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THE LARGE-SCALE ISOLATION OF BRYOSTATIN 1 FROM *BUGULA NERITINA* FOLLOWING CURRENT GOOD MANUFACTURING PRACTICES

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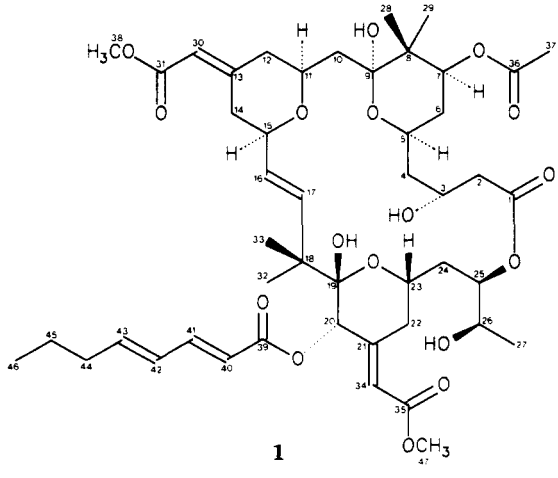
ABSTRACT.—A novel process was designed for the large-scale isolation of bryostatin 1 [**1**] from the bryozoan *Bugula neritina* L. in order to obtain multigram quantities of highly pure material for formulation studies, preclinical toxicology, and clinical trials in cancer patients. Multigram quantities of bryostatin 1 were obtained from a collection of approximately 10,000 gallons of wet animal. A phorbol dibutyrate (PDBu) receptor binding assay and hplc with photodiode array detection were used for the design, validation, and control of the isolation process.

Marine natural products represent a vast potential source of new drugs with diverse and often unique structures, many associated with interesting biological properties. A number of compounds with cytotoxic or antitumor properties have been identified from marine organisms.

One group of very promising compounds is the bryostatins, macrocyclic lactones isolated from the marine bryozoan *Bugula neritina* L. (Bugulidae). Bryostatin 1 [**1**] is



1

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one of the most abundant and certainly the best studied compound of this series (1,2). Its biological properties include cytostatic effects (1–3), binding to and activation of protein kinase C (4–6), stimulation of hematopoietic progenitor cells (7–9), activation of T-cells (10), and immunomodulating properties (11,12); recently, the compound has been shown to have activity against human leukemias in vitro (13,14).

This publication describes a large-scale isolation of multigram quantities of highly pure bryostatin 1 from its natural source. Because the purified compound was intended for use in humans, the compound was required by federal law to be processed following current Good Manufacturing Practices (cGMPs). The implications of production by cGMPs on planning and execution of the isolation process are discussed.

RESULTS AND DISCUSSION

Although the isolation of bryostatin 1 [**1**] has been reported (1,2), the reported process did not appear economic at the scale envisioned for production under cGMPs. The cost of materials, minimization of the number of steps, and the increased scale and throughput required were design factors that needed to be addressed. Our goal was to isolate 10 g of very pure bryostatin 1 from 10,000 gal of wet animal.

We report here a novel isolation scheme designed to maximize the recovery of bryostatin 1 as a bulk pharmaceutical from a crude extract of *B. neritina*. The purification of the crude extract was directed by hplc with photodiode array detection in combination with a ^{3}H-PDBu receptor binding assay (15). The bulk pharmaceutical was transferred to another facility for final formulation.

The EtOAc partition (see Experimental) served as the starting material for the isolation process in the pilot plant. A first large silica batch elution reduced the total mass and concentrated the bryostatins within a single fraction. Next, bryostatin 1 was separated from bryostatin 2 on a smaller silica step gradient of hexane/Me_2CO (2). The remainder of the purification steps were carried out within the laboratory. The bryostatin-1-containing fraction was subjected to reversed-phase flash chromatography on C18 prior to preparative hplc to increase column lifetime. After hplc, pigments were removed by chromatography on Sephadex LH-20. A final precipitation from hexane/Me_2CO yielded pure (>99% by hplc analysis) bryostatin 1 as an off-white powder. Table 1 summarizes these results.

The process development was organized into three stages. The overall isolation scheme and the analytical methodology used to support it were first developed in Phase I. Bryostatin 1 isolated in Phase I was compared to a reference standard and found identical. A portion of this isolated material served as a primary analytical standard for the remainder of the isolation process. Phase II consisted of scale-up of the process and the preparation of the cGMP documentation. The isolation of multigram quantities of pure

TABLE 1. Purification of Bryostatin 1 [**1**].

Step	Mass Range per Aliquot	Bryostatin (%) (hplc 265 nm)[a]	^{3}H PDBu Assay IC_{50} (ng/ml)[a]
EtOAc partition	~2 kg	~0.1%	1130
1. Batch elution	~800 g	~0.4%	530
2. Si gel	24–47 g	8%	11.3
3. RP-flash	11–18 g	17%	5.8
4. Preparative hplc	1.2–2.3 g	90%	1.6
5. Sephadex	1.0–2.0 g	99%	1.6
6. Precipitation	0.9–1.8 g	100%	1.2

[a]Numbers obtained from a representative aliquot.

material under cGMPs was conducted in Phase III. Four lots of pure bryostatin 1, with a total mass of 18 g, were produced from the 15 batches of crude extract. The production phase was completed in 10 months.

The isolation or synthesis of a drug intended for human use must be carried out under cGMPs (16). The guidelines were designed to avoid contamination, mix-ups, and errors during the manufacturing process and to ensure that the final drug product is pure, safe, and effective. The Food and Drug Administration (FDA) has the responsibility of ensuring that the pharmaceutical manufacturer complies with the cGMP regulations. The FDA also has the authority to initiate recalls or to suspend production if the facility is in violation of any cGMP regulations. The product itself does not have to be proven defective or imperfect, but is considered suspect if investigation showed the cGMP regulations were not followed.

We have demonstrated an efficient and economic large-scale process for the isolation of an important anticancer drug for clinical trials while complying with federal law. Our approach to compliance with these guidelines is unique, and we hope it will be beneficial to others interested in producing a drug for clinical studies.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Stainless steel column units were used for the first and second Si gel chromatographies. The first column (60 × 20 in. i.d.) was obtained from Lee Process Equipment Co. (Phillipsburg, PA) and the second column (60 × 12.5 in. i.d.) from Tubetec Co. (Sanford, FL). Other large-scale equipment included a 20-gal drying kettle with Teflon scraper blades (Lee Process Equipment Co., Phillipsburg, PA) and a wiped film evaporator (WFE, 12 ft^2, Pfaudler, Rochester, NY). An Eco (Rochester, NY) G-6 gear pump was used for solvent delivery. Pilot plant solvents were ACS grade (Brand Nu). Diatomaceous earth, Dicalite "Speedplus," was obtained from Grefco, Inc. (Torrance, CA). Si gel 60 Å (grade 22, 75–250 μm) was obtained from Davison Chemical (Baltimore, MD).

MeCN used for preparative hplc was non-spectro grade (Burdick & Jackson); otherwise, laboratory solvents were hplc grade (Burdick & Jackson). Matrex silica C18, 50 μm (Amicon Corp.) was used for reversed-phase flash chromatography. Sephadex LH-20 (25–100 μm) was obtained from Pharmacia.

ANALYTICAL HPLC.—Reversed-phase separations were performed as reported previously (15). This was designated as the standard method.

In addition to the standard method, a normal phase hplc method was used during the final product testing to verify the results of the reversed-phase analysis. Separations were performed on a Microsorb Si gel 3 μm, 100 × 4.6 mm i.d. column with a 15 × 4.6 mm i.d. precolumn (Rainin); the mobile phase was hexane–*n*-PrOH (9:1) at 1 ml/min.

THIN LAYER CHROMATOGRAPHY.—Samples were analyzed by tlc on Merck Si gel 60 F_{254} using hexane-Me_2CO (3:2) as a developing solvent. The plates were visualized under uv light (254 nm), sprayed with *p*-anisaldehyde reagent, and heated. *p*-Anisaldehyde reagent was prepared as a solution of MeOH, 40 ml; H_3PO_4, 85%, 10 ml (Mallinckrodt); and *p*-anisaldehyde, 1 ml (Aldrich).

^{3}H-PDBu DISPLACEMENT ASSAY.—A ^{3}H-phorbol dibutyrate (PDBu) receptor binding assay in conjunction with chromatographic techniques was used for the detection of bryostatins 1 and 2 in the crude extracts. In addition, this assay was used to guide the fractionation of the crude extracts during the methods development process (Phase I). The binding assay was also performed on final product samples from each lot produced to obtain IC_{50} values. A description of this assay and its coupling to the standard hplc method are described elsewhere (15).

COLLECTION AND EXTRACTION.—*Bugula neritina* specimens were collected in shallow waters off the coast of southern California during the spring of 1988 by Marinus, Inc. (Long Beach, CA). This collection consisted of approximately 10,000 gal, corresponding to an estimated damp weight of 28,000 lb of wet animal. A voucher sample was deposited with the Smithsonian Institution, Washington, DC; the voucher number is available upon request. Wet samples were stored in technical grade iPrOH at ambient temperature prior to extraction with MeOH. The combined iPrOH/MeOH extracts were concentrated and partitioned between EtOAc and H_2O. The extraction and EtOAc partitioning of *B. neritina* were performed by Hauser Chemical Research, Inc. (Boulder, CO). The EtOAc extract (130 gal) was concentrated under reduced pressure to a volume of 78 liters and was aliquoted into 15 batches (ca. 5.2 liters each) and

stored at −20°. Each aliquot of the crude extract was processed sequentially according to the isolation procedure described below.

FIRST SI GEL COLUMN.—An aliquot of EtOAc extract was resuspended in 18 gal of EtOAc and coated on 33 kg of Dicalite in a drying kettle. Residual solvent was removed under reduced pressure, and the dry, coated Dicalite was slurried in 20 gal of hexane and added to the top of a slurry-packed Si gel column (silica bed, 53 kg, 50–75 × 50 cm i.d.; total bed height 85–105 cm, 1 bed volume ca. 44–54 gal). The column was eluted with 3–4 bed volumes (150–160 gal) each of CH_2Cl_2 and EtOAc, followed by elution with 100 gal of MeOH. A flow rate of approximately 2.5 gal/min was maintained throughout the entire process. Bryostatins 1 and 2 were present in the EtOAc fraction, which was concentrated and coated on 9 kg of Dicalite.

SECOND SI GEL COLUMN.—The dry, coated Dicalite was suspended in 10 gal of hexane and added to the top of a slurry-packed Si gel column (silica bed, 18 kg, 50 × 30 cm i.d.; total bed height 75–90 cm, 1 bed volume ca. 14–18 gal). The column was eluted with a gradient of hexane-Me_2CO (90:10) 73 gal; (80:20) 91 gal; (70:30) 55 gal, and (50:50) 55 gal. Fractions of approximately 0.5–1.0 bed volumes were collected. Finally, the column was washed with 2–3 bed volumes of MeOH. The flow rate was kept between 0.75 and 1.5 gal/min.

Fractions were analyzed by hplc using the standard method. Bryostatin 1 eluted in the hexane-Me_2CO (80:20) fraction and bryostatin 2 eluted in the hexane-Me_2CO (70:30) and (50:50) fractions. Based on hplc results, fractions were pooled and concentrated under reduced pressure. The bryostatin-2-containing fraction was set aside, and the bryostatin-1-containing fraction was further purified as described below.

REVERSED-PHASE FLASH CHROMATOGRAPHY.—The bryostatin-1-containing fraction (24–47 g) was dissolved in 500 ml of MeOH-CH_2Cl_2 (1:1) and adsorbed on Matrex silica C18 using a ratio of sample to packing material of approximately 1:10 to 1:15 by weight. Additional Matrex silica C18, approximately half that used to adsorb the sample, was dry-packed into a 25 × 10 cm i.d. glass column. After drying in vacuo overnight, the coated material was dry-packed onto the top of the resin bed. The column was eluted with 2 bed volumes (1 bed volume was 1.0–1.4 liters) of MeOH-H_2O (1:1), 3 bed volumes of MeOH-H_2O (9:1), and finally 2 bed volumes of MeOH. Fractions of approximately 1 bed volume each were collected. Bryostatin 1 was eluted in MeOH-H_2O (9:1). The bryostatin-1-containing fractions were pooled, the solvents removed under reduced pressure, and the residue (11–18 g) dried in vacuo.

PREPARATIVE HPLC.—The crude fraction (11–18 g) containing bryostatin 1 was further purified by preparative hplc on a Waters Prep LC 3000 System with a Waters 600E System Controller and a Waters 484 Tunable Absorbance Detector set at 265 nm. Separations were performed on a Dynamax 60Å, 8 μm, C8, 250 × 41.4 mm i.d. column with corresponding precolumn (50 × 41.4 mm i.d., Rainin). The mobile phase was isocratic aqueous MeCN 75% at 60 ml/min. The sample was dissolved in aqueous MeCN 70% (final concentration 100 mg/ml). Approximately 1 g (10 ml) was loaded on the column per run, each run producing 70–75 mg of bryostatin 1. A representative chromatogram is shown in Figure 1. The bryostatin 1 fractions were collected separately and then pooled at the end. The solvents were removed under reduced pressure until a final volume of approximately 400–500 ml was obtained. The aqueous layer was diluted with an equal volume of H_2O and was back-extracted with CH_2Cl_2 (3× the volume of the aqueous layer). The CH_2Cl_2 layers were combined, the solvents were removed under reduced pressure, and the residue was dried to yield 1.0–2.3 g of a dark orange powder.

SEPHADEX COLUMN CHROMATOGRAPHY.—Sephadex LH-20 was used to remove most of the orange pigments from the bryostatin 1 fraction. Sephadex LH-20 (150 g) was soaked for a minimum of 4 h in CH_2Cl_2-hexane (3:1). A 45 × 5 cm i.d. glass column was slurry-packed with the Sephadex LH-20 and the bed washed with approximately 200 ml of mobile phase. The bryostatin-1-containing fraction was dissolved in a minimum amount of the mobile phase (10 ml), loaded on the column, and eluted with CH_2Cl_2-hexane (3:1). Fractions (10–40 ml) were collected and analyzed by tlc, and those containing bryostatin 1 were pooled, the solvents removed under reduced pressure, and the residue dried to yield a gold to light orange powder (0.9–2.1 g).

PRECIPITATION FROM Me_2CO/HEXANE.—Bryostatin 1 was obtained as an off-white powder (0.9–1.8 g) by precipitation (2×) from Me_2CO/hexane. Approximately a 1:5 to 1:10 ratio of Me_2CO to hexane was required.

The identity of bryostatin 1 was confirmed by ir, uv, ^1H and ^{13}C nmr (17), and the ^3H PDBu assay; its purity was defined by normal and reversed-phase hplc (>99%, detection at 265 and 220 nm); residual solvents by thermogravimetric analysis.

CURRENT GOOD MANUFACTURING PRACTICES.—To assure compliance with all aspects of the cGMP regulations in producing bryostatin 1 as a bulk pharmaceutical, a project team was established. The

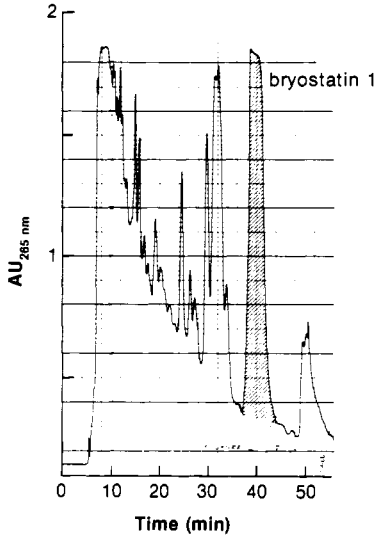

FIGURE 1. Preparative hplc of a bryostatin 1 fraction. Dynamax 60Å, 8 μm, C8, 250 × 41.4 mm i.d. with 100 × 41.4 mm precolumn; aqueous MecN 75%, 60 ml/min; sample: 1 g (70–75 mg bryostatin).

team was composed of the heads of each area representing Research and Development (R&D), Scale-up, Management, Quality Control (QC), and Quality Assurance (QA). The project team met regularly to establish production parameters and deadlines and to review batch records, deviation reports, and final product testing results. Problems which arose during R&D and production were addressed by the project team.

The cGMP documentation required for production of bulk pharmaceutical chemicals was prepared prior to Phase III isolation. Standard Operating Procedures (SOPs) were prepared to define each step in the isolation process as well as for routine laboratory procedures such as verification of cleanliness of equipment and equipment use. SOPs describing maintenance and calibration programs for equipment (i.e., hplc, balance) as well as proper equipment functioning were also necessary. SOPs were subjected to periodic review and updated if necessary.

Instructional Worksheets (IWSs) were prepared by Production prior to beginning Phase III. Worksheets were issued as controlled documents by QA for each step of the purification process. Each batch was assigned a lot number (based on month and year) by QA, and this lot number appeared on each IWS for that batch. The IWS describing each purification step must be strictly followed each time the procedure is repeated. Each completed step was signed and dated by the person who performed it. The completed IWS was reviewed by a supervisory scientist before becoming part of the Master File.

A separate room and the equipment within it were dedicated solely to the GMP process. Environmental monitoring of the area was performed on a regular basis to ensure that the quality of the product was not compromised. Routine cleaning and maintenance of the area were also required. These aspects of area control were described in SOPs, and performance of these procedures was documented in log sheets. Assurance that procedures were followed was accomplished by periodic audits conducted by QA.

The process developed for large scale isolation had to be shown to be functional as well as reproducible before it could be used routinely. The evaluation of the process is termed *validation*. The validation process allows Production to correct any major flaws or unforseen problems in the process. Due to the scarcity of the crude extract, the first three batches were designated as validation runs. With successful validation, the bryostatin obtained from each of these runs was pooled after the final precipitation and reprecipitated to obtain the first final product lot. The remaining batches were processed similarly, producing a total of four final product lots of equivalent purity.

All raw materials for the GMP process were tested and released to production by QC. Raw materials were obtained from approved vendors, and the vendors were required to provide Certificates of Analysis for their products. Representative samples from each manufacturer lot of a raw material (e.g., solvent, silica) were tested prior to use and were required to pass certain purity specifications agreed upon by the Project Team. All incoming raw materials were placed in quarantine until the testing was completed. Raw materials meeting specifications were released and approved for use in the GMP process. Any rejected raw mate-

rials could not be used. Separate storage areas were required for quarantined, rejected, and released raw materials. Each raw material container was labelled with a color-coded label that distinguished among these categories. The labels for the released material provided the following information: product, internal lot number, release date, and the name of the analyst. Samples of every lot of each component were reserved and stored for 3 years. Production was issued inventory cards and kept a balance of the material used. Released material had a one-year expiration date and after that time required retesting before use in the GMP process.

During the isolation process QC provided analytical support to Production. QC verified by hplc analysis the bryostatin 1 content of the fractions collected to ensure that the appropriate fractions were pooled for further processing. The final product of each production lot was tested by QC before release. Testing involved comparing the lot produced to the primary analytical standard. To be released, the product was required to pass specifications agreed upon by the Project Team prior to starting the GMP process.

The function of the QA unit was to ensure that the personnel, facilities, process, and records were in compliance with the cGMP regulations. QA maintained the original batch records, conducted audits, and was responsible for obtaining an establishment registration number from the FDA. All information pertaining to the isolation process was compiled within the Master File. The information contained within the Master File will be used to support an Investigational New Drug Application (IND), a New Drug Application (NDA), or amendments to either of these.

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