Screening Malaysian Marine Fungal Extracts for Cytotoxic Activity

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Abstract: A total of 178 fungal isolates were successfully isolated from marine invertebrates collected in surrounding waters of Pulau Redang and Pulau Payar, Malaysia. All isolates grew on both sea water and non-sea water media, thus all of them can be classified as facultatively marine. No obligate marine fungal were isolated. A number 25 extracts (43.9%) were found to be potentially active by inhibiting >50% of the growth of the P388 cells. Of these, 10 extracts (40%) showed cytotoxicity (IC_{50} <12,500 mg/ml) against the P388 cell lines. HPLC screening using in-house HPLC-UV/Rt library database readily dereplicated four known secondary metabolites from four cytotoxic fungal extracts, while six extracts contained peak(s) that were not readily dereplicated. These metabolites were dereplicated from extracts F6406 (meleagrine), F6415 (cytochalasins), F6430 (indole-3-carboxylic acid) and F6451 (dehydrosterigmatocystin). The results showed naturally occurring metabolites in Malaysian marine fungi have potentials to produce cytotoxic metabolites enabling the discovery of new bioactive compounds, hence merit future studies.

Keywords: marine fungi, marine invertebrates, cytotoxic metabolites, Malaysia

Abstrak: Sejumlah 178 pencilan kulat telah berjaya dipencilkan daripada invertebrata marin yang dikutip di sekitar perairan Pulau Redang dan Pulau Payar, Malaysia. Kesemua pencilan boleh dikelaskan sebagai kulat marin fakultatif kerana mampu hidup di dalam media air laut dan media bukan air laut. Tiada kulat marin obligat yang dipencilkan. Sejumlah 25 ekstrak (43.9%) kulat didapati berpotensi aktif dengan dapat merencat >50% tumbesaran sel P388. Daripada ini, sejumlah 10 ekstrak (40%) menunjukkan sitotoksisiti (IC_{50} <12,500 mg/ml) terhadap sel P388. Penyaringan HPLC mengunakan pangkalan data perpustakaan HPLC-UV/Rt menunjukkan 4 struktur metabolit yang telah diketahui daripada empat ekstrak kulat manakala 6 lagi mengandungi puncak yang tidak *readily direplicated*. Metabolit ini didapati daripada ekstrak F6406 (meleagrine), F6415 (cytochalasins), F6430 (indole-3-carboxylic acid) dan F6451 (dehydrosterigmatocystin). Keputusan yang didapati menunjukkan kulat marin dari perairan Malaysia mempunyai potensi yang baik untuk mengeluarkan metabolit semulajadi yang bersifat sitotoksik dan ini memungkinkan penemuan sebatian bioaktif baru untuk bidang perubatan pada masa hadapan.

Introduction

In the past, marine fungi have often been defined based on their ability to grow at certain seawater concentrations. As the definition cannot be strictly based on physiological aspects (Kohlmeyer, 1979), a broad ecological definition was used namely; *obligate marine fungi* are those that grow and sporulate exclusively in a marine or estuarine habitat; *facultative marine fungi* are those from freshwater or terrestrial environment able to grow (and possibly also to sporulate) in the marine environment (Kohlmeyer, 1979).

After the discoveries of penicillin and cephalosporin C, a further milestone in the history of medicinal fungal products was the discovery of cyclosporin A produced by *Tolypocladium inflatum* and *Cylindrocarpon lucidum* (Dreyfuss *et al.*, 1976). The cholesterol biosynthesis inhibitor, lovastatin, isolated from *Aspergillus terreus* (Alberts *et al.*, 1980) is another further metabolite of great pharmacological importance from fungi.

Overall, research on marine-derived fungi has led to the discovery of some 272 new natural products until 2002 (Bugni and Ireland, 2004), and another 240 new structures from 2002 until 2004 (Ebel, 2006), thus, providing evidence that marine-derived fungi have a potential to be a rich source of pharmaceutical leads. Bugni and Ireland (2004) showed that fungi from marine invertebrates (sponges, corals and tunicates) showed the largest contribution to the total number of compounds described in the literature (40%) with the fungi from marine plants, driftwood or sediments accounting for 37%. The review by Ebel (2006) shows the majority of novel fungal secondary metabolites originate from inorganic matter (soil, sediments, sandy habitats and artificial substrates), while marine invertebrate-derived fungi contributed less than the marine plants (algae, sea grasses, mangrove plants and woody habitats). It noted that even if many of the marine-derived fungi are already known from terrestrial habitats, and have been chemically well studied, novel secondary metabolites continue to be detected. An example is the marine-derived *Penicillium chrysogenum* that was isolated from the sponge, *Ircinia fasciculate*. This isolate produced the sorbicillin-derived alkaloid sorbicillacton A (Bringmann *et al.*, 2003) that showed selective cyctostatic activity against lymphoblasts and was able to protect human T cells against HIV-1. Some of the examples of fungal metabolites isolated from sponges, corals and tunicates are listed in Table 1. Many of these fungi have novel carbon skeletons thus providing further evidence of the potential of marine-derived fungi.

Table 1: Some new cytotoxic metabolites from fungi derived from marine invertebrates

Fungi	Marine Invertebrates	Metabolites	Activities	References
Aspergillus niger	Sponge (*Axinella damicornis*)	3,3'-bicoumarin bicoumanigrin aspernigrin A aspernigrin B pyranonigrins A, B, C and D	moderate cytotoxicity inactive neuroprotective inactive	Hiort *et al.* (2004)
Phomospis asparagi	Sponge (*Rhaphidophlus juniperina*)	chaetoglobosins	cytotoxic	Christian *et al.* (2005)
Gymnascella dankaliensis	Sponge *Halichondria japonica*	gymnastatins F and G	cytotoxic inactive	Amagata *et al.* (2006)
Acremonium sp.	Sponge (*Teichaxinella* sp.)	gymnastatin H RHM1 and RHM2	weak cytotoxicity	Boot *et al.* (2006)
Penicillium aurantiogriseum	Sponge (*Mycale plumose*)	aurantiomides B and C	moderate cytotoxic	Xin *et al.* (2007)
Gymnascella Dankaliensis	Sponge (*Halichondria japonica*)	gymnastatins Q and R dankastatins A and B	cytotoxic to P388 cells(all); inhibited growth of BSY-1 (breast) and MKN7 (stomach) human cancer cell lines (only gymnastatins Q)	Amagata *et al.* (2008)

The culturable microorganisms associated with marine invertebrates from Malaysian waters were explored for bioactivity and chemical interest. Within the Marine Chemistry Group at University of Canterbury, New Zealand, an integrated approach between research groups in microbiology and natural product chemistry is taken for marine natural products discovery. This paper presents the groundwork that was conducted towards the search for biologically active metabolites from extracts of fungi derived from Malaysian marine invertebrates.

Materials and Methods

Marine invertebrate sampling

Samples were collected by scuba diving off Pulau Redang and Pulau Payar Marine Parks, Malaysia in September 2005. Each sample was placed in a sterile bag, and either processed immediately or stored in a freezer at –20°C before isolation of fungi.

Isolation of fungi

Tissue and/or liquid portion of invertebrate samples were used for isolation of fungi. Samples were washed 3-5 times with sterile seawater. Samples were cut into small pieces, and placed in a sterile mortar with sterile seawater (1-2 ml) and homogenized with a pestle. Aliquots of the liquid portion (100-200L) were spread on Peptone Yeast Glucose (PYG) agar plates (0.1% peptone (Becton Dickinson), 0.1% yeast extract (Oxoid), 0.2% glucose (BDH), 2% agar (Oxoid) and 1% chlortetracycline/streptomycin (Sigma) in 100% natural seawater). The remaining tissue in the mortar was pressed to remove liquid and pieces (5-7; 1-3mm^3) were placed onto a PYG agar plate. Inoculated plates were incubated at 20°C. Pure fungal cultures were isolated from these initial plates after repeated inoculation onto fresh PYG agar plates (without antibiotics).

Culture and extraction

Fermentation was carried out in broth media (0.1% peptone, 0.1% yeast, 0.2% glucose, and 4% sea salt (Sigma), dissolved in distilled water, pH 7.5). Cultures were incubated at 28°C for 30 days (15 days, 200 rpm shaking followed by 15 days static). Thirty day cultures were extracted with ethyl acetate (EtOAc) overnight and extracts dried, evaporated to dryness and weighed. For solid media, isolates were cultured on a salt-based agar (0.1% peptone, 0.1% yeast, 0.2% glucose, 2% agar and 4% sea salt, dissolved in distilled water, pH 7.5). Isolates were cultured at 28°C for 30 days. Thirty day cultures were macerated and extracted with EtOAc overnight before being dried, evaporated to dryness and weighed.

P388 assays

All crude extracts (1 mg/ml) were initially screened for cytotoxicity against murine leukaemia cell lines P388 (P388 cells) (ATCC CCL 46, P388D1). An aliquot of 5 L of each crude extracts was pipetted into 90 individual wells of a 96-well microtitre plate. This assay comprised of a serial dilution of the sample of interest followed by incubation for 72 h with P388 cells. Cell viability is determined colorimetrically by the addition of the yellow dye, MTT tetrazolium. Unhealthy or dead cells cannot metabolize this dye, leaving a yellow colour, whereas healthy cells reduce this dye to MTT formazan resulting in an intense purple colour. The concentration of sample required to reduce cell growth by 50% when compared to controls, is expressed as an IC_{50} in ng/mL (Perry *et al.*, 1999). Samples that inhibited 50% of the growth of P388 cells were considered as potentially active and were subjected to a further assay to determine the concentrations required to inhibit the growth of P388 cells by 50% (IC_{50}).

HPLC screening of cytotoxic extracts

Cytotoxic crude extracts were filtered through a 0.45 µm polytetrafluoroethylene (PTFE) membrane filters immediately prior to the HPLC screening. An aliquot of the samples (25 µg) was analyzed by reverse-phase analytical HPLC on a Dionex liquid chromatograph equipped with a UVD 340U diode array detector (DAD), and connected to an Alltech evaporative light scattering detector (ELSD) 800. For reverse-phase HPLC a Phenomenex Luna C18 (10 x 250 mm, 5 m) column was used. The standard gradient used was: 2 min of 10% ACN/H_2O; a linear gradient to 75% ACN/H_2O for 12 min; isocratic at 75% for another 10 min; a linear gradient for 2 min to 100% ACN/H_20 followed by isocratic at 100% ACN for 4 min then returned to 10% ACN/H_20 in 2 min and re-equilibriated for 8 min with a flow rate of 1 ml/min and at 40C.

Retention times (R_t) of the HPLC peaks as well as their UV spectra and molecular masses were used to identify known fungal metabolites by searching in an in-house UV/ R_t library of known metabolites (using the software *Chromeleon, Dionex*), in commercial natural products databases (e.g. Antibase), and published data on fungal metabolites (Nielsen and Smedsgaard, 2003).

Results and Discussion

Isolation of fungi

The isolation of fungi from Malaysian marine invertebrates resulted in 178 isolates obtained in pure culture from a total of 17 marine invertebrates (Table 2).

Table 2: Fungi isolated from Malaysian marine invertebrates

Sampling sites	Marine invertebrates	Portion used	No. of isolates	Total
Pulau Redang Marine Park	Tunicate PR1 (*Didemnum* sp.)	Tissue	3	7
		Liquid	4	
	Soft coral tree PR2 (*Dendronephthya* sp.)	Tissue	10	17
		Liquid	7	
	Soft coral tree PR3 (*Dendronephthya* sp.)	Tissue	18	25
		Liquid	7	
	Nudibranch PR4 (*Phyllidia* sp.)	Tissue	8	8
	Sea fan PR5	Tissue	8	15
		Liquid	7	
	Sea whip PR7 (*Juncella* sp.)	Tissue	3	3
	Yellow sponge PR8	Liquid	8	8
	Soft coral PR9	Tissue	4	5
		Liquid	1	
	Sea fan PR10	Tissue	8	20
		Liquid	12	
	Soft sponge PR11	Tissue	12	18
		Liquid	6	
	Barrel sponge PR12 (*Xestospongia* sp.)	Tissue	1	3
		Liquid	2	
	Brittle star PR13	Tissue	4	4
	Sponge PR14	Tissue	17	21
		Liquid	4	
	Staghorn coral PR17	Liquid	4	4
Pulau Payar Marine Park	Soft coral tree Pp1 (*Dendronephthya* sp.)	Tissue	7	7
	Soft coral tree PP2 (*Dendronephthya* sp.)	Liquid	5	5
	Sea anemone PP4	Tissue	2	8
		Liquid	6	
Total				**178**

This study adopted the isolation technique of Namikoshi *et al.* (2002) for both the tissue and liquid portions of the investigated marine invertebrates. This technique enabled the isolation of 15 fungal isolates from the tissue portions of three marine invertebrates off Pulau Redang namely; nudibranch PR4 (8), sea whip PR7 (3) and brittle star PR13 (4) however, none were isolated from the liquid portions. Liquid portions of yellow sponge PR8 and staghorn coral PR17 yielded 8 and 4 isolates, respectively, while the tissue and liquid portions of the remaining 9 samples off Pulau Redang yielded 131 fungal isolates from both liquid and/or tissue portions. From three marine invertebrates off Pulau Payar, portions of soft coral tree PP1 tissue and soft coral tree PP2 liquid yielded 7 and 5 isolates, respectively. A number of 8 isolates were isolated from liquid (2) and tissue (6) portions of sea anemone PP4, respectively.

Cytotoxicity of fungal extracts

From the 178 isolates obtained from Malaysian marine invertebrates, only 57 were further evaluated for the production of biologically active metabolites. The selection was based on the taxonomy of the isolates, unusual morphology and the diversity of reported secondary metabolite chemistry of related taxa. Some of the isolates were also randomly selected from productive genera such as *Trichoderma* and *Xylaria*.

A number of 16 and 41 of the isolates were cultured on PYGA and PYGB media, respectively. Resultant cultures were extracted with EtOAc to yield a total of 57 extracts (Table 3).

Table 3: Number of extracts produced from selected isolates cultured on agar and/or broth media

Origins of isolate	No. of isolates selected	Agar media	Broth media	Total
Tunicate PR1 (*Didemnum* sp.)	4	1	3	4
Soft coral tree PR2 (*Dendronephthya* sp.)	4	1	3	4
Soft coral tree PR3 (*Dendronephthya* sp.)	9	3	6	9
Nudibranch PR4 (*Phyllidia* sp.)	7	1	6	7
Sea fan PR5	5	1	4	5
Sea whip PR7 (*Juncella* sp.)	2	2	0	2
Sponge PR8	2	1	1	2
Soft coral PR9	2	1	1	2
Sea fan PR10	4	2	2	4
Soft sponge PR11	1	0	1	1
Barrel sponge PR12 (*Xestospongia* sp.)	1	0	1	1
Brittle star PR13	3	1	2	3
Sponge PR14	1	1	0	1
Staghorn coral PR17	2	0	2	2
Soft coral tree PP1 (*Dendronephthya* sp.)	4	0	4	4
Soft coral tree PP2 (*Dendronephthya* sp.)	2	0	2	2
Sea anemone PP4	4	1	3	4
Total	57	16	41	57

The quick screening assay of the Malaysian fungi showed that 25 of 57 extracts (43.9%) assayed were potentially active (growth inhibition >50%). Of the 25 extracts that inhibited the growth of P388 cells in the quick screening assay, only 10 (40%) showed IC_{50}<12,500 ng/ml. The strongest cytotoxic activity was shown by extract F6451, with IC_{50} 3,886 ng/ml. The results of are shown in Table 4.

HPLC screening of bioactive extracts

A total of 10 extracts that showed IC_{50}<12,500 ng/ml in the P388 assay were subjected to HPLC screening. The results of the HPLC screening are shown in Table 5.

Table 4: Cytotoxicity of Malaysian fungi

Isolates	Media	Extracts	A	B
PR1T2 (unidentified)	F6401	PYGB	51.3	4,938
PR1L3 (unidentified)	F6404	PYGB	59.0	na
PR2T2 (unidentified)	F6406	PYGA	82.4	4,126
PR2T4 (unidentified)	F6407	PYGB	74.3	na
PR2T10 (unidentified)	F6408	PYGB	93.4	5,911
PR2L6 (unidentified)	F6409	PYGB	50.4	na
PR3T12 (*Trichoderma* sp.)	F6412	PYGB	79.9	7,512
PR3T19 (unidentified)	F6414	PYGB	68.9	na
PR3L8 (*Xylaria* sp.)	F6415	PYGB	92.0	4,004
PR4T7 (unidentified)	F6423	PYGA	60.3	na
PR4T8 (unidentified)	F6424	PYGB	64.0	na
PR5T2 (unidentified)	F6426	PYGB	78.9	12,133
PR5T4 (unidentified)	F6428	PYGB	53.8	na
PR5L9 (*Paecilomyces* sp.)	F6430	PYGB	66.0	10,445
PR7T3 (unidentified)	F6433	PYGA	58.5	na
PR8L8 (unidentified)	F6435	PYGB	62.5	na
PR10T2 (*Paecilomyces* sp.)	F6439	PYGA	86.8	na
PR10T8 (unidentified)	F6440	PYGB	66.2	na
PR11T4 (unidentified)	F6444	PYGB	59.9	8,726
PR13T1 (unidentified)	F6446	PYGA	53.6	na
PR13T2 (unidentified)	F6447	PYGB	55.7	na
PR17L2 (unidentified)	F6451	PYGB	92.4	3,886
PP1T2 (unidentified)	F6453	PYGB	91.3	na
PP2L4 (unidentified)	F6457	PYGB	88.2	12,500
PP4L6 (unidentified)	F6460	PYGB	93.0	na

A: growth inhibition (%) against P388 cells; B: IC_{50} (ng/mL) against P388 cells; na: growth inhibition <50% or IC_{50}>12,500 ng/mL; Non-active extracts in both A and B assays were not shown in Table.

Table 5: Dereplication of metabolites from fungal extracts using the in-house HPLC-UV/R_t library database

Extracts	Cytotoxicity	Compounds (dereplication)*
F6401	4,938	Unknown metabolites
F6406	4,126	meleagrine
F6408	5,911	Unknown metabolites
F6412	7,512	Unknown metabolites
F6415	4,004	cytochalasins
F6426	12,133	Unknown metabolites
F6430	10,445	indole-3-carboxylic acid
F6444	8,726	Unknown metabolites
F6451	3,886	dehydrosterigmatocystin
F6457	12,500	Unknown metabolites

* in-house HPLC-UV/R_t library database readily

HPLC screening using in-house HPLC-UV/R_t library database readily dereplicated four known secondary metabolites from four cytotoxic fungal extracts. These metabolites were meleagrine (extract F6406), cytochalasins (extract F6415), indole-3-carboxylic acid (extract F6430) and dehydrosterigmatocystin (extract F6451). The results for dereplication were represented by extract F6451 (Fig. 1). The HPLC screening of F6451 showed one significant peak eluting at 16.8 min. A search of the HPLC-UV/R_t library database for this peak showed matches in both UV chromophores and R_t to the known compound, dihydrosterigmatocystin (Fig. 2).

The remaining six extracts (F6401, F6408, F6412, F6426, F6444 and F6457) contained peak(s) that were not readily dereplicated. Further investigation using various dereplication techniques will be carried out to characterize the unknown metabolites.

Rapid identification of natural products is an important step to distinguish between known and unknown compounds and consequently allowing the exclusion of known compounds at an earlier stage. The dereplication of fungal metabolites using the library database works efficiently if the peaks have characteristic UV spectra, simple MS is carried out by establishing whether any of the significant peaks showed matches (both UV chromophore and R_t) with any known compounds already present within the database. Various dereplication techniques using the LC-MS-UV technique in combination with the AntiMarin database or the CapNMR technique together with the AntiMarin database could be applied to characterize the unknown metabolites.

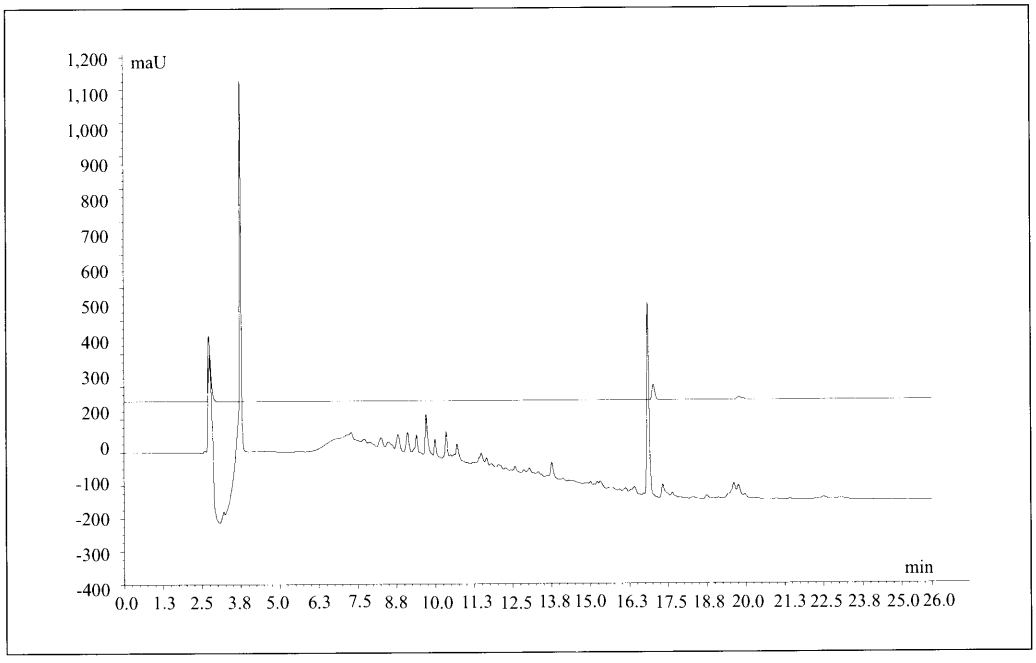

Figure 1: HPLC chromatogram of F6451 showing overlay of ELSD detection (top) for the main peak eluted at 16.8 min

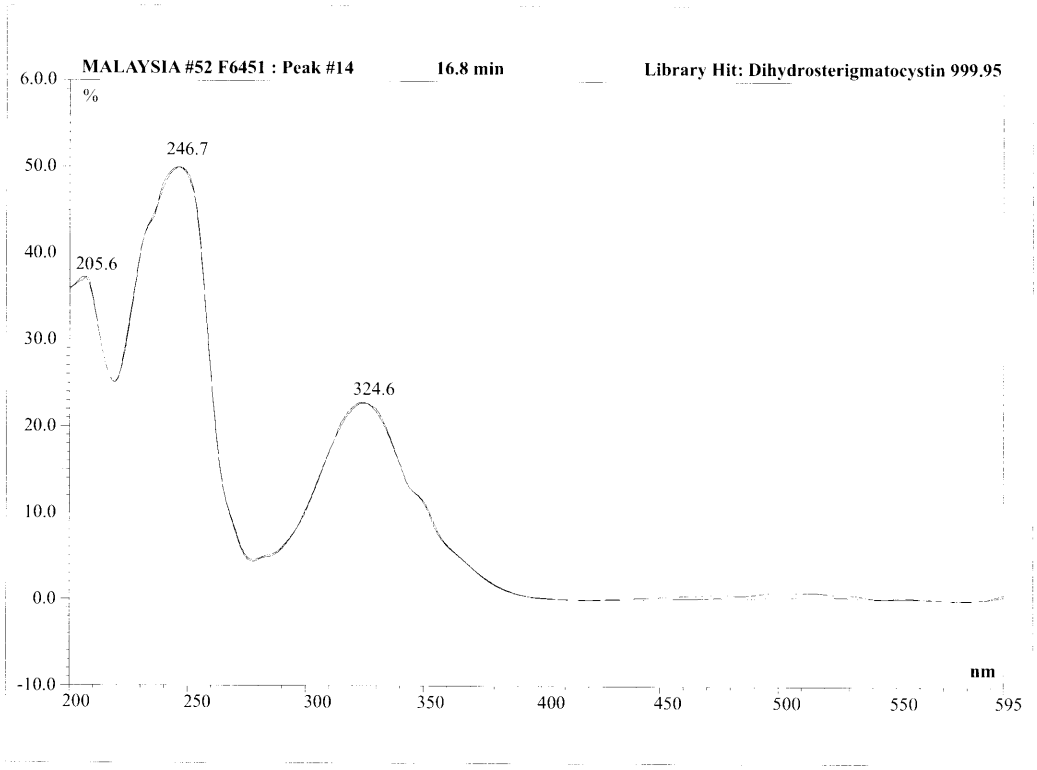

Figure 2: UV spectrum of the main peak eluted at 16.8 min (black) showing the UV library hit (red) with the known dihydrosterigmatocystin in the HPLC-UV/R_t library database

Conclusion

Rapid identification of natural products is an important step to distinguish between known and unknown compounds and consequently allowing the exclusion of known compounds at an earlier stage. This study is focused on fungal metabolites and an in-house HPLC-UV/R_t library database for known fungal metabolites by using the Chromeleon software on the Dionex analytical HPLC that has been established. Further investigation using various dereplication techniques will be carried out to characterize the unknown metabolites.

References

Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffmann, C., Rothcock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E.,Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J. and Springer, J. 1980. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proceedings of the National Academy of Sciences of the United States of America*, **77**: 3957-3961.

Amagata, T., Minoura, K. and Numata, A. 2006. Gymnastatins F-H, cytostatic metabolites from the sponge-derived fungus *Gymnascella dankaliensis*. *Journal of Natural Products*, **69**:1384-1388.

Amagata, T., Tanaka, M., Yamada, T., Minoura, K. and Numata, A. 2008. Gymnastatins and dankastatins, growth inhibitory metabolites of a gymnascella species from a *Halichondria* sponge. *Journal of Natural Products*, **71**: 340-345.

Boot, C.M., Tenney, K., Valeriote, F. A. and Crews, P. 2006. Highly *N*-methylated linear peptides produced by an atypical sponge-derived *Acremonium* sp. *Journal of Natural Products*, **69**: 83-92.

Bringmann, G., Lang, G., Muhlbacher, J., Schaumann, K., Steffens, S., Rytik, P.G., Hentschel, U., Morschhauser, J. and Muller, W.E.G. 2003. Sorbicillactone A: a structurally unprecedented bioactive novel-type alkaloid from a sponge-derived fungus. *Progress in Molecular and Subcellular Biology*, **37**: 231-253.

Bugni, T.S. and Ireland, C.M. 2004. Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Natural Product Reports*, **21**: 143-163.

Christian, O. E., Compton, J., Christian, K. R., Mooberry, S. L., Valeriote, F. A. and Crews, P. 2005. Using jasplakinolide to turn on pathways that enable the isolation of new chaetoglobosins from *Phomospis asparagi*. *Journal of Natural Products*, **68**: 1592-1597.

Dreyfuss, M., Harri, E., Hotmann, H., Kobel, H., Pache, W. and Tscherter, H. 1976. Cyclosporin A and C; new metabolites from *Trichoderma polysporum* (Link ex Pers.) Rifai. *European Journal of Applied Microbiology*, **3**: 125-133.

Ebel, R. 2006. Secondary metabolites from marine derived fungi. *In:* Proksch, P. and Muller, W. E. G. (eds.). Frontiers in Marine Biotechnology. Horizon Bioscience. pp. 73-143.

Hiort, J., Maksimenka, K., Reichert, M., Perovic-Ottstadt, Lin, W. H., Wray, V., Steube, K., Schaumann, K., Weber, H., Proksch, P., Ebel, R., Muller, W. E. G. and Bringmann, G. 2004. New natural products from the sponge-derived fungus *Aspergillus niger*. *Journal of Natural Products*, **67**: 1532-1543.

Kohlmeyer, J. and Kohlmeyer, E. Marine mycology: the higher fungi, Academic Press, New York, 1979.

Namikoshi, M., Kobayashi, H., Yoshimoto, T. and Meguro, S. 2000. Distribution of marine filamentous fungi associated with marine sponges in coral reefs of Palau and Bunaken Island, Indonesia. *Chemistry Letters*, **4**: 308-309.

Nielsen, K.F. and Smedsgaard, J. 2003. Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography-UV-mass spectrometry methodology. *Journal of Chromatography A*, **1002**: 111–136.

Perry, N.B., Benn, M.H., Brennan, N.J., Burgess, E.J., Ellis, G., Galloway, D.J., Lorimer, S.D. and Tangney, R.S. 1999. Antimicrobial, antiviral and cytotoxic activity of New Zealand lichens. *Lichenologist*, **31**: 627-636.

Xin, Z. H., Fang, Y., Du, L., Zhu, T., Duan, L., Chen, J., Gu, Q. and Zhu, W. 2007. Aurantiomides A-C, quinazoline alkaloids from the sponge-derived fungus *Penicillium aurantiogriseum* SPO-19. *Journal of Natural Products*, **70**: 853-855.