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Synthesis of reversible nucleoside amino acid conjugates

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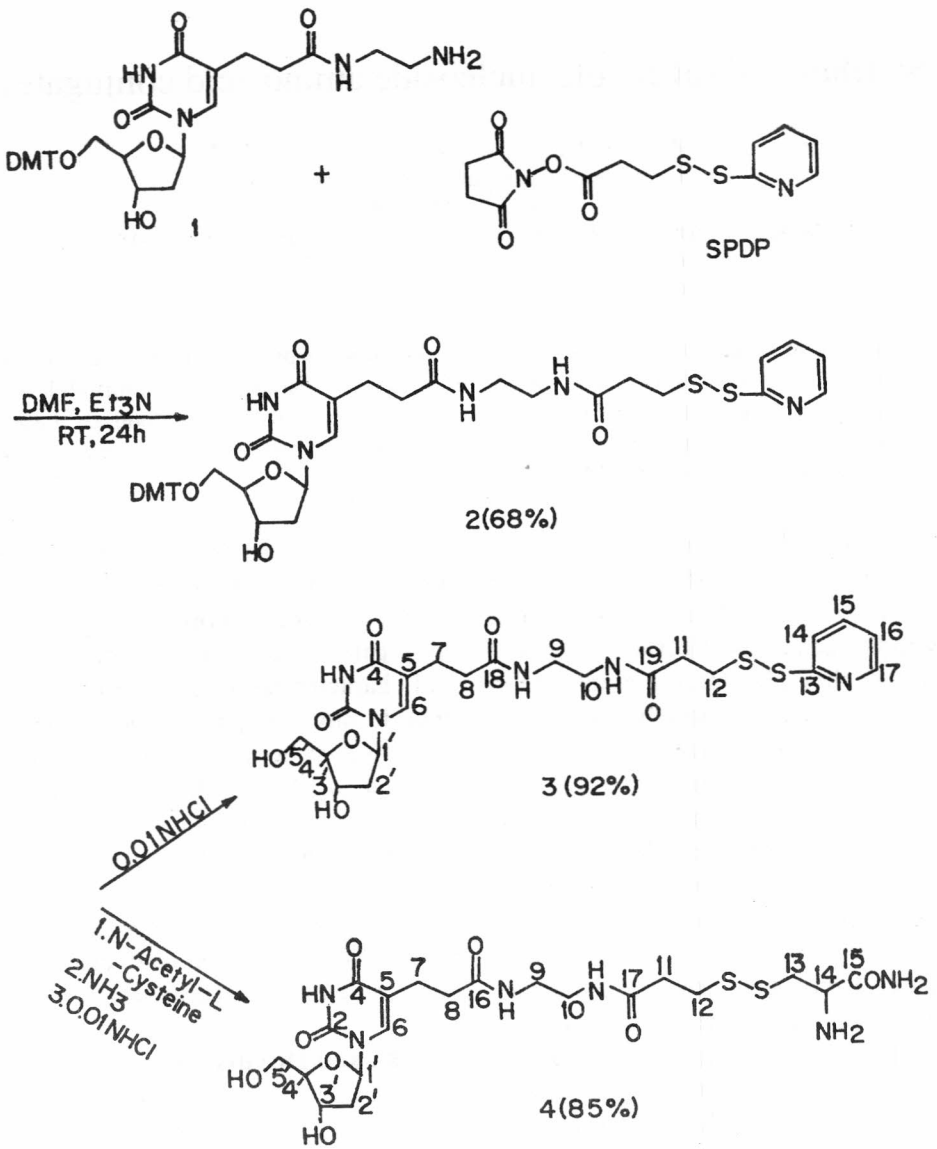
A general synthetic method to conjugate cysteine-containing peptides to nucleosides via a disulfide link has been developed, using the heterobifunctional reagent *N*-succinimidyl-3-(2-pyridyl-thio)propionate(SPDP) and amino-modified nucleosides. Representative compounds based on C-6 modified adenosine, C-5 modified uridine and 2'-deoxyuridine are reported, along with extensive NMR characterizations of these novel nucleosides.

The class of bioconjugates known as nucleoside peptides is of considerable interest because of their established biological activity. These bioconjugates occur naturally in the genomes of certain RNA and DNA viruses, where they play an important role in viral replication. Generally, the proteins are linked to a 5'-phosphate of a nucleic acid moiety via a phosphodiester bond to serine, tyrosine or threonine[1]. Another naturally occurring example of RNA-amino acid bioconjugates is the family of aminoacyl-tRNA molecules in which the amino acid is esterified to the 2'- or 3'-hydroxyl group at the 3'-end of the tRNA chain[2]. Synthetic DNA-enzyme conjugates have been used as sequence-specific, oligonucleotide-directed nucleases[3,4]. Oligonucleotides have been conjugated to peptides such as cationic polylysine[5,6] and hydrophobic polytryptophan[7] to enhance cellular uptake. The sequence-specific inhibitory effect of poly-L-lysine antisense conjugates has been demonstrated[8,9]. Conjugation of oligoarginine peptides or ornithine peptides to oligonucleotides was reported to promote membrane penetration of these bioconjugates and increase their binding affinities to their complementary DNA strands [10-12]. Recently, molecules such as cholesterol and cyclodextrin have been conjugated to synthetic oligonucleotides to enhance their antisense activity, and higher antisense activity has been related to their increased cellular uptake[13,14]. Reversible nucleosides are designed to link cell binding proteins to nucleic acids via disulfide groups. Uptake of these nucleic acid-protein bioconjugates will be enhanced by cell binding protein but free nucleic acid will be released into the cell.Because of the growing importance of nucleic acid-protein conjugates, we have undertaken the preparation and characterization of a series of oligonucleotide-peptide bioconjugates in order to mimic naturally occurring systems, and because oligonucleotides with pendant peptides may have advantageous reactivity or drug delivery properties. We have earlier reported the synthesis and characterization of nucleoside-histidine conjugates and oligonucleotide-histidine and lysine derivatives[15-17]. We report herein the preparation and characterization of 3-nucleoside cysteine conjugates.

Results and Discussion

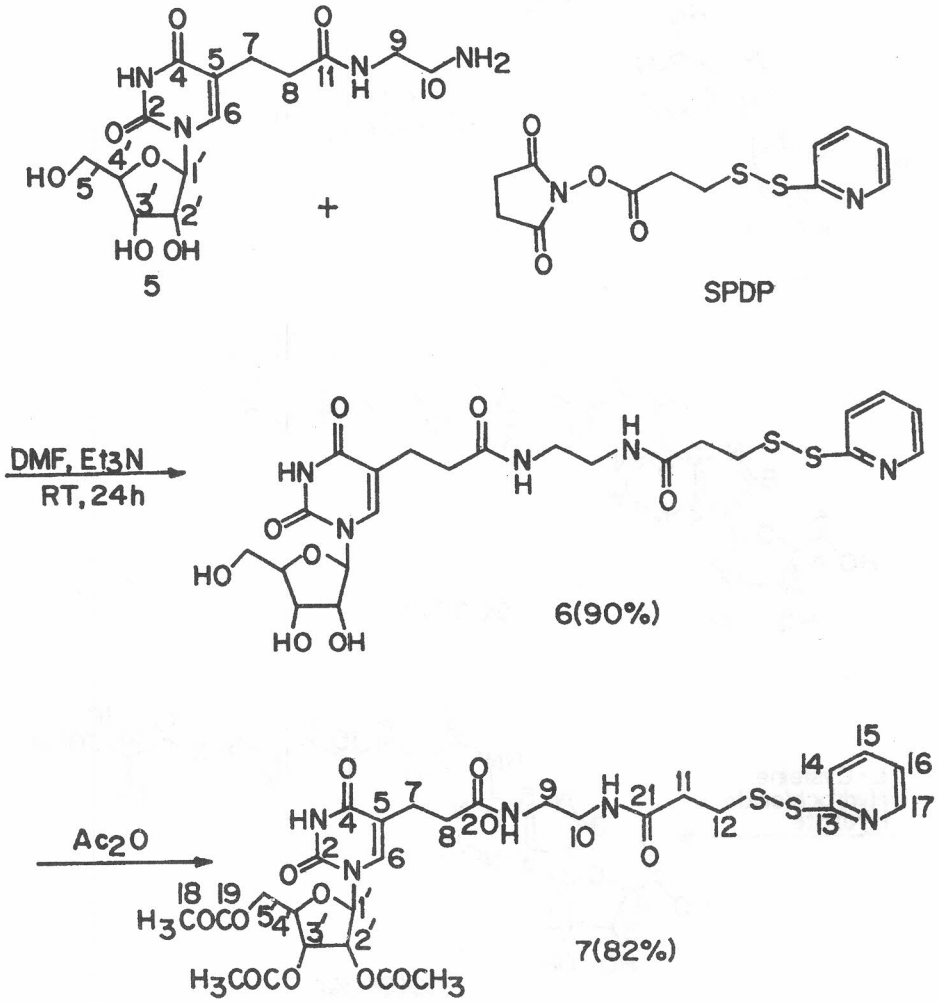
As shown in Scheme I, we chose C-5 substituted 2'-deoxyuridine as our parent synthetic substrate. C-5 modified nucleoside derivatives can be incorporated into oligonucleotides without disrupting with Watson-Crick base pairing to complementary strands. Nucleoside **1** was prepared by the literature method [18]. It was coupled with SPDP to give nucleoside **2** with a purified yield of 68%. Fully assigned ^{13}C and ^{1}H NMR data are given in the Experimental Section. The quaternary carbon C13, as shown in Scheme I, was identified as the resonance at 159.7 ppm from the 1D ^{13}C APT spectrum of **2**. The other ^{13}C assignments of the thiopyridine moiety of the SPDP nucleoside conjugate were initially ambiguous, and because of the many overlapping proton resonances in the aromatic region, heteronuclear correlation



Scheme I

techniques would not have been effective in completing the identification. Therefore, to analyze the ^{13}C spectrum, the INADEQUATE experiment[19] was employed to obtain 2D ^{13}C-^{13}C connectivity information of a model compound, pyridine-2-thiol. The assignment of the remaining carbons in the pyridine ring was made by their connectivity with the previously identified quaternary carbon resonance. This spectral analysis was used to help assign the ^{13}C spectra of compounds **7** and **9**, described below. The disulfide **2**, which can be regarded as a protected thiol, withstood detritylation conditions, as indicated by the 92% yield for the conversion of **2** to **3**. When **2** was treated with one equiv. of *N*-acetyl-L-cysteine, the resulting bioconjugate **4** was isolated in 85% overall yield after deprotection of both the 5'-*O*-trityl and *N*-acetyl groups and chromatographic purification.

We extended the deoxyuridine methodology of Scheme I to the synthesis of the ribonucleoside analogues, shown in Scheme II. Ribonucleoside **5**



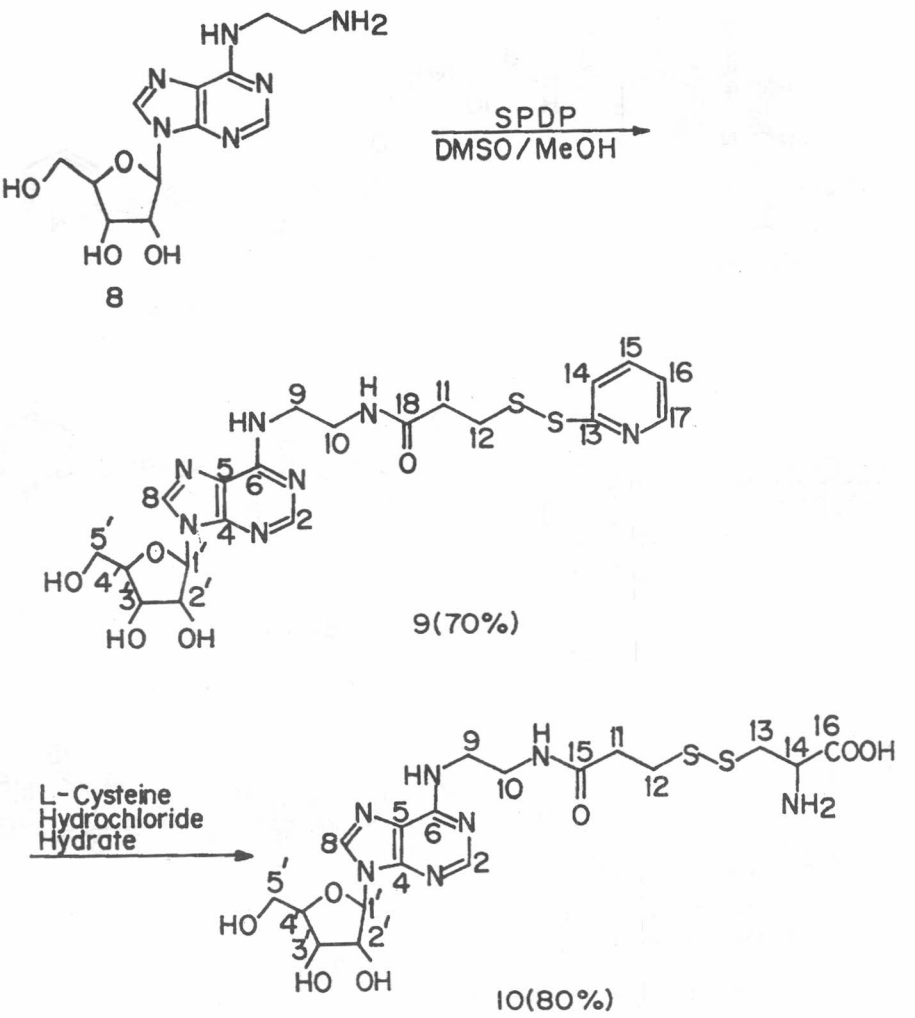
Scheme II

was prepared by treating a known C-5 substituted uridine derivative[20] with ethylenediamine. This ribonucleoside was then treated with a 3-fold excess of SPDP in DMF. The intermediate product **6** was not isolated but was acetylated with acetic anhydride to improve its solubility and lipophilicity for easier isolation, giving **7** in 82% overall yield. The disulfide moiety was stable to the acetylation conditions.

A further example of ribonucleoside-cysteine conjugates is given in Scheme III. Compound **8**, which proved to be a useful substrate, was derived from the alkylation of a large excess of ethylenediamine with 6-chloropurine ribonucleoside[21] in 90% yield. On treatment of **8** with SPDP, the disulfide **9** was obtained in 70% yield, and a disulfide exchange reaction with L-cysteine hydrochloride hydrate gave the nucleoside **10** in 80% yield.

Conclusions

We have developed a methodology for the introduction of disulfide group into ribonucleosides and 2'-deoxyribonucleosides, and we have demonstrated how this procedure can be used to prepare nucleoside-peptide bioconjugates derived from cysteine. No unwanted modifications of the nucleic acid bases occur in the course of this chemistry, as indicated by the extensive characterization of the products, including high



Scheme III

resolution mass spectral data and ^{1}H and ^{13}C NMR data.

Experimental Section

General. NMR spectra were recorded on Varian UNITY plus-500, UNITY-600, and VXR-400 spectrometers. Exchangable protons are labeled (ex.). The high resolution mass spectra were recorded on a Finnegan/MAT90 spectrometer. The FAB^{+} low resolution spectra were run on a VG 40-250T spectrometer where the FAB matrix was a saturated solution of LiI in 3-nitrobenzyl alcohol, which is especially useful for acid-labile, protected nucleosides. Thin layer chromatography was performed on Baker-Flex Silica gel IB2-F plates. Flash column chromatography was performed on silica gel (Merck SG-60, 230-240 mesh). Reverse phase (RP) column chromatography was carried out using an Alltech Econosil C-18 silica gel. RP HPLC was carried out on an Alltech Econosil C-18 preparative column (10 μ, 22.5 x 250 mm) using a linear ternary gradient flowing at 6 mL/min. Solvent A (0.1M $[Et_3NH]OAc$) was kept constant at 25%, while B (MeCN) and C (H_2O) were varied as follows, where time is in min: (time, %B, %C), (O, 5, 70), (3, 5, 70), (33, 35, 40), (45, 70, 5). The HPLC was monitored simultaneously at 260 and 400 nm. SPDP (Pharmacia), *N*-acetyl-L-cysteine (Sigma), and L-cysteine hydrochloride hydrate (Sigma) were used as received.

5-[3- [[[[2-[2-Pyridyldithio]ethyl]amino]ethyl]-amino]- 3-oxopropyl]-5'-*O*-DMT-2'-deoxyuridine 2. A solution of nucleoside[18] (0.322 g, 0.5 mmol) in DMF (5 mL) and Et_3N(0.2 mL) was cooled to 0^0C in an ice-bath and SPDP (0.469 g, 1.5 mmoles) was added to the stirred reaction mixture. After 15 min the ice bath was removed and the mixture was stirred at room temperature for 24 hr. The reaction mixture was diluted with 20 mL CH_2Cl_2 and washed with water (10 mL). The organic layer was dried, concentrated and chromatographed on silica (6% EtOH/CH_2Cl_2) to give the desired product **2** (0.285 g, 68%). R_f 0.4(10% EtOH/CH_2Cl_2). ^{13}C NMR (75 MHz, $CDCl_3$) : 84.9 (C1'), 40.8 (C2'), 72.2 (C3'), 86.2 (C4'), 63.9 (C5'), 164.2 (4CO), 150.7 (2CO), 113.7 (C5), 137.2 (C6), 23.6 (C7), 35.4 (C8), 39.8 & 39.2 (C9 & C10), 35.5 (C11), 34.2 (C12), 159.7 (C13), 137.4 (C15), 120.2 (C14), 121.1 (C16), 149.5 (C17), 55.3 [OCH_3(DMT)], 86.8 [C(Ph)$(PhOMe)_2$], 173.0 (C18), 171.7 (C19); ^{1}H NMR (300 MHz, $CDCl_3$) : δ 6.35 (t, 1H, H1'), 2.2 & 2.4 (m, 2H, H2'), 4.5 (m, 1H, H3'), 4.05 (m, 1H, H4'), 3.4 (m, 2H, H5'), 7.55 (s, 1H, H6), 2.1 & 2.2 (m, 4H, H7 & H8), 3.25 (m, 4H, H9 & H10), 2.55 (t, 2H, H11), 2.95 (t, 2H, H12), 7.65 (t, 1H, H14), 7.60(d, 1H, H15), 7.05 (m, 1H, H16), 8.35 (d, 1H, H17), 3.75 (s, 6H, OCH_3(DMT)). FAB HRMS m/z) $C_{43}H_{47}N_5O_9S_2Li^+$: Calcd 848.2975, Found 848.2841; MS (FAB) : m/z 854 [M+2Li-H], 848[M+Li], 739 [M+Li+H-C_5H_5NS], 551 [M+2Li-H-DMT], 430 [M+Li+H-sugar].

5-[3-[[[[2-Pyridyldithio] ethyl]amino]ethyl]-amino]-3-oxopropyl]-2'-deoxyuridine 3. A solution of **2** (0.42 g, 0.5 mmole) in CH_2Cl_2 (5 mL) was treated with 0.01 N HCl, *p*H 2 (5 mL) for 0.5 h. The mixture was washed with CH_2Cl_2 (2 x 20 mL), then lyophilized twice to yield the final product **3** (0.220 g, 92%). ^{13}C NMR (75 MHz, CD_3OD) : 86.3 (Cl'), 41.2 (C2'), 72.2 (C3'), 88.9 (C4'), 62.9 (C5'), 165.7 (4CO), 152.2, (2CO), 114.1 (C5), 139.1 (C6), 24.2 (C7), 35.8 (C8), 40.0 & 39.9, (C9 & C10), 36.1 (C11), 35.3 (C12), 161.2 (C13), 121.2 (C14), 139.1 (C15), 122.4 (C16), 150.4 (C17), 175.2 (C18), 173.6 (C19); ^{1}H NMR (300 MHz, D_2O) :δ 6.2 (t, 1H, H1'), 2.3 (m, 2H, H2'), 4.4 (m, 1H, H3'), 3.95 (m, 1H, H4'), 3.75 (m, 2H, H5'), 7.6 (s, 1H, H6), 2.5 (2 t's, 4H, H7 & H8), 3.3 (s, 4H, H9 & H10), 2.6 (t, 2H, H11), 3.05 (t, 2H, H12), 8.2 (d, 1H, H14), 8.4 (t, 1H, H15), 7.8 (t, 1H, H16), 8.6 (d, 1H, H17). FAB HRMS m/z $C_{22}H_{29}N_5O_7S_2Li^+$: Calcd 546.5647, Found 546.5673; MS (FAB) : m/z 546 [M+Li], 435 [M+Li-C_5H_5NS], 430 [M+Li+H-sugar].

5-[3-[[[[2- [L-Cystamide]thioethyl]amino]ethyl]-amino]-3-oxopropyl]-2'-deoxyuridine 4. To a solution of **2** (0.084 g, 1.0 mmole) in CH_2Cl_2 (0.5 mL) was added a solution of *N*-acetyl-cysteine(0.17 g, 1.0 mmole) in CH_3CN(0.25 mL). The mixture was stirred at room temperature for 30 min and concentrated to dryness. The residue was chromatographed on silica (8-12% EtOH/CH_2Cl_2). The crude product was treated with conc.aq. NH_3(5 mL) for 24 hr and concentrated to dryness, then treated with 0.01 *M* HCl (*p*H 2) for 0.5 h and washed with CH_2Cl_2 (2 x 5 mL), and purified by RP HPLC (retn. time 25 min) to yield **4** (0.046 g, 85%). ^{13}C NMR (75 MHz, D_2O) : 88.1 (C1'), 43.1 (C2'), 73.4 (C3'), 89.5 (C4'), 64.2 (C5'), 168.4 (4CO), 154.4 (2CO), 116.3 (C5), 141.2 (C6), 24.9 (C7), 37.4 (C8), 41.7 and 41.6 (C9 & C10), 37.8 (C11), 36.3 (C12), 25.8 (C13), 56.8 (C14), 178.3 (C15), 177.3 (C16), 176.7 (C17); ^{1}H NMR (300 MHz, D_2O) : δ 6.30 (t, 1H, H1', J=6.6 Hz), 2.35 (m, 2H, H2'), 4.45 (m, 1H, H3'), 4.05 (m, 1H, H4'), 3.80 (m, 2H, H5'), 7.70 (s, 1H, H6), 2.55 (2 t's, 4H, H7 & H8), 3.31 (s, 4H, H9 & H10), 2.65 (t, 2H, H11, *J*=6.6 Hz), 2.80 (t, 2H, H12), 3.10 (m, H13, 2H), 4.55 (m, H14, 1H). FAB HRMS m/z $C_{20}H_{32}N_6O_8S_2Li^+$: Calcd 555.5724; Found 555.5745; MS (FAB) : m/z 555 [M+Li], 549 [M+H], 439 [M+Li+H-sugar].

5-[3-[2-Aminoethyl)amino]-3-oxopropyl]uridine 5. 5-(2-Carbomethoxyethyl) uridine[20] (3.96 g, 12 mmoles) was stirred with ethylenediamine (7.2 mL) for 60 hr. The reaction mixture was concentrated to dryness, chromatographed on silica (1-3% NH_3/EtOH) to give pure product **5** (3.8 g, 90%). ^{13}C NMR (75 MHz, MeOD) : 90.7 (Cl'), 75.7 (C2'), 71.4 (C3'), 86.4 (C4'), 62.5 (C5'), 167.8 (4CO), 154.2 (2CO), 114.4 (C5), 138.8 (C6), 24.5 (C7), 35.9 (C8), 42.0 & 44.4 (C9 & C10), 175.4, (C11); ^{1}H NMR (300 MHz, D_2O) : δ 5.93 (d, 1H,H1'), 4.25 (m, 1H, H2'), 4.21 (m, 1H, H3'), 4.05 (m, 1H, H4'), 3.85 (m, 2H, H5'), 7.50 (s, 1H, H6), 2.52 (2 t's, 4H, H7 & H8), 3.01 (m , 4H, H9 & H10). FAB HRMS m/z $C_{14}H_{22}N_4O_7Li^+$:Calcd 365.2930; Found 365.2967; MS (FAB) : m/z 365 [M+Li], 359 [M+H].

5-[3-[[[[2-[2- Pyridyldithio]ethyl]amino]-ethyl]amino]-3-oxopropyl]-2', 3', 5'-tri-*O*-acetyluridine 7. Nucleoside **5** (0.178 g, 0.5

mmole) and Et_3N (0.2 mL) in DMF (5 mL) were cooled in an ice-bath. SPDP (0.469 g, 1.5 mmoles) was added. The reaction mixture was stirred for 15min, then the ice-bath was removed. The mixture was stirred at room temperature for another 24hr. Acetic anhydride (0.306 g, 3 mmoles) in CH_2Cl_2 (5 mL) was added to the reaction mixture and left stirring for 2 hr. After quenching with water (2 mL), the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was dried and concentrated to give the crude product. The residue was purified by chromatography on silica (3 % EtOH/CH_2Cl_2) to give the product **7** (0.279 g, 82%). R_f0.48 (5% EtOH/CH_2Cl_2). ^{13C}NMR (75 MHz, $CDCl_3$) ppm 87.8 (C1'), 72.7 (C2'), 70.3 (C3'), 79.8 (C4'), 63.2 (C5'), 163.6 (4CO), 150.3 (2CO), 113.9 (C5), 137.3 (C6), 23.1 (C7), 34.8 (C8), 39.5 & 39.6 (C9 & C10), 35.5 (C11), 34.3 (C12), 159.6 (C13), 137.5 (C15), 120.2 (C14), 121.0 (C16), 149.5 (C17), 172.7 (C20), 171.7 (C21), 169.7, 169.8, 170.6, 19C; 20.5, 20.9, 20.7 18C; ^{1}H NMR (300 MHz, $CDCl_3$) : δ 6.10 (d, 1H, H1'), 5.35 (m, 1H, H2'), 4.35 (m, 1H, H3'), 4.35 (m, 1H, H4'), 4.30 (m, 2H, H5'), 7.35 (s, 1H, H6), 2.51 (2 t's, 4H, H7 & H8), 3.35 (m, 4H, H9 & H10), 2.60 (t, 2H, H11), 3.01 (t, 2H, H12), 7.65 (m, 1H, H14), 7.65(m, 1H, H15), 7.12 (m, 1H, H16), 8.40 (m, 1H, H17), 6.85, 7.15, 9.95 (br, 3H, NH); 2.00 & 2.21 (3 s's, 9H, $COCH_3$). FAB HRMS m/z $C_{28}H_{35}N_5O_{11}S_2Li^+$: Calcd 688.6770, Found 688.6783; MS (FAB) : m/z 682 [M + H], 609 [M+Li-C_5H_5NS], 577[M+Li-C_5H_5NS], 430 [M+Li+H-sugar].

N-[2-[[[2- [2-Pyridyldithio]ethyl]carbonyl]-amino]ethyl] adenosine 9. To a solution of *N*-aminoethyl adenosine[21] **8** (0.310 g, 1 mmole) in DMSO (3.0 mL) was added SPDP (0.313 g, 1 mmole). The reaction mixture was stirred at room temperature for 24 hr under N_2, and concentrated to dryness. Ethyl acetate (10 mL) and MeOH(2 mL) were added to the resulting thick syrup, and the mixture was vigorously mixed with a spatula until a white solid separated, and stored at 0^{0}C for 2hr. The final product **9** was obtained as a white solid (355 mg, 70%). m.p. 160 ^{0}C (d); IR (Nujol) : 3301, 1733, 1632, 1540, 1377 cm^{-1};. ^{13}C NMR (125 MHz, DMSO-d_6) : 88.1 (C1'), 73.7 (C2'), 70.8 (C3'), 86.1 (C4'), 61.8 (C5'), 152.4 (C2), 148.5 (C4), 154.8 (C5), 119.3 (C6), 139.9 (C8), 39.2 (C9), 38.6 (C10), 34.5 (C11), 33.7 (C12), 159.0 (C13), 119.3 (C14), 137.9 (C15), 121.3 (C16), 149.7 (C17), 169.9 (C18); ^{1}H NMR (500 MHz, DMSO-d_6) : δ 5.84 (d, 1H, H1'), 4.57 (m, 1H, H2'), 4.11 (m, 1H, H3'), 3.92 (m, 1H, H4'), 3.63 and 3.50 (dd, 2H, H5'), 8.17 (s, 1H, H2), 8.32 (s, 1H, H8), 3.50 (m, 2H, H9), 3.26 (t, 2H, H10), 2.43 (t, 2H, 11H), 2.94 (m, 2H, 12H), 7.71 (d, 2H, H14), 7.77 (m, 1H, H15), 7.18 (m, 1H, H16), 8.41 (d, 1H, H17); FAB HRMS (m/z) $C_{20}H_{25}N_7O_5S_2Li^+$: Calcd 514.1519, Found 514.1595; MS (FAB) : m/z 514 [M+Li], 508 [M+H], 382 [M+Li+H-sugar], 466 [M+H-C_2H_2O], 403 [M+Li-C_5H_5NS], 355 [382-HCN].

***N*-[2[[[2-{L-Cysteine] ethylthio]carbonyl]amino]ethyl]-adenosine 10.** L-Cysteine hydrochloride hydrate (78.8 mg, 0.5 mmole) was added to a solution of **9** (0.253 g, 0.5 mmole) in 5 mL CH_2Cl_2, 3.5 mL EtOH and 2.5 mL water. The reaction was complete in 1 hr at room temperature and was evaporated to dryness. The residue was chromatographed on reversed phase silica (30% H_2O/MeCN). Final product **10** was obtained as a white solid (0.220 g, 80%). m.p. 140 ^{0}C (d) IR (Nujol) : cm^{-1} 3329; 1969; 1622; 1456 cm^{-1}. ^{13}C NMR (150 MHz, D_2O) : 91.3 (C1'), 76.8 (C2'), 73.6 (C3'), 88.7 (C4'), 64.5 (C5'), 155.2 (C2), 150.2 (C4), 157.5 (C5), 122.3 (C6), 142.9 (C8), 42.7 (C9), 41.7 (C10), 35.5 (C11), 37.6 (C12), 40.7 (C13), 56.2 (C14), 177.2 (C15), 175.4 (C16); ^{1}H NMR (600 MHz, D_2O) : δ 5.94 (d, 1H, H1'), 4.69 (m, 1H, H2'), 4.36 (m, 1H, H3'), 4.24 (m, 1H, H4'), 3.87 and 3.78 (dd, 2H, H5'), 8.07 (s, 1H, H2), 8.19 (s, 1H, H8), 3.62 (m, 2H, H9), 3.45 (t, 2H, H10), 2.78 (t, 2H, 11H), 2.53 (m, 2H, 12H), 3.18 and 2.97 (m, 2H, H13), 3.96 (t, 1H, H14); FAB HRMS (m/z) $C_{18}H_{27}N_7O_7S_2Li^+$: Calcd 524.5329; Found 524.5374.

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