

Extraction and Analysis of Bioactive Agents from Tasmanian Marine Organisms

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KRA305 Biosynthesis and Function of Natural Products
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Abstract

*Numerous terrestrial and marine organisms were chosen for investigation from samples collected on Tasmania's East coast. Several bioassays were performed to determine the antibacterial and allelopathic effects of compounds extracted. After extraction, samples were screened for biological activity using bacteria (*Bacillus cereus*), lettuce (*Lactuca sativa*) and Duckweed (*Lemna minor*) bioassays. The strongest bioactive compounds were found in the sponge *Dendrilla rosea*, *Flavoparmelia rutidota* (lichen), *Steginoporella truncata* (bryozoan), and *Parerythropodium membranecium*, a soft coral. The extracts of these were purified using chromatography and the fractions were screened again for bioactivity and analysed using liquid chromatography-mass spectrometry, gas chromatography-mass spectrometry, proton nuclear magnetic resonance spectroscopy, carbon nuclear magnetic resonance spectroscopy and infrared spectroscopy. Following comparison with similar species that have been previously researched, it is likely that the compounds contained in *D. rosea* are diterpenes, while the bryozoan probably contains a multitude of alkaloid compounds such as amathamides and homarine. The active compound in the lichen was an organic acid, however the active component of *P. membranecium* was not isolated.*

Introduction

Over the last few decades, much scientific interest has been generated around the growing field of natural products in search of new biomedicines and other bioactive compounds (Blackman, 2005(2)). Subsequently, entire institutions have been established such as the Eskitis Institute for Cell and Molecular Therapies at Griffith University, Queensland, which is dedicated to this kind of research. Traditionally these products have come from a terrestrial environment; only in the last 30 years have scientists moved into the marine environment in search of new compounds (Blackman, 2005(2)). Over 10 000 new compounds have been isolated, most stemming from secondary metabolites found in sponges and coelenterates. Many of these are vastly different from known terrestrial compounds, although they frequently bear a resemblance to known structural classes, notably terpenes, polyketides and alkaloids (Blackman, 2005 (1)).

Previous research has succeeded in making comparisons between the secondary metabolites isolated from different marine species. Sponges, for example, have become a leading source of bioactive marine products and are known to produce large amounts of secondary metabolites (Carte, 1996). In recent years, over 500 novel compounds have been identified from this family, many showing bioactivity (Ralph, 1990). In contrast, although bryozoans contain comparatively few compounds (Ralph, 1990), they are well known for their alkaloid content (Narkowicz, 2003). Seaweeds, however, frequently incorporate bromine into their secondary metabolites, which are mostly diterpenes, while ascidians produce mainly amino acid derivatives (Carte, 1996).

The main function of many secondary metabolites still remains unknown but it is thought that the majority are synthesised by microorganisms such as phytoplankton as secondary metabolites and sequestered by higher organisms to provide an advantage, for example, protection from predators or to deter competitors (Mann, 1994).

The purpose of this study was to investigate the bioactivity of compounds extracted from marine organisms collected from Spring Beach, Tasmania. An emphasis was placed on gaining a fundamental understanding on the techniques and theory behind laboratory methods used for the detection, screening and analysis of samples. Advanced spectroscopic techniques for structure elucidation were employed by the Central Science Laboratory to assist with research.

Many different assays are available for bioactivity screening, including cytotoxicity, fish shrimp and reporter gene bioassays in addition to the 3 chosen for this analysis (Narkowicz, 2003). The antibacterial bioassay gives insight into the interaction of the compounds with microorganisms and potential medicinal uses. The lettuce seed bioassay indicates allelopathic behaviour and toxicity and the Duckweed bioassay can display compound selectivity between dicots (lettuce) and monocots (Duckweed). However, the results of these assays are not conclusive as a number of compounds may be present in each extract and cause bioactive interference. A t-test was performed on the results for the lettuce seed bioassays to determine the statistical significance.

Experimental

In February 2005 a variety of intertidal and sub tidal marine and terrestrial organisms were collected by scuba divers from the southern end of Spring Beach, Orford (42° 34.85' South 147° 54.62' East). A voucher specimen of each organism was prepared and preserved in formalin or ethanol and the remainder was placed in a bag and frozen for later use.

A Mayer's test was also performed on a number of bryozoans, including *Steginoporella truncata*, to qualitatively determine the presence or absence of alkaloids (Table 1).

Thin layer chromatography (TLC) screening on aluminium backed silica gel plates was performed using cholesterol and a triglyceride as references. Taking into consideration these results and knowledge of secondary metabolites common to certain phyla, organisms were selected for extraction and bioactivity screening. Each sample was extracted firstly with methanol, followed by 1:1 methanol: dichloromethane and dichloromethane. The residue of the 3 extractions was combined and dissolved in acetone to a concentration of ~100mg/mL then the prepared solutions were screened for the presence of bioactive compounds. The extraction yields are recorded in Table 1.

In order to test for antimicrobial bioactivity, paper susceptibility disks containing extract were placed on agar plates inoculated with the bacterium *Bacillus cereus* (Figure 1). The zone of inhibition was measured and recorded in Table 1.

Two different areas of allelopathic behaviour were investigated using a lettuce seed bioassay, namely inhibition of germination and inhibition of growth. Ten *Lactuca sativa* seeds were placed into a mixture containing 1000ppm extract in 1% agar. Root and hypocotyl (shoot) length were measured and compared to a control after 1 week. Extracts showing inhibition of germination or root/hypocotyl growth were followed up with 3x and 4x dilutions of the original 1% mixture. The average percentage growths of the root and hypocotyl compared to the control are shown in columns 6 - 9 of Table 1. A t-test was performed on the data, assuming 1% significance, as a method for determining the statistical probability of bioactivity.



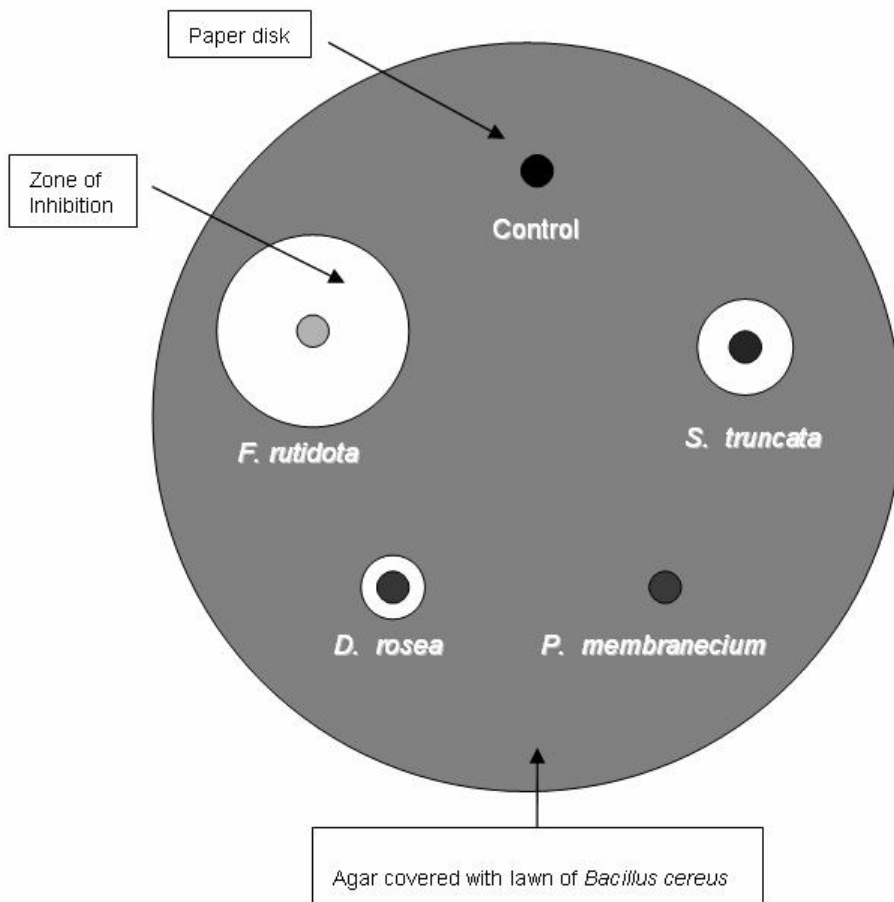


Figure 1.

To determine whether the bioactive compounds in the lettuce seed bioassay showed selectivity, a Duckweed bioassay was also performed. Four mature Duckweed plants were added to a solution of 0.1% extract in growth medium (liquid fertiliser dissolved in water). The number of plants and state of health were compared to a control after 1 week.

The components of *Dendrilla rosea*, *Parerythropodium membranecium* and *Flavoparmelia rutidota* were separated using flash chromatography on a glass column packed with silica. Approximately 500mg of extract was applied to the column and separated with polar solvents, starting with hexane and finishing with ethyl acetate.

The components of *Steginoporella truncata* were separated using reverse-phase chromatography on a pre-prepared Alltech 500mg C₁₈ cartridge. The initial mobile phase was polar and subsequent solvents increasingly non-polar. Fractions of each extract were TLC screened, like-fractions were combined and an antibacterial bioassay was performed to locate the bioactive agent. Finally, each active fraction was analysed using LC-MS, GC-MS, ¹H NMR or IR spectroscopy to establish its composition. In addition, a Mayer's test was performed on each of the combined fractions of *S. truncata* to determine which contained alkaloids.

Results and Discussion

The results from the bioactivity screenings are shown in Table 1.

Table 1: Yields and results of Bioactivity screenings of four marine organisms.

Organism	Voucher #	Extract % Yield	Antibacterial Bioassay Average zone of inhibition (mm)	Lettuce seed bioassay % growth compared to control				Duckweed Bioassay # plants more / less than control		
				3x dilution		4x dilution				
				Root	Shoot	Root	Shoot	Root	Shoot	
Lichen	<i>F. rutidota</i>	15.47	19.0	133	108.2					
Sponge	<i>D. rosea</i>	0.70	4.5	7.7	85.9	41.8	72.8	40	68.7	-5
Soft Coral	<i>P membranecium</i>	0.06	0.0	8.5	79.2					-1
Bryozoan	<i>S. truncata</i>	0.32	7.5	15.3	29.3	7.1	69.5	12.9	63.8	1



Figure 2: *Flavoparmelia rutidota*

The screenings of *Flavoparmelia rutidota* showed the strongest antibacterial activity of the four organisms, with an inhibition zone of 19.0mm. Chromatographic purification showed that the strongest activity was displayed by the fractions eluted with 100% ethyl acetate, indicating a polar compound. Analysis of this combined fraction by proton nuclear magnetic resonance spectroscopy ($^1\text{H NMR}$), carbon nuclear magnetic resonance spectroscopy ($^{13}\text{C NMR}$), gas chromatography-mass spectrometry (GC-MS) and infra red spectroscopy (IR) revealed that the main constituents were 3 independent compounds with possible presence of $\text{CH}_3\text{-C=O}$, $\text{-CH}_2\text{-CH=O}$, -COOH , nitrogen or oxygen substituents but void of halogens. There is also a peak at 6ppm that is indicative of an aromatic ring. From this information, possible functional groups present are esters, amides, acids, aldehydes or ketones.

Unfortunately, no publication or study on this particular lichen was found to support these findings. However, studies by Lawrey (1989) did show that the related lichen, *F. baltimorensis*, also exhibited antimicrobial activity against gram-positive bacteria (e.g. *Bacillus* spp.). Usnic acid (Figure 3), present in this lichen, was quite effective as an antimicrobial defence. Atranorin (Figure 4) and usnic acids contain similar functional groups to those found in *F. rutidota* and derive from similar biosynthetic pathways. More work would be required to determine the true structures of the active compounds but it is most likely related to these structures.

No conclusive evidence of allelopathic behaviour was shown by the lichen extract in either the lettuce seed or Duckweed assays. On the contrary, the extract seems to have promoted growth of the lettuce seed plants quite dramatically. This shows that the extract is non-toxic, at least to the lettuce seed, which is an essential quality of natural products intended for medicinal use.

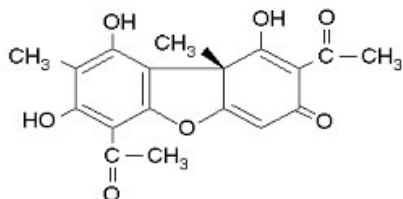


Figure 3. Usnic Acid.

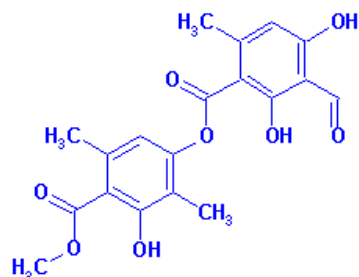


Figure 4. Atranorin.



Figure 5: *Dendrilla rosea*

The extract from *Dendrilla rosea* showed reasonable antibacterial activity with a zone of inhibition of 4.5mm. After chromatographic purification, the fractions eluted with 50:50 dichloromethane:petroleum spirits and 20:80:2 ethyl acetate:dichloromethane:methanol both showed strong antibacterial activity. The former was analysed by GC-MS and ¹H NMR, however as time restrictions did not permit analysis of the latter, future studies could investigate this fraction further. The analysed fraction was reasonably pure, with 3 main constituents, including oxacycloheptadec-8-en-2-one (Ambrettolide), a polyketide; 9,12-octadecadienoic acid methyl ester, a diterpene and -(Z)-9-octadecenamide, the amide of oleic acid, which is a fatty acid derivative (Figure 6). This is consistent with the fact that 50% of secondary metabolites from sponges are terpenes, 25% are acetogenins (polyketides) and 25% are fatty acid derived (Blackman, 2005(1)).

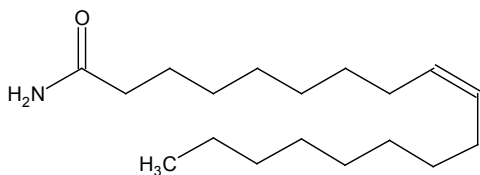


Figure 6: (Z)-9-octadecenamide.

Other studies of this sponge have isolated anti-inflammatory diterpenes such as aplyroseol-1, -5, and -6 (Karuso, 1986). Further analysis of the fractions by MS or ^1H NMR may yield such products. Extensive studies have also been performed on two related sponges, *D. membranosa* and *D. nigra*. The former of these sponges, collected from Antarctic waters, has yielded new diterpenes, namely membranolides B-D (Ankisetty, 2004). These compounds have shown positive results in antibiotic and antifungal bioassays using similar techniques to those used in this study (Ankisetty, 2004). Compounds isolated from *D. nigra* are potent antibacterial and broad-spectrum vibriostatic agents (Selvin, 2004). It seems logical that the extract prepared from *D. rosea* may contain the same or similar diterpenes due to the potent antibacterial effect witnessed in this study.

The lettuce seed and Duckweed bioassays revealed the allelopathic nature of the sponge extract. Positive test values for growth inhibition at the root were returned for the lettuce seed bioassay even after dilution to 250ppm. Duckweed bioassay results showed the sponge extract caused most plants to become yellow or brown in colour and appear unhealthy. The extract also appeared to inhibit reproduction, as only six plants were present, while up to 16 plants were present in control vials. Further screening and analysis would be required to isolate the compounds responsible for this behaviour.



Figure 7: *Steginoporella truncata*

The extract showed strong antibacterial activity, with an inhibition zone of 7.5mm. The active component was traced to the fraction eluted with 70% methanol, which gave a 5mm bacterial inhibition zone and a strongly positive Mayer's test result. ^1H NMR analysis of the fraction showed a complex mixture of peaks indicating many different hydrogen environments. Peaks in the double bond region of the spectrum and the presence of other substituents, such as oxygen, are consistent with the GC-MS analysis, suggesting numerous fatty acid methyl esters are present.



This result indicated that the fraction contained many inactive compounds but the structure of the active compound could not be elucidated from the spectral data.

Studies by Narkowicz (2002), however found an alkaloid present in this species called homarine (Figure 8). This compound, with its aromatic structure, methyl group and carboxylic acid substituent, reflects the ^1H NMR spectrum obtained for the active fraction of *S. truncata* and, despite negative results in antiparasitic assays (Narkowicz, 2003), is possibly the major component of the active fraction from this experiment.

In the lettuce seed bioassay, *S. truncata* returned a positive result for inhibition at both the root and hypocotyl even when diluted to 250ppm. In contrast to the sponge and soft coral, which were effective in stunting the growth of the plants, the bryozoan showed inhibition of germination as 40% of the seeds had not sprouted after 1 week.

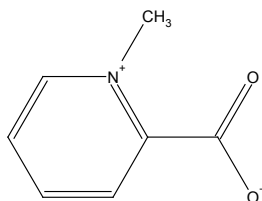


Figure 8: Homarine, Active Constituent in *Steginoporella truncata*.

Since this bryozoan is so heavily calcified, it is likely that the number of secondary metabolites present is relatively few, as chemical defence is not as essential. 'Soft' bryozoans collected in Tasmanian waters have been found to have strong nematocidal properties and many are toxic to ectoparasites (Narkowicz, 2004). *Amathia convoluta*, a relatively rare bryozoan found in Tasmanian waters is known to contain two novel tribrominated alkaloids, namely convolutamine H and convolutindole A, but not bryostatin 1, a bryozoan metabolite which is known for its potent antineoplastic activity (Narkowicz, 2002). Amathamides A-G, a family of alkaloids, are also noted for their presence in Tasmanian bryozoans (Narkowicz, 2002).



Figure 9: *Parerythropodium membranecium*

A total of 90 fractions were obtained from this compound but due to time restrictions, the properties of these could not be fully explored. The fraction chosen for GC-MS gave peaks corresponding to long chain aliphatic compounds consistent with fatty acid methyl esters. It is highly unlikely that such compounds would exhibit a bioactive response when subjected to the tests performed in this study, indicating that the bioactive compound is located in one of the other fractions. As many soft corals contain up to 10% of their weight in terpenes to protect them from predators (Blackman, 2005(1)), it is likely that further analysis may yield some of these compounds. However, similar to bryozoans, the secondary metabolites found in soft corals may not be produced by the coral itself. Coral is usually found living with symbiotic algae (Blackman, 2005 (2)). For this reason, further investigation would be necessary to determine the true origin of any active compounds. It is interesting to note that the colour of the extract was predominantly green, while the coral itself was a purple-grey colour, possibly indicating the presence of such algae.

Recent studies of the related soft coral, *P. fulvum*, have led to the extraction of the novel sesquiterpene 3-acetoxyspathulenol, in addition to the known compounds spathulenol and acetoxyspathulenol (Wessels, 2001). It would not be unreasonable to assume that the soft coral *P. membranecium* may contain similar compounds (Wessels, 2001).

Conclusion

It is reasonable to conclude that the initial aims of the experiment have been reached. The techniques employed during the experiment have resulted in the extraction and purification of a number of secondary metabolites produced by marine and terrestrial organisms, with interesting and potentially useful bioactive properties. Unfortunately, due to time limitations and poor spectral data, the structures of the majority of these compounds could not be conclusively determined. However, research has been undertaken into compounds extracted from similar organisms to those collected and it seems appropriate to suggest similarity between these compounds. Further study and analysis of these compounds may lead to the innovation of new pharmaceutical and industrial products. These initial studies show that further research into the bioactivity of these four organisms is warranted.

Acknowledgements

We thank Gintaras Kantvilas from the Tasmanian Herbarium for lichen taxonomic classification and Christian Narkowicz and Stewart Page for collection of marine organisms.

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