46. Synthesis and Biological Activities of C(5)-N-Spin-Labeled Uridines and Related Derivatives

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Dedicated to Prof. Dr. *Max Viscontini* on the occasion of his 70th birthday

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Summary

Direct introduction of a N-atom in one step at C(5) of 5-hydroxyuridine (**4a**) or 5-hydroxy-2′-deoxyuridine (**4b**) by certain primary amines led to the synthesis of two novel C(5)-N-spin-labeled nucleoside analogs and to several C(5)-N-aryl adducts. Substitution by 4-amino-2,2,6,6-tetramethylpiperidinooxyl (**3**) at C(5) of **4a** or **4b** led to the spin-labeled nucleosides 5-[(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)amino]uridine and -2′-deoxyuridine (**2a** and **2b**, respectively). The analogous C(5)-substituted aniline adducts 5-anilino uridine (**5a**) and 5-anilino-2′-deoxyuridine (**5b**) and the *p*-toluidine adducts 5-(*p*-toluidino)uridine (**6a**) and 5-(*p*-toluidino)-2-deoxyuridine (**6b**) were also prepared. In addition, results of the antiviral and antimetabolic activity of some of these analogs are reported.

A large variety of 5-substituted 2′-deoxyuridines (dUrd) have been synthesized which are all effective inhibitors of virus replication in cell culture [1]. To further delineate the structural parameters that govern the antiviral potential of 5-substituted dUrd, we have synthesized and evaluated an additional series of dUrd-analogs, and their uridine (Urd) counterparts, for their inhibitory effects on *Herpes simplex* virus (HSV) and *vaccinia* virus replication in cell culture. The previously prepared C(5)-spin-labeled synthetic nucleoside gt-dUrd[2]) (**1**) [2] which showed some activity against leukemia P388 cells [3] can be considered as a C(5)-O-substituted dUrd. The newly synthesized compounds ta-Urd (**2a**) and ta-dUrd (**2b**) belong to the class of the C(5)-N-substituted nucleosides. A facile synthesis of **2a** and **2b** was accomplished by condensation of 4-amino-2,2,6,6-tetramethyl-piperidinooxyl (**3**) with 5-hydroxy-Urd (**4a**) or with 5-hydroxy-dUrd (**4b**),

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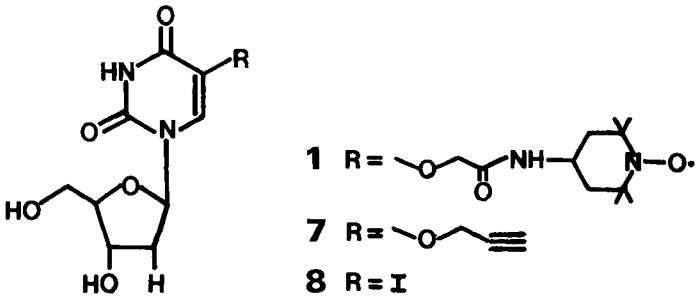
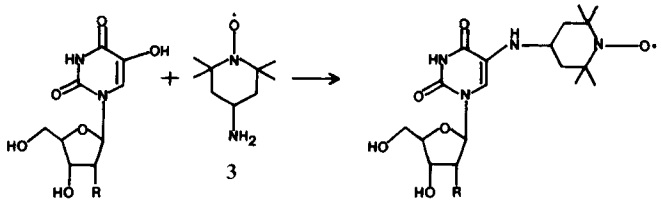
[2]) For convenience 5-[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)carbamoyl]methoxy-2′-deoxyuridine (**1**), and 5-[(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)amino]uridine (**2a**) and -2′-deoxyuridine (**2b**) are abbreviated as gt-dUrd (**1**), ta-Urd (**2a**), and ta-dUrd (**2b**).



respectively, under acidic conditions. In addition, several aromatic analogs of **2a** and **2b** were synthesized to generalize the synthetic approach described here. Condensation with aniline gave 5-anilino-Urd (**5a**) and 5-anilino-dUrd (**5b**), whereas condensation with *p*-toluidine yielded 5-(*p*-toluidino)-Urd (**6a**) and 5-(*p*-toluidino)-dUrd (**6b**). The antiviral and antimetabolic activities of the novel compounds are also compared with those of the established antiviral agents 5-(2-propynyl)oxy-dUrd (**7**), 5-iodo-dUrd (**8**), and (*E*)-5-(2-bromovinyl)-dUrd.

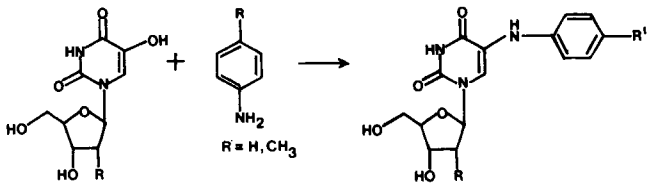
Results. – The spin-labeled nucleosides **2a** and **2b** were readily formed in dilute aqueous acid by condensation of **3** with **4a** or **4b**, respectively, as shown in *Scheme 1*. These compounds were isolated as amorphous orange powders. The long-wavelength absorbance-maxima of their base moieties were red-shifted in comparison with Urd and dUrd. Since conventional NMR. spectra of paramagnetic species are usually not well-resolved [4], the ^{1}H-NMR. spectra of **2a** and **2b** were

Scheme 1



4a R = OH
4b R = H

2a R = OH
2b R = H



4a R = OH
4b R = H

5a R = OH; R′ = H
5b R = H; R′ = H
6a R = OH; R′ = CH_3
6b R = H; R′ = CH_3

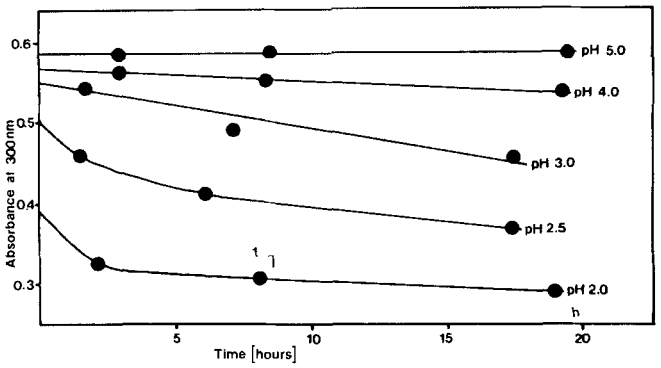


Figure. *Time dependence of the 300-nm UV. absorbance maximum of* **2a** *at various pH-values*

observed in (D_6)DMSO after conversion of the aminooxyl to the corresponding *N*-hydroxylamine derivative with sodium dithionite [5]. A table containing these data has been previously reported [5]. These results indicated monosubstitution by the spin label at C(5).

The spin-labeled nucleosides **2a** and **2b** were stable in alkali, but they were relatively unstable in acid. The *Figure* summarizes the effect of pH on the absorbance maximum of **2a** at 300 nm over a 20 hour time period. No change was observed at pH 5 or greater, but decay of the 300-nm absorbance maximum was accelerated with decreasing pH. Additionally, no new chromophores were observed between 240–360 nm as a result of decay. The chemical events which caused the observed decay were not investigated. For comparison with Urd, the pK_a-values of **2a** were determined spectrophotometrically. The N(3)–H in the ring of **2a** had essentially the same pK_a as that of uridine (pK_a = 9.1), whereas the exocyclic amine group at C(5) of **2a** had a pK_a = 1.6 ± 0.1.

Attempts to form the C(5) adducts of **4a** with cyclohexylamine or with 2-aminoethanol in dilute aqueous acid did not yield the expected products. Since cyclohexylamine and 2-aminoethanol have pK_a values which are greater than 10, whereas **3** has pK_a = 8.6 ± 0.2 (potentiometrically determined), it appears that the pK_a of the starting primary amine must be less than 9–10 under the described reaction conditions. Furthermore, as shown in *Scheme 1*, aniline (pK_a = 4.63) and *p*-toluidine (pK_a = 5.08) reacted readily with **4a** or **4b** to give **5a** and **5b**, and **6a** and **6b**, respectively, and the latter analogs were isolated in the crystalline state. The long-wavelength UV. maxima of the base moieties were also red-shifted. The assigned structures of the latter compounds were corroborated by ^{1}H-NMR., and the observed chemical shift values are listed in *Table 1*. The pK_a-value of the exocyclic amino-group of the aromatic adduct **5a** was found to be below 0, and it was noted that compound **5a**, in contrast to **2a**, was stable in aqueous solutions below pH 5.

The fluorescence emission spectra of **5a** were measured at pH values of 5.6 and 8.0, since certain C(5)-aryl-substituted nucleosides in which the C(5),C(6)-double bond is in conjugation with the aromatic moiety can show strong fluorescence [6]. However, only weak fluorescence was observed.

Table 1. *^{1}H-NMR. assignments in (D_6)DMSO* (chemical shifts in ppm relative to tetramethylsilane (= 0 ppm) *s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet, br. = broad)[a][b])

Compound	H–N(3)	H–C(6)	H-Ar	HN-Ar	H–C(1′)
5a	11.53	7.85	7.0	7.07	5.93
	br. *s*	*s*	*m*, 5 H	*s*	*d*
5b	11.60	7.84	7.0	7.0	6.30
	br. *s*	*s*	*m*, 5 H	br.	*t*
6a	11.53	7.77	6.9	6.86	5.92
	br. *s*	*s*	*m*, 4 H	*s*	*d*
6b	11.50	7.67	6.9	6.85	6.25
	br. *s*	*s*	*m*, 4 H	*s*	*t*

Compound	HO–C(3′)	HO–C(5′)	HO–C(2′)	H–C(2′)	H–C(3′)	H–C(4′)	H_2C(5′)	H_3C-Ar
5a	5.38		5.05		4.0		3.6	
	d		*m*, 2 H		*m*, 3 H		*m*, 2 H	
5b		5.0		2.15	4.3	3.83	3.58	
		b, 2		*m*, 2 H	*m*	*m*	*m*, 2 H	
6a	5.35		5.05		4.0		3.6	2.20
	d		*m*, 2 H		*m*, 3 H		*m*, 2 H	*s*, 3 H
6b	5.22	4.93		2.14	4.3	3.80	3.57	2.20
	d	*t*		*m*, 2 H	*m*	*m*	*m*, 2 H	*s*, 3 H

[a]) Absorption signals indicating more than one H-atom are listed in number below the corresponding chemical shift values.

[b]) Chemical shift values for **2a** and **2b** have been previously reported [5].

The dUrd-analogs **1**, **2b**, **5a**, **5b**, and **6b** were evaluated for their biological properties. Of these nucleoside analogs only one compound, 5-anilino-dUrd (**5b**), exhibited an appreciable antiviral activity. This activity was more pronounced with *vaccinia* virus than with HSV-1 or HSV-2 *(Table 2)*. Moreover, **5b** was much less active against HSV-1 and HSV-2 than the reference compounds 5-(2-propynyl)oxy-dUrd (**7**), 5-iodo-dUrd (**8**), and (*E*)-5-(2-bromovinyl)-dUrd. Also, **5b** inhibited HSV-1 and *vaccinia* virus replication at a concentration which was 2.5- to 5-fold

Table 2. *Antiviral activity of 5-substituted nucleosides in PRK cell cultures*[a])

Compound	HSV-1 (strain KOS)	HSV-1 (strain F)	HSV-1 (strain McIntyre)	HSV-2 (strain Lyons)	HSV-2 (strain G)	HSV-2 (strain 196)	Vaccinia virus
5-Anilino-dUrd (**5b**)	30	30	30	60	30	100	15
5-Anilino-Urd (**5a**)	> 400	> 400	> 400	> 200	> 400	> 400	> 400
5-(*p*-Toluidino)-dURD (**6b**)	> 400	> 400	> 400	> 400	> 400	> 400	> 400
ta-dUrd (**2b**)[2])	> 400	> 400	> 400	> 400	> 400	> 400	> 400
gt-dUrd (**1**)[2])	> 40	> 40	> 40	> 40	> 40	> 40	> 40
5-Iodo-dUrd (**8**)[b])	0.15	0.15	0.15	0.2	0.4	0.2	0.3
(*E*)-5-(2-bromovinyl)-dUrd[b])	0.007	0.007	0.009	1	1	1.5	7
5-(2-Propynyl)oxy-dUrd (**7**)[b])	1	0.7	1	10	40	7	20

[a]) The results are expressed as the minimal inhibitory concentration (μg/ml), *i.e.*, the concentration required to inhibit virus-induced cytopathogenicity by 50%.

[b]) For **8**, (*E*)-5-(2-bromovinyl)-dUrd and **7** the data are taken from [9].

Table 3. *Antimetabolic activity of 5-substituted nucleosides in PRK cell cultures*[a])

Compound	(^{3}H-methyl)dThd incorporation	(^{3}H-1′,2′)dUrd incorporation
5-Anilino-dUrd (**5b**)	75	100
5-Anilino-Urd (**5a**)	175	300
5-(*p*-Toluidino)-dUrd (**6b**)	7.5	42
ta-dUrd (**2b**)[2])	175	350
5-Iodo-dUrd (**8**)[b])	1–2.5	0.25
(*E*)-5-(2-bromovinyl)-dUrd[b])	70	20
5-(2-Propynyl)oxy-dUrd (**7**)[b])	75–150	75

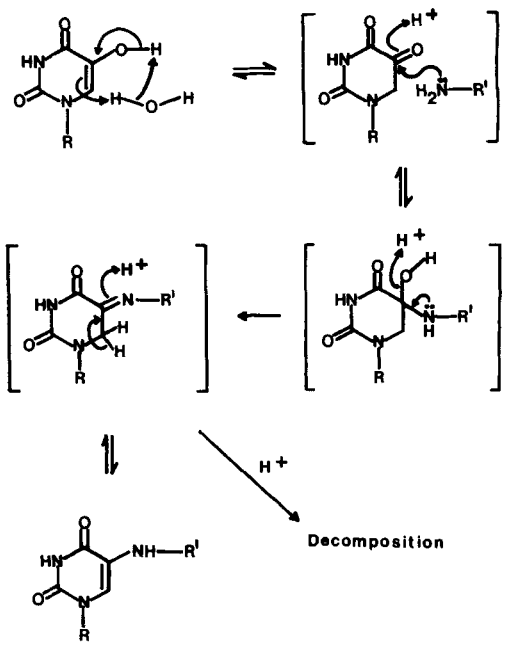
[a]) The results are expressed as the inhibitory dose – 50 (μg/ml), *i.e.*, the concentration required to inhibit (methyl-$^2H_3$)dThd or (1′,2′-$^3H_2$)dUrd incorporation by 50%.

[b]) For **8**, (*E*)-5-(2-bromovinyl)-dUrd and **7** the data are taken from [1].

lower than the concentration required to inhibit (methyl-$^3H_3$)dThd-incorporation and 3.3- to 6.7-fold lower than the concentration required to inhibit (1′,2′-$^3H_2$)dUrd incorporation *(Table 3)*. This contrasted with the behavior of 5-(*p*-toluidino)-dUrd (**6b**), which, despite its inactivity as an antiviral agent, proved inhibitory to dThd- and dUrd-incorporation at a concentration of 7.5 and 42 μg/ml, respectively.

Discussion. – The reaction of **4a** with amines to form the C(5)–N-substituted analogs probably involves the acid-catalyzed formation of the C(5)-keto-tautomer, which then suffers attack at C(5) by the primary amine, as illustrated in *Scheme 2*.

Scheme 2



Since the nucleoside aminooxyl adducts **2a** and **2b** are unstable in acid, the reaction may be expected to occur reversibly. This is not the case, however, since **4a** was not formed from **2a** at low pH. Consequently the decomposition mechanism cannot be the simple reverse case of the proposed reaction scheme. In contrast, the spin-labeled nucleosides **2a** and **2b** are stable at neutral and basic pH, whereas 5-amino-uridine is not [7]. Thus, the stability of the spin-labeled nucleosides above pH 5 should make these molecules valuable spin probes to study biological systems involving nucleic acid residues. This novel procedure may also be useful for the preparation of nucleoside analogs with affinity labels [8] or amino acids at C(5).

Some dUrd-analogs, including 5-fluoro-, 5-bromo-, 5-iodo-, 5-hydroxy- and 5-thiocyano-dUrd, inhibit several DNA viruses, *e.g., herpes simplex* virus type 1 (HSV-1) and type 2 (HSV-2) and *vaccinia* virus, to the same extent. Other dUrd-derivatives, such as 5-cyano-, 5-formyl-, 5-amino- and 5-nitro-dUrd, are more inhibitory to *vaccinia* than HSV, while 5-(2-propynyl)oxy- and 5-(2-halogenovinyl)-dUrd-derivatives inhibit HSV-replication to a greater extent than *vaccinia* virus replication. The 5-(2-halogenovinyl)-dUrd derivatives even discriminate between HSV-1 and HSV-2 in that they inhibit the former to a significantly greater extent than the latter [1] [9]. It is interesting to note that the anti*vaccinia* and anti*herpes* activity of 5-anilino-dUrd (**5b**) was remarkably similar to that of the structurally related 5-styryl-dUrd [10], although **5b** appeared more selective in its antiviral action than 5-styryl-dUrd [10].

The antiviral data of *Table 2* indicate that the relative size of the C(5)-substituent of the aromatic analog is an important factor which influences the antiviral activity. Replacement of the *para*-H-atom in **5b** with the methyl group in **6b** abolishes the antiviral activity. A similar loss of antiviral activity has been observed previously upon addition of a nitro group to the benzene ring of 5-styryl-dUrd [10]. One may conclude, therefore, that the antiviral properties of 5-substituted analogs of dUrd can be influenced by steric and/or configurational aspects of the C(5)-substituent. This notion is further strengthened by the inactivity of 5-benzyloxy-dUrd [11], 5-(*p*-nitrobenzyloxy)-dUrd [11] and gt-dUrd (**1**) *(Table 2),* relative to the marked antiviral activity recorded for 5-(2-propynyl)oxy-dUrd [11] and 5-(cyanomethylene)oxy-dUrd [12].

Finally, it has been postulated that dUrd-analogs which inhibit dUrd-incorporation to a significantly greater extent than dThd-incorporation selectively block thymidylate synthetase [13]. Based on this premise, none of the new nucleoside analogs would qualify as a specific inhibitor of thymidylate synthetase, since neither compound showed a greater inhibitory effect on dUrd- than dThd-incorporation *(Table 3).*

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Experimental Part

Synthesis. – *General remarks.* The 5-Hydroxyuridine and 5-hydroxy-2′-deoxyuridine were prepared as described in [3]. The 4-amino-2,2,6,6-tetramethylpiperidinooxyl was purchased from *Aldrich.* Reagent-grade aniline and *p*-toluidine were obtained from *Fisher,* and were used without further purification. Melting points (m.p.) were determined with a *Mel-Temp* and are uncorrected. UV. spectra (λ_{max} in nm (ε)) were recorded with a *Cary 14,* ^{1}H-NMR. spectra with a *Varian T-60,* fluorescence spectra with a *Perkin-Elmer 650-10S,* mass spectra (*m/z*) with a *Perkin-Elmer RMU-7.*

5-[(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)amino]uridine (**2a**). To 100 mg (0.38 mmol) of **4a** in 0.5 ml of water were added 172 mg (1.0 mmol) of **3**. The solution was adjusted to pH 5 with 1N H_2SO_4, and heated in an open vial in a boiling water bath for a total of 7.5 h, while adding back sufficient water to maintain the volume between 0.5–1 ml. The reaction mixture was adjusted to pH 5 with 1N H_2SO_4 after 3.5 h. After 7.5 h the reaction mixture was cooled, one drop of 6N NaOH was added, and the solvent was removed under diminished pressure at 35–37°. The residue was evaporated from dry MeOH several times, and chromatographed on silica gel (MeOH/$CHCl_3$ 3:17). After rechromatography on silica gel, 30 mg of **2a** were recovered as an amorphous orange powder, m.p. 131–134°. – UV. (pH 7): 238 (8700), 300 (5400). – ^{1}H-NMR.: see [5]. – MS.: 413 (M^+).

5-[(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)amino]-2′-deoxyuridine (**2b**). The same procedure as for the preparation of **2a** was used, starting with 100 mg (0.41 mmol) of **4b**, and silica gel chromatography was done with MeOH/$CHCl_3$ 1:9. 40 mg **2b** as amorphous orange powder, m.p. 126–129°. – UV.: (pH 7): 237 (8700), 299 (5400). – MS.: 397 (M^+).

5-Anilinouridine (**5a**). To 120 mg (0.46 mmol) of **4a** in 0.5 ml of water were added 0.25 ml (2.7 mmol) of aniline (heat to dissolve) and the pH was adjusted to 5 with 1N H_2SO_4. The resulting mixture was heated in an open vial in a 100° water bath for a total of 6 h. Water was added as required to maintain the volume near 1.2 ml, and the solution was adjusted to pH 5–6 (paper) with aniline each hour. The chromatographic procedure described for **2a** was followed, and **5a** was recrystallized from acetone/ethyl acetate 1:1: 44 mg **5a** as white crystals, m.p. 184–186°. – UV. (pH 7): 252 (12400), 322 (3800). – ^{1}H-NMR.: see [5]. – MS.: 335 (M^+).

5-Anilino-2′-deoxyuridine (**5b**). The procedure described for the preparation of **5a** was followed, starting with 250 mg (1.0 mmol) of **4b**, and 0.5 ml (5.4 mmol) of aniline. After silica gel column chromatography (MeOH/$CHCl_3$ 13:37, *v/v*), the crude residue was separated on a prep. TLC. plate (MeOH/$CHCl_3$ 2:23). Recrystallization from methanol-chloroform 1:19 yielded 40 mg of **5b** as light yellow crystals, m.p. 202–203°. – UV. (pH 7): 253 (12300), 321 (3600). – ^{1}H-NMR.: *Table 1.* – MS.: 319 (M^+).

5-(p-Toluidino)uridine (**6a**). The procedure for the preparation of **6a** was analogous to that of **5a** starting with 150 mg (0.58 mmol) of **4a** and 300 mg (2.8 mmol) of *p*-toluidine. Following chromatography on silica gel (MeOH/$CHCl_3$ 3:17), **5c** was recrystallized from the minimum of absolute ethanol. The yield of white crystals was 24 mg, m.p. 202–204°. – UV. (pH 7): 254 (13700), 320 (3900). – ^{1}H-NMR.: *Table 1.* – MS.: 349 (M^+).

5-(p-Toluidino)-2′-deoxyuridine (**6b**). The procedure for synthesis of **6b** was analogous to that of **5a**, starting with 150 mg (0.61 mmol) of **4b**, and 300 mg (2.8 mmol) of *p*-toluidine. After column chromatography on silica gel (MeOH/$CHCl_3$ 1:9), **6b** was recrystallized from ethyl acetate/hexane 1:1. The yield of yellow crystals was 35 mg, m.p. 217–219°. – UV. (pH 7): 255 (13700), 320 (3800). – ^{1}H-NMR.: *Table 1.* – MS.: 333 (M^+).

Biological studies. – *Antiviral activity.* Confluent primary rabbit kidney (PRK) cell cultures in *Falcon* microtiter trays were inoculated with 100 $CCID_{50}$ of virus, 1 $CCID_{50}$ being the virus dose required to infect 50% of the cell cultures. After 1 h of virus adsorption, residual virus was removed and the cell cultures were incubated with maintenance medium (*Eagle's* minimal essential medium supplemented with 3% calf serum) containing varying concentrations (400, 200, 100, ... µg/ml) of the test compounds. Virus-induced cytopathogenicity was recorded as soon as it reached completion in the control virus-infected cultures, and the antiviral effects are expressed as the concentrations of compound required to inhibit virus-induced cytopathogenicity by 50%.

Antimetabolic activity. The antimetabolic effects were monitored by the inhibition of either (methyl-$^3H_3$)dThd- or (1′,2′-$^3H_2$)dUrd-incorporation into DNA of PRK cell cultures. To this end, PRK cells were seeded in *Falcon* microwells in the presence of either (methyl-$^3H_3$)dThd (0.12 µCi/10 pmol/10^5 cells) or (1′,2′-$^3H_2$)dUrd (0.25 µCi/6 pmol/10^5 cells) and varying concentrations (400, 200,

100, ... μg/ml) of the test compounds, and allowed to proliferate for 16 h at 37° in a humidified, CO_2-controlled atmosphere. Acid-insoluble radioactivity was then measured, essentially as described previously [13].

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