Int J Clin Exp Med 2014;7(4):1022-1027
www.ijcem.com /ISSN:1940-5901/IJCEM0000191

Original Article

Synthesis and effects of new caffeic acid derivatives on nitric oxide production in lipopolysaccharide-induced RAW 264.7 macrophages

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Received March 9, 2014; Accepted April 10, 2014; Epub April 15, 2014; Published April 30, 2014

Abstract: In this study, 20 new derivatives of caffeic acid esters were synthesized and their inhibitory activities against the lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 macrophages were determined. Compounds 3l, 3r, 3s and 3t were found to decrease nitrite levels in a dose-dependent manner in LPS-induced cells and showed potent inhibitory activities against the NO production in RAW264.7 macrophages with IC_{50} values of 7.4, 5.9, 3.3 and 2.2 μM, respectively. They could be selected as compromising compounds for the later pharmacological study.

Keywords: Caffeic acid, structure modification, condensation, nitric oxide, anti-inflammatory

Introduction

Caffeic acid is a kind of common phenolic acid, which is frequently present in fruits, coffee and Chinese herbal medicines [1]. Caffeic acid derivatives possess various biological activities, including antioxidant, anti-bacterial, anti-tumor, hepatoprotection and so on [2]. Most of caffeic acid derivatives are existed in the form of esters, such as caffeic acid phenethyl ester (CAPE), chlorogenic acid, rosmarinic acid, these compounds also exhibit multiple biological properties [3].

Nitric oxide (NO) is a signaling molecule, which involved in the immunoregulation intracellular and intercellular [4]. It acts as different roles in living bodies. It can regulate vascular tone, participating in the immune response. Meanwhile, it forms a free oxygen radical ($NO^{\cdot}$) in metabolic process, acts as a cytotoxic agent in inflammatory disorder [5]. The production of NO can be reduced by inhibiting the inducible nitric oxide synthase (iNOS), thus relieving the inflammation [6].

Though the anti-inflammatory activities of caffeic acid and its derivatives have been reported previously, they also exhibiting fine NO inhibitory activities, their mechanisms are not fully understood [7]. In this study, we synthesized a series of caffeic acid ester derivatives by a simple and fast method, all these derivatives were screened for the inhibitory activity against the LPS-induced NO production in RAW264.7 macrophages [8].

Materials and methods

Chemicals

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance 500-NMR spectrometer using TMS as an internal standard and CD_3OD as solvents. Chemical shifts (δ values) and coupling constants (*J* values) were given in ppm and Hz, respectively. ESI-MS was recorded on a Waters Q-TOF MS Premier. Melting points were taken on a MEL-TEMP II apparatus by Laboratory Devices and are uncorrected. All other commercial reagents and solvents were used as received without further purification. Flash column chromatography was performed with Merck silica gel 60 (300 - 400 mesh).

General synthetic procedure

A mixture of the caffeic acid (90 mg, 0.5 mmol), dicyclohexyl carbodiimide (DCC, 103 mg, 0.5

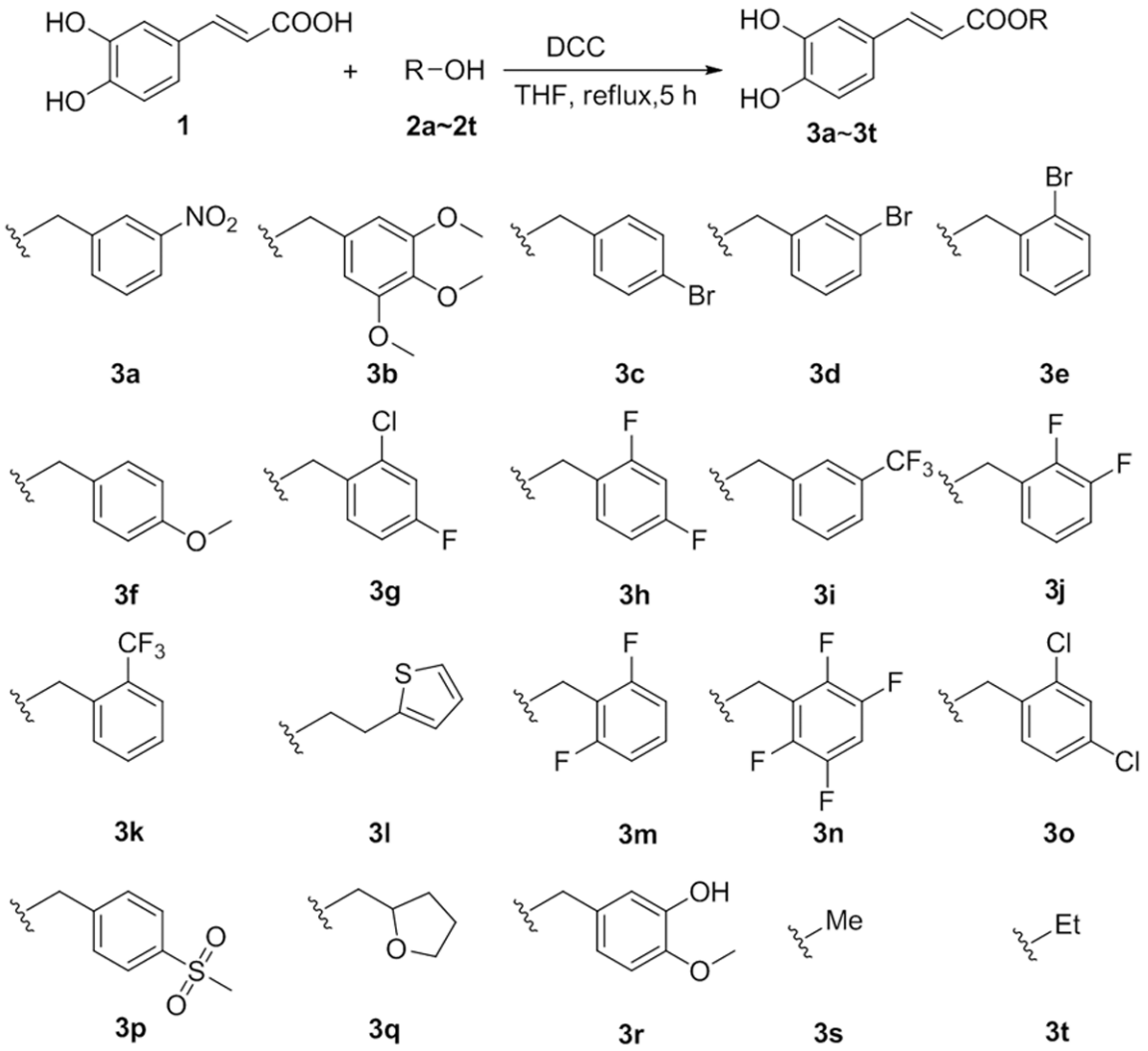

Figure 1. Synthetic route of structural modification. (*E*)-3-nitrobenzyl 3-(3,4-dihydroxyphenyl)acrylate (3a) light yellow solid; yield: 8.9%; mp: 146~148°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 8.30 (1H, s, H-2′), 8.20 (1H, dd, *J* = 8.2, 1.4 Hz, H-5′), 7.82 (1H, d, *J* = 7.6 Hz, H-4′), 7.66 - 7.59 (2H, m, H-7, H-6′), 7.05 (1H, d, *J* = 2.0 Hz, H-2), 6.96 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.34 (1H, d, *J* = 15.9 Hz, H-8), 5.34 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.6 (C-9), 149.8 (C-4), 147.7 (C-3), 146.8 (C-7), 140.3 (C-3′), 135.1 (C-1′), 130.9 (C-6′), 127.6 (C-1), 123.9 (C-5′), 123.7 (C-2′), 123.1 (C-6), 121.4 (C-4′), 116.5 (C-5), 115.2 (C-8), 114.4 (C-2), 65.8 (C-7′). ESI-MS: *m/z* 316 $[M+H]^+$. (*E*)-3,4,5-trimethoxybenzyl 3-(3,4-dihydroxyphenyl)acrylate (3b) light yellow solid; yield: 33.3%; mp: 98~99°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.57 (1H, d, *J* = 15.9 Hz, H-7), 7.04 (1H, d, *J* = 2.0 Hz, H-2), 6.93 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 6.70 (2H, s, H-2′, H-6′), 6.29 (1H, d, *J* = 15.9 Hz, H-8), 5.13 (2H, s, H-7′), 3.82 (6H, s, H-8′, H-10′), 3.75 (3H, s, H-9′); ^{13}C NMR (125 MHz, CD_3OD) δ: 169.0 (C-9), 154.6 (C-3′, C-5′), 149.6 (C-4), 147.2 (C-3), 146.8 (C-7), 139.0 (C-4′), 133.8 (C-1′), 127.7 (C-1), 123.0 (C-6), 116.5 (C-5), 115.2 (C-8), 114.9 (C-2), 106.8 (C-2′, C-6′), 67.4 (C-7′), 61.1 (C-9′), 56.6 (C-8′, C-10′). ESI-MS: *m/z* 361 $[M+H]^+$. (*E*)-4-bromobenzyl3-(3,4-dihydroxyphenyl)acrylate (3c) light yellow solid; yield: 30.3%; mp: 145~147°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.58 (1H, d, *J* = 15.9 Hz, H-7), 7.53 (2H, d, *J* = 8.2 Hz, H-3′, H-5′), 7.34 (2H, d, *J* = 8.2 Hz, H-2′, H-6′), 7.04 (1H, d, *J* = 2.0 Hz, H-2), 6.95 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.30 (1H, d, *J* = 15.9 Hz, H-8), 5.18 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.8 (C-9), 149.7 (C-4), 147.4 (C-3), 146.8 (C-7), 137.2 (C-1′), 132.7 (C-3′, C-5′), 131.0 (C-2′, C-6′), 127.7 (C-1), 123.1 (C-6), 123.0 (C-4′), 116.5 (C-5), 115.2 (C-8), 114.7 (C-2), 66.3 (C-7′). ESI-MS: *m/z* 350 $[M+H]^+$. (*E*)-3-bromobenzyl 3-(3,4-dihydroxyphenyl)acrylate (3d) light yellow solid; yield: 25.7%; mp: 132~134°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.61 - 7.56 (2H, m, H-7, H-4′), 7.47 (1H, d, *J* = 8.0 Hz, H-2′), 7.37 (1H, d, *J* = 7.8 Hz, H-6′), 7.28 (1H, t, *J* = 7.8 Hz, H-5′), 7.04 (1H, d, *J* = 2.1 Hz, H-2), 6.95 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 6.31 (1H, d, *J* = 15.9 Hz, H-8), 5.18 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.8 (C-9), 149.7 (C-4), 147.5 (C-3), 146.8 (C-7), 140.5 (C-1′), 132.2 (C-2′), 132.0 (C-4′), 131.4 (C-5′), 127.8 (C-6′), 127.7 (C-1), 123.4 (C-3′), 123.1 (C-6), 116.5 (C-5), 115.2 (C-8), 114.7 (C-2), 66.1 (C-7′). ESI-MS: *m/z* 350 $[M+H]^+$. (*E*)-2-bromobenzyl 3-(3,4-dihydroxyphenyl)acrylate (3e) light yellow solid; yield: 38.9%; mp: 106~108°C; ^{1}H NMR (500 MHz,

CD_3OD) δ: 7.65 - 7.57 (2H, m, H-7, H-3′), 7.49 (1H, d, *J* = 6.7 Hz, H-5′), 7.38 (1H, t, *J* = 7.2 Hz, H-4′), 7.25 (1H, m, H-6′), 7.05 (1H, d, *J* = 1.9 Hz, H-2), 6.96 (1H, dd, *J* = 8.2, 1.9 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.33 (1H, d, *J* = 15.9 Hz, H-8), 5.29 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.7 (C-9), 149.7 (C-4), 147.5 (C-3), 146.8 (C-7), 137.0 (C-1′), 133.9 (C-6′), 131.1 (C-4′), 131.0 (C-3′), 128.8 (C-5′), 127.7 (C-1), 124.2 (C-2′), 123.1 (C-6), 116.5 (C-5), 115.2 (C-8), 114.6 (C-2), 66.7 (C-7′). ESI-MS: *m/z* 350 $[M+H]^+$. (*E*)-4-methoxybenzyl 3-(3,4-dihydroxyphenyl)acrylate (3f) light yellow solid; yield: 30%; mp: 167~168°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.55 (1H, d, *J* = 15.9 Hz, H-7), 7.35 - 7.32 (2H, m, H-2′, H-6′), 7.03 (1H, d, *J* = 2.1 Hz, H-2), 6.95 - 6.90 (3H, m, H-6, H-3′, H-5′), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 6.27 (1H, d, *J* = 15.9 Hz, H-8), 5.14 (2H, s, H-7′), 3.80 (3H, s, -0CH3); ^{13}C NMR (125 MHz, CD_3OD) δ: 169.1 (C-9), 161.2 (C-4′), 147.0 (C-4), 146.8 (C-3), 145.7 (C-7), 131.1 (C-2′, C-6′), 129.8 (C-1′), 126.1 (C-1), 123.0 (C-6), 116.5 (C-5), 115.2 (C-8), 115.1 (C-3′, C-5′), 114.9 (C-2), 67.0 (C-7′), 55.7 (C-8′). ESI-MS: *m/z* 301 $[M+H]^+$. (*E*)-2-chloro-4-fluorobenzyl 3-(3,4-dihydroxyphenyl)acrylate (3g) light yellow solid; yield: 31.0%; mp: 139~142°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.63 - 7.52 (2H, m, H-7, H-3′), 7.28 (1H, dd, *J* = 8.6, 2.5 Hz, H-6′), 7.12 (1H, td, *J* = 8.5, 2.6 Hz, H-5′), 7.05 (1H, d, *J* = 1.8 Hz, H-2), 6.96 (1H, dd, *J* = 8.2, 1.8 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.31 (1H, d, *J* = 15.9 Hz, H-8), 5.29 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.7 (C-9), 162.2 (C-4′), 149.8 (C-4), 147.5 (C-3), 146.9 (C-7), 135.7 (C-2′), 133.0 (C-1′), 127.6 (C-1), 123.1 (C-6), 121.4 (C-6′), 117.7 (C-3′), 116.5 (C-5), 115.3 (C-5′), 115.2 (C-8), 114.5 (C-2), 63.9 (C-7′). ESI-MS: *m/z* 324 $[M+H]^+$. (*E*)-2,4-difluorobenzyl 3-(3,4-dihydroxyphenyl)acrylate (3h) light yellow solid; yield: 20.9%; mp: 129~131°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.57 (1H, d, *J* = 15.9 Hz, H-7), 7.52 (1H, m, H-6′), 7.03 (1H, d, *J* = 2.1 Hz, H-2), 7.02 - 6.96 (2H, m, H-3′, H-5′), 6.94 (1H, dd, *J* = 8.2, 2.1 Hz, H-6), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 6.28 (1H, d, *J* = 15.9 Hz, H-8), 5.24 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.7 (C-9), 163. 8 (C-2′), 159.9 (C-4′), 149.7 (C-4), 147.5 (C-3), 146.8 (C-7), 133.4 (C-6′), 127.6 (C-1), 123.1 (C-6), 116.5 (C-5), 115.2 (C-8), 114.6 (C-2), 112.5 (C-1′), 112.3 (C-5′), 104.7 (C-3′), 60.4 (C-7′). ESI-MS: *m/z* 307 $[M+H]^+$. (*E*)-3-(trifluoromethyl)benzyl 3-(3,4-dihydroxyphenyl)acrylate (3i) light yellow solid; yield: 20.7%; mp: 120~121°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.74 - 7.55 (5H, m, H-7, H-2′, H-4′, H-5′, H-6′), 7.05 (1H, d, *J* = 2.0 Hz, H-2), 6.96 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.33 (1H, d, *J* = 15.9 Hz, H-8), 5.30 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.8 (C-9), 149.8 (C-4), 147.6 (C-3), 146.8 (C-7), 139.4 (C-1′), 132.8 (C-3′), 130.5 (C-6′), 127.7 (C-1), 125.9 (C-5′), 125.7 (C-2′), 125.4 (C-8′), 125.3 (C-4′) 123.1 (C-6), 116.5 (C-5), 115.2 (C-8), 114.6 (C-2), 66.2 (C-7′). ESI-MS: *m/z* 339 $[M+H]^+$. (*E*)-2,3-difluorobenzyl 3-(3,4-dihydroxyphenyl)acrylate (3j) light yellow solid; yield: 20.9%; mp: 140~142°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.58 (1H, d, *J* = 15.9 Hz, H-7), 7.30 - 7.22 (2H, m, H-4′, H-5′), 7.17 (m, 1H, H-6′), 7.04 (1H, d, *J* = 2.0 Hz, H-2), 6.95 (1H, dd, *J* = 8.2, 2.1 Hz, H-6), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 6.30 (1H, d, *J* = 15.9 Hz, H-8), 5.30 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.6 (C-9), 149.8 (C-4), 147.6 (C-3), 147.3 (C-3′), 146.9 (C-7), 137.8 (C-2′), 127.6 (C-1), 126.6 (C-1'), 125.7 (C-5′), 125.6 (C-6′), 123.1 (C-6), 118.3 (C-4′), 116.5 (C-5), 115.2 (C-8), 114.5 (C-2), 60.4 (C-7′). ESI-MS: *m/z* 307 $[M+H]^+$. (*E*)-2-(trifluoromethyl)benzyl3-(3,4-dihydroxyphenyl)acrylate (3k) light yellow solid; yield: 20.7%; mp: 101~103°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.74 (1H, d, *J* = 7.9 Hz, H-3′), 7.68 - 7.63 (2H, m, H-4′, H-5′), 7.60 (1H, d, *J* = 15.9 Hz, H-7), 7.52 (1H, m, H-6′), 7.05 (1H, d, *J* = 2.0 Hz, H-2), 6.96 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.32 (1H, d, *J* = 15.9 Hz, H-8), 5.40 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.6 (C-9), 149.8 (C-4), 147.6 (3), 146.9 (C-7), 136.0 (C-1′), 133.6 (C-5′), 131.3 (C-6′), 129.6 (C-4′), 127.6 (C-2′), 127.1 (C-1), 127.1 (C-3′), 124.7 (C-8′), 123.1 (C-6), 116.5 (C-5), 115.2 (C-8), 114.5 (C-2), 63.5 (C-7′). ESI-MS: *m/z* 339 $[M+H]^+$. (*E*)-2-(thiophen-2-yl)ethyl 3-(3,4-dihydroxyphenyl)acrylate (3l) light yellow solid; yield: 41.4%; mp: 119~120°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.55 (1H, d, *J* = 15.9 Hz, H-7), 7.22 (1H, dd, *J* = 5.1, 1.2 Hz, H-4′), 7.03 (1H, d, *J* = 2.0 Hz, H-2), 6.97 - 6.90 (3H, m, H-6, H-3′, H-5′), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.25 (1H, d, *J* = 15.9 Hz, H-8), 4.36 (2H, t, *J* = 6.6 Hz, H-7′), 3.22 (2H, t, *J* = 6.6 Hz, H-6′); ^{13}C NMR (125 MHz, CD_3OD) δ: 169.0 (C-9), 149.6 (C-4), 147.1 (C-3), 146.8 (C-7), 141.4 (C-1′), 127.8 (C-5′), 127.7 (C-1), 126.7 (C-4′), 125.0 (C-3′), 123.0 (C-6), 116.5 (C-5), 115.1 (C-8), 114.9 (C-2), 65.9 (C-7′), 30.3 (C-6′). ESI-MS: *m/z* 291 $[M+H]^+$. (*E*)-2,6-difluorobenzyl 3-(3,4-dihydroxyphenyl)acrylate (3m) light yellow solid; yield: 33.0%; mp: 158~161°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.54 (1H, d, *J* = 15.9 Hz, H-7), 7.44 (1H, m, H-4′), 7.07 - 6.98 (3H, m, H-2, H-3′, H-5′), 6.93 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.76 (1H, d, *J* = 8.2 Hz, H-5), 6.25 (1H, d, *J* = 15.9 Hz, H-8), 5.30 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.6 (C-9), 162.3 (C-2′, C-6′), 149.7 (C-4), 147.4 (C-3), 146.8 (C-7), 132.4 (C-4′), 127.6 (C-1), 123.1 (C-6), 116.5 (C-5), 115.1 (C-8), 114.4 (C-2), 112.6 (C-1′), 112.4 (C-3′, C-5′), 54.8 (C-7′). ESI-MS: *m/z* 307 $[M+H]^+$. (*E*)-2,3,5,6-tetrafluorobenzyl 3-(3,4-dihydroxyphenyl)acrylate (3n) light yellow solid; yield: 17.5%; mp: 120~122°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.57 (1H, d, *J* = 15.9 Hz, H-7), 7.47 (1H, tt, *J* = 10.2, 7.5 Hz, H-4′), 7.03 (1H, d, *J* = 2.0 Hz, H-2), 6.95 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 6.26 (1H, d, *J* = 15.9 Hz, H-8), 5.35 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.2 (C-9), 149.8 (C-4), 147.9 (C-3), 147.8 (C-3′, C-5′), 146.9 (C-7), 134.5 (C-2′, C-6′), 127.5 (C-1), 123.2 (C-6), 116.5 (C-5), 115.2 (C-8), 114.0 (C-2), 108.0 (C-1′), 101.1 (C-4′), 54.6 (C-7′). ESI-MS: *m/z* 343 $[M+H]^+$. (*E*)-2,4-dichlorobenzyl 3-(3,4-dihydroxyphenyl)acrylate (3o) light yellow solid; yield: 19.4%; mp: 140~142°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.60 (1H, d, *J* = 15.9 Hz, H-7), 7.52 - 7.48 (2H, m, H-3′, H-6′), 7.37 (1H, dd, *J* = 8.3, 2.1 Hz, H-5′), 7.05 (1H, d, *J* = 2.1 Hz, H-2), 6.96 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.32 (1H, d, *J* = 15.9 Hz, H-8), 5.29 (2H, s, H-7′); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.6 (C-9), 149.8 (C-4), 147.7 (C-3), 146.9 (C-7), 135.8 (C-1′), 135.5 (C-4′), 134.4 (C-2′), 132.2 (C-3′), 130.3 (C-6′), 128.5 (C-5′), 127.6 (C-1), 123.1 (C-6), 116.5 (C-5), 115.2 (C-8), 114.4 (C-2), 63.8 (C-7′). ESI-MS: *m/z* 340 $[M+H]^+$. (*E*)-4-(methylsulfonyl)benzyl 3-(3,4-dihydroxyphenyl)acrylate (3p) white solid; yield: 21.3%; mp: 155~157°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.97 (2H, d, *J* = 8.4 Hz, H-3′, H-5′), 7.67 (2H, d, *J* = 8.4 Hz, H-2′, H-6′), 7.61 (1H, d, *J* = 15.9 Hz, H-7), 7.06 (1H, d, *J* = 2.0 Hz, H-2), 6.97 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.34 (1H, d, *J* = 15.9 Hz, H-8), 5.33 (2H, s, H-7′), 3.12 (3H, s, -CH3); ^{13}C NMR (125 MHz, CD_3OD) δ: 168.6 (C-9), 149.8 (C-4), 147.7 (C-3), 146.9 (C-7), 144.4 (C-1′), 141.6 (C-4′), 129.5 (C-3′, C-5′), 128.7 (C-2′, C-6′), 127.6 (C-1), 123.1 (C-6), 116.5 (C-5), 115.2 (C-8), 114.5 (C-2), 65.9 (C-7′), 44.4 (C-8′). ESI-MS: *m/z* 349 $[M+H]^+$. (*E*)-(tetrahydrofuran-2-yl)methyl 3-(3,4-dihydroxyphenyl)ac-

rylate (3q) light yellow solid; yield: 34.8%; mp: 112~114°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.56 (1H, t, *J* = 12.1 Hz, H-7), 7.04 (1H, d, *J* = 2.0 Hz, H-2), 6.95 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.27 (1H, d, *J* = 15.9 Hz, H-8), 4.25 - 4.07 (3H, m, H-1′, H-6′), 3.89 (1H, m, H-3′), 3.80 (1H, m, H-3′), 2.05 (1H, m, H-5′), 2.00 - 1.87 (2H, m, H-4′), 1.69 (1H, m, H-5′); ^{13}C NMR (125 MHz, CD_3OD) δ: 169.0 (C-9), 149.6 (C-4), 147.2 (C-3), 146.8 (C-7), 127.7 (C-1), 123.0 (C-6), 116.5 (C-5), 115.1 (C-8), 114.8 (C-2), 78.2 (C-1′), 69.4 (C-6′), 67.3 (C-3′), 28.9 (C-5′), 26.6 (C-4′). ESI-MS: *m/z* 265 $[M+H]^+$. (*E*)-3-hydroxy-4-methoxybenzyl 3-(3,4-dihydroxyphenyl)acrylate (3r) light yellow solid; yield: 33.0%; mp: 146~148°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.55 (1H, d, *J* = 15.9 Hz, H-7), 7.03 (1H, d, *J* = 1.7 Hz, H-2), 6.96 - 6.73 (5H, m, H-5, H-6, H-2′, H-5′, H-6′), 6.27 (1H, d, *J* = 15.9 Hz, H-8), 5.08 (2H, s, H-7′), 3.84 (3H, s, -OCH3); ^{13}C NMR (125 MHz, CD_3OD) δ: 169.1 (C-9), 149.6 (C-4), 149.1 (C-4′), 147.6 (C-3), 147.0 (C-7), 146.8 (C-3′), 130.6 (C-1′), 127.7 (C-1), 123.0 (C-6), 121.1 (C-6′), 116.5 (C-2′), 116.5 (C-5), 115.1 (C-5′), 115.1 (C-8), 112.6 (C-2), 67.1 (C-7′), 56.4 (C-8′). ESI-MS: *m/z* 317 $[M+H]^+$. (*E*)-methyl 3-(3,4-dihydroxyphenyl)acrylate (3s) light yellow solid; yield: 81%; mp: 166~167°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.54 (1H, d, *J* = 15.9 Hz, H-7), 7.03 (1H, d, *J* = 2.1 Hz, H-2), 6.94 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 6.26 (1H, d, *J* = 15.9 Hz, H-8), 3.75 (3H, s, H-10); ^{13}C NMR (125 MHz, CD_3OD) δ: 169.8 (C-9), 147.9 (C-4), 147.0 (C-3), 146.9 (C-7), 127.6 (C-1), 122.9 (C-6), 116.5 (C-5), 115.1 (C-8), 114.8 (C-2), 52.0 (C-10). ESI-MS: *m/z* 195 $[M+H]^+$. (*E*)-ethyl 3-(3,4-dihydroxyphenyl)acrylate (3t) light yellow solid; yield: 36.5%; mp: 149~151°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.53 (1H, d, *J* = 15.9 Hz, H-7), 7.03 (1H, d, *J* = 2.0 Hz, H-2), 6.94 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 6.25 (1H, d, *J* = 15.9 Hz, H-8), 4.21 (2H, q, *J* = 7.1 Hz, H-10), 1.31 (3H, t, *J* = 7.1 Hz, H-11); ^{13}C NMR (125 MHz, CD_3OD) δ: 169.3 (C-9), 149.6 (C-4), 146.8 (C-3), 146.7 (C-7), 127.7 (C-1), 122.9 (C-6), 116.5 (C-5), 115.3 (C-8), 115.1 (C-2), 61.4 (C-10), 14.6 (C-11). ESI-MS: *m/z* 209 $[M+H]^+$.

Table 1. Inhibitory Activities of 20 Compounds against LPS-Induced NO Production in RAW264.7 Macrophages (n = 2, means ± S.E.M.)

Compd.	Inhibition (%)	Compd.	Inhibition (%)
3a	25.7 ± 3.95	3k	17.8 ± 0.02
3b	15.9 ± 0.34	3l	59.0 ± 0.61
3c	45.9 ± 2.14	3m	6.5 ± 1.15
3d	25.9 ± 0.90	3n	21.9 ± 0.44
3e	49.4 ± 0.42	3o	12.7 ± 0.57
3f	34.4 ± 1.89	3p	30.9 ± 5.78
3g	16.1 ± 7.04	3q	25.6 ± 9.39
3h	25.6 ± 3.16	3r	59.7 ± 1.52
3i	14.8 ± 0.36	3s	60.1 ± 0.41
3j	16.8 ± 3.53	3t	78.9 ± 0.80

Table 2. Inhibitory Activities of 3l, 3r~3t against LPS-Induced NO Production in RAW264.7 Macrophages (n = 2, means ± S.E.M.)

Compd*.	IC_{50} (μM)
3l	7.4 ± 3.28
3r	5.9 ± 1.35
3s	3.3 ± 0.41
3t	2.2 ± 0.22
aminoguanidine	177.3 ± 24.20

*The concentration gradient of 3l, 3r and 3s was 0.016 to 50 μM, and 3t was 0.0005 to 50 μM.

mmol) and the corresponding alcohol (0.5 mmol) in THF were refluxed for 5 h [9]. The resulting white precipitate N,N'-dicyclohexylurea (DCU) was removed by filtration, and the residue was subjected to flash chromatography, purified by dichloromethane/methanol (70:1) as a eluent. The Synthetic route of structural modification is showed in **Figure 1**.

Cell cultures

RAW 264.7, a mouse macrophage cell line, was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cell were cultured in phenol red-free Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% CO_2 humidified atmosphere at 37°C.

Measurement of nitric oxide

The concentration of NO was assessed as an indicator of NO production with Griess reaction. Briefly, RAW264.7 macrophages were harvested and seeded in 96-well plates (1×10^5 cells/well) for NO production. After 6 h, the plates were pretreated with various concentrations of samples for 30 min and incubated with LPS (1 μg/mL) for 24 h. The amount of NO was determined by the nitrite concentration in the cultured RAW264.7 macrophage supernatants with the Griess reagent. Absorbance was measured at 540 nm using a microplate reader, a standard curve of sodium nitrite solution was obtained to determine the nitrite concentra-

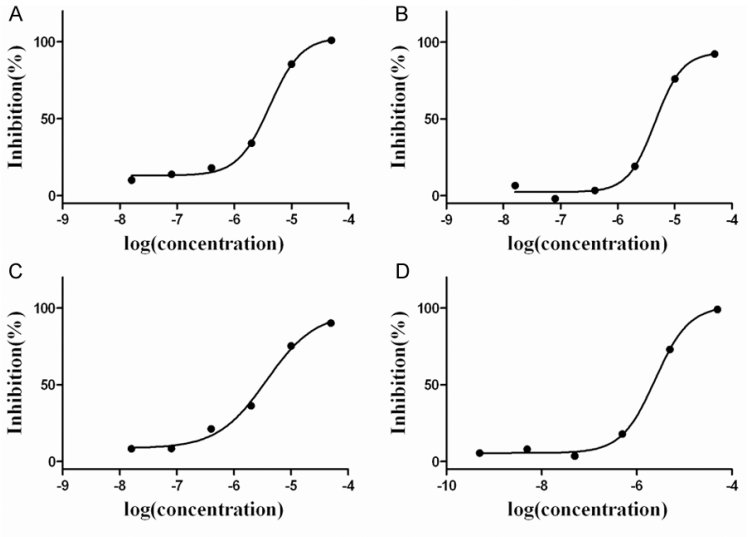

Figure 2. A representative IC_{50} testing curves diagram of 3l, 3r~3t. A. 3l, the concentration gradient was 50, 10, 2, 0.4, 0.08, 0.016 μM, IC_{50} = 4.1 μM; B. 3r, the concentration gradient was 50, 10, 2, 0.4, 0.08, 0.016 μM, IC_{50} = 4.5 μM; C. 3s, the concentration gradient was 50, 10, 2, 0.4, 0.08, 0.016 μM, IC_{50} = 3.7 μM; D. 3t, the concentration gradient was 50, 5, 0.5, 0.05, 0.005, 0.0005 μM, IC_{50} = 2.4 μM.

tion. Inhibition of samples against NO production was calculated using the following formula:

NO inhibition (%) = (NO concentration$_{LPS\text{-}treated}$ - NO concentration$_{sample\text{-}treated}$)/NO concentration$_{LPS\text{-}treated}$ × 100%.

Statistical analysis

Statistical analysis was performed with one-way ANOVA followed by a post hoc LSD test. The data are presented as the means ± S.E.M.

Results and discussion

Preliminarily the 20 compounds were screened for the inhibitory activity against the LPS-induced NO production at the concentration of 10 μM. The results were showed in **Table 1**.

When the testing samples were at a concentration of 10 μM, the inhibition of 3l, 3r, 3s and 3t on LPS-induced NO production in RAW264.7 macrophages were greater than 50%, with the values of 59.0%, 59.7%, 60.1% and 78.9%, respectively. Further assay showed the four compounds' IC_{50} (**Table 2**). **Figure 2** was a representative IC_{50} testing curve diagram of 3l, 3r, 3d and 3t.

As is clearly shown in **Figure 2**, which the curves were obtained from one independent test, the inhibitory activities of the four compounds showed a dose-dependent relationship between different testing concentrations. The shape and tendency of the four curves were nearly approximated, simply we can see that the inhibition was weak at the low concentration, and it came to a rise during a very short range of concentration, then the inhibition level reach its peak. The result revealed that the ester moiety of these compounds was essential to the inhibitory activities against NO production, compared with the lead compound caffeic acid with IC_{50} value of 330 μM in the same pharmacological model [7]. Take the different structures of these compounds into consideration, we can see that compounds synthesized from the substituted benzyl alcohol, showed weak inhibitory activities against NO production. On the other hand, the simple esters derivatives 3l, 3s and 3t showed potent inhibition, the simple and rotatable side chain may match the functional target better than the substituted benzyl esters. It is notable that the compound 3r, also synthesized from the substituted benzyl alcohol, showed potent inhibition, while the analogous compound 3f showed a weak inhibitory activity (IC_{50} > 10 μM), the hydroxyl in the meta-position may cause the difference.

Conclusion

In summary, we have successfully synthesized 20 caffeic acid derivatives by a simple and fast method, they all showed inhibitory activities against the NO production in RAW264.7 macrophages compared with the lead compound caffeic acid. Further study showed that four of the derivatives, 3l, 3r, 3s and 3t, exhibited potent inhibitory activities, with IC_{50} values of 7.4, 5.9, 3.3 and 2.2 μM, respectively, they could be selected as compromising compounds for the later pharmacological study.

It is a new approach for drugs discovery by the mean of modifying the existing natural products, the derivatives could also possess potent biological activities [10]. NO is a hotspot in recent research field of anti-inflammatory, there is a great significance in finding new natural derivatives consisting potent NO inhibitory activities [11]. Caffeic acid derivatives are compromising candidates in recent years' drugs discovery, showed various pharmacological activities, including anti-tumor, antioxidant, anti-inflammatory and immunoregulation. Fortunately, four caffeic acid ester derivatives were determined have potent inhibitory activities against the NO production in RAW264.7 macrophages. The exploration of the pharmacological application of these bioactive compounds represents our future endeavors.

Acknowledgements

The work was supported by the Natural Science Foundation of Shanghai, China (11ZR1446300), the Shanghai Committee of Science and Technology, China (12431900901), the Fundamental Research Funds for the Central Universities of China and in part by National Science and Technology Major Projects of China for "Major New Drugs Innovation and Development" (2012ZX09J12202-004).

Disclosure of conflict of interest

None.

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