Chemodiversity of the Mediterranean sea:
The ECIMAR network

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Biotechnology: an applied science that seeks to enhance human life and environmental quality.

Marine biotechnology seeks to develop goods and services from the organisms and processes of the ocean.

New Products from the Seas

New Processes from the Seas

Understanding and Conserving the Seas
Different steps towards the marketing of a new drug
Natural Products as Pharmaceuticals

- Natural Products: > 50% of NCE between 1981 and 2006
- High molecular diversity
- ‘Designed’ to be bioactive
- Less than 10% do not respect Lipinski rule of 5

Decline in the 90’s
- Combinatorial chemistry
- Infectious diseases less studied
- Rio convention on the biodiversity 1992

Patents on Natural Products for Drug Discovery
Natural Products as Pharmaceuticals

The 21th century: the renewal

- Technological advances: bioassays, NMR, MS
- High ratios of bioactive compounds
- Improvement of the production of complex molecules

Chemical process towards the isolation of a new drug
Marine Natural Products as Pharmaceuticals

- 60’ : Cousteau and the exploration
- Oceans: 70% of the earth surface
- < 10% biodiversity known
- Ara-A and C first marine drugs

Tunicate
Ecteinascidia turbinata

Yondelis® launched september 2007
Advanced soft tissue sarcoma
PharmaMar S.A. (Madrid)
### Marine Natural Products as Pharmaceuticals

#### Low success!
2 compounds on the market after 30 years

#### Complex structures and problem of production

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Source</th>
<th>Chemical class</th>
<th>Company</th>
<th>Disease area</th>
<th>Status</th>
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<tr>
<td>Ziconotide</td>
<td>Cone snail</td>
<td>Peptide</td>
<td>Neuriva</td>
<td>Chronic pain</td>
<td>Phase III</td>
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<td>AM5316</td>
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<td>Nautilis vine</td>
<td>Anabasine derivative</td>
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<td>Novartis</td>
<td>Cancer</td>
<td>Phase I</td>
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<td>Protein kinase inhibitors</td>
<td>Bryostatin-1</td>
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<td>Sponge</td>
<td>Steroid</td>
<td>Inflzyme/Aventis</td>
<td>Asthma</td>
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</table>

Main Marine Natural Products and analogues in development

Biological Activity of Marine Phyla

Main organisms of interest
✓ Benthic marine invertebrates: sponges
✓ Microorganisms

Marine bioactive phyla
How does it work?

Main academic and industrial efforts use bioguided fractionation to identify a compound responsible for the bioactivity of an extract.

Example of the collaboration University Nice/ PharmaMar

The process:
- letter of agreement of the French overseas territories (Nice)
- organisation of the expedition (Nice)
- financial support for recollection (PharmaMar)
- recollection of organisms (>200 g, Nice and PharmaMar)
- antitumoral screening (PharmaMar)
- isolation and structural characterization of bioactive compounds (10% Nice, 90% PharmaMar)
The Results

After 8 expeditions (Mayotte, Guadeloupe, Geyser, Martinique, Wallis) in 6 years of collaboration:
- average of 250 samples of 200 g per expedition
- no identification of the organisms
- around 40% of the samples exhibited antitumoral activity
- no patentable product !!!

Why ?
- false positive (synergy)
- false negative

Other drawbacks
- no respect of the biodiversity
- focus is only on one target
Main problem for the development of therapeutic agents from the marine environments:
- limited or sporadic distribution in the organisms
- variation in the production of the metabolites

Other problem due to the production in small quantities of complex molecules:
- organic synthesis often difficult
- complex biochemical processes inducing difficulty with gene cloning
- the culture is often difficult

ROLE of SYMBIOSIS ??
The new era

Microorganisms: easier to describe and to handle for production
Kerr, Fenical...

Biosynthetic studies: Leadlay, Poulter, Vederas, Piel, Khosla, Moore...
Biotica, Kosan biosciences

Creation of library HPLC-MS-NMR-bioassay: Ireland

Aquaculture: Osinga, Muller...

New NMR and MS tools: 1 to 10 µg of pure compound

Cell localisation of the biosynthesis: Gerwick
The French Paradox

French overseas:

- 11 Millions km² (19 times the metropolitan France)
- 2nd world’s maritime area
- Unique country with access to all oceans
- 10% world’s coral reeves

Marine research:

- Cousteau and diving
- Marine biologists (COM J. Vacelet)
- Small groups of MNP
- Few collaborations
- ‘Industry’ dependence
The ECIMAR Program

Marine Chemical Ecology: Biodiversity Indicators and Development

Duration
4 years since 2007
Coordination: T. PEREZ, O. THOMAS
Marine Biologist, Marine Chemist 100% Academic
The ECIMAR Network

Main advantage: federation of multidisciplinary scientists

MNP Chemists need taxonomists and pharmacologists
Ecologists and taxonomists need MNP Chemists

Secondary Metabolites
The ECIMAR Main Goals

WP 1: Marine Natural Products
- Chemical fingerprints
- Secondary metabolites
- Bioactivity

WP 2: Production of Metabolites
- Culture
- Biosynthesis
- Biomimetic synthesis

WP 3: Fluctuation of the production

Biotic and abiotic Factors
The ECIMAR ‘Playground’: the Mediterranean Sea

Implication of local researchers and formation of students

Recollections 2007 and 2008

Recollections For 2009
The ECIMAR ecosystems

First Area of Interest:
The Mediterranean Sea

Solid bases in ecology and biology of marine invertebrates:
Porifera, cnidarians, tunicates...
High specific diversity
The Mediterranean Sea

0.8% of the surface of the oceans – 5 to 10% of the marine biodiversity - Hotspot

Mediterranean ecosystems:
- coralligenous
- caves

Long bioconstruction:
600 to 8000 years

More than 1600 species (300 algae, 1200 invertebrates set 110 fishes)
Importance of the network

Description of typical ecosystems – Inventory of the marine biodiversity

Respect of the biodiversity - Work on max10 g of wet samples

Strong collaborations and exchanges between research groups

Complete characterization of the secondary metabolites of selected species. No loss of data

Biological activities on pure compounds

Anti activities from antifouling to antitumoral…

Easier to find financial support
Future prospects

Transnational financial supports: EU for example
MNP at the University of Nice

Biosynthesis
- In aquarium studies

Organic synthesis
- Total synthesis
- Biomimetic hypotheses

Marine Natural Products
- Isolation
- Structure determination
- Stereochemistry

Chemical ecology
- Chemotaxonomy
- Global warming
- Fluctuation of expression

Development
- Therapeutics
- Cosmetics
- Fouling

N. Cachet
Main goals of biosynthetic studies:
- Production of metabolites by « bio engineering »
- Chemical reactivity knowledge and discovery of new chemical reactions inspired by Nature
- Alternative approach for species classification

Marine biosynthetic pathways:
close to those used by terrestrial plants

BUT:
experimental studies more difficult
Incorporation experiments:
- *in situ*
- in aquarium (*ex situ*)
- cell cultures

Labelled precursors most often used for the studies
- Radioactive or stable isotopes (MS or NMR)

Preliminary radioactive experiments: optimization of the metabolization conditions using $^{14}\text{C}$ or $^3\text{H}$ labeled precursors ($\mu\text{g.L}^{-1}$)

Confirmation and localization using $^{13}\text{C}$, $^2\text{H}$ or $^{15}\text{N}$ (mg.L$^{-1}$)
• Origin of the anticancer alkaloid ET743 from the ascidian *Ecteinascidia turbinata* (cell-free extract) R. Kerr *Tetrahedron* 2000, 3303
A collaborative project

* Start: well adapted IAEA equipments (LEM – Ross JEFFREE)
  - Aquaria from 20 to 200 L in continuous flow
  - Complete control of the experimental conditions:
    - salinity, light, temperature
  - Hot laboratory (J.-L. TEYSSIER) enabling radioactive experiments: $^{14}$C, $^3$H...

* Biosynthetic hypotheses of the ICSN (Ali AL MOURABIT) on targeted metabolites produced by Mediterranean marine sponges (Agelasidae and Axinellidae)

* Recollection of the organisms and experiments UNS (N. CACHET, M. MEHIRI, O. THOMAS)
Geographic localization: Monaco

Saint Nicolas Rocks

Rare and preserved ecosystem
Biosynthetic studies on a model

What are the amino acid precursors of oroidin?
What are the best conditions to optimize the biosynthesis of the compound?
Chemical fingerprint

LCMS Profile of the organic fraction of *Agelas oroides*

- **Sample concentration:**
  10 mg.mL⁻¹ in CH₂Cl₂/MeOH 1:1

- **Injected volume:**
  20 µL

- **LC-MS Gradient:**
  A = CH₃CN + 0.1% FA
  B = H₂O + 0.1% FA
  t = 0 min 5:95 A / B
  t = 5 min 5:95 A / B
  t = 35 min 100:0 A / B

Chemical fingerprint:

Oroidin

C₁₁H₁₁Br₂N₅O
Aquarium: 20 L of flowing fresh sea water

300 mL of artificial sea water (closed water circuit)

Agelas oroides
Collection: Monaco (Roches St Nicolas) Depth = 25 m

Addition of $^{14}C\text{-Arginine}$ for A and B (0.15 nmol.g$^{-1}$)
Addition of $^{14}C\text{-Proline}$ for C and D (0.2 nmol.g$^{-1}$)

2 days of acclimatization
Incorporation time: 22h

- 90% incorporation after 10 h
- Slower for arginine than proline
Loss kinetics

Incorporation period: 16:30
300 mL of artificial sea water (closed water circuit) + $^{14}$C proline

Aquarium: 20 L of flowing fresh sea water

Metabolization:
- 0 day for A
- 1 day for B
- 2 days for C
- 3 days for E
- 7 days for D

Radioactivity per mg of sponge extract
Radio-TLC profile

Oroidin

TLC plate:
Preparative glass TLC Si60 20x20 mm
Thickness layer: 2 mm

Elution:
Ethyl acetate/Acetone/H₂O/Formic acid
(5:3:0.5:0.5)

Application volume:
100 µL (10 mg) Band width: 10 mm

Derivatization reagent:
H₂SO₄ + anisole

Next steps:
Quantitation, Best conditions of production
MNP at the University of Nice

- **Biosynthesis**
  - In aquarium studies

- **Marine Natural Products**
  - Isolation
  - Structure determination
  - Stereochemistry

- **Organic synthesis**
  - Total synthesis
  - Biomimetic hypotheses

- **Chemical ecology**
  - Chemotaxonomy
  - Global warming
  - Fluctuation of expression

- **Development**
  - Therapeutics
  - Cosmetics
  - Fouling

- **Centra d’océanologie de Marseille**

Authors:
- N. Cachet
- M. Mehiri
Parazoanthus sp.

- Colonial anemone
- Polyps up 1,5 cm tall
- 0,5 cm in diameter
- 26-34 polyps
- All over the world
- Often living on sponges

Cnidarian Anthozoa, Zoantharia
MNPs from *Parazoanthus sp.*

(Marinlit database 2008)
Parazoanthus axinellae from the Mediterranean

Crude extract

Aqueous phase

100% H$_2$O

V = 60 mL

C$_{18}$ SPE

MeOH/CH$_2$Cl$_2$ 1:1

V = 60 mL

Organic Phase

LC-MS profile

Luna, Phenomenex Phenyl-Hexyl (150×4.6 mm ; 5 μm)

Gradient MeOH/H$_2$O + 0.1% FA

5:95 to 100:0 in 30 min
A new molecule

HRESIMS \( \text{C}_{16}\text{H}_{19}\text{N}_5\text{O}_3 \)

\(^3J_{\text{H6-H7}}(E) = 15\ \text{Hz}\)

d\(_4\)-MeOH (18K5025NHH\(_2\))
The parazoanthines: a new family

Parazoanthines A-G
Microtox® bioassay: *Vibrio fischeri*

[Chemical structures and EC₅₀ values]

At this level collaboration with Asepta for cosmetic applications
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