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Selective alkylation of β-anhydroicaritine and their biological evaluation on anticancer

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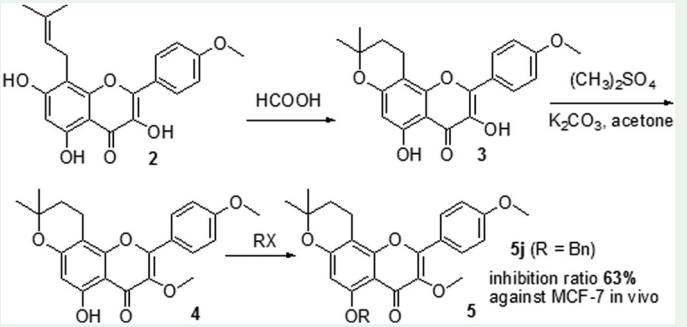
ABSTRACT

A convenient and selective alkylation of icaritin has been developed. The methodology involved initial formation of β-anhydroicaritine (**3**) under acidic conditions followed by selective methylation at the C-3 position and then alkylation at C-5 position. Several alkylated β-anhydroicaritine derivatives were synthesised using this methodology. These newly synthesised derivatives, especially the compounds **5b**, **5c** and **5j**, significantly suppressed cell proliferation when tested against cancer cell lines *in vitro*. Compound **5j** (R = Bn) exhibited a competitive inhibition against MCF7 *in vivo* compared to tamoxifen.



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1. Introduction

Flavones are of great interest due to their profound biological activities and widespread occurrence in nature (Manojit et al. 2003; Bruno et al. 2005; Manojit et al. 2005; Nigel and Renee 2011; Boris et al. 2013; Christian 2020). As a natural allyl flavonoid, icariin (**1**, Figure 1) has low oral absorption and low bioavailability because of its low solubility, which blocks it enter to BBB and limits its use in clinic. Recent pharmacological research shows that icaritin has an inhibitory effect on a variety of tumour cells, and in metabolism, this effect is mainly hydrolysed to icaritin (**2**) (He et al. 2010).

After the perusal of lit, we found that the previous structural modification to icaritin is mainly focused on the esterification of 3- and 7-hydroxy group in order to

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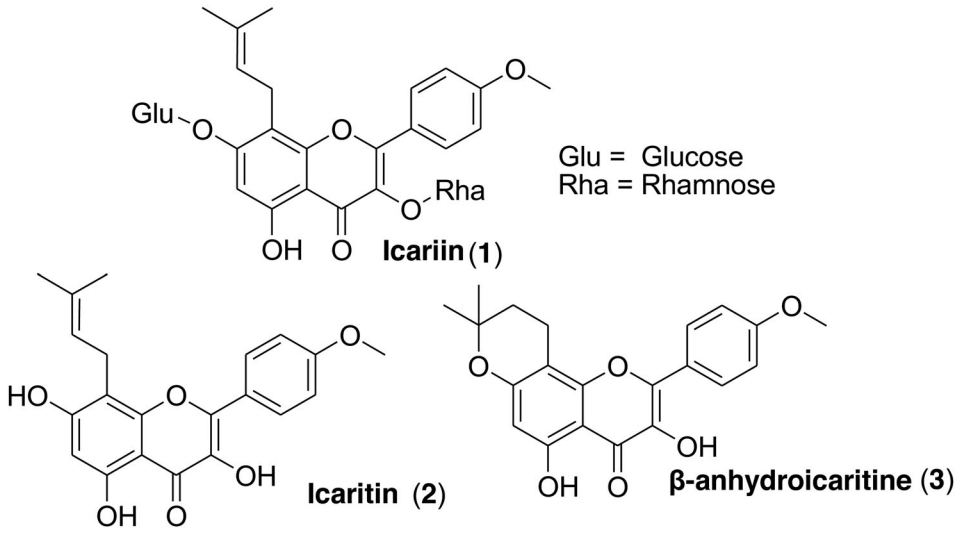

Figure 1. Structure of icariin, icaritin and β-anhydroicaritine.

improve their solubility or anticancer activity (Wang et al. 2000; Liu et al. 2009; Yao et al. 2010; Sun et al. 2013). Studies (Huang et al. 2007; Guo et al. 2011; Wang et al. 2015; Vanson et al. 2017) have shown that a single substitution on the C-3 or C-7 hydroxyl group of icaritin can improve the cytotoxicity of icaritin, and C-3 hydroxyl group may be the preferred site for chemical modification. However, several problems arise to this synthetic route. On the one hand, it is difficult to get mono-substituted esterification product selectively, and always, a mixture of 3- and 7-esterified products was obtained. On the other hand, there are few literatures were reported on the chemical modification of C-5 hydroxyl group, and its anticancer activity is also unknown.

In view of this problem, we envisioned that a selective alkylation for icaritin can be realised according to the difference in activity for three hydroxyl groups. And herein we reported our research results.

2. Research and discussion

It is interesting to find that three phenolic hydroxyl groups exhibit different reaction activity during esterification or alkylation reaction (Figure 2), which makes it possible for us to introduce alkyl group selectively. In fact, the direct alkylation to icaritin will lead to the formation of a mixture of 3- and 3, 7-disubstituted icaritin. To overcome this problem, we decide to protect 7-hydroxy group firstly by intra-molecular cyclisation reaction.

A modified procedure was explored to synthesise the building block β-anhydroicaritine (**3**) (Wang and Lou 2004; Wang et al. 2007; Tong et al. 2019). Upon treatment with formic acid, icaritin underwent cyclisation reaction at reflux to give β-anhydroicaritine in 95% yield (Dell'Agli et al. 2008). This modified procedure is more suitable for the scale up in industrial manufacture compared to the reported microwave reaction method.

In the following, in the alkaline conditions, β-anhydroicaritine was reacted with methylation reagent in a ratio of 1:1, such as dimethyl sulfate or methyl iodide, and

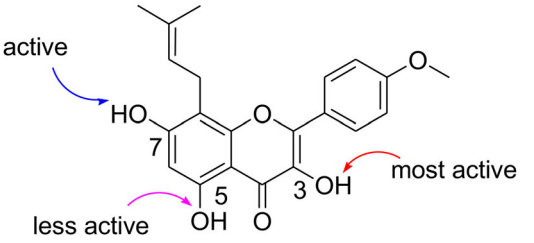

Figure 2. Activity difference to three phenolic hydroxyl groups for icaritin.

Table 1. Selective alkylation of β-anhydroicaritine with RX.

Entry	R	Coupling reagent	Solvent	Temp.	Time	yield
A	CH_3	K_2CO_3	acetone	reflux	2 d	85%
B	C_2H_5	K_2CO_3	acetone	reflux	2 d	60%
C	n-C_3H_7	K_2CO_3	acetone	reflux	2 d	60%
D	n-C_5H_{11}	K_2CO_3	acetone	reflux	2 d	60%
E	$BrCH_2CH_2$	K_2CO_3	acetone	reflux	3 d	44%
F	$BrCH_2CH_2CH_2$	K_2CO_3	acetone	reflux	3 d	44%
G	$BrCH_2CH_2CH_2CH_2$	K_2CO_3	acetone	reflux	2 d	67.3%
H	allyl	K_2CO_3	acetone	reflux	2 d	66.3%
I	$\equiv CH_2$	K_2CO_3	acetone	reflux	2 d	44.6%
J	Bn	K_2CO_3	acetone	reflux	2 d	62.2%

refluxed for 2 h to afford 3-methoxy β-anhydroicaritine (**4**) in good yield. In this step, the molar ratio and the amount of base are essential to affect the reaction yield. Particularly, a side product 3, 5-disubstituted β-anhydroicaritine will be formed when the molar ratio and the amount of base are more than 1:1.

And finally, the above obtained 3-methoxy β-anhydroicaritine was further reacted with different alkyl halides to give β-anhydroicaritine derivatives **5a–j** in a moderate yield. The detailed results are summarised in Table 1.

2.1. Proliferation inhibition assay

The cell proliferation inhibition of the four human cancer cell lines (HepG2, SW480, A549 and MCF7) by all synthetic compounds was evaluated by standard MTT methods. HepG2, SW480, A549 and MCF7 cells were cultured in RPMI 1640 or McCoy's 5 A medium (Invitrogen), respectively, supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin (Thermo Fisher Scientific, China). All cell lines were maintained at 37 °C with 5% CO_2. Cells were seeded in 96-well plates and incubated for 24 h. A range of concentrations of the test compounds were added, and the plates were incubated for 72 h before the addition of 10 μL MTT (5 mg/ml)/well. After 4 h of incubation, the medium was removed and 100 μL DMSO was added to each well. The absorbance was measured using a SpectraMax M5/M5e, USA microplate reader at 550 nm. The results are shown in Table S2. The results showed that compound **5j** had the best anti-cell proliferation effect.

2.1.1. Inhibition tumour cell growth in vivo

Three compounds **5b**, **5c** and **5j** were selected as candidates for the screening test against MCF7 *in vivo*. Nude mice were injected with a solution of MCF7 in Matrigel, and inoculation for 5 days until the tumour size is up to 0.5 cm approximately. Each

mouse was fed with test compound (5 mg per day) for 20 days. The results are shown in Table S3. The inhibition ratio was calculated as follows:

$$\text{Inhibition rate} = [\text{W(in Negative control)} - \text{W(in test compound)}]/\text{W(in Negative control)}.$$

3. Experimental

3.1. Materials and methods

Melting points were determined by a Mettler Toledo FP62 melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 400 MHz on a Varian Unity nova 400 NMR spectrometer using tetramethylsilane (TMS) as an internal standard, and ^{13}C NMR spectra were recorded at 100 MHz, respectively. Mass spectra were run on TOF-SIMS instrument (PHI Nano TOF II). TLC plates (GF 254) were bought from Branch Qingdao Haiyang Chemical Plant. Icariin (98%) was bought from Biopurify Phytochemicals Ltd (Chengdu, China). All the solvents are bought from Sinopharm.

4. Conclusion

In summary, a convenient selective alkylation protocol for icariin was developed. By protection to form β-anhydroicaritine (**3**) under acidic conditions, and followed by the selective alkylation to C-3 and C-5, several β-anhydroicaritine alkylated derivatives were thus easily synthesised. These new synthesised C-5 substituted β-anhydroicaritine derivatives, especially for compounds **5b**, **5c** and **5j**, significantly suppress cell proliferation *in vitro*. Compound **5j** (R = Bn) exhibits a competitive inhibition against MCF7 *in vivo* compared to tamoxifen, which give us much imagination on its further development.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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