ORGANIC LETTERS

2011
Vol. 13, No. 12
3229–3231

A Reactive Conjugated Allene Involved in the Biosynthesis of Volatile Oxylipins in the Moss *Dicranum scoparium*

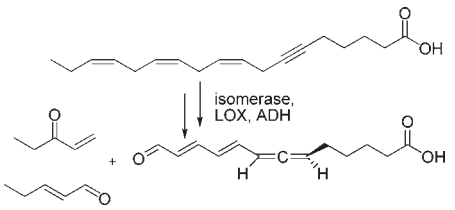
M. Rempt,[†] B. Schneider,[‡] and G. Pohnert*,[†]

Institute for Inorganic and Analytical Chemistry, Bioorganic Analytics, Friedrich Schiller University, Lessingstr. 8, D-07743 Jena, Germany, and Max Planck Institute for Chemical Ecology, Hans Knöll Str. 8, D-07743 Jena, Germany

Georg.Pohnert@uni-jena.de

Received April 27, 2011

ABSTRACT



We addressed the role of the unusual acetylenic fatty acid dicranin as a precursor for volatile oxylipins in the moss *Dicranum scoparium*. Dicranin is transformed immediately after mechanical wounding of moss tissue to volatile C5- and C6-oxylipins. The transformation of synthetic deuterium labeled dicranin was monitored using LC/MS analysis and multivariate statistics to identify polar metabolites produced during volatile formation. Among the newly formed oxylipins is a highly reactive conjugated C13 allene with similar degrees of labeling compared to the C5 volatiles suggesting that it results as second cleavage product from the biosynthesis of pentenal and pentenone.

Lipoxygenases (LOX) are central enzymes in the lipid metabolism of mammals, higher plants, algae, and mosses.[1−4] LOX introduce molecular oxygen into the homoconjugated double bond system of unsaturated fatty acids. The resulting hydroperoxides are intermediates in the formation of important signal and defense chemicals. In plants, algae, and mosses, the wound activated formation of volatiles is characteristic for the action of lipoxygenases. *Dicranum scoparium* is a moss releasing a particularly diverse blend of volatile C5, C6, and C8 oxylipins.[5] Biosynthetic investigation with stable isotope labeled ω-3 linolenic and ω-3 eicosapentaenoic acid revealed that the C8 metabolites are exclusively derived from C20 fatty acids while both C18 and C20 fatty acids are transformed to C6 volatiles.[5] Significantly lower incorporation rates of labeled precursors were observed for the C5 volatiles 1-penten-3-one **12** and 2-pentenal. The moss is particularly rich in the acetylenic C18 fatty acid dicranin **7** that can reach up to 44% of its total fatty acid content.[6] We addressed the possibility that this unusual fatty acid might serve as a precursor in the production of volatile oxylipins and explored the involved pathways. Labeled dicranin **7a,b** with deuterium at the terminus or at the 2 position was used to monitor the formation of volatiles and polar metabolites. Labeling on the fatty acid terminus was achieved by oxidative degradation of the natural product followed by partial synthesis (Scheme 1). Free and lipid bound dicranin from 5.5 kg *D. scoparium* was therefore transformed to the corresponding methyl ester, which was extracted and purified as described.[7] The methyl ester was then oxidized at the terminus based on a modified protocol developed for the oxidation of polyunsaturated fatty acids (Scheme 1).[8] In two steps 14-epoxy dicranin was obtained

[†] Friedrich Schiller University.
[‡] Max Planck Institute for Chemical Ecology.

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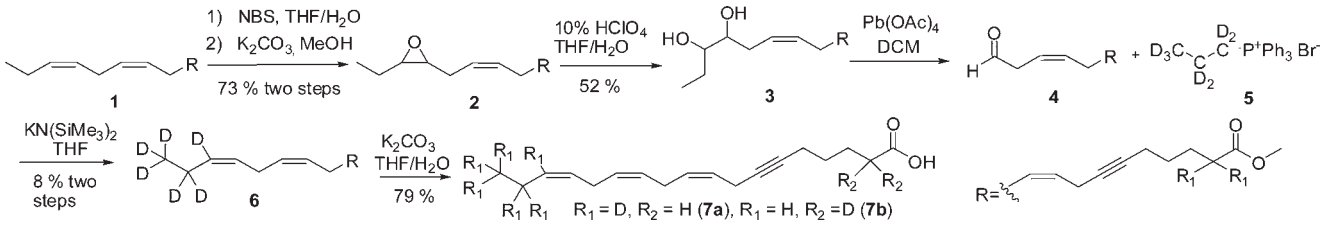
10.1021/ol201114g
Published on Web 05/23/2011

Scheme 1. Synthesis of [16,17,17,18,18,18-$^2H_6$](9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trien-6-ynoic Acid ([$^2H_6$]Dicranin) **7a**



in good yields and could be further transformed to the corresponding diol **3**. $Pb(OAc)_4$ oxidation resulted in an unstable aldehyde that was transformed without further purification by a Wittig reaction to introduce the deuterium labeled fatty acid terminus. Saponification gave [16,17,17,18,18,18-$^2H_6$](9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trien-6-ynoic acid ([$^2H_6$]dicranin **7a**).[9] [2,2-$^2H_2$] (**7b**) was generated according to published procedures.[7] Both metabolic probes were applied to wounded *D. scoparium* in equal amounts, and transformation products were monitored. Volatiles were extracted by solid phase microextraction (SPME) and analyzed by GC/MS,[5] and the nonvolatile oxylipins were monitored by UPLC/MS. [$^2H_6$]dicranin (**7a**) was readily transformed if administered to mechanically wounded *D. scoparium* and deuterated C5 and C6 oxylipins were detected with a degree of labeling for 1-penten-3-one **12** of 9.6% (±1.7%), 2-pentenal of 9.6% (±3.5%), and 2-hexenal of 10.5% (±1.6%). The unusual C6 volatile 2-ethylfurane was also derived from dicranin. The identity of this compound was proven by comparison of its mass spectrum and retention time with those of a commercially available standard. We also applied the same concentration of commercially available labeled [16,17,17,18,18,18-$^2H_6$]-linolenic acid to the same batch of freshly collected moss. Here we obtained incorporation rates for pentenone and pentenal of around 5% and for hexenal of 32.5%. Apparently, the moss enzyme converts dicranin as well as linolenic and eicosapentaenoic acid[5] to volatile oxylipins with a different substrate preference.

Using a chemometric supported identification of nonvolatile fatty acid transformation products we established previously several molecules with 12 or 13 carbons that are derived from dicranin.[7] These metabolites could formally arise as second cleavage products out of the transformation of **7a** to (labeled) C5 and C6 volatiles. Interestingly, the most dominant labeled C13 metabolite identified after administration of [2,2-$^2H_2$]dicranin (**7a**) in LC/MS profiles was a highly unstable oxygenated metabolite that decomposed quantitatively within 5 h in methanol at room temperature. The observed degree of labeling recorded in ESI-MS was 18.8% (±2.8%) (see Figure 1). The UV spectrum of this metabolite (**8**) exhibited an unstructured band at 327 nm, which is indicative of an aldehyde or ketone conjugated to a double bond system. For structure elucidation 6.7 kg of the moss were worked up and extracted under mild conditions to avoid decomposition of the reactive metabolite. Several measures to optimize the workup procedure had to be taken until 7 mg of the metabolite could be isolated (see Supporting Information). The high resolution MS data lead to the molecular formula $C_{13}H_{16}O_3$. Further structure elucidation was based on 1D and 2D NMR experiments. The $^1H,^1H$−COSY spectrum allowed us to follow the entire spin system from C2 to C13, which enabled us to assign the conjugated dienal, a neighboring allene, and the aliphatic protons. HMBC correlations of the carbon atom at 210 ppm with 5-CH_2 and 9-CH allowed us to assign the location of the allene (see Supporting Information). The coupling constants of $J(9,10)$ = 15.0 Hz and $J(11,12)$ = 15.2 Hz proved the *trans*/*trans*-double bond geometry.[10] The stereochemistry of the allene group was assigned by comparison of the obtained circular dichroism spectra (CD) with literature data and calculated values (Supporting Information).[11,12]

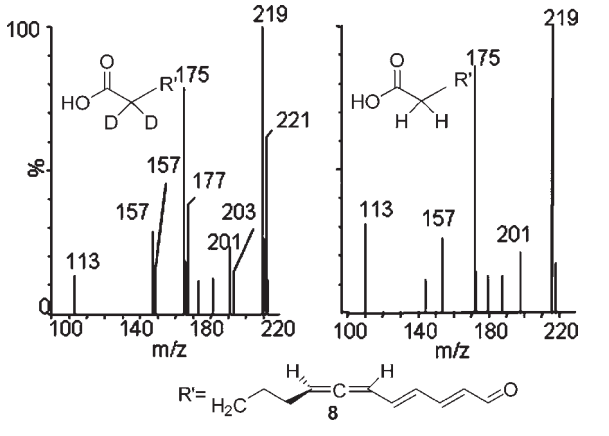


Figure 1. ESI MSMS spectra of deuterated (left) and unlabeled (right) (*S*,9*E*,11*E*)-13-oxotrideca-6,7,9,11-tetraenoic acid **8**.

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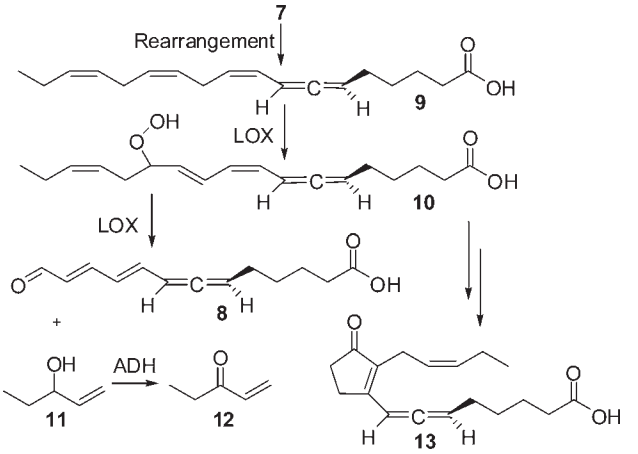
More than 200 allenic natural products have been described, but to our knowledge this is the first one with an aldehyde in conjugation, which results in a highly activated structure.[13] The reactive novel oxylipin contains a Michael acceptor motif, which makes it a likely candidate for a metabolite involved in chemical defense.[14] Interestingly, the crude extract of *D. scoparium* exhibits a pronounced anti feeding activity against slugs, which cannot fully be explained by the activity of the sum of purified components.[7] But due to its reactivity a testing of **8** in feeding assays that last over several hours is not feasible.

A proposed biosynthetic pathway could be initiated by an isomerase acting on **7** (Scheme 2). Such isomerase activity was previously postulated by Davies and Hodge for the formation of allenes from acetylenic fatty acids in fungi.[15] The resulting allene **9** could then be transformed by a lipoxygenase and an alcohol dehydrogenase[16] to give **8** and **12**. Such a 13-lipoxygenase-mediated transformation of **9** could be supported by the formation of the energetically favored conjugated system. Multifunctional lipoxygenases that can catalyze both the introduction of molecular oxygen into the fatty acid and the further transformation to shorter chain length products have been previously described from mosses.[17] Alternatively, the sequence of isomerase/lipoxygenase action could be reversed with the hydroperoxide as the first intermediate that is then converted to the allene **10**. It is also open at this stage if additional enzymes are required for the cleavage of the intermediate hydroperoxide **10**. The formation of C6 volatile oxylipins could accordingly be explained with the concomitant release of previously described dicranin **7** derived C12 oxylipins.[7] In addition to the volatile and short chain oxylipins we found a high degree of labeling in the cyclopentenones dicranenone A 34.1% (±5.8%) and dicranenone B_1 (**13**) 29.2% (±4.2%) after application of $[^2H_2]$dicranin **7b**. This suggests that the allene **9** is also converted to the cyclic C18 oxylipin **13**. It is remarkable that all described oxylipins are formed within 5 min after wounding of the moss. Even the complex transformations leading to allenic cyclopentenones are thus only dependent on enzymes that do not require the intact cellular matrix for their action. Most likely a highly active enzyme cascade, involving isomerases, lipases, lipoxygenases, and reductases, is active in the wounded moss, releasing initially dicranin **7** from lipids and then transforming it to various oxylipins of which at least some have already been identified as active defense metabolites.[7]

Scheme 2. Proposed Biosynthetic Transformation of Dicranin **7**[a]



[a] Note: The sequence of isomerase and LOX could also be reversed.

Acknowledgment. We thank the German Research Foundation (PO 628/6-1) for funding. Financial support by the Volkswagen Foundation is acknowledged. P. Dabrowska and S. Bartram are acknowledged for CD measurements

Supporting Information Available. Experimental procedures and full spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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