Synthesis of 2′-thio-uridine and -cytidine derivatives as potential inhibitors of ribonucleoside diphosphate reductase: thionitrites, disulfides and 2′-thiouridine 5′-diphosphate

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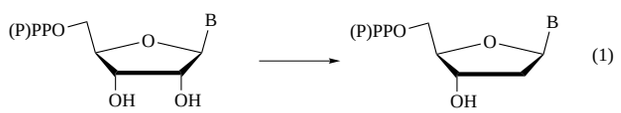
In order to study or/and inhibit ribonucleotide reductase, thio derivatives of uridine and cytidine which can interact with the reducing cysteines at the active site have been prepared. The first nucleosidic thionitrites 12 and 15 have been synthesized from 2′-thiouridine 4 and 2′-thiocytidine 11, respectively and their ability to generate spontaneously nitric oxide, a potent inhibitor of *Escherichia coli* ribonucleotide reductase (RDPR), has been evidenced. The 2′-thiol function in 2′-thiouridine is protected as a mixed disulfide to obtain the stable and useful precursor 18 of 2′-thiouridine 5′-diphosphate 19 which has been found strongly to inhibit RDPR.† The same protection has been successfully used during the conversion of 2′-thiouridine into 2′-thiocytidine.

Introduction

The reduction of ribonucleotides to 2′-deoxyribonucleotides by the enzyme ribonucleotide reductase is considered to be a rate-limiting step in the biosynthesis of DNA [equation (1)].

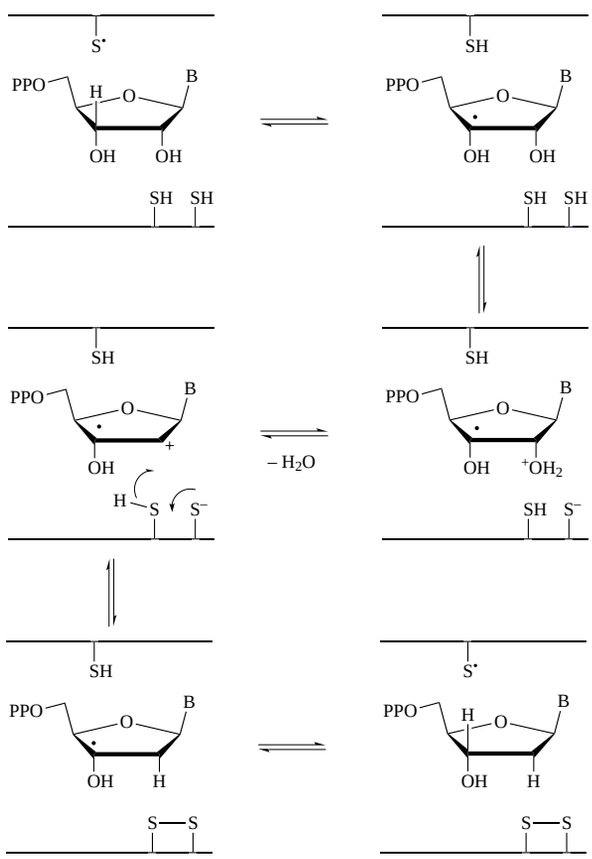


(1)

Equation (1) Reduction of ribonucleotides to 2′-deoxyribonucleotides by the enzyme ribonucleotide reductase

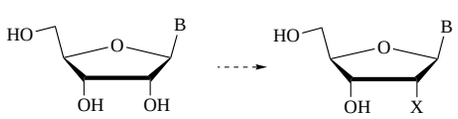
The *E. coli* reductase has been extensively studied as a prototype for mammalian and virally induced reductases.[1–3] It is composed of two homodimeric subunits designated R1 and R2. R1 contains the substrate-binding site and redox active cysteines.[3a] R2 contains a dinuclear iron centre and a stable tyrosyl radical which initiates the reaction through long-range electron transfer.[3b] A cysteinyl radical generated in subunit R1 abstracts the 3′-hydrogen atom of the substrate (Scheme 1).[3a,c] Dehydration of the resulting protonated nucleotidic radical leads to an intermediate radical cation which is then reduced by the thiol groups of a pair of cysteines in the R1 subunit.[3d] Finally, the remaining radical regains the abstracted 3′-hydrogen atom and is converted into the 2′-deoxyribonucleoside diphosphate. The protein disulfide is then converted into a dithiol by 1,4-dihydronicotinamide adenine dinucleotide phosphate (NADPH) with the help of an electron-transfer chain (thioredoxin–thioredoxin reductase).

Modifications of the sugar moiety of nucleotides at the 2′-position have led to a number of mechanism-based inhibitors of ribonucleotide reductase or/and antitumour agents.[2,4] 2′-Azido- and 2′-chloro-2′-deoxynucleotides[4b] are potent inactivators of *E. coli* RDPR, and 2′deoxy-2′,2′-difluorocytidine[4c] (gemcitabine) is now used clinically as an anticancer agent. It is noteworthy that these drugs can be delivered only in the nucleoside form and their activity requires that they are phosphorylated by cellular kinases.



Scheme 1 Mechanism of reduction of nucleoside 5′-diphosphates by *E. coli* ribonucleoside diphosphate reductase (From *J. Chem. Soc., Perkin Trans. 1*, 1995, 395).

An interesting modification which could lead to active compounds is the replacement of the 2′-hydroxy function by a thiol or an *S*-nitroso group [equation (2)]. Attached at the 2′-



(2)

Equation (2) Modifications of ribonucleosides

† **Abbreviations:** DTT, *threo*-1,4-dimercapto-2,3-butanediol (dithiothreitol); Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; NADPH, nicotinamide adenosine diphosphate; NBA, 3-nitrobenzyl alcohol; RDPR, ribonucleoside diphosphate reductase; Tris, tris-(hydroxymethyl)aminomethane.

position of a nucleoside 5′-disphosphate, such groups could interact with the reducing cysteines at the active site and thus modify the course of the reaction.[5]

We report here the synthesis of the first nucleosidic thionitrites **12** and **15**. Their properties, in particular their spontaneous decomposition into nitric oxide, are described. The first synthesis of a very efficient inactivator of the R2 subunit of *E. coli* reductase,[6] 2′-thiouridine 5′-diphosphate **19**, from 2′-thiouridine **4** is also reported.

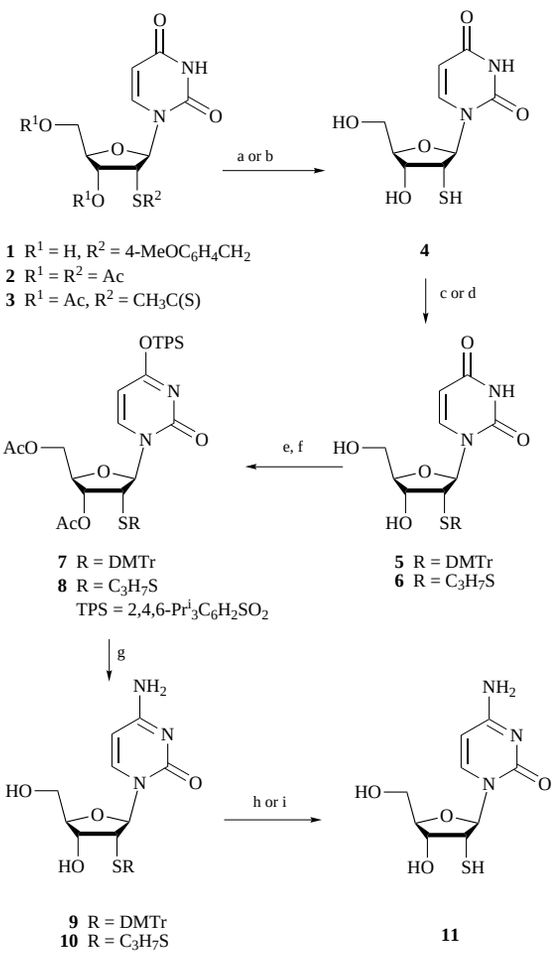
Results and discussion

2′-Thiouridine 4 and 2′-thiocytidine 11

Compound **4** was prepared from 2,2′-anhydrouridine according to the method described by Reese and co-workers *via* 2′-*S*-(4-methoxybenzyl)-2′-thiouridine **1** (Scheme 2).[7] It was also prepared from 3′,5′-di-*O*-acetyl-2,2′-anhydrouridine *via* the method reported by Imazawa *et al.* (Scheme 2).[8]

With this method, a new intermediate was detected and characterized during the preparation of the acetylthio derivative **2**. Heating of 3′,5′-*O*-diacetyl-2,2′-anhydrouridine with thioacetic *S*-acid under the conditions described[8b] afforded a mixture (80 : 20) of two nucleosides. The major component of the mixture was identified as compound **2** as described previously.[8b] The minor product of the reaction was identified as compound **3** by ^{1}H and ^{13}C NMR spectroscopy and [FAB$^+$] mass spectrometry.[9] Attempts to avoid the formation of by-product **3** were unsuccessful. The mixture of products **2** and **3** finally obtained in 79% yield led to the required thiol **4** in 60% yield after treatment with ethanolic KOH.

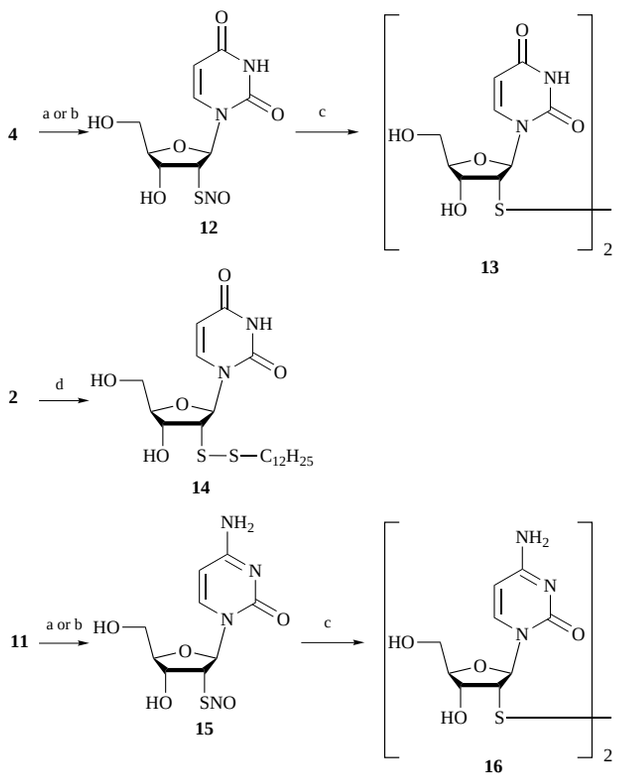
Reese and co-workers prepared 2′-thiocytidine **11** by conversion of the uracil base into cytosine using the relatively easily acid-removable 9-phenylxanthen-9-yl (Px) protective group for the 2′-thiol function and a 4-triazolo intermediate.[7b] We have prepared compound **11** using the acid-labile protective group 4,4′-dimethoxytrityl (DMTr), which was removed under the conditions described (72% overall yield, Scheme 2).



Scheme 2 Synthesis of thiols **4** and **11**: (a) Method A with **1**, trifluoroacetic acid (TFA), phenol, reflux;[7b] (b) Method B with **2**[8b] and **3**, KOH, ethanol; (c) 4,4′-dimethoxytrityl chloride (DMTrCl), $NaHCO_3$, tetrahydrofuran (THF); (d) diethyl azodicarboxylate (DEAD), PrSH[23] or benzothiazol-2-yl propyl disulfide;[24] (e) Ac_2O, pyridine; (f) 2,4,6-triisopropylbenzenesulfonyl chloride, K_2CO_3; (g) NH_3, CH_2Cl_2, NH_4OH; (h) with **9**: 2 M HCl, 2-mercaptoethanol, MeOH; (i) with **10**: dithiothreitol (DTT).

Preparation of the *S*-nitrosonucleosides 12 and 15

Among the different methods available to transform a thiol into a thionitrite,[10] we chose the reaction with *tert*-butyl nitrite (Scheme 3). This method allowed us to prepare selectively *S*-nitroso derivatives of aliphatic thiols possessing a primary amino group such as cysteamine derivatives H_2NCH_2-CRR′SH.[1] Moreover, because of their relatively low boiling points, *tert*-butyl nitrite and 2-methylpropan-2-ol, one of the reaction products, could be removed easily at the end of the reaction.



Scheme 3 Synthesis of thionitrites and disulfides: (a) *tert*-butyl nitrite (8–10 mol equiv.), MeOH; (b) *tert*-butyl nitrite (1.1–1.5 mol equiv.), water; (c) *hν* (365 nm); (d) KOH, ethanol,[8b] HCl, dodecyl thionitrite, *hν* (365 nm)

Preparation of thionitrites **12** and **15** from thiols **4** and **11**, respectively, was first conducted at room temperature in methanol, which can be easily evaporated off. Under these conditions, the reactions were monitored by TLC and were also studied in a NMR tube (CD_3OD). After addition of 1.1 mol equiv. of *tert*-butyl nitrite to a solution of thiol **4**, a new compound was formed but the reaction appeared to be incomplete (10–20%). Addition of an excess of *tert*-butyl nitrite (7–10 mol equiv.) was necessary to complete the reaction in a short time (1 h) and to observe the red colour characteristic of the thionitrite function. A minor reaction product was isolated and identified as the symmetrical disulfide **13** from its characteristics previously described in the literature.[7b] Compound **12** could be separated from dimer **13** by chromatography on silica gel.

Table 1 ^{1}H and ^{13}C chemical shifts in ppm for thiols and their corresponding thionitrites and disulfides (200 MHz) in D_2O (from aqueous preparation, pH 2) or in CD_3OD (number in parentheses)

δ/ppm Nucleoside	2′-H	3′-H	1′-H	C-2′
Thiol **4**	3.66 (3.43)	4.30 (4.11)	6.00 (5.90)	48.2 (47.2)
Thiol **11**	3.66 (3.49)	4.31 (4.16)	6.02 (5.93)	47.0
Thionitrite **12**	5.07 (5.03)	4.57 (4.37)	6.21 (6.18)	56.6 (56.0)
Thionitrite **15**	5.07 (5.04)	4.59 (4.41)	6.20 (6.16)	56.5
Disulfide **13**	3.70	4.41	6.27	58.1
Disulfide **16**	3.70	4.44	6.26	59.1

Unfortunately, it could not be isolated as a pure solid product: it decomposed spontaneously into disulfide **13** during evaporation of the solvent and storage at −18 °C (decoloration). The same behaviour was observed for the cytidine thionitrite **15**.

Thus, the thionitrites **12** and **15** appeared to be unstable as solids and were characterized, stored and used only in solution. They could also be prepared in water at room temperature by reaction of the corresponding thiol with only a slight excess of *tert*-butyl nitrite (1.1–1.5 mol equiv.) to complete the reaction. Under these conditions, the proportion of disulfide could be minimized (5–10%).

During the preparation of the *S*-nitroso cytidine derivative **15**, *N*-nitrosation could have occurred and would have led to uridine derivatives. No trace of such derivatives (thionitrite **12** and disulfide **13**) could be detected by TLC or NMR spectrometry. Thus, the reaction of 2′-thiocytidine with *tert*-butyl nitrite appears to be selective.

Characterization of thionitrites

The FAB[+] high-resolution mass spectra of the thionitrites **12** and **15** were recorded from an aqueous preparation (1.1 mol equiv. of *tert*-butyl nitrite). The molecular-ion peaks $[M + H]^+$ were detected respectively at *m*/*z* 290.0452 and 289.0598. The presence of an SNO group in a molecule can be readily evidenced from UV–visible spectrophotometry.[10–12] The UV–visible spectra of the thionitrites **12** and **15** in water display two bands: in the near-UV region at 341 and 344 nm respectively ($\varepsilon = 550$ and 500 $dm^3\ mol^{-1}\ cm^{-1}$) and in the visible region at 547 nm ($\varepsilon = 11$ and 10 $dm^3\ mol^{-1}\ cm^{-1}$, respectively). These latter light absorptions are responsible for the red colour of solutions of thionitrites **12** and **15**.

Compounds **12** and **15** were characterized by ^{1}H and ^{13}C NMR spectroscopy from homodecoupling and distortionless enhancement by polarization transfer (DEPT) experiments, respectively. ^{1}H NMR characteristics allow a very convenient and reliable proof of nitrosation of thiol compounds (Table 1).[11] The chemical shifts of the C-2′ proton for the thiols **4**, **11** and the disulfides **13**, **16** are close to each other (see the text headed **Preparation of disulfides from thionitrites** in this section, below). For the thionitrites **12** and **15**, a strong deshielding of the signal was observed (~1.6 ppm in CD_3OD). The β protons (1′-H and 3′-H) were affected by the *S*-nitrosation to a minor extent (~0.25 ppm). The ^{13}C NMR spectra were also sensitive to thiol nitrosation. Resonances of α-carbon atoms (C-2′) were deshielded (8–10 ppm) and resonances of β-carbon atoms (C-1′ and C-3′) were slightly affected (~1–3 ppm). The ^{13}C NMR chemical shifts for thionitrites were found to be near those of the corresponding disulfides (Table 1). All these effects of nitrosation of the thiol group were expected from previous studies with aliphatic thionitrites which showed that SNO has a deshielding effect comparable to that of an OH group.[11]

Stability of the thionitrites

Thionitrite decomposition is dependent on a number of parameters, including pH of the solution, oxygen pressure,[13] presence of metal impurities,[14] light,[15] *etc.* As a consequence, decomposition rate constants are difficult to determine accurately.

Decomposition of thionitrite **12** was first studied in acidic solutions obtained after nitrosation of thiol **4** in water ($c \approx 2$ mM; pH 2). The reaction in the dark was monitored by UV–visible spectroscopy since decomposition of thionitrites correlated with the bleaching of the solution and the disappearance of the 330–340 and 500–550 nm bands. Pseudo-first-order kinetics with respect to the thionitrite were observed but the half-life appeared to vary strongly (2–10 days at 25 °C) for different preparations of compound **12** under apparently the same conditions (concentration of reagents, temperature). The half-life of compound **12** appeared to be greatly increased at low temperature (145 days at 6 °C). As a consequence, these compounds were usually stored in the dark and the cold.

Compounds **12** and **15** were found to decompose very rapidly in buffered solution near pH 7 and could be manipulated during the initial dissolution and dilution phases of the kinetic studies by keeping the medium acidic and then mixing rapidly with an excess of pH 7.5 buffer to start the decomposition. Half-lives of compound **12** measured from a same preparation were found to be very dependent on the nature of the buffer (Tris > Hepes > phosphate). Tris = 2-amino-2-(hydroxymethyl)propane-1,3-diol, Hepes = 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid. These results indicate that traces of metal impurities catalyse the decomposition. In the presence of 0.2 mM desferrioxamine, a strong iron chelator, the half-life of compound **12** (1 mM; Hepes 10 mM; pH 7.5; 37 °C) increased from 7 min (rate constant $k = 5.81\ h^{-1}$, $r = 0.992$) to 5 h ($k = 0.143\ h^{-1}$, $r = 0.999$). Treatment of water with Chelex resin to remove metallic impurities increased the half-life of compound **12** (1.7 mM; Hepes 50 mM; pH 7.5; 37 °C) from 10 min to 2.5 days ($k = 0.0120\ h^{-1}$, $r = 0.986$). Catalysis of thionitrite decomposition by copper(II) ions has been reported.[14] Addition of a strong copper chelator, bathocuproine disulfonate, did not improve the stability of a Chelex-treated solution of compound **12** ($k = 0.0118\ h^{-1}$, $r = 0.985$). In conclusion, treatment of aqueous solutions of thionitrites **12** and **15** with Chelex resin appears to be an efficient method to stabilize them by removing traces of transition metal ions which catalyse thionitrite decomposition.

Generation of NO by thionitrites

Decomposition of the thionitrites **12** and **15** in D_2O or CD_3OD was monitored by ^{1}H NMR spectroscopy. In each case, the corresponding symmetrical disulfide **13** or **16** was quantitatively formed as the only organic decomposition product.

The decomposition should produce nitric oxide. NO is a radical but it cannot be detected by electron paramagnetic resonance (EPR) spectroscopy due to the very short relaxation times of the excited electron. Spin traps such as metal complexes are generally used to detect its formation.[16] [Iron(II)] ions are very good traps for NO and the resulting nitrosyl complexes are EPR-active with characteristic signals. Three NO traps were used: the oxyhaemoglobin complex,[17] the iron(II)–diethyldithiocarbamate (DETC) complex[18] and the iron(II)–thiosulfate complex.[18,19] The last assay was used to determine the yield of NO formation.

After incubation of thionitrite **12** or **15** with oxyhaemoglobin at pH 6.5 for 60 s, the characteristic three-line hyperfine EPR signal of the nitrosyl-ferrohaemoglobin complex was observed at 100 K[17] (Fig. 1).

During incubation with yeast, diethyl dithiocarbamate (DETC) penetrates the cell wall and complexes the iron present in hydrophobic membrane compartments.[18] NO generated, for example from the decomposition of an *S*-nitroso thiol, in yeast cell suspensions can be trapped as the nitrosyl $[Fe(DETC)_2NO]$ complex since NO is uncharged and may accumulate in lipophilic compartments. Incubation at 37 °C of thionitrite **12** or **15** with the $Fe(DETC)_2$ complex formed from yeast allows us to observe at 100 K the characteristic EPR signal of the $[Fe(DETC)_2NO]$ complex (Fig. 2).

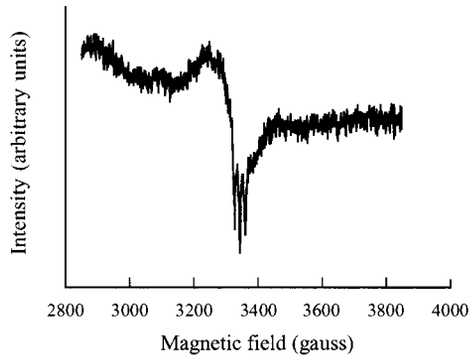


Fig. 1 Trapping of NO by oxyhaemoglobin (HbO_2) during decomposition of compound **15** (0.25 mM aqueous solution. After 1 min of incubation in the presence of 0.25 mM HbO_2 in acetate buffer, pH 6.5, the solution was frozen in liquid nitrogen, and the EPR spectrum was recorded at 100 K.

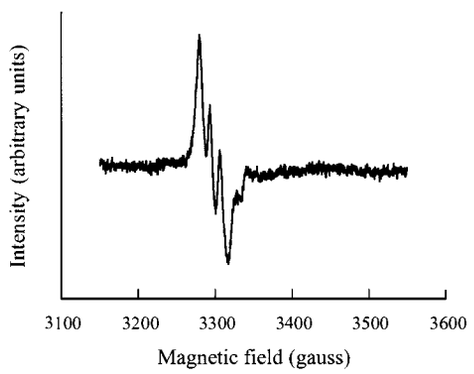


Fig. 2 EPR spectrum of the paramagnetic $Fe(DETC)_2NO$ complex formed during decomposition of compound **15** in aqueous solution (0.5 mM) in suspensions of yeast cells loaded with DETC (recorded at 100 K)

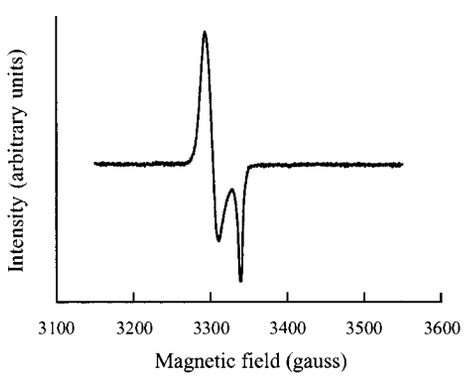


Fig. 3 EPR spectrum of the paramagnetic $Fe(NO)_2(S_2O_3)_2$ complex formed during decomposition of compound **12** in aqueous solution (0.25 mM) in the presence of a mixture of iron(II) sulfate and sodium thiosulfate (pH 7.5) (recorded at 100 K)

The characteristic EPR signal of the $[Fe(NO)_2(S_2O_3)_2]$ paramagnetic complex was also detected at 100 K after addition of an aqueous solution of thionitrite **12** or **15** to a mixture of iron(II) sulfate and sodium thiosulfate (Fig. 3).

Protein R2, the small subunit of ribonucleotide reductase from *E. coli*, contains a stable tyrosyl radical, which is responsible for a characteristic EPR signal of protein solutions at 100 K.[3b] The stability of this protein radical is explained by the fact that it is deeply buried in the interior of the protein, and access to the radical site is greatly constrained. However, because of the small size and the electrical neutrality of NO, on one hand, and because of the high reactivity of phenoxyl radicals towards NO, on the other, a specific reaction between NO and the protein radical takes place, which can be used as an assay for detection of NO. Actually, NO couples to the radical, giving rise to

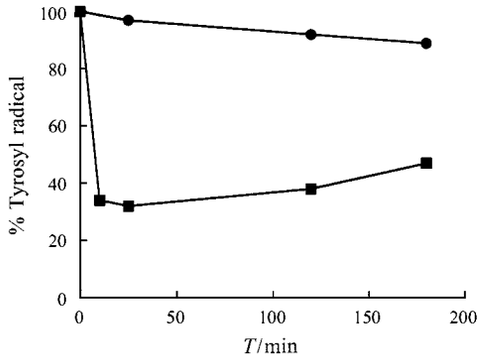


Fig. 4 Loss of the tyrosyl radical EPR signal recorded at 100 K during incubation of protein R2 (1 mg/ml) with 1 mM thionitrite **12** (■) in 50 mM Tris buffer (pH 7.5, 10% glycerol at 37 °C). A control experiment was carried out in the absence of **12** (●).

an EPR-silent nitroso adduct, a process which can be monitored by the disappearance of the tyrosyl radical EPR signal at $g = 2.00$. Furthermore, the reaction is reversible, so that when NO disappears from the reaction mixture, the EPR signal increases back again.[6] A buffered preparation of pure protein R2 was incubated with an aqueous solution of compound **12** or **15**. In both cases, the characteristic signal of the tyrosyl radical EPR signal detected at $g = 2.00$ disappeared and later reappears very slowly (Fig. 4) in agreement with the formation of NO from the thionitrites first with the transient and reversible formation of an EPR-silent nitrosotyrosine.

In conclusion, we have demonstrated unambiguously that *S*-nitrosomercaptonucleosides decompose, in solution, into the corresponding disulfide and NO. The yield of the reaction is high and, as shown below, this may serve as a basis for the preparation of disulfides.

Preparation of disulfides from thionitrites

According to Imazawa *et al.*, compound **4** is oxidized to bis-(2′-deoxyuridin-2′-yl) disulfide **13** in 50% yield by treatment with a stoichiometric amount of iodine in aq. ethanol.[8b] This disulfide has been obtained by Reese and co-workers from compound **1** in two steps [(i) phenol, TFA, reflux 1 h; (ii) iodine, triethylamine, pyridine, room temp., 5 h] with a 64% overall yield.[7b] The yield of compound **13** is probably limited under basic conditions due to the decomposition of compound **4**, which leads to the uracil base.[8,20] The conversion of thionitrites **12** and **15** into the corresponding disulfides reported here led us to consider a one-pot, two-step protocol for preparing a disulfide from a thiol, using the thionitrite as an intermediate (Scheme 3).

Compound **13** was actually obtained in 76% yield from the thiol **4** by using thionitrite **12** as an intermediate. The conversion of thionitrite **12** into disulfide **13** was achieved by irradiation of the solution at 365 nm. The mixed disulfide **14** could be also obtained from the triacetyl derivative **2** in 53% overall yield by the following one-pot reactions (Scheme 3): [(i) deprotection with ethanolic KOH, 0 °C, 1 h; addition of aq. HCl until pH 6; (ii) addition of an excess of dodecylthionitrite (freshly prepared) and then irradiation at 365 nm].

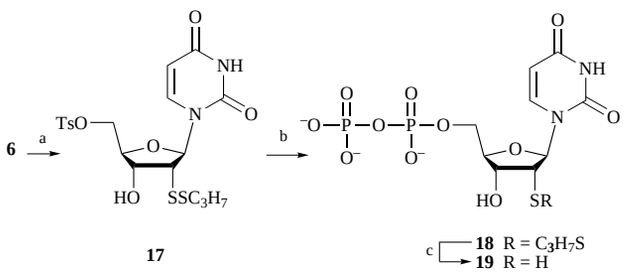
The photochemical decomposition of thionitrites was also used to prepare a small amount (67 mg) of the symmetrical cytidine derivative **16** in 66% yield by irradiation of an aqueous solution of thionitrite **15** prepared from thiol **11**. This compound had been previously prepared by oxidation of compound **11** in the presence of triethylamine in a 90% yield.[7b] The new protocol described may be of general use for preparing symmetrical or mixed disulfides.

2′-Thiouridine 5′-diphosphate 19

A method for preparing 5′-diphosphates of natural nucleosides from their 5′-*O*-tosyl derivative has been developed by Poulter

and co-workers.[21] We have previously applied this procedure to prepare 2′-deoxy-5-fluorouridine 5′-diphosphate[22] and we used this approach to synthesize **19**, the 5′-disphosphate derivative of **4** (Scheme 4).

The formation of mixed disulfides was chosen as a method to protect the reactive thiol group. This protection should be easily removed at the end of the synthesis by using a reducing agent such as dithiothreitol (DTT). Dithiothreitol was chosen since this is one of the electron sources used in the assays for ribonucleotide reductase activity *in vitro*. Protection of compound **4** with alkanethiols appeared to be suitable since the presence of a hydrophobic side-chain in the intermediate 5′-diphosphate disulfide should facilitate its purification by reversed-phase chromatography. Moreover, the presence of a long and bulky alkyl side-chain should allow a more selective tosylation at the 5′-position to occur without protection of the 3′-hydroxy group (Scheme 4).



Scheme 4 Synthesis of diphosphates: (a) TsCl/pyridine, 0 °C; (b) tris-(tetrabutylammonium) hydrogen pyrophosphate;[21] (c) DTT (1.05 mol equiv.)

Thus, the disulfide **6** possessing a propyl side-chain was prepared from compound **4** and propane-1-thiol by two methods described in the literature to prepare usual aliphatic and aromatic disulfides (Scheme 2). With the method using DEAD as an oxidizing reagent,[23] removal of the diethyl hydrazinedicarboxylate formed was difficult and appeared to be a limitation in the preparation of compound **6** obtained in 73% yield. Reaction of compound **4** at room temperature with benzothiazol-2-yl propyl disulfide prepared and isolated as described[24] also afforded the disulfide **6** in 72% yield.

Tosylation of compound **6** in pyridine at 0 °C led to the 5′-*O*-tosyl derivative **17** in 57% yield as well as minor amounts of the 3′,5′-ditosyl derivative (12%) (Scheme 4). The tris-(tetrabutylammonium) salt of the 5′-phosphate disulfide **18** was obtained in 52% yield after reaction of compound **17** with tris-(tetrabutylammonium) hydrogen pyrophosphate in excess at room temperature during 48 h. This salt was characterized by ^{1}H, ^{31}P NMR (respectively 500 and 81 MHz) and mass spectrometry. The tetrabutylammonium counter ions were exchanged with ammonium ions by chromatography on Q-Sepharose. The high-resolution mass spectrum of the ammonium salt displayed a peak at m/z = 512.0353, corresponding to the molecular ion $[M + 3\,H + NH_4]^+$.

Finally, reduction of compound **18** with a nearly stoichiometric amount of DTT (1.05 mol equiv.) allowed us to generate immediately and quantitatively the thiol **19** as observed by ^{1}H, ^{31}P NMR (respectively 500 and 81 MHz, Fig. 5) and HRMS (molecular-ion peak $[M + 3\,H + NH_4]^+$ at m/z = 438.0122). All the signals observed in the ^{1}H NMR spectrum obtained were attributed using phase-sensitive two-dimensional total correlation spectroscopy (TOCSY) experiments. After reduction, the propane-1-thiol formed could be removed from the solution by argon bubbling (characteristic smell, disappearance of the corresponding signals in the ^{1}H NMR spectrum). Compound **19** could be separated from DTT by TLC on C_{18} reversed phase. The presence of a thiol function in compound **19** was evidenced with Ellman's reagent. Under the same conditions of the reduction, the nucleoside disulfide **6** led to the thiol **4** (^{1}H NMR,

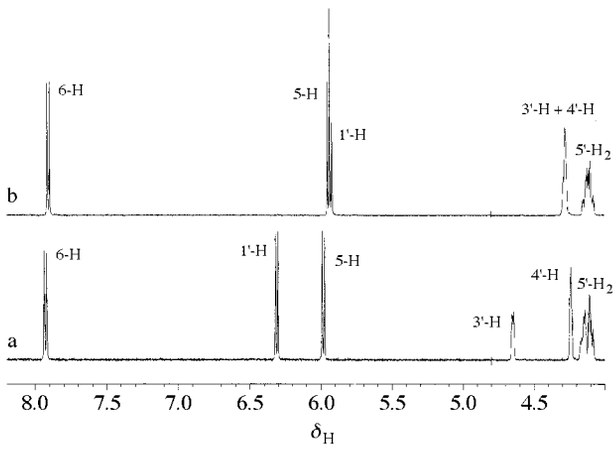


Fig. 5 Reduction, with DTT, of the mixed disulfide diphosphate **18** to 2′-thiouridine 5′-diphosphate **19** monitored by ^{1}H NMR spectrometry (500 MHz): (a) spectrum of **18** in D_2O, (b) spectrum of **19** obtained after addition of 1.05 mol equiv. of DTT (aqueous solution)

TLC, Ellman's reagent). In the absence of DTT, the thiol **19** decomposed to give several products, probably the disulfide and uracil.[20]

In a parallel study, compound **19**, prepared from disulfide **18** as described above, was shown to be a very strong inhibitor of the *E. coli* RDPR.[6] In conclusion, the protection of compound **19** in the form of a stable mixed disulfide **18** is a very convenient method to avoid problems related to the relatively fast decomposition and oxidation of thiol **19** and the resulting required purification. Moreover, compound **19** can be very easily recovered by reduction of disulfide **18**.

Such protection appeared useful also in attempts to convert the mixed disulfide nucleoside **6** into the corresponding cytosine analogue **10** *via* formation of the triisopropylbenzenesulfonyl intermediate **8** (63% overall yield). Reduction of compound **10** with DTT gave 2′-thiocytidine **11** in 79% yield.

The pK_a-value of the 2′-thiol group in 2′-thiouridine **4**, at room temperature, is 7.3.[8a] As a consequence, the thiolate ion of the diphosphate **19** is formed in aqueous neutral buffered solution and should react selectively with electrophiles to lead to new 2′-modified uridine 5′-diphosphate derivatives. Various 2′-thio derivatives could be prepared rapidly from thiol **19** and their inhibitory effects on the *E. coli* ribonucleotide reductase evaluated. From preliminary experiments conducted in an NMR tube (^{1}H; 500 MHz), it appeared that a selective *S*-methylation or *S*-nitrosation of compound **19** occurs in aq. ammonium hydrogen carbonate. The 2′-*S*-nitroso 5′-diphosphate derivative formed under these conditions appeared to be very unstable. This approach is now under investigation.

Experimental

General

Mps were measured on a Buchi 530 apparatus and are uncorrected. NMR spectra were measured on Bruker AC200, AM300 and/or Varian VT 500 spectrometers. Chemical shifts are reported in ppm relative to the residual signal of the solvent. In D_2O, ^{1}H chemical shifts were measured using sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]propionate ([$^2H_4$]TSP) as internal reference. Chemical shifts in ^{31}P NMR spectra are relative to the signal of orthophosphoric acid as external reference. Coupling constants (*J*-values) are given in Hz. IR spectra were measured on a Perkin-Elmer 298 spectrophotometer. UV spectra were measured on a Kontron Unikon 930 spectrophotometer.

Synthesis

3′,5′-Di-*O*-acetyl-2′-*S*-acetyl-2′-thiouridine 2 and 3′,5′-di-*O*-acetyl-2′-thio-2′-*S*-thioacetyluridine 3. Thioacetic *S*-acid (25 ml, 0.35 mol) was added to a stirred suspension of 3′,5′-di-*O*-

acetyl-2,2′-anhydrouridine[8,25] (4.5 g, 14.5 mmol) in 1,4-dioxane (25 ml) and then the mixture was refluxed under argon for 6 h. The resultant solution was cooled and then evaporated. The oily residue was chromatographed on silica gel in dichloromethane and then dichloromethane–methanol (98:2 and 95:5) to yield a mixture of products **2** and **3** (80:20, 4.45 g, 79%). From this mixture, separate compounds **2** and **3** were purified by chromatography on silica gel with diethyl ether as eluent (**2**: 3.25 g, 58%; **3**: 0.72 g, 12%).

3′,5′-Di-*O*-acetyl-2′-*S*′-acetyl-2′-thiouridine **2**: mp 119–120 °C (lit.,[8b] 124–126 °C) [Found: C, 46.95; H, 4.93; N, 7.33; S, 8.68. Calc. for $C_{15}H_{18}N_2O_8S$ (386.38): C, 46.63; H, 4.70; N, 7.25; S, 8.30%]; *m*/*z* [FAB+; glycerol] 387 $[M + H]^+$; δ_H(200 MHz; C^2HCl_3) 9.48 (1 H, br s, 3-H), 7.46 (1 H, d, *J* 8.1, 6-H), 6.24 (1 H, d, *J* 9.6, 1′-H), 5.80 (1 H, d, *J* 8.1, 5-H), 5.25 (1 H, d, *J* 5.9, 3′-H), 4.50-4.20 (4 H, m, 2′-H, 4′-H, 5′-H_2) 2.31 (3 H, s, CH_3COS), 2.17 (3 H, s, CH_3CO_2) and 2.11 (3 H, s, CH_3CO_2); δ_C(50 MHz; C^2HCl_3) 193.0 (SCO), 170.1 (CO), 169.8 (CO), 162.9, 150.7, 138.7, 103.6, 86.3, 81.8, 74.3, 63.8, 46.6 (C-5′), 30.3 (*C*H_3COS), 20.7 (CH_3) and 20.6 (CH_3).

3′,5′-*Di*-O-*acetyl*-2′-*thio*-2′-S-*thioacetyluridine* **3**: mp 67–69 °C [Found: C, 45.02; H, 4.53; N, 6.71; S, 15.90. $C_{15}H_{18}N_2O_7S_2$ (402.45) requires C, 44.77; H, 4.51; N, 6.96; S, 15.94%]; *m*/*z* [FAB+; glycerol] 403 $[M + H]^+$; δ_H(200 MHz; C^2HCl_3) 8.52 (1 H, br s, 3-H), 7.48 (1 H, d, *J* 8.2, 6-H), 6.38 (1 H, d, *J* 9.6, 1′-H), 5.76 (1 H, dd, *J* 8.2 and 2.1, 5-H), 5.33 (1 H, d, *J* 5.7, 3′-H), 4.89 (1 H, dd, *J* 9.6 and 5.7, 2′-H), 4.53–4.25 (3 H, m, 4′-H, 5′-H_2), 2.77 (3 H, s, CH_3CS_2), 2.19 (3 H, s, CH_3CO_2) and 2.12 (3 H, s, CH_3CO_2); δ_C(50 MHz; C^2HCl_3) 230.1 (CS), 170.0 (CO), 169.7 (CO), 162.9, 150.6, 138.7, 103.5, 85.6, 82.3, 73.9, 64.0, 53.9 (CH_2), 38.9 (*C*H_3CS_2), 20.7 (CH_3) and 20.6 (CH_3).

2′-*S*-(4,4′-Dimethoxytrityl)-2′-thiouridine 5. A solution of 2′-*S*-(4-methoxybenzyl)-2′-thiouridine **1** (4.0 g, 10.5 mmol) and phenol (1.5 g, 16 mmol) in TFA (40 ml) was refluxed for 2 h,[7b] then was evaporated to dryness. The residue obtained after addition and evaporation of ethanol and then of anhydrous toluene was dissolved in anhydrous THF (70 ml). To the resultant solution were added $NaHCO_3$ (10 g, 0.12 mol) and DMTrCl (5.0 g, 14.8 mmol). After stirring of the mixture for 3 h at room temperature, ethanol (5 ml) was added and then the solution was evaporated. The resultant oil was dissolved in dichloromethane (100 ml) and the solution was washed with water. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated off. The solid residue was flash chromatographed on silica gel with CH_2Cl_2–pyridine (99.5:0.5) and then CH_2Cl_2–MeOH–pyridine (89.5:10:0.5) as eluent to yield compound **5** (4.98 g, 83%), mp 137–139 °C [Found: C, 62.98; H, 5.46; N, 4.81; S, 5.30. $C_{30}H_{30}N_2O_7S{\cdot}0.5\ H_2O$ (571.645) requires C, 63.03; H, 5.47; N, 4.90; S, 5.61%]; *m*/*z* [FAB+; glycerol] 303 $[DMTr]^+$, [FAB−; glycerol] 562 $[M - H]^-$; δ_H[200 MHz; $(CD_3)_2SO$] 11.33 (1 H, br s, NH), 7.34 (1 H, d, *J* 8.1, 6-H), 7.25–7.19 (9 H, m, ArH), 6.77 (4 H, dd, *J* 8.8 and 1.7, ArH), 5.97 (1 H, d, *J* 9.8, 1′-H), 5.44 (1 H, d, *J* 8.1, 5-H), 5.01 (1 H, d, *J* 4.1, 3′-OH), 4.75 (1 H, br t, 5′-OH), 3.80 (1 H, br s, 4′-H), 3.71 (6 H, br s, 2 × OCH_3), 3.31 (2 H, br s, 5′-H_2), 3.22 (1 H, t, 3′-H) and 2.88 (1 H, dd, *J* 9.8 and 4.1, 2′-H); δ_C(50 MHz; C^2HCl_3) 163.3 (CO), 158.0 (CO), 151.0, 145.4, 140.3, 136.8, 136.6, 130.6, 130.4, 129.0, 128.3, 126.9, 113.5, 102.7, 87.3, 86.1, 72.1 (S*C*Ar_3), 61.8 (C-5′), 55.3 (OCH_3) and 52.3 (OCH_3).

2′-Deoxyuridin-2′-yl propyl disulfide 6. *Method A.*—A solution of 2′-thiouridine **4** (310 mg, 1.19 mmol) and DEAD (200 μl, 1.2 mmol) in THF (10 ml) was kept at room temperature under argon for 18 h and then propane-1-thiol (6.3 ml, 68.9 mmol) was added. The resultant solution was refluxed for 8 h and then was evaporated. Flash chromatography of the residue on silica gel with CH_2Cl_2–MeOH (95:5) as eluent afforded the mixed disulfide **6** (290 mg, 73%).

Method B.—A deoxygenated solution of thiol **4** (argon; 800 mg, 3.07 mmol) in methanol (18 ml) was added dropwise (30 min) to a stirred, deoxygenated solution of benzothiazol-2-yl propyl disulfide[24] (750 mg, 3.11 mmol) in chloroform (90 ml). Stirring was continued at room temperature for 3 h and then the solvents were evaporated off under reduced pressure. The residue was chromatographed on silica gel with dichloromethane–methanol (95:5) as eluent to afford the *disulfide* **6** (738 mg, 72%), mp 135–136 °C [Found: C, 43.24; H, 5.36; N, 8.47; S, 19.25. $C_{12}H_{18}N_2O_5S_2$ (334.41) requires C, 43.10; H, 5.43; N, 8.38; S, 19.17%]; *m*/*z* [FAB+; thioglycerol] 336 $[M + 2H]^+$ and 229 $[M + 2H - C_3H_7SS]^+$; [FAB−; thioglycerol] 333 $[M - 1]^-$; δ_H[200 MHz; $(CD_3)_2SO$] 11.41 (1 H, s, NH), 7.86 (1 H, d, *J* 8.0, 6-H), 6.16 (1 H, d, *J* 9.0, 1′-H), 5.90 (1 H, d, *J* 5.3, 3′-OH), 5.73 (1 H, d, *J* 7.9, 5-H), 5.14 (1 H, t, *J* 5.4, 5′-OH), 4.25 (1 H, t, 3′-H), 3.87 (1 H, br t, 4′-H), 3.62–3.55 (3 H, m, 5′-H_2, 2′-H), 2.61 (2 H, m, SCH_2), 1.60–1.49 (2 H, m, C*H*$_2CH_3$) and 0.86 (3 H, br t, CH_2CH_3); δ_C[75 MHz; $(CD_3)_2SO$] 162.8 (CO), 150.7 (CO), 140.5, 102.4, 87.6, 86.6, 72.3, 61.4, 58.5, 40.0 (SCH_2), 21.4 (*C*H_2CH_3) and 12.7 (CH_3).

2′-*S*-(4,4′-Dimethoxytrityl)-2′-thiocytidine 9. To a stirred, ice-cold solution of 2′-*S*-(4,4′-dimethoxytrityl)-2′-thiouridine **5** (2.85 g, 5.1 mmol) in pyridine (50 ml) was added acetic anhydride (5 ml, 53 mmol). The resultant solution was stirred at room temperature for 18 h. After addition of ethanol at 0 °C, the solvent was evaporated off. Residual pyridine was removed by coevaporation with toluene and then the residue was dissolved in dichloromethane (200 ml). The solution was washed with water and then dried over Na_2SO_4. After evaporation, the residue obtained was dissolved in acetonitrile (100 ml). To the resultant solution were added K_2CO_3 (5 g, 36 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (5.0 g, 16.5 mmol). The mixture was stirred at room temperature overnight under argon and then was filtered and evaporated. The residue (compound **7**) was dried under vacuum and then was dissolved in a saturated solution of ammonia in dichloromethane (200 ml). The solution was kept at room temperature for 48 h and then was evaporated. The residue was dissolved in a conc. aq. ammonia–ethanol mixture (50:50; 100 ml). After 24 h, the solution was evaporated to dryness and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH–pyridine (94.5:5:0.5 to 89.5:10:0.5) as eluent to yield compound **9** (2.05 g, 72%) and the starting compound **5** (0.69 g, 24% recovery), mp 181–183 °C [Found: C, 63.81; H, 5.77; N, 7.70; S, 5.78. $C_{30}H_{31}N_3O_6S$ (561.65) requires C, 64.16; H, 5.56; N, 7.48; S, 5.71%]; *m*/*z* [FAB+; glycerol] 1123 $[2M + H]^+$ and 562 $[M + H]^+$; [FAB−; glycerol] 560 $[M - H]^-$; δ_H[200 MHz; $(CD_3)_2SO$] 7.34–7.15 (12 H, m, 6-H, ArH and NH_2), 6.75 (4 H, dd, *J* 8.0 and 1.5, ArH), 6.08 (1 H, d, *J* 9.7, 1′-H), 5.62 (1 H, d, *J* 7.2, 5-H), 5.01 (1 H, d, *J* 4.1, 3′-OH), 4.75 (1 H, br t, 5′-OH), 3.71 (7 H, br s, 4′-H, 2 × OCH_3), 3.22 (2 H, br s, 5′-H_2) and 2.90–2.76 (2 H, m, 3′- and 2′-H); δ_C(50 MHz; C^2HCl_3) 165.5 (CO), 157.6 (CO), 155.5, 145.5, 145.2, 141.1, 136.6, 136.4, 130.3, 130.1, 128.8, 128.3, 127.9, 126.4, 113.2, 95.1, 86.4, 86.0, 71.6, 66.1, 62.0 (C-5′), 55.1 (OCH_3) and 52.8 (OCH_3).

2′-Deoxycytidin-2′-yl propyl disulfide 10. To a solution of disulfide **6** (225 mg, 0.67 mmol) in pyridine (5 ml) cooled to 0 °C was added acetic anhydride (1 ml, 10 mmol). The solution was stirred at room temperature for 14 h and then ethanol was added to the solution. After evaporation to dryness, the residue was dissolved in CH_2Cl_2 (50 ml). The resultant solution was washed with water, dried over Na_2SO_4, and the organic phase was evaporated. The residue was dissolved in acetonitrile (10 ml). K_2CO_3 (1.60 g, 11.5 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (400 mg, 1.32 mmol) were added. The mixture was stirred at room temperature for 24 h under argon. After filtration, the solution was evaporated and the residue was dissolved in a saturated solution of ammonia in CH_2Cl_2 (50 ml). The resultant solution was stirred at room temperature for 36 h and the solvent was evaporated off. To a solution of the residue in ethanol (20 ml) was added conc. aq. ammonia (30 ml) and the mixture was stirred at room temperature for 8 h. After

evaporation, the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (85:15) as eluent to obtain the *disulfide* **10** (142 mg, 63%), mp 148–150 °C [Found: C, 42.63; H, 5.73; N, 12.36; S, 18.80. $C_{12}H_{19}N_3O_4S_2$·0.25 H_2O (337.92) requires C, 42.65; H, 5.82; N, 12.43; S, 18.97%. *Hydrochloride* Found: C, 39.15; H, 5.56; N, 11.46. $C_{12}H_{19}N_3O_4S_2$·HCl (369.88) requires C, 38.97; H, 5.45; N, 11.36%]; *m/z* [FAB+; thioglycerol] 334 $[M + H]^+$ and 260 $[M + 2H - C_3H_7S]^+$; δ_H[200 MHz; $(CD_3)_2SO$] 7.76 (1 H, d, *J* 6.9, 6-H), 7.20 (2 H, br s, NH_2), 6.18 (1 H, d, *J* 8.9, 1′-H), 5.80 (1 H, d, *J* 4.8, 3′-OH), 5.76 (1 H, d, *J* 6.9, 5-H), 5.06 (1 H, br t, *J* 4.8, 5′-OH), 4.24 (1 H, m, 3′-H), 3.87 (1 H, m, 4′-H), 3.61–3.52 (3 H, m, 5′-H_2, 2′-H), 2.51–2.48 (2 H, m, SCH_2), 1.57–1.46 (2 H, m, CH_2CH_3) and 0.84 (3 H, br t, CH_2CH_3); δ_C(75 MHz; [$^2H_4$]MeOH) 167.5 (CO), 158.6 (CO), 143.7 102.4, 96.7, 91.8, 88.1, 74.0, 63.2 (C-5′), 61.6, 42.0 (SCH_2), 23.1 (CH_2CH_3) and 13.2 (CH_3).

2′-Thiocytidine 11. *Preparation from compound* **9**.—To a solution of 2′-*S*-(4,4′-dimethoxytrityl-2′-thiocytidine **9** (0.60 g, 1.07 mmol) in 2-mercaptoethanol (10 ml) was added 2 M methanolic HCl (6 ml). After 5 h at room temperature under argon, the mixture was treated with methanol (10 ml). The resultant solution was concentrated under reduced pressure to remove methanol and hydrochloric acid. The solution was kept at room temperature for 30 min and 2-mercaptoethanol was evaporated off. The residue was dissolved in a minimum volume of methanol and the solution was added dropwise to stirred diethyl ether (250 ml). Compound **11** was obtained as a hydrochloride after filtration and washing with diethyl ether (285 mg, 88%), mp 187–189 °C (lit.,[7b] 187 °C) [Found: C, 35.82; H, 4.82; N, 13.43; S, 10.61. $C_9H_{13}N_3O_4S$·HCl·0.5 H_2O (304.75) requires C, 35.47; H, 4.96; N, 13.79; S, 10.52%]; *m/z* [FAB+; glycerol or NBA] 260 $[M + H]^+$; ν_{max}(KBr)/cm^{-1} 2550 (SH); δ_H[200 MHz; $(CD_3)_2SO$] 9.79 (1 H, br s, NH), 8.70 (1 H, br s, NH), 8.17 (1 H, d, *J* 7.8, 6-H), 6.19 (1 H, d, *J* 7.8, 5-H), 5.89 (1 H, d, *J* 7.8, 1′-H), 4.15–3.15 (4 H, m, 3′- and 4′-H, 3′- and 5′-OH), 3.58 (2 H, m, 5′-H_2) and 3.48 (1 H, m, 2′-H); δ_C[75 MHz; $(CD_3)_2SO$] 159.5 (CO), 147.6 (CO), 144.1, 94.6, 90.3, 86.7, 71.1, 60.8 and 45.8.

Preparation from the mixed disulfide **10**.—To a deoxygenated solution of disulfide **10** (free base, 200 mg, 0.592 mmol; argon) in methanol (2 ml) was added, under argon, aq. DTT (140 mg, 0.90 mmol in 4 ml). Argon was bubbled through the solution for 20 min to remove propane-1-thiol, and then 2 M methanolic HCl was added (10 ml). The resultant solution was evaporated to dryness and the residue was dissolved in 0.5 M methanolic HCl (2 ml). The solution was added dropwise to stirred diethyl ether. The resultant precipitate was collected by filtration, washed with diethyl ether, and crystallised from aq. ethanol (142 mg, 79%).

2′-*S*-Nitroso-2′-thiouridine 12 and 2′-*S*-nitroso-2′-thiocytidine 15. To a stirred solution of 2′-thiouridine **4** or 2′-thiocytidine **11** (hydrochloride; 0.20 mmol) in deoxygenated water (HPLC grade; 70 ml; argon) was added *tert*-butyl nitrite (98% in weight; 27 μl, 0.22 mmol) in the dark. The solution was stirred at room temperature for 30 min (the complete disappearance of compound **4** was confirmed with Ellman's reagent). The resultant solution was washed with diethyl ether (2 × 35 ml) to remove *tert*-butyl alcohol, and argon was bubbled through the solution for 15 min (for the preparation of compound **12** or **15** in methanol, see the synthesis of the corresponding symmetrical disulfide).

2′-S-*Nitroso*-2′-*thiouridine* **12**.—LRMS *m/z* [FAB+; glycerol] 290 $[M + H]^+$ and 259 $[M - NO]^+$; [FAB−; glycerol] 259 $[M - NO]^-$ and 227 $[M - SNO]^-$; HRMS *m/z* [FAB+; glycerol] 290.0452. $C_9H_{12}N_3O_6S$ $[M + H]^+$ requires *m/z*, 290.0447; λ_{max}(water; pH 2)/nm 342 (550 ε/dm^3 mol^{-1} cm^{-1}) and 547 (11); δ_H(200 MHz; [$^2H_4$]MeOH) 8.16 (1 H, d, *J* 8.1, 6-H), 6.18 (1 H, d, *J* 8.7, 1′-H), 5.93 (1 H, d, *J* 8.1, 5-H), 5.03 (1 H, dd, *J* 8.7 and 5.3, 2′-H), 4.37 (1 H, dd, *J* 5.3 and 1.5, 3′-H), 4.03 (1 H, m, *J* 2.7 and 1.5, 4′-H) and 3.79–3.76 (2 H, m, 5′-H_2); δ_H(200 MHz; ^2H_2O–[$^2H_4$]TSP) 8.06 (1 H, d, *J* 8.1, 6-H), 6.21 (1 H, d, *J* 8.1, 1′-H), 5.98 (1 H, d, *J* 8.1, 5-H), 5.07 (1 H, dd, *J* 8.1 and 6.0, 2′-H), 4.57 (1 H, dd, *J* 6.0 and 3.0, 3′-H), 4.22–4.17 (1 H, m, 4′-H) and 3.93–3.90 (2 H, m, 5′-H_2), δ_C NMR (50 MHz; [$^2H_4$]MeOH) 165.7 (CO), 152.2 (CO), 142.1, 103.6, 89.3, 88.5, 74.2, 63.0 (C-5′) and 56.0 (C-2′).

2′-S-*Nitroso*-2′-*thiocytidine* **15**.—*m/z* [FAB+; glycerol] 289.0598. $C_9H_{13}N_4O_5S$ $[M + H]^+$ requires *m/z* 289.0607; λ_{max}-(water; pH 2)/nm 344 (500) and 547 (10); δ_H(200 MHz; [$^2H_4$]MeOH) 8.57 (1 H, d, *J* 7.7, 6-H), 6.16 (2 H, br d, *J* 7.9, 1′- and 5-H), 5.04 (1 H, m, 2′-H), 4.41 (1 H, dd, *J* 5.3 and 2.3, 3′-H), 4.06 (1 H, d, *J* 2.3, 4′-H) and 3.79 (2 H, m, 5′-H_2); δ_H(200 MHz; ^2H_2O–[$^2H_4$]$_2$TSP) 8.24 (1 H, d, *J* 7.9, 6-H), 6.30 (1 H, d, *J* 7.9, 5-H), 6.20 (2 H, d, *J* 7.7, 1′-H), 5.07 (1 H, br t, 2′-H), 4.59 (1 H, dd, *J* 5.9 and 3.6, 3′-H), 4.19 (1 H, m, 4′-H) and 3.94–3.90 (2 H, m, 5′-H_2); δ_C(75 MHz; ^2H_2O) 161.7 (CO), 150.9 (CO), 146.6, 98.4, 91.0, 89.6, 73.7, 63.5 (C-5′) and 56.5 (C-2′).

Bis(2′-deoxyuridin-2′-yl) disulfide 13. To a stirred solution of 2′-thiouridine **4** (143.5 mg, 0.55 mmol) in methanol (30 ml) was added *tert*-butyl nitrite (90% by weight; 660 μl, 5.0 mmol) at room temperature. After a few minutes a red coloration appeared. The solution was stirred for 1 h and then was irradiated at 365 nm (125 W high pressure mercury lamp, Phillips HPW) for 24 h until decoloration. Methanol and *tert*-butyl alcohol were removed by evaporation and the residue was chromatographed on a short reversed-phase column (C_{18}-Sep-Pak® Waters, 1 g cartridge) in water–methanol mixture (80:20) to obtain the pure symmetrical disulfide **13** (111.5 mg, 76%), mp 157–158 °C (lit., 158 °C[7b] and 161–164 °C[8b]) [Found: C, 40.38; H, 4.39; N, 10.46; S, 11.83. Calc. for $C_{18}H_{22}N_4O_{10}S_2$· H_2O (536.53): C, 40.30; H, 4.51; N, 10.44; S, 11.95%]; *m/z* [FAB+, glycerol] 519 $[M + H]^+$; δ_H[300 MHz; $(CD_3)_2SO$] 11.30 (2 H, br d, *J* 1.6, NH), 7.75 (2 H, d, *J* 8.0, 6-H), 6.15 (2 H, d, *J* 8.3, 1′-H), 5.75 (2 H, br s, 3′-OH), 5.67 (2 H, dd, *J* 8.0 and 1.8, 5-H), 5.02 (2 H, br s, 5′-OH), 4.16 (2 H, d, *J* 4.7, 3′-H), 3.83 (2 H, d, *J* 1.6, 4′-H), 3.61 (2 H, dd, *J* 8.5 and 5.4, 2′-H) and 3.53 (4 H, br d, 5′-H_2); δ_C(50 MHz; D_2O–[$^2H_4$]TSP) 168.7 (CO), 154.6 (CO), 144.5, 105.9, 91.7, 89.0, 74.7, 63.9 (C-5′) and 58.1.

2′-Deoxyuridin-2′-yl dodecyl disulfide 14. A solution of dodecyl thionitrite was prepared by addition of *tert*-butyl nitrite (5.2 ml, 42.5 mmol) to a solution of dodecane-1-thiol (7 ml, 28.3 mmol) in methanol (100 ml) and stirring of this mixture at room temperature for 30 min.

To a stirred solution of the triacetyl derivative **2** (1.10 g, 2.85 mmol) in methanol (15 ml) was added 1 M KOH (ethanol–water 9:1; 9 ml) under argon. After 15 min, pH was adjusted to 6 with 1 M aq. HCl and then the above red solution of dodecyl thionitrite was added to the solution cooled to 0 °C. The resultant solution was stirred at room temperature for 1 h and then was irradiated at 365 nm for 36 h. The resultant solution was concentrated until precipitation of didodecyl disulfide, which was removed by filtration. The filtrate was evaporated to dryness and the residue obtained was chromatographed twice on a reversed-phase column (C_{18}-Sep-Pak®, 10 g cartridge) with water–methanol mixtures (80:20 to 5:95) as eluent to obtain the *mixed disulfide* **14** (707 mg, 53%) and the nucleosidic symmetrical disulfide **13** (314 mg, 43%), mp decomp. [Found: C, 53.67; H, 7.86; N, 5.69; S, 13.71. $C_{21}H_{36}N_2O_5S_2$·0.5 H_2O (469.65) requires C, 53.70; H, 7.94; N, 5.96; S, 13.65%]; *m/z* (DCI; NH_3–isobutane) 461 $[M + H]^+$ and 227 $[M - C_{12}H_{25}SS]^+$; δ_H(200 MHz; $CDCl_3$) 8.95 (1 H, br s, NH), 7.54 (1 H, d, *J* 8.0, 6-H), 5.79 (2 H, br t, 1′- and 5-H), 4.54 (1 H, d, *J* 4.5, 3′-H), 4.17 (1 H, d, *J* 1.9, 4′-H), 3.98–3.76 (3 H, m, 5′-H_2, 2′-H), 3.06 (1 H, br s, OH), 2.90 (1 H, br s, OH), 2.66 (2 H, m, SCH_2), 1.60 (2 H, m, SCH_2CH_2), 1.23 (18 H, br s, 9 × CH_2), 0.86 (3 H, t, CH_2CH_3); δ_C(75 MHz; C^2HCl_3) 162.6 (CO), 150.4 (CO), 142.2, 103.0, 92.3, 86.4, 72.6, 62.7 (C-5′), 58.9, 39.4 (SCH_2), 31.9, 29.6, 29.5, 29.4, 29.3, 29.2, 28.8, 28.4 and 22.6 (all CH_2) and 14.1 (CH_3).

Bis(2′-deoxycytidin-2′-yl) disulfide 16. To a stirred solution of 2′-thiocytidine **11** (99.3 mg, 0.336 mmol) in methanol (30 ml)

was added *tert*-butyl nitrite (90% by weight; 400 μl, 3.0 mmol) at room temperature. After a few minutes, a red coloration appeared. The solution was stirred for 1 h and then was irradiated at 365 nm for 24 h until decoloration. Methanol and 2-methylpropan-2-ol were removed by evaporation and the residue was dissolved in 3 M methanolic HCl. After evaporation to dryness, the residue was dissolved in a minimal volume of methanol and then the solution was added dropwise to stirred acetone (30 ml). The precipitate was filtered and washed with acetone to obtain the hydrochloride of the *symmetrical disulfide* **16** (67 mg, 66%), mp 178–180 °C [Found: C, 35.56; H, 4.44; N, 13.56; S, 10.46. $C_{18}H_{24}N_6O_8S_2{\cdot}2HCl{\cdot}H_2O$ (607.48) requires C, 35.59; H, 4.65; N, 13.83; S, 10.56%] *m/z* [FAB+; glycerol] 517 $[M + H]^+$; δ_H[200 MHz; $(CD_3)_2SO$] 9.61 (2 H, br s, NH), 8.58 (2 H, br s, NH), 8.23 (2 H, d, *J* 7.8, 6-H), 6.19–6.13 (4 H, br t, 1′- and 5-H), 5.80 (2 H, br s, OH), 5.02 (2 H, br s, OH), 4.27 (2 H, br s, 3′-H), 3.87 (2 H, br s, 4′-H) and 3.82–3.60 (6 H, m, 2′-H, 5′-H_2); δ_C[50 MHz; $(CD_3)_2SO$] 159.3 (CO), 147.4 (CO), 144.5, 94.7, 88.9, 87.7, 71.5, 60.8 and 57.2.

2′-Deoxy-5′-*O*-tosyluridin-2′-yl propyl disulfide 17. To a stirred solution of the mixed disulfide **6** (250 mg, 0.75 mmol) in pyridine (15 ml) cooled to 0 °C was added toluene-*p*-sulfonyl chloride (500 mg, 2.62 mmol). The resultant solution was stirred at 0 °C for 10 h and then at room temperature for 2 h. Ethanol (10 ml) was added and the solution was evaporated. The residue was dissolved in dichloromethane, and the solution obtained was washed with water, dried over Na_2SO_4 and evaporated. The residue was flash chromatographed on silica gel with CH_2Cl_2–MeOH (90 : 10) as eluent to yield the 5′-tosyl derivative **17** (210 mg, 57%) and the 3′,5′-di-*O*-tosyl derivative (60 mg, 12%). Required *compound* **17**: mp 72–75 °C [Found: C, 46.90; H, 4.91; N, 5.91; S, 19.28. $C_{19}H_{24}N_2O_7S_3$ (488.59) requires C, 46.71; H, 4.95; N, 5.73; S, 19.69%]; *m/z* [FAB+; NBA] 489 $[M + H]^+$; [FAB−; NMA] 487 $[M - H]^-$, 459 $[M - C_2H_5]^-$, 445 $[M - C_3H_7]^-$, 413 $[M - C_3H_7S]^-$ and 332 $[M - Ts]^-$; δ_H(200 MHz; C^2HCl_3) 8.30 (1 H, br s, NH), 7.78 (2 H, d, *J* 8.2, ArH), 7.42 (2 H, d, *J* 8.0, Ar-H), 7.35 (1 H, d, *J* 8.2, 6-H), 6.09 (1 H, d, *J* 8.6, 1′-H), 5.74 (1 H, d, *J* 8.0, 5-H), 4.42 (1 H, dd, *J* 4.7 and 1.5, 3′-H), 4.23 (3 H, m, 4′-H, 5′-H_2), 3.50 (1 H, dd, *J* 8.6 and 4.7, 2′-H), 2.70–2.61 (3 H, m, SCH_2, 3′-OH), 2.45 (3 H, s, ArCH_3), 1.74–1.60 (2 H, m, CH_2CH_3) and 0.96 (3 H, t, $SCH_2CH_2CH_3$); δ_C(50 MHz; C^2HCl_3) 162.2 (CO), 150.1 (CO), 145.8, 139.4 (C-6), 132.1, 130.2, 127.8, 103.4 (C-5), 87.4, 82.2, 72.0, 68.9 (C-5′), 60.3, 41.3 (SCH_2), 22.1 (CH_2CH_3), 21.7 (ArCH_3) and 13.0 (CH_2CH_3).

2′-Deoxyuridin-2′-yl propyl disulfide 5′-diphosphate 18. To a solution of compound **17** (64 mg, 0.13 mmol) in acetonitrile (250 μl), was added a solution of tris(tetrabutylammonium) hydrogen pyrophosphate[21] (300 mg, 2.41 mmol) in acetonitrile (250 μl). The solution was concentrated by half under argon and then was stirred for 48 h at room temperature. The resultant solution was concentrated under argon and the residue was dissolved in water and chromatographed on a reversed-phase column (C_{18}-Sep-Pak®, 1 g cartridge) with water–methanol (70 : 30) as eluent. After lyophilization of the fractions, the pure *nucleotide* **18** was obtained [0.068 mmol (UV), 52%]. The tetrabutylammonium counter ions were exchanged with ammonium ions on Q-Sepharose by chromatography in ammonium hydrogen carbonate (400 mM), LRMS *m/z* [FAB+; glycerol] 512 $[M + 3H + NH_4]^+$, 495 $[M + 4H]^+$ and 242 $[M + 3H - H_3P_2O_7 - C_3H_7S]^+$; [FAB−; NBA] *m/z* 493 $[M + H]^-$; HRMS *m/z* [FAB+, glycerol] 512.0353. $C_{12}H_{24}N_3O_{11}P_2S_2$ $[M + 3H + NH_4]^+$ requires *m/z* 512.0328; δ_H(500 MHz; ^2H_2O) 7.93 (1 H, d, *J* 8.1, 6-H), 6.31 (1 H, d, *J* 8.4, 1′-H), 5.98 (1 H, d, *J* 8.1, 5-H), 4.65 (1 H, dd, *J* 6.0 and 2.5, 3′-H), 4.24 (1 H, m, 4′-H), 4.20–4.05 (2 H, m, 5′-H_2), 3.74 (1 H, dd, *J* 8.4 and 6.0, 2′-H), 2.70–2.55 (2 H, m, SCH_2), 1.60–1.53 (2 H, m, CH_2CH_3) and 0.85 (3 H, t, *J* 7.3, CH_2CH_3); δ_P(81 MHz; ^2H_2O) −5.6 (1 P, d, *J* 23) and −10.3 (1 P, d, *J* 23).

2′-Thiouridine 5′-disphosphate 19. To an aqueous solution of disphosphate **18** (3.5 mM; 500 μl, 1.7 μmol) was added aq. DTT (700 mM; 5 μl, 2 μmol). Argon was bubbled through the solution for 15 min to remove propane-1-thiol which was formed immediately (characteristic odour), to leave *compound* **19**, *m/z* [FAB+; glycerol] 438.0122. $C_9H_{18}N_3O_{11}P_2S$ $[M + 3H + NH_4]^+$ requires *m/z* 438.0137); δ_H(500 MHz; ^2H_2O) 7.91 (1 H, d, *J* 8.1, 6-H), 5.95 (1 H, d, *J* 8.1, 5-H), 5.93 (1 H, d, *J* 8.1, 1′-H), 4.30 (2 H, m, 3′- and 4′-H), 4.15–4.05 (2 H, m, 5′-H_2), 3.75 (1 H, m, 2′-H); δ_P(81 MHz; ^2H_2O) −5.7 (1 P, d, *J* 22) and −10.1 (1 P, d, *J* 22).

EPR Experiments

The EPR spectra were recorded with a Bruker ESP 300 E spectrometer. Oxyhaemoglobin was prepared from human methaemoglobin purchased from Sigma (86% metHb, 2% desoxyHb, 7% oxyHb). Iron(II) sulfate heptahydrate and sodium thiosulfate were from Sigma. Diethyldithiocarbamic acid sodium salt trihydrate was from Janssen. Baker yeast was bought in a local store. Protein R2 was prepared from overproducing strains of *E. coli*.[6]

Detection of NO by paramagnetic nitrosohaemoglobin complex. [17] To a mixture of an aqueous oxyhaemoglobin solution (1 mM; pH 7.5; phosphate buffer 10 mM; 200 μl) and sodium acetate buffer (pH 6.5; 100 mM; 100 μl) was added an aqueous solution of thionitrite **12** or **15** (1 mM, 100 μl). The mixture was stirred and the EPR spectrum was recorded at 100 K as a function of time at room temperature.

Detection of NO by paramagnetic $Fe(DETC)_2NO$ complex.[18a] A suspension of yeast cells (200 mg/ml) in 0.1 M Hepes (pH 7.5) was incubated with sodium diethyldithiocarbamate (2.5 mg/ml) for 30 min at 37 °C. The suspension (2 × 2 ml) was centrifugated and the solid was resuspended in 2 × 1 ml, 0.1 M Hepes (pH 7.5). To this suspension (200 μl) was added an aqueous solution of thionitrite **12** or **15** (200 μl; 0.5–1 mM). After 15 min at 37 °C, the sample was transferred into an EPR tube, frozen in liquid nitrogen and the EPR spectrum was recorded at 100 K.

Detection of NO by paramagnetic $Fe(NO)_2(S_2O_3)_2$.[18a,19] Iron(II) sulfate heptahydrate (20 mg) and sodium thiosulfate (356 mg) were dissolved in water (20 ml) previously deoxygenated by argon. Argon was bubbled through the solution for 15 min.

NO detection.—To a mixture of freshly prepared iron(II) sulfate and sodium thiosulfate solution (200 μl) were added Hepes buffer (pH 7.5; 100 mM; 100 μl) and thionitrite **12** or **15** (1 mM or 0.1 mM for quantification; 100 μl). The mixture was stirred and the EPR spectrum was recorded at 100 K.

EPR spectroscopy with pure protein R2. The reaction was carried out at 37 °C in an EPR tube containing the small subunit of ribonucleotide reductase named protein R2 (1 mg ml^{-1}) and thionitrite **12** or **15** at various concentrations in 150 μl of 50 mM Tris-HCl buffer, pH 7.5, with 10% glycerol. At time intervals, the tube was frozen in liquid nitrogen and the EPR spectrum of the solution was recorded at 100 K. The amount of tyrosyl radical was determined from the comparison of the amplitude of the typical EPR signal at $g = 2$ to that of a pure sample of protein R2 (1 mg ml^{-1}).

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