A model reaction for assessing the coupling and chiral efficiency of reagents in depside bond formation

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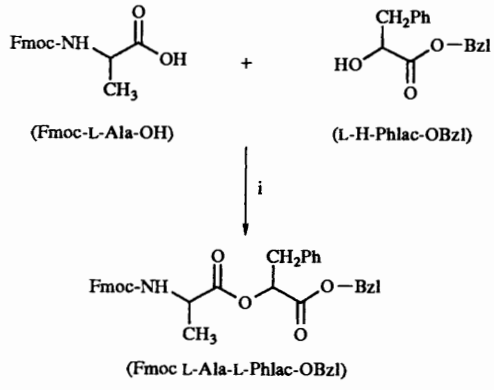
The formation of Fmoc-L-Ala-L-Phlac-OBzl (and Fmoc-D-Ala-L-Phlac-OBzl if there is racemisation) has been monitored by HPLC analysis for a number of coupling conditions between Fmoc-L-Ala-OH and H-Phlac-OBzl.† For this depside link formation it is revealed that CDI, DCC/DMAP and mixed anhydride couplings gave yields near to 50%. Couplings *via* TBTU, TNTU and TSTU gave lower yields. The best yields were achieved by acid chloride (61%), urethane-*N*-carboxyanhydrides (80%) and by PyBroP coupling (82%).

An ester or lactone bond incorporated into the backbone of a peptide or cyclopeptide sequence has traditionally been referred to as a depside link and the resultant compounds are known as depsipeptides. Nature has evolved a wealth of depsipeptide structures ranging from the well known ionophores such as valinomycin[1] and the enniatins,[2] antibiotics such as the actinomycins[3] and more recently marine sponges have been found to be a rich source of cyclodepsipeptides, many with cytotoxic properties.[4]

In the structural elucidation of these depsipeptides confirmatory syntheses of the native forms and their analogues have not been very dissimilar to conventional polypeptide synthesis, in that the basic procedures of protection, activation, coupling and deprotection are required. However, the incorporation of the depside bond into a sequence has highlighted the paucity of suitable protecting groups for the hydroxy group involved in the depside link, and also the need for a much greater degree of activation of the carboxyl group in depside bond formation, when compared with its peptide bond equivalent. As part of an extended study on the influence of the depside link in the conformation of biologically active cyclic peptides and depsipeptides, we discovered that while certain coupling reactions had been used successfully for depside bond formation in the past, in general the tendency in the published literature has been to synthesize pre-formed depside links and then carry out routine couplings to form amide bonds either in the solution or the solid phase. The aim of the present study was to answer the query of whether it was now possible in an era where routine peptide synthetic protocols abound,[5] to carry out such routines on the solid phase to incorporate the depside link as well.

To test protocols in peptide bond formation it is necessary to ascertain not only the coupling efficiency but also the degree of racemisation, or should it be enantiomerisation,[6] which occurs during the activation of the acylating carboxyl species. Many racemisation tests have been used in peptide synthesis.[7] The same criteria should be used for depside bond formation. After some reconnaisance testing of model systems, we eventually found that the coupling reaction summarised in Scheme 1



Scheme 1 (i) Various coupling conditions shown in Table 1

seemed to fit the criteria, since the phenyllactic acid has a secondary alcohol group and the Fmoc-alanine would be a typical residue encountered in a depsipeptide synthesis. In choosing the model reaction in Scheme 1, it was possible to monitor the extent of racemisation by HPLC analysis of the diastereoisomeric ratios since the Fmoc-L-Ala-L-Phlac-OBzl could be separated on a reversed phase column from its diastereoisomer Fmoc-D-Ala-L-Phlac-OBzl. Thus the basis of the test for the various coupling methods became the assessment of the yield of coupling by HPLC analysis to find the calibrated yield of depside product and the amount of diastereoisomer formed.

The choice of coupling agents investigated was governed both by the relative success of certain reagents in the past and the need to assess the most recent activating groups which have found favour in solid phase peptide synthesis. The latter became the thrust of this work, since a routine procedure for adaptation to solid phase synthesis was the main aim of the work.

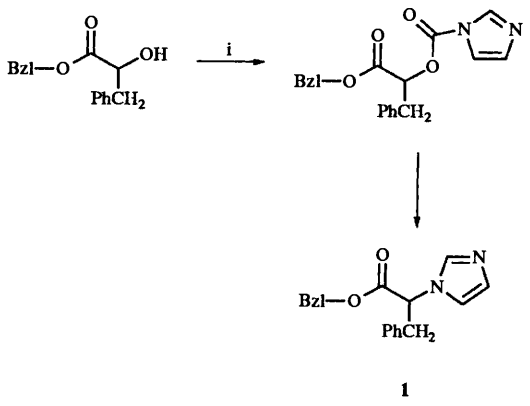
Review of past usage of certain coupling agents

Reports in the literature over the last 30 years[8] have highlighted a few coupling agents capable of forming a depside bond. Amongst the most successful of these have been CDI,[9] active esters,[10] mixed anhydride couplings[11] and DCC in the presence of pyridine or DMAP.[12]

A disadvantage of the CDI method is the moisture sensitivity of the reagent and the length of time needed for a reasonable coupling yield. During a scaled up synthesis of a depside link we

† *Abbreviations used:* Fmoc = fluoren-9-ylmethoxycarbonyl; H-PhLac-OH = phenyllactic acid; H-Phlac-OBzl = phenyllactic acid benzylester; CDI = *N*,*N*′-1,1-carbonyldiimidazole; DCC = *N*,*N*′-dicyclohexylcarbodiimide; DMAP = 4-(dimethylamino)pyridine; BOP = benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; HOBt = *N*-hydroxybenzotriazole; HOSu = *N*-hydroxysuccinimide; TNTU = 2-(5-norbornene-2,3-dicaroximido)-1,1,3,3-tetramethyluronium tetrafluoroborate; TBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TSTU = *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate; PyBroP = bromotrispyrrolidinophosphonium hexafluorophosphate; HOAt = 1-hydroxy-7-azabenzotriazole; IBC = isobutylchloroformate; UNCA = urethane-*N*-carboxyanhydride; DMSO = dimethylsulfoxide; DIEA = diisopropylethylamine. In the Schemes, R′OH represents H-Phlac-OBzl.

found a major side reaction (35% yield) which we identified as compound (1) (Scheme 2), involving the benzyl ester of



Scheme 2 *Reagent*: i, CDI

phenyllactic acid. The side reaction [13] is due to the long time the alcohol component is in contact with the CDI reagent. This problem could be eliminated by allowing a longer activation time for the formation of the acylating intermediate, and a higher temperature was also beneficial.

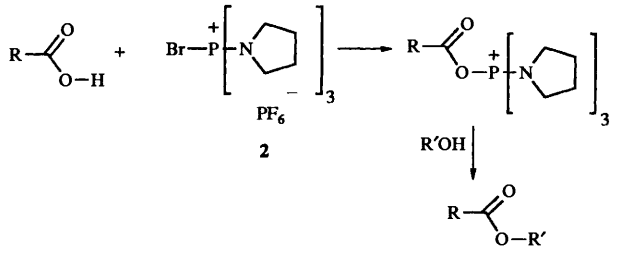
Amongst the 'respected genre' of reactions for depside bond formation have been successful couplings with benzene sulfonyl chloride–pyridine,[14] while most recent reports [15] tend to favour isobutyl chloroformate and isoprenyl chlorocarbonate for activation.

The conditions that best reflect past success with DCC in depside formation is the reaction catalysed by DMAP [16] where DMAP functions both as a Brönsted base to increase the nucleophilicity of the alcohol and activates the carboxyl group *via* the acyl pyridinium salt.

More recent examples of coupling techniques

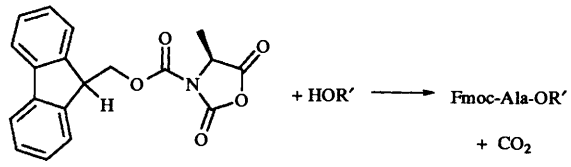
The demands and fine tuning of the solid phase techniques [5] for peptide synthesis have seen the development of new families of coupling agents. The success of the DCC/HOBt combination [17] and to a lesser extent DCC/HOSu,[18] believed to be due to the *in situ* formation of the corresponding active esters *e.g* the hydroxybenzotriazole ester, led to a family of reagents being introduced based on the benzotriazol-1-yloxytrisdimethylaminophosphonium hexafluorate, BOP reagent.[19]

More recently less toxic analogues, *e.g.*, HBTU, TBTU, TSTU and TNTU have been developed.[20] The mechanism of activation is common to all the reagents and the differences often lie only in their solubilities. Recently it has been reported [21] that the intermediate formation of a hydroxybenzotriazole ester may hinder reactions involving *N*-methylamino acids and much better yields have been obtained by the bromophosphonium salt PyBroP (**2**) as shown in Scheme 3.



Scheme 3

Two other methods have their origins very far back in the early work in peptide synthesis. Acid chloride activation of the carboxyl group has long been recognised as having the right level of activation, but was left on the wayside because of racemisation problems, lack of crystallinity and difficulties in the preparation of pure material. However the observation [22] that Fmoc-amino acid chlorides can be prepared pure, and have good chiral stability during numerous peptide couplings, have renewed interest in this coupling method. *N*-Carboxyanhydrides have also found more recent application as urethane analogues,[23] known generally as UNCA's, but used either as the t-Boc-*N*-carboxy anhydrides or Fmoc-*N*-carboxyanhydride derivatives of amino acids. Scheme 4 summarises the reaction

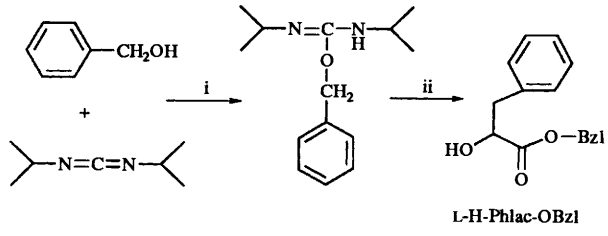


Scheme 4

involved. Good chiral stability is obtained due to the UNCA's inability to form oxazolones and the only by-product of the coupling is carbon dioxide.

Model coupling reaction (Scheme 1)

The Fmoc-L-alanine (and D-isomer) were prepared using standard procedures,[24] but the L-phenyllactic acid benzyl ester was prepared according to Scheme 5 which is a modification of the diisopropyl-*O*-benzylisourea method.[25]



Scheme 5 i, CuCl; ii, L-Phlac-OH–THF

For the coupling reaction most of the conditions involved coupling 4.8×10^{-4} mol Fmoc-L-Ala-OH or its equivalent derivative with 4.8×10^{-4} mol of L-H-Phlac-OBzl under conditions published in the literature for the particular method. After work-up by washing the organic phase with citric acid and hydrogen carbonate solutions, the dried neutral phase was subjected to a quantitative reversed phase HPLC analysis. The HPLC system (ODS column eluted with MeCN–H_2O 70:30 at 1 cm^3 min^{-1}) was calibrated against standard quantities of Fmoc-L-Ala-L-Phlac-OBzl. In an assessment of the sensitivity of the method for detecting the presence of the diastereoisomer it was shown that a mixture containing 1% Fmoc-D-Ala-L-Phlac-OBzl could be readily analysed. We would therefore place a lower detection limit of 0.5–1% racemisation on our method.

The HPLC analytical data also provided us with information on the number of by-products produced and comments in Table 1 reflect a qualitative assessment of the complexity of the reaction products obtained on the basis of the HPLC trace. In most of the values for yields listed in Table 1 they represent the average of duplicate runs. In others, similar methods were rerun under slightly varied conditions. The HPLC analysis was carried out on the neutral fraction, the starting materials having been removed at the extraction stage.

Table 1

Coupling method	Reaction time	Yield of depside (%) L-L	% D-L in mixture[a]	By-products[b] (neutral)	Ref.
CDI	3 days	45	a	None	9
CDI/DMAP	2 days	40	a	None	
DCC/DMAP	24 h	55	b	Two	12
Symmetrical anhydride/DMAP	1 day	40	a	One	28
IBC (mixed anhydride)	1 h	45	c	Two	15
	1 h (at 0 °C)	51	c	Two	
TNTU/DIEA	2–3 days	6	a	One	20*a*
TBTU/DIEA	1 day	21	a	Two	20*b*
TBTU/DMAP	1 day	33	a	Two	
TSTU/DIEA	1 day	9	a	Many	20*c*
Acid chloride/DMAP	6 h	61	a	None	22
Acid chloride/HOBt	1 day	15	a	One	
UNCA/DIEA (2.5 equiv.) dry toluene	4 h	80	b	None	23, 29
PyBroP	3 h (0 °C)	83	a	One (v small)	21
	3 h (ambient)	80	a	None	

[a] For results a, no peak corresponding to the D-L diastereoisomer could be detected and within the detection limits of the methodology can be recorded as ≤0.5–1%. Peaks corresponding to 0.4–0.5% D-L diastereoisomer were recorded in analyses b and up to 0.8% in analyses c. However these values are too near to the detection limit, to be discriminating. [b] Starting material would have been removed at the extraction stage. Survey refers to extra peaks detected in hplc trace.

Discussion of results

Of the coupling reagents traditionally associated with coupling in the solution phase (CDI, DCC/DMAP and mixed anhydride), all gave yields in the medium range (45–55% yield). Much cleaner HPLC traces were obtained with CDI, although there was a longer time (3 days) necessary to achieve a reasonable yield.

The *N*-hydroxybenzotriazole/*N*-hydroxysuccinimide derivatives gave disappointing yields, suggesting that the activation of the carboxyl group was insufficient to induce coupling with the comparatively less nucleophilic hydroxy group. A marginal improvement did occur with the addition of DMAP, but overall they would seem to eliminate themselves from application to the solid phase work. The HOAt analogues have recently become available,[26] and show improved reactivity profiles, but these were not available to us when the current work was in hand.

Three coupling conditions therefore stand out as possible contenders for depside formation in the solid phase. The Fmoc amino acid chloride in the presence of DMAP (61% yield), with few side reactions could be recommended in terms of our results and is free from racemisation. However, it has been reported[27] recently that adapting the acid chloride method to the solid phase is problematical due to the formation of oxazolones when the acid chloride is brought into contact with amines such as diisopropylethylamine. Our results with the urethane *N*-carbonic anhydride, with a yield of 80% and a reaction time of 4 h with reports of few side effects offers good prospects in adaptation to the solid phase. Also the PyBroP conditions (82% yield) gave impressive results, and represent our best candidates for adaptation.

It has also become clear from our studies that the model is quite a demanding test, with the secondary alcohol group of phenyllactic acid proving reasonably difficult to acylate. When glycollic acid benzyl ester with its primary alcohol group was substituted for the phenyllactic acid benzyl ester improved yields were obtained for both DCC/DMAP and the UNCA coupling conditions.

Experimental

^{1}H NMR spectra were recorded at 250 MHz on a Bruker WM 250 spectrometer, tetramethylsilane as internal standard. Abbreviations used, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Mass spectra were determined at the EPSRC Centre Swansea, on a VG 12, 250 for low resolution EI/Cl and a VG Autospec for FAB MS measurements. Elemental microanalysis was performed at University of Wales College Cardiff and mps were determined on a hot stage apparatus and are uncorrected. HPLC analyses were performed using a Milton Roy apparatus equipped with constametric pumps, a spectromonitor D and C1 10β integrator. All analyses utilised a Spherisorb ODS column (25 × 0.43 cm) eluted at 1.0 cm^3 min^{-1} at a pressure of 3000–4000 psi. HPLC solvents were AnalaR grade from Merck or Fisons, and water for HPLC was de-ionised and doubly distilled.

Synthesis of Fmoc-L-Ala-OH and Fmoc-D-Ala-OH

Standard published procedures[24] were used, giving Fmoc-L-Ala-OH from ethyl acetate as white crystals, mp 144–145 °C (lit.,[24] 143–144 °C); δ([$^2H_6$]DMSO), 7.8–7.3 [9 H, m, Ar*H* (Fmoc)], 4.25–4.20 (2 H, d, CH_2O), 4.06–4.0 (1 H, q, NH-C*H*) and 1.31, 1.38 (3 H, d, CH_3). Fmoc-D-Ala-OH, mp 143–144 °C was also produced in the same way starting from D-alanine.

Synthesis of L-phenyllactic acid benzyl ester

***(a)* Preparation of *N,N'*-diisopropyl-*O*-benzylisourea.**[25] Freshly distilled benzyl alcohol (1.04 cm^3, 10 mmol) was added slowly to a stirred mixture of copper(I) chloride (1.3 mg) and *N,N'*-diisopropylcarbodiimide (1.57 cm^3, 10 mmol) at 0 °C and stirred for 1 h. The reaction was then left at ambient temperature for 24 h. Hexane, up to twice the volume of solution, was added and the solution filtered through neutral alumina to remove copper salts. Removal of solvent gave an oil which was left under high vacuum overnight. The oil had a characteristic NMR spectrum, δ($CDCl_3$), 7.3 (5 H, s, C_6H_5), 5.1 (2 H, s, CH_2O), 2.9–4.1 (3 H, m, overlapping NH and CH) and 1.15 (12 H, d, 4 × CH_3).

***(b)* Synthesis of the benzyl ester.** L-Phenyllactic acid (0.7 g, 4.2 mmol) was added slowly to the freshly prepared *N,N'*-diisopropyl-*O*-benzylisourea (0.987 g, 4.2 mmol) and the mixture was stirred vigorously until it became very viscous, due to the precipitation of the urea. The volume was then increased to 8 cm^3 with dry tetrahydrofuran. A white precipitate of diisopropylurea appeared almost immediately. After stirring at room temperature for 48 h, the mixture was cooled to −10 °C

and the precipitated urea removed by centrifugation. The tetrahydrofuran was then removed under high vacuum to leave a slightly blue thick oil (84% yield), which was further purified on a silica gel column using 50% dichloromethane–ethyl acetate as eluent. Pure fraction had a t_R = 10.6 min (50:50 MeCN:water), on HPLC (1 cm^3 min^{-1}) and gave $\delta(CDCl_3)$, 7.3–7.15 (10 H, d, C_6H_5), 5.17 (2 H, s, CH_2O), 4.53–4.48 (1 H, q, CH) and 3.16–2.93 (2 H, dd, CH_2Ar) [Found CI/MS $(M + NH_4)^+$, 274.1443. $C_{16}H_{16}O_3{\cdot}NH_4^+$ requires 174.1470]. Proof that the benzyl ester was chirally pure was obtained from established coupling procedures which gave no evidence of Fmoc-L-Ala-D-Phlac-OBzl which would have been seen on HPLC analysis.

Synthesis of Fmoc-L-Ala-L-Phlac-OBzl and Fmoc-D-Ala-L-Phlac-OBzl (using the CDI method[9])

A solution of either Fmoc-L-Ala-OH (0.8 mmol) in dry dichloromethane (10 cm^3) (or the corresponding Fmoc-D-Ala-OH) was cooled to 0 °C and fresh CDI (0.8 mmol) in dry dichloromethane added. After stirring for 30 min, the L-phenyllactic acid benzyl ester (0.8 mmol) was added dropwise and the reaction mixture was maintained at 0 °C for a further 2 h and at room temperature for 3 days when TLC analysis showed no further change in the reaction. The dichloromethane was then extracted with 10% citric acid solution, 10% hydrogen carbonate solution and water, before drying over magnesium sulfate. Removal of drying agent and solvent gave on recrystallisation from diethyl ether–light petroleum, white crystals of the corresponding products.

Fluoren-9-ylmethoxycarbonyl-L-alanyl-L-phenyllactic acid benzyl ester (Fmoc-L-Ala-L-Phlac-OBzl). Mp 92–94 °C (50% yield) (Found: C, 74.0; H, 5.95; N, 2.3. $C_{34}H_{31}NO_6$ requires C, 74.3; H, 5.6; N, 2.55%); FAB-MS [$(M + H)^+$, 550 $(M + Na)^+$, 572]; $\delta(CDCl_3)$ 7.73–7.15 [19 H, m, overlapping ArH (2 × C_6H_5 + Fmoc ArH)], 5.12–5.10 (2 H, d, CH_2O), 4.5–4.34 (3 H, m $CHCH_2$, CH_2OCONH), 4.23–4.47 (1 H, q, NHCH), 3.19–3.14 (2 H, m, $CH_2C_6H_5$) and 1.39–1.36 (3 H, d, CH_3).

Fluoren-9-ylmethoxycarbonyl-D-alanyl-L-phenyllactic acid benzyl ester (Fmoc-D-Ala-L-Phlac-OBzl). Mp 89–91 °C (55% yield) FAB-MS [$(M + H)^+$, 550 $(M + Na)^+$, 572]; $\delta(CDCl_3)$ 7.72–7.12 [19 H, m, overlapping ArH (2 × C_6H_5 + Fmoc ArH)], 5.12 (2 H, s, CH_2O), 4.48–4.42 (1 H, t, $OCHCH_2$), 4.38–4.35 (2 H, m, CH_2O), 4.22–4.19 (1 H, m, NHCH), 3.2–3.08 (2 H, dd, $CH_2C_6H_5$) and 1.27–1.25 (3 H, d, CH_3).

Other methods used for coupling

Conditions were as reported in the references listed in Table 1, except that in order to establish leads for potential adaptation to the solid phase, dichloromethane and dimethyl formamide were mainly used as solvents,[28] under ambient conditions. For the UNCA method toluene was used as solvent, following reports[29] of slower reaction times and of racemisation in DMF. In the general work-up of the reaction mixtures, organic solutions obtained at the end of the reaction period, were subjected to washing by 10% citric acid solution and 10% hydrogen carbonate solution followed by water. On drying the organic layer (magnesium sulfate) and removing the solvent a crude yield of neutral material could be assessed, but the product yield was based on an HPLC analysis as described below.

Product analysis by HPLC

A calibration graph based on peak areas against concentration of Fmoc-L-Ala-L-Phlac-OBzl was constructed from standard solutions of the depsipeptide run on a ODS C-18 column (25 × 0.4 cm) at a flow rate of 1.0 cm^3 min^{-1}. The most sensitive wavelength for detection was found to be 266 nm, and the separation of peaks due to Fmoc-L-Ala-L-Phlac-OBzl and Fmoc-D-Ala-L-Phlac-OBzl was optimised with an eluent ratio of MeCN–H_2O of 70:30 which gave good diastereoisomeric separation (selectivity factor α = 1.14, resolution R_s = 1.03) for routine checking.

The calibration graph was produced from 20 mm^3 injections of solutions varying in concentration from 0.14–0.86 mg cm^{-3} of Fmoc-Ala-L-Phlac-OBzl. A correlation coefficient of 0.999 97 was obtained and using such a graph the various analyses were carried out by measuring the area under the appropriate peak in the HPLC trace, and reading off the concentration of the solutions. The product yields were then calculated as reported in Table 1.

Acknowledgements

We thank the EPSRC for a Quota Studentship for J. H. and the ERASMUS scheme for a studentship for M. L. B. Peboc Llangefni, Anglesey, kindly donated samples of TBTU, TSTU and TNTU, while Propeptide, France provided a complimentary sample of Fmoc-L-Ala-NCA.

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Paper 5/02351C
Received 12*th April* 1995
Accepted 25*th August* 1995