The Trifluoroacetate Method of Peptide Synthesis. I. The Synthesis and Use of Trifluoroacetate Reagents

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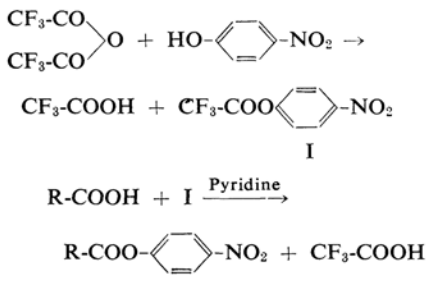
In a preceding paper,[1] a new method for the preparation of the *p*-nitrophenylesters of ordinary carboxylic acids, using *p*-nitrophenyl trifluoroacetate (I) as a reagent, has been reported. Reagent I reacts smoothly with carboxylic acids in pyridine to give the *p*-nitrophenylesters by an ester-exchange reaction. In the present investigation, various active esters of trifluoroacetic acid were synthesized



as possible reagent, and the above-mentioned method was extended to the synthesis of the respective esters of acylated amino acids. In addition, a new procedure for the synthesis of peptides using these new reagents was developed.

First, various compounds, such as phenol, thiophenol and *N*-hydroxyimide derivatives, which are known to be hydroxylic partners of various active carboxylic acid esters, were converted to their respective trifluoroacetates by the procedure used for synthesizing reagent I. The physical constants of and analytical data on the trifluoroacetate reagents obtained are listed in Table I. Since these reagents were rather unstable against water, they had to be kept in sealed tubes or in tightly-closed bottles. Attempts to synthesize the trifluoroacetates of 2, 4-dinitrophenol, 2, 4, 6-trinitrophenol and 2, 4-dinitrothiophenol were unsuccessful under the present experimental conditions. Moreover, the trifluoroacetates of common alcohols, such as ethanol and benzyl alcohol, were found to be inactive as ester-exchange reagents in pyridine.

The reagents cited in Table I were each subjected to reaction with acylated amino acids in pyridine for the ester-exchange reactions;

TABLE I. PHYSICAL CONSTANTS AND ANALYTICAL VALUES OF THE TRIFLUOROACETATE REAGENTS

Compound No.	Name	[m. p.] or (b. p.)[a] °C	Anal. % Found C	Found H	Found N	Calcd. C	Calcd. H	Calcd. N
I	*p*-Nitrophenyl trifluoroacetate[b]	[36—38]	41.11	1.89	5.99	40.86	1.71	5.96
II	Phenyl trifluoroacetate	(123)	50.60	2.61		50.54	2.65	
III	*p*-Chlrophenyl trifluoroacetate	(50/5 mmHg)	43.30	1.89		42.78	1.80	
IV	*p*-Bromophenyl trifluoroacetate	(135—142)	35.71	1.56		35.71	1.50	
V	2, 4, 5-Trichlorophenyl trifluoroacetate	Oil[c]	34.41	0.97		32.74	0.69	
VI	Pentachlorophenyl trifluoroacetate	[64—66]	26.49	0.00		26.51	0.00	
VII	*p*-Methoxyphenyl trifluoroacetate	(150—160)	49.07	3.25		49.10	3.21	
VIII	Thiophenyl trifluoroacetate	(140—150)	46.43	2.45		46.60	2.44	
IX	*p*-Nitrothiophenyl trifluoroacetate	Oil[c]	38.76	1.85		40.51	1.70	
X	*N*-Hydroxysuccinimide trifluoroacetate	Oil[c]	30.59	1.64		34.14	1.91	
XI	*N*-Hydroxyphthalimide trifluoroacetate	Oil[c]	42.71	1.90		46.34	1.56	

a) The boiling point indicates the boiling range, when the material was distilled in a simple distilling flask.
b) S. Sakakibara and N. Inukai, This Bulletin, **37**, 1231 (1964).
c) Purification was unsuccessful and correct analytical values were not obtained because of the instability of this material. Thus, this material should be prepared just before subsequent reactions.

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1) S. Sakakibara and N. Inukai, This Bulletin, **37**, 1231 (1964).

TABLE II. ACTIVE ESTER FORMATION OF ACYLAMINO ACIDS BY ESTER-EXCHANGE REACTION WITH THE TRIFLUOROACETATE REAGENTS IN PYRIDINE

Product	Reagent used	Reaction conditions	Yield %	M. p., °C Found/Cited	$[\alpha]_D$ (°C) *c* 1, in DMF Found/Cited	Anal. % Found/Calcd. C	H	N
Z-L-CyS(MBz)-ONP	I	Room temp. 10—20 min.	85	97—98.5 94—97[a]	−39.3(22) −39.8(21)	60.72 60.48	4.93 4.87	5.59 5.64
BOC-L-Ala-ONP	I	Room temp. 10—20 min.	80	81—82 83[b]	−51.6(26) −52.5(22)	54.08 54.19	5.75 5.85	9.08 9.03
Ac-L-Ala-ONP	I	Room temp. 10—20 min.	83	115—119[c]	0	52.12 52.38	4.80 4.80	10.95 11.11
Phth-L-Phe-ONP	I	Room temp. 10—20 min.	84	169—171	−204.7(23)	66.62 66.34	3.82 3.81	6.83 6.73
Tos-Gly-ONP	I	Room temp. 10—20 min.	80	157—159		51.55 51.42	4.19 4.03	7.95 8.00
Z-L-Phe-OPh	II	Reflux 80 min.	72.5	107—108 106—106.5[d]	−29.5(21) −30.1(25)	73.37 73.54	5.59 5.64	3.68 3.73
Z-Gly-OCP	III	Reflux 35 min.	71	105—107		60.39 60.10	4.62 4.41	4.45 4.38
Z-Gly-OBP	IV	Reflux 25 min.	71	111—114.5		52.83 52.76	3.71 3.87	3.82 3.85
Z-Gly-OTCP	V	Room temp. 10—20 min.	93	105—108 107—108[e]		49.39 49.45	3.05 3.11	3.47 3.60
Z-L-Phe-OTCP	V	Room temp. 10—20 min.	65	140—142 142[f]	−39.4(24) −51 (22)	58.02 57.70	3.85 3.79	2.92 2.93
Z-Gly-OPCP	VI	Room temp. 10—20 min.	78	133—134 185.5—187[g]		41.78 42.00	2.31 2.20	2.98 3.06
Z-L-Phe-OPCP	VI	Room temp. 10—20 min.	74	156—157 153—155.5[d]	−49.4(24) −50.1(26)	50.70 50.44	3.00 2.94	2.48 2.56
Z-L-Val-OPCP	VI	Room temp. 10—20 min.	80	140—141 140—142[d]	−22.8(24) −22.5(26)	45.77 45.67	3.30 3.23	2.76 2.80
Z-L-Glu(OMe)-OPCP	VI	Room temp. 10—20 min.	72.5	118—120 122—123[d]	−27.5(21) −27.1(26)	44.33 44.19	2.98 2.97	2.52 2.58
Z-L-Met-OPCP	VI	Room temp. 10—20 min.	69	129—131 130—131[d]	−30.7(21) −26.9(26)	43.15 42.92	2.95 3.03	2.58 2.63
Z-Gly-OMP	VII	Reflux 20—30 min.	71	86.5—88.5		64.55 64.75	5.55 5.43	4.53 4.44
Z-Gly-SPh	VIII	Reflux 2 hr.	50	70—72 72[h]		63.77 63.77	4.98 5.02	4.63 4.65
Z-L-Phe-SPh	VIII	Reflux 1 hr.	81	128—130[c] 116—118[d]	0 −78.2(26)	70.38 70.56	5.53 5.41	3.52 3.58
Z-Gly-SNP	IX	Room temp. 10—20 min.	83.4	110—112 112.5[i]		55.13 55.48	4.02 4.07	7.98 8.09
Z-L-Phe-SNP	IX	Room temp. 10—20 min.	81	177—180.5 174—176[d]	−71.5(26) −68.5(26)	63.08 63.29	4.43 4.62	6.34 6.42
Z-Gly-OHSI	X	Room temp. 10—20 min.	55	113—114 113—114[j]		54.94 54.90	4.54 4.61	9.07 9.15
Z-L-Phe-OHSI	X	Room temp. 10—20 min.	90	134—136 140—140.5[k]	−53.8(24) −17.3(25)	63.67 63.63	5.14 5.09	7.02 7.07
Z-Gly-OHPI	XI	Room temp. 10—20 min.	23.8	122—125 124[l]		60.82 61.01	3.94 3.98	7.94 7.91
p-Nitrophenyl laurate	I	Room temp. 10—20 min.	86	44.5—47		67.49 67.26	8.51 8.47	4.29 4.36
p-Nitrophenyl palmitate	I	Room temp. 10—20 min.	96.5	60—63		70.18 69.99	9.35 9.35	3.64 3.71
p-Nitrophenyl levulinate	I	Room temp. 10—20 min.	76.5	60—62		55.47 55.69	4.73 4.67	5.97 5.91

a) S. Sakakibara, Y. Nobuhara, Y. Shimonishi and R. Kiyoi, This Bulletin, **38**, 120 (1965).
b) E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1638 (1963).
c) This material racemized during the reaction.
d) These values were obtained from a compound prepared by the dicyclohexylcarbodiimide method.
e) J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1609 (1963).
f) The same lit. as in e). The same compound which was prepared in the laboratory according to the description of Pless and Boissonnas gave the following values; m. p. 139.5—141°C, $[\alpha]_D^{25}$ −39.8° (*c* 1, DMF).

g) *Chem Abstr.*, **57**, 7373 (1962). The cited value may have been a misprint.
h) T. Wieland, W. Schäfer and E. Bokelmann, *Ann.*, **573**, 99 (1951).
i) J. A. Farrington, G. W. Kenner and J. M. Turner, *Chem. & Ind.*, **1955**, 601.
j) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).
k) The same lit. as in j). The optical rotation was determined in dioxan at C=2. This compound when prepared in this laboratory according to the description of Anderson et al. gave the following values; m. p. 134—136.5°C, $[\alpha]_D^{26}$ −52.8° (*c* 1, DMF). This material did not dissolved in dioxan at room temperature at *c*=2.
l) G. H. L. Nefkens, G. I. Tesser and R. J. F. Nivard, *Rec. trav. chim.*, **81**, 683 (1962).
Z=carbobenzoxy; BOC=*t*-butoxycarbonyl; Ac=acethyl; Phth=phthalyl; Tos=tosyl; NP=*p*-nitrophenyl; Ph=phenyl; CP=*p*-chlorophenyl; BP=*p*-bromophenyl; TCP=2, 4, 5-trichlorophenyl; PCP=pentachlorophenyl; MP=*p*-methoxyphenyl; HSI=*N*-hydroxysuccinimide residue; HPI=*N*-hydroxyphthalimide residue; DMF=dimethylformamide.

Table III. Stabilities of optical rotation of carbobenzoxy-L-phenylalanine active esters in pyridine at room temperature

Compound[a]	Optical rotation change in pyridine (*c* 1)		
	Starting value	Storage time at room temp. hr.	Observed value
Z-L-Phe-ONP	−24.3	19	−24.2
Z-L-Phe-OTCP	−40.6	22	−39.6
Z-L-Phe-OPCP	−49.5	1[b]	−45.4
Z-L-Phe-SPh	−67.5	23	−67.5
Z-L-Phe-SNP	−57.1	2[b]	−57.4
Z-L-Phe-OHSI	−31.8	1[b]	−30.7

No decomposition of these compounds was detectable by thin-layer chromatography during the period except in the cases with b) as a suffix.
a) See Table II for the abbreviations used.
b) Partial decomposition of this material was detected during the period by thin-layer chromatography.

the results are listed in Table II. In addition, the *p*-nitrophenylesters of some fatty acids and keto acid were also prepared. In general, the trifluoroacetates of 2, 4, 5-trichlorophenol (V), pentachlorophenol (VI), *p*-nitrothiophenol (IX) and *N*-hydroxysuccinimide (X) gave good results in the reaction. Reagents II, III, IV, VII and VIII, however, were found to be less reactive, and the reaction mixture had to be heated in order to accelerate the reaction. In the case of XI, a remarkable decomposition of the reagent was observed in pyridine, and the yield of the desired product was poor. Since amino acids with free functional groups on the side chains, such as serine, threonine, tyrosine and histidine, showed complicated side reactions with these trifluoroacetate reagents, these functional groups had to be protected before the compounds could be subjected to the reaction. Carbobenzoxy-L-asparagine also gave a poor yield. This may be due to intramolecular dehydration by the reagent.

No racemization was observed during the ester-exchange reaction of these reagents with phthalylamino acids or urethane-type acylamino acids. In the following cases, however, apparent racemization was observed: (1) When carbobenzoxy-L-phenylalanine was treated with VIII in boiling pyridine, and (2) when acetyl-L-alanine was treated with I at room temperature. The latter case seemed to be general for any of the reagents when usual acylamino acids, such as acetyl or benzoyl derivatives, were treated. Therefore, if racemization is to be avoided, the procedure should be limited to amino acids which are acylated through a phthalyl- or urethane-type group, since these are known to be resistant to racemization. Reagent VIII seemed to be exceptional in causing racemization with carbobenzoxy-L-phenylalanine. The thiophenylester of the compound, when once formed, may readily racemize in hot pyridine during the procedure. To check this possibility, authentic carbobenzoxy-L-phenylalanine thiophenylester was prepared by the dicyclohexylcarbodiimide method; the complete racemization of the compound was observed after treatment with boiling pyridine for one hour.

As can be seen in Table III and Fig. 1, the optical stabilities of other carbobenzoxy-L-phenylalanine active esters were measured in pyridine at room temperature; a distinct decrease in rotation was observed only with the pentachlorophenylester. This may be due not only to the partial racemization, but also to

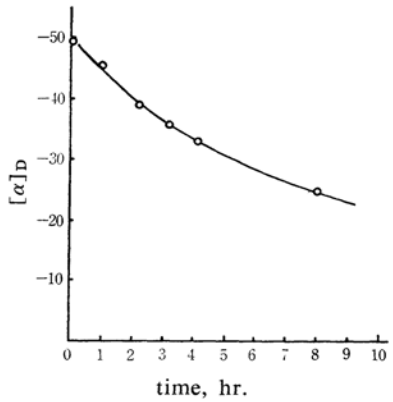

Fig. 1. Optical rotation change of carbobenzoxy-L-phenylalanine pentachlorophenylester in pyridine. $c=1$, 22°C.

the partial decomposition (probably the oxazolon formation) of the compound. Serious decomposition was also observed with the *p*-nitrothiophenylester wtihin two hours. Therefore, care should be taken with prolonged treatment of the materials with VI or IX. Finally, it was concluded that the *p*-nitrophenylesters, 2, 4, 5-trichlorophenylesters and *N*-hydroxysuccinimidesters of phthalyl or urethanetype acyl-L-amino acids can safely be prepared by the new procedure.

Next, the direct syntheses of peptides using the trifluoroacetate reagents I, V and X were studied. One advantage of the trifluoroacetate method in the preparation of active esters of acylamino acids is that the only co-product of the ester-exchange reaction is water-soluble trifluoroacetic acid, which can be removed from the product very easily. Therefore, in preparing peptides, a direct method becomes possible, a method in which the isolation of the active ester of the acylamino acid is not necessary before it is condensed with an amino component. This procedure is considered to be similar to that of the mixed anhydride method, in which the acylamino acid is activated by an acyl chloride reagent, and then the activated intermediate is condensed with an amino acid or peptide ester without being isolated from the reaction mixture.

As examples of the direct method, carbobenzoxy-L-phenylalanyl-L-phenylalanine benzylester, carbobenzoxy-L-phenylalanylglycine ethylester and carbobenzoxyglycyl-L-tyrosine ethylester were prepared, and the optimum conditions of each reaction were investigated. The relationship between the reactivity of each active ester and the amount of triethylamine added to a reaction mixture was interesting. In the cases of reaction with I or V, two equivalents of triethylamine were necessary to proceed the

TABLE IV. DIRECT SYNTHESIS OF PEPTIDES BY THE TRIFLUOROACETATE METHOD FROM CARBOBENZOXYAMINO ACID AND AMINO ACID ESTER HYDROCHLORIDES OR TOSYLATES

Product	Reagent	Mole equiv. of triethylamine used	Reaction period for peptide formation	Yield %	M. p. °C	$[\alpha]_D$ (c , solvent) (°C)
Z-L-Phe-	I	2	1 hr.	85	147—151[a]	+8.2(2, $CHCl_3$) (25)
L-Phe-OBz	V	2	1 hr.	80	149—152	+8.0(2, $CHCl_3$) (25)
	X	0	5 days	80	149—150	+8.1(2, $CHCl_3$) (21)
	X	1	2 days	73	150—151	+8.5(2, $CHCl_3$) (25)
	X	2	1 hr.	96	149—151	+8.4(2, $CHCl_3$) (25)
	X[b]	1	2 days	94	152	+8.2(2, $CHCl_3$) (25)
Z-L-Phe-Gly-	I	2	6 hr.	98	108—110[c]	−17.1(3, EtOH) (21)
OEt	V	2	6 hr.	94	109—111	−17.4(3, EtOH) (21)
	V[b]	1	1 day	83	110—110.5	−16.5(4, EtOH) (25)
	X	1	2 days	81	110—111	−17.2(5, EtOH) (22)
	X[b]	1	2 days	93	110—111	−16.9(5, EtOH) (22)
	X	2	6 hr.	95	109—111	−17.1(3, EtOH) (21)
Z-Gly-L-Tyr-	I	2	6 hr.	89	123—125[d]	+19.1(3, EtOH) (21)
OEt	I[b]	1	1 day	97	125—126	+19.3(3, EtOH) (21)
	V	2	6 hr.	95	125—127	+19.1(3, EtOH) (21)
	X	2	6 hr.	92	125—126	+19.0(3, EtOH) (21)

a) Cited: m. p. 149—150°C, $[\alpha]_D^{25}$ +8.4 (c 2, $CHCl_3$); T. Sugimura and W. K. Paik, unpublished data.

b) The intermediate, carbobenzoxyamino acid active ester, was isolated and then recrystallized before being subjected to reaction with amino acid ester hydrochloride or tosylate in pyridine.

c) Cited: m. p. 110—111°C, $[\alpha]_D^{25}$ −16.9 (c 5, EtOH); R. W. Young, K. H. Wood, R. J. Joyce and G. W. Anderson, *J. Am. Chem. Soc.*, **78**, 2126 (1956).

d) Cited: m. p. 126—127°C, $[\alpha]_D^{24}$ +19.2 (c 5, EtOH); G. W. Anderson, J. Blodinger and A. D. Welcher, ibid., **74**, 5309 (1952).

reaction; these may be consumed for liberating the free ester from the hydrochloride or tosylate and for neutralizing the trifluoroacetic acid formed. In the case of reaction with X, however, the addition of triethylamine was not necessary for the completion of the reaction, even if ester hydrochloride or tosylate was used, and the only contribution of the triethylamine to the reaction was to reduce the reaction period. The pyridine in the reaction mixture should act as the necessary base for the reaction (Table IV). The yields of the reaction products were excellent, and their purities were also quite satisfactory. The procedure should be useful for the step wise elongation method of peptide synthesis.

One advantage of the direct method for peptide synthesis is that the procedure can be applied whether or not the acylamino acid active ester can be crystallized. For the same reason, it should also be useful for the synthesis of long peptides by the fragment-condensation method or for the synthesis of cyclic peptides. Studies of these possibilities will be published in subsequent papers.

Experimental

All melting points given were uncorrected, and all were determined by the capillary method in a sulfuric acid bath. The completion of the reaction was checked by simplified thin-layer chromatography[2] using Merck's silica gel G and with a mixture of chloroform, methanol and acetic acid (95 : 5 : 3 v/v) as the solvent. *N*-Carbobenzoxy or *t*-butoxycarbonyl compounds were developed on the plates by heating with hydrobromic acid, followed by treatment with ninhydrin.

Materials.—Trifluoroacetic anhydride was prepared according to the procedure of Bourne.[3] *p*-Nitrothiophenol was prepared according to the procedure of Price and Stacy,[4] as was 2, 4-Dinitrothiophenol. *N*-hydroxysuccinimide was prepared by the procedure of Anderson et al.[5] *N*-hydroxyphthalimide was prepared by the method of Nefkens and Tesser.[6] *p*-Chlorophenol, *p*-bromophenol, 2, 4, 5-trichlorophenol, pentachlorophenol, *p*-methoxyphenol and thiophenol were obtained from the Tokyo Kasei Co., Ltd., Tokyo. Acyl amino acids were prepared by standard procedures, and their physical constants were checked before the reactions. Pyridine was dried over potassium hydroxide pellets before use.

The General Procedure for the Preparation of Trifluoroacetate Reagents.—A mixture of trifluoroacetic anhydride (0.15 mol.) and a phenolic compound or an *N*-hydroxyimide derivative (0.1 mol.) in benzene (30 ml.) was refluxed for about 5 hr. until all the crystals had disappeared. The solution was then concentrated to dryness in order to obtain the product. The yield was generally quantitative, and the product was used without further purification in subsequent reactions. The trifluoroacetates of 2, 4, 5-trichlorophenol, *p*-nitrothiophenol, *N*-hydroxysuccinimide and *N*-hydroxyphthalimide did not give correct analytical data, because all the purification procedures tested, such as recrystallization and distillation, caused their partial decomposition. Other trifluoroacetates could, however, be purified for analysis by sublimation or distillation. Their physical constants and analytical data are listed in Table I.

The General Procedure for the Preparation of Acylamino Acid-active Esters Using Trifluoroacetate Reagents.—A solution of an acylamino acid (0.005 mol.) in pyridine (3 ml.) was treated with a trifluoroacetate reagent (0.006 mol.) until the starting material had disappeared; this was checked by thin-layer chromatography. After the reaction was complete, water (about 20 ml.) was added to the reaction mixture in order to precipitate the product as crystals; these were then separated by filtration and recrystallized from a suitable solvent system. The yields, properties and analytical data on these products are listed in Table II.

The General Procedure for the Preparation of Carbobenzoxy Dipeptide Esters by the Direct Method.—A solution of carbobenzoxy-amino acid (0.005 mol.) in pyridine (4 ml.) was treated with a trifluoroacetate reagent (0.006 mol.), I, V or X, as has been described above, and then water (0.018 ml.) was added to the reaction mixture in order to destroy the excess reagent. A solution of amino acid ester hydrochloride or tosylate (0.005 mol.) and triethylamine (2 mole equivalents) in chloroform (4 ml.) was added to the reaction mixture. After an adequate period, water was added to the solution, and the oil precipitated was extracted with ethyl acetate. The extract was washed successively with a 10% sodium bicarbonate solution or, if necessary, with N ammonia and N hydrochloric acid, and then dried over anhydrous magnesium sulfate. The dried solution was concentrated to dryness, and the residue was crystallized from a suitable solvent system. The data are listed in Table IV.

Summary

The trifluoroacetates of various phenol, thiophenol and *N*-hydroxyimide derivatives, which are known as hydroxylic partners of various active carboxylic acid ester, have been synthesized, and it has been found that they are good reagents for the preparation of the respective active esters of acylamino acids by the ester-exchange reaction in pyridine. The new method is especially effective in the preparation of *p*-nitrophenyl, 2, 4, 5-trichlorophenyl and *N*-hydroxysuccinimide esters of acylamino acids, which are well known to be useful intermediates for peptide synthesis. The direct

2) K. Morita and F. Haruta, *J. Chromatog.*, **12**, 412 (1963).
3) E. J. Bourne, M. Stacey, J. C. Tatlow and J. M. Tedder, *J. Chem. Soc.*, **1949**, 2976.
4) C. C. Price and G. W. Stacy, *J. Am. Chem. Soc.*, **68**, 498 (1946).
5) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, ibid., **85**, 3039 (1963); ibid., **86**, 1839 (1964).
6) G. H. L. Nefkens and G. I. Tesser, ibid., **83**, 1263 (1961).

syntheses of peptides using these reagents, without the isolation of the intermediates, have also been studied, and the new procedure has been found to be useful for the step wise elongation of peptides.

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