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ortho-Halogen Naphthaleins as Specific Inhibitors of *Lactobacillus casei* Thymidylate Synthase. Conformational Properties and Biological Activity

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Abstract—Thymidylate synthase (TS) (EC 2.1.1.45), an enzyme involved in the DNA synthesis of both prokaryotic and eukaryotic cells, is a potential target for the development of anticancer and antinfective agents. Recently, we described a series of phthalein and naphthalein derivatives as TS inhibitors. These compounds have structures unrelated to the folate (Non-Analogue Antifolate Inhibitors, NAAIs) and were selective for the bacterial versus the human TS (hTS). In particular, halogen-substituted molecules were the most interesting. In the present paper the halogen derivatives of variously substituted 3,3-bis(4-hydroxyphenyl)-1H,3H-naphtho[2,3-*c*]furan-1-one (**1**–**5**) and 3,3-bis(4-hydroxyphenyl)-1H,3H-naphtho[1,8-c,d]pyran-1-one (**6**–**14**) were synthesized to investigate the biological effect of halogen substitution on the inhibition and selectivity for the TS enzymes. Conformational properties of the naphthalein series were explored in order to highlight possible differences between molecules that show species-specific biological profile with respect to non species-specific ones. With this aim, the conformational properties of the synthesized compounds were investigated by NMR, in various solvents and at different temperatures, and by computational analysis. The apparent inhibition constants (K_i) for *Lactobacillus casei* TS (LcTS) were found to range from 0.7 to 7.0 μM, with the exception of the weakly active iodo-derivatives (**4**, **10**, **13**); all] the compounds were poorly active against hTS. The di-halogenated compounds **7**, **8**, **14** showed the highest specificity towards LcTS, their specificity index (SI) ranging between 40 and >558. The di-halogenated 1,8-naphthalein derivatives (**7**–**10**) exhibited different conformational properties with respect to the tetra-haloderivatives. Though a clear explanation for the observed specificity by means of conformational analysis is difficult to find, some interesting conformational effects are discussed in the context of selective recognition of the compounds investigated by the LcTS enzyme.
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Introduction

Thymidylate synthase (TS) (EC 2.1.1.45), an enzyme involved in the DNA synthesis of both prokaryotic and eukaryotic cells, is a potential target for the development of antibacterial, antimycotic, anticancer and, recently, antiviral agents.[1–6] TS catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) by a reductive methylation involving N^5,N^{10}-methylentetrahydrofolate (mTHF) as cofactor.[1,2] In the absence of dTMP formation via Thymidine Kinase (TK) (salvage pathway), the reaction catalyzed by TS is the rate-limiting step in the DNA synthesis, for it is the sole de novo pathway for the synthesis of thymidine triphosphate (dTTP). Inhibition of TS would therefore prevent cell multiplication.

Compounds structurally related to the substrate (dUMP) or to the cofactor (mTHF) have been developed as classical antimetabolites.[3] A huge number of derivatives have been tested and some are on clinical trial or on the market for anticancer therapy, such as FdUMP (5-fluoro-2′-deoxyuridine-5′-monophosphate) and the folate analogues ZD1694, BW1843U89 (Fig. 1). These compounds prevent the substrate or the cofactor from binding the TS active site and, as a consequence, they block the synthesis of dTMP. Nevertheless, the

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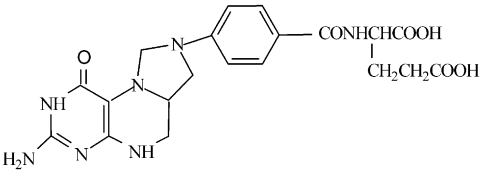
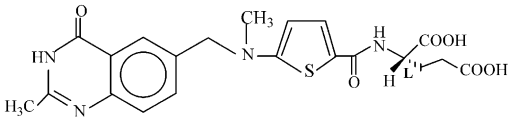
excess of free TS, due to failures of feedback regulation, and its capability to bind mRNA can induce more complex long-term reactions.[7–9] Owing to the structural similarity between classical antimetabolites and natural metabolites, these compounds are generally poorly selective and give rise to resistance through different mechanisms.[10,11]

Recently, Non Antifolate Analogue Inhibitors (NAAIs) compounds not structurally related to the folate cofactor but possessing antifolate activity, have been receiving increasing attention. In an attempt to overcome resistance, especially that arising from the metabolic pathway activation typical of the folate analogues, many inhibitors with non-classical folate structure have been developed, and some of them, such as AG331 (Fig. 1) and AG337[12,13] have been tested in clinical trials.

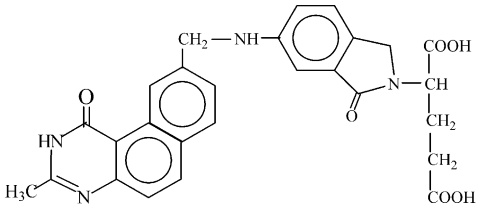
Recently, TS has become an interesting target for anti-infective drugs. That is due to structural differences between TS from various sources, as recently demonstrated by X-ray crystallography of *Lactobacillus casei* TS (LcTS),[14] *Escherichia coli* TS (EcTS),[15] *Pneumocistis carinii* TS (PcTS)[16] and human TS (hTS),[17,18] either as apo-enzymes and complexes with inhibitors.[3] A comparative analysis of the mentioned structures suggests that the folate analogue inhibitors bind in the folate binding site and the binding mode is highly conserved among TS complexes[3] from different species.



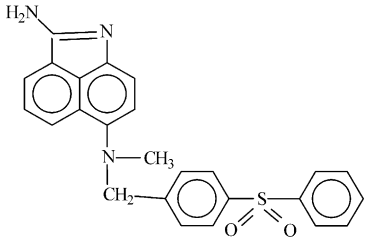
N^5,N^{10}-methylentetrahydrofolate



ZD1694

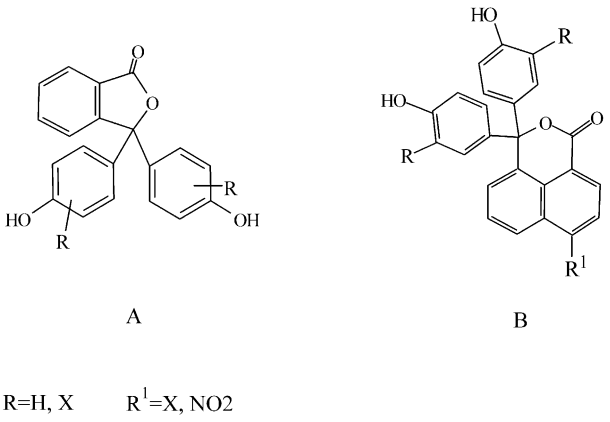


BW1843U89



AG331

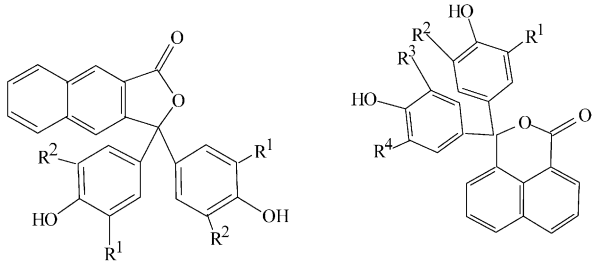
Figure 1. N^5,N^{10}-Methylentetrahydrofolate, folate analogues and **NAAI** of Thymidylate synthase of recent development in anticancer chemotherapy.



A B

R=H, X R^1=X, NO2

Figure 2. Phthalein and naphthalein derivatives.[4]



1 - 5 **6 - 14**

	R^1	R^2
1	H	H
2	F	H
3	Cl	H
4	I	H
5	Br	Br

	R^1	R^2	R^3	R^4
6	H	H	H	H
7	F	H	F	H
8	Cl	H	Cl	H
9	Br	H	Br	H
10	I	H	I	H
11	Cl	Cl	Cl	Cl
12	Br	Br	Br	Br
13	I	I	I	I
14	Br	H	H	H

Figure 3. Chemical structure of the compounds studied in the present paper.

In a previous work,[4] we studied a series of phthalein and naphthalein derivatives as NAAIs, (A,B, Fig. 2). These compounds have structures not related to the folate but display a competitive inhibition pattern with respect to the cofactor. In particular, compound 3,3-bis(3-chloro-4-hydroxyphenyl) - *1H*,*3H* - naphtho[1,8 - *c*,*d*]pyran-1-one (R = Cl, R′ = H, **8**, Fig. 3) was 60 times more active for LcTS with respect to hTS.

The X-ray structure of the complex between compound **8** and LcTS showed that it binds at the entrance of the active site pocket and makes very few specific hydrogen bonds with the enzyme.[4] In particular, the hydrogen bonds between the phenol hydroxyl groups and Trp85, His106 and Val316 are shown in Figure 4. Compound **8** shows some interactions with an area of the enzyme surface called small domain; this area, formed by residues 90–139, is typical of LcTS and other pathogens TS such as *Staphylococcus aureus* and *Enterobacter faecalis*. The ability of the compound **8** to bind to the 'small domain' of TS, which is not present in the human enzyme (Fig. 4), has been suggested to explain the specificity of compound **8** and its derivatives. However, the molecular bases of the observed species-specificity remain unclear, at the moment. Therefore better-designed naphthalein derivatives have to be synthesized to better explore structure–specificity relationships.

In the present paper, two series of halogen derivatives of variously substituted 3,3-bis(4-hydroxyphenyl)-*1H*,*3H*-naphtho[2,3-*c*]furan-1-one (**1**–**5**) and 3,3-bis(4-hydroxyphenyl)-*1H*,*3H*-naphtho[1,8-*c*,*d*]pyran-1-one (**6**–**14**) were synthesized (Fig. 3) and the biological role of the halogen substitution on the inhibitory profile towards TS enzymes was investigated. Conformational properties of the naphthalein derivatives synthesized were explored to highlight possible differences in molecular properties between molecules showing species-specific and non species-specific biological profiles. Studies on the conformational properties of the naphthalein derivatives have been performed by NMR, in various solvents and at different temperatures, and by computational analysis. Inhibition assays versus hTS and LcTS were performed for all the compounds, and their specificity was estimated.

Chemistry

ortho-Halogen substituted naphthaleins can be obtained either through Friedel–Crafts reaction between the naphthalidic compound (2,3-naphthalic anhydride and 1,8-naphthalic anhydride) and the required substituted phenol or through direct halogenation of the appropriate naphthalein compound, namely [3,3-bis(4-hydroxyphenyl)-*1H*,*3H*-naphtho[2,3-*c*]furan-1-one (**1**) and 3,3-bis(4-hydroxyphenyl)-*1H*,*3H*-naphtho[1,8-*c*,*d*]pyran-1-one (**6**)] by bromine, or iodine chloride. It should be noted that in no case examples of substitutions on the naphthalene ring were evidenced.

Compounds **1**–**3** (Scheme 1) were obtained by heating the anhydride at 180 °C for 5 h with the appropriate

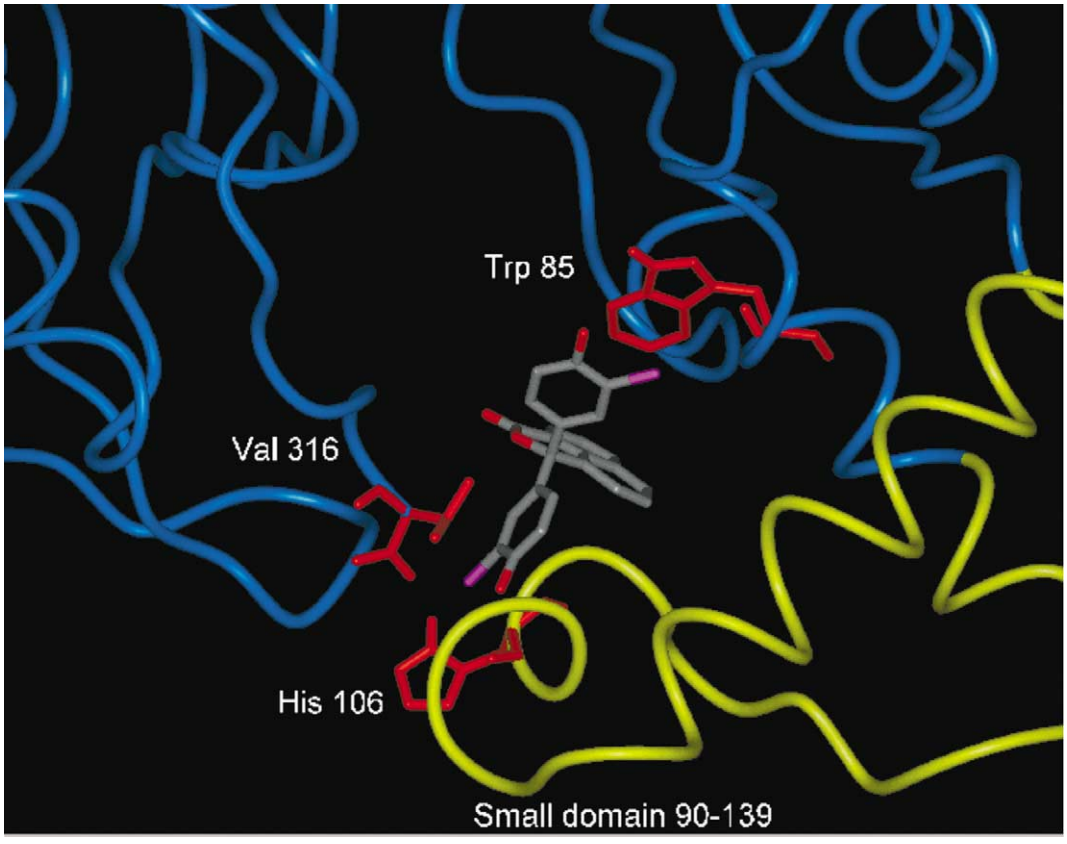


Figure 4. Detail of the X-ray crystal structure of the binary complex *Lactobacillus casei* TS-compound **8**. The small domain is colored in yellow.

phenol (molar ratio 1:2), in the presence of a few drops of sulphuric acid,[19] and purifying the crude by silica-gel chromatography. Compound **4** was obtained by stirring **1** and an excess iodine chloride in glacial acetic acid at room temperature overnight.[20] Compound **5** was prepared by bromination of **1**, using 4 equivalents of bromine in ethanol overnight at room temperature[21,22] (Scheme 1).

Compounds **6–8** were prepared by heating 1,8-naphthalic anhydride at 110–115 °C for 3 days with the appropriate phenol, in the presence of aluminum chloride.[23,24] Treatment of **6** with excess bromine in dichloromethane at room temperature for 6 h gave a mixture of **9** and **14**,[21] which were separated silica-gel chromatography. Compound **14,** the only chiral molecule of the series, was tested as a racemic mixture. The tetra-bromo derivative **12** was prepared from **6** as reported for **5**, while the tetra-chloro derivative **11** was obtained by heating **6** with chlorine in DMF at 60 °C. The iodo derivatives **10** and **13** were synthesized by treating **6** with iodine chloride at room temperature or with a mixture of iodine/mercury acetate at 95 °C, respectively (Scheme 2).

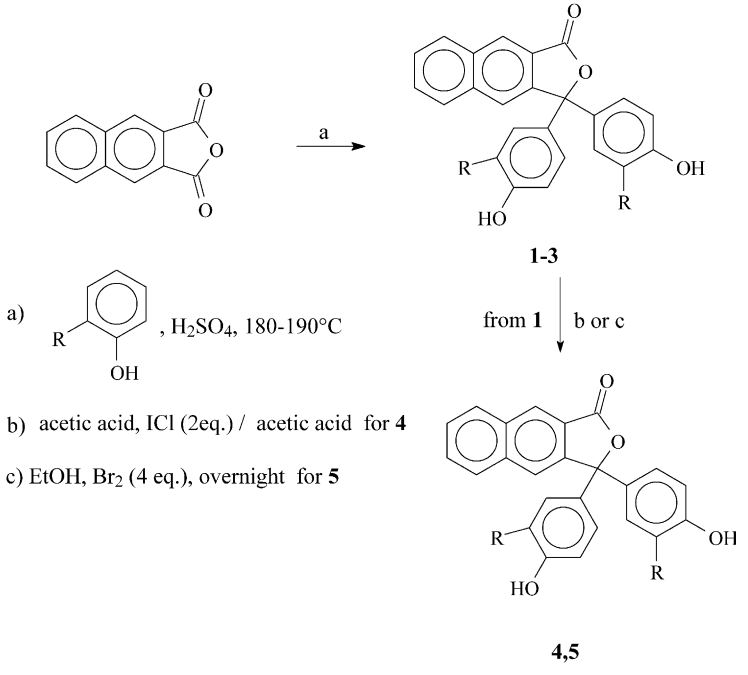
^{1}H NMR Spectroscopy

Room temperature one-dimensional proton NMR spectra of compounds **1–14**, dissolved in DMSO-d_6, were fully assigned on the basis of the chemical shifts and the signal multiplicity analysis. In a few cases, homonuclear scalar and dipolar correlations obtained, respectively, by COSY and NOESY spectra were required. All the data are summarized in Table 1.

In a previous paper,[25] we reported that the chemical shift (δ) of phthalidic proton H4, of phthaleins disubstituted at position 3 by two phenolic rings, is sensitive to the average angle formed by the phthalidic and the aromatic ring planes. Moreover, we reported that the resonances of chemically equivalent protons belonging to the phenolic rings split at low temperature as a result of conformational constrains due to steric hindrance. These aspects were considered relevant also in the present study in order to investigate the conformational properties.

Proton NMR spectra of compounds **1–14** in DMSO-d_6 at 300 K showed small and meaningless differences in the δ of proton H4 (values ranging between 8.36 and 8.50 ppm) and only one signal for each couple of chemically equivalent protons belonging to the phenolic rings. This behaviour was also observed for compounds **1–5**, **6** and **11–13** in acetone-d_6, at temperature ranging between 300 and 170 K (freezing point of the solutions). This demonstrates that in all these cases, the two phenolic rings lie in the same average conformational state in the NMR time scale although, in some cases (see tetra-bromo derivative **5**), bulky substituents are present. As a consequence, it is possible to suggest that the presence of the penta-atomic lactonic ring does not promote any significant steric constrain at the phenolic rings that maintain unimpaired high rotational freedom around the C3–C10 and C3–C17 bonds. On the contrary, the low temperature NMR spectra of compounds **7–10** shown separate peaks for protons H11, H14, H15, H18, H21 and H22 whose differences in chemical shift increased with the lowering of the temperature, as shown in Figure 5.

To verify if the polarity of the solvent could influence the conformational properties of the molecules, $CDCl_3$ and CS_2 were used. Only compound **8** was soluble enough to allow the collection of NMR spectra, so the experiments were limited to this compound. Up to 203

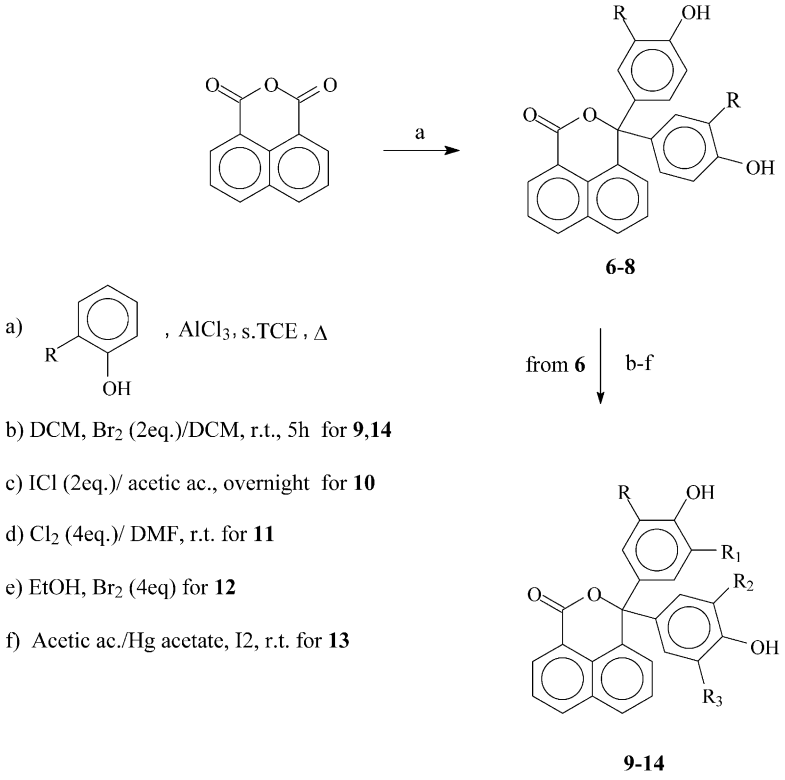


Scheme 1.

and 170 K, the freezing points in $CDCl_3$ and CS_2 respectively, the spectra did not display any splitting of signals. This behavior, compared with that observed in aceton-d_6, suggests that the polarity of the solvent plays a role in conformation. Since in acetone-d_6, compound **8** behaves like all the other di-halogen derivatives **7**, **9** and **10**, one can reasonably suppose compounds **7**, **9** and **10** to behave in similar fashion in $CDCl_3$ and CS_2.

Low temperature NOESY spectra of **8**–**10**, dissolved in acetone-d_6, were also recorded to gain deeper insight into the conformational differences observed among the di-halogen derivatives and all the other compounds. In all cases only positive NOEs were detected, i.e., the molecules maintained their extreme narrowing condition ($\omega \tau_c < < 1$) and signals resulted well detectable although, in some cases, they fall in crowded area of the spectra (in compound **9** all the phenolic protons resonate in only 0.5 ppm of spectral width). In the case of the tetra-iododerivative **10**, all the possible NOEs were observed: between protons H4-H11, H4-H18, H4-H14, H4-H15 and between all the phenolic protons (Fig. 6). It is worth noting that the splitting of oxydrilic protons was also observed. On the basis of these data, a conformation for compound **10** can be drawn in which the two phenolic rings appear as shown in Figure 7.

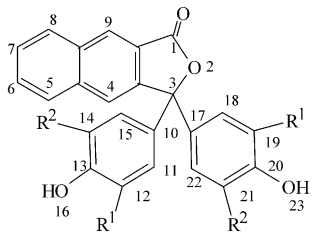
Although a complete set of NOEs was observed only for compound **10**, it is reasonable to suppose that the other di-halogen derivatives **7**–**9** also exhibit, at low temperature,



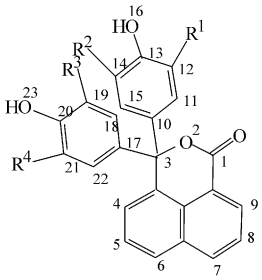
Compound	R	R^1	R^2	R^3
6	H	H	H	H
7	F	H	F	H
8	Cl	H	Cl	H
9	Br	H	Br	H
10	I	H	I	H
11	Cl	Cl	Cl	Cl
12	Br	Br	Br	Br
13	I	I	I	I
14	Br	H	H	H

Scheme 2.

Table 1. NMR data of proton spectra of compounds **1**—**14**



1-5



6-14

p.n.	Chemical shift (ppm)													
	1		**2**		**3**		**4**		**5**		**6**		**7**	
H4	8.36	s	8.46	s	8.50	s	8.45	s	8.19	s	7.17	dd	7.24	dd
H5	8.23	d	8.25	d	8.25	d	8.25	d	8.22	d	7.75	dd	7.77	dd
H6	7.83	td	7.85	td	7.86	td	7.85	t	7.84	t	8.19	dd	8.23	dd
H7	7.77	td	7.78	td	7.79	td	7.78	t	7.87	t	8.47	dd	8.49	dd
H8	8.34	d	8.35	d	8.35	d	8.35	d	8.32	d	7.84	dd	7.86	dd
H9	8.73	s	8.73	s	8.77	s	8.76	s	8.75	s	8.36	dd	8.39	dd
H11	7.24	dt	7.17	dd	7.34	d	7.65	d	7.89	d	6.96	d	6.98	o
H12	6.86	dt	—		—		—		—		6.81	d	—	
H14	=H12		7.06	t	7.09	d	=12		—		7.04	d	6.79	
H15	=H11		7.11	dd	7.26	dd	=11		=11		6.99	dd	=11	
H16	9.72	s	10.10	s	10.61	s	10.73	s	—		9.71	s	9.83	
H18	=H11		=H11		=H11		=H11		=H11		=H11		=H11	
H19	=H12		—		=H12		=H12		—		=H12		—	
H21	=H14		—		=H14		=H14		—		=H14		=H14	
H22	=H15		=H15		=H15		=H15		=H15		=H15		=H15	
H23	=H16		=H16		=H16		=H16		—		=H16		=H16	
	8		**9**		**10**		**11**		**12**		**13**		**14**	
H4	7.24	dd	7.23	dd	7.21	dd	7.36	d	7.33	dd	7.35	d	7.20	dd
H5	7.78	dd	7.78	dd	7.78	dd	7.80	t	7.89	dd	7.95	t	7.77	dd
H6	8.24	dd	8.24	dd	8.23	dd	8.27	d	8.28	d	8.32	d	8.22	dd
H7	8.50	dd	8.50	dd	8.49	dd	8.52	d	8.53	dd	8.57	d	8.48	dd
H8	7.87	dd	7.87	dd	7.87	dd	7.89	t	7.81	dd	7.86	t	7.86	dd
H9	8.39	dd	8.40	dd	8.39	dd	8.42	d	8.43	dd	8.46	d	8.38	dd
H11	7.10	d	7.25	d	7.46	dd	7.26	s	7.31	s	7.54	s	7.24	d
H12	—		—		—		—		—		—		—	
H14	6.97	d	7.04	d	...	d	—		—		—		7.00	o
H15	7.05	dd	6.99	dd	6.96	d	=H11		=H11		=H11		=H14	
H16	10.62	s	10.70	s	10.70	s	10.70		10.50	s	10.06	s	9.76	s
H18	=H11		=H11		=H11		=H11		=H11		=H11		6.82	d
H19	=H12		=H12		=H12		—		—		—		7.00	o
H21	=H14		=H14		=H14		—		—		—		=H19	
H22	=H15		=H15		=H15		=H15		=H15		=H15		=H18	
H23	=H16		=H16		=H16		=H16		=H16		=H16		10.65	s
							Correlations							

1) **C**: H5–H6;H6–H7;H7–H8;H11–H12–N: H4–H11;H4–H5; H8–H9
6) **C**: H4–H5;H4–H6;H5–H6;H7–H8; H7–H9;H8–H9;H11–H12; **N**: H4–(H11,H18); H4–(H15,H22) ; H6–H7
8) **C**:H4–H5;H4–H6;H5–H6;H7–H8;H7–H9;H8–H9;H11–H14; H11–H15;H14–H15; **N**: H4–H11;H4–H15;H6–H7
10) **C**: H4–H5;H4–H6;H5–H6;H7–H8;H7–H9;H8–H9,H11–H14, H11–H15
12) **C**: H4–H5; H5–H6; H7–H8; H8–H9; **N**: H6–H7; H4–H11

Chemical shifts (δ), spin multiplicities (s = singlet, d = doublet, t = triplet), scalar and dipolar correlations (C = correlation in 2D-COSY spectra; N = correlation in 2D-NOESY spectra; Hn–Hm means correlation between proton Hn and proton Hm) of compounds **1**–**14** dissolved in DMSO-d_6. All spectra were recorded at room temperature. All chemical shifts are referred to the residual signal of DMSO-d_6 fixed at 2.6 ppm.

the conformation found for **10** (data not shown), because the splitting pattern of the signals is very similar to that observed for **10**.

Finally, compounds **6** and **14** exhibited neither splitting nor broadening of the resonance of the phenolic protons at low temperature in acetone-d_6. This proofs that the conformation of compounds **1**–**14** also depends on the symmetry/asymmetry of the substitution, as confirmed by the different behavior of di-halogen with respect to the tetra-halogen derivatives.

Quantum chemical calculations

In order to investigate the conformational properties and confirm the interpretation of the NMR results,

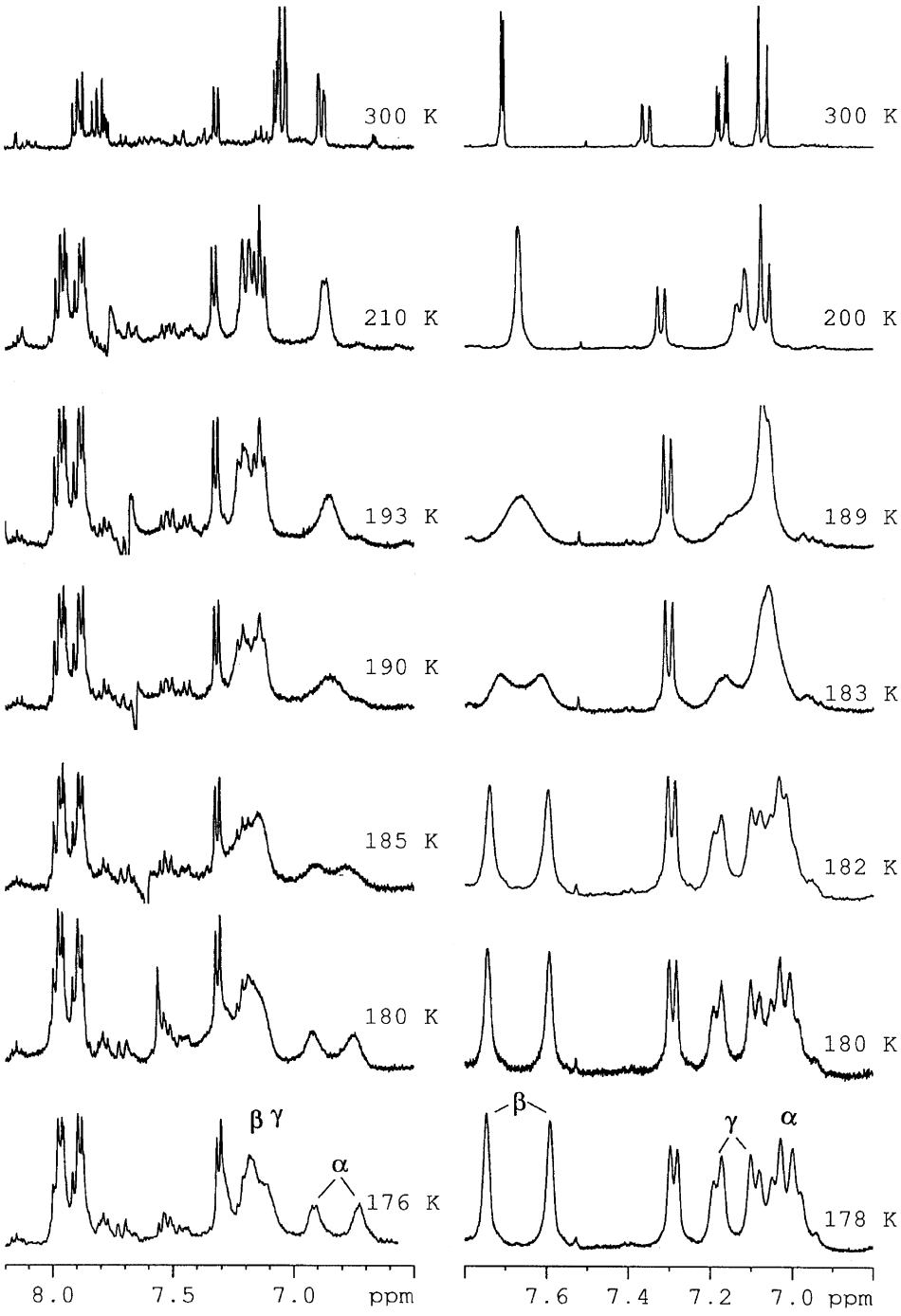


Figure 5. ^{1}H NMR spectra of compounds **7** (left panel) and **10** (right panel) in acetone-d_6 at different temperatures. The splitting protons are indicated as follows: α = H14, β = H11, γ = H15. With decreasing temperature, the splitting of the proton signal of the phenol rings is evident.

conformational analysis was performed on a selected set of compounds (**6**, **8**, **9**, **11** and **12**). These compounds were chosen to investigate the effects of the halogens on conformational barriers in symmetrically- and asymmetrically-substituted compounds. Conformations were investigated by rotating the phenolic rings in steps of 20°, with complete geometry optimization at each step, using the PM3Hamiltonian.

The rotational profiles obtained for these compounds are graphically reported and superimposed in Figure 8. Inspection of these profiles shows that all the compounds have low rotational barriers (close to 1 Kcal/mol), in agreement with the high conformational freedom of the two phenolic rings demonstrated by NMR experiments. Interestingly, one significant difference was found between symmetrically- and asymmetrically-substituted compounds. The symmetrically-substituted compounds **6**, **11** and **12**, have almost identical conformational profiles. Therefore, these compounds exhibit almost identical conformational properties regardless of substitution and of the nature of the halogen substituent (chloro or bromo). The asymmetrically-substituted compounds **8** and **9** still have very similar conformational profiles, thus

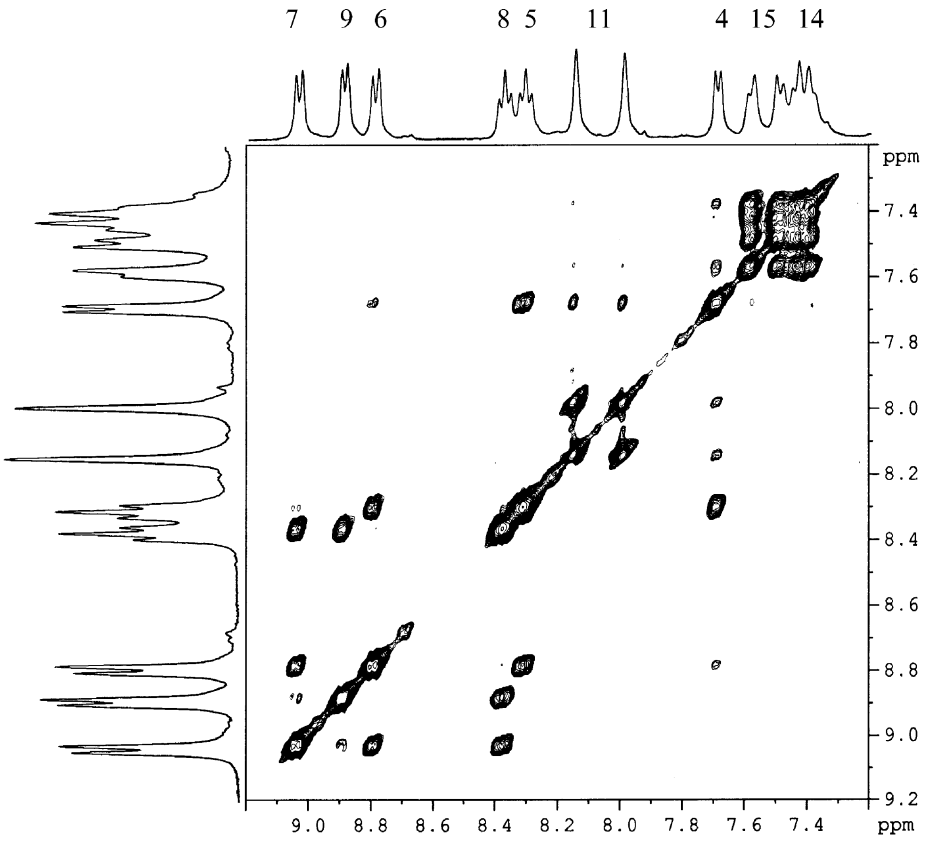


Figure 6. ^{1}H/2D-NOESY spectrum of compound **10** recorded at 170 K in acetone-d_6 using 200 ms of mixing time.

affording further evidence that conformational properties are not significantly affected by the type of halogen substituent, but they exhibit somewhat higher rotational barriers in the 150–270° range of the dihedral angle with respect to the symmetrically substituted compounds. This increase in rotational barrier for compounds **8** and **9** nicely supports the NMR experimental evidence that all the di-halogen derivatives assume a well-defined conformation at very low temperature, at variance with the non-substituted (**6**) and symmetrically substituted (**11**, **12**) compounds. In conclusion, the calculated conformational profiles are in agreement and support the NMR results; they also suggest that conformation, in this type of molecules, is more dependent on symmetry/asymmetry of substitution rather than on the nature of substitution. Similar conclusions have also been reached by calculating the dipole moments of the compounds as a function of the conformational angle (Fig. 9). Even for the dipoles, identical trends were observed for the non-substituted compound **6** and the symmetrically-substituted compounds **11** and **12,** regardless of the substituent, while a completely different trend was observed for the asymmetrically-substituted compounds **8** and **9**.

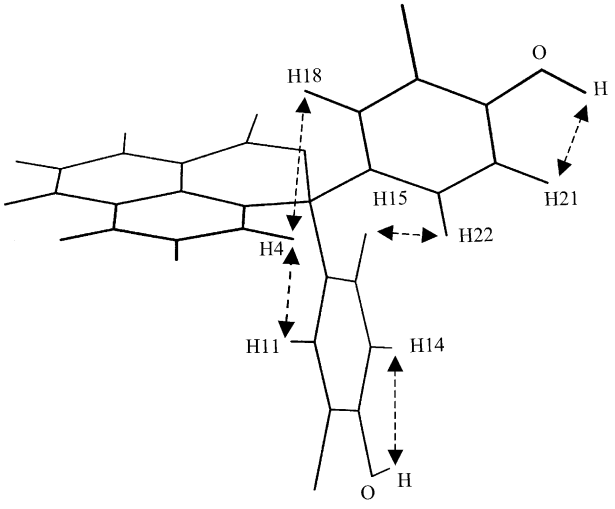


Figure 7. Compound **10** is reported with the conformation supported by ^{1}H/2D-NOESY spectrum at 170 K in acetone-d_6 (see Fig. 6). The protons referring to NOEs are indicated by arrows.

Enzyme assays

All the compounds were evaluated as inhibitors of *L. casei* TS and human TS (Table 2) and IC_{50} values were determined. In order to study their inhibition pattern, apparent inhibition constant values (K_i) were measured against LcTS (Table 2); the specificity indexes (SI) were calculated by the ratio IC_{50}hTS/IC_{50}LcTS (Table 2).

All the compounds proved to be competitive inhibitors of LcTS with respect to the folate cofactor. 2,3-Naphthalein derivatives (**1**–**5**) are generally less active towards LcTS than the 1,8-naphthalein derivatives (**6**–**14**). Their apparent K_i values range from 1.5 μM (**2**), to 7.0 μM (**1**), while no inhibition is seen for compound **4** at 23 μM, which corresponds to the solubility limit for

this compound. Their IC_{50} values at high concentrations towards hTS were also measured; they range from 6.0 to 41 μM; the iodo-derivative (**4**) is inactive at the concentration measured (23 μM). The most selective compound is the *ortho*-fluorine derivative **2**, which shows a specificity index (SI) of 4.

Inhibition data analysis for the 1,8-naphthalidic di-halogen series (**7**–**10**,**14**) (Table 2) showed that the best inhibitor towards LcTS is compound **8** ($K_i = 0.7$ μM); compound **12** and the chiral compound **14** are also good inhibitors with K_i values of 0.9 and 1.0 μM, respectively. All the other compounds had higher K_i values ranging from 1.6 to 6.8 μM; the iodo derivative **10** exhibited the lowest inhibitory activity ($K_i = 31$ μM). All the compounds are weak or very weak inhibitors of hTS, with IC_{50} between 6 and well above 4 mM. Compounds **7**, **10** and **14** did not demonstrate any inhibitory activity at the solubility limit (Table 2); the IC_{50} values were therefore calculated assuming 2% of inhibitory activity to be present at the solubility limit owing to the experimental error.[4] This approximation clearly underestimates the true IC_{50} value of the compounds.

The most specific inhibitors are **7**, **8** and **14**. The specificity range that we were able to measure was higher than 558 for **7**, 298 for **14** and 40 for **8** (Table 2).

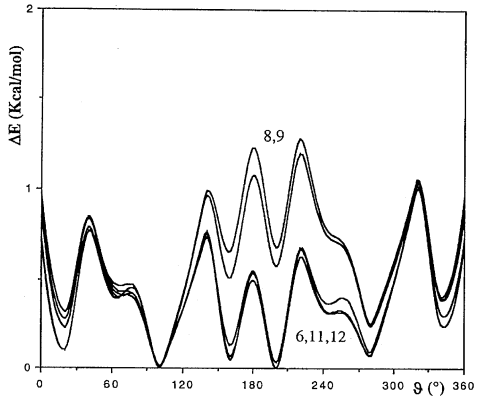


Figure 8. Rotational profile for compounds **6**, **8**, **9**, **11**, **12**. The solid line is obtained from interpolation of the calculation results every 20°, as described in Experimental.

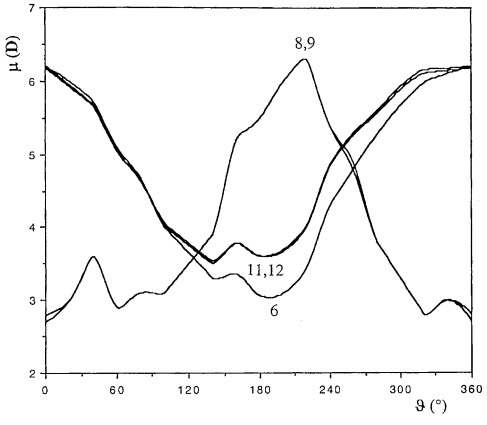


Figure 9. Dipole moment profiles for compounds **6**, **8**, **9**, **11**, **12**.

Table 2. Inhibitory activity of compounds **1**–**14** against LcTS and hTS. Specificity indexes (SI) are given by the ratio of IC_{50} for hTS and IC_{50} for LcTS

Compd	LcTS K_i (μM)	LcTS IC_{50} (μM)	hTS IC_{50} (μM)	SI
1	7.0	20	40	2.0
2	1.5	7.2	29	4.0
3	1.6	5.3	6.0	1.1
4	NI[a]	—	NI[a]	—
5	5.3	20	41	2.1
6[b]	2.8	27	67	2.5
7	1.6[b]	7.2	>4018[c]	>558[c]
8[b]	0.7	1.3	52	40
9	6.8	32	47	1.4
10	31	111	>1317[c]	>12[c]
11	2.0	7.4	57	7.6
12	0.9	4.3	32	6.1
13	—	NI[a]	NI[a]	—
14	1.0	5.4	>1627[c]	>298[c]
CB3717	0.06	0.09	0.03	0.34

[a]NI, no inhibition at 23 μM, corresponding to the solubility limit of the compound.
[b]Ref 4.
[c]Calculated supposing 2% inhibition at the maximum solubility concentration, **7**, 82 μM; **10**, 27 μM; **14**, 33 μM, respectively.

The 1,8-naphthalidic tetra-halogen derivatives (**11**, **12**) are good inhibitors of LcTS but also inhibit hTS, hence the SI value is around 7 for both the compounds. The iodo derivative **13** is inactive at the solubility limit (23 μM). The well-known TS inhibitors with classical folate structure, CB3717[26] (5,8-dideaza folic acid), did not demonstrate any species-specificity among the two species considered (LcTS and hTS), displaying an SI of 0.34.

Discussion

The conformational studies performed with ^{1}H NMR showed that compounds **1**–**5** behave similarly to that of the previously investigated phthalein derivatives,[25] with high rotational freedom at room and low temperature. On the contrary, differences were noted within the series of compounds **6**–**14**. The derivatives having two halogens on both phenolic rings in *ortho* position to the hydroxyl group (**11**–**13**) still exhibit high rotational freedom up to 170 K, regardless of the nature of the halogen atom. In contrast, the analogues having only one halogen substituent on the phenolic ring (**7**–**10**) assume a well-defined conformation when the temperature is lowered to 179 K. This conformation is similar to the one observed from X-ray crystallography of similar compounds.[27] In that conformation the phenolic rings are on perpendicular planes with the chlorine atom on opposite direction. It is noteworthy that, at least in the case of compound **8**, a well-defined conformation could not be observed in less polar solvents such as $CDCl_3$ and CS_2. This compound was the only one soluble in apolar solvents and it was not possible to perform a similar experiment for the other di-halogen derivatives (**7**, **9**, **10**). These data suggest that the 1,8-di-halogen substituted compound, **8**, likely undergoes specific interactions with the polar solvent leading to a well-defined conformation; this effect is not seen in apolar solvents. Since the NMR results proved to be independent

from the nature of the halogen, it is likely that the other di-halogen derivatives (**7**, **9**, **10**) would also behave similarly in apolar solvents.

In agreement with NMR, quantum-chemical calculations suggest that the symmetrically-substituted compounds **6**, **11** and **12**, exhibit almost identical conformational properties regardless of substitution and of the nature of the halogen substituent (chloro or bromo). The asymmetrically-substituted compounds **8** and **9** still have very similar conformational profiles, thus affording further evidence that conformational properties are not significantly affected by the type of halogen substituent. The low conformational barriers are in agreement with the high conformational freedom observed by NMR. Interestingly, calculations suggest that conformation is more dependent on symmetry/asymmetry of substitution rather than on the nature of substitution. Similar conclusions have also been reached by calculating the dipole moments of the compounds as a function of the conformational angle.

Considerations on structure–activity and structure–specificity relationships show that the 2,3-naphthalein series (**1**–**5**) is less interesting than the 1,8-naphthalein series (**6**–**14**). In the first series the K_i ranged between 1.5 and 7.0 μM, and no species-specificity could be observed (selectivity index, SI = 4, being the best result). The second series is more interesting in terms of species-specificity and, in particular, the di-halogen derivatives (**7**–**10**). Their K_i values range between 0.7 and 31 μM, and the SI values were 100 times higher with respect to the SI values found in the first series, the SI being >558 for compound **7**. One exception is the chiral compound **14**, that shows a K_i higher than 298.

The findings that di- and tetra-halogenated compounds display different conformational behaviour in polar/apolar solvents, and that their dipole moments follow opposite trends as a function of conformation, suggest that these two classes of compounds could have different molecular states also in a biological environment. In a previous work,[4] it was proposed that the molecules showing a species-specific inhibitory profile bind to non-conserved binding site such as the small domain in LcTS, as showed by the X-ray structure of the binary complex LcTS-a156. The different molecular state of the present molecules might influence the binding conformation, shifting the molecule from a well-conserved site to a non-conserved one, therefore contributing to the observed specificity.

Obviously, conformational behavior is not the only factor determining activity and specificity. The importance of one *ortho* substituent can also affects the polarity of the phenolic hydroxyls and influences the interactions with polar aminoacids of the active site.

Conclusions

In the present study, some *ortho*-halogen di- and tetra-substituted derivatives were synthesized, NMR were performed at room and low temperature in different solvents and quantum chemical calculations were run to study their conformational properties. Their biological activity profile against LcTS and hTS was then determined.

We found an agreement between the conformational behaviour of the di-halogen derivatives in solution as determined by ^{1}H NMR analysis and the quantum chemical calculations performed on the conformational properties of the molecules. Low-temperature NMR in acetone showed that compounds **7**–**10** with only one halogen on the phenol ring have a preferred conformation corresponding to the low-energy conformation, even though in NMR terms it is an average conformation. Correspondingly, conformational analysis based on quantum chemical calculations demonstrated that no preferred conformation exists for the di-halogen derivatives with respect to the tetra-halogen substituted derivatives and that rotational freedom is allowed at room temperature. Thus, these compounds exhibit almost identical conformational properties regardless of the substitution and the nature of the halogen substituent (chloro or bromo). The asymmetrically substituted compounds **8** and **9** still display very similar conformational profiles. This adds further evidence that the type of halogen does not significantly affect conformational properties. The compounds exhibit somewhat higher rotational barriers in the 150–270° range of the dihedral angle with respect to the symmetrically substituted compounds.

This suggests that the behaviour of the compound **8** in solution at low temperature is governed by specific interaction with the solvent. In fact, unlike the other di-halogens derivatives, compound **8** is soluble in two solvents with different polarity, namely, acetone and carbon disulfide.

In acetone, a highly polar solvent, preferred conformations were found at low temperature. On the other hand, these preferred conformations were not detected in the apolar solvent.

Structure–specificity analysis was difficult to be assessed because factors other than conformation can play a role. It is also true that the conformational analysis was performed in conditions far from those found in the biological environment. Nevertheless, it was clearly highlighted that some molecules (**7**–**10**) have conformational spaces that are different from the others. We suggest that those molecules with different conformational properties can bind to different sub-sites in the recognition process, thus expressing a different biological activity profile.

This conclusion agrees with the results of a previous study on the binding mode of naphthalein derivatives: the folate pocket surface is a highly plastic area in the LcTS enzyme where the naphthalein derivatives can easily bind in more than one mode (multiple binding mode). A deeper analysis of the mode of interaction of the mentioned compounds is required to clearly explain the observed specificity.

Table 3. Elemental analyses of compounds 1–14

Compd	Calcd %			Found %		
	C	H	X	C	H	X
1	78.25	4.38		78.37	4.11	
2	71.29	3.49	9.40 (F)	70.98	3.56	
3	65.92	3.23	16.22 (Cl)	66.01	3.11	16.09 (Cl)
4	46.48	2.28	40.93 (I)	46.37	1.99	41.05 (I)
5	42.14	1.77	46.73 (Br)	42.23	1.86	46.55 (Br)
6	78.25	4.38		78.37	4.44	
7	71.29	3.49	9.40 (F)	71.09	3.27	
8	65.92	3.23	16.22 (Cl)	66.01	3.41	16.35
9	54.78	2.68	30.37 (Br)	54.67	2.79	30.51 (Br)
10	46.48	2.28	40.93 (I)	46.52	2.32	41.05
11	56.95	2.39	28.02 (Cl)	57.09	2.24	27.95 (Cl)
12	42.14	1.77	46.73 (Br)	42.28	1.95	46.03 (Br)
13	33.06	1.39	58.22 (I)	33.11	1.29	58.34 (I)
14	64.45	3.38	17.86 (Br)	64.51	3.46	17.54 (Br)

Experimental

Compounds commercially available were used without further purification. Br_2 (Merck), ICl (Aldrich), mercury acetate (J.T. Baker), I_2 (Acros) were bought as indicated in parentheses. Melting points were determined on a Büchi 510 apparatus and are uncorrected. Thin-layer chromatography (TLC) was carried out with pre-coated silica gel F254 plates (Merck).

The IR spectra were recorded in Nujol suspension on a Perkin-Elmer Spectrophotometer Mod 681. Silica Gel 60 (Merck 70–230 mesh) was used for purification of the synthesized compounds using the solvent system indicated in the section dealing with synthesis.

Microanalysis was carried out by Rossella Gallesi in the Microanalysis Laboratory of Dipartimento di Scienze Farmaceutiche (Università di Modena e Reggio Emilia). The results (Table 3) were within ±0.4% of the theoretical value and the structure of the compounds were consistent with their spectroscopic data.

Chemistry

3,3-Bis(4-hydroxyphenyl)- (1), 3,3-bis(3-fluoro-4-hydroxyphenyl)-(2) and 3,3-bis(3-chloro-4-hydroxyphenyl)-*3H*-naphtho[2,3-*c*]furan-1-one (3). General procedure. A mixture formed by 2,3-naphthalic anhydride (0.01 mol), the appropriate phenol (0.02 mol) and a few drops of H_2SO_4 concd was heated at 180–190 °C for 5 h with constant stirring. After cooling, water (30 mL) was added to the reaction mixture; the new mixture was then extracted with dichloromethane (3 × 20 mL). The organic phase was dried on anhydrous Na_2SO_4 and concentrated in vacuum. The solid residue was purified by silica gel chromatography with dichoromethane/methanol (95/5 v/v) as eluent. The first collected compound was the unreacted anhydride, followed by the desired compound. The final compounds crystallized from dichloromethane as a pale-brown powder.

1: yield 35%; mp 298–300 °C. 2: yield 38%; mp 138–140 °C. 3: yield 40%; mp 200–203 °C.

3,3-Bis(4-hydroxyphenyl)- (6), 3,3-bis(3-fluoro-4-hydroxyphenyl)- (7) and 3,3-bis(3-chloro-4-hydroxyphenyl)-*1H*,*3H*-naphtho[1,8-*c*,*d*]pyran-1-one (8). Anhydrous aluminum chloride (0.02 mol) was added portionwise to a mixture formed by 1,8-naphthalic anhydride (0.01 mol) and the appropriate phenol (0.02 mol) in anhydrous s-tetrachloroethane (100 mL); the suspension was stirred for three days at 100–115 °C. The hot mixture was then poured onto ice and dichloromethane (200 mL) was added. After stirring for 0.5 h the precipitate was filtered off and 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 v/v) to give, first, the unreacted anhydride, followed by an unidentified compound and, finally, the desired product. The final compounds were crystallized from dichloromethane as a pale-brown solid.

6: yield 18%; mp 265–267 °C. 7: yield 32%; mp 248–250 °C. 8: yield 38%; mp 190–195 °C.

3,3-Bis(3,5-dibromo-4-hydroxyphenyl)-*1H*,*3H*-naphtho[2,3-*c*]furan-1-one (5) and 3,3-bis(3,5-dibromo-4-hydroxyphenyl)-*1H*,*3H*-naphtho[1,8-*c*,*d*]pyran-1-one (12). Bromine (0.60 mmol) was added dropwise at room temperature to compound 1 or 6 (0.14 mmol) dissolved in 2 mL of ethanol and the reaction mixture was stirred overnight. After evaporation of the solvent, the residue was purified by silica gel chromatography eluting with cyclohexane/ethyl acetate (70/40 v/v). Crystallization from ethylacetate gave the desired compounds as a yellowish crystalline solid.

5: yield 50%; mp 155–158 °C. 12: yield 65%; mp 257–260 °C.

3,3-Bis(3-bromo-4-hydroxyphenyl)-*1H*,*3H*-naphtho[1,8-*c*,*d*]pyran-1-one (9) and 3-(3-bromo-4-hydroxyphenyl)-3-(4-hydroxyphenyl)-*1H*,*3H*-naphtho[1,8-*c*,*d*]pyran-1-one (14). Bromine (0.27 mmol), dissolved in dichloromethane, was added dropwise to a stirred solution of 6 (0.14 mmol) in 3 mL of dichloromethane at room temperature. After stirring for 5 h at rt the solvent was evaporated and the residue purified by silica gel chromatography (dichloromethane/methanol 98/2 v/v). 14 was eluted as the first run followed by 9. Compounds were crystallized from ethyl acetate as yellowish crystalline solids.

9: yield 28%; mp 98–100 °C. 14: yield 25%; mp 228–230 °C.

3,3-Bis(3-iodo-4-hydroxyphenyl)-*3H*-naphtho[2,3-*c*]furan-1-one (4) and 3,3-bis(3-iodo-4-hydroxyphenyl)-*1H*,*3H*-naphtho[1,8-*c*,*d*]pyran-1-one (10). Iodine monochloride (0.61 mmol), dissolved in 1 mL of glacial acetic acid, was added to a mixture of 1 or 6 (0.14 mmol) suspended in 2.5 mL of glacial acetic acid, and the mixture was stirred at room temperature overnight. After evaporation of the solvent, the residue was purified by silica gel chromatography (dichloromethane/methanol 98/2 v/v). Compounds were crystallized from ethyl acetate as brown crystalline solids.

4: yield 59%; mp 230–232 °C. **10**: yield 71%; mp 143–145 °C.

3,3-Bis(3,5-diiodo-4-hydroxyphenyl)-*1H,3H*-naphtho[1,8-*c,d*]pyran-1-one (13). Iodine (0.54 mmol) was added portionwise to a stirred mixture of **6** (0.14 mmol) and mercury acetate (0.54 mmol) in 2 mL of glacial acetic acid heated to 95 °C; the mixture was allowed to react under stirring at the same temperature for 30 min. After cooling, the mixture was diluted with water and extracted with ethyl ether. The organic phase was dried over anhydrous Na_2SO_4, evaporated and the residue purified by silica gel chromatography (dichloromethane/methanol 98/2 v/v). **13**: yield 8.5; mp 160–163 °C (ethyl acetate, dec).

3,3-Bis(3,5-dichloro-4-hydroxyphenyl)-*1H,3H*-naphtho[1,8-*c,d*]pyran-1-one (11). A solution of **6** (2.72 mmol) in anhydrous DMF (10 mL) was saturated with chlorine, stirred at room temperature for 60 min and then decomposed with ice. The precipitate was crystallized by acetone/petroleum as a pale brow solid. Yield 32%; mp 95–100 °C.

NMR spectra

All NMR measurements were performed on a Bruker AMX 400 WB spectrometer operating at 9.395 Tesla. Spectra were performed on 16 mM samples dissolved in DMSO-d_6 for analysis at 303 K, or in acetone-d_6, $CDCl_3$ and CS_2 for studies at low temperatures. 1D proton, carbon-13 and carbon-13 DEPT spectra were obtained by optimizing the spectral width and the digital resolution. No weighting of the FIDs was performed before the Fourier transformation.

2D-COSY[28] spectra were recorded using 512 or 1024 time-domain points and 128 or 256 increments (F_1 dimension), depending on the spectral width employed, 1 s of relaxation delay and 8 transients per increment. Data were doubled in the F_1 dimension by zero filling and weighted by a sine-bell function in both dimensions before magnitude-mode Fourier transformation.

2D-NOESY[29–32] spectra were obtained with TPPI Phase Cycle, 256(F_1)\1024(F_2) or 512(F_1)\1024(F_2) data points, depending on the spectral width used, 1 s of relaxation delay, 8 scans per transient and using mixing times of 200 ms. Data were weighted before Fourier transformation using a sine square-bell function shifted by ∨2 radiants in both dimensions.

Quantum chemical calculations

A subset of compounds in Figure 3 was selected for quantum chemical calculations: **6** (unsubstituted), **11** (tetra-chloro) and **12** (tetra-bromo) were chosen to investigate the effects of halogen substitution on conformational barriers in symmetrically substituted compounds. The di-bromo (**9**) and di-chloro (**8**) derivatives were chosen to investigate asymmetrical substitutions, particularly as the di-chloro compound is specific for Lc TS.

Conformational analysis was performed using the MOPAC[33] program in the PM3 parameterization.[34] The rotational profiles were obtained by rotating one of the two substituted phenyl rings in steps of 20°, with all other geometric parameters completely optimized.

All calculations were performed on Silicon Graphics O2 workstations.

Enzyme assay

Plasmids that express *L. casei* TS in the Thy$^-$ *Escherichia coli* strain χ2913 have been previously described.[35] The enzyme was purified by column chromatography using phosphocellulose (P11, Biorad) and hydroxyapatite (HAP, Biorad) resin, with phosphate buffer as eluent.[35,36] Human Thymidylate synthase (hTS) was purified, as reported, through affinity column chromatography.[37] The enzyme preparations were >95% homogeneous, as shown by SDS-polyacrylammide gel electrophoresis. The LcTS purified enzyme was stored at −80° C in 10 mM phosphate buffer, pH 7.0, 0.1 mM EDTA until use. hTS was used immediately after purification.

Enzymatic assays were run with a Beckman DU-640 UV-Vis spectrophotometer, equipped with multicell system thermostated with a Haake F3C circulating bath.

The activity of Thymidylate synthase was determined spectrophotometrically by steady-state kinetic analysis, following the increasing absorbance at 340 nm due to the oxidation reaction of N^5,N^{10}-methylenetetrahydrofolate to dihydrofolate at 5,6 bond.[38] Assays were performed at 20 °C in the standard assay buffer, which contained 50 mM TES (tris ethylaminosulfonic acid) at pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA (ethylendiaminetetracetic acid) and 75 mM 2-mercapto-ethanol. 1 mL of reaction mixture consists of standard TES buffer pH 7.4, dUMP 120 μM, 6-(R,S)-l-CH_2CH_4-folate 180 μM, enzyme 0.07 μM.

The stock solutions of the inhibitors were prepared in DMSO (dimethylsulfoxide) and kept at −20 °C. Control reactions were run in order to measure the effect of DMSO on enzyme activity. DMSO never exceeded 10% in the enzyme assay mixture.[39]

TS inhibition studies were carried out under the condition of the standard assay. Enzyme concentration was 0.07 μM, dUMP concentration was 120 μM, while CH_2H_4folate concentration was 60 μM for IC_{50} calculations and was varied for K_i calculations. Reactions were initiated by the addition of the enzyme. Steady-state kinetic analysis of the Lineweaver–Burk plot of the enzyme activity upon different inhibitor concentrations was carried out by varying the folate concentration in the case of compounds **6** and **8**. All the compounds displayed a competitive inhibition pattern. When the inhibition pattern was competitive, K_i values were determined with the appropriate equation.[40] All the experiments were repeated at least three times, and the standard errors from the linear least-squares fit of the experimental data are within the 20%.

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