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Substituted 1,8-Naphthyridin-2(1*H*)-ones Are Superior to Thymine in the Recognition of Adenine in Duplex as Well as Triplex Structures

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Abstract: The synthesis and evaluation of a series of novel nucleobases based on substituted 1,8-naphthyridin-2(1*H*)-ones are reported. The nucleobases were designed to meet the requirements for incorporation into peptide nucleic acids (PNAs) and were evaluated as part of PNA duplex and triplex nucleic acid recognition systems. Of the various nucleobases tested, only the 7-chloro-1,8-naphthyridin-2(1*H*)-one (7-Cl-bT) nucleobase led to consistently increased affinity in all recognition systems, duplex (Watson−Crick) as well as triplex (Hoogsteen). For multiply modified systems, the increase in thermal stability per modification was dependent on the sequence context, ranging from 2.0 °C (in separate positions) to 3.5 °C (in adjacent positions) in PNA−DNA duplexes and from 1.2 °C (in separate positions) to 3.2 °C (in adjacent positions) in PNA−RNA duplexes. Singly mismatched oligonucleotide targets were employed to demonstrate uncompromised sequence discrimination. When part of multiply modified triplex (Hoogsteen) recognition systems, the 7-Cl-bT unit gave rise to increases in the thermal stability ranging from 2.7 to 3.5 °C when incorporated into separated and adjacent positions, respectively. Our results furthermore indicate that the duplex stabilization is predominantly enthalpic and therefore most likely not a consequence of single-strand preorganization. Finally, and most surprisingly, we find no direct correlation between the end-stacking efficiency of this type of nucleobase and its helix stabilization when involved in Watson−Crick base pairing within a helix.

Introduction

Peptide nucleic acids (PNAs)[1] have potential to become third generation antisense and/or antigene therapeutics and are also widely used in research. Single-stranded nucleic acids can be effectively targeted by Watson−Crick complementary PNAs, whereas efficient targeting of duplex DNA requires triplex invasion of homopurine tracts within the DNA by homopyrimidine PNAs or double duplex invasion by pseudo complementary PNA oligomers.[2]

Modification of the natural nucleobases represents an obvious way to control recognition between nucleic acids. The surface area of a nucleobase is a decisive parameter for the stacking energy of neighboring base pairs.[3] Hence, the capacity of modified pyrimidines with increased surface area to stack effectively with neighboring nucleobases has motivated numerous attempts to synthesize pyrimidines containing substituents that extend the pyrimidine surface area in the trace of the C4 and C5 pyrimidine positions (the *d* face of the pyrimidine).[4] Elaboration of existing N-nucleosides has been the most convenient route to nucleosides containing nucleobases with increased surface area, as synthesis of so-called C-nucleosides requires a more extensive synthetic effort. The achiral nature

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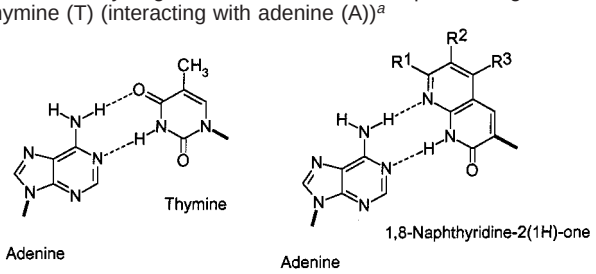
of PNA makes this nucleic acid recognition system an attractive skeleton onto which novel nucleobases can be incorporated, the only structural requisite for incorporation into a *tert*-butyloxycarbonyl (Boc) PNA monomer being the presence of an appropriately positioned acetic acid linker on the nucleobase. As part of a continuing effort to exploit this feature of PNA, we recently reported the synthesis and hybridization properties of PNA recognition systems containing the 1,8-naphthyridin-2(1*H*)-one (bT) and benzo[*b*]-1,8-naphthyridin-2(1*H*)-one (tT) heterocycles.[5] The bT heterocycle was shown to be an effective mimic of the thymine nucleobase, generally allowing for efficient targeting of single as well as double-stranded oligonucleotides, through formation of duplex and triplex structures, respectively, of uncompromised (but not increased) stability and sequence discrimination. We now report the synthesis and properties of a series of (Boc)PNA monomers containing substituted naphthyridinones: 7-chloro-1,8-naphthyridin-2(1*H*)-one (7-Cl-bT), 6-chloro-1,8-naphthyridin-2(1*H*)-one (6-Cl-bT), 7-methyl-1,8-naphthyridin-2(1*H*)-one (7-CH_3-bT), 6-methyl-1,8-naphthyridin-2(1*H*)-one (6-CH_3-bT), and 5-methyl-1,8-naphthyridin-2(1*H*)-one (5-CH_3-bT), designed to mimic the function of thymine in duplex and triplex (Hoogsteen) recognition systems by specific interaction with adenine. The 1,8-naphthyridin-2(1*H*)-one (bT) heterocyclic system should retain the distribution of hydrogen-bond-donor and -acceptor sites present in thymine by supporting hydrogen-bond formation between the adenine C-6 amino group and the 1,8-naphthyridin-2(1*H*)-one *N*-8, in addition to the formation of a hydrogen bond between the adenine *N*-1 and the 1,8-naphthyridin-2(1*H*)-one *N*-1−H.[5]

Furthermore, within an A- or B-type double helix, the overlap between substituents in the 6- and 7-positions of 1,8-naphthyridin-2(1*H*)-one and the neighboring nucleobases[5] is expected to provide additional stability to the helical structure. We now show that 7-chloro-1,8-naphthyridin-2(1*H*)-one does indeed stabilize PNA−DNA, −RNA, and −PNA duplexes as well as PNA_2–DNA triplex. Our results furthermore indicate that the stabilization is predominantly enthalpic and therefore most likely not a consequence of single-strand preorganization. Finally, and most surprisingly, we find no direct correlation between the end-stacking effeiciency of this type of nucleobase and its helix stabilization when involved in Watson−Crick base pairing within a helix.

Results and Discussion

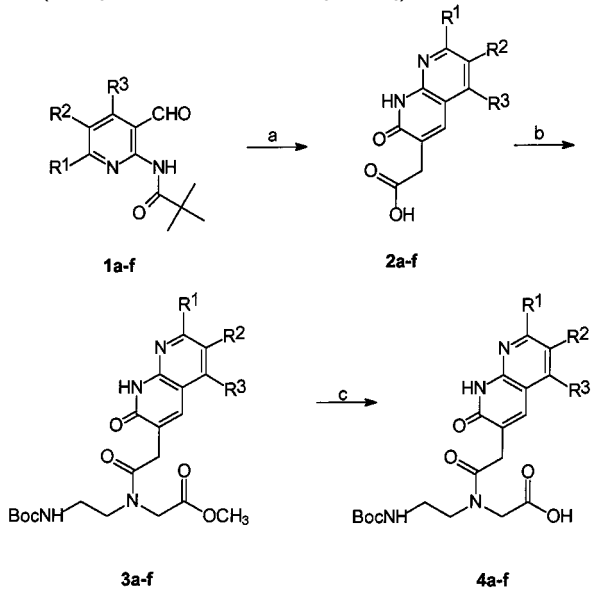
For the synthesis of the Boc-PNA monomer containing the novel 7-Cl-bT, 6-Cl-bT, 7-CH_3-bT, 6-CH_3-bT, and 5-CH_3-bT nucleobases (**4b**−**f**, Scheme 2), the starting material was 6-chloro-3-formyl-2-(pivaloylamino)pyridine[6] (**1b**), 5-chloro-3-formyl-2-(pivaloylamino)pyridine[6] (**1c**), 3-formyl-6-methyl-2-(pivaloylamino)pyridine[6] (**1d**), 3-formyl-5-methyl-2-(pivaloylamino)pyridine[6] (**1e**), and 3-formyl-4-methyl-2-(pivaloylamino)pyridine (**1e**), respectively. The precursor to the formyl derivative **1b** was conveniently prepared from 2,6-diaminopyridine through monoacylation with pivaloyl chloride and subsequent diazotation in concentrated aqueous HCl to yield the 6-chloro-2-(pivaloylamino)pyridine, which was formylated according to literature procedures.[6] However, other more laborious procedures to obtain the 6-chloro-2-(pivaloylamino)-pyridine have been described.[7] The previously unknown 3-formyl-4-methyl-2-(pivaloylamino)pyridine (**1e**) was prepared from 4-methyl-2-(pivaloylamino)pyridine[6] by simple lithiation with *tert*-BuLi in diethyl ether at −78 °C. The mandatory acetic acid derivatives (**2b**−**f**) were prepared from **1b**−**f** by condensation with the enolate of di-*tert*-butyl succinate[5] and subsequent cyclization under acidic conditions. The general procedure for these conversions includes the preformation of the enolate of di-*tert*-butyl succinate using LDA and di-*tert*-butyl succinate in diethyl ether at −78 °C. The enolate was added to a precooled solution (−78 °C) of **1b**−**f** in THF and initially stirred at −78 °C and then at room temperature. The crude product obtained

Scheme 1. Hydrogen-Bond-Donor and -Acceptor Arrangement in Thymine (T) (interacting with adenine (A))[a]



[a] Similar interactions are possible with various substituted derivatives of the 1,8-naphthyridin-2(1*H*)-one ring system (R_1, R_2, or R_3 =/H), as N-8 of the 1,8-naphthyridin-2(1*H*)-one heterocycle functions as an efficient hydrogen-bond acceptor.

Scheme 2. General Scheme Describing the Synthesis of Boc-PNA Monmers **4a**−**f** Containing Various Substituted 2(1*H*)-Oxo-1,8-naphthyridine Nucleobases: **2a**−**4a** (bT; R_1 = R_2 = R_3 = H),[5] **2b**−**4b** (7-Cl-bT; R_1 = Cl, R_2 = R_3 = H), **2c**−**4c** (6-Cl-bT; R_2 = Cl, R_1 = R_3 = H), **2d**−**4d** (7-CH_3-bT; R_1 = CH_3, R_2 = R_3 = H), **2e**−**4e** (6-CH_3-bT; R_2 = CH_3, R_1 = R_3 = H), and **2f**−**4f** (5-CH_3-bT; R_1 and R_2 = H, R_3 = CH_3)[a]



[a] (a)Preformed enolate of di-*tert*-butyl succinate using LDA and di-*tert*-butyl succinate in diethyl ether at −78 °C was added to a precooled solution (−78 °C) of **1b**−**f** in THF, initially stirred at −78 °C and then at room temperature; the crude product from this reaction was treated with 3 M aqueous HCl at reflux. (b) Methyl *N*-(2-Boc-aminoethyl)glycinate, DHBTOH, and DCC, or HBTU in DMF, rt, overnight. (c) 2 M LiOH in THF at room temperature. tT is composed of three aromatic rings, i.e., compound **4** with R_1, R_2 = −CH=CHCH=CH−.

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Table 1. Thermal Stability (T_m) of PNA−DNA, PNA−RNA, and PNA−PNA Duplexes Assessed Using a 10-mer PNA Containing One 7-Cl-bT, 6-Cl-bT, 7-CH_3-bT, 6-CH_3-bT, or 5-CH_3-bT Unit and a 10-mer Otherwise Watson−Crick Complementary DNA (5′-dAGTGATCTAC-3′), RNA (5′-dAGUGAUCUAC-3′), or PNA (H-AGTGATCTAC-LysNH_2)[a]

10-mer PNA sequence	T_m (°C) DNA/RNA/PNA	ΔT_m (°C) per modification[b] DNA/RNA/PNA
H-GTAGATCACT-LysNH_2 (PNA 1)	51.0/56.0/68.5	
H-GTAGA(bT)CACT-LysNH_2 (PNA 2)	51.0/54/68.5	0.0/−2.0/0.0
H-GTAGA(7-Cl-bT)CACT-LysNH_2 (PNA 3)	54.0/58.5/72.5	3.0/2.5/4.0
H-GTAGA(6-Cl-bT)CACT-LysNH_2 (PNA 4)	53.0/56/69.0	2.0/0.0/0.5
H-GTAGA(7-CH3-bT)CACT-LysNH_2 (PNA 5)	54.0/58/71.0	3.0/2.0/2.5
H-GTAGA(6-CH3-bT)CACT-LysNH_2 (PNA 6)	51.0/55.0/68.0	0.0/−1.0/−0.5
H-GTAGA(5-CH3-bT)CACT-LysNH_2 (PNA 7)	53.0/56/69.0	2.0/0.0/0.5

[a] Similar sequences containing thymine or bT are included for comparison. [b] ΔT_m is indicated per modification, relative to the thermal stability of the thymine-containing control.

Table 2. Thermal Stability (T_m) of PNA−DNA and PNA−PNA Duplexes Assessed Using a 10-mer PNA Containing Three 7-Cl-bT, 6-Cl-bT, 7-CH_3-bT, 6-CH_3-bT, or 5-CH_3-bT Units in Isolated Positions and a 10-mer Otherwise Watson−Crick Complementary DNA (5′-dCATCATCTAC-3′), RNA (5′-dCAUCAUCUAC-3′), or PNA (H−CATCATCTAC-LysNH_2)[a]

10-mer PNA sequence	T_m (°C) DNA/RNA/PNA	ΔT_m (°C)[b] per modification DNA/RNA/PNA	ΔT_m (°C)[c] for hybridization X=G RNA	X = C RNA	X = U RNA
H-GTAGATGATG-LysNH_2 (PNA 8)	57.0/58.5/67.5		10	15	12
H-G(bT)AGA(bT)GA(bT)G-LysNH_2 (PNA 9)	51.0/51.5/63.0	−2.0/−2.1/−1.5	6	14	4
H-G(7-Cl-bT)AGA(7-Cl-bT)GA(7-Cl-bT)G-LysNH_2 (PNA 10)	63.0/62/69.5	2.0/1.2/0.7	14	16	15
H-G(6-Cl-bT)AGA(6-Cl-bT)GA(6-Cl-bT)G-LysNH_2 (PNA 11)	54.0/54.0/62.5	−1.5/−1.5/−1.7	11	16	8
H-G(7-CH3-bT)AGA(7-CH_3-bT)GA(7-CH3-bT)G-LysNH_2 (PNA 12)	58.0/57.5/65.0	−0.2/−0.3/−0.8	5	16	12
H-G(6-CH3-bT)AGA(6-CH_3-bT)GA(6-CH_3-bT)G-LysNH_2 (PNA 13)	49.0/50.5/60.5	−3.2/−2.7/−2.3	5	13	5
H-G(5-CH_3-bT)AGA(5-CH_3-bT)GA(5-CH_3-bT)G-LysNH_2 (PNA 14)	53.0/54.0/63.5	−1.8/−1.5/−1.3	5	15	7

[a] Similar sequences containing thymine or bT are included for comparison. Data for complexes with a singly mismatched RNA (5′-dCAUCXUCUAC-3′) are also presented as a change in thermal stability (ΔT_m) from the fully matched duplex. [b] ΔT_m is listed per modification, relative to the thermal stability of the thymine containing control. [c] ΔT_m is listed for hybridization to an RNA oligonucleotide containing one mismatch (5′-CAUCUXCUAC) compared to the full match (X = A).

from this reaction was treated with refluxing 3 M aqueous HCl, for some preparations using dioxane as cosolvent, to give the desired acetic acid derivatives **2b**−**f**. Condensation of the resulting acetic acid derivatives with methyl *N*-(2-Boc-aminoethyl)glycinate,[8] using standard peptide coupling reagents in DMF at room temperature, proceeded to give the fully protected PNA monomers **3b**−**f** and was followed by alkaline hydrolysis using LiOH in THF to afford the desired Boc-PNA monomers (**4b**−**f**, Scheme 2).

PNA oligomerization was performed analogously to the described procedure,[9] and a C-terminal lysine amide was included for comparison with previously synthesized PNA oligomers.[5] All Boc-PNA monomers **4b**−**f** showed coupling efficiencies comparable to those found for the conventional PNA monomers. The recognition properties of the various 1,8-naphthyridin-2(1*H*)-one heterocycles derived from the Boc-PNA monomers **4b**−**f** were analyzed by thermal denaturation of complexes between the corresponding PNA oligomers and complementary oligonucleotides or PNA oligomers. All thermal denaturation curves showed monophasic, well-defined transitions from which the T_m was determined.[10] The ability to recognize adenine was assessed in a duplex (Watson−Crick) as well as in a triplex (Hoogsteen) recognition system, and sequence discrimination in the duplex system was analyzed in selected cases.

The Watson−Crick recognition properties of the 1,8-naphthyridin-2(1*H*)-one heterocyclic systems was studied by incorporation of the monomers **4b**−**f** into 10-mer oligomers of PNA in isolated as well as in adjacent positions. Duplexes of these PNAs with DNA, RNA, or PNA targets were designed as to have adenine facing the 1,8-naphthyridin-2(1*H*)-one units in otherwise Watson−Crick complementary oligomers (Tables 1−3). The thermal stability of complexes containing differently substituted 1,8-naphthyridin-2(1*H*)-one units was compared to that of the corresponding unsubstituted 1,8-naphthyridin-2(1*H*)-one[5] and to a thymine-containing control. In general, the thermal stability of the duplexes formed with PNA containing one modified nucleobase, H-GTAGA**X**CACT-Lys-NH_2 (PNAs 3−7), was increased relative to the thymine- (PNA 1) and bT-containing (PNA 2) controls. This effect was most pronounced for the 7-substituted derivatives, at best leading to an increase in T_m per modification of 4.0 °C in the PNA−PNA duplex (7-Cl-bT) (PNA3). For the multiply modified system, H-GXAGAXGAXG-LysNH_2 (PNAs 10−14)—containing three modified nucleobases in isolated positions—only the 7-Cl-bT derivative (PNA10) gave rise to increased thermal stability. The effect was most pronounced for the PNA−DNA system (ΔT_m = 2.0 °C), while the increase was lower in the PNA−RNA (ΔT_m = 1.2 °C) and the PNA−PNA (ΔT_m = 0.7 °C) system. Incorporation of all other derivatives led to uncompromised or decreased thermal stability of the resulting duplexes. In a duplex-forming system of similar composition, containing

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(10) The T_m was determined as the maximum of the first derivative. In the present case, this results in a slight systematic overestimation of the T_m as compared to the theoretically more correct value obtained via the half-maximum hyperchromicity determination (or a curve-fitting procedure). However, the value is more accurately determined, since uncertainties in defining postmelting and especially premelting baselines are not an issue. Most importantly, this difference is systematic (corresponding to 1−2 °C when comparing the T_m values recorded here with those we obtained by half-maximum hyperchromicity determination) and therefore does not affect our conclusions regarding relative thermal stabilities between homologous PNA oligomer complexes.

Table 3. Thermal Stability (T_m) of PNA−DNA, PNA−RNA, and PNA−PNA Duplexes Assessed Using a 10-mer PNA Containing Three 7-Cl-bT, 6-Cl-bT, 7-CH_3-bT, 6-CH_3-bT, or 5-CH_3-bT Units in Adjacent Positions and a 10-mer Otherwise Watson−Crick Complementary DNA (5′-dCTCAAACTCT-3′) or PNA (H−CTC AAA CTC T-Lys-NH_2)[a]

10-mer PNA sequence	T_m (°C) DNA/RNA/PNA	ΔT_m (°C)[b] per modification DNA/RNA/PNA
H-AGAGTTTGAG-LysN H2 (PNA 15)	59.5/63/66.5	
H-AGAG(bT)(bT)(bT)GAG-LysNH_2 (PNA 16)	58.0/61.5/64.5	−0.5/−0.5/−0.7
H-AGAG(7-Cl-bT)(7-Cl- bT)(7-Cl-bT)GAG-LysN H2 (PNA 17)	70.0/72.5/76.0	3.5/3.2/3.2
H-AGAG(6-Cl-bT)(6-Cl-bT)(6-Cl-bT)GAG-LysN H2 (PNA 18)	64.0/66/67.0	1.5/1.0/0.2
H-AGAG(7-CH_3-bT)(7-CH_3-bT)(7-CH_3-bT)GAG-LysNH_2 (PNA 19)	63.5/66/68.0	1.3/1.0/1.5
H-AGAG(6-CH_3-bT)(6-CH_3-bT)(6-CH_3-bT)GAG-LysNH_2 (PNA 20)	55.0/61/66.0	−1.5/−0.7/−0.2
H-AGAG(5-CH_3-bT)(5-CH_3-bT)(5-CH_3-bT)GAG-LysNH_2 (PNA 21)	60.5/63/66.0	0.3/0.0/−0.2

[a] Similar sequences containing thymine or bT are included for comparison. [b] ΔT_m is indicated per modification, relative to the thermal stability of the thymine containing control.

Table 4. Thermal Stability (T_m) of Singly Mismatched PNA−DNA, PNA−RNA, and PNA−PNA Duplexes Assessed Using 10-mer PNAs Together with 10-mer DNA (5′-dAGT GXT CTA C-3′), RNA (5′-dAGU GXU CUA C-3′), or PNA (H-AGT GXT CTA C-NH_2) Oligomers Containing a Single Mismatch Facing the 7-Cl-bT Unit, All Other Positions Consisting of Watson−Crick Complementary Base Pairs[a]

10 mer PNA sequence	ΔT_m[a] for DNA/RNA/PNA X = G	X = C	X = T
H-GTAGATCACT-LysNH_2 (PNA 1)	14/10/8	5/20/16	10/17/14
H-GTAGA(bT)CACT-LysNH_2 (PNA 2)	5/9/6	15/16/18	8/10/20
H-GTAGA(7-Cl-bT)CACT-LysNH_2 (PNA 3)	8/14/12	6/22/22	7/25/23

[a] ΔT_m is indicated relative to the thermal stability of the adenine-containing control. The estimated accuracy of these values as judged from reproducibility and the shape of the transition curves is ±2 °C.

Table 5. Thermal Stability (T_m) of PNA·DNA−PNA Triplexes Using a Bis-PNA Containing Three 7-Cl-bT, 6-Cl-bT, 7-CH_3-bT, 6-CH_3-bT, or 5-CH_3-bT Units in Isolated Positions Together with a 13-mer DNA Target Containing the 7-mer Target (5′-dCGCAAGAAAGCGC-3′)[a]

bis-PNA sequence[b]	T_m (°C)	ΔT_m (°C)[c] per modification
H-TTJTTTJ-$(eg1)_3$CTTTCTT-NH_2 (PNA 22)	59.5	
H-T(bT)J(bT)T(bT)J-$(eg1)_3$CTTTCTT-NH_2 (PNA 23)	63.0	1.2
H-T(7-Cl-bT)J(7-Cl-bT)T(7-Cl-bT)J-$(eg1)_3$CTTTCTT-NH_2 (PNA 24)	65.5	2.0
H-T(6-Cl-bT)J(6-Cl-bT)T(6-Cl-bT)J-(eg1)3CTTTCTT-NH_2 (PNA 25)	67.5	2.7
H-T(7-CH_3-bT)J(7-CH_3-bT)T(7-CH_3-bT)J-$(eg1)_3$CTTTCTT-NH_2 (PNA 26)	57.5	−0.7
H-T(6-CH_3-bT)J(6-CH_3-bT)T(6-CH_3-bT)J-$(eg1)_3$CTTTCTT-NH_2 (PNA 27)	63.0	1.2
H-T(5-CH_3-bT)J(5-CH_3-bT)T(5-CH_3-bT)J-$(eg1)_3$CTTTCTT-NH_2 (PNA 28)	64.5	1.7

[a] Adenines are positioned to face the modified nucleobases, all other positions consisting of conventional T·A−T and J·G−C triplets. Similar sequences containing thymine or bT are included for comparison. [b] Pseudoisocytosine (J) was used instead of protonated cytosine in the Hoogsteen strand of the bis-PNA. Three units of 8-amino-3,6-dioxaoctanoic acid (eg1) connect the two antiparallel 10-mer PNA strands. [c] ΔT_m is indicated per modification, relative to the thermal stability of the thymine containing control.

three modified nucleobases in adjacent positions, H-AGAGXXX-GAG-LysNH_2 (PNAs 17−21), the 7-Cl-bT nucleobase (PNA 17) was still superior to all other derivatives, leading to an increase of 3.5 °C per modification in the PNA−DNA system, while the increase in thermal stability amounted to 3.2 °C in both the PNA−PNA and in the PNA−RNA duplex systems.

For the oligomers containing a single 7-Cl-bT unit, sequence discrimination was assessed by introduction of a mismatch opposing the 7-Cl-bT position (Table 4). The results presented in Table 4 show a ΔT_m sequence discrimination on the order of 6−8 °C in the PNA−DNA duplex system, while in the PNA−RNA and PNA−PNA systems, discrimination was more efficient (12−23 °C). These values are similar in magnitude to those obtained from the thymine-containing controls, although a detailed interpretation requires more comprehensive data and analyses. All new derivatives were also tested for mismatch discrimination toward RNA (Table 1), and all showed clear recognition of uracil, although with varying efficiency.

To study the Hoogsteen recognition properties of the modified nucleobases, the monomers **4b**−**f** were incorporated into three positions in the parallel strand of bis-PNAs[11] in separated (PNAs 24−28) as well as adjacent (PNAs 31−35) positions (Tables 5 and 6). The DNA target was chosen such that the three modified consist of conventional T·A−T and J·G−C triplets.[11] The thermal stability of complexes formed between these bis-PNAs and the respective DNA targets was compared to that of corresponding thymine- (PNAs 22 and 29) and bT-containing (PNAs 23 and 30) controls. For sequences in which three modified nucleobases were incorporated into isolated positions, H-TXJXTXJ-$(eg1)_3$-CTTTCTT-NH_2, the 6-Cl-bT (PNA 25) derivative, was slightly superior to the 7-Cl-bT (PNA 24) derivative, giving rise to an increase in thermal stability per modification of 2.7 and 2.0 °C, respectively. All other derivatives gave rise to a more moderate increase in the thermal stability of the resulting triplex structures (1.2−1.7 °C) as compared to the thymine-containing control (PNA 22). In an isomeric sequence, where the modified units were in adjacent positions, H-TJXXXTJ-$(eg1)_3$-CTTTTCT-NH_2, this order was reversed, and the 7-Cl-bT derivative (PNA 31) (ΔT_m=3.5 °C) now performed slightly better than the 6-Cl-bT derivative (PNA 32) (ΔT_m=3.2 °C). For the remaining derivatives, the gain in thermal stability, ΔT_m, ranged from 0.8 to 2.2 °C.

The procedure outlined in Sheme 2 provides a general route to Boc-PNA monomers containing various substituted deriva-

(11) Egholm, M.; Christensen, L.; Dueholm, K.; Buchardt, O.; Coull, J.; Nielsen P. E. *Nucleic Acids Res.* **1995**, *23*, 217−222.

Table 6. Thermal Stability (T_m) of PNA·DNA−PNA Triplexes Using a Bis-PNA Containing Three 7-Cl-bT, 6-Cl-bT, 7-CH_3-bT, 6-CH_3-bT, or 5-CH_3-bT Units in Adjacent Positions Together with a 13-mer DNA Target Containing the 7-mer Target (5′-dCGCAGAAAAGCGC-3′)[a]

bis-PNA sequence[b]	T_m (°C)	ΔT_m (°C)[c] per modification
H-TJTTTTJ-(eg1)$_3$CTTTTCT-NH_2 (PNA 29)	61.0	
H-TJ(bT)(bT)(bT)TJ-(eg1)$_3$CTTTTCT-NH_2 (PNA 30)	65.5	1.5
H-TJ(7-Cl-bT)(7-Cl-bT)(7-Cl-bT)TJ-(eg1)$_3$CTTTTCT-NH_2 (PNA 31)	71.5	3.5
H-TJ(6-Cl-bT)(6-Cl-bT)(6-Cl-bT)TJ-(eg1)$_3$CTTTTCT-NH_2 (PNA 32)	70.5	3.2
H-TJ(7-CH_3-bT)(7-CH_3-bT)(7-CH_3-bT)TJ-(eg1)$_3$CTTTTCT-NH_2 (PNA 33)	63.5	0.8
H-TJ(6-CH_3-bT)(6-CH_3-bT)(6-CH_3-bT)TJ-(eg1)$_3$CTTTTCT-NH_2 (PNA 34)	67.5	2.2
H-TJ(5-CH_3-bT)(5-CH_3-bT)(5-CH_3-bT)TJ-(eg1)$_3$CTTTTCT-NH_2 (PNA 35)	66.5	1.8

[a] Adenines are positioned to face the modified nucleobases, all other positions consisting of conventional T·A−T and J·G−C triplets. Similar sequences containing thymine or bT are included for comparison. [b] Pseudoisocytosine (J) was used instead of protonated cytosine in the Hoogsteen strand of the bis-PNA. Three units of 8-amino-3,6-dioxaoctanoic acid (eg1) connect the two antiparallel 10-mer PNA strands. [c] ΔT_m is indicated per modification, relative to the thermal stability of the thymine-containing control.

Table 7. Thermodynamic Parameters for PNA−DNA Duplex Formation as Derived from the Experiments Shown in Figure 1

PNA	ΔG_{37},[a] kJ/mol	ΔH, kJ/mol	ΔS, J/mol
H-AGAGTTTGAG-LysNH_2 (PNA 15)	−51.0	−304	818
H-AGAG(7-Cl-bT)(7-Cl-bT)(7-Cl-bT)GAG-LysNH_2 (PNA 17)	−61.2	−315	818

[a] ΔG was calculated at 37 °C.

tives of the 1,8-naphthyridin-2(1*H*)-one (bT) ring system. Although the majority of the described derivatives shows significantly increased affinity for adenine in singly modified sequences, only the 7-Cl-bT derivative shows consistently increased affinity in all sequences (relative to the thymine- and bT-containing controls). All other nucleobases seem more dependent on the sequence context for their binding to adenine. The cause for this dependency is yet unknown and has to be further analyzed.

As previously mentioned, substituents in the 7 and 6 (and 5) positions of the bT ring system would be expected to affect the affinity for the "complementary" nucleobase, adenine, both through alteration of the electron density in the ring system (and thereby the pyridine nitrogen's ability to function as an efficient hydrogen-bond acceptor) and by affecting van der Waal interactions between neighboring nucleobases as indicated by X-ray crystallographic data for a bT-containing PNA duplex.[5]

While in duplex structures 6-Cl-bT performed consistently worse than 7-Cl-bT, this nucleobase performed as well or better than the corresponding 7-substituted analogue in triplex (Hoogsteen) structures. The reason for this difference could relate to differences in overlap between neighboring nucleobases in duplexes and triplexes, respectively. It is, however, not obvious from structural considerations, as the structure of a PNA_2–DNA triplex is much closer to a PNA−PNA duplex (P-form) than to a PNA−DNA or a PNA−RNA duplex,[12] and this is not reflected in the T_m results.

The thermal stability of the H-GXAGAXGAXG-LysNH_2- and H-AGAGXXXGAG-LysNH_2-containing DNA−PNA duplexes is rather similar for thymine (58.5 and 59.5 °C). However, for oligomers containing the bicyclic derivatives, cooperativity is much more pronounced, as indicated by the difference in thermal stability between the two sequences ranging from 5.5 to 10 °C. This could indicate that stacking forces were a dominating factor for the increased stability, and mechanistically, such increased stacking could help preorder the single-stranded PNA for hybridization, thereby decreasing the entropy loss in the process. To address this question, we derived the enthalpy and entropy values (and thus the free energy of binding) for the binding process from T_m data (Figure 1, Table 7). These results clearly show that the increased binding free energy provided by the 7-Cl-bT PNA monomer is predominantly enthalpic and therefore must be ascribed to base pair stabilization per se as opposed to single-strand preorganization.

Table 8. Thermal Stability of PNA−DNA Duplexes Having a Nonhybridized PNA Nucleobase Stacking at the 3′-End[a]

	T_m (°C)	
PNA	(a)	(b)
H(bT)GTAGATCACT-NH_2 (PNA 36)	57.0	59.0
H(7-Cl-bT)GTAGATCACT-NH_2 (PNA 37)	57.5	60.0
H(6-Cl-bT)GTAGATCACT-NH_2 (PNA 38)	58.0	60.0
H(7-CH3-bT)GTAGATCACT-NH_2 (PNA 39)	57.5	60.0
H(tT)GTAGATCACT-NH_2 (PNA 40)	60.0	63.0
H-TGTAGATCACT-LysNH_2 (PNA 41)	52.0	57.0

[a] The complementary DNA oligonucleotide was (a) 5′-AGTGATCTAC or (b) 5′-AGTGATCTACA. tT = benzo[*b*]-1,8-naphthyridin-2(1*H*)-one (cf. Scheme 2).

It is well-described that stacking forces alone can stabilize DNA helices and that an estimate of stacking energy can be obtained by studying DNA duplexes where the nucleobase in question is just stacked at the end of the helix without being hydrogen bonded to another nucleobase.[3] Performing analogous experiments with the most interesting of the present 1,8-naphthyridin-2(1*H*)-ones, as well as with an analogous tricyclic thymine analogue (benzo[*b*]-1,8-naphthyridin-2(1*H*)-one)[5] (cf. Scheme 2) (Table 8), clearly shows a correlation of thermal stabilization with nucleobase gross surface area, but not at all with the Watson−Crick type stabilization by these nucleobases. This is most dramatically illustrated by comparing the bicyclic 1,8-naphthyridin-2(1*H*)-one (bT) with the tricyclic benzo[*b*]-1,8-naphthyridin-2(1*H*)-one (tT) (and with thymine). According to the data of Table 8, the stacking stabilization by tT is—as expected—significantly higher than that of any of the bicyclic derivatives, which give very similar stabilization. However, as shown above, when part of Watson−Crick base pairs inside PNA−DNA, PNA−RNA, or PNA−PNA double helices, these nucleobases behaves quite differently, and specifically, the tT

(12) (a) Brown, S. C.; Thomson, S. A.; Veal, J. M.; Davis. D. G. *Science* **1994**, *265*, 777−780. (b) Betts, L.; Josey, J. A.; Veal, J. M.; Jordan, S. R. *Science* **1995**, 270, 1838−1841. (c) Eriksson M.; Nielsen, P. E. *Nat. Struct. Biol.* **1996**, *3*, 410−413. (d) Eriksson M.; Nielsen, P. E. *Q Rev Biophys* **1996**, *29*, 369−394.(e) Rasmussen H.; Kastrup, J. S.; Nielsen, J. N.; Nielsen, J. M.; Nielsen, P. E. *Nat Struct Biol* **1997**, *4*, 98−101.

nucleobase does not stabilize the duplex significantly.[5] It could be argued that the absence of hydrogen bonding to a complementary nucleobase could allow a more optimal position for stacking. However, enforcing such hydrogen bonding by including an adenine in the DNA strand did not change the result (Table 8). The molecular interactions that determine the intrahelix base-pairing properties of a nucleobase are therefore—not surprisingly—more complex than simply just combined hydrogen bonding and general stacking properties, and further studies are required to unravel these intricate molecular-recognition processes.

Finally, it is worth noting that the thermal stability of the H-AGAG(7-Cl-bT)(7-Cl-bT)(7-Cl-bT)GAG-LysNH_2/5′-dCT-CAAACTCT-3′ duplex is as high as 70 °C, which is comparable to that of a duplex-forming homopurine PNA previously shown to strand invade dsDNA.[13] Further investigations are in progress to fully elucidate the potential of the 1,8-naphthyridin-2(1*H*)-one ring system as a means to obtain strand invasion of dsDNA by duplex formation, thereby circumventing the sequence limitations related to the targeting of dsDNA by PNAs.

Conclusion

The results presented herein clearly show that increasing the surface area of a nucleobase per se in a Watson−Crick base pair is not sufficient to stabilize nucleic acid duplexes when this base pair is within the helix. Adversely, our results indicate a good correlation between nucleobase suface and helix-stabilizing effect when positioned at the end of the helix. Therefore, neither simple stacking considerations nor end-stacking experiment will predict the helix stabilization of a Watson−Crick base pair within a nucleic acid helix, and other still unresolved parameters play a decisive role. Nonetheless, even though these parameters are not identified at this stage, our results show that very simple substitutions in nucleobases with increased surface area such as the 1,8-naphthyridin-2(1*H*)-one system can produce significantly improved nucleobases, such as the 7-chloro-1,8-naphthyridin-2(1*H*)-one. These could be very useful in the design of PNA- (and DNA-)based antisense and antigene reagents, as well as hybridization probes, and also by further analyses help in gaining a quantitative understanding of the forces involved in nucleic acid helix formation.

Experimental Section

General Remarks. 6-Chloro-3-formyl-2-(pivaloylamino)pyridine,[6] 3-formyl-6-methyl-2-(pivaloylamino)pyridine,[6] 3-formyl-5-methyl-2-(pivaloylamino)pyridine,[6] di-*tert*-butyl succinate,[5] and methyl *N*-(2-Boc-aminoethyl)glycinate[8] were prepared by previously described methods. Diethyl ether (LAB-SCAN) and tetrahydrofuran (LAB-SCAN) were dried over molecular sieves. Dichloromethane (LAB-SCAN) and dimethylformamide (LAB-SCAN) were used as received. The following chemicals were used as received: Butyllithium (2.5 M in hexanes) (Aldrich), dicyclohexylcarbodiimide (DCC) (Aldrich), diisopropylamine (Aldrich), and 3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one (DhbtOH) (Aldrich). Phosphoramidites for DNA synthesis were obtained from CruaChem. TLC was performed on silica 60 (Merck aluminum sheet) and column chromatograpy on silica 60 (230−400 mesh ASTM) (Merck) (Figure 1). ^{1}H and ^{13}C NMR spectra were obtained at either 250 MHz (Bruker AMX 250) or 400 MHz (Varian Unity 400) in 5 mm tubes; chemical shifts are positive in the low-field direction. FAB mass spectra were recorded on a JEOL Hx110/110 mass spectrometer.

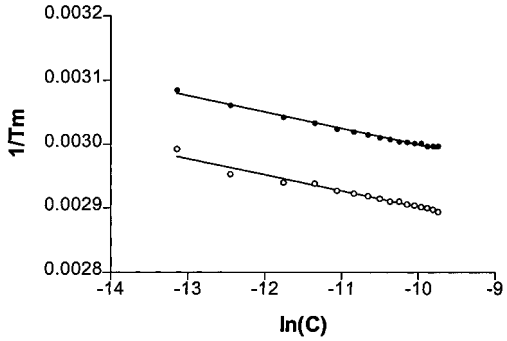

Figure 1. Plot of $1/T_m$ versus ln C for the duplexes formed between PNA15 (o) or PNA17 (o) and the complementary DNA oligonucleotide. From the slope of the curve and the y-axis intercept (ln $C = 0$), ΔH and ΔS were determined from the equation $1/T_m = R/\Delta H \ln C + (\Delta S - R \ln 4)/\Delta H$ (where C is the total oligomer concentration) and ΔG is determined at 37 °C from the equation $\Delta G = \Delta H - RT\Delta S$. The values are given in Table 8.

All PNA oligomers were characterized by MALDI-TOF mass spectrometry recorded on a Kratos Compact MALDI II instrument operating in the positive ion mode, using 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix. T_m values were obtained on a Gilford Response spectrophotometer and measured on solutions ca. 3 μM in PNA and DNA at pH 7.0 in 100 mM NaCl, 10 mM Na_2HPO_4, 0.1 mM EDTA; absorptions at 260 nm were recorded with 0.5 °C intervals from 5 to 90 °C. PNA oligomerization was performed as previously described.[9] DNA oligomerization was performed according to standard protocols on a Biosearch 8750 DNA synthesizer.

6-Amino-2-(pivaloylamino)pyridine. To a solution of 2,6-diaminopyridine (50.0 g, 0.46 mol) in dioxane (250 mL) was added pivaloyl chloride (27.9 g, 0.23 mol) dissolved in dioxane (50 mL) over 1 h. The resulting mixture was stirred for 2 h and the white precipitate (2,6-diaminopyridine hydrochloride) filtered off. The organic phase was evaporated in vacuo, and the crude product recrystallized from *n*-hexane/ethyl acetate to give the desired product (32.83 g, 74%) as colorless crystals (mp 140−141 °C). ^{1}H NMR (DMSO-d_6): δ 8.93 (s, 1H, NH), 7.34 (t, 7.9 Hz, 1H, arom), 7.19 (d, 7.6 Hz, 1H, arom), 6.18 (d, 7.6 Hz, 1H, arom), 5.75 (br s, 2H, NH_2), 1.19 (s, 9H, CH_3). ^{13}C NMR (DMSO-d_6): δ 176.5, 158.5, 150.4, 138.4, 103.6, 101.5, 39.4, 27.1. FAB$^+$MS (m/z): 193.88 M + H$^+$, calcd for $C_{10}H_{15}N_3O$ + H$^+$ 194.1293.

6-Chloro-2-(pivaloylamino)pyridine (1b). To a precooled (−10 °C) suspension of 6-amino-2-(pivaloylamino)pyridine (28.00 g, 0.145 mol) in concentrated aqueous HCl (150 mL) was added potassium nitrite (14.81 g, 0.174 mol) dissolved in water (8 mL) over a 1.5 h period. the resulting reaction mixture was stirred for 5 h additionally at −10 °C under nirogen, before adjustment of the pH to 9 by addition of concentrated aqueous NaOH. The aqueous solution was extracted with ethyl acetate (3 × 100 mL), and the organic fractions were washed with 2 M NaOH (3 × 40 mL). The organic phase was dried ($MgSO_4$), evaporated in vacuo, and recrystallized from *n*-hexane/ethyl acetate to give the desired product (17.70 g, 58%) as a white powder (mp 86−87 °C). ^{1}H NMR (DMSO-d_6): δ 10.08 (s br, 1H, NH), 8.04 (d, 8.1 Hz, 1H, arom), 7.81 (t, 8.1 Hz, 1H, arom), 7.17 (d, 8.1 Hz, 1H, arom), 1.22 (s, 9H, CH_3). ^{13}C NMR (DMSO-d_6): δ 177.4, 152.5, 147.8, 141.4, 119.0, 112.8, 39.4, 26.8. FAB$^+$MS (m/z): 213.06 M + H$^+$, calcd for $C_{10}H_{13}ClN_2O$ + H$^+$ 213.0795.

(7-Chloro-2(*1H*)-oxo-1,8-naphthyridin-3-yl)acetic Acid (2b). To a precooled (−78 °C) solution of *N*,*N*-diisopropylamine (10.11 g, 100 mmol) in diethyl ether (150 mL) was added BuLi (2.5 M in hexanes) (40 mL, 100 mmol), and the solution was stirred at this temperature for 15 min before slow addition of di-*tert*-butyl succinate (10.83 g, 50.0 mmol) dissolved in diethyl ether (20 mL). After 20 min at −78 °C, 6-chloro-3-formyl-2-(pivaloylamino)pyridine (11.32 g, 50.0 mmol) dissolved in dry THF (20 mL) was added slowly. The yellow solution was stirred at −78 °C for 30 min and then allowed to warm to room

(13) Nielsen, P. E.; Christensen, L. *J. Am. Chem. Soc.* **1996**, *118*, 2287−2288.

temperature. The solution was then poured into NH_4Cl (sat., aqueous) (200 mL), and the aqueous layer was separated and extracted twice with diethyl ether (2 × 100 mL). The combined organic layers were washed once with water (100 mL) and once with brine (100 mL), dried over $MgSO_4$, and evaporated in vacuo. The crude products of diastereomeric alcohols were dissolved in dioxane (150 mL) and HCl (3 M, aqueous) (150 mL) and refluxed for 6 h. The resulting yellow precipitate was filtered off, washed with water and diethyl ether, and dried in vacuo to give the desired product as a yellow solid (10.91 g, 89%). ^{1}H NMR (DMSO-d_6): δ 12.43 (s, 1H, NH), 8.12 (d, 8.1 Hz, 1H, arom), 7.87 (s, 1H, arom), 7.29 (d, 8.1 Hz, 1H, arom), 3.55 (s, 2H, CH_2). ^{13}C NMR (DMSO-d_6): δ 171.7, 162.6, 149.9, 149.1, 139.3, 136.4, 129.6, 118.4, 113.5, 35.6. FAB$^+$MS (*m*/*z*): 239.04, M + H$^+$, calcd for $C_{10}H_7ClN_2O_3$ + H$^+$ 239.0223. Anal. Calcd for $C_{10}H_7ClN_2O_3$: C 50.33, H 2.96, N 11.74. Found: C 50.49, H 2.98, N 11.96.

Methyl *N*-(2-(*tert*-Butyloxycarbonyl)aminoethyl)-*N*-(7-chloro-2(*1H*)-oxo-1,8-naphthyridin-3-yl)acetylglycinate (3b). (7-Chloro-2(1*H*)-oxo-1,8-naphthyridin-3-yl)acetic acid (0.50 g, 2.10 mmol) and DhbtOH (0.34 g, 2.10 mmol) were dissolved in dry DMF (15 mL). The mixture was cooled on ice, DCC (0.43 g, 2.10 mmol) was added, and the resulting mixture was stirred for 40 min. Methyl *N*-(2-*tert*-butyloxycarbonyl)aminoethyl)glycinate (0.46 g, 2.10 mmol) dissolved in DMF (5 mL) was added. After 1 h the ice bath was removed and the mixture was stirred overnight at room temperature. The mixture was evaporated in vacuo, redissolved in ethyl acetate (25 mL), and filtered and washed with $NaHCO_3$ (5%, aqueous) (3 × 25 mL) and with brine (2 × 25 mL). The organic phase was dried ($MgSO_4$), 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 desired product (0.61 g, 64%) as a slightly yellow solid (mp 68−69 °C). ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.45 (br s, 1H, NH), 8.11 (8.16) (d, 8.1 Hz, 1H, arom), 7.79 (7.81) (s, 1H, arom), 7.32 (d, 8.1 Hz, 1H, arom), 6.91 (6.81) (m, 1H, NH), 4.05 (4.36) (s, 2H, CH_2), 3.68 (3.67) (s, 2H, CH_2), 3.64 (s, 3H, CH_3O), 3.49−3.42 (m, 2H, CH_2), 3.20−3.15 (3.05−3.00) (m, 2H, CH_2), 1.36 (1.35) (s, 9H, CH_3(Boc)). ^{13}C NMR (DMSO-d_6): δ 170.33, 170.08, 162.39 (162.48), 155.77, 149.72, 149.04, 139.06, 135.89 (136.16), 130.29 (130.00), 118.32, 113.45, 78.01, 51.79 (52.20), 47.61 (48.15), 46.7, 38.43, 33.45 (33.78), 28.25. FAB$^+$MS (*m*/*z*): 453.1553 (M + H$^+$, calcd for $C_{20}H_{25}N_4O_6Cl$ + H$^+$ 453.1540).

***N*-2-((*tert*-Butyloxycarbonyl)aminoethyl)-*N*-(7-chloro-2(*1H*)-oxo-1,8-naphthyridin-3-yl)acetylglycine (4b).** Methyl *N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)-*N*-(7-chloro-2(1*H*)-oxo-1,8-naphthyridin-3-yl)-acetylglycine (0.607 g, 1.34 mmol) was dissolved in THF (10 mL), and LiOH (0.428 M, aqueous) (6.90 mL, 2.95 mmol) was added. After 30 min at room temperature. additional water (10 mL) was added and the THF was removed in vacuo. The pH of the aqueous solution was adjusted to 3.0 by addition of HCl (2 M, aqueous). The white precipitate was filtered off, washed with water (2 × 10 mL), and dried in vacuo to give the product (355 mg, 60%) as a slightly yellow powder (mp 176−78 °C). ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.42 (br s, 1H, NH), 8.07 (8.14) (d, 8.1 Hz, 1H, arom), 7.84 (7.78) (s, 1H, arom), 7.30 (d, 8.1 Hz, 1H, arom), 6.88 (6,71) (m, 1H, NH), 3.97 (4.23) (s, 2H, CH_2), 3.61(s, 2H, CH_2), 3.47−3.43 (3.33−3.30) (m, 2H, CH_2), 3.19−3.16 (3.05−3.03) (m, 2H, CH_2), 1.36 (1.34) (s, 9H, CH_3 (Boc)). ^{13}C NMR (DMSO-d_6): δ 170.92 (171.21), 169.88 (170.22), 162.37 (162.46), 155.74, 149.67, 149.00, 138.95 (139.13), 135.82 (136.06), 130.35 (130.00), 118.28, 113.45 (113.51), 77.99 (77.75), 48.10 (50.29), 47.57 (46.75), 38.41 (37.97), (33.75) 33.51, 28.27. FAB$^+$MS (*m*/*z*): 439.1371 (M + H$^+$, calcd for $C_{19}H_{23}N_4O_6Cl$ + H$^+$ 439.1384).

(7-Methyl-1,8-naphthyridin-2(*1H*)-on-3-yl)acetic Acid (2c). To a precooled (−78 °C) soultion of *N*,*N*-diisopropylamine (4.84 g, 48.0 mmol) in diethyl ether (125 mL) was added BuLi (2.5 M in hexanes) (19.0 mL, 48.0 mmol), and the solution was stirred at this temperature for 15 min before slow addition of di-*tert*-butyl succinate (5.75 g, 25.0 mmol) dissolved in diethyl ether (10 mL). After 20 min at −78 °C, 3-formyl-6-chloro-2-(pivaloylamino)pyridine (5.00 g, 23.0 mmol) dissolved in dry THF (10 mL) was slowly added. The resulting yellow solution was stirred at −78 °C for 30 min and then allowed to warm to room temperature. The solution was then poured into NH_4Cl (sat., aqueous) (100 mL). The aqueous layer was separated and extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed once with water (50 mL) and once with brine (50 mL), dried over $MgSO_4$, and evaporated in vacuo. The crude products of diastereomeric alcohols were dissolved in HCl (3 M, aqueous) (80 mL) and refluxed for 3.5 h. The aqueous phase was washed with diethyl ether (3 × 75 mL) and with chloroform (3 × 50 mL) and then adjusted to pH 7 by addition of K_2CO_3. The resulting yellow precipitate was filtered off, washed once with water and once with diethyl ether, and dried in vacuo to give the desired product (1.25 g, 27%) as a yellow solid (mp >250 °C). ^{1}H NMR (D_2O/NaOH): δ 7.53 (d, 8.1 Hz, 1H, arom), 7.31 (s, 1H, arom), 6.69 (d, 8.1 Hz, 1H, arom), 3.20 (s, 2H, CH_2), 2.25 (s, 3H, CH_3). ^{13}C NMR (D_2O/NaOH): δ 180.4, 172.1, 159.0, 155.7, 136.5, 135.9, 126.6, 116.6, 114.8, 39.6, 23.3. FAB$^+$MS (*m*/*z*): 218.0768 (M + H$^+$, calcd for $C_{11}H_{10}N_2O_3$ + H$^+$ 219.0770).

Methyl *N*-((7-Methyl-1,8-naphthyridin-2(*1H*)-on-3-yl)acetyl)-*N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate (3c). To a precooled (0 °C) solution of (7-methyl-1,8-naphthyridin-2(1*H*)-on-3-yl)acetic acid (0.995 g, 4.60 mmol) and DhbtOH (0.744 g, 4.60 mmol) in DMF (25 mL) was added DCC (1.04 g, 5.00 mmol), and the mixture was stirred for 40 min at 0° before addition of ethyl *N*-(2-(*tert*-butyloxycarbonyl)-aminoethyl)glycinate (1.10 g, 5.0 mmol). This mixture was stirred at room temperature overnight, evaporated in vacuo, redissolved in ethyl acetate (75 mL), and washed with $NaHCO_3$ (5%, aqueous) (3 × 25 mL) and with brine (2 × 25 mL). The organic phase was dried over $MgSO_4$, filtered, and evaporated in vacuo. The crude product was purified on silica using dichloromethane/methanol (90:10 v/v) as the eluent. Fractions containing the product were pooled and evaporated in vacuo to yield the desired product (790 mg, 41%) (mp 98−100 °C). ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.05 (s br, 1H, NH), 7.91 (7.96) (d, 8.0 Hz, 1H, arom), 7.74 (7.71) (s, 1H, arom), 7.10 (d, 8.0 Hz, 1H, arom), 6.90 (6.99) (m, 1H, NH), 4.05 (4.36) (s, 2H, CH_2), 4.09 (m, 2H, CH_2), 3.63 (s, 3H, OCH_3), 3.47 (m, 2H, CH_2), 3.17 (s, 2H, CH_2), 3.16 (s, 3H, CH_3), 1.36 (1.35) (s, 9H, CH_3 (Boc)). ^{13}C NMR (DMSO-d_6): δ 170.44 (170.66), 170.11, 162.65 (162.73), 159.22, 155.83 (155.71), 148.99, 136.43 (136.77), 136.04 (136.17), 128.61 (128.29), 118.21, 112.00 (112.07), 78.04, 51.81 (52.20), 48.7, 47.63 (48.20), 38.59 (38.45), 33.42 (33.75), 28.27, 24.22. FAB$^+$HRMS: 433.2086, calcd $C_{21}H_{29}N_4O_6$ 433.2087.

***N*-((7-Methyl-1,8-naphthyridin-2(*1H*)-on-3-yl)acetyl)-*N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycine (4c).** To a solution of methyl *N*-((7-methyl-1,8-naphthyridin-2(1*H*)-on-3-yl)acetyl)-*N*-(2-*tert*-butyl-oxycarbonylaminoethyl)glycinate (0.775 g, 1.79 mmol) in THF(10 mL) was added LiOH (2 M, aqueous) (2.0 mL). The resulting solution was stirred at room temperature for 30 min, additional water was added, and the THF was removed in vacuo. The pH of the aqueous solution was adjusted to pH 3.0 and the precipitate collected and washed (2 × 10 mL) by filtration, to yield the desired product (424 mg, 56%) as a colorless precipitate (mp 94−95 °C). ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.05 (s br, 1H, NH), 7.90 (7.96) (d, 7.9 Hz, 1H, arom), 7.78 (7.71) (s, 1H, arom), 7.11 (d, 7.9 Hz, 1H, arom), 6.90 (6,73) (m, 1H, NH), 3.97 (4.23) (s, 2H, CH_2), 3.84 (s, 2H, CH_2), 3.59 (s, 3H, CH_3), 3.44 (3.32) (m, 2H, CH_2), 3.04 (3.15) (m, 2H, CH_2), 1.37 (1.36) (s, 9H, CH_3 (Boc)). ^{13}C NMR (DMSO-d_6): δ 170.94 (171.22), 170.00 (170.16), 162.6, 159.1, 155.7, 148.8, 136.30 (136.57), 135.90 (136.09), 128.28 (128.62), 118.12, 111.96 (112.00),

77.9, 48.08 (48.29), 47.5, 38.39 (38.05), 33.44 (33.66), 28.3, 24.2. FAB$^+$HRMS (*m*/*z*): 417.1794, calcd $C_{20}H_{25}N_4O_6$ 417.1774.

6-Methyl-2(*1H*)-oxo-1,8-naphthyridine-3-acetic Acid (2d). To a precooled (−78 °C) solution of diisopropylamine (35.7 mmol, 3.61 g) in diethyl ether (75 mL) was added BuLi (2.5 M in hexanes) (31.5 mmol, 12.6 mL), and the resulting solution was stirred at room temperature for 15 min, whereafter di-*tert*-butylsuccinate (18.7 mmol, 3.85 g) dissolved in diethyl ether (50 mL) was slowly added. After 30 min at −78 °C, 2,2-dimethyl-*N*-(3-formyl-5-methyl-2-pyridinyl)propanamide (17 mmol, 3.74 g) dissolved in THF (30 mL) was slowly added, and stirring of the resulting yellow mixture was continued at −78 °C for additionally 30 min. The solution was then allowed to warm to room temperature and was poured into NH_4Cl (sat., aqueous) (100 mL). The aqueous phase was extracted three times with diethyl ether (50 mL), and the organic extracts were washed with brine (50 mL), dried over $MgSO_4$, and evaporated in vacuo. The crude product was recrystallized from ethyl acetate/hexane to yield the diastereomeric mixture (3.36 g, 44%), which was used as such after drying in vacuo. To a solution of the above diastereomeric mixture (7.0 mmol, 3.15 g) in dioxane (25 mL) was added HCl (3M, aqueous) (25 mL), and the resulting solution was stirred under reflux for 10 h. The dioxane was evaporated in vacuo, additional water (25 mL) was added, and the pH was adjusted to 8.0 before extraction with dichloromethane (50 mL). The aqueous phase was stirred vigorously for 15 min to remove remaining dichloromethane, and the pH of the aqueous phase was adjusted to 4.0 by addition of HCl (2 M, aqueous). The precitate was filtered off, washed with water (2 × 5 mL), and dried in vacuo to yield the desired product (878 mg, 57%) as a colorless powder (mp >250 °C). ^{1}H NMR (DMSO-d_6): δ 12.10 (s br, 1H, NH), 10.46 (s, 1H, COOH), 8.33 (s, 1H, arom), 7.89 (s, 1H, arom), 7.79 (s, 1H, arom), 3.34 (s, 2H, CH_2), 2.33 (s, 3H, CH_3). FAB$^+$HRMS: 219.0773 (M + H$^+$, calcd for $C_{11}H_{10}N_2O_3$ + H$^+$ 219.0770).

Methyl *N*-(2-(*tert*-Butyloxycarbonyl)aminoethyl)-*N*-(6-methyl-2(*1H*)-oxo-1,8-naphthyridin-3-yl)acetylglycinate (3d). To a solution of 6-methyl-2(1*H*)-oxo-1,8-naphthyridine-3-acetic acid (3.30 mmol, 719 mg) in DMF (20 mL) was added DIEA (3.30 mmol, 427 mg) and HBTU (3.30 mmol, 1.25 g), followed by methyl *N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate (3.00 mmol, 696 mg), and this mixture was stirred for 2 h at room temperature. 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 over $MgSO_4$, filtered, and evaporated in vacuo, and the crude product was purified on silica using dichloromethane/methanol (95:5 v/v) as the eluent. Fractions containing the product were evaporated in vacuo to yield the desired product (551 mg, 41%) as a colorless foam. ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.08 (s br, 1H, NH), 8.34 (s, 1H, arom), 7.84 (7.91) (s, 1H, arom), 7.73 (7.70) (s, 1H, arom), 6.91 (6.75) (m, 1H, NH), 4.05 (4.36) (s, 2H, CH_2), 3.56 (m, 2H, CH_2) 3.53 (s, 2H, CH_2), 3.64 (s, 3H, CH_3O), 3.20−2.99 (m, 2H, CH_2), 2.34 (s, 3H, CH_3), 1.36 (1.37) (s, 9H, CH_3 (Boc)). ^{13}C NMR (DMSO-d_6): δ 170.31 (170.54), 170.06, 162.45 (162.53), 155.77 (155.67), 150.29, 147.45, 136.17 (136.47), 135.42 (135.57), 129.87 (129.55), 127.35 (127.30), 113.96 (114.02), 78.01 (77.66), 51.80 (52.18), 48.20 (46.63), 47.58 (50.12), 40.60, 33.53 (33.84), 28.60, 17.38. FAB$^+$MS: 433.2093 (M + H$^+$, calcd for $C_{21}H_{28}N_4O_6$ + H$^+$ 433.2087).

***N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)-*N*-(6-Methyl-1,8-naphthyridin-2(*1H*)-on-3-yl)acetylglycine (4d).** To a solution of methyl *N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)-*N*-(6-methyl-2(1*H*)-oxo-1,8-naphthyridin-3-yl)acetylglycinate (1.0 mmol, 405 mg) dissolved in THF (8 mL) was added LiOH (2 M, aqueous), and the resulting solution was stirrred at room temperature for 15 min. Additional water (8 mL) was added, the THF was evaporated in vacuo, and the pH of the aqueous phase was adjusted to 3.0 by addtion of HCl (2 M, aqueous). The colorless powder was filtered off and washed with water (2 × 5 mL) to give the desired product (228 mg, 84%) as a colorless powder, pure according to HPLC (260 nm). ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.08 (s br, 1H, NH), 8.33 (s, 1H, arom), 7.81 (7.90) (s, 1H, arom), 7.76 (7.70) (s, 1H, arom), 6.89 (6.73) (m, 1H, NH), 3.97 (4.23) (s, 2H, CH_2), 3.59 (s, 2H, CH_2), 3.30−3.50 (m, partly covered by water signal, 2H, CH_2), 3.18−3.00 (m, 2H, CH_2), 2.34 (s, 3H, CH_3), 1.36 (1.34) (s, 9H, CH_3 (Boc)). ^{13}C NMR (DMSO-d_6): δ 170.96 (171.22), 170.14 (170.47), 162.44 (162.51), 155.74, 150.22, 147.41 (147.38), 136.13 (136.35), 135.36 (135.55), 129.90 (129.55), 127.31 (127.27), 113.97 (114.03), 78.00 (77.77), 48.19 (46.71), 47.51 (50.29), 38.98 (38.40), 33.60 (33.81), 28.26, 17.40.

6-Chloro-2(1*H*)-oxo-1,8-naphthyridine-3-acetic Acid (2e). To a precooled (−78 °C) solution of diisopropylamine (46.2 mmol, 4.67 g) in diethyl ether (100 mL) was added BuLi (2.5 M in hexanes) (46.2 mmol, 18.5 mL), and the resulting solution was stirred at room temperature for 15 min, whereafter di-*tert*-butyl succinate (24.2 mmol, 4.99 g) dissolved in diethyl ether (20 mL) was slowly added. After 20 min at −78 °C, 2,2-dimethyl-*N*-(5-chloro-3-formyl-2-pyridinyl)propanamide (22.0 mmol, 5.03 g) dissolved in THF (50 mL) was slowly added, and stirring of the resulting yellow mixture was continued at −78 °C for 60 min before the solution was allowed to warm to room temperature and poured into NH_4Cl (sat., aqueous) (100 mL). The aqueous phase was extracted three times with diethyl ether (50 mL), and the organic extracts were washed with brine (50 mL), dried over $MgSO_4$, and evaporated in vacuo. The crude product was recrystallized from ethyl acetate/hexane to yield the diastereomeric mixture, which was used as such after drying in vacuo. The crude diastereomeric mixture was dissolved in dioxane (75 mL) and HCl (3 M, aqueous) (75 mL) was added. The resulting solution was stirred under reflux for 6 h, cooled to room temperature, and filtered. The dioxane was evaporated in vacuo and the pH of the aqueous phase was adjusted to 8.0 by addition of K_2CO_3, and the aqueous phase was extracted with diethyl ether (25 mL). The pH of the aqueous phase was then adjusted to pH 3.0 by addition of HCl (4 M, aqueous), and the slightly tan precipitate was filtered off and washed with water (2 × 20 mL). The solid was dried in vacuo to yield the desired product (3.21 g, 58.2%) as a white solid (mp >250 °C). ^{1}H NMR (DMSO-d_6): δ 12.42 (s br, 1H, NH), 10.79 (s, 1H, COOH), 8.52 (d, 2.5 Hz, 1H, arom), 8.28 (8.19) (d, 2.5 Hz, 1H, arom), 7.84 (7.74) (s, 1H, arom), 3.50 (s, 2H, CH_2). ^{13}C NMR (DMSO-d_6): δ 171.6, 162.5, 148.0, 145.2, 135.9, 133.9, 130.9, 124.5, 115.3, 35.6. FAB$^+$MS: 239.00 (M + H$^+$, calcd for $C_{10}H_7N_2O_3Cl$ + H$^+$ 239.0223).

Ethyl *N*-(2-(*tert*-Butyloxycarbonyl)aminoethyl)-*N*-(6-chloro-2(*1H*)-oxo-1,8-naphthyridin-3-yl)acetylglycinate (3e). To a solution of 6-chloro-2(1*H*)-oxo-1,8-naphthyridine-3-acetic acid (313 mg, 1.25 mmol), triethylamine (304 mg, 3.0 mmol), DhbtOH (204 mg, 1.25 mmol), and ethyl *N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate hydrochloride (283 mg, 1.0 mmol) in DMF was added DCC (258 mg, 1.25 mmol). The resulting solution was stirred overnight at room temperature and evaporated in vacuo. The mixture was redissolved in dichloromethane (150 mL), filtered, and washed with 5% aqueous $NaHCO_3$ (2 × 50 mL) and then with brine (50 mL). The organic phase was dried over $MgSO_4$, filtered, and evaporated in vacuo. The crude product was purified on silica using ethyl acetate (95:5 v/v) as the eluent. Fractions containing the product were evaporated in vacuo to yield the desired product (294 mg, 63%) as a colorless foam. ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.40 (s br, 1H, NH), 8.51 (d, 2.5 Hz, 1H, arom), 8.23 (8.29) (d, 2.5 Hz, 1H, arom), 7.77 (7.74) (s, 1H, arom), 6.90 (6.73) (m, 1H, NH), 4.07 (4.33) (s, 2H, CH_2), 4.09 (4.13) (q, 7.1 Hz, 2H, CH_2), 3.64 (3.50) (s, 2H, CH_2), 3.48 (3.29) (m, 2H, CH_2), 3.18 (3.05), (m, 2H, CH_2), 1.37 (1.35), (s, 9H, CH_3 (Boc)), 1.18 (t, 7.1 Hz, 3H, CH_3). ^{13}C NMR (DMSO-d_6): δ 169.99 (170.25), 169.47 (169.80), 162.40, 155.77, 147.96, 147.82, 135.61, 134.64 (134.79), 131.41 (131.06), 124.07, 115.32, 78.01 (77.78), 60.51 (61.05), 48.16 (50.26), 47.81 (46.66), 38.46 (37.99), 33.48

(33.89), 28.25, 14.11. FAB$^+$MS: 467.1712 (M + H$^+$, calcd for $C_{21}H_{27}N_4O_6Cl$ + H$^+$ 467.1697).

***N*-(2-(*tert*-Butyloxycarbonyl)aminoethyl)-*N*-(6-Chloro-1,8-naphthyridin-2(*1H*)-on-3-yl)acetylglycinate (4e).** To a solution of methyl *N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)-*N*-(6-chloro-2(1*H*)-oxo-1,8-naphthyridin-3-yl)acetylglycinate (374 mg, 0.8 mmol) was added LiOH (2 M, aqueous). The resulting mixture was stirred at room temperature for 15 min. Then additional water (8 mL) was added, the THF was evaporated in vacuo, the pH of the aqueous phase was adjusted to 3.0 by addition of HCl (4 M, aqueous), and the precipitate was filtered off and washed with water (2 × 5 mL). The resulting solution was strirred at room temperature for 15 min, additional water (8 mL) was added, the THF was evaporated in vacuo, and pH of the aqueous phase was adjusted to 3.0 by addtion of HCl (2 M, aqueous). The white precipiate was filtered off, washed with water (2 × 5 mL), and dried in vacuo to give the desired product (281 mg, 80%) as a colorless powder, pure according to HPLC (260 nm). ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.70 (s vbr, 1H, COOH), 12.39 (s br, 1H, NH), 8.51 (d, 2.2 Hz, 1H, arom), 8.20 (8.28) (d, 2.2 Hz, 1H, arom), 7.80 (7.74) (s, 1H, arom), 6.88 (6.74) (m, 1H, NH), 4.21 (3.97) (s, 2H, CH_2), 3.63 (3.48) (s, 2H, CH_2), 3.45 (m, 2H, CH_2), 3.16 (3.04), (m, 2H, CH_2), 1.37 (1.35), (s, 9H, CH_3 (Boc)). ^{13}C NMR (DMSO-d_6): δ 170.96 (171.26), 169.88 (170.22), 162.42 (162.48), 155.78 (155.67), 147.94, 147.82, 135.52, 134.61 (134.79), 131.47, 131.17, 115.33, 78.00 (77.78), 47.45 (50.27), 48.07 (46.71), 38.40 (37.97), 33.42 (33.56), 28.26. FAB$^+$MS: 439.1380 (M + H$^+$, calcd for $C_{19}H_{23}N_4O_6Cl$ + H$^+$ 439.1384) and 445.1 (M + Li$^+$, calcd for $C_{19}H_{23}N_4O_6Cl$ + Li$^+$ 445.1466).

3-Formyl-4-methyl-2-(pivaloylamino)pyridine (1f). To a precooled (−78 °C) solution of 4-methyl-2-(pivaloylamino)pyridine (6.87 g, 36.0 mmol) in diethyl ether (100 mL) was slowly added *tert*-BuLi (50.0 mL, 75.0 mmol). The resulting solution was stirred at −78 °C for 3.5 h prior to the addition of DMF (5.19 g, 71.0 mmol), stirred for an additional 30 min at −78 °C, allowed to warm to room temperature, and poured into 2 M HCl (aqueous, 100 mL). After stirring of this mixture for 15 min, the pH was adjusted to 7.0 by addition of K_2CO_3. The aqueous phase was washed with diethyl ether (3 × 75 mL), and the combined organic fractions was washed with brine (100 mL), dried ($MgSO_4$), and evaporated in vacuo. The crude product was recrystallized from ethyl acetate/hexane to give the desired product (4.66 g, 59%) as slightly tan crystals (mp 117−20 °C). ^{1}H NMR(DMSO-d_6): δ 11.19 (s br, 1H, CHO), 10.41 (s, 1H, NH), 8.49 (d, 5.0 Hz, 1H, arom), 6.92 (d, 5.0 Hz, arom), 2.69 (s, 3H, CH_3), 1.37 (s, 9H, CH_3(Boc)). ^{13}C NMR (DMSO-d_6): δ 192.02, 176.97, 154.15, 152.82, 152.69, 121.70, 116.09, 40.69, 27.46, 18.77. FAB$^+$MS (*m*/*z*): 221.1300 (M + H$^+$, calcd for $C_{12}H_{16}N_2O_2$ + H$^+$ 221.1290).

(5-Methyl-1,8-naphthyridin-2(1*H*)-on-3-yl)acetic Acid (2f). To a precooled solution of *N*,*N*-diisopropylamine (6.2 mL., 0.044 mol) in diethyl ether (125 mL) was added BuLi (2.5 M in hexanes) (17.8 mL, 0,044 mol), and the resulting solution was stirred at room temperature for 15 min, whereafter di-*tert*-butylsuccinate (5.35 g, 23 mmol) dissolved in diethyl ether (10 mL) was slowly added. After 30 min at −78 °C, 3-formyl-4-methyl-2-(pivaloylamino)pyridine (4.65 g, 21.0 mmol) dissolved in THF (10 mL) was slowly added, and stirring at this temperature was continued for 30 min, before the solution was allowed to warm to room temperature and poured into NH_4Cl (sat., aqueous) (100 mL). The aqueous phase was extracted three times with diethyl ether (3 × 50 mL), and the organic extracts were washed with water (50 mL) and brine (50 mL), dried over $MgSO_4$, and evaporated in vacuo. The crude product was recrystallized from ethyl acetate/hexane to yield the diastereomeric mixture, which was used as such after drying in vacuo: The diastereomeric mixture was dissolved in HCl (3 M, aqueous) (50 mL), and the solution was stirred under reflux for 3.5 h, cooled to room temperature, washed with diethyl ether (2 × 50 mL), neutralized with K_2CO_3, and again washed with chloroform (3 × 50 mL). The desired product (1.39 g, 37%) precipitated from this solution upon cooling (mp >250 °C). ^{1}H NMR (D_2O/NaOD): δ 8.25 (d, 4.9 Hz, 1H, arom), 7.71 (s, 1H, arom), 6.84 (d, 4.9 Hz, 1H, arom), 2.36 (s, 2H, CH_2), 1.05 (s, 3H, CH_3). ^{13}C NMR (D_2O/NaOD): δ 180.30, 171.68, 155.44, 148.84, 146.04, 132.61, 127.08, 117.60, 116.21, 27.22, 16.98. FAB + MS (*m*/*z*): 219.10 (M + H$^+$, calcd for $C_{11}H_{10}N_2O_3$ + H$^+$ 219.0770).

Methyl *N*-((5-Methyl-1,8-naphthyridin-2(*1H*)-on-3-yl)acetyl)-*N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate (3f). To a precooled (0 °C) solution of (5-methyl-1,8-naphthyridin-2(1*H*)-on-3-yl)acetic acid (1.20 g, 5.50 mmol) and DhbtOH (0.987 g, 6.10 mmol) in DMF (25 mL) was added DCC (1.25 g, 6.10 mmol), and the resulting mixture was stirred for 40 min prior to the addition of methyl *N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate (1.32 g, 6.10 mmol). The mixture was stirred at room temperature overnight, evaporated in vacuo, and redissolved in ethyl acetate (75 mL), and the DCU was filtered off. The organic phase was washed with $NaHCO_3$ (3 × 25 mL) and with brine (2 × 25 mL), dried ($MgSO_4$), and evaporated in vacuo. The crude product was purified on silica using MeOH/dichloromethane (95:5 to 90:10 v/v) as the eluent. Fractions containing the product were pooled and evaporated in vacuo to yield the desired product (752 mg, 33%) (mp 175−77 °C). ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.10 (12.08), (s br, 1H, NH), 8.33 (8.33) (d, 4.8 Hz, 1H, arom), 7.96 (7.90) (s, 1H, arom), 7.09 (d, 4.8 Hz, 1H, arom), 6.92 (6.70) (m, 1H, NH), 4.07 (4.37) (s, 2H, CH_2), 3.67 (s, 2H CH_2), 3.64 (s, 3H CH_3), 3.46 (m, 2H, CH_2), 3.17 (m, 2H, CH_2), 2.54 (2.53) (s, 3H, CH_3), 1.37 (1.35) (s, 9H, CH_3 (Boc)). ^{13}C NMR(DMSO-d_6): δ 170.30 (170.50), 170.03, 162.30, 155.73, 149.47, 149.25, 145.16, 132.92 (133.56), 129.12 (129.28), 119.87, 113.35, 77.97, 52.13, 51.76 (50.12), 47.47 (48.16), 38.98 (38.39), 33.81 (33.99), 28.24, 17.66. FAB + MS (*m*/*z*): 433.2087 (M + H$^+$, calcd for $C_{21}H_{28}N_4O_6$ + H$^+$ 433.2086)

***N*-((5-Methyl-1,8-naphthyridin-2(*1H*)-on-3-yl)acetyl)-*N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycine (4f).** To a solution of methyl *N*-((5-methyl-1,8-naphthyridin-2(1*H*)-on-3-yl)acetyl)-*N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate (0.72 g, 1.70 mmol) in THF (10 mL) was added LiOH (aqueous, 2 M) (2.0 mL). The resulting solution was stirred at room temperature for 45 min, then additional water was added, the THF was evaporated in vacuo, and the pH of the aqueous solution was adjusted to 3.0. The resulting precipitate was washed with water (2 × 10 mL), collected by centrifugation, and dried in vacuo to yield the desired product (366 mg, 53%) as a white powder (mp 133−36 °C). ^{1}H NMR (DMSO-d_6; this compound exists as two rotamers, and chemical shifts for the minor rotamer are given in parentheses): δ 12.12 (s br, 1H, NH), 8.33 (d, Hz, 1H, arom), 8.00 (s, 1H, arom), 7.09 (d, 4.9 Hz, 1H, arom), 6.92 (6.70) (m, 1H, NH), 3.99 (4.26) (s, 2H, CH_2), 3.62 (s, 2H, CH_2), 3.45 (m, 2H + H_2O, CH_2), 3.17 (m, 2H, CH_2), 2.53 (2.52) (s, 3H, CH_3), 1.36 (1.34) (s, 9H, CH_3 (Boc)) ^{13}C NMR (DMSO-d_6): δ 171.07 (171.31), 170.23 (170.54), 162.36, 155.80, 149.53, 149.27, 145.30, 132.67 (133.55), 129.35 (129.20), 119.92, 113.44, 78.01, 48.13, 47.33, 38.60 (38.36), 34.07, 28.27, 17.76. FAB−HRMS (*m*/*z*): 417.1798, calcd $C_{20}H_{26}N_4O_6$ − H 417.1774.

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