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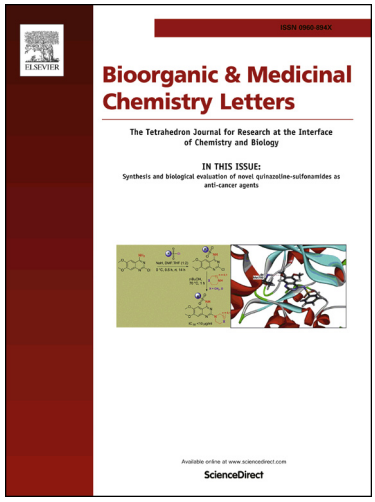

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PII: S0960-894X(17)30413-4
DOI: http://dx.doi.org/10.1016/j.bmcl.2017.04.039
Reference: BMCL 24892

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 1 December 2016
Revised Date: 22 February 2017
Accepted Date: 12 April 2017

Please cite this article as: Pang, G.X., Niu, C., Mamat, N., Aisa, H.A., Synthesis and *in vitro* Biological Evaluation of Novel Coumarin Derivatives containing Isoxazole Moieties on Melanin Synthesis in B16 cells and Inhibition on Bacteria, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: http://dx.doi.org/10.1016/j.bmcl.2017.04.039

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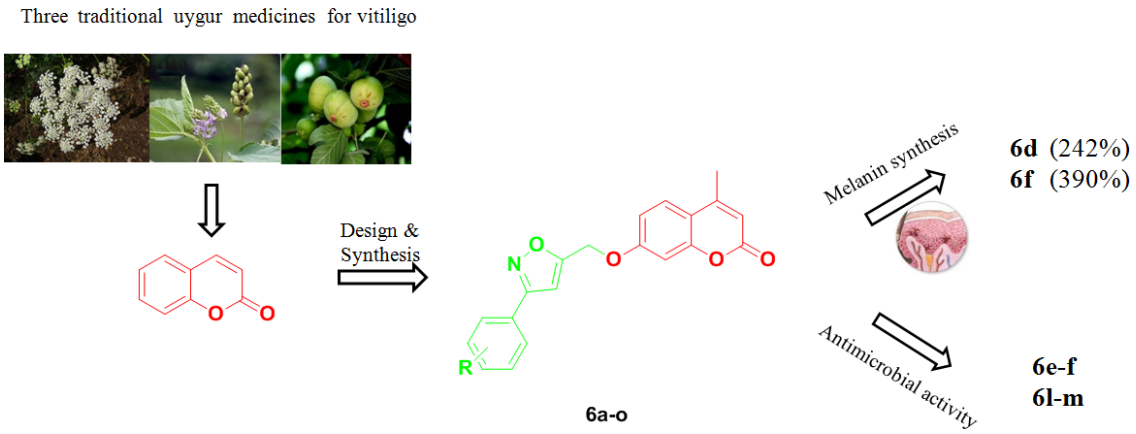

Bioorganic & Medicinal Chemistry Letters
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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
vitiligo
melanin synthesis
antimicrobial activity
coumarin derivatives
SAR

ABSTRACT

A novel series of coumarin derivatives **6a-o**, bearing isoxazole moieties were designed and synthesized. After that, they were evaluated for melanin synthesis in murine B16 cells and inhibitory effect on the growth of CA (*Candida albicans*), EC (*Escherichia coli*), SA (*Staphylococcus aureus*). It was found that eleven compounds (**6b-f**, **6j-o**) showed a better activity on melanin synthesis than positive control (8-MOP). Among them, compounds **6d** (242%) and **6f** (390%), with nearly 1.6 and 2.6-fold potency compared with 8-MOP (149%) respectively, were recognized as the most promising candidate hits for further pharmacological study of anti-vitiligo.

Seven halogen substituted compounds exhibited moderate antimicrobial activity against CA. It is interesting that **6e-f** and **6l-m**, which had two halogens on the benzene showed a comparable activity with Amphotericin B against CA.

The evaluation of melanin synthesis in B16 cells and inhibitory effect on bacteria of above structurally diverse derivatives had also led to an outline of structure-activity relationship.

Vitiligo is a chronic acquired disease of pigmentation characterized by prominent white patches of the skin.[1] The estimated prevalence is 0.5% to 2%.[2] Half of patients develop vitiligo before the age of 20 years. The origin of vitiligo is thought to be a complex interplay of genetics, environment, oxidative stress, and autoimmunity.[3,4] It is believed that the disease was mainly resulted from destruction or damage of the melanocyte and obstruction of the melanin synthesis.[5,6]

Melanin is synthesized following exposure to UV radiation by the oxidation of tyrosine in melanocytes. Although multiple signaling cascades are involved in melanin biosynthesis, it is critically regulated by tyrosinase family enzymes, specifically tyrosinase, which is the rate-limiting enzyme.[7,8] The family of tyrosinase genes in mammals comprises tyrosinase, tyrosinase-related protein-1 (TRP-1) and TRP-2 and all are essential for melanogenesis.[9] In addition, melanogenesis is regulated by microphthalmia-associated transcription factor (MITF), which directly regulates the expression of melanogenic genes, including tyrosinase and TRP-1.[10,11]

Since thousand years ago, the plant species *Ammi majus* L., *Psoralen corylifolia L.* and *Ficus carica* L. (**Figure 1**)[12] were often used for repigmentation of vitiligo with natural sunlight in India, Egypt and other oriental countries.[13] Similarly, The extract of them were popular Uygur medicines used for vitiligo alone or in combination and initially recorded in '*Yao Yong Zong Ku*' around 300 years ago.

Figure 1. The plant of the *Ammi majus* L., *Psoralen corylifolia* L. and *Ficus carica* L.[12]

Coumarins are widely distributed in these plants and have been isolated from their seeds, leaves and fruits.[14] Continuous researches proved that these compounds showing strong

photosensitivity, [15] which may be used for the treatment of vitiligo with subsequent exposure to long-waved ultraviolet radiation.[16,17] Although the therapy was accompanied with some undesired side effects, [18-20] it is still the most successful one for the disease today. Nonetheless, few coumarin derivatives possessed anti-vitiligo activity were reported. Recently, our group have done considerable research on discovery of lead compound of vitiligo and related mechanism of the disease. [21]

Previous studies have also indicated that coumarins combined with pyrazole and 1,2,4-triazole moieties exhibited antibacterial activity (**Figure 2**).[22,23] The isoxazoles were widely used in medicinal chemistry, which can be regarded as isostere of the pyrazole or triazole and possessed an extensive range of biological activities.[24] Beside, it could actively participate in hydrogen bonding and was very stable in most reaction conditions. In the continuing developing a better medication for the vitiligo and bacteria infection, fifteen coumarin derivatives containing isoxazole moiety (**6a-o**) were prepared and submitted to the activity assay of melanogenesis in B16 cells and inhibitory effect on three kinds of strains.

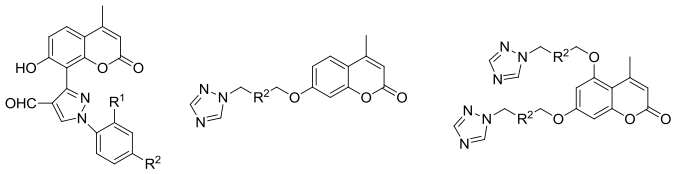


Figure 2. Structures of coumarin derivatives with potent antimicrobial activities

The synthetic procedure of the target compounds was shown in **Scheme 1**. Commercially available aromatic aldehydes were condensed with hydroxyl amine hydrochloride to obtain their corresponding oximes (**2a-o**). They were then converted to hydroxymethylisoxazoles (**3a-o**) by intermolecular cycloaddition with propynol in presence of N-chlorosuccinimide. The intermediates **3a-o** were finally brominated to give the desired 5-(bromomethyl)isoxazoles (**4a-o**) by PBr_3 in dichloromethane at 0 °C. [25] Compound **5** (4-methylumbelliferone) was prepared from resorcine via Pechmann reaction.[26,27] 4-methylumbelliferone was then refluxed with **4a-o**, catalyzed by K_2CO_3 in acetone to yield the final compounds **6a-o**.

In order to avoid the possibility that inhibition of melanin synthesis was due to cytotoxicity, we first performed CCK-8 assay to determine whether these coumarins derivatives (**6a-o**) were cytotoxic to B16 cells. The result illustrated that the cells treated with **6d-f** and **6j** for 24 h caused mild cytotoxicity as compared with the control at the dosage of 50 μM (**Figure 3**).

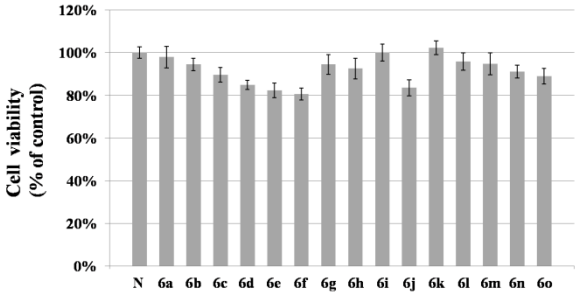


Figure 3. Effect of coumarin derivatives on B16 cells viability

N means negative control; The B16 cells were incubated with 50 μM of different coumarin derivatives (**6a-o**) for 24h and the cell viability was assayed by adding CCK-8 solution. Values are expressed as the mean ± SD of six separate experiments.

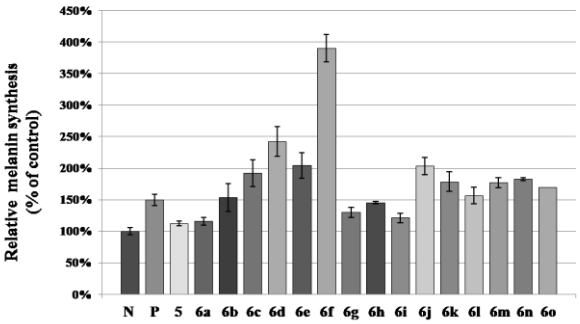


Figure 4. Stimulation of melanin content of B16 cells by coumarin derivatives **6a-o**

N means negative control; P means positive control (8-MOP); The B16 cells were treated with 50 μM of different coumarin derivatives for 48 h. After that, melanin content was measured directly. Values are expressed as the mean ± SD of three separate experiments.

All the synthesized compounds were screened for their activity on melanin synthesis in murine B16 cells, with a known method (**Figure 4**).[28] According to the screening data, all the tested compounds showed a stronger activity than the negative control and eleven (**6b-f**, **6j-o**) were more potent than 8-MOP (positive control).

The position, number and nature of the substituent on benzene ring were varied in order to identify the most appropriate group. Compounds with -Cl (**6c-f**) and -OCF_3 (**6j**) demonstrated higher activity compared with -F (**6a**, **6i**, **6l-m**), -OCH_3 (**6g-h**) and un-substituted (**6b**).

Among these halogenated compounds, the shift of -Cl substituent from the *para* (**6c**, 192%) into the *meta* position led to **6d** (242%), which had a higher activity. The similar result was observed in compounds **6a** and **6i**, which were substituted with -F group as well. The replacement of the -Cl group with -F led to a dramatical decrease for the activity (**6c** and **6a**, **6d** and **6i**, **6e** and **6n**), indicating that the type and the position of the halogen was the most important factor for their efficacy.

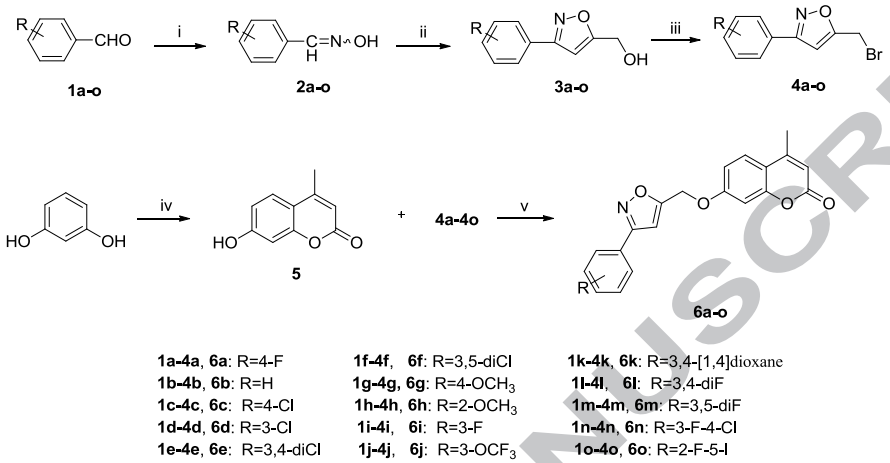
In addition, the number of the halogen atom on benzene made a great influence on activity. Introduction of a second -Cl or -F to the benzene strongly increased activity, such as **6c** and **6d**, **6a** and **6i** compared with **6e** and **6f**, **6l** and **6m**. It is interesting that 3,5-disubstituted compound **6f** and **6m** were more active than 3,4-disubstituted compound **6e** and **6l**, which can be inferred that the second halogen on the *para*-position of the first one could obviously enhance the activity.

However, the melanin content increased to 178% when the benzene was substituted by oxygen heterocycle ([1,4] dioxane) (**6k**), which suggested that benzene was not fundamental for the activity.

Meanwhile, compounds **6a-o** were evaluated for their antimicrobial activities against *Candida albicans* ATCC 10231 (Fungi), *Escherichia coli* ATCC 11229 (Gram negative bacteria) and *Staphylococcus aureus* ATCC 6538 (Gram positive bacteria) strains. As shown in **Table 1**, it was apparent that compounds may exhibited moderate antimicrobial activity against CA, only when substituted by halogen groups on *meta*- (**6d**, **6i**), 3,4- (**6e**, **6l**) and 3,5-positon (**6f**, **6m**) of benzene. And a better inhibitory

activity on CA was identified when a second halogen was constructed.

In conclusion, a series of coumarin derivatives had been prepared via structural modification in present work. Compounds **6d** (242%) and **6f** (390%), which demonstrated the best activity with nearly 1.6 and 2.6-fold potency compared with 8-MOP (149%) respectively in melanin synthesis, were recognized as the most promising drug candidates, and deserved for the further research of vitiligo. In addition, only seven halogen substituted derivatives exhibited antimicrobial activity on CA *in vitro* and similar rules was observed as in the former.



Scheme 1. Synthetic route for the coumarin derivatives

(i) $NH_2OH{\cdot}HCl$, Na_2CO_3, 30% methanol aqueous solution, rt (ii) NCS, propynol, triethylamine, DCM, reflux(iii) PBr_3, DCM, 0°C (iv) ethyl acetoacetate, H_2SO_4, 60°C (v) K_2CO_3, acetone, reflux

Table 1. Stimulation of melanin content in B16 cells and antibacterial activity of **6a-o**

Compounds	R	Melanin synthesis (%)	Inhibition zone diameter (mm)		
			CA[a]	EC[b]	SA[c]
6a	4-F	115.44±6.50	-	-[d]	-
6b	-	153.35±22.04	-	-	-
6c	4-Cl	191.96±21.33	-	-	-
6d	3-Cl	242.18±23.73	7	-	-
6e	3,4-diCl	204.12±20.60	10	-	-
6f	3,5-diCl	389.95±21.63	10	-	-
6g	4-OCH_3	129.45±7.89	-	-	-
6h	2- OCH_3	145.05±1.85	-	-	-
6i	3-F	121.15±7.55	7	-	-
6j	3-OCF_3	203.27±13.55	-	-	-
6k	3,4-[1,4] dioxane	178.33±15.61	-	-	-
6l	3,4-diF	156.64±13.01	10	-	-
6m	3,5-diF	176.55±8.01	10	-	-
6n	3-F-4-Cl	182.27±2.13	7	-	-
6o	2-F-5-I	169.71±0.02	-	-	-
5	-	112.2±4.2	7	-	-
Ampicillin	-	-	-	12.5	19
Amphotericin B	-	-	11	-	-
P[e]	-	149.39±8.64	-	-	-
N[f]		100±5.97	-	-	-

[a] CA: *Candida albicans* (ATCC 10231) fungi strain.
[b] EC: *Escherichia coli* (ATCC 11229) bacteria strain.

[c] SA: Staphylococcus aureus (ATCC 6538) bacteria strain.
[d] "-": inactive for the bacteria (the diameter of inhibition zone≤7 mm)
[e] P measn positive control 8-MOP.

Acknowledgments

This work was supported by the Funds for the Xinjiang Key Research and Development Program (2016B03038-3); "Personalized Medicines-Molecular Signature-based Drug Discovery and Development", Strategic Priority Research Program of the Chinese Academy of Sciences (XDA12050301);West Light Foundation of The Chinese Academy of Science (No. XBBS201403).

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Supplementary Material

Supplementary data (experimental procedures, spectroscopic characterizations and the original spectra of the compounds) associated with this article can be found.

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