Synthesis, Metabolism, and Antitumor Activity of Deuterated Analogues of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea[1]

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Analogues of CCNU have been synthesized in which the cyclohexyl residue is partly (2,2′,6,6′-d_4, 3,3′,4,4′,5,5′-d_6) or wholly deuterated (2,2′,3,3′,4,4′,5,5′,6,6′-d_{10}) at positions susceptible to metabolic hydroxylation. Each deuterated analogue alone or in admixture with CCNU was incubated with rat liver microsomes, and the yields of each hydroxy derivative (*trans*-2-, -3-, and -4-OH, *cis*-3- and 4-OH) were measured by HPLC (individual derivatives) or by HPLC and mass spectrometry (mixtures). Increased retention times in HPLC due to deuterium substitution on the cyclohexyl ring were observed with the OH-CCNU isomers and nearly complete resolution of *cis*- and *trans*-4-OH-CCNU-d_0 and -d_9 was achieved. The total yield of OH derivatives from CCNU-d_{10} was 74% of that from CCNU, but the relative proportions of each OH-CCNU were very similar. CCNU-d_4 yielded much less *trans*-2-OH-CCNU (0.2% of the total OH derivatives) than did CCNU (2.3%), whereas the yield (17%) of this isomer from CCNU-d_6 was correspondingly greater. Thus, selective deuteration directs metabolism away from the sites of isotopic substitution (i.e., induces metabolic switching), although there is only a small isotope effect for the overall hydroxylation process. CCNU and its deuterated analogues had similar antitumor activities against the TLX-5 lymphoma in mice.

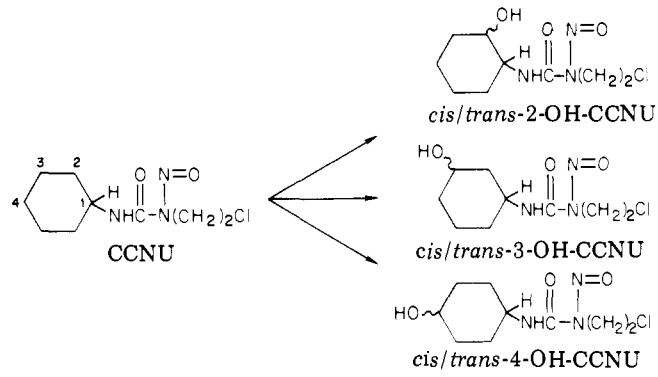
1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) (1) is one of the most effective of the antitumor nitrosoureas against intraperitoneal and intracerebral leukemia L1210 in mice[2] and is of use in the treatment of many human malignancies, particularly those of the brain[3] where it is now often the drug of choice. CCNU decomposes rapidly in aqueous solution and among the many products are the reactive carbamylating agent, cyclohexyl isocyanate,[4] and 2-chloroethanol, the formation of which suggests the intermediacy of an alkylating moiety, possibly a 2-chloroethyl carbonium ion.[5,6]

However, it is now considered likely that the antitumor effects of CCNU in vivo are due to its metabolites, as extensive and rapid hydroxylation of the cyclohexyl ring is mediated by microsomal enzymes in the liver.[7-9] Seven isomers of ring-monohydroxylated derivatives of CCNU (OH-CCNU) are theoretically possible (1-OH-, *cis*-2-OH-, *trans*-2-OH-, *cis*-3-OH-, *trans*-3-OH-, *cis*-4-OH-, and *trans*-4-OH-CCNU). The formation of four (*cis*-3-OH-, *trans*-3-OH-, *cis*-4-OH-, and *trans*-4-OH-CCNU) was established by HPLC and mass spectral and NMR characteristics.[9,10] In previous work, a minor metabolite had HPLC and mass spectral properties similar to those of *cis*-2-OH-CCNU,[9] but work presented in this paper has demonstrated that this metabolite is *trans*-2-OH-CCNU and that *cis*-2-OH-CCNU is formed in even smaller amounts than the trans 2-isomer (Figure 1, Scheme I). Other workers have found 3-OH- and 4-OH-CCNU isomers and *trans*-2-OH-CCNU but were unable to show the formation of the cis 2-isomer by liver microsomes from the rat[11] and mouse.[12] In the plasma of humans to whom CCNU had been administered, *cis*-4-OH-CCNU (the major in vitro metabolite) and *trans*-4-OH-CCNU have been found[13] in approximately equal amounts.

The cis and trans forms of 2-, 3-, and 4-OH-CCNU have been synthesized[9,12,14] and although the half-lives for chemical breakdown of the isomers were similar, each showed a greater therapeutic index (LD_{10}/ED_{50}) than did CCNU against ip and ic L1210 leukemia in mice.[12] The alkylating and carbamylating activities of the various OH-CCNU derivatives also showed significant differences from those of CCNU.

Whereas high alkylating activity is observed with *cis*-2- and *trans*-2-OH-CCNU and *cis*-3-OH-CCNU, these isomers have almost no carbamylating activity; this property is also characteristic of chlorozotocin and streptozotocin

Scheme I. Metabolic Hydroxylation of CCNU



which is presumably explained by intramolecular carbamylation.[15] The low bone marrow toxicity of streptozotocin[16] and chlorozotocin[17] may be related to their low carbamylating activity, a suggestion that implies a low myelosuppressive activity of the *cis*- and *trans*-2-OH-CCNU isomers.

The contributions of the various OH-CCNU metabolites to the biological activity of CCNU are presently unknown but might be assessed if the pattern of hydroxylation could be substantially changed and/or the rate of hydroxylation significantly reduced. In seeking to achieve this objective we have synthesized and studied the metabolism and antitumor activity of a series of CCNU derivatives variously deuterated in the cyclohexyl moiety.

When C-H bond cleavage in a metabolic process is rate determining, replacement of the hydrogen by deuterium will, in general, retard this process[18] although the factors which control the occurrence and magnitude of H/D isotope effects in metabolism remain to be fully defined. The selective and limited substitution in a drug molecule by deuterium may substantially alter the metabolism profile.

The term "metabolic switching" was coined by Horning et al.[19] to describe this effect, in relation to the influence of selective deuteration on the metabolism profiles of antipyrine and caffeine. Apparently, the first report of this phenomenon was by Mitoma et al.[20] who noted that selective deuteration of each carbon atom in the propyl group of *n*-propyl *p*-nitrophenyl ether decreased oxidative metabolism at the site of deuteration and caused increased formation of the other metabolites. Thus, by appropriate

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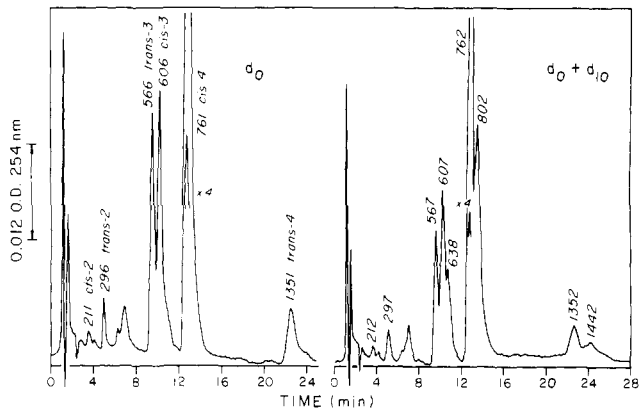


Figure 1. Metabolism profiles for CCNU-d_0 and CCNU-d_0 + -d_{10}. HPLC conditions are as described in the text. The elution profile for the OH-CCNU isomers from CCNU-d_0 is given, with retention time in seconds indicated above each designated peak. The CCNU-d_0 + -d_{10} profile of metabolites with indicated resolved peaks and their retention time in seconds is given.

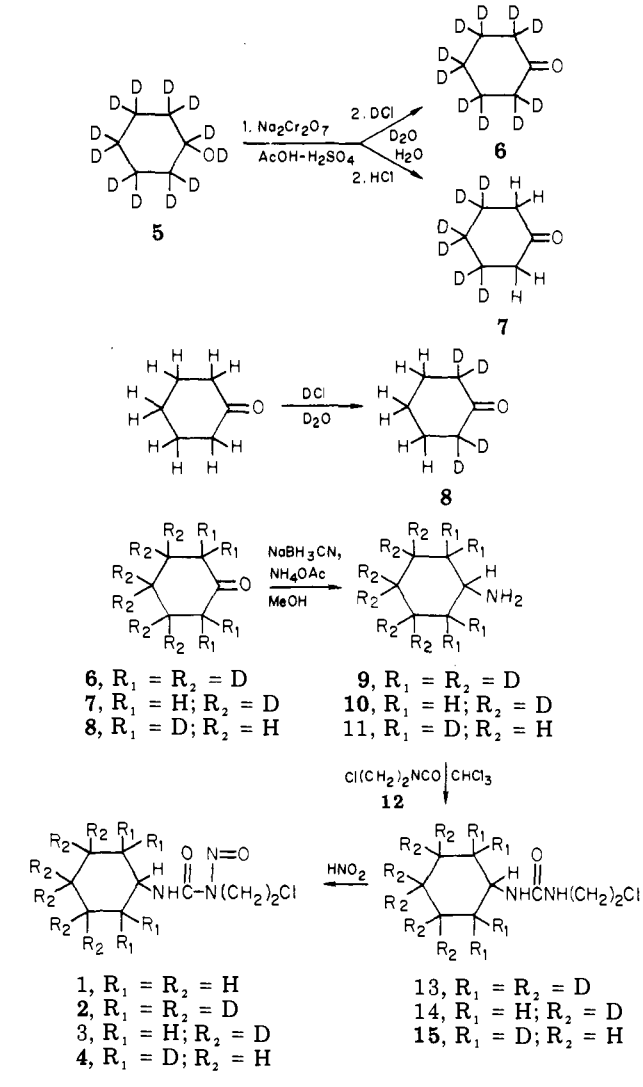
deuterium substitution the biological significance of particular transformations in a metabolism profile may be probed.

The conversion of $-CH_2- \rightarrow -CH(OH)-$ occurs on hydroxylation of the cyclohexyl moiety of CCNU. Hence, the d_6 (**3**, CCNU-3,3′,4,4′,5,5′-d_6) and d_4 derivative (**4**, CCNU-2,2′,6,6′-d_4) were selected to investigate the possibility of metabolic switching of hydroxylation toward position 2(6) and away therefrom, respectively. Additionally, the d_{10} derivative (**2**, CCNU-2,2′,3,3′,4,4′5,5′,-6,6′-d_{10}) was selected in order to determine the effect of perdeuteration of the cyclohexyl moiety on the overall rate of hydroxylation. These studies are part of a program concerned with applications of deuterium labeling in studies of drug metabolism.[21-26]

Synthesis. CCNU is conventionally synthesized[2] by the reaction of 2-chloroethyl isocyanate (**12**) with cyclohexylamine, followed by nitrosation of the resulting urea (Scheme II). The synthesis of d_4, d_6, and d_{10} derivatives therefore requires three specifically deuterated cyclohexylamine derivatives. Cyclohexanol-d_{12} (**5**), which is commercially available, was used for the synthesis of cyclohexylamine-d_{10} and -d_6. Oxidation with chromic acid gave cyclohexanone-d_{10} (**6**) but some deuterium was lost by exchange during the reaction since the d_{10}:d_9 ratio for the product was 100:45 compared to a d_{12}:d_{11} ratio of 100:9.5 for the starting material. This loss was easily reversed by treating the product with boiling 1 M DCl-D_2O; the d_{10}:d_9 ratio then became 100:9. This exchange procedure was carried out immediately before using the ketone, which underwent gradual deuterium loss on storage. Conversion of the keto function in cyclohexanone-d_{10} into an amine group using commercial sodium cyanoborohydride[27] in the presence of ammonium acetate was difficult to achieve without exchange of the deuterium atoms adjacent to the carbonyl function. Even when fully deuterated reagents were used some back exchange occurred. However, removal of trace (presumably basic) impurities from the sodium cyanoborohydride and use of fresh reagent, rigorously purified via its dioxane complex, yielded an acceptable product. The resulting cyclohexylamine-d_{10} (**9**) was converted into CCNU-d_{10} (**2**), the mass spectrum of which contained peaks at m/e 243 (M^+· for CCNU-d_{10}) and 242 (M^+· for CCNU-d_9) in the ratio 100:20.

Oxidation of cyclohexanol-d_{12} with chromic acid and treatment of the product with boiling 1 M HCl completed exchange of the deuterium at C-2 and C-6 and gave cyclohexanone-d_6 (**7**). CCNU-d_6 (**3**) derived therefrom via cyclohexylamine-d_6 gave a mass spectrum with a peak intensity ratio of 100:10 for the ions at m/e 239 (M^+· for CCNU-d_6) and 238.

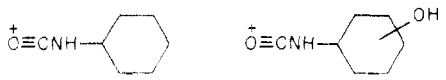
Scheme II



Two treatments of cyclohexanone with boiling 1 M DCl-D_2O gave cyclohexanone-d_4 (**8**) which, with the same precautions to avoid deuterium exchange as were used for the d_{10} compound, was converted into cyclohexylamine-d_4 and thence into CCNU-d_4 (**4**), the mass spectrum of which contained signals at m/e 237 (M^+· for CCNU-d_4) and 236 in the ratio 100:11. The d_4, d_6, and d_{10} derivatives of CCNU appeared homogeneous by TLC and showed the same TLC properties and melting point as CCNU. The position of the label in cyclohexanone-d_4 and -d_6 (**7** and **8**) was confirmed by NMR spectroscopy.

Analytical Methods. The choice of 12 eV as the ionizing voltage in the mass spectrometry of CCNU and its metabolites was based on several considerations. The percentage of each deuterium-deficient species (CCNU-d_9 in CCNU-d_{10}, CCNU-d_5 in CCNU-d_6, and CCNU-d_3 in CCNU-d_4) was determined from the intensity of the signal for the appropriate molecular ion which was relatively greater at 12 eV than at 70 eV. Even so, the signal for the molecular ion was too weak to be utilized in determining ratios between deuterated and nondeuterated species for CCNU and its hydroxy derivatives following metabolism. The abundant fragment ions, **16** and **17**, and their deuterated counterparts (Chart I), used for this purpose, had

Chart I



16 17

Table I. Bioassay[a] Results

	% increase in survival time			
	4 μg/mL	1 μg/mL	0.5 μg/mL	0.25 μg/mL
CCNU + microsomes + cofactors	∞[b]	145	106	21
CCNU + cofactors	∞[b]	64	60	9

[a] Conditions are as described in ref 28. Control animals lived for 6-7 days; 220% increase in survival time corresponds to 99.8% cell kill, 83-99% cell kill, and 47-85% cell kill.[35] [b] Animals survived to the end of the experiment (30 days).

a higher relative abundance at 12 eV than at 70 eV. The mass spectra of CCNU-d_4 illustrates these points. At 70 eV the relative intensities were 1.2% for $M^{+\cdot}$ (m/e 237) and 26.6% for **16**-d_4 (m/e 130); the corresponding values at 12 eV were 3.5 and 42.1%, respectively.

HPLC Experimental Data. As shown previously[9,10] HPLC is a rapid and precise method for separation and quantitation of CCNU and the various hydroxylated metabolites. Increase of the deuterium content of the cyclohexyl ring increased the retention of the OH-CCNU isomers. Particular care was taken in those experiments in which mixtures of CCNU with its d_4 or d_6 analogues were metabolized to ensure that partially resolved metabolic isomers were recombined prior to mass spectrometry. As discussed later, nearly complete resolution of certain d_{10} analogues from OH-CCNU isomers was achieved by HPLC (Figure 1).

Metabolism and Biological Results. Cytotoxicity Assay. CCNU was incubated, alone and in the presence of rat liver microsomes and an NADPH generating system, with Walker 256 ascites tumor cells for 2 h prior to injection of these cells (10^6) into rats.[28] The survival times of the animals were used to estimate the degree of cell kill in the incubation; the results in Table I indicate that there is little change in the cytotoxicity of CCNU consequent upon metabolism. However, the situation in vivo may be affected by the increased hydrophilicity of the metabolites compared to that of CCNU which could result in differences in tissue distribution.

Rates of Metabolism of Deuterated Derivatives of CCNU and Analysis of Metabolites. Relative Rates of Metabolism of CCNU and CCNU-d_{10}. In order to determine the overall effect of deuteration on the rate of metabolism of CCNU, CCNU-d_{10} was mixed with an equimolar amount of unlabeled drug and incubated with a rat liver microsomal system. The amount of each isotopic species of starting material remaining at various times was measured by the addition of aliquots of the incubation mixture to known amounts of a differently labeled (d_4) internal standard, followed by mass spectrometry of the recovered drug. The fragment ions at m/e 126 (**16**) and its d_4 and d_{10} counterparts (m/e 130 and 136) were used. Calibration lines showed linear relationships between the relative intensities of these ions and known quantities of each species present.

The results thus obtained (Table II) show that, within 20 min, metabolism of each compound was essentially complete and indicate an approximately twofold slower initial rate for the d_{10} compound. In control experiments using heat-inactivated microsomes, only 10–15% chemical breakdown occurred during this time.

Table II. Comparative Rates of Metabolism by Rat Liver Microsomes of CCNU and Its d_{10} Analogue in a Mixture

Time, min	% metabolized	
	CCNU-d_{10}	CCNU
2.5	16.1	26.8
5	38.3	47.9
10	69.5	72.5
20	91.9	91.8

The metabolism of CCNU occurs mainly to ring-hydroxylated products[7-12] and was confirmed in the present study, and it appears that a small isotope effct is operating in the hydroxylation of CCNU-d_{10}. This observation may be compared with the report[29] that hydroxylation of cyclohexane-d_{12} proceeds without an isotope effect and with the many examples of microsomal aliphatic hydroxylation which show H/D isotope effects of 2–3.[21,30,31]

Metabolite Analyses. Following the incubation of CCNU with rat liver microsomes (see the Experimental Section), six OH-CCNU isomers could be extracted and separated by HPLC, and the structures were assigned using the data of May et al.[9] Identification of the *cis*-2- and *trans*-2-OH-CCNU metabolites was effected by comparison with authentic synthetic compounds.[9] Because the microsomal yield of *cis*-2-OH-CCNU was extremely low, it was characterized only by UV and HPLC properties; a satisfactory mass spectrum was not obtained. The metabolism profile of each deuterated CCNU was determined by incubation with the microsomal enzyme system and quantification of each metabolite and residual starting material by HPLC. Since the results of these experiments indicated metabolic switching (see below) for the d_4 and d_6 analogues, equimolar mixtures of CCNU-(d_0 + d_4) and -(d_0 + d_6) were metabolized. The metabolites were quantified and isolated by HPLC and subjected to mass spectrometry in order to determine isotope ratios and, hence, the relative rate of production of each labeled metabolite under conditions of competitive metabolism.

Increased retention times in HPLC due to deuterium substitution on the cyclohexyl ring were observed with the OH-CCNU isomers. This effect is illustrated in Figure 1 for the metabolites from a mixture of CCNU-(d_0 + d_{10}). It appears that nearly complete resolution of the *cis*- and *trans*-4-OH-CCNU-d_0 isomers from their *cis*- and *trans*-OH-CCNU-d_9 counterparts was possible, whereas less resolution of the 3-OH isomers was achieved (Figure 1). Isotopic separations have been achieved by reverse-phase HPLC with hydrophobic effects being implicated[32] in the complete resolution of $C_{15}H_{31}COOH$ from $C_{15}D_{31}COOH$. Other isotopic fractionations have been reported,[33] including those of tritiated dimethylbenzanthracene–nucleoside conjugates.[34]

Table III shows the amount of CCNU and of OH-CCNU derivatives isolated after incubation of each compound for 10 min with rat liver microsomes. As the deuterium content of the CCNU increases, there is a progressive decrease in the amount of OH-CCNU derivatives formed (for CCNU-d_{10} the yield is only 74% of that from CCNU). However, this decrease was not consistently reflected in an increase of residual CCNU, suggesting that other metabolic pathways may be operative.

The metabolism profiles as reflected by the hydroxy derivatives for CCNU-d_0, -d_4, -d_6, and -d_{10} showed marked differences; the relevant quantitative analysis data are shown in Table IV. For CCNU-d_0, *cis*-4-OH-CCNU was

Table III. Metabolism of Deuterated CCNU Derivatives[a]

	CCNU recovered, μg	Total OH-CCNU derivatives isolated, μg
CCNU-d_0 (1)	337	1230
CCNU-d_4 (4)	272	1089
CCNU-d_6 (3)	538	994
CCNU-d_{10} (2)	498	911
1 + 4	1170	2587
1 + 3	800	2642

[a] Conditions are as described in the Experimental Section. Incubations of individual isotopic variants were in 20-mL total volume and contained 2.8 mg (12 μmol) of substrate. For mixtures (1 + 4, 1 + 3) 2.8 mg of each substrate was present in 40-mL total volume.

the most abundant metabolite (63% of the total OH-CCNU derivatives) and hydroxylation at the 2 position was a very minor process (2.3% *trans*-2-OH-CCNU). Even less (0.2%) *trans*-2-OH-CCNU was formed from CCNU-d_4, and there were small changes in the levels of the other isomers. However, there was a marked increase in production of *trans*-2-OH-CCNU (17%) from CCNU-d_6 with corresponding decreases in the amounts of all other OH-CCNU derivatives, whereas the metabolism profile for CCNU-d_{10} was broadly similar to that from CCNU-d_0.

These data are readily explicable in terms of isotope effects and the resulting metabolic switching. With CCNU-d_4 an isotope effect is operative for 2-hydroxylation, reducing by tenfold the production of *trans*-2-OH-CCNU. Switching of the metabolism to other pathways is not observable because of the low original level of 2-hydroxylation. With CCNU-d_6 there is an isotope effect for 3- and 4-hydroxylation, reducing the amounts of each of the cis and trans isomers of 3- and 4-OH-CCNU, and metabolic switching leads to a 7.5-fold increase in 2-hydroxylation. This type of switching cannot occur with CCNU-d_{10}, and the extent of 2-hydroxylation is similar to that for CCNU-d_0. Thus, by selective deuterium substitution the extent of 2-hydroxylation can be varied from 0.2 to 17%.

In the experiments in which mixtures of CCNU with its d_4 or d_6 analogues were metabolized, it should be noted that the 1:1 (w/w) mixtures actually both contained a molar ratio of 100:89 (d_0:d_4 or d_0:d_6 forms), owing to differences in molecular weight and to the presence of CCNU-d_3 and -d_5 as minor constituents in the d_4 and d_6 analogues, respectively. Additionally, a 1:1 relationship between the intensities of characteristic fragment ions in the mass spectra and molar proportions of protium and deuterium forms in the products cannot be assumed and should be validated where possible. Although appropriately deuterated OH-CCNU derivatives were not accessible via synthesis, they could be isolated after metabolism of the appropriately deuterated CCNU. The yield of each deuterated *cis*-3- and -4-OH-CCNU was sufficient for 1:1 mixtures with the protium forms to be constituted on the basis of equivalence of UV absorbance at 230 nm. The ratios were corrected for the fact that the deuterated hydroxy derivatives also contained analogues derived from

Table IV. Metabolism Profiles from Deuterated CCNU Derivatives[a]

	% total OH-CCNU derivatives				
	trans-2-OH	*cis*-3-OH	*trans*-3-OH	*cis*-4-OH	*trans*-4-OH
CCNU-d_0 (1)	2.28	19.95	6.31	62.61	5.71
CCNU-d_4 (4)	0.23	19.50	6.18	63.77	6.34
CCNU-d_6 (3)	17.01	18.21	3.53	55.38	3.18
CCNU-d_{10} (2)	2.84	20.25	3.57	66.01	4.65

[a] HPLC conditions are as described in the text. Elution times: *cis*-2-OH-CCNU, 2.8 min; *trans*-2-OH-CCNU, 3.8 min; *trans*-3-OH-CCNU, 7.1 min; *cis*-3-OH-CCNU, 7.8 min; *cis*-4-OH-CCNU, 9.6 min; and *trans*-4-OH-CCNU, 16.9 min.

Table V. Relative Intensities for Characteristic Ions in the Mass Spectra of 1:1 (w/w) Mixtures of CCNU + CCNU-d_4 and of CCNU + CCNU-d_6[d] and 1:1 (UV, 230 nm) Mixtures of *cis*-3- and -4-OH-CCNU Derived Therefrom[e]

Composition of mixture	Molar ratios of protium:deuterium form	Ion intensity ratios[c]
CCNU + CCNU-d_4	100:89[a,b]	100:90 (83:87)
CCNU + CCNU-d_6	100:89[a,b]	100:91 (83:89)
cis-3-OH-CCNU-d_0 + -d_4	100:90[a]	100:86.5 (142:146)
cis-3-OH-CCNU-d_0 + -d_5	100:91[a]	94:100 (142:147)
cis-4-OH-CCNU-d_0 + -d_4	100:90[a]	100:90 (142:146)
cis-4-OH-CCNU-d_0 + -d_5	100:91[a]	100:97 (142:147)

[a] Corrected for the presence of other deuterated species. [b] Corrected for the molecular weight difference between protium and deuterium forms. [c] *m/e* values in parentheses. [d] Cyclohexyl ions at *m/e* 83, 87, or 89.

[e]  ions at *m/e* 142, 146, or 147.

Table VI. Isotopic Composition and Yield of CCNU and Hydroxy Derivatives Recovered Following Incubation of Mixtures of CCNU and Labeled (d_4 or d_6) CCNU with Rat Liver Microsomes

Composition of mixture	Ratio of d_0:d_4 or -d_6 admin	Ratio of nondeuterated:deuterated compd recovered[a]					
		CCNU	*trans*-2-OH	*cis*-3-OH	*trans*-3-OH	*cis*-4-OH	*trans*-4-OH
CCNU + CCNU-d_4	100:90	72.5:100 (20.9)	100:10 (1.8)	100:92 (20.7)	100:67 (8.5)	100:91 (59.9)	100:97 (5.8)
CCNU + CCNU-d_6	100:91	68:100 (14.3)	22:100 (9.1)	100:80 (20.3)	100:36 (6.0)	100:70 (57.1)	100:76 (4.8)

[a] Yields in parentheses are relative: for CCNU, to the amount of starting material; for each OH-CCNU, to the total OH derivative recovered. The values did not total 100% owing to a small contribution from components suspected to be *cis*-2-OH-CCNU derivatives and possibly some 3-nitroso isomers, but this was not confirmed by mass spectrometry.

Table VII. TLX-5 Antitumor Tests

	% increase in survival time[a]					
	5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	160 mg/kg
CCNU-d_0 (1)		54.0	244.0	125.0 (3)[b]	-22.0	-20.0
CCNU-d_{10} (2)		20.0	42.0	217.0 (1)[b]	-22.0	-28.0
CCNU-d_0 (1)	21.6	64.9	95.8 (1)[b]	85.5 (2)[b]	-11.4	-21.7
CCNU-d_6 (3)	38.1	38.1	95.8	81.4 (2)[b]	-7.3	-32.0
CCNU-d_0 (1)	1.0	76.8[c]	69.6	76.8 (1)[d]	-23.3	-31.4
CCNU-d_4 (4)	-5.1	63.6	79.7	178.7	-9.1	-21.3

[a] Control animals died in 9-11 days, five animals per group. Long-term survivors were excluded from calculation of percent increase in survival time. [b] Figures in parentheses denote number of long-term survivors (>160 days). [c] One animal had 273% increase in survival time. [d] One long-term survivor (>70 days).

CCNU-d_3 and -d_5 and thereafter were compared with the intensity ratios for characteristic ions in the mass spectrum of each mixture (Table V). The correlation was sufficiently good to justify the use of corresponding intensity ratios (Table VI) for ions **17** to determine molar ratios between protium and deuterium forms for these and for other hydroxy derivatives of which insufficient quantities were available from metabolism of deuterated CCNU derivatives to constitute analogous 1:1 mixtures.

Thus, for mixtures of CCNU with its d_4 or d_6 analogues (Table VI), metabolic switching is most marked for the deuterated *trans*-2-OH-CCNU derivatives. A ratio of 5:1 between the d_6 and d_0 forms resulted from switching of metabolism away from C-3 and C-4 consequent on deuteration at these positions (cf. aforementioned ratio of 7.5:1 observed when the d_0 and d_6 analogues were metabolized separately). Conversely, a ~10:1 ratio between the d_0 and d_3 derivatives formed from CCNU and CCNU-d_4 reflected the operation of the isotope effect at C-2. The total yield of *trans*-OH-CCNU derivatives expressed as a percentage of total OH-CCNU derivatives is greater from the mixture containing CCNU-d_6 than from CCNU (Table IV), whereas, for the d_4 isomer, the reverse is true. There is no evidence for metabolic switching favoring C-3 and C-4 hydroxylation as a consequence of deuteration at C-2 (CCNU-d_4) (although anomalously *trans*-3-OH-CCNU formed on metabolism of CCNU + CCNU-d_4 was actually depleted in the deuterated analogue), but there is a switching away from 3- and 4-hydroxylation for the d_6 analogue. Similar conclusions were reached from the results of experiments on the separate metabolism of CCNU and its d_4 and d_6 analogues. There is a small isotope effect for the overall metabolism since the CCNU recovered following metabolism of each mixture was depleted in the protium form.

The studies reported here confirm that the effect of deuteration in the cyclohexyl moiety on the total yield of hydroxy derivatives is slight but that selective deuteration can markedly alter the relative proportions of individual metabolites.

Antitumor Testing. Each of the deuterated derivatives of CCNU was tested in parallel with CCNU against the TLX-5 lymphoma in mice by the previously described protocol[35] and the results are given in Table VII. No significant differences in antitumor activity were observed, the compounds all giving high extensions of survival time at doses from 10 to 40 mg/kg. It is possible that the changes in metabolic profile induced by deuteration of CCNU may not be sufficiently large to cause observable differences of activity in vivo.

Experimental Section

Melting points were determined with a Kofler hot-stage apparatus and are corrected. NMR spectra were recorded with a Perkin-Elmer R-10 spectrometer operating at 60 MHz (internal Me_4Si) and UV spectra with a Pye Unicam SP 800A spectrophotometer. Mass spectra were determined using an AEI MS-12 instrument by the direct insertion method with a source temperature of 80 °C and an ionizing voltage of 12 eV; for isotope ratio measurements repeated scans were taken over a limited mass range. Merck Kieselgel GF_{254} coated on glass plates was used for thin-layer chromatography (TLC).

HPLC was performed at room temperature with a Spectra Physics Model 3500B liquid chromatograph, flow UV monitor, recorder, and System I integrator utilizing an analytical column (3.2 × 250 mm) packed with LiChrosorb Si 60, 5-μ particle size.[9] CCNU substrates were isolated and quantified by elution with *n*-heptane–dichloromethane–propan-2-ol (980:15:8) at 1.9 mL/min. Analytical HPLC of OH-CCNU isomers was effected with the same solvents but in a ratio of 960:63:35 and at 1.9 mL/min except for the data in Figure 1 which was obtained using 3,3,5-trimethylpentane–dichloromethane–propan-2-ol (930:63:30) at 1.8 mL/min. Preparative separations of the OH-CCNU isomers were with a column (10 × 250 mm) of LiChrosorb Si 60 (5-μ particle size), elution with *n*-heptane–dichloromethane–propan-2-ol (960:63:35) at 4 mL/min, and a UV monitor at 300 nm.

Cyclohexanone-2,2′,6,6′-d_4 (8). Cyclohexanone (7.5 g, 0.077 mol) was heated under reflux with 1 M DCl–D_2O (40 mL) for 30 min. The organic layer was separated and the aqueous phase was extracted with Et_2O (2 × 30 mL). The combined extracts were washed with saturated aqueous $NaHCO_3$ (10 mL) and then water (10 mL) and dried (Na_2SO_4), and Et_2O was removed by distillation under reduced pressure. The deuterium exchange procedure was repeated and distillation then gave **8** (2.25 g, 30%): bp 40 °C (20 mm) [lit.[36] for cyclohexanone bp 47 °C (15 mm)]; mass spectrum *m/e* 102 (100, M^+·), 101 (8) [cyclohexanone showed *m/e* 98 (100, M^+·), 97 (5)]; NMR ($CDCl_3$) δ 1.82 (m, H-3,3′, H-4,4′, H-5,5′) and 2.57 (m, trace, d_3 analogue). Cyclohexanone showed δ 1.82 (m, 6 H, H-3,3′, H-4,4′, H-5,5′) and 2.35 (m, 4 H, H-2,2′, H-6,6′).

Cyclohexanone-2,2′,3,3′,4,4′,5,5′,6,6′-d_{10} (6). To a cooled (2 °C) solution of cyclohexanol-d_{12} (**5**, Aldrich, 5 g, 0.045 mol, d_{12}:d_{11} ratio 100:9.5) in C_6H_6 (50 mL) a mixture of sodium dichromate (4.76 g, 0.016 mol), AcOH (3.6 mL), H_2SO_4 (6.4 mL), and H_2O (21 mL) was added with stirring at a rate such that the temperature did not exceed 5 °C. The mixture was then stirred overnight at room temperature. The C_6H_6 layer was separated and the aqueous layer was extracted with C_6H_6 (2 × 30 mL). The combined C_6H_6 solutions were washed with water (25 mL), saturated aqueous $NaHCO_3$ (2 × 25 mL), and with water (25 mL) and then dried ($MgSO_4$). The C_6H_6 was removed at <35 °C (20 mm) to give the crude product as an oil (4.5 g, 93%): mass spectrum (12 eV) *m/e* 108 (100, M^+·), 107 (45). This material (3.64 g, 0.034 mol) was subjected to treatment with 1 M DCl–D_2O as described above for the preparation of **8** and distilled to yield **6** (2.26 g, 62%): bp 153–155 °C (lit.[36] for cyclohexanone 155 °C); mass spectrum *m/e* 108 (100, M^+·), 107 (9).

Cyclohexanone-3,3′,4,4′,5,5′-d_6 (7). Crude **6** (2.51 g, 0.023 mol) was heated under reflux with 1 M HCl (300 mL) for 300 min and the product was isolated as described for **8** and distilled to give **7** (1.78 g, 74%): bp 152–155 °C; mass spectrum *m/e* 104 (100, M^+·), 103 (9); NMR ($CDCl_3$) δ 1.60 (m, trace d_5 analogue) and 2.32 (m, H-2,2′, H-6,6′).

Cyclohexylamine-3,3′,4,4′,5,5′-d_6 (10). A solution of **7** (1.78 g, 0.017 mol), NH_4OAc (13.2 g, 0.17 mol), and sodium cyanoborohydride[27] (Aldrich, 0.76 g, 0.012 mol) in MeOH (AR, 60 mL) was stirred at room temperature in the presence of molecular sieves Type 4A. After 48 h, HCl was added to pH <2 and the

solvent was removed under reduced pressure. A solution of the solid residue in H_2O (30 mL) was extracted with Et_2O (2 × 30 mL), then basified (pH >10) with NaOH, saturated with NaCl, and extracted with Et_2O (5 × 50 mL). The combined extracts were dried ($MgSO_4$) and concentrated to a clear oil which was distilled giving **10** (1.26 g, 70%): bp 120–140 °C (lit.[36] 134 °C); mass spectrum ($M^{+\cdot}$) m/e 105:104, 100:16 [cyclohexylamine showed $M^{+\cdot}$ at m/e 99 (78.4% of base peak at m/e 56); ratio m/e 99:98, 100:7].

Cyclohexylamine-2,2′,3,3′,4,4′,5,5′,6,6′-d_{10} (9) and Cyclohexylamine-2,2′,6,6′-d_4 (11). The preparation of **9** and **11** was carried out as described for **10** except that the sodium cyanoborohydride was purified as its dioxane complex[27] and regenerated by drying for 4 h at 110 °C (5 mm) immediately before use: mass spectra for **9**, m/e 109 (100. $M^{+\cdot}$), 108 (28); for **11** m/e 103 (100, $M^{+\cdot}$), 102 (19).

1-(2-Chloroethyl)-3-(cyclohexyl-2,2′,3,3′,4,4′,5,5′,6,6′-d_{10})urea (13), 1-(2-Chloroethyl)-3-(cyclohexyl-3,3′,4,4′,5,5′-d_6)urea (14), and 1-(2-Chloroethyl)-3-(cyclohexyl-2,2′,6,6′-d_4)urea (15). In a typical reaction 2-chloroethyl isocyanate (**12**, Eastman, 1.1 mL, 1.26 g, 0.012 mol) was added dropwise during 30 min to a solution of **10** (1.26 g, 0.012 mol) in dry $CHCl_3$ (80 mL) at 0 °C. The mixture was then stirred overnight at room temperature. Solvent was removed under vacuum and the residual pink solid was triturated with petroleum ether (bp 30–40 °C), dried in vacuo over P_2O_5, and recrystallized from MeCN to give **14** as white prisms (1.05 g, 40%): mp 114–115 °C (lit. 130,[2] 130–132 °C[37]). [Unlabeled 1-(2-chloroethyl)-3-cyclohexylurea, prepared as above and recrystallized from MeCN, had mp 115.5–116.5 °C and was analytically pure. Anal. ($C_9H_{17}ClN_2O$) C, H, Cl, N. The melting point values reported[2,37] for this compound are for material which was uncrystallized[2] or recrystallized from EtOAc–hexane.[37]] Likewise **9** and **11** were converted into **13** (35%, mp 118–119 °C) and **15** (42%, mp 115–116 °C).

1-(2-Chloroethyl)-3-(cyclohexyl-2,2′,3,3′,4,4′,5,5′,6,6′-d_{10})-1-nitrosourea (2), 1-(2-Chloroethyl)-3-(cyclohexyl-3,3′,4,4′,5,5′-d_6)-1-nitrosourea (3), and 1-(2-Chloroethyl)-3-(cyclohexyl-2,2′,6,6′-d_4)-1-nitrosourea (4). Nitrosation of the ureas **13–15** carried out under anhydrous conditions as described by Johnston et al.[2] gave **2–4**, respectively, as light yellow solids which were homogeneous by TLC: R_f 0.65 ($CHCl_3$), 0.54 (CH_2Cl_2), identical with those of CCNU. **2** had mp 88–89.5 °C; ratio m/e 243 ($M^{+\cdot}$):242, 100:20. **3** had mp 89–90 °C; ratio m/e 239 ($M^{+\cdot}$):238, 100:10. **4** had mp 86–87 °C; ratio m/e 237 ($M^{+\cdot}$):236, 100:11. CCNU had mp 90 °C and ratio m/e 233 ($M^{+\cdot}$):232, 100:0.5.

Metabolism and Biological Testing. The preparation of microsomes from the livers of male Wistar rats (pretreated with sodium phenobarbital) and the method for cytotoxicity assays of compounds following their incubation with Walker 256 ascites tumor cells in vitro have been described previously.[28] In vivo antitumor testing against the TLX-5 lymphoma in mice was carried out as previously described;[35] deuterated compounds were administered ip in 10% EtOH in arachis oil and parallel control tests with CCNU were carried out for each compound tested.

Incubations. Incubations (10 mL) were performed under oxygen in stoppered 25-mL flasks, shaken gently at 37 °C. Each flask contained microsomes (active or, for controls, inactivated by heating to 80 °C for 7 min) equivalent to 1 g of liver, NADP (3.4 μmol), glucose 6-phosphate (83 μmol), $MgCl_2$ (49 μmol), and glucose-6-phosphate dehydrogenase (1.4 units) all in phosphate buffer (0.1 M, pH 7.4). The protein concentration was 2.1 mg/mL.

When the comparative rate of metabolism of CCNU and its d_{10} analogue was measured, the concentration of each substrate was 50 μg/mL (0.21 mM). Duplicate samples (1 mL) taken before (0 °C) and at various times during incubation (37 °C) were shaken with hexane (2.1 mL) containing 50 μg of the internal standard (CCNU-d_4). The hexane extracts were dried under a stream of nitrogen and the residue was subjected to mass spectrometry. The intensities of the peaks (average of at least five scans) for the appropriate ions **16** [m/e 126 (d_0) and 136 (d_{10})] compared to that of the standard added [m/e 130 (d_4)] were used to calculate the amount of CCNU-d_0 and -d_{10} present in the mixture, employing calibration lines for known quantities of the pure compounds.

For metabolite isolation, the substrate concentrations were 140 μg/mL (0.6 mM) for incubations of individual isomers or 70 μg/mL (0.3 mM) each for competitive metabolism of CCNU-(d_0 + d_4) and -(d_0 + d_6). After incubation for 10 min under the above conditions the combined products (20–40 mL for each substrate or pair of substrates) were extracted with hexane (3 × 20–40 mL; spectrograde) and then with Et_2O (3 × 30–60 mL). The hexane and Et_2O extracts, which contained CCNU and OH-CCNU derivatives, respectively, were concentrated and subjected to HPLC for quantification (analytical column) and isolation (preparative column) of components. The unchanged starting material and isolated OH-CCNU isomers derived from partial metabolism of CCNU-(d_0 + d_4) and -(d_0 + d_6) were subjected to mass spectrometry and the ratios of the appropriate ions in the ranges m/e 83–89 or 126–132 for each CCNU and m/e 142–148 for OH-CCNU derivatives (**17**) were determined. The last-mentioned ions comprised m/e 142 for the unlabeled OH-CCNU derivatives, m/e 145 (*trans*-2-OH-CCNU) and 146 (*cis*- and *trans*-3- or -4-OH-CCNU) for the products from CCNU-d_4, and m/e 147 (*cis*- and *trans*-3- or -4-OH-CCNU) and 148 (*trans*-2-OH-CCNU) for the products from CCNU-d_6. Where possible, ratios for mixtures of known composition were used to calculate the molar ratio of species present in the mixtures. Thus, 2-OH-CCNU-d_0, -d_3, and -d_6, 3-OH-CCNU-d_0, -d_4, and -d_5, and 4-OH-CCNU-d_0, -d_4, and -d_5 were all available from the metabolism of the individual deuterated CCNU derivatives, and, where quantities were sufficient (*cis*-3- and *cis*-4-OH-CCNU derivatives), mass spectra were determined on appropriate 1:1 mixtures, made up according to UV extinction (230 nm). The mass spectra of the individual OH-CCNU derivatives (both deuterated and nondeuterated) were also checked for the absence of significant cross contributions between diagnostic ions. For example, m/e 146 was absent from the mass spectrum of *cis*-4-OH-CCNU and m/e 142 was only minor (3% of m/e 146) in the spectrum of the d_4 analogue. Analogous conclusions applied to the other more abundant OH-CCNU derivatives and also to 2-OH-CCNU and its d_6 analogue.

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References and Notes

(1) A preliminary report of part of this study was presented at the Symposium on Stable Isotopes: Applications in Pharmacology, Toxicology and Clinical Research, London, 1977 ("Stable Isotopes", T. A. Baillie, Ed., Macmillan, London, in press).
(2) T. P. Johnston, G. S. McCaleb, P. S. Opliger, and J. A. Montgomery, *J. Med. Chem.*, **9**, 892 (1966).
(3) (a) T. H. Wasserman, M. Slavik, and S. K. Carter, *Cancer Treatment Rev.*, **1**, 131 (1974); (b) EORTC Brain Tumor Group, *Eur. J. Cancer*, **12**, 41 (1976).
(4) B. Schmall, C. J. Cheng, S. Fujimura, N. Gersten, D. Grunberger, and I. B. Weinstein, *Cancer Res.*, **33**, 1921 (1973).
(5) D. J. Reed, H. E. May, R. B. Boose, K. M. Gregory, and M. A. Beilstein, *Cancer Res.*, **35**, 568 (1975).
(6) M. Colvin, R. B. Brundrett, W. Cowens, I. Jardine, and D. B. Ludlum, *Biochem. Pharmacol.*, **25**, 695 (1976).
(7) H. E. May, R. Boose, and D. J. Reed, *Biochem. Biophys. Res. Commun.*, **57**, 426 (1974).

(8) D. L. Hill, M. C. Kirk, and R. F. Struck, *Cancer Res.*, **35**, 296 (1975).
(9) H. E. May, R. Boose, and D. J. Reed, *Biochemistry*, **14**, 4723 (1975).
(10) D. J. Reed and H. E. May, *Life Sci.*, **16**, 1263 (1975).
(11) J. Hilton and M. D. Walker, *Biochem. Pharmacol.*, **24**, 2153 (1975).
(12) G. P. Wheeler, T. P. Johnston, B. J. Bowden, G. S. McCaleb, D. L. Hill, and J. A. Montgomery, *Biochem. Pharmacol.*, **26**, 2331 (1977).
(13) J. Hilton and M. D. Walker, *Proc. Am. Assoc. Cancer Res.*, **16**, 103 (1975).
(14) T. P. Johnston, G. S. McCaleb, and J. A. Montgomery, *J. Med. Chem.*, **18**, 634 (1975).
(15) J. A. Montgomery, *Cancer Treatment Rep.*, **60**, 651 (1976).
(16) P. S. Schein, M. J. O'Connell, J. Blom, S. Hubbard, I. T. Magrath, P. Bergevin, P. H. Wiernik, J. L. Ziegler, and V. T. DeVita, *Cancer Res.*, **34**, 993 (1974).
(17) T. Anderson, M. O. McMenamin, and P. S. Schein, *Cancer Res.*, **35**, 761 (1975).
(18) S. D. Nelson and L. R. Pohl, *Annu. Rep. Med. Chem.*, **12**, 319 (1977).
(19) M. G. Horning, K. D. Haegele, K. R. Sommer, J. Nowlin, M. Stafford, and J-P. Thenot, *Proc. Int. Conf. Stable Isot. Chem., Biol., Med., 2nd*, 41 (1975).
(20) C. Mitoma, R. L. Dehn, and M. Tanabe, *Biochim. Biophys. Acta*, **237**, 21 (1971).
(21) A. B. Foster, M. Jarman, J. D. Stevens, P. Thomas, and J. H. Westwood, *Chem.-Biol. Interact.*, **9**, 327 (1974).
(22) T. A. Connors, P. J. Cox, P. B. Farmer, A. B. Foster, M. Jarman, and J. K. MacLeod, *Biomed. Mass Spectrom.*, **1**, 130 (1974).
(23) P. B. Farmer, A. B. Foster, and M. Jarman, *Biomed. Mass Spectrom.*, **2**, 107 (1975).
(24) L. J. Griggs and M. Jarman, *J. Med. Chem.*, **18**, 1102 (1975).
(25) P. J. Cox, P. B. Farmer, A. B. Foster, E. D. Gilby, and M. Jarman, *Cancer Treatment Rep.*, **60**, 483 (1976).
(26) P. J. Cox, P. B. Farmer, A. B. Foster, L. J. Griggs, M. Jarman, R. Kinas, K. Pankiewicz, and W. J. Stec, *Biomed. Mass Spectrom.*, **4**, 371 (1977).
(27) R. F. Borch, M. D. Bernstein, and H. D. Durst, *J. Am. Chem. Soc.*, **93**, 2897 (1971).
(28) T. A. Connors, P. J. Cox, P. B. Farmer, A. B. Foster, and M. Jarman, *Biochem. Pharmacol.*, **23**, 115 (1974).
(29) V. Ullrich, *Hoppe-Seyler's Z. Physiol. Chem.*, **350**, 357 (1969).
(30) C. Mitoma, D. M. Yasuda, J. Tagg, and M. Tanabe, *Biochem. Biophys. Acta*, **136**, 566 (1967).
(31) R. E. McMahon, H. R. Sullivan, J. C. Craig, and W. E. Pereira, *Arch. Biochem. Biophys.*, **132**, 575 (1969).
(32) N. Tanaka and E. R. Thornton, *J. Am. Chem. Soc.*, **98**, 1617 (1976).
(33) P. D. Klein, *Adv. Chromatogr.*, **3**, 3 (1966).
(34) A. M. Jeffrey, S. H. Blobstein, I. B. Weinstein, and R. G. Harvey, *Anal. Biochem.*, **73**, 378 (1976).
(35) T. A. Connors and M. Jones, *Recent Res. Cancer Res.*, **33**, 181 (1970).
(36) "Dictionary of Organic Compounds", 4th ed, Eyre and Spottiswoode, London, 1965.
(37) H. Najer, P. Chabrier, and R. Giudicelli, *Bull. Soc. Chim. Fr.*, 352 (1959).
(38) P. J. Cox, B. J. Phillips, and P. Thomas, *Cancer Res.*, **35**, 3755 (1975).

New Inhibitors of Platelet Aggregation. 5′-Phosphate, 5′-Phosphorothioate, and 5′-*O*-Sulfamoyl Derivatives of 2-Substituted Adenosine Analogues

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Analogues of AMP incorporating modifications of the adenine moiety and the phosphate function were synthesized as potential inhibitors of platelet aggregation. 2-Methoxy-, 2-ethoxy-, 2-methylthio-, 2-ethylthio-, 2-methylamino-, 2-ethylamino-, 2-trifluoromethyl-, 2-chloro-N^6-methyl-, and 2-methylthio-N^6-methyladenosines were converted, via 2′,3′-*O*-isopropylidene derivatives, to the 5′-monophosphates using 2-cyanoethyl phosphate and DCC. The isopropylidene derivatives of adenosine, 2-chloroadenosine, and 2-methylthioadenosine were also used to synthesize the respective 5′-*O*-sulfamoyladenosines by reaction with NaH and sulfamoyl chloride and subsequent deblocking. In addition, 2-chloroadenosine 5′-phosphorothioate, 2-methylthioadenosine 5′-phosphorothioate, and 2-ethylthioadenosine 5′-phosphorothioate were prepared from the unprotected nucleosides by treatment with $PSCl_3$ in triethyl phosphate. With the exception of the 5′-*O*-sulfamates of adenosine and 2-chloroadenosine, all the compounds tested inhibited the ADP-induced aggregation of sheep platelets. The 5′-phosphates and phosphorothioates of 2-methylthio- and 2-ethylthioadenosine were 2–13 times more potent than adenosine; the remaining 2- and N^6-substituted phosphates and phosphorothioates were less potent than adenosine. 5′-*O*-Sulfamoyladenosine and 2-chloro-5′-*O*-sulfamoyladenosine potentiated ADP-mediated platelet aggregation but the three 5′-*O*-sulfamates inhibited serotonin-induced platelet aggregation. In contrast, all the 5′-phosphate and 5′-phosphorothioate analogues tested had negligible activity as inhibitors of serotonin-induced sheep platelet aggregation.

The aggregation of blood platelets in response to vascular insult plays a central role in the formation of arterial thrombi. Collagen exposed by injury to blood vessels, and thrombin and ADP (adenosine 5′-diphosphate) produced as a result of such injury, are the primary agents which cause platelets to clump together in vivo.[1-3] In vitro, platelet aggregation can be induced by a variety of stimuli including collagen, thrombin, serotonin, ADP,[1] and 2-substituted ADP analogues.[4] Drugs that inhibit platelet aggregation have potential for use both as antithrombotic

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