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Biological activity in New Zealand marine organisms

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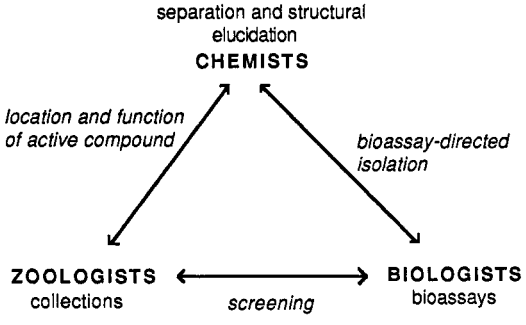
Abstract - The incidence and significance of antiviral and cytotoxic activity in a New Zealand collection of marine organisms has been investigated. Several compounds with potent biological activities have been isolated and characterised.

INTRODUCTION

A research program targeted towards the isolation of potential pharmaceuticals from marine organisms was initiated at the University of Canterbury in 1982. The specific aims of the program were:

-the detection and structure determination of new classes of biologically active compounds
-the modification and synthesis of these new classes of compound
-the determination of the biological role and site of production of these compounds

To implement these aims a multidisciplinary approach was necessary. The three vital areas required were chemistry, biology and marine zoology/phycology. The perceived interactions between these disciplines are indicated.



As only biologically active natural products were of interest to this program the strategy adopted to accomplish the **FIRST AIM** was to rapidly locate 'active' species using appropriate bioassay systems, determine the physical and *in vitro* properties of the biologically active component(s), and test for *in vivo* activity.

The **SECOND AIM**, the synthesis, semi-synthesis and modification of the biologically active compounds, is multifaceted. It is directed at reducing dependence on the inevitably erratic supply of the raw material, and the discovery of the 'pharmacophore' in each new series, so that a rational approach can be taken to the production of derivatives or analogs for biological assessment.

The **THIRD AIM**, the examination of the biological role of these compounds, is accomplished in part by examining trends in the presence and type of biological activity with taxon, ecological and morphological parameters (*vide infra*). The extension of this work is to determine the site or origin of activity in the sample and then to explore the possibilities for culturing the whole organism, specific cell types or any symbiotic organism responsible for the observed biological activity.

SAMPLE COLLECTION

Since 1982, some 3,300 marine organisms, representing close to 1000 different species, have been collected from many locations and differing habitats around the New Zealand coastline and from latitudes as far South as Antarctica to as far North as Western Samoa in tropical waters. Samples have been collected primarily by SCUBA in the depth range of 10-30 m, but a significant number were obtained by inter-tidal collection or by trawling down to depths of 2.5 km. The specimens collected are representative of all the invertebrate phyla, but with an emphasis on sponges, ascidians and bryozoans. The algae are also well represented. A breakdown of the collection percentages is shown:

PORIFERA	55%	HYDROIDS	~10%	UNASSIGNED	3%
ASCIDIANS	23%	CORALS			
BRYOZOA	4%	MOLLUSCS			
MARINE ALGAE	6%	ECHINODERMS			

BIOLOGICAL TESTING

In the screening a disease-oriented approach has been taken. The therapeutic areas of interest are:

ANTIVIRAL	AIDS, *Herpes* spp. infections
ANTITUMOUR	Human lung, colo-rectal, mammary, stomach, etc cancers
ANTIFUNGAL	Systemic *Candida* infections
IMMUNOMODULATORY	Stimulatory - for diseases of the immune system such as AIDS
	Depressive - transplant therapy.

These four disease areas, although seemingly diverse, represent a cohesive grouping of disease types with interactive therapeutic requirements and in some instances representing clinical areas where a suitable range of safe, effective therapeutic agents is currently not available.

The extracts prepared from the sample collections are passed through the biological screens. All extracts have been tested for antiviral properties (DNA and RNA virus) and, where appropriate, activity against the murine leukaemia cell line P388. The immunomodulatory and antifungal screening has been a more recent development. The screening effort is summarised below

ANTIVIRAL	*Herpes simplex* Type I	**ANTIMICROBIAL**	*Escherichia coli*
	Poliovirus		*Pseudomonas aeruginosa*
			Bacillus subtilis
ANTITUMOUR	P388	**ANTIFUNGAL**	*Candida albicans*

INCIDENCE OF ACTIVITY

The approach of noting the incidence of various biological activities in the various phyla is not new or novel and has been carried out by many groups to good effect (refs. 1, 2). These studies have served to highlight variations in type of activity and to demonstrate some trends in the presence of activity as a function of taxonomy. In the main these studies have concentrated on tropical waters. In contrast to tropical environments the New Zealand benthic marine biota is typical of warm temperate to sub-antarctic assemblages and is characterised by a high incidence of sponges, ascidians, bryozoa and algae and a relatively low incidence of cnidarians. This therefore presents a different balance of species than represented in the earlier studies. For the New Zealand collection the data is presented below in Table I. Extracts prepared from each sample were assayed on a volumetric basis for viral inhibition, viral inhibition **and** cytotoxicity, or just cytotoxicity in the antiviral assay, cytotoxicity against a murine leukaemia cell line (P388) and a positive response in the antibiotic assays against the various microorganisms listed above. It should be emphasised that the incidence of activity noted in Table I is based on the number of **SPECIES** tested, not **SAMPLES** assayed. The inclusion of a **SPECIES** as active or not is judged from the results of assaying replicate **SAMPLES** of that particular **SPECIES.**

TABLE I. INCIDENCE OF ACTIVITY ACROSS THE PHYLA [%(# species)]

	HSV[a]			P388[b]		AM[c]	
	AV/Cyt	Cyt					
Porifera	37	47	(563)	52	(275)	28	(302)
Ascidiacea	28	35	(165)	64	(79)	41	(80)
Bryozoa	27	45	(45)	39	(28)	19	(27)
Cnidaria	3	6	(37)	21	(19)	6	(18)
Mollusca	18	24	(17)	42	(12)	8	(12)
Echinodermata	63	74	(19)	33	(12)	38	(13)
Annelida	40	40	(5)	0	(2)	0	(2)
Brachiopoda	0	0	(1)	100	(1)	0	(1)
Chlorophyta	25	33	(12)	17	(12)	17	(12)
Phaeophyta	52	55	(31)	64	(31)	37	(30)
Rhodophyta	22	42	(45)	45	(29)	27	(22)

[a] Antiviral and/or cytotoxic response against *Herpes simplex* Type I virus. [b] Cytotoxicity against P388 murine leukaemia cell line. [c] Response against any of *E. coli, P. aeruginosa, B. subtilis* or *C. albicans*

The Porifera, which made up over half of the **SPECIES** collected, showed a uniformly high incidence of activity. A more detailed analysis of the incidence of activity in the Porifera follows below.

The Ascidiacea are also well represented in the collection and species from this phylum similarly exhibit a relatively high incidence of activity, in particular antimicrobial and against the P388 cell line. Of 165 ascidian species tested only 12 species were 'solitary', and of these only one exhibited any activity. The ascidian species with activity were predominantly colonial and most of these belonged to the family Polyclinidae. These species are generally of massive morphology and were frequently found in densely encrusting communities (*vide infra*).

The 'active' bryozoan species were usually foliose or bushy in structure. In contrast to the ascidians, encrusting bryozoans exhibited a low incidence of bioactivity in all assays.

The low representation of Cnidaria species in the collection reflects the absence of hard and soft corals from New Zealand waters. Most of the species tested were from the Hydroida. Relatively few Mollusca have been examined. This, however, was a function of the sampling approach rather than a lack of available species for collection. Greater than 70% of the 'active' Mollusca were nudibranchs. Shell-bearing Mollusca exhibited low levels of activity. Another group not well represented in New Zealand waters is the Echinodermata. The high incidence recorded for this phylum was almost totally (~90%) attributable to starfish species. As few species of Annelida or Brachiopoda have been examined to date it is difficult to comment sensibly on the incidence of activity in these phyla. It would appear to be low.

Finally, the incidence of activity in the Algae has been grouped according to the three major Divisions. Activity in the Chlorophyta was due mainly to the Caulerpales and similarly, activity in the Rhodophyta was due to predominantly one family, the Gigartinales. A surprisingly high incidence of activity was found in species from the Phaeophyta (>50%). This is in keeping with a similar observation made by Rinehart from his Caribbean collection (ref. 1). Both Laminarian and Fucoid algae were well represented, although species from most families exhibited activity.

As has been previously concluded there is a finite probability of finding biological activity in just about every phylum. This approach then serves only to indicate the areas where there is a higher probability of finding a species with a certain type of activity. It will not guarantee that useful activity will be found in certain phyla, or conversely overlooked by ignoring phyla with an apparently low incidence of activity (ref. 2).

SIGNIFICANCE OF ACTIVITY

To compare the incidence of activity **between** phyla is of little assistance because of the grossness of the comparisons. It is more relevant and productive to examine each phylum separately for trends in the incidence of activity with parameters such as taxonomic grouping, morphology or ecology.

The Porifera has been the phylum most widely studied by chemists. These studies have usually been carried out in conjunction with zoologists, and several hypotheses have been put forward to account for the likely role of the biologically active compounds. For example, sponges may have biologically active compounds:

- *as a chemical defense mechanism against predators, overgrowth, or to prevent recruitment*
- *to fight bacterial, fungal or viral infections*

The general conclusion appears to be that the biologically active chemicals have some defense function (refs. 3-8), although this has not been experimentally tested to date. Similar studies have been carried out on corals (refs. 9,10) and ascidians (refs. 11,12). In these various studies most researchers used toxicity as a criterion for activity and it was found that natural chemical exudates of some species did occur at levels that would affect potential predators or competitors (refs. 7,13). The second hypothesis, dealing with the possibility that the chemicals are natural antibiotic, antitumour or antiviral agents, does not appear to have received any attention.

Several groups have examined the Porifera for trends in those species exhibiting biological activity. It was generally thought that the most toxic sponges were those from the tropics, where such species can be heavily grazed by fish (refs. 3-5). However, opposite latitudinal trends have been reported (refs. 6,14,15). Although these studies are often compared, the researchers had used different experimental criteria - sponge toxicity on one hand and antimicrobial activity on the other - resulting in opposite conclusions. The earlier studies also concluded that there was no pattern in either biological activity or morphology. The only certain association appears to be a tendency for those 'active' species to be free of surface fouling organisms (ref. 6).

The New Zealand collection is heavily biased towards the phyla Porifera and Chordata (~80%). The data from the antiviral and antitumour assays for the Porifera have been examined on a taxonomic basis and the preliminary results presented below (Fig Ia). This shows the relative levels of antiviral and cytotoxic activity between the Demospongia orders. The histograms are presented with 95% confidence levels. These levels are large, indicating a high degree of variability inherent in the data. This was not surprising as all species have been combined within each order ignoring inter- and intra-specific variations. There are no significant differences between the orders when comparing the responses. There is, however, a general pattern. The orders Dictyoceratida, Dendroceratida, Haplosclerida and Poecilosclerida all have relatively higher scores for antiviral activity than cytotoxicity. The other four orders exhibit relatively higher levels of cytotoxicity. This broad division into two groupings is interesting, in that the first group represent those orders whose species are generally associated with dense, encrusting communities, while the other group represents species more commonly found in deeper, quieter waters and include most of the solitary or erect species of sponge. Extrapolating from that ecological clue to trends in relative activity the data were reorganised, based this time on morphological considerations. Four morphological groupings were used:

THIN	- encrusting on vertical walls, associated with shallower, surge conditions
MASSIVE	- encroached by other species
MASSIVE	- solitary in habit, rarely surrounded by competitors
ERECT	- solitary, branching sponges, usually found in deeper quieter water

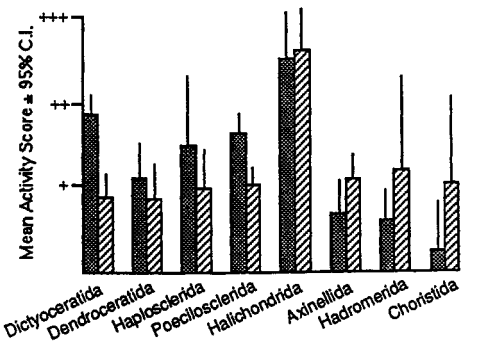


Figure Ia Activity as a Function of Sponge Order

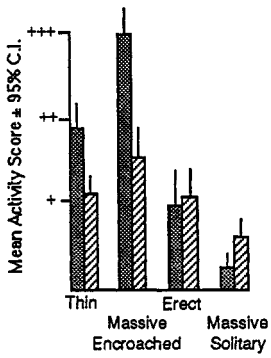


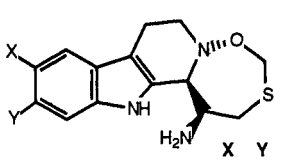
Figure Ib Activity as a Function of Sponge Morphology

The differences in bioassay response are now marked (Fig. Ib). There are significant differences between growth forms for the type of activity (*Herpes*, P388 etc) and the relative responses within each group. Those species that are most frequently encroached exhibit significantly higher antiviral responses than the more solitary species. Levels of antiviral activity are also higher in these groupings than cytotoxicity, while the reverse is true of the erect and solitary sponges. In commenting on this it is interesting to note that the erect and solitary sponges are those most commonly grazed by fish, urchins and opisthobranchs, whereas the thin encrusting and encroached species are those that compete for space and therefore must defend against overgrowth and the settling larvae of other organisms. To comment further is to speculate on the actual role that the biologically active compounds might be playing. Although the degree of antiviral or cytotoxic activity are the parameters being used in this study, it has been our observation that many of the strongly antiviral extracts (compounds) are also strongly immunosuppressive. If there is indeed a correlation between the observed antiviral effects and immunosuppressive properties, then those sponges with that activity are at a considerable advantage for overgrowing other species.

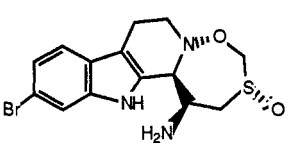
BIOLOGICALLY ACTIVE COMPOUNDS

Several of the 'active' species detected in the New Zealand screening program have been investigated. The biological activity in these species has been tracked by using bioassay-directed techniques, leading ultimately to the component(s) responsible for the activity. In the species investigated to date it was established at an early stage in the isolation procedure that the active components were not only active *in vitro* , but also possessed significant activities *in vivo*. For example, 7 species detected in the program have T/C (**T**est upon **C**ontrol) values in the murine leukaemia P388 assay of >125%. The *in vivo* antiviral program is not nearly as far advanced due to the lack of suitable animal models and only 4 compounds or extracts have been tested in an *in vivo* antiviral model (murine *Coronavirus* A59). One extract was particularly effective leading to a 100% survival rate for an otherwise invariably fatal viral disease.

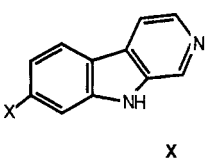
One early lead originated from the compound ascidian *Ritterella sigillinoides.* An interesting series of compounds was recovered from this small, stalked tunicate (refs. 16,17). Although *R. sigillinoides* is phylogenetically a long way removed from the Caribbean tunicate *Eudistoma olivaceum* the compounds with the interesting biological properties (**1**-**6**) were found to be the same as, or similar to the antiviral agents reported from the *Eudistoma* species (ref. 18). As in the earlier work the most potent activity was associated with the oxathiazepine β-carbolines. In addition to the antiviral properties that had been reported earlier by Rinehart *et al* (ref. 18), the eudistomins are also very effective antitumour agents with, in the case of eudistomin K, a T/C value of 137% in the P388 assay. A most interesting additional metabolite was isolated from the *Ritterella* species. This was the sulfoxide of eudistomin K (**4**). Like the other oxathiazepine β–carbolines this compound also has very effective *in vitro* antiviral and antitumour properties. New eudistomins are currently being examined.



		X	Y
1	Eudistomin C	OH	Br
2	Debromoeudistomin K	H	H
3	Eudistomin K	H	Br



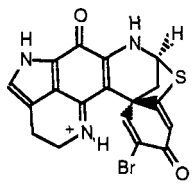
4 Eudistomin K sulfoxide



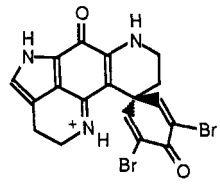
		X
5	Eudistomin O	Br
6	β-carboline	H

A series of strongly cytotoxic pigments, named the discorhabdins A to E (**7**-**11**), have been isolated from three New Zealand species of sponges of the genus *Latrunculia* (refs. 19-21). Each species contained one major pigment (discorhabdins A-C) in addition to other minor discorhabdins, some still to be described. Without exception the discorhabdins have proven to be strongly cytotoxic in the P388 assay system. The discorhabdins A-D are also strongly inhibitory against the gram-negative bacillus *Escherichia coli*, while discorhabdins A, C and D, were also active against the fungus *Candida albicans*. Despite having effective *in vitro* properties, only discorhabdin D of the four checked to date has *in vivo* antitumour activity (T/C 132%).

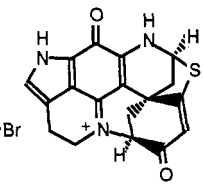
7 Discorhabdin A



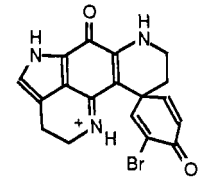
8 Discorhabdin B



9 Discorhabdin C

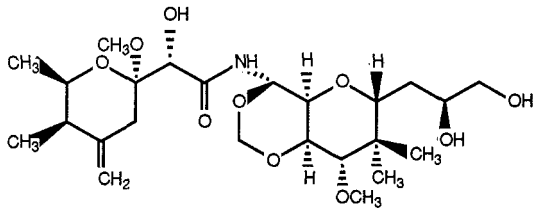


10 Discorhabdin D

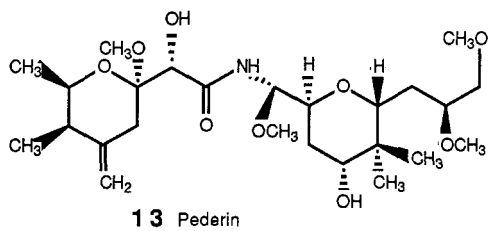


11 Discorhabdin E

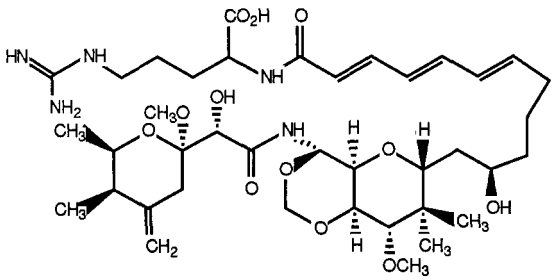
The most effective antiviral and antitumour lead that has come from the New Zealand program has been developed from a sponge of the genus *Mycale*. The crude extract from this sponge was strongly inhibitory against the *Herpes simplex* and *Polio* Type I viruses used in the assay system and was also effective *in vivo* against P388 (T/C 183%). Assay directed purification produced the active agent, the tricyclic amide mycalamide A (**12**) (ref. 22). As with the other compounds described above, the structure of mycalamide A was secured by a combination of spectroscopic techniques, principally mass and 2D-nmr spectroscopy. Remarkably, the structure of mycalamide A is very similar to that of pederin (**13**), a blistering agent isolated in 1953 from the terrestrial beetle, *Paederus fuscipes* (ref. 23), and onnamide (**14**), a biologically active compound (isolated after mycalamide A) from a Japanese sponge of the genus *Theonella* by Higa *et al* (ref. 24). Mycalamide A has a range of very potent biological properties against a wide variety of cancer cell lines, but probably the most significant activity is the *in vivo* activity reported against an RNA virus, the murine *Coronavirus* A59. Mice inoculated with this normally fatal virus were protected by mycalamide A. The experiment was terminated while all the test animals were still alive, making it difficult to estimate a T/C, but it is certainly >350%. The biological data accumulated to date for mycalamide A is shown in Tables IIa and IIb below. It has been established that the mode of action of mycalamide A is that of protein synthesis inhibition, but the mechanism by which the compound acts has yet to be determined. Other mycalamides and their derivatives are currently under investigation.



12 Mycalamide A



13 Pederin



14 Onnamide

TABLE IIa

ANTITUMOUR ACTIVITIES OF MYCALAMIDE A

In vitro P388 IC_{50} 2.6 ng/ml

In vivo tumour	dose regime		µg/kg	T/C %
P388	ip	qd 1-9	ip 10	156
B16	ip	qd 1-9	ip 10	175
M5076	ip	qd 1,5,9	ip 50	233
M5076	sc	q4d	iv 100	21
Lewis Lung	sc	qd 1-9	ip 20	23

TABLE IIb ANTIVIRAL ACTIVITIES OF MYCALAMIDE A

In vitro

Herpes simplex Type I	2 ng/ml*
Vesicular stomatitis	2 ng/ml*
Polio vaccine Type I	2 ng/ml*
Coronavirus A59	10 ng/ml*

In vivo Murine *Coronavirus* A59

~10% myc. A	qd 1-9	ip	100 µg/kg	100% survivors day 14
ribavarin	qd 1-9	ip	100 mg/kg	100% survivors day 14
control				0% survivors day 14

*dose required for 100% virus inhibition

CONCLUSIONS

Adoption of a multidisciplinary approach to the search for biologically active marine natural products has led to the rapid recognition of 'active' species from the New Zealand geographic zone. This approach has markedly assisted the isolation of a variety of compounds active *in vitro* and *in vivo* against a range of viruses and cancer cell lines. In the process it has also generated a wealth of information on the incidence and disposition of biological activity within the marine phyla as well as yielding clues as to the likely role of these biologically active compounds in the host organisms.

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