การสังเคราะห์ไฮดรอกซีแซนโทนและการประเมินฤทธิ์ยับยั้งเอ็นไซม์อะเซติลโคลีนเอสเทอเรสและความเป็นพิษต่อเซลล์ประสาท

Synthesis of Hydroxyxanthones and Evaluations for their Acetylcholinesterase Inhibitory and Neurotoxicity Activities

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บทคัดย่อ

การสังเคราะห์สารกลุ่มไฮดรอกซีแซนโทน และทดสอบฤทธิ์ยับยั้งเอ็นไซม์อะเซติลโคลีนเอสเทอเรส นำไปสู่การค้นพบสารที่แสดงฤทธิ์ชนิดใหม่ในการยับยั้งเอนไซม์อะเซติลโคลีนเอสเทอเรส โดยสารที่แสดงฤทธิ์ยับยั้งสูงสุดมีค่า IC_{50} 69.45±0.23 ไมโครโมลาร์ จากการทดสอบความเป็นพิษต่อเซลล์ประสาทแสดงให้เห็นว่า สารกลุ่มไฮดรอกซีแซนโทน ไม่เป็นพิษกับเซลล์ประสาทนิวโรบลาสโตมาที่ IC_{50} สูงกว่า 94 ไมโครโมลาร์

ABSTRACT

A series of hydroxyxanthones has been synthesized and screened for their anti-AChE activity. This led to the discovery of new AChE inhibitors with the highest inhibitory activity of IC_{50} 69.45±0.23 μM. A neurotoxicity evaluation revealed that hydroxyxanthones represented another class of compound that was non-toxic to the neuroblastoma cells at IC_{50} higher than 94 μM.

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คำสำคัญ: ไฮดรอกซีแซนโทน โรคอัลไซเมอร์ ฤทธิ์ยับยั้งอะเซติลโคลีนเอสเทอเรส ความเป็นพิษต่อเซลล์ประสาท
Keywords: Hydroxythioxanthone, Alzheimer's disease, Anti-acetylcholinesterase, Neurotoxicity

Introduction

Alzheimer's disease (AD) is a condition that causes uncommon changes in the brain mainly affecting memory, other mental abilities, which can interfere with normal daily activities. Accordingly, AD treatment and care constitute a major social and health problem (Alzheimer's, Association, 2011). The cause of AD is still unknown (Jack, 2004), until now there is no cure for AD (Manepalli, 2009). All drugs are temporarily slow progressive of symptoms for about 6–12 months. AD is neuropathologically characterized by the presence of plaques of β-amyloid (Aβ) plaques, neurofibrillary tangles (NFT), and degeneration or atrophy of the basal forebrain cholinergic neurons (Bruhlmann et al., 2001). The loss of basal forebrain cholinergic cells results in an important reduction in acetylcholine (ACh), which is believed to play a role in the cognitive impairment associated with AD. Accordingly, increasing the levels in ACh has been regarded as one of the most promising approaches for the symptomatic treatment of AD. To date, several acetylcholinesterase (AChE) inhibitors are commercially available including galantamine, donepezil, rivastigmine and tacrine, nevertheless, their clinical use is rigorously limited because of adverse effects such as some pharmacokinetic disadvantages and hepatotoxicity (Suh et al., 2005). The search for new pharmacophores for AChE inhibitor with low toxicity remains continue. Phenolic-containing substances are one of the largest classes widely found in natural sources and have been described to hold promise for human health benefits (Cicerale et al., 2010). Naturally occurring and synthetic oxygenated xanthones have revealed a wide range of biological profiles (Dua et al., 2004), including their AChE inhibitory activity (Bruhlmann et al., 2004). However, their neurotoxicity evidence has been limited described. Natural xanthones comprising the mono-, tri-, tetra- and penta-hydroxylated substitutions (Urbain et al., 2008) in addition to the synthetic 3-hydroxyxanthone analogues (Piazzi et al., 2007) have been evaluated as AChE inhibitors. Our interest in the bioactive constituents along with their safety concern (Luerang et al., 2012) prompted us to explore the neurotoxicity of some anti-AChE substances. In this report, xanthones with different hydroxylated pattern were synthesized and screened for their anti-AChE activity. Neurotoxicity study on the active compounds was consequently performed.

Research Methodology

General

The 1D- and 2D-NMR spectra were recorded on a Bruker Avance 300 FT-NMR and are reported in ppm relative to the reference solvent of the sample in which they were run; coupling constants are reported in hertz (Hz). The chemical shifts were referenced to the residual solvent peaks (δ_H 7.24 and δ_C 77.00 for $CDCl_3$, and δ_H 2.04 and δ_C 29.8 for acetone-d_6). ESI-MS Mass spectra were obtained from Finnigan LCQ. All flash column chromatographies (CC) were performed on Merck silica gel 60 (particle size of 230-400 mesh). All reagents were of reagent grade and obtained from commercial sources. Reactions were monitored by TLC, performed on precoated silica gel GF_{254} TLC plates. Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating (Pirrung, 2007). Melting points were determined with Griffin melting point apparatus, and are uncorrected.

Preparation of hydroxyxanthones analogues

Synthesis of 1-hydroxyxanthone (3a)

A mixture of salicylic acid (**1a**, 1.340 g, 9.7 mmol), resorcinol (**2a**, 1.154 g, 10.4 mmol) and anh. $ZnCl_2$ (1 g, 7.3 mmol) was heated at 170 °C in an oil bath for 6 h. After cooling, the crude product was dissolved with EtOAc (50 mL), silica gel (5 g) was then added and evaporated to afford a dark red residue, which was purified by flash CC using 1% EtOAc - hexane as eluting system. 1-Hydroxyxanthone (**3**) was yielded as pale yellow solid (1.59 g, 7.5 mmol, 71 % yield), m.p. 145-146 °C, lit. 142-144°C (Coelho, et al., 2001), R_f 0.62 (30% acetone-hexane); (+ve) *m/z* : 213.2 $[M+H]^+$ (100).

Synthesis of hydroxyxanthones 3b-3g

Freshly prepared methanesulfonic acid (MsOH, 36.5 g, 25 mL, 0.4 mol) was placed in a two-necked flask (500 mL) fitted with an efficient magnetically stirrer and a calcium chloride drying tube. Phosphorus pentoxide (3.6 g, 25.4 mmol) was added in one portion which was generally dissolved in 1-2 h. The reagent could be used immediately or could be stored in a stoppered flask for later use (Eaton et al., 1973). Then the solution was added a mixture of corresponding phenolic compounds (**2a-2b**, 10.0 mmol) and hydroxybenzoic acid derivatives (**1a-1d**, 10 mmol). Heating at 80 °C was continued for 30 min. The reaction progress was monitored by TLC. After completion, the reaction mixture was poured into ice-water. The resulting orange solid was collected by filtration, washed with water, dried in air, and the residue was then purified by flash CC eluting with 10% acetone-hexane to give yellow solid of the desired products (**3b-3g**) in 1.8-71.0 % yield.

1,3-Dihydroxyxanthone (**3b**): Yield 71.0%, m.p. 254-255 °C, lit. 255-256 °C (Fotie

etal., 2003), R_f 0.50 (30% acetone-hexane); ESIMS (-ve) : m/z 227.6 $[M-H]^-$(100).

1,6-Dihydroxyxanthone (**3c**): Yield 10.0 %, m.p. 236-238 °C, lit. 246-248.5 °C (Fletcher and Marlow, 1970), R_f 0.3 (30% acetone-hexane); ESIMS (-ve) : m/z 227.6 $[M-H]^-$(100).

1,8-Dihydroxyxanthone (**3d**): Yield: 1.8 %, m.p.: 190-191°C, lit. 192-194 C° (Mills et al., 1995), R_f 0.37 (30% acetone-hexane); ESIMS (-ve) : m/z 227.7 $[M-H]^-$ (100).

1,3,6-Trihydroxyxanthone (**3e**): Yield 59.0 %, m.p. 260 °C (decomp.), lit. 332°C (decomp.) (Lund et al., 1953), R_f 0.37 (30% acetone-hexane); ESIMS (-ve) : m/z 243.7 $[M-H]^-$ (100).

1,3,8-Trihydroxyxanthone (**3f**): Yield 60.0 %, m.p. 270-272 °C, lit. 269–270°C (Fonteneau et al., 2001), R_f 0.37 (30% acetone-hexane); ESIMS (-ve) : m/z 243.9 $[M-H]^-$ (100).

1,3,6,8-Tetrahydroxyxanthone (**3g**): Yield: 32.0 %, m.p. 343-345°C, lit. 347-348°C (Pillai et al., 1986), R_f 0.30 (40% acetone-hexane); ESIMS (-ve) : m/z 259.6 $[M-H]^-$ (100).

Anti-AChE Assay

The Ellman's method (Ellman et al., 1961) was used to screen *in vitro* anti-AChE activity. Briefly, in the 96-well plates, 140 μL of 10 mM sodium phosphate buffer (pH 8.0), 20 μL of a solution of *Electrophorus electricus* AChE (eeAChE, 0.2 unit/mL in 10 mM sodium phosphate buffer, pH 8.0) and 20 μL of test compound solution dissolved in 80% MeOH (a final concentration of 0.1 mg/mL) were mixed and incubated at RT for 15 min. The reaction was started by adding 20 μL of mixture solution of 5 mM DTNB in 10 mM sodium phosphate buffer (pH 8.0), containing 0.1% bovine serum albumin (BSA) and 5 mM acetylthiocholine (ASCh) in 10 mM sodium phosphate buffer, pH 8.0 (5:1). The hydrolysis of ASCh was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion as result of reaction with DTNB with thiocholines (SCh), catalyzed by enzymes at a wavelength of 405 nm and the absorbance was measured after 2 min of incubation at RT. Percentage of inhibition was calculated by comparing the rate of enzymatic hydrolysis of ASCh for the sample to that of the blank (80% MeOH in buffer). Galantamine was used as a standard.

Cytotoxicity studies

The *in vitro* neurotoxicity testing was carried out according to the procedures described previously using neuroblastoma cell SH-SY5Y (ATCC CRL-2266) (Meesarapee et al., 2013). The cells were grown at 37°C in a 1:1 mixture of minimum essential media (MEM): Ham-F12 supplemented with 10% heat inactivated fetal bovine serum (FBS), 25 mg/mL penicillin, 25 U/mL streptomycin, 1 mM sodium pyruvate and 1 mM nonessential amino acid. After seeding, cells were grown in a humidified atmosphere at 37°C, 5% CO_2 for 24 h to reach 80% confluence.

Cell viability was quantified by resazurin based-cell viability assay. The cells were plated into 96-well culture plates, 10^4 cell/well, and incubated over night at 37 °C, 5% CO_2. Then the cell were treated with various concentrations (200, 150, 125, 100, 80, 40, 20, 10 and 5 μM) of the tested compound for 24 hr, followed by addition of resazurin dye solution (100 μg/mL) in an amount equal to 10% of the culture medium volume. Return cultures to incubator for 2-4 h. depending on cell type and maximum cell density. The plates were measured fluorometrically by monitoring the increase in fluorescence at a wavelength of 590 nm using an excitation wavelength of 530 nm.

Statistical analysis

Neurotoxicity studies were carried out where possible at least in triplicate and on four different occasions. The results are reported as mean ± standard error (SEM). Standard curves were generated and calculation of the 50% inhibitory concentration (IC_{50}) values was done using GraphPad Prism Version 5.00 for Windows (GraphPad Software Inc.). Cytotoxicity results are expressed as the percentage cell survival compared to the control using a dose response curve.

Chemistry

Various oxygenated xanthones **3a-3g** containing different number and position of hydroxyl groups on the hydroxyxanthen-9-one nucleus were prepared in 1.8-71.0 % yields (Figure 1) from accessible starting materials using those previous described oxidative coupling reaction (Pillai et al., 1986). A mixture of appropriate hydroxybenzoic acid: salicylic acid (**1a**), 2,6-dihydroxybenzoic acid (**1b**), 2,4-dihydroxybenzoic acid (**1c**), 2,4,6-trihydroxybenzoic acid (**1d**) and activated phenol, resorcinol (**2a**) or phloroglucinol (**2b**), was heated in the presence of phosphorus pentoxide (P_2O_5) in methanesulfonic acid (MsOH). The structures of these compounds were confirmed mainly by means of NMR (1D- and 2D-NMR) and MS techniques. NMR data of these compounds were in agreement with the structure and were consistent with the literature values.

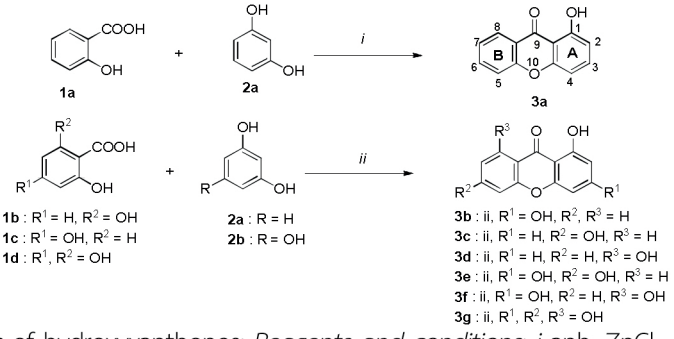


Figure 1 Synthesis of hydroxyxanthones: *Reagents and conditions*: *i* anh. $ZnCl_2$, 160 °C, 6 h, 71.0 %; *ii* P_2O_5/CH_3SO_3H, 80±5 °C, 30-35 min, 1.8-71.0%.

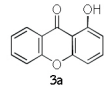
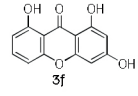
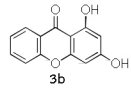
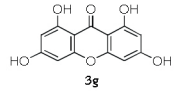
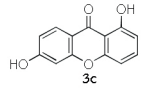
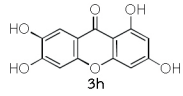
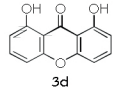
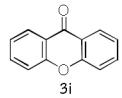
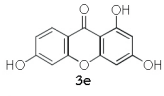
Results and Discussion

Antiacetylcholine esterase and neurotoxicity evaluations

All synthesized xanthones **3a-3g**, together with a naturally isolated 1,3,6,7,-tetrahydroxyxanthone (**3h**) (Suksamrarn and Komutiban, 2011) and a commercially available unsubstituted xanthone **3i** were tested for their anti-AChE activity against the *Electrophorus electricus* AChE (eeAChE) by employing the procedure established by Ellman (Ellman et al., 1961) and their IC_{50} values are shown in Table 1. Among the tested compounds, 1,3,6-trihydroxyxanthone (**3e**, IC_{50}= 69.45 μM) was the highest activity which was comparable to that of 1-hydroxyxanthone (**3a**, IC_{50} = 70.83 μM). A drop in activity was noted for the dihydroxylated series (**3b**-**3d**), trihydroxyxanthone (**3f**) and tetrahydroxyxanthone (**3g**) with their IC_{50} ranges of 79.18–150.61 μM. It should be noted that, the hydroxyl group at peri carbon C-1 seemed crucial as was evident from the activity of 1-hydroxyxanthone (**3a**, IC_{50} = 70.83μM) compared with the inactivity of the unsubstituted one **3i**.

A further study towards the effect on the viability of SH-SY5Y cells using the Resazurin cytotoxicity assay on the anti-AChE compounds was achieved. As shown in Table 1 and Figure 2, the AChE inhibitors **3b**, **3c**, **3e** and **3f** showed no neurotoxicity at the IC_{50} higher than 94 μM, in which **3e** was the least toxic at IC_{50} of 223.28 μM. An insolubility of hydroxyxanthones **3a**, **3d** and **3g** under the condition employed, their IC_{50} values therefore cannot be determined.

Table 1 *In vitro* inhibition against eeAChE and neurotoxicity activity of hydroxyxanthones

Structure	IC_{50}±SD (μM)		Structure	IC_{50}±SD (μM)	
	eeAChE	Neuro-toxicity		eeAChE	Neuro-toxicity
 3a	70.83±1.39	nd[b]	 3f	103.39±2.36	118.36±1.07
 3b	150.61±0.59	162.21±1.04	 3g	131.08±2.36	nd[b]
 3c	79.18±0.91	94.91±1.09	 3h	Inactive[a]	nd[b]
 3d	75.64±0.69	nd[b]	 3i	Inactive[a]	nd[b]
 3e	69.45±0.23	223.28±1.09	Galantamine	1.45±0.04	nd[b]

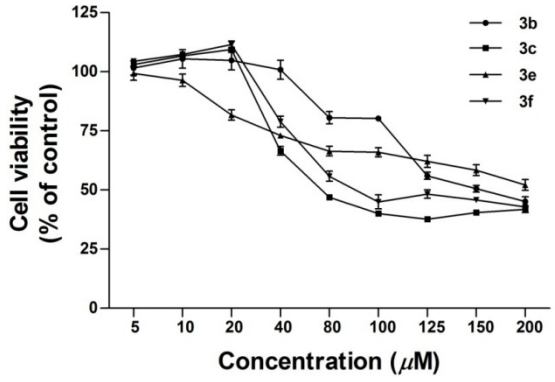


Figure 2 Effects of hydroxyxanthones (**3b, 3c, 3e** and **3f**) on the viability of SH-SY5Y cells. The cells were treated with various concentrations of the tested compounds as measured by resazurin based-cell viability assay

Conclusion

By employing a one-step oxidative cyclization process, a series of hydroxylated xanthones was prepared and evaluated as new moderate to weak inhibitors of AChE without neurotoxicity. Hydroxyxanthones are presented, though in small amount, in many Clusiaceous or Guttifereous and other plants. The obtained results are valuable for health benefit concerning the safety medicinal plant extracts and/or their metabolites utilizations. Furthermore, 1,3,6-trihydroxy xanthone (**3e**), which was the most active AChE inhibitor and has no toxicity against neuroblastoma cells, represents a useful template for further development of new anti-AD agents.

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