[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION[1]]

Antibiotics Against Plant Disease. III. Duramycin, A New Antibiotic from *Streptomyces cinnamomeus* forma *azacoluta*[2]

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Duramycin, a polypeptide antibiotic, has been obtained from culture filtrates of *Streptomyces cinnamomeus* forma *azacoluta*. This antibiotic is active against gram-positive rods and some yeasts and fungi. Initial studies indicate that the antibiotic contains at least nine amino acids, two of which contain sulfur. A crystalline picrate has been prepared.

Duramycin, a new antibiotic, has been isolated from culture broths of *Streptomyces cinnamomeus* forma *azacoluta* (NRRL B-1699),[3,4] an isolate known to produce factors active against plant diseases.[5] The antibiotic, temporarily designated Antibiotic B,[4] has been named duramycin because of its stability.

Preliminary studies on the culture filtrates indicated that duramycin was very stable to heat in *p*H range 3–9. It was not adsorbed appreciably by Amberlites IRC-50, IRA-400, IR-4B, MB-3 and IRA-401[6] probably because of the size and shape of the molecule; however, it passed through a dialysis membrane. Of the many organic solvents tried, only 1-butanol was effective for extraction of duramycin from culture filtrates.

The crude antibiotic isolated by butanol extraction was purified by chromatography on acid-washed alumina columns. Eighty per cent. methanol was used as both solvent and developer; the last fraction was eluted with 50% methanol. The following methods of purification failed to increase the potency as measured by disk assay against *Bacillus subtilis* or *Corynebacterium fascians:* deionization on a monobed ion-exchange resin, countercurrent distribution, large-scale paper chromatography or the formation of a crystalline alcoholate. A crystalline picrate of duramycin was prepared which was insoluble in absolute methanol, but which was crystallized from aqueous ethanol. Also, a helianthate was prepared from which the antibiotic could be regenerated with a monobed ion-exchange resin. Duramycin regenerated from either the helianthate or the recrystallized picrate was no more active than material obtained by chromatography on alumina columns.

Attempts to prepare crystalline duramycin failed. The antibiotic was readily soluble on heating in absolute methanol, water–methanol and water–ethanol, but it had a marked tendency to form gels and amorphous precipitates, especially if cooled rapidly. Crystals could be obtained by cooling 80% methanol solutions over a period of several days. On filtration, however, the solid lost its crystalline form and became an amorphous powder. Crystals formed upon addition of methanol to the powder, indicating that the crystalline product in 80% methanol is an alcoholate. Attempts to prepare more stable alcoholates from alcohols of higher molecular weight failed.

Duramycin obtained from hydrochloric acid-washed alumina columns was probably the hydrochloride. In this form it was soluble in water, and aqueous acetone, methanol and ethanol, but only slightly soluble in absolute alcohols. Deionization decreased its solubility in water. The antibiotic was highly active in lowering surface tension. Duramycin had an infrared spectrum typical of a polypeptide with bands at 3.06, 6.00 and 6.55 μ characteristic of the peptide bond and at 14.33 μ characteristic of a polypeptide.[7] It did not absorb in the ultraviolet, indicating the absence of tyrosine and tryptophan. The antibiotic from alumina columns had a specific rotation of $[\alpha]^{25}D$ $-6.4°$ (*c* 3.9, water) and had no definite melting point. Potentiometric titration indicated that it contained at least one free amino group and several free carboxyl groups. The presence of more than one carboxyl group also was shown by the compound's behavior on paper electrophoresis and a Van Slyke determination established the presence of free amino nitrogen.

Duramycin, of course, gave a positive biuret test. It gave negative ferric chloride, Benedict, Molisch, periodic acid, Millon, nitroprusside, Pauly, Sakaguchi, xanthoproteic and Hopkins–Cole tests. It gave a positive azide–iodine test, which indicates the presence of a sulfur-containing amino acid. Nine or ten spots that reacted with ninhydrin were obtained on paper chromatography of acid hydrolysates. These chromatograms showed that lanthionine, either β-methyllanthionine or cystathionine, aspartic acid, glycine, glutamic acid, proline, valine, phenylalanine and possibly ornithine and hydroxyproline could be present in the antibiotic. β-Methyllanthionine and cystathionine could not be differentiated by paper chromatography. The absence of cystathionine in duramycin was established by microbiological assay using a cystathionine-requiring *Neurospora* mutant.

The organism which forms duramycin is a physiological form of *Streptomyces cinnamomeus* that produces cinnamycin. Closely related in their chemical and antimicrobial properties, duramycin and

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(2) Presented before the Division of Organic Chemistry at the 131st National Meeting of the American Chemical Society, Miami, Fla., April 9, 1957.

(3) L. A. Lindenfelser, T. G. Pridham, O. L. Shotwell and F. H. Stodola, "Antibiotics Annual 1957–1958," Welch and Marti-Ibañez, Medical Encyclopedia, Inc., New York, N. Y., in press.

(4) T. G. Pridham, O. L. Shotwell, F. H. Stodola, L. A. Lindenfelser, R. G. Benedict and R. W. Jackson, *Phytopathol.*, **46**, 575 (1956).

(5) T. G. Pridham, L. A. Lindenfelser, O. L. Shotwell, F. H. Stodola, R. G. Benedict, C. Foley, R. W. Jackson, W. J. Zaumeyer, W. H. Peterson, Jr., and J. W. Mitchell, *ibid.*, **46**, 568 (1956).

(6) Mention of commercial products does not imply that they are recommended over others of similar quality.

(7) E. R. Blout and S. G. Lindsley, THIS JOURNAL, **74**, 1946 (1952).

cinnamycin exhibit similar behavior on paper electrophoresis. Paper chromatograms of acid hydrolysates show that duramycin contains many of the amino acids which are present in cinnamycin.[8] The two antibiotics in admixture could be seprated in 53 hours on paper strips using water-saturated 1-butanol.

Experimental

Assay.—Duramycin was assayed either against *Bacillus subtilis* NRRL B-765 or *Corynebacterium fascians* NRRL B-190 by disk assay.[4]

Isolation of Crude Duramycin.—The fermentation broths[4] produced in Fernbach flasks were filtered through a coarse cloth. The clear filtrate (53 l.) was extracted in a Podbielniak extractor with 1-butanol. To the butanol extract (36 l.) was added heptane (18 l.) causing the separation of a water layer (3.48 l.) that contained most of the antibiotic. The heptane–1-butanol mixture was washed with distilled water (10 l.), and the combined water layer and wash were concentrated *in vacuo* to replace the butanol with water. Lyophilization of the concentrated water solution (3.5 l.) yielded 26.7 g. of a product that was 12 times as active as the dried culture liquor solids.

Alumina Column Chromatography.—Crude duramycin (6.50 g.) was dissolved in 80% methanol (250 ml.), and the solution was filtered. The filtrate was used as a charge for the alumina column. Harshaw alumina was adjusted to *p*H 4.7 with hydrochloric acid, washed with water and packed as a water slurry in a Pyrex column (diameter 2.6 cm., height 39 cm.). The column was washed with water until the eluate was clear and then with 1 l. of 80% methanol. The charge was placed on the column, and two 50-ml. portions of 80% methanol were used to wash the charge into the column. The column was then eluted with 80% methanol. The final fraction was collected using 50% methanol as the influent to remove the last traces of antibiotic.

Fractions 1 and 20–23 were collected under gravity; the remainder were collected under pressure at a rate of 2–3 ml./min. Active material was located by taking a 2-ml. aliquot of each fraction to dryness and dissolving in distilled water (2 ml.) for microbiological assay against *B. subtilis*. Appropriate fractions were combined, concentrated *in vacuo* to remove methanol and lyophilized. This experiment is summarized in Table I. Material (1.80 g.) isolated from fractions 8–20 and products of similar activity from other alumina columns were used in further studies.

TABLE I

PURIFICATION OF DURAMYCIN BY ALUMINA COLUMN CHROMATOGRAPHY

Fractions	Volume, ml.	Original activity, %	Residue from fractions, g.	Concentration
1	890			
2–7	326			
8–20	1054	51	1.80	1.9X
21–23	2710	35	1.45	1.6X
Total	4980	86	3.25	

Countercurrent Distribution of Duramycin.—A countercurrent distribution of duramycin (488 mg.) was carried out in eight separatory funnels using the solvent 2-butanol–0.01 *N* ammonium hydroxide.[9] Equal volumes (40 ml.) of each phase were used and the lower phase was transferred. Contents of the separatory funnels containing a high concentration of duramycin had to be centrifuged to break the emulsions before transfers. The tendency of the antibiotic to foam and to form emulsions indicated that it was highly effective in lowering surface tension. At the end of the distribution the contents of each funnel were concentrated *in vacuo* to remove the butanol, and the resulting water solutions were lyophilized. The results of the distribution are shown in Fig. 1. Separatory funnels 5 and 6 contained most of the antibiotic (311 mg.) and represented 64% of the original activity and 64% of the original weight.

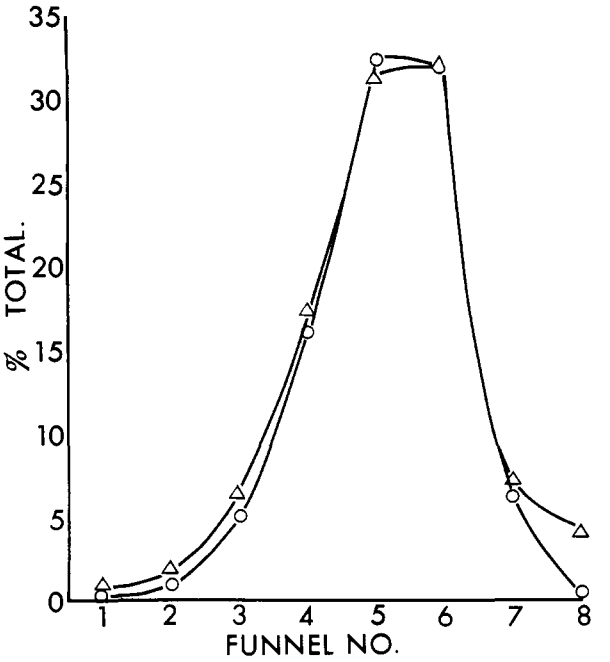

Fig. 1.—Countercurrent distribution of duramycin between 2-butanol and 0.01 *N* ammonium hydroxide: O——O, *B. subtilis* activity; Δ——Δ, weight. The lower phase was moved.

A 54-tube countercurrent distribution of duramycin was performed in the apparatus described by Craig and Post[10] using the solvent system 1-butanol–ammonium acetate (0.2 *M*) (*p*H 5.35).[11] Duramycin (450 mg.) was dissolved in buffer solution (42 ml.) and equilibrated with butanol (42 ml.). The two phases were separated and made up to 45 ml. with equilibrated solvents. Equal volumes (9.68 ml.) of the phases from the above distribution were placed in tubes 0–3. The remainder of the tubes contained 9.68 ml. of each phase. After distribution, the contents of each tube were assayed against *C. fascians* by combining 1-ml. aliquots of each phase, drying and dissolving in ammonium acetate buffer (5 ml.). The distribution curve obtained is shown in Fig. 2; 97% of the antibiotic activity was found in tubes 22–37. The solutions in these tubes were combined, concentrated to remove the butanol and passed through an Amberlite MB-3 column to remove ammonium acetate. Lyophilization of the deionized solution resulted in a product (170 mg.) that was just as active as the starting material.

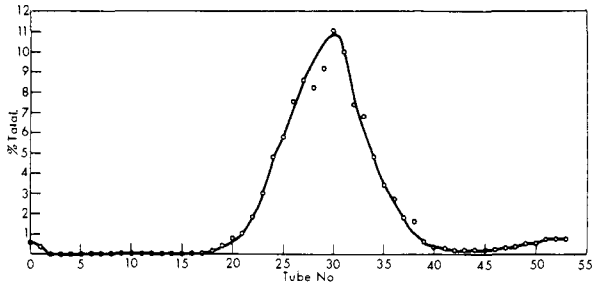

Fig. 2.—Countercurrent distribution of duramycin between 1-butanol and ammonium acetate (0.2 *M*) (*p*H 5.35): O——O, *C. fascians* activity.

Picrate of Duramycin.—Duramycin picrate was formed

(8) W. Dvonch, O. L. Shotwell, R. G. Benedict, T. G. Pridham and L. A. Lindenfelser, *Antibiotics & Chemotherapy*, **4**, 1135 (1954).

(9) J. G. Pierce, S. Gordon and V. du Vigneaud, *J. Biol. Chem.*, **199**, 929 (1952).

(10) L. C. Craig and O. Post, *Anal. Chem.*, **21**, 500 (1949).

(11) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **195**, 669 (1952).

by heating the product from alumina columns (300 mg.) in water (30 ml.) with picric acid-saturated water (4.5 ml.) and hot absolute methanol (3.0 ml.) containing picric acid (160 mg.). The picrate was washed twice with water and with methanol–anhydrous ether (1:5). A crystalline picrate (195 mg.) was obtained by cooling a boiling 50% ethanol solution (6 ml.) of the crude product over a period of several days. Recrystallization resulted in a slightly hygroscopic product (58 mg.), m.p. 212–245° dec., that was insoluble in absolute methanol and soluble in water-saturated 1-butanol.

Anal. Found: C, 51.30; H, 5.76; N, 16.85; S, 3.18.

The recrystallized picrate (19.8 mg.) was triturated with methanol (0.5 ml.) and 6 *N* hydrochloric acid (1 drop) to form duramycin hydrochloride. The white hydrochloride was washed with methanol–anhydrous ether to remove picric acid and excess hydrochloric acid. The regenerated hydrochloride (17.8 mg.) was no more active than the starting material.

Helianthate of Duramycin.—A helianthate of duramycin was prepared by treating the product from alumina columns (28.3 mg.) in 50% methanol (0.6 ml.) with water (2 ml.) containing methyl orange (31.6 mg.) at 70°. The helianthate was washed with hot water to remove excess methyl orange. The antibiotic was regenerated by stirring the helianthate in water with Amberlite MB-3. This water solution was filtered and lyophilized to obtain duramycin (20 mg.) that had the same activity as products obtained from alumina columns.

Alcoholate of Duramycin.—Duramycin (100 mg.) was dissolved in boiling 80% methanol (2 ml.) and was placed in a Dewar flask filled with hot water to cool slowly from an initial temperature of 56°. Long needle-shaped crystals appeared in 19 hours at which time the temperature registered 30°. Crystallization was essentially complete in 3 days. When the solid was collected by filtration, it lost its crystalline form and became an amorphous powder (40 mg.) with no increase in activity over the starting material.

Paper Chromatography and Paper Electrophoresis.—Paper chromatograms of duramycin and cinnamycin in admixture were run by descending technique on Whatman No. 1 paper strips ($1/2'' \times 17\ 3/4''$) using water-saturated 1-butanol. *B. subtilis* was the organism used to locate the antibiotics in the bioautographs. In 15 hours, cinnamycin (R_f 0.17) and duramycin (R_f 0.05) were not separated, but in 33 hours the two antibiotics were separated completely. On being subjected to paper electrophoresis in a Foster-type[12] apparatus, duramycin moved on Munktell No. 20 paper −6.2 cm. at *p*H 8.8, −2.9 cm. at *p*H 7.3, −2.8 cm. at *p*H 6.6, −1.4 cm. at *p*H 5.0 and −0.93 cm. at *p*H 2.8 in 18 hours at 128–131 volts (across paper) and 10 milliamp. The above movements were corrected for electro-osmosis. The barbital buffers of Michaelis[13] were used at an ionic strength of 0.10.

A large-scale paper chromatogram of duramycin (50 mg.) was run by descending technique on Whatman No. 3 paper ($11'' \times 17\ 3/4''$) using 1-butanol–pyridine–water (6:4:3) as developing solvent. Areas containing the antibiotic were located by bioautograph of narrow paper strips using *B. subtilis* as the assay organism. Duramycin eluted from the paper was no more active than the starting material, and only 50% of the antibiotic was recovered.

Acid Hydrolysis of Duramycin.—Acid hydrolysis of duramycin (9.5 mg.) was accomplished by heating at 109° with 6 *N* hydrochloric acid (2 ml.) in a sealed tube for 48 hours. Hydrochloric acid was removed from the hydrolysates by repeated evaporation of water solutions *in vacuo*. The resulting residue was dissolved in water (0.3 ml.) for paper chromatography.

Paper chromatograms using the descending technique on paper strips $1/2'' \times 17\ 3/4''$ (Whatman No. 1) with various solvent systems had 9 to 11 spots that reacted with ninhydrin. Papers were sprayed with 0.1% ninhydrin in ethanol containing 5% v./v. collidine[14] to develop differentiating colors. Results are shown in Table II.

Papers were also sprayed with 0.2% isatin in 4% glacial acetic acid–1-butanol after development to indicate the presence of proline and hydroxyproline. These results are shown in Table III.

Microbiological Determination.—*Neurospora* mutant NRRL A-5222 (36104-A)[15] was used to show the absence of L-cystathionine or its optical isomers in duramycin. The amino acids in hydrochloric acid hydrolysates were racemized[16] by dissolving the hydrolysate from duramycin (60 mg.) in 1 *N* sodium hydroxide (0.6 ml.) and adding acetic anhydride (0.3 ml.). After standing 2 days at 33°, the mixture was refluxed 3 hours with 6 *N* hydrochloric acid (3 ml.). After repeated evaporations from water solution *in vacuo* to remove acid, the residue was dissolved, neutralized and tested on the *Neurospora* mutant. The racemized hydrolysate of duramycin did not show any L-cystathionine activity.

TABLE II

R_f VALUES OF AMINO ACIDS PRESENT IN DURAMYCIN HYDROLYSATES

Amino acid	Color of ninhydrin spot	*t*-Butyl alcohol–acetic acid–water (2:1:1)	Acetone (60 ml.), water (40 ml.), urea (0.5 g.)	Upper layer of 1-butanol–acetic acid–water (4:1:5)	Water-saturated collidine
Unidentified	Purple	0.10			
Lanthionine	Purple	.16 (0.14)[a]	0.20 (0.20)	0.04 (0.05)	
β-Methyllanthionine	Purple	.18 (.19)	.24 (.24)	.07 (.06)	0.06
Unidentified	Yell.-brown	.24			
Ornithine	Purple	.29 (.26)	.31 (.35)	.11 (.11)	
Aspartic acid	Aqua	.34 (.31)	.42 (.43)	.14 (.15)	
Glycine	Brown	.38 (.38)	.46 (.46)	.17 (.17)	.14 (0.16)
Glutamic acid	Purple	.45 (.40)	.50 (.53)	.22 (.24)	
Proline	Yellow	.56 (.51)	.69 (.57)	.30 (.30)	.22 (.21)
Valine	Purple	.65 (.69)	.65 (.63)	.46 (.44)	.30 (.31)
Phenylalanine	Brown	.68 (.68)	.65 (.61)	.62 (.61)	.55 (.53)

[a] The R_f values in parentheses are those of standard amino acids.

TABLE III

R_f VALUES OF AMINO ACIDS PRESENT IN DURAMYCIN HYDROLYSATES[a]

Lanthionine	Brown	0.11 (0.14)[b]
β-Methyllanthionine	Pink	.17 (.20)
Ornithine	Pink	.26 (.30)
Aspartic acid	Blue-green	.31 (.31)
Glycine	Pink	.39 (.37)
Hydroxyproline	Blue[c]	.42 (.41)
Proline	Blue	.52 (.51)
Phenylalanine	Grey[d]	.62 (.63)

[a] Solvent was *t*-butyl alcohol–acetic acid–water (2:1:1). [b] R_f values in parentheses are those of standard amino acids. [c] Tan spot of glutamic acid was obscured by hydroxyproline. [d] Pink spot of valine was obscured by phenylalanine.

Acknowledgment.—We are indebted to C. H. VanEtten and Mrs. Clara McGrew for the micro-

(12) A. B. Foster, *Chemistry & Industry*, 1050 (1952).
(13) L. Michaelis, *Biochem. Z.*, **234**, 139 (1931).
(14) A. L. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).
(15) N. H. Horowitz, *J. Biol. Chem.*, **171**, 255 (1947).
(16) J. C. Lewis and N. S. Snell, THIS JOURNAL, **73**, 4812 (1951).

analyses, to E. H. Melvin and C. A. Glass for the infrared absorption spectrum, to O. L. Brekke, R. E. Beal and E. B. Lancaster for the Podbielniak extraction, and to R. E. Campbell for the Van Slyke nitrogen determination.

PEORIA, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF WISCONSIN]

Piperidine Derivatives. XXX. 1,4-Dialkyl-4-arylpiperidines

BY S. M. MCELVAIN AND DAVID H. CLEMENS[1]

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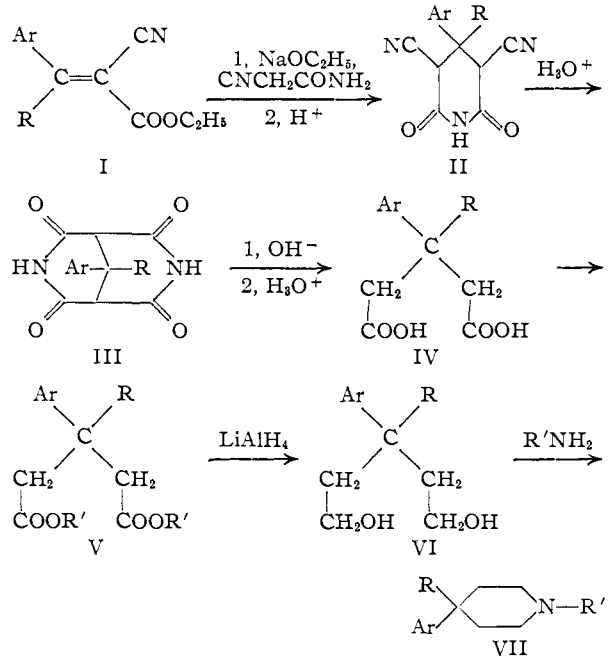
A series of trisubstituted piperidines mentioned in the title has been prepared and submitted for pharmacological screening for analgesic activity. These compounds were synthesized by a sequence of reactions that started with the ethyl 1-arylalkylidenecyanoacetates (I) and went through the β-alkyl-β-arylglutaric acids (IV) to the piperidines (VII). The 1-methyl-4-alkyl-4-phenylpiperidines showed low analgesic activity which appeared to increase as the 4-alkyl substituent was changed from methyl to *n*-propyl. Increase in the size of the 1-alkyl substituent produced no noticeable effect on the analgesic properties. The 1-methyl-4-alkyl-4-(*m*-hydroxyphenyl)-piperidines are potent analgesics, the 4-*n*-propyl compound being comparable to morphine in its analgesic activity. However, when the hydroxyl group is in the *o*- or *p*-position in the 4-phenyl substituent the analgesic activity disappears. Likewise, the replacement of the 4-alkyl substituent of these highly active compounds by hydrogen destroys their activity.

The synthesis of 4,4-disubstituted piperidines has been the subject of many researches, from which have resulted a number of compounds that possess marked analgesic activity.[2-4] Typical of these synthetic products are 1-methyl-4-phenyl-4-carbethoxypiperidine (Demerol) and the highly active 1-methyl-4-phenyl-4-propionoxypiperidine.[2,3]

In most of these synthetic analgesics as well as in the widely used, naturally occurring analgesic morphine, a polar oxygenated function shares the 4-position of the piperidine nucleus with the aryl substituent. It seemed of interest to determine whether such a polar substituent is essential for the analgesic activities of such compounds or whether a non-polar alkyl substituent would suffice. To this end a series of 1,4-dialkyl-4-arylpiperidines (VIIa-q, Tables I and VIII) has been prepared for pharmacological screening.

The synthesis of these compounds was accomplished by a sequence of reactions, which started with the ethyl 1-arylalkylidenecyanoacetate (I), readily prepared in good yield by Cope's procedure, and went through the β-alkyl-β-arylglutaric acids (IV) to the desired piperidines VII.

The first step in this sequence involved considerable study before the proper reaction conditions were determined. All attempts to add malonic ester or cyanoacetic ester to ethyl 1-phenylethylidenecyanoacetate (I, R is CH_3, Ar is C_6H_5) under various conditions for the Michael condensation were uniformly unsuccessful. Phalnikar and Nargund[5] have reported the preparation of the glutarimide (II, R = CH_3) in 2.7% yield by the Guareschi condensation[6] of acetophenone and ethyl cyanoacetate and ammonia. They hydrolyzed this imide to β-methyl-β-phenylglutaric acid (IV, R = CH_3), which is the only β-alkyl-β-phenylglutaric acid reported in the literature.



It seemed likely that the low yield of the glutarimide obtained by the Indian workers was due to the sluggish nature and reversibility of the initial Knoevenagel condensation[7] between acetophenone and ethyl cyanoacetate under the conditions of the Guareschi reaction. Indeed it was found that when the preformed ethylidene ester I (R is CH_3) was allowed to react with ethyl cyanoacetate in ethanol saturated with ammonia, the desired imide II was obtained in 31% yield. A neutral solid XV, m.p. 213–214°, the structure of which is discussed later, also was obtained from this reaction. However, when the homologous alkylidene ester Ib (R is C_2H_5) was allowed to react with ethyl cyano-

(1) Wisconsin Alumni Research Foundation Research Assistant 1954–1956; Procter and Gamble Co. Fellow 1956–1957; E. I. du Pont de Nemours and Co. Summer Research Assistant, 1954, 1955, 1956.

(2) J. Lee in C. M. Suter's "Medicinal Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1951, Vol. I, Chapter 6.

(3) C. M. Suter in F. F. Blicke and C. M. Suter's "Medicinal Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1956, Vol. II, Chapter 3.

(4) H. Krueger in R. H. F. Manske's "The Alkaloids," Academic Press, Inc., New York, N. Y., 1955, Vol. V, Chapter 38.

(5) N. L. Phalnikar and K. S. Nargund, *J. Univ. Bombay*, **6**, Pt. II, 102 (1937); *C. A.*, **32**, 3763 (1938).

(6) I. Guareschi, *Gazz. chim. ital.*, **49**, 124 (1919); A. I. Vogel, *J. Chem. Soc.*, 1758 (1934).

(7) J. Scheiber and F. Meisel, *Ber.*, **48**, 238 (1915), showed that this condensation proceeds to only 30% completion even when the reactants are heated for some time with an aniline–zinc chloride catalyst.