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ISOLATION OF DOLASTATINS 10-15 FROM THE MARINE MOLLUSC DOLABELLA AURICULARIA[1a,b]

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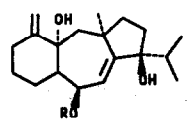
ABSTRACT. A twenty year pursuit of the cell growth inhibitory and antineoplastic constituents of the Western Indian Ocean (Mauritius) sea hare *Dolabella auricularia* has resulted in the discovery of fifteen structurally unique peptide, cyclopeptide, depsipeptide, and cyclodepsipeptide type-substances designated dolastatins 1-15. Solution of the difficult isolation problems leading to discovery of dolastatins 10-15 in 10^{-6} to 10^{-7}% yields required 1,600 kg of *Dolabella auricularia*. To date, this represents the largest scale separation of sea hare components. Of these dolastatin 10 (4) has displayed unprecedented potency in experimental antineoplastic and tubulin assembly systems. Dolastatin 15 (9), and to a lesser extent dolastatin 14 (8) were also found to exhibit unusually strong antineoplastic activity. Both dolastatins 10 and 15 are in advanced preclinical development. Details of the isolation strategies and structural summaries for dolastatins 10-15 have been recorded.

The Aplysiomorpha, Nudibranchia and Sacoglossa constitute the three largest orders of opisthobranch[2] sea slugs (shell-less marine molluscs[3]). Species of aplysiomorphs such as *Aplysia*[4] and *Dolabella*[1,5] feed upon marine algae. The herbivorous Sacoglossa even have the ability to cultivate in their digestive glands ingested chloroplasts and then survive for weeks at a time on solar radiation and dissolved atmospheric gases. In contrast, the nudibranchs[6,7] are carnivorous and free swimming. The dorid nudibranchs[8,9] are even capable of consuming bryozoans, ascidians, acorn barnacles, sponges and tunicates. Such varied dietary selections in turn serve as useful sources of potent compounds for devising powerful chemical defences for these soft bodied and slow moving animals

In 1965-66 we began a systematic and geographically broad evaluation of marine organisms as potential new sources of anticancer drugs representing unprecedented structural types.[10] Early in these investigations certain opistobranch species[11] were found especially promising, most notably the Indian Ocean (Mauritius) sea hare, *Dolabella auricularia*.[12-19]



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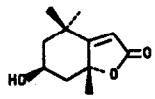

Loliolide
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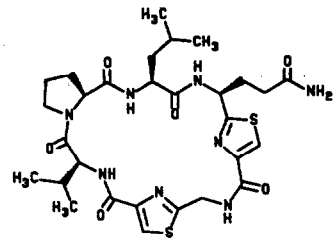

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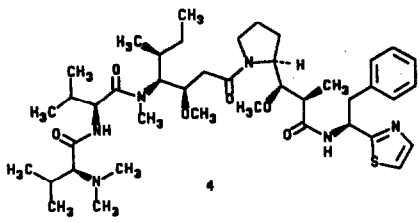

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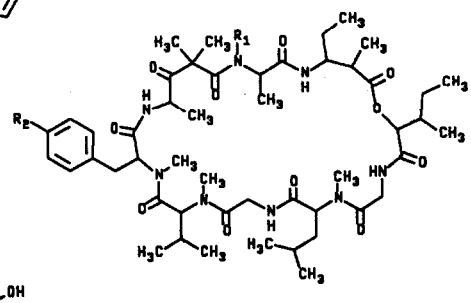

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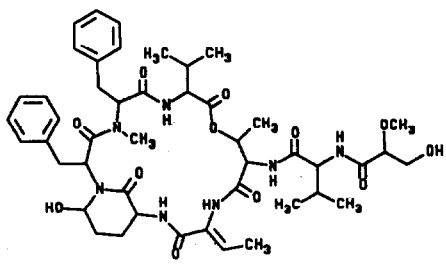

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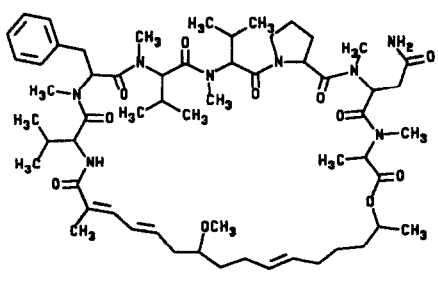

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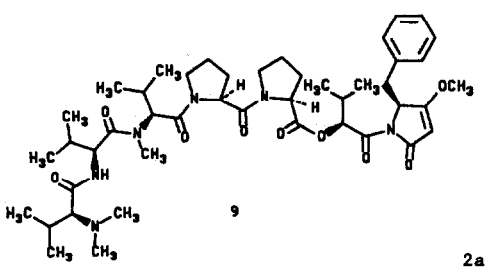

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2a

The sea hare yielded extracts that proved unusually effective (over 100% increase in life span) in the U.S. National Cancer Institute's (NCI) murine P388 lymphocytic leukemia (PS system).[20] Initial isolation studies guided by bioassay using the PS cell line led to the cell growth inhibitory dolatriols[12] (1a) and loliolide.(2)[21] By 1980 we succeeded in isolating nine new (and powerful) antineoplastic and/or cell growth inhibitory peptides designated dolastatins 1-9.[19] Of these, dolastatin 1 was found to be the most active antineoplastic substance. Because of the potency of the dolastatin series the sea hare seems to accumulate only vanishingly small quantities (about 1 mg each from 100 kg), making isolation and structural elucidation of these peptides exceptionally challenging. Due to the small amounts available and sensitivity to decomposition, the more stable dolastatin 3 (3) was selected for detailed structural elucidation.[22,23]

With the P388 leukemia bioassay indicating that additional dolastatins would contain the most powerful antineoplastic activity, all further effort was concentrated on reaching this objective. Increasingly larger recollections of *D. auricularia* failed to provide enough starting material to locate and characterize the next and most extraordinarily potent member of the peptide series, dolastatin 10, until success was realized in 1984 with a 1,600 kg recollection made in 1982. The final isolation procedure (evaluating some 20,000 fractions) found to be most useful has been summarized in Separation Scheme, Parts 1- 5. By 1987[24] we were able to report a structure for the exceptionally potent pentapeptide dolastatin 10 (4). That event was followed (refer to Separation Scheme) using analogous isolation techniques by discovery of the cyclodepsipeptide dolastatins 11 (5), 12 (6),[25] 13(7),[26] 14 (8)[27] and the depsipeptide dolastatin 15 (9).[28] Rigorous applications of high resolution EI, FAB and tandem mass spectrometry, combined with results of extensive two-dimensional ^{1}H- and ^{13}C-NMR, nOe and HMBC NMR experiments, allowed structural assignments[24-28] for dolastatins 10-15. None of these complex peptides could be induced to crystallize which precluded confirmation of the structure determinations by crystallographic techniques. However, the structures of dolastatins 10[29-31] and 15,[32] including stereochemical assignments, were substantiated by total syntheses. The principal evidence for the dolastatin 10-15 structures was presented in a series of communications[24-29,32] and does not require further elaboration. Some of these structures (cf., dolastatins 11 and 12) suggested a possible dietary source among the blue-green algae but the actual genesis of dolastatin 10 and related novel peptides bearing hitherto unknown amino acids will have to await interpretative biosynthesis studies.

As readily ascertained from inspection of the Separation Scheme and the experimental details, dolastatin 10 has, heretofore, been one of Nature's best kept secrets. The activity of dolastatin 10 against key NCI human cancer cell lines, corresponding human xenografts and murine cancer systems proved to be quite remarkable (see Tables 1 and 3). Biological evaluations of dolastatin 15 and, to a somewhat lesser extent, of dolastatin 14 pointed to significant promise.

The *in vivo* (murine) antineoplastic evaluation results for dolastatin 10 (4) serve (Table 1) as a useful illustration of more extensive studies now in progress. An initial comparative analysis of dolastatins 4-9 revealed selective activities against a "minipanel"

TABLE 1

Experimental Anticancer Activity of Dolastatin 10 in Murine *In Vivo* Systems T/C (μg/kg)[a]

P388 Lymphocytic Leukemia	L1210 Lymphocytic Leukemia
toxic (13.0)	152 (13)
155 and 17% cures (6.5)	135 (6.5)
146 and 17% cures (3.25)	139 (3.25)
137 (1.63)	120 (1.63)
B16 Melanoma	**M5076 Ovary Sarcoma**
238 and 40% cures (11.11)	toxic (26)
182 (6.67)	166 (13)
205 (4.0)	142 (6.5)
171 (3.4)	151 (3.25)
142 (1.44)	
LOX Human Melanoma Xenograft (Nude Mouse)	OVCAR-3 Human Ovary Xenograft (Nude Mouse)
toxic (52)	300 (40)
301 and 67% cures (26)	
301 and 50% cures (13)	MX-1 Human Mammary Xenograft (Nude Mouse)
206 and 33% cures (6.5)	
170 and 17% cures (3.25)	toxic (26)
LOX in separate experiments	137 (13)
340 and 50% cures (43)	178 (6.25)
181 and 33% cures (26)	
192 (15)	
138 and 17% cures (9.0)	
	MX-1 Human Mammary Xenograft (Tumor Regression)
	14 (52)
	50 (26)
	61 (13)
	69 (6.25)

a) T/C = Test/Control, both bearing tumor, expressed in time of survival except for the MX-1 system which is recorded as tumor regression (% of the tumor remaining).

of six lines from the NCI's primary screening panel of 60 human tumor cell lines;[33-35] Table 3 shows that dolastatins 10 and 15 exhibited the most promise. The latter two compounds were therefore selected for more detailed analyses in the full 60 cell line screen. Dolastatin 10 proved to be one of the most potent of over 33,000 diverse agents thus far tested in the NCI's new primary screen. The panel average GI_{50} for dolastatin 10 was in the 0.1 nanomolar range, while individual cell lines (e.g. within the brain, ovary, and especially colon tumor subpanels) were 3-10 fold more sensitive than the panel average. Dolastatin 15 was somewhat less potent (e.g. panel average GI_{50} in the 1 nanomolar range), however, a computer analysis[35] of the characteristic differential cellular response profiles for dolastatins 10 and 15 revealed a very high correlation (0.9). Dolastatin 10 has also been found to display an extraordinary ability to prevent tubulin polymerization.[36] Interestingly, dolastatin 15 by comparison is relatively weak by this standard but is still a very strong inhibitor of mitosis.

Presently, dolastatins 10 and 15 are at the preclinical development stage of animal safety studies preparatory to planned Phase I human clinical trails. Doubtlessly, marine organisms contain a vast array of potentially useful anticancer drugs (and drugs for other serious medical problems) awaiting discovery. Illustrative of this potential are the structurally unique and diverse bryostatin,[37] halichondrin[38,39] and cephalostatin[40] series of remarkably potent marine invertebrate antineoplastic substances already discovered in our laboratories and elsewhere.

EXPERIMENTAL

GENERAL PROCEDURES. Solvents used for chromatographic procedures were redistilled. The Sephadex LH-20 (25-100μ) employed for gel permeation and partition chromatography was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Gilson FC-220 race track and FC-80 microfractionators connected to Gilson HM uv-visible detectors were used for chromatographic separation experiments. Column chromatographic procedures with silica gel utilized the 200-400 mesh (Davisil 633), the 70-230 mesh, or silica gel 60 prepacked columns supplied by E. Merck (Darmstadt). A Partisil M9 ODS-2 (C-18 reverse phase) column (10 mm i.d. x 50 cm) was used for hplc and obtained from Whatman, Inc. Clifton, N.J. HPLC separations were performed with Gilson Model 302 pumps using an Apple IIe programmer and Rheodyne 7125 injector or with Altex (Model 110A) solvent metering pumps. Preparative layer plates were also obtained from Whatman, Inc. The silica gel GHLF Uniplates for thin layer chromatography (tlc) were supplied by Analtech, Inc., Newark, Delaware. The tlc plates were viewed with uv light, developed with an anisaldehyde-acetic acid-sulfuric acid spray (heating at approx. 150° for 10 min.) or with ceric sulfate-sulfuric acid (heating for 10 min.).

Amino acid analyses were performed with a Beckman Model 121 unit. Ultraviolet spectra were observed using a Hewlett-Packard 9450A uv/vis spectrophotometer equipped with a HP7225A plotter. The infrared spectra were recorded with a Nicolet MX-1 FT instrument. High resolution SP-SIMS mass spectra were obtained using a Kratos MS-50 triple analyzer mass spectrometer at the NSF Regional Facility, University of Nebraska, Lincoln. The nmr experiments (in various solvents employing a Bruker 5-mm $^{1}H^{13}C$ dual switchable probehead) were

conducted using a Bruker AM-400 narrow bore spectrometer with an ASPECT 3000 computer and pulse programmer operating at 400.13 and 100.62 MHz for ^{1}H-and ^{13}C-nmr respectively.

Animal Collection and Preliminary Experiments

A summary of our 20 year endeavors directed at isolation of antineoplastic agents from the Western Indian Ocean sea hare, *Dolabella auricularia*, follows. The sea hare *Dolabella auricularia* was initially collected in October, 1972, along the Eastern Coast of Mauritius. The approximate location, was 21° S latitude, 56° E longitude, at depths of 1 to 2 m. The *D. auricularia* collected were olive green in color with pear-shaped bodies and an average length of 15-20 cm. The first collection was approximately 1 kg wet weight. Taxonomic identification was provided by the Smithsonian Institute.

By March, 1975 confirmed activity of an ethanol extract against the NCI's P388 lymphocytic leukemia (PS system) was established and showed T/C 235 at 600 mg/kg to T/C 167 at 176 mg/kg. A series of analogous extracts from subsequent recollections during 1975-1979 gave comparable antineoplastic results and are described briefly under Procedure A. The experiments reported in more detail in Procedure B were conducted with a 1982 recollection from the same site. Total volume of the animal (~1,600 kg) and ethanol preservative was 700 gallons which was about ten times the volume of preceding recollections.

Procedure A.

The initial (1972) collection of intact organisms was stored in ethanol. Subsequent recollections from the same site were shipped either as ethanol extracts or again as the whole animal preserved in ethanol. The first ethanol extract (2 kg) was received in October 1975, and subjected to isolation studies. After a series of solvent partition separations (9:1→4:1→3:2 methanol-water with ligroin→carbon tetrachloride→chloroform)[41] the P388 antineoplastic activity was found to reside in the carbon tetrachloride (T/C 174 at 12.5 mg/kg, PS ED_{50} 9.1 x 10^{-3} μg/mL) and chloroform (T/C 177 at 25 mg/kg, PS ED_{50} 0.027 μg/ml) extracts. Numerous attempts at isolation of the active constituent(s) using silica gel chromatography did not provide enough of the active fractions. Thus, additional recollections were required.

A fall 1975 recollection of *D. auricularia* in ethanol was received in February 1976 and extracted to yield 1.58 kg of greenish-black oil. Again, only small quantities of unproductive and very complex fractions were obtained. In November 1976, an ethanol extract (1.6 kg) prepared in Mauritius was received and used to provide PS active (ED_{50} <0.1 μg/mL) carbon tetrachloride (3.26 g) and chloroform fractions (3.33 g). Column chromatographic separation of the carbon tetrachloride fraction on silica gel using hexane→ethyl acetate→methanol as mobile phase yielded dolatriol 6-acetate (1b, 15 mg)[12] and dolatriol (1a, 5 mg).[12] Loliolide (2, 10 mg)[21] and the dolatriols were also isolated from the chloroform fraction. Another supply (12 kg wet wt in ethanol) of *D. auricularia* was collected in the Fall of 1977 and received in March 1978. The ethanol extract weighed 585 g and showed PS ED_{50} 3.3 μg/mL. Solvent partitioning gave 7.9 g (PS ED_{50} 0.2 μg/mL) of a carbon tetrachloride fraction and 16 g (PS ED_{50} 0.043 μg/mL) of a chloroform fraction. Active fractions from the earlier recollections were combined and added to analogous active material from this 1977

recollection. A hot ethanol extraction of the residual ground-up animal (1977 recollection) gave additional carbon tetrachloride (1.8 g, PS ED_{50} 2.9 μg/mL) and chloroform (4.5 g, PS ED_{50} 0.039 μg/mL) fractions. The combined active fractions were separated on a column of Sephadex LH-20 using 4:1 CH_3OH-$CHCl_3$, and again on LH-20 in 5:5:1 hexane-$CHCl_3$-CH_3OH, followed by successive column chromatographic steps employing silica gel with 85:15:1.6 chloroform-methanol-water, 95:5 ethyl acetate-methanol, 90:10:0.8 chloroform-methanol-water and finally using 97:3 chloroform-ethanol, to provide dolastatin 1 (3.76 mg, PS ED_{50} 7.3×10^{-7} μg/mL) and dolastatin 2 (6.02 mg, PS ED_{50} $<10^{-8}$ μg/mL).[19]

The P388 results for dolastatins 1 and 2 proved to be very promising. But consumption of material by the biological screening evaluations combined with unexpected decomposition at ambient temperatures resulted in insufficient product to complete the structure determinations. Meanwhile larger scale recollections were underway and a 100 kg (wet wt) amount of the sea hare in ethanol was completed in early 1979 and received in May, 1979. In addition to the shipping solution extract, ambient and hot ethanol extractions were conducted using the ground animal material. The concentrated aqueous residue was partitioned with methylene chloride in place of chloroform.[41] Next, the solvent partition scheme was modified using 9:1→1:1 methanol-water with ligroin and methylene chloride. The combined active methylene chloride fraction (129 g, PS ED_{50} 0.046 μg/mL) was separated in portions on Sephadex LH-20 in methanol followed by separation of the PS active fraction in 4:1 methanol-methylene chloride. The active fraction from the ambient ethanol extract amounted to 32 g (PS ED_{50} 0.011 μg/mL) and was further separated employing a series of silica gel chromatographic procedures analogous to those utilized for isolation of dolastatins 1 and 2.[19] Dolastatins 3 through 9 were separated in sub-milligram amounts with the exception of dolastatin 3[22,23] (3.5 mg, PS ED_{50} 0.14 μg/mL). After preliminary biological and chemical studies the sample required repurification. As a result about 1 mg remained for structure elucidation. By this time it was clear that the principal antineoplastic constituent(s) had still eluded us and a rather massive scale recollection would be required and carefully investigated as summarized in the following approach (B).

Procedure B.

The final large scale recollection (1,600 kg wet wt in ethanol for a total volume of 2,800 liters) completed in 1982 was received in January 1983. After extraction and solvent partitioning (Separation Scheme Part 1) a 2.75 kg residue from the methanol-water concentrate was obtained and subjected to large-scale preparative hplc. Two 3.0 m x 15 cm (i.d.) columns in series were prepared with silica gel (Davisil 633, 200-400 mesh, slurry packed in 7:3 hexane-ethyl acetate). The 2.75 kg of dark (green-black) concentrate was dissolved in ethyl acetate (7.5 liters), pumped onto the column and chromatographed using the solvent gradients shown in Table 2 at a rate of 60-72 l/h. Each fraction was eluted with 20 liters of solvent and comparable (by tlc, 9:1 methylene chloride-methanol, batyl alcohol as a reference compound) fractions were combined to afford twenty one fractions by June 1983. Fractions A-G in Separation Scheme Part I represents those selected for further separation. Fraction A was found to contain a number of PS inactive to marginally active terpenes while Fraction B

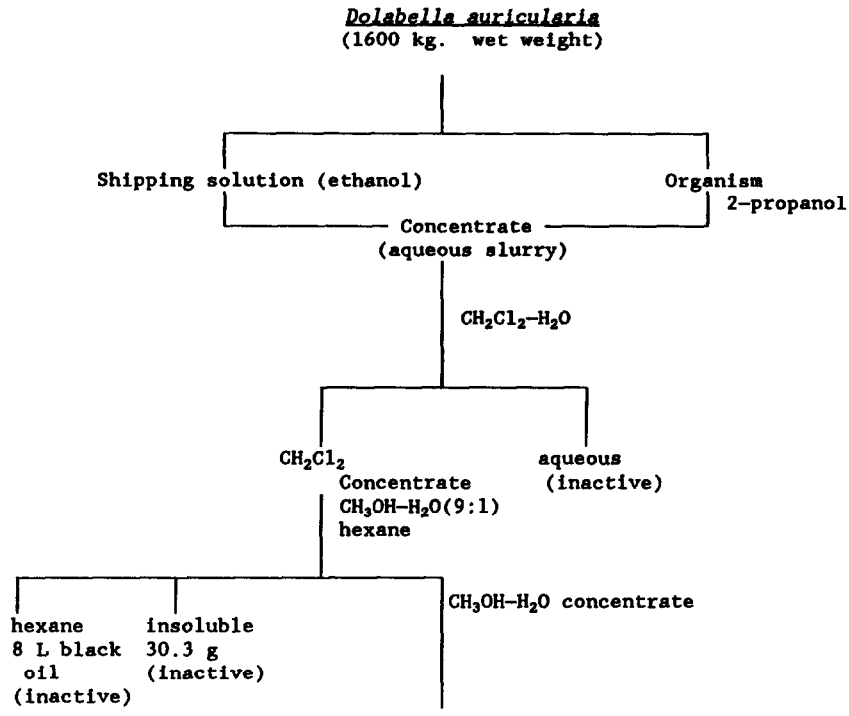
TABLE 2

Preparative HPLC Separation of a 2.75 kg Fraction from *Dolabella auricularia*

Eluent	Eluent[a] Vol. (l)	Fraction Number	Fraction[b] Residue (g)	P388 ED_{50} (μg/ml)	P388 (*in vivo*) T/C (mg/kg)
7:3 hexane: ethyl acetate	200	1	64.9	13	inactive
3:2 hexane: ethyl acetate	120	2-8	282	14	inactive
1:1 hexane: ethyl acetate	240	8-9	78	21	inactive
		10-14 A	160.1	0.46	inactive
		15-16 B	58	0.12	135(7)
		17-18 C	72.2	0.059	toxic(16)
ethyl acetate	120	19-21 C	74.9	0.057	140(12.5)
		22-25 D	70.6	0.055	142(9)
95:5:0.7 ethyl acetate-methanol-water	120	26-28 D	156.4	0.048	135(30)
		29-31 D	50.5	0.02	131(30)
83:17:1.4 ethyl acetate-methanol-water	240	32-35 E	42.7	0.11	131(28
		36-38 E	50.3	0.58	140(30)
		39 E	66.2	0.43	130(30)
		40 F	76	1.3	124(30)
		41-45 G	132	0.03	165(7.5)
67:33:2.5 ethyl acetate-methanol-water	240	46-50 G	72	0.004	147(17.5)
		51	77	0.37	inactive
		52-55	209.5	0.27	toxic(15)
50:50:5 ethyl acetate-methanol-water		56-60	56	1.6	inactive
45:45:10 ethyl acetate-methanol-water		61-65	100	1.9	inactive
		66-69	30.5	1.8	inactive

[a]Each fraction was eluted with 20 l of solvent and comparable (by TLC) fractions were combined.

[b]Weights are approximate, fractions contain trace of solvents



Prep silica gel hplc
1) hexane–ethyl acetate (7:3→6:4→5:5→0:1)
2) ethyl acetate–CH_3OH–H_2O
(95:5:0.7→83:17:1.4→67:33:2.5→
50:50:5→45:45:10)

	A	B	C	D	E	F	G
Wt.(g)	160.1	58.0	147.1	277.5	159.2	76.0	204.0
P388 ED_{50} μg/ml	0.46	0.12	0.057	0.05–0.02	0.1–0.5	1.3	0.03–0.004
P388 T/C(mg/kg)	inactive	122→135 (at 28→7)	toxic→140 (at 50→12.5)	135→131 (at 30→7.5)	140→120 (at 30→7.5)	124→121 (at 30→7.5)	toxic→165 (at 30→7.5)

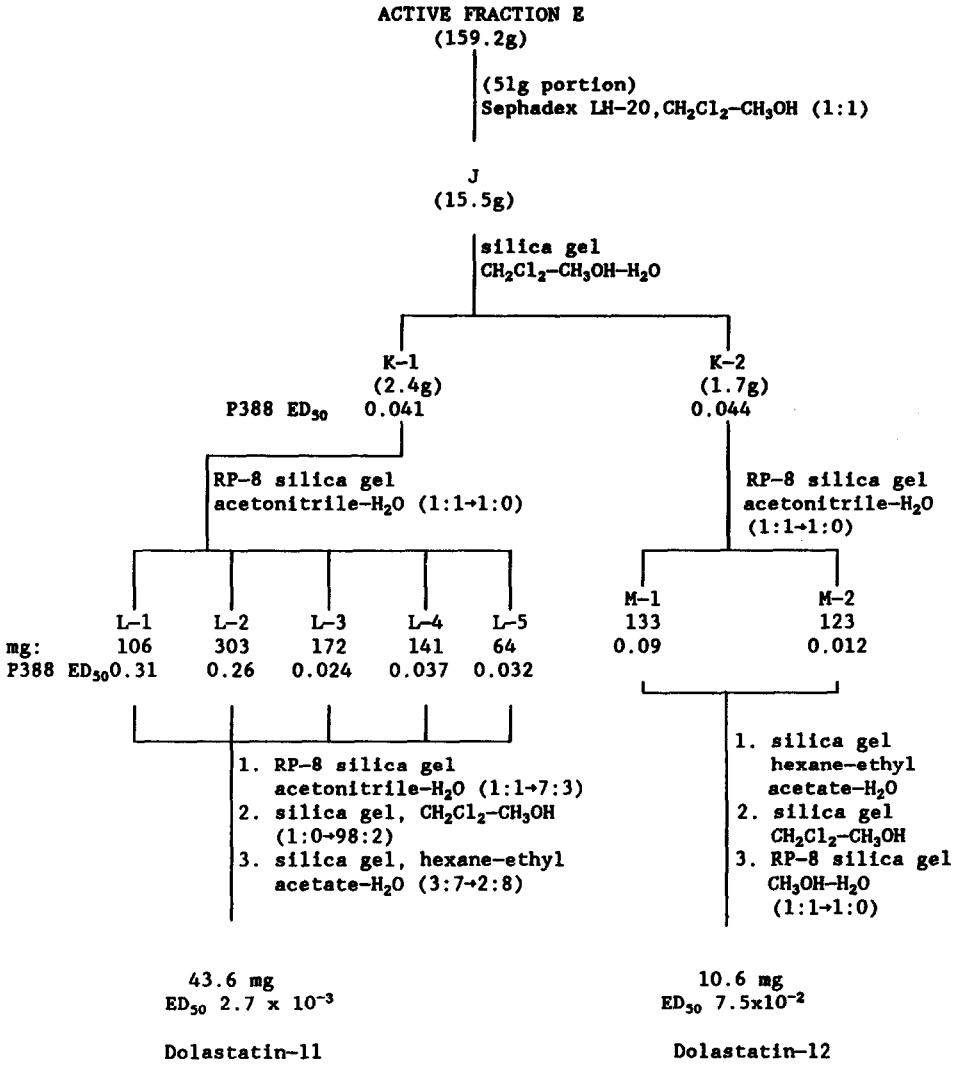
Separation Scheme Part 1

yielded as a major component batyl alcohol (4.5 g).[42] Fractions C, D and F were investigated but none of the dolastatins were located.

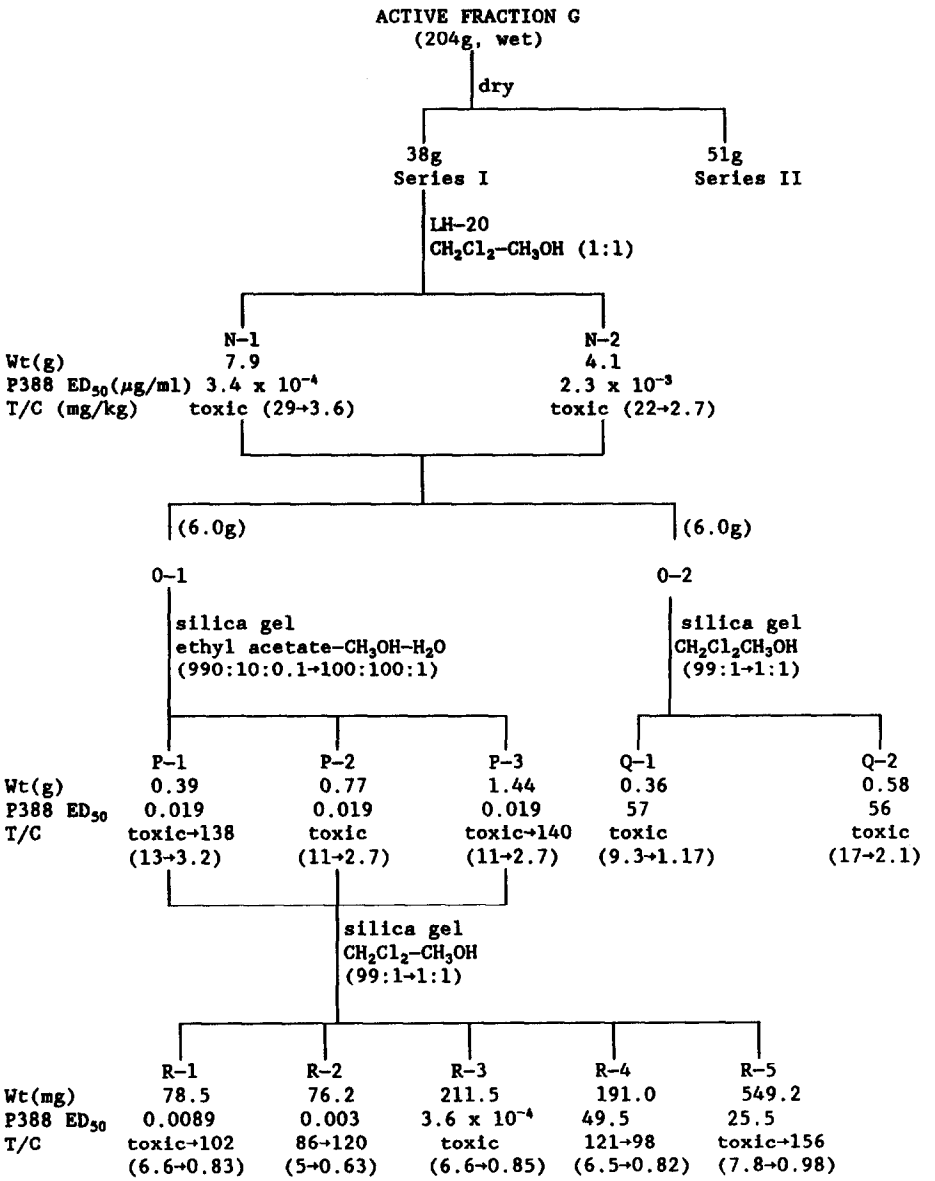
Fraction E (a 51 g portion) was chromatographed on a column of Sephadex LH-20 (10x120 cm) in 1:1 methylene chloride-methanol. Fraction combinations (by tlc comparison) provided active fraction J (15.5 g) which was subjected to silica gel chromatography using 9:1:0.8 methylene chloride-methanol-water. Two adjacent active fractions K-1 (2.4g, PS ED_{50} 0.041 μg/mL) and K-2 (1.7g, PS ED_{50} 0.044 μg/mL) were chromatographed separately, both on RP-8 silica gel, with a 1:1→1:0 gradient of acetonitrile-water. Fraction K-1 afforded active fractions L-1 through L-5 (PS ED_{50} 0.31→0.024 μg/mL), with a combined weight of 786 mg. Fractions L-1 to L-5 were further separated on RP-8 silica gel, using a 1:1→7:3 acetonitrile-water gradient. The resulting crude dolastatin 11 fraction was purified by silica gel column chromatography using a 1:0→98:2 methylene chloride-methanol gradient and, again on silica gel, by eluting with 3:7→2:8 hexane-ethyl acetate-water to provide dolastatin 11 (5, 43.6 mg) with the physical constants recorded in reference 25.

By analogous means, active fraction K-2 gave active fractions M-1 (PS ED_{50}0.098 μg/mL) and M-2 (PS ED_{50} 0.012 μg/mL) which were combined (256 mg) and submitted to column chromatography on silica gel using 3:7:0.5 → 2:8:0.5, upper phase hexane-ethyl acetate-water. By this method crude dolastatin 12 was uncovered and purified by column (silica gel) chromatography using 25:1:0.08 methylene chloride-methanol-water to yield 21.5 mg which was chromatographed on RP-8 silica gel, with a 1:1→1:0 gradient of methanol-water to give pure dolastatin 12 (6, 10.6 mg)[25].

Combined preparative hplc fractions (Table 2) 41-45 (132 g, PS T/C toxic→165 at 30→7.5 mg/kg and $ED_{50}<10^{-2}$) and 46-50 (72 g, PS T/C toxic→141 at 35→8.7 mg/kg and $ED_{50}<10^{-2}$ μg/mL) displayed very good antineoplastic activity. The fractions were combined and dried to give 89 g of fraction G. An aliquot (38 g) was treated as summarized below utilizing a series of column chromatographic separations. The remaining 51 g was investigated separately as described later. In a typical series of experiments, the 38 g fraction was chromatographed on a column of Sephadex LH-20 (10x120 cm) in 1:1 methylene chloride-methanol. Combination of similar fractions gave PS *in vivo* active fractions N-1 (PS T/C toxic, 29→3.6 mg/kg; ED_{50} $3.4x10^{-4}$ μg/mL) and N-2 (PS toxic, 22→2.7 mg/kg; ED_{50} 2.3×10^{-3} μg/mL). Fractions N-1 and N-2 were combined and divided into two equal parts (6 g each), O-1 and O-2, for separation using silica gel column chromatography. The O-1 series was further separated by dry column silica gel chromatography with a gradient of 990:10:0.1 to 100:100:1 ethyl acetate-methanol-water to give fractions P-1 (PS T/C toxic→138, 13→3.2 mg/kg, ED_{50} 0.019 μg/mL), P-2 (PS T/C toxic, 11→2.7 mg/kg, ED_{50} 0.019 μg/mL) and P-3 (PS T/C toxic→140, 11→2.7 mg/kg, ED_{50} 0.019 μg/mL). The parallel O-2 series was also separated by dry column silica gel chromatography using methylene chloride-methanol with a gradient from 99:1 to 1:1 giving fractions Q-1 (0.36 g, PS T/C toxic 9.3→1.17 mg/kg, ED_{50} 57 μg/mL) and Q-2 (0.58 g, PS T/C toxic 17→2.1 mg/kg, ED_{50} 56 μg/mL). Combined active fractions P-1, P-2 and P-3 (2.6 g) were separated using dry column silica gel chromatography and a 99:1 to 1:1 methylene chloride-methanol gradient to afford fractions R-1 through R-5 (1.11 g, PS T/C toxic→156, 7.8→0.98 mg/kg, ED_{50} 25-3.6 $\times 10^{-4}$



Separation Scheme Part 2

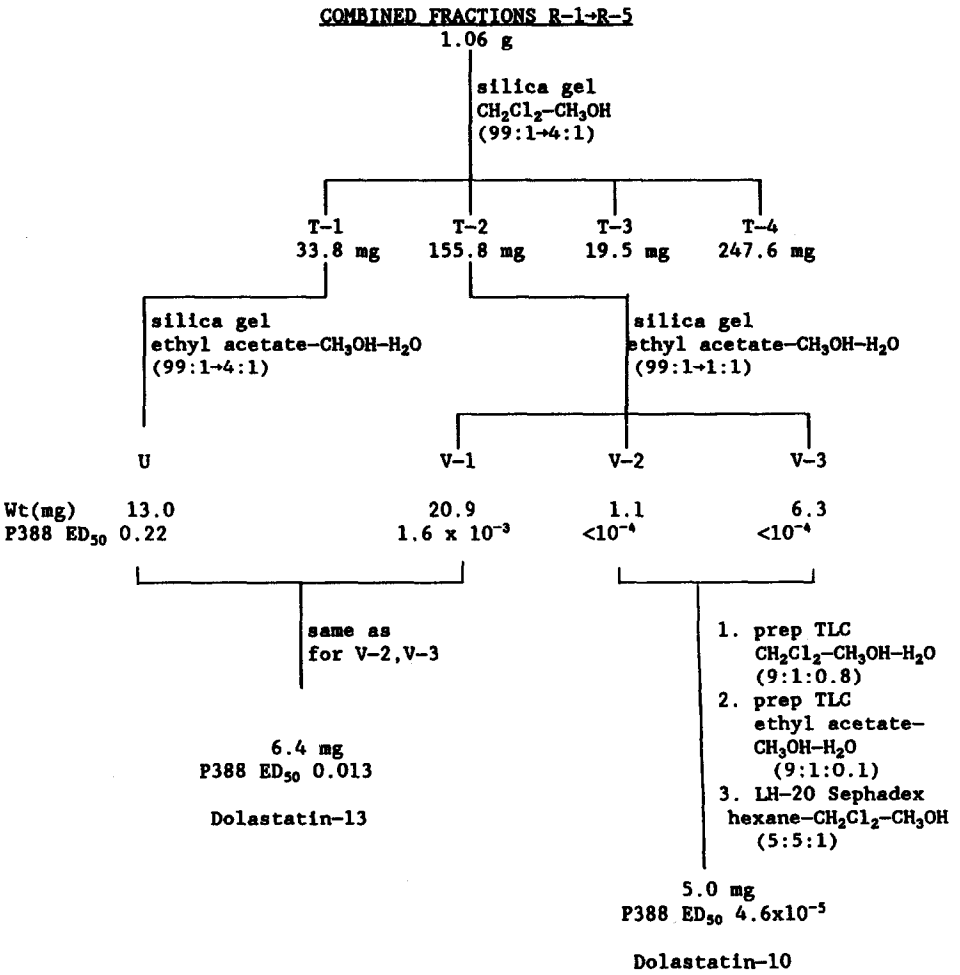


Separation Scheme Part 3
Series I

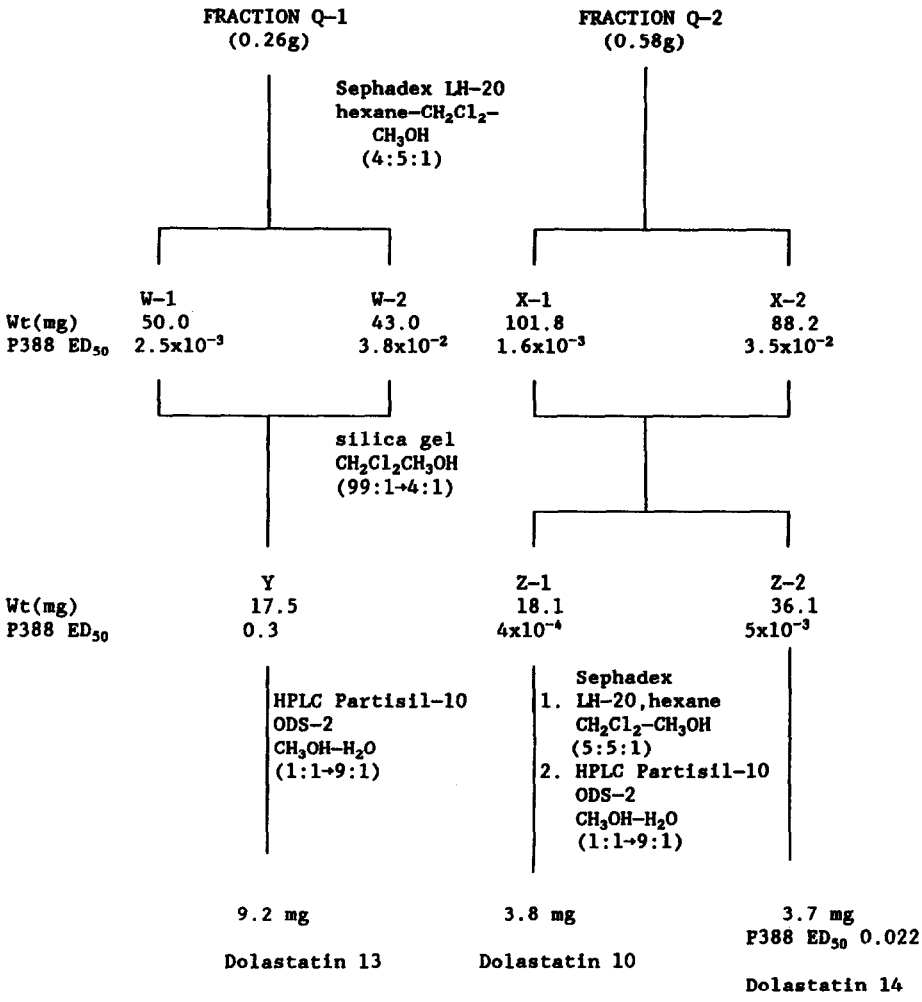
μg/mL). Fractions R-1 through R-5 were further separated on a column of silica gel, using a 99:1 to 4:1 methylene chloride-methanol gradient that resulted in fractions T-1 through T-4. Fraction T-1 was column chromatographed on silica gel employing 99:1→4:1 ethyl acetate-methanol-water to provide fraction U (13 mg, PS ED_{50} 0.22 μg/mL). Fraction T-2 was further separated on a column of silica gel (wet) using ethyl acetate-methanol (99:1 to 1:1) to yield fractions V-1 (PS ED_{50} $1.6x10^{-3}$ μg/mL), V-2 (PS $ED_{50}<10^{-4}$ μg/mL) and V-3 (PS $ED_{50}<10^{-4}$ μg/mL). Combined fraction V-2 and V-3 (7.4 mg) was finally separated by three principal steps: preparative tlc with 9:1:0.8 methylene chloride-methanol-water and again with 9:1:0.1 ethyl acetate-methanol-water followed by partition chromatography using Sephadex LH-20 and 5:5:1 hexane-methylene chloride-methanol as eluent. By this overall procedure series O-1 led to 5 mg of dolastatin 10 (4)[24].

Fraction U was combined with Fraction V-1 to give 33.9 mg of active material which was purified using the final three steps described directly above. In this case the procedure yielded dolastatin 13 (7, 6.4 mg).[26] Continuation of the separation with active fraction O-2 to yield fraction Q-1 and Q-2 required dry column chromatography on silica gel employing 99:1 to 1:1 methylene chloride-methanol gradient solvent system. Active fraction Q-1 gave fractions W-1 (50 mg, PS ED_{50} $2.5x10^{-3}$ μg/mL) and W-2 (43 mg, PS ED_{50} 0.038 μg/mL) which were combined for separation on silica gel using a 99:1→4:1 methylene chloride-methanol gradient. Resultant fraction Y (17.5 mg, PS ED_{50} 0.3 μg/mL) was finally purified by hplc (Partisil-10 ODS-2, methanol-water gradient 1:1 to 9:1) to provide another 9.2 mg of dolastatin 13 (7).[26] An analogous series of separation techniques with fraction Q-2 gave fractions X-1 (101 mg, PS ED_{50} $1.6x10^{-3}$ μg/mL) and X-2 (88 mg, PS ED_{50} 0.035 μg/mL). Combination of fractions X-1 and X-2 and separation on silica gel (column) as before gave fractions Z-1 (18 mg, PS ED_{50} $4x10^{-4}$ μg/mL) and Z-2 (36 mg, PS ED_{50} $5x10^{-3}$ μg/mL). Fraction Z-1 was separated by partition chromatography on Sephadex LH-20 with 5:5:1 hexane-methylene chloride-methanol, followed by final purification by HPLC with a Partisil-10 ODS-2 column and a methanol-water (1:1 to 9:1) gradient, to give a further 3.8 mg of dolastatin 10 (4).[24] Fraction Z-2 was separated in the same way to give 3.7 mg of dolastatin 14 (8).[28] Separation of active fractions T-3 (19.5 mg) and T-4 (247 mg) as separate units yielded 4.5 mg of nearly pure dolastatin 14. A parallel series of chromatographic steps using prep tlc and Sephadex LH-20 as described for separation of combined fractions V-2 and V-3 (above) provided another 2.5 mg of pure dolastatin 14 (8).[28]

The larger quantity of active fraction G (51 g) was chromatographed on columns (10 x 120 cm) of Sephadex LH-20 in five portions in 1:1 methylene chloride-methanol. The active fractions were combined and further separated using a column (4.5 x 80 cm: 1.2 kg) of silica gel and a stepwise gradient of methylene chloride-methanol (49:1 → 23:2 → 9:1 → 22:3 → 17:3 → 4:1 → 1:1 and 0:1) to give fraction d (6.87 g, PS ED_{50} 10^{-2}-10^{-3}μg/mL. Fraction d was rechromatographed on silica gel (dry) using a 99:1 to 1:1 methylene chloride-methanol gradient. The resulting active fractions e-1 to e-6 (4.6 g) were combined and chromatographed (dry column) on silica gel using a 99:1 to 1:1 ethyl acetate-methanol gradient to provide fractions f-1 (578 mg, PS ED_{50} 2.1 x 10^{-3} μg/mL), f-2 (695 mg, PS ED_{50} 3.4 x 10^{-4} μg/mL), f-3 (715 mg, PS ED_{50} 2.2 x 10^{-3} μg/mL), and f-4 (413 mg, PS ED_{50} 1.8 x 10^{-3}



Separation Scheme Part 4
Series I



Separation Scheme Part 5
Series I

μg/mL). Fractions f-1 and f-2 were combined (1.27 g) and chromatographed on Sephadex LH-20 using a 5:5:1 hexane-methylene chloride-methanol partition system to afford fractions g-1 (124 mg, PS ED_{50} 7.2 x 10^{-4} μg/mL) and g-2 (574 mg, PS ED_{50} 2.9 x 10^{-4} μg/mL. Separation of fraction g-2 on silica gel (Size B Merck prepack) with a 99:1 to 1:1 methylene chloride-methanol gradient procedure gave fractions h-1 (20.6 mg, PS ED_{50} 0.015 μg/mL) and h-2 (91.6 mg, PS ED_{50} 1.4 x 10^{-4} μg/mL). At this point, fraction h-2 was treated separately in 39 mg (fraction i) and 44 mg (fraction j) portions, in parallel using preparative tlc (90:10:1 ethyl acetate-methanol-water mobile phase) followed by successive Sephadex LH-20 partition steps with 5:5:1 hexane-methylene chloride-methanol, 5:5:1 hexane-ethyl acetate-methanol and lastly the 5:5:1 hexane-toluene-methanol solvent system. Very importantly, fraction i gave 8.6 mg and fraction j 11.3 mg of pure dolastatin 10 (4).[24] Fraction h-1 was separated by hplc (Partisil-10, ODS-2 with a 1:1 to 9:1 methanol-water gradient) to yield 9.6 mg of dolastatin 13 (7).[26]

Fractions f-3 and f-4 were combined (1.13 g) and subjected to Sephadex LH-20 partition chromatography using 5:5:1 hexane-methylene chloride-methanol to uncover fraction k (288 mg, PS ED_{50} 1.4 x 10^{-3} μg/mL). Separation on a column of silica gel with a methylene chloride-methanol gradient of 99:1 to 1:1 yielded fraction l (76.7 mg, PS ED_{50} 1.8 x 10^{-3} μg/mL). Separation by hplc (Partisil-10 ODS-2, methanol-water gradient of 1:1 to 9:1) afforded 5.8 mg of dolastatin 14 (8).[28] Active fraction g-1 (124 mg) was further separated by silica gel column chromatography using a 99:1 to 1:1 methylene chloride-methanol gradient to give fraction m (12.8 mg, PS ED_{50} 7.2 x 10^{-4} μg/mL). Final purification (hplc) utilizing Partisil 10 ODS-2 with a methanol-water gradient of 1:1 to 9:1 provided 6.2 mg of dolastatin 15 (9).[28]

Dolastatins 10-15 were obtained in the following total yields from the 1,600 kg 1982 recollection: dolastatin 10, 28.7 mg (1.8 x 10^{-6}% yield); dolastatin 11, 43.6 mg (2.7 x 10^{-6}%; dolastatin 12, 10.6 mg (6.6 x 10^{-7}%); dolastatin 13, 25.2 mg (1.6 x 10^{-6}%); dolastatin 14, 12.0 mg (7.5 x 10^{-7}%; and dolastatin 15, 6.2 mg (3.9 x 10^{-7}%). Each of the dolastatins was ascertained to be chromatographically pure (by tlc and hplc) and purity was further substantiated by 400 MHz ^{1}H-nmr and high resolution mass spectral studies.

Dolastatins 10-15 were comparatively evaluated for *in vitro* antitumor activity against a subset of human solid tumor lines selected from the NCI's "disease-oriented" 60-cell line primary screening panel. Because of the very small available amounts of the natural compounds, a "minipanel" of six lines (one each from the ovary, brain, renal, lung, colon and melanoma subpanels of the NCI screen) was used for the initial comparisons. The minipanel screening study was performed using the same assay methodology as described elsewhere and as employed in the NCI primary screen.[43] Table 3 summarizes the results of duplicate, same-week comparisons of dolastatins 4-9 against the minipanel.

Because dolastatins 10 and 15 proved to be the most potent in the minipanel evaluation, and because larger quantities of these compounds subsequently were available by synthesis, they were selected for extensive evaluation in the full 60-cell line primary screen. Each was subjected to quadruplicate screenings in two different concentration ranges (10^{-6}M and 10^{-8}M upper limits; five 10-fold dilutions). Data calculations, graphic displays (e.g., mean

graphs), and analyses were performed as described elsewhere.[33-35,42] Standard errors for the calculated mean panel $GI_{50,s}$ ($0.012x10^{-8}$ M and $0.096x10^{-8}$ M for dolastatin 10 and 15, respectively) averaged less than 10% of the respective means. The compare analysis[34-35] was performed using the averaged, TGI-based mean graphs for each compound.

Table 3

Comparative Activities ($GI_{50,s}$; μg/ml) of Dolastatins 10-15 against a Human Tumor Cell Line Minipanel from the NCI Primary Screen

Compound	Cell Line					
Dolastatin	OVCAR-3[a]	SF-295[b]	A498[c]	NCI-H460[d]	KM20L2[e]	SK-MEL-5[f]
10 (4)	$9.5x10^{-7}$	$7.6x10^{-6}$	$2.6x10^{-5}$	$3.4x10^{-6}$	$4.7x10^{-6}$	$7.4x10^{-6}$
11 (5)	0.04	0.031	0.023	$1.9x10^{-4}$	0.037	0.034
12 (6)	0.14	0.37	0.78	0.088	0.29	0.32
13 (7)	2.5	3.9	4.5	3.5	2.4	2.7
14 (8)	$4.1x10^{-3}$	$2.0x10^{-3}$	0.038	$2.7x10^{-3}$	0.020	$6.4x10^{-5}$
15 (9)	$1.3x10^{-4}$	$4.3x10^{-4}$	$2.7x10^{-4}$	$2.8x10^{-4}$	$1.1x10^{-4}$	$1.7x10^{-4}$

a) ovarian adenocarcinoma, b) brain (glioma), c) renal carcinoma, d) lung large cell carcinoma, e) colon carcinoma, f) melanoma

The averaged individual $-\log_{10} GI_{50}$ values obtained for dolastatin 10 and dolastatin 15, respectively, for each of the 60 cell lines comprising the NCI panel (cell line descriptions available elsewhere[43,44]) are provided as follows, with each cell line identifier: CCRF-CEM (10.21, 9.60), HL-60TB (10.96, 10.00); K-562 (10.43, 9.70), MOLT-4 (10.00, 9.39), RPMI-8226 (10.25, 9.51), SR (9.38, 9.44); A549/ATCC (9.77, 8.31), EKVX (8.82, 8.38), HOP-18 (8.00, 8.00), HOP-62 (9.92, 8.70), HOP-92 (9.17, 8.59), NCI-H266 (9.92, 8.49), NCI-H23 (9.96, 8.85), NCI-H322M (9.08, 8.08), NCI-H460 (10.82, 9.28), NCI-H522 (10.49, 8.89), LXFL 529 (10.07, 9.27); DMS 114 (10.00, 9.37), DMS 273 (10.55, 9.64); COLO 205 (10.54, 9.20), DLD-1 (10.00, 9.27), HCC-2998 (10.17, 9.15), HCT-116 (10.21, 9.30), HCT-15 (9.68, 8.68), HT29 (11.02, 9.42), KM12 (10.36, 8.57), KM20L2 (10.85, 9.19), SW-620 (10.54, 9.48); SF-268 (9.47, 8.51), SF-295 (10.00, 8.77), SF-539 (10.19, 9.33), SNB-19 (9.00, 8.46), SNB-75 (10.12, 8.68), SNB-78 (9.39, 8.85), U251 (9.80, 8.72), XF-498 (9.92, 8.72); LOX IMVI (10.07, 9.38), MALME-3M (10.25, 8.96), M14 (10.39, 8.96), M19-MEL (10.11, 9.41), SK-MEL-2 (10.23, 9.28), SK-MEL-28 (8.89, 8.20), SK-MEL-5 (10.62, 9.06), UACC-257 (9.25, 8.40), UACC-62 (9.68. 9.00); IGROVI

(9.92, 9.17), OVCAR-3 (10.80, 9.70), OVCAR-4 (9.19, 8.46), OVCAR-5 (9.55, 8.00), OVCAR-8 (9.24, 8.80), SKOV-3 (10.25, 8.82); 786-0 (10.24, 9.37), A498 (9.27, 8.31), ACHN (9.25, 8.30), CAKI-1 (9.27, 8.72), RXF-393 (10.02, 8.74), SN12C (10.24, 8.70), TK-10 (8.39, 8.21), UO-31 (9.20, 8.32).

The remarkable potency of dolastatin 10 against the preceding broad selection of human cancer cell lines combined with its extraordinary activity against various *in vivo* experimental cancer systems has allowed it to be selected for preclinical development by the NCI. For analogous reasons dolastatin 15 is also undergoing preclinical investigations. Both dolastatins 10 and 15 are scheduled to enter phase I clinical trial in 1993.

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