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Synthesis, characterization and vasculoprotective effects of nitric oxide-donating derivatives of chrysin

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ABSTRACT

Vascular complications are major causes of disability and death in patients with diabetes mellitus. It is often characterized by endothelial dysfunction. Studies have shown that either the loss of nitric oxide bioactivity or the decreased biosynthesis of NO is a central mechanism in endothelial dysfunction. As such, the delivery of exogenous NO is an attractive therapeutic option that has been used to slow the progress of diabetic vascular complications. In this paper, a novel group of hybrid nitric oxide-releasing chrysin derivatives was synthesized. The results indicated that all these chrysin derivatives exhibited in vitro inhibitory activities against aldose reductase and advanced glycation end-products formation. And some of them were even found to increase the glucose consumption of HepG2 cells. Furthermore, all compounds released NO upon incubation with phosphate buffer at pH 7.4. These hybrid ester NO donor prodrugs offer a potential drug design concept for the development of therapeutic or preventive agents for vascular complications due to diabetes.

1. Introduction

Diabetes is a chronic metabolic disorder characterized by hyperglycemia and followed by micro and macrovascular complications. Vascular endothelial dysfunction has been implicated in the development and progress of diabetic vascular complications.[1] An early marker of endothelial dysfunction is the reduction of endothelium-dependent vasodilation due to the reduced bioavailability of nitric oxide (NO).[2] Many common time-honored drugs (e.g., glycerol trinitrate) act via the release of exogenous NO. Hybrid NO donor drugs represent a novel type of drug that retains the pharmacological activity of the parent compound but also has the biological actions of NO.[3]

Chrysin (5,7-dihydroxyflavone), a natural, widely distributed flavonoid, has been reported to possess diverse biologically active properties, including antioxidant,[4,5] anticancer,[6] anxiolytic,[7] anti-inflammatory,[8,9] anti-diabetic[10] and anti-glucosidase characteristics.[11] Recently, several researchers have attempted to modify the profile of chrysin and found that some chrysin derivatives have diverse activities including anti-diabetic effects.[12–17] Shin et al. synthesized a series of chrysin derivatives in which the butyl compound was found to have the most potent hypoglycemic effect on diabetic mice.[17] This study aims to investigate a synthesis approach based on grafting an NO-releasing moiety to chrysin derivatives via a variable spacer. We suppose that the new pharmacodynamic hybrids will present the combined advantages of hypoglycemic effect with a slow release of NO, thus mimicking the effects of endogenous NO, which can improve endothelial dysfunction and prevent the progression of diabetic complications.

Research has indicated that no matter what is the cause of diabetes, the result is always hyperglycemia.[18] Hyperglycemia increases the risk of both micro and macrovascular diseases in patients with diabetes.[19] In both type 1 and type 2 diabetes, large, prospective clinical studies have shown a strong relation between time-averaged mean values of glycemia, measured as glycated hemoglobin A1c (HbA1c), and diabetic complications.[20,21] These studies are the basis for the American Diabetes Association's current recommended treatment goal that HbA1c should be <7%.[22] Selvin performed a *meta*-analysis of 13 cohort studies and indicated that for type 2 diabetes, a 1% point increase in HbA1c was associated with a significant 18% increase in the risk of coronary heart disease or stroke and a 28% increase in the risk of peripheral vascular disease.[23] The metabolism of excess glucose drives several damage pathways, such as oxidative stress, generation of advanced glycation end-products (AGE), increased aldose reductase (AR)-related polyol pathway flux, activation of protein kinase C, increased hexosamine pathway flux, which leads to endothelial dysfunction. This indicates that hyperglycemia is a major cause of the vascular complications in diabetes cases.

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A great deal of evidence indicates that the non-enzymic glycation of proteins is implicated in a number of biochemical abnormalities associated with diabetes. The glycation reaction occurs between the carbonyl group of sugars and the amino group of proteins, which finally results in the formation of AGE. The increased serum concentration of AGE in patients with diabetes has been associated with the dysfunction of the vascular endothelium.[24] Since studies have shown the role of AGE in promoting diabetic complications, it is believed that the inhibition of AGE formation may prevent the progression of diabetic complications.

AR is the first, rate-limiting enzyme in the polyol pathway of glucose metabolism, which converts glucose into sorbitol. The hyperactivity of AR has been linked to the development of cardiovascular and neurological complications in diabetic patients.[25] In line with this, patients treated with AR inhibitors showed an improvement of vascular and other complications of diabetes, such as peripheral neuropathy, retinopathy, and cataracts.[25] Studies of this nature make the development of a potent AR inhibitor an obvious and attractive strategy to prevent or delay the onset and progression of diabetic complications. In this light, some research has shown that NO donors can prevent AR activation and sorbitol accumulation during the onset of diabetes.[26,27]

We report herein the synthesis and characterization of several NO-donating derivatives of chrysin. The NO-releasing capacities, glucose consumption promotion, AR and AGE inhibitory effects of theses derivatives were explored in vitro. We hope the results to be of value in further understanding the potential uses of chrysin NO-donating derivatives for the development of therapeutic and preventive agents for vascular complications of diabetes.

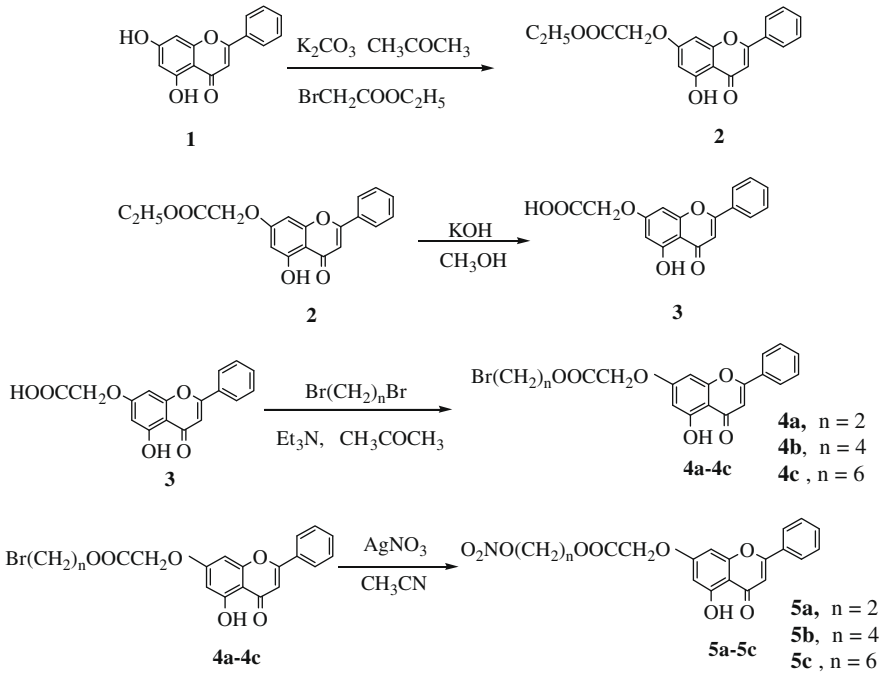
2. Chemistry

The preparation of the nitrate derivatives follows the synthetic routes illustrated in Scheme 1. Chrysin **1** was reacted with bromoacetate to produce compound **2**. Subsequent hydrolysis of this compound and reaction with a dibromo alkane produced compounds **4a–c**. These compounds were then reacted with $AgNO_3$ (silver nitrate), producing products **5a–c**.

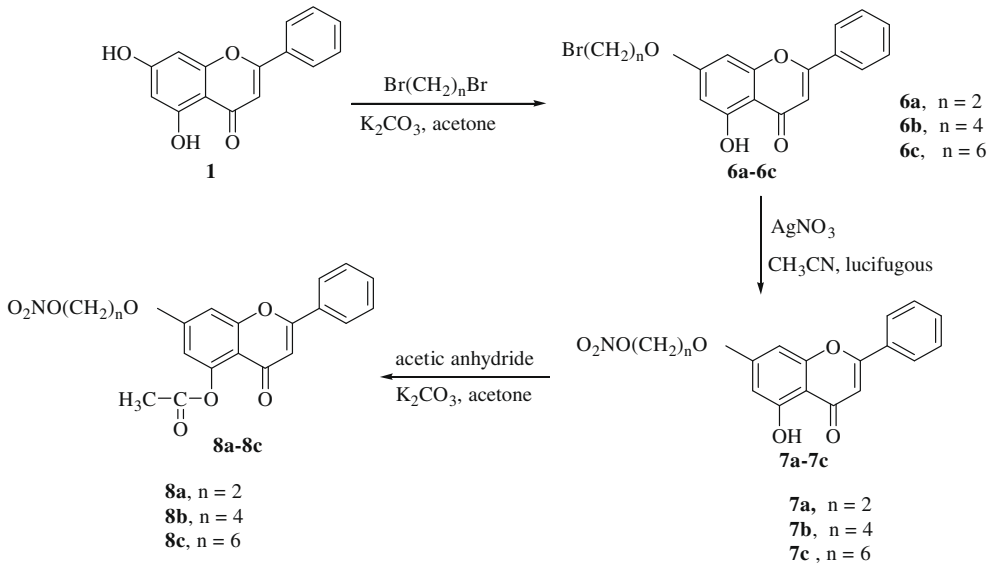
The synthesis of compounds **7a–c** and **8a–c** was accomplished according to the general pathway illustrated in Scheme 2. An alkyl chain with a bromide group was introduced to the 7-position of chrysin **1** by a selective reaction with an appropriate dibromo alkane, yielding the 7-bromo alkane derivatives **6a–c**, which were subsequently converted to the nitro esters **7a–c** via treatment with silver nitrate ($AgNO_3$) in anhydrous acetonitrile. These compounds were then reacted with acetic anhydride to produce the final substituted nitro esters **8a–c**.

3. Results and discussion

In the present study, we investigated AR and AGE assays, NO-releasing capacities were also determined for all the target compounds in vitro. Structure–activity relationships acquired for the assays indicated that the hybrid nitrooxyl carboalkoxy methyl chrysin (**5a–c**) resulted in high inhibitory activities on AGE formation (IC_{50} = 43.89 ± 6.76 to 137.6 ± 8.86 μM, Table 1), as well as high inhibitory effects on AR (IC_{50} = 0.13 ± 0.03 to 0.30 ± 0.01 μM). The potency of AGE formation inhibition was 6–18.8 times that of the positive reference compound aminoguanidine. AR inhibition showed a potency that was over 10 times higher than the positive reference compound quercetin. Derivatives **5b**, **7b**, and **8b** exerted higher AR inhibition activities than the other serial compounds, indicating that modified compounds with butyl groups were more effective in inhibiting AR. NO appeared to be released from all the chrysin derivatives upon incubation with phosphate-buffered saline solution (PBS at pH 7.4) in the presence of L-cysteine. The percentage of released NO varied from 1.21 ± 0.20% to 1.77 ± 0.10%, indicating a slow release. The amount of NO released from sodium nitroprusside (SNP), however, was substantially higher (10.42 ± 1.80%). These results should be assessed based on the actual additional amount of NO needed by the body. The concentrations of NO required to mediate primarily protective effects are extremely low (i.e., picomolar to nanomolar range). At higher concentrations, particularly under conditions of oxidative stress, NO is highly cytotoxic, a feature that is exploited by inflammatory cells in response to invading pathogens.[28] In our

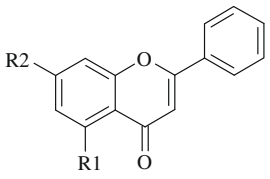


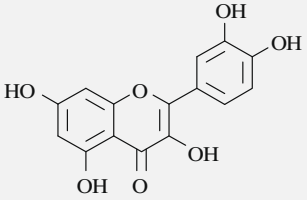
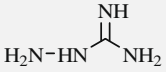
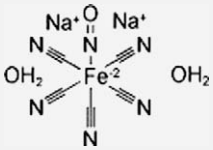
Scheme 1. Synthesis of chrysin derivatives **5a–c**.



Scheme 2. Synthesis of chrysin derivatives **7a–c** and **8a–c**.

Table 1
Structure and in vitro AR, AGE inhibition and NO-releasing properties of the target compounds



Compound	Structure		AR inhibition[a]	AGE inhibition[b]	%NO released[c]
	R1	R2	IC_{50} (μmol/L)		
0.1%DMSO					
Chrysin	OH	OH	7.79 ± 0.57	63.54 ± 6.42	
5a	OH	$NO_3(CH_2)_2COOCH_2O$	0.30 ± 0.01	43.89 ± 6.76	1.77 ± 0.10
5b	OH	$NO_3(CH_2)_4COOCH_2O$	0.13 ± 0.03	52.47 ± 3.73	1.26 ± 0.09
5c	OH	$NO_3(CH_2)_6COOCH_2O$	0.21 ± 0.07	137.6 ± 8.86	1.38 ± 0.06
7a	OH	$NO_3(CH_2)_2O$	0.49 ± 0.006	⩾300	1.67 ± 0.15
7b	OH	$NO_3(CH_2)_4O$	0.29 ± 0.009	75.5 ± 8.07	1.43 ± 0.04
7c	OH	$NO_3(CH_2)_6O$	0.71 ± 0.06	⩾300	1.21 ± 0.20
8a	CH_3COO	$NO_3(CH_2)_2O$	0.86 ± 0.02	⩾300	1.68 ± 0.11
8b	CH_3COO	$NO_3(CH_2)_4O$	0.39 ± 0.04	⩾300	1.48 ± 0.16
8c	CH_3COO	$NO_3(CH_2)_6O$	0.42 ± 0.03	⩾300	1.43 ± 0.22
Quercetin[d]			2.85 ± 0.04		
Aminoguanidine[e]				826.22 ± 9.26	
SNP[f]					10.42 ± 1.80

Each value represents the mean ± S.D. ($n = 3$).

[a] The concentration required for a 50% inhibition of the decrease in the optical density of NADPH at 340 nm relative to 0.1% DMSO. IC_{50} values were calculated from the dose inhibition curve.

[b] The concentration required for a 50% inhibition of the fluorescence intensity of AGE relative to 0.1% DMSO, IC_{50} values were calculated from the dose inhibition curve.

[c] Percent of nitric oxide released based on a theoretical maximum release of 1 mol of NO/mol of the target compounds.

[d] Quercetin was used as positive control for AR inhibition test.

[e] Aminoguanidine (AG) was used as positive control for AGE inhibition test.

[f] SNP (sodium nitroprusside) was used as positive control for NO releasing test.

study, the release of adequate amounts of NO required to provide protective effects were balanced with the concentration range required for the sufficient activity of chrysin derivatives.

Hyperglycemia is the primary cause of vascular complications in diabetes. To date, the benefit of glycemic control with respect to the macrovascular complications in diabetes remains controversial. Nevertheless, the importance of glycemic control in preventing the microvascular complications of retinopathy, nephropathy, and neuropathy in patients with type 2 diabetes has been consistently demonstrated over the past 20 years.[29] Some researchers demonstrated that NO could stimulate glucose metabolism or transport in adipocytes or skeletal muscle.[30–32] Shin et al. have found that a series of chrysin alkyl derivatives have potent hypoglycemic effect in vivo.[17] To evaluate the hypoglycemic effects of the target compounds, we determined the glucose consumption of HepG2 cells treated with chrysin, chrysin derivatives, and positive reference rosiglitazone (Fig. 1). The results showed that **5b**, **5c**, **7a**, **7b**, **7c**, and rosiglitazone significantly promoted the glucose consumption of HepG2 cells compared to the blank vehicle 0.1% DMSO (P <0.05). In contrast, chrysin significantly reduced glucose consumption (P <0.05 vs 0.1% DMSO, Fig. 2). It is of note that **5a**, **8a**, **8b**, **8c** had no effect on the glucose consumption of HepG2 cells.

An O7-nitrooxyalkyl nitric oxide donor moiety is attached to the chrysin parent compound and its O7-[(nitrooxyl)alkyloxycarbonyl] methyl analogs were synthesized (**5a–c**). Furthermore, a novel class of hybrid ester prodrugs (**7a–c**) was synthesized. AR, AGE formation, and glucose consumption assays, as well as NO-releasing capacities, showed that: (i) the [(nitrooxyl)ethoxycarbonyl] methyl derivatives are more potent inhibitors of AR and AGE, (ii) the parent compound chrysin is a potent AGE inhibitor, (iii) all the hybrid ester prodrugs release NO slowly upon incubation with PBS in the presence of L-cysteine, and (iv) the O7-nitrooxyethyl chrysin derivatives (**7a–c**), O7-[(nitrooxyl)butoxycarbonyl] methyl analog (**5b**), and O7-[(nitrooxyl)hexoxycarbonyl] methyl analog (**5c**) significantly promotes the glucose consumption of HepG2 compared to the blank vehicle 0.1% DMSO. In order to test our hypothesis that the 5-OH group contributes to the observed activities, compounds 8a–**c** or O5-acetyl, O5-butyryl, and O5-caproyl, respectively, were designed in a flavone skeleton. The changes made decreased the inhibition of AR and AGE formation, as well as glucose consumption promotion, which suggests that the 5-OH group is the key group that determines the biological activities.

In conclusion, these hybrid ester NO donor prodrugs offer a potential drug design concept for the development of therapeutic or preventive agents for vascular complications of diabetes.

4. Experimental

4.1. Biological activity

4.1.1. Glucose consumption assay[33]

HepG2 cells (obtained from the Cell Culture Center of the Central South University, Changsha, China) grown in high glucose DMEM (purchased from Hyclone) containing 10% FBS (obtained from Sijiqing Biological Engineering Materials, Hangzhou, China) were plated into 96-well tissue culture plates with some wells left blank. After the cells reached 80–90% confluence, the medium was replaced by DMEM supplemented with 1% FBS. Twelve hours later, the medium was removed and the same culture medium containing target compounds, chrysin or rosiglitazone (standard product) was added to wells. After 24 h of treatment, glucose was assayed in 10 μL of medium by enzymatic methods with diagnostic kits (Nanjing Jiancheng Bioengineering Inst.). Data were expressed as glucose concentrations of blank wells subtracting the remaining glucose in the cell plated wells. Following the glucose measurement in the medium, a MTT assay was used to monitor the cell number and to adjust the glucose consumption values.

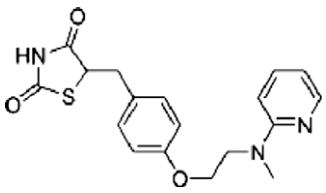


Figure 1. The structure of rosiglitazone.

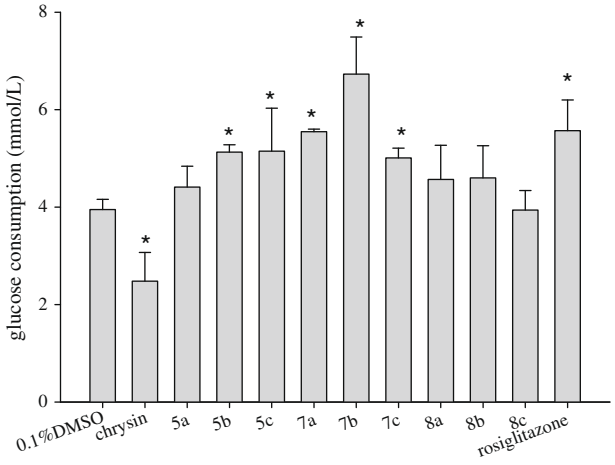


Figure 2. Effects of 10 μmol/L chrysin, chrysin derivatives (**5a–c**, **7a–c** and **8a–c**) and rosiglitazone on glucose consumption of HepG2 cells (values are mean ± S.D.; n = 3; $^{*}P$ <0.05 vs 0.1% DMSO).

4.1.2. AR inhibitory activity[34]

After homogenization, centrifugation, $(NH_4)_2SO_4$ fractionation (30–75%) and dialysis, the crude AR from calf lenses was obtained. The inhibitory activity of the compounds on aldose reductase was carried out using a 200 μL volume optimized amount of enzyme (750 μg/mL protein), and different concentrations of the compounds (0.1–10 μmol/L) in 50 mM PBS (pH 6.0) containing 5 mM β-mercaptoethanol, 0.24 mM NADPH, 0.4 M Li_2SO_4 and 2.5 mM of glyceraldehyde (substrate) were added. The reaction was initiated by addition of glyceraldehyde and the decrease in the optical density of NADPH at 340 nm was recorded for 3 min. IC_{50} of the compounds was calculated using Sigmaplot software and expressed as mean ± S.D. of triplicate experiments. The flavonoid quercetin was used as a reference in the AR assay.

4.1.3. Inhibitory activity of AGE formation[35]

To prepare the AGE reaction solution, 10 mg/mL of bovine serum albumin in 50 mM PBS (pH 7.4) was added to 0.2 M glucose, and 0.02% sodium azide was added to prevent bacterial growth. The reaction mixture (950 μL) was then mixed with various concentrations of the target compounds (1–300 μmol/L). After incubating at 37 °C for 14 d, the fluorescence intensity of AGE was determined by a LS-55 fluorospectrophotometer (PE, USA) with excitation and emission wavelengths at 350 nm and 420 nm, respectively. IC_{50} of the compounds was calculated using sigmaplot software and expressed as mean ± S.D. of triplicate experiments. Aminoguanidine hydrochloride was used as a reference compound.

4.1.4. Detection of nitrite[36]

A solution of the appropriate compound (20 μL) in dimethylsulfoxide (DMSO) was added to 2 mL of 1:1 v/v mixture of

50 mM PBS (pH 7.4) with MeOH, containing of 5×10^{-4} M L-cysteine. The final concentration of target compounds was 10^{-4} M. After 1 h at 37 °C, 1 mL of the reaction mixture was treated with 250 μL of Griess reagent [sulfanilamide (4 g), *N*-naphthylethylenediamine dihydrochloride (0.2 g), 85% phosphoric acid (10 mL) in distilled water (final volume: 100 mL)]. After 10 min at room temperature, the absorbance was measured at 540 nm. Sodium nitrite standard solutions (10–80 nmol/mL) were used to construct the calibration curve. The results were expressed as the percentage of NO released ($n = 3$) relative to a theoretical maximum release of 1 mol NO/mol of test compound.

4.1.5. Statistical analysis

Data were shown as mean ± S.D. Differences between individual groups were analyzed by using ANOVA followed by Dunett's test. A difference with a P value of <0.05 was considered to be significant.

4.2. Chemical synthesis

4.2.1. Materials and instrumentation

All chemicals were of reagent grade and commercially available. Chrysin (>98%) was purchased from ShanXi Huike Co., Ltd, Jiangsu, China, and used without further purification. All the ^{1}H NMR spectra were recorded on a Bruker AV 400 model Spectrometer in DMSO-d_6. Chemical shifts (d) for ^{1}H NMR spectra were reported in parts per million of residual solvent protons. ESI-MS spectra were recorded on a ThermoFinnigan LCD-Advantage mass spectrometer. Melting points were measured using a Beijing Biotech X-4 micromelting point apparatus.

4.2.2. General method for synthesis of compounds 5a–c, 7a–c and 8a–c

4.2.2.1. O7-(Ethoxycarbonyl) methyl chrysin (2). Anhydrous potassium carbonate (0.69 g, 5.0 mmol) was added to a solution of **1** (2.54 g, 10 mmol) in anhydrous acetone (150 mL), followed by refluxing until the solution became clear. Methyl bromoacetate (2.3 mL, 20 mmol) was then dribbled into the mixture, and acetone (10 mL) was added. The solution was refluxed for 24 h and vacuum filtered. The filter liquor was concentrated to obtain a yellow solid, which was subsequently washed with petroleum, 1% NaOH, and water. The solid was dried (2.3 g, 71% yield). Mp 160–162 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.75 (s, 1H), 7.87–7.89 (m, 2H), 7.51–7.57 (m, 3H), 6.68 (s, 1H), 6.53 (d, 1H, $J = 2.3$ Hz), 6.37 (d, 1H, $J = 2.3$ Hz), 4.7 (s, 2H), 4.27–4.32 (m, 2H), 1.30–1.34 (m, 3H).

4.2.2.2. O7-Carboxymethyl chrysin (3). Potassium hydroxide (1.68 g, 30 mmol) was added to a solution of **2** (1.70 g, 5.0 mmol) in anhydrous methanol (120 mL). The solution was then mechanically stirred and heat refluxed for 8 h until a yellow solid that does not dissolve in methanol was obtained. The solid was dried with methanol and dissolved in water. Then, the pH was adjusted to the desired acidity with hydrochloric acid. The solution underwent vacuum filtration, as well as water washing and drying. (**3**: 1.4 g, 90% yield). Mp 284–285 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.78 (s, 1H), 8.09 (d, 2H, $J = 7$ Hz), 7.55–7.63 (m, 3H), 7.03 (s, 1H), 6.81 (d, 1H, $J = 2.3$ Hz), 6.39 (d, 1H, $J = 2.3$ Hz), 4.83 (s, 2H).

4.2.2.3. O7-[(Bromoethoxyl)carbonyl] methyl chrysin (4a–c). Triethylamine (3.03 g, 30 mmol) was added to a solution of **3** (3.12 g, 10 mmol) in acetone (100 mL). The mixture was then refluxed for 30 min. 1,2-Dibromoethane (9.40 g, 50 mmol) was dribbled into the mixture, followed by refluxing for 5 h and filtration to remove any precipitates that may have formed. The filter liquor was concentrated to obtain a solid, which was then washed twice with petroleum and ethanol. A light yellow solid was produced. This solid was dissolved with dichloromethane. Any insoluble solids were discarded. Recrystallization was performed with ethyl acetate/petroleum ether (**4a**: 2.01 g, 48% yield). Mp 122–124 °C. ^{1}H NMR (400 MHz, CDCl3) δ (ppm): 12.75 (s, 1H), 7.86 (d, 2H, $J = 8$ Hz), 7.53–7.55 (m, 3H), 6.64 (s, 1H), 6.53 (s, 1H), 6.36 (s, 1H), 4.71 (s, 2H), 4.46–4.48 (m, 2H), 3.37–3.39 (m, 2H).

Compounds **4b** and **4c** were synthesized according to the method for **4a**.

Compound **4b**: 46.0% yield, mp 127–128 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.76 (s, 1H), 7.85 (d, 2H, $J = 8$ Hz), 7.53–7.57 (m, 3H), 6.63 (s, 1H), 6.54 (s, 1H), 6.38 (s, 1H), 4.68 (s, 2H), 4.39 (s, 2H), 4.14 (m, 2H), 1.76 (s, 4H).

Compound **4c**: 34.7% yield, mp 100–102 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.77 (s, 1H), 7.86 (d, 2H, $J = 8$ Hz), 7.50–7.52 (m, 3H), 6.64 (d, 1H, $J = 2.8$ Hz), 6.50 (s, 1H), 6.33 (s, 1H), 4.68 (s, 2H), 4.37–4.39 (s, 2H), 4.11–4.12 (m, 2H), 1.81 (s, 4H), 1.61–1.68 (m, 4H), 1.23–1.37 (s, 4H).

4.2.2.4. O7-[(Nitrooxyl)ethoxycarbonyl] methyl chrysin (5a–c). A solution of **4a** (2.09 g, 5 mmol) was mixed with anhydrous acetonitrile (30 mL) and heated to 50 °C. To this solution, acetonitrile (20 mL) containing silver nitrate (0.43 g, 2.5 mmol) was dribbled, followed by heating to 70 °C. The reaction was allowed to proceed for 1 h in the dark. Filtration was performed to remove any silver bromide precipitates. The filtrate was concentrated and a yellow solid was produced. This solid was washed in water several times until the runoff was clear. The solid was then dried and dissolved in dichloromethane. Any undissolved solids were discarded. The solution was spun and dried to produce a yellow solid product (**5a**: 0.53 g, 44.9% yield). Mp 127–129 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.76 (s, 1H), 67.89 (d, 2H, $J = 8$ Hz), 7.53–7.58 (m, 3H), 6.69 (s, 1H), 6.55 (s, 1H), 6.39 (s, 1H), 4.77 (s, 2H), 4.54–4.57 (m, 2H), 3.55–3.57 (m, 2H). EI-MS (m/z): 401 $[M]^+$. Anal. Calcd for $C_{19}H_{15}NO_9$: C, 56.86; H, 3.77; N, 3.49. Found: C, 56.53; H, 3.95; N, 3.12.

Compounds **5b** and **5c** were synthesized according to the method for **5a**.

Compound **5b**: 75.2% yield, mp 122–123 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.78 (s, 1H), 7.89 (d, 2H, $J = 8$ Hz), 7.53–7.57 (m, 3H), 6.69 (s, 1H), 6.53 (s, 1H), 6.36 (s, 1H), 4.72 (s, 2H), 4.47 (s, 2H), 4.28 (m, 2H), 1.81 (s, 4H). EI-MS (m/z): 429 $[M]^+$. Anal. Calcd for $C_{21}H_{19}NO_9$: C, 58.74; H, 4.46; N, 3.26. Found: C, 58.57; H, 4.82; N, 3.01.

Compound **5c**: 55.0% yield, mp 97–98 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.77 (s, 1H), 7.89 (d, 2H, $J = 8$ Hz), 7.53–7.57 (m, 3H), 6.67–6.69 (d, 1H, $J = 2.3$ Hz), 6.53 (s, 1H), 6.37 (s, 1H), 4.72 (s, 2H), 4.41–4.44 (s, 2H), 4.22–4.26 (m, 2H), 1.81 (s, 4H), 1.68–1.73 (m, 4H), 1.40 (s, 4H). EI-MS (m/z): 457 $[M]^+$. Anal. Calcd for $C_{23}H_{23}NO_9$: C, 60.39; H, 5.07; N, 3.06. Found: C, 60.02; H, 5.34; N, 3.01.

4.2.2.5. O7-Bromethyl chrysin (6a). Anhydrous potassium carbonate (0.69 g, 5.0 mmol) was added to a solution of **1** (2.54 g, 10 mmol) in 150 mL of anhydrous acetone, followed by refluxing until the solution became clear. Then, 1,2-dibromo ethylene (9.4 g, 50 mmol) was added dropwise, followed by refluxing for 24 h and vacuum filtration. This procedure yielded concentrated filter liquor, from which a yellow solid was obtained. The solids were collected, washed with petroleum, 1% NaOH and water, respectively, then dried (**6a**: 1.5 g, 42% yield). Mp 157–158 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.81 (s, 1H), 8.10 (d, 2H, $J = 8$ Hz), 7.53–7.62 (m, 3H), 7.05 (s, 1H), 6.87 (d, 1H, $J = 2.3$ Hz), 36.43 (d, 1H, $J = 2.3$ Hz), 4.45–4.51 (m, 2H), 3.82–3.84 (m, 2H).

Compounds **6b** and **6c** were synthesized according to the method for **6a**.

Compound **6b**: 74.5% yield, mp 146–147 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.71 (s, 1H), 7.87–7.89 (m, 2H), 7.52–7.56 (m,

3H), 6.74 (s, 1H), 6.50 (d, 1H, J = 2.3 Hz), 6.36 (d, 1H, J = 2.3 Hz), 4.07–4.10 (m, 2H), 3.48–3.51 (m, 2H), 1.98–2.12 (m, 4H).

Compound **6c**: 60% yield, mp 126–127 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.64 (s, 1H), 7.81–7.83 (m, 2H), 7.44–7.51 (m, 3H), 6.60 (s, 1H), 6.43 (d, 1H, J = 2.3 Hz), 6.30 (d, 1H, J = 2.3 Hz), 3.96–3.99 (m, 2H), 3.35–3.38 (m, 2H), 1.76–1.86 (m, 4H), 1.45–1.46 (m, 4H).

4.2.2.6. O7-Nitrooxyethyl chrysin (7a). Compound **6a** (1.81 g, 5 mmol) was dissolved in anhydrous acetonitrile, followed by heating to 50 °C. $AgNO_3$ (2.5 mmol) and acetonitrile (20 mL) were added. The mixture was stirred by heating to 70 °C in the dark for 1 h. The precipitate was filtered out, and the solvent was evaporated under a vacuum. The resulting residue was dissolved in ethyl ether and water (**7a**: 1.44 g, 84% yield). Mp 135–136 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.78 (s, 1H), 8.07–8.09 (d, 2H, J = 8 Hz), 7.55–7.61 (m, 3H), 7.03 (s, 1H), 6.81 (d, 1H, J = 2.3 Hz), 6.39 (d, 1H, J = 2.3 Hz), 4.59(s, 2H), 4.14 (s, 2H). EI-MS (m/z): 343 $[M]^+$. Anal. Calcd for $C_{17}H_{13}NO_7$: C, 59.48; H, 3.82; N, 4.08. Found: C, 59.12; H, 4.01; N, 4.07.

Compounds **7b** and **7c** were synthesized according to the method for **7a**.

Compound **7b**: 93% yield, mp 122–123 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.73 (s, 1H), 7.87–7.89 (m, 2H), 7.53 (d, 3H, J = 8 Hz), 6.67 (s, 1H), 6.493 (d, 1H, J = 8 Hz), 6.354–6.36 (d, 1H, J = 2.3 Hz), 4.56 (s, 2H), 4.09 (s, 2H), 1.96 (s, 4H). EI-MS (m/z): 371 $[M]^+$. Anal. Calcd for $C_{19}H_{17}NO_7$: C, 61.45; H, 4.61; N, 3.77. Found: C, 61.17; H, 4.86; N, 3.59.

Compound **7c**: 91% yield, mp 105–106 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.71 (s, 1H), 7.87–7.89 (m, 2H), 7.50–7.57 (m, 3H), 6.67 (s, 1H), 6.50 (d, 1H, J = 2.3 Hz), 6.36 (d, 1H, J = 2.3 Hz), 4.46–4.4 9 (m, 2H), 4.03–4.06 (m, 2H), 1.74–1.87 (m, 4H), 1.50–1.53 (m, 4H). EI-MS (m/z): 399 $[M]^+$. Anal. Calcd for $C_{21}H_{21}NO_7$: C, 63.15; H, 5.30; N, 3.51. Found: C, 62.83; H, 5.48; N, 3.26.

4.2.2.7. O5-Acetyl-O7-nitrooxyethyl chrysin (8a). The compound **7a** (0.34 g, 1 mmol) was placed into a 250 mL flask and the following were added sequentially: anhydrous potassium carbonate (0.14 g, 1 mmol) and anhydrous acetone (50 mL). The mixture was then heated via reflux. About 4.7 mL acetic anhydride was then dribbled into the mixture. Reflux was continued for 24 h, after which vacuum filtration, de-sludging, filter evaporation, ethanol rinsing, and ethyl acetate recrystallization were carried out. A beige, solid O5-acetyl-O7-nitrooxyethyl chrysin product was obtained (**8a**: 0.11 g, 28.6% yield). Mp 112–113 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.85 (d, 2H, J = 8 Hz), 7.52 (d, 3H, J = 2.3 Hz), 6.90 (s, 1H), 6.64 (d, 2H, J = 2.3 Hz), 4.39 (s, 2H), 3.68 (s, 2H), 2.44 (s, 3H). EI-MS (m/z): 386 $[M+H]^+$. Anal. Calcd for $C_{19}H_{15}NO_8$: C, 59.22; H, 3.92; N, 3.64. Found: C, 59.58; H, 4.12; N, 3.46.

Compounds **8b** and **8c** were synthesized according to the method for **8a**.

Compound **8b**: 51% yield, mp 106–107 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.85 (d, 2H, J = 2.3 Hz), 7.52 (s, 3H), 6.87 (s, 1H), 6.61 (s, 2H), 4.56 (s, 2H), 4.11 (s, 2H), 2.44 (s, 3H), 1.97 (s, 4H). EI-MS (m/z): 414 $[M+H]^+$. Anal. Calcd for $C_{21}H_{19}NO_8$: C, 61.02; H, 4.63; N, 3.39. Found: C, 61.32; H, 4.84; N, 3.12.

Compound **8c**: 36.3% yield, mp 87–88 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.84–7.86 (m, 2H), 7.50–7.51 (m, 3H), 6.87 (d, 1H, J = 2.3 Hz), 6.60 (s, 2H), 4.46–4.49 (m, 2H), 4.05–4.08 (m, 2H), 2.44 (s, 3H), 1.76–1.87 (m, 4H), 1.52 (s, 4H). EI-MS (m/z): 442 $[M+H]^+$. Anal. Calcd for $C_{23}H_{23}NO_8$: C, 62.58; H, 5.25; N, 3.17. Found: C, 62.86; H, 5.56; N, 3.02.

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