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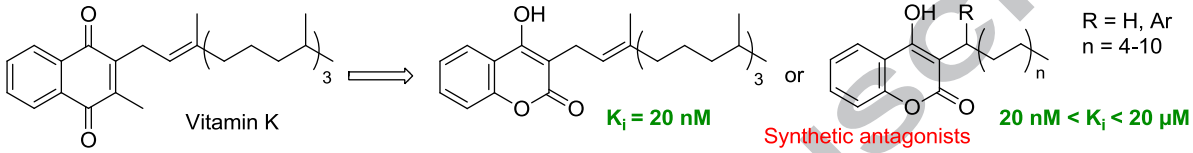

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Synthesis and biological evaluation of C-3 aliphatic coumarins as vitamin K antagonists

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ABSTRACT

Since the discovery of Warfarin in the 1940s, the design of new warfarin-derived anticoagulants for rodent management has been challenging, with mainly structural modifications performed on the C3 position of the coumarin skeleton. In order to better understand the pharmacomodulation of such derivatives, we have synthesized a family of C3 (linear and ramified) alkyl-4-hydroxycoumarins, which conducted to the identification of compounds 5e and 5f as potential short-term active anticoagulants.

Vitamin K 2,3-epoxide reductase (VKORC1) is a key enzyme involved in the vitamin K cycle (Figure 1), catalyzing the conversion of vitamin K 2,3-epoxide to vitamin K. Vitamin K hydroquinone functions as an essential cofactor for vitamin K-dependent carboxylases, which converts a limited number of glutamic acid residues in targeted proteins to γ-carboxyglutamic acid (Gla) residues. These Gla residues enable the proteins to bind Ca^{2+}, as part of the blood coagulation cascade. Concomitantly with γ-carboxylation, the hydroquinone cofactor is converted to the metabolite vitamin K 2,3-epoxide, which is reduced back into vitamin K by VKORC1. Thus inhibition of the activity of VKORC1 with its central position in regulating biosynthesis of biologically active vitamin K-dependent proteins can prevent the formation of vitamin K and subsequently reduced vitamin K. It is well-documented that coumarin-type anticoagulants target the vitamin K cycle by inhibition of VKORC1 activity but their mode of action at the molecular level remains unclear.[1]

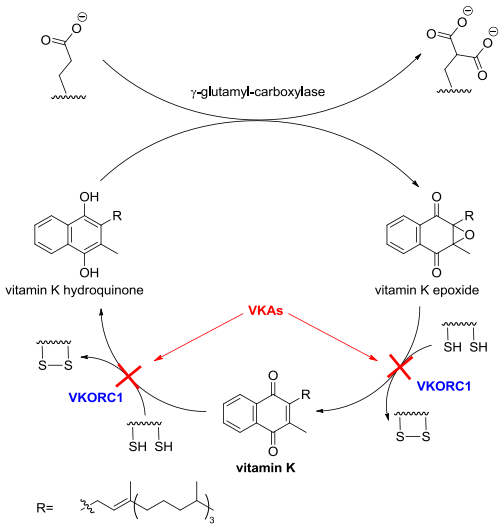
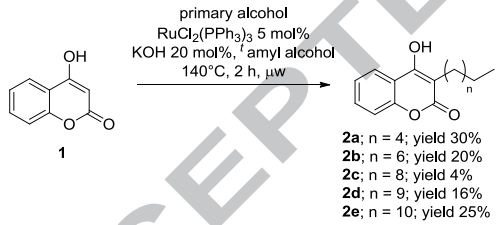


Figure 1. Vitamin K cycle

The data provided by literature preclude a mechanism where warfarin binds covalently to a thiol group at the catalytic site of vitamin K 2,3-epoxide reductase as proposed by Silverman.[2] Up to now, VKORC1 remains the main biological target for thrombosis treatments and for rodenticides but the use of new oral anticoagulants (dabigatran etexilate, rivaroxaban and apixaban) targeting X and II factors are increasing. Compounds that target VKORC1 enzyme are known as VKAs (Vitamin K antagonists), and belong essentially to the 4-hydroxycoumarin or indanedione families.[3]

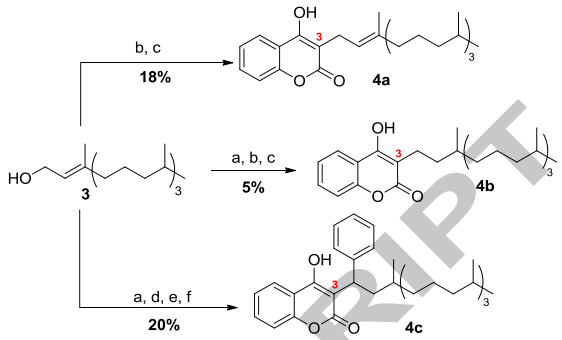
However, despite the use of 4-hydroxycoumarin compounds as VKAs for the last 50 years, very few papers described *in vitro*/*in vivo* structure activity relationship studies.[4] The sole study of SAR was performed in 2007 by Gebauer, pointing out the significant decrease of activity for any structural modification on the 4-hydroxycoumarin nucleus except for the C-3 position substituted by isoprenyl motifs (such as geranyl, ferulenyl or geranylgeranyl).[5] Inspired by this seminal study, we decided to investigate the nature and the length of the lateral chain grafted on 4-hydroxycoumarin and propose a complementary structure-activity relationship study. Simple alkyl chains were introduced *via* a hydrogen auto-transfer methodology. Complexity was introduced with ramified side chains (either from phytol / phytanol or from secondary benzyl alcohols). Biological *in vitro* and *in vivo* results will be collected in tables and discussed at the end of the manuscript.

At the beginning of our project, we decided to take advantage of a recent methodology developed in our group using a borrowing hydrogen reaction between 4-hydroxycoumarin and benzylic alcohol in the presence of ruthenium catalyst.[6] The reaction was easily extended to a large range of substituted benzylic alcohols but limitations with primary alcohols were identified (Scheme 1). With hexanol, octanol, decanol, undecanol and dodecanol, the compounds (**2a-e**) were obtained with rather poor yields but in sufficient amount for *in vitro* biological assays.



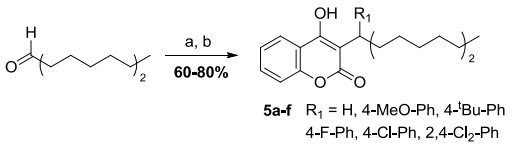
Scheme 1. Dehydrogenative oxidation of primary aliphatic alcohols and coupling with 4-hydroxycoumarin

Structural modifications were performed on C-3 position with introduction of a side chain (with at most one insaturation) structurally related to ferulenol and to vitamin K cofactor. Phytanol / phytol functionalized coumarins were not reported by Gebauer. Three compounds bearing a phytol-derived side chain (Scheme 2) were synthesized. Compounds **4a** and **4b** were prepared by condensation of 4-hydroxycoumarin respectively on phytyl bromide and phytanyl bromide. Phytanol was obtained by alkene reduction of phytol **3** by H_2 and Raney nickel.[7] The competitive C *vs* O alkylation was studied modifying the base (LiOH, NEt_3, $NaOH_{aq}$, ^{t}BuONa), the solvent (H_2O, dioxane, DMSO, THF, EtOH), the concentration, the temperature and / or the nature of the leaving group. Despite this optimization, our efforts failed to provide more than 20% yield of the *C*-alkylated compound at this stage of the synthesis. Nevertheless, we decided to pursue the synthesis with the best conditions in our hands: the use of an alkyl bromide in dry ethanol with 1 eq of freshly crushed NaOH. Compound **4c** was obtained in a four-step sequence. Phenyl magnesium bromide was reacted on phytanal (obtained by classical Swern oxidation of phytanol),[8] to provide the corresponding alcohol, directly submitted to modified Friedel-Crafts alkylation with 4-hydroxycoumarin and iron (III) chloride as a Lewis acid catalyst.[9]



Scheme 2. C_3 alkylation of 4-hydroxycoumarin
a) H_2, Ni(Ra), EtOH, rt, 72 h. b) CBr_4, PPh_3, DCM, rt, 2 h. c) 4-hydroxycoumarin, EtOH, NaOH, reflux, 24 h. d) DMSO, $(COCl)_2$, DCM, 30 min, -60 °C then EtN_3 -60 °C to rt. e) PhMgBr, THF, 0 °C to rt. f) 4-hydroxycoumarin, $FeCl_3$ (0.1 eq), CH_2Cl_2, 100 °C, µW, 1 h.

Combination of a shorter alkyl chain with phenyl substitution was then studied in order to understand the real impact of the lateral chain on the biological activity (importance of R_1 and n in Scheme 3). Thus, by using a simpler linear alkyl chain such as C10 alkyl provided by dodecanal, only two steps of synthesis were necessary to provide the desired coupled branched products **5a-f** (Scheme 3). Addition of Grignard reagents on dodecanal afforded with complete conversion secondary alcohols, directly engaged in previously described coupling conditions with 4-hydroxycoumarin.[9] Following this strategy, six derivatives (**5a-f**) were obtained with good overall yields (60-80%) (Scheme 3).



Scheme 3. Synthesis of ramified coumarins
a) R_1MgBr (3 eq), THF, 0 °C to rt. b) $FeCl_3$ (0.1 eq), CH_2Cl_2, 100 °C, µW, 1 h.

The complete series of these 14 functionalized 4-hydroxycoumarins (**2a-e**, **4a-c**, **5a-f**) (Table 1) were evaluated for their biological activity. For comparison, structurally related phenprocoumon (with R_1 = phenyl and an ethyl group as an alkyl chain), which is used in human medicine in the prevention and the treatment of thromboembolic disorders, was included in the test as an internal standard. The biological activity of the 14 functionalized 4-hydroxycoumarins was first assessed by the *in vitro* determination of their ability to inhibit VKORC1 activity in rat liver microsomes. For this purpose, inhibition constants (K_i) were determined after addition of various concentrations of one of the 4-hydroxycoumarin compounds to the standard solution containing liver microsomes in the presence of increasing amounts of vit K>O (from 0.003 to 0.2 mM). Data were fitted by non-linear regression to the non-competitive inhibition model

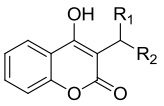
$v= (V_{max}/(1+(I/K_i)))*(S/(K_m+S))$ using the R-fit program.[10]

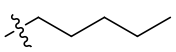
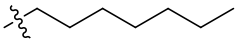
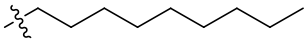
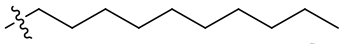
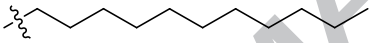
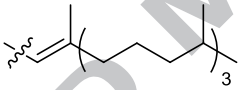
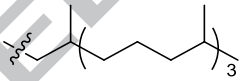
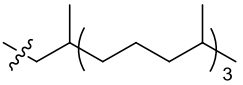
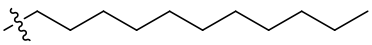
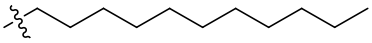
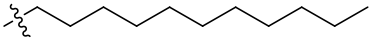
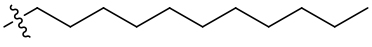
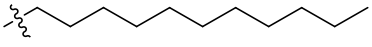
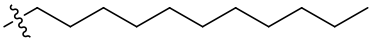
To the exception of the compounds **2a** and **5c**, all the tested compounds displayed sub-micromolar activity varying from 20 nM (for compounds **4a**, **5d** and **5e**) to 200 nM (for **4c**), exhibiting better inhibition than benchmark compound phenprocoumon. The less active compound **2a** is functionalized by a hexyl lateral chain, the length of which seems not sufficient comparing to other related compounds **2**. It is noticeable that increasing the length of the chain brought a significant improvement in the

inhibiting activity. Adding two methylene units led to a 15 fold more potent molecule (**2b**). The optimal activity for simple alkyl chains was observed when undecanol and dodecanol were coupled to 4-hydroxycoumarin (**2d** and **2e**): there was no difference in terms of biological activity as the K_i constants were relatively similar.

In complement of the seminal work from Gebauer, the compounds **4a-c**, bearing isoprenyl fragments also led to excellent activities, especially compounds **4a** and **4b** displaying respectively K_i of 20 nM and 50 nM on VKORC1. Introduction of a phenyl group as R_1 substituent in α-position, led to a 10 fold decrease of the biological activity.

Table 1. Inhibition constants of VKOR activity in the presence of synthetic molecules



Compound	R_1	R_2	K_i (nM)
2a	H		3900± 200
2b	H		260 ± 80
2c	H		90 ± 10
2d	H		60 ± 10
2e	H		50 ± 20
4a	H		20 ± 10
4b	H		50 ± 10
4c	H		200 ± 40
5a	Ph		30 ± 10
5b	4-MeO-Ph		100 ± 40
5c	4-^{t}Bu-Ph		22000 ± 1400
5d	4-F-Ph		20 ± 10
5e	4-Cl-Ph		20 ± 10
5f	2,4-Cl_2-Ph		90 ± 20
Phenprocoumon	Ph	CH_3	200 ± 40

Due to the influence of a steric and hydrophobic substituent in C-3 position, we selected the compound **2e** which was the most efficient in the linear series and decided to study the influence of the nature of R_1 group (compounds **5a-f**) in order to improve the inhibiting power. All the selected molecules displayed nanomolar activities with the exception of **5c** bearing a sterically hindered ^{tert}butyl group in the *para* position of the phenyl group. They were as or even more potent than the linearly substituted compound **2e**. A halogen group in *para* position does not improve *in vitro*-potency of **5d** and **5e**. On the contrary, the presence of a methoxy group in **5b** and of two chlorine atoms in **5f** seems to decrease slightly the biological activity. All these results could be explained by the size and steric hindrance parameters.

Table 2. *In vivo* efficiency and persistence in liver of various synthetic antagonist derivatives

Compound	*in vivo*-test	
	Increase of the prothrombin time (s)	Liver concentration (µg/g of liver)
2e	× 1 [b]	0.7± 0.1 [b]
4a	× 1 [b]	1.3±0.3
4b	× 1 [b]	< LOQ [d]
4c	× 1 [b]	< LOQ [d]
5a	× 1 [b]	0.7±0.2 [b]
5d	× 2 [b]	0.7±0.1 [b]
5e	× 1 [a] / × 4 [b] / × 1 [c]	13±1.2 [a] / 3±0.6 [b] / 1±0.2 [c]
5f	× 2 [b] / × 1 [c]	3.5±0.5 [b] / 0.7±0.1 [c]
Phenprocoumon	× 5.5 [b] / × 5.5 [c]	12.8±0.6 [b] / 1.7±0.3 [c]

PT was determined as the clotting time of a citrated plasma sample to which thromboplastin had been added, according to the methodology described by Damin et al, 2016[12]. For each animal, PT (in seconds) was assessed immediately in duplicate and the PT of one individual was the mean of both measurements. For each time point, PT was determined using 4 different individuals and was the mean of these 4 individuals. Results are presented as the ratio between PT determined for the group of rats receiving one of the compounds and the PT determined for control group not exposed to anticoagulant molecules. (× 1, normal PT was observed; × 2, × 4, × 5.5, PT was 2-, 4- or 5.5-fold increased compared to the control group for which PT was 17.9 ± 2.5)
Liver concentration was determined by HPLC after liquid-tissue extraction as described by Damin et al, 2016[12]. Results are expressed as mean values ±SD of concentration determined in 4 rats. [a]: 7 h / [b]: 24 h / [c]: 72 h after oral injection (10 mg/kg); [d] LOQ : limit of quantification

To go further into the biological evaluation, *ex vivo* tests were performed in 8-weeks old *Sprague Dawley*® male rats. 7, 24 or 72 hours after oral administration (10 mg/kg) of one of the compounds, a group of 4 rats were anesthetized with isoflurane and blood was taken by cardiac puncture into citrated tubes. Finally, rats were euthanized with CO_2 and liver was immediately collected. Ability of compounds to inhibit the blood coagulation was evaluated by their respective ability to increase *in vivo* the prothrombin time. The PT of control group, that is, the basal levels of PT when not exposed to anticoagulant molecules, was 17.9 ± 2.5 for 8-weeks old *Sprague Dawley* male rats male. Increase of prothrombin time after administration of one of the compounds are presented in Table 2 (× 1, means that no significant increase of the prothrombin time was observed. Tissue-persistence of compounds was also evaluated by the determination of residues in liver through HPLC-UV quantitative analysis (Table 2). Residues were quantified in liver, the storage tissue of these molecules and the tissue where vitamin K-dependent clotting factors are synthesized and activated by gamma-carboxylation before secretion in blood. Quantity of secreted activated vitamin K-dependent clotting factors in blood are crucial for normal coagulation and influence PT.

Phytol-derived compounds **4a-c** and compound **5a** were not enough persistent to impact on prothrombin time. Compounds **5b** and **5c** were not evaluated *in vivo* due to their low *in vitro* activity. Among compounds **5** bearing a C12 lateral chain, the presence of a halogen group in the *para* position of the phenyl moiety exhibited promising *in vivo* anticoagulant potency after 24 h (**5d-f**). We could assume that the halogen atom may protect VKA from drug metabolism enzymes attack into liver. The highest anticoagulant activity was obtained with compound **5e** bearing one chlorine atom: prothrombin time was 4-fold increased 24 h after administration of compound **5e**. But 72 h after administration of **5e,** coagulation was no longer increased, as observed for **5f** and contrary to phenprocoumon. Compounds **5e** and **5f** could be good short-term active anticoagulants but to confirm this hypothesis, metabolic study has to be carried out to determine if these molecules are metabolized by CYP2C9.[11] Indeed, in human medicine, dosage variability of VKA is due to polymorphisms of CYP2C9. Selecting a new VKA able to efficiently inhibit VKORC1 with a moderate tissue persistence, which would be not metabolized by CYP2C9, could be a major improvement in the management of patients.

By targeting a synthetic antagonist close to vitamin K (**4a**), anticoagulants displaying interesting *in vitro* potency were prepared. *In vivo* efficiency was confirmed by the introduction of an additional halogen substituted phenyl group on a shortened lateral saturated chain.

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