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Highly cytotoxic and neurotoxic acetogenins of the Annonaceae: New putative biological targets of squamocin detected by activity-based protein profiling

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ABSTRACT

Acetogenins of the Annonaceae are strong inhibitors of mitochondrial complex I but discrepancies in the structure/activity relationships pled the search for other targets within the whole cell proteome. Combining hemisynthetic work, Cu-catalyzed Huisgen cycloaddition and proteomic techniques we have identified new putative protein targets of squamocin ruling out the previously accepted 'complex I dogma'. These results give new insights into the mechanism of action of these potent neurotoxic molecules.

Acetogenins of the Annonaceae constitute a broad group of secondary metabolites with impressive biological activities and have been considered as important leads for new anticancer drugs.[1] But recently, acetogenins of the Annonaceae have been suspected to be involved in neurodegenerative disorders such as fatal atypical parkinsonisms in worldwide areas where annonaceous-derived edible products or traditional medicines are comsummated.[2] The hyperactivity of the acetogenins of the Annonaceae in conjunction with this new public health issue claim for urgent biological studies for a better understanding of their exact mechanisms of action.[3] Therefore, we have embarked on synthetic investigations on squamocin **1**,[4] an ubiquitous acetogenin of the Annonaceae extracted, for example, from the seeds of *Annona reticulata*. Squamocin **1** (Fig. 1) possesses a terminal α,β-unsaturated γ-lactone and a central polar part consisting in two tetrahydrofuran rings and three secondary alcohol functions.

Annonaceous acetogenins are known to be strong inhibitors of mitochondrial complex I (NADH-ubiquinone reductase)[4b] and inducers of cell-apoptosis. One of the main facts resulting from all pharmacomodulation studies conducted by us and many other groups is the absence of any clear link between complex I inhibition and apoptosis and/or cytotoxicity. In this Communication, a program directed towards the identification of acetogenin biological targets is reported. It is inspired by Affinity-Based Protein Profiling (ABPP) as part of chemical proteomic technologies.[5]

In fact, from a chemical reactivity point of view, the work of Duval et al.[4e] suggested that the α,β-unsaturated γ-lactone could constitute a possible electrophilic functional group that may covalently bind to nucleophilic residues in specific protein targets.[6] Among the chemical tools towards the design of chemical probes for functional proteomics developed in the recent years we chose to exploit the Cu^{I}-catalyzed version of the azide-alkyne Huisgen cycloaddition[7] ('Click-chemistry-based ABPP'). To this aim, we needed chemical probes derived from **1**. The first target was molecule **2**, suitable for a 'tag-free' strategy. For this purpose we needed a N_3 group as a viable chemical reporter on an almost complete

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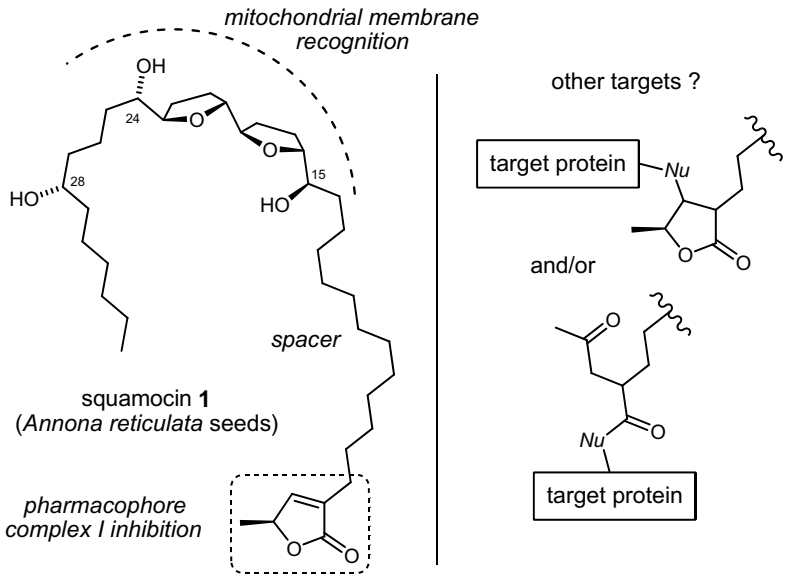


Figure 1. Structure of squamocine **1**, key RSA data and potential protein-reactive sites.

squamocin skeleton (keeping unchanged the lactone ring and the bis-hydroxy/THF part) for detection of substrate-target covalent adducts by tagging with a fluorophore in whole proteomes. Central in our strategy was the search for a selective introduction of an azido group at C-28. Interestingly, the direct introduction of the azido group was successful in our case using Mitsunobu-type reaction with diphenylphosphorazide[8] leading to **2** as a probe for 'tag-free' ABPP. Having reached this key-molecule, a new probe (**3**) was synthesized in the classical conditions described for the catalyzed Huisgen-type cycloaddition of azides with terminal alkynes.[9] In moving on to the next approach, a bifunctional derivative **4** bearing (i) a reactive function for photoaffinity labeling in place of the terminal lactone and (ii) the fluorescent group at position 28 was prepared. [9]

Confocal microscopy with laser scanning allowed us to identify more precisely the organelles targeted by **5** (with the fluorescein moiety in place of the lactone ring).[4a] At first, intracellular localization of probes **3** and **5** was studied (Fig. 2). In Hela cells, after 6 h, **3** and **5** were predominantly, but not solely, addressed to the mitochondria (71% and 62%, respectively, LSM Image Browser software).

Kinetic studies were also performed with **5** and shown a rapid internalization into the cell (5–10 min) and a mitochondrial localization after 15–25 min. This latter is completed after 40 min, that is to say far before the observation of the mitochondrial transmembrane potential (MTP) disruption, an early stage feature of apoptosis (typically 3–6 h with annonaceous acetogenins.[4c] These discrepancies, both in terms of localization and kinetic, prompted us to further search for other targets than mitochondrial complex I on which acetogenins such as **1** could covalently bind.

We first decided to study probe **3**. Jurkat-T cells were incubated with **3** (10 μM for 24 h). The optimal protocol for both 1D and 2D (IEF/SDS–PAGE) electrophoresis was established in denaturating conditions.

Affinity-isolated proteins were first separated and visualized by 1D (SDS–PAGE) and in-gel fluorescence scanning with Coomassie

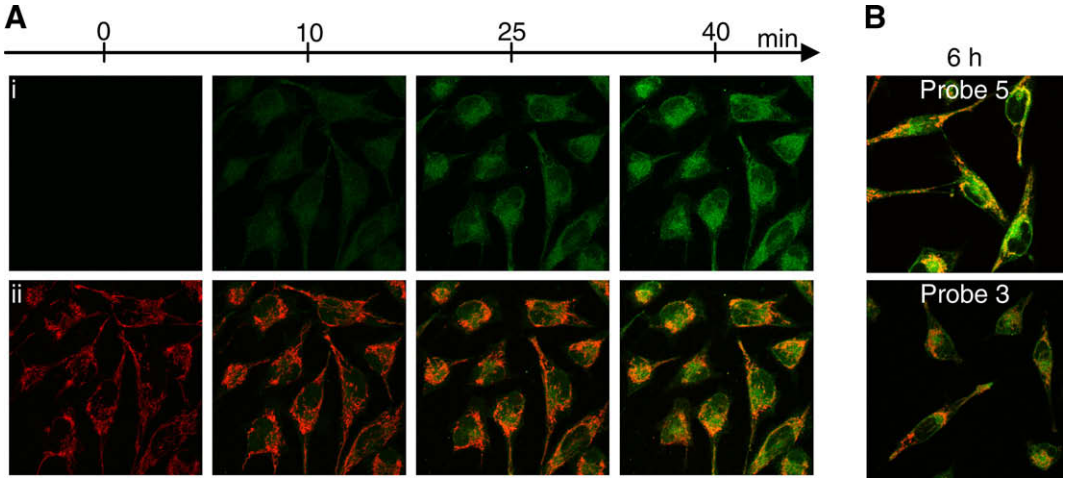


Figure 2. Kinetic study of the penetration of probe **5** in Hela cells (A) and visualization of the colocalisation of **3** and **5** with mitochondria (B). (A) Hela mitochondria were labeled with Mitotracker Red CMXRos®. At $t = 0$, 10 μM of **5** were injected in the culture medium and acquisitions were realized. (i) Hela cells labeled with **5**. (ii) Overlays of CMXRos and **5** stainings. (B) Hela cells were treated with **3** or **5** (green) for 6 h and mitochondria were labeled with CMXRos (red). The overlay indicated that both probes were predominantly addressed to the mitochondria (71% and 62%, respectively, LSM Image® Browser software).

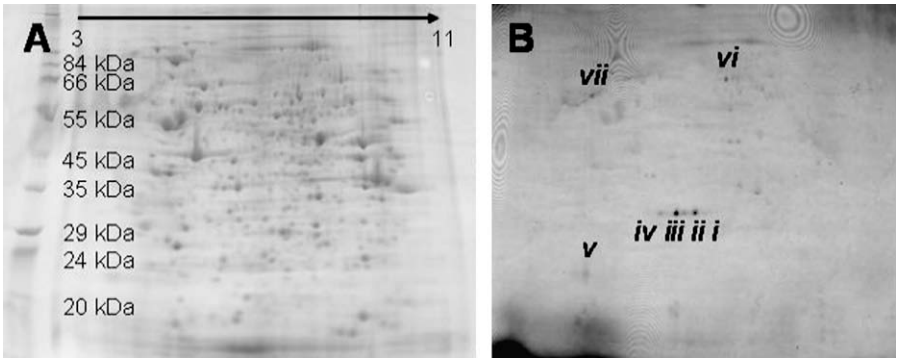
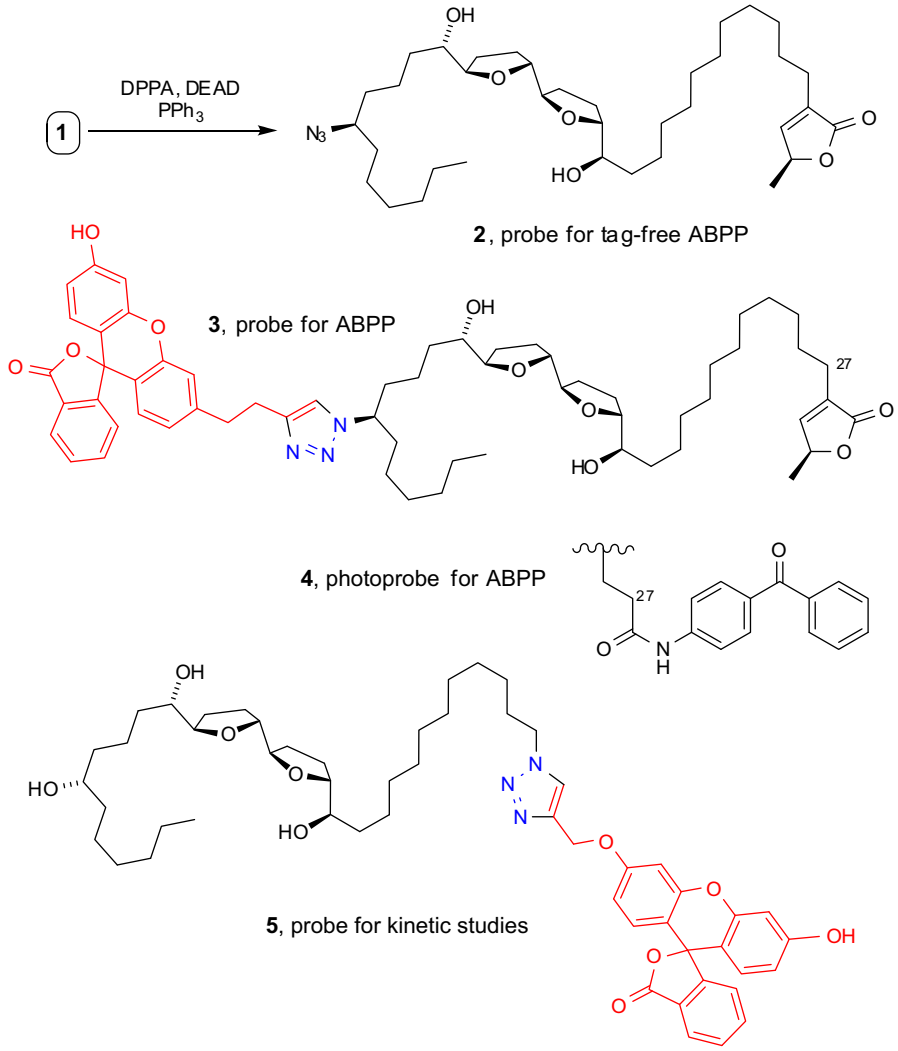


Figure 3. 2D-electrophoresis of total proteins (**A**) and from Jurkat cells treated by probe **3** (**B**). Seven proteins (i–vii) are labeled by probe **3** in a reproducible manner (three independent experiments): i, ii, vi and vii were identified (see text).

co-staining to directly visualize protein bands.[9] Less than 10 protein bands (ranging from 20 to 80 kDa) were labeled in a specific and reproducible manner. Interestingly, the electrophoretic profile was identical when cells were treated with photoprobe **4** for 24 h and then photoactivated to establish a covalent link to its nearby environmental proteins in vivo.[10]

The 2D gel showed seven proteins which were reproducibly fluorescent. Protein spots were excised, digested by trypsin and analyzed by MALDI-TOF/TOF mass spectrometry. Spots (i), (ii), (vi) and (vii) (Fig. 3) correspond, respectively, to the COP9 signalosome complex subunit 7b (MW 30 kDa, p*I* 5.83), an enzymatic complex associated with the ubiquitinylation and phosphorylation of transcription factors (p53 for example),[11] the glutathione transferase omega 1 (MW 27 kDa, p*I* 6.23), an enzyme involved in detoxification,[12] the flavoprotein subunit of succinate dehydrogenase (MW 73 kDa, p*I* 7.06) also known as mitochondrial complex



Scheme 1. Synthesis of squamocin-derived probes. See Supporting Information for experimental details.

II[13] and to the disulfide isomerase (MW 57 kDa, p*I* 4.76), a chaperone protein from endoplasmic reticulum.[14] Proteins (iii)–(v) could not be identified as the protein amount was not sufficient. However their molecular weights around 30 kDa as the ND1-subunit of mitochondrial complex I is promising.[3g]

The ultimate challenge in our approach ideally entailed using probe **2** for tag-free ABPP, that is, in vivo covalent interactions of **2** with its targets and subsequent labeling of the bio-orthogonal azide chemical reporter group in vitro on whole proteomes. Unfortunately, an unspecific labeling of proteins by propargylfluorescein was systematically observed. Possible reasons can be put forward for this failure and are inherent to acetogenin structures and physicochemical properties. Previously reported successes of this latter strategy[15] dealt with the targeting/identification of soluble cytosolic proteins or expressed proteins. In our case, membrane associated targets and a strong affinity of acetogenins to lipids may considerably complicate the task making the 28-azide totally inaccessible for chemical modifications on whole proteome (Scheme 1).

In conclusion, an acetogenin of the Annonaceae was converted to activity-based probes for chemical proteomics. But mainly, we were able to identify new putative targets including mitochondrial (vi), but also cytosolic (i and ii) and reticulum associated (vii) enzymes.[16] This rules out the 'complex I dogma' and opens the way to major new developments in biology for the comprehension of both cytotoxicity and neurotoxicity of this class of secondary metabolites which are found in edible products from the Annonaceae and constitute an important public health issue. These results also validate the particular importance of natural products in the exploration of proteome.[17]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.09.091.

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