Pergamon

Tetrahedron Letters, Vol. 36, No. 38, pp. 6941-6944, 1995
Elsevier Science Ltd
Printed in Great Britain
0040-4039/95 $9.50+0.00

0040-4039(95)01375-X

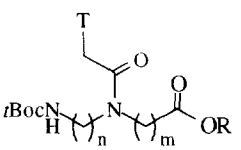
Synthesis of 'Retro-Inverso' Peptide Nucleic Acids: 2. Oligomerization and Stability

Achim H. Krotz[§#], Ole Buchardt[§†] and Peter E. Nielsen*

Center for Biomolecular Recognition, IMBG, Dept. of Biochemistry B, The Panum Institute, Blegdamsvej 3c, DK-2200 Copenhagen N, Denmark, FAX +45-31396042

Abstract: Novel PNA oligomers containing an *N*-(aminomethyl) β-alanine backbone ('retro-inverso' PNA) were prepared *via* blocksynthesis or *via* a combination of solution and solid-phase chemistry. The half-life time of the unprotected N terminus is 38 h at pH 7.0 (20 °C) and is pH dependent.

In the previous communication we reported the preparation of novel PNA monomers of thymine (**1**) and N^6-Cbz adenine with an *N*-(aminomethyl) β-alanine backbone suitable for the synthesis of 'retro-inverso' PNA oligomers.[1] For the initial investigations of the hybridization properties of the new backbone modification we chose three types of poly-T oligomers: *N*-(aminoethyl) glycine PNA with one modification T* (**I**), *N*-(aminoethyl) glycine PNA with one T* and one β-ala-T monomer at the N terminus of T* (**II**) and a polyT* PNA (**III**).

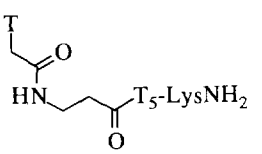


n=1, m=2: T* (R=H: **1**, R=Et **2**)
n=2, m=1: T
n=2, m=2: β-ala-T

$H\text{-}T_4\text{-}T^*\text{-}T_5\text{-}LysNH_2$
I

$H\text{-}T_3\text{-}(\beta\text{-ala-}T)\text{-}T^*\text{-}T_5\text{-}LysNH_2$
II

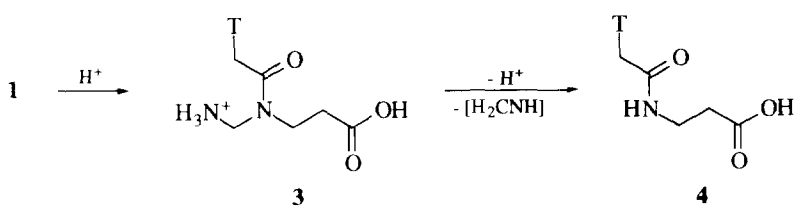
$t\text{Boc-}T^*{}_8\text{-OEt}$
III



Ia

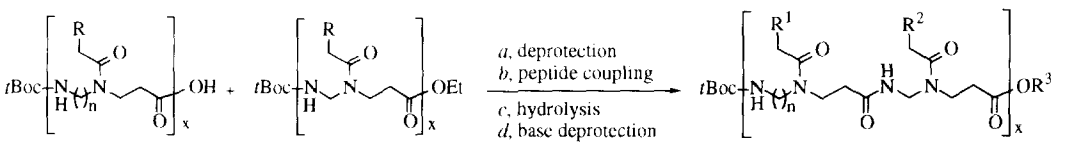
For protection of the N terminus of the monomers we chose an acid-labile carbamate protecting group (*t*Boc) to reduce degradation during deprotection as we expected a higher stability of the ammonium form as compared to the free amine of the unprotected N terminus. Treatment of **1** with trifluoroacetic acid (TFA) at ambient temperature furnished **3** within minutes [95% TFA/cresol (5 min), 50% TFA/CH_2Cl_2 (20 min)]. Heating of **1** at elevated temperature (HCl/1,4-dioxane, 100 °C, 5 min) resulted in quantitative degradation and formation of **4** (Scheme 1).

Scheme 1



3 **4**

Following essentially standard methods of peptide chemistry, PNA oligomers containing the four natural nucleobases are obtained with high coupling efficiency (>99%).[2] The 10mer H-T_4-T*-T_5-LysNH_2 (**I**) was assembled purely by coupling of monomer units on a solid support using our standard protocol. The analytical HPLC profile indicated two main peaks. FAB mass spectroscopy (FAB-MS)[3] of fraction 1 (34 %) showed a peak at *m/z* 1714.1 (calcd 1713.7 $(M+H)^+$), corresponding to a truncated oligomer **Ib**, which we attribute to the degradation of the N terminus of T* after deprotection during solid phase synthesis (acc. Scheme 1). The secondary amide which then forms the new N terminus is no longer reactive in subsequent coupling reactions and hence chain elongation is terminated. FAB-MS of fraction 2 (31%) showed a peak at *m/z* 2808.1 (calcd 2808.0 $(M+H)^+$) corresponding to the full length oligomer **I**. As indicated by the 1:1 ratio of **I** and **Ib**, peptide coupling of the *N*-(aminomethyl) amide terminus of T* takes place to a significant extent, but the competing degradation of the growing chain imposes major restrictions on the synthesis of oligomers containing multiple modifications T*. An attempt to synthesize a 10mer H-T*$_{10}$-LysNH_2 using the same protocol resulted mainly in formation of shorter fragments according to HPLC and FAB-MS.



a, TFA/CH_2Cl_2 1:1, *v/v*, 20 min; *b*, HBTU, DMF/pyridine/iPr_2EtN; *c*, LiOH/THF; *d*, H_2, Pd/C

	x	R^1	R^2	R^3	n	General procedure	Yield[a] [%]	FAB-MS $[M^+]$ *m/z* found (calcd)
5	1	T	T	Et	2	A, B	66	693.6 (693.3)
6	1	T	T	H	2	C	97	671.3 (665.3)
7	1	T	T	Et	1	A, B	56	679.5 (679.3)
8	1	T	T	H	1	C	99	657.3[b] (651.3)
9	2	T	T	Et	1	A, B	42	1211.5 (1211.5)
10	2	T	T	H	1	C	90	1183.2 (1183.5)
11	4	T	T	Et	1	A, B	31	2276.8 (2276.9)
12	1	(Cbz)-A	T	Et	1	A, B	66	822.1 (822.4)
13	1	(Cbz)-A	T	H	1	C	96	795.0 (794.3)
14	2	(Cbz)-A	T	Et	1	A, B	33	1497.4 (1497.6)
15	2	A	T	Et	1	D	99	1229.9 (1229.5)
16	1	(Cbz)-A	(Cbz)-A	Et	1	A, B	62	965.8 (965.4)
17	1	(Cbz)-A	(Cbz)-A	H	1	C	95	938.9 (937.4)

[a] Purity established by HPLC [b] $[M+Li]^+$

Table 1: Preparation and MS characterization of 'retro-inverso' PNA oligomers.

In an attempt to overcome this problem we modified our synthetic strategy for H-T_3-(β–ala-T)-T*-T_5-LysNH_2 (**II**) which for other reasons also contains a β-alanine-T monomer. We prepared a dimer *t*Boc-(β–ala-T)-T*-COOR by peptide coupling in solution (**5**, R = Et) and subsequent hydrolysis (**6**, R = H) (Table 1) which we then included as dimer building block in the solid phase synthesis. β–ala-T with a stable *N*-(2-aminoethyl) amide terminus served as protecting group of the labile *N*-(aminomethyl) amide terminus of T*. As expected, we obtained **II** as the major product by prep. HPLC [FAB-MS: *m/z* 2822.3 (calcd 2822.0 $(M+H)^+$]. The combination of solution and solid phase chemistry would be the method of choice for the synthesis of oligomers containing only a few modifications with an *N*-(aminomethyl) terminus.

For the synthesis of oligoT* PNA, we changed our strategy completely from solid-phase to block synthesis in solution, thus reducing the number of crucial coupling steps from seven to three (Table 1). The N terminus of **2** was deprotected with TFA/CH_2Cl_2 (1:1 *v/v*) and coupled with the activated [*O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU)] C terminus of **1**, affording dimer-ester **7**. Saponification (LiOH, THF/water) of **7** furnished acid **8** and dimerization of **7** and **8** (as described for **1** and **2**) afforded tetramer-ester **9** and, after hydrolysis, tetramer-acid **10**. Condensation of **9** and **10** afforded H-T*$_8$-OC_2H_5 (**11**). Both mixed purine-pyrimidine [*t*Boc-A^{Cbz}T-OR (**12**, **13**), *t*Boc-$A^{Cbz}TA^{Cbz}$T-OEt (**14**)) and purine-purine sequences *t*Boc-$A^{Cbz}{}_2$-OR (**16**, **17**) (R = Et, H) have also been synthesized in solution. The deprotection of N^6 of adenine of **14** by catalytic hydrogenation (Pd/C) under atmospheric pressure furnished **15** in a clean and quantitative reaction.

Coupling of the N terminus of **1** to the C terminus of another monomer affords the corresponding dimer in yields between 56-66%. The decrease in coupling efficiency as the molecular weight of the reactants increased may be attributed to the fact that the rate of the bimolecular coupling reaction under the conditions used in our experiments becomes sufficiently slow, so that the degradation of the N terminus becomes the yield determining factor. The yields of hydrolysis were greater 90%.

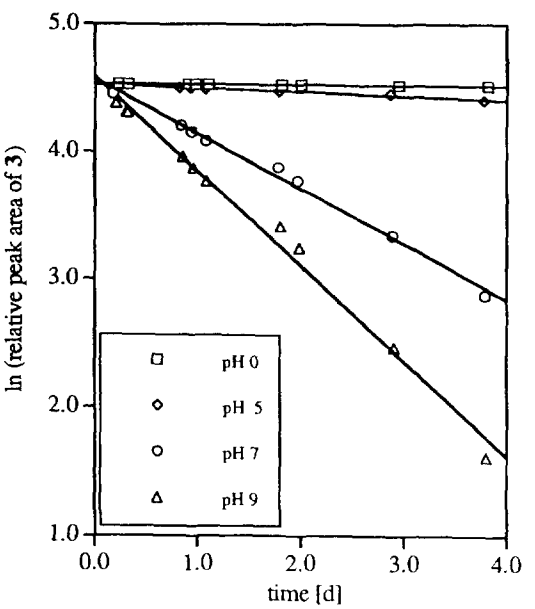


Figure 1. First-order plot of the degradation of **3** in aqueous solution at pH 0, 4, 7 and 9, respectively (Scheme 1). The first-order rate constants are 1.2 x 10^{-6} s^{-1}, 8.8 x 10^{-6} s^{-1}, 1.2 x 10^{-4} s^{-1} and 2.1 x 10^{-4} s^{-1}, respectively. Aliquots of a solution of **3** in buffered aqueous solution were analyzed by reverse-phase HPLC (λ = 260 nm) at the indicated times. Gradient: CH_3CN/H_2O/TFA 1:99:0.1 to 10:90:0.1 in 20 min, *f* = 1.5 ml min^{-1}, t_R(**3**) = 7.05 min (k'= 2.07), min, t_R(**4**) = 8.65 min (k' = 2.76), α = 1.33.

pH variations have profound effects on the degradation pathways and degradation rates for *N*-(aminoalkyl)amides.[4] Due to potential applications of PNA as DNA targeting agent in biological systems we investigated the degradation of **3** in aqueous medium as a function of pH[5] at room temperature (Scheme 1). Reverse-phase HPLC analysis showed a clean and quantitative conversion of **3** to **4**. The time course of the degradation was followed by comparing the relative peak areas of two baseline resolved peaks corresponding to **3** and **4**. A first-order plot of **3** is linear and the half-life times at pH 9 and pH 7 are 0.9 and 1.6 days, respectively (Figure 1). Under acidic conditions stabilization of the N terminus was observed and half-life times at pH 4 and 0 were extrapolated to 22 and 160 days, respectively. These results confirm the expected higher stability of the ammonium form compared to the free base and also show a considerable stability of the unprotected N terminus in the physiological pH range.

In summary, three alternative strategies (solid-phase, combination of solution and solid phase, block synthesis in solution) for the preparation of 'retro-inverso' PNA oligomers have been investigated. PNA oligomers containing only few T* modifications are most efficiently synthesized by a combination of block synthesis and solid phase synthesis. The applicability of a block synthesis approach for the preparation of oligo-T* PNA has been demonstrated. Full experimental details and the hybridization properties of the PNA oligomers with oligodeoxynucleotides will be reported in due course.[6]

Acknowledgement: This work was supported by the Danish National Research Foundation and The Danish Research Academy (a DANVIS fellowship to AHK). We thank Inga Nordstrøm and Annette W. Jørgensen for their skilful technical assistance.

References and Notes

+ The Late
§ Center for Biomolecular Recognition, The H. C. Ørsted Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark.
# Current address: ISIS Pharmaceuticals, 2292 Faraday Av., Carlsbad, CA 92008, USA.

1. See preceding paper in the issue.
2. Dueholm, K. L.; Egholm, M.; Behrens, C.; Christensen, L.; Hansen, H. F.; Vulpius, T.; Petersen, K. H.; Berg, R. H.; Nielsen, P. E.; Buchardt, O. *J. Org. Chem.* **1994**, *59*, 5767-5773.
3. Fast-atom bombardment mass spectra (FAB-MS) were taken on a Masslab VG 12-250 quadropole instrument fitted with a VG-FAB source and a JMS-Hx/Hx 110A High Performance Tandem Mass Spectrometer (JEOL). Peaks at higher *m/z* can be assigned to $[M+Li]^+$ or $[M+Na]^+$.
HPLC was carried out with a Waters 600E System Controller, equipped with a 486 Tunable Absorbance Detector on a reversed phase column (C_{18}) from Vydag using acetonitrile/water/TFA gradients.
4. For an excellent discussion of the mechanism of the hydrolysis of *N*-(aminoalkyl) amides: Loudon, G. M.; Almond, M. R.; Jacob, J. N. *J. Am. Chem. Soc* **1981**, *103*, 4508-4515.
5. Buffers: pH 0: (HCl), pH 4: (56 mM citric acid, 68 mM NaOH, 44 mM NaCl, Fluka), pH 7 (26 mM KH_2PO_4, 41 mM Na_2HPO_4, Fluka), pH 9 (13 mM sodium tetraborate, 4.6 mM HCl, Fluka).
6. Krotz, A. H.; Larsen, S.; Buchardt, O.; Nielsen, P. E., in preparation.

(*Received in UK* 11 *May* 1995; *revised* 17 *July* 1995; *accepted* 21 *July* 1995)