[CONTRIBUTION FROM THE WESTERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION, AGRICULTURAL RESEARCH SERVICE, U. S. DEPARTMENT OF AGRICULTURE]

Isolation of Subtilin A

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A method is presented for isolating subtilin A on a preparative (2-g.) scale by partition chromatography on silica gel. The product was characterized by countercurrent distribution, ultraviolet and infrared spectra, paper electrophoresis, stability and antibiotic activity measurements.

Early published methods[1-3] of preparing subtilin based on partition and solubility properties were of a single-stage nature and thus might be expected to be less efficient in resolving small contents of closely related peptides than more recent multistage processes. By means of paper electrophoresis Sacks and Pence[4] recently showed qualitative heterogeneity of samples of subtilin of both high and medium original biological activity. Brink, Mayfield and Folkers[5] subjected subtilin to separatory-funnel countercurrent distribution in *n*- and *sec*-butyl alcohol–water. They were able to show an approximate 10% increase in specific antibiotic activity at the distribution peak, but judgments of homogeneity were complicated by the small number of transfers and a low recovery of solids with resultant narrowing of the experimental curve (probably arising from a more or less constant weight loss per tube in recovery of solids by a precipitation method)

In this investigation both 10-year-old samples of low original activity and freshly grown subtilin have been examined by the high-resolution multistage processes of chromatography and countercurrent distribution. A pool of the 70% alcohol-soluble portion of a large stock of various samples 10 to 15 years old and of relatively low activity, accumulated during early phases of previously published work, was used in developing the preparative partition chromatographic method and countercurrent distribution systems of this report. This old preparation showed at least 6 components by hanging-curtain electrophoresis.[4] Countercurrent distribution in system 4 of Table I indicated about a 50% content of biologically active subtilin A[6] with several other lesser components spread over the train. By successive countercurrent distribution in systems 4 and 6, retaining only the central section of the main peak each time, it was possible to obtain material with a fair approximation to theoretical behavior but at a great sacrifice in yield. The countercurrent distribution approach to separation of the components of the low-activity preparation revealed severe emulsification which could, however, be controlled by inclusion of a high content of acetic acid in aqueous two-phase systems of the butanols.

From a preparative standpoint the application of the resolving power of a two-phase liquid system in the form of a partition chromatogram has certain advantages over a countercurrent distribution application of the system in that chromatographic columns are readily scaled up and the disturbing effects of emulsification are avoided.

TABLE I

COUNTERCURRENT DISTRIBUTION SYSTEMS

System no.	Composition	*K* for subtilin
1	*n*-BuOH (1); water (1)	0.5
2	20% HOAc, (5); *n*-BuOH, (4)	.56, 0.55
3	20% HOAc, (3); iso-BuOH, (2)	.33, 0.30, 0.28
4	20% HOAc, (1); *n*-BuOH, (1)	.45
5	4% HOAc, (6); *sec*-BuOH, (5)	.33
6	20% HOAc, (3); *n*-BuOH, (2)	.4

The commonly used Celite support failed to hold either phase of butanol–water immobile in the presence of subtilin. However, a special grade of commercially available silica gel did hold about an equal weight of lower phase. Preparative (2-g. loads) partition chromatograms of the old low-activity preparation in water–*n*-butyl alcohol and water–*sec*-butyl alcohol showed heterogeneity similar to that revealed by countercurrent distribution of the preparation in system 4. The prominent (~60%) subtilin A peak was substantially freed of emulsifiers by the chromatography and after two countercurrent distributions in butanol–water showed fair approximation to theoretical behavior.

Partition chromatography of subtilin isolated from a new fermentation by published methods showed it to be a much less complex mixture than the pool of stored low-activity material. As shown in Fig. 1, about 85% of the newly prepared material was subtilin A, which was widely separated (about 8 standard errors, peak to peak) from two faster-moving minor components. The presence of the faster of these, an alcohol-soluble, water-insoluble substance with an ultraviolet spectrum unlike subtilin, is due to not having applied the alcohol extraction step in the isolation procedure.[3] The other minor component of Fig. 1 behaves electrophoretically like the material called subtilin B by Sacks and Pence.[4] The subtilin A provides over 98% of the assayed antibiotic activity of the preparation, since the specific activities of the two minor components are very low.

As shown in Fig. 2A, the chromatographed subtilin A gives a fair approximation to binomial

(1) K. P. Dimick, G. Alderton, H. D. Lightbody and H. L. Fevold, *Arch. Biochem.*, **15**, 1 (1947).

(2) H. L. Fevold, K. P. Dimick and A. A. Klose, *ibid.*, **18**, 27 (1948).

(3) H. Lineweaver, A. A. Klose and G. Alderton, *ibid.*, **16**, 311 (1948).

(4) L. E. Sacks and J. W. Pence, *Anal. Chem.*, **29**, 1802 (1957).

(5) N. G. Brink, J. Mayfield and K. Folkers, THIS JOURNAL, **73**, 330 (1951).

(6) The nomenclature of Sacks and Pence,[4] designating the major component of high biological activity as subtilin A and the next most abundant component with a characteristic slope on paper electrophoresis as subtilin B, is followed here.

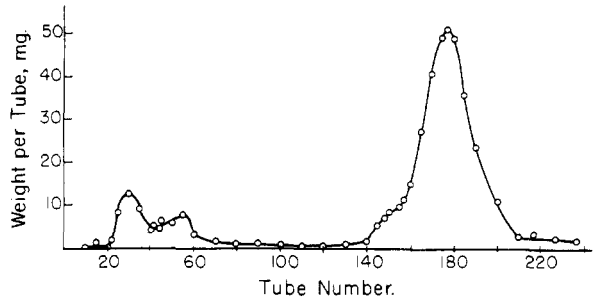

Fig. 1.—Preparative silica gel partition chromatogram of subtilin preparation no. 2.

behavior on countercurrent distribution in system 2. The specific antibiotic activity of the peak (tube 65) and the two shoulders (55 and 75) were identical. Figure 2B shows an analytical redistribution of subtilin A in system 3. In this distribution, which followed the fitted binomial better than Fig. 2A, a trend toward lower specific anti-

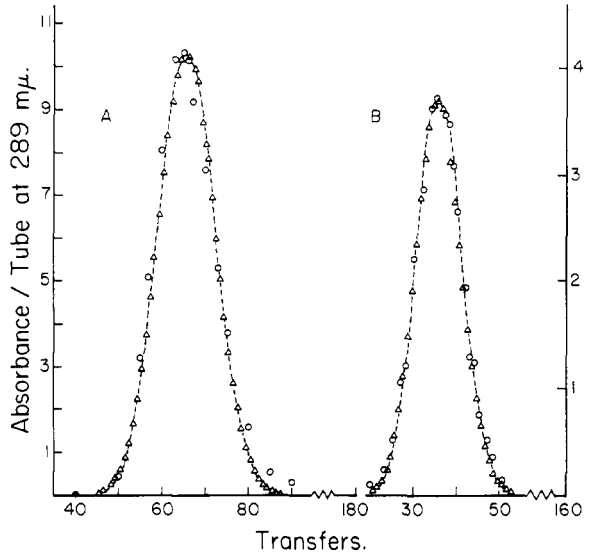

Fig. 2.—A, countercurrent distribution of chromatographed subtilin A; B, analytical redistribution of subtilin A; O, experimental; Δ, binomial curve.

biotic activities on the shoulders appeared which was probably beyond assay variation. The chromatographed subtilin A shows only one component in hanging-curtain electrophoresis under the conditions of Sacks and Pence.[4] A sample of the above chromatographed subtilin A was furnished to Dr. L. C. Craig, who confirmed[7] its one-component behavior in solvent system 2.

Subtilin A is relatively stable, since dried samples stored over 10 years at about 5° retained most of their activity. After 10 years the sample no. 215-0 showed a biological activity 90% as high as that of chromatographed subtilin A. Allowing for the presence of a small content of essentially inactive subtilin B which the former[2,3] methods of isolation left in the preparation, this indicates a near quantitative retention of activity for this extended period.

(7) A. Stracher and L. C. Craig, THIS JOURNAL, **81**, 696 (1959).

The data of Table II indicate a fairly high light stability at 400 μg./ml. for 3 days in water, 70% alcohol, and countercurrent distribution systems 1 and 4. Considering the variability of the bioassay, the effects of the treatments are small. At 36 μg./ml. in water, a 3-day exposure at 25° to strong, south, glass-transmitted sunlight destroyed about half the activity.

TABLE II

STABILITY OF SUBTILIN A

Environment	Activity retained after 3 days, % 70% alcohol	*n*-BuOH–water	*n*-BuOH–20% HOAc
25°, dark	92	83	101
0°, dark	84	111	100
−18°, dark	93	103	110
25°, *north light*	90	87	95

The cylinder plate antibiotic assay has a much higher variability between than within assays. In this method the low activity preparation no. 1, the newly isolated preparation no. 2, and a 10-year-old stored sample of high original activity (no. 215-0) showed about the same slope of dosage response curve as the purified subtilin A. In a liquid medium assay with *Streptococcus agalactiae*, however, differing slopes suggest that extensive interaction of components occurred, as Lewis[8] noted with crude preparations, obviating precise comparisons of specific activities among multicomponent preparations. Within the limitations of the cylinder plate assay variation, the degree of enhancement of antibiotic activity is approximately in agreement with the degree of purification indicated by chromatography. The specific activity of cuts from the central section of the subtilin B peak from a silica gel partition column was only about 3% of that of subtilin A. The other minor component was essentially inactive. By cylinder plate assay the relative antibiotic activities of subtilin A, preparation no. 2, and the crude preparation no. 1 were approximately 130, 100, 70.

In the ultraviolet absorption spectrum of subtilin A (Fig. 3) the tryptophan bands account for the absorption down to about 270 mμ. The absorption in 225–250 mμ region appears too high to be accounted for by peptide bonds and suggests the possibility of an uncommon linkage in the molecule. In bacitracin the absorption of the thiazoline linkage on the long wave length side of the peptide bond absorption is destroyed by heating 20 minutes at 100° in 0.5 *N* HCl.[9] Heating under much more severe conditions (2.2 *N* HCl at 100° for 1 hour) fails to eliminate the unassigned absorption of subtilin A, although it is diminished. Calculation of tryptophan content by a single absorbance reading at 289 mμ gives an average value of 5.9% tryptophan for a series of subtilin A peaks from chromatograms. The minimal molecular weight calculated from the tryptophan analysis is 3,460, in agreement with Lewis and

(8) J. C. Lewis, E. M. Humphreys, P. A. Thompson, K. P. Dimick, R. G. Benedict, A. F. Langlykke and H. D. Lightbody, *Arch. Biochem.*, **14**, 437 (1947).

(9) L. C. Craig, J. R. Weisiger, W. Hausmann and E. J. Harfenist, *J. Biol. Chem.*, **199**, 259 (1952).

Snell[10] from microbiological assays of other amino acids. The spectrophotometric tryptophan content of material from the subtilin B peak is much lower (~3.8%).

Experimental

Starting Materials. Low-activity Stored Material (Preparation No. 1).—Samples which had been stored at about 5° for periods upward of 10 years were pooled and the 70% ethyl alcohol-soluble portion (~85 g.) used as raw material in developing the partition chromatographic methods and countercurrent distribution systems of this work.

Newly Fermented and Isolated Subtilin (Preparation No. 2).—We are indebted to Mr. K. Ijichi of this Laboratory for growing a 150-liter culture of *B. subtilus* (ATCC 6633) on a synthetic medium by the method and in the equipment described by Garibaldi and Feeney.[11] Subtilin was isolated from the culture by a modification of previously published methods from this Laboratory[3] involving NaCl dehydration at *p*H 5 of an acid-*n*-butyl alcohol extract of the culture. After washing out excess solid NaCl from the subtilin precipitate with 10% NaCl the filter cake was mixed with 7 times its weight of water and again filtered to reduce the NaCl content[2] and finally vacuum dried without ethanol extraction to yield 23.5 g. of subtilin, representing a 55% yield of activity based on the butanol-culture emulsion.

Hanging-curtain electrophoresis of this preparation on a Karler Misco apparatus under the conditions of Sacks and Pence[4] showed a major component corresponding to subtilin A and a minor component with the slope of subtilin B. The preparation showed a biological activity about 25% higher than the no. 326 used by Sacks and Pence,[4] which in turn had an original activity at the time of preparation about 75% of that of the best preparations such as the no. 215-0 sample mentioned by them.

Paper Chromatography.—A search for solvent systems which would resolve subtilin components on paper was carried out with a view toward scaling up the process on cellulose columns as well as for analytical purposes. When tried on the old low-activity subtilin (preparation no. 1) 16 out of 43 solvent systems gave some degree of resolution though accompanied by serious streaking or tailing. One solvent [*n*-propyl alcohol (18), benzene (5), ethylene glycol (1.5), HOAc (1), water (6)] did separate the preparation into two clear-cut zones, but on rechromatography either by changing the direction of migration or by elution and rerunning, the major component proved unstable as shown by its separation into the same two zones given by the original sample.

Ion Exchange Chromatography.—No resolution of the stored, low-activity subtilin was obtained by elution chromatography on the carboxymethyl cellulose ion exchanger of Peterson and Sober[12] when developed with ammonium acetate buffers. Likewise, elution chromatography on IRC 50 with dilute aqueous acetic acid developers failed to give clear resolution.

Partition Chromatography on Silica Gel.—Equal volumes of redistilled *n*-butyl alcohol and glass-distilled water were equilibrated at room temperature. Six hundred twenty ml. of the lower phase were mixed by thorough kneading and stirring with 700 g. of silica gel (Mallinckrodt label, prepared by the method of Ramsey and Patterson). When the mixture had a uniform "dry" appearance it was stirred for about 30 minutes with all of the upper phase which would be used as developer (~ 3,500 ml.). After settling under gravity most of the liquid phase was decanted and retained as developer while the gel slurry was poured to give a 7.5 × 36 cm. column. The sample was prepared by dissolving 2 g. of subtilin in 225 ml. of the developer warmed to about 40°. With the 10-year-old, low-potency sample about 350 mg. of material of very low activity remained insoluble and was separated by centrifuging. With the newly isolated preparation no. 2 about 70 mg. remained insoluble, because the preparation had not been extracted with ethyl alcohol. After the sample had drained into the column, developer was allowed to flow at about 70 ml./hour and was collected in 22-ml. cuts in a GME constant-volume fraction collector. Effluent concentrations were followed by dry weights or by ultraviolet absorption scanning in a Cary model 14 Spectrophotometer in 70% ethyl alcohol. Figure 1 shows a typical elution curve of a 2-g. load of the newly isolated preparation no. 2. Although on re-use with water-saturated *n*-butyl alcohol as developer the resolution gradually deteriorated, the columns could be used at least 4 times without serious loss of resolution.

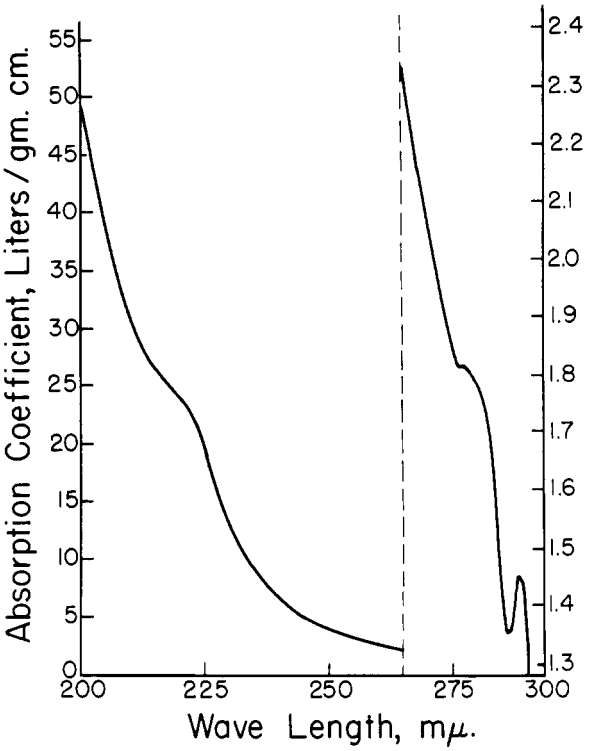

Fig. 3.—Ultraviolet spectrum of subtilin A in 70% ethanol.

Partition Chromatography on Celite.—Trials of partition chromatography on Celite with the system *n*-butyl alcohol (1), 20% HOAc (1) were unsuccessful with either phase mobile in that the presence of subtilin appeared to bring about leaking of the immobile phase.

Countercurrent Distribution.—Countercurrent distributions were carried out in the all-glass automatic apparatus of Craig[13] in a constant-temperature room (72–73°F.). Equal phase volumes (10 ml.) of solvents equilibrated at the operating temperature and robot operation were used. All components of the solvent systems were distilled from glass to eliminate non-volatile ultraviolet absorbers. Table I shows the composition of several systems in which countercurrent distributions have been run. The aqueous HOAc solutions were made on a volume basis, *i.e.*, 20 ml. of glacial HOAc plus water to 100 ml. As used, the butanols, water and HOAc form near-critical two-phase systems[14] in which the ratio of phase volumes is quite sensitive to small changes in both composition and temperature.

Only subtilin A which had been prepared by partition chromatography could be distributed in the water–butanols systems in the absence of a high content (15–20%) of acetic acid without severe emulsification. Even after chromatography, variation of indicated partition coefficients in system 1 between runs suggested emulsification effects though none were detected visually.

For analysis of the subtilin content of a whole tube from the distribution train, sufficient ethyl alcohol was added to give a single phase and the resulting solution evaporated to dryness at <40° under oil pump vacuum with Dry Ice trap. Ultraviolet spectra were scanned in a Cary (model 11 or 14)

(10) J. C. Lewis and N. S. Snell, THIS JOURNAL, **73**, 4812 (1951).

(11) J. A. Garibaldi and R. E. Feeney, *Ind. Eng. Chem.*, **41**, 432 (1949).

(12) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

(13) L. C. Craig and D. Craig, in "Technique of Organic Chemistry," Vol. III, Part I, ed. 2, Chapter II, Interscience Publishers, Inc., New York, N. Y., 1956.

(14) P. Van Tavel and R. Signer in "Advances in Protein Chemistry," Vol. XI, Academic Press, Inc., New York, N. Y., 1956.

spectrophotometer on 70% ethyl alcohol solutions of the dried material.

Figure 2A shows the distribution pattern obtained in the *n*-BuOH (4), 20% HOAc (5) system on a 138-mg. sample from the central section of the subtilin A peak of a silica gel partition chromatogram corresponding to that of Fig. 1. The sample was loaded in two tubes. Figure 2B shows an analytical redistribution from a single tube of a 50-mg. sample of subtilin A in the system isobutyl alcohol (2), 20% HOAc (3). The sample had been chromatographed as above and distributed for 130 transfers in system 2 and for 200 transfers in system 3 where a skewed distribution pattern was obtained at a higher concentration.

Theoretical curves for both single and multiple tube loadings were constructed from a table of the cumulative binomial.[15]

Optical Rotation.—Optical rotation was determined with sodium light in 1% aqueous HOAc at 25° in Rudolph polarimeter with photoelectric field matcher. The newly grown preparation no. 2 showed $[\alpha]^{26}D$ −35.2° (*c* 0.186). For a cut from the center of the subtilin B peak from a chromatogram like that of Fig. 1 $[\alpha]^{25}D$ −44.3° (*c* 0.0524). Chromatographed subtilin A had $[\alpha]^{26}D$ −34.4° (*c* 0.228).

Stability Tests.—Subtilin A isolated by partition chromatography from preparation no. 2 was exposed for 3 days at 400 μg./ml. to the solvents and conditions of light and temperature listed in Table II. After exposure the samples were evaporated to dryness under oil-pump vacuum and assayed for antibiotic activity.

Antibiotic Activity Measurement.—Antibiotic activity of subtilin fractions was measured by cylinder-plate assay with *Arthrobacter citreus*, ATCC 11,624, a bacterium used for some time in this Laboratory for assay purposes. Cells from Nutrient Agar (Difco) slants incubated 20 to 24 hours at 27° were suspended in 0.8% NaCl to an optical density of 0.4 at 650 mμ in 18-mm. test-tubes in a Coleman model 11 spectrophotometer. Assay plates (9-cm. diameter), prepared fresh for each assay, contained two 10-ml. layers of Nutrient Agar (1.5 and 1% agar, respectively, in lower and upper layers) with 3% NaCl added. The upper layer was inoculated immediately before dispensing, 1 ml. to 50 ml., with the 0.8% NaCl suspension of the test organism.

Subtilin was tested in aqueous solution in 8-mm. diameter stainless-steel cylinders. Assays were incubated 16 to 20 hours at 35°. For the most part the assays followed established procedures which have been discussed adequately in the literature.[16] A helpful variation was use of only 1% agar in the upper layer of plate medium, permitting slight sinking and improved sealing of cylinders. Assay sensitivity was increased by retarding growth of the test organism with inclusion of 3% NaCl in the medium and with incubation at higher than optimum temperature. Caution is required in the use of stainless-steel cylinders. We observed considerable destruction of subtilin activity in blackened or in very slightly corroded cylinders. Cylinders discolored by sulfuric acid–dichromate cleaning solution, even after subsequent repeated and prolonged washings, caused reductions in activity as great as 50% when used in assays. Contact with such cylinders for 24 hours preceding assay destroyed almost all the activity in weak aqueous solutions of subtilin. Excessive replication was necessary in assays until this factor was discovered.

Infrared Spectra.—The infrared spectrum of 0.98 mg. of chromatographed subtilin A in a 12.5-mm. (247 mg.) KBr pellet was scanned in a Beckman IR-3 instrument with rock salt optics. Strong bands were found at 3.05, 6.01 and 6.57 μ; medium at 7.21, 7.48, 7.78 and 8.13 μ; weak at 9.55 and 13.40 μ.

Acknowledgment.—We are indebted to Glen Bailey and Edith Gong for many ultraviolet spectra and the infrared spectrum, and to L. E. Sacks for paper electrophoresis.

(15) Harvard University Staff of the Computation Laboratory, "Tables of the Cumulative Binomial Probability Distribution," Harvard University Press, Cambridge, Mass., 1955.

(16) J. J. Gavin, *Appl. Microbiol.*, **5**, 25 (1957).

ALBANY, CALIF.

[CONTRIBUTION FROM THE MEDICINAL CHEMICAL RESEARCH DEPARTMENT OF THE SCHERING CORPORATION]

Parasympathetic Blocking Agents. III. N-Alkylpiperidinecarboxylic Esters[1]

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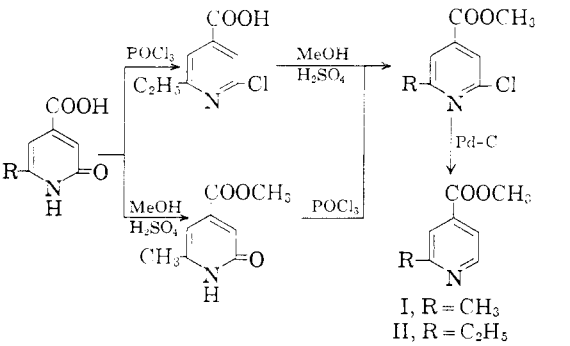
A number of pyridinecarboxylic esters have been prepared and quaternized with alkyl *p*-toluenesulfonates and alkyl halides. The hydrogenation of the crude quaternary salts with platinum oxide or Raney nickel yielded N-alkylpiperidinecarboxylates, intermediates in the synthesis of compounds of potential pharmacological interest.

As part of a program on the synthesis of parasympathetic blocking agents related to N-methyl-4-benzhydrylidenepiperidine,[1a] a number of ring substituted N-alkylpiperidinecarboxylates were required for the synthesis of the intermediate N - alkyl - α,α - diphenylpiperidinemethanols. Although the lower N-alkylpiperidine esters have been described previously, the ring substituted and higher N-alkylpiperidine esters have not been reported.

In general, the requisite pyridine ester (Table I) were prepared according to known procedures with slight modifications. The preparation of methyl 2-methylisonicotinate (I) and methyl-2-ethylisonicotinate (II) is illustrated by a series of reactions similar to those employed by Tracy and Elderfield,[2] for the preparation of 2-ethylisonicotinic acid. In a similar manner ethyl 6-isobutylnicotinate and ethyl 2-chloro-6-isopropylnicotinate were



prepared from the corresponding 3-carboxy-6-isobutylpyridone-2[3] and 3-carboxy-6-isopropylpyridone-2, respectively. The requisite pyridones

(1) (a) Part I, N. Sperber, F. J. Villani, M. Sherlock and D. Papa, THIS JOURNAL, **73**, 5010 (1951); (b) Part II, S. Coan and D. Papa, *J. Org. Chem.*, **20**, 774 (1955).

(2) A. H. Tracy and R. C. Elderfield, *ibid.*, **6**, 70 (1941).

(3) R. P. Mariella, THIS JOURNAL, **69**, 2670 (1947).