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Inhibitory Effect of Umbelliferone Aminoalkyl Derivatives on Oxidosqualene Cyclases from *S. cerevisiae*, *T. cruzi*, *P. carinii*, *H. sapiens*, and *A. thaliana*: a Structure–Activity Study

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Eighteen coumarin derivatives were tested as inhibitors of oxidosqualene cyclases (OSCs) from Saccharomyces cerevisiae, Trypanosoma cruzi, Pneumocystis carinii, Homo sapiens*, and* Arabidopsis thaliana*, all expressed in an OSC-defective strain of* S. cerevisiae.[35] *All the compounds have an aminoalkyl chain bound to an aromatic nucleus; unconventional synthetic procedures (microwave- and ultrasound-promoted reactions) were successfully used to prepare some of them. The most interesting structure-dependent difference in inhibitory activities was observed with an* N*-oxide group replacement of the tertiary amino group at the end of the side chain. An interesting species specificity also emerged:* T. cruzi *OSC was the least sensitive enzyme;* P. carinii *and* A. thaliana *OSCs were the most sensitive. The remarkable activities of three compounds on the* T. cruzi *enzyme and of five of them on the* P. carinii *enzyme suggest the present series as a promising compound family for the development of novel antiparasitic agents.*

Introduction

The most striking reaction step in sterol biosynthesis, the cyclization of oxidosqualene to form the steroid nucleus, is brought about by enzymes that belong to the family of oxidosqualene cyclases (OSCs). These enzymes catalyze the formation of lanosterol (lanosterol synthases, LASs) in non-photosynthetic organisms and cycloartenol (cycloartenol synthases, CASs) in photosynthetic organisms.[1,2] Lanosterol and cycloartenol synthases belong to the lanosterol-type OSCs, which share the same catalytic mechanism up to the formation of the lanosteryl cation, which is then specifically deprotonated at C8 to form lanosterol or at C19 to form cycloartenol (Figure 1).

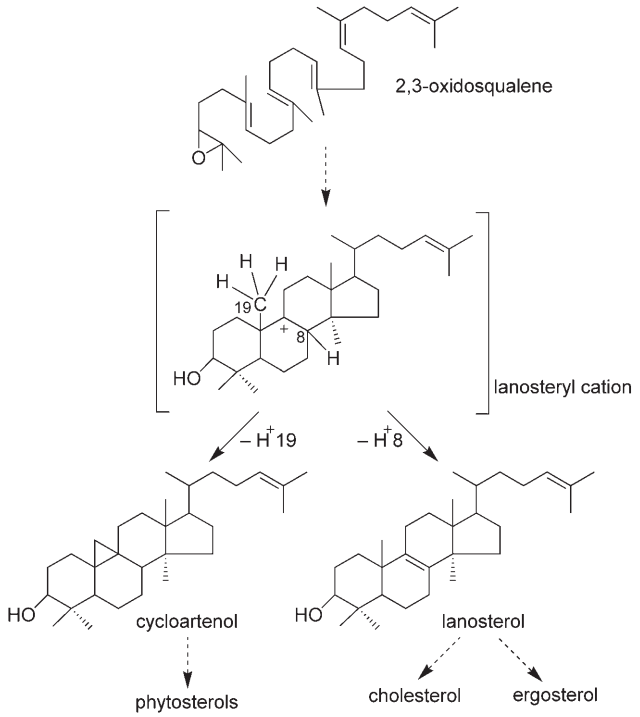

Figure 1. Cyclization of 2,3-oxidosqualene: various fates of the lanosteryl cation.

The close similarities between lanosterol and cycloartenol synthases were thoroughly investigated by Matsuda and coworkers through directed evolution experiments, mutagenesis, and homology modeling.[3,4] Directed evolution experiments identified single amino acid substitutions resulting in CAS mutants that formed lanosterol instead of cycloartenol as the main cyclization product.[4] These results invited further comparative studies of lanosterol and cycloartenol synthases to better clarify the catalytic mechanism and to design more specific inhibitors of these enzymes.

The recent crystallographic work on *Homo sapiens* oxidosqualene cyclase (LAS) complexed with both inhibitor and reaction product provided a better picture of the active site and cyclization mechanism.[5] For decades, OSCs attracted interest

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not only for this fascinating mechanism but also as potential targets in drug development. Selective OSC inhibition might offer a promising approach to the control of blood cholesterol levels in humans[6–9] and to the search for new antifungal agents.[10–14] A variety of OSC inhibitors were recently tested on *Trypanosoma cruzi*, *Trypanosoma brucei*, *Pneumocystis carinii*, and *Leishmania mexicana* with the aim of developing new agents against these microorganisms.[15,16] In fact, diseases caused by these pathogens still lack adequate cures and are responsible for millions of deaths each year worldwide. Among the likely targets for controlling these pathogens, enzymes of sterol biosynthesis such as squalene synthase,[17] 14α-demethylase,[18] and OSC have been taken into consideration.

In a recent study of ours, several umbelliferone aminoalkyl derivatives were tested as inhibitors of OSC (LAS) from yeast (*S. cerevisiae*) and of OSCs (LASs) from *Trypanosoma cruzi* and *Pneumocystis carinii* expressed in yeast.[19] Some of these compounds were found to be effective, which encouraged us to expand the series of derivatives under testing and to include two more target enzymes: human lanosterol synthase and cycloartenol synthase from *Arabidopsis thaliana*, both expressed in yeast. Human lanosterol synthase was included in the present study because it has been recently co-crystallized with a specific inhibitor to provide a clearer picture of the interactions between a hosted molecule and the amino acid residues that form the active site cavity.[5] Cycloartenol synthase, an enzyme that belongs to the lanosterol-type OSCs, was studied because it can be a powerful tool for probing the subtle mechanistic control of lanosterol-generating enzymes.[3,4] Moreover, the active sites of all known cycloartenol synthases share an important structural feature with trypanosomal lanosterol synthases (from *T. cruzi* and *T. brucei*), namely a tyrosine at position 384 (*S. cerevisiae* numbering), where all the other lanosterol synthases share a conserved threonine residue. Matsuda and coworkers showed that this threonine–tyrosine dichotomy has an influence on catalytic activity,[20] and should be regarded as a critical feature in the development of antitrypanosomal drugs.

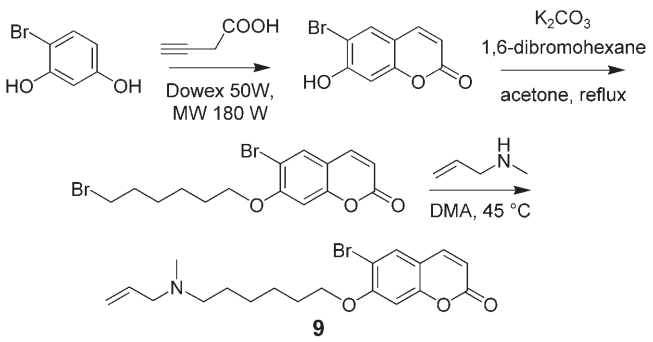
Newly synthesized compounds used for the present study differ from our previous umbelliferone-based OSC inhibitors[19] either in the coumarin nucleus (by halogenation or lactone cleavage) or in the side chain bearing the tertiary amino group (length, flexibility, *N*-oxide function). *N*-oxides were included in particular because our previous work on squalene derivatives[13] had revealed that some interesting differences of inhibitor activity on yeast OSCs are present between amino derivatives and the corresponding *N*-oxides. Moreover, a study of structure–activity relationships in potential antitrypanosomal drugs had recognized the *N*-oxide group as an effective pharmacophore.[21] The results of systematic tests combining 18 novel umbelliferone or resorcinol aminoalkyl derivatives with five OSCs led us to identify important features that might be used to develop specific antiparasitic drugs.

Results and Discussion

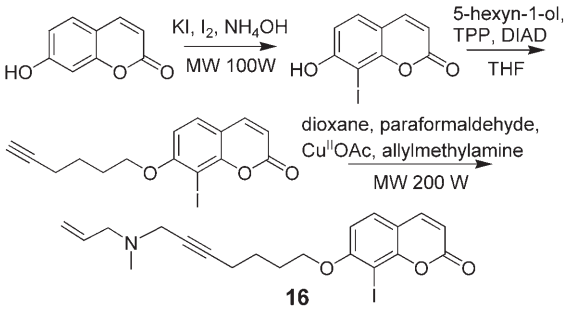
Eighteen coumarin derivatives were tested as inhibitors of OSCs from five different organisms, *S. cerevisiae*, *T. cruzi*, *P. carinii*, *H. sapiens*, and *A. thaliana*, (all expressed in the same OSC-deficient strain of *S. cerevisiae*) with the aim of identifying specific enzyme–inhibitor interactions and providing indirect evidence of architectural differences between the active sites. Compounds **1**–**16** share a basic structure that consists of a coumarin nucleus bearing a side chain with a terminal tertiary amino function or its corresponding *N*-oxide. In compounds **17** and **18**, the lactone ring was opened by nucleophilic attack with dimethylamine. The preparation of compounds **1**–**8**, **10**, and **12** has been described previously[22] as was their inhibitory activity on OSCs from *S. cerevisiae*, *T. cruzi*, and *P. carinii*.[19] Thus, this work is an extension of previous studies that explored the potential of the coumarin nucleus as a basic structure for the design of new OSC inhibitors. In the present study, chemical modulation of coumarin derivatives involved three structural features: 1) the length and flexibility of the side chain, 2) the presence of an *N*-oxide group at the end of the side chain, and 3) the coumarin nucleus itself, by the insertion of a halogen atom or the opening of the lactone ring.

Synthesis of umbelliferone derivatives

We prepared a series of umbelliferone derivatives in which the phenolic function carries, through an ether linkage, a carbon chain with a terminal tertiary amine or the corresponding *N*-oxide. The carbon chains were either flexible (all sp^3-hybridized C atoms) or rigid (containing *sp* C atoms). In some derivatives, the coumarin skeleton was decorated with iodine or bromine. 6-Bromo-7-hydroxy-2*H*-chromen-2-one was prepared by solvent-free MW-promoted cyclization of 1,3-dihydroxy-4-bromobenzene with propiolic acid finely dispersed on an acidic resin (Scheme 1).[23] Within a few minutes, 6-bromoumbelliferone was obtained in good yield, whereas no reaction took place by heating at reflux overnight in dioxane. The iodination of umbelliferone[24] was accelerated by MW irradiation as was the subsequent Mannich-type aminomethylation of the terminal alkyne with *N*-methylallylamine and paraformaldehyde in the presence of copper(II) acetate (Scheme 2). Under conventional heating, both reactions gave much poorer yields even after many hours. The Mitsunobu protocol used for the O-alkylation of 8-iodoumbelliferone was promoted by ultrasound treat-

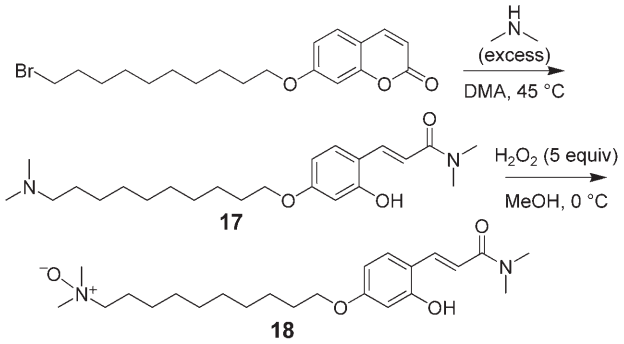


Scheme 1. Synthesis of 6-bromo-7-hydroxy-2*H*-chromen-2-one **9** starting from a solvent-free MW-promoted cyclization. DMA = *N*,*N*-dimethylacetamide.



Scheme 2. Synthesis of 8-iodoumbelliferone aminoalkynyl derivative **16** by MW- and ultrasound-promoted reactions. DIAD = diisopropylazodicarboxylate, TPP = triphenylphosphine.

ment. The *N,N*-dimethylacrylamido derivative **17** was easily prepared by treatment of 7-(10-bromodecyloxy)-2*H*-chromen-2-one with excess *N,N*-dimethylamine, achieving a one-pot nucleophilic substitution of the bromine and amidation of the lactone (Scheme 3). Structural modification of our original um-



Scheme 3. Synthesis of *N,N*-dimethylacrylamido derivative **17** and its *N*-oxide.

belliferone-based OSC inhibitors[19] yielded significant clues for future improvements in molecular design; the coumarin skeleton itself can be conveniently cut down to alkylresorcinol by cleavage of the lactone ring through amidation (Scheme 3).

Effects of coumarin derivatives on OSCs

Inhibitory activities were tested by incubating homogenates prepared from cell cultures of different strains with radiolabeled oxidosqualene in the presence of various inhibitor concentrations. A comparison of the results for 18 coumarin derivatives on five enzymes revealed considerable differences in the activities toward each enzyme and in the susceptibilities of enzymes from different organisms to each inhibitor (Table 1).

Susceptibilities of different cyclases

The OSCs of *P. carinii* and *A. thaliana* were the most susceptible to our inhibitors and that of *T. cruzi*, the most resistant. Twelve out of 18 compounds had IC_{50} values $\leq 0.5\,\mu M$ on the *A. thaliana* enzyme. The question arises, on which architectural feature the lower susceptibility of the *T. cruzi* enzyme may depend. In our previous work on coumarin derivatives we speculated that the presence of a Tyr residue close to the active site of the *T. cruzi* enzyme (position 384, *S. cerevisiae* numbering) might hinder the access of coumarin derivatives. Surprisingly, *T. cruzi* and *A. thaliana* OSCs, which both have a Tyr residue near the active site,[20] showed the greatest difference in susceptibility to individual inhibitors; for instance, compound **12** was 10^3-fold more effective on the *T. cruzi* OSC than on the *A. thaliana* OSC ($IC_{50} = 10$ and 0.01 μM, respectively). This might mean that for lanosterol synthases, Tyr 384 specifically affects the sensitivity to inhibitors, making the *T. cruzi* OSC less susceptible than animal and fungal lanosterol synthases. On the other hand, the presence of this residue at the corresponding position in a cycloartenol synthase would not critically affect its susceptibility to inhibitors. Cycloartenol synthase stood out in our group of OSCs for being the only enzyme sensitive to inhibitor **5**, a compound with a morpholine group on the side chain. Interestingly, compound **4**, which also bears the same group but at the end of a longer side chain, inhibited both *P. carinii* and *A. thaliana* enzymes, while it was inactive on the other OSCs. Morpholine-type derivatives could then provide a useful tool to probe architectural differences in the active sites of the two enzymes.

Structure-dependent inhibitor activity

Both the type of amino group and the flexibility of the side chain had a very strong influence on the inhibitory activity, though to an extent that varied markedly with the species source of the target enzyme.

1. Type of tertiary amino function

With a few exceptions, an acyclic tertiary amine conferred a stronger activity than a cyclic one, thus confirming, as shown by several studies on squalene and oxidosqualene cyclases,[5,25,26] that molecular recognition between enzymes and amino-group-bearing inhibitors largely depends on the interaction between the catalytic Asp 456 (yeast numbering) that initiates the cyclization reaction and an aptly sized protonated amino group belonging to the inhibitor.

Differences among acyclic tertiary amines were especially notable with the OSC from *T. cruzi*. Among compounds bearing the amino group at the end of a flexible chain, allylmethylamino derivatives proved more effective than dimethylamino analogues. Despite the similarity between inhibitors **10** and **12**, IC_{50} values differed widely, from $> 100\,\mu M$ for dimethylamino compound **12** to 4 μM for allylmethylamino derivative **10**. Interestingly, the allylmethylamino group is present in the well-known cholesterol-lowering agent RO048871,[7] as well as in other orally active nonterpenoid OSC inhibitors.[9]

Table 1. Inhibitory effect of umbelliferone derivatives on OSCs of different organisms.

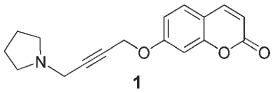
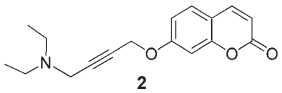
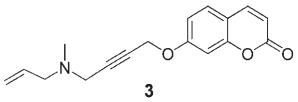
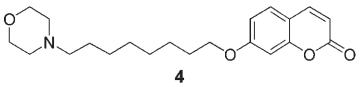
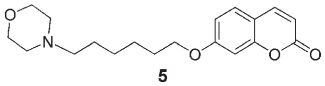
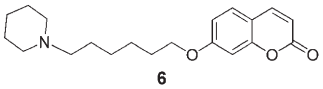
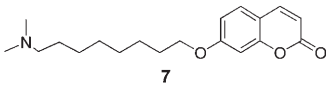
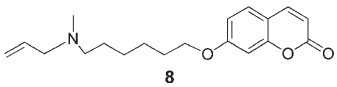
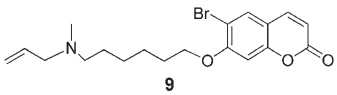
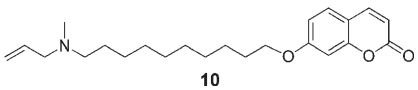
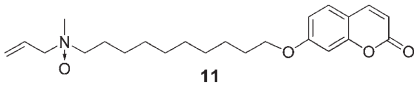
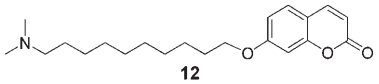
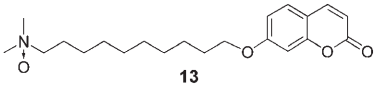
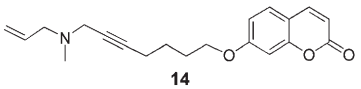
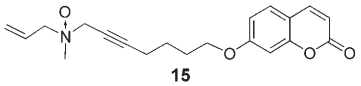
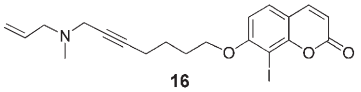
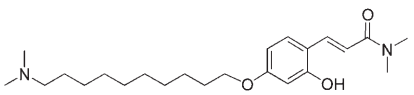
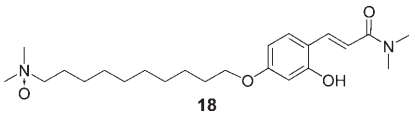
Compound	IC_{50} [μM][a]				
	S. cerevisiae[b]	*T. cruzi*[b]	*P. carinii*[b]	*H. sapiens*[b]	*A. thaliana*[c]
 **1**	*10.28*	*106.28*	*0.52*	50.00	2.80
 **2**	*20.35*	*72.66*	*1.85*	> 100	0.52
 **3**	*3.42*	*≥ 100*	*1.12*	70.00	0.19
 **4**	*30.67*	*102.38*	*5.35*	55.00	2.90
 **5**	*102.82*	*95.83*	*78.76*	> 100	0.14
 **6**	*31.54*	*≥ 100*	*2.48*	7.50	7.50
 **7**	*6.03*	*25.43*	*1.04*	1.35	0.52
 **8**	*1.04*	*4.06*	*0.15*	0.65	0.50
 **9**	1.40	3.60	0.30	3.00	1.40
 **10**	*3.21*	*4.14*	*0.18*	0.85	0.50
 **11**	0.14	0.36	0.05	0.46	0.04
 **12**	2.70	na[d]	0.02	1.25	0.01
 **13**	0.03	0.55	0.16	0.38	0.12
 **14**	0.08	3.80	0.07	4.00	0.07
 **15**	9.00	na[d]	> 10	na[d]	3.20
 **16**	0.19	7.00	0.07	4.50	0.06
 **17**	1.60	na[d]	0.03	8.00	1.20

Table 1. (Continued)

Compound	IC_{50} [μM][a] *S. cerevisiae*[b]	*T. cruzi*[b]	*P. carinii*[b]	*H. sapiens*[b]	*A. thaliana*[c]
 **18**	1.00	0.80	0.15	0.20	0.11

[a] Values are the means of three independent experiments; maximum deviations from the mean were $<10\%$; values in italics are from a previous study.[19] [b] lanosterol synthase. [c] cycloartenol synthase. [d] Not active at 100 μM.

2. Amine versus N*-oxide function*

The most interesting structure-dependent difference arose with the presence of an N–O group at the end of the side chain. Surprisingly, the resulting effect depended on the flexibility of the chain. Within couples **10** and **11**, **12** and **13**, and **17** and **18**, all of which bear a flexible chain, the N–O derivative was generally more active than the corresponding amino compound. This difference could be very large, as was the case for compounds **12** and **13**, and **17** and **18** toward the *T. cruzi* enzyme (the amines were almost inactive, whereas IC_{50} values for the N–O derivatives were <1 μM). The opposite, however, was observed with the couple **14** and **15**, in which the side chain is stiffened by a triple bond. In this case, the effects on the *T. cruzi* and human OSCs were almost identical. Apparently, the N–O group needs a more flexible chain to correctly face the critical residue(s) of the active site. Of course, a satisfactory explanation of the observed differences can only emerge from inhibitor–enzyme modeling studies.

3. Side-chain length and flexibility

Among the compounds bearing a flexible side chain, allylmethylamino derivatives **8** and **10** were almost equally active on all five OSCs, while dimethylamino derivative **12** was 50-fold more active on the OSCs from *P. carinii* and *A. thaliana* than compound **7**, which has a similar but shorter chain. Regarding compounds **3** and **14**, which bear a more rigid side chain, the longer chain conferred a greater activity on all OSCs except that from *A. thaliana*. As observed above, chain flexibility strongly influences the effect of a terminal N–O group: the N–O derivatives bearing a flexible chain were more active, whereas the opposite was observed with compounds **14** and **15**, which have a more rigid chain.

4. Requirement for the coumarin nucleus

With compounds **17** and **18** we intended to ascertain whether the coumarin nucleus was required at all for the compounds to have inhibitory activity. Comparison of IC_{50} values for couples **17** and **18**, and **12** and **13** suggested that the presence of a coumarin skeleton did not make an important contribution, with a few remarkable exceptions (**13** versus **18** on *S. cerevisiae* OSC and **12** versus **17** on *A. thaliana* OSC). Thus, as had been observed with a previous series of OSC inhibitors,[9,27,28] the aminoalkyl chain appeared to be the most essential feature, possibly because of its substrate-mimicking structure, that is, its ability to pass the channel constriction that controls access to the active site cavity[29] and to interact with Asp 456 (yeast numbering), the substrate-protonating group located at the polar cap of the active site cavity.[5] The inhibitory activities of the couple **17** and **18** against *T. cruzi* OSC perfectly overlap those of the couple **12** and **13**, thus confirming that differences between amino and *N*-oxide derivatives depend on the flexibility of the side chain.

Conclusions

We studied the inhibitory activities of 18 coumarin derivatives on five different OSCs, four of which (from *Saccharomyces cerevisiae*, *Trypanosoma cruzi*, *Pneumocystis carinii*, and *Homo sapiens*) were oxidosqualene lanosterol synthases and one (from *Arabidopsis thaliana*) was an oxidosqualene cycloartenol synthase. As a rule, *P. carinii* and *A. thaliana* enzymes were the most susceptible to the inhibitors. *T. cruzi* OSC, although the least susceptible among the enzymes studied, was effectively inhibited by three of our derivatives. This finding, along with the high efficacy of 11 derivatives on the *P. carinii* enzyme, suggests our series is a promising family of compounds for the development of novel antiparasitic agents, although in vitro activity tests on individual pathogens will be required to select the best candidates for drug design. Such experiments are currently in progress on *T. cruzi* cells. The unexpectedly similar and high levels of susceptibility to the majority of the inhibitors observed with *P. carinii* and *A. thaliana* OSCs suggest that the molecular recognition mechanism is similar for these two enzymes, possibly because the architecture of their active sites is similar, despite their different cyclization products. Unfortunately, only the amino acid sequences of the two proteins are available so far. Decisive answers concerning this speculation of ours should come from homology modeling and molecular docking studies, as this approach has become practicable with the availability of the structure of human OSC.[5]

Experimental Section

Chemicals: All chemicals, buffer components, culture media, the bovine serum albumin (used as a standard for determining protein concentration) were obtained from Sigma–Aldrich (Milan, Italy) unless otherwise specified. Reactions were monitored by TLC on

Merck 60 F_{254} plates (0.25 mm), which were visualized by UV light and/or by heating after a spray with 5% H_2SO_4 in ethanol. Merck silica gel was used for column chromatography (CC). IR spectra were recorded with a Shimadzu FT-IR 8001 spectrophotometer. Unless stated otherwise, NMR spectra were recorded with a Bruker Avance 300 at 25 °C; chemical shifts are calibrated to the residual proton and carbon resonance of the solvent: $CDCl_3$ ($\delta_H = 7.26$). MW-promoted reactions were carried out in a multimode oven, MicroSYNTH Milestone (Italy), ultrasound-promoted reactions in a sonochemical apparatus with an immersion horn developed in the authors' laboratory.[30] HPLC separations were performed on an Amersham AKTA purifier 10/100. Coumarin derivatives **1**–**8**, **10**, and **12** (Table 1) and the OSC substrate, 2,3-oxidosqualene (OS), were synthesized as previously reported.[22,31]

7-[6-(allylmethylamino)-hexyloxy]-6-bromo-2*H*-chromen-2-one (9): *6-bromo-7-hydroxy-2H-chromen-2-one*: 1,3-dihydroxy-4-bromobenzene (378 mg, 2 mmol), finely mixed in a mortar with Dowex 50W 8×200 (200 mg) and propiolic acid (246 μL, 4 mmol) was placed in a pressure-resistant tube. The mixture was irradiated in the multimode MW oven for 15 min at 180 W. The reaction outcome was evaluated by TLC ($CHCl_3$/MeOH 95:5); the reaction mixture was extracted with MeOH under sonication, and the solution was filtered on Büchner funnel. The raw product was purified by silica-gel CC to give 205 mg of 6-bromo-7-hydroxy-2*H*-chromen-2-one (0.85 mmol, yield 43%). $R_f = 0.34$ ($CHCl_3$/MeOH 95:5).

7-(6-bromohexyloxy)-6-bromochromen-2-one: In a 50-mL two-necked, round-bottomed flask equipped with magnetic stirrer, condenser, and nitrogen inlet, 6-bromo-7-hydroxy-2H-chromen-2-one (73 mg, 0.3 mmol), K_2CO_3 (83 mg, 0.6 mmol), and anhydrous acetone (20 mL) were added. The stirred mixture was heated at reflux for 2 h under nitrogen atmosphere, then allowed to cool to room temperature. 1,6-Dibromohexane (46 μL, 0.3 mol) was then added, and the resulting mixture was heated at reflux for another 5 h. Eluent used for TLC was $CHCl_3/CH_3OH$ 98:2. 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 ($CHCl_3$) to afford 103 mg of 7-(6-bromohexyloxy)-6-bromochromen-2-one (0.25 mmol, yield 83%). $R_f = 0.51$ ($CHCl_3$/MeOH 98:2).

*7-[6-(allylmethylamino)-hexyloxy]-6-bromochromen-2-one (**9**)*: In a 25-mL round-bottomed flask, 7-(6-bromohexyloxy)-chromen-2-one (80 mg, 0.20 mmol), DMA (5 mL), and *N*-methylallylamine (40 μL, 0.40 mmol) were added. The mixture was stirred for 24 h at 45 °C, and the reaction was monitored by TLC (hexanes/EtOAc 98:2). The product was purified by silica-gel CC (eluent $CHCl_3$/MeOH 98:2) to give 50 mg of **9** (0.12 mmol, yield 63%). $R_f = 0.45$ ($CHCl_3$/MeOH 9:1). White powder; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 7.64$ (1H, s, H-5), 7.57 (1H, d, $J = 9.5$ Hz, H-4), 6.80 (1H, s, H-8), 6.27 (1H, d, $J = 9.50$ Hz, H-3), 5.83 (1H, m, H-2″), 5.14 (2H, m, H-3″a,b), 4.03 (2H, t, $J = 7.0$ Hz, H-1′a,b), 3.00 (2H, d, $J = 6.54$ Hz, H-7′a,b), 2.36 (2H, t, $J = 7.41$ Hz, H-6′a,b), 2.21 (3H, s, NCH_3), 1.91–1.42 ppm (8H, m, CH_2). CIMS: 394 $[M+H]^+$.

Preparation of *N*-oxides 11, 13, 15, and 18: A solution of amine (1 equiv) in MeOH (1 mL mg^{-1} of amine) was stirred at 0 °C, and H_2O_2 (5 equiv, 35% *w/w*) was added. The mixture was stirred for 2 h at the same temperature. The reaction outcome was monitored by TLC ($CHCl_3$/MeOH 98:2) and the product isolated after evaporation of the solvent under vacuum was purified by neutral alumina CC ($CHCl_3$/MeOH 98:2). Yields were 35–40%.

7-[10-(Allylmethylamino)-decyloxy]-chromen-2-one *N*-oxide (11): White powder; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 7.61$ (1H, d, $J = 9.5$ Hz, H-4), 7.33 (1H, d, $J = 9.8$ Hz, H-5), 6.76 (2H, m, H-6, H-8), 6.20 (1H, d, $J = 9.5$ Hz, H-3), 6.14 (1H, m, H-2″), 5.45 (2H, m, H-3″a,b), 3.98 (2H, t, $J = 6.5$ Hz, H-1′a,b), 3.80 (2H, d, $J = 6.0$ Hz, H-1″a,b), 3.03 (3H, s, O←NCH_3), 1.77 (2H, m, H-10′a,b), 1.42–1.21 ppm (16H, m, H-2″, H-9″). CIMS: 388 $[M+H]^+$. $R_f = 0.55$ in alumina ($CHCl_3$/MeOH 9:1).

7-[10′-(Dimethylamino-*N*-decyloxy)]chromen-2-one *N*-oxide (13): White powder; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 7.64$ (1H, d, $J = 9.5$ Hz, H-4), 7.37 (1H, d, $J = 8.5$ Hz, H-5), 6.82 (2H, m, H-6, H-8), 6.25 (1H, d, $J = 9.4$ Hz, H-3), 4.01 (2H, t, $J = 6.5$ Hz, H-1′a,b), 3.27 (2H, m, H-10′a,b), 3.20 (6H, s, O←$N(CH_3)_2$), 1.80 (2H, m, H-2′a,b), 1.46–1.25 ppm (14H, m, H-3′a,b, H-9′a,b). CIMS: 361 $[M+H]^+$. $R_f = 0.4$ in alumina ($CHCl_3$/MeOH 9:1).

7-(7-(allyl(methyl)amino)hept-5-ynyloxy)-2*H*-chromen-2-one (14): *7-(hex-5-ynyloxy)-2H-chromen-2-one*: The ultrasound-promoted Mitsunobu reaction was carried out as described previously.[32] A solution in anhydrous THF (35 mL) containing triphenylphosphine (2.4 g, 9.24 mmol), 5-hexyn-1-ol (1.0 mL, 9.24 mmol), and 7-hydroxy-2*H*-chromen-2-one (1.0 g, 6.16 mmol) was sonicated at 10 °C under an argon atmosphere. Diisopropylazodicarboxylate (DIAD, 1.8 mL, 9.24 mmol) was then added dropwise over 5 min. The orange-red color of DIAD immediately disappeared, and a weakly exothermic reaction occurred. The mixture was sonicated for 40 min at 20 °C. When the reaction was complete as indicated by TLC (eluent: hexanes/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. After silica-gel CC purification, 1.35 g of pure product (0.43 mmol, yield 53%) were obtained.

*7-(7-(allyl(methyl)amino)hept-5-ynyloxy)-2H-chromen-2-one (**14**)*: In a 50-mL two-necked, round-bottomed flask equipped with magnetic stirrer, condenser, and optical fiber thermometer, the following were placed: dioxane (2 mL), paraformaldehyde (25 mg, 0.82 mmol), copper(II) acetate (5 mg), and *N*-methylallylamine (80 μL, 0.82 mmol). The mixture was irradiated with MW (150 W) at 60 °C for 10 min, during which time a violet color developed. A solution of 7-(6-hexynyloxy)coumarin (200 mg, 0.82 mmol) in dioxane (5 mL) was then added, and irradiation was continued at 90 °C (200 W) for 45 min. The raw material was purified by CC ($CHCl_3/CH_3OH$ 98:2) to give 140 mg of **14** (0.43 mmol, yield 53%).

7-(7-(allyl(methyl)amino)hept-5-ynyloxy)-2*H*-chromen-2-one *N*-oxide (15): White powder; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 7.97$ (1H, d, $J = 9.5$ Hz, H-4), 7.63 (1H, d, $J = 9.8$ Hz, H-5), 6.80 (2H, m, H-6, H-8), 6.25 (1H, d, $J = 9.5$ Hz, H-3), 5.70 (1H, m, H-2″), 5.20 (2H, m, H-3″a,b), 4.00 (2H, t, $J = 6.5$ Hz, H-1′), 3.95 (2H, br s, H-7′), 3.90 (2H, d, $J = 6.5$ Hz, H-1″a,b), 2.90 (3H, s, O←NCH_3), 2.0–1.71 ppm (6H, m, H-2′, H-4′). CIMS: 341 $[M+H]^+$. $R_f = 0.53$ in alumina ($CHCl_3$/MeOH 9:1).

7-(7-(allyl(methyl)amino)hept-5-ynyloxy)-8-iodo-2H-chromen-2-one (16): *7-hydroxy-8-iodo-2H-chromen-2-one:* A solution of 7-hydroxy-2*H*-chromen-2-one (5 g, 30.8 mmol), KI (7.68 g, 46.3 mmol), and I_2 (11.75 g, 46.3 mmol) in 30% NH_4OH (100 mL) was irradiated (100 W) for 20 min in a closed-vessel MW apparatus (Milestone). After it was allowed to cool, the mixture was acidified with 10% HCl, extracted with EtOAc, and washed with 5% $Na_2S_2O_3$ until colorless. The residue was purified by silica-gel CC ($CHCl_3$/MeOH 98:2) to afford 3.30 g (11.8 mmol, yield 37.2%) of the product. $R_f = 0.36$ ($CHCl_3$/MeOH 98:2).

7-(6-hexynyloxy)-8-iodo-2H-chromen-2-one: The same ultrasound-promoted Mitsunobu protocol was employed[32] using anhydrous THF (8 mL), triphenylphosphine (682 mg, 2.6 mmol), 7-hydroxy-8-iodo-2*H*-chromen-2-one (500 mg, 1.73 mmol), 5-hexyn-1-ol (286 μL, 2.6 mmol), and DIAD (525 μL, 2.6 mmol). The reaction, monitored by TLC (eluent: $CHCl_3/CH_3OH$ 98:2) was complete after sonication for 50 min. 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. The product was purified by silica-gel CC (394 mg, 1.07 mmol, yield 62%). $R_f = 0.76$ ($CHCl_3$/MeOH 98:2).

7-(7-(allyl(methyl)amino)hept-5-ynyloxy)-8-iodo-2H-chromen-2-one (***16***): 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 (5 mL), paraformaldehyde (27 mg, 0.91 mmol), copper(II) acetate (5 mg), and *N*-methylallylamine (87 μL, 0.91 mmol). When the mixture was irradiated with MW (200 W) for 5 min, the temperature rose to 80 °C, and a violet color developed. After the temperature was allowed to drop to 25 °C, 7-(6-hexynyloxy)-8-iodocoumarin (350 mg, 0.91 mmol) dissolved in dioxane (5 mL) was added, and the mixture was irradiated (200 W) for a further 10 min. The reaction was monitored by TLC using $CHCl_3/CH_3OH$ 95:5 as eluent. 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 CC ($CHCl_3 \rightarrow CHCl_3$/MeOH 9:1) to afford 203 mg of **16** (0.45 mmol, yield 50%). $R_f = 0.30$ ($CHCl_3$/MeOH 98:2); on alumina $R_f = 0.31$ ($CHCl_3$/MeOH 19:1). White powder; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 7.61$ (1H, d, $J = 9.5$ Hz, H-4), 7.35 (1H, d, $J = 8.6$ Hz, H-5), 6.84 (1H, d, $J = 8.6$ Hz, H-6), 6.24 (1H, d, $J = 9.5$ Hz, H-3), 6.05 (1H, m, H-2′), 5.19 (2H, m, H-3′′a,b), 4.13 (2H, t, $J = 6.8$ Hz, H-1′a,b), 3.75 (2H, s, H-7′a,b), 3.19 (2H, d, $J = 6.5$ Hz, H-1′′a,b), 2.64 (3H, s, NCH_3), 2.01–1.70 ppm (6H, m, H-2′a,b - H-4′a,b). CIMS: 452 $[M+H]^+$.

(*E*)-3-(4-(10-(dimethylamino)decyloxy)-2-hydroxyphenyl)-*N,N*-dimethylacrylamide (17): In a 50-mL round-bottomed flask 7-(10-bromodecyloxy)-2*H*-chromen-2-one (300 mg, 0.9 mmol), DMA (15 mL), and *N,N*-dimethylamine (147 μL, 2.77 mmol) were added. The mixture was stirred for 15 h at 45 °C, and the reaction was monitored by TLC ($CHCl_3$/MeOH 9:1). Purification by silica-gel CC (eluent $CHCl_3$/MeOH 19:1) yielded 280 mg of **17** (yield 79%). White powder; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 7.90$ (1H, d, $J = 17.0$ Hz, H-1), 7.33 (1H, d, $J = 8.5$ Hz, H-6′), 7.01 (1H, d, $J = 17.0$ Hz, H-2), 6.37 (2H, m, H-3′, H-5′), 3.94 (2H, t, $J = 6.9$ Hz, H-1′′a,b), 3.15 (3H, s, $CONCH_3$), 3.07 (3H, s, $CONCH_3$), 2.37 (2H, m, H-10′′a,b), 2.30 (6H, s, $N(CH_3)_2$), 1.73 (2H, quint, $J = 7.2$ Hz, H-2′′a,b), 1.55–1.28 ppm (16H, m, H-3′′, H-9′′). CIMS: 346 $[M+H]^+$. $R_f = 0.69$ in alumina ($CHCl_3$/MeOH 9:1).

(*E*)-3-(4-(10-(dimethylamino)decyloxy)-2-hydroxyphenyl)-*N,N*-dimethylacrylamide *N*-oxide (18): White powder; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 7.87$ (1H, d, $J = 17.0$ Hz, H-1), 7.31 (1H, d, $J = 9.5$ Hz, H-6′), 7.03 (1H, d, $J = 17.0$ Hz, H-2), 6.64 (1H, d, $J = 6.9$ Hz, H-3′), 6.33 (1H, dd, $J = 9.5$, 2.7 Hz, H-5′), 3.96 (2H, t, $J = 6.7$ Hz, H-1′′a,b), 3.25 (6H, s, $O \leftarrow N(CH_3)_2$), 3.14 (3H, s, $CONCH_3$), 3.05 (3H, s, $CONCH_3$), 1.99 (2H, m, H-10′′a,b), 1.72 (2H, quint, $J = 6.8$ Hz, H-2′′a,b), 1.60–1.40 ppm (16H, m, H-3′′, H-9′′). CIMS: 362 $[M+H]^+$. $R_f = 0.49$ in alumina ($CHCl_3$/MeOH 9:1).

^{14}C-Labeled (3*S*)-2,3-oxidosqualene was prepared according to Popják[33] by incubating pig liver S_{10} supernatant with (*R,S*)-[2-^{14}C]mevalonic acid (1 μCi, 55 mCi mmol^{-1}, 2.04 GBq mmol^{-1}; Amersham Pharmacia Biotech, UK) in the presence of the OSC inhibitor DMAE-DHA.[34]

Strains of *S. cerevisiae* and culture conditions: The recombinant strains of *S. cerevisiae*, SMY8[pSM61.21] (*MATa erg7:HIS3 hem1:TRP1 ura3-52 trp1-Δ63 LEU2:OSC S. cerevisiae his3-Δ200 ade2 Gal*$^+$) expressing wild-type yeast OSC,[35] SMY8[pBJ1.21] (*MATa erg7:HIS3 hem1:TRP1 ura3-52 trp1-Δ63 LEU2:OSC T. cruzi his3-Δ200 ade2 Gal*$^+$) expressing *T. cruzi* OSC,[36] SMY8[pBJ4.21] (*MATa erg7:HIS3 hem1:TRP1 ura3-52 trp1-Δ63 LEU2:OSC P. carinii his3-Δ200 ade2 Gal*$^+$) expressing *P. carinii* OSC,[36] and SMY8[pSM60.21] (*MATa erg7:HIS3 hem1:TRP1 ura3-52 trp1-Δ63 LEU2:OSC A. thaliana his3-Δ200 ade2 Gal*$^+$) expressing *A. thaliana* OSC,[37] were used. Cells of SMY8[pSM61.21], SMY8[pBJ1.21], and SMY8[pBJ4.21] were grown aerobically at 30 °C to early stationary phase in YPD medium (1% yeast extract, 2% peptone, 2% glucose) supplemented with hemin (0.013 mg mL^{-1})[35] and ergosterol (0.02 mg mL^{-1}). OSC expression was induced in YPG medium (1% yeast extract, 2% peptone, 2% galactose) supplemented with hemin (0.013 mg mL^{-1}). *A. thaliana* OSC expression in SMY8[pSM60.21] was induced in YPG medium in the presence of hemin (0.013 mg mL^{-1}) and ergosterol (0.02 mg mL^{-1}).

For human OSC expression, the recombinant strain of *S. cerevisiae* SMY8[pSOB1.1] (*MATa erg7:HIS3 hem1:TRP1 URA3:OSC H. sapiens trp1-Δ63 leu2-3.112 his3-Δ200 ade2 Gal*$^+$) was prepared in our laboratory using pYES2 yeast plasmid containing the human OSC cDNA (clone pOTB7 from the IMAGE, USA) to transform the SMY8 cells.[35] Cells were grown aerobically at 30 °C to early stationary phase in synthetic complete medium without uracil SC-Ura (0.17% yeast nitrogen base, 0.2% amino acids, 0.5% ammonium sulfate), with glucose (2%) in the presence of hemin (0.013 mg mL^{-1}) and ergosterol (0.02 mg mL^{-1}). Human OSC expression was induced in SC-Ura medium with galactose (2%) in the presence of hemin (0.013 mg mL^{-1}).

All precultures were grown aerobically for 48 h at 30 °C. For OSC expression, inocula (1 mL each) were grown for 48–72 h in 100 mL expression medium. All strains were preserved in 40% glycerol at −80 °C.

Enzyme assays: Cell-free homogenates were obtained as described.[19] Briefly, after lysis of the cell wall with lyticase, the spheroplasts were homogenized with a Potter device. Proteins in the homogenate were quantified with the Sigma Protein Assay Kit based on the method of Lowry modified by Peterson,[38] using bovine serum albumin as a standard.

OSC activity was assayed as described.[19] Briefly, the homogenates were incubated for 30 min with ^{14}C-labeled (3*S*)-2,3-oxidosqualene (1000 cpm). The enzymatic reaction was terminated by the addition of 15% KOH in methanol, lipids were saponified at 80 °C for 30 min, and nonsaponifiable lipids were extracted with petroleum ether. Extracts were spotted on TLC plates using *n*-hexane/ethyl acetate (85:15) as the developing solvent. The conversion of labeled substrate to labeled product was quantified with a System 200 Imaging Scanner. (Hewlett–Packard, Palo Alto, CA, USA).

OSC inhibition was studied by incubating the homogenates with ^{14}C-labeled (3*S*)-2,3-oxidosqualene (1000 cpm) in the presence of inhibitors as described above. IC_{50} values (inhibitor concentrations that decreased enzymatic conversion by 50%) were calculated by nonlinear regression analysis of the residual activity versus the log of inhibitor concentration using statistical software from Genstat (NAG, Oxford, UK).

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