1,8-Naphthyridin-2(1*H*)-ones − Novel Bicyclic and Tricyclic Analogues of Thymine in Peptide Nucleic Acids (PNAs)

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Keywords: Base-pairing / Hybridization / Nucleic acids / Nucleobases / PNA structure

The synthesis of two novel PNA nucleobases derived from 1,8-naphthyridin-2(1*H*)-one (bT) and benzo[*b*]-1,8-naphthyridin-2(1*H*)-one (tT) are reported, together with their incorporation into oligomers of PNA and evaluation as substitutes for thymine. Compound bT is shown to be an effective mimic of the natural thymine nucleobase in PNA-DNA, PNA-RNA, and PNA-PNA duplex structures. A study using singly mismatched target sequences showed bT to be selective for the recognition of adenine. The X-ray structure of a PNA hexamer containing a single bT base (H-GbTATAC-L-lys-NH_2) was determined to 1.8 Å resolution and confirmed the base-pairing capability with adenine. The introduction of a bT base does not alter the P-form double helix structure of PNA, as compared to other PNA structures containing natural nucleobases. With the tricyclic derivative, incorporation of several units of tT resulted in decreased specificity in some systems, while maintaining specificity in others. In PNA·DNA-PNA triplex structures, incorporation of bT into the Hoogsteen strand resulted in enhanced stability relative to control triplexes containing only T·A-T and pseudoisocytosine (J)·G-C triplets (ΔT_m = +1.5 °C/modification). The evaluation of another nucleobase, 3,5-diaza-4-oxophenothiazine (tC), expected to mimic the function of cytosine is similarly reported. Incorporation of tC in place of cytosine in PNA oligomers increased the thermal stability of the corresponding PNA-DNA, PNA-RNA, and PNA-PNA duplexes. However, the sequence specificity was diminished in some PNA-DNA duplex systems containing several tC units. The thermal stability of triplex structures containing tC in the Hoogsteen strand was reduced relative to the cytosine-containing control.

Introduction

Inhibition of gene expression by antisense or antigene approaches relies on the formation of stable duplex and triplex structures.[1,2] In this context, the use of modified nucleobases has several interesting applications,[2−12] including the use of purines and pyrimidines containing extended aromatic moieties as a means of increasing the stacking energy and hence the stability of the hybridized complexes. Recent attempts in this direction, by incorporation of pyrimidines substituted in the 5-position with propynyl or thiazolyl moieties, have resulted in increased affinity for oligonucleotides of complementary sequence and increased in vitro cellular antisense efficacy.[4−7,9,11] Similarly, the synthesis and preliminary evaluation of several tricyclic systems based on phenothiazine, phenoxazine, and carbazolepyrimidine heterocycles have recently been published.[8,10] The phenothiazine/phenoxazine C-analogue appears particularly interesting, providing both higher target affinity and improved cellular uptake.[8,13]

Peptide nucleic acid (PNA) is a DNA mimic with a pseudopeptide backbone, displaying exciting potential for the development of gene therapeutic antisense and antigene drugs, and also for genetic diagnostic and molecular biology reagents.[14,15] Therefore, PNAs with modified backbones and/or containing alternative nucleobases, conferring improved DNA/RNA recognition properties or providing PNAs with improved cellular delivery characteristics, are of interest. Furthermore, recent results with DNA oligonucleotides have shown that duplex formation can be stabilized significantly by increased stacking interactions.[16]

We report here the synthesis of *tert*-butyloxycarbonyl-protected (Boc-protected) PNA monomers containing 1,8-naphthyridin-2(1*H*)-one (bT), benzo[*b*]-1,8-naphthyridin-2(1*H*)-one (tT), and 3,5-diaza-4-oxophenothiazine (tC),[8,13,17,18] allowing for the synthesis of oligomers of PNA containing these extended pyrimidine analogues. The new PNA monomers were incorporated into PNA oligomers including bis-PNAs[19] to evaluate their base-pairing properties in PNA-DNA, PNA-RNA, and PNA-PNA duplexes (Watson−Crick) and PNA·DNA-PNA triplexes (Hoogsteen). For triplex formation, bis-PNAs containing pseudoisocytosine (J) instead of cytosine in the Hoogsteen strand were used.[19] Mismatched target sequences were applied to evaluate the specificity in the recognition of adenine (bT and tT) or guanine (tC) in duplex systems. The X-ray structure of the hexameric self-complementary PNA-PNA duplex (bT-PNA, H-GbTATAC-L-lys-NH_2) was deter-

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  1434−193X/01/0509−1781 $ 17.50+.50/0

mined so as to obtain direct structural information on the base-pairing properties of bT as well as base-pair stacking interactions and helical parameters.

Results and Discussion

The bT and tT moieties (Figure 1) were introduced into PNA aminoethylglycine monomers by a method based on the synthesis of (1,2-dihydro-2-oxo-1,8-naphthyridin-3-yl)acetic acid **3a** (Figure 2) and (1,2-dihydro-2-oxobenzo[*b*][1,8]-naphthyridin-3-yl)acetic acid (**3b**) (Figure 2), respectively. Compound **3a** was obtained from 2-pivaloylaminopyridine-3-carbaldehyde (**1a**) by means of an aldol type condensation with the lithio enolate of di-*tert*-butyl succinate, producing a mixture of diastereomeric alcohols **2a** that was cyclized by reflux in a 1:1 mixture of dioxane and aqueous HCl, affording the desired product **3a**. Compound **1a** was readily available from 2-aminopyridine by means of a pivaloylamino-directed *ortho* lithiation on 2-pivaloylaminopyridine, producing a dianion that was partially quenched with *N*-formylmorpholine or dimethylformamide, according to the procedure published by Turner.[20,21] Similar procedures were employed for the synthesis of the corresponding tricyclic acetic acid derivative **3b**, using 2-aminoquinoline as the commercially available starting material, from which 2-pivaloylaminoquinoline was prepared by treatment with pivaloyl chloride and triethylamine in dichloromethane. As for the bicyclic derivative, the pivaloylamino-directed *ortho* lithiation gave the 3-formyl derivative **1b**, which was converted into **2b** by condensation with di-*tert*-butyl succinate and subsequently ring-closed to yield the tricyclic derivative **3b**.

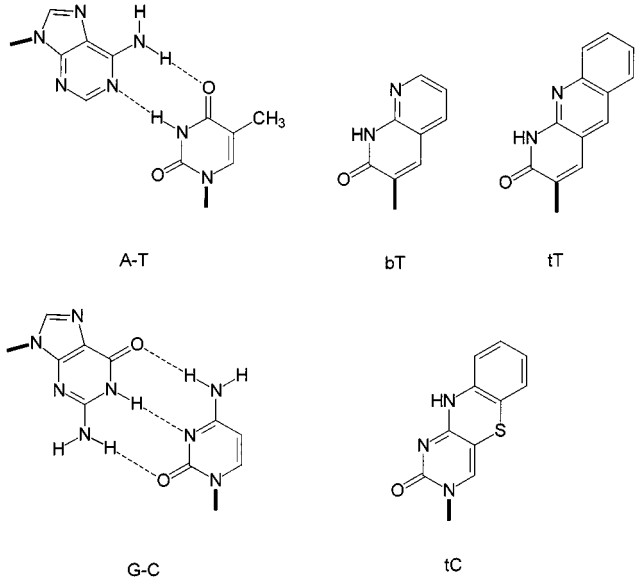

Figure 1. Comparison of the structures of thymine (T) and cytosine (C) (in the A-T and G-C Watson-Crick base pairs) and bicyclic 1,8-naphthyridin-2(1*H*)-one (bT), tricyclic benzo[*b*][1,8]-naphthyridin-2(1*H*)-one (tT), and tricyclic 3,5-diaza-4-oxophenothiazine (tC)

The *N*-Boc-protected PNA monomers **5a** and **5b** were prepared from the acetic acid derivatives **3a** and **3b**, using

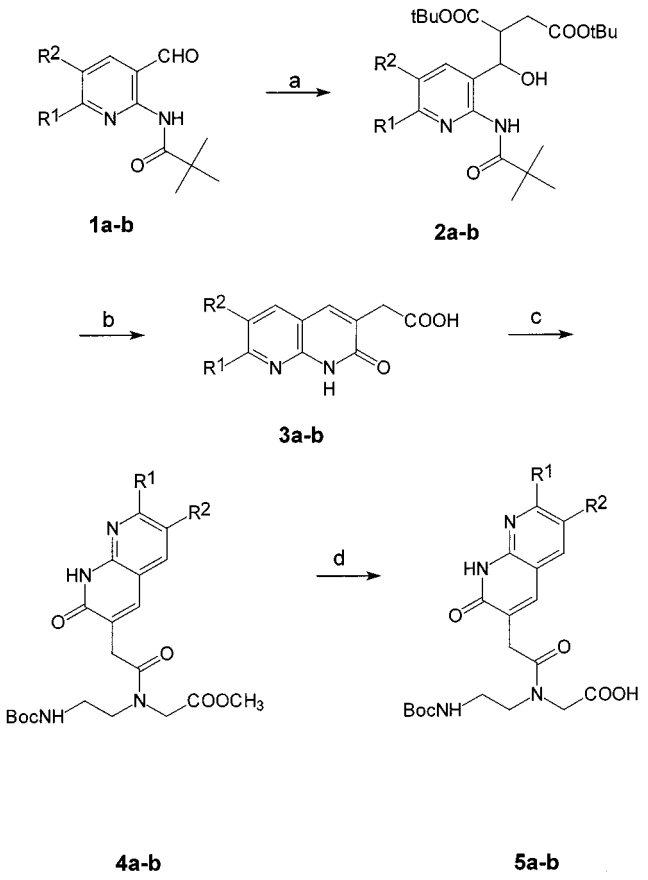

Figure 2. Synthesis of *N*-(2-Boc-aminoethyl)-*N*-(1,2-dihydro-2-oxo-1,8-naphthyridin-3-yl)acetylglycine (**5a**, $R_1 = R_2 = H$) from 2-pivaloylaminopyridine-3-carbaldehyde (**1a**): (a) Preformation of the succinate dianion using 2.0 equiv. of LDA and 2.0 equiv. of di-*tert*-butylsuccinate in THF for 30 min at −78 °C, then **1a**, 15 min at −78 °C, then room temp.; (b) HCl (3 M, aqueous) in dioxane, overnight, reflux, 42%; (c) Methyl *N*-(2-Boc-aminoethyl)glycinate, HOAc, diisopropylethylamine in DMF for 1 h at 0 °C, then overnight at room temp., 58%; (d) 5.0 equiv. LiOH in THF, 20 min at room temp., 88%. Synthesis of the tricyclic derivative *N*-(2-Boc-aminoethyl)-*N*-(1,2-dihydro-2-oxobenzo[*b*][1,8]-naphthyridin-3-yl)-acetylglycine (**5b**) (R_1 and R_2 are joined to form a six-membered aromatic ring) from 2-Pivaloylaminoquinoline-3-carbaldehyde (**1b**): (a) Preformation of the succinate anion, using 2.0 equiv. LDA and 2.0 equiv. of di-*tert*-butyl succinate in THF for 15 min at −78 °C, then **4a**, 15 min at −78 °C, then room temp.; (b) HCl (3 M, aqueous) in dioxane, overnight, reflux, 43%; (c) Methyl *N*-(2-Boc-aminoethyl)glycinate and HBTU in DMF for 1 h at 0 °C, then overnight at room temp., 21% from impure **3b**. (d) 5.0 equiv. LiOH in THF, 15 min at room temp., 91%

the previously published procedure for transformation of acids into activated esters followed by coupling to methyl *N*-(2-Boc-aminoethyl)glycinate,[22] to yield the fully protected PNA monomers **4a** and **4b**. The carboxylic esters **4a** and **4b** were hydrolyzed using aqueous LiOH in THF to give **5a** or **5b**.

The 3,5-diaza-4-oxophenothiazine heterocycle, first synthesized by Roth et al.,[17,18] has previously been incorporated into oligodeoxyribonucleotides.[8] The parent nucleoside was synthesized from 5′-iodo-2′-deoxyuridine, utilizing anchimeric assistance from the 5′-hydroxy group of the nucleoside.[8] A similar methodology was not directly applicable to the synthesis of PNA monomers. However, the phenothi-

azine nucleobase **6** (Figure 3) could be synthesized from 5-bromouracil by the method described by Roth et al.[17,18] Subsequent transformation into **7** was performed by alkylation of the anion of **6** with ethyl *N*-(2-Boc-aminoethyl)-*N*-(bromoacetyl)glycinate in DMF to give the fully protected monomer **7**, which was hydrolyzed using aqueous LiOH in THF to give Boc-protected PNA monomer **8**. All PNA monomers were found to exist in a mixture of the two rotamers around the tertiary amide bond. All compounds were characterized and verified by NMR (^{1}H and ^{13}C) and HRMS analyses.

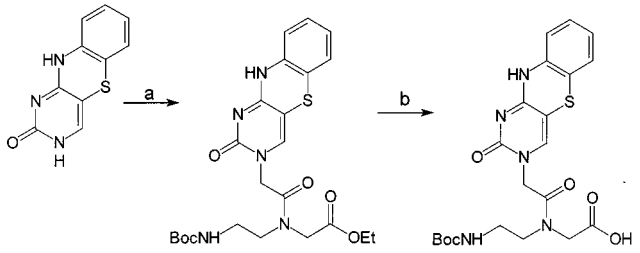

Figure 3. Synthesis of *N*-(2-Boc-aminoethyl)-*N*-[(2-hydroxy-10*H*-pyrimido[5,4-*b*][1,4]benzothiazin-1-yl)acetylglycine (**8**) from the benzothiazine **6**: (a) ethyl *N*-bromoacetyl-*N*-(2-Boc-aminoethyl)-glycinate and 1.1 equiv. of NaH in DMF for 2 h at 0 °C, then overnight at room temp., 57%; (b) 5 equiv. LiOH in THF 15 min at room temp., 91%

PNA 10-mers containing the bT (PNAs 2 and 3) or tT (PNAs 8 and 9) moieties in one or three separate positions were synthesized in order to study the recognition properties of these new PNA units (Table 1). In another PNA sequence, three units of bT or tT were incorporated in separate PNAs (5 and 9) or adjacent (PNAs 7 and 11) positions (Table 1). The binding to 10-mer DNA-, RNA-, or PNA-complementary oligomers was subsequently studied by thermal stability determinations and compared with control oligomers (PNAs 1, 4, and 6) containing the natural A-T Watson−Crick base pairs. For duplexes containing bT (PNAs 2, 3, 5, and 7) (Table 1), the thermal stabilities were generally similar to those of the controls, confirming the ability of this nucleobase to behave like thymine, although the PNA containing three separate bT units (PNA 5) gave slightly less stable duplexes (ΔT_m = −1.5−2.0 °C/modification). The nature of the target (DNA, RNA, or PNA) had only marginal influences on the stabilities of the duplexes relative to the control duplex.

The base recognition specificity of bT was studied by duplex thermal stability measurements, using singly mismatched DNA and PNA targets. In all cases, bT was found to recognize adenine, and the decrease in thermal stability (ΔT_m) upon introduction of bases other than A ranged from 4.5 °C to 15.0 °C in PNA-DNA duplexes, from 8.0 °C to 16.0 °C in PNA-RNA, and from 6.5 to 20.5 °C in the PNA-PNA duplexes (PNA 2) (Table 1). Specificity for adenine was maintained for sequences containing three separate units of bT (PNA 3) [ΔT_m = 6.5−10.5 °C (PNA-DNA)], as well as for the sequence containing three adjacent units of bT (PNA 7) [ΔT_m = 7.0−17.5 °C (PNA-DNA)].

The X-ray structure of a hexameric bT-PNA duplex containing one alternative nucleobase (bT) in each strand has been determined, and is the first structure of a PNA hexamer with a sequence (H-G*bT*ATAC-L-Lys-NH_2) different from that of the other reported structures of PNA duplexes.[24,25] The asymmetric unit of the crystal contains one right-handed and one left-handed double helix, which are coaxially stacked (Figure 4, a). The preference for forming a continuous pseudohelix of alternating left- and right-handed helices seems to be preserved in bT-PNA, despite the different sequence and the modification of one of the nucleobases. The bT-PNA hexamer adopts the P-form helix (Table 2) with helical parameters (except base displacements) close to those of the previously reported PNA-PNA duplexes.[24,25]

The existence of water molecules bridging the backbone and the nucleobases is another characteristic feature of the PNA duplex that recurs in bT-PNA. This clearly indicates the structural importance of the bridging water molecules, independent of PNA sequences. However, in two out of the four bT nucleobases (one in the right-handed and one in

Table 1. Thermal stability (T_m) of PNA/DNA and PNA/PNA duplexes assessed using a 10-mer PNA containing thymine (control), 1,8-naphthyridin-2(1*H*)-one (bT), or benzo[*b*][1,8]-naphthyridin-2(1*H*)-one (tT). The PNA oligomers were hybridized to antiparallel either fully complementary DNA, PNA, or RNA oligomers (X = A), or oligomers containing a single (mismatch) base substitution opposite the middle T, bT, or tT unit

10 mer PNA sequence (PNA no)	X = A DNA/PNA/RNA	X = G DNA/PNA/RNA	X = C DNA/PNA/RNA	X = T(U) DNA/PNA/RNA
H-GTAGATCACT-Lys-NH_2, (1) control	51.0/68.5/56	37.0/60.0/45	45.0/52.5/36	41.0/50.5/38
H-GTAGA(bT)CACT-Lys-NH_2 (2)	51.0/68.5/54	46.5/62.0/46	c. 36/50.5/38	43.0/c.48/45
H-G(bT)AGA(bT)CAC(bT)-Lys NH_2 (3)	52/66.5/55	45.5/nd/nd	41.5/nd/nd	45.0/nd/nd
H-GTAGATGATG-Lys-NH_2 (4) control	57/67.5/58.5	nd/nd/48.5	nd/nd/43.5	nd/nd/45.5
H-G(bT)AGA(bT)GA(bT)G-Lys-NH_2 (5)	51.0/63.0/52	nd/nd/45.5	nd/nd/37.5	nd/nd/46.5
H-AGAGTTTGAG-Lys-NH_2 (6) control	59.5/66.5/63	nd/nd/nd	nd/nd/nd	nd/nd/nd
H-AGAG(bT)(bT)(bT)GAG-Lys-NH_2 (7)	58.0/64.5 /61.5	51.0/nd/nd	40.5/nd/nd	50.0/nd/nd
H-GTAGA(tT)CACT-Lys-NH_2 (8)	51.0/68.0/55	45.5/62.5/48	c. 45/c.52/39	41.0/51.0/40
H-G(tT)AGA(tT)CAC(tT)-Lys NH_2 (9)	59/68/53	57.5/nd/nd	58.0/nd/nd	57.5/nd/nd
H-G(tT)AGA(tT)GA(tT)G-Lys-NH_2 (10)	mt[a]/60/mt	mt/nd/nd	mt/nd/nd	mt/nd/nd
H-AGAG(tT)(tT)(tT)GAG-Lys-NH_2 (11)	58/66.5/63	53.5/nd/nd	mt[b]/ nd/nd	50.0/nd/nd

[a] Multiple transitions were observed, indicating several binding modes to be operating. − [b] No well defined transition could be identified. nd: not determined. mt: multiple transitions.

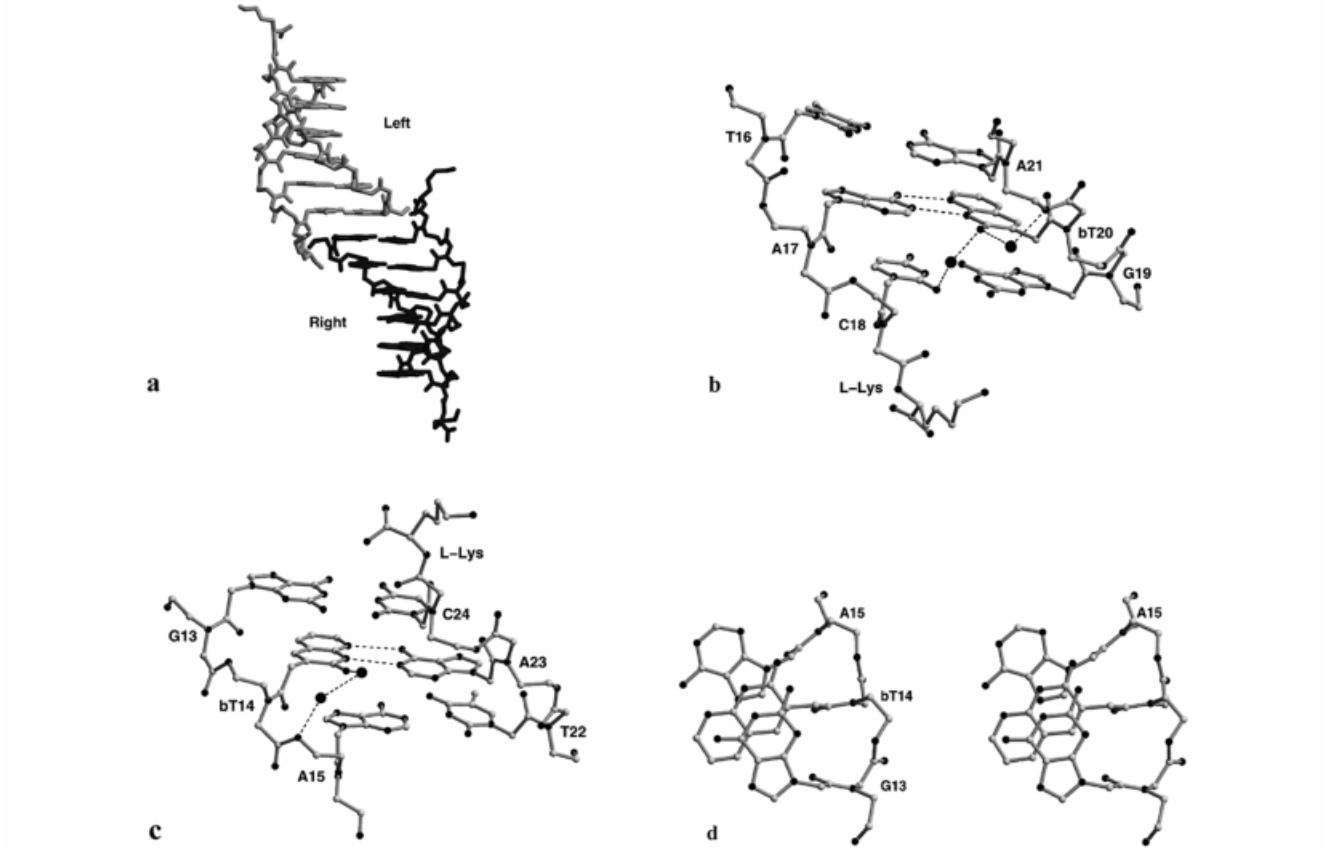

Figure 4. X-ray structure of the bT-PNA hexamer (H-G*bT*ATAC-L-lys-NH_2) **a**. The bT-PNA hexamer crystallizes with one right-handed (dark gray) and one left-handed (light gray) double helix in the asymmetric unit. The right-handed and left-handed helices are coaxially stacked, thereby forming long pseudohelices of interchanging right-handed and left-handed helices. **b**, **c**. Close-up view in the vicinity of the bT nucleobases in the left-handed duplex showing the base-pairing and stacking interactions of bT. One of the bT nucleobases is bridged to the backbone via one water molecule (**b**) and the other through two water molecules (**c**). The water molecules are displayed as large black spheres and the oxygen and nitrogen atoms of PNA are in black. Hydrogen bonds to the bT nucleobases are shown as dotted lines. (**d**) Close-up view showing the nucleobase stacking interactions in the vicinity of the bT nucleobase no. 14 in the left-handed bT-PNA. The view is down the helix axis. Color coding as in **b**, **c**. The stereo figure was generated using the program MOLSCRIPT[32] and rendered using Raster3D.[33]

Table 2. Helical parameters (average) of the bT-PNA duplex

PNA duplexes	Twist[a] [°]	Rise[a] [Å]	Base tilt[a] [°]	Displacement[a] [Å]	Bases per turn
bT-PNA[b] (right)[b]	18	3.7	0.1	3.3	18
bT-PNA (left)[b]	−18	3.8	0.7	2.7	18
N-Me PNA (right)[c]	+19	3.8	0.2	4.8	18
N-Me PNA (left)[c]	−20	3.5	0.0	7.2	18
Unmod PNA (right)[d]	+20	3.2	1.0	8.3	18

[a] The helical parameters were determined using the program CURVES.[31] – [b] bT-PNA: PNA hexamer containing one bT base (H-GbTATAC-L-lys-NH_2). – [c] N-Me PNA: backbone *N*-methylated PNA (H-$C_{Me}GT_{Me}AC_{Me}$G-L-Lys-NH_2).[25] – [d] Unmod PNA: backbone unmodified PNA (H-CGTACG-NH_2).[24]

the left-handed duplex), the nucleobase O-2 atom is bridged to the backbone via two water molecules, instead of one as is usually observed (Figure 4, b and c).[24,25] The two O-2 atoms, bridged to the backbone by only one water molecule, are also involved in hydrogen bonding to the terminal cytosine of the second PNA strand of the double helix, in contrast to the bT O-2 atoms bridged through two water molecules.

The introduction of the base (bT) is easily accommodated in the structure of the hexameric PNA duplex. The base-pairing between bT and A is shown in Figure 4 (b, c). As expected, hydrogen bonds are formed between N-1 of bT and N-3 of adenine, and between N-8 of bT and the C-6 amino group of adenine. The N-1 and N-3 atoms of bT are not involved in further hydrogen bonding. Additional stacking interactions are observed between the bicyclic ring

system of bT and the adjacent guanine, compared to the T-A base pair of the corresponding unmodified PNA (Figure 4, d).[24]

Analogous T_m experiments were carried out using the corresponding tricyclic derivative tT. For the PNA oligomer containing one tT (PNA 8), the thermal stability of PNA-DNA, PNA-RNA, and PNA-PNA duplexes remained unchanged relative to the control (PNA 1). In the same PNA sequence, incorporation of three separate tT units (PNA 9) resulted in a somewhat higher thermal stability of the duplex with DNA (ΔT_m = 2.6 °C/modification) but slightly lower stability for the duplexes with RNA and PNA. In a different sequence context, introduction of three separated units of tT (PNA 10) resulted in multiple thermal transitions in both the PNA-DNA and the PNA-RNA complexes, showing that two-state duplex to single strand transitions did not take place. Thus, for these systems, a simple interpretation of the highest transition is not possible. In the corresponding PNA-PNA system, a decrease in thermal stability (ΔT_m = −2.7 °C/modification) was observed. For the PNA (11) containing three adjacent tT units, in which increased overlap between adjacent tT units is to be expected, hardly any differences were observed for any of the complexes (Table 1).

Again, measurements using singly mismatched oligonucleotides were used to assess the specificity in the recognition of adenine (Table 1). For the sequence containing one tT unit (PNA 8), specificity for adenine was maintained as judged from the decrease in thermal stability [ΔT_m = 6.0−10 °C (PNA-DNA), ΔT_m = 7−16 °C (PNA-RNA), and ΔT_m = 5.5−17.0 °C (PNA-PNA)]. Specificity in the PNA-DNA system with several tT moieties incorporated into three separate positions in the PNA strand (PNAs 9 and 10) is difficult to assess because of the melting behavior (multiple transitions) of these duplexes. However, the results for PNAs 11 hybridized to DNA indicates reasonable base discrimination.

Using the same sequence as used for the bT/tT study, a 10-mer PNA containing one tC was prepared (PNA 12) in order to compare the behavior of this electronically different but sterically isomorphous nucleobase with the behavior of the bT and tT nucleobases. In another sequence, three units of tC were incorporated in separate (PNA 14) or adjacent (PNA 16) positions (Table 3). For the sequence containing tC in one position, the affinity for the DNA, RNA, and PNA complement was slightly increased (ΔT_m ca. 3 °C). For the sequence containing three separate units of tC, significant increases in thermal stability were observed, both in PNA-DNA (ΔT_m = 6.2 °C/modification), and PNA-RNA (ΔT_m = 3.7 °C/modification), and to a lesser degree in the PNA-PNA (ΔT_m = 3.3 °C/modification) system. Similar increases were observed in duplexes containing tC in adjacent positions [ΔT_m = 4.7 °C/modification (PNA-DNA), ΔT_m >2.8 °C/modification (PNA-PNA) >2.7 °C/modification (PNA-RNA)].

For the oligomer containing only a single tC unit, measurements using singly mismatched DNA targets showed slightly impaired selectivity for guanine, as compared to the control PNA (1) containing cytosine. On the other hand, selection for the fully matched targets was maintained in PNA-PNA duplex systems (ΔT_m = 20−24 °C) (Table 3). It is most likely that differences in next-neighbor stacking

Table 3. Thermal stability (T_m, °C) of PNA/DNA, PNA/PNA, and PNA/RNA duplexes assessed using a 10-mer PNA containing phenothiazine (tC) and a 10-mer DNA, PNA, or RNA antiparallel complement with or without a single base pair mismatch (X) opposite the (middle) C or tC unit (nd: not determined)

PNA sequence (PNA no)	DNA/PNA/RNA/X = G	DNA/PNA/X = A	DNA/PNA/X = C	DNA/PNA/X = T
H-GTAGATCACT-Lys-NH_2 (1) control	51.0/68.5/56	44/46	44/44	43/45
H-GTAGAT(tC)ACT-Lys-NH_2 (12)	53.0/71/57	48/47	49/46	47/48
H-GCAGACGACG-Lys-NH_2 (13) control	52.0/66.0/56	nd/ nd	nd/ nd	nd/ nd
H-G(tC)AGA(tC)GA(tC)G-Lys-NH_2 (14)	70.5/76.0/67	nd/ nd	nd/ nd	nd/ nd
H-AGAGCCCGG-Lys-NH_2 (15) control	72.0/81.5/77	48/nd	49/nd	49/nd
H-AGAG(tC)(tC)(tC)GAG-Lys-NH_2 (16)	86.0/>90.0/>85	73/nd	72/nd	76/nd

Table 4. Thermal stability (T_m) of PNA/DNA complexes using a bis-PNA containing 1,8-naphthyridin-2(1*H*)-one (bT) or benzo[*b*]-1,8-naphthyridin-2(1*H*)-one (or phenothiazin, tC) inserted into appropriate positions and a DNA complement containing the 7-mer target

bis-PNA sequence (PNA no)[a]	oligonucleotide target	T_m [°C]
H-TTJTTTJ-eg1-eg1-eg1-CTTTCTT-NH_2[b] (17) control	5′-dCGC**AAGAAAG**CGC-3′	59.5
H-T(bT)J(bT)T(bT)J-eg1-eg1-eg1-CTTTCTT-NH_2[b] (19)	5′-dCGC**AAGAAAG**CGC-3′	63.0
H-T(tT)J(tT)T(tT)J-eg1-eg1-eg1-CTTTCTT-NH_2[b] (21)	5′-dCGC**AAGAAAG**CGC-3′	49.0
H-TJTTTTJ-eg1-eg1-eg1-CTTTTCT-NH_2[b] (18) control	5′-dCGC**AGAAAAG**CGC-3′	61.0
H-TJ(bT)(bT)(bT)TJ-eg1-eg1-eg1-CTTTTCT-NH_2[b] (20)	5′-dCGC**AGAAAAG**CGC-3′	65.5
H-TJ(tT)(tT)(tT)TJ-eg1-eg1-eg1-CTTTTCT-NH_2[b] (22)	5′-dCGC**AGAAAAG**CGC-3′	60.5
H-TCTCTCT-eg1-eg1-eg1-TCTCTCT-NH_2 (23) control	5′-dCGC**AGAGAGA**CGC-3′	42.0/50.5/78.0[c]
H-T(tC)T(tC)T(tC)T-eg1-eg1-eg1-TCTCTCT-NH_2 (25)	5′-dCGC**AGAGAGA**CGC-3′	41/42.0/70.0[c]
H-TTCCCTT-eg1-eg1-eg1-TTCCCTT-NH_2 (24) control	5′-dCGC**AAGGGAA**CGC-3′	36.5/47.5/77.5[c]
H-TT(tC)(tC)(tC)TT-eg1-eg1-eg1-TTCCCTT-NH_2 (26)	5′-dCGC**AAGGGAA**CGC-3′	34.5/44.5/74.5[c]

[a] Three units of 8-amino-3,6-dioxaoctanoic acid (eg1) connects the two antiparallel PNA strands. − [b] Pseudoisocytosine (J) was used instead of protonated cytosine in the Hoogsteen strand of the 7-mer bis-PNA. − [c] The thermal stability was determined at pH 9.0/7.0/5.0.

interactions will be more important for the tC nucleobase as compared to, for example, cytosine.

To supplement the study of the behavior of bT, tT, and tC in duplexes (Watson−Crick), bis-PNAs for triplex binding, containing these bases in the Hoogsteen strand of 7+7-mer bis-PNAs at three positions facing an adenine [bT (PNAs 19 and 20) and tT (PNAs 21 and 22)] or a guanine [tC (PNAs 25 and 26)], were constructed. All other positions consisted of T·A-T or either J·G-C (bT and tT) or C^+·G-C (tC) pairs for recognition of adenine or guanine in the DNA target (Table 4).

The thermal stability of the bis-PNAs was assessed using 13-mer oligodeoxyribonucleotides containing the 7-mer target sequences. The thermal stability of the triple helix complexes was compared with that of controls containing only conventional triplets. Increased thermal stability was observed for the system containing bT in both separated (PNA 19; ΔT_m = 1.2 °C/modification) and adjacent (PNA 20; ΔT_m = 2.0 °C/modification) positions in the Hoogsteen strand (Table 4). When tT was incorporated into the Hoogsteen strand in separated positions (PNA 21), the thermal stability was reduced (ΔT_m = −3.5 °C/modification), whereas incorporation into adjacent positions (PNA 22) resulted in triplexes of comparable stability to that of the control triplex. When tC was used in the Hoogsteen strand of bis-PNAs, decreases in the thermal stability were generally observed [ΔT_m = −2.7 °C/modification (pH = 7) (separated units, PNA 25) and ΔT_m = −1.0 °C/modification (adjacent units, PNA 26)]. However, the effect − not surprisingly − is less pronounced in the PNA 26 system that allows for tC-tC interbase stacking. It is also noted that the pH dependency of the triplex stability (reflecting the requirement of protonation) is more pronounced for the tC-containing PNAs, indicating that the pKa for tC is lower than that of cytosine.

Conclusion

In conclusion, the results presented here demonstrate the bT ring system to be a good mimic of the natural thymine nucleobase, providing increased thermal stability in PNA_2-DNA triplex structures upon incorporation of bT into the Hoogsteen strand. Furthermore, affinity for "Watson−Crick-complementary" oligomers was not impaired relative to the A- and T-containing controls, and both PNA-DNA and PNA-PNA duplexes were formed with a specificity comparable to that of thymine. This was further emphasized by the X-ray structure of the hexameric self-complementary bT-PNA containing one bT nucleobase. The bT nucleobase is easily accommodated in the PNA structure and the P-form double helix structure is preserved.

Extending the 1,8-naphthyridine heterocycle to include yet another aromatic ring, as in tT, should preserve the position and identity of the donor and acceptor hydrogen bonding sites. Indeed, the thermal stability of the duplexes containing *one* tT was identical to that of the thymine control. However, the tT heterocycle performed inconsistently in other sequences, giving rise to multiple thermal transitions, and displaying impaired specificity. When part of the Hoogsteen strand of bis-PNAs, tT led to a decrease in thermal stability.

Duplexes containing tC-G base pairs exhibited significantly enhanced thermal stability relative to duplexes containing only the natural A-T and G-C base pairs. The increase in thermal stability was found, in the very limited sequence context studies here, to be most pronounced when the substituents were adjacent or when both nearest neighbors were purines. The decreased thermal stability observed upon introduction of tC into the Hoogsteen strand of bis-PNAs may be a result of decreased basicity of the N-3 relative to the cytosine control.

The present results very clearly demonstrate (not too surprisingly) that factors other than mere molecular (hydrophobic) stacking overlap − such as (induced) dipole-dipole interactions − contribute significantly to base pair stacking stabilities. However, it should be reasonably uncomplicated to use the naphthyridinone system to explore the influence of such electronic factors by constructing substituted naphthyridinones, and such work is now in progress.

In terms of applications, the bT and the tC extended pyrimidines are interesting substitutes for thymine and cytosine, respectively, for antisense/antigene and diagnostic/molecular biology applications of PNA,[14,15] and the bT moiety may also be of interest in a DNA chemical context. Furthermore, it will be very interesting to examine whether incorporation of tT and/or tC nucleobases into biologically active PNAs improves their potency by improving cellular uptake, as was recently observed for the phenoxazine tC in a DNA context.[13]

Experimental Section

General Remarks: 2-(Pivaloylamino)pyridine-3-carbaldehyde was synthesized from 2-aminopyridine by means of a pivaloylamino-directed *ortho* lithiation on 2-pivaloylaminopyridine according to the procedure published by Turner.[20,21] The alkyl *N*-[2-(*tert*-butyloxycarbonylamino)ethyl]glycinate backbone was prepared by the previously described method.[22] The following chemicals were used as received: Butyllithium (BuLi) (10 M in hexane), *tert*-butyllithium (*t*BuLi) (1.5 M in hexane), lithiumdiisopropylamide (LDA), diisopropylamine, diisopropylethylamine, sodium hydride (60% in mineral oil), 5-bromouracil, 2-aminothiophenol, 1,3-dicyclohexylcarbodiimide, 1-hydroxy-7-azabenzotriazole, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), dichloromethane, ethyl acetate, tetrahydrofuran (THF), and dimethylformamide (DMF). − TLC was performed on silica 60, column chromatography on silica 60 ((Merck 5554 aluminum sheet). − ^1H and ^{13}C spectra were obtained in [D_6]DMSO (if not indicated otherwise) at either 250 MHz (Bruker AM 250) or at 400 MHz (Varian 400) in 5 mm tubes. − Melting points were obtained on a Büchi B-540 melting point apparatus.

Thermal stability (T_m) was measured on solutions ca. 3 μM in PNA and DNA at pH 7.0 in 100 mM NaCl, 10 mM Na-phosphate, and 0.1 mM EDTA. Absorptions at 260 nm were recorded at 0.5 °C

intervals from 5−90 °C. RNA oligonucleotides were obtained from DNA Technologies, Denmark. DNA oligonucleotides were synthesized by Dr. Britta Dahl at the Chemistry Department.

Di-*tert*-butyl Succinate: Succinoyl chloride (0.40 mol, 61 g) was added dropwise to a refluxing mixture of dimethylaniline (0.84 mol, 101 g), *tert*-butyl alcohol (0.75 mol, 57 g), and diethyl ether (200 mL). The resulting solution was stirred under reflux for 2 h and quenched by addition of water (200 mL). The organic phase was separated, extracted twice with 10% H_2SO_4 (25 mL) and once with $NaHCO_3$ (100 mL), dried ($MgSO_4$), and evaporated to an oil in vacuo. Kugelrohr distillation (116 °C, 16 Torr) afforded the desired product (11 g, 22%) as a colorless, low melting solid. − ^{1}H NMR ($CDCl_3$): δ = 2.48 (s, 4 H, CH_2), 1.45 (s, 18 H, CH_3). − ^{13}C NMR ($CDCl_3$): δ = 171.6, 80.5, 30.6, 28.1.

(1,2-Dihydro-2-oxo-1,8-naphthyridin-3-yl)acetic Acid (3a): Di-*tert*-butyl succinate (20.0 mmol, 4.60 g) dissolved in THF (5 mL) was added dropwise to a precooled (−78 °C) solution of lithium diisopropylamide (2 M in heptane/THF/ethylbenzene) (20 mL, 40.0 mmol) in THF. After 30 min, 2-(pivaloylamino)pyridine-3-carbaldehyde (19.0 mmol, 3.92 g) dissolved in THF (10 mL) was added and the clear yellow solution was stirred at −78 °C for 15 min, and then allowed to warm to room temp. The solution was poured into ammonium chloride (sat., aqueous) (200 mL) and extracted with dichloromethane (2 × 100 mL). The organic phase was dried ($MgSO_4$) and evaporated to dryness in vacuo. This crude product of diastereomeric alcohols was heated to reflux for 24 h in a mixture of dioxane (10 mL) and HCl (3 M, aqueous) (40 mL), and then poured into water (200 mL) and neutralized with K_2CO_3. The tan precipitate was washed with water (2 × 20 mL) and dried in vacuo overnight to yield the desired product (1.62 mg, 42%), more than 90% pure according to HPLC (260 nm) and used as such in the synthesis of **4a**.

Methyl *N*-[2-(*tert*-butyloxycarbonylamino)ethyl]-*N*-(1,2-dihydro-2-oxo-1,8-naphthyridin-3-yl)acetylglycinate (4a): Methyl *N*-[2-(*tert*-butyloxycarbonylamino)ethyl]glycinate (4.50 mmol, 1.04 mg) was dissolved in DMF (20 mL) and **3a** (5.00 mmol, 1.02 g), 1-hydroxy-7-azabenzotriazole (5.00 mmol, 680 mg), and diisopropylethylamine (5.0 mmol, 0.87 mL) were added. The mixture was cooled on ice and dicyclohexylcarbodiimide (5.5 mmol, 1.13 g) was added. After 1 h, the ice bath was removed and the mixture was stirred overnight at room temp. The mixture was evaporated in vacuo, redissolved in ethyl acetate (150 mL), and washed with 5% aqueous $NaHCO_3$ (2 × 50 mL) and with water (50 mL). The organic phase was dried over $MgSO_4$, filtered, and evaporated in vacuo. The crude product was purified on a silica column eluted with dichloromethane/methanol (9:1 v/v). Fractions containing the product were evaporated in vacuo to yield the product (1.09 g, 58%) as a slightly tan solid, pure according to HPLC (260 nm). − ^{1}H NMR (this compound exist as two rotamers; chemical shifts for the minor rotamer are given in brackets): δ = 12.18 (br. s, 1 H, NH), 8.49−8.47 (m, 1 H, arom), 8.08−8.02 (8.16−8.08) (m, 1 H, arom), 7.79 (7.76) (s, 1 H, arom), 7.25−7.22 (m, 1 H, arom), 6.91 (6.75) (t, 1 H, NH), 4.06 (4.37) (s, 2 H, CH_2), 3.67 (3.48) (s, 2 H, CH_2), 3.64 (s, 3 H, CH_3O), 3.49−3.46 (m, 2 H, CH_2), 3.20−3.15 (3.08−3.02) (m, 2 H, CH_2), 1.36 (1.35) [s, 9 H, CH_3 (Boc)]. − ^{13}C NMR: δ = 170.3 (170.4), 170.1 (170.5), 162.6, 155.8, 149.9, 149.3, 136.5 (136.8), 135.9 (136.0), 129.9 (129.6), 118.5, 114.4, 78.1 (77.1), 51.8 (52.2), 48.2, 47.6 (46.7), 38.5 (38.0), 33.5 (33.8), 28.3. − M.p. 145−146 °C. − HRMS (FAB^+): 419.1941 (M + H^+, calcd. for $C_{20}H_{26}N_4O_6+H^+$ 419.1930).

***N*-[2-(*tert*-Butyloxycarbonylamino)ethyl]-*N*-(1,2-dihydro-2-oxo-1,8-naphthyridin-3-yl)acetylglycine (5a):** Methyl *N*-[2-(*tert*-butyloxycarbonylamino)ethyl]-*N*-(1,2-dihydro-2-oxo-1,8-naphthyridin-3-yl)acetylglycinate (**4a**) (2.39 mmol, 1.00 g) was dissolved in THF (20 mL) and LiOH (2 M, aqueous, 12.0 mmol, 6.0 mL) was added. After 20 min at room temp., additional water (15 mL) was added and the THF was removed in vacuo. The pH of the aqueous phase was adjusted to 3.0 by addition of HCl (2 M, aqueous). The precipitate was filtered off, washed with water (2 × 10 mL), and dried in vacuo to yield the product (852 mg, 88%) as a colorless powder, pure according to HPLC (260 nm). − ^{1}H NMR (this compound exist as two rotamers; chemical shifts for the minor rotamer are given in brackets): δ = 12.67 (br. s, 1 H, COOH), 12.20 (br. s, 1 H, NH), 8.49−8.46 (m, 1 H, arom), 8.11−8.00 (8.18−8.11) (m, 1 H, arom), 7.82 (7.76) (s, 1 H, arom), 7.26−7.22 (m, 1 H, arom), 6.91 (6.74) (t, 1 H, NH), 3.98 (4.25) (s, 2 H, CH_2), 3.61 (3.47) (s, 2 H, CH_2), 3.47−3.40 (m, 2 H, CH_2), 3.22−3.10 (3.08−3.02) (m, 2 H, CH_2), 1.36 (1.35) [s, 9 H, CH_3 (Boc)]. − ^{13}C NMR: δ = 171.0 (171.3), 170.1 (170.5), 162.61 (162.7), 155.8 (155.7), 155.0, 149.8 (149.3), 136.5 (136.7), 135.8 (136.0), 130.0 (129.7), 118.4, 114.5, 78.0 (77.8), 47.6 (50.3), 48.1 (46.7), 38.4 (38.0), 33.8 (33.5), 28.3. − M.p. 176−178 °C. − HRMS (FAB^+): 405.1776 (M + H^+, calcd. for $C_{19}H_{24}N_4O_6+H^+$ 405.1774).

2-Pivaloylaminoquinoline-3-carbaldehyde (1b): BuLi (10 M in hexane, 54.80 mmol, 5.48 mL) was added dropwise at −78 °C to a solution of 2-pivaloylaminoquinoline (21.92 mmol, 5.00 g) in THF (75 mL). After 2 h at −78 °C, the dianion was quenched by addition of *N*-formylmorpholine (37.88 mmol, 3.79 g), and the reaction mixture was subsequently allowed to warm to room temp. and then poured into HCl (2 M, aqueous, 20 mL). The pH of the aqueous phase was adjusted to 7.0 by addition of HCl (2 M, aqueous) and, after addition of water (100 mL), the aqueous phase was extracted with diethyl ether (2 × 100 mL). The organic phase was dried ($MgSO_4$) and evaporated to dryness in vacuo. The crude product was recrystallized from petroleum ether/ethyl acetate to yield the product (3.14 g, 56%), pure according to HPLC (260 nm). − ^{1}H NMR: δ = 10.70 (br. s, 1 H, NH), 9.82 (s, 1 H, CHO), 8.85 (s, 1 H, arom), 8.19−8.17 (m, 1 H, arom), 7.97−7.95 (m, 1 H, arom), 7.91−7.87 (m, 1 H, arom), 7.66−7.62 (m, 1 H, arom), 1.30 (s, 9 H, CH_3). − ^{13}C NMR: δ = 189.62, 189.55, 178.4, 149.7, 148.2, 140.3, 132.8, 129.8, 127.1, 126.6, 125.5, 123.6, 27.0. − M.p. 161−162 °C. − HRMS(FAB^+) 257.1294 (M + H^+, calcd. for $C_{15}H_{16}N_2O_2+H^+$ 257.1290).

(1,2-Dihydro-2-oxobenzo[*b*][1,8]-naphthyridine-3-yl)acetic Acid (3b): BuLi (10 M in hexane, 30.85 mmol, 3.09 mL) was added at −78 °C to a solution of diisopropylamine (30.85 mmol, 3.12 g) in THF. After 15 min, di-*tert*-butyl succinate (30.85 mmol, 7.10 g) dissolved in THF (10 mL) was added dropwise. After another 30 min at −78 °C, 2-pivaloylaminoquinoline-3-carbaldehyde (**1b**) (7.50 mmol, 1.92 g) dissolved in THF (10 mL) was added over 15 min, and the solution was allowed to warm to room temp. after stirring at −78 °C for 30 min. The solution was then poured into sat., aqueous ammonium chloride (200 mL) and extracted with diethyl ether (2 × 100 mL). The organic phase was washed with sat., aqueous NaCl (100 mL) and dried ($MgSO_4$), and evaporated in vacuo. The crude product of diastereomeric alcohols **2b** (4.18 g, 59%) was heated under reflux for 24 h in a mixture of dioxane (10 mL) and HCl (3 M, aqueous, 10 mL) and then poured into water (100 mL) and neutralized with K_2CO_3. The tan precipitate was washed with water (2 × 25 mL) and dried in vacuo overnight to yield the crude product (1.59 g, 43%), more than 90% pure according to HPLC (260 nm), and used as such in the synthesis of **4b**.

Methyl *N*-[2-(*tert*-Butyloxycarbonylamino)ethyl]-*N*-(1,2-dihydro-2-oxobenzo[*b*][1,8]-naphthyridin-3-yl)acetylglycinate (4b): Methyl *N*-

[2-(*tert*-butyloxycarbonylamino)ethyl]glycinate (3.5 mmol, 812 mg) was dissolved in DMF (10 mL) and (1,2-dihydro-2-oxobenzo[*b*][1,8]-naphthyridine-3-yl)acetic acid (**3b**, 883 mg, 3.5 mmol) was added. The mixture was cooled (ice bath) and HBTU (4.0 mmol, 1.52 g) was added. After 1 h, the ice bath was removed and the mixture was stirred overnight at room temp. The mixture was evaporated in vacuo, redissolved in dichloromethane (100 mL), and washed with 5% aqueous $NaHCO_3$ (2 × 50 mL). The organic phase was dried with $MgSO_4$, filtered, and evaporated in vacuo. The crude product was purified on a silica column eluted with dichloromethane/methanol (95:5 v/v). Fractions containing the product were evaporated in vacuo to yield the product (821 mg, 50%) as a slightly tan solid, more than 95% pure according to HPLC (260 nm). − ^{1}H NMR (this compound exist as two rotamers; chemical shifts for the minor rotamer are given in brackets): δ = 12.13 (br. s, 1 H, NH), 8.70−8.65 (m, 1 H, arom), 8.07−8.01 (m, 1 H, arom), 7.91 (s, 1 H, arom), 7.88 (s, 1 H, arom), 7.79−7.75 (m, 1 H, arom), 7.55−7.49 (m, 1 H, arom), 6.90 (6.72) (br. s, 1 H, NH), 4.05 (4.36) (s, 2 H, CH_2), 4.11 (q, 3 H, CH_3), 3.67 (3.33) (s, 2 H, CH_2), 3.48−3.53 (m, 2 H, CH_2), 3.27−3.04 (m, 2 H, CH_2), 1.35 (1.35) [s, 9 H, CH_3 (Boc)]. − ^{13}C NMR: δ = 170.2 (170.3), 170.0 (170.4), 163.1 (163.2), 155.8 (155.6), 148.7, 147.0, 136.2 (136.5), 136.07 (136.13), 131.2, 130.2, 129.9, 128.7, 126.9, 124.9 (124.7), 115.7 (115. 6), 78.0 (77.8), 51.8 (52.2), 48.18 (48.4, 47.6, 38.4 (38.3), 33.6 (33.9), 28.26. FAB$^+$MS: 469.10 (M + H$^+$, calcd. for $C_{24}H_{28}N_4O_6$+H$^+$ 469.21) and 491.08 (M + Na$^+$, calcd. for $C_{24}H_{28}N_4O_6$+Na$^+$ 491.19).

***N*-[2-(*tert*-Butyloxycarbonylamino)ethyl]-*N*-(1,2-dihydro-2-oxobenzo[*b*][1,8]-naphthyridin-3-yl)acetylglycine (5b):** Methyl *N*-[2-(*tert*-butyloxycarbonylamino)ethyl]-*N*-(1,2-dihydro-2-oxobenzo[*b*]-[1,8]-naphthyridin-3-yl)acetylglycinate **4b** (0.426 mmol, 200 mg) was dissolved in THF (4.0 mL) and LiOH (2 M, aqueous) (2.14 mmol, 1.07 mL) was added. After 15 min at room temp., additional water (7.0 mL) was added and the THF was removed in vacuo. The pH of the aqueous phase was adjusted to 3.0 by addition of HCl (2 M, aqueous). The precipitate was filtered off, washed with water (2 × 5 mL), and dried in vacuo to yield the product (176 mg, 91%) as a slightly tan powder, more than 95% pure according to HPLC (260 nm). − ^{1}H NMR (this compound exist as two rotamers; chemical shifts for the minor rotamer are given in brackets): δ = 12.66 (br. s, 1 H, COOH), 12.15 (br. s, 1 H, NH), 8.63 (8.69) (s, 1 H, arom), 8.02−8.05 (m, 1 H, arom), 7.95 (s, 1 H, arom), 7.87−7.90 (m, 1 H, arom), 7.76−7.81(m, 1 H, arom), 7.50−7.54 (m, 1 H, arom), 6.91 (6.73) (t br, 1 H, NH), 4.00 (4.26) (s, 2 H, CH_2), 3.66 (3.52) (s, 2 H, CH_2), 3.45 (3.35) (t, 2 H, CH_2), 3.16−3.21 (3.02−3.08) (m, 2 H, CH_2), 1.37 (1.35) (s, 9 H, CH_3). − ^{13}C NMR: δ = 170.9 (171.2), 170.0 (170.3), 163.1 (163.2), 155.6 (155.7), 148.7, 147.0, 136.2 (136.4), 136.0 (136.4), 131.2, 120.0, 128.6, 126.9, 124.8, 124.7, 115.69 (115.66), 78.0 (77.7), 50.3 (46.8), 47.5 (48.1), 38.4 (38.0), 33.6 (33.8), 28.26. − M.p. 139−141 °C. − HRMS(FAB$^+$) 455.1922 (M + H$^+$, calcd. for $C_{23}H_{27}N_4O_6$+H$^+$ 455.1931).

Ethyl *N*-(Bromoacetyl)-*N*-(2-*tert*-butyloxycarbonylaminoethyl)glycinate: Bromoacetyl bromide (404 mg, 2.0 mmol) was added to a precooled solution (−78 °C) of ethyl *N*-(2-Boc-aminoethyl)glycinate (464 mg, 2.0 mmol) and 2,4,6-collidine (249 mg, 2.05 mmol) in dichloromethane. The resulting solution was allowed to warm to room temp. and stirred for 30 minutes before evaporation to dryness in vacuo. The crude residue was taken up in ethyl acetate (20 mL) and the 2,4,6-collidine hydrobromide filtered off. The solution was concentrated in vacuo, and the crude product was purified on silica with ethyl acetate as the eluent. Fractions containing the product were pooled and evaporated in vacuo to yield the desired product as a clear oil (538 mg, 73.2%), pure according to TLC (viewed using ninhydrin). − ^{1}H NMR ($CDCl_3$, this compound exists as two rotamers; chemical shifts for the minor rotamer are given in brackets): δ = 5.45 (4.98) (s, 1 H, NH), 4.15 (4.16) (q, 2 H, CH_2), 3.97 (s, 2 H, CH_2), 3.88 (3.75) (s, 2 H, CH_2), 3.49 (q, 2 H, CH_2), 3.25 (m, 2 H, CH_2), 1.39 (1.38) (s, 9 H, CH_3(Boc)), 1.24 (1.26) (t, 3 H, CH_3). − ^{13}C NMR: δ = 169.7, 167.8, 156.1, 79.8, 61.7 (62.1), 50.3 (51.1), 49.2 (48.2), 38.7 (38.5), 28.4, 25.6 (2627), 14.1. − HRMS(FAB$^+$) 367.0869 (M + H$^+$, calcd. for $C_{13}H_{23}N_2O_5$+H$^+$ 367.0869).

Ethyl *N*-[(2-Hydroxy-10*H*-pyrimido[5,4-*b*][1,4]benzothiazin-1-yl)-acetyl]-*N*-[2-(*tert*-butyloxycarbonylamino)ethyl]glycinate (7): 2-Hydroxy-10*H*-pyrimido[5,4-*b*][1,4]benzothiazine (**6**) (PNAs 14, 15) (653 mg, 3.0 mmol) was suspended in DMF (30 mL) and NaH (60% in mineral oil) (132 mg, 3.3 mmol) was added in one portion. After 15 min, ethyl *N*-bromoacetyl-*N*-[2-(*tert*-butyloxycarbonylamino)ethyl]glycinate (1.10 g, 3.3 mmol) was added. The mixture was stirred at room temp. for 2 h, evaporated in vacuo, redissolved in dichloromethane (200 mL), and washed once with sat. aqueous $NaHCO_3$ (100 mL) and once with sat., aqueous NaCl, dried ($MgSO_4$), and evaporated to dryness in vacuo. The crude material was purified on silica, using methanol/dichloromethane (1:9 v/v) as eluent, to yield the product (853 mg, 57%) as a yellow powder. − ^{1}H NMR (this compound exist as two rotamers; chemical shifts for the minor rotamer are given in brackets): δ = 10.37 (br. s, 1 H, NH), 7.51 (s, 1 H, H-6), 7.09−7.05 (m, 2 H, arom), 6.94−6.75 (m, 3 H, arom+NH), 4.66 (4.48) (s, 2 H, CH_2), 4.04 (4.30) (s, 2 H, CH_2), 4.08 (4.18) (q, 2 H, CH_2), 3.41 (m, 2 H, CH_2), 3.20 (3.03) (m, 2 H, CH_2), 1.38 (1.37) (s, 9 H, CH_3(Boc)), 1.18 (1.24) (t, 3 H, CH_3). − ^{13}C NMR: δ = 169.1 (169.4), 167.6 (167.9), 160.2, 155.8, 154.6, 141.2, 136.5, 127.5, 126.1, 124.0, 117.0, 115.8, 93.4, 78.1 (77.9), 60.6 (61.2), 49.0 (49.26), 48.0, 47.1, 38.3, 28.3, 14.1. − M.p. 183−185 °C. − HRMS(FAB$^+$) 504.1917 (M + H$^+$, calcd. for $C_{23}H_{29}N_5O_6S$+H$^+$ 504.1917).

***N*-[(2-Hydroxy-10*H*-pyrimido[5,4-*b*][1,4]benzothiazin-1-yl)acetyl]-*N*-[2-(*tert*-butyloxycarbonylamino)ethyl]glycine (8):** Ethyl *N*-[(2-Hydroxy-10*H*-pyrimido[5,4-*b*][1,4]benzothiazin-1-yl)acetyl]-*N*-[2-(*tert*-butyloxycarbonylamino)ethyl]glycinate (**7**) (201 mg, 0.4 mmol) was dissolved in THF (10 mL) and LiOH (2 M, aqueous) (1.0 mL) was added. After 15 min at room temp., water (10 mL) was added, the THF was removed in vacuo, and the pH of the aqueous phase was adjusted to 3.0 by addition of HCl (2 M, aqueous). The solid yellow material was filtered off, washed with water (2 × 3 mL), and dried in vacuo to yield the product (173 mg, 91%) as a yellow powder, more than 96% pure according to HPLC. − ^{1}H NMR (this compound exist as two rotamers; chemical shifts for the minor rotamer are given in brackets): δ = 10.41 (br. s, 1 H, NH), 7.50 (s, 1 H, H-6), 7.15−7.07 (m, 2 H, arom), 7.00−6.75 (m, 3 H, arom+NH), 4.64 (4.50) (s, 2 H, CH_2), 4.04 (4.28) (s, 2 H, CH_2), 3.41 (m, 2 H, CH_2), 3.25 (3.07) (m, 2 H, CH_2), 1.39 (1.38) [s, 9 H, CH_3(Boc)]. − M.p. 205−207 °C. − HRMS(FAB$^-$) 475.1516 (M$^-$, calcd. for $C_{21}H_{25}N_5O_6S$ 475.1526).

PNA Oligomer Synthesis: PNA oligomers were synthesized as previously described, using commercial A, C, G, and T Boc-protected monomers (PerSeptive Biosystems, Framingham MA, USA).[14,23] All oligomers were purified by reversed phase HPLC, and were characterized by MALDI-TOF mass spectrometry.

X-ray Crystallographic Study − Crystallization of bT-PNA (H-GbTATAC-L-lys-NH_2): A sparse-matrix screening (Crystal Screen I and II, Hampton Research) and the hanging drop vapor diffusion

method were used to find the initial crystallization conditions for bT-PNA, which were optimized to give crystals with a size of 0.25 × 0.20 × 0.02 mm. A 4 μL drop of a 5 mg/ml solution of bT-PNA in water was mixed with a 3 μL drop of a reservoir solution containing 0.6 M ammonium sulfate and 5% 2-propanol and the resulting drop was equilibrated against 0.5 mL of the reservoir solution at 20 °C. Compound bT-PNA crystallizes in the orthorhombic space group $P2_12_12$, with cell dimensions a = 30.34, b = 45.00, c = 49.28 Å.

Data Collection: A complete diffraction data set to 1.82 Å was collected at 293 K and at a crystal-to-image plate distance of 225 mm, using synchrotron radiation, λ = 1.050 Å (EMBL beamline X31 at the DORIS storage ring, DESY, Hamburg). Diffraction data corresponding to 102° rotation were collected on one crystal in steps of 2° oscillations. Autoindexing and processing of diffraction data were performed using DENZO, SCALEPACK,[26] and the CCP4 suite of programs.[27] Data collection statistics are shown in Table 5.

Molecular Replacement: The program AMORE[28] implemented in the CCP4 suite of programs was used to find a molecular replacement solution. One right-handed and one left-handed double helix were located in the asymmetric unit. The coordinates of a right-handed and a left-handed double helix of a previously reported PNA-PNA hexamer (H-CGTACG-NH_2, pdbcode 1PUP) were used as search models in the resolution range 25.0−3.0 Å for the rotation function and in the resolution range 25.0−2.0 Å for the translation function. The left-handed search model was generated by inversion of the deposited right-handed structure using the program SYBYL (Tripos Associates, Inc.). The 20 highest peaks of the rotation solutions were used in the translation searches. The right-handed double helix was located first and its position fixed in the translation search for the position of the left-handed double helix. The correlation coefficient and R-value after the translation search were 43.8 and 68.7%, respectively, for the right-handed double helix, and 54.9 and 66.7% after the translation search for the left-handed double helix. This solution was subjected to 10 cycles of rigid body refinement using the program AMORE. The refinement was performed with each of the left-handed and right-handed double helices as rigid bodies in the resolution range 25.0−2.0 Å. Rigid body refinement of the individual double helices resulted in a correlation coefficient of 70.5 and an R value of 60.4%. The packing of the molecules in the unit cell was inspected using the program O,[29] and revealed coaxial stacking of the two double helices as seen for other PNA structures.[24,25]

Refinement and Model Building: An electron density map was generated using CCP4,[27] and the nucleobases corresponding to the bT-PNA sequence were built into the density. The two double helices were then subjected to a rigid body refinement protocol using the program X-PLOR,[30] and the four individual strands were refined independently. The R factor after rigid body refinement was 58.2% and R-free (10%) was 60.2%. The structure was refined by positional refinement in X-PLOR in the resolution range 6.0−1.82 Å and with a cutoff of 3σ. B factor refinement and addition of water molecules were included. After the first refinement cycle, the C-terminal lysines were built into the electron density using the graphics program O, and the refinement progressed using positional refinement alternating with graphical sessions. Two alternative conformations of the backbone amide groups, connecting A5 and C6 in the right-handed helix and the corresponding A23 and C24 in the left-handed helix, respectively, were identified (occupancies of 2/3 and 1/3). Additional double conformations, although less pronounced, are indicated at seven other backbone amide groups. The final R value is 21.2% and R-free 26.9%. The refinement statistics are listed in Table 5.

The atomic coordinates of bT-PNA have been deposited in the Protein Data Bank http://www.rcsb.org/pdb/, accession code 1HZS.

Table 5. Diffraction data and refinement statistics for bT-PNA (H-G*bT*ATAC-L-lys-NH_2)

Diffraction data	
Space group	$P2_12_12$
Unit cell parameters	a = 30.34 Å, b = 45.00, c = 49.28 Å
Resolution range	30.0−1.82 Å
Unique reflections	6245
Completeness	97.2%
Multiplicity	3.8
R*merge*(*I*) (30.0−1.82 Å)[a]	5.5%
R*merge*(*I*) (1.84−1.82 Å)[a]	28.1%
$<I/\sigma(I)>$ (30.0−1.82 Å)	20.1
$<I/\sigma(I)>$ (1.84−1.82 Å)	2.6
Refinement	
Resolution range	6.0−1.82 Å
Number of reflections	4196
σ cutoff	3
R-factor/*R*-free	21.2/26.9%
Total number of non-hydrogen atoms	626
Water molecules	110
Average B factor of PNA units	11.0 Å^2
Average B factor of L-Lys units	38.7 Å^2
Average B factor of water molecules	29.2 Å^2
R.m.s. deviation of bond lengths	0.018 Å
R.m.s. deviation of bond angles	3.6°

[a] R*merge*: agreement between symmetry related reflections. – [b] *R*-free: cross-validation *R* factor for test set of reflections (10%) omitted during refinement.

Acknowledgments

We thank Ms. Annette W. Jørgensen, Margit Jørgensen, and Brian Rosenberg for skillful technical assistance. EMBL, Hamburg, Germany and MAX-Lab, Lund, Sweden are gratefully acknowledged for support of the work during X-ray data collections. This work was supported by the Danish National Research Foundation, the Lundbeck Foundation, The Danish Cancer Society, PharmaBiotec, the Carlsberg Foundation, and the Dansync Center for Synchrotron Radiation. We thank the European Union for support of the work at EMBL Hamburg through the HCMP Access to Large Installations Project, Contract Number CHGE-CT93-0040.

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Received May 8, 2000
[O00227]