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Xanthones as Inhibitors of Growth of Human Cancer Cell Lines and Their Effects on the Proliferation of Human Lymphocytes In Vitro

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Abstract—Twenty-seven oxygenated xanthones have been assessed for their capacity to inhibit in vitro the growth of three human cancer cell lines, MCF-7 (breast cancer), TK-10 (renal cancer) and UACC-62 (melanoma). The effect of these xanthones on the proliferation of human T-lymphocytes was also evaluated. Differences on their potency towards the effect on the growth of the human cancer cell lines as well as on the proliferation of human T-lymphocytes can be ascribed to the nature and positions of the substituents on the xanthonic nucleus.

Introduction

The xanthone structure is a very interesting scaffold for different groups in different positions leading to a large variety of pharmacological activities reported[1–15] namely inhibitors of growth of a variety of tumor cell lines[6–11] as well as modulators of PKC isoforms.[12] Although the anti-inflammatory activity of this group of compounds is documented,[13,14] little information has been accumulated about its interference with the effectors cells of the specific immunity.[15]

As xanthones from natural origin are relatively limited in type and position of the substituents imposed by the biosynthetic pathways, the syntheses of new compounds can afford to enlarge the possibilities of having different nature and positions of the substituents on the xanthonic nucleus that will allow us to rationalize and characterize the structure features that are important to their activity.

Based on these considerations we have synthesized a series of mono-, di- and trioxygenated simple xanthones and investigated their potential antitumor and immunomodulatory activity.

Consequently, this work reports the synthesis and the study of the in vitro effect of twenty seven xanthone derivatives on the growth of three human cancer cell lines, MCF-7 (breast cancer), TK-10 (renal cancer) and UACC-62 (melanoma) and on the mitogenic response of human lymphocytes to phytohemaglutinin (PHA). The aspect of structure–activity relationship within this class of compounds is also tentatively discussed.

Results and Discussion

Chemistry

Chemically xanthones (9*H*-xanthen-9-ones) are heterocyclic compounds with the dibenzo-γ-pyrone framework (Fig. 1). The xanthone nucleus is numbered according to a biosynthetic convention with carbons 1–4 being assigned to acetate-derived ring A and carbons 5–8 to the shikimate-derived ring B. The other carbons are indicated as 4a, 4b, 8a, 9 and 9a for structure elucidation purposes.

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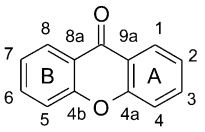


Figure 1. Nucleus of xanthone.

The standard methods for the synthesis of xanthones include the via benzophenone (**I**) and the via diaryl ether (**II**) intermediates (Scheme 1). In the Grover, Shah and Shah classic method the xanthones are obtained by condensation between an *ortho*-oxygenated benzoic acid and an activated phenol, in phosphorous oxychloride and zinc chloride (**a**).[16] The intermediate benzophenone derivatives (**I**) are also accessible through condensation by Friedel–Crafts acylation of appropriately substituted benzoyl chlorides with phenolic derivatives (**b**).[17] While the benzophenone method involves the cyclization of 2,2′-dioxygenated benzophenones (**I**) through a dehydrative or oxidative process (**c**),[17] the diaryl method (Ullman synthesis)[18] was carried out via suitable biphenyl ether intermediate (**II**) with the ring formation by one-step conversion by lithium diisopropylamide (**d**)[19] or by acetyl chloride (**e**).[20]

The compounds **2**, **6** and **24** were synthesized by via (**a**), while the compounds **3**, **4**, **7**, **8**, **13**, **14**, **17**, **18**, **20**–**23**, **25**–**27** were obtained by via (**b**). The diaryl process has led to the synthesis of the compounds **10** and **15** by via (**d**) and the compounds **5**, **9**, **12** and **16** by via (**e**), respectively (see Experimental protocols).

For compounds **10**, **12**, **13**, and **16** are indicated, for the first time, spectral data from INEPT, HETCOR, COSY or HMBC experiments in order to clarify the structural elucidation of these compounds.

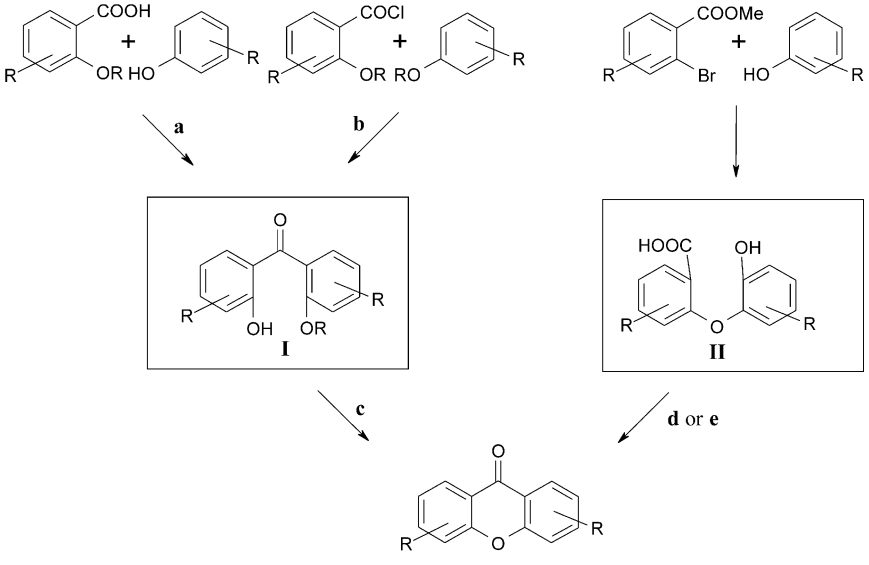
Effect on the growth of human cancer cell lines

The effects of the 27 xanthones on the growth of three human cancer cell lines, MCF-7, TK-10, UACC-62, given in concentrations that were able to cause 50% of cell growth inhibition (GI_{50}), are summarized in Table 1. The growth inhibition effects presented by these xanthones cannot be attributed to a toxic effect, as inferred from the sulforhodamine B (SRB) assay (data not shown).

No capacity to inhibit the growth of the human cancer cell lines was observed with xanthone (**1**) even when tested at concentrations higher than 200 μM, but the introduction of oxygenated groups in the xanthonic nucleus led, in general, to the appearance of a dose dependent growth inhibitory effect. Although this effect showed to be not very strong ($GI_{50} > 50$ μM) for the majority of the xanthone derivatives, some xanthones exhibited interesting growth inhibitory effects on the human cancer cell lines tested with GI_{50} values ≤ 20 μM. Although there was, in general, no discernible cell-type selectivity for the xanthones studied, it was found that 1,2-dihydroxyxanthone (**10**), 3,4-dihydroxyxanthone (**13**), and 2,3-dihydroxy-4-methoxyxanthone (**25**) were signifcantly ($p < 0.01$) much more active to the melanoma UACC-62 cell line than to MCF-7 and TK-10.

In the mono-oxygenated xanthones with substituents on C(2) or C(4) it is possible to observe drastic changes in the potency of the growth inhibitory activity: substituting OH by OCH_3 on C(2) leads to the appearance of activity while the opposite phenomenon occurs with the same change on C(4).

The nature of the substituents also seems to influence the growth inhibitory effect of the dioxygenated xanthones. The dihydroxylation at 1,2 (**10**), 2,3 (**12**), 3,4 (**13**), and 3,5 (**14**) positions of the xanthonic nucleus was associated with compounds that showed growth inhibitory effects that were significantly ($p < 0.05$) stronger to all the three cell lines than those presented by 1,2- (**15**), 2,3- (**16**), 3,4- (**17**), and 3,5-dimethoxyxantones (**18**). Similarly, the hydroxy and methoxy substituents at 2,1 (**19**), 4,3 (**22**), and 5,3 (**23**) positions were associated with a



Scheme 1. General methods for the synthesis of xanthones.

Table 1. Effects of xanthones on the growth of human cancer cell lines and proliferation of human lymphocytes

No. Compounds	GI_{50} (μM)[a]			IC_{50} (μM)[a]
	MCF-7[b] (breast cancer)	TK-10[b] (renal cancer)	UACC-62[b] (melanoma)	Human lymphocytes[c]
1 Xanthone	>200	>200	>200	>200
2 1-Hydroxy	>200	69.3±10.9	142.9±20.1	125.7±21.7
3 2-Hydroxy	>200	>200	>200	>200
4 3-Hydroxy	125.5±23.8	125.8±7.9	117.9±9.4	>200
5 4-Hydroxy	80.2±9.2	53.8±5.0	66.0±2.7	120.8±12.1
6 1-Methoxy	94.4±8.2	106.2±10.2	78.1±14.5	180.5±34.8
7 2-Methoxy	87.4±8.5	118.7±14.7	59.0±8.2	142.5±23.2
8 3-Methoxy	>200	94.4±14.7	73.8±12.1	53.5±5.1
9 4-Methoxy	>200	>200	>200	>200
10 1,2-Dihydroxy	38.4±2.7	65.8±5.1	14.0±0.3	73.3±2.2
11 1,7-Dihydroxy	57.1±11.0	60.1±9.6	51.3±9.6	20.2±0.2
12 2,3-Dihydroxy	40.6±1.3	61.4±4.3	31.7±3.7	31.3±1.5
13 3,4-Dihydroxy	40.5±1.5	59.2[a]	21.6±2.6	12.2±1.3
14 3,5-Dihydroxy	146.5±13.1	101.6±9.1	77.5±9.8	180.2±5.1
15 1,2-Dimethoxy	>200	>200	136.7±16.6	>200
16 2,3-Dimethoxy	174.1±13.8	174.1±14.1	125.9±21.1	59.9±18.5
17 3,4-Dimethoxy	>200	130.1±11.3	162.1±22.2	42.1±6.3
18 3,5-Dimethoxy	>200	>200	>200	>200
19 2-Hydroxy-1-methoxy	24.1±3.0	35.2±7.6	39.3±10.6	11.6±0.8
20 3-Hydroxy-4-methoxy	>200	>200	>200	178.5±8.9
21 3-Hydroxy-5-methoxy	>200	49.6±16.8	36.9±20.2	>200
22 4-Hydroxy-3-methoxy	108.8±22.7	72.7±9.2	78.5±11.9	92.8±11.8
23 5-Hydroxy-3-methoxy	66.1±12.6	30.9±2.3	37.7±6.7	111.2±8.3
24 1,3-Dihydroxy-2-methyl	21.9±0.4	34.3±3.8	20.0±0.5	84.4±12.4
25 2,3-Dihydroxy-4-methoxy	37.2[d]	76.6±7.0	19.8±1.6	17.4±2.7
26 1-Formyl-4-hydroxy-3-methoxy	75.3±8.6	73.1±9.0	66.7±8.5	80.7±11.7
27 2-Formyl-3-hydroxy-4-methoxy	82.7 ±14.8	128.1±14.3	59.3±7.5	19.2±3.3

[a]Results are expressed as GI_{50} (concentrations of compounds that cause 50% inhibition of cancer cell growth) or IC_{50} (concentrations that cause 50% inhibition of lymphocytes proliferation) and show means±SEM of 3–6 independent observations performed in duplicate.
[b]Doxorubicin was used as positive control in cancer cell lines growth (GI_{50} MCF-7 = 42.8±8.2 nM; GI_{50} TK-10 = 548.0±60.0 nM; GI_{50} UACC-62 = 94.0±9.4 nM).
[c]Cyclosporin A was used as positive control in lymphocytes proliferation (IC_{50} = 0.34±0.04 μM).
[d]Data based on two independently run duplicate experiments.

significant ($p<0.05$) increase of activity when compared with dimethoxyderivatives (**15**, **17**, **18**) in the same positions. Interestingly the hydroxy-methoxylation at 3,4 (**20**) caused a complete loss of activity of the compound. The improvement of the activity from 3-hydroxy-4-methoxyxanthone (**20**) to 3,4-dihydroxyxantone (**13**) is also remarkable especially for UACC cell line. When 4-hydroxy-3-methoxyxanthone (**22**) and 3-hydroxy-4-methoxyxanthone (**20**) were compared for their activity, it was observed that the shift of the substituents lead to a complete lost of activity in all the cell lines.

In the case of the trisubstituted xanthones tested, the number of compounds is not sufficient to draw any conclusion. However, it is interesting to point out that 1,3-dihydroxy-2-methylxanthone (**24**) was found to have the best inhibitory activity to all the three cell lines.

Effect on human lymphocytes proliferation

The effects of the 27 xanthones against the proliferation of human lymphocytes to PHA, given in concentrations that were able to cause 50% inhibition of proliferation (IC_{50}), are also shown in Table 1.

Xanthone (**1**) showed to be devoid of activity. But the mono-, di- and tri-substitution of the xanthonic nucleus was shown to be, in general, associated with the appearance of a dose-dependent antiproliferative effect. Although the majority of the xanthone derivatives have exhibited a weak inhibitory effect ($IC_{50}>50$ μM), some oxygenated xanthones, namely 1,7-dihydroxyxanthone (**11**), 3,4-dihydroxyxanthone (**13**), 2-hydroxy-1-methoxyxanthone (**19**), 2,3-dihydroxy-4-methoxyxanthone (**25**), and 2-formyl-3-hydroxy-4-methoxyxanthone (**27**) were found to possess a pronounced inhibitory activity on the mitogenic response of human lymphocytes to PHA ($IC_{50}\leq 20$ μM). No lymphocytoxicity was observed when the human lymphocytes were exposed to the IC_{50} concentrations of these xanthones (cell viability >70%), which leads to the conclusion that their inhibitory activity was associated with cell proliferation rather than to a toxic effect (data not shown).

Table 1 showed that the mono-oxygenated xanthones exhibited in generally weaker antiproliferative effect on T-lymphocytes than di- and trioxygenated xanthones. However, there are interesting structural features within the mono-oxygenated xanthones on their activity.

The nature of the substituents on C(**2**) and C(**4**) of the xanthonic nucleus showed to be very decisive for the antiproliferative effect of these compounds. Thus, 2-hydroxyxanthone (**3**) and 3-hydroxyxanthone (**4**) which OH groups on C(**2**) and C(**3**) were inactive while the presence of OCH3 group in the same positions causes the appearance of the activity in 2-methoxy (**7**) and 3-methoxyxanthone (**8**). In contrast, the activity was lost when substituting OH group on C(**4**) in 4-hydroxy xanthone (**5**) by OCH3 in 4-methoxyxanthone (**9**).

In the dioxygenated xanthones, the nature and positions of the substituents also played an important role in their activity. Thus 1,2-dihydroxyxanthone (**10**) ($IC_{50} = 73.3$ μM) is more than 6-fold less active than 2-hydroxy-1-methoxyxanthone (**19**) ($IC_{50} = 11.6$ μM) when OH was replaced by OCH3 on C(1) ($p < 0.01$). Interestingly, the complete lost of activity was observed when both OH groups were replaced by OCH3 in 1,2-dimethoxy derivative (**15**). The effects of OH and OCH3 substituents were also observed for activity of the xanthones with these substituents on the positions 3,4. As a result, 3,4-dihydroxyxanthone (**13**) ($IC_{50} = 12.2$ μM) was more active than 3,4-dimethoxyxanthone (**17**) ($IC_{50} = 42.1$ μM) and even more than 3-hydroxy-4-methoxyxanthone (**20**) ($IC_{50} = 178.5$), respectively.

Two of the trisubstituted xanthones 2,3-dihydroxy-4-methoxyxanthone (**25**) and the 2-formyl-3-hydroxy-4-methoxyxanthone (**27**) were found to be the most active. The presence of OH or CHO on C(2) seemed to be responsible for the ten-fold increase of the antiproliferative activity of these compounds when compared to 3-hydroxy-4-methoxyxanthone (**20**).

Conclusion

In summary, from all these results it can be inferred that the effects of oxygenated xanthones on the growth of the human cancer cell lines as well as on the proliferation of human T-lymphocytes can be ascribed to the nature, positions and number of substituents on the xanthonic nucleus.

Concerning the effect on the growth of human cancer cell lines, 1,3-dihydroxy-2-methylxanthone (**24**) was found to have the best inhibitory activity to all the three cell lines, while some selectivity for UACC-62 cell line was found for 1,2-dihydroxy (**10**), 3,4-dihydroxy (**13**), and 2,3-dihydroxy-4-methoxyxanthones (**25**).

The dihydroxylation at all the positions of the xanthone derivatives tested was associated with stronger inhibition to all the three cell lines than those presented by the corresponding dimethoxy analogues.

For the effect on human lymphocytes proliferation the dioxygenated derivatives 2-hydroxy-1-methoxyxanthone (**19**) and 3,4-dihydroxyxanthone (**13**) showed the most interesting results for the inhibitory response.

Based on the described results further modifications are underway to improve the activity of these compounds.

Experimental

Chemistry

Purifications of compounds were performed by column chromatography (CC) using Merck silica gel 60 (0.50–0.20 mm), flash chromatography using Merck silica gel 60 (0.040–0.063 mm) and preparative thin layer chromatography (TLC) using Merck silica gel 60 (GF_{254}). Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were recorded on a Perkin–Elmer 257 in KBr. ^1H and ^{13}C NMR spectra were taken in $CDCl_3$ or DMSO-d_6 at room temperature, on Bruker AC 200, DRX 300 and DRX 500 instruments. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. MS spectra were recorded as EI (electronic impact) mode on a Hitachi Perkin–Elmer. Xanthone was purchased from Sigma Chemical Co., St. Louis, USA.

The following xanthone derivatives were synthesized and purified by the described procedures.

The synthesis of the following xanthones has already been described according to the references cited below.

1-Hydroxyxanthone (**2**) (48%), 2-hydroxyxanthone (**3**) (74%), 3-hydroxyxanthone (**4**) (92%), 4-hydroxyxanthone (**5**) (59%), 1-methoxyxanthone (**6**) (86%), 2-methoxyxanthone (**7**) (66%), 3-methoxyxanthone (**8**) (45%), 4-methoxyxanthone (**9**) (14%), 3,4-dimethoxyxanthone (**17**) (61%), 3,5-dihydroxyxanthone (**14**) (55%), 3,5-dimethoxyxanthone (**18**) (35%), 3-hydroxy-5-methoxyxanthone (**21**) (42%), 3-hydroxy-4-methoxyxanthone (**20**) (51%) and 4-hydroxy-3-methoxyxanthone (**22**) (39%) were obtained according to the described procedure.[4] 1,2-Dimethoxyxanthone (**15**) (74%) was obtained[21] and characterized[21,22] according to the described procedures.

2-Hydroxy-1-methoxyxanthone (19) and 1,7-dihydroxyxanthone (11). These xanthones 2-hydroxy-1-methoxyxanthone (**19**) and 1,7-dihydroxyxanthone (**11**) were isolated from *Calophyllum teysmannii* var. *inophylloide* (Guttiferae)[15] and *Cratoxylum maingayi* (Guttiferae),[23] respectively.

1,2-Dihydroxyxanthone (10). The compound **10** was obtained by demethylation of 1,2-dimethoxyxanthone (**15**) according to the described procedure (48%).[24,25] Mp 163–165 °C, methylene chloride/hexane (lit.,[26] 166–167 °C, ethanol aq); UV (EtOH): 261, 243, 232, 203 (33,300, 28,800, 14,200, 11,000); UV (EtOH + NaOH): 311, 236, 222 (76,000, 23,900, 17,000); UV (EtOH + $AlCl_3$): 305, 283, 266, 204 (33,000, 22,900, 87,00); IR (KBr) $\upsilon_{máx}$: 3467, 1606, 1475, 1365, 1288, 1045, 748 cm^{-1}. ^1H NMR (DMSO-d_6, 300.13 MHz) δ:[27,28] 12.45 (1-O*H*), 9.42 (2-O*H*), 8.16 (1H, dd, $J = 7.9$, and 1.6 Hz, H-8), 7.81 (1H, ddd, $J = 8.4$, 7.0, and 1.6 Hz, H-6), 7.60 (1H, dd, $J = 8.4$, and 0.8 Hz, H-5) 7.46 (1H, ddd, $J = 7.9$, 7.0, and 0.8 Hz, H-7), 7.32 (1H, d, $J = 9.0$ Hz, H-4), 6.96 (1H, d, $J = 9.0$ Hz, H-3); ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 182.3 (C-9), 155.9 (C-4b), 148.3 (C-4a), 147.6 (C-1), 140.2 (C-2), 136.4 (C-6), 125.4 (C-8), 124.5 (C-3), 124.2 (C-7), 119.3 (C-8a), 118.0 (C-5), 108.8 (C-9a), 106.2

(C-4); EIMS, *m/z* (% rel. int.): 230 (1, $[M+2]^+$), 229 (1, $[M+1]^+$), 228 (70, $[M^+]$), 199 (13), 126 (5), 115 (4), 78 (95), 63 (100).

2,3-Dihydroxyxanthone (12). The compound **12** was obtained by demethylation of 2,3-dimethoxyxanthone (**17**) according to the described procedure (91%).[25] Mp >330 °C, acetone/hexane (lit.[25] >350 °C ethyl acetate, lit.[29] 293–295 °C chloroform/methanol); UV, IR and EIMS data accord with lit.[29] The unequivocal assignments of carbon resonances, mainly by using 1D selective INEPT and 2D HETCOR experiments, allowed us to correct some ^{1}H and ^{13}C resonances of **12**,[29] namely the assignment of the proton resonances of H-6 to 7.78 (1H, ddd, $J=8.4$, 6.8, and 1.7 Hz) and H-5 to 7.58 (1H, d, $J=8.4$ Hz), and the carbon resonances of C-2 (144.1), C-3 (151.3), and C-4a (154.2).

3,4-Dihydroxyxanthone (13).[25] (78%) Mp above 330 °C (lit.,[16] 240–241 °C, ethanol aq; lit.,[29] 238–240 °C, methanol); UV, IR and EIMS data accord with lit.[29] the analysis of the ^{1}H, ^{13}C, COSY, HETCOR and HMBC spectra of **12** permits correction of the literature[29] assignment of the proton resonances of H-6 and H-5 to 7.81 (1H, ddd, $J=8.6$, 6.9, and 1.7 Hz, H-6), and 7.62 (1H, dd, $J=8.6$, and 0.9 Hz, H-5).

2,3-Dimethoxyxanthone (16). The 2-(3′,4′-dimethoxyphenoxy) benzoic acid obtained by an Ullman reaction[18] underwent an appropriate cyclization to **16** according to the described procedure (92%).[20,30] Mp 170–171 °C, methylene chloride/pentane (lit.,[20] 164–165 °C, acetone; lit.,[29] 157–158 °C, chloroform; lit.,[31] 155–159 °C, methylene chloride/heptane; lit.,[32] 165–167 °C, chloroform/petrol); IR and EIMS data accord with lit.[29] The unequivocal assignments of carbon resonances, mainly by using 1D selective INEPT and 2D HETCOR experiments, allowed us to correct some ^{1}H and ^{13}C resonances of **16**:[17,29] ^{1}H NMR (DMSO-d_6, 200.13 MHz) δ: 8.16 (1H, dd, $J=7.9$ and 1.5 Hz, H-8), 7.82 (1H, ddd, $J=8.4$, 7.4 and 1.5 Hz, H-6), 7.60 (1H, d, $J=8.4$ Hz, H-5), 7.49 (1H, s, H-1), 7.44 (1H, ddd, $J=7.9$, 7.4, and 0.9 Hz, H-7), 7.20 (1H, s, H-4), 3.94 (3H, s, 3-OCH_3), 3.87 (3H, s, 2-OCH_3); ^{13}C NMR (DMSO-d_6, 50.03 MHz) δ: 174.8 (C-9), 155.7 (C-4b), 155.6 (C-4a), 152.0 (C-3), 146.7 (C-2), 134.8 (C-6), 125.9 (C-8), 124.3 (C-7), 120.9 (C-8a), 118.0 (C-5), 114.0 (C-9a), 104.7 (C-1), 100.5 (C-4), 56.6 (3-OCH_3), 55.9 (2-OCH_3).

1,3-Dihydroxy-2-methylxanthone (24). The compound **24** was obtained (28%) and characterized according to the described procedures.[33,34]

2,3-Dihydroxy-4-methoxyxanthone (25), 1-formyl-4-hydroxy-3-methoxyxanthone (26), 2-formyl-3-hydroxy-4-methoxyxanthone (27). The compounds **25** (74%), **26** (36%) and **27** (28%) were obtained according to the described procedure.[34]

Pharmacology

Fetal bovine serum and RPMI-1640 medium were obtained from Gibco BRL. Cyclosporin A, *N,N*-dimethylformamide (DMF), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), doxorubicin hydrochloride, gentamicin, L-glutamine, Histopaque-1077, phytohemagglutinin (PHA), sodium lauryl sulfate (SDS), and sulforhodamine B (SRB) were purchased from Sigma Chemical Co. National Cancer Institute (Bethesda, MD, USA) kindly provided the tumor cell lines.

Stock solutions of xanthones were prepared in DMSO and stored at −20 °C providing uniform samples for re-tests. These frozen concentrates were then diluted to the desired final concentrations with the appropriate solvents immediately prior the biological assays. Final concentrations of DMSO showed no interference with the biological activities tested.

Cell growth assay. The effects of compounds on the growth of human cancer cell lines were evaluated according to the procedure adopted in the NCI's in vitro anticancer drug screening that uses the SRB assay to assess growth inhibition.[35] This colorimetric assay estimates cell number indirectly by staining cellular protein with the protein-binding dye SRB. Three human tumor cell lines were used, MCF-7 (breast cancer), TK-10 (renal cancer), UACC-62 (melanoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 5% heat-inactivated fetal bovine serum, 2 mM glutamine and 50 μg/mL gentamicin at 37 °C in a humidified air incubator containing 5% CO_2. Each cell line was plated at an apropriate density (1.5×10^5 cells/mL to MCF-7 and TK-10, 1.0×10^5 cells/mL to UACC-62) in 96-well plates and allowed to attach overnight. Cells were then exposed for 48 h to serial concentrations of compounds and to the positive control, doxorubicin. Following this incubation period, the adherent cells were fixed in situ washed and dyed with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a microplate reader. Growth inhibition of 50% (GI_{50}) was calculated as described elsewhere.[36] Toxicity of xanthones was inferred from the SRB assay by comparing, after 48 h, the absorbance of the wells with xanthone-treated cells with the absorvance of the wells containing untreated cells that were fixed at time zero (time at which compounds were added). Lower absorbances after 48 h of treatment indicate occurrence of cell death instead of growth arrest.

Lymphocyte assays

The effects of xanthones on the mitogenic response of human lymphocytes to PHA (10 μg/mL) were evaluated using a modified colorimetric MTT assay.[37] This assay was previously described by us.[15] Mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers by Histopaque-1077 density centrifugation. The toxicity of xanthones against human lymphocytes was evaluated by an assay based on the ability of viable cells to reduce the colorless tetrazolium salt MTT to a colored formazan product. Briefly, in flat-bottom 96-well plates, $2–3\times10^6$ mononuclear cells/mL in RPMI-1640 medium containing 10% heat-inactivated

fetal bovine serum, 2 mM glutamine and 50 μg/mL of gentamicin were exposed for 24 h to the various concentrations of each sample. Following this incubation period the MTT solution (1 mg/mL) was added. After incubation for 4 h the MTT formazan products were solubilized with SDS/DMF solution (20% SDS in a 50% solution of DMF, pH 4.7) overnight at 37 °C. Absorbance (OD 550 nm) of the colored solution was measured with a plate reader. Lymphocytoxicity, determined in terms of the percentage of viable cells, was present when the viability of the exposed cells was less than 70% of the non-exposed control cells.

Statistics. Unpaired Student's *t*-tests were used. Differences with *p* values below 0.01 or 0.05 were considered statistically significant. Whenever $GI_{50} > 200$ μM, values of 200 μM were considered to evaluate the statistical significance of differences.

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