(collapsed triplet, 2 H, CH_2OH), and 9.30 (triplet, J = 7 Hz, 3 H, CH_2CH_3); mass spectrum, main peaks, m/e 297 (M − 1), 184, 170, and 156.

Anal. Calcd for $C_{19}H_{26}ON_2$: C, 76.47; H, 8.78; N, 9.39; mol wt 298.204. Found: C, 76.81; H, 8.93; N, 9.15; mol wt 298.204 (high-resolution mass spectrometry).

Mesylation of Alcohols B and D. (a) **Alcohol B** (XXVIII) (206 mg, 0.69 mmol) was dissolved in a mixture of dry triethylamine (2.5 ml) and chloroform (5.0 ml). The reaction mixture was cooled at −10 to −0° (ice–rock salt bath) and freshly distilled methanesulfonyl chloride (approximately 500 mg, 4.37 mmol) was added dropwise with efficient stirring. The resulting mixture was allowed to come slowly to room temperature (moisture was excluded). After 42 hr the solvent was removed under reduced pressure and the resulting deep red solid was dissolved in chloroform and extracted several times with aqueous 4 *N* ammonium hydroxide. The basic solution was concentrated under reduced pressure with gentle heating (water bath) to give a yellow gum. Traces of water were removed from the gum by virtue of several azeotropic distillations with benzene. The resulting solid was treated with warm chloroform several times. The combined chloroform extracts, after removal of the solvent under reduced pressure, gave an amorphous yellowish compound. This substance was the desired mesylate of alcohol A (XXI, 260 mg) and formed in essentially quantitative yield. This substance was usually used directly for the next step. However, fast column chromatography on alumina (neutral Woelm, activity II), followed by elution with chloroform–methanol (3:1), can be used if necessary. It was not important for our purpose to completely characterize this compound.

(b) **Alcohol D** (XXIX) was mesylated exactly as above to give an amorphous yellowish material. Here again, the yield was essentially quantitative.

Preparation of *dl*-Quebrachamine (XXII). The reductive cleavage of the quaternary mesylate was studied under a variety of conditions. The initial investigations involving alkali metals in liquid ammonia were abandoned when it was found that hydride reduction provided markedly higher yields. A few typical experiments are recorded here.

(a) **Using Sodium in Liquid Ammonia.** The quaternary salt (XXI) obtained directly from a mixture of the amino alcohols (100 mg) as mentioned above was dissolved in anhydrous ethanol (2 ml) and transferred to a three-necked flask fitted with a Dry Ice condenser and an ammonia outlet. After condensing approximately 30 ml of liquid ammonia into the flask, small quantities (50 mg each) of freshly cut sodium were added until after the final addition the blue color persisted for 20 min. Ammonium chloride was added and the ammonia allowed to evaporate. The residue was treated with water and extracted with ether. The ether extract was washed with water and saturated sodium chloride solution, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue, a light green gum (32 mg) was chromatographed on alumina (Woelm, activity I, 3 g). *dl*-Quebrachamine (15 mg, 18%—based on mixture of cyclized amino alcohols) was eluted with dichloromethane–ether 20:1. The ir, nmr, and mass spectra of this material were identical with those of natural (−)-quebrachamine.

(b) **Using Lithium Aluminum Hydride.** The mesylate of alcohol B (130 mg, 0.345 mmol) was taken in N-methylmorpholine (50 ml, distilled over lithium aluminum hydride) and lithium aluminum hydride (390 mg, 10.3 mmol) was added in small portions to it under efficient stirring. The entire reaction was performed under oxygen-free nitrogen. The mesylate which was almost insoluble in this solvent formed during the reflux period (11 hr). The reaction mixture was cooled to room temperature and the excess of lithium aluminum hydride was decomposed carefully by slow addition of water in excess with vigorous stirring. The resulting gray sludge was stirred for a further 15 min and then filtered through a bed of Celite. The solid on the funnel was washed several times with warm chloroform and discarded. The filtrate was taken in some water (20 ml) and the organic layer was separated. The aqueous layer was washed with chloroform and the combined organic layers were washed with water. The chloroform layer was dried over anhydrous sodium sulfate, and the solvent removed under reduced pressure to give a viscous colorless oil. This material was chromatographed on alumina (50 g, neutral Woelm, activity I). Elution with benzene–chloroform (1:1) gave 50 mg of a colorless viscous oil which solidified upon standing in a desiccator. Pure *dl*-quebrachamine (50% yield) was obtained by crystallization from wet methanol (mp 141–144°).

(c) The mesylate of alcohol D was also converted to quebrachamine following the above reaction procedure. Similar comparison confirmed the identity of the purified reaction product. The yield was 51%.

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Failure Sequences in the Solid Phase Synthesis of Polypeptides

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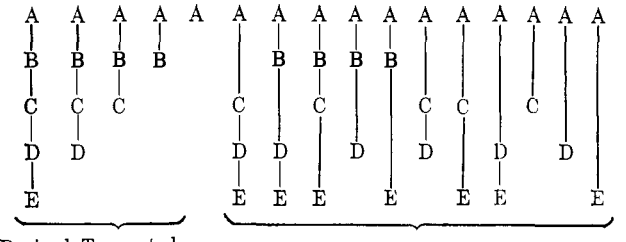
Abstract: Failure sequences occur during solid phase synthesis of polypeptides. The number of these failure sequences can be considerably decreased by acetylation of the amino groups which do not react or by the use of specially prepared, resin-coated glass beads.

One of the limitations of Merrifield's[1-3] solid phase approach to the synthesis of peptides and proteins is the possibility of creating failure sequences, which cannot be separated or even analytically distinguished from the desired sequence. Theoretically the number of failure peptides increases exponentially with increasing chain length. Only 100% yield in every coupling step could prevent the formation of failure sequences.

We can distinguish between the truncated sequences, in which amino acids are missing from the amino end, and the failure sequences, in which amino acids are missing from within the chain. If a truncated sequence cannot couple in later steps, no failure sequences are

(1) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963); **86**, 304 (1964).
(2) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).
(3) R. B. Merrifield, *Science*, **150**, 178 (1965).



Mono-: Di-: Tri-: Tetra-: Pentapeptide = 1:4:6:4:1

Figure 1. Possible wrong sequences during a solid phase synthesis of a pentapeptide A-B-C-D-E.

present, and the isolation of the desired peptide is much more likely to be achieved than in the case when all possible failure sequences are formed. The possible truncated and failure sequences for a pentapeptide are shown in Figure 1.

It had not been previously investigated whether only truncated sequences were occurring or whether all theoretically possible failure sequences were occurring. The main reason for this was that analytical methods are not sensitive enough to detect failure sequences in a solid phase synthesis conducted with high yields in the coupling steps.[4-6]

We therefore synthesized polypeptides of repeating dipeptide sequences $(A\text{-}B)_x$ to improve the detection limit. Failure sequences would be clearly indicated by the occurrence of peptides: A-A or B-B after partial hydrolysis. Truncated sequences and the correct sequence would result in peptides A-B or B-A. The detection limit increases with increasing chain length. The dipeptides obtained after partial hydrolysis were separated as N-trifluoroacetylmethyl esters by gas chromatography and the higher peptides were separated by liquid chromatography and both were then identified by mass spectrometry.[5,7,8]

Results and Discussion

The results for the solid phase synthesis of the dodecapeptides $(Leu\text{-}Ala)_6$[9] and $(Ala\text{-}Phe)_6$,[9] summarized in Tables I and II, clearly indicate that failure sequences occur despite the fact that there were no difficulties in coupling, as high yields have been obtained in every step. Since random hydrolysis does not occur, the values in Tables I and II can be considered only as semiquantitative. These values however are in agreement with the observed reaction rates of more than 99% in every coupling step. With peptide sequences such as Arg-His, Arg-Arg, or Arg-Lys, much lower yields and more failure sequences are detectable.[10] The results are definite experimental proof of failure sequences. In order to define the separation and identification problems caused by failure sequences, it was necessary to know the chain length and amount of contaminating peptides. One can calculate the amount of contaminating peptides for polypeptides of different chain lengths and estimate the optimum chain length to which the Merrifield procedure may be applied. Figure 2 and Table III show the expected yield of myoglobin with 153 amino acid residues, ribonuclease with 124, apoferredoxin with 55, insulin **B** chain, and a dodecapeptide. Figure 2 also shows the yields of the contaminating failure peptides, assuming 90 or 99% average yield in every coupling step. Unfortunately most of the contaminating peptides are nearly identical in chain length with the desired product, especially in the case of 99% yield. In addition, there is little possibility of separating these by existing separation methods when more than 20 amino acid residues are present.

Table I. Observed Sequences in Hydrolysates of $(Leu\text{-}Ala)_6$

Peptide sequence	Normal synthesis, %	Acetylating amino groups, %	Synthesis with pellicular resin, %
Ala-Ala	1.35	0.1	0
Leu-Ala	67.50	71.00	70.55
Ala-Leu	28.53	29.25	30.23
Leu-Leu	2.62	0.1	0

Table II. Observed Sequences in Hydrolysates of $(Ala\text{-}Phe)_6$

Sequence	Amount, %
Ala-Ala	Not detectable
Phe-Ala	39.7
Ala-Phe	51.0
Phe-Phe	9.3

Table III. Expected Yields of Several Polypeptides Assuming Average Coupling Yields of 90 and 99%

Peptide	Number of amino acid residues	Yield assuming 90% coupling rates, %	Yield assuming 99% coupling rates, %
Dodecapeptide	12	31.4	89.5
Insulin B chain	30	4.7	74.1
Ferredoxin	55	0.3	57.5
Ribonuclease	124	0.0002	29.0
Myoglobin	153	<0.00001	21.7

With 90% yield in every coupling step, ribonuclease would be obtained only in 0.0002% yield as is shown in Table III. Polypeptides with one amino acid less, however, would be synthesized in a 15-fold higher yield. Even with 99% yield in every coupling step, the yield of the main product would be 29% as opposed to 36% $n-1$ peptides which could not be separated. In the case of myoglobin, the possibility of a synthesis is even more remote. Assuming 99% yield in every coupling step (and that only the $n-1$ peptides cannot be separated), the limit of the chain length of a peptide, which can be synthesized to 80% purity by the Merrifield method is about 30 amino acid residues. Higher yields or better separation methods would increase this limit. The presence of amino acids that are difficult to couple decreases this value.

There are two possibilities of extending the application of the solid phase method: increasing the yields

(4) E. Bayer, H. Hagenmaier, G. Jung, and W. A. König in "Peptides 1968," E. Bricas, Ed., North-Holland Publishing Co., Amsterdam, 1968, p 162.
(5) E. Bayer and W. A. König, *J. Chrom. Science*, 7, 95 (1969).
(6) E. Bayer in "Proceedings of the 1st American Peptide Symposium," Marcel Dekker, Inc., New York, N. Y., in press.
(7) F. Weygand, A. Prox, H. H. Fessel, and K. Z. Kun Sun, *Z. Naturforsch*, 20b, 1169 (1965).
(8) E. Bayer, H. Hagenmaier, W. A. König, and H. Pauschmann, *Z. Anal. Chem.*, 243, 670 (1968).
(9) $(Leu\text{-}Ala)_6$ and $(Phe\text{-}Ala)_6$ are used as abbreviations for H-L-Leu-(L-Ala-L-Leu)$_5$-L-Ala-OH and H-L-Ala-(L-Phe-L-Ala)$_5$-L-Phe-OH.
(10) E. Bayer and K. Hägele, unpublished results.

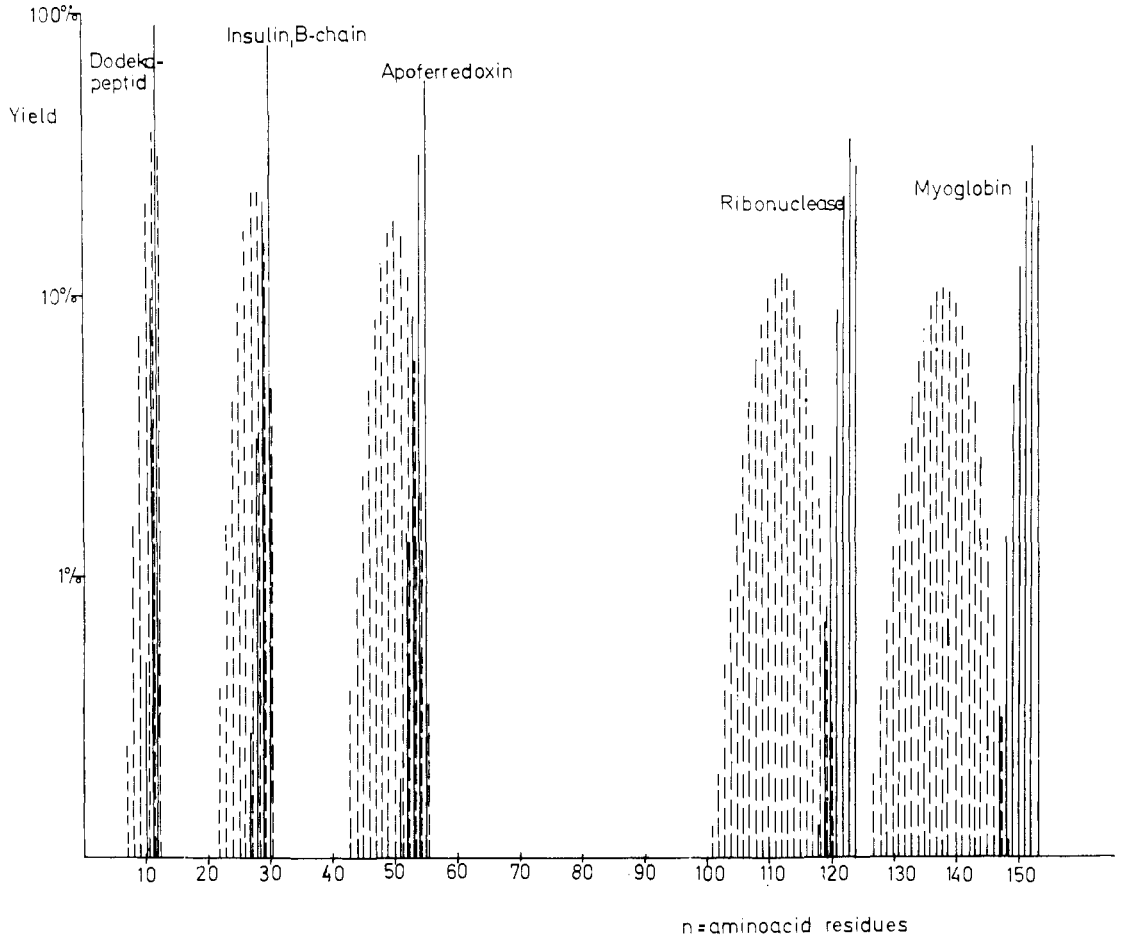


Figure 2. Yield of desired polypeptide and of failure sequences assuming 90% (dotted line) and 99% (solid line) yield in every coupling step.

or preventing truncated sequences from growing into failure sequences. The truncated sequences are easier to separate from the main product as has been shown in the example of the synthesis of the ACTH sequence 1–20 by the solid phase method.[10]

To increase the yield, one must consider that the solid phase method is a heterogeneous reaction which is controlled by the diffusion in the resin. Theoretically, the mass transfer in the resin bead is dependent upon the particle diameter.[11] It is difficult to reduce the particle size because of experimental difficulties in handling such small diameter resin beads. In liquid chromatography, pellicular resins have been introduced for the purpose of decreasing diffusion pathway and thus decreasing the time of chromatography. Such pellicular resins have been prepared by polymerizing a thin layer of resin onto inert glass beads.[12] Using these resins as support, the yield is considerably increased, as the investigation of failure sequences of $(Leu\text{-}Ala)_6$ in Table II shows in comparison to the conventional beads. An additional advantage of the pellicular resin is the low swelling ratio and the possibility of conducting the synthesis in column operations.

To reduce the failure sequences to truncated sequences, the amino groups not coupling in one step must be prevented from further coupling in later steps. Merrifield[1] introduced a procedure for this in his early publications; however, it has not been followed up generally. We acetylated after every coupling step. Table II demonstrates that in this case the failure sequences are considerably reduced.

By using these methods, and by synthesizing peptides with chain lengths up to twenty or thirty amino acids (which are then coupled by fragment techniques), the Merrifield method might be used to synthesize homogeneous proteins.

Experimental Section

Materials. Boc-amino acids were prepared according to the pH–stat method[13] and the purity established by thin-layer chromatography.

Boc-Ala Polymer. Chloromethylated Bio Beads S-X2 (2 g, capacity 1.1 mmolar equiv/g) were stirred in 50 ml of absolute ethanol with 0.34 g (1.8 mmol) of Boc-Ala-OH and 0.13 g (1.8 mmol) of triethylamine for 24 hr at 80°. The resin was then filtered off, and washed with ethanol, DMF, and CH_2Cl_2.

$(Leu\text{-}Ala)_6$ Polymer. The further synthesis of the peptide resin was conducted as described by Bayer, *et al.*[14] The loading on the resin represented 0.39 mmol. The number of free amino groups decreased by approximately 1% per synthesis step.

The following amounts of Boc-amino acids were used for the coupling step: 6×0.46 g (2 mmol) of Boc-Leu-OH dried *in vacuo* over P_2O_5, and 5×0.38 g (2 mmol) of Boc-Ala-OH.

$(Leu\text{-}Ala)_6$ Hydrobromide. The peptide resin was dried *in vacuo* and suspended in 25 ml of trifluoroacetic acid. The suspension was stirred and purified HBr was passed through for 90 min. The resin was then filtered off and washed twice with 20 ml of trifluoroacetic acid. The combined filtrates were concentrated;

(11) J. F. K. Guber and J. A. R. Halsman, *Anal. Chim. Acta*, **38**, 305 (1967).
(12) C. G. Horvath, B. A. Preiss, and S. R. Lipsky, *Anal. Chem.*, **39**, 1422 (1967).
(13) E. Schnabel, *Ann. Chem.*, **702**, 188 (1967).
(14) E. Bayer, G. Jung, and H. Hagenmaier, *Tetrahedron*, **24**, 4853 (1968).

the (Leu-Ala)$_6$-hydrobromide was precipitated with 200 ml of ether. The product was filtered off and washed with ether. After drying *in vacuo*, 415 mg of a white powder was obtained (0.345 mmol = 88% with respect to the amount of alanine esterified onto the resin). A sample of this product was hydrolyzed for 20 hr in 6 *N* HCl at 110°. The amino acid analysis of the hydrolysate indicated a ratio of Ala:Leu = 1:0.97.

(Ala-Phe)$_6$. The synthesis was carried out according to the description given for (Leu-Ala)$_6$. According to amino acid analysis the ratio of Ala–Phe was 0.94:1.0. (Ala-Phe)$_6$-HBr is practically insoluble in all conventional solvents. It is soluble only in trifluoroacetic acid.

Synthesis of (Leu-Ala)$_6$ with Pellicular Resin. Boc-Ala Beads. Glass beads (150 g, 88–105μ) coated with chloromethylated polystyrene[15] (capacity 0.15 mmolar equiv/g) were put into a column (1 × 100 cm) and a solution of 1.9 g of Boc-Ala-OH (10 mmol) and 1 g (10 mmol) of triethylamine in 60 ml of ethanol soaked in the resin bed and allowed to react for 48 hr at 60°. The coated glass beads were then washed with ethanol, DMF, and CH_2Cl_2. The capacity loading on the coated beads represented 0.07 mmolar equiv of Ala/g.

Boc-(Leu-Ala)$_6$ Beads. The further synthesis of the peptide beads was carried out in a column (3 × 10 cm) with 75 g of the Boc-Ala-OCH$_2$ beads using the basic steps and washing procedures as described for (Ala-Phe)$_6$. In every coupling step, 9 mmol of Boc-L-Ala-OH and Boc-L-Leu-OH and 9 mmol of dicyclohexylcarbodiimide, respectively, were used. The product obtained from the beads after hydrolysis showed a ratio of Leu–Ala of 1.0:1.0, and was contaminated with resin.

Synthesis of (Leu-Ala)$_6$ with Acetylation. The synthesis was carried out as described above with 2.5 g of chloromethylated resin (0.7 mmolar equiv/g Bio Beads S-X2, 200–400 mesh). The total quantity of the first amino acid attached to the resin was 0.4 mmol; the amount of the free amino groups after each cleaving step of the *t*-Boc group was constant during the entire synthesis. After each coupling step, the resin was washed three times with dimethylformamide, and the acetylation was carried out with 2.5 ml of acetic anhydride in a mixture of 25 ml of dimethylformamide and 0.75 ml of triethylamine for 20 min. Afterward, the resin was washed three times with dimethylformamide. The yield of the peptide hydrobromide was 321 mg (55%). According to the amino acid analysis, the ratio of Leu–Ala was 1.0:1.0.

Gas Chromatography–Mass Spectrometry and Sephadex Chromatography. The same methods, previously described in ref 5 were used. For gas chromatographic and mass spectrometric investigations, an LKB 9000 gas chromatograph–mass spectrometer was used. All mass spectra were taken at 70 eV. For solid samples, a heated and water-cooled direct inlet was used. The separation of the trifluoroacetyldipeptide methyl esters of the partial hydrolysate was achieved on SF 96 coated steel capillary columns 500 ft × 0.03 in., isothermal at 190°: carrier gas, 15 ml He/min; detector, FID, 300°; injector 280°. For the gas chromatography–mass spectrometry combination, the total ion current detector was used.

Partial Hydrolysis of Peptides. A peptide (10–100 mg) was sealed in a glass tube with 5 ml of concentrated HCl and left for 72 hr at 37°. After evaporation of the HCl *in vacuo* (18 Torr) the peptide mixture was esterified and trifluoroacetylated for gas chromatography or directly submitted to partition chromatography. Under these conditions approximately 50% (Leu-Ala)$_6$ was hydrolyzed to dipeptides, 30% to higher peptides, and 20% to amino acids. Due to this and to the fact that random hydrolysis was not occurring, the results in Table I and II, calculated on the basis of sequences found in di- to pentapeptides, are only semiquantitative.

Esterification of Peptides. The almost neutral peptide hydrolysate was dissolved in methanol. A concentrated solution of diazomethane in diethyl ether was added until the yellow color remained. If any precipitate formed, a few drops of water and more diazomethane were added until the precipitate dissolved. The yellow solution was evaporated to dryness *in vacuo* (18 Torr).

Trifluoroacetylation. The peptide esters were dissolved in 5 ml of dry methanol and brought to a pH of 7.5–8 by adding triethylamine. Methyl trifluoroacetate (2 ml) was added and the solution left at room temperature for 8 hr. After evaporation to dryness *in vacuo* (18 Torr), the residue was shaken with a mixture of equal amounts of ethyl acetate and water. The organic phase was evaporated *in vacuo* (18 Torr), resulting in the TFA–peptide–methyl esters.

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(15) The sample of chloromethylated glass beads was provided by Dr. C. G. Horvath and S. R. Lipsky, New Haven, Conn.

Retention of Configuration in the Solid Phase Synthesis of Peptides

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Abstract: Amino acid enantiomers can be resolved by the use of optically active stationary phases in a gas chromatographic system. This technique has allowed the study of racemization in the solid phase synthesis of polypeptides. The results indicate that almost complete retention of configuration is obtained.

Peptide synthesis by the solid phase method is assumed to proceed without racemization. Merrifield,[2] in his first publication on the subject has reported that the L-leucyl-L-alanyl-glycyl-L-valine prepared by his method was completely digested by leucine aminopeptidase. Furthermore, the high biological activity (450 units/mg, potency on the isolated rat uterus), coupled with high synthetic yield (79%) of the oxytocin prepared by Bayer and Hagenmaier[3] indicates that racemization did not occur to any appreciable extent. Nevertheless, this aspect of solid phase synthesis of peptides has not as yet been thoroughly investigated and the question has been raised whether racemization is completely excluded in all cases. With the advent of new, sensitive gas chromatographic methods for the resolution of enantiomeric amino acids[4–6]

(1) On sabbatical leave from the Weizmann Institute of Science, Rehovoth, Israel.

(2) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963).

(3) E. Bayer and H. Hagenmaier, *Tetrahedron Lett.*, 2037 (1968).

(4) E. Gil-Av, R. Charles-Sigler, G. Fischer, and D. Nurok, *J. Gas Chromatog.*, **4**, 51 (1966).

(5) J. W. Westley and B. Halpern in "Gas Chromatography 1968," S. L. A. Harbourn, Ed., Institute of Petroleum, London, 1969, p 119.

(6) E. Gil-Av, B. Feibush, and R. Charles-Sigler, *Tetrahedron Lett.*,