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Reactive DNA: 6-Methylsulphoxypurine Used for Site-specific and Chemical Crosslinking with Cysteine and its Peptide§

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Abstract: A novel method is described for site-specific and chemical crosslinking of oligodeoxynucleotides containing 6-methylsulphoxypurine with cysteine or peptides containing cysteine. 6-Methylsulphoxy group on purine is stable in aqueous solution, but easily replaceable by the thiol group of cysteine and glutathione. A mechanism of the reaction is proposed, and potential applications are discussed with a focus on DNA-protein interactions.

INTRODUCTION

Interactions between DNA and proteins are the most fundamental reactions in biological chemistry, involved in every essential biological process from DNA replication to gene regulation.[1] The understanding of these reactions has been, and remains, of great interest. One of the techniques used to study DNA-protein interaction has been photo crosslinking.[2] Azide derivatives, halogenated bases and thio-bases are the most frequently used among the many photoactive agents.[3] However an inherent disadvantage of photo crosslinking is that the reaction is generally non-specific.[2,3] An alternative approach is by chemical crosslinking, in which the reaction occurs primarily via nucleophilic or electrophilic substitution. This kind of reaction is well understood and relatively easy to perform. However, there are a great number of potentially reactive sites in any biological macromolecule. For instance, a single DNA has numerous nucleophilic sites, such as exocyclic amino groups. Therefore the control of the specificity of the reaction is a real challenge but potentially of great use in biochemistry. In order to achieve the desired specificity, it is essential to create a unique reactive site, eg. a suitable electrophilic site within the DNA, which would react with a single or limited number of nucleophilic groups within the protein so that the reaction would proceed in a site-specific manner. Although cysteine is one of least abundant amino acids in protein it has uniquely important properties. The SH group in cysteine is readily oxidized to form the S-S bridges, which contribute substantially to the structure of many proteins. Furthermore, the SH group is of high nucleophilicity which is often the driving force for protein containing cysteine to interact with other macromolecules. For example cysteine plays a decisive role in the action of methylguanine methyltransferase (MGMT).[4] This paper presented here describes the development of a novel protocol to chemically crosslink cysteine or peptides containing cysteine with reactive DNA containing 6-methylsulphoxypurine in a site-specific manner. This work may provide a useful method for studies on interactions between DNA or RNA with proteins.

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RESULTS AND DISCUSSION

Preparation and characterization of oligodeoxynucleotide containing 6-methylsulphoxypurine

The synthesis of a pentamer (CG^{MeS}PAT, ^{MeS}P: 6-methylthiopurine) was carried out as previously described.[5] In the preparation phosphoramidites with base-labile protecting groups were used for the natural nucleotides allowing deprotection at room temperature (RT) to avoid possible damage of methylthiopurine. For the oxidation magnesium monoperoxyphthalate (MMPP) was chosen because of its good solubility in water and its mild oxidizing capability.[6] Examination of the course of the oxidation showed that either excessive amount of MMPP or prolonged reaction period could produce a small amount of by-products. However at 0°C and with a limited amount of MMPP, little damage was caused towards normal bases while the methylthio group on the purine was selectively activated and became convertible. To further characterize the oxidized product(s), the ^{MeS}P pentamer, after treatment with MMPP, was purified with HPLC, and the isolated pentamer subjected to nucleoside composition analysis. The result (Fig. 1) shows that the modified nucleoside was the mono-oxidized product (i.e. 6-methylsulphoxypurine-2'-deoxynucleoside) and that no doubly-oxidized product (6-methylsulphonylpurine-2'-deoxynucleoside) was observed.

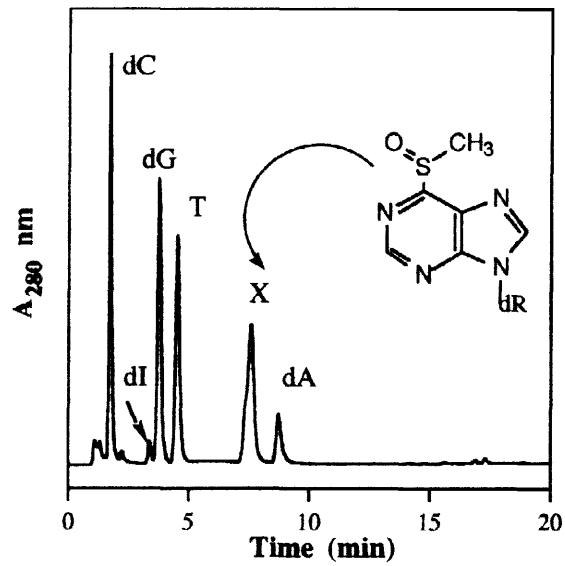


Fig. 1. HPLC profile of nucleoside composition analysis of the pentamer containing oxidized 6-methylthiopurine. The pentamer was digested with phosphodiesterase and alkaline phosphate at 37 °C for 2 hours. The peak X could co-elute with synthesized 6-methylsulphoxypurine-2'-deoxynucleoside. The small amount of deoxyinosine was from enzymatic deamination of deoxyadenosine. For details, see experimental part.

Chemical properties of oligodeoxynucleotide containing 6-methylsulphoxypurine

A detailed investigation was carried out of chemical properties of the pentamer containing 6-methylsulphoxypurine [$^{Me(O)S}$P pentamer] under various conditions and the results are summarized below:

a) stability under alkaline conditions: pH effects

In aqueous solution at pH 6-7, the modified purine in the $^{Me(O)S}$P pentamer was stable at RT and unchanged after its storage for several months at -20°C, even after repeated freezing and thawing. At pH 8 no change was observed after overnight incubation at RT. When the pH of the solution was raised to 9, there was very slow hydrolysis of the modified purine, leading to the formation of hypoxanthine. Even at pH 10, the modified purine pentamer was substantially stable with a half-life of about 18 hours.[7] Obviously the stability of 6-methylsulphoxypurine in aqueous solution at neutral pH is very useful for its reaction with other molecules as the most biological reactions occur under the physiological conditions, i.e. in aqueous solution and at neutral pH.

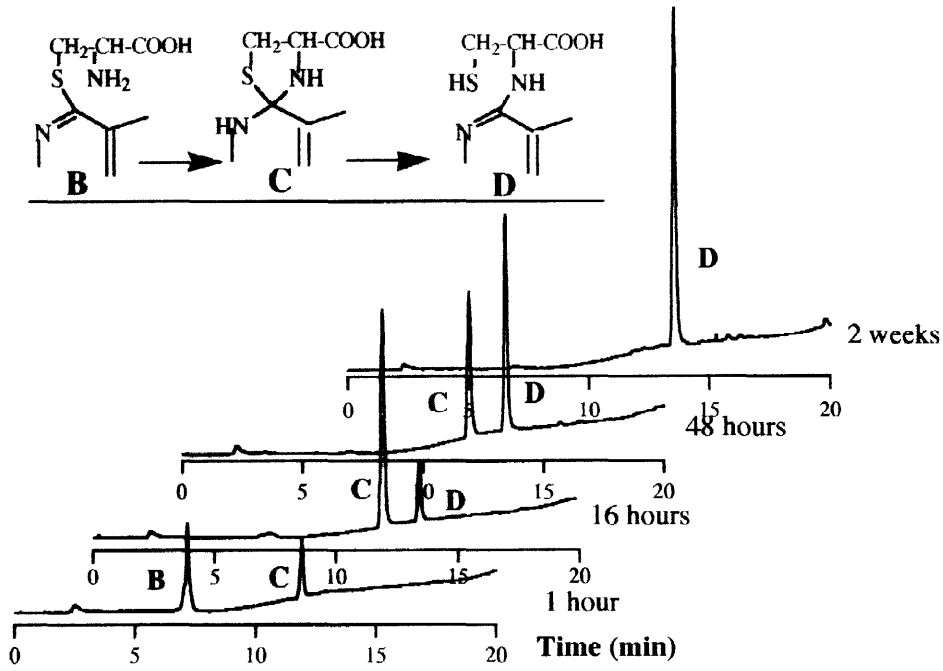


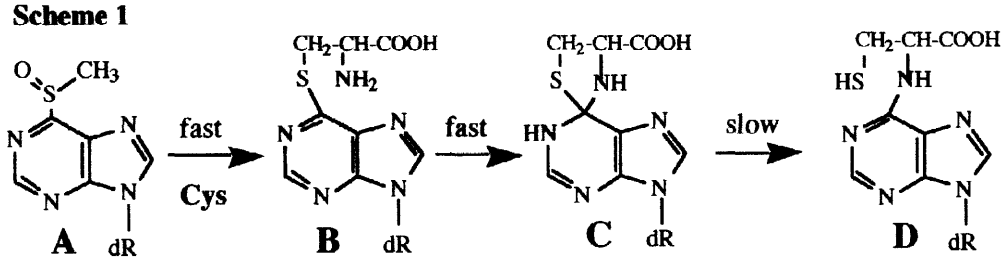
Fig. 2. HPLC profiles of the conversion course of the purified intermediate **B** into the cyclic intermediate **C** and the product **D**. **B** was prepared from a reaction of 6-methylsulphoxypurine-2'-deoxynucleoside with cysteine. For details, see experimental part.

b) reactivity towards cysteine

The reaction course of the Me(O)SP pentamer with cysteine (Cys) was followed up by HPLC. It was found that the reaction (in aqueous solution at pH 6.3) was very rapid even with a dilute cysteine solution (1 mM) and complete within 1 hour. Comparison of the reaction with cysteine with the reaction with glycine (see below), it convincingly suggests that it is the thiol group of Cys that allows the reaction to take place.

However, it was also noted that the product from the reaction between Cys and the pentamer was decreased when it was left for a longer time either in the reaction mixture or in its purified form, indicating that the crosslinked product was not very stable. In an effort to understand the underlying chemistry of this instability, 6-methylsulphoxypurine-2'-deoxynucleoside (**A**) was prepared and treated with a dilute cysteine solution. Compound **A** was quickly converted into an intermediate (**B**), putative S-bonded 6-cysteinylpurine-2'-deoxynucleoside. The intermediate, even after purification, was rapidly converted to another intermediate (**C**), which slowly turned to compound **D**. Compound **D** was believed to be N-bonded 6-cysteinylpurine-2'-deoxynucleoside (Fig. 2) because it had a similar UV spectrum (Fig. 3a) to that of N^6-methyladenosine.[8] A mechanism for this transformation is proposed as shown in Scheme 1:

Scheme 1



To examine further the proposed mechanism, several additional experiments were carried out. When 2-mercaptoethylamine (i.e. 2-aminoethanethiol) was used instead of cysteine, a similar reaction was observed. ie. an S-substituted product (**B**-type) first formed which then changed gradually to a new compound (C-type), then very slowly to another new compound (**D**-type). The cyclization from **B** to **C** is faster with cysteine than with 2-mercaptoethylamine. This may be ascribed to the neighbouring carboxyl group, exclusively present in cysteine, which could enhance nucleophilicity of the vicinal amine by partial de-protonation via a five-member ring (scheme 2).

When 2-mercaptoethanol was used instead of cysteine, only an S-substituted product (**E** in Scheme 3 and Fig. 3a) was observed.[9] No change of this product was found even after 5 days, suggesting that under the conditions used the hydroxyl group in the S-substituted product, although in a favoured position to attack the C-6 of the purine, does not produce cyclization. This probably is due to the weak nucleophilicity of the hydroxyl group.

When the S-substituted compound (**B**) (in Scheme 3) was treated with a large excess of mercaptoethanol, only the cyclic product (**C**), and then its ring-opened product (**D**) (not shown in Scheme 3), were formed and no formation of mercaptoethanol-substituted product (**E**) was observed. This strongly suggested that the intramolecular cyclization is much more favoured than an intermolecular substitution.

The reaction of **A** was also undertaken with an N-blocked cysteine (N-Boc-cysteine) and only the S-substituted product (**F**) was formed (Scheme 4). In contrast to **B**, the product (**F**) was stable in aqueous solution at pH 6.3. The stability may be ascribed to the fact that the attacking primary amine present in **B** is not available. This finding offers a way to form stable crosslinked oligomer-peptide, since the α-amino group of cysteine in peptides (unless the cysteine is the N-terminal residue) is always blocked in the form of peptide amide bond. It may also explain the instability of the crosslinked product formed between the pentamer and free cysteine.

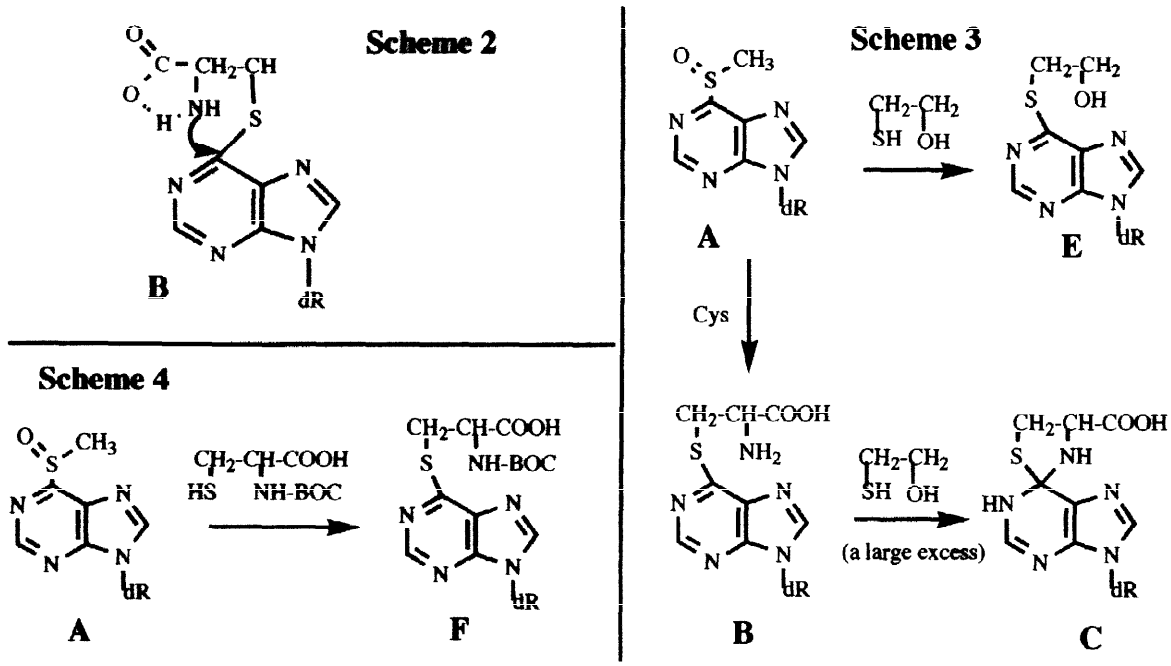
The above work would present a facile route to the preparation of N-alkyladenine derivatives (such as **D**) under much milder conditions than the reported.[10] The current route should be particularly useful for preparation of the RNA oligomer, in which a variety of N-alkyladenine derivatives are naturally present.[11] In addition our method should have an advantage in the use of ^{35}S-radioactive cysteine since the substituting agent is employed in a very low concentration and at the last step. Thus radioactivity and two functionals (carboxyl and thiol groups) could be easily added onto purine residues at both the nucleoside level and the oligomer level for further chemical or enzymatic manipulation.

c) reactivity towards other amino acids

Besides cysteine, other amino acids were also tested in the reaction with oligomer containing 6-methylsulphoxypurine and the results are summarised below:

Glycine: at neutral pH, no reaction with the $^{Me(O)S}P$ pentamer was observed, even with 100 mM glycine (that is 100 times more concentrated than with cysteine) and overnight incubation at RT.

Lysine: the reaction of the $^{Me(O)S}P$ pentamer with lysine was pH-dependent. At neutral pH, the pentamer remained unchanged, even overnight incubation. But at pH 10, the reaction did take place, after 1 hour a third of the starting pentamer was consumed and an overnight incubation gave rise to complete reaction. The reaction produced three new products, one of which is the hydrolysed product (15%), ie. the pentamer containing hypoxanthine, and other two are probably ε-amino and α-amino substituted adenine pentamers respectively (total



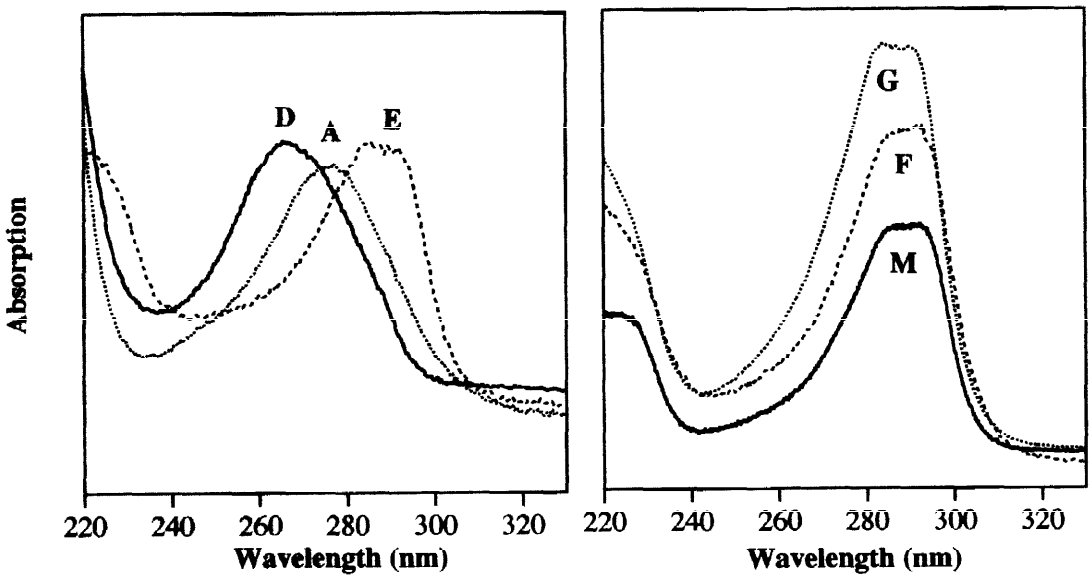


Fig. 3a) UV spectra of nuceloside derivatives showing the effects of the atoms linked with the C6 of the purine upon the maximum wavelength. **A**: 6-methylsulphoxypurine-2'-deoxynucleoside (λmax=277 nm); **D**: N-bonded 6-cysteinylpurine-2'-deoxynucleoside (λmax=266 nm) ; **E**: S-bonded 6-(mercaptoethanol)purine-2'-deoxynucleoside (λmax=282-292 nm).

Fig. 3b) UV spectra of 6-S-alkylated thiopurine nucleosides showing the characteristic maximum wavelengths at about 282-292 nm; **F**: S-bonded 6-(N-Boc-cysteineyl)purine-2'-deoxynucleoside; **G**: S-bonded 6-(glutathionyl)purine-2'-deoxynucleoside; **M**: 6-methylthiopurine-2'-deoxynucleoside. All measurements are made in aqueous solution at neutral pH.

85%).[12] In all cases, the reactions with lysine were much slower than those with cysteine.

Crosslinking with Glutathione -- a tripeptide containing cysteine

a) reaction with a tripeptide

From the above experiments, cysteine behaves as a good candidate for site-specific reaction with Me(O)SP in both the nucleoside and pentamer at neutral pH. It is the mercapto group of cysteine that plays the decisive role. Furthermore, at neutral pH, non-thiol-containing amino acids, such as glycine, histidine or lysine (a highly basic amino acid) are completely inactive, and it would be reasonable to assume that they, when present in peptides or proteins, would not compete with cysteine. Therefore the above results may be of useful potential for site-specific crosslinking between oligomer and peptides of interest. To explore this idea, glutathione was chosen to investigate its reaction with Me(O)SP at both the nucleoside and oligomer level. Glutathione, an important biological molecule, plays significant roles in the detoxification of foreign compounds in human body[13] and has also been found to have anticancer activity.[14] However, the chemical structure of this important molecule is amazingly simple, consisting of three amino acids, ie. γ-Glu-Cys-Gly.

When the Me(O)SP pentamer was reacted with glutathione (reduced form) (1 mM, pH 7) the oligomer was consumed rapidly at RT, with the most being converted within 1 hour (Fig. 4). However, when the same oligomer was treated with 10 mM oxidized glutathione (i.e. the S-S bridged dimer), no reaction was observed after 1 hour incubation. This provides another line of supporting evidence that mercapto group is essential for this crosslinking reaction.

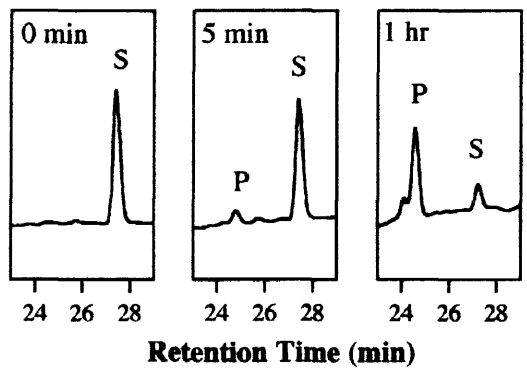


Fig. 4. HPLC profiles of the reaction of the pentamer containing 6-methylsulphoxypurine (Peak **S**) with glutathione (reduced form) monitored at 260 nm at 0 min, 5 min and 1 hour. Peak **P** is the crosslinked product. For details, see experimental part.

b) stability of the crosslinked complex

The crosslinked pentamer-peptide was isolated by HPLC and found to be stable in aqueous solution (pH 6.3) with no significant change after one month's storage at -20 degree. Similarly S-bonded 6-glutathionyl-purine-2'-deoxynucleoside (**G**) (Fig. 3b) was also found to be stable and unchanged after a long incubation (5 days).

c) crosslinkage between the pentamer and glutathione

The reaction course was followed up of the Me(O)SP pentamer with glutathione. The starting oligomer (peak **S** in Fig. 4) was quickly converted into a new peak (peak **P**). This peak, after isolation and purification, was subjected to enzymatic digestion for nucleoside composition analysis.[15] Digestion with phosphodiesterase (exonuclease activity) and phosphatase for a limited time produced chiefly two nucleosides, dT and dA, the nucleosides right end to the modified nucleoside, and the digestion could not be completed. This indicates that the access by digesting enzymes to the modified purine was blocked probably by the linked glutathione.

However, the addition of Nuclease P1 and overnight incubation did force the digestion to completion, resulting in five peaks, four of which corresponded to the four normal nucleosides. The fifth peak was a nucleoside derivative (called **H**), however, did not co-elute with the synthesized S-bonded 6-glutathionylpurine nucleoside **G**. Although the reason for the formation of **H** is not known yet, it might be ascribed to the transformation of **G** by enzymes present in the digestion mixture.

In short, in the view of all of above findings and the supportive evidence from mass spectroscopic analysis,[16] it would be reasonable to conclude that the modified pentamer was crosslinked with glutathione via the sulphur atom of cysteine.

The present work provides a novel method to site-specifically crosslink cysteine (or its peptides) with oligomers (or DNA) containing 6-methylsulphoxypurine. As 6-methylsulphoxypurine is stable in aqueous solution at neutral pH and could be placed at any pre-determined position in oligonucleotides and, this should be of general use for studying interactions of DNA with proteins. As the modification of the purine is kept to a minimum, the modified oligomer (or DNA) should not cause much disturbance to its structure, and hence its interaction with proteins should be virtually the same. Therefore the resultant crosslinking site should, to greater extent, reflect the actual interacting architecture. This may be of particular interest and value in the case of methylguanine methytransferase (MGMT),[4] as thiol group of the cysteine in this protein reacts directly with the methyl on the O6-position to repair methylated guanines. If the cysteine in MGMT would replace the methylsulphoxy group on guanines in oligomer (or DNA), a crosslinked protein-DNA should be formed and, if stable, the crosslinked product could be further characterized or manipulated. Such an approach would also be very useful for studying interaction (recognition) of DNA with DNA-binding proteins. One of immediate concerns is whether the conclusion from this work would hold true for reactions of longer oligomers with longer peptides (or proteins). It has been tested that the reaction of a longer oligomer (12-mer)[17] with glutathione also produced a crosslinked product. Investigation is underway of crosslinking of longer oligomers with longer peptides and proteins

ACKNOWLEDGEMENTS

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EXPERIMENTAL

Chemicals and general methods

Syntheses of oligomer were carried out by ABI 391 DNA synthesizer (Applied Biosystems), using Millipore's Expedite monomers and supports in which amino groups of the bases are protected with t-butylphenoxyacetyl group. All other chemicals were from either Aldrich or Sigma and used directly without further purification unless stated otherwise. General methods such as purification with Nensorb Prep cartridges (Du Pont) or Fast protein liquid chromatography (FPLC) on a Mono Q 5/5 column (Pharmacia), nucleoside composition analysis by reversed phase HPLC were carried as described before.[19]

Preparation of 6-methylsulphoxypurine-2'-deoxynucleoside (**A**)

14.5 mg of 6-methylthiopurine-2'-deoxynucleoside (prepared as previously reported[5]) in 1 mL of DMF

was treated with 0.5 equimole of m-chloroperoxybenzoic acid (MCPBA) in DMF at RT. The reaction course was monitored by both thin layer chromatography (TLC) on silica and HPLC with C_{18} column. After 15 min, nearly half of the starting nucleoside (Rf: 0.5 in 10% MeOH/CH_2Cl_2 by TLC) was converted into a product with a lower Rf of 0.25. After isolation by HPLC [Column: Radial-Pak cartridge (8NVC_{18} 4μ) from Waters, Flow rate: 1 ml/min, Eluent: 0.67 % CH_3CN in 50 mM KH_2PO_4 (pH 6.3) aqueous solution], the product (**A**) showed the following characteristics: a) the product was convertible by various nucleophiles, eg. NaSH, to give its 6-thiopurine derivative (which has the same retention time (Rt) with HPLC and the same UV spectrum as the authentic 6-thiopurine[18]; b) the product could be further oxidized to a new compound (Rf: 0.38 by TLC) -- the doubly-oxidized product, which was also convertible by NaSH to give the same 6-thiopurine 2'-deoxynucleoside.

Reaction of **A** with Cysteine or 2-Mercaptoethylamine

To 200 μL [0.4 OD(A_{260})/ml] of 6-methylsulphoxypurine-2'-deoxynucleoside (**A**) in phosphate aqueous solution (50 mM KH_2PO_4, pH 6.3), was added 20 μL of freshly made 10 mM cysteine in the same buffer. The reaction was followed up by HPLC with dual wavelength monitor at 260 nm and 280 nm (HPLC conditions as the above). The starting nucleoside **A** [Retention time (Rt)=7.3 min and Ratio of absorption at 280 nm over that at 260 nm ($R_{280/260}$)=1.6] was rapidly converted to an intermediate **B** (Rt=7.5 min and $R_{280/260}$ =2.9), then further to another intermediate **C** (Rt=12 min and $R_{280/260}$=0.87) which was changed slowly to a sole product **D** (Rt=13.6 min and $R_{280/260}$=0.87). After isolation, the product showed a UV spectrum (see Fig. 3a) similar to that of 6-N-alkyladenine nucleoside[8]. The same reaction was also monitored with TLC with the final product **D** being on the original line (in 20% MeOH/CH_2Cl_2) and a positive nucleoside response [with the spray of anisaldehyde/sulphuric acid/ethanol (5:5:90)]. In a similar protocol, 6-methylsulphoxypurine-2'-deoxynucleoside (**A**) was reacted with 2-mercaptoethylamine.

Reaction of **A** with 2-Mercaptoethanol or with N-Boc-Cysteine

To 200 μL [0.4 OD(A_{260})/ml] of 6-methylsulphoxypurine-2'-deoxynucleoside (**A**) in a phosphate buffer (50 mM KH_2PO_4, pH 6.3) was added 2-mercaptoethanol in the same buffer with the final conc. being 1 mM. The reaction mixture was analyzed by HPLC (the conditions as above). After half a hour, the starting nucleoside (**A**) was converted completely into a new product (**E**) (Rt= 14 min, $R_{280/260}$=3.3). The product, after isolation by HPLC, showed a UV spectrum (λmax= 282-292 nm) typical of S-substituted derivative.

Similarly Compound **A** was reacted with N-(Boc)-cysteine [prepared by the reduction[20] with $NaBH_4$ of N,N'-bis-(t-Boc)-cystine (from Sigma)] to produce compound **F** (with Rt =16.8 min, $R_{280/260}$=3.4). Compound **F** has a UV spectrum (λmax= 284-293 nm) also typical of S-substituted derivative.

Reaction of **A** with glutathione

Compound **A** was reacted with glutathione under similar conditions as with cysteine and the reaction was followed up by HPLC. The resultant product **G** showed two slightly split peaks (Rt=11.2 and 11.4 min) but with the same $R_{280/260}$ value of 3.0 and a UV spectrum (λmax= 283-293 nm) typical of S-substituted derivative.

Preparation of pentamer containing 6-methylsulphoxypurine

The preparation was carried out in a protocol modified from the published one.[5] In brief: 5 OD (A_{260}) units of a synthetic pentamer containing 6-methylthiopurine (CG^{MeS}PAT) was dissolved in 200 μL of water, to which was added 20 μL of freshly made 4.2 mM MMPP aqueous solution. The reaction mixture was left at 0°C or at RT. For monitoring the reaction progress, aliquot of the reaction mixture was treated with 1 M sodium sulphide aqueous solution to convert the pentamer containing oxidized methylthiopurine to the pentamer containing thiopurine followed by HPLC analysis monitored at 260 nm and 330 nm since the pentamer containing thiopurine has unique absorption at 330 nm.[18] When the oxidation was complete, the product was isolated by HPLC and could be used without further purification for reactions with amino acids (see below).

Characterization of pentamer containing 6-methylsulphoxypurine

The above HPLC-isolated oligomer was desalted using Sep-Pak cartridge as described before[5] and a part of the oligomer was subjected to enzymatic digestion with phosphodiesterase and alkaline phosphatase followed by HPLC analysis of the resultant nucleosides. HPLC conditions. Column: Nova-Pak C_{18} (3.9 x 150 mm) from Waters. Flow rate: 1 ml/min. Eluting system I: Eluent A: 50 mM KH_2PO_4 (pH 4.5); Eluent B: 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. Four standard peaks [dC: 1.8 min, dG: 3.9 min; T: 4.6 min; and dA: 8.9 min] and a new peak (7.7 min) were observed. The new peak was also found to co-elute with the prepared 6-methylsulphoxypurine-2'-deoxynucleoside.

Mass spectroscopic data: 1534 (found); 1534 (calculated) for the product pentamer; 1518 (found) and 1518 (calculated) for the starting pentamer.

Stability of the pentamer containing 6-methylsulphoxypurine under alkaline conditions

100 μL (0.1 OD / mL) of purified $CG^{MeS(O)}PAT$ in 50 mM phosphate buffer (pH 6.3) was mixed with an equal volume of an alkaline solution. The pH of the resultant mixture was measured and the reaction course followed up by HPLC analysis at given times. HPLC conditions. Column: Radial-Pak cartridge (8NVC18 4 μ) from Waters. Flow rate: 1 ml/min. Eluting system II: Eluent C: 50 mM KH_2PO_4 (pH 6.3); Eluent D: 67% 50 mM KH_2PO_4 (pH 6.3) and 33% CH_3CN.

Reaction of the modified pentamer with various amino acids or with glutathione

100 μl of purified $CG^{MeS(O)}PAT$ in 50 mM phosphate buffer (pH 6.3) was mixed first with 10 μL of 1 M phosphate buffer (pH 6.5), then with 10 μL of a specified amino acid (eg. cysteine, glycine, lysine or histidine) of known concentrations. The reaction course was followed up by HPLC analysis (the conditions as the immediately above). With a similar protocol the modified pentamer was reacted with both glutathione (reduced form) and (oxidized from).

Characterization of the crosslinked glutathione-pentamer

Purification and analysis are done in a similar protocol to the one used for the pentamer containing 6-methylsulphoxypurine.

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§. The author wishes to dedicate this paper to the memory of Prof. WANG Yu (1910 - 1997), a former consulting editor of the Journal and the late honorary director of Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences.

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7. At pH 10, the modified purine in the pentamer was slowly hydrolysed to hypoxanthine chiefly, and a minor product was noted resulting probably from the attack of hydroxide ion on the C2-position of the purine in a manner of Dimroth rearrangement (Fujii, T.; Itaya, T. *Reviews on Heteroatom Chem.* **1997**, *16*, 257-285). A similar phenomenon was observed when S^6-dinitrophenylthiopurine oligomer was treated with alkaline aqueous solution (see Ref. 18).
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9. The product has a UV spectrum (λmax=280-290nm), which is characteristic of S-substituted nucleoside (also see Fig. 3b).
10. Our method (1 mM of reagent, 20 °C) is much milder than a reported one (5 M of reagent, 65°C) (Ferentz, A.E.; Verdine, G.L. *J. Am. Chem. Soc.* **1991**, *113* , 4000-4002 and Macmillan, A.M.; Verdine, G.L. *Tetrahedron* **1991**, *47*, 2603-2616).
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15. The digestion of this crosslinked complex was very slow under the same conditions as that for the starting pentamer (see Fig. 1).
16. Mass spectroscopic analysis of the isolated peak (peak **P** in Fig. 4) showed a fragment with m/e of 1648, substantially bigger than the starting pentamer (m/e: 1534).
17. Sequence of the 12 mer is AGG CXG ACG GAT (X: 6-methylsulphoxypurine).
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