Bioactive Molecules from Water Extracts: 15 Years Experience at NCI-Frederick

Thomas G. McCloud*, Natural Products Support Group, SAIC-Frederick, Inc.
Barry R. O'Keefe, Molecular Targets Development Program, CCR, NCI-Frederick
David J. Newman, and Gordon Cragg, Developmental Therapeutics Program, NCI-Frederick, Frederick, MD

Introduction:
There were few precedents for finding water soluble compounds useful in the treatment of human disease conditions in 1986. Most useful drugs identified from natural products sources had come from organic solvent extracts, often ethanol for plants, and ethyl acetate for fermentation antibiotics. Though biological activity was known for many proteins, the development of these macromolecules as human therapeutics had proven an extremely difficult task, though some success had been achieved with interferon.

Water extracts of natural product specimens could easily be made, and biological evaluation was possible, but the ability of chemists to fractionate and isolate pure compounds from water extracts was limited by inadequate chromatographic techniques. Even if pure, solving the structures of water soluble macromolecules by chemical degradation, NMR, MS and X-ray crystallography was an extremely challenging prospect. Consequently, water extracts had infrequently been investigated as sources for new chemotherapeutics, and generally held in low esteem as fruitful locations for drug prospecting. But progress in addressing both the chromatographic and structure solution problems was being made, so when the anticancer drug discovery effort was established at the National Cancer Institute-Frederick in 1986, the decision was made that a water soluble extract would be prepared from each specimen for bioassay.

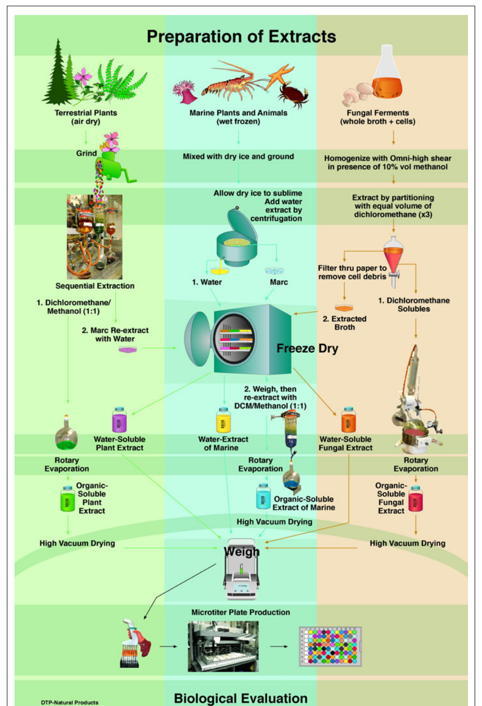
Preparation of Water Soluble Extracts:

Specimens acquired for the drug discovery program were highly varied: higher plants predominantly from tropical rain forests were air dried in the field. Marine organisms, both animal and plant, most from tropical waters of the South Pacific, were frozen promptly after collection. Whole fungal fermentation broths, grown from a wide range of pure fungal cultures, also required extraction. Consequently, equipment and protocols used to process these highly varied specimens and prepare both an organic solvent soluble extract as well as a water soluble extract from each material had to be devised. Very briefly summarized, these methods were:

Plants: Finely ground
Extracted with a dichloromethane/methanol mixture
Re-extracted with water

Marine Organisms: Finely ground while frozen
Water added
Water extract produced by basket centrifugation

Fungal Fermentation Broths: High shear homogenization
Partition broth against dichloromethane
Clarify broth by passing through filter paper



In all cases the water soluble extract was quickly frozen, then lyophilized. The dry material was transferred to a borosilicate bottle, tightly capped, and its weight recorded. Though yield of water solubles from specimens is quite variable, it is not at all unusual to have >10% of the specimen weight in a water extract.

Since the inception of the natural products drug discovery program at NCI-Frederick, approximately 58,000 water extracts have been prepared from higher plant specimens, 13,300 from marine organisms, and 15,500 from fungal fermentations. All of these extracts have been placed into 96 well microtiter plate format, and are available from the DTP Natural Products Repository for screening in new bioassays.

Characterization of Water Soluble Extracts:

1. A question was raised whether these lyophilized extracts could easily and efficiently be resolubilized for testing purposes. Did the extracts contain a lot of particulates? Did the processing protocol create globs of insoluble denatured protein? A random selection of several hundred extracts were weighed into glass vials, water added and the vial shaken. This solution/suspension was passed through a 0.45 micron nylon filter, and the amount of protein in this solution was determined by a colorimetric method. Protein content was found to be in a range from <1% to 20% by weight. The filtrate was re-dried, then weighed. In general, > 90% of the mass of lyophilized water soluble extracts pass the filter, confirming that solubility is very good.
2. There is an appreciable quantity of sea water, and thus sea salt, in the frozen marine specimens, as received, and this will be present in the water extract of marine organisms. While the presence of sea salt does not pose a problem for most bioassays, if it is present in large quantity, it serves to 'dilute out' those organic molecules which could be bioactive. To determine the percentage of salt in lyophilized water extracts of marine organisms, a random series of extracts was weighed into platinum crucibles, then ashed at high temperature in a muffle furnace. The amount of ash which includes sea salt, was determined to lie between ~10 and 70% of the dry weight of the water extract.

A portion of this data has been presented previously as poster #P36, ASP meetings, San Juan, Puerto Rico, 6-10 August, 1989. Additional information on production of extracts is available on the web site of the Natural Products Support Group, at http://npsg.ncifcrf.gov

Bioassay of Water Soluble Extracts:

Information regarding screens and drug discovery resources of the Developmental Therapeutics Program, and the Screening Technologies Branch is available over the web at http://dtp.nci.nih.gov/ branches/npb/repository.html and at http://spheroid.ncifcrf.gov/STB/stb_index.cfm.

Among the high throughput assays performed at the NCI-Frederick are anticancer in human cells in tissue culture, anti-HIV in CEM-SS human lymphocytes cells, and anti-microbial evaluating for toxicity toward *Candida albicans, Staphylococcus aureus* and *Enterococcus faecalis.*

ANTICANCER screening of crude extracts has been a major activity of the program since its inception. Some 85,000 water soluble extracts have been evaluated in at least one of the whole cell anticancer screens. If an extract kills at least 3 cell lines at a dose of 100 ug/ml, it is considered toxic. Of 19,000 fungal broths, ~900, or 5% showed toxicity. 54,000 water soluble extracts of higher plants have been tested, with 6,800 displaying toxicity, 12.6%. From water soluble extracts of marine animals and plants, 12,500 extracts have been tested, with 2,600 showing toxicity, or 20%.

In the CEM-SS ANTI-HIV assay, some ~35,000 water soluble extracts were screened.If an extract is toxic to the CEM cell line, then any protective effect from HIV infection due to an extract component cannot be detected.

	% inactive @ 10 μg/ml	% protective @ 10 μg/ml
plants	85%	15%
marines	82%	18%
fungi	98%	2%

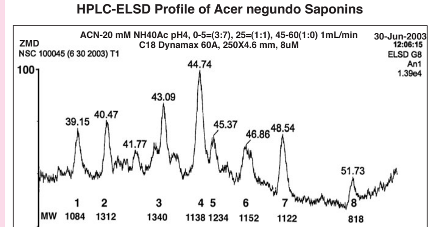
The high hit rate from marine organisms was traced to the sensitivity of this specific bioassay to the presence of high molecular weight poly-anionic molecules, such as polysulfated polysaccharides, which are common in marine organisms. A high throughput dereplication protocol for removal of these high molecular weight substances, consisting of treatment at 50% ethanol in a freezer to induce precipitation, centrifugation, and retesting of both precipitate and soluble portion, was developed. After being subjected to this protocol, protection of the CEM cells from the cytopathologic effect of HIV was localized in the precipitate of ~94% of those extracts which were initially protective. The common occurrence of substances in water extracts which interfere with bioassay is an assay-specific phenomenon. There seems to be no generic problem with macromolecules, lignins, or salts with whole cell assays, but each target-based assay should be examined critically for common interfering substances. Previous publications relating to this include Poster#166, ASP Annual Meetings 27-31 July, 1996, Santa Cruz; "Evaluation of anti-HIV aqueous extracts of Marine Invertebrates", John A. Beutler, et.al.; and in *Antiviral Chemistry and Chemotherapy* (1993) 4 (3), 167-172.

Approximately 80,000 water extracts have been assayed for ANTIMICROBIAL activity by the disc diffusion method. Filter paper discs, each containing 1 mg of crude extract are prepared in 96 well microtiter format, then transferred, in this same 8 x 12 pattern, onto 245 x 245 mm nutrient agar plates containing test organism. 1.2% of extracts show a clear zone of growth inhibition of wild type *Candida albicans*, 0.4% inhibit growth of *Escherichia coli*, and 5.6% inhibit growth of *Bacillus subtilis*.

65,000 water soluble extracts were bioassayed in high throughput mode against three microorganisms: an azole resistant *Candida albicans*, a methicillin resistant *Staphylococcus aureus*, and a multidrug resistant *Enterococcus faecalis*. 652 extracts, 1%, showed toxicity when 90% inhibition of microbial growth was used as the endpoint in these three test systems.

Among extracts identified as inhibiting growth of azole resistant *Candida albicans* are several containing saponins. Because of the potential for hemolysis, and other problems, saponins are not generally considered as suitable for development as drugs. Therefore, rapid dereplication of saponins is desirable. When a crude extract is chromatographed on a C-18 column, eluted with a steep gradient of methanol in water, and the toxicity is eluted at the non-polar end of the chromatogram, and in the MS there are prominent mass ions in the 800-->1200 amu range, these compounds are often saponins. Acid hydrolysis of a sub-milligram aliquot followed by re-injection into the HPLC/MS can often lead to identification of the genin, though the exact identification and linkage of the sugars remain unknown.

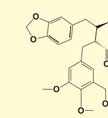
HPLC-ELSD Profile of Acer negundo Saponins



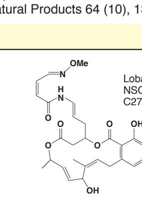
Bioactive Compounds Identified from Water Extracts at NCI-Frederick:

Many of the compounds cited on this poster were found by the Molecular Targets Drug Program. Their website, http://home.ncifcrf.gov/mtdp/contains additional information on both water soluble and organic solvent soluble compounds isolated from crude natural products extracts at NCI-Frederick.The potential for new drugs from proteins has recently been reviewed, (O'Keefe, B.R., Journal of Natural Products 64 (10), 1373-1381, 2001).

Anti-Cancer



Deoxypodorhizon
NSC 332046
C22H24O7



Lobatamide A
NSC 692909
C27H32N2O8

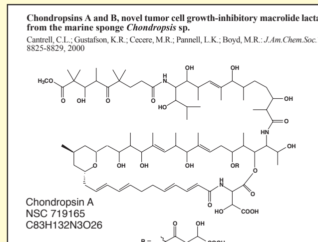
J. Org Chem. ***1998***, 63, 7805–7810

The Lobatamides, Novel Ckytotoxic Macrolides from Southwestern Pacific Tunicates†

Tawnya C. McKee, Deborah L. Galinis, Lewis K. Pannell, John H. Cardellina II, Jodi Laakso, Chris M. Ireland, Leanne Murray, Robert J. Capon, and Michael R. Boyd

Chondropsins A and B, novel tumor cell growth-inhibitory macrolide lactams from the marine sponge *Chondropsis* sp.

Cantrell, C.L.; Gustafson, K.R.; Cecere, M.R.; Pannell, L.K.; Boyd, M.R.: *J.Am.Chem.Soc.* **122:** 8825-8829, 2000

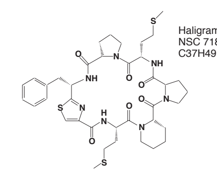


Chondropsin A
NSC 719165
C83H132N3O26

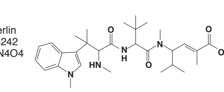
- Potent cytotoxicity (~24 nM) in 60-cell human tumor panel
- Specific human V-ATPase inhibitor

Haligramides A and B, two new cytotoxic hexapeptides from the marine sponge *Haliclona nigra*

Rashid,M.A.; Gustafson,K.R.; Boswell,J.L.; Boyd,M.R.: *J.Nat.Prod.* **63:** 956-959, 2000.



Haligramide A
NSC 718326
C37H49N7O6S3



Hemiasterlin
NSC 695242
C30H46N4O4

Cytotoxic and tubulin-interactive hemiasterlins from *Auletta* sp. and *Siphonochalina* spp. sponges

Gamble, W.R., Durso, N.A., Fuller, R.W., Westergaard, C.K., Johnson, T.R., Sackett, D.L., Hamel, E., Cardellina, J.H. 2nd, Boyd, M.R.

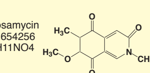
Bioorg. Med. Chem. **7**: 1611-1615, 1999.

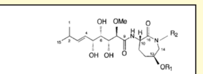
1094 *J. Nat. Prod.* **1997,** *60,* 1094–1099

Isolation of a Novel Kunitz Family Protease Inhibitor in Association with *Tethya* Hemolysin from the Sponge *Tethya ingalli*

Barry R. O'Keefe,† John A. Beutler,† John H. Cardellina II,† Tanya R. Prather,† Robert H. Shoemaker,† Raymond C. Sowder II,‡ Louis E. Henderson,‡ Lewis K. Pannell,§ and Michael R. Boyd*,†

Mimosamycin
NSC 654256
C12H11NO4





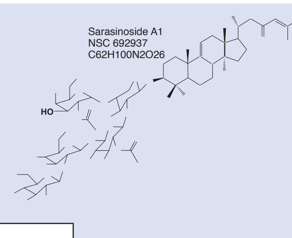
Bengamide:		R_1	R2
NCS 660650 — C17H30N2O7	Y (1)	H	H
	A (2)	$-CO(CH_2)_{12}CH_3$	H
NCS 716230 — CN18H32N2O7	Z (3)	H	CH_3
	B (4)	$-CO(CH_2)_{12}CH_3$	CH_3

Cytotoxic metabolites from an Australian collection of the sponge *Jaspis* species

Groweiss, A., Newcomer, J.J., O'Keefe, B.R., Blackman, A., and Boyd, M.R.: *J.Nat.Prod.* **62:** 1691-1693, 1999.

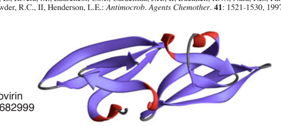
Anti-HIV

Sarasinoside A1
NSC 692937
C62H100N2O26



Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: Potential applications to microbicide development

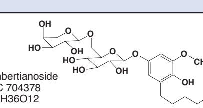
Boyd, M.R., Gustafson, K.R., McMahon, J.B., Shoemaker, R.H., O'Keefe, B.R., Mori, T., Gulakowski, R.J., Wu, L., Rivera, M., Laurencot, C.M., Cardellina, J.H., II, Buckheit, R.W., Nara, P.L., Pannell, L.K., Sowder, R.C., II, Henderson, L.E.: *Antimocrob. Agents Chemother.* **41**: 1521-1530, 1997.



Cyanovirin
NSC 682999

Structure
- 101 amino acids, mostly βsheet
- Purified from the cyanobacterium *Nostoc ellipsosporum*
- Internal sequence homology
- Flexible hinge region
- Can form domain-swapped dimers

Activity
- CV-N inactivates diverse strains of HIV-1, HIV-2 and SIV.
- CV-N prevents the fusion of HIV-infected and non-infected cells.
- Previous studies indicated HIV gp120 is a molecular target.
- Binds to the high-mannose oligosaccharides Man-8 and Man-9.

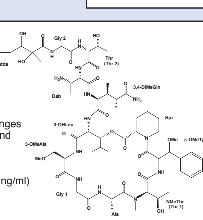


Lambertianoside
NSC 704378
C23H36O12

Lambertianoside, a novel phenylglycoside from *Eugenia lambertiana*

Bokesch, H.R., Young, S.M., McKee, T.C., Blunt, J.W., Boyd, M.R.

Nat.Prod. Lett. **11**: 211-216, 1998.



Papuamide A
NSC 714357
C65H103N13O21

- Isolated from the sponges *Theonella swinhoei* and *T. mirabilis*
- Both HIV inhibitory (EC50 = 4 ng/ml) and cytotoxic (EC50 = 75 ng/ml)

Papuamides A-D, HIV-inhibitory and cytotoxic depsipeptides from the sponges *Theonella mirabilis* and *Theonella swinhoei* collected in Papua New Guinea

Ford, P.W., Gustafson, K.R., McKee, T.C., Shigematsu, N., Maurizi, L.K., Pannell, L.K., Williams, D.E., de Silva, E.D., Lassota, P., Allen, T.M., Van Soest, R., Anderson, R.J., and Boyd, M.R.: *J. Am. Chem. Soc.* **121**: 5899-5909, 1999.

Circulins A and B: Novel HIV-inhibitory macrocyclic peptides from the tropical tree *Chassalia parvifolia*

Gustafson, K.R., Sowder, R.C., II, Henderson, L.E., Parsons, I.C., Kashman, Y., Cardellina, J.H., II, McMahon, J.B., Buckheit, R.W., Pannell, L.K., Boyd, M.R. *J. Am. Chem. Soc.* **116**: 9337-9338, 1994.

disulfide bridges

```
C    C   C    C     C C
1    2   3    4     5 6
NKVCYRNG-IPCGESCVWIPCISAALGCSCK
NKVCYRNGVIPCGESCVFIPCISTLLGCSCK
```

circulin A
NSC 693011

circulin B
NSC 693012

- Cyclic Peptides with Anti-HIV Activity
- Very rigid cyclic peptide structures with three disulfide bonds
- Completely resistant to enzyme attack

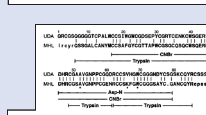
Eur. J. Biochem. 245, 47-53 (1997)

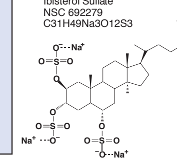
NSC 693017

Isolation and characterization of niphatevirin, a human-immunodeficiency-virus-inhibitory glycoprotein from the marine sponge *Niphates erecta*

Barry R. OKEEFE[1], John A. BEUTLER[1], John H. CARDELLINA II[1], Robert J. GULAKOWSKI[1], Benjamine L. KREPPS[1], James B. MACMAHON[1], Raymond C. SOWDER II[2], Louis E. HENDERSON[2], Lewis K. PANNELL[3], Shirley A. POMPONI[4] and Michael R. BOYD[1]

N-terminal amino acid sequencing of niphatevirin: N_2H-Ala-Val-Pro-Xaa-Pro-Gly-Val-Asn-Ile-Ala-Pro-Ala-Ala-Pro-Gln-Tyr-Met-Leu-Ala-Leu-Ser-His-Pro-Ala-Gly-Tyr-Xaa-Lys-Asn-Asn-Pro-Ala-Arg-Xaa-Pro-Val-Thr-





Ibisterol Sulfate
NSC 692279
C31H49Na3O12S3

Tetrahedron Letters, Vol. 34, No. 3, pp. 389-392, 1993

IBISTEROL SULFATE, A NOVEL HIV-INHIBITORY SULFATED STEROL FROM THE DEEP WATER SPONGE TOPSENTIA SP.[1]

Tawnya C. McKee, John N. Cardellina II, Mark Tischler, Kenneth M. Snader† and Michael R. Boyd*

Laboratory of Drug Discovery Research and Development and †Natural Products Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Building 1052, Room 121, Frederick, MD 21702-1201, USA.

1170 *J. Nat. Prod.* **2000,** *63,* 1170–1174 NSC 714343

Isolation and Characterization of *Myrianthus holstii* Lectin, a Potent HIV-1 Inhibitory Protein from the Plant *Myrianthus holstii*[1]

Romila D. Charan, Murray H. G. Munro,† Barry R. O'Keefe, Raymond C. Sowder II,‡ Tawnya C. McKee,* Michael J. Currens,§ Lewis K. Pannell,⊥ and Michael R. Boyd*

Conclusion:
Water extracts have been the source of a wide diversity of bioactive chemotypes, some of which were known, and some new compounds. Small molecular weight compounds, peptides and proteins have been identified. The potential to find new, useful chemotherapeutics for treatment of human diseases exists in water extracts of natural product origin.

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract #NO1-124000.

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