

ORIGINAL ARTICLE

New rubrolides from the marine-derived fungus *Aspergillus terreus* OUCMDZ-1925

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Two new rubrolides, rubrolides R (1) and S (2), were isolated from the fermentation broth of the marine-derived fungus *Aspergillus terreus* OUCMDZ-1925. Their structures were elucidated on the basis of spectroscopic analysis and X-ray single crystal diffraction. Compound 1 showed comparable or superior antioxidation against 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals to those of trolox and ascorbic acid with an IC₅₀ value of 1.33 mM. Compound 2 showed comparable or superior anti-influenza A (H1N1) virus activity to that of ribavirin with an IC₅₀ value of 87.1 μM. Both compounds 1 and 2 showed weak cytotoxicity against the K562 cell line with IC₅₀ values of 12.8 and 10.9 μM, respectively. *The Journal of Antibiotics* (2014) 67, 315–318; doi:10.1038/ja.2013.135; published online 11 December 2013

Keywords: anti-H1N1 virus; antioxidation; *Aspergillus terreus*; cytotoxicity; marine-derived fungus; rubrolides

INTRODUCTION

Marine-derived fungi have long been recognized as a potential source of structurally novel and pharmacologically active metabolites.^{1–3} Many bioactive compounds including polyketides, terpenes, steroids and peptides have been isolated from cultured fungi.² Rubrolides comprising a central furanone ring substituted at C-3 and C-4 with two 4-hydroxyphenyl moieties,⁴ A–Q, for example, were obtained from marine tunicate.^{5–9} This type of compounds possess antibacterial activity,^{5,6,9} cytotoxicity,⁷ inhibition of aldose reductase,^{10,11} anti-inflammatory activity⁸ and selective inhibition of protein phosphatases 1 and 2A.⁵ As part of our ongoing research on bioactive compounds from marine fungal origin,^{12–17} marine-derived *Aspergillus terreus* OUCMDZ-1925 was isolated from the viscera of the barracuda (*Chelon haematocheilus*) grown in the Yellow River estuary. The Yellow River delta formed from the convergence of the Yellow River mouth and the Bohai Sea possesses a unique wetland environment different from both the sea and the land. The solid state fermented products of *A. terreus* OUCMDZ-1925 showed antibacterial effects on *Bacillus subtilis*, from which four butyrolactones (I–IV) and methyl 2,4-dihydroxy-3,5,6-trimethylbenzoate with antibacterial effects on *Staphylococcus aureus*, *Enterobacter aerogenes*, *Bacillus subtilis* and *Escherichia coli* were identified.¹² To further enrich the chemical diversity of this fungus, *A. terreus* OUCMDZ-1925 was re-fermented in a liquid medium and the ethyl acetate (EtOAc) extract of the products showed 53% inhibition of the growth of P388 cells at a concentration of 100 μg ml⁻¹. Chemical investigation resulted in the isolation of two new rubrolides that we named rubrolides R (1) and S (2).

Rubrolide R (1) showed inferior antioxidation against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical to that of ascorbic acid and comparable or superior antioxidation against 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical to those of ascorbic acid and trolox with IC₅₀ values of 43.4 μM and 1.33 mM, respectively. Rubrolide S (2) exhibited comparable or superior anti-virus activity against influenza A (H1N1) virus to that of ribavirin with an IC₅₀ value of 87.1 μM. Both compounds 1 and 2 showed weak cytotoxic effects on the K562 cell line with IC₅₀ values of 12.8 and 10.9 μM, respectively.

RESULTS

Physico-chemical properties

Rubrolide R (1). Yellow amorphous powder and colorless triclinic crystal after cultured in acetone and the atmosphere of benzene by solvent diffusion method; mp 202–203 °C; HRESIMS *m/z* 349.1432 [M + H]⁺ (calcd for C₂₂H₂₁O₄, 349.1434); UV (MeOH) λ_{max} (log ε) 203 (4.41), 250 (4.03), 378 (4.24) nm; IR ν_{max} (KBr) 3322, 2960, 2923, 2853, 1730, 1701, 1540, 1508, 1457, 1433, 1261, 1091, 1032, 803 cm⁻¹; ¹H and ¹³C NMR (see Table 1).

Rubrolide S (2). Yellow solid; HRESIMS *m/z* 349.1430 [M + H]⁺ (calcd for C₂₂H₂₁O₄, 349.1434); UV (MeOH) λ_{max} (log ε) 203 (4.39), 252 (4.01), 376 (4.22) nm; IR ν_{max} (KBr) 3330, 2972, 2928, 2852, 1719, 1608, 1571, 1495, 1345, 1267, 1236, 1174, 1121, 824 cm⁻¹; ¹H and ¹³C NMR (see Table 1).

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Table 1 ^1H - (600 MHz) and ^{13}C - (150 MHz) NMR data for **1** and **2** in $\text{DMSO}-d_6$

Position	1		2	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1	168.8, C _q		168.8, C _q	
2	110.7, CH	6.34, s	110.9, CH	6.37, s
3	158.2, C _q		158.2, C _q	
4	145.3, C _q		145.7, C _q	
5	113.9, CH	6.27, s	113.4, CH	6.31, s
1'	124.3, C _q		124.9, C _q	
2'	132.5, CH	7.56, d (2.2)	132.5, CH	7.57, d (2.2)
3'	128.2, C _q		121.4, C _q	
4'	156.7, C _q		154.9, C _q	
5'	115.4, CH	6.86, d (8.8)	117.5, CH	6.78, d (8.2)
6'	130.1, CH	7.53, dd (8.8, 2.2)	130.1, CH	7.58, dd (8.2, 2.2)
7'	28.0, CH ₂	3.22, d (7.7)	21.8, CH ₂	2.75, t (6.6)
8'	122.5, CH	5.26, t (7.7)	32.0, CH ₂	1.78, t (6.6)
9'	131.9, C _q		75.1, C _q	
10'	25.6, CH ₃	1.69, s	26.7, CH ₃	1.29, s
11'	17.8, CH ₃	1.68, s	26.7, CH ₃	1.29, s
1''	120.8, C _q		120.5, C _q	
2''(6'')	130.5, CH	7.48, d (7.7)	130.5, CH	7.48, d (8.8)
3''(5'')	116.0, CH	6.93, d (7.7)	116.1, CH	6.92, d (8.8)
4''	159.8, C _q		160.2, C _q	

Biological activity

The antiviral activity against H1N1 virus of compounds **1** and **2** was evaluated by the CPE inhibition assay.^{18,19} The observed IC₅₀ values of **1**, **2** and ribavirin (a positive control) against H1N1 virus were 221.6, 87.1 and 118.8 μM , respectively. Compound **2** showed comparable or superior anti-H1N1 activity to that of ribavirin.

The cytotoxicities of the two compounds against K562, A549 and HL-60, and HeLa and HCT-116 cell lines were evaluated by the MTT method²⁰ and SRB method,²¹ respectively. The results showed that compounds **1** and **2** are cytotoxic on the K562 cell line with IC₅₀ values of 12.8 and 10.9 μM , respectively, as they are not active against the A549, HL-60, HeLa and HCT-116 cell lines (IC₅₀ > 30 μM). The IC₅₀ values of adriamycin (a positive control) against K562, A549, HL-60, HeLa and HCT-116 cell lines were 0.64, 0.16, 0.65, 0.60 and 0.21 μM , respectively.

The anti-oxidative activities of **1** and **2** against DPPH^{22–24} and ABTS^{25–27} radicals were also assayed. Compared with ascorbic acid and trolox (two positive controls), compound **1** displayed weak DPPH radical-scavenging activity and comparable or superior ABTS radical-scavenging activity with IC₅₀ values of 43.4 μM and 1.33 mM, respectively, when compound **2** was not active (IC₅₀ > 100 μM and 50 mM, respectively). The IC₅₀ values of ascorbic acid against DPPH and ABTS radicals and trolox against ABTS radical were 1.7 μM , 2.85 mM and 2.04 mM, respectively. The results showed that phenolic-OH in the benzal nucleus has a key role in antioxidation of **1** against DPPH and ABTS radicals.

Structure determination

Compound **1** was assigned the molecular formula of C₂₂H₂₀O₄ based on the HRESIMS peak at m/z 349.1432 [M + H]⁺. The IR spectrum indicated the existence of hydroxyls (3322 cm⁻¹), an ester or lactone carbonyl (1730 cm⁻¹) and aromatic nucleus (1540 cm⁻¹). The ^1H

NMR spectrum showed an A₂B₂ system at δ_{H} 7.48 (d, 2H, $J = 7.7$ Hz) and 6.93 (d, 2H, $J = 7.7$ Hz), corresponding to a 1,4-disubstituted phenyl ring. The ^1H NMR signals of the ABX system at δ_{H} 7.56 (d, 1H, $J = 2.2$ Hz), 6.86 (d, 1H, $J = 8.8$ Hz) and 7.53 (dd, 1H, $J = 8.8$ Hz, 2.2 Hz) indicated an additional 1,2,4-trisubstituted phenyl ring. These data suggested **1** as a rubrolide.²⁸ The ^1H and ^{13}C NMR spectra (Table 1, Supplementary Figures S1–S4) of **1** were similar to those of rubrolide E,²⁸ with the exception that an aromatic methine signal ($\delta_{\text{C}/\text{H}}$ 117.6/6.84) was replaced by an aromatic quaternary carbon signal (δ_{C} 128.2) and additional prenyl signals that is two methyl ($\delta_{\text{C}/\text{H}}$ 25.6/1.69 and 17.8/1.68), a methylene ($\delta_{\text{C}/\text{H}}$ 28.0/3.22), an olefinic methine ($\delta_{\text{C}/\text{H}}$ 122.5/5.26) and an olefinic quaternary carbon (δ_{C} 131.9), indicating **1** as a 3'-prenylated derivative of rubrolide E. This deduction was confirmed by COSY correlation of H-7' (δ_{H} 3.22) to H-8' (δ_{H} 5.26) (Supplementary Figure S5) and key HMBC correlations of H-11' to C-8'/C-9'/C-10' and H-7' to C-3'/C-4' (Figure 1 and Supplementary Figure S6). The single-crystal X-ray diffraction analysis further confirmed the structure (Figure 2), and the *Z*-configuration of $\Delta^{4(5)}$ double bond was also resolved. Thus, the structure of rubrolide R (**1**) was elucidated to be (*Z*)-5-(4-hydroxy-3-prenylbenzal)-4-(4-hydroxyphenyl) furan-2-one.

Compound **2** was obtained as a yellow solid with the same molecular formula of C₂₂H₂₀O₄ based on the HRESIMS peak at m/z 349.1430 [M + H]⁺, indicating an isomer of **1**. The IR spectrum showed the presence of phenolic OHs at 3330 cm⁻¹, ester/lactone carbonyls peaks at 1719 cm⁻¹ and aromatic rings at 1608 cm⁻¹. Except for a prenylated phenyl nucleus moiety, its ^1H and ^{13}C NMR data (Table 1, Supplementary Figures S7–S10) were very similar to those of **1**, suggesting the same 4(*Z*)-benzal-3-phenyl- γ -butyrolactone skeleton with **1** that was confirmed by an NOE correlation between H-5 (δ_{H} 6.31) and H-2''(6'') (δ_{H} 7.48) (Figure 1 and Supplementary Figure S13). Careful NMR comparison between **1** and **2** revealed that a sp³ methylene signal ($\delta_{\text{C}/\text{H}}$ 32.0/1.78) and an oxygenated quaternary carbon signal (δ_{C} 75.1) in **2** replaced the corresponding trisubstituted ethylene signals ($\delta_{\text{C}/\text{H}}$ 122.5/5.26, δ_{C} 131.9) in **1**, indicating that an intramolecular electrophilic addition of phenolic-OH with the trisubstituted ethylene of **1** occurs to form a dihydropyran ring fused to the trisubstituted benzene ring of **2**. This deduction was further supported by the key COSY correlation between H-7' and H-8' (Supplementary Figure S11) and HMBC correlations of H-7' to C-2'/C-3'/C-4' and of H-8' to C-9'/C-10'/C-11' (Supplementary Figure S12), as well as the upfield shifts of C-3'/C-4'/C-7' and the downfield shifts of C-5'/C-10'/C-11'. Therefore, the structure of rubrolide S (**2**) was identified as (*Z*)-5-((2,2-dimethylchroman-6-yl)methylene)-4-(4-hydroxyphenyl)furan-2-one.

METHODS

General experimental procedures

UV spectra were measured on a Unicco 2802 PCS spectrophotometer. IR spectra were recorded on a Nicolet Nexus 470 spectrophotometer as KBr disks. ^1H , ^{13}C NMR, DEPT and 2D-NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as the internal standard and chemical shifts were recorded as δ values. NOESY spectra were obtained on a Bruker Avance 600 spectrometer. The HRESIMS measurements were taken on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. The semi-preparative HPLC was performed using an ODS column (YMC-pak ODS-A, 10 \times 250 mm, 5 μm , 4.0 ml min⁻¹). TLC and column chromatography were performed on plates precoated with silica gel GF₂₅₄ (10–40 μm , Qingdao Marine Chemical Factory, Qingdao, China) and over Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), respectively. Vacuum-liquid chromatography (VLC) utilized silica gel (200–300 mesh, Qingdao Marine Chemical Factory). Sea salt used was made by

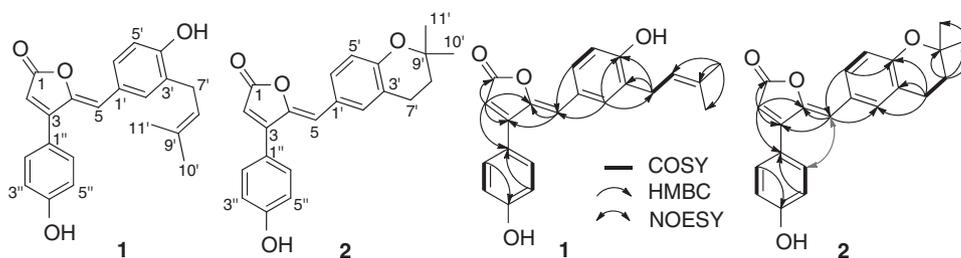


Figure 1 Structures of compounds **1** and **2** and key HMBC, NOESY and COSY correlations of **1** and **2**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

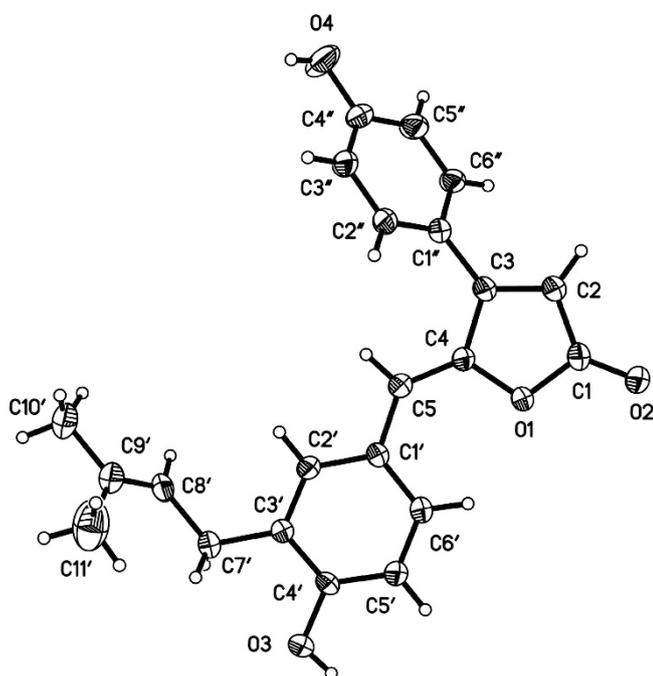


Figure 2 ORTEP drawing of the crystal structure of **1**.

evaporation of seawater collected in Laizhou Bay (Weifang Haisheng Chemical Factory, Weifang, China).

Strain

The fungal strain *A. terreus* OUCMDZ-1925 was isolated from the viscera of *C. haematocheilus* grown in the waters of the Yellow River Delta, and identified by BLAST search of ITS sequence using the GenBank database. ITS sequence of OUCMDZ-1925 strain (GenBank accession no GQ812053) showed 100% of homology with those of *A. terreus* (GenBank accession number KC762934 and KC582297).¹² The voucher specimen was stored in our laboratory at -80°C . The working strain was prepared on potato dextrose agar slants and stored at 4°C .

Fermentation

Spores were directly inoculated into 1000 mL conical flasks containing 300 mL liquid medium (glucose 10 g, maltose 20 g, mannitol 20 g, monosodium glutamate 10 g, CaCO_3 20 g, KH_2PO_4 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3 g, corn steep liquor 1 g, yeast extract 3 g and sea salt 33 g, dissolved in 1 l of tap water, pH 7.5). The fungus *A. terreus* OUCMDZ-1925 was grown under static conditions at 30°C for 45 days in 35 conical flasks.

Extraction and isolation

The whole fermentation broth (10.5 l) was filtered through a cheesecloth to separate the filtrate from the mycelia. The filtrate was extracted three times with an equivalent volume of EtOAc to give an EtOAc solution, as the mycelia were extracted three times with acetone. The acetone solution was concentrated *in vacuo* to afford an aqueous solution. The aqueous solution was extracted three times with an equivalent volume of EtOAc to give another EtOAc solution. Both EtOAc solutions were combined and concentrated *in vacuo* to give a crude gum (35 g).

The crude extract was separated into eight fractions on a silica gel VLC column using a stepwise gradient elution, with CH_2Cl_2 /petroleum ether (50–100%) followed by MeOH/ CH_2Cl_2 (0–50%). Fraction 4 (1 g) was separated into four subfractions (Fr.4-1–Fr.4-4) on Sephadex LH-20, eluting with MeOH. Fraction 4-3 (150 mg) was further separated into four subfractions (Fr.4-3-1–Fr.4-3-4) by VLC on RP-18 silica using a stepwise gradient elution with 5–90% MeOH/ H_2O . Fraction 4-3-1 (45 mg) was finally purified by the semi-preparative HPLC (60% MeOH/ H_2O) to yield **1** (20 mg, t_{R} 77 min). Fraction 5 (2.1 g) was separated into four subfractions (Fr.5-1–Fr.5-4) by VLC on RP-18 silica using a stepwise gradient elution with 5–90% MeOH/ H_2O . Fraction 5-2 (450 mg) was further separated into four subfractions (Fr.5-2-1–Fr.5-2-4) on Sephadex LH-20, eluting with MeOH. Fraction 5-2-3 (80 mg) was finally purified by the semi-preparative HPLC (70% MeOH/ H_2O) to yield **2** (23 mg, t_{R} 31 min).

X-ray structure determination of **1**

Compound **1** was obtained as a colorless triclinic crystal with molecular formula $\text{C}_{22}\text{H}_{20}\text{O}_4$. Space group *P*-1, $a = 9.7055(8) \text{ \AA}$, $b = 13.3599(12) \text{ \AA}$, $c = 14.3396(13) \text{ \AA}$, $\alpha = 85.5280(10)$, $\beta = 90.324(2)$, $\gamma = 90.216(2)$, $V = 1853.6(3) \text{ \AA}^3$, $Z = 2$, $D_{\text{calcd}} = 1.281 \text{ mg m}^{-3}$, $\mu = 0.089 \text{ mm}^{-1}$, $F(000) = 756$, crystal size $0.40 \times 0.17 \times 0.15 \text{ mm}$, $T = 298(2) \text{ K}$. A total of 6463 unique reflections ($2\theta < 50^{\circ}$) were collected on a CCD area detector diffractometer with graphite monochromated MoK α radiation ($\lambda = 0.71073 \text{ \AA}$). The structure was solved by direct methods (SHELXS-97) and expanded using Fourier techniques (SHELXL-97). The final cycle of full-matrix least squares refinement was based on 6463 unique reflections ($2\theta < 50^{\circ}$) and 2865 variable parameters and converged with unweighted and weighted agreement factors of $R_1 = 0.1416$, $R_w = 0.1447$ and $R = 0.0685$ for $I > 2\sigma(I)$ data. Crystallographic data (excluding structure factors) for structure **1** in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 946389. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

Anti-influenza A virus (H1N1) bioassay

The antiviral activity against H1N1 was evaluated by the CPE inhibition assay.^{18,19} Confluent MDCK cell monolayers were firstly incubated with influenza virus (A/Puerto Rico/8/34 (H1N1), PR/8) at 37°C for 1 h. After removing the virus dilution, cells were maintained in an infecting media (RPMI 1640, $4 \mu\text{g mL}^{-1}$ of trypsin) containing different concentrations of test compounds at 37°C . After 48 h incubation at 37°C , the cells were fixed with 100 μl of 4% formaldehyde for 20 min at room temperature. After removal of

the formaldehyde, the cells were stained with 0.1% crystal violet for 30 min. The plates were washed and dried, and the intensity of crystal violet staining for each well was measured in a microplate reader (Bio-Rad, USA) at 570 nm. The IC₅₀ was calculated as the compound concentration required for inhibiting influenza virus yield at 48 h post infection by 50%. Ribavirin was used as the positive control with the IC₅₀ values of 118.8 μM.

Cytotoxicity assay

Cytotoxicity was assayed by the MTT²⁰ and SRB methods,²¹ respectively. In the MTT assay, K562, A549 and HL-60 cell lines were cultured in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Cell suspensions (198 μl) at a density of 5 × 10⁴ cells · ml⁻¹ were plated in 96-well microtiter plates and incubated for 24 h. Then, the test solutions (2 μl in DMSO) were added to each well and further incubated for 72 h. The MTT solution (20 μl, 5 mg ml⁻¹ in RPMI-1640 medium) was then added to each well and incubated for 4 h. The old medium containing MTT (150 μl) was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Spectra Max Plus plate reader at 570 nm. In the SRB assay, the HeLa and HCT-116 cell suspension (200 μl) was plated in 96-well plates at density of 2 × 10⁵ cell per ml. Then, the test solutions (2 μl in MeOH) were added to each well and the culture was further incubated for 24 h. The cells were fixed with 12% trichloroacetic acid and the cell layer stained with 0.4% SRB. The absorbance of the SRB solution was measured at 515 nm. Adriamycin was used as the positive control with IC₅₀ values of 0.64, 0.16, 0.65, 0.60 and 0.21 μM for K562, A549, HL-60, HeLa and HCT-116 cell lines, respectively.

DPPH scavenging assay

The test sample solution (160 μl in MeOH) and DPPH solution (40 μl in MeOH) were plated in 96 cell plates and incubated in the dark for 30 min.^{22–24} Then, absorbance was measured at 520 nm and the inhibition was calculated. IC₅₀ values were calculated from the log-dose inhibition curve as the concentration of sample required to scavenge 50% of DPPH free radicals. Ascorbic acid was used as the positive control with an IC₅₀ value of 1.7 μM.

ABTS decolorization assay

The ABTS radical cation (ABTS^{·+}) was first produced by reacting ABTS stock solution (7 mM) with 2.45 mM potassium persulfate.^{25–27} The mixture was then placed in the dark at room temperature overnight before use. The reaction mixture consisted of 200 μl of sample or positive controls and 6 ml of ABTS^{·+} working solution was vortexed for 30 s. The absorbance was read at 734 nm after reaction for 4 min at 30 °C. The antioxidant activities of the test compounds were expressed as IC₅₀ that was defined as the concentration to inhibit the formation of ABTS radicals by 50%. All determinations were performed in triplicate. Trolox and ascorbic acid were used as the positive controls with IC₅₀ values of 2.04 mM and 2.85 mM, respectively.

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