Bioorganic & Medicinal Chemistry Letters 24 (2014) 1851–1855

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Synthesis and pharmacological evaluation of novel limonin derivatives as anti-inflammatory and analgesic agents with high water solubility

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ARTICLE INFO

Article history:
Received 24 November 2013
Revised 20 January 2014
Accepted 1 February 2014
Available online 10 February 2014

Keywords:
Limonin derivatives
Solubility
Analgesic activity
Anti-inflammatory activity

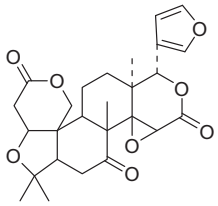
ABSTRACT

A novel series of water-soluble derivatives of limonin were synthesized by introducing various tertiary amines onto the C (7)-position of limonin. Ten target compounds were characterized and screened for their anti-inflammatory and analgesic activity *in vivo*. Compound **3c** exhibited the strongest analgesic and anti-inflammatory activity among the limonin and its derivatives tested; its analgesic activity is more potent than that of aspirin and its anti-inflammatory activity is stronger than that of naproxen.

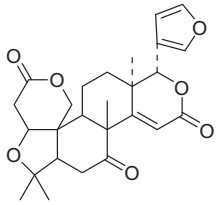
Limonoids are typical secondary metabolites, and are abundant in citrus fruit and other plants found in the Rutaceae and Meliaceae families. Limonin (**1**) is known as the most abundant limonoid in the natural environment and is isolated from navel orange juice in which it works as bitter principle. As limonoids possess various therapeutic effects, such as antitumor,[1–4] anti-inflammatory,[5,6] analgesic,[6] antibacteria,[7] antimalaria,[7] antifeedant[8] etc., the chemistry and pharmacology of these compounds have attracted great interest in medicinal chemistry.

A number of literature reported that Evodiae Fructus, originates from a variety of Evodia species (Rutaceae), has been used in clinics as NSAIDs in the treatment of traditional Chinese medicine.[9] Limonin isolated from Evodiae Fructus is a potent anti-inflammatory and analgesic agent.[10–12] However, these active substances suffered from poor aqueous solubility (<0.005 mg/ml), which results in low bioavailability and poses challenges in its formulation development. Therefore, obtaining the water-soluble derivatives of limonin by structural modification has become one of the key issues before limonoids are applied in clinics.

To increase the water solubility of limonin derivatives, various tertiary amine moieties were introduced onto C (7)-position of limonin to yield five nitrogen-containing derivatives which were treated with a solution of ether saturated with hydrogen chloride to form hydrochloride (**3a**–**3e**). In addition, five tertiary amine derivatives (**6a**–**6e**) of desoxylimonin (**4**)[13] were designed and synthesized to evaluate contributions of the oxygen bridge between C(14) and C(15) to anti-inflammatory and analgesic activity.



(**1**) Limonin



(**4**) Desoxylimonin

The synthetic routes for compounds **3a**–**3e** and **6a**–**6e** are depicted in Schemes 1 and 2. Compound **4** was prepared from limonin according to the literature method.[13] Treatment of **1** or **4** with hydroxylamine hydrochloride yielded exclusively limonin-7-oxime (**2**)[8] or desoxylimonin 7-oxime (**5**). **2** was alkylated with 1-(2-chloroethyl) piperidine to give 7-[2-(piperidin-1-yl)ethoxyimino]

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http://dx.doi.org/10.1016/j.bmcl.2014.02.003
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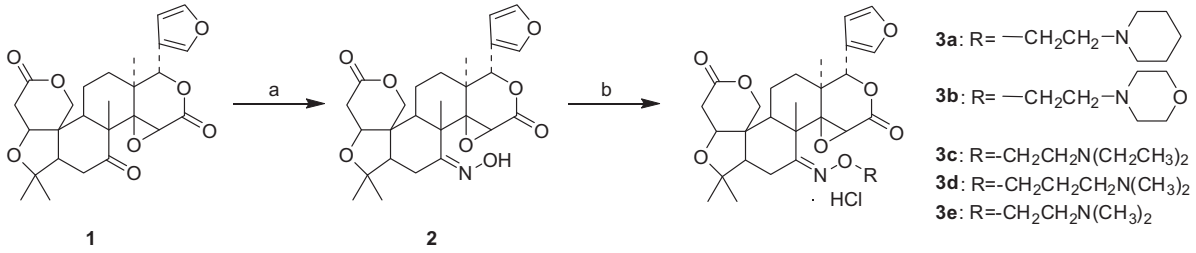
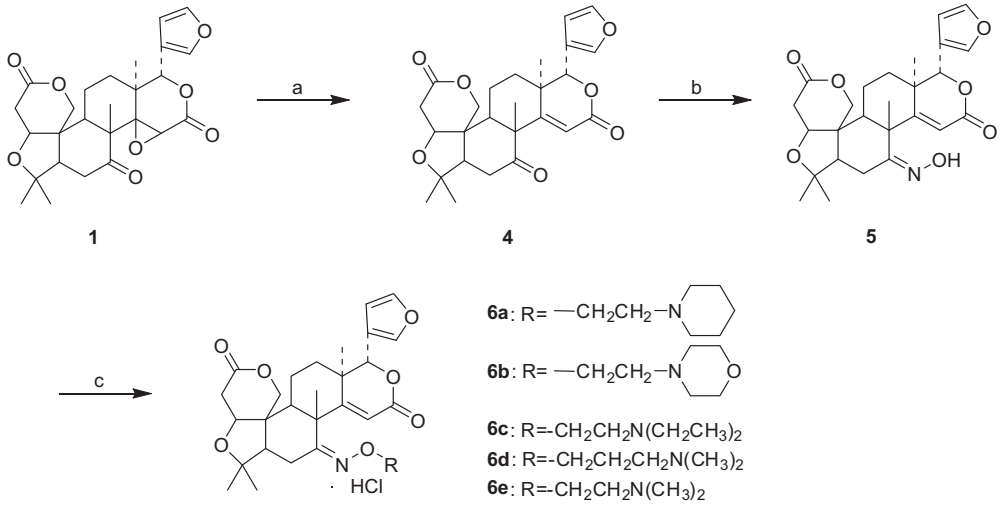


Scheme 1. Reagents and conditions: (a) hydroxylamine hydrochloride, pyridine, anhydrous ethanol, reflux; (b) (i) corresponding alkyl chloride hydrochloride, NaOH, TBAB (Tetrabutylammonium Bromide), anhydrous THF, 80 °C; (ii) hydrogen chloride, anhydrous ether, anhydrous dichlormethane.



Scheme 2. Reagents and conditions: (a) hydroiodic acid, acetic acid; (b) hydroxylamine hydrochloride, pyridine, anhydrous ethanol, acetonitrile, reflux; (c) (i) corresponding alkyl chloride hydrochloride, NaOH, TBAB, anhydrous THF, 80 °C; (ii) hydrogen chloride, anhydrous ether, anhydrous dichlormethane.

Table 1
p*K*a, log$D_{7.4}$, solubility and analgesic activity by acetic acid-induced writhing response in mice of indicated compound

Entry	p*K*a	log$D_{7.4}$	Intrinsic solubility (mg/mL)	Acetic acid induced writhing test		
				Dose (mg/kg)	Number of writhings[a]	Inhibition rate (%)
Control	—[b]	—	—	—	44.0 ± 6.7	—
Aspirin	—	—	—	200	12.4 ± 2.4***	71.82
1	—	—	<0.005[c]	70	23.8 ± 2.8***	45.91
4	—	—	<0.005[c]	70	33.17 ± 5.5	24.61
3a	8.87	0.44	25.3	70	13.0 ± 3.6***	70.45
3b	6.11	2.45	4.5	70	18.5 ± 3.0***	57.95
3c	9.17	0.26	14.5	70	8.8 ± 2.7***, #	80.00
3d	9.28	0.37	9.6	70	15.8 ± 6.2***	64.09
3e	8.78	0.59	17.8	70	13.8 ± 2.2***	68.64
6a	8.52	0.81	19.8	70	24.5 ± 5.9**	44.32
6b	6.84	2.52	2.1	70	22.0 ± 4.8**	50.00
6c	9.49	0.57	1.9	70	29.4 ± 2.0*	33.18
6d	9.39	0.19	9.4	70	17.3 ± 6.2***	60.71
6e	8.88	0.32	28.9	70	30.8 ± 1.7	30.00

[a] Statistical analysis was performed using one-way ANOVA (*p <0.05, **p <0.01, ***p <0.001 as compared with the respective control; #p <0.05 as compared with **1**). The results are expressed as mean ± SEM (n = 8).
[b] —: Not determined.
[c] Equilibrium solubility values measured by the classical saturation shake flask method.

limonin which was treated with a solution of ether saturated with hydrogen chloride to form hydrochloride **3a**. Accordingly, compounds **3b**–**3e** or **6a**–**6e** were obtained respectively by treating **2** or **5** with corresponding alkyl chloride in different yields. Compounds **3a**–**3e** and **6a**–**6e** were characterized by IR, ^{1}H/^{13}C NMR and HRMS spectroscopy.[14,15] The absence of a broad singlet peak at $\delta \sim 7.70$ ppm (C=NOH) and the emergence of a new triplet peak around $\delta \sim 4.20$ ppm which is attributed to the proton of O–CH_2 in the ^{1}H NMR spectrum provided good support for the formation of oxime ether derivatives.

The physiochemical properties including p*K*a (ionization constants), log$D_{7.4}$ (partition coefficient at pH 7.4) and aqueous solubility of ten target compounds were summarized in Table 1. The p*K*a, log$D_{7.4}$ and aqueous solubility were determined according to the method of Avdeef and Tsinman[16] on a Gemini Profiler instrument (pION) by the 'goldstandard' Av-deef-Bucher potentiometric titration method.[17]

All target compounds (**3a**–**3e** and **6a**–**6e**) were evaluated in vivo for their analgesic and anti-inflammatory activity. The analgesic effect was evaluated using acetic acid-induced writhing tests[18,19]

Table 2
The inflammatory activity by xylene-induced ear swelling test in mice of indicated compounds

Entry	Xylene-induced ear swelling test		
	Dose (mg/kg)	Swollen extent (%)[a]	Inhibition rate (%)
Control	—[b]	132.8 ± 16.3	—
Naproxen	150	84.1 ± 6.4***	36.65
1	100	89.3 ± 14.1**	32.74
4	100	111.7 ± 9.9	15.89
3a	100	60.1 ± 16.0***, #	54.77
3b	100	62.4 ± 9.4***	53.01
3c	100	32.6 ± 8.2***, ###	75.46
3d	100	71.6 ± 11.2***	46.08
3e	100	72.4 ± 13.1***	45.46
6a	100	99.1 ± 6.1*	25.36
6b	100	96.1 ± 3.2*	27.61
6c	100	108.2 ± 6.3	18.43
6d	100	97.8 ± 5.1*	26.35
6e	100	100.2 ± 7.4*	24.55

[a] Statistical analysis was performed using one-way ANOVA (*p <0.05, **p <0.01, ***p <0.001 as compared with the respective control; #p <0.05, ##p <0.01, ###p <0.001, as compared with **1**). The results are expressed as mean ± SEM (n = 8).
[b] —: Not determined.

and tail-immersion tests[20–22] in mice. In the acetic acid-induced writhing test, limonin and desoxylimonin (70 mg/kg, i.g.[14]), and aspirin (200 mg/kg, i.g.), used as positive control, and compounds **3** and **6** (70 mg/kg, i.g.) were suspended in 0.5% CMC-Na.[14] The model group was given 0.5% CMC-Na using the same method. One hour after gavage administration of the corresponding drug, mice were injected intraperitoneally with 0.1 ml/10 g body weight of 0.7% acetic acid solution in saline. The number of writhes was recorded 15 min after the chemical stimulus. The results are summarized in Table 1. In the tail-immersion test, all the compounds were administrated in the same way used in the writhing test, using a same single dosage (100 mg/kg, i.g.). Tail-curl latency time was measured before administration and 30, 60, 90 and 120 min afterward.

Ear swelling induced by xylene in mice was used to evaluate the anti-inflammatory activities of the target compounds according to the method reported in previous literature[23] with minor modifications. The limonin, desoxylimonin and their derivatives (100 mg/kg, i.g.) with naproxen (150 mg/kg, i.g.) as positive controls were suspended in 0.5% CMC-Na. The vehicle control group treated with xylene was given 0.5% CMC-Na with the same method. 90 min after administration of the corresponding drugs, 25 μl of xylene was applied to anterior and posterior surfaces of right ear lobe. Left ear was considered as control. 30 min after xylene application, the mice were sacrificed. Circular sections on both ears were taken using a cork borer with a diameter of 8 mm and weighed. Degree of swelling caused by the xylene was measured based on the weight of left ear without stimulus. The results are summarized in Table 2 and are expressed as swollen extent.

As shown in Table 1, the aqueous solubility of ten target compounds was superior to that of **1** and **4**. There is a significant negative correlation between the pKa and log$D_{7.4}$ of the ten tested compounds. Overall, compound **3** series with an oxygen bridge between C(14) and C(15) were more water-soluble than compound **6** series (except compound **6e**) with a double bond. Piperidinoethoxy analogue **3a** showed the most significant increase in water-solubility, whereas morpholinyl ethoxy analogue **3b** showed poor water-solubility among compounds **3a**–**3e**.

The result of acetic acid-induced writhing test (Table 1) revealed that compounds **3a**–**3e**, **6b** and **6d** possessed more potent analgesic activity than that of **1**. Compound **3c** exhibited the most potent analgesic activity and higher inhibition rate of pain threshold compared with aspirin (200 mg/kg, i.g.). As shown in Figures 1 and 2, all the tested compounds exhibited moderate analgesic apacity, and their analgesic effects were more potent than that of **1** in the tail-immersion test; however, compounds **4** displayed no analgesic activities at the administered dose. The tail-immersion latencies of compounds **3a**–**3e** showed a significant increase. The maximal anti-nociceptive response was obtained between 30 and 90 min, while compounds **6a**–**6e** reached maximal anti-nociceptive responses after 60 min of gavage administration. Significant analgesic effects were observed at 60 min for **3a** and **6d**, and 90 min for **3c**.

The results revealed that the inhibition rates on mouse ear swollen (Table 2) of compounds **3a, 3b** and **3c** were higher than

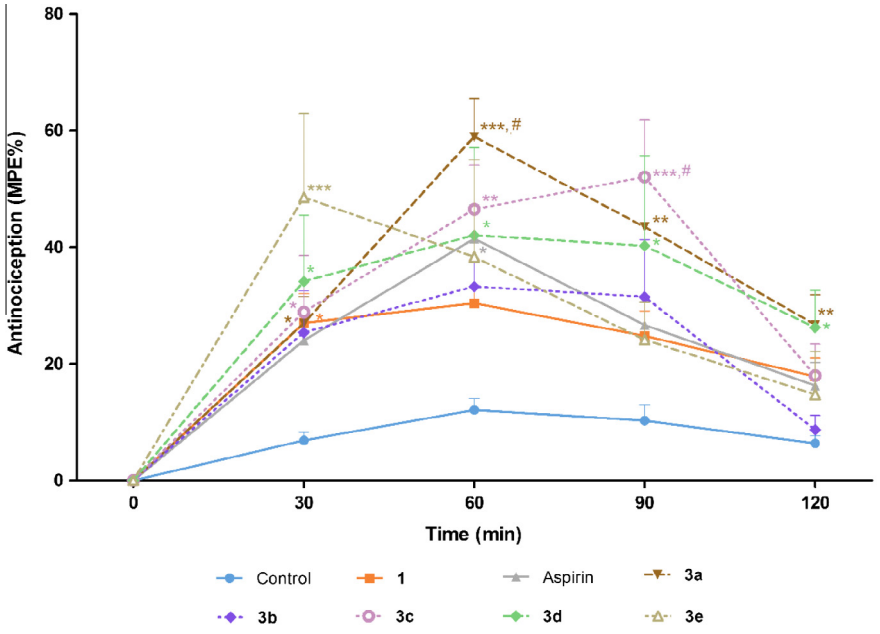


Figure 1. Antinociceptive effect of compounds **3a**–**3e** (100 mg/kg), **1** (100 mg/kg), **4** (100 mg/kg) and Aspirin (100 mg/kg) in the tail-immersion test in mice. Statistical analysis was performed using one-way ANOVA (*p <0.05, **p <0.01, ***p <0.001 as compared with the respective control; #p <0.05 as as compared with **1**). The results are expressed as mean ± SEM (n = 8).

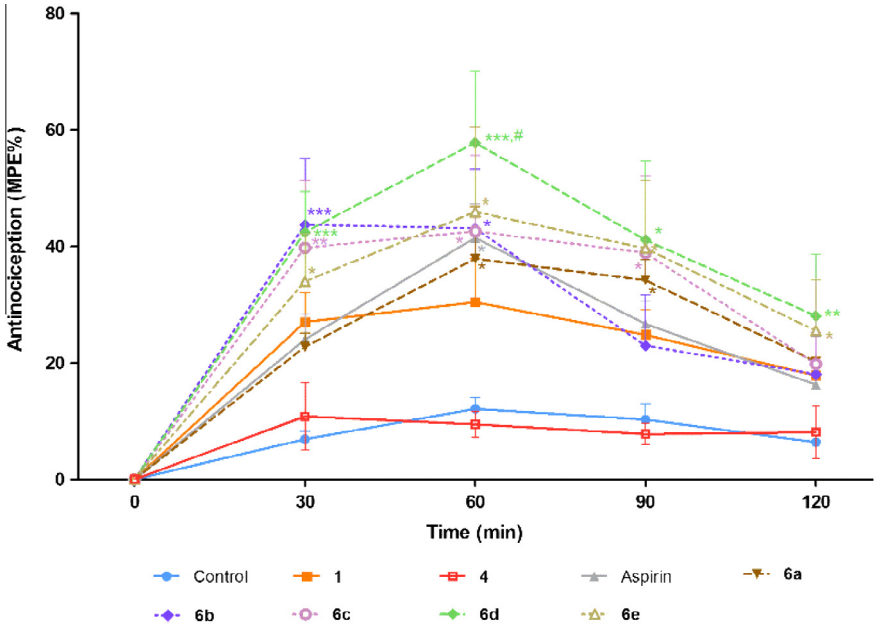


Figure 2. Antinociceptive effect of compounds **6a**–**6e** (100 mg/kg), **1** (100 mg/kg), **4** (100 mg/kg) and Aspirin (100 mg/kg) in the tail-immersion test in mice. Statistical analysis was performed using one-way ANOVA (*p <0.05, **p <0.01, ***p <0.001 as compared with the respective control; #p <0.05 as as compared with **1**). The results are expressed as mean ± SEM (n = 8).

those of **1** and naproxen. The inhibition rates of compounds **3d** and **3e** were equivalent to that of naproxen. However, compounds **6a**–**6e** displayed weaker anti-inflammatory activities at the administered dose.

As a whole, compounds **4** and **6a**–**6e** exhibited less analgesic and anti-inflammatory activities than those of compounds **1** and **3a**–**3e**. This indicated that the oxygen bridge between C(14) and C(15) played an important role in analgesic and anti-inflammatory activity. Among all compounds, **3c** exhibited the highest analgesic activity, the best activity in acetic acid-induced writhing test, and remarkable activity in tail-immersion test with high aqueous solubility (14.5 mg/ml). Compound **3c** also showed the best anti-inflammatory activity in mouse ear swelling test, even stronger than that of naproxen at the administered dosage.

In summary, a series of novel limonin oxime ether derivatives **3a**–**3e** and desoxylimonin oxime ether derivatives **6a**–**6e** were synthesized and evaluated for their analgesic and anti-inflammatory activity and water-solubility. The investigation of analgesic and anti-inflammatory effects and solubility indicated that oximation of limonin and subsequent introduction of tertiary amino group by etherification of limonin oxime improved water solubility and bioactivity. In general, compounds **1** and **3a**–**3e** exhibited more potent analgesic and anti-inflammatory efficacies than those of compounds **4** and **6a**–**6e**, suggesting that oxygen bridge between C(14) and C(15) in limonin derivatives is important for improvement of analgesic and anti-inflammatory activities. In particular, Compound **3c** exhibited the greatest analgesic and anti-inflammatory activity among all the compounds tested; its analgesic activity is more potent than that of aspirin and its anti-inflammatory activity is stronger than that of naproxen. It has been identified as a promising analgesic and anti-inflammatory candidate compound with high water-solubility.

Acknowledgments

The Project was supported by the National Undergraduate Training Programs for Innovation and Entrepreneurship of China.

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14. *General procedure:* To a solution of compounds **2** or **5** (1 mmol) in anhydrous THF (25 ml), NaOH (10 mmol) was added, then the reaction mixture was stirred for 30 min at room temperature. Alkyl chloride hydrochloride (3 mmol) and TBAB (catalytic amount) were added, and the mixture was heated to reflux and monitored by thin layer chromatography (TLC). After completion of the reaction, the solvent was evaporated and the crude reaction mixture was diluted with water (50 ml) and acidified to pH 6 with HCl (1 M). The aqueous layer was extracted twice with dichlormethane and the combined organic layer was washed with brine, dried over $MgSO_4$, and evaporated to dryness. The crude product was purified by column chromatography ($MeOH/CH_2Cl_2$ 1:50). The purified product thus obtained was dissolved in anhydrous dichlormethane (3 ml) and treated with a saturated solution of dry hydrogen chloride in diethyl ether. The solid separated out was filtered, washed with diethyl ether, and dried, to give **3** or **6**. Symbols: CMC-Na: Carboxymethyl Cellulose Sodium; i.g.: Intragastric Administration.
15. *Physical and spectral data of selected compound.* Compound **3c**: Yield 43%, white solid, mp 78 °C (decomposition); IR (KBr, ν cm^{-1}): 3434, 2948, 2648, 1740, 1626, 1464 1023, 810; ^1H NMR ($CDCl_3$, 500 MHz), δ (ppm): 12.21 (s, 1H), 7.45 (d, J = 1.6 Hz, 2H), 6.39 (s, 1H), 5.55 (s, 1H), 4.73 (d, J = 12.4 Hz, 1H), 4.66 (br s, 1H), 4.38 (d, J = 11.4 Hz, 2H), 4.01 (br s, 1H), 3.82 (s, 1H), 3.49 (dt, J_1 = 57.4, J_2 = 28.7 Hz, 2H), 3.27 (br s, 5H), 2.97 (d, J = 16.1 Hz, 1H), 2.71 (d, J = 16.4 Hz, 1H), 2.43 (t, J = 17.0 Hz, 1H), 2.14–1.74 (m, 6H), 1.47 (br s, 6H), 1.35 (s, 3H), 1.29 (s, 3H), 1.21 (s, 3H), 1.04 (s, 3H); ^{13}C NMR ($CDCl_3$, 300 MHz), δ (ppm): 169.34, 167.76, 161.73, 143.27, 140.95, 119.92, 109.58, 80.38, 79.13, 78.57,

67.39, 65.82, 64.67, 60.48, 54.33, 49.86, 49.74, 47.38, 46.84, 46.30, 46.12, 38.14, 35.77, 32.93, 30.26, 21.53, 21.44, 20.43, 19.58, 17.79, 8.36, 8.34; HR-ESIMS *m*/*z* 585.3165 $[M+H]^+$ (calcd for $C_{32}H_{45}N_2O_8$, 585.3170).

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