Studies on the Diastereoisomeric and Conformational Aspects of Benzoyl Dipeptide Esters, as a Means of assessing Racemisation using Nuclear Magnetic Resonance Spectroscopy [1]

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Distinct methyl ester signals in the ^{1}H n.m.r. spectra of diastereoisomeric forms of benzoyl dipeptide methyl esters provide a means of estimating the isomer composition of diastereoisomeric mixtures. Analysis of the mixture derived from peptide-coupling reactions using this n.m.r. technique provides a convenient means of comparing the racemisation potential of a series of coupling reagents. Significant asymmetric induction accompanies racemisation during the model peptide couplings. Conformational effects have been studied using ^{13}C n.m.r. spectroscopy and the structural criteria for observing diastereoisomeric ester signals in the ^{1}H n.m.r. spectra have been rationalised.

METHODS for testing the degree of racemisation in peptide coupling depend on an efficient means of analysing diastereoisomeric or racemic mixtures. Polarimetry, through the approaches of Anderson [2] and Young [3] has proved a valuable technique in routine analysis, with a sensitivity limit of 1—2% racemisation. A modification of these tests by Kemp [4] and his co-workers utilising ^{14}C-carboxy-components has greatly increased the sensitivity to 0.001—0.01% racemisation. Ion-exchange chromatography using an amino-acid analyser has proved successful in the Izumiya method,[5] and more recently by Benoiton [6] in studies on *N*-methylamino-acids. Sensitivity limits of 1 part in 10 000 have been attained in the ion-exchange separation of diastereoisomeric Leu-Ala-Gly-Val by Merrifield and his co-workers.[7]

Separation of diastereoisomers using g.l.c. has been extensively studied [8] since the pioneering work of Weygand [9] on *N*-trifluoroacetyl peptide methyl esters. Optically active stationary phases in g.l.c. also discriminate between enantiomers,[10] and recently h.p.l.c. of diastereoisomeric benzoyl-phenylalanyl-alanyl esters has proved to be an efficient means [11] of assessing racemisation. Scintillation counting linked to a t.l.c. separation of tritium-labelled compounds has been used,[12] while hydrolysis of some peptides with tritiated hydrochloric acid, followed by measurement of the amount of incorporated tritium, has also been developed as a racemisation test.[13] A generally applicable method utilises the stereoselective enzymic hydrolysis of diastereoisomeric peptides by leucine aminopeptidase.[14]

Many examples [15] are known of magnetic non-equivalence of protons in diastereoisomeric dipeptides but only when Weinstein and Pritchard [16] used blocked alanylphenylalanyl dipeptides did a workable racemisation test emerge based on the n.m.r. technique. However the integration of overlapping alanyl methyl doublets in this method is not straightforward. The observation that methyl *N*-benzoyl-L-valyl-L-valinate and methyl *N*-benzoyl-D-valyl-L-valinate showed ester methyl signals at τ 6.29 and 6.36 respectively led to the development of the model reaction outlined in the Scheme as a convenient test [1] for racemisation. It combines the high susceptibility of *N*-benzoylamino-acids (Young test [17]) towards racemisation with the reduced sensitivity of the integration of signals in n.m.r. spectroscopy. Routinely, integration of ^{1}H n.m.r. signals gives a sensitivity limit of about 5% but an accuracy of 1—2% can be achieved by integration of the diastereoisomeric ester methyl signals on an expanded scale and taking the average of at least three integrals. The lower detection limit is also 1—2%, which is a similar figure to that quoted by Benoiton and his co-workers [6] using a 60 MHz spectrometer. In its sensitivity the n.m.r. method compares favourably with the Young [3] and Anderson [2] racemisation tests.

The ester signals are solvent dependent, so that all contaminating solvents need to be removed before adding deuteriochloroform. The location of the ester

$$\text{Bz-L-Val} + \text{L-Val-OMe} \xrightarrow[\text{iii, iv}]{\text{i, ii}} \text{Bz-(?)-Val-L-Val-OMe}$$

SCHEME i, Coupling with suitable reagents; ii, removal of excess of reagents and other by-products by extraction with 1M-HCl and saturated $NaHCO_3$; iii, drying and removal of solvent, followed by dissolving the sample in $CDCl_3$; iv, integration of diastereoisomeric ester methyl peaks

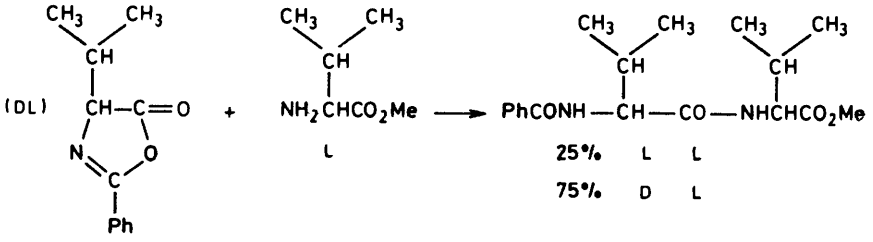
signals is advantageous, in that they are far removed from most contaminating by-products apart possibly from acylated esters formed as by-products during mixed anhydride coupling.

The nature of the aliphatic side-chains does not affect the separation of the ester peaks since diastereoisomeric mixtures of Bz-Ala-L-Val-OMe, Bz-Ala-L-Ala-OMe, and Bz-Val-L-Ala-OMe all individually show separation of approximately 0.07 p.p.m. between the ester methyl signals of their diastereoisomers. During the formation of the alanyl dipeptides the extent of racemisation was noticeably less than in the divalyl peptide formed under the same conditions.

Since the original development [1] of this technique it has been possible (*a*) to assess the degree of asymmetric induction during the peptide coupling process, and (*b*) to define the structural criteria necessary to give rise to the diastereoisomeric ester signals in ^{1}H n.m.r. spectra. Unambiguous assignment of the ester methyl signals in the diastereoisomers has also been checked by an independent synthesis of Bz-L-Val-L-Val-OMe and Bz-D-

Val-L-Val-OMe *via* racemisation-free coupling techniques using the benzyloxycarbonyl group for *N*-protection.

When an intermediate racemic oxazolone formed during the racemisation process [18] reacts with a chiral reagent such as methyl L-valinate, asymmetric preference for one diastereoisomer has been reported.[19] To check this in the present system the coupling between the DL-oxazolone (1) and L-valine methyl ester was studied and then repeated with the oxazolone derived from Bz-DL-alanine which was reacted with L-alanine methyl ester.



(1)

In both systems each diastereoisomeric product was quantitatively assessed using the integration of the methyl ester signals in ^{1}H n.m.r. spectra. For the coupling of the valyl residues there was a 3 : 1 preference for the formation of the D–L diasteroisomer, while in the alanine series there was only a 3 : 2 preference. This observation that the valyl residues give higher racemisation figures is in line with the observations of Benoiton and his co-workers [20] who report that each amino-acid might have its own racemisation characteristics.

The previously published figures [1] for the % D–L diastereoisomer formed using different coupling reagents are reproduced in Table 1. But if a true assessment of racemisation is required which is independent of the nature of the *C*-terminal component, then a correction factor for the asymmetric induction should be applied. For a 3 : 1 preference for D–L form, as in the valyl-valine case, the % D–L figure should be reduced by a third. When this corrected figure is doubled it gives the figures quoted under ' % racemisation ' in Table 1.

For a complete assessment of the n.m.r. technique the structural requirements for separate ester signals in the ^{1}H n.m.r. spectra of L–L and D–L diastereoisomers have been investigated. Mixtures of the *N*-benzyloxycarbonyl-protected (*Z*) dipeptides [21] Z-L-Val-L-Val-OMe and Z-D-Val-L-Val-OMe do not give diastereoisomeric ester signals, thus suggesting that the position of the benzene ring is important. However this difference between the benzyloxycarbonyl group and the benzoyl group could also be interpreted in terms of the reduced nucleophilicity of the urethane carbonyl in the former which would reduce the tendency for H-bonding as suggested in the 7-membered-ring model conformation of dipeptides.[22] But other evidence indicates that the aromatic π-system of the benzoyl group contributes to the shielding effect, since it has been found [23] that if Bz-didehydro-Val-L-Val-OMe is hydrogenated over Adams catalyst (PtO_2), diastereoisomeric cyclohexyl dipeptide derivatives are formed with a reduced separation (τ 6.32 and 6.34) between the diastereoisomeric ester signals, in the ^{1}H n.m.r. spectra. It has also been observed [23] that *N*-trifluoroacetyl-DL-Val-L-Val-OMe shows a similar separation of ester signals (τ 6.30 and 6.32) owing to the presence of diastereoisomers. It is reasonable to deduce that the magnetic shielding effect of the benzoyl aromatic ring contributes *ca.* 0.05 p.p.m. extra separation between ester peaks.

To test whether a secondary amide bond capable of stabilising a 7-membered H-bonded ring structure [22] was a basic requirement for ester signal separation, the n.m.r. characteristics of benzoyl dipeptide esters containing *N*-methyl-L-valine and L-proline as *C*-terminal residues have been studied. *N*-Alkylation of an amide bond lowers the energy barrier to rotation about the C–N bond, so that within the time scale of an n.m.r. determination, signals can originate from peptides depending on whether the tertiary amide is in the *cis*- or *trans*-rotational form.[24]

TABLE 1

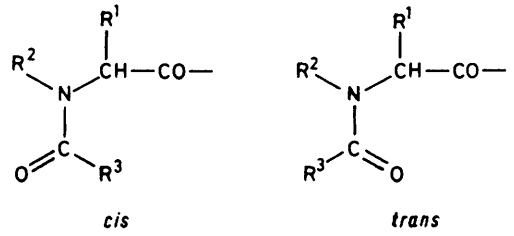
Degree of racemisation during peptide bond formation using the model reaction Bz-L-Val + L-Val-OMe

Coupling agent	% D–L in product	% Racemisation (corrected for asymmetric induction)
Azide [a]	0	0
N'N'-dicyclohexylcarbodi-imide [b] (DCCI)	48	64
DCCI + *N*-hydroxysuccinimide (HONSu) [c]	10	14
DCCI + 1-hydroxybenzotriazole [d]	13	18
Mixed anhydride (Et_3N as base) [e]	46	62
Mixed anhydride (*N*-methylmorpholine base)	21	28
Ethyl 2-ethoxy-1,2-dihydroquinoline-1-carboxylate (EEDQ) [f]	22	30
N-Ethyl-5-(3-sulphonatophenyl)-isoxazolium salt [g] (Woodward's Reagent)	33	44

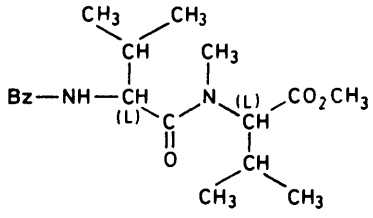
For reaction conditions see: [a] M. W. Williams and G. T. Young, *J. Chem. Soc.*, 1963, 881; [b] J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, 1955, **77**, 1067; [c] F. Weygand, D. Hofmann, and E. Wünsch, *Z. Naturforsch., Teil B*, 1966, **21**, 426; [d] W. König and R. Geiger, *Chem. Ber.*, 1970, **103**, 789; [e] J. R. Vaughan, *J. Am. Chem. Soc.*, 1951, **73**, 3547; [f] B. Belleau and G. Malek, *ibid.*, 1968, **90**, 1651; [g] R. B. Woodward, R. A. Olofson, and H. Mayer, *ibid.*, 1961, **83**, 1010.

Note: All couplings involving DCCI were carried out in dichloromethane.

Therefore for the model peptides, even if the *N*-terminal residue is in the L-form, conformers (2) and (3) would be possible for the *N*-methylvaline case and (4) and (5) for



the proline analogue, and might give rise to ester methyl signals in the ^1H n.m.r. spectrum owing to conformational effects.



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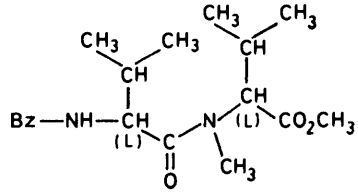

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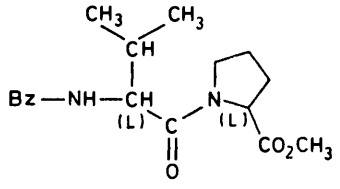

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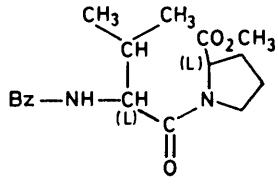

(5)

Bz-Val-L-Pro-OMe diastereoisomers were therefore chosen for the study since the β- and γ-carbon atoms of proline show a characteristic *cis–trans* pattern [25] in their ^{13}C spectra which can be used to monitor the extent of rotational isomerism about the central amide bond. Bz-L-Val-L-Pro-OMe (4/5) was synthesised using both the azide coupling method [26] and the mixed anhydride method from Bz-L-Val and L-proline methyl ester. The product from the mixed anhydride method yielded a crystalline compound which on ^1H n.m.r. analysis gave only one main ester peak at τ 6.37 and a much smaller peak at τ 6.26. Integration of the latter peak was shown to be equivalent to the amount of *cis*-rotamer indicated from the β- and γ-carbons of proline in the ^{13}C spectrum. A similar picture emerged when the same crystalline compound was obtained through coupling Bz-DL-Val with the proline ester. However when the total product of the reaction (crystals + mother-liquor) was analysed, another main ester signal appeared at τ 6.32. A diagrammatical summary of the relevant part of the spectra appears in Figure 1. This can be interpreted as being due to one diastereoisomer (the D–L form) being separated off during the fractional crystallisation.

In order to confirm this interpretation Bz-L-Val-L-Pro-OMe was synthesised by benzoylation of commercially available H-L-Val-L-Pro-OH followed by esterification with diazomethane. This sample (which is a non-crystallisable gum) gave ester signals at τ 6.25 (small intensity) and τ 6.32 which confirms that the crystalline material obtained from the coupling reactions was in fact Bz-D-Val-L-Pro-OMe. It follows that the signal at τ 6.25 arises from conformational isomerism about the acyl prolyl bond. In the diastereoisomeric mixture Bz-DL-Val-L-Pro-OMe, the minor peak at τ 6.25 (6.26) assigned to the *cis*-form always appeared as a singlet, which suggests that magnetic-shielding effects on the ester methyl protons are not very significant in the *cis*-rotamer. Taking the results from Bz-Val-L-Pro-OMe in isolation, a secondary amide bond joining the two residues is not a necessary requirement for showing the differential shielding effect on the ester methyls. But since proline

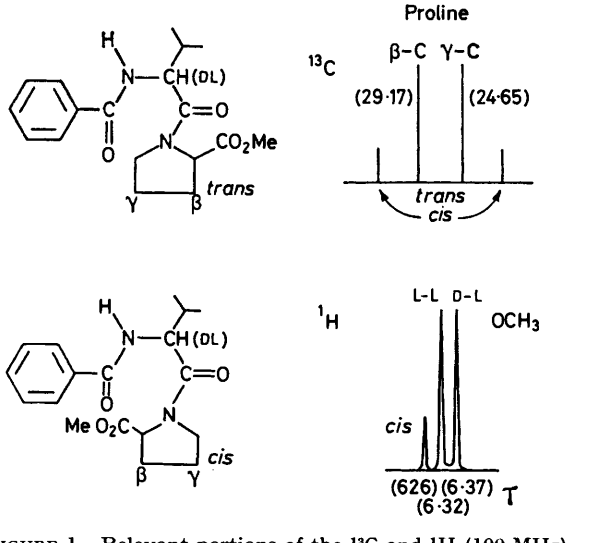

FIGURE 1 Relevant portions of the ^{13}C and ^1H (100 MHz) n.m.r. spectra of Bz-DL-Val-L-Pro-OMe

is often atypical in its properties, a study of a peptide containing an acyclic imino-acid was desirable.

The acyclic *N*-methyl analogue initially chosen for study was Bz-L-Val-L-MeVal-OMe (2/3), but using the same azide coupling conditions as above for Bz-L-Val and L-*N*-methylvaline methyl ester, the main product was the urea Bz-NHCH(CHMe$_2$)NHCON(Me)CH(CHMe$_2$)CO$_2$Me. This implies that the Bz-L-Val azide had rearranged to the isocyanate before attack by the sterically hindered amine, HN-MeVal-OMe. To overcome this problem, less 'hindered' amino-acids were coupled. Mixed anhydride coupling of Bz-L-Ala with L-*N*MeAla-OMe using pivaloyl chloride gave Bz-L-Ala-L-MeAla-OMe (methyl ester signal at τ 6.32 in ^{1}H n.m.r. spectrum). When mixed with authentic Bz-D-Ala-L-MeAla-OMe there was only one ester signal at τ 6.32 in the ^{1}H n.m.r. spectrum. Minor signals, very near to the main signals, could be due to different conformational forms, which seem to be indicated also in the ^{13}C n.m.r. spectrum. This result therefore suggests that in acyclic dipeptides there is a need for a secondary-amide bond to give rise to the diastereoisomeric ester effect.

To complete the study on this series of dipeptides, investigations have also been carried out on the effect of *N*-alkylation of the *N*-terminal amide on the diastereoisomeric ester signals. Concentrating on the ^{1}H n.m.r. spectra of Bz-*N*MeVal-L-Val-OMe and Bz-Pro-L-Val-OMe it has been shown that distinct ester peaks can be identified for both diastereoisomeric pairs, *i.e.* Bz-L-MeVal-L-Val-OMe (τ 6.30) and Bz-D-MeVal-L-Val-OMe (τ 6.36) for the former and Bz-L-Pro-L-Val-OMe (τ 6.28)

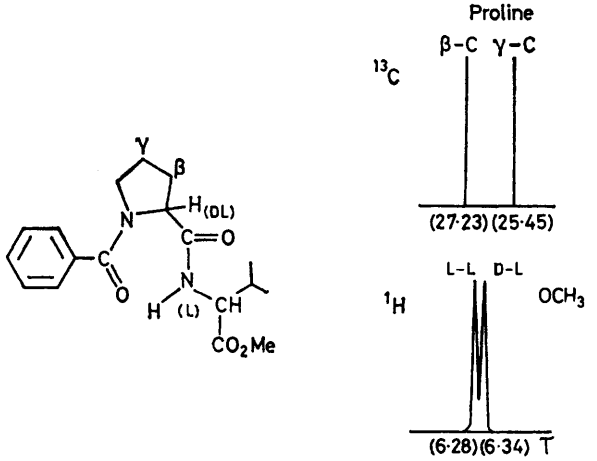

FIGURE 2 Relevant portions of the ^{13}C and ^{1}H (100 MHz) n.m.r. spectra of Bz-DL-Pro-L-Val-OMe

and Bz-D-Pro-L-Val-OMe (τ 6.34) for the latter. The separation of the peaks is less than in the secondary-amide analogues, but the separation is still sufficient for the method to be used for an assessment of the racemisation characteristics of *N*-alkylated amino-acids.[27] Analysis of the ^{13}C n.m.r. spectra confirms that the benzoyl-Pro bond in Bz-Pro-L-Val-OMe is completely in the *trans*-form, since only 2 singlets at δ 27.23 and 25.45 are seen for the β- and γ-carbons of proline. Thus the results can be summarised as in Figure 2. Moreover it it has been found that no racemisation is detectable when benzoyl-L-proline is coupled using the mixed anhydride method.

In summary, our studies show that the siting of the aromatic ring of the benzoyl group in the *N*-terminal position certainly increases the differential shielding effect on the ester group, and is therefore advantageous to the application of n.m.r. in the analysis of diastereoisomeric mixtures. Conformational mobility due to *N*-alkylation of the *N*-terminal amide bond does not inhibit the separation of diastereoisomeric ester signals, but a more complicated pattern emerges if the central dipeptide bond is *N*-alkylated. In dipeptides from acyclic amino-acids the presence of a 7-membered H-bonded model[1] can be implicated from the results. However the more rigid geometry of a prolyl residue also gives a molecular confirmation conducive to separation of diastereoisomeric ester signals.

EXPERIMENTAL

^{1}H N.m.r. spectra were determined at 100 MHz on a Varian HA-100 instrument. ^{13}C N.m.r. spectra were obtained on a Varian XL-100 instrument at 25.20 MHz. Tetramethylsilane was used as internal standard for all spectra. Optical rotations were measured on a Perkin-Elmer 141 automatic polarimeter. M.p.s were determined on a Kofler block. For t.l.c. Kieselgel G plates were developed in chloroform or chloroform–methanol (19 : 1 v/v) and spots were located using iodine vapour.

Synthesis of Optically Pure Bz-L-Val-L-Val-OMe and Bz-D-Val-L-Val-OMe.—Both compounds have been previously reported,[28] but were synthesised without the benefit of the more recent advances in peptide synthetic strategy. The following summarises our synthetic approach.

N-Benzyloxycarbonyl-D-valyl-L-valine methyl ester. A solution of Z-D-valine[29] (25.1 g, 0.1 mol) in tetrahydrofuran (300 ml) was cooled to −20 °C. Triethylamine (10.1 g, 0.1 mol) and isobutyl chloroformate (13.7 g, 0.1 mol) were added and the mixture was stirred at −20 °C for 1 h. A suspension of L-valine methyl ester hydrochloride (16.7 g, 0.1 mol) and triethylamine (10.1 g, 0.1 mol) in tetrahydrofuran (170 ml) was added and the mixture allowed to reach room temperature and then stirred for 20 h. The solvent was removed *in vacuo* and the solid residue dissolved in ethyl acetate. After washing with dilute HCl and sodium hydrogencarbonate solution, the solvent was evaporated to yield *N*-benzyloxycarbonyl-D-valyl-L-valine methyl ester, (26.2, 72%), m.p. 161—162 °C (lit.,[28] 163—163.5 °C), from ethyl acetate–light petroleum, $[\alpha]_D^{20}$ +11.6° (*c*, 1.0 in $CHCl_3$) [lit.,[28] +12.0° (*c*, 1.0 in $CHCl_3$)]; $\nu_{max.}$ 1 738 (ester CO), 1 693 (urethane CO), and 1 650 cm^{-1} (amide CO); τ ($CDCl_3$) 9.1 (12 H, m, CH_3CHCH_3), 7.9 (2 H, m, CH_3CHCH_3), 6.25 (3 H, s, OCH_3), 5.93 (H, q, NHCHCO), 5.50 (H, q, NHCHCO), 4.94 (2 H, s, $PhCH_2$), *ca.* 4.6 (H, d, CONH), *ca.* 3.4 (H, d, CONH), and 2.78 (5 H, s, phenyl).

N-Benzyloxycarbonyl-L-valyl-L-valine methyl ester. Prepared in the same manner as for the D–L isomer above, the L–L ester had m.p. 100—104 °C (lit.,[28] 100—103 °C) from ethyl acetate–light petroleum, $[\alpha]_D^{20}$ −28.2° (*c*, 1.2 in

EtOH) [lit.,[28] −21° (*c*, 1.0 in EtOH)]; i.r. and n.m.r. spectra as for the D–L isomer.

D-Valyl-L-valine methyl ester hydrobromide. 45% Hydrogen bromide in acetic acid (100 g) was added to *N*-benzyloxycarbonyl-D-valyl-L-valyl methyl ester (36.4 g, 0.1 mol) under a calcium chloride guard-tube. The mixture was stirred at room temperature until the evolution of CO_2 ceased (1 h). The peptide hydrobromide was precipitated in dry ether (1.5 l), collected and on recrystallisation from methanol–ether gave *D-valyl-L-valine methyl ester hydrobromide* (29.2 g, 94%), m.p. 207—210 °C, $[\alpha]_D^{20}$ −45.3 (*c*, 1 in MeOH) (Found: C, 42.3; H, 7.45; N, 9.25. $C_{11}H_{23}N_2O_3Br$ requires C, 42.45; H, 7.45; N, 9.0%) $\nu_{max.}$ 1 732 (ester CO) and 1 678 cm^{-1} (amide CO); τ ([$^2H_6$]DMSO) *ca.* 9.0 (12 H, m, CH_3CHCH_3), 7.9 (2 H, m, CH_3CHCH_3), 6.37 (3 H, s, OCH_3), 6.16 (H, m, $\overset{+}{N}H_3CHCO$), 5.76 (H, q, NHCHCO), *ca.* 1.8br (3 H, s, NH_3^+), and 1.21 (H, d, CONHCH).

L-Valyl-L-valine methyl ester hydrobromide. The above procedure was repeated for the L–L analogue to yield *L-valyl-L-valine methyl ester hydrobromide* (5.6 g, 90%), m.p. 156—159 °C, $[\alpha]_D^{20}$ +4.4° (*c*, 1.2 in MeOH) (Found: C, 42.2; H, 7.5; N, 8.8%); i.r. and n.m.r. spectra as for D–L isomer.

N-Benzoyl-D-valyl-L-valine methyl ester. Benzoyl chloride (2.8 g, 0.02 mol) and triethylamine (2.0 g, 0.02 mol) in dichloromethane (40 ml) was cooled to 0 °C. A cold suspension of D-valyl-L-valine methyl ester hydrobromide (6.2 g, 0.2 mol) and triethylamine (2.09 g, 0.02 mol) in dichloromethane (60 ml) was added and the mixture was stirred overnight at room temperature. Work-up of the solution by washing with dilute HCl and sodium hydrogencarbonate solution, and removal of dried solvent *in vacuo* yielded *N*-benzoyl-D-valyl-L-valine methyl ester (3.3 g, 50%), m.p. 185—187 °C (from methanol) (lit.,[28] 187—187.5 °C), $[\alpha]_D^{20}$ +1.3° (*c*, 1 in $CHCl_3$) (lit.,[28] +2.0° (*c*, 1 in $CHCl_3$)], $\nu_{max.}$ 1 760 (ester CO), and 1 640 cm^{-1} (amide CO); τ ($CDCl_3$) 9.0 (12 H, m, CH_3CHCH_3), 7.8 (2 H, m, CH_3CHCH_3), 6.26 (3 H, s, OCH_3), 5.51 (H, q, NHCHCO), 5.28 (H, q, NHCHCO), 2.90 (2 H, d, CONHCH), and 2.70 and 2.22 (5 H, m, phenyl protons).

N-Benzoyl-L-valyl-L-valine methyl ester. The same procedure as for the D–L isomer above gave *N*-benzoyl-L-valyl-L-valine methyl ester (3.5 g, 52%), m.p. 173—174 °C (from methanol) (lit.,[28] 171—172 °C), $[\alpha]_D^{25}$ −9.2 (*c*, 1 in acetone) [lit.,[28] −11.4 (*c*, 0.9 in acetone)].

Coupling of Bz-L-Val to L-Val-OMe.—A series of coupling conditions as outlined in Table 1 were investigated. Each coupling was repeated and an average of the ester integrals (each sample integrated three times) quoted in assessment of racemisation. In a typical procedure benzoyl-L-valine (0.001 mol) and L-valine methyl ester hydrochloride (0.001 mol) were coupled using the appropriate coupling reagent in an inert solvent, and using triethylamine or *N*-methylmorpholine (0.001 mol) to release the amine from the ester hydrochloride. All couplings except the azide (0 °C) and mixed anhydrides (0 °C) methods were carried out at room temperature. On completion of reaction, the reaction mixture was filtered and the filtrate was washed with dilute hydrochloric acid and sodium hydrogencarbonate solution to remove the excess of reagent and starting materials. The solution was dried over magnesium sulphate, filtered, and the solvent removed *in vacuo*. The residue was dissolved in deuteriochloroform and analysed in a 100 MHz n.m.r. spectrometer. Authenticity of product was also checked against standards on t.l.c. plates.

TABLE 2

$CDCl_3$ (0.4 ml) + *x* drops [$^2H_6$]-DMSO	*x* = 0	2	6	10
Separation of ester methyl signals (D–L — L–L) (p.p.m.)	0.08	0.05	0.02	0

TABLE 3

^{13}C N.m.r. spectral characteristics of Bz-Val-L-Pro-OMe in $CDCl_3$ [a]

^{13}C Shifts (p.p.m.) (proton-decoupled spectra)

CO groups	Aromatic	Ester CH_3	α-C	Proline β-C γ-C δ-C	Valine side-chain
Bz-L-Val-L-Pro-OMe					
(*trans*)					
172.36, 171.08, 167.34	134.13, 131.47, 128.37, 127.24	52.02	58.99 (Pro) 56.22 (Val)	29.08, 25.05, 47.38	31.42, 19.23, 18.13
(*cis*)				(31.16), 22.25, 46,38	
cis : *trans* 5 : 95					
Bz-Val-L-Pro-OMe					
(*trans*)					
172.24, 170.64, 167.16	134.28, 131.56, 128.52, 127.14	52.11	58.99 (Pro) 55.93 (Val)	29.17, 24.65, 47.29	31.62, 19.70, 17.75
(*cis*)				30.93, 22.32, 46.48	
cis : *trans* 17 : 83					

[a] Peaks assigned by off-resonance coupling and by comparison with analogues (ref. 24).

Separation of ester peaks in diastereoisomers was found to vary with solvent polarity as shown in Table 2.

Coupling of Bz-Ala with L-Ala-OMe.—Characterisation of diastereoisomeric ester peaks in this case was achieved by independently coupling Bz-L-Ala and Bz-D-Ala (both commercially available) to L-Ala-OMe and taking the major ester peaks in each product to represent the ester signals in Bz-L-Ala-L-Ala-OMe and Bz-D-Ala-L-Ala-OMe respectively. Since each product had a little of its diastereoisomer present, m.p.s were no criteria of purity, so t.l.c. and n.m.r. spectra were routinely used for characterisation and analysis. In a typical experiment, Bz-L-Ala (0.089 g) in acetonitrile containing *N*-methylmorpholine (0.05 g) was added to a suspension of 3-(2-ethylisoxazol-5-ylio)phenylsulphonate (Woodward's Reagent K) (0.125 g) in acetonitrile. The solution was stirred at room temperature until the suspension dis-

solved (*ca.* 1 h), when L-alanine methyl ester hydrochloride (0.07 g) and *N*-methylmorpholine (0.05 g) were added and the mixture stirred overnight. On removal of solvent, the residue was dissolved in ethyl acetate, extracted with dilute acid and sodium hydrogencarbonate solution and worked up in the usual way to yield *crystals*, m.p. 125—132 °C (from ethyl acetate–light petroleum) (Found: C, 60.45; H, 6.6; N, 10.15. $C_{14}H_{18}N_2O_4$ requires C, 60.43; H, 6.52; N, 10.07%) $\nu_{max.}$ 1 749 (ester CO) and 1 635 cm^{-1} (amide CO); τ ($CDCl_3$) 8.55 (6 H, m, CH_3CH), 6.33 (s, CH_3O of L–L diastereoisomer), 6.40 (s, CH_3O of D–L diastereoisomer), 5.48 (H, t, NHC*H*CO), 5.06 (H, t, NHC*H*CO), 2.7 (2 H, m, NH), and 2.6 and 2.2 (5 H, m, phenyl); M^+ 278. Integration of the ester peaks of total product before recrystallisation gave 15% Bz-D-Ala-L-Ala-OMe and 85% Bz-L-Ala-L-Ala-OMe in the product (*cf.* 33% D–L present in the divalyl analogue when prepared under the same conditions). When EEDQ was used for coupling Bz-L-Ala to L-Ala-OMe only 5% D–L form could be detected by n.m.r. spectroscopy (*cf.* 22% for the divalyl analogue).

Reaction of DL-2-*Phenyl*-4-*isopropyloxazol*-5-*one with Valine Methyl Ester*.[19]—DL-2-Phenyl-4-isopropyloxazol-5-one was prepared as follows. *N*-Benzoyl-DL-valine (3 g) in dioxan–acetic anhydride (50 ml; 2 : 1 v/v) was stirred overnight at room temperature. The solvent was removed *in vacuo* and the resultant oil shaken with light petroleum for 2 h at 0 °C, yielding the *oxazolone* (2.5 g, 90%), m.p. 47—50 °C (Found: C, 70.7; H, 6.7; N, 6.7. $C_{12}H_{13}NO_2$ requires C, 70.92; H, 6.45; N, 6.90%), τ ($CDCl_3$) 8.90 (6 H, 2d, CH_3CHCH_3), 7.7 (H, m, CH_3CHCH_3), 5.75 (H, d, NHC*H*CO), and 2.60 and 2.05 (5 H, m, phenyl).

The above oxazolone (0.203 g) and L-valine methyl ester hydrochloride (0.167 g) with triethylamine (0.1 g) in tetrahydrofuran (20 ml) were stirred overnight at room temperature. After removal of solvent, the residue was dissolved in ethyl acetate, washed with dilute acid and sodium hydrogencarbonate solution and worked up in the usual way to yield a residue which was dissolved in $CDCl_3$ for n.m.r. analysis. Integration of the spectrum (peaks at τ 6.29 and 6.36) which was identical with that of Bz-Val-L-Val-OMe showed that the Bz-D-Val-L-Val-OMe : Bz-L-Val-L-Val-OMe ratio in the product was 3 : 1 (average of two experiments and 10 integrations).

Reaction of 2-*Phenyl*-4-*methyloxazol*-5-*one with Alanine Methyl Ester*.—DL-2-Phenyl-4-methyloxazol-5-one [30] (0.167 g), L-alanine methyl ester hydrochloride (0.168 g) in tetrahydrofuran (40 ml), and triethylamine (0.2 ml) were allowed to react together as described for the valine analogue above. The product gave the characteristic n.m.r. spectrum for Bz-Ala-L-Ala-OMe, and on integration of ester peaks at τ 6.40 and 6.33 the ratio of diastereoisomers was found to be 3 : 2 in favour of the D–L form (average of two experiments, 10 integrations).

Coupling of the DL-alanine oxazolone with valine methyl ester under the above conditions gave a diastereoisomeric mixture of Bz-D-Ala-L-Val-OMe and Bz-L-Ala-L-Val-OMe in the ratio 73 : 27.

N-*Benzoylvalyl*-L-*proline Methyl Ester* (4/5).—*Method A. Mixed anhydride coupling*. *N*-Benzoyl-L-valine (0.7 g) and triethylamine (0.45 ml) in tetrahydrofuran (1.5 ml) were stirred at −10 °C. Isobutyl chloroformate (0.4 ml) was added and after further stirring for 2 min, commercially available L-proline methyl ester hydrochloride (0.50 g) in tetrahydrofuran (2.2 ml) and triethylamine (0.45 ml) were added. The reaction mixture was kept at −10 °C for 1 h and then stirred overnight at room temperature. Solvent was removed and the white solid residue was dissolved in ethyl acetate. Work-up of the product as described for preparations above yielded a yellow oil (300 mg, 33%) which was further purified by preparative t.l.c. (Kieselgel G). The main band, R_F 0.2 ($CHCl_3$), was extracted to yield a gummy solid (270 mg) on evaporation of the solvent. Trituration with ether yielded a *solid*, m.p. 104—108 °C (Found: C, 64.67; H, 7.48; N, 8.02. $C_{18}H_{24}N_2O_4$ requires C, 65.04; H, 7.28; N, 8.43%), τ ($CDCl_3$) 8.99 [6 H, t (2 overlapping doublets), CH_3CHCH_3], 7.93br (5 H, m, CH_3CHCH_3 and CH_2CH_2), 6.0—6.4br (2 H, m, NCH_2), 6.26 (minor) and 6.37 (major) (3 H, 2s, OCH_3), 5.57 (1 H, m, α-*H*Pro), 5.01—5.21 (1 H, 2d, α*H*Val), 3.15 (1 H, d, NH), and 2.21 and 2.55 (5 H, m, phenyl); $[\alpha]_D^{28}$ −52° (*c*, 1.8 in $CHCl_3$). An n.m.r. spectrum of the mother-liquor from the crystals revealed an extra peak in the ester region at τ 6.32.

On repeating the same reaction starting with Bz-DL-Val and L-Pro-OMe, the crystalline solid was again obtained giving an identical n.m.r. spectrum to the previous sample. The mother-liquor again revealed an extra ester peak [due to the L–L diastereoisomer (see below)]. Synthesis from Bz-D-Val using the azide method (see below) served to identify the crystalline material as Bz-D-Val-L-Pro-OMe.

Method B. Azide coupling. *N*-Benzoyl-L-valine hydrazide [31] (0.5 g) in water (10 ml), glacial acetic acid (2 ml), and concentrated hydrochloric acid (2 ml) was cooled to 0 °C. Cold saturated sodium nitrite was added until starch–iodide paper showed positive. The mixture was extracted into ethyl acetate (20 ml) and dried over magnesium sulphate. L-Proline methyl ester hydrochloride (0.34 g) and triethylamine (0.2 g) were added to the filtered ethyl acetate solution and the reaction mixture was stirred for 24 h. Removal of the excess of starting materials by extraction gave a gum (240 mg) after evaporation of the ethyl acetate. Preparative t.l.c. (Kieselgel G) yielded as the main fraction (110 mg), a non-crystallisable gum, *Bz-L-Val-L-Pro-OMe* (Found: C, 64.95; H, 7.3; N, 7.9. $C_{18}H_{24}N_2O_4$ requires C, 65.04; H, 7.28; N, 8.43%). Its n.m.r. spectrum in ($CDCl_3$) was identical to that of the above diastereoisomer except that ester peaks were at τ 6.26 (minor) and 6.32 (major); M^+ 332.

Method C. From L-valyl-L-proline. L-Valyl-L-proline (0.107 g) (Sigma Co.) in 2M-sodium hydroxide (0.3 ml) was cooled in an ice-bath and treated with benzoyl chloride (0.08 g) and 2M-sodium hydroxide (0.3 ml) in several portions with shaking and cooling. The solution was acidified using concentrated hydrochloric acid, and the precipitate was collected and washed several times with water and dried. The product (Bz-L-Val-L-Pro) was converted directly into its ester by dissolving the solid in ether and adding ethereal diazomethane until a yellow colour persisted in the solution. Evaporation of the ether yielded a non-crystallisable gum identical to the sample of benzoyl-L-valyl-L-proline methyl ester obtained above.

N-*Benzoylalanyl*-L-N-*methylalanine Methyl Ester*.—L-*N*-Methylalanine methyl ester hydrobromide for this preparation was prepared from *N*-benzyloxycarbonyl-L-*N*-methylalanine,[32] either by methylation with diazomethane, or using the method of Brenner and Huber,[33] followed by removal of the benzyloxycarbonyl group by hydrogenolysis in ethanol containing hydrochloric acid, or using hydrogen bromide–glacial acetic acid.[34] The *N*-benzyloxycarbonyl-*N*-methylalanyl methyl ester was obtained as an oil, τ($CDCl_3$) 8.6 (3 H, d, $CHCH_3$), 7.13 (3 H, s, NCH_3), 6.34 (3 H, s, OCH_3), 5.1—5.3 (1 H, m, $CHCH_3$), 4.89 (2 H, s, OCH_2Ar),

and 2.69 (5 H, s, ArH). Removal of the protecting group yielded the amine ester salt as a gummy solid which was used directly for coupling.

N-Benzoyl-L-alanine (0.193 g) in tetrahydrofuran (2 ml) containing triethylamine (0.15 ml) was stirred at −10 °C. Trimethylacetyl chloride (pivaloyl chloride) (0.13 ml) was added and the solution stirred for 2 min when a cold solution of *N*-methyl-L-alanylmethyl ester hydrochloride (0.153 g) in tetrahydrofuran (2 ml) and triethylamine (0.15 ml) was added. The reaction mixture was kept at −10 °C for 1 h and then overnight at room temperature. Solvent was removed *in vacuo*, and the residue was triturated with ethyl acetate. Undissolved solid was filtered off and the solution washed with acid and base in the usual way. Evaporation of the dried solvent yielded an oily gum which was further purified by preparative t.l.c. (Kieselgel G). The band of R_F 0.2 (in $CHCl_3$) was extracted to yield N-*benzoylalanyl*-L-N-*methylalanine methyl ester* as a gum (0.05 g) (Found: C, 61.8; H, 6.8; N, 9.75. $C_{15}H_{20}N_2O_4$ requires C, 61.6; H, 6.9; N, 9.6%); τ ($CDCl_3$) 8.54 (6 H, m, 2 × CH_3), 6.94 (3 H, s, NCH_3), 6.32 (3 H, s, OCH_3), 6.28 (very minor peak), 4.78—4.95 (2 H, m, α-CHs), and 2.18 and 2.53 (5 H, 2m, phenyl); δ_C ($CDCl_3$) (proton decoupled) 173.25, 171.86, and 166.5 (CO), 134.03, 131.56, 128.46, and 127.15 (aromatic C), 52.54 and 45.98 (α-Cs), 52.3 (ester CH_3), 31.29 (NCH_3), and 18.16 and 14.15 (CCH_3); small signals appeared at δ 54.74, 48.65 (α-C region), and 29.21 (N-CH_3 region) which could be due to conformational isomerism.

N-Benzoyl-D-alanyl-L-*N*-methylalanine methyl ester was synthesised in an analogous manner and gave n.m.r. data almost identical with its diastereoisomer. An equimolar mixture of both diastereoisomers showed a single signal at τ 6.32 for the ester methyl.

N-*Benzoylprolyl*-L-*valine Methyl Ester*.—*N*-Benzoyl-L-proline [35] (1.65 g) and triethylamine (1.2 ml) in tetrahydrofuran were stirred at −10 °C. Isobutyl chloroformate (1.2 ml) was added with stirring, followed by L-valine methyl ester hydrochloride (1.35 g), and triethylamine (1.2 ml) in tetrahydrofuran (6.2 ml). The suspension was kept at −10 °C for 1 h and then overnight at ambient temperature. Removal of solvent and work-up as previously described for analogues gave N-*benzoyl*-L-*prolyl*-L-*valine methyl ester* (0.80 g), m.p. 104—105 °C (from ether) (Found: C, 65.2; H, 7.25; N, 8.45. $C_{18}H_{24}N_2O_4$ requires C, 65.04; H, 7.28; N, 8.43%); $\nu_{max.}$ 1 740 (ester CO), and 1 630 and 1 690 cm^{-1} (amide CO); τ ($CDCl_3$) 9.12 and 9.04 (6 H, 2 d, CH_3CHCH_3), 8.27—7.47 [5 H, m, $CH(CH_3)_2$ and CH_2CH_2], 6.52 (2 H, m, NCH_2), 6.31 (3 H, s, OCH_3), 5.59 and 5.50 (1 H, 2d, α-*H*-Val), 5.20 (1 H, m, α-*H*-Pro), and 2.56 (5 H, m, Ar*H*); δ_C (proton decoupled) 172.16 and 171.07 (CO) ,136.42, 130.20, 128.45, and 126.97 (aromatic C), 59.80 and 57.52 (α-Cs), 52.04 (ester CH_3), 50.38 (NCH_2), 31.11, (β-C-Val), 27.23 (β-C-Pro), 25.45 (γ-C-Pro), 19.11, 17.75 (γ-C-Val). A mixture of diastereoisomers (Bz-DL-Pro-L-Val-OMe) was produced when Bz-DL-Pro was used instead of Bz-L-Pro. The n.m.r. spectrum of this product showed ester peaks at τ 6.31 and 6.34, thus suggesting that the Bz-D-Pro-L-Val-OMe is the source of the signal at τ 6.34.

N-*Benzoyl*-N-*methylvalyl*-L-*valine Methyl Ester*.—*N*-Benzoyl-*N*-methylvaline [6] {2.35 g $[\alpha]_D$ −120° (*c*, 0.03)} in tetrahydrofuran (5 ml) containing triethylamine (1.4 ml) at −10 °C, was treated with isobutyl chloroformate (1.3 ml) and the solution was stirred for 1.5 min. L-Valine methyl ester hydrochloride (1.6 g) in tetrahydrofuran (7.5 ml) containing triethylamine (1.4 ml) was added and the resulting mixture was stirred for 1 h at −10 °C and overnight at room temperature. Solvent was removed *in vacuo* and the residual oil (containing some solid) was dissolved in ethyl acetate (solid insoluble), washed with water, dilute acid, and base, and worked up in the usual way to yield N-*benzoyl*-N-*methylvalyl*-L-*valine methyl ester* as a non-crystallisable yellow oil (2.4 g) (70%) (Found: C, 66.0; H, 7.8; N, 8.0. $C_{19}H_{28}N_2O_4$ requires C, 65.49; H, 8.10; N, 8.04%); τ ($CDCl_3$) 9.03 (12 H, m, 2 × CH_3CHCH_3), 7.77 (2 H, m, 2 × CH_3CHCH_3), 7.09 (3 H, s, NCH_3), 6.30 * and 6.36 * (3 H, 2s, OCH_3-L-L and -D-L forms), 5.83—5.33 (2 H, m, 2α-*H*), 3.21 (1 H, m, NH), and 2.60 (5 H, s, ArH).

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* Assigned by separate synthesis, starting with Bz-DL-MeVal. (Ratio of L-L to D-L form in mixed anhydride coupling starting from Bz-L-MeVal was 75 : 25.

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