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Original article

Synthesis of new chiral xanthone derivatives acting as nerve conduction blockers in the rat sciatic nerve

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ABSTRACT

The synthesis and structure elucidation of three new chiral xanthone (9*H*-xanthon-9-one) derivatives (**2**–**4**) are fully reported. The coupling reactions of the synthesized building block 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylic acid (**1**) with two enantiomerically pure amino alcohols ((*S*)-(+)-valinol and (*S*)-(+)-leucinol) and one amine ((*S*)-(−)-α-4-dimethylbenzylamine), were carried out using the coupling reagent *O*-(benzotriazol-1-yl-)-*N*,*N*,*N*′,*N*′-tetramethylluronium tetrafluoroborate (TBTU). The coupling reactions were performed with yields higher than 97% and enantiomeric excess higher than 99%. The structures of the compounds were established by IR, MS, and NMR (^{1}H, ^{13}C, HSQC, and HMBC) techniques. Taking into account that these new chiral xanthone derivatives have molecular moieties structurally very similar to local anaesthetics, the ability to block compound action potentials (CAP) at the isolated rat sciatic nerve was also investigated. Nerve conduction blockade might result from a selective interference with Na^{+} ionic currents or from a non-selective modification of membrane stabilizing properties. Thus, the mechanism, by which the three chiral xanthone derivatives cause conduction blockade in the rat sciatic nerve and their ability to prevent hypotonic haemolysis, given that erythrocytes are non-excitable cells devoid of voltage-gated Na^{+} channels, are also described. Data suggest that nerve conduction blockade caused by newly-synthesized xanthone derivatives might result predominantly from an action on Na^{+} ionic currents. This effect can be dissociated from their ability to stabilize cell membranes, which became apparent only upon increasing the concentration of compounds **2**–**4** to the higher micromolar range.

1. Introduction

Chiral compounds represent almost two-third of all drug sales worldwide and are of great importance in Medicinal Chemistry and in drug discovery [1]. According to a recently survey, the three top-selling drugs for 2008 and 2009, namely Lipitor™ (atorvastatin calcium), Plavix™ (clopidogrel bisulfate) and Nexium™ (esomeprazole magnesium), are single enantiomeric drugs [2,3]. The differences normally exhibited by the enantiomers in pharmacodynamics, pharmacokinetics and toxicity, makes the therapeutics with single enantiomers, comparing with racemates, of unquestionable advantages [4,5]. Therefore, regulatory authorities recommend that chiral drugs should be marketed as pure enantiomers [6,7]. Consequently, the development of efficient methodologies for synthesis of chiral compounds in high yields and with high enantiomeric purity is becoming one of the most important tasks in the field of Chemistry.

Xanthone (9*H*-xanthon-9-one) derivatives have emerged as a class of compounds with increasing interest, possessing a broad spectrum of biological and pharmacological activities [8] and many have proved to be important building blocks for the synthesis of new interesting compounds [9–13]. Despite the large structural multiplicity of bioactive xanthonic compounds only a handful of

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synthetic chiral derivatives have been reported to date [14–23]. Some chiral xanthone derivatives (CXDs) exhibit heterogeneous activities at the central nervous system (CNS). These compounds can either stimulate neuronal activity, acting as analeptics, or decrease nerve firing like antiepileptic drugs [17]. CXDs have also revealed antiarrhythmic [16,18], antitumoral [14,19], anticonvulsant [15,18,20,21], local anaesthetic [22], antifungal and antibacterial [23] effects.

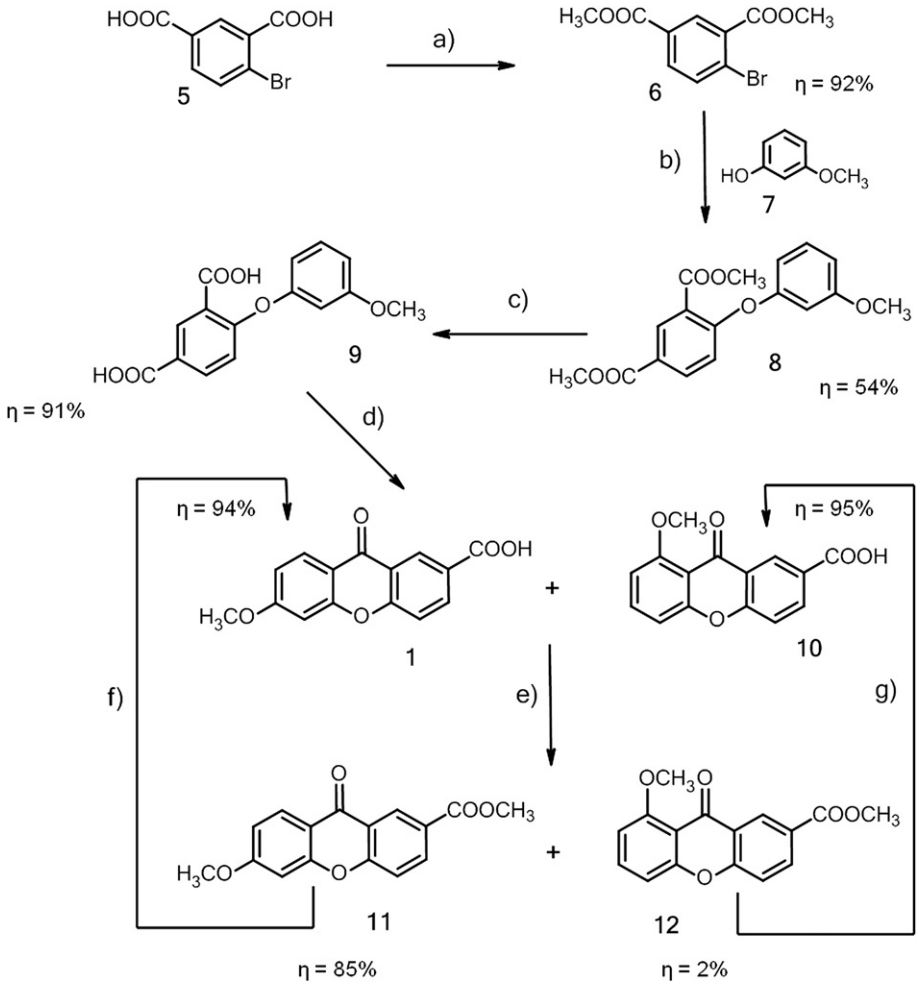
By inhibiting Ca^{2+} influx through receptor-operated and/or voltage-sensitive channels, some of these compounds have been shown to cause antihypertensive and vasorelaxant effects [24]. The reported mechanisms of action are frequently associated with enantioselectivity.

In this paper, we report the synthesis and biological activity as nerve conduction blockers of three new CXDs, namely compounds (*S*)-*N*-(1-hydroxy-3-methylbutan-2-yl)-6-methoxy-9-oxo-9*H*-xanthene-2-carboxamide (**2**), (*S*)-*N*-(1-hydroxy-4-methylpentan-2-yl)-6-methoxy-9-oxo-9*H*-xanthene-2-carboxamide (**3**) and (*S*)-6-methoxy-9-oxo-*N*-(1-(*p*-tolyl)ethyl)-9*H*-xanthene-2-carboxamide (**4**), as well as the synthesis of the building block, 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylic acid (**1**).

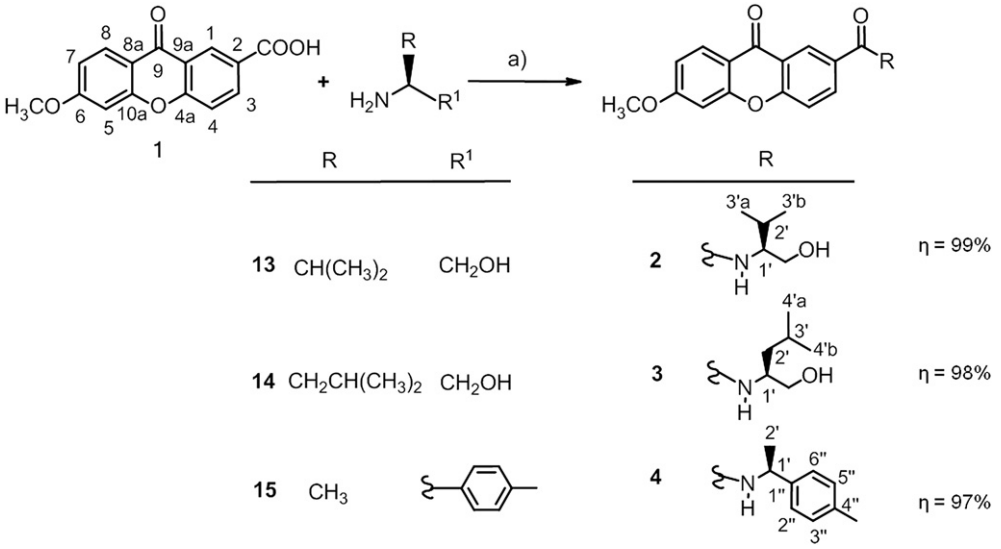
The total synthesis of 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylic acid (**1**) has already been accomplished by a multi-step pathway [25,26]. Herein, the synthetic approach to obtain the compound **1** (Scheme 1) was based on the previous procedure, however the methodologies were improved in order to decrease the reaction time and to improve the final yield. The compound **1** was then used as building block to synthesize CXDs **2**–**4**, by coupling reactions with (*S*)-(+)-valinol (**13**), (*S*)-(+)-leucinol (**14**) and (*S*)-(−)-α-dimethylbenzylamine (**15**), respectively. The reactions were performed with the coupling reagent *O*-(benzotriazol-1-yl-)-*N*,*N*,*N*′,*N*′-tetramethylluronium tetrafluoroborate (TBTU) based on previously reports (Scheme 2) [27,28].

During the last few years, a huge number of coupling reagents has appeared in the literature, which has been widely used for the synthesis of peptides [29–31]. TBTU is one of the reagents that have become popular due to its higher efficiency and lower tendency towards racemization [27]. Thus, we used TBTU for the synthesis of the CXDs by coupling a suitable functionalized xanthonic building block (**1**) to different chiral ligands, in enantiomeric pure form.

The CXDs **2**–**4** (Scheme 2) are structurally similar to many local anaesthetics, namely dibucaine, mepivacaine, lidocaine or bupivacaine, with which they might share common molecular moieties, and, consequently, the same pharmacophore. Local anaesthetics are known to block the peripheral nerve conduction by inhibiting voltage-gated Na^+ channels [32]. The mechanisms of nerve conduction blockade caused by local anaesthetics can be differentiated according to their structure and the way they cross the cell membrane. The hydrophilic compounds diffuse to the axoplasm through opened Na^+ channel pores, whereas lipid soluble drugs



Scheme 1. Reagents and conditions: a) Methanol, H_2SO_4, reflux, 17 h; b) CuI, Cs_2CO_3, *N*,*N*-Dimethyl glycine, Dioxane, N_2, 90 °C, 14 h; c) Methanol/Tetrahydrofuran (1:1 v/v), 5 M NaOH, room temp., 18 h; d) P_2O_5, CH_3SO_3H, room temp., 22 h; e) Methanol, H_2SO_4, reflux, 19 h; f) Methanol/Dichloromethane (1:1 v/v), 5 M NaOH, room temp., 22 h; g) Methanol/Dichloromethane (1:1 v/v), 5 M NaOH, room temp., 22 h.



Scheme 2. Reagents and conditions: a) TBTU, triethylamine, dry tetrahydrofuran, room temp., 30 min–2 h (the numbering used concerns the NMR assignments).

can easily pass the nerve membrane and gain access to the Na^+ channels even if they are closed (hydrophobic pathway).

Due to the structure similarity between the three CXDs (**2**–**4**) and local anaesthetic drugs, these compounds can act putatively as modulators of Na^+ influx, either from selective interference with Na^+ ionic currents or from non-selective modifications of the membrane stabilizing properties. This prompted us to investigate the effect of these CXDs on the amplitude of compound action potentials (CAP) at the isolated rat sciatic nerve, as this electrophysiological parameter is positively correlated with Na^+ transport. We also evaluated the ability of compounds **2**–**4** to prevent hypotonic haemolysis, given that erythrocytes are non-excitable cells devoid of voltage-gated Na^+ channels.

2. Results and discussion

2.1. Chemical synthesis

2.1.1. Synthesis of building block *1*

Xanthone derivative **1** was synthesized, *via* Ullmann reaction, with the formation of diaryl ether intermediate from the aryl bromide **6** and the phenol **7** (Scheme 1). The aryl bromide **6** was previous prepared from the corresponding carboxylic acid **5** by Fisher esterification. The Ullmann condensation between **6** and **7** under the catalytic action of *N*,*N*-dimethyl glycine, CuI and Cs_2CO_3 in dioxane [33] afforded the diaryl ether **8**. This coupling reaction was carried out at 90 °C for 14 h to give the compound **8** in good yield (54%), instead of 115 °C, 24 h and 32%, respectively, used in the traditionally copper-catalyzed Ullmann reaction with pyridine and K_2CO_3 as described before [26]. The *N*,*N*-dimethyl glycine and the stronger base Cs_2CO_3 was preferred to promote Ullmann coupling reaction due to the lower reaction temperature, short reaction time and higher yield was achieved. The diaryl ether **8** was dissolved in a mixture of tetrahydrofuran/methanol and 5M NaOH solution to hydrolyze the methyl esters, providing the compound **9**. An intramolecular acylation of diaryl ether **9**, using phosphorus pentoxide and methane sulfonic acid at room temperature afford two compounds, the xanthone **1** and its isomer **10** in different yields. The separation of the two isomers was easily achieved through a Fisher esterification followed by filtration due to the great solubility difference in methanol. The compound **12** (described here for the first time) presented a higher solubility in methanol than the ester isomer **11**. After separation, compounds **11** and **12** were hydrolyzed in alkaline medium yielding the xanthone derivative **1** and its isomer **10**, respectively (Scheme 1).

2.1.2. Synthesis of chiral xanthone derivatives *2*–*4*

Chiral xanthone derivatives **2**–**4** were obtained by the coupling reaction of the building block **1** with (*S*)-(+)-valinol (**13**), (*S*)-(+)-leucinol (**14**) and (*S*)-(−)-α-dimethylbenzylamine (**15**), respectively, using TBTU and a catalytic amount of triethylamine in dry tetrahydrofuran (Scheme 2). Despite a high amount of coupling reagents described in the literature [29–31], TBTU is widely used due to its higher efficiency and lower tendency towards racemization [27]. Thus, we used TBTU for the synthesis of the CXDs by coupling the xanthone building block **1** to different chiral ligands, in enantiomeric pure form. These reactions were carried out at room temperature and showed high yields (above 97%), short reaction times (30 min–2 h) and no racemization were observed (enantiomeric excess higher than 99%). The purification procedures were easy, involving extraction followed by crystallization.

2.2. Structure elucidation

The structure of all synthesized compounds was established by IR, MS, and NMR techniques. Although the synthesis of compound **1** and its intermediates have already been described before [25,26] (except compounds **11** and **12**) their structural data were not reported.

The IR spectra of compounds **1**–**4** showed the presence of the absorption bands corresponding to the C=O (1652–1687 cm^{-1}) and to aromatic C=C (1575–1433 cm^{-1}) associated with the xanthone scaffold and a band at 1264–1272 cm^{-1} corresponding to the Ar–OCH_3 group. Comparing IR data for the xanthonic building block (**1**) and for the CXDs (**2**–**4**), the main changes are: the presence of two strong bands corresponding to the N–H (3371–3244 cm^{-1}) and C=O of amide (1611–1617 cm^{-1}) groups, as well as the absence of the broad band at 2901 cm^{-1} corresponding to the O–H carboxyl group.

The ^1H NMR and ^{13}C NMR data of CXDs (**2**–**4**) are reported in Tables 1 and 2, respectively. The NMR data of compound **1** are also included. Compounds **1**–**4** showed similar NMR profiles with respect to the six aromatic protons of the xanthone scaffold and to

Table 1
^{1}H NMR chemical shifts of building block **1** and of chiral xanthone derivatives **2**–**4**.

	1	2	3	4
H-1	8.68 (*d*, *J* = 3.2)	8.73 (*d*, *J* = 2.2)	8.72 (*d*, *J* = 2.2)	8.56 (*d*, *J* = 2.2)
H-3	8.29 (*dd*, *J* = 13.3, 3.2)	8.31 (*dd*, *J* = 8.7, 2.2)	8.30 (*dd*, *J* = 8.7, 2.2)	8.32 (*dd*, *J* = 8.7, 2.2)
H-4	7.69 (*d*, *J* = 13.3)	7.71 (*d*, *J* = 8.7)	7.70 (*d*, *J* = 8.7)	7.53 (*d*, *J* = 8.7)
H-5	7.19 (*d*, *J* = 3.3)	7.21 (*d*, *J* = 2.3)	7.21 (*d*, *J* = 2.3)	6.92 (*d*, *J* = 2.3)
H-7	7.07 (*dd*, *J* = 13.3, 3.3)	7.09 (*dd*, *J* = 8.7, 2.3)	7.10 (*dd*, *J* = 8.9, 2.3)	6.99 (*dd*, *J* = 8.9, 2.3)
H-8	8.09 (*d*, *J* = 13.3)	8.14 (*d*, *J* = 8.7)	8.14 (*d*, *J* = 8.9)	8.25 (*d*, *J* = 8.9)
OCH_3	3.93 (*s*)	3.95 (*s*)	3.95 (*s*)	3.93 (*s*)
NH	–	8.38 (*d*, *J* = 8.7)	8.40 (*d*, *J* = 8.7)	6.58 (*d*, *J* = 7.3)
OH	–	4.63 (*t*, *J* = 5.6)	4.75 (*t*, *J* = 5.7)	–
H-1′	–	3.85 (*m*)	4.11 (*m*)	5.33 (*m*)
H-2′	–	1.97 (*m*)	1.64 (*m*)	1.63 (*d*, *J* = 6.9)
H-3′	–	0.90, 0.93 (2 *d*, *J* = 7.0)	1.45 (*m*)	–
H-4′	–	–	0.90, 0.91 (2 *d*, *J* = 6.0)	–
CH_2OH	–	3.54 (*dd*, *J* = 10.4, 5.8)	3.43 (*dd*, *J* = 13.3, 5.3)	–
Ar–CH_3	–	–	–	2.34 (*s*)
H-2″, H-6″	–	–	–	7.31 (*d*, *J* = 8.0)
H-3″, H-5″	–	–	–	7.18 (*d*, *J* = 8.0)

the three protons of the methoxyl group, with similar chemical shifts and coupling constants.

The ^{1}H NMR spectra of all CXDs (**2**–**4**) had in common the presence of the signals corresponding to the proton of the amide group at 6.58–8.40 ppm. The ^{1}H NMR spectra of compounds **2** and **3** contained signals due to a hydroxyl group on the side-chain primary alcohol, at 4.63 and 4.75 ppm respectively, while the spectra of CXD **4** showed signals concerning the presence of a phenyl ring (side-chain). It is also important to point out the following chemical shifts, with respect to the side chain of the CXDs: 1.45–5.33 ppm for the proton of one (**4**) or two (**2** and **3**) methine groups, 1.64–3.54 ppm for the protons of one (**2**) or two (**3**) methylene groups, and 0.90–2.34 ppm for the protons of two (**2**–**4**) methyl groups.

The ^{13}C NMR spectra revealed signals for one (**1**) or two carbonyl groups (**2**–**4**), one methoxyl group (**1**–**4**), two (**1**–**3**) or three aromatic rings (**4**), one (**4**) or two (**2** and **3**) methine groups, one (**2**) or two (**3**) methylene groups and two (**2**–**4**) methyl groups.

Table 2
^{13}C NMR chemical shifts of building block **1** and of chiral xanthone derivatives **2**–**4**.

	1	2	3	4
C-1	124.9	125.4	125.3	124.0
C-2	127.6	130.9	130.7	130.1
C-3	135.7	134.1	134.0	134.3
C-4	116.7	118.0	118.1	118.5
C-5	100.7	100.8	100.8	100.3
C-6	164.9	165.1	164.7	164.8
C-7	113.7	114.0	114.0	113.8
C-8	126.6	127.7	127.7	128.3
C-9	175.3	174.9	174.9	175.9
C-4a	156.3	157.0	157.1	157.8
C-10a	157.6	157.6	157.5	158.0
C-8a	115.0	114.9	114.9	115.5
C-9a	120.2	120.6	120.7	121.0
$CONH_2$	–	167.7	165.2	165.5
COOH	167.6	–	–	–
Ar–OCH_3	56.2	57.0	56.3	55.9
C-1′	–	56.3	49.8	49.3
C-2′	–	28.7	31.9	21.0
C-3′	–	18.8, 19.7	21.9, 24.5	–
C-4′	–	–	23.5	–
CH_2OH	–	61.3	63.9	–
Ar–CH_3	–	–	–	29.7
C-1″	–	–	–	139.8
C-2″, C-6″	–	–	–	126.2
C-4″	–	–	–	137.2
C-3″, C-5″	–	–	–	129.4

For compounds **2**–**4** were provided spectral data from HSQC and HMBC experiments to clarify the structural elucidation of these compounds.

The MS spectra of all compounds showed the respective molecular ion peaks at the expected *m*/*z* values (see Experimental Section).

Thus, the spectral data confirm the success of the coupling of the xanthonic building block (**1**) with the three different chiral units in order to obtain the compounds **2**–**4**.

2.3. Biological activity studies

2.3.1. Chiral xanthone derivatives decrease sciatic nerve conduction

The synthesized CXDs (**2**–**4**) (Scheme 2) show chemical structures related to those found in local anaesthetics, which include an aromatic end (xanthone scaffold) and an amide linkage [34]. The absence of a tertiary amine is the most significant difference regarding the pharmacophoric descriptors for local anaesthetics. The main targets of local anaesthetic drugs are voltage-gated Na^+ channels. Therefore, compounds with anaesthetic-like properties should block the initiation and propagation of nerve action potential. This prompted us to investigate the effect of the newly-synthesized CXDs on the amplitude of compound action potentials (CAP) in the isolated rat sciatic nerve.

CXDs (**2**–**4**) concentration-dependently decrease the amplitude of CAP in the isolated rat sciatic nerve (Fig. 1). Compounds **2**–**4** and the xanthone itself were about equipotent. Nerve conduction blockade caused by CXDs was observed in the low micromolar range (0.1–3 μM), but their effects were hardly washable even when superfusion with the physiological salt solution was prolonged for several hours (data not shown). Blockade of nerve conduction by CXDs mimicked the effect of a prototypical local anaesthetic drug, dibucaine, when this compound was used in the same concentration range (0.1–3 μM) (Fig. 1A). Conversely, no significant effect on CAP amplitude was observed when the rat sciatic nerve was challenged with sucrose to increase the osmotic strength of the physiological salt solution to the levels comparable to the application of the CXDs (Fig. 1A). These results suggest that nerve conduction blockade caused by the newly-synthesized xanthone derivatives might result from an action on Na^+ ionic currents, acting in a similar manner to local anaesthetic drug. The local anaesthetic-like property was shared by all xanthonic compounds tested, yet a higher potency was expected for the CXDs (**2**–**4**) compared to the xanthone itself. The absence of the tertiary amine may contribute to nerve conduction blocking potency of

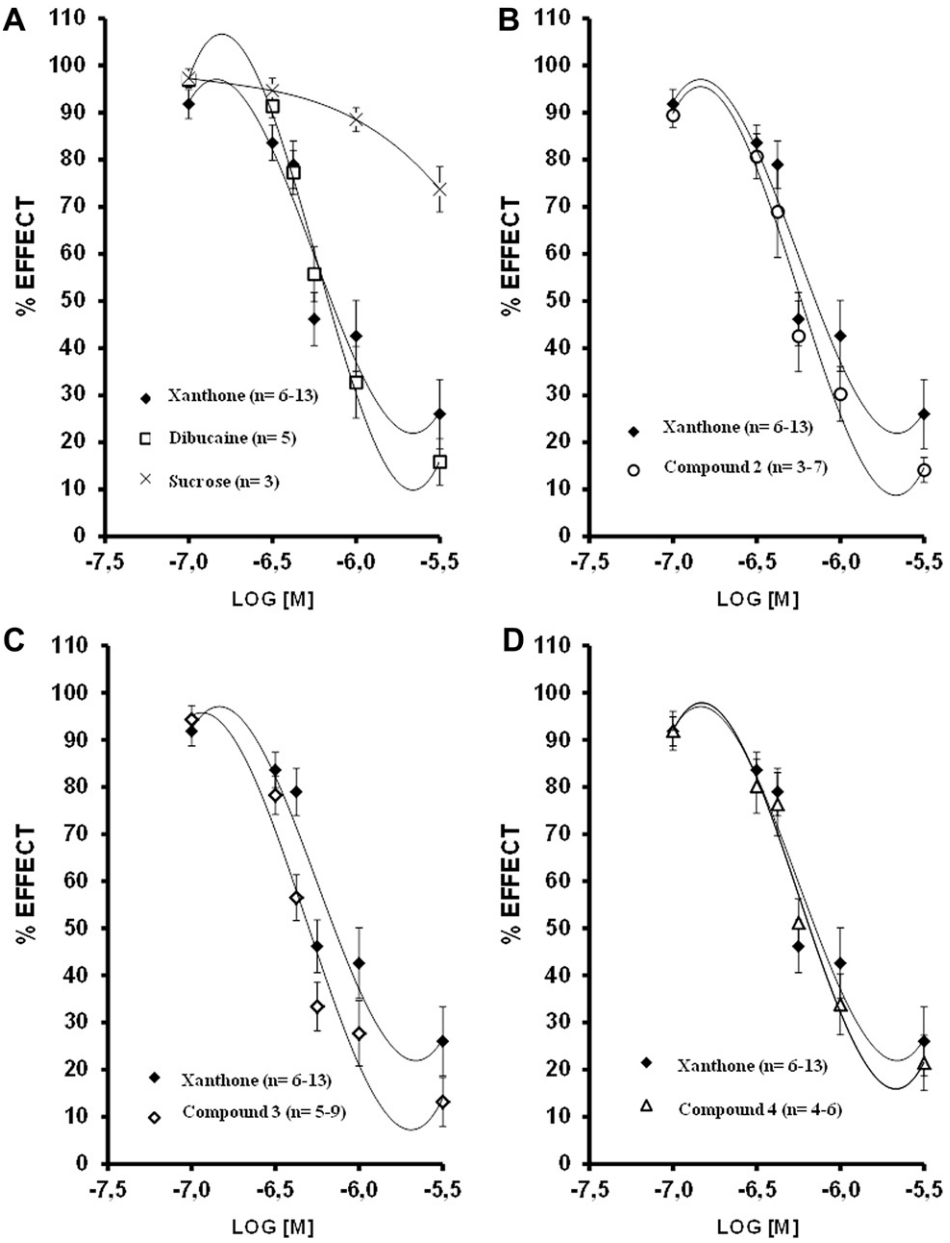


Fig. 1. Effect of chiral xanthone derivatives on rat sciatic nerve conduction. (A–D) Chiral xanthone derivatives (**2**–**4**) concentration-dependently decrease the amplitude of compound action potentials (CAP) in the isolated rat sciatic nerve. Control experiments with sucrose (×), to access the effect of osmolarity, and with the prototypical local anaesthetic, dibucaine (□), were also performed. Drugs were applied in a cumulative manner (from 0.1 to 3 μM) and contacted the sciatic nerve for at least 15 min. The ordinates are percentage of compound action potential amplitude (100%) observed in control conditions (in the absence of test drugs). The vertical bars represent ±S.E.M. of an *n* number of experiments (shown in parenthesis) and are shown when they exceed the symbols in size.

CXDs, while increasing their hydrophobic characteristics that make these compounds long-acting agents as they are hardly washable from nerve membranes within several hours. In spite of this, when used in the low micromolar concentration range compounds **2**–**4** did not cause axonal damage as predicted from the absence of significant changes ($P > 0.05$) in the activity of lactate dehydrogenase (LDH) leaked into the incubation medium over time. Local anaesthetics must penetrate the epineurium, perineurium and endoneurium in order to reach their intended site of action. Consequently, local anaesthetics require much higher concentrations to be effective when used clinically than when tested in isolated nerves [34]. The hydrophobic characteristics of these new molecules (**2**–**4**) may, thus, become an interesting feature for anaesthetic drug improvement.

2.3.2. Chiral xanthone derivatives had no protective effect on hypotonic haemolysis

Local anaesthetics also display non-specific membrane stabilizing properties besides their action on voltage-gated Na^+ channels. This prompted us to investigate if the CXDs **2**–**4** could prevent hypotonic haemolysis on rat erythrocytes, given that these are non-excitable cells lacking voltage-gated Na^+ channels.

The membrane protective effects of sucrose and dibucaine (0.1–100 μM) on hypotonic haemolysis were initially evaluated (Fig. 2A). Sucrose had a protective effect on hypotonic haemolysis, but its effect was not concentration-dependent. Just this fact suggests that osmolarity is not responsible for the membrane stabilizing effect of sucrose in the micromolar concentration range. Membrane stabilizing properties of sugars have been described by

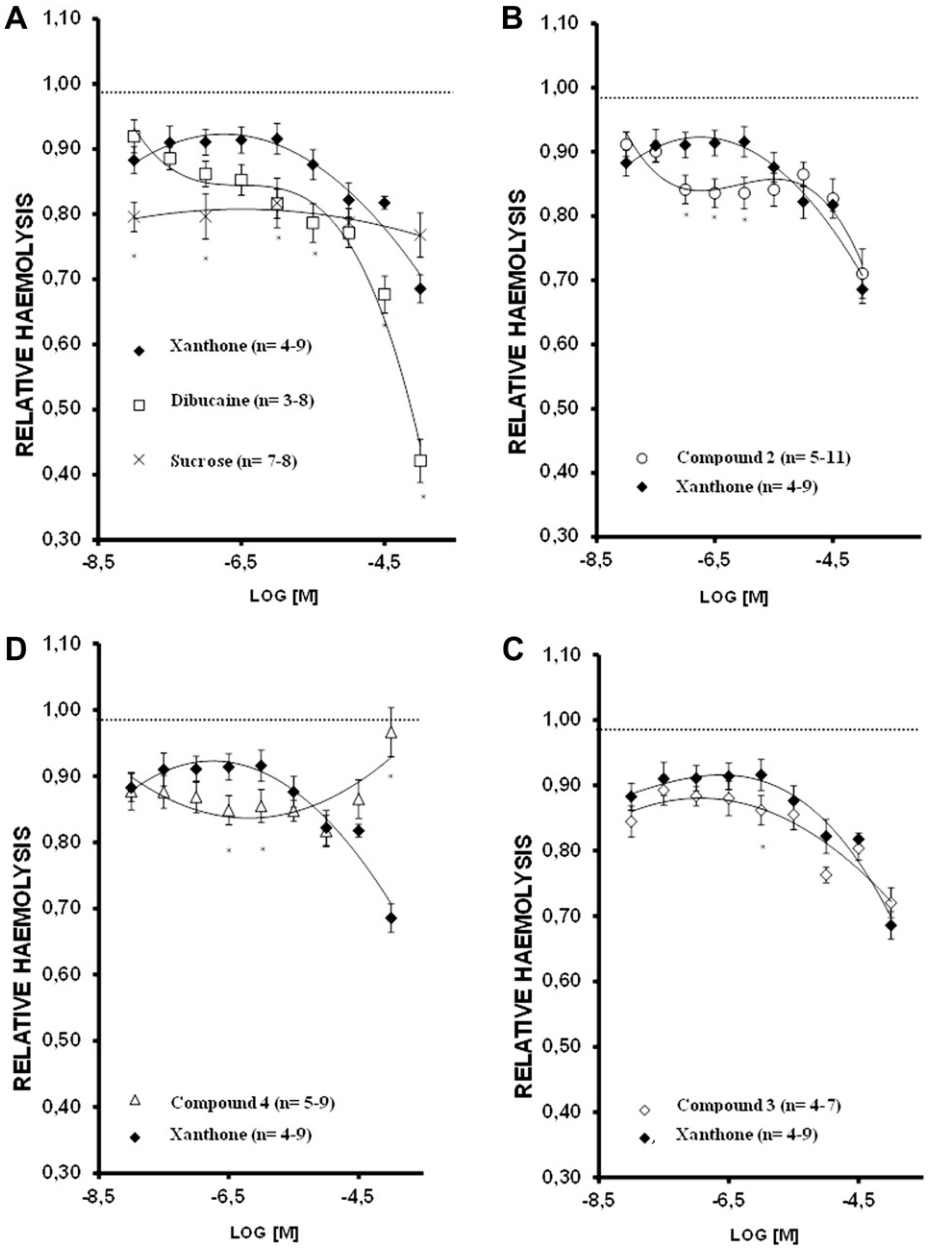


Fig. 2. Anti-haemolytic effects of chiral xanthone derivatives (0.01–100 μM) compared with those produced by the prototypical local anaesthetic drug, dibucaine, and by changes in fluid osmolarity caused by sucrose. Chiral xanthone derivatives (**2**–**4**), as well as dibucaine (□) and sucrose (×), were applied in concentrations ranging from 0.01 to 100 μM. The ordinates are relative haemolysis (RH) caused on rat erythrocytes incubated for 10 min with an haemolytic solution containing a predetermined concentration of NaCl in 10 mM phosphate buffer, at pH 7.0; Value one represents no haemolytic protection and corresponds to 50% erythrocyte haemolysis obtained with NaCl ranging from 63 to 67 mM in the absence of test drugs. The vertical bars represent ±S.E.M. of an *n* number of experiments (shown in parenthesis) and are shown when they exceed the symbols in size. $^{*}p < 0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) when compared with the situation with no haemolytic protection.

Crowe [35], being sucrose one of the most potent compounds of this class. Strauss and Hauser [36] suggested a direct interaction of sucrose with the phosphate group of membrane phospholipids. The local anaesthetic dibucaine (0.1–100 μM) reduced hypotonic haemolysis in a concentration-dependent manner (Fig. 2A). At the highest concentration tested (100 μM), dibucaine reduced relative haemolysis (RH) to 0.421 ± 0.034. Similar results were reported with this compound in a previous study [37].

The xanthone had little or no protective effect on hypotonic haemolysis in the low micromolar concentration range (0.1–30 μM) (Fig. 2A). A significant protective effect was observed only upon increase of the concentration to 100 μM (RH was decreased to 0.686 ± 0.021, $n = 4$), a situation in which the xanthone exceeded the sucrose protective effect. Compound **2** applied in the low micromolar concentration range (0.1–1 μM), increased significantly ($P < 0.05$) haemolytic protection as compared to xanthone (Fig. 2B). Nevertheless, neither of these compounds exceeded the membrane protective effect of sucrose, except when they were used in the 100 μM concentration. Similar results were obtained with compounds **3** and **4** (0.3–1 μM) (Fig. 2C and D). However, in contrast with compounds **2** and **3**, compound **4** was devoid of protective effect when it was applied in the 100 μM concentration; in these circumstances, RH was 0.967 ± 0.037 ($n = 6$, Fig. 2C).

Thus, in contrast with the more potent action of dibucaine, the anti-haemolytic effect of CXDs (**2**–**4**) was only observed when

these compounds were tested in higher micromolar concentrations (30–100 μM). No significant membrane protective effects were verified in the low micromolar concentration range (0.1–3 μM); in these circumstances, CXDs cause blockade of sciatic nerve conduction (see above), but their effects on hypotonic haemolysis were of smaller magnitude than that produced by sucrose.

3. Conclusions

The synthetic approach used to synthesize the xanthonic building block (**1**) was improved when compared to the described procedure in the literature. TBTU demonstrated to be very efficient, allowing the coupling of the xanthone derivative **1** with two chiral amino alcohols and one chiral amine, at room temperature. The three new CXDs were obtained, **2**–**4**, with excellent yields, short reaction times and no racemization. Long-range C, H correlations led to an unambiguous establishment of the structures of the new compounds.

Data from biological activity studies suggest that CXDs (**2**–**4**) cause no significant protection against hypotonic haemolysis when applied in concentrations high enough to block the sciatic nerve conduction in the rat. It, thus, appears that nerve conduction blockade caused by the newly-synthesized CXDs result predominantly from an action on Na^+ ionic currents. This effect can be dissociated from their ability to stabilize cell membranes, which only became apparent upon increasing the concentration of CXDs (**2**–**4**) to the high micromolar range.

4. Experimental

4.1. General methods

Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were measured on an ATIMattson Genesis series FTIR (software: WinFirst v.2.10) spectrophotometer in KBr microplates (cm^{-1}). ^1H and ^{13}C NMR spectra were taken in $CDCl_3$ or DMSO-d_6 at room temperature, on Bruker Avance 300 instrument (300.13 MHz for ^1H and 75.47 or 125.77 MHz for ^{13}C). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. Coupling constants are reported in hertz (Hz). ^{13}C NMR assignments were made by 2D HSQC and HMBC experiments (long-range C, H coupling constants were optimized to 7 and 1 Hz). MS spectra were recorded as EI (electronic impact) mode on a VG Autospec Q spectrometer (m/z) and HRMS mass spectra were measured on a Bruker Daltonics micrOTOF Mass Spectrometer, recorded as ESI (electrospray) mode in Centro de Apoio Científico e Tecnolóxico á Investigation (C.A.C.T.I.), University of Vigo, Spain.

TLC was performed using Merck silica gel 60 (GF_{254}) plates, with appropriate mobile phases. Compounds were visually detected by absorbance at 254 and/or 365 nm. Column chromatography was carried out using Merck silica gel 60 (0.040–0.063 mm). Optical rotation measurements were carried out on a Polartronic Universal polarimeter. Liquid chromatography was performed using a HPLC system consisted of two Shimadzu LC 10-ADvp pumps, a FCV-10AL solvent selector valve, an automatic injector SIL10-Advp, a SPD-10AV UV/VIS detector with a SCL-10Avp interface or a HPLC system consisted of two Shimadzu LC 10-AD pumps, an automatic injector SIL10-AF, a SPD-10A UV/VIS detector with a CBM-10A interface or a System 880-PU Intelligent HPLC Pump (Jasco) equipped with a Rheodyne 7125 injector fitted with a 20 μL loop, a 875-UV Intelligent UV/VIS Detector, and a Chromatography Station for Windows, version 1.7 DLL. Chirobiotic T column (15 × 0.46 cm ID size column) was commercially available from Sigma–Aldrich. Polysaccharide column was prepared in the laboratory as described elsewhere [38,39] and consisted of amylose *tris*-3,5-dimethylphenylcarbamate coated onto APS-Nucleosil (500 Å, 7 μm, 20% w/w), and packed into a stainless-steel 15 × 0.46 cm ID size column. Working solutions of enantiomeric mixtures of CXDs were prepared mixing equal aliquots of each enantiomer at the concentration of 20 μg/mL. Optical pure (*S*)-(+)-valinol, (*S*)-(+)-leucinol and (*S*)-(−)-α-dimethylbenzylamine were commercial reagents, from Fluka and Sigma–Aldrich. Other reagents and solvents were commercially available materials at *pro analysis* or HPLC grade, from Sigma–Aldrich, Merck and Fluka, and used without further purification.

4.2. Synthesis of the building block **1**

The synthetic route used to synthesize the building block **1** is outlined in Scheme 1, which involved a multi-step pathway.

4.2.1. Esterification of 4-bromoisophthalic acid (5). dimethyl 4-bromoisophthalate (**6**)

To a solution of 4-bromoisophthalic acid (**5**) (16.40 g, 66.93 mmol) in methanol (600 mL) was added 12 mL of concentrated H_2SO_4. Then, the reaction mixture was refluxed for 17 h. After evaporation of the methanol, water (100 mL) was added and the crude product was extracted with diethyl ether (3 × 100 mL). The organic layer was washed with water (100 mL), saturated $NaHCO_3$ solution (3 × 150 mL) and water (2 × 100 mL), successively. After drying with anhydrous sodium sulfate and filtered, the solvent was evaporated under reduced pressure. During overnight at room temperature the dimethyl 4-bromoisophthalate (**6**) appeared as a white solid. Yield: 92% m.p.: 56–58 °C; IR ν_{max} (cm^{-1}) (KBr): 1754, 1309, 1253, 929, 565; ^1H NMR ($CDCl_3$, 300.13 MHz) δ: 8.43 (1H, *d*, *J* = 2.2 Hz, H-2), 7.95 (1H, *dd*, *J* = 8.3 and 2.2 Hz, H-6), 7.75 (1H, *d*, *J* = 8.3 Hz, H-5), 3.95 (3H, *s*, C(1″)OOC$\underline{H}_3$), 3.93 (3H, *s*, C(1`)OOC$\underline{H}_3$); $^{13}C$ NMR ($CDCl_3$, 75.47 MHz) $\delta$: 165.7 (C-1″), 165.5 (C-1`), 134.7 (C-4), 133.0 (C-6), 132.3 (C-2), 132.2 (C-5), 129.3 (C-3), 127.0 (C-1), 52.7 (C(1″)OO$\underline{C}H_3$), 52.5 (C(1`)OO$\underline{C}H_3$); MS (EI) m/z (%): 273 $[M]^{+\cdot}$ (100), 256 (9), 240 (13), 221 (6), 209 (8), 203 (11).

4.2.2. Ullmann diaryl ether coupling. dimethyl 4-(3′-methoxyphenoxy) isophthalate (**8**)

A mixture of dimethyl 4-bromoisophthalate (**6**) (10.10 g, 36.98 mmol), 3-methoxyphenol (**7**) (6.88 g, 55.45 mmol), CuI (0.70 g, 3.70 mmol), Cs_2CO_3 (24.09 g, 73.94 mmol), *N*,*N*-dimethyl glycine (1.14 g, 11.09 mmol) and dioxane (74 mL) was heated in a sealed flask at 90 °C under nitrogen atmosphere for 14 h. After cooling water (100 mL) was added and the crude product was extracted with ethyl acetate (100 mL). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (2 × 100 mL). The combined organic layers were washed with brine, dried with anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The oily dark product was purified by column chromatography (silica gel, petroleum ether/diethyl ether in gradient) to provide dimethyl 4-(3′-methoxyphenoxy) isophthalate (**8**). Yield: 54% m.p. 88–90 °C; IR ν_{max} (cm^{-1}) (KBr): 1724, 1719, 1613, 1488, 1274, 1229, 1153, 948, 763; ^1H NMR ($CDCl_3$, 300.13 MHz) δ: 8.58 (1H, *d*, *J* = 2.2 Hz, H-2), 8.07 (1H, *dd*, *J* = 8.7 and 2.2 Hz, H-6), 7.27 (1H, *dd*, *J* = 8.4 and 8.3 Hz, H-5′), 6.95 (1H, *d*, *J* = 8.7 Hz, H-5), 6.74 (1H, *dd*, *J* = 8.3 and 3.1 Hz, H-6′), 6.62 (1H, *dd*, *J* = 3.6 and 3.1 Hz, H-2′), 6.61 (1H, *dd*, *J* = 8.4 and 3.6 Hz, H-4′), 3.93 (3H, *s*, C(1′)OOC$\underline{H}_3$), 3.89 (3H, *s*, C(1″)OOC$\underline{H}_3$), 3.79 (3H, *s*, Ar–OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ: 165.7 (C-1′), 165.3 (C-1″), 161.1 (C-3), 160.4 (C-1), 156.9 (C-4), 134.6 (C-2), 133.6 (C-6), 130.4 (C-5), 124.6 (C-3′), 122.0 (C-1′), 118.7 (C-5′), 111.6 (C-6′), 110.3 (C-4′), 105.6 (C-2′), 55.4 (Ar–O$\underline{C}H_3$), 52.3 (C(1′)OO$\underline{C}H_3$), 52.2 (C(1′)OO$\underline{C}H_3$); MS (EI) m/z (%): 316 $[M]^{+\cdot}$ (100), 285 $[M\text{-}O\underline{C}H_3]^{+\cdot}$ (55), 253

(54), 242 (10), 225 (16), 213 (12), 198 (25), 138 (15), 127 (10), 92 (15), 83 (14), 64 (11).

*4.2.3. Hydrolysis of dimethyl ester. 4-(3′-Methoxyphenoxy)isophthalic acid (**9**)*

Dimethyl 4-(3′-methoxyphenoxy)isophthalate (**8**) (7.28 g, 23.01 mmol) was dissolved in methanol/tetrahydrofuran (1:1 v/v) and stirred at room temperature with 5M NaOH solution (25 mL) for 18 h. After evaporation of the organic solvents, water was added (150 mL) and the crude product was washed with dichloromethane (2 × 200 mL). The organic layer was extracted with water (2 × 150 mL). The aqueous layer was acidified with 5M HCl solution resulting in the formation of a precipitate that was collected by filtration under reduced pressure and washed with water, to provide 4-(3′-methoxyphenoxy)isophthalic acid (**9**) as a white solid. Yield: 91%.

m.p.: 232–234 °C; IR ν_{max} (cm^{-1}) (KBr): 2907, 1694, 1680, 1601, 1486, 1271, 1265, 911, 758; ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ: 8.53 (1H, *d*, *J* = 3.3 Hz, H-2), 8.09 (1H, *dd*, *J* = 13.0 and 3.3 Hz, H-6), 7.29 (1H, *dd*, *J* = 12.3 and 11.9 Hz, H-5′), 6.95 (1H, *d*, *J* = 13.0 Hz, H-5), 6.76 (1H, *dd*, *J* = 11.9 and 3.4 Hz, H-6′), 6.62 (1H, *dd*, *J* = 3.4 and 3.3 Hz, H-2′), 6.59 (1H, *dd*, *J* = 12.3 and 3.3 Hz, H-4′), 3.77 (3H, *s*, Ar–OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ: 179.3 (C-1″), 179.3 (C-1′), 163.5 (C-3), 162.5 (C-1), 159.2 (C-4), 145.3 (C-2), 136.6 (C-6), 135.5 (C-5), 132.3 (C-3′), 127.3 (C-1′), 120.6 (C-5′), 113.4 (C-6′), 112.1 (C-4′), 107.5 (C-2′), 56.7 (Ar–O$\underline{C}H_3$); MS (EI) *m*/*z* (%): 288 $[M]^{+\cdot}$ (100), 271 (10), 257 $[M\text{-}OCH_3]^{+}$ (12), 227 (11), 199 (23), 165 (61), 124 (63), 92 (23), 77 (20), 64 (19).

4.2.4. Intramolecular acilation. Xanthones formation

To a solution of 4-(3′-methoxyphenoxy)isophthalic acid (**9**) (4.15 g, 14.40 mmol) in methane sulfonic acid (60 mL) was added phosphorus pentoxide (6.53 g, 45.99 mmol) and the reaction mixture was stirred at room temperature for 22 h. The mixture was poured over ice, resulting in the formation of a cream-coloured solid that was collected by filtration under reduced pressure and dried at room temperature. The crude product was dissolved in methanol (800 mL) and H_2SO_4 (16 mL) was added. The mixture was refluxed for approximately 19 h. The products were separated by filtration and the solid was washed with cooled methanol, providing methyl 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylate (**11**) with 85% yield. It was also obtained, at lower yield (2%), the compound methyl 8-methoxy-9-oxo-9*H*-xanthene-2-carboxylate (**12**), after the evaporation of the methanol and purification by column chromatography (silica gel, ethyl acetate/n-hexane in gradient). Methyl 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylate (**11**) (3.94 g, 13.86 mmol) was dissolved in methanol/dichloromethane (670 mL, 1:1 v/v) and 5M NaOH solution (54 mL) was added. The mixture was stirred at room temperature for 22 h. After evaporation of the organic solvents, water was added (100 mL) and the solution was acidified with 5M HCl solution resulting in the formation of a white precipitate. The suspension was filtered under reduced pressure and the white solid was washed with water, to afford 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylic acid (**1**). Yield: 94%. The same procedure was followed to hydrolyze the methyl 8-methoxy-9-oxo-9*H*-xanthene-2-carboxylate (**12**) (37 mg, 0.14 mmol) to afford 8-methoxy-9-oxo-9*H*-xanthene-2-carboxylic acid (**10**). Yield: 95%.

*4.2.4.1. Methyl 6-methoxy-9-oxo-9H-xanthene-2-carboxylate (**11**).* m.p.: 176–178 °C; IR ν_{max} (cm^{-1}) (KBr): 1730, 1663, 1581, 1467, 1438, 1270, 1117, 764; ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ: 8.98 (1H, *d*, *J* = 3.2 Hz, H-1), 8.32 (1H, *dd*, *J* = 13.1 and 3.2 Hz, H-3), 8.24 (1H, *d*, *J* = 13.2 Hz, H-8), 7.48 (1H, *d*, *J* = 13.1 Hz, H-4), 6.96 (1H, *dd*, *J* = 13.2 and 3.4 Hz, H-7), 6.89 (1H, *d*, *J* = 3.4 Hz, H-5), 3.95 (3H, *s*, COOC$\underline{H}_3$), 3.93 (3H, *s*, Ar–OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ: 175.5 (C-9), 165.9 (C$\underline{O}$OCH$_3$), 165.3 (C-6), 158.7 (C-10a), 157.8 (C-4a), 134.8 (C-3), 129.1 (C-2), 128.3 (C-8), 125.9 (C-1), 121.5 (C-9a), 118.1 (C-4), 115.6 (C-8a), 113.7 (C-7), 100.4 (C-5), 55.9 (Ar–O$\underline{C}H_3$), 52.3 (COO$\underline{C}H_3$); MS (EI) *m*/*z* (%): 284 $[M]^{+\cdot}$ (89), 253 $[M\text{-}OCH_3]^{+\cdot}$ (100), 225 (32), 197 (14), 182 (30), 169 (17), 154 (16), 142 (14), 126 (28), 111 (12), 75 (12), 63 (14).

*4.2.4.2. Methyl 8-methoxy-9-oxo-9H-xanthene-2-carboxylate (**12**).* m.p.: 206–207 °C; IR ν_{max} (cm^{-1}) (KBr): 1726, 1668, 1611, 1480, 1431, 1264, 1079, 760; ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ: 8.98 (1H, *d*, *J* = 2.1 Hz, H-1), 8.33 (1H, *dd*, *J* = 8.7 and 2.1 Hz, H-3), 7.65 (1H, *dd*, *J* = 8.4 and 8.4 Hz, H-6), 7.47 (1H, *d*, *J* = 8.7 Hz, H-4), 7.09 (1H, *dd*, *J* = 8.4 and 0.8 Hz, H-7), 6.86 (1H, *dd*, *J* = 8.4 and 0.8 Hz, H-5), 4.05 (3H, *s*, COOC$\underline{H}_3$), 3.98 (3H, *s*, Ar–OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125.77 MHz) δ: 175.8 (C-9), 166.0 (C$\underline{O}$OCH$_3$), 160.8 (C-8), 157.9 (C-10a), 157.8 (C-4a), 135.3 (C-3), 134.9 (C-6), 129.4 (C-2), 126.0 (C-1), 122.6 (C-9a), 117.7 (C-4), 112.5 (C-8a), 110.1 (C-7), 106.1 (C-5), 56.5 (Ar–O$\underline{C}H_3$), 52.4 (COO$\underline{C}H_3$); MS (EI) *m*/*z* (%): 284 $[M]^{+\cdot}$ (100), 255 (56), 253 (30), 238 (56), 223 (47), 195 (27), 139 (33), 126 (20), 112 (18), 70 (15), 63 (12).

*4.2.4.3. 6-Methoxy-9-oxo-9H-xanthene-2-carboxylic acid (**1**).* m.p.: >300 °C; IR ν_{max} (cm^{-1}) (KBr): 3411, 1687, 1610, 1575, 1500, 1433, 1271, 766; ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 8.68 (1H, *d*, *J* = 3.2 Hz, H-1), 8.29 (1H, *dd*, *J* = 13.3 and 3.2 Hz, H-3), 8.09 (1H, *d*, *J* = 13.3 Hz, H-8), 7.69 (1H, *d*, *J* = 13.3 Hz, H-4), 7.19 (1H, *d*, *J* = 3.3 Hz, H-5), 7.07 (1H, *dd*, *J* = 13.3 and 3.3 Hz, H-7), 3.93 (3H, *s*, Ar–OC$\underline{H}_3$); ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 175.3 (C-9), 167.6 (C$\underline{O}$OH), 164.9 (C-6), 157.6 (C-10a), 156.3 (C-4a), 135.7 (C-3), 127.6 (C-2), 126.6 (C-8), 124.9 (C-1), 120.2 (C-9a), 116.7 (C-4), 115.0 (C-8a), 113.7 (C-7), 100.7 (C-5), 56.2 (Ar–O$\underline{C}H_3$); MS (EI) *m*/*z* (%): 270 $[M]^{+\cdot}$ (100), 253 $[M - OH]^{+\cdot}$ (26), 226 (27), 199 (15), 182 (15), 169 (8), 154 (7), 139 (7), 126 (17), 115 (10), 63 (16).

*4.2.4.4. 8-Methoxy-9-oxo-9H-xanthene-2-carboxylic acid (**10**).* m.p.: 268–270 °C; IR ν_{max} (cm^{-1}) (KBr): 3460, 1687, 1663, 1603, 1469, 1420, 1266, 763; ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 8.66 (1H, *d*, *J* = 2.1 Hz, H-1), 8.28 (1H, *dd*, *J* = 8.7 and 2.1 Hz, H-3), 7.79 (1H, *dd*, *J* = 8.4 and 8.4 Hz, H-6), 7.67 (1H, *d*, *J* = 8.7 Hz, H-4), 7.20 (1H, *dd*, *J* = 8.4 and 0.7 Hz, H-7), 7.06 (1H, *dd*, *J* = 8.4 and 0.7 Hz, H-5), 3.94 (3H, *s*, Ar–OC$\underline{H}_3$); ^{13}C NMR (DMSO-d_6, 125.77 MHz) δ: 174.3 (C-9), 166.2 (C$\underline{O}$OH), 160.2 (C-8), 157.2 (C-10a), 157.0 (C-4a), 136.1 (C-3), 134.9 (C-6), 127.9 (C-2), 125.0 (C-1), 122.1 (C-9a), 118.2 (C-4), 111.6 (C-8a), 109.7 (C-7), 107.0 (C-5), 56.3 (Ar–O$\underline{C}H_3$); HRMS (ESI) *m*/*z*: calcd for ($C_{15}H_{10}O_5$ + H): 271.16994, found: 271.06010.

*4.3. General procedure for the synthesis of chiral xanthone derivatives **2**–**4***

The 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylic acid (**1**) (100 mg, 0.37 mmol) was dissolved in dry tetrahydrofuran (20 mL) and triethylamine (103 μL, 0.74 mmol) was added. Following, TBTU (120 mg, 0.37 mmol) and an appropriate chiral reagent (0.37 mmol) were added. The mixture was stirred at room temperature for 30 min (**4**) or 2 h (**2**–**3**). After completion of the reaction, the solvent was evaporated under reduced pressure and the crude product was dissolved in dichloromethane (50 mL). This solution was washed with 1M HCl solution (2 × 25 mL), saturated solution of $NaHCO_3$ (2 × 30 mL) and water (3 × 50 mL). The organic layer was dried with anhydrous sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The product was recrystallized from ethanol (**2**) or ethyl acetate/n-hexane (**3**–**4**), to afford the chiral xanthone derivative.

*4.3.1. (S)-N-(1-hydroxy-3-methylbutan-2-yl)-6-methoxy-9-oxo-9H-xanthene-2-carboxamide (**2**)*

Compound **2** was obtained as white solid. Yield: 99%; m.p.: 180–182 °C (ethanol); $[\alpha]_D^{25°C}$ −14.89° (10.08 mg/mL, dichloromethane); IR ν_{max} (KBr): 3371, 3244, 1656, 1616, 1539, 1476, 1444, 1272, 834 cm^{-1}; ^{1}H

NMR (DMSO-d_6, 300.13 MHz) δ: 8.73 (1H, *d*, J = 2.2 Hz, H-1), 8.38 (1H, *d*, J = 8.7 Hz, NH), 8.31 (1H, *dd*, J = 8.7 and 2.2 Hz, H-3), 8.14 (1H, *d*, J = 8.7 Hz, H-8), 7.71 (1H, *d*, J = 8.7 Hz, H-4), 7.21 (1H, *d*, J = 2.3 Hz, H-5), 7.09 (1H, *dd*, J = 8.7 and 2.3 Hz, H-7), 4.63 (1H, *t*, J = 5.6 Hz, OH), 3.95 (3H, *s*, Ar−OCH$_3$), 3.85 (1H, *m*, H-1′), 3.54 (2H, *dd*, J = 10.4 and 5.8 Hz, CH$_2$OH), 1.97 (1H, *m*, H-2′), 0.93 (3H, *d*, J = 7.0 Hz, H-3′a)*, 0.90 (3H, *d*, J = 7.0 Hz, H-3′b)*; ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 174.9 (C-9), 167.7 (C=O, amide), 165.1 (C-6), 157.6 (C-10a), 157.0 (C-4a), 134.1 (C-3), 130.9 (C-2), 127.7 (C-8), 125.4 (C-1), 120.6 (C-9a), 118.0 (C-4), 114.9 (C-8a), 114.0 (C-7), 100.8 (C-5); 61.3 (CH$_2$OH), 57.0 (Ar−OCH$_3$), 56.3 (C-1′), 28.7 (C-2′), 19.7 (C-3′a)*, 18.8 (C-3′b)*; MS (EI) *m*/*z* (%): 357 $[M + H]^{+\cdot}$+1 (15), 356 $[M + H]^{+\cdot}$ (43), 340 $[M - CH_3]^{+\cdot}$ (8), 270 (11), 254 (20), 253 (100), 239 (12), 227 (12), 226 (15); HRMS (ESI) *m*/*z*: calcd for ($C_{20}H_{21}NO_5$ + H): 356.14925, found: 356.14967; Enantiomeric purity: e.e. > 99% (HPLC; Column: Chirobiotic T, Mobile phase: *n*-Hexane:Ethanol (80:20 v:v), 0.5 mL/min, λ_{max} 254 nm).

* Asterisks denote assignments that may be interchanged.

4.3.2. (S)-N-(1-hydroxy-4-methylpentan-2-yl)-6-methoxy-9-oxo-9H-xanthene-2-carboxamide (**3**)

Compound **3** was obtained as white solid. Yield: 98%; m.p.: 156–158 °C (ethyl acetate/n-hexane); $[\alpha]_D^{25°C}$ - 25.00° (11.40 mg/mL, dichloromethane); IR ν_{max} (cm^{-1}) (KBr): 3360, 3285, 1652, 1617, 1546, 1468, 1443, 1264, 830; ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 8.72 (1H, *d*, J = 2.2 Hz, H-1), 8.40 (1H, *d*, J = 8.7 Hz, NH), 8.30 (1H, *dd*, J = 8.7 and 2.2 Hz, H-3), 8.14 (1H, *d*, J = 8.9 Hz, H-8), 7.70 (1H, *d*, J = 8.7 Hz, H-4), 7.21 (1H, *d*, J = 2.3 Hz, H-5), 7.10 (1H, *dd*, J = 8.9 and 2.3 Hz, H-7), 4.75 (1H, *t*, J = 5.7 Hz, OH), 4.11 (1H, *m*, H-1′), 3.95 (3H, *s*, Ar−OCH$_3$), 3.43 (2H, *dd*, J = 13.3 and 5.3 Hz, CH$_2$OH), 1.64 (2H, *m*, H-2′), 1.45 (1H, *m*, H-3′), 0.91 (3H, *d*, J = 6.0 Hz, H-4′a) *, 0.90 (3H, *d*, J = 6.0 Hz, H-4′b)*; ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 174.9 (C-9), 165.2 (C=O, amide), 164.7 (C-6), 157.5 (C-10a), 157.1 (C-4a), 134.0 (C-3), 130.7 (C-2), 127.7 (C-8), 125.3 (C-1), 120.7 (C-9a), 118.1 (C-4), 114.9 (C-8a), 114.0 (C-7), 100.8 (C-5), 63.9 (CH$_2$OH), 56.3 (Ar−OCH$_3$), 49.8 (C-1′), 39.0 (C-2′), 24.5 (C-3′), 23.5 (C-4′a)*, 21.9 (C-4′b)*; MS (ESI) *m*/*z* (%): 371 $[M + H]^{+\cdot}$+1 (28), 370 $[M + H]^{+\cdot}$ (100), 328 (7), 306 (7), 253 (2), 197 (3), 171 (11); HRMS (ESI) *m*/*z*: calcd for ($C_{21}H_{23}NO_5$ + H): 370.16490, found: 370.16488; Enantiomeric purity: e.e. > 99% (HPLC; Column: Amylose *tris*-3,5-dimethylphenylcarbamate coated onto APS-Nucleosil (500 Å, 7 μm, 20% w/w), Mobile phase: Ethanol:Acetonitrile (50:50 v:v), 0.5 mL/min, λ_{max} 254 nm).

* Asterisks denote assignments that may be interchanged.

4.3.3. (S)-6-methoxy-9-oxo-N-(1-(p-tolyl)ethyl)-9H-xanthene-2-carboxamide (**4**)

Compound **4** was obtained as white solid. Yield: 97%; m.p.: 221–223 °C (ethyl acetate/n-hexane); $[\alpha]_D^{25°C}$ + 125.70° (10.70 mg/mL, dichloromethane); IR ν_{max} (cm^{-1}) (KBr): 3333, 1660, 1611, 1530, 1476, 1438, 1266, 831, 827; ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ: 8.56 (1H, *d*, J = 2.2 Hz, H-1), 8.32 (1H, *dd*, J = 8.7 and 2.2 Hz, H-3), 8.25 (1H, *d*, J = 8.9 Hz, H-8), 7.53 (1H, *d*, J = 8.7 Hz, H-4), 7.31 (2H, *d*, J = 8.0 Hz, H-2″ and H-6″), 7.18 (2H, *d*, J = 8.0 Hz, H-3″ and H-5″), 6.99 (1H, *dd*, J = 8.9 and 2.3 Hz, H-7), 6.92 (1H, *d*, J = 2.3 Hz, H-5), 6.58 (1H, *d*, J = 7.3 Hz, NH), 5.33 (1H, *m*, H-1′), 3.93 (3H, *s*, Ar−OCH$_3$), 2.34 (3H, *s*, Ar−CH$_3$), 1.63 (3H, *d*, J = 6.9 Hz, H-2′); ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ: 175.9 (C-9), 165.5 (C=O, amide), 164.8 (C-6), 158.0 (C-10a), 157.8 (C-4a), 139.8 (C-1″), 137.2 (C-4″), 134.3 (C-3), 130.1 (C-2), 129.4 (C-3″ and C-5″), 128.3 (C-8), 126.2 (C-2″ and C-6″), 124.0 (C-1), 121.0 (C-9a), 118.5 (C-4), 115.5 (C-8a), 113.8 (C-7), 100.3 (C-5), 55.9 (Ar−OCH$_3$), 49.3 (C-1′), 21.7 (Ar−CH$_3$), 21.0 (C-2′); MS (ESI) *m*/*z* (%): 389 $[M + H]^{+\cdot}$+1 (27), 388 $[M + H]^{+\cdot}$ (100), 354 (7), 338 (13), 270 (8), 219 (6), 197 (10); HRMS (ESI) *m*/*z*: calcd for ($C_{24}H_{21}NO_4$ + H): 388.15433, found: 388.15357; Enantiomeric purity: e.e. > 98% (HPLC; Column: Amylose *tris*-3,5-dimethylphenylcarbamate coated onto APS-Nucleosil (500 Å, 7 μm, 20% w/w), Mobile phase: Ethanol:Acetonitrile (50:50 v:v), 0.5 mL/min, λ_{max} 254 nm).

4.4. Biological activity assay

4.4.1. Preparation and experimental conditions

In this study, sciatic nerve bundles, 3–4 cm in length, were taken from adult Wistar rats (200–400 g), obtained from Charles Rivers (Barcelona, Spain). Rats were kept at a constant temperature (21 °C) and a regular light (06.30–19.30 h)-dark (19.30–06.30 h) cycle, with food and water *ad libitum*. Animal handling and experiments followed the guidelines defined by the European Communities Council Directive (86/609/EEC).

The rats were killed by decapitation and the sciatic nerves were exposed from the spinal cord to the knee. A portion of the sciatic nerve was rapidly isolated free from surrounding structures and their ends were tied, in order to allow the setting of the nerve in the recording chamber (*Marsh Ganglion Bath)*. This process was performed in the presence of gassed (95% O_2 and 5% CO_2) Tyrode's solution (pH 7.4) containing (mM): KCl 2.7, $CaCl_2$ 1.8, NaH_2PO_4 0.4, $MgCl_2$ 1, NaCl 137, $NaHCO_3$ 11.9 and glucose 11.2, at 25 °C. Before electrophysiological recordings, desheathed sciatic nerves were equilibrated with gassed Tyrode's solution superfused with a flow rate of 1 mL/min for at least 3 h at 25 °C, in order to achieve a stable baseline and reproducible electrically-evoked compound action potentials (see *e.g* [40].). Measurement of lactate dehydrogenase (LDH, EC 1.1.1.27) activity leaked into the incubation medium from nerve fibre bundles was taken as a measure of neuronal cell damage and followed the method described by Bergmeyer [41]. LDH activity showed no significant changes over time of incubation, suggesting that experimental manipulations did not affect sciatic nerve viability.

4.4.2. Electrophysiology experiments

Electrophysiology experiments were performed using an extracellular partition recording technique, in order to monitor changes in membrane polarization associated with electrical sciatic nerve stimulation - compound action potentials (CAP). Sciatic nerve trunks were mounted in a *Marsh Ganglion Bath* recording chamber. The recording chamber was partitioned into three compartments: a) a stimulating pool, containing a pair of silver electrodes connected to a Grass SD9 stimulator (Quincy, MA, USA); b) a test-pool, where drugs were applied; and c) a recording pool, containing a pair of silver electrodes connected to a Hitachi VC-6025A digital storage oscilloscope (Tokyo, Japan) coupled to an ink-recording plotter.

The rat sciatic nerve trunk was stimulated with a 0.2 Hz frequency throughout the experiment. Supramaximal intensity rectangular pulses of 0.03–0.05 ms duration were used to achieve nerve firing synchronization, thus reducing the number of silent units that might confound data interpretation. The stimulation voltage was set 2 times the threshold stimulus required to achieve maximal CAP amplitude, which did not exceed 2–3 V. The pulses were delivered *via* a Grass SD9 stimulator (Quincy, MA, USA).

Solutions were changed by transferring the inlet tube of the peristaltic pump (Gilson, Minipuls3, France) from one flask to another. The flow rate was kept at 1 mL/min throughout the experiments. Test drugs were applied in a cumulative manner and were allowed to contact with the preparations for at least 15 min.

Nerve impulse blockade caused by test drugs was expressed as a percentage decrement in amplitude of the CAP in control conditions, *i.e.* without addition of any drug to the Tyrode's solution.

4.4.3. Hypotonic haemolysis on rat erythrocytes

Experiments were carried out on erythrocytes isolated from rat blood samples. The technique used to produce haemolysis followed the one described by Seeman and Weinstein [42], modified by Timóteo and Ribeiro [43]. Blood samples were obtained by heart

puncture from Wistar rats anaesthetized with urethane (1.2 g/kg). Aliquots of 0.9 mL heparinised (100 units/mL) blood samples were centrifuged for 15 min at 2000 rpm. The plasma, including the leukocyte layer, was carefully removed, and then 154 mM NaCl in 10 mM phosphate buffer pH 7.0 was added, to make a total volume of 12.5 mL. Erythrocytes were re-suspended by a gentle and repeated inversion of the test tube. Aliquots of 0.2 mL of the stock erythrocyte suspension were added to 4 mL of test drug solutions (various concentrations) diluted in haemolytic solution containing a predetermined concentration of NaCl in 10 mM phosphate buffer, at pH 7.0; 50% erythrocyte haemolysis is usually obtained with NaCl ranging from 63 to 67 mM. The mixture was kept at room temperature for 10 min and then it was centrifuged at 3000 rpm for 15 min. The haemoglobin content of the supernatant was evaluated at 540 nm using a spectrophotometer. All experiments were done in duplicate.

4.4.4. Materials and solutions

Stock solutions of chiral xanthone derivatives (**2**–**4**), xanthone (Sigma–Aldrich) and dibucaine (Sigma–Aldrich) were prepared in dimethylsulfoxide (DMSO); solutions were kept protected from the light to prevent photodecomposition. All stock solutions were stored as frozen aliquots at −20 °C. Dilutions of these stock solutions were made daily (0.100, 0.300, 0.420, 0.560, 0.750, 1.00 and 3.00 μM) in Tyrode's solution and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.05% v/v), were observed.

4.4.5. Statistical analysis

The data is expressed as mean ± S.E.M., from an n number of experiments. Statistical significance of experimental results was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's modified t-test. $P < 0.05$ was considered to represent significant differences.

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References

[1] H. Caner, E. Groner, L. Levy, Trends in the development of chiral drugs, DDT 9 (3) (2004) 105–110.
[2] I. Agranat, S.R. Wainschtein, The strategy of enantiomer patents of drugs, DDT 15 (5–6) (2010) 163–170.
[3] http://www.imshealth.com (2011-03-11).
[4] D.R. Brocks, Drug disposition in three dimensions: an update on stereoselectivity in pharmacokinetics, Biopharm. Drug Dispos. 27 (2006) 387–406.
[5] B. Kasprzyk-Hordern, Pharmacologically active compounds in the environment and their chirality, Chem. Soc. Rev. 39 (2010) 4466–4503.
[6] Food and Drug Administration, Federal Register 57, FR 22249 (1992).
[7] Directive 75/318/EEC (1993).
[8] M.M.M. Pinto, M.E. Sousa, M.S.J. Nascimento, Xanthone derivatives: new insights in biological activities, Curr. Med. Chem. 12 (21) (2005) 2517–2538.
[9] M.M.M. Pinto, E.P. Sousa, Natural and synthetic Xanthonolignoids: chemistry and biological activities, Curr. Med. Chem. 10 (2003) 1–12.
[10] R.A.P. Castanheiro, M.M.M. Pinto, A.M.S. Silva, S.M.M. Cravo, L. Gales, A.M. Damas, N. Nazareth, M.S.J. Nascimento, G. Eaton, Dihydroxyxanthones prenylated derivatives: synthesis, structure elucidation, and growth inhibitory activity on human tumor cell lines with improvement of selectivity for MCF-7, Bioorg. Med. Chem. 15 (2007) 6080–6088.
[11] E. Sousa, A. Paiva, N. Nazareth, L. Gales, A.M. Damas, M.S.J. Nascimento, M. Pinto, Bromoalkoxyxanthones as promising antitumor agents: synthesis, crystal structure and effect on human tumor cell lines, Eur. J. Med. Chem. 44 (2009) 3830–3835.
[12] A. Palmeira, M.H. Vasconcelos, A. Paiva, M.X. Fernandes, M. Pinto, E. Sousa, Dual inhibitors of P-glycoprotein and tumor cell growth: (re)discovering thioxanthones, Biochem. Pharmacol. 83 (2012) 57–68.
[13] A. Palmeira, A. Paiva, E. Sousa, H. Seca, G.M. Almeida, R.T. Lima, M.X. Fernandes, M. Pinto, M.H. Vasconcelos, Insights into the *in vitro* antitumor mechanism of action of a new pyranoxanthone, Chem. Biol. Drug Des. 76 (2010) 43–58.
[14] E.P. Sousa, A.M.S. Silva, M.M.M. Pinto, M.M. Pedro, F.A.M. Cerqueira, M.S.J. Nascimento, Isomeric Kielcorins and Dihydroxyxanthones: synthesis, structure elucidation, and inhibitory activities of growth of human cancer cell lines and on the proliferation of human lymphocytes in vitro, Hel. Chim. Acta 85 (2002) 2862–2876.
[15] H. Marona, Synthesis and anticonvulsant effects of some aminoalkanolic derivatives of xanthone, Pharmazie 53 (1998) 672–676.
[16] H. Marona, N. Szkaradek, M. Kubacka, M. Bednarski, B. Filipek, M. Cegla, E. Szneler, Synthesis and evaluation of some xanthone derivatives for antiarrhythmic, hypotensive properties and their affinity for adrenergic receptors, Arch. Pharm. Chem. Life Sci. 341 (2008) 90–98.
[17] M. Jastrzêbska-Wiêsek, T. Librowski, T. Czarnecki, H. Marona, G. Nowak, Central activity of new xanthone derivatives with chiral center in some pharmacological tests in Mice, Pol. J. Pharmacol. 55 (3) (2003) 461–465.
[18] H. Marona, N. Szkaradek, A. Rapacz, B. Filipek, M. Dybała, A. Siwek, M. Cegła, E. Szneler, Preliminary evaluation of pharmacological properties of some xanthone derivatives, Bioorg. Med. Chem. 17 (2009) 1345–1352.
[19] G.W. Rewcastle, G.J. Atwell, B.C. Baguley, M. Boyd, L.L. Thomsen, L. Zhuang, W.A. Denny, Potential antitumor agents. 63. Structure-activity relationships for side-chain analogues of the colon 38 active agent 9-Oxo-9*H*-xanthene-4-acetic acid, J. Med. Chem. 34 (1991) 2864–2870.
[20] H. Marona, Evaluation of some 2-substituted derivatives of xanthone for anticonvulsant properties, Pharmazie 53 (1998) 405–409.
[21] H. Marona, E. Pekala, L. Antkiewicz-Michaluk, M. Walczak, E. Szneler, Anticonvulsant activity of some xanthone derivatives, Bioorg. Med. Chem. 16 (2008) 7234–7244.
[22] M. Jastrzebska-Wiesek, R. Czarnecki, H. Marona, The anticonvulsant, local anesthetic and hemodynamic properties of some chiral aminobutanol derivatives of xanthone, Acta Pol. Pharm.-Drug Res. 65 (5) (2008) 591–601.
[23] H. Marona, N. Szkaradek, E. Karczewska, D. Trojanowska, A. Budak, P. Bober, W. Przepiórka, M. Cegla, E. Szneler, Antifungal and antibacterial activity of the newly synthesized 2-Xanthone derivatives, Arch. Pharm. Chem. Life Sci. 342 (2009) 9–18.
[24] L. Wang, J. Kang, I. Chen, C. Teng, C. Lin, Antihypertensive and vasorelaxing activities of synthetic xanthone derivatives, Bioorg. Med. Chem. 10 (3) (2002) 567–572.
[25] J.R. Pfister, R.W. Ferraresi, I.T. Harrison, W.H. Rooks, A.P. Roszkowski, A. Van Horn, J.H. Fried, Xanthone-2-carboxilic acids, a new series of antiallergic substances, J. Med. Chem. 15 (1972) 1032–1035.
[26] W.T. Jackson, R.J. Boyd, L.L. Froelich, D.M. Gapinski, B.E. Mallett, J.S. Sawyer, Design, synthesis, and pharmacological evaluation of potent xanthone dicarboxylic acid leukotriene B 4 receptor antagonists, J. Med. Chem. 36 (1993) 1726–1734.
[27] F. Albericio, M.A. Bailén, R. Chinchilla, D.J. Dodsworth, C. Nájera, 2-Mercaptopyridine-1-oxide-based peptide coupling reagents, Tetrahedron 57 (2001) 9607–9613.
[28] S. Balalaie, M. Mahdidoust, R. Eshaghi-Najafabadi, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate as an efficient coupling reagent for the amidation and phenylhydrazation of carboxylic acids at room temperature, J. Iran. Chem. Soc. 4 (3) (2007) 364–369.
[29] S. Han, Y. Kim, Recent development of peptide coupling reagents in organic synthesis, Tetrahedron 60 (2004) 2447–2467.
[30] C.A.G.N. Montalbetti, V. Falque, Amide bond formation and peptide coupling, Tetrahedron 61 (2005) 10827–10852.
[31] T.I. Al-Warhi, H.M.A. Al-Hazimi, A. El-Faham, Recent development in peptide coupling reagents, J. Saudi Chem. Soc. 16 (2) (2012) 97–116.
[32] B. Hille, Ionic Channels of Excitable Membranes, second ed., MA: Sinauer Associates, Sunderland, 1992, pp. 390–460.
[33] D. Ma, Q. Cai, *N, N*-Dimethyl glycine-promoted Ullmann coupling reaction of phenol and aryl halides, Org. Lett. 5 (21) (2003) 3799–3802.
[34] E.J. Simons, E. Bellas, M.W. Lawlor, D.S. Kohane, Effect of chemical permeation enhancers on nerve blockade, Mol. Pharmaceutics 6 (2009) 265–273.
[35] J.H. Crowe, L.M. Crowe, J.F. Carpenter, C.A. Wistrom, Stabilization of dry phospholipid bilayers and proteins by sugars, Biochem. J. 242 (1987) 1–10.
[36] G. Strauss, H. Hauser, Stabilization of lipid bilayer vesicles by sucrose during freezing, Proc. Nat. Acad. Sci. USA 83 (1986) 2422–2426.
[37] S.V.P. Malheiros, L.M.A. Pinto, L. Gottardo, D.K. Yokaichiya, L.F. Fraceto, N.C. Meirelles, E. Paula, A new look at the hemolytic effect of local anesthetics, considering their real membrane/water partioning at pH 7.4, Biophys. Chem. 110 (2004) 213–221.
[38] S.A. Matlin, M.E. Tiritan, A.J. Crawford, Q.B. Cass, D.R. Boyd, HPLC with carbohydrate carbamate chiral phases: influence of chiral phase structure on enantioselectivity, Chirality 6 (1994) 135–140.

[39] S.A. Matlin, M.E. Tiritan, Q.B. Cass, D.R. Boyd, Enantiomeric resolution of chiral sulfoxides on polysaccharide phases by HPLC, Chirality 8 (1996) 147–152.
[40] T. Mert, Y. Gunes, D. Ozcengis, I. Gunay, S. Polat, Comparative effects of lidocaine and tramadol on injured peripheral nerves, Eur. J. Pharmacol. 543 (2006) 54–62.
[41] H. Bergmeyer, Lactic Dehydrogenase, Methods of Enzymatic Analysis (1963). pp. 736–743.
[42] P. Seeman, J.I. Weinstein, Erythrocyte membrane stabilization by tranquilizers and antihistamines, Biochem. Pharmac. 15 (1966) 1737–1752.
[43] M.A. Timóteo, J.A. Ribeiro, The effect of sodium tripolyphosphate on erythrocytes, Actas Bioq. 2 (1989) 201–205.