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PREPARATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE SYNTHESIS OF VISCOSIN, A CYCLIC DEPSIPEPTIDE

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SUMMARY

The importance of peptides in biochemical research and the facility with which they can be prepared by solid-phase techniques highlights the need for continuing research in the chromatographic purification of these molecules on a preparative scale. In this regard, synthesis of the cyclic depsipeptide antibiotic, viscosin, has provided the opportunity to demonstrate the use of radial compression cartridge technology in the reversed-phase purification of three key peptide intermediates on a scale of several hundred milligrams. Superior resolution of linear and cyclic peptide mixtures on a Bondapak C_{18} radial compression cartridge by using an aqueous acetonitrile solvent system containing 0.1% trifluoroacetic acid contributed significantly to the first total synthesis of viscosin, and demonstrates the applicability of this system to the purification of peptide mixtures.

INTRODUCTION

Peptides play an ever more important role in many areas of scientific research. One reason for this rapid expansion is the relative ease with which a diversity of peptides and peptide analogues can be prepared by using solid-phase techniques[1]. While products of solid-phase peptide synthesis are often remarkably pure, even the best of synthesis can be contaminated by structurally similar side products. Chromatographic techniques such as counter-current distribution or the Ito coil planet centrifuge can give substantial purification of peptide mixtures[2,3]. However, these suffer from the disadvantage of lengthy separation times and an inability to resolve closely eluting species. High-performance liquid chromatography (HPLC), on the other hand, gives rapid resolution of very complex mixtures, and has established itself as a valuable method not only in the monitoring of the formation of side products during peptide synthesis[4] but also in the purification of synthetic peptide mixtures[5'7]. Various reversed-phase media have found successful application in this regard[5,6,8] and the utility of novel separation schemes has been demonstrated[9]. The commercial availability of high-flow pumps and new high-capacity columns continues to make the preparative HPLC purification of large-scale peptide synthesis an important area of investigation.

Radial compression cartridges offer a less expensive alternative to stainless-steel columns while maintaining high capacity and resolution, and their use has been successfully demonstrated in the purification of hundreds of synthetic peptides[6]. Our research on peptide antibiotics afforded the opportunity to examine the efficacy of the Bondapak C_{18} radial compression cartridge, the PrepPak 1000 compression module and the Model 3000 pumping system in the purification of large amounts of peptide reaction products. Work centered on the peptide antibiotic viscosin which was discovered by Kochi in 1951 and was shown to have antimicrobial[10] and antiviral[11] properties. After an initial incorrect structural assignment[12], the revised structure had been postulated as 1 (Fig. 1), a cyclic peptide lactone composed of alternating D- and L-amino acids, with a D-β-hydroxydecanoyl peptide side chain[13]. The Waters Millipore cartridge system was used for the purification of three key peptide intermediates and served as a significant factor in our successful completion of the first total synthesis of viscosin[14].
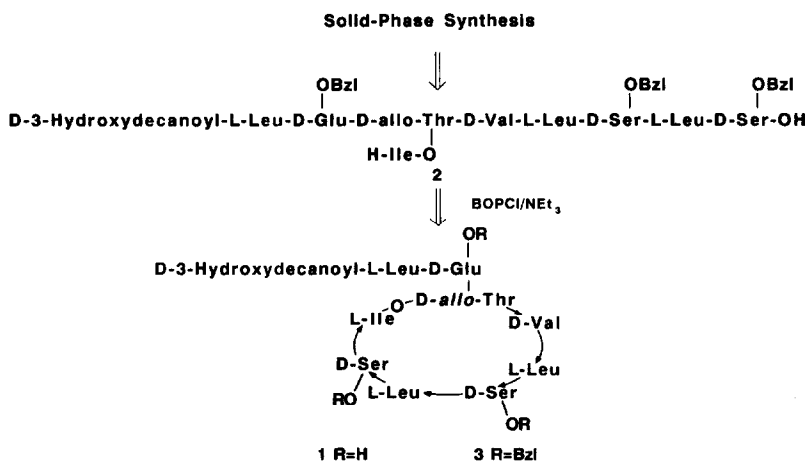
Solid-Phase Synthesis

Fig. 1. Cyclization of precursor fragment 2 to peptide lactone 3. Bzl = benzyl; Et = ethyl.

EXPERIMENTAL

Materials

Solvents were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Acetonitrile was HPLC grade and water was passed through a Barnstead Nanopure II cartridge purification system (Dubuque, IO, U.S.A.) with a 0.2-μm filter (>18 MΩ/cm). Peptides were obtained by synthetic procedures reported elsewhere[14] and lyophilized from reagent-grade tetrahydrofuran (dried over 4-Å sieves).

Methods

Chromatography was performed on a Waters Division of Millipore (Milford, MA, U.S.A.) LC 3000 solvent delivery system equipped with a Rheodyne 7010 injector (having a 7012 filler port and a 5.1-ml sample loop). Additional equipment consisted of a Waters Model 381 variable-wavelength detector and Model 740 data module. Solvent systems were (A) 0.1% trifluoracetic acid (TFA) in water and (B) 0.1% TFA in

acetonitrile with gradients as indicated in Figs. 2–6 (% B shown). Analytical chromatography was carried out on a μBondapak C_{18}, 10 μm, 15 × 0.39 cm I.D. column (Waters) with a flow-rate of 1 ml/min and detection at 214 nm. Samples were typically injected in a volume of 100 μl. Semipreparative chromatography was performed by using a Waters μBondapak C_{18}, 10 μm, 15 × 1.9 cm I.D. column at a flow-rate of 10 ml/min. Samples (75 mg) were injected in 3 ml of dioxane. Preparative chromatography was performed at a flow-rate of 60 ml/min by using a Waters PrepPak 1000 radial compression module equipped with a Waters Bondapak C_{18} prep cartridge, 15–20 μm, 30 × 4.7 cm I.D. maintained under 760 p.s.i. Samples were pumped on in 20–50 ml of dioxane. Both analytical and semipreparative runs utilized UV detection with an analytical cell (10 mm path length), while preparative runs utilized a semipreparative cell (2.1 mm path length). Prior to lyophilization, samples were taken to dryness under rotary evaporation at reduced pressure. Temperatures of 50–60°C were permissible for benzyl-protected peptides, while a temperature of 40°C was used for debenzylated material. Residues were lyophilized from dioxane, giving white solids. Assigned structures were confirmed by amino acid analysis and fast atom bombardment mass spectrometry.

RESULTS AND DISCUSSION

A central problem in the synthesis of cyclic depsipeptides is ring closure. This closure is traditionally achieved through amide bond formation rather than through ester bond formation[15]. For the synthesis of viscosin, a scheme was developed using solid-phase peptide techniques based on an acid-sensitive resin[16] and fluorenyl-methyloxycarbonyl (Fmoc)/butyloxycarbonyl (Boc) amino protection, which produced a linear fragment 2 suitable for cyclization to the benzyl-protected viscosin (3) (Fig. 1). Crude linear 2 was examined by analytical HPLC and was shown to consist primarily of a faster-eluting pyroglutamic acid derivative (2a)[17] and product 2 with a side product of unknown composition (2b) (Fig. 2). Initial semipreparative HPLC
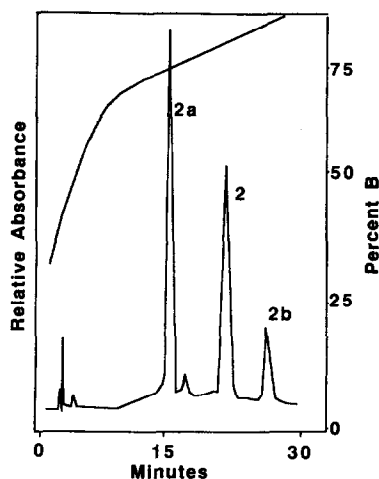

Fig. 2. Analytical HPLC of crude 2 resulting from the TFA cleavage of the solid-phase resin. Absorbance at 214 nm.
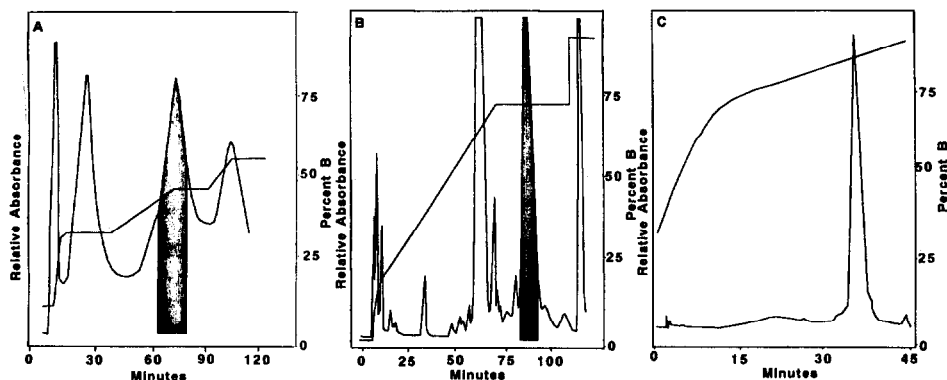
Fig. 3. Purification of crude **2**. Shaded areas indicate portions collected. (A) Semipreparative HPLC purification of a 75-mg sample; (B) preparative purification of an 800-mg sample; (C) analytical HPLC of purified **2** obtained from preparative purification indicated in (B). Absorbance at 220 nm (A) and (B) or 214 nm (C).

purification of a 75-mg sample of crude **2** failed to give baseline resolution (Fig. 3A), however, 21 mg (28% of crude) of pure linear **2** was obtained by this procedure. Superior resolution was achieved in the subsequent preparative HPLC purification of 800 mg of crude **2** (Fig. 3B), where baseline separation yielded 277 mg (35%, w/w) of pure **2** (Fig. 3C). The linear velocity of the preparative run (200 cm/h) was approximately one half that of the analytical scale (500 cm/h), and this in combination with a shallower gradient resulted in preparative retention times approximately three times longer than the analytical retention times. Qualitatively, chromatograms obtained preparatively were remarkably similar to analytical results.

After several hundred milligrams of pure linear **2** had successfully been obtained, cyclization of **2** was carried out under various conditions. Cyclization was finally achieved under high dilution by using the activating agent bis(2-oxo-3-oxazolidinyl)-phosphinic chloride (BOPCl)[18] in dioxane (Fig. 1). The resulting crude reaction mixture contained the desired cyclized product **3** and a faster eluting impurity **3a** (Fig. 4A). Preparative HPLC purification of the crude reaction mixture from a 250-μmol reaction (representing approximately 360 mg of peptide) (Fig. 4B) gave 82 mg of highly enriched benzyl-protected viscosin **3** (Fig. 4C), which was of sufficient purity to carry through the subsequent deprotection step.

Debenzylation of 82 mg of **3** was achieved by stirring with ammonium formate in methanol in the presence of 10% Pd · C[19]. The reaction was followed by analytical HPLC until the mixture of partially debenzylated products coalesced to one unchanging peak. The crude reaction product (Fig. 5A) was then subjected to preparative HPLC purification (Fig. 5B), yielding 52 mg (75%) of pure synthetic viscosin (**1**) (Fig. 5C), which was indistinguishable from natural material[a] both chromatographically (Fig. 6) and by NMR.

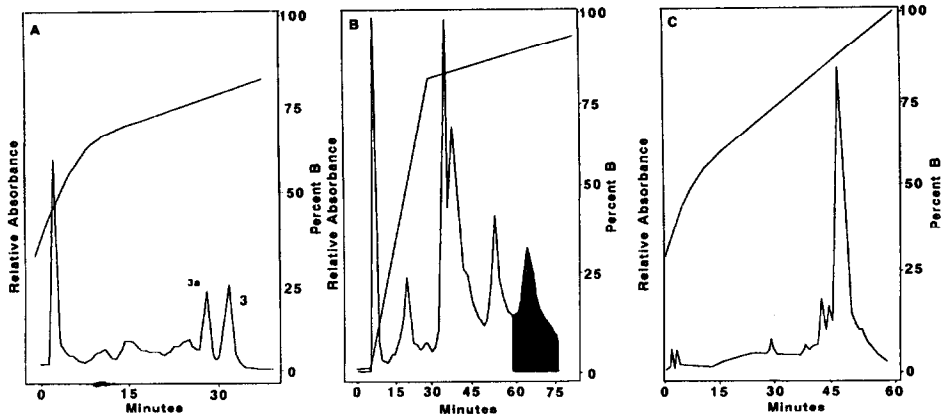[a] A sample of natural viscosin was kindly obtained from Sumitomo Chemical Company, Osaka, Japan.

Fig. 4. Purification of benzyl-protected peptide lactone **3**. (A) Analytical HPLC of crude cyclized **3**; (B) preparative HPLC of approximately 360 mg of crude **3**; (C) analytical HPLC of purified **3** obtained from preparative HPLC (B). Absorbance at 214 nm.
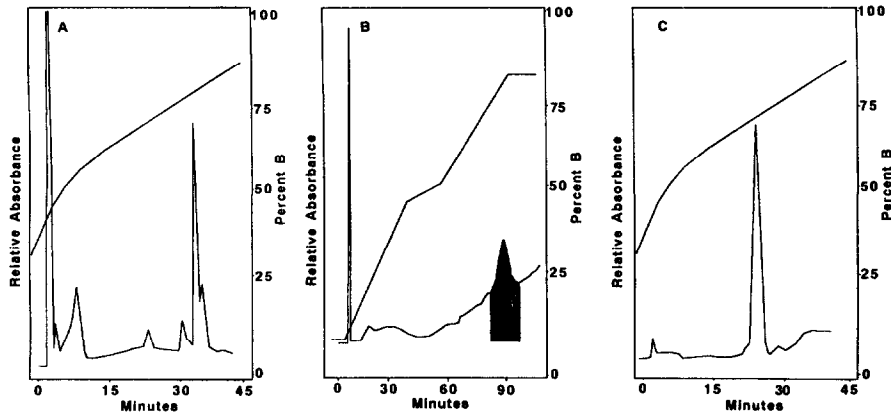

Fig. 5. Purification of synthetic viscosin **1**. (A) Analytical HPLC of crude synthetic viscosin resulting from debenzylation of **3**; (B) preparative HPLC of crude **1**; (C) analytical HPLC of purified viscosin obtained from (B). Absorbance at 214 nm.
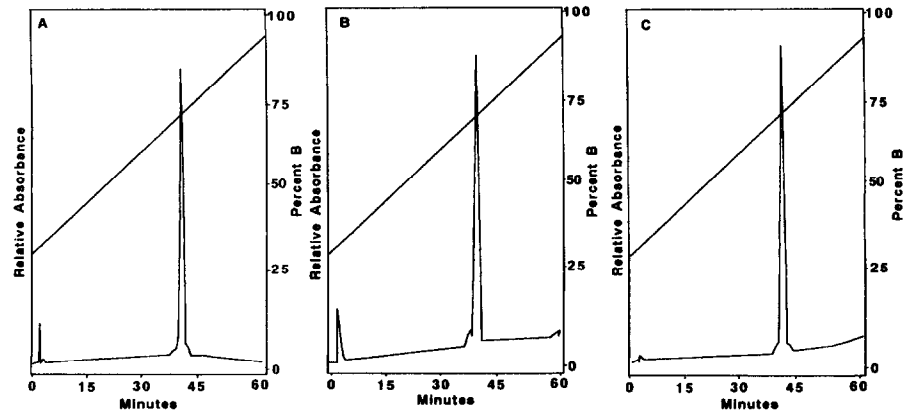

Fig. 6. Comparison of synthetic and natural viscosin by analytical HPLC. (A) Synthetic viscosin; (B) natural viscosin; (C) cochromatography of approximately equal amounts of natural and synthetic viscosin. Absorbance at 214 nm.

While preparative HPLC purification of linear **2** and final product **1** was conducted with good separation, the problematic purification of cyclized, benzyl-protected **3** needed to be re-examined. It was found that debenzylation of crude **3** directly without intermediate purification gave a reaction mixture the components of which could be more easily resolved, yielding the desired **1**. It was therefore found to be more expedient to bypass the intermediate purification of **3** and to debenzylate the crude reaction product directly. Preparative HPLC purification of the resulting crude debenzylated mixture was then achieved in yields (19% overall from linear **2**) comparable to that obtained by using an intermediate purification of benzylated precursor (18% overall yield).

In summary, the first total synthesis of viscosin has afforded the opportunity to examine the utility of the Bondapak C_{18} radial compression PrepPak 1000 system in the large-scale purification of peptide reaction products. The resulting purification of the benzyl-protected linear and cyclized peptides as well as deprotected cyclic viscosin enabled the achievement of the first total synthesis of viscosin.

REFERENCES

1 B. F. Gisin, R. B. Merrifield and D. C. Tosteson, *J. Am. Chem. Soc.*, 91 (1969) 2691–2695.
2 T. R. Burke, Jr. and M. Knight, *J. Chromatogr.*, 411 (1987) 431–435.
3 M. Knight, J. D. Pineda and T. R. Burke, Jr., *J. Liq. Chromatogr.*, 11 (1988) 119–131.
4 G. Szokan, A. Torok and B. Penke, *J. Chromatogr.*, 387 (1987) 267–280.
5 M. Knight, G. D. Mack, R. Perkins and T. R. Burke, *J. Chromatogr.*, 444 (1988) 345–348.
6 J. Rivier, R. McClintock, R. Galyean and H. Anderson, *J. Chromatogr.*, 288 (1984) 303–328.
7 J. X. Huang and G. Guiochon, *BioChromatogr.*, 3 (1988) 140, 143–148.
8 K. Larsson, W. Hermann, P. Moller and D. Sanchez, *J. Chromatogr.*, 450 (19888) 71–80.
9 T. W. L. Burke, C. T. Mant and R. S. Hodges, *J. Liq. Chromatogr.*, 11 (1988) 1229–1247.
10 M. Kochi, D. W. Weiss, L. H. Pugh and V. Groupe, *Bacteriol. Proc.*, (1951) 29–30.
11 V. Groupe, L. H. Pugh, D. Weiss and M. Koch, *Proc. Soc. Exp. Biol. Med.*, 78 (1951) 354–358.
12 T. Ohno, S. Tajima and K. Toki, *J. Agric. Chem. Soc. Jpn.*, 27 (1953) 665–669; *Chem. Abstr.*, 49 (1955) 3012d.
13 M. Hiramoto, K. Okada and S. Nagai, *Tetrahedron Lett.*, 13 (1970) 1087–1090.
14 T. R. Burke, Jr., M. Knight, B. Chandrasekhar and J. Ferretti, *Tetrahedron Lett.*, 30 (1989) 519–522.
15 K. L. Rinehart, V. Kishore, S. Nagarajan, R. J. Lake, J. B. Gloer, F. A. Bozich, K. M. Li, R. E. Maleczka, W. L. Todsen, M. H. G. Munro, D. W. Sullions and R. Sakai, *J. Am. Chem. Soc.*, 109 (1987) 6846–6848.
16 S. S. Wang, *J. Am. Chem. Soc.*, 92 (1970) 5748, 5749.
17 A. J. Hubert, R. Buyle and B. Hargitay, *Helv. Chim. Acta.*, 46 (1963) 1429–1445.
18 R. E. Shute, B. Dunlap and D. H. Rich, *J. Med. Chem.*, 30 (1987) 71–78.
19 M. K. Anwer and A. F. Spatola, *Synthesis*, (1980) 929–932.