detector, DuPont Model 837 spectrophotometer; pump, Altex Model 110A; λ = 222 nm for **3a–d** and 262 nm for **3e**; P = 2500–3500 psi at FR = ca. 2 mL/min. Values of k' under these HPLC conditions were 1.5 for **3a**, 2.2 for **3b**, 0.93 for **3c**, 0.84 for **3d**, 2.5 for **3e**, and 2.8 for **1**.

Ex Vivo Competitive Binding Studies. Male mice, weighing 25–30 g, used in the present study had been kept under constant temperature and diurnal lighting prior to their experimental use, and all were killed nearly at the same time of day to avoid any possible effects of circadian rhythm. Stock solutions of **3a–f** were prepared in 0.9% NaCl at a concentration range of 6×10^{-4} to 10^{-3} M, and that of **1** was made in 2% ethanol at 3×10^{-4} M. All drugs were administered through an iv route using a tail vein. Control mice received equal volume of the vehicle alone.

In the dose–response studies, mice were sacrificed by decapitation 30 min after varying doses of **2** and **3d–f** were administered. In the time–course studies, mice were killed 1, 3, 10, 30, and 60 min after 3 μmol/kg of **1**, 10 μmol/kg of **3a–c**, and 6 μmol/kg of **3e** were administered.

After decapitation, mice brain was quickly removed from the skull. The whole brain minus cerebellum was homogenized for 30 s in 50 vol (ca. 10–15 mL) of 0.05 M Tris buffer at pH 7.4 and 4 °C, using a Brinkman Polytron homogenizer (PCU-2-110) at no. 6 setting. Tritium-labeled flurnitrazepam ([^{3}H]FNZ; New England Nuclear; specific activity 84.8 Ci/mmol) binding was measured by incubating a 1.0-mL aliquot of the homogenate, 0.10 mL of [^{3}H]FNZ, which resulted in a final concentration of about 0.77 nM, 0.1 mL of H_2O or a flurazepam (Hoffman LaRoche) solution, and 0.8 mL of 0.05 M Tris buffer at pH 7.4. This 2.0-mL mixture was incubated for 30 min at 25 °C and then filtered through a Whatman GF/B filter disk under vacuum. The incubation tube was rinsed with 5 mL of ice-cold Tris buffer and filtered through again. The filter disk was finally washed 3 times with 5-mL aliquots of the same buffer. The filter disk was then placed in a scintillation vial containing 15 mL of Amersham-Searle ACS cocktail, and the radioactivity was measured by a liquid scintillation counter (Beckman Model LS3133T).

Specific binding is defined as the total binding minus binding in the presence of 10 μM flurazepam. Specific binding represents over 90% of the total binding.

Registry No. **1**, 28981-97-7; **2**, 85966-74-1; **3a**, 102537-66-6; **3b**, 102537-67-7; **3c**, 102537-68-8; **3d**, 64194-69-0; **3d**·TFA, 102537-73-5; BOC-**3d**, 102537-71-3; **3e**, 102537-69-9; **3e**·TFA, 102537-74-6; BOC-**3e**, 102573-67-1; **3f**, 102537-70-2; **3f**·TFA, 102537-75-7; BOC-**3f**, 102537-72-4; $BOCNHCH_2CO_2H$, 4530-20-5; (L)-$BOCNHCH(i\text{-}Bu)CO_2H$, 13139-15-6; $BOCNH(CH_2)_3CO_2H$, 57294-38-9; succinic anhydride, 108-30-5.

Ribose as the Preferential Target for the Oxidized Form of Elliptinium Acetate in Ribonucleos(t)ides. Biological Activities of the Resulting Adducts

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The covalent binding of the oxidized form of elliptinium acetate, an antitumor drug, to various ribonucleos(t)ides is described. In the absence of a strong nucleophile on the bases, e.g., a sulfhydryl group, the main target of this quinone imine derivative is the sugar moiety. With unmodified regular bases, the first electrophilic addition always occurs on the 2′-oxygen of ribose (more slowly for pyrimidine than for purine); in a second step, cyclization of the reoxidized product leads to a spiro derivative: only one stereoisomer is detected with purine nucleoside; the other stereoisomer appears as a minor product (10–20%) with nucleotides and pyrimidine nucleosides. With modified bases, no change is observed except for bases exhibiting an additional strong nucleophilic center: oxidized elliptinium alkylates thioguanine and thioguanosine on the sulfur atom and in this last case not on the ribose moiety. All spiro derivatives are less cytotoxic than the parent compound even if the base is an antimetabolite (azauridine); however, thio–elliptinium adducts maintain high cytotoxicity.

Elliptinium acetate (**1**; 9-OH-NME or N^2-methyl-9-hydroxyellipticinium acetate) is an antitumor agent currently used for therapeutic treatment of osteolytic metastases of breast cancer.[1,2] In addition to its high affinity for DNA,[3] this molecule can easily be oxidized to a quinone imine derivative **2**.[4,5] The strong electrophilic properties of this oxidized form of 9-OH-NME were shown (i) in vitro with various nitrogen[6,7] (amino acids), sulfur (cysteine, glutathione), and oxygen[5,6] (H_2O_2, CH_3OH) nucleophiles and (ii) in vivo in humans and rats with the recovery of cysteinyl- and glutathionyl–elliptinium adducts as metabolites.[8–10] Previously, it was shown that elliptinium acetate binds to guanosine and adenosine in an unexpected manner, on the sugar moiety of nucleosides.[11,12] This high regioselectivity for the addition of **2** to nucleosides was interpreted in terms of a preliminary stacking between the pyridocarbazole **2** and nucleoside bases.

In the present paper, we report the synthesis and biological properties of various elliptinium adducts with purine and pyrimidine nucleosides and nucleotides and also with other nucleosides bearing modified bases. We intended to modulate the stacking effects between **2** and

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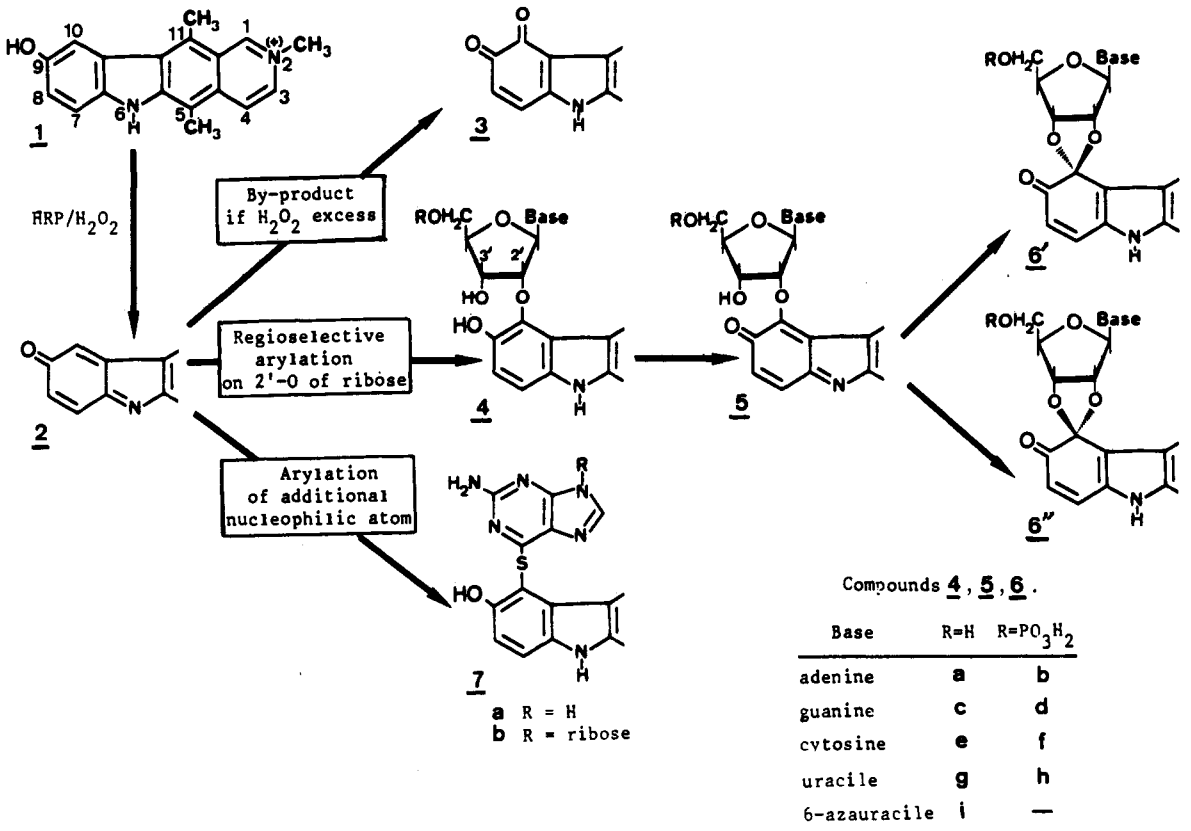
Scheme I. Mechanism for the Formation of the Elliptinium Adducts



Compounds **4**, **5**, **6**.

Base	R=H	R=PO_3H_2
adenine	a	b
guanine	c	d
cytosine	e	f
uracile	g	h
6-azauracile	i	—

Table I. Product Analysis by HPLC during the Reaction between Nucleophile and the Oxidized Form of 2

	adducts					adducts				
starting nucleophile	base	no.	t_R	ratio[a]	yield[b]	base	no.	t_R	ratio[a]	yield[b]
ribonucleoside	adenine	**6a**	1.30	98/2	50	guanine	**6c**	0.85	98/2	80
5'-P-ribonucleotide		**6b**	0.90	92/8	60		**6d**	0.65	98/2	50
ribonucleoside	cytosine	**6e**	0.60	43/57	63	uracil	**6g**	0.64	66/44	60
5'-P-ribonucleotide		**6f**	0.45	24/76	23		**6h**	0.48	34/66	49
free base	azauracil					thioguanine	**7a**	0.85		60
ribonucleoside		**6i**	1.00	64/46	50		**7b**	0.71		12

[a] Ratio of area peak of adduct to that of the *o*-quinone **3** in conditions of test experiments. [b] Isolated yield (%) calculated with respect to initial 9-OH-NME in conditions of preparative experiments.

these various nucleotides in order to obtain different adducts, in particular those resulting from alkylation[13] of the base (classical alkylation sites for electrophilic compounds are bases, phosphates, and, to a minor extent, ribose; for a recent review, see ref 14). Such perturbations in stacking might be obtained by (i) the use of pyrimidines (or modified pyrimidines) instead of purines, (ii) introduction of an anionic phosphate in the 5'-position of ribose (bearing in mind that 9-OH-NME bears a cationic center on nitrogen 2).

Moreover, from in vitro preliminary studies it was demonstrated that RNA can be alkylated by this drug.[15]

For a better knowledge of the binding sites on nucleic acids at the molecular level, it was useful to isolate elliptinium–nucleotide adducts after enzymatic or chemical hydrolysis. Up to now, all attempts to isolate such adducts failed, probably because of our lack of knowledge of the stability and physicochemical properties of the corresponding adducts. We also undertook this work in order to obtain reference compounds in alkylated nucleic acid hydrolysis. Finally the modified nucleos(t)ides synthesized might be endowed with cytotoxic activities.

Results

Oxidation of 9-OH-NME (**1**) by the horseradish peroxidase/hydrogen peroxide (HRP/H_2O_2) system leads to the quinone imine 9-oxo-NME (**2**), which is an highly electrophilic alkylating intermediate:[13] it can react with an excess of H_2O_2 (formation of 9,10-dioxo-NME (**3**) as side product of peroxidase oxidation[5]) or alkylate nucleos(t)ides on the sugar moiety giving the adducts of type 4–6 or on the base moiety (adducts of type 7) when it carries an additional strong nucleophilic center (SH group) (Scheme I).

1. **Comparative Reactivities of Various Nucleos(t)ides with the Quinone Imine Derivative 2 (See Table I).** In all experiments described in Table I the

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(13) The hybridization of carbon 10 of elliptinium adducts can be sp^2 or sp^3 depending on the adduct structure (see Scheme I). In the present paper, we use "alkylation" in both cases, following the conventional meaning adopted in pharmacology to describe the covalent binding of various electrophiles to biological nucleophiles without distinction of the nature of the atom attached to the macromolecules.

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electrophilic quinone imine **2** is generated in situ in the presence of the studied nucleos(t)ides, and the reactions are monitored by high-pressure liquid chromatography (HPLC). Due to the possible formation of the *o*-quinone **3** as the ultimate oxidized product of **1** by HRP/H_2O_2, this compound is always detected in the case of poor nucleophiles; it is, in fact, the only detected reaction product in the case of free bases, 2′-deoxyribo- or arabinonucleosides and 5′-phosphate 2′-deoxyribonucleotides. Thus, adducts are only observed when their formation can kinetically compete with the side oxidation of **2** to **3**. The ratio of peak areas of adduct to *o*-quinone **3** is a good indication of the relative nucleophilicity for all the ribonucleos(t)ides used. From these ratios, given in Table I, it appears that purine derivatives are more reactive than pyrimidine derivatives, since the *o*-quinone **3** is a minor product for adenosine and guanosine while it is the major reaction product in the case of cytidine. Furthermore, the lower nucleophilicity of pyrimidine derivatives compared to that of purine is enhanced when these compounds have a phosphate group attached at the 5′-position.

Due to the oxidizability of thioguanine and thioguanosine, their nucleophilic reactivities may not be compared with the other nucleos(t)ides (in the test experiments HRP/H_2O_2 oxidized RSH to RSSR compounds). Thus a different experimental procedure was used for these thio compounds (see below).

2. Structure of Ribonucleos(t)ide–Elliptinium Adducts. Compounds 6: Adducts on the Ribose Moiety. From a preliminary study on adenosine– and guanosine–elliptinium adducts, we known that the final product resulting from the electrophilic addition of the quinone imine **2** to a ribonucleoside is the spiro derivative **6**, where carbon 10 of the elliptinium skeleton is attached to both the 2′- and 3′-oxygen atoms of the sugar moiety[11,12] (for the detailed mechanism of the formation of **6a** via the intermediate compounds **4a** and **5a**, see ref 16).

In the present case ribonucleos(t)ide–elliptinium adducts exhibit similar ^1H NMR spectra (Table II): (i) on the ellipticine moiety, the absence of a resonance corresponding to H_{10} and two widely separated H_7 and H_8 resonances are interpreted as C_{10} being the fixation site and a conjugated ketone structure from C_7 to O_9 (ref 11, 12), (ii) on the nucleos(t)ide part, the significant modifications on ribose proton resonances (low values for the coupling constants $J_{H'1-H'2}$ and $J_{H'3-H'4}$) indicate that, as for **6a** and **6b**, the ribose stays in the energetically unfavorable O′-exo conformation.[12] For **6a**, **6c**, **6e**, **6g**, and **6i**, the presence of elliptinium–ribose and elliptinium–oxygen MS(DCI) fragments at 407 and 293, respectively, strongly support the fixation of elliptinium on the ribose moiety. Moreover, peaks at M + 2 are observed on FAB spectra for adducts **6a** and **6c**. In the case of **6i**, the introduction of an additional nitrogen atom in the pyrimidine ring seems to modify neither the adduct formation nor its structure.

A nuclear Overhauser effect (NOE) (400 MHz) between Me_{11} and H'_1 and H'_4 established the absolute configuration at carbon 10 for **6b**, **6c**, and **6d**. It corresponds to the one previously established for the adenosine–elliptinium adduct **6a** (absolute configuration *S*, ref 12) and is represented by **6′** in Scheme I. Isomer **6′** is the only spiro derivative detected from the reaction mixture in the case of nucleosides, except for the uridine test. In this last case and with 5′-phosphate ribonucleotides, HPLC profiles

Table II. NMR Data (δ) for Nucleos(t)ides–Elliptinium Adducts (See Formula in Scheme I)[a]

				ellipticine							ribose					base	
adduct	base	R	no.	H_1	H_3[d]	H_4[d]	H_7	H_8	2-CH_3	11-CH_3	5-CH_3	H'_1	H'_2	H'_3	H'_4	$H'_5H'_5$	H8/H6	H2/H5
		H	**6a**	9.83	8.23	8.37	7.67	6.25	4.54	3.38	2.85	6.66	6.12	5.76	4.88	3.92	8.64	8.44
	adenine	PO_3H_2	**6′b**	9.87	8.43	8.27	7.73	6.31	4.58	3.42	2.92	6.71	6.24	5.85	5.07	4.28	8.70	8.48
		PO_3H_2	**6″b**	9.82	8.39	8.21	7.75	6.39	4.51	3.08	2.89	6.92		5.78	4.96	4.39	8.75	8.45
		H	**6c**	9.75	8.17	8.34	7.62	6.20	4.48	3.29	2.82	6.35	6.01	5.59	4.70	3.90	8.15	
	guanine	PO_3H_2	**6′d**	9.86	8.48	8.29	7.79	6.39	4.56	3.41	2.94	6.58	6.16	5.80	5.15	4.35	9.05	
		PO_3H_2	**6″d**	9.83	8.46	8.25	7.82	6.47	4.52	3.11	2.92	6.73			5.02		9.07	
6[b]		H	**6e**	9.67	8.15	8.05	7.61	6.05	4.51	3.21	2.70	6.18	5.75	5.04	4.74	3.89	8.22	6.21	
	cytosine	PO_3H_2	**6′f**	9.85	8.42	8.42	8.42	7.70	6.28	4.55	3.33	2.90	6.20	5.73	5.57	5.05	4.33	8.23	6.38
		PO_3H_2	**6″f**	9.85	8.42	8.42	8.42	7.70	6.28	4.50	3.05	2.87	6.50		4.95	4.28	8.23	6.38	
		H	**6′g**	9.91	8.42	8.22	7.42	5.75	4.51	3.40	2.88	6.16	5.76	5.46	4.71		7.60	5.75	
	uracil	H	**6″g**	9.87			7.88		4.50						4.61		7.64		
		PO_3H_2	**6′h**	9.89	8.41	8.22	7.95	5.94	4.55	3.34	2.89	6.20	5.81	5.53	4.97	4.29	7.68	6.30	
		PO_3H_2	**6″h**	9.85			8.01	5.97	4.52	3.09					4.84		7.74	6.38	
	azauracil	H	**6′i**	9.79	8.33	8.20	7.65	6.23	4.51	3.30	2.81	6.60	5.85	5.43	4.70	3.84		7.66	
		H	**6″i**	9.66					4.46	3.11	2.75								
7	thioguanine	H	**7a**	9.75	8.20	8.34	8.04	7.70	4.40	3.37	2.86						8.04		
		ribose	**7b**[c]	9.70	8.25	8.38			4.48	3.42	2.89	6.03	4.92	4.52	4.34	3.98–3.87	8.36		

[a] Solvents used were as follows: CD_3COOD for **6a**, **6c**, **6e**, **6g**, and **6i**; $CD_3COOD + D_2O$ (50/50) for **6b**, **6d**, **6f**, **6h**, and **7b**; CD_3OD for **7a**. Me_4Si was the internal reference. [b] $J_{H'1-H'2}$ and $J_{H'3-H'4} < 1$ Hz. [c] $J_{H'1-H'2} = 6.5$ Hz. [d] In some cases the attenuation for H_3 and H_4 can be reversed. H_3 should be read as H_3 (or H_4) and H_4 read as H_4 (or H_3).

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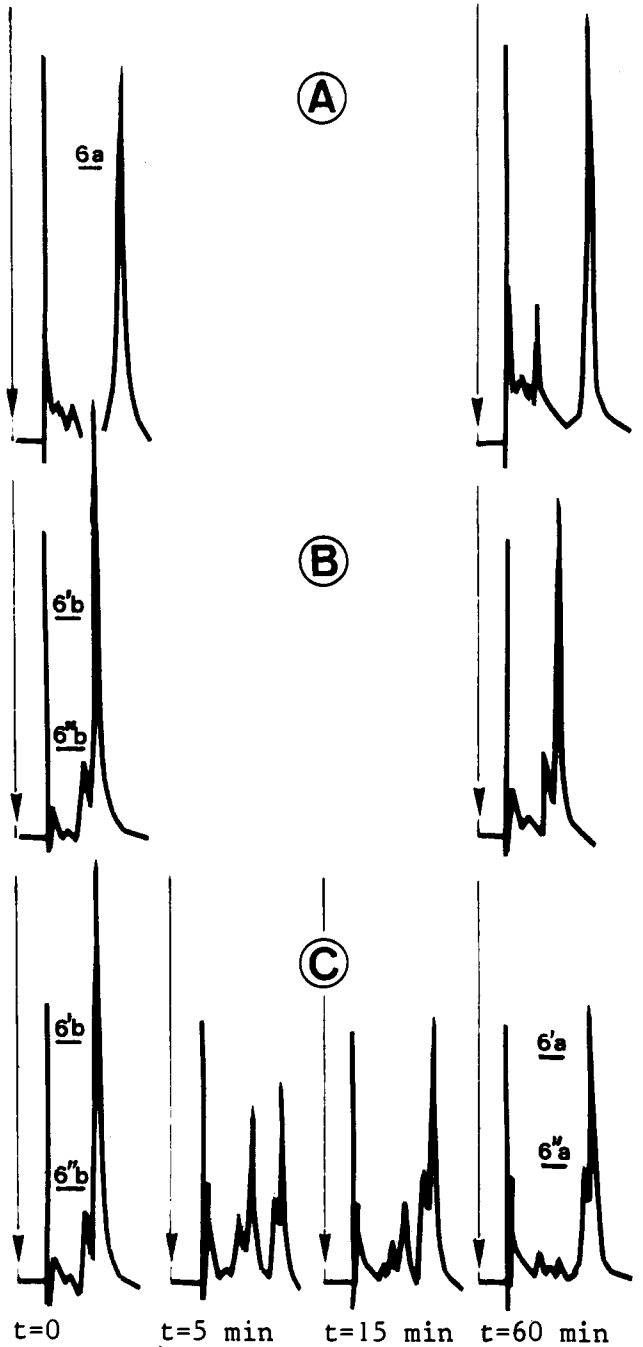

Figure 1. Hydrolysis of the AMP adduct **6b** by *E. coli* alkaline phosphatase (A and B, stability of references **6a** and **6b** respectively; C, hydrolysis experiment).

revealed another peak; this minor adduct (10–20%) could not be separated but is identified from the ^1H NMR spectrum of the crude product as the stereochemical isomer 6″ (absolute configuration *R*). The NMR shifts of 6″ are nearly the same as those of 6′, apart from the high field shift of Me_{11} compared to the value observed in 6′ (see Table II). The absolute configuration at carbon 10 of the minor isomer has been established by NOE in the case of AMP–elliptinium adduct **6″b** (see Figure 2).

Besides these identifications by spectroscopic methods, the structural analogies between nucleotide– and nucleoside–elliptinium adducts were completely confirmed by enzymatic hydrolysis. For example, treating AMP– and GMP–elliptinium adducts **6b** and **6d** with alkaline phosphatase leads to the corresponding nucleoside–elliptinium adducts **6a** and **6c**, respectively (see Figure 1 for hydrolysis experiment on AMP–elliptinium adduct **6b**). The minor isomers **6″b** and 6″d are hydrolyzed, as the corresponding

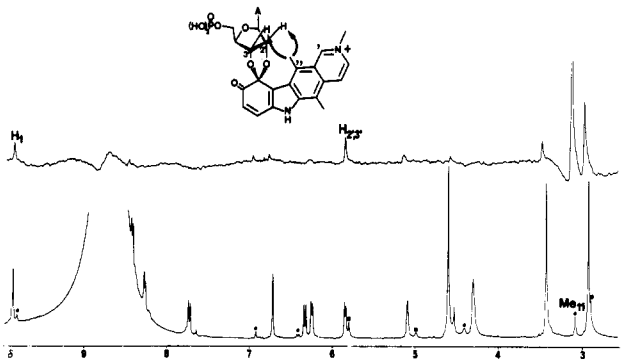

Figure 2. NOE difference spectrum (solvent, $CD_3COOD + D_2O$) obtained upon irradiation of methyl at position 11 of isomer **6″b** along with the reference spectrum of the mixture of both isomers **6′b** and **6″b**; the dots indicate the signals of the minor isomer.

Table III. Cytotoxicity of the Nucleos(t)ides–Elliptinium Adducts and Reference Compounds (L1210 Cells in Vitro)

adduct 6,[a] base =	ID_{50}, μM R = H	ID_{50}, μM R = PO_3H_2
adenine	**a** 0.8	**b** >0.8
guanine	**c** 0.8	**d** >1.0
cytosine	**e** 3.0	**f** 12.8
uracil	**g** >3.0	**h** 1.1
6-azauracil	**i** >3.0	

adduct 7, base =	ID_{50}, μM R = H	ID_{50}, μM R = ribose
6-thioguanine	**a** 0.2	**b** 0.12

ref compd	ID_{50}, μM
9-OH-NME	0.12
6-thioguanine	0.07
6-thioguanosine	0.10
6-azauridine	0.57

[a] See formula in Scheme I.

major adducts **6′b** or **6′d**, by the alkaline phosphatase enzyme, leading to elliptinium adducts that might be the stereoisomers **6″a** and **6″c**, respectively; one can note that these adducts have not been seen before.

Compounds 7: Adducts on the Base Moiety. Adduct structure **6** may be observed with a modified nucleoside such as azauridine (see above), but when an highly nucleophilic SH group is present on the base (i.e., in the case of 6-thioguanine and 6-thioguanosine), the formation of a covalent bond C_{10}–S is always observed (leading to adducts **7a** and **7b**, respectively).

The key aspects of the NMR spectra of the ellipticine skeleton in these adducts **7a** and **7b** are similar to those observed for glutathionyl– or cysteinyl–elliptinium adducts (see ref 8 and 6, respectively): disappearance of the resonance of the H_{10} proton and the short separation between the signals H_7 and H_8 ($\Delta\delta$ = 0.2–0.3 ppm) favors a full aromatic structure for ring A of ellipticine (compared to a quinone form, where the $\Delta\delta$ value is increased up to 1.2 ppm). In the case of the thioguanosine adduct **7b**, the ribose ^1H NMR resonances are not disturbed in contrast with the spiro adducts **6**. Furthermore, mass spectra of adducts **7a** and **7b** show the same fragment at 309 attributed to an elliptinium–sulfur entity.

3. Cytotoxicity Studies on Nucleos(t)ides–Elliptinium Adducts. Murine leukemia L1210 cells cultured in vitro were used for cytotoxicity studies. The ID_{50} values are reported in Table III.

For all the spiro derivatives **6**, ID_{50} values are greater than 0.8 μM, i.e., above the value measured for the parent drug **1** (0.12 μM). But in the case of thioguanine– and thioguanosine–elliptinium adducts, the ID_{50} values are

closer to that of 1, 0.20 and 0.12 μM for **7a** and **7b**, respectively.

Discussion

The antitumor agent 9-OH-NME (1) is easily oxidized to a strong electrophilic quinone imine derivative **2**, which is able to alkylate a large variety of nucleosides and nucleotides in the ribose series. The present results indicate that the substrate reactivity can be slightly lowered by changing the purine base (two rings) to a pyrimidine one (one ring) or by introducing a phosphate residue at 5′-OH (negative charge). Except for thio derivatives, the alkylation always occurs regioselectively on the ribose moiety of nucleos(t)ides (Scheme I, for adduct structures). Thus we have to admit that this unexpected regioselectivity, which is mainly controlled by stacking interactions between 9-oxo-NME and nucleos(t)ides, is nearly unchanged for pyrimidine nucleosides and 5′-monophosphate nucleotides.

However, it must be noted that there are two possible routes for the cyclization of type **5** adducts leading to the two spiro derivatives isomers **6′** and **6″**; the reduction of the base size and the introduction of a 5′-phosphate group can slightly modify the required stacking interaction and consequently increase the proportion of the minor isomer **6″**.

For the other elliptinium adducts **7**, the thiol derivative is always attached at C_{10} by the sulfur atom, even when this reaction may compete with an alternative binding site on ribose (the case of **7b**).

All adducts have been tested for cytotoxicity against murine leukemia L1210 cells in vitro. For adducts **6** the ID_{50} values are greater than or equal to 0.8 μM, i.e., always higher than the value of 0.12 μM measured for the parent drug 1 and consequently less cytotoxic. In contrast, for thioguanine- and thioguanosine–elliptinium adducts, the ID_{50} values are closer to that of 1: 0.2 and 0.12 μM for **7a** and **7b**, respectively. These values are respectively slightly higher than those for thioguanine (0.07 μM) or thioguanosine itself (0.10 μM).

The main conclusion of the present study concerns the surprising reactivity of 9-oxo-NME vs. ribonucleos(t)ides leading to regioselective adducts on ribose. The biological consequence of the alkylation properties of **2** is that both ribonucleic acids and deoxyribonucleic acids have to be considered as possible targets for the antitumor agent 1. We can indeed imagine that elliptinium alkylation of 3′-OH terminal ribose of mRNA or tRNA might, for example, affect protein synthesis. However, our preliminary results for RNA alkylation by **2** in vitro suggest that the terminal ribose is not the only covalent binding site,[15] thus suggesting that with polymeric ribonucleotides, where stacking interactions of more complex nature are developed, arylation sites on bases might be revealed. As an illustration of these rather subtle effects on the alkylation site control, we have to remember that in the present case the stereoisomers ratio **6′/6″** can be significantly modified simply by changing the base from a purine to a pyrimidine or by addition of a phosphate group at 5′-OH. In conclusion, the antitumor agent 9-OH-NME (1) appears to be an original molecule among all DNA intercalators, able to alkylate ribonucleosides after a biochemical oxidation process. We are currently studying the alkylation of oligonucleotide molecules by the electrophilic quinone imine **2** in order to determine the binding sites at the molecular level.

Experimental Section

Materials. 9-OH-NME (1) was donated by Sanofi (France). Horseradish peroxidase (type VI, EC 1.11.1.7), alkaline phosphatase (from *Escherichia coli*, type III, EC 3.1.3.1), nucleosides, and nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO) and other usual laboratory reagents from Prolabo (Paris).

Spectrometric Methods. NMR spectra were recorded on a Bruker (250 or 400 MHz) spectrometer operated in the Fourier transform mode (FT). The chemical shifts are expressed in ppm (δ) with tetramethylsilane (Me_4Si) as internal standard. The NOE difference spectra were essentially the difference between the equilibrium spectrum and the spectrum acquired just after saturation (1s) of a selected resonance. Sixteen on and off resonance scans are alternatively acquired and substracted. This is repeated until a sufficient signal to noise ratio is obtained for the NOE peaks. Two dummy scans are inserted in the sequence to account for any uncomplete relaxation between cycles.

A Riber R10-10 apparatus was used for desorption chemical ionisation (DCI) and MM-ZAB for fast atomic bombardment (FAB) mass spectra (glycerol matrix).

Chromatographic Methods. All HPLC studies were performed on a Waters chromatograph using a μ-Bondapak C_{18} column and a mixture of methanol/10^{-2} M ammonium acetate (50/50, v/v) acidified to pH 4.5 with acetic acid as eluent. The detection of ellipticine derivatives was monitored by a UV spectrometer at 254 or 313 nm. A t_R value was associated with each peak of the HPLC chromatograms and defined as the ratio of the retention time of the unknown peak to the retention time of the peak corresponding to 9-OH-NME (1) (t injection = 0).

Test Experiments (Table I). The following conditions were used: 10 μL of 2×10^{-2} M H_2O_2 was added to 20 μL of 5×10^{-3} M 9-OH-NME 1, 400 μL of 2.5×10^{-3} M purine derivatives, and 10 μL of 5×10^{-6} M HRP in 560 μL of 0.066 M (pH 8.0) phosphate buffer. After a reaction time of 3 min at room temperature, aliquots were analyzed by HPLC.

Preparation of Nucleos(t)ides–Elliptinium Adducts. Adenosine–Elliptinium 6a and Guanosine–Elliptinium 6c Adducts. These two compounds were prepared according to the procedure described in ref 11. A 100-μL sample of 1 M H_2O_2 was added in five fractions to 0.25 mmol of adenosine or guanosine, 0.05 mmol of 9-OH-NMe, and 100 μL of 0.1 mM HRP in 20 mL of 0.05 M (pH 8) phosphate buffer. After 5 min of incubation at room temperature, the adduct precipitated. It was washed with water and dried under reduced pressure. This compound, the phosphate salt of the adduct, was dissolved in 0.5 mL of MeOH and 0.5 mL of acetic acid and precipitated with 4 mL of diethyl ether, washed twice with ether, and dried under reduced pressure. A 15-mg sample of the deep orange adenosine–NMHE adduct 6a (acetate salt) was obtained (yield 50%). The guanosine adduct **6c** was obtained in 81% yield under these experimental conditions.

6a: UV–visible (H_2O) λ 370 nm (ε 7000 M^{-1} cm^{-1}), 315 (13 500), 272 (36 050). Anal. ($C_{30}H_{29}N_7O_7$) C, H, N.

6c: UV–visible (H_2O) λ 372 nm (ε 9350 M^{-1} cm^{-1}), 315 (20 350), 275 (33 450). Anal. Calcd for $C_{30}H_{29}N_7O_8$: C, 58.54; H, 4.72; N, 15.93. Found: C, 57.15; H, 4.45; N, 15.12.

Cytidine–Elliptinium 6e, Uridine–Elliptinium 6g, and Azauridine–Elliptinium 6i Adducts. The acetate salts of these adducts were prepared by using a procedure similar to that described above for **6a** and **6c**, but the molar ratio nucleoside/9-OH-NME was increased from 5 to 25. After workup, the yields were 63%, 60%, and 50% for **6e**, **6g**, and **6i**, respectively.

6e: UV–visible (H_2O) λ 460 nm (ε 1600 M^{-1} cm^{-1}), 372 (8900), 313 (16 000), 275 (35 000). Anal. ($C_{29}H_{29}N_5O_8$) C, H, N.

6g (6′g, 90%; 6″g, 10%): UV–visible (CH_3COOH, H_2O, 50/50) λ 450 nm (ε 1600 M^{-1} cm^{-1}), 368 (11 100), 313 (21 000), 275 (35 800). Anal. ($C_{29}H_{28}N_4O_9$) C, H, N.

6i (6′i, 90%; 6″i, 10%): UV–visible (H_2O) λ 450 nm (ε 1600 M^{-1} cm^{-1}), 370 (9000), 312 (16 100), 274 (31 100). Anal. Calcd for $C_{28}H_{27}N_5O_9$: C, 58.23; H, 4.68; N, 12.13. Found: C, 57.28; H, 4.01; N, 11.85.

5′-Adenylic Acid–Elliptinium 6b, 5′-Guanylic Acid–Elliptinium 6d, 5′-Cytidylic Acid–Elliptinium 6f, and 5′-Uridylic Acid–Elliptinium 6h Adducts. To 0.25 mmol of AMP, GMP, CMP, or UMP, 0.01 mmol of 9-OH-NME and 50 μL of 10^{-4} M HRP in 20 mL of water brought to pH 8 with NaOH (for AMP and GMP) or in 5 mL of 0.05 M (pH 8) phosphate buffer (for CMP and UMP) was added dropwise 200 μL of 0.1 M H_2O_2 over 20 min. After an incubation period of 1 h at room temperature, adducts

were precipitated with an excess (1 mL) of 0.5 M ammonium hexafluorophosphate after acidification with acetic acid (50 μL), isolated, washed with water, and then dessicated; yields were 60%, 50%, 23%, and 49% for **6b**, **6d**, **6f**, and **6h**, respectively (hexafluorophosphate salts).

6b (**6′b**, 90%; **6″b**, 10%): UV–visible (CH_3COOH, H_2O, 50/50) λ 460 nm (ε 1500 M^{-1} cm^{-1}), 370 (8000), 313 (16000), 299 (16500), 274 (25500). Anal. Calcd for $C_{28}H_{27}N_7O_8P_2F_6$: C, 43.92; H, 3.53; N, 12.81. Found: C, 43.21; H, 3.16; N, 11.97.

6d (**6′d**, 90%; **6″d**, 10%): UV–visible (CH_3COOH, H_2O, 50/50) λ 450 nm (ε 2500 M^{-1} cm^{-1}), 366 (11000), 312 (21000), 295 (24500), 274 (38000). Anal. Calcd for $C_{28}H_{27}N_7O_9P_2F_6$: C, 43.02; H, 3.46; N, 12.55. Found: C, 42.54; H, 3.13; N, 11.76.

6f (**6′f**, 80%; **6″f** 20%): UV–visible (CH_3COOH, H_2O, 50/50) λ 375 nm (ε 11100 M^{-1} cm^{-1}), 314 (21500), 277 (44500). Anal. Calcd for $C_{27}H_{27}N_5O_9P_2F_6$: C, 43.72; H, 3.64; N, 9.45. Found: C, 43.14; H, 3.18; N, 8.95.

6h (**6′h**, 80%; **6″h**, 20%): UV–visible (CH_3COOH, H_2O, 50/50) λ 450 nm (ε 4000 M^{-1} cm^{-1}), 373 (17300), 314 (41100), 302 (40000), 276 (54000). Anal. Calcd for $C_{27}H_{26}N_4O_{10}P_2F_6$: C, 43.67; H, 3.50; N, 7.55. Found: C, 42.72; H, 3.12; N, 7.12.

Thioguanine–Elliptinium 7a and Thioguanosine–Elliptinium 7b Adducts. Contrary to the method described above for nucleophiles that are not easily oxidized, thioguanine and thioguanosine were added to an organic solution of 9-oxo-NME (**2**), this quinone imine being generated by HRP/H_2O_2 in the absence of a SH-containing molecule. This procedure was previously used for syntheses of cysteinyl–NMHE adducts.[6] Thus, 1 mL of 0.1 M H_2O_2 was added to a solution of 0.05 mmol of 9-OH-NME and 20 μL of 10^{-4} M HRP in 30 mL of 0.067 M (pH 5) phosphate buffer. After 5 min, the quinone imine was extracted into 100 mL of dichloromethane in the presence of an excess of ammonium hexafluorophosphate (0.5 mmol). A 0.2-mmol sample of thioguanine or thioguanosine in 100 mL of water (acidified with acetic acid to pH 3.5) was then added to the organic extract and the mixture vigorously shaken. The adducts were precipitated from the aqueous phase with an excess of ammonium hexafluorophosphate (0.5 mmol), washed with water, dissolved in acetic acid (10 mL), precipitated again with 50 mL of diethyl ether, and dried under reduced pressure. The adducts (acetate salts) were obtained in 60% and 12% yield for **7a** and **7b**, respectively.

7a: UV–visible (CH_3COOH, H_2O, 50/50) λ 450 nm (ε 3300 M^{-1} cm^{-1}), 386 (7200), 320 (38800). Anal. ($C_{25}H_{23}N_7O_3S$) C, H, N.

7b: UV–visible (CH_3COOH, H_2O, 50/50) λ 450 nm (ε 3700 M^{-1} cm^{-1}), 386 (8000), 323 (42400). Anal. Calcd for $C_{30}H_{31}N_7O_7S$: C, 56.87; H, 4.90; N, 15.48. Found: C, 56.22; H, 4.51; N, 14.96.

See Table II for ^1H NMR data of all these elliptinium adducts.

Enzymatic Hydrolysis of 5′-Phosphate Ribonucleotides–Elliptinium Adducts 6b and 6d. The hydrolysis tests were performed under the following conditions: 0.8 unit of alkaline phosphatase was added to 1 mL of 0.127 M of 5′-phosphate ribonucleotide–elliptinium adducts in 20 mM Tris buffer at pH 8.5 and incubated at 37 °C. HPLC conditions were as described above.

Cytotoxicity Experiments. Inhibition of cell growth was determined with L1210 leukemia cells in vitro as previously described (see ref 17). The inhibitory efficiency of the elliptinium adducts is expressed in terms of ID_{50}, the drug concentration that reduces the number of cells by 50% as compared to the control after 48 h (ID = inhibitory dose).

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Registry No. **6′a**·AcO^-, 93033-93-3; **6′b**·PF_6^-, 102420-10-0; **6″b**·PF_6^-, 102420-12-2; **6′c**·AcO^-, 102434-52-6; **6′d**·PF_6^-, 102420-14-4; **6″d**·PF_6^-, 102420-16-6; **6′e**·AcO^-, 102420-00-8; **6′f**·PF_6^-, 102420-18-8; **6″f**·PF_6^-, 102420-20-2; **6′g**·AcO^-, 102420-02-0; **6″g**·AcO^-, 102420-04-2; **6′h**·PF_6^-, 102420-22-4; **6″h**·PF_6^-, 102434-54-8; **6′i**·AcO^-, 102420-06-4; **6″i**·AcO^-, 102420-08-6; **7a**·AcO^-, 102420-24-6; **7b**·AcO^-, 102434-56-0; 9-OH-NME, 58337-35-2; AMP, 61-19-8; GMP, 85-32-5; CMP, 63-37-6; UMP, 58-97-9; adenosine, 58-61-7; guanosine, 118-00-3; cytidine, 65-46-3; uridine, 58-96-8; azauridine, 54-25-1; thioguanine, 154-42-7; thioguanosine, 85-31-4.

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Catechol Estrogens of the 1,1,2-Triphenylbut-1-ene Type: Relationship between Structure, Estradiol Receptor Affinity, Estrogenic and Antiestrogenic Properties, and Mammary Tumor Inhibiting Activities

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1,1,2-Triphenylbut-1-enes (**26–35**), which are substituted with one or two 3,4-diacetoxy groups or with one 3,4-diacetoxy and one 3- or 4-acetoxy group in two aromatic rings, were synthesized. The occurring *E* and *Z* isomers were isolated, and their identity was established by ^1H NMR spectroscopy. A study on structure–activity relationship was carried out with regard to estradiol receptor affinity in vitro, estrogenic and antiestrogenic properties in the immature mouse, and inhibition of the hormone-dependent MXT mammary tumor of the mouse in vivo. Among the tested compounds, most of the 1,1-disubstituted 1,1,2-triphenylbut-1-enes (**29**, ***Z*-30**, ***Z*,*E*-31**) and (*E*)-1-(3-acetoxyphenyl)-1-phenyl-2-(3,4-diacetoxyphenyl)but-1-ene (***E*-35**) as well as its respective *Z* isomer (***Z*-35**) exerted antiestrogenic properties. Compounds ***Z*-30**, ***Z*,*E*-31**, ***Z*-35**, and ***E*-35** inhibited the growth of the hormone-dependent MXT tumor. The best antitumor effect without estrogenic side effects during therapy was shown by ***E*-35**.

Many compounds of the triarylethylene type have been tested with regard to their mammary tumor inhibiting activity.[1-3] Tamoxifen, one of these compounds, is now routinely used for the treatment of the advanced mammary carcinoma.[1-3] In two former publications,[4,5] we have

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