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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis of nitrogen-containing oleanolic acid derivatives as carbonic anhydrase and acetylcholinesterase inhibitors

Halil Şenol[1] · Gurbet Çelik Turgut[2] · Alaattin Şen[3,4] · Rüya Sağlamtaş[5] · Salih Tuncay[6] · İlhami Gülçin[7] · Gülaçtı Topçu[8]

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Abstract

In this study, a total of 13 compounds **(5–17)** were synthesized starting from oleanolic acid (OA), a natural triterpenoid. Five new compounds **(10, 11, 12, 15** and **17)**, are the main targets of the study, which were synthesized for the first time in this work as oxime, imine and hydrazone derivatives of OA. Other compounds were previously obtained as natural or semi-synthetically. NMR and HRMS analyses were carried out to determine of structures of all the synthesized molecules. The inhibitory effects of the synthesized compounds on acetylcholinesterase (AChE), human carbonic anhydrase I (hCA I) and II (hCA II) were evaluated. Compounds **13** and **15** showed better inhibitory activity than the other compounds against both hCA I and hCA II isoenzymes, which are competing with AZA. In addition, compound **15** showed the strongest AChE inhibitory activity among all the tested compounds, with an IC_{50} value of 34.46 µM.

Keywords Oleanolic acid · Acetylcholinesterase · Oxime · Imine · Hydrazone · Carbonic anhydrase

Introduction

Alzheimer's disease (AD) is a progressive and irreversible neurological illness that causes cognitive impairment, neuroinflammation, glial activation and degeneration of synapses, which are clinically characterized by cognitive decline and memory loss, pathologically neurofibril tangles formed by tau fibrils and amyloid-β (Aβ) plaque deposition [1–5]. Significant clinical problems such as language, memory, attention, or problem-solving ability, and problems related to the mental performance expected from the age of the person, accompanied by neuropsychiatric symptoms and behavioral changes, significantly affect the daily life of the person [6]. AD is the world's most common neurological disorder. The estimated number of Alzheimer's patients will reach 152 million in 2050 [7]. Increases in these rates may be due to improvements in the recognition, identification, diagnosis, and treatment of such disorders. Drugs used to treat AD have been shown to improve functional, cognitive, and behavioral symptoms. However, these drugs are extremely limited, and do not have the efficacy to stop the progression of the disease or to reverse the symptoms and improve the disease [8]. Therefore, it is very important to discover new drugs that can be used in the treatment of AD and to investigate their effects. These results show that it is inevitable to use the compounds and

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✉ Halil Şenol
hsenol@bezmialem.edu.tr

1 Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Bezmialem Vakif University, Fatih, Istanbul 34093, Turkey

2 Faculty of Applied Sciences, Department of Organic Farming Business Management, Pamukkale University, Civril, Denizli 20600, Turkey

3 Faculty of Science, Department of Biology, Pamukkale University, Kinikli, Denizli 20070, Turkey

4 Faculty of Life & Natural Sciences, Department of Molecular Biology and Genetics, Abdullah Gul University, Kocasinan, Kayseri 38080, Turkey

5 Vocational School of Health Services, Department of Medical Services and Technology, Agri Ibrahim Cecen University, Agri 04100, Turkey

6 Vocational School of Health Services, Department of Food Technology, Uskudar University, Uskudar, Istanbul 34664, Turkey

7 Faculty of Science, Department of Chemistry, Atatürk University, Erzurum 25240, Turkey

8 Faculty of Pharmacy, Department of Phytochemistry & Pharmacognosy, Bezmialem Vakif University, Fatih, Istanbul 34093, Turkey

products obtained from natural sources, which are accepted as an important approach to treating AD [9, 10].

Carbonic anhydrases (CAs), which contain Zn^{2+} ions in their active site, catalyze the reversible hydration of carbon dioxide (CO_2) and water (H_2O) to proton (H^+) and bicarbonate (HCO_3^-) anion [11, 12].

$$CO_2 + H_2O \overset{CA}{\Leftrightarrow} H_2CO_3 \rightarrow HCO_3^- + H^+$$

In the direction of hydration, the first step is a nucleophilic attack. Formation of zinc ion (Zn^{2+})-bonded hydroxide ion (OH^-) on CO_2 and consequent HCO_3^-, which is then removed from the active site by a H_2O molecule (**a**) [13, 14].

$$\text{Enzyme} \sim Zn^{2+} \sim OH^- + CO_2 \overset{CA}{\Leftrightarrow} \text{Enzyme} \sim Zn^{2+} \sim HCO_3^- + H^+ \ (\mathbf{a})$$

$$\text{Enzyme} \sim Zn^{2+} \sim HCO_3^- \rightarrow \text{Enzyme} \sim Zn^{2+} \sim H_2O + HCO_3^- \ (\mathbf{b})$$

$$\text{Enzyme} \sim Zn^{2+} \sim H_2O + B \rightarrow \text{Enzyme} \sim Zn^{2+} \sim OH^- + BH^+$$

The second step as a rate-limiting step produces the catalytically active Zn^{2+}-linked hydroxide (OH^-) ion is regenerated via a proton transfer reaction from the Zn^{2+}-bonded H_2O molecule to an exogenous proton acceptor or generally to an active site residue represented by B (**b**) [15, 16].

CA inhibitors, on the other hand, have been shown to be effective in the treatment of glaucoma, ulcers, and osteoporosis [17]. Acetazolamide, diclofenamide, dorzolamide, zonisamide, tolsultazolamide, brinzolamide, methazolamide, and ethoxzolamide are some of the most well-known inhibitors [18, 19]. However, these drugs' side effects have prompted researchers to investigate their metabolism and distribution in a variety of organisms [20]. It has been critical to synthesize high biological value inhibitors with no adverse effects for CA isoenzymes [21–23]. Therefore, in order to avoid the side effects of CA inhibitors, it is better for the inhibitor to have topical activity, so we decided to show that oleanolic acid derivatives can be applied for the treatment of glaucoma disease [24, 25].

Oleanolic acid (OA), which is one of the natural triterpenoids, and its derivatives have a variety of biological activities [26]. Many activities of OA have been determined such as anti-acetylcholinesterase (AChE) [27], anti-arrhythmic [28], anti-tumor [29], anti-cancer [30–32], atherosclerosis [33], anti-inflammatory [34], anti-microbial [35], anti-oxidant [36], anti-HIV [37], anti-viral [38], α-glycosidase [39, 40], hepatoprotective [41], anti-pyretic [42], anti-angiogenic [43], anti-allergic [44], immunomodulatory [45], anti-diabetic [46]. The limited solubility of OA, rapid metabolism, poor bioavailability, and insufficient isolation from natural plants prevent its clinical applications. Therefore, many OA derivatives have been synthesized to overcome these mentioned disadvantages of OA. Despite extensive efforts, the discovery of effective OA derivatives has so far met with only limited success [46]. Due to these disadvantages besides very valuable advantages, it is important to investigate and improve synthetic/semi-synthetic derivatives of natural OA for the treatment of AD and dementia-related diseases [47, 48].

This study aims to discover natural product derivatives of OA those may have more potential effects compared to existing AD drugs and CA inhibitors. For this purpose, new and some known oleanane triterpenoids having oxime, imine, hydrazone and nitrile moiety were synthesized from OA and their inhibitory effects on AChE, hCA I and hCA II enzymes were investigated.

Results and discussion

Alzheimer's is a progressive and degenerative disease of the brain characterized by the accumulation of Aβ plaques and neurofibrillary tangles, leading to memory loss and other cognitive problems [49, 50]. Currently, there is no known treatment method that completely stops the disease or slows the progression of the disease, but pharmacological agents such as AChE inhibitors (rivastigmine, galantamine and donepezil) and N-methyl-D-aspartate receptor antagonists (memantine) are used to reduce its progression [51]. Since these commercially produced agents not provided complete treatment, it is important to investigate and design new drugs that can be used in the treatment of AD. Therefore, pharmaceutical companies and scientists continue to search for new drugs.

The present study aimed to find OA derivatives that will have potential AChE and carbonic anhydrase inhibitory effects to be used in the treatment of AD and CA related disease. For this purpose, first of all, OA derivatives were synthesized, structures of the all compounds were determined by ^{1}H-,^{13}C-NMR and HRMS analyses (Supplementary Figures), and then the AChE and carbonic anhydrase inhibitory activities of these compounds were investigated.

Syntheses

To synthesize the targeted compounds, initially, carbonyl derivatives of OA (**8** and **9**) were synthesized. For this purpose, first, OA was reduced to erythrodiol **(5)** which was then oxidized in a single by pyridinium chlorochromate to obtain dicarbonyl derivative **9**. For the synthesis of the monocarbonyl derivative **(8)**, initially, OA was first converted to 3β-methoxy-olean-12-en-28-oic acid methyl ester **(6)** using MeI in the presence of NaH at a high temp. Compound **6** was then reduced to 3β-methoxy-erythrodiol **(7)** by $LiAlH_4$. Finally, compound **7** was oxidized by PCC to obtain mono carbonyl derivative **8** (Fig. 1).

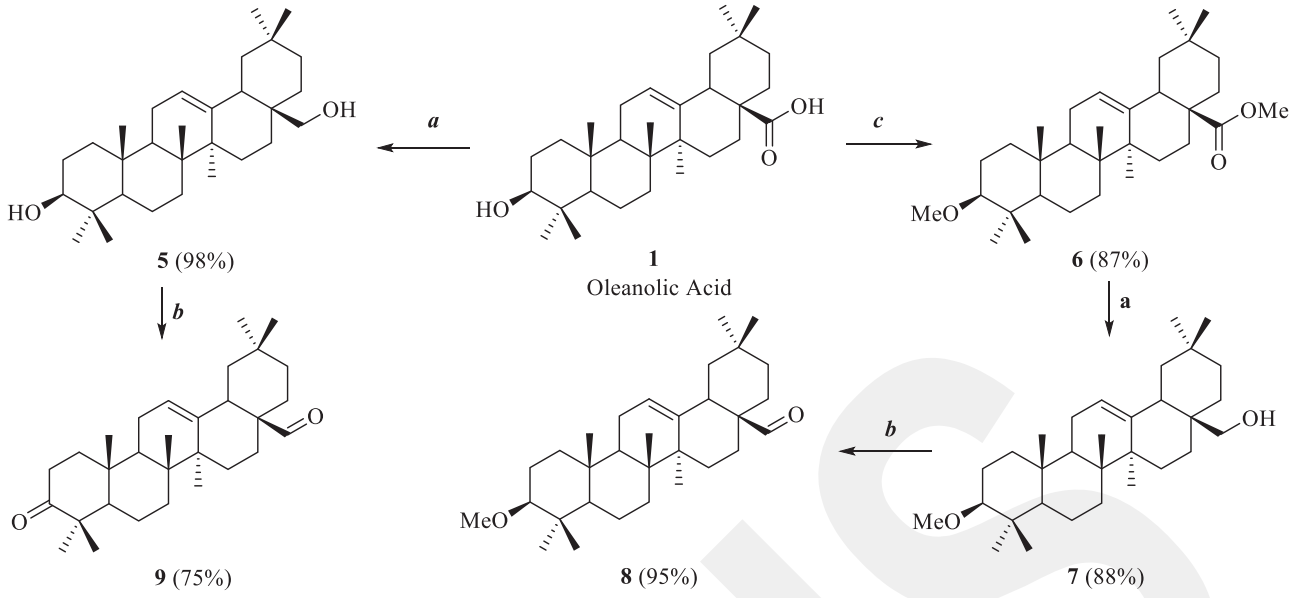

Fig. 1 Synthesis of carbonyl derivatives of OA

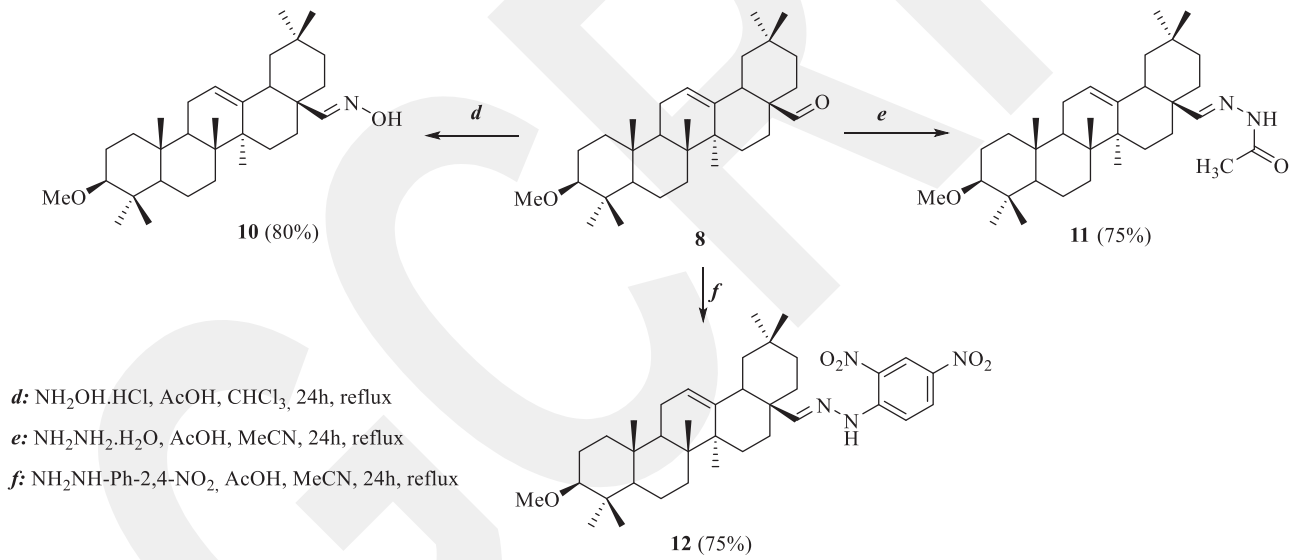

Fig. 2 Synthesis of compounds **10–12**

The oxime derivative **10**, acetohydrazide-hydrazone derivative **11**, and 2,4-dinitrophenylhydrazone derivative **12** were synthesized from the reaction of **8** with hydroxylamine, hydrazine and 2,4-dinitrophenylhydrazine, respectively (Fig. 2). As a result of the self-acetylation of the hydrazone during the reaction, compound **11** was obtained as an acetyl derivative.

Four different condensation products (**13–16**) were obtained from the reaction of compound **9** with hydroxylamine in acetonitrile. In addition, the imine derivative **17** was obtained from the reaction of **9** with aniline in acetonitrile (Fig. 3).

Anti-acetylcholinesterase and anti-carbonic anhydrase I and II activity

OA and all synthesized compounds (**5–17**) were tested against AChE, hCA I, and hCA II enzymes, and the results were summarized in Table 1. The IC_{50} value of OA was calculated as 57.39 µM (r^2: 0.9988) against the AChE

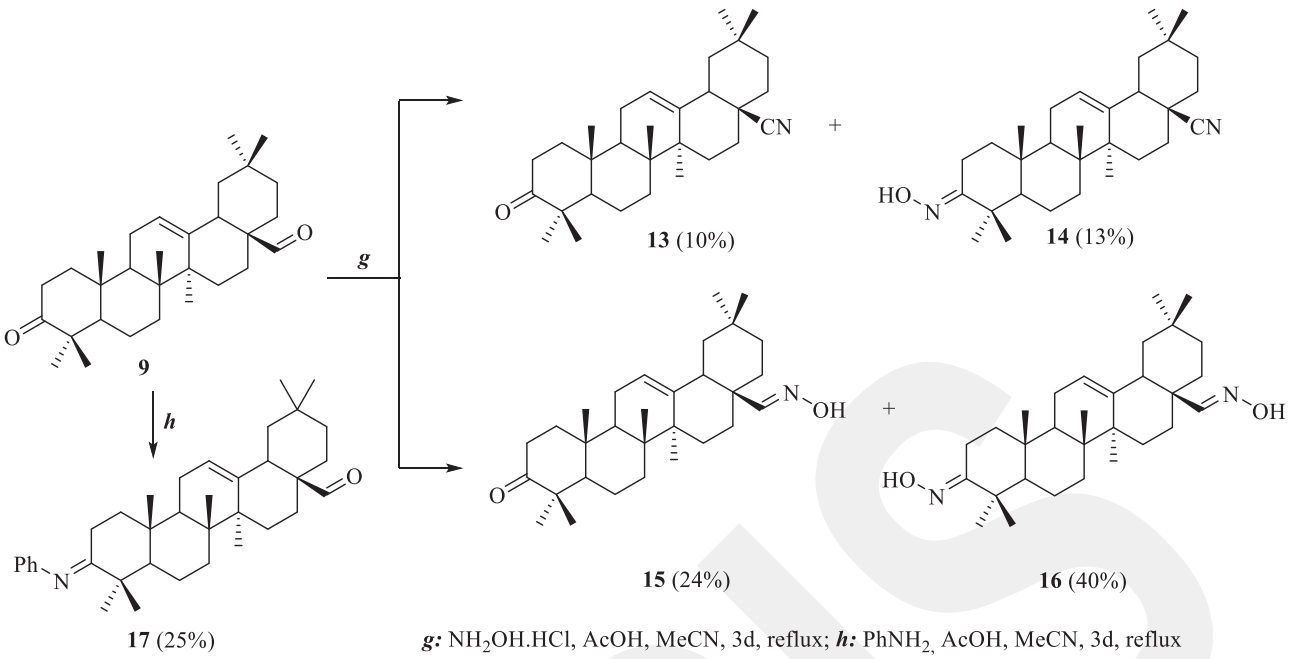

Fig. 3 Synthesis of compounds **13–17**

enzyme. The IC_{50} value of compound **6** is 55.77 µM (r^2: 0.9839). The fact that compound **6**, which has a more non-polar character, has a higher AChE inhibition effect than OA. The fact that this compound can cross the blood-brain barrier more easily than OA makes these results more meaningful. Comparing the carbonyl derivatives **8** and **9** of OA, **9** have better AChE inhibitory activity with an IC_{50} value of 46.99 µM (r^2: 0.9894). Among them, oxime and nitrile derivatives **10** and **13–16** had over 50% inhibition of AChE at 75 µM. The highest inhibition effect against AChE was produced by **15** ($IC_{50} = 34.46$ µM (r^2: 0.9782)) which was synthesized for the first time. Comparing the hydrazone **11**, 2,4-dinitrophenylhydrazone **12**, and benzylidene **17**, compound **11** showed the highest AChE inhibition effect. These findings showed that compound **15**, which was synthesized for the first time, could promisingly block the AChE enzyme.

In addition, oleanolic acid derivatives (**5–17**) effectively inhibited both cytosolic hCA I and II isoenzymes, which are the target enzymes for improving epilepsy, glaucoma, altitude sickness, idiopathic intracranial hypertension, and congestive heart failure, among other diseases, with IC_{50} values of between 10.25–23.03 and 10.23–19.17 µM, respectively (Table 1). Also, AZA, which is extensively used as a positive inhibitor for both CA isoforms, demonstrated IC_{50} values were calculated as 16.58 (r^2: 0.9887) for cytosolic hCA I isoform and 8.37 µM (r^2: 0.9825) for cytosolic and dominant hCA II isoform. Within the scope of studies with hCA I inhibition, compound **13** possessed a nitrile (-CN) functional group and carbonyl group (-C=O), which exhibited the most effective hCA I inhibition profile with IC_{50} value of 10.25 µM (r^2: 0.9879) among the tested molecules. It is well known that the molecules containing these two groups effectively inhibit CA isoenzymes [52]. This inhibition value is about 1.62

Table 1 AChE enzyme and CA I and II isoenzymes inhibition activities of oleanolic acid derivatives

Enzymes	hCA I		hCA II		AChE	
Compounds	IC_{50} (µM)	r^2	IC_{50} (µM)	r^2	IC_{50} (µM)	r^2
OA	17.32	0.9563	19.17	0.9933	55.14	0.9988
5	14.69	0.9779	17.17	0.9507	55.77	0.9984
6	13.11	0.9746	12.23	0.9868	57.39	0.9839
7	11.93	0.9905	10.23	0.9795	51.93	0.9890
8	18.23	0.9831	12.01	0.9836	55.14	0.9691
9	15.22	0.9844	10.45	0.9699	46.99	0.9884
10	18.58	0.9405	17.45	0.9859	56.26	0.9662
11	23.03	0.9691	15.64	0.9969	45.15	0.8077
12	13.09	0.9831	11.46	0.9933	54.59	0.9302
13	10.25	0.9879	11.94	0.9560	62.80	0.9982
14	–	–	–	–	36.59	0.9693
15	13.58	0.9532	10.92	0.9795	34.46	0.9782
16	20.08	0.9635	16.99	0.9795	49.89	0.9003
17	17.97	0.9976	10.62	0.9984	56.82	0.9902
AZA*	16.58	0.9887	8.37	0.9825	–	–
GAL**	–	–	–	–	2.11	0.9032
RIV**	–	–	–	–	228.14	0.8981

**AZA* Acetazolamide was used as a reference inhibitor for hCA I and hCA II isoenzymes

***GAL*, *RIV* Galantamine and Rivastigmine were used as reference inhibitors for acetylcholinesterase enzyme

times higher than AZA (IC_{50}: 16.58 µM, r^2: 0.9887). These results clearly show that six oleanolic acid derivatives (**5, 6, 7, 9, 12** and **13**) had more CA isoenzyme inhibition effects than that AZA. When activity results of CA II were evaluated, it is observed that the majority of the tested oleanolic acid derivatives (**6, 7, 8, 9, 12** and **13**) exhibit an inhibition close to AZA. It was observed that the remaining oleanolic acid derivatives (**5, 10, 11**, and **16**) showed lower inhibition than that of AZA employed as a reference inhibitor. However, the most active molecule against cytosolic hCA II isoenzyme was found in the oleanolic acid derivative of **5**, which exhibited an IC_{50} value of 10.23 µM (r^2: 0.9795). Also, the proposed interaction mechanism between the most powerful oleanolic acid derivatives (**7**) and the CA II isozyme is shown in Fig. 4.

Conclusions

In the present study, the synthesis of potentially bioactive oxime, imine, hydrazone and hydrazone-hydrazide derivatives of a series of OA was performed. The structures of the compounds were determined by NMR and HRMS analyses.

Among the compounds, molecules **13** and **15** were found to be strong enzyme inhibitory agent for both human carbonic anhydrase (CA I and II) enzymes. Molecule **15** further exhibited highest AChE inhibitory activity which showing its potential to be a lead compound in the treatment of some neurological diseases.

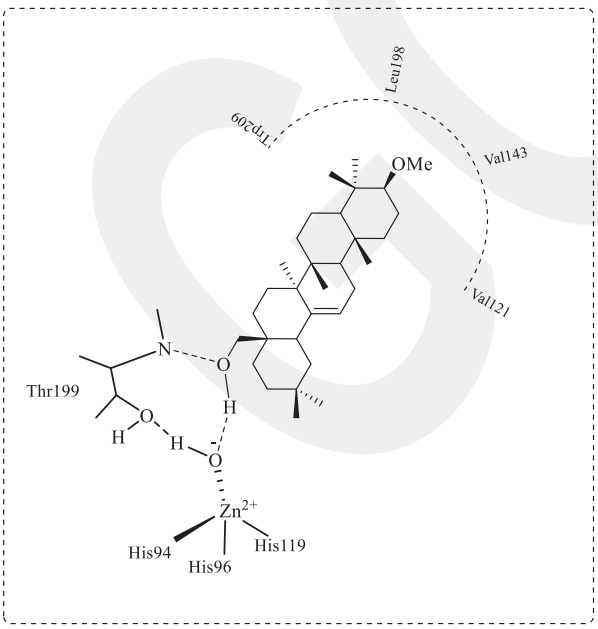

Fig. 4 The proposed binding mechanism between the CA II isoform and oleanolic acid derivatives **(7)** by anchoring to the Zn^{2+} ion coordinated with water / -OH ions

When considering overall carbonic anhydrase inhibitory activity results of the tested compounds, most of them exhibited strong inhibitory activity on both isomers, especially on CA I. As conclusion, some of the synthesized oleanolic acid derivatives, particularly nitrogen containing ones can be potential new drug candidates as CA I and CA II inhibitory agents. Investigations of in vivo toxicities and efficacies of these potential drug candidates should be evaluated with further studies.

Material and methods

Materials

All of the solvents, chemicals, and other supplies utilized in the tests bought from Sigma Aldrich, Merck, TCI Chemicals, and other suppliers. Purification methods were carried out as stated in the literature when appropriate, notwithstanding the excellent purity of commercially accessible chemicals and solvents [53–57].

Column chromatography was used for chromatographic separations. The stationary phase was silica gel, and the mobile phase was a combination of ethyl acetate and hexane. The stationary phase was silica gel, and the mobile phase was a combination of ethyl acetate and hexane. Thin-layer chromatography (TLC) was used to monitor the experiments and column chromatographies, and spots were detected using UV light, a 10% cerium (IV) sulfate solution in sulfuric acid, and heating on a burner at 100 °C. Chemical structures were determined using nuclear magnetic resonance (NMR) analyses (^{1}H NMR, ^{13}C APT NMR, COSY, and HSQC). HRMS analyses were recorded to determine the molecular weight.

The Stuart SMP30 melting point instrument was used to determine melting points. ^{1}H-NMR and ^{13}C APT NMR (500 and 125 MHz, respectively) spectra were run on Bruker Avance NEO Spectrometer. The values of coupling constants were reported in Hertz (Hz). The peak splits were defined as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), and dt (doublet of triplet) relative to the internal standard tetramethylsilane (= 0.00 ppm) (doublet of triplet). HRMS spectra were recorded by ESI technique on Thermo Fischer Scientific Q ExactiveTM Hybrid Quadrupole-OrbitrapTM.

The solvents, chemicals and consumables were used; Acetic acid (Merck), acetonitrile (Merck), dichloromethane (Merck), dimethylsulfoxide D6 (Merck), 2,4-dinitrophenylhydrazine (Sigma Aldrich), ethanol (Merck), ethyl acetate (Merck), hydrazine hydrate (Merck), hydroxyl amine hydrochloride (Merck), chloroform (Merck), chloroform D1 (Merck), methanol (Merck), lithium aluminum hydride

($LiAlH_4$) (Sigma-Aldrich-686034), methyl iodide (Sigma-Aldrich-289566), oleanolic acid (Carbone Scientific-C-22557), pyridinium chlorochromate (PCC) (Sigma Aldrich), potassium hydroxide (KOH) (Merck), cerium(IV)sulfate (Sigma-Aldrich-359009), sodium sulfate (Merck 106649), silica gel 60-column (70-230 mesh ASTM, Merck 107734), silica gel 60-thin layer chromatography (20x20 Al plate - F254, Merck 105554), sodium hydroxide (Merck 106462), sodium hydride (60% in mineral oil, Sigma Aldrich) sodium sulphate (Merck), tetrahydrofuran (THF), (Merck 107025), SH-SY5Y (ATCC-CRL-2266), penicillin/streptomycin (Biological Industries-03-031-1B), DMEM-F12 (Sigma Aldrich - D5796), fetal bovine serum (Sigma Aldrich -F4135), trypsin (Gibco), triton X-100 (Applichem-A4975), EDTA (Bioshop-EDT001), PMFS (Applichem–A0999), Bicinchoninic acid disodium salt hydrate (Sigma Aldrich), acetylthiocholine iodide (Sigma Aldrich), thiocholine-DTNB (Sigma Aldrich), aniline (Sigma Aldrich).

Chemistry

Synthesis of 3β-methoxy-olean-12-en-28-oic-acid methyl ester (6)

Tetrahydrofuran (THF) and sodium hydride (NaH) (10.50 g, 260 mmol, 60 percent, 6 equiv.). were added into a round-bottomed flask. The oleanolic acid (20 g, 44 mmol, 1 equiv.) was added and stirred at room temperature for 30 min. Methyl iodide (MeI) was added (16 mL, 260 mmol, 6 equiv.) and the mixture was stirred overnight in an inert atmosphere. The excess sodium hydride was neutralized with water (15 mL) at the reaction was stopped. The solvent was removed under reduced pressure and the residue was washed with water (3 x 300 mL) and extracted with chloroform (3 x 300 mL). The organic layers were combined, and dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure, and the residue was adsorbed on silica gel. Using an ethyl acetate and hexane mixture, the products were purified using silica gel column chromatography (1:9). Compound **6** was obtained as white solid (20 g, 94% yield).

Compound 6 m.p.: 156°C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.28 (t, $J = 3.40$ Hz, 1H), 3.61 (s, 3H), 3.34 (s, 3H), 2.85 (dd, $J = 4.40$, 13.80 Hz, 1H), 2.65 (dd, $J = 4.00$, 11.60 Hz, 1H), 1.00 (s, 3H), 0.89 (s, 3H), 0.88 (s, 3H), 0.86 (s, 3H), 0.79 (d, $J = 6.50$ Hz, 3H), 0.69 (s, 3H), 0.68 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 177.60, 143.10, 121.70, 87.90, 56.80, 55.00, 50.80, 46.90, 46.00, 45.20, 40.90, 40.60, 38.60, 38.00, 37.60, 36.30, 33.10, 32.40, 31.90, 31.70, 30.00, 27.40, 27.00, 25.20, 22.90, 22.70, 22.30, 21.30, 17.50, 16.10, 15.60, 14.60. ESI-HRMS: Formula: $C_{32}H_{52}O_3$; Exact mass: 484.39165; Calculated *m/z* $[M+H]^+$: 485.39947; Found *m/z* $[M+H]^+$: 485.39954.

General synthesis method of reduction of OA and its methyl ester (5 and 7)

Two-neck round-bottomed flask was charged with freshly distilled THF (300 mL) and $LiAlH_4$ (76 mmol, 2.9 g, 7 eq.) was added in an inert atmosphere. After stirring for fifteen minutes, the solution of OA or methyl ester of OA **(6)** (11 mmol, 1 eq.) was added, and refluxed overnight in an inert atmosphere. The reaction was monitored by TLC and excess $LiAlH_4$ was carefully destroyed with water after completion. The gel form of the white aluminum oxide complex was filtered, and THF was removed under low pressure. The residue was washed with water, and extracted with chloroform (3 x 300 mL). The solvent was removed under reduced pressure after the organic phase was dried over sodium sulfate. The desired products were obtained in their purest form.

Compound 5 White solid, 5.0 g, 98% yield; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.18 (t, $J = 3.55$ Hz, 1H), 3.54 (d, $J = 10.90$ Hz, 1H), 3.20 (d, $J = 10.90$ Hz, 1H), 3.21 (dd, $J = 11.03$, 5.59 Hz, 1H), 0.98 (s, 3H), 0.93 (s, 3H), 0.92 (s, 3H), 0.87 (s, 3H), 0.86 (s, 3H), 0.77 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 144.27, 122.40, 122.38, 79.03, 69.70, 55.22, 47.62, 46.52, 42.38, 41.77, 39.83, 38.82, 38.65, 36.97, 34.14, 33.25, 32.62, 31.08, 31.00, 28.14, 27.25, 25.99, 25.60, 23.64, 23.57, 22.05, 18.40, 16.78, 15.63, 15.56. ESI-HRMS: Formula: $C_{30}H_{50}O_2$; Exact mass: 442.38108; Calculated *m/z* $[M\text{-}H_2O+H]^+$: 425.37864; Found *m/z* $[M\text{-}H_2O+H]^+$: 425.37701.

Compound 7 White solid, 4.74 g, %88 yield; m.p.: 239 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.18 (t, $J = 3.55$ Hz, 1H), 3.53 (d, $J = 10.90$ Hz, 1H), 3.18(d, $J = 10.90$ Hz, 1H), 3.34 (s, 3H), 2.65 (dd, $J = 11.70$, 4.29 Hz, 1H), 0.98 (s, 3H), 0.93 (s, 3H), 0.92 (s, 3H), 0.87 (s, 3H), 0.86 (s, 3H), 0.77 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 144.30, 122.42, 88.72, 69.63, 57.58, 55.78, 47.63, 46.54, 42.40, 41.75, 39.87, 38.76, 38.57, 36.97, 34.15, 33.26, 32.64, 31.08, 30.99, 28.17, 25.98, 25.59, 23.66, 23.61, 22.09, 22.05, 18.29, 16.79, 16.39, 15.54; ESI-HRMS: Formula: $C_{31}H_{52}O_2$; Exact mass: 456.39673; Calculated *m/z* $[M+H]^+$: 457.40456; Found *m/z* $[M+H]^+$: 457.40298.

Synthesis of carbonyl compounds 8 and 9

PCC and dichloromethane (200 mL) were added to a round-bottomed flask (6.60 mmol, 3 eq.). Solution of compound **5** or **7** in DCM (2.20 mmol, 1 eq.) was added, and the mixture was stirred 24 h at room temp. The reaction was monitored

by TLC and terminated after starting material was exhausted. Under reduced pressure, the solvent was removed, and the residue was adsorbed on silica gel. Compounds **8** and **9** were purified using a mixture of EtOAc and hexane in a silica-gel column chromatography (1:9).

Compound 8 White solid, 4.5 g, %90 yield; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 9.33 (s, 1H), 5.30 (t, $J = 3.61$, 3.61 Hz, 1H), 3.30 (s, 3H), 2.64 (dd, $J = 11.77$, 4.32 Hz, 1H), 2.61 (dd, $J = 15.47$, 4.31 Hz, 1H), 1.08 (s, 3H), 1.01 (s, 3H), 0.97 (s, 6H), 0.85 (s, 3H), 0.84 (s, 3H), 0.72 (s, 3H)); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 207.46, 143.02, 123.35, 88.68, 57.55, 55.81, 49.11, 47.60, 45.66, 41.74, 40.47, 39.65, 38.73, 38.45, 37.06, 33.21, 33.12, 32.81, 30.68, 28.18, 27.80, 26.77, 25.59, 23.50, 22.17, 22.04, 18.23, 17.07, 16.38, 15.36; ESI-HRMS: Formula: $C_{31}H_{50}O_2$; Exact mass: 454.38108; Calculated *m/z* $[M+H]^+$: 455.38891; Found *m/z* $[M+H]^+$: 455.38757.

Compound 9 White solid, 1.5 g, %75 yield; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 9.33 (s, 1H), 5.30 (t, $J = 3.61$, 3.61 Hz, 1H), 2.58 (dd, $J = 13.67$, 4.30 Hz, 1H), 2.47 (ddd, $J = 15.94$, 11.14, 7.30 Hz, 1H), 2.29 (ddd, $J = 15.88$, 6.82, 3.67 Hz, 1H), 1.08 (s, 3H), 1.01 (s, 3H), 0.97 (s, 6H), 0.85 (s, 3H), 0.84 (s, 3H), 0.72 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 217.58, 207.31, 143.09, 123.01, 55.33, 49.14, 47.45, 46.83, 45.58, 41.86, 40.47, 39.58, 39.21, 36.75, 34.17, 33.18, 33.09, 32.31, 30.68, 27.73, 26.76, 26.50, 25.46, 23.55, 23.45, 22.10, 21.52, 19.60, 17.00, 15.10; ESI-HRMS: Formula: $C_{30}H_{46}O_2$; Exact mass: 438.34978; Calculated *m/z* $[M-H]^+$: 437.34196; Found *m/z* $[M-H]^+$: 437.34219.

General reaction of carbonyls and hydroxylamine: Syntheses of 10, 13–16

A round-bottomed flask was charged with chloroform (200 mL) and compound **8** or **9** (3.43 mmol, 1 eq.) was added and heated for an hour in the presence of a catalytic amount of acetic acid. Finally, hydroxylamine hydrochloride (20.5 mmol, 6 eq.) was added and refluxed overnight. TLC was used to monitor the reaction, which was stopped when the starting material was depleted. After cooled to ambient temperature the reaction mixture was washed with 1% aqueous NaOH solution and extracted. Then, the mixture was washed with 1% aqueous HCl solution and extracted with chloroform. The organic layers were mixed together and dried over sodium sulfate before being filtered. The solvent was extracted under reduced pressure, and the products were purified on a silica gel column using an ethyl acetate-hexane mixture (1:9). Four different products **(13–16)** were obtained from the reaction of compound **9** and hydroxylamine. Only compound **10** was obtained from the reaction of **8** with hydroxylamine. Instead of the expected aldoxime group in **13** and **14**, the nitrile group was formed via elimination during the reaction.

Compound 10 White solid, 0.5 g %80 yield; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.22 (s, 1H), 5.22 (t, $J = 3.43$ Hz, 1H), 3.35 (s, 1H), 2.65 (dd, $J = 11.69$, 4.29 Hz, 1H), 2.44 (dd, $J = 13.49$, 4.06 Hz, 1H), 1.12 (s, 3H), 0.96 (s, 3H), 0.91 (s, 3H), 0.89 (s, 6H), 0.82 (s, 3H), 0.75 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 159.07, 143.72, 122.89, 88.76, 57.58, 55.81, 47.70, 46.02, 42.99, 41.67, 39.87, 39.75, 38.75, 38.44, 37.07, 33.98, 33.18, 32.92, 32.74, 30.76, 28.19, 26.78, 26.08, 24.86, 23.72, 23.59, 22.07, 18.26, 17.15, 16.35, 15.44; ESI-HRMS: Formula: $C_{31}H_{51}NO_2$; Exact mass: 469.39198; Calculated *m/z* $[M+H]^+$: 470.39980; Found *m/z* $[M+H]^+$: 470.39990.

Compound 13 White solid, 150 mg, %10 yield; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.38 (t, $J = 3.59$ Hz, 1H), 2.60–2.51 (m, 2H), 2.36 (ddd, $J = 15.88$, 6.73, 3.61 Hz, 1H), 1.14 (s, 3H), 1.08 (s, 3H), 1.07 (s, 6H), 1.04 (s, 3H), 0.93 (s, 3H), 0.90 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 216.49, 140.70, 124.53, 123.41, 54.37, 46.44, 45.82, 43.72, 43.06, 41.00, 38.47, 38.23, 37.02, 35.75, 33.14, 31.94, 31.91, 31.47, 31.29, 29.56, 27.04, 25.45, 24.44, 23.05, 22.57, 22.41, 20.51, 18.58, 16.18, 14.09; ESI-HRMS: Formula: $C_{30}H_{45}NO$; Exact mass: 435.35102; Calculated *m/z* $[M+H]^+$: 436.35794; Found *m/z* $[M+H]^+$: 436.35651.

Compound 14 White solid, 200 mg, % 13 yield; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.38 (t, $J = 3.52$ Hz, 1H), 3.12–3.07 (m, 1H), 2.59 (dd, $J = 13.60$, 4.16 Hz, 1H), 1.18 (s, 3H), 1.11 (s, 3H), 1.08 (s, 3H), 1.07 (s, 3H), 1.06 (s, 3H), 0.94 (s, 3H), 0.90 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 167.07, 141.72, 125.64, 124.64, 55.93, 47.17, 44.78, 44.07, 42.02, 40.36, 39.59, 38.61, 38.08, 37.14, 33.01, 32.97, 32.76, 32.37, 30.61, 28.07, 27.25, 25.51, 24.11, 23.60, 23.47, 23.34, 19.08, 17.32, 17.08, 15.03.

Compound 15 White solid, 375 mg, %24 yield; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.22 (s, 1H), 5.23 (t, $J = 3.46$ Hz, 1H), 2.56–2.45 (m, 2H), 2.36 (ddd, $J = 15.82$, 6.81, 3.67 Hz, 1H), 1.13 (s, 3H), 1.07 (s, 3H), 1.04 (s, 3H), 1.03 (s, 3H), 0.98 (s, 6H), 0.88 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 217.79, 159.11, 143.85, 122.49, 55.32, 47.48, 46.95, 45.98, 42.97, 41.81, 39.78, 39.21, 36.78, 34.19, 33.97, 33.15, 32.88, 32.24, 30.77, 26.79, 26.52, 25.95, 24.84, 23.69, 23.63, 21.48, 19.63, 17.08, 15.17.

Compound 16 White solid, 650 mg, %40 yield; ^{1}H-NMR (500 MHz, DMSO-D_6) δ: 10.34 (s, 1H), 10.27 (s, 1H), 7.12 (s, 1H), 5.18 (t, $J = 3.41$ Hz, 1H), 2.93–2.86 (m, 1H), 2.50 (td, $J = 3.60$, 1.79 Hz, 1H), 2.41 (dd, $J = 13.54$, 4.04 Hz, 1H),

1.09 (s, 6H), 0.97 (s, 3H), 0.94 (s, 3H), 0.94 (s, 3H), 0.87 (s, 3H), 0.86 (s, 3H), 0.80 (s, 3H); ^{13}C-NMR (125 MHz, DMSO-D_6) δ: 163.26, 156.67, 143.85, 121.80, 55.30, 46.57, 45.57, 42.95, 41.21, 39.34, 38.79, 37.79, 36.54, 33.45, 32.93, 32.88, 32.03, 30.39, 27.50, 26.12, 25.61, 24.06, 23.44, 23.40, 22.98, 18.58, 16.96, 16.43, 14.45; ESI-HRMS: Formula: $C_{30}H_{48}N_2O_2$; Exact mass: 468.37158; Calculated *m/z* $[M+H]^+$: 469.37940; Found *m/z* $[M+H]^+$: 469.37811.

Synthesis of hydrazone derivative (11)

A round-bottomed flask was charged with acetonitrile (200 mL). Compound **8** (300 mg, 0.6 mmol, 1 eq.) was added and heated for an hour in the presence of a catalytic amount of acetic acid (2 mL). Then hydrazine hydrate (0.1 mL, 0.96 mmol, 5 eq.) was added and refluxed overnight. The reaction was monitored by TLC, and terminated after starting material was exhausted. The residue was adsorbed on silica gel after the acetonitrile was removed under reduced pressure. Compound **11** was purified using an ethyl acetate-hexane mixture (1:9) on a silica gel column chromatography, and it was obtained a white solid (250 mg, 75% yield). Compound **11** was self-acetylated during the reaction.

Compound 11 ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 9.74 (s, 1H), 6.93 (s, 1H), 5.23 (t, $J = 3.47$ Hz, 1H), 3.33 (s, 3H), 2.63 (dd, $J = 11.68$, 4.27 Hz, 1H), 2.49 (dd, $J = 13.62$, 4.02 Hz, 1H), 2.18 (s, 1H), 1.12 (s, 3H), 0.94 (s, 3H), 0.88 (s, 3H), 0.87 (s, 6şlH), 0.73 (s, 3H), 0.71 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 173.81, 154.81, 143.92, 122.58, 88.68, 57.55, 55.79, 47.64, 45.91, 43.10, 41.66, 40.57, 39.84, 38.72, 38.45, 37.04, 33.99, 33.21, 32.79, 32.69, 30.76, 28.18, 26.67, 26.04, 24.41, 23.72, 23.56, 22.05, 20.32, 18.24, 17.08, 16.41, 15.36; ESI-HRMS: Formula: $C_{33}H_{54}N_2O_2$; Exact mass: 510.41853; Calculated *m/z* $[M+H]^+$: 511.42635; Found *m/z* $[M+H]^+$: 511.42514.

Synthesis of 2,4-dinitrophenylhydrazon derivative (12)

Acetonitrile was added into a round bottom flask (200 mL). Compound **8** (300 mg, 0.6 mmol, 1 eq.) was added and heated for an hour in the presence of a catalytic amount of acetic acid (2 mL). Then 2,4-dinitrophenylhydrazine (0.25 g, 2 mmol, 3 eq.) was added and refluxed overnight. The reaction was monitored by TLC, and terminated after starting material was exhausted. Acetonitrile was removed under reduced pressure, and residue adsorbed on silica gel. Compound **12** was purified by silica gel column chromatography using an ethyl acetate-hexane mixture (1:9), and it was obtained as yellow solid (250 mg, 75% yield). Compound **12** was self-acetylated during the reaction.

Compound 12 ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 10.91 (s, 1H), 9.09 (d, $J = 2.54$ Hz, 1H), 8.28 (d, $J = 2.46$ Hz, 1H), 8.27 (d, $J = 2.46$ Hz, 1H), 7.85 (d, $J = 9.59$ Hz, 1H), 7.30 (s, 1H), 5.36 (t, $J = 3.35$ Hz, 1H), 3.34 (s, 3H), 2.64 (dd, $J = 11.68$, 4.24 Hz, 1H), 2.59 (dd, $J = 13.52$, 3.83 Hz, 1H), 1.16 (s, 3H), 0.95 (s, 3H), 0.94 (s, 3H), 0.93 (s, 3H), 0.84 (s, 3H), 0.71 (s, 3H), 0.68 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 160.11, 145.36, 143.83, 137.72, 129.95, 128.80, 123.60, 122.97, 116.59, 88.65, 57.57, 55.75, 47.59, 45.79, 43.38, 41.75, 41.61, 39.90, 38.72, 38.43, 37.02, 33.89, 33.15, 32.72, 30.79, 28.16, 26.82, 26.06, 24.49, 23.69, 23.58, 22.02, 18.17, 17.16, 16.35, 15.38; ESI-HRMS: Formula: $C_{37}H_{54}N_4O_5$; Exact mass: 634.40942; Calculated *m/z* $[M+H]^+$: 635.41725; Found *m/z* $[M+H]^+$: 635.41663.

Synthesis of benzylidene derivative (17)

Chloroform was added to a flask with a round bottom (200 mL). Compound **9** (1.50 g, 3.43 mmol, 1 eq.) was added and heated for 30 min. in the presence of catalytic amount of acetic acid (2 mL). Then aniline (1.43 g, 20.5 mmol, 6 eq.) was added and refluxed overnight. TLC was used to monitor the reaction, which was stopped when the starting material was depleted. The reaction mixture was warmed to room temperature before being extracted with a 1 percent aqueous NaOH solution. The mixture was also extracted with chloroform after being rinsed in a 1 percent aqueous hydrochloric acid (HCl) solution. The organic layers were mixed together, dried with Na_2SO_4, and then filtered. Under reduced pressure, the solvent was extracted and the residue was adsorbed on silica-gel. Compound **17** was purified using an ethyl acetate- hexane mixture (1:9) on a silica-gel column. Only compound **17** (white solid, 250 mg, 25% yield) could be isolated as pure from the mixture.

Compound 17 ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 9.39 (s, 1H), 7.27 (d, $J = 7.79$ Hz, 1H), 7.25 (d, $J = 1.56$ Hz, 1H), 6.99 (d, $J = 8.29$ Hz, 1H), 6.89 (t, $J = 7.35$, 7.35 Hz, 1H), 6.60 (s, 1H), 5.40 (t, $J = 3.49$ Hz, 1H), 2.66 (dd, $J = 13.66$, 4.01 Hz, 1H), 1.21 (s, 3H), 1.12 (s, 6H), 1.15 (s, 3H), 0.92 (s, 3H), 0.91 (s, 3H), 0.80 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 207.30, 201.06, 143.47, 142.25, 132.81, 129.33, 126.36, 122.83, 120.85, 118.50, 52.91, 49.20, 45.44, 44.28, 43.49, 42.13, 40.56, 40.33, 38.10, 33.19, 33.12, 32.56, 30.71, 28.44, 27.73, 26.75, 25.56, 23.69, 23.48, 22.18, 22.05, 20.21, 18.99, 17.47; ESI-HRMS: Formula: $C_{36}H_{51}NO$; Exact mass: 513.39707; Calculated *m/z* $[M-H]^+$: 512.38924; Found *m/z* $[M-H]^+$: 512.38739.

Cell culture

American Type Culture Collection (ATCC) offers SH-SY5Y, a human neuroblastoma cell lines. The cells were

cultivated in Dulbecco's Modified Eagle Medium F12 (DMEM-F12), which contained 10% heat-inactivated fetal bovine serum and 1% antibiotic, and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Cell homogenization

SH-SY5Y cells were plated at the density of 1 x 10^6 cells/mL culture medium in a 75 cm^2 flask. Following 24 h incubation and attachment, cells were washed phosphate-buffered saline and scraped from the flask in lysis buffer (0.1 M phosphate buffer, pH 7.8, 2 mM EDTA, 0.2% Triton X-100, 0.3 mM εACA, 1 mM DTT, and 0.5 mM PMFS). Then, cells were homogenized for 15 s. intervals for four times by sonication on ice. A bicinchoninic acid assay was used to measure total cellular protein, with bovine serum albumin as the reference.

Cholinesterase assay

Acetylcholinesterase (AChE) inhibitory activities of the synthesized compounds were evaluated using acetylthiocholine iodide (AChI) as substrate through the Ellman technique [58], which followed the emergence of the thiocholine-DTNB conjugate spectrophotometrically. In a spectrophotometric cuvette, 80 mM Tris–HCl buffer pH 8.5, 0.6 DTNB, 1 mg protein and the test compound were added and incubated for 5 min at 25 °C. The reaction was started by adding 2.5 mM AChI, and the rate was measured at 412 nm for 150 s at 25 °C using an Analytik Jena Specord 200 UV-1601 spectrophotometer. Inhibition curves were used to calculate the percentage of inhibition value (log inhibitor concentration vs. percent of inhibition). The amount of enzyme required to catalyze the hydrolysis of 1 mmol of substrate per minute at saturating substrate concentration was determined to be one unit activity [59].

Carbonic Anhydrase Assay

The human red blood cells were used as both CA isoenzymes sources. It was centrifuged at 10.000*x*g for 30 min. Then, it was precipitated and the serum was separated and adjusted with solid Tris to pH 8.7 [23]. Both CA isoenzyme was purified using Sepharose-4B-L-Tyrozyne-sulfanilamide affinity column chromatography [60]. Then, this sample was applied to the affinity column and equilibrated with Tris-Na_2SO_4/HCl (22 mM/25 mM, pH: 8.7). Finally, hCA I and II isozymes were eluted with NaCl/Na_2HPO_4 (1.0 M/0.025 M, pH 6.3) and CH_3COONa/$NaClO_4$ (0.1 M/0.5 M, pH 5.6), respectively [61]. The protein content during the purification steps was determined Bradford method [62] as described previously [63]. Bovine serum albumin was used as standard protein [64]. The purity of both hCA isoenzymes was controlled by SDS-PAGE as described in prior studies [65]. During the isoenzyme purification and inhibition process, esterase activity was performed [66]. Both CA isoenzymes activity was determined by following the change in absorbance at 348 nm [67].

Determination of IC_{50} value

For the determination of half-maximal inhibitor concentration (IC_{50}), different concentrations were used for the all oleanolic acid derivatives. In the absence of these derivatives, the control cuvette activity was accepted as 100% [68] Activities (%) – [Oleanolic acid derivatives] graphs in this study were separately plotted for each oleanolic acid derivative. The method of this study has been stated in our earlier studies in detail [69, 70].

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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