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Potential Moracin M Prodrugs Strongly Attenuate Airway Inflammation *In Vivo*

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Abstract

This study aims to develop new potential therapeutic moracin M prodrugs acting on lung inflammatory disorders. Potential moracin M prodrugs (KW01-KW07) were chemically synthesized to obtain potent orally active derivatives, and their pharmacological activities against lung inflammation were, for the first time, examined *in vivo* using lipopolysaccharide (LPS)-induced acute lung injury model. In addition, the metabolism of KW02 was also investigated using microsomal stability test and pharmacokinetic study in rats. When orally administered, some of these compounds (30 mg/kg) showed higher inhibitory action against LPS-induced lung inflammation in mice compared to moracin M. Of them, 2-(3,5-bis((dimethylcarbamoyl)oxy)phenyl)benzofuran-6-yl acetate (KW02) showed potent and dose-dependent inhibitory effect on the same animal model of lung inflammation at 1, 3, and 10 mg/kg. This compound at 10 mg/kg also significantly reduced IL-1β concentration in the bronchoalveolar lavage fluid of the inflamed-lungs. KW02 was rapidly metabolized to 5-(6-hydroxybenzofuran-2-yl)-1,3-phenylene bis(dimethylcarbamate) (KW06) and moracin M when it was incubated with rat serum and liver microsome as expected. When KW02 was administered to rats via intravenous or oral route, KW06 was detected in the serum as a metabolite. Thus, it is concluded that KW02 has potent inhibitory action against LPS-induced lung inflammation. It could behave as a potential prodrug of moracin M to effectively treat lung inflammatory disorders.

Key Words: Arylbenzofuran, 2-(3,5-bis((dimethylcarbamoyl)oxy)phenyl)benzofuran-6-yl acetate, Moracin, Prodrug, Airway inflammation, Lung

INTRODUCTION

Lung inflammation is provoked by various insults such as tobacco smoke, air pollution, and pathological infection such as bacteria and virus. While acute bronchitis is relatively well-cured, it is difficult to control chronic lung inflammatory conditions such as chronic obstructive pulmonary diseases (COPD) (Jeffery, 2001). Several classes of drugs including anti-inflammatory steroids, antitussives, mucolytics, and/or bronchodilators are clinically used for these disorders, resulting in alleviation of some symptoms and attenuating deterioration of disease progress. However, they may not completely cure COPDs. Anti-inflammatory steroids have been frequently used to control symptoms of lung inflammation. However, they can result in serious complications sometimes. In this regard, some phosphodiesterase IV inhibitor and leukotriene receptor antagonist have been clinically introduced recently (Dentener *et al*., 2008; Aaron *et al*., 2013). However, they do not provide satisfactory results. Thus, new anti-inflammatory agents are needed to treat lung inflammatory condition.

Moracins are 2-arylbenzofuran derivatives. Some moracins are constituents of root barks of *Morus alba* L. widely used in traditional medicine for treating various inflammatory conditions including bronchitis in North-East Asia (Bae, 2000). Previously, we have demonstrated the inhibitory action of *M. alba* on airway inflammation. Alcoholic extract of *M. alba* can inhibit bronchitis-like symptoms of lipopolysaccharide (LPS)-induced airway inflammation in mice at oral doses of 100-400 mg/kg (Lim *et al*., 2013). As constituents of *M. alba*, several moracin derivatives also show anti-inflammatory activity including inducible nitric oxide synthase (iNOS)-catalyzed NO and IL-6 production (Yang *et al*., 2011; Lee *et al*., 2016). Among mora-

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cin derivatives, moracin M possesses significant inhibitory action against lung inflammation in mice by oral administration (20-60 mg/kg) (Lee *et al.*, 2016). Since moracin M bears three phenolic hydroxyl groups, its bioavailability might be poor due to phase II metabolism. We hypothesized that prodrugs of moracin M could improve blood concentration level of moracin M and exhibit good anti-inflammatory activity *in vivo*. This could lead to new anti-inflammatory therapeutics. Thus, the objective of this study was to design and synthesize a number of potential moracin M prodrugs and investigate their *in vivo* anti-inflammatory activity and pharmacokinetic profiles to establish their therapeutic potential for lung inflammatory disorders.

MATERIALS AND METHODS

Chemicals

2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT) was purchased from Tocris Cookson Ltd (Bristol, UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dexamethasone, IL-1β and lipopolysaccharide (LPS) (*Escherichia coli* 0127:B8) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 and other cell culture reagents including fetal bovine serum (FBS) were products of Gibco BRL (Grand Island, NY, USA). IL-1β mouse ELISA kit and IL-6 Human ELISA kit were purchased from BD bioscience (San Jose, CA, USA).

Animals

Male ICR mice and SD rats (4-6 weeks old, specific pathogen-free) were obtained from Nara Biotech (Seoul, Korea). The animals were maintained in animal facility (Kangwon National University, Chuncheon, Korea) at 20-22°C under 40-60% relative humidity and a 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiment. The experimental design using the animals was approved by the local committee for animal experimentation, Kangwon National University (KW-170717-1). The ethical guideline described in the Korean Food and Drug Administration guide for the care and use of laboratory animals was followed throughout the experiments.

Synthesis of moracin M and its analogues

Moracin M and its several analogues were synthesized as illustrated in Fig. 1.

2-(3,5-Bis(benzyloxy)phenyl)benzofuran-6-yl acetate (3): To a stirred solution of phosphonium salt 1 (15.0 g, 29.5 mmol), 4-dimethylaminopyridine (DMAP, 0.4 g, 4.9 mmol), and benzoic acid 2 (9.8 g, 29.5 mmol) in anhydrous CH_2Cl_2 (300 mL) was added a solution of dicyclohexylcarbodiimide (DCC, 7.7 g, 37.3 mmol) in anhydrous CH_2Cl_2 (50 mL). The reaction mixture was stirred for 20 h at room temperature, and concentrated at reduced pressure. The residue was dissolved in anhydrous 1,4-dioxane (150 mL). Triethylamine (20.6 mL, 147.8 mmol) was added and the reaction mixture was refluxed for 24 h. The mixture was filtered and the filtrate was concentrated at reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (hexanes/EtOAc, 3:1) to give benzofuran 3 as an amorphous solid (8.5 g, 62%): ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.53 (d, *J*=8.4 Hz, 1H), 7.49-7.31 (m, 11H), 7.10 (d, *J*=2.3 Hz, 2H), 6.98 (dd, *J*=8.1, 1.5 Hz, 2H), 6.63 (t, *J*=2.2 Hz, 1H), 5.11 (s, 4H), 2.34 (s, 3H); LRMS (ESI) *m/z* calcd.

for $C_{30}H_{24}O_5$ ([M+H]$^+$) 465.2, found 465.4.

2-(3,5-Bis(benzyloxy)phenyl)benzofuran-6-ol (4): To a stirred solution of benzofuran 3 (4.0 g, 8.6 mmol) in EtOH/H$_2$O (5:1, 43 mL) was added potassium hydroxide (1.0 g, 17.2 mmol). The mixture was refluxed for 2 h. The mixture was cooled to room temperature and diluted with water. Ethanol was removed by evaporation at reduced pressure. The mixture was acidified to pH 2 with 1N HCl, and the precipitate was filtered to give benzofuranol 4 (3.5 g, 98%) as an off-white solid: ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.48-7.31 (m, 11H), 7.07 (d, *J*=2.2 Hz, 2H), 7.00 (d, *J*=2.0 Hz, 1H), 6.90 (d, *J*=1.0 Hz, 1H), 6.77 (dd, *J*=8.4, 2.2 Hz, 1H), 6.60 (t, *J*=2.2 Hz, 1H), 5.11 (s, 4H); LRMS (ESI) *m/z* calcd. for $C_{28}H_{22}O_4$ ([M+H]$^+$) 423.1, found 423.2.

Moracin M: To a stirred suspension of benzofuranol 4 (3.5 g, 8.4 mmol) in EtOH/EtOAc (5:1, 120 mL) was added Pd/C (5% on activated carbon, 0.3 g) at room temperature. The mixture was stirred for 48 h at room temperature under a hydrogen atmosphere. The mixture was filtered through a pad of Celite and the filtrate was concentrated at reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (hexanes/EtOAc, 1:1) to give moracin M (1.8 g, 90%) as a white solid: meting point 260-262°C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.44 (s, 3H), 7.38 (d, *J*=8.4 Hz, 1H), 7.07 (d, *J*=0.6 Hz, 1H), 6.92 (d, *J*=1.7 Hz, 1H), 6.73 (dd, *J*=8.4, 2.1 Hz, 1H), 6.66 (dd, *J*=8.7, 2.2 Hz, 2H), 6.24-6.17 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 158.8, 155.8, 155.3, 154.0, 131.7, 121.2, 120.8, 112.5, 102.7, 102.3, 101.6, 97.5; LRMS (ESI) *m/z* calcd. for $C_{14}H_{10}O_4$ (M$^+$) 242.1, found 242.3.

2-(3,5-Dihydroxyphenyl)benzofuran-6-yl acetate (KW01): A mixture of benzofuran 3 (520 mg, 1.1 mmol) and Pd/C (5% on activated carbon, 50 mg) in THF (20 mL) was stirred for 40 h at room temperature under a hydrogen atmosphere. The reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated at reduced pressure to yield KW01 (308 mg, 96%) as an off-white solid (The product was pure enough to collect spectral data without further purification): ^{1}H NMR (300 MHz, CD3OD) δ 7.55 (d, *J*=8.4 Hz, 1H), 7.30 (s, 1H), 7.04 (s, 1H), 7.00-6.94 (m, 1H), 6.82 (d, *J*=2.2 Hz, 2H), 6.30 (t, *J*=2.1 Hz, 1H), 2.30 (s, 3H); LRMS (ESI) *m/z* calcd. for $C_{16}H_{12}O_5$ ([M+H]$^+$) 285.1, found 285.2.

2-(3,5-Bis((dimethylcarbamoyl)oxy)phenyl)benzofuran-6-yl acetate (KW02): To a solution of KW01 (850 mg, 3.4 mmol) and DMAP (42 mg, 0.34 mmol) in anhydrous pyridine (10 mL) was slowly added dimethylcarbamoyl chloride (1.57 mL, 17.1 mmol) at 0°C. The reaction mixture was stirred for 24 h at room temperature and concentrated at reduced pressure. The resulting residue was suspended in water and extracted with CH_2Cl_2 (20 mL×3). The combined organic layers were washed with 1N HCl and brine, dried over anhydrous MgSO$_4$, and concentrated at reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (CH$_2$Cl$_2$/MeOH, 99:1) to give KW02 (1.3 g, 88%) as a white solid: meting point 113-115°C; 1H NMR (300 MHz, CDCl$_3$) δ 7.53 (d, *J*=8.4 Hz, 1H), 7.46 (d, *J*=2.1 Hz, 2H), 7.26 (s, 1H), 6.98 (ddd, *J*=5.8, 3.0, 1.4 Hz, 3H), 3.11 (s, 6H), 3.03 (s, 6H), 2.34 (s, 3H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 169.8, 155.6, 154.7, 154.4, 152.3, 148.2, 131.8, 126.9, 121.2, 117.4, 116.0, 115.0, 105.2, 102.3, 36.8, 36.5, 21.2; LRMS (ESI) *m/z* calcd. for $C_{22}H_{22}N_2O_7$ ([M+H]$^+$) 427.1, found 427.2.

5-(6-Acetoxybenzofuran-2-yl)-1,3-phenylene diacetate (KW03): To a stirred solution of moracin M (50 mg, 0.21 mmol), DMAP (3
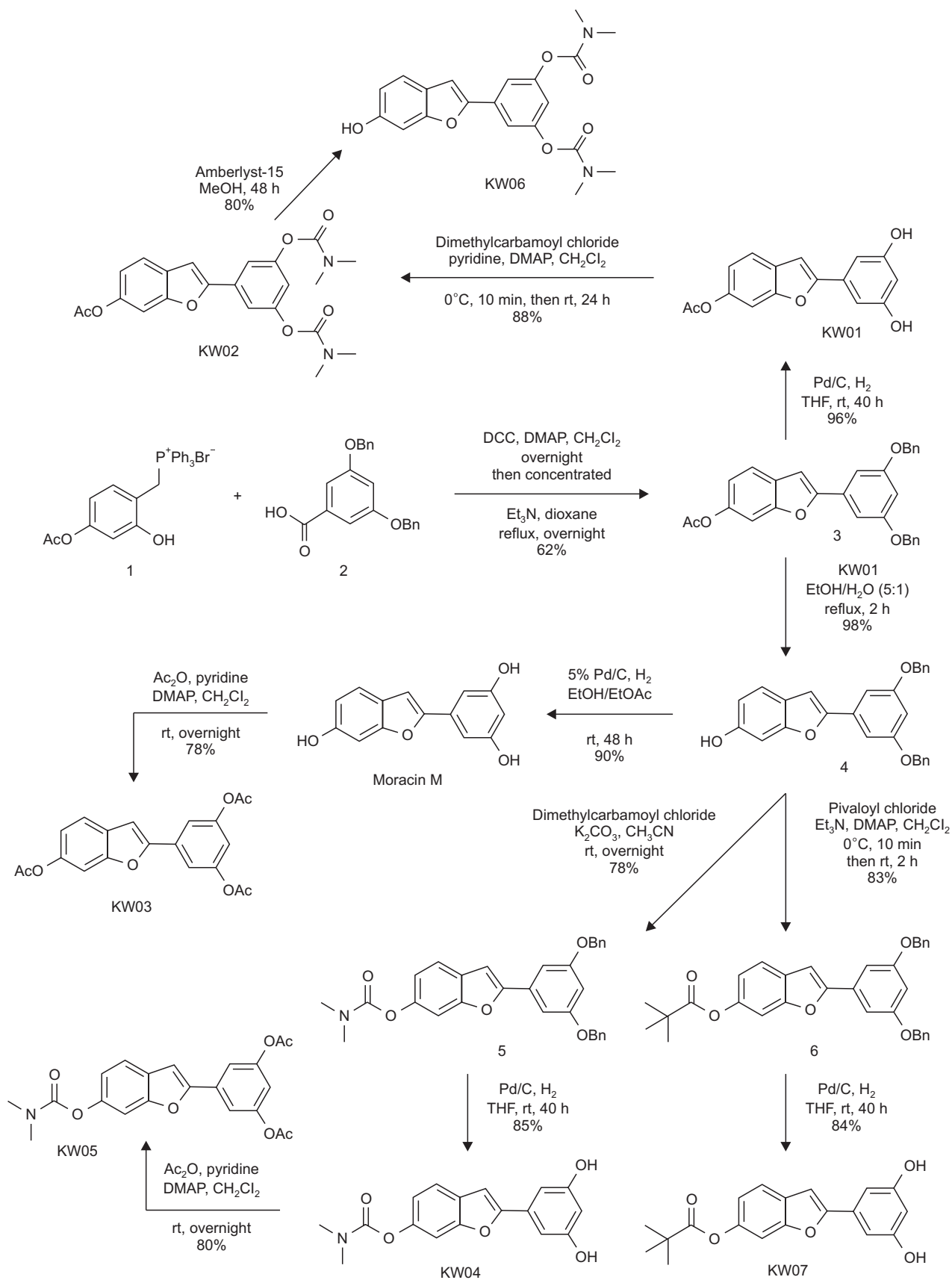
Fig. 1. Synthesis of moracin M and its derivatives KW01-KW07.

mg, 0.02 mmol) and pyridine (166 μL, 2.1 mmol) in anhydrous dichloromethane (3 mL) was added dropwise acetic anhydride (78 μL, 0.83 mmol) at 0°C. The reaction mixture was stirred overnight at room temperature. The mixture was diluted with CH_2Cl_2 (20 mL), and washed with water, 1N HCl and brine. The mixture was dried over anhydrous $MgSO_4$ and concentrated at reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (hexanes/EtOAc, 9:1) to give KW03 (60 mg, 78%) as a white solid: ¹H NMR (300 MHz, CDCl₃) δ 7.54 (d, *J*=8.4 Hz, 1H), 7.45 (d, *J*=1.9 Hz, 2H), 7.27 (s, 1H), 7.03-6.96 (m, 2H), 6.92 (s, 1H), 2.33 (d, *J*=3.2 Hz, 9H); LRMS (ESI) *m/z* calcd. for $C_{20}H_{16}O_7$ ([M+H]⁺) 369.1, found 369.2.

2-(3,5-Bis(benzyloxy)phenyl)benzofuran-6-yl dimethylcarbamate (5): To a mixture of benzofuranol 4 (550 mg, 1.3 mmol) and K2CO3 (360 mg, 2.6 mmol) in anhydrous acetonitrile (10 mL) was slowly added dimethylcarbamoyl chloride (175 mg, 1.6 mmol) at room temperature under a nitrogen atmosphere. The reaction mixture was stirred overnight and concentrated at reduced pressure. The residue was suspended in water (25 mL). The mixture was extracted with CH_2Cl_2 (25 mL×3). The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated at reduced pressure. The residue was purified by flash column chromatography on silica gel (hexanes/EtOAc, 3:2) to afford carbamate 5 (500 mg, 78%) as a white solid: ¹H NMR (300 MHz, CDCl₃) δ 7.50 (d, *J*=8.4 Hz, 1H), 7.48-7.30 (m, 11H), 7.10 (d, *J*=2.3 Hz, 2H), 7.00 (dd, *J*=8.4, 2.1 Hz, 1H), 6.95 (d, *J*=0.9 Hz, 1H), 6.62 (t, *J*=2.2 Hz, 1H), 5.11 (s, 4H), 3.14 (s, 3H), 3.04 (s, 3H); LRMS (ESI) *m/z* calcd. for $C_{31}H_{27}NO_5$ ([M+H]⁺) 494.2, found 494.3.

2-(3,5-Dihydroxyphenyl)benzofuran-6-yl dimethylcarbamate (KW04): To a solution of carbamate 5 (450 mg, 0.91 mmol) in THF (15 mL) was added Pd/C (5% on activated carbon, 50 mg) at room temperature. The mixture was stirred for 40 h at room temperature under a hydrogen atmosphere. The mixture was filtered through a pad of Celite and the filtrate was concentrated at reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (hexanes/EtOAc, 3:2) to give KW04 (215 mg, 85%) as a white solid: ¹H NMR (300 MHz, CD₃OD) δ 7.54 (d, *J*=8.4 Hz, 1H), 7.29 (d, *J*=1.6 Hz, 1H), 7.04 (d, *J*=0.7 Hz, 1H), 6.98 (dd, *J*=8.4, 2.0 Hz, 1H), 6.81 (d, *J*=2.2 Hz, 2H), 6.29 (t, *J*=2.2 Hz, 1H), 3.14 (s, 3H), 3.00 (s, 3H); LRMS (ESI) *m/z* calcd. for $C_{17}H_{15}NO_5$ ([M+H]⁺) 314.1, found 314.1.

5-(6-((Dimethylcarbamoyl)oxy)benzofuran-2-yl)-1,3-phenylene diacetate (KW05): Acetic anhydride (75 μL, 0.80 mmol) was slowly added to a mixture of KW04 (100 mg, 0.32 mmol), DMAP (4 mg, 0.03 mmol) and pyridine (130 μL, 3.2 mmol) in anhydrous CH_2Cl_2 (3 mL) at 0°C. The mixture was stirred overnight at room temperature and diluted with CH_2Cl_2 (20 mL). The mixture was washed with water, 1N HCl and brine, dried over anhydrous $MgSO_4$, and concentrated at reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH, 99:1) to give KW05 (104 mg, 80%) as a white solid: ¹H NMR (300 MHz, CDCl₃) δ 7.52 (d, *J*=8.4 Hz, 1H), 7.45 (d, *J*=2.1 Hz, 2H), 7.29 (d, *J*=1.8 Hz, 1H), 7.02 (dd, *J*=8.2, 1.5 Hz, 2H), 6.91 (t, *J*=2.1 Hz, 1H), 3.14 (s, 3H), 3.04 (s, 3H), 2.33 (s, 6H); LRMS (ESI) *m/z* calcd. for $C_{21}H_{19}NO_7$ ([M+H]⁺) 398.1, found 398.2.

5-(6-Hydroxybenzofuran-2-yl)-1,3-phenylene bis(dimethylcarbamate) (KW06): To a stirred suspension of KW02 (0.90 g, 2.1 mmol) in MeOH (20 mL) was added Amberlyst-15 (450 mg) at room temperature. The mixture was stirred for 48 h at room temperature. The mixture was filtered through a pad of

Celite and washed with MeOH (10 mL×3). The filtrate was concentrated at reduced pressure. The residue was diluted with water. The mixture was extracted with EtOAc (20 mL×3). The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated at reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH, 99:1) to give KW06 (650 mg, 80%) as a white solid: meting point 233-236°C; ¹H NMR (300 MHz, CDCl₃) δ 7.29 (d, *J*=2.0 Hz, 2H), 7.16 (d, *J*=8.4 Hz, 1H), 6.90 (s, 1H), 6.67 (d, *J*=19.4 Hz, 3H), 6.59 (dd, *J*=8.4, 1.9 Hz, 1H), 3.14 (s, 6H), 3.07 (s, 6H); ¹³C NMR (75 MHz, CDCl₃) δ 155.8, 155.0, 154.8, 153.0, 152.1, 132.6, 121.8, 121.0, 115.1, 114.8, 112.5, 102.4, 98.3, 36.9, 36.7; LRMS (ESI) *m/z* calcd. for $C_{20}H_{20}N_2O_6$ ([M+H]⁺) 385.1, found 385.3.

2-(3,5-Bis(benzyloxy)phenyl)benzofuran-6-yl pivalate (6): Pivaloyl chloride (93.0 μL, 0.75 mmol) was added dropwise to a mixture of benzofuranol 4 (265 mg, 0.63 mmol), DMAP (8 mg, 0.06 mmol) and triethylamine (95 mg, 0.94 mmol) in CH_2Cl_2 (20 mL) at 0°C. The mixture was stirred for 2 h at room temperature. Water (5 mL) was added to the reaction mixture. The organic layer was separated and the aqueous phase was extracted with CH_2Cl_2 (10 mL×3). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$ and concentrated at reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (hexanes/EtOAc, 9:1) to afford pivaloate 6 (265 mg, 83%) as a white solid: ¹H NMR (300 MHz, CDCl₃) δ 7.52 (d, *J*=8.4 Hz, 1H), 7.50-7.30 (m, 11H), 7.10 (d, *J*=2.3 Hz, 2H), 6.98-6.91 (m, 2H), 6.63 (t, *J*=2.2 Hz, 1H), 5.11 (s, 4H), 1.39 (s, 9H); LRMS (ESI) *m/z* calcd. for $C_{33}H_{30}O_5$ ([M+H]⁺) 507.2, found 507.2.

2-(3,5-Dihydroxyphenyl)benzofuran-6-yl pivalate (KW07): To a stirred solution of pivalate 6 (220 mg, 0.43 mmol) in THF (15 mL) was added Pd/C (5% on activated carbon, 50 mg) at room temperature. The mixture was stirred for 40 h at room temperature under a hydrogen atmosphere. The mixture was filtered through a pad of Celite and the filtrate was concentrated at reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (hexanes/EtOAc, 1:1) to give KW07 (120 mg, 84%) as an off-white solid: ¹H NMR (300 MHz, CD₃OD) δ 7.55 (d, *J*=8.4 Hz, 1H), 7.25 (d, *J*=1.7 Hz, 1H), 7.04 (d, *J*=0.8 Hz, 1H), 6.92 (dd, *J*=8.4, 2.0 Hz, 1H), 6.82 (d, *J*=2.2 Hz, 2H), 6.30 (t, *J*=2.2 Hz, 1H), 1.37 (s, 9H); LRMS (ESI) *m/z* calcd. for $C_{19}H_{18}O_5$ ([M+H]⁺) 327.1, found 327.4.

IL-1β-induced IL-6 production in human lung epithelial cell line, A549

A549 cells were purchased from American Type Cell Culture (ATCC, Rockville, VA, USA). Cells were maintained in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in a 5% CO_2 atmosphere at 37°C. Cells were pre-incubated for 24 h. IL-1β (10 ng/mL) and test compounds were added simultaneously. Four hours later, media was collected and the concentration of IL-6 in the media was determined using an ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's recommendation. Cell viability was examined using MTT assay as described previously (Mosmann, 1983). Test compounds including reference agents were dissolved in DMSO and diluted with complete RPMI media. The final concentration of DMSO in the cell culture was 0.1% (v/v). This concentration of DMSO did not affect the cell viability or IL-6

LPS-induced airway inflammation in mice

To elucidate inhibitory activities of the synthesized derivatives against lung inflammation, mice were divided into the following groups: control, LPS-treated, LPS/derivatives (30 mg/kg)-treated, and LPS/dexamethasone (30 mg/kg)-treated (n=7). Test compounds were administered orally. One hour later, LPS (*E. coli* 0127:B8, 2 mg/mL saline) was administered intranasally to mice (10 μL/mouse, 5 times) to induce bronchitis according to previously published method (Lim *et al.*, 2013). At 16 h after LPS treatment, mice were sacrificed and bronchoalveolar lavage fluid (BALF) was collected via intratracheal cannulation after 700 μL infusion of saline three times. BALFs collected were approximately 2,000 μL/mouse. Total cell number in BALF was counted with a haemocytometer.

KW02 was selected for further study. To examine the dose-dependency on the same animal model, KW02 (1, 3, 10 mg/kg) was orally administered to mice (n=12). One hour later, LPS was administered. At 16 h after LPS treatment, mice (n=7) were sacrificed and BALF was obtained as mentioned above. From the BALF, total cells were counted with a haemocytometer and cells were differentially counted with FACS (BD bioscience). For histology, the remaining mice (n=2) were sacrificed and lung tissues were excised. Histological samples were prepared by fixing and H&E staining. To check proinflammatory cytokine production, mice (n=3) were sacrificed at 6 h after LPS treatment and BALF was obtained. From the BALF, concentrations of IL-1β were determined with ELISA kit (BD biosciences).

Test compounds including reference drug were dissolved in 0.3% carboxymethylcellulose (CMC) and orally administered. Control and LPS treatment groups also received the same amounts of CMC solution.

Microsomal stability assay

Rat liver microsome (RLM; Corning Gentest; Tweksbury, MA, USA) was thawed and diluted with potassium phosphate buffer (100 mM, pH 7.4, final concentration) that contained an NADPH regenerating system (Corning Gentest) with moracin M, KW02, or KW06 (1.0 μM, final concentration). Reaction mixtures were shaken at 37°C for 30 min. These reaction mixtures (50 μL) were sampled at 0 and 30 min. Reactions were stopped with ice cold acetonitrile (150 μL). These mixtures were centrifuged at 13,000 rpm for 5 min at 4°C. After separation of the supernatant, 5 μL aliquots were injected into an LC-MS/MS instrument.

Pharmacokinetic study

Rats were anesthetized with an intramuscular injection of Rompun® (10 mg/kg) and Zoletil® (50 mg/kg). They were catheterized with polyethylene tubing (PE-50; Intramedic, Franklin Lakes, NJ, USA) which was filled with saline containing heparin (20 I.U./mL) in the femoral vein. Rats were housed individually and fasted for 12 h before dosing. Moracin M, KW02, and KW06 dosing solutions (2.5 mg/1.0 mL) were prepared in a vehicle consisting of 5% dimethyl sulfoxide, 40% polyethylene glycol 400, and 55% injection water. Rats were administered with moracin M, KW02, or KW06 dosing solution intravenously (5 mg/kg, n=3) and orally (5 mg/kg, n=3). Blood samples (approximately 150 μL at each time point) from the femoral vein were collected at 2, 10, 30 min, and 1, 2, 4, 6, 9, and 24 h after intravenous administration, or at 15 and 30 min, and 1, 2, 4, 6, 9 and 24 h after oral administration. Plasma was immediately separated by centrifugation at 3,000 rpm for 5 min and stored at –80°C until LC-MS/MS analysis.

LC-MS/MS analysis

LC-MS/MS system is composed of Agilent 1260 Infinity HPLC system and tandem quadrupole mass spectrometer (Agilent 6460A QQQ LC-MS/MS; Agilent Inc., Lexington, MA, USA). Chromatographic separations were conducted on a reversed phase column (Gemini 3 μM 110A, 3 μm, 50×2.0 mm i.d.; Phenomenex, Torrance, CA, USA). The mobile phase (acetonitrile:water=80:20, v/v, containing 0.1% formic acid) had a flow rate of 0.3 mL/min. Temperatures of the column and autosampler tray were maintained at 40°C and 4°C, respectively. Moracin M, KW02, and KW06 concentrations in both RLM incubation samples and blood samples were determined with the LC-MS/MS system using electrospray ionization (ESI) in positive ion mode. Nebulizer pressure, sheath gas temperature, desolvation gas temperature, and desolvation gas flow rate were set at 15 psi, 400°C, 350°C, and 8 l/min, respectively. Data were analyzed with MassHunter software (Agilent Inc.).

Statistical analysis

Experimental values were represented as arithmetic mean ± SD. One way ANOVA followed by Dunnett's test was used to determine the statistical significance.

RESULTS

Synthesis of moracin M and its analogues

Although the previously reported synthesis of moracin M was concise, its overall yield was not high (Arias *et al.*, 2012). We thus developed a new synthetic route that could provide moracin M and its derivatives (KW01-07) in gram quantities by modifying the previously reported synthesis of a structurally related natural product, moracin C (McAllister *et al.*, 1998). Coupling of readily available phosphonium salt 1 and commercially available benzoic acid 2 followed by cyclization according to McKittrick and Stevenson's protocol in one pot generated benzofuran 3 in 62% yield as shown in Fig. 1 (McKittrick and Stevenson, 1984). Removal of an acetyl group in benzofuran 3 by hydrolysis with KOH produced benzofuranol 4 in 98% yield. Dibenzyl ether 4 was hydrogenolyzed to afford moracin M with Pd/C under a hydrogen atmosphere in 90% yield. With these moracin M and intermediates 3 and 4 in hand, we designed and synthesized several analogues of moracin M that might be more potent and/or work as moracin M prodrugs.

Removal of dibenzyl groups in benzofuran 3 by hydrogenolysis gave KW01 (96%), of which dicarbamoylation produced KW02 in 88% yield. KW02 was selectively hydrolyzed to KW06 in 80% yield. Benzofuranol 4 was carbamoylated to dibenzyl ether 5 which was debenzylated to KW04 (66% for two steps). KW04 was acetylated to KW05 in 80% yield. Acylation of benzofuranol 4 with pivaloyl chloride gave pivaloate 6, from which benzyl groups were removed by hydrogenolysis to afford KW07 (70% for two steps). Acetylation and carbamoylation of moracin M produced KW03 in 78% yield.
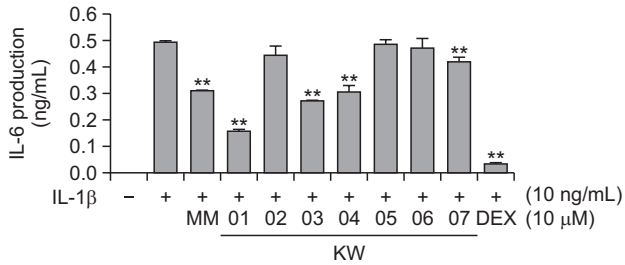
Fig. 2. Effects of moracin M derivatives on IL-1β-induced IL-6 production in A549 cells. All derivatives including dexamethasone were treated at 10 μM. MM, moracin M; DEX, dexamethasone. **p<0.01, Significantly different from the LPS-treated control group.

Table 1. Inhibition of moracin M derivatives on lung inflammation in ALI mice

Compounds	Dose (mg/kg)[a]	% inhibition[b]
Experiment 1		
Moracin M	30	70.5 ± 55.1*
KW01	30	64.9 ± 45.3*
KW02	30	100.0 ± 21.6**
KW03	30	75.0 ± 38.9*
KW04	30	61.8 ± 62.6[+]
Dexamethasone	30	76.8 ± 20.4*
Experiment 2		
Moracin M	30	70.6 ± 17.2*
KW05	30	70.2 ± 23.2*
KW06	30	95.9 ± 14.7**
KW07	30	74.4 ± 10.0*
Dexamethasone	30	77.0 ± 13.5[+]

[a]All compounds were orally administered, [b]% inhibition was calculated based on the total cell numbers in the BALF. The total cell numbers in the BALF of the LPS-treated group were 7.1×10^5 cells/lung and the basal level was 9.8×10^4 cells/lung (n=7). The values are represented as an arithmetic mean ± standard deviation.
[+]p<0.1, *p<0.05, **p<0.01, Significantly different from the LPS-treated control group.

Effects of moracin M derivatives on IL-1β-induced IL-6 production in A549 cells

IL-1β treatment on A549 cells, a human lung epithelial cell line, produced high amounts of proinflammatory IL-6 (0.5 ± 0.0 ng/mL) after 4 h of incubation (n=3) (Fig. 2). Under this condition, KW01-KW07 were treated at 10 μM and IL-6 concentration in the media was measured. Dexamethasone used as a reference agent showed strong inhibition at 10 μM. The ideal prodrugs are not likely to be metabolized in this cell culture. They are expected to be inactive or weakly active. However, they might possess strong *in vivo* activity. As expected, KW02 and KW06 (the dicarbamoylated derivatives at the phenyl ring in moracin M) as well as KW05 failed to inhibit IL-6 production. This finding suggests that KW02, KW06, and KW05 might be prodrug forms of moracin M if they show strong inflammatory action *in vivo*. On the other hand, moracin M (parent compound) and other derivatives (KW01, KW03, KW04, and KW07) significantly inhibited IL-6 production in cultured A549 cells. These moracin M derivatives should be active per se and/or transformed into active metabolites in this condition. KW01, KW03, KW04, and KW07 are not likely prodrugs of moracin M.

Effects of moracin M derivatives (KW01-07) on LPS-induced acute lung injury

KW01-07 including moracin M were orally administered to mice at 30 mg/kg initially and LPS was administered intranasally. Sixteen hours later, mice were sacrificed and BALFs were obtained. In the present study, when 50 μg LPS/mouse was administered, total cell numbers in the BALF as an index of inflammatory response of lung tissues were significantly increased (approximately 5.9-fold). Table 1 demonstrates the inhibitory action of these synthetic derivatives on total cell numbers recruited in the BALF as an index of lung inflammatory response. All derivatives showed significant inhibitory action on lung inflammation. Specifically, KW02 and KW06 possessed potent activities. They showed higher inhibitory activities than parent molecule moracin M and reference drug dexamethasone at the same dose. Considering the inactive nature of KW02 and KW06 in the above cell culture study, these findings indicate that KW02 and KW06 behave as prodrugs of moracin M.

Dose-dependent inhibition of moracin M derivative (KW02) on LPS-induced airway inflammation in mice

The most potent derivative (KW02) was selected for further

dose-dependency study. When KW02 was orally administered (1-10 mg/kg), numbers of total cells in the BALF were significantly reduced. KW02 showed a dose-dependent inhibition (31.6%, 52.1%, and 75.4% inhibition at 1, 3, and 10 mg/kg, respectively) while dexamethasone, a potent anti-inflammatory steroid used as reference drug, showed 88.7% inhibition at 30 mg/kg (Fig. 3A). FACS analysis also revealed that numbers of neutrophils and macrophages in the BALF were reduced by KW02. In addition, these inhibitory actions by KW02 were confirmed by histological comparison. As shown in Fig. 3B and 3C, KW02 (10 mg/kg) treatment almost completely reversed alveolar cell hyperplasia and infiltration of inflammatory cells in the lung lesion induced by LPS. When IL-1β concentration from the BALF was measured, KW02 significantly inhibited proinflammatory IL-1β production in inflamed lungs at 10 mg/kg (86.0% inhibition). In comparison, dexamethasone reduced IL-1β concentration by 70.7% at 30 mg/kg. All these results indicate that the activity of KW02 is more potent than dexamethasone against lung inflammation in mice by oral administration.

Microsomal stability of moracin M, KW02, and KW06

In vitro microsomal metabolic stabilities of moracin M, KW02, and KW06 were evaluated using rat liver microsomes (RLMs) and human liver microsomes (HLMs) and summarized in Table 2. Moracin M was highly stable compared to KW02 and KW06 after 30 min incubation in RLMs (remaining amount, 54%) and HLMs (remaining amount, 74%). In contrast, metabolic stabilities of KW02 were very poor and there was little remaining amount after 30 minutes incubation in RLMs and HLMs. In case of KW06, it was metabolically unstable (remaining amount, <2%) in RLMs, but stable (remaining amount, 53%) in HLMs after 30 min incubation. To determine the possibility of KW02 and KW06 as moracin M prodrugs, conversions to KW06 and moracin M from KW02 were investigated. The conversion ratios to moracin M and
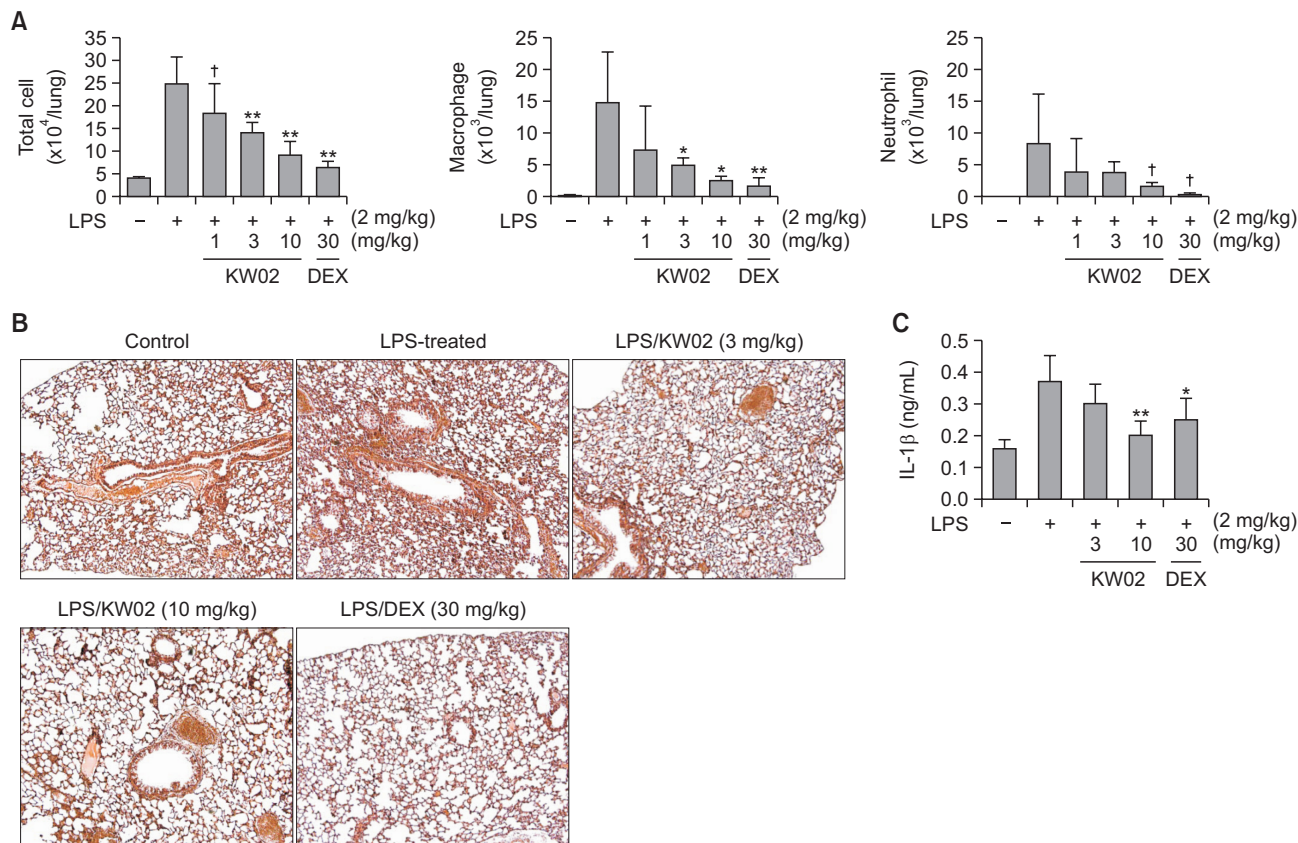
A

B

C

Fig. 3. Dose-dependent inhibition of KW02 in LPS-treated acute lung injury in mice. LPS was intranasally treated to induce airway inflammation. Sixteen hours later, mice were sacrificed and BALF was obtained. All compounds were orally administered 1 h prior to LPS treatment. (A) The numbers of total cells and inflammatory cells in the BALF, Total cells were counted by haemocytometer and each cell type was differentially counted by FACS (n=7), (B) Effects on IL-1β production, From the BALF obtained at 6 h after LPS treatment, IL-1β concentration was measured using ELISA (n=3). DEX, dexamethasone. $^{†}p<0.1$, $^{*}p<0.05$, $^{**}p<0.01$, Significantly different from the LPS-treated control group. (C) Histological observation (×100), Represented here is one of two samples in each group.

Table 2. Microsomal stability of moracin M, KW02 and KW06

	Rat (nM)		Human (nM)	
	0 min	30 min	0 min	30 min
Moracin M	1,726 ± 209	928 ± 130**	1,535 ± 78	1,143 ± 140*
KW02	1,132 ± 638	0.0 ± 0***	1,245 ± 132	0.1 ± 0.2***
KW02→KW06	4.7 ± 0.8	7.0 ± 0.9*	4.2 ± 0.3	342 ± 98.9**
KW02→Moracin M	ND	25.4 ± 3.2***	ND	ND
KW06	625 ± 194	7.6 ± 0.9**	645 ± 78.4	340 ± 69.6**
KW06→Moracin M	ND	32.2 ± 1.2***	ND	ND

*$p<0.05$, **$p<0.01$, ***$p<0.001$, Significantly different from 0 min group. n=3, ND, not detected.

KW06 from KW02 were very low (<1%) in RLMs after 30 min incubation. However, the generation of moracin M (~5%) was observed after incubating KW06 in RLM solutions and conversion to KW06 from KW02 was observed over 30% after 30 min incubation of KW02. Conversion to moracin M from KW02 or KW06 was observed at lower levels in RLMs but not detected in HLMs because of the detection limit of LC-MS/MS analytical method.

Preliminary pharmacokinetic profiling for moracin M, KW02, and KW06 in rats

Pharmacokinetic profiles of moracin M, KW02 and KW06 were determined in rats. Plasma concentration-time curves of KW06 were plotted at 2, 10, 30 min, and 1, 2, 4, 6, 9, and 24 h after intravenous administration of KW02 and KW06 (5 mg/kg, n=3), and at 15 and 30 min, and 1, 2, 4, 6, 9 and 24 h after oral administration of KW02 and KW06 (5 mg/kg, n=3) in Fig. 4. Moracin M showed very short half-life ($t_{1/2}$, 0.08 h)
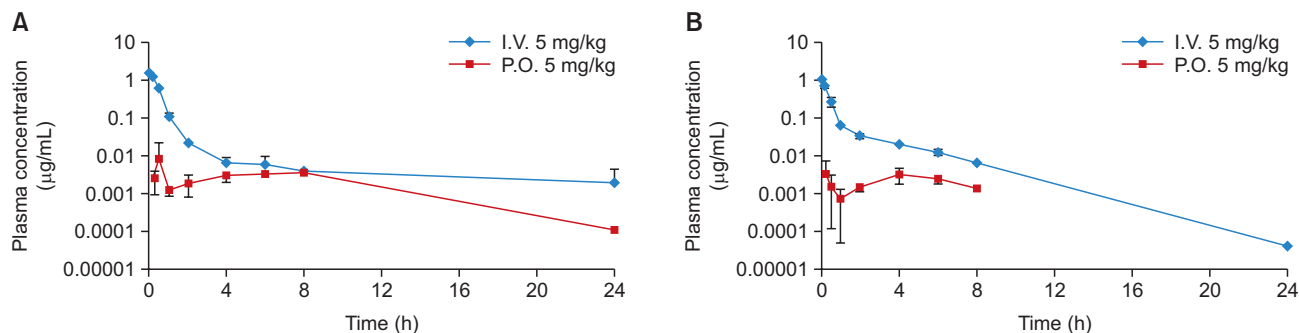
Fig. 4. Plasma concentration-time curves of KW06 after intravenous and oral administration of KW02 (A) and KW06 (B) (5 mg/kg, n=3).

Table 3. Pharmacokinetic parameters after intravenous administration of moracin M (5 mg/kg) in male rats

Pharmacokinetic parameters	Values
$t_{1/2}$ (h)	0.0781 ± 0.0084
AUC_{30min} (μg·h/mL)	0.922 ± 0.0356
AUC_{∞} (μg·h/mL)	0.932 ± 0.041
CL (L/h/kg)	5370 ± 229
V_{ss} (L/kg)	0.393 ± 0.056

$t_{1/2}$, terminal half-life; AUC_{30min}, area under the plasma concentration-time curve from time zero to 30 min; AUC_{∞}, area under the plasma concentration-time curve from time zero to infinity; CL, elimination clearance; V_{ss}, volume of distribution at steady state, Data represent arithmetic mean ± SD (n=3).

Table 4. Pharmacokinetic parameters for KW06 after administration of KW02 in male rats

Pharmacokinetic parameters	i.v. (5 mg/kg)	p.o. (5 mg/kg)
T_{max} (h)	-	1.58 ± 2.1
C_{max} (μg/mL)	-	0.0109 ± 0.0115
		(0.0049-0.0223)
$t_{1/2}$ (h)	2.57 ± 1.44	13.8 ± 10.7
		(8-24)
AUC_{24h} (μg·h/mL)	0.909 ± 0.127	0.0337 ± 0.11
AUC_{∞} (μg·h/mL)	0.917 ± 0.132	0.11 ± 0.0599
CL (L/h/kg)	5.53 ± 0.823	
V_{ss} (L/kg)	6.01 ± 3.33	
F (%)		3.7

T_{max}, the time to reach maximum plasma concentration; C_{max}, the peak plasma concentration; $t_{1/2}$, terminal half-life; AUC_{24h}, area under the plasma concentration–time curve from time zero to 24 h; AUC_{∞}, area under the plasma concentration–time curve from time zero to infinity; CL, elimination clearance; V_{ss}, volume of distribution at steady state; F, bioavailability. Data represent arithmetic mean ± SD (n=3).

after intravenous administration (5 mg/kg, Table 3). All of detection limits for moracin M, KW02, and KW06 using LC-MS/MS analysis were 0.5 ng/mL. However, we could not detect moracin M in blood after oral administration probably due to its lower concentration than the limit of detection value and/

Table 5. Pharmacokinetic parameters for KW06 after administration of KW06 in male rats

Pharmacokinetic parameters	i.v. (5 mg/kg)	p.o. (5 mg/kg)
T_{max} (h)	-	1.58 ± 2.09
C_{max} (μg/mL)	-	0.00557 ± 0.00257
		(0.0032-0.0087)
$t_{1/2}$ (h)	2.33 ± 0.37	4.15 ± 1.72
		(2-6)
AUC_{24h} (μg·h/mL)	0.589 ± 0.0926	0.0177 ± 0.00285
AUC_{∞} (μg·h/mL)	0.604 ± 0.0864	0.0262 ± 0.0014
CL (L/h/kg)	8.39 ± 1.27	
V_{ss} (L/kg)	13 ± 1.23	
F (%)		3.01

T_{max}, the time to reach maximum plasma concentration; C_{max}, the peak plasma concentration; $t_{1/2}$, terminal half-life; AUC_{24h}, area under the plasma concentration–time curve from time zero to 24 h; AUC_{∞}, area under the plasma concentration–time curve from time zero to infinity; CL, elimination clearance; V_{ss}, volume of distribution at steady state; F, bioavailability, Data represent arithmetic mean ± SD (n=3).

or low absorption caused by first-pass metabolism. Although KW02 was not detected in blood samples after intravenous and oral administration of KW02, generation of KW06 was observed as shown in Table 4. KW06 showed longer half-life ($t_{1/2}$, 2.3 h) than moracin M (Table 5). We attempted to detect moracin M in rat plasma samples after administration of KW02 and KW06, but the generation of moracin M could not be confirmed probably due to its lower concentration than the limit of detection value and/or its fast metabolism. Interestingly, the AUC of KW06 was improved around 50% after intravenous administration of KW02 instead of KW06.

DISCUSSION

The present investigation clearly revealed that all tested moracin M derivatives showed significant inhibitory action against lung inflammation *in vivo*. Particularly, KW02 (1-10 mg/kg) possessed potent anti-inflammatory activity by oral administration against lung inflammation compared to its parent molecule moracin M and reference drug dexamethasone.

Under the same experimental condition, moracin M showed much less activity at 20-60 mg/kg (Lee *et al*., 2016). Dexamethasone at three-fold higher doses (3-30 mg/kg) showed a similar inhibitory action when total cell numbers in BALF were compared (Lee *et al*., 2018). These findings clearly showed that KW02 possessed significantly stronger potency than its parent molecule moracin M and steroid anti-inflammatory drug dexamethasone. Given that steroids show some serious adverse effects after prolonged use, KW02 may possess some advantage over steroids as a therapeutic agent on lung inflammatory disorders such as COPD.

As an animal model of airway inflammation, LPS-induced acute lung injury model was employed in the present investigation. It is a frequently used model for establishing anti-bronchitis activities of various compounds (Chapman *et al*., 2007). Intranasal treatment of LPS to ICR mice provokes inflammatory responses including inflammatory cell recruitment, elevated levels of proinflammatory cytokines, and histologic changes in the lung tissue (Chapman *et al*., 2007; Lim *et al*., 2013). KW02 dose-dependently inhibited all these parameters after oral administration.

In this study, we designed and synthesized several potential prodrugs of moracin M. Acetate and carbamate groups are frequently employed to protect first pass metabolism (especially phase II metabolism) of drugs bearing a phenolic hydroxyl group(s) (Tunek *et al*., 1988; Ghosh and Brindisi, 2015). These groups are quickly eliminated in the liver and plasma. Although moracin M was not detected after its oral administration in rats, the compound showed moderate anti-inflammatory activity in an LPS-induced airway inflammation mouse model. The results imply that an active metabolite(s) of moracin M is generated in mice. In our preliminary *in vivo* pharmacokinetic experiments, we could not also observe the generation of moracin M after both oral and intravenous administration of KW02 and KW06, although KW06 was produced after administration of KW02. This result might be attributed to low detection limit of moracin M and/or its fast metabolism. The metabolic formation of both KW06 from KW02 and moracin M from KW06 was proven by performing microsomal stability experiments. In addition, the remaining amount of KW06 or biotransformation of KW02 into KW06 are higher in HLM than in RLM. These can be expected to be more positively effective against LPS-induced lung inflammation in humans than in rodents. The results also showed that KW02 may be metabolized to other metabolites as well as KW06, and KW06 may also be biotransformed into other metabolites including moracin M. Therefore, further study may be needed to determine which metabolic enzymes or biological systems are involved in this metabolic process.

The prodrug nature of KW02 and KW06 was also revealed by findings that they did not exhibit inhibitory action on IL-1β-treated IL-6 production in A549 cells, although they exerted strong inhibition against LPS-induced lung inflammation by oral administration to mice. The longer duration of KW06 (after oral administration of KW02 or KW06) than moracin M in blood and higher bioavailability may explain the stronger potencies of KW02 and KW06 than moracin M. Moreover, more potent anti-inflammatory activity of KW02 than KW06 might be attributed to the fact that larger AUC of KW06 was achieved after oral administration of KW02. All of these results strongly suggest that KW02 and KW06 should act as prodrugs of moracin M and/or its active metabolite(s).

In conclusion, for the purpose of obtaining highly active oral inhibitor of lung inflammation, a number of moracin M derivatives were newly synthesized. They showed inhibitory activities against lung inflammation in an animal model. Particularly, KW02 possessed strong inhibitory activity against LPS-induced lung inflammation by oral administration. This derivative as a prodrug form may have therapeutic potential for treating lung inflammatory disorders. Further extensive pharmacokinetic and metabolism study to prove complete metabolism of KW02 and moracin M will be performed and the results will be reported in due course.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

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