EPIMERIZATION DURING COUPLING TO THE UNNATURAL AMINO ACID IN SOLID PHASE PEPTIDE SYNTHESIS

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Extensive epimerization was observed during the coupling of Fmoc-L-Trp(Boc) and Fmoc-L-Pro to D-Ual, a synthetic α-amino acid with a nucleobase in the side chain. This unnatural amino acid was used extensively in the construction of nucleic acids recognition libraries. Reaction protocols based on the use of DIC–HOAt–DIEA mixture without preactivation of the coupling mixture has been proven to suppress the epimerization reaction significantly.

Keywords: Solid phase synthesis; Epimerization; Peptides; Nucleobases; PNA; Uracil; Amino acids.

A combinatorial library strategy is an effective approach to obtain thousands of structurally different compounds either for lead generation or for lead optimization in drug discovery[1–5]. Although the final compounds of a library are physically mixed in a pool, each compound should be of sufficient purity to generate valuable results in subsequent screening. Side products such as enantiomeric and diastereomeric impurities may seriously hamper the interpretation of biological data and further mislead subsequent research work. This is a serious problem when considering peptide libraries of unnatural amino acids. For a combinatorial peptide library, the control of the absence of any epimerization during each coupling step is important for the overall quality of the library.

It is well known that peptide bond formation requires the activation of the carboxyl group of the incoming amino acid. In this activation step, the α-carbon of the amino acid in particular is prone to epimerization[6,7]. Studies on the configurational integrity of α-amino acids during coupling procedures have resulted in the discovery of new protecting groups, new coupling reagents, new coupling additives and on the selection of different

solvents and bases[8–10]. Usually these studies have been carried out with natural amino acids and little work has been done with unnatural amino acids.

RESULTS AND DISCUSSION

Chemical libraries are often composed of unnatural amino acids. Recently, we developed a strategy to select dsDNA binding peptides using labeled DNA and a library of peptides composed of unnatural amino acids[5]. During our search for the optimal coupling conditions to obtain high yields of the peptides, we observed extensive epimerization while coupling Fmoc-L-Trp(Boc) or Fmoc-L-Pro to D-Ual, an unnatural amino acid with the nucleobase attached to the β-carbon of α-D-alanine[5] (Fig. 1).

By using DIC–HOAt (see footnote[+]) in DMF as coupling conditions for the synthesis of Ac-L-Trp-D-Ual-Inp-β-Ala-Gly-$CONH_2$ and Ac-L-Pro-D-Ual-Inp-β-Ala-Gly-$CONH_2$ (Fig. 1), epimerization was observed by RP-HPLC (Fig. 2) with formation of 9.3 and 11% of the diastereomeric peptides, respectively[11]. Peptides were identified using mass spectrometry. Since among the amino acid residues used only Trp, Ual and Pro have a chiral center, it is most likely that the epimerization took place during either the coupling of D-Ual to Inp or the coupling of L-Trp/L-Pro to D-Ual. Therefore investigations were focused on the amide bond formation between these residues. Because Inp does not possess chiral center, it was replaced with proline, a cyclic amino acid, during the investigations. D-Ual was coupled to both L- and D-proline to form dipeptides on Rink Amide MBHA resin. After cleavage from the resin, the dipeptides were subjected to RP-HPLC and NMR analyses.

However, RP-HPLC was not able to resolve these diastereomeric peptides as a mixture of Ac-D-Ual-L-Pro-$CONH_2$ and Ac-D-Ual-D-Pro-$CONH_2$ showed only one peak. Comparison of NMR spectra of this mixture with the NMR spectrum of the separate compounds, Ac-D-Ual-L-Pro-$CONH_2$ and Ac-D-Ual-D-Pro-$CONH_2$, revealed the absence of any epimeric impurity, indicating that epimerization did not take place upon Ual activation. It has to be noted here that several other dipeptides were prepared, with Ual coupled to the amino acids depicted in Fig. 3. In all cases no epimerization was ob-

+ *Abbreviations used*: Boc, *tert*-butoxycarbonyl; Cbg, (3-chlorobenzylamino)acetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; Inp, isonipecotic acid, piperidine-4-carboxylic acid; Nic, 2-amino-3-[(3-pyridine-3-carbonyl)amino]propinoic acid; PyBOP, *O*-(1*H*-benzotriazole-l-yloxy)tripyrrolidinophosphonium hexafluorophosphate; Ual, 2-amino-3-(2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)propionic acid. For other abbreviations see Experimental.

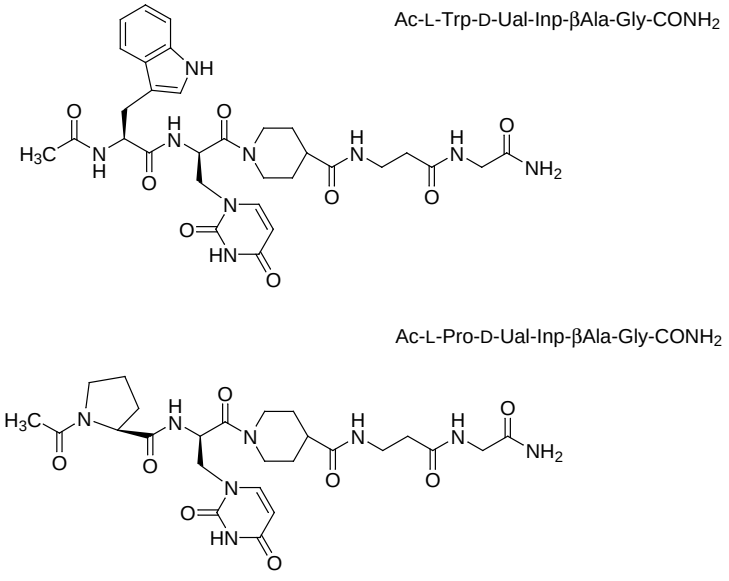


FIG. 1
Structures of two pentapeptides where epimerization is observed during the coupling reaction of the ultimate amino acid to D-Ual

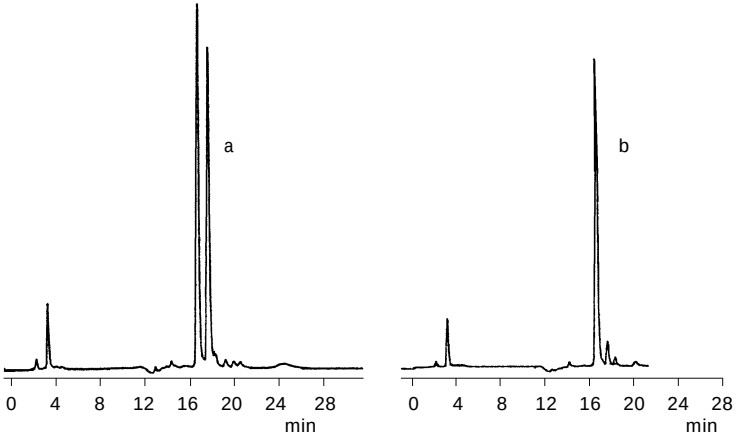


FIG. 2
HPLC system used for the evaluation of the enantiomeric impurity of the peptide. a: mixture of Ac-L-Trp-D-Ual-Inp-β-Ala-Gly-$CONH_2$ and Ac-D-Trp-D-Ual-Inp-β-Ala-Gly-$CONH_2$; b: Ac-L-Trp-D-Ual-Inp-β-Ala-Gly-$CONH_2$ when epimerization is suppressed (a: 50% epimerization, DIC–HOAt–PyBOP–DIEA in DMF with preactivation; b: 3.9% epimerization, DIC–HOAt–DIEA in DMF without preactivation, for details see Experimental)

served. Apparently, epimeric impurities were generated during the activation of L-Trp and/or L-Pro when coupled to D-Ual.

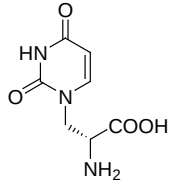
Peptide bond formation may be accelerated by using a cocktail of coupling reagent and additives, displaying different mechanisms of activation. This approach was used in our studies, when we found that neither the classic DIC–HOAt mixture alone nor PyBOP alone was able to successfully couple Ual to other amino acids, both natural and unnatural[5]. However, by using a cocktail of 2 equivalents of PyBOP and 4 equivalents of DIC–HOAt–DIEA in DMF, a high coupling yield (95%) was obtained for the synthesis of Ac-D-Ual-L-Pro-$CONH_2$ without noticeable epimerization.

The rationale for the successful combination of PyBOP, HOAt and DIC is not clear. In the present study, when using this mixture for the coupling of Trp and Pro to Ual, the yield was significantly improved up to 95%, from 82% when PyBOP was used alone. High yields are necessary for library synthesis. However, this was accompanied by an extensive epimerization when 50 or 27% of diastereomers were obtained after coupling with Fmoc-L-Trp(Boc) and Fmoc-L-Pro, respectively (Table I).

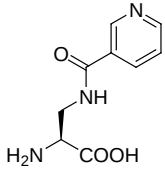
TABLE I

Percentage of epimerization with or without preactivation after the coupling (in DMF) of Fmoc-L-Trp(Boc) or Fmoc-L-Pro to D-Ual-Inp-β-Ala-Gly-$CONH_2$ (all yields were over 95%). For detailed conditions see Experimental

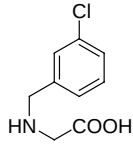
Amino acid	Solvents and bases	Epimerization, %	
		with preactivation	without preactivation
Fmoc-L-Trp(BOC)	DIC–HOAt–DIEA	9.3	3.9
	DIC–HOAt–PyBOP–DIEA	50	12
Fmoc-L-Pro	DIC–HOAt–DIEA	11	5
	DIC–HOAt–PyBOP–DIEA	27	10



D-Ual



L-Nic



Cbg

FIG. 3

Dipeptides were prepared by coupling D-Ual to L-Nic and Cbg, respectively

In a normal condensation procedure, the incoming amino acid is preactivated with the coupling reagents and additives for 10 min at room temperature before it is reacted with the amino function of the previous amino acid that is bound to the resin. Epimerization *via* oxazolone formation is mostly favored by a long contact time between the amino acid and the coupling reagent (DIC) and additives (HOAt) (Fig. 4)[12]. It would be beneficial to reduce this contact time as much as possible and to consume the active ester intermediate at the moment it is formed by the incoming nucleophile.

With this in mind, the coupling solution was prepared and added immediately to resin without any preactivation. The RP-HPLC profiles revealed a significant suppression of epimerization by 50% (from 9.3 to 3.9% in the case of Trp; and from 11 to 5% in the case of Pro) with DIC–HOAt–DIEA in DMF and by more than 60% (from 50 to 12% in the case of Trp; and from 27 to 10% in the case of Pro) using a cocktail of PyBOP/DIC–HOAt–DIEA in DMF for both Fmoc-L-Trp(Boc) and Fmoc-L-Pro coupling to the amino function of D-Ual bound to the resin (Table I).

Further improvement was anticipated by optimizing the choice of solvents and bases. It was recently reported that DCM could accelerate the formation of OAt active ester, and that collidine (TMP), a weaker base than DIEA, might promote the attack by the acid carboxylate in subsequent

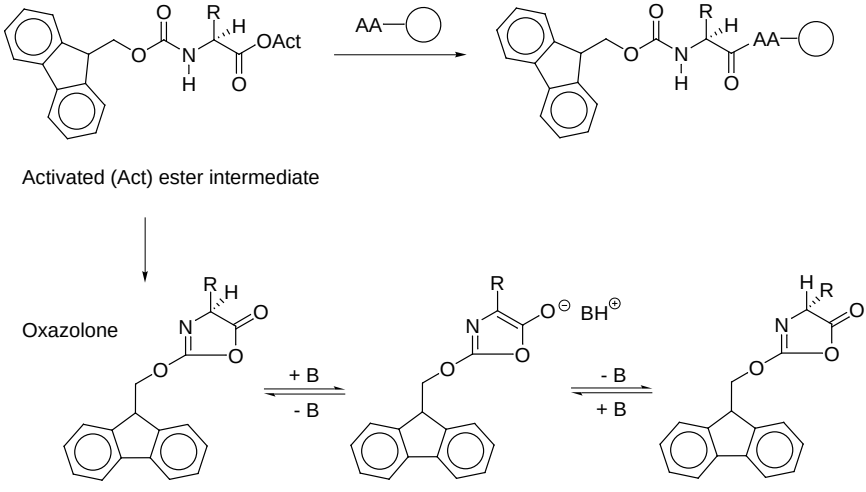


FIG. 4
Epimerization *via* the oxazolone as a competitive reaction to amide formation (B is DIEA)

steps, without unduly interfering with the first step of carbodiimide protonation[10,13]. Therefore we investigated the use of DCM as solvent and TMP as base for the coupling reaction. Fmoc-L-Trp(Boc) and Fmoc-L-Pro were coupled to the target tetrapeptide with D-Ual as N-terminus by using DIC–HOAt–TMP in DCM either with or without preactivation. The results in Table II revealed that with preactivation the epimerization was suppressed by 35% (from 9.3 to 6% in the case of Trp), and by 40% (from 11 to 6.9% in the the case of Pro), if compared with the reaction using DIC-HOAt–DIEA in DMF. Without preactivation, the influence of solvent and base was negligible. Nevertheless, as in the case with DMF–DIEA, the absence of preactivation resulted in a substantial lower degree of epimerization.

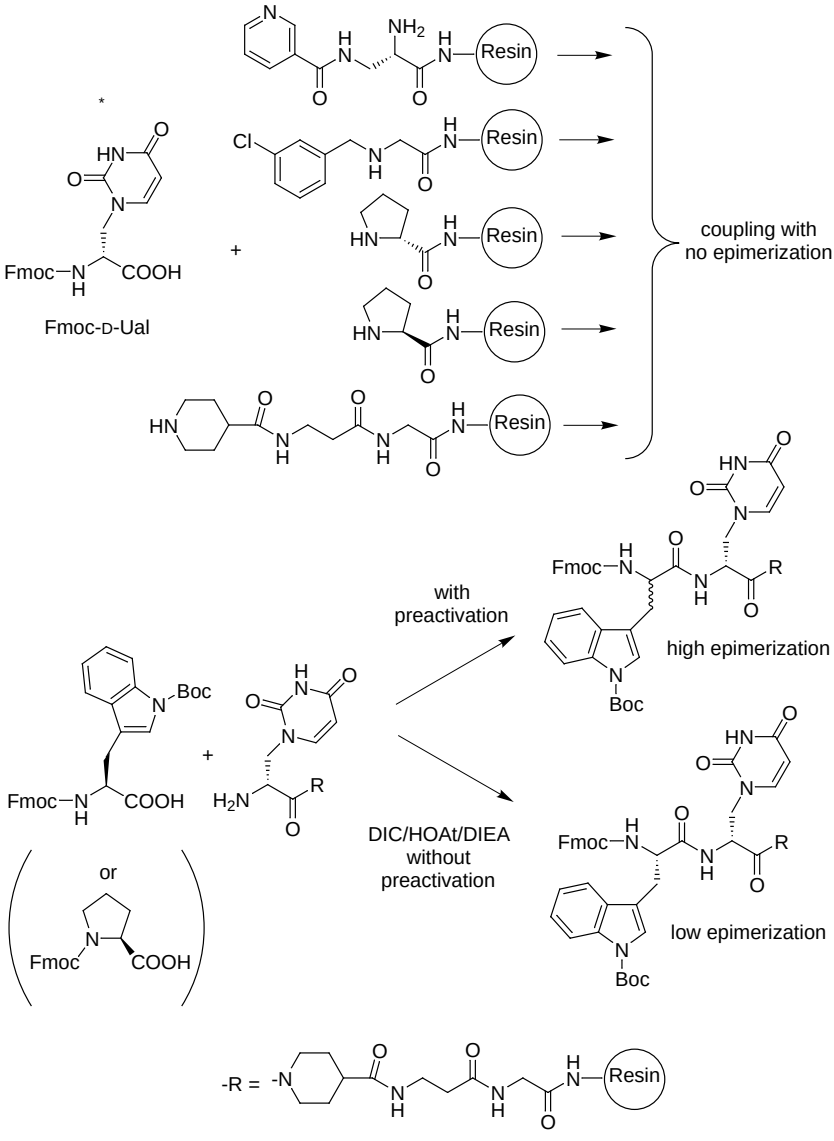
From these data, it is clear that preactivation was responsible for the high amount of epimerization, when either Fmoc-L-Trp(Boc) or Fmoc-L-Pro was coupled to D-Ual-Inp-β-Ala-Gly-$CONH_2$.

The extent of epimerization is dependent on the nature of the amino acids. By using the same coupling reagent and additive (DIC–HOAt), the coupling of Fmoc-L-Trp(Boc) to Fmoc-L-Pro did not generate any epimerization. However, when Fmoc-L-Trp(Boc) was coupled to D-Ual, serious epimerization resulted. The primary amine of Ual is expected to give less steric hindrance, comparing with the secondary amine of proline. Coupling kinetics are dependent on both amino acids and coupling time influences epimerization. In this particular case, the side chain of uracil, which is a

TABLE II
Influence of solvent, base and preactivation on epimerization of Fmoc-L-Trp(Boc) and Fmoc-L-Pro in the coupling (DIC–HOAt) to D-Ual-Inp-β-Ala-Gly-$CONH_2$ (all yields over 95%). For detailed conditions see Experimental

Amino acid	Solvents and bases	Epimerization, %	
		with preactivation	without preactivation
Fmoc-L-Trp(BOC)	DMF–DIEA	9.3	3.9
	DCM–TMP	6	4
Fmoc-L-Pro	DMF–DIEA	11	5
	DCM–TMP	6.9	5.7

nucleobase, may also be involved in catalyzing the epimerization reaction. The summary of reactions investigated in our study is shown in Scheme 1.



The summary of reactions investigated in the present study.
Resin is MBHA solid support with Rink amide linker.

SCHEME 1

CONCLUSION

Epimerization of Fmoc-L-Trp(Boc) and Fmoc-L-Pro upon coupling to D-Ual, an unnatural amino acid with the nucleobase attached to L-alanine, could be significantly suppressed by using a DIC–HOAt–DIEA in DMF protocol without any preactivation. The replacement of solvent and base with DCM–TMP did not further improve the result in the absence of preactivation. Moreover, this reaction protocol resulted in coupling yields over 95%. The increased use of synthetic amino acids for the construction of combinatorial libraries justifies the research and the use of the described coupling conditions for the amide bond formation can be recommended when extensive epimerization is observed.

EXPERIMENTAL

The ^{1}H and ^{13}C NMR spectra were recorded with a Varian Gemini 500 spectrometer in DMSO-d_6. For the ^{1}H spectra, the solvent signal at 2.5 ppm was used as reference. For the ^{13}C spectra, the solvent peak at 39.60 ppm was used as reference. Exact mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qTof 2, Micromass, Manchester, U.K.) equipped with a standard electrospray ionization (ESI) interface. Samples were infused in a 2-propanol–water (1 : 1) mixture at 3 ml/min. For analysis of racemization, the RP-HPLC was performed on a Nova-Pak® C18 column (60 Å, 4 μm), 3.9/150 mm (Waters, U.S.A.). Anhydrous solvents were obtained as follows: pyridine and *N*,*N*-diisopropylethylamine (DIEA) were refluxed overnight over potassium hydroxide and distilled; *N*,*N*-dimethylformamide (DMF) was stored with molecular sieves for 3 days and was tested for the presence of dimethylamine prior to use. CH_3CN for HPLC was purchased from Rathburn (grade S) and water for HPLC purification was distilled twice. Rink amide MBHA resin was supplied by Novabiochem (Laufelfingen, Switzerland). Bio-spin column 732-6008 was used as reaction vessel for the solid phase synthesis (BioRad, U.S.A.). Dichloromethane (DCM), DMF, acetic anhydride (Ac_2O) and pyridine were obtained from BDH (Poole, England). 1-Hydroxy-7-azabenzotriazole (HOAt), Fmoc-β-Ala, Fmoc-Gly, Fmoc-L-Trp(Boc), Fmoc-L-Pro were purchased from Advanced ChemTech (Louisville, Kentucky, U.S.A.). Piperidine, trifluoroacetic acid (TFA), diisopropylcarbodiimide (DIC) and collidine or 1,3,5-trimethylpyridine (TMP) were supplied by ACROS (Geel, Belgium). Fmoc protection of the amine of isonipecotic acid (Inp) was realized in our laboratory. Fmoc-D-Ual, L-Nic and Cbg were synthesized as described[5].

Preparation of Solid Support for Synthesis

Rink amide MBHA resin (50 mg, 27 μmol) was weighed into a reaction vessel and swelled in DCM and DMF each time for 20 min. After filtering off the solvent, the beads were treated with 20% piperidine in DMF over 15 min, followed by washing three times with DMF and DCM.

Coupling of the Fmoc-Amino Acid

A solution of 4 equivalents (relating to the amount of solid support used) of Fmoc-amino acid, 4 equivalents of HOAt, 4 equivalents of DIC, 4 equivalents of DIEA (or TMP) in 500 µl of DMF (or DCM) was prepared either *in situ* or with 10 min preactivation at room temperature and added to the amino functionalized solid support. In some cases 2 equivalents of PyBop were added to the reaction mixture.

Following the overnight reaction at room temperature, the beads were washed three times with DMF and three times with DCM. In the case of preactivation, the solution of Fmoc-amino acids with coupling reagents and base in DMF was stored at room temperature for 15 min before being poured to the functionalized solid support.

Determination of the Coupling Yield

The completion of acylation by the Fmoc-amino acids was determined by UV spectrometry. The Fmoc-amino protecting group was removed with piperidine and the coupling yield was determined on the basis of the absorption of the resulting piperidinyldibenzofulvene adduct having an ε of 7 500 at 300 nm. Briefly, an accurately weighed amount of beads (6–7 mg) was suspended in exactly 25 ml of 20% piperidine in DMF. After 10 min, the absorbance was measured at 300 nm with a solution of 20% piperidine in DMF as a blank. The loading was calculated with the formula: loading (µmol/g) = $A \cdot 10^6/300/\mathrm{wt}$, where A is absorbance at 300 nm and wt is weight in mg.

Capping and Fmoc Deprotection Procedure

A mixture of pyridine–Ac_2O–*N*-methylimidazole (4 : 1 : 0.5) was freshly prepared and added to the reaction vessel with the solid support. The reaction mixture was kept for 10 min at room temperature. The solution was removed and the solid support was washed three times with DMF and DCM. A solution of 20% piperidine in DMF was added to the beads. After 15 min, the mixture was filtered off and the beads were washed three times with DMF and DCM.

Removal of the Protecting Groups and Cleavage of the Peptides from the Solid Support

After the final coupling cycle, the Fmoc-protecting group was removed and the terminal amino group was acetylated. The acid labile side chain protecting groups were removed and oligopeptides were released from the beads by treatment with TFA–H_2O (95 : 5) in the presence of thioanisole (5%) over 2 h at room temperature. The mixture was filtered and the filtrate was evaporated and co-evaporated with toluene, giving the peptides as a solid material.

Determination of the Epimeric Purity of Peptides

The epimeric purity of the peptides was checked with L-6200 A Merck–Hitachi pump using UV monitoring. RP-HPLC analysis was performed on a Nova-Pak® C18 column (60 Å, 4 µm), 3.9/150 mm (Waters, U.S.A.). The peptides were eluted using isocratic conditions of 10% B (H_2O–CH_3CN–TFA, 20 : 80 : 0.1) and 90% A (H_2O–CH_3CN–TFA, 95 : 5 : 0.1), with a flow rate of 1 ml/min, and detected by UV absorption at 254 nm. The isolated peptides were further identified by high-resolution mass spectrometry. Peptide Ac-L-Trp-D-Ual-Inp-β-Ala-Gly-$CONH_2$: exact mass (ESI MS) for $C_{31}H_{39}N_9O_8$ $[M + H]^+$: calculated 666.2999, found

666.3009. Peptide Ac-L-Pro-D-Ual-Inp-β-Ala-Gly-$CONH_2$: exact mass (ESI MS) for $C_{25}H_{36}N_8O_8$ $[M + H]^+$: calculated 577.2734, found 577.2758.

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