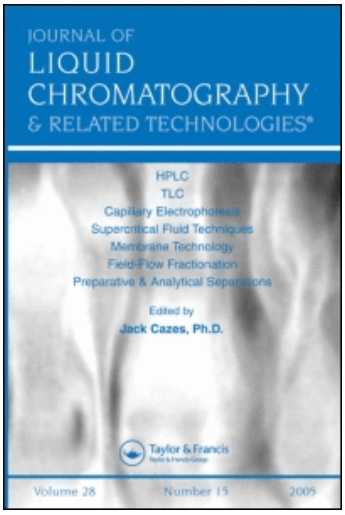

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Detection and Quantitation of Bryostatin 1 and 2 in *Bugula Neritina* by Combined High-Peformance Liquid Chromatography and ^{3}H-Phorbol Dibutyrate Displacement

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DETECTION AND QUANTITATION OF BRYOSTATIN 1 AND 2 IN BUGULA NERITINA BY COMBINED HIGH-PEFORMANCE LIQUID CHROMATOGRAPHY AND ^{3}H-PHORBOL DIBUTYRATE DISPLACEMENT

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ABSTRACT

An HPLC method has been developed to detect and quantitate bryostatins 1 and 2 from crude extracts of B. neritina. This, coupled with a ^{3}H-phorbol dibutyrate binding assay and photodiode array-acquired uv spectra makes possible the evaluation of crude extracts in a rapid, sensitive and specific manner.

INTRODUCTION

The bryostatins comprise a group of more than a dozen macrocyclic lactones isolated from the marine animal Bugula neritina L. (Bryozoa, Bugulidae) [1-3]. Bryostatins activate protein kinase C, bind to the phorbol receptor [4,5], and have antineoplastic [6] and

immunomodulating properties [7,8]. Bryostatin 1 is currently in pre-clinical development as an antitumor compound by the US National Cancer Institute.

Recently, methods for the separation of pure bryostatins 1-12 by HPLC [9] and for the detection of bryostatin 1 and 2 in crude samples by multimodal thin layer chromatography have been developed [10].

In this paper we describe a method combining HPLC, photodiode array detection, and a ^{3}H-phorbol dibutyrate (PDBu) receptor binding assay for the detection of bryostatin 1 and 2 in crude extracts or fractions of B. neritina. The HPLC method is also applicable to the quantitative assay of bryostatin 1 in B. neritina.

EXPERIMENTAL

General:
Diatomaceous earth, Dicalite Speed Flow, was obtained from Grefco Inc. Solvents were HPLC grade (Burdick & Jackson). Silica gel 60 (40-63 μm) was obtained from EM Science.

Bugula neritina:
Specimens of B. neritina L. (Bugulidae) were collected near Long Beach, CA (Marinus Inc.) during the spring of 1988 and in December 1988. (a) Frozen samples were shipped on dry ice and lyophilized. See "quantitation of bryostatin 1" for extraction and work-up procedure.

(b) Wet samples (collected in spring 1988 as part of a large-scale collection operation) were stored in technical grade i-PrOH at ambient temperatures prior to extraction with MeOH. Combined i-PrOH/MeOH extracts were concentrated, then partitioned between EtOAc and water. An aliquot (20 g) of the EtOAc extract was coated on dicalite (200 g) and slurry packed (hexane) on a silica gel flash column (10 x 10 cm). Elution was carried out with methylene chloride, followed by EtOAc and MeOH (3 L each). The EtOAc fraction yielded pure bryostatin 1 and 2 (full details of the large-scale preparative isolation will be published elsewhere). Another aliquot of the EtOAc fraction was used for the HPLC/^{3}H-PDBu displacement assay.

HPLC:

HPLC separations were performed on a Microsorb C-18 3 μm, 100 x 4.6 mm I.D. column with precolumn (15 x 4.6 mm I.D., Rainin); using a Perkin-Elmer Series 410 LC pump, ISS-100 autosampler, SEC-4 solvent environmental control and a Waters 990 photodiode array detector; the mobile phase was aqueous ACN 78%, at 1 ml/min. All sample solutions were filtered through Anotop 0.2 μm filters (Anotec Separations, Ltd.) prior to injection.

HPLC/^{3}H-PDBu displacement assay:

A crude EtOAc fraction (see "B.neritina" (b)) was injected (5 μl of a 10 mg/ml solution) and analyzed by HPLC under the conditions described above. Fractions of six drops each of mobile phase were collected in 120 x 75 mm culture tubes using a Foxy fraction collector (Isco). The fractions were dried in vacuo in the collection tubes which were used directly for the ^{3}H-PDBu binding assay. The binding assay was then performed essentially as reported in [5].

Frozen rat brains (male Sprague Dawley, Zivic Miller Co.) were homogenized with a Polytron at setting 5 for 20 sec. in 10 volumes of 10 mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged for 10 min. at 14,000 rpm (SS-34 rotor, Sorvall RC-5 centrifuge). The pellet was resuspended and rehomogenized in this manner a total of three times. Prepared tissue was stored as a frozen suspension at -70°C. For the assay, an aliquot of stored tissue was resuspended in binding buffer (50 mM Tris-HCl pH 7.4 with 2 mg/ml bovine serum albumin). The suspended tissue was added to the incubation mixture to start the assay.

10 μl (5 nM) of ^{3}H-PDBu in ethanol (Du Pont NEN, specific activity 10-20 Ci/mmol) was added to each tube. Blank tubes (measuring nonspecific binding) received 10 μl (20 μM) of unlabeled PDBu (Sigma) in EtOH as well. The volume was made up to 0.9 ml with binding buffer and 0.1 ml of brain membrane suspension was added. The tubes were incubated at 30°C for 60 min.

The incubation was terminated by filtration over Whatman GF/B glass fiber filters in a Brandel cell harvester. Filters were rinsed twice for 4 sec. with the centrifugation buffer (no BSA) at room temperature. Radioactivity on the filters was solubilized with 4 ml Aquasol (Du Pont NEN) and the vials were shaken at 250 rpm for 20 min. Activity was measured by conventional liquid scintillation counting.

Quantitation of bryostatin 1:

Standards: Bryostatins 1 and 2 were isolated from B. neritina and their identity was confirmed by IR, UV, ^{1}H- and ^{13}C-NMR, and (HR)FAB MS. Solutions (prepared from 1 mg samples, in duplicate) containing 25, 50, 75

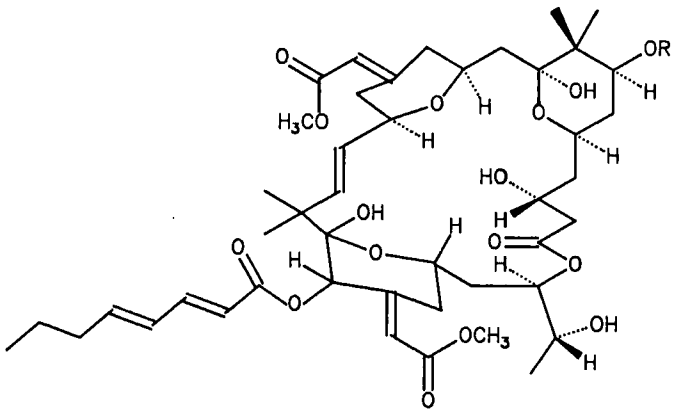
and 100 μg/ml were analyzed by HPLC (10 μl/injection). Bryostatin 1 peak areas were integrated at 265 nm detection wavelength. Results are presented in Fig. 2.

B.neritina: 5.0 g dry lyophilized powdered material was extracted with methylene chloride - MeOH (1:1) (3 x 100 ml, room temperature, 1 h, magnetic stirrer). The extract was coated on dicalite (3 g) by slow evaporation of solvent under reduced pressure. The dry coated material was packed over a silica gel extraction cartridge (Mega Bondelut, 2g/12ml, Analytichem) and eluted with methylene chloride (20 ml), EtOAc (50 ml) and MeOH (50 ml), respectively. The EtOAc fraction was evaporated under reduced pressure and the residue redissolved in 2 ml MeOH. This solution was loaded onto a C-8 cartridge (Bondelut, 3 ml, Analytichem) which had previously been rinsed with ACN (5 ml). The cartridge was eluted with ACN until a total volume of 10 ml was obtained. The ACN solution was evaporated under reduced pressure and the residue dissolved in MeOH (2.0 ml) and the resulting solution filtered prior to injection (10 μl).

RESULTS

HPLC/^{3}H-PDBu Displacement Assay:

The crude extract of Bugula neritina collected in California was partitioned between EtOAc and water, giving 98% recovery of total bioactivity in the EtOAc phase. The organic phase was dried and further purified over silica gel using a batch elution sequence with methylene chloride, EtOAc and MeOH. Analysis of the



Bryostatin 1: R = $COCH_3$

2: R = H

fractions with the ^{3}H-phorbol dibutyrate displacement assay showed that 95% of the binding activity was found in the EtOAc eluant. This fraction was analyzed by reversed phase HPLC with photodiode array detection. Separations (50 μg per injection) were achieved within 20 min run times and good resolution was obtained with a short analytical column (C-18, 3 μm particle size) and isocratic elution (aqueous ACN 78%, 1 ml/min; see Fig. 1). The complex nature of the sample is illustrated by the chromatogram recorded at 220 nm (Fig. 1a), whereas the detection at 265 nm (Fig. 1b) was found to be very selective for bryostatins 1 and 2. UV spectra recorded for the bryostatin 1 elution peak showed the typical absorption maximum at 265 nm,

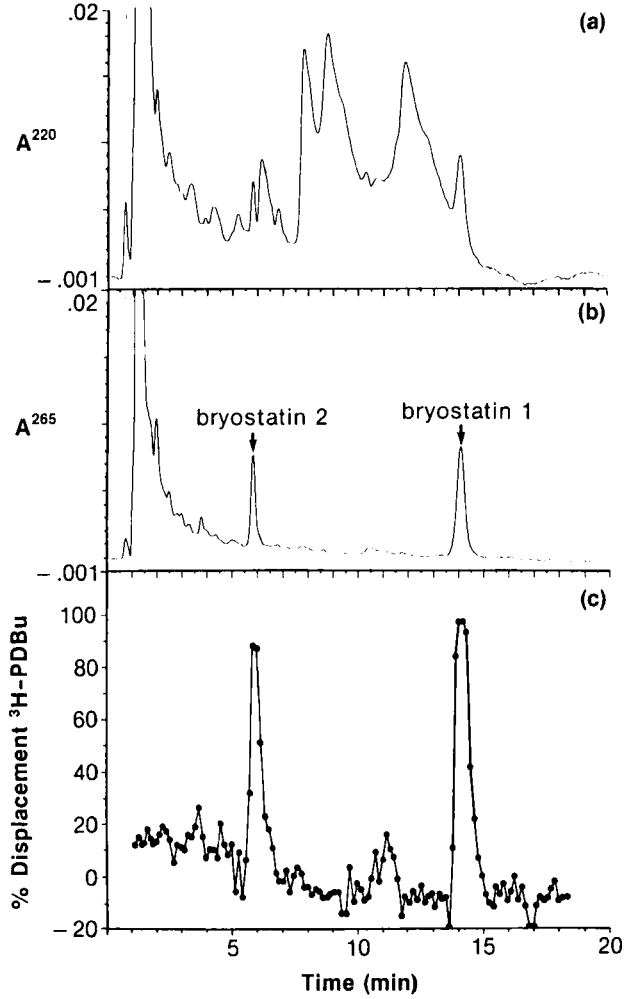


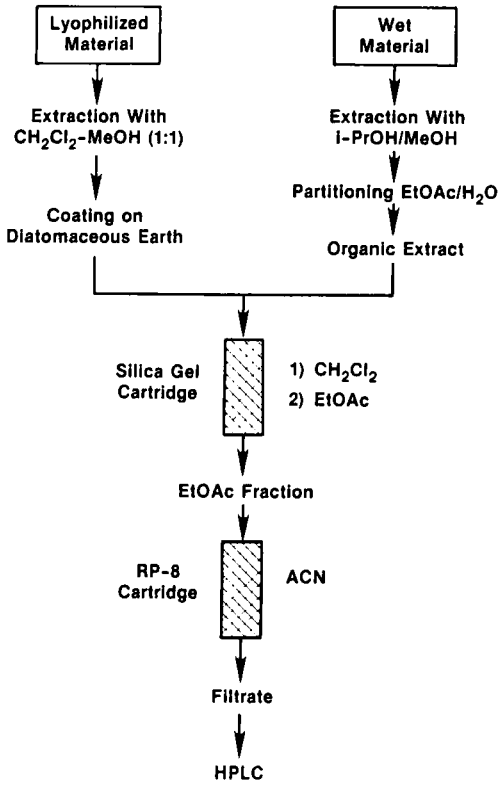
FIGURE 1: Combined HPLC/^{3}H-phorbol dibutyrate (PDBu) displacement assay. Separation of B. neritina fraction (C-18, 3 μm, 100 x 4.6 mm I.D.; ACN 78%, 1 ml/min): detection at 220 nm (a), at 265 nm (b), and resulting synthesized "displacement chromatogram" (c).

whereas the maximum at 235 nm was shifted to a shorter wavelength, indicating interference with peak impurities. Peak identity was confirmed by co-injection with authentic samples of bryostatin 1 and 2. Bryostatin 1 was not detectable by normal or reversed phase TLC of the EtOAc fraction.

The column effluent generated during the above separation was collected in fractions of 6 drops (corresponding to approximately 10 sec. each at the flow rate of 1 ml/min.). The resulting fractions were dried *in vacuo* for one hour in order to remove the organic solvent. Next, buffer, rat brain membranes, and ^{3}H-PDBu were added directly to the tubes and the binding assay performed. Displacement values were plotted as a function of retention time (after adjustment for delay in tubing between detector and fraction collector). The resulting synthesized "displacement chromatogram" is shown in Fig. 1c.

Quantitation of bryostatin 1:

Samples of lyophilized *B. neritina* (5 g dry weight) were extracted with methylene chloride-MeOH (1:1). Bryostatins were enriched by further purification, as summarized in scheme I: the extracts were coated on diatomaceous earth and the coated material was packed over a silica gel solid phase extraction cartridge.



SCHEME I: Sample clean-up procedures for the detection of bryostatin 1 in B. neritina extracts.

The cartridge was successively eluted with methylene chloride, EtOAc and MeOH. This step has been shown (binding assay data, above) to give ca. 95% recovery of bryostatins in the EtOAc fraction. This fraction was evaporated, redissolved in MeOH and further passed over a reverse phase cartridge (MeOH, then ACN) to trap lipophilic compounds which degrade HPLC column lifetime. This step gave quantitative recovery of

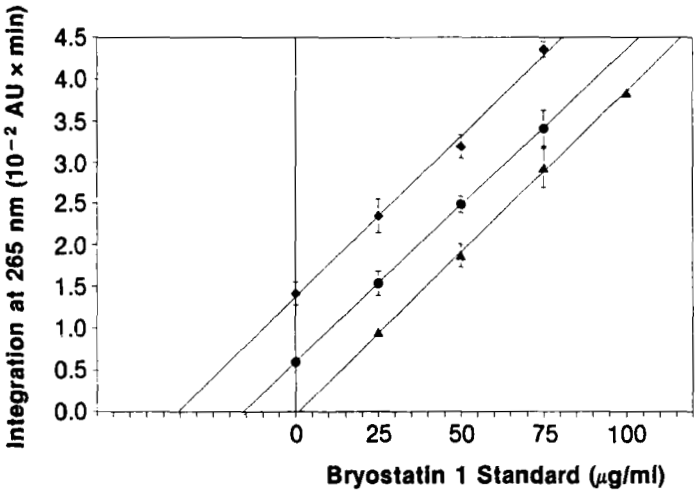


FIGURE 2: Quantitation of bryostatin 1 by standard addition method: standard (▲); B. neritina collected in spring 1988 (●) and in December 1988 (◆).

bryostatins. The MeOH-ACN eluate was evaporated, reconstituted in MeOH and filtered prior to injection. (See experimental section for details). Resulting sample solutions were analyzed (in triplicate) by reversed phase HPLC (conditions see Fig. 1.) and bryostatin 1 peak areas were integrated at 265 nm. Injections were repeated with addition of bryostatin 1 standard solutions to sample solutions (standard addition method).

Results are summarized in Fig.2 and indicate bryostatin 1 concentrations of 6.4 µg/g and 14 µg/g for samples collected during spring 1988 and in December

1988, respectively. This corresponds to an average yield of approximately 0.001% bryostatin 1 (B. neritina dry weight). Bryostatin 2 levels (data not shown) were approximately 50% to 75% of bryostatin 1 concentrations.

DISCUSSION

Receptor displacement assays are highly specific methods and have a large potential in drug discovery. Only a few applications have been reported in natural products chemistry, e.g. for the bioassay guided isolation of the opiate receptor antagonist pericin from Picralima nitida (Apocynaceae) [11].

The phorbol dibutyrate binding assay is a very useful technique for guiding isolation of bioactive molecules such as phorbol esters, debromoaplysiatoxin, teleocidins, and bryostatins. In this paper the first direct combination of the ^{3}H-PDBu displacement assay with analytical HPLC is described. The column effluent resulting from the separation of a complex Bugula neritina fraction containing bryostatin 1 and 2, was collected in small fractions of 6 drops each (see Results). These fractions were directly assayed and the ^{3}H-PDBu displacement plotted to synthesize a

"displacement chromatogram". This chromatogram (Fig. 1c) can be matched to the UV detector peaks of the HPLC analysis (Fig. 1b).

Since by necessity the binding assay must be run in singlicate, the signal to noise in the direct binding assay is poorer than that found in the usual duplicate or triplicate binding assay. This inherent noise of the assay appears to be equivalent to 10 to 15 percent displacement. In such an analysis a peak of binding activity is not considered valid unless several consecutive points all show a significant displacement. With the >30 sec peak widths observed in our analysis by UV we routinely can observe multiple points within that time frame. This assay is quite sensitive; for example, 50 μg of an EtOAc fraction of B. neritina was easily analyzed by our method (Fig.1), even though it contained less than one percent of bryostatins 1 and 2 in a complex matrix. For pure bryostatin 1 and 2 detection limits are in the 1-10 ng/ml range.

The binding assay tolerates up to 50 μl ACN per ml of test solution, whereas protic solvents are less tolerated (e.g. MeOH < 10 μl/ml). We recommend removal of residual organic solvent from the mobile phase since this is conveniently achieved by a rapid exposure to reduced pressure. We have noted some interference with

column material eluted from reversed phase columns, and recommend that cartridges and columns be thoroughly solvent rinsed prior to use.

The combined PDBu displacement/HPLC assay characterizes elution peaks by retention time, UV spectrum and displacement activity resulting in improved peak identification. This is important when crude samples are analyzed which contain interfering compounds. The method is useful for the characterization of active compound(s) in crude samples, and has potential for rapid screening for bryostatins or other compounds.

HPLC analyses of crude extracts and fractions of B. neritina require concentration and purification steps in order to reduce sample loading and increase column life. The purification steps outlined in scheme I were found to be suitable for HPLC samples. The silica gel cartridge is most effective for mass reduction and the reversed phase cartridge traps compounds which do not elute from reversed phase packing material with 100% ACN. Analytical columns were used with precolumns, which were periodically replaced. The method described is used in our laboratory on a routine basis for the evaluation of crude samples derived from a large-scale extraction/isolation process.

Analysis of B. neritina samples collected in California during spring 1988 and December 1988 revealed bryostatin 1 concentrations of 6-14 μg/g dry weight. For interpretation of results several factors have to be taken in consideration: Some B. neritina extracts contain large quantities of orange and red pigments with chromatographic properties similar to those of bryostatin 1 which can interfere with integration results. Specimens contain highly variable amounts of sea sand, salt water, and other organisms; thus for comparative studies it is recommended that B. neritina samples be washed thoroughly with fresh water prior to lyophilization and extraction.

The sample clean-up procedure and the HPLC method described allow a fast determination of bryostatin 1 in crude samples. The combination of HPLC with the ^{3}H-PDBu displacement assay provides information about each separated peak and represents an important method for the detection of compounds responsible for PDBu displacement activity in crude samples.

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