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STUDIES OF SAPONIN STRUCTURES FROM:
A. ECHINODERMS OF THE ST-LAWRENCE ESTUARY
B. PHYTOLACCA DODECANDRA.

by

© Jacqueline M. R. Bélanger, B.Sc., B.Ed., M.Sc.
(Université de Moncton)

A Thesis submitted to the Faculty of Graduate
Studies and Research in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy.

Department of Chemistry
Carleton University,
Ottawa, Ontario
February, 1984

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ABSTRACT

Saponins from some echinoderms of the St-Lawrence estuary comprising the species Cucumaria frondosa, Psolus phantapus, Asterias vulgaris/Asterias forbesi, along with saponins from a terrestrial plant, Phytolacca dodecandra, were isolated and purified using up to date techniques in liquid chromatography. Structural elucidation work was conducted on the underivatized saponins and partial or complete structures are reported for frondoside J, frondoside A, asterosaponin C, and Endod samples from the type-3 and the type-unknown of P. dodecandra.

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DEDICATION

A la mémoire de mon père

*"A tous ceux pour qui la science
représente un espoir"*

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PREFACE

The marine world is out there, waiting to be explored and exploited. Although the number of compounds from the sea has more than doubled since 1973, year at which the first compendium was completed, much remains to be done. Many species remain to be studied, and the best part of it all is that many new compounds of marine origin have demonstrated possibilities in being biologically active. One can only hope that the progress of science will lead to a better future for humanity.

PART A

STUDIES OF SAPONINS STRUCTURES FROM:

ECHINODERMS OF THE ST-LAWRENCE ESTUARY.

CHAPTER 1

HOLOTHURINS: SOME GENERAL COMMENTS.

1.0 INTRODUCTION

It is estimated that about one million animal species live on earth, comprising thirty different phyla. Among these, half a million species are believed to live in the aquatic environment (1). It is therefore not surprising to see that natural product chemists, in their continuous search for new organic compounds, have in recent years, turned their attention towards the sea. There is always the hope that maybe one day, one of these new compounds will demonstrate some biological activity useful to mankind.

In 1979 (1), no more than 50 marine toxins had been discovered whose molecular structure had been elucidated and whose pharmacological activities had been explored. The availability of new materials from the sea is therefore likely

and in the context of this work, the examination of new species of sea cucumbers, in the search for new animal saponins should be fruitful.

1.1 HOLOTHURINS FROM SEA CUCUMBERS.

The general name "holothurin" is used to mean any sea cucumber saponin. Saponins are water soluble natural products, composed of a carbohydrate side chain attached to a triterpenoid (or steroid) aglycone. Although saponins are very often encountered in terrestrial plants, in the animal kingdom they have been found only in the marine phylum Echinodermata.

Sea cucumbers are sedentary, slow and fleshy creatures. According to Hyman (2) the only predator of sea cucumbers is man, although Bakus (3) mentioned that sharks occasionally consume the species Stichopus cloronotus. Sea cucumbers are distinguished from other echinoderms by the absence of a calcarous formation on their external surface.

The living echinoderms can be divided into the five following classes: Crinoidea (sea lilies), Holothuroidea (sea cucumbers), Echinoidea (sea urchins, sand dollars and heart urchins), Ophiuroidea (brittle-stars and basket stars), and Asteroidea (starfishes). Figure 1 (p. 5) schematically summarises these classes. There is evidence that only members of the Holothuroidea and Asteroidea classes possess saponins (4). It is to be noted that the saponins of sea cucumbers (holothurins) have a triterpenoid aglycone, whereas the saponins of starfish (asterosaponins) have a steroidal aglycone.

In some sea cucumbers, the major source of the toxic holothurins are found in the Cuvier glands of the animal, who when disturbed, eject this substance through its anus, this being part of its defense mechanism. Although the very high concentrations of holothurins are within these Cuvierian tubules (5) all parts of a sea cucumber's body contain saponins. In addition many very toxic species (e.g., Holothuria atra and Stichopus japonicus) do not possess any Cuvierian tubules.

FIGURE 1**ECHINODERM PHYLUM****PHYLUM:****Echinodermata****SUB-
PHYLUM:****Eleutherozoa****Pelmatozoa****CLASSES:****Asteroidae****Echinoidae****Holothuroidae****Ophiuroidae****Crinoidae****COMMON
NAMES:****Starfish****Sea
Urchin****Sea
Cucumber****Brittle
Star****Sea
Lily**

The saponin level contained in the Cuvierian tubules of these animals is not constant. Seasonal studies (6,7) have shown a marked elevation of saponin levels during the warmer months and the reproductive period of the animals. Presumably, the female gametes and the larvae stages are protected against predation by high holothurin levels.

Sea cucumbers of the same species, but collected from different locations have also shown differences in the amounts of saponins they contain, along with the composition of those saponins (8,9,10). Although the aglycone part of the saponin does not seem to vary from place to place, variations are noted in the sugar content. An example of this is holothurin A (1) which was the major saponin found in the species Holothuria atra, that was collected at Mara-kei Island, Gilbert Islands. Other specimens of the same species, which were collected in New Guinea, were reported to contain only holothurin B (2), which has two sugar units less than holothurin A (9).

1.2 DISTRIBUTION OF SAPONINS IN SEA CUCUMBERS.

Sea cucumbers are found worldwide and at different

2 X = H, holothurin B

1 X = R, holothurin A

depths in the sea. A recent review on Echinoderm saponins by ApSimon and Burnell (4) covers a range of at least 74 different holothurins for which either the aglycone or the complete structure are reported in the literature.

A close examination of these data indicates that holothurin A or B, or both compounds, are present in almost all species of the Holothuroidae family. The four exceptions are

3 Seychellogenin

$C_{30}H_{46}O_3$, MW 454

mp 234° - 238° C, $[\alpha]_D -70^{\circ}$

(Roller *et al.*, 1969)

from the genus Bohadchia. The sugar composition of these four holothurins was the same as for holothurin A (1), but

the predominant aglycone was seychellogenin (3). A group of these saponins has provisionally been designated holothurin C by Elyakov (9).

Elyakov (9) studied 41 species covering the families Holothuroidae, Stichopoidae, Cucumariidae and Synaptidae. Neither holothurin A or B were detected in the last three families, instead stichoposide A (4) and B (5) were detected in Stichopus japonicus and stichoposide B was also present in Astichopus multifidus after hydrolysis of the saponins. Structure 5 is an acid hydrolysis product that is probably not a genuine aglycone. Neither holothurins nor stichoposides were detected in four species of the family Cucumariidae, i.e. C. japonica, C. frondosa, C. faudatrix and Afrocucumis africana. The saponins derived from these species were all similar in their sugar composition, i.e. quinovose, xylose, glucose, 3-O-methyl glucose but differed in their aglycone moiety.

4 R ; QUINOVOSE - XYLOSE - GLUCOSE - 3-O-METHYL GLUCOSE
and SULPHATE GROUP (1:1:1:1)

AGLYCONE IS HOLOTOXIGENOL (6)

5 R ; QUINOVOSE - XYLOSE - GLUCOSE
3-O-METHYL GLUCOSE

Only one species of the Psolidae family has been studied so far (11). The holothurin, psoluthurin A, from Psolus fabricii, whose carbohydrate side chain bears the four equal sugars found in these saponins, has holotoxi-

6 Holotoxigenol

$C_{30}H_{44}O_4$, MW 468

genol (6) as its aglycone. It was the first example reported of an holothurin bearing two sulfate groups in its sugar side chain.

From the above observations, it can be concluded that family specificity exists in relationship to the occurrence of saponins extracted from species of different families and collected from various parts of the world.

1.3 THE TOXICITY AND PHYSIOLOGICAL ACTIVITY OF SEA CUCUMBER SAPONINS.

The poisonous properties of sea cucumbers have been known for many years and probably even centuries (2,3,12). The first report on this kind of toxicity dates back to 1880, when Cooper (12a) reported that certain species of sea cucumbers could liberate tenacious filaments from their visceral cavities, which on contact with human skin could cause inflammation. Saville-Kent (13) reported an incident involving the poisoning of many Chinese people who consumed the Australian species of the sea cucumber Stichopus variegatus. This observation was contradicted by Cleland (14) who claimed that sea cucumbers were not poisonous. Castellani and Chalmers (15) and Moru (16) reported that a Polynesian species of sea cucumber, Holothuria argus could cause dermatitis or blindness when put into contact with human skin or eyes. On the other hand, Clark (17) insisted that the Cuvierian organ, that was believed to contain large amounts of saponins, was totally harmless. Other reports (1) claim that sea cucumbers cause nausea in humans and this toxic effect is due to the presence of saponins in the animal. The first studies on the toxicity of sea cucumbers were reported by Yamanouchi (18) in 1929, when he

observed 100% fish mortality when a water extract from the body walls of the sea cucumber Holothuria vagabunda was placed in the aquarium. Very dilute solutions from different species of sea cucumbers were also proven to be fatal to several species of marine and freshwater fish (3, 5, 8, 19-22). A toxicity screening of 27 species of sea cucumbers (23, 24) from Japan and Palau reported that ichthyotoxicity was found in 24 species and hemolytic activity in 22 of them.

Yamanouchi (23-25) also examined the toxicity of dried sea cucumber products and concluded that the toxicity was too low to cause poisoning. This suggested that large amounts of saponins were probably lost during the processing of the animals. Sea cucumbers are commonly eaten in the South Pacific (e.g. Palau), in Japan and in China.

Nigrelli (5) also observed that the Bahamian sea cucumbers Actinopyga agassizi are highly toxic to fish and independently of Yamanouchi, he proposed the name holothurin for the toxin. Moreover, he observed that this toxin was

mostly concentrated in the Cuvierian organs of the animals. The general toxicity of dilute holothurin solutions has been demonstrated with coelenterates (19, 25), the planarian Dugesia tigrina (26), nematodes (19), annelids, mollusks (19,25), crustaceans (25), Drosophila larvae (27) and frogs (25).

Among the many reports dealing with the toxicological and pharmacological effects of holothurins, holothurin A (1) seems to be the saponin most often employed in those experiments. There also seems to be a very marked difference between the sulfated and desulfated saponins. For example, holothurin A (1) was reported by Friess et al. in 1967 (28) to elicit toxic signs such as convulsions, stiffness of the limbs, impairment of respiration and more, only a few minutes after administration to laboratory animals. On the other hand, desulfated holothurin A, which has approximately the same LD₅₀ as holothurin A, provoked those signs only after a period of many hours. Although holothurins are highly toxic to animals, Yamanouchi (25) explained that the reason why they could be eaten with impunity by humans is probably due to the fact that they are rapidly decomposed by acid in the stomach.

Sea cucumber saponins have been shown to possess hemolytic activity on mouse, rabbit and human erythrocytes (18, 19). Part of the general toxicity seems to be ascribed to the surface activity of the saponins. Sea cucumbers are not themselves affected by their own toxin, possibly because there is no complexation with membrane cholesterol taking place in these organisms. It is therefore not surprising that, like starfish, sea cucumbers have predominantly Δ^7 -sterols (29-32) for which the saponins may have less affinity (33).

Holothurins have been reported to possess low antibacterial activity (19, 34) but some positive results against specific kind of parasites (35) have been reported. Holotoxin mixtures from Stichopus japonicus were shown to be active against a variety of fungi (34, 36). In a clinical test, a holotoxin extract improved symptoms in 77 of 88 cases of superficial dermatophytosis (34). Members of the genus Bohadschia (family Holothuroidae) and of the family Stichopodidae were generally more active against a variety of fungi than were other members of Holothuroidae and Cucumariidae (37).

Gametes or fertilized eggs from sea urchins (38) when exposed to holothurins demonstrated abnormalities in their subsequent development. The most common abnormality encountered was animalization of the larvae, which is believed to be a consequence of interference with protein metabolism (22, 38).

Antineoplastic (P-388 leukemia) activity was reported for some holothurins such as stichostatin 1 from Stichopus chloronotus and thelenostatin 1 from Thelenota ananas (39). Holothurins from Actinopyga agassizi suppressed the growth of sarcoma 180 in vitro (40). Holothurin A, the thelothurins, actinostatin 1 from Actinopyga mauritiana, and extracts from Holothuria mexicana and Astichopus multifidus were all cytotoxic toward epidermal carcinoma (KB) cells (8, 20, 22, 39). Neurotoxicity studies were carried out by Friess et al. (41, 42). In some of their experiments they showed that holothurin A blocks transmission in nerve preparations from frogs and toads, and that the effect is irreversible, contrary to conventional agents such as procaine, cocaine and physostigmine.

CHAPTER 2

AN OVERVIEW OF SEA CUCUMBER SAPONINS.

2.0 INTRODUCTION

Holothurins are triterpene glycosides, which upon acid hydrolysis liberate monosaccharides and sulfuric acid in different ratios, along with an aglycone. The four monosaccharides usually encountered are: D-xylose (7), D-glucose (8), 3-O-methyl glucose (9) and 6-deoxy-D-glucose (10) (also referred to as quinovose).

7

8 R=H
9 R=CH₃

10

The aglycone is characterised by a γ -lactone ring attached to ring D of a triterpene tetracyclic system, of the lanostane type. Various modifications are observed in the side chain of the aglycone as well as in the basic skeleton.

2.1 GENERAL STRUCTURE AND NOMENCLATURE.

11 Holostane

The nomenclature used here is that of Habermehl and Volkwein (43). Because the lactone ring is common to all known sea cucumber triterpenoids, this nomenclature is based

on "holostane" [20 S-hydroxy-5 α -lanostane-18-carboxylic acid (18 $\rightarrow$ 20) lactone] (11). The stereochemistry is also consistent with recent NMR and x-ray studies. This nomenclature will be used throughout this thesis, unless otherwise indicated.

2.2 PREVIOUS WORK

INTRODUCTION

Chanley et al. (44) first isolated holothurin A (1) from Actinopyga agassizi. Upon acid hydrolysis, it released a mole of sulfuric acid, along with one mole each of D-glucose, D-xylose, quinovose and 3-O-methyl glucose. The steroidal aglycones released had a lanostane-type skeleton and were identified as 22,25-oxidoholothurinogenin (12) and its 17-deoxy derivative (13) whose names are derived from a parent compound, holothurinogenin (14)).

In the meantime, Yasumoto et al. (45) were working on the species Holothuria vagabunda and H. lubria and

12 22, 25-Oxidoholothurinogenin

$C_{30}H_{44}O_5$, MW 484

mp 315.2° – 315.8° C, $[\alpha]_D^{25}$ -21.2° ($CHCl_3$)

(Chanley *et al.*, 1966)

13

$C_{30}H_{44}O_4$, MW 468

mp 285.8° – 286.4° C, $[\alpha]_D^{25}$ -9.3°

(Chanley *et al.*, 1966)

14 Holothurinogenin

$C_{30}H_{46}O_4$, MW 470, mp $277^{\circ}C$

(Habermehl and Volkwein, 1970)

managed to isolate holothurin B(2). Acid hydrolysis of holothurin B released only some D-quinovose and D-xylose as its sugar constituents. Complete structures of Holothurins A and B are reported in section 2.6.

Several other species of sea cucumbers have been studied. The elucidation of structure of their triterpenoid aglycone showed that they were all related to the lanostane skeleton (1, 4, 47, 48).

Today, 38 different triterpenoids, obtained from sea

cucumber saponins are known (4) (Chart 1, Appendix p. 264). It is possible that a majority of those are artifacts obtained by the strong acid hydrolysis of the naturally occurring saponins. Acid hydrolysis often causes double-bond rearrangements, dehydration, or alkylation of hydroxyl groups (e.g. the use of methanol as a co-solvent leads to methylation). Triterpenoids 12 and 13 have now been obtained from many species of sea cucumbers. They were the first of a series of compounds differing only by the substitution in their side chain.

2.2.1 17 α -HYDROXY SERIES

In the 17 α -hydroxy series, holothurinogenin (14) was isolated from Holothuria polii by Habermehl and Volkwein (51). It is the simplest one of the series with no functional group in the side chain.

Griseogenin (15) the 22-hydroxy analog of 14, was isolated from Halodeima grisea by Tursch et al. (52). Praslinogenin (16) was isolated also from H. polii (51) and as a

15 Griseogenin

$C_{30}H_{46}O_5$, MW 486

mp 285° – 287° C, $[\alpha]_D^{26} -22^{\circ}$ ($CHCl_3$)

(Tursch *et al.*, 1967)

16 Praslinogenin

$C_{31}H_{48}O_5$, MW 500

mp 290° – 291.5° C

(Tursch *et al.*, 1970)

minor constituent from Bahadschia koellikeri by Tursch et al. (53). The Δ^{25} compound (17), was isolated from Actinopyga agassizi by Chanley and Rossi (54) and from Bohadschia vitiensis by Clastres et al. (55). (It is to be noted that no physical or spectroscopic data for 17 have been published.)

2.2.2 17-DEOXY SERIES

In the 17-deoxy series, seychellogenin (3) is the simplest one, with no side chain functionality. Seychello-genin was isolated from the Cuvier glands of Bohadchia koellikeri by Roller et al. (46). From the body walls of the same species, koellikerigenin (18) and its methyl ether derivative ternaygenin (19) were also isolated (46). Ternay-genin was later also found in H. polii (51). However, koellikerigenin was converted under acid hydrolysis conditions (MeOH/HCl) to ternaygenin. This led Tursch et al. (53) to speculate that pralinogenin might also be arising from a 25-hydroxy compound. However, on the basis of an experiment by Chanley and Rossi (54) it seems that it is probably arising from a $\Delta^{25(26)}$ precursor.

2.3 HOLOTHURINOGENINS VERSUS LANOSTEROL.

Roller et al. (46) correlated seychellogenin (3) with lanosterol (20) by a series of chemical transformations, to

18 Koellikerigenin
 $C_{30}H_{46}O_4$, MW 470
 mp 213° - $214^{\circ}C$, $[\alpha]_D -8^{\circ}$
 (Roller *et al.*, 1969)

19 Ternaygenin
 $C_{31}H_{48}O_4$, MW 484
 mp 239° - $242^{\circ}C$, $[\alpha]_D 2^{\circ}$
 (Roller *et al.*, 1969)

the common intermediate (21) . Scheme 1 (p. 28) describes these transformations.

This interconversion confirmed the correctness of structure 3 and of all other holothurinogenins (except for the configuration at C-20 , which was determined later; 1975-76) .

2.4 ARTIFACTS OF HOLOTHURINS.

Natural glycosides do not have the 7:8, 9:11 diene system. That was known from the beginning, since the natural holothurins lacked the ultraviolet absorption at 244 nm, characteristic of a heteroannular diene system that was invariably present in the genins. Chanley and Rossi (54) proved that the diene system was generated during acid treatment of the holothurins. These workers also investigated the authenticity of the 22,25-oxido bridge found in

SCHEME 1

12, 13, 22 and 23. Hydrogenation and methanolysis experiments showed that the 22,25-oxido moiety was genuine. They also examined a series of 25-oxygenated triterpenoids (i.e. 16, 18, 19) and concluded that the 25-methoxy moiety was the result of the addition of methanol (or water) across a 25(26) double bond (54).

Since holothurin A contained only one methoxy group (as 3-O-methyl-D-glucose), the methoxy group(s) of the neo holothurinogenins must have arisen during methanolysis. Enzymatic hydrolysis of desulfated holothurin A (1) showed that the native aglycone corresponded to 22,25-oxidoholothurinogenin, structure 24, and was designated 22,25-oxido-neo holothurinogenin. Also recent studies by Kitagawa *et al.* (36) showed that 24 was in fact the genuine aglycone. Nuclear magnetic resonance studies of intact holothurins have demonstrated that the 12 α -hydroxy-triterpenoids are the genuine aglycones, giving rise to the other $\Delta^{7,9(11)}$ compounds. It is now known that both holothurins A and B have 24 as their genuine aglycone (11, 12). The process by which the genuine aglycone gives a $\Delta^{7,9(11)}$ compound is probably through a $\Delta^{8,11}$ diene system as illustrated in Scheme 2 (p.31).

22 23

$C_{31}H_{48}O_5$, MW 500

Acetate mp 281° - 282° C, $[\alpha]_D^{25} -45^{\circ}$ ($CHCl_3$)

(Chamley and Rossi, 1969a)

31

24-Holothurigenol
 $C_{30}H_{48}O_6$, MW 502

SCHEME 2

$C_{30}H_{48}O_4$, MW 472

$C_{30}H_{46}O_3$, MW 454

Acetate mp 172° - $173^{\circ}C$, $[\alpha]_D -299^{\circ}$ ($CHCl_3$)
 (Kitagawa *et al.*, 1981)

2.5 OTHER HOLOTHURINS.

The Holothuroidae are the most heavily studied family of sea cucumbers. Nevertheless, other saponins have been isolated from other families and named according to their origin; i.e. holotoxins, stichoposides, cucumariosides and thelothurins.

Work on holotoxin extracts from Stichopus japonicus resulted in the separation and identification, by different groups (49, 56, 57) of Stichopogenin A₂ (27), Stichopogenin A₄ (28) (holotoxinogenin) and the 25-methoxy derivative (29). The genuine aglycone of these was shown to be holotoxigenol (6) by C-13 NMR experiments on an intact pure saponin from holotoxin extracts. (Holotoxigenol was also found to be the aglycone of psoluthurine A, the major saponin from Psolus fabricii (11, 58).

A genin 30 related to holotoxinogenin, has been obtained from the body walls of S. chloronotus (59). The work of Pettit et al. (39,60) on the same species resulted in the

27 Stichopogenin A₂

C₃₀H₄₄O₄, MW 468

mp 238°-240°C, $[\alpha]_D^{25}$ -48° (CHCl₃)

(Elyakov *et al.*, 1969)

28 Stichopogenin A₄, holotoxinogenin

C₃₀H₄₆O₅, MW 486

mp 238°-241°C, $[\alpha]_D^{20}$ -97.6° (CHCl₃)

(Tan *et al.*, 1975)

29

$C_{31}H_{48}O_5$, MW 500

mp 236° - 238° C, $[\alpha]_D^{20}$ -125 $^{\circ}$ ($CHCl_3$)

(Tan *et al.*, 1975)

isolation of Stichostatin 1, to which structure (31) has been tentatively assigned.

The species Thelenota ananas, upon acid hydrolysis of its holothurins gave six triterpenoids (61), structures 30-35 and 36. Triterpenoid 37 was shown to be the genuine aglycone of these holothurins by C-13 NMR of the intact saponin. Isomerisation of the double bond from Δ^7 to Δ^8 (37--38) followed by Δ^8 to Δ^9 ⁽¹¹⁾ (38--39) was followed by acid hydrolysis experiments, ($HCl/CHCl_3$). Structure 37 was also shown to be the genuine aglycone of the holothurin from Cucumaria frondosa by C-13 NMR (11, 58).

$C_{32}H_{50}O_5$, MW 514

mp 223° - 224° C, $[\alpha]_D^{20}$ -20° ($CHCl_3$)

(Rothberg *et al.*, 1973)

$C_{32}H_{50}O_6$, MW 530

32

$C_{30}H_{48}O_4$, MW 472
 mp 229°-232°C, $[\alpha]_D + 6.2^\circ$ ($CHCl_3$)
 (Kelecom *et al.*, 1976)

33

$C_{32}H_{48}O_5$, MW 512
 mp 225°-227°C, $[\alpha]_D - 4.0^\circ$ ($CHCl_3$)
 (Kelecom *et al.*, 1976)

$C_{30}H_{46}O_4$, MW 470

mp 202° - 203° C, $[\alpha]_D$ 10.5° ($CHCl_3$)

(Kelecom *et al.*, 1976)

$C_{32}H_{50}O_6$, MW 530

mp 203° - 205° C, $[\alpha]_D$ -10° ($CHCl_3$)

(Kelecom *et al.*, 1976)

36

$C_{30}H_{48}O_5$, MW 488

mp 227° - $228^{\circ}C$, $[\alpha]_D -1.3^{\circ}$ ($CHCl_3$)

(Kelecom *et al.*, 1976)

37

$C_{32}H_{50}O_5$, MW 514

38

$C_{32}H_{50}O_5$, MW 514

mp 198° - $201^{\circ}C$, $[\alpha]_D +13.8^{\circ}$ (CH_3OH)

(Kelecom *et al.*, 1976)

Stichlorogenol (39) and dehydrostichlorogenol (40) are hydrolysis products of stichlorosides A₁ and A₂ isolated from Stichopus chloronotus (62).

X-ray studies of 39 demonstrated that configuration at C-9 was β . This led to the explanation of the instability of the system of triterpenoid 37, 39, 40. It is thought that the double bond isomerization relieves the strain imparted on ring C by this unusual configuration. This type of migration has also been observed in abieslactone (41) (63), although this compound is less strained than 40.

Other saponins have been obtained from Cucumaria fau-
datrix and C. japonica, but complete structures have not yet been reported.

39 Stichlorogenol

C₃₀H₄₈O₄, MW 472

mp 225°-226°C, $[\alpha]_D^{20}$ -42° (CHCl₃)

(Kitagawa *et al.*, 1981)

40 Dehydrostichlorogenol

$C_{30}H_{46}O_4$, MW 470

mp 239° - 240° C, $[\alpha]_D^{17} -35^{\circ}$ ($CHCl_3$)

(Kitagawa *et al.*, 1981)

41 Abieslactone

2.6 STUDIES ON COMPLETE STRUCTURES OF HOLOTHURINS.

The advent of sophisticated instrumental techniques such as Carbon-13 Nuclear Magnetic Resonance Spectroscopy and Fast Atom Bombardment Mass Spectrometry (FAB-MS) have led to the study of saponins in their intact forms, thus avoiding the creation of artifacts.

Although there are not very many complete saponin structures available at the present (see Chart 2, Appendix p. 265), the efforts in that direction has been considerable in the last few years, with research groups from different laboratories involved (e.g. ApSimon, Kitagawa and Elyakov's groups).

The complete structure of holothurin B (2) was determined independently by Kitagawa *et al.* (64, 65) and by Stonik *et al.* (66). Enzymatic hydrolysis of holothurin B allowed the determination of the sugar sequence. The anomeric configuration of these sugars was elucidated by proton and carbon-13 NMR. The (1→2) linkage of xylose to quinovose was determined by examination of the hydrolysis pro-

ducts of permethylated desulfated holothurin B (64, 65). The location of the sulfate group was confirmed by replacement of the sulfate by a formyl group (67) on the peracetylated holothurin B (66).

On the other hand, partial enzymatic hydrolysis of holothurin A (1) provided a different sugar sequence than the one proposed by Chanley *et al.* (68). Again the characteristic features of this holothurin, i.e. sugar sequence, sulfate position and the β -anomeric configuration, were determined by examining the hydrolysis products of the permethylated derivatives and the use of NMR (36, 69).

Fairly recent reexamination of holothurin A extracted from *A. agassizi* by Kitagawa *et al.* (70) revealed that this saponin was actually constituted of two holothurins; the known holothurin A (1) and a novel one, 24-dehydroechinoside A (42). Acid hydrolysis provided structures 43 and 44 as artifacts. *Actinopyga echinita* provided echinosides A (45) and B (46). Their carbohydrates were the same as the one

42 24-DEHYDROECHINOSIDE A

43

44

R =

- 45** X = R, echinoside A
46 X = H, echinoside B

for holothurin A and B; their difference being in the side chain of the aglycone (36b). Bohadschia bivittata gave four bivittosides (47), (48), (49) and (50). No sulfate group was found in these holothurins (36c). One of these is suspected to be holothurin C reported by Elyakov et al., from Bohadschia species (9a).

From Stichopus japonicus, two saponins, stichoposides A and C were isolated. Their aglycone is similar, but their sugar sequences differ. Stichoposide A has a sulfate group and an equimolar ratio of xylose, quinovose, glucose, 3-O-methyl glucose. Stichoposide C also has a sulfate group but a different ratio of the same sugars; i.e. 2:1:2:1 (71). Structure (6), holotoxigenol (72) is the genuine aglycone of stichoposide A, but a complete structure is not available at this time.

A complete reexamination of holotoxin A by Kitagawa et al. (10) revealed that this holothurin was constituted of six monosaccharides, all with β -linkages and that structure 6 was the genuine aglycone. It is now believed that structure (51) and (52) are the ones for holotoxin A and holotoxin B respectively.

- 47** X = OH, Y = H, Z = H, bivittoside A
48 X = OH, Y = R, Z = H, bivittoside B
49 X = H, Y = R, Z = R, bivittoside C
50 X = OH, Y = R, Z = R, bivittoside D

51 X = R, Y = R, holotoxin A

52 X = R, Y = R', holotoxin B

The complete structure of psoluthurine A (53) from Psolus fabricii was elucidated by Garneau et al. (73). Again the use of C-13 NMR and FAB-MS were useful tools in providing crucial structural information. The FAB-mass spectral fragmentation pattern of psoluthurine A (53) is presented on p 49.

The structures of thelothurins A (54) and B (55) seem to differ only in the side chain of their aglycones (8). A genuine aglycone 56 from thelenota ananas seems to be a common type in most species of the family Stichopodidae (66, 74, 75, 76). The substituent X in these compounds is still unidentified but is known not to be a sulfate group. These specimens collected near the Seychelles by Kelecom et al. (8) are different from the ones collected near Singapore which lack glucose as a carbohydrate constituent. Also, thelenostatin 1, reported by Pettit et al. (39) and collected from Taiwan and the Marshall Islands does not bear a sulfate group.

Five related holothurins (77-79) from Stichopus chlo-

FAB-MS DATA FOR PSOLUTHURINE A

56 AGLYCONES

ronotus and Astichopus multifidus were assigned structures 57-61. These differ only in their sugar sequence and the double bond was shown to isomerise under acidic hydrolysis (80, 81). Astichoposide C (62) was shown to be the genuine aglycone.

In the Cucumaria species, no complete structure has been established yet. Girard (58) studied the saponins of Cucumaria frondosa and proposed 63 as the aglycone of frondoside A. On the other hand, Elyakov et al. (81) proposed 64 as the aglycone of cucumarioside G from Cucumaria faudatrix. However, a sapogenin isolated as a dixyloside by Daljeet (82) from C. frondosa seemed to have the 16- α -acetoxy structure (65). (A complete structure proposal for frondoside A is presented in this thesis.)

2.7 BIOSYNTHESIS OF HOLOTHURINS.

<u>57</u>	X=H	Y=H	Z=H	STICHOPOSIDE A
<u>58</u>	X=OH	Y=H	Z=H	STICHOPOSIDE B
<u>59</u>	X=H	Y=R	Z=R'	STICHOPOSIDE C
<u>60</u>	X=OH	Y=R	Z=R'	STICHOPOSIDE D
<u>61</u>	X=H	Y=R	Z=R'	Δ^{25} ASTICHOPOSIDE C

To date, there have been only three investigations dealing with the biosynthesis of holothurins. Elyakov *et al.* (83) observed some incorporation of [^{14}C] acetate into saponins from Stichopus japonicus. Kelecom *et al.* (61) observed very low incorporation of [1- ^{14}C] acetate into saponins from Thelenota ananas. Since in both studies most of the radioactivity was found in the aglycone moiety and not in the sugar moiety, this led to the suggestion that the carbohydrates were derived from a dietary source.

Evidence that holothurinogenins are based on a lanostane skeleton, was provided by Sheikh and Djerassi (84) who demonstrated the conversion of labeled lanosterol to holotoxinogenin by Stichopus californicus.

CHAPTER 3

HOLOTHURIN SAPONINS STUDIED IN THIS WORK.

3.0 SPECIES STUDIED

The two species of sea cucumbers studied are from the Cucumariidae family, Cucumaria frondosa and from the Psolidae family, Psolus phantapus.

The species C. frondosa was collected along the North coast of the St-Lawrence estuary. This species of sea cucumbers is easily recognized as it has no calcarous formation on the outside body walls of the animal. Part of these specimens were collected at the Matamek research station of the Woods Hole Oceanographic Institution, near Sept-Iles, Québec. The rest of these specimens were collected at Baie St-Pancrase and Baie-Comeau and at Islets de Mai, Québec, between 1979 and 1982.

FIGURE 2

THE SPECIES: CUCUMARIA FRONDOSA

FIGURE 3

THE SPECIES: PSOLUS PHANTAPUS

The Psolus phantapus specimens were collected at Point Kirwin, Maggoty Cove, Bay Bulls, Newfoundland, and at Baie St-Pancrase, Québec. These were collected during the years 1980 to 1982. Table 1 and figure 4 (p.59,60) summarises the origin and quantities of the sea cucumbers employed in this work.

Due to very large distances to be covered before arriving at destination in our laboratory, the specimens could not be kept alive. They were therefore frozen and shipped by air or surface. Upon arrival in the laboratory, the animals were immediately thawed and dissected.

3.1 EXTRACTION AND PURIFICATION OF HOLOTHURINS.

A. CUCUMARIA FRONDOSA.

Previous work in this laboratory (58, 85, 86) has led to improved methods of extraction and purification of saponins from sea cucumbers. These techniques were therefore used here without major modifications.

TABLE 1

ORIGIN OF SEA CUCUMBER SPECIMENS:
CUCUMARIA FRONDOSA AND PSOLUS PHANTAPUS.

Species	Collection Site	Date	Number of sea cucumbers	Depth
<u>Cucumaria frondosa</u>	Matamek	28-09-79	29	-
<u>Cucumaria frondosa</u>	Baie St-Pancrase & Baie Comeau	30-04-82	24	5-7m
<u>Cucumaria frondosa</u>	Islets de Mai	19-06-82	250	-
<u>Psolus Phantapus</u>	Maggotty Cove, Bay Bulls	04-04-80	31	17-19m
<u>Psolus Phantapus</u>	Point Kirwin	17-05-81	17	18-20m
<u>Psolus Phantapus</u>	Baie St-Pancrase	30-05-82	38	5-7m

Figure 4

60

MAP OF COLLECTION SITES.

The general extraction procedure employed is described schematically in figure 5 (p.62). The use of Amberlite XAD-2 resin was very efficient for the removal of inorganic salts that were extracted along with the saponins. Figure 6 (p.63) summarises the types of columns used in the different stages of purification of the holothurins. High performance liquid chromatography using C-18 reverse phase columns was an ideal technique in the purification of such polar compounds.

The extracts used, in the search of holothurins, were the ones from the 70% ethanol extractions, which were extremely rich in saponins. The methanol extracts were used in a few experiments but they did not prove to contain enough saponins to be purified in a reasonable yield.

In a trial test, the visceral contents of the animals were extracted according to the same procedure used for the body walls of the specimens (Figure 5, p.62). These viscera showed a very low content in saponins. These extracts were therefore not subjected to silica gel column chromatography, as not enough material could be collected in a pure enough

FIGURE 5

EXTRACTION AND PURIFICATION PROCEDURES OF SAPONINS FROM
CUCUMARIA FRONDOSA AND PSOLUS PHANTAPUS

FIGURE 6**COLUMNS****RESIN COLUMN**

COLUMN:	AMBERLITE XAD-2 RESIN
DIMENSIONS:	MALLINCKRODT, 20-50 MESH
SOLVENTS:	4.5 CM X 54 CM
	H ₂ O, H ₂ O/CH ₃ OH, CH ₃ OH

MEDIUM PRESSURE LIQUID CHROMATOGRAPHY

COLUMN:	SILICA GEL LICHROPREP SI 60
DIMENSIONS:	MERCK, 15 - 25 MICRONS
FLOW RATE:	2.5 CM X 50 CM
PRESSURE:	7 ML/MIN
SOLVENT:	ELUTION AT 20 PSI
	GRADIENT OF: CH ₂ Cl ₂ - CH ₃ OH - H ₂ O

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

COLUMN:	μ -BONDAPAC C ₁₈
DIMENSIONS:	MILLIPORE, WATERS ASSOCIATES,
FLOW RATE:	10 MICRONS
PRESSURE:	3.9 MM X 30 CM
SOLVENT:	2.0 ML/MIN
	ELUTION AT 2000 PSI
	H ₂ O --- CH ₃ OH

state to enable structural elucidation work to proceed.

Thin layer chromatography analysis of the 70% ethanol extracts revealed the presence of at least ten constituents in the mixture. The triterpene-glycosides of interest had R_f values ranging inbetween 0.21, 0.24, 0.29 and 0.35, (in the solvent system: CH_2Cl_2 -MeOH- H_2O ; 60-25-5.4 v/v; color of spots: pink to purple). Repeated chromatography using a medium pressure liquid chromatography system, followed by a final purification on a high performance liquid chromatography system (C-18 reverse phase column), enabled purification of a saponin named frondoside J ($R_f=0.22$, solvent system: CH_2Cl_2 -MeOH- H_2O ; 60-25-5.4 v/v) in enough quantity (20.0 mg) to enable structural analysis work. Unfortunately, the other frondosides were not purified in amounts sufficient to permit structural work to proceed.

B. PSOLUS PHANTAPUS

a) SOLID-LIQUID EXTRACTION

Saponins from the species Psolus phantapus were extracted, according to the procedure described in Figure 5 (p.62). Table 2 (p.66) summarises the yield of such a solid-liquid extraction. The purification of those saponins was then accomplished using the same approach as for the species C. frondosa (i.e. figure 6, p.63).

The saponin content of the 70% ethanol extract seemed quite rich. It contained at least five different saponins with Rf varying between 0.15-0.30 (solvent system: CH_2Cl_2 - $\text{MeOH-H}_2\text{O}$; 65-38-11, lower layer, v/v).

Repeated column chromatography yielded different fractions of holothurins that were more or less pure (3 or more spots on TLC). It is to be noted that direct comparison by TLC of saponins from the species collected in Newfoundland and the ones from the St-Lawrence estuary did not show any similarities on a TLC plate. It was therefore impossible to combine these partly purified holothurins.

In the species Psolus phantapus originating from the St-Lawrence estuary, and after a last purification by HPLC,

TABLE 2

YIELD OF A SOLID-LIQUID EXTRACTION OF THE SEA CUCUMBER POWDER FROM THE
SPECIES PSOLUS PHANTAPUS.

PSOLUS PHANTAPUS (358.9g)

765 mL benzene, 7h, 52 °C

1.7978g filtrate A benzene	solid + 550 mL benzene, 7:50h, 54 °C
1.3050g filtrate B benzene	solid + 500 mL benzene, 8h, 54 °C
0.7704g filtrate C benzene	solid + 500 mL benzene, 6:40h, 56 °C
0.4370g filtrate D benzene	solid + 500 mL EtOH 70%, 10h, 54 °C
2.2961g filtrate A EtOH 70%	solid + 550 mL EtOH 70%, 13h, 54 °C
6.8953g filtrate B EtOH 70%	solid + 500 mL EtOH 70%, 8h, 56 °C
4.5245g filtrate C EtOH 70%	solid + 500 mL EtOH 70%, 15h, 55 °C
3.0243g filtrate D EtOH 70%	solid

a pure compound (9.4mg) was obtained. This sample with a R_f of 0.11 was named phanthothurin I. Another sample, phanthothurin II gave two spots on TLC and a yield of 5.0 mg. Table 3 (p. 68) summarises the physico-chemical data available for phanthothurin I. Unfortunately, not enough data could be collected to permit even a partial structure elucidation of this compound. Attempts at recording a C-13 NMR spectrum were unsuccessful, therefore suggesting that the molecule was probably of very high molecular weight, and not enough sample was available to observe NMR signals. The FAB-MS spectrum of phanthothurin I were recorded in the the positive and the negative modes in various solvents such as glycerol, thioglycerol and a trace of methanol, and polyethyleneglycol 200. Unfortunately most of those spectra showed only solvent peaks. The FAB-MS recorded in glycerol is presented in the appendix (p. 249). The highest mass observed in this spectrum is at m/z 679. Lack of more definitive spectroscopic data prevents us from presenting any more information about this structure. Isolation of more material is necessary in order to be able to run an adequate C-13 NMR of the present sample. The proton NMR spectrum is also presented in the appendix (p. 248).

TABLE 3PHYSICO-CHEMICAL DATA FOR PHANTOTHURIN I.

Aspect: White powder

Melting point: 255-260 °C (dec.)

UV (MeOH) transparent above 210 nm

R_f: 0.11 in the solvent system: CH₂Cl₂ - MeOH - H₂O
60 - 25 - 5.4 (v/v)

Coloration on TLC: Pink

¹H NMR: See appendix (p.248)

Only 0.3 mg of pure material was isolated from the species originating from Newfoundland. A FAB-MS of this last sample did not give any satisfying results.

b) LIQUID-LIQUID EXTRACTION (TRIALS)

In an attempt to improve the above solid-liquid extraction procedure (figure 5, p. 62) of holothurins from sea cucumbers, a liquid-liquid extraction method was attempted.

Preliminary work has suggested the extraction of the sea cucumber powder with solvents of increasing polarities, i.e. benzene, methanol, and finally ethanol (70-75%). The benzene extraction would liberate the sterols and less polar components from the sea cucumber powder, while the ethanol would extract the more polar constituents such as saponins and salts. Nevertheless, the presence of less polar components was also observed in the ethanol extracts. This extraction procedure being long and tedious, a liquid-liquid extraction was investigated.

The dry powder from the sea cucumber Psolus phantapus (175.09 g) was extracted with 70% ethanol for 6 hours. After filtration, the filtrate A was extracted three times with benzene. Difficulties were encountered in the second and third extractions, where emulsions occurred. These emulsions were broken upon addition of water.

Thin layer chromatography indicated the inefficiency of this liquid-liquid extraction. In a solvent system suitable for developing sterols (CHCl_3 -MeOH-90-10), the presence of sterols was observed in all three benzene extracts as well as in the remaining ethanol extract. Therefore benzene does extract the sterol components present, but a large proportion seems to remain in the ethanol extracts. In a second solvent system suitable for the development of saponins (CHCl_3 -MeOH- H_2O ; 60-42-10, lower layer) it was observed that the saponins were present in both benzene and ethanol extracts. It is not believed that the addition of water would be responsible for the presence of saponins in the benzene extracts, because these are also present in the first benzene extraction. This method was therefore not effective for the extraction of saponins from sea cucumbers.

Table 4 (p. 72) summarises the liquid-liquid extraction of the specie Psolus phantapus.

3.2 STRUCTURAL ANALYSIS OF CUCUMARIA FRONDOSA.

3.2.1 FRONDOSIDE J FROM C. FRONDOSA.

Our objective was to extract pure holothurin(s) from Cucumaria frondosa and to study the structure of the intact saponins. No chemical derivatization was envisioned. The use of Carbon-13 NMR and of FAB-MS* were the tools of choice for such a task.

C-13 NMR has proved to be a very powerful technique in the structural analysis of new marine organic compounds (87). Provided that sufficient model compounds are available, complete structures can be elucidated without chemical derivatization, therefore saving time, energy and most of all precious samples.

*C-13 NMR is a well established technique and is discussed in the context to reported data. On the other hand, the technique of FAB-MS is new and as such a discussion covering its use and application is enclosed in Appendix A (p. 223).

TABLE 4

YIELD OF A LIQUID-LIQUID SAPONIN EXTRACTION FROM THE POWDER OF THE SPECIES *PSOLUS PHANTAPUS*.

FRONDOSIDE J

Frondoside J (77) was obtained as a yellowish solid; melting at 205 °C with decomposition (see p. 74). The UV spectrum was transparent above 210 nm, therefore excluding the possibility of a conjugated diene system.

The carbohydrates present in frondoside J are responsible for the intense absorption band at ν_{OH} 3380 cm^{-1} in the IR spectrum, recorded as a KBr pellet. The presence of a carbonyl group was indicated by an absorption band at $\nu_{C=O}$ 1740 cm^{-1} , suggesting the presence of a γ -lactone, characteristic in holothurins. The occurrence of a sulfate group in the IR spectrum was evidenced by absorption bands at $\nu_{S=O}$ 1235 cm^{-1} and ν_{C-O-S} at 815 cm^{-1} . Table 5 (p. 74) summarises the physico-chemical properties of frondoside J (77).

THE AGLYCON MOIETY OF FRONDOSIDE J.

The aglycone moiety of frondoside J was studied by C-13 NMR of the intact saponin. Model compounds such as lanost-9en-3 β -ol (78) (88), lanost-8(9)-en-3-one (79) (88)

TABLE 5**PHYSICO-CHEMICAL PROPERTIES OF FRONDOSIDE J (77).**

Melting point: 205 °C

Infrared: $\nu_{\max}$ ⁻¹cm (KBr)
3380, free OH (broad)
1740, C=O, γ -lactone and acetate
1235, S=O, sulfate
815, C-O-S, sulfate

Ultraviolet: $\lambda_{\max}$ ^{nm} (EtOH)
Transparent above 210 nm

C-13 NMR: Tables 8 and 9 (see also appendix, p. 238 & 239).

¹H NMR: Table 7 (see also appendix, p.237)

and structure 80 and 81 reported by Elyakov et al. were used (76) in this structure elucidation work. Frondoside A (89) (58), isolated during previous studies on Cucumaria frondosa in this laboratory, was also used for comparison purposes.

The aglycone moiety of frondoside J is similar to that of stichoposide A (81) (76). The signals at 35.1, 26.0, 88.2, 38.8, 47.3, 28.8 and 16.6 are assigned to carbons C(1), C(2), C(3), C(4), C(5), C(29), and C(30) respectively, taking into account the influence of C(3) glycosidation (89). Quaternary carbons C(10), C(13) and C(14) are assigned the signals at 34.7, 59.8 and 38.6 respectively, based on comparison with model compounds (58, 76, 88). Resonances at 119.5 and 144.9 are indicative of the presence of an endocyclic double bond at position 7,8 of the holostane B ring (81).

* Previous studies on holothurins have shown that double bond migration may occur under acidic solvolysis conditions (61, 81). Different chemical shifts for the various possible endocyclic double bond positions in these kind of systems are reported in table 6. In Frondoside J minor signals are also observed at 128.8 and 132.7 ppm. It therefore would appear that a double bond at position 8,9 suggests the presence of a $\Delta^{8,9}$ artifact in the spectrum. This artifact could probably have arisen during the isolation process of the saponin.

80 R = ACETATE; $\Delta^{9(11)}$

81 R = ACETYLATED SUGAR

SIDE CHAIN; $\Delta^{7,8}$

TABLE 6

POSITION OF THE ENDOCYCLIC DOUBLE BOND.

COMPOUNDS (81)	C-13 CHEMICAL SHIFTS			
	C ₇ - C ₈	C ₈ - C ₉	C ₉ - C ₁₁	
Astichoposide (82)	120.0	146.6		
(82) after acid hydrolysis			130.6	135.4
			151.0	110.7
Cucumarioside G (84)	120.3	144.8		
(84) after acid hydrolysis			130.2	135.5
Cucumarioside A (86)	121.8	144.3		
<u>Other compounds (88)</u>				
Lanost-8(9)-en-3-one (79)			133.1	135.3
Lanost-9-ene-3 β -ol (78)				148.2
				115.0
Lanost-7,9(11)-diene-3 β -ol (87)	120.1	142.7		
			145.9	116.3

2

The signals at 179.6, 82.5, 173.4 and 21.5 ppm are assigned to carbons C(18), C(20), C(31) and C(32) respectively (89, 90, 91, 95). Individual assignment of the side chain carbons is accomplished by chemical shift comparison with 2,6-dimethyl-octane (88) as a model compound where chemical shifts are predictable on the basis of empirical additivity rules (91). Introduction of an acetate group at carbon-23 affects principally C(22), C(23), C(24) and C(25). Signals at 42.8, 68.2, 46.4 and 23.1 are respectively assigned to those carbons (by comparison with the calculated values 43.2, 69.9, 45.7 and 25.3). The assignment of the acetate group at C(23) is also ascertained by ^1H NMR (82). The absence of a signal at 5.96 ppm in the ^1H NMR spectrum of frondoside J suggests that the acetate group is not at C(16), but on the side-chain of the aglycone, at C(23). Table 7 (p.79) reports the principal chemical shifts of the ^1H NMR for Frondoside J. Carbons C(26) and C(27) are assigned the signals at 22.4 and 22.0 ppm respectively. The remaining signals are assigned by comparison with model compounds (58, 76, 81, 88). The C-13 chemical shifts of the aglycone moiety of frondoside J are reported in Table 8 (p. 80).

TABLE 7

¹H NMR OF FRONDOSIDE J.

Chemical Shifts (ppm)	Assignments
0.86 }	{ 26-Me
0.90 }	{ 27-Me
1.15	30-Me
1.20	28-Me
1.22	29-Me
1.31	19-Me
1.54	21-Me
1.69	Quinovose-Me
2.08	Acetate Me
3.90	3-O-methyl glucose-Me

This spectrum was run in pyridine-d₅, with TMS as an internal standard.

TABLE 8

ASSIGNMENT OF C-13 CHEMICAL SHIFTS FOR THE AGLYCONE MOIETY OF
FRONDOSIDE J, COMPARED TO MODEL COMPOUNDS.

Carbon number	Lanost-9- ene-3 β -ol CDCl ₃	Lanost-8(9)- en-3-one CDCl ₃	80 CDCl ₃	81 CDCl ₃	frondoside A C ₅ D ₅ N	frondoside J C ₅ D ₅ N /H ₂ O
1	36.2	36.0	35.7	35.9	35.8	35.1
2	28.3	34.5	24.1	26.6	27.8	26.0
3	79.0	217.6	80.8	90.3	89.2	88.2
4	39.2	47.2	38.0	39.3	39.2	38.8
5	52.6	51.2	52.4	47.8	48.0	47.3
6	22.3	19.4	24.0	n/a	23.0	21.7
7	28.1	26.3	27.8	120.1	120.5	119.5
8	41.9	133.1	39.6	145.6	146.4	144.9
9	148.2	135.3	151.0	47.0	47.1	38.2
10	39.5	36.8	39.4	35.3	35.3	34.7
11	115.0	21.0	110.9	n/a	28.1	27.2
12	37.3	30.8	33.2	n/a	32.1	31.4
13	44.4	44.4	57.9	58.5	59.4	59.8
14	47.1	49.8	47.0	51.1	47.2	38.6
15	34.0	30.8	35.7	n/a	38.8	30.6
16	28.2	28.1	24.4	n/a	31.4	27.9
17	51.1	50.5	52.5	54.0	54.6	53.8
18	14.5	15.8	177.4	179.7	181.6	179.6
19	21.5	18.6	21.8	n/a	22.2	21.8
20	36.2	36.4	82.8	83.2	84.5	82.5
21	18.6	18.6	27.6	27.0	23.7	27.5
22	36.6	36.4	43.9	44.0	39.4	42.8
23	24.2	24.0	68.2	68.3	69.7	68.2
24	39.6	39.4	45.4	45.4	43.5	46.4
25	28.1	27.9	24.4	24.5	26.5	23.1
26	22.6	22.5	22.2	22.2	22.3	22.4
27	22.9	22.7	23.2	23.2	22.5	22.0
28	18.6	24.2	21.3	n/a	22.4	20.5
29	27.9	26.1	171.0	n/a	28.6	28.8
30	15.7	21.2	16.8	n/a	17.2	16.6
31 (CH ₃ COO)			28.1	n/a	172.2	173.4
32 (CH ₃ COO)			19.9	n/a	21.2	21.5

n/a = not available

THE CARBOHYDRATE MOIETY OF FRONDOSIDE J.

The C-13 chemical shifts for the carbohydrate moiety of frondoside J are presented in Table 9 (p.82). The approach taken to suggest a structure for frondoside J was as follows. Six signals appear in the region characteristic of anomeric carbon signals (i.e. 90.0-105.0 ppm), thus presenting the evidence that six sugar units are present in frondoside J. Anomeric carbon signals for sugars with alpha configuration at the anomeric carbon appear at much higher field than the ones with beta configuration (91, 95). Therefore, the single resonance at 92.3 ppm indicates that only one sugar unit has an α -linkage, while the remaining 5 sugar units would be β -anomers.

The sugars most often encountered in these type of holothurins from Cucumaria frondosa are xylose, quinovose, glucose and 3-O-methyl glucose. According to these precedents, we have investigated the spectra (^1H and C-13 NMR) to see if by spectroscopic means, it would be possible to determine the sugars that constitute frondoside J.

In the C-13 NMR, a resonance appearing at 17.1 ppm

TABLE 9

C-13 CHEMICAL SHIFTS FOR THE CARBOHYDRATE MOIETY OF
FRONDOSIDE J (77).

17.1	72.7	81.7
61.1	73.3	84.5
61.2	73.6	84.8
62.1	73.9	85.8
63.0	74.1	86.4
63.5	74.5	92.3
65.2	74.5	101.2
65.6	74.7	103.7
69.5	75.5	103.8
69.8	75.8	104.3
70.3	76.9	104.4
72.0	78.2	
	80.5	

Solvent: C_5D_5N/H_2O

(representing a methyl group) remains to be assigned. The only sugar that could bear such a methyl group is quinovose. We can therefore reasonably assume that quinovose is present in this compound. From the ^1H NMR spectrum, we identified the signal at 3.9 ppm (52) as being a OCH_3 belonging to a 3-O-methyl glucose. In principle integration of this resonance would provide us with the number of 3-O-methyl glucose unit present in the molecule, but since this spectrum was recorded in the Fourier transform mode, we cannot rely entirely on the integration, since relaxation rates are not the same for all protons in the spectrum. Nevertheless, the OCH_3 signal's integration could be compared with the one for the CH_3 resonance of quinovose (1.7 ppm) since these two methyl groups are both rotating with approximately the same degree of freedom and therefore their relaxation time should exhibit similar values. Therefore, it would appear that only one 3-O-methyl glucose is present in frondoside J. Consequently there are six sugars present in the molecule, of which one unit (and only one) is a quinovose, and one unit is a 3-O-methyl glucose.

There are 36 carbon signals in total remaining to be assigned in the sugar region. A mathematical calculation

for the distribution of those signals and based on the evidence presented here, leads us to suggest the following 2 possibilities for the carbohydrate moiety of frondoside J.

Possibility A	Possibility B
1 mole quinovose	1 mole quinovose
1 mole xylose	2 moles xylose
3 moles glucose	1 mole glucose
1 mole 3-O-Me glucose	2 moles 3-O-Me glucose

In addition, the infrared spectrum of frondoside J indicates the presence of a sulfate group (see table 5 p. 74). The sulfate group can be present as HSO_3 , NaSO_3 , KSO_3 or all of these combinations.

Attempts to record Fast Atom Bombardment Mass Spectra of frondoside J were made and this under various conditions. Both positive and negative spectra were recorded and this with various kinds of support matrices (i.e. glycerol, glycerol/methanol, glycerol/ NaCl , glycerol/thioglycerol). Unfortunately none of these combinations gave a satisfactory spectrum; reproducibility in the molecular ion region was poor thus preventing us to ascertain, unequivocally, the molecular weight of the molecule.

Based on all the above considerations, and on the C-13 NMR spectrum of the aglycone moiety of this sample (as discussed in section 3.2.1 p. 71) we therefore suggest structure 77, Figure 7 (p. 86) as a plausible structure for frondoside J. At this time it is not possible to suggest a more complete structure for frondoside J and more work in that direction is still in progress.

FIGURE 7

PROPOSED STRUCTURE FOR FRONDOSIDE .1 (ZZ)

R =

- Quinovose**
- Xylose**
- Glucose (3 moles)**
- 3-O-Methyl Glucose**

$\text{HSO}_3 / \text{NaSO}_3 / \text{KSO}_3$

3.3 THE COMPLETE STRUCTURE OF FRONDOSIDE A.

The partial structure of frondoside A, isolated from *Cucumaria frondosa*, was reported during previous work in this laboratory (58). The complete structure of this saponin is now reported.

The C-13 NMR spectrum of frondoside A revealed that its aglycone moiety is similar to that of stichoposide A (76). More experimental data are reported here. The ^1H NMR spectrum of frondoside A, Table 10 (p. 88) shows 7 signals between 0.85-1.51 ppm assigned to the methyl groups on the genin. The assignment of the acetate group at C(23) is also strengthened by the absence of a signal at around 6 ppm in the ^1H NMR spectrum, which was suggested in recent literature (82) to be indicative of a C(16) acetate group at that position.

The carbohydrate side-chain constitution was studied by gas chromatographic analysis of the trimethylsilylated methyl glycosides obtained from anhydrous acid methanolysis.

TABLE 10¹H NMR OF FRONDOSIDE A.

Chemical Shifts (ppm)	Assignments
0.85)	{ 26-Me
0.88)	{ 27-Me
1.15	28-Me
1.18	30-Me
1.21	29-Me
1.29	19-Me
1.51	21-Me
1.68	Quinovose-Me
2.04	Acetate-Me
3.87	3-O-methyl glucose-Me
5.80	Vinylic hydrogen

This spectrum was recorded in pyridine-d₅. Chemical shifts are in ppm, downfield from TMS.

These results show that frondoside A contains three units of xylose, and one unit each of 3-O-methyl glucose and quinovose. Glucose, usually found in glycosides that contain more than two sugar units, was not detected in this compound as a carbohydrate constituent.

Fast Atom Bombardment Mass Spectrometry (73, 92, 93) is used in the elucidation of the sugar sequence and overall structure of frondoside A and indicates that a xylose unit is bearing the sulfate group and is directly linked to the sapogenin. The positive ion FAB-MS data for frondoside A are presented in Table 11 (p. 90).

Molecular weight information is obtained from the fragment at m/z 1335 $[M + H]^+$. Other quasi-molecular ions are the result of the various possible combinations for the molecular ion, when a trace of sodium salt is present (76, 77). Characteristic ions useful for the sequencing of the sugars are at m/z 1181 $[M - (3-O\text{-methyl glucose}) + Na]^+$, m/z 1025 $[M - (3-O\text{-methyl glucose} + \text{xylose})]^+$, m/z 731 $[\text{aglycone} + \text{xylose} + NaSO_3]^+$ and m/z 497 $[\text{aglycone}]^+$.

TABLE 11

SELECTED FAB-MS DATA FOR FRONDOSIDE A.

m/z	Ion
1335	$[M + H]^+$
1181	$[M - (3\text{-O-methyl glucose}) + Na]^+$
1025	$[M - (3\text{-O-methyl glucose} + \text{xylose})]^+$
731	$[\text{Aglycone} + \text{xylose} + NaSO_3]^+$
497	$[\text{Aglycone}]^+$
473	$[3\text{-O-methyl glucose} + \text{xylose} + \text{quinovose} + H]^+$ and subsequent losses of 3 molecules of water.
455	
437	
419	

The FAB-MS spectrum was recorded on a glycerol support.
Peaks considered were all exhibiting a S/N of at least 4.

Unfortunately the corresponding ions indicative of the sequence of the second and third sugars (a quinovose and a xylose) are not recorded, the region between m/z 800-1000 being of very weak intensity. However, the FAB-MS data indicates that the sugars are not linked in a branched fashion. In fact the characteristic ions normally found in branched oligosaccharide spectra (for example in the spectrum of digitonin (94)), occurring principally at the branching points are not seen, i.e. in this case the loss of a (deoxy or normal) quinovose unit or loss of a (deoxy or normal) xylose unit. Furthermore, intense peaks at m/z values of 473, 455, 437 and 419 can be seen as indicative of a 3-O-methyl glucose - xylose- quinovose arrangement*. The simultaneous presence of these peaks and that of m/z 1025 (see above) precludes branching at the xylose unit attached to the 3-O-methyl glucose moiety. The final assignment for these sugars is done according to the previous discussion. The branching points of the sugar sequence are assigned by comparison with the chemical shifts of model sugar compounds (36). Table 12 (p. 92) reports the C-13 NMR spectrum of frondoside A. The structure of frondoside A is therefore suggested as: 38-O-[3-O-methyl- β -D-glucopyranosyl(1 $\rightarrow$ 4)- β -

* J.W. ApSimon and J.R.J. Paré, unpublished FAB-MS study on model oligosaccharides; for further details see ref. 129.

TABLE 12

C-13 NMR OF PRONDOSIDE A

TRITERPENE AGLYCONE		SUGARS	
CARBONS	CHEMICAL SHIFTS	CARBONS	CHEMICAL SHIFTS
1	35.76	1'	102.17
2	27.79	2'	82.42
3	89.16	3'	76.12
4	39.20 (q)	4'	74.13
5	48.03	5'	63.30
6	23.04		
7	120.49		
8	146.41 (q)	1''	103.72
9	47.08	2''	73.80
10	35.34 (q)	3''	85.65
11	28.13	4''	68.47
12	32.06	5''	66.00
13	59.38 (q)		
14	47.23 (q)		
15	38.80	1'''	104.01
16	31.35	2'''	74.36
17	54.58	3'''	85.78
18	181.63 ^b	4'''	78.34
19	22.20*	5'''	70.97
20	84.53 (q)	6'''	17.70
21	23.72		
22	39.43		
23	69.67	1''''	103.72
24	43.50	2''''	74.67
25	26.54	3''''	76.29
26	22.30*	4''''	81.90
27	22.50*	5''''	65.46
28	22.40*		
29	28.63		
30	17.23	1'''''	104.57
31	172.21 (q)	2'''''	73.72
32	21.17	3'''''	86.00
		4'''''	69.70
		5'''''	76.73
		6'''''	61.39
		3'''''OMe	60.12

a) The spectrum was recorded in C_5D_5N . Chemical shifts are in ppm downfield from TMS.

b) q = quaternary carbon (shown in partially decoupled spectra).

* Those signals can be interchanged.

D-xylopyranosyl (1 → 3)-β-D-quinovopyranosyl (1 → 3)-β-D-xylopyranosyl (1 → 2)-β-D-4-sulfonato-xylopyranoside]-23-5-acetoxystyrene sodium salt (89), Figure 8 (p. 94).

Fragment ions are representative of associated cleavages and/or clusters.
 e.g. $1335 [M + H]^+$, $497 [\text{Genin moiety}]^+$ cleavage

NOMENCLATURE

X: SUGARS

CHAPTER 4

STRUCTURAL ELUCIDATION OF AN INTACT SAPONIN FROM THE STAR- FISH SPECIES ASTERIAS VULGARIS, ASTERIAS FORBESI.

4.0 INTRODUCTION.

Saponins have been shown to be the element responsible for the toxicity of starfish (1). Since many years ago, starfish saponins were also known to possess ichthyotoxic as well as hemolytic activity. A major characteristic of asterosaponins which aroused researchers' curiosity was their ability to evoke escape responses in other marine species (4), although this phenomenon is not yet completely clarified.

4.1 CHEMICAL STRUCTURES OF STARFISH SAPONINS.

A. PREVIOUS WORK ON THE AGLYCONE MOIETY.

Saponins from starfish, or "asterosaponins", are complex mixtures of many different steroidal aglycones, with carbohydrate side-chains varying in length and constitution. The usual sugars encountered in asterosaponins are D-fucose (90), D-quinovose (10), D-xylose (7), D-glucose (8) and D-galactose (91).

90

91

It seems that most asterosaponins possess a sulfate moiety at C-3 of the steroidal aglycone, which is lost during acid hydrolysis. The oligosaccharide side-chain is commonly attached to C-6 oxygen of the sapogenin (96-99). The most remarkable feature of the sapogenin is probably the biosynthetically unusual $\Delta^9(11)$ double bond and all but one have a 6α -hydroxyl group.

To date, over thirty different sapogenins have been reported, of which the most commonly encountered one, in different species, is probably the 3β - 6α -dihydroxy- 5α -pregn-9(11)-en-20-one (commonly named Asterone (92)). The major difference between all the sapogenins reside most often in the side-chain substitution. It must be noted however, that many of these reported sapogenins are artifacts, arising during acid treatment of the saponin.

Chart 3 (see appendix p. 266) reports the structures of all the steroids obtained from asterosaponins (4). Asterone (92), also called asterosapogenin I, is the most commonly reported steroid from asterosaponins. Asterone is always accompanied by its 17α -isomer, isoasterone (93). Asterone

was the principal steroid aglycone recovered after acid hydrolysis of the saponins from Acanthaster planci, Asterias amurensis, and Asterias forbesi, by Ikegami et al. (100), Sheikh et al. (101), Shimizu (102) and ApSimon et al. (103). The trivial name asterone was given by Gilgan et al. (104). This steroid has now been recovered from the asterosaponins of other starfish (105-107) and three different chemical synthesis were reported for asterone (108-110). These synthesis have helped in the final verification of the structure.

From the European species Asterias rubens, a C-21 steroid 94 lacking the 9(11) double-bond of Asterone, was obtained (106). On the other hand, the North American species, Asterias vulgaris and Asterias forbesi, which are very similar to Asterias rubens (111), yielded the triol asterogenol (95).

In the acid hydrolysis products of asterosaponins, the most abundant products are C-27 steroids with the same steroid nucleus as asterone and with an oxygen function at C-23. Example of these are marthasterone (96) and dihydromarthasterone (97). Many compounds from asterosaponins differ from marthasterone only by the position or number of double bonds in the side-chain (compounds 98-104).

96 Marthasterone

97 Dihydromarthasterone

98 $C_{27}H_{42}O_3$, MW 414 **99** $C_{27}H_{42}O_3$, MW 414, mp 117-119°C
(Sheikh *et al.*, 1972a) **100** $C_{27}H_{42}O_3$, MW 414 **101** $C_{27}H_{42}O_3$, MW 414 **102** $C_{27}H_{42}O_3$, MW 414 **103** $C_{27}H_{40}O_3$, MW 412 **104** $C_{27}H_{40}O_3$, MW 412

Kitagawa et al. (112, 113) isolated thornasterol A (105) and thornasterol B (106) from Acanthaster planci. Acid treatment of both of these aglycones furnished asterone (92) as a minor constituent and the $\Delta^{20(22)}$ -steroid as the major one. On the other hand, enzymatic hydrolysis of thornasterol A (105) and thornasterol B (106) gave no asterone (92), but gave a significant amount of the $\Delta^{20(22)}$ -steroid. Also, the UV and the CD spectra of the original saponin did reveal the presence of some α, β -unsaturated ketone. Therefore, asterone from Acanthaster planci, must be considered an artifact, whereas it can be considered that 98 (or 99) is a natural aglycone, although much of it seems to occur during acid hydrolysis (112, 113).

It was suggested that the steroids with a 17(20) double bond (compounds 101-104) could be artifacts arising from direct dehydration of thornasterol, or a genuine aglycone akin to it, or from a deconjugation of a $\Delta^{20(22)}$ -steroid (114) or are derived from a hitherto undetected 17-hydroxy steroid (115).

105 Thornasterol A **106** Thornasterol B

From the species Echinaster sepositus, Minale et al. (116) reported a group of steroids with a 8,14 diene in the steroid nucleus, and lacking the 6 α -hydroxyl group (see compounds 107-110). Depending on whether the saponins are hydrolyzed with HCl or HBr, chlorohydrins or bromohydrins are obtained. A model study confirmed that these compounds are derived from (22S,23S)-epoxides (117). The 8,14-diene steroids 107-110 are known to arise from the 6 β -hydroxy- Δ^7 -steroid (111) via the $\Delta^{6,8(14)}$ intermediate 115 (118). Similarly steroids 112, 113 and 114 have been shown to be the genuine aglycone giving rise to the three minor artifacts 108, 109 and 110 (119).

B. PREVIOUS WORK ON COMPLETE STRUCTURES OF ASTEROSAPONINS.

To date, the complete structures of only a dozen asterosaponins have been reported (see Chart 4 in appendix p. 267). A recent chapter covering the origin of all of these reported structures can be found in reference (4). Of these, sepositoside A, from Echinaster sepositus 116 is the one having the most unusual (or interesting) features, as it bears no sulfate group at C-3 of the aglycone and it

107

108

a X = Cl
b X = Br

32a

X = Cl

109

b X = Br

110

a X = Cl

b X = Br

111

$C_{27}H_{44}O_3$, MW 416

112

$C_{26}H_{42}O_3$, MW 402

113

$C_{27}H_{44}O_3$, MW 416

114

$C_{27}H_{44}O_3$, MW 416

115

$C_{27}H_{42}O_2$, MW 398

mp 120°-122°C, $[\alpha]_D -22^\circ$ ($CHCl_3$)

(DeSimone *et al.*, 1981)

contains no 6-deoxy sugar. There is an unprecedented glucuronic acid moiety attached to C-3. The carbohydrate side-chain is cyclized between C-3 and C-6 of the aglycone. Three minor saponins 117-119 from the same species differed only in the nature of the aglycone (119a). Other cyclic saponins have been isolated also from Echinaster luzonicus (Structures 120-123) (119b).

The complete structures of asterosaponins (except for compounds 116-129 and 135, see Chart 4, Appendix, p. 267) are always seen with a sulfate group at C-3 of the aglycone and a carbohydrate side-chain varying in length (from one to five sugar units, or sometime more) and composition. At least one 6-deoxyhexose unit seems to always be part of the oligosaccharide portion of the molecule.

Asterosaponin A (130), was found (120, 121) to contain an aglycone along with quinovose, fucose and a sulfate group in the molar ratio: 1:2:2:1. This was the first report claiming to have found fucose in the animal kingdom. The complete structure of asterosaponin B has not yet been reported, but it is known to be composed of an aglycone,

quinovose, fucose, xylose, galactose and a sulfate group in the molar ratio 1:2:1:1:1:1 (122).

The major saponin from Acanthaster planci was found to be thornasteroside A (131) (123, 124), composed of the aglycone thornasterol A (105), a sulfate group and one mole of quinovose, 2 moles of fucose, and one each of xylose and galactose, all linked in a β -anomeric configuration. The molecule was rendered more stable by conversion of thornasterol A to an asterone moiety (132). Glycoside B (133) from Asterias amurensis is almost identical to thornasteroside A, except that the terminal fucose unit is replaced by quinovose (125). Structure 134 is a third component of the saponin mixture from Acanthaster planci.

7 From Protoreaster nodosus, nodososide (135), a novel type of glycoside was isolated in small amounts. The carbohydrate includes an α -L-arabinofuranosyl moiety attached via a C-24 hydroxyl of undetermined stereochemistry on the side chain. The aglycone was shown to be a hexahydroxy steroid (126). More glycosides of this type (compounds 125-129) were reported from Hacelia attenuata (126) and Protoreaster nodosus (126).

13.5 NODOSOSIDE

4.2 SPECIES STUDIED IN THIS WORK.

ASTERIAS VULGARIS AND ASTERIAS FORBESI .

Samples of the starfish Asterias vulgaris and Asterias forbesi were collected in the gulf of St-Lawrence and extracted according to a procedure reported by Burnell (127).

* Asterias vulgaris and Asterias forbesi are probably the same species (127).

Figure 9

THE SPECIES: ASTERIAS VULGARIS

Figure 10

Purification of the asterosaponin content was done through the use of a medium pressure liquid chromatography system. The crude extract showed at least seven different constituents on TLC. One major product was isolated, for which we report a C-13 NMR spectrum.

4.3 STRUCTURAL STUDIES ON THE ISOLATED ASTEROSAPONIN C.

THE AGLYCONE MOIETY.

Correlation of the C-13 NMR spectrum of the isolated asterosaponin C (136) with the one reported for a glycoside saponin 137 by Komori *et al.* (128) seems to indicate that the aglycone moiety is the same, except for the side-chain substitution. In the asterosaponin C side-chain, the double bond of 137 is replaced by a CH₃ group as in thornasterol B (106) (113, 128b).

138 $\text{R} = \text{H}$, $\text{R}' = \text{H}$, $\text{R}'' =$

137 $\text{R} = \text{Na}^+\text{SO}_3^-$, $\text{R}' = \text{S}$, $\text{R}'' =$

Assignment of the chemical shifts signals of the intact saponin was done by comparison with model compounds (111, 128). The presence of a $\Delta^{9(11)}$ double bond and of a ketone functional group is obvious by the signals appearing at 148.70, 116.76 and 211.83 ppm. Carbon-3 is assumed to bear a sulfate group due to its shift in the low field region of the spectrum; 79.52 ppm compared to 70.90 ppm for a C-OH signal. Glycosidation at C-6 is evidenced by its signal occurring at 80.61 ppm, a downfield shift of 12 ppm compared to C-6 of asterone (111). The remaining signals fit within less than 1.0 ppm difference with model compounds 137 and 138. The chemical shifts for asterosaponin C are presented in Table 13 (p.119). The methyl group at C-24 is assigned the signal at 30.10 ppm.

TABLE 13

C-13 NMR SPECTRA OF ASTEROSAPONIN C MOIETY
COMPARED TO MODEL COMPOUNDS.

CARBON	ASTERONE (92)	<u>138</u>	<u>137</u>	ASTEROSAPONIN C (136)
1	36.7	36.6	36.2	36.1
2	32.6	32.5	29.7	29.6
3	70.9	70.9	78.8	79.5
4	34.3	34.2	30.9	30.9
5	51.0	51.0	49.6	49.5
6	68.4	68.5	80.6	80.6
7	43.5	43.5	41.7	41.7
8	36.1	35.8	35.5	35.4
9	147.1	146.6	145.4	148.7
10	38.8	38.7	38.5	38.4
11	115.8	116.5	116.7	116.8
12	40.9	42.7	42.7	42.5
13	42.8	41.7	41.8	41.7
14	54.0	54.3	54.1	54.1
15	23.4	23.3	23.6	23.4
16	25.9	25.3	25.3	25.2
17	63.6	59.7	59.8	59.7
18	13.5	13.6	13.8	13.6
19	19.7	19.6	19.5	19.4
20	208.1	73.8	74.3	74.2
21	31.2	27.1	27.6	27.1
22		55.0	56.1	55.0
23		211.7	201.7	211.8
24		54.1	126.0	54.1
25		24.4	154.9	24.4
26		22.7	27.5	22.6
27		22.6	20.9	22.7
Me				30.1

* The spectra of asterosaponin C was recorded in pyridine-d₅.
Chemical shifts are in ppm downfield from TMS.

The following resonances accounted for 2 carbons each due to
their high intensity in the spectrum: chemical shifts at 41.7
and 54.1 ppm.

THE CARBOHYDRATE SIDE CHAIN.

In the C-13 NMR of asterosaponin C (136), five resonances appear in the anomeric region (90.0-105.0 ppm), therefore indicating the presence of 5 sugar units in this compound. The chemical shifts at which they resonate also indicates that the anomeric carbons are all in the β -configuration (91,95).

From the C-13 NMR, 29 signals remain to be assign to the carbohydrate moiety of asterosaponin C (136). Taking into account that the most often encountered sugars in these kind of asterosaponins are 6-deoxy galactose (fucose), quinovose, xylose, galactose and glucose, the following deductions were made. A mathematical calculation indicates that to assign 29 carbons to 5 different sugars, and knowing (from NMR data) that no 3-O-methyl glucose is present, only one of them can be a five-atom pyranose. Xylose being the only such sugar present in our expected sugars, it is therefore reasonable to assume that one xylose unit is present in this compound, along with any of the other 4 sugars mentionned above. Also the signal at 64.7 ppm compares well with the chemical shift usually reported for C-5 of a xylose unit.

A closer look at the chemical shift values, along with a comparison to model compounds (84), indicates the presence of 3 signals at 17.9, 18.6 and 18.6 ppm. These signals are indicative of the presence of CH_3 groups at C-5 belonging either to quinovose or fucose (84). Therefore it is probable that, based on the chemical shift values of CH_3 of our model compounds, the chemical shift at 17.9 ppm belongs to C-6 of the fucose unit and that the ones at 18.6 ppm belong to C-6's of 2 quinovose units. As for the other monosaccharides constituting the carbohydrate side-chain it appears to be either a glucose or a galactose, based on the occurrence of a resonance at 62.1 ppm which are usually reported for CH_2OH of those sugars in such molecules (84).

The sulfate group in this molecule can be present as HSO_3 , NaSO_3 , KSO_3 or a combination of all of them. Assuming that the sugar combination mentioned above is the right one, and taking into account that the aglycone is the same as in thornasterol B (as described in section 4.3) we are left with the following possibilities for the molecular weight of asterosaponin C.

TABLE 14

POSSIBLE C-13 CHEMICAL SHIFT ASSIGNMENT
FOR THE SUGARS OF ASTEROSAPONIN C (136)
AS COMPARED WITH MODEL COMPOUNDS (84).

CARBONS	QUINOVOSE (I)	XYLOSE	QUINOVOSE (II)	GLUCOSE or GALACTOSE	FUCOSE
1	104.7	105.1	106.8	102.6	105.3
2	75.1	81.9	76.5	83.7	73.8
3	90.5	76.4	76.9	77.1	75.9
4	75.7	77.9	76.9	69.4	73.6
5	73.6	64.7	74.7	77.7	72.1
6	18.6		18.6	62.1	17.9

* This spectrum was recorded in pyridine-d₅. Chemical shifts are in ppm downfield from TMS.

The following resonances accounted for 2 carbons each due to their high intensity in the spectrum: chemical shifts at 18.6, 73.6 and 76.9 ppm.

TABLE 15

THEORETICAL FAB-MS DATA OF THE MOLECULAR ION REGION FOR
ASTEROSAPONIN C (136).

m/z	Pseudo-Molecular Ions
1335	$[M + K(\text{salt}) + K]^+$
1319	$[M + K + Na(\text{salt})]^+$
1303	$[M + Na(\text{salt}) + Na]^+$
1259	$[M + H]^+$
1189	$[M - SC + Na(\text{salt}) + Na]^+$
1183	$[M - SC + K]^+$
1003	$[M - (2 \text{ sugars})^* + H]^+$
531	$[\text{Aglycone}]^+ (\text{with } NaSO_3 \text{ salt})$

* Sugars can be quinovose/or/fucose + xylose.

SC = Side-chain

The FAB-MS spectrum was recorded in glycerol and methanol.

M.W. 1258 with HSO_3
1280 with NaSO_3
1296 with KSO_3

Table 15 summarizes the pseudo-molecular ions expected for the FAB-MS of asterosaponin C (136). In the FAB-MS, a quasi-molecular ion would occur at m/z 1259 $[\text{M} + \text{H}]^+$ from the HSO_3 form of the sulfate group. The aglycone moiety might also be present at m/z 531. Other expected pseudo-molecular ions are outlined in Table 15. It is proposed that structure 136 (Figure 11, p. 125) represents the major saponin contained in this extract from the species Asterias vulgaris/Asterias forbesi.

The spectra presented in Appendix (p. 247) are included to demonstrate that although the expected peaks are found in the spectra, their relatively low intensity and the changes in the overall distribution of the peaks (this suggests some form of decomposition) forbid the enunciation of a definitive statement and also prevents direct corroboration of the C-13 NMR spectrum.

FIGURE 11

PROPOSED STRUCTURE FOR ASTEROSAPONIN C.

136

Quinovose I

Glucose or Galactose

Xylose

Quinovose II

Fucose

S = SALTS HSO_3 / NaSO_3 / KSO_3

R = CARBOHYDRATES

EPILOGUE

This research project had for its goal the isolation and study of the complete structures of saponins from marine origin in their underivatized form. Modern spectroscopic methods such as C-13 NMR and FAB-MS have opened the door to promising results. Much work remains to be done, but we believe that with the advent of more model compounds for C-13 NMR and a better understanding of FAB-MS, more complete studies of marine or terrestrial saponins will be a success in the near future. This thesis reports for the first time, a complete structure for a holothurin from the Cucumariidae family. It is also the first time that a carbohydrate side-chain with 5 sugars in a linear fashion has been reported. The fact that only spectroscopic means were used to propose structures such as the ones for frondoside J and asterosaponin C, demonstrates great hope for future work of this kind.

CHAPTER 5

EXPERIMENTAL

Melting points (m.p.) were determined on a Büchi SMP-20 apparatus using capillary tubes or on a Fisher-Johns hot stage melting point apparatus and are uncorrected.

Carbon-13 nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WP-250 spectrometer operating at 62.5 MHz, Agriculture Canada, or on a Bruker CXP-300 spectrometer operating at 75.5 MHz, National Research Council of Canada, or on a Bruker WH-400 spectrometer operating at 100.6 MHz, Université de Montreal, or on a Varian XL-200 spectrometer operating at 50.0 MHz, McGill University and Carleton University. Chemical shifts are expressed in δ scale downfield from TMS.

The Infrared (IR) spectra were recorded on a Beckman Micro Lab 600 MX spectrometer. Ultraviolet (UV) spectra were recorded on a DMS-90 UV-Visible spectrophotometer (Varian), in spectral grade methanol or ethanol.

Fast Atom Bombardment mass spectra (FAB-MS) were recorded on a glycerol support (unless indicated otherwise on the spectra themselves) in the positive and negative modes, on a VG 7070-E instrument (from VG Analytical), at the University of Ottawa and on a Finnigan MAT 312, Agriculture Canada.

Thin layer chromatography (TLC) analysis were carried out on precoated silica gel GF-254 (Merck) and reverse phase KC-18F (Whatman) glass plates. Glycoside fractions were developed in the solvent system CH_2Cl_2 -MeOH- H_2O (relative quantities are indicated in the experimental part for each sample). Detection was accomplished by spraying a 1% ceric ammonium-nitrate in 50% sulfuric acid solution, followed by heating at 100-120 °C. Reverse phase TLC were developed in a solvent system of methanol and water (70/30, v/v) and detected by spraying a solution of acetic anhydride and concentrated sulfuric acid (ratio 9:1), followed by heating at 100 °C for a few minutes.

Medium pressure liquid chromatography separations were carried out on a "home built ApLab" system. The columns were packed with silica gel 60H (Merck) or Whatman silica gel LPS-1 (13-24 microns) or LPS-2 (37-53 microns).

Columns were packed dry and washed with methanol before usage. Regeneration of the silica gel was done by heating it in an oven at 550 °C for 24 hours.

High performance liquid chromatography separations were carried out on a μ -Bondapak C-18 reverse phase column (Waters Associates, 10 microns), using a Liquid Chromatograph (from Waters Scientific Ltd) linked to a variable wavelength UV detector (from Schoeffel Instrument Corp.).

All solvents used were reagent grade and glass distilled before use. The methanol was HPLC grade. Deionized water was used for HPLC runs.

COLLECTION OF SEA CUCUMBERS.

CUCUMARIA PRONDOSA

Animals were captured on June 19, 1982 at Islets de Mai (16 Km south of Sept-Iles, Québec), by SCUBA diving to depths of 5-7 meters during low tide. The sea cucumbers

were kept on ice in ice boxes for 5-7 hours prior to freezing (using the facilities at the Université du Québec à Chicoutimi).

In April 1982, the team of divers from the Université du Québec à Chicoutimi supplied us with some Cucumaria frondosa collected at Baie St-Pancrase and Baie Comeau, Québec. They also provided us with sea cucumbers from Matamek, Québec, collected on September 9, 1979.

PSOLUS PHANTAPUS

Samples of Psolus phantapus were provided by Professor J. Kingston. They were collected by SCUBA diving to depths of ca. 20 m. Some of the animals originate from Point Kirwin and were collected on May 17, 1981; while the others are from Maggotty Cove, Bay Bulls and were collected on April 4, 1980.

The Université du Québec à Chicoutimi also provided us with some specimens collected at Baie St-Pancrase, on May 30, 1982, by SCUBA diving to depths of 5-7 m.

SPECIMEN PREPARATION

The frozen animals were thawed and washed in sea water. All the specimens were dissected and only the body walls of the animals were kept. Each animal was cut into 2-3 pieces and soaked in a bath of acetone for 2-3 h. This helps to get rid of the water that is imprisoned in the tissues. The dried skins were then cut into smaller pieces and reduced to powder with a blender. This powder was then ground to 20 mesh with a small mill to give a finer powder that was air-dried for 24 h.

EXTRACTION AND PURIFICATION OF SAPONINS FROM CUCUMARIA FRONDOSA. (AN EXAMPLE).

The body walls of 250 specimens of the species Cucumaria frondosa were soaked in distilled acetone for 2-3 h and ground to a fine powder (1.52 Kg). The powder was then placed in a round bottom flask equipped with a mechanical stirrer and extracted successively with benzene (2 x 3000 mL, reflux for 24 h), ether (1 x 1500 mL, reflux for

5 h) and methanol (3 x 4000 mL, reflux for 24 h). The benzene extraction allowed elimination of most pigments and lipids, while the methanol extracted some saponins and steroids.

The glycosides were extracted with 3 portions of 3500 mL of ethanol 70% (v/v) at 55-60 °C for 24 h. The combined extracts were evaporated under reduced pressure with a rotatory evaporator. During the evaporation process, 1-butanol had to be added to the flask to help reduce the formation of a foamy substance in the flask, effectively due to the presence of saponins. This residue (185.84 g) represents the crude yield of glycosides.

AMBERLITE XAD-2 RESIN TREATMENT FOR SAPONIN EXTRACTION.

This method is similar to the one reported by Gilgan et al. (66, 67). A column (60 cm x 4.55 cm internal diameter) was filled with an aqueous slurry of Amberlite-XAD-2 resin (Mallinckrodt, 20-50 mesh). The column was washed successively with the following solvents, in order to eliminate any trace of organic contaminants: water (1000

mL), methanol (1000 mL), acetone (1000 mL), 1:1 (v/v) 1N HCl/acetone (2000 mL), acetone (1500 mL), 1:1 (v/v) 1N NaOH/acetone (2000 mL), acetone (2000 mL), water (3000 mL), methanol (1000 mL), water (2000 mL), 1N HCl (500 mL), water (1000 mL). The pH of the last washings was tested and showed to be neutral.

In a typical run, 15 g of the 70% ethanol extract of Cucumaria frondosa were dissolved in 100 mL of water. The saponins were then eluted from the Amberlite-XAD-2 resin column with the following solvents: water (6000 mL, yield 6.5768 g, to remove the salts), 50% methanol/water (1000 mL, yield 1.9817 g), 100% methanol (2000 mL, yield 0.5231 g) and finally a mixture of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 8/2 (v/v). Most of the glycosides were recuperated in the methanol elutions.

ISOLATION OF SAPONINS FROM CUCUMARIA FRONDOSA.

The purification of saponins was done on a medium pressure liquid chromatography "ApLab" system. Part of the saponin mixture (0.9832 g) was dissolved in 8 mL of a solvent mixture of $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ (60/18/3.2, v/v and

injected on a column of silica gel (13-24 microns). Column size: 32 cm x 4.0 cm, internal diameter. The elution rate of the column was set to 3 mL/min at a pressure of 15 psi. The following elution solvents were used: CH_2Cl_2 -MeOH- H_2O , 60-18-3.2 (1100 mL) - 60-20-3.6 (836 mL) - 60-25-5.4 (904 mL) - 60-42-10 (lower layer, 1120 mL); MeOH (1000 mL). Fractions of about 20 mL were collected and analyzed by TLC in CH_2Cl_2 -MeOH- H_2O , and evaporated under reduced pressure. The following scheme A summarises the combination of different fractions.

Scheme A: Fr. 1-13, Fr. 14-28, Fr. 29-40, Fr. 41-63, Fr. 64-79, Fr. 80-95, Fr. 96-104, Fr. 105-115, Fr. 116-171, Fr. 172-188, Fr. 189-197, Fr. 198-215, Fr. 216-264, Fr. 265-MeOH.

Fractions 116-171 contained a saponin named frondoside J (20 mg) which proved to be pur by normal and reverse phase TLC. Frondoside K (3 mg) and frondoside L (2 mg) were also obtained as pure saponins. Unfortunately, due to their small amounts, no characterization of their structure could be performed.

EXTRACTION AND PURIFICATION OF SAPONINS FROM PSOLUS
PHANTAPUS.

The body walls of 31 sea cucumbers of the species Psolus phantapus were soaked in distilled acetone for 5 h, then ground to a fine powder (0.700 Kg). The powder was placed in a round bottom flask equipped with a mechanical stirrer and extracted successively with benzene (2 x 1500 mL, 24 h), ether (1 x 1500 mL, 4 h), methanol (2 x 1500 mL, 20 h), and finally ethanol 70% (3 x 1500 mL, 24 h).

The different extracts were combined and evaporated under reduced pressure. The ethanol extracts contained the glycosides of interest (40.5 g, residu after evaporation). This residue was desalted by running it through a column of Amberlite XAD-2 resin (Mallinchrodt, 20-50 mesh, column dimensions: 60 cm x 4.5 cm internal diameter). The column was eluted successively with water (4000 mL) to get rid of most of the salt, methanol/water 50/50 (v/v) (2000 mL yield 6.9470 g), methanol (2000 mL, yield 0.7232 g) and finally a mixture of $\text{CH}_2\text{Cl}_2/\text{MeOH}$, 8/2 (v/v) (1000 mL, yield 0.0380 g).

ISOLATION OF SAPONINS FROM PSOLUS PHANTAPUS.

The purification of saponins from Psolus phantapus was achieved on a medium pressure liquid chromatography "ApLab" system, followed by a final purification on a HPLC system, equipped with a reverse phase column .

In a typical run, the saponin mixture of Psolus phantapus (463.87 mg) was dissolved in 14 mL of the solvent mixture $\text{CH}_2\text{Cl}_2\text{-MeOH-H}_2\text{O}$, 60-18-3.2. Since the residue proved difficult to dissolve, 1.0 mL of methanol in 0.5 mL of water were added to help solubilise the saponin mixture. This saponin mixture was injected on a column of silica gel (13-24 microns, column size: 32 cm x 4.0 cm internal diameter; pressure 26 psi; elution rate 6 mL/min). The silica gel was always washed before use with methanol, followed by the first elution solvent. The elution solvents used were: $\text{CH}_2\text{Cl}_2\text{-MeOH-H}_2\text{O}$: 60-18-3.2 (1500 mL), 60-20-3.6 (2000 mL), 60-25-5.4 (1500 mL), methanol (2000 mL). Fractions of about 20 mL were collected and monitored by TLC. Similar fractions were combined and evaporated under reduced pressure. The following fraction combinations are shown as an example (Scheme B).

Scheme B: Fr. 1-69, Fr. 70-100, Fr. 101-130, Fr. 131-149, Fr. 150-170, Fr. 171-205, Fr. 206-245, methanol fraction.

Final purification by HPLC of fractions 571-600 from another column furnished 9.4 mg of a pure sample named phanthothurin I, and another sample phanthothurin II (5.0 mg) that showed 2 spots on TLC.

EXTRACTION OF PSOLUS PHANTAPUS.

a) SOLID-LIQUID EXTRACTION

The powder from Psolus phantapus (358.9 g) was extracted successively with benzene (765 mL, 7h, 52 °C), benzene (550 mL, 7:50 h, 54 °C), benzene (500 mL, 8 h, 54 °C), benzene (500 mL, 6:40 h, 56 °C). The powder was the extracted successively with different portions of ethanol 70% (500 mL, 10 h, 54 °C, 550 mL, 13 h, 54 °C, 500 mL, 8 h, 56 °C, 500 mL, 15 h, 55 °C). The yield of these extractions is reported in Table 2 (p.66).

b) LIQUID-LIQUID EXTRACTION.

The powder from *Psolus phantapus* (175.1 g) was extracted with 625 mL of ethanol 70% for 6 h at 50 °C. After filtration, the filtrate A (244 mL) was extracted successively with three portions of benzene. The first benzene extraction gave two well distinguished and easy to separate phases. The second benzene extraction gave an emulsion that was only controllable by adding water. The same phenomenon occurred with the third extraction. The non efficiency of the liquid-liquid extraction was verified by TLC. The yield of these extractions is reported in Table 4 (p. 72).

ASTERIAS VULGARIS, ASTERIAS FORBESI.

The extraction of the saponins from the starfish *A. vulgaris*, *A. forbesi* was accomplished according to a method previously described by Burnell (127), and is considered a "Classical saponin extraction method".

The starfish are first thawed, then blended to give a homogenate. Part of the homogenate is mixed with water and

methanol and left standing for 3 h. The slurry is suction filtered through Whatman 42, then Whatman 50 filter paper. The filtrate is rotoevaporated, followed by dilution of the concentrate with water. The aqueous concentrate is extracted with hexane (to remove fats), then with ether and followed by benzene. All these extractions create emulsions. For the hexane and ether separations, the emulsions require up to 24 h before they are broken up. For the benzene separation, the emulsion is broken by acidification (to pH 4.0 with HCl) of the aqueous layer after standing for 3 days. The aqueous solutions are then made up to 5% (w/v) NaCl and extracted with 1-butanol. The butanol extracts are combined, concentrated down to a volume of about 100 mL and left standing for a few days. From these concentrates, a pale yellow precipitate of crude saponins is collected by suction filtration. These crude saponins are then purified by the same chromatography techniques as described previously in this thesis.

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PART B.

STUDIES OF SAPONINS STRUCTURES FROM:

PHYTOLACCA DODECANDRA.

CHAPTER 1

THE ENDOD PLANT PHYTOLACCA DODECANDRA.

1.0 INTRODUCTION.

Two decades ago, when an interest arose for the plant Phytolacca dodecandra it was because scientists were searching for a molluscicide of natural origin, if possible, to control the disease schistosomiasis. This is still the goal today, and results have shown (1) that fairly small quantities of berries from Phytolacca dodecandra and a simple method of application (1) could be used to control the snails who are the intermediate hosts in the life cycle of the parasite responsible for that plague that is schistosomiasis. The ideal molluscicide required to do this job would be a natural product that can be grown in large quantities and can be used as a crude powder or in a concentrated extract form. Also, since this problem belongs to devel-

oping countries, it should require an inexpensive solution that could be use on a self-help basis.

In the last 15 years (2), the molluscicidal properties of the berries from P. dodecandra have been studied by many agencies of which the following are a few. The Pathobiology Institute of the Addis Ababa University of Ethiopia, the Tropical Products Institute (TPI) in London, the Stanford Research Institute (SRI) in Menlo Park, California, the G.W. Hooper Foundation of the University of California, San Francisco, the Harvard School of Public Health, Boston, the U.S. Public Health Service Laboratory and field stations in Puerto Rico, the U.S. Naval Medical Research unit Number 3 (NAMRU-3) in Cairo, and at other laboratories in different parts of the world.

To date, over 40 scientific articles have been published on the subject (2), and several patents secured on the various aspects of the processing of the plant product. Therefore, there is a world wide interest and need to combat schistosomiasis and the active principle involved in this

battle is believed to be from the plant Phytolacca dodecan-
dra. In this laboratory, the structural elucidation of the
intact saponins, believed to be the active principle, has
been investigated and the results so far obtained are repor-
ted in the following sections of this thesis.

1.1 SCHISTOSOMIASIS.

1.1.1 THE SPREADING OF THE DISEASE.

Schistosomiasis is also known as bilharziasis, and is
considered to be one of the most deadly and rapidly spread-
ing diseases, threatening large-scale agricultural deve-
lopment in many tropical and subtropical parts of Africa,
the Middle East, the Far East, some of the Caribbean Islands
and many parts of South America. It is estimated that 200
million people are affected by it, while another 400 to 500
million are exposed to it (1,2).

Schistosomiasis is a disease for which the important
transmitting vector are aquatic snails (3). These snails
breed rapidly in irrigation canals, water reservoirs, lakes,

slow-flowing streams and other such bodies of water. Schistosomiasis is an example of a man-propagated disease. In countries such as Africa, where agriculture is essential for the development and advancement of the country, often the type of agricultural projects required involve the irrigation of canals. Unfortunately, this type of irrigation results in the creation of new breeding habitats for snails. In these propitious habitats, these blood flukes multiply and then swarm to invade the human skin.

It is in the building of such canals, that infected and non-infected people are brought together. There, they drink, bath and labor in those same canals. Also too often, human wastes are excreted in the same waters. All these combinations are ideal for the rapid spread of schistosomiasis, especially among highly vulnerable children.

1.1.2 THE CYCLE.

The causative organisms of schistosomiasis are flukes
(3). In infected individuals, adult male and female pair of

the parasites reside in small blood vessels, usually in the mesentery or the pelvic region. The parasite, which is called *Schistosoma*, is able to lay a large number of eggs. A single female parasite is known to lay up to 3,500 eggs per day and has a life span of 20 to 30 years. An infected person can carry a large number of such females. The combination of such a great number of eggs, along with the host's reaction to such a foreign material, can cause considerable damages to the liver or the bladder and ureter; depending on the species of the parasite involved. *Schistosoma mansoni* are the parasites that characteristically and preferentially lodge in the mesenteries. *Schistosoma haematobium* are the ones that usually enter the bladder area. The diseases they cause are known as intestinal schistosomiasis and urinary schistosomiasis respectively. There is also a third species known as *Schistosoma japonicus* that is found mainly in Asia and also lodges in the intestinal mesenteries.

The cycle of transmission of the disease is schematically depicted in figure 1 (p. 157). The capulated eggs of the parasites are excreted with feces or urine, as the case may be. These eggs hatch when they come into contact with

TRANSMISSION OF SCHISTOSOMIASIS.

Intermediate Host-Parasite Relationship

the water, and the freely swimming miracidia enters an appropriate fresh-water snail and develops into a cercaria, which is the infective form of the parasite. The infected snails continuously releases cercaria by millions in streams, canals and whatever other water flow. The cercaria can then enter through the skin of the people that come into contact with the infected water, then enter the blood stream and lodge in their preferred sites as male and female pairs. Schistosomiasis is becoming an increasingly more serious health problem and in some countries it is even taking the place of malaria (3).

1.1.3 THE SYMPTOMS.

Schistosomiasis is a chronic disease. It can cause intensive and irreversible damages to the liver and other tissues, along with reducing resistance to other infectious diseases. In children it often causes mortality. The most insidious impact of such a disease is perhaps the loss of energy which along with the weakening effect of other parasitic infections, produces a state of lethargy, an all too common sight in tropical regions. The unfortunate impact that this disease causes to a country, could therefore probably be calculated in terms of socio-economic losses.

1.2 SCHISTOSOMIASIS IN ETHIOPIA.

Schistosomiasis is a relatively new disease in Ethiopia (2). Until fairly recently, it was thought to be non-existent in that country. Many parasitology texts ruled out the disease on the assumption that the low temperatures of

the highland areas would prevent the snail hosts from becoming established. Though true in part, studies over the last 10 to 20 years have shown that both the intestinal and urinary forms of the disease are widely distributed in different parts of that country. It appears that intestinal schistosomiasis is widely spread in patches of isolated foci in most of the highland areas, while urinary schistosomiasis is more restricted to lower and warmer areas, such as the Awash, Webi-Shebelle and Geenale Valleys.

1.3 CONTROL METHODS.

Since the last 15 years (1, 2), many methods to control schistosomiasis have been tried. Among these, effective and relatively non toxic drugs such as metrifonate, oxamquine and praziquantel were developed. Unfortunately, due to economic costs, these treatments are not available to the majority of people who need them. Also, even if a miraculous drug would be found, it would be of limited value, since cured people would promptly be reinfected while their source of infection remained unchecked.

Among other control methods, immunological studies were looked upon but the chances of finding the right vaccine still lies far in the future. The immunological problems involved are immense since schistosomiasis worms are large and complex organisms, with multiple antigens of unidentified and varying immunological potency and protective effects.

For the time being, the best control method is most likely health education and sanitary improvements in the standards of living. The creation of a system of clean water supply to individual houses and effective health education, with warning signs for children not to swim in infected waters, would be a step in the right direction. But, it is clear that something must be done at the village level, where transmission occurs. Therefore snail eradication has to be the focal point of any control program.

1.4 SNAIL CONTROL.

Over the last 50 years (2), molluscicides having several potent snail-killing chemicals have been developed and used in various parts of the world. Among these, the oldest and probably most often used are copper sulphate and sodium pentachlorophenate. In recent years, more effective and efficient molluscicides such as BayluscideTM and FresconTM have become available on the market and replaced the older compounds. As was to be expected none of these molluscicides are free of problems. Copper sulphate, is less potent than the others, and is readily absorbed in water by both organic and inorganic matter; sodium pentachlorophenate is an irritant to handlers and is quickly inactivated by the sun's ultraviolet light. FresconTM, the most potent molluscicide available, is not ovicidal in concentrations sufficient to kill adult snails, it is absorbed by organic and inorganic matter in water, and it is affected by the pH of the water. BayluscideTM, is considered to be the molluscicide of choice, despite the fact that it is partially inactivated by solar ultraviolet rays. The pitfalls of all these chemicals are of course, cost, danger to humans who handle them and

are exposed to the treated waters, lethality to other marine organisms, duration of potency and relative efficacy.

The Chinese Approach to the schistosomiasis problem has been the destruction of the snails by removing individual snails by hand or chopsticks from the rivers, or even crushing them for complete destruction. But this requires the mobilization of many hundreds of peoples, something that is not always realisable, especially when the snail control campaign has to be on a continuing basis.

An alternative solution is then required. The "best" molluscicides might well be those produced from local products, that would be readily available, of low cost and could easily be employed by a permanent cadre of appropriately trained villagers.

1.5 THE ENDOD PLANT.

In 1964, Aklilu Lemma (4) discovered that the plant

Phytolacca dodecandra l'Hérit, from the family Phytolaccaceae, had the ability to kill snails. In Ethiopia (5), the berries of this plant are called ENDOD. For centuries, Endod has been used as a laundry soap, while other parts of the plant have served as a medicine and poison.

The first reports of the use of Endod as a soap in Ethiopia came from Portuguese Jesuits in the early part of the seventeen century (6). At present, folk uses of Endod in Ethiopia, as described by Lemma (7) and Kloos (8), include its use as a purgative, soap, hirudinicide (leechicide) and abortifacient. On the other hand, Watt and Meyer-Brandwijk (9) described its uses as taenicides, emetic, abortifacient, stimulant, tonic, purgative, veterinary medicine, toxin and vermicide in other African countries.

It was during ecology studies on the distribution of bilharzia transmitting snails, in Adwa, northern Ethiopia, that A. Lemma (4) noticed that in streams and along the river, where people washed their clothes with Endod berries (also known as soap berries) that there were fewer snails than in adjacent areas. It was shown subsequently that

Endod possessed snail-killing properties (10). Extensive studies on the Endod plant has since been conducted (1, 2) and some promising results obtained.

Until now, P. dodecandra has not been used in the fight against schistosomiasis (1) because it was difficult to cultivate them on an industrial scale and therefore insufficient quantities of berries with molluscicidal properties were available. A further problem encountered in attempts to grow the plants on a large scale is that stem-borers of the genus Gitona attack young shoots of the plant.

As part of a project in search for effective control over bilharzia transmitting snails, Lugt (1) studied 65 different types of P. dodecandra, all originating from Ethiopia. From these, he found that eleven possessed acceptable molluscicidal potency. Out of those eleven types, five also had favorable agronomic qualities. Lugt estimated that mature plants without shade and with sufficient water gave an average yield of 264g of berries per bush (11). The Endod plants occur in many varieties with respect to morphology, saponin content of the berries and molluscicidal potency.

The most potent part of the Endod plants are the berries, which contain a fairly large amount of saponins. Other parts of the plant were shown to be less potent. Lugt also concluded (11) that it was at their unripe stage that the berries possessed their highest molluscicidal potency. The haemolytic capacity was also at its highest at that point. This potency information was also corroborated by the local people in Ethiopia, in that the unripe stage is always used for washing clothes. However, some varieties produce berries with a high snail-killing potency even at their overripe stage. There are also variations between short staminate plants of *P. dodecandra* and long staminate ones.

In other studies (2) the potency of Endod was found to remain constant at different pH values (5-9), temperatures and ultraviolet radiations. The toxicity was retained in various concentrations of organic and inorganic river mud, but it still proved to be biodegradable. Berries that were stored for more than five years still retained their potency (1). Crude samples of Endod killed fish, tadpoles, and schistosome miracidia and cercariae at molluscicidal concentrations. However, the Endod was nonovicidal for

Bulinus and Biomphalaria. Endod was also shown to be toxic to leeches (12). From these researches, it was concluded that Endod berries possess characteristics that rank them among the important molluscicides. Although not as toxic as very low doses of commercial synthetics, they possess many favorable features and a potential for increased molluscicidal potency.

In laboratory testings, Endod showed no toxic effects in mice, rats, chickens, sheep and rhesus monkeys, after administration of high dosages of Endod. Also no skin irritation was reported either for humans or animals, which was a good indication that the compound could be handled safely (7) by workers. Likewise, no permanent phytotoxicity was found to develop during continuous application of high concentrations to different local plants grown experimentally (13).

As the interest in molluscicides of plant origin grew larger, other plants than P. dodecandra were investigated. Among these the following plants were studied: P. octandra (14), Polygonum senegalense (15), Croton macrostachys (16),

Sesbania sesban (16) and Ambrosia maritima (17). They have all been reported to possess molluscicidal properties. These plants were tested against berries of 3 selected types of P. dodecandra (1). The Endods were by far the most potent molluscicide. A 100% snail-kill at concentrations of 15 or 10 ppm was achieved in all 3 cases. It should also be mentioned that the berry powders of Endods mixed extremely well with water. As for the other kind of plants tried, much higher concentrations were necessary to achieve snail-killing.

The principal conclusions drawn from Lugt's work (1) are that to be effective, berry powder must mix very well with water and a certain waiting period is necessary for the active principle to dissolve. Otherwise, berry powder applied directly is much less active than an extract made from the same berry powder. Lugt's experiments also showed that freeze-dried extracts retained only half of the activity of the original extracts. Extractions with 70% ethanol, gave berrie extracts that did not have any molluscicidal activity. Lugt's also prepared distilled water and saline extracts and found those easy to prepare, while butanol ex-

tracts were not. The saline extract was kept at 20°C, and after 250 days it retained the same molluscicidal level that it exhibited immediately after preparation.

Lugt (1) also concluded that there was almost a linear correlation between concentration and time, as regard to the duration of the molluscicidal activity.

1.6 THE ACTIVE COMPOUNDS IN ENDOD.

The active molluscicidal component of *P. dodecandra* berries are saponins. These saponins are constituted of a triterpenoid aglycone, to which a branched trisaccharide is linked at carbon-3. The four aglycones identified to date are: oleanolic acid, bayogenin, hederagenin and 2-hydroxy-oleanolic acid (18, 19). The derivatives of oleanolic acid occur in considerably greater amounts than do those of bayogenin. The saponin mixture comprises at least eight

different compounds (1), including the four mentioned above. It appears that the saponins vary in their molluscicidal potency and synergism, depending on their component glycosides. The structures of these compounds will be reported in chapter 2 of portion B of this thesis.

1.7 OTHER USES OF ENDOD.

Since the early discovery of the molluscicidal potency of Endod berries, these compounds have also been found to be fungicidal (20), emetic (21), larvicidal for the mosquitos (22) and a potent spermicide (23). Also, purified compounds from Endod have shown antifertility activity in animals.

Besides all these interesting effects, scientists (24) have conducted researches on the active component of Endod, in order to use it as an antidote for overdosage of certain drugs or poisons, as this agent induces vomiting immediately

upon absorption and this without any lasting effects. In fact, Ethiopians make a strong tea out of the Endod berries and use it as an antidote for poison.

Some other researches have shown that weak extracts of Endod are lethal to the fungus responsible for athlete's foot. Harvard researchers have found that the larvae of the mosquito that carries malaria is ten times more susceptible to Endod extracts than are water snails. According to Parkhurst, in the presence of only a trace of Endod, the larvae cannot develop into an adult. Therefore, it seems that there is a very broad spectrum of possible applications for Endod, but the best thing about this, is that Endod being a natural product it could become a cheap and effective means of controlling schistosomiasis in certain parts of the world. Under suitable climatic conditions, the plant grows rapidly and can bear fruits twice a year.

CHAPTER 2.

PREVIOUS STUDIES ON ENDOD.

2.0 EXTRACTION PROCEDURE FOR ENDOD BERRIES.

The extraction process used for Endod berries is highly important, since depending on the kind of solvent used, the extracts will show different levels of molluscicidal activity. An ideal process would be one that could be use on very large scales and not require any expensive organic solvents. Financially this would be the principal advantage for the Ethiopian people. Since the extraction process for Endod developed over the years are few, they will all be reported here.

2.1 JEWERS AND KING.

Over the years, a few patents have been filed in regarding extraction processes. The first one was filed by Jewers and King (25) in 1968. Their process consisted of extracting the berries of P. dodecandra with an alcoholic solvent and subjecting the extracted material to alkaline hydrolysis. Comparative tests had shown (25) that it was possible to recover approximately twice as much molluscicidal saponin from Endod by alcoholic extraction than by aqueous ones. Therefore, the ground berries were defatted with petroleum ether; these extracts were then reextracted with hot methanol and the material so extracted, subjected to alkaline hydrolysis. This solution was then neutralized and precipitated inorganic material removed by filtration. Upon concentration of the solution, and addition of diethylether, the Endod saponin precipitated out, was separated, washed and dried to give an amorphous yellow solid, which was then ready to be purified by chromatography if desired.

2.2 A. LEMMA ET AL.

The second method, which was developed and patented by the Stanford Research Institute investigators in 1972 (26), was simpler and involved a one-step butanol extraction from an aqueous suspension of dried berries. This group also claimed that their procedure provided a molluscicide with slightly more or equal potency than the molluscicide produced by Jewers and King, along with an economy in time and money. A summary of the extraction is as follows: The berries are dried, ground and defatted with petroleum ether. The defatted berries are then extracted twice with hot water. This aqueous solution is then centrifuged and extracted twice with n-butanol. A final washing with diethylether gives a light tan powder. Therefore, this method could be used inexpensively in tropical areas where Endod is found and the need for a cheap and safe molluscicide is great. The only drawback, once more, was that the method depends on imported solvents, which demand hard currency.

2..3 T. LEMMA.

The financial problems involved in the first two extraction processes were overcome in 1975, when Tesfaye Lemma (2), an Ethiopian chemist, developed a third and probably most promising extraction procedure. This procedure was based on fermentation techniques. This fermentation-based extraction procedure depends on practical and simple techniques using yeast cells that are part of the normal flora of the berries. The Endod berries are ground, soaked in water and left in a warm place where they ferment rapidly. Eventually, they separate into a clear supernatant which contains active principles and a residue, which contains yeast cells and debris. The supernatant can easily be separated from the residue and concentrated or dried into a fine powder. This powder can afterward be used for dusting over contaminated water, or use as flakes, or even be prepared as emulsions concentrates for spraying or use as briquettes of different hardness for slow release in water. The refinement and standardization of these methods has not been developed so far.

3

In 1976, at the invitation of the Ethiopian government, Charles B. Lugt (1) conducted agrobotanical studies on Endod. The aim was to select and test Phytolacca dodecandra types for their agronomic qualities and molluscicidal potency, and to propagate and plant selected and tested types as a crop.

For the isolation of the glycosides, Lugt selected P. dodecandra plants of type-2 at their unripe stage. In contrast to Lemma et al. (26) Lugt did not use the butanol extraction process but instead, used distilled water, since the saponins are highly soluble in such aqueous media. The first step was to homogenize the berries with water in a tissue grinder, then centrifuge them at 5° for one hour. This was followed by washing and centrifugation of the pellets twice, and combination of the three supernatants. A verification on snails and by TLC confirmed that the extraction was complete. Water was evaporated at 70-80°C and a brown yellow powder was obtained. Lugt isolated various compounds of the glycoside mixture by TLC. This method has the advantage of being cheap and fast.

The application of P. dodecandra berries for snail control has the advantage of being relatively easy. According to Lugt (1) it can be done in two different ways. The first one is to make a slurry of the dried and ground berries in water. This can then be easily suited for irrigation schemes and rivers. The second one would be to extract and use only the active principle. This method would be more suitable for large bodies of water such as lakes, where focal applications of the molluscicide is required and sprayers have to be used. This allows for selective treatment. It would also be more economic and spare the lives of fish and hopefully of other living organisms.

2.5 TRITERPENOID SAPONINS FROM PHYTOLACCA DODECANDRA.

The toxic principal, which is present in various parts of the plant Phytolacca dodecandra has been suggested to be a saponin (1). The first chemical studies on the structure of saponins from the berries of P. dodecandra dates back to the late sixties, early seventies. Preliminary investigations of these saponins were conducted by Powell

and Whalley (27). The extraction procedure they used, was to crush the dried fruits, defat them with petroleum ether and extract them with methanol. This provided a water soluble gum, bearing the general properties of saponins. Powell and Walley (27) tested these extracts against young adult snails of a Puerto Rican strain of Australerbie glabratus and obtained the following reproducible results.

- a) 1:7 500 parts in water, all snails killed in four days;
- b) 1:3 250 parts in water, all snails killed after 48 hours.

Their structural work was conducted on derivatized crude saponins, i.e. hydrolysis, acetylation and methylation. Separation of the mixed products by chromatography furnished methyl-O-acetyl oleanolate (1) and methyl tri-O-acetyl bayogenin (2), thereby indicating the presence of the oleanolic acid and bayogenin moieties as aglycones in the original saponins. The NMR and mass spectral data of 2 confirmed that structure to be in accord with the one previously assigned to bayogenin (28). These investigations by Powell and Whalley were discontinued as they became aware that more extensive researches were being carried by the Tropical Products Institute, who had also reported in the previous year, the isolation of oleanolic acid (29).

1

2

Parkhurst *et al.* (30) investigated the structure of one of the major molluscicidal saponins of Endod and found it to be 3-[2,4-di-O-(β -D-glucopyranosyl)- β -D-glucopyranosyl]-olean-12-ene-28-oic acid (3). Their isolation procedure consisted of grinding finely the dried Endod berries, defating with light petroleum ether and extracting with warm water. This extract was further partitioned with butanol to provide a biologically active light tan powder, representing 20-25% of the initial ground berries (31). Further examination of the acetylated material by TLC showed a complex mixture of more than ten compounds of which three appeared as being major ones. Deacetylation of one of the three major compounds gave a biologically active substance named oleanoglycotoxin-A. * Upon reacetylation the material was identical to the originally isolated acetate. Acid hydrolysis of oleanoglycotoxin-A gave a compound with the same *R_f* as oleanolic acid (4). Examination of its permethyl derivative by GC-MS demonstrated that the major component (86%) had a retention time and MS identical with those of authentic methyl O-methyl-oleanolate (5), while the minor component (11%) gave a MS identical with authentic methyl di-O-methyl hederagenin (6). Therefore, in spite of TLC homogeneity of its acetate, this would seem to indicate that oleanoglycotoxin-A contains a minor component derived from hederagenin (7). The aqueous phase of the acid hydrolysis of oleanoglycotoxin-A contained only glucose. NMR and MS data of peracetylated and permethylated oleanoglycotoxin-

4 $R_1 = R_3 = \text{OH}; R_2 = \text{H}$ Oleanolic Acid

5 $R_1 = R_3 = \text{OMe}; R_2 = \text{H}$ Methyl-O-Methyl Oleanolate

6 $R_1 = R_2 = R_3 = \text{OMe}$ Methyl di-O-Methyl Hederagenin

7 $R_1 = R_2 = R_3 = \text{OH}$ Hederagenin

A, along with GC-MS analysis of the trimethylsilylated sugar mixtures, confirmed the structure to be a triglucoside of oleanolic acid (3). Parkhurst et al. also concluded that the minor hederagenin containing constituent probably had a similar structure or could possibly be associated with a linear trisaccharide, as this could also be true for a small part of the total oleanolic acid present in the mixture.

The most biologically active saponin of Endod was also isolated by Parkhurst's group (32). It is also a derivative of oleanolic acid, substituted in the 3 position and with a branched trisaccharide, containing 2 glucose units and one galactose unit. This compound, named Lemmatoxin, after Aklilu Lemma, seems to have twice the biological activity (LD_{50} , 1.5 ppm, 24 h, Biomphalaria glabrata) as the major component that was oleanoglycotoxin-A. Studies on Lemmatoxin's structure were done on its acetate derivative, which showed homogeneity on TLC. Lemmatoxin acetate could be deacetylated to give a biologically active saponin and reacylated to give back Lemmatoxin acetate, identical to that of the originally isolated material. Its concentration in the crude material

was estimated to about 16%. Acid hydrolysis of Lemmatoxin, followed by GC studies gave oleanolic acid as its aglycone and glucose and galactose in the ratio of 2:1, respectively. Mass spectra and ^1H NMR spectra data of Lemmatoxin acetate helped in proposing structure 8 for Lemmatoxin, although some doubts still exist as to the position of attachment of the glucose and galactose units. The structure of Lemmatoxin was suggested to be $3\text{-O-}[4'\text{-O-}(\beta\text{-D-glucopyranosyl})\text{-}3'\text{-O-}(\beta\text{-D-galactopyranosyl})\text{-}\beta\text{-D-glucopyranosyl}]$ olean-12-ene-28-oic acid (8)

Among the minor saponin constituents of the Endod berries, Parkhurst et al. (33) isolated and studied the structure of two additional triglycosidic derivatives of oleanolic acid. They called them Lemmatoxin-C. Although Lemmatoxin-C was homogeneous on TLC, it was found to be a mixture of two closely related oleanolic acid derivatives, each with a linear trisaccharide at the C-3 of oleanolic acid and with rhamnose as the end sugar. Its biological activity was shown to be only half of the biological activity of Lemmatoxin. Indications of the non-homogeneous constitution of this compound was evident in the NMR

spectrum where variations in the sugar region of the spectrum could only be due to multiple components. This was confirmed by GC analysis of the pertrimethylsilylated sugars, where total acid hydrolysis of Lemmatoxin-C confirmed the multiple component theory that rhamnose, glucose and galactose were present. Therefore, structure 9 was proposed for the major component of Lemmatoxin-C (at least 70%) 3-O-(α -L-rhamnopyranosyl-2'-O- β -D-glucopyranosyl-2''-O- β -D-glucopyranosyl) olean-12-ene-28-oic acid, and the remaining component as the galactose containing species, structure 10.

In summary, the aglycones of *P. dodecandra* studied so far, consist of oleanolic acid ((66.2%), hederagenin (8.9%), 2-hydroxyoleanolic acid (6.5%) and bayogenin (14.9%), all 28-carboxyoleanenes. The composition determination of the aglycones were done on the crude butanol extract by GC studies of the permethylated hydrolyzed crude saponins (34). Also, Oleanoglycotoxin-A, Lemmatoxin and Lemmatoxin-C were reported to occur in the crude saponin mixture from *P. dodecandra* in a ratio of 18, 16, and 17% respectively (23). Parkhurst *et al.* (33) noted an interesting fact, that the synthetic β -glucoside of

oleanolic acid has only one-tenth the molluscicidal properties of Lemmatoxin-C.

In a structure-activity relationship of a series of monodesmodic and bidesmodic triterpenes, of the dammarane types, primarily those representing oleanolic acid and hederagenin, Hostettmann et al. (34) showed that from their data, it appeared that the number, sequence, point of attachment and configuration of sugars attached to the dammarane aglycone were less important than the fact that sugars attached to 2 different positions on the aglycone render the resulting saponins inactive as molluscicides.

The two main disadvantages to the use of Endod saponins as molluscicides are that i) the mixed saponins are lethal to fish at low concentrations and ii) the snail eggs are not killed by P. dodecandra molluscicides therefore, the berries will not provide a lasting clearance of B. pfeifferi snails.

NOMENCLATURE

CHAPTER 3

STRUCTURAL STUDIES OF ENDOD SAPONINS.

Our interests and involvement in the study of Endod saponins was to: a) Take advantage of our separation techniques that had proven efficient for other kinds of saponins difficult to purify b) Confirm if possible the structure of intact saponins with state-of-the-art techniques such as C-13 NMR and FAB-MS as developed for saponins in Part A of this thesis c) Separate pure saponins in amounts sufficient to allow molluscicidal studies of individual constituents by Prof. D.R. Gardner*.

3.0 TYPE STUDIED.

The species of berries studied were from Phytolacca dodecandra (Phytolaccaceae family). They were of two types;

* Prof. D.R. Gardner, Dept. of Biology, Carleton University

the first one being of Type-3 and the other one of Type-unknown. Both types were supplied by Prof. J.D.H. Lambert* in the form of extracted powders from Ethiopia.

3.1 EXTRACTION AND PURIFICATION OF SAPONINS.

The type-3 samples were in the form of a dark-brown powder. These berries had been extracted according to the procedure used by Lugt (1). This procedure consists of homogenizing the berries with distilled water in a tissue grinder. This is then followed by centrifugation at $g=800$ and a temperature of 5°C for one hour, followed by washing and recentrifugation of the pellets. This procedure is carried out three times and the supernatants are combined. By this mean, complete extraction is obtained; this is verified by a test on snails and by TLC of the pellets. The water is then evaporated at $70-80^{\circ}\text{C}$ and a powder obtained. (In this case, a brown-yellow colored powder was reported for the species of type-2).

The Endod samples were very soluble in water, in which media they foamed abundantly. Type-3 samples showed to be constituted of at least five different compounds, as

* Prof. J.D.H. Lambert, Dept. of Biology, Carleton U.

verified by a triple elution on a thin layer chromatography plate in the solvent system CH_2Cl_2 -MeOH- H_2O ; 60-25-5.4 v/v. Two of these spots appeared as major constituents of the mixture. Since these compounds demonstrated solubility problems in other solvents than water, the powder was first extracted with the above solvent mixture, then filtered and concentrated. Part of that concentrated extract was purified on a medium pressure liquid chromatography system, followed by a final purification on a high performance liquid chromatography system. Repeated chromatography therefore furnished all the samples listed in Table 1, (p. 192) for type-3 Endods.

The type-unknown Endods were in the form of a yellow-brown powder and showed at least eight different compounds on TLC, all with an R_f very close to each other. Five of those spots appeared to be major constituents of the mixture. Repeated chromatography on a medium pressure liquid chromatography system followed by final purification by high performance liquid chromatography gave all the samples listed in Table 2, (p. 193). for the type-unknown Endods.

TABLE 1PHYTOLACCA DODECANDRA

ENDOD TYPE-3

ENDOD TYPE-3	COLOUR ON TLC	Rf CH ₂ Cl ₂ -MeOH-H ₂ O (60-25-5.4)	WEIGHT (mg)	UV MeOH (λ _{max})	m.p. °C
E-3-2	purple	0.22	27.0	T>210	270-275
E-2-201	purple	0.24	57.6	T>210	256-259
E-4-201	pink	0.14	16.0	T>210	220-230
Z-100	purple	0.25	17.2	T>210	262-266
Z-200	lgt. purple	0.26	11.6	T>210	265-268
Z-300	purple	0.29	14.4	T>210	240-244
Z-400	lgt. purple	0.16	7.3	T>210	276-279
Z-500	purple	0.23	11.1	T>210	271-274
Z-600	lgt. pink	0.37	4.1	T>210	276-283

T = transparent above 210 nm.

lgt. = light coloration

TABLE 2PHYTOLACCA DODECANDRA

ENDOD TYPE-UNKNOWN

ENDOD TYPE- UNKNOWN	COLOUR ON TLC	Rf CH ₂ Cl ₂ -MeOH-H ₂ O (60-25-5.4)	WEIGHT (mg)	UV (MeOH) (λ _{max})	m.p. °C
un-A-3	n/a	n/a	1.7	n/a	n/a
un-A-6	dark pink	0.10	12.1	T>210	250-252
un-B-10	lgt. pink	0.14	6.1	T>210	242-245
un-C-10	lgt. pink	0.14	7.3	T>210	249-252
un-D-11	purple	0.14	25.4	T>210	235-238
un-D-13	lgt. purple	0.17	10.6	T>210	236-240
un-B-2	lgt. pink	0.11	4.3	T>210	238-242
un-E2-10	lgt. pink	0.17	8.6	T>210	243-237

n/a = non available

lgt. = light coloration

T = transparent above 210 nm.

Overall, nine pure compounds from the type-3 Endods were obtained, while another eight pure compounds from the type-unknown Endods were purified. The amount of pure material obtained is also reported in Tables 1 and 2. It was interesting to see that on TLC, the compounds char with a very nice pink or purple coloration. All compounds obtained were considered to be homogeneous on the basis of their behavior on both normal and reverse phase TLC. Tables 3 and 4 (p. 195, 196) summarises the average retention times of the Endod samples on a μ -Bondapak C-18 reverse phase column, which was used in the last purification step of the samples.

3.2 STRUCTURAL ANALYSIS OF ENDOD SAPONINS.

ENDOD Z-500 (11)

The compound Endod Z-500 (11) was obtained as a white solid, having a melting point between 271-274 °C. The UV spectrum of 11 was recorded in spectral grade methanol and showed no maximum absorbance above 210 nm.

TABLE 3

RETENTION TIMES OF ENDOD TYPE-3 ON μ -BONDAPAK C₁₈
REVERSE PHASE COLUMN.

SAMPLE	ELUTION SOLVENT methanol/water (%, v/v)	AVERAGE RETENTION TIME (minutes, seconds)
Z-600	67	22,32
Z-100	70	9,57
Z-200	70	36,14
Z-300	70	51,08
Z-500	70	21,18
Z-400	70	24,42

Conditions: Flow rate: 1 mL/min
Detector set at 206 nm.

TABLE 4

RETENTION TIMES OF ENDOD SAMPLES OF TYPE-UNKNOWN ON
A μ -BONDAPAK C₁₈ REVERSE-PHASE COLUMN.

SAMPLE	ELUTION SOLVENT methanol/water (%, v/v)	AVERAGE RETENTION TIME (minutes, seconds)
un-A-3	61	14,17
un-A-6	61	35,40
un-B-2	61	14,06
un-B-10.	61	41,47
un-C-10	62	31,06
un-D-11	62	28,11
un-D-13	62	38,23
un-E2-10	64	31,05

Conditions: Flow Rate: 1 mL/min
Detector set at 206 nm.

The intact saponin Z-500 (11) was studied by C-13 NMR in deuterated methanol. Signal assignments for the aglycone part of this molecule were carried out by means of known chemical-shift rules (35), such as hydroxyl substitution shifts, glycosidation shifts (36), steric γ and δ effects (38) and chemical-shift comparisons from compound to compound, and especially in this case, spectral comparison with β -amyrin (12) (39) and the methyl ester of oleanolic acid 13 (40), were very useful.

The aglycone moiety of Z-500 (11) was shown to be similar to oleanolic acid. The signals at 38.2, 25.0, 92.0, 40.7, 57.4, 28.8 and 16.2 ppm are assigned to carbons C(1), C(2), C(3), C(4), C(5), C(23) and C(24) respectively, taking into account the influence of C(3) glycosidation (36). The signal at 92.0 ppm for C(3) might appear somewhat downfield, if it is considered that usually the α -position of the OH group in which the glycosidation took place are shifted downfield by about +8.3 ppm (36). This could probably be explained by a solvent effect, since the spectrum is recorded in deuterated methanol and not in deuterated chloroform like the model compounds 12, 13 and 14.

Doddrell et al. (40) have reported a series of chemical shifts for olefinic carbons at C(12) and C(13) for olean-12-ene type of compounds. Comparison of the present data with Doddrell's seems to indicate that the double bond is also present at position Δ^{12-13} in Z-500. The signal at 184.1 ppm is indicative of the presence of a carboxylic acid group (41) and is assigned to C(28). The remaining signals for the aglycone moiety are assigned by direct comparison of the chemical shifts with the ones of the model compounds (42). The C-13 NMR chemical shifts for Z-500 are reported in Table 5 (p. 199).

TABLE 5

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C-13 CHEMICAL SHIFTS FOR Z-500 COMPARED TO MODEL COMPOUNDS.

CARBONS	Z-500 (11) (CD ₃ OD)	β -AMYRIN (12) (CDCl ₃)	ME ESTER OF OLEANOLIC ACID (13) (CDCl ₃)	ME-OLEANOLATE β -D- GLUCO- PYRANOSIDE (14) (CDCl ₃)
1	38.2	38.6	38.5	38.7
2	25.0	27.2	27.1	25.9
3	92.0	78.9	78.7	90.5
4	40.7	38.7	38.7	39.0
5	57.4	55.1	55.2	55.9
6	18.3	18.4	18.3	b
7	32.0	32.6	32.6	b
8	40.9	39.7	39.3	b
9	a	47.6	47.6	b
10	35.5	36.9	37.0	b
11	24.5	23.5	23.1	b
12	123.4	121.7	122.1	b
13	146.2	145.1	143.4	b
14	43.3	41.6	41.6	b
15	27.5	26.1	27.7	b
16	24.6	26.9	23.4	b
17	a	32.4	46.6	b
18	43.4	47.1	41.3	b
19	a	46.8	45.8	b
20	29.4	31.0	30.6	b
21	34.0	34.7	33.8	b
22	34.4	37.1	32.3	b
23	28.8	28.0	28.1	27.9
24	16.2	15.6	15.6	16.4
25	17.2	15.6	15.3	b
26	19.7	16.7	16.8	b
27	26.8	26.1	26.0	b
28	184.1	28.4	177.9	b
29	34.5	33.3	33.1	b
30	24.9	23.6	23.6	b

a = Those signals are masked by the deuterated methanol signals.

b = Only the chemical shifts for the carbons affected by the glycosidation were published. The remaining signals should be at almost similar chemical shifts as those for 13. For Z-500, the methanol signal was taken as the internal reference at 49.3 ppm.

THE CARBOHYDRATE MOIETY OF Z-500 (11).

The C-13 NMR spectrum of Endod Z-500, Table 6 (p.201) indicates that this compound contains 3 anomeric carbons, therefore containing 3 sugar units in its carbohydrate side-chain. These anomeric carbons are also in the β -configuration, being in the 100.0-105.0 ppm region. The presence of 18 signals in the carbohydrate region of the C-13 NMR indicates that the 3 sugars present contain each 6 carbons. The most commonly encountered sugars in these type of molecules are glucose, galactose, rhamnose (6-deoxy mannose), xylose and arabinose. Since no chemical shift in Table 6 (p. 201) is in the region where chemical shifts of methyl groups appear, no rhamnose unit is present in this compound. Also, since there are 18 carbons present in the spectrum, mathematically no xylose and/or arabinose can be present in the compound. Therefore, Z-500 is probably constituted only of glucose and/or galactose units. It is impossible at this time to distinguish between these 2 sugar isomers, as neither C-13 NMR nor FAB-MS can discriminate between them. These glucose/galactose units can also be linked in a branched or linear manner. The FAB-MS spectrum for Endod Z-500 is presented in Appendix (p. 252). The

TABLE 6

C-13 CHEMICAL SHIFTS FOR THE CARBOHYDRATE MOIETY OF ENDOD
Z-500.

62.3	76.5	78.6
62.7	76.6	80.6
63.4	77.3	81.1
71.6	78.2	104.7
72.2	78.3	104.9
75.3	78.4	105.7

* The spectrum was recorded in deuterated methanol.

aglycone moiety is present as the peak at m/z 439. The quasi molecular ion peak is observed at m/z 965 $[M + Na + H]^+$. Plausible structure for Endod Z-500 is presented in Figure 2 (p.203).

3.3 OTHER PARTIAL STRUCTURAL ANALYSIS OF TYPE-3 ENDOD SAPONINS.

Although many Endod samples were obtained, it is difficult to obtain C-13 NMR spectra because of the very

Figure 2

PROPOSED STRUCTURE FOR ENDOD Z-500.

small amounts of pure material available and also because of the insolubility of these samples in most deuterated solvents. Large solvent volumes are often required to avoid precipitation of the samples. C-13 NMR spectra attempts were made with samples Z-100, Z-300, E-2-201 and E-4-201, but unfortunately very noisy spectra were obtained. Due to these factors, we report here only on partial C-13 NMR spectra for these four compounds. More studies are obviously required for these compounds.

Table 7 (p. 209) summarises some of the C-13 NMR signals for compounds 15, 16, 17 and 18 (that could be distinguished from noise).

ENDOD 4-201 (18)

A partial analysis of the spectrum for the Endod compound E-4-201 seems to indicate that this sample, although it shows homogeneity on normal and reverse phase TLC, could be a mixture of at least 2 compounds. This is so because of the occurrence of 4 olefinic signals at 116.9, 123.7, 130.0 and 144.2 ppm. This spectrum, when compared against the C-13 NMR spectrum of the methyl ester of

hederagenin (19) (40) seems to bear some similarities. This is seen by the chemical shift at 11.9 ppm, which could correspond to the chemical shift of C(24) in a hederagenin type of molecule. This is our only Endod spectrum for which a chemical shift at such a high field is observed. This compound also has at least 3 sugar units present in the molecule, as indicated by the presence of the anomeric carbons at 101.2, 103.5 and 104.7 ppm. Several other sugar signals are missing, since they cannot be distinguished from noise in the spectrum. In hederagenin, the C(3) is at 76.4 ppm. Again, if we refer to the glycosidation shift rule (37), where a downfield shift of +8.3 ppm should be observed for the glycosidic carbon, then a theoretical value of 84.7 ppm should be observed for C(3) in our spectrum. For C(3) of Endod E-4-201, a value of 84.2 ppm can be assigned. The signal at 179.4 ppm seems to be indicative of the presence of a carboxylic acid in this compound. Another fact to be noted, is the absence of a signal between 56-58 ppm in this spectrum. This fact led us to believe that the signal for C(5) is therefore different in this spectrum from the one for C(5) in all the other spectra.

ENDOD Z-100 (15) AND Z-300 (16)

The spectra for compounds Endod Z-100 and Z-300 bear some similarities with the spectrum for compound Z-500. In this regard, the presence of olefinic carbons at 122.8 and 146.3 (for Z-100) and at 125.9 and 146.0 (for Z-300) are indicative of the presence of a Δ^{12-13} double bond in these samples. Compound Z-100 also has a carboxylic acid present as indicated by the signal at 183.8 ppm. Any signal of this kind remains doubtful in Z-300, as it cannot be distinguished from noise. The presence of signals at 57.1 (Z-100) and 57.9 ppm (Z-300) are comparable with the C(5) signal at 57.4 ppm in Z-500. Also, the presence of signals at 91.7 (Z-100) and 92.4 (Z-300) are comparable with the signal at 92.0 ppm for C(3) in Z-500. Both of these compounds also have at least three sugar units present in their molecules and these have β -linkages at the anomeric carbons.

Samples prefixes "Z" were isolated by Mr Zhun Liu in collaboration with this author.

The FAB-MS of the Endod samples type-3 are presented in Appendix C (p. 250). Although they are difficult to interpret at this stage, we want to present them here as preliminary results, indicating that there is a promising future in FAB-MS for elucidation of structures of underivatized saponins. The partial C-13 NMR are also presented as a bank of data which will eventually become useful in future work of this kind. The idea has been launched here, it is still in the stages of development but soon, we anticipate that FAB-mass spectrometry will be the tool of choice for this type of work.

ENDOD 2-201 (17)

Although the C-13 NMR spectrum of compound E-2-201 was recorded in pyridine- d_5 , this spectrum has no more remarkable features than do the other C-13 NMR spectra of this series of saponins. It appears that compound E-2-201 is made up of at least two sugar units, having anomeric carbons at 105.0 and 105.8 ppm, also indicating once more some β -linkages. A carboxylic acid signal is present at 180.7 ppm. Only one olefinic carbon signal is present at 145.2 ppm, the other one being masked by the pyridine signals.

More work is required on these Endod samples, before a complete structure can be assigned. Running the C-13 NMR spectra in D_2O , while heating the probe could have advantageous effects on the spectra.

ENDOD TYPE-UNKNOWN.

Attempts were made at recording C-13 NMR spectra of the 'type-unknown' Endod compounds, but unfortunately no

TABLE 7

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PARTIAL C-13 NMR SPECTRA FOR 15, 16, 17, 18.

Z-100 (15) CD ₃ OD*	Z-300 (16) CD ₃ OD*	E-2-201 (17) C ₅ D ₅ N	E-4-201 (18) CD ₃ OD*/H ₂ O
16.0	15.5	15.4	11.9
17.0	16.8	16.8	15.1
18.1	17.5	17.4	15.5
24.3	19.0	23.8	16.6
24.6	24.0	26.1	17.6
26.5	26.2	28.1	18.0
27.2	27.1	30.9	19.0
28.5	28.0	32.7	21.0
29.2	28.4	33.1	23.0
31.8	28.9	36.9	23.8
33.9	31.3	39.4	25.3
34.2	33.4	40.0	25.5
38.0	33.8	42.2	26.1
40.4	34.2	46.8	28.5
40.6	35.1	48.1	30.5
43.0	39.3	56.1	33.2
57.1	40.1	62.4	37.2
62.0	40.5	62.8	37.6
62.4	40.9	71.7	40.0
71.3	43.2	71.9	41.1
72.0	57.9	75.1	42.3
75.0	62.9	76.3	60.5
76.3	63.3	76.9	68.4
77.0	63.7	77.3	71.2
77.9	72.2	78.1	71.5
78.1	72.7	78.3	71.9
78.3	78.1	81.6	72.7
80.3	78.5	81.8	73.1
80.7	78.9	89.4	73.5
91.7	83.0	105.0	84.2
104.5	83.0	105.8	101.2
104.6	92.4	145.2	103.5
105.4	104.0	180.7	104.7
122.8	104.4		116.9
146.3	107.6		123.7
183.8	125.9		130.0
	146.0		144.2
			179.4

* For those spectra, the methanol signals are masking any other signals that could be present in that region.

positive results were obtained. Very poor ratio of signal to noise was obtained, for which it is difficult to give an analysis. ^1H NMR spectra gave very broad and undefined signals.

ENDOD TYPE-UNKNOWN

The Endod of type-unknown have shown peculiar behavior in the FAB-MS, in the sense that for most of them, masses higher than 500 amu were not reached. This fact has aroused our curiosity as to what these compounds, that we were suspecting of being saponins, could be. Since no C-13 NMR gave any satisfactory result and therefore no indication at all as to what the skeleton of the isolated Endod products could be, we wondered if these type of compounds were actually triterpenes-glycosides or not. For the time being these questions cannot be answered, but more work is actually under way in order to identify and characterize the purified samples from Endod type-unknown.

DISCOVERIES

The work on the Endod plants as presented in this thesis, has shown that the separation system developed is very efficient for these saponins. It also showed that some structural work is possible, either to confirm all ready existing structures or to point out differences, or to identify other constituents of the plants and study their structures in the intact form of the saponins. The Endod samples that were sent for physiological testing have shown promising results.* Three chromatographically distinct fractions from Endod extracts of the type- 3 were tested. Two of the fractions had no effect on the whole animal after a one hour exposure to 25 ppm, or on the tension of the penis retractor muscle preparation from *L. stagnalis*. No change occurred to the high K induced contracture either. The third fraction (which appeared to be composed of 2 closely related compounds) caused mortality at 25 ppm after one hour exposure and a temporary increase in tension in smooth muscle which declined to control levels over a 45 minutes exposure. The high K contracture was reduced by this fraction but could be restored by washing in normal saline.

* D.R. Gardner and A. Elia, private communication, Dept. of Biology, Carleton University, 1984.

CHAPTER 4

EXPERIMENTAL

INSTRUMENTATION

The instrumentation used in the analysis of the Endod saponins was the same as those used for the holothurin saponins and reported in Chapter 5 (p.127) of Part A of this thesis. The Chromatotron is the only technical novelty introduced in Part B of this work.

ISOLATION AND PURIFICATION OF SAPONINS FROM PHYTOLACCA DODECANDRA - ENDOD.

Two types of Endod berries from the plant Phytolacca dodecandra were studied. One was identified as Type-3 and the other as Type-unknown. Type-3 Endod showed at least 5 compounds on TLC after a triple elution with CH_2Cl_2 -MeOH- H_2O , 60-25-5.4, v/v, while type-unknown Endod showed nine compounds with R_f very close to each other in the above mentioned solvent system.

All the saponins from the Endod berries were purified using the MPLC "ApLab" system, with a final purification on the HPLC, using a reverse phase column (Waters Associates, μ -Bondapak C-18, column dimensions: 3.9 x 30 cm).

In a typical run, 490 mg of the crude brown powder extract were dissolved in 8 mL of the solvent system CH_2Cl_2 -MeOH- H_2O , 60-18-3.2 (v/v). In most cases, more water had to be added to the sample because of the very low solubility of saponins in the solvent system. The solubilised saponins were then injected on a column of silica gel (15-25 microns, LiChroprepTM Si60, column size, 50 cm x 2.5 cm internal diameter, elution rate 5 mL/min, pressure 20 psi, actual column volume 245.44 mL, void volume 160 mL, weight of silica gel 98.17 g).

The column was eluted with the following solvent system: CH_2Cl_2 -MeOH- H_2O , 60-18-3.2 (1200 mL), 60-20-3.6 (1500 mL), methanol (1000 mL). Fractions of about 15 mL each were collected and verified by TLC. Fractions containing similar compounds were combined and evaporated under reduced pressure. The following is an example of

typical fraction combinations: Fr. 1-19, Fr. 20-39, Fr. 40-69, Fr. 70-89, Fr. 90-149, Fr. 150-185.

In a typical HPLC run, about 5 mg of sample is dissolved in 1-1.5 mL of the elution solvent, methanol/water (70/30, v/v) and injected on the column. Detection of the compounds was monitored with a variable wavelength UV detector set at 206 nm. The flow rate was 1.0 mL/min and the samples collected as they were detected. The amount of samples obtained is reported in Table 1 (p. 192).

The type-unknown Endod were purified using the same procedure as described above. In total, 9 pure compounds from the type-3 Endod were obtained as well as 8 pure compounds from the type-unknown Endod.

CHROMATOTRON RUNS.

Purification of the saponins from the type-3 Endod were also tried on a chromatotron system. A brief description of a run is given here.

A 2 mm thick silica gel plate was used, on which 50 mg of type-3 Endod dissolved in 2 mL of CH_2Cl_2 -MeOH- H_2O , 60-25-5.4 (v/v), were injected. Forty different fractions of about 3 mL each were collected, while the plate was being eluted with the solvent mentioned above. All fractions were again monitored by TLC, the ones containing similar compounds were combined and evaporated under reduced pressure. The following is an example of the fraction combination: Fr. 1-18, Fr. 19-23, Fr. 24, Fr. 25-34, Fr. 35-41. Although good separation was obtained, we did not pursue experiments with the chromatotron because of our solvent system which contained water and after a few runs, the silica gel would disaggregate.

FUTURE WORK

A lot of work remains to be done before any conclusion can be reached as to the authenticity and characterization of the Endod samples. Based on the experience acquired, we would like to make a few suggestions on the directions to be followed for the future.

ISOLATION

Another purification technique that would be more adequate in order to avoid contamination of the isolated samples would be welcome. In this line of thinking we would like to suggest into looking at the possibility of using Droplet Counter Current Chromatography, at least for the last purification stages of the samples. DCC has proven to be a good technique for the isolation and purification of very polar compounds including saponins (34). It is believed that DCC is a method that could be used with success in the purification process of Endod saponins. This technique would also certainly be an improvement in the percentage recovery of the pure material.

STRUCTURE

FAB-MS is still at a very young age, but it is developing rapidly. It is believed that as more data are compiled, and as more understanding of the technique comes forward, this method will be a precious and useful one for the study of saponins isolated from either marine or terrestrial sources. It is recommended that more FAB- mass spectra be recorded for the Endod samples and this in various matrices, in order to establish optimum conditions for this kind of molecules. It is a method that most certainly will be used to study the sugar sequence of underivatized saponins. Eventually, as more understanding of the method is made, it could also lead to good indications as to where linkages occur in the carbohydrate chain of the triterpene-glycosides.

FAB-MS has the disadvantage of not being able to distinguish between isomeric sugars. This is a sad point, but it could easily be resolved by a quick check by gas chromatography of the sugars in their trimethylsilyl ether form. This requires minimum amounts of material and therefore, not too much of precious samples are lost. The

improvement of techniques requiring very small quantities for hydrolysis and silylation of sugars is therefore something to be looked into.

All of the above considerations are of general interest and therefore could be applied to unknown saponins and lead to fruitful isolation, identification and/or characterization of compounds that could eventually become useful molecules with biological activity.

One of the major goal of this research project was to rely almost entirely on spectroscopic methods to determine the structures of intact saponins, without resorting to degradation procedures that frequently produce artifacts. With these data in hand (mainly C-13 NMR and FAB-MS), structures for fondoside A, frondoside J, asterosaponin C and Endod Z-500 are proposed.

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APPENDIX A

FAST ATOM BOMBARDMENT MASS SPECTROMETRY

FAST ATOM BOMBARDMENT MASS SPECTROMETRY.

"The Use of Fast Atom Bombardment Mass Spectrometry in Structural Elucidation Studies of Sugars in Saponins."

The characterization, sequencing and determination of linkage point positions of carbohydrate units in oligo- and polysaccharides from saponins has always been the source of severe limitations in structural elucidation.

The fact that usually, only minute amounts of purified material are generally available, often precludes the use of classical wet methods, where derivatization is a necessity, and, relatively speaking, larger amounts of sample are necessary. These techniques also have the disadvantage of often giving often ambiguous and poorly reproducible results.

In order to remedy to these type of problems, what is required is an analytical method that would be all together fast, easy, sensitive, reproducible and require a minimum amount of sample. Among these, mass spectrometry seems to

bear all these qualities and therefore could be the method of choice.

The usual ionization techniques in mass spectrometry have so far precluded many types of compounds mainly because of their thermal lability, their polar nature, their general involatility and molecular size. Chemical derivatization to increase the volatility and the thermal stability of a compound can sometimes be used, but it also has its own problems. More material is then needed, the sample experiences an increase in its molecular weight and often purification steps are required.

To overcome these problems, and mainly that of volatility, solid state ion sources have been examined. Among these, field desorption (1) is the technique that has been most widely adopted. This technique has a few disadvantages. It often gives molecular weight information, but little structural information. It is also notorious for the transient nature of the spectra.

Other techniques such as laser-induced desorption (2) of ions from surfaces and desorption chemical ionization (3)

have been described, but not reached their full potential as yet as routine analytical methods.

Therefore, the use of general methods of ionizing materials from the solid state was necessary. Recently, Secondary Ion Mass Spectrometry (SIMS) (4-8) and Fast Atom Bombardment Mass Spectrometry (FAB-MS) (9-12) have made their appearance. These techniques are both based on the "sputtering phenomenon", which is not new, as it was first reported by Grove in 1852 (11).

FAST ATOM BOMBARDMENT MASS SPECTROMETRY: THE TECHNIQUE.

Fast Atom Bombardment Mass Spectrometry has become a powerful structural tool in the last four years. It has shown great potential for high molecular weight compounds, many of which are highly polar and of generally low volatility. Molecular weight information (13) as well as structural information (14, 15) have been recorded for various compounds.

The phenomenon of FAB-MS can be described very simply.

A beam of ions such as Xe^{+} is produced by ionizing xenon atoms and accelerating the resulting ions through an electric field. The resulting (fast) ions are directed through a xenon gas chamber where charge-exchange occurs (Eq. 1) to give fast atoms (most of the original direction and kinetic energy of the fast ions is maintained in the resulting fast atoms).

Equation 1.

Excess fast xenon ions can be deflected leaving a beam of fast atoms. It has been shown that, when such beams of fast atoms bombard a metal plate (Figure 1, p 228) coated with a sample of the substance being investigated, the large amount of kinetic energy in the atoms is dissipated in various ways, some of which lead to volatilisation and ionization of the sample. By maintaining a suitable electric gradient from the plate, either positive or negative ions can be directed into the analyser of the mass spectrometer (16).

COMMONLY ENCOUNTERED INFORMATION FROM FAB MASS SPECTRA.

The samples used are usually underivatized. No true

Figure 1

molecular ions are observed in the spectra, but the molecule forms a stable ion by addition or loss of a proton (depending if the instrument is in the positive or negative mode). This phenomenon is generally observed for organic molecules ionized by this method. Molecules that are alkali metal salts form similar stable species, in high abundance, by addition or loss of the alkali metal cation such as for example Na^+ or K^+ . Cationized species can also be formed from mixtures of organic molecules with salts present as impurities in the sample (11).

Fast Atom Bombardment Mass Spectra are always recorded with a solvent matrix which is usually glycerol (11, 12). Glycerol has the advantage of providing enhanced sensitivity, compared with solid sample preparations. Glycerol gives peaks at mass numbers corresponding to $(92n + 1)^+$ and $(92n - 1)^-$ with values of n up to 15. When glycerol is not effective, other solvent matrices such as thioglycerol, triton X100, tetragol, etc. can be used. For maximum sensitivity, the samples should form a perfect monolayer at the surface of a substrate having low volatility. It should be noted that it is essential that

the sample has some solubility in the low volatility solvent, not only to provide the diffusion mechanism but also as a reservoir of non-irradiated material.

Improvements in the spectrum can be observed by judicious doping of the sample with alkali metal salts or protonating agents. These tend to increase the intensity of the protonated molecular ion or cationized species.

The FAB-MS method can provide spectra of difficultly volatile substances such as sugars (14, 17), peptides (13), nucleotides (13) and other thermally unstable compounds (18).

The FAB-MS source has the advantage of being simple and robust. Both positive and negative spectra can be run with equal facility (19). Also, although either positive or negative quasi-molecular ions, $(M + X)^+$ or $(M - X)^-$ where X can be H, Na, K etc., can be formed in large abundance, there are usually sufficient fragment ions to give valuable structural information. In some cases of metal-containing material a true molecular ion M^+ can be observed.

FAST ATOM BOMBARDMENT MASS SPECTROMETRY AS APPLIED TO SUGAR STUDIES.

Very recently, Dell et al. (17, 20) have presented some data showing that it was possible to record mass spectra of underivatized polysaccharides under FAB-MS conditions. ApSimon et al. (14, 21) have also used the technique to elucidate the sugar sequence contained in saponins of marine origin.

Dell et al. (20) have presented three different fragmentation patterns to account for the various fragment ions found in the Fast Atom Bombardment Mass Spectra of polysaccharides (20). These fragmentation patterns are presented in Scheme 1 (p.232). It is to be noted that in those fragmentation patterns, Dell et al. have not taken into account the stereochemistry of the glycosidic bond involved, neither have they presented any mechanism.

In very recent studies of FAB mass spectral data of a series of alpha and beta anomers of different polysaccharides, Paré* has suggested that in polysaccharides with the β -configuration, mechanism A will predominate,

* J.R.J. Paré, Ph.D. Thesis, Carleton University, 1984.

SCHEME 1

while in polysaccharides with the α -configuration, mechanism B will have predominance. These favored mechanisms can be explained by stereoelectronic effects.

Fast Atom Bombardment Mass Spectrometry was explored for different saponins of marine and terrestrial origin. Results are presented in this thesis, along with discussions pertaining to structural elucidation and sugar sequencing of these type of compounds (i.e. Part A, Chapters 3 & 4, Part B, Chapter 3).

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APPENDIX B

SPECTROSCOPIC DATA

FOR

FRONDOSIDE J

FRONDOSIDE A

ASTEROSAPONIN C

AND

PHANTOTHURINE I

238

20

60

100

140

180

^{13}C -NMR Spectrum of Frondoside J (77) recorded in $\text{CD}_3\text{OD}/\text{D}_2\text{O}$.

TMS external reference.

^{13}C -NMR Spectrum of Frondoside J (77) recorded in Pyridine- $\text{d}_5/\text{H}_2\text{O}$.
(Chemical Shifts are in ppm from Pyridine as internal standard.)

IR Spectrum of Frondoside J (77)
 KBr pellet

Frondoside A (89), Chemical Shifts are in ppm.
 Instrument: XL-200 Varian (50.0 MHz).

Instrument: Bruker CXP-300 (75.5 MHz)

IR Spectrum of Frondoside A (89).
KBr pellet.

-----, Fast Atom Bombardment Mass Spectrum of Frondoside A (89).

Conditions: Positive Mode; Glycerol

MASS SPECTRUM
02/14/84 9:54:00 + 0:33
SAMPLE: FRONDO-A/DS
COND.: FAB(GLY)

DATA: 84MAT150A #3
CALI: PEGJA20 #2
BASE M/E: 115
RIC: 9306110.

Fast Atom Bombardment Mass Spectrum of Desulfated Frondoside A. (Pos. Mode; Glycerol)

Instrument: Bruker WH-400 (100.6 MHz).

Fast Atom Bombardment Mass Spectrum of Aterosaonin C (136).

Conditions: Positive Mode: Glycerol/Methanol

Fast Atom Bombardment Mass Spectrum of phantothurin I.

Conditions: Positive Mode; Glycerol

APPENDIX C

SPECTROSCOPIC DATA

FOR

ENDODSZ-500, 15, 16, 17, 18.

Instrument: Varian XL-200 (50.0 MHz).

MASS SPECTRUM
12/21/83 11:32:00 +1:17
SAMPLE: E/Z-500
COND.: FAB (GLY)

DATA: 83MAT299 #7
CALI: PEGDE20 #8

BASE M/E: 115
RIC: 11812800.

Fast Atom Bombardment Mass Spectrum of Endod Z-500 (11).

Conditions: Positive Mode; Glycerol

EXPI PULSE SEQUENCE: S2PUL

ACQUISITION	DEF. & UT
SN 11.500	DN 1.500
SW 12004.8	DO 0.000
AT 1.333	DM 0.000
AP 32000	DM 1.000
PU 6.0	DM 0.000
PI 2.000	DM 0.000
D1 0.000	DM 0.000
D2 0.000	DM 0.000
D3 0.000	DM 0.000
D4 0.000	DM 0.000
D5 0.000	DM 0.000
D6 0.000	DM 0.000
D7 0.000	DM 0.000
D8 0.000	DM 0.000
D9 0.000	DM 0.000
D10 0.000	DM 0.000
D11 0.000	DM 0.000
D12 0.000	DM 0.000
D13 0.000	DM 0.000
D14 0.000	DM 0.000
D15 0.000	DM 0.000
D16 0.000	DM 0.000
D17 0.000	DM 0.000
D18 0.000	DM 0.000
D19 0.000	DM 0.000
D20 0.000	DM 0.000
D21 0.000	DM 0.000
D22 0.000	DM 0.000
D23 0.000	DM 0.000
D24 0.000	DM 0.000
D25 0.000	DM 0.000
D26 0.000	DM 0.000
D27 0.000	DM 0.000
D28 0.000	DM 0.000
D29 0.000	DM 0.000
D30 0.000	DM 0.000
D31 0.000	DM 0.000
D32 0.000	DM 0.000
D33 0.000	DM 0.000
D34 0.000	DM 0.000
D35 0.000	DM 0.000
D36 0.000	DM 0.000
D37 0.000	DM 0.000
D38 0.000	DM 0.000
D39 0.000	DM 0.000
D40 0.000	DM 0.000
D41 0.000	DM 0.000
D42 0.000	DM 0.000
D43 0.000	DM 0.000
D44 0.000	DM 0.000
D45 0.000	DM 0.000
D46 0.000	DM 0.000
D47 0.000	DM 0.000
D48 0.000	DM 0.000
D49 0.000	DM 0.000
D50 0.000	DM 0.000
D51 0.000	DM 0.000
D52 0.000	DM 0.000
D53 0.000	DM 0.000
D54 0.000	DM 0.000
D55 0.000	DM 0.000
D56 0.000	DM 0.000
D57 0.000	DM 0.000
D58 0.000	DM 0.000
D59 0.000	DM 0.000
D60 0.000	DM 0.000
D61 0.000	DM 0.000
D62 0.000	DM 0.000
D63 0.000	DM 0.000
D64 0.000	DM 0.000
D65 0.000	DM 0.000
D66 0.000	DM 0.000
D67 0.000	DM 0.000
D68 0.000	DM 0.000
D69 0.000	DM 0.000
D70 0.000	DM 0.000
D71 0.000	DM 0.000
D72 0.000	DM 0.000
D73 0.000	DM 0.000
D74 0.000	DM 0.000
D75 0.000	DM 0.000
D76 0.000	DM 0.000
D77 0.000	DM 0.000
D78 0.000	DM 0.000
D79 0.000	DM 0.000
D80 0.000	DM 0.000
D81 0.000	DM 0.000
D82 0.000	DM 0.000
D83 0.000	DM 0.000
D84 0.000	DM 0.000
D85 0.000	DM 0.000
D86 0.000	DM 0.000
D87 0.000	DM 0.000
D88 0.000	DM 0.000
D89 0.000	DM 0.000
D90 0.000	DM 0.000
D91 0.000	DM 0.000
D92 0.000	DM 0.000
D93 0.000	DM 0.000
D94 0.000	DM 0.000
D95 0.000	DM 0.000
D96 0.000	DM 0.000
D97 0.000	DM 0.000
D98 0.000	DM 0.000
D99 0.000	DM 0.000
D100 0.000	DM 0.000

¹³C-NMR Spectrum of (methyl 2-100 (13))

recorded in CD₃CO.

100% internal reference.

100% internal reference.

Fast Atom Bombardment Mass Spectrum of Endod Z-100 (15).

Conditions: Positive Mode; Glycerol

Fast Atom Bombardment Mass Spectrum of Endod Z-300 (16).

Conditions: Positive Mode; Glycerol/Methanol

Instrument: Varian XL-200 (50.0 MHz)

Instrument: Varian XL-200 (50.0 MHz).

8:50

40

SIR: D., 80 SCAN

CAL: CAL

07-JUN-83

0733R
FAB/6117/MEOH

Fast Atom Bombardment Mass Spectrum of Endod E-2-201 (17).

Conditions: Positive Mode; Glycerol/Methanol

0

2

4

6

Instrument: Varian XL-200 (50.0 MHz).

Instrument: Varian XL-200 ((50.0 MHz)).

Fast Atom Bombardment Mass Spectrum of Endod E-4-201 (18).
Conditions: Positive Mode; Glycerol

APPENDIX D

CHART 1:

SAPOGENINS FROM SEA CUCUMBERS

CHART 2:

COMPLETE STRUCTURES OF SAPONINS FROM SEA CUCUMBERS

CHART 3:

SAPOGENINS FROM STARFISH

CHART 4:

COMPLETE STRUCTURES OF SAPONINS FROM STARFISH

1 of 1

Chart 1

Sapogenins from Sea Cucu

12 22, 25-Oxidoholothurinogenin
 $C_{30}H_{44}O_5$, MW 484
 mp 315.2°-315.8°C, $[\alpha]_D^{25} -21.2^\circ$ (CHCl₃)
 (Chanley *et al.*, 1966)

13 Holothurinogenin
 $C_{30}H_{44}O_4$, MW 468
 mp 285.8°-286.4°C, $[\alpha]_D^{25} -9.3^\circ$
 (Chanley *et al.*, 1966)

14 Griseogenin
 $C_{30}H_{44}O_4$, MW 470, mp 277°C
 (Habermehl and Volkwein, 1970)

15 Seychellogenin
 $C_{30}H_{44}O_5$, MW 486
 mp 285°-287°C, $[\alpha]_D^{25} -$
 (Tursch *et al.*, 1967)

16 Koelikerigenin
 $C_{30}H_{44}O_3$, MW 454
 mp 234°-238°C, $[\alpha]_D^{25} -70^\circ$
 (Roller *et al.*, 1969)

17 Ternaygenin
 $C_{30}H_{44}O_4$, MW 470
 mp 213°-214°C, $[\alpha]_D^{25} -8^\circ$
 (Roller *et al.*, 1969)

67
 $C_{32}H_{46}O_6$, MW 532
 Acetate mp 277°-273°C, $[\alpha]_D^{25} -52^\circ$ (CHCl₃)
 (Chanley and Rossi, 1969a)

68
 $C_{31}H_{44}O_5$, MW 500
 Acetate mp 245°-247°C (dec), $[\alpha]_D^{25} -83^\circ$ (CHCl₃)
 (Chanley and Rossi, 1969a)

69
 $C_{30}H_{44}O_5$, MW 486
 Diacetate mp 240°-243°C (dec)
 (Chanley and Rossi, 1969b)

70
 $C_{31}H_{44}O_5$, MW 500
 Acetate mp 205°-208°C
 (Chanley and Rossi, 1969a)

71
 $C_{30}H_{44}O_5$, MW 488

72 Holothurinogenol
 $C_{30}H_{44}O_6$, MW 502

73 Stichapogenin A₂
 $C_{30}H_{44}O_4$, MW 468
 mp 238°-240°C, $[\alpha]_D^{25} -48^\circ$ (CHCl₃)
 (Elyakov *et al.*, 1969)

74 Stichapogenin A₁, holothurinogenol
 $C_{30}H_{44}O_5$, MW 486
 mp 238°-241°C, $[\alpha]_D^{25} -87.6^\circ$ (CHCl₃)
 (Tan *et al.*, 1976)

Chart 1

Sapogenins from Sea Cucumbers

14 Holothurinogenin
 $C_{30}H_{48}O_4$, MW 470, mp 277°C
 (Häbermehl and Volkwein, 1970)

15 Griseogenin
 $C_{30}H_{48}O_5$, MW 486
 mp 285°-287°C, $[\alpha]_D^{25} -22^\circ (CHCl_3)$
 (Tursch *et al.*, 1967)

16 Praslinogenin
 $C_{31}H_{48}O_5$, MW 500
 mp 290°-291.5°C
 (Tursch *et al.*, 1970)

17
 $C_{30}H_{44}O_4$, MW 468

29

19 Ternaygenin
 $C_{31}H_{48}O_4$, MW 484
 mp 239°-242°C, $[\alpha]_D^{25} 2^\circ$
 (Roller *et al.*, 1969)

22

23

$C_{31}H_{48}O_5$, MW 500
 Acetate mp 281°-282°C, $[\alpha]_D^{25} -45^\circ (CHCl_3)$
 (Chanley and Rossi, 1969a)

26

$C_{31}H_{50}O_6$, MW 518
 Diacetate mp 310°C (dec)
 (Chanley and Rossi, 1969a)

69

$C_{30}H_{48}O_5$, MW 486
 Diacetate mp 240°-243°C (dec)
 (Chanley and Rossi, 1969a)

70

$C_{31}H_{48}O_5$, MW 500
 Acetate mp 205°-208°C
 (Chanley and Rossi, 1969a)

71

$C_{30}H_{48}O_5$, MW 472
 mp 209°-210°C, $[\alpha]_D^{25} -5^\circ (CHCl_3)$
 (Clastres *et al.*, 1978)

72

$C_{30}H_{48}O_4$, MW 472

27 Stichopogenin A₂

$C_{30}H_{48}O_4$, MW 468
 mp 238°-240°C, $[\alpha]_D^{25} -48^\circ (CHCl_3)$
 (Etyakov *et al.*, 1969)

28 Stichopogenin A₄, holotoxinogenin

$C_{30}H_{48}O_5$, MW 486
 mp 238°-241°C, $[\alpha]_D^{25} -87.6^\circ (CHCl_3)$
 (Tan *et al.*, 1975)

29

$C_{31}H_{48}O_5$, MW 500
 mp 238°-238°C, $[\alpha]_D^{25} -126^\circ (CHCl_3)$
 (Tan *et al.*, 1975)

8 Holotoxinogenin
 $C_{30}H_{44}O_4$, MW 468

67
 $C_{32}H_{50}O_6$, MW 532
 Acetate mp 272°-273°C, $[\alpha]_D^{25} -52^\circ$ (CHCl₃)
 (Chanley and Rossi, 1969a)

68
 $C_{31}H_{48}O_6$, MW 500
 Acetate mp 245°-247°C (dec), $[\alpha]_D^{25} -53^\circ$ (CHCl₃)
 (Chanley and Rossi, 1969a)

69
 $C_{30}H_{46}O_6$, MW 486
 Diacetate mp 240°-243°C (dec)
 (Chanley and Rossi, 1969a)

70
 $C_{31}H_{48}O_6$, MW 500
 Acetate mp 205°
 (Chanley and Rossi, 1969a)

73
 $C_{30}H_{48}O_6$, MW 488

74 Holothurigenol
 $C_{30}H_{46}O_6$, MW 502

77 Stichopogenin A₂
 $C_{30}H_{44}O_6$, MW 468
 mp 238°-240°C, $[\alpha]_D^{25} -48^\circ$ (CHCl₃)
 (Elyakov et al., 1969)

78 Stichopogenin A₂, holotoxin
 $C_{30}H_{46}O_6$, MW 486
 mp 238°-241°C, $[\alpha]_D^{25} -97.6^\circ$
 (Tan et al., 1975)

31
 $C_{32}H_{50}O_6$, MW 530

30
 $C_{32}H_{50}O_6$, MW 514
 mp 223°-224°C, $[\alpha]_D^{25} -20^\circ$ (CHCl₃)
 (Rothberg et al., 1973)

32
 $C_{30}H_{48}O_6$, MW 472
 mp 228°-232°C, $[\alpha]_D^{25} +6.2^\circ$ (CHCl₃)
 (Kelecom et al., 1976c)

33
 $C_{32}H_{50}O_6$, MW 530
 mp 225°-226°C
 (Kelecom et al., 1976c)

35
 $C_{30}H_{48}O_6$, MW 488
 mp 227°-228°C, $[\alpha]_D^{25} -1.3^\circ$ (CHCl₃)
 (Kelecom et al., 1976c)

36
 $C_{30}H_{48}O_6$, MW 484
 Acetate mp 172°-173°C, $[\alpha]_D^{25} -299^\circ$ (CHCl₃)
 (Kitagawa et al., 1981a)

37
 $C_{32}H_{50}O_6$, MW 514
 mp 198°-201°C, $[\alpha]_D^{25} -10^\circ$ (CHCl₃)
 (Kitagawa et al., 1981a)

38 Stichlerogenol
 $C_{30}H_{48}O_6$, MW 472
 mp 226°-228°C, $[\alpha]_D^{25} -42^\circ$ (CHCl₃)
 (Kitagawa et al., 1981a)

39
 $C_{32}H_{50}O_6$, MW 530
 mp 231°-233°C, $[\alpha]_D^{25} -16^\circ$ (CHCl₃)
 (Kitagawa et al., 1981a)

34

69

$C_{30}H_{48}O_5$, MW 486
Diacetate mp 240°-243°C (dec)
(Chanley and Rossi, 1969a)

70

$C_{31}H_{48}O_5$, MW 500
Acetate mp 205°-208°C
(Chanley and Rossi, 1969a)

71

$C_{30}H_{48}O_4$, MW 472
mp 209°-210°C, $[\alpha]_D^{25} -5^\circ (CHCl_3)$
(Clasres *et al.*, 1978)

72

$C_{30}H_{48}O_4$, MW 472

27 Stichopogenin A₂

$C_{30}H_{44}O_4$, MW 468
mp 238°-240°C, $[\alpha]_D^{25} -48^\circ (CHCl_3)$
(Elyakov *et al.*, 1969)

28 Stichopogenin A₁, holotoxinogenin

$C_{30}H_{46}O_5$, MW 486
mp 238°-241°C, $[\alpha]_D^{25} -87.6^\circ (CHCl_3)$
(Tan *et al.*, 1975)

29

$C_{31}H_{48}O_6$, MW 500
mp 236°-238°C, $[\alpha]_D^{25} -125^\circ (CHCl_3)$
(Tan *et al.*, 1975)

8 Holotoxinogenol
 $C_{30}H_{44}O_4$, MW 468

32

$C_{30}H_{48}O_6$, MW 472
mp 229°-232°C, $[\alpha]_D^{25} +6.2^\circ (CHCl_3)$
(Kelecem *et al.*, 1976c)

33

$C_{32}H_{48}O_5$, MW 512
mp 225°-227°C, $[\alpha]_D^{25} -4.0^\circ (CHCl_3)$
(Kelecem *et al.*, 1976c)

34

$C_{30}H_{48}O_4$, MW 470
mp 202°-203°C, $[\alpha]_D^{25} +10.5^\circ (CHCl_3)$
(Kelecem *et al.*, 1976c)

35

$C_{32}H_{50}O_6$, MW 530
mp 203°-206°C, $[\alpha]_D^{25} -10^\circ (CHCl_3)$
(Kelecem *et al.*, 1976c)

26

$C_{30}H_{48}O_5$, MW 454
Acetate mp 172°-173°C, $[\alpha]_D^{25} -200^\circ (CHCl_3)$
(Kitagawa *et al.*, 1981a)

36

$C_{32}H_{50}O_5$, MW 514
mp 198°-201°C, $[\alpha]_D^{25} +13.8^\circ (CH_3OH)$
(Kelecem *et al.*, 1976c)

75

$C_{32}H_{48}O_5$, MW 512
mp 198°-199°C
(Kelecem *et al.*, 1976c)

37

$C_{32}H_{48}O_5$, MW 514

76

$C_{31}H_{48}O_5$, MW 488
mp 231°-233°C, $[\alpha]_D^{25} -16^\circ (CHCl_3)$
(Kitagawa *et al.*, 1981a)

18 Dehydrestichlerogenol

$C_{30}H_{48}O_4$, MW 470
mp 238°-240°C, $[\alpha]_D^{25} -35^\circ (CHCl_3)$
(Kitagawa *et al.*, 1981a)

4 of 4

Chart 2

Complete Structures of Saponins from Sea Cucumb

2 X = H, holothurin B

1 X = R, holothurin A

42 24-DEHYDROECHINOSIDE A

104

Chart 2

f Saponins from Sea Cucumbers

42 24-DEHYDROECHINOSIDE A

45 X = R, echinoside A

46 X = H, echinoside B

29

63 PSOLUTHURIN A

- 47 X = OH, Y = H, Z = H, bivittoside A
 48 X = OH, Y = R, Z = H, bivittoside B
 49 X = H, Y = R, Z = R, bivittoside C
 50 X = OH, Y = R, Z = R, bivittoside D

- 51 X = R, Y = R, holotoxin A
 52 X = R, Y = R', holotoxin B

- 57 X=H Y=H Z=H STICHOPOSIDE A
 58 X=OH Y=H Z=H STICHOPOSIDE B
 59 X=H Y=R Z=R' STICHOPOSIDE C
 60 X=OH Y=R Z=R' STICHOPOSIDE D
 61 X=H Y=R Z=R' Δ²⁰ ASTICHOPOSIDE C

FAB-MS DATA FOR PROXOSIDE A

Fragment ions are representative of associated cleavages and/or clusters.
 e.g. 1336 [M + H]⁺, 497 [Biosin moiety]⁺ cleavage.

53 PSOLUTHURIN A

X = R, Y = R, holotoxin A
 X = R, Y = R', holotoxin B

54 R = Thelothurin A

55 R = Thelothurin B

X = unidentified substituent

63

64

65

ent ions are representative of associated cleavages and/or clusters.

1336 [H + H]⁺, 497 [benzyl moiety]⁺ cleavage

4 of 4

44
OF / DE

Chart 3

Sapogenins from Starfish

$C_{26}H_{42}O_3$, MW 402

$C_{27}H_{44}O_3$, MW 416

$C_{27}H_{42}O_2$, MW 398
mp 120°-122°C, $[\alpha]_D -22^\circ$ (CHCl₃)
(DeSimone *et al.*, 1981)

Chart 3

ogenins from Starfish

29

$C_{27}H_{42}O_2$, MW 398
mp 120°-122°C, $[\alpha]_D -22^\circ$ (CHCl₃)
(DeSimone *et al.*, 1981)

$C_{26}H_{42}O_3$, MW 402

$C_{27}H_{42}O_2$, MW 398
mp 120°-122°C, $[\alpha]_D -22^\circ$ (CHCl₃)
(DeSimone *et al.*, 1981)

$C_{27}H_{44}O_3$, MW 416

$C_{27}H_{44}O_3$, MW 416

$C_{27}H_{44}O_3$, MW 416

$C_{27}H_{42}O_2$, MW 398
mp 120°-122°C, $[\alpha]_D -22^\circ$ (CHCl₃)
(DeSimone *et al.*, 1981)

Chart 4 Complete Structures of Saponins from Starfish

267

116 R = A, sepositoside A

117 R = B

118 R = C

119 R = D

120 R = A, LUZONICOSIDE

121 R = B

122 R = C

123 R = D

124 NODOSOSIDE

125 X = H Y = R

126 X = H Y = H

127 X = OH Y = R

128 X = OH Y = H

129

130 Asterosaponin A

131 R = a, X = OH, Y = H, thornasteroside A

132 R = b, X = OH, Y = H

R = a, X = H, Y = OH, glycoside B

134

END

0	1	H	1	1	H	8	5
---	---	---	---	---	---	---	---

FIN