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Chimeric (*aeg*-pyrrolidine)PNAs: synthesis and stereo-discriminative duplex binding with DNA/RNA†

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The design and facile conversion of naturally occurring 4-hydroxyproline to all four diastereomers of thymine pyrrolidine PNA monomer, (2*R*,4*S*)-adenine, -guanine and -cytosine monomers and their incorporation into duplex forming PNA oligomers is reported. The interesting results of the hybridization studies with complementary DNA/RNA sequences in either parallel or antiparallel orientation reveal the stereochemistry-dependent DNA *vs.* RNA discriminations and parallel/antiparallel orientation selectivity.

Introduction

Peptide nucleic acids, in which the sugar phosphate backbone of natural nucleic acids is replaced by the pseudopeptide backbone derived from *N*-(2-aminoethyl)glycyl units (*aeg*PNA **II**), have emerged as promising DNA/RNA (**I**) mimics (Fig. 1).[1] Several analogues and derivatives of *aeg*PNA have been synthesized to overcome the limitations of PNA, such as poor water solubility, cellular permeability and orientational ambiguity in complementary DNA recognition[2] by making them cationic and chiral. As part of our continuing studies of conformationally constrained chiral analogues of PNA based on the pyrrolidine core structure,[3] we have recently reported[3f] a pyrrolidine PNA **III** derived by introducing a methylene bridge between the α carbon atom of the nucleobase acetamide linker and the β′ carbon atom of the aminoethyl segment of *aeg*PNA backbone. The introduction of a single modification of (2*R*,4*S*)-pyrrolidine-T unit **III** in *aeg*PNA-T_8 largely stabilized the PNA_2 : DNA complex ($\Delta T_m = +16$ °C) while the (2*S*,4*S*)-**III** diastereomer destabilized the complex ($\Delta T_m = -16$ °C) compared to unmodified *aeg*PNA. The binding efficiency of chimeric (*aeg*-pyrrolidine)PNA oligomers with DNA, in triplex mode, seems to be influenced by the stereochemistry of the backbone chiral unit.

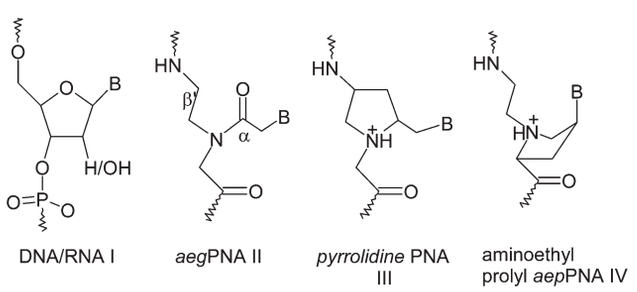

Fig. 1 DNA/RNA/PNA structures.

In view of the above encouraging results on the (2*R*,4*S*)-pyrrolidine thymine isomer **III**, we considered the synthesis of the corresponding monomers of the other nucleobases and other diastereomeric pyrrolidine PNA thymine monomers. In the *aeg*PNA backbone, substitution with a pyrrolidine modification such as *aep*PNA **IV** was reported to exhibit nucleobase dependent selectivity in DNA recognition properties.[3c] The direct attachment of the nucleobases with different electronegativities to the pyrrolidine ring as in *aep*PNA **IV**, perhaps affected the five-membered ring conformation and stability of the resulting complexes. It was thought that the design of the (pyrrolidine)PNA **III** where the nucleobase attachment to the backbone is through a flexible methylene linker might prove to be more appropriate for uniform results. The introduction of nucleobases besides thymine will allow access to PNAs with mixed purine–pyrimidine sequences for examining the consequence of the chiral constraints and the nucleobase effects on PNA : DNA duplex stability.

We herein report the synthesis of (2*R*,4*S*)-pyrrolidine monomers of the natural nucleobases cytosine (**5a**), adenine (**5b**) and guanine (**5d**). The synthetic strategies to obtain the other two diastereomeric pyrrolidine thymine monomers **14** (2*S*,4*R*) and **15** (2*R*,4*R*) (Fig. 2) requires an additional Mitsunobu reaction in the scheme to invert the stereochemistry at C4. The synthesis of chimeric (*aeg*-pyrrolidine)PNAs and their binding to complementary DNA/RNA sequences is also presented in this paper. The results presented indicate that the preferred stereochemistry of the pyrrolidine ring varied with the different structural requirements for the PNA_2 : DNA triplexes, PNA : DNA duplexes and PNA : RNA duplexes.[4] The DNA : PNA duplexes were found to be uniformly more stable in antiparallel orientation.

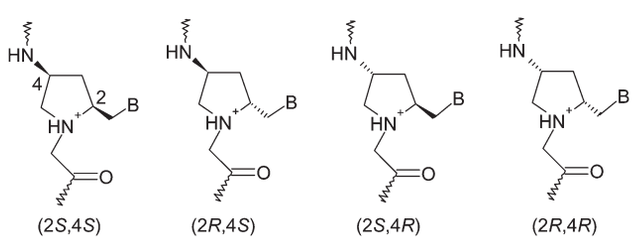

Fig. 2 (Pyrrolidine)PNA diastereomeric structures.

Results and discussion

Synthesis of (pyrrolidine)PNA monomers 5a,b,d and 14, 15

The previously reported[3f] intermediate (2*R*,4*S*)-**1** was reacted with individual protected nucleobases, N^4-benzyloxycarbonylcytosine, N^6-benzoyladenine and 2-amino-6-chloropurine in the presence of K_2CO_3 and 18-crown-6 in DMF to give the desired pure nucleobase derivatives **2a**,**b**,**c** in 30, 40 and 45% isolated yields respectively (Scheme 1).[3d,5] The ring nitrogen was deprotected and the free amine was immediately alkylated with ethyl bromoacetate in the presence of diisopropylethyl amine to obtain **3a**–**c**. The C-4 azide in **3** was subjected to catalytic hydrogenation over Pd–C to obtain the primary amine that was immediately protected as a *tert*-butyl carbamate to give **4a**–**c**. The ethyl ester in **4a**–**c** was hydrolyzed using sodium hydroxide in aqueous methanol to give free acids of the pyrrolidine–cytosine, –adenine and –guanine monomers (**5a**,**b**,**d**).

† Electronic supplementary information (ESI) available: HPLC profiles and MALDI-TOF mass spectra of the chimeric (*aeg*-pyrrolidine)PNAs **17**–**23**. UV-mixing Job's plot of PNA : DNA **19** : **24** indicating 1 : 1 complex formation. First derivative plots of (*aeg*-pyrrolidine)PNA (**17**–**23**) : DNA (**24**) complexes. See http://www.rsc.org/suppdata/ob/b4/b407292h/

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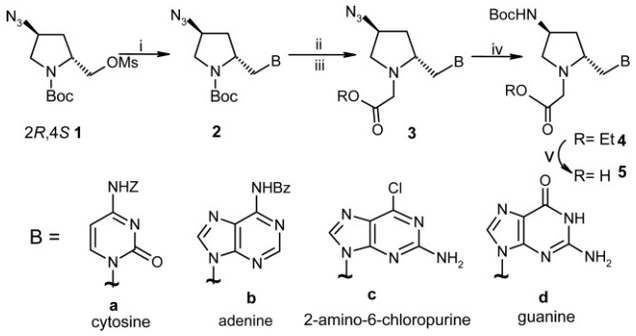


Scheme 1 Reagents and conditions: (i) K_2CO_3, 18-crown-6, BH, DMF; (ii) TFA, DCM; (iii) $BrCH_2COOEt$, DIPEA, THF (92%); (iv) H_2, Pd/C or Raney-Ni, $(Boc)_2O$ (90%); (v) NaOH/water–MeOH.

The synthesis of (2*R*,4*R*)-**14** and (2*S*,4*R*)-**15** pyrrolidine thymine diastereomers was accomplished by a set of reactions shown in Scheme 2. The (2*R*,4*R*)-*N*-benzyloxycarbonyl-4-hydroxyproline methyl ester **6** was converted to (4*S*)-tosylate **7** with inversion at C4 under Mitsunobu conditions using DIAD/PPh_3 and methyl tosylate. The (4*S*)-tosylate **7** was treated with sodium azide to give the (4*R*)-azide **8**, the reaction proceeding by inversion of configuration at C-4. The azide **8** was selectively reduced by catalytic hydrogenation in presence of Raney-Ni to give free (4*R*)-amine, which was *in situ* protected as a Boc derivative **9**. The sodium borohydride reduction of the ester function in **9** to alcohol **10** was followed by its conversion to *O*-mesyl derivative **11** using mesyl chloride in the presence of triethylamine. *N*-1 alkylation of thymine was effected by its reaction with the mesylate **11** in the presence of K_2CO_3 and 18-crown-6 to give (2*R*,4*R*)-*N*-1 thyminylmethyl derivative **12** in 30% yield. The pyrrolidine ring nitrogen in **12** was deprotected by hydrogenation and immediately alkylated with ethyl bromoacetate to obtain **13**. Subsequent hydrolysis of the ethyl ester **13** with sodium hydroxide in aqueous methanol gave (4*S*)-(*N*-Boc-amino)-(2*R*)-(thymin-1-ylmethyl)pyrrolidin-*N*-1-acetic acid **14**. Starting from 1-(*N*-benzyloxycarbonylamino)-(4*R*)-hydroxy-(2*S*)-proline methyl ester gave (4*R*)-(*N*-Boc-amino)-(2*S*)-(thymin-1-ylmethyl)pyrrolidin-*N*-1-acetic acid **15**. During the alkylation of nucleobases, minor by-products are formed that could be the regiomers due to alkylations at N^3/N^7 ring nitrogens. These were carefully separated during column chromatography and the yields correspond to the isolated pure products. Fig. 3 represents the mirror image relationship in CD spectroscopy of the enantiomeric thymine pyrrolidine-PNA monomer pairs (2*S*,4*S*[3f] and 2*R*,4*R*) and (2*S*,4*R* and 2*R*,4*S*[3f]). The monomers **5a**,**b**,**d** and **14**, **15** were used for solid phase synthesis of (*aeg*-pyrrolidine)PNAs **17**–**23**. All new compounds were characterized using ^1H, ^{13}C NMR and mass spectrometry analysis. The pK_a of the pyrrolidine ring nitrogen atom in the monomeric unit was determined by acid–base titration to be 6.8, indicating that in the modified oligomers the pyrrolidine ring nitrogen could be at least partially protonated at physiological pH.

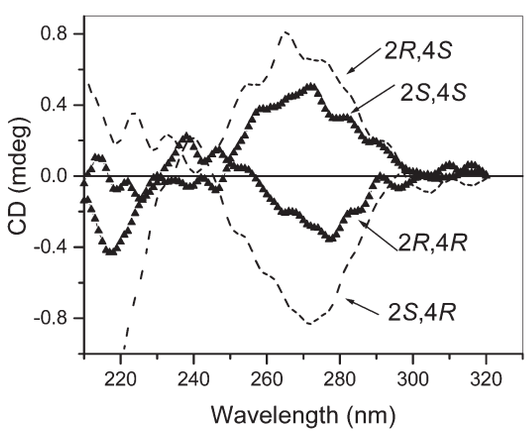
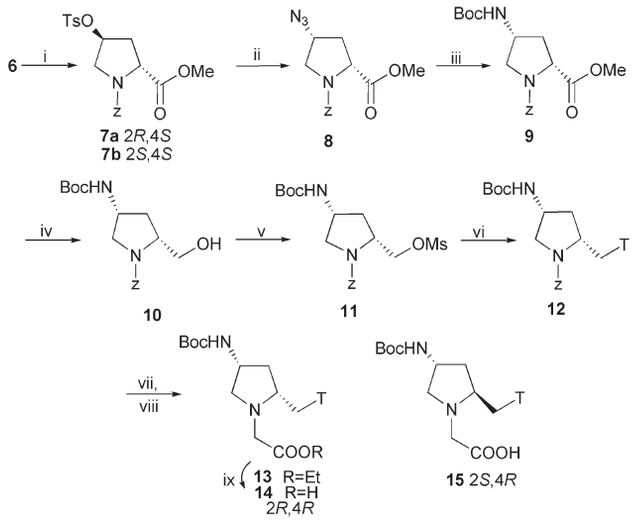


Fig. 3 CD spectra of diastereomeric thymine pyrrolidine-PNA monomers.



Scheme 2 Reagents and conditions: (i) methyl tosylate, PPh_3, DIAD, THF (70%); (ii) NaN_3, DMF (85%); (iii) Raney-Ni, H_2, $(Boc)_2O$ (85%); (iv) $NaBH_4$, LiCl, EtOH, THF (80%); (v) MsCl, DCM, Et_3N (95%); (vi) thymine, K_2CO_3, 18-crown-6, DMF (35%); (vii) H_2,Pd/C; (viii) $BrCH_2COOEt$, DIPEA, THF (80%); (ix) NaOH/water–MeOH (90%).

Solid phase synthesis of chimeric (*aeg*-pyrrolidine)PNA oligomers

The modified (2*S*,4*S*), (2*S*,4*R*), (2*R*,4*R*), (2*R*,4*S*) thymine monomers and (2*R*,4*S*) cytosine, adenine and guanine monomers, were individually introduced at pre-defined positions into duplex forming *aeg*PNA sequence **16**. The (*aeg*-pyrrolidine)PNA oligomers **17**–**20** were synthesized to study the effect of stereochemistry of the thymine monomer at the same position, while PNAs **20**–**23** were required for examining the effect of nucleobases with the same stereochemistry. The *aeg*PNA sequence **16** was used as control. The synthesis of all PNA oligomers was carried out using standard solid-phase synthesis protocols.[6] Appropriate modified PNA monomers were used for coupling at the desired positions. The oligomers were cleaved from the support using trifluoroacetic acid–trifluoromethanesulfonic acid[7] to yield the PNA oligomers carrying β-alanine at their carboxy termini. The oligomer **22** required an additional deprotection of N^6-benzoyl group.[3d] The oligomers were purified by FPLC on a C8-PepRPC column. The oligomers were rechecked for purity by HPLC on an RP-C18 column and were characterized by MALDI-TOF mass spectrometry (Table 1) (ESI†). No precipitation was observed in samples of chimeric (*aeg*-pyrrolidine)PNA even after prolonged storage, in comparison to the control *aeg*PNA **16**, indicative of their water solubility possibly as a consequence of ring *N*-protonation. The complementary oligonucleotides DNA **24, 25,** RNA **26** and DNA **27** with a mismatched base were synthesized on automated DNA synthesizer and were used for complementation studies. All synthesized PNA, DNA and RNA sequences are listed in Table 1.

UV-T_m studies

UV-T_m data of duplexes of PNAs **16**–**23** in both antiparallel (*ap*) and parallel (*p*) modes with DNA (**24** and **25**, respectively) and (*ap*) RNA (**26**) was obtained as a measure of duplex stability (Figs. 4 and 5). The 1 : 1 stoichiometry of PNA : DNA complexation was confirmed by UV-mixing data (Job's plot,[8] ESI†). The T_m data is summarized in Table 2. The T_m values were obtained by the first derivative curves from individual melting curves (ESI†).

(*aeg*-Pyrrolidine)PNA : DNA duplexes. The control *aeg*-PNA : DNA antiparallel duplex (**16 : 24**) was only marginally more stable (ΔT_m = +1 °C) than the parallel duplex (**16 : 25**).

All (*aeg*-pyrrolidine)PNA oligomers **17**–**23** formed stable duplexes preferentially in the *ap* mode with complementary DNA. The PNAs **18** (**t** 2*S*,4*R*) and **19** (**t** 2*R*,4*R*) were found to stabilize the complexes with *ap* DNA **24** (ΔT_m = +11 and +5 °C,

Table 1 PNA, DNA and RNA oligomer sequences

No.	PNA Sequence	M (Calc.)	M (Found)[a]
16	H–GTAGATCACT–NH$(CH_2)_2$COOH		
17	H–GTAGA **t** $_{(2S,4S)}$ CACT–NH$(CH_2)_2$COOH	2796	2796.0
18	H–GTAGA **t** $_{(2S,4R)}$ CACT–NH$(CH_2)_2$COOH	2796	2798.0
19	H–GTAGA **t** $_{(2R,4R)}$ CACTNH$(CH_2)_2$COOH	2796	2796.0
20	H–GTAGA **t** $_{(2R,4S)}$ CACT–NH$(CH_2)_2$COOH	2796	2796.75
21	H–GTA **g** $_{(2R,4S)}$ATCACT–NH$(CH_2)_2$COOH	2796	2797.0
22	H–GT **a** $_{(2R,4S)}$ GATCACT–NH$(CH_2)_2$COOH	2796	2797.7
23	H–GTAGAT **c** $_{(2R,4S)}$ ACT–NH$(CH_2)_2$COOH	2796	2796.0

T, C, G, A represent *aeg*PNA unit and **t**, **c**, **g**, **a** represent pyrrolidine PNA unit. **24** 5′-AGT GAT CTA C-3′ DNA (*ap*), **25** 5′-CAT CTA GTG A-3′ DNA (*p*), **26** 5′-AGU GAU CUA C-3′ RNA (*ap*), **27** 5′-AGT GT**T** CTA C-3′ DNA (*ap*) with a **T** mismatch. [a] Molecular weights as obtained by MALDI-TOF mass spectrometry.

Table 2 UV T_m (in °C) studies of PNA : DNA/RNA duplexes

PNA	DNA **24** (*ap*)	DNA **25** (*p*)	RNA **26** (*ap*)
H–GTAGATCACT–NH$(CH_2)_2$COOH **16**	47	46	41
H–GTAGA **t** $_{(2S,4S)}$ CACT–NH$(CH_2)_2$COOH **17**	34	30	76
H–GTAGA **t** $_{(2S,4R)}$ CACT–NH$(CH_2)_2$COOH **18**	58 (44)	48	44
H–GTAGA **t** $_{(2R,4R)}$ CACT–NH$(CH_2)_2$COOH **19**	52 (48)	47	78
H–GTAGA **t** $_{(2R,4S)}$ CACT–NH$(CH_2)_2$COOH **20**	39	38	43
H–GTA **g** $_{(2R,4S)}$ATCACT–NH$(CH_2)_2$COOH **21**	29	27	33
H–GT **a** $_{(2R,4S)}$ GATCACT–NH$(CH_2)_2$COOH **22**	36	34	38
H–GTAGAT**c** $_{(2R,4S)}$ ACT–NH$(CH_2)_2$COOH **23**	34	31	36

Buffer: 10 mM sodium cacodylate, 100 mM NaCl, 0.01 mM EDTA. pH 7.3. T, C, G, A represent *aeg*PNA units and **t**, **c**, **g**, **a** represent pyrrolidine PNA unit. Melting experiments were performed with a PNA : DNA strand concentration of 1 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 for DNA. Values in parenthesis indicate melting temperature with DNA sequence **27** having one base mismatch.

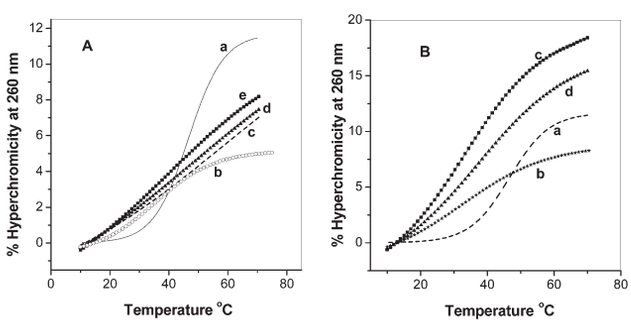


Fig. 4 UV melting profiles of PNA : DNA complexes: (A) (a) **16 : 24**, (b) **17 : 24**, (c) **18 : 24**, (d) **19 : 24**, (e) **20 : 24**; (B) (a) **16 : 24**, (b) **21 : 24**, (c) **22 : 24**, (d) **23 : 24**.

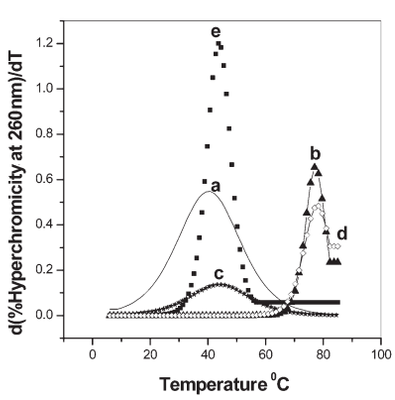


Fig. 5 The first derivative plots of % hyperchromicity *vs*. temperature for PNA : RNA complexes: (a) **16 : 26**, (b) **17 : 26**, (c) **18 : 26**, (d) **19 : 26**, (e) **20 : 26**.

respectively). Complexes formed by PNA **17** (**t** 2*S*,4*S*) and PNA **20** (**t** 2*R*,4*S*) with DNA **24** were quite destabilized (ΔT_m = −13 and −8 °C, respectively) compared to control *aeg*PNA **16** : DNA hybrids. The stereochemistry of the pyrrolidine ring in **18** (**t** 2*S*,4*R*) could thus exercise preferential structural pre-organization of (*aeg*-pyrrolidine)PNA that stabilized PNA : DNA **18 : 24** duplex in *ap* orientation. The diastereomeric (**t** 2*R*,4*R*) pyrrolidine monomer in **19** also stabilized the *ap* duplex with DNA **24** (ΔT_m = +4 °C) but to a lesser extent. PNA oligomers **18** and **19** with DNA **27** having a single mismatch at the site complementary to the pyrrolidine unit were destabilized by 14 and 4 °C, respectively. This indicates that the recognition process could be primarily through WC base pairing aided by steric fit and not overruled by steric fitting or electrostatic interactions. The oligomers **21**–**23** comprising other bases **g**, **a** and **c** with (2*R*,4*S*) stereochemistry of the pyrrolidine ring, destabilized the corresponding DNA duplexes indicating that this stereochemistry indeed is not suitable for duplex formation independent of the nucleobase.

(*aeg*-Pyrrolidine)PNA : RNA duplexes. Considering the differential structural requirements[4] for PNA : RNA duplexes compared to PNA : DNA duplexes, we studied the duplex binding properties of the (*aeg*-pyrrolidine)PNAs **17**–**23** with *ap* RNA (Fig. 5, Table 2). The control *aeg*PNA : RNA antiparallel duplex (**16 : 26**) was found to be less stable compared to control PNA : DNA duplex with a lower T_m of 41 °C. Interestingly, all four (*aeg*-pyrrolidine) PNA thymine oligomers comprising different diastereomers (**17**–**20**) formed more stable duplexes than control *aeg*PNA. PNA oligomers with *cis* diastereomers as in (**t** 2*S*,4*S*) **17** and (**t** 2*R*,4*R*) **19** which destabilized the corresponding duplexes with DNA, conferred very large stabilization (ΔT_m = +35, +37 °C, respectively) of the resulting RNA duplexes. This is an interesting observation and the reason could be either effective better steric fitting of PNA stereochemistry with RNA or other complex intrinsic structural differences in DNA and RNA. The oligomers **20**–**23** comprising other bases **a**, **g** and **c** with (2*R*,4*S*) stereochemistry of the pyrrolidine ring, destabilized the corresponding RNA duplexes. Apart from the stereochemical requirements, the reason for the destabilization could also be the position of the modification in the sequence as observed in case of fluorinated olefinic PNAs.[9] The (2*R*,4*S*) stereochemistry that stabilized the PNA_2 : DNA triplex structures was thus found to be unsuitable to form duplexes with RNA in *ap* orientation. The PNAs with an L-lysine unit at the *C*-terminus is known to bind better to RNA over DNA.[10] In our studies with the unmodified *aeg*PNA **16**, the presence of β-alanine at *C*-terminus allowed better binding to DNA over RNA.

Parallel/antiparallel (*p*/*ap*) orientation selectivity. The largest *ap*/*p* differentiation (ΔT_m = +12 °C) was observed for PNA **18** having a (2*S*,4*R*) pyrrolidine–thymine unit. This stereochemistry was thus found to be better suiting for *ap* binding than binding in *p* orientation. The diastereomer in **19** (**t** 2*R*,4*R*), also stabilized *ap* duplex with DNA **24** with comparatively less discrimination of *ap*/*p* orientation selectivity. The results reported here show the differential *p*/*ap* DNA and RNA binding properties conferred by a single pyrrolidine unit in an *aeg*PNA sequence as a consequence of the stereochemistry of the pyrrolidine ring. Such an effect was earlier observed when a chiral cyclic monomer was present in the middle of the sequence along with an L-lysine unit at the *C*-terminus or *N*-terminus of the sequence.[11] We have used β-alanine at the *C*-terminus so that the *C*-terminus remains achiral and the results obtained are solely because of the presence of the chiral pyrrolidine unit in the middle of the PNA sequence and not due to the positive charge or chirality of the terminal lysine unit.

Thus, the different structural features of the PNA_2 : DNA, PNA : DNA *vs.* PNA : RNA complexes seem to have an impact on the selection of the stereochemistry of the pyrrolidine unit while binding to either RNA or DNA complementary sequence, in duplex binding mode. It is interesting to note that the pyrrolidine backbone with (2*R*,4*S*) stereochemistry in the homopyrimidine sequence stabilized the triplex with complementary DNA,[3f] but was found to destabilize the derived DNA/RNA duplexes. Further work that will reveal the suitable stereochemistry in sequence context and with homo-pyrrolidine backbone is currently undergoing in our laboratory.

Gel-shift assay

The formation of PNA : DNA *ap* duplexes was confirmed by the non-denaturing gel-shift assay (Fig. 6) which is useful to establish the binding of (*aeg*-pyrrolidine)PNAs to complementary oligonucleotides by observed mobility retardation of the complexes on the gel. The PNAs modified with one unit of (pyrrolidine)PNA and the control PNA were individually treated with complementary DNA and complexation were monitored by non-denaturing gel electrophoresis at 10 °C. The spots were visualized on a fluorescent TLC background. The PNA : DNA complexes derived from (pyrrolidine)PNA with single modified unit were significantly retarded in the gel (lanes 3, 4; PNA : DNA **18 : 24, 19 : 24**).

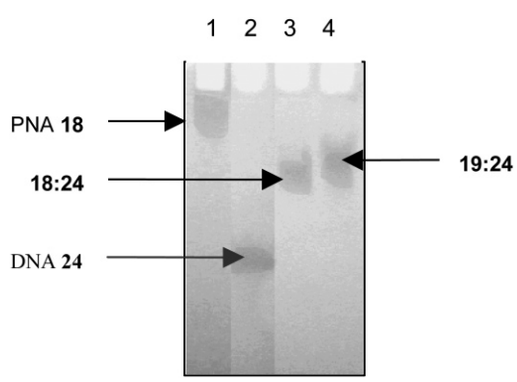


Fig. 6 Non-denaturing gel: lane 1: PNA **18** ss; lane 2: DNA **24**, lane 3: PNA **18** : DNA **24**, lane 4: PNA **19** : DNA **24**.

These results were in accordance with the data obtained from the UV-thermal melting studies with these oligomers, which also underline the specificity of the hydrogen bonding between the complementary base pairs.

In summary, this paper presents detailed studies on the synthesis and comparative complexation of PNA oligomers incorporating diastereomeric pyrrolidine monomers having nucleobases T, A, G and C with complementary DNA and RNA sequences. It is found that (i) (2*R*,4*S*)-PNA oligomers that stabilize PNA_2 : DNA homopyrimidine : homopurine triplexes destabilize the mixed pyrimidine : homopurine duplexes, (ii) the presence of (2*S*,4*R*) and (2*R*,4*R*) stereoisomers affects enhanced DNA duplex stability, (iii) *cis*-(2*S*,4*S*) and -(2*R*,4*R*) remarkably enhance PNA : RNA duplex stability, (iv) all pyrrolidine modifications show preference for antiparallel DNA binding. The interesting stability differences seen in the complexes of different pyrrolidinyl PNAs with DNA/RNA and stereochemical preferences will be useful to design and arrive at PNA analogues having selective DNA/RNA recognition properties.[12] Such studies are currently under progress in our laboratory.

Experimental

General

All the reagents were purchased from Sigma-Aldrich and used without purification. DMF and pyridine were dried over KOH and 4 Å molecular sieves. THF was passed over basic alumina and dried by distillation over sodium. Ethanol was dried over Mg/iodine. TLCs were run on Merck 5554 silica 60 aluminium sheets. All reactions were monitored by TLC and usual work-up implies sequential washing of the organic extract with water and brine followed by drying over anhydrous sodium sulfate and evaporation under vacuum. Column chromatography was performed for purification of compounds on silica gel (100–200 mesh, LOBA Chemie). TLCs were performed using dichloromethane–methanol or petroleum ether–ethyl acetate solvent systems for most compounds. Purity of the free acids was checked by TLC using isopropanol–acetic acid–water (9 : 1 : 1, v/v) solvent system. Compounds were visualized with UV light and/or by spraying with ninhydrin reagent subsequent to Boc-deprotection (exposing to HCl vapourS) and heating. ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectra were recorded on a Bruker ACF 200 spectrometer fitted with an Aspect 3000 computer and all the chemical shifts (δ/ppm) are referred to internal TMS for ^{1}H and chloroform-*d* for ^{13}C NMR. Optical rotations were measured on a JASCO DIP-181 polarimeter. Mass spectra were recorded on a Finnigan-Matt mass spectrometer, while MALDI-TOF spectra were obtained from a KRATOS PCKompact instrument. UV experiments were performed on a Perkin Elmer λ35 UV-VIS spectrophotometer fitted with a Peltier temperature programmer and a Julabo water circulator. The DNA oligomers were synthesized on CPG solid support using a Pharmacia GA plus DNA synthesizer by β-cyanoethyl phosphoramidite chemistry followed by ammonia treatment[13] and their purities checked by HPLC prior to the use. *aeg*PNA monomers were synthesized according to literature procedures.[6]

Synthesis, cleavage and purification of the PNA oligomers from the solid support

The PNA oligomers were synthesized manually by solid-phase peptide synthesis using the Boc-protection strategy and employing HBTU and 1-hydroxybenzotriazole (HOBT) as the coupling agents. The solid support used was Merrifield resin that was derivatized with β-alanine (0.19 meq./g resin) as the spacer amino acid. Synthesis involved repetitive cycles, each comprising (i) deprotection of the N-protecting Boc-group using 50% trifluoroacetic acid (TFA) in CH_2Cl_2, (ii) neutralization of the TFA salt formed with diisopropylamine (5% solution in CH_2Cl_2, v/v) and (iii) coupling of the free amine with the free carboxylic acid group of the incoming monomer (3–4 equivalentS) in the presence of HBTU and HOBt, in DMF or NMP as the solvent. The deprotection of the *N*-Boc protecting group and the coupling reaction were monitored by Kaiser's test.[14] Since the coupling efficiencies were found to be >98%, capping of the unreacted amino groups was not found necessary.

The PNA oligomers were cleaved from the solid support using the TFA–trifluoromethanesulfonic acid (TFMSA) method to yield oligomers with free carboxylic acids at their carboxy termini.[7] The resin-bound PNA oligomer (10 mg) was stirred in an ice-bath with thioanisole (20 µl) and 1,2-ethanedithiol (8 µl) for 10 min. TFA (120 µl) was then added and the stirring was continued for another 10 min. TFMSA (16 µl) was added while cooling the reaction in an ice-bath and stirring was continued for 2 h. The

reaction mixture was filtered through a sintered funnel, the residue washed with TFA (3 × 2 ml) and the combined filtrate and washings were evaporated under vacuum. The residual pellet was re-dissolved in methanol (~0.1 ml) and re-precipitated by adding ether to obtain the crude PNA oligomer. (In the case of oligomer **22**, the exocyclic benzoyl protection of adenine was removed by treating the resin with ethylene diamine–ethanol[3d,15] in 1 : 1 ratio overnight at room temperature prior to TFA–TFMSA cleavage.) This was purified by gel filtration over Sephadex G25. The purity of the PNA oligomer was checked by analytical RP HPLC on a C18 column and found to be >90% (ESI†).

UV-melting studies

The concentration of the PNA oligomers was calculated on the basis of the absorption at 260 nm, at 80 °C[6] assuming the molar extinction coefficients of the nucleobases to be as in DNA, *i.e.*, T, 8.8 cm^2 μmol^{-1}; C, 7.3 cm^2 μmol^{-1}; G, 11.7 cm^2 μmol^{-1} and A, 15.4 cm^2 μmol^{-1}. Molar extinction coefficients for the (pyrrolidine)PNA monomers were calculated and found to be almost the same as that of the nucleobases in DNA (molar extinction coefficients for T with (2*R*,4*S*), (2*R*,4*R*), (2*S*,4*S*) and (2*S*,4*R*): 8.7, 8.6, 9.0 and 9.1 cm^2 μmol^{-1}; (2*R*,4*S*) G, 11.9 cm^2 μmol^{-1}; C, 7.8 cm^2 μmol^{-1}; A, 16.0 cm^2 μmol^{-1}) The PNA oligomers (**16**–**23**) and the relevant complementary DNA/RNA oligonucleotide (**24**–**26**) were mixed together in a 1 : 1 molar ratio in 10 mM sodium cacodylate, 100 mM NaCl, 0.01 mM EDTA, (pH 7.3) to obtain a final strand concentration of 1 μM. The samples were annealed by heating at 85 °C for 1–2 min, followed by slow cooling to room temperature, kept at room temperature for ~30 min and then, refrigerated overnight. The samples were heated at a rate of 0.5 °C min^{-1} and the absorbance at 260 nm was recorded at every 1 min. The percent hyperchromicity at 260 nm was plotted as a function of temperature and the melting temperature was deduced from the peak in the first derivative plots.

1-(*N*-Butyloxyoxycarbonyl)-(4*S*)-azido-(2*R*)-(*O*-mesylmethyl)-pyrrolidine (1)

To a stirred, cooled mixture of 1-(*N*-Boc)-(4*S*)-azidopyrrolidin-(2*S*)-methanol[3f] (1.0 g, 4.1 mmol) and triethylamine (1.44 ml, 10.3 mmol) in dry dichloromethane (10 ml), methanesulfonyl chloride (0.8 ml, 10.3 mmol) was added dropwise. Stirring was continued at room temperature overnight. The solvent was completely removed under vacuum. The residue was taken up in water and the crude product obtained by extraction in dichloromethane, followed by drying of the organic layer sodium sulfate and evaporation under vacuum. Silica gel column chromatography yielded the pure product in the form of a gum (0.6 g, 90% yield).

^{1}H NMR ($CDCl_3$) δ: 4.45 (dd, 1H), 4.15 (br m, 3H), 3.65 (m, 1H), 3.35 (m, 1H), 3.03 (s, 3H), 2.30 (m, 1H), 2.13 (m, 1H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 164.9, 154.2, 152.2, 152.1, 149.4, 143.2, 133.3, 132.2, 128.2, 127.7, 122.4, 80.5, 58.3, 55.8, 51.4, 44.5, 34.4, 27.9. ESIMS: calc. for $C_{22}H_{25}N_9O_3$ (M^+) *m*/*z* 463.6, found: 464 (M + 1).

1-(*N*-Butyloxyoxycarbonyl)-(4*S*)-azido-(2*R*)-(*N*^{4}-benzyloxy-carbonylcytosin-1-ylmethyl)pyrrolidine (2a)

A mixture of 1-(*N*-Boc)-(4*S*)-azido-(2*R*)-(*O*-mesylmethyl)-pyrrolidine **1** (0.80 g, 2.50 mmol), anhydrous potassium carbonate (0.86 g, 6.25 mmol), *N*^{4}-benzyloxycarbonylcytosine (0.60 g, 3.00 mmol) and 18-crown-6 (0.13 g, 0.75 mmol) in dry DMF was stirred at 60 °C under argon atmosphere overnight. The solvent was completely removed under vacuum and the crude residue was purified by column chromatography subsequent to filtering and evaporation of the filtrate to obtain the pure product in the form of liquid gum **2a** (0.27 g, 30% yield).

$[\alpha]^{24}_D$ −25.0 (*c* 0.04, MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.65 (d, 1H), 7.40 (s, 5H), 7.15 (d, 1H), 5.20 (s, 2H), 4.20 (m, 2H), 4.05 (m, 1H), 3.80 (m, 1H), 3.55 (m, 1H), 3.35 (m, 1H), 2.15 (m, 2H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 162.3, 155.8, 154.3, 152.6, 148.7, 134.9, 128.2, 128.0, 127.7, 95.0, 80.3, 67.2, 58.5, 55.7, 51.3, 36.0, 34.3, 27.8. MS: calc. for $C_{22}H_{28}N_7O_5$ (M^+) *m*/*z* 470.5, found 470.0.

(4*S*)-Azido-(2*R*)-(*N*^{4}-benzyloxycarbonylcytosin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid ethyl ester (3a)

To a solution of 1-(*N*-Boc)-(4*S*)-azido-(2*R*)-(*N*^{4}-benzyloxy-carbonylcytosin-1-ylmethyl)pyrrolidine (0.27 g, 0.57 mmol) in dry CH_2Cl_2 (1 ml) was added TFA (1 ml) and the reaction was stirred at room temperature for 10 min, when TLC indicated a complete absence of starting material. The resulting free amine was immediately subjected to alkylation using ethyl bromo-acetate (0.07 ml, 0.63 mmol) in dry THF (2 ml) in the presence of DIPEA (0.060 ml, 0.35 mmol). The amine was completely consumed within 3 h, upon which, the solvents were removed under vacuum and the crude product purified by silica gel column chromatography to obtain the pure product **3a** in the form of a gum (0.24 g, 93%).

^{1}H NMR ($CDCl_3$) δ: 7.92 (d, 1H, *J* = 7.32 Hz), 7.35 (s, 5H), 7.16 (d, 1H, *J* = 7.33 Hz), 5.19 (s, 2H), 4.14 (m, 4H), 3.89 (m, 1H), 3.70 (m, 1H), 3.40 (m, 2H), 3.29 (m, 1H), 2.58 (m, 1H), 2.04 (m, 1H), 1.85 (m, 1H), 1.24 (t, 3H, *J* = 7.32 Hz). ^{13}C NMR ($CDCl_3$) δ: 170.4, 162.4, 155.5, 152.7, 149.8, 135.0, 128.2, 128.0, 127.7, 94.4, 67.2, 60.5, 59.2, 58.3, 55.4, 51.6, 40.3, 34.3, 13.7. ESIMS: calc. for $C_{21}H_{25}N_7O_4$ (M^+) *m*/*z* 439.4, found 440 (M + 1).

(4*S*)-(*N*-Butyloxyoxycarbonylamino)-(2*R*)-(*N*^{4}-benzyloxy-carbonylcytosin-1-ylmethyl)pyrrolidin-*N*-1-acetic acid ethyl ester (4a)

(4*S*)-Azido-(2*R*)-(*N*^{4}-benzyloxycarbonylcytosin-1-ylmethyl)-pyrrolidin-*N*-1-ethyl acetate (0.24 g, 0.55 mmol) was reduced to the (4*S*)-amine by hydrogenation using Raney–Ni as a catalyst (0.16 g) in ethyl acetate at room temperature for 2 h, which was converted *in situ* to the corresponding *tert*-butyl carbamate by Boc-anhydride (0.14 g, 0.66 mmol). The product was purified by silica gel column chromatography to obtain pure **4a** in the form of a gum (0.25 g, 90% yield).

$[\alpha]^{24}_D$ −33.1 (*c* 0.024, MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.72 (br s, 1H), 7.37 (s, 5H), 7.17 (br s, 1H), 5.20 (s, 2H), 5.08 (m, 1H), 4.13 (m, 4H), 3.79 (m, 2H), 3.69 (m, 1H), 3.42 (m, 2H), 3.31 (m, 1H), 2.55 (m, 1H), 2.03 (m, 1H), 1.41 (s, 9H), 1.24 (br s, 3H). ^{13}C NMR ($CDCl_3$) δ: 171.1, 162.8, 156.0, 155.6, 152.9, 150.0, 135.3, 128.7, 128.5, 128.3, 94.8, 79.6, 67.8, 60.8, 59.1, 58.7, 54.1, 53.5, 49.8, 36.1, 28.4, 14.2. ESIMS: calc. for $C_{26}H_{36}N_5O_7$ *m*/*z* 530.6, found 530.0.

(4*S*)-(*N*-Butyloxyoxycarbonylamino)-(2*R*)-(*N*^{4}-benzyloxy-carbonylcytosin-1-ylmethyl)pyrrolidin-*N*-1-acetic acid (5a)

To a solution of (4*S*)-(*N*-Boc-amino)-(2*R*)-(*N*^{4}-benzyloxy-carbonylcytosin-1-ylmethyl)pyrrolidin-*N*-1-ethyl acetate **4a** (0.1 g, 0.44 mmol) in methanol–water, 1 : 1 (3 ml) was added 1 M NaOH (1 ml) and stirred for 20 min at room temperature. The excess alkali was neutralized using Dowex H^+ resin, which was then filtered off to obtain the product as a white foam (90% yield).

$[\alpha]^{24}_D$ −5.0 (*c* 0.002 MeOH); ^{1}H NMR (D_2O) δ: 7.41–7.31 (m, 2H), 6.99–6.91 (m, 1H), 5.18 (m, 1H), 2.55 (s 1H), 2.31–1.68 (m, 2H), 1.46 (s, 2H), 1.45–1.32, 2.3 (s, 2H). ^{13}C NMR (D_2O) δ: 172.13, 170.97, 158.79, 155.63, 155.28, 153.95, 150.84, 142.66, 138.64, 137.54, 128.63, 128.48, 128.29, 128.17, 128.06, 127.97, 127.85, 127.80, 126.77, 126.58, 85.06, 63,07, 62.97, 59.61, 58.59, 57.72, 53.85, 52.56, 51.66, 40.38, 34.33, 28.37, 27.97. ESIMS: calc. for $C_{25}H_{35}N_5O_6$ *m*/*z* 501.5, found 502 (M^+); λ_{max} = 270 nm, ε = 7.8 cm^2 μmol^{-1}.

1-(*N*-Butyloxyoxycarbonyl)-(4*S*)-azido-(2*R*)-(*N*^{6}-benzoyl-adenin-9-ylmethyl)pyrrolidine (2b)

A mixture of *N*^{6}-benzoyladenine (0.80 g, 3.15 mmol) and K_2CO_3 (0.86 g, 6.25 mmol) in dry DMF was stirred at 60 °C for 1 h. A solution of 1-(*N*-Boc)-(4*S*)-azido-(2*R*)-(methyl-*O*-mesyl)-

pyrrolidine (0.84 g, 2.63 mmol) in DMF was added dropwise to this mixture and stirring was continued at 60 °C for 5 h, when TLC inspection indicated absence of the starting material. The solvent was completely removed under vacuum and the crude residue purified by column chromatography to obtain the pure product **2b** in the form of a gum (0.26 g, 40% yield).

$[\alpha]^{24}_{D}$ +35.7 (*c* 0.014, MeOH); ^{1}H NMR ($CDCl_3$) δ: 8.77 (s, 1H), 8.02 (m, 3H), 7.54 (m, 3H), 4.60 (m, 2H), 4.31 (m, 1H), 3.79 (m, 1H), 3.50 (m, 1H), 2.06 (m, 2H), 1.49 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 164.9, 154.2, 152.2, 152.1, 149.4, 143.2, 133.3, 132.2, 128.2, 127.7, 122.4, 80.5, 58.3, 55.8, 51.4, 44.5, 34.4, 27.9. ESIMS: calc. for $C_{22}H_{25}N_9O_3$ (M^+) *m*/*z* 463.5, found 464.0 (M + 1).

(4*S*)-Azido-(2*R*)-(*N*^{6}-benzoyladenin-9-ylmethyl)pyrrolidin-*N*-1-ethyl acetate (3b)

To a solution of 1-(*N*-Boc)-(4*S*)-azido-(2*R*)-(*N*^{6}-benzoyladenin-9-ylmethyl)pyrrolidine **2b** (0.39 g, 0.84 mmol) in dry CH_2Cl_2 (1 ml) was added TFA (1 ml) and the reaction was stirred at room temperature for 10 min, when TLC indicated a complete absence of starting material. The resulting free amine was immediately subjected to alkylation using ethyl bromoacetate (0.1 ml, 0.93 mmol) in dry THF (4 ml) in the presence of DIPEA (0.22 ml, 1.26 mmol). The amine was completely consumed within 2.5 h, upon which, the solvents were removed under vacuum and the crude product purified by silica gel column chromatography to obtain the pure product **3b** in the form of a gum (95% yield).

$[\alpha]^{24}_{D}$ +35.7 (*c* 0.004, MeOH); ^{1}H NMR ($CDCl_3$) δ: 8.70, (s, 2H), 8 (m, 3H), 7.6 (m, 4H), 4.2, (m, 2H), 3.5 (m, 2H), 3.4 (m, 2H), 2.7, (m, 1H), 2, (m, 1H), 1.3 (t, 3H). ^{13}C NMR ($CDCl_3$) δ: 176.5, 170.2, 146.7, 146.3, 145.5, 140.9, 135.2, 131.6, 129.4, 127.7, 120.7, 60.8, 60.5, 58.6, 58.1, 54.1, 45.4, 34.3, 13.8. ESIMS: calc. for $C_{22}H_{25}N_9O_3$ *m*/*z* 463.6, found 464.0 (M^+).

(4*S*)-(*N*-Butyloxyoxycarbonylamino)-(2*R*)-(*N*^{6}-benzoyladenin-9-ylmethyl)pyrrolidin-*N*-1-ethyl acetate (4b)

(4*S*)-Azido-(2*R*)-(*N*^{6}-benzoyladenin-9-ylmethyl)pyrrolidin-*N*-1-ethyl acetate **3b** (0.34 g, 0.76 mmol) was reduced to the (4*S*)-amine by hydrogenation using 10% Pd–C as catalyst at room temperature for 2 h. The resulting amine (0.30 g, 0.71 mmol) was converted *in situ* to the corresponding *tert*-butyl carbamate by Boc-anhydride (0.19 g, 0.85 mmol). The crude product was purified by silica gel column chromatography to obtain pure product **4b** in the form of a gum (0.37 g, 98% yield).

^{1}H NMR ($CDCl_3$) δ: 8.10 (m, 2H), 7.90 (s, 1H), 7.45 (m, 3H), 7.25 (s, 1H), 5.10 (m, 1H), 4.20 (m, 4H), 4.05 (m, 1H), 3.75 (m, 1H), 3.40 (m, 4H), 2.60 (m, 1H), 1.45 (s, 9H), 1.28 (t, 3H). ^{13}C NMR ($CDCl_3$) δ: 171.2, 162.7, 155.5, 152.4, 152.0, 149.7, 144.6, 133.7, 132.4, 128.4, 128.2, 12.8.0, 122.5, 78.9, 60.7, 60.1, 58.8, 53.1, 49.0, 45.9, 35.7, 28.2, 14.1. ESIMS: calc. for $C_{26}H_{25}N_7O_5$ *m*/*z* 515.5, found 515.0.

(4*S*)-(*N*-Butyloxyoxycarbonylamino)-(2*R*)-(*N*^{6}-benzoyladenin-9-ylmethyl)pyrrolidin-*N*-1-acetic acid (5b)

To a solution of (4*S*)-(*N*-Boc-amino)-(2*R*)-(*N*^{6}-benzoyladenin-9-ylmethyl)pyrrolidin-*N*-1-ethyl acetate **4b** (0.34 g, 0.76 mmol) in methanol–water, 1 : 1 (3 ml) was added 1 M NaOH (1 ml) and the reaction mixture was stirred for 20 min at room temperature when TLC indicated complete absence of starting material. The excess alkali was neutralized using Dowex H^+ resin which was then filtered off. The filtrate was then evaporated to obtain the product as a white foam (90% yield).

$[\alpha]^{24}_{D}$ +20 (*c* 0.002, MeOH); ESIMS: calc. for $C_{24}H_{29}N_7O_5$ *m*/*z* 495.5, found 497 (M + 1); λ_{max} = 270 nm, ε = 16.0 cm^2 μmol^{-1}.

1-(*N*-Butyloxyoxycarbonyl)-4-(*S*)-azido-2(*R*)-(2-amino-6-chloropurin-9-ylmethyl)pyrrolidine (2c)

A mixture of 1-(*N*-Boc)-(4*S*)-azido-(2*R*)-(methyl-*O*-mesyl)-pyrrolidine **1** (0.2 g, 0.6 mmol), anhydrous potassium carbonate (0.22 g, 1.56 mmol), 2-amino-6-chloropurine (0.13 g, 0.78 mmol) and 18-crown-6 (0.03 g, 0.09 mmol) in dry DMF were stirred at 65 °C under argon atmosphere overnight. The solvent was completely removed under vacuum and the crude residue, purified by column chromatography subsequent to filtering and evaporation of the filtrate to obtain the pure product **2c** in the form of a gum (45% yield).

$[\alpha]^{24}_{D}$ +5.83 (*c* 0.012, MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.60 (s, 1H), 5.15 (br, 2H), 4.50 (m, 1H), 4.20 (m, 2H), 3.73 (m, 1H), 3.40 (m, 1H), 3.10 (m, 1H), 1.95 (m, 2H), 1.40 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 159.2, 153.9, 150.6, 142.4, 123.9, 80.3, 58.2, 55.5, 51.4, 44.1, 34.1, 27.6. ESIMS: calc. for $C_{15}H_{20}N_9O_2Cl$ *m*/*z* 393.3, found 394 (M + 1).

(4*S*)-Azido-(2*R*)-(2-amino-6-chloropurin-9-ylmethyl)-pyrrolidin-*N*-1-ethyl acetate (3c)

To a solution of 1-(*N*-Boc)-(4*S*)-azido-(2*R*)-(2-amino-6-chloropurin-9-ylmethyl)pyrolidine **2c** (0.08 g, 0.2 mmol) in dry CH_2Cl_2 (1 ml) was added TFA (1 ml) and the reaction was stirred at room temperature for 10 min, when TLC indicated a complete absence of starting material. The TFA salt was neutralized with DIPEA and the free amine was immediately subjected to alkylation using ethyl bromoacetate (0.025 ml, 0.20 mmol) in dry THF (1 ml) in the presence of DIPEA (0.053 ml, 0.31 mmol). The amine was completely consumed within 2 h, upon which, the solvents were removed under vacuum and the crude product purified by silica gel column chromatography to obtain the pure product **3c** in the form of a gum (89% yield).

$[\alpha]^{24}_{D}$ +36.66 (*c* 0.012, MeOH), ^{1}H NMR ($CDCl_3$) δ: 8.05 (s, 1H), 5.25 (s, 2H), 4.20 (m, 2H), 4.05 (m, 2H), 3.70 (m, 1H), 3.40 (m, 2H), 2.65 (m, 1H), 2.00 (m, 1H), 1.75 (m, 1H), 1.30 (t, 3H). ^{13}C NMR ($CDCl_3$) δ: 170.3, 159.4, 150.7, 143.4, 124.2, 60.9, 60.5, 58.8, 58.3, 54.7, 45.4, 34.4, 31.1, 13.8. ESIMS: calc. for $C_{15}H_{20}O_2N_9Cl$ *m*/*z* 393.8, found 394 (M^+).

(4*S*)-(*N*-Butyloxyoxycarbonylamino)-(2*R*)-(2-amino-6-chloropurin-9-ylmethyl)pyrrolidine-*N*-1-ethyl acetate (4c)

(4*S*)-Azido-(2*R*)-(2-amino-6-chloropurin-9-ylmethyl)pyrrolidine-*N*-1-ethyl acetate **3c** (0.07 g, 0.18 mmol) was reduced to the (4*S*)-amine by hydrogenation using 10% Pd–C as catalyst (0.05 g) in methanol, which was converted *in situ* to the corresponding *tert*-butyl carbamate by Boc-anhydride (0.08 g, 0.46 mmol) to obtain the crude product that was purified by silica gel column chromatography to obtain pure **4c** in the form of a gum (85% yield).

^{1}H NMR ($CDCl_3$) δ: 7.95 (s, 1H), 5.28 (s, 2H), 5.13 (m, 1H), 4.14 (m, 5H), 3.48 (m, 1H), 3.28 (m, 2H), 2.93 (m, 2H), 2.51 (m, 1H), 2.28 (m, 1H), 1.37 (s, 9H), 1.26 (t, 3H). ^{13}C NMR ($CDCl_3$) δ: 171.2, 159.3, 155.4, 154.3, 151.2, 143.5, 124.7, 77.9, 61.0, 59.8, 59.2, 58.8, 55.1, 46.0, 36.5, 28.4, 14.2. ESIMS: calc. for $C_{19}H_{27}O_4N_2Cl$ *m*/*z* 439.8, found 456 (M + 17, M + NH_3).

(4*S*)-(*N*-Butyloxyoxycarbonylamino)-(2*R*)-(guanin-9-ylmethyl)pyrrolidin-*N*-1-acetic acid (5d)

To a solution of (4*S*)-(*N*-Boc-amino)-(2*R*)-(2-amino-6-chloropurin-9-ylmethyl)pyrrolidin-*N*-1-ethyl acetate (0.2 g, 0.5 mmol) in methanol–water, 1 : 1 (5 ml) was added 1 M NaOH (2 ml) and stirred overnight at room temperature. The excess alkali was neutralized using Dowex H^+ resin which was then filtered off. The filtrate was then concentrated to obtain the pure product as a white foam (88% yield).

$[\alpha]^{24}_{D}$ −5.0 (*c* 0.002, MeOH); ^{1}H NMR (D_2O) δ: 8.37–8.16 (m, 1H), 4.47–4.19 (m, 2H), 4.26–3.96 (m, 5H), 3.71–3.66 (m, 1H), 3.38–3.21 (m, 1H), 2.36–2.21 (m, 1H), 2.15–1.89 (m, 1H), 1.57–1.30 (m, 9H). ^{13}C NMR (D_2O) δ: 170.6, 169.9, 159.5, 156.9, 154.2, 140.5, 139.7, 69.5, 65.4, 65.0, 63.0, 62.4, 59.6, 59.2, 58.6, 57.3, 56.6, 55.2, 54.8, 47.1, 42.9, 30.3, 29.5, 27.3. ESIMS: calc. for $C_{16}H_{24}O_5N_7$ *m*/*z* 406.2, found 408 (M + 1); λ_{max} = 270 nm, ε = 11.9 cm^2 μmol^{-1}.

(2*R*/*S*,4*S*)-*N*-1-(Benzyloxycarbonyl)-4-(*p*-toluenesulfonyloxy)-proline methyl ester (7)

(2*R*/*S*,4*R*)-*N*-1-(benzyloxycarbonyl)-4-hydroxyproline methyl ester **6** (5 g, 7.85 mmol), PPh_3 (3.97 g, 19.6 mmol) and methyl-*p*-toluene sulfonate (3.65 g, 19.4 mmol) were taken in dry THF (100 ml) and the mixture was cooled to −10 °C in an ice-salt bath. To this, a solution of diisopropylazodicarboxylate (5.14 ml, 25 mmol) in dry THF (10 ml) was added using a syringe over a period of 1 h. The reaction mixture was further stirred for 1 h at −10 °C followed by 12 h at room temperature, when the TLC indicated the complete conversion of starting material. The reaction mixture was concentrated and was subjected to silica gel column chromatography. The separation proved to be difficult as the diisopropyl-hydrazodicarboxylate moves very closely with the product.

7a: $[\alpha]^{24}_D$ +15.31 (*c* 0.322, MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.9–7.5 (m, 2H) 7.3–7.1 (m, 2H) 5.1–4.8 (m, 3H) 4.5–4 (m, 2H) 3.7–3.5 (s, 3H), 3.7 3.2–3.0 (s, 2H), 2.5–2.3 (s, 3H), 2.2–2.0 (m, 3H). ^{13}C NMR ($CDCl_3$): δ 172.5, 156.0, 143.0, 136.2, 132.6, 132.1, 130.4, 129.9, 126.3, 125.6, 65.36, 62.0, 51.2, 22.5, 20.1, 13.4. ESIMS: calc. for $C_{20}H2_3O_8NS$ *m*/*z* 433.4, found 434.0 (M + 1).

7b: $[\alpha]^{24}_D$ −1.1 (*c* 0.012, MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.9–7.10 (m, 2H), 7.4–7.1 (m, 2H), 5.4–4.8 (m, 2H), 4.6–3.9 (m, 2H), 3.8–3.49 (m, 4H), 2.5 (s, 3H), 2.2–1.9 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 173.5, 156.7, 145.0, 136.2, 133.6, 132.1, 131.5, 129.8, 127.3, 127.6, 67.36, 62.0, 52.2, 21.5, 20.6, 14.3. ESIMS: calc. for $C_{20}H2_3O_8NS$ *m*/*z* 433.4, found 434.0 (M + 1).

(2*R*/*S*,4*R*)-*N*-1-(Benzyloxycarbonyl)-4-azidoproline methyl ester (8)

The mixture from the above step was taken in dry DMF (50 ml) and to this was added NaN_3 (7.64 g, 117 mmol). The mixture was stirred at 60 °C for 12 h when TLC indicated complete conversion of starting material, DMF was removed under vacuum on a rotary evaporator, 100 ml of water was added to the mixture and extracted with ethyl acetate (30 ml × 3). The organic layers were pooled, washed with water (20 ml) and concentrated to give the crude product that was further purified by column chromatography as a thick liquid (85% yield).

8a: $[\alpha]^{24}_D$ +20.45 (*c* 0.022, MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.8–7.4 (m, 2H), 7.3–7.2 (m, 2H), 5.2–4.7 (m, 3H), 4.45 (m, 2H), 3.66 (s, 3H), 3.42 (m, 2H), 2.13–1.48 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 172.5, 154.2, 153.8, 128.3, 128.2, 127.8, 127.8, 127.6, 69.5, 58.9, 52.2, 51.1, 50.8, 35.8, 21.7. ESIMS: calc. for $C_{14}H_{16}O_4N_4$ *m*/*z* 304.3, found 305.0 (M + 1).

8b: $[\alpha]^{24}_D$ −50.9 (*c* 0.022, MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.4 (s, 5H), 5.35–5 (m, 2H), 4.5–4.32 (m, 1H), 4.25–4.15 (m, 1H), 3.75 (s, 3H), 3.6–3.4 (m, 2H), 2.4–2.3 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 171.5, 171.2, 154.2, 153.8, 136.0, 128.1, 127.6, 67.1, 67.0, 58.9, 57.5, 57.2, 52.2, 51.1, 50.8, 35.8, 34.8, 21.6. ESIMS: calc. for $C_{14}H_{16}O_4N_4$ *m*/*z* 304.2, found 321 (M + 17, M + NH_3).

(2*R*/*S*,4*R*)-*N*-1-(Benzyloxycarbonyl)-4-(*tert*-butyloxycarbonyl-amino)proline methyl ester (9)

Compound **8** (4.74 g, 15 mmol) was dissolved in a dry ethyl acetate and to this was added Raney-Ni (4 ml) and Boc-anhydride (4 ml, 18 mmol). The mixture was subjected to hydrogenation for 2 h at a pressure of 40 psi in a Parr-hydrogenation apparatus when TLC indicated complete conversion of starting material. The crude product was purified by column chromatography to obtain **9** as a gum (95% yield).

9a: $[\alpha]^{24}_D$ +9.0 (*c* 0.02 MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.5 (s, 5H), 5.20–5.1 (m, 2H), 4.27 (m, 1H), 3.87 (m, 1H), 3.6–3.4 (m, 3H), 3.39–3.32 (m, 1H), 2.46–2 (m, 2H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 173.5, 154.8, 154.4, 153.7, 136.0, 128.1, 127.7, 127.5, 79.36, 67.0, 57.7, 57.3, 53.0, 52.8, 51.9, 49.7, 48.7, 36.6, 35.5, 28.0. ESIMS: calc. for $C_{19}H_{26}N_2O_6$ *m*/*z* 378.2, found 378.0.

9b: $[\alpha]^{24}_D$ −17.20 (*c* 0.022 MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.4–7.29 (m, 5H), 5.22–5.11 (m, 2H), 5.08–5.04 (m, 1H), 4.66 (br s, 1H), 4.88–4.40 (m, 1H), 4.37–4.26 (m, 1H), 3.89–3.84 (m, 1H), 3.76 (s, 2H), 3.58 (s, 1H), 3.45–3.34 (m, 1H), 2.28–2.15 (m, 2H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 172.2, 172.0, 154.8, 154.5, 153.8, 135.9, 128.1, 127.7, 127.6, 77.0, 76.7, 67.0, 57.5, 52.1, 51.9, 51.5, 36.6, 35.5, 28.0. ESIMS: calc. for $C_{19}H_{26}N_2O_6$ *m*/*z* 378.2, found 379.0 (M + 1).

(2*R*/*S*,4*R*)-*N*-(Benzyloxycarbonyl)-4-(*tert*-butyloxycarbonylamino)-pyrrolidine-2-methanol (10)

To a stirred mixture of dry ethanol (20 ml) and dry THF (30 ml), was added lithium chloride (2.16 g, 50 mmol) and sodium borohydride (1.88 g, 50 mmol) and stirring continued for ~10 min under argon atmosphere. A solution of (2*R*,4*R*)-*N*-(benzyloxycarbonyl)-4-(*tert*-butyloxycarbonylamino)proline methyl ester **9** (5.49 g, 14 mmol) in THF (10 ml) was added dropwise and stirring was continued at room temperature under an argon atmosphere for 6 h. When TLC indicated complete disappearance of starting material, the pH of the reaction mixture was adjusted to 4 with aqueous ammonium chloride. The reaction mixture was stirred for 10 min and the solvents were removed on a rotary evaporator. The residue was taken up in water and extracted with ethyl acetate (4 × 20 ml). The organic layer was then dried over sodium sulfate and evaporated to obtain the crude product. Silica gel column chromatography yielded the pure product in the form of a gum (80% yield).

10a: $[\alpha]^{24}_D$ +5.0 (*c* 0.024 MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.42–7.30 (s, 5H), 5.18–5.12 (m, 2H), 4.86 (br s, 1H), 4.27–3.96 (m, 4H), 3.87–3,79 (m, 1H), 3.76–3.52 (m, 3H), 3.49–3.39 (m, 1H), 2.46–2.0 (m, 2H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 154.0, 128.2, 127.8, 127.5, 79.5, 67.0, 65.4, 55.7, 52.7, 51.9, 48.8, 34.2, 33.6 28.0. ESIMS: calc. for $C_{18}H_{26}O_5N_2$ *m*/*z* 350.4, found 350.0.

10b: $[\alpha]^{24}_D$ −27.5 (*c* 0.004, MeOH); ^{1}H NMR ($CDCl_3$) δ 7.40–7.32 (m, 4H), 5.16 (br s, 2H), 4.72–4.64 (m, 1H), 4.27–4.03 (m, 2H), 3.79–3.6 (m, 3H), 3.5–3.4 (s, 2H), 2.01–1.75 (m, 2H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 156.0, 154.9, 136.0, 128.2, 127.9, 127.6, 79.5, 66.0, 58.8, 52.8, 51.0, 34.4, 28.0. ESIMS: calc. for $C_{18}H_{26}O_5N_2$ *m*/*z* 350.4, found 350.0.

***N*-1-(Benzyloxycarbonyl)-(4*R*)-(*tert*-butyloxycarbonylamino)-(2*R*/*S*)-(*O*-mesylmethyl)pyrrolidine (11)**

To a stirred, cooled mixture of alcohol **10** (1.64 g, 4.6 mmol) and triethylamine (1.95 ml, 19.30 mmol) in dry dichloromethane (10 ml) was added dropwise methanesulfonyl chloride (0.64 ml, 5.5 mmol). Stirring was continued for further 30 min when TLC indicated absence of starting material. The solvent was removed under vacuum, the residue was taken up in water and extracted with ethyl acetate (2 × 10 ml). The organic layer was dried over sodium sulfate, and then evaporated to dryness. Silica gel column chromatography yielded the pure product in the form of a gum (95% yield).

11a: $[\alpha]^{24}_D$ +25.5 (*c* 0.02, MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.4 (s, 5H), 5.0 (s, 2H), 4.9–4.8 (m, 1H), 4.35–3.7 (m, 4H), 2.8 (s, 3H), 2.49–2.3 (m, 1H), 1.8–1.7 (m, 1H), 1.4 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 154.0, 155.0, 136.0, 128.2, 127.7, 80.3, 68.0, 67.3, 66.0, 55.1, 52.2, 48.5, 36.7, 33.2, 28.0, 21.6. ESIMS: calc. for $C_{19}H_{28}O_8N_2S$ *m*/*z* 446.3, found 448.0 (M + 1).

11b: ^{1}H NMR ($CDCl_3$) δ: 7.4 (s, 5H), 5.0 (s, 2H), 4.9–4.8 (m, 1H), 4.35–3.7 (m, 4H), 2.8 (s, 3H), 2.49–2.3 (m, 1H), 1.8–1.7 (m, 1H), 1.4 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 154.8, 155.6, 154.1, 136.0, 128.6, 79.7, 68.9, 66.8, 60.1, 54.8, 54.3, 52.2, 36.9, 36.7, 33.7, 31.2, 28.0. ESIMS: calc. for $C_{19}H_{28}O_8N_2S$ *m*/*z* 446.3, found 448.0 (M + 1).

***N*-1-(Benzyloxycarbonyl)-(4*R*)-(*tert*-butyloxycarbonylamino)-(2*R*/*S*)-(thymin-1-ylmethyl)pyrrolidine (12)**

To compound **11** (1 g, 2.7 mmol) in dry DMF was added anhydrous potassium carbonate (0.6 g, 5 mmol), thymine (0.51 g, 4 mmol) and 18-crown-6 (0.26 g, 0.98 mmol). The reaction mixture was allowed to stir at 60 °C for 4 h. When TLC indicated complete conversion of starting material, solvent was removed under vacuum. Silica gel column chromatography yielded the pure product in the form of a gum (0.4 g, 35%).

12a: $[\alpha]^{24}_D$ +41.25 (*c* 0.008, MeOH); ^{1}H NMR ($CDCl_3$) δ: 7.3 (s, 5H), 5.1 (s, 2H), 4.2–3.6 (m, 5H), 3.49–3.3 (m, 1H), 2.3–2.1 (m, 1H), 1.9–1.7 (m, 5H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 163.8, 161.0, 155.4, 154.9, 151.7, 140.1, 135.9, 128.2, 127.9, 127.6, 127.5, 66.9, 56.6, 52.7, 49.5, 48.5, 36.1, 32.8, 28.0, 11.8. ESIMS: calc. for $C_{23}H_{30}O_6N_4$ *m*/*z* 458.7, found 458.0.

12b: $[\alpha]^{24}_D$ −10.0 (*c* 0.01, MeOH); ^{1}H NMR ($CDCl_3$) δ 7.3 (s, 5H), 5.1 (s, 2H), 4.2–3.6 (m, 5H), 3.49–3.3 (m, 1H), 2.3–2.1 (m, 1H), 1.9–1.7 (m, 5H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 163.83, 155.4, 154.9, 151.7, 140.1, 135.9, 128.2, 127.9, 127.6, 127.5, 79.0, 66.9, 56.6, 52.7, 49.5, 48.5, 36.1, 32.8, 28.0, 11.8. ESIMS: calc. for $C_{23}H_{30}O_6N_4$ *m*/*z* 458.7, found 458.0.

(2*S*,4*S*)-4-(*tert*-Butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1-ethyl acetate (13)

Compound **12** (0.35 g, 0.8 mmol) was taken in methanol and to this 10% Pd–C (0.3 g) was added. The mixture was subjected to hydrogenation at a pressure of 50 psi for 6 h when TLC indicated complete absence of starting material. The free amine was immediately used for alkylation using ethyl bromoacetate (0.2 g, 1.6 mmol) in the presence 2.5 equivalents of DIPEA (0.08 ml, 0.35 mmol). The reaction was complete after 2 h as evident from TLC. The solvent was completely removed under vacuum and the crude product was purified by silica gel column chromatography to obtain the pure product in the form of a gum (90% yield).

13a: $[\alpha]^{24}_D$ +5.0 (*c* 0.002, MeOH); ^{1}H NMR ($CDCl_3$) δ: 9.39 (s, 1H), 7.29 (s, 1H), 4.96 (s, 1H), 4.18 (q, 2H), 4.02–3.94 (m, 1H), 3.6–3.37 (m, 2H), 3.26–3.23 (s, 1H), 3.14–3.04 (m, 1H), 3.00–2.91 (m, 1H), 2.80–2.69 (m, 1H), 2.34–2.20 (m, 1H), 1.89 (s, 3H), 1.63–1.52 (m, 1H), 1.39 (s, 9H), 1.28 (t, 3H). ^{13}C NMR ($CDCl_3$) δ: 154.9, 128.3, 127.9, 127.6, 66.9, 55.8, 51.9, 48.9, 34.4, 29.4, 28.0, 13.8, 11.8. ESIMS: calc. for $C_{19}H_{30}N_4O_6$ *m*/*z* 410.4, found 411.

13b: $[\alpha]^{24}_D$ −12.01 (*c* 0.004, MeOH); ^{1}H NMR ($CDCl_3$) δ: 9.41 (s, 1H), 7.29 (s, 1H), 4.77–4.58 (q, 2H), 4.33–3.99 (m, 3H), 3.90–3.28 (m, 3H), 2.97–2.83 (m, 2H), 2.64–2.48 (br s, 1H), 1.95 (s, 3H) 1.76–1.59 (br s, 2H), 1.45 (s, 9H) 1.31–1.26 (t, 3H). ^{13}C NMR ($CDCl_3$) δ: 167.5, 162.8, 154.9, 151.1, 139.7, 63.4, 61.2, 60.9, 59.4, 58.6, 52.8, 52.1, 49.1, 41.8, 36.4, 28.1, 13.8, 12.6. ESIMS: calc. for $C_{19}H_{30}N_4O_6$ *m*/*z* 410.4, found 410.0.

(2*R*/*S*,4*R*)-4-(*tert*-Butyloxycarbonylamino)-2-(thymin-1-ylmethyl)pyrrolidin-*N*-1-acetic acid (14, 15)

To a solution of **13** (0.11 g, 0.27 mmol) in methanol (1 ml) was added 2 M aqueous NaOH (1 ml). The reaction was stirred at room temperature for 10 min, when TLC indicated complete conversion of starting material. The excess of alkali was neutralized by Dowex H^+ resin, which was then filtered off. The filtrate was then evaporated to obtain the product as a white foam (0.10 g, 90%).

(2*R*,4*R*)-4-(*tert*-Butyloxycarbonylamino)-2-(thymin-1-ylmethyl)pyrrolidin-*N*-1-acetic acid (14)

^{1}H NMR (D_2O) δ: 7.28 (s, 1H), 4.47–4.39 (m, 1H), 4.12–4.09 (m, 1H), 4–3.91 (m, 2H), 3.77–3.64 (m, 3H), 3.49–3.39 (m, 1H), 3.22 (br s, 1H), 2.54–2.48 (m, 1H), 1.64 (s, 4H), 1.14 (s, 9H). ^{13}C NMR (D_2O) δ: 170.04, 166.51, 157.43, 153.63, 143.07, 111.26, 81.65, 65.70, 60.37, 55.92, 47.75, 47.28, 46.55, 34.44, 32.72, 31.83, 29.53, 27.34, 11.10. ESIMS: calc. for $C_{17}H_{26}N_4O_6$ *m*/*z* 382.4, found 383.0 (M + 1); λ_{max} = 270 nm, ε = 8.6 cm^2 μmol^{-1}.

(2*S*,4*R*)-4-(*tert*-Butoxycarbonylamine)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid (15)

^{1}H NMR (D_2O) δ: 7.31 (s, 1H), 4.45 (s, 1H), 4.12–3.9 (m, 5H), 3.04 (m, 1H), 2.78 (s, 1H), 2.06 (m, 2H), 1.70 (s, 3H), 1.20 (s, 9H). ^{13}C NMR (D_2O) δ: 172.18, 169.52, 164.79, 156.97, 152.76, 140.83, 110.88, 81.88, 65.20, 62.98, 60.10, 57.47, 57.18, 55.20, 48.53, 43.06, 36.72, 32.87, 29.50, 27.41, 11.75. ESIMS: calc. for $C_{17}H_{26}N_4O_6$ *m*/*z* 382.4, found 383.0 (M + 1); λ_{max} 270 nm, ε = 9.0 cm^2 μmol^{-1}.

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