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Intracellular Compartmentation in the Biosynthesis of Caulerpenyne: Study on Intact Macroalgae Using Stable-Isotope-Labeled Precursors

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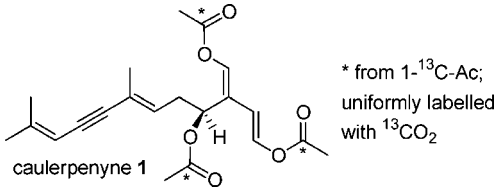
ABSTRACT



The biosynthesis of caulerpenyne 1 was studied in the invasive green alga *Caulerpa taxifolia*. The investigation was performed on intact algae with stable-isotope-labeled precursors administered under mixotrophic growth conditions. According to the labeling pattern, after incorporation of 1-^{13}C-acetate and $^{13}CO_2$, respectively, the biosynthesis of the sesquiterpene backbone occurs in the chloroplast and follows the methyl-erythritol-4-phosphate (MEP) pathway. In contrast, the acetyl residues of caulerpenyne 1 are derived from a cytosolic resource.

The invasive tropical green alga *Caulerpa taxifolia* spread rapidly after its accidental introduction into the Mediterranean and was recently also detected on the North American Pacific coast.[1,2] By outcompeting the natural flora it causes massive alterations of the ecosystem, densely covering large areas of the coastal regions.[3] Introduced *C. taxifolia* reduces the species diversity and also threatens fishery yields in the regions of introduction.[3,4] The alga's success is attributed to its high temperature and substrate tolerance but also to efficient chemical defense.[5] This is assumed to be based mainly on the sesquiterpene caulerpenyne **1** or caulerpenyne-derived degradation products released after wounding of Caulerpales.[6,7] Caulerpenyne **1** is the major secondary metabolite of *C. taxifolia* and can account for more than 1.3% of the wet weight of the alga.[7] Until now, nothing was known about the biosynthesis of this dominant sesquiterpene.

Since the discovery of the coexistence of two different pathways towards isoprenoids, numerous studies have addressed the metabolic source of the central intermediate isopentenyl-pyrophosphate in different organisms.[8] We have now a broad knowledge about the distribution of the mevalonate (MVA) and methyl-erythritol-4-phosphate **2** (MEP) pathway in different phyla, but few studies have addressed the origin of terpenes in marine macroalgae. This is partly due to the difficulties arising out of the morphology of macroalgae. In contrast to vascular higher plants, where externally applied precursors are often taken up readily, the incorporation of metabolites into the nonvascular macroalgae has rarely been achieved.[9] Thus, most studies of macroalgal

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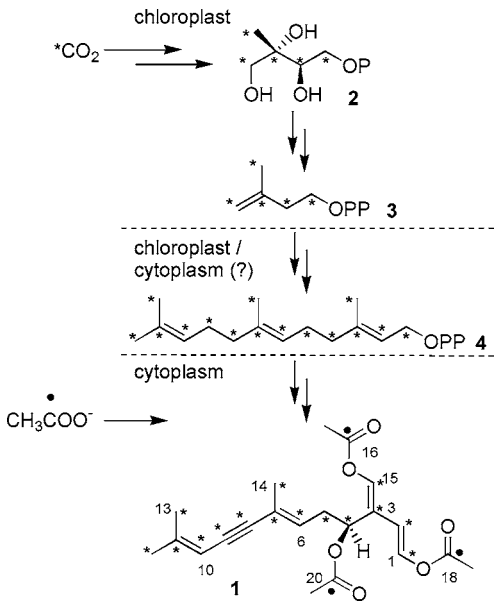
secondary metabolites have focused on the investigation of cell-free preparations,[10] suspension cultures,[11] or reproductive cells that have not yet developed a tough cell wall.[12] The siphonous green alga *C. taxifolia* represents an exception among the generally autotrophic macroalgae as it has been demonstrated with radioactive tracers that it is capable of taking up organic precursors from the aqueous environment.[13] We show that this property can be exploited to investigate the biosynthesis of secondary metabolites in intact *C. taxifolia*. The incorporation of stable-isotope-labeled precursors into caulerpenyne **1** with a significant degree of labeling made it possible to deduce the early steps of isoprenoid biosynthesis in this alga and to identify cellular resources for the acetyl moieties of the metabolite.

For incorporation of 1-^{13}C-acetate, a unialgal *C. taxifolia* isolate kept in artificial seawater was used. Experiments supplying externally labeled precursors under normal growth conditions[6] failed even with DMSO-pretreated algae, as did microinjection experiments of labeled metabolites. Because mixotrophic uptake was reported mainly under low light conditions over the rhizoids (filamentous, unpigmented structures of the alga),[13] these were enclosed in plastic tubes filled with a 1 mg mL^{-1} solution of 1-^{13}C-acetate in seawater. Enclosure of rhizoids proved beneficial because it reduced the amount of labeled precursor needed and did not promote bacterial growth due to exposure of the entire algae to increased nutrient levels in the growth medium. Algae with treated rhizoids were put into aerated aquaria and grown under a 1 h/23 h light/dark regime for 1 week before workup.

Caulerpenyne isolation and purification followed an established procedure,[7a] and incorporation of label was monitored by ^{13}C NMR in comparison with an unlabeled standard. Significant label incorporation was found only for the C1-carbon atoms of the acetyl residues of caulerpenyne (**1**), and no enrichment of ^{13}C signals was observed in the terpene backbone (Scheme 1).[14] This proves clearly that labeled acetate was taken up by the macrophyte and incorporated into caulerpenyne **1**. The precursor nevertheless did not serve as a building block in the terpene biosynthesis as would have been expected for metabolites produced via the MVA pathway.[8] Since incorporation of later intermediates of the respective pathways to terpenes failed presumably as a result of extensive bacterial metabolism in the growth medium, the uptake of $^{13}CO_2$ was monitored to get further insight into the biosynthesis of caulerpenyne **1**. Fragments of *C. taxifolia* were transferred into fresh medium, saturated with $^{13}CO_2$. After 7 days under standard growth conditions the algae were worked up, and the incorporation of label

Scheme 1. Biosynthesis and Labeling Pattern of Caulerpenyne **1**



was verified with GC−MS. High incorporation rates of $^{13}CO_2$ were observed. Besides the signals from unlabeled caulerpenyne **1**, presumably generated before the administration of $^{13}CO_2$, clusters of ions corresponding to labeled caulerpenyne **1** can be detected in the mass spectrum (Figure 1). The characteristic fragmentation pattern of caulerpenyne **1** made it possible to assign the incorporation rates for both the terpene backbone and the acetyl residues separately. Caulerpenyne ($M^+ = 374$) loses the three acetyl groups under EI conditions to form a $C_{15}H_{16}O$ fragment ion at $m/z = 212$ corresponding to the monooxygenated sesquiterpene unit.[15] In the labeled sample a cluster of additional ions can be observed between masses 223 and 228. This arises from the terpene backbone of caulerpenyne **1** produced during the incubation with $^{13}CO_2$. Modeling of the mass spectra made it possible to deduce an incorporation of 88% $^{13}CO_2$ (only the degree of labeling for caulerpenyne that was generated after treatment with $^{13}CO_2$ was calculated for the evaluation of the incorporation success) (Figure 1). This value was also found for other fragment ions from the terpene backbone of **1**. It can be clearly deduced from the C_2H_3O fragment that stems from the acetate units of caulerpenyne[15] that $^{13}CO_2$ was significantly less incorporated into acetate compared to the terpene moiety of caulerpenyne (76% of ^{13}C) (Figure 1).[16] This preferential incorporation of CO_2 as carbon source into terpenes is indicative for the biosynthesis via the MEP pathway[17] and confirms the finding after administration of labeled acetate. According to the general model the MEP-dependent biosynthesis occurs in the chloroplasts where CO_2 is fixed by photosynthesis (Scheme 1). In contrast to the

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(14) C16, 12.5%; C18, 9.1%; C20, 39.6% apparent enrichment in ^{13}C. The high enrichment of C20 might be explained with the reversible acetylation/deactetylation of **1** with an esterase[6] in the presence of elevated internal 1-^{13}C-acetate levels. Because of enolization this reversible process cannot occur with the other acetyl residues.
(15) Fragment ions were verified by high-resolution GC−MS (43, C_2H_3O; 44, $^{12}C^{13}CH_3O$; 45, $^{13}C_2H_3O$; 212, $C_{15}H_{16}O$).
(16) Mass spectra were modeled using the Bernoulli scheme (Zachmann, H. G. *Mathematik für Chemiker*, 5th ed.; VCH: Weinheim, 1994).
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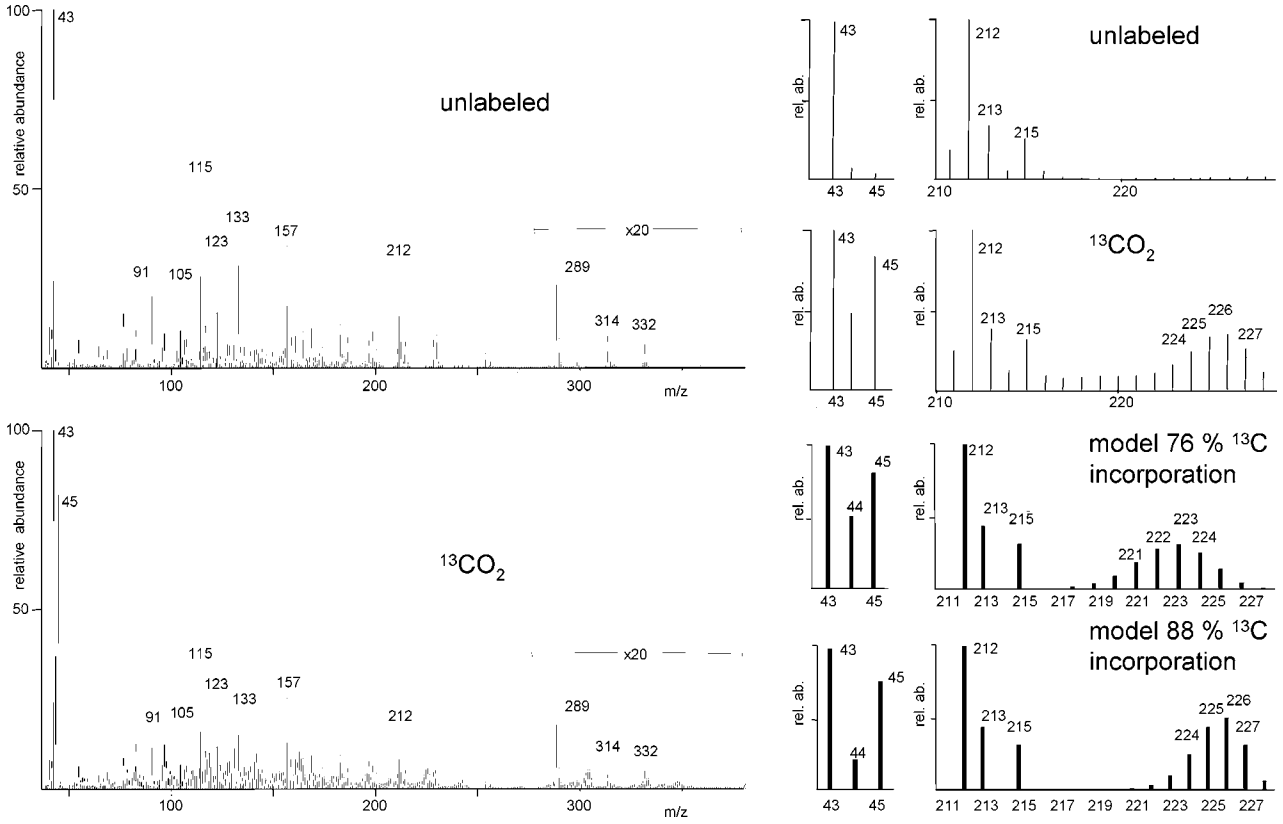

Figure 1. (Left) Mass spectra of unlabeled (top) and labeled caulerpenyne **1** extracted after treatment of the alga with $^{13}CO_2$ (below). (Right) Regions of the mass spectra corresponding to acetyl ($m/z = 43$) and terpene ($m/z = 212$) fragments (top). Modeled mass spectra[16] for C_2H_3O from acetate and $C_{15}H_{16}O$ from the terpene backbone with 76% and 88% ^{13}C incorporation, respectively (below; incorporation refers to the labeled cluster only; unlabeled signals that arise from **1** present before $^{13}CO_2$ treatment are not included in the calculation).

biosynthesis of the terpene moiety of **1**, our findings indicate that acetate from caulerpenyne **1** is cytosol-derived, since it shows lower degree of labeling after $^{13}CO_2$ treatment but is labeled when externally applied ^{13}C-acetate is taken up. Accordingly, biosynthesis of caulerpenyne requires isopentenyl-pyrophosphate **3** or farnesyl-pyrophosphate **4** synthesized in the chloroplast, whereas the introduction of acetyl groups as further tailoring reactions relies on cytoplasm-derived acetate (Scheme 1).

Caulerpa taxifolia uses thus the MEP pathway for the biosynthesis of its major sesquiterpene caulerpenyne **1**. The alga does not follow the general route of sesquiterpene biosynthesis observed in higher plants, which occurs in the cytoplasm via the MVA pathway.[8] In a survey of green microalgae it was found that usage of the MEP pathway for the biosynthesis of terpenes was a common feature of the chlorophyta investigated.[18] Our study shows that this pathway is also the only source for the biosynthesis of the sesquiterpene backbone of caulerpenyne **1** in the green macroalga *C. taxifolia* but that subsequently resources from different cellular locations are used for the introduction of acetyl functionalities into caulerpenyne **1**. To our knowledge, the methods applied here allowed for the first time the investigation of the biosynthesis of macroalgal secondary metabolites in intact adult organisms using stable isotope incorporation.

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