin B_{12}.

EXPERIMENTAL: Label incorporation experiments with whole cells of *P. shermanii* were conducted according to previously published procedures[2]. Mass spectral observations were made on a VG Analytical 70-S double focusing magnetic sector instrument with DS-11/250J datasystem. Samples were dissolved in a few microliters of *m*-nitrobenzyl alcohol matrix (Aldrich, 95%), and introduced on the FAB insertion probe. Neutral argon at 6-8 KeV was used as the bombarding particle, produced by an Ion Tech saddle field source operated at 1.5 - 2 mAmp. The mass spectrometer was adjusted to approximately 2000 resolving power, and scanned at 10 sec/decade over a typical mass range of 90-1500 mass units. Mass calibration was achieved using concentrated H_2SO_4[3].

RESULTS: We have thus far examined 50 porphyrin compounds -- isolated intermediates or derivatives thereof -- with 100% success and considerable ease, using positive ion FAB-MS and a *m*-nitrobenzyl alcohol matrix, first reported by Meili and Seibl[4]. Some typical spectra are shown in Figure 1. The compounds examined include metal-free porphyrins, as well as species containing metal atoms. Structures belonging to the coproporphyrin, uroporphyrin and isobacteriochlorin groups have been examined, as well as mono- and dilactone derivatives of, for example, Factor II and Factor III. Prominent $[M+H]^+$ is observed for all metal-free porphyrins, and is the principal feature of the spectrum, constituting a large proportion of the total ion current ascribable to the target analyte. Those metal-containing compounds, however, show $[M]^{+\cdot}$. A relatively small amount of fragmentation is seen, consisting of losses of small functional groups from the molecular ion. These conditions, however, are ideal for conducting experiments in which the molecules are

derivatized or otherwise chemically transformed, and the changes in molecular weight used to infer the presence and number of functional sites on the original molecule. We have conducted a number of experiments using this strategy, and shall describe several cases.

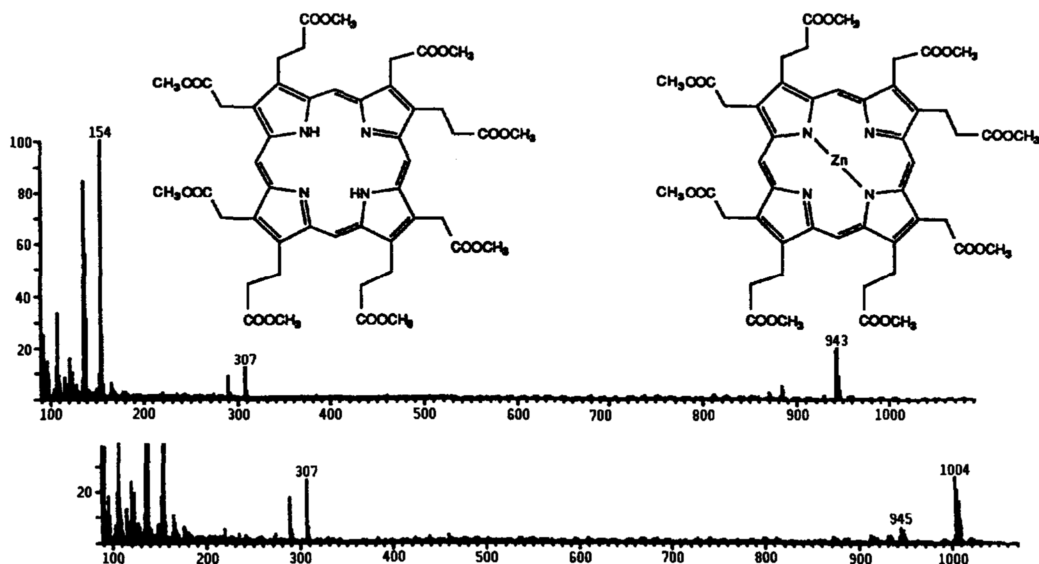

Figure 1: Positive ion FAB mass spectra of uroporphyrin III octamethyl ester (m.w. 942, upper) and the zinc complex (m.w. 1004, lower) using *m*-nitrobenzyl alcohol (m.w. 153) as matrix. Major peaks below m/z 307 (inclusive) are from the matrix: m/z 136, $[M+H-H_2O]^+$; m/z 154, $[M+H]^+$; m/z 307, $[M_2+H]^+$; m/z 289, $[M_2+H-H_2O]^+$.

Factor III, an isobacteriochlorin, is the last identified porphyrin-derived ring structure on the pathway to vitamin B_{12} before contraction of the ring system to the corrin structure. Preparation of Factor III with strategically placed isotopic labels is key to defining the chemical steps, presently unknown, whereby Factor III is converted, ultimately, to vitamin B_{12}. The scarcity of starting materials, and the small structural changes expected demand an accurate and sensitive way to analyze products. FAB-MS has demonstrated itself able to accomplish this task.

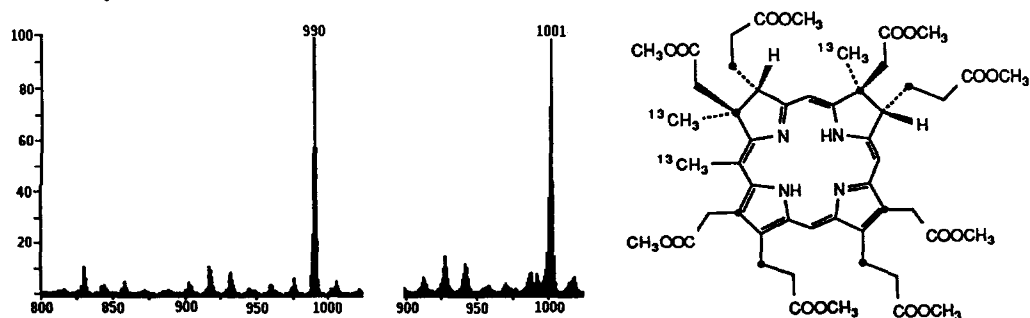

Figure 2: Molecular ion region for Factor III octamethyl ester (left, $[M+H]^+$ 990) and ^{13}C-labeled Factor III (right). Labeling is shown in the structure as black dots (from aminolevulinic acid) and $^{13}CH_3$ (from methionine).

^{13}C-labeled Factor III was prepared by incubating whole cells of *P. shermanii* with [3-^{13}C]aminolevulinic acid (90 atom%) and [methyl-^{13}C]methionine (90 atom%). According to the known labeling pattern, Factor III should contain eight labeled sites in the ring and propionate side chains (shown by the black dots in the structure of Figure 2), derived from aminolevulinic acid, and three methyl groups (indicated as $^{13}CH_3$) derived from methionine[5]. The FAB-MS analysis, shown in Figure 2, indicates that the [^{13}C]-Factor III was, as expected, 11 mass units heavier than the unlabeled material.

A similar experiment was conducted by incubating *P. shermanii* cells with [methyl-C^2H_3]methionine in an attempt to introduce multiple -C^2H_3 groups into Factor III at positions 2, 7 and 20. NMR data showed that a certain level of labeling had been achieved in each desired position, but did not indicate whether the sample consisted of molecules carrying only one -C^2H_3 group per molecule, or a mixture of molecules, some carrying no C^2H_3 and some being triply labeled. The molecular ion region of the FAB mass spectrum, shown in Figure 3, clearly indicated that the sample consisted of a mixture of approximately 33% unlabeled Factor III, 34% $(-C^2H_3)_1$, 23% $(-C^2H_3)_2$ and 9% $(-C^2H_3)_3$. This example emphasizes the complimentarity of mass spectrometry and NMR in determining the "molecularity" of labeling -- *i.e.* how many labels per molecule, in addition to total label content and position.

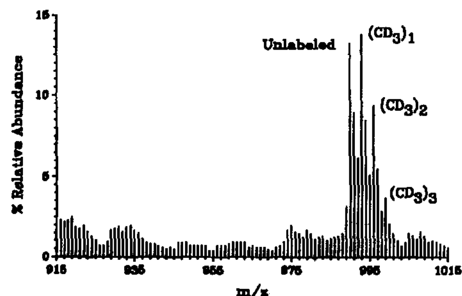

Figure 3: Molecular ion region for Factor III octamethyl ester (m/z 990, $[M+H]^+$) which has been multiply labeled with -C^2H_3 from [methyl-C^2H_3]methionine: approximately 33% unlabeled Factor III at m/z 990, 34% $(-C^2H_3)_1$ at m/z 993, 23% $(-C^2H_3)_2$ at m/z 996, and 9% $(-C^2H_3)_3$ at m/z 999.

[^2H]-Cobester was prepared by methanolysis (in CH_3OD/D_2SO_4) of Vitamin B_{12}. Under the esterification conditions, ^{13}C and ^1H NMR spectroscopy[6] showed that only the proton at C-10 had been exchanged. Confirmation of this minute change was possible by comparison of the FAB-MS spectra of labeled and unlabeled material (Figure 4), showing a difference of only one mass unit.

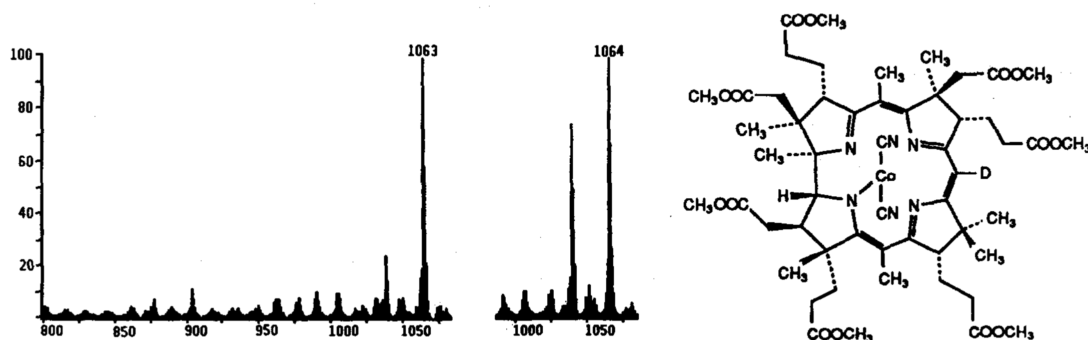

Figure 4: Molecular ion règion for cobester (left, $[M+H-CN]^+$, m/z 1063) and deuterated cobester (right, $[M+H-CN]^+$, m/z 1064). Position of acid-exchangeable hydrogen, at carbon C-10, is shown in the structure at right.

DISCUSSION: Since the first description of the FAB ionization technique[7,8] vitamin B_{12} has become one of the *de facto* standards upon which various principles and performance specifications of FAB have been

demonstrated. Indeed, it was one of the first organometallic compounds to be examined in detail by FAB mass spectrometry[9], and substantial attention has been focused on finding the optimum observation conditions. Schiebel and Schulten[10] have reviewed such efforts by comparing ten different ionization methods which have been applied to the corrin ring system.

Schwarz *et al.*[11] describe their early results on eight dicyanocobyrinic acid heptamethyl ester derivatives using a glycerol matrix. While they obtained very abundant $[M-H]^-$ in negative ion FAB, the positive ion mode did not provide any detectable $[M]^{+\cdot}$ or $[M+H]^+$, but rather loss of both -CN ligands. Schiebel and Schulten[10] show previously unpublished spectra (their Figure 36) of vitamin B_{12}, by both positive ion and negative ion, out of triethanolamine, where they observe intact $[M+H]^+$ and $[M-H]^-$ for the dicyano compound. In our work, the main peak in the spectrum of cobester (Figure 4) corresponds to loss of one of the cyano ligands. Meili and Seibl[4] first report the use of *m*-nitrobenzyl alcohol and *o*-nitrophenyl octyl ether as matrices, and compare spectra of B_{12} in *o*-nitrophenyl octyl ether and glycerol, but not in *m*-nitrobenzyl alcohol. Forest *et al.*[12] have used *m*-nitrobenzyl alcohol for their study of hydrolytically labile dihalotitanium porphyrins. Musselman *et al.*[13] report having to use mixed matrices of thioglycerol, dithiothreitol and dithioerythritol adulterated with trichloroacetic acid in order to produce preformed ions of certain "difficult" synthetic porphyrins. Gallegos and Sundararaman[14] report having to use concentrated H_3PO_4 for certain geoporphyrins. Brown and Wilkins avoid the problem of matrix selection by performing laser desorption Fourier transform mass spectrometry[15].

Reports on *m*-nitrobenzyl alcohol as a FAB matrix for a wide variety of compounds are now appearing[16,17]. Sweetman and Blair[18] even suggest that it could become a more general purpose matrix than glycerol - a suggestion consistent with our experience with these and a variety of other organic and organometallic compounds[16,19]. Spectra from the *m*-nitrobenzyl alcohol matrix seem ideal as starting points for subsequent collision-induced decomposition tandem mass spectrometry (CID MS-MS) studies aimed at exploring what fragmentation information can be obtained which can be related to and used in further structure confirmations, such as label positions in Figure 3. We are continuing to examine these areas.

ACKNOWLEDGEMENT: This work was supported by grants from the National Institutes of Health, and the Robert A. Welch Foundation. Instrumentation for the Mass Spectrometry Applications Laboratory, Dept. of Chemistry, Texas A&M University, was purchased in part with funds from NSF grant CHE-8705697 and the TAMU Board of Regents Research Program.

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(Received in USA 20 July 1990)