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# Synthesis and cardiomyocyte protection activity of crocetin diamide derivatives

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Abstract

A series of novel diamide derivatives (**2**–**8**) of crocetin (**1**) were synthesized and evaluated for their cardioprotective activity *in vitro*. Using well-established model of hypoxia-induced injury in H9c2 cells, we investigated the effects of 9 compounds and positive drug nicorandil on cellular cytotoxicity by MTT assay, mitochondrial viable staining, LDH activity and mitochondrial membrane potential (MMP). Among the new derivatives, compounds **3** and **4** with good liposolubility showed significantly potent activity than crocetin (**1**) against hypoxia-induced cytotoxicity. Further mechanisms studies indicated that the cardioprotective effect of compounds **3** and **4** was due to these abilities by decreasing LDH release, preserving mitochondrial viabilities and reducing oxidative stress-induced depolarization of MMP. Our results demonstrated that compounds **3** and **4** as a new class of crocetin diamide derivatives could be developed as potential agents in our further drug development studies for ischemic heart disease.

## 1. Introduction

Ischemia heart disease is the leading cause of death world-wide and acute myocardial infarction viewed as a manifestation of cardiomyocyte death is currently one of the most prevalent cardiovascular diseases with a high mortality rate. Different forms of cardiomyocyte death have been identified as necrosis, apoptosis and necroptosis, and are proposed to lead to final myocardial infarct size [1]. Due to the high energy demand of the heart, mitochondria make up at least 30% of the cardiomyocyte volume. It is evident then that mitochondria plays roles in a number of signal transduction mechanisms and ultimately determination of cardiomyocyte survival or death. Especially in response to ischemia/reperfusion, the opening of the mPTP (mitochondrial permeability transition pore) appears to be decisive for severity or progression of cardiomyocyte death, and preventing mPTP opening using either pharmacological inhibitors or genetic ablation has been reported to reduce myocardial infarct [2,3]. Since the emerging pursuit of mitochondria-directed therapies in cardiovascular diseases, it is urgent to identify and develop safe and effective agents against ischemic myocardial injury [4]. From the clinical perspective, cardiomyocyte death is equally essential to how a mechanism works. Therefore, it is reasonable to firstly evaluate the protective effect of selected compounds against cardiomyocyte death induced by ischemia.

Recently, many studies have focused on crocetin which was extracted from a famous and precious plant *Crocus sativus* Linn [5–10]. Saffron, a spice and a food colorant present in the stigmas of *Crocus sativus* Linn, had been used as folk medicine to treat various ailments including cardiovascular diseases in ancient Arabia, India and China [11,12]. Crocetin, an important carotenoid constituent of saffron, had been reported to have anti-oxidative [10,13], hypolipidaemic [14], anti-cancer [7] and anti-atherosclerorotic [15] effects. Recently, anti-ischemia effect of crocetin has been attracted a considerable interests. Wang et al. first reported that crocetin exhibited potential as a cardioprotective agent in myocardial ischemia reperfusion injury model [9]. However, it clearly showed the important role of crocetin as a lead compound preventing, treating or alleviating ischemia cardiac diseases.

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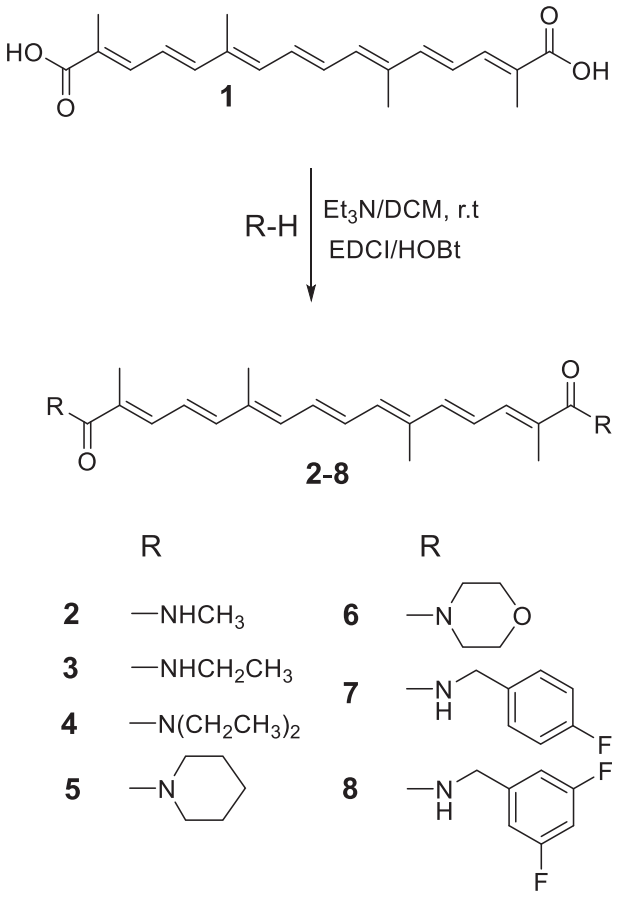


**Scheme 1.** Synthesis of crocetin diamide derivatives **2–8**.

## 2. Results and discussion

### 2.1. Chemistry

Crocetin is a polyene dicarboxylic acid (8,8′-diapocarotene-8,8′-dioic acid) with two hydrophilic free carboxylic groups and hydrophobic polyene structure which has poor solubility in many solvents. It was reported that neutralization of the carboxyl group by amidation could increase liposolubility and permeability by adding lipophilic alkyl chain to amine group [16–19]. Moreover, it was demonstrated that amides are found in a large array of biologically important compounds and many pharmaceutical substances [20–24]. Herein, we report the chemical synthesis of crocetin amide derivatives and biological evaluation of their cardioprotective activity. The synthesis of compounds **2–8** is illustrated in Scheme 1. Treatment of crocetin (**1**) with corresponding amines in the presence of 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDCI), 1-hydroxybenzotrizole (HOBt) and triethylamine in dry dichloromethane produced derivatives **2–8**, respectively, in good yields. Among these substrates, both simple primary amine (methylamine, ethylamine) and secondary amine (diethylamine, piperidine, morpholine) could form the amides with crocetin (**1**) in mild conditions. Aromatic ring was introduced by amidation with 4-fluorobenzylamine, 3,5-difluorobenzylamine to afford compounds **7** and **8**. All synthesized compounds were purified by column chromatography and their structures were elucidated based on $^{1}$H NMR, $^{13}$C NMR and HRESIMS spectroscopic evidence (SI).

### 2.2. In Vitro ischemic myocardial injury assay

To examine the cardioprotective effects of crocetin (**1**) and its derivatives (**2–8**), we employed a well-established *in vitro* ischemic model that causes cardiomyocyte death [25,26]. The induction of myocardial ischemia by hypoxia was designed to mimic the *in vivo* conditions of the ischemic myocardium. As shown in Fig. 1, compounds **1**, **3** and **4** at 0.2 μM significantly increased the cell viability against the hypoxia-induced cardiomyocyte injury. Their cardioprotective activity at 0.2 μM exhibited an equal effect to positive drug nicorandil at 100 μM. The activity of compounds **3** and **4** which are amide derivatives of **1** displayed stronger activity than **1**. While the activity of compounds **5** and **6**, nonaromatic ring (piperidine and morpholine) diamide derivatives is slightly lower than **1**. However, compounds **7** and **8**, containing aromatic rings, sharply decreased the activity at the same concentration. As for the structure-activity relationship (SAR), the *N*-ethyl derivative (**3**) and *N,N*-diethyl derivative (**4**) had a higher potency than crocetin and other derivatives. Replacing ethyl with piperidine or morpholine rings or shorting the alkyl chain from ethyl to methyl decreased the potency. It's noted that incorporation of fluoro-substituted benzene rings into crocetin significantly decreased bioactivity.

### 2.3. Mitocghondrial viability staining

The activity of compounds **1**, **3** and **4** was further evaluated by mitochondrial viability staining. Hypoxia stimuli significantly reduced the mitochondrial viability in H9c2 cells compared with normal group ($p < 0.001$). Treatment with compounds **1**, **3** and **4** at 1, 0.2 and 0.04 μM significantly protected the mitochondrial viability in dose-dependent manners (Fig. 2).

### 2.4. LDH activity detection

For the cellular toxicity detection, compounds **1**, **3** and **4** were

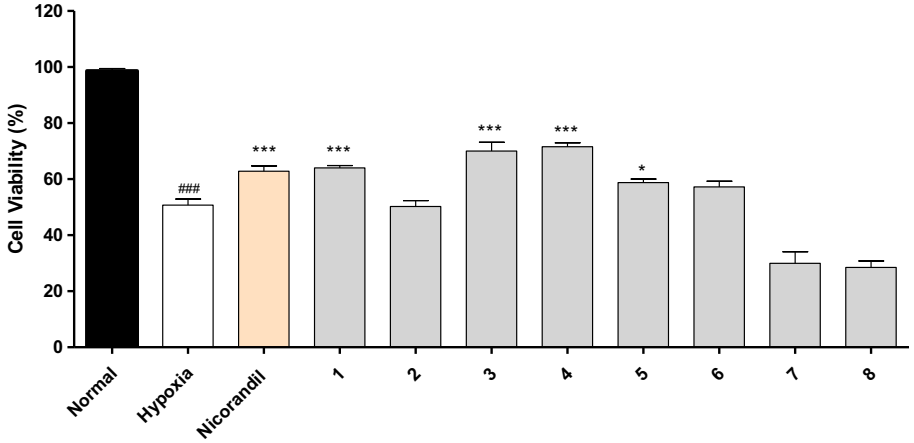


**Fig. 1.** Effects of crocetin (**1**) and its diamide derivatives (**2–8**) on hypoxia-induced injury in H9c2 cells subjected to ischemia. *In vitro* ischemic myocardial injury was induced by incubating H9c2 cells in Krebs-Ringer Bicarbonate buffer without serum, glucose and glutamate for 3 h in a Billups-Rotenberg hypoxia. H9c2 cells were pretreated with compounds **1–8** (0.2 μM), nicorandil (100 μM), respectively, for 5 min before hypoxia. Cell viability was determined by MTT assay. Cell viability of normal group was considered as 100%. Data represent mean ± SEM. The results were reproduced by six independent experiments. ### indicates $p < 0.001$ for hypoxia group *vs.* normal group; *** indicates $p < 0.001$ for each treatment group *vs.* hypoxia group.