ISOLATION AND CHARACTERIZATION OF PALYSTATINS A–D[1]

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ABSTRACT.—Four new and relatively low molecular weight (~3,000–5,000), cell growth (PS) inhibitory peptides designated palystatins A–D have been isolated from the Indian Ocean *Palythoa liscia* Haddon and Duerden. The isolation utilized chromatographic procedures (Amberlite XAD-7 to Sephadex G-100) guided by bioassay with the P388 murine lymphocytic leukemia (PS system). Palystatins A–D exhibited PS cytotoxicity in the range ED_{50} 0.02–0.0023 μg/ml.

An eight year investigation (1) of the Western Indian Ocean (Mauritius) *Palythoa liscia* Haddon and Duerden directed at isolating and characterizing the antineoplastic and/or cytotoxic constituents has led to the discovery of three cell growth (*in vitro* P388 murine lymphocytic leukemia, the PS system) inhibitory proteins ranging in mass from 128,000 to $>2 \times 10^7$. The present study was initiated to uncover the substance(s) responsible for PS cell growth inhibition shown by certain lower molecular weight fractions from an ethanol extract of this *Palythoa sp.*

As noted earlier (1) we were concerned that our separation guided by the PS *in vitro* cell line might lead to palytoxin (2), a prominent toxin of *Palythoa toxica*, *P. caribaeorum*, *P. mammilosa*, and *P. tuberculosa*.[2] Palytoxin has presented (3, 4) an intravenous lethality from LD_{50} (μg/kg) 0.45 (mouse, lateral tail vein) to 0.033 (dog, median cubital vein) and 0.078 (monkey, saphenous vein) and is one of the most toxic compounds known. Indeed, palytoxin by intraperitoneal injection is sixteen times more potent than tetrodotoxin (5). Except for certain lower plant proteins such as the botulins, diphtheria, and tetanus toxins, and the higher plant ricin, it is the most toxic substance presently known (6). Also palytoxin has proved to be effective against the murine Ehrlich ascites carcinoma (7) and corresponds to the relatively low mass 2681 (8). The fractions from *Palythoa liscia* that appeared most promising were those from Sephadex G–50 gel permeation chromatographic steps that appeared to be in the 3,000–5,000 molecular weight range. Pressing ahead with bioassay-directed separation of these fractions fortunately afforded, instead of the nonpeptide (8, 9) palytoxin, four new and low molecular weight (~3,000–5,000) cell growth inhibitory peptides named palystatins A–D. A summary of the isolation and preliminary characterization procedures now follows.

Although our chemical study of *Palythoa liscia* began in 1973 and a variety of methods were pursued to isolate palystatins A–D, the two most workable solutions were realized with recollections made in 1975 and 1978. In both cases the animals were extracted with ethanol, and the extract was partitioned between methylene chloride-water. The methylene chloride fraction was successively partitioned between (9:1→4:1→3:2) methanol-water and ligroin→carbon tetrachloride→methylene chloride as previously described (1). By this means PS *in vitro* activity was concentrated in the 3:2 methanol-water fraction.

Further concentration of the PS cytotoxicity by chromatography on Sephadex LH–20 (2:3 methylene chloride-methanol as eluant) followed by Sephadex G–50 (water or Tris hydrochloride buffer at pH 7) gave a high molecular weight (excluded) fraction which, when finally separated as already reported (1), provided palystatins 1–3. A 1975 lower mass G–50 fraction selected for detailed separation

[1]Antineoplastic Agents 79; For part 78 refer to (1).
[2]For leading references consult (1).

was chromatographed in water on a macroreticular resin (Amberlite XAD-7). The active methanol fraction was further purified by a gel permeation sequence using Sephadex G–50 (water) and G–100 (99.5:0.5 water-*n*-butanol). An active Sephadex G–100 fraction was separated on the ion exchange resin DEAE–Sephadex A–50 (linear gradient of sodium chloride) to afford palystatins A–D.

With the 1978 lower mass G–50 fraction, the isolation of palystatins A–D was again simplified by going directly to Sephadex G–100 (water) chromatography, to remove high mass components, followed by careful separation on Sephadex G–50 (water). When Tris hydrochloride buffer (pH 7.0) was employed as eluant for the final step, palystatins A–D formed a single chromatographic peak and were not separable.

Amino acid and carbohydrate analyses of palystatins A–D indicated that A and B are glycopeptides and C and D related peptide conjugates. Molecular weight determinations (by gel permeation) led to estimated masses of 4,500, 4,000, 3,300, and 3,000, respectively, for palystatins A–D. Since these substances are well within the range of amino acid sequential analyses, eventual structural elucidation may be feasible and should substantially add to our knowledge of structural requirements for inhibitory growth of the PS cell line. The structures of palystatins A–D may be of use eventually in the design of small peptides (10, 11) with antineoplastic and/or cytotoxic properties. Also, the discovery of palystatins 1–3 (1) and A–D illustrates the splendid versatility of *Palythoa liscia* in biosynthesis of potentially useful cell growth inhibitory substances. Obviously a good number of such compounds remain to be discovered among the *Palythoa*, related *Zoanthidae* (*Zoanthus* and *Isaurus*) and *Epizoanthidae* (*Epizoanthus* and *Parazoanthus*).

Presently, palystatins A–D have been evaluated by the PS *in vitro* system and have exhibited inhibition (ED_{50} μg/ml) at 0.0023 (A), 0.020 (B), 0.0018 (C) and 0.022 (D). The National Cancer Institute is now exploring the effects of palystatins A–D on selected *in vivo* tumor systems. In the initial results, palystatin A showed a 22% life extension at a dose of 150 μg/kg (a toxic dose was not recorded up to 600 μg/kg), and palystatin B showed a 32% life extension (at a dose of 300 μg/kg and 22% at 80 μg/kg) against the PS *in vivo* leukemia. In sharp contrast, palystatin C displayed only toxicity down to the lowest dose (100 μg/kg) used while palystatin D showed no PS *in vivo* activity to the highest dose (350 μg/kg) employed.

EXPERIMENTAL

GENERAL METHODS.—All solvents employed for chromatography were redistilled. The Sephadex LH–20, G–50, and G–100 used for gel permeation chromatography and the DEAE–Sephadex A–50 were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Rohm and Haas Co. supplied the macroreticular resin Amberlite XAD–7. Gilson FC–220 racetrack and FC–80 micro-fractionators equipped with the Gilson HM UV–Visible Holochrome detector (280 nm) were utilized for careful chromatographic fractionation. Thin-layer chromatography (tlc) was performed with silica gel GF Uniplates obtained from Analtech, Inc. The tlc plates were visualized by spraying with concentrated sulfuric acid followed by heating. Ultraviolet spectra were recorded on a Hewlett-Packard 8450A UV/VIS spectrophotometer and recorded on a HP 7225A plotter.

Amino acid analyses were provided by Drs. J. R. Cronin and A. M. Yates. Trace metal analyses were obtained by Dr. M. L. Parsons using a Jarrell-Ash Model 3.4M spectrograph. Elemental microanalyses were determined by Galbraith Laboratories, Inc., Knoxville, Tennessee.

ANIMAL COLLECTION AND EXTRACTION.—The Western Indian Ocean *Palythoa liscia* Haddon and Duerden (Coelenterata phylum, Anthozoa subphylum, Zoantharia class, Zoanthiniaria order and Zoanthidae family) was first collected in September and October, 1972, at Tombeau Bay, Mauritius. A definitive taxonomic study was nicely completed by Prof. C. E. Cutress (University of Puerto Rico), and a specimen has been deposited with the Smithsonian Institution. Later recollections of this colonial Zoanthiniarian were made in the same location. Ethanol extracts of the whole animal from 1975 (25 kg wet weight) and 1978 (125 kg wet weight) recollections described previously (1) formed the starting point for experiments reported herein.

PRELIMINARY SEPARATION.—The 1975 and 1978 ethanol extracts (835 g and 4.52 kg) were partitioned between methylene chloride-water. To further separate the methylene chloride fraction, the 9:1→4:1→3:2 methanol-water with ligroin→carbon tetrachloride→methylene

chloride partition sequence used earlier was employed (1). Generally chloroform was employed prior to 1976, and that variation was performed with the 1975 extract. Whereas the 1978 extract was treated as just noted with the safer methylene chloride replacing chloroform. In both cases PS *in vitro* activity was concentrated in the 3:2 methanol-water fraction (33 g and 44 g), and further concentration of 19 g (1975) and 30.5 g (1978) samples was effected, as before (1), by chromatography on Sephadex LH-20 (2:3 methylene chloride-methanol as eluant) followed by Sephadex G-50 (water or 0.02 M Tris hydrochloride buffer at pH 7.0 containing 0.5 M sodium chloride).

PURIFICATION OF AMBERLITE XAD-7.—Commercial Amberlite XAD-7 was placed in a column with an aqueous slurry. The resin was washed successively with five bed volumes of water and five of methanol and allowed to remain at room temperature 3 hr. The washing process was continued with four bed volumes of acetone, six of methanol and, finally, with twenty of water.

ISOLATION OF PALYSTATINS A-D. *Method A.* Fractions (7 ml each) 77-240 prepared by the Sephadex G-50 chromatography (using a 3.9 g fraction from the Sephadex LH-20 separation in water) procedure (see above) were combined and lyophilized to yield 3.6 g of brown powder. A solution of this fraction in water was chromatographed on a column (2x45 cm) of Amberlite XAD-7. Elution with water was continued until the effluent was colorless and then with methanol until colorless. Evaporation of the solvent from the methanol fraction gave a tan powder (0.755 g) that was rechromatographed in water on a column (5x120 cm) of Sephadex G-50. The first 700 ml of water collected was discarded, and chromatography was continued with 8 ml fractions. Combination and lyophilization of fractions 43-103 provided a 0.15 g (PS ED_{50} <0.01 μg/ml) residue that was further separated on a column of Sephadex G-100 (2.6x87 cm) with water-*n*-butanol (99.5:0.5) as eluant. After elution of 150 ml of this solvent, 5.6 ml fractions were collected. Fractions 19-39 were combined and lyophilized to afford 74 mg (PS ED_{50}<0.001 μg/ml) of yellow powder. Final purification was achieved by ion exchange chromatography (6 ml fraction) on a column of DEAE-Sephadex A-50 (1x43 cm, particle size 40-120 μ) using a linear gradient (500 ml of water to 500 ml of 1 M sodium chloride). Fractions 27, 29 and 30 were desalted (in water) on a column (1x30 cm) of Amberlite XAD-7 and eluted with methanol. By this means palystatins A (6 mg PS ED_{50} 0.00078 μg/ml), B, (8 mg PS ED_{50} 0.0097 μg/ml) and C (5 mg PS ED_{50} 0.1 μg/ml) were obtained as tan amorphous powders from DEAE-Sephadex A-50 fractions 27, 29 and 30, respectively. Palystatin D was not isolated by this method but by the improved method B.

Method B. A 3.1 g amount of the PS *in vitro* active Sephadex LH-20 fraction from the 1978 recollection was chromatographed on Sephadex G-50 in 0.02 M Tris hydrochloride buffer (pH 7.0) containing 0.5 M sodium chloride. After elution of 817 ml, the next series of fractions corresponding to 838 ml were combined, desalted (Sephadex G-10), and lyophilized; 0.644 g of very active (PS ED_{50} $<10^{-2}$ μg/ml) brown powder[3] was obtained. The 0.644 g fraction was dissolved in 20 ml of water and applied to a column of Amberlite XAD-7 (2x57 cm). Elution of the column with water proceeded until the effluent was colorless; after lyophilization, 0.31 g of PS *in vitro* inactive material was obtained. Subsequent elution of the column with methanol until the effluent was colorless yielded 0.24 g of brown powder (PS ED_{50} $<10^{-2}$ μg/ml). The 0.24 g fraction was dissolved in 8.0 ml of 0.02M Tris hydrochloride buffer (pH 7.0, 0.5 M sodium chloride) and applied to a column of Sephadex G-100 (2.5x88 cm). Fractions 15-46 were collected (7.0 ml each, after 126 ml of void volume was discarded), desalted (Sephadex G-10) and lyophilized; 0.13 g of tan powder (PS ED_{50} $<10^{-2}$ μg/ml was obtained). Careful Sephadex G-50 (2x98 cm column) chromatography of this fraction (40 mg portion) in water and monitoring by uv absorbance at 280 nm afforded (137 ml of void volume were discarded and 7.0 ml fractions were collected), palystatins A (fractions 1-20, 8.5 mg, PS ED_{50} 0.0023 μg/ml); B (fractions 21-27, 3.6 mg, PS ED_{50} 0.020 μg/ml); C (fractions 28-33, 7.5 mg, PS ED_{50} 0.0018 μg/ml) and D (fractions 34-36, 7.5 mg, PS ED_{50} 0.022 μg/ml). The yield of palystatins A-D was 0.2-0.3% of the methanol-water (3:2) fraction. Application of water as eluant in the final purification step allowed circumvention of a subtle pitfall. When the Tris hydrochloride buffer (see above) was used as eluant, palystatins A-D were found to comprise the same chromatographic peak.

Molecular weights of the palystatins were estimated by gel filtration on Sephadex G-25 (1.6x30 cm) with Tris hydrochloride buffer (see above) as eluant (fig. 1). Elution volumes were 22.5 ml, 26.3 ml, 29.5 ml and 35.8 ml, respectively, for palystatins A-D and corresponded to approximate molecular weights of 4,500 (A), 4,000 (B), 3,300 (C) and 3,000 (D). Because of the molecular weight similarity with that of palytoxin, authentic specimens[4] of this substance were obtained and compared by tlc (with 7:7:6 pyridine-*n*-pentanol-water as mobile phase).[5] The palystatins A-D were found to move somewhat faster on silica gel, give a pale orange color during development of the plate with sulfuric acid, and display much weaker and completely different ultraviolet absorption spectra (fig. 2)[6] than palytoxin (fig. 3)[7].

[3]When water was used as eluant for this procedure a major portion of the high molecular weight components of the LH-20 fractions were retarded by the Sephadex G-50 and were eluted with the lower mass 0.644 g fraction.

[4]We wish to thank Professors Y. Hirata and P. J. Scheuer for these specimens of palytoxin.

[5]Thoughtfully suggested by Professor Y. Hirata in a private communication.

[6]Palystatins B-D gave ultraviolet absorption curves essentially identical to that shown for palystatin A in Fig. 2.

[7]While the present contribution was in press, the marvelously complex and heretofore elusive polyalcohol structure of palytoxin was reported by Moore (14) and Hirata (15)

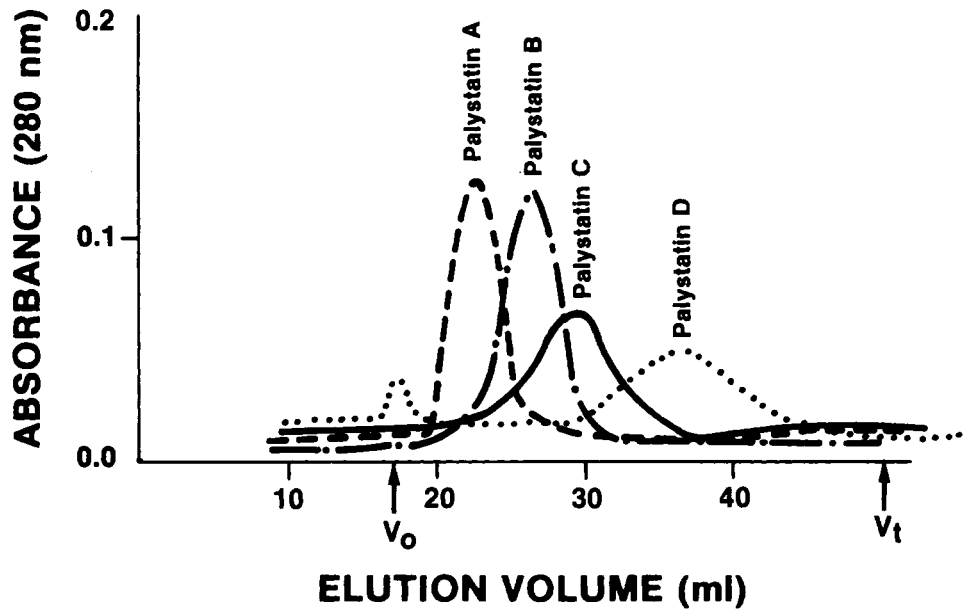

FIG. 1. Molecular weight estimations of palystatins A–D by gel permeation chromatography (0.25–0.5 mg sample in 0.25 ml of buffer applied separately to Sephadex G–25).

Amino acid analyses (for the procedure, consult table 1 and for pertinent references consult reference 1) revealed that palystatins A–D were peptides. The Lowry method (12) indicated 62%, 75%, 10% and 12% peptide, respectively, for palystatins A–D. Elemental analyses (see below) for nitrogen, if reasonably accurate, suggested a lower percent of peptide for palystatins A and B and somewhat higher values for palystatins C and D. The results of carbohydrate analyses by the phenol-sulfuric acid method (13) showed 10% carbohydrate for palystatins A and B and 3% for palystatins C and D. Metal analyses were essentially negative as only trace (<0.01%) quantities of aluminum, calcium, copper, iron, magnesium, manganese, and sodium were detected.

Elemental analyses gave the following results: Palystatin A; Anal. found: C, 51.05; H, 6.43; N, 5.41. Palystatin B; C, 47.23; H, 6.10; N, 5.80. Palystatin C; N, 2.62. Palystatin D; N, 2.25. Carbon and hydrogen analyses for palystatins C and D proved to be erratic, and insufficient amounts of each substance were available to reach a definite conclusion.

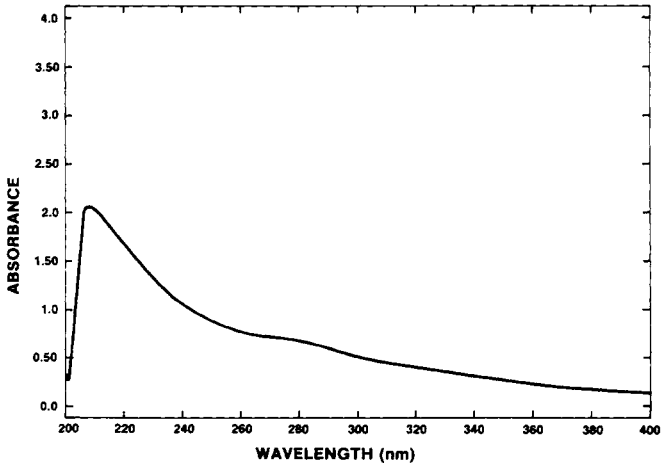

FIG. 2. Ultraviolet spectrum of palystatin A (0.2 mg/3 ml of 0.02 M Tris hydrochloride buffer (pH 7.0) containing 0.5 M sodium chloride.

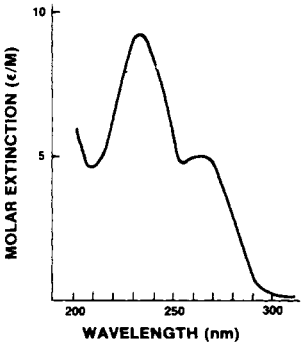

FIG. 3. Ultraviolet spectrum of palytoxin in water (2).

TABLE 1. Amino acid compositions (Mole %) of palystatins A–D.

Amino Acid	A	B	C	D
Ala	8.4	4.9	5.0	5.6
Arg	1.8	2.7	3.9	4.1
Asx	8.7	7.3	5.1	5.7
Cys	2.6	1.0	—	—
Glx	6.7	6.8	9.6	7.5
Gly	14.7	15.6	20.0	19.2
His	0.7	0.8	0.6	0.5
Ile	5.2	6.5	5.3	4.8
Leu	7.3	8.4	6.0	6.4
Lys	3.1	3.2	5.8	6.0
Met	2.1	2.2	1.8	1.5
Phe	4.3	4.4	4.7	5.1
Pro	13.2	16.8	12.0	11.6
Ser	6.5	4.8	6.4	6.5
Thr	6.0	5.5	4.2	5.4
Tyr	2.4	2.4	3.6	3.5
Val	6.4	7.0	6.0	6.6

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