SYNTHESIS OF 1,4-BENZOXATHIIN-9*H*-PURINE DERIVATIVES AS ANTIPROLIFERATIVE AGENTS

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Abstract. *The synthesis and anticancer activity of a series of substituted 9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9H-purine, 9-(2,3-dihydro-1,4-benzoxathiin-2-ylmethyl)-9H-purine and homochiral (R)- and (S)-9-(2,3-dihydro-1,4-benzoxathiin-3-yl and 2-ylmethyl)-9H-purine derivatives is reported. Their antiproliferative effect against the MCF-7 human breast cancer cell line was carried out. The most active compounds were subjected to cell cycle and apoptosis studies. In order to elucidate the mechanism of action at the molecular level of such benzannelated six-membered derivatives several targets were also studied.*

Contents

1. Introduction

Six-membered heterocycles containing two heteroatoms in a 1,4-relationship fused to an aromatic ring has elicited much research activity due to their interest in both chemical and biological properties. The 2,3-dihydro-1,4-benzoxathiin system is present in a variety of therapeutic agents that possess important biological activities. The *in vitro* bioactivation of a selective estrogen receptor modulator (2*S*,3*R*)-(+)-3-(3-hydroxyphenyl)-2-[4-(2-pyrrolidin-1-ylethoxy)phenyl]-2,3-dihydro-1,4-benzoxathiin-6-ol (**1**, Figure 1) in liver microsomes has been reported.[1] High-throughput screening of Merck sample collections against human H_3 receptor led to the identification of the novel dihydrobenzoxathiin lead **2**, which has an IC_{50} value of 6.7 nM.[2] Aryl substituted 2,3-dihydrobenzo[b][l,4]oxathiine derivatives (**3**) act as agonists or partial agonists of the 5-HT_{2C}.[3] The 2,3-dihydro-1,4-benzoxathiin skeleton was also evaluated in binding assays to determine their activity on melatonin receptors, the 2,3-dihydro-*N*-methyl-1,4-benzoxathiin-5-butanamide (**4**) being the most potent agonist.[4]

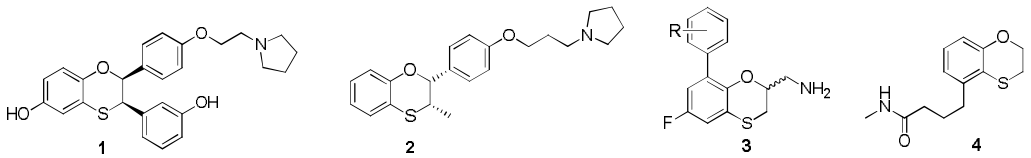


Figure 1. Chemical structure of several 2,3-dihydro-1,4-benzoxathiin derivatives with diverse therapeutic activities.

The development of new drugs against cancer is among the priorities of the development of science and fundamental research. The use of traditional cancer chemotherapy is still very limited because it is difficult to discover novel agents that selectively kill tumour cells or inhibit their proliferation without general toxicity. Paradoxically, despite the considerable development of biologically active compounds with the 2,3-dihydro-1,4-benzoxathiin moiety, their implication in cancer has not been studied.

The importance of 5-fluorouracil (5-FU, Figure 2) as the first-choice drug in carcinomas of the gastrointestinal tract is well known despite its side-effects. With the aim of diminishing the toxicity and obtaining biologically active derivatives of 5-FU suitable for oral administration, great effort has been made in the preparation of 5-FU prodrug derivatives.[5,6]

We previously designed and synthesized a series of 5-FU-linked benzene-fused seven-membered *O,N*-acetals (**5**).[7,8] 5-FU moiety was substituted by the natural pyrimidine, uracile (**6**). The ability of these structures to modulate the activity of p53 and bcl-2 for the induction of apoptosis and also to accumulate the cancerous cells in the G_1/G_0-phase[9] shows that such compounds may be considered as drugs in their own right, with antitumor activity independent of that of 5-FU.[10] Later, the pyrimidine base was substituted for a purine group (**7**) with the objective of increasing the lipophilicity, and the oxygen atom at position 1 was replaced by its isosteric sulfur atom to enlarge the structural diversity of the target molecules.[10-12]

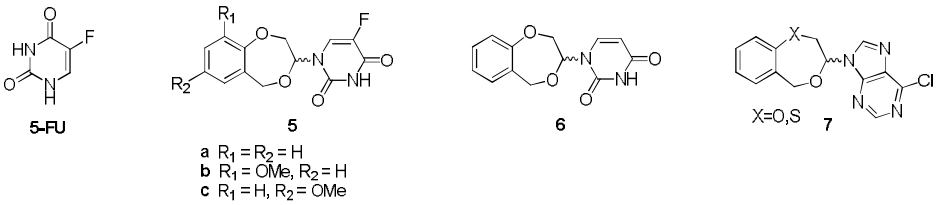


Figure 2. Several benzene-fused seven-membered ring pyrimidine and purine *O,N*-acetals reported by our research group.

If the previously described compounds are not prodrugs, it is not necessary to maintain the *O,N*-acetal characteristics with the corresponding weakness of the *O,N*-acetal bond. Therefore, from this point onwards, we were involved in the design of molecules in which both structural entities (the benzene-fused heterocyclic ring and the purine base) are linked by a heteroatom-C-C-base-N-atom bond. We then decided to synthesize a new series of 1,4-benzoxathiin-9*H*-purine in order to evaluate their antiproliferative activity (Figure 3).

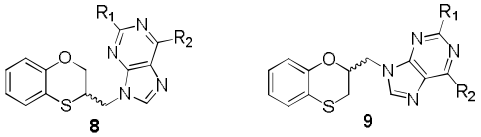


Figure 3. General structure of the 1,4-benzoxathiin-9*H*-purine derivatives.

2. 9-(2,3-Dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purines

A series of eleven 2- and 6-substituted 9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purine derivatives (Figure 4) were obtained by applying a standard Mitsunobu protocol that led to a six-membered ring contraction from 3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol *via* an episulfonium intermediate.[13]

2.1. Synthesis

Cabiddu *et al.* obtained 1,5-benzoxathiepine derivatives in good yields by the reaction of epichlorohydrins with 2-hydroxybenzenethiols in an aqueous alkaline hydroxide medium.[14] They described the formation of **11** as a consequence of nucleophilic attack by the phenoxide ion at the secondary epoxide carbon of **10**; nevertheless, when these same experimental conditions were applied, we also observed the

formation of the six-membered ring in **12** (Scheme 1), caused by the attack of the phenolic oxygen atom at the more hindered position of the epoxide ring. A comparison of the results obtained for catechols[15] that produce the 1,4-benzodioxin rings with those given by 2-hydroxybenzenethiols suggests that the larger atomic radius of the sulfur atom causes the attack to occur at the secondary carbon atom of the epoxide moiety rather than the tertiary one, thus producing the seven-membered ring as the major product.

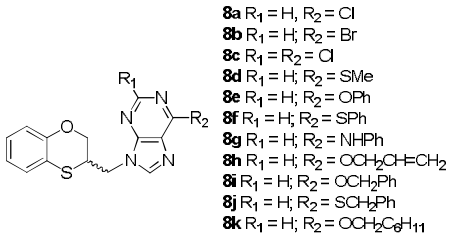
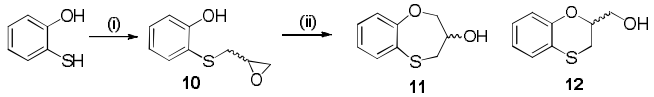
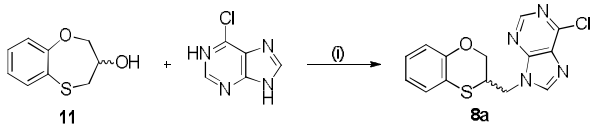


Figure 4. Chemical structures of the 9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purines.



Scheme 1. Reagents and conditions: (i) epichlorohydrin, pyridine, H_2O, r.t.; (ii) NaOH, H_2O, 100 °C.

The conventional Mitsunobu conditions employing diisopropyl azodicarboxylate (DIAD) and triphenylphosphine in anhydrous THF were applied in the reaction between **11** and 6-chloropurine. Surprisingly and interestingly we got the six-membered derivative **8a** (Scheme 2).[13]



Scheme 2. Reagents and conditions: (i) Ph_3P, DIAD, anhydrous THF, 45 °C.

The structure of **8a** was determined by ^{1}H- and ^{13}C-NMR, HMQC, HMBC, and X-ray diffraction (the crystallization was carried out in a $CHCl_3$/acetone mixture). In the HMBC (Heteronuclear Multiple Bond) correlations between carbons and protons that are separated by three bonds, the interaction between the hydrogen atom of the aliphatic CH and the quaternary carbon atom of the benzene ring was observed. Nevertheless, no interaction from the methylene groups with such a carbon atom was found, as would be the case if the seven-membered ring were formed (Figure 5).

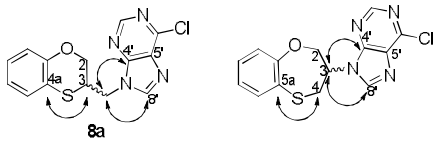


Figure 5. Representation of the HMBC interactions (with double-tipped arrows) that was observed in the six- and seven-membered rings and purine group atoms.

Importantly, the correlation between the methylene group that links the six-membered and the purine rings (δ=4.54 ppm, dd, J_{gem}=14.3 Hz, J_2=8.0 Hz, 1 H) and the quaternary carbon at δ=151.82 ppm of **8a**, has

the following two consequences: a) this signal can be assigned to C4′ and b) this correlation proves unequivocally that the linkage between the six-membered moiety and the purine base takes place through N9′ in compound **8a** (the atoms of the benzoxaheteroin ring are tagged with numbers without primes, while those of the purine bases are numbered with primes). The chemical shift of C4′ in **8a** agrees with previous findings on related N9′ purine *O*,*N*-acetals.[7-9,11] Alkylation of purine nucleobases and analogues is rarely regiospecific, and mixtures of N9′ and N7′ isomers are usually obtained. The N9′ compound is normally the major product, but formation of significant amounts of the N7′ isomer is often observed.[11]

The asymmetric unit contains two independent molecules (**A** and **B**) which correspond to the *R* and *S* enantiomers. The lowest numeration corresponds to the **B** molecule (Figure 6). In the crystal, enantiomers have a closely similar conformation, in which both contain the 6-chloropurine fragment in the axial position. Such enantiomers build infinite chains of **A***n* or **B***n* running along the *a* axis. There is a π–π stacking interaction that involves the benzene ring of one molecule and the six-membered ring of the 6-chloropurine moiety of the next molecule in each chain. The stacking parameters of this interaction reveal an appreciable strength in both chains of enantiomers, with the planes of the involved rings nearly parallel, slipping angles of 10–16°, and distances between centroids and between ring planes close to 3.5 Å.[13]

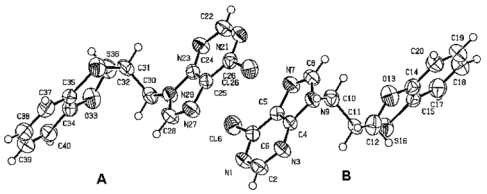
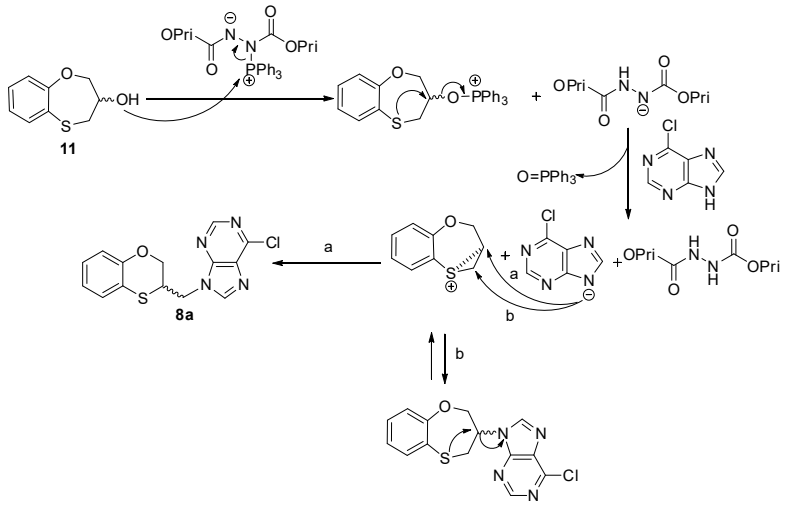


Figure 6. A view of the asymmetric unit of **8a** containing both enantiomers.

The formation of **8a** could be explained by the steric hindrance caused by the triphenylphosphine linked to the secondary OH group of **11** that affects attack by bulky nucleophiles such as 6-chloropurine during the course of the Mitsunobu reaction (Scheme 3).

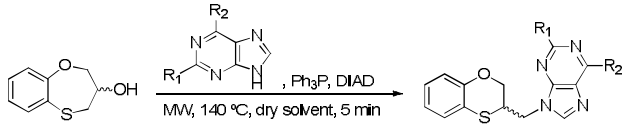


Scheme 3. Possible mechanism for the formation of **8a** under Mitsunobu conditions.

Hence, the reaction would deviate because the sulfur atom competes as an alternative nucleophile. The S neighbouring participation implies the formation of an episulfonium ion intermediate. Nevertheless, the formation of the expected seven-membered ring from this three-membered ring would be possible. Not the slightest trace was detected, and therefore its instability can be presumed because of the strong nucleophilic character of the sulfur atom and good leaving ability of 6-chloropurine. The formation of episulfonium rings by intramolecular attack of a nucleophilic sulfur atom at electrophilic positions in the same molecule is the cause of the instability of compounds and deviation of reactions towards unexpected products.

The Mitsunobu reaction between **11** and 6-chloropurine was next carried out under microwave conditions at 140 °C for 5 min at a pressure of 100 kPa. The yield of **8a** increased from 15% under classical conditions to 80% in dry THF, or 85% in dry MeCN under microwave heating (Table 1). Because of all the advantages of microwave heating, the remaining target molecules were obtained under the same conditions, and the structures and yields are shown in Table 1. The non-commercially available purine moieties (6-cyclohexylmethoxypurine, 6-allyloxypurine, 6-benzyloxypurine, 6-phenoxypurine, 6-phenylthiopurine, 6-benzylthiopurine and 6-anilinopurine) were synthesized according to published procedures.[16-18]

Table 1. Microwave-assisted synthesis of 9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purines.



11 8a-k

Compound	R_1	R_2	Solvent	Yield (%)
8a	H	Cl	THF	80
8a	H	Cl	MeCN	85
8b	H	Br	MeCN	62
8c	Cl	Cl	THF	81
8d	H	SMe	THF	37
8e	H	OPh	THF	41
8f	H	SPh	THF	36
8g	H	NHPh	THF	23
8h	H	$OCH_2CH{=}CH_2$	THF	34
8i	H	OCH_2Ph	THF	60
8j	H	SCH_2Ph	THF	52
8k	H	$OCH_2C_6H_{11}$	THF	31

Assignments of N9′ versus N7′ isomers can be readily made from the ^{13}C-NMR signal of the C4′ peaks ($CDCl_3$):[12] the signal δ=∼151 ppm is characteristic of the C4′ atom of the N9′ regioisomers, whilst the signal δ=∼160 ppm is characteristic of the C4′ atom of the N7′ regioisomers. In our case, the signals for C4′ appear at the following chemical shifts for compounds **8a**–**8k**: δ=151.82 (**8a**), 150.70 (**8b**), 152.29 (**8c**), 148.34 (**8d**), 152.54 (**8e**), 148.95 (**8f**), 149.77 (**8g**), 151.90 (**8h**), 152.73 (**8i**), 148.53 (**8j**) and 152.16 (**8k**) ppm. The nature of the substituent at position 6 of the purine ring causes a slight down- or upfield shift of about 2 ppm (148 ppm for the thio-substituted derivatives, 149 ppm for the amino-substituted one, and 152 ppm for the oxy-substituted analogues).

2.2. Biological assays

Table 2 shows the antiproliferative activities[13] against the MCF-7 human breast cancer cell line for the target compounds, including 5-FU as a reference drug. In general, it seems that bulky substituents at position 6′ are detrimental to anticancer activity. The presence of a halogen atom at position 6′ (compounds **8a** and **8b**) or two chlorine atoms at both 2′ and 6′ positions (compound **8c**) lead to the most active compounds. Compound **8b** is nearly equipotent to 5-FU.

Table 2. Antiproliferative activities against the MCF-7 cell line for 5-FU and **8a**-**8k**.

Compound	IC_{50} (μM)
5-FU	4.32 ± 0.02
8a	10.6 ± 0.66
8b	6.18 ± 1.70
8c	8.97 ± 0.83
8d	20.5 ± 1.81
8e	20.5 ± 1.11
8f	10.5 ± 1.06
8g	11.2 ± 2.73
8h	17.5 ± 0.25
8i	23.2 ± 1.26
8j	16.7 ± 3.03
8k	17.4 ± 1.60

The three most potent compounds **8b**, **8c**, and **8a**, were subjected to cell cycle and apoptosis studies on the MCF-7 human breast cancer cell line.[13] Our results show that compounds **8a-8c**, in contrast to 5-FU, provoke a G_0/G_1-phase cell-cycle arrest upon treatment of MCF-7 cells with the compounds at their respective IC_{50} concentrations over the course of 48 h, mainly at the expense of the S-phase populations (Table 3). The fact that the novel derivatives at similar doses exhibit different sequences of cell-cycle perturbations in comparison with 5-FU indicates that these compounds act through different pathways.[19] Moreover, in the case of **8b**, there is an increase in the G_2/M phase of the cancerous cells. The apoptotic indices of the target compounds are very significant, especially for **8c** (58.29% for **8a**, 63.05% for **8b**, and 76.22% for **8c**).

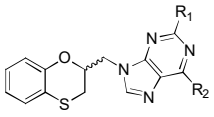
Table 3. Cell-cycle distribution and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h for the 5-FU and **8a**-**8c** as antiproliferative agents.

Compound	Cell cycle[a]			Apoptosis[b]
	G_0/G_1	S	G_2/M	
Control	58.62 ± 0.74	33.82 ± 0.72	7.55 ± 1.34	0.22 ± 0.16
5-FU	58.07 ± 0.11	39.38 ± 0.98	2.10 ± 0.12	52.81 ± 1.05
8a	69.71 ± 1.50	23.73 ± 1.65	6.56 ± 0.17	58.29 ± 0.75
8b	62.85 ± 0.87	26.71 ± 1.25	10.43 ± 0.38	63.05 ± 0.26
8c	70.30 ± 0.32	23.67 ± 2.40	6.06 ± 2.72	76.22 ± 2.02

[a]Determined by flow cytometry.[19] [b]Apoptosis was determined using an Annexin V-based assay.[19] Data indicate the percentage of cells undergoing apoptosis in each sample.

3. 9-(2,3-Dihydro-1,4-benzoxathiin-2-ylmethyl)-9*H*-purines

A fundamental approach that guides the design of drugs is bioisosterism, which we had carried out as suitable structural modifications of the seven-membered building block.[12,20] Therefore, we proposed the preparation and study of the anticancer activity of isomers of **8a**, **8b**, and **8c** (**9a**-**9c**, Figure 7).[21] The methylene linker that connects the six-membered ring and the purine moiety was changed from position 3 to 2**.** In addition, the adenine natural fragment linked to the benzo-fused six-membered ring through a methylene linker was also included (**9d**).

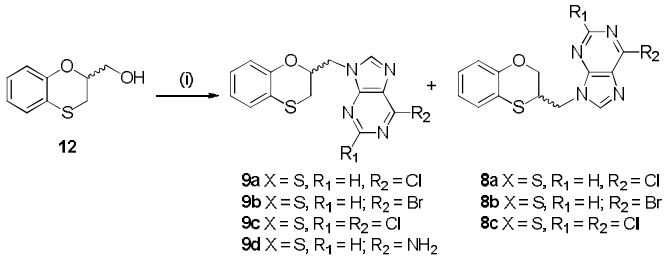


9a R_1 = H; R_2 = Cl
9b R_1 = H; R_2 = Br
9c R_1 = R_2 = Cl
9d R_1 = H; R_2 = NH_2

Figure 7. Chemical structures of the 9-(2,3-dihydro-1,4-benzoxathiin-2-ylmethyl)-9*H*-purines.

3.1. Synthesis

2,3-Dihydro-2*H*-1,4-benzoxathiin-2-methanol (**12**) was prepared as previously described.[13] Target compounds **9a**–**9d** were synthesized by the Mitsunobu reaction in dry THF between **12** and the corresponding purines (6-chloropurine, 6-bromopurine, 2,6-dichloropurine and adenine) under microwave-assisted conditions (Scheme 4).[21] It must be pointed out that when starting from **12** and using 6-chloro-, 6-bromo-, and 2,6-dichloro-purines, their corresponding previously reported isomers **8a-8c** were also obtained as side-products, apart from target compounds **9a-9c**.



Scheme 4. Reagents and conditions: (i) substituted purines, Ph_3P, DIAD, anhydrous THF, microwave irradiation, 140 °C, 5 min.

The formation of such "abnormal" products may explained through a neighbouring-group mechanism. The neighbouring-group mechanism occurs with appropriate ring sizes. For example, it was previously observed that in the case of $MeO(CH_2)_nOBs$ [Bs: brosylates or *p*-bromobenzene-sulfonates], neighbouring-group participation occurred with n=4 or 5 (corresponding to a five- or six-membered intermediates), but not with n=2, 3, or 6.[22] However, optimum ring size is not the same for all reactions. In general, the most rapid reactions occur when the ring size is three, five, or six, depending on the reaction type. The likelihood of four-membered ring neighbouring-group participation is increased when there are alkyl groups α or β the neighbouring group.[23,24] The OR group is more important as a neighbouring group than the SR one.[25] Scheme 5 might explain the formation of **8a-8c**.

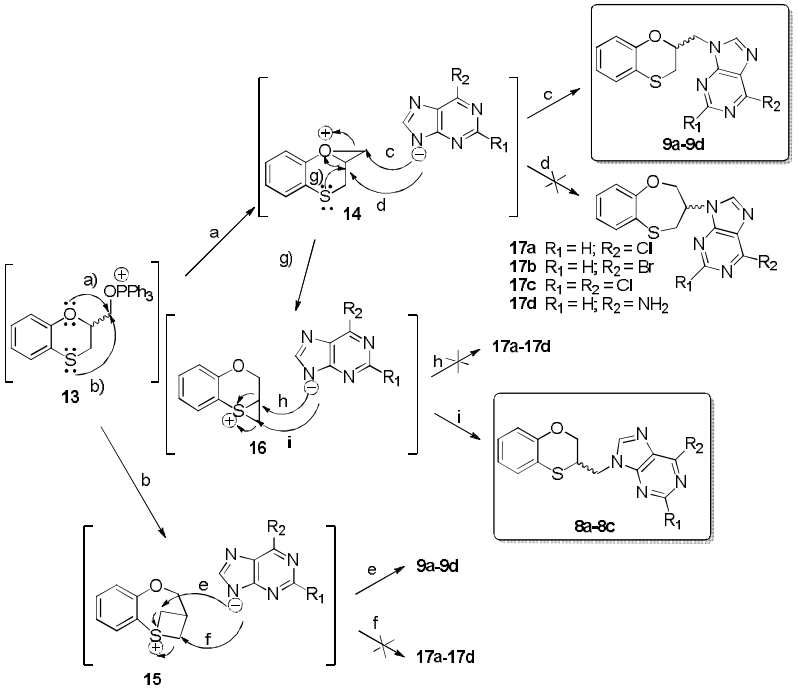
The starting triphenylphosphonium salt **13** *via* routes (a),(c) and (b),(e) gives rise to the target molecules **9a**-**9d** through intermediates **14** and/or **15**. Nevertheless, following the formation of the oxyranium ion **14** and after the sulfur neighbouring-group participation with the concomitant generation of the episulfonium ring **16** [route (g), Scheme 5], the nucleophilic attack of the purine anion to the less hindered α carbon atom of the thiiranium intermediate would give rise to molecules **8a-8c**. Accordingly, a formal 1,4-thio migration (from the 2,3-dihydro-1,4-benzoxathiin-2-ylmethyl to the 2,3-dihydro-1,4-benzoxathiin-3-ylmethyl moieties) can proceed through two consecutive O-*3* and S-*3* (when describing nucleophilic participation it is frequently convenient to use the symbol *G*-*n*, where *G* is the participating group and *n* the size of the ring that is formed in the transition state) neighboring-group participations in a six-membered ring system. In the case of compound **9d** (bearing the adenine moiety) there is no neighbouring-group participation.

A successful Mitsunobu displacement does not depend on the nucleophilicity of the incoming nucleophile but rather on the pK_a associated with the *N*–H bond.[26] Adenine is a poor nucleophile with a low solubility in the most common solvent, THF.[27]

Although the *N'*-9 alkylation of the purine ring takes place at two sites of the benzoxathiin moiety (through intermediates **14** and **16**, Scheme 5) to lead to a mixture of products, the overall yields of the target and "rearranged" compounds (**9a**+**8a**; **9b**+**8b**; and **9c**+**8c**) are good [88%=78% (**9a**)+10% (**8a**), 60%=54% (**9b**)+6% (**8b**), and 70%=55% (**9c**)+15% (**8c**)] in the ratios **9a**:**8a**=7.8:1, **9b**:**8b**=9.0:1, and **9c**:**9c**=3.7:1. Starting from adenine, the target compound **9d** is the only one obtained, although with only a modest yield (30%).

The effects of structures on reactivity need to be analyzed in order to rationalize all these experimental facts. In relation to the nucleophile it must be pointed out that the yields for the non-rearranged purine

derivatives having electron-withdrawing groups, such as 6′-Cl (**9a**), 6′-Br (**9b**), and 2′,6′-diCl (**9c**) are higher than that of the adenine derivative (**9d**), bearing an electron-releasing group (6′-NH_2). The electrophile character of the two intermediates, such as the oxyranium **14** and the episulfonium **16** ions must be also taken into account. Although in both cases the attack of the nucleophile takes place to the less hindered side of the three-membered ring [routes (c) and (i) on intermediates **14** and **16**, respectively], the higher electronegativity of the positively charged oxygen atom (in **14**) in relation to the positively charged sulfur (in **16**) would explain the greater yields of the target derivatives **9a**–**9c** compared to the rearranged ones **8a-8c**. In the case of the adenine derivative (**9d**), since the target compound is formed in a moderate yield (30%), the possible rearranged one is not detected under our experimental conditions. Finally, the attack of the bulky purine nucleophile against the more hindered position of intermediates **14** and **16** [routes (d) and (h), respectively, Scheme 5] does not take place and not the slightest trace of the seven-membered rings **17a**–**17d** is detected.[21] As mentioned before the quaternary signals δ=∼151 ppm, which correspond to the C-4′ carbon atom, is a proof of the *N*-9′ regioisomers.[13] This chemical shift of C-4′ in compounds **9a**–**9d** agrees with previous findings on related *N*-9′ purine *O,N*-acetals.



Scheme 5. Proposed mechanism for the formation of **8a-8c** through neighbouring-group participation.

3.2. Biological assays

The antiproliferative effect of the target molecules **9a-9d** was reported against the MCF-7 human breast cancer cell line.[21] As shown in Table 4, the biological activity depends on the substituent of the purine ring. In general, compounds bearing halogen atoms on the purine ring (**9a-9c**) present better activity than the compound substituted bearing an amino group (**9d**). The most active compound **9c**, bearing two chlorine atoms at positions 2 and 6 of the purine ring, shows an IC_{50}=2.75 μM.

The effects on the cell cycle distribution of the most active compounds **9a-9c** were analysed by flow cytometry (Table 5).[21] DMSO-treated cell cultures contain a 62.79% of the cells in the G_0/G_1-phase, and a 19.29% of the cells in the S-phase, a 13.26% of the cells in the G_2/M-phase. In contrast, MCF-7 cells treated

during 48 h with **9a-9c** show important differences in the cell cycle progression compared with DMSO-treated control cells. Compounds **9a-9c** accumulate the cancerous cells in the G_2/M-phase (23.35, 31.37 and 43.89, respectively) at the expense of the S-phase cells (13.77, 17.06 and 10.83, respectively) and of the G_0/G_1-phase cells in the case of compounds **9b** and **9c** (51.56 and 45.28, respectively), except in the case of **9a**, which induces a cell cycle arrest in the G_2/M-phase cells (23.35) at the expense of the S-phase cells (13.77).

Table 4. Antiproliferative activities against the MCF-7 cell line for the 9-(2,3-dihydro-1,4-benzoxathiin-2-ylmethyl)-9*H*-purines.

Compound	IC_{50} (μM)
9a	9.24 ± 0.01
9b	4.87 ± 0.02
9c	2.75 ± 0.03
9d	>30

Table 5. Cell cycle distribution and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h with the three most active compounds as antiproliferative agents.

Compound	Cell cycle[a]			Apoptosis[b]
	G_0/G_1	S	G_2/M	
Control	62.79 ± 1.30	19.29 ± 1.68	13.26 ± 2.98	0.92 ± 1.29
9a	62.87 ± 0.60	13.77 ± 1.13	23.35 ± 1.97	37.99 ± 8.56
9b	51.56 ± 1.06	17.06 ± 0.75	31.37 ± 1.45	14.33 ± 1.23
9c	45.28 ± 2.73	10.83 ± 4.70	43.89 ± 1.96	70.08 ± 0.33

[a]Determined by flow cytometry.[19] [b]Apoptosis was determined using an Annexin V-based assay.[19] The data indicate the percentage of cells undergoing apoptosis in each sample.

The protein expression analysis by western blot showed that **9a-9c** have an important role in the activation and phosphorylation of the initiation factor eIF2α. The initiation factor eIF2α was phosphorylated in the MCF-7 human breast cancer cell line after treatment with **9a-9c** (Figure 8). It is well established that eIF2α phosphorylation correlates with a translational block and consequently produces inhibition of protein synthesis.[28] These results are in concordance with the delay in the G_2/M cell cycle phase produced by compounds. Furthermore, a prolonged induction of eIF2α finally triggers the cell cycle arrest and/or the apoptosis phenomena.[29,30]

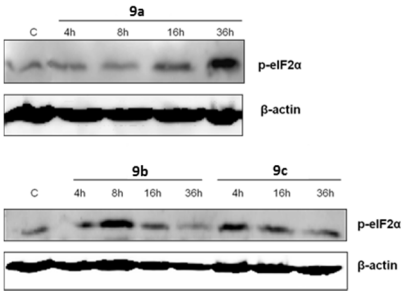


Figure 8. Human breast cancer MCF-7 cells were control-treated or treated with **9a-9c** during 4, 8, 16 and 36 hours. Total proteins were extracted for immunoblot analysis using anti-phospho eIF2α, and anti-β-actin antibodies.

MCF-7 cells treated for 48 h with compounds **9a-9c** induced apoptosis, **9c** being the compound that showed a significant increase of apoptotic cells in relation to the control culture with a percentage of 70.08 ± (Table 5).[21] Apoptosis is a major form of cell death characterized by changes in signalling pathways that lead to the recruitment and activation of caspases, a family of cysteine-containing, aspartate-specific proteases. Caspases exist as inactive proenzymes in cells, and are activated through their processing into two subunits in response to apoptotic stimulation. Activated caspases cleave a variety of important cellular proteins, other caspases, and Bcl-2 family members, leading to a commitment to cell death. Caspase-9 is involved in one of the relatively well-characterized caspase cascades. It is triggered by cytochrome C release from the mitochondria, which promotes the activation of caspase-9 by forming a complex with Apaf-1 in the presence of dATP. Once activated, caspase-9 initiates a caspase cascade that finally induces cell death.[31] Western blot assays showed that compounds **9a-9c** induced activation of caspase 9 at late times (16 h and 36 h of treatment) similarly to paclitaxel used as control compound (Figure 9). These data confirm that levels of apoptosis showed by annexin V assays that are dependent of intrinsic pathway of cell death. p53 was not activated by the compounds which indicate that apoptosis was induced in a p53 independent manner (data not shown).

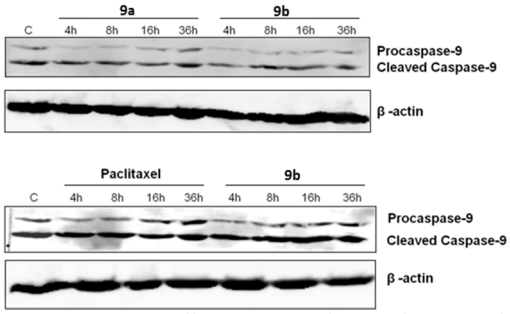


Figure 9. Human breast cancer MCF-7 cells were control-treated or treated with **9c-9c** or paclitaxel during 4, 8, 16 and 36 hours. Total proteins were extracted for immunoblot analysis using anti- caspase 9 and anti-β-actin antibodies.

4. (*R*)- and (*S*)-9-(2,3-Dihydro-1,4-benzoxathiin-3-yl and 2-ylmethyl)-9*H*-purines

The issue of drug chirality is a major theme in the design and development of new drugs, underpinned by a new understanding of the role of molecular recognition in many pharmacologically relevant events.[32] Racemic compounds **8a-8c** and **9a-9c** (Figures 4 and 7, respectively) were selected because of their notable anticancer activity against the human breast cancer cell line MCF-7. Herein, we reported an efficient enantiospecific synthesis of 9-(2,3-dihydro-1,4-benzoxathiin-2 and 3-ylmethyl)-9*H*-purine derivatives (Figure 10) and their antitumour activity against the human breast cancer cell lined MCF-7 and SKBR-3.[33]

4.1. Synthesis

An interesting fringe benefit to the study of the chemistry of the chiral glycidyl *m*-nitrobenzenesulfonyl and the chiral epichlorohydrin, was that it is possible to manipulate stereochemistry by judicious juxtaposition of the nucleophile and substrate. Nucleophilic displacement on the glycidyl *m*-nitrobenzenesulfonyl occurs by direct displacement of the carbon atom bearing the *m*-nitrobenzenesulfonyl group, the chiral centre not being affected. This does not occur with epichlorohydrin, where the epoxide furnishes an alkoxide that effects a second displacement on the Cl-bearing carbon atom to reproduce the three-membered ring, but with complete inversion of its stereochemistry. We reported herein the enantiospecific synthesis of both enantiomers of **8a-8c** and **9a-9c** from 2-mercaptophenol and enantiomeric pure (*S*)-glycidyl *m*-nitrobenzenesulfonate **18** or (*S*)-epichlorohydrin **25** (Schemes 6 and 7).[33]

Three-carbon (C-3) epoxides bearing halide substituents are highly versatile synthetic building blocks because each carbon is functionalized and is a potential site of nucleophilic attack. Sharpless *et al.* described

the preparation of a series of crystalline arenesulfonate derivatives of enantiomerically enriched glycidol and their application to the synthesis of homochiral α-adrenergic blocking agents.[34] Epichlorohydrin is a readily available C-3 unit widely employed in organic and polymer synthesis.[35] We thus decided to compare both (*S*)-glycidyl sulfonate (*S*)-**18** and (*S*)-epichlorohydrin (*S*)-**25** as reagents for the preparation of the enantiomeric compounds **8a-8c** and **9a-9c** (Schemes 6 and 7). Thiolate displacements of sulfonates of glycidyl epoxides have been reported.[36,37]

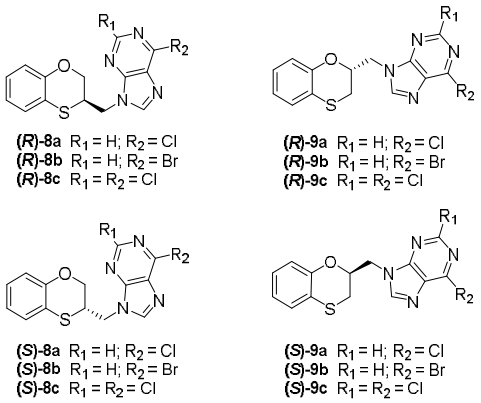
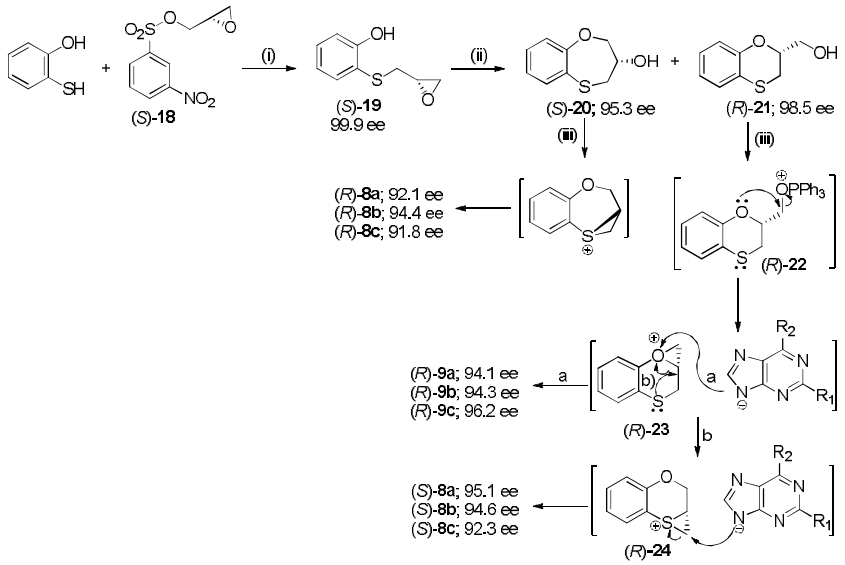


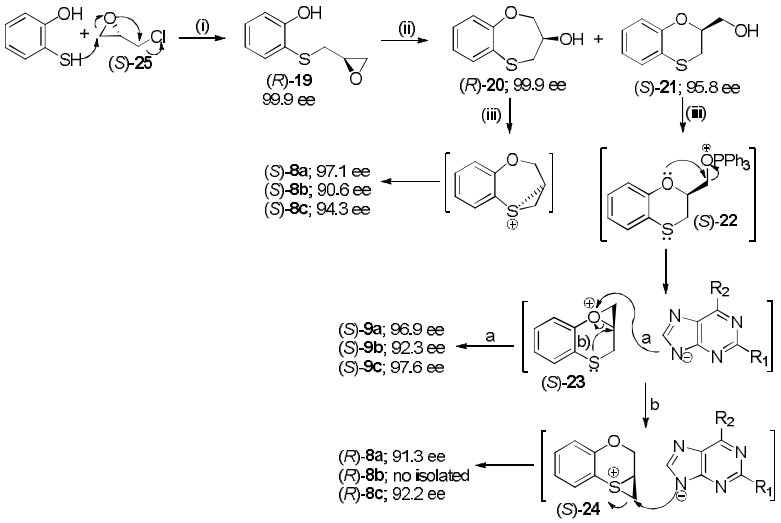
Figure 10. Chemical structures of the (*R)*- and *(S*)-9-(2,3-dihydro-1,4-benzoxathiin-3-yl and 2-ylmethyl)-9*H*-purines.



Scheme 6. Reagents and conditions: (i) NaH, DMF, from -30 ºC to -5 ºC, 95 min; (ii) NaOH, H_2O, 95 ºC, 6 h; (iii) substituted purines, Ph_3P, DIAD, anhydrous THF, microwave irradiation, 140 ºC, 5 min.

As expected, the attack by the (*S*)-glycidyl sulfonate (*S*)-**18** takes place with retention of configuration giving a single enantiomer of the epoxide (*S*)-**19** (step i, Scheme 6). Interestingly, when (*S*)-epichlorohydrin (*S*)-**25** is used instead of (*S*)-**18** the inverted homochiral epoxide (*R*)-**19** is obtained with complete inversion

of its configuration (step i, Scheme 7). The cyclization step from both (*S*)-**19** and (*R*)-**19** occurs with configuration retention to produce the seven-membered derivative (*S*)- and (*R*)-**20** and the opposite configuration of the six-membered ring (*R*)- and (*S*)-**21** (step ii, Schemes 6 and 7, respectively). The absolute configurations of (*S*)-**20**, (*R*)-**20**, (*S*)-**21** and (*R*)-**21** have been determined by the X-ray crystal structure of (*S*)-**20**, (*R*)-**21** (Figure 11A and B) and by the HPLC analysis of the four intermediates. Finally, the Mitsunobu reaction of both (*S*)-**20** and (*R*)-**21** (step iii, Scheme 6) and (*R*)-**20** and (*S*)-**21** (step iii, Scheme 7) resulted in target compounds (**8a-8c** and **9a-9c**) with excellent enantiomeric excess (ee). Mitsunobu reaction normally takes place with inversion of the configuration of the substrate alcohol. Nevertheless, the product of retention of configuration was reported with a series of hindered alcohols.[38-40]



Scheme 7. Reagents and conditions: (i) Pyr, H_2O, r.t., 24 h; (ii) NaOH, H_2O, 95 ºC, 6h; (iii) substituted purines, Ph_3P, DIAD, anhydrous THF, microwave irradiation, 140 ºC, 5 min.

It must be pointed out that when starting from (*R*)- and (*S*)-**21**, apart from the target compounds (*R*)- and (*S*)-**9a-9c**, their corresponding regioisomers (*S*) and (*R*)-**8a-8c** with the opposite configuration are also obtained as side-products through a neighbouring-group mechanism. The absolute configuration of (*R*)-**8c** has been determined by X-ray crystal structure (Figure 11C) and by chiral HPLC analysis of all target intermediates.[33]

4.2. Biological assays

Compounds (*R*)-**8a-8c**, (*R*)-**9a-9c**, (*S*)-**8a-8c** and (*S*)-**9a-9c** were subjected to antiproliferative, apoptosis (Table 6) and cell cycle studies in the MCF-7 and SKBR-3 human breast cancer cell lines.[33]

Compounds **8a**, **8c** and **9a-9c** show one major bioactive enantiomer against both MCF-7 and SKBR-3 human breast cancer cells whereas compound **8b** has presented equally bioactive enantiomers. In general, the IC_{50} values of the racemates **8** and **9** are similar to the average IC_{50} of the corresponding enantiomers (*R*)-**8** and (*R*)-**9** and (*S*)-**8** and (*S*)-**6**. Structure-activity relationship between the configuration of the enantiomers and the antiproliferative effect indicates that, in general, (*S*)-enantiomers are more active in the MCF-7 cell line. Thus, (*S*)-**8a**, (*S*)-**8c**, (*S*)-**9b** and (*S*)-**9c** are more potent than their corresponding racemates while (*R*)-**9a** is more active than **9a** in the MCF-7 cell line. However, (*S*)-**8a**, (*S*)-**8c**, and (*R*)-**9a-9c** and show more cytotoxicity than in the SKBR-3 cell line.

In the MCF-7 cell line racemic and homochiral compounds **9a-9c**, with the purine moiety at position 2, are more active than their corresponding regioisomers **8a-8c**, with the purine moiety at position 3, except for (*S*)-**9a**. The most active compound (*S*)-**9c**, with 2,6-dichloropurine moiety at position 2, shows an IC_{50}=1.85

μM. In contrast, in the SKBR-3 cell line both racemic and homochiral compounds **9a-9c** are more active than their corresponding regioisomers **8a-8c**, except for (*S*)-**8a** and (*S*)-**8c**. The most active compound in this case is (*R*)-**9c** with 2,6-dichloropurine moiety at position 2, that shows an IC_{50}=4.34 μM.

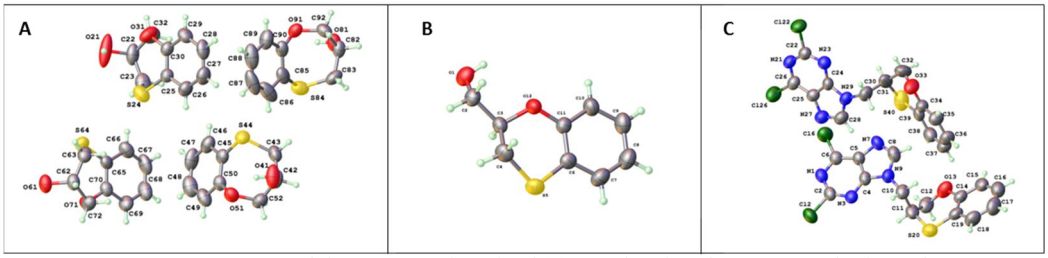


Figure 11. **A)** Content of the asymmetric unit of (*S*)-**20**, showing the atom-numbering schemes. Displacement ellipsoids are drawn at the 50% probability level. **B)** Content of the asymmetric unit of (*R*)-**21**, showing the atom-numbering schemes. Displacement ellipsoids are drawn at the 50% probability level. **C)** Content of the asymmetric unit of (*R*)-**8c**, showing the atom-numbering schemes. Displacement ellipsoids are drawn at the 50% probability level.

Table 6. Antiproliferative effect and apoptosis induction for the target compounds in the MCF-7 and SKBR3 cell lines.

Compound	MCF-7			SKBR-3		
	IC_{50} (μM)[a]	Apoptosis (3x IC_{50})[b]	Apoptosis (IC_{50})[b]	IC_{50} (μM)[a]	Apoptosis (3x IC_{50})[b]	Apoptosis (IC_{50})[b]
8a[13]	10.60 ± 0.66	73.80 ± 0.42	22.55 ± 0.07	8.17 ± 0.005	40.80 ± 0.12	13.40 ± 0.14
(*R*)-**8a**	15.20 ± 0.03	71.95 ± 0.21	20.25 ± 0.21	12.10 ± 0.04	29.25 ± 0.11	9.35 ± 0.12
(*S*)-**8a**	3.30 ± 0.02	31.55 ± 1.40	13.95 ± 0.60	4.50 ± 0.12	42.05 ± 2.31	18.43 ± 0.44
8b[13]	6.18 ± 1.70	63.45 ± 1.50	30.60 ± 6.78	8.98 ± 0.003	28.60 ± 0.50	7.62 ± 0.70
(*R*)-**8b**	6.17 ± 0.07	55.75 ± 0.12	26.55 ± 0.20	9.24 ± 0.003	42.72 ± 0.15	7.95 ± 0.02
(*S*)-**8b**	6.32 ± 0.04	60.50 ± 9.00	41.80 ± 0.56	9.05 ± 0.14	26.60 ± 1.30	27.23 ± 0.05
8c[13]	8.97 ± 0.83	51.35 ± 0.21	15.75 ± 0.49	5.73 ± 0.22	59.85 ± 0.11	20.25 ± 0.04
(*R*)-**8c**	10.30 ± 0.01	27.35 ± 0.07	6.25 ± 3.30	7.52 ± 0.01	37.52 ± 0.05	10.58 ± 0.32
(*S*)-**8c**	6.93 ± 0.09	58.75 ± 2.75	60.35 ± 2.40	4.35 ± 0.002	68.95 ± 0.57	27.46 ± 0.60
9a[21]	9.24 ± 0.01	67.35 ± 0.90	10.30 ± 0.14	8.04 ± 0.001	55.21 ± 0.70	23.60 ± 0.10
(*R*)-**9a**	4.73 ± 0.02	42.95 ± 0.63	9.70 ± 0.42	6.56 ± 0.11	60.05 ± 1.13	11.70 ± 0.23
(*S*)-**9a**	11.40 ± 0.06	89.50 ± 0.70	19.05 ± 0.63	9.46 ± 0.005	37.20 ± 0.11	12.35 ± 0.87
9b[21]	4.87 ± 0.02	99.45 ± 0.07	38.45 ± 4.73	7.25 ± 0.003	95.80 ± 0.21	36.25 ± 1.03
(*R*)-**9b**	4.45 ± 0.07	63.75 ± 6.00	15.95 ± 2.33	5.18 ± 0.002	47.51 ± 2.11	8.42 ± 0.41
(*S*)-**9b**	3.33 ± 0.13	50.20 ± 1.13	25.15 ± 0.49	7.78 ± 0.002	25.68 ± 0.55	10.60 ± 0.09
9c[21]	2.75 ± 0.03	97.70 ± 0.56	29.35 ± 0.30	5.00 ± 0.001	78.20 ± 1.26	27.53 ± 0.33
(*R*)-**9c**	3.33 ± 0.04	99.10 ± 0.65	77.00 ± 2.80	4.34 ± 0.002	87.40 ± 0.35	37.20 ± 0.30
(*S*)-**9c**	1.85 ± 0.05	89.40 ± 1.50	33.15 ± 0.20	7.03 ± 0.002	56.10 ± 0.09	4.85 ± 0.19

[a]All experiments were conducted in duplicate and gave similar results. Data are means ± SEM of three independent determinations. IC_{50} was determined after 6 days of treatment. [b]Apoptosis was detected after 48 h of treatment.

In the MCF-7 cell line compounds are more potent as programmed cell-death inducers than in the SKBR-3 one, and more specifically our homochiral compounds (*S*)-**8c** and (*R*)-**9c** are the more effective

apoptotic inducers (60% and 77% at their IC_{50}, respectively) in the MCF-7 cell line. In the SKBR-3 cell line the best apoptotic values are observed at their $3\times IC_{50}$ concentrations. Compounds **9b**, **9c** and (*R*)-**9c** present the best apoptotic percentages in both cancerous cell lines at their $3\times IC_{50}$ concentrations (99%, 98%, and 99%, respectively in MCF-7, and 96%, 78%, and 87%, respectively, in SKBR-3).

The cell cycle does not show significant differences among the compounds (data not shown). We also analyzed the protein activation of eIF2α factor by western blot. eIF2α is significantly phosphorylated in MCF-7 cancer cells after treatment with (*R*)-**8a**, (*S*)-**8b** and (*S*)-**9c**, and at 16 h and 36 h (Figure 12A). Interestingly, (*S*)-**9c** induces high eIF2α phosphorylation in the MCF-7 cell line in comparison with its racemate and its enantiomer**,** where no activation is shown (Figure 12A). These results support the highest antiproliferative activity displayed by (*S*)-**9c** and suggest that this activity is in part due to the suppression of protein synthesis provoked by eIF2α phosphorylation.[41] Furthermore, a prolonged induction of eIF2α finally triggers the apoptosis phenomena.[42,43] The homochiral compounds included in this study show a different apoptosis effect between the two enantiomers.

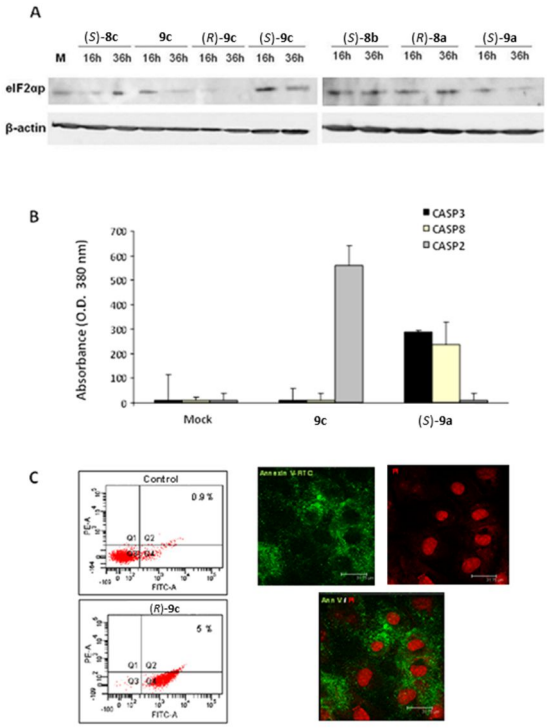


Figure 12. Effects on the eIF2α transcription factor and apoptotic activity in the MCF-7 and SKBR-3 human breast cancer cell lines. **A)** Modification of the expression of eIF2α kinase analysed by western blot. **B)** Fluorometric detection for caspase-3, caspase-2 and caspase-8. **C)** Cytometry analysis and confocal microscopy for control cells treated with 0.5% DMSO and (*R*)-**9c**. **D)** Cell viability metabolic-analysis of SKBR-3 human breast cancer cell line control cells treated with 0.5% DMSO or treated with **9b** and **9c** compounds in absence (black square) or presence (white square) of 20 μM of pan caspase inhibitor z-VAD-fmk.

All target compounds included in this study induce strong levels of cell death measured by citotoxicity analysis and by phosphatidylserine externalization (Annexin V binding) (Table 6 and Figure 12C) even in the MCF- 7 breast cancer cells that have shown deficiency in the caspase-activation mechanisms.[33,44]

Whereas compound (*S*)-**9a** activates the canonical intrinsic caspase-8/caspase-3 apoptotic pathway on the MCF-7 cell line, compound **9c** induces caspase-2 activation (Figure 12B). However, a strong apoptosis induction is also detected in the rest of the compounds analysed. To further confirm the involvement of caspases, including caspase-3, in the apoptosis induced by the most apoptotic compounds in the caspase-3 wild type SKBR-3 cell line, cells were pre-treated with the pan-caspase inhibitor z-VAD-fmk for 2 h, followed by the **9b** and **9c** treatment, and cell viability metabolic-analysis was carried out (Figure 12D). Our results show that **9b** and **9c** were sensible to the effect of the z-VAD-fmk caspase inhibitor, which could rescue SKBR-3 cells from the cytotoxicity of compounds. These results demonstrate the involvement of caspase activation during cell death induced by the compounds in the SKBR-3 cells as previously described for numerous antitumour apoptotic drugs.[45-47]

These and other antitumour effects such as autophagy or senescence events could be involved in the caspase-dependent and caspase-independent cell death induced by the compounds included in this study. This fact opens an important line of research that is yet to be explored.

5. Conclusions

A series of 9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purine derivatives (**8a-8k**) were obtained from a Mitsunobu reaction that led to a six-membered ring contraction from a secondary alcohol in a seven-membered cycle under microwave heating. The most active compounds **8b** and **8c** present an IC_{50} of 6.18 μM and 8.97 μM, respectively, against the MCF-7 cancer cell line and are correlated with their capability to induce apoptosis (63.05 % for **8b** and 76.22 % for **8c**).

A bioisosteric series of substituted 9-(2,3-dihydro-1,4-benzoxathiin-2-ylmethyl)-9*H*-purines (**9a-9d**) were synthesized. During the preparation of **9a-9c**, isomerization products were isolated (**8a-8c**). A formal 1,4-thio migration (from the 2,3-dihydro-1,4-benzoxathiin-2-ylmethyl to the 2,3-dihydro-1,4-benzoxathiin-3-ylmethyl moieties) proceeded through two consecutive O-*3* and S-*3* neighbouring-group participations in the six-membered ring system. The most active compound **9c** with IC_{50}=2.75 μM is 3.3-fold higher as antiproliferative agent against the MCF-7 cell line than its bioisostere **8c**. Apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h for compounds **9c** and **8c** are in the same range (70.08% and 76.22%, respectively). Our results also demonstrate that compounds **9a-9c** have the inhibition of translation by eIF-2α phosphorylation, and induction of cell apoptosis in a p53-independent manner as molecular targets.

Finally, we reported an efficient enantiospecific synthesis of (*R*)- and (*S*)-9-(2,3-dihydro-1,4-benzoxathiin-2 and 3-ylmethyl)-9*H*-purine. The contraction into the six-membered ring takes place with concomitant inversion at the stereocentre with excellent enantiomeric excesses giving rise to the homochiral 9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purines. Starting from both enantiomers of 2,3-dihydro-1,4-benzoxathiin-2-methanol, the 2- and 3-isomers of benzo-fused six-membered heterocycles linked to purines through a methylene group are obtained with excellent enantiospecificities. In general, (*S*)-enantiomers are more active in the MCF-7 cell line. The most active compounds are (*S*)-**9c** and (*R*)-**9c**, with 2,6-dichloropurine moiety at position 2, show an IC_{50} of 1.85 μM and 4.34 μM, respectively. Interestingly, (*S*)-**9c** induces high eIF2α phosphorylation in the MCF-7 cell line in comparison with its racemate and its enantiomer**,** where no activation is shown. Our results support the highest antiproliferative activity displayed by (*S*)-**9c** and suggest that this activity is in part due to the suppression of protein synthesis provoked by eIF2α phosphorylation. The enantiomers of all the compounds included in this study show different apoptotic effects and may serve as prototypes for the development of more potent structures endowed with an apoptotic mechanism of action so far unknown. At present we are studying several targets in order to solve the mechanism of action at the molecular level of such benzannelated six-membered derivatives.

Acknowledgements

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