nium coupling in alkaline media. Although this is also an electrophilic substitution, this result is not contradictory since apparently the initial attack occurs at the nitrogen of the ring.[15]

Experimental Section

The nmr spectra were determined with a Varian Associates A-60 spectrometer with the samples dissolved in 1 *N* NaOD solution. Chemical shifts (δ) are given in parts per million relative to the internal reference, the sodium salt of 3-trimethylsilyl-1-propane sulfonic acid. Infrared spectra were obtained with the Beckman IR-8 spectrophotometer. Ultraviolet absorption spectra were measured with the Perkin-Elmer Model 202 spectrophotometer. All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Samples for analysis were dried under vacuum over phosphorus pentoxide at 62° for 12–18 hr and the elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Imidazole, obtained from Aldrich Chemical Co., Inc., was recrystallized from benzene (mp 89°).

4(5)-Iodoimidazole was prepared according to our procedure[12] by deiodinating triiodoimidazole with sodium sulfite, mp 137–138° (lit.[3a] 136°).

4(5)-Bromoimidazole was prepared by reducing tribromoimidazole with sodium sulfite in aqueous solution according to the method of Balaban and Pyman.[9] The compound was recrystallized from a mixture of chloroform and petroleum ether to give colorless leaflets, mp 130–131° (lit.[9] 131°).

Anal. Calcd for $C_3H_3N_2Br$: C, 24.51; H, 2.06; N, 19.06; Br, 54.38. Found: C, 24.59; H, 2.02; N, 18.90; Br, 54.32.

4,5-Diiodoimidazole was prepared in alkaline solution by iodinating imidazole with an equimolar amount of iodine. The imidazole (2 g, 29 mmol) was dissolved in 600 ml of 0.04 *N* sodium hydroxide. About 150 ml of pure hexane was added and the flask chilled to 0°. Iodine (7.44 g, 29 mmol) in 600 ml of hexane was added dropwise with vigorous stirring. Simultaneously, 600 ml of 0.2 *N* sodium hydroxide was added dropwise to prevent any precipitation of the reaction product. The addition took about 2 hr and the temperature of the reaction mixture rose to 10°. The reaction mixture was neutralized to pH 7 and left overnight in the refrigerator. The colorless, crystalline material which separated out was collected (3.4 g). The filtrate on evaporation gave 0.4 g of additional material. Recrystallization from dilute alcohol gave colorless, granular crystals, mp 197–198° (lit.[3a] 180°).

Anal. Calcd for $C_3H_2N_2I_2$: C, 11.30; H, 0.63; N, 8.76; I, 79.34. Found: C, 11.42; H, 0.71; N, 8.77; I, 79.40.

4,5-Diiodoimidazole was also prepared by iodinating imidazole at pH 7 according to Ridd[4] and by the removal of a single iodine from triiodoimidazole by refluxing with an equimolar amount of sodium sulfite, according to Brunings.[11] The products from these two preparations showed no depression of melting point with the analyzed preparation described above. The infrared spectra of the three diiodo compounds were identical.

4,5-Dibromoimidazole was prepared according to the procedure of Balaban and Pyman[9] by reducing tribromoimidazole with sodium sulfite in aqueous solution. The compound recrystallized from dilute ethanol and dilute acetic acid appeared as colorless, needle-shaped crystals, mp 229–230° (lit.[9] 225°).

Anal. Calcd for $C_3H_2N_2Br_2$: C, 15.95; H, 0.90; N, 12.41; Br, 70.80. Found: C, 16.12; H, 1.04; N, 12.44; Br, 70.86.

4(5)-Methylimidazole was prepared according to the procedure of Yabuta and Kambe.[17] The vacuum-distilled product has been further purified according to the procedure of Koessler and Hanke.[18] Fine, colorless, crystalline material, mp 56° (lit.[19] 56–56.5°).

Anal. Calcd for $C_4H_6N_2$: C, 58.51; H, 7.37; N, 34.13. Found: C, 58.28; H, 7.52; N, 33.90.

4(5)-Iodo-5(4)-methylimidazole was prepared by iodinating 4(5)-methylimidazole in 0.04 *N* NaOH with a 0.5 *M* amount of iodine and isolating the product from the solution at pH 7.4. Recrystallization from hot water gave colorless crystals, mp 176–177° (lit.[3c] 171°).

Anal. Calcd for $C_4H_5N_2I$: C, 23.10; H, 2.42; N, 13.47; I, 61.01. Found: C, 22.44; H, 2.13; N, 13.25; I, 62.09.

4(5)-Bromo-5(4)-Methylimidazole was prepared according to the procedure of Pyman.[7] The compound, recrystallized from ethyl acetate–*n*-hexane, gave fine, silky needles, mp 154–155° (lit.[7] 155°).

Anal. Calcd for $C_4H_5N_2Br$: C, 29.85; H, 3.13; N, 17.41; Br, 49.65. Found: C, 29.46; H, 3.08; N, 16.41; Br, 51.00.

2-(5-Tetrazolylazo)imidazole was prepared by the slow addition of 5-diazo-1-H-tetrazole (prepared from 2.6 g of 5-amino-1-H-tetrazole[16]) to a solution of 3.4 g of imidazole in 100 ml of 6% sodium carbonate at 0° with stirring. The dark orange material which separated was found to be the bisazo derivative with a maximal absorption at 475 mμ. The filtrate on concentration under vacuum yielded some bisazo derivative and then appreciable amounts of a crystalline, orange-yellow material. The orange-yellow material was recrystallized twice from water to yield light orange-yellow globules (3.5 g) which decomposed explosively at 240°. The absorption spectrum of the product in bicarbonate buffer, pH 8.8, showed a sharp absorption band at 376 mμ, indicating that it is a monoazo compound.

Anal. Calcd for $C_4H_4N_8 \cdot 2H_2O$: C, 24.00; H, 4.00; N, 56.00. Found: C, 24.34; H, 3.14; N, 57.39.

Registry No.—4(5)-Bromoimidazole, 6967-66-2; 4(5)-methylimidazole, 872-33-3; 4(5)-iodo-4(5)-methylimidazole, 15813-07-7; 4(5)-bromo-4(5)-methylimidazole, 15813-08-8; 4,5-diiodoimidazole, 15813-09-9; 2(5-tetrazolylazo)imidazole, 15813-10-2; 4,5-dibromoimidazole, 4150-74-5.

(17) Y. Yabuta and K. Kambe, *Bull. Agr. Chem. Soc. Jap.*, **4**, 74 (1928); *Chem. Abstr.*, **24**, 3509 (1930).

(18) K. K. Koessler and M. T. Hanke, *J. Biol. Chem.*, **39**, 497 (1919).

(19) A. Windaus and F. Knoop, *Ber.*, **38**, 1166 (1905).

Configuration of the Asparaginyl and Aspartyl Residues of Bacitracin[1a]

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Recently this laboratory reported a micromethod for identifying residues of asparagine and glutamine in *endo* position in peptides.[2] In this method, the peptide amides are dehydrated with ethylene chlorophosphite to the corresponding β-cyanoalanyl and γ-aminobutyryl derivatives, which are reduced with sodium–methanol–ammonia, then hydrolyzed to the easily recognizable 2,4-diaminobutyric acid (2,4-DAB) and ornithine. In a limited number of model compounds, residues of isoasparagine and isoglutamine were partly converted into β-alanine and γ-aminobutyric acid by reductive fission of the intermediate α-aminonitrile derivatives.[2,3]

When the dehydration–reduction procedure was applied to commerical bacitracin, approximately 0.7 mol of 2,4-DAB formed per mole of leucine or lysine, which identified the amide-bearing group as

(1) (a) This work was aided by Grant NB-04316 from U. S. Public Health Service and by Muscular Dystrophy Associations of America. (b) Recipient of a general research support summer fellowship, 1966.

(2) C. Ressler and D. V. Kashelikar, *J. Amer. Chem. Soc.*, **88**, 2025 (1966).

(3) Recently this technique proved useful in identifying, for the first time in a natural product, isoglutamine and isoasparagine residues as constituents of the cell wall peptidoglycans of certain bacteria: E. Muñoz, J.-M. Ghuysen, M. Leyh-Bouille, J.-F. Petit, H. Heymann, E. Bricas, and P. Lefrancier, *Biochemistry*, **5**, 3748 (1966); D. Jarvis and J. L. Strominger, *ibid.*, **6**, 2591 (1967); J.-M. Ghuysen, E. Bricas, M. Leyh-Bouille, M. Lache, and G. D. Shockman, *ibid.*, **6**, 2607 (1967).

an asparaginyl rather than an isoasparaginyl residue.[4a] The single amide-bearing group of bacitracin has continued to evoke interest since 2 mol of aspartic acid are derived from the antibiotic, one of L, the other of D configuration,[5] and a definite assignment of these to the asparaginyl and aspartyl residues has not been made. It was known that L-aspartic acid was derived from the residue adjacent to the lysine residue, whereas the aspartic acid more easily liberated on partial acid hydrolysis had the D configuration.[5b,c] Results of lithium borohydride reduction of esterified bacitracin A and commercial bacitracin indicated that the residue giving rise to D-aspartic acid had a free β-carboxyl group.[6] The asparaginyl residue was thereby inferred to have the L-configuration.[2]

The validity of this inference now has been examined by applying the dehydration–reduction technique to commercial bacitracin on a millimole scale, isolating the formed 2,4-DAB, and determining its optical configuration.

After dehydration–reduction, the treated bacitracin was freed of salts with a cation-exchange resin and hydrolyzed in acid. The 2,4-DAB was purified by chromatography on the automatic amino acid analyzer[7] in a slightly modified system that separated it well from lysine, ornithine, and the other amino acids present in the hydrolysate. Buffer salts were removed from the eluent with a column of Dowex 50W resin. The 2,4-DAB was purified further by paper electrophoresis, then converted into the crystalline monohydrochloride which was recrystallized. This isolated material had the same infrared spectrum and the same behavior on chromatography and cochromatography on the amino acid analyzer and on paper electrophoresis at several different pH conditions as the authentic amino acid. Although slightly lower in magnitude, its optical rotation agreed in sign with that of reference L-2,4-DAB monohydrochloride. This confirms the L configuration of the asparaginyl residue of bacitracin and implies that the aspartyl residue has the D configuration. As concluded earlier,[2] of the structures for bacitracin suggested by the studies of Abraham and Craig and their coworkers,[5c,6] the various findings on amide and carboxyl groups appear to favor that one which contains an ϵ-(D-aspartyl-L-asparaginyl)-L-lysine sequence[4b] within a cyclopeptide moiety of seven rather than six amino acid residues.

Experimental Section[8]

Bacitracin (1 mmol) was heated with ethylene chlorophosphite (47 ml) in triethyl phosphite (160 ml) at 97–100° for 24 hr, as described previously for a microscale.[2] The dehydrated peptide in liquid ammonia (700 ml) and methanol (21 ml) was treated with sodium (8.1 g) in portions, after which ammonium chloride (16 g) was added. To the residue left after evaporation of ammonia and methanol were added 17 ml of 2 *N* HCl and 50 ml of warm water. The tan solid was filtered off and hydrolyzed in 20 ml of 6 *N* HCl. The aqueous filtrate at pH 2 was applied to a column (36 × 4 cm) of AG 50W-X2 (H^+ cycle) resin. This was washed with water until the effluent was chloride free, then eluted with 3 *N* NH_3. Ninhydrin-positive basic fractions were concentrated to dryness and hydrolyzed in 50 ml of 6 *N* HCl. The two hydrolysates were taken to dryness. The residues were dissolved in a minimum volume of water and, in batches of 18 μmol each of 2,4-DAB, applied at pH 2 to the 50-cm resin column (newly regenerated) of the amino acid analyzer. The material was chromatographed in system C^8 at a flow rate of 0.5 ml/min. At an effluent volume of 50 ml, 1-ml fractions were collected with a fraction collector and analyzed with ninhydrin.[9] The 2,4-DAB was eluted at 167–179 ml and was well separated from the preceding peak containing ornithine and lysine at 145–160 ml. Ten such runs afforded 127 μmol of 2,4-DAB. The eluate was freed of most of the salts with a column (26 × 1.7 cm) of Dowex 50W-X8 (H^+ cycle) resin,[10] and the material was eluted with 6 *N* NH_3 in the manner already described. Fractions appearing 8–16 ml after the breakthrough of base yielded 72 mg of solid containing 21% 2,4-DAB (125 μmol). This was electrophoresed in portions as a band on paper in pyridinium acetate buffer, pH 5.6.[8] The 2,4-DAB was located with ninhydrin and eluted with water; the eluate was then taken to dryness. The material was precipitated from aqueous solution at pH 5 with ethanol. The solid was dried and extracted into a small volume of water. The solution had 22 mg of solid containing 37% 2,4-DAB (69 μmol). To it was added 0.3 ml of 2 *N* HCl. It was then taken to dryness, and the residue was dissolved in water and adjusted to pH 5 with dilute pyridine. Crystallization was initiated from several drops of aqueous solution by addition of hot ethanol to give 7.5 mg of 2,4-DAB of 76% purity. Recrystallization from the same solvent gave 4.8 mg of light colored prisms of 2,4-DAB monohydrochloride of 98% purity on amino acid analysis in system B. On cochromatography with the authentic material on the analyzer in system A, a single peak emerged with 95% recovery. The material traveled as a single spot with the mobility of authentic 2,4-DAB when electrophoresed on paper in formate–acetate buffer, pH 1.8; pyridinium acetate buffer, pH 4.0 and 5.6; and sodium barbital buffer, pH 8.5. It had $[\alpha]^{25}D$ +21.6° (*c* 0.3, 5 *N* HCl); reference L-2,4-DAB·HCl had $[\alpha]^{25}D$ +24.2° (*c* 0.4) (lit.[11] $[\alpha]^{25}D$ +24.2° (*c* 2)).

Registry No—L-2,4-DAB, 1758-80-1; L-2,4-DAB monohydrochloride, 15765-10-3.

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(4) (a) In view of the presence of ϵ-(aminosuccinyl)lysine in partial acid hydrolysates of bacitracin A [D. L. Swallow and E. P. Abraham, *Biochem. J.*, **70**, 364 (1958); see also ref 5c], carbobenzoxy-L-aminosuccinimide was also subjected to dehydration, reduction, and hydrolysis, but formation of 2,4-DAB was not detected. (b) ϵ-(Aminosuccinyl)lysine is thus derived originally from the ϵ-(L-asparaginyl)-L-lysine sequence in the antibiotic, although ϵ-(α-L-aspartyl)-L-lysine and ϵ-(β-L-aspartyl)-L-lysine are capable of cyclizing to this succinimide derivative (see reference cited in 4a).

(5) (a) L. C. Craig, W. Hausmann, and J. R. Weisiger, *J. Biol. Chem.*, **199**, 865 (1952); (b) I. M. Lockhart and E. P. Abraham, *Biochem. J.*, **62**, 645 (1956); (c) for a review, see E. P. Abraham, "Biochemistry of Some Peptide and Steroid Antibiotics," John Wiley and Sons, Inc., New York, N. Y., 1957.

(6) D. L. Swallow and E. P. Abraham, *Biochem. J.*, **72**, 326 (1959).

(7) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(8) Bacitracin was the commercial preparation described previously.[2] Ethylene chlorophosphite was a gift of Hooker Chemical Co., Niagara Falls, N. Y. Dowex 50W-X8 and AG 50W-X2 cation-exchange resins (both 100–200 mesh) were obtained from Bio-Rad Laboratories, Richmond, Calif., and Calbiochem, Los Angeles, Calif. Infrared spectra were recorded on a Perkin-Elmer Model 137 spectrophotometer. Optical rotations were measured in a Perkin-Elmer Model 141 photoelectric polarimeter read against air. Amino acid analyses were obtained on the Beckman-Spinco Model 120 automatic amino acid analyzer with sulfonated styrene–8% divinylbenzene copolymer resin. Complete analyses of basic amino acids in hydrolysates of treated bacitracin were made on the 50-cm column, Type 50A resin, 25–31 μ, 0.38 *N* sodium citrate buffer, at pH 4.25 and 50°[2] (system A). Routine determinations of 2,4-DAB in isolates were done on the 15-cm column, Type 15A resin, 19–25 μ, with 0.35 *N* sodium citrate buffer, at pH 5.28 and 50°[7] (system B). Preparative chromatography was on the 50-cm column, Type 50A resin, with the latter buffer at pH 5.28 and 50° (system C). Preparative paper electrophoresis was carried out on 10-cm-wide strips of Whatman No. 3MM paper at 9 V/cm for 3.5 hr. Acid hydrolyses were carried out in 6 *N* HCl under nitrogen in an oil bath at 120° for 16 hr.

(9) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

(10) The resin was regenerated as described [S. Moore and W. H. Stein, *ibid.*, **192**, 663 (1951)], then converted with HCl into H^+ cycle and washed free of acid before use.

(11) S.-C. J. Fu, K. R. Rao, S. M. Birnbaum, and J. P. Greenstein, *ibid.*, **199**, 207 (1952).