Phytochemistry Letters 25 (2018) 118–121

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Chemical structures and antimutagenic effects of unusual oximes from the peels of *Citrus limon*

Takahiro Matsumoto, Maoko Koike, Chika Arai, Takahiro Kitagawa, Eriko Inoue, Daisuke Imahori, Tetsushi Watanabe*

Kyoto Pharmaceutical University, Misasagi-Shichono-cho 1, Yamashina-ku, Kyoto 607-8412, Japan

ARTICLE INFO

Keywords:
Citrus limon
Limonoxime
Mesaconic acid
Antimutagenic effect

1. Introduction

Citrus fruits were reported to contain many antioxidants, antimutagens, anticarcinogens, and antiallergic agents, such as flavonoids, furanocoumarins, and limonoids (Bruno et al., 2014) (Campelo et al., 2011). Among the *Citrus* fruits, lemons (*Citrus limon*) have been used for centuries in a variety of applications including medicine, perfumery, and the food industry. The peels of a lemon, used in beverage and cooking applications, can provide a bright, citrusy flavor without the sourness (Cannon et al., 2015). In the course of our study to determine the antimutagenic compounds derived from foods (Matsumoto et al., 2017a) (Matsumoto et al., 2017b), we previously reported the structures of four new coumarins, wakayamalimonol A–D and a new furanocoumarin, wakayamalimonol E isolated from the peels of *Citrus limon* (L.) Burm.f. together with their antimutagenic effects (Matsumoto et al., 2017c). As a continuing study, we isolated two new oximes, named limonoxime I (**1**) and II (**2**), from the peels of *Citrus limon* (L.) Burm.f. The chemical structures of new compounds were elucidated on the basis of chemical and physicochemical evidence such as NMR, HR-EIMS, and UV spectra and chemical synthesis. In addition, there are only a few reports of oxime moiety compounds isolated from natural plants, which encourage us to investigate the antimutagenic effects of isolated new oximes, limonoxime I (**1**). The in antimutagenic test (Ames test) of limonoxime I (**1**) and its derivatives showed antimutagenic effects against Trp-P-1 and PhIP. On the other hand, limonoximes I did not show antimutagenic effects against BaP.

The methanolic extract of the fresh peels of *C. limon* was partitioned into an EtOAc-H_2O (1:1, v/v) mixture to furnish an EtOAc-soluble fraction and an aqueous layer as described previously (Matsumoto et al., 2017c). The EtOAc-soluble fraction was subjected to normal- and reversed-phase gel column chromatography and finally HPLC to isolate two new oximes named limonoximes I (**1**, 0.000017%) and II (**2**, 0.00001%).

2. Results and discussion

Limonoxime I (**1**) was isolated as an amorphous powder. In the EI-MS of **1**, a molecular ion peak $[M]^+$ was observed at *m/z* 249 and the molecular formula $C_{13}H_{15}NO_4$ was determined by HRMS measurement of the molecular ion peak. The ^1H NMR ($CDCl_3$) and ^{13}C NMR (Table 1) spectra of **1**, which were assigned by various NMR experiments, showed signals assignable to 4-substituted phenyl group [δ_H 8.08 (d, J = 8.9 Hz, H-2, 6), 6.95 (d, J = 8.9 Hz, H-3, 5)], carbonyl group [δ_C 186.6 (C-7)], oxime group [$\delta_{C/H}$ 148.9 (C-8), 1.39 (s, H-8)], and 2-methyl-but-2-ene-1,4-diol group [δ_H 4.68 (d, J = 6.2 Hz, H-1′), δ_H 5.78 (t, J = 6.2 Hz, H-2′), δ_H 4.10 (s, H-4′), δ_H 1.78 (s, H-5′)]. These ^1H and ^{13}C NMR spectra of **1** closely resembled those of 4-(3-methyl-2-butenoxy)-isonitrosoacetophenone except for 3′, 4′, and 5′ position (Ito et al., 1990) (Dubery et al., 1988). Therefore, the planar structure of **1** was determined as 4-(4-hydroxy-3-methyl-2-butenoxy)-isonitrosoacetophenone. In previous report, in the crystal structure of 4-(3-methyl-2-butenoxy)-isonitrosoacetophenone, the aldoxime moiety had the *E* configulation and hydrogen bonding occurs in infinite chains between the hydroxyl hydrogen atom and the carbonyl oxygen atom of a neighbouring molecule (Dubery et al., 1988). The similarity of ^1H and ^{13}C NMR spectra suggested that the geometry of **1** at aldoxime moiety is *E* configuration. As shown in Fig. 2, the DQF COSY and HMBC experiments suggest the planar structure of **1**. The geometry of **1** at 2-

* Corresponding author.
E-mail address: watanabe@mb.kyoto-phu.ac.jp (T. Watanabe).

https://doi.org/10.1016/j.phytol.2018.04.016
Received 17 January 2018; Received in revised form 15 March 2018; Accepted 3 April 2018

Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data for **1** and **2**.

Position	**1**		**2**	
	δ C	δ H (*J* in Hz)	δ C	δ H (*J* in Hz)
1	128.8		128.8	
2	132.5	8.08 (d, 8.9)	132.4	8.08 (d, 8.9)
3	114.5	6.95 (d, 8.9)	114.4	6.94 (d, 8.9)
4	163.2		163.2	
5	114.5	6.95 (d, 8.9)	114.4	6.94 (d, 8.9)
6	132.5	8.08 (d, 8.9)	132.4	8.08 (d, 8.9)
7	186.6		186.5	
8	148.9	8.03 (s)	149.0	8.02 (s)
1′	64.7	4.68 (br-s)	64.6	4.66 (d, 6.1)
2′	119.0	5.78 (t, 6.1)	121.6	5.77 (t, 6.1)
3′	140.8		136.2	
4′	67.7	4.10 (s)	67.5	4.56 (s)
5′	14.2	1.78 (s)	14.5	1.79 (s)
1”			166.2	
2”			115.6	5.73 (s)
3”			157.6	
4”			20.3	2.18 (s)
5”			27.5	1.91 (s)

Measured in $CDCl_3$.

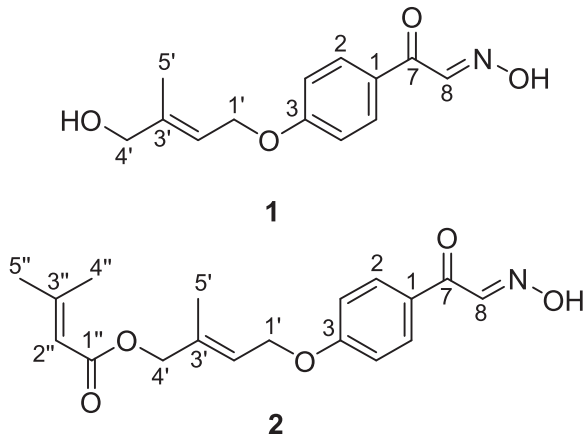

Fig. 1. Chemical structures of **1** and **2.**

methyl-but-2-ene-1,4-diol group was determined as *E* configuration by the NOESY experiment. Correlations were observed between H-1′ and H-5′, H-2′ and H-4′. For identification of the chemical structure and bioassay, limonoxime I (**1**) was synthesized from mesaconicacid. Specifically, mesaconicacid was dissolved in MeOH and treated with sulfuric acid followed by refluxed 6 h to give mesaconic acid dimethyl ester, which upon treatment with DIBAL-H in DMF afforded **1a**. Treatment of **1a** with NaH and 4′-fluoroacetophenone followed by HPLC separation gave **1b**. Which was treated with NaH and *tert*-butylnitrite to give **1** (Fig. 2). The ^{1}H NMR and ^{13}C NMR spectra and the result of HR-EIMS measurement of synthesized compound **1** was identical with isolated limonoxime I (**1**). On the basis of all this evidence, the chemical structure of limonoxime I (**1**) was determined to be shown (Fig. 1).

Limonoxime II (**2**) was isolated as an amorphous powder. In the EI-MS of **2**, a molecular ion peak $[M]^{+}$ was observed at *m/z* 331 and the molecular formula $C_{18}H_{21}NO_5$ was determined by HRMS measurement of the molecular ion peak. The ^{1}H NMR ($CDCl_3$) and ^{13}C NMR (Table 1) spectra of **2**, which were assigned by various NMR experiments, showed signals assignable to 4-(4-hydroxy-3-methyl-2-butenoxy)-isonitrosoacetophenone moiety same as **1** and 3-methylcrotonicacid moiety [δ_H 5.73 (s, H-2”), δ_H 2.18 (s, H-4”), δ_H 1.91 (s, H-5”)]. As shown in Fig. 2, the 2D-NMR experiments of **2** indicated that the 3-methylcrotonicacid moiety was attached to 4′ position. Namely, correlations were observed between H-5′ and C-1”. On the basis of all this evidence, the chemical structure of limonoxime II (**2**) was determined to be shown (Fig. 1).

In previous report, oxime derivative in *Citrus* peel had been expected to have a specific function in the oxidative stress metabolism of the plant tissue (Dubery et al., 1988). In the course of our study to find new compounds that have cancer prevention effects, antimutagenic activities of isolated limonoxime I (**1**), methaconic acid and intermediates (**1a** and **1b**) for the synthesis were evaluated by *in vitro* test against Trp-P-1, PhIP, and BaP. As a result, limonoxime I (**1**), and intermediate (**1b**) showed antimutagenic effects against Trp-P-1 (Table 2) and PhIP (Table 3) without antimicrobial activity at tested concentrations. On the other hand, methaconic acid and intermediate (**1a**) did not show antimutagenic effects against Trp-P-1 (Table 2) and PhIP (Table 3). These results suggest that the aryl moiety is necessary for antimutagenic effects on these compounds. In addition, all tested compounds did not show effects against BaP (data not shown).

3. Conclusion

Two new oximes, limonoximes I (**1**) and II (**2**) were isolated from the peels of *Citrus limon*. To investigate the antimutagenic effects of isolated new oximes, limonoximes I (**1**) was synthesized from mesaconic acid. As the results of antimutagenic test, limonoximes I (**1**) and its derivatives showed antimutagenic effects against Trp-P-1 and PhIP. On the other hand, limonoximes I did not show antimutagenic effects against BaP. Results suggests that the isolated new compounds have a potency of antimutagen against heterocyclic amines.

4. Experimental section

4.1. General experimental procedures

The following instruments were used to obtain physical data: specific rotations, a Horiba SEPA-300 digital polarimeter (l = 5 cm); EIMS and HREIMS, a JEOL JMS-GCmateII mass spectrometer; ^{1}H NMR spectra and ^{13}C NMR spectra, JEOL ECS400 (400 MHz) and JNM-ECA 600 K (600 MHz) spectrometer; 2D-NMR spectra, JEOL JNM-ECA 600 K (600 MHz) spectrometer; HPLC, SPD-10A*vp* fitted with UV–vis detectors. COSMOSIL 5C18-MS-II (250 × 4.6 mm i.d. and 250 × 20 mm i.d.) columns were used for analytical and preparative purposes. The following experimental conditions were used for chromatography: normal-phase silica gel column chromatography (CC), silica gel 60-N (Kanto Chemical Co., Inc., 163–210 μm); reversed-phase silica gel CC, Cosmosil 140C_{18}-OPN (Nacalai Tesque Inc., 75–140 μm); TLC, precoated TLC plates with Silica gel 60F_{254} (Merck, Darmstadt, Germany; 0.25 mm) (ordinary phase) and Silica gel RP–18 F_{254S} (Merck, 0.25 mm) (reverse phase); reversed-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm). Detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

4.2. Plant material

The fruit of *C.limon* (Eureka varieties), which was cultivated in Wakayama prefecture, Japan, were purchased from Kannonyama fruit garden (Wakayama, Japan) in 2016.

4.3. Extraction and isolation

The fresh peels of *C. limon* (2.1 kg) were extracted three times with methanol under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a MeOH extract (2059.1 g, 9.8%). The MeOH extract was partitioned into an EtOAc-H_2O (1:1, v/v) mixture to furnish an EtOAc- soluble fraction (54.3 g, 0.3%) and an aqueous phase. The EtOAc-soluble fraction was subjected to normal phase silica gel column chromatography [2 kg, hexane–$CHCl_3$ (4:1 → 1:1 → 1:4, v/v) →