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Original article

Umbelliferone aminoalkyl derivatives, a new class of squalene-hopene cyclase inhibitors

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Abstract

The synthesis is described of several aminoalkyl derivatives of coumarin, obtained in good yields under microwave or high-intensity ultrasound irradiation. These compounds proved uniformly active as inhibitors of squalene-hopene cyclase (SHC) from *Alicyclobacillus acidocaldarius*. Their design stemmed from our recent finding that the umbelliferone nucleus acquires inhibitory properties towards SHC when functionalized with a suitable chain such as the ω-epoxyfarnesyl group. Under our experimental conditions the most active ones, such as 7-(4′-allylmethylamino-but-2-ynyloxy)chromen-2-one (IC_{50} 0.75 mM), approached the potency of anticholesteremic drug Ro 48-8071 (IC_{50} 0.35 mM), an effective inhibitor of both squalene- and oxidosqualene-cyclases (OSC). Tests are in progress to determine their efficacy on different eukaryotic OSCs.
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Keywords: Coumarin derivatives; Squalene-hopene cyclase; Oxidosqualene cyclase; Microwave; High-intensity ultrasound

1. Introduction

One of the most intensively studied enzymes of sterol biosynthesis, oxidosqualene cyclase (OSC), catalyzes the conversion of an acyclic compound, 2,3-oxidosqualene, to the first cyclic intermediate along the pathway. This is lanosterol in all non-photosynthetic organisms, cycloartenol in plants [1]. Several mammalian OSCs have been isolated, characterized, cloned and sequenced [2–9]; in the same spate hundreds of OSC inhibitors have been designed and tested [10–14] as potential anti-fungal [15] or cholesterol-lowering drugs [16]. 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 [17]. OSC inhibitors are also under investigation as potential antifungal [18] and anti-trypanosomal agents [19–21].

As no detailed crystal structure has been published yet for eukaryotic OSCs, a straightforward structure-based drug design is not feasible for the development of novel inhibitors. However, the elucidation of the crystal structure of squalene-hopene cyclase (SHC) [22], the functional analogue of OSCs in bacteria, opened the road to understanding the catalytic mechanism of OSCs and provided a good model for the preliminary testing of potential inhibitors. Experiments of co-crystallization of SHC with squalene-like [23] and other molecules [24,25], in conjunction with homology-modelling studies [26], have shed light on the structural requirements for a molecule to fit the active site of these enzymes. One such required feature is a tertiary amino group linked to a hydrophobic moiety. Crystallographic studies have established that the positive charge of the amine directly interacts with the aspartate residue of the conserved DDTA motif at the active site of SHC [23–25]. Recently, after investigating a group of meroterpenoids that proved efficient inhibitors of SHC [27], we proceeded to simplify the most active structures (**1**,**2**, Fig. 1), gathering useful clues to improve molecular design. We later found [28] that 2,4-dihydroxyacetophenone also provides a convenient starting skeleton: compounds **3** and **4** tested as moderately active. The present work elaborates further on the umbelliferone nucleus (7-

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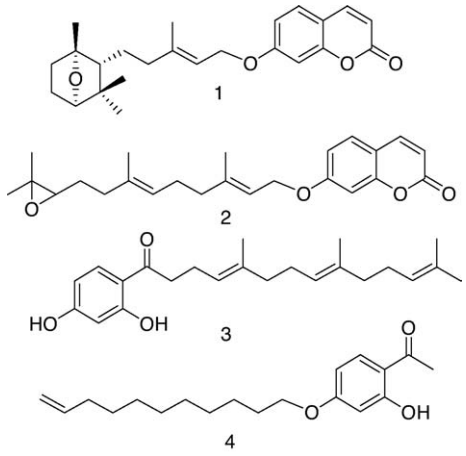


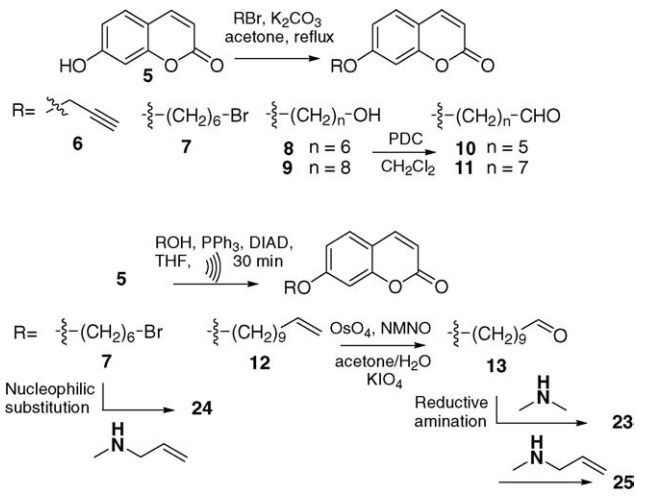
Fig. 1. SHC inhibitors derived from umbelliferone and 2,4-dihydroxyacetophenone: farnesyferol C (**1**) (IC50 7.0 μM), umbelliprenine-10′,11′-monoepoxyde **(2)** (IC50 2.5 μM), secoammoresinol (**3**) (IC50 30 μM), and 4-(10-undecenyl) resacetophenone **(4)** (IC50 48 μM).

hydroxycoumarin) by the insertion of alkyl chains bearing a terminal amino group; this appears in fact to be most promising feature for OSC inhibitory activity.

2. Chemistry

O-alkylation of umbelliferone **(5)** was achieved by treatment with alkyl bromides and base or, more efficiently, by the Mitsunobu reaction (alcohol, DIAD, TPP, THF) carried out under sonochemical conditions as previously described [29]. High-intensity ultrasound (HIU) considerably reduced reaction times and gave uniformly good yields (Scheme 1).

Among the strategies we explored to insert the amino function in the side chain, the most efficient ones were microwave (MW)-promoted Mannich aminomethylation of a terminal alkyne, reductive amination under HIU and classic nucleophilic substitution with alkyl bromides. *O*-propargylation of **5** afforded the well-studied compound **6** [30], a



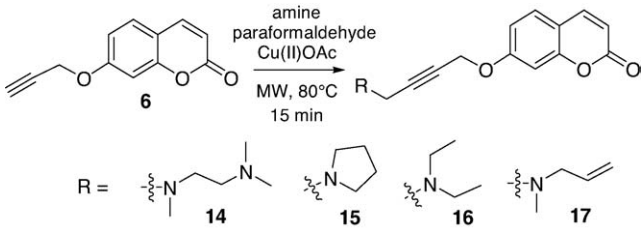
Scheme 1. Conditions for umbelliferone alkylation and further modifications of the side chain.

suitable substrate for the Mannich reaction of terminal C(*sp*) with putative methyliminium species originating from the condensation of formaldehyde with secondary amines such as *N,N,N′*-trimethylethylenediamine, pyrrolidine, diethylamine and allylmethylamine (**14**–**17**, Scheme 2). Our previous protocol [31] was much improved working in tightly sealed pressure-resistant tubes and irradiating with MW. Table 1 shows the isolated yields for both methods.

Umbelliferone **5** was *O*-alkylated with 6-bromohexanol [32], 8-bromooctanol and 1,6-dibromohexane by refluxing in acetone in the presence of potassium carbonate [33]. The primary alcoholic groups were successively oxidised to aldehyde by stirring at room temperature with pyridinium chlorochromate in CH_2Cl_2. A longer alkyl chain (C_{10}) was inserted by Mitsunobu dehydroalkylation of umbelliferone with 10-undecen-1-ol [29], then exploiting the C=C double bond as a latent carbonyl function. Oxidative demolition of the terminal olefin with osmium tetroxide/*N*-methylmorpholine *N*-oxide and potassium periodate afforded the 10-formyl derivative [34]. Products easily underwent reductive amination under HIU. This reaction was carried out in a sonochemical reactor developed in the authors' laboratory [35] using methanol–water 7:3 containing 3% CH_3COOH and excess amine (benzylamine, piperidine, morpholine, dimethylamine or allylmethylamine) followed by treatment with $NaBH_3CN$ (Scheme 3). After about 1 h sonication, amino derivatives **18**–**25** were isolated in good yields (70–85%).

Products **19**, **20** and **24** were obtained in comparable yields but after much longer reaction times (36–48 h) by nucleophilic substitution of 7-(6-bromohexyloxy)coumarin **(7)** [36] prepared by *O*-alkylation of umbelliferone with 1,6-dibromohexane.

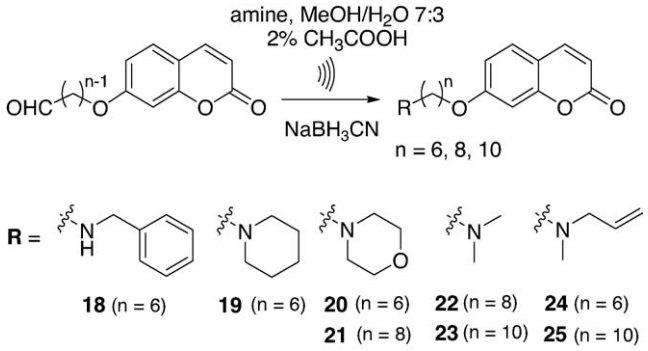
Although we had previously found that derivatives of umbelliferone were generally more active than those of 4-hydroxycoumarin bearing the same chain [27], we decided to determine the effect of introducing an amino group into



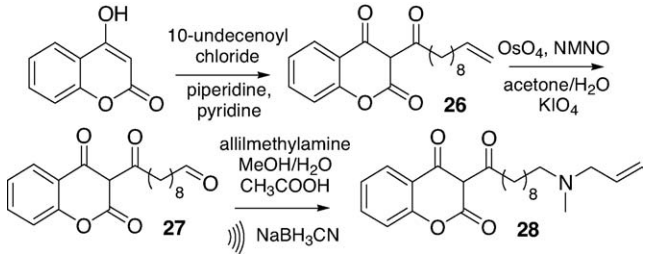
Scheme 2. MW-promoted Mannich aminomethylations of *O*-propargyl umbelliferone.

Table 1
Yield % of Mannich aminomethylations

Compound	MW (90 °C) (15 min), yield %	Heating under reflux (9 h), yield %
14	81	34
15	92	54
16	96	53
17	94	60



Scheme 3. Sonochemical reductive amination of umbelliferone formyl derivatives.



Scheme 4. Synthesis of 3-aminoacyl derivatives of 4-hydroxycoumarin.

one of the latter. The allylmethylamino derivative of 4-hydroxycoumarin (**28**) was prepared in three steps: acylation with 10-undecenoyl chloride, oxidative demolition of the terminal double bond and reductive amination under HIU (Scheme 4).

3. Biology

Inhibition tests carried out on SHC from *Alicyclobacillus acidocaldarius* at concentrations up to 100 μM showed that, excepting the adduct of umbelliferone with the *N,N,N′*-trimethylethylenediamine **(14)** and the 3-aminoacyl derivative of 4-hydroxycoumarin **(28)**, all compounds listed in Table 2 had good and comparable inhibitory activities. Most active were **17** (IC_{50} 0.75 μM) and **25** (IC_{50} 2 μM), that shared a methylallylamino group.

Table 2
Inhibitory activities on SHC for compounds **14**–**28** in comparison with Ro 48-8071 (Hoffman-La Roche)

Compound	IC50 (μM) [a]	Compound	IC50 (μM) [a]
14	na [a]	**21**	7
15	5	**22**	5–7
16	5	**23**	5
17	0.75	**24**	4–5
18	8	**25**	2
19	8	**28**	100
20	6	**Ro 48-8071**	0.35

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

4. Result and discussion

As described in paragraph 2, several aminoalkyl derivatives of coumarin were prepared in good yields by MW- and HIU-promoted reactions. Umbelliferone derivatives proved uniformly effective on SHC from *Alicyclobacillus acidocaldarius*. Their design stemmed from our recent finding that the umbelliferone nucleus acquired inhibitory properties towards SHC when functionalized with a suitable side chain such as the ω-epoxyfarnesyl group [27]. The tip of the prenyl moiety plays here a crucial role by mimicking the portion of the squalene molecule that interacts with the substrate-protonating groups of the enzyme [37], while the coumarin moiety interacts with the aromatic residues at the active site that are involved in stabilizing the high-energy intermediates. As the literature abundantly documents the importance of an amino group in prenyl- and non prenyl-type inhibitors of squalene and oxidosqualene cyclases, we replaced the epoxyfarnesyl arm with a 6–10 C chain bearing a tertiary amino group.

The majority of derivatives tested in the present work proved to be active with IC_{50} values of 2–7 μM. The type of amino group, the length and carbon unsaturation of the chain influenced activity only slightly. Most active was **17** (IC_{50} 0.75 μM), in which the rigid acetylenic arm bears an allylmethylamino function. Under our experimental conditions the activity of **17** approached that of the anticholesteremic drug Ro 48-8071 (IC_{50} 0.35 μM), an effective inhibitor of both squalene- and oxidosqualene cyclases [16,17,25] that has a very different structure but also contains the same amino group.

5. Conclusion

In summary, we showed that umbelliferone aminoalkyl derivatives are a new promising class of SHC inhibitors, and pinpointed the structural details of the aminoalkyl group that are critical for molecular recognition. These compounds are easier to prepare and much more stable than monoepoxyfarnesyl derivatives previously studied by us [27]. Tests are in progress to determine their efficacy on different eukaryotic OSCs.

6. Experimental protocols

6.1. General methods

Anhydrous conditions were achieved (when indicated) by flame-drying flasks and other equipment. Reactions were monitored by TLC on Alugram Sil—Macherey-Nagel F254 (0.25 mm) plates, which were visualized by UV inspection and stained with a 5% $KMnO_4$ solution or by heating after a spray of 5% H_2SO_4 in ethanol. Macherey-Nagel silica gel was used for column chromatography (CC). A Waters mi-

croPorasil column 7.8-300 was used for semipreparative HPLC, a Gilson 133 refractive index refractometer serving for peak detection. Melting points were obtained on a Büchi SMP-20 apparatus and are uncorrected. ^{1}H NMR (300 MHz) spectra were recorded on a Bruker 300 Advance spectrometer at 25 °C. CIMS were performed on a Finnigan-MAT TSQ70 with isobutane as reactant gas. Unless otherwise noted commercially available reagents and solvents were used without further purification. Immediately prior to use CH_2Cl_2 and THF were distilled under N_2 from P_4O_{10} and sodium-benzophenone ketyl, respectively.

The sonochemical apparatus used in the present work was designed for stringent reaction conditions. The transducer, consisting of two high-efficiency pre-stressed piezoelectric rings, is lodged in a Delrin® housing that can be cooled by a flow of refrigerated oil. Moreover, the whole probe system (comprising transducer, booster and immersion horn) is refrigerated by an oil forced-circulation circuit connected to an oil–freon heat exchanger; this is cooled in turn by a refrigerator unit. Frequency can be tuned between 17 and 45 kHz and power output can be varied up to 200 W/cm^2. To achieve optimal acoustic efficiency the reactor can rotate eccentrically around the horn and the probe can be made to move alternatively up and down by a predetermined excursion at a chosen speed. Transducer resonance is maintained by a true motional feedback network. Electrical and acoustic parameters are monitored. Reactions were carried out in a Teflon® tube (1 mm thick) inserted in a Delrin® reactor that was thermostatted by four Peltier modules.

For microwave-promoted reactions a modified version of a domestic oven (Candy MSA 20M) was used. The oven was placed in a protective cage. CAUTION: reaction vessels must be allowed to cool down below the boiling point of the solvent before they are removed from the oven and opened. Analyses for C, H, N were within ±0.3% of the theoretical value.

6.2. General procedures for O-alkylation of umbelliferone

6.2.1. Conventional protocol

In a 50 ml two-necked, round-bottomed flask equipped with a magnetic stirrer, a condenser and a nitrogen inlet, umbelliferone (891 mg, 5.5 mmol), K_2CO_3 (1.52 g, 11.0 mmol) and 33 ml of anhydrous acetone were added. The stirred mixture was heated under reflux for 2 h under nitrogen atmosphere, then allowed to cool down to room temperature before the alkylbromide or bromoalcohol (5.5 mmol) was added dropwise. The resulting mixture was heated under reflux for another 7 h. Eluents used for TLC were hexane/EtOAc 1:1 or 7:3.

Work-up: the reacted mixture was quenched with water (20 ml), extracted with ethyl acetate (50 ml) and washed with brine (20 ml). After drying ($MgSO_4$) and removal of the solvent, the residue was purified by silica gel CC (hexane/EtOAc, 9:1) to afford the corresponding 7-alkyloxycoumarin in high yield.

6.2.2. Mitsunobu reaction under HIU

A solution in anhydrous THF (35 ml) containing triphenylphosphine (2 mmol), the alcohol (2 mmol) and umbelliferone (2 mmol), was sonicated (18.0 kHz, 100 W/cm^2) at 10 °C under an argon atmosphere. Diisopropyl azodicarboxylate (DIAD) (2 mmol) was added dropwise over 5 min. The orange-red colour of DIAD immediately disappeared and a weakly exothermic reaction occurred. The mixture was sonicated for 35–55 min at 20 °C. When the reaction was complete, as indicated by TLC (eluent: hexane/EtOAc, 9:1), the mixture was evaporated to dryness. The residue was diluted with hexane–ether 3:1 v/v, filtered through a thin pad of Celite® to remove the precipitate of triphenylphosphine oxide and concentrated under reduced pressure. Finally the product was purified by silica gel CC. The reaction was carried out under nitrogen in a Teflon tube inserted in the thermostatted reactor.

6.2.3. 7-(6-Bromohexyloxy)chromen-2-one (7)]

White powder; m.p. 45 °C. IR (KBr) 3422, 1722, 1624, 1400, 1293, 1236, 829 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ = 7.64 (1H, d, J = 9.5 Hz, H-4), 7.37 (1H, d, J = 8.4 Hz, H-5), 6.85–6.81 (2H, m, H-6, H-8), 6.25 (1H, d, J = 9.46 Hz, H-3), 4.03 (2H, t, J = 6.34 Hz, H-1′), 3.44 (2H, t, J = 6.72 Hz, H-6′), 1.90–1.52 (8H, m, CH_2). CIMS: 325 (M + H)$^+$. R_f = 0.50 (hexane/EtOAc, 1:1).

6.2.4. 7-(6′-hydroxyhexyloxy)chromen-2-one (8)]

White powder; m.p. 49 °C. IR (KBr) 3450, 1720, 1620, 1540, 1295, 1232, 1120 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ = 7.64 (1H, d, J = 9.5 Hz, H-4), 7.36 (1H, d, J = 8.4 Hz, H-5), 6.85–6.79 (2H, m, H-6, H-8), 6.24 (1H, d, J = 9.5 Hz, H-3), 4.02 (2H, t, J = 6.5 Hz, H-1′), 3.67 (2H, t, J = 6.5 Hz, H-6′), 1.83 (2H, m, H-2′) 1.60 (2H, m, H-5′) 1.55–1.44 (4H, m, H-3′ and 4′). CIMS: 263 (M + H)$^+$. R_f = 0.46 (hexane/EtOAc, 1:1).

6.2.5. 7-(8′-hydroxyoctyloxy)chromen-2-one (9)]

White powder; m.p. 101 °C. IR (KBr) 3450, 1738, 1646, 1473, 1410, 1298, 838 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ = 7.58 (1H, d, J = 9.5 Hz, H-4), 7.29 (1H, d, J = 8.4 Hz, H-5), 6.77–6.69 (2H, m, H-6, H-8), 6.16 (1H, d, J = 9.5 Hz, H-3), 3.57 (2H, t, J = 6.5 Hz, H-8′), 3.32 (2H, t, J = 6.4 Hz, H-1′), 1.70 (2H, m, H-2′) 1.50 (2H, m, H-7′), 1.50–1.25 (8H, m, CH_2). CIMS: 291 (M + H)$^+$. R_f = 0.55 (hexane/EtOAc, 1:1).

6.3. General procedure for the oxidation of primary hydroxyls in the side chain

In a 100 ml round-bottomed flask containing the alcohol solution (2 mmol in 40 ml CH_2Cl_2), pyridinium chlorochromate (PCC, 4 mmol) and Celite® (0.2 g) were added. After 2 h stirring at room temperature the reaction was monitored by TLC (eluent: hexane/EtOAc, 9:1). The residue was filtered through a pad of Celite® and concentrated under vacuum. Products were purified by silica gel CC.

*6.3.1. 7-(6′-oxohexyloxy)chromen-2-one **(10)]***

Gum; IR (liquid film) 1732, 1614, 1556, 1506, 1350, 1230, and 1122 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 9.79 (1H, s, CHO), 7.64 (1H, d, J = 9.5 Hz, H-4), 7.37 (1H, d, J = 8.4 Hz, H-5), 6.84–6.79 (2H, m, H-6, H-8), 6.25 (1H, d, J = 9.5 Hz, H-3), 4.02 (2H, t, J = 6.5 Hz, H-1′), 2.50 (2H, td, J = 7.2, 1.6 Hz, H-5′), 1.87–1.80 (2H, m, H-2′), 1.75–1.68 (2H, m, H-4′), 1.58–1.50 (2H, m, H-3′). CIMS: 261 $(M + H)^+$; R_f = 0.60 (hexane/EtOAc, 1:1).

*6.3.2. 7-(8′-Oxooctyloxy)chromen-2-one **(11)]***

White powder, m.p. 53 °C. IR (KBr) 1732, 1618, 1402, 1290, 1242, 1132, and 845 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 9.77 (1H, s, CHO), 7.63 (1H, d, J = 9.5 Hz, H-4), 7.36 (1H, d, J = 8.4 Hz, H-5), 6.77–6.69 (2H, m, H-6, H-8), 6.24 (1H, d, J = 9.5 Hz, H-3), 4.01 (2H, t, J = 6.5 Hz, H-1′), 2.44 (2H, td, J = 7.2 Hz, J = 1.6 Hz, H-7′), 1.82 (2H, m, H-2′) 1.65 (2H, m, H-6′), 1.48–1.37 (6H, m, H-3′-5′). CIMS: 289 $(M + H)^+$. R_f = 0.51 (hexane/EtOAc, 1:1).

*6.3.3. 7-(10-undecenyloxy)chromen-2-one **(12)***

White powder; m.p. 85 °C. IR (KBr) 1726, 1624, 1134, 837 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.60 (1H, d, J = 9.5 Hz, H-4), 7.35 (1H, d, J = 8.4 Hz, H-5), 6.80 (2H, m, H-6, H-8), 6.25 (1H, d, J = 9.5 Hz, H-3), 3.95 (2H, t, J = 6.50 Hz, H-1), 2.75 (2H, t, J = 4.90 Hz, H-11′), 2.45 (1H, m, H-10′), 1.80 (2H, m, H-2′), 1.65 (2H, m, H-9′), 1.50–1.20 (12H, m, CH_2). CIMS: 315 $(M + H)^+$; R_f = 0.45 (hexane/EtOAc, 4:1).

*6.3.4. Oxidative demolition of **12***

In a 50 mL round-bottomed flask equipped with a magnetic stirrer 628 mg of **12** (2.0 mmol), water/acetone, 1:1 mixture (15 ml), 0.2 M OsO_4 in toluene (100 μl, 0.025 eq/mol) and *N*-methylmorpholine-*N*-oxide (232 mg) were added. After 1 h stirring at room temperature, $NaIO_4$ (422 mg, 1.97 mmol) was added portionwise and the mixture was stirred for another 3 h, monitoring the reaction course by TLC (eluant: hexane/EtOAc, 7:3). The reacted mixture was quenched with water (20 ml), extracted with CH_2Cl_2 (40 ml), and washed with brine (20 ml). After drying ($MgSO_4$) and removal of the solvent, the residue was purified by silica gel CC yielding 405 mg of **13** (yield 63%).

*6.3.5. 7-(10′-Oxodecyloxy)chromen-2-one **(13)***

White powder. m.p. 55 °C. IR (KBr) 3343, 2980, 2853, 1736, 1616, 1128, and 833 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 9.78 (1H, s, CHO), 7.64 (1H, d, J = 9.46 Hz, H-4), 7.37 (1H, d, J = 8.4 Hz, H-5), 6.85–6.81 (2H, m, H-6, H-8), 6.25 (1H, d, J = 9.46 Hz, H-3), 4.02 (2H, t, J = 6.47 Hz, H-1′), 2.44 (2H, t, J = 7.27 Hz, H-10′), 1.82 (2H, m, H-9′) 1.66–1.27 (12H, m, CH_2). CIMS: 317 $(M + H)^+$. R_f = 0.22 (hexane/EtOAc, 7:3).

6.4. General procedure for Mannich aminomethylation

6.4.1. Microwave-assisted (N.B. always work behind a shield)

In a 50 ml Carius-type pyrex tube (heavy-wall borosilicate glass, ACE Glassware) stoppered with a pressure-resistant screw cap, the following were placed: dioxane (10 ml), paraformaldehyde (4.0 mmol), copper(II) acetate (5 mg) and the amine (1.5 mmol). The mixture was irradiated with microwave (200 W) for 5 min. Temperature rose to 80 °C and a violet color developed. After cooling down to 25 °C, 7-propargyloxycoumarin (300 mg, 1.5 mmol) dissolved in dioxane (5 ml) was added and the mixture was irradiated further (200 W) for 10 min. The reaction was monitored by TLC using $CHCl_3/CH_3OH$ (9:1) as eluent (starting material R_f = 0.8, products R_f all below 0.6). The reacted mixture was quenched with water (20 ml), extracted with ethyl acetate (50 ml) and washed with brine (20 ml). After drying ($MgSO_4$) and removal of the solvent, the residue was purified by silica gel column chromatography ($CHCl_3$ → $CHCl_3$/MeOH, 9:1) to afford the corresponding Mannich adduct.

6.4.2. Heating under reflux

In a 50 mL two-necked, round-bottomed flask equipped with a magnetic stirrer and a condenser, the following were placed: dioxane (10 mL), paraformaldehyde (4.0 mmol), copper (II) acetate (5 mg) and the amine (1.5 mmol). The stirred mixture was heated at 60 °C for 1 h, during which time a violet color developed. Then 7-(propargyloxy)coumarin (300 mg, 1.5 mmol) in dioxane (5 ml) was added, and heating continued at 90 °C for 8 h.

*6.4.3. 7-[4′-(*N,N,N′*-trimethylethylendiamino)-but-2-ynyloxy]chromen-2-one **(14)***

Yellow oil; IR (liquid film) 1721, 1607, 1566, 1283, 1231, 1123, 1086 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.61 (1H, d, J = 9.5 Hz, H-4), 7.38 (1H, d, J = 8.7 Hz, H-5), 6.96–6.87 (2H, m, H-6, H-8), 6.24 (1H, d, J = 9.5 Hz, H-3), 4.79 (2H, s, H-1′), 3.78 (2H, s, H-4′), 2.71 (4H, s, H-1″, 2″), 2.37 (6H, s, 2 × CH_3), 2.26 (3H, s, CH_3). CIMS: 315 $(M + H)^+$; R_f = 0.19 ($CHCl_3$/MeOH, 9:1).

*6.4.4. 7-[4′-(*N-*pyrrolidyn)-but-2-ynyloxy]chromen-2-one **(15)***

White powder; m.p. 66 °C; IR (KBr) 1725, 1616, 1277, 1232, 1123, 999, and 831 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.62 (1H, d, J = 9.5 Hz, H-4), 7.36 (1H, d, J = 8.3 Hz, H-5), 6.88 (2H, m, H-6, H-8), 6.23 (1H, d, J = 9.5 Hz, H-3), 4.76 (2H, s, H-1′), 3.43 (2H, s, H-4′), 2.56 (4H, t, J = 5.4 Hz, H-1″, 4″), 1.75 (4H, m, H-2″, 3″). CIMS: 284 $(M + H)^+$; R_f = 0.51 ($CHCl_3$/MeOH, 85:15).

*6.4.5. 7-[4′-(*N-*diethylamino)-but-2-ynyloxy]chromen-2-one **(16)***

Gum; IR (KBr) 1744, 1730, 1618, 1607, 1275, 1207, 1120, 1007, and 839 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.33 (1H, d, J = 8.3 Hz, H-5), 6.59 (1H, d, J = 9.5 Hz, H-4), 6.83 (2H, m, H-6, H-8), 6.18 (1H, d, J = 9.5 Hz, H-3), 4.71 (2H, s, H-1′), 3.37 (2H, s, H-4′), 2.41 (4H, q, J = 7.2 Hz, CH_2N), 0.95 (6H, t, J = 7.2 Hz, CH_3). CIMS: 286 $(M + H)^+$; R_f = 0.52 ($CHCl_3$/MeOH, 4:1).

*6.4.6. 7-(4′-Allylmethylamino-but-2-ynyloxy)chromen-2-one **(17)***

Yellow oil; IR (liquid film) 1721, 1618, 1279, 1107, and 812 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.63 (1Η, δ, J = 9.5 Hz, H-4), 7.37 (1H, d, J = 8.3 Hz, H-5), 6.90 (1H, s, H-8), 6.88 (1H, overlap, H-6), 6.24 (1H, d, J = 9.5 Hz, H-3), 5.76 (1H, m, H-7′), 5.11 (2H, m, H-8′), 4.77 (2H, s, H-1′), 3.33 (2H, s, H-4′), 2.97 (2H, d, J = 6.6 Hz, H-6′), 2.24 (3H, s, CH_3). CIMS: 284 $(M + H)^+$; R_f = 0.58 ($CHCl_3$/MeOH, 1:1).

6.5. General procedure for reductive amination

Reactions were performed in a 1-mm thick Teflon® tube inserted in a Delrin® reactor thermostatted by two Peltier modules. 7-(formylalkyloxy)coumarin (0.4 mmol) and an excess of the amine (1.5 mmol) dissolved in methanol–water (7:3) containing 3% CH_3COOH (20 ml) were sonicated (18.1 kHz, 80 W/cm^2) for 15 min at 30 °C. Then $NaBH_3CN$ (0.4 mmol) was added portionwise and the mixture was sonicated for an additional 45 min. TLC: eluent hexane/EtOAc, 1:1. Work-up: the mixture was diluted with water (20 ml), extracted with CH_2Cl_2 (30 ml) and washed with brine (20 ml). After drying ($MgSO_4$) and removal of the solvent the residue was purified by silica gel column chromatography (hexane/EtOAc, 7:3 → 1:1) to afford the corresponding amino derivatives.

*6.5.1. 7-[6′-(benzylamino-hexyloxy)]chromen-2-one **(18)***

Yellow oil; IR (liquid film) 3300, 1732, 1614, 1271, 1230, 1122, 835, and 698 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 8.11 (1H, d, J = 9.5 Hz, H-4), 7.82 (5H, m, Ph), 7.80–7.71 (1H, m, H-5), 7.34–7.27 (2H, m, H-6, H-8), 6.71 (1H, d, J = 9.5 Hz, H-3), 4.49 (2H, t, J = 6.5 Hz, H-1′), 4.29 (2H, s, $C\underline{H}_2$-Ph), 3.15 (2H, t, J = 6.9 Hz, H-6′), 2.31 (2H, m, H-2′), 2.08–1.87 (6H, m, H-3′, H-4′, H-5′). CIMS: 352 $(M + H)^+$; R_f = 0.45 ($CHCl_3$/MeOH, 9:1).

*6.5.2. 7-(piperidinyl-N-hexyloxy)chromen-2-one **(19)***

Yellow oil; IR (liquid film) 1732, 1614, 1350, 1280, 1230, 1122, and 835 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 8.10 (1H, d, J = 9.5 Hz, H-4), 7.63 (1H, d, J = 8.4 Hz, H-5), 6.84–6.79 (2H, m, H-6, H-8), 6.23 (1H, d, J = 9.5 Hz, H-3), 4.02 (2H, t, J = 6.4 Hz, H-1′), 2.55 (4H, br s, H-2″, H-6″), 2.31 (2H, t, J = 7.6 Hz, H-6′), 1.81 (2H, m, H-2′), 1.63–1.36 (12H, m, CH_2), CIMS: 330 $(M + H)^+$; R_f = 0.24 ($CHCl_3$/MeOH, 9:1).

*6.5.3. 7-(Morpholinyl-*N*-hexyloxy)chromen-2-one **(20)***

Yellow oil; IR (liquid film) 1732, 1614, 1350, 1280, 1242, 1120, and 835 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.54 (1H, d, J = 9.5 Hz, H-4), 7.25 (1H, d, J = 8.4 Hz, H-5), 6.73–6.63 (2H, m, H-6, H-8), 6.11 (1H, d, J = 9.5 Hz, H-3), 3.90 (2H, t, J = 6.5 Hz, H-1′), 3.60 (4H, t, J = 4.4 Hz, H-3″, H-5″), 2.24 (2H, t, J = 7.4 Hz, H-6′), 2.33 (4H, s, H-2″, H-6″),1.71 (2H, m, H-2′), 1.48–1.09 (6H, m, CH_2). CIMS: 332 $(M + H)^+$; R_f = 0.40 ($CHCl_3$/MeOH, 9:1).

*6.5.4. 7-(Morpholinyl-*N*-octyloxy)chromen-2-one **(21)***

Yellow oil; IR (liquid film) 1732, 1614, 1352, 1280, 1230, 1121, and 837 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.61 (1H, d, J = 9.5 Hz, H-4), 7.34 (1H, d, J = 8.4 Hz, H-5), 6.81–6.75 (2H, m, H-6, H-8), 6.20 (1H, d, J = 9.5 Hz, H-3), 3.97 (2H, t, J = 6.5 Hz, H-1′), 3.72 (4H, m, H-3″, H-5″), 2.52 (2H, t, J = 7.6 Hz, H-2″, H-6″), 2.39 (2H, m, H-8′), 1.75 (2H, m, H-2′), 1.52–1.31 (10H, m, CH_2). CIMS: 360 $(M + H)^+$; R_f = 0.13 (hexane/EtOAc, 1:9).

*6.5.5. 7-[8′-(Dimethylamino-*N*-octyloxy)]chromen-2-one **(22)***

White powder; m.p. 50 °C; IR (KBr) 1732, 1614, 1470, 1363, 1124, and 854 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.59 (1H, d, J = 9.5 Hz, H-4), 7.32 (1H, d, J = 8.4 Hz, H-5), 6.80–6.73 (2H, m, H-6, H-8), 6.18 (1H, d, J = 9.5 Hz, H-3), 3.95 (2H, t, J = 6.5 Hz, H-1′), 2.63 (2H, t, J = 7.8 Hz, H-8′), 2.49 (6H, s, H-1″, H-2″), 1.75 (2H, m, H-2′), 1.59 (2H, m, H-7′), 1.40–1.20 (8H, m, CH_2). CIMS: 318 $(M + H)^+$; R_f = 0.53 ($CHCl_3$/MeOH, 4:1).

*6.5.6. 7-[10′-(Dimethylamino-*N*-decyloxy)]chromen-2-one **(23)***

White powder; m.p. 57 °C; IR (KBr) 1732, 1614, 1470, 1364, 1126, and 854 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.59 (1H, d, J = 9.5 Hz, H-4), 7.32 (1H, d, J = 8.4 Hz, H-5), 6.80–6.73 (2H, m, H-6, H-8), 6.18 (1H, d, J = 9.5 Hz, H-3), 3.95 (2H, t, J = 6.5 Hz, H-1′), 2.64 (2H, t, J = 7.8 Hz, H-10′), 2.50 (6H, s, H-1″, H-2″), 1.75 (2H, m, H-2′), 1.61 (2H, m, H-9′), 1.46–1.20 (12H, m, CH_2). CIMS: 318 $(M + H)^+$; R_f = 0.56 ($CHCl_3$/MeOH, 4:1).

*6.6. Amination of **7** with N-methylallylamine*

In a 50 ml round-bottomed flask **7** (300 mg, 0.92 mmol), DMA (15 ml) and *N*-methylallylamine (177 μl, 1.85 mmol) were added. The reaction mixture was stirred 24 h at 45 °C and monitored by TLC ($CHCl_3$/MeOH, 9:1). CC (eluent $CHCl_3$/MeOH, 19:1) yielded 210 mg of **24** (yield 72%).

*6.6.1. 7-[6-(Allylmethylamino)-hexyloxy]-chromen-2-one **(24)***

White powder. m.p. 145 °C. IR (KBr) 1728, 1626, 1473, 1400, 1298, and 843 cm^{-1}; ^1H-NMR ($CDCl_3$) δ = 7.64 (1H, d, J = 9.5 Hz, H-4), 7.37 (1H, d, J = 8.4 Hz, H-5), 6.85–6.81 (2H, m, H-6, H-8), 6.25 (1H, d, J = 9.46 Hz, H-3), 6.19–6.07 (1H, m, H-2″), 5.55–5.46 (2H, m, H-3″), 4.02 (2H, td, J = 2.22, 3.88 Hz, H-1′), 3.56 (2H, d, J = 6.64 Hz, H-1″), 2.91 (2H, t, J = 7.79 Hz, H-6′), 2.67 (3H, s, CH_3), 1.93–1.46 (8H, m, CH_2). CIMS: 316 $(M + H)^+$. R_f = 0.29 ($CHCl_3$/MeOH, 9:1).

*6.6.2. 7-[10-(Allylmethylamino)-decyloxy]-chromen-2-one **(25)***

White powder; m.p. 99 °C. IR (KBr) 3450, 1724, 1622, 1300, 1134, and 851 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.64 (1H, d,

J = 9.5 Hz, H-4), 7.37 (1H, d, J = 8.4 Hz, H-5), 6.85–6.81 (2H, m, H-6, H-8), 6.25 (1H, d, J = 9.46 Hz, H-3), 6.18–6.05 (1H, m, H-13′), 5.59–5.49 (2H, m, H-14′), 4.01 (2H, t, J = 12.9 Hz, H-1′), 3.63 (2H, d, J = 6.99 Hz, H-12′), 2.98–2.93 (2H, m, H-10′), 2.73 (3H, s, H-11′), 1.83–1.32 (16H, m, CH_2). CIMS: 372 $(M + H)^+$; R_f = 0.68 ($CHCl_3$/MeOH, 1:1).

6.6.3. 3-(10′-Undecenoyl)chroman-2,4-dione ***(26)***

In a 50 ml two-necked round-bottomed flask equipped with a magnetic stirrer and a nitrogen inlet, 4-hydroxycoumarin (5.0 g, 30.8 mmol) and a catalytic amount of piperidine were dissolved in anhydrous pyridine (35 ml). The solution was placed under stirring, cooled down to 0 °C and 10-undecenoyl chloride was added dropwise. The mixture was stirred 8 h under nitrogen at 40 °C and the reaction monitored by TLC. Work-up: the reacted mixture was diluted with EtOAc and washed with 5% HCl and with $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to dryness. CC (eluent hexane/EtOAc, 19:1) yielded 7.0 g of **26** (21.3 mmol, 70%).

White powder, m.p. 98 C. IR (KBr) 1717, 1607, 1547, 1225, 980, and 901 cm^{-1}. ^1H NMR ($CDCl_3$) δ = 8.08 (1H, d, J = 7.90 Hz, H-5), 7.73 (1H, td, J = 7.14 Hz, J' = 1.39 Hz, H-7), 7.38–7.28 (2H, m, H-6, H-8), 5.90–5.76 (1H, m, H-10′), 5.03–4.93 (2H, m, H-11′), 3.21 (2H, t, J = 7.45 Hz, H-2′), 1.83–1.30 (14H, m, CH_2). CIMS: 329 $(M + H)^+$. R_f = 0,4 (hexane/EtOAc 4:1).

6.6.4. 3-(10′-oxodecanoyl)chroman-2,4-dione ***(27)***

In a 50 ml round-bottomed flask **26** (350 mg, 1.07 mmol), 30 ml of H_2O/acetone 1:1, 54 μl (0.02 eq/mol) of 0.2 M OsO_4 in toluene and *N*-methylmorpholine-*N*-oxide (125 mg, 1 eq/mol, 1.067 mmol) were placed and the mixture was magnetically stirred for a few minutes before adding 502 mg (2.2 eq/mol, 2.34 mmol) of $NaIO_4$ in small portions over an interval of 40 min at 24–26 °C. The stirring was continued for 3 h and the reaction was monitored by TLC on silica gel plates, eluent hexane/EtOAc, 7:3.

Work up: the reacted mixture was diluted with H_2O, poured into a separatory funnel and extracted with CH_2Cl_2. The organic phase was washed with saturated NaCl solution, dried with Na_2SO_4, filtered and evaporated under vacuum. Products were purified by CC, eluent petrol ether/EtOAc, 9:1. 102 mg of product were obtained (yield: 30%).

White powder. m.p. 119 °C. IR (KBr) 1717, 1609, 1549, 1225, 982, 768 and cm^{-1}; ^1H NMR ($CDCl_3$) δ = 9.78 (1H, s, CHO), 8.08 (1H, d, J = 7.90 Hz, H-5), 7.73 (1H, td, J = 1.39, 7.14 Hz, H-7), 7.38–7.28 (2H, m, H-6, H-8), 3.21 (2H, t, J = 7.45 Hz, H-2′), 2.44 (2H, td, J = 5.98, 1.40 Hz, H-9'), 1.77–1.27 (12H, m, CH_2). CIMS: 331 $(M + H)^+$. R_f = 0.52 (hexane/EtOAc, 7:3).

6.6.5. 3-[10′-(Allylmethylamino)decanoyl]-chroman-2,4-dione ***(28)***

White powder. m.p. 101 °C. IR (KBr) 1651, 1599, 1520, 1390, and 1036 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 8.02 (1H, d, J = 7.76 Hz, H-5), 7.41 (1H, t, J = 6.88 Hz, H-7), 7.28–7.18 (2H, m, H-6, H-8), 6.04–5.91(1H, m, H-2″), 5.42 (2H, m, H-3″), 3.45 (2H, d, J = 6.91 Hz, H-1″), 2.79 (2H, t, J = 10.60 Hz, H-2'), 2.59 (3H, s, CH_3), 1.65-1.18 (16H, m, CH_2). CIMS: 386 $(M + H)^+$. R_f = 0.40 ($CHCl_3$/MeOH, 9:1).

6.7. SHC inhibition tests

[^{14}C]Squalene, the substrate of squalene-hopene cyclase, was obtained by incubating 1 μCi of [^{14}C]mevalonolactone with S_{10} supernatant of a pig liver homogenate (25 mg of protein) following the method of Popják [38], in the presence of oxidosqualene cyclase inhibitor U-18666A. Under these conditions the bulk of radioactivity in the unsaponifiable extract was shared between squalene and oxidosqualene, which could be easily separated by TLC [39].

E. coli cells expressing the SHC gene were provided by Prof. G. Schulz (Universität Freiburg, Germany). For enzyme preparation we modified the procedure of Ochs et al. [40]. Briefly, a colony was suspended in 8 mL of Luria broth containing 30 mg ampicillin per liter. After incubation for 15 h at 37 °C (pH 7.0), the culture was diluted to 100 ml with the same medium. After growing to mid-log phase (OD_{578} 0.6–0.8), the cells were induced for 5–7 h with 1 mM isopropylthio-β-galactoside, after which they were harvested and resuspended in 200 mM Tris buffer pH 8.0 containing 10 mM β-mercaptoethanol and 5 mg/ml lysozyme. The suspension was frozen at—80 °C for 2 h, then the cells were thawed at room temperature and lysed. The lysate was centrifuged at 40 000 × g for 60 min and membrane-bound proteins were solubilized by adding 1% Triton X-100.

The enzymatic activity of SHC was assayed as previously reported [41].

IC_{50} values (the concentration of inhibitor that reduced by 50% the enzymatic conversion of squalene to hopene) were determined at 10 μM substrate concentration.

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