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Synthesis, in vitro inhibitory activity towards COX-2 and haemolytic activity of derivatives of Esculentoside A

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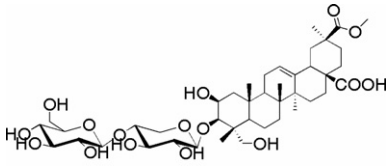
Abstract—Esculentoside A (EsA) has been reported to possess anti-inflammatory activity and selective inhibitory activity towards cyclooxygenase-2. A series of derivatives of EsA were synthesized by converting the C-28 carboxylic acid group into amides. The haemolytic activity and inhibitory activity towards cyclooxygenase-2 were evaluated in vitro. The SAR study of the derivatives was conducted and showed that introducing aromatic ring to EsA greatly enhanced its biological activity. Compound **23** showed higher inhibitory activity than Celecoxib and EsA, but lower haemolytic toxicity than EsA.

The Chinese herb *Phytolacca esculenta* has been proved to have striking therapeutic effects on a number of diseases such as rheumatoid arthritis, oedema and cancer. Esculentoside A (EsA), a kind of triterpene saponin isolated from roots of *Phytolacca esculenta*, has been identified as 3-*O*-[β-D-glucopyranosyl-(1,4)-β-D-xylopyranosyl] phytolaccagenin (**1**).[1] Our previous research showed EsA has strong inhibition on acute and chronic inflammation in various kinds of animal models.[2–5] We have demonstrated that the mechanism of its anti-inflammation effect might be associated with the reduction of several key inflammatory mediators. In vivo, EsA dose-dependently decreased the TNF, IL-1 and IL-6 levels in the sera of mice following LPS challenge. In vitro, EsA significantly reduced the release of TNF, IL-1 and IL-6 from the peritoneal macrophage of mice.[6–11] Furthermore, EsA diminished the functions of activated macrophages such as phagocytosis and antibody production and secretion of cytokines. A recent literature also disclosed that EsA inhibited cyclooxygenase-2 (COX-2) in a dose-dependent manner, but had no effect on COX-1.[12] EsA exhibits great anti-inflammatory activity, however, EsA also has great haemolytic toxicity. Therefore, we aimed at optimizing the structure of EsA and exploring the structure–activity relationship of EsA in order to seek the derivatives with increased biological activity and lower toxicity.



1

In the study of structure–activity relationship of triterpene saponin, both the aglycone and the sugar moiety play important roles in the evaluation of biological activity.[13] However, to our current knowledge, there was no literature describing the SAR of EsA. We herein report the structure optimization of EsA and the structure–activity relationship of EsA derivatives.

The first step of the optimization is to convert the carboxylic acid group in the aglycone of EsA (**1**) into an amide by a coupling reaction as described in Scheme 1. EsA was reacted with HOBt in the presence of DCC in DMF at room temperature for 1hr to give ester **2**, characterized by ^{1}H NMR, ^{13}C NMR and HRMS.

Keywords: Esculentoside A; Anti-inflammatory; Derivatives; Synthesis; Structure–activity relationship.
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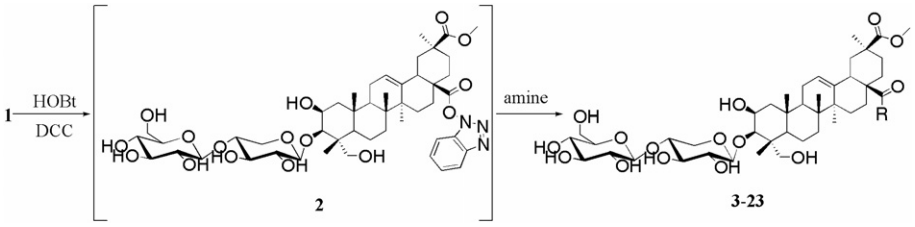


Scheme 1. General synthetic route for compounds **3**–**23**.

The amines (aliphatic amines, aromatic amines and amino acid esters) were added to the reaction mixture and heated at 60 °C for 1 h to give the amides **4**–**23**, which were purified by HPLC and identified by ^{1}H NMR, ^{13}C NMR and HRMS. The compound **3** was formed as a by-product in the reactions and it was confirmed by ^{1}H NMR, ^{13}C NMR and HRMS to be the product of EsA coupling with DCU. The by-product formation appears to be related with the high solubility of DCU in DMF. This suggested that DMF is not a favourable solvent for this reaction. When DMF–THF (1:2, v:v) was used as the solvent, good yields of the desired amides were achieved and there was no trace of by-product **3** found in the reaction mixtures.

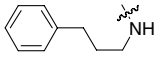
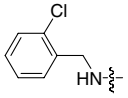
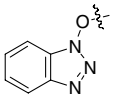
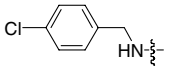
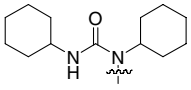
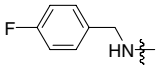
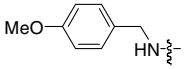
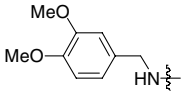
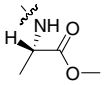
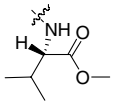
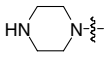
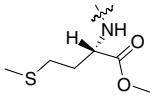
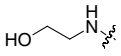
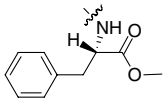
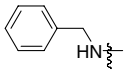
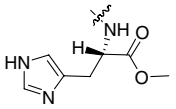
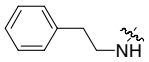
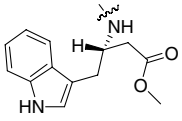
The measurement of the inhibitory effects on reactive oxygen species fluorescence in hCOX-2 expressing sf-9 cells is a rapid method to screen COX-2 inhibitors and is more effective, less expensive, and does not have isotope contamination in contrast to RIA or ELISA.[14] The inhibitory effects of the compounds were determined by the method described by Zhang.[15] Briefly, recombinant human COX-2 (hCOX-2) was expressed in insect sf-9 cells and harvested cloned sf-9 cells were stored in liquid nitrogen until use. Reactive oxygen species production was stimulated by arachidonic acid in sf-9 cells and was measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCDHF-DA) fluorescence. DCDHF-DA can rapidly permeate into the cells and is converted into 2′,7′-dichlorodihydrofluorescein (DCDHF). DCDHF is not fluorescent, but it can rapidly react with a reactive oxygen species to produce fluorescence.[16] The sf-9 cells (1×10^5/mL) containing hCOX-2 protein were preincubated with the tested compounds, including **1**, **3**–**23**, and Celecoxib (10 μM), for 30 min in a 96-well black plate, followed by adding DCDHF-DA (2.5 μM, final concentration) and arachidonic acid (5 μM, final concentration). The fluorescence in the cell suspension was immediately detected using a PolarStar plate reader with excitation wavelength of 485 nm, emission wavelength of 520 nm. The rate of fluorescence generation in the first 10 min was recorded. The biological evaluation of the compounds was investigated at the concentration of 10 μM in hCOX-2 expressing sf-9 cells in vitro. Four parallel experiments were performed for each sample. The inhibitory activities of the tested compounds are listed in Table 1.

Haemolysis assay was carried out following the procedure reported by Wang.[17] Non-heparinized blood of healthy New Zealand rabbit (Experimental Animal Center of Second Military Medical University, China) was used in the experiments. The erythrocytes were washed three times in PBS (phosphate-buffered saline: NaCl = 8 g/L, KCl = 0.2 g/L, Na_2HPO_4 = 1.44 g/L and KH_2PO_4 = 0.24 g/L; pH 7.4) and then diluted with PBS to obtain a 10% suspension. All the tested compounds were dissolved in DMSO at a concentration of 500 μg/mL, and then PBS was added to prepare the testing concentrations ranging from 2.5 to 250 μg/mL. The final volume of the sample was 1.0 mL. The erythrocyte suspension (100 μL) was added to the samples to be tested then the samples were rapidly stirred and incubated at 37 °C with periodic stirring during a 60-min incubation period. The solutions were then centrifuged at 3000 rpm for 5 min and the absorbance of the supernatant was measured at 540 nm using Thermo Multiskan MK3 (Labsystems Dragon, Finland, model: 353). The haemolysis percentage was calculated by comparison with the 100% haemolysis caused by distilled water as maximal haemolytic controls. The haemolytic percent developed by the PBS control was subtracted from all groups. The concentration inducing 50% of the maximum haemolysis is abbreviated as HD_{50}. Each experiment included triplicate at various concentrations and the results are listed in Table 1.

Over the past two decades, we have revealed that EsA has strong anti-inflammatory activity through effects on several important inflammatory mediators including TNF, IL-1 and COX-2. Haemolytic activity is the main toxicity of EsA, which needs to be overcome. We initiated the optimization of EsA, which aims at increasing the anti-inflammatory activity and lowering the haemolytic activity.

The compounds **2**, **11**–**16** and **23** bearing aromatic ring showed higher potency towards COX-2 than EsA and Celecoxib, but also showed higher haemolytic activity than EsA. The compounds **4**–**9** containing aliphatic groups lost their activity towards COX-2 and also showed lower haemolytic activity. From this result, we can make a conclusion that aromatic ring can promote the biological activity of EsA derivatives. Most noticeable is compounds **12** and **14** exhibited the highest inhibition rates, 158.2% and 126.1%, respectively, while EsA and Celecoxib exhibited 21.7% and 40.0%, respectively. However, the haemolytic activities of the compounds **12** and **14** are also the highest. Interestingly, our research showed correlativity between COX-2 inhibitory activity and haemolytic activity of EsA, and there was no report in the literature, which is worth further research. However, the results suggested us that the C-28 carboxylic acid group plays an important role in biological activity.

Table 1. Haemolytic activity and inhibitory activity of the compounds **1**, **3**–**24** on reactive oxygen species fluorescence in hCOX-2 expressing sf-9 cells

	R	Biological activity			R	Biological activity	
		Inhibition (%,10 μM)	HD_{50} (μM)			Inhibition (%,10 μM)	HD_{50} (μM)
Celecoxib[a]		40.0		**12**		158.2	<2.6
EsA (1)	–OH	21.7	185.5	**13**		82.9	42.8
2		/[b]	55.2	**14**		126.1	<2.6
3		75.0	<2.4	**15**		58.1	94.5
4	MeNH–	—[c]	276.2	**16**		56.8	12.7
5	EtNH–	—	/	**17**		19.4	215.4
6	*n*-PrNH–	—	191.0	**18**		—	>274.1
7	*n*-BuNH–	—	15.8	**19**		—	>266.0
8		4.6	/	**20**		—	202.7
9		—	>287.4	**21**		—	204.3
10		26.9	136.5	**22**		—	/
11		94.7	21.3	**23**		47.7	>243.4

[a] Celecoxib, a selective inhibitor towards COX-2, as a positive control.
[b] Not tested.
[c] No inhibition activity observed.

The compounds **18**–**23**, in which amino acid esters were introduced, lost their activity towards COX-2 and decreased their haemolytic toxicity. However, compound **23** showed 47.7% inhibitory rate towards COX-2, which was higher than EsA and Celecoxib, and showed lower haemolytic activity (HD_{50} > 243 μM) than EsA (HD_{50} = 185.5 μM). These findings suggest that the modification of the C-28 carboxylic acid may yield higher potent COX-2 inhibitors with lower haemolytic activity by coupling with appropriate groups.

The screening tests gave us a clear SAR on the modification of C-28 carboxylic acid group. The conversion of the C-28 carboxylic acid into an amide affected their inhibitory activity towards COX-2 and haemolytic activity. Especially, introducing aromatic ring to EsA

enhanced its biological activity. Further research on the structure optimization of EsA is in progress to investigate its structure–activity relationship.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.10.006.

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