CHEMPLUSCHEM
COMMUNICATIONS

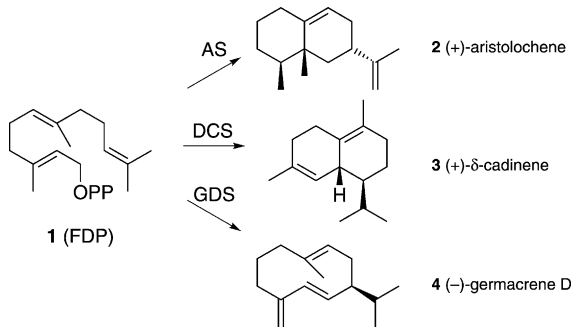
ChemPubSoc
Europe

DOI: 10.1002/cplu.201300303

Efficient Terpene Synthase Catalysis by Extraction in Flow

Oscar Cascón, Gerald Richter, Rudolf K. Allemann,* and Thomas Wirth*[a]

Sesquiterpenes are an important family of natural products that play many roles in nature. They provide the hydrocarbon scaffolds for terpenoids with medicinal and agrochemical applications. Chemical synthesis of the structurally complex hydrocarbons is usually not a viable option. In nature sesquiterpenes are biosynthesized by terpene synthases from the water-soluble substrate farnesyldiphosphate (FDP) **1** (Scheme 1). Catalysis takes place with high regio- and stereoselectivity to afford enantiomerically pure bioactive compounds; the turnover numbers of these enzymes are however usually rather low.[1] Examples are *aristocholene synthase* (AS), which is responsible for the production of the PR-toxin precursor aristolochene **2** in *Penicillium roqueforti*,[2] *δ-cadinene synthase* (DCS) from *Gossypium arboreum* synthesizing δ-cadinene **3** as the precursor of the cotton phytoalexine gossypol,[3] and *germacrene D synthase* (GDS) from *Solidago canadensis*. (−)-Germacrene D **4** has been reported to act as an alarm pheromone for several species of aphids.[4]



Scheme 1. Sesquiterpenes obtained with terpene synthases used in this study.

The synthetic application of terpene synthases such as α-pinene synthase or germacrene synthase targeting incubation and work-up conditions has been reported earlier.[5] Recently, high-throughput screening essays of terpene synthases have been developed.[6] The overall rate of the enzymatic reaction is limited by the release of the hydrophobic product into the aqueous solvent; pre-steady-state kinetic studies of trichodiene synthase revealed that the enzymatic steps are about 40 times faster than product release.[7] The solubility of monoterpenes in water is typically very low (10–30 mg L^{-1}).[8] Therefore, the aqueous incubation media will be saturated almost from the beginning of the enzymatic reaction and the rate of product formation is controlled by the mass transfer ratio between aqueous and organic phases.

The high current interest in continuous-flow processes performed in microfluidic systems[9] is driven by their advantages over classical batch approaches. Miniaturisation of flow devices allows an enhanced mass and heat transfer owing to a very large surface-to-volume ratio. Apart from organic synthetic protocols, biocatalytic reactions have been facilitated by biphasic flow. Esterifications in ionic liquid/heptane mixtures,[10] hydrolysis of esters in water/decane solvents,[11] and reductions of carbon–carbon double bonds[12] or of aldehydes[13] are accelerated in biphasic flow systems because of efficient product removal from the aqueous phase. Microwave- and ultrasound-facilitated biphasic reactions have already been reported.[14]

In this study, we present a new incubation methodology, using continuous flow with sonication that speeds up the enzymatic reaction by increasing the mass transfer between two phases of the biphasic mixture. The method has been tested with the three terpene synthases GDS, AS, and DCS. These three terpene synthases were produced in *E. coli* and purified to apparent homogeneity as described previously.[5b,15] Subsequently, the method was also used for the enzymatic conversion of FDP analogues into the corresponding modified products.

The sesquiterpene synthases used in this study require Mg^{2+} for activity. In batch experiments, the optimal metal concentrations for these enzymes were found to be in the range of 3–10 mM. These data are in agreement with previous kinetic experiments with radiolabeled FDP.[16] With FDP concentrations above 0.4 mM, an insoluble, unreactive precipitate was observed in the presence of Mg^{2+}[17] and hence a substrate concentration of 0.35 mM was selected for this study.

Initial flow experiments were performed to evaluate optimal protocols for this study. In initial flow experiments, the substrate FDP, enzyme (GDS), and pentane were injected into the flow reactor as three separate solutions. The experiment was repeated after premixing the enzyme and substrate solutions. Identical conversions to germacrene D were found in both experiments indicating that the reaction rate is solely ruled by the removal of product from the aqueous phase and that the substrate conversion in the enzyme solution is negligible. Another reaction was performed with ethylenediamine tetraacetic acid (EDTA) in the collecting vial to quench any reaction after the incubation time. The conversion in this case was also iden-

[a] O. Cascón, Prof. Dr. G. Richter, Prof. Dr. R. K. Allemann, Prof. Dr. T. Wirth
School of Chemistry, Cardiff University
Park Place, Main Building
Cardiff CF10 3AT (United Kingdom)
Fax: (+44) 29-2087-6968
E-mail: allemannRK@cf.ac.uk
wirth@cf.ac.uk
Homepage: http://www.cardiff.ac.uk/chemy/staffinfo/allemann
http://www.cf.ac.uk/chemy/wirt

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cplu.201300303.

tical with the original experiment performed without quenching indicating that any improvement observed in flow is not due to extra incubation time in the loading syringe or in the collecting vial.

The first set of reactions in flow was performed with a GDS solution at a fixed enzyme concentration of 9 μM and a substrate concentration of 0.35 mM using pentane as the organic solvent for product extraction. The diameter of the tubing reactor was changed while the tubing reactor volume (length of tubing) and the flow rate were adjusted to a 30 minute reaction time (see the Supporting Information). In reactions performed in tubing with a small internal diameter (0.175 mm) enzyme inactivation was observed. A precipitate was formed and a conversion of $<1\%$ suggested enzyme denaturation. No precipitate was observed when bigger tubing diameters were used indicating that the turbulences generated inside the tubing are able to extract the product and are mild enough to avoid enzyme denaturation. A conversion of 17% was observed with a tubing reactor of 0.45 mm internal diameter. To further improve the mass transfer between the organic and aqueous phase, the tubing reactor was immersed in a sonication bath. The conversion was increased to 52%.[18] However, when the flow rate was decreased and the reaction time increased to 1 hour, a large decrease in the conversion to only 10% was observed. The decrease in the turbulences generated inside the tubing resulting from the decrease in the flow rate lead to a greatly diminished extraction of the product and, therefore, to lower conversion. A reaction performed in a tubing reactor with 0.8 mm internal diameter led to almost the same conversion (47%) whereas a further increase in diameter to 1.6 mm resulted in a less efficient extraction and, therefore, in a decreased conversion (22%) as shown in Figure 1.

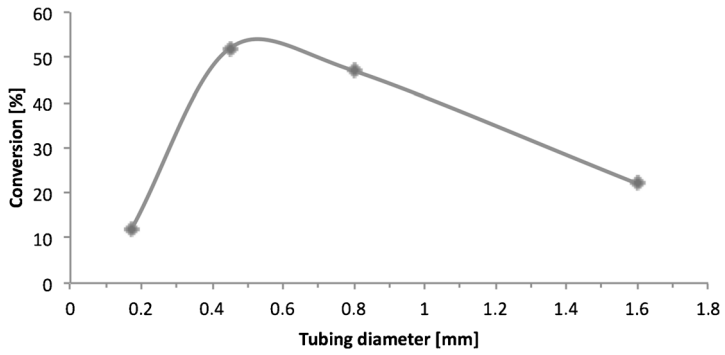


Figure 1. Conversions with different tubing internal diameters (30 min reaction time).

The general applicability of this methodology with other terpene synthases was established by extending the study to DCS and AS. These two enzymes were tested at different concentrations in batch and in flow. All experiments (reaction time: 30 min at 30 °C) were performed without sonication to determine the optimal enzyme concentration for batch and flow reactions as shown in Table 1. A setup sketched out in Figure 2 was used for the flow reactions.

All these experiments revealed an improved conversion by using a flow reactor in comparison with batch experiments.

Table 1. Conversions in batch and flow at different enzyme concentrations.

Entry	Concentration [μM] (enzyme)	Conversion batch [%]	Conversion flow [%][a]
1	6 (AS)	15	21
2	9 (AS)	27	34
3	12 (AS)	43	49[b]
4	6 (DCS)	6	14
5	9 (DCS)	11	26
6	12 (DCS)	15	42
7	9 (GDS)	24	48[c]
8	12 (GDS)	n.d.	52[c]

[a] Tubing internal diameter: 0.45 mm. [b] 21% conversion when performed at 40 °C, enzyme precipitation observed. [c] Reaction performed with sonication. n.d. = not determined.

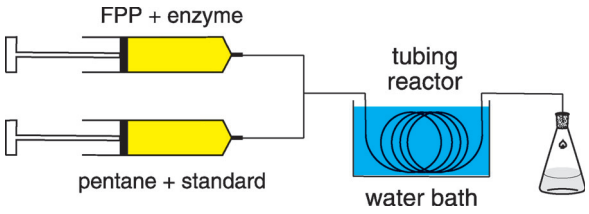


Figure 2. Flow setup for enzymatic reactions.

With AS producing (+)-aristocholene, the improvement was rather modest (Table 1, entries 1–3) whereas in the case of synthesizing (+)-δ-cadinene with DCS, the use of flow more than doubled the conversion (Table 1, entries 4–6). The batch experiments also showed enzyme aggregation at higher concentrations (Table 1, entries 5 and 6). In previous batch studies,[2] the conversion after 24 hours incubation was decreased owing to inactivation of the enzyme by aggregation. The flow approach now allows much shorter incubation times where the (slow) aggregation process is negligible. Further experiments were performed at an enzyme concentration of 12 μM using sonication and varying the tubing internal diameters, which gave the highest conversions with sonication (Table 2). For AS, almost quantitative conversion (88%) was observed with 0.8 mm tubing, meanwhile DCS performed optimally (56% conversion) with tubing of 0.45 mm internal diameter.

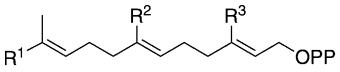
Engineering metabolic pathways in yeast have already achieved large-scale production of valuable sesquiterpene products. However, this approach uses the yeast metabolic pathways for the generation of **1** and

Table 2. Comparison of batch and flow reactions with sonication.

Entry	Conc. [μM] (enzyme)	Conversion batch [%]	Conversion flow 0.45 mm[a] [%]	Conversion flow 0.8 mm[a] [%]
1	12 (AS)	43	66	88
2	12 (DCS)	30	56	27

[a] Tubing internal diameter.

therefore only the production of natural compounds can be achieved; this is because the alkyl chains R^1–R^3 (Scheme 2) derive from acetyl-CoA.[19] Some unnatural FDP analogues have been shown to act as substrates of these enzymes generating



5 (12-Me-FDP) R^1=Et, R^2=Me, R^3=Me
6 (14-Me-FDP) R^1=Me, R^2=Et, R^3=Me
7 (15-Me-FDP) R^1=Me, R^2=Me, R^3=Et

Scheme 2. FDP analogues as substrates for terpene synthases.

a range of unnatural sesquiterpenes. Three methyl-substituted analogues were tested here with our approach to generate a library of potentially bioactive unnatural compounds. The syntheses of the FDP analogues **6** (14-Me-FDP) and **7** (15-Me-FDP) were performed as described previously.[7] The synthesis of the novel compound **5** (13-Me-FDP) is described in the Supporting Information. Using the reaction conditions identified above, the enzymes DCS and AS were used to transform the three FDP analogues **5**–**7** shown in Scheme 2. The results of these reactions are summarized in Table 3 and show that the methodology is also applicable to the conversion of FDP analogues

Table 3. Conversions in batch and flow at 12 μM enzyme concentration.

Entry	Substrate (enzyme)	Conversion batch [%]	Conversion flow [%]
1	**5** (AS)	22	63[a]
2	**6** (AS)	6	11[a]
3	**7** (AS)	9	15[a]
4	**5** (DCS)	2.5	5[b]
5	**6** (DCS)	9	14[b]
6	**7** (DCS)	1	3.5[b]

[a] Tubing internal diameter: 0.45 mm. [b] Tubing internal diameter: 0.8 mm.

into aristocholene and δ-cadinene derivatives. The difficulty with such FDP analogues in batch reactions is largely overcome because the reactions are about two to three times more efficient when performed in flow.

In summary we have shown that the use of a biphasic continuous flow system increases the enzymatic conversion of terpene synthases through improved extraction of the hydrophobic cyclic reaction products. This effect is not limited to a specific enzyme and is especially useful when the overall conversion is low as in the case of substrate analogues.

Experimental Section

Enzymatic conversion of 1 into (+)-aristolochene 2 in flow

Incubation buffer (20 mM Tris, 5 mM 2-mercaptoethanol, 15% glycerol, 3 mM $MgCl_2$, pH 7.5, 422.5 μL) with enzyme solution in buffer (Tris 20 mM, pH 7.5; 100 μM, 60 μL) and a solution of **1** (10 mM, 17.5 μL) were premixed to a final total volume of 500 μL. The mixture was placed in a 1 mL syringe. Pentane (1 mL) was spiked with a solution of α-humulene (1 mM, 35 μL) and placed in a 1 mL syringe. Both syringes were placed separately in two syringe pumps and connected via a T-piece to a tubing reactor (length: 4 m, internal diameter: 0.8 mm). The loop was immersed in a thermocontrolled sonication water bath at 30 °C. Flow rates were adjusted to a total flow rate of 66 μL min^{-1} (22 μL min^{-1} for the aqueous phase and 44 μL min^{-1} for the organic phase) leading to a residence time of 30 min. Direct analysis of the organic phase with GC analysis allowed the determination of the conversion by comparison of the product peak area with the area of the standard.

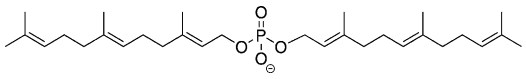
Acknowledgements

We thank Dr. Sabrina Touchet and Dr. Verónica González for the preparation of the enzymes and Dr. Rob Jenkins for assistance with the GC analysis and valuable scientific discussion. We thank the EPSRC National Mass Spectrometry Facility, Swansea, for mass spectrometric data and Cardiff University for financial support.

Keywords: continuous flow synthesis · extraction · sesquiterpenes · sonication · terpene synthases

[1] D. J. Miller, R. K. Allemann, *Nat. Prod. Rep.* **2012**, *29*, 60–71.
[2] D. Cane, *Chem. Rev.* **1990**, *90*, 1089–1103.
[3] J. A. Faraldos, D. J. Miller, V. González, Z. Yoosuf-Aly, O. Cascón, A. Li, R. K. Allemann, *J. Am. Chem. Soc.* **2012**, *134*, 5900–5908.
[4] a) R. Mozuraitis, M. Stranden, M. I. Ramirez, A. K. Borg-Karlson, H. Mustaparta, *Chem. Senses* **2002**, *27*, 505–509; b) T. J. A. Bruce, *Pest Manage. Sci.* **2005**, *61*, 1115–1121; c) M. A. Birkett, *Phytochemistry* **2008**, *69*, 1710–1715; d) J. A. Pickett, R. K. Allemann, Michael A. Birkett, *Nat. Prod. Rep.* DOI: 10.1039/C3NP70036D.
[5] a) Z. Yoosuf-Aly, J. A. Faraldos, D. J. Miller, R. K. Allemann, *Chem. Commun.* **2012**, *48*, 7040–7042; b) O. Cascón, S. Touchet, D. J. Miller, V. González, J. A. Faraldos, R. K. Allemann, *Chem. Commun.* **2012**, *48*, 9702–9704.
[6] R. Lauchli, K. S. Rabe, K. Z. Kalbarczyk, A. Tata, T. Heel, R. Z. Kitto, F. H. Arnold, *Angew. Chem.* **2013**, *125*, 5681–5684; *Angew. Chem. Int. Ed.* **2013**, *52*, 5571–5574.
[7] a) D. E. Cane, H. T. Chiu, *Biochemistry* **1997**, *36*, 8332–8339; b) J. R. Mathis, K. Back, C. Starks, J. Noel, C. D. Poulter, J. Chappell, *Biochemistry* **1997**, *36*, 8340–8348.
[8] J. D. Weidenhamer, F. A. Macias, N. H. Fisher, B. Williamson, *J. Chem. Ecol.* **1993**, *19*, 1799–1807.
[9] a) *Microreactors in Organic Synthesis and Catalysis* (Ed.: T. Wirth), Wiley-VCH, Weinheim, **2013**; b) J. Yoshida, A. Nagaki, T. Yamada, *Chem. Eur. J.* **2008**, *14*, 7450–7459; c) P. Watts, C. Wiles, *Org. Biomol. Chem.* **2007**, *5*, 727–732; d) B. Ahmed-Omer, J. C. Brandt, T. Wirth, *Org. Biomol. Chem.* **2007**, *5*, 733–740; e) B. P. Mason, K. E. Price, J. L. Steinbacher, A. R. Bogdan, D. T. McQuade, *Chem. Rev.* **2007**, *107*, 2300–2318.
[10] A. Pohar, I. Plazl, P. Žnidaršič-Plazl, *Lab Chip* **2009**, *9*, 3385–3390.
[11] P. He, G. Greenway, S. J. Haswell, *Process Biochem.* **2010**, *45*, 593–597.
[12] S. Mohr, K. Fisher, N. S. Scrutton, N. J. Goddard, P. R. Fielden, *Lab Chip* **2010**, *10*, 1929–1936.
[13] a) R. Karande, A. Schmid, K. Buehler, *Langmuir* **2010**, *26*, 9152–9159; b) R. Karande, A. Schmid, K. Buehler, *Adv. Synth. Catal.* **2011**, *353*, 2511–2521.
[14] a) B. Ahmed-Omer, D. Barrow, T. Wirth, *Chem. Eng. J.* **2008**, *135S*, S280–S283; b) B. Ahmed, D. Barrow, T. Wirth, *Adv. Synth. Catal.* **2006**, *348*, 1043–1048; c) S. Aljbour, H. Yamada, T. Tagawa, *J. Ind. Eng. Chem.* **2009**, *15*, 829–834; d) S. Hübner, S. Kressirer, D. Kralisch, C. Bludszuweit-Philipp, K. Lukow, I. Jänich, A. Schilling, H. Hieronymus, C. Liebner, K. Jähnisch, *ChemSusChem* **2012**, *5*, 279–288.

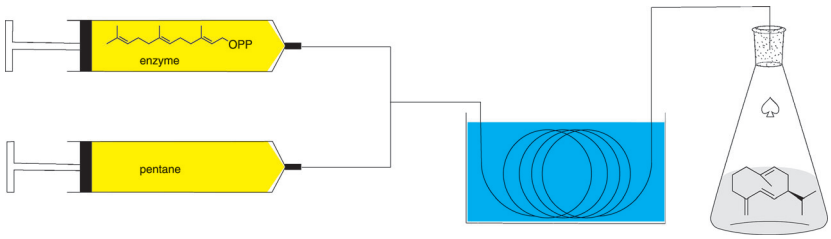
[15] a) H. A. Gennadios, V. Gonzalez, L. Di Costanzo, A. A. Li, F. L. Yu, D. J. Miller, R. K. Allemann, D. W. Christianson, *Biochemistry* **2009**, *48*, 6175 – 6183; b) M. J. Calvert, P. R. Ashton, R. K. Allemann, *J. Am. Chem. Soc.* **2002**, *124*, 11636 – 11641.
[16] T. M. Hohn, R. D. Plattner, *Arch. Biochem. Biophys.* **1989**, *272*, 137 – 143.
[17] When optimizing the substrate concentration, we obtained a precipitate at FDP concentrations above 0.4 mM. Mass spectrometry analysis of the insoluble precipitate gave a mass of 505 *m/z*. The formation of this side-product depends on the presence of Mg^{2+} ions; in water FDP concentrations up to 30 mM could be obtained. Difarnesyl phosphate **A** is the side product as identified by ^{31}P NMR and ^{1}H NMR spectroscopy: V. Birault, G. Pozzi, N. Plobeck, S. Eifler, M. Schumutz, T. Palanché, J. Raya, A. Brisson, Y. Nakatani, G. Ourisson, *Chem. Eur. J.* **1996**, *2*, 789 – 799.



A

[18] A control experiment with sonication in batch gave a conversion of 24%.
[19] a) D. K. Ro, E. M. Paradise, M. Ouellet, K. J. Fisher, K. L. Newman, J. M. Ndungu, K. A. Ho, R. A. Eachus, T. S. Ham, J. Kirby, M. C. Y. Chang, S. T. Withers, Y. Shiba, R. Sarpong, J. D. Keasling, *Nature* **2006**, *440*, 940 – 943; b) C. J. Paddon, P. J. Westfall, D. J. Pitera, K. Benjamin, K. Fisher, D. McPhee, M. D. Leavell, A. Tai, A. Main, D. Eng, D. R. Polichuk, K. H. Teoh, D. W. Reed, T. Treynor, J. Lenihan, H. Jiang, M. Fleck, S. Bajad, G. Dang, D. Dengrove, D. Diola, G. Dorin, K. W. Ellens, S. Fickes, J. Galazzo, S. P. Gaucher, T. Geistlinger, R. Henry, M. Hepp, T. Horning, T. Iqbal, L. Kizer, B. Lieu, D. Melis, N. Moss, R. Regentin, S. Secrest, H. Tsuruta, R. Vazquez, L. F. Westblade, L. Xu, M. Yu, Y. Zhang, L. Zhao, J. Lievense, P. S. Covello, J. D. Keasling, K. K. Reiling, N. S. Renninger, J. D. Newman, *Nature* **2013**, *496*, 528 – 532.

Received: August 28, 2013
Published online on ■■ ▮▮, 0000



O. Cascón, G. Richter, R. K. Allemann, T. Wirth**

■■ – ■■

Efficient Terpene Synthase Catalysis by Extraction in Flow

Flowing enzymes: Continuous extraction of products enhances the enzymatic productivity of sesquiterpenes (see figure). Even unnatural substrates are tolerated leading to valuable unnatural target molecules in superior yields compared with batch protocols.