Synthesis of 1-Hydroxy-3-*O*-Substituted Xanthone Derivatives and their Structure-activity Relationship on Acetylcholinesterase Inhibitory Effect

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Abstract

Xanthenes are valuable compounds in drug design and development, attributed to their multi-dimensional pharmacological properties, including anti-cancer, anti-bacterial, anti-malarial, anti-inflammatory and anti-cholinesterase. This study focused on the synthesis of 1,3-dihydroxyxanthone (**1**) and its new 1-hydroxy-3-*O*-substituted derivatives with alkyl (**2a-2f**), alkenyl (**2g-2k**), alkynyl (**2l-2n**) and alkylated phenyl (**2o-2r**) groups and were synthesised in a high percentage yield of >70%, except for **2l** and **2p**. Their structures were confirmed by MS, NMR and FTIR spectroscopic techniques. The evaluation of acetylcholinesterase (AChE) inhibitory activities showed that all the substituted xanthenes (**2a-2r**) are more potent than **1**. Compounds **2g** and **2j** are the strongest AChE inhibitors with the IC₅₀ values of 20.8 and 21.5 μ M and their enzyme kinetic analyses indicated that these derivatives possess a mixed-mode inhibition, where they targeted both the active sites and allosteric sites of AChE. Molecular docking study revealed that **2g** binds favourably to the active site of AChE via π - π stacking and hydrogen bonding, in addition to π -alkyl interaction and alkyl interaction from the substituent group. The xanthone derivatives are identified as potential lead compounds for further development of Alzheimer's disease treatments.

Introduction

Every process in the human body, including sensory and motoric, are coordinated and regulated by the complex and sophisticated nervous system. The nervous system is vulnerable to various disorders, and even a slight interference to the neuron's structural pathway can induce dysfunction in the nervous system¹. Among neurological disorders, Alzheimer's disease (AD) has the highest prevalence, with an estimated 47 million cases worldwide in 2020². The widely recognised pathological mechanisms of AD include progressive loss of cholinergic neurotransmission, aggregation of extracellular beta-amyloid plaques (A β -plaques), and intracellular neurofibrillary tangles (NFT) from abnormal hyperphosphorylations of tau proteins³⁻⁵. The most successful approach to managing AD is based on enhancing the cholinergic activity by administering acetylcholinesterase (AChE) inhibitors^{6,7}. These agents inhibit the hydrolysis of a neurotransmitter, acetylcholine, to improve the associated cognitive function of AD patients^{8,9}. Unfortunately, the current cholinesterase inhibitors, donepezil¹⁰, galantamine¹¹ and rivastigmine¹², have been associated with adverse effects, including nausea, dizziness, blurred vision, loss of appetite, vomiting and diarrhoea^{10,13,14}. Meanwhile, another cholinesterase inhibitor, namely tacrine, was the first anti-Alzheimer's drug approved by Food and Drug Administration (FDA)¹⁵, but its clinical use has been discontinued due to its shortcomings in acute liver toxicity concerns toxicity^{16,17}. Thus, the development of drugs for AD is highly imperative due to the increased prevalence and irreversible consequences of AD, besides the vastly ageing world population.

A substantial amount of literature shows that extensive studies have been dedicated to searching for new leads of AChE inhibitors by screening bioactive compounds from plant sources^{18,19} and synthetic sources^{20,21}, as well as by computational-aided approaches²². Among the bioactive compounds, xanthenes are notable for their pharmacological benefits including anti-cancer^{23,24}, anti-bacterial^{25,26},

anti-inflammatory^{27,28}, anti-malarial^{29,30} and anti-cholinesterase^{22,31–37}. In particular, xanthenes as secondary plant metabolites^{32,34,35} and their synthetic derivatives^{31,38,39} have received attention due to their AChE inhibitory activity. Xanthenes are heterocyclic compounds with a symmetrical dibenzo- γ -pyrone as a basic scaffold, which is deemed "privileged" due to its planar tricyclic nucleus with a carbonyl moiety in the central skeleton that can carry a wide variety of substituents (Fig. 1). Its structural features allow xanthenes to bind to various biomolecular targets, giving rise to a plethora of pharmacological benefits.

Therefore, the current study aimed to synthesise a series of new xanthenes derivatives from 1,3-dihydroxyxanthone with different substituents, including alkyl (**2a-2f**), alkenyl (**2g-2k**), alkynyl (**2l-2n**) and alkylated phenyl (**2o-2r**) groups (Scheme 1). The AChE inhibition effects of these xanthone derivatives were evaluated, and their structure-activity relationship (SAR) was elucidated. Furthermore, enzyme kinetic analysis was carried out on the xanthone derivatives with the most potent activities and followed by molecular docking simulations to illustrate their modes of inhibition and binding interactions with AChE.

Results And Discussion

Synthesis & characterisation of 1,3-dihydroxyxanthone (1)

1,3-Dihydroxylated xanthone (**1**) was obtained as a yellow crystal with 82% of yield through the acylation-dehydration reaction between salicylic acid and phloroglucinol. The Eaton's reagent used in the reaction is an excellent acylation catalyst and condensation agent, providing a high yield of **1** with no observable concentration of benzophenone intermediate^{40–42}.

The structure of **1** was confirmed by the spectral data obtained from FT-IR, MS, NMR and FT-IR spectroscopies. The mass spectrum revealed a molecular ion peak at m/z 228, which coincides with the molecular weight of **1**. The FTIR spectrum demonstrated multiple essential peaks, including absorptions of free O-H at 3398 to 3478 cm^{-1} , -OH...O at 2600 to 3100 cm^{-1} , sp^2 C-H stretch at 3073 cm^{-1} , sp^3 C-H stretch at 2940 cm^{-1} , C = O stretch at 1651 cm^{-1} , aromatic C = C stretch at 1610 and 1454 cm^{-1} , C-O-H bend at 1341 cm^{-1} , C-O stretch at 1312 and 1076 cm^{-1} . In the NMR spectra, the solvents of acetone- d_6 resonated at 2.02ppm in ^1H -NMR and 29.0 and 205.4 ppm in ^{13}C -NMR, respectively. The ^1H -NMR spectrum recorded signals that were integrated for eight protons. The presence of two -OH groups at position C-1 and C-3 were observed as two singlet peaks at δ 12.90 and 9.92 ppm, respectively. The electron withdrawing effects of the carbonyl group at position C-9 leads to de-shielding effect and results in a higher chemical shift for 1-OH than 3-OH.. Six signals were observed in the range of δ 6.21–8.39 ppm that belong to the aromatic protons present in the tricyclic skeleton of xanthone. Two doublets were recorded at δ 8.17 and 7.51 for H-8 and H-5, respectively, while two triplets were detected at δ 7.82 and 7.44 for H-6 and H-7 respectively. Additionally, two singlet peaks at δ 6.42 and 6.25 were assigned to H-4 and H-2. Meanwhile, the ^{13}C NMR showed the presence of thirteen carbons which are in agreement with

the molecular formula of **1**, C₁₃H₈O₄. A highly de-shielded quaternary signal at δ 180.5 was assigned to the carbonyl carbon at C-9, and followed by the two hydroxylated carbons at C-3 (δ 165.7) and C-1 (163.9). Next, two signals at δ 158.1 and 156.0 were assigned to the two oxygenated aromatic carbons at C-4a and C-5a. Lastly, six methine carbons and two quaternary aromatic carbons of the xanthone skeleton were found in the range of δ 103.0 to 135.5 ppm. The spectral data assignment for **1** are consistent and in good agreement with the reported literature values^{39,43}

Synthesis & characterisation of xanthone derivatives (**2a-2r**)

The parent compound, **1** was reacted with alkyl, alkenyl, alkynyl and alkylated-phenyl bromides through nucleophilic substitution reaction to afford eighteen new xanthone derivatives, **2a-2r**. Sixteen derivatives were obtained in good yield with more than 70%, except for **2l** and **2p**, which were obtained at 67.0% and 44.6% of yield.

The mass spectra of **2a-2r** revealed molecular ion peaks that are corresponded to the molecular weight of respective xanthone derivatives. For the FT-IR, the structural characteristics of main skeleton of xanthenes for **2a-2r** are similar to **1**, including OH...O absorptions (2700 to 3500 cm⁻¹), *sp*² C-H stretch (3062–3095 cm⁻¹), *sp*³ C-H stretch (2900–2960 cm⁻¹), C = O stretch (1640–1660 cm⁻¹), aromatic C = C stretch (1434–1468 cm⁻¹), C-O-H bend (1292–1318 cm⁻¹) and C-O stretch (1073–1082 cm⁻¹), except for the broad O-H peak at the region of 3398–3478 cm⁻¹ were absent in **2a-2r**. The results indicate that the hydroxyl group at position C-3 was replaced by substituents groups successfully.

The etherification of **2a-2r** was further confirmed by ¹H NMR, where a singlet at δ 9.9 ppm corresponding to 3-OH in the structure of **1** has disappeared. Furthermore, additional signals were found at the region of δ 0.9–4.1 ppm across four series of derivatives (**2a-2r**). These are the peaks belong to alkoxy protons of the side chains substituted at position C-3 of **1**. Specifically, the presence of C = C groups in the derivatives of alkenyl-series (**2g-2k**) were confirmed by the observation of two proton signals located at higher chemical shift region of δ 5.1–5.9 ppm. Meanwhile, the shielding effect of C $\equiv$ C in the alkynyl-series (**2l-2n**) resulted in a slightly downfield proton chemical shift at δ 4.7–4.9 ppm. For the alkylated-phenyl series (**2o-2r**), the presence of an aromatic ring in the side chain were confirmed by the observation of three additional methine proton peaks at the region of δ 7.1–7.3 ppm, in comparison to that of **1**. On the other hand, the substitution of 1-OH was omitted due to a robust intramolecular hydrogen bonding with the adjacent carbonyl group⁴⁴. The evidence is the presence of a downfield singlet at around δ 12.8 ppm, which is associated with the chelated hydroxyl group at position C-1 in the ¹H NMR spectra of **1** and **2a-2r**.

Similarly, the alkoxy peaks appeared at the region of δ 57–75 ppm in ¹³C NMR spectra of xanthone derivatives **2a-2r** prove that the substitution reaction is successful through the formation of an ether bonding. In addition, the C = C carbon in **2g-2k** were confirmed by the observation of two signals at δ 115–136 ppm. Another two signals at around δ 85 – 73 ppm are associated with the C $\equiv$ C group in **2l-2n**.

For the derivatives **2o-2r**, three additional signals related to the methine carbon were detected at δ 126, 128 and 139 ppm, validating the presence of an additional aromatic ring in their structures.

Acetylcholinesterase Inhibitory Activities

The parent compound, 1,3-dihydroxyxanthone (**1**), and the synthesised derivatives (**2a-2r**) were investigated for their AChE inhibitory activities using slightly modified Ellman's method. The standard drug, tacrine, was found to inhibit AChE with an IC_{50} value of 0.87 μ M. The result is in good agreement with the reported value of tacrine against AChE in past literature^{20,45}. All the xanthenes, **1** and **2a-2r** were firstly evaluated for their AChE inhibition activities at a concentration of 45 μ M. The parent compound **1** did not show any inhibition activity towards AChE at this concentration. Interestingly, all eighteen derivatives, **2a-2r** demonstrated moderate-to-good inhibitory effects. In general, most of the derivatives could inhibit 50% of AChE at 45 μ M, except for **2c**, **2l**, **2m** and **2n**. Subsequently, the IC_{50} values of the **1** and the derivatives (**2a-2r**) were determined at the micromolar level, as presented in Table 1. The IC_{50} value obtained for **1** was 157.47 μ M, which is close to that of previously published data by Thongchai et al. (2014) with 150.61 μ M)³⁶. The inhibition activity of **1** is significantly weaker than 3-hydroxyxanthone, which has an IC_{50} value of 2.4 μ M against AChE³³. This finding shows that the hydroxyl group at position C-1 of xanthone failed to contribute favourable effects to anti-AChE activities. This might be due to its close distance to the adjacent carbonyl group, resulting in a significant chelating effect^{37,44}.

As expected, the eighteen xanthone derivatives (**2a-2r**) showed significantly stronger anti-AChE effects than **1** with IC_{50} values ranging from 20.81 to 71.22 μ M. The results indicate that substituting hydrophobic moiety at position C-3 of **1** improved AChE inhibition activity significantly. Among the derivatives, **2g** and **2j** appeared as the most potent AChE inhibitors with IC_{50} values of 20.81 and 21.51 μ M. Notably, the inhibition effects of these two derivatives are approximately seven-fold stronger than **1**. In agreement with our findings, Qin et al. (2013) revealed that additional hydrophobic nature is exceptionally favourable for the anti-AChE effects of hydroxy xanthenes, evidenced by having an allyloxy group at C-3 resulted in an approximately eighteen times more potent activity. The authors also reported that the activity was further increased by forty and forty-five times when the C-3 of xanthone was substituted with a prenyloxy and methoxyl groups, respectively³⁹.

Subsequently, the AChE inhibition effects of different types of substituents at position C-3 of xanthenes were elucidated. The xanthone derivatives, which are substituted with alkenyl (**2g-2k**) and alkylated-phenyl (**2o-2r**) groups, were observed to be significantly more potent than the other groups of derivatives, including alkyl (**2a-2f**) and alkynyl (**2l-2n**) groups (refer to Table 1). The pronounced AChE inhibitory effects of **2g-2k** clearly showed the importance of C = C in contributing to the activity. The results are consistent with a recently published report that described the C = C moieties in prenylated and geranylated xanthenes forms favourable π - σ and π -alkyl interaction with Trp82 in the choline-binding pocket and Trp86 in the anionic active site of the AChE, respectively³⁵.

Besides that, the positive effect of the other aromatic ring in **2o-2r** is apparent (Table 1). The xanthenes bearing phenylpropyl (**2o**) and phenylbutoxyl (**2p**) groups showed a significant increase in AChE inhibition levels, which are about 80%-85% stronger than **1**. Similar findings were observed in our previous study, whereby the substitution of the hydroxyl group at C-3 with a phenylpropoxyl or phenylbutoxyl group resulted in 48%-63% stronger anti-AChE effects compared to its parent, 3-hydroxyxanthone³³. This observation is in agreement with several previous reports indicating that arene groups in xanthenes allow stronger binding affinity to AChE through hydrophobic π - π interactions with Trp84 in the choline-binding pocket⁴⁶⁻⁴⁹.

The structure-activity relationship (SAR) elucidated for the anti-AChE activity of xanthone derivative (**2a-2r**) revealed that the chain length and linearity of the hydrocarbon side chain at C-3 did play a role in the inhibition effects. Generally, the xanthone derivatives with a substituent group constituted of four carbons straight-chain have shown to possess a more substantial AChE inhibition effect throughout the alkyl (**2a-2f**), alkenyl (**2g-2k**), alkynyl (**2l-2n**) and alkylphenyl series (**2o-2r**). For instance, **2a** is a derivative that carries four methylene carbons in straight-chain and exhibited the most potent inhibition amongst the alkyl series (**2a-2f**). A reduction in the chain length of hydrocarbon side-chain to three methylenes has significantly weaker AChE inhibition effects, as observed in **2b**. The other detrimental effect was seen for **2c**, which has a 3-methylene branched-chain substituent group. Similarly, the most potent xanthone derivatives in each series bear four methylene carbons in straight-chain in their structures, which are **2g**, **2m** and **2p** for alkenyl, alkynyl and alkylated phenyl series, respectively.

The influence of carbon chain length on AChE inhibition effects was studied on xanthone derivatives previously^{22,33,50}. Particularly, Alawi et al. (2020) reported the *in vitro* AChE inhibition effects of several types of aliphatic chains in different lengths that substituted the hydroxyl groups at C-3 and C-6 of 3,6-dihydroxyxanthone. The authors revealed that among three to six hydrocarbon chains, a butoxyl (4-carbons) substitution resulted in a potent inhibitory effect with an IC_{50} value of 3.2 μ M and an optimum binding affinity to AChE (-8.19 kcal/mol)⁵⁰. Our previous study also observed similar findings on 3-*O*-substituted xanthenes, where those bearing 4-carbons chain length exhibited stronger inhibition activity than other chain lengths. For instance, 3-butoxyxanthone (IC_{50} value = 1.40 μ M) demonstrated stronger anti-AChE effects than 3-propoxyxanthone (IC_{50} value = 1.86 μ M), and 3-(but-3-en-1-yloxy)xanthone (IC_{50} value = 2.09 μ M) is more potent than 3-(pent-4-en-1-yloxy)- and 3-(hex-5-en-1-yloxy)-xanthone (IC_{50} : >3.40 μ M).

In summary, eighteen xanthone derivatives (**2a-2r**) showed stronger AChE inhibitory activity compared to its parent, 1,3-dihydroxyxanthone (**1**). In agreement with the previous reports, SAR analysis in the present work suggested that the hydrophobic characteristic of xanthone, especially at position C-3, is of great importance for its anti-AChE activity. Particularly, linear unsaturated C = C hydrocarbon or linear saturated hydrocarbon and phenyl group are the most favourable substituents to anti-AChE effects. Moreover, 1-hydroxy-3-*O*-substituted xanthenes bearing four methylene carbons straight-chain lengths are anticipated to have the optimum structural characteristics for the AChE inhibition activity.

Enzyme Kinetics Analysis

The xanthone derivatives, **2g** and **2j**, that exhibited the strongest AChE inhibition were investigated for their enzyme inhibition mode. The results of enzyme kinetics analysis were presented by the Lineweaver-Burk plot in **Figs. 2 and 3**. Both the reciprocal plots of **2g** and **2j** showed the lines that intersect at the secondary quadrant, which indicates a mixed-mode inhibition. The indication of mixed-mode inhibition was further supported by the Michaelis-Menten parameter tabulated in **Table S1 & S2**, which showed both the maximal velocity of the AChE-ATCl enzyme-substrate reaction (V_{max}) and affinity (K_m) were affected by the addition of **2g** and **2j**. A mixed-mode inhibition allows the binding of a substrate (ATCl) to an enzyme (AChE) but with reduced affinity. The results revealed that the xanthone derivatives are likely to bind to either the active site of AChE, including esteratic site (ES) and anionic catalytic site (AS), as well as allosteric sites such as peripheral active site (PAS).

Binding Interactions of 1-hydroxy-3-(but-3-en-1-yloxy)-9H-xanthen-9-one (**2g**) with AChE

The flexible docking results, as shown in **Figure 4**, suggest that **2g** could bind favourably (-9.0 kcal/mol) to the active site of *Electrophorus electricus* AChE. The planar geometry of the xanthone ring allowed it to enter the gorge and bind deeply into the anionic catalytic sites. The ring forms π - π stacking with the phenol side-chain of Tyr341. On the other hand, the carbonyl group directed towards the hydration site comprised of D74, T83, W86, N87 and S125 residues and formed hydrogen bonding interactions with one of the buried water molecules and hydroxyl side-chain of Tyr124. The hydroxyl group in the first position of the xanthone ring failed to improve the binding interactions with either the crystal water molecules or the adjacent residues. This could be due to the strong chelating effect of the hydroxyl group with the adjacent carbonyl group, hence preventing hydrogen bonding interaction. On the other hand, we observed that **2g** exhibited a similar binding pose in the human AChE (-13.9 kcal/mol). The but-1-ene group appeared to be flexible and could interact with Leu289 and Ser293 (*Electrophorus electricus* AChE) and Tyr72, Ser298 and Tyr124 (human AChE), further validating the importance of C=C group in the AChE inhibition effects of xanthenes.

Material And Methods

All chemicals and solvents used in the synthesis reactions were analytical grade (> 95%) and used as received from Sigma Aldrich (Germany). Silica gel 60 (40–63 μ m) and thin-layer chromatography (TLC) (silica gel 60 F₂₅₄) were obtained from Merck (United States). Acetylcholinesterase from *Electrophorus electricus* type VI-S, 500U (AChE, EC 3.1.1.7), acetylthiocholine iodide ($\geq 98\%$), 5,5'-dithiobis(2-nitrobenzoic) acid ($\geq 98\%$) were obtained from Sigma-Aldrich (Malaysia). Sodium phosphate monobasic ($\geq 99\%$) and sodium phosphate dibasic ($\geq 99\%$) were obtained from Systerm Chemicals (Malaysia). Tacrine hydrochloride ($\geq 98\%$) was purchased from Cayman Chemical (United States).

Melting points (m.p.) were determined on digital melting point apparatus IA9000 (Electrothermal™). Fourier transform infrared spectroscopy (FTIR) spectra were recorded on Spectrum™ 100 Optica FT-IR

Spectrometer (Perkin Elmer) with potassium bromide (KBr) pellet technique. ^1H - and ^{13}C -nuclear magnetic resonance (NMR) spectra were recorded in chloroform- d or acetone- d_6 at 600 MHz and 125 MHz, respectively, using ECZ600R/S1 NMR spectrometer (JEOL). Mass spectra (EI) were recorded on Agilent 7890A GC instrument equipped with Agilent 5975C MSD single quadrupole mass detector (Agilent Technologies). The GC system was equipped with helium as a carrier gas and HP-5MS (5%-phenyl-methylpolysiloxane) capillary column as stationary phase (diameter: 0.25 mm; length: 30 m; film thickness: 0.25 μm). The optically active compound was identified with polarimetry analysis on Polarimeter POL-1 (Optika Microscopes) at 25 °C with the optical measurement corrected and converted to specific rotation. All enzymatic reactions were carried out in triplicate on Epoch 2 UV-Vis microplate spectrophotometer (BioTek Instruments)

Synthesis of xanthones

The synthesis of 1,3-dihydroxyxanthone (**1**) was carried out through the condensation of salicylic acid and phloroglucinol with Eaton's reagent^{40,51}. The reaction mixture was heated at 80 °C for 30 mins (Scheme 1). The synthesis reaction was monitored by TLC for reaction completion. After completion, the mixture was introduced into ice-cool water and stirred for an hour. The mixture was vacuum filtered, and the resulted orange solid crudes were dried overnight at room temperature. The solid precipitate was introduced to a stepwise gradient column chromatographic purification with silica gel 60 using *n*-hexane, dichloromethane and chloroform.

Compound **1** was reacted with respective bromides of alkyl, alkenyl, alkynyl, and alkylated phenyl groups in acetone and potassium carbonate, as shown in Scheme 1. The mixture was refluxed at 60°C for hours. Then, the reaction mixture was mixed with distilled water and extracted using chloroform. The organic layer was collected and washed with a diluted hydrochloric acid solution (10% v/v), followed by saturated sodium carbonate solution and distilled water. The crude product was purified by column chromatography technique over silica gel through a stepwise gradient system to obtain pure derivatives (**2a–2r**). The pure xanthone derivatives were structurally elucidated using MS, NMR and FT-IR spectroscopic analyses.

1,3-Dihydroxy-9*H*xanthen-9-one (**1**): Yield: 82%; Yellow crystalline; M.P. 259–260°C (lit.^{ref} 259°C); m/z , $\text{C}_{13}\text{H}_8\text{O}_4$: 228, 200, 171, 155, 131, 115, 93, 77, 51; IR ν_{max} cm^{-1} : 3073, 2940, 1651, 1610, 1454, 1341, 1312, 1076; ^1H NMR (600 MHz, acetone- d_6): δ_{H} 12.90 (s, 1H, OH-1), 9.92 (s, 1H, OH-3), 8.17 (d, J = 8.3 Hz, 1H, H-8), 7.82 (t, J = 8.3 Hz, 1H, H-6), 7.51 (d, J = 8.3 Hz, 1H, H-5), 7.44 (t, J = 8.3 Hz, 1H, H-7), 6.42 (s, 1H, H-4), 6.25 (s, 1H, H-2); ^{13}C NMR (150 MHz, acetone- d_6): δ_{C} 180.5 (C-9), 165.7 (C-3), 163.9 (C-1), 158.1 (C-4a), 156.0 (C-5a), 135.5 (C-6), 125.5 (C-8), 124.3 (C-7), 120.5 (C-8a), 117.7 (C-5), 103.0 (C-9a), 98.2 (C-2), 94.1 (C-4).

1-Hydroxy-3-butoxy-9*H*xanthen-9-one (**2a**): From **1** and 1-bromobutane; Yield: 93%; Pale yellow crystalline; M.P. 119–121°C (lit.^{ref} 118–120°C); m/z , $\text{C}_{17}\text{H}_{16}\text{O}_4$: 284, 254, 228, 200, 172, 155, 131, 115, 93,

77, 51; IR ν_{max} cm^{-1} : 3074, 2935, 2700, 1656, 1608, 1467, 1346, 1297, 1080; ^1H NMR (600 MHz, CDCl_3): δ_{H} 12.81 (s, 1H, OH-1), 8.20 (t, J = 1.8 Hz & 8.3 Hz, 1H, H-8), 7.67 (td, J = 1.8 Hz & 8.3 Hz, 1H, H-6), 7.38 (d, J = 8.3 Hz, 1H, H-5), 7.33 (t, J = 8.3 Hz, 1H, H-7), 6.37 (d, J = 1.8 Hz, 1H, H-4), 6.29 (d, J = 1.8 Hz, 1H, H-2), 4.02 (t, J = 6.4 Hz, 2H, H-1'), 1.69 (m, 2H, H-2'), 1.49 (m, 2H, H-3'), 0.98 (t, J = 7.4 Hz, 3H, H-4'); ^{13}C NMR (150 MHz, CDCl_3): δ_{C} 180.8 (C-9), 166.4 (C-3), 163.6 (C-1), 157.8 (C-4a), 157.1 (C-5a), 135.0 (C-6), 125.9 (C-8), 124.0 (C-7), 120.7 (C-8a), 117.6 (C-5), 103.9 (C-9a), 97.5 (C-2), 93.3 (C-4), 68.5 (C-1'), 31.1 (C-2'), 19.2 (C-3'), 13.9 (C-4').

1-Hydroxy-3-propoxy-9*H*-xanthen-9-one (**2b**): From compound **1** and 1-bromopropane; Yield: 91%; Pale yellow crystalline; M.P. 154–155°C; m/z , $\text{C}_{16}\text{H}_{14}\text{O}_4$: 270, 255, 228, 200, 172, 155, 131, 115, 92, 77, 51; IR ν_{max} cm^{-1} : 3073, 2934, 1658, 1605, 1466, 1341, 1292, 1079; ^1H NMR (600 MHz, CDCl_3): δ_{H} 12.83 (s, 1H, OH-1), 8.22 (dd, J = 1.8 Hz & 8.3 Hz, 1H, H-8), 7.68 (td, J = 1.8 Hz & 8.3 Hz, 1H, H-6), 7.39 (d, J = 8.3 Hz, 1H, H-5), 7.35 (t, J = 8.3 Hz, 1H, H-7), 6.40 (d, J = 1.8 Hz, 1H, H-4), 6.32 (d, J = 1.8 Hz, 1H, H-2), 3.99 (t, J = 6.4 Hz, 2H, H-1'), 1.80 (m, 2H, H-2'), 1.05 (t, J = 7.3 Hz, 3H, H-3'); ^{13}C NMR (150 MHz, CDCl_3): δ_{C} 180.8 (C-9), 166.5 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 135.0 (C-6), 125.9 (C-8), 124.04 (C-7), 120.7 (C-8a), 117.7 (C-5), 103.9 (C-9a), 97.5 (C-2), 93.3 (C-4), 70.3 (C-1'), 22.4 (C-2'), 10.5 (C-3').

1-Hydroxy-3-isobutoxy-9*H*-xanthen-9-one (**2c**): From compound **1** and 1-bromo-2-methylpropane; Yield: 86%; Light green crystalline; M.P. 134–136°C; m/z , $\text{C}_{17}\text{H}_{16}\text{O}_4$: 284, 254, 228, 200, 172, 155, 131, 115, 93, 77, 57; IR ν_{max} cm^{-1} : 3065, 2962, 1656, 1605, 1468, 1343, 1294, 1077; ^1H NMR (600 MHz, CDCl_3): δ_{H} 12.82 (s, 1H, OH-1), 8.22 (d, J = 8.2 Hz, 1H, H-8), 7.69 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-6), 7.40 (d, J = 8.2 Hz, 1H, H-5), 7.35 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-7), 6.40 (s, 1H, H-4), 6.32 (s, 1H, H-2), 3.79 (d, J = 6.9 Hz, 2H, H-1'), 2.12 (m, 1H, H-2'), 1.03 (d, J = 6.9 Hz, 6H, H-3'); ^{13}C NMR (150 MHz, CDCl_3): δ_{C} 180.8 (C-9), 166.6 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 135.0 (C-6), 125.9 (C-8), 124.0 (C-7), 120.7 (C-8a), 117.6 (C-5), 103.9 (C-9a), 97.6 (C-2), 93.3 (C-4), 75.0 (C-1'), 28.2 (C-2'), 19.2 (C-3').

1-Hydroxy-3-((4-methylpentyl)oxy)-9*H*-xanthen-9-one (**2d**): From compound **1** and 1-bromo-4-methylpentane; Yield: 81%; Light green crystalline; M.P. 120–122°C; m/z , $\text{C}_{19}\text{H}_{20}\text{O}_4$: 312, 293, 251, 228, 200, 172, 144, 121, 101, 77, 55; IR ν_{max} cm^{-1} : 3067, 2953, 1656, 1604, 1466, 1347, 1298, 1077; ^1H NMR (600 MHz, CDCl_3): δ_{H} 12.83 (s, 1H, OH-1), 8.22 (d, J = 8.2 Hz, 1H, H-8), 7.69 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-6), 7.40 (d, J = 8.2 Hz, 1H, H-5), 7.35 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-7), 6.40 (d, J = 2.7 Hz, 1H, H-4), 6.32 (d, J = 2.7 Hz, 1H, H-2), 4.01 (t, J = 6.9 Hz, 2H, H-1'), 1.81 (m, 2H, H-3'), 1.61 (m, 1H, H-4') 1.33 (m, 2H, H-2'), 0.92 (d, J = 6.9 Hz, 6H, H-5'); ^{13}C NMR (150 MHz, CDCl_3): δ_{C} 180.8 (C-9), 166.5 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 135.0 (C-6), 125.9 (C-8), 124.0 (C-7), 120.7 (C-8a), 117.7 (C-5), 103.9 (C-9a), 97.5 (C-2), 93.3 (C-4), 69.1 (C-1'), 35.1 (C-3'), 27.9 (C-4'), 27.0 (C-2'), 22.6 (C-5').

1-Hydroxy-3-(isopentyloxy)-9*H*-xanthen-9-one (**2e**): From compound **1** and 1-bromo-3-methylbutane; Yield: 82%; Light green crystalline; M.P. 122–123°C; m/z , $\text{C}_{18}\text{H}_{18}\text{O}_4$: 298, 279, 255, 228, 200, 172, 144, 115, 96, 77, 55; IR ν_{max} cm^{-1} : 3071, 2956, 1656, 1606, 1467, 1345, 1296, 1078; ^1H NMR (600 MHz, CDCl_3): δ_{H}

12.83 (s, 1H, OH-1), 8.22 (d, J = 8.2 Hz, 1H, H-8), 7.69 (dd, J = 2.7, 8.2 Hz, 1H, H-6), 7.40 (d, J = 8.2 Hz, 1H, H-5), 7.35 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-7), 6.40 (d, J = 2.7 Hz, 1H, H-4), 6.32 (d, J = 2.7 Hz, 1H, H-2), 4.06 (t, J = 6.9 Hz, 2H, H-1'), 1.83 (m, 1H, H-3'), 1.70 (m, 2H, H-2'), 0.97 (d, J = 6.9 Hz, 6H, H-4'); ^{13}C NMR (150 MHz, CDCl_3): δ_{C} 180.8 (C-9), 166.5 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 135.0 (C-6), 125.9 (C-8), 124.0 (C-7), 120.7 (C-8a), 117.6 (C-5), 103.9 (C-9a), 97.5 (C-2), 93.3 (C-4), 67.2 (C-1'), 37.7 (C-2'), 25.1 (C-3'), 22.6 (C-4').

(*R*)-(-)-1-Hydroxy-3-(2-methylbutoxy)-9*H*-xanthen-9-one (**2f**): From compound **1** and (*S*)-(+)-1-bromo-2-methylbutane, Yield: 81%; Light green crystalline; M.P. 125–126°C; $[\alpha]_{\text{D}}$: -42.8° (in ethanol); m/z , $\text{C}_{18}\text{H}_{18}\text{O}_4$: 298, 269, 253, 228, 200, 172, 155, 139, 121, 105, 77, 55; IR ν_{max} cm^{-1} : 3088, 2964, 1657, 1607, 1468, 1359, 1318, 1078; ^1H NMR (600 MHz, CDCl_3): δ_{H} 12.82 (s, 1H, OH-1), 8.22 (d, J = 8.2 Hz, 1H, H-8), 7.68 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-6), 7.40 (d, J = 8.2 Hz, 1H, H-5), 7.35 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-7), 6.40 (s, 1H, H-4), 6.32 (s, 1H, H-2), 3.89 (dd, J = 5.5 Hz & 9.6 Hz, 1H, H-1'a), 3.81 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-1'b), 1.89 (m, 1H, H-2'), 1.57 (m, 1H, H-3'a), 1.28 (m, 1H, H-3'b), 1.02 (d, J = 6.9 Hz, 3H, H-5'), 0.95 (t, J = 6.9 Hz & 8.2 Hz, 3H, H-4'); ^{13}C NMR (150 MHz, CDCl_3): δ_{C} 180.8 (C-9), 166.6 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 135.0 (C-6), 125.9 (C-8), 124.0 (C-7), 120.7 (C-8a), 117.6 (C-5), 103.9 (C-9a), 97.6 (C-2), 93.3 (C-4), 73.6 (C-1'), 34.6 (C-2'), 26.1 (C-3'), 16.5 (C-5'), 11.4 (C-4').

1-Hydroxy-3-(but-3-en-1-yloxy)-9*H*-xanthen-9-one (**2g**): From compound **1** and 4-bromo-1-butene, Yield: 82%; Light green crystalline; M.P. 105–107°C; m/z , $\text{C}_{17}\text{H}_{14}\text{O}_4$: 282, 253, 228, 200, 172, 144, 121, 101, 77, 55; IR ν_{max} cm^{-1} : 3068, 2957, 1659, 1601, 1439, 1362, 1323, 1078; ^1H NMR (600 MHz, CDCl_3): δ_{H} 12.83 (s, 1H, OH-1), 8.22 (d, J = 8.2 Hz, 1H, H-8), 7.69 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-6), 7.40 (d, J = 8.2 Hz, 1H, H-5), 7.35 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-7), 6.40 (d, J = 2.7 Hz, 1H, H-4), 6.32 (s, 1H, H-2), 5.89 (m, 1H, H-3'), 5.19 (d, J = 19.2 Hz, 1H, H-4'a), 5.13 (d, J = 8.2 Hz, 1H, H-4'b), 4.09 (t, J = 6.9 Hz, 2H, H-1'), 2.57 (m, 2H, H-2'); ^{13}C NMR (150 MHz, CDCl_3): δ_{C} 180.9 (C-9), 166.2 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 135.1 (C-6), 133.8 (C-3'), 125.9 (C-8), 124.1 (C-7), 120.7 (C-8a), 117.7 (C-5, C-4'), 104.0 (C-9a), 97.6 (C-2), 93.4 (C-4), 67.9 (C-1'), 33.4 (C-2').

1-Hydroxy-3-((2-methylallyl)oxy)-9*H*-xanthen-9-one (**2h**): From compound **1** and 3-bromo-2-methylpropene, Yield: 75%; Light green crystalline; M.P. 127–128°C; m/z , $\text{C}_{17}\text{H}_{14}\text{O}_4$: 282, 267, 241, 225, 207, 190, 165, 139, 121, 93, 77, 51; IR ν_{max} cm^{-1} : 3095, 2967, 1655, 1606, 1466, 1342, 1293, 1081; ^1H NMR (600 MHz, CDCl_3): δ_{H} 12.81 (s, 1H, OH-1), 8.20 (d, J = 8.2 Hz, 1H, H-8), 7.66 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-6), 7.38 (d, J = 8.2 Hz, 1H, H-5), 7.33 (t, J = 6.9 Hz & 8.2 Hz, 1H, H-7), 6.40 (d, J = 2.7 Hz, 1H, H-4), 6.32 (d, J = 2.7 Hz, 1H, H-2), 5.10 (s, 1H, H-4'a), 5.03 (s, 1H, H-4'b), 4.49 (s, 2H, H-1'), 1.83 (s, 3H, H-3'); ^{13}C NMR (150 MHz, CDCl_3): δ_{C} 180.8 (C-9), 165.9 (C-3), 163.6 (C-1), 157.7 (C-4a), 156.1 (C-5a), 139.8 (C-2'), 135.0 (C-6), 125.9 (C-8), 124.0 (C-7), 120.7 (C-8a), 117.6 (C-5), 113.6 (C-4'), 104.0 (C-9a), 97.8 (C-2), 93.6 (C-4), 72.2 (C-1'), 19.4 (C-3').

1-Hydroxy-3-(hex-5-en-1-yloxy)-9*H*-xanthen-9-one (**2i**): From compound **1** and 6-bromo-1-hexene, Yield: 70%; Light green crystalline; M.P. 107–108°C; *m/z*, C₁₉H₁₈O₄: 310, 293, 267, 251, 228, 200, 172, 155, 139, 121, 105, 77, 55; IR ν_{max} cm⁻¹: 3065, 2962, 1640, 1601, 1460, 1342, 1295, 1074; ¹H NMR (600 MHz, CDCl₃): δ_H 12.83 (s, 1H, OH-1), 8.22 (d, *J* = 8.2 Hz, 1H, H-8), 7.69 (t, *J* = 6.9 Hz & 8.2 Hz, 1H, H-6), 7.40 (d, *J* = 8.2 Hz, 1H, H-5), 7.35 (t, *J* = 6.9 Hz & 8.2 Hz, 1H, H-7), 6.40 (dd, *J* = 2.7 Hz, 1H, H-4), 6.32 (dd, *J* = 2.7 Hz, 1H, H-2), 5.82 (m, 1H, H-5'), 5.04 (d, *J* = 15.1 Hz, 1H, H-6'a), 4.98 (d, *J* = 8.2 Hz, 1H, H-6'b), 4.04 (t, *J* = 5.5 Hz & 6.9 Hz, 2H, H-1'), 2.13 (m, 2H, H-4'), 1.83 (m, 2H, H-2'), 1.57 (m, 2H, H-3'); ¹³C NMR (150 MHz, CDCl₃): δ_C 180.8 (C-9), 166.4 (C-3), 163.3 (C-1), 157.8 (C-4a), 156.1 (C-5a), 138.4 (C-5'), 135.0 (C-6), 125.9 (C-8), 124.1 (C-7), 120.7 (C-8a), 117.7 (C-5), 115.1 (C-6'), 103.9 (C-9a), 97.5 (C-2), 93.3 (C-4), 67.9 (C-1'), 33.4 (C-4'), 28.5 (C-2'), 25.3 (C-3').

1-Hydroxy-3-(pent-4-en-1-yloxy)-9*H*-xanthen-9-one (**2j**): From compound **1** and 5-bromo-1-pentene, Yield: 91%; Light green crystalline; M.P. 63–65°C; *m/z*, C₁₈H₁₆O₄: 296, 267, 251, 228, 200, 172, 155, 139, 121, 105, 77, 53; IR ν_{max} cm⁻¹: 3070, 2958, 1655, 1607, 1466, 1345, 1296, 1079; ¹H NMR (600 MHz, CDCl₃): δ_H 12.82 (s, 1H, OH-1), 8.22 (d, *J* = 7.8 Hz, 1H, H-8), 7.68 (t, *J* = 7.2 Hz & 7.8 Hz, 1H, H-6), 7.39 (d, *J* = 7.8 Hz, 1H, H-5), 7.34 (t, *J* = 7.2 Hz & 7.8 Hz, 1H, H-7), 6.39 (s, 1H, H-4), 6.31 (s, 1H, H-2), 5.84 (m, 1H, H-4'), 5.07 (d, *J* = 13.2 Hz, 1H, H-5'a), 5.02 (d, *J* = 9.6 Hz, 1H, H-5'b), 4.04 (t, *J* = 5.4 Hz & 7.2 Hz, 2H, H-1'), 2.24 (m, 2H, H-3'), 1.92 (m, 2H, H-2'); ¹³C NMR (150 MHz, CDCl₃): δ_C 180.8 (C-9), 166.3 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 137.5 (C-4'), 135.0 (C-6), 125.9 (C-8), 124.0 (C-7), 120.7 (C-8a), 117.6 (C-5), 115.7 (C-5'), 103.9 (C-9a), 97.5 (C-2), 93.3 (C-4), 67.9 (C-1'), 30.0 (C-3'), 28.1 (C-2').

1-Hydroxy-3-((4-methylpent-3-en-1-yl)oxy)-9*H*-xanthen-9-one (**2k**): From compound **1** and 5-bromo-2-methyl-2-pentene, Yield: 89%; Light green crystalline; M.P.: 110–111°C; *m/z*, C₁₉H₁₈O₄: 310, 293, 267, 251, 228, 200, 172, 155, 127, 101, 83, 55; IR ν_{max} cm⁻¹: 3067, 2973, 1652, 1604, 1466, 1347, 1298, 1077; ¹H NMR (600 MHz, CDCl₃): δ_H 12.82 (s, 1H, OH-1), 8.21 (d, *J* = 8.2 Hz, 1H, H-8), 7.68 (t, *J* = 6.9 Hz & 8.2 Hz, 1H, H-6), 7.39 (d, *J* = 8.2 Hz, 1H, H-5), 7.34 (t, *J* = 6.9 Hz & 8.2 Hz, 1H, H-7), 6.39 (s, 1H, H-4), 6.31 (s, 1H, H-2), 5.19 (t, *J* = 6.9 Hz & 8.2 Hz, 1H, H-3'), 3.99 (t, *J* = 6.9 Hz, 2H, H-1'), 2.50 (m, 2H, H-2'), 1.73 (s, 3H, H-5'), 1.67 (s, 3H, H-6'); ¹³C NMR (150 MHz, CDCl₃): δ_C 180.8 (C-9), 166.3 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 135.1 (C-6), 135.0 (C-4'), 125.9 (C-8), 124.0 (C-7), 120.7 (C-8a), 119.0 (C-3'), 117.6 (C-5), 103.9 (C-9a), 97.6 (C-2), 93.3 (C-4), 68.4 (C-1'), 28.0 (C-2'), 25.9 (C-5'), 18.0 (C-6').

1-Hydroxy-3-(pent-2-yn-1-yloxy)-9*H*-xanthen-9-one (**2l**): From compound **1** and 1-bromo-2-pentyne, Yield: 67%; Light green crystalline; M.P. 125–126°C; *m/z*, C₁₈H₁₄O₄: 294, 265, 249, 228, 207, 191, 171, 152, 133, 115, 96, 77, 51; IR ν_{max} cm⁻¹: 3085, 2971, 2244, 1734, 1649, 1601, 1462, 1365, 1318, 1166; ¹H NMR (600 MHz, CDCl₃): δ_H 12.85 (s, 1H, OH-1), 8.23 (t, *J* = 1.8 Hz & 8.3 Hz, 1H, H-8), 7.70 (td, *J* = 1.8 Hz & 8.3 Hz, 1H, H-6), 7.42 (d, *J* = 8.3 Hz, 1H, H-5), 7.36 (t, *J* = 8.3 Hz, 1H, H-7), 6.50 (d, *J* = 1.8 Hz, 1H, H-4), 6.41 (d, *J* = 1.8 Hz, 1H, H-2), 4.74 (t, *J* = 1.8 Hz, 2H, H-1'), 2.25 (m, 2H, H-4'), 1.14 (t, *J* = 7.3 Hz, 3H, H-5'); ¹³C NMR (150 MHz, CDCl₃): δ_C 181.0 (C-9), 165.0 (C-3), 163.6 (C-1), 157.7 (C-4a), 156.2 (C-5a), 135.1 (C-6), 126.0 (C-8),

124.1 (C-7), 120.7 (C-8a), 117.7 (C-5), 104.3 (C-9a), 97.9 (C-2), 90.8 (C-4), 90.8 (C-3'), 73.1 (C-2'), 57.1 (C-1'), 13.6 (C-5'), 12.6 (C-4').

1-Hydroxy-3-(but-2-yn-1-yloxy)-9*H*-xanthen-9-one (**2m**): From compound **1** and 1-bromo-2-butyne, Yield: 86%; Light green crystalline; M.P. 180–181°C; *m/z*, C₁₇H₁₂O₄: 280, 251, 228, 207, 165, 140, 121, 96, 77, 51; IR ν_{max} cm⁻¹: 3081, 2923, 2240, 1734, 1649, 1603, 1462, 1329, 1290; ¹H NMR (600 MHz, CDCl₃): δ_H 12.85 (s, 1H, OH-1), 8.23 (t, *J* = 1.8 Hz & 8.3 Hz, 1H, H-8), 7.70 (td, *J* = 1.8 Hz & 8.3 Hz, 1H, H-6), 7.41 (d, *J* = 8.3 Hz, 1H, H-5), 7.36 (t, *J* = 8.3 Hz, 1H, H-7), 6.49 (d, *J* = 1.8 Hz, 1H, H-4), 6.40 (d, *J* = 1.8 Hz, 1H, H-2), 4.72 (m, 2H, H-1'), 1.88 (t, *J* = 2.8 Hz, 3H, H-4'); ¹³C NMR (150 MHz, CDCl₃): δ_C 181.0 (C-9), 165.0 (C-3), 163.6 (C-1), 157.7 (C-4a), 156.1 (C-5a), 135.2 (C-6), 126.0 (C-8), 124.1 (C-7), 120.7 (C-8a), 117.7 (C-5), 104.3 (C-9a), 97.9 (C-2), 93.8 (C-4), 85.0 (C-2'), 73.0 (C-3'), 57.1 (C-1'), 3.8 (C-4').

1-Hydroxy-3-(but-3-yn-2-yloxy)-9*H*-xanthen-9-one (**2n**): From compound **1** and 3-bromo-1-butyne, Yield: 74%; Light green crystalline; M.P. 174–176°C; *m/z*, C₁₇H₁₂O₄: 280, 265, 228, 207, 181, 152, 132, 104, 77, 51; IR ν_{max} cm⁻¹: 3282, 3074, 3006, 2114, 1650, 1605, 1466, 1331, 1293, 1082; ¹H NMR (600 MHz, CDCl₃): δ_H 12.82 (s, 1H, OH-1), 8.22 (d, *J* = 1.8 Hz & 7.3 Hz, 1H, H-8), 7.70 (td, *J* = 1.8 Hz & 7.3 Hz, 1H, H-6), 7.41 (d, *J* = 8.3 Hz, 1H, H-5), 7.35 (t, *J* = 8.3 Hz, 1H, H-7), 6.52 (d, *J* = 1.8 Hz, 1H, H-4), 6.43 (d, *J* = 1.8 Hz, 1H, H-2), 4.94 (m, 1H, H-1'), 2.55 (d, *J* = 1.8 Hz, 1H, H-3'), 1.70 (d, *J* = 6.4 Hz, 3H, H-4'); ¹³C NMR (150 MHz, CDCl₃): δ_C 181.0 (C-9), 164.4 (C-3), 163.5 (C-1), 157.6 (C-4a), 156.1 (C-5a), 135.2 (C-6), 126.0 (C-8), 124.1 (C-7), 120.7 (C-8a), 117.7 (C-5), 104.4 (C-9a), 98.5 (C-2), 94.3 (C-4), 81.8 (C-2'), 75.0 (C-3'), 64.1 (C-1'), 22.1 (C-4').

1-Hydroxy-3-(3-phenylpropoxy)-9*H*-xanthen-9-one (**2o**): From compound **1** and 1-bromo-3-phenylpropane, Yield: 72%; Light green crystalline; M.P. 105–106°C; *m/z*, C₂₂H₁₈O₄: 346, 228, 200, 172, 144, 117, 91, 65; IR ν_{max} cm⁻¹: 3063, 2959, 1656, 1598, 1336, 1298, 1073; ¹H NMR (600 MHz, CDCl₃): δ_H 12.84 (s, 1H, OH-1), 8.23 (t, *J* = 2.7 Hz & 8.2 Hz, 1H, H-8), 7.69 (t, *J* = 8.2 Hz, 1H, H-6), 7.40 (d, *J* = 8.2 Hz, 1H, H-5), 7.35 (t, *J* = 8.2 Hz, 1H, H-7), 7.29 (dd, *J* = 2.7 Hz & 8.2 Hz, 2H, H-6'), 7.21 (d, *J* = 6.9 Hz, 2H, H-5'), 7.19 (s, 1H, H-7'), 6.39 (d, *J* = 2.7 Hz, 1H, H-4), 6.32 (s, 1H, H-2), 4.03 (t, *J* = 6.9 Hz, 2H, H-1'), 2.82 (t, *J* = 6.9 Hz & 8.2 Hz, 2H, H-3'), 2.14 (m, 2H, H-2'); ¹³C NMR (150 MHz, CDCl₃): δ_C 180.9 (C-9), 166.3 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 141.1 (C-4'), 135.1 (C-6), 128.6 (C-6'), 126.2 (C-5'), 125.9 (C-8, C-7'), 124.1 (C-7), 120.7 (C-8a), 117.7 (C-5), 104.0 (C-9a), 97.6 (C-2), 93.3 (C-4), 67.6 (C-1'), 32.1 (C-3'), 30.5 (C-2').

1-Hydroxy-3-(4-phenylbutoxy)-9*H*-xanthen-9-one (**2p**): From compound **1** and 1-bromo-4-phenylpentane, Yield: 45%; Light green crystalline; M.P. 130–131°C; *m/z*, C₂₃H₂₀O₄: 360, 228, 200, 172, 144, 115, 91, 65; IR ν_{max} cm⁻¹: 3062, 2936, 1661, 1601, 1434, 1332, 1294, 1075; ¹H NMR (600 MHz, CDCl₃): δ_H 12.84 (s, 1H, OH-1), 8.23 (t, *J* = 2.7 Hz & 8.2 Hz, 1H, H-8), 7.69 (t, *J* = 2.7 Hz & 8.2 Hz, 1H, H-6), 7.41 (d, *J* = 8.2 Hz, 1H, H-5), 7.35 (t, *J* = 8.2 Hz, 1H, H-7), 7.29 (t, *J* = 6.9 Hz & 8.2 Hz, 2H, H-7'), 7.20 (dd, *J* = 2.7 Hz & 8.2 Hz, 2H, H-6'), 7.18 (s, 1H, H-8'), 6.39 (s, 1H, H-4), 6.32 (d, *J* = 2.7 Hz, 1H, H-2), 4.04 (t, *J* = 6.9 Hz, 2H, H-1'), 2.70 (t, *J* = 8.2 Hz & 6.9 Hz, 2H, H-4'), 1.82 (m, H-2', 2H, H-3'); ¹³C NMR (150 MHz, CDCl₃): δ_C 180.9 (C-9), 166.4 (C-3),

163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 142.0 (C-5'), 135.0 (C-6), 128.5 (C-7'), 128.5 (C-6'), 126.0 (C-8'), 125.9 (C-8), 124.1 (C-7), 120.7 (C-8a), 117.7 (C-5), 103.9 (C-9a), 97.5 (C-2), 93.3 (C-4), 68.6 (C-1'), 35.6 (C-4'), 28.6 (C-2'), 27.8 (C-3').

1-Hydroxy-3-(phenethoxy)-9*H*-xanthen-9-one (**2q**): From compound **1** and 2-(bromoethyl) benzene, Yield: 90%; Light green crystalline; M.P. 134–136°C; *m/z*, C₂₁H₁₆O₄: 332, 228, 200, 172, 146, 127, 105, 77, 51; IR ν_{max} cm⁻¹: 3064, 2954, 1652, 1601, 1464, 1357, 1318, 1077;

¹H NMR (600 MHz, CDCl₃): δ_H 12.83 (s, 1H, OH-1), 8.22 (dd, *J* = 1.8, 8.3 Hz, 1H, H-8), 7.69 (td, *J* = 1.8 Hz & 8.3 Hz, 1H, H-6), 7.49 (d, *J* = 8.3 Hz, 1H, H-5), 7.35 (t, *J* = 8.3 Hz, 1H, H-7), 7.33 (dd, *J* = 1.8 Hz & 7.3 Hz, 2H, H-5'), 7.29 (d, *J* = 6.4 Hz, 2H, H-4'), 7.26 (d, *J* = 7.3 Hz, 1H, H-6'), 6.41 (d, *J* = 2.8 Hz, 1H, H-4), 6.33 (d, *J* = 2.8 Hz, 1H, H-2), 4.25 (t, *J* = 6.4 Hz & 7.3 Hz, 2H, H-1'), 3.13 (t, *J* = 6.4 Hz & 7.3 Hz, 2H, H-2'); ¹³C NMR (150 MHz, CDCl₃): δ_C 180.9 (C-9), 166.0 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 137.7 (C-3'), 135.0 (C-6), 129.1 (C-5'), 128.7 (C-4'), 126.8 (C-6'), 126.0 (C-8), 124.1 (C-7), 120.7 (C-8a), 117.7 (C-5), 104.0 (C-9a), 97.6 (C-2), 93.4 (C-4), 69.3 (C-1'), 35.5 (C-2').

1-Hydroxy-3-((1-phenylpropan-2-yl)oxy)-9*H*-xanthen-9-one (**2r**): From compound **1** and 2-bromo-1-phenylpropane, Yield: 96%; Light green crystalline; M.P. 106–108°C; *m/z*, C₂₁H₁₆O₄: 346, 228, 200, 172, 144, 117, 91; IR ν_{max} cm⁻¹: 3063, 2960, 1660, 1598, 1456, 1336, 1298, 1073; ¹H NMR (600 MHz, CDCl₃): δ_H 12.84 (s, 1H, OH-1), 8.22 (dd, *J* = 1.8 Hz & 8.3 Hz, 1H, H-8), 7.68 (t, *J* = 1.8 Hz & 8.3 Hz, 1H, H-6), 7.39 (d, *J* = 8.3 Hz, 1H, H-5), 7.34 (t, *J* = 8.3 Hz, 1H, H-7), 7.30 (dd, *J* = 2.8 Hz & 7.3 Hz, 2H, H-6'), 7.21 (d, *J* = 6.4 Hz, 2H, H-5'), 7.20 (s, 1H, H-7'), 6.37 (d, *J* = 1.8 Hz, 1H, H-4), 6.31 (d, *J* = 2.8 Hz, 1H, H-2), 4.02 (t, *J* = 6.4 Hz, 1H, H-1'), 2.82 (t, *J* = 7.3 Hz, 2H, H-2'), 2.14 (m, 3H, H-3'); ¹³C NMR (150 MHz, CDCl₃): δ_C 180.8 (C-9), 166.3 (C-3), 163.6 (C-1), 157.8 (C-4a), 156.1 (C-5a), 141.1 (C-4'), 135.0 (C-6), 128.6 (C-6'), 128.6 (C-5'), 126.2 (C-7'), 125.9 (C-8), 124.0 (C-7), 120.7 (C-8a), 117.6 (C-5), 104.0 (C-9a), 97.6 (C-2), 93.3 (C-4), 67.6 (C-1'), 32.1 (C-2'), 30.6 (C-3').

Acetylcholinesterase Inhibition Assay

The AChE inhibitory activity of the synthesised xanthenes (**1**, **2a-2r**) were measured by referring to Ellman's colourimetric method with minor modifications⁵². Tacrine was used as a standard drug, and the concentrations of the xanthenes used were 0.9, 1.9, 3.8, 7.5, 15, 30 and 45.0 μ M. In brief, 210 μ L of sodium phosphate buffer (0.1 M, pH 7.4) was aliquoted into a 96-well plate. Subsequently, 10 μ L of DTNB (0.3 mM), followed by 20 μ L of the compounds, were dispensed into the wells. Next, 20 μ L of AChE solution (5.32×10^{-3} U/mL) was suspended in each well, and the mixtures were incubated in the dark for 15 minutes at 37°C. The absorbance was measured at 412 nm at 25°C in every minute interval for ten consecutive times. Enzyme activity was calculated by comparing reaction rates for the samples to the control through the following formula:

$$\text{Inhibition (\%)} = \frac{S_{\text{control}} - S_{\text{sample}}}{S_{\text{control}}} \times 100 \%$$

Where **S** blank is the slope of the blank reaction (without test compounds or reference standard), and **S** sample is the slope of the sample reaction (with test compounds or standard reference). The IC₅₀ values were calculated via non-linear regression analysis on GraphPad Prism v. 8.4.0 (GraphPad Software, United States).

Acetylcholinesterase Enzyme Kinetic Study

The AChE enzyme kinetic inhibition modes of the two most active xanthone derivatives, **2g** and **2j**, were evaluated using the method elaborated in the section above. A range of substrate ATCI concentrations of 0.5, 0.6, 0.7, 0.8, and 0.9 mM were used, with and without the presence of the compounds. The concentrations of the **2g** and **2j** used were 7.5, 10, 12.5 and 15 µM. Then, Lineweaver-Burk plots were obtained by plotting reciprocal velocity versus substrate ³³.

Statistical Analysis

The results of the anti-AChE assay were expressed as mean ± standard deviation (SD) and statistically analysed by using ANOVA ($p < 0.05$) in GraphPad Prism v. 8.4.0 (GraphPad Software, United States).

Flexible Docking

The X-ray crystal structure of *Electrophorus electricus* acetylcholinesterase (PDB ID: 1C20) was retrieved from the protein database bank (PDB) ([http:// www.rcsb.org](http://www.rcsb.org)). Although the resolution is 4.2 Å, there was minimal difference in the position of the amino acid residues in the active site as compared with some high-resolution crystal structures available in the PDB. Hence, this protein crystal structure was suitable to be used in our docking study. The ligand-binding site was identified based on the position of the inhibitor-binding site as reported by literature. Residues W108, E224, S225, Y313, Y355, F356 and Y359 were specifically selected as flexible based on the observation by Šinko et al. (2019), in which they superposed 68 AChE complex crystal structures over the crystal structure of human apo-AChE (PDB ID: 4PQE) ⁵³.

Additionally, several conserved water molecules (structural water molecules) were inserted manually to the binding site according to the protein crystal structure (PDB ID: 1ACJ) to create a more realistic environment of the AChE binding site ⁵⁴. Compound **2g** was then prepared and minimised before the docking procedure. The DS Flexible Docking module was adopted for the receptor-ligand docking ³³. The ligand was docked to the active site of each receptor conformation using LibDock. The maximum number of compounds conformations generated were set at 225 numbers, followed by clustering to remove similar ligand pose. The selected protein side-chains were refined in the rigid ligand using ChiRotor, and the final ligand pose was optimised using CDOCKER.

Conclusion

Overall, 1,3-dihydroxyxanthone (**1**) and a known xanthone derivative (**2a**), along with seventeen new derivatives (**2b-2r**) were successfully synthesised. Remarkably, all eighteen xanthone derivatives (**2a-2r**) have four different hydrocarbon substituents, including alkyl, alkenyl, alkynyl, and alkylated-phenyl groups possessed stronger AChE inhibitory effect than **1**. In particular, **2g** and **2j** were found to exhibit an excellent inhibitory effect amongst the derivatives with the lowest IC₅₀ values of 20.8 and 21.5 µM, respectively. Kinetic analysis suggested that 1-hydroxy-3-*O*-substituted xanthenes inhibited AChE in a mixed-mode manner, indicating the possibility of binding to both AS and PAS of AChE. SAR analysis revealed that the hydrophobic interactions of the substituent groups at position C-3 of xanthone gave positive impacts on the inhibition effects. Specifically, xanthone derivatives substituted with C = C or alkylated phenyl in linear chains are favourable for the activity. The molecular docking study of **2g** further confirmed on the importance of the hydrophobic substituents by forming π-alkyl interactions with AChE. Two new xanthone derivatives (**2g** and **2j**) with promising AChE inhibitory properties are strongly recommended to be further studied on the hit-to-lead optimisation for the development of new drug for Alzheimer's disease.

Declarations

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Author Contribution

Conceptualization: SHM Data curation: VVV Formal analysis: VVV, SHM Funding acquisition: SHM Investigation: VVV, SST, SHM Methodology: VVV, SST, KWL, SHM Project administration: SHM Resources: SST, KWL, SHM Supervision: SST, SHM. Validation: SST, SHM Writing – original draft: VVV, SHM Writing – review & editing: SST, KWL, SHM. All authors reviewed the manuscript.

Competing Interests Statement

The authors declare no competing interests.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

The chemical structure of xanthone

Figure 2

Lineweaver–Burk plot of 1-hydroxy-3-(but-3-en-1-yloxy)-9H-xanthen-9-one (2g) against AChE. Bar indicates the standard deviation. Bars indicate standard deviation. All data are presented as mean $\pm$ SD of triplicates run in three independent experiments.

Figure 3

Lineweaver–Burk plot of 1-hydroxy-3-(pent-4-en-1-yloxy)-9H-xanthen-9-one (2j) against AChE. Bars indicate standard deviation. All data are presented as mean $\pm$ SD of triplicates run in three independent experiments.

Figure 4

Orientation of 1-hydroxy-3-(but-3-en-1-yloxy)-9H-xanthen-9-one (2g) (carbon atoms are colored green) in the active sites of (A) *Electrophorus electricus* AChE (PDB ID: 1C20) and (B) human AChE (PDB ID: 4PQE). The atoms in the residues are coloured as follows: carbons in cyan, oxygen in red, and nitrogen in blue. Distance is indicated in angstroms, Å.

Supplementary Files

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