Chinese Chemical Letters 25 (2014) 787–790

Contents lists available at ScienceDirect

Chinese Chemical Letters

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

Original article

Novel liver-specific nitric oxide (NO) releasing drugs with bile acid as both NO carrier and targeting ligand

Xue-Yuan Jin [a], Shi-Yong Fan [b], Hong-Wu Li [b], Wei-Guo Shi [b], Wei Chen [b], Hui-Fen Wang [a,*], Bo-Hua Zhong [b,*]

[a] Chinese PLA General Hospital & Chinese PLA Medical School, Beijing 100853, China
[b] Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China

ARTICLE INFO

Article history:
Received 6 January 2014
Received in revised form 13 March 2014
Accepted 24 March 2014
Available online 13 April 2014

Keywords:
Liver-specific
Nitric oxide releasing drugs
Hepatoprotective activity
Bile acid

ABSTRACT

Novel liver-specific nitric oxide (NO) releasing drugs with bile acid as both the NO carrier and targeting ligand were designed and synthesized by direct nitration of the hydroxyl group in bile acids or the 3-*O*-hydroxyl alkyl derivatives, with the intact 24-COOH being preserved for hepatocyte specific recognition. Preliminary biological evaluation revealed that oral administrated targeted conjugates could protect mice against acute liver damage induced by acetaminophen or carbon tetrachloride. The nitrate level in the liver significantly increased after oral administration of **1e** while nitrate level in the blood did not significantly change. Co-administration of ursodeoxycholic acid (UDCA) significantly antagonized the increase of nitrate in the liver resulted by administration of **1e**.

1. Introduction

Nitric oxide (NO) mediates multiple physiological and pathological processes [1]. Previous studies have demonstrated that NO can protect the liver from injury and ameliorate the progress of liver fibrosis or cirrhosis. NO-releasing drugs may represent an important therapy for liver damage, fibrosis, and cirrhosis [2,3]. However, non-specific NO-releasing drugs such as isosorbide-5-mononitrate induce significant reduction of arterial pressure, which limits their clinical application [4].

Liver targeted therapy can deliver chemotherapeutic drugs selectively to the liver to avoid side effects or toxicities. Bile acids are the only small molecules with high oral availability that are taken in specifically by the liver [5,6]. Conjugating NO releasing components with bile acids such as cholic acid (CA) or ursodeoxycholic acid (UDCA) can deliver NO selectively to the liver for the treatment of liver diseases without significant reduction of arterial pressure. NCX-1000 (Fig. 1) is the first liver-specific NO donor drug that can protect mice against acute liver injury induced by acetaminophen (APAP) or Con A [7,8]. However, NCX-1000 has not met the endpoints in phase 2 clinical trials.

NCX-1000 was prepared by conjugating the NO releasing moiety with the 24-COOH of UDCA by two ester bonds. However, the intact 24-COOH of bile acid is essential for liver specificity [5]. Conjugating with the 24-COOH may abolish the targeting of UDCA, and the ester bond in the conjugate is not stable in human blood.

Novel liver-specific nitric oxide (NO) releasing drugs were designed and synthesized by use of the hydroxyl group on the steroid nucleus at position 3, 7, or 12 as the conjugating group with the preserved 24-COOH for hepatocyte specific recognition. Compounds **1a–1d** were nitrates of 3-OH, 7-OH, or 12-OH. In these compounds, the bile acids are used as both the NO carrier and targeting ligand. Compounds **1e** and **1f** were nitrates of 3-O$(CH_2)_4$ OH and 3-O$(CH_2)_2$OH derivatives of UDCA, respectively. The linkage chain between nitrate and UDCA can modulate the release rate of NO from the conjugates.

Preliminary biological evaluation revealed that the targeted conjugates can protect mice against acute liver injury induced by APAP or carbon tetrachloride (CCl_4). Both the site and number of nitration could affect the biological activity. Compounds **1e** and **1f** are two most active compounds and show more potent protective effects than NCX-1000. Further *in vivo* nitrate distribution investigation revealed that the nitrate level in the liver significantly increased after oral administration of **1e**, while nitrate level

* Corresponding authors.
E-mail addresses: wanghf0501@163.com (H.-F. Wang), bohuazhong@yahoo.com (B.-H. Zhong).

1001-8417/$ – see front matter © 2014 Hui-Fen Wang and Bo-Hua Zhong. Published by Elsevier B.V. on behalf of Chinese Chemical Society. All rights reserved.
http://dx.doi.org/10.1016/j.cclet.2014.04.001

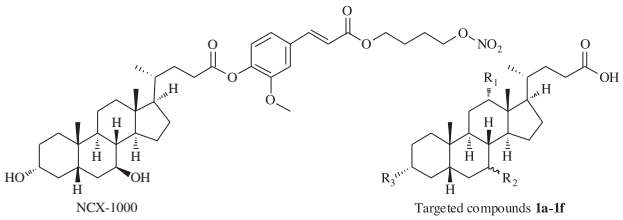


Fig. 1. Structure of NCX1000 and targeted compounds.

in the blood did not significantly changed. Co-administration of UDCA significantly antagonized the increase of nitrate in the liver resulted by **1e**, which demonstrated that the delivery of **1e** to the liver was mediated *via* the bile acid transport system.

2. Experimental

2.1. Chemistry

The synthesis of compounds **1a** and **1b** is outlined in Scheme 1. The 24-COOH of bile acids was first protected by methylation in HCl-methanol solution to give **2a**. Target compound **1a** was obtained by nitration of all three hydroxyl groups of **2a** with fuming nitric acid in Ac_2O and then deprotection with 5% KOH menthol solution. The 3-OH of **2a** was selectively reacted with acetyl chloride to give the 3-OH protected derivative **3b**. Compound **1b** was obtained by nitration of **3b** followed by deprotection of **4b**, similar to **1a**.

Compounds **1c** and **1d** were synthesized as depicted in Scheme 2. The methylated derivatives (**2a** and **2d**) of bile acids were reacted with Ac_2O to give the acetate derivatives **3c** and **3d**; **3c** and **3d** were selectively deprotected to give the 3-OH free derivative **4c** and **4d**. The target compounds **1c** and **1d** were obtained by nitration and then deprotection as that of **1a**.

Compounds **1e** and **1f** were prepared as outlined in Scheme 3. **4d** was reacted with methanesulfonyl chloride to obtain the methanesulfonate derivative **6**. Compound **6** was then reacted with 1, 4-butanediol or ethylene glycol to give the hydroxyl alkylated derivatives **7e** and **7f**, respectively. Compounds **7e** and **7f** were nitrated and then deprotected to afford target compounds **1e** and **1f**, respectively. NCX-1000 was synthesized according to the literature [9].

The structures of compounds **1a–1f** were characterized [10].

2.2. Pharmacology

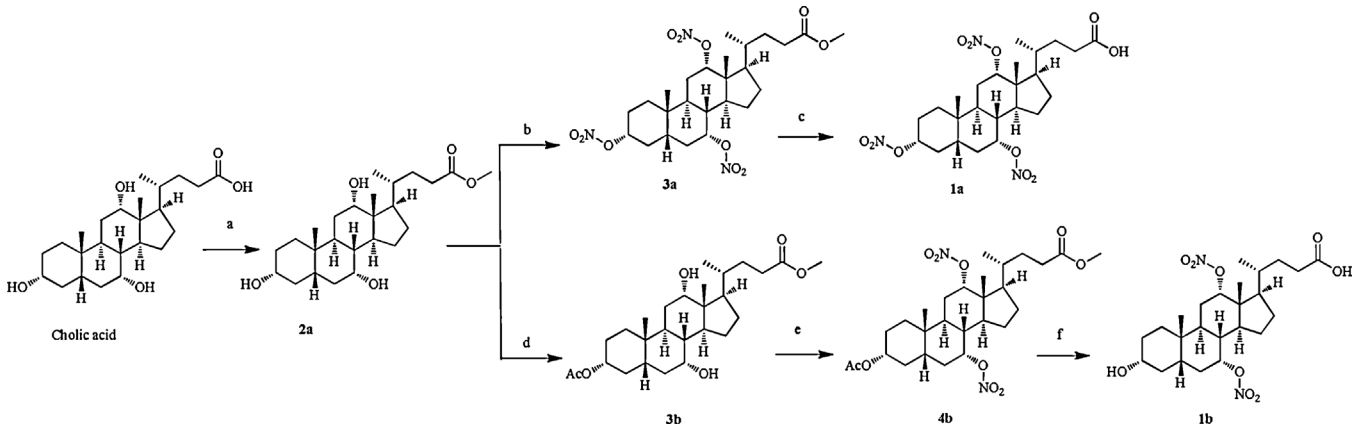
2.2.1. Protective effect against acute liver injury induced by CCl_4 or APAP

Baclb/c mice were randomly divided (n = 10, half male and half female). Compounds were suspended in 1% sodium methoxycellulose and administered by intragastric at dosage of 50 mg/kg. Sixty minutes after administration of the test compounds, 0.1% CCl_4 in olive oil at dose of 10 mL/kg or APAP at dose of 200 mg/kg was administrated by i.p. injection to induce the acute liver injury model. Saline was subcutaneous injected as normal control. Twelve hours after administration of carbon tetrachloride or APAP, same amount of the compounds was administered by intragastric. Twenty-four hours after the second administration of compounds, whole blood samples were taken from the suborbital sinus and the samples were centrifuged, the plasma was frozen and stored immediately at −70 °C. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was determined with the Olympus AU5400 automated biochemistry analyzer (Olympus Corporation, Tokyo, Japan).

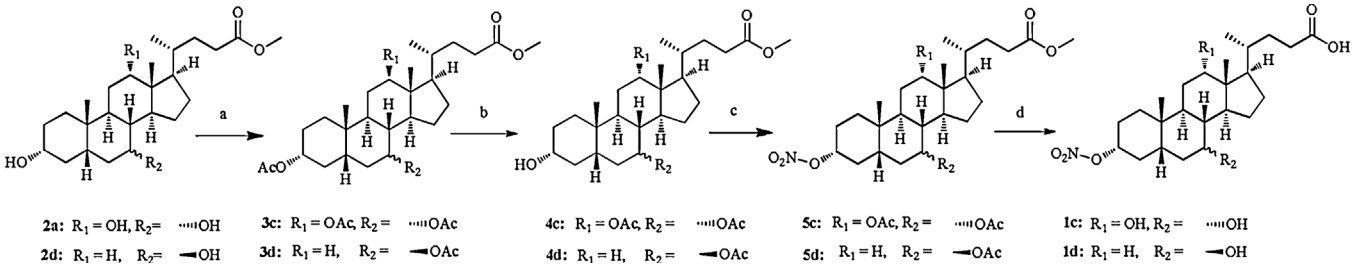
2.2.2. NO distribution in vivo

After overnight fasting, male Baclb/c mice were randomly divided into 4 groups (n = 10). Compounds were suspended in 1% sodium methoxycellulose and administered by intragastric injection. The mice in the first group were treated with saline as control; the mice in the second group were treated with **1e** (10 mg/mL, 20 mL/kg); in the third group, mice were treated with UDCA (50 mg/mL, 20 mL/kg); in the fourth group, mice were treated with UDCA (50 mg/mL, 20 mL/kg) and **1e** (10 mg/mL, 20 mL/kg). Five mice from each group were sacrificed in 2 h or 4 h after administration of the compounds. Whole blood samples were taken from the suborbital sinus, and the samples were centrifuged. The plasma was frozen and stored immediately at −70 °C. The livers were collected at the same time and rapidly frozen in liquid nitrogen and maintained at −80 °C until analysis. Liver lysates were prepared by homogenization in Tris–HCl buffer (0.01 mol/L + NaCl 0.1 mol/L). The homogenates were centrifuged at 15,000 rpm for 15 min and the supernatant was taken for nitrate determination.

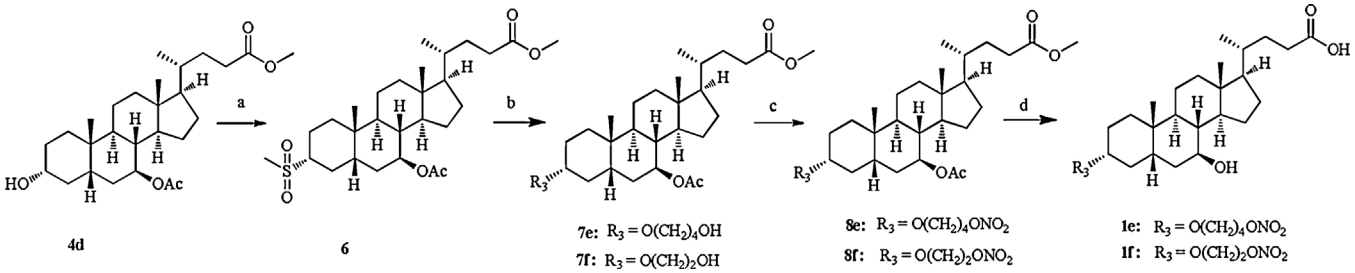
The nitrate concentration was measured by Griess assay using a colorimetric assay kit (Beyotime, China) [11]. Briefly, 50 μL of the sample solutions were mixed with 50 μL Griess reagent I and 50 μL Griess reagent II, and the mixture was then placed at room temperature for 10 min. The absorbance was measured at 540 nm, and sodium nitrate solutions at different concentrations were used as positive controls for the standard curve.



Scheme 1. Synthesis route of compounds **1a–1b.** (a) HCl–methanol, r.t., 12 h; (b, e) acetic anhydride, fuming nitric acid, −5 °C, 1 h; (c, f) 5% KOH menthol solution, reflux, 1 h; (d) acetyl chloride, pyridine, 0 °C, 1 h, then r.t., 2 h.



Scheme 2. Synthesis route of compounds **1c–1d**. (a) acetic anhydride, pyridine, DMAP, r.t., 6 h; (b) HCl–methanol, r.t., 1–3 h; (c) acetic anhydride, fuming nitric acid, −5 °C, 1 h; (d) 5% KOH menthol solution, reflux, 1 h.



Scheme 3. Synthesis route of compounds **1e–1f**. (a) methanesulfonyl chloride, pyridine, 0 °C, 1 h, then r.t.,2 h; (b) butanediol or ethylene glycol, pyridine, 100 °C, 2 h; (c) acetic anhydride, fuming nitric acid, −5 °C, 1 h; (d) 5% KOH menthol solution, reflux, 1 h.

All results are expressed as mean ± SD. For multiple comparisons statistical analysis was performed by one-way ANOVA followed by Tukey multiple comparison tests with SPSS 20.0 software. $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Protective effect against acute liver injury induced by CCl_4 or APAP

Both CCl_4 and APAP treatment resulted in significant increase of ALT and AST in the blood. As shown in Table 1, at doses of 50 mg/kg, compounds **1b**, **1d**, **1e**, **1f** and NCX-1000 significantly inhibited the rise of both plasma ALT and AST in both the CCl_4 and APAP models; compound **1a** and UDCA showed some effects on both plasma ALT and AST in the CCl_4 model but only plasma ALT in the APAP model; compound **1c** significantly inhibited the rise of plasma AST in the CCl_4 model and plasma ALT in the APAP model; CA has no effect on either plasma ALT or AST in the CCl_4 or the APAP model. All the active compounds except **1c** showed greater inhibitory effects on plasma ALT than on plasma AST.

Compounds **1e** and **1f** exhibited much more profound protective effects against both CCl_4 and APAP induced hepatocyte injury than NCX-1000, UDCA, and **1a–1d**. The difference in the activity between **1e** or **1f** and other target compounds may result from the difference in NO releasing rate from these compounds. Nitrate of the axial hydroxyl group on the steroid nucleus may not be easily reached by cytochrome P450 or glutathione-S-transferase, enzymes that are responsible for the hydrolysis of organic nitrate. *In vitro* NO releasing experiments revealed that **1a–1d** release NO much more slowly than **1e** or **1f** after incubation with HepG2 cells (Fig. 2). Ursodeoxycholic acid is a hepatocyte protective agent [12]. The nitrates of ursodeoxycholic acid (**1d–1f**) showed better hepatocyte protective effects than the nitrates of cholic acid (**1a–1c**). This may be explained by the synergistic effect of UDCA and NO released from the target compounds **1d–1f**.

Table 1
Protective activity against CCl_4 and APAP induced acute liver-injury.*

Group	CCl_4 model		APAP model	
	ALT (U/L)	AST (U/L)	ALT (U/L)	AST (U/L)
NC	377 ± 113	489 ± 146	391 ± 159	306 ± 215
MC	2855 ± 765**	3647 ± 861**	3416 ± 575**	3048 ± 722**
NCX-1000	1703 ± 618##	2633 ± 608#	1774 ± 339##	1843 ± 462##
1a	2150 ± 577#	2410 ± 796##	1948 ± 661##	2626 ± 459
1b	2184 ± 564#	2549 ± 675#	2071 ± 1024##	2377 ± 493#
1c	2261 ± 597	2601 ± 745#	2307 ± 751#	2667 ± 606
1d	2061 ± 560#	2088 ± 866##	1558 ± 565##	2348 ± 590#
1e	818 ± 527##	2354 ± 457#	1100 ± 746##	1698 ± 480##
1f	1020 ± 582##	2206 ± 594##	1167 ± 466##	1920 ± 423##
CA	2521 ± 627	3378 ± 756	3243 ± 797	3154 ± 809
UDCA	1940 ± 336##	2965 ± 451#	2240 ± 731#	2805 ± 620

NC: normal control, MC: model control.
* Values are mean ± SD; $n = 10$.
** $P < 0.01$ *vs.* NC group.
# $P < 0.05$ *vs.* MC group.
$P < 0.01$ *vs.* MC.

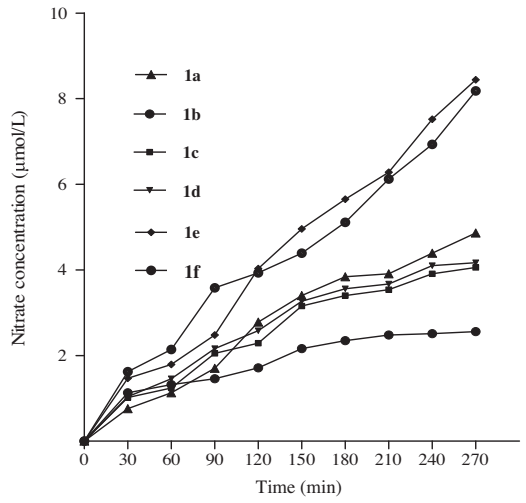


Fig. 2. NO release assay *in vitro* of target compounds. HepG2 cells were incubated in triplicate with each compound. The levels of nitrate produced in the lysates of HepG2 cells were determined using a calorimetric Griess assay [11].

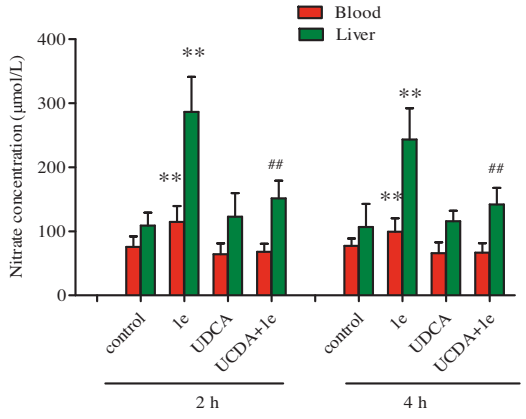


Fig. 3. Nitrate concentration in blood and liver after oral administration of **1e**.

3.2. NO distribution in vivo

After absorption and transportation to the liver *via* the bile acid mediated route, the liver specific NO releasing compounds will release NO by cytochrome P450 or glutathione-S-transferase catalyzed hydrolysis, and the NO released is then transformed into nitrate. So the concentration of nitrate in liver and in blood was determined to evaluate the specificity of the target compound.

As shown in Fig. 3, the nitrate level in the liver is significantly increased at 2 h and 4 h after administration of **1e**, while the change of nitrate level in the blood is not significant. UDCA has no effect on the nitrate levels in the liver. Co-administration of UDCA significantly antagonized the increase of nitrate in the liver resulted by **1e**. These results indicated that the NO-releasing compounds we synthesized were delivered into the liver *via* the bile acid mediated transportation system route and released NO specifically in the liver.

4. Conclusion

In conclusion, six novel liver specific NO releasing compounds with bile acid as both the NO carrier and targeting ligand were designed and synthesized. Compounds **1a–1d** were synthesized by selective nitration of the 3-OH, 7-OH, or 12-OH on the steroid nucleus, while **1e** and **1f** were nitrates of 3-$O(CH_2)_4$ OH and 3-$O(CH_2)_2$OH derivatives of ursodeoxycholic acid. The intact 24-COOH is preserved in all the targeted compounds for hepatocyte specific recognition. All compounds showed protective effects against both CCl_4 and APAP induced acute liver injury in mice models. Compounds **1e** and **1f** exhibited much more profound protective effects against both CCl_4 and APAP induced hepatocyte injury than NCX-1000, UDCA, and **1a–1d**. The nitrate level in the liver is significantly increased after administration of **1e** while the change of nitrate level in the blood is not significant. Co-administration of UDCA significantly antagonized the increase of nitrate in the liver resulted by administration of **1e**, indicating that the NO-releasing compounds were delivered specifically into the liver by the bile acid mediated transportation system.

Acknowledgments

This work was supported by the National High Technology Research and Development (863) Project (No. 2006AA02A4C6) and National Natural Science Foundation of China (Nos. 30572220 and 30972626).

References

[1] D.C. Rockey, V. Shah, Nitric oxide biology and the liver: report of an AASLD research workshop, Hepatology 39 (2004) 250–257.
[2] M. Abu-Amara, S.Y. Yang, A. Seifalian, B. Davidson, B. Fuller, The nitric oxide pathway-evidence and mechanisms for protection against liver ischaemia reperfusion injury, Liver. Int. 32 (2012) 531–543.
[3] N.M. Atucha, F.J. Nadal, D. Iyu, et al., Role of vascular nitric oxide in experimental liver cirrhosis, Curr. Vasc. Pharmacol. 3 (2005) 81–85.
[4] L. Bellis, A. Berzigotti, J.G. Abraldes, et al., Low doses of isosorbide mononitrate attenuate the postprandial increase in portal pressure in patients with cirrhosis, Hepatology 37 (2003) 378–384.
[5] A. Enhsen, W. Kramer, G. Wess, Bile acids in drug discovery, Drug Discov. Today 3 (1998) 409–418.
[6] J. Li, L. Hai, W.J. Liu, X.C. Wu, Y. Wu, Study on synthesis and distribution in vivo of 5-Fu-cholic acid conjugate, Chin. Chem. Lett. 20 (2009) 136–138.
[7] S. Fiorucci, E. Antonelli, E. Distrutti, et al., Liver delivery of NO by NCX-1000 protects against acute liver failure and mitochondrial dysfunction induced by APAP in mice, Br. J. Pharmacol. 143 (2004) 33–42.
[8] S. Fiorucci, A. Mencarelli, B. Palazzetti, et al., An NO derivative of ursodeoxycholic acid protects against Fas-mediated liver injury by inhibiting caspase activity, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 2652–2657.
[9] D.P. Soldato, S.A. Nicox, Pharmaceutical compounds, Patent WO 0061604, 2000-10-19.
[10] Data for new compounds. **1a**: Yield 73%. Mp: 221–223 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.93 (s, 1H), 5.29 (s, 1H) 5.07 (s, 1H), 4.79 (s, 1H), 2.39–2.37 (m, 1H), 2.26–2.23 (m, 1H), 2.23–1.02 (m, 28 H), 0.81 (s, 3H). MS (FAB): *m*/*z* 542.6 (M-1). **1b**: Yield 76%. Mp: 191–193 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.98 (s, 1H), 5.39 (s, 1H), 5.09 (s, 1H), 3.24 (s, 1H), 1.98–0.97 (m, 23H), 0.99–0.78 (m, 11H). MS (FAB): *m*/*z* 497.7 (M-1). **1c**: Yield 67%. Mp: 218–220 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.93 (s, 1H), 4.85 (s, 1H), 4.20–4.18 (d, 2H), 3.79 (s, 1H), 3.63 (s, 1H), 1.99–0.97 (m, 30H), 0.59 (s, 3H). MS (FAB): *m*/*z* 452.5 (M-1). **1d**: Yield 62%. Mp: 186–188 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.84 (s, 1H), 4.85 (s, 1H), 3.60–3.57 (d, 2H), 2.24 (s, 1H), 2.02–0.97 (m, 25H), 0.94 (d, 3H, *J* = 6.5 Hz), 0.85 (s, 3H), 0.63 (s, 3H). MS (FAB): *m*/*z* 436.4 (M-1). **1e**: Yield 47%. Mp: 185–187 °C; ^{1}H NMR (400 MHz, CDCl3): δ 11.96 (s, 1H), 4.56–4.52 (t, 2H), 3.86–3.84 (d, 1H, *J* = 7.0 Hz), 3.48 (s, 1H), 3.34–3.32 (t, 2H), 2.25–0.87 (m, 37H), 0.62 (s, 3H). MS (FAB): *m*/*z* 508.8 (M-1). **1f**: Yield 43%. Mp: 174–176 °C; ^{1}H NMR (400 MHz, CDCl3): δ 12.05 (s, 1H), 4.56–4.54 (t, 2H), 3.90–3.87 (d, 1H, *J* = 6.8 Hz), 3.46 (s, 1H), 3.31–3.28 (t, 2H), 2.26–1.04 (m, 27H), 0.97 (s, 3H), 0.89 (d, 3H, *J* = 6.4 Hz), 0.61 (s, 3H). MS (FAB): *m*/*z* 480.5 (M-1).
[11] M.D. Lu, X. Zhou, Y.J. Yu, et al., Synthesis and in vitro biological evaluation of nitric oxide-releasing derivatives of hydroxylcinnamic acids as anti-tumor agents, Chin. Chem. Lett. 24 (2013) 415–418.
[12] Z. Xiang, Y.P. Chen, K.F. Ma, et al., The role of Ursodeoxycholic acid in non-alcoholic steatohepatitis: a systematic review, BMC Gastroenterol. 13 (2013) 140.