Bioorganic & Medicinal Chemistry 18 (2010) 5391–5399

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Identification of chalcones as in vivo liver monofunctional phase II enzymes inducers

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ARTICLE INFO

Article history:
Received 1 April 2010
Revised 8 May 2010
Accepted 13 May 2010
Available online 20 May 2010

Keywords:
Chalcones
Chemopreventive agents
Phase II enzyme inducers

ABSTRACT

Cancer preventive agents (CPA) are drugs able to suppress the carcinogen metabolic activation or block the formation of ultimate carcinogens. CPA could act through various molecular mechanisms, for example by interfering with the action of procarcinogen. This could be attained by increasing the phase II enzymes levels of quinone reductase (QR) and glutathione *S*-transferase (GST). New flavonoids, especially chalcones, have been identified as in vivo monofunctional phase II enzymes inducers. Oral administration of chalcone, **4**, and both *p*-methoxy-substituted chalcones, **6** and **14**, increased hepatic QR activity with concomitant decrease in CYP1A1 activity, a member of the most important group of phase I enzymes cytochrome P450. Among them, **4** also increased GST activity. While *p*-bromo-substituted chalcone **8** was the best inducer of QR it decreased hepatic GST expression and cytochrome P450, being the most effective decreasing cytochrome P450-expression. Thienyl-chalcone **20** being the bioisostere of chalcone **4** did not display the same in vivo profile in the phase I level modification. As chalcone **4** its bioisostere, chalcone **20**, displayed low DNA strand breakage and absence of mutagenicity. Also, in our preliminary in vivo tumourigenesis/chemopreventive and acute-toxicity studies, chalcones **4**, **6** and **8** showed the best behaviours as CPA justifying additional studies that are ongoing.

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1. Introduction

Carcinogenesis is a complex and protracted multistage process initiated by events wherein an endogenous or exogenous agent damages a cellular macromolecule. Cancer chemoprevention involves prevention, delay, or reversal of the carcinogenic process through administration of drugs named as cancer preventive agents (CPA). These CPA are able to suppress the carcinogen metabolic activation or to block the formation of ultimate carcinogens.[1]

CPA can interfere with the carcinogenic process at various levels by blocking initiation and by suppressing later stages involving promotion, progression, angiogenesis, invasion and metastasis.[2] The possible ways to block the initiation of carcinogenesis could include metabolism alteration of procarcinogens in favour of conjugation and excretion of reactive metabolites.[3] The modification of the procarcinogens metabolism is regulated by two different groups of enzymes, named phase I and phase II enzymes.[4] Phase I enzymes, involves xenobiotics oxidation, reduction and hydrolysis processes. The end-products of these processes are mainly electrophilic entities with enhanced capability to react with DNA and proteins promoting damages and cellular disruption that could initiate carcinogenic process. The most representative enzymes belong to cytochrome family, for example, Cyt P450 (CYP), Cyt b5 and NADPH-cytochrome c reductase. On the other hand, phase II enzymes promote the xenobiotics conjugation with endogenous ligands, like glutathione and glucuronic acid, to facilitate their excretion to diminish their potential carcinogenic effects. Examples of phase II enzymes are UDP-glucuronosyltransferase, NAD(P)H:quinone reductase (QR) and glutathione *S*-transferase (GST).[5] QR is considered a phase II enzyme because it has protective functions, is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those

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doi:10.1016/j.bmc.2010.05.033

that control GST.[6] Recent studies have shown that increase in phase II enzymes activity, such as QR and GST, correlates with protection against chemical-induced carcinogenesis in the initiation and promotion stages.[1,2,6]

The enzyme inducers CPA are of two types: monofunctional and bifunctional.[4] Monofunctional-inducers increase selectively phase II enzymes and activate the antioxidant response element, ARE, through the Keap1-Nrf2 system.[7] Since phase I enzymes can activate procarcinogens to their ultimate reactive species, monofunctional agents that induce selectively phase II enzymes appear to be more desirable candidates for cancer chemoprevention. Bifunctional-inducers increase phase II as well as phase I enzymes and bind with high affinity to the aryl hydrocarbon receptor and to xenobiotic response element in the nucleous.[8] Many structurally unrelated agents including the polyaromatic heterocycles, barbiturates, phenolic antioxidants, curcuminoids, cinnamates, isothiocyanates, 1,2-dithiole-3-thiones, lactones, thiocarbamates and flavonoids were found to induce genes of phase II enzymes (Fig. 1a).[9–11] Specifically, flavonoids are among the most studied CPA, being the natural ones the best-characterized, that is, flavanol quercetin (Fig. 1a).[12] Between the synthetic flavonoids the flavones **1–3** (Fig. 1b) were identified as CPA.[13] Chalcones, bioprecursors of flavonoids bearing the structure of 1,3-diphenyl-2-propen-1-one, have been reported to possess many biological activities, however they have been scarcely studied as CPA and in these cases mainly in vitro studies were performed.[13] In an earlier study, we found that chalcones with substituents on the B ring were good in vitro anti-tumour agents with negligible toxic effects in normal cells, evaluated throughout DNA strand breakage study (alkaline comet assay).[14] For example, chalcones **4–15** (Fig. 2) showed a great variety of in vitro anti-tumour activities against three different tumour cell lines with adequate drug-likeness properties.

As chalcones have not been recognized as CPA yet, we were interested in exploring their in vivo potential in this area. Hence, we selected from our chemical library chalcones **4–15**, flavones **1**, (Fig. 1b), **16** and **17**, and flavanones **18** and **19** with a wide range of cytotoxic activities on tumour cells (Fig. 2).[14] Additionally, we prepared chalcone **20**, bioisostere of derivative **4**, in order to corroborate the continuance of the chemoprevention ability. The compounds were investigated in vivo, after oral administration in a pre-established regimen, for their ability to modify liver phase I (CYP1A1/CYP1A2, EC 1.14.14.1) and phase II (QR, EC 1.6.5.2 and GST, EC 2.5.1.18) enzyme activity. In order to assess their potential use as drugs, some of the most interesting derivatives were also tested for their in vitro mutagenicity and their in vivo toxicity and for their ability to prevent the appearance of *N*-nitroso-*N*-methylurea (NMU)-induced tumours.

2. Results and discussion

2.1. Chemistry

According to their in vitro anti-tumour activities (Fig. 2), four different clusters of flavonoids were selected to study the influence on the in vivo chemo-preventive properties. Active flavonoids against the three studied tumour cells, chalcones **4** and **5**, cluster I; active flavonoids against two of the studied tumour cells, chalcones **6**, **7** and **12–14**, and flavanone **19**, cluster II; active flavonoids against one of the studied tumour cells, chalcone **15**, flavone **17** and flavanone **18**, cluster III; and inactive flavonoids against all of the studied tumour cells, chalcones **8–11**, flavones **1** and **16**, cluster IV.[14] These flavonoids were prepared according to procedures previously described.[13b–16] Briefly, we used the aldolic condensation with NaOH as base for the chalcones **4–15** exemplified in Figure 3a for the new compound studied here, chalcone **20**.[15] Flavones **1**, **16** and **17** and flavanones **18**, **19** and **21** (Fig. 6a) were prepared using traditional methodologies from the corresponding hydroxychalcones, oxidative and non-oxidative cyclization.[17,18] All the compounds were characterized by NMR (^{1}H, ^{13}C, COSY, HSQC and HMBC experiments), IR and MS. The purity was established by TLC and microanalysis. The stereochemistry around the chalcones olefinic carbon–carbon bond was established using H–H coupling constant values or NOE-diff experiments being *E*-isomers in all the cases. Single crystals of derivative **20** adequate for structural X-ray diffraction studies were obtained by slow evaporation from an Et_2O solution. Figure 3b shows the ORTEP[19a] drawing of the chalcone **20** molecule in the solid state.

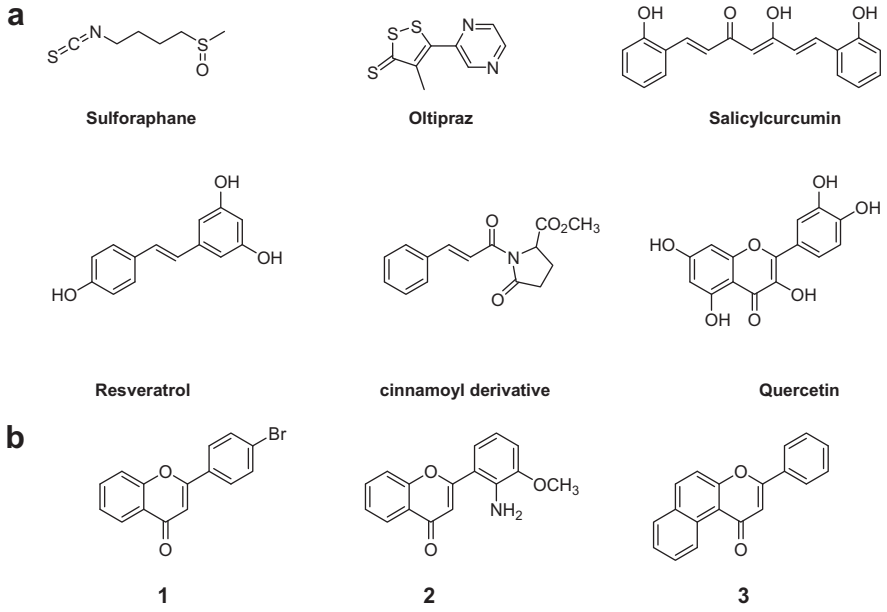
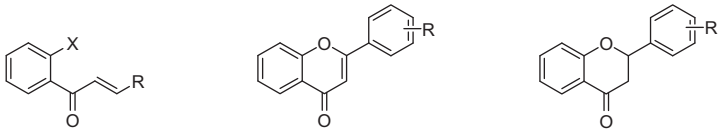


Figure 1. (a) Described phase II-enzymes genes inducers. (b) Flavones studied as chemopreventive agents.



Group	(I): 4-15		(II): 1, 16, 17	(III): 18 and 19		
cpd	-X	-R	cluster	activity against tumour cells		
				TK-10	MCF-7	HT-29
4		-Ph	**I**	Y	Y	Y
5		-2-pyridinyl		Y	Y	Y
6		-Ph-4-OMe	**II**	Y	Y	N
7		-Ph-3,4-diOMe		Y	Y	N
8	-OH	-Ph-4-Br	**IV**	N	N	N
9		-Ph-4-NH_2		N	N	N
10		-Ph-4-$N(CH_3)_2$		N	N	N
11		-Ph-3,4-OCH_2O-		N	N	N
12		-Ph-4-Cl	**II**	Y	N	Y
13		-Ph-4-Cl		Y	N	Y
14	-H	-Ph-4-OMe		Y	N	Y
15		-Ph-4-SMe	**III**	Y	N	N
1		-*p*-Br	**IV**	N	N	N
16		-H		N	N	N
17	-	-*o*-Br	**III**	N	Y	N
18		-*o*-Br		N	Y	N
19		-*p*-OMe	**II**	Y	Y	N
20	-OH	-2-thienyl		nd	nd	nd

Figure 2. Chalcones, flavones and flavanones evaluated as anti-tumour agents.[14] TK-10: human kidney carcinoma; MCF-7: human mammary adenocarcinoma; HT-29: human colon adenocarcinoma. The compounds were evaluated in vitro at 100 μM doses and the percentages of cellular survival were determined. When the percentage of cellular survival is <40% the compounds is classified as active (Y), when the percentage of survival is ⩾40% the compound is classified as inactive (N). nd: not determined.

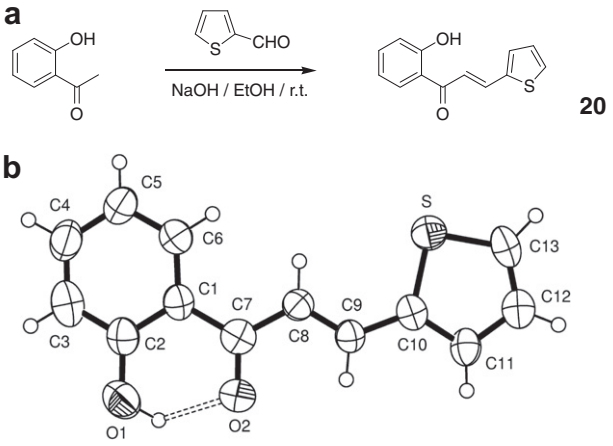


Figure 3. (a) Chalcone **20** synthesis, prepared as bioisostere of chalcone **4**. (b) Drawing of the chalcone **20** molecule, showing the labelling of the non-H atoms and their displacement ellipsoids at the 50% probability level.[19] The intra-molecular H-bond is indicated by dashed lines.

2.2. Biological characterization

2.2.1. Cytotoxicity studies on tumour cells

The chalcone **20**, was tested at 100 μM dose against TK-10 (human kidney carcinoma) (NCI), MCF-7 (human mammary adenocarcinoma) (ATCC HTB-38), and HT-29 (human colon adenocarcinoma) (ATCC HTB-38) tumour cell lines, according to previously described procedures.[14,20] Survival percentages (SP) were determined and the results are gathered Table 1. Derivative **20** was inactive against the three studied tumour cells so classified to pertain to cluster IV (Fig. 2). This marked difference to its bioisostere **4** and the other heterocyclic flavonoid, chalcone **5**, which fit in cluster I (Fig. 2).

2.2.2. In vivo phase I and phase II hepatic enzymes induction

To evaluate the flavonoids capability to induce phase I and phase II hepatic enzymes we performed an in vivo study using rats as the biological model. As a first study, the rats were placed on different experimental oral diets with chalcone **4**, 15.0 or 22.5 mg/kg/day for two or three weeks, to find the best experimental conditions (Table 1). For oral administration schedule see Supplementary data (Fig. S1). For derivative **4**, no differences in the QR-level changes were observed between both studied doses; however, three-weeks-treatment produces differences in the studied findings (data not shown). Consequently, we selected the low doses and the long-time treatment (15.0 mg/kg/day, 15 days-treatment during 3 weeks, Fig. S1, Supplementary data). Secondly, compounds **4–9**, **11–16**, **18** and **20** were orally dosed in the selected experimental condition assaying also flavone **1**, taken as a CPA reference. Finally, after animal sacrification, the livers were collected and QR, GST and CYP activities were assessed spectrophotometrically (Table 1). GST and CYP activities were not measured in flavonoids-treated-animals with lower QR activities than the untreated animals (control).

As it is indicated in Table 1, except derivative **9** the studied flavonoids modified the levels of phase I and phase II liver enzymes in our experimental conditions. Both phase II hepatic QR and GST activities were significantly induced in comparison with the control groups (basal diet) by flavonoids **4**, **5**, **15**, **18** and **20**, while chalcones **6**, **8**, **11–14** were able to increase only QR activity. Tana-

Table 1
Effect of flavonoids on QR, GST and CYP activities in rat liver

Compd	Doses (15 days)	Cluster	Percentage of increment/decrement of enzyme specific activity respect to untreated control[a] (%)		
			Phase II		Phase I
			QR	GST	CYP
4	15.0 mg/kg/day	I	+10 ± 5	+30 ± 10	−60 ± 10
4	22.5 mg/kg/day		+10 ± 5	nd[b]	nd
5			+13 ± 5	+20 ± 10	+10 ± 10
6		II	+60 ± 10	−10 ± 10	−40 ± 10
7			−20 ± 10	nd	Nd
8		IV	+128 ± 50	−30 ± 10	−80 ± 10
9			0 ± 5	nd	nd
11			+30 ± 5	−20 ± 10	−50 ± 5
12	15.0 mg/kg/day	II	+20 ± 5	−20 ± 10	−10 ± 5
13			+10 ± 5	−20 ± 10	+40 ± 10
14			+10 ± 5	−10 ± 10	−30 ± 10
15		III	+20 ± 10	+30 ± 10	+750 ± 20
1		IV	+30 ± 10	nd	nd
16			−30 ± 10	nd	nd
18		III	+10 ± 5	+10 ± 5	+30 ± 10
20[c]		IV	+10 ± 5	+10 ± 5	+20 ± 10

[a] Induction was calculated by comparing the treatment groups with the control group (basal diet). Treatment groups were significantly different from the basal diet control group ($P < 0.05$) using Student's t-test with $n = 5$. Values represent means ± SE. The sign (+) denotes increment and (−) denotes decrement.
[b] nd : not determined (see text).
[c] SP_{TK-10} = 72%, SP_{MCF-7} = 91%, SP_{HT-29} = 95%. SP: percentage of cellular survival at 100 μM doses.

ka et al. obtained the same induction increment for QR and GST with chalcone **4** in a model of colon tumour.[13d] On the other hand, chalcones **4**, **6**, **8**, **11**, **12** and **14** were able of significantly decreasing the hepatic CYP induction in comparison with the control groups (basal diet), in our experimental conditions.

Previously, it has been demonstrated that flavones **1** and **3** (β-naphthoflavone, Fig. 1) are bifunctional agents,[13] inducing both phase I and phase II enzymes, having a similar performance that the flavanone studied in this paper, derivative **18**.

Chalcone **8** was the best hepatic QR inducer and chalcones **4** and **15** were the best hepatic GST inducers. The worst hepatic CYP inducers, and therefore the most interesting compounds, were **8** > **4** > **11** > **6** > **14**.

The best monofunctional inducers are the cycle B-unsubstituted and p-OCH_3-substituted chalcones with a relation of **4** > **6** > **14**. Chalcone **8**, p-Br-substituted, and di-substituted chalcone **11** are as interesting as derivatives **6** and **14** but with lack of GST-inducer capabilities (Fig. 4). Chalcone **15** particularly induced hepatic CYP, maybe as a result of its $-SCH_3$ structural substitution. The bioisosteres **4** and **20** showed different behaviour as CYP inducers, being chalcone **20**, like the other studied heterocyclic flavonoid, that is, chalcone **5**, a moderate CYP inducer.

2.2.3. In vitro QR induction studies

Induction of QR activity was also assessed in an in vitro model using human mammary adenocarcinoma cells (MCF-7),[21,22] the same kind of in vivo neoplastic system, for chalcones **4**, **5** and **8**. Firstly, the previously described flavone **1** was studied in a doses-response assay to select the adequate in vitro doses (Fig. 5a). The in vitro results for derivatives **4**, **5** and **8**, are in complete agreement with the in vivo ones (Fig. 5b).

2.2.4. In vitro toxicity studies

Two different studies were performed to assess flavonoids toxic effects, comet assay for derivative **20** and mutagenicity test for compounds **4**, **5** and **20**.

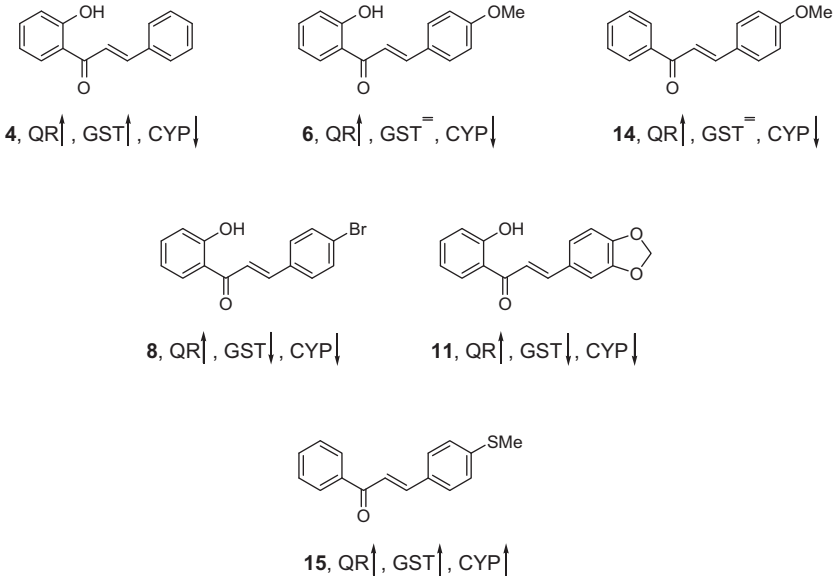


Figure 4. Structures of chalcones and their effects in hepatic enzymes levels modifications (↑: denotes increment; ↓: denotes decrement; =: denotes not change).

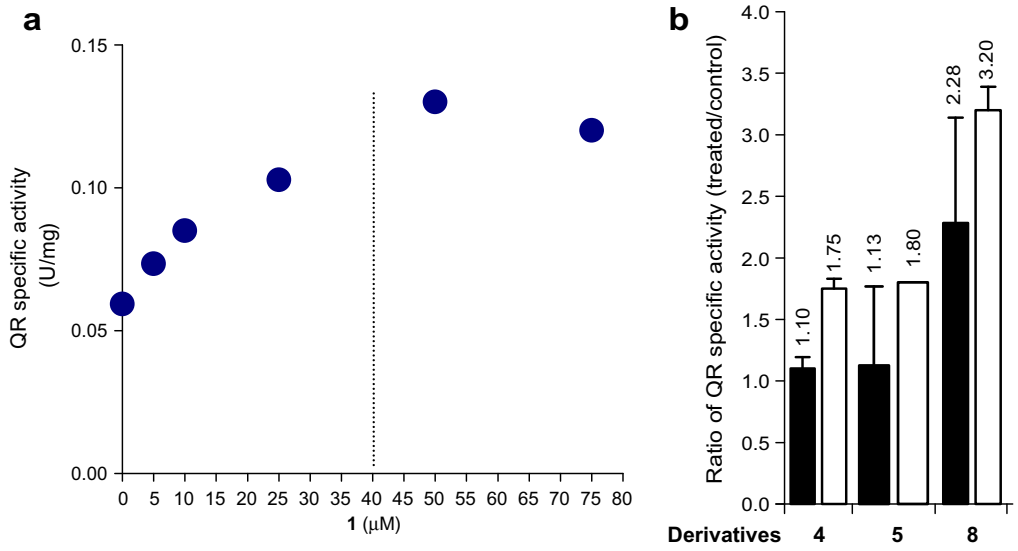


Figure 5. (a) Dose-response of QR induction by flavone **1** on MCF-7 tumour cells. Selected doses 40 μM. (b) In vivo (black), see Table 1, and in vitro (white), on MCF-7 tumour cells at 40 μM, QR induction by chalcones **4**, **5** and **8**.

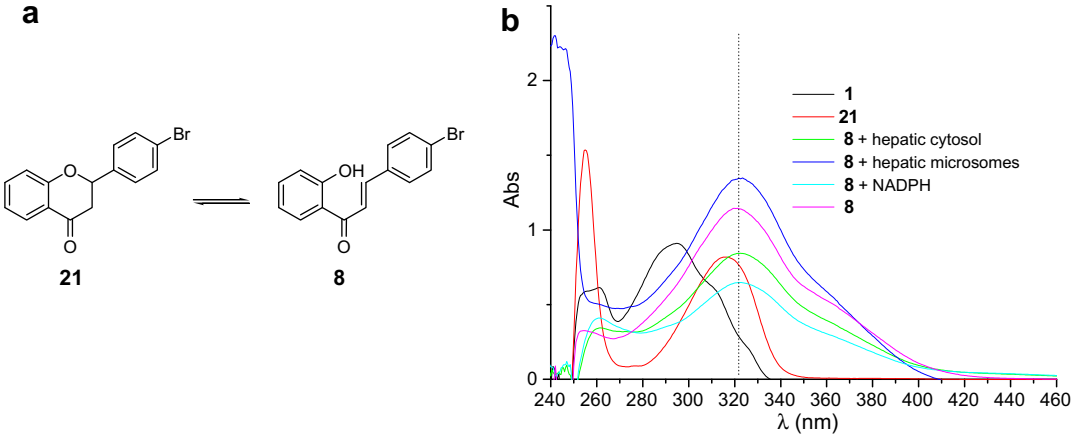


Figure 6. (a) Bond-tautomerism between flavanone **21** and chalcone **8**. (b) Example of UV study in the in vitro metabolism studies of chalcone **8**.

2.2.5. Alkaline comet assay

The effect of compound **20** against DNA of HK-2 cells (human kidney cells) was evaluated through the alkaline single cell gel electrophoresis assay. The compound was submitted to the comet assay at 20 μM. Comets were scored visually, and one hundred nucleoids per slide were classified according to the intensity of fluorescence in the comet tail.[23] Images were given a value of 0–4 (from undamaged to maximally damaged). DNA damage therefore extends from 0 to 400 arbitrary units and covers a range of strand break frequencies (estimated using X-ray calibration) of 0–3 breaks per 109 Daltons. The total score was calculated by the following equation: (percentage of cells in class 0×0) + (percentage of cells in class 1×1) + (percentage of cells in class 2×2) + (percentage of cells in class 3×3) + (percentage of cells in class 4×4). The results are compared with the previous studied compounds in Table 2. Derivative **20** does not produce relevant DNA strand breakage. Its effect was milder than the pyridinyl derivative **5**, the most harmful one, but stronger than the its bioisostere **4**.

2.2.6. Mutagenicity assay

The method of pre-incubation in plate[24] using culture of *Salmonella typhimurium* TA98 strain was performed on derivatives **4**, **5** and **20**. Positive control of 4-nitro-*o*-phenylendiamine (4-NPD) was run in parallel. The revertant colonies were manually counted and compared to the spontaneous revertants (Table 3). The compounds are considered mutagenic when the number of revertant

Table 2
DNA strand breakage (arbitrary units) promoted by chalcones on HK-2 cells

Compd	Total score in comet assay[a,b]
4	56[c]
5	124[c]
6	56[c]
8	49[c]
11	60[c]
14	75[c]
20	83
C (−)[d]	59
C (+)[e]	107

[a] The results are the means of two different experiments with a SD less than 10% in all cases.
[b] According to the calculus the maximum score is 400.
[c] From Ref. 14.
[d] C (−): negative control, DMSO.
[e] C (+): positive control, HK-2 cells treated with 50 μM hydrogen peroxide during 15 min on ice.

Table 3
Number of revertants of derivatives **4**, **5** and **20** on TA98 *S. typhimurium* strain

4		5		20	
D[a]	NR[b,c]	D[a]	NR[b,c]	D[a]	NR[b,c]
0.0	19 ± 3	0.0	19 ± 3	0.0	16 ± 1
3.0	13 ± 1	1.0	13 ± 3	1.0	15 ± 0
8.0	16 ± 1	2.0	17 ± 3	4.0	16 ± 1
25.0	17 ± 1	5.0	16 ± 1	13.0	19 ± 4
75.0	20 ± 6	15.0	17 ± 1	38.0	13 ± 1
225.0	14 ± 2	45.0	18 ± 4	115.0	14 ± 6
	4-NPD[d]				
20.0	1223 ± 237				

[a] D: doses in μg/plate.
[b] NR: number of revertants.
[c] The results are the means of two independent experiments ± SD.
[d] 4-NPD: 4-nitro-*o*-phenylendiamine (positive control).

colonies is at least twofold of the spontaneous revertant frequencies for at least two consecutive dose levels.[25] None of the studied compounds were classified as mutagenics.

2.2.7. In vitro flavonoids 1- and 8-metabolic studies

Being flavonoids **8** and **21** bond-tautomers (Fig. 6a), it could be proposed that the observed biological activity of chalcone **8** is the result of the bio-activity of flavanone **21** after enzymatic/chemical transformation or the activity of flavanone **21** is the result of chalcone **8** after metabolic pathways. Consequently, phase I bio-transformation of chalcone **8** and flavanone **21** by rat hepatocytes microsomes and cytosolic fraction was investigated using thin layer chromatography and UV spectroscopy. After incubation time, neither **8** to **21** transformation nor **21** to **8** were observed (see examples of UV results in Fig. 6b). In these assays flavone **1** was also studied to find that no inter-conversion occurs between the studied flavonoids.

New apolar entities were identified chromatographically in the **8** metabolism, one new compound for hepatic cytosolic fraction and this and another one for hepatic microsomes, but further studies to identify them were not performed. Also, no chemical transformation was observed when chalcone **8** was incubated with the NADPH cofactor. These results showed that the measured bio-activities of compounds **8** were not the result of its bio-transformation to the corresponding bond-tautomer, **21**, or the flavone **1**.

2.2.8. In vivo studies

To evaluate the potential of the studied flavonoids as cancer chemopreventive drugs, we have applied an in vivo model of tumourigenesis, induced by NMU. In this assay we have evaluated the potential chemoprevention properties of studied flavonoids, named flavones **1** and **17**, chalcones **4**, **6–8** and **10**, and flavanone **19**, and their toxic effects in these conditions. The schedule of the flavonoids oral administrations, 10.0 mg/kg/day, for this study is shown in Figure 7.

Three differences between untreated and **4**-, **6**- and **8**-treated animals were observed, namely (i) the apparition time of the first tumour, (ii) the dissected tumours weights and (iii) the incidence of observable tumours (Fig. 8). None of the studied flavonoids had relevant effect on the body weight (see examples in Fig. 9), on the biochemical parameters (Table 4), or on the histopathology of the studied organs in the administration schedule. These results showed that these flavonoids could be administered following this protocol without apparently toxic effects.

Besides, derivatives **4** and **6** were administered orally in one dose of 250.0 mg/kg, corresponding to 25-fold the previous studied one, in order to evaluate their acute toxicity. For that, healthy animals were treated with the single oral dose and at the third post-administration day the animals were weighted and sacrificed.[27,28] Differences in weight, animal-survival's percentages, clinical biochemistry findings, haematological and organ histological results with respect to healthy non-treated animal were analyzed. No differences between treated and non-treated animals were observed.

3. Conclusions

Flavonoids, especially chalcones, have been identified as in vivo monofunctional phase II enzymes inducers. The unsubstituted chalcone **4**, and both 4-methoxysubstituted chalcone **6** and **14**, increase, after oral administration, hepatic NAD(P)H:quinone reductase levels with concomitant decreasing of the phase I enzyme,

Week	0	1	2	3	4	5	6	7	8	9	10	11
Flavonoid oral administration[a]	⊕	⊕	⊕	⊗	⊕	⊕	⊕	⊗	⊕	⊕	⊕	⊗
NMU[b]	•	⊕	•	•	•	⊕	•	•	•	⊕	•	•

[a] 10.0 mg/kg/day, during 5 days with 2 days of rest.
[b] 18.0 mg/kg, one intravenous administration at the beginning of the week (see Experimental Section)
⊕ - administration; ⊗ - basal diet; • – no administration

Figure 7. Flavonoids oral administration schedule and NMU-induction of tumours.

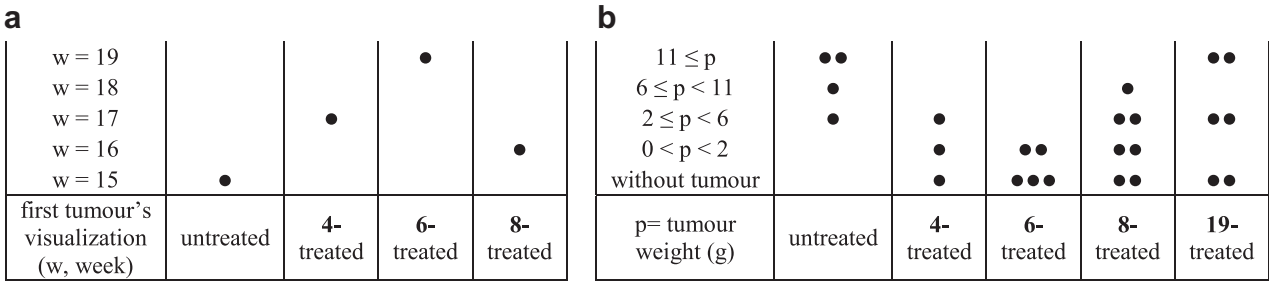


Figure 8. (a) Week of the first visualization of tumour in control (untreated) animals and those receiving **4**, **6**, or **8** according to pre-established schedule (Fig. 7). (b) Dispersion diagrams of the dissected tumours weights, in living animals, at the end of the assays in control (untreated) animals and those receiving **4**, **6**, **8** or **19** according to pre-established schedule (Fig. 7). Results in untreated animals are from different experiments.

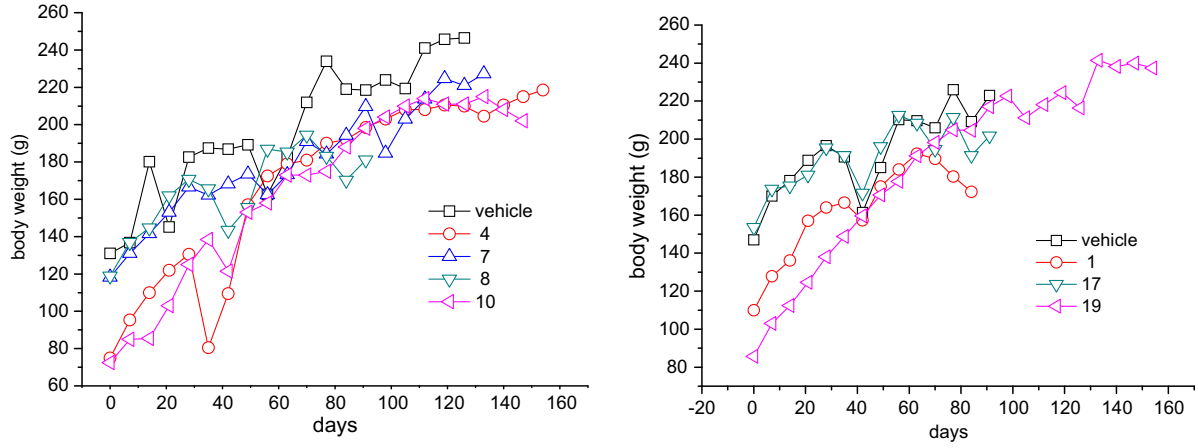


Figure 9. Examples of effects of dietary flavonoids on body weight of rats treated with NMU (flavonoids and NMU administrations according to Fig. 7).

Table 4
Mean values of the biochemical and the haematological findings in NMU-treated animals orally dosage with studied flavonoids

Animal treated with[a]	Leucocyte[b,c] (/μL)	Haemoglobin[c,d] (g/L)	GPT[c,e] (U/L)	GOT[c,f] (U/L)
4	5200	11.3	ND[g]	ND
6	7300	12.3	13.2	52.2
7	9600	9.3	15.0	62.1
8	7700	8.4	26.0	61.7
10	5500	11.5	ND	ND
17	4500	9.6	45.4	52.9
19	8600	11.3	ND	ND
Vehicle	3600	9.6	11.7	48.5

[a] See text for details.
[b] Normal value: 5200 ± 1500/μL.
[c] From Ref. 26.
[d] Normal value: 15.1 ± 4.1 g/dL.
[e] GPT: glutamic-pyruvate transaminase (alanine aminotransferase); normal value: 22.8 ± 13.2.
[f] GOT: glutamic-oxalacetate transaminase (aspartate ketoglutarate aminotransferase); normal value: 71.4 ± 7.8.
[g] ND: not determined.

cytochrome P450. Unlike to chalcones **6** and **14**, chalcone **4** also increases the hepatic glutathione *S*-transferase levels. The best NAD(P)H:quinone reductase inducer is the 2′-hydroxy-4-bromochalcone **8** with a fourfold increased activity compared to the well known phase II-detoxification enzymes inducer, flavone **1**.[13] Although chalcone **8** decreases hepatic glutathione *S*-transferase expression, it concomitantly decreases the expression of the studied phase I-detoxification-enzyme, cytochrome P450. Chalcone **8** is the best cytochrome P450-expression depletor. The developed bioisostere of chalcone **4**, derivative **20**, does not possess the same in vivo profile in the phase I level modification. According to the in vitro toxicity studies, chalcone **20** possesses the same behaviour of bioisostere **4**, that is, low DNA strand breakage and absence of mutagenicity. In this regard, derivatives **4** and **8** are the lowest DNA-damager of the studied chalcones. In our preliminary in vivo tumourigenesis/chemopreventive and acute-toxicity studies, chalcones **4**, **6** and **8** showed interesting behaviours that justify further research, which is currently ongoing in our group.

4. Experimental

4.1. Chemistry

Compounds **1** and **4–19**[14] were prepared according to literature procedures. Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and are uncorrected. Proton and carbon NMR spectra were recorded on a Bruker DPX-400 spectrometer. The chemical shifts values are expressed in ppm relative to tetramethylsilane as internal standard. Mass spectra were determined either in a MSD 5973 Hewlett Packard spectrometer using electronic impact (EI). Microanalyses were performed in a Fisons EA 1108 CHNS-O equipment and were within ±0.4% of the calculated compositions. Column chromatography was carried out using Merck silica gel (60–230 mesh). Most chemicals and solvents were analytical grade and used without further purification. All the reactions were carried out in a nitrogen atmosphere. The typical work-up included washing with brine and drying the organic layer with sodium sulphate before concentration.

4.1.1. (*E*)-3-(Thienyl)-1-phenyl-2-propen-1-one (20)

A mixture of the acetophenone (1 equiv) and thiophene-2-carbaldehyde (1 equiv) in anhydrous EtOH (70 mL/23 mmol of acetophenone) was stirred at room temperature during 5 min. Then, NaOH (3 equiv) was added and the mixture stirred at room temperature until aldehyde consumption in the reaction. After that, HCl (10%) was added to attain neutrality. The precipitate was filtered out and the resulting solid re-crystallized from MeOH as yellow crystals, mp 98–100 °C in accordance with reported data.[15]

4.1.2. X-ray diffraction studies

The crystal structure of compound **20** ($C_{10}H_{10}O_2S$) was determined by X-ray diffraction methods. Bragg's reflections were collected on an Enraf-Nonius CAD4 diffractometer with EXPRESS,[19b] reduced with XCAD4[19c] and corrected numerically for absorption with PLATON.[19d] The structures were solved by direct and Fourier methods with SHELXS[19e] and its non-H atom refined by anisotropic full-matrix least-squares with SHELXL.[19f] The hydrogen atoms were positioned stereo-chemically and refined isotopically with the riding model. The structure was deposited with the Cambridge Crystallographic Data Centre reference number CCDC 734861.

4.1.3. Crystallographic structural results

Compound **20** crystallized in the monoclinic $P2_12_12_1$ space group with $a = 4.966(1)$, $b = 11.380(2)$, $c = 19.367(2)$ Å and $Z = 4$ molecules per unit cell. The structure was solved from 774 reflections with $I > 2\alpha(I)$ and refined to an agreement R1-factor of 0.046. The molecule is planar [rms deviation of atoms from the least-squares plane of 0.0215 Å], a conformation stabilized by a strong intra-molecular O1-H···O2 bond [d(H···O) = 1.748 Å, ∠(O-H···O) = 151.8°].

4.2. Biology

4.2.1. Analysis of QR, GST and CYP activities in rat liver

QR activity was measured by determining the decrement in the absorbance at 600 nm of the substrate DCPIP (2,6-dichlorophenolindophenol). The reaction mixture has 25 mM Tris–HCl buffer pH 7.4, 6% w/v bovine seroalbumin (BSA), 0.01% v/v Tween 20, 5 μM FAD, 0.2 mM NADH and 80 μM DCPIP.

To determine **GST** activity, 1 mM CDNB (1-chloro-2,4-dinitrobenzene) and 1 mM GSH (reduced glutathione) were used as substrates. The increase in the absorbance at 340 nm of the conjugated CDNB-GSH was measured. The reaction took place in 100 mM phosphate buffer at pH 6.5. To assay **CYP1A1**, EROD activity was measured as the fluorescence increment produced by the release of the fluorophore resorufine from the 7-etoxyresorufine used as substrate. The reaction was done in phosphate buffer100 mM, pH 7.6 containing 250 μM NADPH.

4.2.2. Alkaline comet assay

4.2.2.1. Culture of HK-2 cells. HK-2 cells were routinely cultured in DMEM supplemented with 10% FBS (100 g/mL) and 1% of penicillin/streptomycin in a humidified atmosphere of 5% CO_2 in air at 37 °C.

4.2.2.2. Sample preparation. From a stock solution in DMSO. Then, the flavonoids were diluted in serum free medium to obtain a final concentration of 20 μM.

4.2.2.3. Single cell gel electrophoresis (the comet assay). Monolayer HK-2 cells in exponential growth were trypsinized and cell cultures were prepared in a six-well plate: 2.5×10^6 cells/mL in 2 mL of DMEM containing 10% FBS and 1% of penicillin/streptomycin. The cells were exposed to the flavonoid (20 μM in DMSO) for 6 h at 37 °C in 5% CO_2 of air. Control cultures received the equivalent concentration of DMSO to a maximum of 1% of the culture medium. The cultures were washed twice, recovered using trypsin/EDTA and then spun at 200×*g* at 4 °C for 3 min. The pellet (approx. 4.5×10^4 cells) was suspended in LMP agarose for comet analysis. Cells embedded in agarose were lysed with high concentrated salt and detergent to obtain the DNA as a distinct 'nucleoid'. DNA was allowed to unwind under alkaline conditions. Breaks in the DNA molecule disrupt its complex super-coiling allowing free DNA loops to migrate toward the anode during electrophoresis. DNA damage to the cells can thus be visualized as 'comets'. Cells were suspended in LMP agarose [80 mL of a 1% (w/v) solution in PBS] at 37 °C and pipetted onto a frosted glass microscope slide pre-coated with a similar solution and buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8, 4 °C). The slides were held in an electrophoresis tank (260 mm wide) containing buffer (1 mM Na_2EDTA, 0.3 M NaOH, pH 12.7 at 4 °C) for 40 min before electrophoresis at 25 V for 30 min. The slides were neutralized by washing three times for 5 min each in buffer (0.4 M Tris–HCl, pH 7.5, 4 °C) and then stained with DAPI (20 mL of a 5 mg/mL stock solution). DAPI-stained nuclei were evaluated with a Nikon Eclipse TE 300 fluorescence microscope. A total of 100 comets on each comet slide were scored visually and classified as belonging to one of five classes according to the tail intensity. Each experiment was carried out in duplicate and each experiment repeated at least twice. DNA damage therefore extends from 0 to 400 arbitrary units. To check the performance of the comet assay, a positive control was included in all the experiments: HK-2 cells were treated with 50 μM hydrogen peroxide during 15 min on ice.

4.2.3. Mutagenicity assay

The *Salmonella* mutagenicity assay (Ames assay) was performed according to the method described previously.[24] Firstly, we determined the minimum toxic doses (MTD, μg/plate) of the studied compounds against the bacteria (*S. typhimurium* His^-, TA98 strain). The determined MTD was selected as the maximum doses to be assayed in the Ames test. For the test, suspension of 2×10^9 *S. typhimurium*/mL was prepared and the pre-incubation procedure was performed by mixing 50 μL of each test substance solution with 500 μL of phosphate buffer (0.1 M, pH 7.4) and 100 μL of bacteria suspension. Four to six consecutive dose levels were studied beginning with the corresponding MTD and decreasing one third successively (MTD/3, MTD/9, MTD/27, etc.). After 60 min of incubation, 2 mL of molten top agar supplemented with traces of histine and biotine (50 μM each, final concentration) were added, rapidly vortexed and poured on agar plates. As the top agar hardened, plates were inverted and incubated for 48 h, at 37 °C. Controls were tested in duplicate plates for each assay. A solvent control treated with DMSO and a positive control, 4-NPD (20 μg/plate), were always included. After 48 h incubation at 37 °C the revertant colonies were counted. For all the assays the data were analysed using the modified twofold rule,[25] in which a response is considered positive if the average response for at least two consecutive dose levels was more than twice the spontaneous frequencies, and also subjected to multifactor variance analysis.

4.2.4. In vitro metabolic studies

4.2.4.1. Preparation of the rat liver microsomal and cytosolic proteins. Livers were obtained from female Wistar rats (250–300 g), provided by the 'Centro de Investigaciones Nucleares, UdelaR' (Montevideo, Uruguay). The animals were allowed food and water *ad libitum*. The experimental protocols with animals were evaluated and supervised by the local Ethics Committee and the research adhered to the Principles of Laboratory Animal Care.[29] The animals were sacrificed by cervical dislocation and the livers, maintained in a ice bath, were perfused in situ with an ice-cold NaCl (0.9%) solution and washed with three volumes of Tris–HCl (0.05 M)-sucrose (0.25 M) pH 7.4, then they were sliced and homogenised in a Potter-Elvehjem glass-Teflon homogeniser. The homogenates were centrifuged for 30 min at 900 g at 4 °C and the supernatant fraction was centrifuged at 10,000×*g* for 1 h at 4 °C. The pellet was discarded and the supernatant fraction was further centrifuged at 100,000×*g* for 1 h at 4 °C. The cytosolic and microsomal fractions, supernatant and pellet, respectively, were recovered. The pellet was washed twice by re-suspension in the above Tris–HCl buffer solution, re-sedimented by centrifugation for 1 h at 100,000×*g* at 4 °C and finally re-suspended in the Tris–HCl buffer solution. Metabolic assays were carried out with microsomes and cytosol either fresh or frozen in Tris–HCl buffer and stored at −80 °C. Protein content of the microsomal and cytosolic fractions was determined by the bicinchoninic acid assay from Sigma as suggested by the manufacturer.

4.2.4.2. Incubation of flavonoids 1 and 8 with rat microsomal fraction and rat cytosolic fraction[30,31]. The procedure was adapted from published methods.[32] The standard incubation mixture contains $MgCl_2$ (1.3 mM), $NADP^+$ (0.4 mM), glucose 6-phosphate (3.5 mM), 0.5 U/mL glucose 6-phosphate dehydrogenase in a phosphate buffer (0.1 M, pH 7.4) containing EDTA (1.5 mM) and the corresponding flavonoid (400 μM, from stock solutions in DMSO) with 1 mL of final volume. The final concentration of the DMSO in the incubation mixture was below 1.0%. After pre-equilibration of the mixture at 37 °C, appropriate volumes of microsomal or cytosolic fraction were added to give a final protein concentration of 1 mg/mL. The mixtures were incubated for 0.5 h, in 24-well plates open to air, at 37 °C. The experiments were performed by triplicate. Two control incubations were done: (1) without β-nicotinamide adenine dinucleotide phosphate (NADPH)-generating system; (2) using the subcellular fractions inactivated by heating for 5 min at 95 °C. At the end of the incubation, 400 μL of methanol

was added and the mixture was kept at 4 °C for protein precipitation.

4.2.4.3. Chromatography and UV-spectroscopy for monitoring metabolites. The incubated mixtures were extracted with EtOAc (3 × 400 μL) and the organic layer was evaporated to dryness under reduced pressure. The residue was treated twice with acetonitrile (AcCN), 500 μL each, the combined organic layers were filtered through RC regenerated cellulose filters 0.45 μm pore size (Sartorius, Germany). The 100 μL aliquot of the obtained AcCN solution was analysed by TLC or by UV-spectroscopy with a Shimadzu UV-1603 UV–vis spectrophotometer.

4.2.5. In vivo tumourigenesis/chemopreventive studies

4.2.5.1. Formulation of drugs for in vivo trials. Flavonoids were suspended in sterile physiological saline/Tween 80 (4:1) solution (vehicle solution) immediately prior to oral administration. These preparations were made under aseptic conditions and in all cases homogeneous suspensions were obtained by shaking under ultrasound conditions.

4.2.5.2. Animals. The experiments were carried out on two month-old Sprague–Dawley female rat (200 g approximately) bred under specific pathogen-free conditions. Animals were housed in wire mesh cages at 20 ± 2 °C with natural light-dark cycles. The animals were fed 'ad libitum' to standard pellet diet and water and were used after a minimum of 3 days acclimation to the housing conditions.[33] Control and experimental group consisted of 5–7 animals. The experimental protocols with animals were evaluated and supervised by the local Ethics Committee and the research adhered to the Principles of Laboratory Animal Care.[29] Animals were evaluated by supervision of international protocols and they were sacrificed in a humane way in accordance with recognized guidelines on experimentation. At the end of experiments they were anaesthetised with ethyl ether and sacrificed by cervical dislocation.

4.2.5.3. Biological samples. For the in vivo studies, two kinds of biological samples were obtained. (1) Blood for biochemical and haematological studies was drawn by sectioning the subclavian artery and studied immediately or maintained in EDTA or heparin anticoagulant at 0 °C. The biochemical and haematological determinations were carried out no more than 24 h post extraction. (2) Organs (lung, kidney, liver, spleen, heart and intestine) were obtained by autopsy and maintained in aqueous formalin solution (10%) for further histological studies.

4.2.5.4. Treatment of healthy animals with higher doses than the established posological dose. The animals were treated orally with a unique dose of 250 mg/Kg. The oral administration was done via intragastric syringe (1.0 mL). Also, negative control, animals treated with vehicle, were included. The experiments lasted 3 days during which these the animals were daily weighted and observed for alterations in skin, physical aspect, activity and faeces aspect, also the microenvironment was examined. At the end of the experiments the animals were sacrificed and dissected and the organs and blood were submitted for further studies.

Acknowledgements

The authors thank the financial support of PDT 75-04. M.C. thanks to Fundación Carolina for a fellowship. O.E.P. is a Research Fellow of CONICET (Argentina). M.C. and M.L.L. are PEDECIBA students and they thank to PEDECIBA and ANII for scholarships.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.033.

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