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A. TOWARD THE ISOLATION OF A FLOWERING HORMONE

B. FAB-MS AS A STEREOCHEMICAL PROBE APPLICATION TO THE GLYCOSIDIC BOND

C. CHARACTERIZATION AND ANALYSIS OF TRICHOTHECENE MYCOTOXINS BY MS.

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Studies in Natural Products Chemistry

- A. Toward the Isolation of a Flowering Hormone
- B. (FAB-MS as a Stereochemical Probe: Application to the Glycosidic Bond
- C. Characterization and Analysis of Trichothecene Mycotoxins by MS.

by

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A thesis submitted to the Faculty of Graduate
Studies and Research in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

A reexamination of the problems associated with the isolation of a flowering hormone led to the measurement of the kinetics of flowering for photoinduced Xanthium strumarium. It is shown that the rate of flowering can be controlled. After developing an adequate bioassay procedure attempts to isolate a flower-promoting substance from extracts of photoinduced plant material of the same species were not successful. Some extracts however exhibited some flower-accelerating activity in plants that had been previously subjected to one inductive short-day cycle. These results suggest that there does not exist a single hormonal factor capable of inducing flowering. A concerted action enzyme/hormone however could account for the induction/regulation of the flowering process.

Fast atom bombardment mass spectrometry (FAB-MS) has attracted much attention in the past few years because of its ability to allow for high molecular weight determination. A series of oligosaccharides were examined under these conditions with a view to defining rules that would aid in structure elucidation problems (e.g. sequencing) in addition to providing further insight into

the general understanding of the matrix-related phenomena occurring in FAB. The method appears to be highly stereochemistry-dependent and an approach based upon stereoelectronic concepts can be used to correlate the stereochemistry of the glycosidic bond with certain features of the mass spectrum.

Mass spectrometry has been used to analyse a series of fungal toxins in the trichothecene family. Fast atom bombardment (FAB) is an excellent ionization technique for a crude fungal extract and can be used as a fast, efficient and reliable screening test for the presence of the toxins. The fragmentation patterns of the trichothecenes nivalenol, deoxynivalenol, 3-Acetyl and 15-Acetyl deoxynivalenol, T-2, HT-2, diacetylscirpenol and dihydrodiacetyldeoxynivalenol have been established using linked-scan techniques. These data are evaluated in terms of a definitive analytical method for the quantitative analysis and the characterization of the toxins. The fragmentation patterns are discussed in terms of their use in structure elucidation problem.

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affirmer que pendant le cours des huit dernières années où nous avons entretenus une relation amicale et professionnelle, il a été notre père spirituel et que la notation de "Maître" que nous lui avons porté avec tout le respect, n'a jamais été si vraie; il sera toujours, de façon affective, notre maître spirituel et nous lui en sommes reconnaissant.

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DEDICACE/DEDICATION

A mes parents pour le don de la vie, à mon frère Yves, pour le désir de vivre, et à Geoffrey et tous les enfants de sa génération: puisse le travail de ce jour devenir leur ~~voie~~ de demain vers un monde sans douleur et de bien-être. Ce n'est qu'alors que toute l'exaltation de la découverte sera plus qu'un simple rêve.

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TABLE OF CONTENTS

A Title page.....	i
Acceptance sheet.....	ii
Abstract.....	iii
Acknowledgements/Remerciements.....	v
Dedicace/Dedication.....	ix
Table of Contents.....	x
List of Tables.....	xiv
List of Figures.....	xvi
List of Appendices.....	xx
Preface.....	xxii
 <u>Chapter 1</u> : Toward the Isolation of a Flowering Hormone.....	1
1.1 General Concepts of Flowering.....	2
1.1.1 Flower Formation.....	3
1.1.2 Interest in Controlling Flowering.....	8
1.2 Scope of Previous Work.....	10
1.2.1 Previous Experiments and Results.....	11
1.2.2 Methodology.....	19

1.3 Scope of this work.....	22
1.4 Experimental.....	25
1.4.1 Germination and Seedling.....	25
1.4.2 Measurements of the Kinetics of Photoinduced Flowering.....	26
1.4.3 Extraction and Isolation Procedures.....	27
1.4.4 Bioassay Experiments.....	28
1.5 Results and Discussion.....	30
1.5.1 Germination and Growth (Success) Reproducibility.....	30
1.5.2 Kinetics Data.....	31
1.5.3 Extraction Procedures.....	40
1.5.4 Bioassay Data.....	45
1.6 Conclusion.....	56
1.7 References.....	59

<u>Chapter 2:</u> Evaluating Fast-Atom Bombardment (FAB) Mass Spectrometry as a Stereochemical Probe: Application to the Glycosidic Bond.....	66
2.1 General Concepts of FAB.....	67
2.1.1 Principles and Instrumentation.....	68
2.1.2 Sputtering.....	76
2.1.3 Matrix Support.....	77
2.2 Scope of Previous Work.....	82
2.2.1 Applications of Mass Spectrometry to the Characterization of Carbohydrates.....	83

A. Electron Impact Studies.....	85
B. Chemical Ionization.....	98
C. Field Desorption.....	102
D. Laser, Plasma and Flash Desorption.....	111
E. SIMS and FAB.....	114
2.2.2 Stereochemical Considerations in Mass Spectrometry.....	119
2.3 Scope of this Work.....	135
2.4 Experimental.....	137
2.5 Results and Discussion.....	139
2.5.1 Monosaccharide Glycosides.....	143
2.5.2 Disaccharides.....	158
2.5.3 Disaccharide Glycoside and Derivatives.....	184
2.5.4 Higher Oligosaccharides.....	189
2.6 Conclusion.....	200
2.6.1 Conclusions from the Present Study..	200
2.6.2 Future Directions.....	202
2.7 References.....	204
<u>Chapter 3</u> : Mycotoxins in the Trichothecene Family from <u>Fusarium</u> Species: Characterization and Analysis by Mass Spectrometry.....	228
3.1 General Concepts.....	229
3.1.1 Trichothecene Mycotoxins: Sources and Problems.....	229
3.1.2 Structural and Analytical Needs.....	234
3.1.3 Fast Atom Bombardment (FAB).....	244
3.1.4 Linked-Scan Techniques.....	244

3.2 Previous work.....	265
3.3 Scope of this work.....	269
A. Fragmentation Patterns.....	270
B. Screening of Trichothecene Mycotoxins..	272
C. Development of a Definitive Analytical Procedure.....	273
3.4 Experimental.....	274
3.4.1 Mycotoxins.....	274
3.4.2 Linked Scan Spectra.....	274
3.4.3 FAB Spectra.....	275
3.5 Results and Discussion.....	276
3.5.1 Linked Scan Data and Discussion....	276
3.5.2 FAB Data and Discussion.....	296
3.5.3 Scope of Analytical Use: Sugges- tions for future Applications.....	300
3.6 Conclusion.....	303
3.7 References.....	306
Epilogue.....	314
Appendices.....	316

LIST OF TABLES

Chapter 1

Table 1.1:	Floral Development of <u>Xanthium strumarium</u> * Plants after Photoinductive Treatment.....	35
Table 1.2:	Variations* in Apical Bud Size in <u>Xanthium strumarium</u> . Plants at the End of a Typical Bioassay Experiment.....	50
Table 1.3:	Approximate Uptake of the Extracts Used in Bioassay Experiments given in Table 1.2.....	51
Table 1.4:	Variations* in Apical Bud Size in bioassayed <u>Xanthium strumarium</u> plants using the Specific Neutral-Basic Extract.....	52
Table 1.5	Approximate Uptake of Extracts in Bioassay Experiment of Combined Neutral-Basic Fraction...	53

Chapter 2

Table 2.1:	Selected FAB-MS Data (%I) of Some Monosaccharide Glycosides	144
Table 2.2:	Selected FAB-MS Data (%I) of Some Monosaccharide Glycosides	147
Table 2.3:	Selected Positive Ion FAB-MS Data (%I) of Some Disaccharides.....	161
Table 2.4:	Selected Positive Ion FAB-MS Data for <u>beta</u> -phenyl lactoside.....	185
Table 2.5:	Selected Positive Ion FAB-MS Data (%I) of Some Disaccharide Derivatives.....	187

Table 2.6: Selected Positive Ion FAB-MS Data (%I) of
Some Trisaccharides 192

Table 2.7: Selected Positive Ion FAB-MS Data (%I) for
the Tetrasaccharide Stachyose.....193

Chapter 3

Table 3.1: Representative Trichothecene Mycotoxins
from Fusarium spp.....231

Table 3.2: Linked-Scan Laws for Double Focussing Mass
Spectrometers.....257

Table 3.3: Linked-Scan Data (B/E) for Deoxynivalenol
(DON).....277

Table 3.4: Linked-Scan Data (B/E) for 3-Acetyldeoxy-
nivalenol (3 Ac-DON).....278

Table 3.5: Linked-Scan Data (B/E) for Diacetoxyscirpenol (DAS).....279

Table 3.6: Linked-scan Data (B/E) for HT-2 Toxin
(HT-2).....280

Table 3.7: Linked-Scan Data (B/E) for T-2 Toxin (T-2)....281

Table 3.8: Linked-Scan Data (B/E) for Nivalenol (NIV)....282

Table 3.9: Linked-Scan Data (B/E) for Dihydrodiacetoxyl
deoxynivalenol (DDD).....283

LIST OF FIGURES

Chapter 1:

- Figure 1.1: Diagram of Chailakhyan's experiment(s) illustrating the translocation of the floral stimulus.....5
- Figure 1.2: Diagram illustrating a) the effect of long- and short-day cycle on Xanthium strumarium and b) the requirement for uninterrupted photoinductive cycles (reference 9).....7
- Figure 1.3: Pretreated Xanthium strumarium plant.....32
- Figure 1.4: Homogeneity of Xanthium strumarium plants used.....32
- Figure 1.5: Stages of development of the staminate inflorescence primordia of Xanthium strumarium (reference 3).....33
- Figure 1.6: Rate of floral development of Xanthium strumarium plants after 1 or more inductive short-day cycles (floral stages according to Salisbury (3)).....34
- Figure 1.7: Vegetative main apex of Xanthium strumarium plants.....36
- Figure 1.8: Flowering main apex (a,b) and first lateral apex (b) of Xanthium strumarium plants.....37
- Figure 1.9: Fully-blossomed (seed production stage) Xanthium strumarium plant.....38
- Figure 1.10: Typical extraction scheme that yielded a flowering, regulating fraction.....41
- Figure 1.11: Typical extraction scheme I used in preliminary experiments that did not yield any fraction with flower-inducing activity.....42

Figure 1.12: Typical extraction scheme II used in preliminary experiments that did not yield any fraction with flower-inducing activity.....	43
Figure 1.13: Typical split-petiole bioassay experiment....	46
Figure 1.14: Diagram of the leaf-flap bioassay experiment.....	46
Figure 1.15: Diagram of the stem-flap bioassay experiment.....	47

Chapter 2

Figure 2.1: Overall diagram of a fast atom bombardment ion source.....	69
Figure 2.2: Schematic diagram of a FAB ion source.....	72
Figure 2.3: Schematic of the sputtering phenomenon occurring in a FAB source.....	73
Figure 2.4: Primary fragment ions arising by <u>alpha</u> -cleavage in the mass spectra of partially methylated alditol acetates.....	89
Figure 2.5: Mass spectral nomenclature for fragment ions from oligosaccharide derivatives.....	93
Figure 2.6: Some typical mass spectral fragmentation pathways for permethylated glycopyranosides.....	93
Figure 2.7: Examples of kinetically-controlled stereochemical effects on fragmentation pathways in mass spectrometry.....	122
Figure 2.8: Stereoelectronically-controlled fragmentation pattern for epimeric 3-dimethyl-amino-3-methyl-cholestane.....	128
Figure 2.9: Fragmentation patterns of the glycosidic bond under FAB conditions in the positive ion mode.....	165

Figure 2.10: Pyranoside ring conformations, (illustrated for <u>beta-D-glucose</u>).....	167
Figure 2.11: Interresidue linkages in carbohydrate chains (illustrated for some glucose dimers). Linkage conformation is defined by the dihedral angles (ϕ, ψ) or (ϕ, ψ, ω). The intramolecular hydrogen bonds are also indicated.....	170
Figure 2.12: Probable binding sites between glycerol and a (1 $\rightarrow$ 4) disaccharide.....	182
Figure 2.13: Linked-scan (B^2/E) performed on the daughter ion at m/z 163 for raffinose under FAB conditions.....	196
Figure 2.14: Linked-scan (B/E) performed on the precursor ion at m/z 343 for raffinose under FAB conditions.....	197

Chapter 3

Figure 3.1: Stereochemical configuration of deoxynivalenol (DON) based on a Dreiding model....	237
Figure 3.2: High resolution proton NMR spectrum (250 MHz) of deoxynivalenol (<u>24</u>) recorded in dimethyl sulfoxide.....	240
Figure 3.3: Carbon-13 NMR spectrum of deoxynivalenol (<u>24</u>) recorded at 62.5 MHz.....	242
Figure 3.4: Hypothetical sequence ion fragmentations occurring in a mass spectrometer.....	247
Figure 3.5: Schematic representation of a double-focussing mass spectrometer of conventional configuration.....	249
Figure 3.6: Schematic representation of a normal geometry double-focussing mass spectrometer in the Mattauch-Herzog configuration.....	250

- Figure 3.7: Schematic representation of a reversed-geometry double-focussing mass spectrometer in the Nier-Johnson configuration.....251
- Figure 3.8: The B,E plane showing the relationship between the B/E (solid line) and the B^2/E (dashed curve) linked-scans.....264
- Figure 3.9: Typical (B/E) linked-scan spectrum.....284
- Figure 3.10: A three-dimensional image of the fragmentation pattern of deoxynivalenol (DON) as obtained from (B/E) linked-scans.....286
- Figure 3.11: A three-dimensional image of the fragmentation pattern of 3-acetyldeoxynivalenol as obtained from (B/E) linked-scans.....287
- Figure 3.12: A three-dimensional image of the fragmentation pattern of diacetoxyscirpenol (DAS) as obtained from (B/E) linked-scans.....288
- Figure 3.13: A three-dimensional image of the fragmentation pattern of HT-2 Toxin (HT-2) as obtained from (B/E) linked-scans.....289
- Figure 3.14: A three-dimensional image of the fragmentation pattern of T-2 Toxin (T-2) as obtained from (B/E) linked-scans.....290
- Figure 3.15: A three-dimensional image of the fragmentation pattern of nivalenol (NIV) as obtained from (B/E) linked-scans.....291
- Figure 3.16: A three-dimensional image of the fragmentation pattern of dihydrodiacetyldeoxynivalenol (DDD) as obtained from (B/E) linked-scans.....292
- Figure 3.17: (a) Highlight of the locus of primary fragmentation in the EI mass spectrum of DON and (b) the proposed mechanism for the simultaneous loss of water and formaldehyde from the molecular ion radical of DON occurring at that same locus 295
- Figure 3.18: FAB mass spectrum (positive ion in glycerol) of a mixture of trichothecene mycotoxins after a simple pre-separation step.....298

LIST OF APPENDICES

Appendices A through H:	FAB mass spectra (positive ions) of the monosaccharide glycosides used in this study recorded in glycerol.....316
Appendices I through P:	FAB mass spectra (negative ions) of the monosaccharide glycosides used in this study recorded in glycerol.....318
Appendices Q through FF:	FAB mass spectra (positive ions) of the disaccharides used in this study recorded in glycerol.....320
Appendices GG through PP:	FAB mass spectra (negative ions) of the disaccharides used in this study recorded in glycerol.....325
Appendices QQ through XX:	Positive and negative ion FAB mass spectra of some selected disaccharides recorded in diethanolamine.....330
Appendix YY:	FAB mass spectrum (positive ion in glycerol) of phenyl- <u>beta</u> -lactoside.....334
Appendices ZZ through BBB:	FAB mass spectra (positive ions) of the disaccharide derivatives used in this study recorded in glycerol.....335
Appendix CCC:	FAB mass spectrum (negative ions in glycerol) of phenyl- <u>beta</u> -lactoside.....337
Appendices DDD through FFF:	FAB mass spectra (negative ions) of the disaccharide derivatives used in this study recorded in glycerol.....338
Appendices GGG through LLL:	Positive and negative ion FAB mass spectra of some selected disaccharide glycosides and disaccharide derivatives recorded in diethanolamine...339

Appendices MMM through OOO:	FAB mass spectra (positive ions) of the trisaccharides used in this study recorded in glycerol.....	342
Appendices PPP through RRR:	FAB mass spectra (negative ions) of the trisaccharides used in this study recorded in glycerol.....	344
Appendices SSS through VVV:	Positive and negative ion FAB mass spectra of stachyose recorded in gly- cerol.....	345
Appendices WWW through KKKK:	FAB mass spectra of the higher oligo- saccharides used in this study recor- ded under various matrix conditions...	347
Appendices LLLL through RRRR:	FAB mass spectra of various glyco- sides used in this study recorded in polyethyleneglycol 200.....	356
Appendices SSSS through BBBB:	EI mass spectra of representative trichothecene mycotoxins.....	358
Appendix CCCCC:	EI mass spectrum of zearalenone (ZEAR).....	362
Appendices DDDDD through MMMM:	FAB mass spectra of representative trichothecene mycotoxins.....	362

PREFACE

Le temps d'une vie

A l'orée du jour nouveau le soleil brille.
La lumière, cet enfant espiègle, chante.
Bientôt ce sera le temps des camomilles
Et avec lui une étape palpitante.

Les émotions défilent sur toute la gamme.
Là, devant nos yeux, tout est beau et tout grandit
Recherchant sans cesse cette grande Dame
Qui sommeille, là tout au fond de nos cœurs tapie.

Voilà que déjà sonne le coup de midi.
Certes des plus fatidiques s'il en est un
Laissant présager tout ce potentiel tari
Et la vision d'un visiteur inopportun.

Devant nous une ombre floue se dessine.
On se voudrait en mesure de la toucher,
De pouvoir déchirer ce voile de brume.
Hélas c'est la vie, merveille d'un instant passée.

J.R. Jocelyn Paré
21 juin 1984.

CHAPTER 1

TOWARD THE ISOLATION
OF A FLOWERING HORMONE

CHAPTER 1

TOWARD THE ISOLATION OF A FLOWERING HORMONE

1.1. GENERAL CONCEPTS OF FLOWERING

Plants are living organisms which exhibit most of the fundamental biological functions during their life cycle. Among these is the phenomenon of growth which results in the formation of the shoot and root system, flower and fruit formation and so on. Each one of these vital functions is basically controlled by the genome of the plant, but many are also known to be finely regulated by the action of hormonal substances. Today, many plant hormones are known to exist, based on their isolation and characterization by modern chemical procedures, and also from many demonstrations of their regulation of specific growth events; however, much further work remains to be done before a comprehensive understanding of the role of plant hormones is achieved (1).

One of the major unsolved problems in plant biology today is the isolation and chemical characterization of the flowering hormone, or hormonal substances that appear to be responsible for initiating flower formation (2). The concept of a floral stimulus was first enunciated nearly 50 years ago by Chailaklyan (5) based upon observations that the photoperiodic signal is perceived by the leaves, but flower formation takes place at the stem apices.

1.1.1. Flower Formation

It is well known that the flowering process in short-day plants can be initiated by a proper combination of a relatively short photoperiod followed by a longer dark period making up a 24 hour (photoinductive) cycle (2-4). The floral stimulus is produced in the leaves under photoinductive conditions and then transported in the phloem to the stem apices, where it initiates the process of evocation that leads to flower formation (6,7).

Data from several lines of investigation have been presented as evidence for the existence of a floral stimulus in short-day plants. Vince-Prue (8), for example, conducted

studies on plants requiring only a single inductive cycle. By removing the induced leaves at various times after the end of the inductive cycle she was able to provide clear evidence for the gradual movement of the floral stimulus from the leaves to the buds (8). Similarly, plants kept in the light but having their leaves covered with opaque material (e.g. foil paper) that did not allow light to reach the leaves, started flowering while those not covered remained vegetative; on the other hand, no flowering occurred if the stem of the plant was covered while the leaves remained in the light (9), a result which supported the concept that the hormonal material is produced in the leaf. Furthermore, it is possible to induce flowering in a plant that has not received any photoperiodic treatment by grafting one of its petiole to the petiole of another plant that has received a photo-inductive treatment (10), as indicated in figure 1.1.

Another interesting and very significant fact is that in most cases where a successful graft union is obtained between a photoinduced donor plant and a vegetative receptor plant, the latter will flower irrespective of whether the donor and the receptor are, or are not, of the same photo-

Figure 1.1: Diagram of Chalakhyan's experiment (5) illustrating the translocation of the floral stimulus.

response type (i.e. short-day or long-day plants) or whether they are different species or genera (6, 11, 12). An example of considerable interest is the successful transmission of the floral stimulus in an inter-family graft involving the short-day plant Xanthium strumarium and the long-day plant Silene armeria (13). Such results suggest that there may exist a single substance, or possibly 2-3 closely related substances whose flower promoting ability would be fairly universal.

It should also be noted that the photoinductive treatment of plant cannot be interrupted while in progress; an inductive dark period for short-day plants cannot be interrupted with light breaks, no matter how short, without resulting in a non-flowering situation. Similarly, for long-day plants, a light break during a non-inductive dark period will promote stem elongation and spike formation (14). The wavelength of the light used in these experiments was shown to be a major factor in terms of the efficiency of promotion or suppression of flowering (14-16). Figure 1.2 schematically presents these findings.

7
→ no flowers

→ flowers

→ no flowers

→ flowers

a)

- no flowers

- no flowers

- flowers

- no flowers

← one day →

b)

Figure 1.2: Diagram illustrating a) the effect of long- and short-day cycles on Xanthium strumarium and b) the requirement for uninterrupted photoinductive cycles (ref. 9).

1.1.2 Interest in Controlling Flowering.

Regulation of the time of flowering, through the influence of photoperiodic changes in the environment, on the induction of flower-formation is a powerful adaptive mechanism that has attracted considerable attention and investigation in the past. Given the great diversity of plants and ecological niches, and the many steps involved in the flowering process, a large variety of control mechanisms have been suggested and many others, more refined proposals are still to be expected.

For example, Evans (7) reported that during the 6-year period (1965-1971) between the writing of his review on "Flower Induction" and an earlier one written by Searle (17), more than 1200 papers on the physiology of flowering had been published. The number of papers published between Evan's review and the one that followed written by Zeevaart in 1976 (12) was stated by Zeevaart to be even larger. It would therefore seem reasonable to assume that since this last review of the subject, very many more papers have been published describing recent work in the field.

However, the abundance of research papers and reviews, should not be taken as a reflection of progress in the field. On the contrary, no major advances have been made over the past decade and fewer laboratories are currently still engaged in fundamental research on the flowering process. This is not because the major problems have been solved, but rather because they have turned out to be exceedingly complex. In retrospect, this should not come as a surprise since the overall flowering process involves many steps, usually starting with the differentiation at the stem apex of three-dimensional structures, the flower primordia. Since a degree of structural organization is required for flower primordia formation to proceed, biochemical reactions underlying this process cannot be studied in a test tube. Much is known, in a descriptive sense, about the morphological steps involved in flower formation, but direct biochemical approaches to uncover the underlying mechanisms have been few in number and invariably yielded inconclusive or negative results.

All of these earlier results, mostly negative, justify further investigation of this important developmental process, particularly from the academic point of view. However, interests in controlling flowering are numerous and well-diversified; in addition to those of the plant science

research community, the agricultural sciences and the horticultural sciences have also interests in solving the flower hormone problem, since this information could probably be used to enhance crop growth both at the quantitative and qualitative levels. There are also some economic and humanitarian interests as well, since larger, more productive crops would provide more prosperous world trade for Canada and also provide better food supplies for several developing countries who rely largely on their agricultural crops for their own food requirements. In addition, there is always the possibility that a better understanding of the steps involved in the flowering process will provide a breakthrough in our understanding of other important biochemical problems, or that some of the methodologies developed during the course of these investigations might be applicable to other extraction, isolation and characterization problems in other unrelated studies.

1.2 SCOPE OF PREVIOUS WORK

As mentioned above, the literature on the physiology of flowering is very broad and encompasses all aspects of

the process. It would therefore be outside the scope of the present study to attempt to cover all of these data. With this point in mind and realizing that present contribution to this field centers around the fundamentals underlying the floral hormone concept, this thesis will mainly report on past work dealing with evidence for the existence, or not, of a hormonal substance directly related to the flowering of a given species.

1.2.1 Previous Experiments and Results

In any search for the floral stimulus, the development of a reliable and convenient bioassay procedure is essential, but none so far has been presented (2, 12). The situation, unfortunately, has been like a vicious circle, namely, floral stimulus activity could not be tested because of the lack of a suitable assay, yet the selection of a suitable assay plant requires the availability of an active component. Thus, researchers have had to resort to a strictly empirical approach, although general guidelines for testing flower-promoting and flower-inhibiting substances have been proposed (18-21); for example, excised stem tips of Chenopodium (18, 21), intact Xanthium (22-24), Lemna

gibba G3 (25) , and L. perpusilla 6746 (22,23) have all been used as test plants.

Most attempts at the isolation of the floral stimulus have involved the direct extraction of photo-induced leaves and apices of a given species with a variety of organic solvents such as methanol or acetone (24, 26-32). These extracts, after concentration and dissolution in an aqueous medium, are then tested for flower-inducing activity by feeding them back to vegetative test plants by various techniques. The most promising results so far reported have been obtained with Xanthium strumarium plants. Lincoln and co-workers (24) first reported that a methanol extract from lyophilized shoot tips of flowering Xanthium could induce flower formation in up to 50 percent of vegetative Xanthium plants. The active material was claimed to have acidic properties (based on the extraction procedures used) and consequently, was termed "florigenic acid" (29). Hodson and Hamner (22, 23) extracted frozen leaf material with acetone and found that the crude extracts from flowering plants caused flower formation in Lemna. When tested on Xanthium, however, the extracts were active only when supplemented with Gibberellin A3 (GA3) (1). Extracts from vegetative Xanthium, with or without GA3, and GA3 alone, showed no

1

Gibberellin A3 (GA3) = Giberellic Acid.

flower-inducing activity . No data were reported to evaluate the degree of efficiency (partition coefficient) for the extraction of GA3 in acetone versus in methanol, so as to ascertain that the requirement for GA3 spiking was not simply an artefact resulting from the extraction procedures. The most active preparations were obtained from young expanding leaves and buds without mature leaves (31). The culture filtrate of a particular Calonectria strain, originally isolated from a flowering gall of cocoa was also shown to be active in the Xanthium (33) and Lemna assays (22).

Further progress towards the purification and identification of the promotive principle(s) has been slow, mainly because positive results could not be obtained

consistently (2, 12) and because of a need for better physical methods of analysis. However, the results with Xanthium extracts have been confirmed in at least two laboratories (30, 34, 35), although other workers did not succeed at reproducing them (2, 12). It should also be noted that although Gibby's initial experiments were successful, he was unable to duplicate them later (34, 35). Possible reasons for this lack of reproducibility have been discussed (34).

A number of natural plant products including the plant hormones indole-3-acetic acid (IAA, 2) (36), GA3 (37) and zeatin (a cytokinin, 3) (38), are produced by one or more species of fungi. It has been hoped, therefore, that some fungi might produce some active floral stimulus. However, with the exception of the limited success reported by Lincoln and co-workers (33), no other evidence has been presented to warrant further investigations along this line of study.

All available evidence indicates that long-distance movement of the floral stimulus occurs exclusively in the phloem (6). Aphids, for example, are known to feed on phloem sap and produce honeydew that is qualitatively

2

Indole-3-acetic acid (IAA)

3

Zeatin
6-(3-methyl-4-hydroxy-trans-but-2-enylamino)purine

similar to phloem sap (39, 40). Honeydew was therefore collected from aphids feeding on vegetative or flowering Xanthium, dissolved in water and extracted with ethyl acetate (at pH 2.5) both before and after acid hydrolysis. The concentrated and redissolved extracts were then tested on the long-day plant Lemna gibba G3 for their effect on flowering (41-43). The extracts showed some activity and consequently were then examined by thin layer chromatography. The active flower-inducing substance was identified as salicylic acid (4). Authentic samples of salicylic acid (along with analogs such as benzoic acid and acetylsalicylic acid) were subsequently shown to induce a good flowering response in Lemna gibba G3 and several other species of Lemna and in the related genus, Spirodela (41-43). These substances were not active in Xanthium, however. Therefore, although salicylic acid might interact with other unknown substances to influence the flowering process in Lemna, it seems unlikely that it plays any role in the control of flowering in Xanthium.

Although other attempts to isolate the flowering hormone have been reported as being underway (2, 12), to date no success or any meaningful data have been reported.

These investigations include:

- i) extraction of phloem sap,
- ii) diffusion of substances from induced leaves into agar and subsequent extraction of the agar, and
- iii) extraction of xylem exudate collected from induced Xanthium.

4

Salicylic Acid

Another line of investigation that deserves attention is that taken by Chailakhyan and co-workers (44). In 1937, over half a century after the initial hypothesis by Sachs

(45, 46) of the existence of specific flower-forming substance(s) in plants, Chailakhyan (47) was the first to put forward the hypothesis that the florigen is a general complex of flowering hormones of unknown chemical nature. After 20 years of research around this concept, the hypothesis of the bicomponent nature of florigen consisting of two groups of flowering hormones, viz the gibberellins which determine the formation and growth of flower systems, and the so-called "anthesins" which were said to induce flower formation, was first enunciated (48). After a further 20 years of research, Chailakhyan and coworkers suggested mechanisms accounting for the formation of the proposed pair of floral hormones (49). Unfortunately, however, no evidence has ever been presented with respect to the identification of a species which would respond to this definition of the term anthesin. More recently, it has been demonstrated that genetic characteristics, such as long-day or short-day sensitivity for flower induction, can be controlled by hormonal action (50). This idea has led to a series of papers measuring the differences in amounts of some nucleic acids and proteins and related substances in flowering plants compared with the amounts in vegetative plants of the same species (44, 51-55).

In summary, whether one supports the single-component floral stimulus or favors the two- (or multi-) component floral stimulus, the basic problem remains the same, namely, the extraction, isolation and subsequent structural characterization of one or more substances capable by themselves, or in combination with other hormonal factors, of inducing flowering. An acetone or methanolic extract obtained from flowering plants should not, in principle, discriminate between these two concepts. Although much effort has been put into this type of approach, over the last 25 years, no reproducible data have so far been presented. The lack of a good bioassay procedure has undermined most of the extraction techniques used in the past as there did not exist a simple, reliable method to test for flower-inducing (or flower-inhibiting) activity.

1.2.2 Methodology

Another factor of prime importance in earlier work on the flowering stimulus was the lack of physical methods to help in isolation and characterization studies. There also

was a time lapse between the development of modern analytical and physical methods (e.g. mass spectrometry, nuclear magnetic resonance spectroscopy, etc.) and their use by the various workers in the field of Plant Physiology. It is reasonable to assume that this was due in part to a lack of exchange of technical information between the fields and to the limited number of interdisciplinary research groups existing at the time.

In the mid-1960's, however, the advent of mass spectrometry in organic chemistry and the subsequent coupling of a gas-liquid chromatograph to a mass spectrometer led the way to a series of papers on the isolation and characterization of a variety of plant hormonal factors, such as gibberellins (e.g. 1) (56-64), abscissic acid (5) (65-69), phaseic acid (70), cytokinins (e.g. 3) (71-74) and auxins (e.g. 2) (75-81). Similarly, in the late 1970's the development of high performance liquid chromatography had a major impact on the procedures for isolating several hormonal substances (82-86, see ref. 87 for a review on that topic).

5

(cis-trans Abscissic Acid (ABA))

In the light of the statements in the preceding paragraphs, one can understand why numerous efforts, over many years, to isolate the floral stimulus have been unsuccessful, despite the abundance of physiological evidence for its existence.

1.3 SCOPE OF THE PRESENT WORK

This report deals strictly with the concept of the existence of a flowering hormone (where the word hormone is used liberally and can represent any type of molecule present in small amounts that is transported from its site of formation to its site of action). No discussion will be made, at this stage, on the possibility of the existence of a so-called flowering-inhibitor. Although some substances have been shown to be capable of inhibiting flowering (2, 7, 12, 52, 88, 89) it is reasonable to assume, as suggested from successful grafting experiments (1-12), that once flowering has been induced, the presence of inhibiting substances is no longer relevant. At most, it can be suggested that an inhibitor to the biosynthesis of the flowering hormone might exist and that it is destroyed during the long-darkness period (for short-day plants). Therefore, the extraction of flowering plants should circumvent that problem, although it will not provide any evidence to support or reject the concept of the existence of a flowering-inhibitor.

There is good reason to believe that one of the major, if not the most important reason for the lack of success in past experiments attempting to identify the floral stimulus is that the extraction techniques employed were not appropriate. In fact, various workers have expressed the belief that the floral stimulus must be a rather simple organic molecule, or molecules, similar in molecular size to the other known hormonal substances, since its rate of movement through a plant is similar to that shown by auxin and gibberellin substances. If this is the case, then the present availability of several excellent separatory and identification techniques such as gas-liquid chromatography, high performance liquid chromatography, mass spectrometry and, nuclear magnetic resonance spectroscopy, should make these two steps much easier to perform.

Keeping these principles in mind, the present work will focus mainly on the extraction of shoot material from photoinduced and non-induced Xanthium strumarium plants and will try to demonstrate unequivocally, the existence, of a flowering hormone. The overall approach taken to achieve this goal can be summarized as follows:

- Ensure, by developing good germination and seedling cultivation procedures, that the plant material employed is healthy and that natural selectivity is

respected when treating plant specimens so as to avoid artefacts resulting from careless choice of plant specimens to be used in experiments.

- Determine whether or not one can regulate the rate of flowering by different photoperiodic treatments. This would strongly support the existence of a flowering hormone that could be biosynthesized in larger concentrations prior to the performing of the extraction procedures.
- To perform methanolic extractions of photoinduced and non-induced plant material. The crude extracts will be subjected to further fractionation although these will be kept to a minimum so as not to increase the possibility of altering or destroying the flower-inducing substances.
- Develop a reliable bioassay procedure capable of yielding reproducible data, since this was one of the major drawbacks in earlier work.
- To screen all the extracts using the newly developed bioassay procedure and to compare their performance against several types of controls designed to ensure that the plant specimens are not exhibiting a wound response, or that an eventual lack of response to one or all of the extracts is not due to the fact that the plant material is under severe physiological stress.

Should this report be able to conclude positively on these experiments, it should open the door to more rigorous separation, characterization and structure elucidation work. On the other hand, should the conclusions be negative, then, it could indicate that a major reorientation of the directions taken is necessary.

1.4 EXPERIMENTAL

1.4.1 Germination and Seedling

Xanthium strumarium seeds that were stored for at least 6 months at 4 degrees C, were soaked in running water at 23-25 degrees C for 5 days then planted in trays containing a soil:perlite mixture for germination in a controlled environment cabinet under long-day conditions (17h light) for 7 days. The seedlings were then transplanted individually into 13/cm pots and transferred to an air-conditioned greenhouse where they continued their growth under long-day conditions. Plants aged between 6-8 weeks and of 35 cm minimum height, were used as the experimental plants. All specimens were defoliated, except for the four uppermost developing leaves, one week prior to the actual experiment so as to ensure that no excision stress remained in the specimens at the time the experiments were performed.

1.4.2 Measurement of the Kinetics of Photoinduced Flowering.

These experiments were conducted in a controlled environment plant growth cabinet. A long-day cycle consisted of a 17 hour light period followed by a 7 hour dark period, whereas a short-day cycle consisted of a 9 hour light period followed by a 15 hour dark period. The plant specimens were divided in groups, usually of 10-12 plants, and each group was given one, two or more short-day cycles. At daily intervals, after the completion of each short-day cycle, one group of plants was transferred back to the air-conditioned greenhouse and allowed to continue their growth, along with the control (non-induced) plants under long-day cycle conditions. Dissections were performed on 2 plants in each group using a light microscope (X32) within 2 hours following the end of the appropriate dark period. The main shoot apex along with the uppermost 4 lateral apices were dissected and the vegetative or floral stage was recorded according to the scale proposed by Salisbury (3). A minimum of 2 dissections were performed on each group of plants on each day thereafter for a period of at least 4 days following the end of the last short-day cycle (i.e. after the last long-darkness period).

1.4.3 Extraction and Isolation Procedures

The plant specimens used in attempts to extract and isolate the flower hormone were first subjected to a series of 4 short-day cycles. Immediately after the end of the fourth inductive long darkness period, the shoot of each plant was cut off at soil level and transferred immediately to a water filled container. The base of each stem was cut promptly again to ensure that no airlocks could block the vascular system. The excised shoots were then quickly transported to the laboratory where the top portion of each shoot, consisting of the upper stem, apical bud and the first five expanding leaves, was dropped into a prechilled mortar of liquid nitrogen. The resulting slurry was well ground to a fine powder which was then extracted with 80 per cent methanol. (1L of glass-distilled methanol cooled at 0 degree C for approximately 250g of fresh weight). The methanolic slurry was stirred at 4 degrees C in darkness for 30 hours, after which the slurry was filtered and the filtrate concentrated in vacuo to the aqueous phase. The solid plant residue from the filtration was discarded. The aqueous concentrate was filtered over diatomaceous earth 100 g grade III, (Sigma Chemicals) to remove precipitated plant pigments, and the filter pad was washed with hot distilled

water (50mL, 70 degrees C). An aliquot of the amber-green filtrate was set aside for bioassay and the pH of the main portion of the filtrate was successively adjusted to values of ca. 10.5 (with 2N potassium hydroxide) and 2.5 (with 4N phosphoric acid) and extracted three times with redistilled diethyl ether (i.e. 3 X 100 mL) after each pH change to yield a combined neutral-basic ether fraction and an acidic ether fraction, plus the residual extract as an aqueous solution. The ethereal fractions were evaporated to dryness in vacuo and the residue redissolved in 50 mL of glass distilled water adjusted to a pH value of 8.0 for use, along with an aliquot the total extract and residual aqueous extract, bioassay experiments. Identical extraction procedures were performed on shoot tissues from non-induced (non-flowering) plants which provided the control extracts for bioassay.

1.4.4 Bioassay Experiments

Six to eight week old plants that had been continuously maintained under non-inductive, long-day cycles were used in the bioassay experiments to test for flower-inducing activity in the various extracts prepared as

described in the preceding section. The procedure used in these assays was as follows: the uppermost completely expanded leaf was cut off under water at its point of attachment to the petiole and the upper 2 cm portion of the petiole was split with a razor blade, under water, into two halves. The split petiole was then immediately inserted into a scintillation vial containing the solution to be tested (20 mL representing 1, 2 or 3 plant equivalents of extract) and the vial was sealed with Parafilm and attached to the stem with a piece of masking tape. In most cases, the test solution was absorbed by the plant within 24 hours and the vial was then refilled with distilled water. The vial was refilled every 24 hours for the first 4 days of the bioassay period, after which it was removed and the petiole cut off. The quantity of extract consumed by each plant was determined in terms of plant equivalents by recording the volume of distilled water added after each 24 hour period and accounting for the proper dilution factor so-created.

Control plants were supplied with distilled water only to monitor the stress imposed by excision of the leaf prior to the feeding procedure. Some test plants were given one inductive short-day cycle immediately before and some immediately after the bioassay feeding treatment, in order

to monitor for flower-regulating activity in addition to testing for flower-inducing activity in the plants that were not treated at all with an inductive short-day cycle. During the course of these bioassays, watering of the plants was reduced by a factor of 4 to ensure a more rapid rate of extract uptake by the plants. Controls were also set-up to monitor these water-stress conditions but no noticeable effects were recorded as photoinduced plants suffering the same water-stress conditions, flowered normally at the same rate as plants not suffering from any reduction in their daily water supply. Similar treatments to those recorded above were performed on plants used to bioassay extracts obtained from vegetative plants, to monitor once again the possibility of a wound response; no such response however, was observed.

1.5 RESULTS AND DISCUSSION

1.5.1 Germination and Growth

Using the described method for germination, a successful seedling efficiency (seed germination) of above

85 percent was achieved, which is comparable to that found under natural field conditions (90). It is essential to maintain standard germination conditions if acceptable seedling efficiency and good quality plants (figure 1.3) with a high degree of homogeneity size and maturity, are to be obtained (figure 1.4). Plants grown under the conditions described did not need to be preselected for size, shape, or any other criterion since after 6-8 weeks of vegetative growth, over 98 percent of the plants were in excellent condition for the experimental work described in this thesis.

1.5.2 Kinetics Data

As expected from previous investigations (1-4, 6-12), the kinetic data obtained were dependent upon the age of the plants used in each experiment. However, the rate of flower initiation within a given set of plants was extremely constant as the data proved to be reproducible within one percent (i.e. no more than 1 plant out of 100 showed a discrepancy in its floral stage on any day after the photoinductive treatment, as seen from dissections of the main shoot apices. The floral stage scale used in these experiments is shown in figure 1.5, (from ref.3). These

Figure 1.3: Pretreated Xanthium strumarium plant.

Figure 1.4: Homogeneity of Xanthium strumarium plants used.

Figure 1.5: Stages of development of the staminate inflorescence primordium of Xanthium strumarium (ref. 3).

Figure 1.6: Rate of floral development of *Xanthium strumarium* plants after one or more inductive short-day cycles (floral stages after Salisbury (3)).

TABLE 1.1

Floral Development at the stem apex of Xanthium strumarium
and upper nodes after Photoinductive Treatment

No of	I N F L O #	<u>FLORAL STAGES*</u>				
		No. of Long-days after Short-day treatment				
SD	#	0	1	2	3	4
1	apex	0	0	0-1	2-3	2-3
	node 1	0	0	0	0	0
	node 2	0	0	0	0	0
	node 3	0	0	0	0	0
2	apex	0	1-2	2-3	3-4	4-5
	node 2	0	0	0	-	0
	node 3	0	0	1	2-3	4-5
3	apex	0	3-4	4	6	7
	node 2	0	-	-	4	-
	node 3	0	1	1	6	6
4	apex	2	4-5	7	7-8	8+
	node 2	0	0	-	-	-
	node 3	0	0-1	5-6	6+	6-7
5	apex	3-4	5-6	7-8	8+	-
	node 2	-	-	6-7	7	-
	node 3	1	5	7	8	-
	node 4	2	-	-	-	-

* Scale after reference 3.

SD = Short-Days.

REMARKS

- 1- Stages recorded from 2 dissections.
- 2- "-" is for no data.
- 3- "INFLO #" is for inflorescence point.

- 4- Controls remained at 0.
- 5- Main apex showed multiple bud development.

Figure 1.7: Vegetative main apex of Xanthium strumarium plants.

a)

b)

Figure 1.8: Flowering main apex (a, b) and first lateral apex b) of *Xanthium strumarium*.

results provided strong support for our previous claim of good homogeneity within our greenhouse grown test plants.

Figure 1.6 represents graphically the floral induction process observed in one experiment and Table 1.1 summarizes the results from another similar experiment. It also includes some observations made with respect to the dissections performed on lateral apices. Figures 1.7 and 1.8 are representative of the typical morphology of a main shoot apex of a vegetative Xanthium plant, and of a flowering plant, respectively. Figure 1.9 shows a Xanthium specimen at full-blossom where the seed butts can clearly be seen.

Figure 1.9: Fully-blossomed (seed production stage) Xanthium strumarium plant.

From the differential of the slope of the curves obtained by plotting the floral stage vs the number of days after the end of the photoinductive treatment (figure 1.6, table 1.1) it can be seen that the effect of successive short-day treatments on the rate of floral development is additive, at least for the first 4 days, until a plateau is reached. The optimum number of short-days cycles for this strain of Xanthium strumarium plants was 4; this observation was the decisive factor in choosing the length of the inductive photoperiodic treatment for the plants that were used in the flower hormone extraction and bioassay experiments.

Another observation of significance is the fact that plants that were still under short-day treatment (eg. 4 SD cycles), did not flower as quickly as those that had been given fewer short-day cycles and then returned to long-day conditions. This finding fully supports the idea that the flower-promoting substance can be built-up in the leaves during SD treatments. Once removed from the inductive short-day treatment, the plants flowered at a rate proportional to the number of short-day cycles they had been previously subjected to. This phenomenon can be explained in terms of a favored and enhanced translocation mechanism of the stimulus during longer light periods.

A further observation along the same line is that the plants that were treated with several inductive short-day sequences (3 or more) showed a larger number of inflorescence points at the lateral apices (table 1.1).

1.5.3 Attempts to extract the flower hormone

The extraction procedures used (schematized in figure 1.10) to obtain fractions that later proved to be active in flower development was not the procedure first used in these experiments. In fact, several slightly different procedures were used in preliminary experiments and the fractions tested for flower-inducing activity only, but all showed no promotive activity. Figures 1.11 and 1.12 depict schematically two typical extraction procedures that were evaluated during the course of these studies. The failure of these procedures can be summarized in the following specific points, although we must stress that all of the fractions obtained in the early extractions employed were not tested by the flower-regulating bioassay, but rather by the flower-inducing bioassay:

- Timing of defoliation pretreatment. When plants were subjected to a defoliation pretreatment (see

Figure 1.10: Typical extraction scheme that yielded a flower-regulating fraction.

Figure 1.11: Typical extraction scheme I used that did not yield an active fraction.

Figure 1.12: Typical extraction scheme II used that did not yield any active fraction.

experimental, section 1.4.1) just before the photoinductive treatment, treated plants that were as "flowering controls" showed either no response to the photoinductive cycle, or only a very slow flowering response. This weak response was probably due to the amount of physiological stress imposed on each plant by excision of most of its leaves. However, when the defoliation pretreatment was performed one week prior to the photoinductive treatment, all the test plants behaved as expected and responded well to short-day treatment. A period of one week was therefore adopted as the standard period that the defoliation treatment must occur before the plants could be used in bioassay experiments.

- The solvents used in the separatory extraction procedure must all be quite volatile so as to allow their complete removal under mild conditions. For example, when water saturated n-butanol was used, heat had to be applied during concentration of the extract, which increased the possibility of losing or simply altering the hormonal substance under investigation. When chloroform was used, any trace of it left in the residual aqueous extract caused serious damage to the vascular system of the bioassay plants, thus causing severe stress conditions which greatly reduced their flowering potential. This situation was also the case when n-butanol was used as an extraction solvent when all the test plants died during bioassay of the concentrated extracts. Subsequently dissections under a microscope showed the damages caused to the vascular system by trace amounts of n-butanol occurred all the way up to the stem apex, at which point the plants died, as indicated by the brownish coloration of the apex. These observations confirmed the pathway taken by the extract in the vascular system of the test plant.
- Minimization of steps in the separatory procedure. For a preliminary investigation like this one, it is advisable to begin with the minimum number of manipulations possible, so as to reduce the

possibility of altering or destroying the chemical stimulus. This would also have the further advantage of keeping the number of plants required for the bioassay of extracted fractions down to a reasonable level (ca. 300) yet still preserve the statistical nature of the bioassay to be performed.

- Greater fractionation is always possible at a later stage in the work when an "active fraction" has been found and this may have the advantage of allowing one to monitor at which step (if any) in the fractionation procedure is alteration of the "active" constituent occurring.
- Clarity of extract. It has been our experience that the extract solution to be used in bioassay experiments must be free of any particulate material, since any small particles present in the extract will partially or completely block the vascular system and so prevents further uptake of the bioassay extract.

1.5.4 Bioassay Data

Here also the choice of bioassay procedure used in the successful experiments, which is termed the split petiole method (described in the experimental, section 1.4.4 and depicted in figure 1.13), was not the first method employed. In fact, we have also evaluated another bioassay procedure that we call the leaf-flap method (schematized in figure

Figure 1.13: Typical split-petiole bioassay experiment.

Figure 1.14: Diagram of the leaf-flap bioassay experiment.

Figure 1.15: Diagram of the stem-flap bioassay experiment.

1.14) and have attempted to repeat the stem-flap feeding method (figure 1.15) described by Lincoln and Mayfield (24, 28, 29, 31, 32). The leaf-flap method did not yield any positive results; the plants suffered major stress most likely due to the obturation of some pores and some vessels in the main vascular system. The fraction of the leaf remaining was therefore unable to be fed properly and simply died. The stem-flap method, on the other hand, was abandoned since no success was achieved using this procedure and because of the inherent difficulty of the method which requires cutting a flap of stem tissue of consistent depth without weakening the stem too much and preventing it from drying out. Previous workers have also reported little success with this feeding procedure (24, 28, 29, 31, 32).

The successful bioassay procedure used in this work can be seen as a direct extension of the grafting experiments introduced by Chailakhyan in 1936 (5). A comparison of figures 1.1 (Chailaklyan's grafting experiment) and 1.13 (the bioassay technique used in this work) should help in visualizing the relationship between the two methods.

In order to investigate further the possibility of

- i) the existence of a flower-inducing:flower-promoting pair of substances (as suggested by Chailakhyan (5),
- ii) the claim that a photoinductive treatment is a compulsory step for the flowering of Xanthium (91),

the bioassay procedure was applied to both, photoinduced and non-induced plants. Furthermore, plants were bioassayed with various concentrations of extract. These two elements of the experiment should allow for a direct comparison to be made between the present data and those obtained in the physiological experiment described earlier.

Table 1.2 shows the results obtained from one typical bioassay experiment. The combined neutral-basic extract showed activity in terms of an enhancement of the flowering rate whereas the acidic extract did not accelerate

nor decelerate it. On the other hand, the total extract was too dirty and almost none of the solution being bioassayed was taken up by the plants (dissections under a microscope showed clearly that the vascular system had been obturated thus preventing any further extract solution uptake). Table 1.3 gives the extract uptake for the same experiment summarized in table 1.2. Similar experiments were repeated with other, larger, plant populations and clearly demonstrated that the activity exhibited by the combined neutral-basic extract was reproducible (tables 1.4 and 1.5) and was not a wound-related response nor that the plants were under physiological stress that could accelerate or decelerate the flowering process. These conclusions are reached after comparing the data (table 1.2) for the acidic and combined neutral-basic extracts and the corresponding non-induced bioassayed control.

Tables 1.2 and 1.4 include a summary of a statistical analysis of the variations in bud size recorded in Table 1.2. The small standard deviation and variance values should be noted; they indicate good reproducibility of the data. Only in the case of the short-day (SD) treatment followed by bioassay with 1 plant equivalent of the combined

TABLE 1.2

Variations* in apical bud size in Xanthium strumarium plants: at the end of a typical bioassay experiment

Plant Type	No. of Plants	Bud Size (mm)	Standard Deviation	Variance	Variation (%)
V-C	22	0	0	0	veget
F-C	4	1.80	0	0	standard
A1-O	10	0	0	0	veget
A2-O	5	0	0	0	veget
B1-O	10	0	0	0	veget
B2-O	5	0	0	0	veget
T1-O	10	0	0	0	veget
A1-SD	10	1.80	0.120	0.0145	0
A2-SD	4	1.81	0.207	0.0430	0
B1-SD	10	2.15	0.122	0.0150	19.4
B2-SD	5	2.80	0.245	0.0600	55.6
T1-SD	9	1.75	0.118	0.0139	decrease
SD-A1	9	1.78	0.342	0.117	0
SD-A2	5	1.80	0.245	0.0600	0
SD-B1	7	2.57	0.728	0.531	42.8
SD-B2	3	3.33	0.471	0.222	85.0
SD-T1	9	1.75	0.0204	0.0417	decrease

* with respect to F-C.

T = Total extract

A = Acidic extract

B = Combined neutral-basic extract

SD = Short-Day treatment (prior e.g. SD-A1, or after e.g. A1-SD bioassay of extract).

-0 stands for non-induced controls.

The numbers, e.g. 1 or 2, indicates the number of plant equivalents present in the bioassay extract.

TABLE 1.3

Approximate uptake of the extracts used in bioassay
experiment given in Table 1.2.

Plant Type	Plant Equivalent	Plant Type	Plant Equivalent	Plant Type	Plant Equivalent
T1-0	0.58	T1-SD	0.55	SD-T1	0.44
A1-0	0.94	A1-SD	0.97	SD-A1	0.91
A2-0	1.9	A2-SD	2.0	SD-A2	1.8
B1-0	0.88	B1-SD	1.0	SD-B1	0.91
B2-0	1.8	B2-SD	2.0	SD-B2	1.8

NOTES

1. Data refer to plant material used in experiment described in Table 1.2 (refer to Table 1.2 for definitions of plant types).
2. V-C and F-C plants were not bioassayed at all, hence they do not appear in this table.
3. Uptake data above were very consistent within a given plant type (within 10%).

TABLE 1.4

Variations* in apical bud size in bioassayed Xanthium strumarium plants using the specific active neutral-basic extract

Plant Type	No. of Plants	Bud Size (mm)	Standard Deviation	Variance	Variation (%)
V-C	12	0	0	0	veget
F-C	14	2.01	0.146	0.021	4. standard
B1	27	2.32	0.230	0.053	15.4
B2	14	2.35	0.241	0.058	16.9
B3	10	2.05	0.184	0.034	2.0

NOTES

These plant specimens exhibited a larger relative spread value although the larger number of plants used allowed to obtain a better overall standard variation.

B = combined neutral-basic extract.

F = flowering

V = vegetative

C = controls

The number after each extract symbol indicates the number of plant equivalent being bioassayed.

TABLE 1.5

Approximate uptake of extracts in bioassay experiment of
combined neutral-basic fraction

Plant Type	Plant Equivalents
B1	<u>ca.</u> 0.50
B2	<u>ca.</u> 0.50
B3	<u>ca.</u> 0.25

NOTES

1. V-C and F-C plants were not bioassayed at all, hence they do not appear in this table.
2. These data refer to the experiment described in Table 1.4, refer to it for definition of symbols.

neutral-basic (NB) extract are the standard deviation and variance values somewhat high (Table 1.2). However, this is due to the inclusion in the sample of 1 plant whose apical bud size was well outside the range exhibited by all other specimens in the sample. If we were to exclude that particular plant, the values would then read as follows: mean = 2.33, standard deviation = 0.471 and variance = 0.222. We should also note a trend toward larger variations in bud size when more plant equivalents were fed to the plants (e.g. relate data of tables 1.2 and 1.3 together and those of tables 1.4 and 1.5 together). This can be explained simply by the fact that the plant, although absorbing potentially more flowering hormone material, also absorbs more other material which may possibly be somewhat toxic, and thus creates a situation of physiological stress in the test plant.

It must be noted, of course, that no extract was capable of inducing flowering in the non-induced control plants (see the -0 data in table 1.2). However, plants that were pretreated with 1 short-day cycle and then bioassayed with 1 or 2 plant equivalents of the neutral-basic extract showed the highest rate of flowering (Table 1.2), and this finding provides good evidence for the presence in this

fraction of a hormonal factor capable of enhancing flowering. It appears that this factor can be accumulated (by analogy with the physiological data presented in Table 1.1), but that it is unable by itself to initiate the flowering process, thus corroborating earlier reports (91) to the effect that a short-day cycle is a requirement for induction. Some bioassays were performed just prior to and some just after photoinductive treatment of the test plants (Table 1.2) and it seems that the neutral-basic extract was more effective when supplied after the photoinductive cycle. This result is in agreement with the following accepted concepts:

- i) synthesis of the flower hormone factor takes place the dark period of a photoinductive cycle,
- ii) translocation of the hormonal factor is more effective during light periods, and
- iii) the rate of natural synthesis of a hormone (or destruction of an inhibitor) is less during the bioassay long-darkness combination as it competes directly with the unnatural process of extract uptake that is being inflicted upon the plants during the bioassay experiment.

1.6 CONCLUSION

The results gathered in this work suggest that there does not exist just one substance that is responsible for the flowering process. They support the view that a short-day treatment is essential to trigger the flower-induction mechanism (91), but there does exist a hormone-like substance that can act to regulate the rate of flowering. This type of regulation was first suggested by Zeevaart (12) although no critical data were presented.

The present finding that a short-day cycle is essential to induce flowering in Xanthium raises several questions about the data presented in earlier bioassay investigations. It might be that some of the extraction procedures used in these earlier studies were as good, or even better than the method used in the present work, but the fractions isolated were not tested adequately because of the lack of an appropriate bioassay. Also, in most cases, the fractions were tested only for inducing activity rather than for possible flower-regulating activity. The present work, however, has developed a more suitable bioassay

procedure that should allow other future workers to test their extracts for both flower-initiating and flower-regulating activity.

Recently, Kato (92) presented a kinetic study of growth and flowering in Lemna gibba G3 and his results are in full agreement with those presented here. In addition, our results supporting the principle of a flowering regulator, can account for every one of the questions raised in Kato's study (92). In particular, Kato raised questions about the light requirements for a given species and on the possibility that the flower-inducing mechanism might be totally distinct from the flower buds growth. Kato's work is indicative of the specificity, exhibited by the flowering process, to the type of light used (wavelength and intensity), and as the present work does, points out to a phytochrome-connected reaction as the triggering mechanism for flower formation. This action, however, must be very slow in nature and might be seen as a series of reactions of which the first few are reversible thus accounting for the need for an uninterrupted long darkness period (for short-day plants) (figure 1.2 and refs. 14-16, 92-94). Our data however, because of the type of bioassay used, cannot discriminate between the phenomena of hormone synthesis and

inhibitor destruction. It is therefore impossible, from the present work, to favor one or the other of these two possibilities and to suggest which type(s) of mechanism is (are) followed by the phytochrome.

Future work will most likely involve further fractionation of the type of extracts described in the present work, followed by bioassay to monitor the isolation of the flower-regulating activity. The contents of such extracts will likely be monitored by high-performance liquid chromatography and in the event where some new substance, or striking change in concentration of some component is observed, attempts will be made to isolate and identify the substance by gas chromatography-mass spectrometry analysis. Demonstration of the flower-inducing or flower-regulating activity of synthetically prepared sample of the isolated natural compound will provide the ultimate proof for the existence of a flower-regulating hormone.

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65

Chapter 2

EVALUATING FAST ATOM BOMBARDMENT
(FAB) MASS SPECTROMETRY AS A
STEREOCHEMICAL PROBE: APPLICATIONS
TO THE GLYCOSIDIC BOND.

CHAPTER 2

EVALUATING FAST ATOM BOMBARDMENT (FAB) MASS SPECTROMETRY AS A STEREOCHEMICAL PROBE: APPLICATIONS TO THE GLYCOSIDIC BOND

2.1 GENERAL CONCEPTS OF FAB

Fast Atom Bombardment Mass Spectrometry (FAB-MS) can be seen as a renewed old technique although it is most often thought of in terms of a novel mass spectral technique. In fact, as early as in 1966, Devienne and co-workers (1) presented data on a technique that they called Molecular Beam for Solid Analysis (MBSA). The idea was further developed by the same group (2-8) but was ignored by the rest of the scientific community until the announcement by Barber et al. (9) and by Surman and Vickerman (10) of the discovery of FAB stimulated efforts throughout the world as well as a number of symposia and workshops devoted in whole,

or in part to the understanding of FAB and its applications (11-14). Despite all of these efforts and an update by Devienne et al. (15), no definitive reports dealing with FAB fundamentals has been presented to date although the literature describing its applications has seen an explosive growth.

2.1.1 Principles and Instrumentation

The overall diagram of a fast atom bombardment ion source is depicted in figure 2.1. This is fairly simple, consisting of three main elements; i) an atom gun, ii) a sample inlet, and iii) an ion extraction system.

The atom gun is made up of an evacuated chamber that encloses a plate to which a high voltage potential (nominally 8 kV) is applied. The gas to be used as the bombardment gas is allowed in the chamber, through an appropriate inlet, where it is ionized by the high potential plate. The ion beam so created is repelled by the same plate (at 8 kV) and, to a certain extent, regains its neutral

Figure 2.1: Overall diagram of a fast atom bombardment ion source.

character by electron-capture or by charge-resonance. Since the atoms do not suffer much loss in momentum they exit the atom gun and are aimed at the target where the sample is located. A high voltage plate, positioned at 90 degrees with respect to the atom beam, is used to deflect all of the remaining ions that have not been neutralized by charge-resonance. Thus only the atom beam will reach the sample to be analyzed. In the past, workers in the field have used separate charge exchange chambers (16) to carry out the neutralization process described above. However, the source becomes extremely simple once it is realized that any high-pressure confined-discharge ion source will produce large quantities of fast neutral species. These arise by charge-resonance exchange between the ions produced in the discharge and the high-pressure non-ionized gas in the gun itself (equation 2.1).

Saddle field ion sources such as those by Ion Tech (17) are very compact, simple and efficient and have been widely adopted for this purpose. Their characteristics are well documented and they have been used by Barber et al. during the course of their development of FAB⁺ (18, 19).

A typical sample inlet is shown schematically in figure 2.2. The sample is loaded onto the tip of a probe along with a support-matrix (the following section deals in greater detail with the reasoning for the use of such a medium) which is inserted into the conventional ion source of the mass spectrometer at a location such that it intercepts the incoming beam of fast neutrals (from the atom gun). The angle of incidence of the beam is of importance, and an angle of 70 degrees (i.e. 20 degrees with respect to the sample) is a good value (19, 20). This gives rise to a sputtering phenomenon (figure 2.3) and the secondary ions so produced are then subjected to the effect of the ion extraction system which consists of the standard ion extraction system that is already built into the mass spectrometer, i.e. an extraction plate (nominally at 3 kV) and a centering plate to focus the accelerated secondary ion beam into the molecular slit of the mass analyzer.

The technique is very closely related to another condensed state ionization method, secondary ion mass spectrometry (SIMS) (21, 22) but differs from this in that the accelerated inert gas ions used in SIMS (23, 24) are being charge-exchanged prior to the bombardment of the

Figure 2.2: Schematic diagram of a FAB ion source.

2

Figure.2.3: Schematic of the sputtering phenomenon occurring in a FAB source.

sample. On a theoretical basis it can be argued that the bombarding ions in SIMS effectively become bombarding atoms through the Auger effect (25). Experimentally, the same ion gun is used in both methods and it has been demonstrated that the ion beam in SIMS contains many neutral atoms (26), no means being deployed to prevent the spontaneous charge-exchange reactions from taking place. On the other hand turning off the deflector plates (figure 2.1), used in FAB to clean the atom beam from residual ions that have not charge-exchanged, still produces a FAB-like spectrum. Devienne and Roustan, who have performed SIMS vs FAB studies on organics (1-8, 15), did not report on the evidence, or lack thereof of a close similarity between the techniques and more recent SIMS studies indicate that it is possible to obtain SIMS results that are similar to those obtained by FAB (27, 28).

It is not expected that the types of analyzer and detector used in the mass spectrometer should be critical for the successful recording of FAB spectrum and thus far, the literature supports this statement. Single- and double-focusing magnetic instruments as well as triple analyzers have been used (29), as have quadrupole ones (10, 30). By analogy to their use in SIMS experiments (31), it is

reasonable to assume that time-of-flight instruments should also easily convert to FAB. New ion sources can be purchased for older instruments, or a saddle field ion gun can be attached with relative ease and little modification and expense to existing FD, EI or CI sources (32, 33). The most valuable aspect of FAB is its simplicity.

The other standard option of a modern mass spectrometer can also be carried out while using FAB as the ionization mode. Metastable studies and linked-scan techniques (34) to assign ion fragmentation pathways are possible (18, 35, 36) as are collisional activation experiments to enhance fragmentation (35, 37, 38). Mass spectra obtained by FAB (including relative intensities) are reproducible when recorded on a given instrument as well as when recorded on widely different instruments (39). High resolution accurate mass measurements of FAB-generated ions was also shown to be possible (40, 41) but with some difficulty.

2.1.2 Sputtering

The sputtering phenomenon always held out the promise of being used as a general solid-state ionization technique. It has been used for some time as a means of surface and bulk elemental analysis of solids in the ion microprobe (42). The phenomenon was first reported in 1852 (43), it is simply described. If a solid is bombarded by high-velocity particles e.g. rare gas ions of about 8 keV energy, then some of the solid material will be removed into the gas phase. This is the result of momentum transfer from impinging particles to the target, setting up collision chains (some of which occur across the surface (44)) as shown in figure 2.3. The sputtered material can bear either charge, positive or negative. Robb and Lehrle (45) demonstrated the feasibility of such an ion source but credit is to be given to Benninghoven and co-workers (46) along with Macfarlane (47) for sharing the uniquely advantageous mass spectral results that can be obtained with organic compounds with such a source. In each of these studies the quality of the results obtained was limited by the inherent performance of the mass spectrometers that were

used. The technology of producing fast beams of rare gas atoms with controllable kinetic energy is well known (48).

2.1.3 Matrix Support

In early experiments charged particles sputter sources were used, and the sample was deposited as a solution onto the probe and the solvent was evaporated to dryness before analysis (49). This method yielded mass spectra that were transient in nature with a relatively short lifetime (tens of seconds). Subsequently it was noted however that low vapor pressure liquids and oils gave spectra that lasted for hours. Examples were among a whole variety of pumping fluids such as Apiezon oils, Santovac 5, Convolux 10 and also some siloxanes frequently encountered as contaminants in organic samples. These early observations led to the search for low vapor pressure viscous solvents to make a solution of the material under study thus mimicking the fluid behavior with solids (9). One of the first successful solvents used was glycerol (50), it gave enhanced sensitivity (compared with solid sample

preparations) and much longer sample lifetime (hours) provided that enough sample and glycerol was present to continue "mimicking" the fluid conditions. A series of different solvents have also been used in FAB analysis: polyethylene glycol (PEG-200, 400 etc.), thioglycerol, ethanolamine, diethanolamine, macrocyclic ether (e.g. 18-crown-6), etc. Glycerol, however is still, and by far, the most widely used solvent. These solvents exhibit their own characteristic spectra on top of that of the substance under study. This effect is often seen as a drawback as it can mask some of the peaks related to the solid sample and in most cases it complicates interpretation by adding unwanted peaks to the spectrum. For example, glycerol shows peaks of decreasing intensity at every $(n \times 92) + 1$ daltons corresponding to glycerol molecules solvating a proton. If a trace of a sodium salt is present, as it is often the case with samples obtained from some form of chromatographic separation technique, then another series of peaks at every $(n \times 92) + 23$ daltons arise in the spectrum. On the other hand, the molecules under investigation might have the right surfactant properties and bulk solubility to give a mass spectrum in which the solvent background is totally suppressed (19, 50, 51). It has been claimed, after studying chlorophyll A, that for best results, the solid

sample needed to be dissolved in the solvent rather than simply dispersed (19). Although, in our laboratories, this has rarely been true (52). In principle, however, the objective is to submit the sample to the atom beam at a highly mobile surface concentration and for maximum sensitivity the sample should form a perfect monolayer at the surface of a substrate having low volatility. This is a characteristic of compounds that exhibit high surface activity in aqueous media, attributable to the presence of highly polar or ionic groups, giving hydrophilic properties to an otherwise hydrophobic molecule. Monolayer formation at the surface of a dilute solution implies a constant surface excess concentration. Following Gibbs, this arises when the surface tension depends linearly on the logarithmic bulk concentration ($\log_e C$) of the solution (53). The ratio of peaks due to glycerol to those due to the sample cation falls to zero as the monolayer becomes established. Under monolayer conditions, glycerol ions are absent from spectrum, and the solute exhibits a maximum sputter ion yield that is independent of the bulk concentration. Additionally, since there is no increase in ion yield as we increase the solution concentration in this range, we observe an "apparent" decrease in sensitivity measured relative to this concentration.

Since a monolayer of material is completely sputtered in a matter of seconds in a typical FAB ion source, it is essential that the sample surface be continuously regenerated during prolonged examination. This is done "naturally" by diffusion of the sample to the surface of the solution. It is therefore essential that the sample have some solubility in the low volatility solvent, to provide the diffusion mechanism and also to act as a reservoir of material. Ionic groups that render compounds involatile, thus ruling out conventional methods of ionization, are also those groups that frequently lead to solubility in polar solvents, and to the associated surfactant properties that facilitate good sample preparation for FAB ionization. It follows that the detection of solvent substrate peaks in a FAB mass spectrum implies that optimal sample preparation has not been achieved.

The sputtering of neutral species, which are not detected by the mass spectrometer, is probably the major process occurring at the surface. Similarly, the simple sputtering of ions that are naturally present in the sample, undoubtedly, must constitute an important source of ions in FAB mass spectra. Consequently, sample preparation methods such as spiking with various additives which lead to an increase in the concentration of ionic material in the

sample, can lead to an enhanced sensitivity (54). Despite considerable speculation on the subject, the mechanism of ionization in the sputtering of nonionic compounds is uncertain. A recent review paper states that: "Good FAB mass spectra may be obtained with ease from compounds that do not contain ionizable groups. Completely nonionic compounds, such as aliphatic hydrocarbons, do give good FAB mass spectra when the sample is in a condensed phase" (19). This statement contradicts previous reports to the effect that preionization is an essential factor for the obtention of good FAB spectra (55), but complies well with the idea expressed above.

The complex solution and surface chemistry involved in a typical sample can lead to large apparent differential sensitivities between quite similar compounds (56, 57). Nevertheless, the FAB ion source has proven to be a valuable method for the recording of mass spectra of compounds that were previously considered to be intractable by MS because they were too ionic (9) and too involatile. Without doubt the Californium-252 source is the leader in terms of bombardment technique with respect to high molecular weight determination and this situation is likely to persist if we take into account the large amounts of energy available in this sputter process.

The exact role of the support matrix is ill-defined. In addition to the phenomenon just described, the matrix can influence stereochemical aspects by inducing, or forming, a given conformation of the sample and with such a high energy, it is also reasonable to assume that there might be some solvent-solute reactions taking place (58, 59). It appears, therefore, that data on factors such as solubility, ionic character, charge localization, stereochemistry, degree of interaction between solvent and solute, volatility, concentration, etc., are necessary for the accurate prediction of the success, or otherwise, of an attempted FAB mass spectrum. The complexity of the process warrants some careful examination of the stereochemical aspects however, since some of the factors previously mentioned (solubility, ionic character, etc.) are readily accessible and do not seem to be of prime importance in the cases presented to date (19, 55).

2.2 SCOPE OF PREVIOUS WORK

In this section, the emphasis will be on the coverage of earlier work related to the analysis of oligosaccharides

(and polysaccharides) and on stereochemical effects in mass spectrometry although the latter is of very limited range. It is impossible within the limitations of this dissertation to cover all of the FAB-MS data available to date.

2.2.1 Applications of Mass Spectrometry to the Characterization of Carbohydrates.

Mass spectrometry has made significant contributions to structural problems in carbohydrate chemistry, usually by confirming the structure of gas chromatographically separated saccharides. Much research has evolved from techniques of derivatization to prepare carbohydrate materials for analysis providing us with methods capable of resolving most problems in this area (60-65).

Strategies for sequence determination of polysaccharides usually involves the permethylation and some form of limited degradation of a family of oligomers. These heterogeneous mixtures are chromatographically separated and purified for further degradation and analysis by classical procedures to yield monosaccharide derivatives suitable for

gas chromatography/mass spectrometry (GC/MS). Partial degradation frequently entails acetolysis (66-69) or acid-catalyzed hydrolysis and is very sample specific, requiring (66, 68, 70, 71) independent evaluation for each polysaccharide in order to determine an adequate distribution and maximum yield of oligomers. Systematic and reliable procedures have been developed over the years to extract monosaccharide linkage information based on the identification of the resultant permethylated, reduced, and acetylated alditol acetates (72, 73). These procedures have been reviewed (74, 75) and are widely used. They are presented on page 85 under the heading "A. Electron Impact Studies".

Recently, variations of the permethylation approach have appeared which take advantage of instrumental and data system advancements. Improvements in sensitivity and specificity have been reported (76) by using methane chemical ionization mass spectrometry and selected ion monitoring. Another important modification has been the use of capillary GC columns for improved chromatographic resolution. In this approach the permethylated oligosaccharide is degraded by methanolysis to the corresponding methyl glycosides which are subsequently

acetylated and identified by GC/MS (77). The greater complexity of products due to the anomeric methyl glycosides (compared with the alditol acetate procedure in which single derivatives are obtained for each sugar) is offset by the improved chromatographic resolution and the smaller number of chemical manipulations. Although these procedures provide only glycosyl linkage data, considerable structural detail can be obtained from saccharides when this information is combined with composition data and our current understanding of the usual nature of carbohydrate sequence arrays. This is especially the case for oligosaccharides of mammalian glycoproteins and glycolipids.

A. Electron Impact Studies

Mass spectrometry as commonly practiced uses electron impact most frequently as the ionization mode. Carbohydrate derivatives rarely give molecular ions in electron impact spectra, although molecular weights may often be inferred from various fragment ions. Detailed structural information is best obtained from electron impact spectra. Softer methods of ionization, including chemical ionization using

reagent gases such as isobutane (78, 79), field ionization and field desorption (80) may give molecular or quasimolecular ions and in general give much more abundant ions in the high-mass range (500-2000), but their simpler spectra provide less information on structure. These methods could become of greater importance with the development of chromatographic methods for the fractionation of higher oligosaccharides (see later). At present, oligosaccharide derivatives of molecular weights up to 1000 are convenient for mass spectral analysis, and derivatives of molecular weights of up to 2000 have been reported for permethylated glycoconjugates, such as gangliosides (81) and oligosaccharide-containing glycopeptides from extensive proteolytic digestion of glycoproteins (82).

Recently Ballou and collaborators fractionated a polymer-homologous series of 3-O-methylmannose polysaccharides (83) from Mycobacterium smegmatis into discrete compounds by HPLC (84) and then examined the field desorption mass spectra of the unsubstituted compounds (85). Quasimolecular ions were observed up to m/z 2500 for a tetradecasaccharide. The appearance of fragment ions from the cleavage of successive glycosyl bonds provides the basis

for complete sequence determination up to the decasaccharide level.

In terms of thermal stability and ease of interpretation of spectra, permethylated compounds and TMS ethers are the derivatives of choice for mass spectrometry. At present, the separation and characterization of volatile derivatives by combined GLC-mass spectrometry are widely used for disaccharides and some trisaccharides. However, the resolution of mixtures in the trisaccharide range is not always very good. In general, higher oligosaccharides are separated as the parent compounds, and individually prepared derivatives are introduced into the mass spectrometer by direct insertion. With the increasing potential of HPLC for the separation of oligosaccharide derivatives and the availability of commercial instruments for combined HPLC-mass spectrometry, the mass spectral analysis of mixtures of higher molecular weight carbohydrates will, doubtless, be extended in the near future.

The interpretation of the mass spectra of oligosaccharide derivatives follows the general principles that have been elaborated for simple cyclic and acyclic carbohydrates (62, 63).

Many methylated polysaccharides are much less soluble in hot than in cold aqueous solvents. Accordingly, it is usually convenient to carry out partial hydrolysis in an organic solvent such as formic acid and then to complete the hydrolysis in dilute aqueous acid. Partially methylated alditol acetates formed on reduction with sodium borohydride followed by acetylation, are the most widely used derivatives for the characterization of methylated sugars. The mass spectra of these compounds are normally simple to interpret, with characteristic fragmentation patterns (62). Molecular ions are not seen in electron impact spectra taken at 70 eV, but molecular weights can usually be obtained by extrapolation from fragment ions coupled with the use of GLC retention time data. Direct observation of molecular ions is frequently possible in chemical ionization mass spectra (86).

Primary fragment ions from partially methylated alditol acetates (figure 2.4) arise by alpha-cleavage with, in general, preferred formation of:

- (a) ions of lower molecular weight,
- (b) ions from cleavage between two methoxyl-bearing carbon atoms, with no marked preference between the two possible methoxyl-bearing cations, and

Figure 2.4: Primary fragment ions arising by alpha-cleavage in the mass spectra of partially methylated alditol acetates.

- (c) ions from cleavage between a methoxyl-bearing carbon atom and an acetoxyl-bearing carbon atom with a marked preference for the methoxyl-bearing species to carry the positive charge, but
- (d) ions formed by scission between two acetoxyl-bearing carbon atoms are generally of low abundance.

There is little tendency for chain scission to take place adjacent to a deoxy-generated carbon atom (i.e. cleavage of ring carbon-substituent oxygen bond), but the presence of such a unit is usually apparent from the increase in the m/z values by 14 daltons. Primary fragment ions undergo a series of subsequent eliminations to give secondary fragments, including losses of acetic acid (m/z 60) or methanol (m/z 32) by beta-elimination, losses of acetic acid but not of methanol (figure 2.4) by alpha-elimination and losses via cyclic transition states of formaldehyde, methoxymethyl acetate, or acetoxymethyl acetate. A very valuable collection of mass spectra together with retention time data has been published for partially methylated alditol acetates from methylated hexoses, pentoses, 6-deoxyhexoses, and some heptoses and dideoxyhexoses (87). Mass spectra of these and a variety of other carbohydrate derivatives have been reviewed (62) and some salient features are outlined in this report. Recently, an account

has been published of GLC data and mass spectra of derivatives of aminohexoses and aminohexitols (88).

The main limitation of the use of partially methylated alditol acetates for the characterization of methylated sugars lies in the structural symmetry that may exist when the primary hydroxyl group (O-5 in pentoses and O-6 in hexoses) is not etherified. This difficulty, however, can be overcome by introducing deuterium at C-1 by reduction of the sugar with sodium borodeuteride.

Partially methylated, acetylated aldononitriles are acyclic derivatives readily formed from reducing sugars by reaction with hydroxylamine in pyridine, followed by the addition of acetic anhydride to effect elimination of acetic acid from oxime acetates and acetylation of unsubstituted hydroxyl groups. These derivatives, although less extensively used, appear to give good GLC separations, and their mass spectra can be readily interpreted without the problem of structural symmetry (89).

Partially ethylated alditol acetates may be used as alternative sugar derivatives when separation difficulties

are encountered with particular combinations of the corresponding methylated compounds (90, 91).

Permethylated oligosaccharide alditols are often the most convenient derivatives to prepare from reducing oligosaccharides isolated as partial hydrolysis products. As in the case of partially methylated alditol acetates, reduction is best performed with sodium borodeuteride in order to avoid ambiguities arising from otherwise structurally symmetric terminal units. Spectra may be analysed by considering, in turn, fragment ions derived from reducing and nonreducing terminal units and then fragment ions arising from internal units, often by more complex fragmentation pathways.

A mass spectra nomenclature scheme for oligosaccharide derivatives was developed by Chizhov and Kochetkov (63) and later modified by Kovacik et al (92, 93). In this scheme for unbranched oligosaccharides, sugar residues are designated by lowercase letters starting from the nonreducing end group (a) along to the unit derived from the reducing terminus - in the example shown in figure 2.5, the alditol residue (c). In this example fragment ions are

Figure 2.5: Mass spectral nomenclature for fragment ions from oligosaccharide derivatives.

FIGURE 2.6
A series

B series

D → J series

E series

Figure 2.6: Some typical mass spectral fragmentation pathways for permethylated glycopyranosides.

generated by a variety of bond cleavage processes, and the various pathways are denoted by uppercase letters (A, B, etc.). Additional numerical subscripts indicate successive ions along a given pathway. Some examples are shown below and incorporate the further conventions that the first lowercase letter indicates the ring unit that has undergone cleavage and that subsequent lowercase letters denote unaltered residues that remain attached as substituents.

Methylated alditol units in oligosaccharides are cleaved in a manner similar to that described previously for partially methylated alditol acetates. On the other hand, substituted cyclic carbohydrates undergo a variety of types of fragmentation, the most important of which are summarized in figure 2.6 for simple permethylated glycosides.

The most easily recognizable fragment ions in the mass spectra of permethylated oligosaccharide alditols are those from nonreducing units, i.e. fragment ions at m/z 219 for permethylated hexose, m/z 189 for permethylated deoxyhexose, m/z 175 for permethylated pentose, m/z 233 for permethylated hexuronic ester, and m/z 260 for permethylated O- and N-2-acetamido-2-deoxyhexose residues (see fragment

on figure 2.5). Likewise, from 1-d-labeled alditol residues from reducing termini, the following ions are readily recognized: m/z 236, 206, 192 and 277 for units derived from hexose, pentose, deoxyhexose and 2-acetamido-2-deoxyhexose residues, respectively (see fragment C(ald) on figure 2.5). Furthermore, information on the general nature of unbranched internal units can be obtained by detecting, in the m/z values for fragment ions of higher mass, increments of 204, 174, 160, 218 and 245, respectively, for hexose, 6-deoxyhexose, pentose, and 2-acetamido-2-deoxyhexose units (see fragment baA₁ on figure 2.5). These aspects of mass spectrometry, therefore, allow conclusions to be drawn concerning the sequences of sugar units when these are of different types only. On the whole, very little information, if any, can be obtained concerning stereochemistry, either of individual residues or of the configuration of glycosidic linkages. Nevertheless, some information can be obtained on linkage types from an examination of fragmentation pathways.

The use of primary fragment ions is not enough to allow for extensive structural characterization. Careful examination, however, of the secondary fragmentation pathways provide a more precise means to identify some, but

not all, of the structural characteristics for the substance under study. A recent account by Aspinall (94) reviews a useful example where the permethylated alditols from the milk oligosaccharides lacto-N-tetraose (Gal-(1-beta-3)-Glc-(1-beta-3)-Gal-(1-beta-4)-Glc) and lacto-N-neotetraose (Gal-(1-beta-4)-Glc-(1-beta-3)-Gal-(1-beta-4)-Glc) (95, 96) are analysed. The information that can be so obtained in some cases includes evidence for a 3-linked glycosyl substituent (presence of A_1 and A_2 ions, see figure 2.6), the differentiation between 2- and 4-linked residues in methylated di- and trisaccharides (64) and the recognition of branched oligosaccharide alditols in which the alditol unit carried two glycosyl substituents (97). Care should be taken however as the structures presented for the fragment ions are not exhaustive and several others are possible (94).

Newer methods of ionization (sometimes called "soft ionization techniques") now provided techniques for the analysis of polar, high molecular weight materials with relative ease. Molecular stability during these processes has changed the analytical picture completely so that the focus of attention in biopolymer mass spectrometry is now directed toward sequence and high-mass information. In the

sections that follow, some of these techniques are discussed as they relate to the study of carbohydrate materials.

B. Chemical Ionization

The technique of desorption chemical ionization (DCI) (98) is also included in this section and is treated as a variation on the chemical ionization (CI) theme.

Most investigations of the CI mass spectral behavior of saccharides have employed methane, isobutane, ammonia, or a combination of these gases. The reacting forms of these gases are CH_5^+ , C_4H_9^+ , and NH_4^+ , which decrease in their protonating capability in the order shown. Thus, fragment ions are quite abundant in the methane and isobutane spectra of saccharides, whereas the ammonia CI spectra are dominated by ammonia cluster and sample adduct ions (99).

The first carbohydrate-ammonia CI study reported single adduct ions for peracetylated mono- and disaccharides and also indicated that for underivatized materials smaller fragments corresponding to the loss of water from the

molecular ion adduct are observed (100). Since this early work, considerable carbohydrate mass spectral data, obtained by CI with ammonia as a reagent gas, have accumulated (79, 99, 101-112). With few exceptions, however, little work has been published on fragment ion structures and because of the different methods of sample introduction, various reagent gas pressures and mixtures used, and the poor quality of mass spectra reproduced in many journals, it is difficult to judge and compare the contributions of these parameters to the resultant spectra or to draw definitive conclusions.

Specifically, one difficulty in reviewing CI mass spectral work is the separation of CI-induced fragmentation from that due to pyrolysis, a variable which is highly dependent upon the method of sample introduction. This factor is frequently overlooked and fragmentation schemes have been proposed with all ions originating from the adduct or protonated molecular ion (102, 113).

The first detailed look at carbohydrate oligomers using ammonia CI was reported in 1974. The investigators studied a series of peracetylated oligosaccharides introduced on a solid probe and chose to enhance

fragmentation by the addition of isobutane at half the concentration of ammonia (106). The use of a mixed reagent gas complicated identifying the origin of fragments, but nevertheless peracetylated saccharides produced a number of even-mass fragments indicating that they contained nitrogen. This observation led to the proposal of four possible ways in which this nitrogen may be incorporated (106):

- (1) ammonium attachment to neutral fragments (i.e. thermalized intact molecules and pyrolysis products),
- (2) adduct ion degradation via loss of a small molecule (e.g. HOAc, H₂O),
- (3) attachment of neutral ammonia to charged fragment ions (i.e. CI-induced fragments),
- (4) covalent bond formation followed by ionization (e.g. formation of carbinolamines).

Experimental support for one of these mechanisms was obtained by metastable ion studies which followed the loss of acetic acid from the molecular ion adduct (106). Further fragmentation studies were pursued in later work using the same mixtures of isobutane and ammonia as the reagent gases but with reduced and permethylated carbohydrate derivatives (78). The samples analysed included six disaccharides and

two trisaccharides and the spectra provided sequence information and some indication of linkage based on fragment ion abundances. These studies clearly indicate that fragments do arise from the ammonium ion adduct. However, the use of a mixed reagent gas to enhance fragmentation, as already noted, obscures the influence that ammonia itself may contribute to the spectrum.

Several overall features of oligosaccharide ammonia CI spectra are worthy of note;

first: the stability of the molecular ion adduct;

second: the enhanced ion abundance for rupture at each glycosidic linkage;

third: also related to the glycosidic cleavage, the ions differing by 2 daltons which appear at the expected incremental intervals of 204 daltons, which corresponds to the incremental mass of a permethylated glycosidic unit. These ion pairs are of considerable importance for sequence interpretation and their origin appears clear. However, the exact structures of these ions have not been clarified.

Probably the most interesting glycoside work, from the standpoint of carbohydrate sequencing, has been that reported for the cardiac glycosides (107, 114). Using ammonia DCI, spectra were obtained for two trisaccharide-

containing samples, dioscin and digoxin, which exhibited a series of ions for sequential loss of terminal pyran-oxonium sugar residues from the charged aglycone. No sequence ions starting from the terminal carbohydrate end could be detected. The spectra provided ample high-mass and molecular-weight related ions in addition to the sequence information.

Several other glycoconjugates such as saponins (115), cardenolides (110, 114), glucuronides (113), glucosinolates (111, 112), flavone glycosides (115), cyanogenic glycosides (116), and the aminoglycoside/antibiotics (117) have been studied by CI-MS. Again, although molecular weight information was obtained easily, the amount of structural data so obtained was of almost no value.

C. Field Desorption

Field desorption mass spectrometry (FDMS) is today one of the best known soft ionization methods (118, 119). The technique has been important since the early 1970s in biomedical, chemical, and environmental research for the analysis of compounds which are involatile, thermally

labile, and of high molecular weight (for recent reviews, see references 120 and 121). Since FD generates ions related to the molecular weight even for salts and very polar organic materials, derivatization may be avoided. This has the advantage that one need not worry about the stability of the sample during chemical manipulation or whether the weight of the derivative is beyond the mass range of available instrumentation.

From its inception in 1969 (118), FDMS has been applied to the structural characterization of carbohydrates and glycoconjugates (61, 120, 121). The FD spectra of free sugars and di- and trisaccharides (in the absence of alkali salts) are dominated by $(M + H)^+$ and $(M + H - H_2O)^+$ ions and exhibit little or no fragmentation at lower emitter currents (12-15 mA) (80, 122, 123). At higher heating currents (15-18 mA) sequence-informative ions are produced by cleavage on either side of the glycosidic oxygens. For example, the FD mass spectrum of raffinose recorded at 18-19 mA exhibits a base peak at m/z 505 which corresponds to $(M + H)^+$, and abundant fragment ions at m/z 163 and 343. At higher emitter current an additional ion at m/z 325 is observed as well as many low-mass peaks due to fragmentation of component sugar residues.

The mechanism of fragment ion formation (124) may be similar to the acid-catalyzed hydrolysis of carbohydrates in solution. Cleavage on the terminal side of the glycosidic oxygen produces the terminal "1-deoxysaccharides," some pyran-oxonium species (ions at, e.g., m/z 163, 325, ..., etc., for aldohexoses --see A series in figure 2.6, p. 94). Alternatively, cleavage on the reducing side of the glycosidic oxygen with hydrogen and proton transfer results in the protonated terminal saccharide ion, such as at m/z 343 in the spectrum of maltotriose (80). No correlation between stereochemical considerations and the occurrence of one or the other fragmentation has been made. These fragments could also arise through hydrolytic interaction of bound water with the sugars on the emitter surface (125). Charge retention on the reducing end of the molecule would result in a series of fragments which are isobatic with those from the terminal end. Thus, to define more precisely the origin of these fragments, the FD spectra of oligosaccharides could be compared before and after reduction with NaBH_4 . These results indicate that the charge is localized preferentially on the terminal (nonreducing) end of the oligomer for saccharides with fewer than five residues, but with increased chain length there is a gradual shift to charge retention on the reducing end (126).

Early FD studies of saccharides suggested that molecular weight information might be difficult to obtain for oligomers containing more than three residues (80). More recently, however, spectra for larger oligosaccharides have been reported for samples in which alkali metal ions are present or have been added (125, 127-129).

The success of model studies (125, 130) has prompted the application of FDMS to high-molecular weight oligosaccharides of biological significance. Structurally informative fragments were observed in the FD mass spectra of $(\text{Man})_8\text{-Glc-NAcH}_2$ (8 mannose units linked to a N-acetyl glucosamine alditol) (85), which has a calculated molecular weight of 1520.53. In this example, loss of the terminal mannose residue by cleavage on the nonreducing side of the glycosidic oxygen is accompanied by proton transfer resulting in ions corresponding to $(M - 162 + \text{Na})^+$ and $(M - 162 + \text{H})^+$, with calculated masses of 1381.47 and 1359.49, respectively. Loss of the terminal disaccharide fragment is also indicated by the ion at m/z 1219 = $(M - 324 + \text{Na})^+$. However, care must be taken because contamination by shorter oligomers would result in the same series of ions.

Recent developments in magnet technology have provided mass spectrometers that can transmit ions heavier than 3000 daltons at full accelerating potential and high sensitivity. Instrumentation with such extended performance capability was used to obtain FD mass spectra of a mixture of the higher-molecular weight methylmannose oligosaccharides (131). Abundant ions were observed at m/z 2154, 2330, and 2506, which correspond to the $(M + Na)^+$ ions of $\text{Man}(\text{MeMan})_x\text{OCH}_3$, where $x = 10-13$.

Undoubtedly the excellent results obtained for this series of compounds are due, in part, to the increased volatility and stability imparted by the presence of methyl groups on approximately one-third of the available hydroxyl groups. This suggests that FDMS of synthetically permethylated oligosaccharides may allow the molecular weight characterization of even larger polymers. Preliminary work on fully permethylated di- and trisaccharides indicated that the relatively high volatility of these compounds result in poorly reproducible FD mass spectra (80). Recently, however, FDMS has been successfully employed in the analysis of a 6-O-methylglucose polysaccharide isolated from Mycobacterium smegmatis (132) that contains 20 hexose units. Usable FD mass spectra could

not be obtained on the native 6-O-methylated material, whereas the synthetic, fully permethylated eicosasaccharide gave clusters of peaks separated by 14 daltons (due to undermethylation or demethylation during desorption) up to approximately m/z 4250 (calculated MW = 4230, $(M + Na)^+ = 4253$) thereby confirming the degree of polymerization of the oligosaccharide. Masses were assigned by reference to a crystal time marker which had been calibrated under EI conditions with Fomblin oil. It should be noted that the underivatized 6-O-methylglucose polymer was readily analyzed by fast atom bombardment mass spectrometry (section E, p.114).

Peracetylated oligosaccharides have also been studied and these derivatives exhibit abundant $(M - 60)^+$ ions and sequence informative fragments (80). However, peracetylation nearly doubles the weight of the sugar and, therefore, may be of limited practical value for large oligosaccharides.

During the late 1960's and early 1970's, a number of reports appeared which described the successful application of FIMS (118) to the analysis of aromatic (133) and steroidal glycosides (134-136). This work (described in detail in a recent review (61)) clearly demonstrated that FI

could provide the weights and sequence of the component monosaccharides and the molecular weight of the intact molecule and aglycone for underivatized glycosides containing up to three sugar residues.

These early successes suggested that FD would be a promising analytical approach for less volatile glycosides containing even longer carbohydrate moieties. A series of underivatized O-glycosides of varying sugar compositions and genin structures were subsequently studied by FDMS. These results gave comparable information to that obtained by field ionization (FIMS), but the FD spectra were more intense and showed less fragmentation.

Examples of successful experiments include complex molecules such as rutin (1) (126), saponins such as pennogenin - 3 - O - beta - L - rhamnopyranosyl - (1 - beta - 2) - glucopyranoside (2) and its tetrasaccharide counterpart 3 (124, 136-139), and higher molecular weight pentacyclic triterpene saponins such as that presented as structure 4 (124).

Some attempts have been made to correlate the known rates of hydrolysis of various sugars in aqueous acidic

media with the observed relative abundances of ions in the FD mass spectra (124, 137-140). For example, it is known that D-xylosides hydrolyze about five times as fast as D-glucosides in solution. Similarly, the ratio for loss of terminal xylose to terminal glucose in the FD mass spectrum of the glycoside tomatine was found to be 2.8:1 (139). However, there are many exceptions to the analogy (139) and, therefore, more model systems must be studied before conclusions can be made for glycosides of unknown structure.

From these results it is apparent that FDMS is highly successful in obtaining both molecular weight and sequence information for complex carbohydrates and glycoconjugates. Undoubtedly, FDMS will continue to be relied on as a physical method for the structural analysis of these molecules.

D. Laser, Plasma and Flash Desorption

Laser desorption mass spectrometry, like SIMS, was developed primarily as an analytical tool to study inorganic elements on surfaces (141, 142). However, shortly after the

introduction of this technique, salts of simple organic molecules were shown to yield abundant cationized molecular ions by LD (143). Since that time, LD has been shown to have remarkable potential for obtaining molecular and structural information on complex, involatile biomolecules (144-146). These observations, coupled with the recent availability of commercial instrumentation, has sparked a resurgence of interest in LD (147-150).

Under LD conditions, alkali metal cation attachment is the dominant ionization process. Protonated molecular and fragment ions are generally not observed for carbohydrates under these conditions (144). In addition to the abundant pseudomolecular ion $(M + \text{alkali})^+$, fragment ions corresponding to alkali-cationized saccharides (mono-, di-, tri-, etc.), pyran-oxonium saccharides (mono-, di-, tri-, etc.) and ring ruptures are observed. No stereochemically-related influence on the spectra has been reported.

As previously observed for FD, SIMS and FAB, addition of salts shifts both molecular and sequence-informative fragments by the mass difference of the added cation. Salt addition to carbohydrate samples is usually unnecessary for obtaining good LD mass spectra because sufficient quantities

are often present in the sample or on the support surface (144). However, Na^+ doping of ethanol solutions of carbohydrates has been reported to improve the sensitivity for their analysis under LD conditions (150, 151). Other matrix effects may also have a significant contribution to LD mass spectra, but experience with biomolecules is still very limited. Few glycoconjugates have as yet been analysed by LD, but preliminary data for some steroidal glycosides demonstrate that excellent results can be obtained using this technique (144).

A relatively new method that shows promise for the analysis of biomolecules is that of plasma desorption (PD) (e.g., from californium-252) (152-156). However, no commercial instrumentation is available yet and too few applications to carbohydrate structural problems have appeared to allow for a good appraisal of the technique and to warrant any greater coverage of the technique.

Another method, not technically related, that shows promise and that has been used more extensively than PD in oligosaccharides analysis, although still not enough to be fully evaluated, is that of Flash Desorption (157-159). Recent papers by Daves and co-workers (158, 159) have

clearly shown that this technique has some value. It appears unlikely, however, that this will develop into a standard analytical method.

E. SIMS and FAB

The SIMS and FAB mass spectra of oligosaccharides are remarkably similar in appearance to each other and to the corresponding FD spectra. During SIMS operation ions are formed from the sample and the metal substrate and from ion/molecule reactions between the two (160). For example, in the SIMS spectrum of stachyose deposited on a silver target, Ag^+ , (fragment + Ag) $^+$, and $(\text{M} + \text{Ag})^+$ ions were observed (27, 161). Because silver has two nearly equally abundant stable isotopes (m/z 107 = 51.83%, m/z 109 = 48.17%) the argentated ions form readily recognizable doublets. Ions arising from the sample support are not usually evident in FAB mass spectra unless a portion of the target becomes directly exposed to the primary beam as a result of glycerol depletion.

Cationization also occurs with alkali metal ions present in the sample or as impurities on the substrate

surface. In SIMS such ions are usually very abundant, whereas in FAB $(M + H)^+$ and $(\text{fragment} + H)^+$ may dominate because glycerol is a ready source of protons. As observed in FD, doping with salts increases the contribution of cation-containing species in both the FAB and SIMS spectra. However, preliminary evidence indicates that high concentrations of alkali cations decrease the relative abundance of fragment ions in the FAB spectra of many compounds, including carbohydrates, and therefore should be avoided (105, 162).

The nature of the sample matrix also has a decided effect in FAB. For example, glycerol gives rise to a characteristic spectrum of its own which consists chiefly of cationized polymers of the type $((\text{glycerol})_x + H)^+$ (19, 158). In addition, ions are observed at every nominal mass, which presumably arise from fragmentation of glycerol and its polymers at or near the points of primary beam impact. Carbohydrates, like glycerol, exhibit peaks of lower relative height 2 daltons below fragment, molecular, and adduct ions. These ions are also present in the SIMS mass spectra of carbohydrates (163), and may arise from oxidation occurring in the gaseous high-pressure region directly above the probe surface. An earlier report has suggested that

improved results can be obtained for oligosaccharides when 3-mercapto-1,2-propanediol (thioglycerol, MW = 108) is used as the liquid matrix (126) because the contribution of matrix-related ions at higher masses is substantially lower and the intensity of sample-related ions is often greater (two- to fivefold) than observed with glycerol. A hidden trap not recognized by these authors will be discussed later. Furthermore, the greater volatility of thioglycerol reduces the effective observation time to minutes, although cooling of the sample may obviate this problem.

A review of empirical results to late 1982 is available (126), unfortunately, it does not present the data in a way that leads to any interpretative conclusions. The coverage of SIMS and FAB in this section is limited and is discussed in detail under section 2.5.

Although complex oligosaccharides of biological origin have only just begun to be examined by FAB and SIMS, preliminary data suggest that these techniques will have a major impact on carbohydrate mass spectrometry. A partial negative ion FAB mass spectrum of gamma-cyclodextrin (cyclooctylamylose) (5) shows a prominent $(M - H)^-$ along with a fragment ion at m/z 1149 due to excision of a

dideoxyglycoside unit (164). The positive ion FAB mass spectrum of beta-cyclodextrin (cycloheptylamylose) (6) is surprisingly rich in structural detail (105). Abundant sequence ions of the type $((\text{O-hexose})_n + \text{H})^+$ (where $n = 1-6$) are present; the smaller members of this homologous ion series carry a larger percentage of the ion current. The molecular region of the spectrum shows a prominent $(\text{M} + \text{H})^+$ as well as $(\text{M} + \text{H} + \text{glycerol})^+$ and $(\text{M} + \text{H} + (\text{glycerol})_2)^+$ ions.

Other preliminary data indicate that FAB could be useful in obtaining the molecular weights of complex oligosaccharides such as the oligomannosides found in the urine of patients suffering from glycoproteinoses or glycolipidoses (165). In preliminary work the FAB mass spectrum of the branched decasaccharide $(\text{Man})_9\text{Glc-NAc}$ exhibited $(\text{M} + \text{Na})^+$, $(\text{M} + \text{H})^+$, and $(\text{M} + \text{H} - \text{H}_2\text{O})^+$ peaks (at m/z 1702, 1680, and 1662, respectively) with good signal-to-noise ratio after 0.1M HCl had been added to the sample in glycerol. Unfortunately, sequence-informative fragments were of negligible abundance. Both FAB and SIMS have also shown considerable promise for the analysis of glycosides (166-168).

2.2.2 Stereochemical Considerations in Mass Spectrometry

Mass spectrometry has become one of the organic chemist's most powerful tools for structure determination. The basic ionization form (EI) involves the bombardment of gas-phase molecules by an electron beam which is energetic enough to ionize and induce a series of unimolecular fragmentations. Despite the excess energy imparted by this electron beam, several structural features of the neutral molecules are often retained in the decomposing molecular ions, thus enabling deduction of some structural information from the fragmentation pattern.

In early stages of mass spectrometry it was shown that some stereochemical details could be retained in certain systems after electron impact ionization (169-172). Despite these early reports, statements repeatedly appeared in the literature to the effect that mass spectrometry was insensitive to configurational differences. Four reviews, which appeared in 1968 (173), 1976 (174), 1977 (175) and 1983 (176) attracted more interest to the potential of this technique in stereochemical studies.

Selected topics connected with stereochemical studies in mass spectrometry, such as heterocyclic compounds (177), hydrocarbons (178), ionization and appearance energy correlations (179) and the connection of electron impact phenomena with free radical chemistry (180), have been the subject of recent reviews.

From the previous references, it can be seen that to date, there exists no unified theory to explain stereochemically related effects encountered in mass spectrometry. It is possible however, to view these as being the result of two main considerations: thermochemical and kinetic correlations (176).

Under the heading of thermochemical correlations we find discussion topics such as: correlation between the molecular ion-radical ($M^{\cdot+}$) and its configuration, the inverse correlation of abundance of $(M - R)^+$ ions with enthalpy (and its direct counterpart), and its ionization and appearance energy correlations (181-200). Unfortunately these data are not always accessible for biologically important molecules such as oligosaccharides thus reducing greatly their analytical usefulness.

Under the heading "Kinetic Correlations", however, we

can find terms and topics much more common to the practicing organic chemist. In fact, several systems show significant differences in the abundance of some specific fragment ions found in the mass spectra of stereoisomers and these cannot be explained in terms of thermochemical considerations. For example the mass spectra of several cis-diones exhibit retro-Diels-Alder (RDA) fragment ions as their most abundant ions (201). These ions are not found in the mass spectra of the corresponding trans-isomers. In the same vein, molecular ions are most abundant in the trans, but of much lower abundance in the cis. "The relatively small difference in the enthalpy of formation of these isomers cannot account for their extremely different fragmentation behavior under electron impact" (176) (e.g. see figure 2.7a). Another example is that of the methyl esters of cyclohexane-1,3-dicarboxylic acids (figure 2.7b). The cis diester loses a $\text{CH}_3\text{O}^\bullet$ radical from its molecular ion, while the trans isomer mainly eliminates methanol (202). Here again the great difference in the fragmentation pattern cannot be explained by relative stability arguments.

In these and in many other systems, kinetic considerations provide a way to explain stereospecific fragmentation processes detected by mass spectrometry. It is

Figure 2.7: Examples of kinetically-controlled stereochemical effects on fragmentation pathways in mass spectrometry.

expected that there should be a difference between stereoisomers in the accessibility of transition states for fragmentation processes which involve bond formation (e.g. rearrangements or elimination of neutral molecules) or the concerted rupture of several bonds. This situation leads to different energies of activation of specific fragmentations for particular stereoisomers, resulting in different fragmentation patterns. Difference in the magnitude of frequency factors operating in certain fragmentation processes of stereoisomers may also lead to different mass spectra. At least in one case this effect could be isolated (203), but it may be of importance in many systems. This finding shows the importance of structural features of the transition states in the fragmentation of gas-phase ions, a fact that has been neglected in some theoretical treatments. The accessibility of cyclic transition states based on the configuration of the stereoisomers in the ground state is of great value in the prediction of stereospecificity of fragmentation processes involving a transfer of specific atoms or groups. This is believed to be true for the dicarboxylates presented in figure 2.7b, where the cyclic transition state shown is available only for the trans isomer. Such anchimeric assistance has been shown to operate in certain systems, enhancing some fragmentation

processes in stereoisomers having suitable configurational features (204-213).

Electrocyclic reactions such as RDA (e.g. figure 2.7a) can also be explained in terms of kinetic correlations through the arguments that RDA must happen via a one step mechanism; it is really the retro-replica (in the gas phase) of the actual Diels-Alder reaction performed in solution (214-218). Fragmentation patterns of other systems, such as steroidal skeletons, have also been shown to be influenced by the stereochemistry of the molecular ion-radical (219-221).

One of the early reported cases of different mass spectra of stereoisomers was that of deacylcyclindrocarpol 7a and its epimer at C-19, 7b (222, 223). The much more pronounced loss of a hydrogen atom from the molecular ion of 7b ($(M - H)^+$, 10.6 versus 1.7% for 7a) was attributed to the assistance of the anti electron pair on the adjacent nitrogen atom. Deuterium labeling at C-19 showed that the hydrogen was indeed lost from that position (222). A similar argument was used to explain the difference between the abundance of $(M - H)^+$ ions of epimeric quinolizines (224).

7a 7b

The relatively easy elimination of axial anomeric groups in glycosides may also be a demonstration of a similar stereoelectronic effect (63, 225).

An interesting case is provided by the mass spectra of the epimeric 3-dimethylamino-3-methylcholestanes 8 (225). The molecular ion of 8a loses a methyl radical (to give ion E) to a greater extent than its epimer 8b (100 and 37%, respectively), but gives rise to lower abundances of ions F and G whose formation is initiated by ring cleavage at bonds 2-3 and 3-4 (see figure 2.8). These results suggest that the conformation of the amino group plays a role in the fragmentation, so that the activation energy for breaking a given C-C bond adjacent to the nitrogen is lowered when the n orbital of the nitrogen atom is antiparallel to that bond. The populations of the three staggered conformations of 8a (axial dimethylamino group) are presumably unequal, and the highly populated one favors elimination of the alpha-methyl radical. An equal abundance is expected for the staggered conformations of the isomeric 8b, and consequently no preference is predicted for the loss of $\text{CH}_3\cdot$ over the rupture of the other alpha-bonds (226).

Figure 2.8: Stereoelectronically-controlled fragmentation pattern for epimeric 3-dimethylamino-3-methyl-cholestane.

This stereoelectronic effect may explain other cases of different mass spectra of stereoisomers. The more pronounced loss of axial versus equatorial methyl groups in heteroatom-containing decalin systems, which has been attributed to stability factors (191-195), could also be interpreted in terms of a stereoelectronic effect, namely, lower energy of activation in isomers in which the n orbital of the heteroatom is antiparallel to the bond to be broken.

The most frequently reported stereospecific fragmentations are those involving new bond formation via cyclic transition states. These processes include the elimination of neutral HX molecules ($X = OH, RO, RCOO, Cl, R$) and various more complicated fragmentations which involve hydrogen migrations. The elimination of neutral HX has been covered extensively in reviews (173-175). Figure 2.7b shows a related case. A common feature of these processes is the proximity of the migrating hydrogen to its destination in the parent molecule. This simple relationship between structure and stereospecificity in these elimination processes gives them some predictability (174, 175, 227-230). On the other hand, it should be emphasized that a necessary condition for stereospecificity is that the stereoisomeric molecular ions do not undergo isomerization;

to a common structure prior to fragmentation; such isomerizations are difficult to predict.

The first case of a pronounced effect of configuration on the behavior of stereoisomers under chemical ionization (CI) conditions apparently was reported in 1972 (231, 232). 3-Oxo-5alpha-steroids give rise to most abundant $(M + H)^+$ ions, while the most prominent peaks in the CI mass spectra of the 5-beta isomers are due to the elimination of water: $(M + H - H_2O)^+$, 100%; $(M + H - 2H_2O)^+$, 90%. This large difference between the stereoisomers was observed with H_2 as the reagent gas, but it was much smaller when a less energetic gas, e.g. CH_4 , was used. The greater extent of the H_2O elimination in the cis-A/B isomers was explained by the short distance between the C-3 oxygen and the C-9 hydrogen atoms (203). A similar explanation was suggested for the abundant $(M + H - H_2O)^+$ ions detected in the CI (isobutane) mass spectra of some unsaturated endo-bicyclic ketones but not in their exo-isomers (233, 234). An allylic hydrogen atom is accessible to the carbonyl oxygen only in the endo ketones, resulting in an efficient loss of water from these isomers (233, 234) (see structures 9 and 10 for example).

An interesting general feature was discovered in 1974 in the CI mass spectra of stereoisomeric cyclic 1,3- and 1,4-diols (235). The abundance of the $(M + H)^+$ ions is often significantly higher for cis than for trans diols, while the trans isomers yield relatively more abundant $(M - H)^+$ and $(M + H - H_2O)^+$ ions. This behavior is explained by the stabilization of the $(M + H)^+$ ions by an intramolecular proton bridging which is possible only in the cis diols (235) (see structures 11 for an example of a pair of cyclic 1,3-diols). This effect was found in various alicyclic and acyclic diols (236-241), and in other difunctional compounds (242-250). Configurational assignments were made in some substituted bicyclo (3.2.0) heptanes using this relationship (251, 252). Intramolecular ion solvation effects (253) and negative-CI characteristics (254) were also investigated.

Chemical ionization mass spectrometry is a powerful technique for conformational analysis of 2-amino alcohols (255, 256). The extent of dehydration under CI-isobutane conditions in several amino alcohols with fixed conformation is dependent on the distance between the functional groups: It is greatest for maximal distance (dihedral angle 180 degrees), and practically disappears in compounds with the dihedral angle smaller than 90 degrees.

This effect is interpreted in terms of intramolecular proton transfer in the $(M + H)^+$ ions (255). It is claimed that the dehydration peak $(M + H - H_2O)^+$ in the mass spectrum represents the $(M + H)^+$ ions decomposed after protonation of the hydroxyl moiety, and the $(M + H)^+$ peak represents ions protonated on the amino group.

Fragmenting ions obtained by softer ionization techniques, such as field ionization or field desorption (257-261), alkali atom attachment (262), negative ionization (263) and negative chemical ionization (264), in some cases may also exhibit a stereospecific behavior. Stereochemical effects have also been reported in some systems for the slow decomposition of metastable ions, both in the relative abundances of the relevant ions (186, 213, 265-273) and in the magnitude of the kinetic energy released in these metastable ion transitions (188, 273, 274). The retention of configuration in nondecomposing ions was demonstrated in several cases by different collisional activation (CA) spectra of stereoisomers (275-277). Stereospecificity in these systems serves as a reliable probe for the retention of configuration in the ions and as an indication of the mechanistic details of the fragmentation processes. An important outcome of the investigations described above is

the fact that mass spectrometry has become an additional technique for configurational studies for the organic chemist. The high sensitivity of mass spectrometry and the possibility of its application in the investigation of complex mixtures by combination with various separation methods assure an extensive use of this technique in future stereochemical studies.

Few applications to carbohydrates have been made as yet in terms of stereochemical effects, although several intriguing (if not controversial) reports have appeared that discuss the sensitivity of FD mass spectra to differences in the anomeric configuration at carbon-1 of monosaccharides (278, 279). A limited number of aromatic glycoside monosaccharides were studied by FDMS and apparently reproducible differences in relative ion abundance were noted, especially between the alpha- and beta-linked anomers of p-nitrophenyl glycosides. However, the data regarding actual absolute ion count values were not presented (equivalent sensitivity is expected for both anomers) thus leaving the doubt that the differences seen might be related to the (undescribed) normalization procedure used by those authors. There has been no work reported on similar studies involving glycosides with longer sugar chains, although it

has been shown that under FD conditions, protonation occurs at the glycosidic bond (140) thus enabling the glycoside to undergo hydrolysis, which in turn is stereoelectronically-controlled (63, 225).

Another promising field is the investigation of stereochemistry of gas-phase ion-molecule reactions. Steric effects have been reported in several cases (280-288). An interesting effect has been observed in the efficiency of formation of attachment ions $(2M + H)^+$ of optical isomers of dialkyl tartrates by chemical ionization. The abundance of attachment ions consisting of two enantiomeric molecules ("meso ions") is lower than that of the $(2M + H)^+$ ions in which both molecules have the same configuration (289). This was the first report of detection of chirality by mass spectrometry (289).

2.3 SCOPE OF THIS WORK

The work described herein deals mainly with experiments aimed at evaluating FAB as a stereochemical probe. As it was shown in the previous section (section

2.2), no definitive methods or rules are available in mass spectrometry for such analysis although several reports indicate that it should be possible to make such use of this physical method. This might be because almost no data are available on FAB (relative to other techniques).

In order to follow a systematic approach for these investigations, it is necessary to concentrate our efforts on one family of molecules. Our choice is centered on oligosaccharides, with the intention of probing the glycosidic bond. In order to achieve this goal the following experimental design was followed:

- a) Analysis of the FAB spectra of a series of oligosaccharides to determine whether or not some stereochemically dependent characteristics are exhibited.
- b) In order to ensure that logical conclusions can be drawn, efforts will be concentrated on model compounds at first. Ideally, the latter should not have too high a molecular weight so as to allow for ready interpretation of the spectra where the features will be clearly related to the appropriate phenomena. Pairs (alpha and beta) of disaccharides and glycoside monosaccharides meet that criterion, as they possess only one anomeric carbon, they are readily available, and well characterized. They also offer the possibility of looking at other stereochemical considerations at once (e.g., stereochemistry at C-4 for glycopyranoses) and allow for a direct evaluation of the effect of the substituent at the glycosidic bond (aglycone versus carbohydrate moiety) on the spectral characteristics.

- c) Interpretative efforts will bear mainly on positive ions' data recorded in (on) a glycerol support-matrix. All compounds will also be analysed in the negative ion mode and some with other support matrices (with or without doping) to allow for a preliminary insight into the solvent, matrix, and charge effects.
- d) The expected variations in the data will be discussed in terms of the stereochemistry of the glycosides used and correlated to existing stereoelectronic concepts. This could lead to a discussion on the effects glycerol has on the type of compounds studied (and on those being used as a dispersive/dissolution medium).
- e) Analysis of some higher oligosaccharides, derivatives and saponins are described so as to provide for other insights into the universality, or not, of the phenomena encountered during the disaccharide/glycoside monosaccharide analysis, and on the eventual use of the technique in oligo- and polysaccharide sequencing. At this early stage, however, the evaluation of the sequencing possibilities of the technique will be second to the need to establish rules for FAB analysis with respect to stereochemistry whether these rules prove to be totally novel in nature or whether they turn out to be direct extensions to the stereochemical concepts already well established for the glycosidic bond in solutions.

2.4 EXPERIMENTAL

All compounds used in the present work (reagents, solvents, carbohydrates, gases, etc.) were obtained commercially with the exception of the following compounds:

- 1) 1-O-beta-D-mannopyranosyl-L-erythritol
- 2) 3-O-beta-D-galactopyranosyl-D-arabinopyranoside
- 3) 1-O-phenyl-4-O-beta-D-galactopyranosyl-D-glucopyranoside
- 4) 0-alpha-D-glucopyranosyl-(1→3)-0-beta-D-fructofuranosyl-(2→1)-alpha-D-glucopyranoside (melezitose)
- 5) 0-beta-D-glucopyranosyl-(1→3)-0-beta-D-glucopyranosyl-(1→4)-D-glucopyranoside
- 6) 4-O-beta-D-glucopyranosyl-D-glucopyranoside octaacetate (cellobiose octaacetate)
- 7) 4-O-alpha-D-glucopyranosyl-D-glucopyranoside octaacetate (maltose octaacetate)

which were obtained from Prof. A.S. Perlin (McGill University) and of psoluthurin A (290, 291) and frondoside A (292, 293) which have been isolated from marine sources in our laboratories.

Conventional FAB spectra were recorded at room temperature using Xenon as neutral atom source on a VG-7070E equipped with a FAB source and a VG data system, whereas linked-scan experiments on FAB spectra were recorded on a Finnigan MAT312 mounted with a saddlefield fast atom gun and controlled by a Finnigan INCOS data system (scan range 100-1300, scan time 10s). Both instruments were using an Ion Tech

power source and were operating at 8kV (atom gun) and at a Xenon pressure of 1.5×10^{-5} mbar. Accelerating voltage was standard (3kV). Linked scan spectra were recorded from the spontaneous unimolecular decomposition processes and in no cases were they collision-induced. They were recorded in the manual mode at room temperature at a scan rate of 11 and a chart speed of 2 mm/s (10 mV scale).

2.5 RESULTS AND DISCUSSION

Throughout the text of this thesis use will be made of the common names given to the various oligosaccharides under study so as to limit space and for a greater ease of reading. A list of proper names follows however, along with the respective common name in bracket, in order to establish the nomenclature and to avoid any confusion.

Monosaccharide glycosides:

1-O-p-nitrophenyl-alpha-D-galactopyranoside, (alpha-p-nitrophenyl-galactose);

1-O-p-nitrophenyl-beta-D-galactopyranoside, (beta-p-nitrophenylgalactose);

1-O-methyl-alpha-D-glucopyranoside, (alpha-methyl-glucose);

1-O-methyl-beta-D-glucopyranoside, (beta-methyl-glucose);

1-O-p-nitrophenyl-alpha-D-glucopyranoside, (alpha-p-nitro-phenyl-glucose);

1-O-p-nitrophenyl-beta-D-glucopyranoside, (beta-p-nitrophenyl-glucose);

1-O-phenyl-alpha-D-glucopyranoside, (alpha-phenyl-glucose);

1-O-phenyl-beta-D-glucopyranoside, (beta-phenyl-glucose);

Disaccharides:

3-O-beta-D-galactopyranosyl-D-arabinopyranoside

1-O-beta-D-mannopyranosyl-L-erythritol

4-O-beta-D-galactopyranosyl-D-glucopyranoside, (lactose)

6-O-alpha-D-galactopyranosyl-D-glucopyranoside, (melibiose)

1-O-alpha-D-glucopyranosyl-beta-D-fructofuranoside, (sucrose)

3-O-alpha-D-glucopyranosyl-D-fructopyranoside, (turanose)

1 -O- alpha -D- glucopyranosyl - alpha -D- glucopyranoside,
(trehalose)

4-O-alpha-D-glucopyranosyl-D-glucopyranoside, (maltose)

4-O-beta-D-glucopyranosyl-D-glucopyranoside, (cellobiose)

6-O-beta-D-glucopyranosyl-D-glucopyranoside, (gentiobiose).

Disaccharide derivatives and glycosides:

4-O-alpha-D-glucopyranosyl-D-glucopyranoside-1,2,2',3,3',4',
6,6'-octaacetate, (maltose octaacetate)

4-O-beta-D-glucopyranosyl-D-glucopyranoside- 1,2,2',3,3',4',
6,6'-octaacetate, (cellobiose octaacetate).

1-O-alpha-D-glucopyranosyl-alpha-D-fructofuranoside- 1,2',3,
3',4,4',6,6'-octaacetate, (sucrose octaacetate)

1-O-beta-phenyl-(4-O-beta-D-galactopyranosyl) -D- glucopyra-
noside, (beta-phenyl-lactose).

Higher oligosaccharides :

1-O-alpha-D-galactopyranosyl-(1-6)-O-alpha-D-glucopyranosyl-
(1-2)-beta-D-fructofuranoside, (raffinose).

1-O-alpha-D-glucopyranosyl-(1-3)-1-O-beta-D-fructofuranosyl
-(2-1)-alpha-D-glucopyranoside, (melezitose)

1-O-beta-D-glucopyranosyl-(1→3)-beta-D-glucopyranosyl-(1→4)-D-glucopyranoside

1-O-alpha-D-galactopyranosyl-(1→6)-alpha-D-galactopyranosyl-(1→6)-1-alpha-D-glucopyranosyl-(1→2)-beta-D-fructofuranoside, (stachyose)

1-O-beta-(delta 20:22-3beta-O-12beta, 14,21-tetrahydroxynorcholenic acid lactone)-1-O-beta-D-2,6-dideoxyribopyranosyl-(1→4)-beta-D-2,6-dideoxyribopyranosyl-(1→4)-D-2,6-dideoxyribopyranoside, (digoxin)

1-O-beta-(delta 20:22-3beta-O,14,21-trihydroxynorcholenic acid lactone)-1-O-beta-D-2,6-dideoxyribopyranosyl-(1→4)-beta-D-2,6-dideoxyribopyranosyl-(1→4)-D-2,6-dideoxyribopyranoside, (digitoxin)

1-O-beta-(delta 20:22-3-O,14,16,21-tetrahydroxynorcholenic acid lactone)-1-O-beta-D-2,6-dideoxyribopyranosyl-(1→4)-beta-D-2,6-dideoxyribopyranosyl-(1→4)-D-2,6-dideoxyribopyranoside, (gitoxin)

1-O-beta-(delta 20:22-3-O,14,21-trihydroxy-16-formyl norcholenic acid lactone)-1-O-beta-D-dideoxyribopyranosyl-(1→4)-beta-D-2,6-dideoxyribopyranosyl-(1→4)-D-2,6-dideoxyribopyranoside, (gitaloxin)

1-O-beta-D-(5alpha,22a,25a-spirostane-2alpha,3beta O,15beta-triol)-1-O-beta-D-xylopyranosyl-(1→3)-beta-D-glucopyranosyl-(1-O-beta-D-glucopyranosyl-(1→3)-beta-D-galactopyranosyl-(1→2))-1-4)-D-galactopyranoside, (digitonin)

1-O-beta-(3beta-O-16-oxoholosta-delta 9:11, 25-diene)-3-O-methyl-beta-D-glucopyranosyl-(1→4)-beta-D-quinovopyranosyl-(1→3)-beta-D-glucopyranosyl-(1→2)-D-xylopyranoside-6'-O-6'''-O-disodium disulfonate, (psoluthurin A)

1-O-beta-(3beta-O-23-acetoxystrostan-7-ene)-3-O-methyl-beta-D-glucopyranosyl-(1→4)-beta-D-xylopyranosyl-(1→3)-beta-D-

quinovopyranosyl-(1→3)-beta-D-xylopyranosyl-(1→2)-D-xylopyranoside-4-O-sodium sulfonate, (frondoside A).

The complete FAB-mass spectra in glycerol for all of these compounds, both in positive and negative modes can be found in appendix at the end of this thesis along with a selection of spectra recorded in other solvents; selected FAB data are presented however under separate headings so as to accent the main features of the spectra.

2.5.1. Monosaccharide Glycosides.

Tables 2.1 and 2.2 give some selected FAB data for anomeric pairs of monosaccharide glycosides in the positive and negative mode respectively. We note differences in the mass spectral data recorded within a given anomeric pair and this is true for every pair studied.

The FAB mass spectra of the methyl glucose pair (Appendices A, B, I and J) have features that can be interpreted in terms of their stereochemical properties. Beta-methyl glucose, for example, exhibits a much larger

D-Xylose D-Quinovose D-Ribose D-Glucose D-Mannose D-Galactose D-Arabinose D-Fructose

Monosaccharide units present in the polysaccharides used in this study.

TABLE 2.1a

SELECTED FAB-MS DATA (%I) OF SOME MONOSACCHARIDE GLYCOSIDES*

1-O-METHYL-D-GLUCOSE

m/z	ION	alpha	beta
127	$(163 - 2(H_2O))^+$	42.0	39.3
145	$(163 - H_2O)^+$	88.7	92.7
163	$(M + H - CH_3OH)^+$	86.7	100.0
183	?	25.1	13.7
193	$(M - H)^+$	14.0	17.4
194	$(M)^+$ (isotopic for 193)	2.7	-
195	$(M + H)^+$	18.7	46.5
217	$(M + Na)^+$	4.7	14.3
255	$(M + gly + H - CH_3OH)^+$	88.0	64.3
287	$(M + Gly + H)^+$	100.0	50.4
325	$(2M + H - 2(CH_3OH))^+$	17.7	21.1
357	$(2M + H - CH_3OH)^+$	11.5	11.8
377		5.4	1.1
379		6.1	-
389	$(2M + H)^+$	35.4	30.8
481	$(2M + Gly + H)^+$	3.2	-

* positive ions in glycerol; corrected for matrix background
 - not detected

TABLE 2.1b

1-O-phenyl-D-GLUCOSE

m/z	ION	alpha (H ₂ O)	beta
127	(163 - 2 H ₂ O) ⁺	36.5	26.6
145	(163 - H ₂ O) ⁺	81.9	64.7
149	X	25.8	36.1
151	Y	19.2	28.3
163	Z	67.7	51.2
255	(M - H) ⁺ and (163 + gly) ⁺	100.0	100.0
256	isobaric of 255	15.8	11.8
257	(M + H) ⁺	3.8	5.8
325	(2M + H - 2 (C ₆ H ₅ OH)) ⁺	20.0	10.7
349	(M + gly + H) ⁺	50.8	43.1
441	(M + 2 gly + H) ⁺	7.3	14.7
513		11.5	4.9
533	(M + 3 gly + H) ⁺	-	3.2
605		-	3.2

* positive ions in glycerol; corrected for matrix background
 - not detected

(H₂O): indicates spectra recorded in a glycerol/water mixture

TABLE 2.1c

1-O-p-NITROPHENYL-D- GALACTOSE GLUCOSE					
m/z	ION	alpha	beta	alpha (H ₂ O)	beta
123		100.0	35.3	92.4	63.3
124		77.8	23.5	65.5	56.1
127	(163 - 2 H ₂ O) ⁺	20.4	-	23.5	15.3
139		-	-	5.9	5.1
140		61.1	23.5	56.3	29.6
145	(163 - H ₂ O) ⁺	44.4	-	63.9	34.7
149	X	72.2	100.0	100.0	100.0
151	Y	61.1	70.6	79.8	90.8
163	Z	94.4	32.4	37.8	21.4
209		57.4	82.4	55.5	68.4
215	(123 + gly) ⁺	-	-	22.7	20.4
255	(163 + gly) ⁺	51.9	26.5	38.7	26.5
285		25.9	-	27.7	13.3
301	(M) ⁺	13.0	20.6	14.3	16.3
302	(M + H) ⁺	16.7	-	19.3	9.2
394	(M + gly + H) ⁺	55.6	44.1	36.1	18.4
486	(M + 2 gly + H) ⁺	13.0	-	-	10.2

* positive ions in glycerol; corrected for matrix background
 - not detected

For structures X, Y and Z refer to the page 145.

(H₂O): indicates spectra recorded in a glycerol/water mixture

TABLE 2.2a

SELECTED FAB-MS DATA (%I) OF SOME MONOSACCHARIDE GLYCOSIDES*

1-O-METHYL-D-GLUCOSE

m/z	ION	alpha	beta
194	$(M)^-$	100.0	100.0
254	$(M + \text{gly} - \text{CH}_3\text{OH})^-$	4.7	3.1
284	$(M - \text{gly} - \text{H}_2)^-$	8.3	5.9
286	$(M + \text{gly})^-$	19.6	8.7
387	$(2M - H)^-$	0.5	13.6
388	$(2M)^-$	10.8	2.3

* negative ions in glycerol; corrected for matrix background

TABLE 2.2b

1-O-phenyl-D-GLUCOSE

m/z	ION	alpha (H ₂ O)	beta
120		42.1	20.5
162		19.6	94.7
256	(M)	100.0	100.0
345	(M + gly - H - H ₂) ⁻	8.2	10.2
347	(M + gly - H) ⁻	37.8	48.1
438	(M + 2gly - H ₂) ⁻	3.2	6.2
509	(2M + gly - H - phenol) ⁻	22.1	19.6

*negative ions in glycerol; corrected for matrix background
(H₂O): indicates spectra recorded in glycerol/water mixture

TABLE 2.2c

1-O-p-NITROPHENYL-D- GALACTOSEGLUCOSE					
m/z	ION	alpha	beta	alpha (H ₂ O)	beta

139	(p-nitrophenol) ⁻	100.0	100.0	100.0	100.0
151		2.8	13.1	7.0	7.5
152		5.2	20.1	10.7	12.7
165		3.2	14.7	8.0	8.4
166		2.2	13.4	6.1	8.1
181		7.9	9.9	13.0	5.7
182		8.5	41.2	20.7	27.4
223		5.7	-	4.0	-
300	(M - H) ⁻	7.1	9.9	5.6	3.8
301	(M) ⁻	10.0	12.1	11.8	11.6
392	(M - H + gly) ⁻	3.3	6.4	6.1	6.1
393	(M + gly) ⁻	2.8	4.8	3.3	4.3
598	(2M - 2H - 2H) ⁻	1.7	-	-	-
599	(2M - H - 2H) ⁻	2.5	-	-	-
-----					

* negative ions in glycerol; corrected for matrix background
 - not detected
 (H₂O): indicates spectra recorded in a glycerol/water mixture

proton affinity than the alpha anomer yielding, in the positive ion mode (Appendices A and B), a much more intense peak at m/z 195 (the pseudomolecular ion).

Consequently the peaks at m/z 163, the pyranoxonium ion (ring oxygen) (see structure 2, table 2.1) resulting from the loss of methanol (from m/z 195) (294), m/z 145 and m/z 127, the sequential losses of two water molecules from m/z 163, are also more prominent for the beta-anomer. An even more intriguing and promising feature is the very intense peak at m/z 287 for the alpha anomer that is present to a much lesser extent in the beta counterpart. That particular peak corresponds to the cluster of one protonated glycoside molecule attached to a glycerol molecule. The reason for such a discrepancy is not obvious. It is proposed here that it is stereochemically-related. It has been shown that beta methyl glucose exists in a single rotameric state (depicted as 12a) because both steric and electronic effects favor such a conformer (295). In fact this can be explained in terms of steric factors, assuming that the lone pairs are considered bigger or nearly equal in size to the hydrogens (a matter of controversy (296, 297)) and, for this system, of the more important electronic component, the exo-anomeric effect (295, 298-302). That favored conformer, (12a),

12a 12b

seriously restricts the binding of glycerol to the sugar unit through the ring oxygen and those at C1 and C6 because the lone pairs of the oxygen are not both in the proper conformation. Rotamer 12b could allow for better glycerol binding but it has been shown not to exist, or to be present only to limited extent. This phenomenon occurs only in beta-glycosides, thus alpha-methyl glucose exists as several rotamers (around the glycosidic bond) some of which must favor the binding to the glycerol moiety. Molecular models show that no rotamer for any alpha-glycoside favors binding through the glycosidic oxygen, which is necessary to account for the low production of ions at m/z 163 and large production of ions at m/z 287. However, if the glycosidic oxygen is protonated and the glycerol unit attached via that same hydrogen, it yields an aggregate species that accounts for both, the intense peak at m/z 287 (binding to glycerol via a proton through the oxygens at C1, C2 and C3) and the reduced peak, with respect to the beta-anomer, at m/z 163. All of these findings are suggestive of the fact that protonation must occur at the glycosidic oxygen which is in agreement with a previous report based on FD studies (140).

The negative ion-FAB spectra of the methyl glucose glycosidic pair (Appendices I and J) show complementary features; the glycerol adduct at m/z 286 being again much greater in the alpha-anomer. Another peculiar difference resides in the ions $(2M - H)^-$ and $(2M)^-$, at m/z 387 and 388 respectively. Alpha-methyl glucose exhibits a strong peak at m/z 388 and a weak one at m/z 387, whereas its beta counterpart shows a strong ion at m/z 387 and a weak one at m/z 388 (table 2.2).

The positive ion FAB mass spectra for the phenyl glucose pairs (Table 2.1 and Appendices C and D) do not show substantial differences but for the various protonated molecular species-glycerol adducts at m/z 349, 441 and 533 whose intensities vary between the anomers (greater for beta) and for an unusual peak at m/z 325 that is tentatively defined as $(2M + H - 2(C_6H_5OH))^+$ by comparison to the disaccharide analysis reported later in this thesis. A very interesting feature however is the peak at 255 that can be attributed to two ions, $(M - H)^+$ and $(163 + Glycerol)^+$. The former can be the result of either the abstraction of a hydrogen radical or the oxidation (loss of H_2) of the normally expected $(M + H)^+$ species at m/z 257 which is only weakly present. Although no high resolution mass

measurements were performed on this ion it is suggested here that m/z 255 corresponds to the $(M - H)^+$ ion. This statement is based upon the complementary information obtained from the negative ion spectra (Table 2.2 and Appendices K and L) where the molecular anion (or radical?) is recorded (m/z 256) instead of the standard $(M - H)^-$ ion. Furthermore, we have performed a B^2/E linked-scan (34) on a similar $(M - H)^+$ peak found in ergosterol (303) and thus established that the origin of the peak was not $(M + \text{glycerol} + H)^+$ necessary to produce $(163 + \text{Gly})^+$ (for details on linked-scan experiments refer to the following chapter). The two anomers present only one difference in their negative ion FAB spectra, namely a reversal in the intensities of the ions at m/z 120 and 162 (see Table 2.2). This phenomenon can also be accounted for in terms of stereochemical effects. For this pair, steric hindrance is seen as the most important factor affecting the stability of the $(M)^-$ ion. Clearly the rotation of the phenyl moiety around the glycosidic bond and around the $O-C1'$ bond is more difficult for the beta-anomer. Charging the phenyl ring with an extra negative charge increases further its molecular geometry and imposes a stress sufficient to yield an intense peak at m/z 162 (see Table 2.2) (kinetic control) thus reducing the probability of producing an ion m/z 120

which must involve a fission of the carbohydrate ring. The alpha anomer on the other hand does not suffer to the same extent from such an enhanced steric factor since for some rotamers the phenyl ring does not overlap with the carbohydrate ring. Formations of m/z 120 (thermodynamic control) is therefore not inhibited, although not necessarily favoured either.

Tables 2.1 and 2.2 also present the data for the para-nitrophenyl galactose (Appendices E and F for positive ions, M and N for negative ions) and the para-nitrophenyl glucose pairs (Appendices G and H for positive ions, O and P for negative ions). The addition of a nitro group to the phenyl ring did not only enlarge it but also enhanced the overall dipole moment exerted by the aglycone on the carbohydrate residue. This results in a much more extensive overall fragmentation. This series also allows for a first glance at the effect of changing the stereochemistry at C4 of the carbohydrate moiety. Comparisons can then be drawn within a pair and between the pairs for a given configuration.

Differences within a pair are again evident and they are more numerous here. In the positive ion spectra major variations are present throughout the spectra. Peak

intensities for ions at m/z 123, 127, 140, 145, 163, 285 and 302 are especially noteworthy. They all are larger for the alpha-anomers. The first 5 indicate the greater instability of the alpha-anomer which was known to be the case in terms of the lesser strength of the glycosidic bond. These fragments come from a fission at the glycosidic bond (m/z 163, 145, and 127) creating an oxonium ion within the carbohydrate ring (structure Z, table 2.1) or from the fission of the ring (m/z 123 and 140). The ion at m/z 285 and 302 indicate a greater proton affinity for the alpha-anomer. Another result that can be of great significance is the variation in the ionic intensities of m/z 163 and 215 of the galactosyl and of the glycosyl residues. To obtain m/z 163 it is necessary to "hydrolyse" para-nitrophenol from the $(M + H)^+$ species. If however a glycerol unit binds through the glycosidic oxygen, as it is favored in the beta-anomers, then production of m/z 163 is greatly hindered. Furthermore the alpha configuration offers the proper stereochemical arrangement for a stereoelectronically controlled elimination of the molecular aglycone. This factor, i.e. glycerol adduct formation via the glycosidic bond is thought to be of prime importance and, as it will be discussed later, it plays a role that makes it one of the three leading factors governing the fragmentation pattern of

glycosides and the like. The peak at m/z 215 in turns indicates a greater facility for the galactosyl moiety to bind to a glycerol through oxygen other than the glycosidic one (m/z 215 is the adduct of ion of mass 123 attached to a glycerol unit). This is thought to be dependent upon the stereochemistry at C-4; it is seen here as a remote stereochemical effect. The corresponding negative ion spectra provide complementary evidence for these explanations.

In summary, it appears that the fragmentation pattern in FAB mass spectra is governed mainly via three factors whose importance vary as the factors are often directly affecting each other. These are:

- i) nature of the aglycone
- ii) configuration of the anomeric carbon
- iii) remote stereochemical effects (i.e. the relative stereochemistry of the other carbohydrate carbons).

In order to further substantiate and to better understand and evaluate these primary factors, it is necessary to record several other spectra of model compounds. A series of intact disaccharides was used for

that purpose and the results obtained are presented in this section and are discussed in terms of the stereochemically-related factors mentioned above.

2.5.2 Disaccharides.

It is impossible to present these results without a word of caution. To understand and fully evaluate the remote stereochemistry factor would require the recording of an almost limitless number of combinations and permutations of hydroxyl moieties at one, the other, or several carbons at once. Clearly this would be outside the scope of any particular study. We are blessed however by the fact that there does not seem to be any major fragmentation attributable to the reducing end of the disaccharides so that the stereochemistry of the reducing sugar is of little importance, exclusion made of the fact that the reducing sugar competes directly with its non-reducing counterpart for the binding to a glycerol moiety. This brings in a fourth factor of major importance, actually the most important one, the nature of the matrix (whether you call it a support, a matrix or a solvent). This will be discussed

in detail further in the course of this section and the following one. Realizing the limitations imposed by the large number of possibilities for the disaccharides it was compulsory to select only a few representative, but related ones. The choice was therefore made in relation to some variety (to show extent of applicability), the stereochemical relation between them (e.g. alpha and beta anomers of a given disaccharide arrangement) and to some extent, on the availability of the disaccharides since they were to be used as models.

Furthermore, only the positive ion FAB mass spectra are presented under table 2.3 (Appendices Q to FF for actual spectra) and they will all be discussed as a group rather than individually as it was the case for the monosaccharide glycoside section just preceding. Data from the negative ion spectra in glycerol (Appendices GG to PP) and those recorded in diethanolamine (Appendices QQ to XX) will only be used as complementary information for the purpose of this thesis as some more work and time will be necessary before all of the spectra will be fully interpreted and before all the information they contain will be extracted and analysed.

Dell and co-workers presented earlier some overall fragmentation patterns involving the glycosidic bond (304, 305). Their work, based upon a study of large polysaccharides, did not suggest any mechanisms for the formation of their proposed ion structures. Figure 2.9 (to be found on page 165) presents the mechanisms for their reported three main patterns (305) along with a fourth one, closely related, introduced for the first time here and that accounts for major peaks in the spectra. Furthermore, their findings were greatly limited by the fact that the carbohydrate units making up the polysaccharide they studied were almost exclusively of the alpha configuration and were glycosides themselves as they bear some methyl moieties on O-6, a truly "artificial" case that is too specific to be used as a general guideline.

Before discussing the main features of the spectra in terms of stereochemical concepts, it is necessary to review some conformational principles in oligosaccharide chemistry, more specifically in disaccharides. It will then be possible to relate the salient points of the spectra directly to these conformational principles.

TABLE 2.3a

SELECTED POSITIVE ION FAB-MS DATA (ZI) OF SOME DISACCHARIDES

m/z	1- α -D-MANNOPYRANOSYL- L-ERYTHRITOL (gly/H ₂ O)	3- α -D-GALACTOPYRANOSYL- D-ARABINOPYRANOSIDE (gly/H ₂ O)
103	6.5	27.2
115	29.7	70.4
122	-	-
123	100.0	16.7
127	9.6 (B)	18.5 (B)
131	4.8	21.1
133	8.3	92.0 (A)
145	12.0 (B)	39.5 (B)
149 (X)	13.9	47.5
150	1.0 (C)	6.8 (A)
151 (Y)	15.6 (D)	51.2
163 (Z)	24.1 (B)	100.0 (B)
178	-	- (C)
179	-	4.3 (D)
207	12.7	14.8
215	5.5	8.6
241	4.9	18.5 (149 + gly)+
243	4.4	17.9 (151 + gly)+
259	1.5	22.8
285	78.6 (M + H)+	-
295	-	75.3 (M + H - H ₂ O)+
313	-	79.6 (M + H)+
377	9.6	-
387	-	6.2 (M+gly+H-H ₂ O)+
405	-	46.9 (M + gly + H)+
469	1.9	-
497	-	6.8 (M + 2gly + H)+
561	0.9 (M+3gly+H)+	-
569	7.6 (2M + H)+	-
589	-	6.2 (M + 3gly, +H)+
607	-	2.5 (2M + H - H ₂ O)+
625	-	9.9 (2M + H)+

see table 2.3d on page 164 for explanatory footnotes

TABLE 2.3b

m/z	ION	LACTOSE		MELIBIOSE	
		(gly)	(gly/H ₂ O)	monohydrate	dihydrate
				(gly)	
105	(123 - H ₂ O)+	22.2	24.2	11.1	25.7
123		69.3	59.8	21.4	77.4
127	(B)	16.4	33.0	37.6	18.8
145	(B)	30.5	48.0	56.4	37.0
149	X	100.0	54.9	17.9	89.7
151	Y	86.5	46.2	15.8	77.6
153		55.2	42.9	14.4	61.5
163	(B) Z	68.1	100.0	100.0	69.1
180	(A)	-	2.8	1.8	2.6
183		95.6	60.5	18.8	100.0
208	(C)	3.9	2.1	1.0	3.2
209	(D)	72.0	25.9	6.9	47.4
215	(123 + gly)+	59.2	32.2	6.0	61.6
241	(149 + gly)+	56.1	19.7	4.3	39.1
243	(151 + gly)+	39.6	14.8	3.5	29.4
245	(153 + gly)+	32.9	17.2	3.4	35.8
255	(163 + gly)+	15.0	12.4	15.8	25.2
273	(180+ gly +H)+	11.2	8.5	3.3	12.4
275	(183 + gly)+	40.6	16.6	3.0	40.0
307	(M+ H -2H ₂ O)+	13.5	6.2	3.5	13.1
325	(M + - H ₂ O)+	30.2	33.4	30.9	28.3
343	(M + H)+	74.2	68.8	17.5	33.5
417	(M+gly+H-H ₂ O)+	-	1.8	1.3	2.8
435	(M + gly + H)+	18.6	10.4	4.8	31.4
527	(M+ 2gly +H)+	6.2	22.0	-	3.9
685	(2M + H)+	-	5.1	4.8	-

see table 2.3d on page 164 for explanatory footnotes

TABLE 2.3c

m/z	ION	SUCROSE (gly)	TURANOSE (gly)
127	(B)	20.7	26.6
145	(B)	29.8	37.6
149	X	19.8	24.2
151	Y	16.6	19.1
163	(B) Z	100.0	100.0
180	(A)	1.6	1.4
208	(C)	0.6	0.8
209	(D)	13.9	17.6
255	(163 + gly)+	19.5	9.6
273	(180 + gly + H)+	13.0	2.3
307	(M + H - 2 H ₂ O)+	2.5	2.6
325	(M + H - H ₂ O)+	16.0	37.3
343	(M + H)+	25.3	2.3
361	(M + H ₂ O + H)+	3.5	-
417	(M + gly + H - H ₂ O)+	0.9	14.1
435	(M + gly + H)+	16.4	18.4
505	(M + 163)+	2.7	-
527	(M + 2gly + H)+	2.5	3.2
597	(M + 163 + gly)+	0.6	-
619	(M + 3 gly + H)+	1.0	1.1
685	(2M + H)+	3.5	-

see table 2.3d on page 164 for explanatory footnotes

TABLE 2.3d

m/z	TREHALOSE (gly/H ₂ O)	MALTOSE (gly) (gly/H ₂ O)	CELLOBIOSE (gly) (gly/H ₂ O)	GENTIOBIOSE (gly) (gly/H ₂ O)
123	13.5	26.9	31.7	59.1 67.1 8.9 9.9
127	33.4	38.0	45.6	9.6 18.3 34.4 41.1
145	75.5	73.0	84.2	16.4 37.9 81.5 90.4
149 X	31.3	53.1	43.9	56.5 77.6 28.4 25.9
151 Y	27.5	42.3	38.0	50.9 69.0 24.8 24.4
153	13.2	22.6	27.5	55.9 62.4 7.3 8.5
163 Z	100.0	100.0	100.0	17.3 51.0 96.3 100.0
167	15.1	27.6	29.4	57.1 58.2 9.5 11.7
180	2.5	-	2.4	- 3.1 1.9 -
183	-	37.6	44.1	100.0 100.0 12.3 13.1
208	0.9	2.4	1.9	2.1 3.3 - 1.4
209	18.8	35.2	21.3	26.9 44.9 21.7 17.9
215	6.8	16.9	17.2	50.0 56.9 5.6 4.4
255	47.1	52.8	41.6	10.8 18.5 33.6 27.8
275	4.3	11.3	10.1	37.8 37.8 3.4 2.8
325	77.1	97.6	85.2	8.5 47.9 100.0 82.1
343	15.7	15.0	11.0	4.1 19.3 53.2 33.3
417	1.4	8.0	5.4	- 2.5 3.1 1.4
435	41.0	61.7	34.7	5.0 31.2 22.0 11.9
505	4.4	2.8	1.6	- - 3.1 1.8
527	3.7	8.6	4.5	- 6.3 3.7 1.1
685	13.0	19.0	12.7	- 3.6 14.7 6.9

- not detected

spectra are corrected for matrix background

gly indicates spectra recorded in glycerol

gly/H₂O indicates spectra recorded in a glycerol/water mixture

A, B, C and D refer to the general mechanisms (page 165)

X, Y and Z refer to structures presented on page 145

Figure 2.9: Fragmentation patterns of the glycosidic bond under FAB conditions in the positive ion mode.

Discussion of the overall shapes of carbohydrate chains usually starts from knowledge of, or assumptions about, the conformations of the component sugar rings (296, 306, 307). Since six-membered (pyranose) rings are by far the most common in naturally occurring polysaccharides and their individual ring conformations are more stable and well defined, most of the successful work has been done with those systems. The only major exceptions are in the field of polynucleotides for which the interconversion between furanose conformations plays an important role in the determination of the polymer form.

The pyranose ring conformations that are important in polysaccharides are the two chair conformations designed ${}^4C_1$ and ${}^1C_4$ (figure 2.10) to indicate the disposition of atoms above and below the plane of the ring (in older notation the same conformations were denoted by C1 and 1C, respectively). Boat conformations probably have some existence in low proportions in disordered (random coil) polysaccharide chains.

The approaches available for predicting the relative stabilities and hence equilibrium populations of alternative conformations have been thoroughly reviewed (296, 306) and

Figure 2.10: Pyranoside ring conformations (illustrated for β -D-glucose).

for free sugars this can extend to comparison of the relative stabilities of alternative isomers, such as five-membered (furanose) rings or open chains. They begin from two complementary, but quite different approaches:

- a) Measurement of equilibrium constants are used empirically to derive the corresponding free energies (308, 309)
- b) In a more general approach attempts are made to build up a complete picture of all the actual attractions and repulsions between atoms in terms of van der Waals forces, polar interactions, hydrogen bonding, and torsional contributions (310, 311).

The calculations are now refined to a point at which an accurate prediction can usually be made of the conformations that will predominate in sugar solutions, as well as the proportions of each form. They may even indicate minor distortions and deviations from "ideal" chair geometry, although they make no attempt to consider terms involving solvent. It appears that these terms usually cancel when differences in free energy are considered, but exceptions are known in which additional stabilization of particular conformations by water is indicated. In general, however, the shape of naturally occurring pyranose rings in carbohydrate chains may be regarded as fixed in that chair conformation in which C-6 is equatorial (${}^4C_1$ for D-sugars and ${}^1C_4$ in the L series). Overall chain geometry is

3

therefore determined predominantly by the relative orientations of adjacent residues. .

For glycosidic bonds in which linkage is through an oxygen atom (attached to a ring carbon, the relative orientations of the participating residues can be defined completely by the two dihedral angles ϕ and ψ shown in figure 2.11. When the connecting linkage is between C1 of one residue and C6 of its neighbor, there is an extra covalent bond and torsion angle (ω), giving these units markedly increased freedom to adopt a wide variety of orientations relative to each other. The term "linkage conformation" (312) is used to define a distinct set of values for these angles (ϕ, ψ) or (ϕ, ψ, ω).

In considering the overall conformations of carbohydrate chains it is useful to start by distinguishing between:

- (a) ordered conformations in which the values of the torsion angles are fixed by cooperative interactions between residues -- and
- (b) disordered conformations in which continuous fluctuation occurs.

Figure 2.11: Interresidue linkages in carbohydrate chains (illustrated for some glucose dimers). Linkage conformation is defined by the dihedral angles (ϕ, ψ) or (ϕ, ψ, ω) . The intramolecular hydrogen bonds are also indicated.

It is not possible to deal with linkage geometry in polysaccharides by simple extension of the classical methods for conformational analysis of small molecules since we would first have to identify a limited number of discrete conformations that correspond to the important low-energy forms in equilibrium. The alternative approach is to calculate conformational energies directly from energy functions by listing the stereochemical constraints that have been established from structural studies. The computer methods used to do this have been reviewed extensively (312-317).

One source of unambiguous information on the rotational angles between adjacent sugar residues consists of X-ray diffraction studies of crystalline disaccharides and their derivatives. These may then be compared with values predicted from calculation. Irrespective of the method of conformational analysis employed, the strategy is to develop a map showing the variation of internal energy with rotation around the glycosidic angles ϕ and ψ (figure 2.11). Except for (1 $\rightarrow$ 6) linkages all such calculations show that more than 90% of the conformations on the map are energetically disallowed (318).

For cellobiose and maltose and derivatives, a sufficient number of crystal structures have been determined to allow useful comparisons to be made (312, 318). As seen on the map obtained for cellobiose (ref. 318) different forms do not have exactly the same conformation at the glycosidic linkage, but lie close together within the predicted boundary and very close to the overall minimum of the energy map. Presumably, the slight adjustments from one derivative to another occur to facilitate packing in a different crystal environment. Although the cellobiose map shows two troughs of favored conformational energy, only one of these is populated. This zone is larger in area and hence more "probable" than the smaller area; it also contains zones of somewhat lower energy states and allows the formation of a hydrogen bond between O3 on one residue and O5 on its neighbor (figure 2.11). Interpretations of optical rotation studies (319) in dimethyl sulfoxide and water in terms of contributions to the overall optical rotation from the linkage conformation also show good agreement between the observed values and those calculated from the solid-state conformation. This agreement does not imply that the disaccharide in solution is locked in the crystal conformation, but it does suggest a preference for ϕ and ψ values in the neighborhood of the minimum energy conformation.

Analogous calculations (312, 318) for maltose show that the crystal conformations characterized by X-ray diffraction are more scattered but again lie mainly in a single zone around the minimum energy conformation, which is once more stabilized by a hydrogen bond between the two sugar rings. A consequence of the difference in configuration at C-1 between cellobiose and maltose is that the stabilizing hydrogen bond is between different pairs of oxygen atoms. In maltose it is between O3 of one residue and O2 of its neighbor (figure 2.11).

In contrast to cellobiose, optical rotation measurements in solution (319) indicate that maltose does not always oscillate in the neighborhood of the hydrogen-bonded conformation. This linkage conformation is very close to eclipsed positions about both bonds to the glycosidic oxygen, and, although the O2...O3' hydrogen bond evidently offsets this disadvantage in the solid state this is not possible in strongly hydrogen-bonded solvents. In dioxane and dimethyl sulfoxide, both optical rotation and proton nuclear magnetic resonance (^1H NMR) measurements (320-322) suggest that, although the time-averaged conformation is significantly displaced from the crystal structure, a large proportion of molecules exist in the

major zone in which the hydrogen bond is possible. In aqueous solution, by contrast, the evidence suggests that the molecule must spend a much larger portion of its time in the area of the subsidiary minimum. Carbon-13 NMR observations (323) confirm that in water, steric or proximity effects, occur between the sugar residues, as would be expected for the alternative "folded" structure. It is also possible that the arrangement of sugar hydroxyl groups in the alternative zone matches more closely the organization in transiently ordered "clusters" of water molecules to provide the extra stability that the conformational calculations fail to predict (319). The solvent dependence of conformation seen in maltose, however, appears to be a relatively uncommon effect.

For some linkages at least, it is possible to detect a substantial influence on the conformation about the glycosidic bond from the exo-anomeric effect; this evidence is from ^{13}C - ^1H coupling constants (324, 325) and optical rotation (326).

For diequatorial linkages calculations show that the numerically averaged values of ϕ and ψ can be predicted independently, ϕ being determined by the size of the

equatorial substituent in C2' and ψ by those in C3 and C5. On this basis, groups of glucosyl oligomers, for example, could then be assigned the value of ϕ known for cellobiose from both solution and crystal studies, whereas that of lactose could be applied to galactosyl homologues, and the measured optical rotations then analyzed to calculate the values of ψ (326). For a large number of compounds, the results demonstrated with remarkable consistency that:

- (a) each group of oligosaccharides having a similar substitution around the aglycone bond show only very small variations in ψ -- and --
- (b) replacement of a substituent with a less bulky one causes ψ to shift as expected toward the extra space that is created.

The factors affecting ϕ and ψ for alpha linkages are more interdependent, but a similar general picture emerges if it is assumed that, as in the beta series, ϕ is the same for all related compounds. Conformational analysis also shows that increasing the number of axial linkages to the glycosidic oxygen decreases the number of accessible conformations.

With these basic conformational principles established it is now possible to account for the difference in the

fragmentation patterns of the disaccharides studied, as presented in table 2.3. The first major difference lies in the presence of the ring oxonium ion (Mechanism B, figure 2.9, p.165) that seems to occur to a much larger extent in the alpha-linked disaccharides. This can be accounted for by the fact that in order to produce that fragment ion, the lone pair of the ring oxygen (O5') must be able to assist in the elimination of the reducing sugar (this is strictly speaking the analog of a simple hydrolysis reaction). The stereoelectronic effects (anomeric effect) clearly favors this pathway for alpha-configuration non-reducing sugar. Furthermore, in beta-linked (1 → 4) disaccharides it was just established that the interresidue hydrogen-bonding was involving the lone pair of electrons of O5' of the non-reducing end (figure 2.11); these electrons are therefore less able to assist the elimination process as they are already partially localized. This fragmentation can therefore be explained in terms of:

- i) the configuration of the anomeric carbon -- and --
- ii) the remote stereochemistry factor.

Inherently, the nature of the aglycone was primordial in allowing intramolecular hydrogen bond formation. The case

of beta-D-galactopyranosyl-(1→3)-D-arabinoside (table 2.3, column 2 and appendix R) is a spectacular example of the power of the technique. In fact, at first sight this disaccharide seems not to follow the rule that was just established as it is a beta-linked disaccharide and yet it exhibits the oxonium ion (at m/z 163) as its base peak. However, if consideration is taken of the fact that this is a 1 → 3 linkage and that the stereochemistry at C3 (and C2) of D-arabinose is (are) inverted it should then be expected that intramolecular hydrogen bonding via the galactosyl O5 is impossible, thus enabling the lone pair to assist in the elimination of the arabinose residue. In fact, now the spectrum exhibits two peaks of nearly equal intensities at m/z 133 and m/z 163 corresponding to mechanisms A and B respectively.

This effect, the remote stereochemistry factor, is also responsible for the differences between a given galactosyl versus glucosyl pair of disaccharides (e.g. lactose and cellobiose, table 2.3, columns 3 and 12 and appendices S and CC). The only primary difference residing in the stereochemistry at C4 of the non-reducing end of the disaccharide (according to the previously presented conformational principles, these two disaccharides should

exhibit the same overall conformation, i.e. that depicted in figure 2.11, p.170) any variation in the fragmentation pattern might be attributable to that stereochemical difference. In the pair just mentioned there are a few differences. For example m/z 163 (Mechanism B, see figure 2.9, p. 165) is of low intensity in cellobiose and of somewhat higher intensity for lactose. This can also be accounted in terms of the nature of the matrix. In fact, it is possible to attach a glycerol unit through the oxygen atom at C3, C4 and C6 of the galactosyl residue (but not for the glucosyl one) thus distorting the overall conformation of the glycerol-hydrogen-disaccharide adduct so as to again, free somewhat the lone pair of the ring oxygen that can then contribute more easily to the formation of the fragment ion at m/z 163. The increased intensity of the $(163 + \text{Gly})^+$ adduct ion at m/z 255 also supports this statement.

To demonstrate further this argument and to show the importance of the nature of the matrix used, spectra of selected disaccharides have been recorded in other matrices (polyethylene glycol-200 (PEG-200) diethanolamine (DEA), and thioglycerol (TGLV)) in both, positive and negative ions mode. The results are conclusive (see appendices at the end). NO STEREOCHEMICAL DEPENDENCE COULD BE SEEN with a

solvent such as PEG-200 that can bind itself randomly to any other part of the disaccharide, thus losing all the stereospecificity required to exhibit valuable differences in the fragmentation patterns of the oligosaccharides under study. Recently a paper by Rose et al. suggested some stereochemical dependence of the adducts obtained when reacting stereoisomeric monosaccharides with boric acid (327). This was, to our knowledge, the very first report indicating some stereochemical effects in FAB-MS of saccharides although they report data for only four pentoses in their cautious claim. Almost simultaneously, Harrison and Lin (328) reported their findings on the stereochemical dependence of the fragmentation of stereoisomeric methylcyclohexanols under EI versus charge-exchange spectra.

As a last spectacular example and convincing evidence of the stereochemical effect in FAB-MS of disaccharides, attention is drawn to the spectra recorded on a glycerol support in presence or not of water. The reasoning here being the drawing of yet another analogy to the solution chemistry of the disaccharides. In fact, if the production of fragment ions of pattern B occurs via the hydrolysis-like mechanism B depicted in figure 2.9, then the addition of water to the glycerol should affect considerably the overall

fragmentation of the disaccharides. This was found to be the case (table 2.3) as the water molecules, competing directly with:

- i) the glycerol for the protonation of the disaccharides -- and --
- ii) with the intramolecular hydrogen-bond formation yielded spectra in which mechanism B was enhanced for beta-anomers and reduced for alpha-anomers (this is representative of a more random conformation in the environment of the glycosidic bond).

Fragment ions arising from the other pathways varied similarly and the adducts containing glycerol were greatly reduced supporting the concept of a greater affinity for water (greater ease of binding to water) than for the glycerol support. Thus it would appear that glycerol binds itself to the disaccharide and induces a fragmentation pathway which, in turn, is stereochemically dependent since the binding sites of glycerol is of prime importance in the route the fragmentation will take. It has been our experience that binding of the matrix to the molecule under study was primordial to the detection of the molecular ion (and not dissolution as suggested in earlier work (49-51) and the strength of that binding is governing, to a large extent, the degree of fragmentation (thus of structural information that can be obtained from the spectra). For

example, in a survey of a series of corticosteroids, we have noticed that glycerol was often unable to bind to the free hydroxyl groups of the steroids mainly because of steric hindrance. Thioglycerol, however, offers larger orbitals (3p versus 2p) than can reach much easier into the volume of the steroid thus allowing to easily detect the molecular ion where glycerol failed. The "bond" so-produced however is consequently weaker (greater inter-molecular distance) and the spectra in thioglycerol showed much less overall fragmentation than their glycerol counterpart (329). The protonated species-support matrix adduct ions are usually not present in the thioglycerol-based spectra but are quite numerous in other strongly-binding solvents such as glycerol, diethanolamine, etc., thus further supporting the above claim.

Figure 2.12 shows how glycerol can bind to a (1 → 4) beta linked disaccharide via the ring oxygen (O5') thus preventing the involvement of mechanism B; it also depicts the impossibility for glycerol to bind to the alpha counterpart. These statements would also hold true for any values of ϕ and ψ (see figure 2.11, p.170) should there be several rotamers present, contrary to the enunciation of our conformational principles, thus reinforcing further the

Figure 2.12: Probable binding sites between glycerol and a (1-4) disaccharide.

theoretical fundamentals claimed in this study. Although precedents are few, mainly due to the novelty of the technique one report appeared very recently suggesting a similar "charge-transfer complexation" as the ionization mechanism in FAB (330). Furthermore, more data are becoming available everyday on the technique (331), its optimization (332), the development of new sources (333) for better reproducibility of relative abundances of ion currents in FAB (334, 335) so as to ascertain the value of these data. More specifically, data on saccharides by FAB (336-339) compare very advantageously to those obtained via other recent mass spectral techniques (340-343) in all respects, be it reproducibility, structural information, molecular ion detection, cleanliness of the spectra, etc.

The negative ion FAB spectra of all these disaccharides have been recorded in glycerol and are presented as Appendices GG through PP. These spectra also ~~show~~ significant differences between a given pair of anomers (e.g. cellobiose versus maltose) although no attempt will be made to explain these differences as it expands outside the scope of this fundamental study. Further work in that direction is in progress and numerous results are to be analysed along the same terms (e.g. see Appendices QQ

through XX for positive and negative ion FAB spectra of a number of these disaccharides recorded in diethanolamine in order to evaluate the effect of the nature of the matrix.

2.5.3 Disaccharide Glycosides and Derivatives

This subgroup was used as a test for the rules just established and a means to help in assigning a priority to each one of the four factors that were just shown to govern the general appearance of a FAB spectrum. Table 2.4 (Appendix YY) presents the data obtained for beta-phenyl lactoside (positive ion recorded in a glycerol support to which water was added). The data might appear contradictory at first sight, because of a strong ion at m/z 163 normally not seen in beta-linked disaccharides. The addition of water is responsible, in part, for this behavior. The main factor at play here, however, is the nature of the aglycone. In fact these data compare well with the previously discussed ones involving monosaccharide glycosides (section 2.5.1, p. 143). This actually confirms how drastically the nature of the aglycone may influence the actual spectrum.

TABLE 2.4
 SELECTED POSITIVE ION FAB-MS DATA (%I) FOR
 β -PHENYL LACTOSIDE

m/z	ION	(gly/H ₂ O)
123		14.8
127	(B)	17.3
145	(B)	43.8
149	X	56.8
151	Y	59.9
163	(B) Z	100.0
183	?	23.5
209	(D from 343)	40.7
215	(123 + gly)+	14.8
241	(149 + gly)+	22.2
243	(151 + gly)+	21.6
301	(209 + gly)+	7.4
307	(M + H - C ₆ H ₅ OH - H ₂ O)+	4.9
325	(M + H - C ₆ H ₅ OH)+ (B)	51.9
419	(M + H)+	46.9
511	(M + gly + H)+	14.8
603	(M + 2 gly + H)+	3.1

Spectrum corrected for matrix background
 this spectrum was recorded in a glycerol/water mixture
 A, B, C, and D refer to the general mechanisms (page 165)
 X, Y, and Z refer to structures presented on page 145

Table 2.5 presents the positive ion FAB data for three peracetylated disaccharides (Appendices ZZ to BBB). These compounds can be thought of as disaccharides bearing a whole series of (identical) non-carbohydrate aglycones. The first point of interest is that these substances do not offer readily any site for binding to glycerol. This is clearly demonstrated by the absence (or very weak presence) of the pseudomolecular ion $(M + H)^+$ at m/z 679. Following the same principle there is almost no fragmentation observed, suggesting that the glycerol-molecular substrate adduct is very weak, accordingly there are no glycerol-protonated molecular species adducts present either. It was necessary to add some trace of yet another better binding solvent (water and thioglycerol) in order to record good spectra of cellobiose acetate and maltose acetate. Actually the only important fragment ions for these two samples occur at m/z 331 (via mechanism B, p.165) and at m/z 169 and 109 occurring via a, as yet undefined, mechanism. Sucrose acetate was recorded in glycerol and a trace of ethyl acetate and in contrast exhibits a much larger fragment ion at m/z 331 than at m/z 169 and 109. This is easily accounted for in view of the fact that sucrose offers two ways to undergo mechanism B (p. 165), i.e. via O5', the ring oxygen of the glucosyl moiety or via O5, the ring oxygen of the fructofuranoside moiety.

TABLE 2.5

SELECTED POSITIVE ION FAB-MS DATA (%I) OF SOME
DISACCHARIDE DERIVATIVES

m/z	ION	MALTOSE ACETATE (gly/thiogly)	CELLOBIOSE ACETATE (gly/H ₂ O)	SUCROSE ACETATE (gly/EtOAc)
109	(C ₂ O ₅ H ₅) ⁺	90.2	85.9	52.6
127	(169 - CH ₂ =C=O) ⁺	44.6	49.8	21.8
169		100.0	100.0	66.6
271	(B)	4.1	7.7	3.4
317	(377 - CH ₃ COOH) ⁺	1.4	3.4	2.4
331	(B)	35.0	32.0	100.0
348	(A)	-	0.8	-
376	(C)	-	-	-
377	(D)	-	1.0	-
517	(577 - CH ₃ COOH) ⁺	1.1	0.7	-
559	(M + H - 2(CH ₃ COOH)) ⁺	4.5	0.7	-
577	(619 - CH ₂ =C=O) ⁺	8.7	2.2	-
619	(M + H - CH ₃ COOH) ⁺	19.3	3.5	-
679	(M + H) ⁺	-	-	2.2

spectrum corrected for matrix background

(gly/thiogly): indicates spectrum recorded in a glycerol/
thioglycerol mixture(gly/H₂O): indicates spectrum recorded in a glycerol/water
mixture(gly/EtOAc): indicates spectrum recorded in a glycerol/ethyl
acetate mixture

A, B, C, and D refer to the general mechanisms (page 165)

Care must be taken not to assign m/z 109 as the daughter ion of m/z 169 through the obvious loss of acetic acid. In fact a quick survey of all the possible combinations for the elemental compositions corresponding to a nominal mass of 109 and obeying the " $2n + 2$ " rules for the hydrogen-to-carbon ratio yields the following:

None of these compositions are reasonable. Even if one was to assume that m/z 109 actually comes from a doubly-charged ion of 218 daltons it would still not be possible to construct an ion according to standard rules. The fact that these ions arise in glycerol that was spiked with three other, different, solvents seems to preclude the fact that these ions would be solvent-related although no definitive claim can be made to that effect. At best here, for the time being, it can only be concluded that the behavior of these disaccharide derivatives supports our claim for the need that binding be established in order to obtain a good spectrum and that the strength of that association will affect drastically the amount of structural information contained in the spectra (i.e. the degree of fragmentation).

Appendices CCC through LLL present complementary spectral data for these substances.

2.5.4 Higher Oligosaccharides

For higher oligosaccharides the situation in terms of conformational principles is much more complex than for disaccharides (section 2.5.2). In fact even though the majority (more than 90-95%) of linkage conformations for disaccharides are shown by the conformational energy maps to be forbidden by steric considerations alone, the remaining conformational space allows significant freedom of oscillation about the bonds to the glycosidic oxygen. In solution the molecules are not normally constrained in a unique state but oscillate around it because of collisions and thermal energy. A carbohydrate chain typically contains a large number of such linkages, and the overall shape is the result of these independent oscillations. At any instant, such a chain would show a spectrum of linkage conformations, most of them in favorable low-energy orientations, although thermal energy would also induce a few linkages to adopt much higher energy forms and a few

monomer units to adopt twist, boat, and alternative chair conformations. The greater the internal freedom at each linkage, the greater the number of conformations available to each individual segment and the less likely it will be for the chain to adopt a unique ordered shape in which each linkage is fixed close to the minimum energy form. Chain flexibility thus provides a strong entropic drive, which generally overcomes energy considerations and induces the chain to adopt disordered or random coil states in solution (307, 312). This influence is known as the conformational entropy.

Under particular circumstances, however, favorable nonbonded energy terms (hydrogen bonding, dipolar and ionic interactions, and solvent effects) can combine to fix macromolecules in ordered shapes (307, 312, 344). For carbohydrate chains the interactions between individual pairs of monomers is insufficient to do this, and it occurs by the cooperative interaction of energy terms along extended sequences of the chain, which reinforce each other to outweigh the conformational entropy. Since these interactions almost invariably occur between long, regular sequences, the result is a helix, because any interactions favored within a particular repeating unit will also be

avored for neighboring units. Ordered structures are more favored for the solid state because cooperative interactions can then operate between chains as well as within them.

This section deals therefore mainly with the effect of the nature of the aglycone, when recorded on a given support-matrix. Now that a theory has been proposed, it is well worth to analyse a few polysaccharides (Tables 2.6 and 2.7 and Appendices MMM to OOO and SSS) and some higher oligosaccharides to determine the extent to which we can relate to the theory and to size the effect of the nature of the aglycone. In order to achieve this, the results obtained for the trisaccharides and the tetrasaccharide will be discussed separately from those from the non-carbohydrate aglycone-containing oligosaccharides.

The data presented in tables 2.6 and 2.7 agree very well with the proposed model theory derived earlier from the study of disaccharides (pp. 158-184). In fact the main fragment ions are obtained from the same type of mechanisms as those presented in figure 2.9 (p. 165). For trisaccharides (table 2.6) two glycosidic linkages are involved and influence the spectra in their characteristic way. For example the fragment ion at m/z 325 arising from

TABLE 2.6

SELECTED POSITIVE ION FAB-MS DATA (%I) OF SOME.
TRISACCHARIDES*

m/z	ION	RAFFINOSE PENTAHYDRATE	MELEZITOSE	tri-GLC**
117		11.1	27.3	54.7
123		8.1	28.2	12.1
127	(B)	55.1	13.7	19.5
145	(B)	63.1	24.1	39.1
149	X	27.1	79.6	59.0
151	Y	21.1	100.0	100.0
163	(B) Z	100.0	65.4	40.6
181	(A + H)+	4.6	-	-
183		11.3	37.3	19.9
195		2.1	31.6	60.5
209	(D)	16.3	63.0	50.0
241	(149 + gly)+	7.8	36.7	23.8
243	(151 + gly)+	4.9	30.8	31.3
255	(163 + gly)+	13.9	6.4	7.0
301	(209 + gly)+	1.5	12.1	9.0
325	(B)	45.7	15.0	36.7
343*	(A + H)+	32.3	1.1	6.6
435	(343 + gly)+	1.0	-	2.7
487	(M + H - H ₂ O)+	3.7	2.7	4.3
505	(M + H)+	21.8	12.6	11.7
597	(M + gly + H)+	4.2	3.5	5.9
687	(M + 5H ₂ O + gly + H)+ (!)	4.6	-	-

* spectra recorded in a glycerol/water mixture; corrected for matrix background

** O-β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→4)-D-glucopyranoside

- not detected

A, B, C and D refer to the general mechanisms (page 165)

X, Y and Z refer to structures presented on page 145

TABLE 2.7

SELECTED POSITIVE ION FAB-MS DATA (%I) FOR THE
TETRASACCHARIDE STACHYOSE

m/z	ION	(gly)	(gly)	(gly/thiogly)	(gly/NaCl)
115	(gly + Na)+	n.a.	n.a.		100.0
127	(145 - H ₂ O)+	31.1	34.1		2.2
137	(gly - H + 2Na)+	n.a.	n.a.		99.4
145	(163 - H ₂ O)+	45.9	46.7		10.1
149	X	93.2	79.8		2.6
151	Y	71.6	73.3		2.1
153		35.1	46.1		1.7
159		-	8.2		6.9
163	(B) Z	100.0	100.0		3.2
165		28.4	34.5		9.2
167		39.2	53.2		2.1
173	(151 - H + Na)+	n.a.	n.a.		99.9
175		n.a.	n.a.		61.6
183		50.0	84.3		2.0
185		n.a.	n.a.		24.0
209	(D)	56.8	39.3		10.5
215		28.4	43.4		11.6
241	(149 + gly)+	44.6	36.0		1.4
243	(151 + gly)+	31.1	27.0		1.4
255	(163 + gly)+	16.2	17.2		8.6
265		n.a.	n.a.		79.6
307		8.1	10.9		1.5
325	(B)	31.1	30.7		13.3
333		8.1	7.9		1.1
343	(A + H)+	5	10.5		2.1
435	(343 + gly)+		1.5	-	1.1
487	(B)	8	7.1	29.5	1.0
505	(A + H)+	7	25.5	100.0	0.6
667	(M + H)+	1	6.4	24.2	-
684	?		-	28.4	-
689	(M + Na)+	n.a.	n.a.	-	5.0
759	(M + gly + H)+	-	2.6	7.8	-

spectra corrected for matrix background

(gly/thiogly) (and (gly/NaCl)) indicate spectra recorded in
a glycerol/thioglycerol mixture (or a glycerol/NaCl mixture)
blanks: outside mass range

- not detected

n.a.: not applicable

A, B, C and D refer to the general mechanisms (page 165)

X, Y and Z refer to structures presented on page 145

mechanism B at the glycosidic bond involving the reducing end sugar and the first non-reducing sugar whereas fragment ion at m/z 163 is the result of fragmentation B at the glycosidic bond linking the two non-reducing sugars (m/z 487 occurs similarly for stachyose (table 2.7)). The differences in the intensities of m/z 163 for example, between the three trisaccharides can be accounted as follows:

raffinose

m/z 163 can be created from the cleavage of the galactose unit, from the cleavage of the fructose unit and the sequential cleavage of the fructose (creating m/z 325, and that of the glucose; since all the linkages are alpha this is favored.

melezitose

for melezitose however, m/z 163 can arise only from the cleavage of the fructose unit (alpha configuration) since the galactose-glucose linkage being 1 $\rightarrow$ 3 has the property to reverse the mechanism and thus does not favor mechanism B and that despite that it is alpha (see disaccharides, section on pp. 158-184 and table 2.3 on pp. 161-164).

glucose trimer

the glucose trimer on the other hand possesses two beta-linkages, and therefore would not be expected to exhibit mechanism B but for the fact that some water was added to the sample so as to induce mechanism B. The result agrees perfectly with the previous data as the intensity of m/z 163 has almost reached that of melezitose.

Fragment ion at m/z 325 is similar and is allowed only for raffinose since this is the only trisaccharide in this study

that meets both requirements i.e. the proper configuration at the (glucosyl) anomeric carbon and the right stereochemistry at C-2 of the fructose unit (remote stereochemistry effect).

Furthermore, figure 2.13 shows a typical linked-scan of the B^2/E type (34) for daughter ion at m/z 163 of raffinose. This indicates that fragment ion at m/z 163 arise from the spontaneous unimolecular dissociation of ions at m/z 325, 289 (?), 255 and 235 in the first field free region. No collision gas was used and these data do not imply that this list of parent ions is exhaustive. Figure 2.14 on the other hand shows a sample linked-scan of the B/E type (34) for precursor ion at m/z 343 for raffinose. It indicates that, in the first field free region, fragment ion at m/z 343 further decomposes into a daughter ion at m/z 325 and its oxidated form at m/z 323. The linked-scan shown in figure 2.13 showed variations with time. This is interpreted as a warning when analyzing such data so as to take great care not to be measuring artefacts (345). It is also indicative of the large number of processes taking place at one time in the FAB ion source.

Urb 2/163

Raffinose

24 Feb 84

Figure 2.13: Linked-scan (B^2/E) performed on the daughter ion at m/z 163 for raffinose under FAB conditions.

Figure 2.14: Linked-scan (B/E) performed on the precursor ion at m/z 343 for raffinose under FAB conditions.

Stachyose was used as an example of the appearance the spectra may take according to the matrix conditions in which they were recorded. Table 2.7 provides some examples of the results that can be obtained. Again they support some solution chemistry concepts such as preference of cationization of the molecular species over protonation, easier binding to thioglycerol than to glycerol (larger $(M + H)^+$) although much weaker (less fragmentation and no adduct ions). The spectrum recorded in glycerol only shows astonishing similarities with that of raffinose (looking at m/z 505 and lower of course). The same statement can be made when comparing the spectrum of raffinose and that of sucrose (Table 2.3c, p. 163) when looking at m/z 343 and lower. This is indicative of the high degree of uniformity and reproducibility obtained with these compounds and it suggests that the cleavages are sequentially performed (cf. linked-scan data discussed above) thus leading the way to further investigations on sequencing aspects in poly- or oligosaccharides. Such attempts have been made recently (346-350) but none of these reports made any correspondence between the fragments produced and any stereochemical implications.

A series of higher oligosaccharides with a variety of distinct aglycones were used to investigate to which extent the fragmentation pathway taken is affected by the aglycone. Appendices EEEE to KKKK present these data. The number of aglycones, their similarities within a group, their limited variety and the fact that it was not possible to obtain good spectra for all of them in the same solvent make it impossible to state definite conclusions, the data being not truly statistically useful. However it is possible to discern a trend that is the fact that when the aglycone is a non-carbohydrate by nature, it induces a fragmentation pathway quite different and that seems to overcome the stereochemical effects noted earlier. This appears to be true certainly for the first sugar from the aglycone, and in most cases the second one as well. After the first two sugars however, the effect of the nature of the (aglycone seems to revert back to "normal" that is to the highly stereochemistry dependent pattern described above. Some noteworthy examples include samples of the digoxin series which are very important in themselves and that are easily sugar sequenced (351). The same holds true for some natural saponins from marine sources such as psoluthurin A and frondoside A (290-293).

2.6 CONCLUSION

7

2.6.1 Conclusions from the Present Study.

The present study clearly highlights four independent parameters that govern a FAB spectrum in terms of whether or not ions will be observed and also whether structurally useful fragments ions will be produced or not. With respect to carbohydrate material, these four parameters are:

1. Nature of the Matrix:

It is proposed that binding of the matrix to the species under study is necessary (and NOT dissolution) in order to obtain a spectrum. The strength of that binding directs the degree of fragmentation that will be present in the spectrum (i.e. the amount of structural information available). The stronger the bond between the matrix and the species, the more it will induce fragmentations (329).

2. Nature of the aglycone:

For polysaccharides, fragmentation patterns B and D (figure 2.9, p. 165) are predominant (along with the protonated ionic species corresponding to mechanism A) thus supporting very strongly that in FAB, the major ionization process is direct protonation (or cationization) and not the production of such a species via an ion radical by electron abstraction from the neutral molecular species. When the aglycone is non-carbohydrate in nature, it induces different

fragmentation patterns up to at least the second sugar unit that is linked to that particular aglycone. In monosaccharide glycosides, the appearance of the spectra is almost entirely governed by the nature of the aglycone as the aglycone represents more than one-half the entire molecule.

3. Configuration of the Anomeric Carbon:

The spectra are very sensitive to the configuration of the anomeric centers in the molecule. This is a truly distinctive attribute of FAB over other mass spectral techniques that should make it a powerful conformational probe if we take into account the very minute quantities required to obtain a good spectrum. This effect however is less important than that of the nature of the matrix. Care must be exercised therefore to choose an appropriate matrix. The sites of binding appear to be primordial since the use of other matrices that offered some binding and some fragmentations, both in FAB and in SIMS totally destroyed the stereochemical dependence of the spectra (352-355). Even the substitution of glycerol for PEG-200 yielded a drastic loss in stereochemical information to be derived from the spectra (Appendices LLLL to RRRR for examples) and that despite the evident similarity between these matrices and a fair binding of PEG-200 to the species under study (59). The type of stereoelectronic effect regulating the fragmentation pathways taken has been suggested only once previously in the literature and it involved CI spectra of some acetate (oxygen) and were computed using MINDO/3 (356).

4. Remote Stereochemical Effect:

The stereochemistry of the various chiral centers in a given carbohydrate ring may affect the spectra. This is mainly the result of the degree of binding that can be accomplished and the number of binding sites so-offered (cf. discussion on disaccharides involving lactose and cellobiose, p. 172). As such this effect can be seen as somewhat dependent itself on the nature of the matrix. But here by nature it is meant the stereochemical considerations brought in by the matrix. Although glycerol does not possess a chiral center it offers a large number of conformers (rotamers around C-C and C-O bonds). Clearly a more rigid matrix could not offer such a versatility in terms of binding sites to the carbohydrate structure.

Furthermore, it is suggested that FAB will become a valuable method for the sequencing of polysaccharides. The real challenge will lie around whether or not it will be possible to correctly assign the configurations of the carbohydrate units making up the polysaccharide and the linkage points between the carbohydrate residues. In the case of oligosaccharide containing a non-carbohydrate aglycone the task will be more difficult for the first and second carbohydrate residues directly attached to the aglycone, although there are ways of circumventing the problem (291-293, 351).

The initial goal of providing a better insight into FAB and its fundamentals has been met. The theory presented here has been tested against other families of compounds and the basic statements with respect to binding, strength of binding and the ionization process appear to be well founded.

2.6.2. Future Directions

Questions arising from the present study and that will, to some extent, direct the orientation of future

experiments include the refinement of the proposed theory regarding the binding aspects of FAB so as to evaluate better matrices that will provide a good handle on the molecule of interest and will allow the analyst to literally induce fragmentation. In this laboratory it was possible to achieve this with well over 400 different substances using more than 10 different matrices. Ironically the only compound that presented some real difficulties was a natural product (frondoside-A (292, 293)) and the problem centered around the presence of some residual sodium salt (chloride) which was found to be responsible for the transient nature of the spectra. Future work is also necessary to show the extent to which one can use the effect of the nature of the aglycone to characterize the same (e.g. a series of steroidal glycosides, etc.). Of course a study like this will be incomplete until some definitive interpretations on the negative ion spectra in glycerol and all the spectra recorded in various matrices, given here as appendices, will be presented. The future of the technique itself seems assured. It is impossible to conclude this discussion on FAB without mentioning the possibility of recording a spectrum directly from a thin layer chromatography (TLC) plate (357, 358) as this will have direct applications (on paper chromatography) in carbohydrate analysis.

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CHAPTER 3

MYCOTOXINS IN THE TRICHOTHECENE
FAMILY FROM FUSARIUM SPECIES:
CHARACTERIZATION AND ANALYSIS
BY MASS SPECTROMETRY.

CHAPTER 3

MYCOTOXINS IN THE TRICHOTHECENE FAMILY FROM FUSARIUM SPECIES: CHARACTERIZATION AND ANALYSIS BY MASS SPECTROMETRY.

3.1 GENERAL CONCEPTS

3.1.1 Trichothecene Mycotoxins: Sources and Problems.

Species of the Fusarium genus are common and widespread in nature. They occur as saprophytes in soil and in decaying vegetation of all kinds. They are also found as parasites of wild and cultivated plants where they are the cause of a variety of diseases in their hosts such as wilts, blights and rots. Some species cause scab, or blight in various cereals (barley, oats, wheat) and cobrot or ear rot in maize. Although much of the early work on mycotoxins caused by Fusarium has been done with the F. roseum, F. tricinctum and F. moniliforme complexes or groups from maize (a major world livestock feed) it is now known that others

involved in decays of cereals and vegetables produce equally potent toxins (e.g. F. oxysporum and F. graminearum).

As early as 1936 it was known that grain invaded by some species of Fusarium, under some conditions, was toxic (1). In the 1940's, Russia suffered outbreaks of alimentary toxic aleukia in man (ATA) and this was later reported by Joffe to have been caused by the consumption of millet that had been invaded by F. poae and F. sporotrichioides.

The diagnosis of the disease(s) in animals is sometimes complicated by the fact that some species of Fusarium produce more than one toxin when colonizing plants or cereal grains. For example, F. roseum, F. tricinctum and F. roseum Gibbosum produce various members of the trichothecene group of toxins (see table 1 on following page for some representative members of the group) as well as zearalenone (29). In swine the latter causes the hyperestrogenic syndrome whereas some of the trichothecenes can cause extensive hemorrhaging, emesis and abortion. When occurring together they can exhibit some synergistic action, or the signs of one toxin might predominate and lead one to overlook the presence of others. Chronic toxicity, which can lead to a predisposition of the animal to an infectious

Table 3.1

Representative Trichothecene Mycotoxins from Fusarium spp.

COMPOUND	R1	R2	R3	R4	R5	R6	Ref
<u>1</u> Verrucarol	H	OH	OH	H	H	H	-
<u>2</u> Diacetylverrucarol	H	OAc	OAc	H	H	H	3
<u>3</u> Trichodermol	H	OH	H	H	H	H	4
<u>4</u> Trichodermin	H	OAc	H	H	H	H	5
<u>5</u> Calonectrinol	OH	H	H	H	H	H	-
<u>6</u> Calonectrin	OAc	H	OAc	H	H	H	6
<u>7</u> 15-Deacetylcalonectrin	OAc	H	OH	H	H	H	6
<u>8</u> Scirpentriol	OH	OH	OH	H	H	H	7
<u>9</u> Diacetoxyscirpenol	OH	OAc	OAc	H	H	H	8-11
<u>10</u> Monoacetoxyscirpenol	OH	OH	OAc	H	H	H	7
<u>11</u> T-2 tetraol	OH	OH	OH	H	OH	H	12
<u>12</u> T-2 toxin	OH	OAc	OAc	H	a	H	11-13
<u>13</u> HT-2 toxin	OH	OH	OAc	H	a	H	11,12
<u>14</u> Neosolaniol	OH	OAc	OAc	H	OH	H	12,14
<u>15</u> Isoneosolaniol	OH	OAc	OAc	OH	H	H	15

COMPOUND	R1	R2	R3	R4	R5	R6	Ref
<u>16</u> Pentahydroxy-scirpene	OH	OH	OH	OH	OH	H	-
<u>17</u> Triacetoxyscirpendiol	OH	OAc	OAc	OH	OAc	H	16
<u>18</u> Diacetoxyscirpentriol	OH	OAc	OAc	OH	OH	H	15
<u>19</u> Trichothecolon	H	OH	H	H	R5=R6=O		-
<u>20</u> Trichothecin	H	b	H	H	R5=R6=O		5,17
<u>21</u> Nivalenol	OH	OH	OH	OH	R5=R6=O		11,18
<u>22</u> Fusarenone	OH	OAc	OH	OH	R5=R6=O		11,19-22
<u>23</u> Nivalenol diacetate	OH	OAc	OAc	OH	R5=R6=O		23,24
<u>24</u> Deoxynivalenol	OH	H	OH	OH	R5=R6=O		25
<u>25</u> 3-Acetoxydeoxynivalenol	OAc	H	OH	OH	R5=R6=O		25,26
<u>26</u> 15-Acetoxydeoxynivalenol	OH	H	OAc	OH	R5=R6=O		-
<u>27</u> Dihydrodeoxynivalenol	OH	H	OH	OH	OH	H	-
<u>28</u> Dihydrodiacetyldeoxynivalenol	OAc	H	OAc	OH	OH	H	-

a = $O-C(O)-CH_2-CH-(CH_3)_2$

b = $O-C(O)-CH=CH(CH_3)$

Zearalenone (ZEAR)

disease is even more difficult to diagnose. In that case, intoxication could go entirely unnoticed and simply be diagnosed as due to an infectious microorganism; thus the veterinary diagnostician has to be aware of the latter condition and must consider that there might be multiple causality, i.e. more than a single causal agent may be involved.

Fusarium species are not very resistant to environmental changes. In fact, with the exception of F. moniliforme which may be involved in advanced decay of high-moisture shelled corn, Fusarium does not grow in shelled grain after harvest. Even if the high moisture requirement (23% wet weight basis/30% dry weight basis) is met, Fusarium cannot compete with the other varieties of fungi that then take over. However, if high-moisture ear corn is stored in cribs then Fusarium that has become established on the ears in the field may be able to continue its growth. The major problem, therefore, can be expressed as a chemical one since although Fusarium present in grains harvest will die out relatively rapidly at the normal moisture content of commercial storage (12-15%) the toxic components it produced and contaminated the grains with will remain -- and those are the essence of the problem as in most cases, their

toxicological implications in man are not well defined as yet.

It must be noted that fungi are not the only sources of trichothecenes. In fact some have also been isolated from plants such as the brazilian shrub Baccharis megapotamica Spreng (Asteraceae). In these species however, the trichothecene nucleus bears a polyunsaturated macrocycle through its carbons C4 and C15 (e.g. $O-C(O)-CH=CH-CH=CH-C(O)-O-CH_2-CH_2-CH(CH_3)-CH(OH)-C(O)-O$ linked at C4 and C15 named verrucarín A. (after verrucarol, 1). That particular type of trichothecene is not covered in this dissertation and the reader is referred to particular papers added at the end of the current references relevant to this chapter (100-105) for further information on their isolation, structures, synthesis and biological significance.

3.1.2 Structural and Analytical Needs.

As shown in table 3.1, the trichothecenes (named after Trichothécium), possess the tetracyclic 12,13-epoxytrichothec-9-ene skeleton (see table 3.1 for numbering system). All of the naturally occurring 12,13-

epoxytrichothecenes known to date have at least one hydroxyl or ester function at C-3, C-4, C-7, C-8 and/or C-15. They are often divided into the following major groups:

- Group A: Trichothecenes that do not have a ketone function at C-8, e.g. diacetoxyscirpenol (DAS) (9)
- Group B: Trichothecenes with a ketone functionality at C-8, e.g. deoxynivalenol (DON) (24)
- Group C: Trichothecenes that have their positions at C-7 and C-8 linked together through an epoxide (7,8 epoxide), e.g. crotocol (30).

Each group can be further subdivided on the basis of the number of substituents, etc.

The majority of the mycotoxicoses reported to date were caused by trichothecenes of groups A and B. For the purpose of the present study, efforts will be directed strictly at the trichothecenes belonging to these two groups.

The trichothecenes offer some unusual structural characteristics. The stereochemical representation (figure 3.1) of deoxynivalenol (24, table 3.1), also called vomitoxin, indicates some interesting structural features. For instance, the epoxide is shielded against a backside attack from any nucleophile, which accounts, in part, for the stability of trichothecenes (they can be stored for a long time without any significant degradation) and for the fact that they remain unaffected under reflux conditions with various organic solvents and mild acidic conditions. However, the prolonged action of boiling water or highly acidic medium (pH below 3) leads to a skeletal rearrangement involving the opening of the 12,13 epoxide (27).

The available spectral data on the trichothecenes are very limited at this time. The infrared spectra provide useful information about the presence or not of various functional groups, although they are not very diagnostic.

DEOXYNIVALENOL, (VOMITOXIN), (DON)

Figure 3.1: Stereochemical configuration of deoxynivalenol (DON) based on a Dreiding model.

For example, the acetyl groups display strong absorptions at ca. 1720 cm^{-1} (C=O) and 1250 cm^{-1} (C-O). An absorption band at 1680 cm^{-1} found simultaneously with a medium-intensity one at 825 cm^{-1} can be taken as an indication of the presence of the trisubstituted double bond (C9-C10) and near infrared can be used to detect the presence of the epoxide group (absorption bands at 4545 and 6060 cm^{-1}) (28).

Ultraviolet spectroscopy is ruled out in several cases by the absence of a conjugated unsaturation. End absorption, due to the unsaturation at C-9, can be seen (ca. 200 nm). Also, trichothecenes belonging to the verrucarol-derived group that bear an unsaturated aliphatic chain at C-4 and C-15 (see table 3.1) give characteristic UV spectra (28).

Proton nuclear magnetic resonance (NMR) spectroscopy provides some diagnostic information in elucidating the skeleton and the stereochemistry of the trichothecenes as the latter exhibit characteristic chemical shifts and coupling constants. For example, the presence of an AB quartet in the region of 2.7 to 3.3 ppm with a vicinal coupling constant of ca. 4 Hz is diagnostic of the methylene protons of the epoxide ring (C-13). A strong singlet at

high field (ca. 0.85) is due to the methyl group attached at C-5 (protons on C-14). Because of allylic and long range coupling, with protons at C-10 and C-11 (J less than 1 Hz) respectively, the methyl protons on C-16 give a broadened singlet at ca. 1.75 ppm. Figure 3.2 presents the proton NMR spectrum of deoxynivalenol (24) where the above statements are clearly depicted. Trichothecenes having a substituent other than H at C-3 possess another characteristic feature in the doublet (or broad doublet, J ca. 5 Hz) centered at ca. 3.5 ppm due to the proton at C-2. Finally the proton on C-10, which can appear either as a doublet or a quartet, is centered at ca. 5.5 ppm. That signal is shifted downfield to ca. 6.6 ppm when a ketone is found at C-8. The chemical shifts remaining for protons at C-3 and C-4 vary considerably depending upon the types of substituents found at these positions (28, 29). It must be noted however that although several assignments of the proton NMR spectra have been reported, they were obtained at frequencies of 100 MHz or less, which did not allow for the full resolution of all of the coupling fine structure (13, 23, 25, 27, 30). Much work remains to be done in this area in terms of the complete characterization of the already isolated trichothecenes that are now available in a pure form. This type of work is underway in our laboratories (31).

Figure 3.2: High resolution proton NMR spectrum (250 MHz) of deoxynivalenol (24) recorded in chloroform- d_1

Carbon-13 NMR is still in its infancy with respect to trichothecenes (31), although it has been used successfully in secondary metabolism studies in fungi other than Fusarium (32-35) where it provided a suitable replacement for radioactive (tritium and/or carbon-14) labelling studies. Reports to date on the assignment of the Carbon-13 NMR spectra of trichothecenes were based upon low field spectra (25 MHz or less) (30) using technique such as off-resonance decoupling and single-frequency off-resonance decoupling (SFORD) (36, 37). These techniques can produce artefacts when the signal-to-noise ratio is poor and when the resonances are close to each other. This led to some disagreement around the assignment of the Carbon-13 chemical shifts of the 12,13 epoxytrichothecene skeleton (37, 38). Until very recently (31), the most definitive study on these assignments was from the work of Ellison and Kotsonis (39) and was based upon the Birdsall plot method of correlating proton- and carbon-13 chemical shift data (40).

While the use of Carbon-13 NMR is possible for the identification of metabolites in crude fungal extracts without the need for extensive chemical purification, proton NMR is also required for the full characterization of the structures of new metabolites. Figure 3.3 presents the

DEOXYNIVALENOL

(62.8 MHz ^{13}C)

Figure 3.3: Carbon-13 spectrum of deoxynivalenol (24) recorded at 62.5 MHz.

Carbon-13 NMR spectrum of deoxynivalenol (24) recorded at 62.5 MHz.

To obtain these trichothecenes is no easy task. The extraction, separation and purification steps are long and costly, both in time and in terms of possible sample loss. The quantities required for good Carbon-13 NMR analysis are fairly large whereas proton NMR requires less sample but asks for greater purity. Very often all of that separation work leads to the isolation of an already known metabolite, or yields quantities too small for full characterization, despite several weeks of chemical manipulations. It is impossible to rely strictly on basic chromatographic techniques, such as thin layer chromatography (TLC), to indicate, unequivocally, the presence, or not, of new trichothecene metabolites. Clearly a spectral technique that could allow for a quick screening, early in the separation step, for new or known trichothecenes would be an asset. The development of such a technique into a structural tool would be even more desirable. A strong candidate to achieve not one, but both of the goals mentioned above is mass spectrometry. This chapter of the dissertation describes such a study using fast atom bombardment (FAB) and linked-scanning techniques; these are first outlined.

3.1.3 Fast Atom Bombardment (FAB)

Fast atom bombardment (FAB) mass spectrometry was described in great detail in the preceeding section 2.1 (p.67 and following) to which the reader is therefore referred.

3.1.4 Linked-Scan Techniques.

Over the past fifteen years, organic mass spectrometry has been transformed by a series of truly remarkable developments. Among them we note the interfacing of both gas-liquid, and liquid chromatographs to the mass spectrometer, the development of quadrupole mass filters, that became relatively inexpensive, reliable and fast-scanning mass analysers, the introduction of a whole series of ionization techniques (such as those mentioned briefly in the previous section 2.2.1. p. 83), the extension of mass ranges and of an increase in scan speeds through new developments in laminated magnet technology and ion optics and, finally, the introduction of a full range of both,

hardware and software for computer-based systems. All of these together, gave such a "new look" to mass spectrometry that it might be hardly recognisable to someone who has not followed these trends as they arose over these years.

This section is concerned with just one aspect of only one of these developments that arose in the second half of that period of changes. The relatively new development has been termed in various ways such as mass separation/mass spectrometry or mass spectrometry/mass spectrometry (both offering the same acronym, MS/MS), two-dimensional mass spectrometry, tandem mass spectrometry, etc. The acronym MS/MS seems to be the most appropriate (for the above latter version) and is certainly the most widely used. The present discussion will deal almost exclusively with MS/MS techniques practiced on double-focussing mass spectrometers with a greater emphasis on instruments of so-called reversed geometry, as opposed to conventional geometry, mainly because this is the type of instrumentation used through the work involved in this project. The definitions and the nomenclature pertinent to these terms will be presented in the following paragraphs.

A mass spectrum, as normally recorded, is the result of several unimolecular dissociation processes. These can be represented by making an analogy to a genealogical tree such as that presented in figure 3.4. In a conventional mass spectrum, all of the genealogical information is lost. There exist, however, some empirical procedures that allow for the generation of such a connectivity scheme between the ions, once one variable has been chosen, namely a precursor ion, a fragment ion or a common neutral loss. Such a scheme can be seen as a two dimensional mass spectrum in which the ion current is now a function of two variables namely the m/z of both, the precursor and the fragment ions. This can explain, in part, why the acronym MS/MS was adopted; it is a direct analogy to GC/MS since in both cases the final characterization is carried out as by mass spectral technique, the difference lying in the initial separation of the species (i.e. gas chromatography versus mass spectrometry).

These MS/MS techniques can be performed on double-focussing sector instruments as well as on quadrupole or hybrid instruments (i.e. sector instrument to which is added one or several quadrupole). This thesis will concentrate on a quick survey of double-focussing sector instruments and

Figure 3.4: Hypothetical sequence ion fragmentations occurring in a mass spectrometer.

the reader is referred to references 42 to 48 for reports and accounts on the quadrupole and hybrid instruments used for MS/MS.

The main two extra advantages of double-focussing instruments over quadrupole and hybrids lie in the extended mass range and in the high resolution that can be obtained from such instruments. These factors alone can guide the choice in the purchase of a first instrument to be devoted to organic applications. The latter is even more important that accurate mass measurements can be performed on ions of the operator's choice prior/after the recording or linked-scan spectra, where the resolution is greatly reduced, thus offering the means to know very accurately the elemental composition of each peak of interest in the spectrum (some instruments offer a resolution of the order of 250 000, although it is difficult to operate them at a nominal resolution greater than 100 000 at 10% valley).

Figures 3.5, 3.6 and 3.7 present diagrams of the double-focussing instruments of so-called:

- i) conventional configuration (figure 3.5) (47)
- ii) Mattauch-Herzog configuration (figure 3.6) (49)

Figure 3.5: Schematic representation of a double-focussing mass spectrometer of conventional configuration (reprinted from reference 47 with permission of the copyright owner).

Figure 3.6: Schematic representation of a normal-geometry double-focussing mass spectrometer in the Mattauch-Herzog configuration.

Figure 3.7: Schematic representation of a reversed-geometry double-focussing mass spectrometer in the Nier-Johnson configuration.

- iii) the reversed geometry instrument in the Nier-Johnson configuration (figure 3.7) (50).

As it can be seen from these figures, an instrument of normal or conventional geometry is one in which the electric sector precedes the magnetic sector (i.e., an arrangement of the type: ion source-FFR-E-FFR-B-detector, where FFR stands for field free-region, E and B for electric and magnetic sector respectively) (figures 3.5 and 3.6), whereas the positions of these sectors are interchanged in an instrument of reversed geometry (figure 3.7) (i.e. arrangement of the type: ion source-FFR-B-FFR-E-detector).

It is not necessary to develop a full ion optics theory of ion trajectories in a double-focussing mass spectrometer to appreciate the fundamental principles involved in the development of linked-scan laws. For our purposes it is only necessary to use a one-dimensional model which considers only ions whose velocities are restricted to the main beam axis, usually denoted as the x-axis. In other words we ignore the other velocity components in the y-direction (i.e. the direction of the radial electric sector field and perpendicular to the direction of the magnetic field) and in the z-direction (the so-called slit height

direction). This approximation is acceptable however, if we consider the large difference in the kinetic energy ranges involved, nominally in the kilovolts for the x-axis versus a maximum of one volt for the y- and z-directions. The order of the two sectors does not affect the overall double-focussing action of the pair, the relatively small velocity dispersions for ions produced in the ion source can be fairly easily arranged to cancel exactly.

The magnetic sector creates a dispersion of ions according to their momentum-to-charge ratios, while the radial electric sector disperses them according to their ratios of kinetic energy-to-charge. In a one-dimensional model these results yield the following set of equations:

For the magnetic sector:

$$B (zev) = mv^2/R \quad (3.1)$$

$$mv/ze = BR \quad (3.2)$$

$$m/ze = B^2 R^2 / 2V \quad (3.3)$$

(m = mass, ze = electric charge, v = velocity of ion (in x-direction), R = magnet radius, V = accelerating potential).

For the electric sector:

$$mv^2/2 = zeV \quad (3.4)$$

$$E ze = mv^2/r \quad (3.5)$$

(E = electric field strength, r = radius or circular trajectory in E).

Equation 3.1 represents the equivalence of the force created by B over the beam of ions crossing the magnetic sector and the centrifugal force associated with the resulting trajectory. Equation 3.2 is simply a rearranged version of equation 3.1 whereas equation 3.3 is also a rearranged equation where the velocity was removed from the equation. This same equation is the basis for the use of the magnetic sector as a mass analyzer. Equation 3.4 is a simple expression of the kinetic energy that the ions acquire when accelerated by the potential V and equation 3.5 is the expression of the equivalence between the force exerted by the electric field on the moving electrons and the resulting centrifugal force.

The last information needed to understand the origins of the scan laws is the fact that the velocity of the fragments is the same as that of the precursor ion that

fragmented to produce them (that is if we ignore any contribution to the kinetic energy of the fragments from the internal energy of the precursor ion). This result is derived briefly below for any system such as that described by equation 3.6 and the associated classical mechanics equations 3.7, 3.8 and 3.9.

$$m_1 = m_2 + m_3 \quad (3.7)$$

$$m_1 v_1 = m_2 v_2 + m_3 v_3 \quad (3.8)$$

$$m_1 v_1^2 = m_2 v_2^2 + m_3 v_3^2 \quad (3.9)$$

Equation 6: Dissociative process

Equation 7: Conservation of mass

Equation 8: Conservation of linear momentum. Holds only if the dissociative process takes place in a FFR.

Equation 9: Conservation of kinetic energy. Holds only if the internal energies of the products equal that of the reactant prior to dissociation (i.e. a zero kinetic energy release process).

To be meaningful, m_1 , m_2 and m_3 must have real positive values and in the centre-of-mass system, the kinetic energy balance of equation 3.9 becomes 0 (i.e. the left term of the equation is 0). Then the only way to

satisfy equation 3.9 is for:

$$v_1 = v_2 = v_3 \quad (3.10)$$

If there is kinetic energy release however, the peak shapes will be affected but the basic findings will remain the same and the mathematical treatment is not necessary for experimental work to be performed adequately (51). This report will not make any use of the changes in internal energy (T) occurring during some dissociative processes. Reviews on the subject are available (52-53).

The method used to derive scan laws is a systematic procedure that can generate a large number of possibilities (47, 54-56). However, if we accept as feasible only the scan laws that meet the following two criteria:

- i) scan laws in which at most two of the three fields (V,B,E) are varied simultaneously --and--
- ii) scan laws where the functional relationships between the fields is relatively simple so that it can be performed accurately and precisely enough to be useful.

The recent review by Boyd (47) describes the mathematical derivation of all the scan laws (known to date) that meet these criteria. They are summarized in table 3.2.

Table 3.2*

Linked-Scan Laws For Double Focussing Mass Spectrometers

A. First Field Free Region Fragmentations**			
Preselected Mass	Linked-Scan Law	Velocity Discrimination	References
m_1	$(V/E^2) = V_o/E_o^2$ $B = B_1$	yes	57,58
m_1	$(B/E) = B_1/E_o$ $V = V_o$	yes	59-61
m_2	scan V only $E = E_o; B = B_2$	no	62
m_2	$(B^2/E) = (B_2^2/E_o)$ $V = V_o$	no	59
m_3	$(B/E) \times (1 - (E/E_o))^{1/2}$ $= (B_3/E_o)$ $V = V_o$	yes	63-65

Table 3.2* (end)

Linked-Scan Laws for Double Focussing Mass Spectrometers

B. Second Field Free Region Fragmentations***

Preselected Mass	Linked-Scan Law	Velocity Discrimination	References
m_1	scan E only $V = V_0; B = B_1$	no	66
m_2	$(B^2 \times E) = (B_2^2 \times E_0)$ $V = V_0$	yes ⁺	67
m_2	$(E/V^2) = (E_0/V_0^2)$ $B = B_2$	yes ⁺	67
m_3	$B^2 \times (1 - (E/E_0)) = B_3^2$ $V = V_0$	yes	67

* adapted from reference 47 (with permission of the copyright owner)

** valid for instruments of either configuration

*** valid only for instruments of reversed geometry

+ very high velocity discrimination

A few comments about table 3.2 are necessary at this point. The list of linked-scan laws found in that table is complete (although not exhaustive) with respect to the two criteria that were set previously. Furthermore for the case where several linked-scan laws yield exactly the same information, only the one that offers the most advantages was included. For example a linked-scan law of B^2/V can be derived (for preselected mass m_1 and fragmentation occurring in the second FFR). But since the operation would require both, control of the magnetic field and ion-source detuning and that none of these disadvantages applies to the widely used "MIKES" scan (mass analysed in kinetic energy spectrum, i.e. scanning E only, table 3.2B) it follows that the scan law was omitted from the table. Furthermore, scan laws accounting for successive fragmentations (e.g. first dissociative process in the first FFR and a subsequent one occurring in the second FFR) can also be derived (55, 56) but have not been included in table 3.2.

It is interesting to note the greater versatility of the reversed-geometry instruments over their conventional geometry counterparts. It lies principally in their ability to study dissociative processes taking place in the second FFR via linked-scan techniques (in conventional geometry

these fragmentations can only be studied in a fashion identical to metastable ion studies in a single focussing instrument)...

Finally it is important to stress (again) that if a particular scan law involves no velocity discrimination then the shapes of the peaks obtained by such a scan will reflect the full kinetic energy release distributions. This information can be of value but is obtained at the cost of resolving power (typically a few hundred) (52). That information would not be directly useful for the goals that are to be set in this study (section 3.3) and will not be discussed further here.

The two scan laws that were chosen for the purpose of this study are the B/E and the B^2/E . Reasons for this choice are numerous but the most important ones are their universality (applicable to instruments of either configuration) and their accessibility on commercial instruments without excessive extra costs.

The B/E Scan

If the standard dissociative process of equation 3.6

occurs in the first FFR and V_1 , B_1 , and E_1 specify the conditions that allow the transmission of m_1^+ . then:

$$\text{From equation (3.4): Energy of } m_2^+ = (m_2/m_1)V_1ze \quad (3.11)$$

$$\text{From equation (3.2): Momentum of } m_2^+ = (m_2/m_1)m_1v \quad (3.12)$$

In order to collect the m_2^+ ions, E must be adjusted to a value E_2 such that:

$$E_1/E_2 = m_1/m_2 \quad (\text{from equations 3.5 and 3.10}) \quad (3.13)$$

Similarly for the magnetic field B :

$$B_1/B_2 = m_1/m_2 \quad (\text{from equations 3.2 and 3.10}) \quad (3.14)$$

From the last two equations 3.13 and 3.14:

$$B_1/B_2 = E_1/E_2 \quad \text{or} \quad B/E = \text{constant} \quad (3.15)$$

The constant in equation 3.15 is determined by the preselected precursor ion m_1^+ . Hence a scan of B and E where the relation B/E is held constant yields all the daughter ions m_2^+ that arose from unimolecular dissociation of m_1^+ . This technique does not detune seriously the ion-source.

The B^2/E Scan

Considering the same dissociative process (equation 3.6) it is possible to rewrite equations 3.11 and 3.12 as follows (respectively):

$$\text{Energy of } m_2^+ = (m_2/m_1) v_{1ez} = m_2 v^2/2 \quad (3.16)$$

$$\text{Momentum of } m_2^+ = (m_2/m_1) \cdot m_1 v = m_2 v \quad (3.17)$$

Therefore, to collect the m_2^+ ions the following must be set:

$$\text{Electric sector: } m_2 v^2/r = E_2 z e \quad (3.18)$$

$$\text{Magnetic sector: } m_2 v = .B_2 R z e \quad (3.19)$$

Combining equations 3.18 and 3.19 yield:

$$B^2/E_2 = (m_2/z e) (r/R^2) = \text{constant} \quad (3.20)$$

The constant in equation 3.20 is determined by the preselected daughter ion m_2^+ . Hence a scan of B and E where the relation B^2/E is held constant yields all the precursor ions m_1^+ that unimolecularly dissociated to produce the daughter ion m_2^+ .

From figure 3:8 it is obvious that the various linked-scans of B and E are essentially different methods of investigating the intensity distribution within the B,E plane. Therefore they are not directly amenable to data system although alternative methods of obtaining this information have been developed that allow the collection of data by a data system that stores them for subsequent use (68-70). Lacey and Macdonald have fully discussed the various topologies these scans can yield when the proper set of three-dimensional coordinates has been used (61, 63, 71-76). The same authors have presented a review very recently on graphical aids and scanning modes for tandem mass spectrometry (77).

It was the choice of this author however to select a somewhat more conventional graphical representation of the linked-scan spectra recorded throughout this study. They are similar to those presented by Dawson and co-workers (44) and are thought to provide a clear view of the connectivity precursor-daughter ions within one whole mass spectrum (see section 3.5 for actual figures).

Figure 3.8: The B, E plane showing the relationship between the B/E (solid line) and the B^2/E (dashed curve) linked-scans.

3.2 PREVIOUS WORK

Past work involving linked scans are too numerous to review fully in this presentation and in most cases inapplicable to our needs for the chemical system under consideration here, the trichothecene mycotoxins. Instead the reader is referred to the most recent reviews on MS/MS available (47, 51). It should be noted however that at the time when this report is being written further developments in term of linked-scans are underway (78) and it can be expected that this will remain the case until all of the possibilities, in terms of fragmentation processes, can be covered by one or another scan law.

In terms of FAB, the previous chapter (p. 66 and following) without being exhaustive, offers a relatively detailed account of the technique as it was prior to this report and a good review of what had been done until then and the most recent indications (78) suggest that although several other classes of compounds have been studied under FAB conditions, little progress has been made with respect

4

to the theory underlying the fundamentals of the technique itself with regard to ionization. The present report is likely to be unique and represents, to the best of our knowledge, the most detailed account of a model developed to determine the factors governing ionization and fragmentation in FAB.

This section will now review all aspects of mass spectral work involving trichothecene mycotoxins since relatively little mass spectrometry work has been performed with these compounds. We also hope to demonstrate the novelty of the approach shown in this study which allows for a rapid screening of the mycotoxins and for their rapid unequivocal identification (once the mass spectral characterization has been carried out for each component).

The EI mass spectra of the trichothecenes are very complex no single characteristic cleavage pattern emerges for the family as a whole that would allow for the readily identification of the trichothecene ring system. The fragmentation patterns seems to vary between compounds and, at first sight, appears to be dependent upon the nature and the position of the ring substituents (27, 79). Only one feature, involving a proton or acyl transfer from C-4 to the

epoxide oxygen has been reported to be occurring in several trichothecenes (27, 79). This can explain largely why most reports to date deal with the direct probe EI spectra of trichothecenes (80) or with the detection of various synthetic derivatives (trifluoroacetate, trimethylsilyl ethers, heptafluorobutyrate, etc) and their indirect identification by comparing the spectra obtained from GC-MS analysis with those recorded from reference sample mixtures (28, 81-83). Such derivatization procedures are not the most desirable; our sister laboratory at Agriculture Canada was successful at developing capillary GC conditions that allowed for the GC/MS analysis of underivatized trichothecene mixtures (84). A review on the limited use of mass spectrometry for toxic substances, including some trichothecene mycotoxins is available (85) as well as an extensive compilation of EI mass spectra of mycotoxins (86). Despite a few papers reporting some promises for negative ions both under EI (87) and CI (88, 89) conditions, most of the work done to date is still using GC/MS; field desorption (FD) mass spectrometry has also been used (96) with no real advantages over other techniques. In other attempts to overcome the problems of gas chromatography of the trichothecenes (highly limited by the high polarity of several of them) and of their thermal decomposition under

direct probe EI conditions (86) Smith and Udseth have reported mass spectral data on trichothecenes when the latter were subjected to direct supercritical fluid injection (DFL-MS) conditions (91, 92) a technique interfacing super-critical fluid chromatography (SCF) to mass spectrometry. Previous attempts in that direction were made over 10 years ago (93) and more recently by Randall and Wahrhaftig (94). Although this latter group succeeded in their attempts, the complexity of their instrumental design and rather extensive solvent cluster formation reduced greatly the applicability of the technique to complex matrix mixtures. The design of Smith et al. (95) is more promising but the technique is still in its infancy and its future as a widespread routine analytical technique is still greatly hampered by its instrumental complexity (92, 95).

MS/MS studies involving trichothecenes are very few, most likely because of the previously mentioned complexity of the EI spectra. It is the view of this author that this is why most earlier MS/MS work was performed strictly as a means of detection of a given substance (48, 96) in the mixture or on other ionization modes (88, 92) where, of course, the fragmentation is greatly reduced thus facilitating the elaboration of the parent-daughter ions

V

relationship. Actually, only one MS/MS study, that of Brumley and co-workers (88), performed via a negative ion chemical ionization mass spectral analysis, attempted to link parent-daughter ions together and to assess some structures to the daughter ion, thus drawing the first formal (although incomplete) fragmentation patterns for trichothecenes. Their study (88), however, is of limited analytical value mainly because of the non-readily accessible techniques they used (hydroxide anion ionization, produced in situ by combining dinitrogen monoxide (N_2O) and methane (CH_4) (97) and chloride attachment spectra (98) where the chloride anion is also produced in situ from difluorodichloromethane (CF_2Cl_2)). Nevertheless, that report is a clear indication that it should be possible to extract the fragmentation pathways of trichothecenes under EI, no matter how complex they are.

3.3. Scope of this work.

This study has three main goals:

- A- To determine the fragmentation patterns of trichothecenes mycotoxins under electron impact (EI) mass spectral conditions.

B- To develop a rapid screening and characterization routine of the components of trichothecenes mycotoxins mixtures.

C- To investigate a novel approach for a definitive analytical procedure for trichothecene mycotoxins.

A brief overview of each of these goals follows.

A. Fragmentation Patterns

This study can be seen firstly as an academic problem although the spinoffs could be of great analytical importance. It consists in attempting to fully characterize the fragmentation patterns by which the trichothecenes dissociate. To achieve this, the linked scans of type B/E and B^2/E were chosen for the following reasons:

1. They are applicable to double-focussing instruments of either geometry (normal or reversed).
2. They apply to unimolecular dissociation reactions occurring in the first field-free region (most basic commercially available instruments require little or no modifications to perform them).
3. When combined together the data from these two linked-scan offer a means of ensuring that all the

peaks recorded are from the sample and not artefacts inherent to a particular linked-scan (47)

4. One of them, the B/E, scan offers relatively good resolution.

Once fragmentation patterns are clearly defined and well-established, it should be possible to use them in later studies as starting points for the definition of some structure-characteristic fragmentation pathways found in the EI mass spectra, which thus far was not possible due to the lack of any parent-daughter ion relations. The linked-scan data will establish such relations and will then allow for the direct comparison and the spectral analysis of all the higher-mass ions recorded in the EI mass spectra of trichothecene mycotoxins. For the purpose of this study, the mycotoxins used in linked-scan experiments, limited to a few representative structures:

- 9 (diacetoxyscirpenol, DAS),
- 12 (T-2 toxin, T-2),
- 13 (HT-2 toxin, HT-2),
- 21 (nivalenol, NIV),
- 24 (deoxynivalenol, DON),
- 25 (3-acetoxydeoxynivalenol, 3-AcDON)
- 28 (dihydroacetyldeoxynivalenol, DDD).

B- Screening of Trichothecene Mycotoxins

This part of the work was aimed at developing a means to quickly characterize mycotoxins and to ascertain the presence or not of individual species in a mixture. To achieve this goal use was made of FAB-MS, and the behaviour of various trichothecenes noted from the spectra recorded under such conditions. The rationale behind this procedure lies on the principle that the trichothecenes having a fairly rigid skeleton should exhibit a strong pseudo-molecular ion with limited fragmentation with the exception of the expected losses of water (or acetic acid) since the hydroxyl moieties (or acetyl) are basically the only "loose ends" that can be lost under such mild conditions. It is therefore expected that the FAB mass spectra of these mixtures should be extremely simplified when compared, for example, to their EI counterparts, where it is, for all practical purposes, impossible to determine unambiguously the presence, or not, of any one toxin in a mixture; the fragmentation being by far too extensive.

C- Development of a Definitive Analytical Procedure

This third and last goal finds its "raison d'être" in the previous two studies. The aim was to be able to make use of the simplified FAB spectra to determine the molecular weight of the components of trichothecene mixtures. It should then be possible to use that molecular weight information to return to the EI spectra of the mixture and to perform some linked-scan experiments on preselected ions. Repeating this procedure for the newly defined daughter ions (in the case of B/E scan) it should, in principle, be possible to extract the whole mass spectrum of a given trichothecene.

The present study limits itself mainly to the first two goals but attempts to identify the fundamentals and to propose an experimental route, to be followed in future studies towards the third goal.

3.4 EXPERIMENTAL

3.4.1 Mycotoxins

All biological materials used in this study were kindly provided by the Mycotoxin Group of the Chemistry and Biology Research Institute, Agriculture Canada, K.W. Neatby Building, Ottawa, ON, Canada, K1A 0C6. They were used as received without any further purification. Purity was ascertained by GC/MS or by monitoring the total ion current resulting from a programmed temperature increment, when using the direct probe inlet.

3.4.2 Linked-scan Spectra

Linked-scan spectra were recorded on a Finnigan MAT 312 double-focussing reversed geometry instrument using the direct probe inlet, operating at 70 eV. The acceleration potential was kept constant at a value of 3 kV and the electron multiplier was kept at 2.2 kV. When collision-

induced dissociation, in the first field free region, was necessary, it was performed by creating a molecular leak (air as a collision gas) in the field free region until a pressure of ca. 7×10^{-7} mbar was obtained, the pressure in the source being kept below 1×10^{-7} mbar. The hard copy display was obtained from a Fisher Instruments Recordall potentiometer-type recorder. Spectra were recorded between mass 50 and the preselected precursor (B/E) or daughter (B^2/E) ion. The scan rate was linear and was kept constant for all the spectra. The mass scale was recorded at the same scan rate, under the same linked-scanning conditions between masses 50 and 500 before and after the recording of linked-scan spectra so as to ensure the uniformity of scan rates and the precision of the mass scale.

3.4.3 FAB Spectra

Fast atom bombardment (FAB) mass spectra were recorded on a Finnigan MAT 312 double-focussing reversed geometry instrument coupled to a INCOS data system and mounted with a saddlefield atom gun (Ion Tech.). High purity research grade Xenon (Matheson) was used as the bombardment gas. The atom gun was operated at nominally 8 kV and the resulting

positive ions were extracted into the mass spectrometer (acceleration potential of 3 kV). The scan ranged from 110 to 1350 a.m.u. at the rate of 10 seconds per scan (electron multiplier at 2.2 kV). The samples were introduced onto the target source as solution, or dispersion, in glycerol. The glycerol used as a support matrix was obtained from Fisher Scientific and was not subjected to any further purification prior to its use in the analysis. Other support matrices were used as well (e.g. polyethylene glycol, ave. mol. wt. 200 = PEG-200) but did not prove to be of any greater value in any of the attempts made. On the contrary, generally speaking, the spectra contained a lot more peaks due to the solvent and they exhibited much more noise than for those recorded in glycerol.

3.5 RESULTS AND DISCUSSION

3.5.1 Linked Scan Data and Discussion.

Tables 3.3 to 3.9 summarize the mass spectral data obtained for various trichothecene mycotoxins when submitted to a B/E-type linked-scan. (Figure 3.9 depicts a typical

Table 3.3

Linked-Scan Data⁺ (B/E) for Deoxynivalenol (DON), 24

<u>m₁</u>	<u>m[*]</u>	<u>m₂</u>	<u>m₁</u>	<u>m[*]</u>	<u>m₂</u>	<u>m₁</u>	<u>m[*]</u>	<u>m₂</u>
	261	278		196	213		146	160
	239	266		178	203		143	158
	237	265	231	155	189	175	123	149
296	228	260		133	175		120	147
	209	249		115	163		98	131
	208	248					78	117
	180	231						
	161	218		183	200			
				164	189		140	151
	243	260		144	177	163	135	135
278	221	248	218	140	175		90	121
	118	181		122	163			
	96	163		102	149			
				84	135			
							117	133
265	232	248		176	189	151	100	123
				172	187		79	100
				169	185		55	91
	214	231						
	191	218	203	151	175		107	121
	165	203		131	163		105	119
249	143	189		106	147	135	101	117
	123	175		82	129		85	107
	107	163		67	117		82	105
	78	139					61	91
				147	163			
				126	151			
	215	231	181	104	137			
	192	218		81	121			
	166	203		66	109			
248	144	189						
	123	175						
	107	163						
	92	151						
	78	139						

⁺ all data were rounded off to the nearest integral value

Table 3.4Linked-Scan Data⁺ (B/E) for 3-Acetyl-Deoxynivalenol, 25

m_1	m^*	m_2	m_1	m^*	m_2
338	303	320	290	263	276
	270	302		214	249
	251	291		212	248
	249	290		184	231
	219	274 (?)		164	218
	200	260		159	215
	183	249		156	213
	182	248		151	209
	172	241		142	203
	158	231		139	201
	147	223		123	189
	122	203		106	175
	81	165			
291	262	276	248	215	231
	213	249	223	147	181
	211	248		119	163
	183	231			
	163	218			
	159	215			
	156	213			
	150	209			
	142	203			
	139	201			
	123	189			
	105	175			
	89	161			

⁺ all data were rounded off to the nearest integral value

Table 3.5

Linked-Scan Data⁺ (B/E) for Diacetoxyscirpenol (DAS), 9

m_1	m^*	m_2
306	277	291
	253	278
	226	263
	199	247
278	243	260
	199	235
	171	218
	144	200
	106	172
263	228	245
	139	191
233	204	218
	198	215
	157	191
218	189	203
	183	200
	164	189

m_1	m^*	m_2
203	169	185
	151	175
	82	129
200	171	185
	148	172
187	135	159
	146	160
175	120	145
	94	128 (?)
	76	115
	159	108
124	96	109
	74	96

⁺ all data were rounded off to the nearest integral value

Table 3.6

Linked-Scan Data⁺ (B/E) for HT-2 Toxin, 13

m_1	m^*	m_2
424 [#]	389	406
	369	396 (?)
	312	364
	296	354
	273	340
	262	333
	246	323
	245	322
322	213	262
	193	249
	169	233
	131	205
	128	203
263	228	245
	206	233
	186	221
	157	203
	123	180

⁺ all data were rounded off to the nearest integral value

[#] the sensitivity obtained for this spectrum was relatively poor; the data presented here are not exhaustive and care must be exercised when assessing the interpretative value of this particular scan.

Table 3.7

Linked-Scan Data⁺ (B/E) for T2-Toxin, 12

m_1	m^*	m_2	m_1	m^*	m_2
466 [#]	462	464	291	256	273
	302	375		183	231
	284	364			
382	261	316 (?)	278	243	260
	90	185		199	235
				171	215
				144	200
364	329	346	231	196	213
	285	322		178	203
	254	304		175	201
	233	291		148	185
	208	275		130	173
		63		121	
322	287	304	205	171	187
	212	261		153	177
				136	167
				71	121
304	275	289	185	158	171 (?)
	249	275		133	157
	224	261		111	142
	197	245		84	125
	179	233			
	144	209			
	133	201			
118	189				
			180	106	138
				81	121

⁺ all data were rounded off to the nearest integral value

[#] the sensitivity obtained for this spectrum was relatively poor; the data presented here are not exhaustive and care must be exercised when assessing the interpretative value of this particular scan

Table 3.8

Linked-Scan Data⁺ (B/E) for Nivalenol (NIV), 21

m_1	m^*	m_2	m_1	m^*	m_2
312	277	294	187	135	159
	234	270		106	141
	205	253		92	131
	177	235		89	129
253				73	117
	218	235	177		
	186	217		143	159
	178	212		125	149
	166	205		122	147
	138	187		100	133
	124	177		97	131
235	100	159		83	121
				77	117
	200	217		62	105
	179	205		60	103
	159	193	159		
	152	189		155	157
	149	187		130	144
205	133	177		125	141
	108	159		108	131
				105	129
	171	187		83	115
	153	177		69	105
	149	175		52	91
	130	163			
	123	159			
	108	149			
	103	145			
	85	132			
	67	117			
	56	107			

⁺ all data were rounded off to the nearest integral value

Table 3.9

Linked-Scan Data⁺ (B/E) for
Dihydrodiacetyldeoxynivalenol (DDD), 28

m_1	m^*	m_2	m_1	m^*	m_2
382	347	364	276	247	261
	324	352		197	233
	303	340		167	215
	271	322		142	198
	130	223		127	187
364	306	334	261	229	245 (?)
	285	322		187	221
	254	304		155	201
	233	291	223	181	201 (?)
348	294	320		147	181
	266	304		119	163
	238	288	187	158	172
	219	276		135	159
322	287	304		112	145
	265	292		109	143
	243	280		106	141
	182	242		89	129
304	275	289		72	116
	269	286			
	251	276			
	224	261			
	196	244			
	161	221			
	157	218			
	152	215			
	149	213			
	136	203			
	118	189			

⁺ all data were rounded off to the nearest integral value

Figure 3.9: Typical (B/E). Linked-scan spectrum.

example of such a scan). These data are best visualized by plotting them in three dimensions as shown in figures 3.10 through 3.16. The absolute intensities were not determined exactly as they are of no real value for the purpose of this study. The ion intensities presented in figures 3.10 to 3.16 are only representative of a limited scale constituted by the basic elements weak, medium and strong, and are depicted to give an overview of the relative intensities of the peaks and to provide a better field depth to the spectra. The latter is a characteristic that enhances favorably the visualization of the relationships between the various masses as established by the linked-scan data. The actual linked-scan presented in figure 3.9 however should suffice to provide the reader with a good feeling of the actual relative intensities. Furthermore, that same figure 3.9 demonstrates quite spectacularly the excellent resolution that was achieved during the course of this study when performing B/E-type linked-scan.

Linked-scan data such as those presented in figures 3.10-3.16 are self-explanatory and allow for the ready establishment of all the parent-daughter ion relationships. This study concentrated on the top half of the mass spectra

Figure 3.10: A three-dimensional image of the fragmentation pattern of deoxynivalenol (DON) as obtained from (B/E) linked-scans.

Figure 3.11: A three-dimensional image of the fragmentation pattern of 3-acetyl-deoxynivalenol (3-Ac-DON) as obtained from (B/E) linked-scans.

Figure 3.12: A three-dimensional image of the fragmentation pattern of diacetoxyscirpenol (DAS) as obtained from (B/E) linked-scans.

Figure 3.13: A three-dimensional image of the fragmentation pattern of HT-2 Toxin (HT-2) as obtained from (B/E) linked-scans.

Figure 3.14; A three-dimensional image of the fragmentation pattern of T-2 Toxin (T-2) as obtained from (B/E) linked-scans.

Figure 3.15: A three-dimensional image of the fragmentation pattern of nivalenol (NIV) as obtained from (B/E) linked-scans.

Figure 3.16: A three-dimensional image of the fragmentation pattern of dihydrodiacetyldioxynivalenol (DDD) as obtained from (B/E) linked-scans.

(from molecular weight down) corresponding to the primary fragmentation process. These parent-daughter ion relationships are expected to be exhaustive because of the high sensitivity that was achieved during the course of the study (figure 3.9) (remember that ions need not to be seen in a normal EI spectrum to be detected by the linked-scan method; refer to section 3.1.4, pp. 244-265 for derivations). Linked-scan of the B^2/E were performed only on those peaks corresponding to masses not seen in the conventional EI spectrum, and/or on those peaks that did not appear to be reasonable. In both cases the aim was to ascertain that the peaks recorded were not artefacts due to the linked-scan itself (47, 99). Full EI mass spectra of several trichothecene mycotoxins are included as appendices (Appendices SSSS through BBBB) for further reference. Appendix CCCCC is the full EI mass spectrum of zearalenone (ZEAR), the mycotoxin often encountered as a contaminant in deoxynivalenol samples.

It can be seen that it is possible to fully reconstruct the EI mass spectrum of the trichothecenes starting from the linked-scan data. This should serve as the starting point in assigning structures to the various daughter ions and in studying the relationship (if any)

between the mass spectral fragmentation and the structure of the trichothecene under study. A summary inspection of the EI spectra of DON, 3-Ac-DON and 15-Ac-DON, for example, strongly suggests that such a relationship exists. In fact the loss of 48 daltons (simultaneous elimination of water and formaldehyde) from the molecular ion of DON (Appendix SSSS) and from that of 3-Ac-DON (Appendix TTTT) but not from that of 15-Ac-DON (Appendix UUUU) where a loss of acetic acid is seen instead indicates that the locus of fragmentation occurs firstly at the volume defined by C6, C7 and their substituents. This statement holds true for the other trichothecenes (refer to Appendices). Figure 3.17 introduces the proposed mechanism by which the losses of water and formaldehyde occur simultaneously and highlights the locus of fragmentation. It is interesting to note that the simultaneous loss of two neutral molecules is peculiar in itself and that, to our knowledge, this is the first report to demonstrate it unequivocally in this type of molecule and to show its importance in mass spectral analysis of trichothecenes since the resulting ion (at m/z 248 for DON) is a major precursor of other daughter ions and is very intense in the spectrum.

Figure 3.17: (a) Highlight of the locus of primary fragmentation in the EI mass spectrum of DON and (b) the proposed mechanism for the simultaneous loss of water and formaldehyde from the molecular ion radical of DON occurring at that same locus.

The same ion also occurs via the sequential loss of water and of formaldehyde, but not the reverse, as can be concluded from the B/E scans of the appropriate ions; namely at m/z values corresponding to the molecular weight (M^+ 296 for DON) and to $(M - 18)^+$. (278 for DON). Further structure-fragmentation studies of this type are more likely to be performed now that the fundamentals of the fragmentation process have been demonstrated and that the various parent-daughter ion relationships have been fully characterized.

3.5.2 FAB Data and Discussion

The FAB spectra (positive ions recorded in glycerol) of individual trichothecene mycotoxins were deceptively simple (Appendices DDDDD through MMMMMM). In fact the spectra exhibit the pseudomolecular ion $(M + H)^+$ and for all practical purposes no fragmentation in the top half of the mass spectra, but for the loss of water (or acetic acid for the acetoxy-containing species).. The rest of the spectrum can be easily deciphered as it consists strictly in the spectrum of glycerol itself (at m/z corresponding to: $((n \times 92) + 1)^+$ where n is an integer) and to glycerol adducts of

the pseudomolecular ion (e.g. at $(M + 92 + 1)^+$). In terms of structural information, the spectra have no value but for an indication whether or not the trichothecenes under study possess or not an acetoxy group.

However, the simplicity of the spectra and the ability to detect the molecular ion (as $(M + H)^+$ and/or as $(M + 92 + H)^+$) even for the most unstable trichothecene such as T2-toxin, HT-2 and T2-tetraol (Appendices KKKKK, LLLLL and MMMMM respectively) are in themselves some rather interesting features in terms of detection studies. Furthermore, the fact that the spectra are so simple led us to attempt the introduction of a crude mixture of trichothecene mycotoxins as a solution/dispersion in glycerol directly into the mass spectrometer without proceeding to the standard isolation and purification steps that are so tedious. By this experiment it should be possible to perform a quick screening of a crude extract for the presence of trichothecene mycotoxins. A crude extract mixture that was simply pretreated by passing the total crude extract over a small column of silica gel so as to get rid of macromolecular biological material, chlorophyll and the likes, was analysed by FAB-MS. Figure 3.18 presents the data for that experiment. The spectrum is very clean with

Figure 3.18: FAB mass spectrum (positive ion in glycerol) of a mixture of trichothecene mycotoxins after a simple pre-separation step.

very little noise if we take into account the fact that several component mixtures usually tend to create artefacts due to intermolecular interactions they might suffer. The spectrum obtained has very good resolution and allowed for the ready identification of the trichothecene mycotoxins present in the mixture by direct comparison with the FAB spectra of the pure materials. In the case depicted in figure 3.18, the toxins present were found to be DAS (m/z 367), DDD (m/z 383 and 365), 3-Ac-DON (m/z 339). 15-Ac-DON was not present as indicated by the absence of m/z 321 recorded in the spectrum of a standard sample of 15-Ac-DON). In addition, culmorin (31) was found in the sample (m/z 477 corresponding to the protonated cluster of two molecules of culmorin (this was double-checked by a high resolution mass measurement yielding a molecular formula of $C_{30}H_{53}O_4$ for m/z 477; furthermore, culmorin exhibits a strong $(M + H - H_2O)^+$ peak at m/z 221 also present in the spectrum and corresponding to the elemental composition $C_{15}H_{25}O$ according to a high resolution mass measurement). Another substance that has not been identified as yet (m/z 323 and 263 from loss of acetic acid, thus suggesting an acetylated material). The FAB spectra of mixtures therefore appear to be a valuable source of information with respect to the detection of trichothecenes and to the quick identification of some members of that family.

3.5.3 Scope of Analytical Use: Suggestions for Future Applications

This study would not be complete without a brief discussion of the scope of these data and an overview of the field of applications they could bring about.

The example presented above allowed the identification of some trichothecene because the spectra of the latter under similar FAB conditions were available. Therefore the establishment of a library of FAB spectra of mycotoxins appears highly desirable. The problem resides mainly in the unequivocal identification of these trichothecenes and with the structure elucidation of novel ones for which FAB data are not available. The former problem can be circumvented by using high resolution mass measurements on the masses suspected to represent the pseudomolecular ions and by performing a linked-scan of the B^2/E -type on that same mass so as to ascertain that it is not a daughter ion. Both of these techniques have been performed in this laboratory without much difficulty despite some inherent problems with using them under FAB conditions (see section 2.1.1 p. 67 and following for a review of these problems). The latter

however requires much more work and normally one would return to more conventional techniques in order to isolate and purify the compound in question. This study however, suggests that there is a way by which one could obtain the maximum amount of structural information prior to performing any other wet chemistry. By following the steps itemized below, the unidentified substances could yield the maximum of structural information:

- 1) Record a conventional FAB spectrum of the mixture.
- 2) Assign all peaks already characterized by direct comparison with a library of FAB spectra.
- 3) Identify peaks due to "unknowns".
- 4) Record a linked-scan (B^2/E) on these peaks to ensure that they are pseudomolecular ions; if not calculate the pseudomolecular ion from the linked-scan data.
- 5) Determine the molecular weight from FAB data in 4) above.
- 6) Record conventional EI spectrum of the mixture.
- 7) Record a linked-scan spectrum (B/E-type) on the mass corresponding to the molecular ion of the unknowns whether the mass in question is observed or not in the spectrum recorded in 6) above.

- 8) Record a series of linked-scan spectra (B/E-type) on the daughter ions obtained from the data of 7) above.
- 9) Repeat for grand-daughter and great-grand-daughter ion (this should suffice).
- 10) Reconstruct, from the data in 7), 8) and 9) above, the EI mass spectrum of the unknown.

This scheme has one limitation - the quantity of material. In fact, linked-scans require time and therefore material. While that factor is not crucial in FAB measurements where the support matrix creates a reduced evaporation rate of the sample, it can be the dominant factor in EI spectra. For the purposes of this study, for example, as much as 1 mg was required to characterize T-2 toxin by linked-scan techniques and it was difficult, if not almost impossible, to fully characterize HT-2 because of this limitation. However, if we take into account the fact that within a time-span of 3 hours one can identify the components present in a fairly complex mixture, then it is obvious that further work in this direction is well worth the efforts and the sample consumed.

3.6 CONCLUSION

This study has achieved its three main goals and has set the foundations for further studies in the field of structure elucidation of trichothecene mycotoxins in addition to provide a good insight into mass spectral applications to the analysis of these compounds. Several conclusions can be drawn with respect to the three main goals that were set earlier in this chapter; they are summarized below:

A- Fragmentation Pattern

The linked-scan results presented in section 3.5.1 pp. 276-296 clearly demonstrate the power of the approach used to unequivocally establish the fragmentation patterns of trichothecenes. Prior to this study, these fragmentation patterns had resisted elucidation, mainly because of their complexity. Furthermore, the data gathered here should enable a detailed study aimed at defining some structure-

fragmentation pathway relationships which should further facilitate structure elucidation work.

B- Trichothecene Mycotoxins Screening

It was demonstrated that fast atom bombardment mass spectrometry is a method of choice for the rapid screening of complex mixtures for the presence, or not, of trichothecene mycotoxins. This conclusion meets our initial goal. Furthermore, our assumption that the FAB spectra of trichothecenes would be much simpler than their EI counterparts⁸ proved to be valid and hence FAB spectra can even allow the direct identification of several trichothecenes if they were previously characterized by FAB and by performing a simple B^2/E linked-scan on the suspected pseudomolecular ion (the original assumption was based on the model developed in section 2.5, pp. 139-200, of the previous chapter). These findings open the way for the elaboration of a FAB spectral data bank and for further studies involving complex biological matrices such as growth culture medium or the direct analysis of total blood samples.

C- Development of a Definitive Analytical Procedure

Finally, the combination of the conclusions above lead directly to the proposal for an experimental protocol aimed at the full characterization and, even structure elucidation. of any unknown trichothecene mycotoxin present in a sample to be analysed. This proposed experimental design, derived from the conclusions of this study, remains to be fully tested for more complex biological matrices and against the more conventional wet chemical methods currently used in natural product chemistry.

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EPILOGUE

"Je ne sais pas ce que j'ai représenté aux yeux des gens, mais quant à moi je n'ai été qu'un enfant, jouant sur la plage à la recherche d'un caillou plus rond que les autres ou d'un coquillage plus joli que les autres..."

*Sir Isaac Newton
(adaptation libre)*

Puissions nous trouver le courage de toujours nourrir cet enfant qui sommeille au plus profond de nous et, de ce fait, de lui permettre de croître davantage, et ce, sans jamais se retourner pour regarder derrière..."

APPENDICES

0891A1. 0 12-JUL-83 CAL:CAL STR:A. 88 SCAN = 0

0:31

0893A1. 0 12-JUL-83 CAL:CAL STR:A. 88 SCAN = 0

0:30

0890A1. 0 12-JUL-83 CAL:CAL STR:A. 88 SCAN = 0

1: 0

0892A1. 0 12-JUL-83 CAL:CAL STR:A. 88 SCAN = 0

0:31

0899A2: 0 12-JUL-83 CAL:CAL STR:R. 88 SCAN = 0
ME-A-GLU FIB/GLY NEG/ION

2:48

0897A2: 0 12-JUL-83 CAL:CAL STR:R. 88 SCAN = 0
ME-B-GLU FIB/GLY NEG

0:50

0899A2: 0 12-JUL-83 CAL:CAL STR:R. 88 SCAN = 0
PH-A-GLU FIB/GLY/H2O NEG

2:18

0899A2: 0 12-JUL-83 CAL:CAL STR:R. 88 SCAN = 0
PH-B-GLU FIB/GLY NEG

2:24

0891A2. 0 12-JUL-83 CAL:CAL STA:R. 86 SCAN 14
PM02PNA-GAL FAB/GLY NEG

1: 1

0893A2. 0 12-JUL-83 CAL:CAL STA:R. 86 SCAN 0
PM02PNA-GAL FAB/GLY NEG

2: 21

0890A2. 0 12-JUL-83 CAL:CAL STA:R. 86 SCAN 0
PM02PNA-GLU FAB/GLY/H2O NEG

3: 31

0892A2. 0 12-JUL-83 CAL:CAL STA:R. 86 SCAN 0
PM02PNA-GLU FAB/GLY NEG

2: 47

ES800A 0 20-OCT-83 CAL:CAL
 1-0-8-D-mannopyranosyl-L-erythritol
 FAB/SLY/H2O

STR: A. 00 SCAN = 0

0:27

ES800A 0 20-OCT-83 CAL:CAL
 1-0-8-D-galactopyranosyl-(1-3)-D-arabinose
 FAB/SLY/H2O

STR: A. 00 SCAN = 0

0:20

072401 0 07-JUN-83 CAL:CAL STA:R. 88 SCAN = 0

0:32

072402 0 07-JUN-83 CAL:CAL STA:R. 88 SCAN = 0

1:51

0722A 0 07-JUN-83 CAL:CAL STA:R. 88 SCAN = 0

2:34

0721A 0 07-JUN-83 CAL:CAL STA:R. 88 SCAN = 0

0:34

0823A 0 25-MAY-83 CAL:CAL STA:R. 88 SCAN = 0

0:28

0723A1. 0 07-JUN-83 CAL:CAL STA:A. 86 SCAN = 0

0:33

0723A2. 0 07-JUN-83 CAL:CAL STA:A. 86 SCAN = 0

2:11

0725A1. 0 07-JUN-83 CAL:CAL STA:A. 86 SCAN = 0

0:33

0725A2. 0 07-JUN-83 CAL:CAL STA:A. 86 SCAN = 0

2:18

E215A1 0 18-JUL-83 CAL:KCAL STR:A. BG SCAN = 0 0:27

E215A2 0 18-JUL-83 CAL:KCAL STR:A. BG SCAN = 0 1:38

F292A 0 13-FEB-84 CAL:CAL STR:A. BG SCAN = 0 0:27

F304A3. 0 14-FEB-84 CAL:CAL STA:A. 88 SCAN = 0

0:28

F280A 0 13-FEB-84 CAL:CAL STA:A. 88 SCAN = 0

0:28

F289A 0 13-FEB-84
S-A-8AL-GLU NEG/FAB/CLY

CAL:CAL

STR:A. 80 SCAN = 0

0:28

F281A 0 13-FEB-84
A-GLU-B-FRU NEG/FAB/CLY

CAL:CAL

STR:A. 80 SCAN = 0

0:27

F303A1. 0 14-FEB-84 CAL:CAL
3-A-GLU-FRU NEG/FAB/OLY

STR: A. 00 SCAN = 0

0:28

F200A 0 13-FEB-84 CAL:CAL
A-GLU-A-GLU NEG/FAB/OLY

STR: A. 00 SCAN = 0

0:28

F302A3. 0 14-FEB-84 CAL:CAL STA:A. 00 SCAN = 0

0:28

F305A1. 0 14-FEB-84 CAL:CAL STA:A. 00 SCAN = 0

0:27

F287A 0 13-FEB-84 CAL:CAL STA:R. 80 SCAN = 3
 8-8-GLU-GLU NEG/FAB/CI

0:24

F304A1 0 14-FEB-84 CAL:CAL STA:R. 80 SCAN = 0
 8-GAL-3-ARA POS/FAB/DEA

0:28

F302A3. 0 14-FEB-84 CAL:CAL
 9-A-GLU-FRU POS/FAB/DEA

STR:A. 00 SCAN = 0

2:38

F302A1. 0 14-FEB-84 CAL:CAL
 4-A-GLU-GLU POS/FAB/DEA

STR:A. 00 SCAN = 0

0:27

F305A3. 0 14-FEB-84 CAL:CAL STA:A. 86 SCAN = 0

2:38

F304A2. 0 14-FEB-84 CAL:CAL STA:W. 86 SCAN = 0

2:32

F303A2. 0 14-FEB-84 CAL:CAL STA:R. 00 SCAN = 0
3-A-GLU-FRU NEG/FAB/DEA

6:28

F302A2. 0 14-FEB-84 CAL:CAL STA:R. 00 SCAN = 0
4-A-GLU-GLU NEG/FAB/DEA

2:23

F30502 0 14-FEB-84
4-B-GLU GLU NEG/FAB/HE

CAL: CAL

STAR: A. BG SCAN = 0

0: 97

ES98A 0 20-OCT-83
PHE-8-0-LAC FAB/OLY/H2O

CAL: CAL

STAR: A. BG SCAN = 0

0: 28

ES98A1 0 20-OCT-83

CAL: CAL

STAR: A. BG SCAN = 0

0: 20

E801A 0 20-OCT-83 CAL:CAL
MALTOSE AC 189 FAB/BLT/H10

STA:A. 88 SCAN = 0

2:19

E599A 0 20-OCT-83 CAL:CAL
CELLOBIOSE-AC 185 FAB/BLT/H20

STA:A. 88 SCAN = 0

0:34

E599A1. 0 20-OCT-83 CAL:CAL

STA:A. 88 SCAN = 0

0:34

08228 0 25-MAY-83 CAL:CAL STA:R. 88 SCAN = 4

2:37

F30083 0 13-FEB-84 CAL:CAL STA:R. 88 SCAN = 8

0:28

F374A3 0 20-FEB-84 CAL:CAL STA:A 88 SCAN = 0

0:25

F373A1 0 20-FEB-84 CAL:CAL STA:A 88 SCAN = 0

0:25

F293A 0 13-FEB-84 CAL:CAL STA:A. 80 SCAN = 0

0:31

F380A1 0 13-FEB-84 CAL:CAL STA:A. 80 SCAN = 0

0:27

F374R1 0 20-FEB-84
MALTOSAC FMS/DEB/POS

CAL:CAL

STA:R. 86 SCAN = 0

0:27

F373R3 0 20-FEB-84
CELLOBIOSEAC FMS/DEB/POS

CAL:CAL

STA:R. 86 SCAN = 0

2:28

F300A2. 0 13-FEB-84
NEG/FAB/DEA

CAL: CAL

STA: A. 86 SCAN = 12

2: 8

F373A2. 0 20-FEB-84
CELLOBIOSE-AC FAB/DEA/NEG

CAL: CAL

STA: A. 86 SCAN = 0

0: 25

F3/482. 0 20-FEB-84
MALTOSE AC FOR/DEA/NEG

CAL: CAL

STA: A. 80 SCAN

12

2: 1

RAFA 0 08-JUN-83
RAFFINOSE/504 FAB/ELY

CAL: CAL

STA: A. 80 SCAN = 0

0: 52

F288A1 0 13-FEB-84
RAFFINOSE NEG/FAB/SLY

CAL:CAL

STR:A. 88 SCAN = 0

0:27

F371A 0 20-FEB-84
MELEZITOSE FAB/SLY/NEG

CAL:CAL

STR:A. 88 SCAN = 0

0:25

F372A 0 20-FEB-84 CAL:CAL
B-GLU-3-B-GLU-4-GLU FAB/GLY/NEG

STR:A. 88 SCAN = 0

0:24

E205A4 0 18-JUL-83 CAL:KCAL
STACHYOSE FAB/GLY

STR:A. 88 SCAN = 0

0:19

E20582. 0 18-JUL-83 CAL:KCAL STA:R. 86 SCAN = 0
 STACHYOSE FAB/SLY-THIOGLY

1:51

APPENDIX ITT

stachyose

E20583. 0 18-JUL-83 CAL:KCAL STA:R. 86 SCAN = 0
 STACHYOSE FAB/SLY-HACL

0:35

APPENDIX UAU

stachyose

29882. 0 13-FEB-84
RAFFINOSE NEG/FAB/DEA

CAL: CAL

STA: A. 88 SCAN = 0

0: 27

31281. 0 15-FEB-84
STACHYOSE FAB/DEA/++

CAL: CAL

STA: A. 88 SCAN = 0

0: 28

E521R1. 0 30-SEP-83 CAL:CAL STR:R. 88 SCAN = 0
Pos FAB/PEG 200

0:18

E521R2. 0 30-SEP-83 CAL:CAL STR:R. 88 SCAN = 14
Neg FAB/PEG 200

2:30

E522R. 0 30-SEP-83 CAL:CAL STR:R. 88 SCAN = 0
STACHYOSE FAB/PEG200/Pos

0:17

129582 0 13-FEB-84 CAL: CAL STD: 0 86 SCAN 0

1:17

129581 0 13-FEB-84 CAL: CAL STD: 0 86 SCAN 0

0:28

F11303 0 15-FEB-84 CAL: CAL STA: A. BG SCAN 0
8 SACCH FNB/OER/+

2:37

F11302 0 15-FEB-84 CAL: CAL STA: A. BG SCAN 0
8 SACCH FNB/OER/+

0:31

MASS SPECTRUM
02/28/84 10:33:00 + 0:22
SAMPLE: DIGITOXIN
CONDOS.: PAB (TGLY)

DATA: 84MAT201 #2
CALI: PEGFE22 #1

BASE M/E: 113 351
PIC: 2437110.

MASS SPECTRUM
02/27/84 11:25:00 + 0:44
SAMPLE: DIGOXIN
CONDOS.: FAB /TGLY

DATA: 84MAT196 #4
CALI: PEGFE22 #1

BASE M/E: 217
RIC: 3076090.

MASS SPECTRUM
02/14/84 11:29:00 + 0:55
SAMPLE: GITALINE
CONDOS.: FAB (GLY)

DATA: 84MAT154 #5
CALI: PEGJ420 #2

BASE M/E: 185
RIC: 681088.

352

MASS SPECTRUM
02/14/84 11:46:00 + 0:11
SAMPLE: GITALINE
CONDOS.: FAB (GLY-EA)

DATA: 84MAT155 #1
CALI: PEGJ420 #2

BASE M/E: 131
RIC: 1253378.

MASS SPECTRUM
12/20/83 11:45:00 + 1:06
SAMPLE: DIGITONIN
COND.: FAB (GLY)

DATA: 83MAT291 #6
CAL: PEGOE20 #8

BASE M/E: 185
RIC: 2916350.

MASS SPECTRUM
02/14/84 21:54:00 + 0:33
SAMPLE: FRONDO-A/OS
COND.: FAB (GLY)

DATA: 84MAT388 #3
CAL: PEGJ20 #2

BASE M/E: 115
RIC: 5306118.

MASS SPECTRUM
82/14/84 9:54:00 + 9:33
SAMPLE: FRONDO-A/05
COND.: FAB(GLY)

DATA: 84MAT158A 03
CALI: PEGJAZ8 02

BASE M/E: 115
RIC: 9306110.

APPENDIX KKKK

Selected FAB-MS⁺ data positive ions in glycerol
for psoluthurin A.

m/z	m/z
1327	583
1313	575
1311	559
1305	467
1289	451
1283	441
1267	425
1209	369
1107	279

E521A1. 0 30-SEP-83 CAL:CAL STA:A. 86 SCAN = 0
Pos FAB/PEG 200

0:18

APPENDIX PPPP

raffinose

E521A2. 0 30-SEP-83 CAL:CAL STA:A. 86 SCAN = 14
Neg FAB/PEG 200

2:30

APPENDIX QQQQ

raffinose

E522A STACHYOSE 0 30-SEP-83 CAL:CAL STA:A. 86 SCAN = 0
FAB/PEG200/Pos

0:17

APPENDIX RRRR

stachyose

E1878
00N

0 11 AUG 83 001 101

SIN 1 10- 01 11N

70

1-2357

MASS SPECTRUM
07/13/83 14:54:00 + 16:50
SAMPLE: TOKINS STD
CONDOS.: CAP. DBS, 20M, 150 TO 250 @ 5 C/MIN
ENHANCED (S 150 24 0T)

DATA: 03M1104 0404
CALI: PFKJUL13 83

BASE M/E: 98
RIC: 263600.

MASS SPECTRUM
07/13/93 14:54:00 + 16:07
SAMPLE: TOXINS STD
COND.: CAP. DBS, 20M, 150 TO 250 @ 5 C/MIN
ENHANCED (5 130 24 07)

DATA: 02MAY194 0387
CALI: PPKJUL13 93

BASE M/E: 90
RIC: 78288.

358

3228.

3228.

MASS SPECTRUM
07/13/93 14:54:00 + 17:05
SAMPLE: TOXINS STD
COND.: CAP. DBS, 20M, 150 TO 250 @ 5 C/MIN
ENHANCED (5 130 24 07)

DATA: 02MAY194 0410
CALI: PPKJUL13 93

BASE M/E: 124
RIC: 282144.

16804.

MASS SPECTRUM
03/02/83 15:53:00 + 16:51
SAMPLE:
CONDOS: GC OV-3 , 6', 170 TO 230 @ 3 C/MIN
ENHANCED (S 158 2N 0T)

DATA: 83MAT45A #289
CALI: FLOF28 03

BASE M/E: 163 360
RIC: 267264.

MASS SPECTRUM
03/02/83 15:53:00 + 20:11
SAMPLE: TRIACETYL UOH.
CONDOS: GC OV-3 , 6', 170 TO 230 @ 3 C/MIN
ENHANCED (S 158 2N 0T)

DATA: 83MAT45A 0346
CALI: FLOF28 03

BASE M/E: 163
RIC: 11632.

MASS SPECTRUM
07/13/83 14:54:00 + 22:15
SAMPLE: TOXINS STD
COND.: CAP. DBS, 20M, 150 TO 250 @ 5 C/MIN
ENHANCED (S 150 2M 0T)

DATA: 83MAY184 0534
CALI: PFKJUL13 83

BASE M/E: 188
RIC: 130400.

361

MASS SPECTRUM
12/19/83 11:22:00 + 1:10
SAMPLE: DOM
COND.: FAB (GLY)
010 TO 016 AVERAGED

DATA: 83MAY287 013
CALI: T5T2 83

BASE M/E: 185
RIC: 386368.

MASS SPECTRUM
12/28/83 14:38:00 + 1:17
SAMPLE:
COND.: FAB (GLY)

DATA: 83MAT294 87
CALI: PEGDE20 88

BASE M/E: 185
RIC: 4751358.

362

MASS SPECTRUM
12/15/83 14:10:00 + 0:20
SAMPLE: 15-A-DON
COND.: FAB GLYCEROL

DATA: 83MAT283 85
CALI: PFKDE15 83

BASE M/E: 185
RIC: 632832.

31456

55

OF / DE

MASS SPECTRUM
03/21/84 9:07:00 + 0:22
SAMPLE: DHD
COND.: FAB (GLY)

DATA: 84MAT238 #2
CALI: PEGFE22 #1

BASE M/E: 185
RIC: 2396158.

363

68488.

68488.

MASS SPECTRUM
12/15/83 13:34:00 + 1:28
SAMPLE: DDD
COND.: FAB GLYCEROL
#21 TO #23 AVERAGED

DATA: 83MAT281 #22
CALI: PFDIE15 #3

BASE M/E: 185
RIC: 347648.

8944.

8944.

MASS SPECTRUM
12/19/83 11:43:00 + 0:42
SAMPLE: NIVALENOL
COND.: FAB (GLY)
#5 TO #10 AVERAGED

DATA: 83MAT288A #7
CALI: TST2 #3

BASE M/E: 185
RIC: 345088.

364

MASS SPECTRUM
12/15/83 13:56:00 + 1:32
SAMPLE: DAS
COND.: FAB GLYCEROL
#21 TO #25 AVERAGED

DATA: 83MAT282 #23
CALI: PFIDE15 #3

BASE M/E: 185
RIC: 751616.

MASS SPECTRUM
01/04/84 15:10:00 + 0:11
SAMPLE: HT-2
COND.: FAB (GLY)

DATA: 84MAT7 #1
CALI: PEGJ4 #2

BASE M/E: 185
RIC: 10027000.

365

404490.

404490.

MASS SPECTRUM
12/15/83 11:45:00 + 0:16
SAMPLE: T-2
COND.: FAB GLYCEROL
#3 TO #6 AVERAGED - #30 X1.00

DATA: 83MAT279 #4
CALI: PFYDE15 #3

BASE M/E: 185
RIC: 261632.

11392.

277/BASE
=0.1385

3796.

MASS SPECTRUM
01/04/04 15:33:00 + 0:11
SAMPLE: T2-TETRAOL
COND: FAS (GLY)

DATA: 84MAT8 #1
CALI: PEGJ4 #2

BASE M/E: 185
RIC: 1769470.

366

END

2	9	H	1	0	H	8	5

FIN