How to cite: *Angew. Chem. Int. Ed.* **2022,** *61,* e202211285
International Edition: doi.org/10.1002/anie.202211285
German Edition: doi.org/10.1002/ange.202211285

VIP **Renewable Resources** **Very Important Paper**

In Vivo Olefin Metathesis in Microalgae Upgrades Lipids to Building Blocks for Polymers and Chemicals

Natalie S. Schunck and Stefan Mecking**

Abstract: Sustainable sources are key to future chemicals production. Microalgae are promising resources as they fixate carbon dioxide to organic molecules by photosynthesis. Thereby they produce unsaturated fatty acids as established raw materials for the industrial production of chemical building blocks. Although these renewable feedstocks are generated inside cells, their catalytic upgrading to useful products requires in vitro transformations. A synthetic catalysis inside photoautotrophic cells has remained elusive. Here we show that a catalytic conversion of renewable substrates can be realized directly inside living microalgae. Organometallic catalysts remain active inside the cells, enabling in vivo catalytic olefin metathesis as new-to-nature transformation. Stored lipids are converted to long-chain dicarboxylates as valuable building blocks for polymers. This is a key step towards the long-term goal of producing desired renewable chemicals in microalgae as living "cellular factories".

Introduction

To achieve carbon neutral production schemes, renewable feedstock sources are a key for the production of chemicals. Microalgae are a particularly promising renewable resource as they fixate atmospheric carbon dioxide to organic compounds by photosynthesis, with sunlight as energy and without the need for additional carbon sources. Unlike higher plants, microalgae do not require arable land and fresh water resources, but can be cultivated with minimal space requirements also in brackish or salt water. By photosynthesis, microalgae build up fatty acids as intracellular storage substances. This photoautotrophic generation of lipids is one of many examples of the extreme efficiency of the chemical machinery of cells. Yet, the scope of cellular chemistry is restricted and synthetic catalysts can allow for transformations complimentary to those found in nature. Traditionally, such synthetic reactions are carried out in vitro. Thus, established schemes for the valorization of the naturally produced lipids as feedstocks for chemicals and polymers employ tedious and energy-consuming extraction, the extracts being catalytically upgraded separate from the cellular sources of the substrates. Implementing a bioorthogonal synthetic catalysis in vivo could be a key step to enable new concepts for producing renewable-sourced chemicals directly in "cellular factories". However, a synthetic catalysis inside living photoautotrophic cells has remained elusive to date.

Unicellular microalgae can generate high amounts of unsaturated fatty acids. Such fatty acids, sourced today from palm oil or other seed oils, are an important feedstock of the chemical industry.[1] For their conversion to desirable products, catalytic olefin metathesis has emerged as an advanced industrial process. The redistribution of fragments of carbon-carbon double bonds of unsaturated substrates (cf. Figure 1a) in olefin metathesis occurs by a unique mechanism,[2] unparalleled in transition metal as well as biocatalysis. The versatility and synthetic capability of olefin metathesis have opened a wide field of applications.[1,3] Especially during the past decade, the conversion of renewable raw materials by olefin metathesis has been implemented industrially for the production of biodiesel, polymers or chemical intermediates.[1,3c]

Despite its broad range of applications in vitro, only two examples of olefin metathesis in living cells have been reported: Michel et al. used metathesis catalysts as sensors for the detection of ethylene in *C. reinhardtii*, aiming at a stoichiometric rather than a catalytic process, releasing a fluorophore via one cycle of metathesis with ethylene.[4] Additionally, Ward et al. assembled artificial metalloenzymes in the periplasm of *E. coli*.[5] The activity of these enzymes was confirmed by ring-closing metathesis of an added reactive substrate that generates a fluorescent product, which enables detection of conversion by virtue of a high sensitivity. In contrast to *E. coli* cells, microalgae can serve as source of valuable fatty acid feedstocks that provide substrates for olefin metathesis and can thereby be converted into the desired renewable chemicals. However, microalgae are unicellular eukaryotic organisms that are surrounded by a complex cellular barrier consisting of a cell wall and membrane. Therefore, intracellular uptake of catalysts into these organisms is much more challenging than for the highly permeable bacterial cells. Notably, neither catalytic olefin metathesis, nor any other type of synthetic

[*] N. S. Schunck, Prof. Dr. S. Mecking
Department of Chemistry, University of Konstanz
Universitätsstrasse 10, 78464 Konstanz (Germany)
E-mail: natalie.schunck@uni-konstanz.de
stefan.mecking@uni-konstanz.de

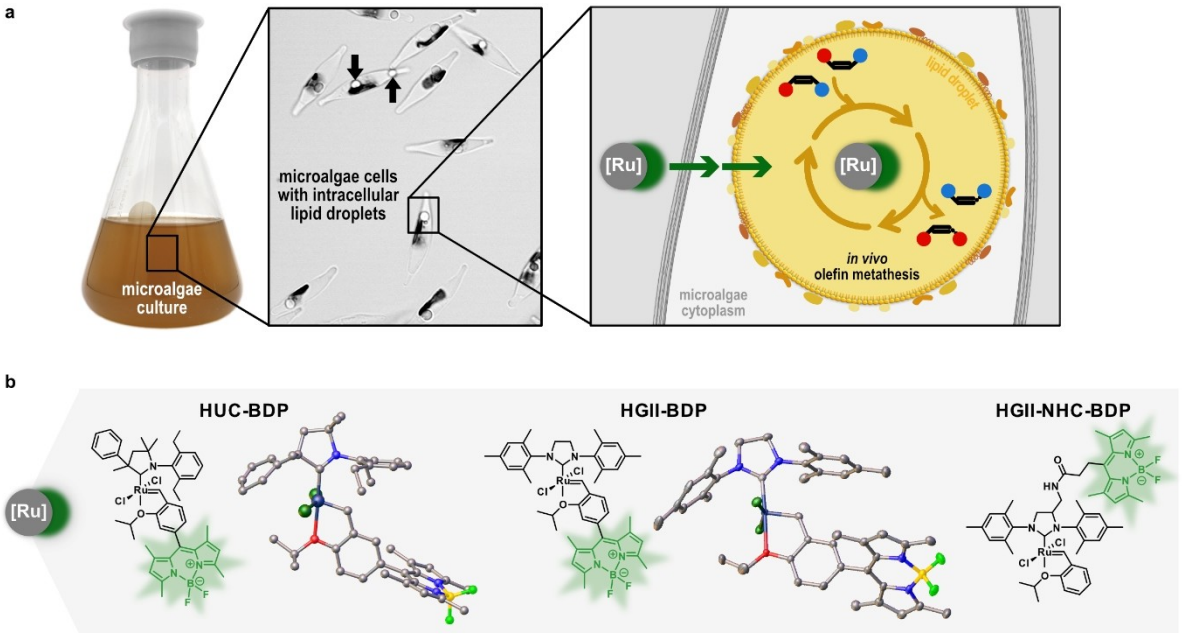

Figure 1. Concept of the in vivo synthetic olefin metathesis in microalgae. a) In microalgae cells, unsaturated fatty acids susceptible to olefin metathesis are stored in intracellular organelles, so-called lipid bodies. These compartments are surrounded by a phospholipid monolayer with associated proteins (depicted in shades of yellow). For intracellular catalysis, olefin metathesis catalysts need to be internalized by the cells through their cell wall and membrane and targeted to these intracellular lipid organelles while retaining their catalytic activity. b) Olefin metathesis catalysts applied in this work are modified at the benzylidene ligand (HUC-BDP and HGII-BDP) or at the N-heterocyclic carbene ligand (HGII-NHC-BDP) by tagging with a green fluorescent BODIPY dye as transport and labelling agent. Solid-state structures of HUC-BDP and HGII-BDP obtained from single crystal X-ray diffraction, hydrogen atoms are omitted for clarity.

catalysis has to date been realized in living photoautotrophic cells producing renewable feedstocks. This is however a key prerequisite for the aforementioned vision of cellular factories producing renewable chemicals.

Therefore, this work aims at the ambitious implementation of catalytic olefin metathesis in photoautotrophic microalgae. We report that small molecule[1] organometallic catalysts can cross the cell wall of unicellular microalgae and remain active in the intracellular environment, enabling in vivo catalytic olefin metathesis as new-to-nature transformation. This converts stored fatty acids to desirable non-natural long-chain alkenes and dicarboxylates with high conversion.

Results and Discussion

Catalyst Design and Cell Viability

Inside the microalgae, the unsaturated fatty acid components are stored in specific organelles surrounded by a phospholipid monolayer with associated proteins, so-called lipid bodies or lipid vesicles (Figure 1a). For intracellular catalysis of the valuable fatty acid substrates, the olefin metathesis catalyst has to be transported past the cellular barrier (wall and membrane), through the whole aqueous cytoplasm as intracellular medium containing potentially detrimental components, to these lipid storage compartments. Since the fatty acid substrates for the conceived in vivo olefin metathesis approach are stored in these intracellular organelles, the lipid bodies were identified as target for the abiotic intracellular reaction.

To enable intracellular uptake and targeting to these lipid organelles,[6] we furnished single component metal alkylidene catalysts with lipophilic fluorescent BODIPY (boron difluoride-dipyrromethene) motifs (Figure 1b). For many other intracellular organelles like mitochondria or the nucleus, targeting motifs are known that direct substances to this specific location. For example, a triphenylphosphonium group on a compound of interest leads to a directed transport of this substance into mitochondria. However, no such targeting signal has yet been identified for the lipid bodies of interest in this work.[7]

The choice of BODIPY motifs was therefore based on their use for staining microalgal lipid organelles for the purpose of lipid quantification and of studying lipid body growth and dynamics by microscopy.[8]

Ruthenium-based and phosphine-free catalysts were employed due to their general tolerance towards functional groups as well as their anticipated cell compatibility.[9] The state-of-the-art cyclic alkyl amino carbene (CAAC) catalyst motif was endowed with a BODIPY vehicle (HUC-BDP, Figure 1b) via ligand exchange of the chloride-bridged dimer[10] with a BODIPY-substituted styrene (for synthesis

[1] As opposed to macromolecular catalytic systems like metalloenzymes or complexes embedded in polymeric scaffolds.

and characterization data see the Supporting Information Figure S1–S5). As a reference, the well-established benchmark Hoveyda-Grubbs II N-heterocyclic carbene (NHC) catalyst motif[11] was also employed (HGII-BDP[4] and HGII-NHC-BDP, Figure 1b, synthesis and characterization see Supporting Information Figure S6–S13). For the NHC-type complexes a fluorophore tag was introduced on the benzylidene as well as the NHC ligand, since only the latter remains strongly coordinated to the ruthenium center throughout the entire catalytic cycle of olefin metathesis (see below, cf. Figure 3b). The catalytic performance of these modified catalysts was confirmed by in vitro experiments with extracted microalgae oil as a test substrate[12] (Supporting Information Figure S16). All complexes are active for self-metathesis, with a decreased activity of HGII-BDP vs. its non-modified parent analogue while for HUC-BDP the activity is increased compared to the parent unmodified cyclic amino alkyl carbene-substituted catalyst (Supporting Information Table S5).

As model organism, the unicellular microalga *Phaeodactylum tricornutum* is widely used in research on renewable feedstocks due to its high growth rates, robust cultivation with minimum space requirements and production of high amounts of fatty acids, therefore being a promising target for in vivo catalysis. Please note that although this microalga is a diatom, no siliceous cellular barrier is present in these organisms if cultivated in absence of an appropriate Si source, as performed in this work (see Supporting Information for details on cultivation conditions).[13] For the approach pursued here, tolerance of the microalgae towards the catalyst is essential. The viability of *Phaeodactylum tricornutum* cells exposed to the catalysts was studied by MTT assays (colorimetric viability assays based on methylthiazol tetrazolium bromide). No adverse effect on microalgal cell viability was found for HUC-BDP or HGII-NHC-BDP (Figure 2a), while for unmodified HGII and HGII-BDP slightly reduced cell viability was observed. The modification of the benzylidene ligand with BODIPY does not affect cell viability (HGII and HGII- BDP comparable), whereas the nature of the NHC/CAAC ligand appears to have a decisive influence. Catalysts with carbene ligands differing from the parent HGII complex (HUC-BDP and HGII-NHC-BDP) show significantly higher cell viabilities compared to catalysts with unmodified carbenes (HGII and HGII-BDP), which is in accordance with previous findings for other NHC-Ru complexes where the structure of the NHC ligand was identified as a decisive factor for viability of mammalian cells.[14] In microalgae, the HUC-BDP catalyst is particularly well tolerated and does not adversely affect cell viability over a broad range of concentrations, an effect also confirmed for prolonged incubation (Supporting Information Figure S18).

Catalyst Uptake and in Cell Catalytic Activity

To investigate the intracellular catalytic activity of the labelled metathesis catalysts in the microalgae cells, an established pro-fluorescent substrate was chosen (Figure 2b, black).[15] This non-fluorescent precursor is very reactive in ring-closing metathesis, yielding umbelliferone as product (Figure 2b, blue) whose formation can readily be monitored via fluorescence intensities. For catalyst uptake, the microalgae were incubated with aqueous catalyst solutions (containing 0.5 v % of water-miscible co-solvent to dissolve the catalyst) and washed thoroughly prior to addition of the non-fluorescent substrate to avoid formation of fluorescent product by potential extracellular catalyst in the surrounding medium (cf. Supporting Information for details). Monitoring of the fluorescence intensity over time reveals an increase for microalgae precedingly incubated with catalyst, relative to control samples without added catalyst (Figure 2b, results

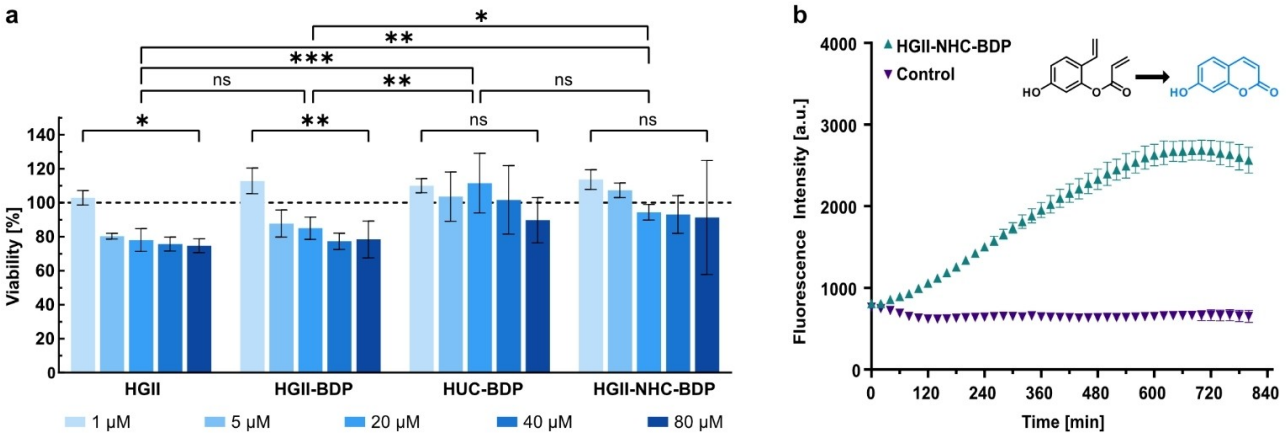

Figure 2. Cell viability and in cell catalytic activity of modified olefin metathesis catalysts. a) Cell viability of microalgae treated with BODIPY-labelled catalysts. Determined by MTT assays, referenced to controls without added catalyst. b) Intracellular catalytic activity of labelled catalysts. For cells incubated with olefin metathesis catalysts, fluorescent umbelliferone is formed as indicated by an increase in fluorescence intensity over time. Ex.: 322 nm, Em.: 440 nm. Error bars represent mean ± standard deviation of three independent biological replicates (N = 3). Two-way ANOVA (with Tukey's multiple comparisons test for viability assays). $p < 0.0002$ (***), $p < 0.0021$ (**), $p < 0.0332$ (*), $p > 0.1234$ (ns); p-values < 0.0001 for catalytic activity (relative to control sample with substrate only, no catalyst).

for HGII-NHC-BDP, other catalysts see Supporting Information Figure S21). The olefin metathesis catalysts promote ring-closing metathesis in microalgae cells, resulting in the formation of the fluorescent umbelliferone. Notably, even though olefin metathesis catalysts are well known to be sensitive to cellular components like water and nucleophiles,[16] the modified catalysts remain active in the intracellular environment of microalgae in the presence of the manifold cellular components.

Elucidation of the uptake and localization of the catalysts by confocal fluorescence microscopy reveals that the BODIPY-modified catalysts are internalized by the microalgae cells. No additional permeabilization is required and the catalysts are located inside the intracellular lipid bodies (Figure 3a HUC-BDP and HGII-NHC-BDP, green: fluorescence of catalysts, red: chlorophyll autofluorescence, for HGII-BDP and comparison with the free BODIPY dye see Supporting Information Figure S22). The intracellular lipid organelles treated with HGII-BDP show a higher fluorescence intensity compared to lipid bodies with HGII-NHC-BDP (Supporting Information Figure S23), which is expected taking into account the catalytic cycle of olefin metathesis (Figure 3b).[2] The fluorescence of the BODIPY-modified catalyst precursors is quenched due to the vicinity of the dye moiety to the ruthenium center.[17] Catalyst initiation by reaction with olefinic substrates results in the release of the labile benzylidene ligand and therefore in higher fluorescence intensities for benzylidene-modified catalysts like HGII-BDP. In contrast, in HGII-NHC-BDP the modified NHC ligand is strongly coordinated to the ruthenium center throughout the entire catalytic cycle. Therefore, particularly the observation of intracellular fluorescence for this permanently labelled HGII-NHC-BDP (Figure 3a), showing the same distribution as the HUC-BDP catalyst, clearly demonstrates catalyst uptake into the lipid storage compartments.

Olefin Metathesis of Intracellular Lipid Substrates

With the catalysts taken up in lipid bodies, they are localized in the storage compartment of intracellular fatty acids. In the investigated microalga *Phaeodactylum tricornutum*, mainly unsaturated palmitoleic acid (16:1), oleic acid (18:1) and eicosapentaenoic acid (20:5) are stored in these lipid compartments (Figure 4a, black), as well as saturated fatty acids not susceptible to olefin metathesis (myristic (14:0) and palmitic acid (16:0), grey in Figure 4a). Conversion of the unsaturated compounds via self-metathesis would result in 7-tetradecene (A14:1), 7-hexadecene (A16:1) and 9-octadecene dioate (DE18:1) as main products (Figure 4a, blue).

To probe the catalytic activity of the internalized catalysts for the conversion of these intracellular substrates, the cellular components were extracted after catalyst incubation to verify product formation (note that the stored fatty acids and their corresponding self-metathesis products do not possess any tags for in vivo investigation). Gas chromatographic analysis of microalgae treated with HUC-BDP catalyst reveals substantial formation of the self-metathesis products of the intracellular fatty acids (Figure 4b, cf. Supporting Information section 7 for full exper-

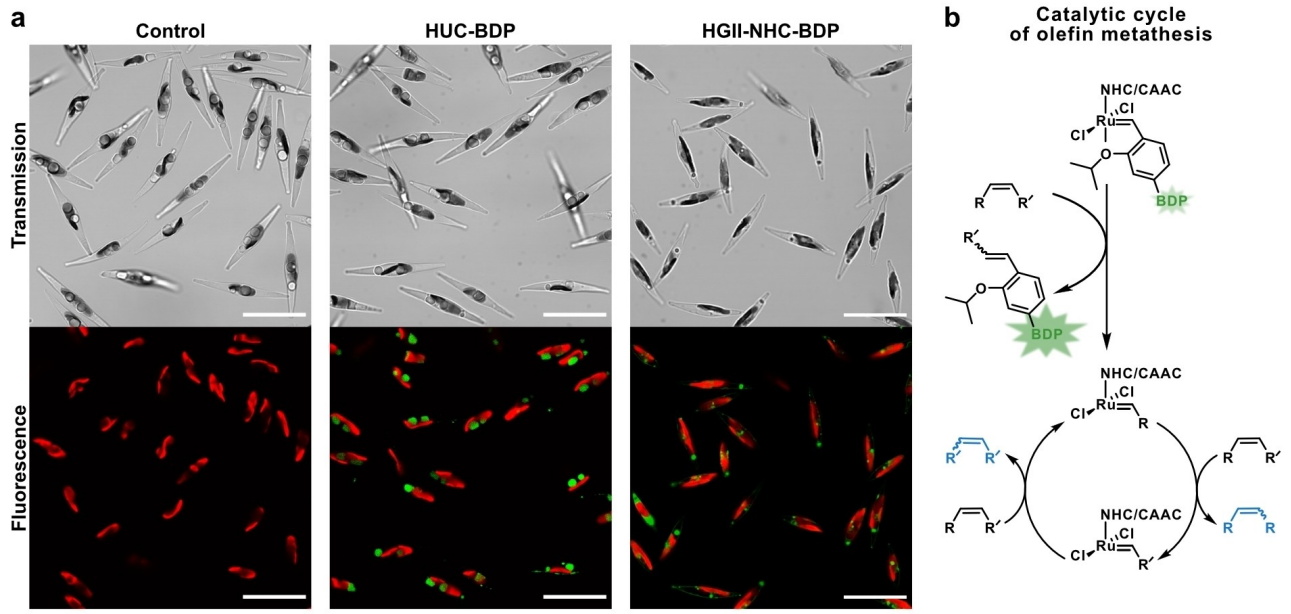

Figure 3. Cellular uptake of modified olefin metathesis catalysts. a) Microalgae cells of *Phaeodactylum tricornutum* (Control) treated with modified olefin metathesis catalyst HUC-BDP and HGII-NHC-BDP. Green = BODIPY, red = chlorophyll, scale bar = 20 µm, Ex.: 488 nm, brightness and contrast adjusted on each color channel individually. b) Mechanistic scheme of initiation and catalytic cycle of olefin metathesis with HGII/HUC catalysts labelled at the benzylidene ligand. The BODIPY fluorescence is quenched by the proximity of the dye to the ruthenium center and upon initiation of the catalyst the BODIPY is released and the fluorescence quantum yield increased. Green = fluorescence, blue = olefinic substrate, for example intracellular unsaturated fatty acids.

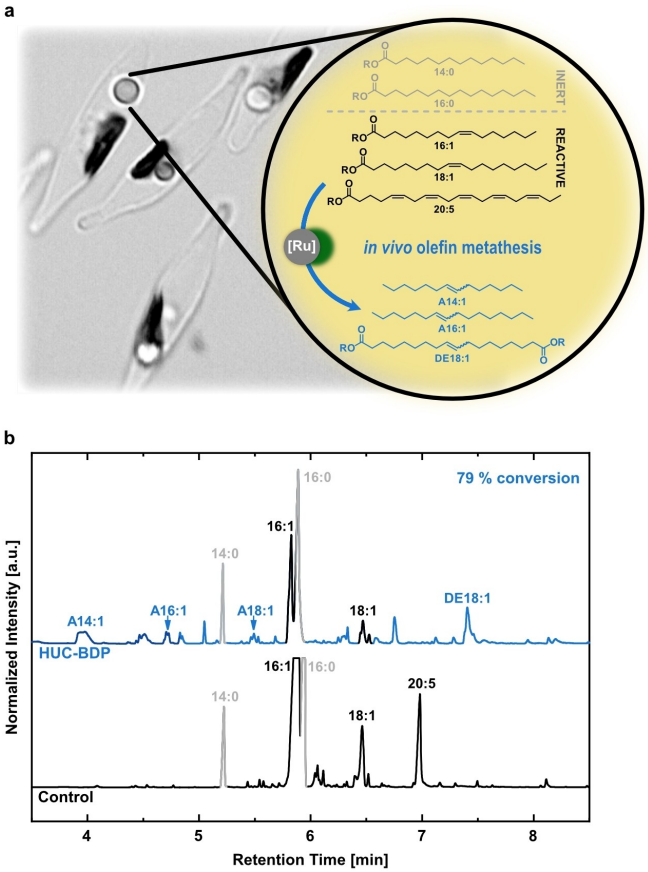

Figure 4. In vivo olefin metathesis in living photoautotrophic microalgae for the conversion of intracellular substrates. a) Unsaturated fatty acids in the lipid bodies of *Phaeodactylum tricornutum* microalgae are converted into value-added chemicals by olefin metathesis. Saturated fatty acids myristic acid (14:0) and palmitic acid (16:0) are not susceptible to olefin metathesis (grey), whereas self-metathesis of palmitoleic acid (16:1), oleic acid (18:1) and eicosapentaenoic acid (20:5, black) yields mainly the long-chain alkenes 7-tetradecene (A14:1), 7-hexadecene (A16:1) as well as the unsaturated diester 9-octadecene dioate (DE18:1, blue). [Ru] = ruthenium-based olefin metathesis catalyst, R = triglycerides in lipid organelles (storage of fatty acids), R = Me for fatty acid methyl esters (derivatization for analysis via gas chromatography). b) Gas chromatogram of extracted microalgae after in vivo olefin metathesis (top) indicates the formation of self-metathesis products. The chromatogram of the control (bottom) is shown with relatively amplified intensity to demonstrate the absence of product peaks. 6 mol% catalyst loading, 72% conversion. Samples transesterified with MeOH/1 v% H_2SO_4 for analysis via gas chromatography.

imental details including reference experiments). All expected major products are identified in the extracts by GC-FID and GC-MS analysis (Supporting Information Figure S24–S25). Remarkably, the self-metathesis products are formed with high conversions (up to 79%, Figure 4b, for details on calculation see Supporting Information), similar to the conversions observed in in vitro self-metathesis (cf. Supporting Information). The small molecule catalysts remain active in the intracellular environment, even though the catalysts are exposed to the incubation medium, internalized by the cells, transported through the microalgal cytoplasm and targeted to the lipid body storage organelles. In terms of different catalysts, the benzylidene modified derivatives HUC-BDP and HGII-BDP show comparable in vivo catalytic activity (cf. Supporting Information), but in general the HUC-BDP catalyst is nevertheless favored due to its lower cytotoxicity (see above). The catalytic activity of HGII-NHC-BDP is lower (cf. Supporting Information), which is in accordance with in vitro self-metathesis experiments of extracted algae oil as model substrate (Table S5, entry 3 vs. 4).

Conclusion

Reported examples of in cell transition metal catalysis to date were limited to bacterial or mammalian cancer cells.[18] Unlike these cells, the unicellular microalgae studied here are autotrophically grown single cell organisms that do not require external carbon and energy sources except for carbon dioxide and sunlight. Thereby, they produce unsaturated fatty acids, compounds which serve as a feedstock today for in vitro transformations to valuable chemicals. Our findings reveal the feasibility of intracellular non-natural transition-metal catalysis in photoautotrophic organisms, and also show that unprotected small molecule olefin metathesis catalysts can operate inside cells. The complex microalgae cell walls can be overcome by the catalysts studied, and particularly the novel HUC-BDP catalyst does not adversely affect cell viability, at the same time it is also tolerant towards the cell environment. This enables high intracellular conversions of unsaturated fatty acids on par to reference in vitro transformations. The in cell olefin metathesis approach demonstrated here converts natural intracellular fatty acid substrates into value-added chemicals. Specifically, the generated dicarboxylates are valuable building blocks for polymers.[3d] They originate from efficient natural carbon dioxide fixation complemented by bioorthogonal synthetic catalysis.

The implementation of synthetic catalysis in photoautotrophic organisms demonstrated here enables a potentially game-changing approach toward a sustainable chemical industry—as a first key step towards the long-term goal of using microalgae as cell factories for the direct production of renewable chemicals. An obvious further challenge is a simultaneous release of desirable products from the living cells, by biological or simple physical pathways. We anticipate the feasibility of non-natural catalytic transformations in unicellular microalgae revealed here will additionally be inspiring and useful to other fields beyond the production of chemicals.

Acknowledgements

N.S.S. gratefully acknowledges financial support from the Carl Zeiss Foundation in form of a PhD scholarship. Support of our studies by the Federal Ministry of Education and Research (BMBF, INTEXCAT, 031B0676) is gratefully acknowledged. We thank Inigo Göttker-Schnetmann and

Peter Kroth for helpful discussions. Support with X-ray crystallographic analysis by Inigo Göttker-Schnetmann is acknowledged. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Catalysis · Homogeneous Catalysis · Polymers · Renewable Resources · Sustainable Chemistry

[1] U. Biermann, U. T. Bornscheuer, I. Feussner, M. A. R. Meier, J. O. Metzger, *Angew. Chem. Int. Ed.* **2021**, *60*, 20144–20165; *Angew. Chem.* **2021**, *133*, 20304–20326.

[2] a) P. Jean-Louis Hérisson, Y. Chauvin, *Makromol. Chem.* **1971**, *141*, 161–176; b) Y. Chauvin, *Angew. Chem. Int. Ed.* **2006**, *45*, 3740–3747; *Angew. Chem.* **2006**, *118*, 3824–3831.

[3] a) Y. Zhu, C. Romain, C. K. Williams, *Nature* **2016**, *540*, 354–362; b) X. Zhang, M. Fevre, G. O. Jones, R. M. Waymouth, *Chem. Rev.* **2018**, *118*, 839–885; c) S. Chikkali, S. Mecking, *Angew. Chem. Int. Ed.* **2012**, *51*, 5802–5808; *Angew. Chem.* **2012**, *124*, 5902–5909; d) M. Häußler, M. Eck, D. Rothauer, S. Mecking, *Nature* **2021**, *590*, 423–427; e) M. J. Benedikter, J. V. Musso, W. Frey, R. Schowner, M. R. Buchmeiser, *Angew. Chem. Int. Ed.* **2021**, *60*, 1374–1382; *Angew. Chem.* **2021**, *133*, 1394–1402.

[4] S. N. W. Toussaint, R. T. Calkins, S. Lee, B. W. Michel, *J. Am. Chem. Soc.* **2018**, *140*, 13151–13155.

[5] M. Jeschek, R. Reuter, T. Heinisch, C. Trindler, J. Klehr, S. Panke, T. R. Ward, *Nature* **2016**, *537*, 661–665.

[6] Y. Maeda, D. Nojima, T. Yoshino, T. Tanaka, *Philos. Trans. R. Soc. London Ser. B* **2017**, *372*, 16.

[7] S. Louzoun-Zada, Q. Z. Jaber, M. Fridman, *Angew. Chem. Int. Ed.* **2019**, *58*, 15584–15594; *Angew. Chem.* **2019**, *131*, 15730–15740.

[8] M. S. Cooper, W. R. Hardin, T. W. Petersen, R. A. Cattolico, *J. Biosci. Bioeng.* **2010**, *109*, 198–201.

[9] T. Matsuo, *Catalysts* **2021**, *11*, 359.

[10] D. L. Nascimento, A. Gawin, R. Gawin, P. A. Guńka, J. Zachara, K. Skowerski, D. E. Fogg, *J. Am. Chem. Soc.* **2019**, *141*, 10626–10631.

[11] S. B. Garber, J. S. Kingsbury, B. L. Gray, A. H. Hoveyda, *J. Am. Chem. Soc.* **2000**, *122*, 8168–8179.

[12] J. Zimmerer, L. Williams, D. Pingen, S. Mecking, *Green Chem.* **2017**, *19*, 4865–4870.

[13] A. D. Martino, A. Meichenin, J. Shi, K. Pan, C. Bowler, *J. Phycol.* **2007**, *43*, 992–1009.

[14] G. Lv, L. Guo, L. Qiu, H. Yang, T. Wang, H. Liu, J. Lin, *Dalton Trans.* **2015**, *44*, 7324–7331.

[15] R. Reuter, T. R. Ward, *Beilstein J. Org. Chem.* **2015**, *11*, 1886–1892.

[16] a) C. O. Blanco, J. Sims, D. L. Nascimento, A. Y. Goudreault, S. N. Steinmann, C. Michel, D. E. Fogg, *ACS Catal.* **2021**, *11*, 893–899; b) B. J. Ireland, B. T. Dobigny, D. E. Fogg, *ACS Catal.* **2015**, *5*, 4690–4698.

[17] a) J. Godoy, V. García-López, L.-Y. Wang, S. Rondeau-Gagné, S. Link, A. A. Martí, J. M. Tour, *Tetrahedron* **2015**, *71*, 5965–5972; b) P. Kos, H. Plenio, *Chem. Eur. J.* **2015**, *21*, 1088–1095.

[18] a) A. H. Ngo, S. Bose, L. H. Do, *Chem. Eur. J.* **2018**, *24*, 10584–10594; b) H. M. Key, P. Dydio, D. S. Clark, J. F. Hartwig, *Nature* **2016**, *534*, 534–537; c) Y. Liu, Y. Bai, *ACS Appl. Bio Mater.* **2020**, *3*, 4717–4746; d) M. Szponarski, F. Schwizer, T. R. Ward, K. Gademann, *Commun. Chem.* **2018**, *1*, 84; e) J. G. Rebelein, T. R. Ward, *Curr. Opin. Biotechnol.* **2018**, *53*, 106–114.

Manuscript received: August 1, 2022
Accepted manuscript online: September 5, 2022
Version of record online: October 5, 2022