Separation, Structural Determination and Biological Evaluation of the Thymidylate Synthase Inhibitor 3,3-Di-(4'-hydroxyphenyl)-6(7)-chloro-1-oxo-1*H*,3*H*-naphtho[1,8-*cd*]pyran

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The chloro substituted 3,3-di-(4'-hydroxyphenyl)-1-oxo-1*H*,3*H*-naphtho[1,8-*cd*]pyran was synthesized in a 40/60 mixture of C_6 or C_7 substituted isomers, respectively. The two isomers were separated by hplc. The X-ray crystal structure of the mixture was obtained. Both the mixture and the single isomers were tested against *Lactobacillus Casei* thymidylate synthase. The X-ray analysis clearly revealed co-crystallization of the two isomeric species. The apparent Ki of the mixture was 0.8 mu*M*, while those of the C_6 and C_7 substituted isomers were 0.42 and 0.52 mu*M*, respectively, thus showing that the position of the chlorine in the naphthalene ring was not critical for enzymatic activity.

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Introduction.

Thymidylate synthase is an enzyme of the pyrimidine bases pathway that catalyzes the conversion of 2'-deoxyuridine-5'-monophosphate to thymidine-5'-monophosphate. This is the only *de novo* step in thymidine-5'-monophosphate synthesis and its inhibition causes a thymineless death of the cell [1]. This enzyme is found throughout living organisms so it can be regarded as an interesting target for cancer chemotherapy, and the possibility of antibacterial, antifungal and antiviral chemotherapy by thymidylate synthase inhibition has also been investigated [2,3]. We have recently described a new class of phthalein derivatives acting as thymidylate synthase inhibitors [4,5], which could be considered as interesting models to be developed, mainly in view of their selectivity towards non human thymidylate synthase. Therefore a structure-based synthetic program [6,7] was started, mainly referring to the X-ray crystal structure of the binary complex formed by *Lactobacillus casei* thymidylate synthase and the inhibitor phenolphthalein (Figure 1) [4]. The study of the above mentioned crystal structure showed that three specific hydrogen bonds contribute to the binding of phenolphthalein to the active site of the enzyme and that the phthalidic part of phenolphthalein was exposed to an empty space of the binding site. With the aim of maintaining

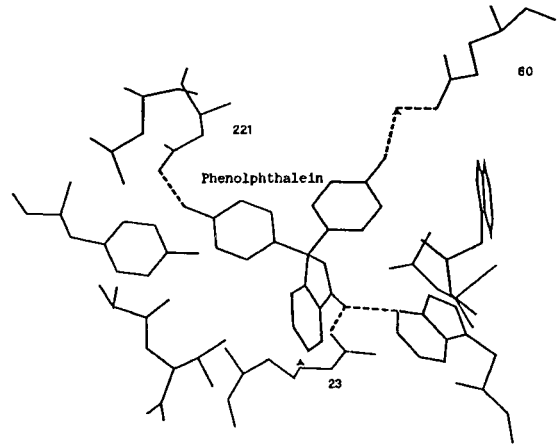
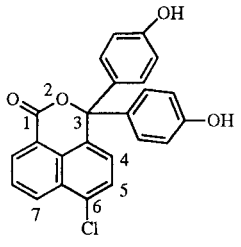


Figure 1. X-ray crystal structure of the binary complex between *Lactobacillus casei* thymidylate synthase and phenolphthalein. 23 = arginine; 60 = glutamic acid; 221 = aspartic acid.

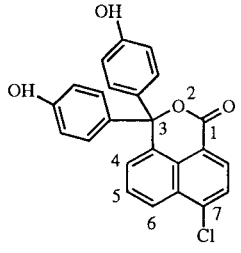
these anchorages, the synthesis of several naphthalene derivatives substituted at the phenols was performed [6,8]. Moreover, assuming that the naphthalene derivatives would bind in the same way as phenolphthalein, we thought that substituents at both rings of the naphthalene moiety could contribute to a stronger interaction with the enzyme. However, it was not predictable if the substitution at one ring could give better inhibition than at the

other. To explore the effects on the inhibitory activity of the 6/7 substituted isomers we have now synthesized **1**, where a chlorine atom is present on the naphthalene ring (see Chart 1).

Chart 1



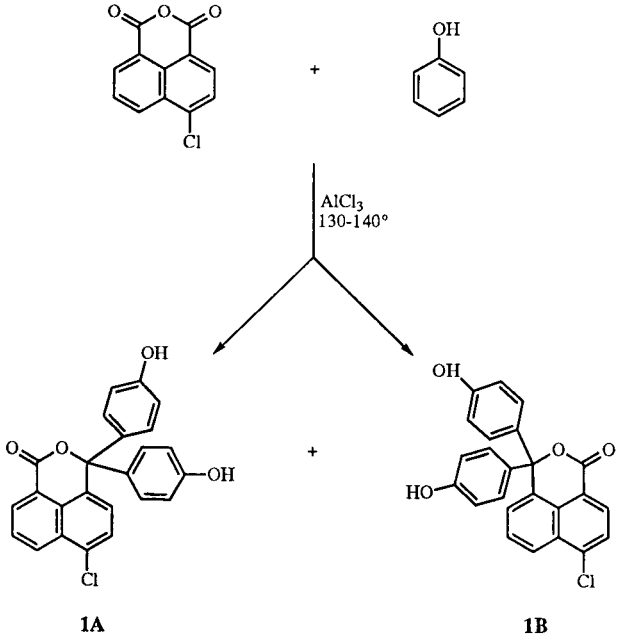
1A



1B

The compounds were synthesized as shown in Scheme 1. Accordingly, the 4-chloro-1,8-naphthoic acid anhydride was heated at 130-140° with a twofold amount of phenol and aluminum chloride for three days. The hot mixture was then poured onto ice and extracted with dichloromethane. The insoluble material was filtered, the filtrate concentrated under vacuum and the residue purified by silica gel chromatography to give as the first run the unreacted anhydride followed by **1**, as a 40/60 mixtures of 6- (**1A**) and 7-substituted (**1B**) isomers, respectively, as clearly revealed by ^{1}H nmr spectra. However, owing to the difficulty of their separation, the mixture was initially tested as such in the biological assays. Based on the significant inhibitory potency of the mixture, we thought it of interest to verify if a different substitution pattern on the naphthalene ring could affect the activity of these compounds. We therefore isolated the two isomers and tested them separately. This information would be useful to explore the binding site for the naphthalide moiety and therefore to address the future design of compounds in this series in a more rational way. This paper describes the results we had with the 3,3-di-(4'-hydroxyphenyl)-6(7)-chloro-1-oxo-1*H*,3*H*-naphtho[1,8-*cd*]pyran **1**. The X-ray crystal structure of the co-crystallized isomeric mixture (Figure 2) was also obtained in order to compare the experimental structure with the *Lactobacillus casei* thymidylate synthase-phenolphthalein binary complex.

Scheme 1



1A **1B**

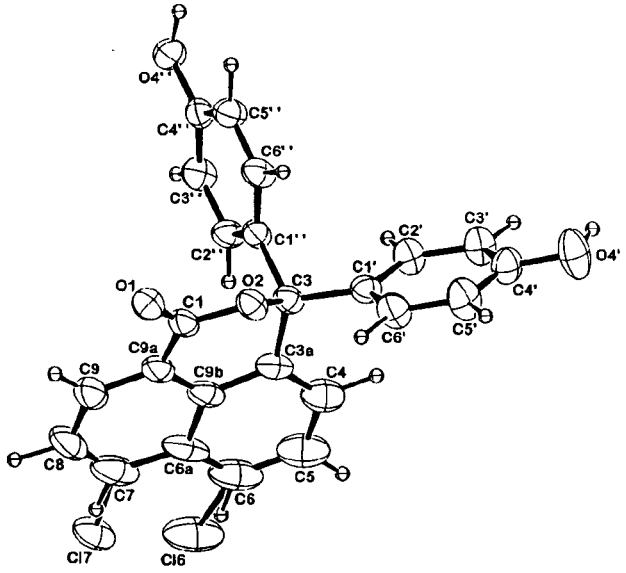


Figure 2. ORTEP drawing of the molecular structure of 3,3-di-(4'-hydroxyphenyl)-6/7-chloro-1-oxo-1*H*,3*H*-naphtho[1,8-*cd*]pyran (**1**) [21]. Thermal ellipsoids for non-H atoms enclose 40% probability. The C6 and C7 atoms (and the protons bonded to the same C atoms) have alternative half site occupancy factors.

Results and Discussion.

X-Ray Analysis.

The main result of the X-ray analysis is the experimental evidence of the co-crystallization of two isomeric species, chloro substituted at the C6 and C7 atoms, respectively. The molecular structure is shown in Figure 2 along with the atom numbering scheme used. One chloroform solvate molecule, whose chlorine atoms exhibit large thermal motion parameters (perhaps indicative of possible disorder) is present in the asymmetric unit. All

bond distances, although affected by rather large standard deviations, are in good agreement with those reported for mean bond lengths in organic molecules [9]. The conformation of the heterocyclic six-membered ring is characterized by an expected coplanarity of the C1-C9a-C9b-C3a atoms, whereas O2 and C3 are displaced -0.111 (8) and 0.485 (8) Å, respectively, on opposite sides from the mean plane through the other ring atoms (Figure 2). The mean planes through the hydroxyphenyl groups form a dihedral angle of 81.2 (2)°, and their orientations appear mainly determined by the strong hydrogen bonding interactions which involve their OH function. These interactions (reported in Table 1, along with other selected molecular dimensions) make the major contribution to the crystal-packing forces, but also many short van der Waals contacts, mainly involving C1 and O atoms, could play an important role in determining either the molecular conformation or the molecular packing.

Table 1

Selected Structural Dimensions

Bond Distances (Å)

C(6)-Cl(6)	1.735(7)	C(7)-Cl(7)	1.801(8)
C(3)-O(2)	1.475(6)	C(1)-O(2)	1.349(6)
C(1)-O(1)	1.213(7)	C(4')-O(4')	1.365(7)
C(4")-O(4")	1.381(6)		

Torsion Angles (deg)

C(3)-C(3a)-C(9b)-C(9a)	-13.5(6)	C(1)-C(9a)-C(9b)-C(3a)	-9.3(7)
C(9b)-C(9a)-C(1)-O(2)	1.9(6)	C(3)-O(2)-C(1)-C(9a)	29.6(6)
C(3a)-C(3)-O(2)-C(12)	-49.5(5)	C(9b)-C(3a)-C(3)-O(2)	40.3(5)
O(2)-C(3)-C(1')-C(2')	-154.7(4)	O(2)-C(3)-C(1')-C(6')	24.0(6)
C(3a)-C(3)-C(1')-C(2')	86.5(6)	C(3a)-C(3)-C(1')-C(6')	-94.7(6)
O(2)-C(3)-C(l")-C(2")	-127.3(5)	O(2)-C(3)-C(1")-C(6")	54.0(5)
C(3a)-C(3)-C(1")-C(2")	-9.4(7)	C(3a)-C(3)-C(1")-C(6")	171.8(5)

Hydrogen Bonding Interactions

Distance O-H		Distance H•••O		Distance O•••O		Angle O-H•••O
O(4')-H(O4')	1.01	H(O4')••O(4") [a]	1.86	O(4')••O(4")	2.851(6)	164.9
O(4")-H(O4")	0.98	H(O4")••O(1) [b]	1.74	O(4")••O(1) [b]	2.714(6)	171.3

[a] Symmetry transformation -x, 1-y, 2-z of the reference coordinates; [b] Symmetry transformation -x, -y, 2-z of the reference coordinates.

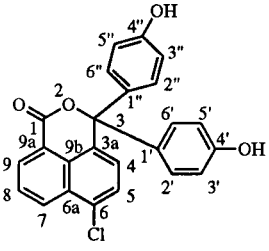
HPLC Separation and ^{1}H nmr Analysis.

The hplc analysis of the isomeric mixture was performed with a Supersphere column 100RP-18, using 50 water:50 acetonitrile as the mobile phase. The mixture was eluted at 9.0 minutes retention time. The hplc separation of the isomers **1A** and **1B** was performed with a Chiralcel OJ 250 column using *n*-hexane/ethanol 66/34 as mobile phase. The two isomers were eluted respectively at 9.3 minutes and 11.2 minutes retention time, the first eluted being the 7-chloro substituted (**1B**), which suggests its slightly lower polarity with respect to **1A**. Their relative structures were assigned by ^{1}H nmr analysis, using 2D ^{1}H nmr. (see Table 2). For both samples, resonances due to H2',6' and H3',5' were easily identified since their integrated areas were double with respect to those of the other signals. In both spectra an AX and an AMX system appeared respectively due to protons H4-H5, H7-H8-H9 in **1A**, and protons H8-H9, H4-H5-H6 in **1B**, whose scalar coupling patterns were unambigously assigned by COSY spectra. The final identification of the two isomers were obtained by the NOESY spectra. In a case, a NOE between one of the protons belonging to the AMX system and one of H2"-H3" system was observed. This NOE was attributed to the dipolar interaction between proton H4 and proton H2", which led us to assign the spectrum to the isomer **1B** and at the same time to recognize the resonances of H4, H5, H6, H2" and H3". It is to be noted that the chemical shifts of H4, H5 and H6 are in agreement with a shielding effect on the H4 proton by the ring current promoted by the phenolic moieties. However, since this effect is not very strong, it could be inferred that in dimethylsulfoxide and at room temperature the averaged dihedral angles between the naphthalene and the phenolic rings deviate from 90°, still being far from 0°. This observation, together with the magnetic equivalence shown by the protons of the two phenolic rings, suggests that in solution the latter undergo a free rotations around the C3-C1" and C3-C1' bonds. Proton nmr spectra performed in acetone-d_6 confirmed that this behavior remained unchanged up to 179 K, the freezing point of the solution, which indicates that the rotational barriers are very low.

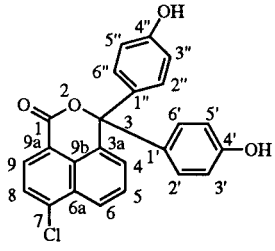
Similar behavior was observed for low temperature nmr spectra of phenolphthaleines, whose rotational barriers, evaluated by quantum chemical (PM3) calculations resulted less than 1 kcal/mol [5]. Finally, the assignement of protons H8 and H9 was done on the bases of the higher chemical shift of H9, due to the presence of the carboxyl group. NOESY spectrum of the other sample showed a NOE between one of the protons belonging to the AX system and one of H2"-H3" system. That was attributed to the dipolar interaction between protons H4 and H2" which allowed us to conclude that this sample was the isomer **1A**. The pattern of the ^{1}H nmr spectra at lower temperature paralleled that observed for **1B**, therefore allowing us to extend to **1A** what the above suggested for **1B**. Finally H7 and H9 were identified on the bases of their chemical shifts, H9 being more shielded.

Table 2

^{1}H NMR and 2D ^{1}H NMR Data for Compounds **1A** and **1B**



1A



1B

Compound **1A**			Compound **1B**	
Position	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)
H4	7.13 d	7.6	7.28 dd	7.0
H5	7.93 d	7.7	7.91 t	7.6
H6	—	—	8.40	8.3
H7	8.66 dd	7.9	—	—
H8	8.01 t	7.7	8.03 d	7.7
H9	8.48 dd	6.7	8.43 d	7.7
H2'	6.82 d	8.7	6.82 d	8.7
H3'	6.99 d	8.7	6.98 d	8.7
H5'	6.99 d	8.7	6.98 d	8.7
H6'	6.82 d	8.7	6.82 d	8.7
4'-OH	9.71 *s*	—	9.71 *s*	—
H2"	6.82 d	8.7	6.82 d	8.7
H3"	6.99 d	8.7	6.98 d	8.7
H5"	6.99 d	8.7	6.98 d	8.7
H6"	6.82 d	8.7	6.82 d	8.7
4"-OH	9.71 *s*	—	9.71 *s*	—

Correlations.

1A C: H4-H5; H7-H8; H8-H9; H2'-H3' N: H4-H2'; H6'; H2"; H6"
1B C: H4-H5; H7-H8; H8-H9; H2'-H3' N: H4-H2'; H6'; H2"; H6"

Biological Assays.

The evidence that the X-ray structure was stabilized by short-distance hydrogen bonds formed between the two hydroxy groups and between a phenolic and a carbonyl oxygen of a second lactone ring, suggested that the functional groups of the molecule might also establish strong hydrogen bonds with specific residues of the enzymatic folate binding pocket. Indeed, this was clearly shown in the X-ray crystal structure of the complex between phenolphthalein and thymidylate synthase [4] (Figure 1), which would suggest that the same binding structure might contribute to stabilize the enzyme-inhibitor complex of **1**. The inhibitory activity of compound **1**, both as a mixture and as single isomers, was determined (see Experimental). An apparent inhibition constant (Ki) value of 0.80 ±0.07 mu*M* was found for the mixture, while the two isomers had almost the same Ki values (0.42 ±0.03 mu*M* for **1A** and 0.52 ±0.025 mu*M* for **1B**), thus indicating that the presence of the substituent in 6 or 7 position is not critical for the activity. A competitive inhibition pattern with respect to the folate cofactor was apparent as already observed in previous studies [8]. Finally, the X-ray crystal structure of compound **1A**, as obtained from the experimental results, was matched with phenolphthalein inside the enzyme pocket, assuming that the two compounds could bind in the same site and that the conformation of the inhibitor would be the same (Figure 3) [10]. The main specific bonds observed in phenolphthalein were maintained in compound **1A**, the matching indicating that the presence of an additional benzene ring could modify the binding mode slightly but that the same orientation was kept. In particular, the phenolic rings were rotated with respect to those of phenolphthalein, but they retained the right direction to hydrogen bond the residues 221 (aspartic acid) and 60 (glutamic acid), while the lactone ring hydrogen bonded arginine 23. A small steric hindrance was due to Tryptophan 85, which was too close to the chlorine atom (1.47 Å) When the matching was performed with **1B**, almost the same results were obtained.

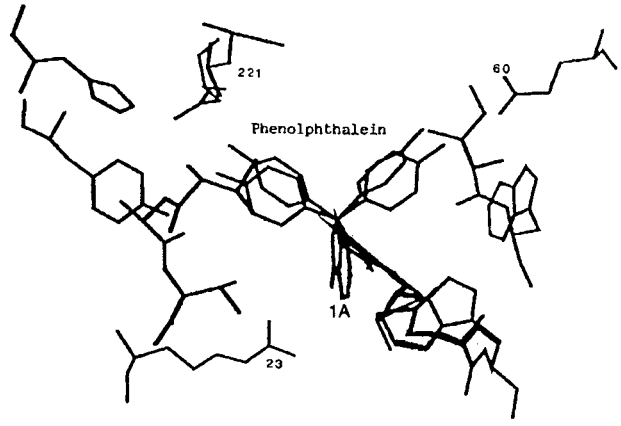


Figure 3. Rigid matching of the binary complex *Lactobacillus casei* thymidylate synthase phenolphthalein and compound **1A** 23 = arginine; 60 = glutamic acid; 221 = aspartic acid.

All the data seem to suggest that small substituents on the naphthalene moiety cannot affect the binding energy, no specific or non-specific interactions being made by the substituent in position 6 or 7 with the enzyme binding site. However, shifting the substituent to different positions of the ring or introducing bulkier groups could give very different results. Further studies in this direction are in progress.

Table 3

Crystal Data and Summary of Data Collection and Structure Refinement

Formula	$C_{24}H_{15}ClO_4 \cdot CHCl_3$
mol wt	522.18
crystal size, mm	0.20 x 0.10
crystal system	triclinic
space group	Pī
a, Å	9.616
b, Å	10.184 (1)
c, Å	13.012 (1)
α, deg	89.59 (1)
β, deg	85.18 (1)
λ, deg	68.45
V, Å^3	1180.6 (2)
Z	2
D_c, Mg m^{-3}	1.469
F (000)	532
2 ϑ range, deg	2-27
radiation (λ, Å)	MoKα (0.71069)
μ, mm^{-1}	0.532
no. reflections collected	6590
no. observed [I ≥ 2σ (I)]	3381
no. varied parameters	308
R (F)	0.096
R_w (F^2)	0 267
weighting scheme w	$[\sigma^2(F_o^2 + (0.1432P^{a})^2 + 1.756P^{a}]^{-1}$
goodness of fit	1.118
largest diff. peak and hole, eÅ^3	1.08 and -0.73

[a] $p = \max (F_o^2 + 2F_c^2)/3$

EXPERIMENTAL

Chemistry.

Melting points were determined on a Büchi 510 capillary melting points apparatus, and are uncorrected. Analyses indicated by the symbols were within ±0.4 of the theoretical values and were determined in the Department of Pharmaceutical Chemistry (Faculty of Pharmacy, Modena). Analysis (tlc) on silica gel plates was used to check product purity. Silica gel 60 (Merck; 70-230 mesh) was used for column chromatography. The structure of the compounds was consistent with their analytical data.

3,3-di-(4'-hydroxyphenyl)-6(7)-chloro-1-oxo-1*H*,3*H*-naphtho-[1,8-*cd*]pyran (**1**).

To a mixture of 4-chloro-1,8-naphthalic acid anhydride (2.32 g, 0.01 mole) and phenol (1.88 g, 0.02 mole) in *s*-tetrachloroethane (100 ml) aluminum chloride (2.67 g, 0.02 mole) was added portionwise at room temperature and the suspension stirred for three days at 130-140°. The mixture while still hot was then poured onto ice and dichloromethane (200 ml) was added. After stirring for 0.5 hour, the precipitate was filtered, the organic layer separated and dried over sodium sulfate. After evaporation of the solvent the residue was purified by silica gel chromatography, eluting with dichloromethane/methanol 95/5. The unreacted anhydride was collected as first run, followed by the desired **1** (as a 40/60 mixture of the 6 and 7 substituted isomers, yield 25%). (see Scheme 1 and Table 2) ($\varepsilon_{321\ nm}$ ethanol 7068, $\varepsilon_{220\ nm}$ dimethyl sulfoxide 8107).

Anal. Calcd. for $C_{24}H_{15}ClO_4$: C, 71.55; H, 3.75. Found: C, 71.35; H, 3.94.

X-ray Measurements and Structure Determination.

Great difficulties were encountered in obtaining a single crystal suitable for X-ray diffraction studies. Repeated attempts at recrystallization from a number of different solvents afforded only very small crystals, which were also largely affected by extensive gemination. Only one crystal, from those grown from chloroform solution, proved to be of sufficient quality for X-ray diffraction measurements. Crystal data and summary of intensity data collection and refinement are reported in Table 3. Intensity data were collected at room temperature using a rotating-anode Siemens P4RA-M18X diffractometer and graphite-monocromated MoKα radiation (52 KV, 100mA). Cell dimensions were derived from least-squares fit to the setting angles of 35 reflections. The intensities of 3 standard reflections monitored at 100-reflections intervals showed no significant changes. The intensities were corrected for Lorenz and polarization effects, but in view of the low absorption coefficient and small crystal size, not for absorption. The centrosymmetric space group $\bar{P}1$ was initially assumed on the basis of E-statics consistent with a centric prediction. The centric assumption implies a disordered model with half site occupancy by two Cl atoms. Following the suggestion of Marsh [11], we opted for the centrosymmetric description to avoid the poor convergence problems typical of nearly centrosymmetric cases, when treated as non-centric. The structure was solved by direct methods [12]. Refinement was carried out by full-matrix least-squares with $\Sigma w (F_o^2-F_c^2)^2$ being minimized. All non-hydrogen atoms were refined anisotropically. The H atoms were placed in calculated positions with fixed isotropic thermal parameters, except the O-bonded ones, which were located in ΔF maps. The structure was refined with the SHELXL-93 program [13] and the scattering factors enclosed therein. Lists of crystal data and structure refinement, atomic coordinates and equivalent isotropic temperature factors, complete bond lengths and angles, anisotropic displacement parameters, torsion angles and selected least-squares planes are available on request from the authors.

HPLC Analysis.

The chromatographic title (from the sum of the two isomers) was determined on the following systems: Superspher 100RP-18 column 250 x 4 mm 5 μm, eluting with 50 water:50 acetonitrile mobile phase and 0.8 ml/minute flux; uv detector 254 nm; the sample concentration was 0.1 mg/ml, 20 μl injection volume. The two isomers were separated on the following system: Perkin-Elmer Binary LC pump 250, Perkin-Elmer LC290 detector, Helwett Packard 3396A integrator, Reodyne 7125, 20 μl injection valve using a Chiracel column OJ 250 x 4.6 mm 10 μm,

mobile phase 66 *n*-exane:34 ethanol, 1 ml/minute flux, uv detector at 254 nm, 20 µl injection volume. Several experiments allowed the detection of the limit injectable concentration which proved to be 3 mg/ml (maintaining a satisfying α value of 1.48). After 40 injections of 20 µl, 435 µg of compound **1A** and 593 µg of compound **1B** were isolated. The purity of the two fractions was controlled by injecting the collected isomers, and the fractions were finally dried in a stream of nitrogen.

NMR Spectra.

All ^1H nmr measurements were performed on a Bruker AMX 400 WB spectrometer operating at 9.395 Tesla. Spectra were performed at a 8 m*M* concentration in dimethyl-d_6 sulfoxide. 2D-COSY [14] spectra were recorded using 512 time-domain points and 128 increments (F_1 dimension), 1s of relaxation delay and 8 transients per increment. Data were doubled in F_1 dimension by zero filling and weighted by a sine-bell functions in both dimensions before Fourier transformation. This last was run in magnitude mode. 2D-NOESY [15] spectra were acquired with Time Proportion Phase Increment Phase Cycle [16] 512 (F_2) x 128 (F_1) data points, 1s of relaxation delay, 8 scans per transient and of 200 ms mixing times.

Biological Evaluation.

The uv spectra were determined using a Perkin Elmer UV spectrophotometer mod. "lambda 16", equipped with multicell system thermostated with an Haake F3C circulating bath. The molar extinction coefficients were measured in dimethyl sulfoxide and in ethanol at a concentration about 10^{-4} *M*. The stock solutions of the inhibitors were prepared in dimethylsulfoxide at about 2 m*M* concentration, spectrophotometrically determined. They were stored at -20° until use and their stability was checked both in dimethyl sulfoxide and in the buffer used in the enzymatic assays (standard 2-[[tri(hydroxymethyl)methyl]-amino]-1-ethanesulfonic acid buffer) at 20°. The enzyme *Lactobacillus casei* thymidylate synthase was obtained from plasmids that express it in the Thy-*Escherichia coli* strain X2913 (kind gift of Daniel V. Santi, University of California, San Francisco) as previously reported [17,18]. The inhibitory activity of the compounds was determined by studying the effect on the catalytic activity of increasing inhibitor concentrations at fixed folate concentration. The apparent Ki was measured by the non linear regression analysis of the experimental data according to Segel [19]. The inhibition assays were performed at 20° on 0.8 ml of reaction mixture formed by standard 2-[[tri(hydroxymethyl)methyl]amino]-1-ethanesulfonic acid buffer *p*H 7.4, 2'-deoxyuridine-5'-monophosphate 120 mu*M*; the folate concentration was 104 mu*M*. The Ki values were obtained from the mean value of at least three determinations; the standard error from non linear least squares fit of the experimental data is reported [20].

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