From fungi to microalgae: UHPLC-HRMS based dereplication and preparative isolation of bioactives

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- **S. cerevisiae**
  - None conversional yeasts

- **Aspergillus Penicillium Fusarium**

- **CHO Cells**
- **Roseobacter Vibrio Lactococcus lactis Bacillus**

- **Microalgae**
  - SCCAP K-0081 Prymnesium parvum

Metabolic pathway:

\[ A \rightarrow B \rightarrow C \rightarrow D \]
Mycotoxin work at Center for Microbial Biotechnology (Mycology group)
The TLC-agar-plug method

Cut a 3 mm plug and add a drop of solvent. Place the plug with the wetted mycelium facing down on a TLC plate and elute.

Secondary metabolites for identification of fungi

• TLC profiling (Filtenborg & Frisvad, 1980)
• HPLC-DAD reversed phase (Frisvad & Thrane, 1987)
  • Library of profiles for 10 000 strains
  • 3000 UV spectra in manual library
• Direct ESI-MS+
• LC-DAD-TOF-MS
Besides sensitivity and specificity, MS detection is vital for $^{13}$C and $^{15}$N labelling studies.
First vacuum stage – where the differences start.

Volatile compound can be lost

Selection between small/large Fragile / stable....

Dual Dry Rough Pumps

Dual ion funnels

No skimmer

Collision Cell / Ion Beam Compressor

Hi Speed Detector Assembly

Shorter desolvation/hexabore capillary

Dual Agilent Jet Stream
LC-ESI⁺-TOF spectra of neosolaniol with different ion-source fragmentations

![Spectra diagram showing m/z values and molecular ions for neosolaniol at 24 V and 6 V, including labels for [M+H-H₂O-CH₃COOH]⁺, [M+H-H₂O-CH₃COOH+CH₃CN]⁺, [M+H]⁺, [M+NH₄]⁺, and [M+Na]⁺]
### Table 2: Common "Jumps" (Δ) observed in Electro spray and the frequency of adducts in 719 reference standards

<table>
<thead>
<tr>
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<td>[M-H]^−</td>
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<td>ND</td>
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<tr>
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<td>ND</td>
<td>1.0</td>
<td>[M+H2O]^−, eg. opening of lactones</td>
<td>18.0106</td>
<td>ND</td>
<td>2.6</td>
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<tr>
<td>[M+H+2Na]^+ (acids, phenols and enoles)</td>
<td>43.9640</td>
<td>ND</td>
<td>0.2</td>
<td>[M+H+Fe]^+ , isotope m/z 2 lower ca. 4%</td>
<td>53.9193</td>
<td>ND</td>
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<td>[M+HCOO]^− to [M+Cl]^−</td>
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<td>ND</td>
<td>0.2</td>
<td>[M+H+Fe]^+ , isotope m/z 2 lower ca. 4%</td>
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<td>ND</td>
<td>0.2</td>
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<td>H2O</td>
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<td>CO2</td>
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<td>[M+H2O]^−, eg. opening of lactones</td>
<td>18.0106</td>
<td>ND</td>
<td>2.6</td>
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</table>

- a mass shift
- b increased by high in-source fragmentation settings
- c loss or addition of NH3 can usually be determined looking for [M+Na]^+
- d if MeCN is used as solvent. MeCN forms NH3 upon acidic hydrolysis
- e sideophors and artefact from Fe^2+ liberation ESI+
- f Often seen in peptides > 1000 Da
- g if HCOOH is added as buffer
- h not observed since acetate was not added

# Mass defect

<table>
<thead>
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<th>Element</th>
<th>Mass</th>
<th>Mass defect (mDa)</th>
<th>Relative to mono isotopic mass (%)</th>
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<tbody>
<tr>
<td>H</td>
<td>1.0078</td>
<td>7.825</td>
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<tr>
<td>$^{12}$C</td>
<td>12.0000</td>
<td>0</td>
<td>100</td>
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<tr>
<td>$^{35}$Cl</td>
<td>34.9689</td>
<td>-31.147</td>
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<tr>
<td>$^{37}$Cl</td>
<td>36.9659</td>
<td>-34.097</td>
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<tr>
<td>$^{16}$O</td>
<td>15.9949</td>
<td>-5.085</td>
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<td>$^{18}$O</td>
<td>17.9992</td>
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<td>$^{15}$N</td>
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<td>$^{32}$S</td>
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<tr>
<td>$^{34}$S</td>
<td>33.9679</td>
<td>-32.133</td>
<td>4.519</td>
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</tbody>
</table>

$\text{C}_{16} \text{H}_{28} \text{N}_3 \text{O}_2$ mass $\text{m/z} 294.2182$

$\text{C}_{15} \text{H}_{24} \text{N}_3 \text{O}_3$ mass $\text{m/z} 294.1818$
T-2 toxin (C_{24}H_{34}O_9) found in naturally contaminated sample (oats)
Why are both mass accuracy & isotopic pattern important?

Table 1. Distribution of compounds in Antibase 2008 with different elemental compositions that cannot be distinguished at selected mass-differences\(^a\), carbon isotope ratio, and A+2 elements (S, Cl, Br) determination

<table>
<thead>
<tr>
<th>Mass range (Da)</th>
<th>Total compounds</th>
<th>Number of compounds</th>
<th>Mass difference (ppm)</th>
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<tr>
<td></td>
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<td>Nominal(^b)</td>
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<td></td>
<td>10 5 5 5 5 5 2 2 2 2 2 1</td>
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<td>Isotope constrain</td>
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<td>None (\pm 5\ C)  (\pm 2\ C)  (\pm 1\ C)  (\pm 1\ C)</td>
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<tr>
<td>60-99</td>
<td>133</td>
<td>45 45 38 31 0</td>
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<tr>
<td>100-199</td>
<td>2969</td>
<td>738 717 461 342 13</td>
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<tr>
<td>200-299</td>
<td>6167</td>
<td>1532 1376 811 538 104</td>
<td>48 43 40 32 19 0 0 0 0 0 0</td>
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<tr>
<td>300-399</td>
<td>6494</td>
<td>2119 1694 899 518 264</td>
<td>84 60 53 45 27 0 0 0 0 0 0</td>
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<tr>
<td>400-499</td>
<td>5125</td>
<td>2208 1636 872 567 434</td>
<td>195 163 138 114 92 0 0 0 0 0 0</td>
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<tr>
<td>500-599</td>
<td>3764</td>
<td>1791 1237 600 458 326</td>
<td>132 95 71 64 50 58 46 41 36 32 0</td>
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<tr>
<td>600-699</td>
<td>2065</td>
<td>1234 815 392 288 203</td>
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<td>700-799</td>
<td>1590</td>
<td>985 669 352 273 145</td>
<td>69 55 26 25 23 23 20 16 16 16 0</td>
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<td>800-899</td>
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<td>Total</td>
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<td>13024 9587 5187 3571 1775</td>
<td>730 1775 730 344 254 130 103 89 82 77 0</td>
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</table>

Chemical database → "ask" if each metabolite is in extract.....and the supervise this?
Sorting out compounds

<table>
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<tr>
<th>Standards</th>
<th>Include Penicillium With RT</th>
<th>With RT</th>
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<td>6</td>
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<td>YES</td>
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<tr>
<td>22</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tbody>
</table>

Sub number of compounds in group

- A. nidulans
- F. niger
- Gladosporum
- Alternaria
- Fumigatus
- Mucor Bacteria
- Penicillium

Confirmed in strain: YES, Likely from: YES, Possible: YES, Possible from: No, Aspergillus sp.: No, No, No, No, No, No.
List ready for Target analysis

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<td>C30H37O7N1</td>
<td>S806-19.20Epoxyctochalasin D</td>
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<td>C27H33O6N1</td>
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<tr>
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<td>285</td>
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<td>Pyrophen</td>
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</table>

S means standard available
(UK unidentified)
Elemental composition searched (corrected for adduct and charge state)
Target analysis (3000 compounds in 2 minutes all from *Aspergillus* and *Penicillium*.

- *Aspergillus niger* and *A. carbonarius* - historic data 80% hit rate first run
- *Aspergillus fumigatus* – 80% hit rate first run
- *Aspergillus nidulans* – 50% of peaks due to many with same elemental composition
- Several penicillia – ID’s (unbiased) misidentification.....
Sort out appropriate target compounds (1-3000 compounds).

Results display:

- S848-Pyranopyrrol A
- Unknown A carbonarious no 6
- S793-TMC-256A1
- Tensyuic acid A
- Tensyuic acid F
- Unknown A carbonarious no 4
- Chloramphenicol (internal std)
- S133-Dihydrofusarubin A
- S710-Altenusin
- Fonsecin
- Fonsecin B
- Aurasperone C
- Nigerasperone B
- Aurasperone B
- HCOONa infused for mass calibration
- Citric acid
- Unidentified peak for manual inspection
- S793-TMC-256C1
- TMC-256A1
- Asperxanthone
- Rubrosarin B
- Flavaspperone
- S115-Ochratoxin A
- Unknown A carbonarious

Target result analysis list:

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<th>Name</th>
<th>Mol. Formula</th>
<th>PMI</th>
<th>d RT</th>
<th>m/z measured</th>
<th>m/z, calc.</th>
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<td>S710-Altenusin</td>
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<td>Fonsecin</td>
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</tbody>
</table>

Explorative Solid-Phase Extraction (E-SPE)

N = 34390 compounds

Thus are we running low pH separations

Explorative Solid-Phase Extraction (E-SPE-II)

SAX

MAX

SCX

LH-2

Diol

Amino

Carboxylic acid, phosphate...

Non acidic

Hydrophobicity

Acidic

Basic amines


Hydrophilic functional groups
**E-SPE for strain prioritisation**

*Two Pseudoalteromonas luteoviolaceae strains with different bio-activity profile*

<table>
<thead>
<tr>
<th></th>
<th>SAX</th>
<th>MAX</th>
<th>SCX</th>
<th>LH-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indolmycin</td>
<td><img src="image" alt="Indolmycin" /></td>
<td></td>
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</tr>
<tr>
<td>Pentabromopseudilin</td>
<td></td>
<td><img src="image" alt="Pentabromopseudilin" /></td>
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</tr>
<tr>
<td>Violacein</td>
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<td><img src="image" alt="Violacein" /></td>
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</tbody>
</table>

**Methylation profile**

- **SAX**
  - Indolmycin: Methylated
  - Pentabromopseudilin: Methylated
- **MAX**
  - Indolmycin: Methylated
  - Violacein: Methylated
- **SCX**
  - Indolmycin: Methylated
  - Pentabromopseudilin: Methylated
  - Violacein: Methylated
- **LH-20**
  - Indolmycin: Methylated
  - Violacein: Methylated
  - Pentabromopseudilin: Methylated
Makes LC-MS interpretation easier
- Possible to enhance HPLC conditions for different fractions
HABFISH – Harmful Algae and Fish Kills

- *Prymnesium parvum*
- *Karlodinium armiger*
- *Chrysochromulina polylepis*

- *Alexandrium tamaranese*
- *Karenia mikimotoi*

KU Marine Biological Section
Algal cultivation
+ DNA probes

AU Animal Science
Fish Immunology

DTU Systems Biology
Natural Products

DTU Food
Chemical Food Analysis
Prymnesium parvum

- Cosmopolitan algae
- Blooms in brackish waters
- Has been linked to severe fish kills worldwide
- Re-occurring blooms and loss of fish stocks in Norway
The known chemistry of *P. parvum*

- Hemolysin 1 (Kozakai 1981)
- Prymnesin -1 and -2 (Igarashi 1996)
- Golden algal toxin (Henrikson 2010)
- Fatty acid amides (Bertin 2012)
Galactolipids and fatty acid amides are not ichthyotoxins!

- Large variation in strain toxicity
- GATs did not correlate to the observed ichthyotoxicity
- Oleamide was not detected in a $^{13}$C enriched culture
- The Danish isolate was the most toxic (has been in collection for 30 years)
Analytical sample preparation

1. Desalting
2. Elution MeOH

Centrifuge
Biomass extraction
Supernatant extraction

Acetone + MeOH
Dereplication of prymnesin-2 in *P. parvum* (+90% found in the biomass)
Prymnisin 2

Triply charged

Measured

Calculated

\[ \text{C}_{65} \text{H}_{136} \text{Cl}_{3} \text{NO}_{35} \]
The known prymnesins are not produced by highly toxic Danish strain.
Potential new high molecular weight compounds in K-0081!

2 high molecular weight compounds with the elemental composition of:

1) $\text{C}_{90}\text{H}_{130}\text{ClNO}_{33}$  
2) $\text{C}_{91}\text{H}_{132}\text{ClNO}_{34}$

Same aglycone – new prymnesin type
Cultivation, harvesting and isolation of novel prymnesins from \textit{P. parvum}

Biomass extracted with 1) acetone, 2) MeOH

3000 g, 40 mL/min, 100 L total (also $^{13}$C enriched analogue)

Amino-propyl functionalised silica
Large scale harvesting

Continuous Centrifuge

C_{18} Column

Algal Culture
Workflow – isolation of natural products

100 L culture!

Crude extract

Preparative fractionation
bio-assay

10 mg – 100 mg

Semi-preparative
HPLC

1.8 mg

Pure compound
Workflow – isolation of natural products

100 L culture! → Crude extract (300 mg) → Preparative fractionation → bio-assay → Semi-preparative HPLC → Pure compound (1.8 mg) → 10 mg – 100 mg
Purification of prymnesins (bimass)

- Crude extract
  - NH2 - EtOAc:MeOH (2:1)
  - NH2 - EtOAc:MeOH (1:1)
  - NH2 - MeOH

Semi-prep HPLC

Pure compound
Complex $^1$H NMR spectrum for new compound

- Low solubility of the pure toxin hampered the detection of the $^{13}$C isotope

- Cultivation of a 90% $^{13}$C enriched version in artificial seawater
Challenges using highly $^{13}$C enriched material

- Loss of spectral resolution due to $^{13}$C-$^{13}$C homonuclear coupling
- Constant time HSQC can “decouple” this $^{13}$C-$^{13}$C coupling
From 2D to 3D NMR

Tools usually used for proteins

Complex and congested

$^1\text{H} - ^1\text{H}$ TOCSY

Overlap in $^1\text{H}$ dimension

Separated in $^{13}\text{C}$ dimension

Extension to 3D NMR
And from 3D back to 2D planes
Backbone assembly

- Calc. DBE = 26

- How to form the many ether rings?

- Difficult to distinguish between ether carbon and hydroxyl carbons by $^{13}$C chemical shifts (both between 65-80 ppm)
Deuterium exchange experiment distinguished ether carbons from hydroxyl bearing carbons.

- $\delta_H = 4.05$ ppm, $\delta_C = 70.5$ ppm
- $\delta_H = 3.13$ ppm, $\delta_C = 80.4$ ppm
- $\delta_H = 3.62$ ppm, $\delta_C = 71.4$ ppm
- $\delta_H = 4.00$ ppm, $\delta_C = 66.4$ ppm

Measurement of the induced shift
Backbone assembly > a new B type

Prymnesin-B1

Prymnesin-2
Elucidation of the carbohydrates by Chiral GC-MS

Prymnesin-B1/B2
R = α-D-galactopyranoside

Prymnesin-B2
R = α-D-ribofuranoside

D-galactose
L-galactose
D-ribose
L-ribose

70°C

150°C
Waters semi-prep

36-46 % MeCN (25 ppm TFA) in 30 min
Injection: 100 µl MeOH

Dionex Ultima

36-46 % MeCN (25 ppm TFA) in 10 min
Injection: 1 µl MeOH

Dionex Ultima

36-46 % MeCN (25 ppm TFA) in 30 min
Injection: 100 µl MeOH

Dionex Ultima

36-46 % MeCN (25 ppm TFA) in 30 min
Injection: 100 µl H2O:MeOH:iPrOH (50:25:25)
Evaluation of the toxicity

Fluorescent method for quantification of prymnesins in solution (Waters Aqu Tag)

Fish (rainbow trout) exposure to purified compounds will be conducted in the spring 2016

\[
\begin{align*}
\text{NH}_2 & \rightarrow \text{NH} \\
\end{align*}
\]
Dereplication showed a even further C-type

- Screening of 10 strains we found prymnesin analogues in all!
- Defining another C-type

MS/MS

C-type $\text{C}_{88}\text{H}_{125}\text{Cl}_{4}\text{NO}_{35}$

B-type $\text{C}_{90}\text{H}_{130}\text{Cl}\text{NO}_{33}$

Original type $\text{C}_{96}\text{H}_{136}\text{Cl}_{3}\text{NO}_{35}$
Dereplication showed an even further C-type
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KU Marine Biological Section
- Per Juel Hansen
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- Jens Øllgaard Duus

DTU Systems Biology
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- Livia Soman De Medeiros

NMR time at Danish NMR Center for Macro Biomolecules Carlsberg Research Centre (CRC)