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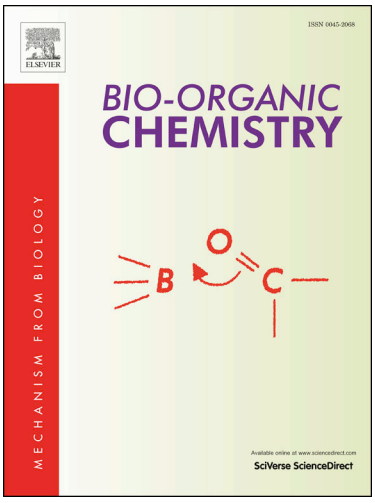

Discovery of new benzhydrol biscarbonate esters as potent and selective apoptosis inducers of human melanomas bearing the activated ERK pathway: SAR studies on an ERK MAPK signaling modulator, ACA-28

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Color should be used for Graphical abstract and Table 1.

Discovery of new benzhydrol biscarbonate esters as potent and selective apoptosis inducers of human melanomas bearing the activated ERK pathway: SAR studies on an ERK MAPK signaling modulator, ACA-28

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Highlight

・ ACA-28 (**2a**), an ERK-dependent apoptosis inducer preferentially kills melanoma cells.

・Fifteen analogs of **2a** were synthesized and analyzed for the anti-melanoma activity.

・This is the first SAR study to enhance selectivity and potency of **2a**.

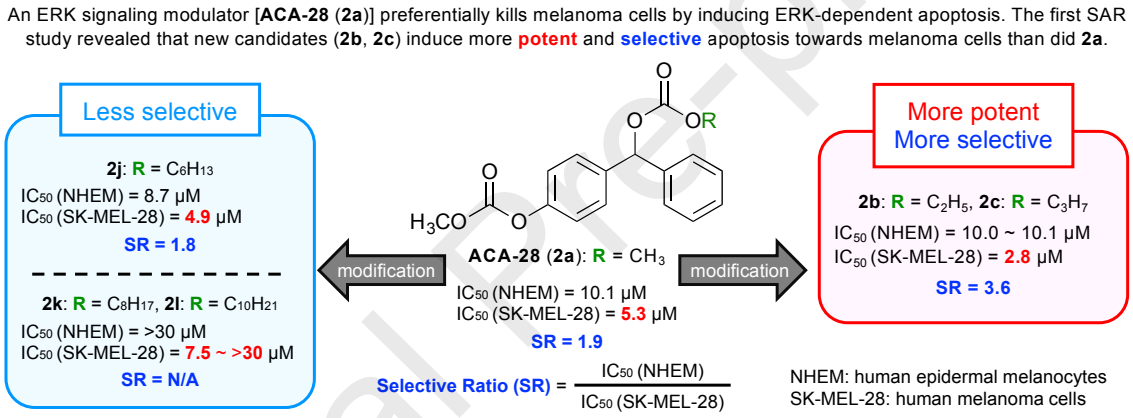
・**2b** and **2c** have much higher selectivities and potencies to kill melanoma cells than **2a**.

・The size of alkyl group at Cα-*O*-carbonate unit was crucial for cancer selectivity.

ABSTRACT

The recent discovery that an ERK signaling modulator [ACA-28 (**2a**)] preferentially kills human melanoma cell lines by inducing ERK-dependent apoptosis has generated significant interest in the field of anti-cancer therapy. In the first SAR study on **2a**, here, we successfully developed candidates (**2b**, **2c**) both of which induce more potent and selective apoptosis towards ERK-active melanoma cells than **2a**, thus revealing the structural basis for inducing the ERK-dependent apoptosis and proposing the therapeutic prospect of these candidates against ERK-dependent cancers represented by melanoma.

Graphical abstract



Keywords:

ERK MAPK; Anti-melanoma activity; Apoptosis; ACA-28; SAR Study; Benzhydrol derivatives

Abbreviations

ERK	Extracellular signal-regulated kinase
ACA	1'-Acetoxychavicol acetate
SAR	Structure-activity-relationship
MAPK	Mitogen-activated protein kinase
RAS	Rat sarcoma
MEK	Mitogen-activated protein/extracellular signal-regulated kinase kinase
BRAF	B-Raf proto-oncogene, serine/threonine kinase

NRAS	Neuroblastoma ras viral oncogene homolog
HER2	Human epidermal growth factor receptor 2
ErbB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2
NHEM	Normal human epidermal melanocytes
SR	Selective ratio
IC_{50}	50% inhibitory concentration
PTSA	Polar total surface area
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum

1. Introduction

The RAS/RAF/MEK/ERK pathway plays a key role in regulating cell proliferation, differentiation, and growth by transducing extracellular signals [1]. This pathway is often activated in various tumours. Hyperactivation of the RAS/RAF/MEK/ERK MAPK pathway has been reported in human cancer as a result of the abnormal activation of receptor tyrosine kinases or gain-of-function mutations, mainly in the RAS or RAF genes [2]. In fact, activation of ERK leads cells to acquire many of the hallmarks of cancer, including uncontrolled proliferation and resistance to apoptosis. Therefore, targeting and inhibiting the ERK signaling pathway is seen as an attractive option to overcome the malignant phenotypes.

In particular, constitutive activation of RAF/MEK/ERK signaling is prevalent in human melanomas, largely due to activating mutations in BRAF or NRAS genes, which cause hyperactivation of ERK [2-4]. Melanoma represents the most aggressive and the deadliest form of skin cancer, and the prognosis of patients with metastatic melanoma is grim, with a 5-year survival rate between 5–19%. The poor prognosis is partly due to its resistance to chemotherapies, such as Dacarbazine. Recently, the use of compounds targeting components of the ERK signaling pathway, including two RAF inhibitors [Vemurafenib (Zelboraf®)] and [dabrafenib (Tafinlar®)], and a MEK inhibitor [Trametinib (Mekinist®)] has led to substantial improvements in clinical outcomes in the treatment of melanoma. However, response rates are highly variable, and the efficacy of these drugs is primarily limited by the development of drug resistance. Both intrinsic and acquired resistance to RAF and MEK inhibitors are frequently associated with the persistence of ERK signaling in the presence of this spectrum of drugs. Therefore, the development of innovative approaches that exert selective cytotoxicity for cancer cells is highly desirable.

Recently, we have established a cell-based yeast phenotypic screen [5-7] for identifying compounds that modulate MAPK signaling by utilizing the phenotype of calcineurin knockout cells and its reversal by MAPK modulation [8]. Our chemical screening system successfully identified a benzhydrol-type analog [ACA-28 (**2a**)] of 1'-acetoxychavicol acetate [ACA (**1**)], a natural seed with various biological properties including antitumor and anti-inflammatory activities [9,10]. Notably, [ACA-28 (**2a**)] turned out to serve as an agent to preferentially kill melanoma cells with hyperactive ERK, including SK-MEL-2 and SK-MEL-28 [11]. Importantly, **2a** specifically induced

apoptosis via selective stimulation of the ERK phosphorylation in melanoma cells and HER2/ErbB2 overproducing cells wherein ERK signaling is hyperactivated [11]. The unique feature of ACA-28 to induce "ERK-dependent apoptosis" represents a conceptual breakthrough, which contrasts with the current strategy to develop ERK signaling inhibitors for cancer therapy. To the best of our knowledge, no studies focusing on the structure-activity-relationship (SAR) aspect of the ERK-dependent apoptosis inducers have been studied to date. Here, we report, as the first SAR studies of **2a**, the pharmacological evaluation of **2a** analogs on their abilities to selectively inhibit cell growth of melanoma cells by inducing ERK-dependent apoptosis.

Although **2a** preferentially inhibited the growth of melanoma cells, it also affects the cell viability of normal human epidermal melanocytes (NHEM), particularly at concentrations higher than 10 μM (Fig. 1) [11]. This prompted us to improve the potency and selectivity of **2a** towards cancer cells and thus spare normal cells. For this purpose, it is imperative to analyze the structural basis of ACA-28, which provides selective ERK-dependent apoptosis. In order to evaluate the selectivity of the compound, the ratio of IC_{50} values of a compound in terms of growth inhibition against melanoma and NHEM cells was calculated (hereafter referred to as "selective ratio (**SR**)"). By focusing on two carbonate areas at the position of C4 and Cα in the structure of **2a**, fifteen analogs (**2b–2n**, **3a,** and **3n**) were synthesized (Fig. 1). As the results of their biological evaluation, seven derivatives (**2b–2e, 2g, 2i**, and **3a**) were shown to inherit the unique biological activity of **2a** to preferentially inhibit the growth of melanoma cells, but with enhanced potency and selectivity than did **2a**. Importantly, **2b** and **2c** induced cancer-specific ERK-dependent apoptosis with much higher potencies than did **2a.** These findings suggest **2b** and **2c** as new lead compounds to combat ERK-dependent cancers such as melanomas.

2. Results and discussion

First, fifteen analogs (**2b–2n**, **3a,** and **3n**) were synthesized following procedure depicted in Scheme 1. Commercially available 4-hydroxybenzophenone (**4**) was treated with methyl chloroformate and propionyl chloride to give the corresponding esters [**5a** (R = OCH_3), **5b** (R = C_2H_5)] in good yields. Subsequently, $NaBH_4$ reduction of **5a** and **5b** were conducted under the pH-controlled conditions, because the yield of a target (**6a**) was reduced by hydrolysis of the base labile phenolic ester function when **5a** was treated under

the usual $NaBH_4$ reduction conditions. As a result, the benzhydrol intermediates (**6a**, **6b**) were obtained in 80%-91% yields. Finally, acylation of the secondary hydroxyl of **6a** and **6b** with appropriate acylating reagent afforded targets (**2b–2n**, **3a**) with different acyl moieties at the two oxygens. The other candidate (**3n**) having two propionyl moieties was synthesized in good yield by $NaBH_4$ reduction of **4**, followed by acylation of the resultant diol (**7**) with propionyl chloride. All the synthetic compounds were characterized by the combination of ^1H and ^{13}C NMR spectroscopy, infra-red spectroscopy, and high-resolution mass spectrometry.

To evaluate and compare the potency and selectivity of the synthesized ACA-28 analog compounds (**2b–2n**, **3a,** and **3n**), IC_{50} values, and selective ratios (**SRs**) of each compound in SK-MEL-28 cells and NHEM cells were calculated (Table 1). Although the effects on cell growth inhibition of **2a** against SK-MEL-28 cells have previously been reported,[11] its activities were re-evaluated in comparison with newly synthesized candidates under the same experimental conditions. As shown in Fig. 2, **2a** killed approximately 60% of SK-MEL-28 cells at 6 μM concentration (IC_{50} = 5.3 μM), whereas about 80% of NHEM cells maintained viability (IC_{50} = 10.1 μM), indicating that **2a** exhibited selectivity for the growth inhibition of melanoma cells (**SR** = 1.9). Also, the preferential growth inhibition of SK-MEL-28 cells over the NHEM cells was observed when cells were treated with 6 to 15 μM concentrations of **2a**.

Analysis of the IC_{50} values for the series of C4-methyl carbonate-type analogs (**2b**–**2n**) revealed that three compounds (**2b, 2c,** and **2e**), which have C2 or C3 alkyl moiety as a component of the carbonate unit at the Cα carbon, exhibited stronger inhibition of SK-MEL-28 cells than did **2a** at 6 μM concentration (average IC_{50} = 2.8–3.2 μM). In contrast, the NHEM cells treated with **2b, 2c,** and **2e**, maintained viability at a similar degree as that with **2a** (IC_{50}: **2a** 10.1 μM **2b** 10.0 μM, **2c** 10.1 μM, **2e** 10.9 μM), indicating that **2b, 2c,** and **2e** achieved higher selectivity against melanoma cells (**SR** >3.0). Especially, the potencies of **2b** and **2c** to inhibit melanoma cell growth were outstanding with IC_{50} values of less than 3 μM (IC_{50}: **2b** 2.8 μM, **2c** 2.8 μM). Approximately 60% of melanoma cells were killed upon 3 μM treatment of **2b** and **2c**, whereas NHEM cells exhibited a markedly high survival rate of up to 90% (IC_{50}: **2b** 10.0 μM, **2c** 10.1 μM) (**SR** = 3.6). Interestingly, despite **2f** having a propargyl group, which is constructed by three carbons, **2f** exhibited a low potency and a poor selectivity for the inhibition of melanoma cells (IC_{50} = 5.5 μM and the **SR** = 2.2). Although substitution

with the C4 or C6 alkyl group maintained moderate anti-melanoma activities of **2g**, **2h**, **2i**, and **2j** (IC_{50}: **2g** 3.8 μM, **2h** 4.2 μM, **2i** 4.3 μM, **2j** 4.9 μM), their potencies were inferior to those of **2b**-**2e**. Further elongation by the introduction of n-octyl (C8) and n-decyl (C10) groups led to a significant reduction in anti-melanoma activity as evidenced by the high IC_{50} values of **2k** (7.5 μM) and **2l** (>30 μM) on melanoma cell growth. Thus, it was noteworthy that the introduction of C2 or C3 alkyl groups to the carbonate moiety at the Cα carbon, by enhancing its hydrophobic property, contributes to preferentially improve the activity of this series of compounds against melanoma cells while maintaining the viability of NHEM cells. The size of alkyl groups (C2 or C3) and/or their hydrophobic property of alkyl groups were seemed to involve in the onset of good cell selectivity. The boiled egg analysis [12] using calculated physicochemical properties, such as lipophilicity (logP) and polar total surface area (PTSA), of the thirteen candidates (**2a**-**2m**) by using the SwissADME server [13] suggested that nine (**2a**-**2i**) compounds were blood-brain-barrier permeable and the remaining four (**2j**-**2m**) were absorbable in the gastrointestinal tract (Fig. S1, S2). This would suggest that a delicate balance between WlogP (from 3.38 to 4.16) and PTSA (71.1) values was important for achieving both excellent potency and selectivity towards SK-MEL-28 cells (Table S1). Additionally, although WlogP (4.16) and PTSA (71.1) values of **2c** and **2d** were the same with each other, a significant difference was detected with respect to their **SR** (**2c**/**2d** = 3.6/2.9). The bulkiness of the substituent was suggested to contribute to the intensity of the activity and selectivity.

Next, three isosteres (**2n**, **3a,** and **3n**) of **2a** were tested. The results showed the following potencies: **3a** showed substantially similar anti-melanoma activity (IC_{50} = 4.1 μM) and a cell-selectivity (**SR** = 2.1) compared with those of **2a**, thus indicating that bioisosteric replacement of the oxygen atom of the carbonate unit at the C4 carbon of **2a** with methylene unit has little effect on the potency and cell-selectivity.

In contrast, a similar modification regarding the carbonate unit at the Cα carbon of **2a** resulted in a significant reduction in the cell growth inhibition ability of **2n** towards SK-MEL-28 (IC_{50} = 13.3 μM) and NHEM cells (IC_{50} = 29.0 μM). Compound (**3n**) also showed similar cytotoxic properties to that of **2n**.

Although the potencies of **2n** and **3n** against melanoma cells were weakened as compared with **2a** by replacing the oxygen atom of carbonate unit at the Cα carbon with methylene unit, the **SR** was maintained (≒ 2). We have previously shown that **2a** induced selective

cell growth inhibition against multiple melanoma cell lines, including SK-MEL-28, SK-MEL-2, and MeWo, all of which have the highly activated ERK MAPK signaling as compared with the normal melanocyte. To investigate if the newly synthesized fifteen analogs (**2b–2n**, **3a,** and **3n**) of **2a** also exhibited selectivity against melanomas, we calculated the IC_{50} values and SRs of the fifteen analogs against SK-MEL-2 and MeWo cells in comparison with NHEM cells. The results are substantially similar to those obtained with SK-MEL-28 cells and further corroborated the conclusion that **2b** and **2c** have superior anti-melanoma activity with a good cell-selectivity (Fig. S3 and Table S2).

As aforementioned, four C4-carbonate analogs **2b** and **2c** exhibited prominent cytotoxicity for melanoma cells (IC_{50} <3 μM) with a high **SR** (=3.6). We, therefore, selected **2b** and **2c** to further analyze their ability to induce ERK-dependent apoptosis in comparison with those of the prototype compound ACA-28 (**2a**), and a less potent and non-selective derivative (**2l**). As shown in Figure 3A (upper panel), the immunoblotting analysis showed that **2b** and **2c** significantly stimulated ERK and ELK1 (a transcription factor as an established ERK substrate) phosphorylation in SK-MEL-28 cells, but not in NHEM cells. The levels of ERK and ELK1 phosphorylation upon **2b** and **2c** treatment in SK-MEL-28 cells seemed higher than those upon **2a**. In contrast, the activation of ERK by a less-potent and non-selective compound **2l** remained unchanged. In order to quantitatively evaluate the ability of these compounds to induce ERK and ELK1 phosphorylation, the relative phosphorylation levels in SK-MEL-28 cells were quantified (Fig. 3A lower panel). The effect of **2b** on ERK activation was far exceeded 150% (up to 180%), as compared with **2a,** which induced approximately 150% activation when phosphorylation levels upon DMSO treatment were set as 100%. In particular, **2c** achieved approximately 210% ERK activation. More importantly, the ERK activation induced by **2b** and **2c** in NHEM cells was almost undetectable, again confirming the highly selective nature of **2b** and **2c** to induce melanoma-specific ERK activation.

Moreover, significant cleavage of the Caspase-3 concomitant with ERK activation was observed in SK-MEL-28 cells treated with **2a**, **2b,** or **2c,** but not with DMSO or **2l**, indicating that **2b** or **2c**, similar to **2a** induced ERK-dependent apoptosis. The induction of apoptosis was stronger in cells treated with **2b** or **2c** as compared with **2a**, further proving that **2b** and **2c** are more potent inducers of ERK-dependent apoptosis in SK-MEL-28 cells than **2a**. In contrast, the cleaved Caspase-3 was barely detectable in NHEM cells treated with all the compounds examined, thus confirming that **2b** and **2c**,

similarly to **2a**, induced cancer-specific apoptosis. Importantly, MEK inhibitor U0126 attenuated the cleavage of Caspase-3 by **2b** and **2c**, suggesting these compounds induce apoptosis through the activation of ERK signaling (Fig 3B). We also performed cell-based assays of apoptotic cells to investigate the effects of the ACA derivatives (**2a**, **2b**, and **2c**) and Cisplatin (positive control) as an apoptosis inducer (Fig. S4). As a result, both of the two cell-based assays, (Caspase-Glo® 3/7 Assay System: Fig. S4, upper panel, and RealTime Glo Annexin V Apoptosis Assay: Fig. S4, lower panel), reproduced the results of the caspase cleavage assays confirming the impact of ACA derivatives (**2a**, **2b**, and **2c**) to induce apoptosis, which is significantly stronger than that by Cisplatin (at 10 μM). Altogether, **2b** and **2c** were more specific and potent inducers of ERK-dependent apoptosis than **2a**.

Finally, we also performed the *in vitro* kinase assay experiments to investigate the effects of the ACA derivatives (**2b** and **2l**, a good and a poor ERK signaling stimulator, respectively), on the ERK kinase activity using ELK1, a transcription factor as an established ERK substrate (Fig. 4). As a result, we obtained the data showing the effect of **2b**, but not **2l,** to stimulate ERK kinase activity *in vitro*, which correlated well with the ERK phosphorylation levels in melanoma cells. Thus, although the mechanism and targets of ACA derivatives remain unknown, these results confirmed that ACA derivatives modulate ERK phosphorylation levels, which leads to biochemical ERK kinase activity. Regarding the experiments to investigate the binding ability of the compounds on the components of the ERK signaling pathway, including ERK MAPK, will be important issues for future study.

3. Conclusion

In summary, with the aim of developing new therapeutic agents for targeting the ERK signaling pathway, here we performed the first SAR study of ACA-28 (**2a**) to develop its ability to selectively inhibit cancer cell growth by inducing apoptosis with ERK hyperactivation, by focusing on two methyl carbonate moieties at C4- and Cα-oxygens of **2a.** The substitution of the methyl group of the carbonate unit at the Cα-oxygen with the other alkyl groups longer than eight carbons (**2k** and **2l**) was not preferable for the anti-melanoma activity. Compounds (**2a**–**2j**) bearing the alkyl groups with the carbons-length less than six were favorable for the onset of the potent activity. The optimal substitution pattern for the activity was obtained when the alkyl group was

two or three carbons (**2b**–**2e**) in length, indicating that **2b**–**2e** were potent apoptosis inducers in melanoma. Particularly, the ethylated and *n*-propylated analogs (**2b** and **2c**) showed good activity (IC_{50} = 2.8 μM) in this series of candidates. It was noteworthy that **2b** and **2c** exhibited higher selectivity ratios between SK-MEL-28 and NHEM cells than **2a**. Regarding the bioisosteric replacement of oxygen atoms at both carbonate units of **2a** with methylene moiety, the bioisostere (**3a**) was revealed to show potent activity, with the same degree as those of **2b**. On the other hand, regarding the bioisosteric replacements of oxygen atoms at Cα-carbonate units of **2a** and **3a** with methylene moiety, the bioisosteres (**2n** and **3n**) significantly decreased the activity against SK-MEL-28.

As aforementioned, this first SAR study on ACA-28 (**2a**) is a significant step forward for the development of new anti-cancer measures against ERK-dependent cancers. However, critical questions remain, including the identification of far more potent and selective inducers of ERK-dependent apoptosis than **2b** and **2c**, as well as clarification of the mechanisms underlying ACA-28-induced cancer cell killing to establish therapeutic measures to combat incurable diseases associated with deteriorated ERK signaling. Performing further SAR studies, and creating appropriate probes to find out what are the potential molecular targets of ACA-28 (**2a**) are currently underway in our laboratory.

4. Experimental

4.1. General experimental procedures

Mps were determined on a hot-stage melting point apparatus and are uncorrected. IR spectra were measured on an FT-IR spectrophotometer. NMR spectra were recorded on an FT-NMR spectrometer (^{1}H, 400, 500, or 800 MHz; ^{13}C, 125, or 200 MHz). Chemical shifts (δ) and coupling constants (J) are given in ppm and Hz, respectively. Tetramethylsilane (TMS) was used as an internal standard for ^{1}H NMR measurements in $CDCl_3$, whereas ^{13}C NMR measurements utilized the solvent signal (77.0 ppm) of $CDCl_3$ for this purpose. For the measurement of ^{1}H and ^{13}C NMR spectra in CD_3OD, the solvent signals 33.1 ppm and 49.0 ppm were used, respectively, as internal standards. Low-resolution and high-resolution mass spectra were recorded on an orbitrap mass spectrometer (ESI). All the organic extracts were dried over anhydrous Na_2SO_4 prior to evaporation.

*4.1.1. Acylation of 4-hydroxybenzophenone (**4**)*

*4.1.1.1. Benzophenone-4-yl methyl carbonate (**5a**)*

Methyl chloroformate (1.6 mL, 20.7 mmol) was added to a solution 4-hydroxybenzophenone (**4**, 1.98 g, 10 mmol) in dry pyridine (20 mL) at 0 °C. After being stirred at 0 °C for 1 h, the reaction mixture was poured into with ice-water (100 mL) and the resulting mixture was extracted with ethyl acetate (50 mL x 1, 30 mL x 2). The extract was successively washed with 10% ice-cooled sulfuric acid, aqueous sodium hydrogen carbonate and brine and condensed *in vacuo* to give a colorless solid (2.57 g), which on recrystallization from a mixture of *n*-hexane and ethyl acetate, gave the title compound (**5a**, 2.42 g, 95%) as colorless needles, mp. 95–96 °C, lit. [14] mp. 94–94 °C. IR (KBr): 1762, 1651 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 3.94 (3H, s, OCH_3), 7.31 (2H, dm, J = 8.9, arom.), 7.49 (2H, tm, J = 7.4, arom.), 7.60 (1H, tm, J = 7.4, arom.), 7.80 (2H, dm, J = 7.4, arom.), 7.87 (2H, dm, J = 8.9, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 55.6 (OCH_3), 120.9/128.3/129.9/131.7/132.5 (d, arom.), 135.3/137.4/153.6/154.1 (s, arom. and $CH_3OCO_2C_6H_4$), 195.4 ($C_6H_4COC_6H_5$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{15}H_{12}O_4Na$ 279.0628; Found 279.0631.

*4.1.1.2. Benzophenone-4-yl propionate (**5b**)*

According to the method used for the preparation of **5a**, 4-hydroxybenzophenone (**4**, 300 mg, 1.52 mmol) was treated with propionyl chloride (264 mL, 3.02 mmol) in dry pyridine (3 mL) at 0 °C for 30 min. Work-up gave a colorless oil (395 mg), which on column chromatography ($CHCl_3$) gave the title compound (**5b**, 369 mg, 96%) as a colorless viscous oil. IR (neat): 1767, 1659 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.29 (3H, t, J = 7.6, CH_2CH_3), 2.64 (2H, q, J = 7.6, CH_2CH_3), 7.22 (2H, dm, J = 8.9, arom.), 7.49 (2H, tm, J = 7.4, arom.), 7.60 (1H, tm, J = 7.4, arom.), 7.80 (2H, dm, J = 7.4, arom.), 7.86 (2H, dm, J = 8.9, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 9.0 (CH_2CH_3), 27.8 (CH_2CH_3), 121.5/128.3/129.9/131.7/132.4 (d, arom.), 134.9/137.5/154.0 (s, arom.), 172.5 ($C_2H_5CO_2C_6H_4$), 195.6 ($C_6H_4COC_6H_5$) HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{16}H_{14}O_3Na$ 277.0835; Found 277.0837.

*4.1.2. $NaBH_4$ reduction of 4-acyloxybenzophenone (**5a** and **5b**)*

*4.1.2.1 Benzhydrol-4-yl methyl carbonate (**6a**)*

To a mixture of benzophenone-4-yl methyl carbonate (**5a**, 1.09 g, 4.26 mmol),

tetrahydrofuran (24 mL) and acetate buffer (pH = 5, 4 mL) was added $NaBH_4$ (177 mg, 4.65 mmol) at 0 °C. The mixture was stirred at 0 °C for 1 h and then stirred at room temperature for another 30 min. The reaction mixture was poured into with ice-water (50 mL), and the resulting mixture was extracted with ethyl acetate (50 mL x 1, 30 mL x 2). The extract was washed with brine and condensed *in vacuo* to give a colorless oil (1.06 g), which on column chromatography ($CHCl_3$) gave the titled compound (**6a**, 1.00 g, 91%) as a colorless oil. IR (neat): 3421, 1767 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 2.34 (1H, d, J = 3.1, OH), 3.89 (3H, s, OCH_3), 5.83 (1H, d, J = 3.1, C*H*OH), 7.13 (2H, dm, J = 8.7, arom.), 7.25–7.41 (7H, m, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 55.4 (O*C*H_3), 75.6 (*C*HOH), 121.0/126.5/127.65/127.73/128.6 (d, arom.), 141.6/143.4/150.3 (s, arom.), 154.2 (CH_3OCO_2). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{15}H_{14}O_4Na$ 281.0784; Found 281.0788.

*4.1.2.2. Benzhydrol-4-yl propionate (**6b**)*

According to the method used for the preparation of **6a**, benzophenone-4-yl propionate (**5b**, 350 mg, 1.38 mmol) was treated with $NaBH_4$ (52 mg, 1.38mmol) in a mixture of tetrahydrofuran (8 mL) and acetate buffer (pH = 5, 2 mL) at 0 °C for 2 h. Work-up gave a colorless oil (430 mg), which on column chromatography ($CHCl_3$→$CHCl_3$/CH_3OH, 20:1) gave the titled compound (**6b**, 282 mg, 80%) as a colorless oil. IR (neat): 3449, 1759 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.25 (3H, t, J = 7.6, $COCH_2CH_3$), 2.31 (1H, d, J = 3.0, OH), 2.57 (2H, q, J = 7.6, $COCH_2CH_3$), 5.83 (1H, d, J = 3.0, C*H*OH), 7.40 (2H, dm, J = 8.6, arom.), 7.27 (1H, m, arom.), 7.31–7.41 (6H, m, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 9.0 ($COCH_2CH_3$), 27.7 ($COCH_2CH_3$), 75.7 (*C*HOH), 121.5/126.5/127.6/127.7/128.5 (d, arom.), 141.2/143.5/150.0 (s, arom.), 173.0 ($C_2H_5CO_2$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{16}H_{16}O_3Na$ 279.0992; Found 279.0995.

*4.1.3. Acylation of compound (**6a**)*

*4.1.3.1. Ethyl 4-(methoxycarbonyloxy)benzhydryl carbonate (**2b**)*

Ethyl chloroformate (200 μL, 2.1 mmol) was added to a solution of **6a** (271 mg, 1.05 mmol) in dry pyridine (2 mL) at 0 °C. After being stirred at 0 °C for 1.5 h, the reaction mixture was poured into ice-water (20 mL), and the resulting mixture was extracted with ethyl acetate (10 mL x 3). The extract was successively washed with 10% ice-cooled

sulfuric acid, aqueous sodium bicarbonate, and brine and condensed in vacuo to give a colorless solid (320 mg), which on column chromatography (*n*-hexane–AcOEt, 10/1) gave the title compound (**2b**, 298 mg, 86%) as colorless needles (from *n*-hexane), mp. 69–70 °C. IR (KBr): 1774, 1751 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.31 (3H, t, J = 7.0, OCH_2CH_3), 3.89 (3H, s, OCH_3), 4.02 (2H, q, J = 7.0, OCH_2CH_3), 6.70 (1H, s, $CHOCO_2$), 7.16 (2H, dm, J = 8.7, arom.), 7.27–7.41 (7H, m, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.2 (OCH_2CH_3), 55.4 (OCH_3), 64.3 (OCH_2CH_3), 79.9 ($CHOCO_2$), 121.1/126.9/128.2/128.3/128.6 (d, arom.), 137.6/139.3/150.7 (s, arom.), 154.1 ($CH_3OCO_2C_6H_4$), 154.4 ($CHOCO_2C_2H_5$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{18}H_{18}O_6Na$ 353.0996; Found 353.0996.

*4.1.3.2. 4-(Methoxycarbonyloxy)benzhydryl n-propyl carbonate (**2c**)*

According to the method used for the preparation of **2b**, **6a** (208 mg, 0.81 mmol) was treated with *n*-propyl chloroformate (280 μL, 2.48 mmol) in dry pyridine (2 mL) at 0 °C for 1 h. Work-up gave a colorless oil (282 mg), which on column chromatography ($CHCl_3$) gave the title compound (**2c**, 239 mg, 86%) as a colorless viscous oil. IR (neat): 1767, 1748 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.95 (3H, t, J = 7.4, $OCH_2CH_2CH_3$), 1.69 (2H, qt, J = 7.4, 6.7, $OCH_2CH_2CH_3$), 3.90 (3H, s, OCH_3), 4.10 (2H, t, J = 6.7, $OCH_2CH_2CH_3$), 6.70 (1H, s, $CHOCO_2$), 7.15 (2H, dm, J = 8.5, arom.), 7.28–7.37 (5H, m, arom.), 7.39 (2H, dm, J = 8.5, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 10.1 ($OCH_2CH_2CH_3$), 22.0 ($OCH_2CH_2CH_3$), 55.4 (OCH_3), 69.9 ($OCH_2CH_2CH_3$), 79.9 ($CHOCO_2$), 121.1/126.9/128.2/128.3/128.6 (d, arom.), 137.6/139.3/150.7 (s, arom.), 154.1 ($CH_3OCO_2C_6H_4O$), 154.6 ($CHOCO_2{}^nC_3H_7$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{19}H_{20}O_6Na$ 367.1152; Found 367.1147.

*4.1.3.3. Isopropyl 4-(methoxycarbonyloxy)benzhydryl carbonate (**2d**)*

According to the method used for the preparation of **2b**, **6a** (207 mg, 0.80 mmol) was treated with isopropyl chloroformate (290 μL, 2.53 mmol) in dry pyridine (2 mL) at 0 °C for 2 h. Work-up gave a colorless oil (280 mg), which on column chromatography ($CHCl_3$) gave the title compound (**2d**, 229 mg, 83%) as a colorless viscous oil. IR (neat): 1767, 1744 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.286/1.294 [each 3H, d, J = 6.2, $OCH(CH_3)_2$], 3.89 (3H, OCH_3), 4.88 [1H, sept, J = 6.2, 6.2, $OCH(CH_3)_2$], 6.69 (1H, s, $CHOCO_2$), 7.15 (2H, dm, J = 8.7, arom.), 7.27–7.36 (5H, m, arom.), 7.39 (2H, dm, J =

8.7 arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.8 [OCH(CH_3)$_2$], 55.4 (OCH_3), 72.4 [OCH(CH$_3$)$_2$], 79.7 ($CHOCO_2$), 121.1/126.9/128.2/128.3/128.6 (d, arom.), 137.7/139.5/150.7 (s), 154.0/154.1 ($CH_3OCO_2C_6H_4$ and $CHOCO_2{}^{iso}C_3H_7$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{19}H_{20}O_6Na$ 367.1152; Found 367.1148.

*4.1.3.4. Allyl 4-(methoxycarbonyloxy)benzhydryl carbonate (**2e**)*

According to the method used for the preparation of **2b**, **6a** (200 mg, 0.78 mmol) was treated with allyl chloroformate (270 μL, 2.56 mmol) in dry pyridine (2 mL) at 0 °C for 2 h. Work-up gave a colorless oil (278 mg), which on column chromatography ($CHCl_3$) gave the title compound (**2e**, 225 mg, 85%) as a colorless waxy solid, mp. 54–55 °C. IR (KBr): 1767, 1751 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.89 (3H, s, OCH_3), 4.63 (2H, ddd, *J* = 5.9, 1.4, 1.1, CH_2CH=CH$_2$), 5.26 (1H, ddt, *J* = 10.5, 1.4, 1.1, CH$_2$CH=CH*H*), 5.35 (1H, ddt, *J* = 17.2, 1.4, 1.4 CH$_2$CH=C*H*H), 5.92 (1H, ddt, *J* = 17.2, 10.5, 5.9 CH$_2$C*H*=CH$_2$), 6.70 (1H, s, $CHOCO_2$), 7.16 (2H, dm, *J* = 8.7, arom.), 7.28–7.36 (5H, m, arom.), 7.39 (2H, dm, *J* = 8.7, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 55.4 (OCH_3), 68.7 ($CH_2CH=CH_2$), 80.1 ($CHOCO_2$), 119.1 ($CH_2CH=CH_2$), 121.1 ($CH_2CH=CH_2$), 126.9/128.2/128.3/128.6/131.4 (d, arom.), 137.4/139.2/150.8 (s, arom.), (s), 154.1 ($CH_3OCO_2C_6H_4$), 154.3 ($CHOCO_2CH_2CH=CH_2$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{19}H_{18}O_6Na$ 365.0996; Found 365.0996.

*4.1.3.5. 4-(Methoxycarbonyloxy)benzhydryl propagyl carbonate (**2f**)*

According to the method used for the preparation of **2b**, **6a** (160 mg, 0.62 mmol) was treated with propagyl chloroformate (180 μL, 1.88 mmol) in dry pyridine (2 mL) at 0 °C for 1 h. Work-up gave a colorless oil (263 mg), which on column chromatography (*n*-hexane–AcOEt, 5/1) gave the title compound (**2f**, 197 mg, 93%) as a colorless microcrystalline solid, mp. 59–60 °C. IR (KBr): 3291, 2129, 1761 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.53 (1H, t, *J* = 2.6, CH$_2$C≡C*H*), 3.89 (3H, s, OCH_3), 4.73 (2H, d, *J* = 2.6, CH_2C≡CH), 6.71 (1H, s, $CHOCO_2$), 7.16 (2H, dm, *J* = 8.9, arom.), 7.28–7.36 (5H, m, arom.), 7.39 (2H, dm, *J* = 8.9, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 55.4 (OCH_3), 55.5 ($CH_2C≡CH$), 75.9 ($CH_2C≡CH$), 76.7 ($CH_2C≡CH$), 80.6 ($CHOCO_2$), 121.1/126.9/128.29/128.8.34/128.6 (d, arom.), 137.1/138.9/150.8 (s, arom.), (s), 153.8/154.0 ($CH_3OCO_2C_6H_4$ and $CHOCO_2CH_2C≡CH$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{19}H_{16}O_6Na$ 363.0839; Found 363.0827.

*4.1.3.6. n-Butyl 4-(methoxycarbonyloxy)benzhydryl carbonate (**2g**)*

According to the method used for the preparation of **2b**, **6a** (200 mg, 0.78 mmol) was treated with *n*-butyl chloroformate (330 μL, 2.54 mmol) in dry pyridine (2 mL) at 0 °C for 1 h. Work-up gave a colorless oil (272 mg), which on column chromatography ($CHCl_3$) gave the title compound (**2g**, 252 mg, 91%) as a colorless viscous oil, IR (neat): 1763, 1745 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.92 (3H, t, J = 7.4, $OCH_2CH_2CH_2CH_3$), 1.34–1.43 (2H, m, $OCH_2CH_2CH_2CH_3$), 1.61–1.68 (2H, m, $OCH_2CH_2CH_2CH_3$), 3.89 (3H, s, OCH_3), 4.14 (2H, t, J = 6.7, $OCH_2CH_2CH_2CH_3$), 6.69 (1H, s, $CHOCO_2$), 7.15 (2H, dm, J = 8.7, arom.), 7.27–7.36 (5H, m, arom.), 7.38 (2H, dm, J = 8.7, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 13.6 ($OCH_2CH_2CH_2CH_3$), 18.9 ($OCH_2CH_2CH_2CH_3$), 30.6 ($OCH_2CH_2CH_2CH_3$), 55.4 (OCH_3), 68.2 ($OCH_2CH_2CH_2CH_3$), 79.9 ($CHOCO_2$), 121.0/126.9/128.2/128.3/128.6 (d, arom.), 137.6/139.4/150.8 (s, arom.), 154.1 ($CH_3OCO_2C_6H_4$), 154.6 ($CHOCO_2{}^nC_4H_9$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{20}H_{22}O_6Na$ 381.1309; Found 381.1307.

*4.1.3.7. tert-Butyl 4-(methoxycarbonyloxy)benzhydryl carbonate (**2h**)*

A mixture of **6a** (100 mg, 0.39 mmol), di-*tert*-butyl carbonate (170 mg, 0.78 mmol), 4-dimethylaminopyridine (14.2 mg, 0.12 mmol) and dry pyridine (2 mL) was stirred at 0 °C for 3 h. The reaction mixture was poured into ice-water (20 mL) and the resulting mixture was extracted with ethyl acetate (10 mL x 3). The extract was successively washed with 10% ice-cooled sulfuric acid, aqueous sodium bicarbonate and brine and condensed in vacuo to give a colorless oil (156 mg), which on column chromatography (*n*-hexane–AcOEt, 10/1) gave the title compound (**2h**, 122 mg, 89%) as a colorless microcrystalline solid, mp. 87–88 °C. IR (neat): 1767, 1743 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.47 [9H, s, $OC(CH_3)_3$], 3.88 (3H, s, OCH_3), 6.64 (1H, s, $CHOCO_2$), 7.15 (2H, dm, J = 8.6, arom.), 7.27–7.36 (5H, m, arom.), 7.38 (2H, dm, J = 8.6, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 27.7 [$OC(CH_3)_3$], 55.4 (OCH_3), 79.1 ($CHOCO_2$), 82.6 [$OC(CH_3)_3$], 121.0/126.9/128.0/128.2/128.6 (d, arom.), 137.9/139.6/150.6 (s), 152.8/154.1 ($CH_3OCO_2C_6H_4$ and $CHOCO_2{}^{tert}C_4H_9$) HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{20}H_{22}O_6Na$ 381.1309; Found 367.1297.

*4.1.3.8. Isobutyl 4-(methoxycarbonyloxy)benzhydryl carbonate (**2i**)*

According to the method used for the preparation of **2b**, **6a** (200 mg, 0.78 mmol) was treated with isobutyl chloroformate (330 μL, 2.51 mmol) in dry pyridine (2 mL) at 0 °C for 1 h. Work-up gave a colorless oil (275 mg), which on column chromatography ($CHCl_3$) gave the title compound (**2i**, 236 mg, 85%) as a colorless viscous oil, IR (neat): 1767, 1748 cm^{-1}. ^1H NMR (800 MHz, $CDCl_3$) δ: 0.94 [6H, d, *J* = 6.7, $OCH_2CH(CH_3)_2$], 1.98 (1H, nonet, *J* = 6.7, $OCH_2CH(CH_3)_2$], 3.90 (3H, s, OCH_3), 3.93 (2H, d, *J* = 6.7, $OCH_2CH(CH_3)_2$], 6.70 (1H, s, $CHOCO_2$), 7.16 (2H, dm, *J* = 8.7, arom.), 7.29–7.37 (5H, m, arom.), 7.39 (2H, dm, *J* = 8.7, arom.). ^{13}C NMR (200 MHz, $CDCl_3$) δ: 18.9 [$OCH_2CH(CH_3)_2$], 27.8 [$OCH_2CH(CH_3)_2$], 55.4 (OCH_3), 77.4 [$OCH_2CH(CH_3)_2$], 79.9 ($CHOCO_2$), 121.1/126.9/128.2/128.3/128.6 (d, arom.), 137.6/139.4/150.8 (s), 154.1 ($CH_3OCO_2C_6H_4$), 154.7 ($CHOCO_2{}^{iso}C_4H_9$. HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{20}H_{22}O_6Na$ 381.1309; Found 367.1309.

*4.1.3.9. n-Hexyl 4-(methoxycarbonyloxy)benzhydryl carbonate (**2j**)*

According to the method used for the preparation of **2b**, **6a** (202 mg, 0.78 mmol) was treated with *n*-hexyl chloroformate (407 μL, 2.50 mmol) in dry pyridine (2 mL) at 0 °C for 1 h. Work-up gave a colorless oil (317 mg), which on column chromatography ($CHCl_3$) gave the title compound (**2j**, 268 mg, 89%) as a colorless viscous oil. The oil turned into a waxy solid on keeping in the freezer, mp. 38–39 °C. IR (neat): 1767, 1748 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.88 [3H, t, *J* = 7.0, $OCH_2CH_2(CH_2)_3CH_3$], 1.24–1.39 [6H, m, $OCH_2CH_2(CH_2)_3CH_3$], 1.62–1.70 [2H, m, $OCH_2CH_2(CH_2)_3CH_3$], 3.89 (3H, s, OCH_3), 4.13 [2H, t, *J* = 6.8, $OCH_2CH_2(CH_2)_3CH_3$], 6.69 (1H, s, $CHOCO_2$), 7.15 (2H, dm, *J* = 8.7, arom.), 7.27–7.37 (5H, m, arom.), 7.39 (2H, dm, *J* = 8.7, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 13.9 [$OCH_2(CH_2)_4CH_3$], 22.5/25.3/28.6/31.4 [$OCH_2(CH_2)_4CH_3$], 55.4 (OCH_3), 68.5 [$OCH_2(CH_2)_4CH_3$], 79.9 ($CHOCO_2$), 121.1/126.9/128.2/128.3/128.6 (d, arom.), 137.6/139.4/150.8 (s, arom.), 154.1 ($CH_3OCO_2C_6H_4$), 154.6 ($CHOCO_2{}^nC_6H_{13}$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{22}H_{26}O_6Na$ 409.1622; Found 409.1617.

*4.1.3.10. 4-(Methoxycarbonyloxy)benzhydryl n-octyl carbonate (**2k**)*

According to the method used for the preparation of **2b**, **6a** (202 mg, 0.78 mmol) was treated with *n*-octyl chloroformate (500 μL, 2.54 mmol) in dry pyridine (2 mL) at 0 °C for 1 h. Work-up gave a colorless oil (329 mg), which on column chromatography

($CHCl_3$) gave the title compound (**2k**, 272 mg, 84%) as a colorless viscous oil, IR (neat): 1767, 1748 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.88 (3H, t, *J* = 7.2, $OCH_2CH_2(CH_2)_5CH_3$], 1.22–1.38 (10H, m, $OCH_2CH_2(CH_2)_5CH_3$], 1.62–1.70 (2H, m, $OCH_2CH_2(CH_2)_5CH_3$], 3.90 (3H, s, OCH_3), 4.13 (2H, t, *J* = 6.7, $OCH_2CH_2(CH_2)_5CH_3$], 6.70 (1H, s $CHOCO_2$), 7.15 (2H, dm, *J* = 8.7, arom.), 7.27–7.37 (5H, m, arom.), 7.39 (2H, dm, *J* = 8.7, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.1 [$OCH_2(CH_2)_6CH_3$], 22.6/25.6/28.6/29.10/29.13/31.7 [$OCH_2(CH_2)_6CH_3$], 55.4 (OCH_3), 68.5 [$OCH_2(CH_2)_6CH_3$], 79.8 ($CHOCO_2$), 121.1/126.9/128.2/128.3/128.6 (d, arom.), 137.6/139.3/150.7 (s, arom.), 154.0 ($CH_3OCO_2C_6H_4$), 154.6 ($CHOCO_2{}^nC_8H_{17}$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{24}H_{30}O_6Na$ 437.1935; Found 437.1933.

*4.1.3.11. n-Decyl 4-(methoxycarbonyloxy)benzhydryl carbonate (**2l**)*

According to the method used for the preparation of **2b**, **6a** (200 mg, 0.78 mmol) was treated with *n*-decyl chloroformate (575 μL, 2.50 mmol) in dry pyridine (2 mL) at 0 °C for 1 h. Work-up gave a colorless oil (350 mg), which on column chromatography ($CHCl_3$) gave the title compound (**2l**, 308 mg, 90%) as a colorless viscous oil, IR (neat): 1763, 1748 cm^{-1}. ^1H NMR (800 MHz, $CDCl_3$) *δ*: 0.88 (3H, t, *J* = 7.2, $OCH_2CH_2(CH_2)_7CH_3$], 1.21–1.36 (14H, m, $OCH_2CH_2(CH_2)_7CH_3$], 1.63–1.69 (2H, m, $OCH_2CH_2(CH_2)_7CH_3$]), 3.89 (3H, s, OCH_3), 4.13 (2H, t, *J* = 6.7, $OCH_2CH_2(CH_2)_7CH_3$], 6.69 (1H, s, $CHOCO_2$), 7.15 (2H, dm, *J* = 8.7, arom.), 7.27–7.37 (5H, m, arom.), 7.38 (2H, dm, *J* = 8.7, arom.). ^{13}C NMR (200 MHz, $CDCl_3$) *δ*: 14.1 [$OCH_2(CH_2)_8CH_3$], 22.7/25.6/28.6/29.2/29.3/29.46/29.48/31.9 [$OCH_2(CH_2)_8CH_3$], 55.4 (OCH_3), 68.5 [$OCH_2(CH_2)_8CH_3$], 79.9 ($CHOCO_2$), 121.1/126.9/128.2/128.3/128.6 (d, arom.), 137.6/139.4/150.8 (s, arom.), 154.1 ($CH_3OCO_2C_6H_4$), 154.6 ($CHOCO_2{}^nC_{10}H_{21}$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{26}H_{34}O_6Na$ 465.2248; Found 465.2249.

*4.1.3.12. 4-(Methoxycarbonyloxy)benzhydryl phenyl carbonate (**2m**)*

According to the method used for the preparation of **2b**, **6a** (200 mg, 0.78 mmol) was treated with phenyl chloroformate (316 μL, 2.50 mmol) in dry pyridine (2 mL) at 0 °C for 1 h. Work-up gave a colorless viscous oil (312 mg), which on column chromatography ($CHCl_3$) gave the title compound (**2m**, 244 mg, 83%) as a colorless microcrystalline solid, mp. 86–87 °C. IR (neat): 1763 cm^{-1}. ^1H NMR (800 MHz, $CDCl_3$) δ: 3.90 (3H, s, OCH_3), 6.79 (1H, s, $CHOCO_2$), 7.16 (2H, dm, *J* = 7.3, arom.), 7.19 (2H, dm, *J* = 8.7, arom.), 7.23

(1H, tm, *J* = 7.3, arom.), 7.33 (1H, tm, *J* = 7.3, arom.), 7.35–7.39 (4H, m, arom.), 7.41 (2H, dm, *J* = 7.3), 7.44 (2H, dm, *J* = 8.7, arom.). ^{13}C NMR (200 MHz, $CDCl_3$) δ: 55.4 (O*C*H_3), 80.9 (*C*HOCO_2), 121.0/121.2/126.1/127.0/128.4/128.5/128.7/129.4 (d, arom.), 137.1/138.9/150.9/151.1 (s, arom.), 152.9/154.1 ($CH_3OCO_2C_6H_4$ and CHO*C*$O_2C_6H_5$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{22}H_{18}O_6Na$ 401.0996; Found 401.0996.

*4.1.3.13. 4-(Methoxycarbonyloxy)benzhydryl propionate (**2n**)*

Propionyl chloride (203 μL, 2.32 mmol) was added to a solution of **6a** (200 mg, 0.78 mmol) in dry pyridine (2 mL) at 0 °C. After being stirred at 0 °C for 1 h, the reaction mixture was poured into ice-water (30 mL), and the resulting mixture was extracted with ethyl acetate (10 mL x 3). The extract was successively washed with 10% ice-cooled sulfuric acid, aqueous sodium bicarbonate, and brine and condensed in vacuo to give a colorless oil (380 mg), which on column chromatography ($CHCl_3$) gave the title compound (**2n**, 219 mg, 90%) as a colorless viscous oil. IR (neat): 1767, 1740 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.17 (3H, t, *J* = 7.6, $COCH_2CH_3$), 2.45 (3H, t, *J* = 7.6, $COCH_2CH_3$), 3.89 (3H, s, O*C*H_3), 6.89 (1H, s, C*H*OCO), 7.14 (2H, dm, *J* = 8.6, arom.), 7.27–7.39 (7H, m, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 9.02 ($COCH_2CH_3$), 27.8 ($COCH_2CH_3$), 55.4 (O*C*H_3), 75.9 (*C*HOCO), 121.0/127.0/128.0/128.3/128.5 (d, arom.), 138.2/139.9/150.5 (s, arom.), 154.1 ($CH_3OCO_2C_6H_4$), 172.3 (CHO*C*OC_2H_5). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{18}H_{18}O_5Na$ 337.1046; Found 337.1047.

*4.1.3.14. Methyl 4-propionyloxybenzhydryl carbonate (**3a**)*

According to the method used for the preparation of **2b**, **6b** (240 mg, 0.94 mmol) was treated with methyl chloroformate (180 μL, 2.32 mmol) in dry pyridine (2 mL) at 0 °C for 1 h. Work-up gave a colorless oil (302 mg), which on column chromatography ($CHCl_3$) gave the title compound (**3a**, 246 mg, 84%) as a colorless viscous oil. IR (neat): 1755 (absorptions due to two carbonyl stretching overlap) cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.24 (3H, t, *J* = 7.6, $COCH_2CH_3$), 2.56 (2H, t, *J* = 7.6, $COCH_2CH_3$), 3.78 (3H, s, O*C*H_3), 6.70 (1H, s, C*H*OCO_2), 7.06 (2H, dm, *J* = 8.6, arom.), 7.27–7.39 (7H, m, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 8.98 ($COCH_2CH_3$), 27.7 ($COCH_2CH_3$), 54.9 (O*C*H_3), 80.2 (*C*HOCO_2), 121.6/126.8/128.2(2C)/128.6 (d, arom.), 137.0/139.3/150.4 (s, arom.), 155.0 (CHO*C*OCH_3), 172.8 ($C_2H_5CO_2C_6H_4$). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{18}H_{18}O_5Na$ 337.1046; Found 337.1051.

4.1.4. 4-Hydroxybenzhydrol (7)

4-Hydroxybenzophenone (**4**, 1.98 g, 10 mmol) was treated with $NaBH_4$ (1.51 g, 40 mmol) in a mixture of THF (30 mL) and H_2O (10 ml). While the mixture was heated under reflux for 9 h, the color of the mixture was gradually changed from pale yellow to colorless. The reaction mixture was poured into with ice-cooled water (100 mL), and the reaction was quenched by the addition of NH_4Cl. The resulting mixture was extracted with ethyl acetate (100 mL x 1, 50 mL x 2). The extract was washed with brine and condensed *in vacuo* to give the practically pure title compound (**7**, 1.99 g, quant.) as a colorless microcrystalline solid, mp. 169–170 °C, lit. [15] mp. 159–162 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.14 (1H, d, J = 2.8, OH), 4.77 (1H, s OH), 5.80 (1H, d, J = 2.8, C*H*OH), 7.20–7.40 (9H, m, arom.).

*4.1.5. 4-Hydroxybenzhydrol dipropionate (**3n**)*

According to the method used for the preparation of **2n**, **7** (200 mg, 1.0 mmol) was treated with propionyl chloride (262 μL, 3.0 mmol) in dry pyridine (2 mL) at 0 °C for 2 h. Work-up gave a colorless oil (319 mg), which on column chromatography ($CHCl_3$) gave the title compound (**3n**, 284 mg, 91%) as a colorless viscous oil. IR (neat): 1759, 1743 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.17 (3H, t, J = 7.6, $CHOCOCH_2CH_3$), 1.25 (3H, t, J = 7.6, $C_6H_4OCOCH_2CH_3$), 2.44 (2H, t, J = 7.6, $CHOCOCH_2CH_3$), 2.57 (2H, t, J = 7.6, $C_6H_4OCOCH_2CH_3$), 6.89 (1H, s, $CHOCO_2$), 7.05 (2H, dm, J = 8.6, arom.), 7.26–7.36 (7H, m, arom.). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 9.03 ($CHOCOCH_2CH_3$), 27.7/27.8 ($CHOCOCH_2CH_3$), 76.3 ($CHOCO_2$), 121.5/127.0/127.9/128.2/128.5 (d, arom.), 137.8/140.1/150.3 (s, arom.), 172.8/173.3 (COC_2H_5). HRMS (ESI) *m/z*: $[M+Na]^+$ Calcd for $C_{19}H_{20}O_4Na$ 335.1254; Found 335.1256.

4.2. Cell culture

SK-MEL-28 cells were obtained from the JCRB Cell Bank of National Institutes of Biomedical Innovation, Health and Nutrition (Japan). NHEM cells were purchased from KURABO Bio-Medical department (Japan). SK-MEL-28 and NHEM cells were fed with the culture medium of Dulbecco's Modified Eagle Medium (DMEM) (Nacalai tesque, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco® Thermo Fisher Scientific, USA) and that of DermaLife® M Comp kit (KURABO, Japan), respectively.

The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

4.3. WST-8 assay

WST-8 assay was performed as previously described [11].

4.4. Protein expression and Western blot analysis

Protein expression and Western blot analysis were performed as previously described [11]. The following primary antibodies were used anti-α-tubulin (Sigma-Aldrich, USA), anti-caspase-3 (full length and cleaved Caspase-3), anti-phospho-p44/42 MAPK Thr202/Tyr204 (phospho-ERK1/2), anti-p44/42 MAPK (total ERK1/2), anti-phospho-ELK1 Ser383 (phospho-ELK1) and anti-ELK1 (total ELK1) (Cell Signaling Technology, USA). "Relative quantification (%)" was calculated by dividing the phosphorylated protein (phospho-ERK1/2 or phospho-ELK1) intensity by the total protein (total ERK1/2 or total ELK1) intensity.

4.5. Cell-based apoptosis assay

Apoptosis-inducing activity of the compounds was detected using the Caspase-Glo® 3/7 Assay System (Promega, USA) and the RealTime Glo Annexin V Apoptosis Assay (Promega, USA) according to the manufacturer's instructions. 2×10^4 cells/well were plated in a 96-well white microplate and incubated at 37°C for 24 hours. After the incubation, 2× compounds diluted by the medium were added into each well at a final concentration of 1×. After the additional 48 hours incubation, Caspase-Glo® 3/7 Reagent or 2× Detection Reagent was added into each well, and the plate was incubated for 30 min or 180 min, respectively. After the incubation, the luminescence was measured using a CentroXS^3 LB960 plate reader (Berthold, Germany).

4.6. Immunoprecipitation

HeLa cells that have been co-transfected with pFLAG-CMV2-ERK1 and pFLAG-CMV2-ERK2 were lysed in TNE buffer (20 mM Tris– HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 50 mM sodium fluoride) with a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin) and a mixture of phosphatase inhibitors (5 mM sodium fluoride, 5 mM Na_3PO_4, 10 mM $Na_2H_2P_2O_7$, 10 mM Na_2MoO_4). The lysates were centrifuged and FLAG-

fused proteins in the supernatants were immunoprecipitated using an EZview™ Red ANTI-FLAG® M2 Affinity Gel (Merck Millipore, USA).

4.7. In vitro kinase assay

In vitro kinase reactions consisted of immunoprecipitated proteins (FLAG-ERK1 and FLAG-ERK2), 5 μg/mL recombinant GST-His fused human ELK1 protein (Sino Biological, China), 1× kinase buffer (Cell Signaling Technology, USA) and 20 μM ATP (Nacalai tesque, Japan). Mixtures were incubated at 37°C for 30 min then the reactions were stopped by adding 3× SDS-PAGE loading buffer.

Author contributions

GT and R Sugiura designed this project. R Satoh, NH, AY, FI, TT, GT carried out experiments. R Satoh, NH, AY, YK, FI, TT, GT and R Sugiura analyzed the data. R Satoh, GT and R Sugiura wrote the manuscript. All authors reviewed the manuscript.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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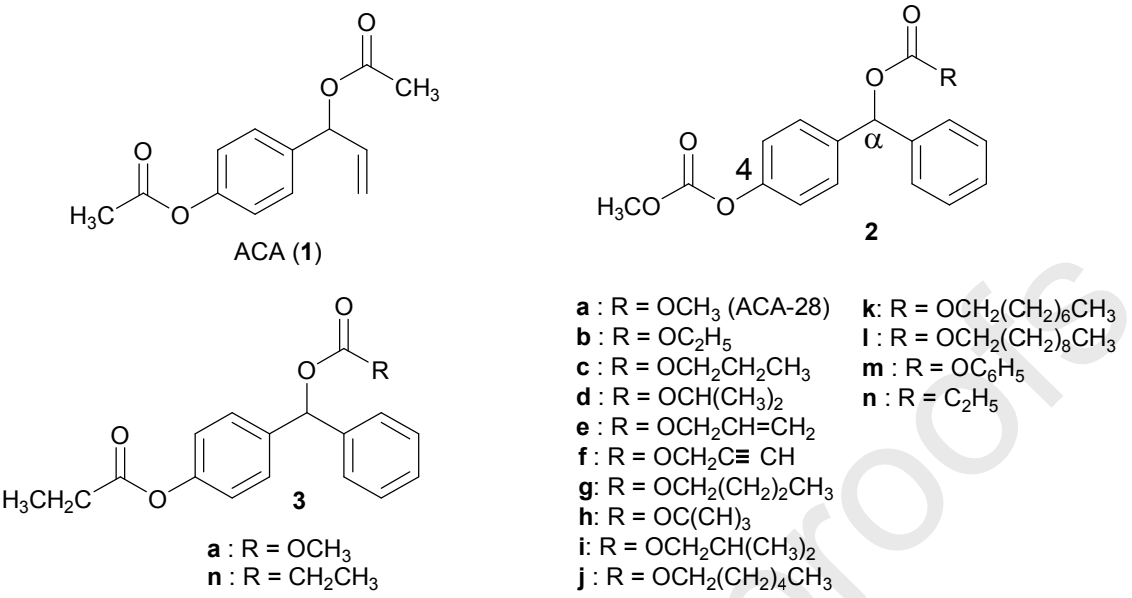
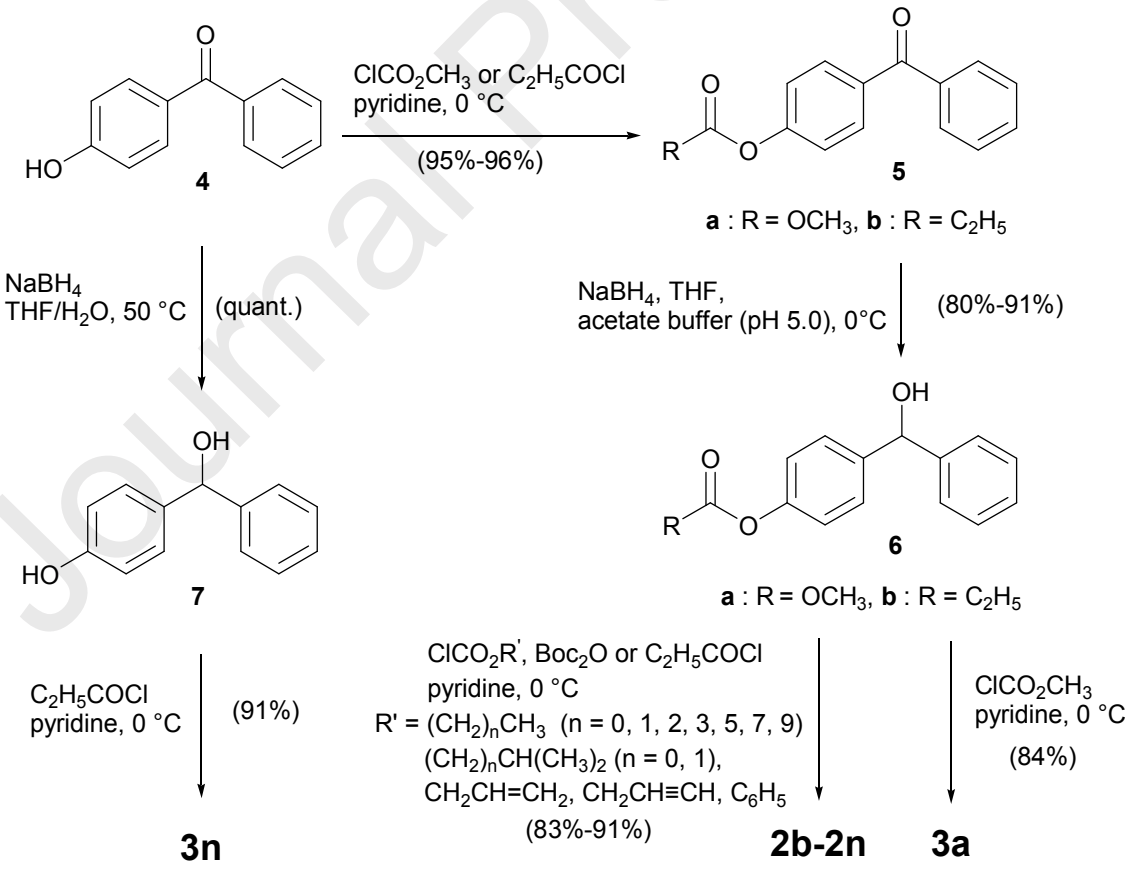


Fig. 1. Chemical structures of ACA (**1**), ACA-28 (**2a**) and analogs (**2b–2n**, **3a,** and **3n**) of **2a**



Scheme 1. Syntheses of analogs (**2b–2n**, **3a,** and **3n**) of **2a**

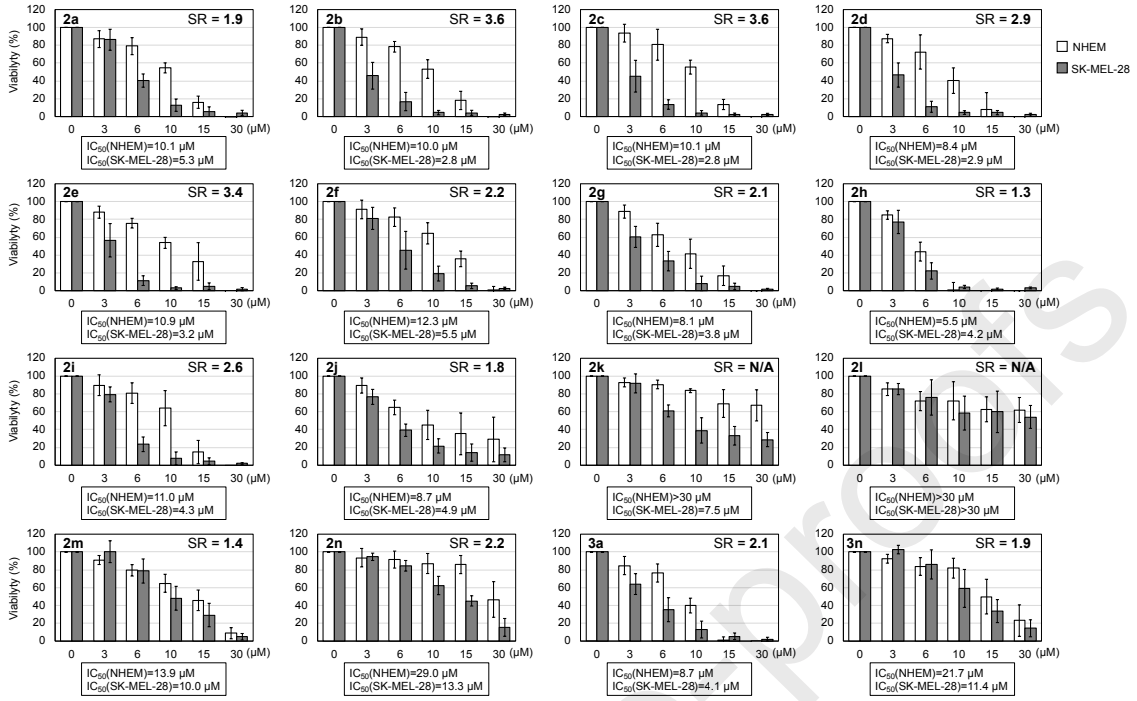


Fig. 2. Cell growth inhibition induced by the compounds against melanoma cells (SK-MEL-28) and normal melanocytes (NHEM). Viability of NHEM and SK-MEL-28 after exposure to the indicated concentrations of the compounds for 48 h, expressed as the relative percentage against that of DMSO treatment (0 μM). Columns, means; bars, SD; SR, selective ratio; $n = 7$.

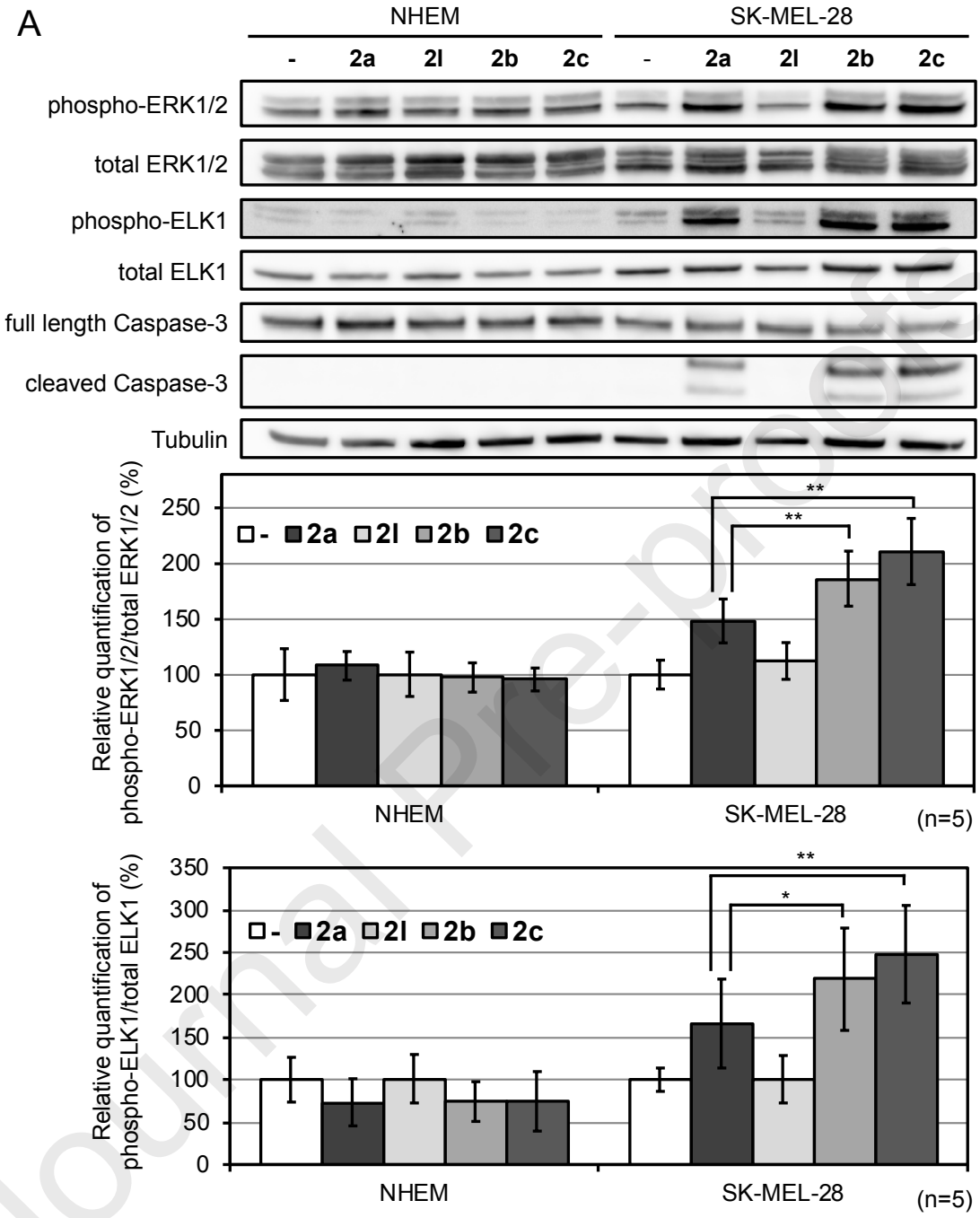


Fig. 3. 2b and **2c** are superior apoptosis inducers than **2a** (ACA-28) in melanoma cells. (A) Cleavage of Caspase-3 was more effectively induced after treatment with **2b** or **2c** as compared with that with **2a** in melanoma cells (upper panel). Relative quantification of ERK1/2 MAPK phosphorylation and ELK1 phosphorylation in NHEM and SK-MEL-28 cells upon each compound treatment (lower panel). Phosphorylation levels (phosphorylated protein intensity/total protein intensity) after treatment with DMSO (-) were set as 100% in each cell. Columns, means; bars, SE; $n = 5$. **P < 0.01, *P < 0.05, significantly different from **2a** by Williams' test. (B) MEK inhibitor U0126 attenuates **2b**- and **2c**-induced apoptosis. Simultaneous treatment of 10 μM U0126 reversed **2b**- and **2c**-induced phosphorylation of ERK1/2 and apoptosis in SK-MEL-28 cells.

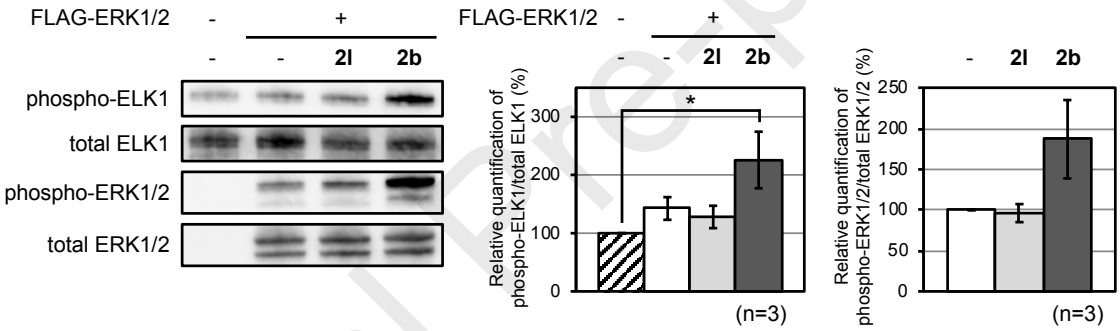


Fig. 4. Immunoprecipitated FLAG-ERK1/2 derived from HeLa cells that were treated with **2b** phosphorylates the target protein ELK1 *in vitro*. Relative quantification of ELK1 phosphorylation (center panel). Phosphorylation levels (phosphorylated protein intensity/total protein intensity) of no addition of FLAG-ERK1/2 were set as 100%. Relative quantification of ERK1/2 phosphorylation (right panel). Phosphorylation levels of using FLAG-ERK1/2 derived from DMSO treated HeLa cells were set as 100%. Columns, means; bars, SE; $n = 3$. *P < 0.05, significantly different from **2a** by Williams' test.

Table 1. IC_{50} values of compounds

Compound	IC_{50} (NHEM)	IC_{50} (SK-MEL-28)	Selective Ratio (SR)
2a (ACA-28)	10.1 μM	5.3 μM	1.9
2b	10.0 μM	2.8 μM	3.6
2c	10.1 μM	2.8 μM	3.6
2d	8.4 μM	2.9 μM	2.9
2e	10.9 μM	3.2 μM	3.4
2f	12.3 μM	5.5 μM	2.2
2g	8.1 μM	3.8 μM	2.1
2h	5.5 μM	4.2 μM	1.3
2i	11.0 μM	4.3 μM	2.6
2j	8.7 μM	4.9 μM	1.8
2k	>30 μM	7.5 μM	N/A
2l	>30 μM	>30 μM	N/A
2m	13.9 μM	10.0 μM	1.4
2n	29.0 μM	13.3 μM	2.2
3a	8.7 μM	4.1 μM	2.1
3n	21.7 μM	11.4 μM	1.9

IC_{50} (SK-MEL-28)= ~3.0 μM
IC_{50} (SK-MEL-28)= 3.1~4.0 μM
IC_{50} (SK-MEL-28)= 6.1~10.0 μM
IC_{50} (SK-MEL-28)= 10.1 μM~

SR= 3.5~
SR= 2.5~3.4
SR= 1.5~1.9
SR= ~1.4

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: