Peptide Nucleic Acids: Synthesis of Thymine, Adenine, Guanine, and Cytosine Nucleobases

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Highly stable analogs of DNA or RNA are promising candidates for inhibiting gene expression.[1] When introduced into cells, these analogs pair with DNA (antigene) or mRNA (antisense) in a sequence-dependent manner to prevent the production of functional protein in a cell. Chemical modifications of either the nucleotide base or the phosphodiester linkage may improve entry of the analog into cells and prevent intracellular degradation. Nielsen and colleagues[2] used computer modeling to design a new nucleic acid analog, peptide nucleic acid (PNA), which is based on a (2-aminoethyl)glycine backbone with the four standard nucleic acid bases as side chains. PNAs are synthesized into a peptide bond-linked linear sequence using standard Merrifield chemistry. The spacing between pyrimidine or purine side chains in a PNA approximates the spacing between bases in DNA or RNA (Figure 1). As a result, a PNA can form normal hydrogen bonded duplexes with a DNA or RNA strand. PNAs have ideal properties for an antigene drug. For example, because of their nonstandard backbone, PNAs are resistant to degradation by cellular proteases and nucleases. PNAs can also inhibit the synthesis of large T-antigen in cells.[3] PNAs hybridize with complementary DNA sequences with high specificity[4] and can form triple-stranded complexes through nonstandard Hoogstein base pairing with a second PNA strand.[5] In a triple-stranded form, short PNAs (<12 subunits) are able to form sequence specific complexes with target DNA under conditions in which DNA duplexes cannot form.

We now report a general synthesis to obtain the Boc-derivatives of the PNA thymine, adenine, guanine, and cytosine monomers on a preparative scale. The synthesis employs a common intermediate, **4**, to which any nucleobase or modified nucleobase can be attached.

The approach developed by Egholm et al.[6,7] for the synthesis of their protected thymine and cytosine monomers was based upon coupling of a protected *N*-(2-aminoethyl)glycine (Aeg) side chain with an N-carboxymethylated base. This route suffers from a number of experimental difficulties. We therefore embarked on the development of an alternate and more general route to protected monomers. We anticipated that the coupling of a suitably protected *N*-(chloroacetyl)-Aeg with the bases themselves would provide the desired end products directly. Specifically, we selected the N-Boc protected ester, **4**, for synthesis (Scheme 1).

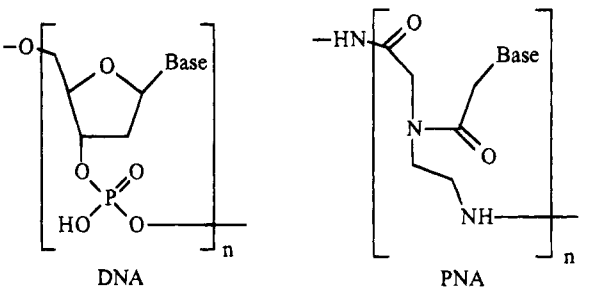

Figure 1. Comparison of DNA and PNA structures.

Results and Discussion

Synthesis. Aminoacetonitrile was treated with di-*tert*-butyl dicarbonate to provide the (*N*-Boc-amino)acetonitrile, **1**, in 83% yield. Hydrogenation at high pressure in a 10% NH_3/EtOH mixture with Raney nickel as catalyst provided the amine **2** in quantitative yield. The ethyl glycinate, **3**, was then obtained in 81% yield upon reaction of **2** with ethyl bromoacetate in acetonitrile in the presence of KF/Celite. The target ester, **4**, was then obtained in 64% yield upon reaction of **3** with chloroacetyl chloride in methylene chloride in the presence of triethylamine.

With **4** in hand, we explored alkylation of the four bases. Thus, thymine, adenine, cytosine, and 2-amino-6-chloropurine were each reacted with **4** in dimethylformamide. Purification by flash chromatography provided **5**, **6**, **7**, and **8**, respectively, in 45–95% yield. Hydrolysis of the esters to provide the required Boc protected acids was effected with sodium hydroxide, as was conversion of the chloropurine **8** to the guanine **12**. After purification, the analytically pure products, **9**, **10**, **11**, and **12**, were obtained in 72%, 93%, 45%, and 36% yields, respectively.

Alkylation of thymine and cytosine is regiospecific. Thus reaction of **4** with both bases provided the 1-substituted products.[8] However, alkylation of adenine is notoriously nonregiospecific[9] although the product of 9-substitution is often dominant.[10] In our hands, this alkylation showed some regiospecificity in that as little as 10% of an undesired regioisomer was obtained. Column chromatography readily provided the desired compound **6** free of any other regioisomers. The position of substitution on the adenine ring can be determined by examination of the ^1H NMR spectra of the two isomers since, in general, the signal for the H-8 proton in the N9 isomer is shifted upfield relative to the corresponding H-8 signal for the N7 isomer. This analysis requires that

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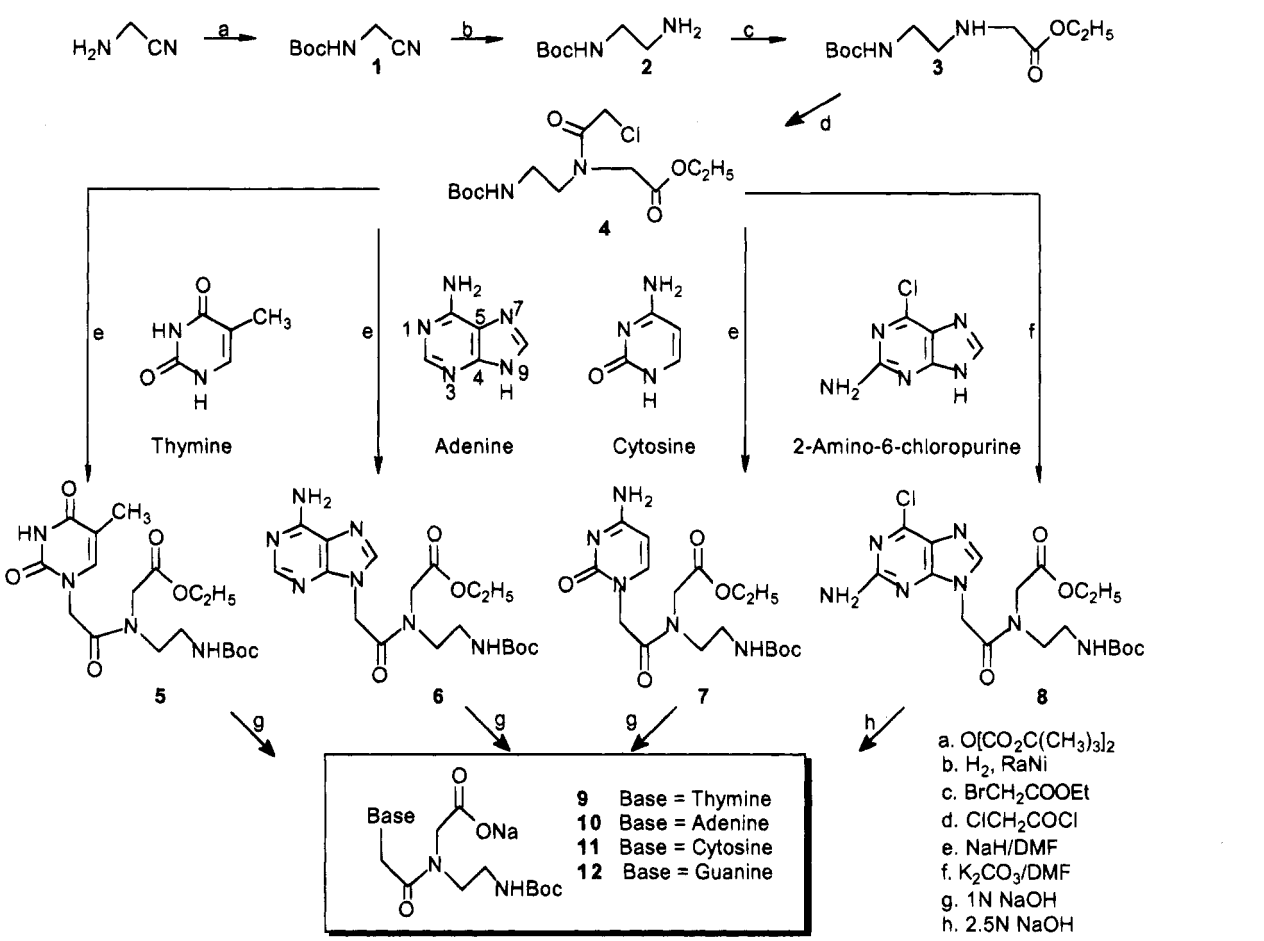
Scheme 1



both N7 and N9 isomers be in hand.[11] In the absence of one of the isomers, the assignment cannot be unambiguously made by these means and Osterman et al.[12] have solved this problem by the use of the selective Insensitive Nuclei Enhanced by Polarization Transfer experiment. We have solved the problem by the comparison of the ^{13}C NMR spectrum measured in DMSO-d_6 for our product, **10**, with those reported by Chenon et al.[13] for 7-methyladenine and 9-methyladenine in the same solvent. An analysis of the ^{13}C NMR spectrum shows complete agreement with their values for 9-methyladenine with sufficient distinction from 7-methyladenine to make an unequivocal assignment (Table 1).

Alkylation of 2-amino-6-chloropurine with **4** was conducted following the general procedure of Harnden et al.[14] The ^1H-NMR spectra obtained for both **8** and **12** proved consistent with the reported data, thus supporting substitution at the 9-position.

The 400 MHz ^1H NMR spectra of compounds **9**–**12** displayed a doubling caused by an equilibrium mixture of the *E* and *Z* isomers. This equilibrium is solvent dependent such that for **10** in D_2O there is a 50:50 mixture of isomers, while in DMSO the *E* isomer predominates (80:20).

An NOE correlation experiment with **10** (Figure 2) served to confirm the assignment of proton resonances. Thus, irradiation of the protons at the methylene position (a) of the *E* isomer caused enhancement for both the C-8 aromatic proton as well as for the *N*-methylene protons (b). Irradiation of the methylene position (a) of the *Z* isomer caused enhancement for the C-8 aromatic proton, but this time the signal for the methylene protons at (c) was enhanced while no effect was evident for the (b) protons.

Table 1. Comparative ^{13}C NMR Assignments for 7- and 9-Methyladenine and Compound 10

compound[a]	C-2	C-4	C-5	C-6	C-8
7-methyladenine[b]	152.4	159.8	111.7	151.9	145.9
9-methyladenine[b]	152.5	149.9	118.7	155.9	141.4
compound **10**	152.4	149.9	118.1	155.9	141.9

[a] Spectra in DMSO-d_6 relative to TMS as internal standard. [b] Data from Chenon et al. (ref 13).

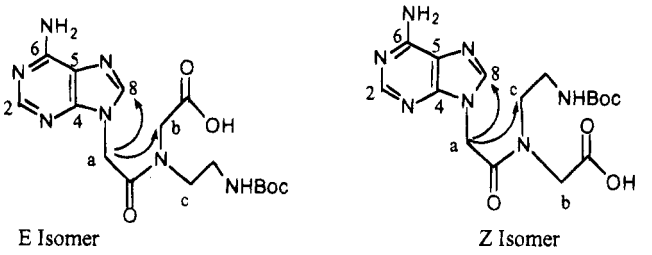


Figure 2. NOE correlation for compound **10**.

Conclusion

A general route for the synthesis of the PNAs for four nucleobases has been described.

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Experimental Section

General Procedures. ^{1}H NMR spectra were recorded at 60 MHz, 100 MHz, or 400 MHz with TMS as internal standard. Melting points are uncorrected. Thin layer chromatography (TLC) was carried out on Baker Si 250F plates. Visualization was accomplished with either iodine vapor, UV exposure, or treatment with phosphomolybdic acid (PMA). Flash chromatography was carried out on Baker silica gel, 40 μM, and Bakerbond Octadecyl. Starting materials were purchased from Aldrich Chemical Co. and solvents were ACS grade. Elemental analyses were performed by Atlantic Microlab, Atlanta, GA.

Ethyl *N*-(Boc-aminoethyl)glycinate (3). Triethylamine (125 mL, 0.9 mol) was added dropwise to a mixture of aminoacetonitrile hydrochloride (99%, 30.0 g, 0.325 mol) and di-*tert*-butyl dicarbonate (70.9 g, 0.325 mol) in CH_2Cl_2 (1 L) at 0 °C. The mixture was stirred at room temperature overnight. Volatile material was removed by rotary evaporation at reduced pressure, and the residue was dissolved in ether (800 mL), washed consecutively with water (250 mL) and saturated aqueous NaCl, dried over $MgSO_4$, filtered, and concentrated to dryness on a rotary evaporator to afford 42.25 g (83%) of (*N*-Boc-amino)acetonitrile (**1**) as a yellow viscous oil: R_f 0.55 (EtOAc/hexane 1:1); ^{1}H NMR (60 MHz, $CDCl_3$) δ 5.3 (br s, 1H), 4.05 (d, 2H, J = 6.0 Hz), 1.46 (s, 9H). This material was used in the next reaction without further purification.

(*N*-Boc-amino)acetonitrile, **1** (20.2 g, 0.129 mol), Raney nickel (Aldrich Chem. Co., 2 teaspoons) in 10% NH_3/EtOH (350 mL) was hydrogenated at room temperature overnight in a Parr pressure vessel at 50 psi. The mixture was filtered through Celite and concentrated to dryness on a rotary evaporator to afford 20.5 g (99%) of *N*-Boc-ethylenediamine (**2**) as a green oil: R_f 0.35 (MeOH/EtOAc 1:1 + 2% NH_4OH); ^{1}H NMR (60 MHz, $CDCl_3$) δ 5.2 (br s, 1H), 3.4 (t, 2H, J = 6.0 Hz), 2.9 (t, 2H, J = 6.0 Hz), 1.46 (s, 9 H), 1.35 (s, 2H). This material was used in the next reaction without further purification.

Ethyl bromoacetate (17.3 g, 0.104 mol) was added in one portion to a mixture of **2** (23.3 g, 0.145 mol) and KF/Celite (60.2 g, 0.518 mol) in acetonitrile (dried over mol. sieves 3 Å; 500 mL) at 60 °C. The reaction mixture was cooled to room temperature after 15 min and then filtered through Celite and concentrated to dryness on a rotary evaporator. The residue was purified by gradient flash chromatography with simultaneous monitoring by TLC (eluent: EtOAc/hexane 1:1; EtOAc; 10% MeOH/EtOAc) to afford 20.8 g (81%) of **3** as a pale yellow viscous oil: R_f 0.4 (10% MeOH/EtOAc); ^{1}H NMR (60 MHz, $CDCl_3$) δ 5.2 (br s, 1H), 4.2 (q, 2H, J = 7.0 Hz), 3.4 (s, 2H), 3.2 (t, 2H, J = 5.0 Hz), 2.7 (t, 2H, J = 5.0 Hz), 1.7 (s, 1H), 1.46 (s, 9H), 1.3 (t, 3H, J = 7.0 Hz). Anal. Calcd for $C_{11}H_{22}N_2O_4$: C, 53.63; H, 9.00; N 11.38. Found: C, 53.38; H, 9.03; N 11.31.

Ethyl *N*-(Boc-aminoethyl)-*N*-(chloroacetyl)glycinate (4). Chloroacetyl chloride was added dropwise over a period of 30 min to a mixture of **3** (58.2 g, 0.24 mol) and triethylamine (140 mL) in CH_2Cl_2 (500 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h. Water (250 mL) was then added, and the aqueous layer was extracted with 100 mL of CH_2Cl_2. The combined CH_2Cl_2 layer was washed with saturated aqueous NaCl, dried over K_2CO_3, filtered through Celite, and concentrated to dryness on a rotary evaporator. The residue was purified by gradient flash chromatography (eluent: EtOAc/hexane 1:1; EtOAc; 10% MeOH/EtOAc) with simultaneous monitoring by TLC to afford 48.7 g (64%) of **4** as a yellow viscous oil: R_f 0.63 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 (br s, 1H), 4.21 (q, 2H, J = 7.2), 4.14 (s, 2H), 4.00 (s, 2H), 3.53 (t, 2H, J = 5.7 Hz), 3.28 (q, 2H, J = 5.7 Hz), (m, 4H), 1.46 (s, 9H) 1.3 (t, 3H, J = 7.2 Hz). Anal. Calcd for $C_{13}H_{23}ClN_2O_5$: C, 48.37; H, 7.18; N 8.68; Cl, 10.98. Found: C, 48.47; H, 7.21; N 8.71; Cl, 11.07.

1-(Boc-Aeg)thymine Ethyl Ester (5). A mixture of thymine (4.19 g, 0.033 mol) and NaH (1.33 g, 0.033 mol) in DMF (100 mL) was heated at 75 °C until effervescence ceased. Compound **4** (11.7 g, 0.033 mol) in DMF (60 mL) was added. The reaction mixture was heated at 75 °C for 1 h. DMF was removed at reduced pressure. The residue was purified by gradient flash chromatography with simultaneous monitoring by TLC (eluent: EtOAc; 2% MeOH/EtOAc; 10% MeOH/EtOAc) to afford 7.5 g (55%) of **5** as a white solid: mp 162–164 °C; R_f 0.38 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 (s, 1H), 6.98 (s, 1H), 5.63 (m, 1H), 4.58 (s, 2H), 4.18 (q, 2H, J = 7.2 Hz), 4.02 (s, 2H), 3.51 (t, 2H, J = 5.6 Hz), 3.30 (q, 2H, J = 5.6 Hz), 1.89 (s, 3H), 1.42 (s, 9H), 1.26 (t, 3H, J = 7.2 Hz). Anal. Calcd for $C_{18}H_{28}N_4O_7$: C, 52.41; H, 6.84; N 13.59. Found: C, 52.44; H, 6.90; N 13.53.

1-(Boc-Aeg)adenine Ethyl Ester (6). A mixture of adenine (5.0 g, 0.037 mol) and NaH (1.48 g 0.037 mol) in DMF (100 mL) was heated at 75 °C until effervescence ceased. Compound **4** (12.0 g, 0.037 mol) in DMF (60 mL) was added. The reaction mixture was heated at 75 °C for 1 h. DMF was removed at reduced pressure. The residue was purified by gradient flash chromatography with simultaneous monitoring by TLC (eluent: 3% NH_4OH, 5% MeOH/EtOAc; 3% NH_4OH, 10% MeOH/EtOAc) to afford 6.8 g (44%) of **6** as a cream colored solid: mp 111–113 °C; R_f 0.38 (10% MeOH/EtOAc + 2% NH_4OH); ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.10 (s 1H), 7.97 (s, 1H), 7.22 (bs, 3H), 5.20 (s, 2H), 4.06 (q 2H, J = 7.1 Hz), 4.04 (s, 2H), 3.52 (t, 2H, J = 6.3 Hz), 3.25 (q, 2H, J = 6.1 Hz), 1.37 (s, 9H). 1.15 (t, 3H, J = 7.1 Hz). Anal. Calcd for $C_{18}H_{27}N_7O_5$: C, 51.29; H, 6.46; N 23.27. Found: C, 51.14; H, 6.50; N 23.19.

1-(Boc-Aeg)cytosine Ethyl Ester (7). A mixture of cytosine (4.26 g, 0.038 mol) and NaH (1.53 g 0.038 mol) in DMF (100 mL) was heated at 75 °C until effervescence ceased. Compound **4** (12.4 g, 0.038 mol) in DMF (60 mL) was added. The reaction mixture was heated at 75 °C for 1 h. DMF was removed at reduced pressure. The residue was purified by gradient flash chromatography with simultaneous monitoring by TLC (eluent: 10% MeOH, 3% NH_4OH/EtOAc) to afford 7.66 g (50%) of **7** as a white solid: mp 118–120 °C; R_f 0.28 (10% MeOH + NH_4-OH/EtOAc; two elutions); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.38 (d, 1H, J = 7.2 Hz), 7.05 (m, 2H), 6.90 (m, 1H), 5.64 (d, 1H, J = 7.2 Hz), 4.63 (s, 2H), 4.08 (q, 2H, J = 7.1 Hz), 4.02 (s, 2H), 3.41 (t, 2H, J = 6.4 Hz), 3.17 (q, 2H, J = 6.4 Hz), 1.37 (s, 9H), 1.18 (t, 3H, J = 7.1 Hz). Anal. Calcd for $C_{17}H_{27}N_5O_6 \cdot 0.25H_2O$: C, 50.80; H, 6.90; N 17.42. Found: C, 50.71; H, 6.90; N 17.39.

9-(Boc-Aeg)-2-amino-6-chloropurine Ethyl Ester (8). A mixture of 2-amino-6-chloropurine (1.73 g, 12.4 mmol), **4** (4.0 g, 12.4 mmol), pulverized K_2CO_3 (2.0 g, 14.5 mmol), and DMF (30 mL) was stirred at room temperature for 4 h. The solution was filtered, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (eluent: 10% MeOH, 3% NH_4OH/EtOAc) to afford 4.4 g (95%) of **8** as a pale yellow foam: ^{1}H NMR (100 MHz, $CDCl_3$) δ 7.90 (s, 1H), 5.70 (m, 1H), 5.21 (m, 2H), 5.00 (s, 2H), 4.20 (q, 2H, J = 7.5 Hz), 4.06 (s, 2H), 3.61 (m, 2H), 3.39 (m, 2H), 1.44 (s, 9H), 1.27 (t, 3H, J = 7.5 Hz). Anal. Calcd for $C_{18}H_{26}N_7O_5Cl$: C, 47.42; H, 5.75; N, 21.51; Cl 7.78. Found: C, 47.32; H, 5.76; N, 21.41; Cl 7.85.

1-(Boc-Aeg)thymine, Sodium Salt (9). A mixture of **5** (7.5 g, 18 mmol) and 1 N NaOH (40 mL) was stirred at room temperature until all starting material was consumed (TLC: eluent EtOAc). The reaction mixture was washed with CH_2Cl_2 (30 mL). The aqueous layer was neutralized with 1 N HCl to pH 6 and concentrated to dryness. The residue was triturated with MeOH/EtOH (1:9) and filtered. The filtrate was concentrated to a small volume on a rotary evaporator and left at 4 °C in a refrigerator overnight. The precipitated white solid was filtered, washed with EtOH and ether, and dried in a vacuum oven at 50 °C to afford 5.0 g (72%) of **9** as a white solid: mp 200 °C dec; R_f 0.47 (nBuOH/H_2O/AcOH 3:1:1); ^{1}H NMR (major [*E*] isomer; 400 MHz, DMSO-d_6) δ 7.22 (s, 1H), 7.16 (t, 1H), 4.40 (s, 2H), 3.60 (s, 2H), 3.28 (t, 2H, J = 6.1 Hz), 3.00 (m, 2H), 1.73 (s, 3H), 1.34 (s, 9H).

^{1}H NMR (minor [*Z*] isomer; 400 MHz, DMSO-d_6): 7.35 (t, 1H), 7.22 (s, 1H), 4.52 (s, 2H), 3.58 (s, 2H), 3.28 (t, 2H, J = 6.1 Hz), 3.11 (m, 2H), 1.73 (s, 3H), 1.37 (s, 9H). Anal. Calcd for $C_{16}H_{23}N_4O_7Na \cdot H_2O$: C, 45.28; H, 5.94; N 13.20; Na, 5.42. Found: C, 45.50; H, 5.98; N 13.03; Na, 5.37.

1-(Boc-Aeg)adenine, Sodium Salt (10). A mixture of **6** (6.6 g, 16 mmol) and 1 N NaOH (40 mL) was stirred at room temperature until all starting material was consumed (TLC: eluent: 10% MeOH/EtOAc + 2% NH_4OH). The reaction mixture was washed with CH_2Cl_2 (30 mL). The aqueous layer was neutralized with 1 N HCl to pH 6 and concentrated to dryness. The residue was triturated with MeOH/EtOH (1:9) and filtered. The filtrate was concentrated to a small volume on a rotary evaporator and left in a refrigerator overnight. The precipitated white solid was filtered, washed with EtOH and ether, and dried in a vacuum oven at 50 °C to afford 5.72 g (93%) of **10** as a white solid: mp 228 °C dec; R_f 0.4 (nBuOH/H_2O/AcOH 3:1:1);.^{1}H NMR

(major [*E*] isomer; 400 MHz, DMSO-d_6) δ 8.09 (s, 1H), 7.94 (s, 1H), 7.18 (m, 3H), 4.96 (s, 2H), 3.75 (s, 2H), 3.31 (t, 2H, J = 6.2 Hz), 3.02 (m, 2H), 1.33 (s, 9H).

^{1}H NMR (minor [*Z*] isomer; 400 MHz, DMSO-d_6): δ 8.09 (s, 1H), 7.95 (s, 1H), 7.15 (m, 3H), 5.05 (s, 2H), 3.61 (s, 2H), 3.45 (m, 2H), 3.18 (m, 2H), 1.38 (s, 9H).

^{13}C NMR (starred peaks are due to the minor isomer; DMSO-d_6): δ 172.3*, 171.7, 167.3, 165.7*, 155.9, 155.7, 152.4, 149.9, 141.9, 118.1, 78.0*, 77.5, 56.0*, 52.6, 50.3*, 47.5, 43.8, 37.7, 28.3. Anal. Calcd for $C_{16}H_{22}N_7O_5Na\cdot H_2O$: C, 44.34; H, 5.58; N 22.63; Na, 5.30. Found: C, 44.31; H, 5.62; N 22.41; Na, 5.32.

1-(Boc-Aeg)cytosine, Sodium Salt (11). A mixture of **7** (7.66 g, 19 mmol) and 1 N NaOH (40 mL) was stirred at room temperature until all starting material was consumed (TLC: eluent EtOAc). The reaction mixture was washed with CH_2Cl_2 (30 mL). The aqueous layer was neutralized with 1 N HCl to pH 6 and concentrated to dryness. The residue was triturated with MeOH/EtOH and filtered. The filtrate was concentrated to a small volume on a rotary evaporator and left in a refrigerator overnight. The precipitated white solid was filtered, washed with EtOH and ether, and dried in a vacuum oven at 50 °C to afford 3.16 g (45%) of **11** as an off-white solid: mp 237 °C dec; R_f 0.3 (nBuOH/H_2O/AcOH 3:1:1); ^{1}H NMR (major [*E*] isomer; 400 MHz, DMSO-d_6) δ 7.33 (d, 1H, J = 7.2 Hz), 7.13 (m, 1H), 6.98 (bs, 2H), 5.64 (d, 1H, J = 7.2 Hz), 4.39 (s, 2H), 3.64 (s, 2H), 3.27 (t, 2H, J = 6.3 Hz), 3.00 (m, 2H), 1.34 (s, 9H).

^{1}H NMR (minor [*Z*] isomer; 400 MHz, DMSO-d_6): δ 7.35 (d, 1H, J = 7.2 Hz), 7.26 (m, 1H), 7.05 (bs, 2H), 5.66 (d, 1H, J = 7.2 Hz), 4.51 (s, 2H), 3.59 (s, 2H), 3.34 (t, 2H, J = 6.3 Hz), 3.13 (m, 2H), 1.36 (s, 9H). Anal. Calcd for $C_{15}H_{22}N_5O_6Na\cdot 0.75H_2O$: C, 44.49; H, 5.85; N 17.30; Na, 5.68. Found: C, 44.63; H, 5.81; N 17.23; Na, 5.51.

9-(Boc-Aeg)guanine (12). A mixture of **8** (2.35 g, 5.15 mmol) and 2.5 N NaOH (25 mL) was stirred at room temperature for 48 h. The yellow solution was neutralized with 4 N HCl and concentrated to dryness. The residue was purified by flash chromatography over Bakerbond Octadecyl (H_2O, 20% MeOH/H_2O) to afford 0.81 g (36%) of **12** as an off white solid: mp 280 °C dec; ^{1}H NMR (major [*E*] isomer; 400 MHz, DMSO-d_6): 11.25 (bs, 1H), 7.49 (s, 1H), 7.03 (m, 1H), 6.87 (s, 2H), 4.77 (s, 2H), 3.79 (s, 2H), 3.32 (t, 2H, J = 6.4 Hz), 3.05 (m, 2H), 1.35 (s, 9H). ^{1}H NMR (minor [*Z*] isomer; 400 MHz, DMSO-d_6): 11.25 (bs, 1H), 7.75 (s, 1H), 7.16 (m, 1H), 6.94 (s, 2H), 4.90 (s, 2H), 3.75 (s, 2H), 3.445 (m, 2H), 3.20 (m, 2H), 1.37 (s, 9H). Anal. Calcd for $C_{16}H_{22}N_7O_6Na$: C, 43.63; H, 5.26; N, 22.27; Na 5.22. Found: C, 43.60; H, 5.21; N, 22.23; Na 5.15.

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Additions and Corrections

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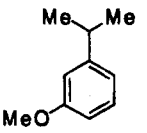
Ranjit C. Desai,* Dennis J. Hlasta, George Monsour, and Manohar Saindane. An Efficient Large Scale Synthesis of 4-Isopropyl- and 4-Isopropyl-6-methoxybenzisothiazolones.

Page 7162, Scheme 2. Structure **5** should be drawn as follows:



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