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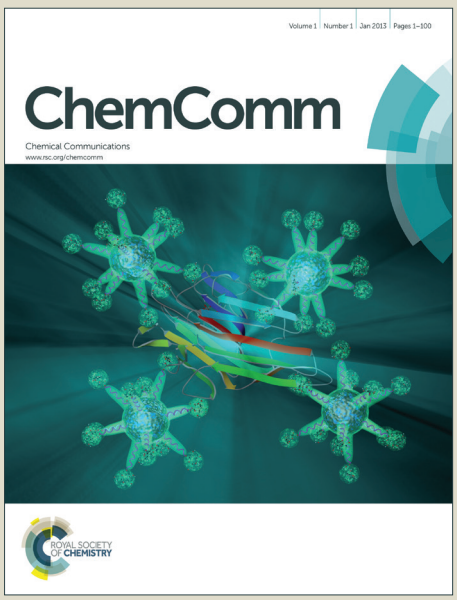

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ARTICLE TYPE

β,γ-bis-substituted PNA with configurational and conformational switch: Preferred binding to cDNA/RNA and cell-uptake studies

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The (*S,S*)- and (*R,R*)- β,γ-bis-substituted PNAs were synthesized from *C*-2 symmetric vicinal diamine system embedded in 1,4 dihydroxybutane and 1,4-dimethoxybutane scaffold. The (*R,R*)–β,γ-bis-methoxymethyl-PNA derived from D- tartaric acid was found to be in the right configuration and conformation to be an excellent mimic of PNA, endowed with superior ability to enter in cells.

The peptide nucleic acids (aegPNA, Figure 1), designed by Nielsen et al, are known for their superior properties such as strong and sequence specific binding to both DNA and RNA following Watson-Crick base pairing rules.[1-3] In aegPNA, the anionic sugar-phosphate- backbone of DNA is replaced by repeating *N*-(2-aminoethyl)glycyl units, carrying adenine, guanine, cytosine and thymine nucleobases *via* methylene carbonyl linkages.[2]

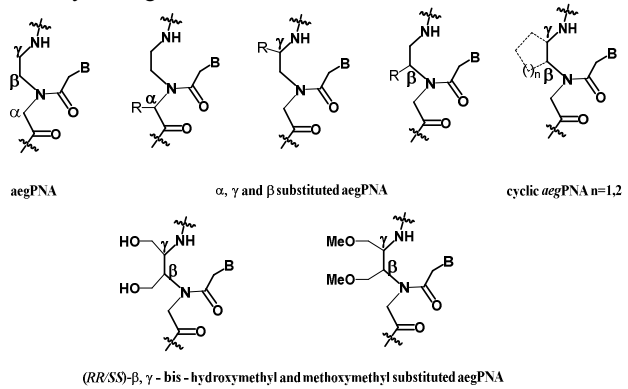
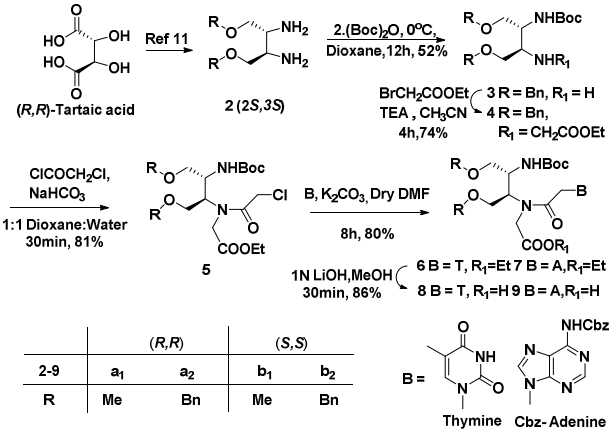


Figure 1: aegPNA, substituted PNA modifications and the proposed β,γ- bis-PNA analogues

Unfortunately, PNA falls short of some crucial properties for biological applications.[1,2] The desired additional attributes for PNA are sufficient aqueous solubility,[3a] efficient cellular uptake[3b] and the ease of synthesis of modified PNAs, while maintaining the specificity of binding to complementary RNA sequences. The substitutions of aegPNA, at α, β or γ positions (Figure 1) have been studied earlier to fulfill some of these additional attributes. The use of D/L-amino acids instead of glycine resulted in α-PNA[4] and α-GPNA[5], substitution of γ-position in aminoethyl segment of aegPNA generated γPNA[6a] and γGPNA[6b]. Increased binding strength was observed in the case of positively charged PNAs depending upon the stereochemistry in the backbone and also the position of the substitution. The L-γ-GPNA exhibited higher T_m, whereas D-α-GPNA exhibited higher sequence selectivity.[7] Substitution of PNA at both α- and γ- positions were also explored that gave excellent improvement in the desired properties of aegPNA.[8] The β-substitution was recently studied and was found to be acceptable in PNA:DNA/RNA duplex structures depending upon stereochemistry.[9] The β, γ substituted cyclic-PNA containing both five- and six-membered rings showed good results in terms of directional selectivity of binding and discrimination in binding to DNA versus RNA complementary sequences.[14] We thought of exploring the possibility of β, γ-bis-substitution of PNA (Figure 1) without involving cyclic structures that can be easily synthesized from natural chiral pool. In this communication, we disclose for the first time the synthesis of chiral acyclic β, γ- bis-hydroxymethyl and methoxymethyl substituted PNA. We use the synthesized oligomers to study their DNA/RNA binding and cellular uptake properties.

To get the PNA with β,γ-bis-hydroxymethyl substitutions, the synthesis of protected modified β, γ- bis- benzyloxymethyl substituted thymine and adenine PNA monomers with (*S,S*)- and (*R,R*)- stereocentres was accomplished from L/D tartaric acids (Scheme 1). The bis-*O*-Bn C_2-symmetric vicinal diamine (2*S*, 3*S*) **2b$_2$** was derived from naturally occurring (*R, R*)-L-tartaric acid as reported earlier.[11] Mono-Boc-protection of the vicinal diamine **2b$_2$** gave compound **3b$_2$** in 52% yield. This was further monoalkylated using ethylbromo acetate to get **4b$_2$**. Compound **4b$_2$** was monoacylated using choroacetyl chloride to get **5b$_2$** in good yield. This intermediate was further used to alkylate thymine and adenine nucleobases to get the ester forms **6b$_2$** and **7b$_2$** respectively. Hydrolysis of **6b$_2$** and **7b$_2$** yielded the protected thymine and adenine modified PNA monomers



Scheme 1: Synthesis of (*R,R*)/(*S,S*) β,γ-substituted PNA monomers

(2*S*, 3*S*)-**8b$_2$** and (2*S*, 3*S*)-**9b$_2$** respectively. The synthesis of (2*R*, 3*R*)-**8a$_2$** and (2*R*, 3*R*)-**9a$_2$** was accomplished using same set of reactions from D-tartaric acid.

To get the PNA with β,γ-bis-methoxymethyl substitutions, the synthesis of protected modified β,γ- bis-methoxymethyl substituted thymine and adenine PNA monomers **8b$_1$**/**9b$_1$**and **8a$_1$**/**9a$_1$** with (*S,S*)/(*R,R*) stereocentres respectively, was accomplished from L/D tartaric acids (Scheme 1) using the same set of reactions from **2b$_1$** and **2a$_1$** as described for *O*-benzyl protected monomers. The compounds were all characterized by ^{1}H, ^{13}C, NMR, HRMS and optical rotation analysis. The chiral purity of the products was also ascertained by chiral HPLC of the enatiomeric pair **6a$_1$**/**6b$_1$**.

The modified thymine monomers (*R,R*)/(*S,S*)- **8a$_1$**/**8b$_1$** and **8a$_2$**/**8b$_2$** and the adenine monomers **9a$_1$**/**9b$_1$** and **9a$_2$**/**9b$_2$** were incorporated in aegPNA oligomer sequence aaccgatttcag-K (PNA1) at defined positions and the sequences are tabulated in Table 1**.** Unmodified PNA1 was used as a control sequence in the present study. We used standard solid phase synthesis using Boc chemistry and L-lysine derivatised MBHA resin. The sequences were cleaved from the support and purified by reverse phase HPLC. All the sequences were characterized by MALDI-ToF mass analysis (Table 1 and SI). The benzyl-protection of the side chain -OH groups was indeed cleaved during TFA-TFMSA cleavage conditions[16a] whereas the OMe groups were intact. The T_m values of the modified PNAs hybridized with cDNA in antiparallel (*ap*) and parallel (*p*) orientations, antiparallel complementary RNA (cRNA) and mismatched RNA (mmRNA) in 1:1 stoichiometry were determined by temperature-dependent UV absorbance plots. The T_m values were determined at 10mM and 100mM salt concentrations. The change in salt concentration showed slight destabilizing effect –3-4 ℃ uniformly for the modified and unmodified PNA oligomers, when four lysine units were attached at N-terminus. The unmodified acyclic flexible aegPNA (PNA1, Figure 2) binds to the *ap*-cDNA and *ap-c*RNA sequences to form stable PNA:DNA and PNA:RNA duplexes (Table 1, entry 1). The PNA1 and PNA2 also bind to the *p*-cDNA but with much lower T_m compared to the *ap*-cDNA. Use of bis-OMe-thymine (**T^{OMe}**) and bis-OMe-adenine (**A^{OMe}**) monomers at defined positions during the synthesis of PNA sequences gave the modified PNA sequences containing bismethoxymethyl

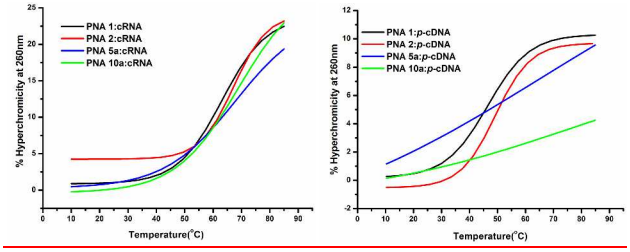


Figure 2: Representative UV-melting curves of antiparallel PNA:cRNA duplexes, parallel PNA:DNA duplexes

substitution in either (*R,R*) or (*S,S*) stereochemistry on the β, γ-carbons of the aminoethyl segment of aegPNA. It is seen from the data in Table 1 that the T_m of the modified PNAs containing (*R,R*) stereochemistry were considerably higher compared to those with (*S,S*) stereochemistry while binding with both *ap*-cDNA and cRNA sequences. The replacement of aegPNA-thymine units in the centre by one or two (*R,R*)- **T^{OMe}** units stabilized the complexes with cRNA by 1.6°C/modification (Table 1, entries 4 and 5). The (*S,S*)- **T^{OMe}** units completely destabilized the duplexes when present in the internal positions. The (*R,R*)- **A^{OMe}** units in the sequences formed stable complexes in the centre or in the end of the sequences (Table 1, entries 7 and 9). The stereochemistry dependent destabilization of the duplexes was less at the *C*-terminal end of the sequence where two adenine aegPNA units were replaced by two (*S,S*)- or (*R,R*)- **A^{OMe}** units (Table 1, entry 9). The PNA sequence with two modified units (*R,R*)- **T^{OMe}** in internal positions were organized such that unlike aegPNA (Table 1, entry 1), they did not show any binding with *p*-cDNA (Table 1, entry 5 Figure 2).

Use of bis-OBn protected monomers at defined positions during the synthesis of PNA sequences allowed us the access to the bis-hydroxymethyl substitution in PNA (Table 1, entry 3 and 6). The two hydroxymethyl groups were expected to largely improve the aqueous solubility of PNA.[12a,b] Unfortunately, it was observed that the modified PNAs with a single (*S,S*)/(*R,R*)- **T^{OH}**/ **A^{OH}** modification in the centre of the sequence, completely destabilized the duplexes. The 1,4 butanediol system containing free hydroxyl groups instead of ethylene segment of PNA thus

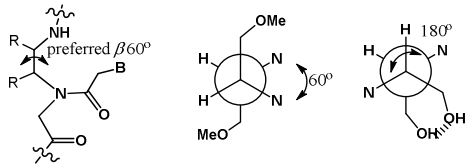
Table 1:Sequences synthesized, their MALDI-ToF analysis and DNA/RNA binding studies

No	Sequences	MALDI ToF mass analysis			*ap*-cDNA		*p*-cDNA	cRNA(mmRNA)	
		Calcd.	Obsvd.						
1. PNA1	aaccgatttcag-K	3381.4	3384.09		58.3		46.4	63.1(46.3)	
2. PNA2	K_4-aaccgatttcag-K	3893.9	3895.98		61.5		49.5	66.6 (53.6)	
			(*R,R*)*	(*S,S*)*	(*R,R*)*	(*S,S*)*	(*R,R*)*	(*R,R*)*	(*S,S*)*
3. PNA3a/b	aaccga**T^{OH}**ttcag-K	3439.4	3478.6(M+K)	3477.9(M+K)	n.t.	n.t.	n.t.	n.t.	n.t.
4. PNA4a/b	aaccga**T^{OMe}**ttcag-K	3467.5	3468.1	3469.1	59.2	43.6	38.5	64.7(51.8)	51.7(42.8)
5. PNA5a/b	aaccga**T^{OMe}**t**T^{OMe}** cag-K	3555.5	3558.2	3597.3(M+K)	60.0	n.t.	n.t	66.3(53.6)	n.t.
6. PNA6a/b	aaccg**A^{OH}**ttcag-K	3439.4	3464.9(M+Na)	3478.2(M+K)	n.t	n.t.	n.t.	n.t.	n.t.
7. PNA7a/b	aaccg**A^{OMe}**ttcag-K	3467.5	3491.8(M+Na)	3490.7(M+Na)	58.2	38.1	38.8	61.7(52.0)	49.2(40.4)
8. PNA8a/b	**A^{OH}A^{OH}**ccgatttcag-K	3499.4	3505.0	3497.5	51.5	45.7	38.6	60.8(47.8)	50.8(40.6)
9. PNA9a/b	**A^{OMe}A^{OMe}**ccgatttcag-K	3555.5	3598.0(M+K)	3593.4(M+K)	57.6	50.6	38.5	63.2(50.3)	57.3(37.8)
10.PNA10a	K_4-aaccga**T^{OMe}**t**T^{OMe}**cag-K	4067.9	4104.7(M+K)	-	-	n.t.	61.5	68.5(53.7)	-

In sequence listing, lower case nucleobase indicates aegPNA monomer, bold and upper case nucleobase indicates modified monomers as designated with bis-CH_2OH or bis- CH_2OMe substitution. The stereochemistry related to the monomers is indicated in the columns showing the *T*m values at 10mM NaCl salt concentration. cDNA: 5'-CTGAAATCGGTT; cRNA: 5'-CUGAAAUCGGUU. n.t.: no sigmoidal transition; Values in bracket indicate *T*m with mismatched RNA: CUGAA***U***UCGGUU. *(*R,R*) modified monomer unit obtained from D-(*S,S*) tartaric acid; (*S,S*) modified monomer unit obtained from L-(*R,R*) tartaric acid. PNA3a-10a contain modified units with (*R,R*) stereochemistry. PNA3b-9b contain modified units with (*S,S*) stereochemistry.

had large destabilizing effect on the stability of the duplex in either (*S,S*) or (*R,R*) stereochemistry when the modification was in the centre of the sequence(Table 1, entries 3 and 6). We further confirmed this result using gel-shift assay for the (*R,R*)- $\mathbf{T^{OH}}$/ $\mathbf{T^{OMe}}$ (Table 1, entries 3 and 4) containing PNA sequences (Please see SI Page S69). The conformationally driven destabilizing effect would be expected to be much less if the modified units are in the end of the sequence. The sequence in which the modified (*R,R*)-$\mathbf{A^{OH}}$ units were at N-terminus (Table 1, entry 8) could form duplexes with *ap*-cDNA and RNA with slight destabilization. As expected, the (*S,S*)-$\mathbf{A^{OH}}$ units at N-terminus of the sequence led to further destabilization (Table 1, entry 8).

We considered to rationalize this change in PNA-DNA/RNA duplex stability on changing the substitution from β,γ-bis-CH_2OMe to β,γ-bis-CH_2OH on the basis of recent work on conformational analysis carried out in 1,4-butanediol system as a prototype.[13a] Theoretical calculations suggested that the preferred lowest energy conformation in the 1,4 butanediol system is largely confined to a lowest energy conformation which can be attributed to the possible linear H--O--H hydrogen bonding in a 7-membered ring.[13b] This conformation also holds good in the presence of added water molecules. Such hydrogen bonded conformations containing linear H--O--H hydrogen bonding in a 7-membered ring are shown to be preferred conformations even in aqueous medium for glycerol[14]. Our results in fact give a chemical evidence for the preference of the hydrogen bonded



N-CHRCHR-N dihedral angle (β) in ethylene diamine segment of modified PNA

Figure 3: PNA and Newman projection of β,γ-substituted PNA monomers

structure for 1,4-butanediol segment of our modified β,γ-bis-CH_2OH-substituted PNA, in solution, considering the known facts about the geometries in PNA:RNA structure. The previous structural analysis, based on NMR and X-ray crystallography studies, pointed out that the preferred dihedral angle β, in the ethylenediamine segment (N –CH_2 –CH_2 –N , Figure 3) would be close to 60-70º in PNA:RNA duplex and the same was ~140º in PNA:DNA duplex. The designed (*R,S*)-*cis*-cyclohexyl[10e,f,g] (Figure 1) based PNA, in which, the dihedral angle β - was locked at 60-70º, was highly successful in stabilizing the PNA:RNA duplexes. The *trans/cis* cyclopentyl PNA[10b,c,d] however could bind to both DNA/RNA successfully due to the relatively flexible cyclopentyl ring system in which the dihedral angle in ethylenediamine segment could span the required range more easily. In *trans*-cyclohexyl system due to closed rigid ring, the dihedral angle β could be in the range to form the duplex in at least one of the chair conformations. The (*S, S*)-*trans*-cyclohexyl[10a] unit caused marginal duplex destabilization effect whereas the (*R, R*)-*trans*-cyclohexyl (Figure 1) based PNAs showed large destabilization of complexes with DNA and RNA. The rigid nature of cyclohexyl ring was very effective in binding with RNA in stereoselective manner when the geometry of substitution was *cis* and the dihedral angle β was restricted to the preferred value (~ 60º) in either of the chair conformations of cyclohexane in RNA: PNA duplexes. In the present modification, the possible hydrogen bonding interactions in vicinal hydroxymethyl substitution could restrict the conformation of modified bis- hydroxymethyl PNA in which this angle would be far away (Figure 2, β =180º) than is suitable for PNA: RNA/DNA duplex formation due to free rotation around C-C single bond and thus formation of such duplexes is precluded. In the bis-methoxymethyl PNA, in the absence of hydrogen bonding interactions, and free C-C bond rotation, a conformation suitable for RNA/DNA binding is maintained (Figure 3) and the formation of stable duplexes with RNA as well as DNA were observed. We therefore propose that the difference in binding properties of bis-hydroxymethyl and bis-methoxymethyl substitution could arise from the possible intramolecular hydrogen bonding in adjacent free hydroxyl groups in bis-hydroxymethyl monomers and thus freezing the conformations of bis-hydroxymethyl units that is not suitable for binding with either RNA or DNA (Figure 3). It is also noteworthy to find that the (*R,R*) stereoisomer is preferred over (*S,S)* isomer while binding to the natural nucleic acids. In this particular modification, the change in preferred stereochemistry compared to cyclohexyl-PNA is due to the change in priority rules while assigning the *R/S* stereochemistry. It would be indeed interesting to study the stability of PNA:RNA/DNA duplexes when the disposition of the substituents would be suitable for RNA binding in the hydrogen bonded conformation, possibly when the starting material is meso-tartaric acid (*R,S/S,R*) as in the case of *cis*-cyclohexyl-PNA.

Further, we wanted to examine the effect of the modification of PNA on their cellular uptake properties. It was shown recently that the attachment of four lysine units helped to increase the water solubility of PNA due to positively charged residues and decreased self aggregation in unmodified PNA in biological experiments.[15a, 15b] We therefore synthesized PNA sequences with four lysine units at *N*-terminus (Table 1, entries 2, 10, PNA2, PNA10a respectively) to study the comparative cell-uptake properties PNA (Table 1, entry 1, PNA1) *versus* PNA with two $\mathbf{T^{OMe}}$ units (Table 1, entry 5, PNA5a). These sequences were chosen for the cellular uptake studies as their binding to the target cRNA was found to be the best in the present series of oligomers (Figure 2). The ability of the unmodified (Table 1, PNA1 and PNA2) and modified PNA sequences (Table 1, PNA5a and PNA10a) to enter the cells without the help of transfection agent was studied using flow cytometry experiments. Sequences PNA1, PNA2, PNA5a and PNA10a were tagged at *N*-terminus with carboxyfluorescein (CF) through amide linkage to get the PNA1-CF to PNA2-CF, PNA5a-CF and PNA10a-CF respectively (SI Pg S64-S67). HCT-116 cells were treated with CF-labled PNAs at 1 μM concentration and incubated for 10 hrs at 37°C. In order to remove the cell surface bound PNAs, the cells were washed with heparin (1 mg/ml) before flow cytometric analysis.[16] Thus, the fluorescent positive cells that were obtained after FACS analysis were indicative of internalized PNAs and not those externally associated with the cell membrane. Figure 4 shows that PNA 10a-CF was most efficiently internalized without the aid of transfection agent with ~ 57% fluorescent positive cells with high mean intensity as compared to untreated cells (~5.5%). Unmodified PNA (PNA1-CF) showed ~5.4% fluorescent positive cells similar to the untreated cells (5.1%). PNA2-CF with four lysine residues showed ~35 % internalization, whereas PNA5a-CF showed ~15 % cellular uptake, respectively. The sequence PNA5a-CF, having bis-methoxyethyl substitution of two aegPNA units was internalized better than unmodified PNA1-CF even without the attachment of K_4. Attachment of lysine units further enhanced the property of modified PNA10a-CF for cellular uptake (Figure 4).

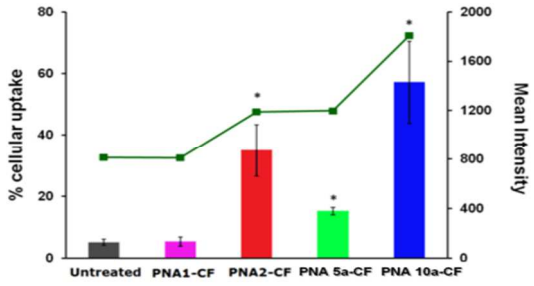


Figure 4: Comparative intake of PNA1-CF, PNA2-CF, PNA5a-CF and PNA10a-CF in HCT-116 (Human colorectal carcinoma) cells

Conclusions

The results presented here reveal for the first time a β,γ-substituted PNA and the specificity of stereochemical requirements of the modified PNA while binding to target DNA and RNA. PNA substituted with (*R,R*)-β,γ- bis- methoxymethyl thymine and adenine residues stabilize the duplexes while the (*S,S*)- stereochemistry completely destabilizes the duplexes. Also, the change from bis-methoxymethyl to bis-hydroxymethyl substitution is unsuitable for duplex formation. This is the first report that a possible intramolecular hydrogen bonding in 1,4 butanediol system, as in the present case, might exert control over acyclic PNA conformations in aqueous medium. Furthermore, the PNAs with (*R,R*)-β,γ- bis- methoxymethyl substituted thymine significantly improve the access for PNA to intracellular space.

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Notes and references

† Electronic Supplementary Information (ESI) available: [Experimental procedures for synthesis and cell uptake experiments, ^{1}H and ^{13}C NMR of synthetic intermediates, HPLC, MALDI-ToF spectra of synthesized PNA oligomers, CD Jobs plot, UV-T_m data, cell uptake results, gel-shift assay]. See DOI: 10.1039/b000000x/

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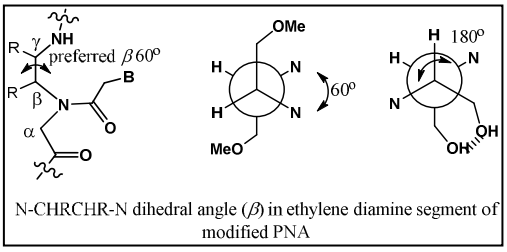
Graphical Content

β,γ-bis-substituted PNA with configurational and conformational switch: Preferred binding to cDNA/RNA and cell-uptake studies

β,γ-bis-methoxymethyl and β,γ-bis-hydroxymethyl PNA show highly differential binding properties while interacting with cDNA/cRNA and can access intracellular space.



N-CHRCHR-N dihedral angle (β) in ethylene diamine segment of modified PNA