The Occurrence of Bound Serine in Acetone Extracts of *Serratia marcescens* Exclusively in Compounds of a Cyclic Depsipeptide Structure Related to Serratamolide

By MARGARET A. C. BERMINGHAM, B. S. DEOL and J. L. STILL

Department of Biochemistry, University of Sydney, Sydney, N.S.W. 2006, Australia

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The lipoamino acid serratamic acid has been isolated in considerable yield by alkaline extraction of *Serratia marcescens* (Cartwright, 1955) and shown to consist of a linear amide of L-serine and D-3-hydroxy-C_{10:0} acid (Cartwright, 1957). A neutral substance, serratamolide, isolated by acetone extraction of the same organism (Wasserman, Keggi & McKeon, 1961), proved to have two molecules of serratamic acid combined in a cyclic depsipeptide structure, which has been confirmed by synthesis (Shemyakin *et al.* 1964). Serratamolide gives serratamic acid in 85% yield on mild alkaline hydrolysis (Wasserman *et al.* 1961). No biochemical studies have been reported on either substance, and it is not known whether serratamic acid is a metabolite of *S. marcescens* or merely an artifact of alkaline extraction.

The present communication reports that in five different strains of *S. marcescens* 'acetone-soluble' serine *in vivo* is contained exclusively in a 14-membered cyclic depsipeptide structure of which a number of types exist; linear amides may be produced from the latter by mild alkaline hydrolysis, but the free lipoamino acids do not accumulate as such in the organism.

S. marcescens red-pigmented strains N.T.C.C. Colindale 1377 and A.T.C.C. 8195, pink strain 122 (locally isolated) and white mutants 214 (u.v. mutant, locally isolated) and N.T.C.C. Colindale 4612 were grown on a medium containing inorganic nitrogen with glycerol as the source of carbon (Bunting, 1946) in 600 ml conical flasks on a rotary shaker at 30°C. Cells were harvested by centrifugation at 20000g, washed with distilled water and freeze-dried. Dried cells (1g) were extracted successively with (a) acetone (3 × 30 ml) for 1 h under reflux and (b) chloroform–methanol (2:1, v/v). Each extract was freed from non-lipid contaminants by passage through Sephadex G-25 (Wuthier, 1966). T.l.c. was carried out on Silica gel G and Silica gel H.R. (E. Merck A.-G., Darmstadt, Germany) at 0.25 mm thickness (qualitative) and 0.75 mm (preparative) in the following systems: (I) chloroform–methanol–7 M-ammonia (65:25:4, by vol.); (II) chloroform–methanol–water (65:25:4, by vol.); (III) chloroform–methanol–acetic acid–water (100:25:8:4, by vol.). Amino acids and

water-soluble phosphate esters were chromatographed on Whatman no. 1 paper in phenol–water (5:2, w/v) (first dimension) and butan-1-ol–acetic acid–water (3:1:1, by vol.) (second dimension). Lipids were detected (a) by viewing under u.v. light after staining with Rhodamine 6G and (b) by spraying successively with 1% ninhydrin in acetone, acid ammonium molybdate (Vaskovsky & Kostetsky, 1968) and 50% (v/v) H₂SO₄ and heating at 150°C. G.l.c. of methyl esters of fatty acids was carried out by using a diethylene glycol succinate column and components were identified by comparison of retention times with those of authentic samples. Lipids were identified by co-chromatography with authentic samples (when available) and by the partial-hydrolysis techniques of Dawson (1967). Serratamic acid was isolated and purified exactly as described by Cartwright (1955). Free amino N was measured by the method of Rosen (1957), with serine as standard. Melting points were determined on a Kofler hot-stage apparatus. I.r. spectra were determined in carbon tetrachloride on a Perkin-Elmer model 221 spectrophotometer, and mass spectra by using an A.E.I. model MS-9 mass spectrometer at the Department of Chemistry, University of Sydney.

Systematic extraction of various amounts of cells with acetone under reflux established that a constant weight of acetone-soluble material was obtained per unit weight of cells. Measurement of amino N of the lipid extracts before and after hydrolysis with 10 M-HCl for 30 min at 100°C (conditions that cleave both serratamic acid and serratamolide to serine and fatty acid) indicated a large increase in amino N after hydrolysis of the acetone extract from all five strains at stationary-growth phase, and during all phases of growth of strain 1377; the chloroform–methanol (2:1, v/v) extract and additional more rigorous extraction procedures released only traces of material giving rise to an increased amino N after acid hydrolysis. Quantitative two-dimensional paper chromatography indicated that the increased amino N from the acetone extract was due entirely to serine. No serine was obtained on mild alkaline hydrolysis of the acetone extract before treatment with 10 M-HCl, indicating the absence of phosphatidylserine from

Fig. 1. T.l.c. of lipids of *S. marcescens* separated on Silica gel G in system (I). Detection was by spraying with 50% H_2SO_4 and heating at $150^\circ C$. O, Origin; SF, solvent front. (a) Acetone-soluble lipids (200 μg); (b) radioautogram of (a); (c) chloroform-methanol (2:1, v/v)-soluble lipids (200 μg); (d) area X from (a) purified by preparative t.l.c.; (e) mild-alkali hydrolysate of area X; (f) serratamic acid, isolated as described by Cartwright (1955); (g) and (h) bands 7 and 9 purified by preparative t.l.c. 1, Unidentified phospholipid; 2, unidentified ninhydrin-positive phospholipid; 3, phosphatidylethanolamine; 4, phosphatidylglycerol; 5, acetone complex of phosphatidylethanolamine obtained on extraction of freeze-dried cells with acetone in the complete absence of water (Helmy & Hack, 1966); 6, unidentified, possibly cardiolipin; 7, 8 and 9, cyclic depsipeptides.

this extract. (The only other ninhydrin-positive compound present in any but trace amounts was ethanolamine, which did not increase.) It is clear that in this organism compounds of the serratamic acid-serratamolide type are extracted almost quantitatively in acetone and the amino acid moiety consists exclusively of serine.

The acetone extract was analysed in several t.l.c. systems, system (I) proving most suitable for separation of bands in one dimension (Fig. 1). Each band indicated was purified as far as possible by preparative t.l.c. in system (I) and hydrolysed for complete identification. As commonly found (Dvorkin & Shemelev, 1968), small amounts of phospholipids are soluble in acetone. No compound that co-chromatographs with serratamic acid prepared by the Cartwright method (which in our hands always gives a double spot) was detected on t.l.c. of any of the lipid extracts. Most of the acetone extract consisted of a series of bands 7, 8 and 9,

which were negative to ninhydrin and phosphate reagents but on hydrolysis (10M-HCl for 30 min at $100^\circ C$) yielded serine and a mixture of 3-hydroxy fatty acids (3-hydroxy- $C_{10:0}$ acid, 3-hydroxy- $C_{12:0}$ acid and 3-hydroxy- $C_{12:1}$ acid). Initial incomplete purification of bands 7, 8 and 9 from each other by preparative t.l.c. gave fractions that all yielded serine on hydrolysis, but differed as to the proportion of the fatty acids present. Mild alkaline hydrolysis of all fractions yielded material identical (R_F value, m.p.) with serratamic acid (Fig. 1). It thus appears that there is present in bands 7, 8 and 9 a series of related compounds similar to serratamolide that differ as to the nature of the hydroxy fatty acids bound to serine, rather than a single entity containing only 3-hydroxy- $C_{10:0}$ acid as previously envisaged. This would explain why preparations of serratamic acid always appear to contain more than one compound (Fig. 1). If the hypothesis is correct, at least three lipamino acids might be possible.

When L-[U- ^{14}C]serine (153 mCi/mmol) was given to a growing culture of *S. marcescens* (1 $\mu Ci/60$ ml of medium) at zero time and cells were harvested at the exponential phase, radioactivity was found to have been incorporated into spots 7, 8 and 9 (Fig. 1). The area occupied by each spot was scraped off directly into Packard vials, mixed with thixotropic gel and counted in a Packard Tri-Carb model 3375 liquid-scintillation spectrometer; the proportions of radioactivity found in spots 7, 8 and 9 respectively were 70:25:4. The proportions in which 3-hydroxy- $C_{10:0}$ acid, 3-hydroxy- $C_{12:0}$ acid and 3-hydroxy- $C_{12:1}$ acid are found in lipids of *S. marcescens* are 70:23:16 (Bishop & Still, 1963). I.r. analysis indicated that the material occupying area X contains all the absorptions expected of a cyclic depsipeptide structure (Hassall, Martin, Schofield & Thomas, 1967). This might suggest that bands 7, 8 and 9 represent cyclic depsipeptides in which serine is esterified to 3-hydroxy- $C_{10:0}$ acid, 3-hydroxy- $C_{12:0}$ acid and 3-hydroxy- $C_{12:1}$ acid respectively, but the following work indicates that a more complex explanation is necessary.

Ten new t.l.c. systems and column chromatography on silicic acid failed to improve the resolution of bands 7, 8 and 9 over that obtained with system (I); consequently they were separated by preparative t.l.c. in that system after initial purification from pigment and phospholipids on an alumina column. Bands 7 and 9 were finally isolated and found to be pure on t.l.c. in systems (I), (II) and (III) (Fig. 1). Compound 7 had m.p. $167-168^\circ C$ (uncorr.) [Wasserman *et al.* (1961) give m.p. $159-160^\circ C$ for serratamolide], and its ion of highest m/e in the mass spectrum was 540 [calc. mol.wt. for serratamolide, 514; found by Wasserman *et al.* (1961), 556]. If band 7 includes a cyclic depsipeptide containing

one 3-hydroxy-C_{10:0} acid residue and one of 3-hydroxy-C_{12:1} acid instead of two residues of 3-hydroxy-C_{10:0} acid (as in serratamolide), then its calculated molecular weight would be 540. I.r. analysis of band 7 showed all the peaks expected of serratamolide, but in addition gave distinct evidence of a carbon-carbon double bond such as would be contained in 3-hydroxy-C_{12:1} acid. G.l.c. of the fatty acid methyl esters obtained on hydrolysis of band 7 material indicates both 3-hydroxy-C_{10:0} acid and 3-hydroxy-C_{12:1} acid, although more of the former than the latter is present. Initial studies on band 9 indicate that the ion of highest *m/e* in the mass spectrum is 570 (calc. for 14-membered cyclic depsipeptide containing two molecules of 3-hydroxy-C_{12:0} acid, 570). It appears not unlikely that a family of 14-membered cyclic depsipeptides exists in *S. marcescens* consisting of different combinations of hydroxy fatty acids with serine. Serratamolide has weak antibiotic activity against a number of organisms (Wasserman, Keggi & McKeon, 1962), and it is found that band 7 material is active against *Proteus*. Antibiotic activity of cyclic depsipeptides is thought to depend on their ability to sequester ions such as K⁺ (Dobler, Dunitz & Krajewski, 1969); it remains to be seen whether the nature of

the fatty acid substituent has any effect on this activity with the present compounds.

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