

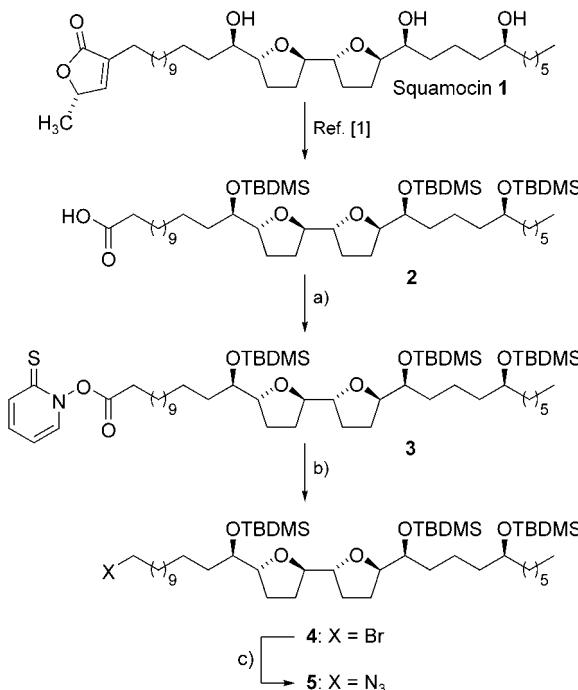
Annonaceous Acetogenins: The Hydroxyl Groups and THF Rings Are Crucial Structural Elements for Targeting the Mitochondria, Demonstration with the Synthesis of Fluorescent Squamocin Analogues

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Acetogenins of the Annonaceae are a group of hyperactive secondary metabolites with interesting and promising antitumor properties.^[1] The acetogenins exert a wide variety of biological activities, among which are an impressive cytotoxicity (10^{-6} – 10^{-14} M) reported on various tumor cell lines,^[1] and the most powerful inhibition of complex I (NADH/ubiquinone oxidoreductase) in mitochondrial transport systems.^[1] In addition, cell death induced by some mono- and bis-THF acetogenins and their analogues has been described in the past few years as an apoptotic process^[2] suspected of being linked to complex I inhibition.

The intriguingly highly potent biological properties coupled with a lack of clear structure–activity relationship studies establishing the exact role of each distinctive part of the acetogenins, prompted us to initiate a program directed towards the synthesis of hybrids consisting of an acetogenin tail connected to a fluorescent tag. In this context, information from such derivatives should shine light on the role of the hydroxyl/THF part of these natural products as a specific (or not) recognition site directed towards mitochondrial internal membranes.

Squamocin, isolated from *Annona reticulata* seeds, was chosen as a starting material for the hemisynthesis of our fluorescent analogues. Our laboratory has previously described an efficient ruthenium-catalyzed periodic oxidation of the terminal lactone of squamocin **1** that enabled the formation of a carboxylic acid–squamocin derivative **2** (Scheme 1),^[3] which might be the cornerstone for the preparation of fluorescent acetogenin derivatives. The synthesis of two of them was envisaged according to two different strategies. The first analogue was obtained under classical coupling conditions. Acid **2** was treated with dansyl derivative **6** to give amide **7** after deprotection of the secondary alcohols with fluoride anions in an alkaline



Scheme 1. Synthesis of intermediates **2** and **5**. Reagents and conditions: a) thiopyridine *N*-oxide (1.2 equiv), DCC (2 equiv), dimethylaminopyridine (cat.), CH_2Cl_2 , RT, 24 h (80%); b) BrCCl_3 (4 equiv), CH_2Cl_2 , $h\nu$, 30 min, RT (50%); c) NaN_3 (excess), DMSO, RT, 48 h (95%). DCC = dicyclohexylcarbodiimide.

medium (Scheme 2). As we feared possible hydrolysis of amide **7** in the cell, giving biased results in terms of fluorescence localization, we sought to design a stable, nonhydrolyzable analogue. In this respect, a 1,4-disubstituted 1,2,3-triazole connection between the acetogenin core and the fluorescent part should provide a virtually nonhydrolyzable link and should be easily introduced by using “click chemistry”^[4] Huisgen-type cycloaddition.^[5] With a reliable and robust method for the attachment of a fluorogenic probe at our disposal, we then needed to prepare a squamocin analogue bearing a terminal azido group. In the search for reliable reactions that would allow the modification of small amounts of acetogenins in a minimum of synthetic steps, we turned our intention to Barton’s radical decarboxylation.^[6] This free-radical chain reaction provides a highly efficient method of replacing a carboxylic acid by a wide variety of functional groups.^[7] In this paper, we report on the exploitation of the radical decarboxylative halogenation.

Starting from acid **2**, esterification with thiopyridine *N*-oxide afforded the photo- and heat-sensitive thiohydroxamic ester **3** (Scheme 1). Decarboxylative bromination was successfully applied to ester **3** when it was exposed to visible light with BrCCl_3 as a bromine source in dry CH_2Cl_2 . Thus, bromo derivative **4** was isolated with a more or less satisfactory yield of 50%. Bromine was displaced by an azido group upon treatment of **4** with excess sodium azide in DMSO. The formation of **5** was followed by using the useful TLC method recently described by Finn.^[8] The stage was set for the triazole formation: cycloaddition with fluoresceine alkyne **8** in water/tert-butanol, catalyzed by ascorbic acid/copper sulfate^[9] gave, after desilyla-

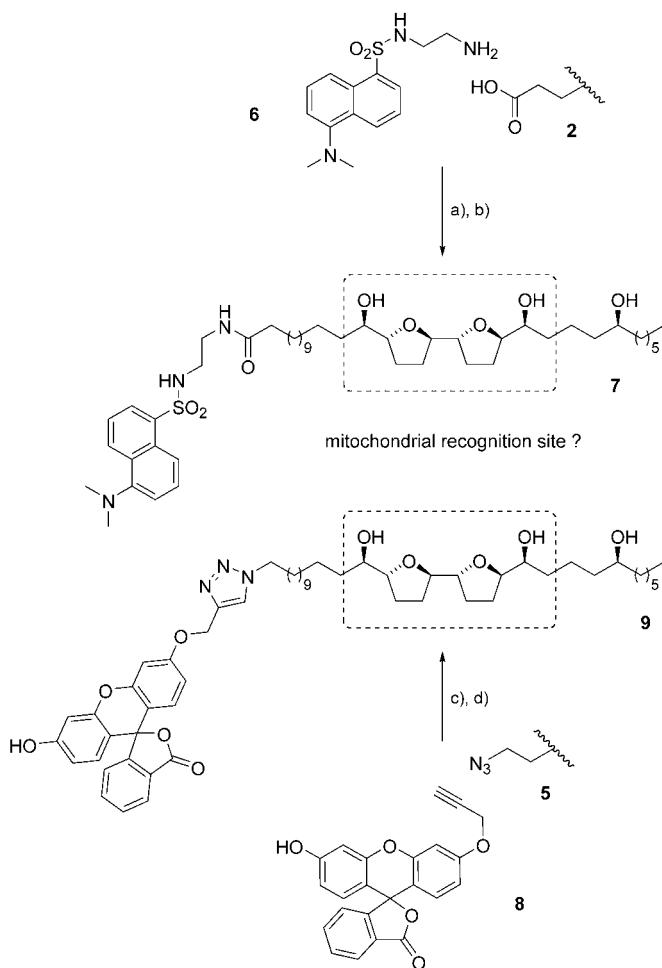
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Scheme 2. Synthesis of fluorescent probes **7** and **9**. Reagents and conditions: a) DCC (2 equiv), HOEt (1 equiv), CH₂Cl₂, RT, 24 h (96%); b) nBu₄NF (150 equiv), THF, RT, 24 h (29%); c) CuSO₄·5H₂O (1 mol %), ascorbic acid (10 mol %), H₂O/tBuOH (1:1), RT, 72 h; d) HF (75 equiv), CH₃CN/CH₂Cl₂ (1:5), RT, 45 min (60%, two steps).

tion with HF, the target compound **9** in 60% yield from **5** (Scheme 2).

Cell death induced by acetogenins was described as apoptosis.^[2] As a way to study the influence of the butenolide replacement by either dansylethylene diamine or fluorescein bulky groups on the biological activity, the proapoptotic potentials of **7** and **9** were evaluated on Jurkat cells. Apoptosis can be defined as a cell-death mechanism in which the activation of catabolic processes and enzymes occurs prior to cytolysis. The dying cells manifest characteristic biochemical hallmarks such as the permeabilization of the external mitochondrial membrane and the exposure of phosphatidylserine (PS) residues on the plasma membrane surface.^[10] In the laboratory, proapoptotic potential is usually evaluated by screening the disruption of the mitochondrial transmembrane potential by means of the cationic lipophilic fluorochrome DiOC₆(3). Since the emission wavelengths of the fluorochromes incorporated in derivatives **7** and **9** synthesis were almost the same as DiOC₆(3), we preferentially chose in this study to detect aberrant PS exposure by using annexin V coupled to allophycocyanin (APC).^[11]

This assay indicated that both **7** and **9** induced apoptosis as squamocin does, even if **9** was less active (Figure 1). Moreover, the cytometric data showed that, even if all cells were not apoptotic, they all incorporated the squamocin derivatives, as revealed by a shift of the cell population in FL-1 channel from nonfluorescent to stained cells.

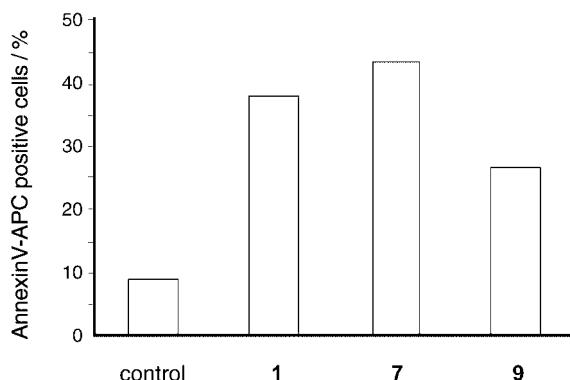


Figure 1. Evaluation of the proapoptotic potential of the fluorescent squamocin derivatives **7** and **9** on Jurkat T cells. Jurkat T cells grown in RPMI 1640 medium with Glutamax supplemented with FCS (10%), penicillin (100 U mL⁻¹), and streptomycin (100 µg mL⁻¹) were treated for 24 h with 15 µM of **1**, **7** or **9** and labeled with annexin V-APC to measure external PS exposure. Control: untreated cells.

The annexin V-APC-stained cells treated with **7** or **9** were visualized by using fluorescent microscopy. After 24 h of treatment, our probes could be properly identified in Jurkat cells, but a pronounced diffusion of fluorescent squamocin derivatives was noted above all in apoptotic cells (Figure 2). However

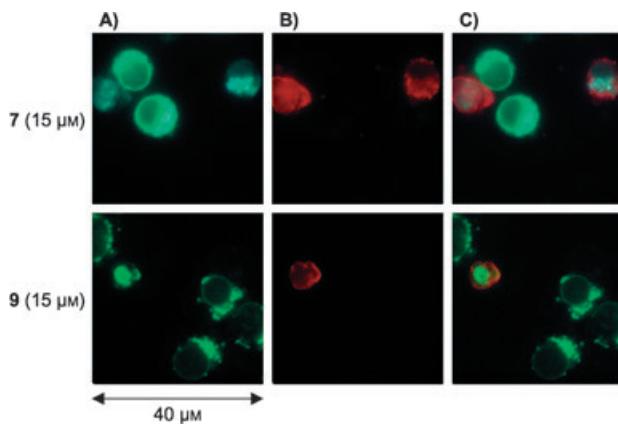


Figure 2. Visualization of **7** and **9** by using fluorescent microscopy. Jurkat T cells were pretreated with 15 µM of **7** or **9**. After 24 h of incubation at 37°C, an annexin V-APC conjugate was used for the assessment of aberrant PS exposure, which characterizes apoptotic cells. After being washed in PBS, cells were incubated at 4°C for 20 min in the presence of fluorescent conjugate (2 µM, Bender Medsystems). Cells were washed three times with PBS and immediately mounted in PBS for examination. Preparations were observed with a Nikon Eclipse TE2000-U microscope, and analyzed by using Nikon ACT-1 software. A) A Jurkat T cell labeled with fluorescent derivatives viewed by using the appropriate excitation filter, UV-2A or B-2A filters, respectively, for **7** and **9**. B) The same cell viewed by using the appropriate filter for annexin V-APC visualization. C) Both **7** and **9** are visualized in Jurkat cells mitochondria but diffused in cytosol in apoptotic cells, as confirmed by superimposition of both stainings.

earlier observations (6 h) of cells treated with higher doses of **7** and **9** and stained with MitoTracker® Red CMXRos, a specific mitochondrial probe, allowed us to see that both fluorescent derivatives localized almost exclusively in mitochondria, as confirmed by the overlapping patterns of the green and red fluorescences (Figure 3).

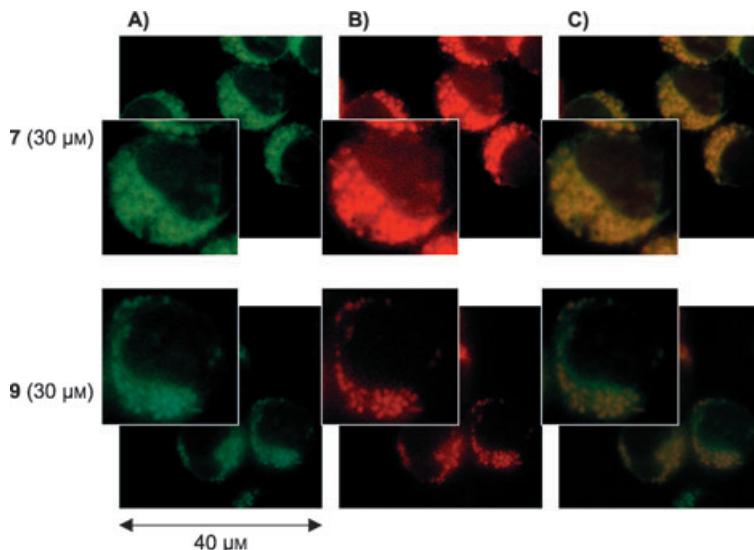


Figure 3. Visualization of **7** and **9** by using fluorescent microscopy. Jurkat T cells were pretreated with 30 μ M of **7** or **9**. After 5 h 45 min of incubation at 37°C, mitochondria were stained with 5 nM of mitochondrial potential fluorochrome MitoTracker® Red CMXRos for 15 min at 37°C. Cells were washed three times with PBS and immediately mounted in PBS for examination. Preparations were observed with a Nikon Eclipse TE2000-U microscope, and analyzed by using Nikon ACT-1 software. A) Mitochondria of a Jurkat T cell labeled with CMXRos visualized by using a G-2A filter. B) The same cell with the fluorescent derivatives' appropriate excitation filter. Both **7** and **9** labeled mitochondria as confirmed by superimposition (C).

As demonstrated by Shimada et al.,^[12] with their U-shape, mono- and bis-THF acetogenins, which are fatty acid derivatives, were able to penetrate dimyristoylphosphatidylcholine liposomal membranes, thus mimicking phospholipids. The THF sequences were localized at the interfacial region of membranes. For the first time, our studies were performed on living, nonfixed cells. We can state that squamocin passes through the plasma membrane and targets the mitochondria. Indeed both hemisynthesized fluorescent derivatives were shown to be potent apoptosis inducers, and to be addressed to this organelle. The lactone replacement does not seem to interfere with the mitochondria targeting but appears to be implied in the activity. The previous synthesis of quinone-annoneaceous acetogenins by Koert et al. supported those data as α,β -unsaturated butenolide replacement by a quinone ring increased the mitochondrial complex I inhibition.^[13] The bis-THF sequence flanked with hydroxyl groups and lipophilic chains (C_{12} and C_{10}) is involved in the addressing of acetogenins to mitochondria membranes. However it does not explain why these membranes are the only one targeted. Cardiolipins, with their dimeric structure, are the only phospholipids exclusively found in mitochondria. This specific component is present at

the inner membrane and at intermembrane contact sites.^[14] Moreover, cardiolipins interact with a large number of mitochondrial proteins, most of which reside in the inner membrane,^[14] and are required for the enzymatic activity of complexes I and III.^[15] Therefore, we can also imagine an interaction between the lipophilic sequence of squamocin and the mitochondrial cardiolipin. The butenolide could be directed onto the proteic environment of these cardiolipins and would be able to interact with its target. This concept, already described to explain the interaction of the terminal lactone with complex I,^[12] is supported by our results. However, cardiolipins interact with other inner-membrane proteins, which therefore appear as potential acetogenins target (e.g., complex III). Indeed the cell death induced by acetogenins and their analogues is not always clearly correlated with complex I inhibition. In addition, acetogenins, like squamocin, induce programmed cell death,^[2a] and modifications of cardiolipins levels or structure have been implicated in apoptosis.^[16]

The data described herein (including new developments in chemistry, such as applications of Barton's radical decarboxylation in hemisynthesis) confirm some of the previous hypothetical proposals regarding acetogenins' biological behavior. Those products penetrate the cells and, after 6 h, target the mitochondrial membrane, where a protein interaction is likely to occur and the inhibition of complex I is supposed.

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