Sequence dependent *N*-terminal rearrangement and degradation of peptide nucleic acid (PNA) in aqueous solution

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The stability of the PNA (peptide nucleic acid) thymine monomer {*N*-[2-(thymin-1-ylacetyl)]-*N*-(2-aminoaminoethyl)glycine} and those of various PNA oligomers (5–8-mers) have been measured at room temperature (20 °C) as a function of pH. The thymine monomer undergoes *N*-acyl transfer rearrangement with a half-life of 34 days at pH 11 as analyzed by ^{1}H NMR; and two reactions, the *N*-acyl transfer and a sequential degradation, are found by HPLC analysis to occur at measurable rates for the oligomers at pH 9 or above. Dependent on the amino-terminal sequence, half-lives of 350 h to 163 days were found at pH 9. At pH 12 the half-lives ranged from 1.5 h to 21 days. The results are discussed in terms of PNA as a gene therapeutic drug as well as a possible prebiotic genetic material.

The synthetic nucleic acid mimic PNA (peptide nucleic acid) exhibits several properties that make this molecule an attractive lead compound for genetic therapeutics.[1–4] The stability of PNA in aqueous media is important for storage, handling and biological applications. It has been shown that PNA is very stable towards biological enzymes[5] as well as under normal chemical handling. However, during extended storage under high pH conditions slow decay of unprotected PNA oligomers has been observed, and has indeed been exploited in a sequencing strategy.[6] Furthermore, PNA has been suggested as a model for[7–9] or even as a *bona fide* primordial genetic material.[10] The early evolution of molecules carrying genetic information must have depended on the accumulation of proper building blocks. Therefore, such building blocks must have sufficient chemical stability in an aqueous environment to reach 'a critical concentration', and the resulting information carrying oligomers must survive long enough to 'pass on' their information. We here report on two competing reactions, an isomerization and a degradation, that lead to alterations in the *N*-terminus of the PNA strand. The conditions at which these reactions occur at observable rates have been characterized, the kinetics have been measured and reaction mechanisms are proposed.

Experimental

Methods

The PNA oligomers used were synthesized as described previously[11] and purified by RP-HPLC. The molecular mass of the PNA oligomers was confirmed by MALDI-TOF (matrix assisted laser desorption ionization-time of flight) mass spectrometry using a Kratos MALDI II instrument. PNA monomers were obtained from PerSeptive Biosystems (Framingham, MA). The pH values quoted for PNA solutions were measured after PNA was dissolved.

NMR experiments. NMR experiments were conducted on samples of 1 mM PNA thymine monomer (**5**) in D_2O solution containing 10 mM phosphate buffer adjusted to various pH by addition of NaOH. One and two dimensional ^{1}H NMR spectra were recorded on a Varian Plus 500 MHz spectrometer, using pre-saturation for suppression of the residual D_2O/H_2O peak. Two dimensional NMR spectra were recorded using standard procedures. A mixing time of 100 ms was used in the NOESY experiments. All NMR data was processed and analyzed with the Varian VNMR software package.

HPLC experiments. Analytical HPLC was carried out using a 3.9 × 150 mm Delta Pak 5 mm C-18 100 reverse phase column (Waters). Buffer A: 99.9% H_2O, 0.1% TFA; Buffer B: 10% H_2O, 89.9% CH_3CN, 0.1% TFA. The solvents were heated to 50 °C and flow rate was 1.0 ml min^{-1}. Alternatively, HPLC experiments were performed on a RPC-5 column using aqueous buffers A (20 mM NaOH, 1 mM Tris-$HClO_4$ buffer) and B (20 mM NaOH, 1 mM Tris-$HClO_4$, 0.1 mM $NaClO_4$) applied in a linear gradient.[12] The concentration of injected samples was approximately 1 mM.

Syntheses

The synthetic pathway for *N*-[2-(thymin-1-ylacetyl)aminoethyl]glycine described below is shown in Scheme 1. All reagents were obtained from commercial suppliers and used without further purification. Melting points are uncorrected. Flash chromatography was performed using Merck silica gel 60 (230–400 mesh ASTM). ^{1}H and ^{13}C NMR spectra were obtained in DMSO-d_6 using a Varian Plus 400 MHz or a Bruker AMX 250 MHz instrument.

Ethyl *N*-(benzyloxycarbonyl)-*N*-[2-(thymin-1-ylacetyl)-aminoethyl]glycinate (2). To a stirred solution of ethyl *N*-(2-aminoethyl)glycinate (**1**) [(420 mg, 2.86 mmol) (^{1}H NMR δ: 8.60 (br s, 4H), 4.19 (q, J = 7.1 Hz, 2H), 3.91 (s, 2H), 3.22 (m, 4H), 1.22 (t, J = 7.1 Hz, 3H); ^{13}C NMR δ: 166.46, 61.82, 46.78, 44.06, 35.04, 14.08] and Pr^i_2NEt in dry DMF (10 ml) was added thymine acetic acid pentafluorophenyl ester (1.00 g, 2.86 mmol). After reaction at room temperature for 15 h the solvent was removed *in vacuo*. EtOAc (40 ml) was added and the solution washed with aqueous $NaHCO_3$ followed by brine. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was then dissolved in dry DMF (10 mL) and *N*-benzyloxycarbonyl-*N*′-methylimidazolium triflate (2.86 mmol) was added. After 6 h the DMF was removed *in vacuo*,

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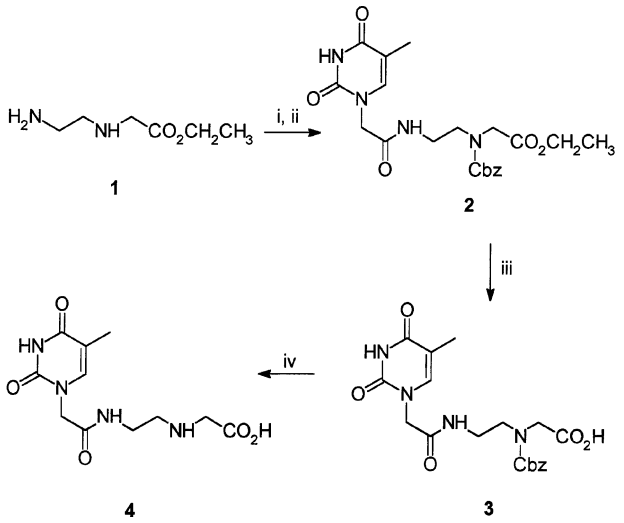


Scheme 1 Synthetic pathway for *N*-[2-(thymin-1-ylacetyl)aminoethyl]glycine (**4**)

the residue taken up in EtOAc (30 ml) and washed sequentially with saturated aqueous $KHSO_4$, saturated aqueous $NaHCO_3$ and brine. Drying (Na_2SO_4) and evaporation of the solvent resulted in a clear oil, which slowly solidified to a waxy solid (720 mg, 56%). ^{1}H NMR δ: 11.27 (s, 1H), 8.17 (m, 1H), 7.37 (m, 5H), 5.15 (m, 2H), 4.23 (m, 2H), 4.00 (m, 4H), 1.74 (s, 3H), 1.19 and 1.11 (t, J = 7.1 Hz, 3H); ^{13}C NMR δ: 169.92 and 169.74, 167.21, 164.54, 155.69 and 155.42, 151.09, 142.29, 128.51, 128.10, 127.30, 108.14, 66.59 and 66.53, 60.73, 49.62 and 49.48, 47.84 and 47.22, 37.62 and 37.21, 14.12, 11.98; IR (KBr): 3350, 1735, 1700, 1665, 1585, 1485, 1385 cm^{-1}; MS (FAB+): m/z 447.2 (requires 447.2).

***N*-(Benzyloxycarbonyl)-*N*-[2-(thymin-1-ylacetyl)aminoethyl]glycine (3).** To a solution of **2** (300 mg, 0.67 mol) in MeOH (10 ml) was added 1 M LiOH (2 ml in MeOH). After 10 min the MeOH was evaporated *in vacuo* and water (10 ml) was added. The aqueous solution was then neutralized with 1 M HCl (2 ml) to produce a white solid. After isolation by centrifuge and washing with water (3×) the solid was dried under high vacuum (to yield 240 mg of **3** 86%). ^{1}H NMR δ: 11.27 (s, 1H), 8.32 (br s, 1H), 7.41–7.28 (m, 5H), 5.10 and 5.05 (s, 2H), 4.25 (s, 2H), 3.98 and 3.95 (s, 2H), 1.73 (s, 3H); ^{13}C NMR δ: 171.27 and 171.11, 167.18, 164.55, 155.69 and 155.08, 151.08, 142.43 and 142.34, 136.84, 128.39, 127.76, 127.14, 108.10, 66.62, 49.55 and 49.50, 47.80 and 47.16, 37.67 and 37.25, 12.01; IR (KBr): 3450, 1700, 1670, 1585, 1482, 1455, 1430 cm^{-1}; MS (FAB+): m/z 419.33 $(M + H)^+$.

***N*-[2-(Thymin-1-ylacetyl)aminoethyl]glycine (4).** A solution of **3** (100 mg, 0.24 mmol) in MeOH (10 ml) containing 10% Pd–C (20 mg) was subjected to hydrogenation (20 °C, 1 atm) for 3 h after which the catalyst was removed by filtration through celite. Evaporation of the solvent gave a white solid (50 mg, 74%). ^{1}H NMR δ: 12.52 (br s, 1H), 8.60 (m, 1H), 7.49 (s, 1H), 4.32 (s, 2H), 3.34 (m, 4H), 3.20 (s, 2H), 1.74 (s, 3H); ^{13}C NMR δ: 167.65, 167.52, 164.54, 151.14, 142.44, 108.08, 49.56, 49.34, 35.72, 11.98; MS (FAB+): m/z 307.0 $(M + Na)^+$.

***N*-[2-(Thymin-1-ylacetyl)]-*N*-(2-aminoaminoethyl)glycine (5).** **5** was prepared by treatment of commercial tBoc-**5** with trifluoroacetic acid (TFA), evaporation of the TFA under reduced pressure and thorough washing of the resulting residue with diethyl ether.

Results

NMR studies of a PNA T monomer

The PNA thymine (T) monomer (**5**) was dissolved in phosphate buffer at pH 7.0 immediately prior to recording its ^{1}H NMR spectrum [Fig. 1(*a*)]. Spectra recorded over the following several weeks showed no significant changes. Likewise, after the pH of the sample was raised to 9.0 or 10.0 no spectral changes were observed over several weeks. The constant appearance of the NMR spectrum over time can be taken as evidence that no chemical changes occur in the molecule.

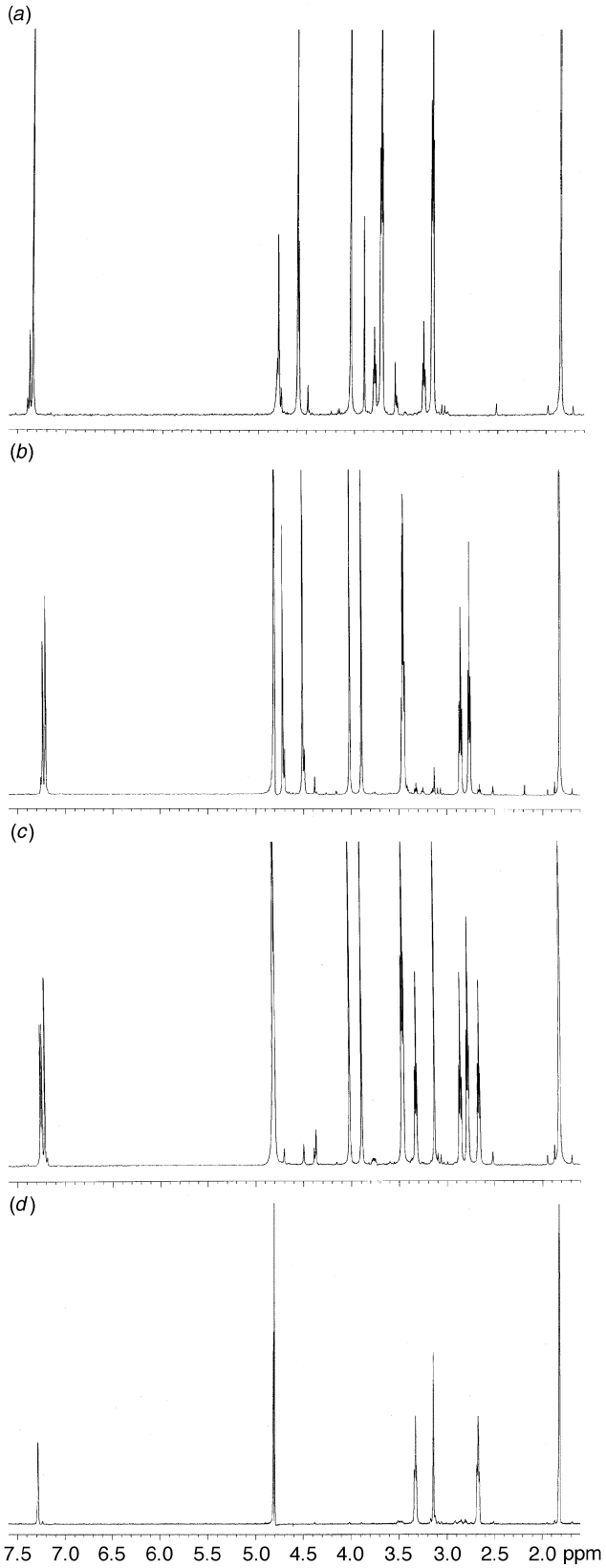


Fig. 1 ^{1}H NMR spectra of PNA thymine monomer (**5**) in 10 mM phosphate buffer (D_2O) at 20 °C. (*a*) Spectrum at pH 7.0 and spectra recorded (*b*) 6 min, (*c*) 17 days and (*d*) 9 months after raising the pH to 11.0

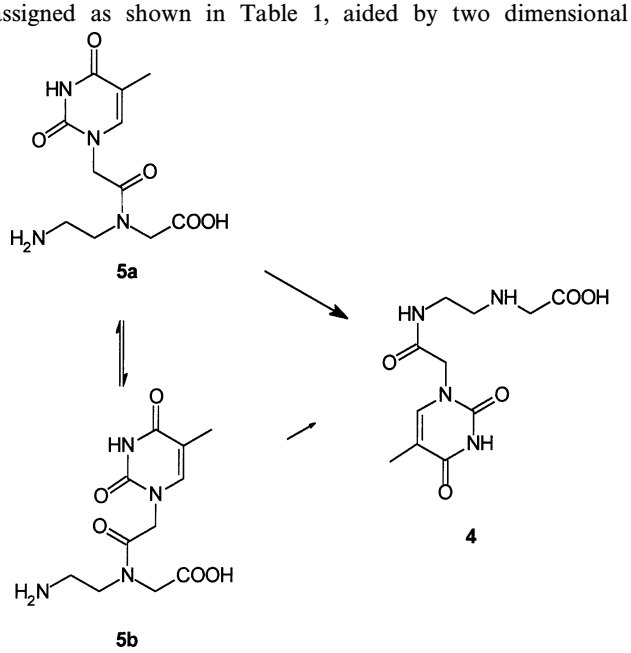
Table 1 Nonexchangeable ^{1}H NMR assignments for PNA thymine monomer (**5**) and the arranged product (**4**) in D_2O, 20 °C

	5		**4**
Proton	Chemical shift/ppm	Conformation	Chemical shift/ppm
pH 11			
H6	7.24	*cis*	7.26
	7.21	*trans*	
H2′, H2″	4.02	*trans*	3.13
	3.09	*cis*	
H4′, H4″, H5′, H5″	3.46	*cis* + *trans*	3.32
	3.45	*cis* + *trans*	2.67
	2.86	*cis*	
	2.76	*trans*	
H7′, H7″	4.72	*cis*	[a]
	4.51	*trans*	[a]
Methyl	1.82		1.82
pH 7 (only **5** present)			
H6	7.38	*cis*	
	7.34	*trans*	
H2′, H2″	4.04	*trans*	
	3.90	*cis*	
H4′, H4″, H5′, H5″	3.79	*cis*	
	3.72	*trans*	
	3.28	*cis*	
	3.19	*trans*	
H7′, H7″	4.59	*cis*	
	4.58	*trans*	
Methyl	1.84		

[a] Proton exchanged with solvent.

A spectrum of the thymine monomer solution was recorded shortly (6 min) after the pH was raised from 7.0 to 11.0 [Fig. 1(*b*)]. Subsequently recorded spectra show the appearance of a second set of peaks, which over time grow more intense while the peaks of the original spectrum lose intensity [Fig. 1(*c*,*d*)]. This observation indicates that a reaction of the thymine monomer (**5** → **4**) (Scheme 2) takes place at pH 11.0 (see Fig. 1). For comparison, the expected rearrangement product, *N*-[2-(thymin-1-ylacetyl)aminoethyl]glycine (**4**), was synthesized, and the spectrum of this authentic control compound (with the exception of a few peaks arising from impurities in the control sample, data not shown) was identical to that of rearranged **5**.

Spectra of the sample both at pH 7 and at pH 11 were assigned as shown in Table 1, aided by two dimensional NOESY and COSY experiments (data not shown). The spectrum recorded at pH 7.0 demonstrates that the original T monomer molecule is present in two forms (indicated **a** and **b** in Scheme 2), which were assigned to the *trans* and *cis* rotamers of the tertiary amide bond as previously observed.[13–15] At neutral pH the *trans* rotamer was found to dominate, whereas at pH 11 an ≈60 : 40 mixture of *trans* : *cis* was observed.



Scheme 2 *N*-acyl transfer reaction in the PNA thymine monomer (**5** → **4**). The *cis* and *trans* conformers of the original compound (**5a** and **5b**, respectively) are indicated

The kinetics of the PNA T monomer rearrangement reaction from **5** to **4** was followed. The intensities of various peaks, both of **5** and **4**, were measured as a function of time. Since the chemical shift of the methyl proton resonance is the same in **5** and **4**, the methyl peak could be used as an internal peak intensity standard. The decay of the amount of **5** was found to correspond closely to the buildup of **4**, as expected. The average half-life for the PNA T monomer was measured to be 34 days at 20 °C at pH 11 (Table 2).

Stability of PNA T_5 oligomers

The stability of the PNA H—T_5—Lys—NH_2 oligomer under various pH conditions at room temperature was followed by reverse phase HPLC for up to 60 days after dissolving the

Table 2 Rate of *N*-acyl transfer reaction as determined from ^{1}H NMR peak intensity changes of the PNA thymine monomer, **5a** and **5b**

	Resonance/ppm	$t_{1/2}$/h, days
Decaying peaks	2.77	791, 33
	2.86	943, 39
	3.46	850, 35
	3.86	766, 32
	4.02	651, 27
Increasing peaks	2.67	882, 37
	3.12	883, 37
	3.33	798, 33
	7.22	851, 35
	7.24	891, 37
	7.26	777, 32
Average		826 h, 34 days

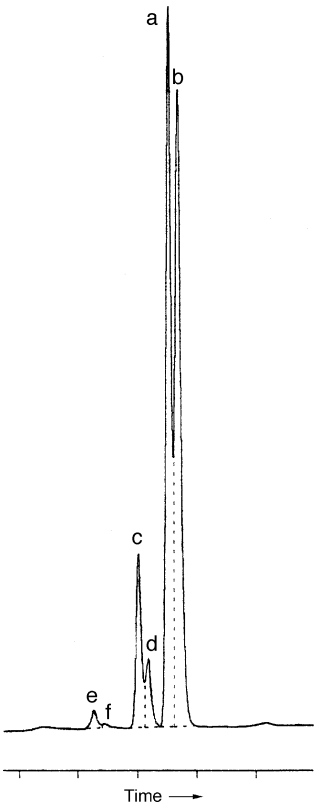


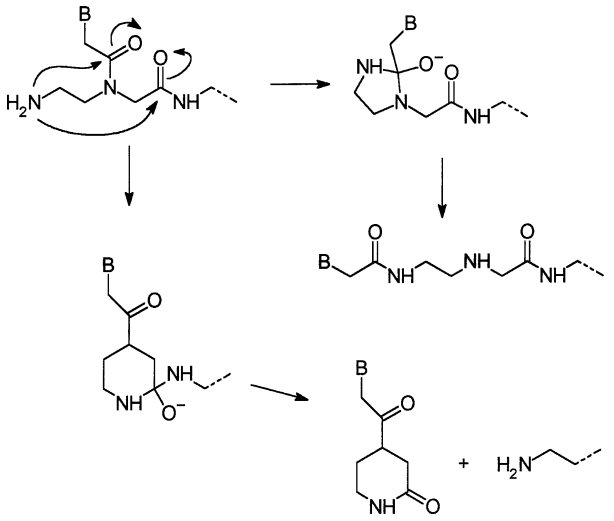
Fig. 2 Representative HPLC trace from H—T_5—Lys—NH_2 at pH 11 after 7 days. Peak a: H—T_5—Lys—NH_2, peak b: H—T_5—Lys—NH_2, peak c: H—T_4—Lys—NH_2, peak d: rearranged H—T_4—Lys—NH_2, peak e: H—T_3—Lys—NH_2 and peak f: rearranged H—T_3—Lys—NH_2. A co-injection with an authentic sample of rearranged H—T_5—Lys—NH_2 (T_{rearr}—T_4—Lys—NH_2) confirmed the assignment of peak b

sample in solutions of pH 5, 7, 9, 10 or 11. Fig. 2 shows a representative chromatogram obtained at pH 11 after 7 days. Besides the H—T_5—Lys—NH_2 (peak a) we also observe a later eluting species, which by MALDI-TOF mass spectrometry was assigned to be the *N*-acyl transfer product (no change in mass; rearrangements). The identity of the transfer product

Table 3 Half-lives, $t_{1/2}$, for the *N*-acyl transfer reaction and the sequential degradation of H—T_5—Lys—NH_2 estimated from HPLC experiments

	$t_{1/2}$/days	
pH	*N*-Acyl transfer	*N*-Terminal degradation
11	30[a]	22[a]
10	114[b]	70[b]
9	ND	120[b]
7	ND	ND
5	ND	250[b]

(ND = not detectable). [a] The degradation reaction results at pH 11 were estimated from the initial 48 h data. [b] $t_{1/2}$ values of over 50 days were obtained from extrapolation.



Scheme 3 Proposed mechanisms for the *N*-acyl transfer and degradation reactions

(peak b) was confirmed by co-injection with the authentic compound. We also observe two faster eluting peaks (c and e) which by mass spectrometry were assigned to be the sequentially hydrolyzed products H—T_3—Lys—NH_2 (c) and H—T_4—Lys—NH_2 (e). These products are both followed in the HPLC chromatogram by their (tentatively assigned) respective *N*-acyl transfer products (peaks d and f). The degradation rate of H—T_5—Lys—NH_2 was measured (Table 3). The rate of the transfer reaction was estimated from the peak area of peak b, while the combined area of peaks c, d, e and f were counted as degradation. Tentative mechanisms for the *N*-acyl transfer and degradation reactions are presented in Scheme 3. In both cases the reaction is initiated by a nucleophilic attack by the terminal free amine on either the tertiary 'nucleobase' amide, leading to the acyl transfer product *via* a five-membered intermediate, or on the secondary 'backbone' amide, leading to the first step of the 'degradation', and the liberated nucleobase unit as a lactam *via* a six-membered intermediate.

Since degradation requires a free *N*-terminus, capping or migration of the sidechain from the secondary to the primary amine prevents further degradation. Accordingly, the *N*-acetylated PNA, Ac—T_5—Lys—NH_2, exhibited no rearrangement or degradation within the pH range studied (5–12) and was used as an internal standard in the HPLC experiments. In addition, H—$T_{Me}T_4$—Lys, carrying a methyl group on the *N*-terminus, was studied at high pH. This secondary amine showed extremely slow degradation rates (less than 1% was degraded after 4 weeks). Increased degradation of the H—T_5—Lys—NH_2 PNA was also observed under acidic conditions (relative to neutral pH), occurring with $t_{1/2}$ of 250 days at pH 5 (Table 3). This reaction, however, must be mechanistically different from the processes observed at high pH.

Stability of PNA G_6

The stability of the homopurine PNA H—G_6—Lys—NH_2 was also studied at different pH. At pH 10 and 9 the half-life

Table 4 Stability ($t_{1/2}$, in days except as noted otherwise) of PNA oligomers at various pH at room temperature

pH	H—T_5—Lys—NH_2	H—G_6—Lys—NH_2	H—ACT_6—NH_2	H—CCT_6—NH_2	H—GCT_6—NH_2	H—TCT_6—NH_2
12	—	2.1 h	1.5 h	20	21	21
11	12	—	18 h	50	57	62
10	40	182 h, 7.6	70 h, 3	163	145	169
9	120	28	350 h, 14.6	148	159	163

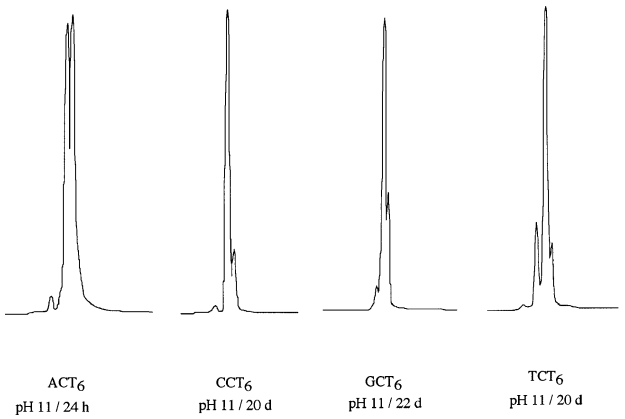


Fig. 3 HPLC chromatographs of $H-ACT_6-NH_2$, $H-CCT_6-NH_2$, $H-GCT_6-NH_2$ and $H-TCT_6-NH_2$ at pH 11. The samples were analyzed on an RPC-5 column at pH 12 (*cf.* ref. 12). Elapsed times are indicated

was measured to be 7.6 and 28 days, respectively, which is approximately five times shorter than the corresponding half-lives of $H-T_5-Lys-NH_2$ (*cf.* Table 4), suggesting that the degradation mechanism is dependent on the sequence at the PNA amino-terminus.

Stability of PNA XCT_6

In order to address the importance of the sequence on the stability of PNA oligomers at high pH, four PNA octamers of the sequence XCT_6, X being either of the four regular nucleobases, were studied. Representative HPLC chromatogram traces are shown in Fig. 3. The PNAs with C, G, or T at the *N*-terminus undergo slow acyl transfer, with $t_{1/2}$ of a few months at pH 11, and $t_{1/2}$ of several weeks at pH 12 (Table 4). In contrast, the PNA with adenine at the *N*-terminus, $H-ACT_6-NH_2$, surprisingly degrades much faster, with $t_{1/2}$ in the range of hours at pH 11 and 12, and a few days at pH 8 or 10. Similarly, the sequence ATT_6 shows a half-life of 20 h at pH 11, whereas $H-AAT_6-NH_2$ and $H-AGT_6-NH_2$ were too slow to measure, indicating that the nature of the second base is of importance for the stability. It should be noticed that the degradation rates quoted for the $H-XCT_6-NH_2$ sequences include both the degradation and *N*-acyl transfer reactions.

Discussion

The present results demonstrate the high chemical stability of PNA and also show that the previously reported *N*-terminal acyl transfer reaction is only taking place at a significant rate under strongly basic conditions (pH > 11) whereas it is virtually undetectable for several months at neutral pH. Thus, under physiological conditions or upon storage of PNA oligomers (or monomers) this reaction should not interfere with the use of PNA.

Two processes are taking place at the PNA *N*-terminus: *N*-acyl rearrangement and degradation. Both reactions are due to attack by the free *N*-terminal amino group and the rates of the two reactions differ. Autodegradation appears to proceed at a higher rate than the rearrangement under the conditions studied. However, the relative rate of the rearrangement increases at higher pH. Only the *N*-acyl transfer reaction can be observed in the monomer, where it is observed at pH 11 or above. We note that the *cis–trans* rotamer equilibrium shifts with pH, increasing the *cis* population at higher pH, and therefore it could be that the *N*-acyl transfer reaction proceeds exclusively from the *cis* rotamer. Analagously, the remarkably poor stability observed for $H-ACT_6-NH_2$, as compared to the oligomers starting with C, G or T, may be due to a shift in the rotamer equilibrium, in favor of the *cis* form, in the *N*-terminal A residues. We have, however, no further evidence for such a mechanism, and can only speculate that the stacking interactions between the two *N*-terminal bases are determinant, and that the AC group strongly favors the *cis* rotamer in a PNA single strand. In order to verify the importance of stacking interactions an extensive study of the sequence dependence of the degradation kinetics is required.

The results also have implications for the discussion concerning PNA and PNA-like molecules as a primordial genetic material. Monomers of a putative primordial genetic material must have sufficient stability in an aqueous environment to accumulate to a concentration that permits polymerization. A simple extrapolation—assuming a base-catalyzed reaction—of the data presented in Table 2 indicates a half-life of a PNA T monomer at pH 7 of approximately 3×10^5 days (about 1000 yr), which seems rather too short on the evolutionary timescale for sufficient accumulation. Monomers could in principle be stabilized by lactam formation (*cf.* Scheme 3), but it is very difficult—if not impossible—to imagine an efficient polymerization reaction on the basis of these. On the other hand, although some oligomers seem very fragile (for instance, an extrapolated half-life of approximately 4 yr at pH 7 is obtained for the $H-ACT_6-NH_2$ oligomer), these would be tremendously stabilized by the first rearrangement reaction or by another simple end-capping (alkylation or acylation). The *N*-methyl-PNA oligomers showed stabilities at pH 11 estimated to exceed 5 yr, which would extrapolate to 50,000 yr at pH 7 and for the *N*-acetyl-PNA oligomer we could not detect any degradation. These times would not be an obstacle to replication, if a suitable replication mechanism exists. Thus, from the perspective of chemical stability, PNA monomers may and PNA oligomers certainly could have played a role in prebiotic life. It should be emphasized, however, that PNA *monomers* are not likely to oligomerize efficiently (but *dimers* can[8]) by simple condensation chemistry due to competing intramolecular lactam formation, and therefore a plausible prebiotic oligomerization mechanism for aminoethyl glycine PNAs must be presented for these to be considered *bona fide* candidates for a prebiotic genetic material.

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