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Esterification of Model Peptides in Aqueous Solution[1]

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Acyl amino acids and peptides were esterified with triethyloxonium fluoroborate in neutral or slightly basic aqueous solution at room temperature, and this simple and convenient method may provide a potential new method in the area of peptide and protein chemistry.

In connection with the previous paper in which it was demonstrated that sodium borohydride is a good reagent for the selective reduction of ester groups of some peptides,[3] it seems to be very desirable that esters of peptides and proteins are prepared under mild conditions in aqueous solution.

Although the treatment with hydrogen chloride in absolute methanol or ethanol provide a generally applicable esterification procedure of amino acids and peptides[4] and diazo compounds are useful for the esterification of carboxyl groups in some enzymes under specified conditions,[5] some limitation in each case are unavoidable.[6]

Recently, the chemical modification of carboxyl groups in protein and enzyme has been a very important subject in chemical studies of protein. It would be preferred, of course, to carry out the desired modification in an aqueous medium. However, there are many difficulties in such a procedure, the most of which is the competition of the water with the protein for the reagent. Meerwein, *et al.* reported, in the course of chemical studies on triethyloxonium fluoroborate (I), that a concentrated aqueous solution of sodium benzoate was treated with I to yield ethyl benzoate in good yield, whereas I is rather unstable in water.[7] Kemp, *et al.* reported briefly that carbobenzoxyglycyl-L-phenylalanylglycine was dissolved in aqueous base and converted with triethyloxonium ion to its ester in 60% yield.[8]

In the present paper, various acyl amino acids and peptides were subjected to the reaction with I in aqueous solution.

As a preliminary experiment, hippuric acid (IIa) in aqueous solution containing sodium bicarbonate was treated with various amounts of I in solid or in acetonitrile solution at room temperature for 30 min. The reaction mixture was extracted with ethyl acetate. Evaporation of the solvent gave ethyl hippurate (IIb) in almost pure state. Indicating the results in Table I, fifteen to twenty fold excess of I was enough to give satisfactory results. This simple esterification reaction in aqueous solution was extended to acyl dipeptides, such as N-benzoyl-DL-alanylglycine (IIIa) and N-benzoylglycyl-L-valine (IVa) to give quite similar results.

1) The preliminary communication of this paper appeared in *Tetrahedron Letters*, **1969**, 1819.
2) Location: *Kita-12, Nishi-6*, Sapporo.
3) O. Yonemitsu, T. Hamada, and Y. Kanaoka, *Tetrahedron Letters*, **1968**, 3575; *Chem. Pharm. Bull.* (Tokyo), **17**, 2075 (1969).
4) J.P. Greenstein and M. Winitz, "Chem. of Amino Acids," J. Wiley, N.Y., 1961, p. 925.
5) A.C. Chibnall, J.L. Mangan, and M.W. Rees, *Biochem. J.*, **68**, 114 (1958); M.S. Duscher and P.E. Wilcox, *J. Biol. Chem.*, **236**, 1328 (1961); J.P. Riem and H.A. Scheraga, *Biochemistry*, **4**, 772 (1965); V.M. Stepanov and T.I. Vaganova, *Biochem. Biophys. Res. Commun.*, **31**, 825 (1968).
6) P.E. Wilcox, "Methods in Enzymology," Vol. 11, Academic Press, 1967, p. 605.
7) H. Meerwein, G. Hinz, P. Hofmann, E. Kroning, and E. Pfeil, *J. prakt. Chem.*, **147**, 257 (1937).
8) D.S. Kemp and S.W. Chien, *J. Amer. Chem. Soc.*, **89**, 2743 (1967).

$$RCO_2^- + Et_3O^+BF_4^- \longrightarrow RCO_2Et + Et_2O + BF_4^- \quad Et=-C_2H_5$$

Chart 1

Table I. Esterification with Various Amounts of I

Mole equivalents of I	4.5	9.0	13.5	18.0
Acyl amino acid and peptide		yield (%)		
Hippuric acid (IIa)	30	64	83	82[a]
	51	62	82	82[b]
N-Bz-DL-Ala-Gly (IIIa)		57	79	89
N-Bz-Gly-L-Val (IVa)		65	77	91

a) I was added in solid. *b*) in acetonitrile solution

The pH dependence of this esterification was then examined, for example, Table II shows the esterification of hippuric acid (IIa) and it is indicated that the carboxylate form is a better species for this reaction.

Table II. The pH Dependence of the Esterification of IIa

pH	2.0	3.0	4.0	5.5	6.0	7.0
Yield (%)	18	48	73	79	82	82

Since the above preliminary experiments gave fairly good results, various acyl amino acids and dipeptides were subjected to the reaction for the purpose of demonstrating the general applicability of the esterification in the area of peptide chemistry, that is, an aqueous solution of an acyl amino acid or peptide was treated with eighteen fold excess of I at room temperature for 30 min. The mixture was extracted with ethyl acetate which was evaporated to leave the corresponding ester in almost pure state. Some results are summerized in Table III.

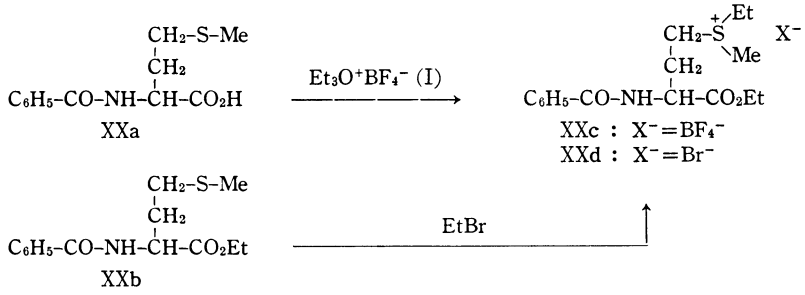

Chart 2

Functional groups, such as the alcoholic hydroxy, phenolic hydroxy, ε-amino and guanidyl groups of side chains of amino acids, as well as benzoyl, phthaloyl, carbobenzoxy groups and the amide bonds, were completely unreactive in the above conditions. However the imidazole ring in histidine and the sulfur atom in methionine still have nucleophilic activities to form the ammonium salt and the sulfonium salts by the alkylation with I.

The sulfonium salt, (3-DL-benzamido-3-carboethoxypropyl)ethyl methylsulfonium fluoroborate (XXc) was easily confirmed by nuclear magnetic resonance (NMR) (see Ex-

Table III. Esterification of Acyl Amino Acids and Peptides

Substrate	Product	Yield (%)
Bz-Gly (IIa)	Bz-GlyOEt (IIb)[9]	82—92
Bz-L-Glu (Va)[10]	Bz-L-GluOEt$_2$ (Vb)[11]	90
Bz-L-Asp (VIa)[10]	Bz-L-AspOEt$_2$ (VIb)[12]	83
Bz-L-Thr (VIIa)[13]	Bz-L-ThrOEt (VIIb)	93
Bz-L-Lys (VIIIa)[14]	Bz-L-LysOEt (VIIIb)[15]	82
Bz-L-Arg (IXa)[16]	Bz-L-ArgOEt (IXb)[17]	90
Bz-L-Tyr (Xa)[18]	Bz-L-TyrOEt (Xb)[19]	86
Cbz-DL-Ser (XIa)[20]	Cbz-DL-SerOEt (XIb)	91
Cbz-DL-Thr (XIIa)[21]	Cbz-DL-ThrOEt (XIIb)	94
Cbz-L-Arg (XIIIa)[20]	Cbz-L-ArgOEt (XIIIb)	96
Bz-DL-Ala-Gly (IIIa)[22]	Bz-DL-Ala-GlyOEt (IIIb)[3]	89
Bz-Gly-L-Val (IVa)[3]	Bz-Gly-L-ValOEt (IVb)[3]	91
Cbz-DL-Ala-Gly (XIVa)[23]	Cbz-DL-Ala-GlyOEt (XIVb)[23]	90
Cbz-Gly-Gly (XVa)[24]	Cbz-Gly-GlyOEt (XVb)[25]	87
Cbz-Gly-L-Ala (XVIa)[26]	Cbz-Gly-L-AlaOEt (XVIb)[26]	88
Cbz-L-Leu-Gly (XVIIa)[27]	Cbz-L-Leu-GlyOEt (XVIIb)[28]	92
Phth-Gly-Gly (XVIIIa)[29]	Phth-Gly-GlyOEt (XVIIIb)[30]	92
L-Trp (XIXa)	Di-Et-L-TrpOEt (XIXb)	62.5
Bz-DL-Met (XXa)[31]	(XXc)	98
Bz-L-His (XXIa)[32]	(XXIc)	58
Ac-DL-His (XXIIa)[32]	(XXIIc)	79

perimental), in which the methylene of S–CH_2CH_3 appears at δ 3.40 (quartet, J=7 Hz). N-Benzoyl-DL-methionine ethyl ester (XXb) was ethylated with ethyl bromide to yield the sulfonium salt (XXd),[33] which is identical to the above one by thin layer chromatography and NMR spectra.

The structure of the ammonium salts (XXIc, XXIIc) were also determined by NMR, in which three ethyl groups appeared. The reactivity of the protonated imidazole ring was next examined. At various pH, N-acetyl-DL-histidine ethyl ester (XXIIb) was treated with

9) E. Fischer, *Chem. Ber.*, **38**, 613 (1905).
10) E. Fischer, *Chem. Ber.*, **32**, 2451 (1899).
11) E. Abderhalden and E. Rossner, *Z. Physiol. Chem.*, **152**, 277 (1926).
12) J. Max, *Ann*,. **369**, 284 (1904).
13) D.F. Elliott, *Biochem. J.*, **45**, 429 (1949).
14) W.F. Ross and L.S. Green, *J. Biol. Chem.*, **137**, 105 (1941).
15) J. Heidberg, E. Holler, and H. Hartmann, *Ber. Bunsenges. Phys. Chem.*, **71**, 19 (1967).
16) K. Felix, H. Müller, and K. Dirr, *Z. Physil. Chem.*, **178**, 192 (1928).
17) G.W. Schwert amd M.A. Eisenberg, *J. Biol. Chem.*, **179**, 665 (1949).
18) E. Fischer, *Chem. Ber.*, **33**, 3638 (1900).
19) T. Curtius and W. Donselt, *J. prakt. Chem.*, **95**, 327 (1918).
20) M. Bergman and L. Zervas, *Chem. Ber.*, **65**, 1192 (1932).
21) G. Riley, J.H. Turnbull, and W. Wilson, *J. Chem. Soc.*, **1957**, 1373.
22) L. Velluz, G. Amiard, and R. Heymes, *Bull. Soc. Chim. France*, **1954**, 1012.
23) S. Goldschmidt and H. Lautenschlager, *Ann.*, **580**, 68 (1953).
24) O. Süs, *Ann.*, **572**, 96 (1951).
25) T. Wieland and B. Heinke, *Ann.*, **599**, 70 (1956).
26) B.F. Erlanger and E. Brand, *J. Amer. Chem. Soc.*, **73**, 3508 (1951).
27) M. Bergmann, L. Zervas, and J.S. Fruton, *J. Biol. Chem.*, **111**, 225 (1935).
28) T. Wieland and R.W. Young, *J. Amer. Chem. Soc.*, **74**, 5307 (1952).
29) J.C. Sheehan and J.J. Hlavka, *J. Org. Chem.*, **21**, 439 (1956).
30) F.E. King, J.W. Clark-Lewis, R. Wade, and W.A. Swindin, *J. Chem. Soc.*, **1957**, 873.
31) E. M. Hill and W. Robson, *Biochem. J.*, **30**, 248 (1936).
32) M. Bergman and L. Zervas, *Biochem. Z.*, **203**, 280 (1928).
33) cf. M.J. Hawkins, J. R.Knowles, L. Wilson, and D. Witcher, *Biochem. J.*, **104**, 762 (1967).

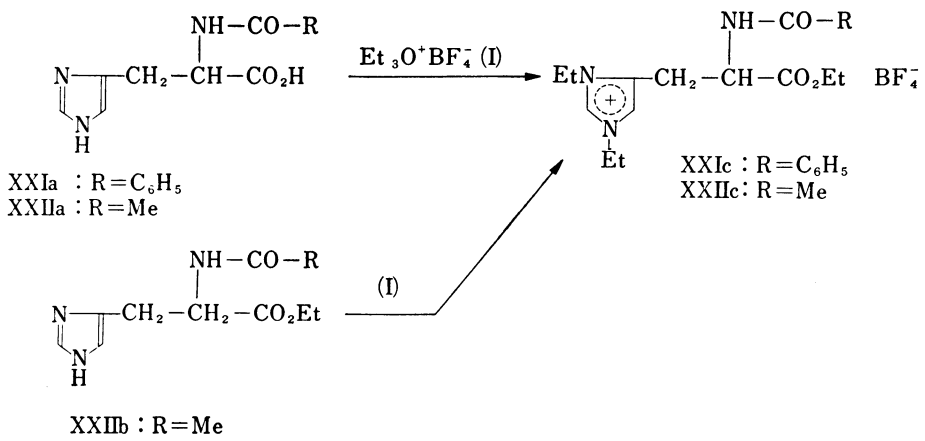

Chart 3

excess of I and analyzed semi-quantitatively by thin-layer chromatography (Fig. 1). The result shows when the esterification of peptide is carried out at pH 3—4, the alkylation of imidazole ring may be avoidable.

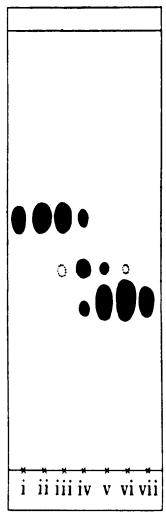

Fig. 1. Alkylation of N-Acetyl-DL-histidine Ethyl Ester (XXIIb) at Various pH

(silica-gal plate, 5×20 cm: solvent; *n*-BuOH, AcOH, H_2O; 4,1,2)
i, XXIIb; ii, with I at pH 3.0; iii, pH 4.0; iv, pH 5.0; v, pH 6.0; vi, pH 7.0; vii, XXIIc

Finally the reactivity of free amino acids were examined and for example L-tryptophan (XIXa) was converted to N,N-diethyl-L-trytophan ethyl ester (XIXb) in faily good yield.

In view of the above facts the esterification and the alkylation with I may provide a potential new method in the area of peptide and protein chemistry, particularly in chemical modification of enzymes, because it is carried out in aqueous solution by the simple and convenient procedure.

Recently, Raftery, *et al.*[34] utilized triethyloxonium fluoroborate (I) to achieve mild selective esterification of carboxylates in lysozyme. Nakayama, *et al.*[35] also demonstrated selective esterification of trypsine in acidic condition. We are now examining the above reaction on lysozyme and other enzymes under various conditions, and for example when the reaction was carried out at neutral pH, the alkylation on imidazole ring of histidine residue in lysozyme was observed.[36] A study of this interesting application to enzyme chemistry is currently in progress.

Experimental

Esterification of Hippuric Acid (IIa) with Various Amounts of Triethyloxonium Fluoroborate (I)——To a stirred solution of 179 mg (1 mmole) of hippuric acid (IIa) in 10 ml of water containing excess sodium

34) S.M. Parsons, L. Jao, E.W. Dahlquist, C.L. Borders, Jr., T. Groff, J. Rack and M.A. Raftery, *Biochemistry*, **8**, 70 (1969).
35) H. Nakayama, K. Tanizawa, and Y. Kanaoka, *Biochem. Biophy. Res. Commun.*, **40**, 537 (1970).
36) T. Hamada to be published.

bicarbonate, various amounts of triethyloxonium fluoroborate (I)[37], *i.e.* 0.86 g (4.5 mmole), 1.71 g (9 mmole), 2.57 g (13.5 mmole) and 3.42 g (18 mmole), in solid or in the solution of 1 ml of acetonitril were added portionwise at room temperature over a period of 10 min. After stirring for an additional 20 min, the reaction mixture was extracted with ethyl acetate, the ethyl acetate solution was washed with 10% sodium carbonate and sodium chloride saturated water, and dried over anhydrous sodium sulfate. Evaporation of the solvent *in vacuo* left colorless crystals of ethyl hippurate (IIb) in almost pure state, mp 58—60°. The result are summarized in Table I.

Esterification of N-Benzoyl-DL-alanylglycine (IIIa) and N-Benzoylglycyl-L-valine (IVa)——N-Benzoyl-DL-alanylglycine (IIIa) and N-benzoylglycyl-L-valine (IVa) were estirified as described above. The results are as follows: substrate, molar equivalents of I, (% yield of ester); N-benzoyl-DL-alanylglycine, 10 (57), 15, (79), 20, (89); N-benzoylglycyl-L-valine, 10, (65), 15, (77), 20, (91).

Esterification of Hippuric Acid (IIa) at Varions pH——An aqueous solution of 179 mg (1 mmole) of hippuric acid (IIa) was treated with 3.42 g (18 mmole) of I at various pH as described above. During the reaction proceeded, the pH of the reaction mixture was kept constant by the continuous addition of 5 N sodium hydroxide from an automatic titrator (Radiometer Titrator TTT 1). The results are summarized in Table II.

General Procedure for the Esterification of Acyl Amino Acids and Acyl Peptides——A stirred solution of 1 mmole of acyl amino acids or acyl peptides in 10 ml of water containing 100 mg of sodium bicarbonate was treated with the portionwise addition of 3.42 g (18 mmole) of I in 1 ml of acetonitrile and 1.7 g (20 mmole) of sodium bicarbonate over a period of 10 min. Stirring was continued for an additional 20 min and the reaction mixture was extracted with ethyl acetate. The extract was washed with 10% sodium carbonate and saturated sodium chloride solution, and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* to yield the corresponding ethyl ester in almost pure state (Table III).

The structures of the unknown ethyl esters were confirmed as follows.

N-Benzoyl-L-threonine Ethyl Ester (VIIb)——A colorless oil. IR ν^{-1}: 3440, 1750, 1650. NMR δ^{CDCl_3}: 7.25—7.95 (6H, m), 4.2—4.85 (2H, m), 4.14 (2H, q, $J=7$ Hz), 3.85 (1H, s), 1.23 (3H, d, $J=7$ Hz), 1.22 (3H, t, $J=7$ Hz). N-Benzoyl-L-threonine (VIIa) was esterified with hydrogen chloride in ethanol to give the authentic sample in 82% yield.

N-Carbobenzoxy-DL-serine Ethyl Ester (XIb)——A colorless oil. IR ν cm^{-1}: 1733, 1718. NMR δ^{CDCl_3}: 7.30 (5H, s), 5.8 (broad s, 1H), 5.09 (2H, s), 4.4 (1H), 4.20 (2H, q, $J=7$ Hz), 3.92 (2H, d, $J=7$ Hz), 2.55 (1H,s), 1.26 (3H, t, $J=7$ Hz).

A solution of 186 mg of N-carbobenzoxy-DL-serine ethyl ester (XIb) in 6 ml of ethanol was hydrogenated in the presece of 200 mg of 10% palladium on charcoal for 6 hr. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo* to give DL-serine ethyl ester, which was converted to the hydrochloride, mp 103—104° (77%).[21]

N-Carbobenzoxy-DL-threonine Ethyl Ester (XIIb)——A colorless oil. IR ν cm^{-1}: 1740 (broad). NMR δ^{CDCl_3}: 7.31 (5H, s), 5.7 (1H), 5.10 (2H, s), 4.3 (2H, m), 4.18 (2H, q, $J=7$ Hz), 2.53 (1H, s), 1.28 (3H, t, $J=7$ Hz), 1.22 (3H, d, $J=6$ Hz).

N-Carbobenzoxy-DL-threonine ethyl ester (XIIb) was hydrogenated as described the proceeding experiment. DL-Threonine ethyl ester hydrochloride, mp 115—116° (64.5%).[21]

N-Carbobenzoxy-L-arginine Ethyl Ester (XIIIb)——A colorless oil. IR ν cm^{-1}: 3380, 1720, 1670, 1533. NMR δ^{CDCl_3}: 7.25 (5H, s), 6.1—7.0 (5H), 5.03 (2H, s), 3.2 (1H), 3.1 (2H, q, $J=7$ Hz), 3.25 (2H), 1.73 (2H), 1.18 (3H, t, $J=7$ Hz).

N-Carbobenzoxy-L-arginine ethyl ester (XIIIb) was catalytically reduced with palladium on charcoal to arginine ethyl ester hydrochloride.[38]

(3-DL-Benzamido-3-carboethoxypropyl) ethyl Methylsulfonium Fluoroborate (XXc)——To a stirred solution of 526 mg (2 mmole) of N-benzoyl-DL-methionine (XXa) in 10 ml of water containing 200 mg of sodium bicarbonate, 3.42 g of I and 1.9 g of sodium bicarbonate was added portionwise. After 20 min the clear reaction mixture was evaporated to dryness at room temperature. The residue was extracted with dry ethanol and ethanol was removed *in vacuo* to yield 785 mg (98%) of a colorless oil. IR ν cm^{-1}: 1740, 1650, 1060 (very strong). NMR δ^{D_2O}: 7.5—7.9 (5H, m), 4.31 (2H, q, $J=7$ Hz), 3.40 (2H, q, $J=7$ Hz), 3.3—3.5 (2H, m), 2.94 (3H, s), 2.3—2.7 (2H, m), 1.48 (3H, t, $J=7$ Hz), 1.30 (3H, t, $J=7$ Hz). The picrate and the perchlorate are also oily compounds.

A solution of N-benzoyl-DL-methionine ethyl ester (XXb) (218 mg, 1 mmole) and 327 mg (3 mmole) of ethyl bromide in 6 ml of 50% aqueous ethanol was heated at 40° in a sealed tube for 24 hr. The reaction mixture was examined with thin-layer chromatography on silica gel (solvent: *n*-BuOH, AcOH, H_2O; 4,2,1) to give the same spot (*Rf* 0.2) with the above sample in *ca.* 20% yield.

4-(L-Benzamido-2-carboethoxyethyl)-1,3-diethylimidazolium Fluoroborate (XXIc)——N-Benzoyl-L-histidine (XXIa) (518 mg, 2 mmole) was treated with I as described above. The ethanol-soluble oil was

37) H. Meerwein, *Org. Syn.*, **46**, 113 (1966).

38) A. Nagamatsu, T. Okuma, M. Watanabe, and Y. Yamamura, *J. Biochem.* (Tokyo), **54**, 491 (1963).

chromatographed on silica gel column and elution with *n*-BuOH, AcOH, H_2O (4,1,1) to give 480 mg (56%) of colorless oil. Pauli reaction is negative. IR ν cm^{-1}: 1740, 1650, 1065. NMR δ^{D_2O}: 8.7, (1H, s), 7.5—7.8 (5H, m), 7.35 (1H, s), 4.95—5.1 (1H), 4.27 (2H, q, J=7 Hz), 4.20 (2H, q, J=7 Hz), 4.12 (2H, q, J=7 Hz), 3.25—3.5 (2H), 1.53 (3H, t, J=7 Hz), 1.38 (3H, t, J=7 Hz), 1.3 (3H, t, J=7 Hz).

4-(DL-2-Acetamido-2-carboethoxyethyl)-1,3-diethylimidazolium Fluoroborate (XXIIc)——N-Acetyl-DL-histidine (XXIIa) (384 mg, 2 mmole) was treated with I as described above. A colorless oil, 581 mg (79%). IR ν cm^{-1}: 1760, 1660, 1050. NMR δ^{D_2O}: 8.75 (1H, s), 7.4 (1H, s), 4.75—5.1 (1H), 4.30 (2H, q, J=7 Hz), 4.25 (4H, q, J=7 Hz), 3.2—3.4 (2H), 2.05 (3H, s), 1.58 (3H, t, J=7 Hz), 1.53 (3H, t, J=7 Hz), 1.30 (3H, t, J=7 Hz).

N-Acetyl-DL-histidine Ethyl Ester Hydrochloride (XXIIb)——N-Acetyl-DL-histidine (XXIIa) (1 g) was esterified with 1.3 N hydrogen chloride in ethanol to yield 1.15 g (90%) of a colorless solid, mp 153—155° (from ethanol–ether). *Anal.* Calcd. for $C_{10}H_{16}O_3N_3Cl$: C, 46.07; H, 5.80; N, 16.16; Cl, 13.60. Found: C, 45.65; H, 6.39; N, 16.29; Cl, 13.63. IR ν^{Nujol} cm^{-1}: 3160, 1735, 1660. NMR δ^{C_2O}: 8.63 (1H, d, J=1 Hz), 7.32 (1H, d, J=1 Hz), 4.9 (1H), 4.21 (2H, q, J=7 Hz), 3.2—3.4 (2H), 2.01 (3H, s), 1.24 (3H, t, J=7 Hz).

Alkylation of N-Acetyl-DL-hystidine Ethyl Ester (XXIIb) at Various pH——N-Acetyl-DL-histidine ethyl ester (XXIIb) was treated with ten-fold excess of I in aqueous acetonitrile at various pH using an automatic titrator. After 30 min the reaction mixture was examined with thin-layer chromatography on silica gel plate, 5×20 cm, (solvent; *n*-BuOH, AcOH, H_2O; 4,1,2). The results were shown in Fig. 1.

N,N-Diethyl-L-tryptophan Ethyl Ester (XIXb)——To an aqueous solution (10 ml) of 189 mg (0.92 mmole) of L-tryptophan (XIXa), 1.9 g of sodium bicarbonate and 3.42 g of I in 1 ml of acetonitrile was added. After stirring for 30 min the reaction mixture was extracted with ethyl acetate. The extract was dried over sodium sulfate and the solvent was evaporated *in vacuo* to give crude N,N-diethyl-L-tryptophan ethyl ester as a colorless oil; NMR δ^{CDCl_3}: 7.0—7.75 (6H, m), 4.05 (2H, q, J=7 Hz), 3.5—3.85 (1H), 2.8—3.3 (2H), 2.67 (4H, q, J=7 Hz), 1.10 (3H, t, J=7 Hz), 1.08 (6H, t, J=7 Hz); which was converted to the hydrochloride, crude yield 275 mg (92%). Recrystallization from ethanol–ether gave 187 mg (62.5%) of a colorless crystal, mp 162—165°. Ninhydrin test; negative. Erhlich test; positive. *Anal.* Calcd. for $C_{17}H_{25}O_2N_2Cl$: C, 62.86; H, 7.76; N, 8.62. Found: C, 63.34; H, 7.99; N, 8.19. NMR δ^{D_2O}: 7.1—7,7 (5H, m), 4.1—4.45 (1H), 4.00 (2H, q, J=7 Hz), 3.28 (4H, q, J=7 Hz), 3.2—3.5 (2H), 1.28 (6H, t, J=7 Hz), 0.93 (3H, t, J=J Hz).

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