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INVESTIGATIONS ON THE STEREOSPECIFICITY OF PEPTIDE ACTIVE PHENYL ESTER FORMATION AND COUPLING*

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Abstract—Phenolic active esters were investigated for stereospecificity during their formation and subsequent coupling. The different esters of Z—Gly—Phe—OH, Z—Gly—Gly—Phe—OH and Bz—Leu—OH were prepared either by: (a) the backing-off procedure, (b) the usual DCC method, (c) the reverse DCC procedure, or (d) by use of a crystalline "complex" consisting of the isourea derivative "A" and 2 PCPOH. In the preparation of active esters by methods (b), (c), and (d), it was found that the more acidic the phenol component the greater was the optical purity of the ester formed. Method (d) generally afforded the highest optical purity. Under coupling conditions similar to those employed for the preparation of sequential polypeptides using pentachlorophenyl esters, no racemization was encounted as shown by the Anderson racemization test.

In esterification of Z—Gly—Phe—OH with PCPOH using DCC (1 : 1 : 1), the oxazolone formed rapidly, and the rate of ring opening showed the following order: DNPOH > PCPOH > NPOH. In the presence of triethylamine, the opening by the phenols was reversed. However, the rate of oxazolone ring opening by DNPOH and PCPOH was faster in the absence of base. The faster rate of ring opening by the more acidic phenols suggests a pathway indicated by Scheme 1. The rate of active ester formation from Z—Gly—OH using DCC gave the following order: DNPOH (p*K* 4·1) > PCPOH (p*K* 5·3) > NPOH (p*K* 7·2). The major path to active ester is through direct attack of the phenols on the acylisourea intermediate, while the minor path is through $(Z—Gly)_2O$.

THE usefulness of activated esters in peptide synthesis is well-established.[1] The active ester method for the synthesis of sequential polypeptides was first introduced in 1953 by Wieland.[2a] The applicability of this general approach has since been demonstrated by several other investigators.[2b–e] In recent years pentachlorophenyl

TABLE 1. PER CENT DISSOCIATION OF "COMPLEX" I IN VARIOUS SOLVENTS AT 20°

Solvent	Percent* dissociation	Solvent	Percent* dissociation
Tetrahydrofuran	91	Benzene	63
Dioxane	87	Chloroform	61
Ethyl acetate	79	Dichloromethane	51

* These values were determined by infrared spectroscopy for 9·95 × 10^{-2}M solutions, based on the appearance of the 4·72 μ (—N=C=N—) band.

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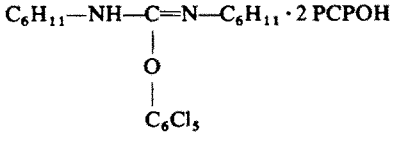
esters have been intensively investigated in this laboratory, including their use in the synthesis of N-protected peptides and polypeptides with known repeating sequence of amino acids.[3] The optical purity of these sequential polypeptides is of utmost importance when they are used as protein models.

In this study, peptide synthesis using pentachlorophenyl active esters, was therefore examined with respect to stereospecificity during active ester preparation and coupling.

Racemization during preparation of N-*protected peptide pentachlorophenyl and other active esters*

The pentachlorophenyl esters synthesized in this report were: Z—Gly—Phe—OPCP, Z—Gly—Gly—Phe—OPCP and Bz—Leu—OPCP.* Also prepared for the sake of comparison were Z—Gly—Phe—ODNP and Z—Gly—Phe—ONP. The various esters were prepared by one or more of the following methods: (a) the backing-off procedure of Goodman,[4] (b) the usual DCC[5] method, i.e. from the N-protected peptides and phenol components, to which was added DCC as condensing agent, (c) the reverse DCC procedure, i.e. DCC and 2 to 3 equivalents of phenol component were allowed to stand in solution before addition of the acid, or (d) the use of a conveniently synthesized crystalline "complex" of DCC and PCPOH. A "complex" prepared from DCC and PCPOH in ethyl acetate, had a composition of 1 DCC and 3 PCPOH and is designated as "complex" I; however, when prepared in dimethylformamide "complex" II formed which is "complex" I solvated with 1 mole of DMF. This composition is supported by its mass spectrum. A partial structure of the "complex" can be considered as consisting of the isourea† derivative "A" and 2 PCPOH.



"A"

The IR spectrum of this "complex" in the solid state shows little or no absorption at 4·72 μ (—N=C=N—) and a strong band at 5·95 μ (>C=N—).

In solution, as shown in Table 1, "complex" I dissociates to different extents, depending on the solvent, into DCC and PCPOH.

The results of the preparation of the various esters are summarized in Table 2. The optical purity of the peptide active esters obtained by procedures (b), (c) or (d) was determined by comparing the specific rotations of the crude esters with those of the pure compounds prepared by the backing-off procedure.[4] Preparation of Z—Gly—Phe—ODNP by the backing-off procedure was not successful. The optical purity of Z—Gly—Phe—ODNP listed in the table was established when no racemic Z—Gly—Phe—Gly—OEt was obtained on coupling in the Anderson test.[7–9]

* Abbreviations for amino acids, peptides and their derivatives are those recommended in *Peptides, Proc. Fifth European Peptide Symp., Oxford*, 1962, Edited by G. T. Young, p. 261. Pergamon Press, Oxford (1963). PCPOH = Pentachlorophenol; DNPOH = 2,4-dinitrophenol; NPOH = p-nitrophenol.

† Structures similar to "A", of phenols and DCC have been reported.[6]

From Table 2 it can be seen that contrary to a recent report,[10] N-protected peptide pentachlorophenyl active esters can be prepared by methods employing DCC as condensing agent, in substantially higher optical purity than 0–25%.* Reaction temperature and solvent noticeably influence the optical purity of the resulting active ester. In addition, the nature and concentration of the phenol component play an important role in the optical purity of the ester. Use of the "complexes" at room temperature in ethyl acetate afforded crude Z—Gly—Phe—OPCP in 78–93% yield with 92–100% optical purity, indicating greater stereospecificity than the usual DCC procedure used in a 1:1:1 ratio of Z—Gly—Phe—OH, PCPOH and DCC. When the "complex" reaction was compared to the usual DCC method using a 2 mole excess of PCPOH, the ester from the "complex" procedure was not significantly better. The greater stereospecificity of the "complex," then, is attributed in the main to the large excess of PCPOH. Preparation of Z—Gly—Gly—Phe—OPCP using "complex" II was also superior to the usual (1:1:1) DCC method.

Noteworthy is the optical purity of crude Z—Gly—Phe—OPCP and Z—Gly—Phe—ODNP, prepared by the usual (1:1:1) DCC method at −10°, when compared to that of Z—Gly—Phe—ONP. Table 3 summarizes the results of these three preparations. It was concluded from these results that the optical purity of the crude phenyl esters of Z—Gly—Phe—OH is related to the acidity of the phenol used, the lower the pK of the phenol the higher the optical quality of the product obtained by the usual DCC procedure.

TABLE 3. COMPARISON OF pK OF PHENOL AND OPTICAL PURITY OF CORRESPONDING CRUDE ESTER OF Z—Gly—Phe—OH PREPARED AT −10°

Ester	pK*	Yield (%)	$[\alpha]_D$ of crude	Optical Purity (%)
Z—Gly—Phe—ODNP	4·1	79	−29·7°	99·0
Z—Gly—Phe—OPCP	5·3	72	−33·9°	90·0
Z—Gly—Phe—ONP	7·2	5·9	−1·5	23·0

* The pK values were calculated from the dissociation constants (25°) listed in *Landolt-Bornstein*, Vol. II, Part 7, pp. 878, 885. Springer Verlag, Berlin (1960).

Racemization during coupling using Anderson[7–9] *and Young tests*[11]

Z—Gly—Phe—OPCP and Z—Gly—Phe—ODNP were coupled with HCl·H—Gly—OEt in the presence of triethylamine to form Z—Gly—Phe—Gly—OEt. The resulting crude tripeptide ester was then subjected to the Anderson solubility test. Results show that in dimethylformamide and in dioxane no Z—Gly—DL—Phe—Gly—OEt, was formed, indicating that the coupling is stereospecific, and that chloride ion does not cause racemization.[11,12]

Z—Gly—Phe—ODNP appeared to be superior to Z—Gly—Phe—OPCP for peptide synthesis since it could be prepared in higher optical purity by the usual

* We assume the differences between our results and those reported in ref. 10 are due to differences in reaction conditions.

TABLE 2. PREPARATION* OF PEPTIDE ACTIVE ESTERS

Peptide active ester	No.	Method	Solvent	Temp. (°C)	Time (hr)	Crude			Recrystallized		% Opt. purity	
						Yield (%)	M.p. (°C)	$[\alpha]_D$ 1% $CHCl_3$†	M.p. (°C)	$[\alpha]_D$ 1% $CHCl_3$†	Crude	Recryst.
Z—Gly—Phe—OPCP	1	Backing-off	EtOAc	−10	3·5	89	154–156·5	—	160–161	−37·7°	—	100
Z—Gly—Phe—OPCP	2	Usual	EtOAc–DMF (20:1)	−10	22	72	158·5–159	−33·9°	161–162	−37·0°	90·0	98·0‡
Z—Gly—Phe—OPCP	3	Usual	EtOAc	−10	22	77	163–164	−29·0°§	—	—	76·8	—
Z—Gly—Phe—OPCP	4	Usual	EtOAc–Diox. (1:1)	−10	22	60	164–165	−26·0°§	—	—	70·0	—
Z—Gly—Phe—OPCP	5	Usual	EtOAc	R.T.	22	65	164·5–166	−26·4°§	—	—	70·0	—
Z—Gly—Phe—OPCP	6	Usual‖	EtOAc	R.T.	22	72	162–163	−36·1°§	—	—	95·7	—
Z—Gly—Phe—OPCP	7	"Complex" I	EtOAc	R.T.	20	93	161–168	−34·7°§	—	—	92·1	—
Z—Gly—Phe—OPCP	8	"Complex" I	DMF	0	20	80	167–168	−18·8°§	—	—	49·8	—
Z—Gly—Phe—OPCP	9	Reverse (3:1)	EtOAc	R.T.	22	75	161·5–162	−36·1°§	—	—	95·7	—
Z—Gly—Phe—OPCP	10	"Complex" II¶	EtOAc	R.T.	48	92	159–162	−37·9°§	—	—	100	—
Z—Gly—Gly—Phe—OPCP	11	Backing-off	EtOAc	−10	3	84	131·5–136	—	171–172**	−34·8°††	—	100
Z—Gly—Gly—Phe—OPCP	12	Usual	EtOAc–Diox. (1:1)	−10	20	69	170–171	−30·3°§, ††	—	—	87·0	—
Z—Gly—Gly—Phe—OPCP	13	"Complex" II	EtOAc	R.T.	20	86	173–174·5	−34·5°§, ††	—	—	99·1	—
Bz—Leu—OPCP	14	Backing-off	Et_2O–EtOAc	0	2	72	127–128	—	125–126	−34·2°	—	100

Bz—Leu—OPCP	15	Usual	EtOAc	−10	18	90	122–124	−11·6°§	—	—	34·0	—
Bz—Leu—OPCP	16	Reverse (2:1)	EtOAc	−15	23	71	119–121	−19·0°§	125–126·5	−33·8°	55·7	99·0
Z—Gly—Phe—ODNP	17	Usual‡‡	EtOAc–Diox.	−10	2·5	75	84·5–86	−29·1°§, §§	84–85	−30·0°§§	97·6	100
Z—Gly—Phe—ODNP	18	Usual	EtOAc–Diox. (1:1)	−10	20	79	86–89	−29·7°§	—	—	99·0	—
Z—Gly—Phe—ONP	19	Usual	EtOAc–DMF (20:1)	−10	20	5·9‖‖	127–130	−1·5°§	—	—	23·0	—
Z—Gly—Phe—ONP	20	Reverse (3:1)	EtOAc	0	2	57 (1st crop)	133–135	−0·9°§, §§	—	—	13·8	—
						18 (2nd crop)	110–130	—	145–146	−6·3°§§	—	100

* Additional pertinent information not included in the table is given in the experimental section.

† Rotations were taken at room temperature which varied between 23 and 33°.

‡ Three recrystallizations gave 100% optically pure product.

§ Elemental analysis for this material was correct.

‖ Z—Gly—Phe—OH, PCPOH and DCC reacted in 1:3:1 molar ratio. A control experiment run at the same time using "complex" I gave 78·5% crude ester, m.p. 162–163°, $[\alpha]_D$ −36·8° (97·6% optical purity)§.

¶ 50% excess of "complex" II used. These results were the best obtained.

** This material exhibits polymorphism; when crystallized from EtOAc m.p. is 171–172°, from $CHCl_3$ – petroleum ether m.p. is 137–138·5°. A previously reported preparation (3f) of m.p. 112–113° and $[\alpha]_D^{25}$ −10° (*c*, 1, chloroform) is considered to be partially racemic.

†† Rotations read in DMF since pure material is very insoluble in $CHCl_3$.

‡‡ 1 molar excess of DNPOH used.

§§ Concentration 2%.

‖‖ The major reaction product was Z—Gly—DL—Phe—OH (60%). The DL-free acid formation can be explained by the generation of the oxazolone intermediate, which reacted only partially with the phenol. The unreacted oxazolone was racemized and opened during the work-up, which included sodium bicarbonate treatment. This isolation is similar to that used by Goodman[4] in the preparation of Z—Gly—Phe—ONP.

DCC method; however, it displayed a noticeable instability.* When stored under the same conditions Z—Gly—Phe—OPCP showed no change in melting point or specific rotation over a 6-month period.

Bz—Leu—OPCP† was coupled with H—Gly—OEt in the very sensitive Young test in a number of solvents, under the conditions described in Table 4. The effect of solvent and temperature on the degree of racemization is obvious from the table. In chloroform, carbon tetrachloride, ethyl acetate and benzene, within a few minutes after addition of H—Gly—OEt, a precipitate of the PCPOH salt of H—Gly—OEt formed. The longer reaction periods listed for several of the experiments in the table were used because the rate of coupling was slowed down by the formation of the sparingly soluble salt.

A comparison was made polarimetrically of the relative rates of racemization with triethylamine and coupling of Z—Gly—Phe—OPCP with H—Gly—OEt. The racemization curves did not show any change in rotation for 45 min; after 2 hr the ester still retained 95% of the optical activity. An approximate racemization half-life of 22 hr was calculated. The coupling half-life of Z—Gly—Phe—OPCP was found to be approximately 5 min.

Racemization of Bz—Leu—OPCP in the presence of triethylamine was also studied polarimetrically. Results showed an "induction"‡ period of 75 min and a racemization half-life of approximately 13 to 14 hr.

The racemization studies with Z—Gly—Phe—OPCP and Bz—Leu—OPCP show that in the presence of triethylamine, both go through an "induction" period prior to any observable racemization. Under favorable conditions, coupling of the highly reactive pentachlorophenyl esters is almost completed during this "induction" interval. It was concluded, therefore, that under the proper conditions, peptide pentachlorophenyl active esters can form sequential polypeptides without racemization.

Pathway of phenolic active ester formation

The path to N-protected peptide active pentachlorophenyl esters using the DCC

* A crude preparation melting at 83–84° initially, with an optical purity of 88%, after standing in a vial at room temperature for 2 months was partially (45%) converted to Z—Gly—DL—Phe—OH. Recrystallization from ethyl acetate–petroleum ether, gave a m.p. 155–158° (Lit.[13] m.p. 159·5–160·5°); its IR spectrum was identical with that of authentic Z—Gly—Phe—OH. The appearance of the DL-dipeptide free acid can be explained by the formation of the oxazolone, subsequent racemization and hydrolysis by atmospheric moisture.

† Our preparation of Z—Leu—OPCP used in the synthesis of Bz—Leu—OPCP by the "backing-off" procedure, melted at 127–128°, $[\alpha]_D^{25}$ −33·3° (c, 0·45, methanol); reported values G. Kupryszewski and M. Formela, *Roczniki Chem.* 35, 1533 (1961); *Zeszyty Nauk, Mat. Fiz. Chem. Wyzsza Szkola Pedagog. Gdansku* 1, 99 (1961) were m.p. 122–124°, $[\alpha]_D^{18}$ −44 ± 4° (c, 0·43, methanol). Comparison of $[\alpha]_D$ values implied out analytically correct ester to be only 76% optically pure. However, coupling of our ester with specific rotation of −33·3° with H—Gly—OEt gave 90·4% crude Z—Leu—Gly—OEt, m.p. 98·5–101° and on recrystallization, 81% of the theoretical yield of dipeptide ester, m.p. 103–104° (Lit. value: 104–105°[7]), $[\alpha]_D^{24}$ −27·2° (c, 5·02, ethanol), Lit value: $[\alpha]_D^{25}$ −26·4° (c, 5·0, ethanol).[7] The analogous coupling reported in the literature, (Kupryszewski *et al., loc. cit.*) where the Z—Leu—OPCP with specific rotation of −44 ± 4° was used, gave a crude protected dipeptide ester in 75% yield with a melting point of 94–97°. On recrystallization, those investigators raised the melting point to 100–101°, and obtained a specific rotation of −27 ± 1°. On the basis of the foregoing results the reported values for the melting point and specific rotation of Z—Leu—OPCP should be corrected.

‡ The term "induction period" used here is intended to mean that no measureable change in specific rotation took place.

TABLE 4. RESULTS OF YOUNG TEST ON Bz—Leu—OPCP

No.	Conditions*	Solvent	Time, (hr)	Temp., (°C)	Crude ester†						% DL-Acid isolated
					Yield, (%)	M.p., (°C)	$[\alpha]_D$ (c 2, EtOH)	Found (%)			
								C	H	N	
1	A	dioxane	22	R.T.	77	155·5–156	−32·0°	63·53	7·56	8·58	0‡
2	A	CCl_4	22	R.T.	85	145–152·5	−28·5°	62·86	7·77	8·54	0‡
3	A	benzene	22	R.T.	75	148–153·5	−27·8°	63·73	7·59	8·80	9·5
4	A	$CHCl_3$	22	R.T.	72	146–152·5	−28·0°	63·40	7·57	8·69	12·5
5	A	EtOAc	22	R.T.	79	151–154·5	−30·7°	63·37	7·55	8·70	5·5
6	A	THF	22	R.T.	78	138–150	−24·4°	63·66	7·46	8·57	19·0
7	A	DMF	22	R.T.	78	139–141·5	−14·6°	63·66	7·32	8·60	43·9
8	B	$CHCl_3$	0·5	R.T.	80	156–156·5	−33·2°	63·89	7·47	8·68	0‡
9	C	$CHCl_3$	24	R.T.	72	154·5–156	−30·0°	63·89	7·38	8·78	3·6
10	A	$CHCl_3$	3·5	0	63	156·5–157·5	−32·8°	—	—	—	—
			+16	+4							
11	D	$CHCl_3$	16	R.T.	76	135·5–152	−18·6°	—	—	—	—
12	A	THF	1	0	79	144–153·5	−28·1°	63·83	7·54	8·74	9·5
			+16	+4							

* Conditions: (A) 6 mmoles each of Bz—Leu—OPCP and freshly distilled H—Gly—OEt reacted; (B) 6 mmoles each of Bz—Leu—OPCP and freshly distilled H—Gly—OEt reacted initially. The insoluble salt of PCPOH and H—Gly—OEt filtered after 5 min and another 6 mmoles of H—Gly—OEt added. The second crop of salt was filtered, followed by isolation of Bz—Leu—Gly—OEt; (C) 6 mmoles of Bz—Leu—OPCP and 7 mmoles of freshly distilled H—Gly—OEt reacted; (D) 1 mmole amounts of HCl·H—Gly—OEt, triethylamine and Bz—Leu—OPCP reacted.

† Anal. Calcd. for Bz—Leu—Gly—OEt: C, 63·7; H, 7·6; N, 8·8; m.p. 156·5–157°, $[\alpha]_D^{20}$ −34·0° (*c*, 3·1, ethanol)[11] and 157–158°, $[\alpha]_D^{20}$ −32·5 ± 0·5° (*c*, 3, ethanol).[14]

‡ M.p. and $[\alpha]_D$ values of Bz—Leu—Gly—OH isolated were: for No. 1, m.p. 133·5–136°; for No. 2, m.p. 135–136°, $[\alpha]_D^{22}$ −27·1° (*c*, 2·1, ethanol); for No. 8, m.p. 135–136°, $[\alpha]_D^{22}$ −27·6° (*c*, 3·0, ethanol). Lit. values for Bz—Leu—Gly—OH are: m.p. 134–135°, $[\alpha]_D^{20}$ −26·4° (*c*, 4·1, ethanol)[11]; m.p. 135·5–136°, $[\alpha]_D^{23}$ −27·5° (*c*, 1·0, ethanol)[15]. M.p. of Bz—DL—Leu—Gly—OH reported: 165°.[11]

method was investigated by DeTar,[10] who showed by IR spectroscopy that the major intermediate was the oxazolone which was responsible for the extensively racemized ester. Since the oxazolone has also been identified as the intermediate responsible for racemization during peptide formation,[16–21] it was of interest to investigate how this intermediate could result in the formation of active pentachlorophenyl and 2,4-dinitrophenyl esters of high optical quality in our experiments, while the corresponding NPOH ester was shown to be extensively racemized.

Goodman's work showed that the oxazolone, once formed at pH's greater than 7, would almost completely racemize before it was nucleophilically attacked to form peptide or other derivatives.[16, 18, 21] Later, however, Goodman[20] and Schnabel[22] prepared several optically active oxazolones in crystalline form, and in addition, Goodman[21] showed that an oxazolone can be opened by hydrazine without racemization, indicating that under certain conditions the oxazolone does not necessarily racemize.

DeTar *et al.*,[10] during the preparation of Z—Gly—Phe—OPCP, observed that the intermediate oxazolone was formed rapidly while the ester appeared slowly with extensive racemization. Our results with Z—Gly—Phe—OPCP differed from DeTar's in that we obtained this ester in high optical purity. In an exploratory rate study on the reactions of Z—Gly—Phe—OH, PCPOH and DCC in a 1:1:1 molar ratio in dioxane and tetrahydrofuran, the percentage formation of oxazolone (5·47 μ) and active ester (5·60 μ) were calculated and are given in Table 5. Data for the reaction of Z—Gly—Phe—OH with "complex" I are also included in this table. The 5·47 μ absorption is due mainly to oxazolone since 86% of the DCU was isolated in 8 min. If the 5·47 μ peak was due primarily to acylisourea, then only 7% DCU would have been obtained in 8 min by conversion of this amount into the 7% ester. If the anhydride was mainly responsible for this peak, the yield of DCU in this time period would have been less than 50%.

The data in Table 5 show that in the usual procedure the oxazolone forms somewhat more slowly in tetrahydrofuran than in dioxane; once formed it reacts faster in tetrahydrofuran than in dioxane to form the active ester. Contrasting the results obtained with the "complex" method in dioxane, there is a twofold increase in the rate of ester formation versus the usual procedure. Although the acylisourea goes primarily to oxazolone, attack on the acylisourea by PCPOH is not completely excluded, neither is the symmetrical anhydride.

The relative reactivity of the oxazolone with NPOH, PCPOH and DNPOH was investigated. The oxazolone of Z—Gly—Phe—OH[22] was reacted in tetrahydrofuran with the phenols in 1:1 and 1:2 molar ratios, and, in a 1:1:1 molar ratio of oxazolone, phenol component and triethylamine. The disappearance of the 5·47 μ oxazolone peak was used as the basis of quantitation. The results are presented in Figs. 1 and 2. In Fig. 1 the relative order of oxazolone disappearance with the three phenols is: DNPOH > PCPOH > NPOH. When a 1:2 ratio of oxazolone to phenol reactant was used, the reaction rates increased, showing a dependence of the rate on the phenol concentration. Interesting is the marked difference in rate and extent of reaction between the two more acidic phenols, DNPOH and PCPOH, and the less acidic NPOH. DNPOH initially was the most reactive in both the 1:1 and 1:2 ratio reactions, but within 10 min an equilibrium was reached; however PCPOH did not appear to reach any equilibrium. When the oxazolone reacted with phenols in the

TABLE 5. ACTIVE ESTER AND OXAZOLONE FORMATION DURING REACTION OF Z—Gly—Phe—OH WITH PCPOH AND DCC (1:1:1 MOLAR RATIO) AND "COMPLEX" I RESPECTIVELY*

Time (min)	Per cent active† ester present			Per cent oxazolone† present			Per cent DCU isolated		
	Dioxane		THF	Dioxane		THF	Dioxane		THF
	Usual	"Complex"	Usual	Usual	"Complex"	Usual	Usual	"Complex"	Usual
8	7	16	20	82	74	64	86	85	82
20	18	31	38	86‡	74	52	—	—	—
60	24	48	69	76	52	29§	—	—	—
181	—	86	84	—	17	12	—	—	—
185	47	—	—	58	—	—	—	—	—

* Initial concentration of each reactant in the usual procedure was 0·25M and in the "complex" method 0·19M.

† These values represent the per cents of the theoretical amounts, which are based on the assumption of complete conversion of the dipeptide to active ester or oxazolone, respectively.

‡ At 20 min DCC was still present; at 60 min, DCC peak was no longer present.

§ Some DCC was still present at 60 min.

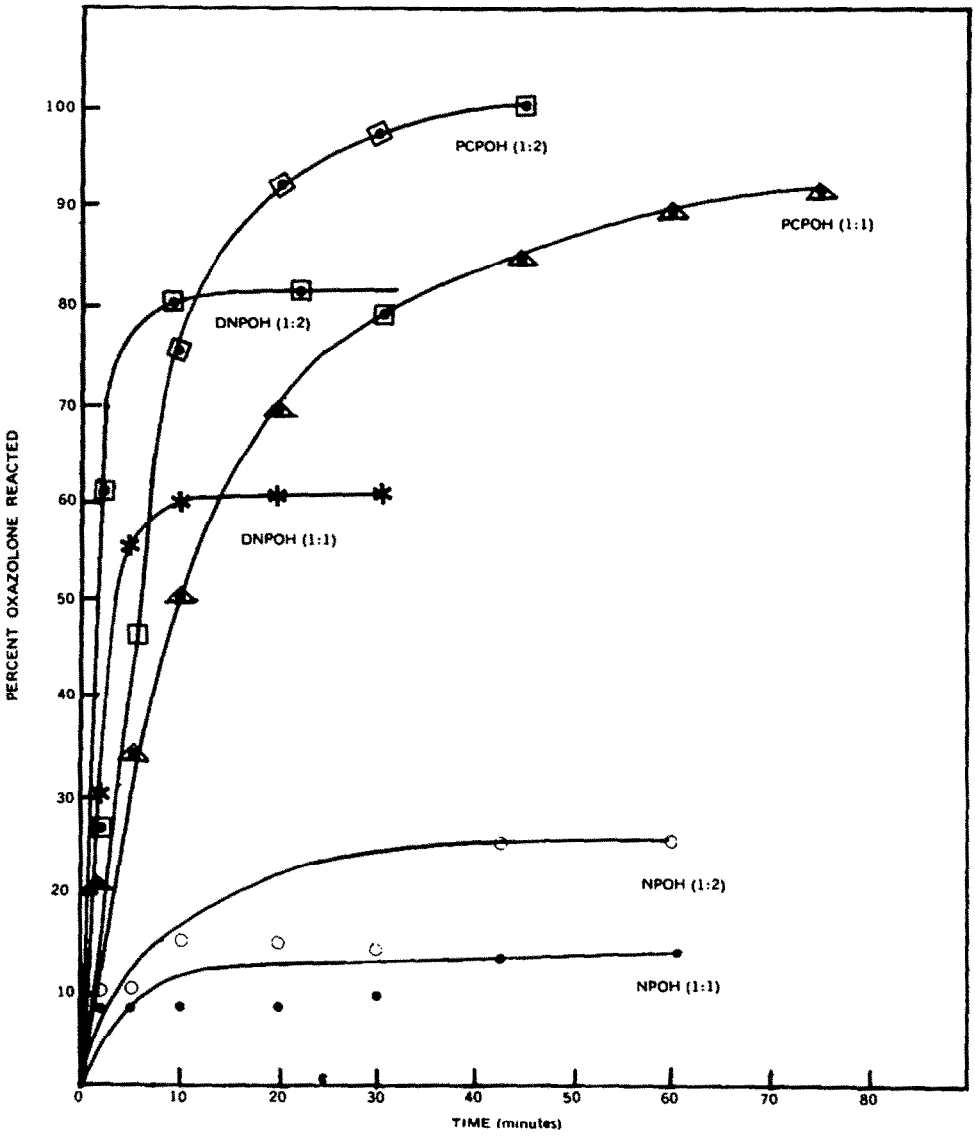


FIG. 1 Per cent oxazolone reacted in reaction of oxazolone and phenols in 1:1 and 1:2 ratios.

presence of triethylamine the relative order of oxazolone disappearance was reversed, NPOH > PCPOH > DNPOH as shown in Fig. 2. Of added interest is the fact that all three of the reactions presented in Fig. 2 appear to reach an equilibrium in less than one hour, the NPOH equilibrium being attained most rapidly. Similar equilibria for the reaction of oxazolones with NPOH in the presence of tertiary amines have been reported previously.[17–19]

The results in Fig. 2, as expected demonstrate that the stronger the nucleophile,

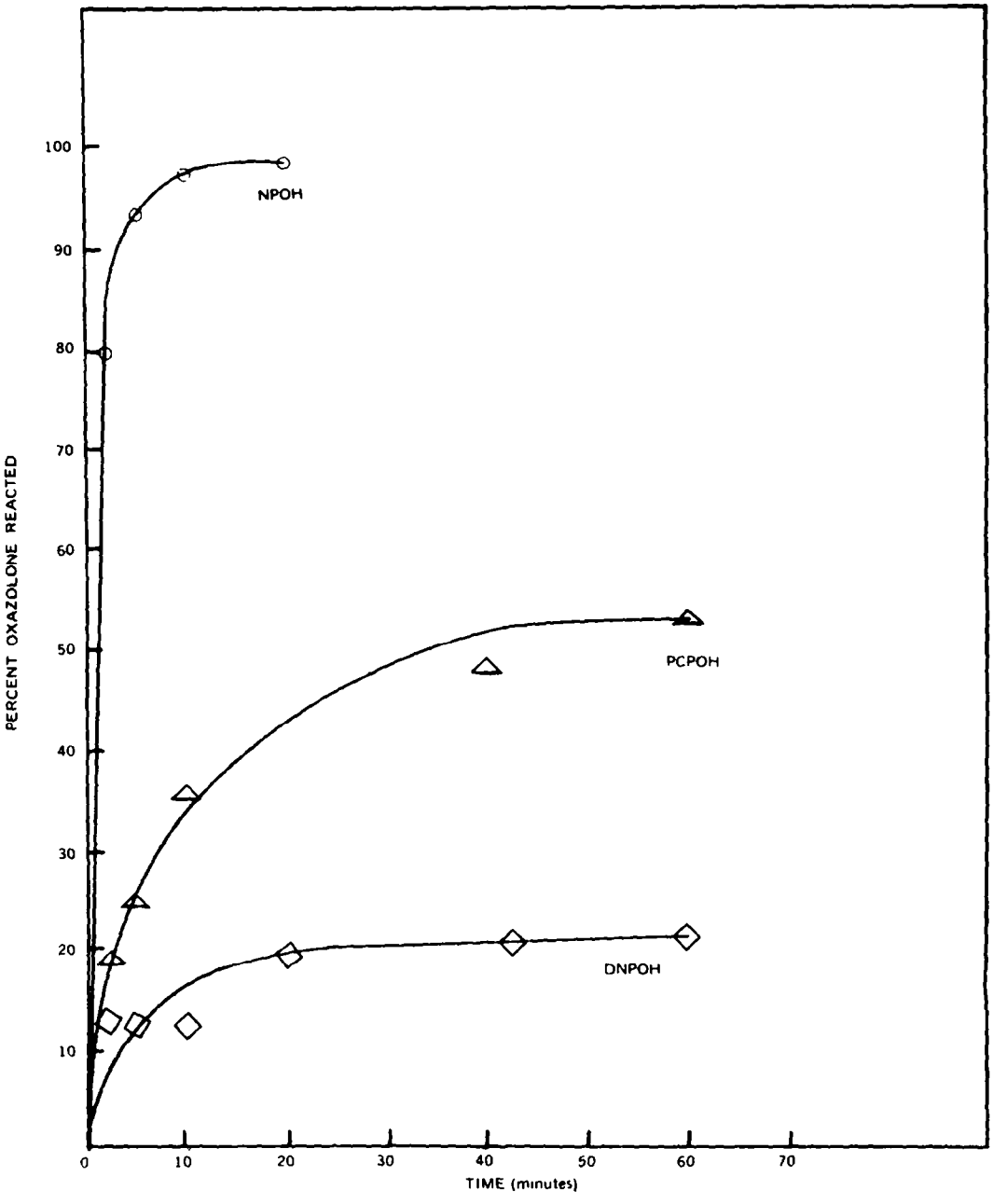
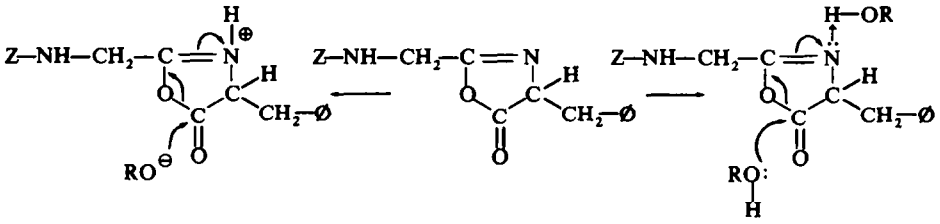


FIG. 2 Per cent oxazolone reacted in reaction of oxazolone phenols and triethylamine (1 :1 :1).

the faster is the rate of oxazolone ring opening. However, on comparing Figs. 1 and 2, it can be seen that DNPOH and PCPOH actually react faster with the oxazolone in the absence of triethylamine. This implies that these two acidic phenols open the oxazolone by a mechanism other than by direct nucleophilic attack of the phenolate

anions. A possible explanation for the faster ring opening of the oxazolone intermediate by DNPOH and PCPOH, obtained by the usual DCC method is presented in Scheme 1. The L-oxazolone is protonated by the acidic phenols on the nitrogen of

SCHEME 1



the C=N, followed by attack of the corresponding phenolate ion. The driving force for the reaction may be attributed to the electron withdrawing effect of the positively charged nitrogen. Alternatively, a concerted mechanism could be considered as shown in the Scheme. In either case, NPOH, because of its lower acidity, would not protonate the oxazolone to the same extent as the more acidic phenols and would therefore react more slowly, allowing the oxazolone to racemize to a greater extent to give a more racemic ester. At low temperatures and/or high phenol concentrations, the racemization of the oxazolone is retarded.

On the basis of the foregoing discussion it was concluded that the acidity of the phenols influenced the rate and mode of ring opening of the oxazolone intermediate. To determine if parallel effects occur in the formation of Z-amino acid active esters through the acylisourea intermediate, the reaction of Z—Gly—OH with DCC and NPOH, PCPOH or DNPOH was investigated. Figure 3 shows the amount of anhydride and corresponding active ester present in these reaction mixtures. Values for the amount of Z—Gly—ODNP are not given since we have not been able to obtain this ester in pure form. These results illustrate that as the acidity of the phenol component increased, less symmetrical anhydride was present at 8 min reaction time (initial reading). However, the disappearance of the anhydride is slower, the more acidic the phenol. Reaction of Z—Gly—OH and DCC (1 :1) under the same conditions showed more than 90% anhydride formation, whereas reaction of $(Z—Gly)_2O$ and PCPOH (1 : 1) showed no active ester over a 73 min period, indicating that direct attack by the phenol on the anhydride cannot contribute significantly to active ester formation.* To estimate the effect of unreacted DCC as a base catalyst, $(Z—Gly)_2O$ was reacted with phenols and 2 moles of DCC. The results showed that this reaction proceeded significantly slower to form active esters than the reaction of Z—Gly—OH with phenols and 1 mole of DCC.

The general conclusions to be drawn from the above results are: (1) the major path to active ester is through direct attack of these phenols on the acylisourea intermediate; (2) the extent to which this path predominates is related to the acidity of the phenol, the lower the pK value the faster the attack on the acylisourea (significantly, with DNPOH the reaction proceeds almost exclusively through this path); (3) the minor path is formation of the anhydride which is attacked by the phenols in

* Similar results were observed by De Tar[23] on reacting acetic anhydride with NPOH. However, on addition of DCC as a base catalyst, he observed that some acylation occurred.

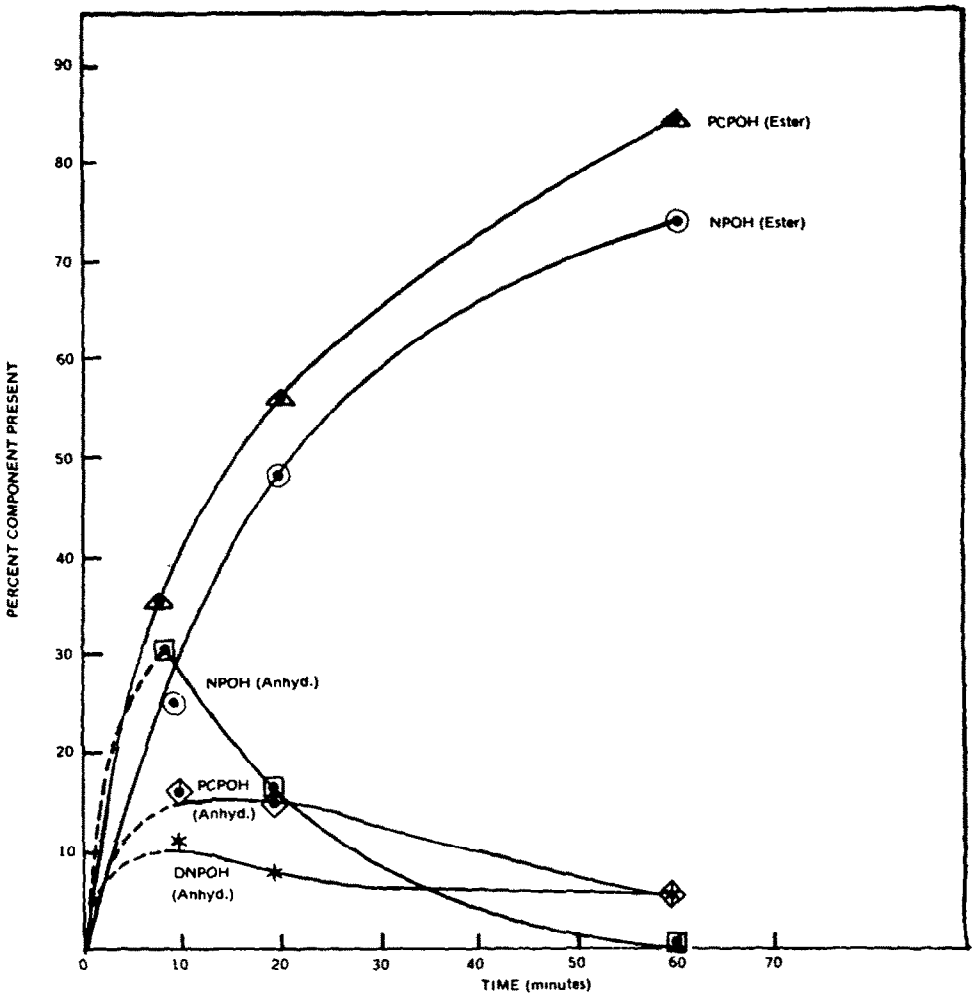


FIG. 3 Per cent anhydride and active ester present in reaction of Z—Gly—OH, phenols and DCC(1:1:1).

the presence of DCC, the relative reactivities being NPOH > PCPOH > DNPOH.

The PCPOH—DCC "Complex" in active ester formation offers an additional pathway, namely, the possibility of direct attack by the N-protected amino acid or peptide on the pentachlorophenyl moiety of the isourea core "A" of the "complex" (see footnote on p. 2556). This was investigated using Z—Gly—OH which contained 0·76% O^{18} per oxygen in the carboxyl group (as determined by mass spectrometry).† The labeled Z—Gly—OH reacted with the "complex" to give DCU with 0·76% O^{18}, thus eliminating the possibility of direct attack of the acid on the pentachlorophenyl ring.

† Mass spectral analyses were carried out by Dr. James E. Morgan, Morgan and Schaffer Corp., Montreal, Canada.

The data presented in this paper is used for the selection of methods and reaction conditions for the preparation of sequential polypeptides with high optical purity.

EXPERIMENTAL

All melting points are uncorrected and were determined in either the "mel-temp" apparatus, Laboratory Devices, Cambridge, Mass., or "uni-melt)' apparatus, Arthur H. Thomas Co., Philadelphia, Pa. Optical rotations were determined in the Rudolph Photoelectric Polarimeter, Model 200S-340-80Q6, O. C. Rudolph and Sons, Inc., Caldwell, N. J. For infrared spectra the Beckman IR-8 Spectrometer was used.

"Complex" I

To a cold stirred solution of PCPOH (8·78 g, 0·033 mole) at 0° in ethyl acetate (10 ml), DCC (2·06 g, 0·010 mole) was added. The mixture was allowed to stand in the freezer (−10°) overnight during which time a crystalline material deposited. Cold hexane (10 ml, 0°) was added to the reaction mixture, the solid filtered and washed with cold hexane. Recrystallization from hot hexane (130 ml) yielded 82% "complex" I, m.p. range 115–162°. (Found: C, 37·25; H, 2·39; N, 3·07; Cl, 52·67. Calcd. for $C_{31}H_{25}O_3N_2Cl_{15}$: C, 37·00; H, 2·50; N, 2·80; Cl, 52·90%).

"Complex" II

To a cold stirred solution of PCPOH (8·77 g, 0·033 mole) at 0° in dimethylformamide (20 ml), DCC (2·06 g, 0·010 mole) was added. The mixture was stirred at room temperature until a clear solution was formed (approx. 5 min), and then stored in the freezer (−10°) for two days. The solid which formed was filtered and washed twice with 5 ml portions of cold dry ether (−10°). Yield 10·8 g, m.p. 75–80°. Recrystallization from hexane (100 ml), yielded 6·0 g, 58% "complex" II, m.p. 104–105°. (Found: C, 37·52; H, 3·03; N, 3·78; Cl, 49·83. Calcd. for $C_{34}H_{32}O_4N_3Cl_{15}$: C, 37·86; H, 2·99; N, 3·90; Cl, 49·31%).

By the same procedure using dimethylacetamide as solvent a "complex" was isolated in 66% yield, m.p. 121–122°. (Found: C, 38·91; H, 2·81; N, 3·81; Cl, 48·38. Calcd. for $C_{35}H_{34}O_4N_3Cl_{15}$: C, 38·48; H, 3·14; N, 3·85; Cl, 48·65%).

Dissociation Study of "Complex" I

The dissociation of "complex" I was followed by IR spectroscopy and the absorbancies at 4·72 μ (−N=C=N−) were calculated by the baseline method.

The DCC standard curves were evaluated by an unweighted least-squares treatment of the data for plots of absorbance vs. concentration. Statistical analysis data indicate that the probable maximum relative errors in the various determinations of the dissociated DCC concentrations are: in dioxane ±3·3%, in ethyl acetate ±1·9%, in tetrahydrofuran ±3·6%, in benzene ±2·1% and in methylene chloride ±3·7%. The least squares calculations were programmed in Fortran II and computed with an IBM 1620.

HBr · H—Phe—OPCP. Z—Phe—OPCP was converted by the usual hydrogen bromide procedure to 93·4% crude HBr · H—Phe—OPCP, m.p. 192·5–193°. Recrystallization from methanol–ether gave the analytical sample, m.p. 193·5° (dec.), $[\alpha]_D^{21}$ −12·6° (*c*, 1·01, methanol). Infrared spectrum (KBr) showed the active ester peak at 5·60 μ and a doublet characteristic of active PCPOH esters at 7·25 and 7·33 μ. (Found: C, 36·46; H, 2·24; N, 2·74. Calcd. for $C_{15}H_{11}O_2NCl_5Br$: C, 36·44; H, 2·24; N, 2·83%).

HBr · H—Gly—Phe—OPCP. Z—Gly—Phe—OPCP through the above procedure gave 98% crude product, m.p. 195–196° (dec.), which after recrystallization melted at 195° (dec.), $[\alpha]_D^{22}$ −39·9° (*c*, 1·00, methanol). (Found: C, 37·19; H, 2·36; N, 5·02. Calcd. for $C_{17}H_{14}O_3N_2Cl_5Br$: C, 37·03; H, 2·56; N, 5·08%).

Z—Gly—Gly—Phe—OH was prepared by coupling Z—Gly—Gly—OH and HCl·H—Phe—OMe through the mixed anhydride procedure. A 74% crude oily methyl ester was isolated. Hydrolysis with N sodium hydroxide yielded 61% crude acid, m.p. 136–139°. Two recrystallizations from water raised the m.p. to 141·5–143°, $[\alpha]_D^{21}$ +21·6° (*c*, 2·0, methanol). For analysis a sample was recrystallized from methanol and then from ethyl acetate. (Found: C, 60·86; H, 5·54; N, 10·17. Calcd. for $C_{21}H_{23}O_6N_3$: C, 61·01; H, 5·61; N, 10·15%).

HBr·H −Leu −OPCP. Z−Leu −OPCP[24] was converted to HBr·H—Leu −OPCP, as previously described for HBr·H—Phe—OPCP, in 91·4% yield, m.p. 174° (dec.). Recrystallization from methanol–ether raised the melting point to 179·5–180° (dec.), $[\alpha]_D^{27}$ +29·3° (*c*, 2·01, ethanol). (Found: C, 31·59; H, 2·68; N, 3·20. Calcd. for $C_{12}H_{13}O_2NBrCl_5$: C, 31·30; H, 2·85; N, 3·04%).

Preparation of peptide active esters appearing in Table 2

A. Z—Gly—Phe—OPCP. *Backing-off Method* (*No.* 1). Z—Gly—OH and HBr·H—Phe—OPCP were coupled through the mixed anhydride procedure. The crude ester was recrystallized from chloroform–petroleum ether, and twice from ethyl acetate. (Found: C, 49·92; H, 3·32; N, 4·48; Cl, 29·22. Calcd. for $C_{25}H_{19}O_5N_2Cl_5$: C, 49·66; H, 3·17; N, 4·63; Cl, 29·31%).

"*Complex*" *Method* (*No.* 7). "Complex" I (1·005 g, 0·001 mole) was dissolved in ethyl acetate (10 ml) at room temperature and Z—Gly—Phe—OH (0·356 g, 0·001 mole) was added and allowed to stand for 20 hr. The resulting solid cake was converted to a finely divided suspension after addition of anhydrous ether (10 ml). The suspension was cooled in ice for 1 hr and filtered. This solid, which was a mixture of ester and DCU, was suspended in dioxane (10 ml), filtered, and washed twice with 2 ml of dioxane. The combined filtrate and washes were taken to dryness *in vavuo* at room temperature. The residue was again treated with dioxane to remove traces of DCU, and evaporated as above. The residue was suspended in anhydrous ether (10 ml) and refrigerated at −10° overnight. The crude ester was then filtered and without subsequent washing was used for physical measurements reported in Table 2. When 0·4 mole excess of "complex" was used, 78% ester was obtained in 100% optical purity.

Experiment (Nos. 6, 8, 9 and 10) follow the procedure described for (No. 7).

B. Z—Gly—Gly—Phe—OPCP. *Backing-off Method* (*No.* 11). Z—Gly—OH and HBr·H—Gly—Phe—OPCP were coupled by the procedure described for Z—Gly—Phe—OPCP in No. 1. The crude ester after recrystallization from chloroform–petroleum ether melted at 136·5–139°. Recrystallization from ethyl acetate gave m.p. 171–172°. A small sample of the material which melted at 171–172°, on recrystallization from chloroform–petroleum ether gave back the lower melting form, m.p. 137–138·5°. De Tar[25] reported $[\alpha]_D^{25}$ −32 (*c*, 1, $CHCl_3$). (Found: C, 48·85; H, 3·34; N, 6·22; Cl, 26·50. Calcd. for $C_{27}H_{22}O_6N_3Cl_5$: C, 49·01; H, 3·35; N, 6·35; Cl, 26·79%).

"*Complex*" *Method* (No. 13). "Complex" II (0·555 g) was dissolved in ethyl acetate (5 ml) at room temperature and a solution of Z—Gly—Gly—Phe—OH (0·207 g, 0·0005 mole) in ethyl acetate (12 ml) was added. After 20 hr at room temperature, anhydrous ether (10 ml) was added, the reaction mixture cooled in ice for 1 hr and the solid filtered. The solid was triturated with tetrahydrofuran (10 ml) and dioxane (20 ml) and filtered, leaving behind the DCU. The combined filtrates were taken to dryness, the residue suspended in anhydrous ether (10 ml), refrigerated overnight and filtered. This solid was then refluxed briefly with methanol (6 ml), cooled to room temperature, filtered and washed with cold methanol (6 ml), to give crude Z—Gly—Gly—Phe—OPCP.

C. Bz—Leu—OPCP. *Backing-off Method* (*No.* 14). HBr·H—Leu—OPCP (32·0 g, 0·05 mole) was suspended in ether (500 ml) at 0°, and benzoyl chloride (8·5 ml, 0·074 mole) was added. To this vigorously stirred suspension a pre-cooled sodium bicarbonate solution (10·0 g, in 150 ml of water) was added over 20 min. Ethyl acetate (350 ml) was added and stirring continued for 1 hr in ice, and 1 hr after removal of the cooling bath. Ethyl acetate (250 ml) was added, and the aqueous phase separated. The ethyl acetate solution was washed with water, dried over magnesium sulfate and the solvent removed *in vacuo* below 35°. Ethyl acetate (150 ml) was added to the resulting solid to form a fine slurry, to which 16 volumes of petroleum ether was added. Overnight refrigeration and filtration gave the crude ester. Recrystallization from chloroform–petroleum ether gave the analytically correct product. (Found: C, 47·13; H, 3·43; N, 2·93; Cl, 36·97. Calcd. for $C_{19}H_{16}O_3NCl_5$: C, 47·19; H, 3·33; N, 2·90; Cl, 36·65%).

Reverse Method (*No.* 16). PCPOH (1·60 g, 0·006 mole) was dissolved in ethyl acetate (30 ml) at 0°. DCC (0·62 g, 0·003 mole) was added, and the solution stirred at 0° for 30 min. At this point the solution became turbid and ethyl acetate (10 ml) was added. The temperature was lowered to −15° and Bz—Leu—OH (0·706 g, 0·003 mole) added. After 23 hr the DCU was filtered, the filtrate concentrated to a small volume at 20°, returned to the freezer for 3 hr, and the additional DCU filtered. The filtrate was concentrated to 5 ml, refrigerated and petroleum ether (40 ml) was added. After 8 hr of refrigeration the crude ester was isolated. A crude sample, after three fractional crystallizations from chloroform–petroleum ether yielded the Bz—Leu—OPCP listed in the table.

Racemization of Z—Gly—Phe—OPCP *in the presence of triethylamine. Polarimetry.*

Z—Gly—Phe—OPCP (0·125 g, 0·000207 mole) was dissolved in ethyl acetate containing triethylamine (0·030 ml, 0·000214 mole), to give a 0·5% solution. The changes in specific rotation with time were recorded.

Rate of coupling of Z—Gly—Phe—OPCP *with* H—Gly—OEt.

Polarimetry. A 25·0 ml solution of Z—Gly—Phe—OPCP (0·1256 g, 0·000207 mole) in ethyl acetate gave

an observed rotation of −0·201. A solution (1·0 ml) of H—Gly—OEt (21·3 mg, 0·000207 mole) in ethyl acetate was added directly to the Z—Gly—Phe—OPCP solution in the polarimeter tube. Addition of the H—Gly—OEt solution introduced a dilution factor which, when compensated for, gave a corrected observed rotation of −0·193 for 0 time. Changes in the observed rotation were used to estimate the ½-life of Z—Gly—Phe—OPCP during coupling as shown in Table 6, based on the derived equation:

$$\text{Observed } \alpha \text{ (of reaction mixture)} = -0{\cdot}193\,n + [-0{\cdot}072\,(1-n)] = -0{\cdot}121\,n - 0{\cdot}072$$

where n = the fraction of unreacted Z—Gly—Phe—OPCP, $(1-n)$ = the fraction of Z—Gly—Phe—Gly—OEt formed, −0·193 = the observed α of Z—Gly—Phe—OPCP before addition of H—Gly—OEt, −0·072 = the observed α of Z—Gly—Phe—Gly—OEt + PCPOH at the end of the reaction (22 hr).

A synthetic mixture of Z—Gly—Phe—Gly—OEt ($[\alpha]_D$ −13·1°) and PCPOH, each in the same concentration as the theoretically completed coupling, gave an observed α of −0·067, which fits quite closely to the experimental value of −0·072 after 22 hours of reaction. The close agreement of these two values indicates that no racemization took place during coupling, otherwise the final experimental value of −0·072 would have been lower than −0·067.

TABLE 6

n	$(1-n)$	Calculated α	Nearest experimental α	Corresponding time period (min)
½	½	−0·133	−0·134	5
¼	¾	−0·102	−0·108 to −0·099	40–47

PCPOH *Salt of* H—Gly—OEt. PCPOH (1·596 g, 0·006 mole) was dissolved in chloroform (40 ml). Freshly distilled H—Gly—OEt was added, and within 5 min a solid mass of crystals formed. The crystals were filtered, washed with chloroform and then with petroleum ether. The yield of salt was 98%, m.p. 172°. (Found: C, 32·46; H, 2·92; N, 3·53. Calcd. for $C_{10}H_{10}O_3NCl_5$: C, 32·51; H, 2·73; N, 3·79%).

Racemization of Bz—Leu—OPCP *in the presence of triethylamine*

Bz—Leu—OPCP (0·250 g, 0·000517 mole) was dissolved in dioxane at 23° to give a 1% solution (0·021M), to which purified triethylamine (0·0725 ml, 0·000517 mole) was added. Results are presented in the text.

Infrared spectrophotometric rate studies

Standard curves of $(Z—Gly)_2O$, Z—Gly—OPCP, Z—Gly—ONP, Z—Gly—Phe—OPCP and the oxazolone of Z—Gly—Phe—OH: Solutions of each of the above compounds were prepared in four concentrations in the same solvents used in the subsequent rate studies. The wavelengths used for quantitation of the above materials were: 5·43 μ for $(Z—Gly)_2O$; 5·6 μ region for Z—Gly—OPCP, Z—Gly—ONP and Z—Gly—Phe—OPCP; 5·47 μ for the oxazolone of Z—Gly—Phe—OH.

(a) Reactions of Z—Gly—OH, phenols and DCC. Z—Gly—OH and NPOH were dissolved in dioxane at room temperature, and DCC in dioxane was mixed with the first solution to give 0·13M concentration for each reactant. Aliquots were taken, after centrifugation in each case, and infrared spectra recorded in 0·10 mm sodium chloride cells. The time period recorded is that time when the recorder pen was between 5·4 to 5·5 μ. The identical reaction was performed using PCPOH and DNPOH (concentration of each 0·14M) in place of NPOH. The foregoing results are shown in Fig. 3.

The formation of $(Z—Gly)_2O$ from Z—Gly—OH and DCC and the reaction of $(Z—Gly_2)O$ and PCPOH at a concentration of 0·14M for each reactant were studied as above and the results are discussed in the text.

Reaction of $(Z—Gly)_2O$, phenols and DCC (1:2:1 molar ratio). The reaction of $(Z—Gly)_2O$ (0·07M) and phenols (0·14M) in the presence of DCC (0·08M) were studied as described earlier. The phenols used were DNPOH, PCPOH and NPOH respectively. The results are discussed in the text.

(b) Reaction of Z—Gly—Phe—OH, PCPOH and DCC. Z—Gly—Phe—OH and PCPOH were dissolved in dioxane or THF at room temperature and DCC in dioxane was mixed with the first solution to give 0·25M concentration for each reactant. The DCC was centrifuged before each reading and an aliquot of the supernatant was measured in the IR spectrophotometer. The results are presented in Table 5.

(c) Reactions of the oxazolone of Z—Gly—Phe—OH and phenols in the absence and presence of triethylamine.

(1) 1 : 1 Molar ratio of oxazolone and phenols. The oxazolone (0·37M) and phenols (0·37M) were dissolved in anhydrous tetrahydrofuran at room temperature. The phenols used were DNPOH, PCPOH and NPOH respectively. The results of these three experiments are presented in Fig. 1. Another series of experiments were run using a 1 :2 molar ratio of oxazolone (0·37M) and phenols (0·74M). The data are included in Fig. 1.

(2) 1 :1 :1 Molar ratio of oxazolone, phenols, and triethylamine were reacted exactly as above except that the tetrahydrofuran contained 0·1 mmole of purified triethylamine. These results are presented in Fig. 2.

O^{18}-*labeled* Z—Gly—OH. To Z—Gly—OMe (8·3 g, 0·037 mole) at room temperature, sodium hydroxide (1·495 g, 0·0374 mole) in H_2O^{18} (1·69 at. % excess O^{18}, 75 ml) was added, and the reaction mixture stirred for 20 min. The resulting solution was acidified to pH 1 with 6 N hydrochloric acid, to give crude labeled Z—Gly—OH which was filtered and washed with water; yield 75%, m.p. 113–118°. Recrystallization from chloroform raised the melting point to 120–121°. Mass spectral analysis: a small quantity of labeled Z—Gly—OH was pyrolyzed under vacuum. The non-condensible material at −80° was found to be CO_2 + some CO. Isotopic abundance measurements on the CO_2 gave $CO^{16}O^{18}/CO^{16}O^{16}$ = 0·76% (above natural abundance). Since the CO_2 is derived in equal amounts from the labeled carboxyl and unlabeled benzyloxycarbonyl groups then the total O^{18} is 1·52% over natural abundance in the labeled carboxyl and is equally distributed over both oxygens.

O^{18}-*labeled* H—Gly—OH. Z—Gly—OH labeled with O^{18} in the carboxyl group, (0·5 g, 0·0024 mole; containing 1·52% O^{18}) was catalytically hydrogenated in acetic acid in the usual way. The catalyst was filtered and the filtrate evaporated *in vacuo*. The residue was treated with boiling ethanol (325 ml) and filtered. The insoluble amino acid (106 mg), was twice recrystallized from water–methanol and melted at 237·5–238·5°. Mass spectral analysis of this material showed that on isotopic analysis of the CO_2 produced by thermal decomposition yielded $CO^{18}O^{16}/CO^{16}O^{16}$ = 1·48% above natural abundance. These results confirm the O^{18} to be equally distributed between both oxygens in the carboxyl group.

Reaction of O^{18}-*labeled* Z—Gly—OH *with "Complex" I*

"Complex" I (1·004 g, 0·001 mole) was dissolved in dioxane (4·0 ml) at room temperature and the labeled Z—Gly—OH (0·209 g, 0·001 mole) was added. The reaction was stirred for 1 hr, the DCU filtered and washed twice with dioxane (2 ml) to give a 93·3% yield, m.p. 226–228°. The filtrate was evaporated *in vacuo* and the residue was recrystallized from ethanol to give 67·5% of active ester, m.p. 133–134°. Another recrystallization from ethanol raised the melting point to 134–134·5°. The DCU was recrystallized twice from ethanol, m.p. 233–234°.

Mass spectral analysis of the DCU showed no parent ion. However, the DCU decomposed in the heated inlet to cyclohexylamine and cyclohexylisocyanate, and isotopic analysis gave 0·75 ± 0·05% O^{18} above natural abundance.

TABLE 7. ANHYDRIDE AND ACTIVE ESTER FORMATION DURING REACTION OF Z—Gly—OH AND "COMPLEX" II IN DIOXANE*

Time (min)	Per cent anhydride present†	Per cent ester formed†
7·5	6	50
19·5	6	69
61	6	78
93	0	81

* Initial concentration of Z—Gly—OH was 0·13M and of "complex" II 0·14M.

† Same as footnotes † and ‡ in Table 5.

Infrared spectrophotometry of the reaction of "Complex" II with Z—Gly—OH

Z—Gly—OH was dissolved in dioxane at room temperature and "complex" II was added. This reaction mixture was used in the IR to evaluate the formation of anhydride and active ester. The results are summarized in Table 7.

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