Azlactone Formation in the Isoxazolium Salt Method of Peptide Synthesis

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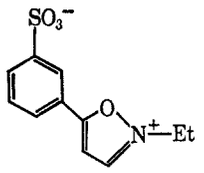
The reaction of hippuric acid with isoxazolium salts to give enol esters has been found to be accompanied by azlactone formation. The extent of the side reaction with N-*t*-butyl-5-methylisoxazolium perchlorate (**7**) decreases with increasing basicity of the medium, although the ester (**12**) from **7** itself decomposes to azlactone in the presence of strong base. The ester **12** free of azlactone may be obtained by use of 2-picoline as the reaction solvent to maintain a medium of intermediate base strength. The peptide reagent N-ethyl-5-phenylisoxazolium 3′-sulfonate (**1**) does not give appreciable azlactone in the normal procedure for its use because the low rate of solution of the reagent fortuitously controls the basicity of the reaction mixture.

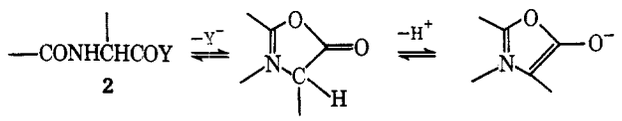
In the investigation[1] of the use of N-ethyl-5-phenylisoxazolium 3′-sulfonate (**1**) for the synthesis of peptides, conditions were defined under which no racemization was observed in the Anderson test.[2] Since then,



1

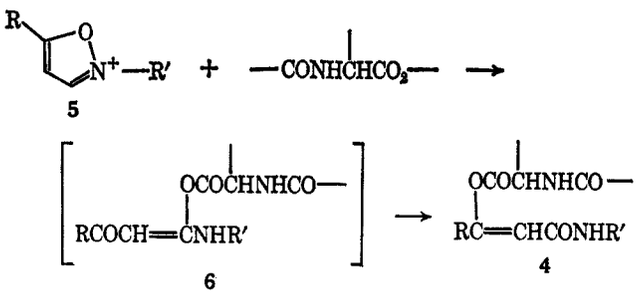
other workers have reported the detection of some degree of racemization in different tests with **1**.[3-5] Our further studies of the reaction of isoxazolium salts with N-acylamino acids have now provided clarification of the potential for racemization in this method of peptide synthesis.

The common mechanism established[6,7] for racemization during peptide synthesis involves fragmentation of an acylating agent (**2**) derived from a peptide acid to give an azlactone (**3**), which may then ionize with loss of chirality at the α carbon. Although with other acylat-



2 **3**

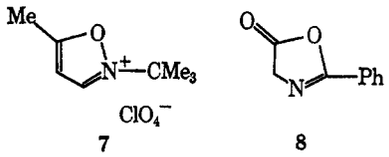
ing agents of the active ester type racemization *via* azlactones usually does not compete effectively with acylation of an amine group during the peptide bond formation reaction itself, preparation of the optically pure active esters of peptide acids by acyl transfer from a racemization-prone acylating agent of greater activity is a common problem.[8] However, it has been proposed that the enol ester acylating agents (**4**) from isoxazolium salts (**5**) are formed from more highly reactive, intermediate acylating agents (**6**) by an intramolecular acyl migration process.[9] On the basis of the initial favorable



5 **6** **4**

racemization results with **1**, it appeared likely that the decomposition of **6** to azlactone was not so rapid as the facile rearrangement of **6** to **4** and that the use of isoxazolium salts might, therefore, eliminate the problem of racemization during the formation of active esters of peptide acids.

Infrared spectral studies of the reaction of hippuric acid with a variety of isoxazolium salts have now revealed that, actually, enol ester preparation generally is complicated by azlactone formation.[10-12] The spectrum of the product mixture from hippuric acid, triethylamine, and the new reagent, N-*t*-butyl-5-methylisoxazolium perchlorate (**7**),[13] in acetonitrile contained a strong peak at 5.45 μ attributable to 2-phenylazlactone (**8**),[14] with only weak enol ester absorption at 5.65 μ. A



7 **8**

similar result was obtained with N-*t*-butyl-5-phenylisoxazolium perchlorate (**9**), while N,5-diphenylisoxazolium

(1) R. B. Woodward, R. A. Olofson, and H. Mayer, *J. Amer. Chem. Soc.*, **83**, 1010 (1961); *Tetrahedron Suppl.*, **8**, 321 (1966).

(2) G. W. Anderson and F. M. Callahan, *J. Amer. Chem. Soc.*, **80**, 2902 (1958).

(3) F. Weygand, A. Prox, L. Schmidhammer, and W. König, *Angew. Chem.*, **75**, 282 (1963).[4]

(4) The observation of racemization in this test was not unexpected since under comparable conditions racemization also was observed in the Anderson test.

(5) M. W. Williams and G. T. Young, *J. Chem. Soc.*, 881 (1963).

(6) M. Goodman and K. C. Stueben, *J. Org. Chem.*, **27**, 3409 (1962); M. Goodman and L. Levine, *J. Amer. Chem. Soc.*, **86**, 2918 (1964); M. Goodman and W. J. McGahren, *ibid.*, **87**, 3028 (1965).

(7) M. W. Williams and G. T. Young, *J. Chem. Soc.*, 3701 (1964); I. Antonovics and G. T. Young, *Chem. Commun.*, 398 (1963).

(8) M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, New York, N. Y., 1966, p 150.

(9) R. B. Woodward and R. A. Olofson, *J. Amer. Chem. Soc.*, **83**, 1007 (1961); *Tetrahedron Suppl.*, **7**, 415 (1966).

(10) The extent of racemization, since it depends on the relative rates of acylation and racemization for the azlactone, would be less than the amount of azlactone formed. Thus, in cases where the azlactone is stable, direct spectral assay of the actual amount of azlactone provides an especially sensitive measure of the potential hazard of racemization associated with the formation of enol esters.

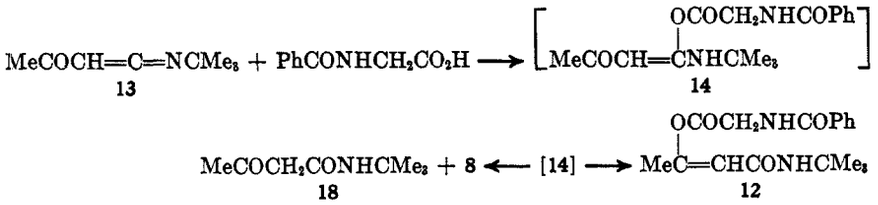
(11) Related results were previously obtained in a study of the N-ethylbenzisoxazolium cation.[12]

(12) D. S. Kemp, Ph.D. Thesis, Harvard University, 1964.

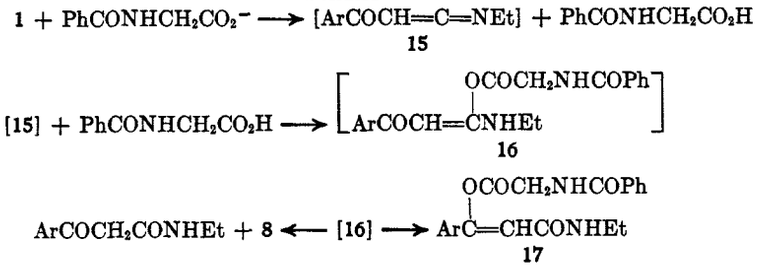
(13) R. B. Woodward and D. J. Woodman, *J. Amer. Chem. Soc.*, **90**, 1371 (1968).

(14) Assignment confirmed by isolation of **8**.

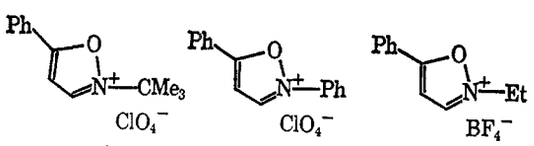
SCHEME I



SCHEME II



perchlorate (**10**) gave an azlactone band of lesser intensity than that of the enol ester. Azlactone absorption was negligible only with the original reagent **1**, and even the closely related compound, N-ethyl-5-phenylisoxazolium fluoroborate (**11**), gave an azlactone peak comparable in intensity with that of the enol ester.



It was also found that the relative amounts of azlactone (**8**) and enol ester (**12**) from the reaction of hippuric acid and the ketoketenimine **13**, from **7**, varied with the basicity of the reaction mixture. In a series of spectral tests in acetonitrile containing progressively greater concentrations of pyridine, the proportion of ester in the product mixture increased steadily, but some azlactone was formed even when pyridine was used as the solvent. The effect of pyridine concentration on the product ratio cannot be attributed to an equilibrium between the enol ester and azlactone since control experiments established that there is no interconversion of **8** and **12** in the presence of pyridine. Therefore azlactone and enol ester must be formed by competing reactions.

The observation of azlactone formation under conditions where the product ester itself does not give **8** provides support for the proposal[9] that the reaction of carboxylic acids with isoxazolium salts proceeds *via* a highly reactive intermediate acylating agent such as **6**. Presumably, then, alternate modes of decomposition of the corresponding intermediate **14**, from hippuric acid and **13**, give either **8** or **12** (Scheme I), and increasing basicity favors the pathway to enol ester.[15]

A similar dependence of the reaction course upon basicity of the medium can be invoked to explain why in the spectral tests only the reagent **1** gave the enol ester of hippuric acid relatively free of azlactone. The crucial difference between **1** and the other isoxazolium salts tested is that **1** is a zwitterion which is relatively insoluble in acetonitrile. All the other isoxazolium salts dissolve immediately in the triethylammonium hippurate solution with rapid ring opening to give hippuric acid and ketoketenimine,[16] which then combine in the slow step of the reaction. In contrast **1** dissolves so slowly (45 min) that the rate of solution is rate determining, and the acid and ketoketenimine (**15**) do not build up to detectable concentrations. Thus, while the intermediates from the other isoxazolium salts decompose in a medium containing acid generated by the fast prior ring opening and give azlactone, the intermediate (**16**) from **1** is exposed to unconsumed base which favors the formation of enol ester (**17**) (Scheme II). That the avoidance of azlactone with **1** is the result of the rate of solution, rather than some other factor, was confirmed in a test in which the order of combination of the reactants was altered. By allowing **1** to react with a solution of triethylamine before combination with the hippuric acid, a reaction mixture similar in acidity to those with the other isoxazolium salts was obtained, and, as expected, the product spectrum contained an azlactone peak comparable in intensity with that of the enol ester.

In view of the potential advantages of the enol esters from the new reagent **7** for synthetic use,[13] it was of interest to determine if the basicity of the reaction medium could be maintained at such a level that azlactone-free enol ester could also be obtained from **7**. Attempts to preserve a basic environment by very slow addition of a solution of **7** to triethylammonium hippurate in acetonitrile resulted in some enrichment of the product mixture in enol ester, but, even when **7** was added at a constant rate over a period of 6 hr, the enol ester absorption was not so great as that of **8**. The failure of the slow addition approach with **7** and the fact that a greater proportion of azlactone is obtained from **7** and triethylammonium hippurate than from the reverse addition experiment with **1** suggest that azlactone formation is more favorable, relative to enol ester formation, from **14** than from **16**.

Although the use of more strongly basic conditions still offered the possibility of channeling the decomposition of **14** exclusively to **12**, such conditions also pre-

(15) This effect of basicity on the reaction course, which could be rationalized on the basis of preferential rearrangement of the anion of **14** to **12**, was originally proposed by D. S. Kemp as a result of his study of the N-ethylbenzisoxazolium cation.[10]

(16) Detected by the characteristic band in the cumulene region of the infrared spectrum.

sented the hazard of equilibration of enol ester with azlactone. Racemization of the closely related enol esters from peptide acids and benzisoxazolium salts in the presence of triethylamine has recently been explained on the basis of such an equilibrium.[17] In the present system the equilibrium actually favors azlactone as shown by spectral tests in acetonitrile containing a catalytic amount of triethylamine in which **12** slowly gave **8** but no reverse reaction was observed with a mixture of **8** and **18**. Complete avoidance of azlactone therefore required that conditions be found more basic than the tests with pyridine to prevent the formation of **8** from **14** yet not so basic that **8** would be formed from **12**. Since the equilibrium favors **8** relative to **12**, direct spectral assay could be used to detect azlactone from either source in further tests designed to find conditions of appropriate intermediate basicity.

Substantial reduction in the proportion of azlactone was achieved in a test of the reaction of **13** and hippuric acid with 10% excess triethylamine in dimethylformamide (DMF) under vacuum on a rotary evaporator. It was hoped that the excess strong base liberated during the reaction would be continually removed by evaporation along with the high boiling solvent. However, all of the azlactone could not be eliminated by this approach even when the more volatile trimethylamine or only an exact equivalent of triethylamine was employed. A more promising method for controlling the basicity was to use a tertiary base intermediate in strength between triethylamine (pK_a of the conjugate acid in water = 10.65[18]) and pyridine (pK_A = 5.17[19]). In a test with 10% excess N-methylmorpholine (pK_A = 7.14[18]) in acetonitrile, some azlactone absorption was observed in the initial product spectrum, and, later in the reaction, the azlactone peak began to increase as that of the ester diminished. It is likely that in this test the basicity at the outset was insufficient to prevent the formation of **8** from **14** and that increasing basicity as the reaction progressed with consumption of acid subsequently brought about conversion of **12** into **8**. Apparently, then, a base weaker than N-methylmorpholine was needed to avoid the latter problem while the base would have to be present in large excess so that decomposition of **14** would give only **12**. The range of base strength in question spans the picolines and lutidines, but with 3-picoline (pK_A = 5.68[17]) as the solvent the result was the same as with pyridine. Finally, tests with either 2-picoline (pK_A = 5.97[17]) or 2,6-lutidine (pK_A = 6.75[17]) as the solvent did give product spectra which contained no azlactone peak, establishing that in this range of basicity both pathways to azlactone are inoperative.[20]

The practical consequence of the spectral tests of azlactone formation with **7** is that special precautions would have to be taken for the preparation of enol esters with the new reagent and N-acylamino acids or peptide acids which are likely to form azlactones. Vacuum evaporation of a solution of equivalents of hippuric acid and the ketoketenimine **13** from **7** in dry 2-picoline after the reaction is largely complete (20 hr) forces the enol ester preparation to completion and azlactone can not be detected in the spectrum of the residue. However, azlactone is apparent with only minor variations on this technique, such as using the isoxazolium salt **7** instead of **13** or evaporating the solvent immediately. In both these modifications the basicity of the reaction medium is diminished slightly and some decomposition of **14** to **8** presumably occurs.[21]

Finally, while the rate of solution of the original peptide reagent **1** has been shown to favor enol ester formation under the optimum conditions previously described[1] for its use, it must be stressed that modifications of the procedure which might upset this accidental control mechanism can lead to competing azlactone formation and the attendant danger of racemization. For example increased azlactone absorption was detected in a spectral test in which 10% excess hippuric acid was present in acetonitrile and also when exact equivalents were used in the solvent nitromethane, in which the reagent **1** dissolves more rapidly. Therefore any changes in the conditions for enol ester preparation with **1** which might increase the rate of solution of the reagent and/or increase the acidity of the medium should be avoided.

Experimental Section

Melting points were taken on a Kofler hot-stage microscope, calibrated with melting point standards from Arthur H. Thomas Co. The nmr spectra were run on a Varian A-60 spectrometer and chemical shifts are reported in τ values relative to tetramethylsilane as an internal standard (τ 10.00). The ir spectra were recorded with a Perkin-Elmer Infracord spectrophotometer, using fixed 0.2-mm path length cells. All amines were dried over type 5A molecular sieves. The analysis was performed by Scandinavian Microanalytical Laboratories.

Spectral Tests of Azlactone Formation.—A solution of 0.179 g (1 mmol) of hippuric acid and 0.101 g (1 mmol) of Et_3N in 10 ml of MeCN (spectral grade) was stirred vigorously while 0.240 g (1 mmol) of **7** was added rapidly. The ir spectrum of the resulting solution was scanned repeatedly from 4 to 7 μ until the ketenimine absorption near 4.85 μ disappeared in about 20 min (t), and the absorbance of the carbonyl peak of **8** at 5.45 μ in the product spectrum was 0.58 (A).[22]

The test was repeated with 1 mmol each of **9**[23] (t = 20 min, A = 0.63), **10**[23] (t = 5 min, A = 0.13), and **11**[1] (t = 7 min, A = 0.20). With 1 stirring was continued until all but traces of the reagent had dissolved (45 min), and no ketenimine absorption could be detected in the spectrum of the solution (A < 0.02).

2-Phenylazlactone (8).—A duplicate of the test reaction mixture with **7** was allowed to stand overnight, diluted with 20 ml of CH_2Cl_2, washed with water, dried (Na_2SO_4), and evaporated under reduced pressure. The residue was washed with three 5-ml portions of cold water to remove the bulk of the by-product **18**, and the remaining solid was dried under vacuum. Partial sublimation (80–90°, 0.1 mm) gave 0.034 g (21%) of **8**, mp 88–88.5° (lit.[12] mp 90.0–91.2°). Further sublimation gave **8** contaminated with **18**. The ir spectrum was identical with that of an authentic sample of **8**;[12] nmr ($CDCl_3$) showed τ 5.65 (s, 2) and 2.83–2.00 (m, 5).

Tests with Pyridine.—A mixture of 1 mmol of hippuric acid and 0.155 ml (1 mmol) of **13**[24] in 10 ml of MeCN was swirled

(17) D. S. Kemp and S. W. Chien, *J. Amer. Chem. Soc.*, **89**, 2745 (1967).

(18) H. K. Hall, Jr., *ibid.*, **79**, 5441 (1957).

(19) H. C. Brown, D. H. McDaniel, and O. Hafliger, in "Determination of Organic Structures by Physical Methods," E. A. Braude and F. C. Nachod, Ed., Academic Press, New York, N. Y., 1955, p 567.

(20) The possibility that the absence of the azlactone peak can be attributed to rapid, selective decomposition of **8** by reaction with traces of nucleophilic impurities in these solvents is ruled out by the detection of azlactone in 2-picoline in the modified procedures below.

(21) The yield of **12** with the successful technique could not be determined accurately because the ester failed to crystallize, but with carbobenzoxyglycine the crude enol ester (**19**) was obtained in 90% yield, albeit in a low state of purity. Since carbobenzoxyglycine is not subject to the azlactone problem and pure **19** can be recovered from the reaction conditions, additional side reactions must be responsible for the low purity of the product obtained from the reaction in 2-picoline.

(22) For 0.1 M **8** in MeCN, A = 1.0.

(23) R. B. Woodward and D. J. Woodman, *J. Org. Chem.*, **31**, 2039 (1966).

(24) R. B. Woodward and D. J. Woodman, *J. Amer. Chem. Soc.*, **88**, 3169 (1966).

until all the acid had dissolved. The ir spectrum of the solution was scanned from 4 to 7 μ until **13** had been consumed completely (t = 30 min, A = 0.77). The product spectrum was identical after 24 hr. No change was observed in the ratio of intensities of the azlactone and enol ester peaks after a duplicate of the test mixture was diluted with pyridine (final concentration 2.0 M).

The test was repeated with reaction mixtures 0.1 M (A = 0.50), 0.2 M (A = 0.39), 0.5 M (A = 0.33), and 2.0 M (A = 0.17) in pyridine. A final test was conducted in pyridine as the solvent (A = 0.05).

Reverse Addition with 1.—A mixture of 0.202 g (2 mmol) of Et_3N in 10 ml of MeCN and 0.507 g (2 mmol) of **1** was stirred until all but traces of the solid had dissolved. Then 5 ml of the resulting solution was added rapidly to a vigorously stirred suspension of 1 mmol of hippuric acid in 5 ml of MeCN. The acid dissolved within 2 min, and the product spectrum was recorded (A = 0.35).

Slow Addition with 7.—A solution of 1 mmol of **7** in 0.5 ml of MeCN was added during 75 min at a constant rate with a motor driven syringe control to a well-stirred solution of 0.102 g (1.01 mmol) and 0.181 g (1.01 mmol) of hippuric acid in 9.5 ml of the solvent. The product spectrum was recorded when the addition was complete (A = 0.35). The test was repeated with addition times of 150 min (A = 0.32) and 375 min (A = 0.29).

Interconversion of 12 and 8.—The ir spectrum of a 0.1 M solution of **12** (isolated below) in MeCN containing a catalytic amount (1 drop/100 ml) of Et_3N showed an azlactone band at 5.45 μ (A = 0.04) within 1 hr at room temperature. The spectrum of the solution the next day contained a diminished enol ester peak and increased azlactone absorption (A = 0.18). No ester absorption was detected on long standing of an MeCN solution 0.1 M in both **8** and **18** (from below) with a catalytic amount of Et_3N.

N-*t*-Butylacetoacetamide (18).—The isoxazolium salt **7** (2.40 g, 10 mmol) was dissolved in 50 ml of 8% $NaHCO_3$. After 24 hr the solution was extracted with three 25-ml portions of CH_2Cl_2. The combined organic extracts were dried (Na_2SO_4) and filtered. Removal of the solvent under reduced pressure left 1.2 g of crystalline solid, mp 45–47.5°. Two recrystallizations from ether gave pure **18**: mp 46.5–48° (lit.[25] mp 44–45°); nmr (CCl_4) τ 8.70 (s), 7.83 (s), 6.95 (s), and 2.97 (broad).

Tests with Other Bases.—A mixture of 1 mmol each of hippuric acid and **13** in 10 ml of DMF containing 0.154 ml (1.1 mmol) of Et_3N was swirled until all the acid had dissolved, and the solution was evaporated in the course of 3 hr at room temperature with a rotary evaporator attached to a mechanical vacuum pump. After 2 hr more on the evaporator, the residue was taken up in 10 ml of MeCN and the ir spectrum recorded (A = 0.15). The test was repeated with 1 mmol of Et_3N (A = 0.17) and with excess (<0.06 g) of Me_3N (A = 0.12).

The spectrum of a solution of 1 mmol each of hippuric acid and **13** in 10 ml MeCN containing 1.1 mmol of N-methylmorpholine recorded 2 min after combining the reactants contained enol ester absorption and a weaker azlactone peak (A = 0.03). After 10 hr both bands had increased in intensity (A = 0.07), and after 60 hr the ester peak had diminished while that of the azlactone had further increased (A = 0.13).

Spectra were scanned from 4 to 7 μ until all the ketenimine had been consumed in test reactions of 1 mmol each of **13** and hippuric acid in 3-picoline (t = 25 hr, A = 0.06), 2-picoline (t = 30 hr, A < 0.01), and 2,6-lutidine (t = 30 hr, A < 0.01).

N-*t*-Butyl-β-hippuryloxycrotonamide (12).—A duplicate of the spectral test solution of **13** and hippuric acid in 2-picoline was allowed to stand 20 hr and then evaporated under vacuum at room temperature. The residue was taken up in 10 ml of MeCN, and the ir spectrum showed enol ester absorption at 5.65 μ free of any azlactone peak. Attempts to crystallize the oil from a variety of solvents were unsuccessful. The procedure was repeated, evaporating the solution immediately (A = 0.02) and substituting 1 mmol of **7** for **13** (A = 0.04).

N-*t*-Butyl-β-carbobenzoxyglycyloxycrotonamide (19).—A solution of 1.05 g (5 mmol) of carbobenzoxyglycine in 40 ml of 2-picoline was combined with 0.696 g (5 mmol) of **13** in 10 ml of the solvent under dry nitrogen. The next day the solution was evaporated and the residue was taken up in CH_2Cl_2 and washed with water, 8% $NaHCO_3$, and water. Each aqueous extract was washed with two small portions of CH_2Cl_2. The combined organic phases were dried (Na_2SO_4), filtered, and evaporated. Precipitation of the residue from benzene with petroleum ether (bp 40–60°) gave 1.57 g (90%) of yellow crystals, mp 76–83°, contaminated with a small amount of tarry material. Recrystallization from benzene–petroleum ether gave pure, colorless **19**:[15] mp 84–85°; nmr ($CDCl_3$) τ 8.72 (s, 8.8), 8.10 (3), 5.92 (d, 1.9; J = 6 Hz), 4.91 (s, 2.1), 4.60 (1.1), 4.26 (broad, 1.9), 2.73 (s, 5.1).

Anal. Calcd for $C_{18}H_{24}N_2O_5$: C, 62.05; H, 6.94; N, 8.04. Found: C, 62.26; H, 7.06; N, 8.07.

When pure **19** was dissolved in 2-picoline and isolated by the above procedure, evaporation of the CH_2Cl_2 left material (93%) of mp 84–85°.

Modifications of the Procedure with 1.—The spectral test with **1** was repeated in the solvent $MeNO_2$ (solution in 15 min, A = 0.07) and with 1.1 mmol of hippuric acid in MeCN (solution in 30 min, A = 0.06).

Registry No.—Hippuric acid, 495-69-2; **7**, 10513-45-8; **12**, 20122-51-4; **19**, 19625-78-6.

Acknowledgment.—This work was generously supported in part by the National Institutes of Health.

(25) C. H. Eugster, L. Leichner, and E. Jenny, *Helv. Chim. Acta*, **46**, 543 (1963).