Pergamon

Tetrahedron: *Asymmetry* 10 (1999) 2063–2066

TETRAHEDRON: *ASYMMETRY*

Direct enantiomeric separation of *N*-aminoethylamino acids: determination of the enantiomeric excess of chiral peptide nucleic acids (PNAs) by GC

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Received 9 April 1999; accepted 24 May 1999

Abstract

Direct chiral separation of *N*-aminoethylamino acids has been performed for the first time by gas chromatographic (GC) analysis with a Chirasil-Val column, after derivatisation with trifluoroacetic anhydride in dichloromethane, which led to the corresponding piperazin-2-one derivatives, as identified by NMR and GC/MS analysis. The method was used for the analysis of the enantiomeric excess of chiral peptide nucleic acid (PNA) monomers and oligomers after hydrolysis with 6N HCl. © 1999 Elsevier Science Ltd. All rights reserved.

Peptide nucleic acids are oligonucleotide mimics in which the sugar–phosphate backbone has been replaced by a pseudopeptide chain of *N*-aminoethylglycine monomers (Fig. 1, R=H). PNAs were shown to bind to complementary DNA or RNA sequences with high affinity and specificity.[1–3] On account of these properties, they are widely used in molecular biology and biotechnology, as tools in genetic diagnostics, for specific regulation of gene expression, and are currently being investigated as potential antiviral and anticancer drugs.[4] Chiral PNAs derived from *N*-aminoethylamino acids[5] are particularly interesting because they induce a preferred handedness in the oligomers[6] and may introduce other functionalities into the PNA backbone, by means of the amino acid side chain.

However, the solid phase peptide synthesis (SPPS) procedures used to date (Boc-protection and coupling with HBTU in the presence of dicyclohexylethylamine)[6] may not be adequate for the synthesis of chiral PNAs since the monomers, being *N*-acylated amino acids, can undergo racemisation during coupling, as shown for some PNA analogues.[7] The *N*-aminoethylamino acids (Ae-AA, **1**, Fig. 1) used as synthons for the PNA monomers are reduced dipeptides (pseudopeptides) of the Gly-r-AA type. Pseudopeptides have been used as antiviral (e.g. anti-HIV) drugs, as potential synthetic vaccines, or

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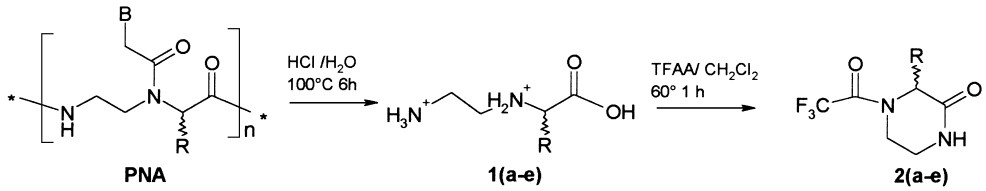
PII: S0957-4166(99)00210-4



R =CH_2-Ph (**a**); CH_3 (**b**); $CH(CH_3)_2$ (**c**); $CH_2CH(CH_3)_2$ (**d**); $(CH_2)_4$-NH_2 (**e**)

Figure 1. General formula and derivatisation of PNA oligomers and *N*-aminoethylamino acids Ae-AA **1a–e**

as components of peptide libraries.[8] However, the enantiomeric separation of *N*-aminoethylamino acids by direct methods has not been reported in the literature.

In recent years, we have developed chromatographic methods (HPLC[9] and GC[10]) for the separation of D- and L-amino acids, and have applied them to the determination of D-amino acids in foods and biological samples.[11,12] *N*-Aminoethylamino acid enantiomers could not be separated by these or other conventional methods, due to the interference of the additional aminoethyl group in the recognition mechanism.

However, during the standard derivatisation conditions used in GC for the separation of amino acid enantiomers by treatment with trifluoroacetic anhydride in dichloromethane (60°C, 1 h), *N*-aminoethylphenylalanine **1a** was found to undergo cyclisation to yield the trifluoroacetylated 3-benzyl-4-trifluoroacetylpiperazin-2-one **2a** (Fig. 1). Enantiomers of **2a** could be separated by gas chromatography using a Chirasil-Val (Chrompack, NL) column, with MS detection.

Each enantiomer of **2a** was isolated by flash chromatography and fully characterised.† The enantiomeric separation of **2a** by GC was very good, as shown in Fig. 2a. Neither enantiomer showed any trace of racemisation above the detection limit (0.3% in scan mode), indicating that the derivatisation procedure does not affect the enantiomeric composition of the analytes.

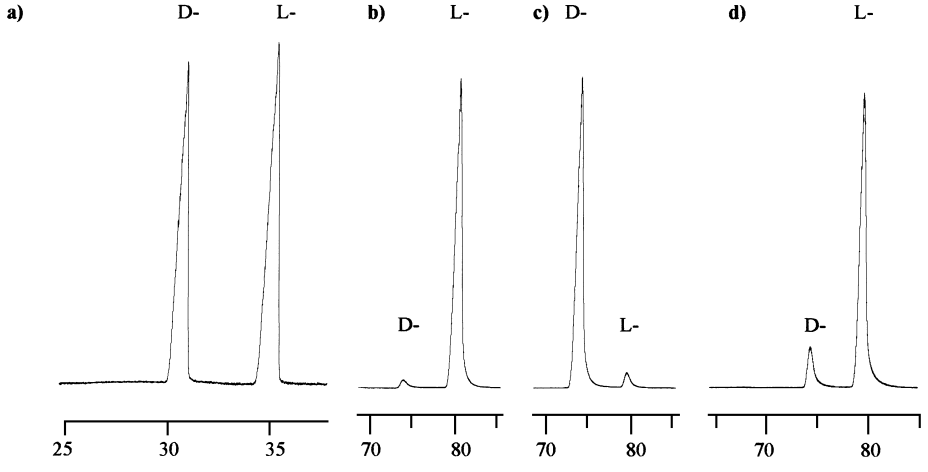


Figure 2. Enantiomeric analysis of: (a) D,L-Ae-Phe; (b) $T_{L\text{-}Lys}$; (c) $T_{D\text{-}Lys}$; (d) PNA $GTAGAT_{L\text{-}Lys}CACT$. Conditions: column Chirasil-Val 25 m, i.d. 0.25 mm, carrier: He 1.1 mL/min, temperature programme: (a) 150°C (3 min), 150–180°C 4°C/min, 180°C final isotherm, detector: MS scan mode (40–500 a.m.u.); (b); (c); (d): 60°C (3 min), 60–190°C 20°C/min, 190°C final isotherm, detector: MS SIM mode (*m/z*=196)

† Compound **2a** (both enantiomers) was fully characterised by IR, NMR and MS analysis.

Table 1
Retention times and enantioselectivity factors ($\alpha=t'_L/t'_D$) observed for the separation of piperazine-2-one derivatives obtained from *N*-aminoethylamino acids (Ae-AA) by GC under isothermal conditions

Ae-AA	t (°C)	t'_D (min)	t'_L(min)	α
Ae-Ala (**1b**)	140	2.45	2.90	1.184
Ae-Val (**1c**)	170	6.65	7.51	1.129
Ae-Leu (**1d**)	170	9.64	10.75	1.115
Ae-Phe (**1a**)	170	42.38	51.16	1.207
Ae-Lys (**1e**)	190	71.28	79.95	1.122

Conditions: Chromatograph HP5890; column: Chirasil-Val (Chrompack, 25 m, i.d. = 0.25 mm, film thickness= 0.12 μm); carrier: He, flow rate = 1.1 mL/min.

Enantiomeric separation of other *N*-aminoethylamino acids **1b**–**e** (obtained from *N*-Boc-protected aminoethylamino acid benzyl esters by acid hydrolysis) was performed using the same derivatisation procedure.[‡] The enantioselectivity factors $\alpha=t'_L/t'_D$ are reported in Table 1.

Very good separations were obtained for Ae-Ala **1b**, Ae-Val **1c**, Ae-Leu **1d** and Ae-Phe **1a**, although at higher temperatures than those commonly used for the separation of amino acid enantiomers. The separation of the piperazine-2-one derivative obtained from Ae-Lys **1e** required even higher temperatures (190°C), and showed considerable peak broadening.

In order to verify if this method can be applied to detect racemisation in PNA monomers, we analysed *N*-(Boc-aminoethyl)-*N*-thyminylethanoyl-L-leucine (Boc-$T_{L\text{-}Leu}$-OH), which was synthesised according to the literature method.[5] The monomer was first hydrolysed with 6N HCl and then treated with trifluoroacetic anhydride as described above and was found to contain 7% (s.d.=1%) of the undesired D-enantiomer.[§] The present method proved to be useful for detecting enantiomeric contamination in a Boc-$T_{D\text{-}Leu}$-OH monomer, which was found to contain 44.1% (s.d.=0.5%) of the L-enantiomer.[¶]

The same procedure was used for evaluating the enantiomeric purity of Boc-protected thymine containing monomers derived from D- or L-N^{α}-aminoethyl-N^{ϵ}-(2-chloro-*Z*)Lys ($T_{D\text{-}Lys}$ and $T_{L\text{-}Lys}$, Fig. 2c and b), which turned out to contain low percentages of the other enantiomer (4.4% for $T_{D\text{-}Lys}$ and 2.4% for $T_{L\text{-}Lys}$, s.d.=0.5%). Thus, racemisation takes place during the preparation of the monomers, the relative percentage depending on the amino acid side chain.

Moreover, racemisation also occurs during the solid-phase synthesis of oligomers, as shown by a PNA decamer (GTAGAT$_{L\text{-}Lys}$CACT) containing only one chiral monomer synthesised from L-lysine by solid phase peptide synthesis (Fig. 2d) which showed a higher degree of racemisation (14.3% of D-Ae-Lys, s.d.=1.4%).[§]

In conclusion, it is worth noting that the SPPS protocols used for PNAs,[6] which are generally satisfactory for amino acids, do not prevent racemisation when using chiral PNA monomers. Thus, protocols for the solid-phase synthesis of chiral PNAs need to be optimised. Only by using enantiomerically pure PNAs will it be possible to clarify the role of chirality in the molecular recognition of nucleic acids. The enantiomeric separation of monomers containing other amino acids and other nucleic bases is in progress.

[‡] Typical sample size for the analysis was 0.3–3 mg. Hydrolysis was performed with 6N HCl as described. Products were identified by their mass spectra.

[§] Hydrolysis with DCl in D_2O, performed under the same conditions (100°C for 6 h) indicated that only 0.7% of the D-isomer was due to racemisation during hydrolysis.

[¶] The same percentage of racemisation was found in the original sample of D-Leu used for the synthesis of the monomer.

Acknowledgements

This work was partially supported by a grant from CNR, Target Project on Biotechnology, and from MURST 'Application of PNAs, Rybozymes and Peptides for the Study of Gene Expression and Development of New Diagnostic and Therapeutic Strategies'.

References

1. Egholm, M.; Behrens, C.; Christensen, L.; Berg, R. H.; Nielsen, P. E.; Buchardt, O. *J. Chem. Soc., Chem. Commun*. **1993**, 800–801.
2. Jensen, K. K.; Orum, H.; Nielsen, P. E.; Nordén, B. *Biochemistry* **1997**, *36*, 5072–5077.
3. Dueholm, K. L.; Nielsen, P. E. *New J. Chem*. **1997**, *21*, 19–31.
4. Knudsen, H.; Nielsen, P. E. *Anti-Cancer Drugs* **1997**, *8*, 113–118. Good, L.; Nielsen, P. E. *Antisense and Nucleic Acid Drug Development* **1997**, *7*, 431–437.
5. (a) Haaima, G.; Lohse, A.; Buchardt, O.; Nielsen, P. E. *Angew. Chem., Int. Ed. Engl*. **1996**, *35*, 1939–1942. (b) Puschl, A.; Sforza, S.; Haaima, G.; Dahl, O.; Nielsen, P. E. *Tetrahedron Lett*. **1998**, *39*, 4707–4710.
6. Sforza, S.; Haaima, G.; Marchelli, R.; Nielsen, P. E. *Eur. J. Org. Chem*. **1999**, 197–204.
7. (a) Sforza, S. *Tesi di Dottorato*; Università di Parma, Italy, 1997. (b) van der Laan, A. C.; van Amsterdam, I.; Tesser, G. I.; van Boom, J. H.; Kuyl-Yeheskiely, E. *Nucleosides and Nucleotides* **1998**, *17*, 219–231.
8. Wen, J. J.; Spatola, A. F. *J. Pept. Res*. **1997**, *49*, 3–14.
9. Galaverna, G.; Corradini, C.; De Munari, E.; Dossena, A.; Marchelli, R. *J. Chromatogr.* **1993**, *657*, 43–54.
10. Corradini, R.; Marchelli, R.; Palla, G. *Chromatographia* **1994**, *38*, 173–176.
11. Gandolfi, I.; Palla, G.; Delprato, L.; De Nisco, F.; Marchelli, R.; Salvadori, C. *J. Food Sci*. **1992**, *57*, 377–379.
12. Marchelli, R.; Dossena, A.; Palla, G. *Trends in Food Science and Technology* **1996**, *7*, 113–119.