Pergamon

Tetrahedron: *Asymmetry* 13 (2002) 1629–1636

TETRAHEDRON: *ASYMMETRY*

Racemization of chiral PNAs during solid-phase synthesis: effect of the coupling conditions on enantiomeric purity

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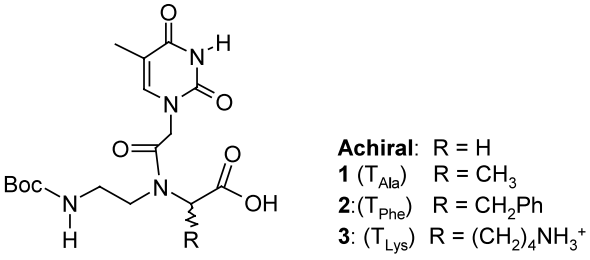
Received 27 May 2002; accepted 19 July 2002

Abstract—Chiral peptide nucleic acid (PNA) monomers based on the amino acids Ala, Phe and Lys were synthesized, and their enantiomeric purity was checked either by RP-HPLC after reaction with L-ValOMe or by GC–MS using a Chirasil-Val column after hydrolysis and conversion of PNAs to the corresponding piperazin-2-ones. A model coupling reaction of these monomers with ValOMe was carried out under various conditions in order to evaluate the effect of synthetic parameters (coupling agent, base, preactivation time) on epimerization. The enantiomeric purity of the products decreased in the order: DEPBT>TDBTU> HBTU>HATU. The use of *sym*-collidine (TMP) as a base produced higher racemization than with DIEA. PNAs containing one or three chiral monomers were subsequently synthesized with different coupling protocols, and the results were found to be consistent with those obtained in solution. High enantiomeric purity was obtained using a DIC/HOBt coupling protocol. A rationale for the observed effects is proposed based on NMR studies.

1. Introduction

Peptide nucleic acids (PNAs) are DNA mimics, first described in 1991,[1] in which the sugar–phosphate backbone has been replaced by an achiral polyamide chain composed of aminoethylglycine covalently linked to DNA bases (Scheme 1). PNAs were shown to form hybrids with complementary DNA or RNA strands with remarkable affinity and selectivity.[2,3] On account of these properties, PNAs are currently used as very powerful tools in molecular biology for the detection of defined DNA sequences or mutations.[4] PNAs are also promising as antisense or anti-gene drugs for the inhibition[5] and regulation of gene expression.[6–10]



Scheme 1.

Many PNA analogs have been described in recent years in order to understand and improve the properties of this class of molecules.[11–14] The use of PNAs based on *N*-aminoethyl-α-amino acid monomers (functional PNAs, Scheme 1) has been shown to be an effective means for the introduction of functional groups which can modulate PNA:DNA interactions[13] and for studying the role of chirality in nucleic acid recognition.[14]

Abbreviations: DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; DEPBT, 3-(diethyloxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one; DIC, *N*,*N*′-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazol[4,5-*b*]pyridine-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[1*H*-1,2,3-(benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt, 1-hydroxy-1,2,3-benzotriazole; PyFNOP, [4-nitro-6-trifluoromethylbenzotriazol-1-yloxy]-tris(pyrrolidino)phosphonium hexafluorophosphate; TDBTU, *O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazine-3-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TMP, 2,4,6-trimethylpyridine (sym-collidine).

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0957-4166/02/$ - see front matter
PII: S0957-4166(02)00413-5

Based on circular dichroism studies and melting experiments, some of us proposed that D-amino acid containing PNAs (regardless of the nature of the amino acid) promote the formation of right-handed structures.[15] The effect of chirality on the sequence selectivity has been studied by introducing one or few distant chiral monomers in an achiral PNA strand,[15] and highly constrained PNAs, containing three consecutive chiral monomers ('chiral box'), were shown to increase sequence selectivity (direction control and mismatch recognition).[16,17]

PNA oligomers can be easily obtained in milligram to gram scale from monomeric units by solid-phase peptide synthesis (SPPS).[18]

A critical factor in the synthesis of chiral PNAs is the control of the enantiomeric purity of both the monomers and oligomers, since epimerization can occur during solid-phase synthesis. In peptide chemistry, the epimerization problem is particularly important in syntheses carried out in solution,[19] and could be a serious problem in the case of coupling with *N*-methyl-α-amino acids,[20] in segment coupling,[21] convergent solid-phase peptide synthesis (CSPPS),[22] or in the synthesis of conformationally constrained cyclic peptides.[23] By careful selection of the coupling reagents, the base, and the reaction conditions, epimerization can be reduced to very low levels, as shown in several studies by Carpino and co-workers.[24] The use of 12 different onium salt-based coupling reagents in peptide synthesis has recently been discussed.[25] Some of us have recently reported that severe epimerization problems occur during the synthesis of oligomers of ornithine-based chiral PNAs. However, careful control of the coupling conditions led to a dramatic increase in the enantiomeric purity.[26] In the case of chiral PNAs derived from *N*-aminoethyl-α-amino acids, the racemization issue during monomer synthesis was thoroughly investigated by reaction with *t*-butyl alaninane as chiral derivatizing agent. HPLC analysis showed only 0.4% of the undesired diastereomer in the case of the L-Ala monomer. The racemization of the monomers was found to be mainly dependent on the conditions used in the removal of the carboxyl protecting group, in particular basic treatments.[27]

We have recently developed a chromatographic method for the enantiomeric analysis of chiral PNAs, which allowed us to monitor not only the enantiomeric purity of the monomers, but also that of the PNA oligomers. Using this method, it was possible to demonstrate that the chiral monomers can be synthesized with high enantiomeric purity, but that the conditions used for the solid-phase synthesis induced a considerable degree of epimerization.[28]

With the aim of preventing epimerization, the present paper describes a systematic study of the enantiomeric purity of chiral PNA monomers and oligomers based on *N*-aminoethyl-α-amino acid backbone as a function of the synthetic procedures, in particular of the coupling conditions used in the solid-phase synthesis.

2. Results and discussion

2.1. Enantiomeric purity of the PNA monomers

The chiral monomers used in this study are shown in Scheme 1 and in Table 1. The monomers **1** and **2** were prepared using allyl or benzyl esters cleaved by hydrogenation; the monomer **D-3** was prepared using allyl ester, which was cleaved, in the final step, with Pd(0). **L-3** was prepared as the methyl ester, which was then cleaved to the free acid with LiOH in THF.

The enantiomeric purity of the monomers was monitored via specific rotation values and HPLC and GC methods (see Table 1).

In the HPLC method, the six monomers were linked to L-ValOMe by coupling with DIC/HOBt with 3 min preactivation (Fig. 1a); the resulting diastereomers were separated in the case of **1** (T_{Ala}) and **2** (T_{Phe}) but not of **3** (T_{Lys}).

The same analysis was performed with the direct GC–MS method which is based on hydrolysis of the monomers to the corresponding *N*-aminoethylamino acids and subsequent reaction with trifluoroacetic anhydride to yield the trifluoroacetylated piperazin-2-ones (Fig. 1b), which can be separated in GC using a Chirasil-Val commercial column.[29]

A series of chromatograms showing the enantiomeric separation of the monomers is reported in Fig. 2.

As reported in Table 1, **3** (T_{Lys}) was the most contaminated with the undesired enantiomer. In particular in the case of **L-3** ($T_{L\text{-}Lys}$) the racemization degree is probably caused by the final synthetic step: the cleavage of the methyl ester under basic conditions.

Table 1. Specific rotation and enantiomeric purity of the monomers used in the present study

Monomer	$[\alpha]_D^{25}$	% Minor enantiomer[a]	
		HPLC	GC–MS
L-1 ($T_{L\text{-}Ala}$)	−18.1	1.2	0.9 (0.4)
D-1 ($T_{D\text{-}Ala}$)	+18.1	1.2	0.8 (0.4)
L-2 ($T_{L\text{-}Phe}$)	−120.3	0.7	0.5 (0.1)
D-2 ($T_{D\text{-}Phe}$)	+120.7	0.4	0.6 (0.1)
L-3 ($T_{L\text{-}Lys}$)	−18.5	N.d.	4.6 (0.7)
D-3 ($T_{D\text{-}Lys}$)	+19.1	N.d.	2.2 (0.8)

[a] Standard deviations are given in parentheses.

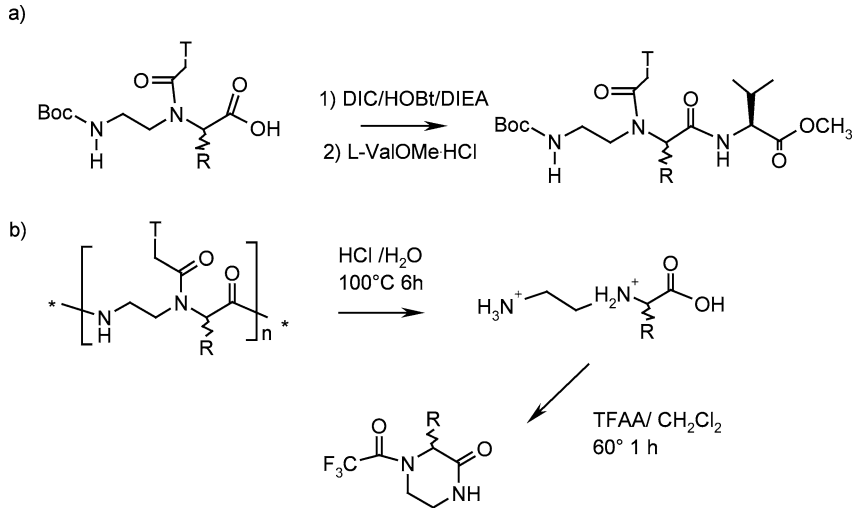


Figure 1. Derivatization methods used in (a) HPLC analysis and (b) GC–MS analysis.

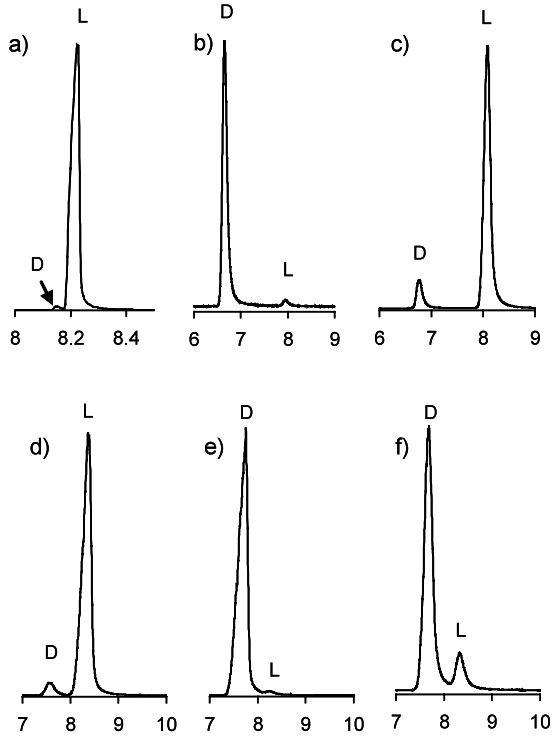


Figure 2. GC–MS enantiomeric analysis of chiral PNA monomers and oligomers performed on a Chirasil-Val column: (a) **L-1**; (b) **D-2**; (c) H-GTAGAT$_{\text{L-Phe}}$CACT-NH$_2$ synthesized using HATU/DIEA coupling; (d) **L-3**; (e) **D-3**; (f) H-GTAGAT$_{\text{D-Lys}}$CACT-NH$_2$ synthesized using DEPBT/DIEA coupling.

2.2. Study of the coupling conditions on model systems

In order to study the process of racemization during the activation of the monomers, we performed the coupling of **L-1** and **L-2** to L-ValOMe with different coupling reagents (shown in Scheme 2) and bases. HBTU and HATU were chosen since they are widely used in peptide chemistry, and in particular in PNA synthesis. TDBTU is an analogous uronium salt containing a 3,4-dihydro-4-oxo-1,2,3-benzotriazine residue. DEPBT is a new coupling reagent claimed to give very low racemization[30] and PyFNOP has been reported to be a fast chemoselective coupling reagent.[31,32]

DIC is a carbodiimide reagent commonly used in combination with HOBt.

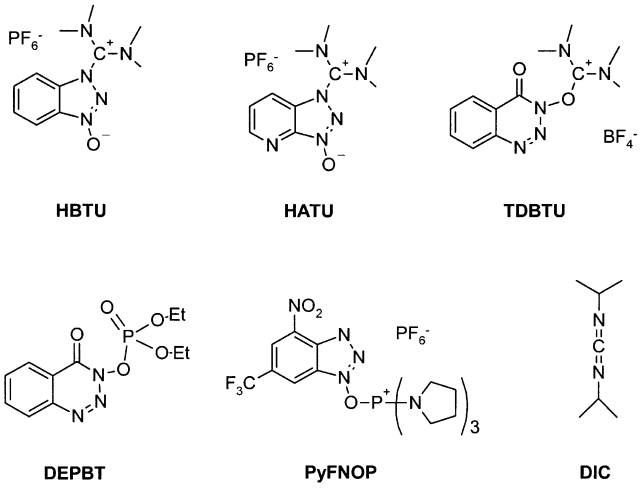
HBTU, TDBTU, HATU, DEPBT, PyFNOP are used in combination with a base. For these experiments we used the stronger base DIEA (pK_a 10.1) and the weaker TMP (collidine, pK_a 7.43), since for uronium coupling reagents (HBTU and HATU) they were found to induce different degrees of epimerization.[19,33]

The coupling conditions employed a mixture of two different solvents: DMF and DCM, which have been shown to give much higher yields and to reduce racemization in peptide synthesis. After quenching the reaction with a saturated aqueous $NaHCO_3$ solution, the organic phase was analysed by RP-HPLC to check the diastereomeric purity. The results obtained are reported in Table 2.

The phenylalanine monomer **L-2** was found to be somewhat less prone to racemization than alanine **L-1** (compare entries 2 and 8 in Table 2). However, the influence of the coupling conditions on racemization was found to be similar for both monomers.

As expected, racemization increased with the preactivation time and was dependent on the coupling reagent. In the case of PyFNOP a high level of racemization was found even after 1 min of preactivation (entry 10, Table 2).

Coupling with HATU and HBTU resulted in relatively high levels of racemization, in contrast with observations in peptide synthesis, where low-racemization couplings can usually be performed using these reagents.



Scheme 2.

Furthermore, HATU was found to give rise to more rapid racemization. In fact while for short preactivation times only a slight difference was found for these two reagents, very long preactivation (30 min) gave rise to dramatic differences (compare entries 15 and 16 in Table 2). The best results were obtained with the reagents TDBTU and especially DEBPT containing a 3,4-dihydro-4-oxo-1,2,3-benzotriazine with 1 min of preactivation time.

Using Collidine instead of DIEA doubled the amount of racemization in the case of HBTU and TDBTU (compare entries 11, 19 and 13, 21 in Table 2). However, coupling with HATU or DEPBT was not influenced by the base (entries 12, 20 and 14, 22).

2.3. Racemization during oligomer synthesis

In order to investigate the racemization occuring in the solid-phase synthesis of chiral PNAs, oligomers with the sequences H-GTAGAT$_{\text{L-Phe}}$CACT-NH$_2$, H-GTAGAT$_{\text{D-Lys}}$CACT-NH$_2$, H-T$_{\text{L-Phe}}$ACT$_{\text{L-Phe}}$CAT$_{\text{L-Phe}}$-ACTCT-NH$_2$ were synthesized by manual standard SPPS on a MBHA resin (loading 0.2 mmol/g) using a Boc protocol.[18] The modified monomers were introduced in the middle of the oligomers using different coupling reagents and conditions, chosen according to the previous preactivation experiments. A mixture of DCM and DMF was used to dissolve TDBTU and DEPBT; DIEA was added as a base. To avoid the risk of guanidinium formation and of racemization, the preactivation time was kept as short as possible (50 s). Double coupling was always performed. Successful couplings were monitored with the Kaiser test; all the reagents gave negative tests after the first coupling except DIC/HOBt. All raw PNAs were obtained in good yields (>97%). The amount of racemization was checked by using the GC–MS method described above, since the HPLC method is not applicable in this case.

As shown in Table 3, the performance of the coupling reagents in solid-phase synthesis is similar to that observed in the preactivation experiments (Table 2) (except for DEPBT).

The best result was obtained with DIC/HOBt, for which a higher enantiomeric purity of the final products was obtained (although a double coupling was necessary). Under these conditions a high chemical yield and a minimum epimerization were achieved.

Table 2. Effect of the coupling conditions on racemization in the model reaction with L-ValOMe[a]

Entry	Monomer	Preactivation time (min)	Coupling conditions	D/(D+L) (%)
1	**L-1** (T$_{\text{L-Ala}}$)	0	TDBTU/DIEA	1.1
2	**L-1** (T$_{\text{L-Ala}}$)	1	TDBTU/DIEA	5.7
3	**L-1** (T$_{\text{L-Ala}}$)	1	DEPBT/DIEA	1.9
4	**L-1** (T$_{\text{L-Ala}}$)	30	DEPBT/DIEA	36.5
5	**L-1** (T$_{\text{L-Ala}}$)	3	HATU/collidine	21.4
6	**L-2** (T$_{\text{L-Phe}}$)	1	HBTU/DIEA	5.0
7	**L-2** (T$_{\text{L-Phe}}$)	1	HATU/DIEA	6.5
8	**L-2** (T$_{\text{L-Phe}}$)	1	TDBTU/DIEA	2.7
9	**L-2** (T$_{\text{L-Phe}}$)	1	DEPBT/DIEA	1.4
10	**L-2** (T$_{\text{L-Phe}}$)	1	PyFNOP/DIEA	32.7
11	**L-2** (T$_{\text{L-Phe}}$)	3	HBTU/DIEA	8.2
12	**L-2** (T$_{\text{L-Phe}}$)	3	HATU/DIEA	11.4
13	**L-2** (T$_{\text{L-Phe}}$)	3	TDBTU/DIEA	4.1
14	**L-2** (T$_{\text{L-Phe}}$)	3	DEPBT/DIEA	2.1
15	**L-2** (T$_{\text{L-Phe}}$)	30	HBTU/DIEA	11.7
16	**L-2** (T$_{\text{L-Phe}}$)	30	HATU/DIEA	31.9
17	**L-2** (T$_{\text{L-Phe}}$)	30	TDBTU/DIEA	7.0
18	**L-2** (T$_{\text{L-Phe}}$)	30	DEPBT/DIEA	22.9
19	**L-2** (T$_{\text{L-Phe}}$)	3	HBTU/collidine	16.1
20	**L-2** (T$_{\text{L-Phe}}$)	3	HATU/collidine	11.5
21	**L-2** (T$_{\text{L-Phe}}$)	3	TDBTU/collidine	8.6
22	**L-2** (T$_{\text{L-Phe}}$)	3	DEPBT/collidine	2.2

[a] Reactions were carried out for 20 min at room temperature after preactivation; yields were in all cases quantitative, as detected by HPLC.

Table 3. Enantiomeric purity of PNA oligomers obtained by SPPS using different coupling conditions

Sample	Coupling conditions	% Minor isomer[a]
H-GTAGA$\mathbf{T_{L\text{-}Phe}}$CACT-$NH_2$	DEBPT/DIEA	5.9 (0.3)
	TDBTU/DIEA	4.6 (0.4)
	HBTU/DIEA	5.7 (0.4)
	HATU/DIEA	8.9 (0.3)
	DIC/HOBt	3.4 (0.4)
H-GTAGA$\mathbf{T_{D\text{-}Lys}}$CACT-$NH_2$	DEPBT/DIEA	10.5 (0.4)
	TDBTU/DIEA	6.2 (0.6)
H-$\mathbf{T_{L\text{-}Phe}}A\mathbf{CT_{L\text{-}Phe}}C\mathbf{AT_{L\text{-}Phe}}$-ACTCT-$NH_2$	DIC/HOBt	2.3 (0.3)

[a] Standard deviations are given in parentheses.

2.4. NMR study of the epimerization mechanism

It is well known that N^{α}-acylated chiral amino acids undergo extensive racemization during peptide synthesis.[34] At least two possible mechanisms operate: racemization occurs either through the abstraction of the α proton from the activated intermediate or through 2-alkyl-5(4*H*)-oxazolones. Recently it has been shown that urethane (Boc, Z, Fmoc) protected amino acids also undergo cyclization to 2-alkoxy-5(4*H*)-oxazolones, but this does not usually result in significant racemization, probably because 2-alkoxy-5(4*H*)-oxazolones react faster with the nucleophilic amine than 2-alkyl-5(4*H*)-oxazolones.[35] Urethane protected amino acids are therefore resistant to racemization. Functional PNAs have no urethane protecting group but do have an alkyl and an acyl group on the corresponding N^{α}-nitrogen and are therefore more prone to racemization. However, *N*-alkylated-*N*-acylamino acids, when treated with carbodiimide reagents, gave rise to oxazolonium ions,[36] which were detected by NMR.[37] These species are prone to racemization even without added base but it was possible to couple *N*-benzoyl-*N*-methyl-L-alanine with L-valine methyl ester by means of HOBt/DCC when the reaction was performed at 0°C without preactivation. Z-Gly-L-MeAla-OBt was shown to be more resistant to racemization implying that the benzoyl group exacerbates racemization more than glycine.

The most surprising data from the results obtained for the coupling of chiral PNAs is the poor performance of HATU as compared to HBTU. Furthermore, while the extent of racemization for HBTU and TDBTU was dependent on the base used (DIEA gave considerably better results than collidine), this was not the case when HATU or DEPBT was used. These observations suggest a different racemization mechanism for these coupling reagents.

In order to clarify the racemization mechanism, we performed NMR measurements of the monomer **L-2** ($T_{L\text{-}Phe}$) after activation with HATU and DIEA in $CDCl_3$/DMSO-d_6 = 1:1 mixture. In Fig. 3 the chemical shifts observed in this experiment are reported. At room temperature, a species consistent with the activated 7-azabenzotriazolyl ester of the monomer **7** was

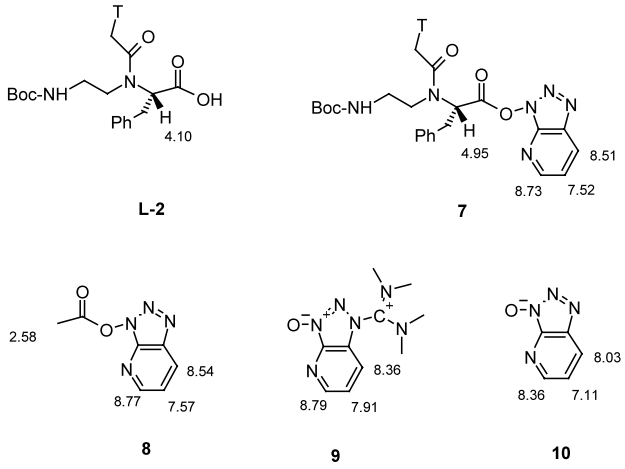


Figure 3. NMR chemical shifts (ppm) of α- and 7-azabenzotriazolyl protons for species involved in activation using HATU in $CDCl_3$/DMSO-d_6 = 1:1 at 300 K.

detected, whose assignment was confirmed by comparing its aromatic moiety with that of the activated ester **8** obtained from acetic acid. A downfield shift (0.85 ppm) of the α-hydrogen signal is also consistent with this hypothesis, since a similar shift was observed in the case of acetic acid (1.88 ppm for the free acid, 2.58 ppm for the activated ester).

This indicates that the oxazolonium salt (Fig. 4a) is not formed under these conditions, since this should give rise to the appearance of the anion **10**.

Similar results were obtained using HBTU, although the rate of formation of the activated ester was significantly lower. Though acylated benzotriazole can exist in both *N*-acyl or *O*-acyl forms,[38] only the *O*-acyl form of the activated ester of **L-2** was detected by NMR, according to benzotriazole chemical shifts.

The conditions used in the coupling with the carbodiimide derivative DIC were also studied by NMR. We found that during preactivation with DIC and HOBt a very low amount of the activated ester is present after 5 min, which is in agreement with a recently reported work.[39] Therefore, in this protocol, only partial preacti-

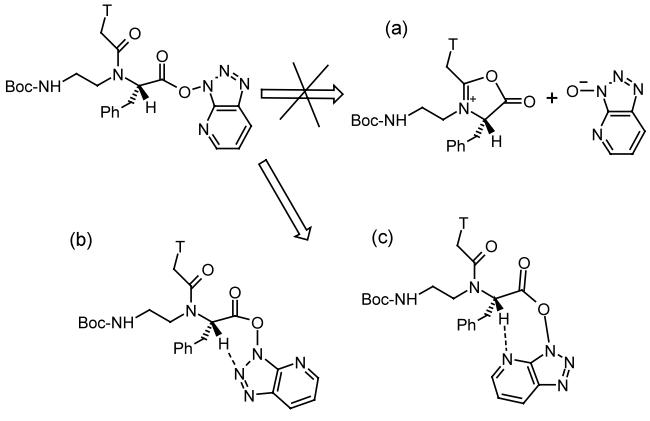


Figure 4. Racemization mechanisms proposed.

vation takes place before the reagents are put in the reactor, where the amino groups are present. Therefore, racemization is low but also the coupling yields turned out to be lower than in other protocols.

All of the above considerations are consistent with the previously proposed mechanism of racemization of *N*-alkyl-*N*-acylamino acids hydroxybenzotriazolyl esters, which proceeds through abstraction of the α-hydrogen from the activated ester (Fig. 4). There is no evidence of the formation of oxazolonium ion intermediates (Fig. 4a), as previously reported for *N*-alkyl-*N*-acylamino acid in the presence of HOBt, for which an intramolecular mechanism was proposed (Fig. 4b).[37] The observation that racemization with HATU is independent of the base used suggests a more efficient intramolecular mechanism for hydrogen abstraction from the activated ester **7**, maybe due to the assistance of the N(7) nitrogen atom (Fig. 4c).

Furthermore, HBTU reacts with the amino groups of the growing chain more slowly than HATU. Therefore, a significant decrease of the base strength can considerably increase the lifetime of the active ester, which is prone to racemization. A similar effect, but with lower racemization rates was observed with the 3,4-dihydro-4-oxo-1,2,3-benzotriazine derivative TDBTU.

3. Conclusions

In the present study we have shown that racemization of chiral PNA monomers during solid-phase synthesis is strongly dependent on the reaction conditions used. The use of HATU, which is strongly recommended in the case of peptide segment coupling, is not optimal in the case of chiral PNAs, due to the higher racemization rate. The racemization process has been found to proceed through α-hydrogen abstraction from the activated ester formed. By carefully choosing the coupling conditions, chiral PNAs of high enantiomeric purity can be obtained. In particular, the best results were obtained using a DIC/HOBt combination, without preactivation in the presence of a base. Studies are now in progress to investigate the effect of PNA enantiomeric purity on the binding properties towards DNA.

4. Experimental

4.1. General

TDBTU was purchased from Fluka; DIC and HOBt from Aldrich; HBTU, HATU, MBHA resin and achiral PNA monomers were obtained from Perseptive Biosystems; DEPBT was a gift from Dr. Xavier Doisy; PyFNOP was a gift from Dr. Thomas Boesen. HCl-ValOMe, DIEA and collidine were purchased from Aldrich. All the six PNA chiral monomers were synthesized according to literature.[14] All solvents were of HPLC grade purity from LAB-SCAN. *N*,*N*′-Dimethylformamide, dichloromethane and pyridine were dried over 4 Å molecular sieves prior to use.

4.2. Test coupling reactions

All the coupling tests in solution were carried out using an 'in situ neutralization' method: the L-Ala /L-Phe monomers (0.05 mmol) were dissolved in DMF (0.2 ml) and added to the coupling reagent dissolved/suspended in DCM (0.12 ml). The appropriate base (0.14 mmol) was added and the solution was allowed to stand for the preactivation time at room temperature and then added to HCl·Val-OMe (0.08 mmol). The reaction was quenched after 20 min by addition of a saturated solution of $NaHCO_3$ (0.5 ml) and DCM (0.5 ml). The dimer was purified by multiple extractions with sat. $NaHCO_3$, brine, citric acid and brine.

The products were obtained in quantitative yields, according to the HPLC analysis.

4.3. HPLC analysis

The diastereomers of the dimer were separated by RP-HPLC using RP-C18 column. The system was equipped with a UV detector. A mixture of two eluents was used: A: 0.1% TFA in H_2O; B: CH_3CN 90%, H_2O 10%, TFA 0.1%. The gradient was: 0–2 min: 100% A, 2–35 min: 50% A-50% B, 35–40 min: 100% B. The flow rate was 1 ml/min.

4.4. Synthesis of PNA oligomers

PNA oligomers with the sequences: H-GTAGAT$_{\text{L-Phe}}$-CACT-NH_2, H-GTAGAT$_{\text{D-Lys}}$CACT-NH_2, H-T$_{\text{L-Phe}}$-ACT$_{\text{L-Phe}}$CAT$_{\text{L-Phe}}$ACTCT-NH_2 were synthesized by using the SPPS standard procedure with Boc protocol. The normal HBTU/DIEA protocol on 50 mg of MBHA–polystyrene resin for PNA monomers[18] was used except in the coupling of the chiral PNA monomers.

The modified monomers, T$_{\text{L-Phe}}$ and T$_{\text{D-Lys}}$ (0.08 mmol) were dissolved in DMF (0.31 ml). The solution of each monomer was added to the coupling reagent; TDBTU/DEPBT/HATU/HBTU (0.07 mmol) were suspended in CH_2Cl_2 (0.2 ml) and then DIEA was added (0.03 ml, 0.176 mmol).

In the case of the DIC/OHBt coupling method, the resin was neutralized (after deprotection with TFA) using 5% of DIEA in DCM. HOBt was dissolved in DMF (0.2 ml), DIC was added and the solution was mixed with that containing the modified monomers.

The coupling mixtures were vortexed for 50 s and then added to the resin. The coupling was allowed to proceed for 20 min in all the reactions. A double coupling (second coupling was allowed to proceed for 1 h) was performed even though the Kaiser test indicated complete reaction.

The oligomers synthesized were purified by RP-HPLC and characterized by MALDI-TOF: H-GTAGAT$_{\text{L-Phe}}$-CACT-NH_2 MW: calculated: 2815.2, found: 2816.9 (DEPT/DIEA), 2817.2 (TDBTU/DIEA), 2817.1

(HBTU/DIEA), 2816.5 (HATU/DIEA), 2815.7 (DIC/HOBt); H-GTAGAT$_{D\text{-}Lys}$CACT-NH$_2$ MW: calculated 2796.2, found: 2798.1 (DEBPT/DIEA), 2797.8 (TDBTU/DIEA); H-T$_{L\text{-}Phe}$ACT$_{L\text{-}Phe}$CAT$_{L\text{-}Phe}$ACTCT-NH$_2$ MW: calculated: 3576, found: 3577.

Crude yields were >97% in all cases.

4.5. GC–MS analysis

The GC–MS experiments were performed by following the derivatization method previously reported in literature for the direct separation of *N*-aminoethyl-α-amino acids and applied to the determination of the enantiomeric excess of chiral PNA monomers and oligomers.[28]

Using the same procedure, the PNA monomers and oligomers (1–2 mg) were hydrolysed at 100°C with 6N HCl (2 ml) for 6 h to the corresponding *N*-aminoethyl-α-amino acids, then they were suspended in DCM (2 ml) and treated with TFAA (0.3 ml) for 1 h at 60°C. After removal of excess reagent by evaporation under vacuum, the samples were dissolved in DCM and injected for GC analysis (1 μl). Analyses were performed with a chiral capillary column: Chirasil-Val, 10 m, ID = 0.25 mm, film thickness: 0.12 μm, carrier: He, flow: 1.1 ml/min, injector temperature: 230°C, detector temperature: 230°C, detection: MS, SIM mode.

Different temperature conditions and different detection modes were chosen for each *N*-aminoethyl-α-amino acid: for Ala derivatives: 60°C (3 min), 60–180°C (20°C/min), SIM detection (m/z = 113, 167, 210); for Phe derivatives: isotherm 180°C, SIM detection (m/z = 91, 286, 167); for Lys derivatives: isotherm 190°C, SIM detection (m/z = 140, 167, 196).

4.6. NMR measurements

^{1}H NMR measurements were performed at 300 MHz on a Bruker AC-300 instrument using a mixture of DMSO-d_6: $CDCl_3$ = 1:1 with TMS as internal standard at 300 K.

In the acetic acid activation experiments, HATU or HBTU (0.05 mmol) were dissolved in DMSO-d_6: $CDCl_3$ = 1:1, then CH_3COOH (0.05 mmol) and DIEA (0.05 mmol) were added and the tube was shaken for less then 50 s. The spectra were collected immediately, after 30 and 60 min for each sample. Alternatively, DIC (0.06 mmol) was dissolved in DMSO-d_6: $CDCl_3$ = 1:1, then CH_3COOH (0.06 mmol) and HOBt (0.06 mmol) were introduced directly in the NMR tube; the mixture was shaken for less then 50 s. The spectra were collected immediately and after 5, 10, 30, and 60 min.

In the experiments involving the activation of the PNA monomer, **L-2** (0.03 mmol) was dissolved in DMSO-d_6: $CDCl_3$ = 1:1, then HATU or HBTU (0.03 mmol) and DIEA (0.03 mmol) were introduced directly in the NMR tube and the mixture was shaken for less then 50 s. The spectra were collected immediately and after 10, 30, and 60 min for each sample.

Alternatively, DIC (0.032 mmol) was dissolved in DMSO-d_6: $CDCl_3$ = 1:1, **L-2** monomer (0.032 mmol) and HOBt (0.06 mmol) were introduced directly into the NMR tube; the mixture was shaken for less then 50 s. The spectra were collected immediately and then after 5, 10, 30, and 60 min.

Acknowledgements

This work was supported by a grant from CNR (Consiglio Nazionale delle Ricerche, Rome Target Project 'Biotechnology') and 'Forkningsstyrelsen' (PEN). The NMR facilities were made available by the Centro Interfacoltà Misure of the University of Parma. We thank Miss Jolanta Ludvigsen for HPLC and MALDI analyses.

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