Comparative Study of the Structure and Conformation in Aqueous Solution of the Antileukemic Agent 6-Thiopurine Ribonucleoside 5′-Phosphate to That of Common Purine 5′-Nucleotides

Frederick E. Evans and Ramaswamy H. Sarma*

Contribution from the Department of Chemistry, State University of New York at Albany, Albany, New York 12222. *Received July 17, 1974*

Abstract: Fast Fourier transform hydrogen-1 nuclear magnetic resonance spectroscopy is used to investigate the molecular framework of 6-thiopurine ribonucleoside (6-TPR) and the corresponding 5′-nucleotide (6-TPR-5′-P). Several lines of evidence have been presented which indicate that the base exists in the *thio* rather than the *mercapto* tautomeric form in aqueous solution. Both the nucleoside and nucleotide have flexible conformations in aqueous solution. The preferred conformation for 6-TPR is anti ($^2E^* \rightleftarrows {}^3E$) gg and that of 6-TPR-5′-P is anti ($^2E^* \rightleftarrows {}^3E$) gg-g′g′; the star indicates a bias for ^2E ribose ring pucker. It is shown that the time-average conformations of 5′-IMP and 6-TPR-5′-P in aqueous solution are *essentially identical;* this finding provides a conformational basis for understanding why the antileukemic agent 6-TPR-5′-P is a competitive inhibitor of the biosynthetic conversion of 5′-IMP to 5′-AMP and 5′-GMP, thus blocking the biosynthesis of nucleic acids.

One of the most valuable purine analogs for the treatment of acute human leukemia is 6-thiopurine (commonly called 6-mercaptopurine) and its derivatives.[1] It is believed that in cell tissues 6-thiopurine undergoes conversion to the corresponding nucleotide, i.e., 6-thiopurine ribonucleoside 5′-phosphate (6-TPR-5′-P, I), which in turn inhibits nucleic acid synthesis. It has been shown that 6-TPR-5′-P inhibits the enzymatic conversion of 5′-IMP to 5′-AMP and to 5′-GMP.[2-4] In the present paper we present our findings on the aqueous solution conformation of 6-TPR-5′-P and compare this conformation to that of 5′-IMP, 5′-AMP, and 5′-GMP. It is believed that the present study provides some stereochemical basis for the chemotherapeutic action of the purine analogs.

Experimental Section

Materials and Method. The ^1H and $^1H\{^{31}P\}$ NMR spectra were recorded at 100 MHz using a Varian HA 100D spectrometer interfaced to a Digilab FTS-3 Fourier transform data system. The internal reference was tetramethylammonium chloride (TMA); TMA (internal) = DSS (internal) + 3.1760 ppm. All samples were commercial preparations. They were lyophilized from 99.8% D_2O and spectra were taken in 100% D_2O. The pD reported are pH meter readings (+0.4). Spectra were analyzed by the computer program LACOON3 (Figure 1). The details of the experimental procedure are discussed elsewhere.[5,6]

Results and Discussion

(a) Tautomeric Form of the Base. There are two possible tautomeric forms in which the 6-sulfur-substituted purine base may exist; these are the thio and mercapto forms. Below we provide three lines of argument which suggest that the 6-thiopurine system shows an outspoken preference for the thio tautomer. (i) The data in Table I show that the C(8)-H and C(2)-H resonances of 6-thiopurine ribonucleoside (6-TPR) appear at significantly higher field than that of 6-methylmercaptopurine ribonucleoside, a nucleoside frozen in the mercapto form. Such an observation, based on ring current arguments presented elsewhere,[7] is indicative of strong preference for the thio form. (ii) Data in Figure 2 show the striking similarity in the pD profiles of 6-TPR and inosine, which in turn suggests that the bases in both nucleosides exist in comparable tautomeric forms. Insofar as inosine is known to exist in the keto form,[7,8] one may make a reasonable conclusion that 6-TPR exists in the thio form. (iii) The third line of evidence that the 6-thiopurine system at neutral pD preferably exists in the thio form comes from a comparison of the concentration profiles of 6-TPR-5′-P, 5′-IMP, 5′-GMP, and 5′-AMP (Table II). We have employed the nucleotides, rather than the nucleosides, because of the necessity of obtaining a fairly wide range of concentration for comparison. The solubility limitations of nucleosides in aqueous solutions are such that data for a meaningful range of concentration cannot be obtained. The data in Table II show that the base protons and also C(1′)-H of 5′-AMP are highly sensitive to concentration changes as have been reported elsewhere.[9,10] In the case of 5′-GMP, 5′-IMP, and 6-TPR-5′-P, the observed concentration shifts (Table II) for the base protons are much smaller than those of 5′-AMP. One may argue that the small concentration shift experienced by 5′-GMP, 5′-IMP, and 6-TPR-5′-P compared to 5′-AMP is due to a lesser degree of stacking interactions or due to different time-average stacking orientations than that in 5′-AMP. However, the data are better rationalized on the ground that the bases in 5′-GMP, 5′-IMP, and 6-TPR-5′-P exist in the thio or keto forms and that vertical base stackings do not cause as significant a perturbation in the magnitude of the chemical shifts because of the smaller ring current fields in the thio and keto forms.[11-15] The substantially larger concentration shifts in 5′-AMP are expected to be due to the pronounced ring current fields in the amino tautomer of adenine.[13-15]

(b) Conformation of the Exocyclic C(5′)–O(5′) and C(4′)–C(5′) Bonds. Estimates of the conformer populations about the C(5′)–O(5′) bond (II–IV) can be made from the magnitude[16-18] of $J_{5'P}$ and $J_{5''P}$. The magnitude of the coupling constants (Table III) indicates[16-18] that the gauche′-gauche′ conformer II is 85% populated. The data in Table III further show that the magnitude of $J_{5'P} + J_{5''P}$ (Σ') in 6-TPR-5′-P is independent of the state of ionization of the base, indicating that the conformer populations about the C(5′)–O(5′) bond have not been perturbed by such ionizations. Ionization of the phosphate group does cause a small variation in Σ', but this is difficult to interpret, because one does not know precisely whether the ionization of the phosphate has some influence on the Karplus relationship of the H–C–O–P bond system.

The conformer populations about the C(4′)–C(5′) bond (V–VII) can be estimated from[16-18] the magnitude of $J_{4'5'}$

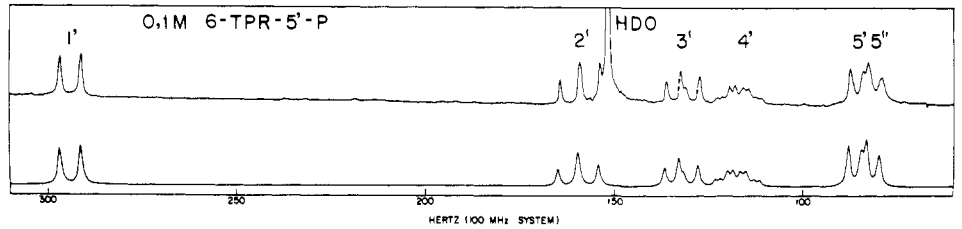
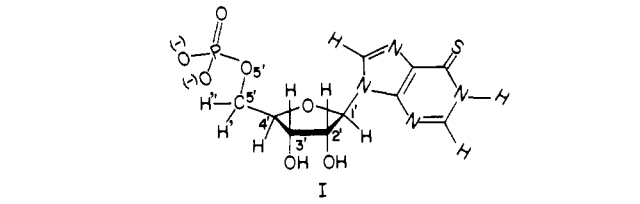
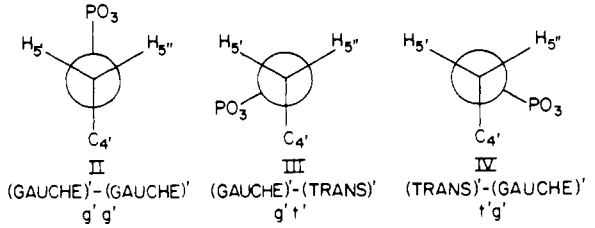
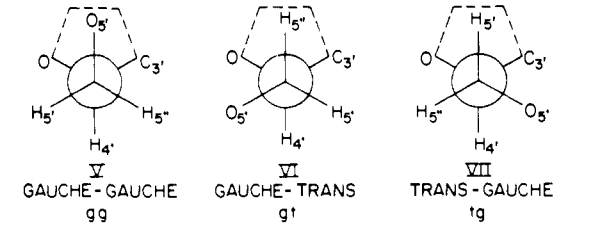
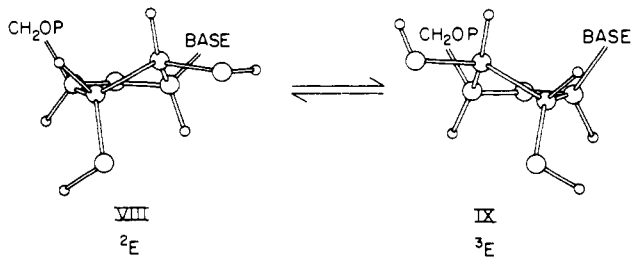


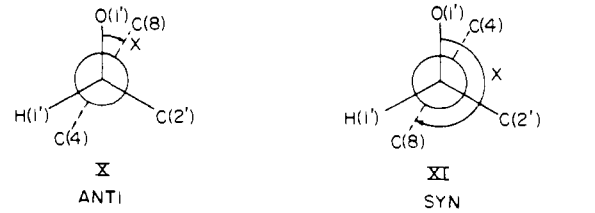
Figure 1. The observed ^1H NMR spectrum of 0.1 *M* 6-TPR-5′-P, pD 8.0, taken at 100 MHz. The computer-simulated spectrum is the bottom one. Note the fine structure of the 4′ region indicating the four-bond coupling between P(5′) and H(4′). The scale is in hertz (100-MHz system) from internal tetramethylammonium chloride (TMA).











and $J_{4'5''}$. The magnitude of $J_{4'5'} + J_{4'5''}$ (Σ) indicates[16-18] that the gauche-gauche (V) population is 60–65% at pD values of 9.9, 7.9, and 5.0. Thus, there is a bias for the gauche-gauche (V) conformer in 6-TPR-5′-P whether the base is anionic (pD 9.9) or neutral (pD 7.0 and 5.0), or whether the phosphate is a dianion (pD 9.9 and 7.9) or a monoanion (pD 5.0). The magnitude of the four-bond[19] coupling $^4J_{4'P}$ further substantiates the conclusion that gauche-gauche and gauche′-gauche′ are the preferred but not the exclusive conformations in 6-TPR-5′-P. The increase in $^4J_{4'P}$ with protonation of the phosphate group (Table III) appears to be due mostly to electronegativity effects rather than conformational changes (Wood, Hruska, and Sarma, unpublished results).

Table I. Observed C(8)-H and C(2)-H Chemical Shifts[a]

Compound	C(8)-H[b]	C(2)-H
Adenosine	516	508
1-Methyladenosine	489	498
6-Thiopurine ribonucleoside	531	518
6-Methylmercaptopurine ribonucleoside	548	536

[a] Chemical shifts of the nonexchangeable base protons are expressed in hertz downfield from internal tetramethylammonium chloride (100-MHz NMR system). Spectra were taken at 0.002 *M* at pD such that the base of each nucleoside did not carry a formal (+) charge from protonation; pD 7.0 for each case except 1-methyladenosine for which pD 11.0 was used.[7] The chemical shifts are accurate to ±1.0 Hz. [b] The C(8)-H resonance was identified by hydrogen exchange.

Table II. Chemical Shift Dependence on Concentration[a]

	6-TPR-5′-P	5′-IMP	5′-GMP	5′-AMP
Δδ2	5.0	7.5		24.5
Δδ8	5.5	6.0	5.0	13.5
Δδ1′	5.0	6.5	4.5	10.0
Δδ2′	3.5	6.5	8.0	5.0
Δδ3′	0	0.5	1.0	0
Δδ4′	−1.0	0	−2.0	−2.0
Δδ5′	−4.0	−3.0	−5.0	−6.0

[a] The difference in chemical shifts between the 0.25 *M* concentration level and the 0.003 *M* concentration level is determined for each nucleotide respectively. A positive value indicates more shielding at high concentration, and the chemical shift differences are expressed in hertz (100-MHz system) which are accurate to ±1 Hz. Temperature is 30°; pD is 7.9, except 6-TPR-5′-P for which pD 6.5 is employed so that the base will be neutrally charged like the other bases. Phosphate ionization has no significant effect on the chemical shift dependence on concentration.

Ionization of the base does cause conformational adjustments about the C(4′)-C(5′) bond in the nucleoside 6-TPR. The value of Σ in 6-TPR is 6.3 Hz when the base carries a negative charge, and it is 7.3 Hz when the base is neutral. According to equations developed elsewhere,[16-18] this suggests a decrease of 10% in the gauche-gauche population in going to the neutral form. The gauche-gauche (V) conformer is still preferred over the gauche-trans (VI) and trans-gauche (VII) conformers. The bias for the gauche-gauche conformation about the C(4′)-C(5′) bond and the gauche′-gauche′ conformation about the C(5′)-O(5′) bond is the same general trend observed in several related compounds in the solution and solid states.[20-28]

(c) Conformation of D-Ribose Ring. The ribose ring conformation in aqueous solution may be treated as an equilibrium between C(2′) endo (^2E) and C(3′) endo (^3E) conformations (VII $\rightleftarrows$ IX).[5,18,29,30,31] Whether one used the traditional Karplus approach[5,18,29,32] of a $^2E \rightleftarrows {}^3E$ equilibrium, or the pseudorotational approach of a $N \rightleftarrows S$ equilibri-

Table III. NMR Parameters for 6-TPR, 6-TPR-5′-P, 5′-IMP, 5′-GMP, and 5′-AMP[a]

	6-TPR		6-TPR-5′-P						
pD	6.0[b]	9.9[b]	5.0[b]	7.9[b]	9.9[b]	7.9[c,d]	5′-IMP, 7.9[c]	5′-GMP, 7.9[c]	5′-AMP, 7.9[c]
$J_{1'2'}$	5.2	6.1	5.3	5.8	5.9	5.8	5.8	6.1	6.0
$J_{2'3'}$	5.2	5.3	5.1	5.1	5.1	5.1	5.2	5.2	5.1
$J_{3'4'}$	4.3	3.4	4.1	3.6	3.6	3.6	3.6	3.4	3.5
$J_{4'5'}$	3.0	2.6	3.0	2.9	3.0	4.0	3.8	4.4	3.9
$J_{4'5''}$	4.3	3.7	3.6	4.0	4.0	2.9	2.9	3.0	2.6
Σ^a	7.3	6.3	6.6	6.9	7.0	6.9	6.7	7.4	6.5
$J_{4'\text{-P}}$			2.1	1.5	1.4	1.5	1.5	1.4	1.7
$J_{5'5''}$	−12.0	−12.0	−12.0	−12.0	−12.0	−12.0	−12.0	−12.0	−12.0
$J_{5'\text{-P}}$			5.0	4.8	4.8	4.8	4.8	4.8	4.6
$J_{5''\text{-P}}$			5.0	4.7	4.7	4.7	4.7	4.8	4.6
Σ'^a			10.0	9.5	9.5	9.5	9.5	9.6	9.2
∂1′	291.8	278.2	296.6	296.6	294.9	297	296	275	297
∂2′	157.7	163.0	157.0	161.7	163.1	163	162	161	163
∂3′	125.3	125.4	132.4	133.5	133.8	133	133	131	134
∂4′	107.9	110.3	120.1	118.7	118.5	118	118	113	118
∂5′	72.9	72.9	97.8	85.3	84.4	84[e]	84[e]	81[e]	82[e]
∂5″	65.8	65.4	93.2	82.9	82.0				
∂2	516.3	512.1	516.8	516.8	515.2	518	504		508
∂8	529.4	514.3	540.4	551.0	542.8	553	539	502	544

[a] Coupling constants were obtained from 0.02 *M* spectra and are expressed in hertz, and further dilution has no significant effect on them. Their accuracy is ±0.1 Hz except for $J_{4'5'}$, $J_{4'5''}$, and $J_{5'\text{-P}}$ where only the sums, $\Sigma = J_{4'5'} + J_{4'5''}$ and $\Sigma' = J_{5'\text{-P}} + J_{5''\text{-P}}$, are significant. The chemical shifts are expressed in hertz (100-MHz system) downfield from internal standard tetramethylammonium chloride. [b] Chemical shifts obtained at 0.02 *M*. [c] Chemical shifts obtained at infinite dilution, ± 1 Hz. [d] Phosphate group is a dianion at pD 7.9. The base is approximately 50% anion and 50% neutral. This is usable for comparison with the neutral bases of 5′-IMP, 5′-GMP, and 5′-AMP since ionization of the base in 6-TPR-5′-P has little effect on the coupling constants and chemical shifts. [e] The chemical shifts given are the average for the 5′ and 5″ protons. The precise individual chemical shifts for these protons cannot be obtained because they are obtained by extrapolation to infinite dilution.

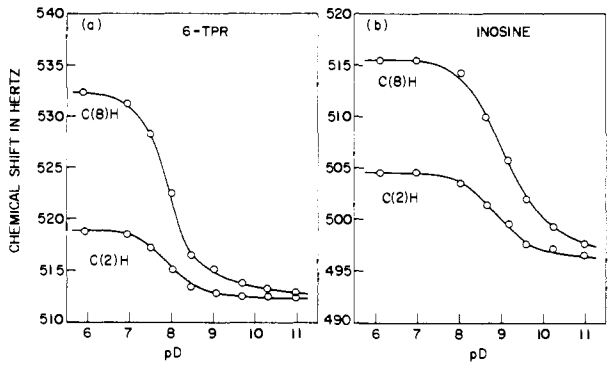


Figure 2. The pD profiles for the C(2)-H and C(8)-H chemical shifts in 6-TPR and inosine. The scale is in hertz relative to internal TMA (100-MHz system). The concentration is 0.003 *M*, and this is low enough that further dilution did not produce a significant change in the chemical shifts.

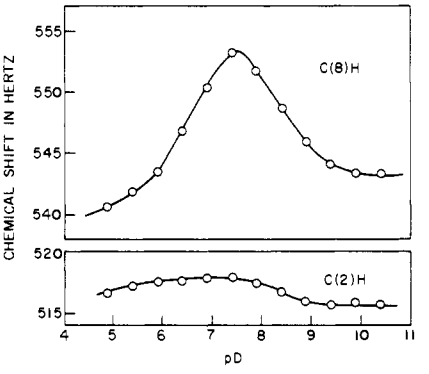


Figure 3. The pD profile for the C(8)-H and C(2)-H chemical shifts of 6-TPR-5′-P at 0.02 *M*. For a nucleotide, 0.02 *M* is a low enough concentration that there is little base stacking.[42]

um,[30,33] one cannot pinpoint the dynamic conformation of the sugar ring in aqueous solution. Estimates of conformer populations have an error of at least ±10%.[18]

The magnitudes of $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$ for the 6-thio compounds (Table III) indicate that, in these compounds, the ^2E pucker is populated 50–65% under the various pD conditions used.[5,18,29,30] The ring coupling constants are slightly dependent on pD (Table III); it is seen that as $J_{1'2'}$ decreases, $J_{3'4'}$ correspondingly increases and the sum $J_{1'2'} + J_{3'4'}$ remains essentially constant. This suggests that the dihedral angle relationships in the "pure" ^2E and ^3E conformers may not be affected by pD changes and that the observed changes in the coupling constants represent mostly changes in ^2E and ^3E populations. Hence, one can compute fairly accurately the magnitudes of relatively small shifts in the $^2E \rightleftarrows {}^3E$ equilibrium. In the case of 6-TPR, the data in Table III show that, in going from pD 6.0 to 9.9, $J_{3'4'}$ changes by −0.9 Hz and $J_{1'2'}$ by +0.9 Hz. This indicates an increase in ^2E population of 9 ± 2%.[34] Further analysis of the data shows that the time-average ribose ring conformation for the nucleotide, unlike the nucleoside, is not perturbed by ionization of the base. However, one observes that the protonation of the phosphate group causes a 5% increase in the ^3E conformer population.

(d) Sugar–Base Torsional Preference. The orientation about the glycosyl bond falls into two possible regions called the anti (X) and syn (XI) conformation. Schweizer et al.[35] observed that in 5′-AMP phosphate ionization perturbed the C(8)-H chemical shift, but not C(2)-H, indicating the accessibility of anti conformation for 5′-AMP. We have recently found that in the syn compound 8-Br-5′-AMP, the C(2)-H chemical shift is relatively insensitive to phosphate ionization;[36,37] this is because in 8-Br-5′-AMP the C(4′)–C(5′) bond is preferentially gauche-trans, and in such an arrangement the phosphate group and C(2)-H of the base are not close.[37]

The pD profile for 6-TPR-5′-P is illustrated in Figure 3. In the range pD 8–10, the base is ionized as has already been discussed for the nucleoside (Figure 2). Lowering the pD from 8 to 5 causes C(8)-H to shift to much higher field, while C(2)-H is only slightly perturbed. This shows the accessibility of anti conformation, but the data do not neces-

Table IV. Comparison of the C(2)-H and C(8)-H Chemical Shifts in the Base, Nucleoside, and Nucleotide[a]

Compound	C(2)-H	C(8)-H
6-Thiopurine	518	521
6-TPR	518	531
6-TPR-5'-P	518	553

[a] The chemical shifts of C(2)-H and C(8)-H are expressed in hertz downfield from tetramethylammonium chloride (100 MHz) at infinite dilution.

sarily mean that the anti conformation is preferred, because the C(2)-H is not a sensitive probe of syn conformer.[36,37] Insofar as the magnitude of the phosphate-induced perturbation in 6-TPR-5'-P is similar to that reported in 5'-AMP,[35,38] and other methods indicate a preference for anti conformation in 5'-AMP,[36,37] it is likely that 6-TPR-5'-P also prefers anti conformation in aqueous solution.

Chan and Nelson[10] have shown that the strongly distant dependent nature of the broadening induced by the paramagnetic Mn(II) ion can be used to distinguish between syn and anti conformation. We have monitored the effect of the Mn(II) ion on the base proton line widths of 6-TPR-5'-P and have obtained similar results to that reported for 5'-AMP under similar conditions.[10,36,37] The line width of the C(8)-H resonance was considerably broadened, while that of C(2)-H was only slightly affected. In the syn compound 8-Br-5'-AMP, C(2)-H undergoes substantial broadening in the presence of the Mn(II) ion.[36,37] The data show a low probability of syn conformer for 6-TPR-5'-P in the presence of the Mn(II) ion and support the earlier conclusion that 6-TPR-5'-P is preferentially anti. However, it is cautioned that the bound Mn(II) ion, which binds at the PO_3^{2-} group and tends to be positioned toward N(7), may perturb the conformation of the nucleotide. In Table IV are presented data which show the effect of introducing the ribose and ribose phosphate moieties on the chemical shifts on C(2)-H and C(8)-H. The changes one observes in going from the base to the nucleoside and to the nucleotide, according to arguments presented elsewhere,[36] serve as another piece of data which support the foregoing conclusions. In addition, it suggests that in aqueous solution the nucleoside 6-TPR may also be preferentially anti. This contrasts to the situation in the solid state where 6-TPR has been crystallized in two different molecular conformations, and both molecules are in the syn conformation.[39] This is similar to the case of the pyrimidine nucleoside 4-thiouridine in which the anti conformation is reported to be the favored one in solution,[40] but in the crystal the molecule exists in the syn conformation.[41] Some limitations in extending nucleoside and nucleotide conformation from the solid state to aqueous solution are discussed elsewhere.[42,43]

(e) Comparison of the Conformation of 6-TPR-5'-P to That of 5'-IMP, 5'-AMP, and 5'-GMP. Since it is generally believed that the antileukemic effect of 6-thiopurine involves its conversion to 6-TPR-5'-P, which in turn inhibits the enzymatic conversion of 5'-IMP to 5'-AMP and 5'-GMP,[2-4] we have undertaken to compare their solution conformation to that of 6-TPR-5'-P. In Table III are presented the ^1H NMR data for all the relevant compounds. There is noticable similarity in all the corresponding coupling constants and chemical shifts in the four purine nucleotides (Table III), and this can only mean a similarity in the ribose ring, exocyclic, and glycosyl conformations between these compounds. The similarity between 6-TPR-5'-P and 5'-IMP (Table III) is most striking, and it indicates that the *entire time-average conformation of 6-TPR-5'-P and 5'-IMP in aqueous solution is essentially identical.* This finding provides a conformational basis for understanding why 6-TPR-5'-P is an effective inhibitor in the biosynthetic conversion of 5'-IMP to 5'-AMP and 5'-GMP.

Acknowledgment. This research was supported by grants from the National Cancer Institute of NIH (CA 12462) and from the National Science Foundation (B028015-001).

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