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Phytochemistry

A Desulfatation–Oxidation Cascade Activates Coumarin-Based Cross-Linkers in the Wound Reaction of the Giant Unicellular Alga *Dasycladus vermicularis***

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Plants and algae protect themselves against environmental threats by fast, wound-activated processes in which metabolites that are stored within the tissue are transformed enzymatically.[1] For example, it was recognized early on that plants and algae rely on the wound-activated action of lipases and lipoxygenases to produce a structural variety of oxylipins that can play important roles in chemical defense.[2] Wound-activated enzymatic reactions can not only be employed to generate efficient chemical defenses against herbivore attack or pathogenic invasion, but may also serve to mechanically protect the tissue or cells.[3] Wound sealing is particularly important for siphonous green macroalgae because of their remarkable organization: they comprise a single giant cell that can reach several meters in length. An impressive example is the wound reaction of the siphonous green alga *Caulerpa taxifolia.* Upon tissue disruption this alga transforms the acetylated sesquiterpene caulerpenyne to oxytoxin 2, a potent protein cross-linker that plays an integral part in forming a protective polymer material that seals the wound.[4] The rapid assimilation of cellular contents into an insoluble wound plug prevents detrimental cytoplasmic loss and limits the intrusion of extracellular components, which could otherwise prove fatal. A number of other marine siphonous-algal lineages also rely on activated cellular metabolites for biopolymer formation, but little is known about the underlying mechanisms.[5] Herein we explore the chemical basis of wound-plug formation in a member of the order Dasycladales, *Dasycladus vermicularis* (Scropoli) Krasser. This evolutionary ancient alga apparently lacks caulerpenyne type molecules but is still capable of wound-plug formation. Previous reports have described the chronological events involved in the wound-healing process in this alga: an initial rapid gelling process followed by a delayed hardening and browning process.[6,7] The overall process leads to the establishment of a rigid biopolymer (Figure 1). Herein we provide evidence for the biochemical basis of this biopolymerization event.

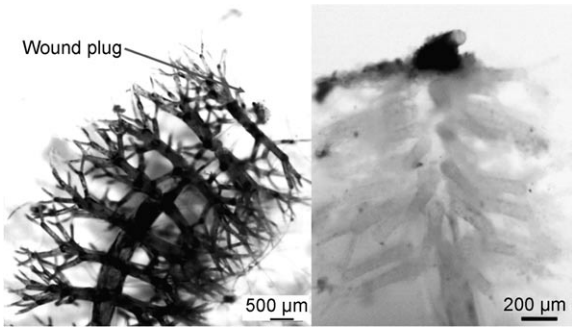

Figure 1. Wound-plug browning in the apical thallus of the unicellular green algae *D. vermicularis* (left 2 h and right 24 h after wounding).

We undertook chemical profiling of the metabolites found in intact and wounded algae to monitor changes in secondary metabolites potentially involved in biopolymerization. Surprisingly, 3,6,7-trihydroxycoumarin (THyC) that has been reported as the major secondary metabolite from *D. vermicularis*[8] was not detected in ultra performance liquid chromatography (UPLC)-MS chromatograms of MeOH extracts of intact algae. Utilizing NMR spectroscopy, HR/MS, and MS/MS the dominant metabolite in the algal extract was determined to be 6,7-dihydroxycoumarin-3-sulfate (DHyCS). The structure was confirmed by synthesis of an authentic standard (see the Supporting Information). Previous studies on the secondary metabolites of *D. vermicularis* have thus most likely been measuring DHyCS concentration and not THyC as the UV-absorbance maximum falls at approximately 345 nm for both compounds.[9,10] No other sulfated coumarins or higher substituted derivatives of THyC were detected by monitoring the characteristic loss of $\Delta m/z = 80$ (SO_3) within fragmentation patterns using UPLC-MS/MS techniques. Interestingly, while DHyCS is stable at room temperature the concentration of this compound in wounded algal tissue rapidly diminishes as a function of time (Figure 2 a–c). We reasoned that mechanical disruption of the cell might result in the decompartmentalization of cellular sulfatases which could transform DHyCS into THyC.

Hydrolysis of sulfate esters by sulfatase enzymes regulates the activity of a broad range of biomolecules and thus controls

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Supporting information for this article (a detailed experimental section mentioning sample collection, extraction procedures, and all experiments as well as the spectroscopic data of DHyCS) is available on the WWW under http://dx.doi.org/10.1002/anie.201100908.

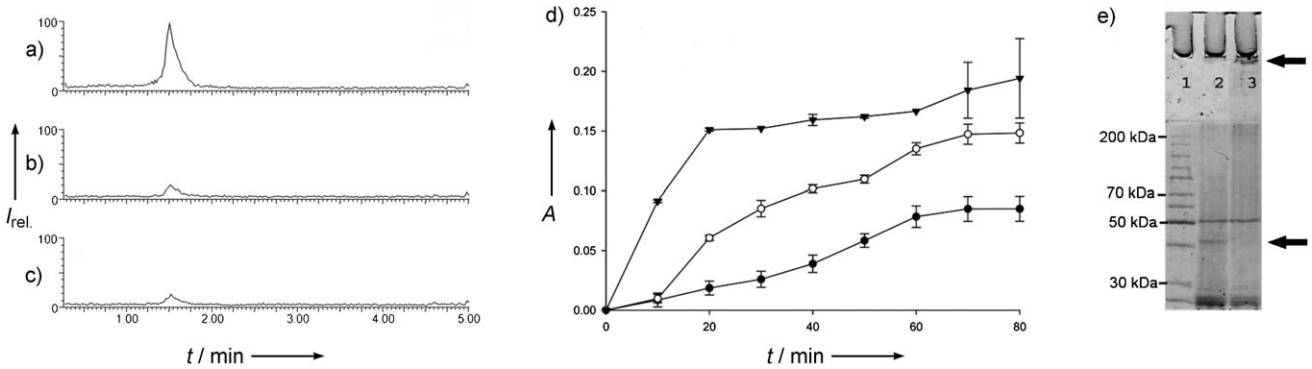


Figure 2. a)–c) UPLC-ESI-MS ion trace of DHyCS (*m*/*z* 273) in algal extracts at 0, 2, and 20 min after injury, respectively. d) THyC mediated α-lactalbumin aggregation under oxidative conditions monitored by light-scattering assays at 350 nm (● 20 μM THyC, ○ 40 μM THyC, ▼ 80 μM THyC). e) SDS PAGE (10%), lane 1: protein ladder, lane 2 : Boiled *D. vermicularis* cells exhibiting protein content of intact cells, lane 3: *D. vermicularis* cells boiled 4 h after wounding. The arrows indicate polymer in the loading pocket (top) and proteins in intact algae.

important functions including hormone regulation and signaling pathways.[11] Sulfatase activity could indeed be detected in an in-vitro assay using the model substrate 4-methylumbelliferone sulfate which was rapidly hydrolyzed by wounded algae (see the Supporting Information). Whereas the product 4-methylumbelliferone could be readily detected, THyC was only found in trace amounts during and after the wound reaction. We therefore concluded that THyC might be further transformed in the wounded algae. Because hydroxycoumarins can be easily oxidized,[12] we reasoned that reactive oxygen species, which were previously detected in wounded *D. vermicularis*,[7] could serve as an oxidant for THyC. If the kinetics of H_2O_2 production[7] and DHyCS transformation are compared it becomes clear that the oxidant is only detected after the complete transformation of DHyCS (see the Supporting Information). In model reactions it could be demonstrated that H_2O_2 only slowly oxidized THyC. This oxidation was considerably accelerated in the presence of horseradish peroxidase (HRP) and was complete after 4 min. The HRP was utilized to mimic the presence of oxidase activity in wounded algae (see the Supporting Information).[7] The observations are in accordance with a mechanism in which H_2O_2 is produced continuously after wounding and acts as substrate for the enzymatic oxidation of THyC. The oxidant would then only be detected once the substrate THyC is quantitatively transformed.

To assess the ability for THyC to serve as a cross-linking agent under oxidizing conditions, model reactions using this compound, H_2O_2, HRP, and selected amino- and sulfhydryl-bearing model compounds were employed. Under these conditions THyC is rapidly oxidized and the product can be trapped by glutathione (GSH), cysteine, and dithiothreitol. Metabolites resulting from the transformation with one or two GSH, or with the other thiol-containing nucleophiles, could be detected and characterized by UV spectroscopy, HR/MS and MS/MS analysis (Figure 3, see the Supporting Information). The ability of the oxidized THyC to form covalent bonds to amino groups was evaluated by using the probe Bodipy FL STP ester, which has an available reactive amine group. In the presence of oxidants, the percentage of free reactive amino groups decreased as the THyC concentration increased (see the Supporting Information). These data provide evidence that ubiquitous thiol- and amine-bearing functionalities in algal tissue (e.g. proteins), could readily react under ambient conditions with oxidized THyC. Moreover, when *D. vermicularis* cells were wounded in the presence of GSH, THyC/GSH adducts were detected (Supporting Information). This observation suggests that conditions necessary for the quinone chemistry to take place are met in vivo. The transformations involved most likely proceed by a radical mechanism, which is also supported by the fact that reactive oxygen species, generated in the presence of a lipoxygenase and its substrate linolenic acid, also lead to THyC oxidation (see the Supporting Information). Since the sulfated DHyCS is not transformed by H_2O_2 or the peroxidase

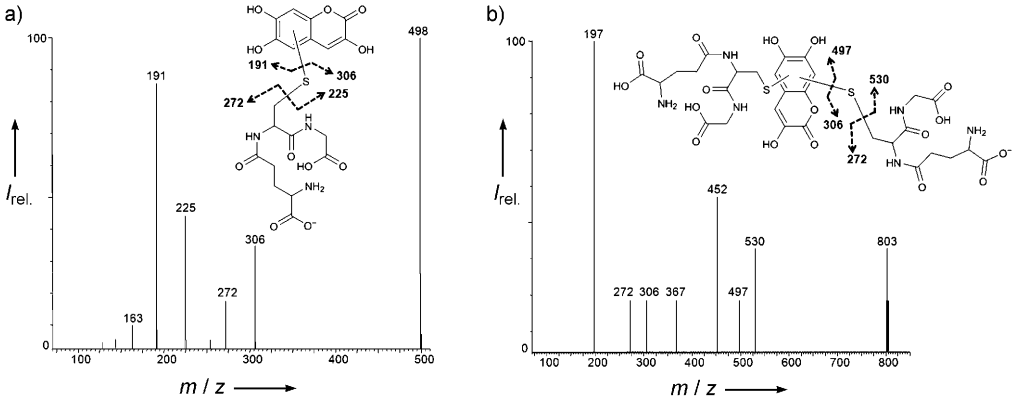
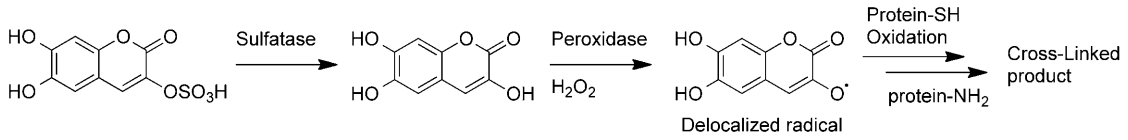


Figure 3. ESI-MS/MS of a) a THyC–glutathion adduct and b) a THyC–(glutathione)$_2$ adduct with indicative fragments.



Scheme 1. Process by which THyC is transformed in to a potent protein cross-linker. Stage 1: Sulfate ester hydrolysis by sulfatase upon cellular decompartmentalization. Stage 2: Radicals formed from THyC oxidation react with amino acid side chains. Stage 3: Further oxidation and reaction with amino acid side chains leads to cross-linked products increasing the stability of the wound plug.

treatment (see the Supporting Information) it can be assumed that the radical attack preferentially occurs at the C3-OH group by formation of a mesomeric radical which adds a nucleophile to form first addition products (Figure 3, see the Supporting Information). These can then undergo further transformation to higher functionalized THyC derivatives (Figure 3, Scheme 1). Alternatively, the reactions could follow the oxidation/addition pathways involving quinones which have been described for the thiol addition to dopamine and the protein cross-linking in privet trees under oxidative conditions.[13,14]

To provide additional evidence for the involvement of THyC in an oxidative biopolymerization process, the metabolite was incubated with HRP and H_2O_2 in the presence of the test protein α-lactalbumin. 30–40 min after the start of incubation the mixture turned brown and a precipitate was clearly visible. This reaction was also observed with other randomly selected test proteins, indicating an unspecific activity. Acid hydrolysis and automated amino acid analysis revealed that this precipitate was rich in α-lactalbumin. Although solution browning occurred even in the absence of a test protein, precipitation occurred only upon addition of α-lactalbumin. A light-scattering assay was used to quantify the degree of polymerization when oxidized THyC was combined with α-lactalbumin.[15] Three concentrations of THyC were incubated with α-lactalbumin, HRP, and H_2O_2 and polymerization was monitored for 80 min (Figure 2d). 20 μM of THyC was capable of promoting protein polymerization and the amount of aggregation increased with 40 μM and 80 μM of THyC. Control experiments showed no turbidity when THyC was omitted from the reaction mixture thus indicating its essential involvement in aggregation. Cellular DHyCS concentrations above 100 μM can be estimated based on photometric quantifications.[10] According to our data this concentration would clearly be sufficient for protein polymerization. To verify if the observed protein aggregation is also found in Dasycladus cells, we used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to monitor the distribution of protein content before and after wounding. Protein analysis of ground cells revealed a band in the loading pocket indicative for a cross-linked protein-rich polymer.[4] Clearly this polymer is formed from algal proteins, since bands arising of algal proteins are strongly depleted compared to those from non-cross-linked controls (Figure 2e).

Taken together these observations suggest that a complex cascade of desulfatation, oxidation, cross-linking occurs after wounding of the giant-celled green alga *D. vermicularis*. This sequence ultimately leads to the formation of a hardened wound plug, and survival of the alga (Scheme 1). UV and fluorescence investigations of the wound plug show that the THyC fluorescence and the characteristic UV absorption are still observed (see the Supporting Information). Nevertheless MS-analysis, which is more sensitive than UV spectroscopy, does not indicate any THyC or low molecular weight derivatives of this metabolite. This result is in accordance with the presence of a coumarine-based polymer in the plugs. Cross-linking based on the oxidation of hydroxylated aromatic metabolites is a common process in wounded plant tissue and also the basis for insect cuticle sclerotization. In these cases substrates and amino acids are usually in separate compartments to prevent uncontrolled reactions.[16] The process observed in *D. vermicularis* follows a novel biopolymerization regulation principle based on desulfatation of stable storage metabolites. Sulfatation is often found in nature as protection for reactive metabolites. Thus sulfatation and desulfatation processes are involved in the regulation of important cellular activities.[11] Sulfatation of THyC at the C3 position might prevent oxidation and minimize autotoxicity during regular growth. It is of note that wound-plug formation based on protein cross-linking in siphonous green algae can be mediated by fundamentally different processes. While the siphonous Caulerpales rely on a terpenoid acetate that is transformed by esterases to form a highly active protein cross-linker,[4] *D. vermicularis* accomplishes protein cross-linking in a fundamentally different manner. A further screening of siphonous algae is required for an in depth understanding of the evolution of wound-repair mechanisms which are essential for such giant-celled organisms.

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