Tesi doctoral

PRODUCTES NATURALS COM A FONT DE NOUS FÀRMACS:

SÍNTESI EN FASE SÒLIDA DE DEPSIPÈPTIDS CÍCLICS I AÏLLAMENT

D'AGENTS ANTITUMORALS D'ESPONGES MARINES

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Per a vosaltres, família

Índex General

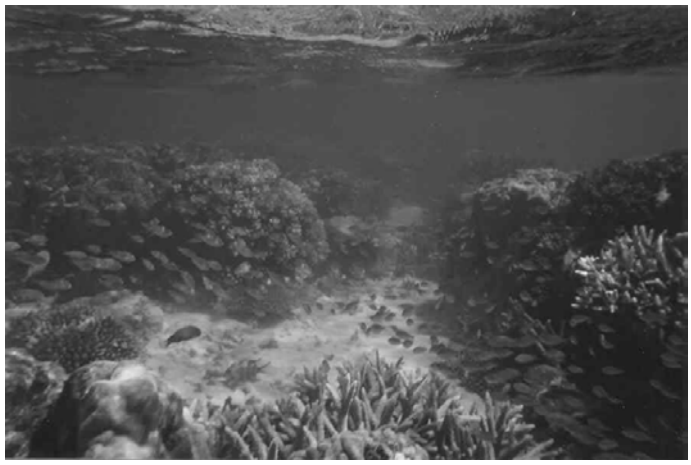
ABREVIACIONS

AA; aa*	Aminoàcid
AAA	Anàlisi d'aminoàcids
Acm	Acetamidometil
Ac_2O	Anhídrid d'acètic
AcOEt	Acetat d'etil
AcOH	Àcid acètic
Alloc	Al·liloxicarbonil
Alloc-Cl	Cloroformiat d'al·lil
Boc	*tert*-butoxicarbonil
Boc_2O	Anhídrid de *tert*-butoxicarbonil
CCF	Cromatografia en cap fina
Cis	Cistina
COSY	Espectrometria de correlació
^{13}C-RMN	Ressonància magnètica nuclear de carboni 13
CTC-PS	Resina 2-clorotritil o resina de Barlos
d	Dublet
δ	Desplaçament químic
DAST	Trifluorur de dietilaminosulfur
DBU	1,8-Diazabiciclo[5.4.0]unde-7-ene
DCM	Diclormetà
DEAD	Dietilazodicarboxilat
Dhb	Àcid α,β-didehidroaminobutíric
DHB	Àcid 2,5-dihidroxibenzoic
DIEA	*N,N'*-Diisopropiletilamina
DIPCDI	*N,N'*-Diisopropilcarbodiimida
DKP	2,5-Dicetopiperazina
CMB	Center of Molecular Biodiversity (Brisbane, Australia)
DMAP	4-Dimetilaminopiridina
DMF	*N,N'*-Dimetilformamida
DSC	Carbonat de *N,N'*-disuccinimil
DTT	Ditiotreitol
EDC·HCl	Hidroclouru de *N*-(3-dimetilaminopropil)-*n'*-etilcarbodiimida
EDTA	Àcid etilendiaminotetraacètic
EM	Espectrometria de masses
EM-IQ	Espectroscopia de masses amb ionització química
eq	Equivalent

* Les abreviacions emprades per als aminoàcids i pèptids segueixen les regles de la IUPAC-IUB descrites al *J. Pep. Sci.*, **2003**, *9*, 1-8. Tot i que les abreviacions de la IUPAC-IUB per un *N*-metil aminoàcid és MeXX, per evitar confusions amb els *N*-metil aminoàcids, emprarem l'abreviació *N*Me-XX.

ESI	Ionització per electrosprai
Et_3N	Trietilamina
Et_2O	Èter dietílic
EtOH	Etanol
f	Funcionalització del suport polimèric
Fmoc	9-Fluorenilmetoxicarbonil
Fmoc-Cl	Clorur de 9-fluorenilmetoxicarbonil
GP	Grup protector
HATU	Tetrafluorofosfat de *N*-òxid de *N*-[(dimetilamino)-1*H*-1,2,3-triazolo-[4,5-b]piridin-1-il-metilen]-*N*-metilmetanamini
HBTU	Hexafluorofosfat de *N*-òxid de *N*-[(1H-benzotriazol-1-il)-dimetilamino-metilen]-*N*-metilmetanamini
HFA	Hexafluoroacetona
HOAt	7-Aza-1-hidroxibenzotriazol
HOBt	1-Hidroxibenzotriazaol
HOSu	*N*-hidroxisuccinimida
HOPfp	Pentafluorofenol
HPLC	Cromatografia líquida d'alta pressió
HPLC-EM	Cromatografia líquida d'alta pressió amb detecció per espectrometria de masses
HPLC-ELSD	Cromatografia líquida d'alta pressió amb detecció per dispersió de la llum
3HQA	Àcid 3-hidroxiquinàldic
^{1}H-RMN	Ressonància magnètica nuclear de protó
Hz	Herz
IC_{50}	Concentració de mostra que causa el 50 % de mort cel·lular
IG_{50}	Concentració de mostra que inhibeix el 50 % del creixement cel·lular
IR	Espectroscopia d'infraroig
J	Constant d'acoblament
λ	Longitud d'ona
m	Multiplet
MALDI-TOFF	Espectrometria de masses de desorció iònica provocada per làser, assistida per matriu i anàlisis de temps de vol
MeCN	Acetonitril
MeOH	Metanol
MFS	*N*-metilació en fase sòlida segons Miller i Scanlan
MS	Espectrometria de masses
Me	Metil
Mmt	Metoxitritil
MSNT	1-(mesitilé-2-sulfonil)-3-nitro-1,2,4-triazol
MST	Àcid mirístic (ácid tetradecanoic)
MTBD	Metil-4-nitrobenzenesulfonat
***n*-BuOH**	*n*-Butanol
***N*Me**	Aminometil
NMeI	*N*-metilimidazol
NOESY	Espectrometria d'efecte nuclear Overhauser
Npys	3-nitro-2-piridilsulfenil

OMS	Organització Mundial de la Salut
oNBS	o-Nitrobenzenesulfonil
OSu	Èster de *N*-hidroxisuccinimida
PDA	Fotodiode array
Pip	piperidina
***p*NZ**	*p*-Nitrobenziloxicarbonil
***p*NZ-Cl**	Cloroformiat de *p*-nitrobenzil
PPh_3	Trifenilfosfina
ppm	Parts per milió
PS	Poliestirè
PTSA	Àcid p-toluensulfònic
PyOAP	Hexafluorofosfat de (7-azabenzotriazol-1-iloxi)-tris(pirrolidin)fosfoni
QNA	Àcid quinàldic
QXA	Àcid 2-quinoxalinecarboxílic
R_f	Factor de retenció a la cormatografia de capa fina
s	Singulet
Scm-Cl	Clorur de metoxicarbonilsulfenil
^{t}Bu	*tert*-Butil
TBAF	Fluorur de n-butilamoni
TBDMS	*tert*-Butildimetilsilil
TBME	*tert*-Butilmetilèter
TBTU	Tetrafluoroborat de *N*-òxid de *N*-[(1*h*-benzotriazol-1-il)-dimetilaminometilen]-*N*-metilmetanamini
Tce	Èster de 2,2,2-tricloroetanol
TFA	Àcid trifluoroacètic
THF	Tetrahidrofurà
TES	Trietilsilà
TGI	Concentració de mostra que causa la inhibició total del creixement
TIS	Triisopropilsilà
TMS	Trimetilsilil
TMS-Cl	Clorur de trimetilsilil
t_R	Temps de retenció
Troc	2,2,2-tricloroetoxicarbonil
Troc-Cl	Cloroformiat de 2,2,2-tricloroetil
Trt	Tritil
Trt-Cl	Clorur de tritil
Ts	Tosil
UV	Ultraviolat
Z	Benziloxicarbonil
&	Enllaç



Capítol 4

Antitumoral compounds isolation from marine sponges

RESULTATS I DISCUSSIÓ

4.1 BIOACTIVE COMPOUNDS FROM MARINE SPONGES

Marine sponges have the potential to be the source of future drugs against important diseases, such as cancer, a broad range of viral diseases, malaria and inflammations diseases. Pharmaceutical interest in sponges aroused in the early 1950s by the discovery of two nucleosides, spongothymidine and spongouridine in the marine sponge *Cryptotethia crypta*[183]. These compounds were the basis for the synthesis of Ara-C, the first marine-derived anticancer agent, and the antiviral drug Ara-A (Figure 4.1).

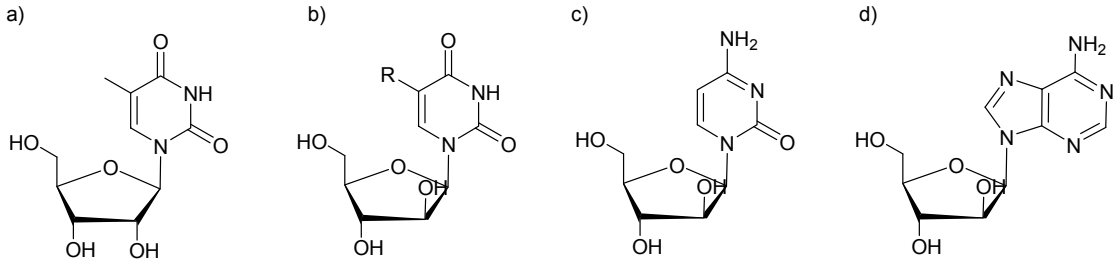


Figure 4.1 a) Human nucleoside thymidine, b) Nucleoside isolated from sponge, c) and d) Derived nucleosides

[183] Bergmann, W.; Feeney, R. J., The isolation of a new thymine pentoside from sponges, *J. Am. Chem. Soc.*, **1950**, *72*, 2809-2810.

Marine sponges are sessile invertebrate filter feeders with a very little cell differentiation that are considered to be among the first multicellular animals. They often lack of physical defense instead, they have evolved chemical defense against predators and larval settlement of other sessile organism. The unusual metabolites produced as chemical defense agents that present selective toxicity could have a value as therapeutic agents. Historically, marine sponges have been the one of the richest sources of interesting chemicals among marine organisms, most likely due to their cosmopolitan nature, and the ease in which specimens of a reasonable mass (10 g – 1000 g) can be secured.

The level of cytotoxicity of some sponge products is high enough to even create a bare zone around the sponge that is maintained by the emission of mucus in which the toxins are contained. This allows the conquest of densely populated rocks or corals and the competition with faster-growing organisms, but it is striking that the sponge can selectively use its poison without self-destruction.

Traditionally, the role of symbiotic microorganisms in sponges was considered to be related to their ability to recycle nutrients or in some cases (i.e. cyanobacterias) to supplement the diet of the sponge by fixing carbon and nitrogen, however their role have been extended to the possibility that bacteria produce some of the potential pharmaceuticals that have been isolated from sponges[184]. These bioactive compounds will be the responsible to enhance the ability of sponges to compete with other organisms. In this symbiotic relationship, the sponge tolerates or encourages bacteria to grow within its tissues in return for some degree of protection. This relationship of mutual benefit separates the symbiotic bacteria from all the bacteria that may be more loosely associated with bacteria. Since most sponges contain any microorganism, the most difficult task is to distinguish symbiotic microbes from those being consumed by the sponge and those that are surface contaminants. The mutual adaptation of the sponge and microbe that defines a symbiotic relationship also suggests that symbionts will be difficult, if not impossible, to culture in the absence of the host. But if bacteria are indeed the producers of bioactive metabolites of interest, transfer of gene clusters responsible for the biosynthesis of the respective natural products to a vector suitable for large-scale fermentation could provide an alternative strategy.

[184] König, G. M.; Kehraus, S.; Seibert, S. F.; Abdel-Lateff, A.; Müller, D., Natural products from marine organisms and their associated microbes, *ChemBioChem,* **2006**, *7*, 229-238.

4.1.1 AUSTRALIAN MARINE SPONGES

It is estimated that a 30 % of Australina marine sponges are indigenous. Furthermore, of those endemic species from Australia, it is estimated that less than 30 % have been taxonomically classified. This is because Australia has a vast coastline (> 69000 km) and a wide variety of climate zones. Other exceptional feature of Australian environment is that it remains still almost intact from millions years ago. In order to study the relationship between sponge's species, localization, bioactivities and specific chemistry, sponges are collected from vastly different geographic places. Amazingly, the number of publications on the isolation of new chemistry from Australian sponges is estimated to represent less than 2 % of the total number of Australian species. It is reasonable to suppose that current efforts in studies of Australian sponges will contribute to increase substantially the number of new compounds biologically active.

4.2 ANTICANCER BIODISCOVERY

Bioassay is a relevant tool to focus the isolation of marine compounds. Each specimen produces a huge number of compounds but only specific species produce active compounds. Antutimoral bioassay allows screening of massive samples and guides the isolation scheme.

The Center of Molecular Biodiversity (CMB) has assembled a broad range of marine and terrestrial extract libraries featuring organisms from many Australian environments. CMB is particularly interested in searching these libraries for new anticancer agents. CMB is collaborating with the pharmaceutical company Pharma Mar S.A which offers its expertise in anticancer therapy and antitumoral bioassays.

Australian marine sponges are excellent candidates in containing antitumoral compounds as is mentioned above. My investigation into anticancer agents from Australian marine sponges will be described in this chapter.

4.2.1 CMB SPONGES COLLECTION

CMB collection of sponges is constituted by more than two thousand specimens stored in ethanol in plastic boxes (250-1000 mL) at -20 ºC. The specimens were collected in the last 20 years from different locations along Australian coast at depth between 5 and 800 m. Aliquots from all ethanol extracts were subjected to water and *n*-BuOH partition. Both fractions were tested in three cell tumoral lines by PharmaMar S.A. This chapter describes the isolation products from eight high active sponge speciments. Anticancer activity is shown in the next table (Table 4.1):

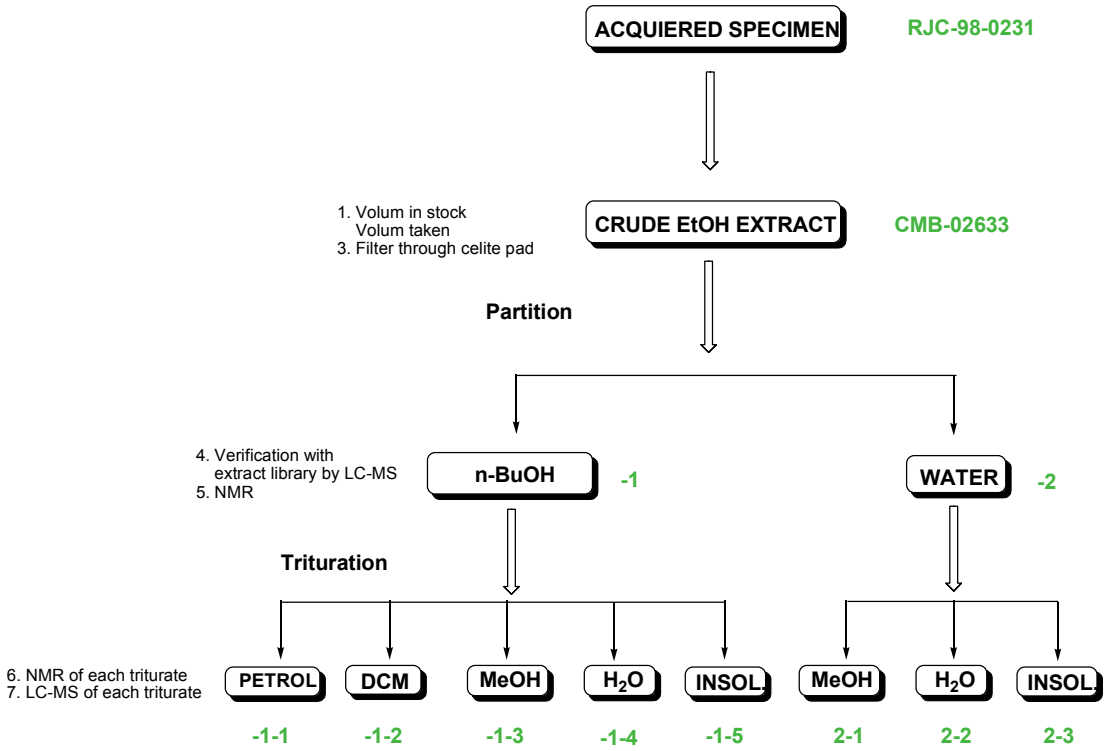
Specimen	Fraction	HT29*			A549*			MDA-MB-231*		
		50	15	5	50	15	5	50	15	5
CMB-02633	*n*- BuOH	-93	-92	101	-92	-91	103	-91	-73	76
CMB-02667	*n*- BuOH	-93	-93	42	-91	-92	48	-91	-92	45
CMB-02709	*n*- BuOH	-93	-93	42	-91	-92	48	-91	-92	45
CMB-02754	*n*- BuOH	-49	-16	79	-30	-11	77	-76	68	86
CMB-02765	*n*- BuOH	-77	-56	-25	-6	11	40	-61	-7	18
CMB-02550	*n*- BuOH	-89	-88	-58	-84	-88	-84	-78	-61	-61
CMB-03235	*n*- BuOH	-21	11	95	-87	-48	78	7	48	85
	H_2O	-46	-34	-5	-90	-89	-86	-46	-16	-8
CMB-03288	H_2O	-34	-31	80	-50	49	87	-51	17	82

Table 4.1 Pharma Mar anti-cancer assay result

* HT29 – Colon cancer cell line; A549 – Lung cancer cell line; MDA-MB-231 – Breast cancer cell line (mammary gland).$^{\Phi}$ Growth percentage scale: <0% implies highly active; >0 - <50% implies moderately active; >50% implies so-so to not active.

4.2.2 ISOLATION PROCESS

An isolation scheme is essential to simplify the process of fractionating a complex EtOH extract. A good isolation scheme readily fractionates common marine compounds such as aliphatic acids, amino acids, steroids and sugars. Approximately 40 % of total EtOH extract from each specimen entered above was dried under vacuum and liophylized to obtain the starting mass. The crude extracts were processed following the trituration/partition process as detailed in Scheme 4.1. In all processes samples were never heated over 40 ºC, and were stored at < - 30 ºC in the dark.



Scheme 4.1 Isolation scheme

- **Partition Process:** The partition process consists on spliting the crude extract into two fractions: *n*-BuOH soluble compounds and water soluble compounds.
- **Trituration Process:** The trituration process consists on a progressive polar solvent treatment. The first petrol treatment will contain fatty acidswhich are present in almost all natural crude extracts.

Mass balance must be constant along the process because only small quantities are available from unique samples. The table below (Table 4.2) contains a wide range of mass from all fractions. Some of them were abundant with more than 1 g and others were based just in few milligrams.

Specimen	aq EtOH Extract Partition		*n*-BuOH Sols Trituration					H_2O Sols Trituration		
	n-BuOH (mg)	H_2O (mg)	Petrol (mg)	DCM (mg)	MeOH (mg)	H_2O (mg)	Insol (mg)	MeOH (mg)	H_2O (mg)	Insol (mg)
CMB-02633	352.9	687	178	11.5	34.2	2.3	-	422	243	2.1
CMB-02667	346.8	1.5 g	31.4	106	75.2	4	-	818	668	-
CMB-02709	1163.6	2.0 g	364.9	250	245.5	7.9	-	1079	762	-
CMB-02754	309.3	960	112.8	14.3	49.2	6.9	-			
CMB-02765	108.5	722	20.9	1.3	42.3	1.8	-			
CMB-2550	135	721	94	8.8	19.4	1.3	12.4	650	30	-
CMB-03235	496	3.5 g	274	11	205	10	19	2.1 g	1.3 g	-
CMB-03288	572	3.6 g*	333	69.4	11	19	35	620	60	-

Table 4.2 Masses obtained from isolation scheme of CMB-02633, CMB-02667, CMB-02709, CMB-02754, CMB-02765, CMB-02550, CMB-03235, CMB-03288. * only 20 % H_2O solvent partition was triturated.

Each fraction was analyzed by ^1H NMR, HPLC-MS [ESI (+), ESI (-)], HPLC-DAD, HPLC-ELSD and they were subjected to bioassay in 6 cell tumoral lines.

4.2.2.1 Spectroscopic data analysis

Spectroscopic and mass data provides information about the identity and structure of compounds. With this information we determined if the compounds contained in each fraction were novel or related with already known compounds. Spectroscopic data was compared with data obtained from the CMB pure metabolites library, and by searching databases such as MariLit[185], SciFinder Scholar, ACD, Web of Science, etc… Early knowledge of the structure classes present in any given extract provided useful information to improve further purification of the active metabolites.

Spectroscopic data analysis from first fractions (*n*-BuOH and water fractions) is complex because of mixtures of different compounds but it offers us preliminary information about class-contained compounds and allows us to quickly identify common usual sponge components; *n*-BuOH fractions contain common fatty acids and water fractions present amino acids, sugars and steroids.

[185] MarinLit database, Department of Chemistry, University of Canterbury: http://www.chem.canterbury.ac.nz/marinlit/marinlitt.shtml.

- ^{1}**H NMR data**. Reveal the abundance of fatty acids and the presence of interesting structures. The most relevant signals are those localized in the aromatic region and also in the region between 2-4 ppm. Both regions are free from fatty acids signals and they indicate the presence of aromatic compounds and functional groups.
- **LC-DAD-MS data**. Chromatographic instrument with DAD and MS detector is an important tool to detect the presence of interesting structures at lower concentrations. DAD offers the possibility to detect and characterize aromatic structures and families of species with common UV spectrum as a fingerprint. MS in ESI(+) and ESI(-) mode offers information about molecular weight and detects the presence of halogen atoms, nitrogens, acids and basic functional groups,… Chromatographic data also give information about the polarity of compounds; compounds with lower retention times will be more interesting because their solubility in water will be higher.
- **LC-DAD-ELSD**. In this case it is possible to detect some compounds that have no active UV group. It also determines the purity of samples.

4.2.2.2 Bioassay data

All fractions were subjected to bioassay test in six tumor cell-lines and one normal cell-line as control.

1- A549: human lung carcinoma
2- HT29: human colon adenocarcinoma grade II cell line
3 - MM96L: human melanoma cell line
4 - DU145: human prostatic carcinoma cell line
5 – SkMel 28: human metastatic melanoma
6 – MCF7: breast cancer cell line
7 - NFF: Neonatal Foreskin Fibroblasts (control normal cell line)

Bioassay data indicate bioactivity route along the isolation scheme. The bioactivity level determines the priority fraction which deserves to be further studied.

4.2.3 COMPOUNDS IN THE SPONGES SUBJECT OF STUDY

The procedures described above outline in a rapid and effective means to assess the chemistry present in each specimen, to detect novel bioactive metabolites. Isolation scheme provided pure compounds in specimen CMB-03235 (Figure 4.2): Damirone A[186] in the DCM soluble fraction (CMB-03235-1-2) and Makaluvamine (J or K)[187] in the MeOH soluble fraction (CMB-03235-1-3). Both compounds are described in the literature as well as their cytotoxicity against esphageal cancer cells[188]. Their chemical syntheses have been also published.[189,190]

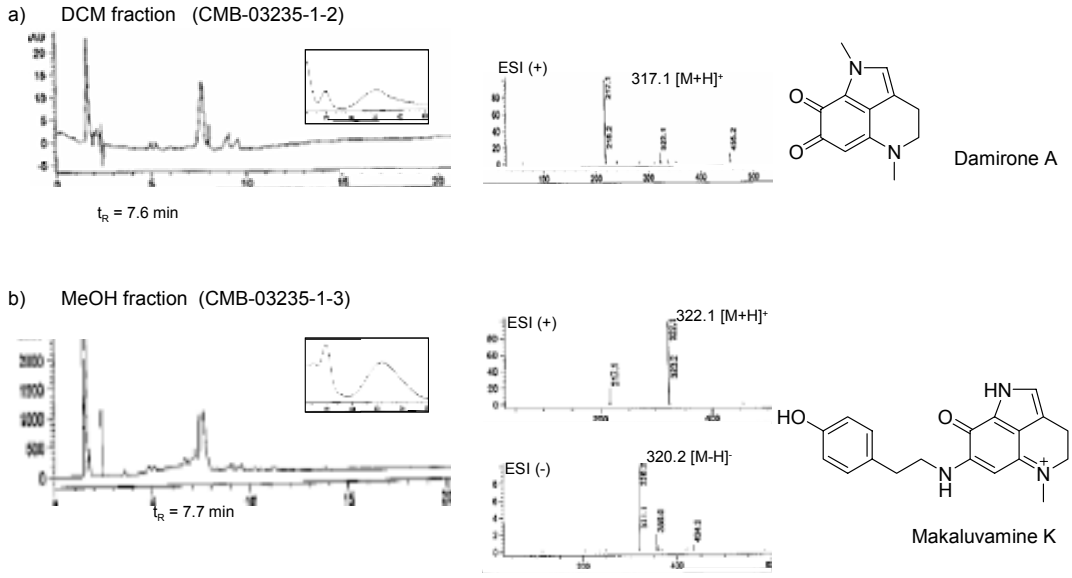


Figure 4.2 HPLC-DAD-EM data and structure of CMB-03235 compounds, a) DCM soluble fraction and b) MeOH soluble fraction. HPLC conditions: column C_8, gradient from 9:1 to 0:10 in 20 min (H_2O : MeCN with 0.5 % HCOOH)

[186] Stierle, D. B.; Faulkner, D. J., Two new pyrroloquinoline alkaloids from the sponge *Damiria sp. J. Nat. Prod.,* **1991**, *54*, 1131-1133.

[187] Schmidt, E. W.; Harper, M. K.; Faulkner, D. J., Makaluvamines H-M and daminorone C from the Pohnpeian sponge *Zyzzya fuliginosa*, *J. Nat. Prod*., **1995**, *58*, 1861-1867.

[188] a) Radisky, D. C.; Radisky, E. S.; Barrows, L. R.; Copp, B. R.; Kramer, R. A.; Ireland, C. M., Novel cytotoxic topoisomerase II inhibiting pyrroloiminoquinones from Fijian sponges of the genus *Zyzzya*, *J. Am. Chem. Soc.,* **1993,** *115*, 1632-1638. b) Barrows, L. R.; Radisky, D. C.; Copp, B. R.; Swaffer, D. S.; Kramer, R. A.; Warters, R. C.; Ireland, C. M., Makaluvamines, marine natural products, are active anti-cancer agents and DNA topo II inhibitors, *Anti-Cancer Drug Des*., **1993***, 8,* 333-347.

[189] a) Sadanandan, E. V.; Cava, M. P., Total syntheses of damirone A and damirone B, *Tetrahedron Lett.,* **1993**, *34*, 2405-2408. b) Roberts, D.; Joule, J. A.; Bros, M. A.; Álvarez, M., Synthesis of pyrrolo[4,3,2-de]quinolines from 6,7-dimethoxy-4-methylquinoline. Formal total synthesis of damirones A and B, batzelline C, isobatzelline C, discorhabdin C, and makaluvamines A-D, *J. Org. Chem*., **1997**, *62*, 568-577.

[190] a) Iwao, M.; Motoi, O.; Fukuda, T.; Ishibashi, F., New synthetic approach to pyrroloiminoquinone marine alkaloids. Total synthesis of makaluvamines A, D, I and K, *Tetrahedron,* **1998,** *54*, 8999-9010.

In CMB-02667 specimen, bioactive compounds were related to fatty acids. CMB-02550 specimen probably contains an attractive family of novel bioactive compounds. It showed really interesting spectroscopic features: presence of Bromide, of low molecular weigh compounds, an UV spectra that revealed an aromatic structure, and presence of compounds with high polarity. However, the low supply prevents the characterization of its constituents. This sample was reserved to be analysed on the new HPLC-NMR instrument which is able to characterize compounds from an analytical HPLC system. The rest of specimens contain known compounds with antitumoral activity: Bromotryptophan, *p*-hydroxibenzaldehyde, cyclostellettamines[191] and trunculins[202] as it is shown in Figure 4.3.

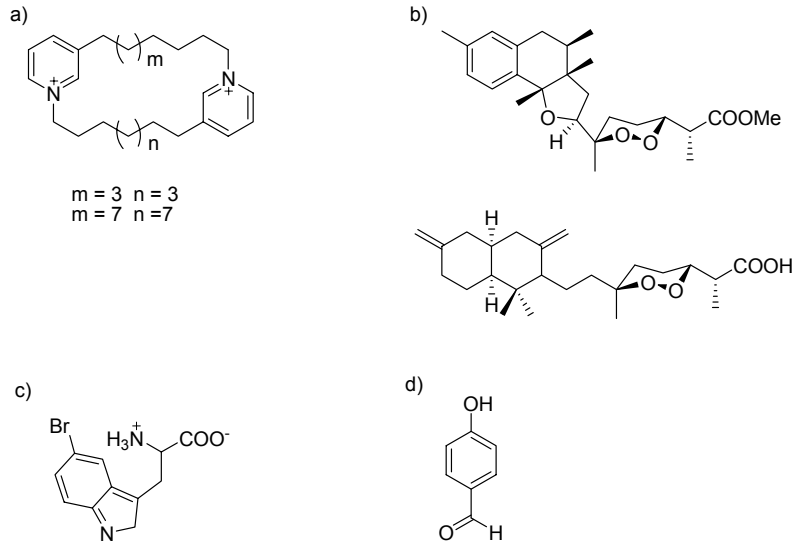


Figure 4.3 Compounds identified in studied specimens, a) Cycloestelletamines, b) Trunculins, c) Bromotryptophan and d) p-Hydroxibenzaldehyde

Cyclostellettamines and pyridine alkyl related compounds were isolated from other CMB specimens and they have been object of further studies as antitumoral compounds. Products isolated in specimen CMB-03288 improved activity-structure relationship studies for this family. We were really interested in exploring trunculins compounds as new antitumoral agents. Trunculins were isolated from two specimens: CMB-02633 and CMB-02709. Each specimen contained several trunculins-related compounds.

[191] Fusetani, N.; Asai, N.; Matsunaga, S., Cyclostellettamines A-F, pyridine alkaloids which inhibit binding of methyl quinuclidinyl benzilate (QNB) to muscarinic acetylcholine receptors, from marine sponge *Stelletta maxima*, *Tetrahedron Lett.*, **1994**, *35*, 3967-3970.

4.3 TRUNCULINS IN SPONGES CMB-02633 AND CMB-02709

Specimens CMB-02633 and CMB-02709 revealed the presence of a similar class of compounds. Both specimens were collected on the south part of Australia. ^{1}H NMR data and LC-MS analysis from the *n*-BuOH soluble fraction revealed the presence of trunculins. Trunculins are a specific family of norsesterpene cyclic peroxides isolated exclusively from south of Australia. Although in 1993 the activity of two trunculins A and B as antitumoral compounds was confirmed by the National Cancer Institute,[202] there is no published synthesis in the literature. Both sponges will allow exploring antitumoral activity from this class of compounds.

4.3.1 NORSESTERPENE CYCLIC PEROXIDES FROM AUSTRALIAN SPONGES

Norsesterpene cyclic peroxides are a unique class of metabolites common in sponges that inhabit temperate waters. The first compound isolated was muqubilin in 1979 from a Red Sea sponge *Xestospongia sp.*[192] (Figure 4.4). From then on, twenty-six new compounds containing the peroxide moiety have been reported in the literature in twelve publications. These have included compounds possessing acyclic, monocyclic, and bicyclic carbon skeletons (Figure 4.4). All of them have been isolated just from five genera of sponges (*Xestospongia, Diacarmus, Lantrunculia, Sigmosceptrella and Mycale*). The norsesterpene cyclic peroxides can be isolated as a carboxylic acid or as methyl ester. Prolonged storage in EtOH also result in formation of the artefact ethyl ester. It has been described that some trunculins are unstable as carboxylic acid.[193] Usually crude extracts were previously methylated with CH_2N_2 in order to stabilize compounds. Methyl ester formation allowed the characterisation of a wide family of norsesterpene cyclic peroxides. However, generally, norsesterpenes cyclic peroxides in the methyl ester form were biologically inactive and methyl ester hydrolysis is not compatible with peroxide moiety stability.

Norsesterpene cyclic peroxides generally show antibiotic activity. Some of them have been reported to have antimalarial activity, the ability to inhibit cell division and anticancer activity. Trunculin A and B were tested in carboxylic form and both of them were identified to possess activity against human melanoma cell lines.[202] Norsesterpene cyclic peroxides contain different stereochemistry on the cyclic peroxide moiety (Figure 4.4——) and a wide spread variety of forms in the aliphatic region (Figure 4.4- -).

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[192] Kashman, Y.; Rotem, M., Muqubilin, a new C_{24}-isoprenoid from a marine sponge, *Tetrahedron Lett.*, **1979**, *19,* 1707-1708.

[193] Oveden, S. P. B.; Capon, R. J., Trunculins G-I: new norsesterterpene cyclic peroxides from a southern Australian marine sponge *Latrunculia sp.*, *Aus. J. Chem.*, **1998**, *51*, 573-579.

Each sponge contains usually several structural related compounds. It is possible that sponges throw out several compounds to defense from a vast range of micro-invaders.

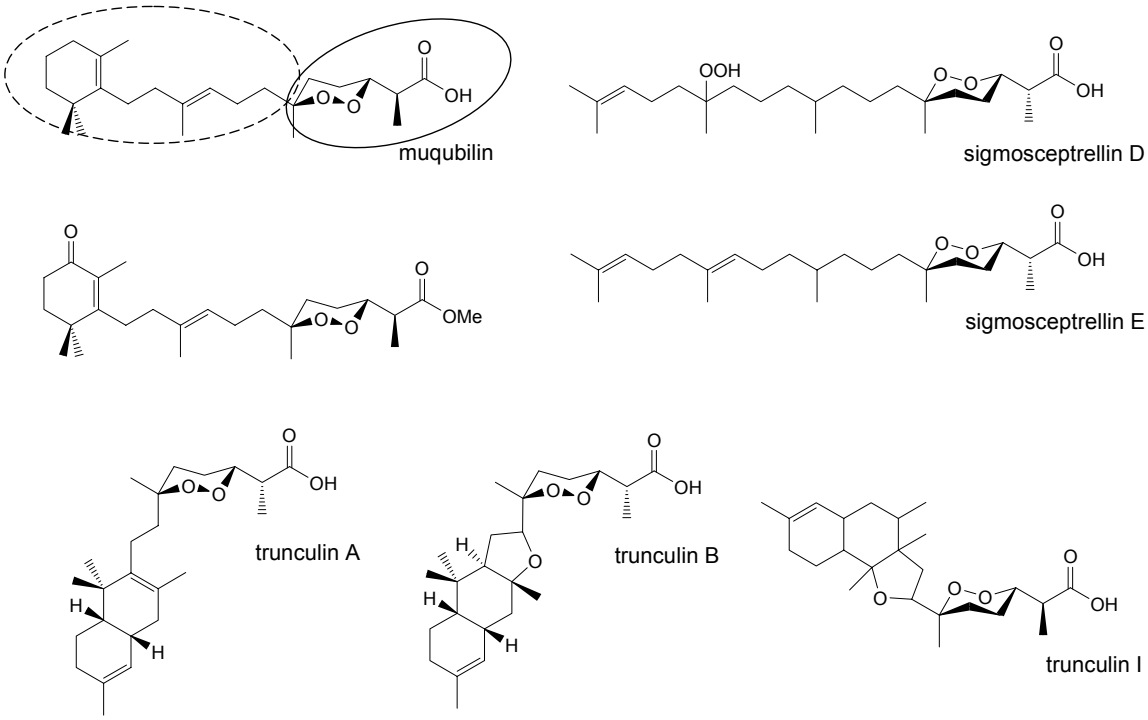

Figure 4.4 Norsesterpene cylic peroxides isolated from marine sponges

Many norsesterpenes cyclic peroxides incorporate two chiral subunits that are effectively independent, lacking through-space interactions and being attached through a rotationally flexible linkage. The assignment of the total relative stereostructures has often proved to be very difficult. Capon *et al*. reported a compendium of empirical rules for assigning relative and absolute stereochemistry about the cyclic peroxide moiety of this family of compounds.[194] Relative stereochemistry was determined by ^{1}H and ^{13}C NMR data and absolute stereochemistry required specific methods as molar rotations,[195] and

[194] Capon, R. J.; Macleod, J.K., Structural and stereochemical studies on marine norterpene cyclic peroxides, *Tetrahedron*, **1985**, *41,* 3391-3404.

[195] Capon, R. J.; Macleod, J. K., Structural and stereochemical studies on marine norterpene cyclic peroxides, part 2, *J. Nat. Prod.***, 1987***, 50*, 225-229.

Horeau,[196] Mosher[197,198] and CD analysis, plus chemical degradation and asymmetric synthesis.[199] The later new isolated compounds were characterized using this guide at the same time that they to reinforce it.

The discovery of sigmosceptrellin D (Figure 4.4), with a hydroperoxide moiety strategically positioned in such a way as to mimic the cyclic peroxide moiety at the opposite end of the molecule, was relevant to propose the biosynthetic pathway of norsesterpene cyclic peroxides.[200]

The information extracted from all this family of compounds facilitates identification of compounds from specimens CMB-02633 and CMB-02709 determining wether they are known or new active marine compounds.

4.3.2 TRUNCULINS IN SPONGE CMB-02633

n-BuOH fraction of CMB-02633 specimen (CMB-02633-1) showed relevant signals in ^{1}H-NMR spectrum (Figure 4.5) which indicated the presence of trunculins: singlet signals in the region 6-5 ppm, doublet signals in the aliphatic region and also multiplet signals in 4.2 ppm. The hypothesis was confirmed with LC-MS data analysis of fractions from the partition process (Table 4.3).

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[196] Weidmann, R.; Horeau, A., Determination of the configuration of secondary alcohols by partial resolution. IX. Nonpolarimetric semimicromethod., *Tetrahedron Lett*., **1973***, 31*, 2979-82.

[197] Dale, J. A.; Mosher, H. S., Nuclear magnetic resonance enantiomer regents. Configurational correlations via nuclear magnetic resonance chemical shifts of diastereomeric mandelate, O-methylmandelate, and α-methoxy-α-trifluoromethylphenylacetate (MTPA) esters., *J. Am. Chem. Soc*.**, 1973***, 95,* 512-519.

[198] Kusumi, T.; Ohtani, I,; Inouye, Y.; Kakisawa, H., Absolute configurations of cytotoxic marine cembranodines: consideration of Mosher's method, *Tetrahedron Lett*., **1988**, *29*, 4731-4734.

[199] Capon, R. J.; Macleod, J. K., Stereochemical studies on marine cyclic peroxides: an unequivocal assignment of absolute stereochemistry by asymmetric synthesis, *Tetrahedron***, 1988***, 44*, 1637-1650.

[200] Ovenden, S. P. B.; Capon, R. J., Nuapapuin A and sigmosceptrellins D and E: new norterpene cyclic peroxides from southern Australian marine sponges *sigmosceptrella sp*., *J. Nat. Prod*., **1999***, 62*, 214-218.

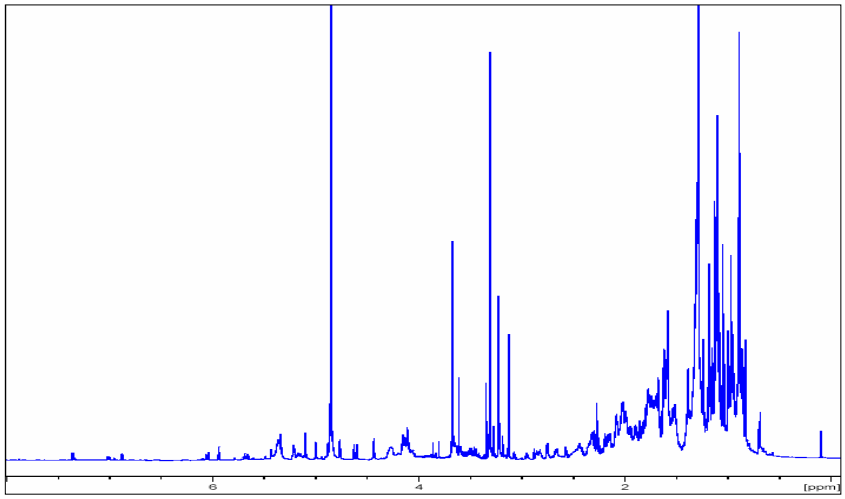


Figure 4.5 ^{1}H NMR spectrum (CD_3OD, 600 MHz) of CMB-02633-1

R_t (min)	-1-1	-1-2	-1-3	-2-1	ESI(+)MS	ESI(-)MS	MW	Compds
			√	ϕ	138 $[M+H]^+$	#	137	6
			√	ϕ	155 (M^+)	#	155	7
17.9	**x**	√	√		437 $[M+H]^+$ 459 $[M+Na]^+$	#	436	5
24.4	√	√	√		417 $[M+H]^+$ 434 $[M+NH_4]^+$ 439 $[M+Na]^+$	#	416	1
24.8	√	√	√		391 $[M+H]^+$ 413 $[M+Na]^+$	389 $[M-H]^-$	390	3
25.0	√	√	√		407 $[M+H]^+$ 429 $[M+Na]^+$	405 $[M-H]^-$	406	2
25.6	√	√	**x**		419 $[M+H]^+$ 436 $[M+NH_4]^+$ 441 $[M+Na]^+$	#	418	4

* **LCMS condition:** Zorbax C_8 analytical column, 150 x 4.6 mm, eluting with 1.0 mL/min 90% H_2O/MeCN to MeCN (0.5% HCO_2H modifier) over 25 min. then held for 5 min., and detecting at 210 and 254 nm (DAD). #Complex

Table 4.3 LC-MS* data for CMB-02633-1-1, CMB-02633-1-2, CMB-02633-1-3 and CMB-02633-2-1

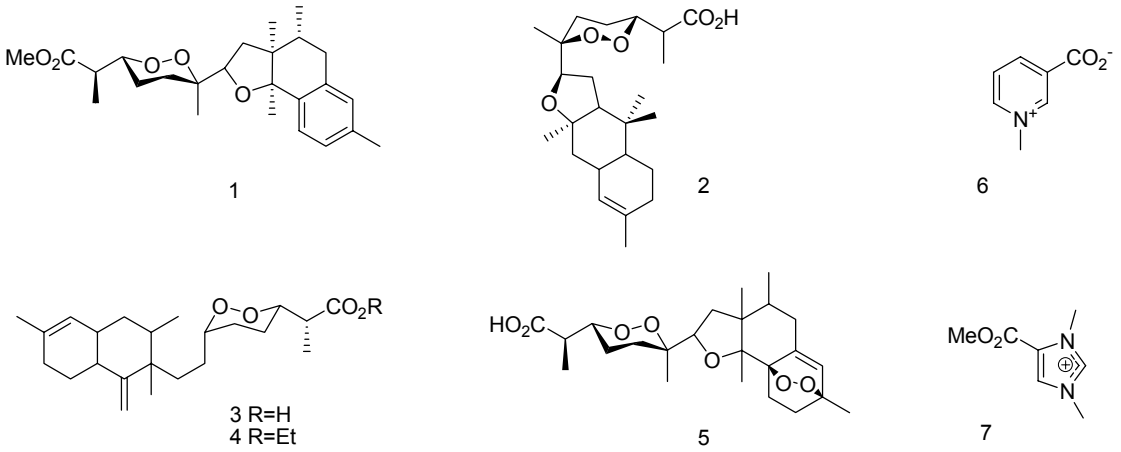


Figure 4.6 Preliminary identification of compounds in CMB-02633-1

The trigonelline (**6**) and norzooanemonin methyl ester (**7**) which appeared to be present in the aqueous extract of CMB-02633 (CMB-02633-2) are quaternary ammonium compounds that may have an important role in cellular osmotic activity of marine invertebrates. These compounds are typically regarded as nuisance compounds, and previous evaluation suggests that they possess no anticancer potential. It is worthwhile noting that whereas the *n*-BuOH partition of CMB-02633 (CMB-02633-1-1) displayed anticancer potential in Pharma Mar, S.A. primary assays, the water soluble partition was inactive. Compounds **(1-4)** were related with known trunculins and compound **5** would be a new trunculin which could offer new prespectives to trunculin's family. Most trunculin related compounds were present in their carboxylic acid form. In order to evaluate antitumoral activity of natural compounds methylation treatment was not performed.

The isolation of trunculins from CMB-02633 specimen was made from petrol solubles fraction (CMB-02633-1-1) because it contained enough mass material (178 mg) and also 4 different trunculins.

4.3.3 6 COLUMNS SWITCHING HPLC SYSTEM. OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

The chromatographic analysis is a relevant step in the process of natural compounds isolation. Sponges offer a wide diversity of structural compounds which require specific chromatographic conditions. Often, sponges generate a family of similar structural compounds. 6 Columns switching HPLC system allows the exploration of different analytical conditions in an automatic and efficient way. The instrument is based on a HPLC system with a DAD and ELSD detector and six different analytical columns with different packed material. The instrument offers the possibility to analyse the same sample in 6 different analytical columns. The analytical columns used were:

1- Zorbax SB-C18, 4.6 x 150 mm, 5 μm
2- Zorbax SB-C8, 4.6 x 150 mm, 5 μm
3- Zorbax SB-C3, 4.6 x 150 mm, 5 μm
4- Zorbax SB-CN, 4.6 x 150 mm, 5 μm
5- Zorbax SB-Ph, 4.6 x 150 mm, 5 μm
6- Zorbax SB-Eclipse XDB C8, 4.6 x 150 mm, 5 μm

Nucleosil columns (C18, C8, C3) offer high resolution in aliphatic compounds that differs in the number on carbon atoms. The ciano column (CN) separates compounds with different dipolar affinity. The phenacyl column (Ph) is especially useful in the resolution of aromatic compounds. The system also offers the possibility to work with different solvents to improve the chromatographic development. Results obtained in the 6 column switching HPLC system for CMB-02633-1-1 are presented below in Figure 4.7.

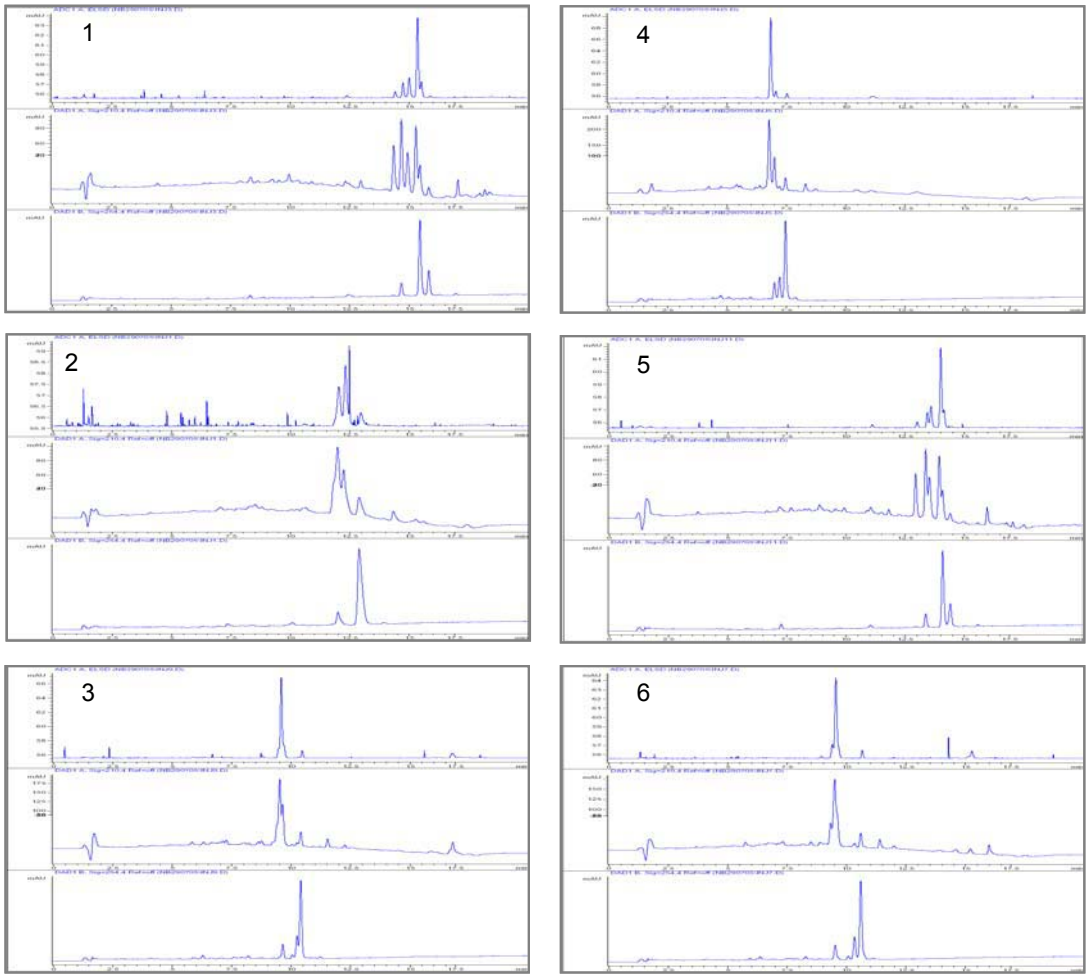


Figure 4.7 HPLC chromatograms from 6 columns switching HPLC system

Nucleosil C18 column offered more resolution in the analysis of CMB-02633-1-1. The use of acidic conditions in front of conditions free of acid allowed better resoluted chromatograms.

4.3.4 ISOLATION COMPOUNDS FROM CMB-02633-1-1

In order to prevent lacking of antitumoral activity information from unstables trunculins, a preliminary bioassay was made directly from 1.5 mg of purified fraction CMB-02633-1-1. Antitumoral activity was detected in acid compounds **2-3** and ethanol ester **4**. Subsequents large-scale purifications (24 mg were performed in HPLC semi-preparative system to characterize active compounds (Figure 4.8). Six pure compounds were collected, but only compounds 1 & 3 (CMB-02633-A and CMB-02633-B) were characterized by ^{1}H NMR. The rest of CMB-02633-1-1 constituents degraded prior to ^{1}H NMR analysis.

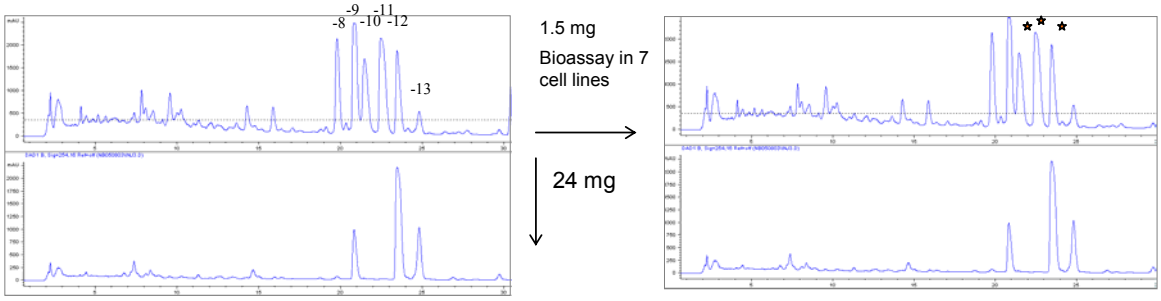


Figure 4.8 Bioassay from an aliquot of CMB-02633-1-1 and large-scale purification

4.3.4.1 CMB-02633-A. New trunculin

CMB-02633-A was isolated as a stable oil which gave a molecular ion in EIMS 417 $[M+H]^+$, 439 $[M+Na]^+$, 455 $[M+K]^+$ consistent with a molecular formula isomeric with trunculin C methyl ester (1). The ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of CMB-02633-A is shown in Figure 4.9. Application of empirical rules[194] (^{1}H NMR chemical shift for 2-CH_3 (δ 1.08), a large $J_{3\text{-}4ax}$ 8.8 Hz and the ^{1}H NMR chemical shift for the 6-CH_3 (δ 1.19) were consistent with a 2R*, 3R*, 6S* relative stereochemistry. Further examination of the ^{1}H NMR data revealed resonances consistent with an aromatic methyl (δ 2.30), ether moiety (δ 4.27) featuring tertiary and quaternary termini, a secondary methyl (δ 1.08), two tertiary methyls (δ 1.39, 1.01). The chemical shift for H-7 (δ 4.25) in CMB-02633-A differed from H-7 in trunculin C methyl ester (δ 3.76) and but it was closer to trunculin B methyl ester (δ 4.54) (Figure 4.10). That means that CMB-02633-A might be an extended version of trunculin C methyl ester.

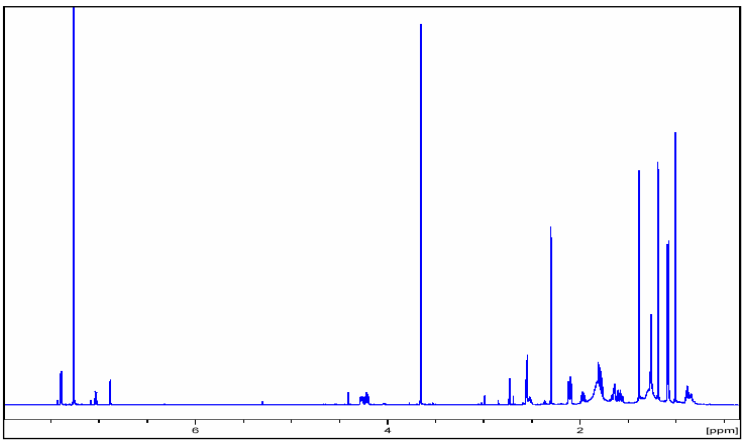
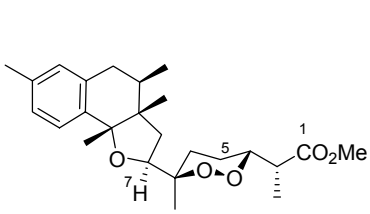
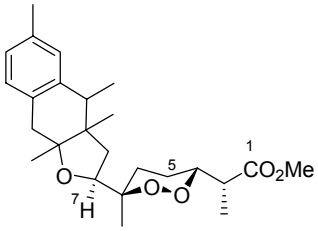


Figure 4.9 ^{1}H NMR spectrum (CD_3OD, 600 MHz) of CMB-02633-A



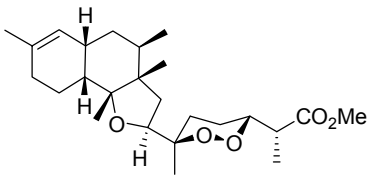
trunculin C methyl ester

δ_{H7} = 3.76



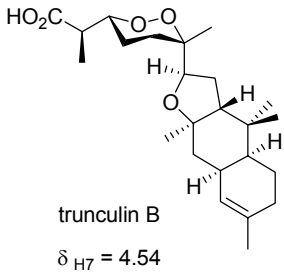
CMB-02633-A

δ_{H7} = 4.24



trunculin F methyl esther

δ_{H7} = 3.66



trunculin B

δ_{H7} = 4.54

Figure 4.10 Trunculins related to CMB-02633-A

4.3.4.2 CMB-02633-B. Trunculin G

CMB-02633-B was isolated as an unstable oil which gave a molecular ion in EIMS 390 $[M+H]^+$, 413 $[M+Na]^+$, 389 $[M-H]^-$ consistent with a molecular formula isomeric with trunculin G. The ^1H NMR ($CDCl_3$, 600 MHz) spectrum of CMB-02633-B is shown in Figure 4.11. The resonances observed were close to those reported in the literature for trunculin G methyl ester.[7] CMB-02633-B conserved the same relative stereochemistry than trunculin G methyl ester. The relative stereochemistry was assigned using the empirical rules:[194] (^1H NMR chemical shift for 2-CH_3 (d 1.23) and 6-CH_3 (d 1.09), a large $J_{3,4ax}$ (7.1 Hz) were consistent with a 2R*, 3S*, 6S* relative stereochemistry. Further examination of the ^1H NMR data revealed resonances consistent with trisubstituted double bond (δ 5.17), a 1,1-disubstituted double bond (δ 4.92, 4.68), a tertiary methyl (δ 1.09), a secondary methyl (δ 0.84), and an olefinic methyl (δ 1.60). The chemical shifts for the 1,1-trisubstituted doble bond differ slightly from the corresponding trunculin G methyl ester (δ 5.09, 4.81 4.65). The upfiled proton (δ 4.68) shows a J 2.3 Hz chemical shift that reveals a neighbour methylene group which differs from trunculin G. Results indicated that CMB-02633-B might consists on an isomeric form from trunculin G. However, the lack of sample does not allow us further spectroscopic studies to confirm that the new structure is related to trunculins.

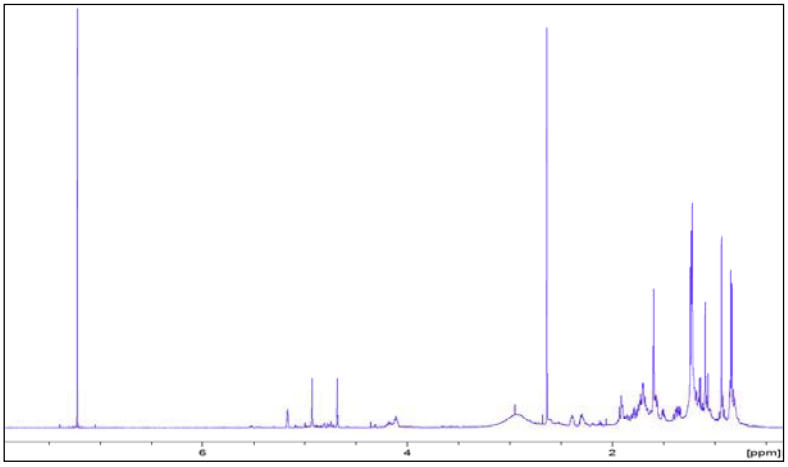
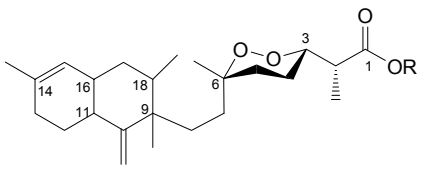
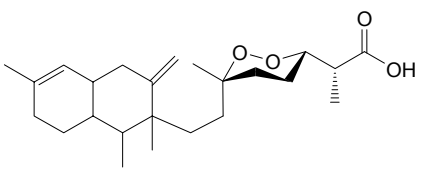


Figure 4.11 ^1H NMR spectrum ($CDCl_3$, 600 MHz) of CMB-02633-B



trunculin G methyl ester



CMB-02633-B

4.3.5 TRUNCULINS IN CMB-02709

The presence of trunculins in specimen CMB-2709 was evident in ^{1}H NMR spectrum from the *n*-BuOH fraction (Figure 4.12). It showed singlets at 6-5 ppm in the NMR, doublets in the aliphatic region and also multiplet signals at 4.2 ppm. In the aromatic region there were also signals that indicated the possible presence of aromatic trunculins.

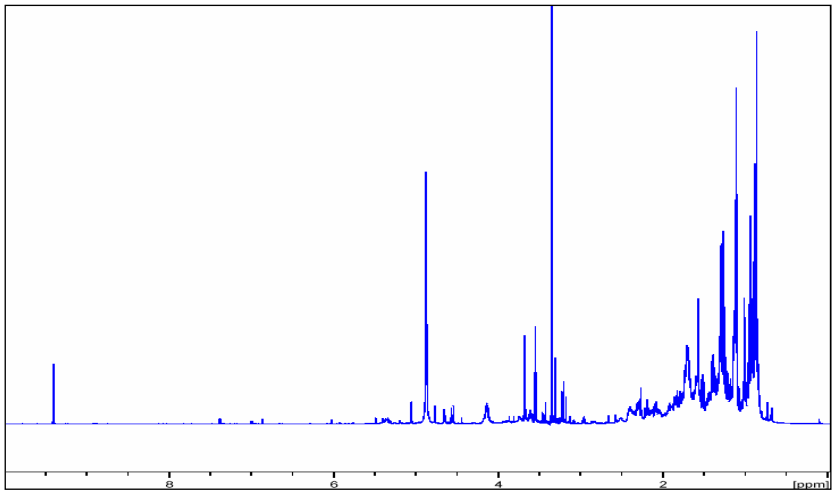


Figure 4.12 ^{1}H NMR spectrum (CD_3OD, 600 MHz) of CMB-02709-1

The LC-MS data analysis gives us the possible trunculins present in the specimen CMB-02709:

R_t (min)	-1-1	-1-2	-1-3	-2-1	ESI(+)MS	ESI(-)MS	MW	Compds
				ϕ	138 $[M+H]^+$	#	137	**6**
				ϕ	155 $[M+H]^+$	#	155	**7**
10.5	**x**	√	**x**					
17.9	**x**	√	√		437 $[M+H]^+$ 459 $[M+Na]^+$	#	436	**5**
22.8	**x**	√	√		405 $[M+H]^+$ 427 $[M+Na]^+$	#	404	**11**
23.9	√	√	√		417 $[M+H]^+$ 439 $[M+Na]^+$	#	416	**8**
24.8	√	√	√		391 $[M+H]^+$ 413 $[M+Na]^+$	#	390	**10**
25.0	√	√	√		407 $[M+H]^+$ 429 $[M+Na]^+$	#	406	**9**
25.2	√	√	**x**		419 $[M+H]^+$ 441 $[M+Na]^+$	#	418	**12**

Table 4.4 LC-MS* data for CMB-02709-1-1, CMB-02709-1-2 and CMB-02709-1-3. * LC-MS conditions: Zorbax C_8 analytical column, 150 x 4.6 mm, eluting with 1.0 mL/min 90% H_2O/MeCN to MeCN (0.5% HCO_2H modifier) over 25 min. then held for 5 min., and detecting at 210 and 254 nm (DAD). #Complex, ϕFIA

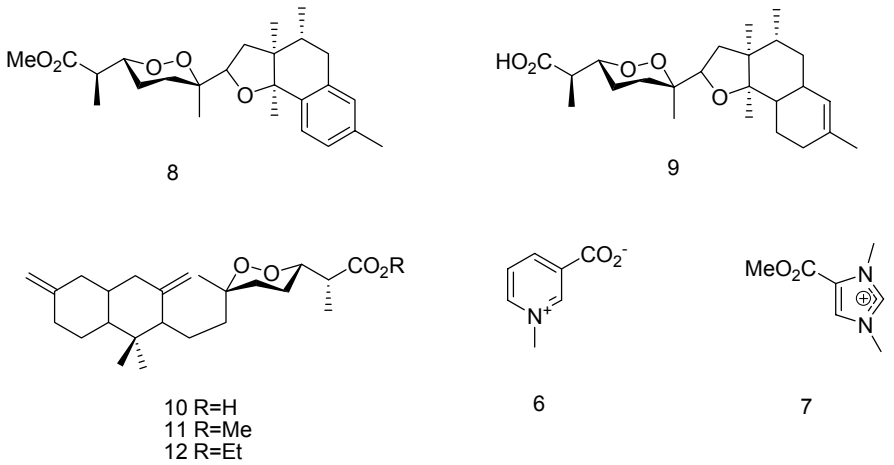
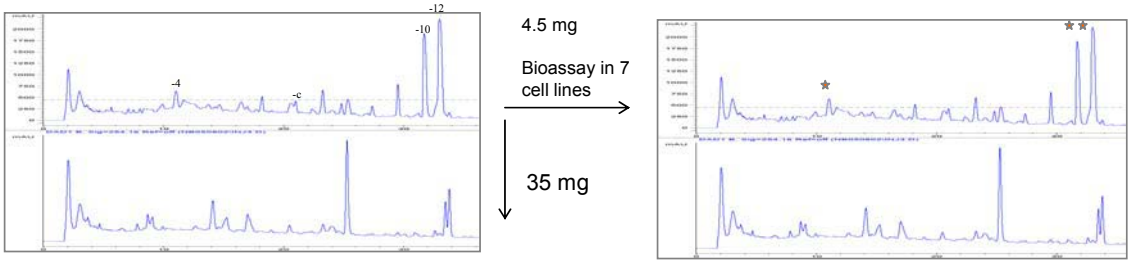


Figure 4.13 Preliminary identification of compounds in CMB-02709

The trigonelline (**6**) and norzooanemonin methyl ester (**7**) which appeared to be present in the aqueous extract of CMB-02709 are quaternary ammonium compounds that may have an important role in cellular osmotic activity of marine invertebrates. These compounds are typically regarded as nuisance compounds, and previous evaluation suggests that they possess no anticancer potential. It is worthwhile noting that whereas the *n*-BuOH partition of CMB-02709 displayed anticancer potential in PharmaMar S.A. primary assays, the water soluble partition was inactive.

The 250 mg DCM solubles fraction contained trunculins compounds, it was clean from aliphatic acids, and it was also active in most of cell lines. In the chromatographic development in the 6 switching columns it was obtained again a good resolution with the zorbax C18 column. As in CMB-02633-1-1, a preliminary bioassay was made directly from 4.5 mg of purified fraction CMB-02709-1-2. Antitumoral activity was detected in acid compounds **9-10**. Subsequents large-scale purifications (35 mg) were carried out in HPLC semi-preparative system to characterize active compounds. It was collected three compounds (CMB-02709-A, CMB-02709-B and CMB-02709-C) and characterized by ^{1}H NMR.



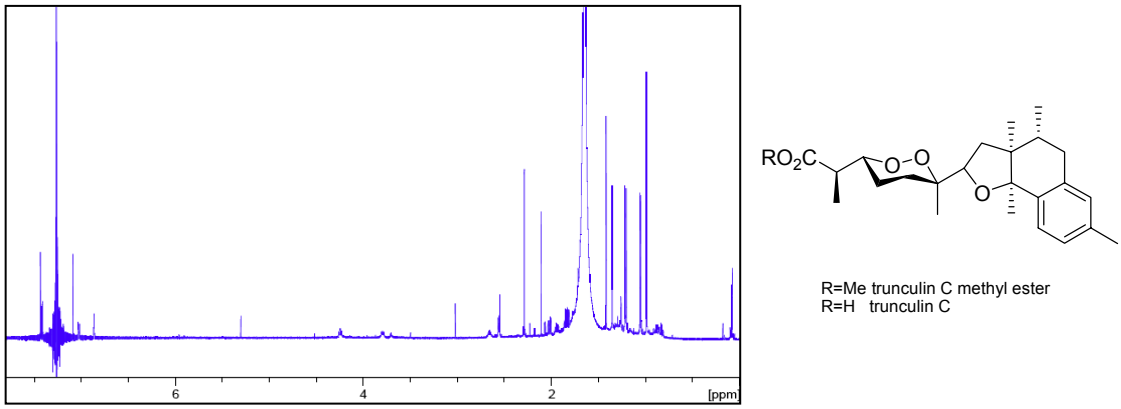
Semiprep #3, C18 semiprep column
Gradient from 50% H_2O to 0% H_20 in 40 min

0.3 mg CMB-02709-A
2.9 mg CMB-02709-B (-10)
6.4 mg CMB-02709-C (-12)

Figure 4.14 Bioassay from an aliquot of CMB-02709-1-2 and large-scale purification

4.3.5.1 CMB-2709-A. Trunculin C

CMB-02709-A was isolated as an unstable oil which gave a molecular ion in EIMS 402 $[M+H]^+$, 425 $[M+Na]^+$, consistent with a molecular formula related with trunculin C methyl ester in the carboxylic free form. The ^1H NMR ($CDCl_3$, 600 MHz) spectrum of CMB-02709-A is shown in Figure 4.. Application of empirical rules:[194] ^1H NMR chemical shift for 2-CH_3 (δ 1.15), a large $J_{3\text{-}4ax}$ 7.6 Hz and the ^1H NMR chemical shift for the 6-CH_3 (δ 1.35) were consistent with a 2R*, 3R*, 6S* relative stereochemistry. Further examination of the ^1H NMR data revealed resonances consistent with an aromatic methyl (δ 2.29), ether moiety (δ 3.79) featuring tertiary and quaternary termini, a secondary methyl (δ 0.95), two tertiary methyls (δ 1.41, 0.97). The resonances observed are closed to those reported in the literature for trunculin C methyl ester.[7] CMB-02709-A would be trunculin C isolated as a carboxylic free form. The lack of sample did not allow to perform more experiments to confirm the absolute stereochemistry.



R=Me trunculin C methyl ester
R=H trunculin C

Figure 4.15 ^1H NMR spectrum ($CDCl_3$, 600 MHz) of CMB-02709-A and identified trunculin C related to trunculin C methyl ester

Trunculin C Me ester[201] ($CDCl_3$)	Posit	CMB-02709-A ($CDCl_3$, 600 MHz)
7.41 (d, 1H, 7.9Hz)	H-16	7.42 (d, 1H, 8.3 Hz)
7.02 (br d, 1H, 7.9 Hz)	H-15	7.02 (br d, 1H, 8.0 Hz)
6.86 (br s, 1H)	H-13	6.86 (br s, 1H)
4.24 (td, 1H, J=8.2, 4.1 Hz)	H-3	4.27 (td, 1H. J=9.0, 3.2)
3.76 (dd, 1H, J=10.8, 6.2 Hz)	H-7	3.79 (dd, 1H, J=10.9, 5.4 Hz)
3.70 (s, 3H)	OMe	-
2.59 (dq, 1H, J= 7.6, 7.2 Hz)	H-2	2.65 (m, 1H)
2.54 (m, 2H)	H-11, H-11'	2.56 (m, 2H)
2.28 (br s, 3H)	Me-23	2.29 (s, 3H)
2.01 (dd, 1H, J=12.6, 10.8)	H-8'	2.02 (dd, 2H, J=12.8, 6.1)
1.93 (m, 1H)	H-10	1.93 (m, 2H)
1.82 (dd, 1H, J=12.6, 10.8)	H-8'	1.83 (dd, ~3H, J=12.9, 10.7)
1.41 (s, 3H)	Me-24	1.42 (s, 4H)
1.34 (s, 3H)	Me-20	1.35 (br s, 4H)
1.15 (d, 3H, J=7.2)	Me-19	1.21 (d, 4H, J=8.0Hz)
0.97 (s, 3H)	Me-21	1.05 (d, 4H, J=5.6)
0.95 (d, 3H, J=6.4 Hz)	Me-22	0.98 (s, 4H)

Table 4.5 ^{1}H NMR data for CMB-02709-A and trunculin C methyl ester

4.3.5.2 CMB-02709-B. Trunculin E

CMB-02709-B was isolated as an unstable oil which gave a molecular ion in EIMS 391 $[M+H]^+$, 413 $[M+Na]^+$, consistent with a molecular formula related with trunculin E. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of CMB-02709-B is shown in Figure 4.16. Application of empirical rules[194] (^{1}H NMR chemical shift for 2-CH_3 (δ 1.19), a large $J_{3\text{-}4ax}$ 8.3 Hz and the ^{1}H NMR chemical shift for the 6-CH_3 (δ 1.27) were consistent with a 2R*, 3R*, 6S* relative stereochemistry. Further examination of the ^{1}H NMR data revealed resonances consistent with two 1,1-disubstituted double bonds [δ 4.74 (*J* 2.3 Hz), 4.66, 4.57,

[201] He, H. Y.; Faulkner, D. J.; Lu, H. S. M.; Clardy, J., Norsesterterpene peroxides from the sponge Latrunculia sp., *J. Org. Chem.*, **1991**, *56*, 2112-15.

4.53 (*J* 2.3 Hz)] and two tertiary methyls (δ 0.97, 0.98). The resonances observed were identical to those reported in the literature for trunculin E.[7]

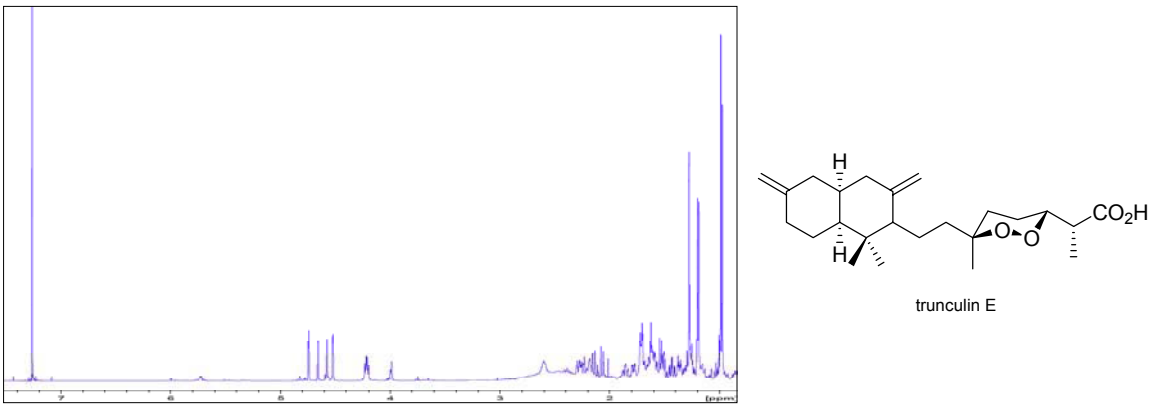


Figure 4.16 ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of CMB-02709-B and trunculin E

Trunculin E ($CDCl_3$)[201]	CMB-02633-B ($CDCl_3$, 600MHz)
4.74 (br d, 1H, 2.3 Hz)	4.74 (br dd, 1H, J=2 Hz)
4.66 (br s, 1)	4.66 (br d, 1H, J= 1.7 Hz)
4.57 (br s, 1H)	4.58 (br s, 1H)
4.52 (br d, 1H, J=2.3 Hz)	4.52 (br d, 1H, J=2.2 Hz)
4.21 (td, 1H, J=8.3, 4.7)	4.21 (td, 1H, J=8.7, 4.4 Hz)
2.59 (dq, 1H, J=8.3, 7.2 Hz)	2.60 (m, 1H ~ 2H)
1.27 (s, 3H)	1.27 (s, 3H)
1.19 (d, 3H, J=7.2)	1.19 (d, 3H, J= 7.1)
0.98 (s, 3H)	0.99 (s, 3H)
0.97 (s, 3H)	0.97 (s, 3H)

Table 4.6 ^{1}H NMR data for CMB-02709-B and trunculin E

4.3.5.3 CMB-02709-C. Trunculin I, mixture of stereoisomers

Even though CMB-02709-C was obtained as a single compound by LC analysis, ^{1}H NMR data revealed the presence of two stereoisomers *erytro/ threo* (1:1) related from trunculin I. The LC-MS analysis showed a molecular ion mass of 407.6 $[M+H]^+$, 429.6 $[M+Na]^+$. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of CMB-02709-C is shown in Figure 4.17. Application of empirical rules:[194] ^{1}H NMR chemical shift for both 2-CH_3 (δ 1.16, 1.19), a large $J_{3\text{-}4ax}$ 8.3 Hz and the ^{1}H NMR chemical shift for the 6-CH_3 (δ 1.11) were consistent with an *erytro* isomer and *threo* isomer. Further examination of the ^{1}H NMR data revealed resonances consistent with a trisubstituted doble bond (δ 5.07), ether moiety (δ 3.65) featuring tertiary and quaternary termini, a secondary methyl (δ 0.84-0.85), two tertiary methyls (δ 0.825-0.833, 1.28) and an olefinic methyl (δ 1.58). Signals from *threo* isomer corresponded with published data from tunculin I methyl ester.

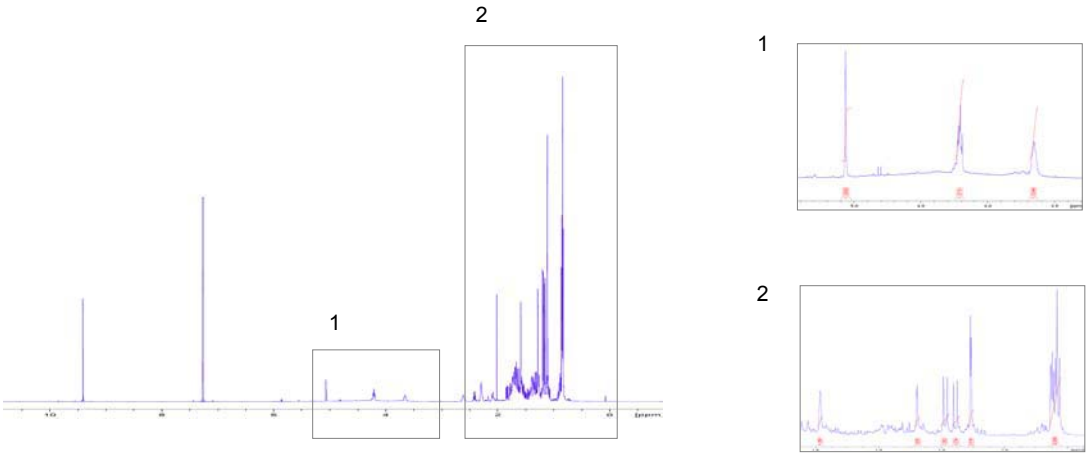
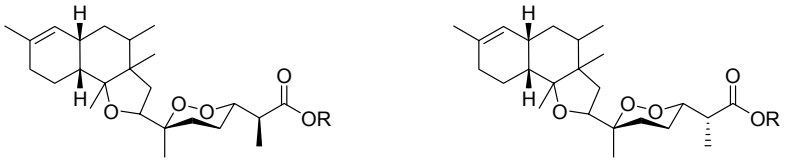


Figure 4.17 ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of CMB-02709-C



R = H *threo*-CMB-02709-C
R = Me Trunculin I Methyl ester

erytro-CMB-02709-C

Figure 4.18 Proposed structures for CMB-02709-C related with trunculin I methyl ester

4.4 NEW CHEMISTRY AFFORDABLE TRUNCULINS

The bioassay results determined that trunculins could be anticancer compounds. However, the supply of these unusual cycloperoxides is limited to natural supplies as no total synthesis currently exists. Consequently it is difficult to fully explore the anticancer potential of trunculins. For this reason the trunculins are not useful drugs. One solution is to chemically interconvert natural trunculin (ie **a**) into more synthetically accessible analogues (ie **c**) through an interesting intermediate (ie **b**). If **c** derivatives are active as anticancer agents, resupply can be assured by synthetic methods. Tetrahydrofuran is present in some antibiotic natural products. The conversion of trunculins in a chemistry synthesis affordable compounds is the main requirement for trunculins to became a useful drug.

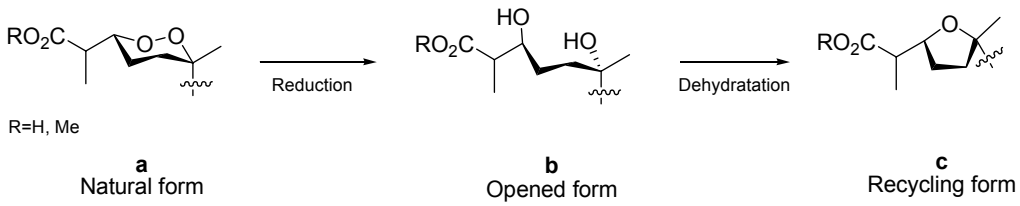


Figure 4.19 New chemistry affordable proposed for trunculins

For this purpose we can make use of supplies of the antitumoral trunculin A (1.34 g) and trunculin B (500 mg) (Figure 4.20). Both compounds were isolated in 1986 by Capon et al.[202] and they were stored in CMB library.

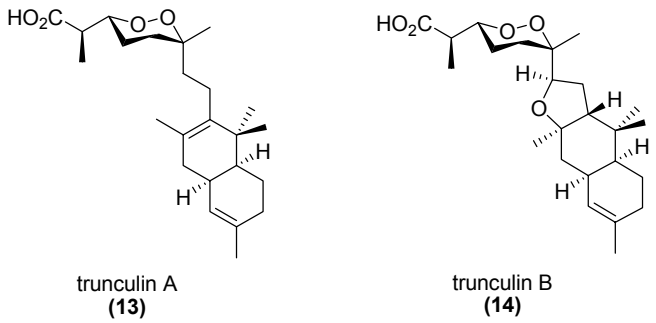


Figure 4.20 Starting material: trunculin A (13) and trunculin B (14)

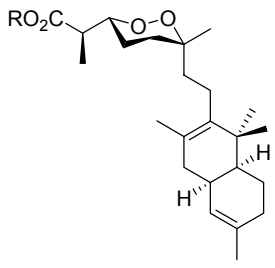
[202] Capon, R. J.; McLeod, J. K.; Willis, A. C., Trunculins A and B, norsesterpene cyclic peroxides from marine sponge, *Lantrunculia brevis*, *J. Org. Chem*., **1987**, *52*, 339-341.

The synthetic scheme is based on two steps: a peroxide reduction to form the diol analogue and a subsequent dehydration reaction. In a first attempt the peroxide reduction was perfomed using H_2 with Pd/C (cat.) as was used in the stereochemistry trunculins assignment. Those conditions are able to reduce both peroxide moiety and double bonds present in the molecule, giving rise to a mixture of compounds that make more difficult the total stereochemistry assigment. In order to preserve completely the aliphatic structure, a selective reduction using Mg/I_2 was tested. Dehydrations were carried out with catalytic *p*-toluenesulfonic acid in benzene. Moreover, we were interested in studying the antitumoral effect of the methyl ester form of each analogue. Methyl ester protects the carboxylic moiety in the dehydration reaction. Methyl ester is formed by reaction with diazomethane in a fast quantitative reaction.

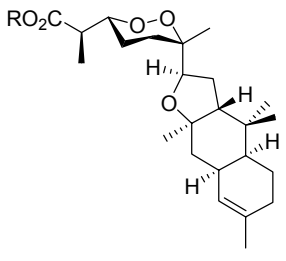
4.4.1 TRUNCULIN A & B

Trunculin A and trunculin B are two norterpene peroxide isolated from marine Australian sponges with important cytotoxic activity. Trunculin A is a white solid product and trunculin B is preserved in an EtOH solution. Both compounds were characterised and tested in tumoral cell-lines.

Trunculin A and Trunculin B were subjected to methylation with diazomethane an ethereal solution to obtain the methyl ester. The reactions were yield quantitative. High pure trunculin A methyl ester and trunculin B methyl ester were obtained as white solid products (Figure 4.21)). Both compounds were subjected to bioassay analysis.



trunculin A **(13)** R = H
trunculin A methyl ester **(15)** R =Me



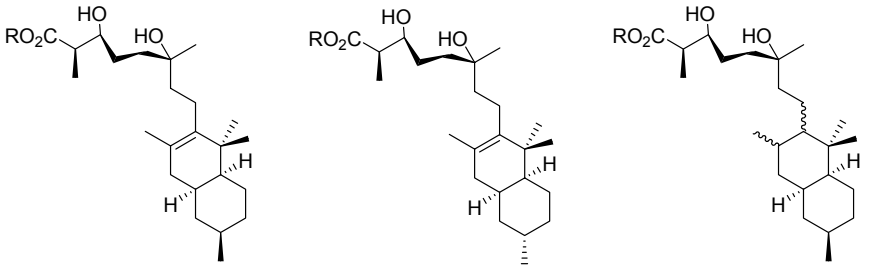
trunculin B **(14)** R = H
trunculin B methyl ester **(16)** R =Me

Figure 4.21 Natural compounds and methyl ester derivatives

4.4.2 OPEN CYCLE TRUNCULINS ANALOGUES

In order to open the peroxide cycle, two reduction conditions were used. The first method used H_2 /Pd (cat) and was able to reduce also double bonds present in the aliphatic chain. The second method was more selective for the peroxide moiety and the rest of aliphatic chain remained intact.

Trunculin A was reacted overnight with a catalytic amount of 10 % Pd/C under H_2 pressure (30 psi) in MeOH. After filtering the catalyst, the crude was analysed by LC-MS and ^{1}H NMR. The LC-MS indicated the partial reduction of trunculin A and the formation of two isomers (3:2). Most probably the compounds obtained were **17** & **18** because the trisubstituted double bond is more labile than the tetrasubstituted double bound. A small amount seemed to be totally reduced (**19**). The ^{1}H NMR spectrum indicated that the main compound corresponded to the diol compound. And the region of methyls between 0.8-1.6 ppm showed the main differences between both compounds that revealed that effectively two steroisomers were present. The methyl ester of the reduced compound from both isomers (**20** & **21**) was also observed. All isomers were purified by semipreparative system. Finally, the purified carboxylic form stereoisomers were methylated in order to study their antitumoral activity.



(17) R= H
(20) R =Me

(18) R = H
(21) R =Me

(19)

Figure 4.22 Compounds obtained from hydrogenation of trunculin A with with H_2/Pd (cat.)

Trunculin B was subjected to the same procedure. The crude was analysed by LC-MS and ^{1}H NMR. The LC-MS indicated the total reduction of Trunculin B (**22**) (65 %) and the presence as a minor component of one isomer for partial reduced compound (**23**) (22 %). It was also observed the formation of methyl ester reduced compound from 10 (**24**) (7 %) and the formation of side products (MW 404, 438). The ^{1}H NMR spectrum indicated that the main compound corresponed to the diol compound (**22**).

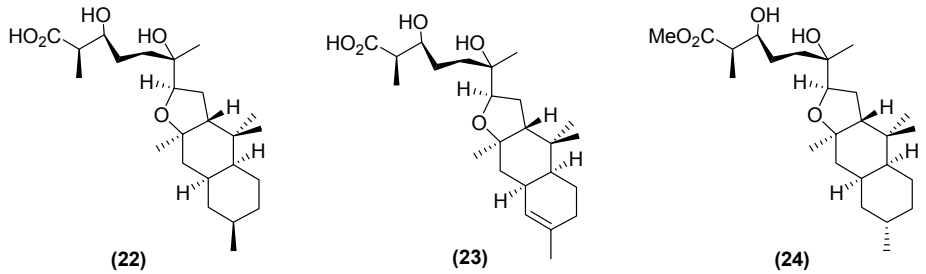


Figure 4.23 Compounds obtained from hydrogenation of trunculin A with with H_2/Pd (cat.)

Dai and col. described the selective reduction of the peroxide moiety with magnesium/methanol with catalytic amount of iodine.[203] That conditions were described to be compatible with the presence of double bounds in the molecule. Alternatively, the use of Mg/MeOH (I_2 cat.) as reducing agent allows access to new analogues with an open cycle while the aliphatic chain remanins intact. Trunculin A was reduced by Dai conditions to obtain the desired compound **(25)** in 89 % purity and quantitative yield. An aliquot of the open cycle trunculin A was methylated with diazomethane. Trunculin B was subjected to the same previous procedure and the desired compound **(23)** was obtained with a 85 % purity and a 75 % yield. An aliquot of the open cycle trunculin B was also methylated with diazomethane.

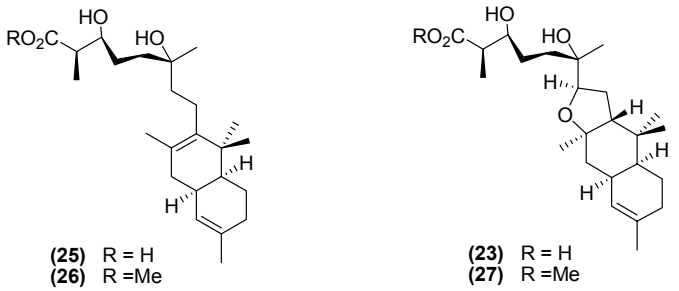


Figure 4.24 Reduced compounds from trunculin A & B using Mg / MeOH and I_2 (cat.)

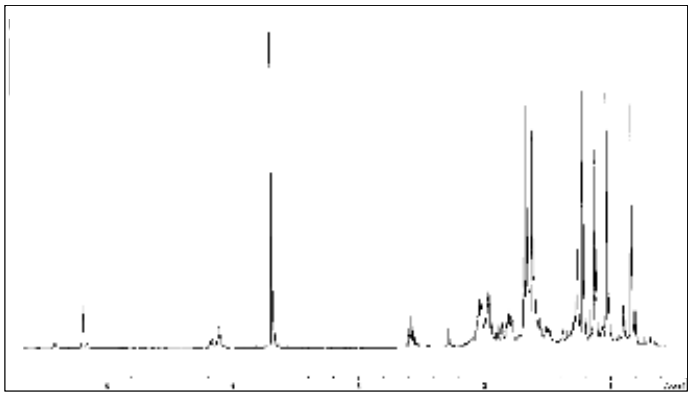
Magnesium/methanol reducing method is proposed as a new method to determine the absolute stereochemistry in norterpene peroxide cyclic natural compounds.

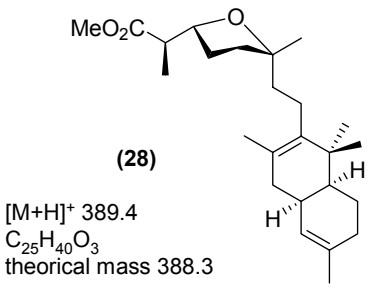
[203] Dai, P.; Dussault, P. H.; Trullinger, T. H., Magnesium/Methanol: an effective reducing agent for peroxides, *J. Org. Chem.* **2004**, *69*, 2851-2852.

4.4.3 RECYCLING TRUNCULIN A

The closure of trunculin A open cycle as a tetrahydrofuran form was achieved through a dehydration reaction. The new compound pretends to be a useful drug derived from natural trunculin A.

Dehydration reaction was carried out in dried benzene with catalytic *p*-toluenesulfonic acid (90 ºC, 16 h). Several attempts to use the reduced carboxylic forms were unsuccessful because the desired product was observed only in the MS detector. 20 mg of methyl ester form **(26)** was subjected to dehydration reaction and the conversion to the desired compound **(28)** was achieved in 90 % yield. Two more isomers were also detected. The desired compound was purified in a silica-gel column to obtain 9.2 mg (48 % yield).



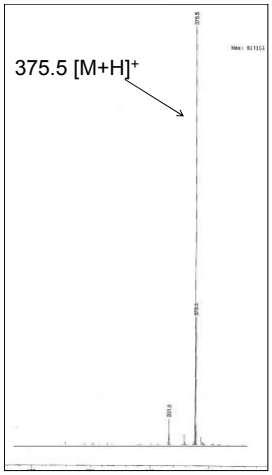


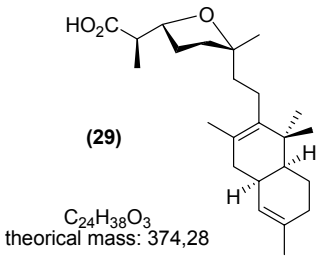
(28)

$[M+H]^+$ 389.4
$C_{25}H_{40}O_3$
theorical mass 388.3

Figure 4.25 ^{1}H NMR spectrum (CDCl3, 600 MHz) of dehydration reaction

The last step was the deprotection of the carboxylic group to obtain the tetrahydrofuran trunculin A derivative **(29)**. Next, methyl ester was removed by base treatment. Several attempts were performed with 2.3 mg of the protected analogue. Overnight reaction with NaOH, 5 eq, gave only 50 % of conversion. Overnight reaction with LiOH, 5 eq, achieved 70 % of conversion. The product, though, was not isolated by basic-acid washings.





(29)

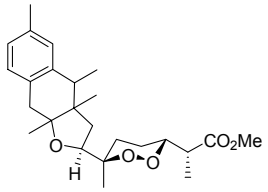
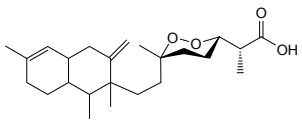
$C_{24}H_{38}O_3$
theorical mass: 374,28

Figure 4.26 ESI (+) spectrum of compound 29 in carboxylic removal reaction

In order to obtain information about the new analogues antitumoral activities, all samples were subjected to bioassay analysis.

4.5 ANTITUMOR RESULTS FROM TRUNCULINS COMPOUNDS

The trunculins derivatives obtained were prepared in 1mg/mL MeOH solution in order to obtain a Structure-Activity relationship. The compounds were testes again three tumor cell lines: HT-29 (human colon adenocarcinoma grade II), A549 (human lung carcinoma) and MM96L (human melanoma cell line), using NFF (neonatal foreskin fibroblasts) as a control cell lines. Human colon carcinoma and human lung carcinoma are the most extended cancer. The third cell line is because Australia has the highest rate of skin cancer in the world.

Trunculins in CMB-02633							
CMB-02633-A				CMB-02633-B			
							
NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L
10	10	10	30	10	30	10	100

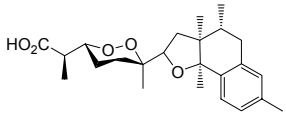
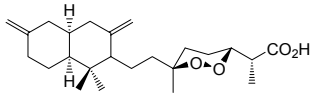
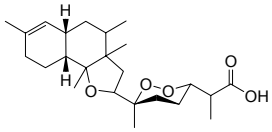
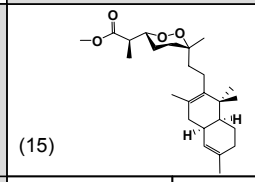
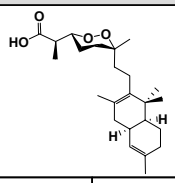
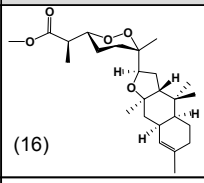
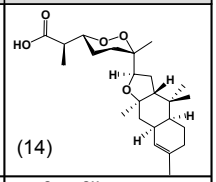
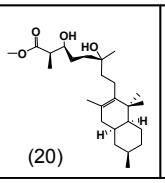
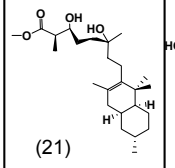
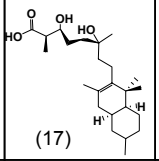
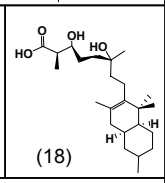
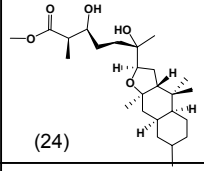
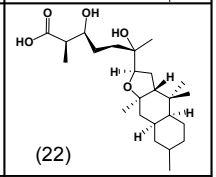
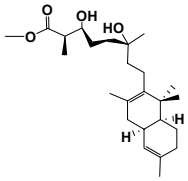
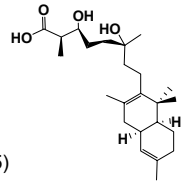
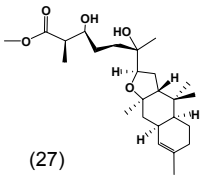
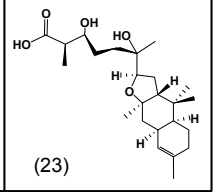
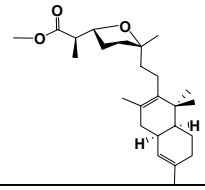
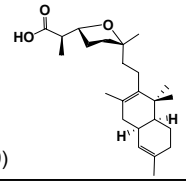
Trunculins in CMB-02709											
CMB-02709-A				CMB-02709-B				CMB-02709-C			
											
NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L
10	10	10	10	10	10	10	10	10	10	10	10

Table 4.7 Bioassay data from trunculins in speciemen CMB-02633 and CMB-02709. Data is presented as a percentage of dyed control cell protein

1 mg/mL	Trunculin A		Trunculin B	
	OMe	OH	OMe	OH
	 (15)	 (13)	 (16)	 (14)
Total Reduction	 (20)  (21)	 (17)  (18)	 (24)	 (22)
Partial Reduction	 (26)	 (25)	 (27)	 (23)
Tetrahydrofuran cycle	 (28)	 (29)		

µg/mL	Trunculina A								Trunculina B							
	OMe				OH				OMe				OH			
Natural	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L
					1	1	1	1					1	0.1	0.1	0.1
Total Reduct.	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L
	20	20	10	7	20	>30	20	20					7	7	7	7
	10	7	7	7	>30	>30	>30	20								
Partial Reduct.	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L
	20	20	20	7	20	10	10	10	20	7	10	7	10	7	7	7
Recyc.	NFF	HT29	A549	MM96L	NFF	HT29	A549	MM96L								
	20	20	10	20	20	20	20	20								

Table 4.8 Bioassay data from trunculin A & B derivatives. Data is presented as a percentage of dyed control cell protein

From bioassay data we can extract a preliminary structure-activity relationship:

- Trunculin B derivatives are more cytotoxic than their trunculin A parteners.
- Contrary it was expected, methyl ester forms are not always the least reactive. In one hand, total reduced derivatives are more cytotoxic in methyl ester form. In the other hand, partial reduced derivatives are more cytotoxic in carboxylic acid form. The recycling forms do not present differences in function protected or unprotected carboxylic form. This analysed is valid for trunculin A derivatives and it is followed by trunculin B derivatives in tested compounds.
- There are significant differences between the two total reduced isomers in the methyl ester form.
- Compound (22) is the most potent trunculin derivative and compound (27) the most selective from all tested.
- Almost trunculin derivative show cytotoxicity again human melanoma cell line (MM96L).
- Recycling form in trunculin A does not show similar cytotoxicity respect natural compound.

We could conclude that aliphatic chain and its stereochemistry can be the responsible to modulate the cyctotoxic activity and the presence of polar compounds are necessary. It is confirmed that peroxide cycle is related with the pharmcaphore molecular part. Even diols compounds have presented some activity and they are susceptible to chemical modifications so they could be an interesting starting point to desing new affordable trunculins as antitumoral compounds.