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Neighbouring-group participation as the key step in the reactivity of acyclic and cyclic salicyl-derived *O*,*O*-acetals with 5-fluorouracil. Antiproliferative activity, cell cycle dysregulation and apoptotic induction of new *O*,*N*-acetals against breast cancer cells

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Abstract—The reaction between *o*-(hydroxymethyl)phenoxyacetaldehyde dimethyl acetals, or 3-methoxy-2,3-dihydro-5*H*-1,4-benzodioxepins with 5-fluorouracil (5-FU), has been studied. The intramolecular cyclization may be explained through a neighboring group attack to give a 2-(5-fluorouracil-1-yl)oxyranium ion that can be attacked by the silylated benzylic hydroxy group to yield the benzannelated seven-membered *O*,*N*-acetals. The formation of a macrocyclic *trans*-bis(5-FU *O*,*N*-acetal) is also reported. Such a compound arrested the human MCF-7 breast cancer cells at the G_o/G_1 phase of the cell cycle. On the contrary, the acyclic nitro *O*,*N*-acetal seems to work as a 5-FU prodrug, because it arrested cancer cells at the S phase as the well-known prodrug Ftorafur does.

1. Introduction

Breast cancer ranks just behind lung cancer as the second leading cancer in women, and is the leading cause of cancer death among women in the 35–54 age group and the second cause of cancer death for women aged 55–74.[1–3] The disseminated nature of breast cancer and the development of cross-resistant tumours are the primary causes of failure of current therapies. By the time a tumour is detected, there is a high probability that methastatic lesions will be present, and many of these will already contain a resistant subpopulation of cells. Not surprisingly, there is a substantial interest in the identification of novel anticancer agents for the treatment of breast cancer.[1–4]

Apoptosis or programmed cell death is an innate mechanism by which unwanted, defective, or damaged cells are rapidly and selectively eliminated from the body. It occurs during tissue remodeling, embryonic development, and immune regulation.[5] Apoptosis is the principle mechanism employed by the immune system and chemotherapeutic drugs in eradicating tumour cells. Resistant tumour cells evade the action of anticancer agents by increasing their apoptotic threshold. This has spurred the development of novel chemical compounds capable of inducing apoptosis in chemo/immune-resistant tumour cells. Although breast cancer is most often treated with conventional cytotoxic agents it has proven difficult to induce apoptosis in breast cancer cells using these drugs.[6] By identifying therapies that are particularly effective in activating apoptosis, improved clinical responses may be obtained.

The non-naturally occurring base with known antitumour activity, 5-fluorouracil (5-FU, **1**), is highly toxic and this fact has motivated important research directed to the synthesis and biological evaluation of 5-FU derivatives with less toxicity than the parent compound. Within the extensive therapeutical arsenal the prodrug Ftorafur **2** occupies an outstanding position.[7] As part of a programme for the development of 5-FU derivatives, we published the synthesis and biological activities of several acyclonucleosides analogues.[8,9] Later on, the synthesis of a wide range of 5-FU derivatives linked to saturated seven-membered moieties through *N*-1 was carried out,[10] and this methodology allowed us access to non-classical nucleosides with a

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3-hydroxymethyl-1,4-dioxepan-5-yl group as the 'sugar' moiety.[11] In a recent article[12] we have reported the preparation, antiproliferative activities and apoptotic induction against the MCF-7 human breast cancer cell line of the benzannelated seven-membered 5-FU *O*,*N*-acetals **3a**–**c**. On one hand, we report herein their reaction mechanism and, on the other, the preparation of the open 5-FU *O*,*N*-acetalic analogues, to assess their biological activities against the breast cancer cells (Chart 1).

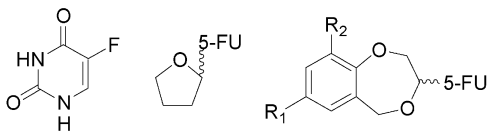


Chart 1.

2. Results and discussion

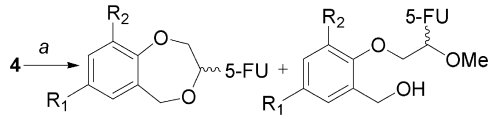
2.1. Chemistry

The synthesis of the *O*,*N*-acetals was carried out in a two-step process: (a) preparation of the intermediate acyclic and cyclic *O*,*O*-acetals, **4a**–**f** and **5a**–**f**, respectively, and (b) reaction of these acyclic and cyclic compounds with the pyrimidine base to give the title structures. 5-chloro-2-hydroxybenzyl alcohols were obtained by reduction of the corresponding salicylaldehyde.[13] Hydroxyacetals **4d**–**f** were prepared in a similar way to the obtention of **4a**–**c**[12] by alkylation of the salicyl alcohols with bromoacetaldehyde dimethyl acetal, using sodium hydride as a base in anydrous dimethylformamide (DMF). Finally, the 3-methoxy-2,3-dihydro-5*H*-1,4-benzodioxepins **5d**–**f** were obtained under an acid-catalyzed intramolecular cyclization process on the hydroxyacetals **4d**–**f**, under the conditions previously reported for **5a**–**c**[12] (Scheme 1).

The substitution of the acetalic OMe group by the 5-FU moiety in the *O*,*O*-acetals **4a**–**f** and **5d**–**f** has been carried out by reaction with 5-FU in the presence of the silylating agents 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and trimethylchlorosilane (TCS), under acid catalysis of stannic chloride. The aminalic bond is established through *N*-1 of the 5-FU moiety unless otherwise stated. In spite of the fact that the starting materials (**4**, **5**) are dissimilar, in both cases the same final compound type was obtained, with different yields.

2.1.1. Condensation between *o*-(hydroxymethyl)phenoxyacetaldehyde dimethyl acetals 4 and 5-FU. The condensation between the acyclic *O*,*O*-acetals **4** and 5-FU, under the previously described conditions,[12] gave rise to the cyclic **3** and/or the acyclic *O*,*N*-acetals **6**, respectively through a process whose regioselectivity depended on the presence and nature of the substituents on the benzene ring (Table 1), named R_1 and R_2. In general, in the majority of cases the attachment of the 5-FU moiety occurred at *N*-1, and rarely at the *N*-3 position of the uracil ring. These facts were confirmed through ^{1}H NMR data. According to Ozaki et al.[14] the H_6 coupling pattern of the pyrimidine ring is indicative

Table 1. Formation of cyclic and acyclic 5-FU *O*,*N*-acetals starting from the acyclic *O*,*O*-acetals **4**, the reaction time being 24 h[a]



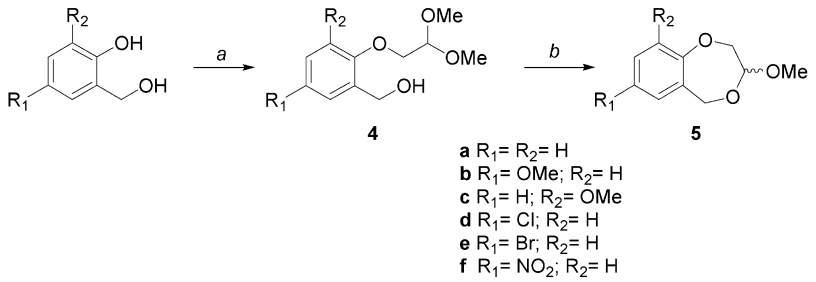
Entry	Starting acetal	R_1	R_2	Yield (%) of **3**	Yield (%) of **6**
1	**4b**	OMe	H	26 (5)[b]	27
2	**4c**	H	OMe	–	37
3	**4d**	Cl	H	–	17
4	**4e**	Br	H	–	4 (8)[b]
5	**4f**	NO_2	H	–	35

[a] 5-FU, HMDS, TCS, $SnCl_4/CH_2Cl_2$, CH_3CN.
[b] All the yields refer to compounds in which the 5-FU moiety is linked through N_1, except in $\mathbf{6e}^{N\text{-}3}$ and $\mathbf{3b}^{N\text{-}3}$, in which the link is through N_3 and whose yields are reported between brackets.

of the substitution model on the 5-FU moiety. Briefly, (a) a broad triplet due to C_6-H at around $\delta \approx 7-8$ ppm indicates that 5-FU has a substituent at its *N*-1 position, and (b) a broad triplet due to C_6-H at around $\delta \approx 7-8$ ppm indicates that 5-FU is 3-substituted. Compound $\mathbf{3b}^{N\text{-}3}$ (this nomenclature indicates that the 5-FU moiety is linked to the carbon chain through its *N*-3 atom) showed a normal behaviour when its ^{1}H NMR spectrum was recorded in a DMSO-d_6 solution. Nevertheless, the situation is different for $\mathbf{6e}^{N\text{-}3}$: C_6-H resonated as a sharp doublet when the spectrum was recorded in CD_3OD due the rapid exchange of the hydrogen atom of the N^1-H group with the deuterium atom of the solvent. Nevertheless, the aspect of the H-1′ casts light on the subject: this proton resonates as a pseudotriplet whereas the same proton of **6e** resonates as a double doublet of doublet (ddd), the latter being due to the long-range coupling with the fluoro atom,[8,10,11] which showed unambiguously that the 5-FU is linked through *N*-1 to the *o*-(hydroxymethyl)phenoxyethyl-1-methoxy moiety. In the former case ($\mathbf{6e}^{N\text{-}3}$), the long-range coupling with the F atom was not observed because its electronic effect was not transmitted through the carbonyl group at position 4 of the uracil ring.

In relation with the condensation reaction between the acyclic *O*,*O*-acetals **4** and 5-FU, the following can be stated:

(1) The nature and position of substituents R_1 and R_2 showed clear influence on both the yields and regioselectivity of the process. Thus, the substitution in position 5 of **4** (equivalent to 7 of **5**) affected in the following manner (only R_2=H is considered):
  1.1 The electron-withdrawing substituents (Cl, Br and NO_2) induced the preferent formation of acyclic *O*,*N*-acetals **6** (entries 3–5).
  1.2 The only electron-donating substituent used, the OMe group, halted the regioselectivity of the reaction forming both the cyclic **3** and acyclic **6** *O*,*N*-acetals and, moreover, in an approximately 1/1 ratio (entry 1).
  1.3 In some cases the presence of substituents in one or other type of *O*,*O*-acetals permitted the isolation of derivatives in which the 5-FU moiety

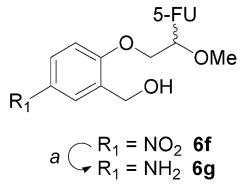


Scheme 1. *Reagents*: (a) $BrCH_2CH(OMe)_2$, HNa, anhydrous DMF. (b) $BF_3{\cdot}OEt_2$ in anhydrous Et_2O.

was linked through *N*-3 of the pyrimidine base (entries 1 and 4).

(2) Finally, the substitution in C-3 of **4** by the only group studied (OMe) clearly favoured the formation of the acyclic *O*,*N*-acetal (entry 2).

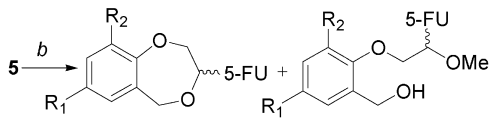
The nitro group of **6f** has been reduced with tin(II) chloride dihydrate in refluxing ethanol to yield compound **6g** (Scheme 2).



Scheme 2. *Reagents*: (a) $SnCl_2{\cdot}2H_2O$, EtOH.

2.1.2. Condensation between 3-methoxy-7-substituted-2,3-dihydro-5*H*-1,4-benzodioxepins 5 and 5-FU. The results obtained when the cyclic *O*,*O*-acetals were used as starting materials in the reaction with 5-FU are shown in Table 2, which reproduces the reaction time and the yields obtained. In this study, the following may be generalized:

Table 2. Formation of cyclic and acyclic 5-FU *O*,*N*-acetals[a] starting from cyclic *O*,*O*-acetals **5**, the reaction time being 24 h[b]



Entry	Starting acetal	R_1	R_2	Yield (%) of **3**[c]	Yield (%) of **6**[c]
1	**5a**	H	H	43	–
2	**5b**	OMe	H	27 (26)	21 (27)
3	**5b**	OMe	H	40[d]	–
4	**5c**	H	OMe	15.5	5
5	**5d**	Cl	H	–	77 (17)
6	**5e**	Br	H	–	33 (4)
7	**5f**	NO_2	H	–	5 (35)

[a] All the yields refer to compounds in which the 5-FU moiety is linked through N_1.
[b] 5-FU, HMDS, TCS, $SnCl_4/CH_2Cl_2$, CH_3CN.
[c] The yields obtained from the acyclic *O*,*O*-acetals **4** are shown between brackets.
[d] Reaction time: 144 h (Ref. 12).

1. The better yield was obtained when R_1 and R_2 were hydrogen atoms (entry 1). The process is regioselective in the cyclic *O*,*N*-acetal **3a** and the 5-FU moiety was linked through its *N*-1 atom, no traces of the *N*-3 substituted compound having being observed.
2. Substituents R_1 and R_2 similarly influenced the regioselectivity of the previously described reaction; thus, the R_1 electron-withdrawing substituents (Cl, Br and NO_2) induced the regioselective formation of the acyclic *O*,*N*-acetals (entries 5–7).

2.1.3. Experimental study of the condensation reaction between the acyclic *O*,*O*-acetals 4, and the cyclic *O*,*O*-acetals 5 with 5-FU: influence of other factors on the progress of the reaction. As has been explained previously, the nature of the substituent on the aromatic ring is determinant for the obtention of the cyclic or acyclic *O*,*N*-acetals. Now the influence of the reaction time and the type of the Lewis acid will be described. For this study the most simple acetals have been selected. The reaction between the cyclic *O*,*O*-acetal **5a** and 5-FU is strongly affected by the reaction time (Table 3). Thus, a short reaction time (4 h) favours the formation of the acyclic *O*,*N*-acetal **6a**, whereas its increase (24 h) provokes the regioselective synthesis of the cyclic *O*,*N*-acetal **3a** (entries 2 and 3), as was described previously.[12] From these experimental data it can be deduced that compound **3a** is formed from the structure **6a**, as will be depicted in Scheme 4.

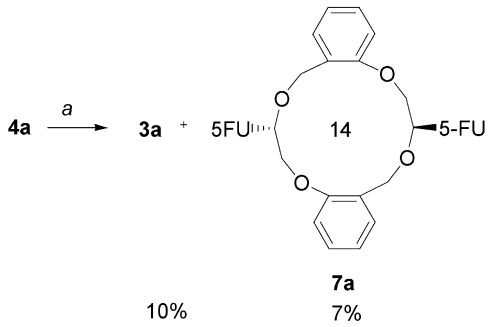
Table 3. Several conditions in the condensation reaction between the cyclic *O*,*O*-acetal **5a** and 5-FU

Entry	Lewis acid	Reaction time (h)	Yield (%) of **3a**	Yield (%) of **6a**
1	$SnCl_4/CH_2Cl_2$	4	4	15
2	$SnCl_4/CH_2Cl_2$	24	43	–
3	$BF_3{\cdot}OEt_2$	24	36	–

All the yields refer to compounds in which the 5-FU moiety is linked through N_1.

Nevertheless, in the reaction between the acyclic *O*,*O*-acetal **4a** and 5-FU with a reaction time of 24 h and using $SnCl_4/CH_2Cl_2$ as the Lewis acid, the two following reaction products have been identified (Scheme 3): **3a** (10%) and its corresponding dimer **7a**† (7%) that shows a 14-crown-4 ether structure with two pendant 5-FU fragments. Although

† This compound will be assigned to have a *trans* isomerism (Section 2.2.2).



Scheme 3. *Reagents*: [a] 5-FU, HMDS, TCS, $SnCl_4/CH_2Cl_2$, CH_3CN, reaction time 24 h.

the yield for the obtention of **7a** was very low, the structure is very attractive and will give it a long pedigree as a result of its notable biological properties (see Section 2.4).

2.2. Conformational analysis

The conformational study of the macrocyclic compound **7a** was carried out in two well differentiated phases. In the first one, the study of the base ring, without the two 5-FU moieties, was tackled to identify the most stable conformation of this compound. In the second one, from the less energetic conformation of this compound, the two 5-FU fragments were added to give rise to the *cis*- and *trans*-**7a** and the conformational analysis on both isomers was carried out.

2.2.1. Conformational analysis of the 4,10-dimethoxy-2,5,8,11-tetraoxa-1,7(1,2)dibenzenadodecaphane.[15,16] The initial geometry of the non-substituted macrocycle was generated from standard fragments from the Sybyl libraries.[17] Once the structure was built up, its optimization was undertaken using the Tripos force field,[18] and the charges were generated by means of the Gasteiger–Hückel methodology.[19] The minimization of the structure was carried out by means of the Powell method,[18] up to the point where the energy gradient is less than 0.005 kcal/mol $Å^2$.

Once the initial molecule was optimized a conformational search was begun to find the most stable conformation. The large size of the saturated ring involves an important restriction on carrying out the conformational search by means of random search or systematic techniques, because the number of bonds that would have to be investigated using these two methodologies is very high. Accordingly, a conformational search by means of molecular dynamics was undertaken using the 'simulated annealing' technique. This technique simulates a heating of the molecule to an elevated temperature allowing conformational changes to take place. Once a certain time has elapsed the molecule is cooled down to a low temperature, that makes the last conformation 'freeze'. Next, such a conformation is saved and the procedure is repeated many times. In our case, a heating to 1000 K was simulated for 1000 ps and, after this time had elapsed such a temperature was kept for further 1000 ps before saving the last conformations. The cycle was repeated 5000 times, which allowed the identification of 5000 conformations of the base ring. Once minimized, the 5000 conformations were compared and those which were geometric and energetically equal were removed. By means of the procedure were identified 307 different conformations whose relative energies range between 0.00 and 14.84 kcal/mol.

Figure 1 shows the four most stable conformations. It can be observed that there is a trend in the molecule to arrange the aromatic rings parallel to each other. Moreover, in three of these conformations both aromatic rings are oriented towards the same side of the macrocycle, probably due to a π-π stacking between them. From these conformations, the most stable one was selected for the following phase of this study.

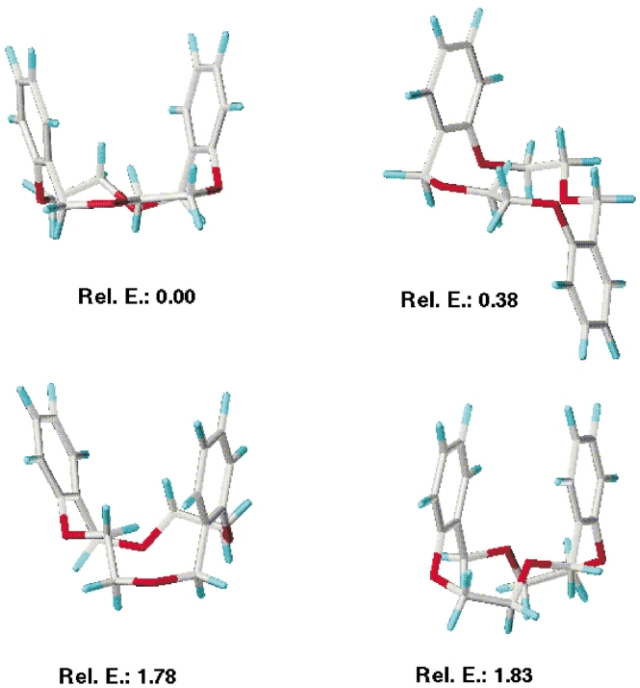


Figure 1. The four most stable conformations of the base ring, together with their relative energy values (kcal/mol) calculated by means of molecular mechanics (Tripos). It is worth noting the nearly parallel arrangement of the aromatic rings in all of them.

2.2.2. Conformational analysis of *cis*- and *trans*-4,10-bis[5-(fluorouracil-1-yl)]-2,5,8,11-tetraoxa-1,7(1,2)dibenzenadodecaphane. Once the most stable conformation of the macrocycle was identified, the two 5-FU *cis* and *trans* isomers were built up. For this the adequate hydrogen atoms on positions 4 and 10 of the base ring were substituted by standard fragments from the Sybyl libraries. The partial atomic charges for each isomers were calculated by means of the Gasteiger–Hückel method and their optimization was carried out until the gradient was less than 0.005 kcal/mol $Å^2$. Following the same procedure explained for the base ring, in the *cis* isomer were identified 392 conformations whose relative energies ranged between 0.00 and 23.13 kcal/mol, whereas for the *trans* isomer were identified 427 conformations whose relative energies ranged between 0.00 and 22.67 kcal/mol. On each compound the most stable conformations were studied.

Figure 2 shows the four most stable conformations of the *cis* isomer, in which it can be observed that at least two parallel rings exist, possibly due to π stacking interactions between them. The differences in energy between such conformations is not very high and accordingly, the molecule could adopt either of them without any difficulty. Although only four conformations were represented, our data show that in this isomer a very complex conformational equilibrium exists with the participation of many conformations of similar energy.

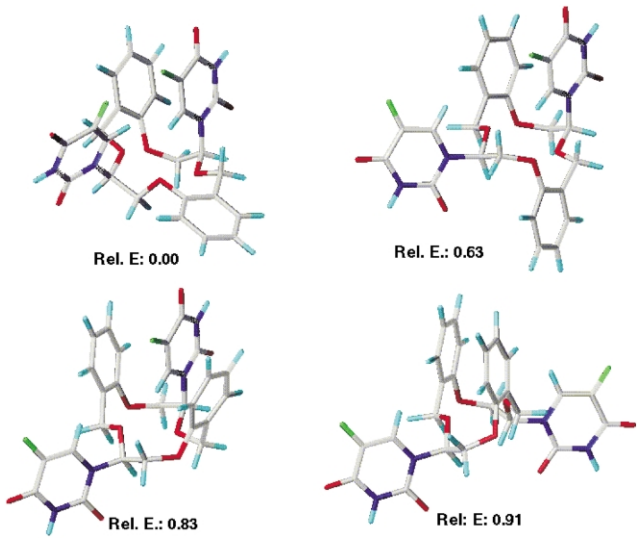


Figure 2. Representation of the four most stable conformations of compound *cis*-**7a**. The relative energy values calculated by means of molecular mechanics (Tripos) are expressed in kcal/mol. It is worth noting the nearly parallel arrangement of at least two aromatic rings in all of them.

Figure 3 shows the four most stable conformations of *trans* isomer. In this compound has been found a great predominance of a conformation in which each benzene ring is located parallel to a 5-FU ring. The great stability of this conformation is probably due to the two π-stacking interactions present in it. Far from isomer *cis*, the *trans* one showed a more simple behaviour from a conformational point of view because the energetic differences between the different conformations are very important. It can be stated that the most stable conformation (upper left conformation in Figure 3) is practically the only one in the conformational equilibrium and, accordingly, the molecule properties would be explained from this conformer. This conformation has an inversion centre, which makes the symmetrical atoms equivalent and, hence, its NMR behaviour should be very simple and should give rise to spectra that contain only half the molecule signals. In the ^{1}H and ^{13}C NMR spectra of this compound this kind of behaviour was found and consequently the *trans* derivative might be the isolated isomer, instead of the *cis* one that should identify each proton or carbon of the molecule. Moreover, if it were a mixture of both isomers, the NMR spectra should have been even more complex.

2.3. Mechanism of the condensation reaction from *O*,*O*-acetals and 5-FU: neighbouring-group participation

The mechanism represented in Scheme 4 (routes 1 and 3), might explain the nature of the reaction products. The

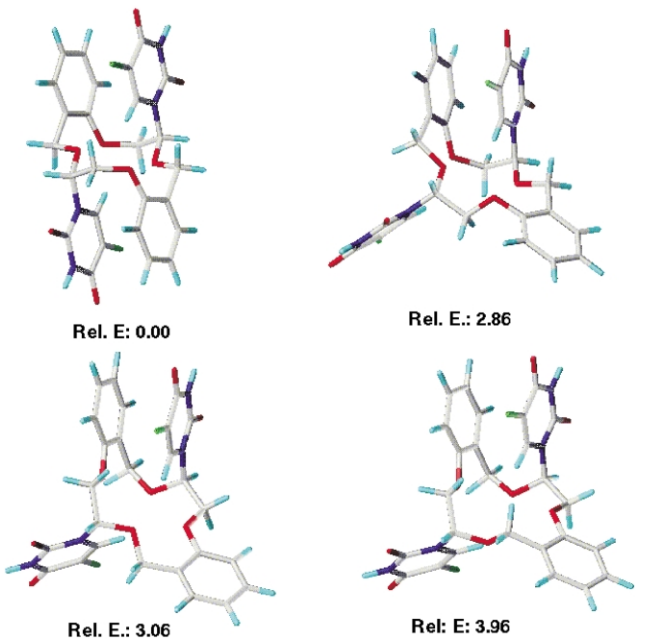
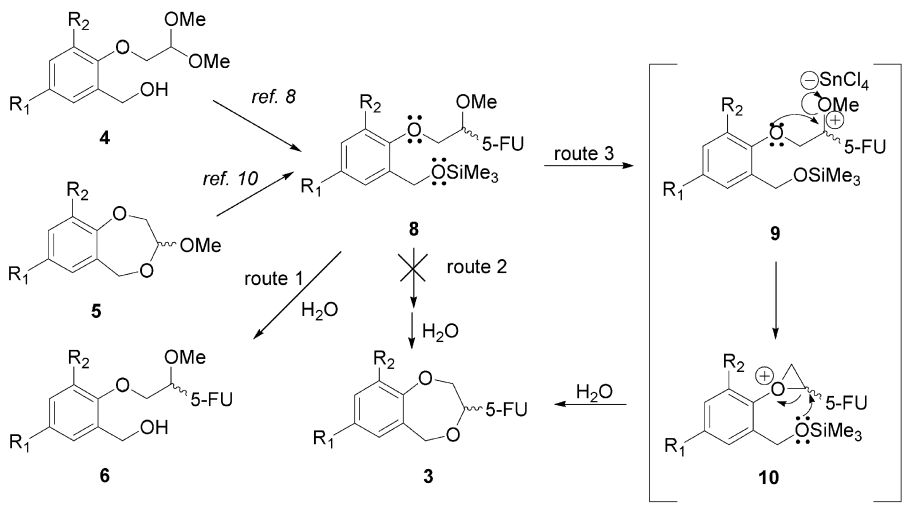


Figure 3. Representation of the four most stable conformations of compound *trans*-**7a**. The relative energy values calculated by means of molecular mechanics (Tripos) are expressed in kcal/mol. It is worth noting the parallel arrangement of the aromatic rings in the most stable conformation, in which the symmetry centre of the molecule can be observed.

substitution of the acetalic OMe group by the 5-FU takes places as we have previously reported for bis-acetals[20] leading to the intermediate **8**. The intramolecular attack of the silylated benzylic hydroxy group might explain the formation of **3** (route 2). Nevertheless, although the mechanism is simple and logical, it does not justify the influence of the R_1 substituent over the course of the reaction. A possible solution might involve a different pathway for the intramolecular cyclization in which the phenolic oxygen atom should intervene as a neighboring group, whose nucleophilicity may be strongly influenced by the electronic character of the substituent R_1. In fact, route 3 shows a possible mechanism for the intramolecular cyclization. According to this route, the intermediate **9** suffers the neighboring group attack to give the oxyranium ion **10**, much more reactive than its predecessor and that can be attacked by the silylated benzylic hydroxy group. Finally, the aqueous work-up renders **3**. Accordingly, the intramolecular cyclization product will depend on the stability of the intermediate **10** which will in turn be influenced by the electronic character of the substituent R_1 (and R_2); in fact, electron-withdrawing groups destabilize the positive charge of the phenolic oxygen atom on generating an electronic deficiency in the carbon atom that carries the oxygen atom, making the intramolecular closing impossible. The contrary holds true for the electron-donating groups such as the methoxy moiety.

In relation with the condensation reaction between the cyclic *O*,*O*-acetals **5** and 5-FU the results seem to suggest that, in the presence of $SnCl_4$, the rest of reaction conditions and in accordance with our previous findings,[8] **8** was formed. This intermediate is the precursor of the two types of target compounds according to Scheme 4. Consequently, the formation of the seven-membered ring reveals that the



Scheme 4.

attack of the benzylic trimethylsilyloxy group leads to a favoured 7-*exo-Tet* process, in accordance with Baldwin's rules.[21] It does not follow that because a process is 'favored' it will necessarily occur readily in every case. The other factor such as the presence of an electron-withdrawing group at position *para* in relation to the phenolic ethereal atom exerts a negative influence and, accordingly, the formation of the seven-membered ring does not take place. This mechanism is supported by the following fact: when the reaction was carried out in a short time (4 h, Table 3, entry 1) the preferential formation of the acyclic *O*,*N*-acetal **6a** over the cyclic one **3a** was observed.

2.4. Antiproliferative activities, cell cycle distribution and apoptosis induction in the human breast cancer MCF-7 cell line

The IC_{50} values of compounds are shown in Table 4. The most active compounds are **6f** (5.42±0.26 μM) and **7a** (5.5±0.58 μM), with antiproliferative activities in the same order as that of Ftorafur (3±0.11 μM).

Cell cycle regulation has attracted a great deal of attention as a promising target for cancer research and treatment.[22,23] The use of cell-cycle-specific treatments in cancer therapy has greatly benefited from the major advances that have been recently made in the identification of the molecular actors regulating the cell cycle and from the better understanding of the connections between cell cycle and apoptosis. As more and more 'cell cycle drugs' are being discovered, their use as anticancer drugs is being extensively investigated.[23] To study the mechanisms of the antitumour and antiproliferative activities of the compounds, the effects on the cell cycle distribution were analyzed by flow cytometry. DMSO-treated cell cultures contained 68.39% G_0/G_1-phase cells, 12.04% G_2/M-phase cells and 19.57% S-phase cells. In contrast, MCF-7 cells treated during 48 h with the IC_{50} concentrations of **6a**,**c**–**g** and **7a** showed important differences in cell cycle progression compared with DMSO-treated control cells. Ftorafur, **2** treatment showed a decrease of the G_0/G_1-phase cells and a corresponding accumulation of S-phase cells (45.62% G_0/G_1-phase cells and 54.38% S-phase cells). Moreover, there was an almost total disappearance in the G_2/M population of the cells treated with this drug. In general the cell cycle regulatory activities for the newly synthesized compounds can be divided into the following three groups: (a) the exposure of MCF-7 cells during 48 h to the novel derivative **7a**, caused strong differences in the cell cycle progression in comparison with Ftorafur, **2**: the breast cancer cells treated with the IC_{50} doses showed a significant accumulation in the G_0/G_1-phase, up to 82.48% of the cells, mainly at the expense of the G_2/M-phase population that decreased to a percentage of 5.13% of the cells; (b) compounds **6d** and **6e** accumulated the cancerous cells in the G_2/M-phase, in the former compound at the expense of the S-phase cells, and (c) compound **6f** induced a S-phase cell cycle arrest (50.24%) in a similar percentage to that caused by Ftorafur, **2** (54.28%) and, accordingly, it can be affirmed that the nitro derivative (**6f**) may act as a 5-FU prodrug. Nevertheless, this hypothesis needs to be corroborated by further assays.

Table 4. Antiproliferative activities, cell cycle dysregulation, and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 24 and 48 h for the compounds

Compound	IC_{50} (μM)	Cell cycle (48 h)			Apoptosis (h)	
		G_0/G_1	G_2/M	S	24	48
Control		68.39	12.04	19.57	1.24	1.24
Ftorafur, **2**	3±0.11	45.62	0	54.38	52.2	58.06
6a	18.5±0.95	67.18	4.67	28.16	59.9	40.23
6c	29±1.63	62.72	1.59	35.69	33.35	37.87
6d	18±0.85	71.01	28.99	0	44.36	50.64
6e	16±1.18	51.45	20.66	27.88	42.24	36.37
6f	5.42±0.26	46.92	2.84	50.24	40.73	48.22
6g	21±1.02	67.32	9.40	23.28	41.15	37.81
7a	5.5±0.58	82.48	5.13	12.40	14.37	19.05

In response to **6a**, the percentage of apoptotic cells increased, from 1.24% in control cells to a maximum of 59.9% apoptotic cells (24 h) at a concentration equal to its IC_{50} against the MCF-7 cell line. This is a remarkable property because the demonstration of apoptosis in MCF-7 breast cancer cells by known apoptosis-inducing agents has proved to be difficult and only few cytotoxic agents act

preferentially through an apoptotic mechanism in human breast cancer cells.[6,24,25] Finally, a fact that is worth emphasizing is that **6f** and **7a** (the only two compounds tested) induce neither toxicity nor death in mice after one month's treatment when administered intravenously twice a week, with a 50 mg/kg dose each time (results not shown).

3. Conclusion

We have completed the synthesis and biological evaluation of novel 1-[*o*-(hydroxymethyl)phenoxyethyl-1-methoxy]-5-fluorouracil compounds, starting from several salicyl alcohols. In this study we have investigated the Lewis acid-promoted transformation of the acyclic *O*,*N*-acetals to the corresponding cyclic *O*,*N*-acetals when the R_1 group is either a hydrogen atom or a methoxy group. All the compounds exhibited an IC_{50} against the MCF-7 human breast cancer cell line in the micromolar range, among which the nitro derivative **6f** was found to be the most cytotoxic. We have demonstrated that compound **6a** induced apoptosis and G_0/G_1 cell cycle arrest in the MCF-7 human breast cancer cell line, whereas **6f** seems to act as the prodrug Ftorafur on the basis that both compounds accumulate the tumoural cells in the S phase of the cell cycle. Taken together, the experimental findings provide evidence of specific antitumour activity of these new substances and warrant further evaluation in *in vivo* models of breast cancer to future clinical applications.

4. Experimental

4.1. Chemistry

For a description of general information, see Ref. 8. 5-Chloro-2-hydroxybenzyl alcohol was obtained by reduction of the corresponding salicylaldehyde.[13]

4.1.1. Starting materials

4.1.1.1. Synthesis of *o*-(hydroxymethyl)phenoxyacetaldehyde dimethyl acetals 4. The preparation of **4a**–**c** was previously reported[12] and we proceeded with the same method to obtain **4d**–**f**.

1-(Hydroxymethyl)-5-chloro-2-phenoxyacetaldehyde dimethyl acetal **4d**. Yield: 34%. R_f (2/1, diethyl ether/hexane): 0.4. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (d, 1H, H-6; J=2.5 Hz); 7.21 (dd, 1H, H-4; J=2.5, 8.6 Hz); 6.80 (d, 1H, H-3, J=8.6 Hz); 4.72 (t, 1H, H-1′, J=5.2 Hz); 4.63 (s, 2H, CH_2OH); 4.04 (d, 2H, H-2′, J=5.2 Hz); 3.45 (s, 6H, (O*Me*)$_2$); 3.12 (bs, 1H, CH_2OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.13 (C-2); 131.91 (C-5); 128.89 (C-4); 128.44 (C-6); 126.37 (C-1); 113.37 (C-3); 101.73 (C-1′); 68.04 (C-2′); 61.38 (CH_2OH); 54.15 ((O*Me*)$_2$). HR LSIMS calcd for, $C_{11}H_{15}O_4NaCl$ $(M+Na)^+$ 269.0556, found 269.0555. Anal. for $C_{11}H_{15}O_4Cl$: calcd: C 53.56; H 6.13. Found: C 54.00; H 6.15.

1-(Hydroxymethyl)-5-bromo-2-phenoxyacetaldehyde dimethyl acetal **4e**. Yield: 44%. R_f (2/1, diethyl ether/hexane): 0.37. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37 (d, 1H, H-6, J=2.5 Hz); 7.33 (dd, 1H, H-4, J=2.5, 8.6 Hz); 6.73 (d, 1H, H-3, J=8.6 Hz); 4.69 (t, 1H, H-1′, J=5.2 Hz); 4.59 (d, 2H, CH_2OH, J=6.2 Hz); 4.01 (d, 2H, H-2′, J=5.2 Hz); 3.41 (s, 6H, (O*Me*)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.68 (C-2); 132.30 (C-1); 131.72 (C-4); 131.49 (C-6); 113.87 (C-3); 113.67 (C-5); 101.68 (C-1′); 67.94 (C-2′); 61.41 (CH_2OH); 54.17 ((O*Me*)$_2$). HR LSIMS calcd for $C_{11}H_{15}O_4NaBr$ $(M+Na)^+$ 313.0051, found: 313.0057. Anal. for $C_{11}H_{15}O_4Br$: calcd: C 45.38; H 5.19. Found: C 45.40; H 5.22.

1-(Hydroxymethyl)-5-nitro-2-phenoxyacetaldehyde dimethyl acetal **4f**. Yield: 26%. R_f (2/1, diethyl ether/hexane): 0.2. ^{1}H NMR (300 MHz, CD_3COCD_3) δ 8.34 (d, 1H, H-6, J=2.9 Hz); 8.13 (dd, 1H, H-4, J=2.9, 9.0 Hz); 7.17 (d, 1H, H-3, J=9.0 Hz); 4.76 (t, 1H, H-1′, J=5.2 Hz); 4.70 (s, 2H, CH_2OH); 4.16 (d, 2H, H-2′, J=5.2 Hz); 3.41 (s, 6H, (O*Me*)$_2$). ^{13}C NMR (75 MHz, CD_3COCD_3) δ 161.11 (C-1); 142.49 (C-5); 133.21 (C-1); 124.74 (C-4); 122.84 (C-6); 111.94 (C-3); 102.79 (C-1′); 69.26 (C-2′); 58.98 (CH_2OH); 54.48 ((O*Me*)$_2$). HR LSIMS calcd for $C_{11}H_{15}NO_6Na$ $(M+Na)^+$ 378.0808, found: 378.0882. Anal. for $C_{11}H_{15}NO_6$: calcd: C 51.36; H 5.88; N 5.45. Found: C 52.21; H 6.22; N 5.46.

Synthesis of (RS)-3-methoxy-2,3-dihydro-5H-1,4-benzodioxepins **5**. The preparation of **5a**–**c** was previously reported by us[12] and we proceeded with the same method to obtain **5d**–**f**.

(*RS*)-7-*Chloro*-3-*methoxy*-2,3-*dihydro*-5*H*-1,4-*benzodioxepin* **5d**. Yield: 44%. R_f (4/1, diethyl ether/hexane): 0.7. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.14 (dd, 1H, H-8, J=2.4, 8.5 Hz); 7.07 (d, 1H, H-6, J=2.4 Hz); 6.91 (d, 1H, H-9, J=8.5 Hz); 5.17 (d, 1H, H-5β, J_{gem}=14.5 Hz); 4.88 (dd, 1H, H-3α, J=3.9, 5.9 Hz); 4.33 (d, 1H, H-5α, J_{gem}=14.5 Hz); 4.09 (m, 2H, H-2α, H-2β); 3.50 (s, 3H, O*Me*). ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.49 (C-10); 131.10 (C-7); 128.57 (C-8); 128.23 (C-6); 127.53 (C-11); 121.41 (C-9); 101.08 (C-3); 72.23 (C-2); 62.34 (C-5); 55.57 (O*Me*). HR LSIMS calcd for $C_{10}H_{11}O_3NaCl$ $(M+Na)^+$ 237.0294, found: 237.0298. Anal. for $C_{10}H_{11}O_3Cl$: calcd: C 55.96; H 5.17. Found: C 55.94; H 5.19.

(*RS*)-7-*Bromo*-3-*methoxy*-2,3-*dihydro*-5*H*-1,4-*benzodioxepin* **5e**. Yield: 35%. R_f (3/1, diethyl ether/hexane): 0.6. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.26 (dd, 1H, H-8, J=2.4, 8.5 Hz); 7.20 (d, 1H, H-6, J=2.4 Hz); 6.84 (d, 1H, H-9, J=8.5 Hz); 5.16 (d, 1H, H-5β, J_{gem}=14.5 Hz); 4.87 (dd, 1H, H-3α, J=4.1, 5.8 Hz); 4.31 (d, 1H, H-5α, J_{gem}=14.5 Hz); 4.07 (m, 2H, H-2α, H-2β); 3.48 (s, 3H, O*Me*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.01 (C-10); 131.57 (C-8); 131.52 (C-11); 131.15 (C-6); 121.85 (C-9); 114.98 (C-7); 101.01 (C-3); 72.16 (C-2); 62.24 (C-5); 55.61 (O*Me*). HR LSIMS calcd for $C_{10}H_{11}O_3NaBr$ $(M+Na)^+$ 280.9789, found: 280.9794. Anal. for $C_{10}H_{11}BrO_3$: calcd: C 46.36; H 4.28. Found: C 46.40; H 4.19.

(*RS*)-3-*Methoxy*-7-*nitro*-2,3-*dihydro*-5*H*-1,4-*benzodioxepin* **5f**. Yield: 24%. R_f (2/1, diethyl ether/hexane): 0.6. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (dd, 1H, H-8, J=2.7, 8.8 Hz); 7.92 (d, 1H, H-6, J=2.7 Hz); 6.96 (d, 1H, H-9, J=8.8 Hz); 5.25 (d, 1H, H-5β, J_{gem}=15.0 Hz); 4.94 (t, 1H, H-3α, J=5.8 Hz); 4.37 (d, 1H, H-5α, J_{gem}=15.0 Hz); 4.17 (m,

2-H, H-2α, H-2β); 3.43 (s, 3H, O*Me*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.78 (C-10); 142.54 (C-7); 129.05 (C-11); 124.51 (C-8); 120.58 (C-6); 100.50 (C-9); 100.48 (C-3); 71.28 (C-2); 61.91 (C-5); 55.64 (C-3-O*Me*). HR LSIMS calcd for $C_{10}H_{11}NO_5Na$ $(M+Na)^+$ 248.0534, found: 248.0538. Anal. for $C_{10}H_{11}NO_5$: calcd: C 53.33; H 4.92; N 6.22. Found: C 53.52; H 5.31; N 6.34.

4.1.2. Final products

4.1.2.1. Reaction between the *o*-(hydroxymethyl) phenoxyacetaldehyde dimethyl acetals 4 and 5-Fluorouracil. General procedure. A 1.0 M solution of $SnCl_4$/CH_2Cl_2 (1.25 mmol) was added dropwise to a suspension of **4** (1 mmol) and 5-fluorouracil (1 mmol), which contains trimethylchlorosilane (TCS, 0.8 mmol) and 1,1,1,3,3,3-hexamethyldisilazane (HMDS, 0.8 mmol) in dry acetonitrile (10 ml/mmol of **4**), with stirring under argon at rt. After stirring, the reaction was quenched by the addition of a concentrated aqueous solution of Na_2CO_3. The solvent was removed with a rotary evaporator. The sticky residue was dissolved in MeOH, and silica gel was added. The solvent was removed with a rotary evaporator, and the residue was applied to the top of a flash chromatography column packed with CH_2Cl_2/MeOH (100/1). Elution with mixtures of CH_2Cl_2/MeOH by gradient elution (100/1→100/5) and concentration with a rotary evaporator gave the target molecules **3** and **6**. The yields of the different compounds are shown in Table 1 and Scheme 3.

(RS)-1-[2-(2-Hydroxymethyl-4-methoxyphenoxy)-1-methoxyethyl]-5-fluorouracil **6b**. See Table 1, entry 1. White solid, mp 85°C. R_f (9/1, CH_2Cl_2/MeOH): 0.45. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.49 (s, 1H, NH); 7.5 (d, 1H, $H_{5\text{-FU}}$, J_{H-F}=5.75 Hz); 6.85 (d, 1H, H-6, *J*=1.6 Hz); 6.69 (d, 2H, H-4, H-3, *J*=1.6 Hz); 5.89 (dt, 1H, H-1′, *J*=1.5, 5.6 Hz); 4.55 (s, 2H, *CH*$_2$OH); 4.12 (dd, 2H, H-2′, *J*=5.6, 10.2 Hz); 3.69 (s, 3H, C-5-O*Me*); 3.42 (s, 3H, O*Me*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.19 (d, C-$4_{5\text{-FU}}$; 35.1); 154.3 (s, C-$2_{5\text{-FU}}$); 150.32 (C-5); 149.5 (C-2); 141.13 (d, C-$5_{5\text{-FU}}$; *J*=316 Hz); 130.81 (C-1); 123.21 (C-$6_{5\text{-FU}}$; 44.5); 114.85 (C-4); 112.63 (C-6); 84.76 (C-1′); 68.40 (C-2′); 60.78 (CH_2OH); 57.30 (O*Me*); 55.62 (C-5-*OMe*). HR LSIMS calcd for $C_{15}H_{17}N_2O_6FNa$ $(M+Na)^+$ 363.0968, found 363.0969. Anal. for $C_{15}H_{17}N_2O_6F$: calcd: C 52.94; H 5.04; N 8.23. Found: C 51.78; H 4.69; N 8.44.

(RS)-3-(7-Methoxy-2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-5-fluorouracil **3b**$^{N\text{-}3}$. See Table 1, entry 1. White solid, mp 235–237°C. R_f (9/1, CH_2Cl_2/MeOH): 0.48. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.16 (d, 1H, NH, *J*=5.3 Hz); 7.85 (t, 1H, $H_{5\text{-FU}}$, J_{H-F}=5.6 Hz); 6.97 (d, 1H, H-9, *J*=8.7 Hz); 6.88 (d, 1H, H-6, *J*=3.1 Hz); 6.80 (dd, 1H, H-8, *J*=3.1, 8.7 Hz); 6.05 (d, 1H, H-3α, *J*=8.1 Hz); 4.81 (d, 1H, H-5α, *J*=13.7 Hz); 4.65 (d, 1H, H-5β, *J*=13.7 Hz); 4.60 (dd, 1H, H-2α, *J*=8.1, 12.4 Hz); 4.35 (dd, 1H, H-2β, *J*=1.5, 12.4 Hz); 3.7 (s, 3H, C-7-O*Me*). ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.10 (d, C-$4_{5\text{-FU}}$; 25.5); 154.99 (C-7); 152.74 (s, C-$2_{5\text{-FU}}$); 149.34 (C-10); 139.18 (d, C-$5_{5\text{-FU}}$; *J*=224.5 Hz); 132.43 (C-11); 125.90 (C-$6_{5\text{-FU}}$; 31.4); 121.17 (C-8); 114.5 (C-9); 114.16 (C-6); 84.91 (C-3); 73.52 (C-2); 70.32 (C-5); 55.43 (C-7-*OMe*). HR LSIMS calcd for $C_{14}H_{13}N_2O_5FNa$ $(M+Na)^+$ 331.0706, found 331.0705. Anal for $C_{14}H_{13}N_2O_5F$: C, 54.55; H, 4.25; N, 9.09. Found: C 54.21; H 3.98; N 9.28.

(RS)-1-[2-(2-Hydroxymethyl-6-methoxyphenoxy)-1-methoxyethyl]-5-fluorouracil **6c**. See Table 1, entry 2. White solid, mp 130–133°C. R_f (9.5/0.5, CH_2Cl_2/MeOH): 0.4. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.46 (s, 1H, NH); 7.5 (d, 1H, $H_{5\text{-FU}}$, J_{H-F}=5.8 Hz); 6.96 (d, 1H, H-5, *J*=7.7 Hz); 6.86 (dd, 1H, H-6, *J*=1.6, 7.7 Hz); 6.79 (dd, 1H, H-4, *J*=1.6, 8.1 Hz); 5.88 (dt, 1H, H-1′, *J*=1.6, 4.7 Hz); 4.59 (d, 2H, *CH*$_2$OH, *J*=1.5 Hz); 4.19 (q, 2H, H-2′, *J*=5.3 Hz); 3.75 (s, 3H, C-3-O*Me*); 3.42 (s, 3H, O*Me*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.33 (d, C-$4_{5\text{-FU}}$; 34.9); 151.88 (s, C-$2_{5\text{-FU}}$); 150.32 (C-3); 144.94 (C-2); 140.93 (d, C-$5_{5\text{-FU}}$; *J*=315 Hz); 134.35 (C-1); 124.56 (C-5); 124.12 (C-$6_{5\text{-FU}}$; 44.6); 121.16 (C-6); 112.04 (C-4); 85.03 (C-1′); 71.77 (C-2′); 60.76 (CH_2OH); 57.19 (C-3-O*Me*); 55.56 (O*Me*). HR LSIMS calcd for $C_{15}H_{17}N_2O_6FNa$ $(M+Na)^+$ 363.0968, found 363.0972. Anal for $C_{15}H_{17}N_2O_6F$: C 52.94; H 5.04; N 8.23. Found: C 53.22; H 5.33; N 8.44.

(RS)-1-[2-(2-Hydroxymethylphenoxy)-1-methoxyethyl]-5-fluorouracil **6a**. See Table 3, entry 1. White solid, mp 100–105°C. R_f (9.5/0.5, CH_2Cl_2/MeOH): 0.57. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.61 (s, 1H, NH); 7.51 (d, 1H, $H_{5\text{-FU}}$, J_{H-F}=5.8 Hz); 7.23 (dd, 1H, H_{Ar}, *J*=1.6, 7.4 Hz); 7.16 (dd, 1H, H_{Ar}, *J*=1.7, 7.8 Hz); 6.88 (t, 1H, H_{Ar}, *J*=7.3 Hz); 6.74 (d, 1H, H_{Ar}, *J*=8.1 Hz); 5.90 (dt, 1H, H-1′, *J*=1.5, 4.3 Hz); 4.54 (s, 2H, *CH*$_2$OH); 4.15 (dd, 1H, H-2′, *J*=4.2, 10.2 Hz); 4.03 (dd, 1H, H-2′, *J*=5.8, 10.2 Hz); 3.41 (s, 3H, O*Me*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.20 (d, C-$4_{5\text{-FU}}$; *J*=26.45 Hz); 155.42 (C-1); 150.30 (s, C-$2_{5\text{-FU}}$); 141.10 (d, C-$5_{5\text{-FU}}$; *J*=239.16 Hz); 129.42 (C-2); 129.02, 128.73 (C-5 y C-3); 123.16 (d, C-$6_{5\text{-FU}}$; *J*=33.72 Hz); 121.55 (C-4); 111.13 (C-6); 84.66 (C-1′); 67.54 (C-2′); 60.72 (PhCH$_2$OH); 57.26 (O*Me*). HR LSIMS calcd for $C_{14}H_{15}N_2O_5FNa$ $(M+Na)^+$ 310.1034, found 310.0965. Anal. for $C_{14}H_{15}N_2O_5F$: calcd: C 54.19; H 4.87; N 9.03. Found: C 54.02; H 4.93; N 9.23.

(RS)-trans-4,10-Bis[5-(fluorouracil-1-yl)]-2,5,8,11-tetraoxa-1,7(1,2)dibenzenadodecaphane **7a**. See Scheme 3. Yellowish solid, mp 80–83°C. R_f (9.5/0.5, CH_2Cl_2/MeOH): 0.6; R_f. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.85 (d, 1H, $H_{5\text{-FU}}$, J_{H-F}=5.5 Hz); 7.28 (t, 2H, H-3 and H-12, *J*=6.5 Hz); 7.27 (d, 2H, H-1 and H-10, *J*=5.9 Hz); 7.06 (t, 2H, H-2 and H-11, *J*=6.6 Hz); 7.04 (d, 2H, H-4 and H-13); 6.12 (d, 2H, H-7 and H-16, *J*=7.9 Hz); 4.95 (d, 2H, H-9 and H-18, J_{gem}=13.9 Hz); 4.76 (dd, 2H, H-6 and H-15, *J*=9, 12.5 Hz); 4.68 (d, 2H, H-9 and H-18, *J*=13.9 Hz); 4.41 (dd, 2H, H-6 and H-15, *J*=1.4, 12.6 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.90 (C-20 and C-22); 157.17 (d, C-$4_{5\text{-FU}}$; *J*=25.41 Hz); 149.40 (s, C-$2_{5\text{-FU}}$); 139.2 (d, C-$5_{5\text{-FU}}$; *J*=224.67 Hz); 131.08 (C-19 and C-21); 129.53, 129.49 (C-1, C-10, C-3 and C-12); 125.92 (d, C-$6_{5\text{-FU}}$; 31.51); 123.39 (C-4 and C-13); 120.31 (C-2 and C-11); 84.50 (C-7 and C-16); 73.04 (C-9 and C-18); 70.05 (C-6 and C-15). HR LSIMS calcd for $C_{26}H_{22}N_4O_8F_2Na$ $(M+Na)^+$ 579.1303, found 579.1305. Anal. for $C_{26}H_{22}N_4O_8F_2$: calcd: C 56.12; H 3.98; N 10.07. Found: C 55.93; H 4.12; N 10.02.

(RS)-1-[2-(4-Chloro-2-hydroxymethylphenoxy)-1-methoxyethyl]-5-fluorouracil **6d**. See Table 1, entry 3. White solid, mp 115–110°C. R_f (9/1, CH_2Cl_2/MeOH): 0.73. ^{1}H NMR

(300 MHz, DMSO-d_6) δ 11.93 (s, 1H, NH); 8.00 (d, 1H, $H_{5\text{-FU}}$, J_{H-F}=6.8 Hz); 7.32 (d, 1H, H-6, J=2.6 Hz); 7.23 (dd, 1H, H-4, J=2.6, 9.4 Hz); 6.97 (d, 1H, H-3, J=9.4 Hz); 5.79 (dt, 1H, H-1′, J=1.3, 4.7 Hz); 5.18 (t, 1H, CH_2OH, J=5.4 Hz); 4.35 (d, 1H, CH_2OH, J=5.4 Hz); 4.29 (dd, 1H, H-2′, J=5.2, 10.5 Hz); 4.18 (dd, 1H, H-2′, J=6.2, 10.5 Hz); 3.33 (s, 3H, O*Me*). ^{13}C NMR (75 MHz, DMSO-d_6) δ 157.02 (d, C-$4_{5\text{-FU}}$; 34.7); 152.86 (s, C-$2_{5\text{-FU}}$); 149.79 (C-2); 143.39 (d, C-$5_{5\text{-FU}}$; J=307 Hz); 133.02 (C-5); 126.79 (C-4); 126.12 (C-6); 124.61 (C-1); 124.39 (C-$6_{5\text{-FU}}$; 44.9); 113.01 (C-3); 83.47 (C-1′); 66.79 (C-2′); 57.05 (CH_2OH); 56.26 (O*Me*). HR LSIMS calcd for $C_{14}H_{14}N_2O_5FNaCl$ $(M+Na)^+$ 367.0473, found 367.0472. Anal. for $C_{14}H_{14}N_2O_5FCl$: calcd: C 48.78; H 4.09; N 8.13. Found: C 48.53; H 3.84; N 7.99.

(RS)-1-[2-(4-Bromo-2-hydroxymethylphenoxy)-1-methoxyethyl]-5-fluorouracil **6e**. See Table 1, entry 4. White solid, mp 74°C. R_f (10/0.25, CH_2Cl_2/MeOH): 0.16. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.47 (d, 1H, $H_{5\text{-FU}}$, J_{H-F}=5.6 Hz); 7.37 (d, 1H, H-6, J=2.5 Hz); 7.31 (dd, 1H, H-4, J=2.5, 8.7 Hz); 6.64 (d, 1H, H-3, J=8.7 Hz); 5.94 (dt, 1H, H-1′, J=1.6, 4.0 Hz); 4.5 (s, 2H, CH_2OH); 4.18 (dd, 1H, H-2′, 4.0, 10.0); 4.01 (dd, 1H, H-2′, J=6.3, 10.0 Hz); 3.44 (s, 3H, O*Me*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.15 (d, C-$4_{5\text{-FU}}$; J=35 Hz); 154.44 (s, C-$2_{5\text{-FU}}$); 150.40 (C-2); 141.31 (d, C-$5_{5\text{-FU}}$; J=316.9 Hz); 131.75 (C-4); 131.70 (C-6); 122.91 (C-$6_{5\text{-FU}}$; 44.5); 114.08 (C-1); 112.83 (C-3); 84.67 (C-1′); 67.82 (C-2′); 60.29 (CH_2OH); 57.54 (O*Me*). HR LSIMS calcd for $C_{14}H_{14}N_2O_5FNaBr$ $(M+Na)^+$ 410.9956, found 410.9941. Anal. for $C_{14}H_{14}N_2O_5FBr$: calcd: C 43.21; H 3.63; N 7.20. Found: C 43.09; H 3.26; N 7.48.

(RS)-3-[2-(4-Bromo-2-hydroxymethylphenoxy)-1-methoxyethyl]-5-fluorouracil **6e**$^{N\text{-}3}$. See Table 1, entry 4. White solid, mp 105–108°C. R_f (10/0.25, CH_2Cl_2/MeOH): 0.13. ^{1}H NMR (300 MHz, CD_3OD) δ 7.61 (d, 1H, $H_{5\text{-FU}}$, J_{H-F}=5.1 Hz); 7.49 (d, 1H, H-6, J=2.5 Hz); 7.33 (dd, 1H, H-4, J=2.5, 8.7 Hz); 6.86 (d, 1H, H-3, J=8.7 Hz); 6.25 (t, 1H, H-1′, J=5.9 Hz); 4.89 (s, 2H, CH_2OH); 4.55 (dd, 2H, H-2′, J=5.9, 18.5 Hz); 3.44 (s, 3H, O*Me*). ^{13}C NMR (75 MHz, CD_3OD) δ 155.66 (s, C-$4_{5\text{-FU}}$); 139.93 (C-2); 133.87 (s, C-$2_{5\text{-FU}}$); 132.39 (C-1); 131.86 (d, C-$5_{5\text{-FU}}$; J=401.1 Hz); 131.76 (C-4); 131.19 (C-6); 126.57 (C-$6_{5\text{-FU}}$; 32.1); 114.50 (C-3); 85.45 (C-5); 85.45 (C-1′); 68.70 (C-2′); 59.40 (CH_2OH); 57.78 (O*Me*). HR LSIMS calcd for $C_{14}H_{14}N_2O_5FNaBr$ $(M+Na)^+$ 410.9967, found 410.9965. Anal. for $C_{14}H_{14}N_2O_5FBr$: calcd: C 43.21; H 3.63; N 7.20. Found: C 43.34; H 3.86; N 6.98.

(RS)-1-[2-(2-Hydroxymethyl-4-nitrophenoxy)-1-methoxyethyl]-5-fluorouracil **6f**. See Table 1, entry 5. Yellow solid, mp 230–233°C. R_f (9.5/0.5, CH_2Cl_2/MeOH): 0.8. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.6 (bs, 1H, NH); 8.23 (d, 1H, H-6, J=2.8 Hz); 8.16 (dd, 1H, H-4, J=2.8, 7.5 Hz); 8.03 (d, 1H, H-3, J=7.5 Hz); 7.76 (d, 1H, H_{5FU}, J_{H-F}=6.08 Hz); 5.89 (t, 1H, H-1′, J=5.0 Hz); 5.45 (bs, 1H, CH_2OH); 4.43 (m, 4H, CH_2OH, H-2′); 3.36 (s, 3H, O*Me*). ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.12 (d, C-$4_{5\text{-FU}}$; 26); 150.06 (s, C-$2_{5\text{-FU}}$); 149.86 (C-2); 140.45 (d, C-$5_{5\text{-FU}}$; J=230.2 Hz); 141.21 (C-5); 132.32 (C-1); 124.38 (C-$6_{5\text{-FU}}$; J=33.5 Hz); 123.99 (C-4); 121.69 (C-6); 111.60 (C-3); 83.37 (C-1′); 67.33 (C-2′); 57.01 (CH_2OH); 56.44 (O*Me*). HR LSIMS calcd for $C_{14}H_{14}N_3O_7FNa$ $(M+Na)^+$ 378.0713, found 378.0708. Anal. for $C_{14}H_{14}N_3O_7F$: calcd: C 47.33; H 3.97; N 11.83. Found: C 46.98; H 4.02; N 11.98.

4.1.2.2. Reaction between the benzoannelated seven-membered compounds 5 and 5-Fluorouracil. General procedure. The procedure was similar to the one described in Section 4.1.2.1 but changing **4** for **5**. The yields and the nature of the compounds are shown in Table 2. Compounds **3a–c** have been previously reported by us.[12]

4.1.2.3. Reduction of 6f: obtention of (*RS*)-3-[2-(4-amino-2-hydroxymethylphenoxy)-1-methoxyethyl]-5-fluorouracil 6g. After dissolving **6f** (1.7 g, 4.8 mmol) in ethanol (24 mL) and adding $SnCl_2$ $2H_2O$ (5.41 g, 24 mmol), the suspension was refluxed for 2 h. After cooling, the mixture was neutralized, rotaevaporated off and the resulting crude was purified by flash chromatography (10/1, CH_2Cl_2/MeOH) to give **6g** (1 g, 65%). Brown solid, mp 100–103°C. R_f (9/1, CH_2Cl_2/MeOH): 0.3. ^{1}H NMR (400 MHz, CD_3COCD_3) δ 7.69 (d, 1H, H_{5FU}, J_{H-F}=5.2 Hz); 6.86 (d, 1H, H-3, J=8.4 Hz); 6.76 (d, 1H, H-6, J=2.5 Hz); 6.48 (dd, 1H, H-4, J=2.5, 8.4 Hz); 6.22 (t, 1H, H-1′, J=5.8 Hz); 4.55 (m, 4H, CH_2OH, H-2′); 3.41 (s, 3H, O*Me*). ^{13}C NMR (100 MHz, CD_3COCD_3) δ 157.12 (s, C-$4_{5\text{-FU}}$); 152.09 (s, C-$2_{5\text{-FU}}$); 146.37 (C-2); 140.45 (d, C-$5_{5\text{-FU}}$; J=230.2 Hz); 132.27 (C-5); 132.20 (C-1); 125.40 (C-$6_{5\text{-FU}}$; J=32.2 Hz); 119.71 (C-4); 119.02 (C-6); 112.94 (C-3); 84.88 (C-1′); 68.95 (C-2′); 59.69 (CH_2OH); 57.36 (O*Me*). HR LSIMS calcd for $C_{14}H_{16}N_3O_5FNa$ $(M+Na)^+$ 348.0971, found 348.0980. Anal. for $C_{14}H_{16}N_3O_5F$: calcd: C 51.69; H 4.96; N 12.92. Found: C 51.88; H 5.32; N 13.21.

4.2. Biological activity

The antiproliferative activities[26] and apoptosis induction[25] in the MCF-7 human breast cancer cell line were followed in accordance with the protocols previously reported.

4.2.1. Cell cycle distribution analysis. Cells at 70% confluence were treated with either DMSO alone or with concentrations of the compounds determined by their IC_{50} values. After 48 h of treatment the medium was aspirated, the cells were harvested, washed twice with sample buffer (1 g glucose, 1 L PBS without Ca^{2+} or Mg^{2+}) and fixed in 70% (vol/vol) cold ethanol for up to one week. Cells were pelleted, washed once with sample buffer and resuspended in PI solution (50 μg/mL PI, 0.5 mg/mL RNase in sample buffer, pH 7.4) for 30 min in the dark. Then, fluorescence-activated cell sorter analysis was performed and the data from 10,000 cells per sample were collected and analyzed using a VANTAGE flow cytometer (Becton Dickinson, San Jose, CA, USA) by CellFIT cell cycle analysis software. All experiments were performed in duplicate and yielded similar results.

The results of the antiproliferative activities, cell cycle distribution and apoptosis induction are recorded in Table 4.

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