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4-Hydroxy-3-methyl-6-phenylbenzofuran-2-carboxylic acid ethyl ester derivatives as potent anti-tumor agents

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Abstract—Based on the structure of 4-hydroxy-3-methyl-6-phenylbenzofuran-2-carboxylic acid ethyl ester (**1**), which exhibits selective cytotoxicity against a tumorigenic cell line, (2,4-dimethoxyphenyl)-(4-hydroxy-3-methyl-6-phenylbenzofuran-2-yl)-methanone (**18m**) was designed and synthesized as a biologically stable derivative containing no ester group. Although the potency of **18m** was almost the same as our initial hit compound **1**, **18m** is expected to last longer in the human body as an anticancer agent.

In our project to develop new anticancer drugs as small molecules, 4-hydroxy-3-methyl-6-phenyl-benzofuran-2-carboxylic acid ethyl ester (**1**) was selected as one of the most promising lead compounds,[1] which showed selective and potent cytotoxicity against a tumorigenic cell line, WI-38 VA-13 subline 2RA (VA13) (EC50 = 40 ng/mL) compare to the normal parental cell line, WI-38 (EC50 > 4000 ng/mL). In general, the ethyl ester group in **1** is readily hydrolyzed by esterases especially in the serum and liver, and the resultant carboxylic acid (**2**) completely loses its activity to kill tumor cells (Fig. 1). Replacing the ester group by biologically stable functional groups was considered to produce more potent chemotherapeutic agents. Therefore, further studies were conducted to synthesize such derivatives in search of more potent compounds containing no ester group.

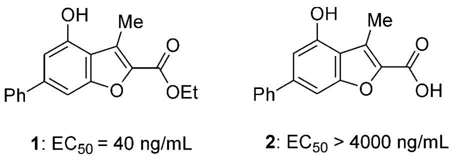

Figure 1. Initial lead compound **1** and corresponding carboxylic acid **2**.

Libraries with a vast number of derivatives were desired for lead expansion and optimization, and combinatorial chemistry, which is a powerful tool used to generate huge libraries, was effective for this purpose. We wish to report herein the discovery of highly potent derivatives of **1** that have no ester group produced by the techniques of combinatorial chemistry as solid phase chemistry and solution phase parallel synthesis.

1. Evaluation of biological activity

Human tumorigenic cell line, VA13 (CCL-75.1), was purchased from ATCC, and was passaged in MEM-E (11095-080, Gibco BRL) containing 100 U/mL of penicillin and 100 μg/mL of streptomycin supplemented with 10% fetal calf serum (Hyclone). Exponentially growing VA-13 cells were collected and distributed into 96-well plates (3598, Corning-Coaster) at a density of 1.5–3.0×10^3 cells/well. After cultivation for two or three days, test compounds dissolved in DMSO were diluted in fresh medium and then added to the plates. The final concentrations of DMSO were always kept at less than 0.5%. The plates were incubated for 24 h. Then the culture medium was removed and washed. Finally, 150 μL/well of fresh medium was added to the cells. After

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cultivation for three days, 50 μL/well of MEM-E containing 1 mg/mL of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanide (XTT: X-4251, Sigma) and 25 mM of phenazine methosulfate (P-9625, Sigma) was added and the plates were incubated for 2.5 h. OD450 was measured by SPECTRA MAX 250 (Molecular Devices) and cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = (\text{WC} - \text{BG}) \times 100/(\text{RF} - \text{BG})$$

WC:	OD450 for the well containing cells treated with compounds
RF:	OD450 for the well containing cells and no compounds
BG:	OD450 for the well containing neither cells nor compounds

A dose–response curve was drawn and the 50% effective concentration (EC_{50}) was determined as an indicator of compound cytotoxicity.

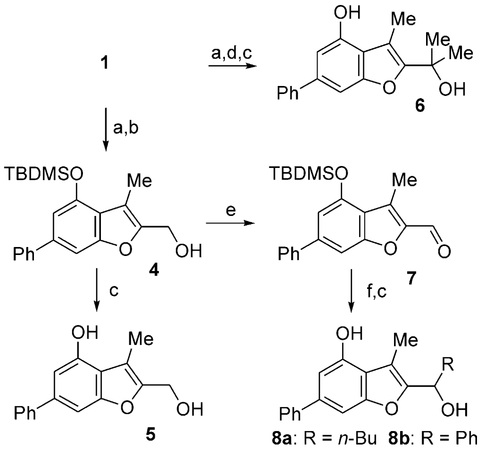
2. Chemistry

Prior to the replacement of the ester group of **1** with another biologically stable functional group, several ester derivatives were prepared to investigate the possibility of modifying this part of the molecule without losing biological activity. Ester derivatives (**3a**–**d**) were synthesized from ethyl ester **1** by employing solid-phase synthesis with the same procedure as that for the following amide synthesis using 2-chloro-1,3-dimethylimidazolinium chloride (DMC) as a dehydrating agent.[2] Various types of esters were newly synthesized and tested. All the compounds had biological activity (Table 1). This result demonstrated a prospect for further modifying the ester group.

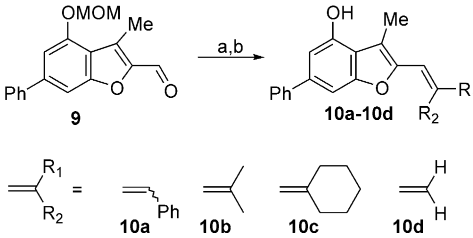
A modification of the ethyl ester moiety of **1** into an alcohol or related functional group was initially attempted. The phenolic hydroxy group of **1** was protected with *tert*-butyldimethylsilyl (TBDMS) group, and then, the ethyl ester was reduced to an alcohol with lithium aluminum hydride (LAH). After removal of the protective group, alcohol **5** was obtained, and efforts to convert the hydroxy group of **4** to an amino group were unsuccessful due to its instability. Tertiary alcohol **6** was prepared using the Grignard reagent, and secondary alcohols **8** were prepared from aldehyde **7** with Grignard reagents (Scheme 1).

For olefin synthesis, aldehyde **9** was prepared by a procedure similar to that for aldehyde **7** synthesis using the methoxymethyl (MOM) group instead of TBDMS as a protective group for the phenolic hydroxy group. By the Wittig reaction, the aldehyde was converted to olefins **10a**–**d** (Scheme 2).

For these alcohols and olefins, which lacked a carbonyl group, cytotoxicity was no longer observed. This result suggested that the carbonyl group of **1** is essential for the biological activity.



Scheme 1. Conversion of ethyl ester to alcohols. (a) 1.5 equiv Bu^t-Me$_2$SiCl, imidazole in DMF, 25 °C, 1 h (quant.); (b) 1.0 equiv $LiAlH_4$ in THF, 25 °C, 30 min (73%); (c) 3.0 equiv *n*-Bu_4NF, AcOH in THF, 25 °C, 14 h (quant.); (d) 10 equiv MeMgBr in THF, 25 °C, 10 min (93%); (e) 10 equiv MnO_2 in DCM, 25 °C, 1 h (82%); (f) 1.5 equiv RMgBr in THF, 0 °C, 15 min (**8a**: 87%, **8b**: quant.).



Scheme 2. Conversion of ethyl ester to olefins. (a) 1.5 equiv Wittig reagent in THF at 0 °C to 25 °C 20 min; (b) 0.05 equiv HCl in MeOH at 55 °C 1 h (**10a**: 99% 2 steps).

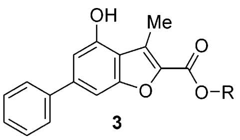
Although most of the attempts to replace the ester group of **1** with alcohols or olefins failed, as mentioned above, amide derivatives were still expected to maintain biological activity as both amides and esters contain a carbonyl group. Although amides could be hydrolyzed biologically, they were still thought to be more stable than esters.

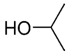
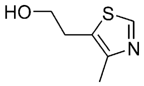
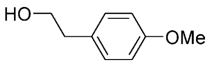
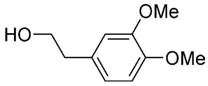
A high-throughput solid-phase synthesis (in parallel with Quest Synthesizer™, Algonaut Technology) based on a technique of combinatorial chemistry was devised to generate amide derivatives of **1**. As shown in Scheme 3, lead compound **1** was introduced on Wang resin purchased from Advanced Chemtech (100–200 mesh). After hydrolysis of the ethyl ester with potassium trimethylsilanolate, various types of amines were introduced using *N*-chlorosuccinimide and triphenylphosphine,[3] and the following acid cleavage from the polymer support under acidic conditions yielded the desired amides. This solid-phase synthesis conducted in only two steps was still effective to prepare large-size compound libraries.

Although various types of amides with aliphatic, aromatic, and heterocyclic amines were synthesized, only a few derivatives (**15a** for example) that showed very weak activity were discovered (Fig. 2).

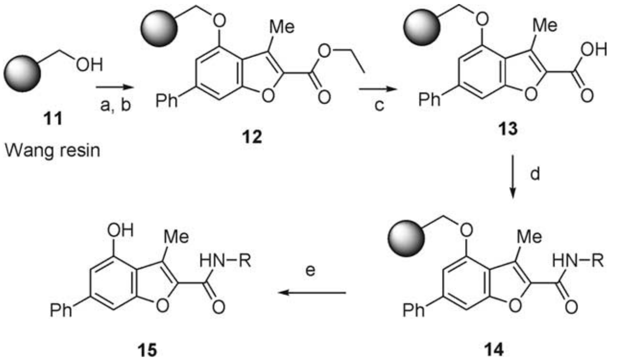
During our attempt to replace the ester group of **1** with a more stable functional group, ketones were finally

Table 1. Ester derivatives and biological activity

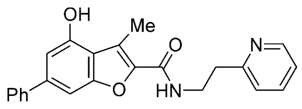


Esters	ROH	Yield[a] (%)	Cytotoxicity[b] EC_{50}, (ng/mL)
3a		16	60
3b		10	55
3c		27	20
3d		30	21

[a] Overall yields based on used solid support after purification by silica gel chromatography.
[b] Selectivity against tumor cells was confirmed.

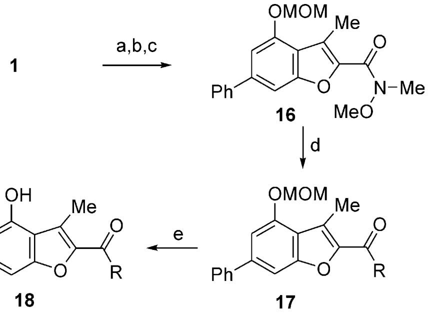


Scheme 3. Solid-phase synthesis of amides. (a) 10 equiv methanesulfonyl chloride, 10 equiv NEt_3 in DCM, 25 °C, 18 h; (b) 3 equiv **1**, 5 equiv K_2CO_3 in DMF, 60 °C, 22 h; (c) 10 equiv Me_3SiOK in THF, 25 °C, 20 h; (d) 10 equiv RNH_2, 10 equiv Ph_3P, 10 equiv *N*-chlorosuccinimide, pyridine in DCM, 25 °C, 10 min; (e) 20% TFA in DCM, 25 °C, 2 h.



15a: EC_{50} = 1500 ng/mL

Figure 2. Cytotoxicity of amide derivatives.



Scheme 4. Solution-phase synthesis of ketones. (a) 2 equiv methoxymethyl chloride, NaH in DMF, 0 °C, 1 h; (b) 4 equiv 1 N NaOH in EtOH, 60 °C, 1 h; (c) 1.5 equiv *N,O*-dimethylhydroxylamine hydrochloride, 1.5 equiv 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 1 equiv 1-hydroxybenzotriazole hydrate, 2 equiv triethylamine in DMF, 25 °C 16 h (93% from **1**); (d) 3.0 equiv RMgCl, 3.0 equiv RLi, or 1–40 equiv RMgBr in THF; (e) HCl in THF–MeOH (1:1).

tested. Scheme 4 shows the solution phase synthetic method employed for the ketone synthesis. *N,O*-dimethylhydroxylamide **16** was prepared from ethyl ester **1** via carboxylic acid using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt), and, then, **16** was allowed to react with Grignard reagents or alkyl (or aryl) lithium reagents to be converted into corresponding ketones **17**.[4] The methoxymethyl protective group was removed under acidic conditions to generate the desired ketones **18**. As shown in Table 2 (phenyl ketones) and in Table 3 (other ketones), quite a few ketone derivatives showed biological activity.

Although phenyl ketone **18a** with no substituent on the benzene ring showed relatively weak cytotoxicity, its derivatives, which had a substituent at the *ortho* position (**18b**, **c**, and **d**) were moderately active. The substituent groups at the *meta* position (**18e**, **f**, and **g**) were relatively less effective even with an *ortho* (**18o**) or a *para* (**18q** and **r**) substituent, and derivatives that had a *para* substituent (**18i**, **j**, **k**, and **l**) had completely lost their activity except for those with a methoxy group substituent (**18h**). The methoxy group was considerd to be a desirable substituent for this series of compounds, and, as expected, the phenyl ketone **18m** (EC_{50} = 40 ng/mL),[5] which has two methoxy substituents at the *ortho* and *para* positions, was the most potent compound.

Phenyl ketone **18m** does not contain any functional groups suspected to be biologically unstable as esters, and its potency was appeared to be comparable to original ethyl ester **1**. Although ethyl ester **1** was readily decomposed in mouse liver microsomes, phenyl ketone **18m** was confirmed to be very stable (Fig. 3).

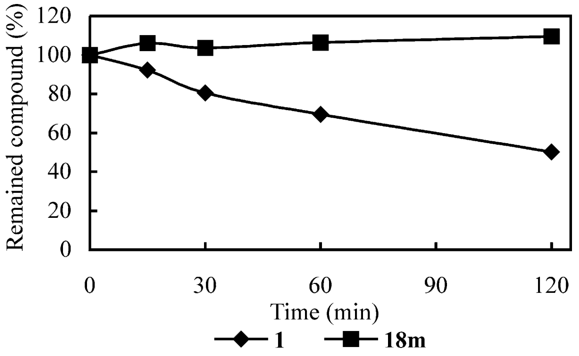
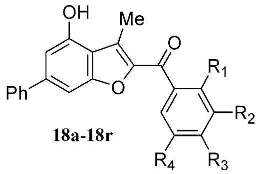


Figure 3. Stability test of ethyl ester **1** and ketone **18m** in mouse liver microsomes. The stability of each compound (20 µmol) was tested in mouse (BDF1) liver microsomes (1 mg protein in 1 mL). The concentration of remained compounds was determined by HPLC.

Table 2. Cytotoxicity of substituted phenyl ketone derivatives

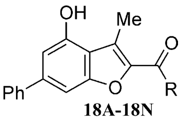


18a-18r

Compd	R_1	R_2	R_3	R_4	Yield (%)		Cytotoxicity EC_{50}, ng/mL[b]
					to **16**	to **18**	
18a[a]	H	H	H	H	—	—	600
18b	OMe	H	H	H	88	81	90
18c	CH_2OMe	H	H	H	68	Quant.	100
18d	Ph	H	H	H	79	Quant.	100
18e	H	CF_3	H	H	72	98	>4000
18f	H	OPr^i	H	H	Quant.	97	350
18g	H	CH_2NMe_2	H	H	Quant.	83	90
18h	H	H	OMe	H	Quant.	89	280
18i	H	H	OPh	H	Quant.	29	>4000
18j	H	H	Et	H	Quant.	81	>4000
18k	H	H	NMe_2	H	Quant.	45	>4000
18l	H	H	CH_2NMe_2	H	79	51	>4000
18m	OMe	H	OMe	H	Quant.	73	40
18n	OMe	H	CF_3	H	Quant.	51	260
18o	OMe	H	H	OMe	90	89	90
18p	CF_3	H	OMe	H	71	89	400
18q	H	OMe	OMe	H	Quant.	93	450
18r	H	F	F	H	92	47	400

[a] Synthesized from **8b**, which was oxidized by MnO_2 in DCM at 25 °C for 4 h (47%) and deprotected using n-Bu_4NF under standard conditions (23%).
[b] Selectivity against tumor cell was confirmed.

Table 3. Cytotoxicity of other ketone derivatives



18A-18N

Compd	R	Yield(%)		Cytotoxicity EC_{50}, ng/mL[a]
		to **16**	to **18**	
18A	Me	88	85	80
18B	Et	88	54	270
18C	1-Pr	Quant.	48	590
18D	2-Pr	46	25	410
18E	1-Bu	54	70	2000
18F	*tert*-Bu	12	73	410
18G	*cyclo*-Hex	34	79	450
18H	2-pyridyl	57	45	600
18I	2-thiophenyl	88	88	2000
18J	2-thiazolyl	62	91	400
18K	5-pyrimidyl	59	62	700
18L	3-quinolyl	53	44	>4000
18M	CH_2Ph	34	51	>4000
18N	CH_2CH_2Ph	Quant.	76	>4000

[a] Selectivity against tumor cell was confirmed.

Introduction of a trifluoromethyl group instead of a methoxy group at the *para* position (**18n**) weakened the biological activity, and placing a methoxy group at the *meta* position (**18o**) did not change the cytotoxicity of **18b**. Methyl ketone **18A**[6] ($EC_{50}=80$ ng/mL) was a compound with potencies similar to **18m**. Derivatives of heterocycles (**18H**, **J**, and **K**) had almost same activity as unsubstituted phenyl ketone **18a**.

In conclusion, phenyl ketone **18m** was discovered from the derivatives of a screening lead **1**. As the structure of **18m** was designed by replacing biologically unstable ester group of **1** with phenyl ketone, it is predicted to be a more promising compound as an anticancer drug.

Acknowledgements

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4. Nahm, S.; Weinreb, S. M. *Tetrahedron Lett.* **1981**, *22*, 3815.
5. Compound **18m**: colorless crystal. Mp 200–202 °C, ^1H NMR (270 MHz, $CDCl_3$) δ 7.59–7.57 (m, 2H), 7.51 (d, 1H, $J=8.3$ Hz), 7.44 (t, 2H, $J=7.3$ Hz), 7.38–7.35 (m, 1H), 7.25 (s, 1H), 6.81 (s, 1H), 6.58 (dd, 1H, $J=1.9$ Hz, 8.7 Hz), 6.53 (d, 1H, $J=2.2$ Hz), 5.43 (s, 1H), 3.89 (s, 3H), 3.77 (s, 3H), 2.71 (s, 3H); MS (EI, m/z) 388 $(M)^+$.
6. Compound **18a**: colorless crystal. Mp 247–249 °C (dec.), ^1H NMR (270 MHz, $CDCl_3+CD_3OD$) δ 761 (d, 2H, $J=7.1$ Hz), 7.48–7.35 (m, 4H), 7.24 (s, 1H), 6.81 (s, 1H), 2.81 (s, 3H), 2.60 (s, 3H); MS (EI, m/z) 266 $(M)^+$.