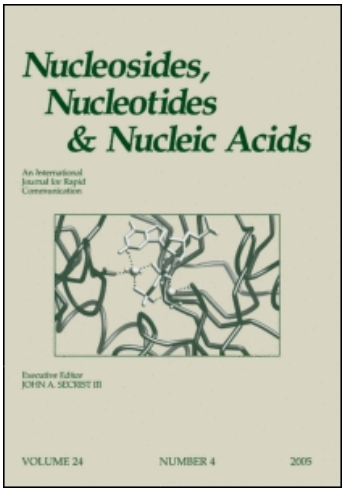

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

SYNTHESIS AND HYBRIDIZATION STUDIES OF A 5-AMINOPENTANOIC ACID NUCLEOBASE (APN) DIMER

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Online publication date: 25 March 2002

To cite this Article Donaldson, Susan F. , Bergmeier, Stephen C. , Hines, Jennifer V. and Gerdeman, Melinda S.(2002) 'SYNTHESIS AND HYBRIDIZATION STUDIES OF A 5-AMINOPENTANOIC ACID NUCLEOBASE (APN) DIMER', Nucleosides, Nucleotides and Nucleic Acids, 21: 2, 111 — 123
To link to this Article: DOI: 10.1081/NCN-120003176
URL: http://dx.doi.org/10.1081/NCN-120003176

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 21(2), 111–123 (2002)

SYNTHESIS AND HYBRIDIZATION STUDIES OF A 5-AMINOPENTANOIC ACID NUCLEOBASE (APN) DIMER

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ABSTRACT

We have prepared a 5-aminopentanoic acid nucleobase (APN) dimer and investigated its hybridization capabilities to complementary DNA using both UV melting and NMR techniques.

The principle of antisense/antigene inhibition of gene expression requires the binding of a complementary oligonucleotide to either DNA (antigene)[1] or RNA (antisense)[2]. Binding of the oligonucleotide to target DNA or RNA ultimately results in inhibition of transcription, translation, or RNA processing through a variety of mechanisms[3]. Due to severe limitations in stability and utility of natural oligonucleotides as drugs, most antisense and antigene agents are based on modified oligonucleotides.

Peptide nucleic acids (PNAs, **2**) are a unique type of oligonucleotide analog in which both the phosphodiester backbone and the sugar moiety have been replaced with a peptide based structure[4]. PNAs have shown excellent hybridization properties to both DNA and RNA targets[5]. Since their introduction in 1991, many analogs have been synthesized and

*Corresponding author.

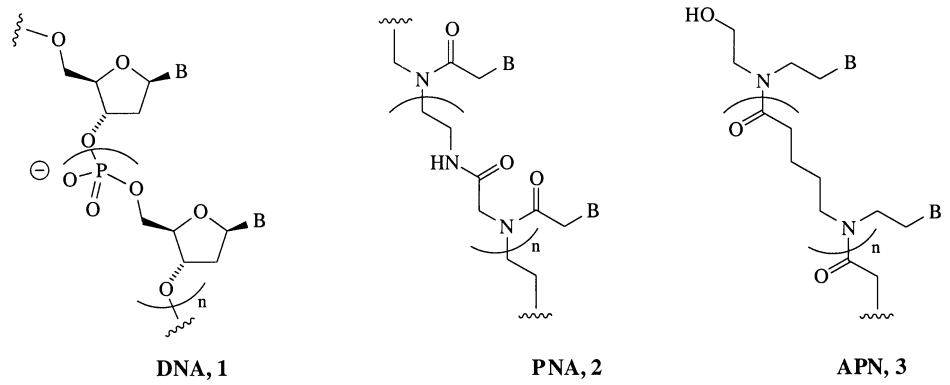
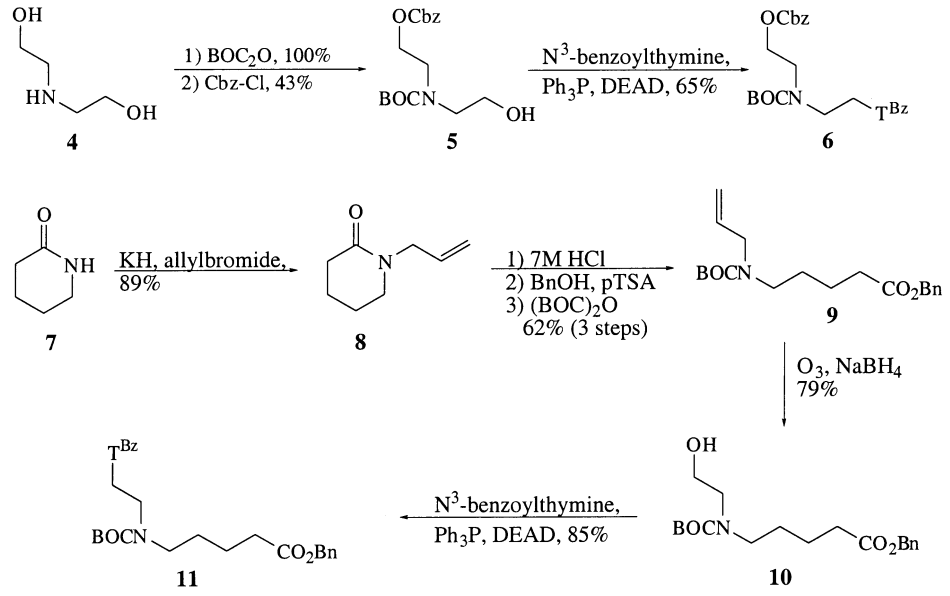


Figure 1.

evaluated for their ability to bind DNA and RNA[6]. Of particular note to our studies has been the preparation of a variety of very flexible PNA type molecules[7]. While some of these molecules have shown decreased hybridization ability, others have shown very similar hybridization properties to PNAs. In order to further investigate the structural requirements of PNAs, we reported[8] the synthesis of a PNA of the general structure **3** in which we oligomerized 5-aminopentanoic acid nucleobase (APN) monomers. We now wish to report the results of UV melting and NMR hybridization studies with



Scheme 1.

an APN-T_2 dimer as well as full experimental details for the synthesis of these novel oligonucleotide analogues.

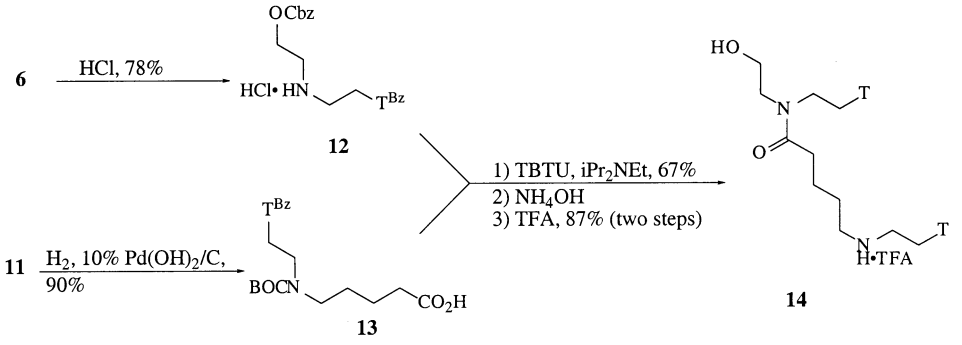
Synthesis of the dimer was carried out as previously described[8]. As reported, to obtain APN-T_2, it was necessary to synthesize two separate pieces, the 5′-end starter unit **6** and the 3′-end monomer unit **11**. Pieces **6** and **11** could be deprotected and coupled together to form the APN dimer. This dimer was then used to study the hybridization capabilities of the APN molecule.

The synthesis of the 5′-end of the oligomer is shown in scheme one. Starting from diethanolamine (**4**), the nitrogen is protected as a *t*-butyl carbamate and one of the alcohols is protected as a benzyl carbonate to produce **5**. The remaining alcohol is coupled to N^3-benzoylthymine[9] via Mitsunobu conditions[10] to give **6**[11].

The route to the 3′-end monomer unit **11** starts from δ-valerolactam (**7**) with an *N*-allylation to provide **8**. The lactam was hydrolyzed, the acid esterified and the nitrogen protected as the *t*-butyl carbamate derivative to give **9** in 62% yield from **8**. The olefin was then cleaved with O_3, and a reductive workup with $NaBH_4$ gave alcohol **10**. Finally the thymine was introduced via a Mitsunobu reaction as before to give **11**.

With monomer units **6** and **11** in hand, the amine of **6** was deprotected to provide **12** using HCl[12]. The carboxyl of **11** was deprotected with hydrogen and Pd/C to provide **13**. For the coupling of **12** and **13** both 2-(1H-Benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU)[13] and 2-(1H-Benzotriazol-1-yl)-1,1,3,3 tetramethyl uronium tetrafluoroborate (TBTU)[14] were examined, with TBTU providing the highest yield (67%)[15].

In order to examine the hybridization capabilities of APN-T_2 to target DNA, it was necessary to deprotect the dimer. Using ammonium hydroxide in dioxane, the benzoyl groups of the dimer were removed. Not suprisingly, the Cbz group was also removed under these conditions thus, eliminating the need for a catalytic hydrogenation step. Treatment of the crude material with triflouroacetic acid to remove the remaining BOC group, gave salt **14** in 87% yield over two steps.



Scheme 2.

With fully deprotected dimer in hand, hybridization studies using both UV and NMR techniques were carried out. Hybridization studies are not usually carried out on such a small oligonucleotides. The T_m of a hybrid system decreases with sequence length because there are less hydrogen bonds involved in the duplex structure[17]. Similarly the stacking of the bases, which produces the change in hypochromicity upon melting is a function of oligonucleotide length. Therefore, the T_m of a very short sequence occurs at a low temperature and may be difficult to detect by UV. However, others have used small synthetic oligonucleotide analogues and successfully studied their annealing with UV melting.

UV melting studies[18] were carried out in a buffer containing 1 M NaCl, 10 mM Tris and 10 mM $MgCl_2$. Three samples were prepared with the following concentrations: (a) 4.30×10^{-7} M in polydA (control sample), (b) 4.30×10^{-7} M in polydA and 2.19×10^{-4} M in dT_2, (c) 4.30×10^{-7} M in polydA and 2.19×10^{-4} M in **15**. Samples (b) and (c) were prepared so as to result in a 1 base to 1 base ratio between the adenines in the polydA and the thymines in either the native dT_2 or APN-T_2.

The melting experiment was conducted over a temperature range from 4°C to 80°C with a ramp rate of 0.5°C/min. First derivatives of the melt data (Fig. 2) were performed using the OD Deriv program[19]. As expected, melting transitions for the dimers with polydA were observed at temperatures less than 20°C. While the data was noisy (due to the small hypochromicity change) it appeared to indicate that our synthetic dimer APN-T_2 might be hybridizing to polydA with only a slightly reduced T_m relative to dT_2 (polydA: T_2, $T_m \sim 16$°C, polydA: APN-T_2, $T_m \sim 11$°C). While inconclusive, these promising results prompted further investigation into APN hybridization utilizing NMR experiments.

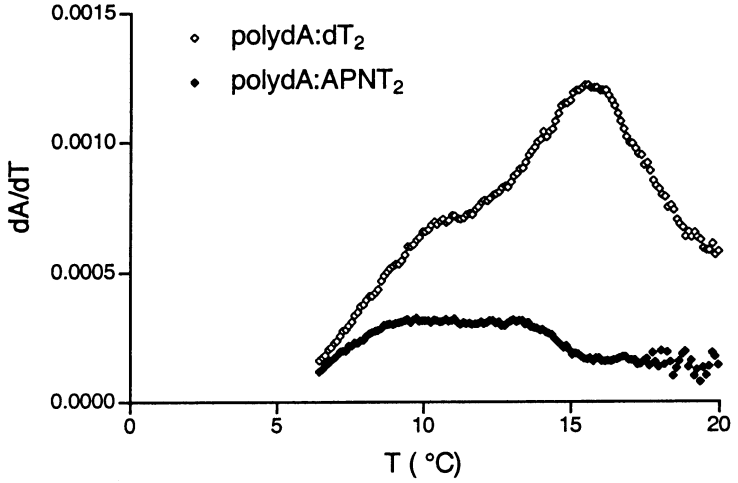


Figure 2. First derivative of melt data for polydA:T_2 and polydA:APN-T_2.

NMR experiments were conducted to observe the imino protons of the APN dimer in the presence of dA_4 (note: polydA was too large to use in these NMR experiments). Observation of an imino proton indicates base pair formation as H-bonding protects the imino proton from exchange. This type of experiment has been carried out previously to provide information regarding base pair stability of an oligonucleotide containing 5-fluorouracil[20]. In our experiments, water supression was achieved using a WATERGATE pulse sequence[21]. The aqueous buffer used was 1 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA. Magnesium was not included in the experiments in order to minimize degradation of the dA_4. Samples were prepared as 90% buffer and 10% D_2O with DSS as an internal standard. Five samples were prepared: (a) 1.0×10^{-3} dA_4 (control sample), (b) 2.0×10^{-3} APN-T_2 (control sample) (c) 2.0×10^{-3} T_2 (control sample) (d) 1.0×10^{-3} dA_4 and 2.0×10^{-3} APN-T_2 (e) 1.0×10^{-3} dA_4 and 2.0×10^{-3} T_2. Samples of (d) and (e) were prepared so as to result in a 1 base to 1 base ratio. Experiments were conducted at 1° increments from 1°C to 12°C. As expected, no imino proton signals were observed with control samples (a), (b), or (c). Figs. 3 and 4 show the results obtained with samples (d) and (e) containing the two sets of complementary strands.

Figure 3 shows the imino region of the dA_4:APN-T_2 experiment. At 1°C an imino peak is clearly visible. As the temperature is slowly increased from 1° to 8°C the imino peak gradually disappears. At 5°C the imino peak has virtually disappeared. Figure 4 shows the imino peak of the dA_4:T_2 sample. Again at low temperature the imino peak is clearly visible. Upon warming the imino peak gradually disappears. In the dA_4:T_2 sample the imino peak is visible until 6–7°C. This again indicates that the dT_2 forms dimers that are only slightly more stable than our synthetic analogue, APN-T_2. The overall lower T_m values in the NMR experiments vs. the UV experiments is most likely due to differences in the buffers.

Based on the UV melting experiments and the NMR observations of the imino protons we can conclude that our APN analogues of oligonucleotides should have similar hybridization properties as native DNA-DNA hybridization. Further experiments with longer, solid-phase synthesized oligomers will investigate this possibility.

EXPERIMENTAL

^1H and ^{13}C spectra were recorded on a Bruker AF 250, Bruker AF 270 or a Bruker DRX 400 model spectrometer. Chemical shifts are reported in ppm relative to tetramethylsilane unless otherwise noted. Melting points were taken using a Thomas Hoover capillary melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on Whatman pre-coated silica gel F_{254} aluminum foils. Purification of the

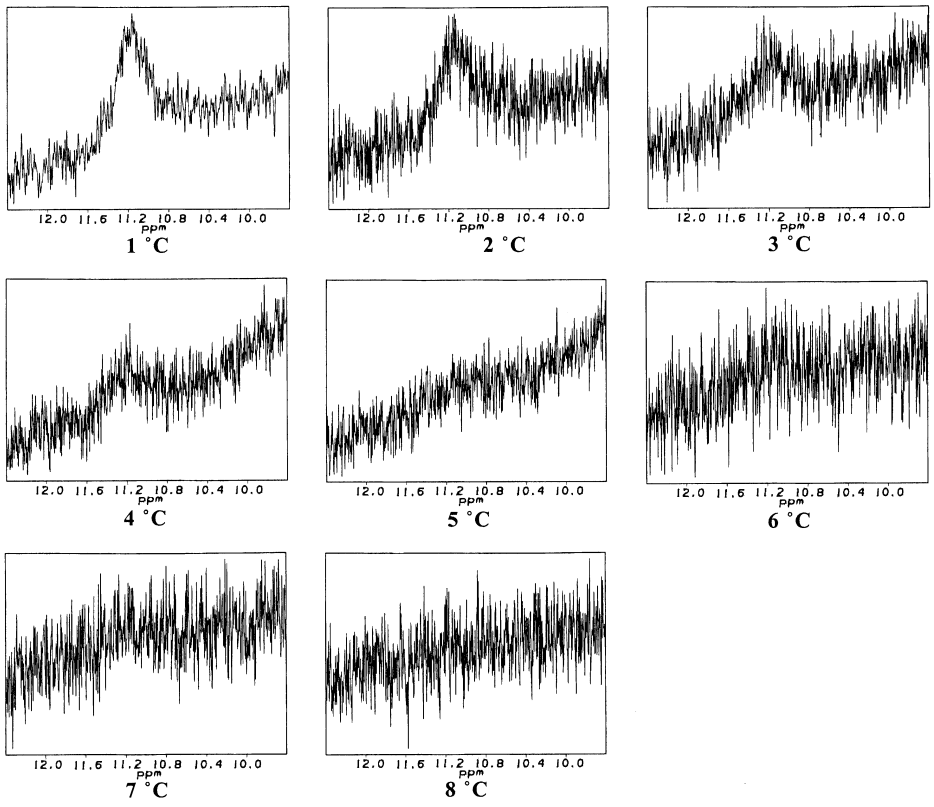


Figure 3. Imino region of dA_4:APN-T_2.

reaction products was carried out by flash column chromatography using a glass column dry packed with silica gel (230–400 mesh ASTM) according to the method of Still[21]. Visualization was accomplished with UV light and/or ninhydrin solution followed by heating. Exact mass measurements recorded in the electron impact (EI) mode were determined at The Ohio State University Chemical Instrument Center with a Kratos MS-30 mass spectrometer. UV spectra were recorded on a Beckman DU-40 spectrometer. Combustion analyses were performed at Quantitative Technologies, Inc., Whitehouse, New Jersey. Infrared spectra were obtained on salt plates in CCl_4 or as a KBr pellet, using a Nicolet Protégé 460 model spectrometer. Peaks are reported in cm^{-1}. UV melting studies were carried out using a Perkin Elmer Lambda 10 UV/Vis Spectrometer equipped with a Peltier Temperature Programmer. NMR hybridization studies were carried out on a Bruker DRX 400 model spectrometer. THF was distilled from sodium and benzophenone. CH_3CN was distilled from phosphorus pentoxide before use. CH_2Cl_2 was distilled from CaH_2. Et_3N, iPr_2NEt, and pyridine were distilled from CaH_2 and stored over KOH pellets. Benzoyl chloride was distilled

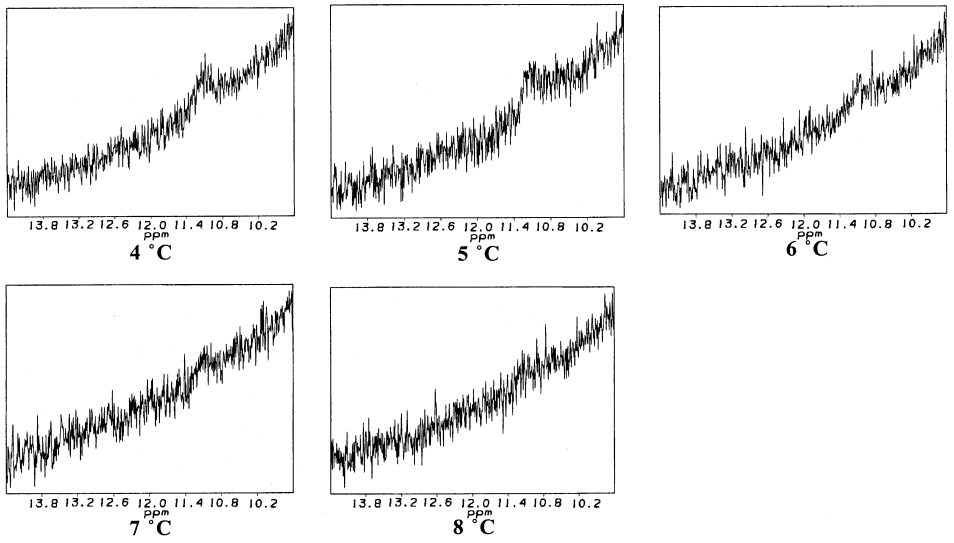


Figure 4. Imino region of dA_4:T_2.

under vacuum just before use. Acetyl chloride and EtOAc were distilled under N_2 before use. All reactions were carried out under an N_2 atmosphere unless otherwise specified.

***O*-Benzyloxycarbonyl-*N*-*tert*-butoxycarbonyl diethanolamine (5).** A solution of diethanolamine (4.8 g, 45.4 mmol) in THF (45.4 mL) was cooled to 0°C. To this solution, di-*tert*-butyl dicarbonate (9.9 g, 45.4 mmol) was added. The reaction was stirred at room temperature for 24 h. Concentration followed by column chromatography (EtOAc) gave 9.3 g of *N*-BOC diethanolamine (100) as an oil: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.46 (s, 9H), 3.43 (t, 4H), 3.76 (t, 4H). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 28.02, 51.58, 61.04, 79.72, 155.93. IR (CCl_4) 1669, 2977, 3385 cm^{-1}. HRMS calculated for $C_9H_{19}NO_4$ was 205.1315, found 205.1319. Next, *N*-BOC diethanolamine (5.0 g, 24.4 mmol), DMAP (300 mg, 2.43 mmol), and Et_3N (3.4 mL, 24.36 mmol) were dissolved in CH_2Cl_2 (49 mL). The mixture was cooled to 0°C and benzylchloroformate (3.5 mL, 24.4 mmol) was slowly added. The reaction was stirred at room temperature for 18 h. The reaction was washed with brine, extracted with CH_2Cl_2, dried over $MgSO_4$, filtered and concentrated. Chromatography (25% EtOAc/hexanes) gave 4.44 g of 5 (54%) as an oil: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.43 (s, 9H), 3.39 (m, 2H), 3.51 (m, 2H), 3.66 (m, 2H), 4.25 (m, 2H), 5.17 (s, 2H), 7.34 (m, 5H). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 27.85, 46.98, 50.46, 60.79, 65.81, 69.12, 79.71, 127.74, 129.10, 134.88, 154.58, 155.51. IR (CCl_4) 1693, 1747, 2975, 3450 cm^{-1}. Anal. Calcd. for $C_{17}H_{25}NO_6$: C, 60.16; H, 7.42; N, 4.13. Found: C, 59.82; H, 7.44; N, 4.03.

1-(5-*O*-Benzyloxycarbonyl-3-aza-(*N*-tert-butoxycarbonyl)-pentyl)-N^3-benzoyl thymine (6). A solution of **5** (4.3 g, 12.6 mmol), N^3-benzoyl thymine[9] (2.9 g, 12.6 mmol), and Ph_3P (4.3 g, 16.4 mmol) in THF (79 mL) was cooled to 0°C and diethylazodicarboxylate (2.6 mL, 16.4 mmol) was slowly added. The reaction was stirred at room temperature for 2.5 h. Concentration followed by chromatography (first in 30% iPrOH/hexanes then 1% MeOH/$CHCl_3$) gave 5.62 g of **6** (65%) as an oil: ^{1}H NMR ($CDCl_3$) δ 1.39 (s, 9 H), 1.77 (s, 3 H), 3.45 (m, 4H), 3.74 (m, 2H), 4.22 (m, 2H), 5.13 (s, 2H), 7.03 (s, 1H), 7.32 (m, 5H), 7.42 (t, 2H), 7.57 (t, 1H), 7.85 (m, 1H), 8.08 (d, 1H). ^{13}C NMR ($CDCl_3$) δ 11.94, 28.08, 46.25, 47.29, 65.86, 69.64, 80.54, 109.73, 128.14, 128.42, 128.82, 130.36, 131.74, 134.58, 134.92, 140.53, 149.71, 154.68, 155.14, 163.06, 169.05. IR (CCl_4) 1651, 1694, 1747, 2977, 3482 cm^{-1}. UV (EtOH) $\lambda_{max} = 251.0$ FABMS (M+H) calculated for $C_{29}H_{33}N_3O_8$ was 551.2377, found 552.2360.

N-Allyl δ-valerolactam (8). KH (9.85 g of a 30% suspension in oil, 150 mmol) was suspended in THF (181 mL). In a second flask, δ-valerolactam (4.95 g, 50 mmol) was dissolved in THF (61 mL). This solution was slowly added to the KH suspension and stirred for 2 h. The reaction was cooled to 0°C and freshly distilled allyl bromide (5.25 mL, 60 mmol) was slowly added. The reaction was stirred for an additional 20 h after which time it was diluted with a 6:1 solution of EtOAc:MeOH. The solution was further diluted with H_2O and extracted with EtOAc. Column chromatography (10% EtOAc/hexanes→5% MeOH/$CHCl_3$) gave 6.21 g (89%) of **8** as a yellow oil. ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.70 (m, 4H), 2.31 (t, 2H), 3.15 (t, 2H), 3.88 (d, 2H), 5.14 (m, 2H), 5.65 (m, 1H). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 21.0, 22.8, 31.7, 46.8, 48.8, 116.5, 132.6, 166.8. IR (CCl_4) 1637.3 cm^{-1}. HRMS calculated for $C_8H_{13}NO$ was 139.0998 found 139.0995.

Benzyl N-allyl-N-*tert*-butoxycarbonyl-5-aminopentanoate (9). Lactam **8** (5.3 g, 38 mmol) was refluxed in 7M HCl (38 mL) for 24 h. The crude material was concentrated and suspended in benzene (38 mL). To this suspension, *p*-toluenesulfonic acid (9.0 g, 47.5 mmol) and benzyl alcohol (25.0 mL, 241.7 mmol) were added. The solution was refluxed for 24 h, after which time it was extracted with ether, and the aqueous phase concentrated. The crude concentrate was resuspended in CH_2Cl_2 (38 mL) and di-*tert*-butyl dicarbonate (12.4 g, 57 mmol) was added. The solution was cooled to 0°C for addition of Et_3N (21.2 mL, 152.1 mmol). The reaction was stirred at room temperature for 24 h after which time additional di-*tert*-butyl dicarbonate (4.1 g, 19 mmol) was added. The reaction was stirred for another 24 h and washed with H_2O. Concentration of the organic phase followed by column chromatography (10% EtOAc/hexanes) gave 8.2 g (62%) of **9** as a colorless oil. ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.45 (s, 9H), 1.59 (m, 4H), 2.36 (t, 2H), 3.16 (t, 2H), 3.76 (t, 2H), 5.05 (m, 4H), 5.71 (m, 1H), 7.31 (m, 5H). 13C NMR ($CDCl_3$, 62.5 MHz) δ 21.9, 27.5, 28.1, 33.6, 45.8, 49.2, 65.7, 115.8, 127.8, 128.2, 134.1, 135.9, 155.1, 172.6.

IR (CCl_4) 1162, 1247, 1693, 1736, 2975 cm^{-1}. Anal Calcd. for $C_{20}H_{29}NO_4$: C, 69.13; H, 8.41; N, 4.03. Found C, 69.23; H, 8.52; N, 4.00.

Benzyl-N-*tert*-butoxycarbonyl-N-(2-hydroxyethyl)-5-aminopentanoate (10). Olefin **9** (7.0 g, 20.2 mmol) was dissolved in CH_2Cl_2 (20 mL) and THF (80 mL) and cooled to $-78°C$. O_3 was bubbled through the solution for 45 min followed by N_2 purging for an additional 45 minutes. Next, $NaBH_4$ (0.76 g, 20.2 mmol) was added and the reaction was stirred 1 h after which time an additional portion of $NaBH_4$ (1.53 g, 40.3 mmol) was added. The reaction was warmed to room temperature, stirred for 1.5 h, and partitioned between 1 M HCl and EtOAc. Column chromatography (50% EtOAc/hexanes) gave 5.7 g (79%) of **10** as a colorless oil. ^1H NMR ($CDCl_3$, 250 MHz) δ 1.45 (s, 9H), 1.59 (m, 4H), 2.36 (t, 2H), 3.25 (m, 4H), 3.66 (m, 2H), 5.05 (s, 2H), 7.29 (m, 5H). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 22.1, 27.9, 28.3. 29.7, 33.8, 48.0, 50.0, 62.0, 66.1, 128.1, 128.5, 136.1, 156.7, 173.2. IR (CCl_4) 1167, 1366, 1693, 1736, 2974, 3445 cm^{-1}. Anal Calcd. for $C_{19}H_{29}NO_5$: C, 64.94; H, 8.32; N, 3.99. Found C, 65.32; H, 8.53; N, 3.83.

Benzyl-N-(*tert*-butoxycarbonyl)-N-(ethyl-2-(N^3-benzoyl thymine)-5-aminopentanoate (11). A solution of **10** (1.3 g, 3.8 mmol), N^3-benzoyl thymine[9] (0.86 g, 3.8 mmol), and Ph_3P (1.30 g, 4.9 mmol) in THF (79 mL) was cooled to 0°C and diethylazodicarboxylate (0.77 mL, 4.9 mmol) was slowly added. The reaction was stirred at room temperature for 2.5 h. Concentration followed by chromatography (50% EtOAc/hexanes) gave 1.81 g of **11** (85%) as an oil: ^1H NMR ($CDCl_3$, 250 MHz) δ 1.53 (s,m 13H), 1.88 (s, 3H), 2.35 (t, 2H), 3.18 (t, 2H), 3.43 (t, 2H), 5.09 (s, 5H), 7.01 (s, 1H), 7.31 (m, 5H), 7.46 (t, 2H), 7.61 (t, 1H), 8.02 (d, 1H). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 11.9, 21.8, 27.7, 28.1, 33.5, 44.8, 47.0, 65.8, 109.6, 127.9, 128.3, 128.8, 130.2, 131.9, 134.5, 135.9, 140.6, 149.6, 155.3, 163.0, 168.9, 172.6. IR (CCl_4) 1155, 11,249, 1656, 1697, 1739, 2958 cm^{-1}. UV (EtOH) $\lambda_{max} = 251.5$ Anal Calcd. for $C_{31}H_{37}N_3O_7$: C, 66.06; H, 6.62; N, 7.45. Found C, 65.79; H, 6.45; N, 7.40.

1-(5-*O*-Benzyloxycarbonyl-3-aza-pentyl)-N^3-benzoyl thymine hydrochloride (12). A solution of **6** (0.55 g, 1 mmol) in EtOAc (10 mL) was added to anhydrous HCl (10 mmol, prepared by adding acetyl chloride (0.71 mL, 10 mmol) to anhydrous MeOH (0.41 mL, 10 mmol) in EtOAc (2 mL)) at 0°C. The ice bath was removed and the mixture was stirred at room temperature for 18 h. Concentration gave 0.38 g (78%) of crude **12** as a yellow solid: ^1H NMR (DMSO-d_6, 250 MHz) δ 1.83 (s, 3H), 3.29 (m, 7H), 4.07 (m, 2H), 4.40 (m, 2H), 5.16 (s, 2H), 7.38 (m, 5H), 7.55 (m, 2H), 7.76 (m, 2H), 8.03 (d, 2H), 9.35 (br s, 1H).

N-(*tert*-butoxycarbonyl)-N-(ethyl-2-(N^3-benzoyl thymine)-5-aminopentanoic acid (13). To benzyl ester **11** (0.65 g, 1.15 mmol) was added 5% $Pd(OH)_2$/C (0.03 g) and THF (4 mL). The reaction stirred under an atmosphere of H_2

(balloon) for 18 h. The reaction was filtered through celite with EtOAc and extracted using sat. aq. $NaHCO_3$. The aqueous layer was acidified with 1 M HCl to pH 3 and then extracted with EtOAc. Concentration of the organic phase gave 0.49 g (90%) of **13** as a white solid. ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.48 (s,m 13H), 1.82 (s, 3H), 2.39 (m, 2H), 3.12 (m, 2H), 3.39 (m, 2H), 3.75 (m, 2H), 7.06(s, 1H), 7.42 (m, 2H), 7.86 (m, 1H), 7.99 (m, 1H). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 11.9, 21.6, 27.7, 28.1, 33.2, 45.0, 47.0, 80.2, 109.7, 128.8, 130.3, 131.6, 134.7, 140.8, 149.7, 155.5, 163.1, 168.9, 177.6. IR (CCl_4) 1161, 1249, 1436, 1654, 1697, 1747, 2976 cm^{-1}. UV (EtOH) $\lambda_{max} = 254.5$. HRMS (M+Na) calculated for $C_{24}H_{31}N_3O_7$•Na was 496.2060 found 496.2027.

APN Dimer (14). To a cold (0°C) suspension of amine hydrochloride **12** (0.38 g, 0.77 mmol) in CH_3CN (2 mL), was added iPr_2EtN (0.27 mL, 1.54 mmol). This solution was added to a solution of acid **13** (0.37 g, 0.77 mmol) and TBTU (0.25 g, 0.77 mmol) in CH_3CN (2 mL). The mixture was stirred at room temperature for 24 h. Concentration followed by chromatography (1% MeOH/$CHCl_3$) gave 0.47 g (67%) of fully protected **15** as a white solid: mp 91–93°C ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.42 (m, 13H), 1.73 (m, 2H), 1.89 (m, 5H), 2.29 (m, 2H), 3.15 (m, 2H), 3.48 (m, m, 6H), 3.83 (m, 4H), 4.25 (m, m, 2H), 5.12 (m, 2H), 6.97 (m, 1H), 7.11 (s, 1H), 7.34 (m, 5H), 7.44 (m, 4H), 7.60 (m, 2H), 7.91 (m, 3H). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 0.4, 12.5, 12.7, 22.4, 23.1, 28.8, 32.8, 45.5, 45.9, 46.8, 47.1, 47.6, 65.5, 66.8, 70.5, 80.6, 111.2, 111.6, 128.8, 128.9, 129.07, 129.14, 129.2, 129.5, 129.6, 130.8, 130.9, 132.0, 135.3, 135.4, 135.6, 140.6, 140.9, 150.3, 150.5, 155.2, 163.6, 169.4, 173.9 IR (CCl_4) 1162, 1257, 1437, 1653, 1698, 1746, 2955, 3067 cm^{-1}. UV (EtOH) $\lambda_{max} = 252.5$. FABMS (M+Na) calculated for $C_{48}H_{54}N_6O_{12}$•Na was 929.3699, found 929.3693. The fully protected APN dimer (0.34 g, 0.37 mmol) was dissolved in dioxane (14.8 mL), and conc. aq. NH_4OH (37 mL, 28–30%) was slowly added. The reaction was stirred at room temperature for 45 min and concentrated. Chromatography (5% MeOH/$CHCl_3$→10% MeOH/$CHCl_3$) resulted in 0.21 g. The crude material was dissolved in CH_2Cl_2 (1 mL) and TFA (0.34 mL, 4.4 mmol) was added at 0°C. The reaction was stirred at room temperature for 3 h. Concentration gave 0.19 g (82%) of **14** as a yellow solid: mp 91–110°C (decomposes) ^{1}H NMR (D_2O, 400 MHz, 85°C) δ 1.59 (m, 5H), 1.82 (m, 6H), 2.37 (m, 2H), 3.01 (m, 2H), 3.40 (m, 4H), 3.64 (m, 4H), 4.02 (m, 1H), 7.37 (m, 2H). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 11.6, 11.7, 22.0, 25.5, 32.3, 43.9, 45.4, 46.6, 46.8, 47.3, 48.0, 49.3, 59.3, 62.7, 110.7, 112.0, 142.6, 143.6, 152.7, 153.2, 167.3, 176.6. FABMS (M+Na) calculated for $C_{21}H_{32}N_6O_6$•Na was 487.2283, found 487.2299.

ACKNOWLEDGMENTS

We would like to acknowledge support of this work by the American Cancer Society, Ohio Division, Inc.

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Received June 12, 2001
Accepted November 20, 2001