Assessment of Racemisation in *N*-Alkylated Amino-acid Derivatives during Peptide Coupling in a Model Dipeptide System [1]

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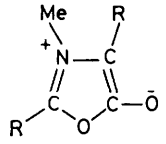
N.m.r. analysis of diastereoisomeric benzoyl dipeptide esters has been used to monitor racemisation during the coupling of benzoyl-*N*-alkylated amino-acids to alanine and valine methyl esters. Results confirm the greater susceptibility of these amino-acids to racemisation, but conditions have been found which eliminate racemisation. The additives HONSu and HOBt vary in their capability as ' chiral stabilisers.' N.m.r. techniques provide a facile means for monitoring the involvement of oxazolonium ions during coupling. A possible mechanism for racemisation is proposed.

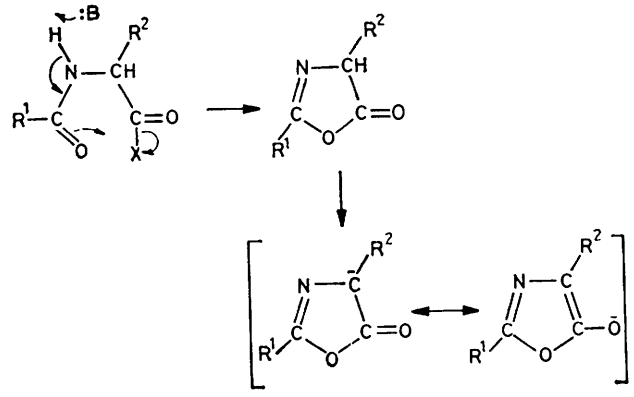
THE *N*-alkylated amino-acid proline has long been recognised as being chirally stable on ' activation ' for peptide coupling, and there are only very few examples [2,3] reporting its racemisation. It has therefore played an important role as a terminal residue at the coupling bond when larger peptide fragments are being coupled (*i.e.* segment condensation). Taking cognisance of one of the accepted mechanisms for racemisation (Scheme 1) it was



(1)

plausible to explain this special stability of proline as being due to *N*-alkylation preventing the initial loss of proton required to initiate the process. However, this hypothesis is unable to explain the rather surprising discovery of Benoiton and his co-worker [3] who reported that *N*-methylamino-acids are very susceptible to racemisation. The pseudoaromatic [4] oxazolonium ion (1) has been proposed [3] as the explanation for the racemisation.

Alternative mechanisms for the racemisation of amino-acid derivatives have been proven for some amino-acids. Direct ionisation of the α-hydrogen has been implicated [5] in the racemisation of derivatives of serine, tyrosine, phenylalanine, and cysteine.[6] More recently there have been strong indications [7] that cysteinyl residues racemise *via* an enolisation mechanism catalysed by the presence of sulphur in the side chain. The chiral instability of histidine derivatives [8] has been explained by proton abstraction catalysed by the basicity of the side-chain imidazole ring, while *N*-phthalyl derivatives which cannot be involved in azlactone formation must involve direct proton abstraction.[9]



SCHEME 1

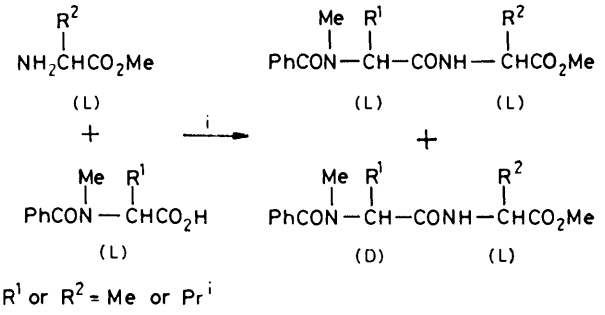
It is now recognised [10,11] that the structural parameters influencing racemisation are quite complex and that the degree of racemisation is often influenced by the structure of the amino-acid, solvents, the nature of the group attached to the *N*-terminal end of the amino-acid, and the organic base used.

Current interest in *N*-methylamino-acid-containing antibiotics and the incorporation of *N*-methylated residues into biologically active peptides for structure–activity studies has prompted a detailed study of the racemisation of these imino-acids to define conditions conducive to minimal racemisation, even when they are used as terminal residues in segment condensation. In the light of the complex choice of mechanisms for racemisation it was of interest also to study the racemisation process.

Recent developments [12,13] in the application of n.m.r. methods to the study of racemisation, had shown that it is possible to identify separate ester signals for diastereoisomeric benzoyl-*N*-methylated dipeptide esters, and this technique was used throughout this work. The principle of the method is outlined in Scheme 2.

N.m.r. monitoring was also advantageous in determining the presence of the oxazolonium ion (1). When either benzoyl-L-*N*-methylalanine or benzoyl-L-*N*-methylvaline are mixed with DCCI (*NN'*-dicyclohexylcarbodi-imide) in deuteriochloroform or [$^2H_8$]tetrahydrofuran, once the dicyclohexylurea starts to precipitate (within the time needed to mix and place a sample in the spectrometer) signals appear in the ^1H n.m.r. spectra (100 MHz) at τ 6.23 (s) and τ 7.87 (s) for (1; R = Me) and τ 6.29 (s) and 8.54 (d) for (1; R = Pr^i). At low temperature (−5 to −20 °C), these oxazolonium species were stable for up to 3 h in the n.m.r. sample tube, and this led to the possibility of checking the degree of asym-

metric induction obtained when the planar ion (1) is coupled with L-alanine or L-valine methyl esters. As shown in Scheme 3 the values for induction are quite significant and are different in each case. Thus when

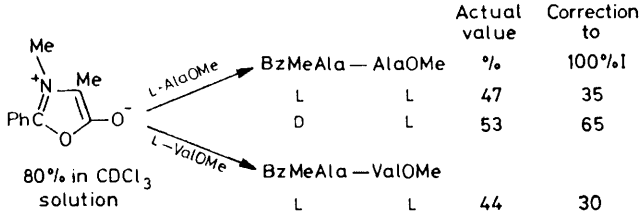


SCHEME 2 i, Miscellaneous coupling conditions (Table 1); ii, excess reagents and other by-products removed by washing with acid and base; iii, solvent, after drying, is removed and the residue dissolved in $CDCl_3$; iv, integration of diastereoisomeric ester peaks after scale expansion to give the ratio of diastereoisomers.

racemisation *via* the oxazolonium ion (1) mechanism is implied the amount of D–L diasteroisomer in the product will be exaggerated by a proportional amount due to chiral induction. The corrected figure to 100% of (1) in Scheme 3 assumes that the 20% of Bz-L-MeAla still in solution would react with *C*-terminal ester without racemisation.

In a series of experiments to study conditions and reagents for peptide coupling, the model reaction outlined in Scheme 2 was investigated as recorded in Table 1.

The results quoted in Table 1 can only be meaningful if the starting materials are optically pure. Earlier results [1] did in fact indicate a 7% D–L content when a sample of BzMeAla was coupled *via* the racemisation free azide method. The sample of BzMeVal used for coupling could not be tested using the azide method, since numerous attempts to convert BzMeValOMe into the hydrazide failed, presumably owing to steric hindrance in the molecule. Synthesis of BzMeVal *via* the removal of the t-butoxycarbonyl group from Boc-L-MeVal (which can be obtained optically pure [14]), followed by benzoylation gave a product no better in optical purity than those obtained by direct methyl-



SCHEME 3

ation [14] of benzoyl-L-valine. Fractional crystallisation of salts formed with optically active bases also failed to raise the optical rotation values of the BzMeVal. The only avenue left open was to assess the optical purity of the starting materials, by comparison with substances known to be optically pure.

BzMeAla and BzMeVal were hydrolysed to HNMeAlaHCl and HNMeValHCl under identical conditions to optically pure [14] Boc-L-MeAla and Boc-L-MeVal respectively and the optical rotations of the products compared. The results indicate that BzMeVal, $[\alpha]_D$ −99° (in $CHCl_3$), contains 15% D-form, while BzMeAla, $[\alpha]_D$ −62.5 (in $CHCl_3$), is optically pure. The purity of the BzMeAla has also been confirmed by the formation of the pure L–L diastereoisomer when coupling was carried out under certain conditions. The results recorded in Table 1 for BzMeVal are therefore higher because of the presence of 8% D-form in the starting material having $[\alpha]_D$ −120° (in $CHCl_3$). Comparisons are still possible within the series of couplings carried out on BzMeVal but the main work

TABLE 1

N-Terminal component	Coupling agents	*T*/°C [a]	Addition of L-alanine methyl ester	Addition of L-valine methyl ester	% D–L Diastereoisomer in product (each entry represents the average of 3 runs/3 integrations each)
BzMeVal($[\alpha]_D$ −120°)	DCCI	A		directly [b]	50
BzMeVal($[\alpha]_D$ −120°)	DCCI + HONSu	A		after 1 h	35
BzMeVal($[\alpha]_D$ −120°)	DCCI + HOBt	A		after 1 h	65
BzMeVal($[\alpha]_D$ −120°)	EEDQ	A		directly [b]	40
BzMeVal($[\alpha]_D$ −120°)	Bu^tOCOCl	0—5		after 60 s	25
BzMeAla($[\alpha]_D$ −62.5)	DCCI	A	directly [b]		50
BzMeAla($[\alpha]_D$ −62.5)	DCCI	−5 (±2)	directly [b]		23
BzMeAla($[\alpha]_D$ −62.5)	DCCI + HONSu	A	directly [b]		10
BzMeAla($[\alpha]_D$ −62.5)	DCCI + HONSu	−5 (±2)	directly [b]		0
BzMeAla($[\alpha]_D$ −62.5)	DCCI + HONSu	A	after 1 h		10
BzMeAla($[\alpha]_D$ −62.5)	DCCI + HONSu	A		after 1 h	35
BzMeAla($[\alpha]_D$ −62.5)	DCCI + HOBt	A	directly [b]		20
BzMeAla($[\alpha]_D$ −62.5)	DCCI + HOBt	−5 (±2)	directly [b]		0
BzMeAla($[\alpha]_D$ −62.5)	DCCI + HOBt	A	after 1 h		60
BzMeAla($[\alpha]_D$ −62.5)	DCCI + HOBt	A		after 1 h	70

[a] A = Ambient ≃ 23 °C. [b] Implies that the *C*-terminal component was already present in the solution at the time of activation.

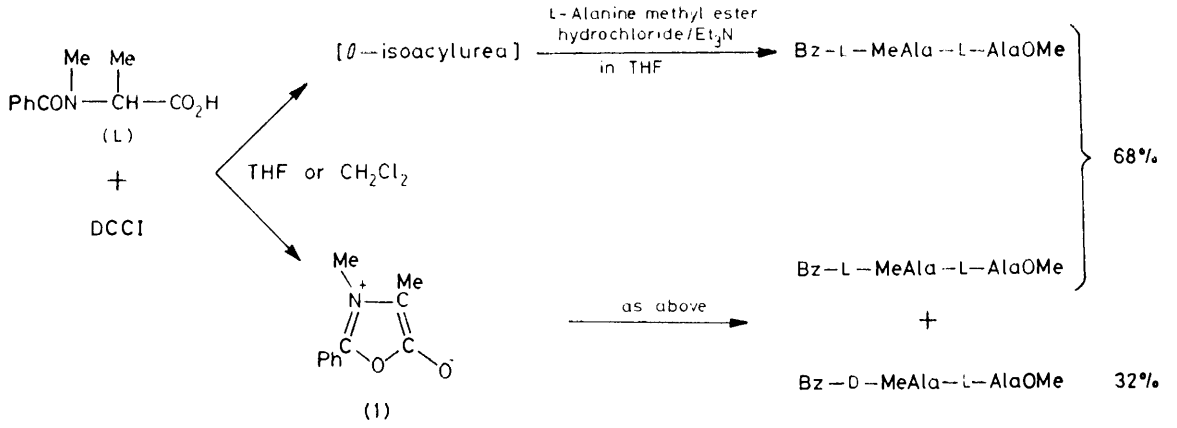
in this paper has concentrated on using the optically pure Bz-L-MeAla.

The results in Table 1 confirm the greater susceptibility towards racemisation of the *N*-methylated compounds. N.m.r. monitoring of selected reactions, for peaks attributable to the ion (1) also confirms that the source of the racemisation involving DCCI is the oxazolonium intermediate. It is also possible to detect (1) using its yellow band at $\lambda_{max.}$ 400 nm. A summary of the reactions with DCCI appears in Scheme 4.

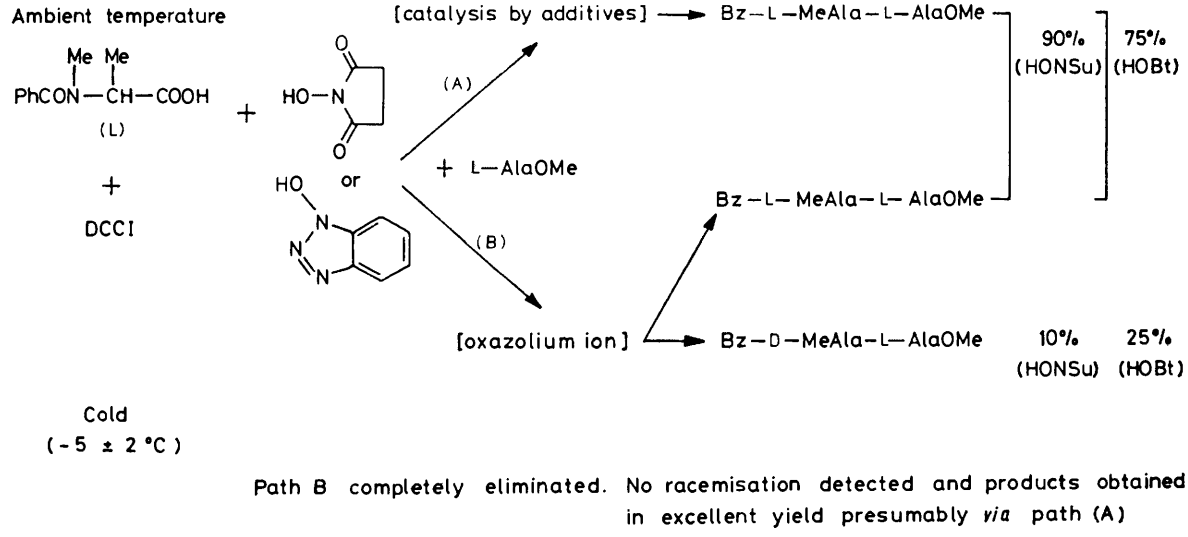
The formation of the oxazolonium ion (1) is temperature-dependent and its formation can be reduced by carrying out DCCI coupling reactions at *ca.* −5 °C, but racemisation is only completely eliminated on addition of the additives *N*-hydroxysuccinimide (HONSu) and *N*-hydroxybenzotriazole (HOBt). N.m.r. and spectroscopic studies confirm that at the lower temperatures with additives present, no oxazolonium ion (1) is produced. At ambient temperatures under the same conditions the oxazolonium pathway competes with direct coupling as summarised in Scheme 5.

A plausible explanation for the efficiency of *N*-hydroxy additives in catalysing peptide-coupling reactions is that the reactions proceed *via* an active ester pathway,[15] and often it has become customary to allow time for the active ester to form by delaying the addition of the *C*-terminal unit at the coupling bond. When this was done in the present project it can be seen from Table 1 that quite different racemisation figures were obtained for HONSu- and HOBt-catalysed reactions. In all cases the latter gave higher amounts of racemisation. The absence of characteristic peaks in the n.m.r. spectra confirmed that the increased racemisation figures for HOBt was not due to formation of oxazolonium ion (1).

A detailed investigation of the source of the ' HOBt ' racemisation was carried out by studying the optical purity of the intermediate *N*-hydroxysuccinimide and *N*-hydroxybenzotriazole esters (2) and (3) respectively. Careful manipulation of reactants at temperatures below 0 °C at all stages (including removal of solvent) enabled us to prepare samples of (2) and (3) (Scheme 6). While (2) was obtained optically active, zero optical rotation was recorded for (3). When (2) and (3) were individually coupled with L-alanine methyl ester hydrochloride the



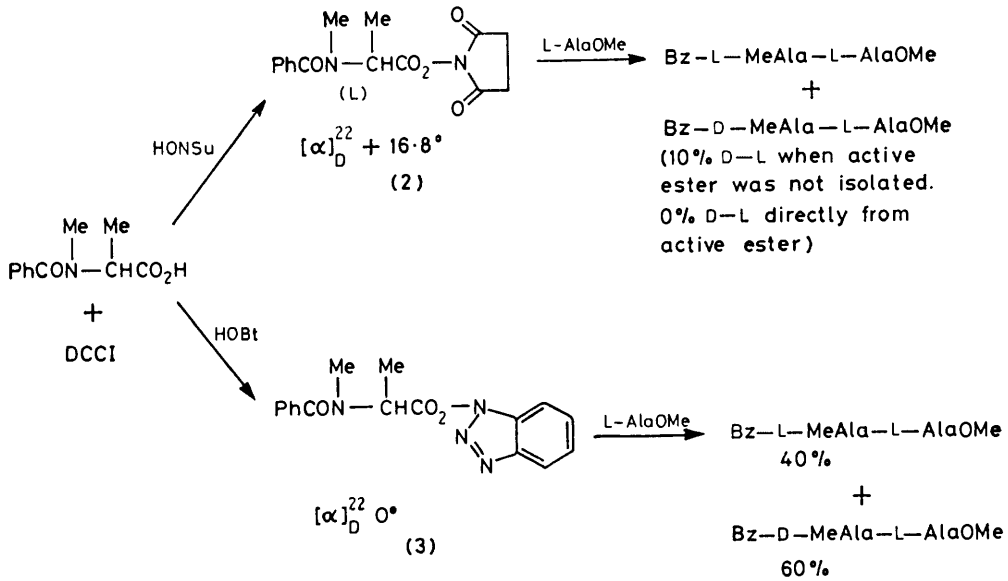
SCHEME 4 Summary of reaction with DCCI as coupling agent



SCHEME 5 Summary of reaction with DCCI + additives

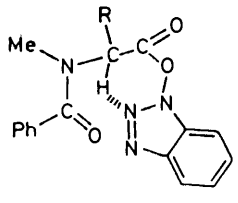
same distribution of diastereoisomeric dipeptide esters as for ' delayed ' coupling conditions was obtained. The source of the racemisation in the HOBt case must therefore be the chiral instability of the ester (3), which results in the situation summarised in Scheme 6.

A possible explanation for the racemisation of the ester (3) comes from the observation that its α-proton is a ' direct abstraction ' of the α-proton as proposed in (4) is considered, then the ' acidity ' of the α-proton might be influenced by the nature of the neighbouring ester carbonyl group, and could have a different activation in the HOBt as compared to the HONSu ester. But this difference may be ruled out from the observation that the ester carbonyls in both (2) and (3) appear at 1 820 cm^{-1}



SCHEME 6 Summary of events *via* the ' active ester ' pathway (equivalent to delaying addition of C-terminal ester for 1 h)

readily exchangeable with deuterium in D_2O–$CDCl_3$ solution. In the ^1H n.m.r. spectrum of the ester (3) in $CDCl_3$ solution the α-proton is split into two multiplets at τ 5.04 and 4.70 because of *cis–trans* rotational isomerism around the benzimido-bond. The alanine methyl group in the same spectrum appears as a doublet at τ 8.22. On addition of D_2O the signals for the α-proton disappeared immediately and the methyl group doublet at τ 8.22 was reduced to a singlet, thus confirming a rapid deuterium exchange of the α-proton. In the absence of any other base it is plausible for the triazole nitrogen to catalyse intramolecularly the abstraction of the α-proton as in (4) or *via* the stabilisation of the enol form [16] (5). If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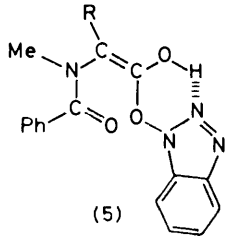
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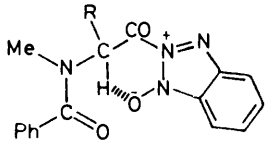
(6)

for spectra run in KBr. However in chloroform solution the 1 820 cm^{-1} peak for BzMeAlaOBt (3) is greatly reduced in intensity and the main carbonyl absorption appears at 1 740 cm^{-1}, which according to König and Geiger [15] is due to species (6). BzMeAlaONSu (2) preserves its 1 820 cm^{-1} absorption even in the solution phase. By studying molecular models and comparing bond distances *etc.* we conclude that species (6) is a prime candidate for explaining the exchangeability of the α-proton.

Veber and his colleague,[17] using theoretical calculations have indicated that the degree of *cis*-rotamer character in the *N*-terminal bond influences the acidity of the α-proton. We have studied the *cis–trans* rotamer ratios for (2) and (3) and conclude that there is little evidence of rotamers in (2), while the spectrum of the HOBt ester (3) shows distinct *cis–trans* rotamers [18] in the ratio 30% *cis* and 70% *trans*. Taking into account the infrared spectral evidence it has to be assumed that these are rotamers of species (6) due to rotation in the benzimido-bond. The significant *cis*-rotamer content would therefore lend support to the Veber argument but there is no obvious means of rigorously testing the possibilities. Having considered all the options we tend to favour some involvement of the species (6), and the readiness of the various HOBt esters to racemise, as seen later, could well reflect the ease of formation of (6).

In trying to estimate the significance of this phenomenon to the application of HOBt as an additive for

peptide coupling in general, it is conceivable that the benzoyl group enhances the exchangeability of the α-proton in excess of what would be expected in more normal peptide coupling conditions. The urethane-protected HOBt derivative, *N*-benzyloxycarbonyl-L-*N*-methylalanyl benzotriazolate (Z-L-MeAlaOBt), was therefor synthesised and the exchangeability of its α-proton studied under the same conditions as the benzoyl analogue. No deuterium exchange of the α-proton could be detected, which agrees with the chiral stabilising influence of the urethane protecting group. The ester carbonyl absorption at 1 825 cm^{-1} in the infrared spectrum was present in chloroform solution and when Z-*N*-methylamino-acids are coupled to L-alanine methyl ester using only DCCI as reagent, followed by removal of the Z-group and re-introduction of the *N*-benzoyl group for chiral assessment using n.m.r., the peptides obtained have always been found to be of pure L–L-form. This confirms the superior chiral stabilisation offered by the urethane-protected *N*-methylamino-acid derivatives.[3]

It was still important to consider the chiral fate of *N*-methylamino-acids protected only by an *N*-acyl group, a situation which could arise in the segment condensation approach to peptide synthesis. As a model, Z-Gly-L-MeAlaOBt was synthesised and when subjected to n.m.r. analysis there was a slow exchange of the alanyl α-proton when D_2O was added. The exchange of the α-proton was monitored by the changes in the multiplicity of the alanine methyl signal. The exchange was much slower than for the *N*-benzoyl analogue, but it could be significant if a slow coupling of the *N*-methylamino-acid was encountered during segment condensation. No comparable exchange was detected when the HONSu ester was studied.

We conclude that acylated *N*-methylamino-acids (not urethane-protected), when activated *via* the HOBt esters, are susceptible to racemisation *via* an intramolecular mechanism, and great care should be taken in their use in segment condensation. However, when making comparisons between HONSu and HOBt, it should be emphasised that for direct coupling over a short time, the yields and chemical purity of product are superior using the HOBt additive.

Recently it has become apparent [19] that certain sterically difficult segment condensations involving leucine at the *C*-terminal activation position and using DCCI–HOBt gave high racemisation figures, but could be successfully carried out with no racemisation using DCCI–HONSu. A key coupling step between tyrosine and leucine in the synthesis of insulin [20] gave 30% racemisation when DCCI–HOBt activation was used. The question therefore arises whether the evidence presented above for *N*-methylamino-acids is equally applicable to the non-methylated analogues. Bz-L-AlaOBt and Bz-L-AlaONSu have been synthesised and subjected to D_2O exchange, and the results show a rapid exchange in the case of the HOBt ester while the HONSu ester showed no tendency to exchange under similar conditions. This strongly suggests a similar chiral instability in HOBt esters of both *N*-methylated and ordinary amino-acids.

EXPERIMENTAL

^1H N.m.r. spectra were determined at 100 MHz on a Varian HA-100 instrument with tetramethylsilane as internal standard. Optical rotations were measured on a Perkin-Elmer 141 automatic polarimeter and u.v. measurements on a Perkin-Elmer 402 spectrophotometer. C,H,N microanalyses were carried out using an F & M 185 analyser, while mass spectra were determined on an AEI MS9 instrument. I.r. spectra were obtained on a Pye Unicam SP 1050 spectrophotometer using KBr discs, as thin films, or in solution. Purity was routinely checked using Kieselgel G t.l.c. plates developed in 1—5% MeOH–$CHCl_3$ (compounds either located using I_2 vapour) or GF_{254} Kieselgel G (u.v. light). M.p.s were determined on a Kofler block.

N-Benzoyl-N-methyl-L-alanine and N-Benzoyl-N-methyl-L-valine.—These were synthesised from the corresponding benzamido-L-acids [21] using the method of Cheung and Benoiton.[14] Samples used had the following physical data. Bz-L-MeAla: m.p. 120—122 °C (lit.,[22] 133 °C), $[\alpha]_D^{28}$ −62.5° (*c*, 1.3 in $CHCl_3$) (lit.,[22] −31.7° in DMF), $[\alpha]_D^{25}$ −52.8° (*c*, 1.26 in DMF); Bz-L-MeVal: m.p. 85—86 °C, $[\alpha]_D^{28}$ −99° (*c*, 1 in $CHCl_3$), m.p. 86—88 °C, $[\alpha]_D^{28}$ −120° (*c*, 0.03 in $CHCl_3$).

Optical Purity Check using Hydrolysis.—*N*-Benzoyl-methylamino-acid (1 mmol) in 6M-HCl (2.5 cm^3) was refluxed for 3 h, cooled, and extracted with ether to remove benzoic acid, and the aqueous layer was evaporated to yield the corresponding *N*-methylamino-acid hydrochloride which was recrystallised from methanol–ether. The identical methodology was repeated using samples [14] of Boc-L-MeAla and Boc-L-MeVal. Optical rotation results: $[\alpha]_D^{28}$ (*c*, 1 in H_2O): MeAlaHCl + 5.5° (from BzMeAla), +5.7° (from BocMeAla); MeValHCl + 13.3° (from BzMeVal); + 18.5° (from BocMeVal).

Within experimental error, Bz-L-MeAla can be considered optically pure, but the sample of BzMeVal, $[\alpha]_D^{28}$ −99° (in $CHCl_3$), contained 15% D-form.

General Coupling Method using NN′-Dicyclohexylcarbodiimide (DCCI).[23]—DCCI (0.5 mmol) in tetrahydrofuran (2 cm^3) was added to the *N*-benzoyl-*N*-methylamino-acid (0.5 mmol), the *C*-terminal methyl ester hydrochloride (0.5 mmol), and triethylamine (0.1 cm^3) in tetrahydrofuran (7 cm^3) at ambient temperature. The mixture was stirred at room temperature for 12 h, and after cooling and filtration of the precipitated urea, the filtrate was washed successively with 1M-HCl, 1M-$NaHCO_3$, and water before drying over magnesium sulphate followed by evaporation of the solvent to yield a gum (yields of purified material ranged from 60% to 70%). The total residue from each coupling was analysed in the n.m.r. spectrometer (100 MHz) as a deuteriochloroform solution, with ester-peak integrations averaged out for signals analysed on expanded scale.

When coupling reactions were carried out at −5 °C the solutions of the reactants were cooled to −10 °C before mixing.

Physical data on pure diastereoisomers of the reaction products have been reported later under conditions that give pure products: the n.m.r. spectra of the diastereoisomeric mixture were as follows. BzMeAla-L-AlaOMe, τ ($CDCl_3$) 8.57 (6 H, 2d, 2 × $CHCH_3$), 7.1 and 7.09 (3 H, 2s, NCH_3 as conformational isomers), 6.28 and 6.32 (3 H, 2s, OCH_3, L–L and D–L diastereoisomers), 5.47 (1 H, m, α-C*H*Ala),

4.93 (1 H, m, α-C*H*MeAla), 3.12 (1 H, br, N*H*), and 2.63 (5 H, s, phenyl); BzMeVal-L-ValOMe, τ ($CDCl_3$) 8.99 [12 H, m, 2 × CH(CH_3)$_2$], 7.6—7.9 [2 H, m, 2 × C*H*(CH_3)$_2$], 7.17 (3 H, s, NCH_3), 6.30 and 6.35 (3 H, 2s, OCH_3 L–L and D–L diastereoisomers), 5.54 (1 H, m, α-C*H*-Val), 5.32 (1 H, d, α-C*H*MeVal), 2.84 (1 H, br, N*H*), and 2.6 (5 H, s, Ar*H*).

Coupling using DCCI–HONSu [24] *and DCCI–HOBt.*[15]—*N*-Hydroxysuccinimide (57 mg, 0.5 mmol) in tetrahydrofuran (2 cm^3) [for HOBt: *N*-hydroxybenzotriazole (66 mg, 0.5 mmol) in tetrahydrofuran (2 cm^3)] was added to the *N*-benzoyl-*N*-methylamino-acid (0.5 mmol), *C*-terminal amino-acid methyl ester hydrochloride (0.5 mmol) in tetrahydrofuran containing triethylamine (0.1 cm^3). DCCI (110 mg, 0.5 mmol) was then added, and the reaction mixture stirred at room temperature for 12 h. Work-up for the reactions was as described for the DCCI coupling conditions above.

For the lower-temperature conditions all reactants were cooled to −10 °C before addition of the DCCI and the reaction mixture was kept at −5 °C for 12 h. All results have been summarised in Table 1. Using the low-temperature conditions it was possible to prepare optically pure samples of the MeAla-Ala dipeptide derivative but the presence of BzD-MeVal in the starting material, prevented the preparation of the pure Bz-L-MeVal-L-ValOMe.

N-*Benzoyl-*L*-methylalanine-*L*-alanine methyl ester* was obtained as crystals m.p. 106—108 °C (from ether–light petroleum) (Found: C, 61.9; H, 7.0; N, 9.8. $C_{15}H_{20}N_2O_4$ requires C, 61.8; H, 6.9; N, 9.9%); $[\alpha]_D^{28}$ −128.5° (*c*, 0.92 in $CHCl_3$), $\nu_{max.}$ 1 740—1 760 (ester CO) and 1 650—1 690 cm^{-1} (amide/imide CO), τ ($CDCl_3$) 8.58 (6 H, 2d, 2 × CH-C*H*$_3$), 7.09 (3 H, s, NC*H*$_3$), 6.28 (3 H, s, OC*H*$_3$), 5.48 (1 H, m, α-*H*Ala), 4.9 (1 H, br, α-*H*MeAla), 3.0 (1 H, br, NH), and 2.60 (5 H, s, phenyl).

Production and Characterisation of the Oxazolonium Ions, Type (1).—*N*-Benzoyl-*N*-methyl-L-amino-acid (38 mg, 0.18 mmol) in deuteriochloroform (0.2 cm^3) was cooled to −20 °C. DCCI (41 mg) in deuteriochloroform (0.2 cm^3) (cooled) was added and the mixture was immediately transferred to an n.m.r. sample tube and a spectrum was measured at −20 °C. Signals due to oxazolonium ion appeared immediately, and reached maximum intensity after 3 h. *Ca.* 20% of unchanged starting material was present in the BzMeAla sample after this time. Only small traces of starting material were found in the BzMeVal case. Characteristic signals in the n.m.r. (100 MHz) were: BzMeVal oxazolonium ion, τ ($CDCl_3$) 8.54 [6 H, d, CH(C*H*$_3$)$_2$] 6.29 (3 H, s, $=\overset{+}{N}-CH_3$) and 2.6 (5 H, broad m, phenyl), with some broad signals at τ 8—9 due to DCCI and starting material; BzMeAla oxazolonium ion, τ ($DCCl_3$) 7.87 (3 H, s, $=C-CH_3$), 6.23 (3 H, s, $=\overset{+}{N}-CH_3$) and 2.50 (5 H, broad m, phenyl), together with peaks (20% relative intensity) due to unchanged BzMeAla. To achieve direct comparison with a model coupling reaction oxazolonium ion formation was also monitored in [$^2H_8$]THF, and the same results were obtained as for the deuteriochloroform solutions. When samples were monitored at ambient temperatures, there was evidence of decomposition of the oxazolonium ions with appearance of new peaks in the n.m.r. spectra—these were not studied. No evidence of oxazolonium ions was obtained when Z-MeAla and BocMeAla were treated with DCCI.

Asymmetric Induction during the Coupling of Oxazolonium Ion to C*-Terminal Amino-acid Esters.*—A sample of oxazolonium ion from BzMeAla (40 mg) was prepared as above, and after 3 h at −20 °C, an equimolar quantity of L-alanine methyl ester hydrochloride and triethylamine in chloroform (2 cm^3) were added. After 12 h at ambient temperature, the reaction mixture was worked up as described for the general procedure to yield BzMeAla-L-AlaOMe (28 mg) as a gum which gave the same spectral and chromatographic characteristics as those for an authentic sample, except for the methyl ester region at τ ($CDCl_3$) 6.28 and 6.32 (L–L and D–L ratio 47 : 53).

The experiment was repeated except for the addition of L-valine methyl ester hydrochloride instead of its alanine analogue to give BzMeAla-L-ValOMe as an oil (27 mg) which showed identical characteristics to authentic material with two singlet signals at τ ($CDCl_3$) 6.27 and 6.32 (L–L and D–L ratio 2 : 3).

Authentic BzMeAla-L-ValOMe was synthesised from Bz-L-MeAla and L-ValOMe using DCCI in THF solution using the general procedure described before, to give N-*benzoylmethylalanyl-*L*-valine methyl ester* as a non-crystallisable yellow oil (50%) (Found: C, 63.4; H, 7.8; N, 9.15. $C_{17}H_{22}N_2O_4$ requires C, 63.7; H, 7.55; N, 8.75), $\nu_{max.}$ 1 760 (ester CO) and 1 640—1 660 cm^{-1} (strong amide CO), τ ($CDCl_3$) 9.15 [6 H, 2d (overlapping) CH(C*H*$_3$)$_2$], 8.55 (3 H, d, CHC*H*$_3$), 7.81 [1 H, m, C*H*(CH$_3$)$_2$], 7.10 [3 H, s (+ shoulder peak), NCH$_3$], 6.27, 6.33 (3 H, 2s, OCH$_3$ L–L and D–L forms), 5.48 [1 H, m, C*H*CH(CH$_3$)$_2$], 4.94 (1 H, m, C*H*CH$_3$), 2.9 (1 H, broad, NH), and 2.61 [5 H, s (+ shoulder peak), phenyl].

Analysis of Oxazolonium Ion Formation using U.v. Spectroscopy.—BzMeAla (10 mg) in THF (10 cm^3) was treated with DCCI (10 mg) in THF (10 cm^3) and the solution was placed immediately in the sample chamber of a u.v. spectrophotometer. The absorption band at $\lambda_{max.}$ 265 nm was gradually replaced by an absorption at $\lambda_{max.}$ 400 nm which has been assigned to the yellow colour of the oxazolonium ion. Formation of this band was monitored under the conditions summarised in Table 2. For experiments using additives, HOBt (7 mg) and HONSu (5.5 mg) were added before addition of DCCI in the appropriate cases.

N-*Benzoyl-*N*-methyl-*L*-alanine-*N*-hydroxysuccinimide Ester* (2).—*N*-Benzoyl-*N*-methyl-L-alanine (414 mg, 2 mmol), and *N*-hydroxysuccinimide (230 mg, 2 mmol) in tetrahydrofuran (8 cm^3) were cooled to −5 °C, and a cooled solution of DCCI (453 mg, 2.2 mmol) in tetrahydrofuran (5 cm^3) was added with thorough mixing. The mixture

TABLE 2

Oxazolonium ion formation, monitored as intensity of u.v. absorption band at $\lambda_{max.}$ 400 nm

Reagents	DCCI		DCCI + HOBt		DCCI + HONSu	
Conditions	(Ambient)	(−5 to 15 °C)	(Ambient)	(−5 to 15 °C)	(Ambient)	(−5 to −15°)
Sampling time						
5 min	0.03	0.00	0.00	0.00	0.00	0.00
25 min	0.13	0.01	0.12	0.00	0.09	0.00
1 h	0.43	0.08	0.20	0.00	0.18	0.00
3 h	$\lambda_{max.}$ shifts	0.34	0.28 (4 h)	0.00	0.23	0.00
5 h	to 370 nm	0.40	0.70 (24 h)	0.00	0.27	0.00

was stirred for 1 h at −5 °C, the precipitated dicyclohexylurea was filtered off, and the solution was evaporated in a rotary evaporator (<10 °C). The residue was washed several times with cold ethyl acetate to dissolve the active ester, and the washings were combined to give on evaporation a solid residue (414 mg, 68%) of *N-Benzoyl-*N*-methyl-*L*-alanine-*N*-hydroxysuccinimide ester* (2) m.p. 100—102 °C (from dichloromethane–ether) (Found: C, 59.15; H, 5.4; N, 9.3. $C_{15}H_{16}N_2O_5$ requires C, 59.2; H, 5.3; N, 9.2%), $[\alpha]_D^{28}$ +16.8 (*c*, 0.77 in $CHCl_3$), $\nu_{max.}$ (KBr and $CHCl_3$) 1 825 (ester CO), 1 795, 1 750 (imide CO), and 1 640 cm^{-1} (amide CO), τ ($CDCl_3$) 8.40 (3 H, d, $CHCH_3$), 7.16 [4 H, s, CH_2CH_2 (succinimido)] 6.97 (3 H, s, NCH_3), 4.34 [1 H, m, $CHCH_3$; a weak signal (broad) appeared at τ 4.98 which could suggest a small amount of *cis* α-H], and 2.56 (5 H, s, ArH). On addition of D_2O, the signal at τ 8.40 only became singlet (but retaining some doublet character) after 16 h. There was no immediate exchange of α-H (*cf.* HOBt analogue): 30% α-H signal was retained after 16 h.

*N-Benzoyl-*N*-methyl-*L*-alanine Hydroxybenzotriazole Ester* (3).—The same procedure was used as for the *N*-hydroxysuccinimide ester but using *N*-hydroxybenzotriazole (270 mg). The *ester* was obtained as crystals, m.p. 134—136° (545 mg, 80%) (from dichloromethane–ether) (Found: C, 62.7; H, 5.3; N, 17.8. $C_{17}H_{16}N_4O_3$ requires C, 62.95; H, 5.0; N, 17.3%), $[\alpha]_D^{28}$ 0° (*c*, 1.2 in $CHCl_3$), $\nu_{max.}$ (KBr) 1 820 (strong, ester CO), 1 740 (weak, CO–N⁺=C(N)–N), and 1 640 cm^{-1} (amide CO), $\nu_{max.}$ ($CHCl_3$) 1 820 (weak) and 1 740 (strong band), τ ($CDCl_3$) 8.22 (3 H, d, $CHCH_3$), 6.84 and 6.81 (3 H, 2s, NCH_3, *cis*- and *trans*-rotamers), 5.04 and 4.70 (1 H, 2m, *cis* α-*H* and *trans* α-*H*), 2.54 (5 H, s, ArH), and 2.08 (4 H, m, benzotriazole Ar*H*). On addition of D_2O, signals at τ 5.04 and 4.70 disappeared immediately and the signal at τ 8.22 was reduced to a singlet.

*N-Benzyloxycarbonyl-*N*-methyl-*L*-alanine Hydroxybenzotriazole Ester* (Z-L-MeAlaOBt).—*N*-Benzyloxycarbonyl-*N*-methyl-L-alanine [22] (474 mg, 2 mmol), *N*-hydroxybenzotriazole (270 mg, 2 mmol) in tetrahydrofuran (8 cm^3) at −5 °C, were allowed to react with DCCI (453 mg, 2.2 mmol) in cold tetrahydrofuran (5 cm^3) by the procedure for analogues (2) and (3), to give the product as a hygroscopic unstable gum which was decomposed on t.l.c. plates. All n.m.r. spectra showed the product to be contaminated with Z-MeAla (*ca.* 20%) but signals were clearly identifiable for the active ester, $\nu_{max.}$ ($CHCl_3$) 1 825 (ester CO), and 1 710 cm^{-1} (urethane); τ ($CDCl_3$) 8.35 (3 H, d, $CHCH_3$), 6.93 and 6.89 (3 H, 2s, NCH_3 *cis*- and *trans*-rotamers), 4.80 (2 H, s, $PhCH_2$), 4.5—4.80 (broad m; most probably α-*H*, partially under 4.80 peak), 2.70 (5 H, br s, Ar*H*), and 1.98—2.15 (2 H, m, 2 *ortho H* benzotriazole). Remaining 2*H* of benzotriazole ring overlapped by other Ar*H* protons.

On addition of D_2O, there was no evidence of exchange of α-H, nor was there any change in CH_3 signal multiplicity. On leaving for 3 days in contact with D_2O, the active ester had almost completely decomposed to Z-MeAla, but there was still no evidence of α-H exchange.

Preparation of Bz-L-MeAla-L-AlaOMe via *coupling using* Z-L-MeAla.—Z-L-MeAla [22] (1.2 g, 5 mmol), L-alanine methyl ester hydrochloride (0.7 g, 5 mmol), and an equimolar quantity of triethylamine were mixed in tetrahydrofuran (5 cm^3) at ambient temperature. DCCI (1.03 g, 5 mmol) in tetrahydrofuran (2 cm^3) was added and the mixture stirred at room temperature for 12 h. Using the general procedure already described, Z-L-MeAla-L-AlaOMe was obtained as a non-crystallisable *gum* (71%) (Found: C, 59.2; H, 6.9; N, 8.4. $C_{16}H_{22}N_2O_5$ requires C, 59.6; H, 6.9; N, 8.7%, $[\alpha]_D^{28}$ −54.5° (*c*, 1.66 in $CHCl_3$), $\nu_{max.}$ 1 770—1 740 (ester CO) and 1 680 cm^{-1} (amide/imide CO); τ ($CDCl_3$) 8.7 [6 H, dd, 2 × $CH(CH_3)$], 7.13 [3 H, s, NCH_3], 6.27 [3 H, s, OCH_3, L–L], 5.49 [1 H, m, $NHCHCH_3$], 4.94 [1 H, q, $NCHCH_3$], 4.83 [2 H, s, $PhCH_2$], 3.44 (1 H, br, NH], and 2.63 [5 H, s, ArH].

Z-L-MeAla-L-AlaOMe (711 mg, 3 mmol) was added to 36% HBr–acetic acid (3 g) under anhydrous conditions. The mixture was shaken at ambient temperature until evolution of CO_2 ceased (1 h). The solvent was removed under reduced pressure and the residue was washed several times with ether to yield L-MeAla-L-AlaOMe hydrobromide, as an oil, dried under vacuum over P_2O_5; $\nu_{max.}$ 1 770—1 740 (ester CO) and 1 680 cm^{-1} (CO amide); τ ($CDCl_3$), 8.55 [6 H, dd, $2CH(CH_3)$], 7.44 [3 H, d, $HNCH_3$], 6.36 [3 H, s, OCH_3], 6.10 [1 H, m, $NHCHCO$], 5.72 [1 H, q, $CH_2\overset{+}{N}HCH$], and 1.04 [2 H, br, $H\overset{+}{N}CH_3$].

L-MeAla-L-AlaOMe hydrobromide (2 mmol) and triethylamine (2 mmol) in dichloromethane (10 cm^3) were added to a pre-cooled solution of benzoyl chloride (2 mmol) and triethylamine (2 mmol) in dichloromethane (10 cm^3). After 2 h at room temperature the solution was filtered and the filtrate washed with 1M-HCl, 1M-$NaHCO_3$, and water. The dried solvent, after evaporation and purification on a silica gel column using [99 : 1 $CHCl_3$–MeOH] for elution, yielded Bz-L-MeAla-L-AlaOCH$_3$ (420 mg, 72%). Physical data on this compound were identical with authentic samples with only one ester methyl signal at τ 6.28 (L–L-diastereoisomer).

Coupling of Ester (2) *with* L-*Alanine Methyl Ester.*—The *N*-hydroxysuccinimide ester (2) (76 mg, 0.25 mmol) in tetrahydrofuran (2 cm^3) at −5 °C was mixed with L-alanine methyl ester hydrochloride (35 mg, 0.25 mmol) in tetrahydrofuran (2 cm^3) containing triethylamine (0.05 cm^3). The reaction mixture was stirred for 12 h and worked up as described for the general coupling method to give a gum (48 mg, 46%) with physical data identical to BzMeAla-L-AlaOMe synthesised directly using DCCI–HONSu; τ ($CDCl_3$) 6.28 (3 H, s, OCH_3, L–L diastereoisomer form only).

The same conditions were used to couple L-valine methyl ester hydrochloride instead of the alanine analogue above, when Bz-L-MeAla-L-ValOMe was obtained as a gum (131 mg, 65%), τ ($CDCl_3$) 6.29 (3 H, s, OCH_3 for L–L form only; other signals as per authentic compound).

Coupling of Ester (3) *with* L-*Alanine Methyl Ester.*—*N*-Hydroxybenzotriazole ester (3) (324 mg, 1 mmol) in tetrahydrofuran (2 cm^3) at −5 °C was mixed with L-alanine methyl ester hydrochloride as described above to yield BzMeAla-L-AlaOMe (73 mg, 25%) with the same physical data and isomer distribution as for product obtained by direct coupling (of BzMeAla and L-AlaOMe); τ ($CDCl_3$) 6.28 and 6.32 (3 H, 2s, OCH_3, L–L 76%, D–L 24%). The same conditions, when applied to coupling with *C*-terminal L-valine methyl ester hydrochloride gave BzMeAla-ValOMe (64 mg, 20%), τ ($CDCl_3$) 6.27 and 6.33 (3 H, 2s, OCH_3 L–L 20%, D–L 80%). A similar distribution of diastereoisomers was obtained by replacing (3) with Bz-DL-MeAlaOBt, synthesised from Bz-DL-Ala in 80% yield.

*Z-Gly-*L*-MeAla.*—An ethereal solution of diazomethane was added gradually to Z-L-MeAla (1 g) in ether (10 cm^3) until the solution remained yellow in colour. After a

further 2 h, the ether was removed to yield *Z*-L-*MeAlaOMe* as an oil (0.98 mg, 92%) (Found: C, 62.3; H, 7.0; N, 5.4. $C_{13}H_{17}O_4N$ requires C, 62.1; H, 6.8; N, 5.6%); $[\alpha]_D^{28}$ −24.7° (*c*, 2.2 in MeOH), $\nu_{max.}$ 1 760 (br) (urethane and ester CO); τ ($CDCl_3$) 8.6 (3 H, d, $CHCH_3$), 7.13 (3 H, s, NCH_3), 6.34 (3 H, s, OCH_3), 5.15 (1 H, q, $CHCH_3$), 4.90 (2 H, s, $ArCH_2$), and 2.70 (5 H, s, ArH).

Z-L-MeAlaOMe (0.753 g) was added to 36% HBr–AcOH (3 g) under anhydrous conditions. Work-up of the reaction mixture as previously described for a similar deprotection yielded L-MeAlaOMe hydrobromide as crystals, m.p. 108—109 °C (lit.,[22] 108—109.5 °C) (0.405 g, 80%); $[\alpha]_D^{28}$ −5.64° (*c*, 1.05 in DMF) (lit.,[22] −5.7°); τ ($[^2H_6]$DMSO) 8.52 (3 H, d, $CHCH_3$), 7.41 (3 H, s, NCH_3), 6.26 (3 H, s, OCH_3), 5.83 (1 H, q, $CHCH_3$), and 8.1 (3 H, br, $\overset{+}{N}H_3$).

The above L-MeAlaOMe hydrobromide (1.39 g) in tetrahydrofuran (5 cm^3) was added to Z-glycine (1.463 g), *N*-hydroxybenzotriazole (0.945 g), and triethylamine (1.1 cm^3) in tetrahydrofuran (10 cm^3). DCCI (1.586 g) was added and the reaction mixture was stirred at ambient temperature for 12 h to yield, after work-up, *Z-Gly-*L-*MeAlaOMe* (1.5 g, 73%) as an oil (Found: C, 58.4; H, 6.8; N, 9.2. $C_{15}H_{20}N_2O_5$ requires C, 58.4; H, 6.5; N, 9.1%; $[\alpha]_D^{28}$ −37° (*c*, 1.47 in $CHCl_3$); $\nu_{max.}$ 1 750 (ester and urethane CO) and 1 670 cm^{-1} (amide CO); τ ($CDCl_3$) 8.61 (3 H, d, $CHCH_3$), 7.13 (3 H, s, NCH_3), 6.32 (3 H, s, OCH_3), 5.98 (2 H, d, CH_2CO), 4.90 (2 H, s, $PhCH_2$) 4.82 (1 H, s, $CHCH_3$), 4.16 (1 H, br, NH) and 3.67 (5 H, s, ArH).

Z-Gly-L-MeAlaOMe (0.924 g) in methanol (18 cm^3) and 1M-NaOH (6 cm^3) were left to react at ambient temperature for 3 h. The solution was evaporated, and after acidification and extraction with ethyl acetate, the dried extracts yielded *Z-Gly-*L-*MeAla* as a non-crystallisable gum (615 mg, 70%) (Found: C, 57.5; H, 5.8; N, 9.2. $C_{14}H_{18}N_2O_5$ requires C, 57.1; H, 6.2; N, 9.5%); $[\alpha]_D^{28}$ −13.8° (*c*, 1.07 in $CHCl_3$), $\nu_{max.}$ 1 730 (urethane and ester, CO) and 1 650 cm^{-1} (amide CO); τ ($CDCl_3$) 8.62 (3 H, d, $CHCH_3$), 7.13 (3 H, s, NCH_3), 5.98 (2 H, d, $NHCH_2$), 4.92 (2 H, s, $PhCH_2$), 4.84 (1 H, q, $CHCH_2$), 3.98 (1 H, br, NH), 2.69 (5 H, s, ArH), and 0.49 (1 H, s, CO_2H).

*Preparation of Active Esters Z-Gly-*L-*MeAlaONSu and Z-Gly-*L-*MeAlaOBt*.—Both esters were prepared from the Z-Gly-L-MeAla above using the same procedures documented above for other *N*-methylalanine derivatives. *Z-Gly-*L-*MeAlaONSu* was obtained as crystals, m.p. 57—58 °C (from ether) (233 mg, 77%) (Found: C, 55.3; H, 5.4; N, 10.55. $C_{18}H_{21}N_3O_7$ requires C, 55.2; H, 5.4; N, 10.75%); $[\alpha]_D^{28}$ −12° (*c*, 1 in EtOH); $\nu_{max.}$ (KBr and $CHCl_3$) 3 350 (NH), 1 820 (ester CO), 1 790 (imide CO), 1 750—1 730 (urethane CO) and 1 670 cm^{-1} (amide CO); τ ($CDCl_3$), 8.38 (3 H, d, $CHCH_3$) 7.22 [4 H, s, $(CH_2CO)_2N$] 7.04 (3 H, s, NCH_2), 5.94 (2 H, d, $NHCH_2$), 4.90 (2 H, s, $PhCH_2$), 4.36 (1 H, q, $CHCH_3$), 4.20 (1 H, br, NH], and 2.68 [5 H, s, ArH]; upon addition of D_2O, no evidence for exchange of C–H was observed even after 24 h.

*Z-Gly-*L-*MeAlaOBt* was obtained as yellowish crystals, m.p. 46—48 °C (305 mg, 74%) (Found: C, 58.3; H, 6.8; N, 17.2. $C_{20}H_{21}N_5O_5$ requires C, 58.4; H, 5.15; N, 17.0%). $[\alpha]_D^{28}$ 0° (*c*, 1.1 in $CHCl_3$); $\nu_{max.}$ (KBr) 1 830 (ester CO), 1 750—1 730 (urethane CO), and 1 670 cm^{-1} (amide CO); the absorption at 1 830 cm^{-1} was much weaker in solution.

Great difficulty was found in obtaining a pure sample of this active ester and the n.m.r. spectra always showed the presence of Z-Gly-L-MeAla, which was readily formed from the ester, τ ($CDCl_3$) 8.36 (3 H, d, $CHCH_3$), 6.88 (3 H, s, NCH_3) 5.96 (2 H, d, CH_2CO), 4.86 (1 H, q, $CHCH_3$), 4.04 (1 H, br, NH), and 1.76—2.5 (9 H, m, 5 ArH and 4 aromatic protons of benzotriazole). On addition of D_2O, there was no immediate exchange of α-proton, but on leaving for 24 h in $CDCl_3$–D_2O the n.m.r. spectrum showed complete conversion of ester to the acid and the $CHCH_3$ doublet at τ 8.62 for the acid had been reduced to a singlet. In an independent experiment, it was proved that the α-proton in Z-Gly-MeAla itself did not exchange with D_2O. When D_2O–trifluoroacetic acid was added to a $CDCl_3$ solution of the ester, an immediate exchange of α-H occurred and conversion into the acid.

*Bz-*L-*AlaOBt*.—Bz-L-Ala [21] (396 mg, 2 mmol), HOBt (270 mg, 2 mmol), and DCCI (953 mg, 2.2 mmol) were allowed to react together under the conditions previously described for the *N*-methyl analogue. *Bz-*L-*AlaOBt* (413 mg, 67%) was obtained as crystals, m.p. 118—120 °C (from CH_2Cl_2–ether) (Found: C, 62.2; H, 4.9; N, 18.1. $C_{16}H_{14}N_4O_3$ requires C, 61.9; H, 4.55; N, 18.1%), $[\alpha]_D^{28}$ 0° (*c*, 1.04 in $CHCl_3$); $\nu_{max.}$ (KBr) 1 820 (weak, ester CO) and 1 750 (strong) ($-CO-\overset{+}{N}$) cm^{-1}; $\nu_{max.}$ ($CHCl_3$) 1 820 (strong, ester CO) and 1 750 (sharp, $-CO-\overset{+}{N}$) cm^{-1}; τ ($[^2H_6]$DMSO) 8.61 [3 H, d, $CHCH_3$ starting material], 8.22 [3 H, d, $CHCH_3$ active ester], 5.44 [1 H, t, CH starting material], 4.96 [1 H, t, CH active ester], 2.84—2.26 [m, (4 H) HOBt + (5 H) ArH], 1.60—2.16 [m, (4 H) HOBt + (5 H)ArH active ester], 1.30 (1 H, d, NH starting material), and 0.54 (1 H, d, active ester NH).

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