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PEPTIDE SYNTHESIS WITH BENZISOXAZOLIUM SALTS—III

UTILITY OF 7-HYDROXY-2-ETHYLBENZISOXAZOLIUM FLUOROBORATE IN THE SYNTHESIS OF PEPTIDES

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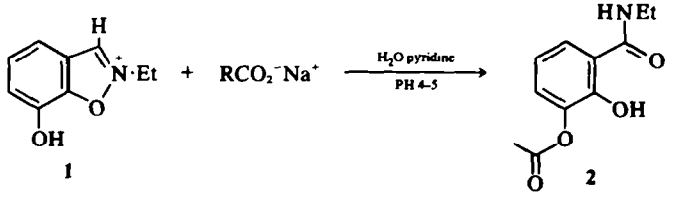
Abstract—The scope and limitations of the 7-hydroxy-2-ethylbenzisoxazolium salt method of forming amide bonds are outlined through the synthesis of a variety of simple peptide derivatives containing all of the common amino acids with the exceptions of arginine and histidine. The 3-acyloxy-2-hydroxy-N-ethylbenzamides derived from C-terminal serine or threonine containing peptides are found to react with amines at anomalously slow rates and with the formation of transesterified byproducts; a mechanistic explanation is offered. The utility of the method for the synthesis of medium sized peptides is examined by synthesis of oligomers of Gly-*L*-Leu-Gly.

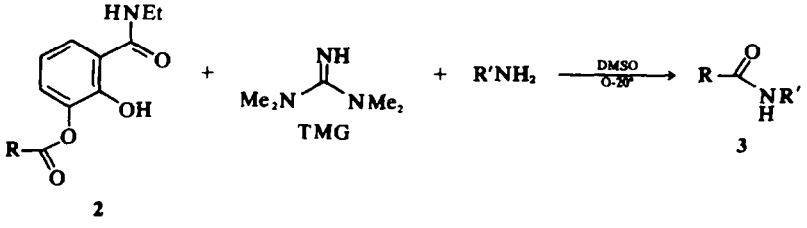
In the preceding papers,[1,2] we have presented the results which have led us to apply the reaction sequences **1**→**2**, and **2**→**3** to peptide synthesis. In this paper, we present the results of a study which is aimed at defining the utility of these reactions when applied to practical synthesis of small to medium sized peptides.

Because the reagent potentially allows convenient, racemization free conversion of peptide acids to active esters, it may offer an alternative to the azide and HBT–DCC coupling methods which are presently the methods of choice for peptide synthesis by fragment condensation. Moreover, since Step 2 occurs most rapidly in the presence of an equivalent of strong base, the method is most naturally used with salts of amino acids or peptides, which though rarely compatible with other activated acyl derivatives, still provide the simplest and most versatile C-protected peptide nucleophiles. The unusual features of Steps 1 and 2 require a careful study of feasibility, part of which is described in the preceding paper and can be summarized as follows:

1. *Activation.* The conversion of peptide acids to esters, **2**, by reaction with **1** is best carried out in a



STEP 1.



STEP 2.

vigorously stirred two phase mixture of water and a suitable organic solvent. The acid is converted to its sodium (or other alkali metal) salt, pyridine is added, and the pH is adjusted to 4·5–5. Optimally, the concentration of acid anion should be greater than 0·3 M; reduced yields are observed if the initial concentration falls below 0·2 M. If solubility problems arise, the mixture can be diluted with acetonitrile (or hexafluoroisopropanol) to a concentration in the range of 0·05–0·2 M. Other diluting solvents are less satisfactory. Yields under optimum conditions lie in the range of 90–94%; yields remain high with hindered acids, and with peptide acids, roughly 0·01% of oxazolone is formed during the activation step. The crude product from an activation contains traces of a yellow impurity, and in the case of ZGlyOH, it was noted that 1·6% of the acid was converted to an unidentified neutral substance.

2. *Coupling.* Although good yields of amides can be obtained by allowing esters, **2**, to react with amides in aprotic solvents, the rates are slow, and reaction times of 10–20 h are typically required. Rates of aminolyses of esters **2** are strongly accelerated by strong bases, and reactions are therefore most conveniently carried out in the presence of an equivalent of tetramethylguanidine (TMG). Control experiments established that low levels of racemization at the site α to the acyl carbon are observed for couplings carried out under these conditions, that use of an excess of TMG results in no increase of racemization rate, and that TMG does not act as an acyl acceptor. These favorable results can be rationalized in terms of a protecting effect of the 2-oxyanion of the conjugate base of the ester. The question of the possible effect of TMG on peptide sites remote from the site of activation has been partially resolved by the observation[3] that ZGly-L-Phe-GlyOEt can be recovered in 77% yield after 48 h at 22° in DMSO containing 0·1 M TMG; racemization in this time was less than 0·01%. The nature of the decomposition was not investigated and is quite possibly ester saponification resulting from traces of water.

Coupling rates for the TMG catalyzed aminolyses of esters **2** are comparable to those of *p*-nitrophenyl esters, and in DMSO at 22°, at 0·2 M concentrations, times for 90% reaction lie in the range of 15–90 min for average peptide couplings. Hindered amino acids react more slowly, the extreme probably being Val + Val with a comparable reaction time of 4 h. Proline reacts very sluggishly as a nucleophile, requiring a reaction time of 9 h for 90% yield in coupling with ZGly and presumably appreciably longer for reactions with more hindered active esters.

The racemizing tendency of **2** under the coupling conditions is markedly increased as the reaction temperature is increased or the initial reactant concentrations are decreased. Satisfactory results are expected above 0·05 M concentrations and at 0–10°.

1. *Preparation of activated esters*, **2**. Properties of activated esters of simple amino acid derivatives which have been completely characterized are reported in Table 1; the reported yields are for recrystallized material of satisfactory purity. Active esters prepared from carbobenzoxy derivatives of L-Leu, L-Pro, L-Ile, β-tBu-L-Asp, as well[a] BOC[a] Z-L-Lys and BOC-L-Ile could not be crystallized and were used directly in couplings, without purification.

Two general points may be noted. Although isotopic dilution and NMR analysis reveals no detectable differences, the yield of crystallizable active ester is often observed to be much higher when prepared from acids which have been subjected to several recrystallizations beyond those necessary to realize the literature mp; this effect is particularly marked with the esters of Z-L-Ser and Z-L-Cys-SBz, and presumably can be attributed to the familiar peptide problem that crystallization ceases once a threshold concentration of certain impurities is reached. For the less water soluble amino acid derivatives, activations must be carried out in the pH range 5·0–5·5 to avoid precipitation; under these conditions the formation of traces of yellow polymer can seriously complicate purification. For the lipophilic esters of derivatives of valine, leucine, and lysine, for which the solubility problem is most severe, the yellow impurities can be efficiently removed by filtration through a short column of alumina.

Although yields were examined by isotopic dilution for the model case, ZGlyOH,[2] it seemed advisable to apply this technique to several other cases as well. Accordingly, the conversions of Z-L-SerOH, Z-L-ThrOH, and Z-L-GluOH, to their esters, **2**, were carried out using ^{14}C-labeled acids. With Z-L-SerOH,91% of the initial activity was converted to ester **2**, 2% was recovered as starting material, and 7% was observed to be present as a neutral byproduct remaining in the mother liquors of ester recrystallizations. For Z-L-ThrOH, the corresponding numbers were 92% ester, **2**, 6% recovered acid, and 2% neutral impurity. For Z-L-GluOH, the values were 88% ester, **2**, 9·5% acid, and 1% impurity. That the large amount of byproduct observed for the Z-L-SerOH activation need not be general for C-terminal serine is revealed by the activation of Z-L-Phe-L-SerOH, from which a 90% yield of recrystallized ester can be obtained.

2. *Formation of simple peptide drivatives by TMG catalyzed aminolysis of esters* **2**. A survey of the generality of the TMG catalyzed synthesis of peptides from active esters, **2**, must begin with a demonstration that the method is compatible with simple amino acids. Of the common amino acids, S-BzCys, Glu, Met, Phe, Pro, Ser, Thr, and Try are soluble in DMSO in the presence of TMG, while Asn and Gly, though only partly soluble, dissolve rapidly

Table 1. Active Esters, 2, derived from L-amino acids

Amino acid Deriv	Ester Yield (%)	Recryst Solvent	mp °	$[\alpha]_D$ °	Elemental analysis C	H	N	S
Z-Ala	93·7	EtOAc cyclohexane	116–117	−65·4 (2·0, EtOAc)	62·10 62·17	5·83 5·74	7·24 7·25	— —
		$C_{20}H_{22}N_2O_6$ requires:						
Z-Asn	90·0	EtOAc	170–171	−32·5 (2·0, DMF)	58·69 58·74	5·47 5·40	9·67 9·79	— —
		$C_{21}H_{23}N_2O_7$ requires:						
Z-Cys–SBz	83·2	Me_2CO–Pet Ether	101–103	−48·8 (2·3, EtOAc)	63·87 63·77	5·64 5·55	5·32 5·51	6·31 6·31
		$C_{27}H_{28}N_2O_6S$ requires:						
Z-Glu-γ-tBu	81	CH_2Cl_2–cyclohexane	159–160	−35·2 (2·0, EtOAc)	61·85 62·39	6·37 6·44	5·46 5·60	— —
		$C_{26}H_{32}N_2O_8$ requires:						
Z-Gln	86·8	EtOAc	145–146	−43·5 (1·7, EtOAc)	59·54 59·98	5·75 5·68	9·52 9·48	— —
		$C_{22}H_{25}N_3O_7$ requires:						
ZGly	91·3	EtOAc	121–122	—	61·50 61·26	5·54 5·42	7·54 7·53	— —
		$C_{19}H_{20}N_2O_6$ requires:						
Z-Met	89·0	EtOAc cyclohexane	106–107	−33·8 (2·0, EtOAc)	59·21 59·19	5·94 5·87	6·23 6·27	7·54 7·18
		$C_{22}H_{26}N_2O_6S$ requires:						
Z-Phe	90·0	EtOAc-cyclohexane	93–94	−47·0 2·0, EtOAc)	67·44 67·52	5·66 5·67	5·98 6·02	— —
		$C_{26}H_{26}N_2O_6$ requires:						
Z-Ser	86·0	EtOAc-cyclohexane	116–117	−40·2	59·63 59·67	5·47 5·51	6·99 6·96	— —
		$C_{20}H_{22}N_2O_7$ requires:						
Z-Thr	86·8	EtOAc-cyclohexane	112–113	−45·7 (1·5, EtOAc)	60·63 60·57	5·92 5·81	6·73 6·73	— —
		$C_{20}H_{22}N_2O_7$ requires:						
Z-Val	75	EtOAc-Pet Ether	77–79	−48·5 (2·0, EtOAc)	63·69 63·76	6·32 6·32	6·83 6·76	— —
		$C_{22}H_{26}N_2O_6$ requires:						

in the presence of active ester. The amino acids Ala, Asp, Gln, His, Ile, Leu, ε-BOCLys, OBzTyr, and Val must be converted to their tetramethyl or tetraethylammonium salts in prior step. Lyophilization is the method which we have most commonly used to prepare these salts, and interestingly, while L-Tyr is insoluble in an equivalent of aqueous 10% tetramethylammonium hydroxide, it is observed to dissolve to yield the desired salt during the course of the lyophilization. In most cases, lyophilization becomes very slow before all traces of water have been removed, and the resulting heavy syrup is

dissolved in DMSO (or DMF), treated with an equivalent of TMG and a solution of the active ester, **2**. For most cases except those involving C-terminal Val or Ile with hindered nucleophiles, C-terminal Ser or Thr, or N-terminal Pro, reaction times of 40 min at 20° or 1·5 hr at 0° are sufficient. For the latter cases, reaction times of 20 h at 20° are suggested. An alternative to lyophilization is azeotropic distillation of water with benzene; this procedure has been explored with Gly, Ala, and Val and found to yield essentially water-free tetraalkylammonium salts; 100% of L-Val was assayed by isotopic dilution after acidification of its tetramethylammonium salt, prepared by azeotropic distillation (3 h). The results of couplings of the ester **2**, R = Zgly, with salts of 14 amino acids are reported in Table 2; the amino acids Asp, Ser, and Thr were found to yield water soluble products which were difficult to recover, Z-Gly-L-ValOH could not be induced to crystallize, while His and Arg were not examined.

Also reported in the Table are results of coupling reactions between amino acids bearing simple side chains. Reported yields are for isolated material of established purity, and some perspective on their significance is given by isotopic dilution experiments for ZGly-L-PheOH and ZGly-L-MetOH; in both cases, yields were found to lie in the range of 98–100%, and identical results were obtained when the isotopic label was placed in the reactant or in the diluent, thereby establishing the absence of artifacts.

The coupling of valine with valine lies at the extreme of steric hindrance, and the reaction of the TMG salt of **2**, R = Z-L-Val with the tetramethylammonium salt of L-Val was therefore studied in some detail. The first problem posed by this reaction is homogeneity. If an amino acid has such a high crystal lattice energy that it is thermodynamically unstable in DMSO solution as its TMG salt, then addition of the TMG salt of an ester **2** to a solution of the tetraalkylammonium salt of the amino acid is expected to result in precipitation of the amino acid and formation of an equivalent of TMG. In fact, a finely divided or gel-like precipitate which quickly

Table 2. Simple peptides prepared by coupling with esters, **2**

Peptide[a]	Yield (%)[b]	mp°	Rep mp°	$[\alpha]_D$[c]	Rep$[\alpha]_D$°
1. Zgly-X-OH Peptides					
ZglyAlaOH	87	135–136	135[4]	−9·6	−9·5
ZGlyAsnOH	50	131–132	130–131[5]	+21·5 (2·0, EtOH)	—
ZGly(SBz)CysOH	82	121–122	120–121[6]	−22·3	−24
$ZGlyGlu(OH)_2$	83	160–161	160–162[7]	+4·8	+4·0
ZGlyGlnOH	90	158·5–159	—	+2·4	—
ZGly|GlyGlyOH	92	197–198			
ZGyIleOH	85	119–120	114–115[8]	+14·7	+14·3
ZGlyLeuOH	92	143–144	143–144[9]	−9·3	−9·5
ZGly-L-(ε Z)LysOH	73	73–75	74–75[10]	—	—
ZglyMetOH	86	112–113	110–111[11]	+7·1	+4·2
ZGlyPheOH	88	129–130	130–131[12]	+38·9	+39·2
ZGlyProOH	86	156–157	155[12]	−69·7	−60·4
ZGlyTryOH	83	141–142	142[12]	+33·2	+33·2
Zgly(OBz)TyrOH	89	142–143	142[13]	+41·5 (2·0, EtOH)	—
2. Other Peptide Acids					
ZGlyLeu|LeuOH	92	142–143	143–144[14]	−9·5	−9·5
$ZGly(Leu)_2\|(Gly)_2OH$	74	124–126	124–126[14]	−36·7	−36·5
ZAlaAlaOH	92	150–151	152–153[15]	−23·1 (2·0, EtOH)	—
ZValValOH	70	137–138	134–135[16]	−34·6 (3·2, IN KOH)	—
ZValGlyOH	84	165–166	166–167[17]	—	—
ZProAlaOH	68	160–162	161–162[18]	−75·4 (2·8, HOAc)	—
ZGlyLeu|GlyOH	94	109–111	110[19]	−15·2	−14·7
ZPro|LeuOH	65	119–121	119–120[20]	−57·4	−56·5[21]
ZPro|PheOH	80	129–130	126–127[21]	−46·1	−49

[a] All amino acids have the *L* configuration.
[b] Yields are reported for recrystallized products of listed mp.
[c] When compared with literature values, rotations were taken under literature conditions.

$$\text{TMG} + \underset{\text{(crystal)}}{\text{Val}} \rightleftharpoons \text{TMG} + \underset{\text{(solution)}}{\text{Val}} \rightleftharpoons \text{TMGH} + \text{HValO}^-$$

dissolves with stirring is occasionally observed with these reactions, and presumably in its finely divided form, a precipitated amino acid readily redissolves and reacts with a non-hindered ester. In the case of Val + Val, a precipitate is immediately formed which remains for several hours; immediate removal and characterization identifies the material as a quantitative yield of valine. Over a period of hours, reaction occurs and after 20 h, a 70% yield of coupling product is observed. The combination of precipitation and slowness of coupling creates a uniquely stringent situation in which the anion of an active ester, **2**, is susceptible to hydrolysis and racemization because the concentration of amine nucleophile is abnormally low. To test the extent of racemization, an isotopic dilution with Z-D-Val-L-ValOH was carried out, with the finding that less than 0·1% of the product is thus accounted for. A coupling conducted in DMSO, in the presence of 3 M water, was observed to yield 61% of Z-L-Val-L-ValOH. No data are available for the racemization expected for a Val − Val coupling in which oxazolone formation is possible at the C-terminus, and it is recommended that this coupling procedure be used with caution in cases in which larger peptides are required to undergo hindered coupling (e.g., combinations of Val, Ile, and Thr, or N-terminal Pro).

3. *Coupling reactions involving amino acids with side chain reactivity.* Results of coupling reactions involving most of the remaining amino acids are presented in Table 3. Cases 1–4 provide examples of clean couplings in which tyrosine is used without O-protection. We observe no evidence suggesting O-acyl transfer, and attribute this cleanness to the low degree of thermodynamic activation of the conjugate bases of esters, **2**. The two cases of methionine couplings reported in Tables 2 and 3

Table 3. Peptide acids prepared by couplings with esters, **2**

Product[a]	Yield (%)[b]	mp	lit mp	$[\alpha]_D$	lit $[\alpha]_D$
1. ZVal|TyrOH	86	161–163	161–163[22]	+12·2	+16
2. ZValTyr|ValOH	79	219–220	218–219[22]	—	—
3. Z(SBz)Cys| TyrOH	76	200–201	200–201[23]	−14·1	−14·8
4. Z(SBz)CysTyr| IleGlnAsnOH	83	244–246	245–247[23]	−23·4	−23·5
5. ZPhe HisOH	83	205–207	198–200[24]	−5·1 (1·0, DMF)	—
6. Z(SBzCys)| GlyOH	80	88–90	88–90[25]	−36·6	−35
7. Z(SBz)Cys| (SBz)CysOH	81	153–154	152[27]	−50·1	−50·6
8. Z(SBz)Cys| ProLeuGlyNH$_2$	88	170–171	170–171[43]	−58·5	−60·0[43] −58[44]
9. ZMet|GlnOH	86	159–163	159–161[28]	−10·7	−13·6
10. ZIle|GlnOH	89	189–190	184–185[29]	−21·4	−18·8
11. ZGln|AlaOH	86	219–220	209–213[30]	−1·5 (1·5, DMF)	—
12. ZGln|PheOH	97	199–200	180[24]	+5·2 (1·0, DMF)	—
13. ZGln|AsnOH	78	209–211	206[31]	+7·8 (1·0, DMF)	—
14. ZAsn|ThrOH	87	207–208	207[32]	+9·8	+10
15. ZAsn|(SBz)Cys OH	89	202–203	198–199[31]	−24·2 (2·0, DMF)	—
16. BOCAla(γOBz) Glu|PheOH	64	97–100	—	−11·0 (1·0, HOAc)	—
17. Z($^{\beta}$tBuO) Asp| SerOH	73[c]	134–135	135[20]	+8·4	+8·6
18. ZPhe|SerOH	76	154–156	155–156[33]	—	—
19. ZSer|AlaOH	65	157–158	161–162[34]	−4·6 (2·0, DMF)	—
20. ZSer|PheOH	67	141–142		+30·3	—
21. ZThr|PheOH	88	154–155		+23·4	—

[a] All amino acids have the L configuration.
[b] Yields correspond to recrystallized material of indicated properties.

proceed to give a single product cleanly; however, it may be noted that the observed optical rotations differ from those previously reported. The NMR spectra of these substances showed methyl thioether resonances in the range 2·0–2·1 δ, and were otherwise clean. Sulfoxide formation, a conceivable difficulty for reactions run in DMSO, can therefore be excluded. The issue was not explored further. Case 5. reports our single observation with histidine.

Cases 3, 4, 6, 7, and 8 provide evidence concerning S-benzyl cysteine in both N and C-terminal roles. The well-established ease of base-catalyzed racemization of cysteine derivatives[35] is clearly of major concern for our salt coupling procedure, yet the yields and optical integrity of the products, particularly in cases 6 and 7, support the notion that β-elimination from the active ester is discouraged by the internal buffering effect of the 2-oxyanion. This critical issue has not been put to the definitive test of isotopic dilution analysis, but the high optical rotation observed for case 6 is nonetheless significant.

Given the exceedingly ready base-catalyzed imide formation reactions of unactivated glutamine and asparagine esters,[36] it would not be surprising to find that the salt coupling procedure is unsuited to esters, **2**, bearing these amino acids at the C-terminus. The results of experiments 9–15 indicate that this concern is unfounded. An isotopic dilution carried out in the case of ZGlnPheOH revealed the yield to be 99·9%, and the rate constant for the coupling reaction in DMSO at 25° in the presence of TMG was found to be 0·35 $M^{-1}min^{-1}$, which implies a reaction time of 3·5 h to realize a yield of 94%, starting with 0·2 M reactants. Although only two cases are available, (16 and 17), the available evidence implies that no difficulty arises with these amino acids. In all these cases, the buffering effect of the 2-anion must be invoked to explain the absence of intramolecular participation.

It was immediately apparent with the first coupling conducted with the ZSer active ester that side reactions complicate its aminolysis, for thin layer scrutiny revealed several products. For example, reaction of the TMG salts of L-Phe and **2**, R = Z-L-Ser, yielded after workup a first crop of 67% of the expected dipeptide acid, and a second crop of material which was resolved by prep tlc into dipeptide and a substance with formula $C_{31}H_{33}N_3O_{10}$, which corresponds to two ZSerOH residues, one Phe, less two water molecules, and which was tentatively assigned the structure Z(O-ZSer)SerPheOH, by analogy with other observations of ester formation from active serine esters.[37] Since this was the first coupling reaction of esters, **2**, observed to give detectable impurities, it was examined in more detail. The above reaction was repeated using a 20% excess of L-Phe at 0·1 M in DMSO at 22° for 24 h. Isotopic dilutions were carried out for remaining active ester, Z-L-SerOH, Z-L-Ser-L-PheOH, and Z-D-Ser-L-PheOH, with the result that 0·25%, 2·7%, 90·0% and 0·3% of starting ester had been converted to these respective products; 96·1% of the initial radioactivity appeared in the $NaHCO_3$ extracts.

Very similar behavior was observed for the reaction of the TMG salt of **2**, R = Z-L-Ser, and Gly-L-AlaOEt. Two products were formed, separable by prep tlc; the major product was the desired, known tripeptide ester; the minor product had composition $C_{29}H_{36}N_4O_{11}$, which corresponds to the esterification product of the tripeptide ester and ZSerOH, and in this case, the product ratio was *ca* 5:1.

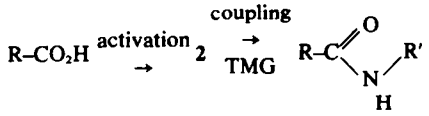
A clue to the causes of complexity of this reaction was provided when kinetics were measured for the reaction of the TMG salts of L-Phe and **2**, R = Z-L-Ser. (Rates of disappearance of ester and appearance of Z-L-PheOH were followed by isotopic dilution.) A rate constant of 0·1 $M^{-1}min^{-1}$ was observed, which may be noted to be half as fast as the similar coupling of two valine residues.[2] This unexpectedly slow coupling rate clearly allows intervention of other processes which are ordinarily too slow to affect product composition. For example, the recovery of **2**, R = Z-L-Ser, from a DMSO solution containing an equivalent of TMG is 96·4% after 12 h and 91·5% after 18 h.

Although the coupling reaction of the TMG salts of L-Phe and **2**, R = Z-L-Thr, proceeds in high yield, without formation of side products, the rate constant for this reaction is 0·04 $M^{-1}min^{-1}$, which is again abnormally and impractically slow.

The ready formation of transesterified products has been documented and rationalized by workers concerned with the catalytic role of serine in the mechanism of the hydrolytic enzymes,[38] and presumably the failure of threonine to partake in these side reactions can be attributed to steric hindrance at its alcohol site. The anomaly for both amino acids is then the slowness of their coupling rates, which appear to be roughly 20-fold smaller than expected. We attribute this slowness to the formation of an intramolecular H-bond between the alcohol function and the 2-anion of the catechol moiety. Models suggest that an assisted aminolysis mechanism should not be possible for a transition state retaining this hydrogen bond. That the alcohol function is in fact responsible for the rate retardation is demonstrated by the observation that the coupling reactions of the TMG salt of **2**, R = ZDL(OBz)Ser, are complete within an hour. Clearly esters, **2**, bearing C-terminal serine or threonine must be O-protected if rapid, clean coupling chemistry is to be observed.

4. *Preparation of larger peptides.* Although our main emphasis in this study was on the initial problem of activation and coupling chemistry of the common amino acids in their two possible roles of C and N-terminal components, some preliminary work is available on the application of these reactions to

Table 4. Preparation of Leucine Homopolymers



Starting Material	Yield, Activation (%)	Amine	Yield, Coupling (%)
ZGly-L-LeuGlyOH	78	Gly-L-LeuGly	73
Z(Gly-L-LeuGly)$_2$OH	84	(Gly-L-LeuGly)$_2$	69
Z(Gly-L-LeuGly)$_4$OH	77	(Gly-L-LeuGly)$_4$[a]	57

[a] The coupling reaction was run with triethylamine, rather than TMG; reaction time was 7 days.

the synthesis of larger peptides and is reported in Table 4.

Although the coupling steps are atypical since they always involve a pair of glycines, the results of the Table clearly indicate that the method we have developed can be applied successfully to large peptides.

The results of one approach to oxytocin revealed the consequences of the solubility limitations described in section 1. Preparation of the tripeptide Z-L-Pro-L-LeuGlyNH$_2$ was achieved in four steps from Z-L-ProOH in a yield of 59%, for product with literature properties. As indicated in Table 3, coupling to form the C-terminal tetrapeptide amide proceeded in 88% yield, and the N-terminal dipeptide acid (Table 3) was converted to its ester 2 in 75% yield. The sequence IleGlnAsn occasioned purification problems. Activation of Z-L-IleLGlnOH yielded an ester which was difficult to purify, and 80% crude yield was obtained which was reduced to 60% after several recrystallizations. Coupling with L-Asn gave 71% of tripeptide acid, whose properties compare favorably with those reported in the literature. As an alternative procedure, BPOC-L-IleOH was carried through this sequence of activations and coupling reactions without purification of intermediates, and the resulting tripeptide acid, after precipitation from CH_2Cl_2 with ether, was deblocked to yield L-Ile-L-Gln-L-Asn which was chromatographically homogeneous. The yield for this procedure was essentially identical with that obtained for the initial preparation. The N-terminal pentapeptide acid reported in Table 3 was subjected to an aqueous activation procedure. Unfortunately, the necessary concentrations of this exceedingly insoluble substance can only be achieved by use of THF-water mixtures, a solvent combination previously noted to give low activation yeilds. Not surprisingly, the product was chromatographically inhomogeneous and was estimated to be a 1:3 mixture of starting material and desired ester.

5. *Summary*

With the exception of arginine and histidine, the 20 common amino acids have been examined in activations and couplings with the 2-ethyl-7-hydroxybenzisoxazolium cation, 1. The following generalizations can be offered from this study, which remains preliminary in the sense that no synthesis of typical medium sized peptides have been seriously attempted.

(a) The major limitation to the activation reaction, 1 → 2, is the solubility requirement. Detectable, but small amounts of neutral impurities are formed in this step.

(b) Coupling reactions involving N-terminal proline, C-terminal threonine or serine with unprotected hydroxyl groups, or C-terminal valine or isoleucine with other hindered amino acids are slow reaction which are expected to result in greater formation of byproducts. These couplings should probably not be attempted if the C-terminal function is larger than a single amino aicd residue.

(c) Although definitive product analysis has not been carried out, the available data imply that couplings with cysteine, aspartic and glutamic mono esters, tyrosine, methionine, lysine, and tryptophane proceed without complication.

(d) A reasonably careful scrutiny of activation and coupling steps indicates that asparagine and glutamine are satisfactorily handled.

(e) In our hands, for most small peptides of the type reported in Tables 2 and 3, this method has proved to be cleaner, more reproducible, and more convenient than the azide, carbodiimide, and mixed anhydride methods. It is somewhat less clean than the *p*-nitrophenyl ester method, which, however, is not as suited for work with salts of amino acids or peptides and which cannot easily permit use of active esters of peptide acids. With larger peptides, our experience is limited, but we have observed some difficulties with yield and purification. Probably for these cases, the method offers little or no advantage over the HBT-DCC procedure or the classical azide method.

EXPERIMENTAL

Solvents and reagents were reagent grade; amino acids were Cal Bio Chem A grade, with the exception of tyrosine which was A grade, purified by solution in

NH_4OH, treatment with charcoal and Celite, and precipitation with HOAc. Tetramethylguanidine were distilled in vacuum from CaH_2 and stored over Molecular Sieves. Carbobenzoxy amino acids were prepared by literature procedures and recrystallized several times after the lit mp was achieved. IR spectra were determined with a Perkin–Elmer 237 spectrometer, NMR spectra with Varian A–60 and T–60 spectrometers, and cpm were measured using a dioxane-based counting solution in a Packard 3375 liquid scintillation spectrometer. Efficiencies were determined by internal or external standardization.

7-Hydroxy-2-ethylbenzisoxazolium fluoroborate

(a) 2,3-*Dihydroxybenzaldehyde.* A modification of the procedure of Merz and Fink[39] was used. In a 1 l round bottomed flask equipped with oil bath, magnetic stirrer, and reflux condenser was placed 100 g (0·66 mole) of *o*-vanillin (Aldrich, technical), 400 ml glacial acetic acid, and 120 ml 48% hydrobromic acid. The mixture was stirred and refluxed 12 h at a bath temperature of 135°. The mixture was cooled, the condenser was replaced by a distilling head and receiver, and the bulk of the solvent was removed on the water aspirator at 60–120°. When solid product was observed in the distilling head, the flask was cooled, and the head was replaced by a short path distilling tube (Ace no, 5085) connected to a three necked flask equipped with stopper and take off tube. The pot was surrounded by a spherical heating mantle and the distilling tube, by a heating tape. The product was distilled at aspirator pressure (pot temp 220–230°). The solid yellow–brown aldehyde was dissolved in hot benzene and the resulting solution concentrated and cooled. Further concentration and dilution with carbon tetrachloride yielded a total of 45 g, 49%, mp 104–106°; rep 105°. Recently, a marked increase in the price of *o*-vanillin has necessitated relying on commercial material available as a black syrup. Vacuum distillation of this material results in *ca* 40% recovery of usuable *o*-vanillin; it is recommended that the bituminous pot residues be removed while they are warm and semifluid.

(b) 7-*Hydroxybenzisoxazole.* A mixture of 53·0 g (0·39 mole) powdered 2,3-dihydroxybenzaldehyde, 60 g (0·53 mole) O-hydroxylammonium sulfonate, and 300 ml water was stirred at 25° until soln was complete, whereupon 300 ml EtOAc was added, the mixture was chilled in ice, and 80 g (1·0 mole) $NaHCO_3$ was added slowly with vigorous stirring. After an hour, the phases were separated, and the aqueous layer was extracted with 3 × 50 ml EtOAc. The combined organic phases were dried over $MgSO_4$ and concentrated *in vacuo* to 150 ml. Solid was collected, and the filtrate was diluted with CCl_4 to yield a second crop; total, 44·5 g, mp 124–127°. (Found: C, 62·40; H, 3·73; N, 10·49. $C_7H_5NO_2$ requires: C, 62·21; H, 3·73; N, 10·37%).

(c) 7-*Hydroxy-2-ethylbenzisoxazolium fluroooborate.* A soln of 17·5 g, (0·13 mole) 7-hydroxybenzisoxazole and 29·8 g (0·16 mole) triethyloxonium fluoroborate in 150 ml dichloromethane was stirred at 25° for 1 h, then chilled for several hours at 3°. The resulting solid was collected and recrystallized from acetonitrile–dichloromethane to yield 29·3 g fine needles, 89·7%, mp 137–138°. The salt is not hygroscopic or light sensitive, although it slowly releases HF when exposed to moist air; UV (H_2O, pH 1)λ max 307 (2640), 248 (8630), 214 nm (2000). (Found: C, 43·19; H, 4·07; N, 5·14; $C_9H_{10}NO_2BF_4$ requires: C, 43·05; H, 4·02; N, 5·57%).

General procedure for preparation of 3-acyloxy-2-hydroxy-N-ethylbenzamides, 2

The peptide acid was dissolved in an equivalent amount of 0·5 N NaOH, and an equal volume of EtOAc added, together with sufficient pyridine to make the mixture 2–4 vol% in pyridine. The mixture was chilled in an ice bath, and the pH adjusted to 4·5–5·0 with short range pH paper and 3 N HCl. A 5–10% excess of 2-ethyl-7-hydroxybenzisoxazolium fluoroborate, 1, was slowly added in portions over a span of 5–30 min. As each portion was added, the mixture became briefly yellow; the color was allowed to fade before more reagent was added. Toward the end of the addition, a pale yellow color persisted. When the addition was complete, the mixture was diluted with an addition portion of EtOAc, stirred from 5–15 min, and transferred to a separatory funnel. The layers were separated, the aqueous phase reextracted, and the pooled organic phases extracted with 3 N CHl (citric acid was substituted for BOC or other acid labile protective groups), $NaHCO_3$, and water. The aqueous phases were back extracted, and all organic phases pooled, dried over $MgSO_4$, and evaporated (in cases for which salt contamination occurs, drying was achieved by evaporation with several portions of EtOAc). In the case of Z-L-Ser, a cleaner product was obtained with a buffer consisting of a 4:1 mixture of pyridine hydrochloride–pyridine. Active esters bearing lipophilic side chains (e.g., leu, val, ile) were freed from all traces of yellow impurities by passage in EtOAc or CH_2Cl_2 soln through a short column of alumina; roughly a 10% drop in yield was then observed. Most active esters crystallized satisfactorily from EtOAc–cyclohexane.

Preparation of tetramethyl or tetraethylammonium salts of amino acids

1. *Lyophilization.* The amino acid was dissolved in a titrated equivalent of a commercial 10% soln of tetramethyl or tetraethylammonium hydroxide in water, and the resultinig soln transferred to a pear-shaped lyophilization flask and quickly frozen as a thin film over the inner surface of the flask by rapid rotation in a Dry Ice-acetone bath. The flask was connected to a lyophilization apparatus bearing a Dry Ice trap and evacuated to 0·5 mm. After several hours, the external ice film melted and the flask contents were observed as a friable foam or as a heavy oil. Evaporation was allowed to proceed for an additional hour; the resulting material was dissolved with vigorous stirring or trituration in cold DMF or DMSO and used directly.

2. *Azeotropic distillation.* A mixture of 33·9 g (37·0 mM) 10% tetramethylammonium hydroxide soln and 4·37 g L-valine (37·2 mM) was overlayered with 200 ml benzene and heated to boiling for 2–3 h in a Dean–Stark apparatus. Roughly 95% of the theoretical volume of water was collected during the first hour. The benzene soln was cooled, and the glistening plates of salt were collected by filtration and transferred to a desiccator; yield, 6·9 g, 97%. The corresponding salt of L-alanine was obtained as a fine powder; that of glycine as a wax-like solid. This procedure should not be employed with amino acids bearing reactive side chains.

General coupling procedure. The amino acids (S-Bz)Cys, Met, Phe, Pro, Ser, Thr, and Try and most free peptides dissolved in DMSO in the presence of 1·1 equiv of TMG in 20 min to 1 h. The amino acid should be finely powdered. To such a soln or to a soln of a tetraalkylam-

monium salt of an amino acid TMG was added to bring the excess base to an equivalent. The soln was cooled (dipeptide couplings run at 20°), and a soln of the active ester added. A slow stream of N_2 was passed through the vessel which contained a magnetic stirrer bar; the vessel was sealed and stirring continued for 40 min to 1 h in the case of normal amino acids, and up to 20 h in the case of hindered amino acids, N-terminal Pro, or C-terminal Ser of Thr.

The reaction mixture was quenched with water and distributed between 0·5 N HCl and EtOAc (a citrate buffer is used for BOC blocked peptides). The layers were separated, and the aqueous phase extracted several times. The pooled organic phases extracted with water to remove DMSO, then with $NaHCO_3$. The $NaHCO_3$ extracts are combined, acidified to pH 1 and extracted with several portions of EtOAc. The pooled extracts are dried, either over $MgSO_4$ or by 2–3 concentrations *in vacuo* with addition of dry EtOAc, and evaporated. The residue is recrystallized.

Illustrative procedure preparation of ZGly(L-Leu)$_2$Gly$_2$OH

1. *ZGly-L-LeuOH.* The ester **2**, R = Zgly, 2·44 g (6·6 mM) was added to Me_4N^+ L-LeuO$^-$ (16·4 mM) in 15 ml DMF. The mixture became viscous and was diluted with 15 ml DMF. After 35 min, 40 ml satd $NaHCO_3$ soln and 40 ml EtOAc were added. The organic phase was extracted with 2 × 30 ml $NaHCO_3$ and discarded. The combined $NaHCO_3$ extracts were acidified to pH 1 with 3 N HCl and extracted with 4 × 30 ml EtOAc. The pooled extracts were again extracted with 4 × 25 ml $NaHCO_3$, and after pooling, acidification, extraction with EtOAc, an organic phase was obtained which was dried over $MgSO_4$ and evaporated. Crystallization from EtOAc-pet ether yielded 1·94 g, 91·5% of product, mp 141–143°, recrystallization gave 1·85 g, 87·4%, mp 142·5–143°. $[\alpha]_D$ −9·5° (*c*, 5·0, EtOH), lit[9] 143–144, −9·5°.

2. *ZGly-L-Leu-L-LeuOH.* Following the general procedure, 1·80 g (5·6 mM) ZGly-L-LeuOH was activated using 1·68 g 1 (6·7 mM). The oily ester so obtained was combined with TMG and 6·0 mM of Me_4N^+-L-LeuO$^-$ in 30 ml DMF for 40 min at 20°. The workup was identical with that given in 1, except that the product crystallized upon acidification of the second $NaHCO_3$ extract; yield, 2·26 g, 92·6%, mp 131–133°. Recrystallization from EtOAc-pet ether yielded 2·24 g, 91·7%, mp 133–134° $[\alpha]_D$ −38·6° (*c* 1·3, EtOH), lit[14] mp 131–132, −38·3.

3. *ZGly-(L-Leu)$_2$Gly$_2$OH.* The above acid, 2·11 g (4·9 mM) was converted to its ester **2** using 1·47 g (5·8 mM) of **1**. The ester was combined without purification in 25 ml DMSO with Me_4N^+ GlyGLyO$^-$ (10·6 mM) and after 1 h at 22° was worked up as usual. Crystallization from EtOAc–light petroleum yielded 2·34 g, 88%, product, and recrystallization from EtOAc-MeCN yielded 1·96 g, 73·7%, mp 124–126·5°, $[\alpha]_D$ −36·7° (*c*, 4·0, EtOH); lit,[14] mp 124–126, −36·5.

Studies of the coupling of **2**, *R = Z-L-Ser with L-Phe.* A soln of L-Phe, 0·595 g (3·60 mM) in 7 ml DMSO containing 0·760 g TMG (6·6 mM) was treated with a soln of 1·207 g **2**, R = Z-L-Ser in 4 ml DMSO (3·0 mM). The resulting soln was stirred for 23 h under N_2, then was diluted with 100 ml ether, and the organic phase was decanted. The residue was distributed between 12 ml of 1·5 N HCl and 20 ml EtOAc. The usual workup procedure yielded an acidic residue which gave 0·903 g, 78% product, mp 134–136·5°, and a second crop of 0·127 g, mp 116–121°. Recrystallization from EtOAc-cyclohexane yielded 0·774 g, 67%, dipeptide acid, mp 140–142°, $[\alpha]_D$ +30·3° (2·0, EtOH). Preparative tlc on Mallinckrodt chromAr sheet 1000, developing with 95:5:1 $CHCl_3$ -MeOH- HOAc separated the second crop into two bands; that with the lower mobility yielded a few mg of a substance, mp 178–180°, after recrystallization from EtOAc–cyclohexane. (Found: C, 60·93; H, 5·18; N, 6·75; $C_{31}H_{33}N_3O_{10}$ requires: C, 61·28; H, 5·47; N, 6·92%). From an analogous coupling of **2**, R = Z-L-Ser, and Gly-L-AlaOEt was isolated a substance, mp 151–152·5°, 3·4% yield, IR (KRr): 1755, 1740, 1655 cm^{-1}. (Found: C, 56·45; H, 5·99; N, 8·97. $C_{29}H_{36}N_4O_{11}$ requires: C, 56·49; H, 5·98; N, 9·09%).

Isotopic dilution was preceded by two control experiments. A soln of Z-L-Ser-L-PheOH, 9·4 mg, 212 dpm/mg, and Z-L-SerOH, 48·8 mg in 1 ml hot EtOAc was diluted with 5 ml $CHCl_3$ and chilled. The resulting solid was recrystallized to yield Z-L-SerOH, mp 116·5–117·5°, 2·0 dpm/mg. Similarly, Z-L-Ser-L-PheOH, 11·7 mg, 212 dpm/mg and Z-D-Ser-L-PheOH, 28·0 mg, in 2 ml hot EtOAc was diluted with 3 ml cyclohexane, and seeded with the DL isomer. Recrystallization yielded Z-D-Ser-L-PheOH, mp 143–144°, 0·6 dpm/mg.

For the isotopic dilution analysis, **2**, R = Z-L-Ser, 80·4 mg, 0·20 mM, $7·76 \times 10^4$ dpm, in 0·5 ml DMSO was treated with 2 ml DMSO containing L-Phe, 39·6 mg, 0·24 mM, and TMG, 50·7 mg, 0·44 mM. After 24 h under N_2 the soln was diluted with 4 ml 1·5 N HCl and 20 ml EtOAc containing 72·0 mg **2**, R = Z-L-Ser, 41·4 mg Z-L-Ser-L-PheOH. After the usual workup and recrystallization, the neutral fraction yielded 26·7 mg **2**, R = Z-L-Ser, mp 113–115°, 2·8 dpm/mg, and the acidic fraction yielded 62·2 mg Z-L-Ser-L-PheOH, mp 138–140°, $2·44 \times 10^5$ dpm/mM. Before these isolations, the total activity of the neutral and acidic fractions were assayed through aliquots and found to be $2·6 \times 10^3$ dpm and $7·46 \times 10^4$ dpm. The mother liquors from the acidic fraction were concentrated and divided in four portions: the first was counted, to the remainder were added, respectively, 49·6 mg Z-L-Ser-L-PheOH, 54·4 mg Z-D-Ser-L-PheOH, and 61·1 mg Z-L-SerOH. The total activity in the mother liquors was $2·77 \times 10^4$ dpm; recrystallization of the three diluents gave respective activities of $3·25 \times 10^4$, 266, and $1·55 \times 10^3$ dpm/mM.

*Preparation of sequence polymers of Gly-L-*LeuGly. The properties of peptides and active esters on the synthetic route to Z(Gly-L-LeuGly)$_n$OH are given in Tables 4 and 5. Characterization of the peptide acids is reported elsewhere. To introduce a further criterion of identity, an isotopic label was introduced into the N-terminal free peptide glycine.

1. **2**, *R = Zgly-L-LeuGly.* A 1·89 g sample of ZGly-L-LeuGlyOH (5 mM) was activated by the general procedure, using 1·38 g (5·5 mM) of **1**. During the activation, a thick gel formed, and more EtOAc was added to facilitate stirring. After 25 min, 8 ml 3 N HCl was added, stirring was continued for 20 min, and the ppt was collected and washed with EtOAc; 1·6 g, 59%, mp 187–189°. From the organic phases was isolated 0·78 g, 29%, mp 187–189°.

2. **2**, *R = Z(Gly-L-LeuGly)$_4$.* A 0·323 g (0·3 mM) sample of Z(Gly-L-LeuGly)$_4$OH was subjected to the usual activation procedure. A gel formed as EtOAc was added to the aqueous soln of the Na–salt, and NaOH aq was added dropwise until it disappeared, whereupon the usual procedure was followed. The product precipitated from

Table 5. Characterization of Peptides and Peptide Active Esters

A. *Esters*, 2.

2, R	Yield	mp°	$[\alpha]_D$°	Composition C	H	N
ZGly-L-Pro	85	103–105	−139·0 (1·6, HOAc)	61·18	5·82	8·92
			$C_{24}H_{27}N_3O_7$ requires:	61·40	5·80	8·95
BOC-L-Ala-*L*(γOBz)Glu	87	93–95	−37·2 (1·1, HOAc)	60·08	6·48	7·22
			$C_{29}H_{37}N_3O_9$ 1/2H_2O req:	59·98	6·60	7·24
Z-L-Ile-L-Gln	73	191–193	—	60·30	6·44	9·96
			$C_{28}H_{36}N_4O_8$ requires:	60·42	6·52	10·07
ZGly-L-Leu-Gly	78	187–189	−17·4 (2·6, DMF)	59·83	6·30	10·35
			$C_{27}H_{34}N_4O_8$ requires:	59·77	6·32	10·33
Z(Gly-L-Leu Gly)$_2$	84	216–218	−20·5 (1·2, HOAc)	57·50	6·74	12·68
			$C_{37}H_{51}N_7O_{11}$ req:	57·72	6·68	12·74
Z(Gly-L-Leu Gly)$_4$	77	dec	−20·7 (0·15, DMF)	54·87	7·17	14·84
			$C_{57}H_{85}N_{13}O_{17} \cdot H_2O$ req:	55·09	7·67	14·65

B. *Peptide acids*

Substance	mp°	$[\alpha]_D$°	C	H	N
ZGly-L-GlnOH	158·5–159·5	+3·0 (546) (2·0, MeOH)	53·31	5·77	12·29
		$C_{15}H_{19}N_3O_6$ requires:	53·40	5·68	12·46
Z-L-Ile-L-GluOH	189–190 lit: 184–185	−21·4 (1·3, 0·5 *N* $KHCO_3$)	57·80	7·05	10·81
		$C_{19}H_{27}N_3O_6$ requires:	58·00	6·92	10·68
Z-L-Glu-L-PheOH	199–200 lit: 200	+5·2 (1·0, DMF)	61·95	5·90	9·75
		$C_{22}H_{25}N_3O_6$ requires:	61·82	5·90	9·83
Z-L-Gln-L-AlaOH	219–220 lit: 209–213	−1·5 (1·5, DMF)	54·68	6·04	11·94
		$C_{16}H_{21}N_3O_6$ requires:	54·70	6·02	11·96
Z-L-Ser-L-PheOH	141–142	+30·3 (2·0, EtOH)	61·95	5·59	7·02
		$C_{20}H_{22}N_2O_6$ requires:	62·17	5·74	7·25
Z-L-Thr-L-PheOH	154–155	+23·4 (2·0, EtOH)	62·82	6·07	7·04
		$C_{21}H_{24}N_2O_6$ requires:	62·99	6·04	7·00

the mixture, which was stirred for 2 h at 22° to complete precipitation. Collection, washing with EtOAc, and trituration with CH_3CN gave 0·281 g ester, 77% mp 240° dec. The analytical sample was thrice precipitated from HOAc.

3. *Z(Gly-L-LeuGly)$_8$OH.* A solution of 54·7 mg, 0·056 mM of Gly-L-LeuGly · HBr and 10·3 mg triethylamine in 2·5 ml DMSO was treated with 62·0 mg, 0·051 mM, **2**, R = Z(Gly-L-LeuGly). After 7 days at 22°, 20 ml ether was added and the mixture was triturated with water, 3 ml 1 N HCl, water, and EtOAc to yield 57 mg product, 57%, mp > 250°, $[\alpha]_D$ −40° (*c* 0·14, formic acid), −55·9° (0·15, dichloroacetic acid). (Found: C, 52·33; H, 7·3 7; N, 16·54. $C_{88}H_{144}N_{24}O_{27} \cdot 3H_2O$ requires: C, 52·20; H, 7·48; N, 16·61%).

Synthesis of peptides derived from oxytocin

1. *Z-L-Pro-L-LeuGlyNH$_2$.* Z-L-ProOH, 3·74 g (15·0 mM) was activated using 39·2 g **1** (15·6 mM); the oily product was mixed with 2 ml DMSO and a soln of 10 ml DMSO containing Me_4N^+ L-LeuO$^-$ (16·5 mM) and TMG (1·73 g) was added. After 30 h, the mixture was worked up in the usual way to yield 3·86 g, 71·2%, of Z-L-Pro-L-LeuOH, mp 115–117°, rep mp, 118–119°. Activation 2·71 g (6·0 mM) in the usual manner (1·57 g **1** (6·25 mM) yielded an oil which was added to a solution of 0·73 g (6·6 mM) $GlyNH_2 \cdot HCl$ in 4 ml DMSO containing 0·76 g (6·6 mM) TMG. After 10 h, the mixture was worked up using a NaOH extraction. The neutral phase yielded a residue which was recrystallized from EtOAc–cyclohexane to yield crop 1, 1·86 g mp 162–164°, and crop 2, 0·08 g, mp 158–160°, total: 77·3%. Recrystallization from water yielded 1·70 g, mp 162–163·5°, $[\alpha]_D$ −69·4° (2·5, EtOH) and 0·12 g, mp 161–162, $[\alpha]_D$ −70·4°,. Lit mp[40] 163–163·5° $[\alpha]_D$ −73·3°; 163°,[41] −71·9; 165–166°[42]–70·6°. When the above reaction sequence was repeated without purification of intermediates a yield of 62·0% of material, mp 161–162° was obtained, based on Z-L-ProOH.

2. *Z-L-(SBz)Cys-L-LeuGlyNH₂.* To a stirred soln of L-Pro-L-LeuGlyNH₂, (formed from 1·25 g, 3·0 mM of the above product by HBr in HOAc treatment followed by Dowex 1-X4 and lyophilization) in 3 ml DMF was added 0·35 g TMG (30 mM) and 1·68 g (3·3 mM) **2**, R = Z(SBz)Cys. After 18 h, the mixture was diluted with 20 ml EtOAc and extracted with 3 × 10 ml 0·5 N NaOH. The organic layer was washed with H_2O, dried, concentrated to 15 ml, seeded, and chilled to yield 1·65 g product, 90%, mp 164–167°. Recrystallization from MeOH–H_2O yielded 1·62 g, 88·3%, mp 170–171°, $[\alpha]_D$ −58·5° (2·0, DMF). Lit mp 170–171·5°,[43] −60·0°; 160–163°,[44] −58°.

3. *Z-L-Ile-L-Gln-L-AsnOH.* A soln of 3·93 g (10·0 mM) of Z-L-Ile-L-GlnOH in 12 ml 1 N NaOH was treated with 1·5 ml pyridine and enough 3 N HCl to bring the pH to 6. EtOAc, 30 ml, and water, 10 ml, were added, and the stirred, chilled soln was treated with 2·61 g (10·4 mM) of **1**. After the addition of **1**, 30 ml EtOAc was added and the mixture was stirred for 30 min and filtered. The organic phase of the filtrate was subjected to the usual workup. The ppt was dissolved in 250 ml warm EtOAc, and the resulting soln was worked up in the usual way. Chilling of the dried EtOAc solutions yielded 4·46 g, 80·3%, ester. Recrystallization from MeCN with slow cooling yielded 3·67 g ester, mp 190–192°. When 0·724 g (1·30 mM) of this ester was added and allowed to react for 3 h with a mixture obtained by slurrying 0·215 g (1·43 mM) L-Asn hydrate and 0·314 g (2·73 mM) TMG in 2 ml DMSO, addition of 15 ml EtOAc, 20 ml H_2O, and HCl to pH 1 gave a heavy ppt which was collected, washed with water, EtOAc, and a small volume of EtOH-water, yield: 0·60 g, 91·5%. Recrystallization from 2:1 MeCN-water (35 ml) gave 0·494 g solid mp 218–220°, $[\alpha]_D$ −43·1° (1·0, 0·5 N $KHCO_3$). Lit:[29] mp 203–204°, −35·5°. When a less carefully purified sample of active ester was used, tripeptide acid with $[\alpha]_D$ − 38·5° was obtained.

4. *Z-L-(SBz)Cys-L-Tyr-L-Ile-L-Glu-L-AsnOH.* Z-L-(SBz)Cys-L-TyrOH when subjected to the usual activation procedure gave a residue which could be precipitated from EtOAc-light petroleum as an amorphous solid in 98% yield, mp 98–101°. The crude ester was dissolved in EtOAc, treated with cyclohexane until an oil separated, then vigorously triturated in a mortar and allowed to stir overnight. Filtration gave 75% of a white, crystalline solid, mp 140–143°. A soln of 0·477 g L-Ile-L-Gln-L-Asn (1·22 mM) and 0·29 g TMG (2·5 mM) in 2 ml DMSO and 0·5 ml DMF was mixed with 0·797 g (1·19 mM) of the above ester in 2 ml DMSO. The mixture was stirred for 10 h at 5–8° under N_2, at which time no ester could be detected by TLC. The mixture was diluted with 15 ml water and 3 ml 0·5 N citric acid, triturated, and filtered. The resulting solid was washed with 30 ml ether and 12 ml water, then crystallized from 30 ml THF and 10–15 ml water to give 0·733 g, mp 244·5–245·5°, and a second crop of 0·130 g, mp 228–237°. Total: 84%. $[\alpha]_D$ −23·2° (1·0, DMF). Lit: 245–247°,[43] −23·5°. 238–240,[45] −23·9°.

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REFERENCES

[1]D. S. Kemp, S-W. Wang, R. C. Mollan, S-L. Hsia, and P. N. Confalone, *Tetrahedron* **30**, 3689 (1974)
[2]D. S. Kemp, S-W. Wang, J. Rebek, Jr., R. C. Mollan, C. Banquer, and G. Subramanyam, *Tetrahedron* **30**, 3955 (1974)
[3]G. Busby, III, B. S. Thesis, M.I.T. 1967
[4]M. Bergmann and J. S. Fruton, *J. Biol. Chem.* **117**, 189 (1937)
[5]S. J. Leach and H. Lindley, *Austral. J. Chem.* 7, 173 (1954)
[6]J. A. MacLaren, *Ibid.* **11**, 360 (1958)
[7]K. Hofmann and M. Bergmann, *J. Biol. Chem.* **134**, 225 (1940)
[8]D. W. Clayton, J. A. Farrington, G. W. Kenner, and J. M. Turner, *J. Chem. Soc.*, 1398 (1957)
[9]F. Weygand and W. Steglich, *Chem. Ber.* **93**, 2983 (1960)
[10]H. Zahn and H. R. Falkenburg, *Leibigs Ann.* **636**, 117 (1960)
[11]S. Golschmidt and H. Lautenschlaer, *Ibid.* **580**, 68 (1953)
[12]F. Weygand and W. Steglich, *Chem. Ber.* **93**, 29 (1960)
[13]M. Bergmann, L. Zervas, H. Sehleich, and F. Leinert, *Z. Physiol Chem.* **212**, 72 (1932)
[14]P. M. Hardy, G. W. Kenner, and R. C. Sheppard, *Tetrahedron* **19**, 104 (1963)
[15]W. H. Stein, S. Moore, and M. Bergmann, *J. Biol Chem.* **154**, 191 (1944)
[16]E. Schröder and K. Lubke, *Liebigs Ann.* **655**, 211 (1962)
[17]G. C. Stelakatos, *J. Am. Chem. Soc.* **83**, 4222 (1961)
[18]E. Schröder, H. Petras, and E. Klieger, *Liebigs Ann.* **679**, 221 (1964)
[19]J. A. Farrington, P. J. Hextall, G. W. Kenner, and J. M. Turner, *J. Chem. Soc.* 1407 (1957)
[20]G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.* **82**, 3359 (1960)
[21]W. D. Cash. *J. Org. Chem.* **26**, 2136 (1961)
[22]R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *Helv. Chim. Acta* **41**, 1273 (1958)
[23]M. Bodanszky and V. duVigneaud, *J. Am. Chem. Soc.* **81**, 2504 (1959)
[24]D. Theodoropoulos and L. C. Craig, *J. Org. Chem.* **20**, 1169 (1955)
[25]J. A. MacLaren, *Austral J. Chem.* **11**, 360 (1958)
[26]M. Bergmann and L. Zervas, *J. Biol. Chem.* **113**, 341 (1936)
[27]N. Izumiya and J. Greenstein, *Arch. Biochem. Biophys.* **52**, 203 (1954)
[28]K. Hofmann, *et al.*, *J. Am. Chem. Soc.* **82**, 3715 (1960)
[29]V. duVigneaud *et al.*, *Ibid.* **76**, 3115 (1954)
[30]E. Sondheimer and R. Holley, *Ibid.* **76**, 2816 (1954)
[31]J. Rudinger, J. Honzl, and M. Zaoral, *Coll. Czech. Chem. Comm.* **21**, 202 (1956)
[32]H. C. Beyerman and J. S. Bontekoe, *Rec. Trav. Chim.* **81**, 699 (1962)
[33]M. Bodanszky, J. T. Sheehan, M. Ondetti, and S. Lance, *J. Am. Chem. Soc.* **85**, 991 (1963)
[34]J. S. Fruton, *J. Biol. Chem.* **146**, 463 (1942)
[35]J. A. MacLaren, W. E. Savige, and J. M. Swan, *Austral. J. Chem.* **11**, 345 (1958); V. duVigneaud, M. F. Bartlett, and A. Jöhl, *J. Am. Chem. Soc.* **79**, 5572 (1957); See also ref. 25 and J. Kovacs, G. L. Mayers, R. Johnson, and U. Ghatak, *Chem. Comm.* 1066 (1968)
[36]For a review, see: E. Schröder and K. Lübke, *The Peptides* Vol. I, Academic Press, New York 1965, pp. 181–206; also M. A. Ondetti, A. Deer, J. T. Sheehan, J. Pluscec, and O. Kocy, *Biochemistry* **7**, 4069 (1968)
[37]E. D. Nicolaides and H. A. DeWald, *J. Org. Chem.* **26**, 3872 (1961); M. Bodanszky and M. A. Ondetti, *Chem. Ind.* 26 (1966)
[38]B. M. Anderson, E. H. Cordes, and W. P. Jencks, *J. Biol. Chem.* **236**, 455, (1961)
[39]K. W. Merz and J. Fink, *Arch. Pharm.* **289**, 353 (1956)

[40]C. Ressler and V. duVigneaud, *J. Am. Chem. Soc.* **76**, 3107 (1954)
[41]R. A. Boissonnas, St. Guttmann, P. Jaquenaud, and J. P. Waller, *Helv. Chim. Acta* **38**, 1491 (1955)
[42]F. H. C. Stewart, *Austral. J. Chem.* **19**, 2361 (1966)
[43]M. Bodanszky and V. duVigneaud, *J. Am. Chem. Soc.* **81**, 2504 (1959)
[44]A. C. Beyerman, J. Bontekoe, and A. Koch, *Rec. Trav. Chim.* **78**, 935 (1959)
[45]A. P. Fosker and H. D. Law, *J. Chem. Soc.* 4922 (1965)