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Synthesis and biological evaluation of alkoxycoumarins as novel nematicidal constituents

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ABSTRACT

We synthesized all of the monomethoxycoumarins, 5-alkoxycoumarins and their derivatives, and investigated their nematicidal activity against the phytopathogenic nematode, *Bursaphelenchus xylophilus*. Among the compounds, 5-ethoxycoumarin showed the highest nematicidal activity. Furthermore, 5-ethoxycoumarin was comparatively harmless against both the brine shrimps, *Artemia salina*, and the Japanese killifish, *Oryzias latipes*.

In Japan, China and the surrounding countries, a serious wilting disease is epidemic in pine wood.[1] This disease is caused by a nematode, *Bursaphelencus xylophilus*,[2] and the nematode is transmitted by a longhorn beetle, *Monochamus alternatus*.[3] Previously, killing the vector by aerial application of insecticides predominated as a way of controlling such diseases. Recently, due to its comparatively safer environmental impact, the trunk-injectable nematicides[4] are progressively replacing the conventional airborne pesticides. Moreover, in trunk injection, it is not necessary for nematicidal components to be highly water-soluble; hence, organic solvents, such as ethanol, acetonitrile and acetone, can also be used.[5] Although some powerful nematicidal chemicals such as milbemectin (**1**, Fig. 1)[6] and morantel tartrate[7] have been used in this approach, they are expensive and ecological unsafe. Moreover, it is feared that the nematodes are developing resistant against these compounds;[8] therefore, it is important that we discover new types of nematicidal compounds that are safer, more effective and economical.

Our previous paper proposed a convenient screening method for nematicidal activity against *B. xylophilus*.[9] We found by this method that 3-undecylphenol (**3**) from *Knema hookeriana*, a Sumatran rainforest plant[10] and coumarin (**4**) from *Ageratum conyzoides*, a native of the South American rainforest (Fig. 1),[11] both had pronounced activity with a minimum effective dose (MED, discussed in more depth later) of 5 μg/cotton ball (μg/bl.), 75 μg/bl., respectively. We then set about systematically modifying these compounds in order to identify molecules with greater activity. First, regarding 3-alkylphenols, our interest focused on how the modification of the length of alkyl group would influence the activity. In order to investigate the structure–activity relationship (SAR), we therefore synthesized several 3-alkylphenols, including **3**. We discovered 3-nonylphenol (**2**) showed remarkable activity (2.5 μg/bl.).[12] On the other hand, coumarin and its many derivatives are chiefly isolated from higher plants, especially citrus fruits, and have been found to exhibit various biological, pharmacological activities, for example, anti-bacterial, anti-inflammatory and analgesic properties.[13] Until now, however, study of their nematicidal activities has not been performed. In a pilot study, we investigated

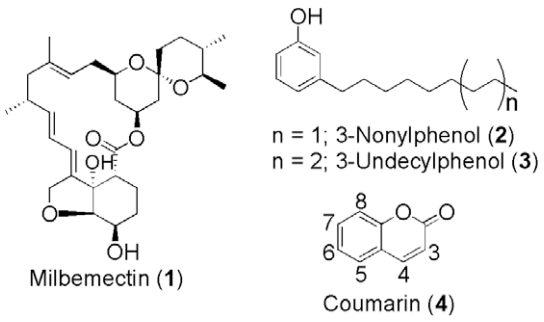

Figure 1. Structures of nematicidal compounds **1**–**4**.

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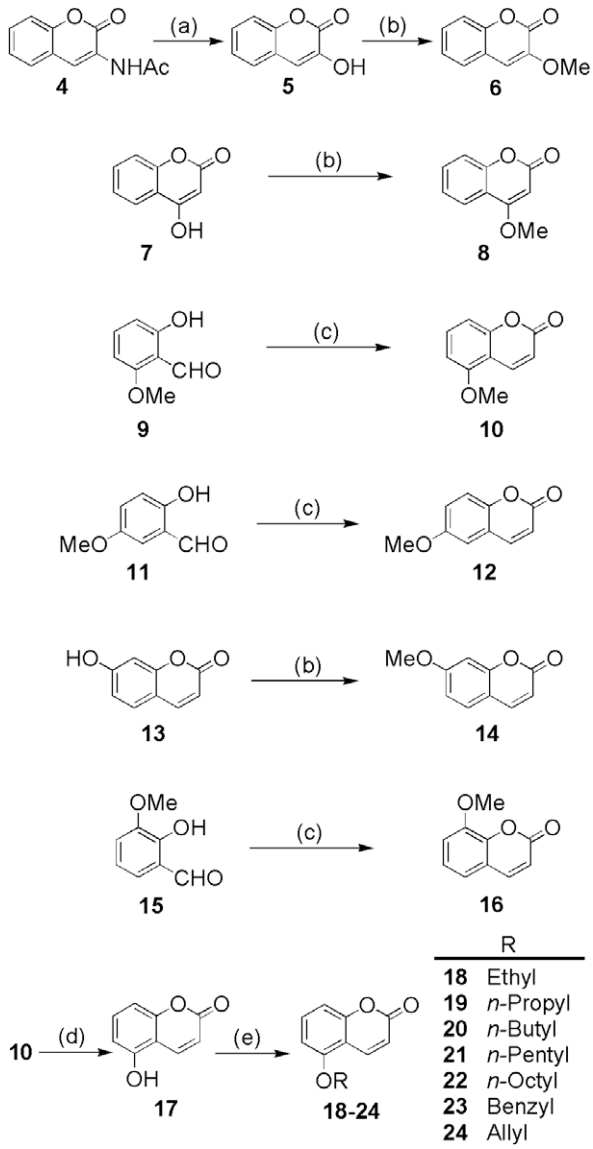
the activity of several simple coumarin derivatives, for example, hydroxycoumarins, methylcoumarins, chlorocoumarins and 4-methoxycoumarin (**8**) and 7-methoxycoumarin (**14**). We found that **14** showed higher activity (MED = 40 μg/bl.) than coumarin, whereas **8** and other related compounds lost the activity. Therefore, we were interested in investigating the SAR, especially the effects of the methoxy group position and the length of the alkoxy group carbon chains. Most of the variant compounds are not commercially available, however, we synthesized the monomethoxycoumarins and their analogs. We planned to investigate their nematicidal activities as well as toxicity to fish and crustaceans, as an ecologic safety assessment index.

Scheme 1 illustrates the synthetic routes to all monomethoxycoumarins, 5-alkoxycoumarins and their analogs. 3-Methoxycoumarin (**6**) was synthesized by a series of two reactions, hydrolysis and methylation, namely 3-acetamidocoumarin (**4**) was hydrolyzed with methanolic 3 N hydrochloric acid,[14] yielding 3-hydroxycoumarin (**5**), which was methylated with diazomethane to **6**. 4-Methoxycoumarin (**8**) and 7-methoxycoumarin (**14**) were prepared by methylation with diazomethane of 4-hydroxycoumarin (**7**) and 7-hydroxycoumarin (**13**), respectively, by standard methods. 5-Methoxycoumarin (**10**), 6-methoxycoumarin (**12**) and 8-methoxycoumarin (**16**) were synthesized by the Wittig reaction of methoxy group substituted 2-hydroxybenzaldehydes with the Wittig reagent, ethyl (triphenylphosphoranylidene)acetate in *N*,*N*-diethylaniline under reflux.[15] This method afforded the desired methoxycoumarins in high yield, though Kitamura et al. report that these coumarins were synthesized using K_2PtCl_4/AgOTf in low yield.[16] Commonly the stereoselectivity of the product in the Wittig reaction depends strongly on stability of ylides. Ethyl (triphenylphosphoranylidene)acetate is stabilized ylides, so (*E*)-alkenes are generated as intermediates. In our case, however, the desired compounds are (*Z*)-alkenes. Both the height of *N*,*N*-diethylaniline's boiling point (217 °C at 1 atm) and the stability of δ-lactone contribute to isomerization of (*Z*)-alkenes from (*E*)-alkenes. 5-Alkoxycoumarins (**18**–**24**) were prepared in two steps from **10**. In the first step, 5-hydroxycoumarin (**17**) was obtained by demethylation of **10** with tribromoborane; in the second step, the desired **18**–**24** were prepared by coupling **17** with the appropriate bromides.



Scheme 1. Reagent and conditions: (a) 3 N methanolic HCl, reflux, 99%; (b) CH_2N_2, Et_2O, rt (35% for **6**, 23% for **8**, 25% for **14**); (c) $Ph_3P=CHCO_2Et$, *N*,*N*-diethylaniline, reflux (71% for **10**, 98% for **12**, 82% for **16**); (d) BBr_3, CH_2Cl_2, −78 °C, 84%; (e) RBr, K_2CO_3, acetone, rt (quant. for **18**, 92% for **19**, 90% for **20**, 97% for **21**, 83% for **22**, 88% for **23**, 83% for **24**).

We tested the synthesized coumarins, two alkylphenols and milbemectin for three kinds of biological activities; nematicidal, toxicity to crustaceans and toxicity to fish. The nematicidal assay previously reported was used with some modifications. A cotton ball (5 mm in diameter) containing a certain test concentration or corresponding equivalent solvent as the control was dried in vacuum and placed on a Petri dish (4 cm diameter) containing full grown mycelia of *Botrytis cinerea*, which had been cultured on Czapek-Dox agar medium at 21 °C for 4 days. Hundred microliters of a distilled-water suspension of nematodes (*B. xylophilus,* ca. 15,000/ml) was injected into the cotton ball, and the Petri dish was kept at 26 °C for 4 days. The minimum effective dose (MED) was determined by observing whether the mycelia were consumed by nematodes or not, and is denoted by the sign − (completely consumed), ± (partly consumed) or + (not consumed).[17] The highest dose of coumarins was 160 μg/bl., and the assay was performed to obtain MED values by the serial dilution method. The values are each defined as the lowest dose of the test compound to show activity (±). If the mycelia are not consumed, it is conceivable that the tested compound has a temporary antifeedant or paralytic activity, as opposed to genuine nematicidal activity. Therefore, nematodes were promptly recovered about those in which active was observed after evaluation, survival was evaluated by methylene blue staining, and nematicidal activity was confirmed in all instances. Crustacean lethality was tested using a brine shrimp, *Artemia salina*, which is widely used for experiments on the safety of agricultural chemicals,[18] as follows: the eggs of *A. salina* were placed in seawater; they hatched within 48 h to provide large number of larvae for experimental use. Compounds dissolved in dimethyl sulfoxide (50 μl) were tested in vials containing 5 ml of seawater and ten shrimps in each of three replicates. Survivors were counted after 24 h, and LC_{100} and LC_{50} were determined. The evaluation system that contained only dimethyl sulfoxide was used as a control in this case. A test of fish toxicity was done using Japanese killifish, *Oryzias latipes*, fasted for 24 h before the test. *O. latipes* is a freshwater fish which, like *A. salina*, is commonly used for evaluation the safety of compounds.[19] Tested compounds were dissolved in acetone (1.0 ml) and added to beakers containing 150 ml of demineralized oxygen-rich water and five fish in each of three replicates. Survivors were counted after 24 h and judged LC_{100} and LC_{50}. The evaluation system that contained only acetone was used as a control in this case. The highest concentration of test compounds was 80 ppm in the *A. salina* lethality test, 100 ppm in the *O. latipes* lethality test; both assays were performed by the serial dilution method.

Table 1
Nematicidal activity of coumarin derivatives, and toxicity against crustacea and fish.

Compound	Substituent site-group of coumarin	Nematicidal activity[a] (MED) (μg/bl.)	Lethal effect on *A. salina* (ppm)			Lethal effect on *O. latipes* (ppm)		
			LC_{100}	LC_{50}	Selectivity index[b]	LC_{100}	LC_{50}	Selectivity index[b]
1		0.6	0.6	0.1	0.17	0.25	0.06	0.10
2		2.5	1.3	0.6	0.24	10	7.5	3.01
3		5.0	1.3	1.0	0.20	5	3.8	7.06
4	Nothing	80	>80	>80	>1.00	50	38	0.48
6	3-OMe	80	>80	>80	>1.00	>100	>100	>1.25
8	4-OMe	na[c]	>80	60	—	100	75	—
10	5-OMe	20	20	30	1.50	50	38	1.90
12	6-OMe	80	>80	>80	>1.00	100	75	0.94
14	7-OMe	40	80	20	0.50	75	50	1.25
16	8-OMe	80	>80	>80	>1.00	>100	100	1.25
25	5,7-Di-OMe	20						
18	5-OEt	10	>80	>80	>8.00	100	75	7.52
19	5-OPr	20						
20	5-OBu	40						
21	5-*O*-Pentyl	40						
22	5-*O*-Octyl	40						
23	5-OBn	80						
24	5-*O*-Allyl	na[c]						
17	5-OH	na[c]						
26		na[c]						

[a] Maximum dose of the test sample is 160 μg/bl.
[b] (1/MED)/(1/LC_{50}).
[c] No activity.

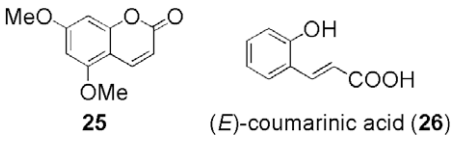


Figure 2. Structures of **25** and **26**.

The result of the bioassay is shown in Table 1. Among the coumarin derivatives, behaviors depend strongly on the position and the type of substituent. Among the monomethoxycoumarins, **10** showed the highest nematicidal activity (MED = 20 μg/bl.). The activity of **14** was comparatively high (MED = 40 μg/bl.) while the other monomethoxycoumarins did not have high activities. 5,7-Dimethoxycoumarin (**25**, Fig. 2), bears both substitution found in **10** and **14**, and was predicted to have higher activity; however, the compound was comparable to **10**. Therefore, we planned to investigate the nematicidal activity of alkoxycoumarins and their analogs with a focus on the 5-position; prior to this study, the bioactivity of these compounds had been hardly studied. Among the 5-alkoxycoumarins, 5-ethoxycoumarin (**18**) showed a higher activity (MED = 10 μg/bl.) than **10**. The activities of **17**, 5-benzyloxycoumarin (**23**) and 5-allyloxycoumarin (**24**) were much lower than **18**; leading us to conclude that in addition to position and the length of the alkoxy group, saturation grade is also important. Moreover, we confirmed that (*E*)-coumarinic acid (**26**, Fig. 2) ((*Z*)-**26** is unstable), the open form of coumarin, did not show nematicidal activity. In the both the *A. salina* and *O. latipes* lethality tests of the main compounds, **10** and **14** showed higher toxicity than other coumarins, in agreement with their increased nematicidal activity. The toxicity of **18**, however, was low against both *A. salina* and *O. latipes*, though it showed high nematicidal activity. On the other hand, **1** showed relatively high nematicidal activity (MED = 0.6 μg/bl.); it also showed hundreds of times higher toxicity than coumarins against *A. salina* and *O. latipes* (LC_{50} = 0.1, 0.06 ppm, respectively). Alkylphenols also had a powerful effect on nematodes (MED = 2.5–5.0 μg/bl.), *A. salina* and *O. latipes* (LC_{50}s were less than 10 ppm) though not to the extent of **1**. In addition, we evaluated selectivity using a simple index expressed as the 1/MED (μg/bl.) divided by 1/LC_{50} (ppm). High values of this metric reflect a high degree of selectivity for nematodes. The selectivity index values of **1**, taken as a benchmark for that of other compounds were 0.17 (against *A. salina*) and 0.10 (against *O. latipes*). Meanwhile, the selectivity values of the coumarins were much higher than those of **1**–**3**. In particular, **18** was 75 (or more) times more selective than **1**.

Systematic evaluation of coumarin derivatives for nematicidal activity has revealed a SAR, with activity associated with methoxy group and 5-alkoxy groups. We have discovered that 5-ethoxycoumarin shows high nematicidal activity. Both *A. salina* and *O. latipes* lethality tests suggested that this compound is much safer than milbemectin and alkylphenols. From this work, we conclude that 5-ethoxycoumarin is comparatively potent and it should be considered a lead compound for the development of new class of the nematocides.

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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.2008.08.102.

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