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Synthesis, in vitro Antimicrobial, and Cytotoxic Activities of New 1,3,4-Oxadiazin-5(*6H*)-one Derivatives from Dehydroabietic Acid

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A series of new 1,3,4-oxadiazin-5(*6H*)-one derivatives (**6a–n**) of dehydroabietic acid were designed and synthesized as potential antimicrobial and antitumor agents. Their structures were characterized by IR, ^{1}H NMR, ^{13}C NMR, MS, and elemental analyses. All the title compounds were evaluated for their antimicrobial activity against four bacterial and three fungal strains using the serial dilution method. Among them, compound **6e** showed the highest antibacterial activity against *Bacillus subtilis* with a minimum inhibitory concentration (MIC) value of 1.9 μg/mL. In addition, the in vitro cytotoxic activities of the title compounds were also assayed against three human carcinoma cell lines (MCF-7, SMMC-7721, and HeLa) through the MTT colorimetric method. As a result, compounds **6b**, **6g**, **6k,** and **6m** exhibited significant inhibition against at least one cell line with IC_{50} values below 10 μM. Compound **6m** was especially found to be the most potent derivative with IC_{50} values of 2.26 ± 0.23, 0.97 ± 0.11, and 1.89 ± 0.31 μM against MCF-7, SMMC-7721, and HeLa cells, respectively, comparable to positive control etoposide.

Keywords: Dehydroabietic acid; 1,3,4-Oxadiazin-5(*6H*)-one; Synthesis; Antimicrobial activity; Cytotoxic activity.

INTRODUCTION

As one of the leading causes of mortality all over the world, cancer poses a tremendous threat to human's health.[1] In spite of the significant advances in cancer therapy in recent years, there still exists an urgent need for the discovery of new anticancer agents. In addition, the rapid development of multidrug-resistant microbial pathogens has made the treatment of infectious diseases an escalating problem, which exerts a continuous need for new antibiotics with better efficacy.[2] In recent years, there has been increased interest in the search of new antimicrobial and anticancer drugs from natural products. Many clinically used antibiotics, such as penicillin, tetracycline, cephalosporin, etc,[3] and antitumor drugs, such as vinblastine, doxorubicin, and paclitaxel, were discovered from natural products or natural product-derived compounds.[4,5] Dehydroabietic acid (DAA, **1**) is a natural-occurring diterpene resin acid obtained from *Pinus* rosin or commercial disproportionated rosin. Its derivatives have drawn much attention for their broad spectrum of biological activities, including antimicrobial, antiprotozoal, antitumor, antiviral, anti-aging, gastroprotective, antifeedant, and BK-channel opening activities.[6–13] These results suggest that DAA is a promising starting material for the discovery of new antimicrobial or anticancer agents.

Nitrogen-containing heterocyclic compounds have drawn the interest of chemists for their extensive biological activities, which can afford extremely versatile building blocks for the manufacture of bioactive compounds in the pharmaceutical drug design and agrochemical industry.[14–16] The 1,3,4-oxadiazines represent an important type of six-membered heterocycles with two adjacent nitrogen atoms. Many 1,3,4-oxadiazine derivatives exhibit a great diversity of biological effects, such as antibacterial, antitumor, antileishmanial, miticidal, insecticidal, and monoamine oxidase (MAO) inhibitory activities.[17–21] Some 1,3,4-oxadiazine

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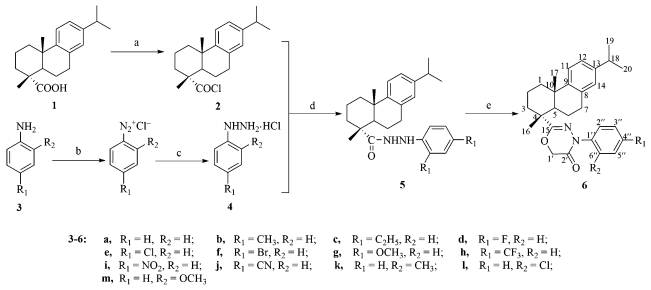
derivatives, e.g., indoxacarb, have been developed as a potent voltage-dependent sodium channel blocker and have been widely used as a commercially successful insecticide.[20,22] These findings suggest that the introduction of the 1,3,4-oxadiazine motif into the scaffold of DAA may probably produce new derivatives with improved antimicrobial and anticancer properties. In continuation of our research on the bioactive derivatives of resin acid,[23–25] a series of new 1,3,4-oxadiazin-5(*6H*)-one derivatives of DAA have been designed and synthesized, and their in vitro antimicrobial and cytotoxic activities are also presented.

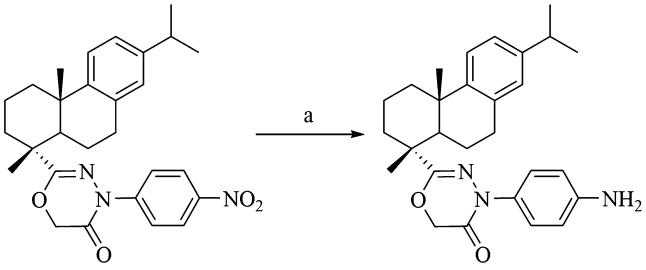
RESULTS AND DISCUSSION

Chemistry

The synthetic routes for the target compounds **6a–p** were depicted in Schemes 1 and 2. Briefly, DAA (**1**) was converted to dehydroabietate chloride (**2**) through a common chloridization reaction. Then, diazotization of different substituted anilines (**3a–m**) followed by reduction with $SnCl_2$ afforded the corresponding substituted phenylhydrazine hydrochlorides (**4a–m**), which were further reacted with the dehydroabietate chloride **2** to give the substituted *N*′-phenylcarbohydrazides (**5a–m**). Subsequently, the title compounds (**6a–m**) were obtained in moderate to good yields by treating compounds **5a–m** with chloroacetyl chloride and K_2CO_3 to form 1,3,4-oxadiazin-5(*6H*)-one rings. In addition, the nitro group of compound **6i** could be reduced by Fe powder to afford the *p*-aminophenyl derivative **6n**. All the title



Scheme 1. Synthetic pathway to target compounds **6a–m** from dehydroabietic acid. Reagents and conditions: (a) $SOCl_2$, benzene, reflux for 3 h; (b) $NaNO_2$, HCl, 0~5 °C, 1 h; (c) $SnCl_2$, HCl, 0~5 °C, 1–2 h; (d) different substituted phenylhydrazine hydrochloride **4**, Et_3N, cyclohexane, 0 °C for 1 h and 3 h at rt.; (e) chloroacetyl chloride, 2-butanone, K_2CO_3, reflux for 3 h.



Scheme 2. Synthetic route for compounds **6n** from **6i**. Reagents and conditions: (a) Fe powder, HCl, EtOH, H_2O, 85 °C, 2 h.

compounds (**6a–n**) were purified by silica gel column chromatography and recrystallization.

The structures of **6a–n** were characterized on the basis of MS, IR, ^{1}H NMR, ^{13}C NMR spectra, and elemental analyses. For example, the ESIMS of compound **6b** displayed a peak at *m*/*z* 445.3, corresponding to the quasimolecular ions $[M + H]^+$, which confirmed the molecular formula ($C_{29}H_{36}N_2O_2$) in combination with the data of the elemental analysis. The absorption bands at 1693 and 1648 cm^{-1} in the IR spectrum of **6b** suggested the presence of C=O and C=N groups. In the ^{1}H NMR spectrum of **6b**, the isopropyl group could be confirmed by a doublet at δ 1.23 ppm corresponding to six methyl protons (H-19 and H-20) together with a multiplet at δ 2.83 ppm due to the vicinal methine proton (H-18). Three singlets at δ 1.25, 1.31, and 2.36 ppm could be assigned to methyl protons at C-17, C-16, and 4″-Me, respectively. Two doublets, each containing one proton, at δ 4.54 and 4.59 ppm were due to the methylene protons at C-1′ on the 1,3,4-oxadiazin-5(*6H*)-one ring. One double doublet at δ 7.01 (1H, J = 8.2, 1.7 Hz) and two doublets at δ 6.90 (1H, J = 1.1 Hz) and δ 7.18 (1H, J = 8.2 Hz) could be attributed to three aromatic protons at C-12, C-14, and C-11, respectively. Protons on the *p*-methylphenyl ring appeared as two doublets at δ 7.20 (H-3″, H-5″) and δ 7.55 (H-2″, H-6″). In the ^{13}C NMR spectrum of **6b**, there were 26 peaks appearing in the range from δ 17.2 to 168.3 ppm, corresponding to 29 carbon atoms. The presence of 1,3,4-oxadiazin-5(*6H*)-one ring could especially be confirmed by three signals at δ 67.4, 158.8, and 168.3 ppm due to C-1′, C-15, and C-2′, respectively. The assignments of the signals in the ^{1}H and ^{13}C NMR spectra of **6b** were in good accordance with its structure.

Antimicrobial activity

The in vitro antimicrobial activities of all the title compounds were evaluated using a modified twofold serial dilution method to obtain the minimum inhibitory concentrations (MICs) for different microorganisms.[26] The strains used in the study were two Gram-positive bacteria (*Staphylococcus aureus* CGMCC 1.1361, *Bacillus subtilis* CGMCC 1.1162), two Gram-negative bacteria (*Escherichia coli* CGMCC 1.1571, *Pseudomonas fluorescens* CGMCC 1.1828), and three fungi (*Candida albicans* CGMCC 2.2086, *Candida tropicalis* CGMCC 2.3967, and *Penicillium citrinum* CGMCC 3.825). All strains were bought from the China General Microbiological Culture Collection Center (CGMCC). Amikacin sulfate and ketoconazole were coassayed as a positive control for tested bacteria and fungi, respectively.

The in vitro antibacterial and antifungal data of the synthesized compounds are shown in Table 1. From the results, most of the title compounds showed inhibitory activity against both Gram-positive and Gram-negative bacteria with MIC values <50 μg/mL. Among them, compounds **6d**, **6e**, **6g**, **6j**, **6l,** and **6n** displayed considerable antibacterial activity against at least one bacterial strain, with MIC values ranging from 1.9 to 15.6 μg/mL. Specifically, compound **6e** exerted the highest inhibitory effect against *B. subtilis,* with an MIC value of 1.9 μg/mL, close to that of the positive control amikacin (0.9 μg/mL). This compound also showed strong inhibition against *E. coli* and *S. aureus* (MIC: 3.9 and 7.8 μg/mL, respectively), indicating its antibacterial potential against both Gram-positive and Gram-negative strains. In addition, compound **6j** exhibited considerable inhibitory activity against two Gram-negative bacteria, with an MIC value of 7.8 μg/mL. On the other hand, the rest of the title compounds only showed weak (MIC: 31.2 μg/mL) or no inhibition (MIC >50 μg/mL) against the four tested bacteria. Concerning antifungal activity, most of the title compounds showed mild or no inhibitory activity against the three tested fungi. Specifically, compounds **6d**, **6e**, **6i,** and **6j** displayed weak antifungal activity (MIC: 31.2 μg/mL) against *C. albicans* and/or *C. tropicalis*, while all the compounds were inactive to *P. citrinum.*

The antimicrobial results indicated that this series of derivatives possessed considerably superior antibacterial properties compared to their antifungal properties. The antibacterial activities of these compounds were substantially influenced by the substituents on the benzene rings. In general, derivatives with electron-withdrawing

Table 1. The antimicrobial activity of the compounds (**1**, **6a–n**) against four test bacteria and three test fungi

	MIC (μg/mL)						
	Gram-negative bacteria		Gram-positive bacteria		Fungi		
Compd.	*E. coli*	*P. fluorescens*	*S. aureus*	*B. subtilis*	*C. albicans*	*C. tropicalis*	*P. citrinum*
1	> 50	> 50	> 50	> 50	> 50	> 50	> 50
6a	> 50	> 50	> 50	31.2	> 50	> 50	> 50
6b	31.2	> 50	31.2	> 50	> 50	> 50	> 50
6c	31.2	> 50	> 50	31.2	> 50	> 50	> 50
6d	31.2	> 50	15.6	15.6	31.2	> 50	> 50
6e	3.9	15.6	7.8	1.9	31.2	> 50	> 50
6f	> 50	31.2	31.2	31.2	> 50	> 50	> 50
6g	31.2	> 50	> 50	15.6	> 50	> 50	> 50
6h	31.2	> 50	> 50	31.2	> 50	> 50	> 50
6i	31.2	> 50	31.2	31.2	31.2	> 50	> 50
6j	7.8	7.8	15.6	15.6	31.2	31.2	> 50
6k	> 50	> 50	> 50	31.2	> 50	> 50	> 50
6l	31.2	> 50	31.2	15.6	> 50	> 50	> 50
6m	31.2	> 50	> 50	> 50	> 50	> 50	> 50
6n	15.6	31.2	31.2	15.6	> 50	> 50	> 50
Amikacin	1.9	0.9	0.9	0.9	–	–	–
Ketoconazole	–	–	–	–	3.9	3.9	7.8

substituents, such as —F, —Cl and —CN groups, displayed stronger antibacterial activities than those with electron-donating ones (—Me, —Et and —OMe). Among them, compounds **6e** and **6j** with *p*-Cl and *p*-CN groups showed stronger activities against tested bacteria than derivatives with —F, —Br, $-CF_3$, and $-NO_2$ groups, indicating that these two substituents were the most beneficial to the antibacterial activity of target derivatives. In addition, replacement of the *p*-Cl group (**6e**) with the *o*-Cl group (**6l**) significantly reduced the antibacterial activity. Similar results could also be observed for —Me or —OMe groups. These phenomena suggested that the position of the substituents would also influence the antibacterial activity. For the same substituent, *para* substitution was generally more favorable than *ortho* substitution.

Cytotoxic activity

All the title compounds were also evaluated for their in vitro cytotoxic activity against the human breast cancer cell line (MCF-7), hepatocarcinoma cell line (SMMC-7721), and cervical carcinoma cell line (HeLa) through the MTT assay method.[27] The anticancer drug etoposide was coassayed as the positive control. The results of the tested compounds expressed as IC_{50} values (concentration required to inhibit tumor cell proliferation by 50%) are presented in Table 2.

As illustrated in Table 2, the target compounds exhibited varying degrees of cytotoxicity against three cancer cell lines. It was found that compounds **6b**, **6g**, **6k,** and **6m** showed significant cytotoxic activities against at least one cancer cell line ($IC_{50} < 10$ μM). Among them, compound **6m** exhibited the most potent cytotoxic activity against MCF-7, SMMC-7721, and HeLa cells with IC_{50} values of 2.26 ± 0.23, 0.97 ± 0.11, and 1.89 ± 0.31 μM, respectively, nearly equipotent to the positive control etoposide. In addition, compound **6a**, **6c–e**, **6l,** and **6n** displayed moderate inhibitory activities to three cancer cell lines. On the other hand, compounds **6f** and **6h** only showed weak inhibition, while **6i** and **6j** were inactive to all three cell lines ($IC_{50} > 50$ μM).

From these results, it could be observed that the cytotoxic activity of most title compounds with 1,3,4-oxadiazin-5(*6H*)-one moieties were stronger than the starting material DDA, indicating that the introduction of this heterocyclic moiety could enhance the cytotoxicity. Moreover, the substituents on the benzene ring could also exert a significant effect on the cytotoxic activity of the title compounds. For example, derivatives with electron-donating substituents (—Me, —Et, —OMe and $-NH_2$) exhibited considerably stronger cytotoxic activity than those derivatives with strong electron-withdrawing ones ($-NO_2$, —CN and $-CF_3$), which suggested that, contrary to antibacterial activity, the electron-donating substituents could significantly increase the cytotoxic activity of these derivatives. Among these electron-donating substituents, the —OMe group was shown to be the most beneficial to the cytotoxicity. Derivatives with halogen substituents (—F, —Cl, and —Br) exhibited moderate antiproliferative activities, and their cytotoxic activities decreased in the order of F > Cl > Br. In addition, the cytotoxic activities of compounds **6k–m** with *ortho*-substituents were generally more potent than their analogs containing *para*-substituents, indicating that the positions of the substituents on the benzene rings could affect the interaction with their potential targets in cancer cells, therefore considerably influencing the antiproliferative potency of these derivatives.

Table 2. The cytotoxicity of the compounds (**1**, **6a–n**) against MCF-7, SMMC-7721, and HeLa cells

Compd.	IC_{50} (μM)		
	MCF-7	SMMC-7721	HeLa
1	> 50	> 50	> 50
6a	27.43 ± 2.31	21.32 ± 1.89	18.79 ± 2.58
6b	17.39 ± 3.07	8.16 ± 0.73	12.71 ± 1.98
6c	18.87 ± 2.16	12.38 ± 2.11	18.03 ± 1.34
6d	17.21 ± 1.33	24.71 ± 2.66	27.03 ± 2.43
6e	33.72 ± 3.79	20.14 ± 2.05	22.31 ± 1.75
6f	43.78 ± 3.20	> 50	> 50
6g	7.21 ± 0.37	5.91 ± 0.20	7.39 ± 0.18
6h	> 50	37.76 ± 3.69	> 50
6i	> 50	> 50	> 50
6j	> 50	> 50	> 50
6k	6.53 ± 0.86	3.03 ± 0.42	3.78 ± 0.28
6l	19.78 ± 2.48	13.21 ± 2.02	11.26 ± 1.59
6m	2.26 ± 0.23	0.97 ± 0.11	1.89 ± 0.31
6n	14.75 ± 1.95	12.37 ± 1.34	18.72 ± 1.13
Etoposide	0.77 ± 0.12	0.62 ± 0.16	0.83 ± 0.22

CONCLUSIONS

In summary, a series of new 1,3,4-oxadiazin-5(*6H*)-one derivatives of DAA was synthesized and

evaluated for their in vitro antimicrobial and cytotoxic activities. As a result, some title compounds exhibited promising antimicrobial and/or cytotoxic activities. Among them, compound **6e** possessed prominent antibacterial properties against four tested bacteria, while compound **6m** exhibited potent cytotoxic activity against three cancer cell lines. The activities of the title compounds were significantly influenced by the properties and positions of the substituents on the benzene ring. Further studies will be carried out to explore the in-depth structure–activity relationships (SAR) and the mechanisms of action of these compounds.

EXPERIMENTAL

Chemistry

Melting points were measured on an XT-4 apparatus (Taike Corp., Beijing, China) and were uncorrected. IR spectra were measured on a Nexus 870 FT-IR spectrometer, and the absorption bands were expressed in cm^{-1}. The ESI-MS spectra were recorded on a Mariner System 5304 mass spectrometer. ^{1}H NMR and ^{13}C NMR spectra were accomplished in $CDCl_3$ on Bruker AV-300, AV-500, and DRX-600 NMR spectrometers using TMS as the internal standard. Elemental analyses were carried out using the Elementar Vario El cube elemental analyzer. Reactions and the resulted products were monitored by TLC, which was carried out on silica gel IB-F flexible sheets obtained from Mallinckrodt Baker Inc., Germany and visualized in UV light (254 nm). Silica gel (300–400 mesh) for column chromatography was purchased from Qingdao Marine Chemical Factory, China. DAA (98%) was bought from Yijing Industrial Co., Ltd. (Shanghai, China). The reagents (chemicals), all being of A.R. grade, were purchased from Shanghai Chemical Reagent Company (Shanghai, China).

General procedure for the preparation of phenylhydrazine hydrochlorides (4a–4m)

A solution of $NaNO_2$ (0.73 g, 10.5 mmol) in 2 mL of H_2O was added dropwise to a solution of substituted aniline (**3**, 10 mmol) in 10 mL of 20% HCl while cooling with an ice water bath. The reaction mixture was stirred at 0~5 °C for 1 h to give a clear solution. Then, a solution of $SnCl_2$ (20 mmol) in 6 mL of 35% HCl at 0~5 °C was added dropwise to this solution. The mixture was then stirred at room temperature for 1–2 h. The solid product was filtered, washed with 35% HCl thrice, and dried in a vacuum desiccator containing anhydrous $CaCl_2$. The product could be used in subsequent reactions without further purification.

General procedure for the preparation of acylhydrazine derivatives (5a–5m)

To a solution of DAA (30 g, 0.1 mol) in 100 mL of benzene, 10.9 mL of $SOCl_2$ (17.85 g, 0.15 mol) was added slowly. The mixture was then refluxed for 3 h. After cooling, the solvent and excess $SOCl_2$ were removed in vacuo to yield dehydroabietate chloride (**2**) as a yellow oily product, which was used in the next step without further purification.

To a solution of dehydroabietate chloride (**2**, 0.638 g, 2 mmol) in 10 mL of cyclohexane, different substituted phenylhydrazine hydrochloride (3 mmol) and triethylamine (0.606 g, 6 mmol) at 0 °C were added. The mixture was stirred for 1 h at 0 °C and then for 3 h at room temperature. At the end of reaction, the mixture was filtered to remove the precipitate. The filtrate was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by recrystallization in MeOH to afford compound **5a–5 m** as yellow resin-like solids.

General procedure for the synthesis of 1,3,4-oxadiazin-5 (*6H*)-one derivatives (6a–6m)

Chloracetyl chloride (40 μL, 0.5 mmol) was added to a solution of compound **5** (0.26 mmol) in 10 mL of anhydrous 2-butanone. The mixture was refluxed at 85 °C for 1 h. After cooling, anhydrous K_2CO_3 (0.415 g, 3 mmol) was added, and the mixture was refluxed for another 3 h. At the end of the reaction, the solvent was removed in vacuo, and the residue was dissolved in 20 mL of ethyl acetate. The solution was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was then purified by column chromatography on silica gel and eluted with petroleum ether-acetone (200:1~10:1, v/v) to afford compounds **6a–6m**.

2-Dehydroabietyl-4-phenyl-4*H*-1,3,4-oxadiazin-5(*6H*)-one (6a)

Yellow solid; yield: 56%; mp 130~133 °C; IR (KBr, cm^{-1}): 2954, 2925, 2867, 1694, 1646, 1595, 1493, 1374, 1309, 1274, 1052, 817, 758, 685; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.23 (d, J = 7.0 Hz, 6H), 1.25

(s, 3H), 1.32 (s, 3H), 1.51 (dt, J = 12.8, 3.6 Hz, 1H), 1.60~1.90 (m, 6H), 2.20 (dd, J = 12.5, 2.0 Hz, 1H), 2.33 (brd, J = 13.2 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.56 (d, J = 15.1 Hz, 1H), 4.61 (d, J = 15.1 Hz, 1H), 6.90 (d, J = 1.1 Hz, 1H), 7.02 (dd, J = 8.1, 1.7 Hz, 1H), 7.19 (d, J = 8.2 Hz, 1H), 7.25 (t, J = 7.4 Hz, 1H), 7.41 (t, J = 7.6 Hz, 2H), 7.70 (d, J = 7.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.9, 24.1, 25.3, 29.8, 33.5, 36.7, 37.4, 37.9, 41.1, 46.3, 67.4, 120.1, 123.9, 124.1, 125.2, 126.9, 128.6, 134.5, 138.6, 146.0, 146.4, 158.8, 168.3; MS (ESI): m/z $[M + H]^+$: 431.3; Anal. Calc. for $C_{28}H_{34}N_2O_2$: C 78.10; H 7.96; N 6.51; found: C 78.16; H 7.91; N 6.58.

2-Dehydroabietyl-4-(*p*-tolyl)-4*H*-1,3,4-oxadiazin-5(*6H*)-one (6b)

Yellow gum; yield: 49%; IR (KBr, cm^{-1}): 2953, 2927, 2863, 1727, 1693, 1648, 1506, 1460, 1374, 1300, 1274, 1210, 1172, 1116, 1063, 967, 819, 711, 634; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.23 (d, J = 6.9 Hz, 6H), 1.25 (s, 3H), 1.31 (s, 3H), 1.50 (dt, J = 12.8, 3.8 Hz, 1H), 1.60~1.89 (m, 6H), 2.20 (dd, J = 12.5, 2.1 Hz, 1H), 2.33 (brd, J = 13.1 Hz, 1H), 2.36 (s, 3H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.54 (d, J = 15.1 Hz, 1H), 4.59 (d, J = 15.1 Hz, 1H), 6.90 (d, J = 1.1 Hz, 1H), 7.01 (dd, J = 8.2, 1.7 Hz, 1H), 7.18 (d, J = 8.2 Hz, 1H), 7.20 (d, J = 8.3 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.4, 20.8, 21.0, 24.0, 25.2, 29.8, 33.5, 36.6, 37.4, 37.9, 41.1, 46.3, 67.3, 121.8, 123.8, 124.1, 126.9, 129.5, 134.5, 137.4, 138.7, 146.0, 146.4, 158.9, 168.2; MS (ESI): m/z $[M + H]^+$: 445.3; Anal. Calc. for $C_{29}H_{36}N_2O_2$: C 78.34; H 8.16; N 6.30; found: C 78.39; H 8.10; N 6.24.

2-Dehydroabietyl-4-(4-ethylphenyl)-4*H*-1,3,4-oxadiazin-5(*6H*)-one (6c)

Yellow gum; yield: 37%; IR (KBr, cm^{-1}): 2958, 2928, 2866, 1734, 1693, 1650, 1507, 1460, 1375, 1309, 1288, 1283, 1209, 1179, 1118, 1076, 1057, 968, 899, 829, 706; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.23 (d, J = 6.9 Hz, 6H), 1.24 (t, J = 7.6 Hz, 3H), 1.25 (s, 3H), 1.31 (s, 3H), 1.50 (dt, J = 12.7, 3.8 Hz, 1H), 1.60~1.90 (m, 6H), 2.20 (dd, J = 12.4, 2.1 Hz, 1H), 2.33 (brd, J = 13.2 Hz, 1H), 2.66 (q, J = 7.6 Hz, 2H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.55 (d, J = 15.1 Hz, 1H), 4.59 (d, J = 15.1 Hz, 1H), 6.90 (d, J = 1.2 Hz, 1H), 7.01 (dd, J = 8.2, 1.7 Hz, 1H), 7.18 (d, J = 8.2 Hz, 1H), 7.23 (d, J = 8.5 Hz, 2H), 7.57 (d, J = 8.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 15.2, 17.2, 18.5, 20.9, 24.0, 25.3, 28.0, 29.9, 33.5, 36.7, 37.4, 37.9, 41.1, 46.4, 67.3, 122.2, 124.0, 124.1, 129.8, 126.9, 146.2, 134.5, 138.7, 146.0, 146.5, 158.8, 168.3; MS (ESI): m/z $[M + H]^+$: 459.3; Anal. Calc. for $C_{30}H_{38}N_2O_2$: C 78.56; H 8.35; N 6.11; found: C 78.61; H 8.39; N 6.18.

2-Dehydroabietyl-4-(4-fluorophenyl)-4*H*-1,3,4-oxadiazin−5(*6H*)-one (6d)

Yellow gum; yield: 44%; IR (KBr, cm^{-1}): 2949, 2927, 2862, 1695, 1649, 1604, 1504, 1460, 1375, 1285, 1229, 1207, 1180, 1099, 1059, 969, 833, 723, 694, 633; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.23 (d, J = 7.0 Hz, 6H), 1.25 (s, 3H), 1.31 (s, 3H), 1.50 (dt, J = 12.5, 2.0 Hz, 1H), 1.60~1.90 (m, 6H), 2.20 (dd, J = 12.5, 2.1 Hz, 1H), 2.34 (brd, J = 12.8 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.55 (d, J = 15.2 Hz, 1H), 4.60 (d, J = 15.2 Hz, 1H), 6.90 (brs, 1H), 7.02 (dd, J = 8.1, 1.3 Hz, 1H), 7.09 (t, J = 8.7 Hz, 2H), 7.18 (d, J = 8.2, 1H), 7.67 (dd, J = 9.1, 4.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.8, 24.0, 25.3, 29.8, 33.5, 36.7, 37.5, 37.9, 41.1, 46.3, 67.4, 115.4 (d, J = 8.9 Hz), 116.2 (d, J = 22.8 Hz), 123.9, 124.1, 126.9, 134.5, 138.8 (d, J = 2.3 Hz), 146.0, 146.5, 158.7, 160.1 (d, J = 243.9 Hz), 168.4; MS (ESI): m/z $[M + H]^+$: 449.3; Anal. Calc. for $C_{28}H_{33}FN_2O_2$: C 74.97; H 7.42; N 6.25; found: C 74.90; H 7.36; N 6.32.

4-(4-Chlorophenyl)-2-dehydroabietyl-4*H*-1,3,4-oxadiazin−5(*6H*)-one (6e)

Yellow gum; yield: 45%; IR (KBr, cm^{-1}): 2949, 2927, 2854, 1696, 1490, 1461, 1369, 1282, 1209, 1179, 1137, 1089, 1059, 1012, 968, 826, 753, 708, 631; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.23 (d, J = 7.0 Hz, 6H), 1.25 (s, 3H), 1.32 (s, 3H), 1.50 (dt, J = 12.7, 3.5 Hz, 1H), 1.59~1.90 (m, 6H), 2.19 (dd, J = 12.6, 2.1 Hz, 1H), 2.34 (brd, J = 12.5 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.55 (d, J = 15.2 Hz, 1H), 4.60 (d, J = 15.2 Hz, 1H), 6.90 (d, J = 1.1 Hz, 1H), 7.02 (dd, J = 8.2, 1.7 Hz, 1H), 7.18 (d, J = 8.2 Hz, 1H), 7.36 (d, J = 8.9 Hz, 2H), 7.69 (d, J = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.9, 24.0, 25.3, 29.8, 33.5, 36.5, 37.3, 38.0, 41.1, 46.3, 67.4, 120.6, 123.9, 124.1, 126.9, 129.3, 130.4, 134.5, 138.5, 146.0, 146.4, 158.6, 168.2; MS (ESI): m/z $[M + H]^+$: 465.2; Anal.

Calc. for $C_{28}H_{33}ClN_2O_2$: C 72.32; H 7.15; N 6.02; found: C 72.37; H 7.09; N 6.09.

4-(4-Bromophenyl)-2-dehydroabietyl-4*H*-1,3,4-oxadiazin−5(6*H*)-one (6f)

Yellow gum; yield: 47%; IR (KBr, cm^{-1}): 2955, 2926, 2861, 1696, 1649, 1488, 1460, 1369, 1282, 1210, 1179, 1110, 1074, 1010, 969, 877, 823, 783, 719, 621; ^1H NMR (600 MHz, $CDCl_3$): δ 1.23 (d, J = 6.9 Hz, 6H), 1.25 (s, 3H), 1.32 (s, 3H), 1.50 (dt, J = 13.0, 3.5 Hz, 1H), 1.59~1.90 (m, 6H), 2.19 (dd, J = 12.5, 2.0 Hz, 1H), 2.33 (brd, J = 12.5 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.54 (d, J = 15.2 Hz, 1H), 4.60 (d, J = 15.2 Hz, 1H), 6.90 (d, J = 1.1 Hz, 1H), 7.02 (dd, J = 8.2, 1.6 Hz, 1H), 7.18 (d, J = 8.2 Hz, 1H), 7.51 (d, J = 8.9 Hz, 2H), 7.64 (d, J = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.8, 24.0, 25.3, 29.8, 33.5, 36.7, 37.4, 37.9, 41.1, 46.2, 67.4, 116.9, 121.7, 124.0, 124.1, 126.9, 131.6, 134.5, 138.3, 146.0, 146.4, 158.8, 168.3; MS (ESI): m/z $[M + H]^+$: 509.2; Anal. Calc. for $C_{28}H_{33}BrN_2O_2$: C 66.01; H 6.53; N 5.50; found: C 66.07; H 6.58; N 5.43.

2-Dehydroabietyl-4-(4-methoxyphenyl)-4*H*-1,3,4-oxadiazin-5(6*H*)-one (6g)

Yellow gum; yield: 53%; IR (KBr, cm^{-1}): 3444, 2949, 2929, 2836, 1799, 1730, 1679, 1598, 1514, 1461, 1389, 1331, 1248, 1214, 1176, 1121, 1078, 1029, 972, 876, 821; ^1H NMR (600 MHz, $CDCl_3$): δ 1.22 (d, J = 6.9 Hz, 6H), 1.26 (s, 3H), 1.37 (s, 3H), 1.49 (dd, J = 12.7, 4.0 Hz, 1H), 1.60~1.88 (m, 6H), 2.20 (dd, J = 12.5, 2.2 Hz, 1H), 2.34 (brd, J = 13.4 Hz, 1H), 2.82 (m, 1H), 2.87~2.92 (m, 2H), 3.89 (s, 3H), 4.56 (d, J = 15.8 Hz, 1H), 4.65 (d, J = 15.8 Hz, 1H), 6.91 (d, J = 2.0 Hz, 1H), 6.94 (dd, J = 8.7, 2.2 Hz, 2H), 7.02 (dd, J = 8.2, 2.1 Hz, 1H), 7.18 (d, J = 8.3, 1H), 7.56 (d, J = 8.7, 2.1, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.8, 24.0, 25.2, 29.7, 33.5, 36.6, 37.4, 37.9, 41.1, 46.3, 55.7, 67.4, 115.2, 122.1, 124.0, 124.1, 126.9, 132.6, 134.5, 146.0, 146.4, 158.8, 161.3, 168.3; MS (ESI): m/z $[M + H]^+$: 461.3; Anal. Calc. for $C_{29}H_{36}N_2O_3$: C 75.62; H 7.88; N 6.08; found: C 75.57; H 7.84; N 6.12.

2-Dehydroabietyl-4-(4-(trifluoromethyl)phenyl)-4*H*-1,3,4-oxadiazin-5(6*H*)-one (6h)

Yellow gum; yield: 52%; IR (KBr, cm^{-1}): 2958, 2931, 2867, 1702, 1648, 1613, 1514, 1496, 1461, 1365, 1349, 1324, 1312, 1284, 1213, 1165, 1125, 1068, 1015, 968, 843, 822; ^1H NMR (600 MHz, $CDCl_3$): δ 1.23 (d, J = 6.9 Hz, 6H), 1.26 (s, 3H), 1.34 (s, 3H), 1.51 (dt, J = 13.0, 3.4 Hz, 1H), 1.60~1.89 (m, 6H), 2.21 (dd, J = 12.5, 2.1 Hz, 1H), 2.34 (brd, J = 12.6 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.57 (d, J = 15.3 Hz, 1H), 4.62 (d, J = 15.3 Hz, 1H), 6.91 (d, J = 1.2 Hz, 1H), 7.02 (dd, J = 8.2, 1.7 Hz, 1H), 7.19 (d, J = 8.2 Hz, 1H), 7.66 (d, J = 8.6, 2H), 7.92 (d, J = 8.5, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.4, 20.8, 24.0, 25.2, 29.7, 33.5, 36.6, 37.4, 37.9, 41.2, 46.3, 67.3, 123.9, 124.1, 122.5 (q, J = 271.2 Hz, CF_3), 126.6 (q, J = 3.8 Hz), 126.9, 127.1 (q, J = 4.1 Hz), 131.1 (q, J = 32.5 Hz), 134.3, 141.2, 146.0, 146.3, 158.8, 168.3; MS (ESI): m/z $[M + H]^+$: 499.3; Anal. Calc. for $C_{29}H_{33}F_3N_2O_2$: C 69.86; H 6.67; N 5.62; found: C 69.82; H 6.70; N 5.68.

2-Dehydroabietyl-4-(4-nitrophenyl)-4*H*-1,3,4-oxadiazin-5(6*H*)-one (6i)

Yellow solid; yield: 58%; m.p. 120~124 °C; IR (KBr, cm^{-1}): 2952, 2924, 2852, 1704, 1650, 1591, 1520, 1493, 1458, 1335, 1297, 1110, 1056, 852, 823, 745, 688; ^1H NMR (600 MHz, $CDCl_3$): δ 1.23 (d, J = 6.9 Hz, 6H), 1.27 (s, 3H), 1.36 (s, 3H), 1.52 (dt, J = 12.5, 3.7 Hz, 1H), 1.60~1.90 (m, 6H), 2.21 (dd, J = 12.5, 2.1 Hz, 1H), 2.35 (brd, J = 12.1 Hz, 1H), 2.84 (m, 1H), 2.87~2.92 (m, 2H), 4.59 (d, J = 15.4 Hz, 1H), 4.64 (d, J = 15.5 Hz, 1H), 6.91 (d, J = 1.0 Hz, 1H), 7.03 (dd, J = 8.2, 1.6 Hz, 1H), 7.19 (d, J = 8.2 Hz, 1H), 8.06 (d, J = 9.3 Hz, 2H), 8.27 (dd, J = 9.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.9, 24.0, 25.3, 29.8, 33.5, 36.6, 37.4, 37.9, 41.1, 46.3, 67.5, 115.2, 124.0, 124.1, 124.5, 127.0, 134.5, 140.9, 142.6, 146.0, 146.4, 158.7, 168.4; MS (ESI): m/z $[M + H]^+$: 476.3; Anal. Calc. for $C_{28}H_{33}N_3O_4$: C 70.71; H 6.99; N 8.84; found: C 70.78; H 6.96; N 8.80.

4-(2-Dehydroabietyl-5-oxo-5,6-dihydro-4*H*-1,3,4-oxadiazin-4-yl)benzonitrile (6j)

Yellow gum; yield: 41%; IR (KBr, cm^{-1}): 2948, 2927, 2853, 2223, 1703, 1646, 1601, 1494, 1458, 1368, 1343, 1163, 835, 819; ^1H NMR (600 MHz, $CDCl_3$): δ 1.23 (s, J = 6.9 Hz, 6H), 1.26 (s, 3H), 1.34 (s, 3H), 1.51 (dt, J = 12.6, 2.9 Hz, 1H), 1.60~1.90 (m, 6H), 2.20 (dd, J = 12.5, 2.1 Hz, 1H), 2.35 (brd, J = 12.3 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.57 (d, J = 15.4 Hz, 1H), 4.62 (d, J = 15.4 Hz, 1H), 6.90 (d, J = 1.1 Hz, 1H),

7.02 (dd, J = 8.2, 1.6 Hz, 1H), 7.19 (d, J = 8.2 Hz, 1H), 7.69 (d, J = 8.9 Hz, 2H), 7.99 (d, J = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.9, 24.0, 25.3, 29.8, 33.5, 36.7, 37.4, 37.9, 41.1, 46.3, 67.4, 118.6, 120.1, 123.9, 124.1, 125.2, 126.9, 128.6, 134.5, 138.6, 146.0, 146.4, 158.8, 168.3; MS (ESI): m/z $[M + H]^+$: 456.3; Anal. Calc. for $C_{29}H_{33}N_3O_2$: C 76.45; H 7.30; N 9.22; found: C 76.51; H 7.23; N 9.20.

2-Dehydroabietyl-4-(*o*-tolyl)-4*H*-1,3,4-oxadiazin-5(6*H*)-one (6k)

Yellow solid; yield: 44%; m.p. 134~136 °C; IR (KBr, cm^{-1}): 2953, 2928, 2868, 1695, 1639, 1495, 1459, 1385, 1283, 1219, 1176, 1131, 1057, 971, 879, 822, 761, 717, 628; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.22 (d, J = 7.0 Hz, 6H), 1.23 (s, 6H), 1.49 (dt, J = 13.0, 3.8 Hz, 1H), 1.60~1.89 (m, 6H), 2.19 (dd, J = 12.5, 2.2 Hz, 1H), 2.25 (s, 3H), 2.32 (brd, J = 13.1 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.59 (d, J = 15.1 Hz, 1H), 4.64 (d, J = 15.1 Hz, 1H), 6.89 (d, J = 1.7 Hz, 1H), 7.01 (dd, J = 8.2, 2.0 Hz, 1H), 7.17 (d, J = 8.1 Hz, 1H), 7.28 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 17.6, 18.4, 20.8, 24.0, 25.3, 29.8, 33.5, 36.7, 37.5, 37.8, 41.1, 46.3, 67.3, 123.8, 124.1, 124.5, 126.3, 126.9, 127.6, 131.3, 133.9, 134.5, 138.1, 146.0, 146.5, 158.8, 168.3; MS (ESI): m/z $[M + H]^+$: 445.3; Anal. Calc. for $C_{29}H_{36}N_2O_2$: C 78.34; H 8.16; N 6.30; found: C 78.39; H 8.20; N 6.27.

4-(2-Chlorophenyl)-2-dehydroabietyl-4*H*-1,3,4-oxadiazin −5(6*H*)-one (6l)

Yellow solid; yield: 54%; m.p. 166~168 °C; IR (KBr, cm^{-1}): 2953, 2928, 2867, 1702, 1639, 1582, 1481, 1447, 1386, 1287, 1219, 1176, 1137, 1071, 1031, 821, 763, 751, 736, 722, 621; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.22 (d, J = 8.6 Hz, 6H), 1.23 (s, 6H), 1.49 (dt, J = 12.6, 3.8 Hz, 1H), 1.60~1.87 (m, 6H), 2.19 (dd, J = 12.5, 2.2 Hz, 1H), 2.32 (brd, J = 13.1 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.61 (d, J = 15.2 Hz, 1H), 4.66 (d, J = 15.1 Hz, 1H), 6.90 (d, J = 2.0 Hz, 1H), 7.01 (dd, J = 8.2, 2.0 Hz, 1H), 7.17 (d, J = 8.1 Hz, 1H), 7.33 (dt, J = 7.4, 2.0 Hz, 1H), 7.37 (dt, J = 7.5, 1.8 Hz, 1H), 7.41 (dd, J = 7.4, 2.0 Hz, 1H), 7.50 (dd, J = 7.4, 2.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.9, 24.1, 25.3, 29.8, 33.5, 36.6, 37.3, 38.0, 41.1, 46.3, 67.5, 123.2, 123.8, 124.1, 126.9, 127.3, 130.1, 130.5, 131.1, 134.5, 137.2, 146.0, 146.4, 158.7, 168.2; MS (ESI): m/z $[M + H]^+$: 465.2; Anal. Calc. for $C_{28}H_{33}ClN_2O_2$: C 72.32; H 7.15; N 6.02; found: C 72.34; H 7.19; N 5.98.

2-Dehydroabietyl-4-(2-methoxyphenyl)-4*H*-1,3,4-oxadiazin-5(6*H*)-one (6m)

Yellow solid; yield: 59%; m.p. 123~126 °C; IR (KBr, cm^{-1}): 3311, 2955, 2929, 2867, 1690, 1597, 1498, 1463, 1380, 1274, 1170, 1112, 1074, 1024, 977, 821, 797, 751, 635, 610; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.22 (d, J = 7.0 Hz, 6H), 1.23 (s, 6H), 1.49 (m, 1H), 1.60~1.90 (m, 6H), 2.19 (dd, J = 12.7, 2.1 Hz, 1H), 2.31 (brd, J = 13.2 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 3.87 (s, 3H, OCH_3), 4.57 (d, J = 15.2 Hz, 1H), 4.62 (d, J = 15.2 Hz, 1H), 6.90 (d, J = 2.0 Hz, 1H), 6.96 (m, 2H), 7.02 (m, 2H), 7.19 (d, J = 8.1 Hz, 1H), 7.58 (d, J = 8.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.8, 24.0, 25.3, 29.7, 33.5, 36.6, 37.4, 37.9, 41.1, 46.3, 55.8, 67.5, 112.8, 121.5, 123.9, 124.1, 126.9, 128.1, 130.7, 140.8, 134.5, 146.0, 146.5, 150.3, 158.7, 168.3; MS (ESI): m/z $[M + H]^+$: 461.3; Anal. Calc. for $C_{29}H_{36}N_2O_3$: C 75.62; H 7.88; N 6.08; found: C 75.57; H 7.83; N 6.13.

General procedure for the synthesis of 1,3,4-oxadiazin-5(6*H*)-one derivatives (6n)

Reduced Fe powder (0.112 g, 2 mmol) and 0.6 mL of concentrated HCl was added to a solution of compound **6i** (0.12 g, 0.26 mmol) in 15 mL of EtOH and 1.5 mL of water. The mixture was refluxed for 2 h at 85 °C and monitored by TLC. After cooling, the mixture was poured into 20 mL of ice-cold water, which was extracted with CH_2Cl_2 (3 × 20 mL). The organic layer was combined; washed with water, saturated $NaHCO_3$, and brine; dried over anhydrous Na_2SO_4; and concentrated in vacuo. The residue was purified by column chromatography on silica gel, eluting with petroleum ether-acetone (5:1, v/v) to afford compound **6n** as a yellow gum.

4-(4-Aminophenyl)-2-dehydroabietyl-4*H*-1,3,4-oxadiazin −5(6*H*)-one (6n**)**

Yellow gum; yield: 91%; IR (KBr, cm^{-1}): 3431, 3368, 2949, 2925, 2857, 1731, 1682, 1629, 1511, 1460, 1382, 1283, 1210, 1184, 1125, 1084, 1057, 1021, 969, 884, 825, 750, 712, 685; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.24 (d, J = 7.0 Hz, 6H), 1.27 (s, 3H), 1.34 (s, 3H), 1.49 (dt, J = 12.6, 3.6 Hz, 1H), 1.60~1.86 (m,

6H), 2.18 (dd, J = 12.5, 2.2 Hz, 1H), 2.32 (brd, J = 12.9 Hz, 1H), 2.83 (m, 1H), 2.87~2.92 (m, 2H), 4.53 (d, J = 15.1 Hz, 1H), 4.58 (d, J = 15.1 Hz, 1H), 5.30 (s, 2H), 6.69 (dt, J = 8.8, 2.1 Hz, 2H), 6.89 (d, J = 1.6 Hz, 1H), 7.00 (dd, J = 8.1, 1.6 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H), 7.40 (dd, J = 8.8, 2.1 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.2, 18.5, 20.8, 24.0, 25.2, 29.7, 33.5, 36.6, 37.4, 37.9, 41.1, 46.3, 67.5, 112.2, 122.3, 124.0, 124.1, 126.9, 128.9, 134.5, 143.8, 146.0, 146.4, 158.7, 168.3; MS (ESI): m/z $[M + H]^+$: 446.3; Anal. Calc. for $C_{28}H_{35}N_3O_2$: C 75.47 H 7.92; N 9.43; found: C 75.51; H 7.86; N 9.38.

Antimicrobial assay

All the target compounds were screened for their antibacterial and antifungal activity. Seven strains were used as test microorganisms, including four bacteria: *Escherichia coli* (CGMCC 1.1571), *Pseudomonas fluorescens* (CGMCC 1.1828), *Staphylococcus aureus* (CGMCC 1.1361), and *Bacillus subtilis* (CGMCC 1.1162) and three fungi: *Candida albicans* (CGMCC 2.2086), *Candida tropicalis* (CGMCC 2.3967), and *Penicillium citrinum* (CGMCC 3.825). The test microbes were obtained from the China General Microbiological Culture Collection Center (CGMCC), China. The antimicrobial activity was assessed in terms of MICs by a modified microdilution method.[26] Compounds were dissolved in DMSO, and serial double dilutions of each compound (75 μL) were prepared in 96-well micro trays. The same amount of test microorganisms in Martin's (for bacteria) or PD (for fungi) broth (~ 10^5 colony-forming unit (CFU)/mL) was added to each well to give a final volume of 150 μL. After incubation at 37 °C for 24 h (for bacteria) or 28 °C for 48 h (for fungi), the trays were examined for growth of the test microorganisms. The MIC was defined as the lowest concentrations of a compound at which microbial growth was inhibited. Amikacin sulfate and ketoconazole were included as positive controls, and DMSO was used as a negative control. All assays were performed in duplicate.

In vitro cytotoxic assay

The in vitro cytotoxic activities of the target compounds were evaluated against the human breast cancer cell line (MCF-7), human hepatocarcinoma cell line (SMMC-7721), and human cervical cancer cell line (HeLa) via the MTT colorimetric method.[27] Briefly, different tumor cells were grown in a DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (50 μg/mL). Cells were harvested at the log phase of growth and seeded in 96-well plates (100 μL/well at a density of 2×10^5 cells/mL). After 24 h incubation at 37 °C and 5% CO_2 to allow cell attachment, cultures were exposed to various concentrations of the isolated compounds for 48 h. Finally, MTT solution (2.5 mg/mL in PBS) was added (40 μL/well). Plates were further incubated for 4 h at 37 °C, and the formazan crystals formed were dissolved by adding 150 μL/well of DMSO. Absorption at 570 nm was measured with an ELISA plate reader. The results were expressed as IC_{50} values with standard deviations, which were defined as the concentration at which 50% survival of cells could be discerned. Etoposide was coassayed as positive control.

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