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Glycolipides d'éponges et d'un échinoderme : isolement, caractérisation et évaluation des activités antiproliférative et antipaludique

THÈSE DE DOCTORAT
Discipline : Science de la vie et de la santé
Spécialité : Physicochimie des lipides marins biologiquement actifs

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CHAPITRE I

INTRODUCTION

1. Introduction générale

L'utilisation d'organismes marins en médecine est connue en Chine depuis l'antiquité mais reste limitée car il n'existe pratiquement aucune tradition orale ou de pharmacopée traditionnelle qui fasse référence aux algues, aux invertébrés ou aux poissons. L'étude de la biodiversité marine à des fins thérapeutiques n'a commencé que vers 1950.

Mais à l'heure actuelle, moins d'une dizaine de médicaments préparés avec des modèles issus de la mer sont disponibles sur le marché.

Cependant, les perspectives offertes pour le futur, restent importantes. En effet, si tous les phyla sont actuellement étudiés, il existe des pistes, comme la chimiotaxonomie, qui permettent de choisir certains organismes plutôt que d'autres. Par ailleurs, les techniques contemporaines de récolte, d'analyse et de criblage pharmacologique ont atteint un haut degré de sophistication, mais avec des coûts élevés qui expliquent que la plupart des découvertes récentes proviennent d'instituts spécialisés ou de sociétés privées de pharmacologie marine.

1.1. Les organismes marins comme source de molécules bioactives

Les produits naturels ont évolué pendant des années pour acquérir des effets biologiques très ciblés, ce qui les rend très intéressants pour la découverte de nouveaux médicaments. De nombreux produits naturels et analogues ont été développés pour traiter des maladies humaines dans beaucoup de domaines de la santé (1) : 63% des 1000 molécules environ mises sur le marché ces 25 dernières années (Fig. 1) (2). Néanmoins, ces dix dernières années, les entreprises pharmaceutiques ont sensiblement réduit leurs financements de projets dédiés à la découverte de produits naturels (3).

Les principales causes de ce désengagement sont :

- les difficultés d'accès et d'approvisionnement en sources de produits naturels
- la complexité chimique des produits naturels
- les problèmes de droits de propriété intellectuelle trans-frontalière (4).
- et l'incapacité pour la plupart des programmes de recherche de produits naturels de fournir suffisamment de molécules nouvelles pour le criblage à haut débit (HTS ou High Throughput Screening) (5).

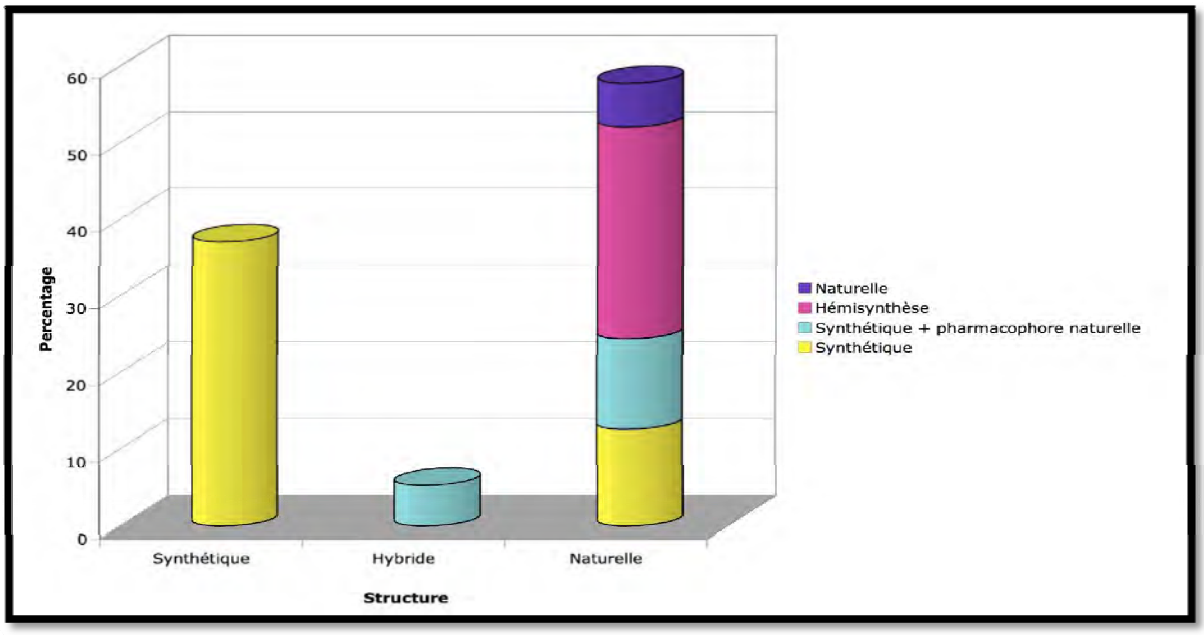


Figure 1 : Sources des principes actifs mis sur le marché pendant la période 1981-2006, inspirés de produits naturels (63%) ou d'origine purement synthétique (37%) (2)

Cependant, plus de 100 composés qui en sont dérivés subissent actuellement des tests cliniques (4). La figure 2 montre la répartition des composés isolés en fonction des organismes (6).

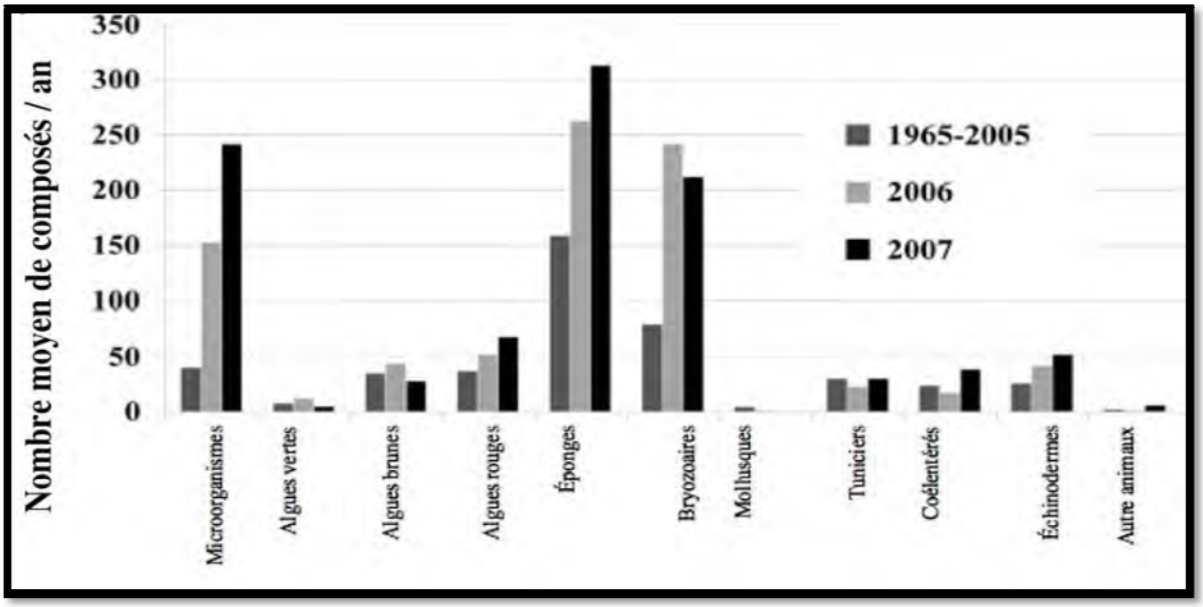


Figure 2 : Distributions des produits naturels marins par phyluma (6)

Les produits naturels ont inspiré beaucoup de développements en chimie organique, menant notamment aux avancées dans les méthodologies de synthèse et à la possibilité de faire des analogues ayant des propriétés pharmacochimiques ou pharmaceutiques améliorées (2, 7, 8).

Les premières découvertes de produits naturels issus de la mer peuvent être attribuées à Bergmann *et al.* (9, 10), qui a isolé et identifié la Spongouridine et la Spongothymidine de l'éponge des Caraïbes *Cryptotethia crypta*. Cette découverte a abouti à la commercialisation de leurs analogues synthétiques : l'antiviral ara-A et l'anticancéreux ara-C (Fig. 3).

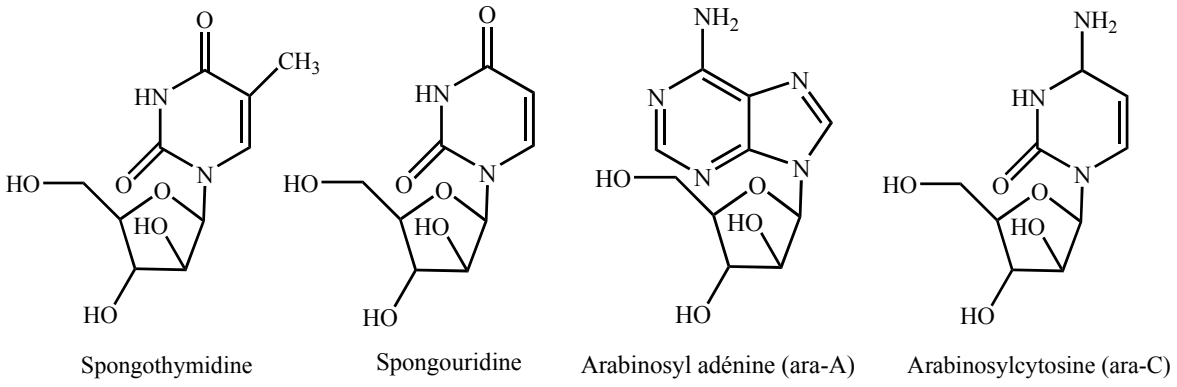


Figure 3. Premières molécules naturelles marines d'intérêt thérapeutique et leurs dérivés synthétiques

1.2. Biologie des Spongiaires

1.2.1 Généralités

Connues depuis l'Antiquité les éponges (Spongiaires, Porifères) existent au moins depuis le Précambrien. Rondelet, au XVIème siècle, dans son « Histoire Naturelle des Animaux », en parle comme des animaux marins privés de sentiments et de mouvements. En effet, les éponges ne possèdent ni organes, ni système nerveux, et les cellules ne forment pas à proprement parler de tissus. Longtemps considérées comme de simples colonies cellulaires, l'évolution des techniques d'observation a permis de découvrir une organisation très simple de cellules spécialisées. De par cette organisation rudimentaire, elles sont actuellement reconnues comme étant les plus simples et les plus primitifs des Métazoaires et constituent un embranchement bien délimité à la base du règne animal, juste au dessus des Protozoaires (Fig. 4) (11).

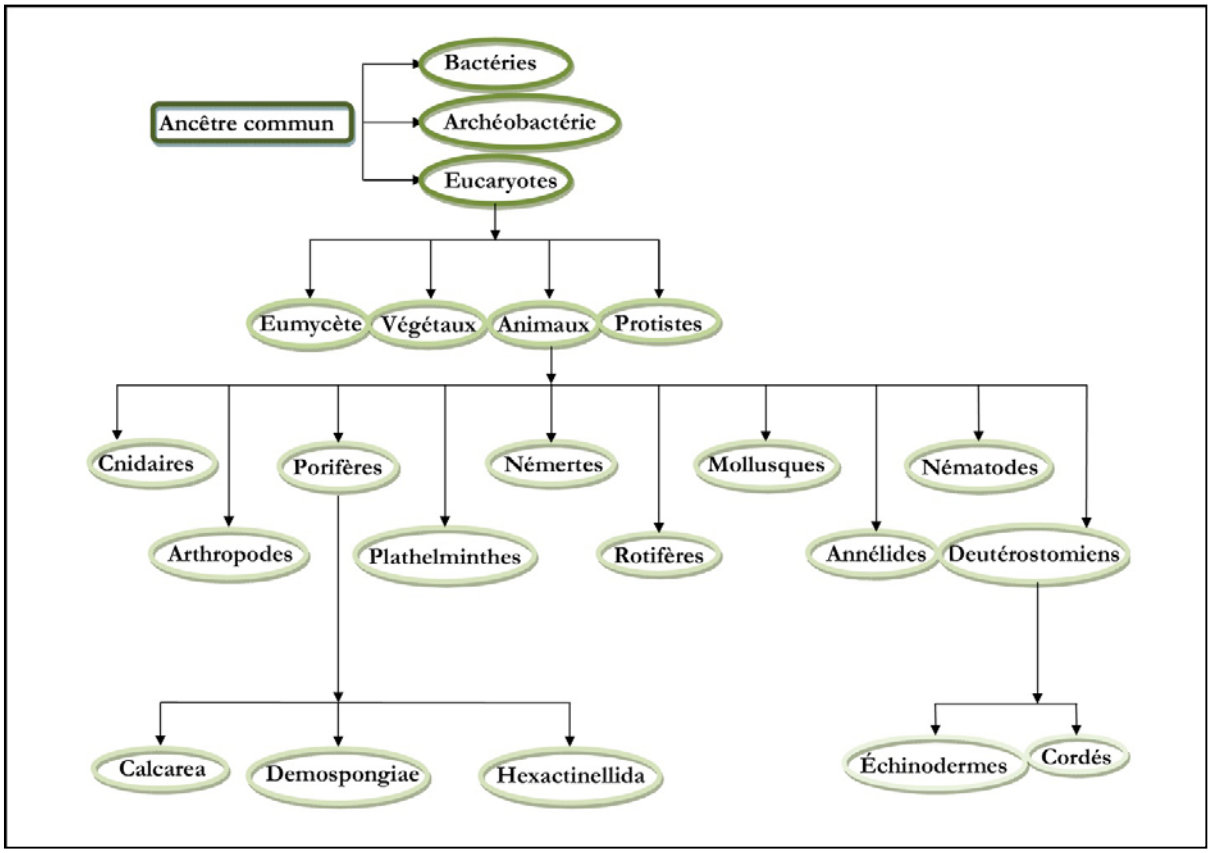


Figure 4. Arbre phylogénique (12)

Les éponges sont des animaux aquatiques, de mer ou d'eau douce, d'une grande diversité morphologique. Elles se sont adaptées a une diversité de niches écologiques, depuis le littoral marin superficiel jusqu'aux zones abyssales et aux eaux douces. Sédentaires à l'âge adulte, ce sont des filtreurs actifs qui jouent un rôle écologique important. L'organisme est parcouru par un courant aquifère dans lequel l'oxygène et la nourriture sont prélevés (surtout du phytoplancton : dinoflagellés), et les déchets rejetés. Un caractère remarquable des éponges est leur étonnante faculté de régénération (13). Des cellules dissociées en suspension, obtenues par filtration de broyats d'éponges, peuvent se regrouper et redonner chacune une petite éponge semblable à la souche (Fig. 5).

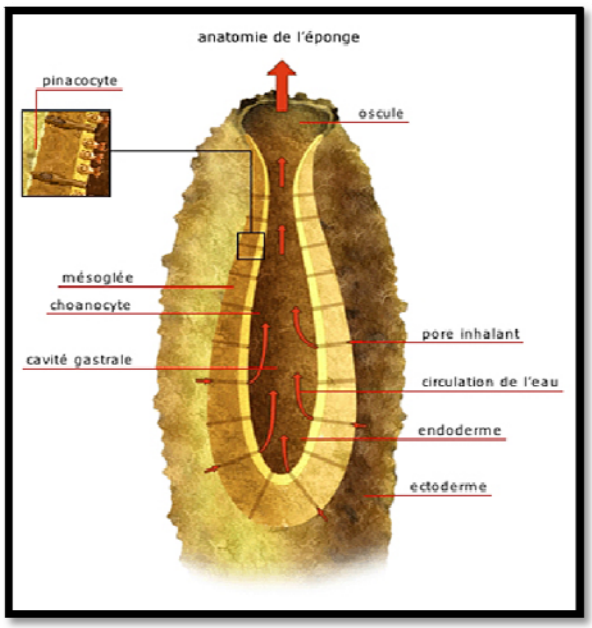


Figure 5. Anatomie d'une éponge. (14)

1.2.2 Morphologie

Les éponges se présentent sous différentes couleurs et formes : encroûtante, perforante, en forme d'arbuste, de balle, de coupe, de 'lobe de cerveaux', de tube ou de vase. Les éponges ont la faculté d'adapter leur forme aux conditions des courants et à la nature du support. La structure de l'ensemble des éponges repose sur un aggrégat de cellules qui forment de 'petits sacs'. Cette structure en sac comporte une paroi, plus ou moins complexe suivant la présence ou non d'un réseau de canaux de circulation d'eau périphérique, constituée de deux membranes :

- ✓ la membrane externe ou ectoderme
- ✓ la membrane interne ou endoderme

Qui sont séparées par une couche semblable à de la gelée et renforcée par des spicules, appelée mésoderme. De la composition chimique des spicules dépend la famille d'appartenance de l'éponge. De la complexité de la circulation de l'eau au travers de la paroi est née une classification en 3 types d'éponges (Fig. 6) :

- ✓ paroi simple: type **Ascon**
- ✓ paroi avec réseau simple de canaux de circulation d'eau périphérique : type **Sycon**
- ✓ paroi avec réseau complexe de canaux de circulation d'eau périphérique : type **Leucon**

Les structures sont simples (Ascon et Sycon) ou complexe (Leucon).

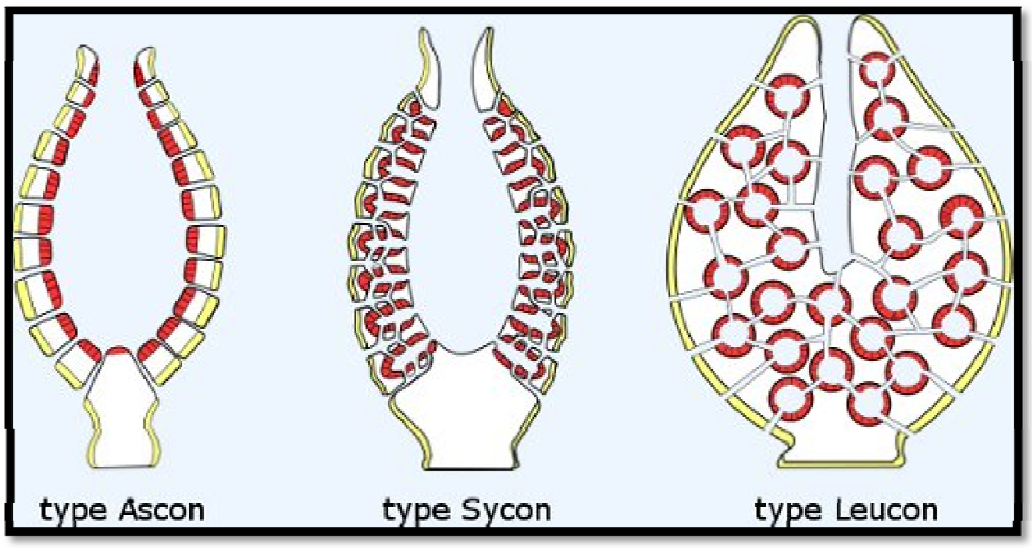


Figure 6. Trois types d'éponges (15)

La paroi de ce sac est également perforée grâce à la présence de cellules perforées ou porocytes réparties sur l'ensemble de sa surface afin de laisser pénétrer l'eau à l'intérieur (16).

1.2.3. Squelette

La structure des éponges est maintenue par un squelette qui peut être organique (collagène fibreux ou spongine), minéral (spicules de carbonate de calcium ou de silice), ou les deux. La spongine est élaborée par des cellules appelées spongocytes. Les spicules sont formés par des cellules du mésophyle dérivant de l'ectoderme : les sclérocytes. Si les spicules sont avant tout des constituants du squelette, ils peuvent parfois jouer le rôle d'organites de défense ou de fixation. Les spicules siliceux sont classés en deux classes par les systématiciens. Les premiers, les mégasclères, relativement simples et grands, peuvent dépasser les 4000 μm et être visibles à l'œil nu. Les seconds, les microsclères, beaucoup plus petits et souvent plus complexes, ne dépassent pas les 20 μm (17).

1.2.4. Systématique

- ✓ **Classe : CALCAREA, Eponges Calcaires**

Leur squelette est composé de spicules calcaires ou d'une structure calcaire continue. Les trois types d'éponges : ascon, sycon et leucon y sont représentés. Les spicules sont de taille homogène, contrairement à celles des deux autres classes.

- ✓ **Classe : DEMOSPONGIAE, Demosponges**

Les représentants de cette classe présentent une structure « leucon ». Les spicules sont siliceux et sont différenciés en mégasclères (grands spicules) et en microsclères (petits spicules). Les spicules existent sous différentes formes, présentant entre 1 et 4 pointes (monactines à tétractines). Ces spicules et le collagène présent dans le parenchyme constituent le squelette des éponges.

- ✓ **Classe : HEXACTINELLIDA, Hexactinellides**

Les « Éponges de verre » au squelette siliceux sont des espèces qui vivent au delà du plateau continental (à environ -200 m et plus). Les spicules se subdivisent également en mégasclères et en microsclères. Ils sont composés de 6 pointes : hexactines, suivant 3 axes (18).

1.2.5. Reproduction

La reproduction des éponges peut se faire par voie sexuée ou par voie asexuée :

- ✓ voie sexuée : les éponges mâles libèrent les spermatozoïdes en pleine eau. Une filtration active va rendre possible la rencontre avec les ovules, à l'intérieur des femelles. L'œuf, une fois libéré, commencera sa division puis, la larve, portée par les courants, ira se fixer sur le fond. Ce type de reproduction permet la dissémination spatiale des espèces.
- ✓ voie asexuée : l'éponge produit un bourgeon qui va se développer. Celui-ci pourra soit se détacher soit rester fixé à l'éponge mère. Ce type de reproduction permet l'extension des colonies d'éponges (19).

1.2.6. Nutrition

Les éponges sont des filtreurs actifs. L'eau pénètre dans l'éponge par des orifices de petite taille, appelés pores ou ostioles, et en ressort par des orifices de grande taille, appelés oscules. Les pores sont rarement visibles, alors que les oscules sont généralement bien visibles. Les éponges sont capables de réguler dans une certaine mesure les débits pompés, grâce à la contraction de certains orifices. Cette contraction est due à certaines cellules aux propriétés contractiles (porocytes, mais aussi myocytes). Par ailleurs, les éponges sont également capables d'arrêter tout mouvement d'eau, par exemple à l'approche d'un intrus. Ceci semble être dû à une simple transmission de médiateurs chimiques (20). L'eau est filtrée par les cellules. Celles-ci conservent les particules en suspension et l'oxygène dissous. Les éponges sont donc des organismes filtreurs. Leur capacité de filtration dépend de l'espèce mais elle peut aller jusqu'à 20000 fois le volume de l'éponge par jour. Lorsque les particules en suspension sont trop importantes (hypersédimentation), elles peuvent obturer les pores et limiter cette circulation. Certaines espèces sont plus sensibles que d'autres à cette matière en suspension. Cela étant, nombre d'entre elles vivent en symbiose avec des bactéries et surtout avec des petites algues qui par photosynthèse, leur procurent également de l'énergie (comme le corail). L'importance de cette symbiose pour la nutrition des éponges varie d'une espèce à l'autre et également d'un océan à l'autre. Elle est beaucoup plus importante pour les éponges de la Grande Barrière de Corail en Australie que pour les éponges des Caraïbes (21).

1.3. Biologie des Echinodermes

1.3.1. Généralités

Les **Echinodermes** sont apparus, il y a près de 600 millions d'années. Le phylum des Echinodermes constitue un groupe ancien d'animaux marins comprenant environ 6 000 espèces vivantes (Fig.7). Le terme échinoderme signifie « peau de hérisson » dont l'aspect est dû à l'**endosquelette** situé juste sous le mince tégument et composé de plaques dures riches en calcium (Fig. 8). Ils possèdent un système hydraulique contribuant au mouvement ou à l'alimentation : appelé **système ambulacraire** (ou aquifère). Il consiste en un réseau de canaux hydrauliques comprenant un anneau central à partir duquel partent cinq tubes radiaires s'étendant dans le corps et les bras. De nombreux animaux familiers des rivages marins, les étoiles de mer, les ophiures, les oursins, les dollars des sables et les concombres de mer sont des échinodermes. Tous ont une symétrie radiaire lorsqu'ils sont adultes. Alors que d'autres types d'animaux ont une symétrie radiaire, aucun n'est doté des organes complexes des échinodermes adultes. À cause de cette symétrie radiaire, les termes habituels utilisés pour décrire le corps d'un animal ne sont pas applicables : dorsal, ventral, antérieur ou postérieur n'ont pas de sens en absence de tête ou de queue. Pour décrire la structure corporelle des échinodermes, on se réfère à l'emplacement de la bouche et l'on parle de surface orale. La plupart des échinodermes rampent sur leur surface orale. Cependant, chez les concombres de mer et certains autres échinodermes, l'axe de l'animal est disposé horizontalement et ces animaux rampent avec la surface orale dirigée vers l'avant. Les échinodermes ont un plan d'organisation corporelle en cinq parties correspondant aux bras d'une étoile de mer ou au dessin visible sur la « coquille » d'un dollar des sables. Ces animaux n'ont ni tête ni cerveau. Leur système nerveux consiste en un **anneau nerveux** central d'où partent des branches. Les animaux sont capables de réagir par des attitudes complexes, mais la coordination des fonctions n'est pas centralisée.

Les échinodermes se retrouvent dans les eaux marines peu profondes, mais aussi dans les profondeurs abyssales. Dans les endroits les plus profonds des océans, les concombres de mer représentent plus 90 % de la biomasse ! La plupart vivent sur le fond, mais quelques espèces se déplacent librement. Les échinodermes adultes ont une taille qui va de quelques millimètres à plus d'un mètre de diamètre, pour une espèce d'étoile de mer, ou de longueur, pour une espèce de concombre de mer.

Les échinodermes attachés au fond de la mer par un pédoncule central furent jadis fréquents, mais actuellement il ne survivrait qu'environ 80 espèces de ce type (22).

Figure 7. **(*a*)** **(*b*)** **(*c*)**
Diversité chez les échinodermes. (*a*) Étoile de mer, *Oreaster occidentalis* (classe des astérides), le golfe de Californie, Mexique. (*b*) Concombre de mer verruqueux, *Parastichopus parvimensis* (classe des holothurides), Philippines. (*c*) Oursin (classe des échinides)

1.3.2. Morphologie

L'eau entre dans le système aquifère à travers un **madréporite**, une plaque filtrante à la surface de l'animal, et s'écoule vers le canal en anneau par un tube, dit canal hydrophore, appelé en anglais « stone canal » (canal de pierre) à cause des anneaux de carbonate de calcium qui l'entourent. Les cinq canaux radiaires se ramifient dans les pieds ambulacraires. Chez certains échinodermes, chaque pied se termine par une ventouse ; chez d'autres, les ventouses sont absentes. À la base de chaque pied se trouve une vésicule musculeuse, l'**ampoule ambulacraire**, remplie de liquide. Ce liquide ne peut être propulsé que dans le pied, ce qui provoque son extension et lui permet de s'attacher au substrat.

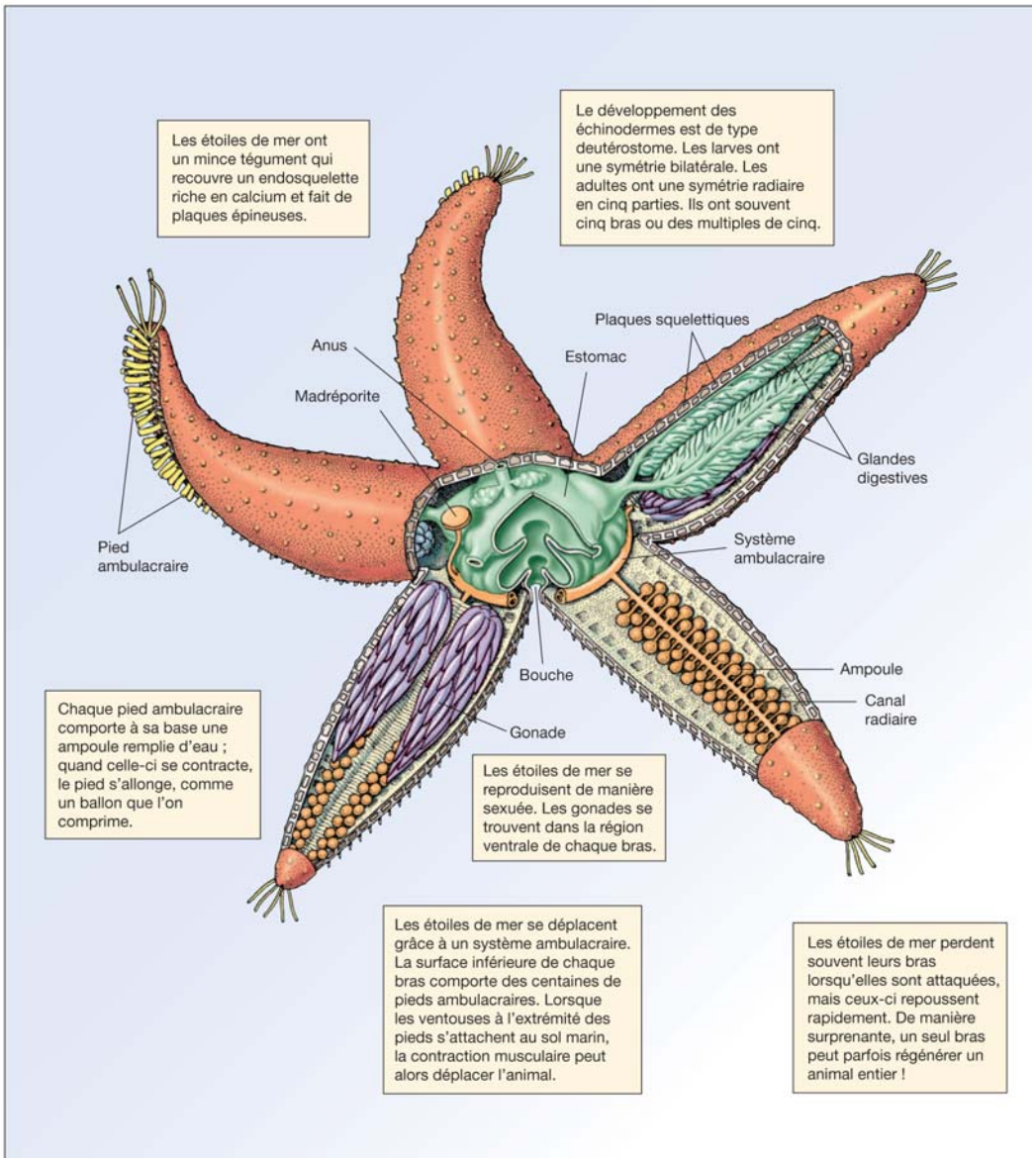


Figure 8. Phylum des échinodermes. Les échinodermes, comme les étoiles de mer, sont des cœlomates dont le développement est celui des deutérostomes. Un mince tégument recouvre un endosquelette fait de plaques riches en calcium, souvent fusionnées et formant ainsi une couche continue, dure et épineuse (22)

1.3.3. Squelette

Chez les échinodermes, un mince épiderme, riche en milliers de cellules neurosensorielles, recouvre un endosquelette composé de plaques soit mobiles, soit fixes, riches en calcium et appelées ossicules. Chez certains échinodermes, comme les astérides et les holothurides, les ossicules sont largement dispersés et la paroi du corps est flexible. Chez d'autres, spécialement les échinides, les

ossicules fusionnent et forment une coquille rigide. Dans de nombreux cas, ces plaques portent des épines.

Les plaques dans certaines portions du corps de certains échinodermes comportent des pores par où sortent les **pieds tubulaires**, qui font partie du système ambulacraire, une structure propre à ce phylum.

1.3.4. Systématique

Il y a plus de 20 classes éteintes d'échinodermes et six sont encore vivantes :

- ✓ **les crinoïdes, lis de mer et comatules**
- ✓ **les astérides, étoiles de mer**
- ✓ **les ophiurides, ophiures**
- ✓ **les échinides, oursins et dollars des sables**
- ✓ **les holothurides, concombres de mer**
- ✓ **les concentrocycloïdes, xyloplax**

Toutes les classes d'échinodermes sont formées sur la base du plan d'organisation corporelle en cinq parties, bien que la symétrie de base du phylum ne soit pas évidente chez certains. Les concombres de mer, ont cinq sillons radiaires courant le long de leur corps mou semblable à celui d'une limace (23).

1.3.5. Reproduction

De nombreux échinodermes sont capables de régénérer des parties de leur corps, et certains, particulièrement les étoiles de mer et les ophiures, se défont de diverses parties de leur corps lorsqu'elles sont attaquées. Chez quelques rares échinodermes, la reproduction asexuée s'effectue par fission, et les parties rompues des étoiles de mer peuvent parfois régénérer un animal entier. Certaines des ophiures les plus petites, surtout les espèces tropicales, se reproduisent régulièrement en se divisant en deux parties égales, chaque moitié régénérant un animal entier. Malgré la capacité de nombreux échinodermes de se diviser en plusieurs parties et de régénérer de nouveaux animaux, le mode de reproduction dans ce phylum est avant tout sexuel et externe. Les sexes chez la plupart des échinodermes sont séparés, bien qu'il y ait peu de différences apparentes. Les oeufs fécondés des échinodermes se développent habituellement en larves à symétrie bilatérale qui sont capables de nager librement, ce qui les différencie des larves trochophores des mollusques et des annélides. Ces

larves font partie du plancton jusqu'à ce qu'elles passent par les différents stades de la métamorphose pour aboutir à la forme adulte sédentaire (22).

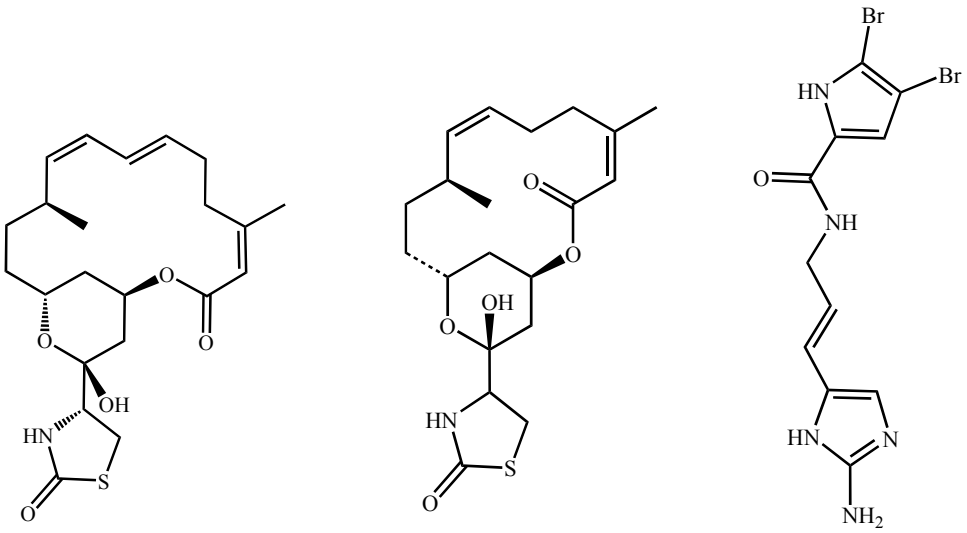
1.3.6. Nutrition

Les étoiles de mer sont des prédateurs. Leurs déplacements lents ne leur permettent d'attraper que des proies qui sont encore plus lentes qu'elles, comme les moules et les huîtres. Elles arrivent à entrouvrir la coquille de ces mollusques en se servant de leurs pieds ambulacraires, et évaginent ensuite leur estomac cardiaque à l'intérieur de la coquille de leur proie. Les enzymes digestives produites par l'estomac digèrent et liquéfient les tissus de la proie, et la « soupe » produite est aspirée à l'intérieur du tube digestif de l'étoile de mer où la digestion se poursuit (dans l'estomac pylorique et les caeca). Le tube digestif se ramifie dans les bras de l'étoile de mer, et cette ramification favorise le transport des éléments nutritifs vers toutes les parties de l'animal (24).

1.4. Les molécules actives des éponges

De nombreuses éponges sont toxiques pour de nombreux prédateurs (25), en particulier sous les tropiques. Par exemple, on a montré que les métabolites comme les Latrunculines A et B et les pyrroles-2-aminoimidazoles comme l'Oroïdine (Fig. 9) protègent l'éponge contre les poissons prédateurs *Gambusia affinis* et *Thalasssoma bifasciatum* (26, 27).

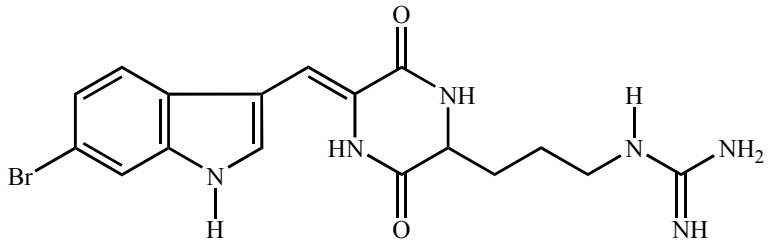
Il s'avère que les éponges de mer sont la source du plus grand nombre de métabolites secondaires isolés et caractérisés jusqu'à présent (28). Ces métabolites peuvent êtres présents à de fortes concentrations, ce qui amène les spécialistes à penser qu'ils jouent un rôle dans des fonctions biologiques importantes comme la communication, la régulation, la défense … etc. Plusieurs revues de la littérature (29) mettent en évidence la grande diversité de molécules chez les éponges : on trouve des dérivés d'acides aminés et de nucléosides, des macrolides, des porphyrines, des terpénoïdes, des stéroïdes, des polycétides … etc (26, 27).



Latrunculine A Latrunculine B Oroïdine

Figure 9 : Métabolites d'éponges toxiques pour les poissons

L'éponge *Aplysina aerophoba* exsude de l'Aerothionine et de l'Homo-aerothionine dans l'eau environnante pour se protéger contre certains dommages mécaniques causés par l'épibiose (30). D'autres études ont montré un rôle anti-biofouling de certains dipeptides comme la Barettine (Fig. 10) par l'inhibition de la métamorphose des larves des épibiontes (31).



Barettine

Figure 10 : Dipeptide à activité anti-biofouling

1.4.1. Activités biologiques des métabolites

Les éponges marines sont considérées comme un gisement sans équivalent, au vu de leur nombre et de la diversité structurale des métabolites secondaires qu'elles produisent. Des centaines d'articles

décrivent leurs activités biologiques diverses et variées telles que les activités anticancéreuses, antivirales, anti-inflammatoires, antifongiques, immunosuppressives, neurosuppressives, relaxantes, antimalariques ou encore antibiotiques (32-35).

Peu de métabolites secondaires d'origine marine sont actuellement utilisés comme médicament. Par exemple il faudra attendre 2006 pour que Yondelis TM, nom commercial de l'ET 743 (Fig. 11), isolé du tunicier *Ecteinascidia turbinata* en 1990 (36), obtienne son AMM (Autorisation de mise sur le marché). Cette molécule est utilisée actuellement dans le traitement de plusieurs types de cancers. Le ziconotide (utilisé comme antalgique) (Prialt TM), isolé du venin du mollusque *Conus magus* (37), a obtenu son AMM en 2004.

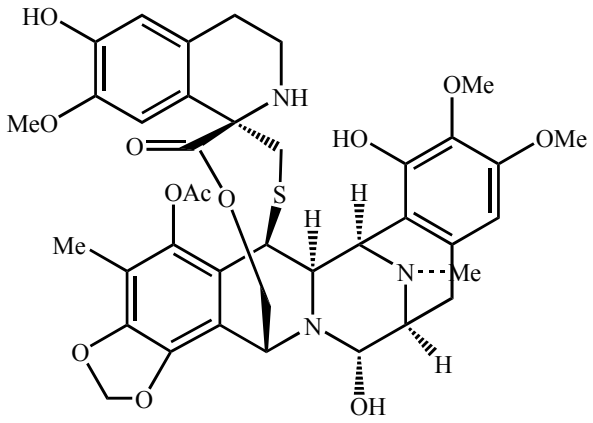


Figure 11 : Ecteinascidine 743 (Yondelis) de *Ecteinascidia turbinata*

La plupart des molécules d'origine marine en essais cliniques sont actuellement testées comme médicament anticancéreux (Tab. 1) (38, 39).

Tableau 1 : Substances naturelles d'origine marine (ou leurs analogues) en essais cliniques comme médicaments anticancéreux

Composé	Origine	Statut	Modèle moléculaire	Cible
E7389 (inspiré par halichondrine B) [a]	Éponge	Phase* III	Polyéther macrocyclique	Tubuline
Déhydro-didemnine B (Aplidine)	Tuniciers	Phase II	Depsipeptide	Ornithine décarboxylase (ODC)
Soblidotine (alias TZT1027) [a]	Lièvre de mer	Phase II	Peptides	Tubuline
Synthadotine (alias ILX651) [a]	Lièvre de mer	Phase II	Peptides	Tubuline
Bryostatine 1	Bryozoaire	Phase II	Macrocyclique lactone	Protéine kinase C (PKC)
Squalamine	Requin	Phase II	Alcaloïde stéroïdien	L'angiogenèse
Kahalaide F	Mollusque	Phase II	Desipeptide	Multiple
KRN-7000 (inspiré par les agelasphines)[a]	Éponge	Phase II	Glycolipide	« NKT » Natural Killer T
Zalypsis (inspiré par jourmycine)[a]	Ascidie	Phase I	Alcaloïde	ADN
E7974 (inspiré par hemiasterline)[a]	Éponge	Phase I	Tripeptide	Tubuline
Taltobuline (alias HTI286 ; inspiré par hemiasterline)[a]	Éponge	Phase I	Tripeptide	Tubuline
Salinosporamide A (alias NPI0052)	Bactérie	Phase I	Lactone cyclique	Protéasomes
Spisulosine (alias ES285)	Mollusque	Phase I	Phospholipide sphingosine	Protéines « Rho » (Ras-homologues)
PMO2734 (inspiré par kahalaide) [a]	Mollusque	Phase I	Desipeptide	Tumeur solide
NPI 2358 (inspiré par l'halimide) [a]	Mycosite	Phase I	Dioxopipérazine phényl-ahistine	Tubuline
LBH 589 (inspiré par la psammapline)[a]	Éponge	Phase I	Acide Hydroxamique	Histone désacétylase (HDAC)

[a] **Synthétique**
*** Tests cliniques sur l'homme (tolérance) : Phase I**
Tests cliniques sur l'homme (intérêt thérapeutique) : Phase II
Tests cliniques sur l'homme (essais à grande échelle) : Phase III

1.4.2. Les métabolites des éponges marines et leur intérêt thérapeutique potentiel

La découverte massive de nouvelles molécules au cours des deux dernières décennies n'a pas encore abouti au stade du médicament mis sur le marché. Plusieurs métabolites secondaires bioactifs (ou leurs analogues) isolés d'éponges sont actuellement en essais cliniques (Tab. 2).

Tableau 2 : Métabolites anticancéreux et antiviraux (ou leurs analogues) isolés d'éponges

Composés	Origine	Traitement	Statut
HTI286	*Siphonochalina* sp.	Anticancéreux	Phase I
LBH 589	*Pseudoceratina purpurea*	Anticancéreux	Phase I
Zalypsis	*Reniera* sp.	Anticancéreux	Phase II
KRN7000	*Agelas mauritianus*	Anticancéreux	Phase II
E7389	*Halichondria okadaï*	Anticancéreux	Commercialisé
Ara-A	*Cryptotethya crypta*	Antiviral	Commercialisé
Ara-C	*Cryptotethya crypta*	Anticancéreux	Commercialisé

L'HTI-286 actuellement en essai clinique son activité contre le cancer de la prostate (40), est un analogue synthétique de l'hémiasterline (Fig. 12). L'Hémiasterline a été isolée des éponges *Cymbastela* sp. et *Hemiasterella minor* (41,42). C'est un puissant inhibiteur de la croissance cellulaire qui empêche l'assemblage normal des microtubules (41).

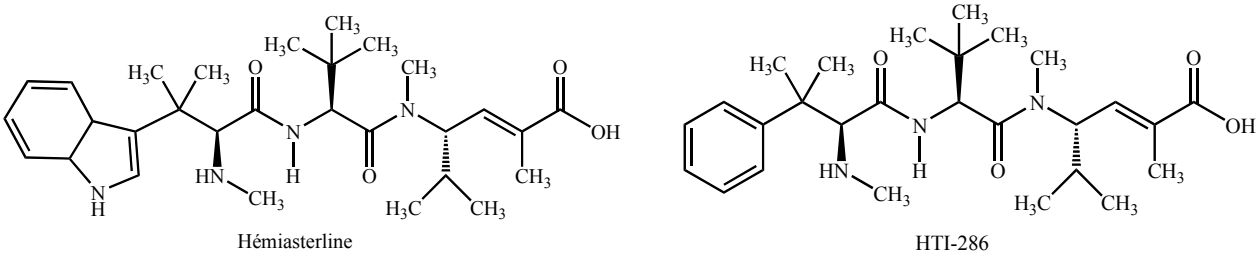


Figure 12 : L'hémiasterline isolée *Cymbastela* sp. et de *Hemiasterella minor* et son analogue HTI-286

L'E7389 est un analogue synthétique et simplifié de l'Halichondrine B (Fig. 13), isolée en 1985 de l'éponge *Halichondria okadai* (43). L'E7389 est actuellement commercialisé comme agent anticancéreux (cancer du sein).

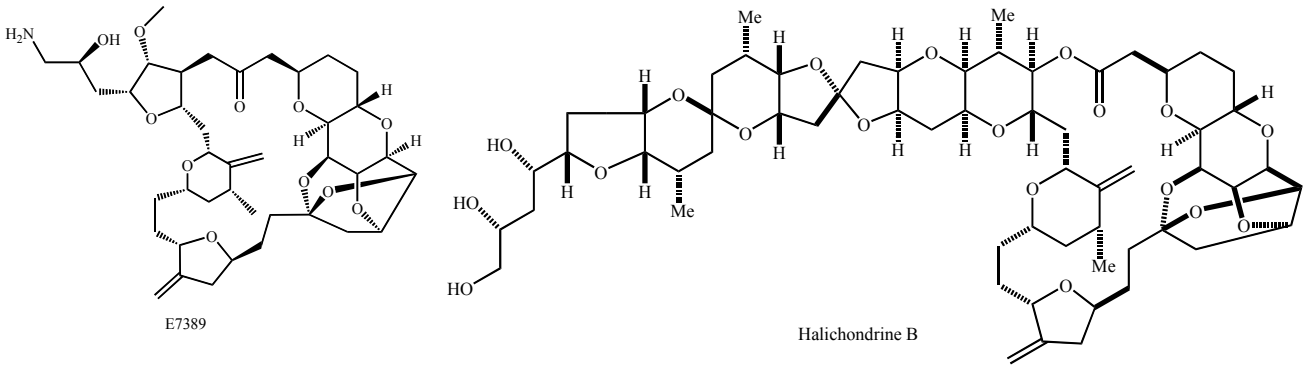
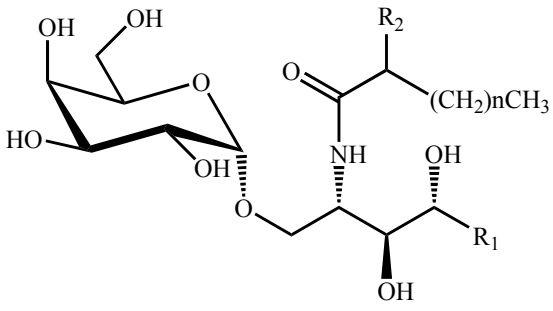


Figure 13 : L'E7389 et l'halichondrine B d'*Halichondria okadai*

Les molécules décrites ci-dessus ne sont que quelques exemples choisis parmi les nombreux composés isolés à partir d'éponges marines rapportés dans la littérature et qui possèdent des activités biologiques.

Le KRN7000 (Fig. 14) est un analogue de synthèse des Agélasphines qui limite l'apparition de métastases hépatiques. Il est également en essais cliniques sur d'autres cibles que le cancer comme la « réaction lymphocytaire mixte – MLR » (44). Il est également en phase II des essais cliniques contre des maladies du greffon contre l'hôte (graft-versus-host disease ou GVHD) (45). Les Agélasphines (Fig. 14), isolées de l'éponge *Agelas mauritianus* se sont révélées être des substances très fortement actives sur certaines tumeurs cancéreuses comme le mélanome B16 (46).



	n	R_1	R_2
Agélasphine 7a	21	$C_{12}H_{25}$	OH
Agélasphine 9a	21	$C_{13}H_{27}$	OH
Agélasphine 13	22	$C_{15}H_{31}$	OH
KRN 7000	23	$C_{14}H_{29}$	H

Figure 14 : Agélasphines *de Agelas mauritianus* et KRN7000

1.4.3. Les glycolipides des éponges

La chimiodiversité considérable des phospholipides et des stérols d'éponges s'accompagne d'une chimiodiversité analogue pour les glycolipides, justifiant les nombreuses et intenses recherches en cours, notamment depuis la mise en évidence des propriétés antitumorales et immunomodulatrices des agélasphines et de leurs analogues de synthèse comme le KRN7000 (Fig. 14). Plusieurs revues bibliographiques ont été consacrées aux glycolipides d'éponges, en particulier celles de Fattorusso & Mangoni, 1997 ; Natori *et al.*, 2000 ; Costantino *et al.*, 2001.

Il est courant de distinguer deux grandes familles de glycolipides selon qu'ils possèdent ou non un atome d'azote. Parmi ceux qui n'en possèdent pas, les glycoglycérolipides sont construits à partir d'une molécule de glycérol qui est diversement reliée à un nombre variable de sucres, la partie aglycone étant liée à une ou à deux chaînes grasses par une liaison éther ou ester. Il exsite des glycoglycérolipides sulfatés sur la partie glucidique, en général en position 6. Les glycolipides azotés se construisent à partir d'une « base sphingoïde » qui n'est pas toujours la sphingosine elle-même, et qui est liée à une chaîne grasse par une liaison amide, l'ensemble formant un céramide. Les glycosylcéramides, 1*a* ou 1*β*, portent le nom de cérébrosides ou de glycosphingolipides (Fig. 15) (47). Les gangliosides sont des glycolipides acides dont les chaînes oligosaccharidiques sont terminées par des résidus d'acides sialiques. Mais, ceux-ci sont non décrits jusqu'ici chez les éponges.

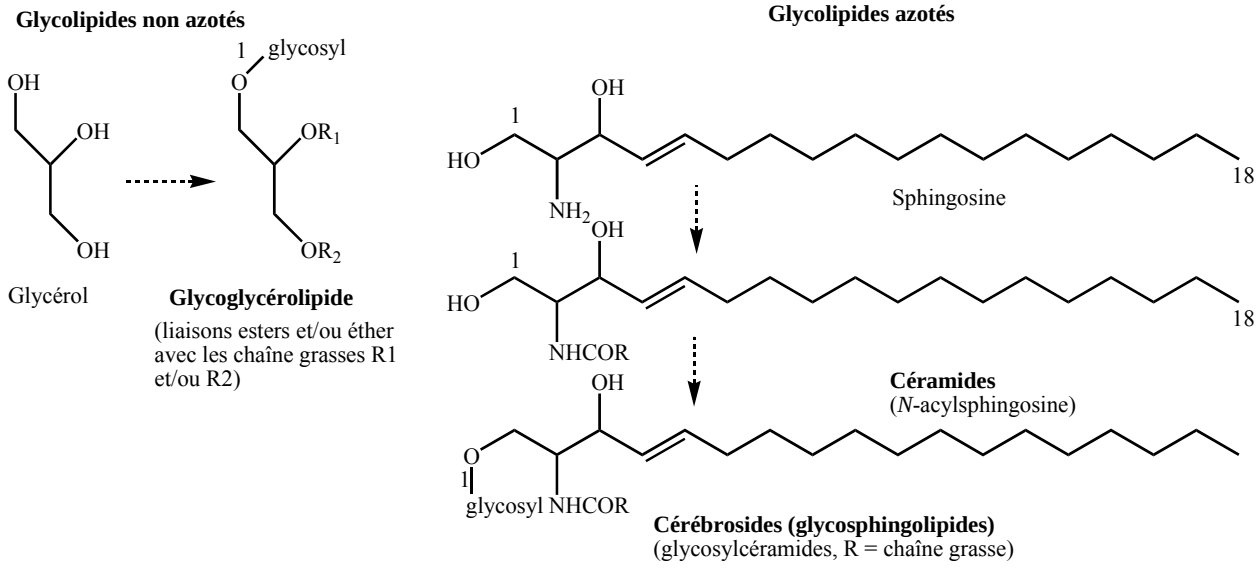


Figure 15. Structures générales des glycolipides d'éponges (47)

Parmi les premiers exemples des glycoglycérolipides isolés d'éponges, il faut citer un galactolipide anti-inflammatoire et un glucolipide portant une fonction acide sulfonique qui possède des propriétés anticomplémentaires. Ces deux glycolipides ont été isolés de *Phyllospongia foliascens* (Dictyoceratida), et dans les deux cas les groupes R_1 et R_2 sont des hydrogènes ou des groupes acyl à 14 et à 16 atomes de carbone (Fig. 16) (48).

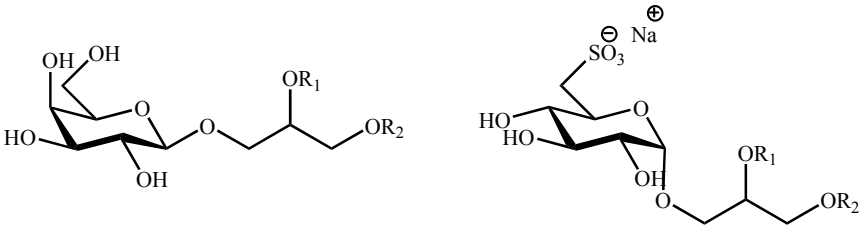


Figure 16. Les glycoglycérolipides de *Phyllospongia foliascens*

L'espèce *Trikentrion laeve (= loeve)* récoltée au Sénégal a permis d'isoler un 1-*O*-alkyl-glycéryl-glycoside dont la partie glycosylée ne contient que du *β*-xylose, et la partie grasse une chaîne linéaire insaturée à 24 atomes de carbone (Fig. 17) (49).

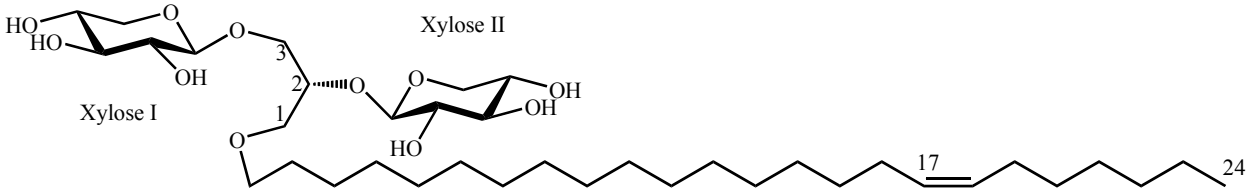


Figure 17. Trikentroside de *Trikentrion laeve (= loeve)*

L'éponge *Pseudoceratina crassa* récoltée dans les Caraïbes contient une série de six analogues glycoglycérolipides, les Crassérides, dans lesquels la « partie glucidique » est en fait un cyclitol à cinq éléments (cyclopentanepentaol). Ces substances fortement anti-appétantes pourraient servir de répulsif contre les prédateurs (50). En 2002, la même équipe italienne a montré que plusieurs espèces de Démosponges contiennent des Crassérides (majoritaires) et des Isocrassérides dans lesquels l'une des fonctions esters du glycérol est fixée sur le cyclitol. La présence simultanée de Crassérides et d'Isocrassérides pourrait être considérée comme caractéristique du phylum des Spongiaires (Fig. 18) (51).

En 1993, la découverte des Agélasphines dans *Agelas mauritianus,* qui appartient maintenant au nouvel ordre des Agelasida (52), a été le point de départ de nombreuses recherches sur les propriétés immunomodulatrices des *a*-galactosylcéramides, qui ont conduit à la synthèse du KRN7000 (Fig. 14) (53).

Des études de structure-activité ont montré que la nature du sucre (galactose), la conformation *a* de la liaison oside, la présence de l'hydroxyle en 3, ainsi que la longueur des chaînes grasses dans la partie amide comme dans la base sphingoïde, avaient un rôle prépondérant sur l'activité antitumorale par immunostimulation, ce qui a conduit à la synthèse du KRN7000 (53). L'étude des galactocérébrosides de plusieurs espèces d'*Agelas* a montré que la liaison 1*a* était fréquente, mais d'autre Démosponges possèdent également des *a*-galactocéramides, comme *Stylissa flabelliformis* (Halichondrida, Axinellidae) (54, 55).

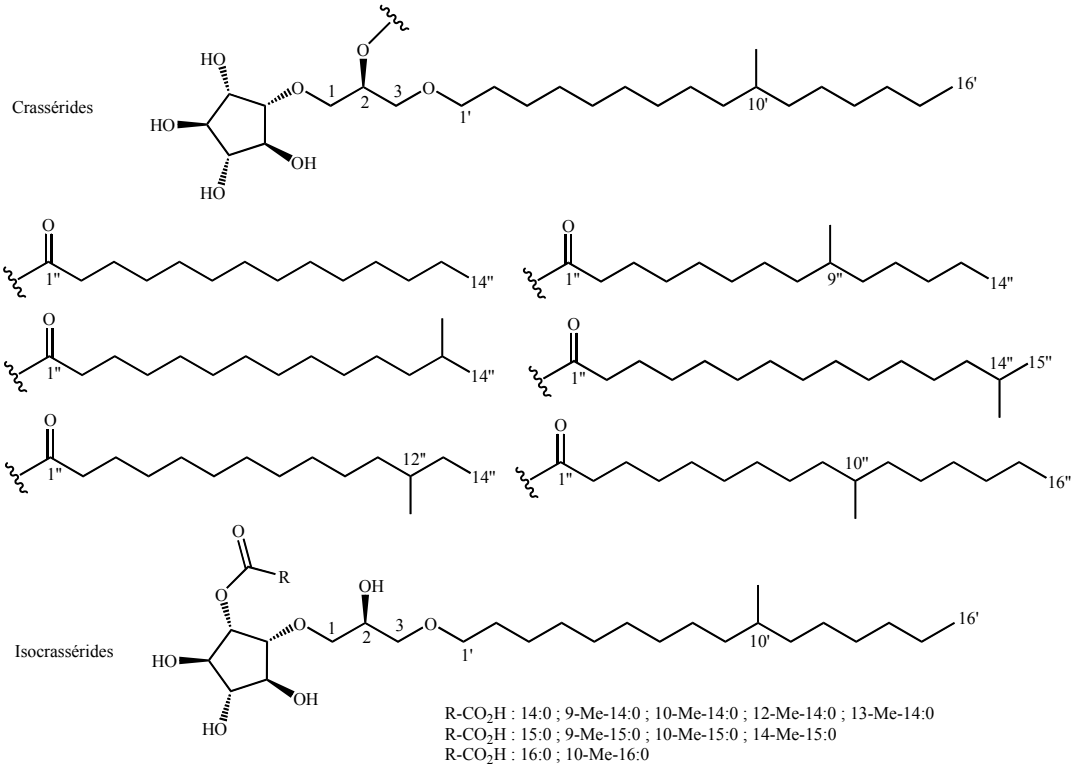


Figure 18. Structure de quelques Crassérides et Isocrassérides chez certaines Démosponges

Une nouvelle classe de glycolipides qui semble être caractéristique d'éponges marines. Les Clathrosides A-C et Isoclathrosides D-F ont été isolés de l'éponge des Caraïbes *Agelas clathrodes* (Fig. 19). Les six composés diffèrent dans la configuration et dans la composition des chaînes alkyles (56).

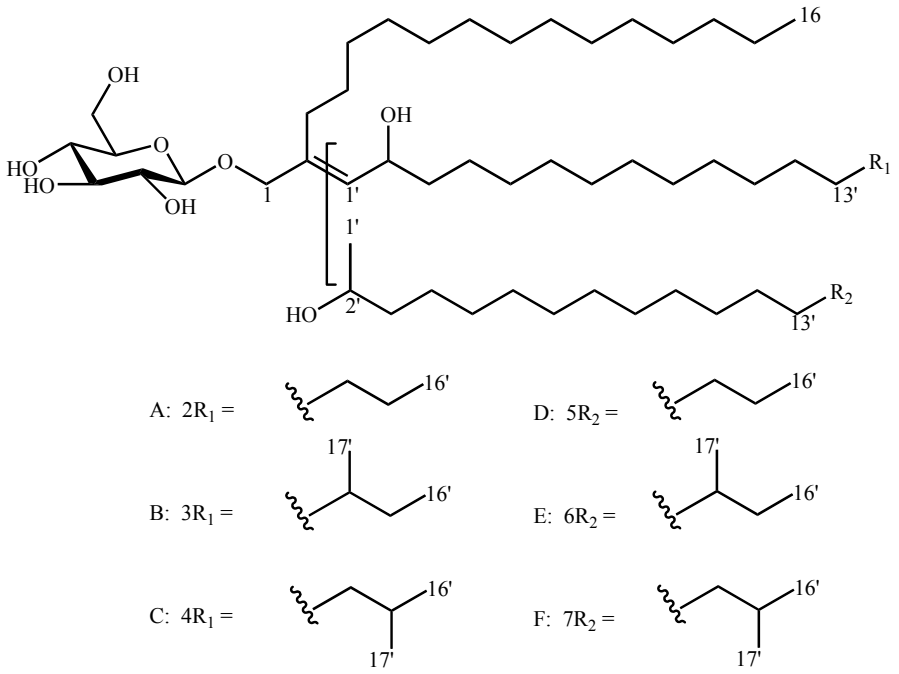


Figure 19. Les Clathrosides A-F de *Agelas clathrodes*

Une famille de glycolipides antimicrobiens (les inhibiteurs du type bactérien III) les Caminosides A-D, ont été isolés de l'éponge des Caraïbes *Caminus sphaeroconia* (Fig. 20) (57).

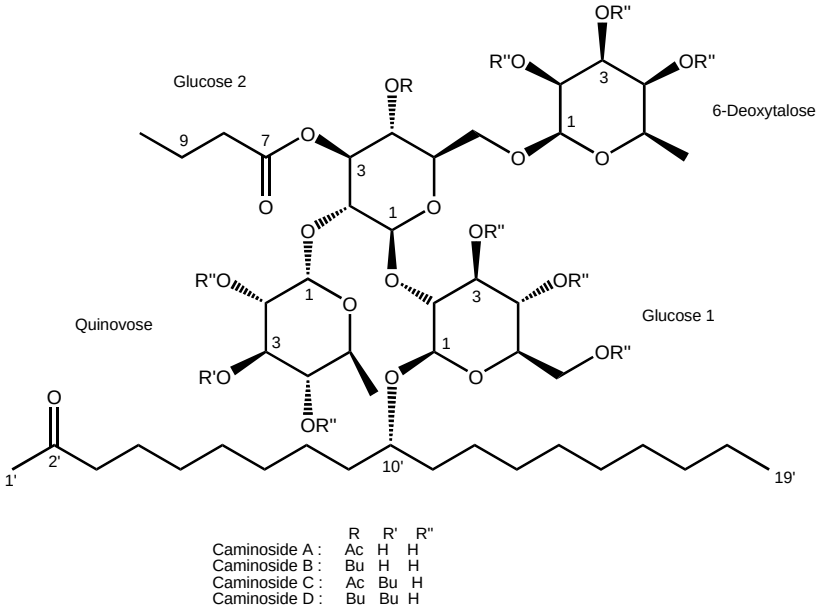


Figure 20. Les Caminosides A-D de *Caminus sphaeroconia*

L'Ectyocéramide est un autre exemple de *β*-galactosylcéramide qui a été isolé de l'espèce *Ectyoplasia ferox* (Poecilosclerida, Raspailiidae) en 2003. C'est le premier exemple connu d'un monohexofuranosylcéramide (Fig. 21) (55).

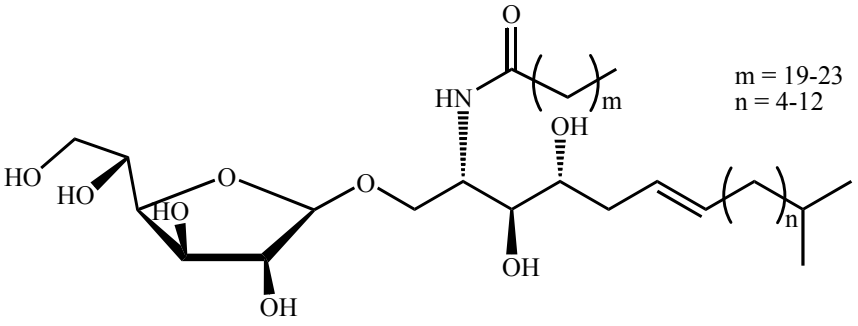


Figure 21. Ectyocéramide d'*Ectyoplasia ferox*

Les Plakosides A-D forment un groupe particulier de *β*-galactosylcéramides par la présence d'un groupe prényle sur l'hydroxyle en 2 du galactose, et l'existence d'un cyclopropane sur la chaîne grasse da la partie amide (Fig. 22). Ces composés ont été isolés des espèces *Plakortis simplex* (Homosclerophorida) et *Ectyoplasia ferox* (Poecilosclerida) récoltées dans les Caraïbes. Les Plakosides A et B sont des immunosuppresseurs (54, 58).

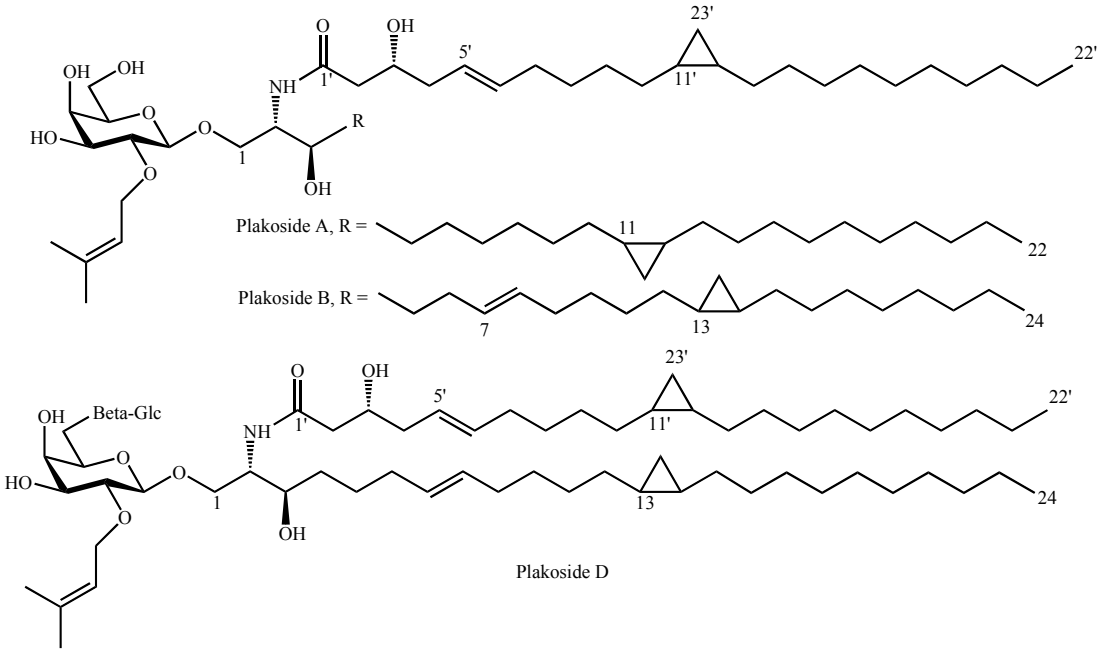


Figure 22. Plakosides A, B et D de *Plakortis simplex* et de *Ectyoplasia ferox*

Les Amphicérébrosides sont probablement les premiers exemples connus d'*a*-glucosylcéramides. Isolés de l'espèce *Amphimedon viridis* récoltée dans le golfe d'Eilat (mer Rouge), ils contiennent une *N*-glucosamine de configuration *a* ou *β*. Contrairement aux agélasphines, les Amphicérébrosides ne présentent aucune activité biologique particulière (Fig. 23) (59).

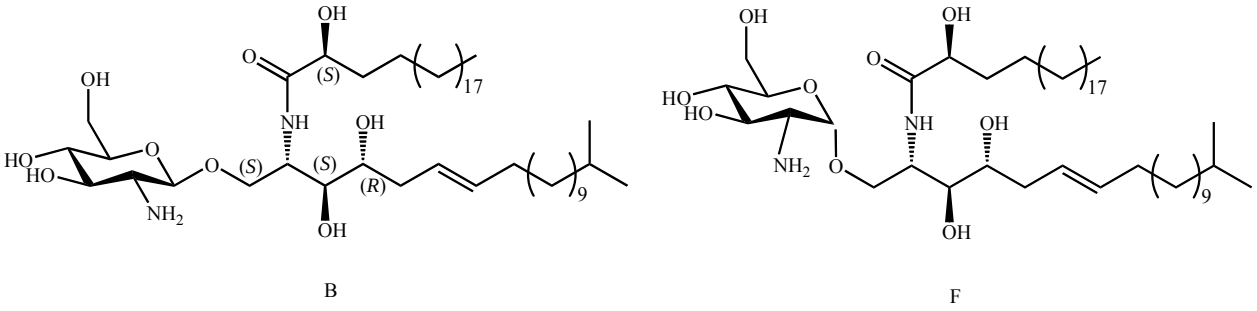
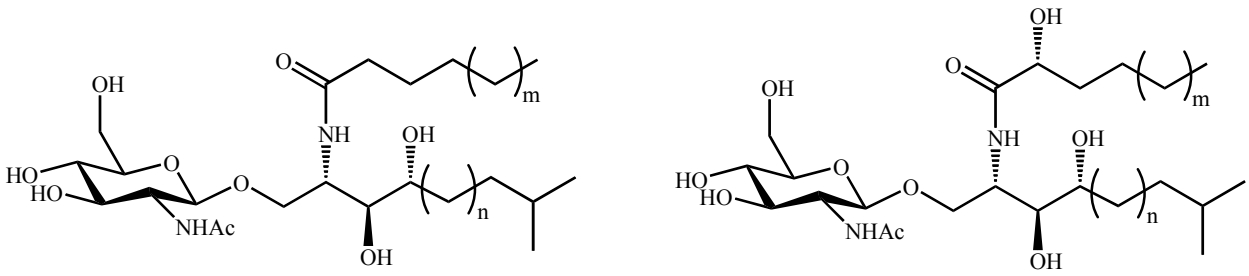


Figure 23. Amphicérébrosides B et F de *Amphimedon viridis*

Les Halicylindrosides sont des *β*-*N*-acétylglucosaminyl-céramides isolés de l'espèce *Halichondria cylindrata* (Halichondrida, Halichondriidae), récoltée sur les côtes japonaises au sud de Tokyo. Les Halicylindrosides B_1-B_2 ne diffèrent des Halicylindrosides A_1-A_4 que par la présence d'un hydroxyle en 2' sur la chaîne grasse de la partie amide (Fig. 24) (60). Les Halicylindrosides sont modérément antifongiques et cytotoxiques pour les cellules de la leucémie murine P388.



Halicylindroside A_1 : m = 16, n= 10
Halicylindroside A_2 : m = 16, n= 11
Halicylindroside A_3 : m = 17, n= 11
Halicylindroside A_4 : m = 18, n= 11

Halicylindroside B_1 : m = 16, n= 8
Halicylindroside B_2 : m = 17, n= 8
Halicylindroside B_3 : m = 16, n= 9
Halicylindroside B_4 : m = 16, n= 10
Halicylindroside B_5 : m = 17, n= 9
Halicylindroside B_6 : m = 16, n= 11

Figure 24. Halicylindrosides d'*Halichondria cylindrata*

Depuis la découverte des galactosylcéramides par Natori *et al.* en 1993, d'autres *a*-galactosylcéramides avec des parties glycosylées plus complexes que celle des agélasphines ont été caractérisés. Le Longiside est un digalactosylcéramide dont les deux galactoses sont simultanément présents avec les configurations *a*-pyranose et *β*-furanose (Fig. 25) (61).

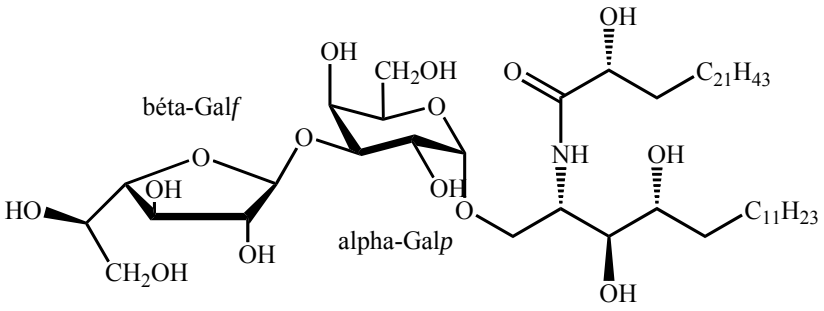


Figure 25. Longiside d'*Agelas longissima*

L'Agélagalastine (Fig. 26) est un trigalactosylcéramide qui contient un *β*-galactopyranose, un *β*-galactofuranose, et le 1*a*-galactofuranose. Ce composé apparaît ainsi comme un « galactosyl-longiside », ce glycolipide est fortement antitumoral pour les cancers du poumon (lignée NCI-H460) et de l'ovaire (lignée OVCAR-3), avec des CI_{50} respectives de 0,77 et 2,8 μg/mL (62).

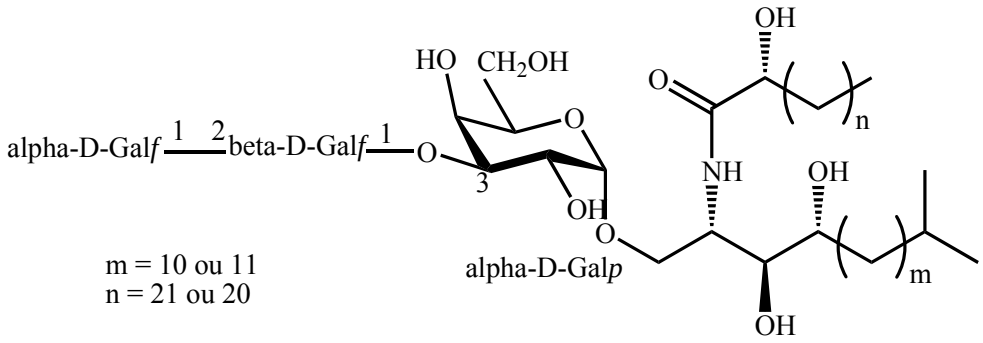


Figure 26. Agélagalastine d'*Agelas* sp.

Les premiers trigalactosylcéramides ont été mis en évidence en 1996 dans l'espèce *Agelas dispar*. Ils ont la particularité de posséder une *N*-acétylgalactosamine (Fig. 27) (63). Certains de ces composés

sont immunostimulants, et les premiers résultats suggèrent que cette activité est due à la glycosylation de l'hydroxyle en 2 de l'ose directement lié à la partie céramide, l'activité étant maximale lorsque cet hydroxyle est libre.

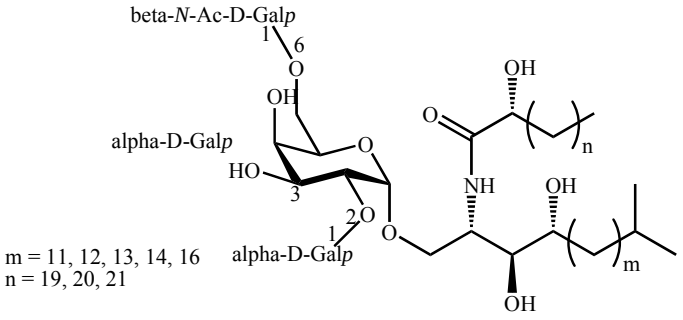
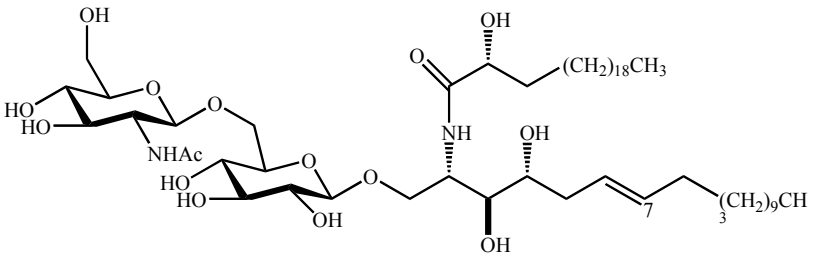
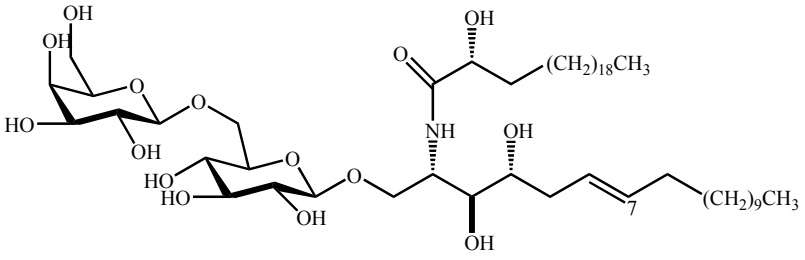


Figure 27. Les trigalactosylcéramides d'*Agelas dispar*

L'analyse des glycolipides de l'éponge des Caraïbes *Amphimedon compressa* par Costantino *et al.* a montré deux nouveaux glycosphingolipides, les Amphicéramides A et B, qui possèdent une inhabituelle Δ^6-phytosphingosine. La chaîne de saccharide d'Amphicéramide A est composée d'un résidu de ß-glucose glycosylé en position 6 par une *N*-acétyl-ß-glucosamine. Ceci n'a jamais été trouvé auparavant dans un produit naturel (Fig. 28) (64).



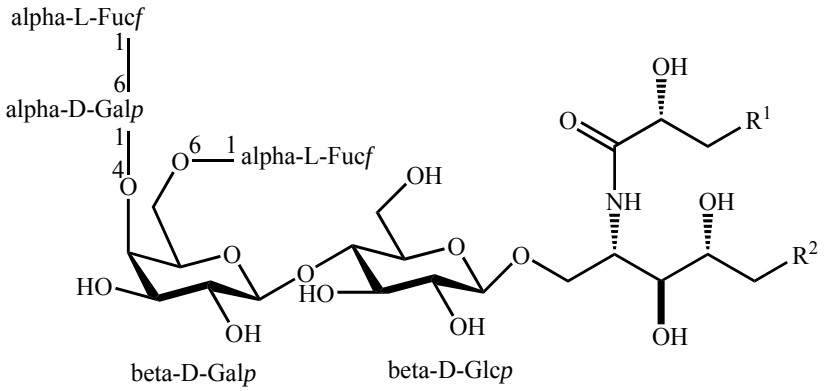
Amphicéramide A



Amphicéramide B

Figure 28. Amphicéramide A et B d'*Amphimedon compressa*

Le Terpioside B, glycolipide unique contenant deux résidus fucose en forme furanose parmi sa chaîne pentasaccharidique, a été isolé de l'éponge marine *Terpios sp*. Le Terpioside B est un puissant inhibiteur du monoxyde d'azote NO produit par le LPS (lipopolysaccharide), il est considérablement plus actif que les glycosphingolipides plus simples comme les monoglucosylcéramides (Fig. 29) (65).



R^1 = 22-26
R^2 = 18-19

Figure 29. Terpioside B de *Terpios* sp

1.5. Les molécules actives des Echinodermes

Diverses substances biologiquement actives ont été isolées des Echinodermes. Kuznetsova *et al.* (66) ont démontré qu'un triterpène glycosylé (Holothurine A et B), isolé de 19 espèces d'holothurie de la zone tropicale du Pacifique, possédait une activité cytotoxique contre levure et cellules de tumeur (la ligne cellulaire de Sarcome 37) (Fig. 30). Haug *et al.* (67) ont démontré des activités antibactériennes des extraits de plusieurs mouchoirs en tissus de l'oursin *Strongylocentrotus droebachiensis*, du concombre de mer *Cucumaria frondosa* et de l'étoile de mer *Asterias rubens*. L'activité antibactérienne détectée indique que les échinodermes peuvent être une source utile comme nouveaux antibiotiques (68).

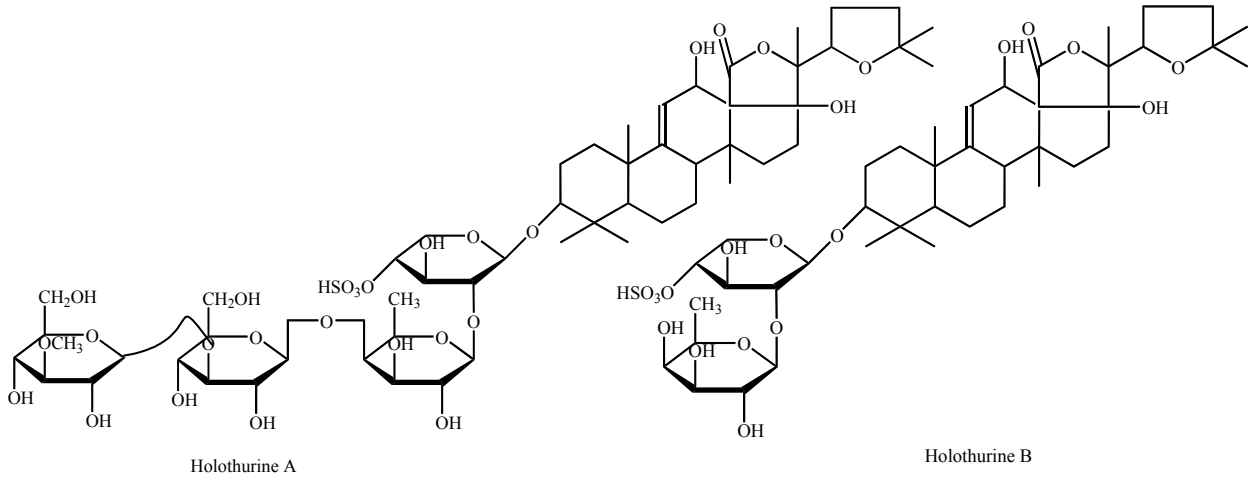


Figure 30. Holothurine A et B

Il a été démontré que de nouveaux polysaccharides présents sur la surface d'échinoderme semblent capables de stimuler la défense des hôtes de produire des bactéries un microbien, mais pas pendant les phases postérieures de blessure tissulaire inflammatoire associée à la septicémie. Ceux-ci sont actuellement considérés comme traitements alternatifs les plus prometteurs lors du développement d'une pneumonie (69, 70). Les lectines sont des protéines qui possèdent des sites de liaison spécifiques pour des mono- et oligosaccharides (71). Chez les invertébrés marins, les lectines agissent dans l'immunité humorale, aboutissant à l'activation de phagocytes (68). Après fixation aux chaînes de glucides spécifiques sur la surface d'érythrocyte, ces lectines endommagent la membrane cellulaire, provoquant la lyse de la cellule (68). Plusieurs lectines uniques en leur genre, ont été trouvées dans des échinodermes (68). Une lectine avec des activités biologiques mitogènique et des caractéristiques chimiotactiques a été décrite dans le venin du pédicellaire de l'oursin *Toxopneustes pileolus* (72). Une autre lectine, ayant une cytotoxicité contre des cellules cancéreuses humaines de poumon, a été isolée de concombres bruns de mer (73). Ces études montrent que ces lectines particulières d'échinodermes ont un potentiel énorme contre plusieurs types de tumeur. Un gène d'oursin montre une homologie structurelle très proche du codage de gène défectueux présent dans la dystrophie musculaire Duchenne (70). La caractérisation partielle de ce gène a permis la construction d'un arbre évolutionnaire connectant la dystrophine chez les vertébrés à celle des gènes liés chez les invertébrés (74,70). Les polysaccharides sulfatés abondent dans les tissus des vertébrés. Certaines espèces d'invertébrés, comme les holothuries, sont une source riche de glycosaminoglycanes sulfatés de structure nouvelle (71). Tapon-Bretaudiere *et al.* (75) ont constaté que la chondroitine fucosylée et sulfatée (FucCs) d'un concombre de mer a permis la prolifération

de vaisseaux sanguins et a une capacité d'empêcher une thrombose veineuse et artérielle chez les mammifères. Mourao *et al.* (76, 77) et Li *et al.* (78) indiquent que la FucCs des concombres de mer possède une activité anticoagulante *in vivo* et Li *et al.* (78) ont suggéré que la FucCs peut être une alternative potentielle à l'héparine, pour bloquer les métastases et les réactions inflammatoires sans les effets secondaires indésirables de l'héparine anticoagulante. Basée sur sa puissance, son meilleur index thérapeutique, des effets secondaires indésirables moindres, son origine naturelle, son abondance et sa facilité de purification, la FucCs pourrait être utilisée comme un agent thérapeutique dans le traitement de cancer (79). La médecine chinoise traditionnelle utilise généralement le concombre de mer dans le traitement de la faiblesse, de l'impuissance, de la démence sénile, de la constipation, des problèmes urinaires. La médecine occidentale utilise le concombre de mer pour traiter la polyarthrite chronique évolutive, l'arthrose et la spondylarthrite ankylosante. L'utilisation du concombre de mer, en vente libre pour le traitement de l'arthrite est approuvée en Australie et au Japon un brevet utilisant la FucCs du concombre de mer pour la thérapie anti-HIV a été déposé (80). Les échinodermes peuvent devenir utiles même dans la bionique (68). Récemment, *Aizenberg et al.* (81) ont découvert que l'étoile fragile *Ophiocoma wendtii* possède des cristaux de calcites simples arrangés pour fonctionner comme des objectifs photographiques. En somme, des milliers d'objectifs forment un œil à facettes qui couvre la surface supérieure de l'animal, aboutissant à une fonction semblable à un appareil photo numérique qui crée une image pixel par pixel (81). À présent, les ingénieurs dans l'industrie photonique essayent d'imiter les calcites de cette étoile pour une utilisation dans la réception du signal (68).

1.5.1. Les molécules actives des étoiles de mer

Les étoiles de mer sont largement étudiées pour leur composition en métabolites de toutes sortes tels que les stérols et les saponines. Ces molécules semblent avoir des rôles de défense de l'organisme contre les agressions extérieures de types fongiques ou bactériennes. Par ailleurs, d'autres études rapportent des propriétés anti-inflammatoires et anticancéreuses de glycosides stéroïdiens et des saponines. Un bilan de ces molécules est présenté dans le Tableau 3.

1.5.1.1. Propriétés antitumorales, antifongiques et antibactériennes

Chludil *et al.* (82) ont isolé des glycosides stéroïdiens à activité antifongique contre le champignon pathogène *Cladosporium cucumerinum*. Certains glycosides stéroïdiens sulfatés, isolés de *Culcita novaeguinenae*, ont démontré une activité cytotoxique légère contre certaines lignées cellulaires tumorales comme la leucémie humaine K-564 et l'hépatome humain BEL-7402 (83, 84). Des travaux ont permis l'isolement de 22 saponines et 36 stérols à partir de *Certonardoa semiregularis*. Plusieurs de ces molécules possèdent des activités cytotoxiques contre un petit panel de lignées cellulaires humaines de tumeurs solides ainsi que des activités antivirales contre le HSV (herpes simplex virus) (85-89). Des extraits provenant *d'Asterina pectinifera* provenant d'une activité antimicrobiens contre les souches de micromycètes et les levures (90). Des activités antibactériennes ont été mises en évidence dans les extraits d'organes gastro-intestinaux d'*Asteria rubens*. L'extrait polaire de *Luidia clathrata* est capable d'inhiber la fixation de certaines larves de bryozoaires et de balanes ainsi que d'inhiber la croissance de diverses bactéries (91).

1.5.1.2. Propriétés adhésives et anti-adhésives

Bavington *et al.* (92) ont isolé des glycoprotéines, appelés mucines, provenant du mucus de *Marthasterias glacialis* et *Porania pulvillus*. Les mucines isolées de *M. glacialis* empêchent l'adhésion bactérienne tandis que celles provenant de *P. pulvillus* la favorise. Ces mucines pourraient avoir un rôle industriel par leur capacité à réguler l'adhésion bactérienne comme agent « anti-fouling ». Inversement, une autre étude rapporte que des substances adhésives retrouvées dans les podies d'étoiles de mer permettraient l'adhésion aux substrats grâce aux propriétés adhésives des protéines produites par deux types de non-ciliés sécrétoires (NCS1 et NCS2) cellules et un adhésif matériau est publié par sécrétoire cilié (CS) cellules (93).

1.5.1.3. Propriétés régénératrices

D'autres travaux ont porté sur l'isolement de molécules de *Asterias rubens* mimant l'action de l'héparine. Les tissus régénérant de cette étoile de mer ont été étudiés afin de mettre en évidence la présence de facteurs de croissance susceptibles de stimuler la prolifération des fibroblastes et des cellules épithéliales. Les résultats montrent que les peptides sont riches en lysine et leur composition en acides aminés est comparable au facteur de croissance de fibroblaste (b-FGF) (94). Une autre étude traite de l'isolement de deux gangliosides à partir de *Luidia maculata* dont un est de structure

nouvelle. Un premier ganglioside de type GD3 (aNeu5Ac(2-8)aNeu5Ac(2-3)bDGalp(1-4)bDGlcp(1-1)Cer) a été isolé d'un échinoderme. De plus, un autre ganglioside s'est montré neuritogénique contre les cellules de rat PC12 (95-97). Ces travaux suggèrent l'existence de composés à fort potentiel régénérateur tissulaire similaire aux facteurs de croissance. Ceci pourrait servir dans des applications cosméceutiques.

1.5.1.4. Propriétés anticoagulantes

Koyama *et al.* (98) ont isolé un peptide d'*Acanthaster planci*, la plancinine. Cette molécule possède des propriétés anticoagulantes vraisemblablement par l'activation de la prothrombine et du facteur X. Les auteurs *Kicha et al.* (99) ont étudié la distribution de plusieurs stéroïdes polaires, dans l'étoile de mer *Asterina pectinifera*. Certains de ces composés auraient des activités hémolytiques.

1.5.1.5. Les glycolipides des étoiles de mer

L'essentiel des publications sur les lipides des Echinodermes en général et des Astérides en particulier concerne les glycolipides, les céramides et les gangliosides. Apparues à la fin des années 1980, les publications dans ce domaine ont augmenté de façon significative depuis le milieu des années 1990, probablement du fait des propriétés biologiques de cette catégorie de lipides. Les différents types structuraux de glycolipides ont déjà été présentés dans la figure 15 mais compte tenu de l'augmentation des travaux sur ces molécules complexes isolées des Echinodermes, il est utile de préciser quelques points de vocabulaire. Le terme de cérébroside désigne un glycosphingolipide dont la partie osidique se limite à un seul hexose, en général le glucose. Les autres composés ayant des parties osidiques complexes sont désignés en faisant référence à cette partie glucidique, ce qui est notamment le cas des céramides lactosides. Les gangliosides sont des glycosylcéramides contenant de l'acide sialique (acide *N*-acétyl-neuraminique) et enfin le terme de sulfatide indique qu'un ou plusieurs sucres sont sulfatés mais les sulfatides n'ont été isolés d'une Astéride qu'en 1997.

Jusqu'à présent très peu de glycolipides simples ont été isolés d'étoiles de mer. Le premier rapport important sur les glycosphingolipides d'étoiles de mer vient d'un groupe de chercheurs japonais qui a isolée six cérébrosides (céramide monohexosides) : les Acanthacérébrosides d'*Acanthaster planci* (Fig. 31) (100), et aussi l'un des plus originaux pourrait être la Forbésine qui associe deux quinovoses au 1,16-docosanediol-1-sulfate et qui a été isolée de l'espèce *Asterias forbesi* récoltée au Canada (New Brunswick) (Fig. 32). Huit glucosylcéramides ont été isolés de l'espèce *Anasterias minuta*, un

des constituants a été identifié comme un nouvel glucosylcéramide, l'Anasterocérébroside A. (Fig. 33) (101), ainsi que six glucocérébrosides de l'espèce *Luidia maculata* (Fig. 34) (95). Maier *et al.* ont isolé deux nouveaux glucosylcéramides de l'étoile de mer *Cosmasterias lurida* (Fig. 35) (102). Aucune activité particulière n'a toutefois été signalée pour ces dérivés.

Peu d'activités biologiques significatives semblent avoir été mises en évidence dans un glycosphingolipide isolé d'une Astéride *Astropecten latespinosus*, hormis une cytotoxicité (*in vitro*) de certains gangliosides pour la leucémie murine L1210. Les Ophidiastérosides A-E (Fig. 36) ont ainsi des DI_{90} de l'ordre de 2 μg/mL sur la leucémie murine L1210 (103-105). L'espèce *Stellaster equestris* contient des glucosylcéramides, des *a*-galactosylcéramides, dont l'agélasphine-10. Quelques gangliosides augmentent la durée de vie des neurones de rat en cultures (106).

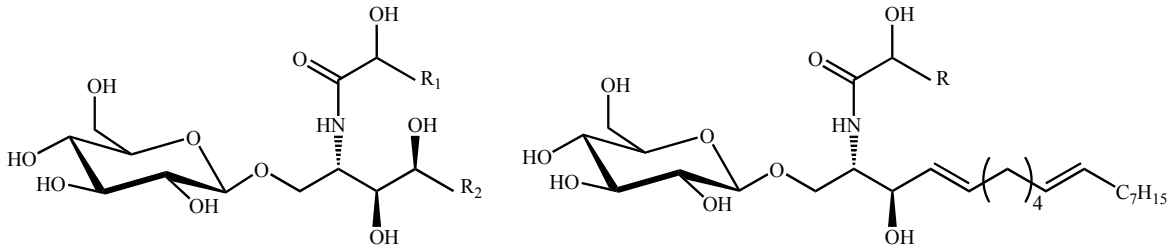


Figure31. Les Acanthacérébrosides d'*Acanthaster planci*

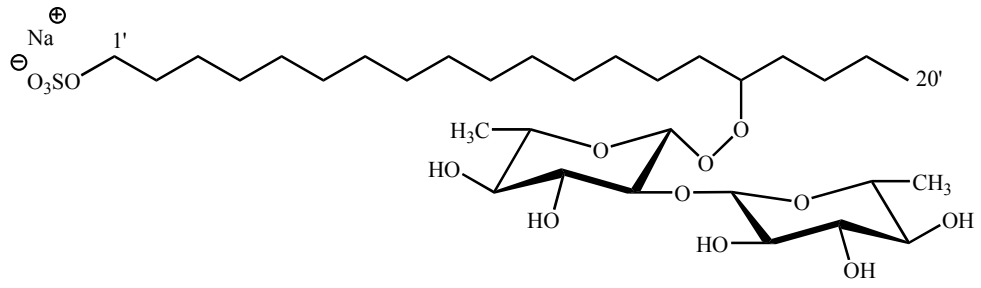


Figure32. Forbésine d'*Asterias forbesi*

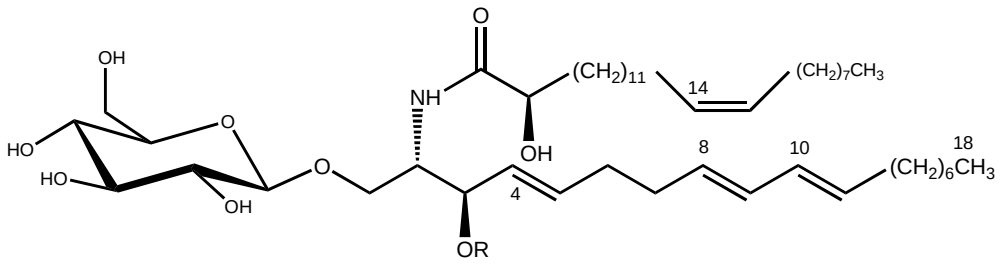


Figure 33. Anasterocérébroside A d'*Anasterias minuta*

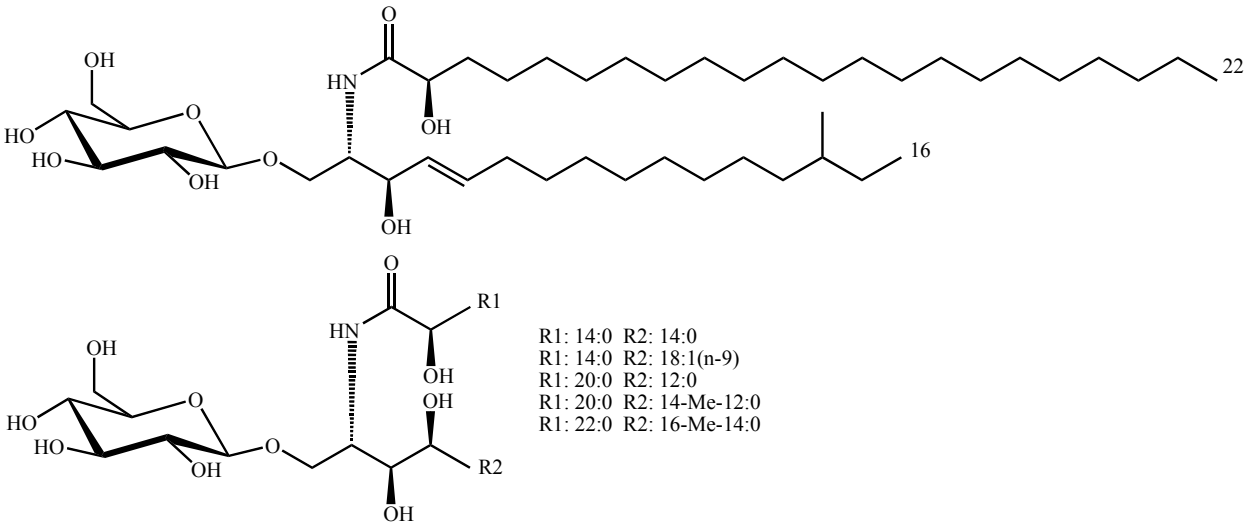
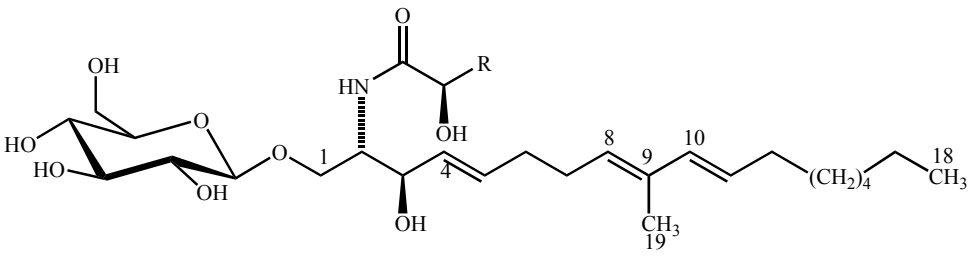


Figure 34. Glucocérébrosides de *Luidia maculata*



1. R = $(CH_2)_{14}CH_3$
2. R = $(CH_2)_{15}CH_3$

Figure 35. Glucosylcéramides de *Cosmasterias lurida*

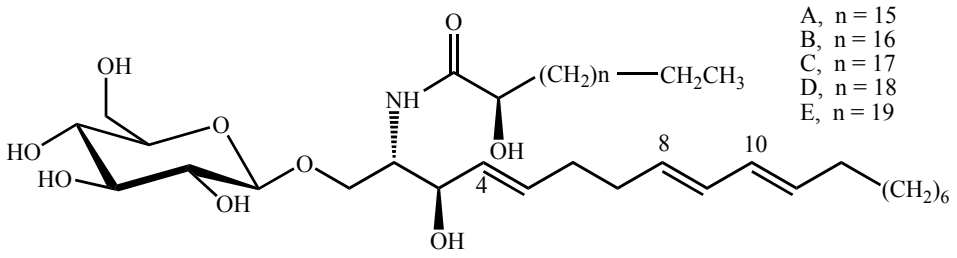
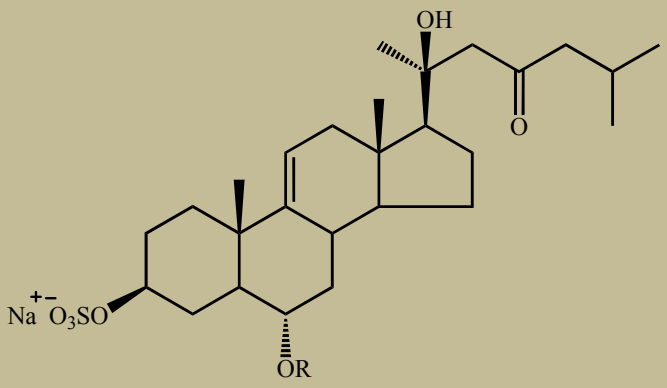
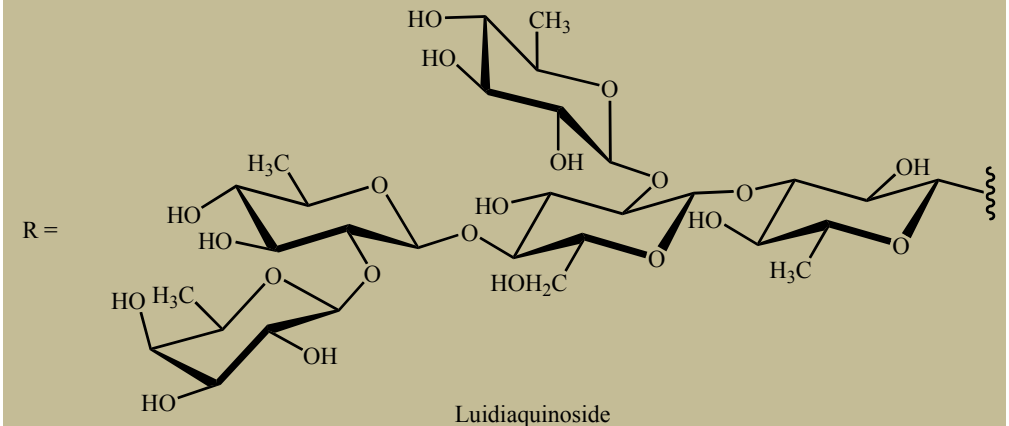
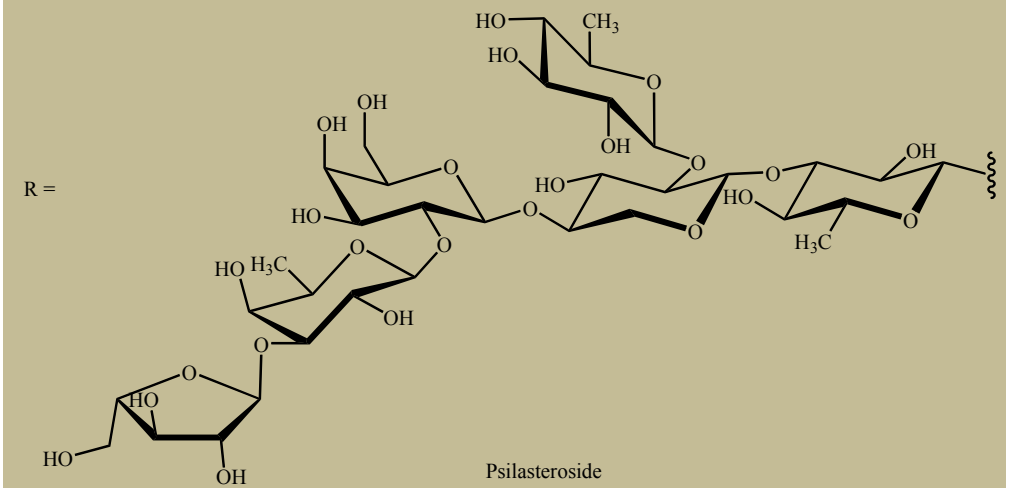
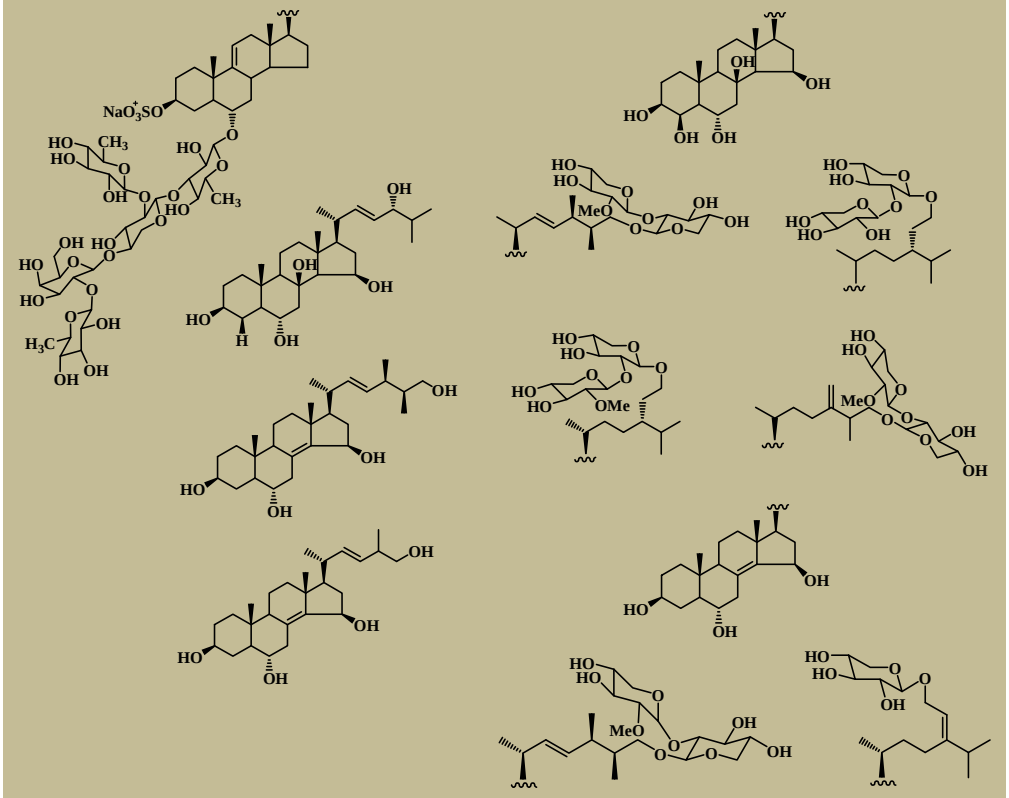
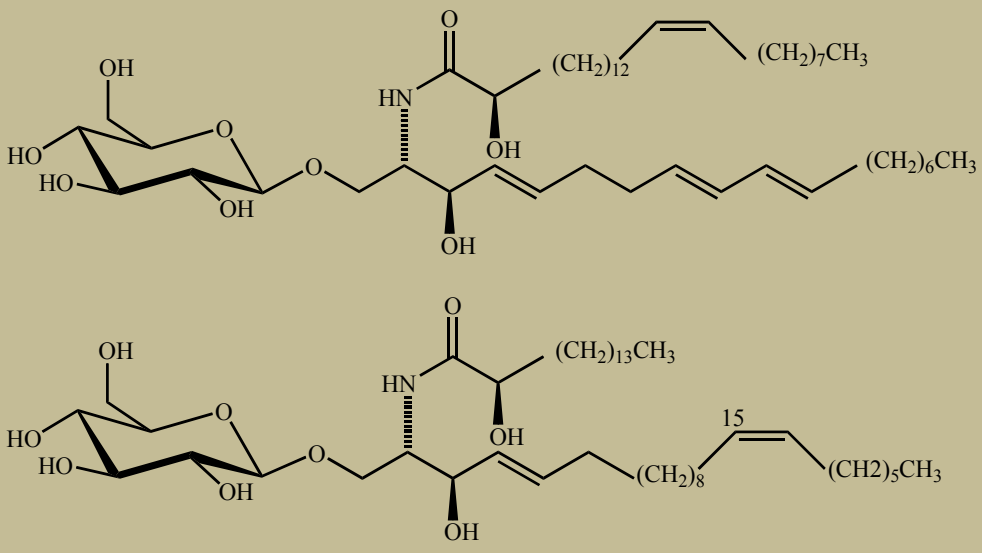


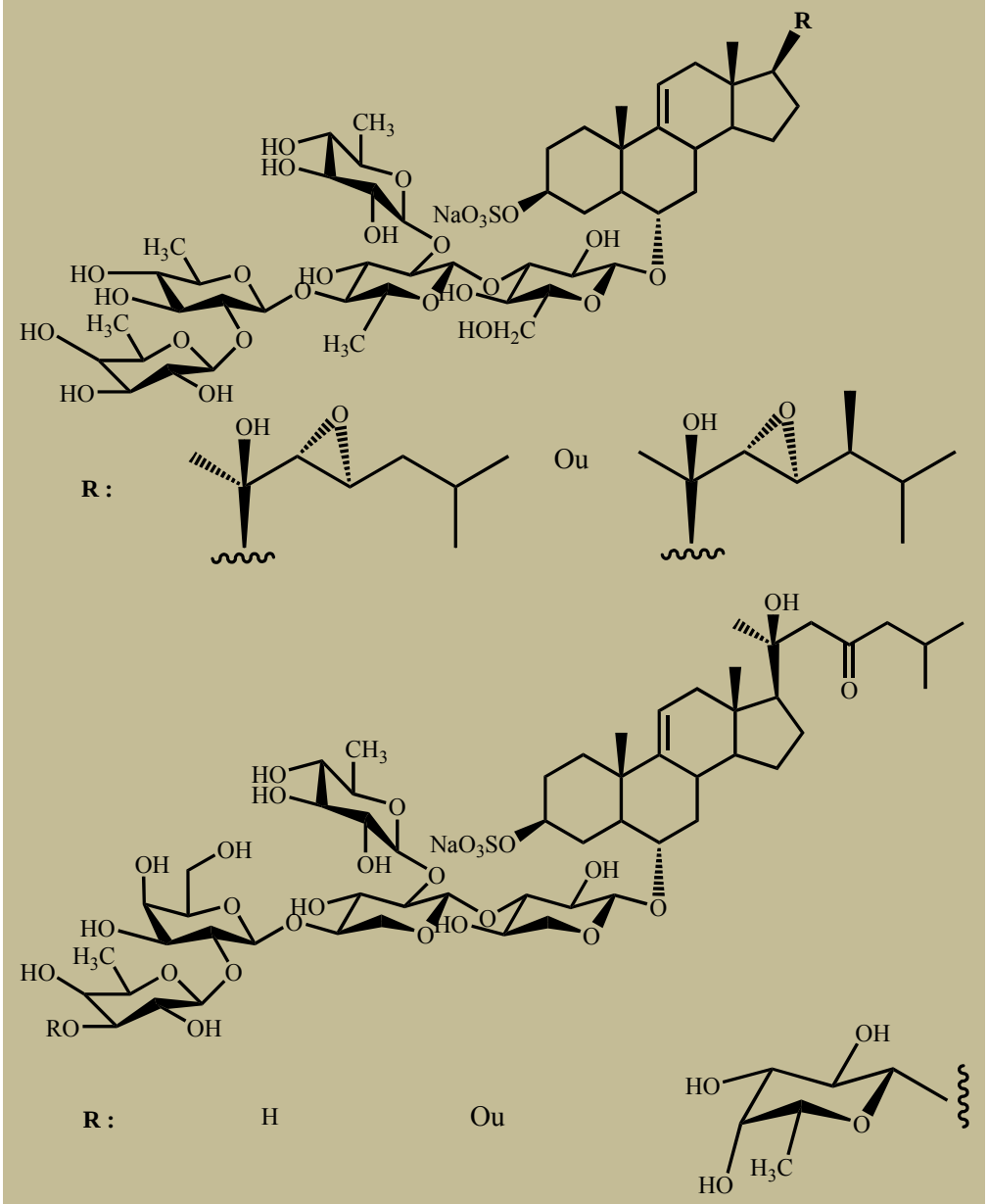
Figure 36. Ophidiastérosides A-E d'*Astropecten latespinosus*

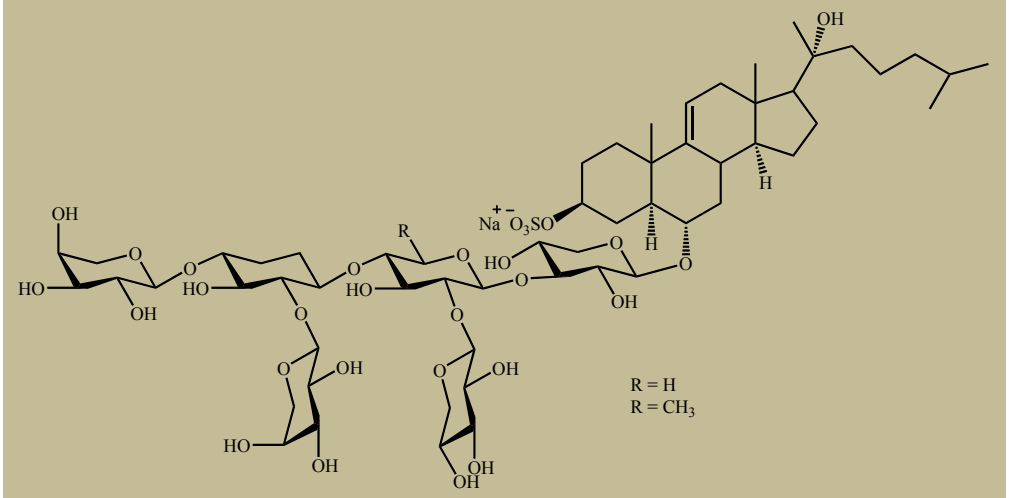
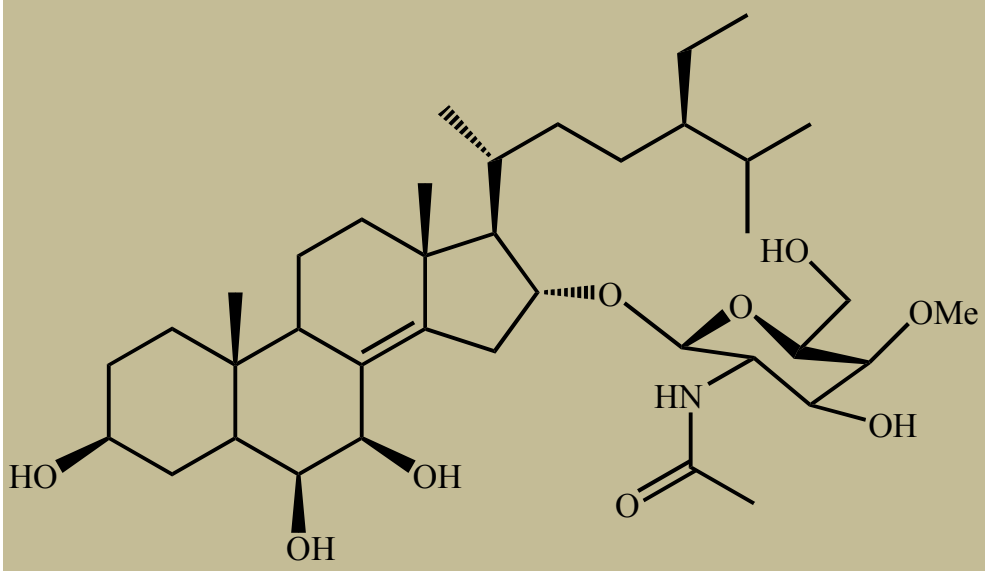
Le sujet qui m'a été proposé s'inscrit dans la poursuite de travaux du Laboratoire et s'appuie sur des premiers résultats prometteurs obtenus avec plusieurs glycolipides marins à activité antitumorale. L'expérience et la connaissance des fonds marins du Sénégal, ainsi que les relations du Laboratoire de avec l'Océanium de Dakar, ont permis de disposer de nombreux organismes originaires de cette région. Le programme européen MAST III (Marine Science and Technology), *Bioactive marine natural product in the field of antitumoral, antiviral and immunomodulant activity* (1996-1999), nous a permis d'avoir accès à des régions du monde très peu étudiées en matière d'éponges (ex : Djibouti). Notre choix s'est porté sur des éponges, *Axinyssa djiferi* (mangrove du Sénégal) et *Myrmekioderma dendyi* (Vanuatu, Pacifique sud) et une étoile de mer, *Narcissia canariensis* (côtes sénégalaises). Depuis 2004, le Laboratoire est partenaire du Cancéropôle Grand Ouest, il s'agit de passer de la molécule isolée au malade ce qui suppose, après le travail des chimistes et des biochimistes, un stade préclinique sur la voie de nouveaux médicaments. Les GL sont évalués pour leurs propriétés antiprolifératives et anti-angiogéniques au sein du Cancéropôle Grand Ouest.

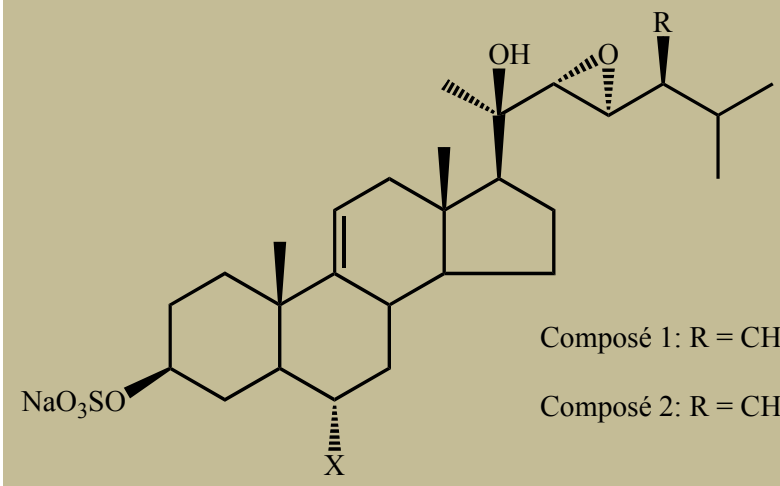
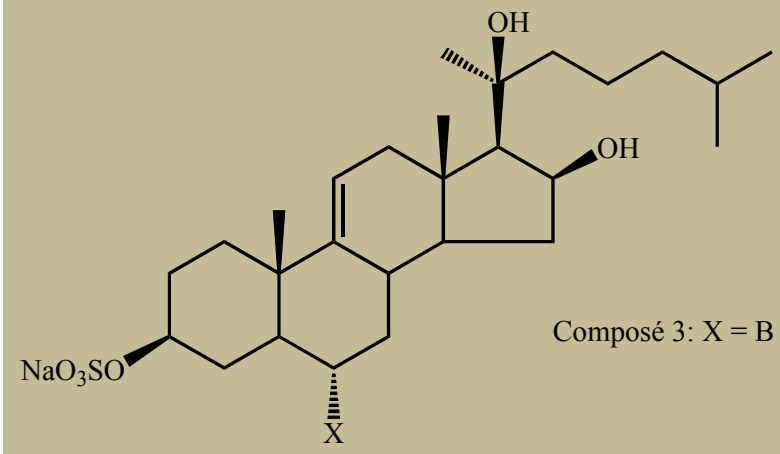
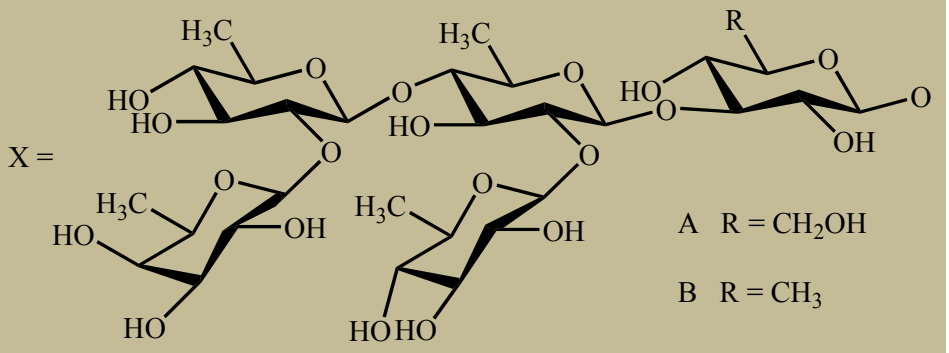
Tableau 3. Les molécules actives des étoiles de mer

Espèces (nom latin) et Biomolécules	Intérêt
***Luidia quinaria Psilaster cassiope* (107)** **Luidiaquinoside et psilastéroside (Astérosaponines)**   Luidiaquinoside  Psilasteroside	Molécules légèrement cytotoxiques contre les cellules sanguines de types leucocytes.

***Acodontaster conspicuus* (108)** **Stéroïdes oligoglycosides et polyhydroxystéroïdes (les plus actifs)** 	19 stéroïdes, dont la moitié sont capables d'inhiber la croissance de bactéries marines.
***Allostichaster inaequalis* (109)** **Glucosylcéramides** 	Les céramides peuvent être utilisés dans certaines crèmes cosmétiques réparatrices.

***Culcita novaeguineae* (110)** **Astérosaponines**  	Molécules cytotoxiques contre les cellules cancéreuses K-562 (leucémie humaine) et BEL-7402 (hépatome humain)

***Novodinia antillensis* (111)** **Saponines astéroïdales**  	Le composé, identifié comme le NaV1.8 (NaV1.8, fait partie d'une famille d'inhibiteurs de canaux sodiques potentiel dépendants) avec IC_{50} = 9 µM.
***Anthenea chinensis* (112)** **Glycosides polyhydroxystéroides**  	Cytotoxiques puissants contre la leucémie humaine K-562, l'hépatome BEL-7402 et le glioblastome, U87MG promouvant l'activité de cellules exposées à la polymérisation de la tubuline.

***Archaster typicus* (113)** **Astérosaponines**  Composé 1: R = CH_2CH_3 X = A Composé 2: R = CH_3 X = A  Composé 3: X = B X =  	Cytotoxique contre HeLa (carcinome cervical humain) et celui de l'épiderme de souris JB6 P+ Cl41.

Chapitre II

Cytotoxicity on Human Cancer Cells of Ophidiacerebrosides Isolated from the African Starfish *Narcissia canariensis*

Activité cytotoxique sur les cellules cancéreuses humaines d'Ophidiacerebrosides isolés de l'Étoile de mer africaine *Narcissia canariensis*

Article publié dans le journal *Marine Drugs* (numéro spécial *Marine Lipids*)

Cytotoxicity on Human Cancer Cells of Ophidiacerebrosides Isolated from the African Starfish *Narcissia canariensis*

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2.1. Abstract

The starfish *Narcissia canariensis* harvested from the coasts off Dakar, Senegal, was investigated for glycolipids (GL). This report deals with the isolation, characterization and biological activity of a fraction F13-3 separated from the GL mixture and selected according to its ability to inhibit KB cell proliferation after 72 hours of treatment. Firstly, a GL mixture F13 was obtained that accounted for 1.36% of starfish biomass (dry weight) and 0.36% of total lipids. The fraction F13-3 obtained from F13 contained three homologous GL identified as peracetylated derivatives on the basis of chemical and spectroscopic evidence. These contained a *β*-glucopyranoside as sugar head, a 9-methyl-branched 4,8,10-triunsaturated long-chain aminoalcohol as sphingoid base and amide-linked 2-hydroxy fatty acid chains. The majority (63%) had an amide-linked 2-hydroxydocosanoic acid chain and was identified as the ophidiacerebroside-C, firstly isolated from the starfish *Ophidiaster ophidiamus*. The minor components of F13-3 differed by one more or one less methylene group, and corresponded to ophidiacerebroside-B and -D. We found that F13-3 displayed an interesting cytotoxic activity over 24 hours on various adherent human cancerous cell lines (multiple myeloma, colorectal adenocarcinoma and glioblastoma multiforme) with an IC_{50} of around 20 μM.

Keywords: glycosylceramides; human cancer cell lines; *in vitro* anticancerous activity; *Narcissia canariensis*; starfish

2.2. Résumé

L'étoile de mer *Narcissia canariensis* récoltée sur les côtes de Dakar (Sénégal) a été examinée pour ses glycolipides (GL). L'isolement, la caractérisation et l'activité biologique d'une fraction F13-3 séparée du mélange de GL a été choisie pour sa capacité à inhiber la prolifération des cellules KB après 72 heures de traitement. La première fraction F13 obtenue représente 1.36 % de la biomasse de l'étoile de mer (poids sec) et 0.36 % de lipides totaux. La fraction F13-3 obtenue de F13 contient trois GL homologues identifiés sous forme peracétylée à partir des données spectroscopiques. La partie sucre est un ß-glucopyranoside, la chaîne longue est tri-insaturée en 4, 8, 10 et possède une ramification méthyle en 9. L'aminoalcool est une base sphingoïde à chaînes d'acides gras 2-hydroxylés. L'Ophidiacerebroside-C, isolé la première fois de l'étoile de mer *Ophidiaster ophidiamus* a été retrouvé chez *Narcissia* et possède en majorité (63 %) l'acide 2-hydroxydocosanoïque lié en amide à l'aminoalcool. Les composants mineurs de F13-3 diffèrent par un groupe méthylène en plus ou en moins et correspondent aux à l'Ophidiacérébrosides B et D. Nous avons constaté que F13-3 possède

une activité cytotoxique intéressante sur des lignées cellulaires cancéreuses humaines (myélome multiple, carcinome colique et gliobestome multiple) avec une CI_{50} de 20 μM.

Mots-clés : Glycosylcéramides ; lignées de cellules cancéreuses humaines ; activité *in vitro* anti-prolifération ; *Narcissia canariensis* ; étoile de mer

2.3. Introduction

Nowadays, myeloma, glioblastoma and carcinomas are a real public health problem in the world with increasing mortality rates in developing countries.

Multiple myeloma (MM) is a cancer of the white blood cells known as plasma cells; it is characterized by skeletal destruction, renal failure, anemia and hypercalcemia. Despite progress in the management of patients, MM remains an incurable disease, with a five-year survival rate not exceeding 50%. Recent advances in the understanding of the pathobiology of multiple myeloma (MM) have provided the basis for a more comprehensive effort to develop novel therapies for this disease (114, 115). As myeloma cells develop mechanisms of resistance to most known treatments (116), the search for new efficient anti-cancerous compounds is needed.

Glioblastoma is the most common and most aggressive type of primary brain tumor in humans, involving glial cells. Glioblastoma multiforme (GBM) is the highest grade glioma (grade 4) tumor and the most malignant form of astrocytomas. In most European and North American countries, incidence is approximately 2–3 new cases per 100,000 people per year. This malignancy of the central nervous system is fatal despite treatment with surgery and adjuvant therapy. In the United States, GBM occurs at a frequency of approximately 5,000 cases annually, and constitutes up to 80% of all malignant gliomas. Long-term control of these tumors is rarely achieved, despite surgical resection and external beam radiation therapy, and GBM recurs within 6–10 months with a median survival of approximately 12 months (117).

Carcinomas are invasive malignant tumors consisting of transformed cells arising from epithelial origin. Epithelial cells cover the external surface of the body, line the internal cavities and form the lining of glandular tissues. Carcinomas are classified by their histopathogical appearance referring to the putative cell of origin or primary organ. With more than 600,000 deaths worldwide per year, colorectal carcinoma is the fourth most common form of cancer in the United States, while approximately 36,000 persons will be newly diagnosed with oral cancer in 2010 (118).

Glycosphingolipids (GSL) are ubiquitous membrane constituents in animals, which play a fundamental role in major phenomena such as cell-cell recognition and antigenic specificity (119, 120). In general GSL exhibit a wide range of biological functions that might be related to the amphipathic nature of the molecule. Several GSL and other various glycolipids (GL) have been isolated from a number of marine sources, mainly including sponges and echinoderms. Glycolipids

are known to possess immunomodulating and antitumor activity in particular those isolated from sponges (119-122). Among them, GSL represent a large group of biomolecules containing two basic structural units: a sugar linked to a ceramide. The hydrophobic ceramide portion involves a sphingoid base and an amide-linked fatty acyl chain. Various cerebrosides, glycosylceramides with a single sugar head, were isolated from sponges (119-122), tunicates (123), octocorals (124), and echinoderms (125-138). The phylum Echinodermata comprises approximately 7,000 living species and their cerebrosides in particular have been chemically studied but little is known regarding their biological and pharmacological properties.

In the search for new efficient GL against cancer, we investigated a not yet studied starfish, *Narcissia canariensis*, harvested off Dakar, Senegal. This paper reports on the isolation of a GL fraction containing particular GSL named ophidiacerebrosides and the evaluation of their cytotoxic activity against various human cancer cell lines.

2.4. Results and Discussion

2.4.1. Glycolipid Isolation and Structure Determination

The common African starfish *Narcissia canariensis* was investigated for lipids and GL fractions. The lipid extract (8.97 g) obtained with CH_2Cl_2/MeOH was subjected to lipid class separation by column chromatography affording a crude GL fraction (0.92 g). A subsequent column chromatography enabled obtaining a bioactive GL fraction named F13 selected for its ability to inhibit KB cell proliferation significatively. The F13 fraction was in turn subjected to a further preparative chromatography that enabled obtaining a purified GL fraction named F13-3 as a white amorphous powder. It showed a single spot on thin layer chromatography with an Rf value similar to that of a commercial standard galactocerebroside with a hydroxylated acyl chain. Interestingly, fraction F13 represented 1.36% of the starfish biomass (dry weight), 0.36% of total lipids and 3.58% of the total GL. Fraction F13-3 contained a major GSL (63%) and two minor homologous ones as shown by NMR and mass spectrometry studies. Thus, the peracetylated F13-3 exhibited the characteristic signals of a sphingoid base and a *β*-glucopyranose in the ^{1}H-NMR spectrum (Fig. 37, Table 4). Its electrospray ionization mass spectrometry (ESI) showed three molecular ion peaks, corresponding to three glycosylceramides with three different *a*-hydroxylated fatty acyl chains. Indeed, the peracetylated major GSL component displayed an adduct ion $[M + Na]^+$ at m/z 1084.6880 (high resolution ESI) in accordance with the formula $C_{59}H_{99}NO_{15}Na$ (a molecular mass of 809.7 amu for

the intact GSL). The minor GSL of peracetylated F13-3 displayed sodiated molecular ions at *m/z* 1070.6702 and 1098.7051 in accordance with a methylene less or more than the major one. The structure of the major cerebroside was determined on the basis of chemical and spectroscopic evidence. Thus, this glycosylceramide contains a triunsaturated long-chain aminoalcohol as the principal sphingoid base. The optical rotation value of peracetylated F13-3 was determined as $[\alpha]_D^{30}$ = +0.44 (*c* = 0.4, CH_2Cl_2).

Figure 37. Glucosylceramides for *Narcissia canariensis*: Ophidiacerebroside-B (*n* = 16), ophidiacerebroside-C (*n* = 17) and ophidiacerebroside-D (*n* = 18)

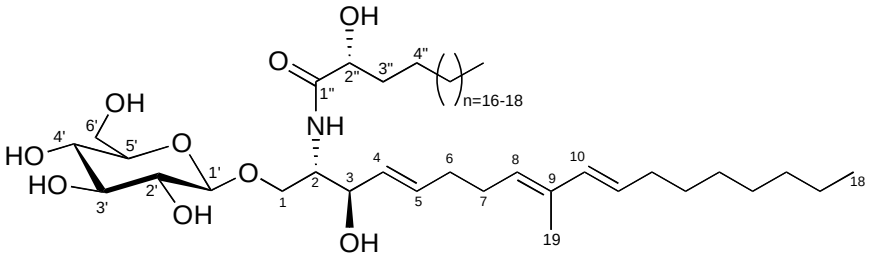


Table 4. ^{1}H, ^{13}C and COSY NMR spectroscopy data for peracetylated F13-3 glycolipids in $CDCl_3$

Position	δ_H ppm, mult., *J* in Hz	δ_C ppm	COSY correlation
NH	6.35 (d, *J* = 9.0)	--	
1a	3.95 (dd, *J* = 10.0/3.8)	67.2	2, 1b
1b	3.62 (dd, *J* = 10.0/3.8)	67.2	2, 1a
2	4.32 (m)	72.7	1a, 1b, 3, NH
3	4.27 (d, *J* = 4.6)	50.6	4, 2
4	5.34 (m)	128.7	3, 5
5	5.43 (dd, *J* = 6.5/15.0)	124.8	4, 6
6	2.07 (s)	32.3	5, 7
7	2.20 (s)	31.9	6, 8, 19
8	5.83 (m)	136.4	7
9	--	134.2	
10	6.04 (d, *J* = 15.4)	134.4	11
11	5.59 (m)	127.8	10, 12
12	2.10 (m)	32.9	11, 13
19	1.73 (s)	12.5	7, 8
CH_2	1.27 (m)	22.7–29.7	
CH_3 acetates	2.02/2.04/2.05/2.08/2.11/2.19 (6s)	20.6–21.0	
C=O acetates		169.3/169.4/169.6/160.7/	

		169.8/169.9	
terminal CH_3	0.90 (t, J = 6.8)	14.1	
1’	4.49 (d, J = 7.9)	100.5	2’
2’	4.97 (dd, J = 8.0/9.5)	71.2	1’, 3’
3’	5.20 (t, J = 9.5)	72.7	2’, 4’
4’	5.10 (t, J = 9.7)	68.2	3’, 5’
5’	3.71 (m)	71.9	4’, 6’a, 6’b
6’a	4.24 (d, J = 4.5)	61.8	5’, 6’b
6’b	4.16 (dd, J = 12.5/2.3)	61.8	5, 6a
1”	--	172.2	
2”	5.36 (m)	73.0	
3”	1.83 (m)	31.8	
4”	1.40 (m)	29.5	
terminal CH_3	0.83 (m)	14.1	

All the chemical shifts of the ceramide are given in the ^{13}C and ^{1}H NMR spectra (Table 4). The sugar linked to the ceramide was identified as glucose by NMR spectroscopy (Fig. 38, 39). First of all, the anomeric proton of the β-glucopyranoside (δ = 4.49, d, J = 7.9 Hz) was correlated with the anomeric carbon at δ = 100.5 ppm in the HMQC spectrum. Starting from this proton, all the ^{1}H and ^{13}C NMR signals of the sugar were assigned by using the COSY, HMQC and HMBC spectra, and the vicinal proton-proton coupling constants were determined (Table 4). The *gluco* configuration of the sugar, as well as its β anomeric configuration, was established on the basis of the ring proton coupling constants ($J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.5 Hz, $J_{3,4}$ and $J_{4,5}$ = 9.6 Hz). The linkage of the glucopyranoside to the ceramide was confirmed by the three bond ^{13}C-^{1}H couplings of anomeric C-1’ with H-1a and H-1b observed in the HMBC spectrum. The presence of signals for five olefinic protons and the singlet at δ = 1.73 ppm indicated that a methyl branch is linked to an olefinic carbon atom. The unsaturation pattern in the ^{1}H NMR spectrum showed a multiplet at 5.34 ppm and a double doublet at 5.43 ppm, characteristic of a Δ^4 sphingosine with *trans* configuration (J = 15.0 Hz), a doublet at 6.04 ppm (J = 15.4 Hz) for a *trans* Δ^{10} double bond, and multiplets at δ 5.59, 5.83 ppm. COSY correlations were observed between H-7 and H-8 and H-19 (Table 4). Further correlations were observed between olefinic signals at δ 5.83 (H-8) and 5.59 (H-11), and vicinal methylene groups at positions 7 and 12. And also we observed no correlation between the olefinic carbon C-9 and an olefinic proton in the HSQC spectrum. The key HMBC correlations from H_3-19 to C-8, C-9, and C-10, and from C-11 to C-9, confirmed the location of the positions of the double bonds. These data allowed us to establish the olefinic pattern of the sphingoid unit as a 9-methyl-4, 8, 10-triene.

To determine the structure of the ceramide, F13-3 was subjected to an acidic methanolysis and the resulting reaction mixture was separated by partitioning between CH_2Cl_2 and H_2O/MeOH into an aqueous phase containing methylglycosides and an organic phase containing 2-hydroxylated fatty acid methyl esters (FAME) and sphingoid bases. Thereafter the latter mixture was analyzed by GC/MS. Only one sphingoid base was observed.

The FAME mixture from the active fraction F13 was analyzed by GC-MS (Fig. 40). The 2-hydroxy FAME produced the characteristic ions at *m/z* 90 (McLafferty) and *m/z* 103. The results were as follows:

2-OH-21:0, t_R = 33.9 min (15.4%), *m/z* 356 (M^+); 2-OH-22:0, t_R = 36.4 min (63.6%), *m/z* 370 (M^+); 2-OH-23:0, t_R = 38.8 min (21.0%), *m/z* 384 (M^+). These fatty acid structures were confirmed by GC-MS analysis of *N*-acyl pyrrolidides showing fragment ions at *m/z* 129 (McLafferty) and the expected molecular ions. The peracetylated methylglycoside from F13-3 was analyzed by GC-MS (Fig. 41) [column temperature 110 °C (2 min) and then (temp. increasing at 3 °C/min until 240 °C)]; t_R = 31.6 min (methylglucopyranoside) similar to that of an authentic sample. Other diagnostic ions were observed at *m/z* 331 $(M\text{-}OMe)^+$, 303 $(M\text{-}OAc)^+$, 243, 200, 157, 145 and 115.

These data showed that F13-3 contained a ceramide composed of the known 4, 8, 10-triunsaturated, 9-methyl branched C_{18} sphingoid base and 2-hydroxylated fatty acyl chains like cerebrosides isolated from other invertebrates (121, 123, 124) including starfish (129, 132, 133, 135, 138).

2.4.2 Cytotoxic Activity

The cytotoxic activity of F13-3, including Ophidiacerebroside-C as major component, was detected and followed using KB cells (human oral epidermoid carcinoma) (IC_{50}: around 20 µM after 72 h of treatment). Thereafter it was investigated on three human cancerous cell lines, KMS-11 (adherent plasma cells obtained from patients with multiple myeloma (139)), GBM (astrocytoma cells obtained after tumor resection of patients with glioblastoma multiforme-primary culture (140)), and HCT-116 (colorectal adenocarcinoma cells derived from a patient with Lynch's syndrome (141) and as described in the experimental section). Results are shown in Table 5.

Table 5. IC_{50} measures for F13-3 (μM ± S.E., 24 h of treatment)

IC_{50}	KMS-11	HCT-116	GBM
F13-3	15.2 ± 4.0	18 ± 3.9	34.6 ± 5.1

The activities observed, mainly on KMS-11 and HCT-116, are interesting as ophidiacerebrosides have not yet been evaluated on human cancer cells. Cytotoxicity on these three cell lines was already found in the same range of concentration for some synthetic bile acid derivatives (LD_{50}: 8.5 μM) in a recent study (142). A mixture of ophidiacerebrosides with C_{20} to C_{24} 2-hydroxyacyl chains, including the major ophidiacerebroside-C with an acyl chain C_{22} occurring at 40%, has been described to display strong cytotoxicity against L1210 murine leukemia cells *in vitro* (129). Cerebrosides isolated from a tunicate, phalluside-1 and -2 (Fig. 42), contain the same triunsaturated sphingoid base and sugar head, but they differ in 2-hydroxyacyl chain lengths, C_{16} and C_{18} respectively. Interestingly, the latter cerebrosides were found inactive against human cancer cells including lung carcinoma (A 549), colon carcinoma (HT 29), and melanoma (MEL 28) (123). In addition, cerebrosides named renierosides with the same sphingoid base and various monounsaturated 2-hydroxylated fatty acyl chains were found inactive against five human solid tumor cell lines (121). These results suggest that the nature of the 2-hydroxylated fatty acyl chain (chain length and possible double bonds) seems to be important for the cytotoxic activities of this type of cerebrosides. Recently, it was shown that the nature of the sugar residue may be relevant for the biological activity of this type of GSL; those with glucopyranosides showing stronger cytotoxicity than those with galactocerebrosides (138). Due to its potential biological interest, phalluside-1 found in the ascidian *Phallusia fumigata* (123), the sea stars *Allostichaster inaequalis* (133) and *Cosmasterias lurida* (132), has recently been recently synthesized (143).

In conclusion, this study provides an additional source (another starfish) for ophidiacerebrosides and points out the potential of these compounds against human cancerous cells. It would be of interest to investigate other GL fractions of *N. canariensis* for glycosylceramide isolation, in particular those with the same sphingoid base but differing by 2-hydroxylated acyl chain length and to compare their cytotoxic activities using the same panel of human cancer cell lines.

2.5. Experimental Section

2.5.1. General Procedures

High resolution electrospray ionization mass spectrometry (HR-ESI-MS, positive mode, ion-source acceleration 4.5 kV, ion-source temperature 200 °C, methanol as solvent) mass spectra were recorded with a Micromass Zab Spec Tof spectrometer. ^{1}H- and ^{13}C-NMR as well as 2D-NMR spectra were obtained on a NMR Bruker Avance-500 spectrometer with triple Probe TBI multinuclear in $CDCl_3$ at 500.13 MHz and 125.76 MHz respectively, with reference to an internal standard of tetramethylsilane. Chemical shifts and coupling constants were expressed in δ (ppm) and Hz respectively. GC-MS spectra were performed on a Hewlett-Packard 6890 gas chromatograph with a mass selective detector MS HP 6890 MS, Little polar column DB-1, 60 m length × 0.25 mm i.d. × 0.25 μm phase thickness. The temperature of the column was varied, after a delay of 2–4 min from the injection, from 110 to 310 °C with a slope of 3 °C.min^{-1}. Optical rotations were measured in CH_2Cl_2 solutions with a Polartronic NH8 Schmidt/Haensch polarimeter at 30 °C. Analytical TLC was performed on precoated silica gel F_{254} plates. After development, the dried plates were sprayed with 50% H_2SO_4-vanillin and orcinol reagents.

2.5.2. Animal Material

The starfish *Narcissia canariensis*, shown below in Figure 43 (photo taken by Dr. Patrice Petit de Voize, Dakar) is found on rocks along the Senegalese coasts off Dakar, at a depth range of 23–38 m and were collected by hand during a scuba diving expedition organized by Oceanium of Dakar in 2009, on the sites named Petit Seminole, Fayss and Thi Wa. The specimens were identified by Professor Peter Wirtz, University of Madeira, Portugal.

Figure 43. *Narcissia canariensis*

2.5.3. Lipid Extraction and F13-3 Isolation

Whole bodies of the collected specimens (241.37 g dry weight) were chopped and twice extracted with CH_2Cl_2/MeOH (1:1, vol/vol) at room temperature. The combined extracts were concentrated *in vacuo* to give the crude extract, which was partitioned between H_2O and CH_2Cl_2/MeOH. The organic layer was concentrated *in vacuo*, and the residue (8.97 g, 3.7%) was chromatographed on silica gel column with pure solvents as successive eluents: Dichloromethane (neutral lipids, 6.30 g), acetone (GL, 0.92 g) and methanol (phospholipids, 1.68 g). The GL mixture was separated on silica gel column to give 14 fractions. Among them, fraction 1 was subjected to a silica gel column chromatography (CH_2Cl_2/MeOH, 95:5 to 80:20, vol/vol) affording 23 fractions. From the latter fractions, fraction 13 (F13, 33 mg) gave a positive test on KB cells, and presented a similar polarity to a commercial standard (galactocerebroside with 2-hydroxy fatty acyl chain type I) (Rf = 0.35 on silica gel thin layer chromatography, CH_2Cl_2/MeOH, 88:12, vol/vol). Then F13 was subjected to silica gel chromatography with a solvent system of CH_2Cl_2 with 5% to 15% MeOH vol/vol) to give seven fractions. The following fraction 3 (21 mg), designated as F13-3, was obtained as a white amorphous powder and was used for biological studies. In order to determine the chemical structure, fraction F13-3 was peracetylated and studied by NMR and ESI-MS.

2.5.4. Acetylation of F13-3

A part of F13-3 (9 mg) was dissolved in 1 mL of acetic anhydride and some drops of dry pyridine. The reaction was allowed to proceed for 18 h in darkness at room temperature, and then the reaction mixture was partitioned between water and dichloromethane. The organic layer was washed with HCl 1 M, neutralized with a Na_2CO_3 solution, and dried on anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was weighed.

2.5.5. Methanolysis of F13-3

A part of F13 (3 mg) was heated with 0.9 mL of MeOH/H_2O/HCl (29:4:3, vol/vol/vol) at 80 °C for 18 h. The reaction mixture was extracted with H_2O/CH_2Cl_2 (3:9, vol/vol), the aqueous layer was concentrated to give methylglycosides, whereas the organic layer contained a mixture of fatty acid methyl esters (FAME) and sphingoid bases. A part (1/3) of the FAME was preserved, the other one was transformed into *N*-acylpyrrolidides (NAP) by heating in a mixture pyrrolidine/acetic acid (10:2, vol/vol, 1 mL) during 1 h at 85 °C. The reaction mixture was separated with H_2O/CH_2Cl_2 and the

organic layer was dried on anhydrous Na_2SO_4, filtrated and weighed after solvent evaporation. The aqueous layer was neutralized by NaOH 1 M and extracted twice with diethyl ether. The organic layer, containing sphingoid base was dried and then acetylated. The aqueous layer containing methylglycosides was evaporated *in vacuo* and then acetylated before GC-MS analysis.

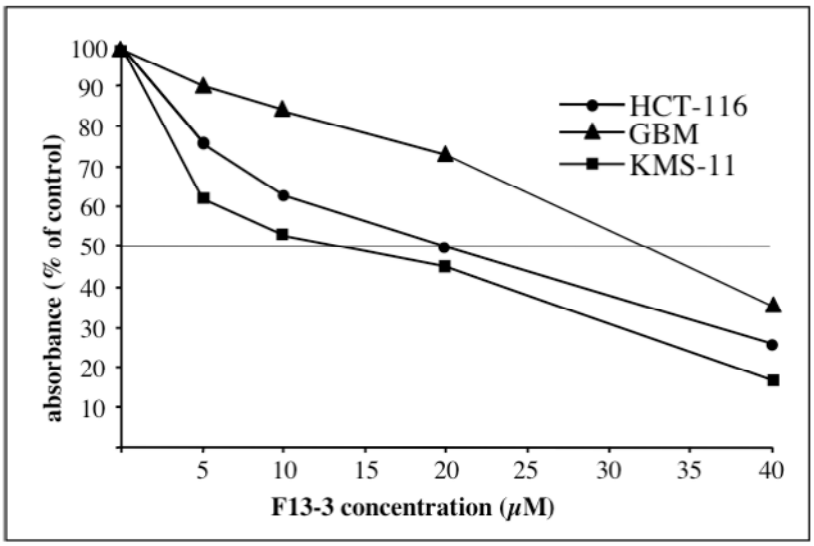
2.5.6. Cell Cultures

Cells were cultured in RPMI 1640 medium (KMS-11, GBM, HCT-116) or BME (KB) supplemented with 10% foetal calf serum, 2 mM glutamine, antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) (Life Technologies). Cells were subcultured at confluency after dispersal with 0.025% trypsin in 0.02% EDTA. Cells were maintained in plastic culture plate at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments cells were used at 70–80% confluency.

2.5.7. Neutral Red Assay

For cytotoxicity tests, 20,000 cells (GBM, HCT-116) and 50,000 cells (KMS-11) (200 μL) were plated in 96-well culture microtiter plates (Falcon) and incubated at 37 °C in 5% CO_2. After 24 h, drugs were added in 50 μL fresh medium, then after 21 h cells were loaded for 3 h with neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) (Sigma-Aldrich, St Quentin, France) at a final concentration of 50 μg/mL in culture medium. Thereafter (24 h of treatment) the medium was removed, cells were fixed for 5 min with a mixture of 1% formaldehyde-1% $CaCl_2$ and the dye extracted with 0.2 mL of 1% acetic acid in 50% ethanol. Plates were left overnight at 4 °C and absorbance was recorded at 570 nm (Multiskan EX-Thermo-Electron Corporation). Experiments were performed at least in triplicate, 4 wells per F13-3 concentration being used. IC_{50} (inhibition of cell viability of 50%) values were calculated from the dose-response curves, an example is given in Figure 44. Statistics: Values are expressed as the mean of three independent experiments ± standard error.

Figure 44. Dose-response curve of F13-3 (24 h of treatment)



2.5.8. MTT Assay

After trypsinization KB cells were suspended as a 200,000 cells/mL suspension and 50 µL were dropped in each well of 96-well microplates (Costar, Corning, NY, U.S.). Tests were performed once the cells had settled at the bottom of the wells (48 h cultures) by incorporating 50 µL of test solutions. After 72 h of incubation, cell viability was determined using the colorimetric MTT assay according to Denizot and Lang (144). This test was mainly used for the detection and follow-up of active fractions in the course of purification.

2.6. Acknowledgements

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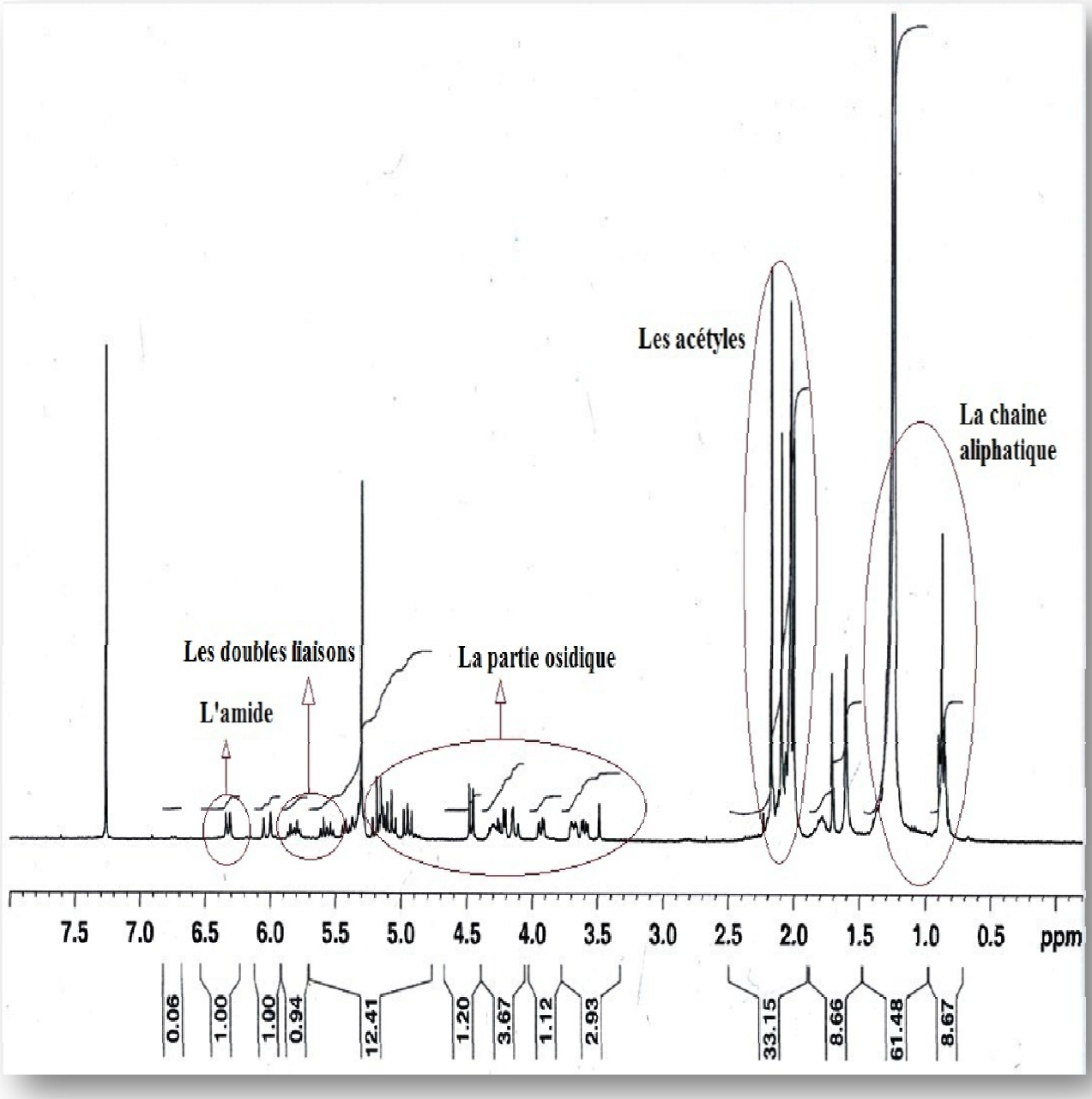

Figure 38. Spectre RMN ^{1}H de F13-3 peracétylé dans $CDCl_3$

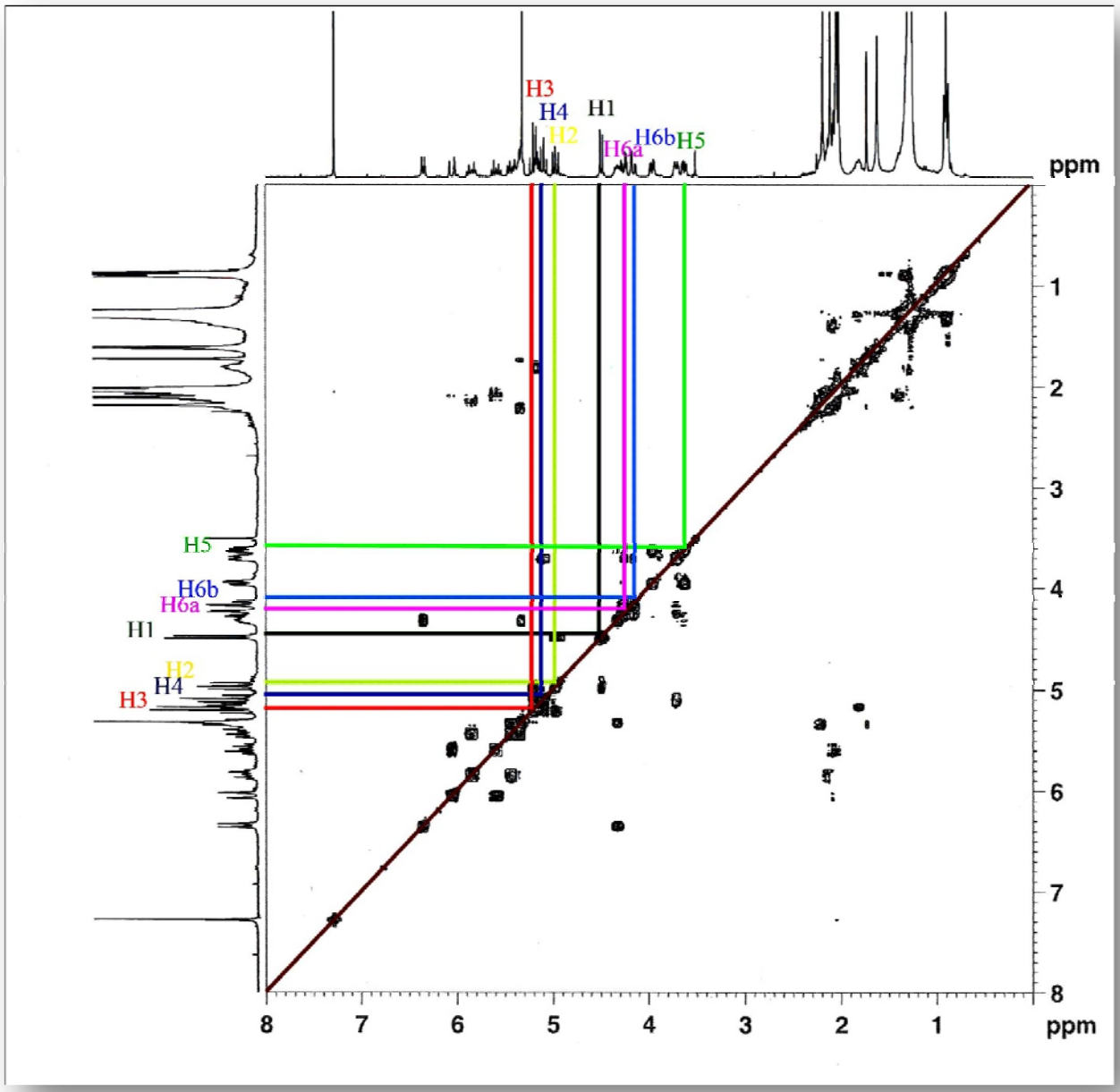

Figure 39. Zone de corrélation des protons du sucre (COSY) de F13-3

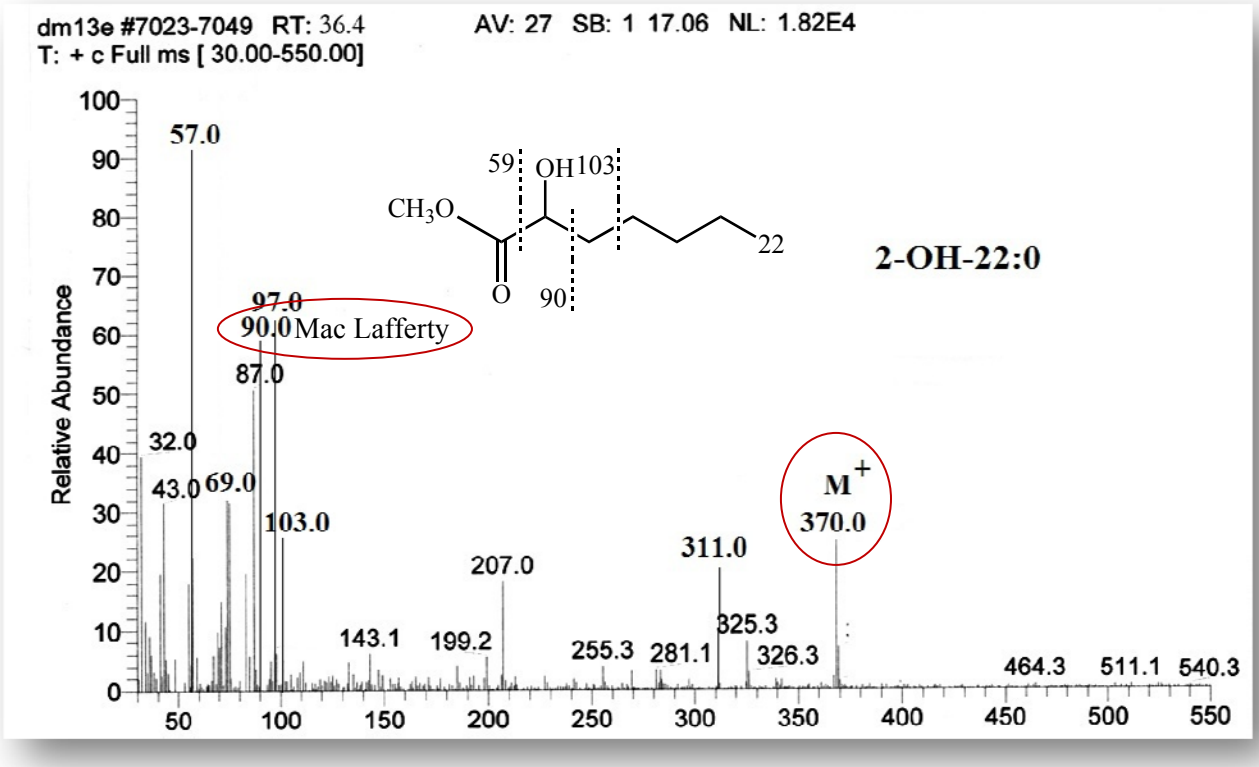

Figure 40. Spectre de l'EMAG de F13-3

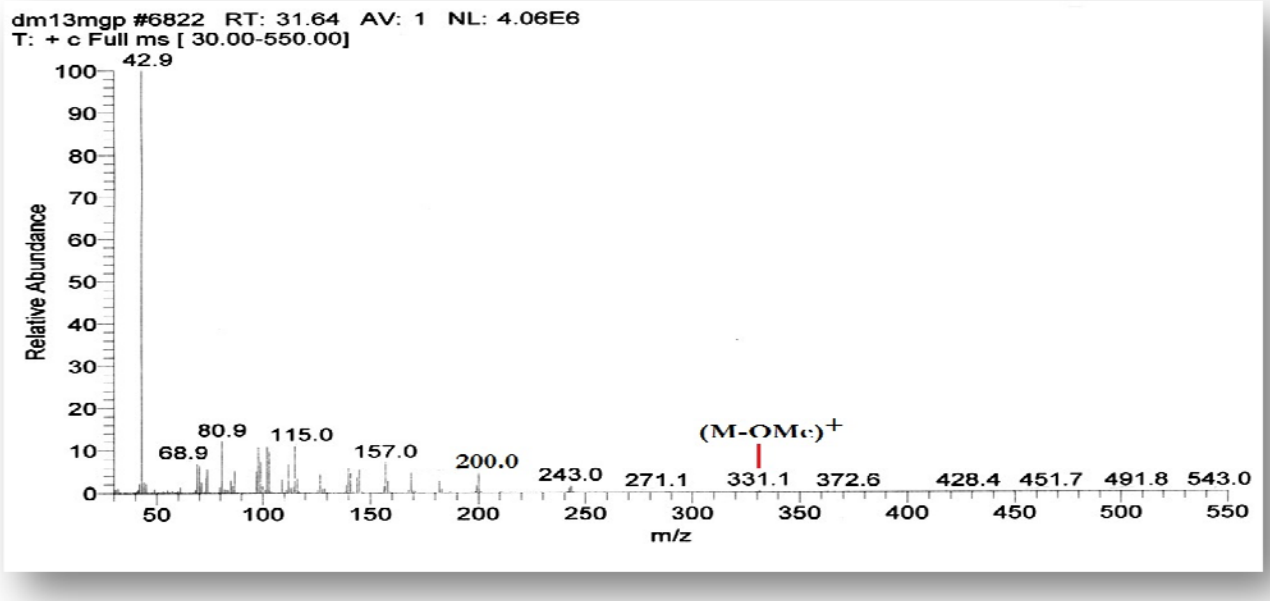

Figure 41. Spectre de méthylglycosides de F13-3

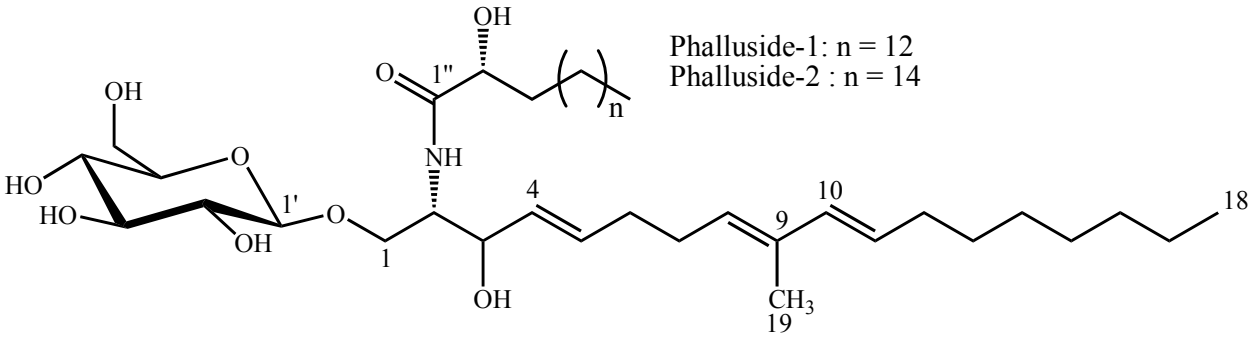

Figure 42 : Phalluside-1 et 2 de *Phallusia fumigata*

Chapitre III

Antiproliferative activity against human non-small cell lung cancer of two *O*-alkyl-diglycosylglycerols from the marine sponges *Myrmekioderma dendyi* and *Trikentrion laeve*

Activité antiprolifération contre les cellules humaines du cancer du poumon non à petites cellules de deux *O*-alkyl-diglycosylglycérols des éponges marines *Myrmekioderma dendyi* et *Trikentrion laeve*

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Antiproliferative activity against human non-small cell lung cancer of two *O*-alkyl-diglycosylglycerols from the marine sponges *Myrmekioderma dendyi* and *Trikentrion laeve*

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3.1. Abstract

Glycolipids of *Myrmekioderma* sponges contain Myrmekiosides, a new family of glycolipids with a unique structure of mono-*O*-alkyl-diglycosylglycerols. This report deals with the identification and biological activity of the new Myrmekioside E from *M. dendyi*. Its structure has been elucidated from spectroscopic data and chemical degradation studies. It contained a glycerol backbone linked to xylose and *N*-acetylglucosamine, and an alkyl long-chain with a terminal alcohol group. A related glycolipid, Trikentroside, known in the sponge *Trikentrion laeve,* was subjected to a comparative biological evaluation. Both glycolipids inhibit proliferation of two human non-small cell lung cancer cell lines (NSCLC-N6 and A549).

Keywords: alkyldiglycosylglycerols; glycolipids; human lung cancer; marine sponges; Myrmekioderma; Trikentrion.

3.2. Résumé

Les glycolipides d'éponges *Myrmekioderma* contiennent des Myrmékiosides, une nouvelle famille de glycolipides avec une structure unique de mono-*O*-alkyl-diglycosylglycérols. Nous avons pu en identifier un nouveau, Myrmekioside E, de *M. dendyi*. Sa structure a été élucidée par les données spectroscopiques et des études de dégradation chimique. Il contient une unité de base glycérol liée à un xylose et à une *N*-acétylglucosamine, et à une chaîne longue alkyle avec un groupe alcool terminal. Le Trikentroside, autre éther de glycérol glycosylé de l'éponge *Trikentrion laeve*, a été soumis à une évaluation biologique comparative. Ces glycolipides inhibent la prolifération de deux lignées cellulaires de cancer de poumon humain (NSCLC-N6 et A549).

Mots-clés : alkyldiglycosylglycérols ; glycolipides; cancer du poumon humain ; éponges marines; *Myrmekioderma* ; *Trikentrion*.

3.3. Introduction

Lung cancer is the leading cause of cancer-related mortality in both men and women in the world. The prevalence of lung cancer is the second only after that of the prostate cancer in men and breast cancer in women. Lung cancer recently surpassed heart disease as the leading cause of smoking-related mortality. Most lung carcinomas are diagnosed at an advanced stage, conferring a poor prognosis. Lung cancers are generally divided into small cell lung cancer and non-small cell lung cancer (NSCLC). Non-small cell lung cancer accounts for approximately 85% of all lung cancers (145).

Marine organisms represent an interesting source of new molecules potentially useful as therapeutic agents. Thus, glycolipids (GL) are known to possess immunomodulating and antitumor activity, especially those from marine organisms (120, 122, 146, 147). They are known to play fundamental roles in major phenomena such as cell-cell recognition and antigenic specificity. Thus, marine invertebrates have been proven to be a source of promising bioactive GL. The most frequent encountered GL are glycosphingolipids, which display a wide range of biological functions that might be related to the amphipathic nature of the molecule (120, 122, 146, 147). Various cerebrosides, glycosylceramides with a single sugar moiety, were isolated from sponges (120, 122, 146) and echinoderms like starfishes (147).

With the aim of discovering new and biologically-active GL from marine sponges, two additional genera were investigated, namely *Myrmekioderma* and *Trikentrion*. Several bioactive compounds were already isolated from *Myrmekioderma* and *Trikentrion* species. Several new terpenic products have been reported from *Myrmekioderma* sponges, e.g. *M. styx,* with various biological activities including those against hepatitis, HIV, tuberculosis, and also antifouling and hemagglutinating activities. In particular, cytotoxicity was shown against human primary tumor cells such as P-388 and A-549 cancer cell lines (148-155). The sponge *Trikentrion laeve* (= *loeve*) contained two new compounds: the Trikentramine and the anti-HIV-1 pigment Trikentriol, whereas *T. flabelliforme* contained the new antimicrobial indolic Trikentrins (156-158). The ether-linked GL, Myrmekiosides A and B, isolated from *Myrmekioderma* sp. were able to reverse the phenotype of melanoma H-ras transformed NIH3T3 cells (159).

They contained two glucoses and one xylose as the sugar moiety and an *O*-alkyl chain. Our previous work on polar lipids of *M. dendyi* showed that phospholipids accounted for about 15.5% of

total lipids (160) and that the ether-linked Myrmekiosides C and D isolated from the same sponge showed an antitumor activity on THP1 cells (161). Two new glycolipid ethers named Myrmekiodermins were isolated from the n-BuOH extract of *M. dendyi*, among them one had a methyl branched alkyl chain (162). The location of the methyl branch was not determined and no biological property was reported for the latter compounds. It is noteworthy, to the best of our knowledge, that such unusual alkyl-diglycosylglycerol GL structure was only found in the sponge genera *Myrmekioderma* and *Trikentrion* (49, 159, 161, 162).

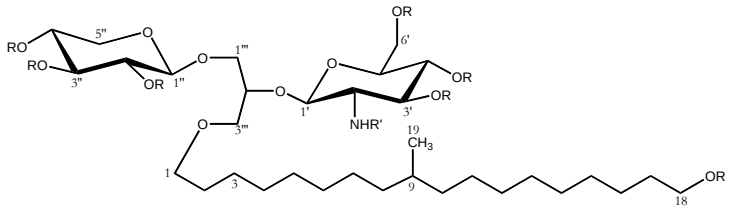
As a part of our continuing search for new efficient marine GL against cancer, we report here the identification of a new active alkyl-diglycosylglycerol from *M. dendyi* harvested from South Pacific. On the other hand, the major ether-linked GL of *T. laeve* was again isolated in order to study its activity.

3.4. Results and discussion

3.4.1. Chemistry

During the investigation of the complex glycolipid (GL) fractions from *M. dendyi*, a new compound was detected. Lipid fractions eluted with acetone and methanol displayed characteristic glycolipid spots on TLC. Among them, the first methanolic fraction, accounting for 3.2% of total lipids and seemed to be potentially interesting to find out GL with unusual structures, taking into account their relatively high polarities. This fraction was acetylated and displayed two spots on TLC. It was subjected to a silica gel column chromatography and then purified by repeated reverse phase HPLC. Two compounds were isolated and the least polar one named Myrmekioside E, accounting for 0.15% of total lipids, was investigated for biological activity. To facilitate we named the natural Myrmekioside E as Myrmekioside E-1, the peracetylated one as Myrmekioside E-2 and the deacetylated one as Myrmekioside E-3. Myrmekioside E-1 showed a quasi-molecular ion (ESI-MS) at m/z 732.4400 ($[M+Na]^+$) in accordance with the molecular formula $C_{35}H_{67}NO_{13}Na$. ^1H NMR spectrum of the Myrmekioside E showed two anomeric protons at δ 4.24 (d, J=5.9 Hz) and δ 4.42 (d, J=7.9 Hz), and signals at δ 6.80 (d, 8.6 Hz) and δ 1.85 (s) revealing the presence of two sugars, one of them being a *N*-acetylated amino sugar, and a long-chain alkyl group indicated by a large signal at δ 1.17. Further NMR experiments were performed using the peracetate derivative to take advantage of the better signal dispersion in the proton spectrum as usually observed. Myrmekioside E-2 showed a quasi-molecular ion at m/z 1026.5250 ($[M+Na]^+$), in accordance with the molecular

formula $C_{49}H_{81}NO_{20}Na$ corresponding to a structure of alkyl-glycosylglycerol with a saturated branched chain possessing a terminal alcohol group (Fig. 45).



Myrmekioside E-1 (natural) : R = H, R' = Ac
Myrmekioside E-2 (acetylated) : R = Ac, R' = Ac
Myrmekioside E-3 (deacetylated) : R = H, R' = H

Figure 45. Myrmekioside E and its peracetylated and deacetylated derivative

Determination of the length of the alkyl chain and location of the methyl branch could not be carried out by NMR analysis and required a controlled chemical degradation. Acid methanolysis afforded his two parts as useful derivatives for gas chromatography-mass spectrometry (GC-MS) analysis: sugar as methylglycosides and *O*-alkylglycerol.

The ^{1}H NMR spectrum of Myrmekioside E-2 (Table 6), exhibited two doublets at δ 4.48 (J = 7.08 Hz) and δ 4.73 (J = 8.4 Hz), identified as the anomeric protons of two sugar residues by their correlation peaks in the HMQC spectrum with the anomeric carbons at δ 100.9. The large coupling constant of these protons in each sugar indicated the β-glycosidic linkage. Using each of these protons as a starting point, examination of the COSY spectrum allowed the sequential assignment for the sugar protons of a xylose and a *N*-acetylglucosamine. The presence of this amide group was indicated by a doublet at δ 5.64 (J = 8.5 Hz) and a singulet at δ 1.88 (acetyl group). Another remarkable signal was a large peak at δ 1.18 due to a long alkyl chain. In addition, a doublet at δ 0.75 (J = 6.39 Hz) was present, corresponding to a methyl attached on this chain. The ^{1}H NMR spectrum also exhibited a signal at δ 1.97 due to 7 *O*-acetyl groups. As only seven acetyl signals were present on the sugar residues, it remained one acetyl group on the alkyl chain (Fig. 46, 47). Furthermore, adjacencies among the three partial structures were revealed by the COSY and HMBC spectra of a xylose, *N*-acetylglucosamine, and an alkyl chain were attached to C-1''', C-2''', and C-3''' of glycerol (Fig. 45).

To determine the structure of the *O*-alkylglycerol, Myrmekioside E-1 was subjected to an acidic methanolysis and the resulting reaction mixture was partitioned between CH_2Cl_2 and H_2O/MeOH to

give an aqueous phase containing methylglycosides and an organic phase containing *O*-alkyl-glycerol. Thereafter the latter mixture was analyzed by GC/MS.

The structure of the sugar residues was confirmed as peracetylated *N*-acetyl-pyranohexosamine and pyranopentose by mass spectral (ESI) data. Ion peaks at *m/z* 259 ($[M\text{-}OCH_3]^+$) and 330 (and *m/z* 362 ($[M+H]^+$), respectively. The other characteristic fragment ions were present at *m/z* 86, 116 and 144 for the peracetylated pyranopentose and at *m/z* 115, 143, and 158 for the peracetylated *N*-acetylpyranohexosamine. The spectrum of the peracetylated *O*-alkylglycerol displayed the fragment ions *m/z* 441 ($[M\text{-}59]^+$), 397, 381, 355, 341, 325 ($[M\text{-}159]^+$), and 159, corresponding to a C_{19} chain with an acetylated terminal alcohol group, and the position of the methyl branch on C-9 was shown by the ion peaks at *m/z* 213 and 185 and those resulting from a loss of acetic acid (Fig. 48, 49).

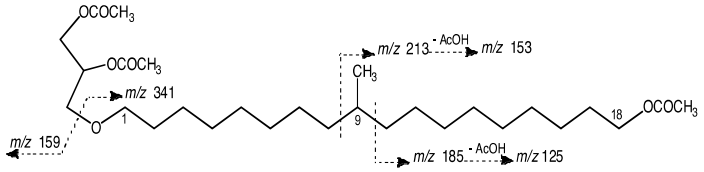


Figure 48. Fragmentation pattern (e.i.) of the peracetylated O-alkylglycerol obtained from Myrmekioside E

The position of the methyl branch on the alkyl chain, observed in the ^1H NMR spectrum, was determined by the study of the fragmentation patterns. The characteristic signals at *m/z* 213 and *m/z* 185 and those resulting from the loss of acetic acid indicated that the methyl group was attached at C-9 in this alkyl chain. The optical rotation value of Myrmekioside E-2 was determined as $[\alpha]_D^{30} = -0.05$ ($c = 0.4$, CH_2Cl_2).

Table 6. ^{1}H, ^{13}C and COSY NMR data of Myrmekioside E-2 in $CDCl_3$ (300 MHz)

Position	δ_H ppm, mult., *J* in Hz	$\delta_C$ppm	COSY correlation
	***N*-acetyl-glucosamine**		
NH	5.64 (d, *J* = 8.5)		2
1'	4.73 (d, *J* = 8.4)	100.9	2
2'	3.78 (m)	54.70	NH,1,3
3'	5.11 (m)	72.85	4
4'	4.99 (t, *J* = 9.6)	68.49	3
5'	3.58 (t, *J* = 5.2)	124.8	2,4,6a,6b
6'a	4.18 (dd. *J* = 12.03/4.8)	63.08	5,6b
6'b	4.08 (dd, *J* = 12.03/2.4)	63.08	5,6a
	Xylose		
1"	4.48 (d, *J* = 7.08)	100.9	2
2"	4.80 (dd, *J* = 7.05/7.95)	71.51	1,3
3"	5.15 (t, *J* = 8.9)	71.90	2,4
4"	4.88 (dd, *J* = 9.0/5.1)	71.39	3,5a
5" axial	3.33 (t, *J* = 6.6)	63.08	5e,4
5" equatorial	4.06 (m)	63.08	5a,4
	***O*-alkyl**		
1	3.98 (d, *J* = 6.9)	64.66	2
2	1.52		1
3-8 et 10-16	1.1 – 1.46	37.08/32.75/29.76/29.69/29.50/ 29.25/28.58/27.04/ 26.19/25.20	
9	1.54 (m)	20.7	
17	1.48	29.69	18
18	3.29 (t, *J* = 11.1)	72.85	17
19	0.75 (d, *J* = 6.39)		
	Glycerol		
1'"a	3.43 (m)	71.51	2
1'"b	3.60 (m)	71.51	2
2'"	3.85 (tt, *J* = 9.97/15.1)	68.49	1a,3a,2b,3b
3'"a	3.43 (m)	71.51	2
3'"b	3.60 (m)	71.51	2
	Acetyl groups		
7-$OCOCH_3$	1.97 (m)	19.66/19.68/20.62/20.7/ 20.75/21.02/21.02	
-$NHCOCH_3$	1.88 (s)	23.25	

Interestingly regarding the product availability, we investigated lipids of *T. laeve* several times and observed that the ether-linked GL Trikentroside was always present (49). The mass spectrum of the peracetylated Trikentroside showed a quasi-molecular ion at *m/z* 965.5441 [(M+Na)$^+$], in accordance with the molecular formula $C_{49}H_{82}O_{17}$ corresponding to the unusual GL which includes a glycerol unit, two xylopyranoses, and a C_{24} alkenyl ether chain and one double bond (49) (Fig. 50). In this work we examine its inhibitory effect on NSCLC-N6 and A549 cell lines.

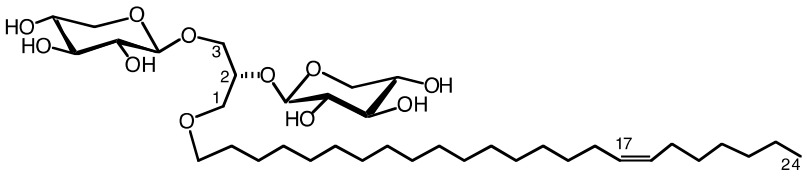


Figure 50. Trikentroside from *Trikentrion leave*

3.4.2. Antiproliferative activity against human non-small cell lung cancer of Myrmekioside E and Trikentroside

Peracetylated Myrmekioside E (Myrmekioside E-2) from *M. dendyi* showed a significant activity on NSCLC-N6 cells with IC_{50} = 7.3 ± 0.2 μM and on lung tumor cells A549 with IC_{50} = 9.7 ± 0.2 μM, and also the native Trikentroside from *T. laeve* showed a significant activity on NSCLC-N6 cells with IC_{50}=12.1 μM (Table 7).

Table 7. IC_{50} values (μM) for Myrmekioside E derivatives and Trikentroside

product	NSCLC-N6	A549
peracetylated Myrmekioside E (E-2)	7.3 ± 0.2	9.7 ± 0.2
deacetylated Myrmekioside E (E-3)	126.3 ± 2.4	Inactive
natural Myrmekioside E (E-1)	61.7 ± 3.4	54.6 ± 1.4
natural Trikentroside	12.1 ± 0.2	--

3.5. Conclusion

This work confirms previous results that showed the interest of ether-linked glycoglycerolipids as potentially useful therapeutic agents (159). Here, we compared the cytotoxicity of Myrmekioside E in three forms with natural Trikentroside, whose structure has been already reported (49). Firstly, having five times more toxicity of the Trikentrioside towards the Myrmekioside E seems to show the importance of the two xyloses. Of this observation, that the nature of the sugar residue appears to be relevant to the biological activity of these glycoglycerolipids, we decided to modify only the sugar residue by peracetylation of Myrmekioside E-1 followed by a deacetylation reaction. The toxicity of Myrmekioside E-2 is nearly eight times more than the native one (this increase occurred on both cell lines), but Myrmekioside E-1 lost the half of its toxicity by deacetylation. On the other hand, the presence or absence of the *N*-acetyl group in the GL structure has no effect in cytotoxicity. Furthermore, the *N*-acetyl-pyranohexosamine of the GL showed stronger cytotoxicity than the pyranohexosamine. We can also explain the difference between the cytotoxicity of Myrmekioside E-1, E-2 and E-3 by their polarity. Myrmekioside E-2, E-1 and E-3, respectively had lower polarity compared to each other on TLC, this event leads to pass more easily of Myrmekioside E-2 than E-1 and E-1 than E-3 from cellular membranes and show more toxicity. Otherwise, comparing the toxicity of Trikentroside and Myrmekioside E can also shows the importance of relationship between the total number of carbon atoms, double bonds and terminal alcohol group of the lipophilic part with the biological activity of these glycoglycerolipids.

The second GL isolated in this work, accounting for around 0.01% of total lipids, was another alkyl-glycosylglycerol with one pyranopentose and two pyranohexosamines as sugar units. Complete identification and study of its biological activity are in progress in the laboratory. Thus, Myrmekiosides, like most natural marine GL, are a complex mixture of homologues differing by their sugar moiety, the length and branching of their alkyl chains.

3.6. Experimental section

3.6.1. General Procedures

High-resolution electrospray ionization mass spectrometry (HR-ESI-MS, positive mode, ion-source acceleration 4.5 kV, ion-source temperature 200°C, methanol as solvent) mass spectra were recorded with a Micromass Zab Spec TOF spectrometer. ^{1}H- and ^{13}C-NMR as well as 2D-NMR

spectra were obtained on a NMR Bruker Avance-300 spectrometer with triple Probe TBI multinuclear in $CDCl_3/CD_3OD$ (70:30, v/v) for natural GL, and $CDCl_3$ for peracetylated GL at 300.13 MHz and 75 MHz respectively, with tetramethylsilane as internal reference. Chemical shifts and coupling constants were expressed in δ (ppm) and Hz respectively. GC-MS spectra were performed on a Hewlett-Packard HP 5890 Series II coupled with the mass spectrometer HP 5989A (electronic impact 70 eV), integrator HP 98785, non-polar column DB-1 30 m length × 0.32 mm i.d. × 0.25 μm phase thickness. The temperature of the column was varied, after a delay of 2-4 min from the injection, from 110 to 310 °C with a slope of 3°C.min^{-1}. Optical rotations were measured in CH_2Cl_2 solutions with a Polartronic NH8 Schmidt/Haensch polarimeter at 30°C. Analytical TLC was performed on precoated silica gel F_{254} plates. After development, the dried plates were spraying with 50% H_2SO_4-vanillin and orcinol reagents. High-performance liquid chromatography (HPLC) was carried out with a Intersphere 5 μm ODS2, 250 × 4.6 mm, *Interchim,* with a 240 nm refractive or UV detector.

3.6.2. Sponge collection and glycolipid isolation

M. dendyi (Burton, 1959) was collected in Vanuatu archipelago, Lamen Bay, Epi Island, in 1997 at 30 m depth by the French Institute of Research for Development, IRD, New Caledonia during the European Programme Marine Science and Technology MAST III. *Trikentrion laeve* (= *loeve*) (Carter, 1879) was obtained from rocky bottoms off Dakar, Senegal, in 2005 at 25-40 m depth. Specimens of both sponges were kept at -80°C. Whole bodies of the collected specimens were chopped and extracted twice with CH_2Cl_2/MeOH (1:1, v/v) at room temperature. The combined extracts were concentrated *in vacuo* to give the crude extract, which was partitioned between H_2O and CH_2Cl_2. The lipid extract obtained in the CH_2Cl_2/MeOH phase was subjected to a lipid class separation by column chromatography affording a crude GL fraction. Lipid class separation was accomplished by open column chromatography on silica gel (70-230 mesh) successively eluted by dichloromethane (neutral lipids), acetone (glycolipids) and methanol (phospholipids and alkyl-glycosylglycerol ethers). The first methanolic fraction was purified by repeated reverse phase HPLC.

3.6.3. Acetylation of Myrmekioside E–1

To prepare samples for NMR analysis, Myrmekioside E-1 was dissolved in acetic anhydride (1:0.002 mol/mol) and some drops of dried pyridine. The reaction was allowed to proceed for 18 h

in darkness at room temperature, and then the reaction mixture was partitioned between water and dichloromethane. The organic layer was washed with 1M HCl, neutralized with a Na_2CO_3 solution and dried on anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was weighed. Myrmekioside E-2 was obtained.

3.6.4. Deacetylation of Myrmekioside E-1

To obtain the deacetylated alkyldiglycosylglycerol a part of Myrmekioside E-1 (8.8 mg) was dissolved in 0.45 mL of MeOH, and 0.200 mL of 0.5 M MeONa in MeOH was added. The reaction was allowed to proceed for 18 h at 25ºC, then the reaction mixture was dried and the residue partitioned between water and chloroform. After removal of the solvent, the deacetylated alkyldiglycosylglycerol, which was named Myrmekioside E-3, was obtained (122).

3.6.5. Methanolysis of Myrmekioside E-1

In order to provide samples for GC-MS, a part of Myrmekioside E-1 (6 mg) was heated with 0.9 mL of MeOH/H_2O/HCl (29:4:3, v/v/v) at 80°C for 18 h. The reaction mixture was extracted with H_2O/CH_2Cl_2 (3:9, v/v), the aqueous layer was concentrated to give methyl glycosides, whereas the organic layer contained fatty acid methyl esters. A third of the latter esters was preserved, the remaining one was transformed into *N*-acylpyrrolidides (NAP) by heating in a pyrrolidine/acetic acid mixture (10:2, v/v, 1 mL) during 1 h at 85°C. The reaction mixture was separated with H_2O/CH_2Cl_2. The aqueous layer was neutralized by 1M NaOH and extracted twice with diethyl ether. The aqueous layer containing methyl glycosides was evaporated *in vacuo* and then acetylated before GC-MS analysis.

3.6.6 Cellular studies (NSCLC-N6 and A549)

The antiproliferative activity of Myrmekioside E-1, E-2 and E-3 was evaluated. The NSCLC-N6 cell line (163), derived from a human non-small-cell bronchopulmonary carcinoma (moderately differentiated, rarely keratinized, classified as T2N0M0), and A549 obtained from ATCC collection reference CCL-185 (164) were used for all experiments. Both cell lines were cultured in RPMI 1640 medium with 5% fetal calf serum, to which were added 100 IU penicillin.mL^{-1}, 100 µg streptomycin.mL^{-1} and 2 mM glutamine, at 37°C in an air/carbon dioxide atmosphere (95:5, v/v).

Cytotoxicity was determined by continuous drug exposure. Experiments were performed in 96 wells microtiter plates (10^5 cells.mL^{-1} for NSCLC-N6 and 2 x 10^4 cells.mL^{-1} for A549). Cell growth was estimated by a colorimetric assay based on the conservation of tetrazolium dye (MTT) to a blue formazan product by live mitochondria (165). Eight repeats were performed for each concentration. Control growth was estimated from 8 determinations. Optical density at 570 nm corresponding to solubilized formazan was read for each well on a Titertek Multiskan MKII.

3.7. Acknowledgments

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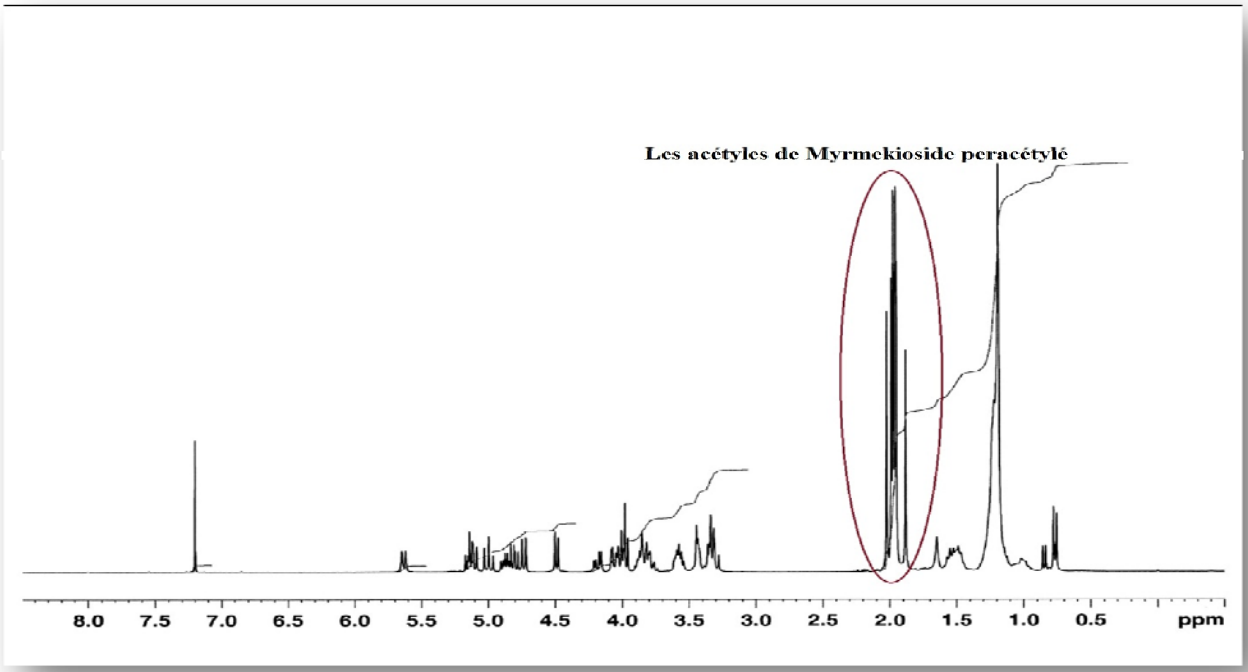

Figure 46. Spectre RMN ^{1}H de Myrmekioside peracétylé

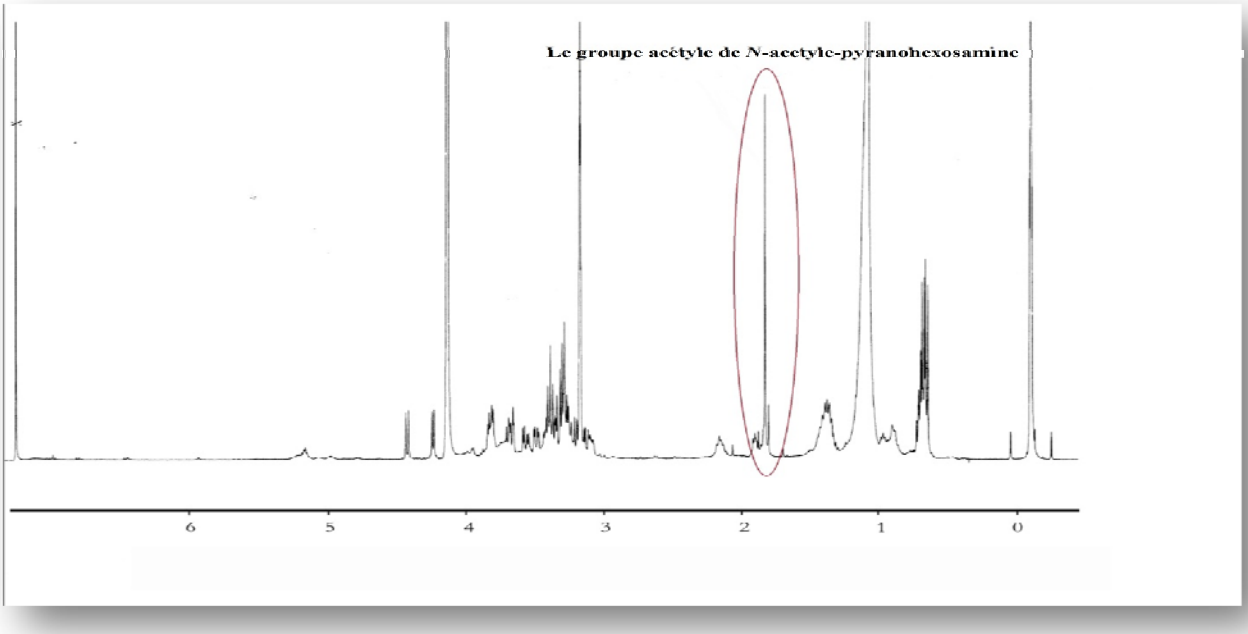

Figure 47. Spectre RMN ^{1}H de Myrmekioside non acétylé

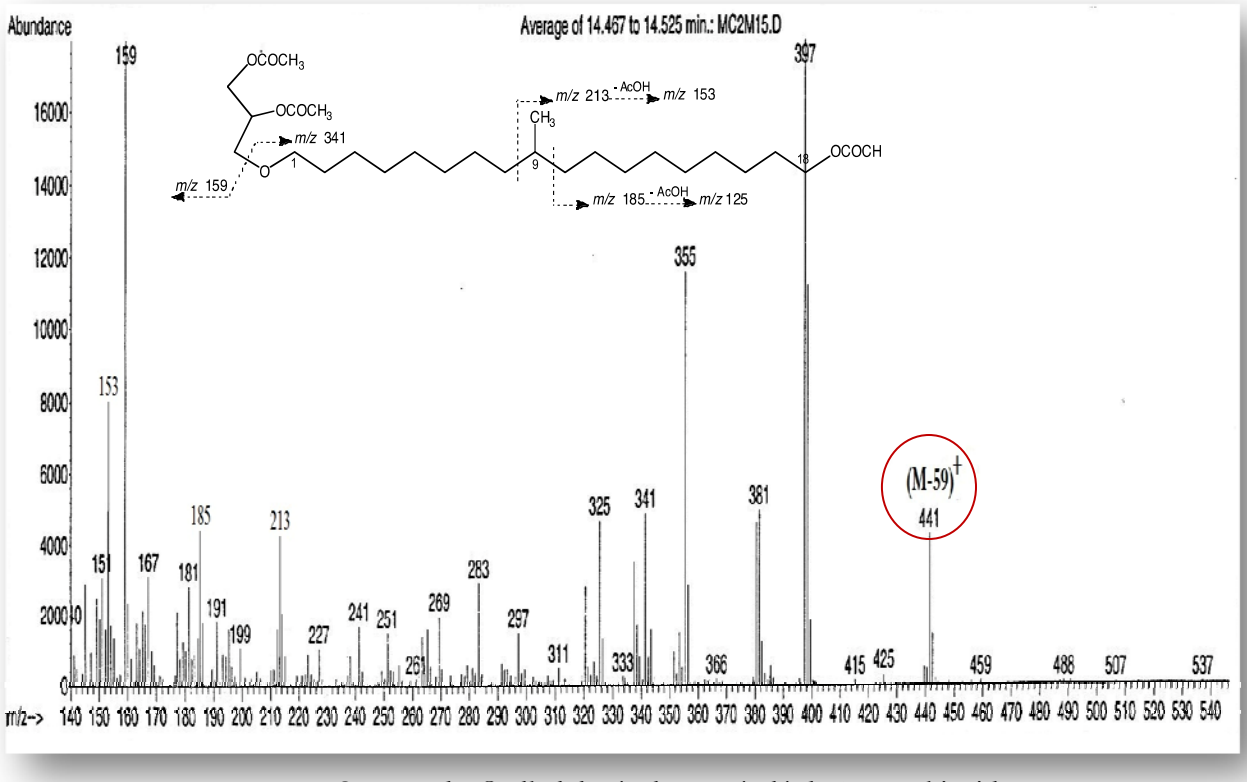

Figure 49. Spectre du *O*-alkylglycérol peracétylé de Myrmekioside

Chapitre IV

Antimalarial activity of an unusual glycosylceramide from the African sponge *Axinyssa djiferi*

Activité antipaludique d'une glycosylcéramide inhabituel de l'éponge africaine *Axinyssa djiferi*

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Antimalarial activity of an unusual glycosylceramide from the African sponge *Axinyssa djiferi*

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4.1. Abstract

The marine sponge *Axinyssa djiferi* collected on mangrove tree roots, Senegal, was investigated for glycolipids (GL). A GL fraction containing glycosphingolipid analogues, named Axidjiferoside-A, accounted for 0.07% of sponge biomass (dry weight) and for 2.16% of total lipids. It showed a significative antimalarial activity with an IC_{50} of 0.53 ± 0.2 μM against a chloroquine-resistant strain of *Plasmodium falciparum*. Its major component was characterized as *β*-galactopyranosylceramide composed of 2-amino-(6*E*)-octadec-6-en-1,3,4-triol and 2-hydroxytetracosanoic acid. Cytotoxicity was studied *in vitro* on human cancer cell lines (multiple myeloma, colorectal adenocarcinoma, glioblastoma and two lung cancer NSCLC-N6 and A549). Results of these investigations demonstrate that Axidjiferoside-A is of interest because it proved a good antiplasmodial activity with only a low cytotoxicity against various human cell lines and no significative antitrypanosomial and antileishmanial activity.

Keywords: *Axinyssa*, sponge glycolipids, glycosphingolipids, antimalarial activity, cytotoxicity.

4.2. Résumé

L'éponge marine *Axinyssa djiferi* récoltée sur des racines de palétuviers de la mangrove au Sénégal, a été étudiée pour les glycolipides (GL). Une fraction GL contenant des glycosphingolipides analogues, nommés Axidjiferoside-A, représentait 0.07 % de la biomasse de l'éponge (poids sec) et 2.16 % des lipides totaux. Cette fraction a montré une activité antipaludique significative avec une CI_{50} de 0.53 ± 0.2 μM sur une souche de *Plasmodium falciparum* résistante à la chloroquine. Son composant majeur a été caractérisé comme ß-galactopyranosylcéramide composé de 2-amino-(6*E*)-octadéc-6-èn-1,3,4-triol et d'acide 2-hydroxytétracosanoïque. La cytotoxicité a été étudiée *in vitro* sur des lignées cellulaires cancéreuses humaines (myélome multiple, adénocarcinome du colon, glioblastome multiforme et sur des cellules de cancer de poumon humain N6 et A549). Les résultats de ces investigations ~~enquêtes~~ démontrent qu'Axidjiferoside-A est intéressant en raison d'une activité antiplasmodiale significative et d'une faible cytotoxicité contre les lignes cellulaires humaines diverses et pas d'activité antitrypanosomiale et antileishmaniale significatives.

Mots-clés : *Axinyssa*, éponges, glycolipides, glycosphingolipides, activité antipaludique, cytotoxicité.

Abbreviations: COSY: homonuclear correlation spectroscopy; FAME: fatty acid methyl esters; LCB: long chain base; GC-MS: gas chromatography-mass spectrometry; GL: glycolipid(s); GSL: glycosphingolipid(s); amu: atomic mass; unit HMBC: heteronuclear multiple bond coherence; HSQC: heteronuclear single quantum coherence; IC_{50}: 50% inhibitory concentration; ESI-MS: electrospray ionization mass spectrometry; TLC: thin layer chromatography; dw: dry weight; MEC: The minimum effective concentration.

Malaria caused by the Anopheles mosquito-transmitted parasite *Plasmodium* is one of the leading causes of mortality and morbidity in more than 100 tropical and subtropical countries of the world (166). *Plasmodium falciparum* is the most dangerous species and causes the most lethal form of malaria (166). It is estimated that 350–500 million clinical malaria cases occur annually, resulting in 1–3 million deaths each year (166, 167).

In the absence of clinically proven vaccine against malaria, there is only a limited number of drugs in widespread use for the treatment of malaria (167). Compounding this paucity of drugs is the rapid development of resistance of this parasite to standard antimalarial drugs, leading to doubling in the number of deaths from malaria in many parts of sub-saharan Africa (168). Therefore, the rational development of novel pharmacophores for the purpose of malaria intervention requires the identification of new chemotherapeutic targets.

The new breakthrough in malaria treatment could come with the development of a marine lead compound. The incredible potential of even a single marine organism (from invertebrates as sponges, tunicates and soft corals) to produce a large array of secondary metabolites can be interpreted by considering the common features of the secondary metabolism in all the living organisms as well as some peculiar features of the marine environment. In addition, the contribution of the symbiotic population to the metabolic work of a marine invertebrate is an important point to be taken into account (169, 170). The parent compound of the small class of isonitrile-containing marine secondary metabolites isolated from the marine sponge *Axinella cannabina* and *Acanthella klethra* were found to possess a potent antimalarial activity both on chloroquine-sensitive (142 ng/mL) and chloroquine resistant (17 ng/mL) *P. falciparum* strains (171-173). Chemical analysis of *Cymbastela hooperi* (Axinellidae) sponge metabolites afforded a series of diterpenes which displayed a significant (low nM ranges) and selective (cytotoxicity in μM ranges) *in vitro* antimalarial activity. The co-occurrence of several strictly related analogues suggested some structure-activity relationships (174). Manzamines are undoubtedly the most important and potent antimalarial alkaloids isolated

from marine sources. Manzamine A and its 8-hydroxy derivative were found to potently inhibit the growth of *P. falciparum* both *in vitro* (IC_{50} ~ 5.0 ng/mL) and *in vivo* (a parasitemia suppression in the same order of magnitude of that of artemisinin) (175-177). Salinosporamide A is a γ-lactam alkaloid isolated from a marine bacterium of the new genus *Salinispora*. This molecule has been found to be a potent parasite proteasome inhibitor and to possess a quite significant antimalarial activity *in vitro* (IC_{50} = 11.4 nM). Salinosporamide A was determined to act on the erythrocytic stage and maintained its potent activity in a malaria mouse model with inhibition of the parasite growth in treated mice at extremely low doses (130 μg/kg) (178-181). Plakortin was isolated more than 25 years ago from *Plakortis halichondroides* and recently re-isolated in remarkable amounts from the Caribbean sponge *Plakortis simplex*. This molecule exhibited a good antimalarial activity against chloroquine-sensitive strain, and chloroquine-resistant strain of *P. falciparum* (IC_{50} ~ 180 ng/mL), devoid of cytotoxicity (182-184). Interestingly, a review on marine antimalarial published in 2009 does not mention any glycolipid (GL) (185), but four new ether diglycosides, named matayosides A-D, were isolated from the root bark of *Matayba guianensis* and inhibited the growth of *P. falciparum in vitro* with IC_{50} values ranging from 25 to 8.9 μg/mL (186).

Glycolipids are ubiquitous cell membrane constituents in animals, which play a fundamental role in major phenomena such as cell-cell recognition and antigenic specificity (119, 120). In general, GL exhibit a wide range of biological functions that might be related to the amphipathic nature of the molecule. Several GL have been isolated from a number of marine sources, mainly sponges. They are known to possess immunomodulating and antitumor activities, with emphasis to those isolated from sponges (119-122). Among them, glycosphingolipids (GSL) represent a large group of biomolecules containing two basic structural units: a sugar linked to a ceramide. The hydrophobic ceramide portion involves a sphingoid base and an amide-linked fatty acyl chain. Various cerebrosides, glycosylceramides with a single sugar head, were isolated from sponges (119-122, 147) and echinoderms like starfishes (187).

In a previous work on the sponge *Axinyssa djiferi* we isolated and identified a mixture of several GSL, named Axidjiferosides, whose biological activities were evaluated (161). In this work, a very similar GSL mixture was isolated from *A. djiferi* and an active fraction containing a major GSL was obtained to specify the biological activities and to characterize its major component. *A. djiferi* (188) is found along the Senegalese coasts in south of Dakar, Senegal, on mangrove tree roots, near the surface at low tide, and was collected by hand during an expedition organized by Oceanium of Dakar in 2007,

on the site named Sine-Saloum. Whole bodies of the collected specimens were chopped and investigated for their GL fractions. They were twice extracted with CH_2Cl_2/MeOH (1:1) at room temperature. The combined extracts were concentrated *in vacuo* to give the crude extract, which was partitioned between H_2O and CH_2Cl_2. The organic layer was concentrated *in vacuo*, and the residue was submitted to column chromatography over silica gel (230-400 mesh) eluting with pure solvents of increasing polarity, to separate the different lipid classes: dichloromethane (neutral lipids, 50.2%), acetone (GSL, 17.8%) and methanol (phospholipids, 32%). The total GL mixture was separated on silica gel column to give 19 fractions. Two of them were pooled and subjected to a silica gel column chromatography (CH_2Cl_2/MeOH, 98:2, with an increasing amount of methanol to 90:10) affording 14 fractions. The purification by silica gel column chromatography and then by repeated reverse phase HPLC (eluent: MeOH/H_2O, 95:5) (achieved on a 250 × 4,6 mm, Intersphere 5 µm ODS1, Interchim column) was continued until we obtained the fraction, named Axidjiferoside-A that contained a major GSL. It presented a similar polarity to a commercial standard (galactocerebroside with a 2-hydroxy fatty acyl chain type I) (Rf = 0.42 on silica gel thin layer chromatography, CH_2Cl_2/MeOH/CH_3CN, 99:1:2). Axidjiferoside-A, accounting for 0.07% of the sponge biomass (dw), 2.16% of total lipids and 17.45% of the total GL, was used for biological studies. Chemical structure and composition of Axidjiferoside-A was obtained by controlled chemical degradation and by electrospray ionization mass spectrometry (ESI-MS) and NMR studies of the peracetylated GL.

A part of Axidjiferoside-A was peracetylated with acetic anhydride in pyridine for 18 h. The peracetylated Axidjiferoside-A exhibited the characteristic signals of a sphingoid base and a *β*-galactopyranose in the ^{1}H-NMR spectrum (Figure 51, Table 8). The ESI-MS showed three molecular ion peaks, corresponding to galactosylceramides. Indeed, the peracetylated major component displayed an adduct ion $[M + Na]^+$ at *m/z* 1160.7436 (high resolution ESI-MS) in accordance with the formula $C_{62}H_{107}NO_{17}Na$ (a molecular mass of 843.7430 amu for the intact GSL). In addition to the major GSL, two minor GSL displayed sodiated molecular ions at *m/z* 1174.7593 and 1146.7280 in accordance with a methylene less or more than the major one. The structure of the major cerebroside was determined on the basis of chemical and spectroscopic evidence. Thus, it contains an unsaturated long-chain aminoalcohol as sphingoid base. The optical rotation value of peracetylated Axidjiferoside-A was determined as $[\alpha]_D^{30} = +0.45$ (c = 0.4, CH_2Cl_2). All the chemical shifts of the ceramide are given in the ^{13}C and ^{1}H NMR spectra (Table 8).

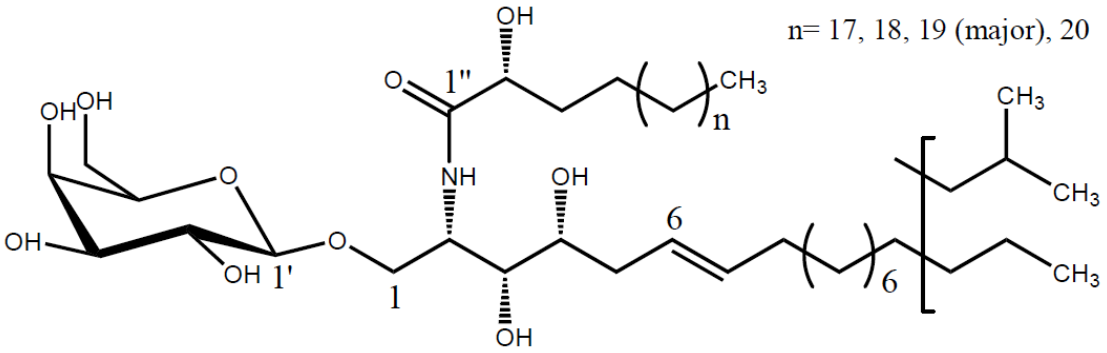

Figure 51. Structure of Axidjiferoside-A from *Axinyssa djiferi*; n = 17, 18, 19 (major), 20

Table 8. ^{1}H (500 MHz) and ^{13}C (125.75 MHz) NMR data of Axidjiferoside-A in $CDCl_3$

Position	δ_H ppm, mult., *J* in Hz	$\delta_C$ppm
1a	3.88 (dd, *J* = 10.6/3.1)	66.19
1b	3.70 (dd, *J* = 10.6/2.7)	-
2	4.33 (m)	48.09
2-NH	6.81 (d, *J* = 9.0)	-
3	5.12 (m)	71.95
4	4.95 (m)	72.64
5	2.40 (m)	20.75
6	5.50 (dt, *J* = 6.8/15.0)	124.03
7	5.28 (dt, *J* = 7.0/15.0)	134.58
8	1.85 (m)	24.86
terminal methyl	0.87 (t, *J* = 6.8)	19.23
terminal isopropyl	0.85 (d, *J* = 6.6)	22.66
7 Ac	2.05/2.25/2.17/2.06/2.11/1.99/2.07 (7 s)	169.44/169.86/170.0/170.06/170.18/170.36/170.74
1’	4.47 (d, *J* = 7.8)	100.66
2’	5.17 (m)	73.88
3’	5.02 (dd, *J* = 10.4/3.3)	70.79
4’	5.38 (d, *J* = 3.3)	66.96
5’	3.95 (t, *J* = 6.7)	70.79
6’	4.15 (d, *J* = 6.7)	61.06
1”	-	171.08
2”	5.14 (m)	68.6
3”	1.31 (m)	34.41

The sugar linked to the ceramide was identified as galactopyranose by NMR spectroscopy. First of all, the anomeric proton of the *β*-galactopyranose (δ_H = 4.47, d, *J* = 7.8 Hz) was correlated with

the anomeric carbon at δ_C = 100.66 ppm in the HMQC spectrum. Starting from this proton, all the ^{1}H and ^{13}C NMR signals of the sugar were assigned by using the COSY, HMQC and HMBC spectra, and the vicinal proton-proton coupling constants were determined (Table 8). The *galacto* configuration of the sugar, as well as its *β* anomeric configuration, was established on the basis of the ring proton coupling constants ($J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.4 Hz, $J_{3,4}$ = 3.3 and $J_{4,5}$ = 6.7 Hz). The linkage of the galactopyranoside to the ceramide was confirmed by the three bond ^{13}C-^{1}H couplings of anomeric C-1' with H-1a and H-1b observed in the HMBC spectrum. The location of the double bond, and its configuration, in the dihydrosphingosine moiety was determined from the ^{1}H-NMR spectrum in $CDCl_3$ with a double triplet at 5.50 ppm (J = 15.0, 7.0 Hz) and a double triplet at 5.28 ppm (J = 15.0, 6.8 Hz), characterizing a (*E*)-Δ^6 sphingosine. This was supported by two carbon signals at δ 20.75/24.86 for the carbons next to the double bond in the ^{13}C-NMR spectrum (189-191), which were assigned by means of COSY and HMQC. COSY correlations were observed between H-5 and H-6 and also H-7 and H-8 (Table 8). Also the key HMBC correlations from H-5 to C-6, C-7, and C-8, confirmed the location of the double bond. In addition, NMR spectrum showed the presence of terminal methyl and isopropyl groups.

To confirm the structure of the fatty acid methyl esters (FAME) and the long-chain base (LCB), Axidjiferoside-A was subjected to an acid methanolysis and the resulting reaction mixture was partitioned between CH_2Cl_2 and H_2O/MeOH. Thus, 4 mg of Axidjiferoside-A was heated with 0.9 mL of MeOH/H_2O/HCl (29:4:3) at 80 °C for 18 h. The reaction mixture was extracted with H_2O/CH_2Cl_2 (3:9), the aqueous layer was concentrated to give methylglycosides, whereas the organic layer contained a mixture of FAME and sphingoid bases. *N*-acylpyrrolidides were prepared from a part of FAME by heating in a mixture pyrrolidine/acetic acid (10:2, 1 mL) during 1 h at 85 °C. The aqueous layer was neutralized by 1M NaOH and extracted twice with diethyl ether. The organic layer, containing sphingoid base was dried and then acetylated. The aqueous layer containing methylglycosides was evaporated *in vacuo* and then acetylated before GC-MS analysis. GC-MS analysis of the FAME indicated that the fatty acid moiety was C_{22-25} methyl 2-hydroxylated (mainly C_{24}). Indeed, the 2-hydroxy FAME produced the characteristic ions at *m/z* 90 (McLafferty) and *m/z* 103. The results were as follows: 2-OH-24:0, t_R = 44.3 min (53.5%), *m/z* 398 (M^+); 2-OH-22:0, t_R = 39.6 min (19.8%), *m/z* 370 (M^+); 2-OH-23:0, t_R = 42.0 min (14.8%), *m/z* 384 (M^+); 2-OH-25:0, t_R = 46.5 min (11.9%), *m/z* 412 (M^+). These fatty acid structures were confirmed by GC-MS analysis of *N*-acyl pyrrolidides showing fragment ions at *m/z* 129 (McLafferty) and the expected molecular ions. The peracetylated methylglycoside from Axidjiferoside-A was analyzed by GC-MS [column

temperature 110 °C (2 min) and then (temp. increasing at 3 °C/min until 240 °C)]; t_R = 37.1 min (methylgalactopyranoside) similar to that of an authentic sample. Other diagnostic ions were observed at m/z 331 $(M\text{-}OMe)^+$, 303 $(M\text{-}OAc)^+$, 243, 200, 157, 145 and 115 (Figure 52). The sphingoid base was observed as 2-amino-1,3,4-trihydroxy-octadecene, t_R = 34.12 min, m/z 482 (M^+) including the *iso* form at 23,8%. It produced the characteristic ions at m/z 43 (base peak) and m/z 144 (Figure 53). The 2*S*,3S,4*R* stereochemistry was assumed by comparison of the ^{13}C-NMR chemical shifts of C-2 and C-3 with those of Plakosides C and D (192, 193). In conclusion, the major component which was present in Axidjiferoside-A was established to be 1-*β*-D-galactopyranoside of a (2*S*,3*S,4R*,8*E*)-3,4-dihydroxy-octadecene ceramide possessing a 2-hydroxytetracosanoic acid.

The antimalarial activity of Axidjiferoside-A was evaluated against the chloroquine-resistant FcB1/Colombia strain of *Plasmodium falciparum* (IC_{50} value for chloroquine of 0.1 μM) (194). The antiplasmodial activity was determined according to Labaied et al. (195) Extracts were prepared in DMSO at a concentration of 10 mg/ml and serially diluted with culture medium before to be added to asynchronous parasite cultures (1% parasitemia and 1% final hematocrite) in 96-well microplates. Plates were maintained for 24 h at 37°C. 0.5 μCi of [^{3}H]hypoxanthine was then added to each well and parasites were maintained for further 24 h. Growth inhibition was determined by comparison of the radioactivity incorporated into the treated culture with that in the control culture maintained on the same plate. Concentrations causing 50% inhibition of parasite growth (IC_{50}) were calculated from the drug concentration–response curves. Each experiment was performed in triplicate.

Antileishmanial activity was evaluated on *Leishmania donovani* (MHOM/ET/67/HU3) line, called LV9. Promastigote forms were grown in M-199 medium supplemented with 10 % inactivated foetal calf serum, 40 mM HEPES, 100 μM adenosine, and 0.5 mg L^{-1} hemin in the presence of 50 μg mL^{-1} gentamicin at 26°C in 5 % CO_2. Peritoneal macrophages were harvested from female CD1 mice three days after an intraperitoneal injection of 1.5 mL of sodium thioglycolate (Biomérieux) and were dispensed into eight-well chamber slides (LabTek Ltd.) at a density of 5.10^4 cells per well (400 μL per well) in RPMI 1640 medium supplemented with 10 % FCS, 25 mM HEPES, and 2 mM glutamine. Four hours after, wells were washed to eliminate fibroblasts. After a 24 h incubation period, the macrophages were infected with promastigotes at a stationary phase in a ratio of 10 parasites per macrophage. After 18 h free promastigotes were eliminated and intramacrophagic amastigotes were treated with various concentrations of compounds. Pentamidine and amphotericin B were used as reference compounds. The culture medium was renewed 48 h later and a new culture medium

containing the drug was added. The experiment was stopped after five days and the percentages of infected macrophages were evaluated microscopically after Giemsa staining. IC_{50} values were determined by linear regression analysis. Each experiment was performed in triplicate.

Antitrypanosomal evaluation was performed as follows: the bloodstream forms of *T. brucei* were purified by centrifugation from the blood of an infected mouse and were maintained *in vitro* for 24 h at 37 °C in a 5% CO_2 atmosphere in a minimum essential medium supplemented with 25 mM HEPES, Earle's salts, 2 mM glutamine, 1 g L^{-1} of glucose, minimum essential medium non essential aminoacids, 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.016 mM thymidine, 15 % heat-inactivated horse serum and 50 μg mL^{-1} of gentamycin. Drug evaluation was carried out in 96-well plates in a final volume of 200 μL containing 2.10^5 trypomastigotes and the compounds to be tested. Pentamidine was used as reference compound. The minimum effective concentration (MEC) was defined as the minimum concentration at which no viable parasite was observed microscopically. This value was confirmed by injecting intraperitoneally the culture from the well corresponding to the MEC into a mouse to confirm that the non-motile parasites were really killed and not able to divide within mice.

The antiproliferative activity of Axidjiferoside-A was investigated upon the cell lines: NSCLC-N6, (derived from a human non-small-cell bronchopulmonary carcinoma, moderately differentiated, rarely keratinized, classified as T2N0M0) (163), A549 (obtained from ATCC collection reference CCL-185) (164), GBM (astrocytoma cells obtained after tumor resection of patients with glioblastoma multiforme-primary culture) (140), HCT-116 (colorectal adenocarcinoma cells derived from a patient with Lynch's syndrome) (141) and KMS-11 (adherent plasma cells obtained from patients with multiple myeloma) (139) as previously described (147, 187). Experiments were performed at least in triplicate, 4 wells per Axidjiferoside-A concentration being used. IC_{50} values were calculated from the dose-response curves.

Interestingly regarding the product availability, we investigated GL of *A. djiferi* two times and observed that Axidjiferoside-A was always present.

Axidjiferoside-A showed a significant activity against *P. falciparum* FcB1 with an IC_{50} of 0.53 ± 0.2 μM. Cytotoxicity and antitumor activity of Axidjiferoside-A, with $IC_{50} > 35$ μM on two human non-small cell lung cancer cell lines (NSCLC-N6 and A549), and with $IC_{50} > 60$ μM on three human cancerous cell lines (KMS-11, GBM and HCT-116), were non significative. Axidjiferoside-A was evaluated *in vitro* for other antiparasitic activities against *L. donovani* (amastigote forms), and *T. brucei*

(bloodstream forms) and showed no activity against these parasites (IC_{50} > 100 μM and MEC > 100 μM, respectively).

It is of interest to compare the antiproliferative activity of Axidjiferoside-A with those of several GSL having also a single sugar, which is generally *β*-D-glucopyranose or *β*-D-galactopyranose. The biological activity of such GSL is nowadays little documented. Halicerebrosides from the sponge *Haliclona* sp. are glucosylceramides with a sphingoid base unsaturated at C-6/C-7 like Axidjiferoside-A (Figure 54). Thus, the only notable difference in their structure is the presence of a glucose instead of a galactose. Halicerebrosides displayed a cytotoxic activity against murine leukemia cells P388 (IC_{50} of 20 μg/mL) (59). Halicylindrosides from the sponge *Halichondria cylindrata* are GSL containing *N*-acetyl-glucosamine as the sugar head (Figure 55). These GSL showed a cytotoxic activity on leukemia cells P388 with IC_{50} of 6.8 μg/mL (60). Furthermore, Ophidiacerebrosides isolated from some starfishes showed an antiproliferative activity against murine leukemia cells L1210 at 2 μg/mL (Figure 56) (103). These GSL contain a *β*-glucopyranoside as sugar head, a 9-methyl-branched 4,8,10-triunsaturated long-chain aminoalcohol as sphingoid base. One of them, Ophidiacerebroside-C from the starfish *Narcissia canariensis* displayed an interesting cytotoxic activity on adherent human cancerous cell lines (multiple myelome, colorectal adenocarcinoma, multiforme glioblastoma) with an IC_{50} around 20 μM (147). Interestingly, the IC_{50} values found for Axidjiferoside-A on the same cancer cells were found superior to 60 μM. We could then conceive that the presence of a galactose decreases or cancels the cytoxicity of the GSL. Unfortunately, data on the cytotoxicity of a galactosylceramide are missing to date. Plakosides A and B, from the sponge *Plakortis simplex*, are *β*-galactosylceramides possessing a prenylated sugar hydroxyl group and cyclopropane-containing alkyl chains (Figure 57). They are immunosuppressors with a non-cytotoxic mechanism of action (58).

Activity of Axidjiferoside-A against *P. falciparum* is six times less than those of chloroquine (IC_{50} = 0.10 μM) but, on the other hand, Axidjiferoside-A has no high cytotoxicity effect. In addition, the compound exhibited a specific antimalarial activity since no activity was observed against Kinetoplatids. So, it is of interest for further research work on malaria treatment.

4.3. Acknowledgements

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4.4. General procedures

High resolution electrospray ionization mass spectrometry (HR-ESI-MS, positive mode, ion-source acceleration 4.5 kV, ion-source temperature 200 °C, methanol as solvent) mass spectra were recorded with a Micromass Zab Spec Tof spectrometer. ^{1}H- and ^{13}C-NMR as well as 2D-NMR spectra were obtained on a NMR Bruker Avance-500 spectrometer with triple Probe TBI multinuclear in $CDCl_3$ at 500.13 MHz and 125.76 MHz respectively, with reference to an internal standard of tetramethylsilane. Chemical shifts and coupling constants were expressed in δ (ppm) and Hz respectively. GC-MS spectra were performed on a Hewlett-Packard 5890II gas chromatograph linked to a HP 5989A spectrometer and a HP 98785A integrator, moderately polar column DB-1, 30 m length × 0.25 mm i.d. × 0.33 μm phase thickness. The column temperature was varied, after a pause of 2–4 min from the injection, from 110 to 310 °C with a slope of 3 °C.min^{-1}. Optical rotations were measured in CH_2Cl_2 solutions with a Polartronic NH8 Schmidt/Haensch polarimeter at 30 °C. Analytical TLC was performed on precoated silica gel F_{254} plates. After development, the dried plates were sprayed with 50% H_2SO_4-vanillin and orcinol reagents. High-performance liquid chromatography (HPLC) was carried out with a Intersphere 5 μm ODS1, C_{18}, 250 × 4.6 mm, *Interchim,* with a 230 nm refractive or UV detector.

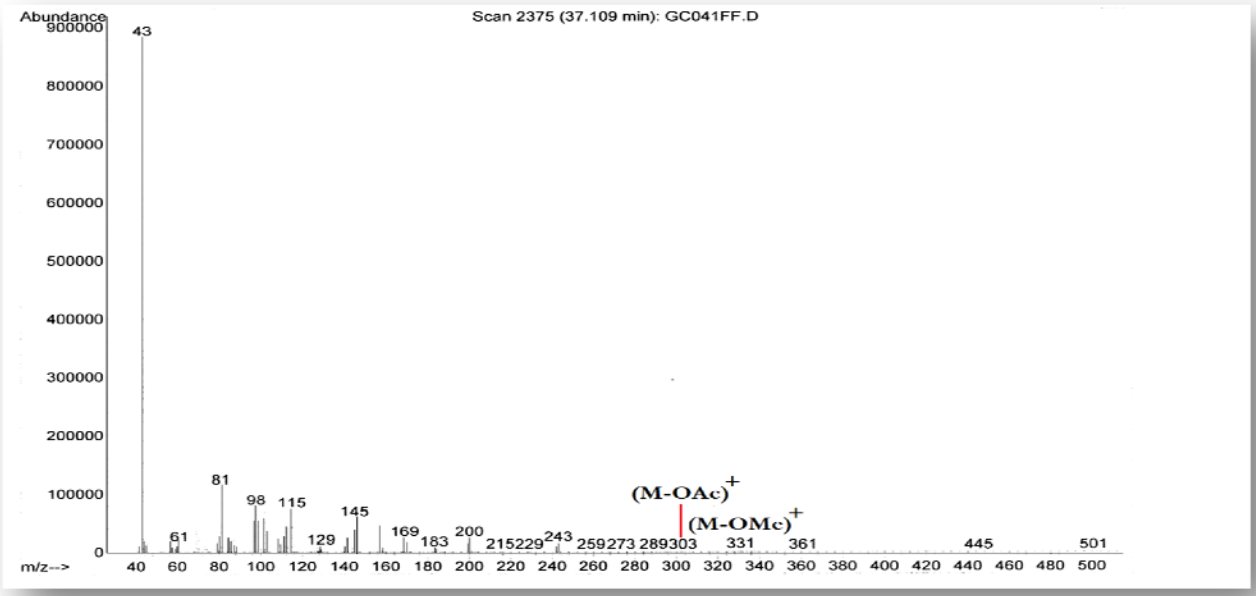


Figure 52. Spectre de mass du méthyl-*β*-galactopyranoside peracétylé preparé à partir des Axidjiferosides

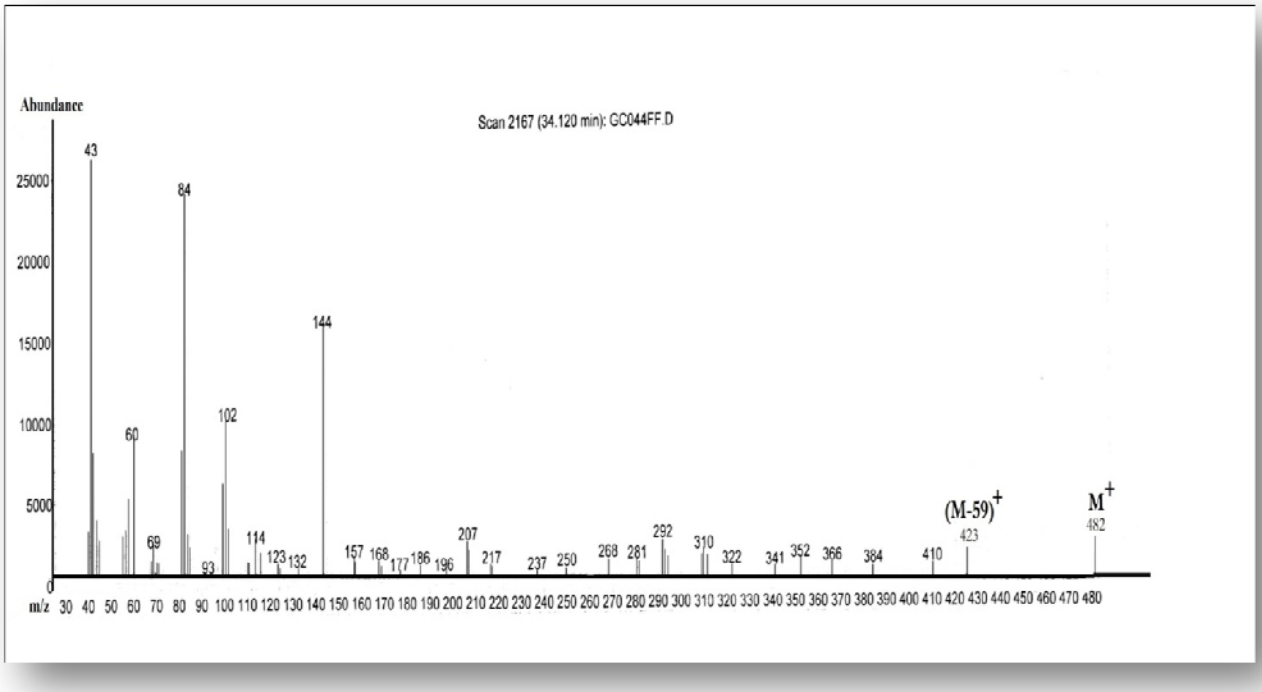


Figure 53. Spectre de l'amino-alcool d'Axidjiferosides

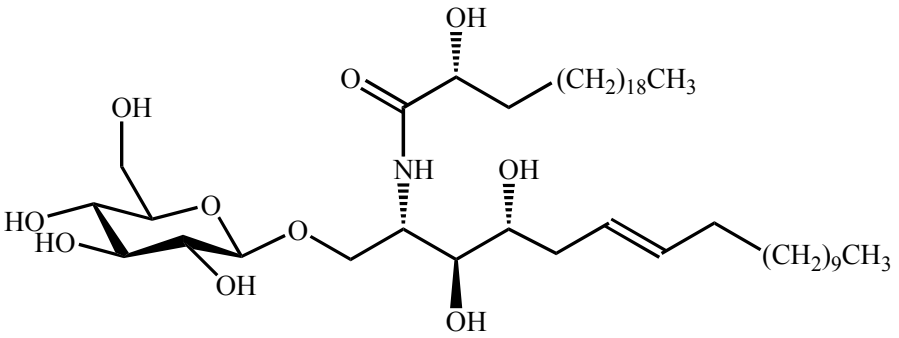
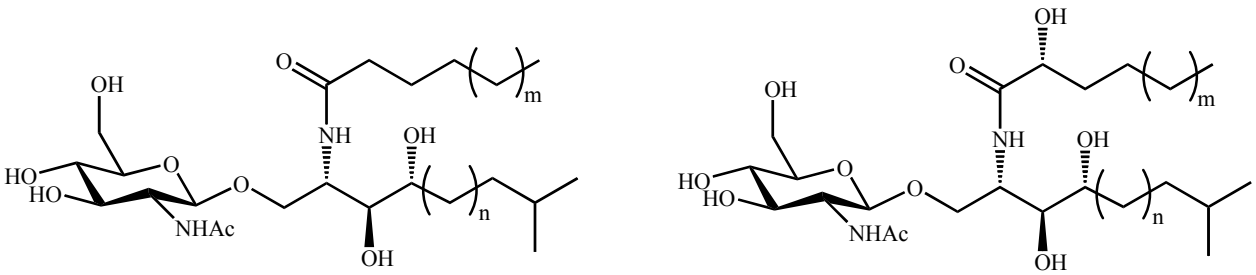


Figure 54. Halicerebroside-A de *Haliclona* sp.



Halicylindroside A_1 : m = 16, n= 10
Halicylindroside A_2 : m = 16, n= 11
Halicylindroside A_3 : m = 17, n= 11
Halicylindroside A_4 : m = 18, n= 11

Halicylindroside B_1 : m = 16, n= 8
Halicylindroside B_2 : m = 17, n= 8
Halicylindroside B_3 : m = 16, n= 9
Halicylindroside B_4 : m = 16, n= 10
Halicylindroside B_5 : m = 17, n= 9
Halicylindroside B_6 : m = 16, n= 11

Figure 55. Halicylindrosides de *Halichondria*

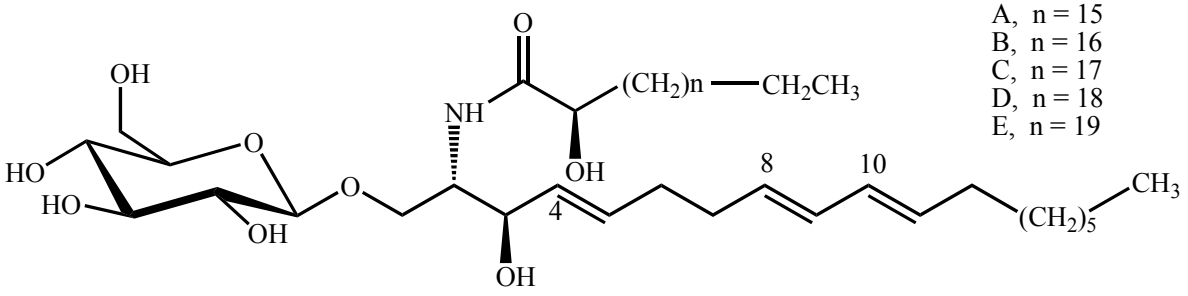


Figure 56. Ophidiacerebrosides

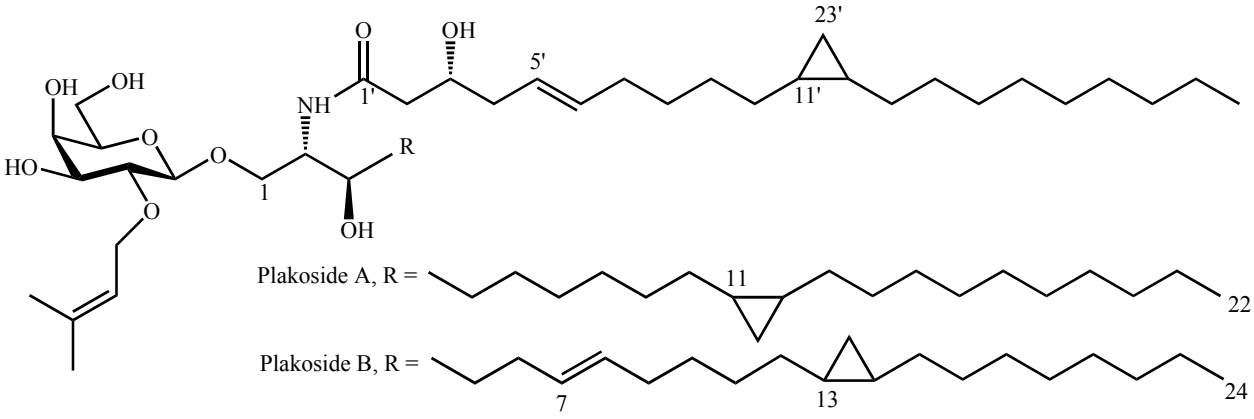


Figure 57. Plakosides A et B de Plakortis simplex

CONCLUSIONS

Le sujet qui m'a été proposé s'inscrit dans la poursuite de travaux du Laboratoire et s'appuie sur des premiers résultats prometteurs obtenus avec plusieurs glycolipides marins à activité antitumorale. Notre choix s'est porté sur des éponges, *Axinyssa djiferi* (mangrove du Sénégal) et *Myrmekioderma dendyi* (Vanuatu, Pacifique sud) et une étoile de mer, *Narcissia canariensis* (côtes sénégalaises). Les GL sont évalués pour leurs propriétés anti-prolifératives et anti-angiogéniques au sein du « Cancéropôle Grand Ouest ». Les laboratoires impliqués sont

- l'EA1155 pour les tests de cytotoxicité sur cellules cancéreuses humaines de cancer du poumon
- l'INSERM CRNA U892 pour les tests de cytotoxicité sur cellules cancéreuses humaines de myélome multiple, carcinome colique et glioblastome
- Le Centre de Biophysique Moléculaire CNRS 4301 pour les tests anti-angiogéniques

L'évaluation antipaludique est effectuée en deux temps :

- ***in vitro***, au Muséum d'Histoire Naturelle de Paris sur une souche de *Plasmodium* résistante à la chloroquine
- ***in vivo*** (souris) dans l'équipe EA1155, Faculté de Pharmacie de Nantes.

Les GL d'intérêt sont sélectionnés à partir de fractions particulières ayant présenté une certaine activité.

- *Narcissia canariensis* : le GL majoritaire (F13-3) que j'ai isolé et identifié a montré une bonne activité cytotoxique contre des lignées cellulaires cancéreuses humaines avec une valeur de CI_{50} de 15,2 ± 4,0 μM sur KMS-11 (myélome multiple), 18 ± 3,9 μM sur HCT-116 (carcinome colique) et 34,6 ± 5,1 μM sur GBM (glioblastome multiple). Le F13-3 peracétylé possède une bonne activité sur cellules cancéreuses du poumon non-à-petites-cellules NSCLC-N6 avec IC_{50} = 23,54 ± 0.2 μM et A549 avec une CI_{50} = 31,08 ± 0.2 μM. De plus le F13-3 non acétylé a montré une activité cytotoxique plus importante sur NSCLC-N6 et A549 avec une CI_{50} inférieure à 12,35 μM. De nos jours, le myélome multiple, le carcinome colique et le glioblastome multiple représentent des problèmes de santé publique avec un taux de mortalité croissant dans les pays développés, alors que par exemple le myélome qui représente environ 1 % de tous les cancers et 2 % de tous les décès par cancer.
- *Axinyssa djiferi*: le GL majoritaire (Axidjiferoside A) a montré une activité significative contre *Plasmodium falciparum* FCB1 *in vitro* avec une CI_{50} = 0,53 ± 0,2 μM. Le Trikentroside et

l'Axidjiféroside permettent une diminution de la parasitémie de 44% in *vivo* (à 8mg/Kg) et le second a une activité anti-plasmodiale (à 30 mg/Kg) quasi équivalente à celle de la chloroquine utilisée à 5 mg/kg (parasitémie diminuée de 85%). Ces résultats, non encore publiés pour les tests effectués *in vivo*, permettent de proposer deux nouveaux modèles pharmacologiques antipaludiques dont l'un, l'Axidjiféroside A, semble spécifique de ce type d'activité car non cytotoxique sur de nombreuses autres lignées cancéreuses humaines et parasitaires (trypanosome et leishmania).

- *Myrmekioderma dendyi*: le glycolipide majoritaire peracétylé possède une bonne cytotoxicité sur cellules cancéreuses du poumon non-à-petites-cellules NSCLC-N6 avec $IC_{50} = 7.3 \pm 0.2$ μM et sur celles A549 avec $IC_{50} = 9.7 \pm 0.2$ μM.

La comparaison structures-activités des glycolipides obtenus dans ce travail nous orientent vers les résultats indiqués ci-dessous :

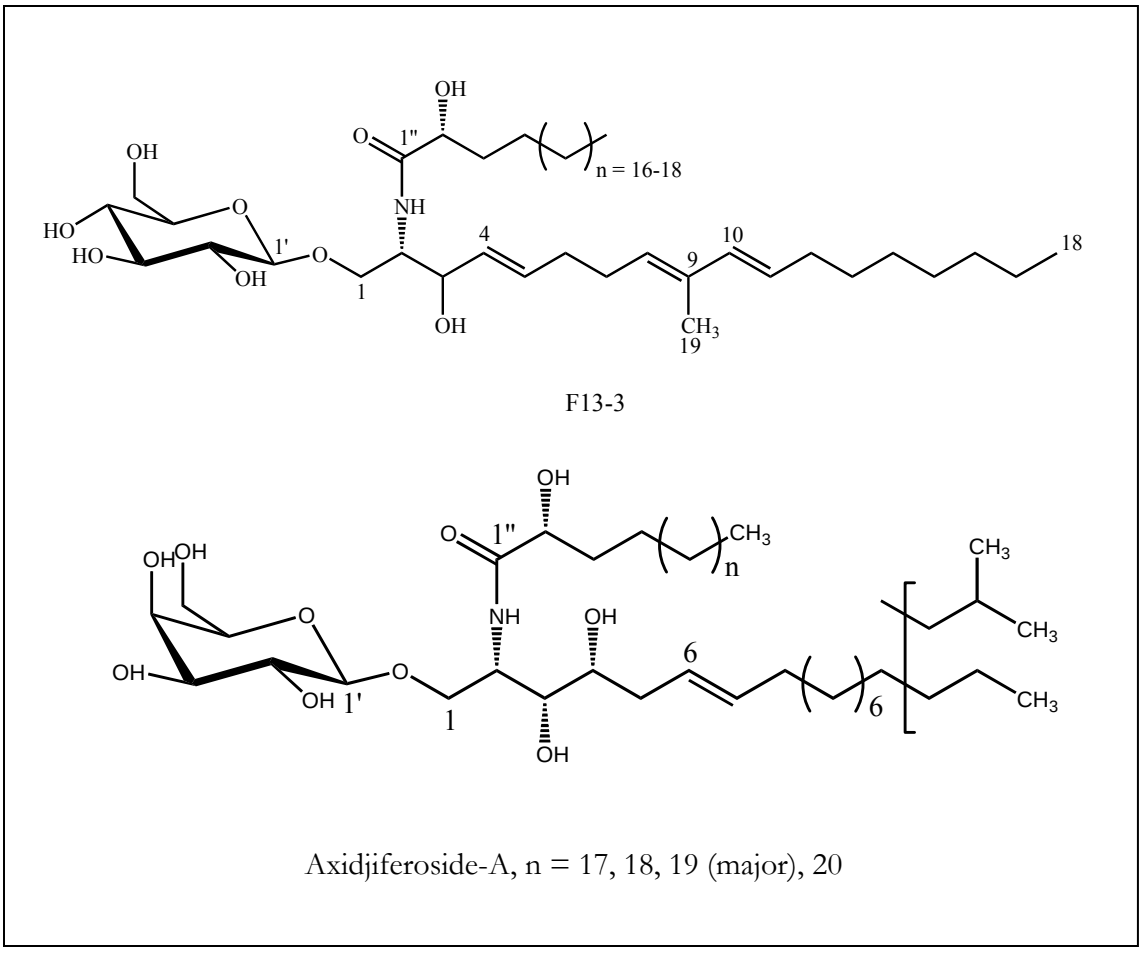
- Comparaison structure-activité entre Axidjiferoside A et F13-3 sur les deux lignées cancéreuses du poumon non-à-petites-cellules NSCLC-N6 et A549, sur KMS-11 (myélome multiple), GBM (glioblastome multiple) et sur HCT-116 (carcinome colique) résumée Tableau 9.

	Axidjiferoside A (*Axinyssa djiferi*)	**F13-3 (*Narcissia canariensis*)**
NSCLC-N6	CI_{50} > 35 µM	CI_{50} < 12,3 µM
A549	CI_{50} > 35 µM	CI_{50} < 12,3 µM
KMS-11	CI_{50} > 60 µM	CI_{50} : 15,2 ± 4,0
GBM	CI_{50} > 60 µM	CI_{50} : 34,6 ± 5,1 µM
HCT-116	CI_{50} > 60 µM	CI_{50} : 18,0 ± 3,9 µM

Tableau 9. Comparaison d'activité anticancéreuse entre Axidjiferoside A et F13-3 (non acétylé)

Il faut regarder les activités antiprolifératives pour des GSL avec un seul sucre (GSL « simple »). Mettons de côté la configuration en alpha de ce sucre, car nous savons qu'elle permet une activité bien plus importante que celle en béta. C'est ce qui a été démontré pour le KRN 7000 et pour l'alphaGalGSL de *Stylissa fulabelliformis* qui inhibe fortement les cellules leucémiques murines P388 dès 10 µg/mL (196). Les GSL possèdent généralement un glucopyranose ou un galactopyranose. Peu d'activités biologiques ont été décrites pour ces GSL « simple ». Les halicérébrosides isolés de l'éponge *Haliclona* sp. possèdent une double liaison en position 6 sur la base sphingoïde comme l'Axidjiferoside A. La seule différence notable dans leur structure est la présence d'un glucose à la place d'un galactose. Ces composés inhibent les cellules leucémiques murines P388 à 20 µg/mL (59). Les halicylindrosides de l'éponge *Halichondria cylindrata* diffèrent de l'Axidjiferoside A du point de vue structural, par le sucre qui est une *N*-acétylglucosamine. Ces composés possèdent une activité antiproliférative sur les cellules leucémiques murines P388 à 6,8 µg/mL (60). Enfin les Ophidiastérosides isolés de quelques étoiles de mer sont des GSL dont la partie sucre est un ß-glucopyranoside, la chaîne longue est tri-insaturée en 4, 8, 10 avec une ramification méthyle en C-9. Ces composés ont une activité antiproliférative sur cellules leucémiques murines L1210 à 2µg/mL (103). Un d'entre eux, l'Ophidiacerebroside-C, isolé de l'étoile de mer *Narcissia canariensis* a montré une activité cytotoxique intéressante sur des lignées cellulaires cancéreuses humaines (myélome multiple KMS11, carcinome colique HTC-116 et

gliobastome multiple GBM) avec une CI_{50} de 20 μM. L'Axidjiferoside-A testé sur ces trois dernières lignées possède une CI_{50} supérieure à 60 μM. On peut se demander si le fait d'avoir un galactose ou un glucose semble un élément important dans l'activité cytotoxique. Malheureusement, aucun GSL avec un galactose en position beta, n'est répertorié dans la littérature comme cytotoxique.



Axidjiferoside-A, n = 17, 18, 19 (major), 20

Figure 58. F13-3 et Axidjiferoside-A

Notre résultat semble indiquer que l'activité biologique est optimale si la chaîne d'acide gras est longue et sans double liaison, si l'ose est un glucose et si l'aminoalcool à longue chaîne possède des doubles liaisons (Fig. 58).

Composé	NSCLC-N6	A549
Myrmekioside E (E-2) peracétylé	7.3 ± 0.2	9.7 ± 0.2
Myrmekioside E (E-3) désacétylé	126.3 ± 2.4	Inactive
Myrmekioside E (E-1) natif	61.7 ± 3.4	54.6 ± 1.4
Trikentroside natif	12.1 ± 0.2	--

Tableau 10. .Les mesures de CI_{50} (μM) pour les Myrmekioside E et Trikentroside

- La comparaison structure-activité entre Myrmekioside E et Trikentroside (Tab. 10) sur les lignées cancéreuses du poumon non-à-petites-cellules NSCLC-N6 et sur des cellules de tumeur de poumon A549 a montré que, tout d'abord, la présence des 2 pyranoses semble importante : le Trikentroside est 5 fois plus cytotoxique que le Myrmekioside E natif sur NSCLC-N6. De cette observation, la partie sucre qui semble importante pour l'activité a été modifiée en l'acétylant et desacétylant ce Myrmekioside E-1. La cytotoxicité de Myrmekioside E-2 est presque huit fois supérieure à celle du Myrmekioside E-1 (cette augmentation a eu lieu sur les deux lignées de cellules), mais Myrmekioside E-1 a perdu la moitié de sa toxicité par desacétylation. D'autre part, la présence ou l'absence du groupe *N*-acétylé dans la structure du sucre n'a pas d'effet sur la cytotoxicité. La différence entre la cytotoxicité de Myrmekiosides E-1, E-2 et E-3 peut être expliquée par leurs polarités différentes. Il peut être suggéré que le caractère liposoluble du Myrmekioside peracétylé E-2 explique que cette molécule est plus en mesure de pénétrer la bicouche lipidique et de traverser plus facilement la membrane cellulaire. D'autre part, la comparaison de la toxicité de Trikentroside et Myrmekioside E peut également montrer l'importance de la relation entre un nombre total d'atomes de carbone plus important et une toxicité plus grande. De même la présence d'une double liaison peut jouer un rôle dans l'augmentation de la toxicité. Par contre la présence du groupe alcool terminal qui va diminuer légèrement la lipophilie va

plutôt amoindrir la toxicité. (Tab. 10 et Fig. 59).

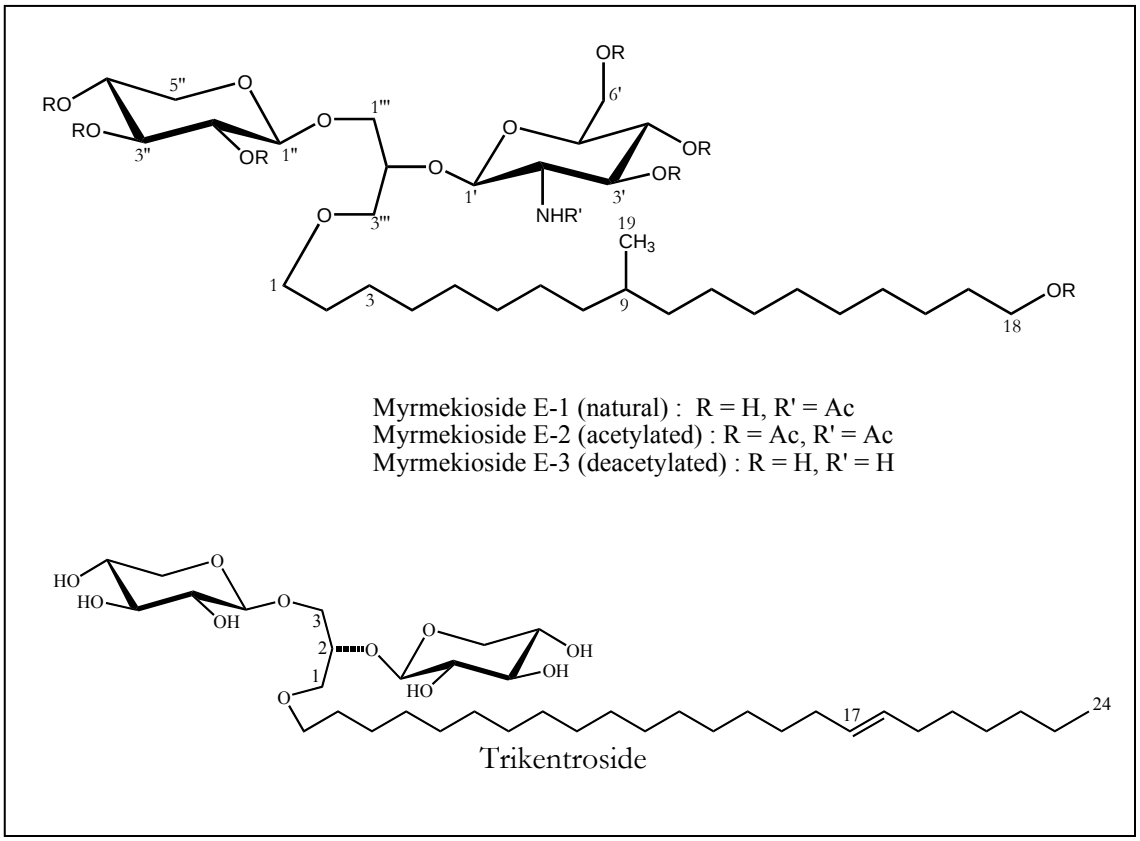


Figure 59. Myrmekioside E et Trikentroside

- L'angiogenèse tumorale conditionne le développement tumoral et les métastases. Formée par bourgeonnement des vaisseaux vicinaux et le recrutement des précurseurs de la moelle osseuse, elle est anormale, immature, chaotique et favorise la dissémination des cellules tumorales. L'évaluation est faite sur lignée humaine de hautes cellules endothéliales de ganglions lymphatiques périphériques, clone B3 (HPLNEC B3) et filmée pendant 20h. On peut alors apprécier la présence ou non du bourgeonnement des vaisseaux. En ce qui concerne l'activité anti-angiogénique, le Myrmekioside E-1 est en cours d'évaluation. Nous pouvons cependant donner les résultats d'étude sur le F13-3 et sur le Myrmekioside peracetylé E-2. Le F13-3 est très faiblement anti-angiogénique. Même si le Myrmekioside peracetylé E-2 montre une activité anti-angiogénique légèrement supérieure à celle de F13-3,

ceci n'est pas comparable à l'activité fortement anti-angiogénique obtenue jusqu'ici par le Laboratoire avec le Trikentroside (résultats non publiés).

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Annexe : Les articles

1. Cytotoxicity on Human Cancer Cells of Ophidiacerebrosides Isolated from the African Starfish *Narcissia canariensis*

2. Antiproliferative activity against human non-small cell lung cancer of two *O*-alkyl-diglycosylglycerols from the marine sponges *Myrmekioderma dendyi* and *Trikentrion laeve*

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Article

Cytotoxicity on Human Cancer Cells of Ophidiacerebrosides Isolated from the African Starfish *Narcissia canariensis*

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Abstract: The starfish *Narcissia canariensis* harvested from the coasts off Dakar, Senegal, was investigated for glycolipids (GL). This report deals with the isolation, characterization and biological activity of a fraction F13-3 separated from the GL mixture and selected according to its ability to inhibit KB cell proliferation after 72 hours of treatment. Firstly, a GL mixture F13 was obtained that accounted for 1.36% of starfish biomass (dry weight) and 0.36% of total lipids. The fraction F13-3 obtained from F13 contained three homologous GL identified as peracetylated derivatives on the basis of chemical and spectroscopic evidence. These contained a β-glucopyranoside as sugar head, a 9-methyl-branched 4,8,10-triunsaturated long-chain aminoalcohol as sphingoid base and amide-linked 2-hydroxy fatty acid chains. The majority (63%) had an amide-linked 2-hydroxydocosanoic acid chain and was identified as the ophidiacerebroside-C, firstly isolated from the starfish *Ophidiaster ophidiamus*. The minor components of F13-3 differed by one more or one less methylene group, and corresponded to ophidiacerebroside-B and -D. We found that F13-3 displayed an interesting cytotoxic activity over 24 hours on various adherent human cancerous cell lines (multiple myeloma, colorectal adenocarcinoma and glioblastoma multiforme) with an IC_{50} of around 20 μM.

Keywords: glycosylceramides; human cancer cell lines; *in vitro* anticancerous activity; *Narcissia canariensis*; starfish

Abbreviations: COSY: homonuclear correlation spectroscopy; FAME: fatty acid methyl ester; GC-MS: gas chromatography-mass spectrometry; GL: glycolipid(s); GSL: glycosphingolipid(s); HMBC: heteronuclear multiple bond coherence; HSQC: heteronuclear single quantum coherence; IC_{50}: 50% inhibitory concentration; ESI-MS: electrospray ionization mass spectrometry; TLC: thin layer chromatography

1. Introduction

Nowadays, myeloma, glioblastoma and carcinomas are a real public health problem in the world with increasing mortality rates in developing countries.

Multiple myeloma (MM) is a cancer of the white blood cells known as plasma cells; it is characterized by skeletal destruction, renal failure, anemia and hypercalcemia. Despite progress in the management of patients, MM remains an incurable disease, with a five-year survival rate not exceeding 50%. Recent advances in the understanding of the pathobiology of multiple myeloma (MM) have provided the basis for a more comprehensive effort to develop novel therapies for this disease [1,2]. As myeloma cells develop mechanisms of resistance to most known treatments [3], the search for new efficient anti-cancerous compounds is needed.

Glioblastoma is the most common and most aggressive type of primary brain tumor in humans, involving glial cells. Glioblastoma multiforme (GBM) is the highest grade glioma (grade 4) tumor and the most malignant form of astrocytomas. In most European and North American countries, incidence is approximately 2–3 new cases per 100,000 people per year. This malignancy of the central nervous system is fatal despite treatment with surgery and adjuvant therapy. In the United States, GBM occurs at a frequency of approximately 5000 cases annually, and constitutes up to 80% of all malignant gliomas. Long-term control of these tumors is rarely achieved, despite surgical resection and external beam radiation therapy, and GBM recurs within 6–10 months with a median survival of approximately 12 months [4].

Carcinomas are invasive malignant tumors consisting of transformed cells arising from epithelial origin. Epithelial cells cover the external surface of the body, line the internal cavities and form the lining of glandular tissues. Carcinomas are classified by their histopathogical appearance referring to the putative cell of origin or primary organ. With more than 600,000 deaths worldwide per year, colorectal carcinoma is the fourth most common form of cancer in the United States while approximately 36,000 persons will be newly diagnosed with oral cancer in 2010 [5].

Glycosphingolipids (GSL) are ubiquitous membrane constituents in animals, which play a fundamental role in major phenomena such as cell-cell recognition and antigenic specificity [6,7]. In general GSL exhibit a wide range of biological functions that might be related to the amphipathic nature of the molecule. Several GSL and other various glycolipids (GL) have been isolated from a number of marine sources, mainly including sponges and echinoderms. Glycolipids are known to possess immunomodulating and antitumor activity in particular those isolated from sponges [6–9].

Among them, GSL represent a large group of biomolecules containing two basic structural units: a sugar linked to a ceramide. The hydrophobic ceramide portion involves a sphingoid base and an amide-linked fatty acyl chain. Various cerebrosides, glycosylceramides with a single sugar head, were isolated from sponges [6–9], tunicates [10], octocorals [11], and echinoderms [12–25]. The phylum Echinodermata comprises approximately 7000 living species and their cerebrosides in particular have been chemically studied but little is known regarding their biological and pharmacological properties.

In the search for new efficient GL against cancer, we investigated a not yet studied starfish, *Narcissia canariensis*, harvested off Dakar, Senegal. This paper reports on the isolation of a GL fraction containing particular GSL named ophidiacerebrosides and the evaluation of their cytotoxic activity against various human cancer cell lines.

2. Results and Discussion

2.1. Glycolipid Isolation and Structure Determination

The common African starfish *Narcissia canariensis* was investigated for lipids and GL fractions. The lipid extract (8.97 g) obtained with CH_2Cl_2/MeOH was subjected to lipid class separation by column chromatography affording a crude GL fraction (0.92 g). A subsequent column chromatography enabled obtaining a bioactive GL fraction named F13 selected for its ability to inhibit KB cell proliferation significatively. The F13 fraction was in turn subjected to a further preparative chromatography that enabled obtaining a purified GL fraction named F13-3 as a white amorphous powder. It showed a single spot on thin layer chromatography with an Rf value similar to that of a commercial standard galactocerebroside with a hydroxylated acyl chain. Interestingly, fraction F13 represented 1.36% of the starfish biomass (dry weight), 0.36% of total lipids and 3.58% of the total GL. Fraction F13-3 contained a major GSL (63%) and two minor homologous ones as shown by NMR and mass spectrometry studies. Thus, the peracetylated F13-3 exhibited the characteristic signals of a sphingoid base and a β-glucopyranose in the ^{1}H-NMR spectrum (Figure 1, Table 1). Its electrospray ionization mass spectrometry (ESI) showed three molecular ion peaks, corresponding to three glycosylceramides with three different α-hydroxylated fatty acyl chains. Indeed, the peracetylated major GSL component displayed an adduct ion $[M + Na]^+$ at *m/z* 1084.6880 (high resolution ESI) in accordance with the formula $C_{59}H_{99}NO_{15}Na$ (a molecular mass of 809.7 amu for the intact GSL). The minor GSL of peracetylated F13-3 displayed sodiated molecular ions at *m/z* 1070.6702 and 1098.7051 in accordance with a methylene less or more than the major one. The structure of the major cerebroside was determined on the basis of chemical and spectroscopic evidence. Thus, this glycosylceramide contains a triunsaturated long-chain aminoalcohol as the principal sphingoid base. The optical rotation value of peracetylated F13-3 was determined as $[\alpha]_D^{30} = +0.44$ (*c* = 0.4, CH_2Cl_2).

Figure 1. Glucosylceramides for *Narcissia canariensis*: ophidiacerebroside-B (n = 16), ophidiacerebroside-C (n = 17) and ophidiacerebroside-D (n = 18).

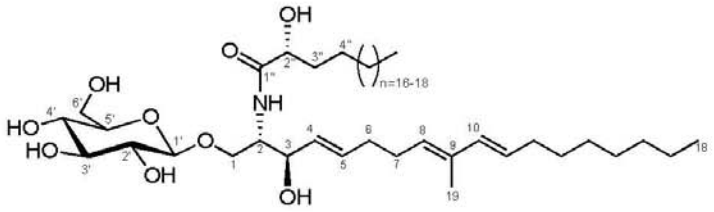


Table 1. ^{1}H, ^{13}C and COSY NMR spectroscopy data for peracetylated F13-3 glycolipids in $CDCl_3$.

Position	δ_H ppm, mult., J in Hz	δ_C ppm	COSY correlation
NH	6.35 (d, J = 9.0)	-	
1a	3.95 (dd, J = 10.0/3.8)	67.2	2, 1b
1b	3.62 (dd, J = 10.0/3.8)	67.2	2, 1a
2	4.32 (m)	72.7	1a, 1b, 3, NH
3	4.27 (d, J = 4.6)	50.6	4, 2
4	5.34 (m)	128.7	3, 5
5	5.43 (dd, J = 6.5/15.0)	124.8	4, 6
6	2.07 (s)	32.3	5, 7
7	2.20 (s)	31.9	6, 8, 19
8	5.83 (m)	136.4	7
9	-	134.2	
10	6.04 (d, J = 15.4)	134.4	11
11	5.59 (m)	127.8	10, 12
12	2.10 (m)	32.9	11, 13
19	1.73 (s)	12.5	7, 8
CH_2	1.27 (m)	22.7–29.7	
CH_3 acetates	2.02/2.04/2.05/2.08/2.11/2.19 (6s)	20.6–21.0	
C=O acetates		169.3/169.4/169.6/160.7 /169.8/169.9	
terminal CH_3	0.90 (t, J = 6.8)	14.1	
1′	4.49 (d, J = 7.9)	100.5	2′
2′	4.97 (dd, J = 8.0/9.5)	71.2	1′, 3′
3′	5.20 (t, J = 9.5)	72.7	2′, 4′
4′	5.10 (t, J = 9.7)	68.2	3′, 5′
5′	3.71 (m)	71.9	4′, 6′a, 6′b
6′a	4.24 (d, J = 4.5)	61.8	5′, 6′b
6′b	4.16 (dd, J = 12.5/2.3)	61.8	5, 6a
1″	-	172.2	
2″	5.36 (m)	73.0	
3″	1.83 (m)	31.8	
4″	1.40 (m)	29.5	
terminal CH_3	0.83 (m)	14.1	

All the chemical shifts of the ceramide are given in the ^{13}C and ^{1}H NMR spectra (Table 1). The sugar linked to the ceramide was identified as glucose by NMR spectroscopy. First of all, the anomeric proton of the β-glucopyranoside (δ = 4.49, d, J = 7.9 Hz) was correlated with the anomeric carbon at δ = 100.5 ppm in the HMQC spectrum. Starting from this proton, all the ^{1}H and ^{13}C NMR signals of the sugar were assigned by using the COSY, HMQC and HMBC spectra, and the vicinal proton-proton coupling constants were determined (Table 1). The *gluco* configuration of the sugar, as well as its β anomeric configuration, was established on the basis of the ring proton coupling constants ($J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.5 Hz, $J_{3,4}$ and $J_{4,5}$ = 9.6 Hz). The linkage of the glucopyranoside to the ceramide was confirmed by the three bond ^{13}C-^{1}H couplings of anomeric C-1′ with H-1a and H-1b observed in the HMBC spectrum. The presence of signals for five olefinic protons and the singlet at δ = 1.73 ppm indicated that a methyl branch is linked to an olefinic carbon atom. The unsaturation pattern in the ^{1}H NMR spectrum showed a multiplet at 5.34 ppm and a double doublet at 5.43 ppm, characteristic of a Δ^4 sphingosine with *trans* configuration (J = 15.0 Hz), a doublet at 6.04 ppm (J = 15.4 Hz) for a *trans* Δ^{10} double bond, and multiplets at δ 5.59, 5.83 ppm. COSY correlations were observed between H-7 and H-8 and H-19 (Table 1). Further correlations were observed between olefinic signals at δ 5.83 (H-8) and 5.59 (H-11), and vicinal methylene groups at positions 7 and 12. And also we observed no correlation between the olefinic carbon C-9 and an olefinic proton in the HSQC spectrum. The key HMBC correlations from H_3-19 to C-8, C-9, and C-10, and from C-11 to C-9, confirmed the location of the positions of the double bonds. These data allowed us to establish the olefinic pattern of the sphingoid unit as a 9-methyl-4,8,10-triene.

To determine the structure of the ceramide, F13-3 was subjected to an acidic methanolysis and the resulting reaction mixture was separated by partitioning between CH_2Cl_2 and H_2O/MeOH into an aqueous phase containing methylglycosides and an organic phase containing 2-hydroxylated fatty acid methyl esters (FAME) and sphingoid bases. Thereafter the latter mixture was analyzed by GC/MS. Only one sphingoid base was observed.

The FAME mixture from the active fraction F13 was analyzed by GC-MS. The 2-hydroxy FAME produced the characteristic ions at *m/z* 90 (McLafferty) and *m/z* 103. The results were as follows: 2-OH-21:0, t_R = 33.9 min (15.4%), *m/z* 356 (M^+); 2-OH-22:0, t_R = 36.4 min (63.6%), *m/z* 370 (M^+); 2-OH-23:0, t_R = 38.8 min (21.0%), *m/z* 384 (M^+). These fatty acid structures were confirmed by GC-MS analysis of *N*-acyl pyrrolidides showing fragment ions at *m/z* 129 (McLafferty) and the expected molecular ions. The peracetylated methylglycoside from F13-3 was analyzed by GC-MS (column temperature 110 °C (2 min) and then (temp. increasing at 3 °C/min until 240 °C)); t_R = 31.6 min (methylglucopyranoside) similar to that of an authentic sample. Other diagnostic ions were observed at *m/z* 331 $(M\text{-}OMe)^+$, 303 $(M\text{-}OAc)^+$, 243, 200, 157, 145 and 115.

These data showed that F13-3 contained a ceramide composed of the known 4,8,10-triunsaturated, 9-methyl branched C_{18} sphingoid base and 2-hydroxylated fatty acyl chains like cerebrosides isolated from other invertebrates [8,10,11] including starfish [16,19,20,22,25].

2.2. Cytotoxic Activity

The cytotoxic activity of F13-3, including ophidiacerebroside-C as major component, was detected and followed using KB cells (human oral epidermoid carcinoma) (IC_{50}: around 20 μM after 72 h of

treatment). Thereafter it was investigated on three human cancerous cell lines, KMS-11 (adherent plasma cells obtained from patients with multiple myeloma [26]), GBM (astrocytoma cells obtained after tumor resection of patients with glioblastoma multiforme-primary culture [27]), and HCT-116 (colorectal adenocarcinoma cells derived from a patient with Lynch's syndrome [28] and as described in the experimental section). Results are shown in Table 2.

Table 2. IC_{50} measures for F13-3 (μM ± S.E., 24 h of treatment).

IC_{50}	KMS-11	HCT-116	GBM
F13-3	15.2 ± 4.0	18 ± 3.9	34.6 ± 5.1

The activities observed, mainly on KMS-11 and HCT-116, are interesting as ophidiacerebrosides have not yet been evaluated on human cancer cells. Cytotoxicity on these three cell lines was already found in the same range of concentration for some synthetic bile acid derivatives (LD_{50}: 8.5 μM) in a recent study [29]. A mixture of ophidiacerebrosides with C_{20} to C_{24} 2-hydroxyacyl chains, including the major ophidiacerebroside-C with an acyl chain C_{22} occurring at 40%, has been described to display strong cytotoxicity against L1210 murine leukemia cells *in vitro* [16]. Cerebrosides isolated from a tunicate, phalluside-1 and -2, contain the same triunsaturated sphingoid base and sugar head, but they differ in 2-hydroxyacyl chain lengths, C_{16} and C_{18} respectively. Interestingly, the latter cerebrosides were found inactive against human cancer cells including lung carcinoma (A 549), colon carcinoma (HT 29), and melanoma (MEL 28) [10]. In addition, cerebrosides named renierosides with the same sphingoid base and various monounsaturated 2-hydroxylated fatty acyl chains were found inactive against five human solid tumor cell lines [8]. These results suggest that the nature of the 2-hydroxylated fatty acyl chain (chain length and possible double bonds) seems to be important for the cytotoxic activities of this type of cerebrosides. Recently, it was shown that the nature of the sugar residue may be relevant for the biological activity of this type of GSL; those with glucopyranosides showing stronger cytotoxicity than those with galactocerebrosides [25]. Due to its potential biological interest, phalluside-1 found in the ascidian *Phallusia fumigata* [10], the sea stars *Allostichaster inaequalis* [20] and *Cosmasterias lurida* [19], has recently been recently synthesized [30].

In conclusion, this study provides an additional source (another starfish) for ophidiacerebrosides and points out the potential of these compounds against human cancerous cells. It would be of interest to investigate other GL fractions of *N. canariensis* for glycosylceramide isolation, in particular those with the same sphingoid base but differing by 2-hydroxylated acyl chain length and to compare their cytotoxic activities using the same panel of human cancer cell lines.

3. Experimental Section

3.1. General Procedures

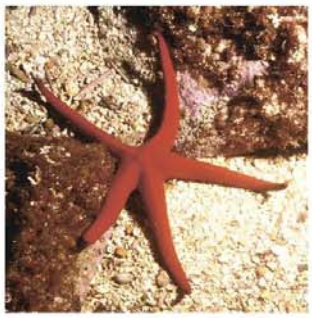
High resolution electrospray ionization mass spectrometry (HR-ESI-MS, positive mode, ion-source acceleration 4.5 kV, ion-source temperature 200 °C, methanol as solvent) mass spectra were recorded with a Micromass Zab Spec Tof spectrometer. ^{1}H- and ^{13}C-NMR as well as 2D-NMR spectra were obtained on a NMR Bruker Avance-500 spectrometer with triple Probe TBI multinuclear in $CDCl_3$ at 500.13 MHz and 125.76 MHz respectively, with reference to an internal standard of tetramethylsilane.

Chemical shifts and coupling constants were expressed in δ (ppm) and Hz respectively. GC-MS spectra were performed on a Hewlett-Packard 6890 gas chromatograph with a mass selective detector MS HP 6890 MS, Little polar column DB-1, 60 m length × 0.25 mm i.d. × 0.25 μm phase thickness. The temperature of the column was varied, after a delay of 2–4 min from the injection, from 110 to 310 °C with a slope of 3 °C min^{-1}. Optical rotations were measured in CH_2Cl_2 solutions with a Polartronic NH8 Schmidt/Haensch polarimeter at 30 °C. Analytical TLC was performed on precoated silica gel F_{254} plates. After development, the dried plates were sprayed with 50% H_2SO_4-vanillin and orcinol reagents.

3.2. Animal Material

The starfish *Narcissia canariensis*, shown below in Figure 2 (photo taken by Dr. Patrice Petit de Voize, Dakar) is found on rocks along the Senegalese coasts off Dakar, at a depth range of 23–38 m and were collected by hand during a scuba diving expedition organized by Oceanium of Dakar in 2009, on the sites named Petit Seminole, Fayss and Thi Wa. The specimens were identified by Professor Peter Wirtz, University of Madeira, Portugal.

Figure 2. *Narcissia canariensis.*



3.3. Lipid Extraction and F13-3 Isolation

Whole bodies of the collected specimens (241.37 g dry weight) were chopped and twice extracted with CH_2Cl_2/MeOH (1:1, vol/vol) at room temperature. The combined extracts were concentrated *in vacuo* to give the crude extract, which was partitioned between H_2O and CH_2Cl_2/MeOH. The organic layer was concentrated *in vacuo*, and the residue (8.97 g, 3.7%) was chromatographed on silica gel column with pure solvents as successive eluents: Dichloromethane (neutral lipids, 6.30 g), acetone (GL, 0.92 g) and methanol (phospholipids, 1.68 g). The GL mixture was separated on silica gel column to give 14 fractions. Among them, fraction 1 was subjected to a silica gel column chromatography (CH_2Cl_2/MeOH, 95:5 to 80:20, vol/vol) affording 23 fractions. From the latter fractions, fraction 13 (F13, 33 mg) gave a positive test on KB cells, and presented a similar polarity to a commercial standard (galactocerebroside with 2-hydroxy fatty acyl chain type I) (Rf = 0.35 on silica gel thin layer chromatography, CH_2Cl_2/MeOH, 88:12, vol/vol). Then F13 was subjected to silica gel chromatography with a solvent system of CH_2Cl_2 with 5% to 15% MeOH vol/vol) to give seven fractions. The following fraction 3 (21 mg), designated as F13-3, was obtained as a white amorphous powder and

was used for biological studies. In order to determine the chemical structure, fraction F13-3 was peracetylated and studied by NMR and ESI-MS.

3.4. Acetylation of F13-3

A part of F13-3 (9 mg) was dissolved in 1 mL of acetic anhydride and some drops of dry pyridine. The reaction was allowed to proceed for 18 h in darkness at room temperature, and then the reaction mixture was partitioned between water and dichloromethane. The organic layer was washed with HCl 1 M, neutralized with a Na_2CO_3 solution, and dried on anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was weighed.

3.5. Methanolysis of F13-3

A part of F13 (3 mg) was heated with 0.9 mL of MeOH/H_2O/HCl (29:4:3, vol/vol/vol) at 80 °C for 18 h. The reaction mixture was extracted with H_2O/CH_2Cl_2 (3:9, vol/vol), the aqueous layer was concentrated to give methylglycosides, whereas the organic layer contained a mixture of fatty acid methyl esters (FAME) and sphingoid bases. A part (1/3) of the FAME was preserved, the other one was transformed into *N*-acylpyrrolidides (NAP) by heating in a mixture pyrrolidine/acetic acid (10:2, vol/vol, 1 mL) during 1 h at 85 °C. The reaction mixture was separated with H_2O/CH_2Cl_2 and the organic layer was dried on anhydrous Na_2SO_4, filtrated and weighed after solvent evaporation. The aqueous layer was neutralized by NaOH 1 M and extracted twice with diethyl ether. The organic layer, containing sphingoid base was dried and then acetylated. The aqueous layer containing methylglycosides was evaporated *in vacuo* and then acetylated before GC-MS analysis.

3.6. Cell Cultures

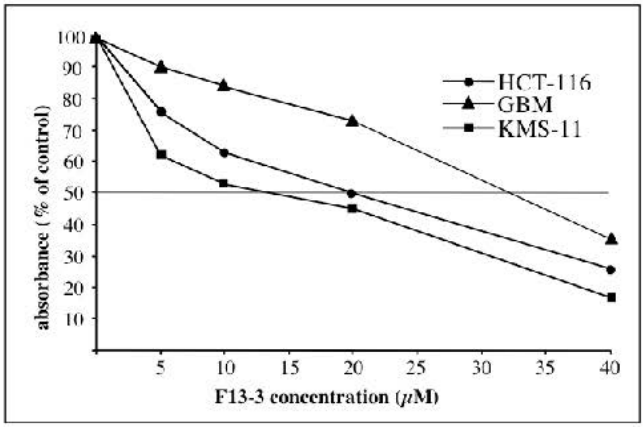
Cells were cultured in RPMI 1640 medium (KMS-11, GBM, HCT-116) or BME (KB) supplemented with 10% foetal calf serum, 2 mM glutamine, antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) (Life Technologies). Cells were subcultured at confluency after dispersal with 0.025% trypsin in 0.02% EDTA. Cells were maintained in plastic culture plate at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments cells were used at 70–80% confluency.

3.7. Neutral Red Assay

For cytotoxicity tests, 20,000 cells (GBM, HCT-116) and 50,000 cells (KMS-11) (200 µL) were plated in 96-well culture microtiter plates (Falcon) and incubated at 37 °C in 5% CO_2. After 24 h, drugs were added in 50 µL fresh medium, then after 21 h cells were loaded for 3 h with neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) (Sigma-Aldrich, St Quentin, France) at a final concentration of 50 µg/mL in culture medium. Thereafter (24 h of treatment) the medium was removed, cells were fixed for 5 min with a mixture of 1% formaldehyde-1% $CaCl_2$ and the dye extracted with 0.2 mL of 1% acetic acid in 50% ethanol. Plates were left overnight at 4 °C and absorbance was recorded at 570 nm (Multiskan EX-Thermo-Electron Corporation). Experiments were performed at least in triplicate, 4 wells per F13-3 concentration being used. IC_{50} (inhibition of cell

viability of 50%) values were calculated from the dose-response curves, an example is given in Figure 3. Statistics: Values are expressed as the mean of three independent experiments ± standard error.

Figure 3. Dose-response curve of F13-3 (24 h of treatment).



3.8. MTT Assay

After trypsinization KB cells were suspended as a 200,000 cells/mL suspension and 50 µL were dropped in each well of 96-well microplates (Costar, Corning, NY, U.S.). Tests were performed once the cells had settled at the bottom of the wells (48 h cultures) by incorporating 50 µL of test solutions. After 72 h of incubation, cell viability was determined using the colorimetric MTT assay according to Denizot and Lang [31]. This test was mainly used for the detection and follow-up of active fractions in the course of purification.

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Short communication

Antiproliferative activity against human non-small cell lung cancer of two *O*-alkyl-diglycosylglycerols from the marine sponges *Myrmekioderma dendyi* and *Trikentrion laeve*

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ABSTRACT

Glycolipids of *Myrmekioderma* sponges contain Myrmekiosides, a new family of glycolipids with a unique structure of mono-*O*-alkyl-diglycosylglycerols. This report deals with the identification and biological activity of the new Myrmekioside E from *Myrmekioderma dendyi*. Its structure has been elucidated from spectroscopic data and chemical degradation studies. It contained a glycerol backbone linked to xylose and *N*-acetylglucosamine, and an alkyl long-chain with a terminal alcohol group. A related glycolipid, Trikentroside, known in the sponge *Trikentrion laeve*, was subjected to a comparative biological evaluation. Both glycolipids inhibit proliferation of two human non-small cell lung cancer cell lines (NSCLC-N6 and A549).

1. Introduction

Lung cancer is the leading cause of cancer-related mortality in both men and women in the world. The prevalence of lung cancer is the second only after that of the prostate cancer in men and breast cancer in women. Lung cancer recently surpassed heart disease as the leading cause of smoking-related mortality. Most lung carcinomas are diagnosed at an advanced stage, conferring a poor prognosis. Lung cancers are generally divided into small cell lung cancer and non-small cell lung cancer (NSCLC). Non-small cell lung cancer accounts for approximately 85% of all lung cancers [1].

Marine organisms represent an interesting source of new molecules potentially useful as therapeutic agents. Thus, glycolipids (GL) are known to possess immunomodulating and antitumor activity, especially those from marine organisms [2,3]. They are known to play fundamental roles in major phenomena such as cell–cell recognition and antigenic specificity. Thus, marine invertebrates have been proven to be a source of promising bioactive GL. The most frequent encountered GL are glycosphingolipids which display a wide range of biological functions that might be related to the amphipathic nature of the molecule [2,3]. Various cerebrosides, glycosylceramides with a single sugar moiety, were isolated from sponges [2] and echinoderms like starfishes [3].

With the aim of discovering new and biologically-active GL from marine sponges, two additional genera were investigated, namely *Myrmekioderma* and *Trikentrion*. Several bioactive compounds were already isolated from *Myrmekioderma* and *Trikentrion* species. Several new terpenic products have been reported from *Myrmekioderma* sponges, e.g. *Myrmekioderma styx*, with various biological activities including those against hepatitis, HIV, tuberculosis, and also antifouling and hemagglutinating activities. In particular, cytotoxicity was shown against human primary tumor cells such as

Abbreviations: COSY, Homonuclear correlation spectroscopy; GC–MS, gas chromatography-mass spectrometry; GL, glycolipid(s); HMBC, Heteronuclear multiple bond coherence; HSQC, Heteronuclear single quantum coherence; IC_{50}, 50% inhibitory concentration; ESI-MS, Electrospray ionization mass spectrometry; FAB-MS, Fast atom bombardment mass spectrometry; HPLC, High-performance liquid chromatography; TLC, Thin layer chromatography.

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P-388 and A 549 cancer cell lines [4]. The sponge *Trikentrion laeve* (= *loeve*) contained two new compounds: the Trikentramine and the anti-HIV-1 pigment Trikentriol, whereas *Trikentrion flabelliforme* contained the new antimicrobial indolic Trikentrins [5]. The ether-linked GL, Myrmekiosides A and B, isolated from *Myrmekioderma* sp. were able to reverse the phenotype of melanoma H-ras transformed NIH3T3 cells [6].

They contained two glucoses and one xylose as the sugar moiety and an *O*-alkyl chain. Our previous work on polar lipids of *Myrmekioderma dendyi* showed that phospholipids accounted for about 15.5% of total lipids [7] and that the ether-linked Myrmekiosides C and D isolated from the same sponge showed an antitumor activity on THP1 cells [8]. Two new glycolipid ethers named Myrmekiodermins were isolated from the n-BuOH extract of *M. dendyi*, among them one had a methyl branched alkyl chain [9]. The location of the methyl branch was not determined and no biological property was reported for the latter compounds. It is noteworthy, to the best of our knowledge, that such unusual alkyldiglycosylglycerol GL structure was only found in the sponge genera *Myrmekioderma* and *Trikentrion* [6,8–10].

As a part of our continuing search for new efficient marine GL against cancer, we report here the identification of a new active alkyldiglycosylglycerol from *M. dendyi* harvested from South Pacific. On the other hand, the major ether-linked GL of *T. laeve* was again isolated in order to study its activity.

2. Results and discussion

2.1. Chemistry

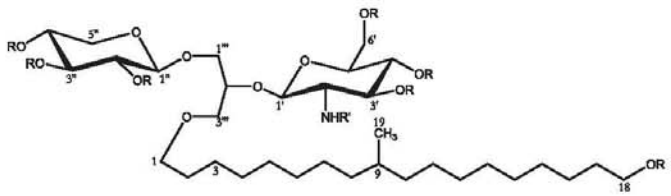
During the investigation of the complex glycolipid (GL) fractions from *M. dendyi*, a new compound was detected. Lipid fractions eluted with acetone and methanol displayed characteristic glycolipid spots on TLC. Among them, the first methanolic fraction, accounting for 3.2% of total lipids and seemed to be potentially interesting to find out GL with unusual structures, taking into account their relatively high polarities. This fraction was acetylated and displayed two spots on TLC. It was subjected to a silica gel column chromatography and then purified by repeated reverse phase HPLC. Two compounds were isolated and the least polar one named Myrmekioside E, accounting for 0.15% of total lipids, was investigated for biological activity. To facilitate we named the natural Myrmekioside E as Myrmekioside E-1, the peracetylated one as Myrmekioside E-2 and the deacetylated one as Myrmekioside E-3. Myrmekioside E-1 showed a quasi-molecular ion (ESI-MS) at m/z 732.4400 ($[M + Na]^+$) in accordance with the molecular formula $C_{35}H_{67}NO_{13}Na$. ^1H NMR spectrum of the Myrmekioside E showed two anomeric protons at δ 4.24 (d, $J = 5.9$ Hz) and δ 4.42 (d, $J = 7.9$ Hz), and signals at δ 6.80 (d, 8.6 Hz) and δ 1.85 (s) revealing the presence of two sugars, one of them being a *N*-acetylated amino sugar, and a long-chain alkyl group indicated by a large signal at δ 1.17. Further NMR experiments were performed using the peracetate derivative to take advantage of the better signal dispersion in the proton spectrum as usually observed. Myrmekioside E-2 showed a quasi-molecular ion at m/z 1026.5250 ($[M + Na]^+$), in accordance with the molecular formula $C_{49}H_{81}NO_{20}Na$ corresponding to a structure of alkyl-glycosylglycerol with a saturated branched chain possessing a terminal alcohol group (Fig. 1).

Determination of the length of the alkyl chain and location of the methyl branch could not be carried out by NMR analysis and required a controlled chemical degradation. Acid methanolysis afforded his two parts as useful derivatives for gas chromatography-mass spectrometry (GC-MS) analysis: sugar as methyl glycosides and *O*-alkylglycerol.

The ^1H NMR spectrum of Myrmekioside E-2 (Table 1), exhibited two doublets at δ 4.48 ($J = 7.08$ Hz) and δ 4.73 ($J = 8.4$ Hz), identified as the anomeric protons of two sugar residues by their correlation peaks in the HMQC spectrum with the anomeric carbons at δ 100.9. The large coupling constant of these protons in each sugar indicated the β-glycosidic linkage. Using each of these protons as a starting point, examination of the COSY spectrum allowed the sequential assignment for the sugar protons of a xylose and a *N*-acetylglucosamine. The presence of this amide group was indicated by a doublet at δ 5.64 ($J = 8.5$ Hz) and a singulet at δ 1.88 (acetyl group). Another remarkable signal was a large peak at δ 1.18 due to a long alkyl chain. In addition, a doublet at δ 0.75 ($J = 6.39$ Hz) was present, corresponding to a methyl attached on this chain. The ^1H NMR spectrum also exhibited a signal at δ 1.97 due to 7 *O*-acetyl groups. As only seven acetyl signals were present on the sugar residues, it remained one acetyl group on the alkyl chain. Furthermore, adjacencies among the three partial structures were revealed by the COSY and HMBC spectra of a xylose, *N*-acetylglucosamine, and an alkyl chain were attached to C-1‴, C-2‴, and C-3‴ of glycerol (Fig. 1).

To determine the structure of the *O*-alkylglycerol, Myrmekioside E-1 was subjected to an acidic methanolysis and the resulting reaction mixture was partitioned between CH_2Cl_2 and H_2O/MeOH to give an aqueous phase containing methyl glycosides and an organic phase containing *O*-alkyl-glycerol. Thereafter the latter mixture was analyzed by GC/MS.

The structure of the sugar residues was confirmed as peracetylated *N*-acetyl-pyranohexosamine and pyranopentose by mass spectral (ESI) data. Ion peaks at m/z 259 ($[M\text{-}OCH_3]^+$) and 330 (and m/z 362 ($[M + H]^+$)), respectively. The other characteristic fragment ions were present at m/z 86, 116 and 144 for the peracetylated pyranopentose and at m/z 115, 143, and 158 for the peracetylated *N*-acetylpyranohexosamine. The spectrum of the peracetylated *O*-alkylglycerol displayed the fragment ions m/z 441 ($[M - 59]^+$), 397, 381, 355, 341, 325 ($[M - 159]^+$), and 159, corresponding to a C_{19} chain with an acetylated terminal alcohol group,



Myrmekioside E-1 (natural) : R = H, R' = Ac
Myrmekioside E-2 (acetylated) : R = Ac, R' = Ac
Myrmekioside E-3 (deacetylated) : R = H, R' = H

Fig. 1. Myrmekioside E and its peracetylated and deacetylated derivatives.

Table 1
^1H, ^{13}C and COSY NMR data of Myrmekioside E-2 in $CDCl_3$ (300 MHz).

Position	δ_H ppm, mult., J in Hz	δ_C ppm	cosy correlation
N-acetyl-glucosamine			
NH	5.64 (d, J = 8.5)		2
1′	4.73 (d, J = 8.4)	100.9	2
2′	3.78 (m)	54.70	NH,1,3
3′	5.11 (m)	72.85	4
4′	4.99 (t, J = 9.6)	68.49	3
5′	3.58 (t, J = 5.2)	124.8	2,4,6a,6b
6′a	4.18 (dd, J = 12.03/4.8)	63.08	5.6b
6′b	4.08 (dd, J = 12.03/2.4)	63.08	5,6a
Xylose			
1″	4.48 (d, J = 7.08)	100.9	2
2″	4.80 (dd, J = 7.05/7.95)	71.51	1,3
3″	5.15 (t, J = 8.9)	71.90	2,4
4″	4.88 (dd, J = 9.0/5.1)	71.39	3,5a
5″ axial	3.33 (t, J = 6.6)	63.08	5e,4
5″ equatorial	4.06 (m)	63.08	5a,4
O-alkyl			
1	3.98 (d, J = 6.9)	64.66	2
2	1.52		1
3–8 et 10–16	1.1–1.46	37.08/32.75/29.76/ 29.69/29.50/29.25/ 28.58/27.04/26.19/25.20	
9	1.54 (m)	20.7	
17	1.48	29.69	18
18	3.29 (t, J = 11.1)	72.85	17
19	0.75 (d, J = 6.39)		
Glycerol			
1‴a	3.43 (m)	71.51	2
1‴b	3.60 (m)	71.51	2
2‴	3.85 (tt, J = 9.97/15.1)	68.49	1a,3a,2b,3b
3‴a	3.43 (m)	71.51	2
3‴b	3.60 (m)	71.51	2
Acetyl groups			
7-$OCOCH_3$	1.97 (m)	19.66/19.68/20.62/ 20.7/20.75/21.02/21.02	
-$NHCOCH_3$	1.88 (s)	23.25	

and the position of the methyl branch on C-9 was shown by the ion peaks at m/z 213 and 185 and those resulting from a loss of acetic acid (Fig. 2).

The position of the methyl branch on the alkyl chain, observed in the ^1H NMR spectrum, was determined by the study of the fragmentation patterns. The characteristic signals at m/z 213 and m/z 185 and those resulting from the loss of acetic acid indicated that the methyl group was attached at C-9 in this alkyl chain. The optical rotation value of Myrmekioside E-2 was determined as $[a]_D^{30} = -0.05$ (c = 0.4, CH_2Cl_2).

Interestingly regarding the product availability, we investigated lipids of *Trikentrion laeve* several times and observed that the ether-linked GL Trikentroside was always present [10]. The mass spectrum of the peracetylated Trikentroside showed a quasi-molecular ion at m/z 965.5441 [$(M + Na)^+$], in accordance with the molecular formula $C_{49}H_{82}O_{17}$ corresponding to the unusual GL which includes a glycerol unit, two xylopyranoses, and a C_{24} alkenyl ether chain and one double bond [10] (Fig. 3). In this work we examine its inhibitory effect on NSCLC-N6 and A549 cell lines.

2.2. Antiproliferative activity against human non-small cell lung cancer of Myrmekioside E and Trikentroside

Peracetylated Myrmekioside E (Myrmekioside E-2) from *M. dendyi* showed a significant activity on NSCLC-N6 cells with IC_{50} = 7.3 ± 0.2 μM and on lung tumor cells A549 with IC_{50} = 9.7 ± 0.2 μM, and also the native Trikentroside from *Trikentrion laeve* showed a significant activity on NSCLC-N6 cells with IC_{50} = 12.1 μM (Table 2).

3. Conclusion

This work confirms previous results that showed the interest of ether-linked glycoglycerolipids as potentially useful therapeutic agents [6]. Here, we compared the cytotoxicity of Myrmekioside E in its three forms with natural Trikentroside, whose structure has been already reported [10]. Firstly, having five times more toxicity of the Trikentrioside towards the Myrmekioside E seems to show the importance of the two xyloses. Of this observation, that the nature of the sugar residue appears to be relevant to the biological activity of these glycoglycerolipids, we decided to modify only the sugar residue by peracetylation of Myrmekioside E-1 followed by a deacetylation reaction. The toxicity of Myrmekioside E-2 is nearly eight times more than the native one (this increase occurred on both cell lines), but Myrmekioside E-1 lost the half of its toxicity by deacetylation. On the other hand, the presence or absence of the *N*-acetyl group in the GL structure has no effect in cytotoxicity. Furthermore, the *N*-acetyl-pyranohexosamine of the GL showed stronger cytotoxicity than the pyranohexosamine.

The difference between the cytotoxicity of Myrmekiosides E-1, E-2 and E-3 can be explained by their different polarities. It can be suggested that the liposoluble character of the peracetylated Myrmekioside E-2 explains that this molecule is more able to penetrate the lipid bilayer and crosses more readily the cell membrane.

Otherwise, comparing the toxicity of Trikentroside and Myrmekioside E can also show the importance of relationship between the total number of carbon atoms, double bonds and terminal alcohol group of the lipophilic part with the biological activity of these glycoglycerolipids.

The second GL isolated in this work, accounting for around 0.01% of total lipids, was another alkyl-glycosylglycerol with one pyranopentose and two pyranohexosamines as sugar units. Complete identification and study of its biological activity are in progress in the laboratory. Thus, Myrmekiosides, like most natural marine GL, are a complex mixture of homologs differing by their sugar moiety, the length and branching of their alkyl chains.

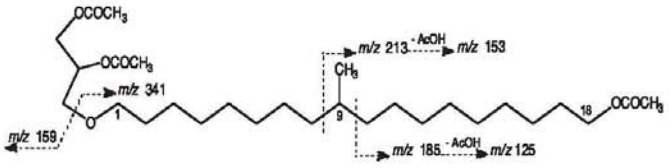


Fig. 2. Fragmentation pattern (e.i.) of the peracetylated O-alkylglycerol obtained from Myrmekioside E.

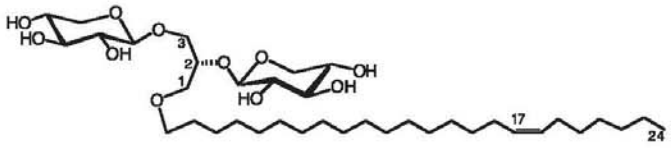


Fig. 3. Trikentroside from *Trikentrion laeve*.

4. Experimental section

4.1. General Procedures

High-resolution electrospray ionization mass spectrometry (HR-ESI-MS, positive mode, ion-source acceleration 4.5 kV, ion-source temperature 200 °C, methanol as solvent) mass spectra were recorded with a Micromass Zab Spec TOF spectrometer. ^{1}H and ^{13}C NMR as well as 2D-NMR spectra were obtained on a NMR Bruker Avance-300 spectrometer with triple Probe TBI multinuclear in $CDCl_3/CD_3OD$ (70:30, v/v) for natural GL, and $CDCl_3$ for peracetylated GL at 300.13 MHz and 75 MHz respectively, with tetramethylsilane as internal reference. Chemical shifts and coupling constants were expressed in δ (ppm) and Hz respectively. GC–MS spectra were performed on a Hewlett–Packard HP 5890 Series II coupled with the mass spectrometer HP 5989A (electronic impact 70 eV), integrator HP 98785, non-polar column DB-1 30 m length × 0.32 mm i.d. × 0.25 μm phase thickness. The temperature of the column was varied, after a delay of 2–4 min from the injection, from 110 to 310 °C with a slope of 3 °C min^{-1}. Optical rotations were measured in CH_2Cl_2 solutions with a Polartronic NH8 Schmidt/Haensch polarimeter at 30 °C. Analytical TLC was performed on precoated silica gel F_{254} plates. After development, the dried plates were spraying with 50% H_2SO_4-vanillin and orcinol reagents. High-performance liquid chromatography (HPLC) was carried out with an Intersphere 5 μm ODS2, 250 × 4.6 mm, *Interchim*, with a 240 nm refractive or UV detector.

4.2. Sponge collection and glycolipid isolation

M. dendyi (Burton, 1959) was collected in Vanuatu archipelago, Lamen Bay, Epi Island, in 1997 at 30 m depth by the French Institute of Research for Development, IRD, New Caledonia during the European Programme Marine Science and Technology MAST III. *T. laeve* (= *loeve*) (Carter, 1879) was obtained from rocky bottoms off Dakar, Senegal, in 2005 at 25–40 m depth. Specimens of both sponges were kept at −80 °C. Whole bodies of the collected specimens were chopped and extracted twice with CH_2Cl_2/MeOH (1:1, v/v) at room temperature. The combined extracts were concentrated *in vacuo* to give the crude extract, which was partitioned between H_2O and CH_2Cl_2. The lipid extract obtained in the CH_2Cl_2/MeOH phase was subjected to a lipid class separation by column chromatography affording a crude GL fraction. Lipid class separation was accomplished by open column chromatography on silica gel (70–230 mesh) successively eluted by dichloromethane (neutral lipids), acetone (glycolipids) and methanol (phospholipids and alkyl-glycosylglycerol ethers). The first methanolic fraction was purified by repeated reverse phase HPLC.

Table 2
IC_{50} values (μM) for Myrmekioside E derivatives and Trikentroside.

Product	NSCLC-N6	A549
Natural Myrmekioside E (E-1)	61.7 ± 3.4	54.6 ± 1.4
Peracetylated Myrmekioside E (E-2)	7.3 ± 0.2	9.7 ± 0.2
Deacetylated Myrmekioside E (E-3)	126.3 ± 2.4	Inactive
Natural Trikentroside	12.1 ± 0.2	–

4.3. Acetylation of Myrmekioside E-1

To prepare samples for NMR analysis, Myrmekioside E-1 was dissolved in acetic anhydride (1:0.002 mol/mol) and some drops of dried pyridine. The reaction was allowed to proceed for 18 h in darkness at room temperature, and then the reaction mixture was partitioned between water and dichloromethane. The organic layer was washed with 1 M HCl, neutralized with a Na_2CO_3 solution and dried on anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was weighed. Myrmekioside E-2 was obtained.

4.4. Deacetylation of Myrmekioside E-1

To obtain the deacetylated alkyldiglycosylglycerol a part of Myrmekioside E-1 (8.8 mg) was dissolved in 0.45 mL of MeOH, and 0.200 mL of 0.5 M MeONa in MeOH was added. The reaction was allowed to proceed for 18 h at 25 °C, then the reaction mixture was dried and the residue partitioned between water and chloroform. After removal of the solvent, the deacetylated alkyldiglycosylglycerol, which was named Myrmekioside E-3 was obtained [2c].

4.5. Methanolysis of Myrmekioside E-1

In order to provide samples for GC–MS, a part of Myrmekioside E-1 (6 mg) was heated with 0.9 mL of MeOH/H_2O/HCl (29:4:3, v/v/v) at 80 °C for 18 h. The reaction mixture was extracted with H_2O/CH_2Cl_2 (3:9, v/v), the aqueous layer was concentrated to give methyl glycosides, whereas the organic layer contained fatty acid methyl esters. A third of the latter esters was preserved, the remaining one was transformed into *N*-acylpyrrolidides (NAP) by heating in a pyrrolidine/acetic acid mixture (10:2, v/v, 1 mL) during 1 h at 85 °C. The reaction mixture was separated with H_2O/CH_2Cl_2. The aqueous layer was neutralized by 1 M NaOH and extracted twice with diethyl ether. The aqueous layer containing methyl glycosides was evaporated *in vacuo* and then acetylated before GC-MS analysis.

4.6. Cellular studies (NSCLC-N6 and A549)

The antiproliferative activity of Myrmekioside E-1, E-2 and E-3, and Trikentroside, was evaluated. The NSCLC-N6 cell line [11], derived from a human non-small-cell bronchopulmonary carcinoma (moderately differentiated, rarely keratinized, classified as T2N0M0), and A549 obtained from ATCC collection reference CCL-185 [12] were used for all experiments. Both cell lines were cultured in RPMI 1640 medium with 5% fetal calf serum, to which were added 100 IU penicillin.mL $^{-1}$, 100 μg streptomycin.mL $^{-1}$ and 2 mM glutamine, at 37 °C in an air/carbon dioxide atmosphere (95:5, v/v).

Cytotoxicity was determined by continuous drug exposure. Experiments were performed in 96 wells microtiter plates (10^5 cells mL^{-1} for NSCLC-N6 and 2×10^4 cells mL^{-1} for A549). Cell

growth was estimated by a colorimetric assay based on the conservation of tetrazolium dye (MTT) to a blue formazan product by live mitochondria [13]. Eight repeats were performed for each concentration. Control growth was estimated from 8 determinations. Optical density at 570 nm corresponding to solubilized formazan was read for each well on a Titertek Multiskan MKII.

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Résumé

Les glycolipides (GL) des éponges suscitent un intérêt croissant en raison de leurs propriétés immunomodulatrices et antitumorales et ceux des échinodermes restent peu documentés. L'isolement et la caractérisation de deux types de GL originaux biologiquement actifs est décrit : glycosphingolipides (GSL) et éthers de glycérol di-glycosylés alkylés. De l'étoile de mer *Narcissia canariensis* (Sénégal) a été isolé le GSL, Ophidiacérébroside-C, associant β-glucopyranoside et céramide (aminoalcool 9-méthyl-4,8,10-triéthylènique et chaîne acyle α-hydroxylée). Il est cytotoxique sur des lignées cellulaires cancéreuses humaines : CI_{50} de 15µM sur myélome multiple, 20µM sur adénocarcinome du colon et 35µM sur glioblastome multiforme. Les GSL majeurs de l'éponge *Axinyssa djiferi* (Sénégal), l'Axidjiferoside-A comportant un β-galactopyranoside, 2-amino-octadéc-6-èn-1,3,4-triol et une chaîne α-hydroxylée est prometteur, associant activité antipaludique (CI_{50} = 0.53 µM) sur une souche de *Plasmodium falciparum* résistante à la chloroquine, et absence de cytotoxicité sur cinq lignées cellulaires cancéreuses humaines. Les GL des éponges *Myrmekioderma,* Myrmekiosides, possèdent un nouveau type structural de mono-*O*-alkyldiglycosylglycérols. Le nouveau Myrmekioside-E a été isolé (Pacifique sud) : un glycérol mono-*O*-alkylglycérol lié à un xylose, à une *N*-acétylglucosamine et à une chaîne alkyle C_{18} méthylée en C-9 et hydroxylée en C-18. Le Myrmekioside-E peracétylé inhibe la prolifération des cellules de cancer de poumon humain N6 et A549 (CI_{50} = 7,3 µM et 9,7 µM respectivement). Un mono-*O*-alkylglycérol lié à deux xyloses isolé de *Trikentrion laeve* (Sénégal) est actif sur ces deux dernières lignées.

Mots-clés : activité anti-proliférative ; activité antipaludique ; *Axinyssa djiferi ;* échinodermes ; éponges ; éthers de glycérol glycosylés ; glycolipides ; *Narcissia canariensis*.

Abstract

Glycolipids (GL) from marine sponges give rise to a growing interest because of their immunomodulating and antitumoral properties, while those of the echinoderms remain little documented. This work describes the isolation and the characterization of two biologically active types of GL: glycosphingolipids (GSL) and ether-linked GL (mono-*O*-alkyl-diglycosylglycerols). A major GSL of the starfish *Narcissia canariensis* (Senegal), ophidiacerebroside-C, was isolated and identified. It contained a β-glucopyranoside, a 9-methyl-4,8,10-triunsaturated long-chain aminoalcohol amide-linked to a C_{22} α-hydroxylated acyl chain. This GSL and its two minor homologous ones displayed a cytotoxic activity over 24 h on various adherent human cancerous cell lines (multiple myeloma, colorectal adenocarcinoma and multiforme glioblastoma) with an IC_{50} of around 20 µM. The major GSL, Axidjiferoside-A, from the sponge *Axinyssa djiferi* (Senegal) contained β-galactopyranoside and 2-amino-octadec-6-en-1,3,4-triol linked to an α-hydroxytetracosanoic acyl chain. It seemed of interest, associating an antiplasmodial activity (IC50 = 0.53 µM against a chloroquine-resistant strain of *Plasmodium falciparum*) with a low cytotoxicity on five cancer cell lines. The new Myrmekioside-E from *Myrmekioderma dendyi* (south Pacific) contained a glycerol backbone linked to xylose and *N*-acetylglucosamine, and an alkyl long-chain with a terminal alcohol group. A GL named Trikentroside, with a glycerol, two xyloses and an unsaturated C_{24} chain, from the sponge *Trikentrion laeve* (Senegal) was subjected to a comparative biological evaluation. Both GL inhibit proliferation of two human lung cancer cell lines.

Key words: antiproliférative activity; antiplasmodial activity; *Axinyssa djiferi ;* echinoderms ; sponges ; ether-linked mono-*O*-alkyl-diglycosylglycerols; glycolipids ; *Narcissia canariensis*.