PATEAMINE: A CASE STUDY FOR THE ISOLATION OF COMPOUNDS WITH BIOLOGICAL ACTIVITY

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Résumé : *la décision essentielle à faire dans la recherche de métabolites à activité biologique est le choix de la plante ou de l'animal à travailler. Afin d'aider cette prise de décision, un protocole de* ***"criblage chimique"*** *d'extrait a été développé. Le criblage chimique met en jeu les propriétés physiques du ou des principes actifs, et donne une indication claire sur la voie la plus efficace pour l'isolement des principes actifs. En plus de cet avantage, le criblage chimique est aussi particulièrement utile pour éviter la duplication des extraits.*

L'utilité du criblage chimique est illustré ici par l'exemple type de la pateamine. La pateamine, diamine macrocyclique, est une cytotoxine potentielle, apparemment sélective, isolée d'une éponge néo-zélandaise Mycale *sp. Les travaux récents de détermination structurale, de chimie, de biologie et de modélisation moléculaire sont présentés.*

Abstract : *the most critical decision to be made in the search for biologically active metabolites is the actual choice of plant or animal species to commence work on. To assist in this decision-making a protocol for the* ***"chemical screening"*** *of an extract has been developed. The chemical screening process establishes the physical properties of the active component(s) and gives a clear indication as to the most efficient pathway to the isolation of the active compounds. If this was not advantage enough chemical screening is also particularly useful as an aid in the dereplication of extracts.*

The utility of chemical screening will be illustrated using pateamine as the prime example. Pateamine, a macrocyclic diamine, is a potent, but apparently selective, cytotoxin isolated from a New Zealand Mycale *sp. Recent work on the structure, chemistry, biology and molecular modelling of pateamine will be presented.*

The diversity of marine natural products research has constantly expanded in recent years and now virtually every aspect of this field is being addressed by at least one research group. The particular area that has held our attention for the last decade has been the prospect of finding compounds with pharmaceutical potential. Since 1982, when an intensive collection programme was initiated, nearly 4000 marine samples have been collected from New Zealand waters. The collecting area ranges from latitudes as high as 80° South, up into the sub-tropical waters off the northern Coast of New Zealand. The Antarctic collections were made close to the the New Zealand research station in McMurdo Sound, and collections have also been made at several of the Sub-Antarctic Islands, at Stewart Island and systematically throughout the North and South Islands of New Zealand. Extracts from each sample have been tested for specific biological activities. The collection, and the testing of the pharmaceutical potential of these samples required the combined skills of a multidisciplinary team. Such a group was assembled at the outset of the collecting effort and incorporated marine zoologists, cell biologists, virologists, microbiologists and organic chemists. Overall our aims are:

the detection of marine species with defined biological activities,
the isolation, identification and chemical modification of the active compounds, and
the chemical ecology of marine species.

The 4000 specimens collected represent some 1000 species, and is the most thorough and comprehensive collection of Southern Pacific shallow water benthic organisms to date.

The areas of biological testing of interest to us 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 areas represent a group of disease types still with an inadequate range of safe, effective therapeutic agents. All samples have been screened against a DNA and an RNA virus, and most have been screened against the P388 murine leukemia cell line and *Candida albicans*. All recent extracts have been screened for immunomodulatory potential and for inhibitory activity against a complementary range of enzyme inhibition assays. The enzymes include dihydrofolate reductase, adenosine deaminase and S-glutathione transferase.

By implementing this approach we have, over the past few years, been able to isolate a number of new classes of compounds with interesting biological properties (1). The emphasis has always been on the isolation of NEW compounds with useful biological activity. As the interest in marine natural products continues to grow, especially in the area of bioassay-directed initiatives, the probability of finding unique compounds diminishes. In order to maximise the return for the effort invested, it is necessary to undertake the selection of species with great care and forethought. ***It is our conclusion that the most critical decision in the overall process is the selection of species to examine.*** Unless a "fingerprint" of each extract of interest is obtained before proceeding to a full-scale investigation it is likely that there will be a considerable wastage of effort as various known compounds are re-isolated, often against a considerable concentration gradient. In earlier years we invested considerable effort into the re-isolation and characterisation of tedanolide (2), halichondrin B (3), various eudistomins (4), girolline (5), and variabilin (6).

How can this "fingerprinting" be achieved? There are at least two aspects that can be considered:

-Dereplication
-Chemical Screening

We use the term **dereplication** in the sense of resolving similarities and use biology, taxonomy and the chemical literature to obtain important information on the likely nature of potential metabolites. The dereplication process could perhaps be summed up as the following series of questions.

Is the taxonomy known?
Literature survey?
Compounds in this genus?
in vitro biological activity?

But, to succeed in the selection process it is also necessary to have information on the stability and physical properties of the active component in the extract. For this aspect of the selection process we use the term **chemical screening*.**

The chemical screening process should be capable of assessing

	aqueous as well as organic extracts,
giving an indication of the	stability
	physical, and
	chromatographic properties of the active component(s)
and, the process should be	cheap
	rapid
	simple
	non-consumptive of extract, and
	non-intensive of assay facilities.

Chemical screening is simply the chemical equivalent of biological screening and answers questions such as :

	Is the active compound polar/non-polar, or is it Ionic/non-ionic?
	What is the stability and molecular weight range for the active compound ?

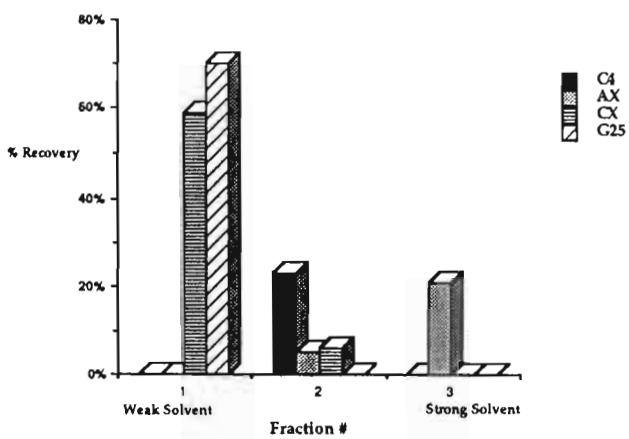
To achieve this we have developed a protocol, based on the use of small bonded-phase cartridges, that very effectively gives an insight into the properties of the active component. The protocol uses a combination of reverse-phase, normal-phase and ion-exchange cartridges and more specialist phases where applicable. Limited fractions, usually three, are taken from each phase and assayed. From a consideration of the pattern and the recovery of activity obtained, direct information on the physical properties and stability of the active component is obtained as well as indications of the best approach to use in an isolation scheme. The combination of taxonomy, biological profile, literature survey, polarity and chromatographic properties and approximate molecular weight allows a rational decision to be made on the likely novelty of the active component in an extract.

Two sample profiles are examined.

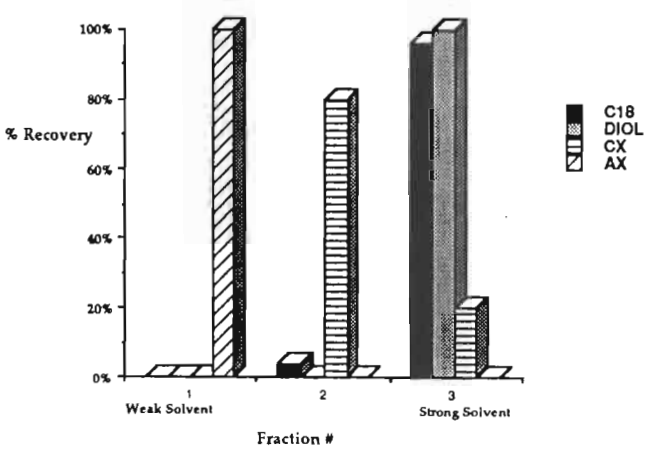
In Case I there was loss of activity on an anion exchange cartridge suggesting that the active component contains a strongly retained anionic grouping such as sulphate or sulphonate. The sample was retained on C4 reverse phase, but was not retained at all on G25 or by a cation exchange cartridge. From this it was concluded that the active component(s) was most likely a high MW sulphated saponin and therefore not of further interest.

Case II illustrates the chemical screening results from a sponge extract. This plot suggests that the active compound is well-retained on both normal and reverse phases, but can be recovered in high yield. Furthermore, the component of interest is retained on cation, but is not retained by anion exchangers. The sponge in question had been collected from the Fiordland area of New Zealand. The initial biological activity of the extract was very high, unprecedented in fact in its potency against the P388 cell line. The **dereplication** process was ambiguous in this case, as this species of *Mycale* was identical to a *Mycale* found on the East coast of the South Island from which the

Case I: Aqueous Plant Extract



Case II: Organic Extract from Sponge



mycalamides had previously been isolated (7). The mycalamides A and B each have IC_{50} values less than 1 ng/ml. The **chemical screening,** however, did lead to an unambiguous answer. The physical properties of the mycalamides are such that they would be eluted by medium, not strong solvents on DIOL and C18, and lacking any ionisable grouping they would not be retained on either of the ion exchange resins. It can be concluded that the active component in the Fiordland *Mycale sp* must have a polar functionality, an aliphatic section, and have a cationic grouping such as an amino functionality.

While there are a number of compounds that meet these physical requirements, none had the necessary very strong biological activity or had previously been isolated from a *Mycale sp.* It was therefore possible to proceed with this extract and be confident that the active component would most likely be a new compound.

Advantage was also taken of the known physical properties of the active component that had been defined by the chemical screening protocol. As the compound was strongly retained on both C18 and DIOL these were selected as the first steps following liquid/liquid partitioning (see Figure 1). The 37g of crude extract was reduced to 240mg with an IC_{50} of just 1.5ng/ml. The graphs (Figure 1) illustrate the shedding of mass while the activity is concentrated. In the original scheme chromatography on an amino phase was followed by a final clean-up on LH20 to give the pure compound. Later these two steps were replaced by chromatography on a carboxylic acid phase. So, purification could be achieved from the crude extract by just four well chosen steps with each of the steps having been defined by the chemical screening procedure.

The new compound was named **pateamine*** and was characterised as follows. The MH^+ ion at m/z 556.3199 in the HRFAB mass spectrum of pateamine was consistent with a molecular formula of $C_{31}H_{45}N_3O_4S$ (dm 1.0 mmu). The presence of sulphur was indicated by the high intensity of the $(M+2)^+$ peak (11% of M^+) and confirmed by X-ray fluorescence analysis. APT and HMQC NMR experiments revealed 43 protons attached to the 31 carbons observed in the ^{13}C NMR spectrum. The remaining two protons required by the formula were confirmed as being exchangeable from a CI mass spectrum with ND_3 as the ionizing gas (8).

Four substructures A-D (Figure 2) could be identified by NMR spectroscopy. Extensive use was made of phase sensitive COSY, difference nOe, NOESY and HMBC experiments to establish these substructures. The largest fragment was A with thirteen carbons. The presence of a terminal *N,N*-dimethyl grouping was indicated by the downfield shift of both the 6H singlet and the allylic methylene resonances upon formation of the TFA salt from the free base and confirmed by HMBC experiments.

The nine carbon fragment B was determined in a similar manner. COSY correlations and nOe enhancements established the presence of a trisubstituted diene moiety with methyl substitution and geometry as shown. HMBC correlations and chemical shift arguments attached the carbonyl to the Z substituted double bond, while another HMBC correlation connected the remaining system to the other end of the diene. The sulphur required by the molecular formula was found in the form of a disubstituted thiazole ring (fragment C), as was suggested by the C7, H7 pair (δ112.41, δ6.75) with a $^1J_{CH}$ value of 190 Hz. The remaining fragment of pateamine, D, was required to have a formula of $C_6H_{11}ON$, and contain the two exchangeable protons. COSY correlations established an aliphatic sequence of a methyl, methine, methylene, methine, and methylene. An HMBC correlation from a methylene proton resonance to a carbonyl carbon resonance established the attachment of a carbonyl to this system. The attachment of an amino group to the methine position C3 was suggested by both the chemical shift data (δ_H 2.58, δ_C 45.48) and the $^1J_{CH}$ value (135 Hz) at this position (9). This completed the atom count, including the two exchangeable protons that had initially been established by DCI/ND_3 mass spectroscopy.

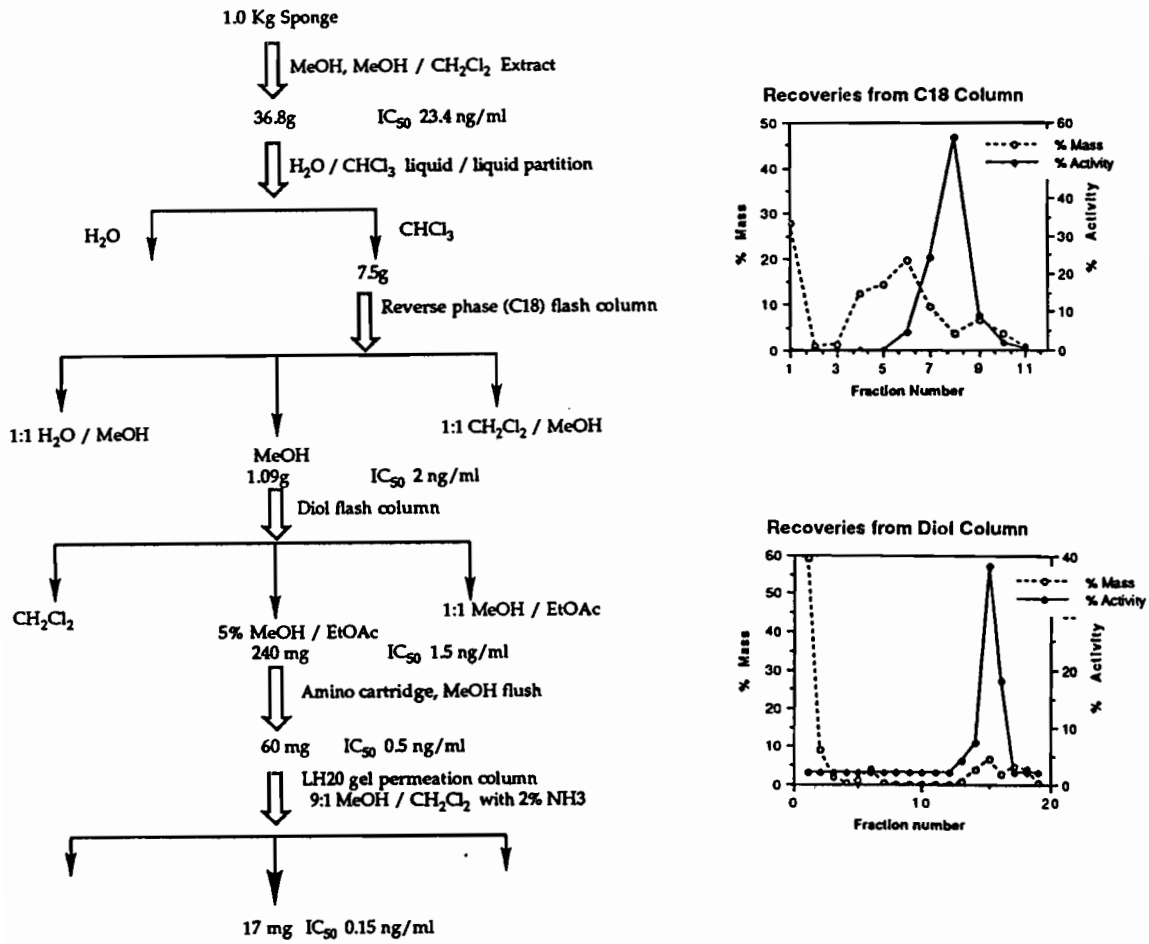


Figure 1 Activity Directed Isolation Scheme

Figure 1 : Activity directed isolation scheme

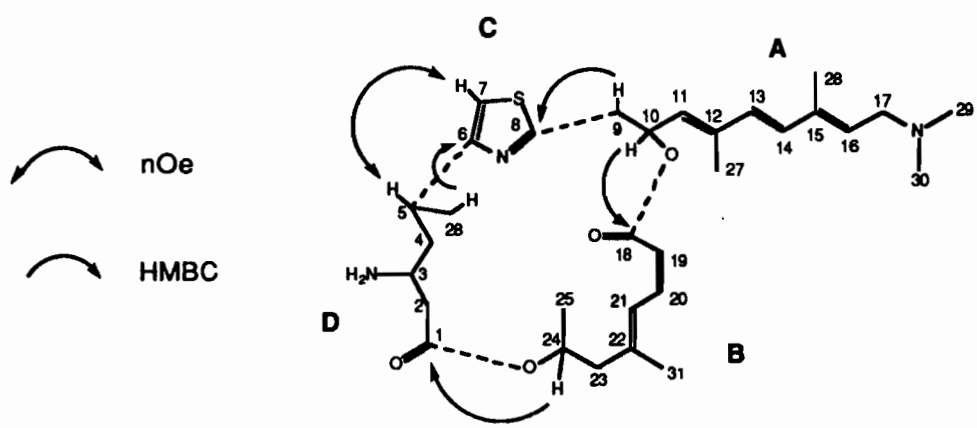


Figure 2 : Substructures A-D and Assembly

These four fragments accounted for all of the atoms in the molecular formula, and 10 of the 11 degrees of unsaturation, thereby implying a macrocyclic structure for pateamine. Further HMBC and nOe correlations guided the assembly of the macrolide structure. These are indicated in Figure 2 to give the structure of pateamine **1** (Figure 3).

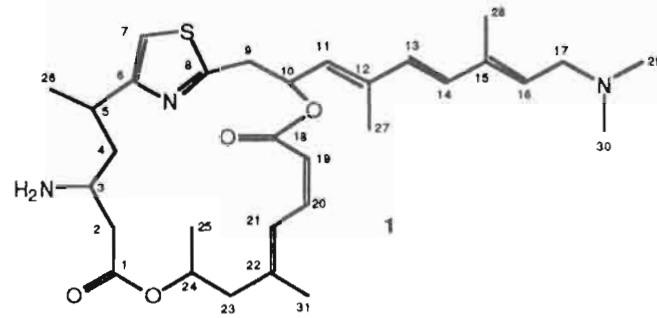


Figure 3 : The Structure of Pateamine 1

Pateamine **1** has four chiral centres at C3, C5, C10 and C24. Despite considerable efforts to crystallise pateamine either directly, or as a salt, the compound refuses to oblige and it has therefore not yet been possible to determine the relative, let alone, the absolute stereochemistries of the chiral centres. Degradative methods will be used once we have isolated further pateamine and the crystallisation attempts will continue. In the meantime our present approach to determining the stereochemistry is based on nmr and molecular modelling techniques. From direct measurement of coupling constants, spectral simulation and phase sensitive COSY, detailed coupling constants at all centres have been obtained, while the spatial relationships between the protons came from difference nOe and NOESY experiments. Using these data we have generated constraints for the spatial relationships for the C20-C24 and C2-C6 regions of pateamine. With these constraints distance geometry methods (10) have been used to generate the possible conformations for these regions. The conformations generated can then be examined to ascertain those that conform to the requirements imposed by the nOe and coupling constant data. Results for the C20-C24 region are shown in Figure 4.

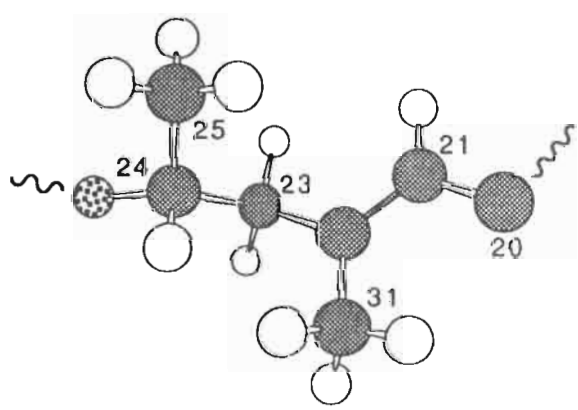


Figure 4 : Probable conformation for the C20-C24 region of pateamine

The major problem associated with this approach is that of conformational mobility, especially with a macrolide molecule, where a number of conformations may be available. For example, in the region of C2-C6 there can be two quite distinct arrangements for each combination of chiralities at C3 and C5 which are each consistent with the coupling constant data. This is shown in Figure 5 for the 3R/5S situation. If the requirements of the nOe observations are now added the situation can become confused. It was observed that H7 had

strong nOe interactions with H5 and H4a and weak interactions with H3 and H26. It is not possible to correlate all these interactions **and** the coupling constant data with one conformation. To assist in visualising the complexities of these requirements the thiazole ring, which contains H7, was rotated about C5-C6 and the interproton distances plotted for all possible combinations of chirality. There is only **one** situation where the interproton distance from C7 to more than one of H3, H4a or H5 is about, or less than 3Å. This is for low rotation angles in PATEAMINE FRAGMENT 1, shown in Figure 5, where it might be anticipated that there would be nOe's observed between H7/H5 and H7/H3. The medium nOe observed between H4a and H7 can only arise from an alternative rotamer and may well be a strong interaction, but arising from a minor population.

It seems likely that it will be possible to generate some of the relative stereochemistry by this approach, but the definitive answer may have to await the X-ray crystallographic data on a suitable crystal.

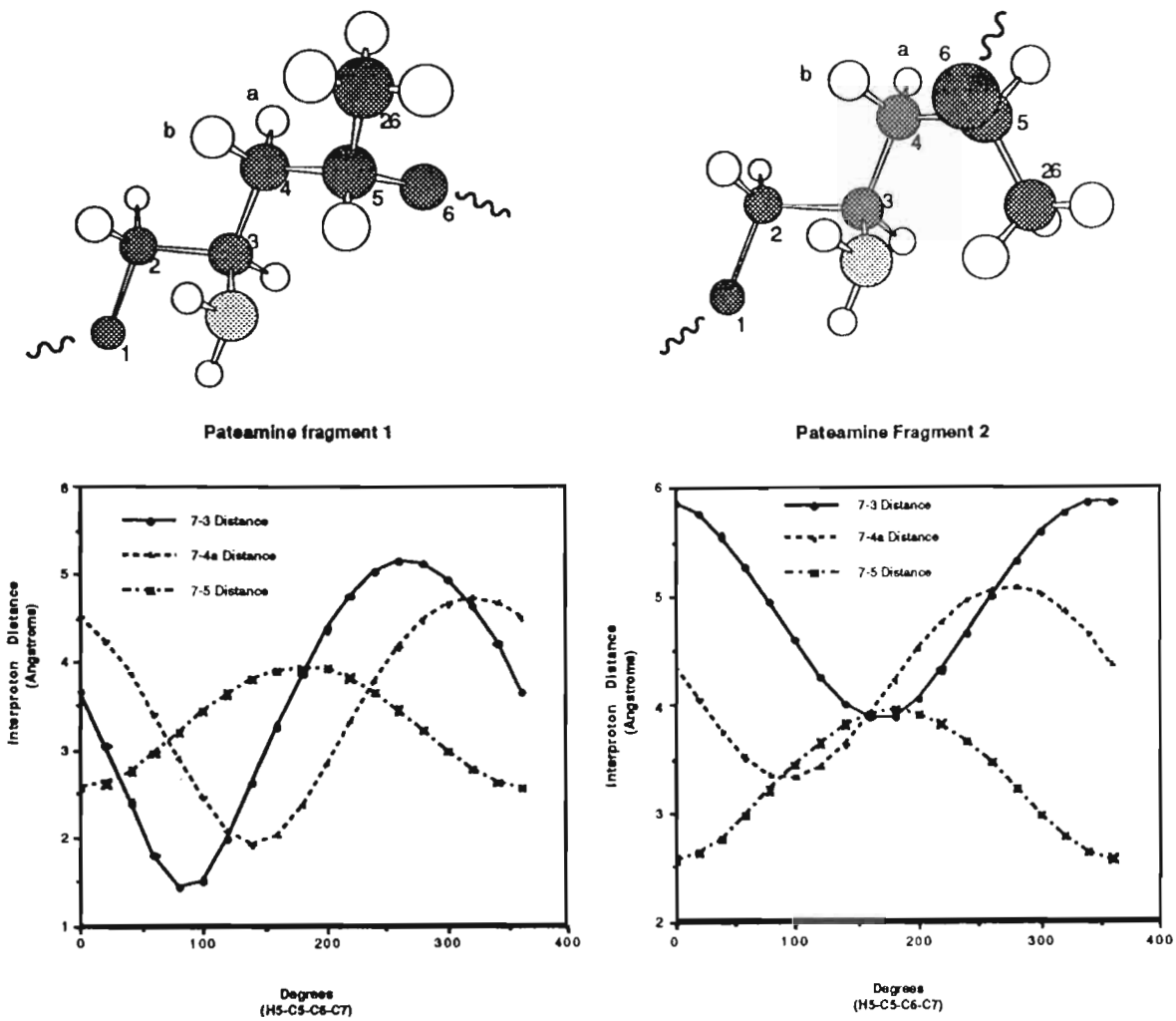


Figure 5 : Possible conformations for the C2-C6 region of pateamine

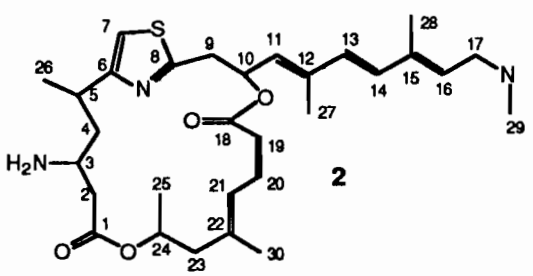
The structure of pateamine **1** represents a unique combination of functionalities, including the relatively rare feature in macrolides of a dilactone, and results in a molecule which is an extreme cytotoxin. The biological evaluation of pateamine against a variety of murine and human cell lines, bacteria, fungi and viruses was undertaken (Table 1). From this it can be concluded that pateamine has antifungal properties, but most importantly it is an extreme cytotoxin. Extreme cytotoxins, while of some interest, are not useful unless they

demonstrate selectivity in their mode of action against various cell lines. Encouragingly, pateamine shows quite a variation in IC_{50} against the various cell lines (Table 1). The most significant result is the cytotoxicity assays where there is a very large distinction in the efficacy of pateamine against cells in log phase growth compared with stationary phase. This indicates that the CV-1 and BHK cell lines, under essentially static growth conditions, have a high tolerance towards pateamine. This relative insensitivity of pateamine against a cell line under static growth conditions is in stark contrast to the situation with fast growing cells, such as found for the P388 or KB cell lines. This degree of selectivity is very high indeed, and is in fact at least 2 orders of magnitude greater than the effect observed with many current drugs used currently for chemotherapy. Overall pateamine may well be efficacious as a **selective** agent against certain classes of tumour cells. The further biological evaluation of pateamine, especially the *in vivo* testing, is currently underway.

Antitumour Assays		
	IC_{50}	
P388	0.15ng/ml	
A-549	0.7ng/ml	
HT-29	2ng/ml	
K B	<0.05 ng/ml	
Cytotoxicity Assays		
	Log Phase	**Stationary Phase**
CV-1	2.4ng/ml	>lOOng/ml
B H K	3ng/ml	>lOOng/ml
Antiviral Assays		
Herpes simplex I	3+ at 20 ng/ml	
Polio	3+ at 20 ng/ml	
Antimicrobial Assays		
	MIC	
B. subtilis	>5μg/ml	
E. coli	>5 μg/ml	
P. aeruginosa	>5μg/ml	
C. albicans	lμg/ml	
T. mentogrophytes	20 μg/ml	
C. resinae	0.4μg/ml	

Table 1 : Biological data on Pateamine 1

Co-occurring with pateamine were two minor compounds. One of these is simply the N-demethyl derivative **2** and the biological activity of this is slightly less than for pateamine itself.



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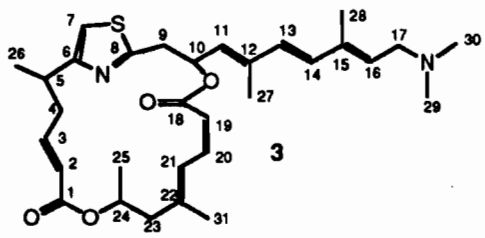
The other minor component has considerably lower activity, but is still selective in its mode of action. It differs from pateamine by one oxygen and perhaps an additional sulphur. Work is continuing on the structure of this derivative.

As the biological properties of pateamine and its derivatives are so promising we have embarked on a program of hemisynthesis. Initial attempts to form N-acyl derivatives with a variety of agents under differing conditions were all unsuccessful as pateamine undergoes a very facile elimination of the C3 amino group to form the Δ^2 unsaturated lactone **3**. Conditions have since been established that allow the formation of suitable N-acyl derivatives. This elimination reaction, and the formation of other suitable derivatives of pateamine form part of the on-going chemistry of this very interesting heterocyclic macrolide. For example the Δ^2-*N*, *N*, *N*-trimethyl quaternary ammonium derivative **4**, formed by treatment of pateamine with methyl iodide under mildly basic conditions, has quite different biological properties from the parent. The IC_{50} against P388 is about 200x less than pateamine, but it still displays selective activity against the range of cell lines, while the inhibitory effect against the enzyme adenosine deaminase is about 600x greater than that measured for pateamine.



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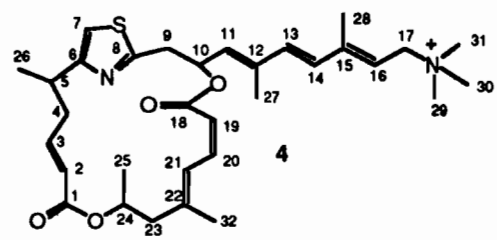

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In the future a wide range of pateamine derivatives will be synthesised to more fully explore the biological potential of the pateamine system.

We anticipate that the chemical screening procedure will allow us to make further rational choices of the active species to work on in the future. This perhaps will guide the isolation of compounds with biological properties comparable to, or better than pateamine.

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* Thanks to Dr M R Boyd and Dr J H Cardellina II, NCI for coining this most apt, descriptive term : Chemical screening

* Pateamine is a name of Maori origin to commemorate 1990, the 150th anniversary of the signing of the Treaty of Waitangi. PATEA was the name of the earliest inhabitants of the Fiordland area on the West coast of the South Island of New Zealand, the area from which the *Mycale sp.* sponge was collected.

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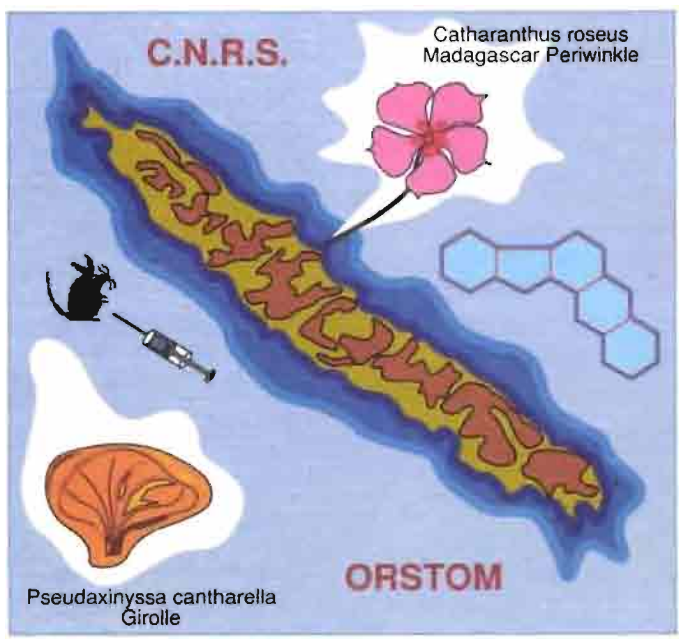

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