Radiation-induced binding of 2,2,6,6-tetramethyl-1,4-piperidone-*N*-oxyl to thymidine in oxygen-free aqueous solutions. Isolation and characterization of the adducts

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Steady-state γ-radiolysis of deaerated aqueous solutions of thymidine has been carried out in the presence of 2,2,6,6-tetramethyl-1,4-piperidone-*N*-oxyl (TAN), a well-known radiosensitizing agent. The eight main radiation-induced TAN addition products to thymidine have been isolated and characterized by ^1H and ^{13}C nmr, cd, and fast-atom bombardment mass spectrometry measurements.

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La radiolyse γ en régime stationnaire de solutions aqueuses désaérées de thymidine a été effectuée en présence de tétraméthyl-2,2,6,6-pipéridone-4-*N*-oxyl (TAN) qui est un agent de radiosensibilisation. Les huit produits d'addition radio-induite de la molécule de TAN avec la thymidine ont été isolés et identifiés par diverses méthodes spectroscopiques: rmn ^1H et ^{13}C, dc et spectrométrie de masse par bombardement avec des atomes rapides.

Introduction

Various nitroxyl free radicals including nor-pseudopelletierine-*N*-oxyl (NPPN) and 2,2,6,6-tetramethyl-1,4-piperidone-*N*-oxyl (TAN) have been shown to sensitize hypoxic living cells to the lethal action of ionizing radiations (1, 2). The radiosensitizing ability of TAN appears to be higher in bacterial cells than in mammalian cells (3, 4). Two mechanisms which involve DNA as the major cellular target have been proposed for the biological action of several classes of radiosensitizers (5). The first postulates the oxidation of DNA anionic radicals (generated as anion–cation radical pairs by the direct action of ionizing radiation in purine and pyrimidine bases) through electron transfer reactions with TAN or NPPN. This would prevent the recombination of anion and cation radicals, resulting in permanent damage to the biopolymer. A second reaction is the covalent binding with radiation-induced DNA radicals (6). The formation of covalent TAN–DNA adducts has been reported upon irradiation either in aqueous solutions of DNA (7) or within living cells (8). Such lesions, the structures of which remain unknown, have been detected in Chinese hamster cells as *M. luteus* sensitive sites (9). Pulse radiolysis experiments have shown that the second-order reaction rate of TAN with the radicals resulting from the addition of OH radicals across the 5,6 bond of pyrimidine DNA components is an efficient process (10). The two main addition products of TAN to radiation-induced 5-hydroxy-5,6-dihydrothymin-6-yl and 6-hydroxy-5,6-dihydrothymin-5-yl radicals have been isolated and characterized (11). In the present study, thymidine has been used as a DNA model compound for a further investigation of the binding of TAN with the various nucleoside radicals induced by the water radiolysis species. We show that the bulk of the adducts result from the binding reaction of TAN with the radicals derived from addition of OH radicals at either carbon C(5) or carbon C(6).

Results

Steady-state γ-radiolysis of oxygen-free aqueous solutions of 1 m*M* thymidine containing 2 m*M* 2,2,6,6-tetramethyl-1,4-piperidone-*N*-oxyl (TAN) led to the formation of a complex mixture of nucleosides and thymine derivatives. These compounds were separated by two-dimensional thin-layer chromatography on silica gel plates (12). Eight nucleosides which exhibit high chromatographic mobility gave a coloration characteristic of carbonyl-containing compounds by spraying the chromatoplates with the 2,4-dinitrophenylhydrazine reagent (13). This is consistent with the presence of the piperidone moiety in the above nucleosides. These TAN–thymidine adducts were further purified and separated from the radiation-induced diastereoisomers of 5,6-dihydrothymidine and 5,6-dihydroxy-5,6-dihydrothymidine by preparative thin-layer chromatography on silica gel, followed by reversed–phase high performance liquid chromatography. These TAN–thymidine adducts, which represent about 50% of the overall radiation-induced degradation products (Table 1), were further characterized on the basis of various spectroscopic measurements. Further confirmation was provided by comparison with the adducts generated in alternate chemical routes.

The eight thymidine derivatives **2**–**9** showed no absorption around 260 nm, in line with the saturation of the 5,6-pyrimidine bond of these nucleosides (14). The six more stable thymidine–TAN adducts **2**–**7** were analyzed by fast-atom bombardment (FAB) mass spectrometry. This soft ionization technique is particularly suitable for the mass spectrometry measurement of polar and fragile molecules, such as the TAN adducts (Fig. 1). A prominent pseudo-molecular ion $(M + Na)^+$ at $m/z = 452$ was observed in the positive mass spectrum of each of the six nucleosides **2**–**7**. The pseudo-molecular ion $(M - H)^-$ exhibited in the negative ionization mode at $m/z = 428$ is of relatively low intensity. These spectroscopic data strongly suggest that these compounds result from the addition of a hydroxyl radical and of a TAN molecule to the starting thymidine. The same conclusions can be deduced from the observation of a pseudo-molecular ion in the field desorption (FD) and the desorption by chemical ionization (DCI) spectra of compounds **2** and **3** respectively. In addition, the fragmentation patterns of these adducts, particularly those obtained in

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TABLE 1. Yield of the radiation-induced TAN addition products **2**–**9** to thymidine in oxygen free aqueous solution[a]

Nucleosides	*G* value[b]
trans-(5*S*,6*S*)-5-hydroxy-6-(2,2,6,6,-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (**2**)	0.11
trans-(5*R*,6*R*)-5-hydroxy-6-(2,2,6,6,-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (**3**)	0.10
cis-(5*S*,6*R*)-5-hydroxy-6-(2,2,6,6,-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (**4**)	0.05
cis-(5*R*,6*S*)-5-hydroxy-6-(2,2,6,6,-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (**5**)	0.06
trans-(5*S*,6*S*)-6-hydroxy-5-(2,2,6,6,-tetramethyl-4-oxo-1-piperidinyl-*N*-oxide)-5,6-dihydrothymidine (**6**)	0.05
trans-(5*R*,6*R*)-6-hydroxy-5-(2,2,6,6,-tetramethyl-4-oxo-1-piperidinyl-*N*-oxide)-5,6-dihydrothymidine (**7**)	0.03
(5*R**) and (5*S**)-5-(2,2,6,6,-tetramethyl-4-oxo-1-piperidinyl-*N*-oxide)-5,6-dihydrothymidine (**8**, **9**)	0.07
Thymidine (**1**)	−0.89

[a]Absorbed dose: 1.5 kGy; 1 m*M* thymidine and 2 m*M* TAN aqueous solutions.

[b]Number of molecules formed or destroyed per an absorbed dose of 1 Gy. The *G* values are the average of four independent measurements (accuracy to ±5%).

FD-ms, provide significant structural information. Thus the base peak at $m/z = 259$ corresponds to the loss of the TAN radical, whereas major fragments can be assigned to the TAN residue ($m/z = 170$) and the deoxyribose residue ($m/z = 117$) respectively. Mass spectrometric analyses of the two other nucleosides **8** and **9** were not so conclusive due to extensive thermal decomposition of the samples.

The 250-MHz ^{1}H nmr spectra of the thymidine–TAN adducts **2**–**7** in D_2O confirm the presence of the piperidine moiety. In particular, they exhibit four singlets in the range 1.04–1.44 ppm which correspond to the resonance signals of the methyl substituents of the piperidone ring. We also note the presence at a slightly higher field (1.55–1.70 ppm) of an additional singlet which was assigned as the pyrimidine methyl group. The chemical shift of this signal and those of the H(6) pyrimidine proton (5.23–5.40 ppm) are characteristic of 5,6-dihydrothymine derivatives. Similar ^{1}H nmr features are observed for the nucleosides **6** and **7**, suggesting an isomeric relationship for the adducts **2**–**7**. On the other hand, the two last products **8** and **9** have a different structure with a methylene group ($J_{gem} = -14$ Hz) at the C(6) carbon and the TAN residue attached to the C(5) carbon.

Site of attachment of the TAN moiety

Considerations of the ^{1}H nmr chemical shift values of the H(6) proton and of the thymine methyl group are not of interest for determining the site of attachment of the piperidone ring, either at the C(5) or the C(6) positions (Fig. 2). The comparison of the chemical shift values of H(6) for the adducts **2**–**7** does not show any significant differences which could be related to the α or β position of the TAN substituent. The lack of any selective downfield shift effect associated with other electronegative α substituents such as a hydroperoxide group has been previously noted for 5,6-dihydrothymidine derivatives (15). It must also be mentioned that the chemical shift of the pyrimidine H(6) proton is sensitive to the orientation of the aglycone about the *N*-glycosidic bond (16). On the other hand, ^{13}C nmr has been shown to be a suitable spectroscopic method for investigating the effects of substituents at carbons C(5) and C(6) in various 5,6-dihydrothymine derivatives (17). The relevant ^{13}C chemical shift values of the six thymidine–TAN adducts **2**–**7** and of various 5,6-dihydrothymidine derivatives are listed in Table 2. The comparison of the C(5) and C(6) chemical shifts of the compounds **2**–**7** with the corresponding carbons of the *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine shows that the C(6) resonance signal of compounds **2**–**5** is shifted downfield by about 9 or about 13 ppm. A similar deshielding effect is observed for the C(5) of the thymidine–TAN adducts **6** and **7**. This strongly suggests that the TAN substituent is at position 5 in nucleosides **6** and **7** and at position 6 in adducts **2**–**5**. The magnitude of the α effect of the TAN substituents (as determined by using 5-hydroxy-5,6-dihydrothymidine and 6-hydroxy-5,6-dihydrothymidine as the reference compounds) is 41–45 ppm for the C(6) carbon and about 38 ppm for the C(5) carbon. The lower value for the latter carbon may be due to the more severe crowding of this carbon which is suggested by the molecular model. The variation in the magnitude of the α effect at the C(6) carbon could be the result of changes in the *syn*–*anti* conformation of the nucleobase with respect to the sugar moiety, as discussed in more detail in the second paper (18). Polarization effects resulting from the close vicinity of the O(1′) atom with the C(6) carbon in compounds **2** and **5** would be at the origin of this shielding effect.

Further confirmation of the assignment of the position of the TAN substituent was provided by the radiation-induced synthesis of the various thymidine–TAN adducts **2**–**9** from 5,6-saturated thymidine derivatives. In the first series of reactions, the γ-radiolysis of oxygen-free aqueous solutions of (−)-*trans*-(5*S*,6*S*)-5-bromo-6-hydroxy-5,6-dihydrothymidine (**12**) (18) in the presence of 4.7 m*M* TAN and 0.1 *M* *tert*-butanol gives rise to the adduct **6**, whereas irradiation of the (+)-*trans*-5-bromo-6-hydroxy-5,6-dihydrothymidine (**13**) generates the adduct **7** (Fig. 3). A reasonable mechanism for their formation would involve, in the initial step of the reaction, the departure of the Br atom through an electron capture dissociation process (20). A recombination reaction between the resulting 6-hydroxy-5,6-dihydrothymid-5-yl radical and the TAN molecule would generate, with retention of configuration at the C(6) carbon (21), the adducts **6** and **7** respectively.

In the second series of reactions, evidence is provided for the adducts **2**–**5**. The two pairs of *cis* and *trans* diasteroisomers of C(6) addition products, **5**,**3** and **4**,**2** respectively, can be prepared in a selective way by using a radiation-induced procedure. γ-Irradiation of deaerated aqueous solutions of the 5*R* diasteroisomer of 5-hydroxy-5,6-dihydrothymidine (**11**) containing 2 m*M* TAN generates adducts **3**,**5**, whereas the two other adducts **2**,**4** are obtained from (5*S*)-5-hydroxy-5,6-dihydrothymidine (**10**). In addition, a mixture of both the 5*R** and 5*S** adducts, **8** and **9**, the absolute configuration of which has not been determined, is produced in both irradiated solutions. Under the experimental conditions used, the hydroxyl radical is the main radiolysis-reactive species which may abstract a hydrogen at carbon C(6) or lead to the loss of a hydroxyl group at carbon C(5). Similar abstraction of a hydroxyl group has been observed in X-irradiated 5-hydroxy-5-methylbarbituric acid (22). A recombination reaction of the resulting oxyl radical with TAN (23) gives rise to a pair of *cis* and *trans* diastereoisomers of C(6) adducts **5**,**3** or **4**,**2** with retention of the C(5) configuration (Fig. 4). It should be noted that the addition of the TAN molecule to the carbon C(6) is less stereoselective than to carbon C(5). This loss of stereospecificity is

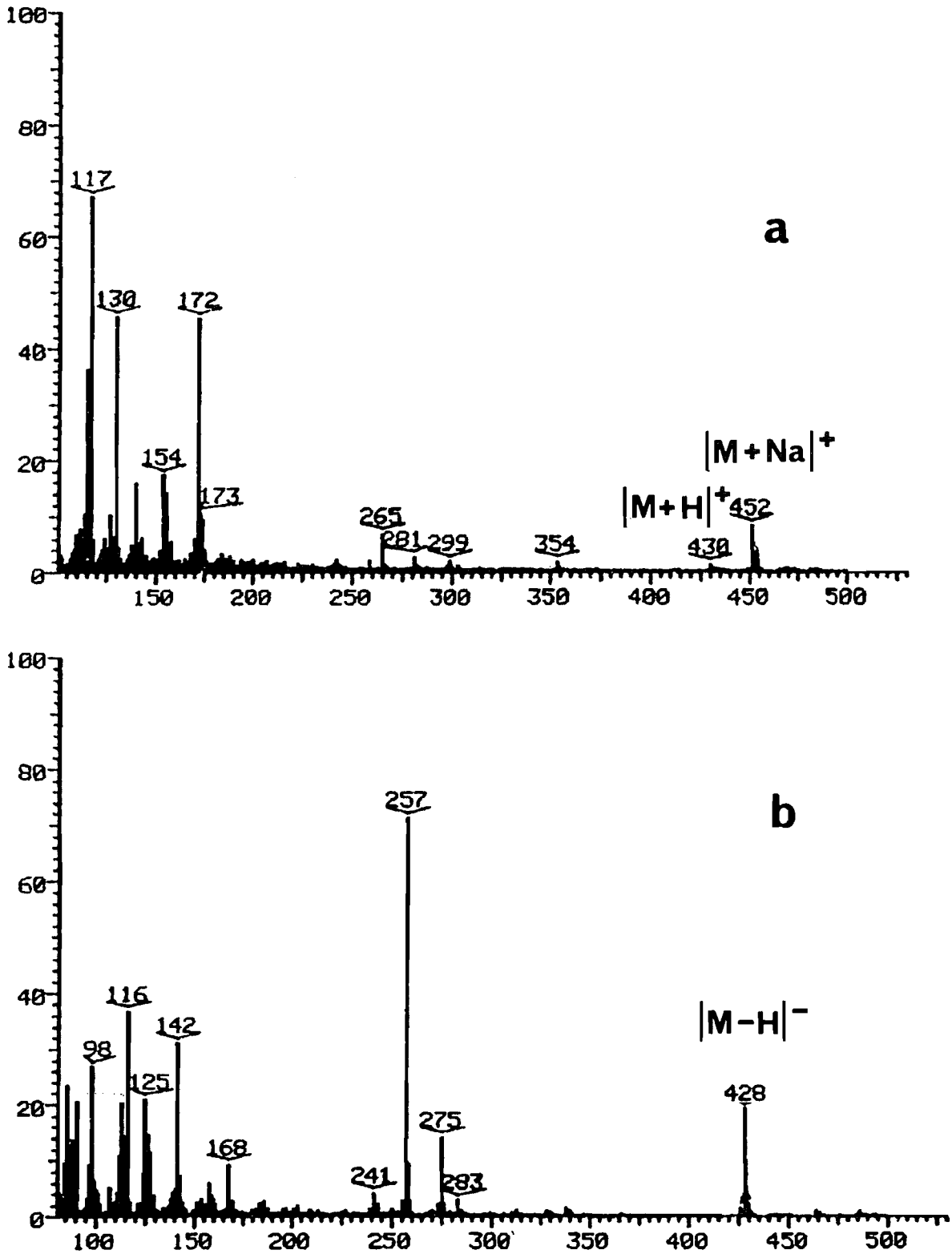

FIG. 1. FAB-ms spectra of the TAN–thymidine adduct **5**: (a) positive mode; (b) negative mode.

probably due to the presence of the two bulky substituents on the carbon C(5) preventing the preferential *trans* radical addition which is usually observed with respect to the 5- or 6-monosubstituted carbon within the pyrimidine ring (24).

Nature of the linkage to the TAN substituent

The covalent addition of nitroxyl radicals to carbon-centered radicals may generate either hydroxylamine derivatives (25) or *N*-oxide type compounds (26). Chemical transformations of the adducts **2**–**9** provided the most convincing evidence that the TAN moiety is linked via the nitroxyl oxygen to carbon C(6) in nucleosides **2**–**5** (hydroxylamine) and via the nitroxyl nitrogen to carbon C(5) in nucleosides **6**–**9** (*N*-oxide). Catalytic hydrogenation is expected to convert the substituted hydroxyl-

TABLE 2. ^{13}C Chemical shifts (δ, ppm) of relevant pyrimidine carbons of TAN–thymidine adducts and various diastereoisomers of 5,6-dihydrothymidine derivatives in D_2O[a]

Nucleosides	CH_3	C(5)	C(6)
trans-(5*S*,6*S*)-5-hydroxy-6-(2,2,6,6-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (**2**)	20.1	70.7	89.1
trans-(5*R*,6*R*)-5-hydroxy-6-(2,2,6,6-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (**3**)	19.8	70.7	93.7
cis-(5*S*,6*R*)-5-hydroxy-6-(2,2,6,6-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (**4**)	23.8	73.2	93.4
cis-(5*R*,6*S*)-5-hydroxy-6-(2,2,6,6-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (**5**)	24.0	73.6	89.1
trans-(5*S*,6*S*)-6-hydroxy-5-(2,2,6,6-tetramethyl-4-oxo-1-piperidinyl-*N*-oxide)-5,6-dihydrothymidine (**6**)	14.6	80.9	79.5
trans-(5*R*,6*R*)-6-hydroxy-5-(2,2,6,6-tetramethyl-4-oxo-1-piperidinyl-*N*-oxide)-5,6-dihydrothymidine (**7**)	15.0	80.1	80.8
(−)-(5*S*)-5-hydroxy-5,6-dihydrothymidine (**10**)	22.3	69.5	48.3
(+)-(5*R*)-5-hydroxy-5,6-dihydrothymidine (**11**)	22.7	69.6	48.2
(+)-(5*R*)-5,6-dihydrothymidine	13.3	35.8	43.2
(−)-(5*S*)-5,6-dihydrothymidine	12.8	35.8	43.1
(+)-*cis*-(5*S*,6*R*)-6-hydroxy-5,6-dihydrothymidine	10.5	42.2	77.6
(−)-*cis*-(5*R*,6*S*)-6-hydroxy-5,6-dihydrothymidine	10.4	42.5	76.7
(+)-*cis*-(5*S*,6*R*)-5,6-dihydroxy-5,6-dihydrothymidine	22.8	73.6	80.7
(−)-*cis*-(5*R*,6*S*)-5,6-dihydroxy-5,6-dihydrothymidine	22.9	73.5	79.9
(+)-*trans*-(5*R*,6*R*)-5,6-dihydroxy-5,6-dihydrothymidine	19.2	72.2	80.8
(−)-*trans*-(5*S*,6*S*)-5,6-dihydroxy-5,6-dihydrothymidine	19.3	72.1	80.2

[a]All chemical shifts were referenced to external TMS.

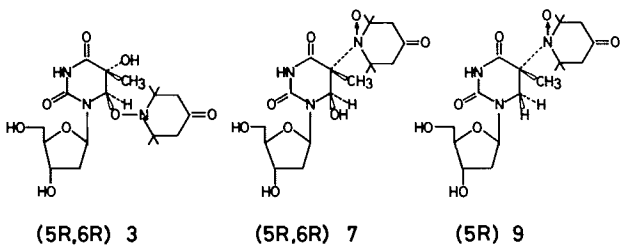

FIG. 2. Structure of three possible TAN–thymidine adducts with a 5*R* stereoconfiguration.

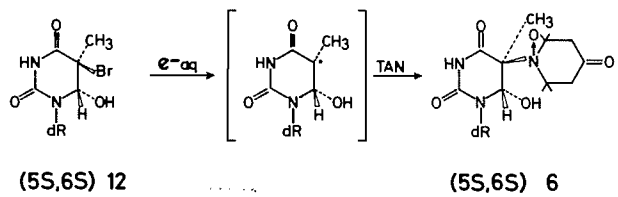

FIG. 3. Specific synthesis of the thymidine–TAN adduct **6** by γ-radiolysis of deaerated aqueous solution of (−)-*trans*-5-bromo-6-hydroxy-5,6-dihydrothymidine **12**.

amine derivative to an alcohol and an amine through heterolysis of the N—O bond according to an S_N2 mechanism (27). Hydrogenolysis of the four adducts **2**–**5**, in the presence of Pd/C, generated in a quantitative way the thymidine glycols and the 2,2,4,4-tetramethylpiperidone. However, the two C(5) thymidine–TAN addition products **6** and **7** remain unchanged under these hydrogenolysis conditions.

The latter compounds **6** and **7** are unstable in solutions and undergo various transformations which can be rationalized on the basis of the postulated *N*-oxide structure. In particular, they show extensive decomposition after standing for 48 h in neutral

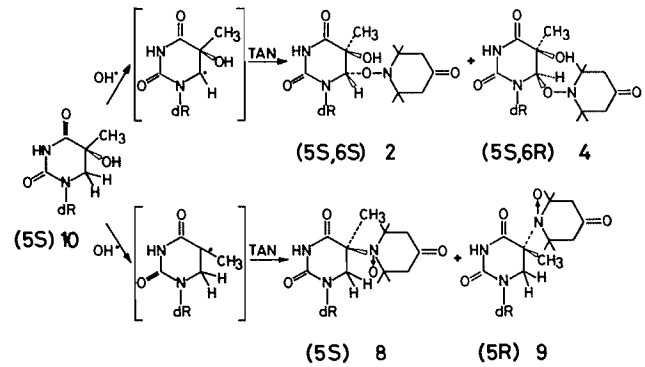

FIG. 4. Preparation of the thymidine–TAN adducts **2**, **4**, **8**, and **9** by γ-irradiation of oxygen-free aqueous solutions of (5*S*)-5-hydroxy-5,6-dihydrothymidine (**10**).

aqueous solution at 20°C. The major degradation product was characterized as 5-hydroxymethyl-2′-deoxyuridine (**14**) by comparison of some of its spectroscopic features (uv, EI-ms, ^1H and ^{13}C nmr) with those of the authentic sample synthesized according to Baker *et al.* (28). A likely mechanism for its formation would involve a Cope rearrangement, which is characteristic of *N*-oxide type compounds (29). Hydrogen abstraction from the methyl by the N—O function through a planar pentagonal transient state and subsequent departure of the piperidone substituent would generate a nucleoside with a methylene group (Fig. 5). Elimination of the hydroxyl at carbon C(6) would give rise to a reactive carbonium ion, which by reaction with water is converted to 5-hydroxymethyl-2′-deoxyuridine (**14**). In H_2O_2 the corresponding hydroperoxide **15** is obtained as the result of the nucleophilic attack by the perhydroxyl ion on the carbonium ion intermediate (30). It is interesting to note that similar reaction mechanisms have been proposed in the biosynthetic pathway of 5-hydroxy-

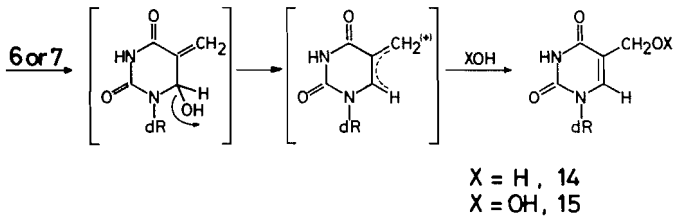

FIG. 5. Cope rearrangement of the thymidine–TAN adduct **6** or **7** to hydroxymethyl-2′-deoxyuridine (**14**) or hydroperoxymethyl-2′-deoxyuridine (**15**).

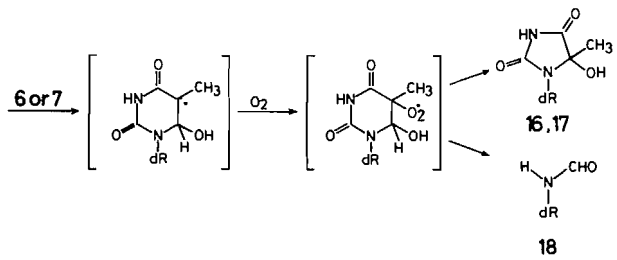

FIG. 6. Rearrangement of the thymidine–TAN adduct **6** or **7** to 5*R** and 5*S**-1(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methyl hydantoin (**16**, **17**) and *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl) formamide (**18**).

methyl-2′-deoxyuridine-5′-monophosphate which is catalyzed by dUMP hydroxymethylase (31). We may note that *N*-1-(2-deoxy-β-D-*erythro*-pentofuranosyl) barbituric acid (32), which would result from initial hydrogen abstraction reaction at carbon C(6), is produced, but in very low yield. This lower yield could be explained by the lack of planarity for the pentagonal transient state, due to the half-chair conformation of the 5,6-saturated pyrimidine ring. The formation of thymidine (**1**) from the other C(5) thymidine–TAN adducts **8** and **9** in aqueous solution would also strongly suggest a *N*-oxide type structure for these compounds. A Cope rearrangement appears as a likely mechanism for the generation of **1**.

Radical reactions, as the result of homolytic scission of the C—N bond of adduct **6** or **7** (*vide infra*) are predominant in aerated aqueous pyridine solutions. The major decomposition products were characterized as thymidine (**1**) (10%), the four *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine (20%), the *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl) formamide (**18**) (33), and the 5*R** and 5*S** diastereoisomers of *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methyl-hydantoin (**16**, **17**) (34). The formation of the two latter classes of products may involve a Meisheimer mechanism (35) which would generate the 6-hydroxy-5,6-dihydrothymid-5-yl radical from homolytic scission of the C(5)—N bond. Subsequent fast reaction of this pyrimidine radical with molecular oxygen would give rise to the corresponding hydroxyhydroperoxyl thymidine radical (36). Disproportionation reactions between these peroxyl radicals are expected to produce oxyl radicals (37), which through subsequent β scission reactions would lead to the opening of the pyrimidine ring (Fig. 6). N^1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-N^1-formyl-N^2-pyruvylurea is the expected product of this reaction. Hydrolysis of this unstable intermediate gives rise to the formamide compound **18** in 40% yield, whereas recyclization generates the hydantoin nucleosides **16**, **17** in about 20% yield.

The loss of oxygen from the molecular ion in electron impact mass spectrometric analysis may be diagnostic for the *N*-oxide structure (38). This particular ion ($M - 16$) has not been observed in the FAB mass spectrometry measurements of adducts **2**–**7**, which show little fragmentation.

Determination of the absolute configuration of adducts 2–7

As mentioned above, the radiation-induced degradation of the 5*R* diastereoisomer of 5-hydroxy-5,6-dihydrothymidine (**11**) in the presence of TAN gives rise to the *trans* and *cis* C(6) adducts **3** and **5** with retention of the initial 5*R* configuration. In the same way, the two 5*S* diastereoisomers **2** and **4** have been prepared from the (5*S*)-diastereoisomer of 5-hydroxy-5,6-dihydrothymidine (**10**) (39). The *cis* and *trans* configuration of the two nucleosides of each pair of 5*R* and 5*S* diastereoisomers could be deduced by considering the γ-effect of the C(6) TAN substituent on the ^{13}C nmr chemical shift value of the pyrimidine methyl group. An upfield shift effect of about 3 ppm is observed for the *trans* thymine glycol (11) and the corresponding 2′-deoxyribonucleoside derivatives (Table 2) when the C(6) hydroxyl group is in a *gauche* relationship with the C(5) methyl substituent. A similar upfield shift effect is noted for the adducts **2** and **3** by comparison with the second 5*S* and 5*R* diastereoisomers **4** and **5** respectively. The magnitude of this γ-effect is higher than for the 5,6-dihydroxy-5,6-thymidine, as expected for a bulky C(6) substituent. As a result, a 6*R* configuration and 6*S* configuration can be deduced for **3**,**4** and **2**,**5** respectively.

The assignment of the C(6) configuration of the TAN–thymidine adducts **6** and **7** is also based on their selective preparation in 90% yield from the corresponding (5*S*,6*S*) and (5*R*,6*R*) 5-bromo-6-hydroxy-5,6-dihydrothymidine (**12** and **13**). As discussed above, a *trans* addition of the TAN molecule to the 6-hydroxy-5,6-dihydrothymid-5-yl radical is expected to take place preferentially, giving rise to the (5*S*,6*S*) and (5*R*,6*R*) TAN–thymidine adducts **6** and **7**, respectively.

Mechanisms of the radiation-induced formation of the adducts 2–9

Interaction of the γ-rays with dilute aqueous solutions of thymidine and TAN leads to the formation of three reactive species (40): OH radicals ($G = 2.72$), solvated electrons ($G = 2.63$), and hydrogen atoms ($G = 0.55$). Under the experimental conditions used, about 40% of the OH radicals will react with thymidine (41): $k_{OH+dThd} = 3.9 \times 10^9\ M^{-1}\ s^{-1}$. The remaining 60% are scavenged by the TAN molecules (42): ($k_{OH+TAN} = 6.3 \times 10^9\ M^{-1}\ s^{-1}$). The pyrimidine ring of the nucleoside is the preferential site of reaction of the OH· radicals through addition at the carbons C(5) and C(6) as shown by esr flow experiments (43). Recombination reactions of these radicals with the TAN molecule give rise to the C(5) adducts and the four *cis* and *trans* diastereoisomers of the C(6) addition products. The reaction of the solvated electrons with thymidine takes place mostly on the pyrimidine moiety (41). The resulting anionic radical is exposed to be quantitatively oxidized to the starting thymidine, since pulse radiolysis experiments have shown that electron transfer reaction from electron pyrimidine adduct to TAN is an efficient process (6, 8). The formation of the two adducts **8** and **9** may also be accounted for by the recombination reaction of 2,2,6,6-piperidone-*N*-oxyl with the radiation-induced 5,6-dihydrothymid-5-yl radical (42). This radical arises from the preferential addition of the hydrogen atom to the carbon C(6) within the thymine moiety (44). It should be noted that, in the present work, adducts with TAN covalently bound to the sugar moiety of thymidine were not

detected. On the other hand, this latter class of adducts has been shown to be relatively important when purine 2′-deoxyribonucleosides are exposed to γ-rays in deaerated aqueous solution containing *N*-oxyl radicals (45).

Conclusion

The main radiation-induced TAN adducts to thymidine have been isolated and characterized as recombination products between TAN and transient 5-hydroxy-5,6-dihydrothymid-6-yl or 6-hydroxy-5,6-dihydrothymid-5-yl radicals. This constitutes a first step to further studies dealing with the radiation-induced binding of TAN to the thymine moiety within isolated DNA. In particular, the knowledge of the structure and chemical properties of these various thymidine–TAN adducts will facilitate their detection in enzymatic hydrolysates of DNA which has been irradiated in the presence of TAN.

Experimental

Materials

Thymidine was purchased from Sigma (St. Louis, Missouri) and used without further purification. 2,2,6,6-Tetramethylpiperidone was obtained from Aldrich (Beerse, Belgium). 2,2,6,6-Tetramethylpiperidone *N*-oxyl was synthesized according to Briere *et al.* (46). The two *trans* diastereoisomers of 5-bromo-6-hydroxy-5,6-dihydrothymidine (**12**, **13**) were prepared by addition of Br_2 to an aqueous solution of thymidine (19) and separated by preparative reversed-phase high performance liquid chromatography.

The four *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine (47), the two *cis* (5*S*,6*R*) and (5*R*,6*S*) diastereoisomers of 6-hydroxy-5,6-dihydrothymidine (48), and the 5*R* and 5*S* diastereoisomers of 5-hydroxy-5,6-dihydrothymidine (**11**, **10**) (49) were prepared according to literature procedures. The (−)-(5*S*)-dihydrothymidine has been obtained according to Kondo and Witkop (14), whereas the (+)-(5*R*)-diastereoisomer has been prepared by hydrogenation of an aqueous solution of thymidine in the presence of Pd/C as the catalyst (50). 5-Hydroxymethyl-2′-deoxyuridine has been synthesized according to Baker *et al.* (28). [$^{14}CH_3$]thymidine has been obtained from the Service des Molécules Marquées du Commissariat à l'Energie Atomique (Saclay, France). The radioactive nucleoside was purified prior to use on a Nucleosil octadecylsilyl silica gel column to remove self-radiolysis decomposition products.

Spectroscopic measurements

The uv absorption spectra were registered on a Beckman spectrophotometer Model 5230. The ir spectra using the KBr micropellet method were obtained on a Perkin–Elmer spectrophotometer Model 177. Field desorption mass spectra were obtained on a Varian-Mat 311 spectrometer. (The intensity of the current of desorption was 10 mA.) Fast-atom bombardment (FAB) mass spectrometry was carried out in a Model MS 50 spectrometer equipped with a commercially available FAB gun. Desorption of the molecules was obtained by exposure to a beam of 8-keV xenon atoms in a glycerol mull. Chemical ionization mass spectra using NH_3 as the reactant gas were performed on a Nermag R 10-10 C apparatus. The ^1H nmr and ^{13}C nmr spectra operating at 250 MHz and 62.83 MHz respectively were obtained on a CAMECA TSN 250 apparatus in the Fourier transform mode. Deuterium oxide was used as the solvent with 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 as the internal reference. Circular dichroïsm spectra (cd) were registered on a Roussel Jouan III dichrograph using methanol as the solvent.

Irradiation procedure

The γ-radiolysis experiments were carried out with three ^{60}Co sources located in a pool. The dose rate, which was determined according to Fricke's method, was 110 Gy/min. The flasks which were used for the irradiation experiments were previously filled with bidistilled water (pH 6.5) and exposed to high doses of γ-rays (10^5Gy) in order to destroy the organic impurities within the glass. The aqueous solutions of thymidine were deaerated by bubbling nitrogen for 15 min prior to irradiation.

Chromatographic analyses

Analytical two-dimensional separations of radiation-induced decomposition products of thymidine were carried out on precoated silica gel 60 F_{254} plates (Merck, Darmstadt, G.F.R.) with the two following solvent systems (12): I. lower phase of chloroform–methanol–water (4:2:1) to which was added 5% of methanol; II. ethyl acetate – 2-propanol – water (75:16:9). Preparative thin-layer chromatography was performed on thick precoated silica gel plates (Merck). Detection of far-uv absorbing compounds was made by fluorescence quenching with a 254-nm emitting Desaga mineral lamp. The 2′-deoxyribonucleosides were visualized as pink spots after spraying the plates with the cysteine – sulfuric acid reagent and subsequent heating for 3 min at 100°C. The carbonyl-containing compounds were detected on the chromatogram by using the 2,4-dinitrophenylhydrazine spray reagent (13).

Quantitative analysis

The [^{14}C] radio-labelled compounds were detected on the thin-layer silica gel plates by autoradiography using Kodak NS-2T X-ray sensitive films. Detection of radioactivity as low as 0.01 μCi/cm^2 was made after overnight exposure. The detected radioactive compounds were further scraped off the silica gel and suspended in 1 mL water for 2 h. Under these conditions, the radioactive recovery was nearly quantitative. The β scintillation counting was accomplished on a Packard model 2425 Tricarb spectrometer.

Isolation and characterization of the radiation-induced TAN–thymidine adducts

A solution of 1 m*M* thymidine (484 mg) and 2 m*M* TAN (680 mg) in 2 L of deaerated water was irradiated for 45 min with ^{60}Co γ-rays (absorbed dose: 4950 Gy). The solution was evaporated to dryness under reduced pressure. The resulting residue was dissolved in 2 mL of aqueous methanol (1:1) and applied to 5 preparative thick-layer chromatoplates. The developing solvent was a mixture of chloroform and methanol (9:1). The faster-moving broad uv-absorbing zone (R_f 0.35), which gives rise to a yellow coloration with the 2,4-dinitrophenylhydrazine spray reagent, was extracted with 50% aqueous methanol (3 × 10 mL). Evaporation of the solution to dryness gave a syrup (129 mg) which was deposited on 5 thin-layer precoated silica gel plates. Two uv absorbing zones were resolved (R_f 0.53 and 0.43 respectively) by using chloroform–methanol (9:1) as the developer. The fastest eluting uv absorbing zone (R_f 0.53) was shown to contain a mixture of three thymidine–TAN adducts **4**, **8**, and **9** (46 mg) after being extracted with 50% aqueous methanol (3 × 6 mL). The slower eluting uv absorbing zone (R_f 0.43) was scraped off and the silica gel was further extracted with 50% aqueous methanol (3 × 6 mL). Evaporation to dryness of the resulting solution yielded 67 mg of a colorless syrup which contains the four thymidine–TAN adducts **2**, **3**, **5**, and **7**.

*cis-(5S,6R)-5-hydroxy-6-(2,2,6,6-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (**4**)*

The oily residue (46 mg) which is a mixture of adducts **4**, **8**, and **9** was extracted from the faster uv-absorbing zone and applied to 3 precoated silica gel plates. The tlc plates were developed with ethyl acetate as the solvent. Two main zones which give a positive coloration with the cysteine–H_2SO_4 or the 2,4-dinitrophenylhydrazine spray reagents were detected by fluorescence quenching at 254 nm. The fastest eluting zone (R_f 0.62) was scraped off and the resulting silica gel was extracted with 3 × 5 mL of water–methanol (1:1). Evaporation to dryness of the filtrates yields 14 mg (1.7%) of an oily compound which was shown to be homogeneous by reversed-phase high performance liquid chromatography analysis on a C-18 Nucleosil column using a mixture of water–methanol (7:3) as the solvent (k' = 1.50); uv (λ_{max} H_2O): 206 nm; ^1H nmr (D_2O, TSP) δ: 1.14 (s, 3, CH_3), 1.21 (s, 3, CH_3), 1.32 (s, 3, CH_3), 1.44 (s, 3, CH_3), 1.59 (s, 3, CH_3 thymine), 2.42 (ddd, 1, H-2″), 2.94 (m, 1, H-2′), 3.74 (dd, 1, H-5″), 3.82 (dd, 1, H-5′), 4.0 (m, 1, H-4′), 4.47 (m, 1, H-3′), 5.41 (s, 1,

H-6), 5.78 (dd, 1, H-1′); ^{13}C nmr (D_2O, TMS) δ: 19.8 (q, CH_3 thymine), 23.7, 24.3, 32.3, 33.8 (q, 4 × CH_3 piperidone), 38.7 (t, C-2′), 53.6 (t, CH_2 piperidone), 53.7 (t, CH_2 piperidone), 63.0 (t, C-5′), 66.1 (s, C-2, C-6 piperidone), 70.7 (s, C-5 thymine), 72.3 (d, C-3′), 87.0 (d, C-4′), 92.8 (d, C-1′), 93.7 (d, C-6 thymine); cd (*c* 1.4, methanol): $[\theta]_{280}$ ±0, $[\theta]_{270}$ −140, $[\theta]_{260}$ −1720, $[\theta]_{249}$ −4650, $[\theta]_{240}$ −1720, $[\theta]_{288}$ ±0, $[\theta]_{230}$ +3500; FAB-ms, *m/e* (relative intensity), positive mode: 452 (30, M^+ + Na), 430 (8, M^+ + H), 265 (20), 172 (15, TAN + 2H), 117 (58, 2-deoxyribose); negative mode: 428 (12, M^- − H), 283 (17), 257 (41, M^- − TAN), 241 (6, thymidine-H), 142 (15,5-hydroxy-5,6-dihydrothymine), 125 (18, thymine-H), 116 (14, 2-deoxyribose-H).

(5R) and (5S*)-5-(2,2,6,6-tetramethyl-4-oxo-1-piperidinyl-*N*-oxide)-5,6-dihydrothymidine (8, 9)*

Extraction of the slowest uv-absorbing zone (R_f 0.31) yielded 23 mg of a mixture of thymidine−TAN adducts **8** and **9** which were further separated on the C-18 Nucleosil octadecylsilyl silica gel column under the above conditions. Two main fractions corresponding to nucleosides **8** (k' = 4.36) and **9** (k' = 4.85) were collected. Evaporation to dryness of the aqueous methanol solutions gave respectively 9 mg of **8** (1.1%) and 8 mg of **9** (1%) as oily compounds.

Compound 8: ^{1}H nmr (D_2O, TSP) δ: 1.18 (s, 3, CH_3), 1.21 (s, 3, CH_3), 1.22 (s, 3, CH_3), 1.27 (s, 3, CH_3), 1.57 (s, 3, CH_3 thymine), 2.18 (ddd, 1, H-2″), 2.35 (ddd, 1, H-2′), 3.57 (d, 1, H-6a), 3.70 (dd, 1, H-5″), 3.76 (dd, 1, H-5′), 3.92 (m, 1, H-4′), 4.17 (d, 1, H-6b), 4.41 (m, 1, H-3′), 6.31 (dd, 1, H-1′).

Compound 9: ^{1}H nmr (D_2O, TSP) δ: 1.16 (s, 3, CH_3), 1.22 (s, 3, CH_3), 1.23 (s, 6, 2 × CH_3), 1.56 (s, 3, CH_3 thymine), 2.16 (ddd, 1, H-2″), 2.28 (ddd, 1, H-2′), 3.48 (d, 1, H-6a), 3.73 (dd, 1, H-5′), 3.76 (dd, 1, H-5″), 3.92 (m, 1, H-4′), 4.12 (d, 1, H-6b), 4.41 (m, 1, H-4′), 6.29 (dd, 1, H-1′).

cis-*(5R,6S)-5-hydroxy-6-(2,2,6,6-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (5)*

The mixture (67 mg) of the four thymidine−TAN adducts **2**, **3**, **5**, and **7** was extracted from the second 2′-deoxyribonucleoside-containing band (R_f 0.42), applied to 5 silica gel precoated plates, and eluted with ethyl acetate as the solvent. Extraction of the fastest eluting band (R_f 0.61) provided a mixture (25 mg) of the TAN-addition products **5** and **7**, which were further separated by reversed-phase hplc on the C-18 Nucleosil column as reported above. Evaporation to dryness of the methanolic aqueous solution containing the fastest eluting compound (k' = 1.14) yielded 12 mg of **5** (1.4%) as an oily compound; uv (λ_{max} H_2O): 216 nm; ^{1}H nmr (D_2O, TSP) δ: 1.09 (s, 3, CH_3), 1.24 (s, 3, CH_3), 1.29 (s, 3, CH_3), 1.42 (s, 3, CH_3), 1.54 (s, 3, CH_3 thymine), 2.35 (ddd, 1, H-2″), 3.15 (ddd, 1, H-2′), 3.76 (m, 2, H-5′ and H-5″), 3.93 (m, 1, H-3′), 4.48 (m, 1, H-4′), 5.36 (dd, 1, H-1′); ^{13}C nmr (D_2O, TMS) δ: 24.0 (q, CH_3 thymine), 24.2 (q, 2, × CH_3 piperidone), 32.4 (q, 2 × CH_3 piperidone), 38.6 (t, C-2′), 53.5 (t, 2 × CH_2 piperidone), 63.0 (t, C-5′), 66.0 (s, C-2 and C-6 piperidone), 72.5 (d, C-3′), 73.6 (s, C-5 thymine), 85.5 (d, C-1′), 86.3 (d, C-4′), 89.1 (d, C-6 thymine); cd (*c* 1.2, methanol): $[\theta]_{310}$ ±0, $[\theta]_{280}$ +310, $[\theta]_{260}$ +2480, $[\theta]_{250}$ +6470, $[\theta]_{246}$ +7100, $[\theta]_{240}$ +4920, $[\theta]_{234}$ ±0; FAB-ms *m/e* (relative intensity), positive mode: 452 (10, M^+ + Na), 430 (3, M^+ + H), 265 (7), 172 (46 TAN + 2H), 117 (67, 2-deoxyribose); negative mode: 428 (20, M^- − H), 275 (16), 257 (73, M^- − TAN), 241 (4, thymidine-H), 142 (31, 5-hydroxy-5,6-dihydrothymine), 125 (22, thymine-H), 116 (37, 2-deoxyribose-H).

trans-*(5R,6R)-6-hydroxy-5-(2,2,6,6-tetramethyl-4-oxo-1-piperidinyl-*N*-oxide)-5,6-dihydrothymidine (7)*

The slowest eluting fractions (k' = 3.50) were collected and evaporated to dryness, yielding 11 mg of **7** (1.3%) as an oily compound; uv (λ_{max} H_2O): 215 nm; ^{1}H nmr (D_2O, TSP) δ: 1.08 (s, 6, 2 × CH_3), 1.17 (s, 3, CH_3), 1.29 (s, 3, CH_3), 1.64 (s, 3, CH_3 thymine), 2.27 (ddd, 1, H-2″), 2.36 (ddd, 1, H-2′), 3.74 (dd, 1, H-5″), 3.79 (dd, 1, H-5′), 3.95 (m, 1, H-4′), 4.44 (m, 1, H-3′), 5.32 (d, 1, H-6), 6.25 (dd, 1, H-1′); ^{13}C nmr (D_2O, TMS) δ: 15.0 (q, CH_3 thymine), 23.6 (q, CH_3), 23.7 (q, CH_3), 33.9 (q, CH_3), 34.0 (q, CH_3), 39.1 (t, C-2′), 53.9 (t, CH_2 piperidone), 54.3 (t, CH_2 piperidone), 62.6 (t, C-5′), 67.3 (s, C-2 or C-6 piperidone), 68.0 (s, C-6 or C-2 piperidone), 72.0 (d, C-3′), 80.1 (d, C-6), 80.8 (s, C-5), 86.2 (d, C-1′), 86.7 (d, C-4′); cd (*c* 1.6, methanol): $[\theta]_{320}$ ±0, $[\theta]_{300}$ −200, $[\theta]_{294}$ −220, $[\theta]_{289}$ ±0, $[\theta]_{270}$ +2900, $[\theta]_{253}$ +6230, $[\theta]_{240}$ 4450; FAB-ms *m/e* (relative intensity), positive mode: 452 (15, M^+ + Na), 430 (6, M^+ + H), 172 (48, TAN + 2H), 154 (18), 130 (48), 117 (2-deoxyribose); negative mode: 428 (22, M^- − H), 275 (18), 257 (76, M^- − TAN), 142 (32), 125 (22, thymine − H), 116 (36, 2-deoxyribose − H).

trans-*(5S,6S)-5-hydroxy-6-(2,2,6,6-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (2)*

The silica gel containing the slowest eluting thymidine−TAN adducts **2** and **3** (R_f 0.30) was extracted with 50% aqueous methanol (3 × 6 mL). The evaporation of the resulting solution to dryness gave 26 mg of the two nucleosides **2** and **3** which were applied to the C-18 Nucleosil column and eluted with methanol−water (7:3) as the solvent. The fractions containing the fastest eluting nucleoside (k' = 3.43), as monitored by its uv absorption at 230 nm, were collected. Evaporation to dryness of the aqueous methanol solution yielded 15 mg of **2** (1.8%) as an oily compound; uv (λ_{max} H_2O): 206 nm; ^{1}H nmr (D_2O, TSP) δ: 1.04 (s, 3, CH_3), 1.07 (s, 3, CH_3), 1.23 (s, 3, CH_3), 1.43 (s, 3, CH_3), 1.69 (s, 3, CH_3 thymine), 2.34 (ddd, 1, H-2″), 3.14 (ddd, 1, H-2′), 3.72 (dd, 1, H-5″), 3.80 (dd, 1, H-5′), 3.88 (m, 1, H-4′), 4.47 (m, 1, H-3′), 5.24 (d, 1, H-6), 6.29 (dd, 1, H-1′); ^{13}C nmr (D_2O, TMS) δ: 20.1 (q, CH_3 thymine), 23.6 (q, 2 × CH_3), 32.8 (q, CH_3), 33.0 (q, CH_3), 53.8 (t, CH_2 piperidone), 53.9 (t, CH_2 piperidone), 62.5 (t, C-5′), 64.4 (s, C-2 or C-4 piperidone), 65.8 (s, C-4 or C-2 piperidone), 70.7 (s, C-5 thymine), 71.7 (d, C-3′), 85.5 (d, C-1′), 86.0 (d, C-4), 89.1 (d, C-6 thymine); cd (*c* 1.2, methanol): $[\theta]_{310}$ ±0, $[\theta]_{280}$ +190, $[\theta]_{260}$ +2840, $[\theta]_{253}$ +3800, $[\theta]_{246}$ +2830, $[\theta]_{240}$ ±0, $[\theta]_{230}$ −8400; FAB-ms, *m/e* (relative intensity), positive mode: 452 (35, M^+ + Na), 430 (12, M^+ + H), 354 (6), 265 (20), 172 (22, TAN + 2H), 117 (40, 2-deoxyribose); negative mode: 428 (22, M^- − H), 283 (15), 257 (42, M^- − TAN), 241 (8, thymidine − H), 142 (16, 5-hydroxy-5,6-dihydrothymine − H), 125 (16, thymine − H), 115 (15, 2-deoxyribose − H); FD-ms, *m/e* (relative intensity): 430 (40, M^+ + H), 259 (100, M^+ − TAN), 170 (75, TAN), 117 (15, 2-deoxyribose).

trans-*(5R,6R)-5-hydroxy-6-(2,2,6,6-tetramethyl-4-oxo-1-piperidinoxy)-5,6-dihydrothymidine (3)*

The fractions containing the slowest eluting thymidine−TAN adduct **3** (k' = 4.57) were collected and evaporated to dryness, yielding 14 mg (1.7%) of an oily compound; uv (λ_{max} H_2O): 214 nm; ^{1}H nmr (D_2O, TSP) δ: 1.07 (s, 3, CH_3), 1.17 (s, 3, CH_3), 1.26 (s, 3, CH_3), 1.44 (s, 3, CH_3), 1.66 (s, 3, CH_3 thymine), 2.42 (ddd, 1, H-2″), 2.96 (ddd, 1, H-2′), 3.73 (dd, 1, H-5″), 3.79 (dd, 1, H-5′), 3.99 (m, 1, H-4′), 4.45 (m, 1, H-3′), 5.34 (s, 1, H-6), 5.75 (dd, 1, H-1′); ^{13}C nmr (D_2O, TMS) δ: 19.8 (q, CH_3 thymine), 23.7 (q, CH_3), 24.3 (q, CH_3), 32.3 (q, CH_3), 33.8 (q, CH_3), 53.6 (t, CH_2 piperidone), 53.7 (t, CH_2 piperidone), 63.0 (t, C-5′), 66.1 (s, C-2 and C-6 piperidone), 70.7 (s, C-5 thymine), 72.3 (d, C-3′), 87.0 (d, C-4′), 92.8 (d, C-1′), 93.7 (d, C-6 thymine); cd (*c* 1.8, methanol): $[\theta]_{290}$ ±0, $[\theta]_{270}$ −300, $[\theta]_{260}$ −1480, $[\theta]_{255}$ −1780, $[\theta]_{249}$ −1290, $[\theta]_{244}$ ±0, $[\theta]_{230}$ +8200; FAB-ms, *m/e* (relative intensity), positive mode: 452 (28, M^+ + Na), 430 (11, M^+ + H), 265 (18), 172 (51, TAN + 2H), 117 (82, 2-deoxyribose); negative mode: 428 (29, M^- − H), 257 (58, M^- − TAN), 241 (8, thymidine − H), 142 (26, 5-hydroxy-5,6-dihydrothymine − H), 125 (37, thymine − H), 115 (25, 2-deoxyribose − H); DCI-ms (NH_3) *m/e* (relative intensity), positive mode: 430 (40, M^+ + H), 276 (10, M^+ − TAN + NH_3), 243 (11, thymidine + H), 172 (42, TAN + 2H).

trans-*(5S,6S)-6-hydroxy-5-(2,2,6,6-tetramethyl-4-oxo-1-piperidinyl-*N*-oxide)-5,6-dihydrothymidine (6)*

The main uv absorption band (R_f 0.22), which contained the starting thymidine (**1**) and the thymidine−TAN addition product **6**, was scraped off and the two nucleosides were extracted with 50% aqueous methanol (3 × 6 mL). The resulting solution was evaporated

to dryness and applied to the C-18 Nucleosil reversed-phase column. The eluent was a mixture of water and methanol (7:3) and the detection of the nucleosides was monitored at 230 nm. The latest eluting nucleoside consisted of thymidine ($k' = 1.05$). The fractions containing the thymidine−TAN adduct ($k' = 4.58$) were collected and evaporated to dryness, giving 12 mg of a colorless oily compound (1.4%); uv (λ_{max} H_2O): 214; ^{1}H nmr (D_2O, TSP) δ: 1.10 (s, 6, 2 × CH_3), 1.21 (s, 3, CH_3), 1.31 (s, 3, CH_3), 1.65 (s, 3, CH_3 thymine), 2.20 (ddd, 1, H-2″), 2.44 (ddd, 1, H-2′), 3.74 (dd, 1, H-5″), 3.79 (dd, 1, H-5′), 3.96 (m, 1, H-4′), 4.46 (m, 1, H-3′), 5.39 (s, 1, H-6), 6.26 (dd, 1, H-1′); ^{13}C nmr (D_2O, TMS) δ: 14.6 (q, CH_3 thymine), 23.6 (q, 2 × CH_3), 33.8 (q, 2 × CH_3), 37.8 (t, C-2′), 53.8 (t, CH_2 piperidone), 54.3 (CH_2 piperidone), 63.1 (t, C-5′), 63.8 (s, C-2 or C-4 piperidone), 64.2 (C-4 or C-2 piperidone), 72.5 (d, C-3′), 79.5 (d, C-6 thymine), 80.9 (s, C-5 thymine), 85.1 (d, C-4′), 86.6 (d, C-1′); cd (*c* 1.3, methanol): $[\theta]_{319}$ ±0, $[\theta]_{309}$ +80, $[\theta]_{283}$ +600, $[\theta]_{276}$ ±0, $[\theta]_{269}$ −1500, $[\theta]_{240}$ −3870, $[\theta]_{229}$ −3760; FAB-ms *m/e* (relative intensity), positive mode: 452 (18, $M^+ + Na$), 430 (10, $M^+ + H$), 172 (40, TAN + 2H); negative mode: 428 (20, $M^- - H$), 257 (70, $M^- -$ TAN), 116 (40, 2-deoxyribose − H).

Decomposition studies of the adducts 2−7 in aqueous solutions

The TAN−thymidine adduct **6** (45 mg) was dissolved in 100 mL of water (pH 6.5) and the resulting aerated solution was stirred for 48 h at 30°C. Two-dimensional thin-layer chromatographic analysis of an aliquot of the solution shows, besides the starting compound, the presence of a far-uv absorbing nucleoside. Preparative separation of this nucleoside was made on two thick silica gel tlc plates by using solvent system II. Extraction of the silica gel (R_f 0.35) with methanol (3 × 6 mL) provided 11.5 mg of a nucleoside which was characterized as 5-hydroxymethyl-2′-deoxyuridine (**14**) by comparison of its uv, ^{1}H nmr, and FAB mass spectrometric features with those of the authentic sample (28).

Under the same experimental conditions, the partial decomposition of **7** (~60%) generated 5-hydroxymethyl-2-deoxyuridine (**14**) in 45% yield as the major product.

It has to be pointed out that the other TAN−thymidine adducts **2−5** did not undergo any detectable degradation in the above conditions, even at a higher temperature (60°C).

Conversion of the adducts 6 and 7 to 5-hydroperoxymethyl-2′-deoxyuridine (15)

The solution of the adduct **6** (35 mg) in 80 mL of 30% aqueous hydrogen peroxide was stirred for 15 h at 30°C. The solution was evaporated to dryness under reduced pressure (10^{-3} Torr; 1 Torr = 133.3 Pa) to remove H_2O_2. The resulting residue was deposited on three thin-layer precoated silica gel plates which were further eluted with the solvent system II. The silica gel containing the main uv absorbing band (R_f 0.50), which gave a positive test for a peroxidic compound by spraying the chromatogram with a methanolic solution of KI, was scraped off and extracted with 3 × 6 mL of methanol. Evaporation of the methanolic solution yielded 8.8 mg of 5-hydroperoxymethyl-2′-deoxyuridine (39%), which showed uv and ^{1}H nmr spectroscopic properties identical to those of the authentic sample (30). Work-up in a similar way of the minor uv absorbing band gave 1.2 mg of 5-hydroxymethyl-2′-deoxyuridine (**14**) (5%).

Treatment of the adduct **7** under the above conditions yielded 5-hydroperoxy-2′-deoxyuridine (**15**) and 5-hydroxymethyl-2′-deoxyuridine (**14**) in 35% and 6% yields, respectively.

Stability studies of the thymidine adducts 2−7 in pyridine−water solutions

The TAN addition product to thymidine, **6** (85 mg), was dissolved in 100 mL of 50% aqueous pyridine. The resulting solution was stirred, open to the air, for 3 h at 60°C. The solution was evaporated to dryness and the resulting residue was applied on two precoated silica gel plates. Solvent system II was used as the developer. Six main bands, which gave a pink coloration after spraying the chromatoplates with the cysteine − sulfuric acid reagent, were extracted with 3 × 5 mL of methanol. Evaporation to dryness of the resulting solutions yielded six homogeneous nucleosides which were characterized by comparison of their uv, ^{1}H nmr, cd, and mass spectrometric features with those of the corresponding authentic samples: *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl) formamide (**18**) (R_f 0.22; 22.2 mg, 40%); *(+)-cis*-(5*S*,6*R*)-5,6-dihydroxy-5,6-dihydrothymidine (R_f 0.30; 9.8 mg, 10%); (−)-*cis*-(5*R*,6*S*)-5,6-dihydroxy-5,6-dihydrothymidine (R_f 0.33; 9.4 mg, 10%); thymidine (**1**) (R_f 0.52; 7.9 mg, 9%); (+)-(5*R**)-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (**16**) (R_f 0.57; 7.9 mg, 9%); (−)-(5*S**)-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (**17**) (R_f 0.61; 8.5 mg, 10%).

Under similar experimental conditions, the degradation of the adduct **7** gave rise to the following compounds, which were separated by thin-layer chromatography on precoated silica gel plates as reported above: *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl) formamide (**18**) (R_f 0.22; 29.3 mg, 41%); (+)-*cis*-(5*S*,6*R*)-5,6-dihydroxy-5,6-dihydrothymidine (R_f 0.30; 8.4 mg, 9%); (−)-*cis*-(5*R*,6*S*)-5,6-dihydroxy-5,6-dihydrothymidine (R_f 0.33; 8.6 mg, 9%); (+)-(5*R**)-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (**16**) (R_f 0.57; 7.7 mg, 9%); (−)-(5*S**)-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (**17**) (R_f 0.61; 8.4 mg, 10%).

The other adducts **2−5** are stable in pyridine−water at 60°C. In particular, no detectable decomposition of these adducts was observed after storage under the above conditions over a period of 24 h.

γ-Radiolysis of deaerated aqueous solutions of 5-bromo-6-hydroxy-5,6-dihydrothymidine (12) or (13) in the presence of TAN

A 0.1 *M tert*-butanol solution of 200 mg of (+)-*trans*-5-bromo-6-hydroxy-5,6-dihydrothymidine (**13**) and 200 mg of TAN in 250 mL of bi-distilled water (pH 6.5) was deaerated by bubbling with a stream of nitrogen for 15 min and subsequently exposed to γ-rays from the ^{60}Co source of 2.5 h (absorbed does 16.5 kGy). The solution was evaporated to dryness and the resulting residue was applied on a preparative high performance silica gel column SI 500 (Waters, Milford). Elution was carried out with solvent system II at a flow-rate of 50 mL per minute. The separation of the major adduct **7** from the starting bromohydrin and TAN was achieved after one recycle. Evaporation of the corresponding fraction ($k' = 2.24$) as detected by refractive index yielded 154 mg of **7** (61%) which was shown to be homogeneous by analytical reversed-phase high performance liquid chromatography and two-dimensional silica gel thin-layer chromatography (eluents I and II). Its ^{1}H nmr and FAB mass spectrometric data were identical to those obtained for the adduct **7** (*vide supra*).

γ-Irradiation of the aqueous solution of the (−)-*trans* diastereoisomers of 5-bromo-6-hydroxy-5,6-dihydrothymidine (**12**) in the presence of 4.7 m*M* TAN and 0.1 *M tert*-butanol gave rise to the TAN−thymidine adduct **6** in a 64% yield.

γ-Irradiation of an aqueous solution of 5-hydroxy-5,6-dihydrothymidine containing TAN

Two hundred milliliters of a degassed aqueous solution of 52 mg of (5*S*)-5-hydroxy-5,6-dihydrothymidine (**10**) and of 70 mg of TAN was irradiated with the γ-rays of ^{60}Co for 1 h (absorbed dose 6600 kGy). The solution was evaporated to dryness and the resulting syrup was deposited on a preparative silica gel thin-layer plate. The developing solvent was a mixture of chloroform and methanol (9:1). The broad band (R_f 0.42) which gave a positive coloration with both the 2,4-dinitrophenylhydrazine and cysteine − sulfuric acid sprays, which are characteristic of carbonyl containing compounds and 2′-deoxyribosides respectively, was extracted with 3 × 4 mL of methanol. Evaporation to dryness of the resulting solution yielded 11 mg of a yellow syrup. This residue was dissolved in 1 mL of 70% aqueous methanol and 0.2 mL of this solution was applied per analysis on the ODS-3 reversed-phase hplc column. Elution was carried out with a mixture of water and methanol (7:3) as the solvent. Four main fractions were detected by their uv absorption at 220 nm and collected. Evaporation to dryness yielded the four homogeneous TAN−thymidine adducts, which were characterized by comparison of their ^{1}H nmr and FAB mass spectroscopic features with those obtained for the authentic samples (*vide supra*): fraction I ($k' = 1.50$), 3.2 mg of

4; fraction 2 (k' = 3.42), 3.5 mg of **2**; fraction 3 (k' = 4.34), 1.2 mg of **8**; and fraction 4 (k' = 4.84), 1.4 mg of **9**.

Under similar experimental conditions, the γ-irradiation of 52 mg of the 5*R* diastereoisomer of 5-hydroxy-5,6-dihydrothymidine (**11**) in the presence of TAN generated four thymidine–TAN adducts. Initial thin-layer analysis, followed by reversed-phase high performance liquid chromatography, provided four main fractions: fraction 1 (k' = 1.15), 3.4 mg of **5**; fraction 2 (k' = 4.57), 3.7 mg of **3**; fraction 3 (k' = 4.35), 1.5 mg of **8**; and fraction 4 (k' = 4.85), 1.4 mg of **9**.

Hydrogenolysis of thymidine–TAN adducts

The nucleoside (10 mg) to be hydrogenolysed was dissolved in 10 mL of methanol; 300 mg of previously hydrogenated Pd/C (Merck, Darmstad, GFR) were added to this solution. Hydrogenolyses were carried out under 50 bars (1 bar = 100 kPa) for 5 h, while stirring the solution. The methanolic solution was then filtered through a Celite pad and evaporated to dryness. The oily residue was analyzed by tlc on silica gel and (or) hplc on the C-18 reversed phase column.

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