ORGANIC LETTERS

2001
Vol. 3, No. 9
1281−1284

Aminoethylprolyl (*aep*) PNA: Mixed Purine/Pyrimidine Oligomers and Binding Orientation Preferences for PNA:DNA Duplex Formation

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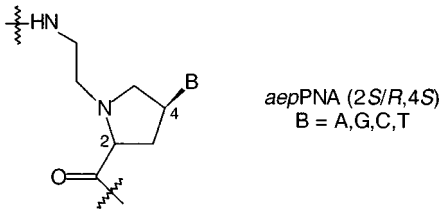
Received January 11, 2001

ABSTRACT



The synthesis of (2*S*,4*S*)- and (2*R*,4*S*)-*aep*PNA monomers of adenine, guanine, and cytosine (3–5) and their incorporation at appropriate positions into *aeg*PNA sequence 7 leads to mixed *aeg-aep* backbone/mixed nucleobase PNAs 8–11. The thermal stabilities of the derived duplexes with DNA are found to be dependent on nucleobase and backbone stereochemistry.

Peptide nucleic acids wherein the nucleobases A, G, C, and T are linked to acyclic, achiral *N*-(2-aminoethyl)glycyl (*aeg*PNA) backbone through a methylene carbonyl linker have emerged as successful DNA mimics.[1] Several analogues and derivatives of *aeg*PNA[2] have been synthesized to overcome the limitations of PNA such as poor aqueous solubility and orientational ambiguity in complementary DNA recognition by making them cationic and chiral. Recent attempts toward designing newer PNA analogues are based on constraining the flexibility of the acyclic backbone to pre-organize the PNA structure for favorable and selective DNA/RNA recognition. These have resulted in a novel class of PNA analogues (Figure 1) derived from cyclic, chiral monomers of either neutral prolyl residues[3] or the cationic pyrrolidine core.[4,5] The two chiral centers of the five-membered pyrrolidine ring along with the different possible ring substitutions in such monomers lead to a combinatorial library of regio- and stereoisomers with the derived PNA oligomers possessing wide conformational diversity. The foremost analogue in this series, the prolyl PNA,[3] was derived by the introduction of a methylene bridge between the β-C of ethylenediamine unit with the α'-C of the glycyl unit on the PNA backbone. These marginally improved the affinity and selectivity in DNA:PNA binding, but were found to be less soluble in aqueous medium. The replacement of the glycyl segment of the PNA backbone by a prolyl unit to which the nucleobase is directly linked led to the cationic and chiral aminoethylprolyl PNAs (*aep*PNA).[4] The mixed backbone *aeg-aep*PNA T_8 oligomers derived from either

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10.1021/ol015546k CCC: $20.00
Published on Web 04/04/2001

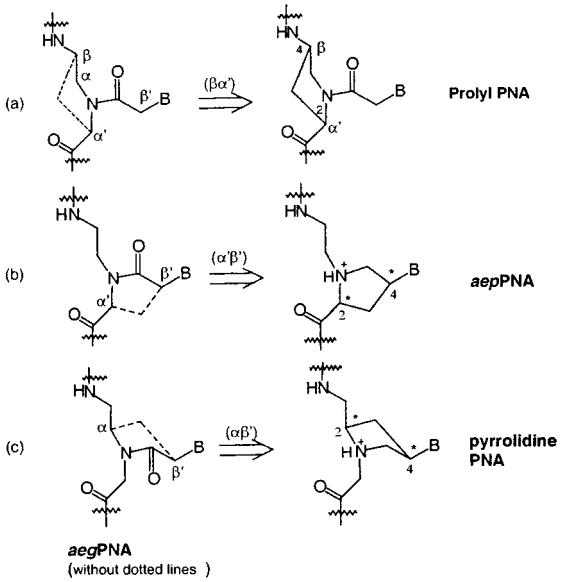


Figure 1. Strategies for the design of conformationally constrained PNA analogues.

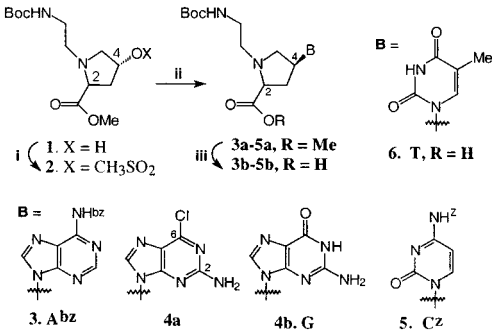
(2*R*,4*S*) or (2*S*,4*S*) backbone stereochemistry exhibited high affinity and sequence specificity to form DNA:PNA_2 triplexes. Further, in such homopyrimidine *aep*PNA oligomers, the nature of the backbone chirality was inconsequential in differentiating the binding of complementary DNA in both parallel (HG) and antiparallel (WC) orientations.[6]

In view of the above results, it was thought worthwhile to examine the effect of *aep* backbone chirality on the binding orientation in duplexes with mixed purine/pyrimidine sequences. This and the combined characteristics of high sequence specificity, affinity, improved aqueous solubility, intrinsic positive charge, and the ease of synthesis of *aep*PNA prompted us to construct *aeg*-*aep*PNA oligomers comprised of pyrimidine-purine units and study preferences in duplexation with DNA. We herein report the synthesis of (2*S*,4*S*)- and (2*R*,4*S*)-*aep*PNA monomers of cytosine, adenine, and guanine (**3**−**5**) and their incorporation, along with thymine[4] **6**, in a control PNA sequence **7** at appropriate positions to generate *aeg*-*aep*PNAs **8**−**11**. Preliminary UV−T_m studies of their binding with antiparallel and parallel complementary DNA sequences (**12**, **13**) reveal interesting stereochemical and orientation preferences in PNA:DNA duplex formation.

Synthesis of *aep*PNA Monomers and *aeg*-*aep*PNAs. The (2*S*,4S)-*aep*-PNA monomers **3**−**5** were synthesized (Scheme 1) by N-alkylation of protected nucleobases A^{bz}, G^{iBu}, and C^{Z} with the common intermediate 1-(*N*-Boc-aminoethyl)-4(*R*)-*O*-mesyl-2(*S*)-proline methyl ester **2**. This was obtained from mesylation of the known (2*S*,4*R*) 4-hydroxyl compound[4] **1** (45% yield). While A^{bz} and C^{Z} gave the expected products **3a** and **5a** in 65% and 60% yield, respectively, G^{iBu} gave a mixture of N7:N9 alkylated products in a 5:1 ratio

(6) parallel (*p*): N-end of PNA facing the 5′-end of DNA in the complex, antiparallel (*ap*): C-end of PNA facing the 5′-end of DNA.

Scheme 1[a]



[a] Reagent: (i) methanesulfonyl chloride, pyridine; (ii) B (A^{bz}/2-amino-6-chloropurine/C^{z}), K_2CO_3, DMF; (iii) NaOH, CH_3OH, H_2O.

with the undesired N7 isomer in higher proportion. To overcome this, **2** was alkylated with 2-amino-6-chloropurine, which gave the N9 isomer **4a** as a major product (90%). The (2*R*,4*S*)-*aep*PNA monomers were likewise synthesized from the diastereomer **1**(2*R*,4*R*) with similar yields. The hydrolysis of the methyl esters was effected with 1 N NaOH in aqueous MeOH to get the desired PNA monomer synthons **3b**−**5b** (2*R*/*S*,4*S*) in 95% yield. These and the corresponding T monomer[4] **6** were individually incorporated into the PNA sequences **8**−**11** by solid-phase synthesis using the standard Boc-chemistry as previously reported.[4] The unmodified PNA **7** and DNA **12**, **13** sequences required for constituting the duplexes were synthesized by standard procedures.

All the modified PNA monomers were characterized by ^1H and ^{13}C NMR and mass spectrometry.[7] The PNA oligomers were purified by C18 reversed-phase HPLC and

(7) Characteristic signals. **3a** (2*S*,4*S*): ^1H NMR ($CDCl_3$) δ 9.27 (br s, 1H, Benzamide-N*H*), 8.76 (s, 1H, *A*-*H*2), 8.70 (s, 1H, *A*-*H*8), 8.20 (m, 2H, Bz, *o*-C*H*), 7.56 (m, 3H, Bz, *p*-C*H*, *m*-C*H*), 5.40 (m, 1H, *H*4), 5.15 (m, 1H, Boc-N*H*), 3.74 (s, 3H, OCH_3), 3.60−2.60 (m, 9H, *H*5, *H*5′, *H*3, *H*3′, *H*2, Boc-NH-(CH_2)$_2$), 1.45 (s, 9H, C(CH_3)$_3$); $[\alpha]^{25}_D$ = +12° (*c* = 0.1, CH_3OH). **3a** (2*R*,4*S*): ^1H NMR ($CDCl_3$) δ 9.27 (br s, 1H, benzamide-N*H*), 8.76 (s, 1H, *A*-*H*2), 8.10 (s, 1H, *A*-*H*8), 8.00 (d, 2H, Bz, *o*-C*H*), 7.56 (t, 1H, Bz, *p*-C*H*), 7.45 (t, 2H, Bz, *m*-C*H*), 5.27 (m, 2H, *H*4, Boc-N*H*), 3.93 (m, 1H, *H*2), 3.74 (s, 3H, OCH_3), 3.60 (m, 2H, *H*5, *H*5′), 3.20 (m, 2H, *H*3, *H*3′), 2.83 (m, 2H, Boc-NH-CH_2), 2.60 (m, 2H, Boc-NH-CH_2CH_2), 1.42 (s, 9H, C(CH_3)$_3$); $[\alpha]^{25}_D$= +20.2° (*c* = 0.1, CH_3OH); MALDI-TOF M^+= 509.6. **4a** (2*S*,4*S*): ^1H NMR ($CDCl_3$) δ 8.35 (s, 1H, 2-amino-6-chloropurine, *H*8), 5.20 (br s, 2H, N^2H_2), 5.10 (m, 2H, *H*4, Boc-N*H*), 3.75 (s, 3H, OCH_3), 3.30 (m, 4H, *H*5, *H*5′, Boc-NH-CH_2), 2.90 (m, 3H, *H*2, Boc-NHCH_2CH_2), 2.70 (m, 1H, *H*3), 2.15 (m, 1H, *H*3′), 1.45 (s, 9H, C(CH_3)$_3$); $[\alpha]^{25}_D$ = −24° (*c* = 0.1, CH_3OH). **4a** (2*R*,4*S*): ^1H NMR ($CDCl_3$) δ 7.85 (s, 1H, 2-amino-6-chloropurine, *H*8), 5.20 (m, 4H, N^2H_2, *H*4, N*H*), 3.92 (t, 1H, *H*2), 3.76 (s, 3H, OCH_3), 3.60−3.00(br m, 4H, *H*5, *H*5′, *H*3, *H*3′), 2.84 (t, 2H, Boc-NH-CH_2), 2.56(t, 2H, Boc-NH-CH_2CH_2), 1.46 (s, 9H, C(CH_3)$_3$); $[\alpha]^{25}_D$ = −4.8° (*c* = 0.19, CH_3OH); MALDI-TOF M + H = 441.6. **5a** (2*S*,4*S*): ^1H NMR ($CDCl_3$) δ 8.45 (d, 1H, *C*-*H*6), 7.40 (s, 5H, Ph), 7.25 (d, 1H, *C*-*H*5), 5.35 (t, 1H, Boc-N*H*), 5.20 (s, 3H, Ph-CH_2, *H*4), 3.75 (s, 3H, OCH_3), 3.20 (br m, 4H, *H*5, *H*5′, Boc-NH-CH_2), 2.80 (m, 2H, Boc-NH-CH_2-CH_2), 2.60 (m, 1H, *H*2), 1.90 (m, 2H, *H*3, *H*3′), 1.45 (s, 9H, C(CH_3)$_3$); $[\alpha]^{25}_D$ = +9.6° (*c* = 0.3, CH_3OH). **5a** (2*R*,4*S*): ^1H NMR ($CDCl_3$) δ 7.90 (d, 1H, *C*-*H*6), 7.35 (s, 5H, Ph), 7.25 (d, 1H, *C*-*H*5), 5.40 (m, 1H, Boc-N*H*), 5.20 (s, 2H, Ph-CH_2), 4.90 (m, 1H, *H*4), 3.95 (dd, 1H, *H*5), 3.74 (s, 3H, OCH_3), 3.40 (m, 1H, *H*5′), 3.25 (m, 2H, Boc-NH-CH_2), 2.95 (m, 1H, *H*2), 2.77 (m, 2H, Boc-NH-CH_2CH_2), 2.60 (m, 1H, *H*3), 2.15 (m, 1H, *H*3′), 1.45 (s, 9H, C(CH_3)$_3$); $[\alpha]^{25}_D$ = +24.2° (*c* = 0.1, CH_3OH); MALDI-TOF M + H = 517.7. PNA **8**, **9**, **10**, **11:** (MALDI-TOF) M_{calc}=2796, M_{obs}=2796.

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characterized by MALDI-TOF mass analysis. The mixed PNA sequences **8**−**11** constituted by systematic replacement of specific *aeg* units in control *aeg*PNA **7** by appropriate *aep* unit were synthesized to test the explicit effect of each nucleobase in (2*S*/*R*) *aep* backbone on duplex formation (Scheme 2).

Scheme 2. DNA and PNA Sequences[a]

PNA-7	*H*-GTA GAT CAC T-*NH(CH$_2$)$_2$COOH*
PNA-8	*H*-GT**a** GAT CAC T-*NH(CH$_2$)$_2$COOH*
PNA-9	*H*-GTA GA**t** CAC T-*NH(CH$_2$)$_2$COOH*
PNA-10	*H*-GTA **g**AT CAC T-*NH(CH$_2$)$_2$COOH*
PNA-11	*H*-GTA GAT **c**AC T-*NH(CH$_2$)$_2$COOH*
DNA-12	5'- *AGT GAT CTA C* -3' (*ap*)
DNA-13	5'- *CAT CTA GTG A* -3' (*p*)

[a] **a**, **t**, **g**, and **c** represent (2*S*/*R*,4*S*)-*aep*PNA units.

UV−T_m and CD Studies of PNA:DNA Duplexes. The UV−T_m data of complexes of **8**−**11** with DNA in both orientations (**12**, antiparallel; **13**, parallel) obtained as a measure of duplex stability are summarized in Table 1 and

Table 1. UV−T_m (°C) of PNA:DNA Duplexes[a]

	DNA **12** antiparallel		DNA **13** parallel	
PNA	(2*S*,4*S*)	(2*R*,4*S*)	(2*S*,4*S*)	(2*R*,4*S*)
PNA **7**	43.8		40.3	
PNA **8** (**a**)	53.8	53.1	50.2	50.2
PNA **9** (**t**)	56.6	33.0[b]	34.0[b]	26.2[b]
PNA **10** (**g**)	43.2	53.1	58.3	28.3
PNA **11** (**c**)	55.2[b]	62.3[b]	31.2[b]	34.0[b]

[a] Buffer: 10 mM sodium phosphate, pH 7.4. Tm values are accurate to (±1 °C). Experiments were repeated at least three times, and the T_m values were obtained from the peaks in the first derivative plots. [b] Low hyperchromicity (~6%), for others 9−12%.

shown in Figure 2. In the unmodified *aeg*PNA **7**, the antiparallel duplex was more stable than the parallel duplex by 3.5°. The *aeg*-*aep*PNAs **8**, **9**, and **11** having (2*S*,4*S*)-*aep*-T, A, and C units uniformly stabilized the antiparallel duplexes (DNA **12**) by 10−13°/unit compared to the control PNA **7**. The complex formed by *aep*PNA-G **10**(2*S*,4*S*) was as good as that of the control. The (2*S*,4*S*)-*aep* purine A/G units (**8**/**10**) also stabilized the parallel PNA:DNA duplexes (DNA **13**), whereas the corresponding PNAs with T/C units (**9**/**11**) destabilized the parallel duplexes. In the other stereochemistry (2*R*,4*S*), the *aep*PNAs show base related variations in both parallel and antiparallel orientations; the *aep* units with A/G/C stabilized the antiparallel duplexes (PNA**8**/**10**/**11**:DNA **12**) and T *aep*PNA **9** had detrimental effects in both parallel and antiparallel orientations. The effect of stereochemistry on stability was most prominent in case of guanine *aep*PNA **10**, where the (2*S*,4*S*) isomer preferred a parallel orientation ($\Delta T_{m(p-ap)}$ +15°) and (2*R*,4*S*) isomer was more stable in the antiparallel orientation ($\Delta T_{m(ap-p)}$ +24°).

To examine whether the observed higher stabilities of *aeg*-*aep*PNAs were accompanied by any loss in base specificity, UV−T_m's were determined for hybrids having single base mismatches on the antiparallel DNA sequences at the site complementary to the *aep*PNA unit. While any single mismatch in the control PNA lowered the duplex T_m by 4−8°, the mismatches in *aep*-PNA induced larger destabilization in the range 9−30° (Table 2). The mismatch

Table 2. UV−T_m (°C) of Mismatched PNA:DNA Duplexes[a]

DNA (5′→ 3′)	*aeg*PNA **7**	*aep*PNA
14 AGT GAT CCA C	35.4 (−8.4)	**8** (**a**), 44.4 (−9.4)
15 AGT G*T*T CTA C	36.8 (−7.0)	**9** (**t**), 26.9 (−29.7)
16 AGT GAT *A*TA C	39.6 (−4.2)	**10** (**g**), 24.7 (−18.5)
17 AGT *T*AT CTA C	36.8 (−7.0)	**11** (**c**), 43.6 (−11.6)

[a] Buffer: 10 mM sodium phosphate, pH 7.4. The mismatch data reported above are for the (2*S*,4*S*)-*aep*PNA units, **a**, **t**, **g**, and **c** respectively. Values in parentheses indicate the ΔT_m values compared to the fully matched PNA:DNA **12** complexes.

effects followed the destabilization order **t**:*T* (**9**:**15**) > **g**:*A* (**10**:**16**) > **c**:*T* (**11**:**17**) > **a**:*C*(**8**:**14**) and the results indicate that the higher affinity of *aeg*-*aep*PNA for complementary DNA was achieved without any loss of selectivity.

The magnitude of percent hyperchromicity changes in the melting transitions was also base dependent. The purine substitutions exhibited hyperchromicity changes (9−12%) as good as the control *aeg*PNAs (11−13%) and higher than pyrimidines (6−9%). The parallel and antiparallel PNA:DNA

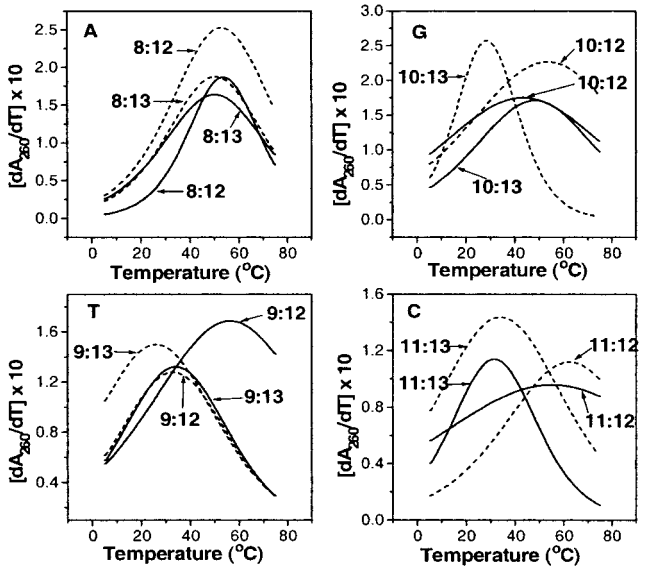

Figure 2. UV−T_m first derivative plots of *aeg*-*aep*PNA:DNA duplexes. (2*S*,4*S*)-PNA (—); (2*R*,4*S*)-PNA (- - -). Melting experiments were performed with a PNA:DNA strand concentration of 0.5 μM each. The concentrations were calculated on the basis of the absorbance at 260 nm using the molar extinction coefficients of A, T, G, and C as for DNA.[8] *Y*-axis data is derived from change in percent hyperchromicity with temperature.

duplexes are expected to differ in the base stacking patterns, and this should be reflected in their circular dichroic (CD) spectra. Figure 3 shows the CD profiles of control *aeg*PNA and selected *aeg-aep*PNAs in parallel and antiparallel orientations.

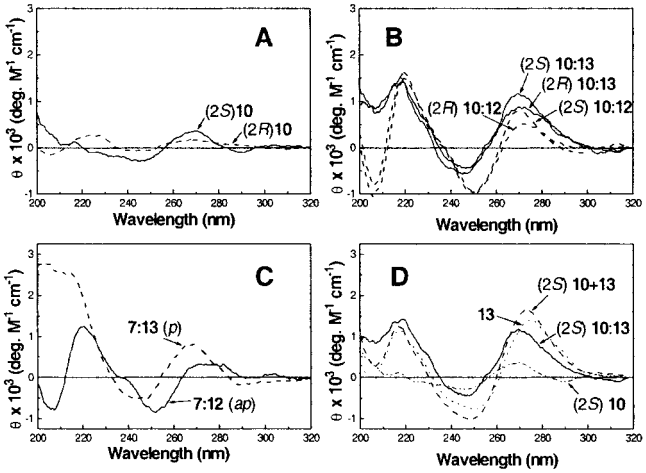

Figure 3. CD profiles of (A) PNA **10** single strands, (B) Selected *aeg-aep*PNA:DNA hybrids, (C) *aeg*PNA:DNA hybrids, and (D) observed (—) and addition (- - -) spectra of PNA **10** and DNA **13**. Each strand was taken in a concentration of 1 μM, and each CD spectrum was recorded as an accumulation of eight scans.

Considerable differences were seen in CD signals of the parallel and antiparallel duplexes, such as the wavelengths and amplitudes of the positive/negative bands in both 210−230 nm and the 240−280 nm regions and the crossover wavelength in 240−280 nm region. These differential features were preserved in the parallel and antiparallel duplexes from *aeg-aep*PNAs. The observed CD effects arise from duplexation since the experimental CD profiles differ from that of the sum of the components. The slightly enhanced amplitudes of both positive and negative bands suggests an improved base stacking in the modified PNAs, but with overall retention of conformational integrity. The backbone configuration of (2*S*,4*S*/*2R*,4*S*)*aep* units did not have any dramatic effect on the geometry of either parallel or antiparallel PNA:DNA complexes and the CD patterns of the duplexes were similar to those of parallel/antiparallel complexes of achiral PNA and DNA. This is perhaps due to the fact that CD of PNA:DNA complexes are dominated by DNA contributions.

The overall results indicate that single *aep* modifications are satisfactorily accommodated in the *aeg* PNA backbones leading to significant thermal stabilization effects. The stereochemistry at C2 has considerable effect on the stability of hybrids since *R* isomers except **9** lowered the duplex stability (with DNA **13**). The fact that *aep* modifications in mixed sequence duplexes exhibit stabilization effects depending on the nucleobase and orientation suggests that the positive charge on the pyrrolidine may not be entirely responsible for the properties of *aep*PNA.

The pyrrolidine ring pucker and/or syn-anti orientation of the nucleobases directly attached to the ring perhaps dictate the individual parallel/antiparallel preferences seen for the nucleobases on chiral *aep* units. It is known that the nature of the 4-substituent plays an important role in defining the pucker of the pyrrolidine ring in 4-substituted prolines.[9] The individual purines or pyrimidines present at the 4-position in *aep*PNA monomers may cause differential pyrrolidine ring puckers and consequent backbone conformational changes. The stereochemistry at C4 of the proline ring (4*S*) to which the nucleobase is attached is same as the spatial disposition in natural DNA.[4]

In summary, the incorporation of single (2*S*,4*S*)-*aep*-A/T/G/C units in **7** stabilize the antiparallel duplexes with DNA. The individual nucleobases in (2*R*,4*S*)-*aep*PNA display interesting binding preferences and affinity with complementary DNA. In view of these interesting results, it emerges that mixed purine-pyrimidine sequences with a homogeneous (2*S*,4*S*) *aep* backbone may be appropriate to study the cumulative effects on the stability of PNA:DNA duplexes. Such studies are currently in progress.

Acknowledgment. M.D. thanks CSIR, New Delhi, for a research fellowship. V.A.K. and K.N.G. thank the Department of Biotechnology, New Delhi, for a research grant.

Supporting Information Available: Mass spectra for key compounds (2*S*,4*S*)-**3a**−**5a**, HPLC profiles, MALDI-TOF mass spectra of (2*S*,4*S*)-PNAs **8**−**11** and UV−T_m plots of *aeg-aep*PNA:DNA duplexes. This material is available free of charge via the Internet at http://pubs.acs.org.

OL015546K

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