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Reactive DNA. 2. Thioguanine used as a peg site for direct and specific introduction of biologically useful functional groups§

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Abstract

An efficient method is described for one-step and specific introduction onto thioguanine in DNA of a variety of biologically useful functional groups, including carboxymethyl, hydroxyethyl, aminoethyl groups and fluorescent tag. Reaction mechanisms were examined. The method could provide new tools for biochemical studies, such as DNA repair and sister chromatid exchange, and the method may be also applicable to RNA.

Keywords: DNA; fluorescence; labelling; nucleosides.

1. Introduction

Thioguanine is a drug used for the treatment of acute leukaemia and as an immunosuppressive agent.[1] It produces a delayed cytotoxicity which is believed to be associated with thioguanine incorporated in DNA. Our recent work has provided a convincing explanation for the delayed toxicity of thioguanine.[2] During the course of our studies, efficient methods were developed for chemical syntheses of thioguanine deoxynucleoside[3] and DNA containing thioguanine.[4] The phosphoramidite monomer for thioguanine used for our solid phase synthesis was commercialized shortly after the publication of our method,[4b] which has made it much easier to obtain DNA containing 6-thioguanine at pre-determined position and to allow one to carry out biological studies relating to thioguanine.[5] With the techniques for the preparation[4] and the analysis[6] available, it is now possible to explore at the oligomer level the chemical properties of the incorporated thioguanine.

Thioguanine incorporated in DNA also causes chromosome damage[7] although its mechanisms are not yet understood. A route via sister chromatid exchange could be possible as thioguanine is an exceptionally good target for alkylating agents of S_N2 type. It has been demonstrated that the highest incidence of sister chromatid exchange was achieved with bifunctional alkylating agents.[8] Thus studies on reactions of thioguanine contained in DNA with bifunctional agents would be of interest. On the other hand, the thio-keto is unique and most nucleophilic within the whole molecule of the thioguanine DNA, thus thioguanine would provide a specific site for pegging desired functional groups. Furthermore introduced groups could be used for chemical and enzymatic manipulation to provide new tools for biochemical studies, such as DNA repair, sister chromatid exchange induction and DNA-protein interaction.

Here I wish to report an efficient and straightforward method for preparation of DNA containing various functional groups including carboxymethyl, hydroxyethyl, aminoethyl and fluorescent groups by one-step introduction of required functional groups onto the thio-keto of thioguanine within synthesized and fully purified deoxyoligonucleotides.

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2. Results and Discussion

Carboxyl Group

To establish an approach to preparation of DNA containing S^6-carboxymethylthioguanine, a pentamer was studied as a model. The pentamer containing thioguanine **A** (CGX°AT, X°: 6-thioguanine), prepared and purified with the reported method,[4b] was reacted with 2-iodoacetic acid in alkaline solution.[9] The reaction course was monitored by reversed phase HPLC. As shown in Fig. 1, the starting pentamer (peak **A**) was quickly converted to a new peak (**B**) with no detectable amount of by-oligomers formed. An UV spectrum of the isolated product (peak **B**) together with that of the starting pentamer **A** are shown in Fig. 2, which clearly shows the presence of S^6-carboxymethylthioguanine (λmax=310 nm) and the disappearance of thioguanine (λmax=345 nm). The product was enzymatically digested and found to have the correct nucleoside composition (Fig. 3).

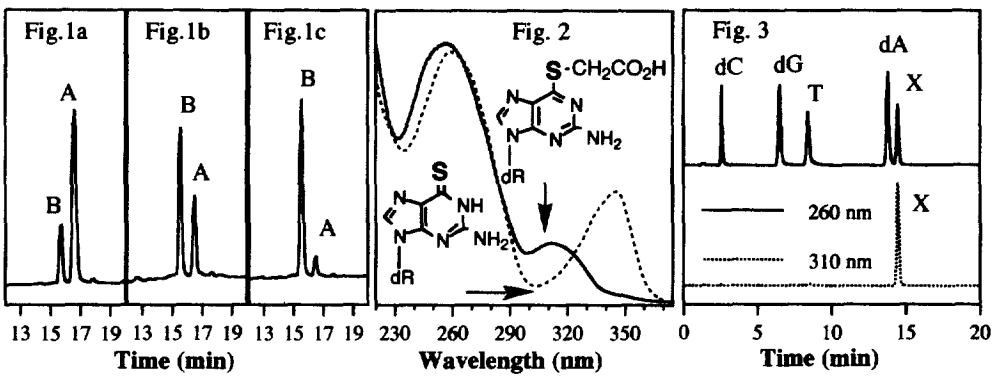
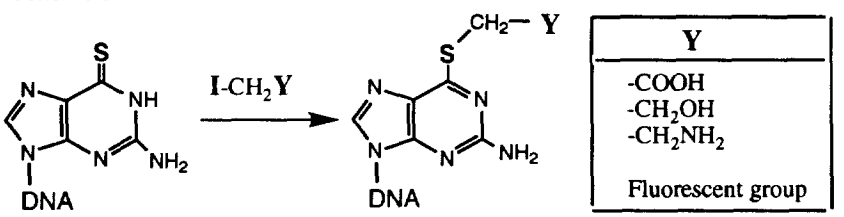


Fig. 1. HPLC traces of the conversion of the pentamer **A** (CGX°AT, X°: 6-thioguanine) into the pentamer **B** (CGXAT, X: S^6-carboxymethylthioguanine) by 2-iodoacetic acid at different times: (1a: 10 min; 1b: 1 hr; 1c: 3 hr) (see note 9); Fig. 2. UV spectra of pentamer **A** (broken line) and pentamer **B** (solid line); Fig. 3. HPLC profiles of enzymatic digest of pentamer B monitored at 260 nm (upper) and 310 nm (lower) (see note 10).

It is worth mentioning that under the conditions used the agent (I-CH_2COONa) did not react with other nucleophilic sites, eg. exocyclic nitrogen atoms of the bases (C, A, G), and the carboxyl group in the agent did not interfere with the reaction. The ease and effectiveness of the approach prompted the author to extend the chemistry for pegging other biologically useful functional groups on DNA (see Scheme 1).

Scheme 1

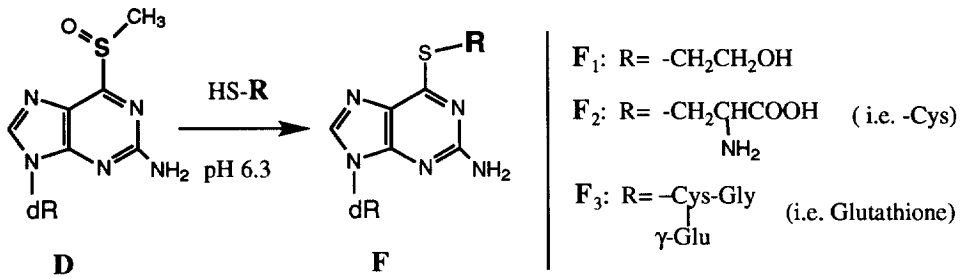


Hydroxyl Group

For site-specific introduction of hydroxyl (alcohol) group, 2-iodoethanol was used in the place of 2-iodoacetate. Under similar conditions to that used for 2-iodoacetate,[9] the reaction of the pentamer **A** with 2-iodoethanol was very slow, and only a half of the starting pentamer **A** was converted into pentamer **C** even with a greatly excessive amount of the agent and overnight incubation at RT.[11] However increasing the pH of the reaction medium from 9.2 to 10.5 speeded up the reaction which was complete within 2 hr.[12] The product, after isolation, also showed the presence of the distinctive UV absorption (around 315 nm) for S-alkylated

thioguanine, confirming that hydroxyethylation indeed occurred on the sulphur atom of thioguanine in the pentamer. Enzymatic digestion of the oligomer produced four normal nucleosides and a modified nucleoside. The modified nucleoside was found to co-elute with synthetic 2'-deoxy-S^6-hydroxyethylthioguanosine prepared in two different routes. One is by direct hydroxyethylation of 2'-deoxythioguanosine by 2-iodoethanol in alkaline solution.[13] The other is by substitution of oxidized 2'-deoxy-S^6-methylthioguanosine with 2-mercaptoethanol[14,16] to produce the target compound ($\mathbf{F_1}$) as showed in Scheme 2. The latter route has been successfully extended for other functional moieties, including cysteine ($\mathbf{F_2}$) and glutathione ($\mathbf{F_3}$).[15]

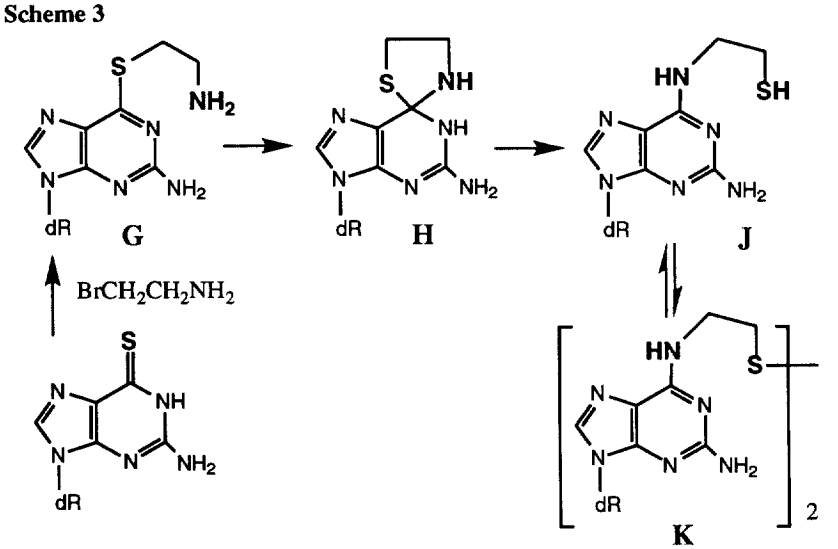
Scheme 2



Amino group

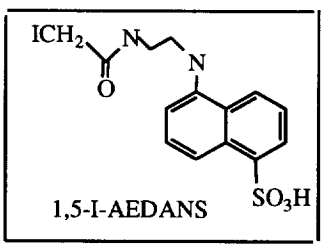
Then it was attempted to introduce an aminoalkyl group onto the sulphur atom of thioguanine of the pentamer **A**. In the reaction with halogenated ethylamines,[17,18] a new pentamer was formed and found to have a UV absorption around 315 nm, characteristic of S-alkylated thioguanine (ie. pentamer containing S^6-aminoethylthioguanine, cf: Fig. 2). However during a long storage the product pentamer was slowly converted into other oligomers. The conversion in neutral aqueous solution (pH 6.3) was slow with 10% being converted after a month storage at RT,[19] but was accelerated in alkaline aqueous solution. This prompted the author to investigate the chemistry involved in the conversion. Thus, 2'-deoxy-6-thioguanosine was treated with 2-bromoethylamine in aqueous solution (pH 8) and the reaction course was followed by HPLC. The starting nucleoside was converted to an alkylated product which, from its UV spectrum and our previous work,[20] was believed to be 2'-deoxy-S^6-aminoethylthioguanosine (**G**). The newly formed compound (**G**) was then slowly changed to another intermediate, probably a cyclic product (**H**). The latter was converted to **J** (minor product) and finally to **K** (major product). Compound **K** could be reduced to **J** by addition of thiol-containing agents, such as mercaptoethylamine.[15] A possible mechanism of the reaction is proposed (Scheme 3).

Scheme 3



Very recently I reported that 6-methylsulphoxypurine-2'-deoxynucleoside (its structure analogous to compound **D** in Scheme 2), when reacted with cysteine or mercaptoethylamine, could form adenine derivatives via cyclization.[20] It is readily envisioned that 2'-deoxy-6-methylsulphoxyguanosine (**D**) would go through similar transformations. In a similar approach to the reported one,[20] compound **D**, when treated with mercaptoethylamine, produced the intermediate **G**, which then turned to **H** and finally to **J**. No compound **K** was observed in this reactions because there was thiol-containing agent (mercaptoethylamine) present.[15] These observations are supportive to the mechanism proposed in Scheme 3.

The above description reports a facile method to introduce functional (carboxymethyl, hydroxyethyl and aminoethyl) groups on thioguanine in DNA as shown in Scheme 1. Among these three functional groups of two-carbon unit, the carboxymethyl group was most readily pegged on the sulphur atom of thioguanine. This may be due to the presence of the strong electron-withdrawing carboxyl group, facilitating the replacement of iodo group on its neighbouring α–carbon. One the other hand, the substitution of the sulphoxyl group on the modified guanine (in the reaction shown in Scheme 2) by mercaptoacetic acid (sodium salt) is much slower[15] than by the other two (mercaptoethanol and mercaptoethylamine). This could also be ascribed to the same electron-withdrawing effect of the carboxyl, reducing nucleophilicity of the mercaptol group on the α–carbon. However this effect was not observed on the β–carbon as demonstrated in the case of cysteine.



1,5-I-AEDANS

The finding that iodoacetic acid can react rapidly and almost quantitatively with thioguanine in oligomers (in Scheme 1) is evidently useful for biological studies, as there is a vast range of commercially available functional moieties, such as fluorescent chromophore (eg. 1,5-I-AEDANS),[21a] spin label (eg. I-A-PROXYL)[21b] or photo-activatable group (eg. azidophenacyl bromide)[21c] linked with iodoacetamido or bromoacetyl group. Their introduction would provide useful utility groups without a need for multi-step synthesis of the required base-adducts. To test this idea, a fluorescent compound (1,5-I-AEDANS) was examined. In a similar protocol to that applied for iodoacetic acid, the fluorescent group was readily pegged on thioguanine in the pentamer **A**.[22] And the fluorescence introduced could be used for investigation of DNA-protein interaction by energy transfer approach.[23] It could be foreseen that other functionalizing moieties could be readily introduced in DNA in similar ways and that introduced functional groups on DNA could find a variety of applications.

For instance, the guanine-modified oligomers could be used as substrates for DNA repair studies. DNA itself is a target of many chemical agents from environmental pollutants or from endogenously produced toxic compounds (eg. nitrosamines). The formation of O^6-carboxymethylguanine, a major DNA adduct, by carcinogenic *N*-nitrosoglycocholic acid was recently reported.[24] Interestingly the guanine adduct was not repaired by O^6-alkylguanine-DNA-alkyltransferase,[25] and it has also been suspected that O^6-carboxymethylguanine may be associated with an increased risk of gastric cancer [24]. The availability of DNA containing S^6-carboxymethylthioguanine, an analogue to O^6-carboxymethylguanine, would be very useful for the cancer studies. To be biologically useful, oligomers with length longer than 10 are often required. Therefore a 34 mer containing S^6-carboxymethylthioguanine was successfully prepared from the 34 mer containing thioguanine with a similar protocol.[26] An anion exchange column (FPLC)[27] was used instead of reversed phase HPLC to follow the progress of conversion and to purify the product. Biological studies of DNA containing S^6-carboxymethylthioguanine are underway.

In summary the work presented here illustrates a facile and versatile method for preparation of DNA containing functionalized thioguanine, including S^6-carboxymethylthioguanine, S^6-hydroxyethylthioguanine, S^6-aminoethylthioguanine and fluorescence-bearing guanine. It can be envisioned that other biologically useful groups could be readily pegged on thioguanine in longer oligodeoxynucleotides or even in DNA fragments as thioguanine can be very efficiently incorporated into cell DNA by cell culture and that the functional groups introduced on the thioguanine in oligomer could be used for further chemical or enzymatic manipulation. The described method, after some modification, could be applied to and would be particularly useful to RNA which is rich in functionalized bases and abundant in unusual but biologically essential structures.

3. Acknowledgements

The author is most grateful to Prof. Peter F. Swann for his inspiring discussion and continuous support and to Dr. Peter Karran for his interest in the work of S^6-carboxymethylthioguanine. Financial support from the Cancer Research Campaign (UK) is acknowledged.

4. Experiments

General methods and chemicals

Syntheses of oligomers containing thioguanine were carried out by ABI 391 DNA synthesizer (Applied Biosystems), using the thioguanine phosphoramidite [4b] (Glen Research) in which the thioketo is protected by dinitrophenyl group, and Millipore's Expedite monomers and supports in which amino groups of the bases are protected with t-butylphenoxyacetyl group. The deprotection of synthetic thioguanine-oligomers were effected with concentrated ammonia containing 10% mercaptoethanol at RT for 2 days.

Separation, purification and analysis of nucleosides and pentamers with HPLC on a Waters C_{18} cartridge and purification of longer oligonucleotides with FPLC on a Mono Q column were carried as described before.[4,16b,20]

All chemicals were from either Aldrich or Sigma and used directly without further purification unless stated otherwise.

Typical reaction of a bifunctional agent with pentamer containing thioguanine

For analytic scale, about 0.1 OD (A_{260}) of a purified pentamer (CGX°AT: X° = 6-thioguanine) in 100 µl of 0.4 M phosphate buffer (pH 9.2) was mixed with 10 µl of a required iodo-derivative (about 10 mM) in the same buffer. The reaction was followed by HPLC equipped with a dual wavelength monitor (Shimadzu SPD-10A) set at 260 and 310 nm or 335 nm. For preparation purpose, a larger scale (about 2 OD) of the oligomer was employed and the product, after HPLC-isolation, was subject to nucleoside composition analysis of its enzymatic digest.

5. References and Notes

§: The previous paper in the series is in Tetrahedron (1998, vol 54, pp187-196). The author wishes to dedicate this work to the memory of Prof. WANG Yu (1910-1997), a renowned Chinese organic chemist.

1. Elion GB. Science, 1989; 244: 41-47.
2. Swann PF. Waters TR. Moulton DC. Xu Y-Z. Zheng Q. Edwards M. Mace R. Science, 1996; 273: 1109-1111.
3. a) Xu Y-Z. Zheng Q. Swann PF. Tetrahedron Lett. 1991; 32: 2817-2820. b) Kung P-P. Jones RA. Tetrahedron Lett. 1991; 32: 3919-3922.
4. a) Christopherson MS. Broom AD. Nucleic Acids Research, 1991; 19: 5719-5724. b) Xu Y-Z. Zheng Q. Swann PF. Tetrahedron, 1992; 48: 1729-1740. c) Waters, TR. Connolly BA. Nucleosides and Nucleotides, 1992; 11: 985-998 and 1561-1574. d) Rappaport HR. Nucleic Acids Res. 1988; 16: 7253-7267. (In this paper, the thio-keto of the thioguanine monomer was not protected and the synthesis was less satisfactory).
5. a) Griffin S. Branch P. Xu Y-Z. Karran P. Biochemistry, 1994; 33: 4787-4793. b) Waters TR. Swann PF. Biochemistry, 1997; 36: 2501-2506.
6. Xu Y-Z. Swann PF. Analytical Biochemistry, 1992; 204: 185-189.
7. Tidd DM. Paterson AP. Cancer Res. 1974; 34: 738-746.
8. Latt SA. Annual Review of Genetics, 1981: 11-55.
9. 2.0 OD (A_{260}) of a purified pentamer (CGX°AT: X° = 6-thioguanine) in 100 µl of 0.4 M aqueous phosphate buffer (pH 9.2) was mixed with 5 µl of 0.1 M 2-iodoacetic acid (Sodium form) in the same buffer. The reaction was followed up by HPLC analyses and complete in about 3 hr. [HPLC conditions: Column: Radial-Pak cartridge (8NVC18 4 µ); Flow rate: 1 ml/min; Eluent A: 50 mM KH_2PO_4 (pH 6.3); Eluent B: 67% 50 mM KH_2PO_4 (pH 6.3) and 33% CH_3CN; Gradient: Eluent B increased from 0 to 30% within 30 min.)
10. The nucleoside composition analysis was carried out using a protocol described before [4b]. In brief: Pentamer **B** was digested with venom phosphodiesterase I (Sigma) and calf alkaline phosphatase (Sigma) and the resultant nucleosides were analyzed with reversed phase HPLC [HPLC conditions: Column: Nova-Pak C_{18} (3.9 x 150 mm); Flow rate: 1 ml/min; Eluent C: 50 mM KH_2PO_4 (pH 4.5); Eluent D: 67% 50 mM KH_2PO_4 (pH 4.5) and 33% CH_3CN. Gradient: first 4 min, 2% Eluent B, then increased to 20% (eluent B) over the next 16 min]. In a separate run, the peak X was found to co-elute with synthetic S^6-carboxymethylthio-2'-deoxyguanosine prepared as below: 2 mg of 6-thio-2'-deoxyguanosine in 100 µl of phosphate aqueous solution (0.4 M, pH. 9.2) was mixed with 100 µl of 0.1 M 2-iodoacetic acid (sodium form) in the same buffer. The reaction was monitored by both TLC and HPLC. After 1 hr, almost all the starting nucleoside was converted into

a new product (TLC: Rf from 0.33 to 0.0 in 20% MeOH/CH_2Cl_2; HPLC: Rt from 3 min to 6 min. [eluting with 0.67% acetonitrile in 50 mM phosphate buffer (pH 4.5)]. The product was purified by HPLC, and its UV data (λmax=310 nm and λmin=275 nm) are substantially different from the UV data (λmax=340 nm, and λmin =290 nm) of the starting nucleoside, 6-thio-2'-deoxyguanosine. Additional supporting evidence is from the fact that the synthetic compound (S^6-carboxymethylthio-2'-deoxyguanosine) is undistinguishable, in the term of UV spectrum and HPLC and TLC mobilities, from the product (S^6-carboxymethylthio-2'-deoxyguanosine) prepared via the reaction of 2-mercaptoacetic acid with **D** (2'-deoxy-6-methylsulphoxyguanosine) (see Notes 14 and 15).

11. During a prolonged reaction period, besides the starting and product peaks an additional peak was observed in the HPLC analysis. The peak was believed to be the S-S bridged dimer of the starting pentamer **A**, since it was returned to the starting pentamer **A** upon addition of thiol-containing agents, such as glutathione. Formation of S-S bridged dimers of oligomers containing thiobase has been previously reported (Milton J. Connolly BA. Nikiforov TT. Cosstick R J. Chem. Soc. Chem. Comm. 1993; 779-780).
12. The protocol for conversion of pentamer **A** into pentamer **C** is similar to that for pentamer **B** except that the pH of the solution was 10.5 and the agent (2-iodoethanol) was pre-dissolved in CH_3CN and greatly excessive.
13. Route a: 2'-deoxythioguanosine was dissolved in dilute NaOH (0.1 M) aqueous solution and vigorously mixed with 2-iodoethanol in CH_3CN (20% w/v) for 2 hr. The new spot (Rf: 0.6 in 20% MeOH/CH_2Cl_2) was isolated by preparative TLC and further purified by reversed phase HPLC [Conditions for the purification, see note 10]. The product showed its characteristic UV spectrum (λmax=310 nm).
14. Route b: 2'-deoxythioguanosine was converted to 2'-deoxy-S^6-methylthioguanosine in a similar way to that described before [16]. Then the product was oxidized with MCPBA to 2'-deoxy-6-methylsulphoxyguanosine, **D** (Rf: 0.46 in 20% MeOH/CH_2Cl_2 for TLC and λmax=325 nm) and 2'-deoxy-6-methylsulphonylguanosine, **E** (Rt: 0.56 in 20% MeOH/CH_2Cl_2 for TLC and λmax=330 nm). The confirmation of **D** was based on the fact that it could be further oxidized to **E** with additional amount of MCPBA and that when treated with NaSH aqueous solution, both compounds (**D** and **E**) were converted into the same final product, which has the characteristic UV absorption at 340 and which co-eluted with authentic 2'-deoxythioguanosine in HPLC analysis.
15. Typical protocol for preparation of S-substituted 2'-deoxy-6-thioguanosine is as below: **D** (or **E**) was reacted with equal volume of an agent [10 mM 2-mercaptoethanol, 2-mercaptoethylamine, cysteine or glutathione) in phosphate buffer (pH 6.3). After 1 hr, HPLC showed that the starting nucleoside (Rm=14 min) was completely converted to a new peak (Rm=23.6 min for 2'-deoxy-S^6-hydroxyethylthioguanosine ($\mathbf{F_1}$), Rm=18 min for 2'-deoxy-S^6-aminoethylthioguanosine (**G**), Rm= 15.5 min for 2'-deoxy-S^6-cysteinylthioguanosine ($\mathbf{F_2}$) or Rm=19 min for S^6-glutathionyl-2'-deoxythioguanosine ($\mathbf{F_3}$)]. Each of $\mathbf{F_1}$ and **G** has the same mobility as the respective compound prepared via direct alkylation of 2'-deoxythioguanosine with 2-iodoethanol or 2-bromoethylamine. Compound $\mathbf{F_1}$ and $\mathbf{F_3}$ are stable, but compound **G** and $\mathbf{F_2}$ were intramolecularly cyclized and then ring-opened to give a final product, N^6-β-mercaptoethyl-2,6-diaminopurine deoxynucleoside (**J**) or N^6-cysteinyl-2,6-diaminopurine deoxynucleoside respectively. Interestingly, the formation of 2'-deoxy-S^6-carboxymethylthioguanosine by this route was exceptionally slow.
16. a) Xu Y-Z. Zheng Q. Swann PF. Nucleosides and Nucleotides, 1995; 14: 929-932. b) Xu Y-Z. Tetrahedron, 1996; 52: 10737-10750.
17. Both 2-bromoethylamine (commercial) and 2-iodoethylamine (synthetic) were employed, and the latter is slightly more efficient.
18. 2-iodoethylamine was prepared by replacement of bromo group of 2-bromoethylamine with dry sodium iodide in acetone under reflux. (see Gabriel S. Berichte Der Deutschen Chemischen Gesellschaft, 1888; 21: 1049-1057)
19. While under the same conditions the pentamer **C** (i.e. the pentamer containing S^6-hydroxyethylthioguanine) remains unchanged even after 2 months.
20. Xu Y-Z. Tetrahedron, 1998; 54: 187-196.
21. a) 1,5-I-AEDANS is a short form of N-iodoacetyl-N'-(5-sulpho-1-naphthyl)ethylenediamine. This fluorescent probe has been extensively used for sulfhydryl determination (see ref. 23). b) I-A-PROXYL is a short form of 3-[2-(2-iodoacetamido)acetamido]-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, which is a free radical. c) Azidophenacyl has been coupled to phosphorothioate-containing oligonucleotides and the resulting oligomers used for photo-crosslinking with a protein (integrase) (see Heuer TS. Brown PO. Biochemistry, 1997; 36: 10655-10665).
22. The pentamer containing thioguanine was rapidly converted to the product with longer retention time. To get satisfactory conversion, freshly prepared solution of 1,5-I-AEDANS should be used as the agent is sensitive to light.
23. Wu CW. Stryer L. Proc. Nat. Acad. Sci. USA, 1972; 69: 1104-1108.
24. Shuker DEG. Margison GP. Cancer Research, 1997: 57: 366-369 (more references therein)
25. For reviews, see Lindahl T. Sedgwick B. Sekiguchi M. Nakabeppu Y. Annu. Rev. Biochem. 1988; 57: 133-157 and Swann PF. Mutation Research, 1990; 233: 81-94.
26. The sequence of the 34 mer is 5'-AGCTTGGCTGCAGGTXGACGGATCCCCGGGAATT 3' (X: modified base). S-carboxymethylation of the 34 mer was slightly slower than that of the pentamer above.
27. Since sulphur atom on thioguanine was alkylated and its imino proton lost, the S^6-carboxymethylated thioguanine oligomer was easily separated from its starting oligomer under alkaline condition (see ref. 6).