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Note

Resolution of linear gramicidins by preparative reversed-phase high-performance liquid chromatography

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In the course of X-ray diffraction studies[1,2] of single crystals of the naturally occurring mixture of gramicidins A, B and C from *Bacillus brevis*, we have found it necessary to purify and modify chemically individual component gramicidins, in order to prepare isomorphous derivatives suitable for phasing either X-ray or neutron-diffraction patterns. Gramicidins A, B and C (Fig. 1) were first separated by counter-current distribution[3], and the Val and Ile variants were isolated when the counter-current procedure was continued for a total of 2000 transfers[4]. More recently, microgram quantities of these component gramicidins have been resolved by reversed-phase high-performance liquid chromatography (HPLC) on octadecyl-silica at 60°C[5]. We have found that a similar separation of Val- and Ile-gramicidins A and C, and of mixed gramicidin B can be achieved on phenyl-silica at room temperature.

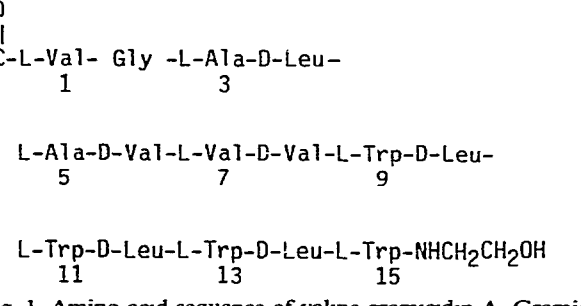

Fig. 1. Amino acid sequence of valine gramicidin A. Gramicidins B and C differ in having Phe and Tyr, respectively, in place of Trp at position 11. The Val–Ile variation within each of the species A, B and C occurs at position 1.

In order to obtain larger quantities of each of the pure component linear gramicidins, we have used a 3.0 m long column of phenyl-silica operating at room temperature under medium pressure (medium-pressure liquid chromatography, MPLC). The procedure allows us to chromatograph 100 mg of commercially

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available gramicidin in 12 h, and from a single injection to obtain immediately 50 mg of Val-gramicidin A, which is 98% pure. The other species can be extracted in hundred mg quantities by pooling peaks from several primary chromatograms, reinjecting and then recycling through the column to achieve high purity. The availability of pure materials has enabled: (a) the growth of crystals of Val-gramicidin A which are large enough to be easily amenable to neutron diffraction; (b) for the first time, the growth of crystals of Ile-gramicidin A, and (c) the isolation of gramidicin B of greater than 95% purity, as judged by both chromatographic and single channel conductance criteria. The crystals of Ile-gramicidin A were found to be expanded along the *b*-axis by 0.3 Å, or 1% when compared to crystals of Val-gramicidin A.

EXPERIMENTAL

Gramicidin was from ICN Life Sciences (Irvine, CA, U.S.A.), methanol from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Solvents were prepared by mixing methanol with deionized, glass distilled water, and filtering through a 0.45-μm pore-size "Fluoropore" membrane (Millipore, Bedford, MA, U.S.A.).

Chromatography was performed using a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A liquid chromatograph, equipped with a 254 nm absorbance detector. The analytical HPLC column was a pre-packed Waters μBondapak Phenyl column, while the MPLC work was done using Waters' Bondapak Phenyl/Porasil B (37–75 μm particles). Although the latter is no longer available, we have recently obtained equivalent results using the Johns-Manville (Denver, CO, U.S.A.) "LC-5" packing. Conditions for separating components on analytical and semi-preparative columns are described in the captions to the figures. Val-gramicidin A and Ile-gramicidin A were crystallized using our previous procedure[1].

RESULTS AND DISCUSSION

Using microgram quantities of the ICN product, it is now a relatively routine matter to separate substantially Val- and Ile-gramicidin A and C, as well as gramicidin B, at ambient temperature in a few minutes (Fig. 2). It is possible to prepare milligram quantities by using a longer column (Fig. 3), but this necessitates using a packing material of larger particle size and operating under lower pressure, both of which tend to decrease the resolving power. Nevertheless, each of the individual components can eventually be purified to greater than 90% purity by repeated passage through the 3.0 m long column, as, for example, has been done with Ile-gramicidin A (Fig. 4).

We routinely run 100 mg of commercial gramicidin per day through our large column, using about 12 h for the chromatography, and 12 h to first wash the column with 100% methanol and then re-equilibrate with the elution solvent. The resulting fractions from several such "primary" separations are then reinjected and recycled to obtain the desired pure species, as in Fig. 4. This system has also been used to purify 50 mg quantities of the semisynthetic Gly-1, L-Ala-1, L-Met-1, L-seleno-Met-1, L-methyl-Cys-1 and L-perdeutero-Val-1 gramicidins, variants in position one of the sequence made according to a modification of the procedure of Morrow *et al.*[6], to be reported elsewhere.

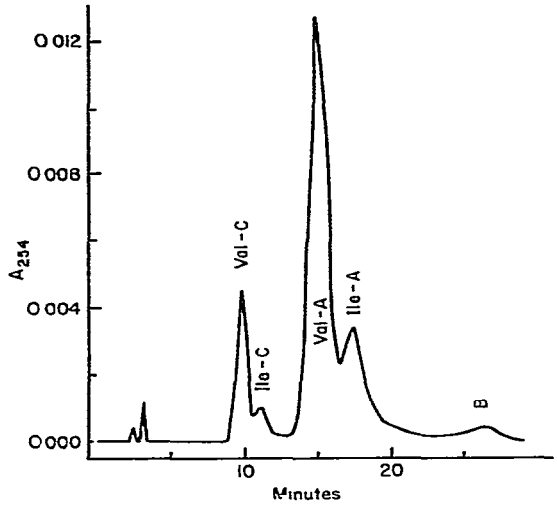

Fig 2. Separation of linear gramicidins by analytical HPLC on phenyl-silica. Column dimensions, 300 × 3 9 mm I D ; particle size, 10 μm; sample size, 10 μg; solvent, methanol–water (75·25); flow-rate, 1.2 ml/min at 2000 p s i

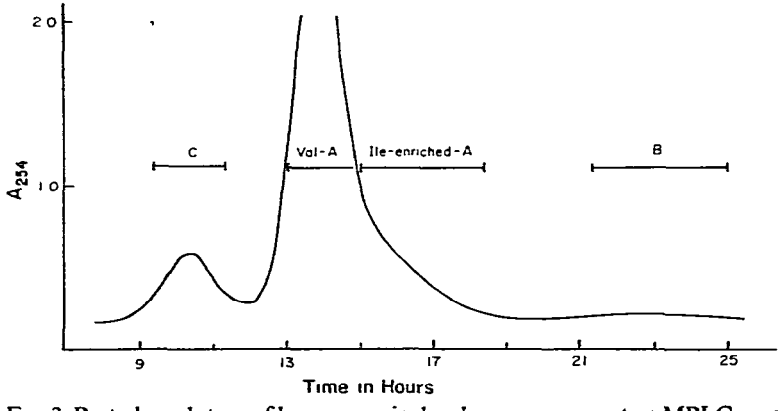

Fig 3 Partial resolution of linear gramicidins by semi-preparative MPLC on phenyl-silica. Column dimensions, 3000 × 7.8 mm I.D.; particle size, 37–75 μm; sample size, 100 mg; solvent, methanol–water (73·27); flow-rate, 1.0 ml/min at 200 p.s.i. Fractions were pooled as indicated

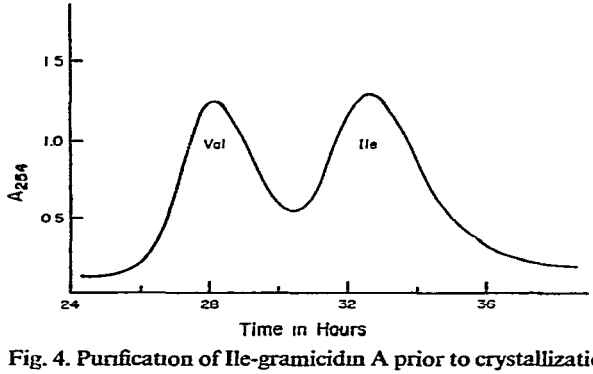

Fig. 4. Purification of Ile-gramicidin A prior to crystallization. The Ile-enriched peak of Fig. 3 from several batches was pooled, reinjected and recycled through the column two additional times. Experimental conditions as in Fig 3. Each peak represents about 50 mg of gramicidin A

TABLE I
UNIT CELL PARAMETERS FOR ETHANOL-GROWN CRYSTALS OF Val- AND Ile-GRAMICIDIN A IN THE SPACE GROUP $P2_12_12_1$

Species	*a (Å)*	*b (Å)*	*c (Å)*
Val	24.67	32.28	32 51
Ile	24 68	32.58	32 54
Commercial mixture*	24.61	32.28	32.53

* Ref. 1.

A comparison of the cell dimensions of the Val- and Ile-gramicidin A crystals (Table I) shows that the extra methyl group forces the molecular packing to change, specifically to expand the *b*-axis from 32.28 Å to 32.58 Å. These numbers result from a least squares fit of 25 machine-centered reflections (Enraf-Nonius CAD IV diffractometer), and are accurate to ±0.02 Å. In contrast, the *a* and *c* crystallographic repeat distances remain the same. The expansion along the *b*-axis suggests that the added methyl group may protrude from the core of the gramicidin helix in the *b*-direction, or alternatively, that the methyl group induces an intra- or intermolecular rotation or other rearrangement which affects packing. The resulting 1% change in crystal volume is rather large for such a small chemical change. In fact, the intensities of the diffracted X-ray reflections are affected to a greater extent by the lack of isomorphism than by the additional electrons introduced by the methyl group.

Gramicidin B is of interest because it exhibits only 2/3 of the single channel conductance of either gramicidin A or C[7]. When the gramicidin B fraction from a medium pressure chromatogram (Peak B of Fig. 3) is analyzed, it is found to contain a substantial amount of gramicidin A (Fig. 5, I). Nevertheless, an additional pass through either our analytical or semipreparative column yields gramicidin B of greater than 90% purity (Fig. 5, II). Therefore, the previous contamination is probably due to "tailing" of the large peak of gramicidin A, and not any effect of physical association between the A and B moieties (as in a dimer) on their chromatographic properties. It is interesting to note that we observe no separation of Val and Ile forms

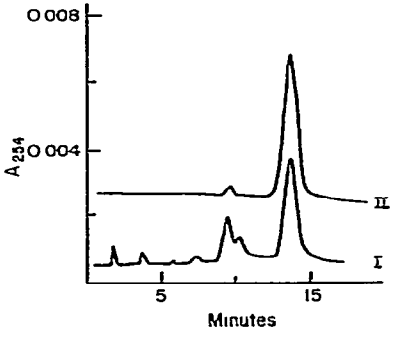

Fig. 5 Purity of isolated gramicidin B by analytical HPLC (I) Peak B from Fig 3. (II) Peak B from Fig 3 after one additional pass through a phenyl-silica column. Column dimensions, 300 × 3.9 mm I.D.; particle size, 10 μm; sample size, 6 μg; solvent, methanol–water (80·20). flow-rate, 1 0 ml/min at 1500 p s i

of gramicidin B (Fig. 5). The gramicidin B sample shown in Fig. 5 (II) shows a very clean single-channel conductance profile, substantially devoid of gramicidin A^8. We are currently using this material to synthesize and characterize the conductance properties of covalent malonyl hetero A–B and B–C dimers.

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