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Synthesis of polyalkoxy-3-(4-methoxyphenyl)coumarins with antimitotic activity from plant allylpolyalkoxybenzenes

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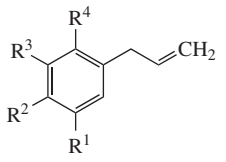
Novel polyalkoxy-3-(4-methoxyphenyl)coumarins – analogues of natural antimitotic compounds – were synthesized from plant allylpolyalkoxybenzenes and tested *in vivo* in the phenotypic sea urchin embryo assay for antiproliferative antitubulin activity.

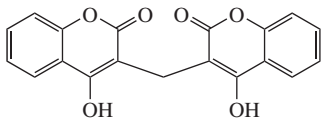
Pharmacologically active allylpolyalkoxybenzenes elemicin **1a**, myristicin **1b**, apiol **1c**, and dillapiol **1d** (Figure 1) are the widespread metabolites of plants belonging to the *Umbelliferae* family.[1] Previously, we have developed a technology for isolation of these compounds from parsley and dill seeds by liquid CO_2-extraction and high-performance rectification.[2]

Synthesis of analogues of natural cytostatic agents containing polyalkoxybenzene moieties, in particular, 3-arylcoumarins, can be a promising approach in synthetic application of allylpolyalkoxybenzenes.

Coumarins with three and four alkoxy groups at the benzene ring and the pyran ring (Figure 1, **I**) are widespread in nature.[3–6] Some of them possess strong fluorescent properties.[3] These compounds are also known to inhibit the growth and stimulate the differentiation of myeloid promonocyte cells U-937.[7,8] Certain di- and trialkoxy-3-phenylcoumarins demonstrate an antiproliferative effect toward human promyelocytic leukemia HL-60 and human lung adenocarcinoma epithelial A549 cells.[9] Active cytostatic agents have been found among polyalkoxy-3,4-dihydro-4-phenylcoumarins that suppress the growth of tumour cells,[10–14] induce apoptosis,[12] and inhibit tubulin polymerization.[12,13]

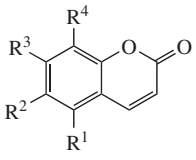


1a–d

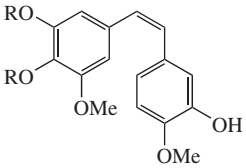


Dicoumarol

a $R^1 = R^2 = R^3 = OMe$, $R^4 = H$
b $R^1 = OMe$, $R^2 + R^3 = OCH_2O$, $R^4 = H$
c $R^1 = R^4 = OMe$, $R^2 + R^3 = OCH_2O$
d $R^1 + R^2 = OCH_2O$, $R^3 = R^4 = OMe$



I



Combretastatin A2: R + R = CH_2
Combretastatin A4: R = Me

Figure 1

It is interesting that dicoumarol (Figure 1) lacking methoxy groups blocked the first division of the fertilized eggs of sea urchins *Strongylocentrotus purpuratus*, *S. franciscanus*, and *Lytechinus pictus* at mitosis with $IC_{50} = 10$ μM[15] by a different mechanism. It has been shown that the cytostatic effect of this compound resembled the effect of taxol, resulting from binding with tubulin and alteration (stabilization) of the microtubules dynamics, while inhibition of tubulin polymerization was not observed. Similar results for dicoumarol obtained in our assay with embryos of sea urchin *Paracentrotus lividus* (see below) suggested that active cytostatics acting both by microtubule destabilization and stabilization mechanisms could be found among polyalkoxy-substituted coumarin analogues.

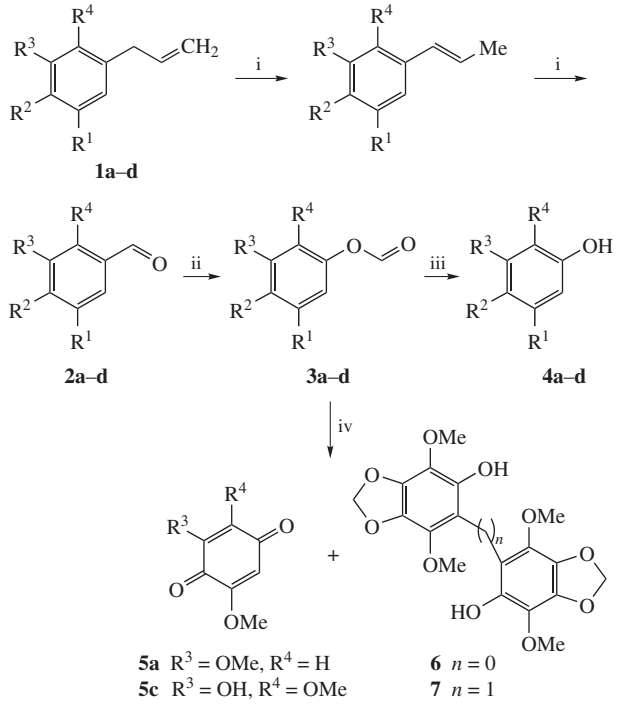
Considering 3-arylcoumarins as bioisosteric analogues of combretastatins (Figure 1), introduction of a 4-methoxyphenyl substituent to 3-position can lead to highly active structures since 4-methoxyphenyl pharmacophore is essential for the antimitotic antitubulin effect of combretastatin.[16]

Polyalkoxyphenols **4** required for the synthesis of intermediate salicyl aldehydes **8** were synthesized by rearrangement of aldehydes **2** by the Baeyer–Villiger reaction at –20 °C (Scheme 1).[†] However, the reaction was complicated by a number of side oxidation reactions yielding quinonoid structures **5**.[17] According to our data, carrying out the reaction in a glass vessel caused partial decomposition of hydrogen peroxide, possibly due to the presence of admixtures in the glass, so a part of the aldehyde remained unconsumed. In a plastic reactor, the aldehyde was com-

[†] Polyalkoxybenzaldehydes were synthesized by the known procedure.[23]

Synthesis of polyalkoxyphenols (general procedure). Hydrogen peroxide (35%, 14.3 ml) was added to formic acid (99%, 29 ml), stirred for 1 h at room temperature, cooled to –10–15 °C, followed by dropwise addition of aldehyde **2a–d** (47.6 mmol) in formic acid (100 ml). The reaction mixture was kept for 24 h at –20 °C and diluted with water (200 ml). Excess H_2O_2 was quenched with $Na_2S_2O_3$. The mixture was extracted with CH_2Cl_2 (4×100 ml), washed with water (3×40 ml), and dried with Na_2SO_4 to afford crude aryl formates **3a–d**. A solution of aryl formate (18.0 mmol) and KOH (2.6 g, 67 mmol) in methanol (50 ml) was stirred for 2 h at room temperature and acidified with 2 N HCl. Methanol was evaporated; the residue was diluted with water (100 ml) and extracted with CH_2Cl_2 (3×100 ml). The solvent was dried and evaporated to afford the target phenol **4**.

3,4,5-Trimethoxyphenol **4a**: yield 4.38 g (50%), mp 144–145 °C (AcOEt). (lit.,[24] mp 145–147 °C). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.55 (s, 3H, OMe), 3.69 (s, 6H, OMe), 6.05 (s, 2H, H_{Ar}), 9.18 (s, 1H, OH). EI-MS, *m/z* (%): 184 $[M]^+$ (73), 169 (100), 141 (28). Found (%): C, 58.73; H, 6.44. Calc. for $C_9H_{12}O_4$ (%): C, 58.69; H, 6.57.



Scheme 1 *Reagents and conditions*: i, see ref. 23; ii, HCOOH, H_2O_2, –20 °C; iii, KOH, MeOH, 2 h, room temperature; iv, AcOH, H_2O_2, 24 h; room temperature, 8 days.

pletely consumed. However, subsequent oxidation of the intermediate *o*-acylphenols **3** occurred at room temperature to give side quinones and dimerization products.

Upon detailed studying the oxidation of apiol aldehyde **2c** under Baeyer–Villiger rearrangement conditions at room temperature as an example, we have shown that considerable amounts of side products were formed: phenol was oxidized to quinone **5c** with opening of the dioxolane ring and formaldehyde extrusion. Subsequently, oxidative dimerization of phenol **4c** led to polymethoxybiaryl **6**, and diarylmethane **7** was formed in a reaction with formaldehyde.

The required salicylic aldehydes **8a–d** were prepared by the treatment of phenols **4** with the formylating mixture PCl_5–HCOOEt at room temperature. Formylation of myristicin derivative **4b** resulted in a mixture of regioisomers **8b** and **8′b** in a ratio of 3:1. The structure of aldehyde **8b** isolated by column chromatography was confirmed by comparison with samples obtained by alternative methods.[6,18]

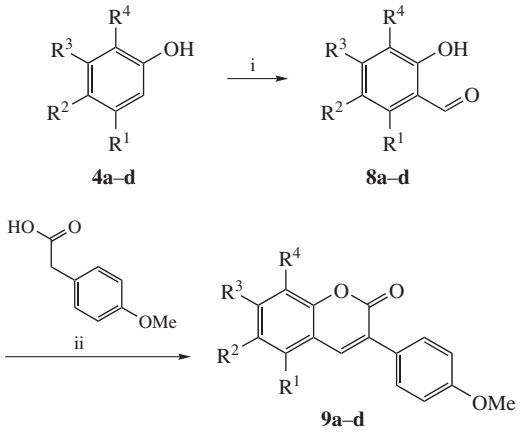
The target polyalkoxy-3-phenylcoumarins **9a–d** were synthesized by the Perkin reaction[19] using carbonyldiimidazole (CDI) by heating equimolar amounts of aldehyde **8** and 4-methoxyphenylacetic acid (Scheme 2).‡

The compounds obtained were tested on embryos of Mediterranian sea urchin *Paracentrotus lividus*.

The sea urchin embryos are widely used as a model object to identify compounds with antiproliferative activity. This study was carried out using the phenotypic sea urchin embryo assay developed in our lab.[20–22] The assay allows for identification of

Reaction of apiolaldehyde **2c** *with H_2O_2–AcOH*. A solution of aldehyde **2c** (2.12 g, 10.1 mmol) in a mixture of AcOH (20 ml) and H_2O_2 (35%, 2.35 ml) was kept for 8 days at room temperature. The reddish-violet deposit of quinone **5c** was filtered off, washed with AcOH (2.3 ml) and dried. The filtrate was evaporated *in vacuo* and dried to afford 1.42 g of a dark oil. Three compounds, namely phenol **4c** (0.14 g), biphenyl **6** (0.44 g) and diarylmethane **7** (0.33 g), were separated from the oil by column chromatography (SiO_2, benzene–AcOEt).

For characteristics of compounds **4b–d**, **5a**,**c**, **6** and **7**, see Online Supplementary Materials.



Scheme 2 *Reagents and conditions*: i, [PCl_5, HCOOEt], $SnCl_4$, CH_2Cl_2, 5 h, 20 °C; ii, CDI, MeCN, reflux, 3 h.

molecules affecting cell division and provides information on the mechanism of antimitotic activity. The experimental procedure includes (i) fertilized egg test for antimitotic activity displayed by cleavage alteration/arrest, and (ii) behavioral monitoring of a free-swimming blastulae treated immediately after hatching. The lack of forward movement, settlement to the bottom of the culture vessel, and rapid spinning of embryos around the animal–vegetal axis suggests a microtubule destabilizing activity caused by a molecule (video illustrations are available at http://www.chemblock.com). The effective concentrations of compounds causing cleavage alteration of sea urchin embryos are comparable with IC_{50} values for mammalian and human cancer cell lines.

‡ *Synthesis of polyalkoxysalicylic aldehydes* **8a–d** *(general procedure)*. (i) Synthesis of the formylation reagent (dichlorodimethyl ether). Ethyl formate (1.32 g, 17.9 mmol) was added at room temperature to a suspension of PCl_5 (3.38 g, 16.28 mmol) in dry CH_2Cl_2 (20 ml). The resulting mixture was refluxed for 2 h with stirring. (ii) Formylation. A mixture of the formylation reagent and phenol (10.85 mmol) was added dropwise to a solution of $SnCl_4$ (1.3 g, 43.4 mmol) in dry CH_2Cl_2 (20 ml) at room temperature. The reaction mixture was stirred for 5 h and diluted with ice-water, followed by extraction with CH_2Cl_2 (3×30 ml). The organic extract was washed with water (4×50 ml), dried and concentrated *in vacuo*. The residual salicylic aldehyde had the purity of about 85%, and was further purified by crystallization or column chromatography (SiO_2) to afford the pure target aldehyde (20–80% yield, R_f 0.6–0.7, AcOEt : light petroleum = 1 : 6).

6-Hydroxy-2,3,4-trimethoxybenzaldehyde **8a**: yield 0.9 g (column chromatography, 45%), mp 54–55 °C (AcOEt–light petroleum) (lit.,[25] 55–57 °C). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.69 (s, 3H, OMe), 3.87 (s, 3H, OMe); 3.98 (s, 3H, OMe), 6.37 (s, 1H, H_{Ar}), 9.98 (s, 1H, CHO) 11.98 (s, 1H, OH). EI-MS, *m/z* (%): 212 $[M]^+$ (100), 197 (91), 195 (1), 182 (5), 179 (10), 169 (58), 167 (12), 137 (48), 109 (10), 95 (8), 69 (51).

Synthesis of 3-arylcoumarins **9a–d** *(general procedure).* A solution of 4-methoxyphenylacetic acid (3.0 mmol) in dry MeCN (5 ml) was treated with carbonyl diimidazole (3.92 mmol) at 20 °C and stirred for 30 min at room temperature. Three portions of a salicylic aldehyde (3.3 mmol) solution in dry MeCN (3 ml) were added in succession. The reaction mixture was refluxed for 1 h after addition of each portion. The target 3-arylcoumarin (yield 3–35%) was separated by column chromatography (SiO_2, R_f 0.4–0.5, AcOEt : light petroleum = 1:4).

*5,6,7-Trimethoxy-3-(4-methoxyphenyl)-*2H*-chromen-2-one* **9a**: yield 0.36 g (35%), mp 112–114 °C (AcOEt–light petroleum, 1:4). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.78 (s, 3H, OMe), 3.81 (s, 3H, OMe), 3.91 (s, 3H, OMe), 3.98 (s, 3H, OMe), 6.91 (s, 1H, H_{Ar}), 7.01 (d, 1H, H_{Ar}, *J* 9.0 Hz), 7.66 (d, 1H, H_{Ar}, *J* 9.0 Hz), 7.98 (s, 1H, C=CH). EI-MS, *m/z* (%): 342 $[M]^+$ (100), 327 (16), 312 (2), 299 (13), 284 (16), 271 (8), 256 (37), 243 (14), 241 (32), 213 (32), 211 (13), 185 (26), 171 (18), 169 (12), 157 (30), 142 (27), 135 (17), 128 (27), 126 (11), 121 (26), 114 (11), 101 (8), 77 (7), 69 (28). Found (%): C, 66.72; H, 5.38. Calc. for $C_{19}H_{18}O_6$ (%): C, 66.66; H, 5.30.

For characteristics of compounds **8b–d** and **9b–d**, see Online Supplementary Materials.

Table 1 Effects of dicoumarol and polyalkoxy-3-phenylcoumarins on sea urchin embryos.

Compound	EC/μM[a]		
	Cleavage alteration	Cleavage arrest	Embryo spinning
Dicoumarol	10	40	>50
9a	0.2	4	>5
9b	4	>4	>4
9c	1	>4	>4
9d	>4	>4	>4

[a]The sea urchin embryo assay was conducted as described in ref. 18. Fertilized eggs/embryos were exposed continuously to 2-fold decreasing concentrations of a compound. Duplicate measurements showed no differences in effective threshold concentration (EC) values. At these concentrations, all tested molecules caused 100% cleavage alteration and embryo death before hatching, whereas at 2-fold lower concentrations, the compounds failed to produce any effect.

The results obtained in the sea urchin embryo assay for the target compounds are shown in Table 1. Figure 2 illustrates morphological changes of arrested eggs caused by dicoumarol and 3-phenylcoumarin **9a**.

Dicoumarol caused cleavage alteration and arrest; in this case, a mitotic spindle was distinctly visible as a light rounded spot in the egg center [Figure 2(*b*)]. The results confirmed the previously described mechanism of dicoumarol cytostatic effect based on cell cycle arrest in mitosis due to microtubule stabilization and alteration of the mitotic spindle dynamics.[15]

Among 3-(4-methoxyphenyl)coumarins **9a**–**d**, only the compound with three methoxy groups, *i.e*. **9a**, had a noticeable antiproliferative activity (EC = 0.2 μM). The structures with a myristicin (**9b**) or apiol (**9c**) moieties altered cell division at higher concentrations of 4 or 1 μM, respectively, whereas dillapiol derivative **9d** was found to be inactive. None of the compounds tested caused changes in embryo motility typical of microtubule destabilizers. Meanwhile, the antimitotic effect of compound **9a** could be considered as a result of targeting tubulin, since cleavage arrest at a concentration of 4 μM was accompanied by formation of tuberculate eggs [Figure 2(*c*)] characteristic of microtubule destabilizers.[20] Thus, both dicoumarol and trimethoxy-3-(4-methoxyphenyl)coumarin **9a** exhibited antimitotic activity affecting tubulin/microtubules. However, unlike dicoumarol, compound **9a** most probably displayed its antiproliferative properties due to microtubule destabilization.

The most active coumarin with an elemicin moiety, **9a**, was further tested in NCI60 cytotoxicity screen against 60 human cancer cell lines at the National Cancer Institute (NCI, USA). It was found that **9a** caused the inhibition of cancer cell growth with the mean GI_{50} = 3.981 μM. The screening results are presented in Online Supplementary Materials.

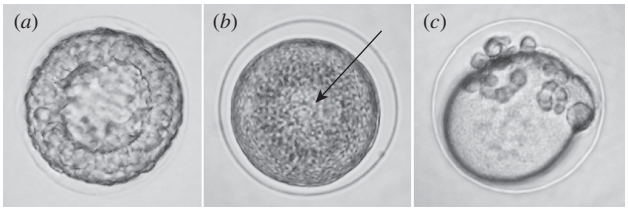


Figure 2 Effects of dicoumarol and compound **9a** on the sea urchin embryo development. (*a*) Intact early blastula. (*b*),(*c*) Arrested eggs. Fertilized eggs were exposed continuously to (*b*) 40 μM of dicoumarol or (*c*) 4 μM of **9a**. Time after fertilization, 6 h. The average embryo diameter is 115 μm. In (*b*), note a light rounded spot in the egg center marked by an arrow.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2013.05.009.

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