ISOLATION AND CHARACTERIZATION OF LYTECHINASTATIN[1]

GEORGE R. PETTIT,* JOHN A. RIDEOUT, JULIA A. HASLER, DENNIS L. DOUBEK, and PETER R. REUCROFT

Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, Arizona 85287

ABSTRACT.—The venomous marine animal *Lytechinus variegatus* (Lamarck) has been found to contain an antineoplastic glycoprotein. Separation of an aqueous extract of this Western Atlantic sea urchin by a series of macroreticular resin (or centrifugation) and gel permeation chromatographic sequences led to the new glycoprotein designated lytechinastatin.

The sea urchin family Toxopneustidae (Echinodermata phylum, Echinacea superorder) is well known for its venomous species, especially the lethal Western Pacific (Japan) *Toxopneustes elegans* and *T. pileolus* (2). The ubiquitous Caribbean (ranges from Brazil to North Carolina) *Lytechinus variegatus* (Lamarck), another toxic member of this family, also contains a pedicellarial (1) venom. In this case the venom was found to be dialyzable and presented an acetylcholine-type pharmacological response (3). Other biochemical studies of this species have been limited to the sperm ribosomal DNA (4–7), egg jelly coat (8), a tubulin-like protein [regulation of microtubule function (9)], and a secretion with fertilization-inhibiting properties (10).

Marine invertebrates such as *L. variegatus* have been of considerable interest to us during the past fifteen years (11) for evaluation as new sources of potentially useful cancer chemotherapeutic agents. For example, we recently described (1,12) the isolation of strongylostatins 1 and 2 from the venomous green sea urchin *Strongylocentrotus droebachiensis* (Müller). Strongylostatin 1 was found to be a glycoprotein of exceedingly high molecular weight ($\geq 4 \times 10^7$) which exhibited significant antineoplastic activity (35–53% life extension at 5–10 mg/kg dose levels) in the National Cancer Institute's (NCI) murine P388 lymphocytic leukemia (PS) *in vivo* system (13). Strongylostatin 2 proved to be a related, albeit lower molecular weight, glycoprotein with similar anticancer activity (1). In 1968 we began to investigate *L. variegatus* collected in the Gulf of Mexico (Apalachee Bay, Florida) and an ethanol extract reached the confirmed active level (PS T/C 150 at 400 mg/kg) in the NCI exploratory biological program. The antineoplastic activity (PS T/C 127 at 60 mg/kg) was next observed in an aqueous fraction obtained from a 1971 recollection. In subsequent recollections made in 1975 and 1977 the activity was found to again vary between the ethanol (1975) and aqueous (1977) extracts. Extensive efforts were made by employing bioassay (PS) and various separation techniques to locate the anticancer agent(s). Only the route (guided by PS *in vivo* bioassay) that proved most successful with the 1977 recollection has been summarized in the sequel.

An aqueous extract of the Caribbean *L. variegatus* was triturated with methanol, and the methanol insoluble fraction was dissolved in water and chromatographed on the macroreticular resin XAD-2. Three fractions (determined by uv monitoring) were eluted by water, and a fourth was obtained by combining material from aqueous methanol (1:1) and methanol elutions. The first three fractions all produced PS *in vivo* activity (the first was most active and displayed T/C 143 at 37 mg/kg), and the fourth was inactive. As the latter fraction amounted to <2% of the total weight, chromatography on XAD-2 was eventually found unnecessary and the following method proved to be more convenient. After dissolution in water and centrifugation, the methanol insoluble fraction was

[1]Contribution 77 of the series Antineoplastic Agents: refer to (1) for Part 76.

chromatographed on a column of Sephadex G-50. The excluded fraction obtained by eluting the column with water was rechromatographed on a Sephacryl S-200 column. The excluded fraction eluted by water showed PS *in vivo* activity (T/C 125 at 5 mg/kg). Other fractions from both the G-50 and S-200 columns were inactive against the PS *in vivo* system.

The active fraction from the Sephacryl S-200 column was next chromatographed on Sepharose 2B. Three fractions, as evidenced by uv detection, were eluted from this column. Only the first fraction was PS active (T/C 121-122 at 12-25 mg/kg). Since no other substance with better PS inhibitory activity was located, the high molecular weight glycoprotein obtained as the first Sepharose 2B fraction was designated lytechinastatin. Larger-scale isolations of lytechinastatin were performed as described above, except that the Sephacryl S-200 step was eliminated.

Lytechinastatin was obtained as a tan or beige fluffy powder, slightly soluble in water. Dissolution in water was facilitated by addition of a detergent (SDS). Gel permeation chromatography of lytechinastatin in 3M guanidine hydrochloride on an analytical column of Sepharose 2B gave evidence (by the use of molecular size indicators) that lytechinastatin corresponded to a maximum molecular weight of about two million. Since the elution curves from both the preparative (water as eluent) and analytical column were essentially identical, it was apparent that, if aggregation was occurring, 3M guanidine hydrochloride had no apparent effect on the process.

Trace metal analysis of lytechinastatin did not reveal any significant metal component. Elemental analyses for carbon, hydrogen, nitrogen, and sulfur gave results consistent with the range expected for a glycoprotein. A small amount of phosphorus (0.54%) was also detected. A carbohydrate analysis (14) showed 18% carbohydrate, based on a glucose standard. Amino acid analyses (15) indicated that Asx, Glx and Gly were the most abundant constituents.

While lytechinastatin appears to account for the relatively low level of PS *in vivo* activity experienced with the *L. variegatus* aqueous extracts, it may not be responsible for the higher inhibitory activity given by the ethanol extracts. Since the latter biological results were not reproducible (and lytechinastatin has not been evaluated above 25 mg/kg to a toxic dose), a future study would be required to answer such remaining questions.

EXPERIMENTAL[2]

ANIMAL COLLECTION.—The initial specimens and all recollections (except for the one noted below) of *Lytechinus variegatus* (Lamarck) preserved in 2-propanol were provided by Mr. Jack J. Rudloe from the Gulf of Mexico in or near Apalachee Bay, Florida. The first collection was made in July 1968 and yielded an ethanol extract with confirmed PS *in vivo* activity (T/C 150 at 400 mg/kg). A 25 kg (wet weight here and in the sequel) recollection received in May 1971 gave an ethanol extract that led (solvent partitioning) to a water-soluble fraction with T/C 127 at 60 mg/kg. In December 1975, a 43 kg recollection was supplied by Dr. R. E. Schroeder from Niles Channel and Newfound, Florida. The ethanol extract of this collection was PS active (T/C 148 at 45 mg/kg). A December 1977 recollection received from Mr. Rudloe amounting to about 250 kg was used to finally isolate lytechinastatin as reported herein. The 1977 collection did not give a PS active ethanol extract, but did give an active (T/C 131 at 15 mg/kg) aqueous extract.

ANIMAL EXTRACTION.—The December 1977 recollection was received in two 55 gallon barrels (approximately 250 kg of wet animal) containing 2-propanol as preservative. The 2-propanol was decanted and filtered. The *L. variegatus* was removed, crushed, and wrapped (in about 2 kg batches) in muslin cloth and placed in a modified (stainless steel) Soxhlet extraction

[2]Distilled water was employed for all chromatographic procedures. The Sephadex G-50, Sephacryl S-200 superfine, Sepharose 2B and special columns were obtained from Pharmacia Fine Chemicals. Amberlite XAD-2 was supplied by Rohm and Haas Co. Column eluates were collected with a Gilson FC-80 microfractionator and fractions were monitored with a Gilson Holochrome spectrophotometer (uv).

Amino acid analyses were performed by Dr. John R. Cronin and Dr. Ann Yates using a model 121 Beckman-Spinco amino-acid analyzer according to procedures (15) that do not detect cysteine and tryptophan. Carbohydrate content was determined by the phenol-sulfuric acid method (14). Microanalyses were provided by Spang Microanalytical Laboratory, Eagle Harbor, Michigan. Trace metal analyses were obtained by Dr. M. J. Parsons employing a Jarrell-Ash 3.4M spectrograph.

apparatus. The animals were extracted with ethanol for 48 hr, followed by water for 48 hr. After removal of the solvents by distillation *in vacuo* and lyophilization of the residues to completely remove solvent the following extracts were obtained: 2-propanol, 5.3 kg; ethanol, 186 g; water, 308 g.

ISOLATION OF LYTECHINASTATIN.—A 10 g specimen of the aqueous extract was triturated with methanol (2 x 300 ml) for 72 hr (total). The methanol-insoluble fraction (5.9 g) was dissolved in water (50 ml), the solution was centrifuged (1 hr) at 37,000 g to remove suspended particles that would otherwise block (and substantially reduce the flow rate) the inlet filter of a Pharmacia column, and the supernatant solution was carefully decanted. The residue was washed with a small volume of water, recentrifuged at 37,000 g for 1 hr, and the wash solution was carefully decanted and combined with the initial supernatant solution. By this means it was possible to eliminate an XAD-2 chromatographic step (elution with water followed by water-methanol and methanol) that was originally employed at this stage of the separation. Next, the supernatant solution from the centrifuge procedure was applied to a column (5 x 75 cm) of Sephadex G-50, and the column was developed with water. A 1.17 g amount (0.21 g prepared as above and the remainder by repeating the procedure) of the excluded band recovered by evaporation of the water (*in vacuo*) was dissolved in water (50 ml) and applied to a column (5 x 75 cm) of Sephacryl S-200 superfine. The first band (0.70 g, light-brown void volume fraction) eluted by water was found to contain the PS *in vivo* active (T/C 125 at 5 mg/kg component. A portion (0.25 g) of this fraction was chromatographed in water (30 ml) on a column (5 x 75 cm) of Sepharose 2B to yield three fractions (monitored with a uv detector). After desalting (a Bio-filter 80 beaker was used) and subsequent lyophilization, the first fraction yielded the antineoplastic (PS T/C 121–122 at 12–25 mg/kg) glycoprotein lytechinastatin (83 mg) as a tan or beige fluffy powder slightly soluble (0.6 mg/ml) in water upon remaining 1 hr at room temperature.

In a large-scale isolation of lytechinastatin, the aqueous extract (60 g) was triturated with methanol, the insoluble portion was dissolved in water (400 ml) and centrifuged at 37,000 g, and the supernatant solution was chromatographed with a Pharmacia K100/100 column (7 liter volume) packed with Sephadex G-50. The excluded band (3.8 g) was finally chromatographed (as described above) on Sepharose 2B. However, the yield (0.15 g) of lytechinastatin was reduced by this simplified procedure. Based on the weight of aqueous extract employed, this corresponds to a yield of 0.25% (by weight) of lytechinastatin. Because of lytechinastatin's molecular size, polyacrylamide gel electrophoresis could not be used to evaluate the extent of purification achieved by these methods.

CHARACTERIZATION OF LYTECHINASTATIN.—An analytical column (1 x 40 cm) was prepared containing Sepharose 2B in 3M guanidine hydrochloride. The fractionation range of this column was calibrated for upper and lower limits by chromatography of molecular weight markers (monitored with a uv detector). Particles removed from the methanol insoluble portion of the aqueous extract by centrifugation proved to be a convenient marker for the exclusion limit (2×10^7 for carbohydrate) and column void volume. Low molecular weight material from the G-50 columns (see above) provided a useful marker for the lower end of the fractionation range (10^5) and total bed volume. Chromatography of lytechinastatin (1 mg) on this column produced an elution curve which fell between the two calibrated limits. Assuming that lytechinastatin obeyed the usual linear relationship between elution volume *vs.* molecular weight (and that it behaved as a carbohydrate rather than a protein) it corresponded to a maximum molecular weight of approximately 2×10^6. Lytechinastatin was found to be a glycoprotein (18% carbohydrate, based on a glucose standard, 14) and amino acid analyses (15–17) showed the following composition: Ala 9.36, Arg 3.41, Asx 12.15, Glx 11.24, Gly 10.72, His 1.44, Ile 5.47, Leu 7.49, Lys 4.33, Met 0.59, Phe 3.96, Pro 5.69, Ser 6.13, Thr 7.79, Tyr 2.49, Val 7.73 in mole % amino acid.

Anal. found: C, 45.05; H, 6.52; N, 5.25; S, 1.68; P, 0.54. No significant amounts of any metal were detected.

The composition of 5.25% nitrogen corresponds to a protein content of about 33% (a nitrogen content of 100% protein was approximated to be 16%). The results of a Lowry protein assay indicated 27% protein.

ACKNOWLEDGMENTS

For support of this investigation, we are pleased to thank the National Cancer Institute (performed pursuant to Contract NO1-CM-97262 with the Division of Cancer Treatment, NCI, National Institutes of Health, DHW), and the National Cancer Institute, DHW, for Grants Nos. CA-16049-01 through 07; the Fannie E. Rippel Foundation, Mrs. Mary Dell Pritzlaff, and the Olin Foundation (Spencer T. and Ann W.). We also wish to thank Messrs. T. S. Krupa and L. D. Vanell for technical assistance, and Drs. J. Douros and M. Suffness for other valuable assistance.

Received 4 May 1981

LITERATURE CITED

1. G. R. Pettit, J. A. Hasler, K. D. Paull and C. L. Herald, *J. Nat. Prod.*, **44**, 701 (1981).
2. B. W. Halstead, *Poisonous and Venomous Marine Animals of the World, 1*, U. S. Government Printing Office, Washington, D.C., 1965, p. 549.
3. E. G. Mendes, L. Abbud and S. Umiji, *Science*, **139**, 408 (1963).
4. N. K. Mishra, *Experientia*, **35**, 1161 (1979).
5. F. E. Wilson, N. Blin and D. W. Stafford, *Chromosoma (Berl.)*, **65**, 373 (1978).
6. N. K. Mishra, *Molec. Biol. Rep.*, **4**, 241 (1978).

7. J. M. Pollock, Jr., M. Swihart and J. H. Taylor, *Nucleic Acids Res.*, **5**, 4855 (1978).
8. G. K. SeGall and W. J. Lennarz, *Developmental Biol.*, **71**, 33 (1979).
9. R. W. Rubin, R. Kahn and J. J. Prior, *Tissue and Cell*, **8**, 209 (1976).
10. R. E. Daniel, *Trans. Kentucky Acad. Sci.*, **28**, 53 (1967).
11. G. R. Pettit, Y. Kamano, Y. Fujii, C. L. Herald, M. Inoue, P. Brown, D. Gust, K. Kitahara, J. M. Schmidt, D. L. Doubek and C. Michel, *J. Nat. Prod.*, **44**, 482 (1981).
12. G. R. Pettit, C. L. Herald and L. D. Vanell, *J. Nat. Prod.*, **42**, 407 (1979).
13. R. I. Geran, N. H. Greenberg, M. M. Macdonald, A. M. Schumacher and B. J. Abbott, *Cancer Chemother. Rep., Part 3*, 3, No. 2 (1972).
14. R. G. Spiro, "Methods in Enzymology," Vol. 8, Academic Press, New York, N.Y., 1966, pp. 93–95.
15. D. H. Spackman, W. H. Stein and H. More, *Anal. Chem.*, **30**, 1190 (1958).
16. R. W. Hubbard and D. M. Kremen, *Anal. Biochem.*, **12**, 593 (1965).
17. J. V. Benson, Jr. and J. A. Patterson, *Anal. Chem.*, **37**, 1108 (1965).