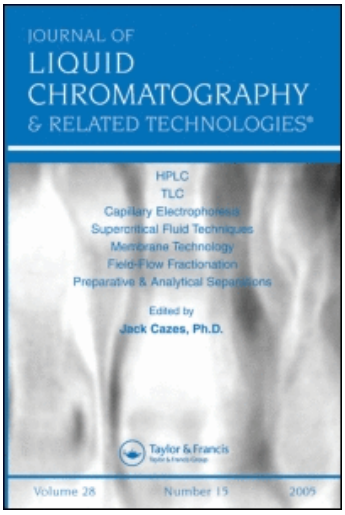

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PROCEDURES FOR THE ANALYSES OF DOLASTATINS 10 AND 15 BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[1]

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ABSTRACT

A series of HPLC procedures were evaluated for assessing the purity of dolastatin 10 (1) and dolastatin 15 (2) samples. Interestingly two readily interconvertible (ambient temperature) dolastatin 10 (1) conformers were detected using a potassium dihydrogen phosphate buffered solvent (methanol-water) with a C8 reversed-phase column. A solvent system composed of acetonitrile-2-propanol-water containing sodium 1-hexanesulfonate was found especially useful for evaluating the purity of dolastatin 10 and 15 specimens. Useful HPLC procedures were also found for detecting diastereomeric isomers in the key dolastatin 10 synthetic intermediate Boc-(S,R,R)-Dap-(S)-Doe using β-cyclodextrin in 3:2 methanol-water.

In 1972 we found extracts of the Western Indian Ocean sea hare *Dolabella auricularia* to produce over 100% life extension in the U.

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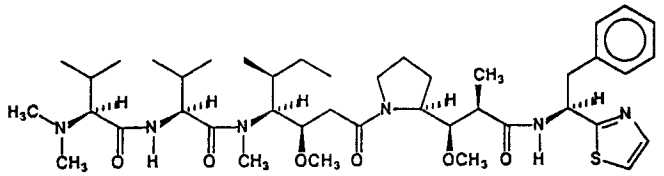
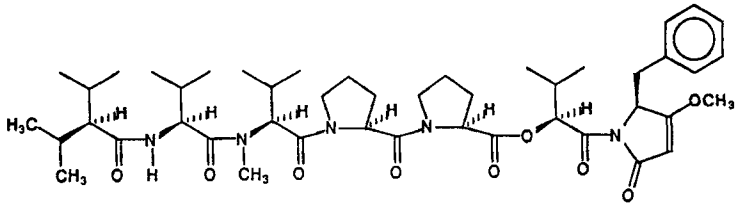
S. National Cancer Institute murine P388 lymphocytic leukemia. Because of this very promising antineoplastic activity we proceeded to isolate and determine structures for the various cytostatic and antineoplastic components.[2-6] The more potent of these components proved to be the two linear depsipeptides dolastatin 10 (1) and dolastatin 15 (2). The isolation,[2,5,6] structure determination[2,7] and initial syntheses[7-10] of these two remarkable peptides have been reported. Because both have been selected for clinical trials, gram-scale quantities were required. Since the marine animal produces only trace ($\sim 10^{-6}$% yields) quantities of these substances, synthetic routes were devised and completed for producing both dolastatins 10[7] and 15[8]. Development of suitable HPLC techniques for ascertaining purity became a very necessary part of these investigations. A variety of HPLC conditions were investigated and resulted in suitable methods for analyses of dolastatins 10 and 15 as well as for several key synthetic intermediates.

EXPERIMENTAL

The β-Cyclodextrin (2.7 g) used in the mobile phase was obtained from Sigma-Aldrich Co. and dissolved in water (120 ml) at 40-50°C. Upon cooling to room temperature, methanol (180 ml) was added. The solution became warm and was allowed to cool (overnight) to room temperature. The precipitated β-cyclodextrin (1.04 g, dry) was removed. The filtrate was a saturated solution of β-cyclodextrin (0.57%) in CH_3OH-H_2O and was used as a mobile phase. The HI-Chrom (reversible) HPLC column (250 x 4.6 mm) containing Pirkle covalent phenylglycine packing (modified Spherisorb S5NH 5 μm particle) was supplied by REGIS Chemical Co.



1, Dolastatin 10



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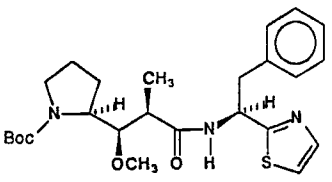

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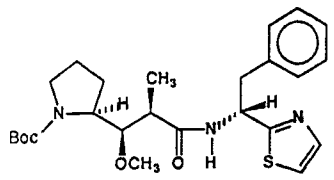

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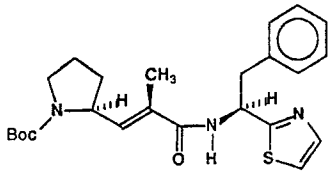

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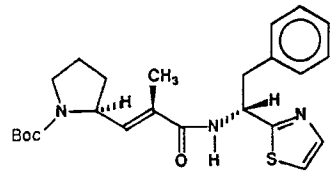

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The HPLC analyses were performed (unless noted otherwise) using a reversed phase Phenomenex Ultremex 3 C8 column (100 x 4.6 mm) with an analytical Gilson HPLC (802B, 811, 2 x 302) instrument equipped with a Rheodyne injection valve (7125 with a 20 μl loop working, pressure 94-101 bar). Control of the HPLC unit was performed with an Apple IIe gradient manager (V 1.2 Gilson). Detection was accomplished with the Hewlett-Packard 1040A, and 9000-300 UV diode array detection system set at 230 nm. Chromatographic displays and data analyses were plotted with a Hewlett-Packard ColorPro plotter.

METHODS AND DISCUSSION

In the stereoselective synthesis of dolastatin 10 (1),[7] synthesis of the (S)-dolaphenine unit proved to be suprisingly challenging. In addition, some reaction conditions led to racemization. Poor yields were usually obtained in a penultimate dehydrogenation step. Protection of (S)-dolaphenine as the BOC derivative improved stability somewhat, but this derivative was still sensitive to oxidation and polymerization upon exposure to air. Due to this instability, HPLC analyses were complicated. However, when (S)-dolaphenine was immediately coupled with BOC-(S)-dolaproine, the resulting dipeptide, BOC-(S,R,R)-dolaproyl-(S)-dolaphenine [Boc-(S,R,R)-Dap-(S)-Doe], (3), was considerably more stable and allowed HPLC analysis of this dolastatin 10 intermediate. Thus, our efforts were initially directed not only to the HPLC analyses of dolastatin 10 and dolastatin 15 alone, but also to the resolution and analysis of dipeptide 3 and its diastereomeric

isomer, Boc-(S,R,R)-Dap-(R)-Doe (4). In addition, two minor side products formed in the dolastatin 10 synthesis, the dehydration products Boc-(S)-dehydro-Dap-(S)-Doe (5) and Boc-(S)-dehydro-Dap-(R)-Doe (6), were also examined. Finally, we explored various HPLC conditions for the resolution of dolastatin 10 and its diastereoisomer, (R)-Doe-*iso*-dolastatin 10.

Initial HPLC analysis of synthetic dolastatin 10 (1) was conducted with a reverse-phase C8 column using a solvent system of 3:1 acetonitrile-H_2O containing 1% acetic acid. Although this eluting system often seemed to provide good resolution between peptide 1 and impurities, reproducible results proved difficult to obtain. The lack of consistent results was attributed to precipitation of the compound(s) during elution. As a consequence, buffered mobile phases containing phosphate salts were investigated. Such mobile phases have been used in chromatographic separations of various synthetic and natural peptides.[11] Use of KH_2PO_4 as a buffer on the C8 column (mobile phase 3:1 CH_3OH-H_2O containing 50mM KH_2PO_4) for chromatography of dolastatin 10 yielded a well defined peak with a longer retention time (3.8 min) than in the previous solvent system (Rt 1.6 min). But this solvent system was found to have limitations. A major problem was precipitation of the phosphate in the pumps, tubing and column during a slight change in the ratio between methanol and buffer, causing severe blockage of the system with concomitant high working pressure (>160 bar). An even more severe drawback was the complete failure of the buffer system to resolve a mixture of authentic dolastatin 10 and the important

diastereoisomer, (R)-Doe-*iso*-dolastatin 10. Since this chiral isomer might be a possible contaminant in synthetic dolastatin 10, the phosphate buffer system was discontinued for evaluating dolastatin 10 purity. However, it was of use in recognizing two room temperature conformers of dolastatin 10, as described below.

During HPLC analysis of dolastatin 10 (1) using the phosphate buffer system, two peaks with Rt of 3.8 min and 4.6 min respectively, would often be observed. The relative ratios of these two peaks would change as shown in Fig. 1, depending upon the samples history. HPLC analysis of several synthetic dolastatin 10 specimens in the CH_3OH phosphate buffer system (reversed phase column) all revealed a single major peak, Rt of ~3.8 min, with a purity of >98%. These fractions were subsequently combined and dissolved in methanol. The solution was passed through a column of Sephadex LH-20 for the purpose of obtaining a pure homogeneous sample for anticancer evaluation. The resulting homogeneous dolastatin 10 was reexamined by HPLC using the phosphate buffer mobile phase. As shown in Fig. 1b, two distinct peaks were initially exhibited, the longer (Rt 4.6 min) peak predominating over the first in the approximate ratio of 2:3. After allowing the HPLC sample solution to stand over various time intervals followed by reinjection, a change in the ratios of the two peaks was noted. Eventually, over time, the shorter (Rt 3.8 min) peak became the dominant peak in a ratio of 95:5. The 3.8 min peak was identical both in Rt and UV spectrum to that of the original dolastatin 10 samples prior to the steric exclusion chromatography on Sephadex LH-

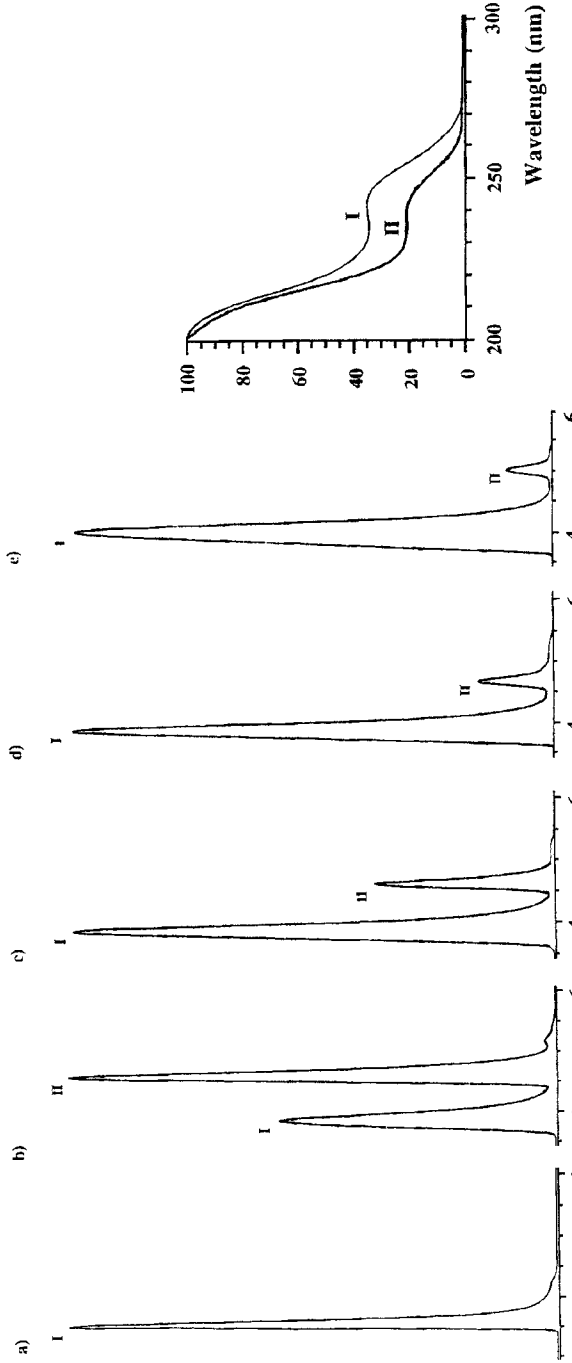

Figure 1. The HPLC (phosphate buffer) of dolastatin 10 (1) showing conformational changes with time in the 3.8 to 4.6 min. retention time range.

a) Pure dolastatin 10 prior to LH-20 chromatography.
b) Same sample immediatly after LH-20 chromatography and solution in eluting solvent (MeOH).
c) Same sample as in b) but after standing for 30 minutes.
d) Same sample as in b) but after standing for 90 minutes.
e) Same sample as in b) but after standing for 14 hours.

20. Such phenomena is typical of the conversion of one conformational isomer to another.

Different obstacles were encountered during the HPLC analysis of synthetic dolastatin 15 (2). Elution of peptide 2 with the 3:1 CH_3OH-H_2O buffer (50mM KH_2PO_4) system on the C8 reversed phase column gave a Rt of 5.5 min, as compared to dolastatin 10 (3.8 min). Because of the problems noted above, the phosphate buffer was abandoned and other systems were investigated. With dolastatin 15, a mobile phase consisting of 1:1 CH_3CN-H_2O on the reversed phase column seemed to provide excellent resolution of dolastatin 15 (2) and its impurities. Final purification of synthetic peptide 2 produced a single major peak with a Rt of 4.3 min and >97% purity.

To find improved analytical HPLC conditions for ascertaining purity of both dolastatin 10 and 15, as well as their synthetic dipeptide intermediates, attention was focused on other mobile phase additives used in the HPLC analyses of peptides.[11] The possibility of substituting 0.1% H_3PO_4 or CF_3COOH for the phosphate buffer system was abandoned when it was found that dolastatin 10 could not be eluted from the column. Sodium dodecyl sulfate (SDS) and β-cyclodextrin[12] (β-CD) were next examined as mobile phase additives. Again dolastatin 10 was not eluted when 1mM SDS in CH_3OH was used as the mobile phase on the reverse phase C8 column. A mobile phase containing β-cyclodextrin (0.57%) produced very broad peaks and this inclusion-complex modifier was generally unsatifactory.

The use of sodium 1-hexanesulfonate ($HexSO_3Na$) as a mobile phase additive gave the most promising results with both dolastatins

10 and 15. A solvent mixture composed of 3:1 CH_3OH-5mM $HexSO_3Na/H_2O$ gave broad but well defined peaks. By substituting acetonitrile for methanol, the peak shape was improved along with the retention time. Further experimentation using the simplex optimization procedure[13] resulted in a much improved solvent system consisting of CH_3CN-2-propanol-5mM $HexSO_3Na/H_2O$ 65:15:20. Because it can be mixed with various solvents in a broad range of concentrations without precipitation, use of $HexSO_3Na$ has distinct advantages over KH_2PO_4.

Optimal concentrations of $HexSO_3Na$ in the CH_3CN- 2-propanol-H_2O (65:15:20) solvent system were determined (Table 1). The optimal concentration of $HexSO_3Na/H_2O$ was about 10 mM or less for separation of dolastatin 10 from dolastatin 15. But this solvent system did not resolve a mixture of dolastatin 10 and (R)-Doe-*iso*-dolastatin 10. When analyzed separately, a ΔRf of 0.19 min was noted for these two compounds. However, when an equimolar mixture of the two was chromatographed, even at low sample concentrations, only peak broadening was observed. A concentration of 20 mM $HexSO_3Na$ led to the best resolution of synthetic dolastatin 10 from its impurities.

A prime objective in the synthesis of dolastatin 10 was optical purity and it became necessary to determine the extent of any racemization of Boc-(S)-dolaphenine. HPLC of a Boc-(R,S)-dolaphenine racemic mixture using CH_3OH-H_2O (3:2) containing 0.57% β-CD[12] (0.8 ml/min) gave no resolution of the R and S isomers. On the other hand, when racemic dolaphenine was converted to Boc-(S,R,R)-Dap-(R,S)-Doe (derivatives 3 and 4), excellent resolution of the two diastereoisomers was achieved, providing a useful method for measuring optical purity of the starting Boc-dolaphenine (Table II).

TABLE I

Retention times for dolastatin 10 and 15 *vs* the concentration of sodium 1-hexanesulfonate in 65:15:20 acetonitrile-2-propanol-water-$HexSO_3Na$

	Rt(min)	
mM $HexSO_3Na$	D-10	D-15
5	10.23	9.40
10	6.15	5.78
15	4.27	4.57
20	3.91	3.75

TABLE II

Retention times for dipeptides 3-6 in CH_3OH-H_2O containing 0.57% β-CD (0.8 ml/min).

Compound	R_t(min)
3	11.11
6	11.84
4	12.23
5	12.75

Here it should be noted that resolution of Boc-(R,S)-dolaphenine was also not realized using a Pirkle chiral column with either *n*-hexane- 2-propanol (9:1, Rt 10.89 min, 0.6 ml/min, 37 bar) or with a 95:5 ratio (Rt 15.23 min, 0.60 ml/min, 47 bar). In addition, dolastatins 10 and 15 were not eluted at all from this column using *n*-hexane 2-propanol (4:1) as mobile phase.

In addition to HPLC analyses, other methods must be employed to accurately evaluate purity of the synthetic dolastatins, *i.e.*, optical rotation and careful high field nmr analyses. The new procedure described for the analysis of dolastatin 15 using $HexSO_3Na$ as an additive in the CH_3CN- 2-propanol (65:15:20) solvent system should assist significantly in detection of possible diastereomeric contaminants. In addition, the HPLC conditions described using β-cyclodextrin and use of the Pirkle type chiral column for resolution of closely related dipeptides should prove to be generally useful.

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