Synthesis of a Peptide Nucleic Acid with a Novel 1-Methyl-6-mercaptopurine Base [1]

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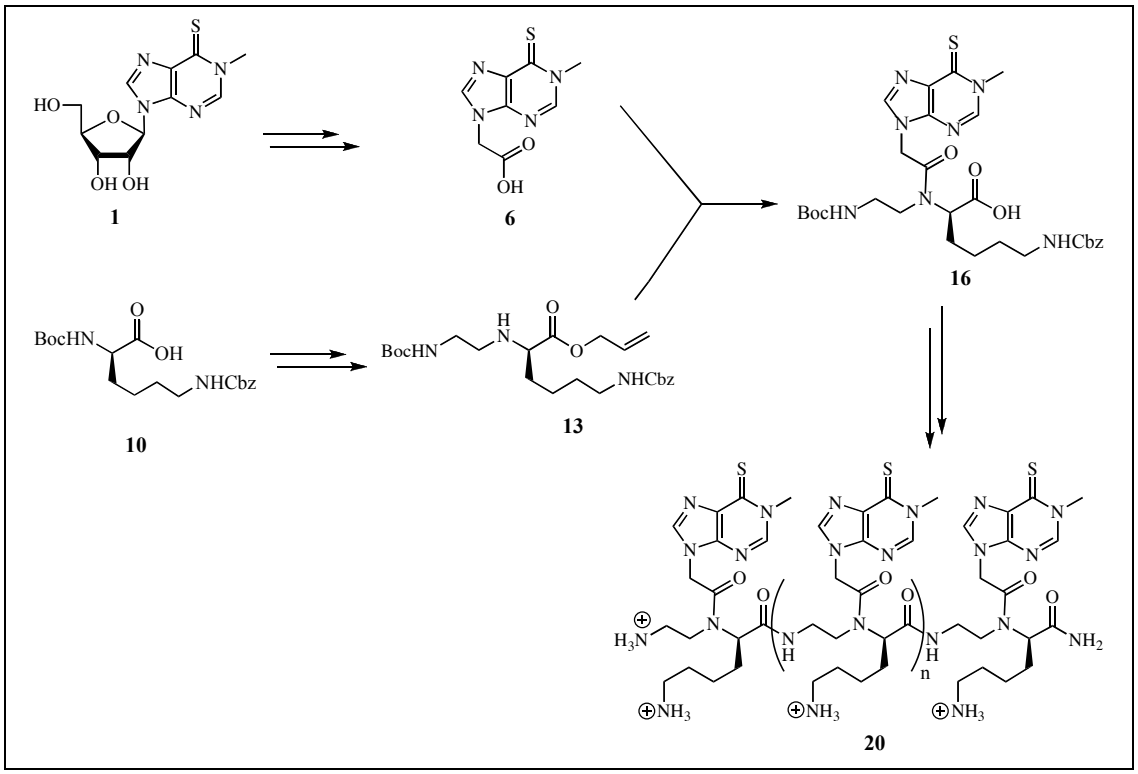

A novel peptide nucleic acid (PNA) monomer **16** containing a novel 1-methyl-6-mercaptopurine base was synthesized by coupling the *in situ* generated acid chloride of (1-methyl-6-mercaptopurin-9-yl)acetic acid (**6**) into an L-lysine backbone (**13**) using 10% CCl_4 in pyridine and Ph_3P. Compound **6** was synthesized from 6-mercapto-1-methylpurine and ethylbromoacetate in the presence of NaH followed by alkaline hydrolysis and subsequent neutralization with a cation exchange resin. The L-lysine backbone (**13**) was obtained by the reaction of Nε-CBZ-L-lysine allyl ester with Boc-aminoactaldehyde in the presence of $NaBH_3CN$ under reductive amination conditions. Oligomerization of the monomer **16** to PNA analogues was achieved using BOC-BHA-PEG-PS resin as a solid support and the *in situ* generated acid chloride of **16** by 10% CCl_4 in DCM in the presence of Ph_3P.

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INTRODUCTION

Peptide nucleic acids (PNAs) are nucleic acid mimics that have potential for modulating gene expression *via* antisense or antigene mechanism or can be used as diagnostic and molecular biology tools [3,4]. Moreover, PNAs may also find various interesting applications in chemistry and technology, *e.g.* as electrochemical biosensors, and in optical data storage [5-7]. In PNA the deoxyribose phosphate backbone of the nucleic acid is replaced by a chiral or achiral pseudopeptide backbone and nucleobases are attached *via* methylene carbonyl linkers (Figure 1). This structure gives an increased stability of PNA-containing duplexes with DNA or RNA, and increased resistance of PNA oligomers to nucleases and proteases compared with natural nucleic acids [8].

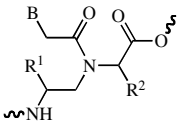


Figure 1

The main limitations of the usefulness of PNAs are their poor solubility in physiological solutions and low

permeability through cellular membranes. The PNA structure is easy to modify and it is probable that synthesis of altered monomers would subsequently result in oligomers with improved properties [9], *e.g*. with better permeability through cellular membranes [10], or with better solubility in physiological fluids [11]. Modified PNA oligomers may constitute a substrate for DNA polymerases [12], and may have numerous other interesting properties [13].

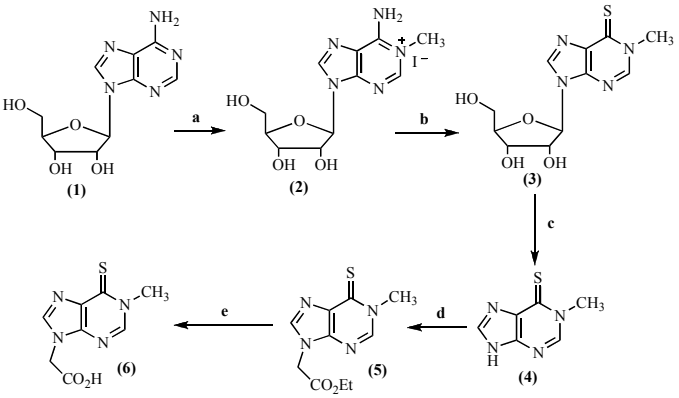
The neutral character of the PNA backbone is an important feature that has many consequences. One of the most impressive is the stronger binding of PNA to DNA and RNA, due in part to the rigidity of the peptide backbone, which arises from the sp2 hybridization as well as the presence of tertiary amide linkages [14,15]. The backbone of PNA lends itself to further chemical modifications, *e.g*. the glycine units can easily be envisaged as replaced by a host of amino acids. One goal has been to test the limits of what the PNA structure will allow without compromising the hybridization properties of PNA. More interestingly, can one tailor specific properties of PNA, such as solubility or electrophoretic mobility, by adding negatively or positively charged amino acid side chains. A large number of chemical modifications of the original aminoethyl glycine PNA backbone have been reported [11,16-22] with only a few appearing to have any practical interest. One of these is the lysine derived backbone; introducing a few lysine-based monomers into a PNA oligomer has been demonstrated to greatly increase water solubility [11].

Accordingly the current work aimed to incorporate 1-methyl-6-thiopurine as a new base to L-lysine backbone based upon earlier work showing that a polyribonucleotide containing this base is a potent inhibitior of HIV and HCMV replication [23,24].

RESULTS AND DISCUSSION

The key intermediate (1-methyl-6-mercaptopurin-9-yl)acetic acid (**6**) was synthesized according to the general procedures of Ueda as subsequently modified for large scale preparations [25,26]. Methylation of adenosine (**1**) by methyl iodide in DMA at room temperature afforded 1-methyladenosine (**2**) in 87% yield. Using the amino-thiono exchange reaction followed by depurination with TFA gave 1-methyl-6-mecaptopurine (**3**) in 85% yield. 1-Methyl-6-mercaptopurine (**3**) could be alkylated with ethyl bromoacetate in DMF by first generating the anion with NaH at room temperature. This procedure yielded one product which was identified as the expected, ethyl (1-methyl-6-mercaptopurin-9-yl)acetate (**5**). Alkaline hydrolysis of **5** followed by neutralization with cation exchange resin afforded the target base **6** in quantitative yield (Scheme 1).

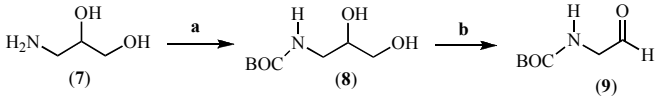
Scheme 1



a, CH_3I/DMA; b, H_2S/pyridine and water, 80ºC steel bomb/3days; c, 50%TFA, 50ºC/24 hrs; d, 1. NaH/DMF, 2. $BrCH_2CO_2Et$; e, 1. NaOH/5 hrs, 2. Dowex50WX8.200 (H^+).

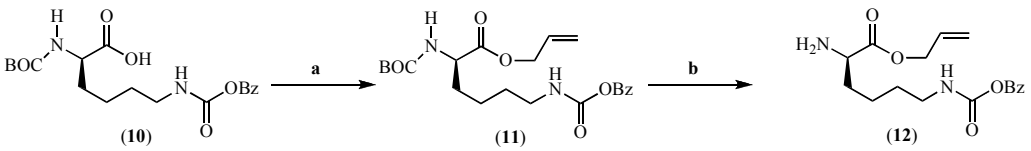
In order to obtain large amounts of starting materials for the synthesis of the monomer employed in preparation of the target PNA, an efficient synthetic route to the L-lysine backbone was needed. The most common method of reduced peptide bond synthesis is the reductive amination of N-protected α-amino aldehydes with amino acid esters [3]. This could be achieved *via* a multi-step reaction described herein. Protection of the amino group of 3-amino-1,2-propandiol (**7**) with *tert*-butyloxycarbonyl (Boc) was undertaken in 86% yield by treatment with di-*tert*-butyl dicarbonate and NaOH. Subsequent periodate oxidation of the crude protected diol **8** yielded almost quantitatively pure BOC-aminoactaldehyde **9** (Scheme 2).

Scheme 2



a, *tert*-$(BOC)_2O$, NaOH/H_2O, 24 hrs; b, $NaIO_4$/H_2O, 2hrs

Scheme 3

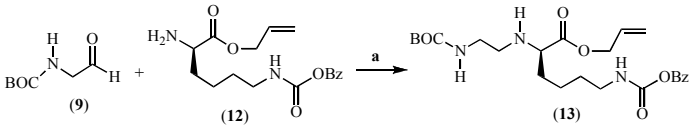


a, Allyl bromide, aliquat 336/ aq. $NaHCO_3$/DCM, 3days; b, 50%TFA/DCM, 20 mins.

It was reported that allyl esters can be used in peptide synthesis as a carboxy protecting group. They could be selectively removed in the presence of the benzyloxy-carbonyl (Z) and the Boc group under mild and neutral conditions [27]. Accordingly, fully protected L-lysine (**11**) was obtained in excellent yield by reacting Nα-Boc-Nε-CBZ-L-lysine (**10**) with allyl bromide under phase-transfer reaction conditions. Boc-Deprotection of the fully protected L-lysine **11** was achieved with 50% TFA in DCM to give **12** (Scheme 3).

The other key intermediate **13** can be prepared in 70% yield by reductive amination of Nε-CBZ-L-lysine allyl ester (**12**) with Boc-aminoactaldehyde (**9**) in the presence of $NaBH_3CN$ (Scheme 4).

Scheme 4

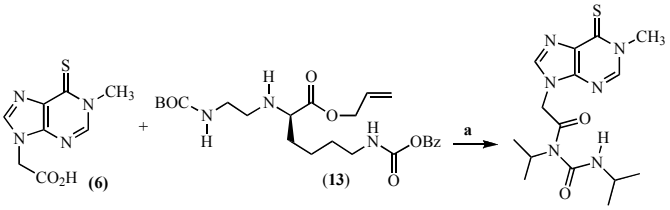


a, $NaBH_3CN$/MeOH.

Coupling of **6** with **13** in presence of standard peptide coupling agents such as DCC and HOBT, DICC and HOBT, BOP, HATU, PyBROP, *etc*. was unsuccessful. When coupling was performed in DMF in presence of DICC, the isolated product **14** was a substituted urea derivative characterized by NMR and mass spectrometry (Scheme 5).

Formation of the urea derivative indicates that the activation of the carboxyl group of **6** occurred, however, coupling to the backbone did not take place. This may be attributed to the steric hindrance of the α-carbon side chain of the backbone **13**. Coupling by using activated ester was tried through formation of the pentafluoro-phenyl ester of **6**, however no reaction took place. Fully

Scheme 5

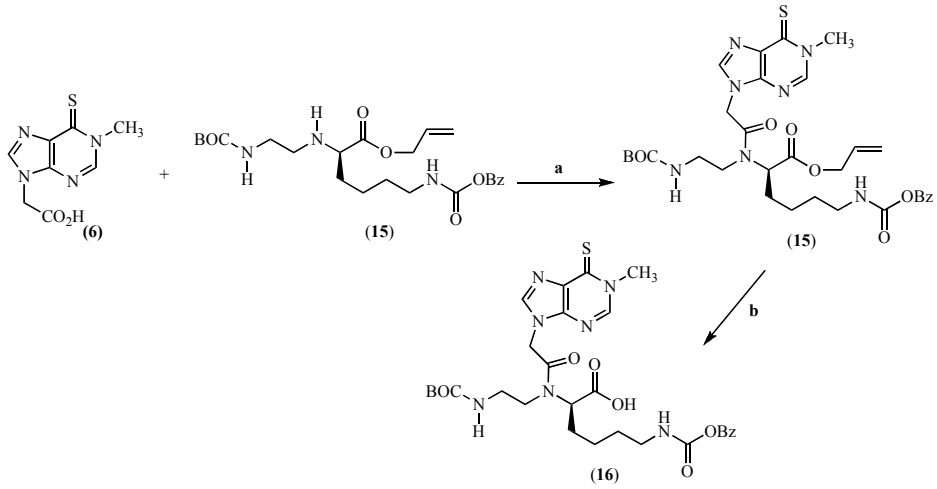


a, DICC/DMF.

protected PNA monomer, **15**, was obtained in 65% yield by coupling **6** with **13** *via in situ* generation of the acid chloride of **6** using 10% CCl_4 in pyridine in the presence of triphenylphosphine. A better yield was obtained when the reaction preformed in DMF and in presence of DIEA, however, the mild basicity of pyridine is preferable in order to minimize racemization. Selective cleavage of the allyl group was undertaken by palladium(0)-catalyzed allyl transfer to morpholine as the accepting nucleophile. In the presence of 10 mol % of tetrakis(triphenyl-phosphine)palladium(0) the allyl residue of the PNA ester **15** was transferred to morpholine at room temperature within 4 hrs quantitatively, however, the other protecting groups, Boc and Z, were completely conserved. Thus the free PNA acid **16** was obtained quantitatively after treatment of its morpholine salt with cation exchange resin (Scheme 6).

Again, attempts to synthesize a homooligomer of **16** under the standard peptide coupling procedures were unsuccessful. Thus, the oligomerization to higher PNA analogues of **16** was accomplished as shown in Scheme 7. Initially the resin (BHA-PEG-PS) was soaked in DCM overnight. Boc protection of the solid support was then removed by treatment with 50% TFA in anhydrous DCM for 30 minutes followed by washing with 10% DIEA in DCM for 3 min. The support bound free amine was then

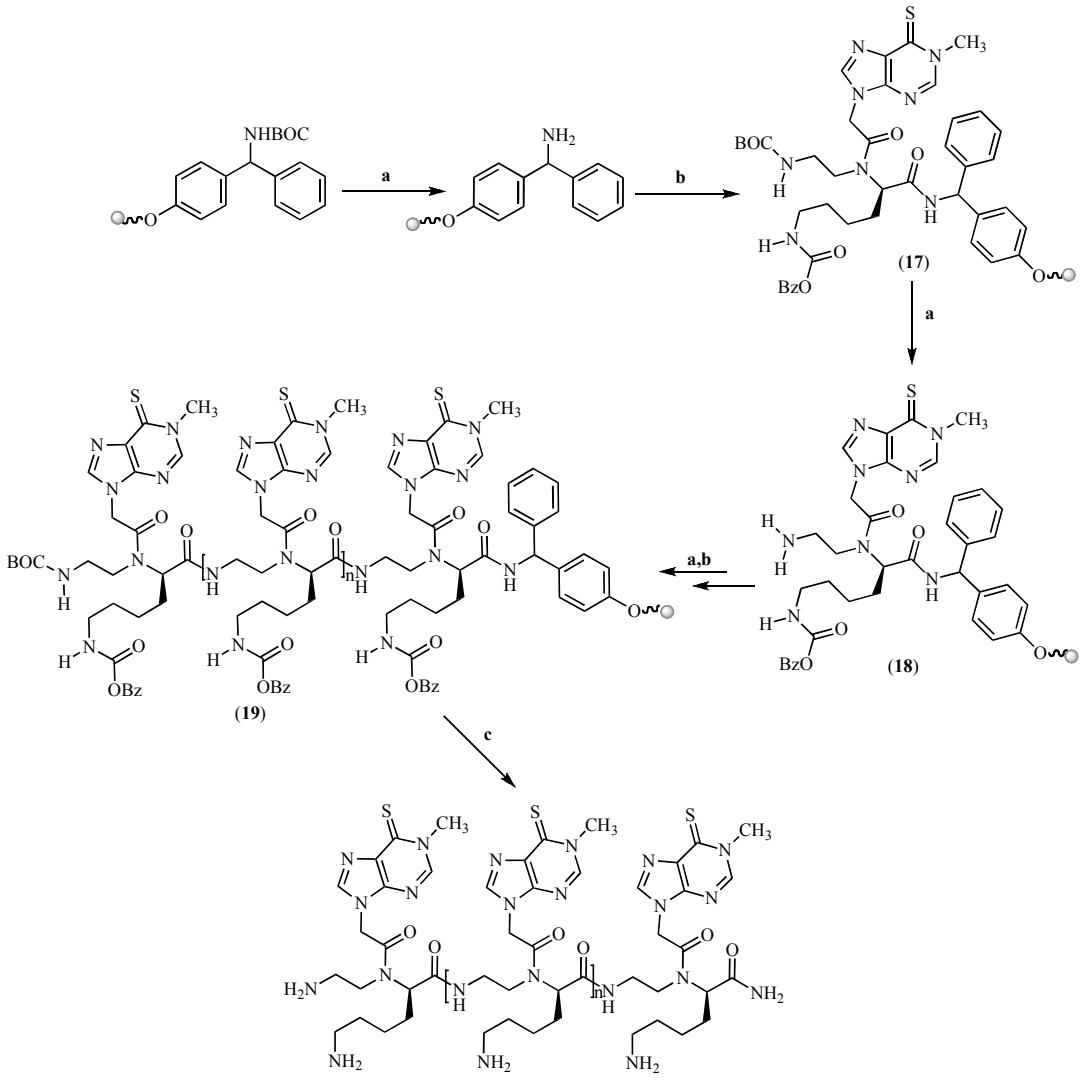
Scheme 6



a, Ph_3P,CCl_4/pyridine; b, 1(Ph_3)$_4$Pd(0)/THF 4hrs, 2. Dowex50WX8-200 (H^+).

coupled with **16** by using 2.5 molar equivalent of **16** and 4 equivalent of Ph_3P in DCM containing 10% CCl_4 under argon atmosphere. DIEA (one equivalent) was added prior to the addition of Ph_3P to trap the liberated HCl during the reaction of acid chloride to amine. After 45 min. the resin was washed thoroughly with DCM, at which time the Kaiser test for amine was negative. The cycle was repeated 3 times and the support was treated with HF/Pyridine containing 5% *m*-cresol for 5 hr at ambient temperature followed by standard peptide deprotection protocol and the crude residue was purified by HPLC. In spite of a purified single compound obtained from HPLC (Figure 2) and a UV absorbance maxima at 328nm (Figure 3) both electrospray and MALDI-TOF MS analysis failed to give molecular ions. This is not unusual in PNA mass spectrometry [28].

Scheme 7

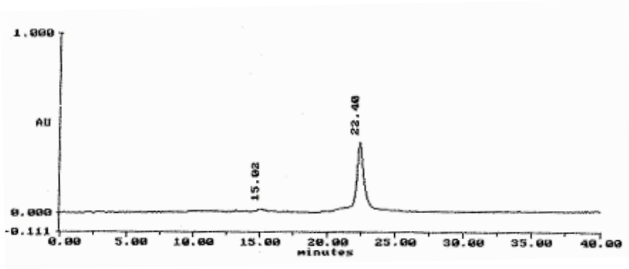


a, 1. 50%TFA/DCM, 30 min., 2. 10%DIEA/DCM; b, 1. (**16**), 2. DIEA(1 eq.), 3. Ph_3P,CCl_4/DCM 45 min.; c, 5%*m*-cresol in HF/pyridine, RT, 5hrs.

containing 5% *m*-cresol for 5 hr at ambient temperature followed by standard peptide deprotection, and the crude residue was purified by HPLC.

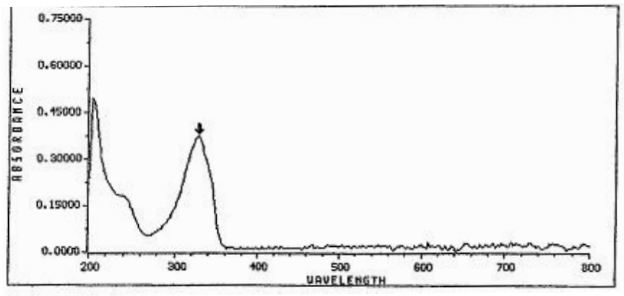
The MALDI-TOF MS of the purified sample gave a peak at m/z 1149.2, which corresponds to a calculated molecular weight of the trimer. This result encouraged us to repeat the synthetic cycle 12 times. Again the support

In conclusion *in situ* generation of the acid chloride of a PNA monomer under neutral conditions may ease the solid phase synthesis of standard PNA. If the methodology is successful the amount of monomer required and the coupling time will be reduced.



HPLC chromatogram of **20** using RP-C8 column and gradient elution of 0-60% ACN in water both containing 0.1% TFA over 20 minutes.

Figure 2



UV Absorption of the HPLC Fraction of **20**.

Figure 3

EXPERIMENTAL

Dry solvents were obtained from Aldrich; however, pyridine was distilled from Na immediately before use. All liquid reagents were purified by distillation. Solid support resin was purchased from PerSepetive Biosystems GmbH Hamburg, Germany. Unless otherwise noted, reactions were conducted under argon. Evaporations of solutions were carried out under vacuum with a rotary evaporator. Flash chromatography was carried out with silica gel (230-400 mesh). Chromatographic separations using HPLC were carried out on a Hitachi L6200 pump equipped with an L3000 photo diode array detector. The reverse-phase chromatography protocol utilized a Rainin Microsorb MV C8 or C18 columns, 0-60%, 20 minutes, or 0-20% 30 minutes, phase a: 0.1% TFA in acetonitrile; phase b: 0.1% TFA in water. Melting points were measured with Thomas Hoover capillary melting point apparatus and were uncorrected. UV spectra were measured with a Hewlett-Packard 8452A diode array spectrophotometer. ^{1}HNMR spectra were recorded with a Varian Mercury 400 MHz FT-NMR. Spectra were measured either in $CDCl_3$ or DMSO-d_6 using TMS as internal standard and the chemical shifts are given in δ ppm. ESI mass spectra were recorded with Sciex API III spectrometer. MALDI spectra were measured with Voyager-DE STR MALDI/TOF. High resolution mass spectra were recorded with a MAT 95 spectrometer. Elemental analyses were performed by M-H-W laboratories, Phoenix, AZ, USA.

Ethyl (1-methyl-6-mercaptopurin-9-yl)acetate (5). NaH (1 g 60% in paraffin, 42 mmol) was washed thoroughly with hexane and suspended in anhydrous DMF under an argon atmosphere. The suspension chilled then 1-methyl-6-mercaptopurine (4g, 24 mmol) was added portion wise while stirring. The reaction mixture was stirred for 2 hours at ambient temperature. Subsequently, ethyl bromoacetate (3.72 ml, 34.7 mmol) was added dropwise at ambient temperature and the stirring was continued for further 2 hours. A few drops of methanol were added to the reaction mixture then the solvent was evaporated under vacuum. The residue was taken up in ethyl acetate (200 ml) and washed twice with water (40 ml each). The organic phase dried over anhydrous sodium sulfate, filtered, evaporated to a gray colored residue and purified by flash chromatography (DCM/EtOAc, 2:3) to afford 3.9 g (65%) of the title compound. ^{1}HNMR ($CDCl_3$): 8.30(s, 1H), 7.92(s, 1H), 5.54(s, 2H), 4.29-4.18(q, 2H, J1=6.3, J2=7.26), 3.88(s, 3H), 1.30(t, 3H). HRMS (FAB): 253.07592(calcd), 253.07557 (found); Anal. Calcd. for $C_{10}H_{12}N_4O_2S$: C, 47.61; H, 4.80; N, 22.22; Found: C, 47.80; H, 4.76; N, 22.42.

(1-Methyl-6-mercaptopurin-9-yl)acetic acid (6). A solution of **5** (2.5 g, 10 mmol) in a mixture of ethanol (50 ml) and water (10 ml) was stirred with sodium hydroxide (0.5g, 12.5 mmol) at ambient temperature for 6 hours. Methanol was then removed by evaporation under vacuum and the residue was dissolved in water (25 ml) and treated with Dowex®50Wx8.200 resin (H^+ form). The resin was filtered and washed with 50 ml DMF/water (1:1), then with DMF (20 ml). The filtrate was evaporated under reduced pressure to give 2.1 g of a pale yellow solid (95%). ^{1}HNMR (DMSO-d_6): 8.72(s, 1H), 8.40(s, 1H), 5.56(s,2H), 3.82(s, 3H). EIMS: m/z = 225 (M+1) HRMS (FAB): 225.04462 (calcd), 225.04533 (found).

3-(Boc-Amino)-1,2-propandiol (8). To a stirring solution of 3-amino-1,2-propandiol **7** (8 g, 880 mmol) in water (150 ml) cooled to 0°C was added di-*tert*-butyl dicarbonate (23 g, 1050 mmol) in one portion. The reaction mixture was warmed to room temperature and the pH was maintained at 10.5 with a solution of sodium hydroxide (7.02 g, 1760 mmol) in water (48 ml). The reaction mixture was stirred overnight at room temperature. Ethyl acetate (100 ml) was added to the reaction mixture followed by cooling to 0°C. The pH was adjusted to 2.5 with 4 *N* hydrochloric acid with vigorous stirring and the phases were then separated. The aqueous phase was extracted with further ethyl acetate (8x50 ml). The pooled organic phase was washed with a saturated aqueous solution of potassium hydrogen sulfate (150 ml) then with saturated aqueous sodium chloride (100 ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure. The residue was further evaporated with 2 portions of DCM under reduced pressure then refrigerated overnight to afford 14.5 g of the titled compound (86%) as a white solid. The product was not further purified and was used directly for the next reaction; ^{1}HNMR ($CDCl_3$): 1.43(s, 9H), 3.25(m, 2H), 3.57(m, 2H), 3.73(m, 1H). EIMS: m/z = 192.1 (M+1).

Boc-Aminoacetaldehyde (9). To a suspension of **8** (8.6 g, 45 mmol) in water (125 ml) sodium *m*-periodate (10.5 g, 45.65 mmol) was added and the reaction mixture was stirred at room temperature for 2 hours. The aqueous phase was extracted with ethyl acetate (3x100ml). The combined organic extract was dried over anhydrous Na_2SO_4 and evaporated to dryness to afford 6.75 g (94%) of a golden semisolid oil which used without any further purification. ^{1}HNMR ($CDCl_3$): 1.45(s, 9H), 4.06(d, 2H, J=4.4 Hz), 5.19(bs, 1H, exchangeable with D_2O), 9.64(s, 1H).

Nα-Boc-Nε-CBZ-L-lysine allyl ester (11). To a solution of Nα-Boc-Nε-CBZ-L-lysine, **10**, (9.5g, 25 mmol) and $NaHCO_3$

(2.11g, 25.125 mmol) in water (50 ml), solution of aliquat 336 (10.65 g, 26.35 mmol) and allyl bromide (2.37 ml, 27.45 mmol) in DCM (100 ml) was added. The mixture was stirred vigorously for 3 days at ambient temperature. The aqueous layer was then separated and extracted three times with DCM (each of 100 ml). The pooled organic layers were dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (Pet. ether/EtOAc, 3:1) to give 10.3g (96%) of the title compound. ^{1}HNMR ($CDCl_3$): 1.43(s, 9H), 1.45-1.66(bm, 4H), 1.73-1.89(bm, 2H), 3.07-3.26(bm, 2H), 4.24-4.35(m, 1H), 4.70-4.55(m, 2H), 5.09 (s, 2H), 5.21-5.38 (m, 3H), 5.75-6.03 (m, 1H), 7.33(bs, 5H). EIMS: m/z = 421.3 [M+1]

Nα-(2-Boc-Aminoethyl)-Nε-CBZ-L-lysine allyl ester (13). Compound **11** (8.25 g, 19.27 mmol) was stirred with 50% TFA (100 ml) in DCM for 20 minutes. The solvent was evaporated under reduced pressure. To the solution of the residue in dry methanol (100 ml), a solution of Boc-aminoacetaldehyde (3.45 g, 21.6 mmol) in dry methanol (25 ml) was added and the mixture stirred for 15 minutes. $NaBH_3CN$ (1.38 g, 22 mmol) was then added and the mixture stirred for 24 hours at ambient temperature. Subsequently, water (30 ml) was added followed by 10% solution of $NaHSO_4$ in water (10 ml). Methanol was evaporated and the aqueous residue was extracted with DCM (3x75 ml), the combined organic layer was dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue was purified by flash chromatography (5% MeOH in DCM) to give 7.1 g (80%) of the titled compound. ^{1}HNMR ($CDCl_3$): 1.44(s, 9H), 1.46-1.60(m, 4H), 1.75-1.93(m, 2H), 3.14-3.22(m, 2H), 3.27-3.29(m, 2H) 4.24-4.35(m, 1H), 4.70-4.55(m, 2H), 5.09 (s, 2H), 5.26-5.37 (m, 4H), 5.75-6.03 (m, 1H), 7.26-7.38 (m, 5H). EIMS: m/z = 464.35 (M+1); HRMS (FAB): 464.27606 (calcd), 464.27627 (found)

Allyl Nα-(2-Boc-Aminoethyl), Nα-(1-methyl-6-mercapto-purin-9-methylcarbonyl)-Nε-CBZ-L-lysinate (15). Compounds **6** (2.24 g, 10 mmol) and **13** (4.64 g, 10 mmol) were dissolved with stirring in anhydrous pyridine (50 ml) containing 10% CCl_4 under an argon atmosphere at ambient temperature. Ph_3P (5.06 g, 19.28 mmol) was added to the reaction mixture and the stirring continued for 4 hours. Pyridine was evaporated under reduced pressure and the residue dissolved in DCM (150 ml), washed twice with water (each of 50 ml). The organic layer was dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue was purified by flash chromatography (1% MeOH in DCM) to give 6.3 g (65%) of the title compound, mp 72°C. ^{1}HNMR ($CDCl_3$): 1.40-1.57 (m, 13H), 1.98-2.08 (m, 2H), 3.17-3.50 (m, 6H), 3.70-3.93(m, 4H), 4.04-4.11(m, 1H), 4.59-4.63(d, 2H), 5.02 (bm, 1H), 5.09 (s, 2H), 5.22-5.57 (m, 4H), 5.82-5.99 (m, 1H), 7.32-7.38 (m, 5H), 7.94 (s, 1 H), 8.24 (s, 1H). EIMS: m/z = 670.50 (M+1); HRMS (FAB): 670.30229(calcd), 670.30015 (found); Anal. Calcd . for $C_{32}H_{43}N_7O_7S$: C, 57.37; H, 6.47; N, 14.65; Found: C, 57.16; H, 6.24; N, 14.75.

Nα-(2-Boc-Aminoethyl)-Nα-(1-methyl-6-mercaptopurin-9-methylcarbonyl)-Nε-CBZ-L-lysine (16). $(Ph_3P)_4Pd(0)$, (0.23 g, 0.2 mmol) was added to a solution of **15** (1.338 g, 2 mmol) in THF (100 ml) under argon atmosphere with stirring. Morpholine (1.76 ml, 20 mmol) was then added drop wise and the stirring was continued for 4 hours at ambient temperature. The solvent was evaporated under reduced pressure and the residue washed with ether (25 ml) then dissolved in ethyl acetate (100 ml). Ether (50 ml) was added to the resulting solution and the formed precipitate was collected by filtration. The precipitate dissolved in methanol (100 ml) and treated with Dowex®50Wx8.200 resin (H^+ form) for 10 minutes. The resin was filtered, washed with methanol (20 ml) and the filtrate was evaporated under reduced pressure to dryness to give 1.2 g (98%) of the title compound. ^{1}HNMR (DMSO-d_6): 1.25-1.55 (m, 12H), 1.92-2.04 (m, 2H), 3.03-3.43 (m, 6H), 3.53-3.99(m, 4H), 4.07-4.15 (t, 1H), 5.09 (s, 2H), 5.29-5.94 (m, 2H), 7.33-7.38 (m, 5H), 7.95 (s, 1H), 8.25 (s, 1H). EIMS: m/z = 630.31 (M+1); HRMS (FAB): 630.27099 (calcd), 630.27052 (found).

General procedures for synthesis of Nα-(aminoethyl)-Nα-(1-methyl-6-mercaptopurin-9-methylcarbonyl)-L-lysine polymers, (20). BOC-BHA-PEG-PS resin (60 mg, 0.12 mmol/g, 0.0036 meq.) was soaked in DCM (5 ml) for overnight then stirred with 50% TFA in DCM (5 ml) for 30 minutes. The resin was filtered, then washed with the following: DCM (2 ml), 10% DIEA in DCM (1 ml) and dry DCM (2 ml). The resin was suspended in dry DCM (5 ml) containing 10% CCl_4 and stirred gently with **16** (20 mg, 0.0317 mol), Ph_3P (40 mg, 0.1525 mol), and DIEA (40 μl, 0.1148 mol) for 45 minutes under an argon atmosphere at ambient temperature. The resin was filtered, washed with DCM (2 ml), suspended in DCM (1 ml) containing 10% Ac_2O (1 ml) and 10% DIEA in DCM with shaking for 5 minutes, then filtered and washed with DCM. The resin was stirred with 50% TFA in DCM (5 ml) for 30 minutes followed by washing and repeating the previous steps for 3 cycles (trimer) and 12 cycles (dodecamer). The resin was then treated with HF/pyridine mixture containing 5% *m*-cresol (0.5 ml) for 5 hours at ambient temperature, filtered and the solvent removed by lyophilization. The lyophilized product was purified by HPLC using RP-C18 column for trimer PNA and RP-C8 column for dodecamer PNA and gradient elution system consisting of solvent (A), 0.1% TFA/water, and system (B), 0.1% TFA/ACN. The percent of B is varied from 20% over a period 30 minutes for 3-mer and 60% over a period of 20 minutes for 12-mer. HPLC fractions of the targets were lyophilized to obtain the products as yellowish white fluffy powder.

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REFERENCES AND NOTES

[1] This work dedicated to Prof. Arthur D. Broom in the occasion of his retirement.

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