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Farnesyloxycoumarins, a new class of squalene-hopene cyclase inhibitors

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Abstract—A few naturally occurring prenyl- and prenyloxycoumarins and several new related synthetic derivatives were evaluated as inhibitors of squalene-hopene cyclase (SHC), a useful model enzyme, to predict their interactions with oxidosqualene cyclase (OSC). Umbelliprenin-10′,11′-monoepoxide (IC_{50} 2.5 μM) and the corresponding 6′,7′-10′,11′ diepoxide (IC_{50} 1.5 μM) were the most active enzyme inhibitors.

Sterol synthesis is one of the essential pathways in eukaryotic cells. Over the last two decades an impressive research effort has been mustered to characterize the enzymes responsible for its several steps.[1,2] Some of them have been chosen as targets for new inhibitors that are being proposed for the treatment of numerous diseases. Among the best known compounds we may quote are the statins, powerful inhibitors of HMG-CoA reductase that are used to control hypercholesterolemia,[3,4] such antifungal agents as the allylamines, inhibitors of squalene epoxidase,[5,6] and azole drugs, inhibitors of 14α-demethylase.[7–9]

One of the most intensively investigated enzymes of sterol synthesis, oxidosqualene cyclase (OSC), catalyzes the remarkably complex conversion of an acyclic compound, 2,3-oxidosqualene, into the first cyclic intermediate along the pathway. This compound is lanosterol in all non-photosynthetic organisms, cycloartenol in plants.[10]

After dozens of OSCs were purified, characterized, cloned and sequenced,[11–18] hundreds of inhibitors have been designed and tested[19–23] as promising candidates for use as antifungal[24] or cholesterol-lowering drugs.[25] Recently, a novel series of *orally active* inhibitors have been tested on human liver microsomal OSC, and their effectiveness as cholesterol-lowering drugs has been evaluated in hyperlipidemic hamsters.[26] OSC inhibitors are also under investigation as potential anti-trypanosomal drugs.[27,28]

The design of new OSC inhibitors received a further impulse when the complete structure of a squalene-hopene cyclase (SHC) was elucidated.[29] This bacterial enzyme, isolated from *Alicyclobacillus acidocaldarius*, proved particularly interesting because its sequence revealed a homology of 17–38% and an identity of 20–26% with eukaryotic OSCs.[30,31] Bacterial and eukaryotic cyclases contain a highly conserved repeating motif, called the QW motif, rich in glutamine and tryptophan residues, and a conserved aspartate-containing domain involved in the catalytic action.[29] SHC may therefore be used as a model to predict how newly designed inhibitors will interact with OSCs. Recently, the interaction of some novel inhibitors of human OSC[26] with the active site of SHC was studied quantitatively by co-crystallization experiments.[32] Because comparative studies[33,34] have shown a close correspondence of inhibitor activities on the two enzyme types, we decided to test oxyprenylcoumarins using a recombinant SHC from *A. acidocaldarius* expressed in *Escherichia coli*. The encouraging results that are reported below may be taken as a preliminary indication of the inhibitory potency of these compounds on eukaryotic OSCs.

Our screening began with a series of naturally occurring prenyl- and prenyloxycoumarins (Fig. 1) isolated from

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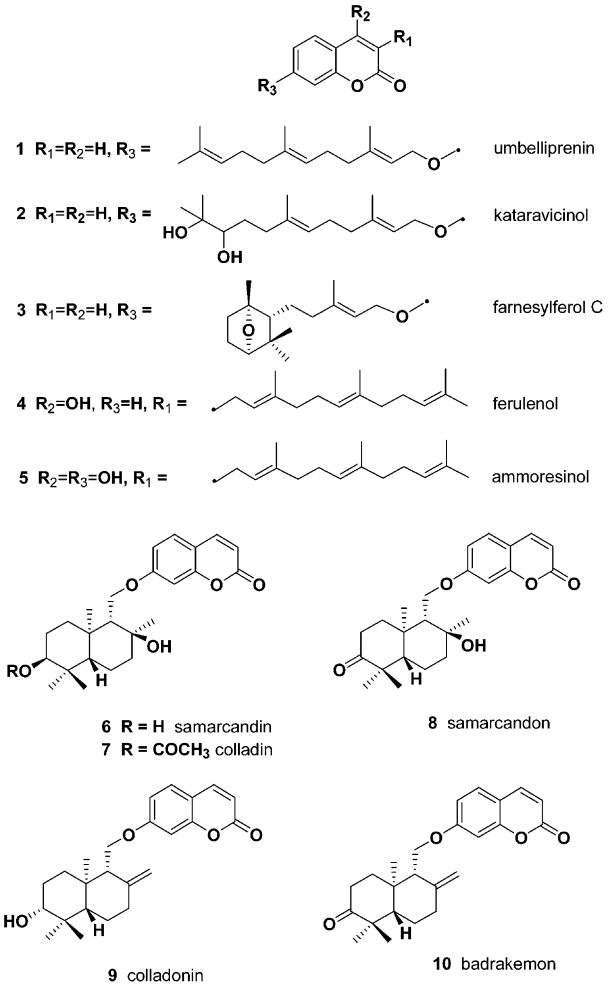


Figure 1. Natural products from the resin *Ferula assa-foetida*, *Ferula communis* and from gum ammoniac resin.

the resin of *Ferula assa-foetida* (**1**–**3**, **6**–**10**),[35] a plant that grows wild in Iran, in Afghanistan and other neighboring countries.[36] The resin is obtained after incision of the root by evaporating to dryness the collected lymph. Ferulenol (**4**) was extracted from the toxic variety of *Ferula communis*,[37] common in Mediterranean areas, and ammoresinol (**5**) from gum ammoniac resin.[38]

Inhibition tests carried out on SHC from *A. acidocaldarius* at concentrations up to 100 μM showed that only four among these compounds have inhibitory activity: umbelliprenin (**1**) (IC_{50} 70 μM), kataravicinol (**2**) (IC_{50} 65 μM), ferulenol (**4**) (IC_{50} 90 μM) and especially farnesylferol C (**3**) (IC_{50} 7 μM) (Table 1), that accordingly was chosen for a more detailed study. The enzyme activity was measured in the presence of several concentrations of both substrate and inhibitor, and data were plotted according to Lineweaver-Burk and Dixon. The result showed with reasonable certainty that farnesylferol C behaves as a noncompetitive inhibitor of SHC.

In order to investigate the structure–activity relationship, we prepared a small library of farnesyloxycoumarins,

Table 1. SHC inhibitory activity[48] for compounds **1**–**14**, **16**, **17**, **19**, **21** and **22**

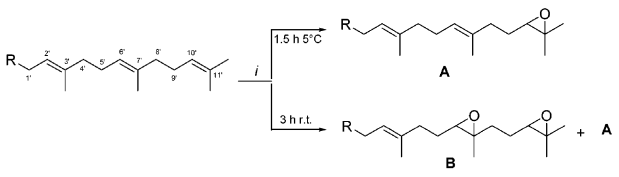
Compd	IC_{50} (μM)[a] or % inhibition	Compd	IC_{50} (μM)[a] or % inhibition
1	70	**11**	2.5
2	65	**12**	1.5
3	7.0	**13**	na[a]
4	90	**14**	40% (100 μM)
5	na[a]	**16**	100
6	na[a]	**17**	38% (100 μM)
7	na[a]	**19**	67
8	na[a]	**21**	46% (100 μM)
9	na[a]	**22**	7% (100 μM)
10	na[a]		

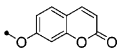
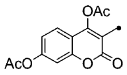
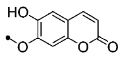
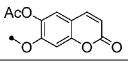
[a] Values are means of three experiments, (na = not active at 100 μM).

starting from hydroxy coumarins; 4- and 7-hydroxycoumarin were easily *O*-alkylated with farnesyl bromide after deprotonation with K_2CO_3 in anhydrous acetone whereas 6,7-dihydroxycoumarin (esculetin) was regioselectively monoalkylated with farnesol at position 7 under the Mitsunobu protocol, as previously reported by us.[39]

The considerable inhibitory activity of farnesylferol C (**3**) prompted us to synthesize several related compounds carrying a terminal epoxy group. We had in fact observed previously[33] with terpenoid OSC inhibitors that their activities increased when a terminal double bond was replaced with an epoxy group. On the other hand, as oxidosqualene binds more strongly than squalene to SHC,[40] it was likely that a terminal epoxy group would increase the affinity of isoprenyl compounds for the enzyme.

Thus, we proceeded to investigate selective monoepoxidation of farnesylcoumarins. Since most acyclic terpenes possess only non-conjugated, trisubstituted double bonds that are approximately sterically and chemically equivalent, selective oxidation of the terminal double bond is not an easy task. Among the known methods for the selective epoxidation of the terminal C=C in terpenes, a particularly efficient one is the reaction with *N*-bromosuccinimide (NBS) in THF/H_2O followed by conversion of the bromohydrin to epoxide by treatment with base.[41] In 1962, van Tamelen first reported the oxidation of umbelliprenin (**1**) to the ω-monoepoxide (**11**) using this method.[42] In our hands epoxidations carried out with MCPBA or with Oxone® in phosphate-buffered acetone–CH_2Cl_2[43] yielded in all cases complex epoxide mixtures. We resorted to a one-pot epoxidation with methyltrioxorhenium (MTO) (CH_3ReO_3) and urea-hydrogen peroxide adduct (UHP). MTO-catalyzed epoxidations have been thoroughly documented[44,45] and the UHP adduct works as an anhydrous oxygen atom donor.[46] This reaction, when carried out at 5 °C, selectively yielded the terminal monoepoxide, whereas at 20 °C with excess oxidant mainly yielded the 6′,7′-10′,11′ diepoxide (Scheme 1). The structural assignment for 7-farnesylesculetin has been already included in our previous paper.[39]



Starting compd	R	Compd A	Compd B
1		11	12
13		14	-
15		16	17
18		19	-
20		21	22

i: CH_3ReO_3 (MTO), urea-H_2O_2 adduct (UHP), $CHCl_3$

Scheme 1.

Although the chemical method employed to obtain the monoepoxides was specific for the terminal double bond, we confirmed the structure assignments of mono- and di-epoxides via 1D and 2D ^{1}H NMR spectroscopy. The g-DQCOSY spectra of the diepoxides showed a connectivity between the unique olefinic proton with the easily recognized O-CH_2 doublet (in compounds **12** and **22**) and with the C_{sp2}-CH_2 resonance (doublet at 3.18 ppm) in **17**, thus confirming the 6′,7′-10′,11′ structures. In these spectra the 3′-Me signal was also clearly recognized. As regards the monoepoxides, the terminal 10′,11′ location was confirmed by the same experiments showing besides the previously mentioned connectivities for H-2′, that the new olefinic proton must be located at position 6′ because it exhibited a cross-peak with one methyl group only (the C_{sp2}-Me at position 7′), and none with either the 11′- or 12′-methyl groups.

As expected, umbelliprenin-10′,11′-monoepoxide (**11**) and the corresponding 6′,7′-10′,11′ diepoxide (**12**)[47] proved the most active enzyme inhibitors (Table 1). The relative activity values[48] of monoepoxides **14** (deriving from 4-farnesiloxycoumarin **13**) and **16** as well as of the diepoxide **17** (prepared from ammoresinol diacetate **15**) indicated that activity strongly depends on the location of the oxyprenyl chain. The spontaneous intramolecular cyclization of ferulenol ω-monoepoxide prevented the testing of this compound; we could however test the ω-monoepoxide of ammoresinol diacetate (**16**). We also found that even small modifications of the 7-hydroxy coumarin moiety were deleterious for inhibitor activity, as demonstrated by the monoepoxide **19** (prepared from 7-*O*-farnesylesculetine **18**) and the corresponding acetylated mono- and di-epoxides **21** and **22** deriving from 6-acetyl-7-*O*-farnesylesculetine **20** (Table 1).

The tip of the prenyl moiety probably plays a crucial role in molecular recognition by mimicking the portion of the squalene molecule that interacts with the substrate-protonating groups of the enzyme, while the coumarin moiety interacts with the aromatic residues at the active site that are involved in stabilizing the high-energy intermediates.

In summary, we proved that the 7-hydroxycoumarin moiety is a good starting structure for the design of new SHC inhibitors. An epoxide function at the end of the prenyl chain increased the inhibitory effect, revealing a critical detail for molecular recognition. The present study is to be extended to eukaryotic oxidosqualene cyclases.

Acknowledgements

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47. *General procedure for regioselective mono- and diepoxidation with UHP/MTO*. Farnesylcoumarin (1 mmol) was added to a magnetically stirred suspension of methyltrioxorenium (0.2 mmol) and urea–H_2O_2 adduct (2 mmol) in $CHCl_3$ (8 mL/mmol) previously cooled to 0 °C. After stirring for about 90 min at 5 °C to complete disappearance of the starting material (TLC), the mixture was diluted with $CHCl_3$, washed with brine, and dried (Na_2SO_4) giving in all cases the terminal monoepoxide (yields 60–65%). Using more methyltrioxorenium (0.3 mmol) and urea–H_2O_2 adduct (2.5 mmol), after 3 h stirring at 20 °C we obtained the corresponding 6′,7′:10′,11′-diepoxide as major product. Mono- and diepoxide were easily separed by column chromatography (hexane/EtOAc 9:1 v/v).
All mono- and diepoxides have been spectroscopically characterized and analytical data have been obtained. For example, (*E*)-7-(5-{3-[2-(3,3-dimethyloxiranyl)-ethyl]-3-methyloxiranyl}-3-methylpent-2-enyloxy)-chromen-2-one (**12**) was isolated as a colorless oil (56%, yield); IR (liquid film) 1732, 1614, 1555, 1506, 1436, 1235, 1120 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.61 (1H, d, $J = 9.5$ Hz, H-4), 7.33 (1H, d, $J = 8.5$ Hz, H-5), 6.84–6.79 (2H, m, H-6 and H-8), 6.22 (1H, d, $J = 9.5$ Hz, H-3), 5.48 (1H, tq, $J = 6.8$ Hz, $J = 1.0$ Hz, H-2′), 4.58 (2H, d, $J = 6.3$ Hz, OCH_2), 2.38–2.24 (2H, m, H-6′ and H-10′), 2.12 (2H, m, 4′-CH_2), 1.77–1.47 (6H, m, 5′-CH_2, 8′-CH_2, and 9′-CH_2), 1.75 (3H, d, $J = 1.0$ Hz, 3′-CH_3), 1.24 (3H, s, 11′-CH_3), 1.16 (3H, s, 11′-CH_3), 1.10 (3H, s, 7′-CH_3); MS (CIMS) *m*/*z* (%) 399 (MH^+, 20), 381 (58), 162 (100). Anal. calcd for $C_{24}H_{30}O_5$: C, 72.34; H, 7.59. Found: C, 72.40; H, 7.38.
48. IC_{50} values (inhibitor concentrations that reduce by 50% the enzymatic conversion of squalene to hopene) were determined at 10 μM substrate concentration.[33] [^{14}C]squalene was obtained by incubating 1 microCi of [^{14}C]mevalonolactone with S_{10} supernatant of pig liver homogenate (25 mg of proteins) following the method of Popják,[49] in the presence of the oxidosqualene cyclase inhibitor U-18666A. Under these conditions the bulk of radioactivity of nonsaponifiable extract was shared between squalene and oxidosqualene, which can be easily separated by TLC.[50]
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