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Biocatalysis

Accelerating Biphasic Biocatalysis through New Process Windows

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Abstract: *Process intensification through continuous flow reactions has increased the production rates of fine chemicals and pharmaceuticals. Catalytic reactions are accelerated through an unconventional and unprecedented use of a high-performance liquid/liquid counter current chromatography system. Product generation is significantly faster than in traditional batch reactors or in segmented flow systems, which is exemplified through stereoselective phase-transfer catalyzed reactions. This methodology also enables the intensification of biocatalysis as demonstrated in high yield esterifications and in the sesquiterpene cyclase-catalyzed synthesis of sesquiterpenes from farnesyl diphosphate as high-value natural products with applications in medicine, agriculture and the fragrance industry. Product release in sesquiterpene synthases is rate limiting due to the hydrophobic nature of sesquiterpenes, but a biphasic system exposed to centrifugal forces allows for highly efficient reactions.*

Flow chemistry is changing the way chemical reactions are performed. The development of catalytic processes in flow chemistry has already led to major advances in the synthesis of fine chemicals and pharmaceuticals. The involvement of heterogeneous catalysts in flow systems has a long history and different catalyst-containing reactors have been developed for these reactions.[1] Homogenously catalyzed reactions have high value also from the industrial perspective,[2] but are more difficult to control in flow reactions as catalyst recycling methods usually have to be implemented to obtain sustainable protocols. Different approaches have been reported for the immobilization (heterogenization) of homogeneous catalysts but often the catalytic reactivity and selectivity are compromised.

Introduced by V. Hessel,[3] the concept of "novel process windows" describes flow procedures operating under unusual reaction conditions. They can overcome traditional thermodynamic or kinetic limitations and, therefore, result in intensified processes or even novel reaction outcomes.

Efficient mixing can sometimes be challenging to achieve, especially in an industrial setting. Mixing can influence the outcome of chemical reactions as product selectivities can be altered through competitive parallel or competitive consecutive reactions.[4] Many such reactions have been described in the literature; the trapping of reactive and unstable intermediates though rapid mixing is one prominent example.[5]

Liquid/liquid counter current chromatography (CCC) has been developed for the separation of mixtures of natural compounds or for the purification of pharmaceuticals on analytical to industrial scales.[6] Recent developments have made robust and reliable CCC instruments commercially available.[7] This technique relies on the partition of solutes between two immiscible liquid phases therefore avoiding the solid phases used in many other chromatographic procedures. One liquid phase is held stationary in a coiled tube through centrifugal forces by spinning the whole coil. The second (mobile) liquid phase, immiscible with the stationary liquid phase, is pumped through the system.[8] The high-performance counter current chromatography (HPCCC) system uses a polytetrafluoroethylene (PTFE) tube wrapped in a coil onto a drum (bobbin) which rotates in a planetary motion (Figure 1). The bobbin revolves around its central axis while simultaneously rotating around its own axis at the same velocity to create an effect similar to wave mixing with more than two million partitioning steps per hour.[9] As this dramatically increases the liquid/liquid interfacial area, we hypothesized that it can provide an ideal platform to improve synthetic processes and opening up alternative and new process windows that rely on biphasic mixing.

The use of small-scale capillaries to improve mixing of biphasic systems through segmented flow as opposed to rapid stirring in a flask has already been characterized by mass transfer coefficients and modelled by engineers.[10] Even the geometry of the capillary can improve mixing by up to 20% through Dean forces.[11] Modelling of HPCCC systems however, is more complex and different models have been proposed for their theoretical investigation.[12]

Phase-transfer catalysis has become a well-established synthetic technique since its discovery in the 1960s.[13] With two reactants being present in two separate immiscible phases, the role of a phase-transfer catalyst (PTC) is to enable the movement of one reactant from one phase into the other to increase the reaction rate substantially. For stereoselective reactions, chiral PTCs can be used to generate products in high selectivity.[14] Enhanced mixing in microreactors has already been exploited for phase-transfer catalyzed reactions.[15] Here, we report the first stereoselective phase-transfer catalyzed reaction in a flow system using

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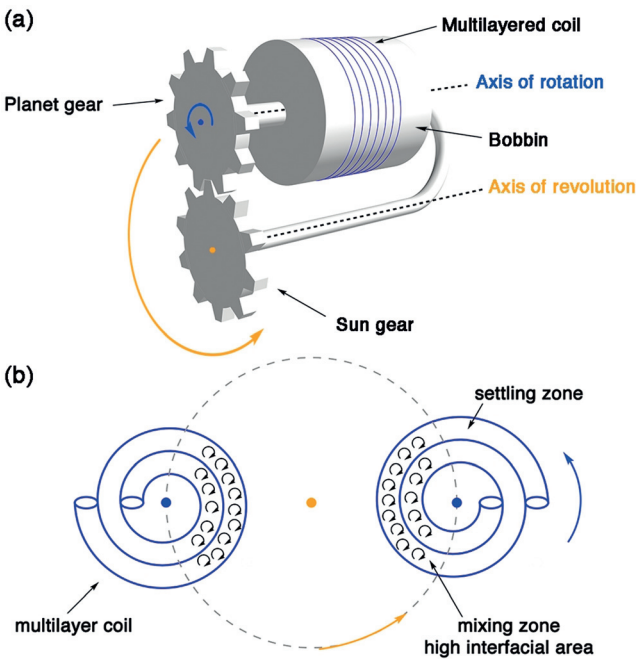
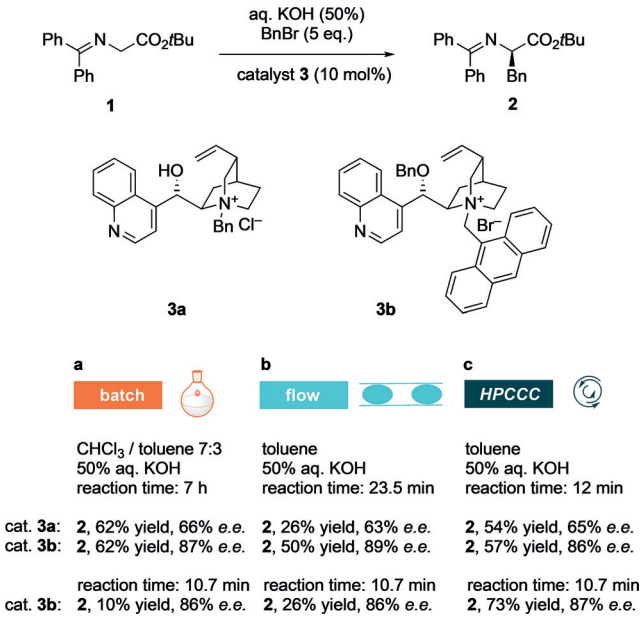


Figure 1. High performance counter current chromatography (HPCCC) device. (a) Schematic view showing only one of the two bobbins for clarity. (b) Settling and mixing zones in the HPCCC column generated by variable centrifugal forces induced by the planetary motion of the bobbin. The mixing zone is situated toward the centre of the axis of revolution coinciding with a low acceleration field and the settling zone coinciding with the high acceleration field away from the centre of revolution.



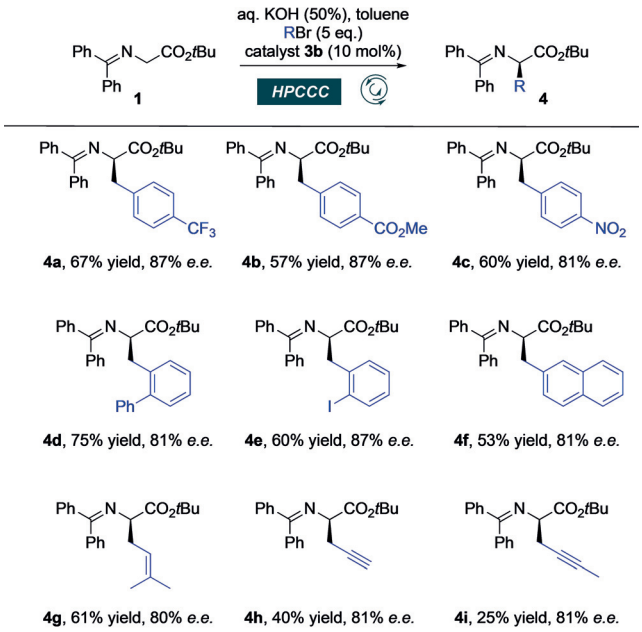
Scheme 1. Stereoselective phase-transfer catalysis. (a) Batch reaction. (b) Segmented flow reaction. (c) HPCCC reaction.

intensified mixing with the HPCCC device. The stereoselective alkylation of *N*-(diphenylmethylene)glycine *tert*-butyl ester **1** (Scheme 1) uses 50% aqueous KOH as base, 5 equivalents of benzyl bromide (BnBr) and a chiral PTC. This is a well-explored reaction leading to product **2** in good yields and selectivities where cinchona-derived catalysts have been successfully used.[16] Products of type **2** can be hydrolyzed to optically active amino acid derivatives as useful building blocks for further synthesis.

Initial investigations of the batch reactions showed a low solubility of catalyst **3a**. Therefore, the reported solvent system (toluene:chloroform 7:3) was changed to dichloromethane where the solubility of **3a** is 0.005 M. In a biphasic reaction in segmented flow, comparable yields and selectivities were obtained with an optimized internal diameter (0.5 mm), flow rate (0.32 mL min^{-1}) and residence time (21 min). Unfortunately, this solvent system cannot be used in the HPCCC as the interfacial tension between the two phases is too small and an emulsion is formed. Toluene would be a suitable organic solvent in the HPCCC due to the large density difference to 50% aq. KOH, but the catalyst is not soluble in either of these solvents. Although the catalyst is soluble in 25% aq. KOH, only trace amounts of product were formed. Preforming the enolate of **1** and exchanging it with the chloride anion of **3a** allowed the formation of a homogenous solution of the catalyst in toluene and the reaction under segmented flow conditions provided product **2** in 26% yield and with 63% *ee* with a residence time of 23.5 minutes. The same solvents were used for experiments in the HPCCC where 54% yield of **2** (65% *ee*) was obtained in a reaction with 12 minutes residence time. All reactions were performed at room temperature and the obtained enantioselectivities were similar (Scheme 1). With catalyst **3b**, which shows enhanced solubility (0.003 M in toluene) and which had been used previously in epoxidation reactions[17] but not in the stereoselective alkylation, selectivity was greatly improved. Further optimization was performed (see Supporting Information) with regards to flow rate/ retention time in the machine. The optimal reaction time of 10.7 minutes was then applied to the batch and segmented flow protocols showing unambiguously the large improvement obtained in HPCCC with 73% yield and 87% *ee* of **2**.

Different alkyl halides were investigated in the reaction using the HPCCC (Scheme 2). With residence times between 10 and 16 minutes, products **4** were all obtained with high enantioselectivities characteristic for catalyst **3b**. Allylic bromides and propargylic bromides were suitable electrophiles too (**4g**–**4i**), while alkyl bromides or benzyl chlorides were unreactive under the conditions used. Even alkyl iodides were unreactive, although these had been reported previously to be used successfully in long (30 hours) batch alkylations.[18] Reactions with extremely long reaction times cannot be performed on the HPCCC. The biphasic stereoselective epoxidation of chalcone with sodium hypochlorite, for which catalyst **3b** was originally prepared, takes 48 hours in batch and this reaction could not be performed successfully by HPCCC.[17]

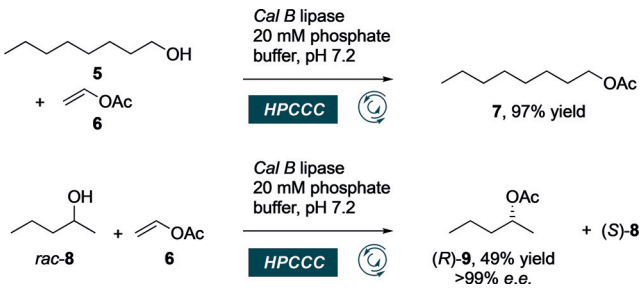
In a previous publication, the use of counter current extraction devices in biocatalysis was described. However, only little details were given in that seminal publication[19] and later reports describe only the use of the centrifugal partition chromatography equipment as an intensified reactor for biocatalytic reactions.[20] We investigated HPCCC as an



Scheme 2. Different electrophiles in the stereoselective phase-transfer catalyzed alkylation.

alternative method to overcome the limitations of traditional batch and flow approaches in biocatalysis.

In initial experiments we used *Cal B* lipase for the transesterification of octanol with vinyl acetate. The organic phase (heptane) contained octanol **5** and vinyl acetate **6** while the aqueous buffer contained the *Cal B* lipase. Both solutions were pumped through the HPCCC. Rotation speed in the HPCCC, residence time and temperature were varied in a face-centered design of experiments (DoE) approach (see supporting information), taking into account that only a smaller amount of the stationary phase is retained under these conditions. A three-fold excess of vinyl acetate with an enzyme concentration of $1\,mg\,mL^{-1}$ and operating the HPCCC at maximum rotation speed (1600 rpm) led to an almost quantitative isolated yield (97%) of octyl acetate **7** with a 6 minutes reaction time (Scheme 3). Compound **7** is an industrially important solvent but is also found in citrus fruits and used as the basis for artificial flavors. If the same reaction is carried out in a stirred flask, only 40% yield of **7** is obtained after 4.5 hours reaction time. The reaction of *rac*-pentan-2-ol **8** with vinyl acetate and *Cal B* lipase led to a racemic



Scheme 3. Transesterifications with *Cal B* lipase.

resolution of **8** and the quantitative formation of enantiomerically pure (*R*)-**9** (Scheme 3). While the reported batch reaction takes 4 hours to complete,[21] the reaction time in the HPCCC is only 6 minutes.

Sesquiterpenes belong to a class of natural products with more than 300 known hydrocarbon backbones. Many sesquiterpene derivatives exhibit important bioactivities with medicinal or agricultural applications.[22] In nature, these compounds are synthesized by sesquiterpene synthases in a single step from farnesyl diphosphate (**10**) in exquisitely regioselective and stereospecific reactions (Figure 2a).[23] Despite their high value, most sesquiterpenes are not available in large quantities as they have to be extracted from plants. They are not easily available through total synthesis due to challenges associated with stereo- and regioselectivity and they are often unstable to heat and acidic conditions. Although recent synthetic work has shown promising progress,[24] nature is much more efficient in producing these complex compounds.[25] Therefore, recombinant sesquiterpene synthases expressed in E. *coli* have been used to synthesize natural sesquiterpenes and to expand the terpenome in the search for novel compounds. Unnatural analogues of farnesyl diphosphate have been successfully converted to new structures including oxygenated products such as aldehydes and cyclic ethers.[26] The bottleneck of this approach is the typically high hydrophobicity of the sesquiterpene products, which can lead to slow reactions. Pre-steady state kinetic measurements have shown that product release is normally the rate-limiting step of the overall reaction.[27] An initial solution was the creation of a rapidly stirred biphasic mixture to release the product into an organic phase, a method which has been used with success also on other enzymes such as lipases.[28] However, this has proven to be inefficient for sesquiterpene synthases due to a low mass transfer rate and a deactivation of the enzyme through long exposure to organic solvents. Typical yields range from 10 to 30% and reaction times are often long (1–2 days). For optimum yields the substrate needs to be added in stages and reaction products extracted several times with organic solvents, which often leads to the formation of emulsions requiring centrifugation for separation. As an alternative, we have reported the use of sesquiterpene synthases in segmented flow systems to accelerate mixing and to shorten reaction times.[29] Optimization through design of experiments improved the yields and shortened the reactions times for the conversion of farnesyl diphosphate **10** to (+)-aristolochene (**11**) and amorpha-4,11-diene (**13**) by aristolochene and amorphadiene synthase in 96% and 69% yields, respectively in 90 minutes.[30] Despite improved yields and reaction rates, segmented flow systems can still result in emulsions at the reactor outlet with occasional blockages during extended reactions due to enzyme precipitation through contact with pentane.

Here we examined the conversion of **10** to (+)-aristolochene by aristolochene synthase from *Penicillium roqueforti* (AS). Purified enzyme and **10** in a buffer solution were loaded on the HPCCC analytical column (22 mL volume) to form a stationary phase (see supporting information). Buffer, substrate and enzyme concentration were identical to those

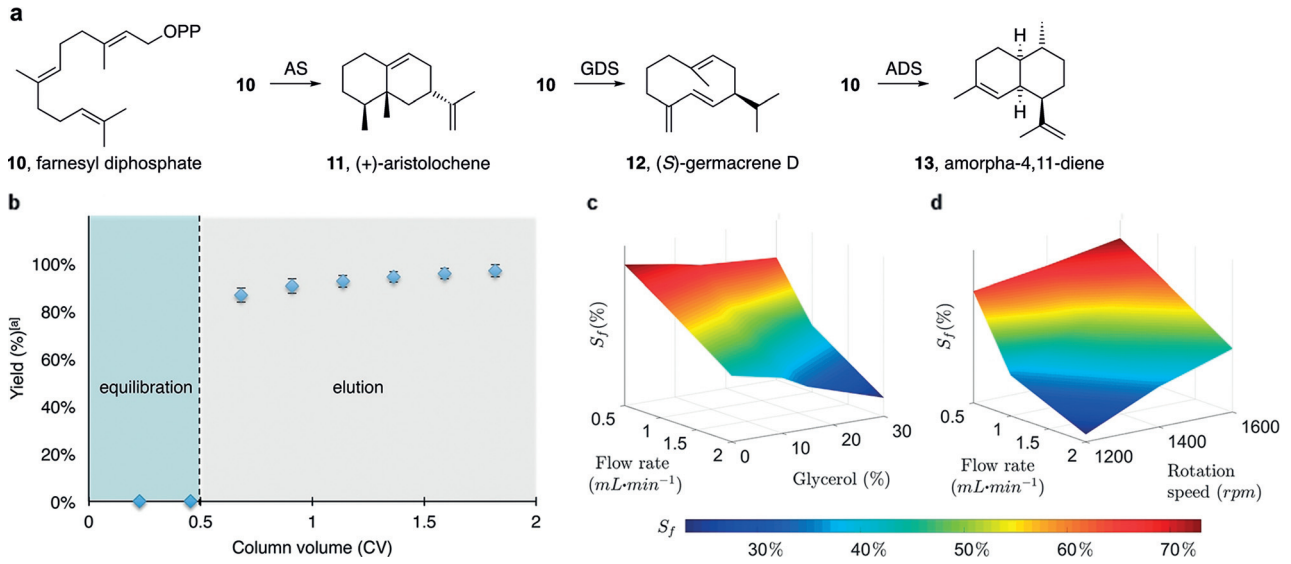


Figure 2. Sesquiterpene cyclases in HPCCC. (a) Conversion of farnesyl diphosphate **10** to **11**, **12** and **13** catalyzed by aristolochene (AS), (*S*)-germacrene D (GDS) and amorphadiene (ADS) synthases. (b) Time course for the AS-catalyzed conversion of **10** to **11** in the HPCCC system (6 μM AS, 0.35 mM **1**, 0.5 mL min^{-1} pentane flow rate), determined by GC-FID.[a] Yield of **11** in total eluted volume. (c) Characterization of the relationship between the pentane flow rate and glycerol content of the aqueous phase on the stationary phase retention S_f. The surface highlights that S_f is severely reduced with glycerol in the buffer and a high flow rate. (d) Characterization of the relationship between the pentane flow rate and rotation speed of the bobbin on the stationary phase retention S_f. Surface highlights that S_f is severely reduced with low rotation speed and high flow rate. (Surface rendered using Matlab 2018b). S_f: Percentage retention of the stationary phase relative to total column volume.

previously used for batch and segmented flow experiments. To assess the efficiency of HPCCC, it was first determined how long the mobile phase needed to be flowed through the system to reach a plateau in product extraction. Surprisingly, > 95 % yield (measured by GC) was reached in less than one column volume (CV) at a flow rate of the mobile phase of 0.5 mL min^{-1}. The first phase shown in blue in Figure 2 b is the equilibration of the biphasic system (definition of equilibration and elution step in the HPCCC are detailed in the supporting information). To investigate further the potential of HPCCC for synthesis, the concentration of substrate **10** was doubled (0.7 mM) to increase the scale of the reaction. This had no impact on the turnover as a similar yield was obtained after 0.5 CV (Figure S4 in the Supporting Information).

The force exerted on the retained phase is closely correlated to its density, the revolving speed of the coil and the flow rate of the mobile phase. When the flow rate was increased from 0.5 mL min^{-1} to 2 mL min^{-1} (reducing the extraction time to a quarter), the increased flow rate affected the retaining force exerted on the aqueous phase leading to a loss of the stationary phase. The percentage retention of the stationary phase relative to the total column volume is defined as S_f. Glycerol is usually required to maintain the stability of sesquiterpene synthases in flow and batch, but the additional density it provides reduced S_f (Figures 2 c,d). However, the reaction time when using HPCCC is so short that glycerol can be omitted. With the modified buffer conditions and a rotation speed of 1600 rpm, approximately half of the column volume ($S_f = 50\,\%$) can be retained as stationary phase at a pentane flow rate of 2 mL min^{-1}. This increased flow rate did not negatively impact on the yield (Table S7) and reduced the reaction time from 44 to 11 minutes. Thus, HPCCC was clearly more efficient than segmented flow and is characterized by a ~10-fold reduced reaction time. We then applied these optimized HPCCC conditions to different sesquiterpene cyclases using the natural substrate **10** as well as modified analogues of **10** such as 12-hydroxy farnesyl diphosphate (12-OH FDP). Vastly improved yields of up to 99 % were observed compared to batch synthesis, where yields were typically between 20 and 40 % (Figure 3 and Supporting Information). HPCCC has been previously reported to scale well when used for purification[31] and the optimized conditions for synthesis were investigated with alarger HPCCC column (135 mL). A similar extraction profile to the smaller column was obtained (Figure S3) with excellent isolated yields of 70–94 % (30–35 mg) (Figure 3),[32] in good agreement with those measured by GC for the smaller HPCCC column.

In summary, we have demonstrated here for the first time that HPCCC provides a unique and general platform to perform biphasic reactions, enabling extremely rapid mass-transfer between two immiscible phases. This method was applied to phase-transfer catalyzed chemical alkylations and biocatalytic transformations. The reactions with *Cal B* lipase and with sesquiterpene synthases produce valuable products with high yields and with extremely short reaction times. The sesquiterpene catalyzed reactions were approximately 70 times faster in HPCCC than in batch. The short reaction times avoid enzyme denaturation alleviating emulsion formation seen in batch and segmented flow systems. This method is readily scaled up with larger HPCCC columns and will find utility with many other reactions where products and/ or substrates are poorly water soluble.

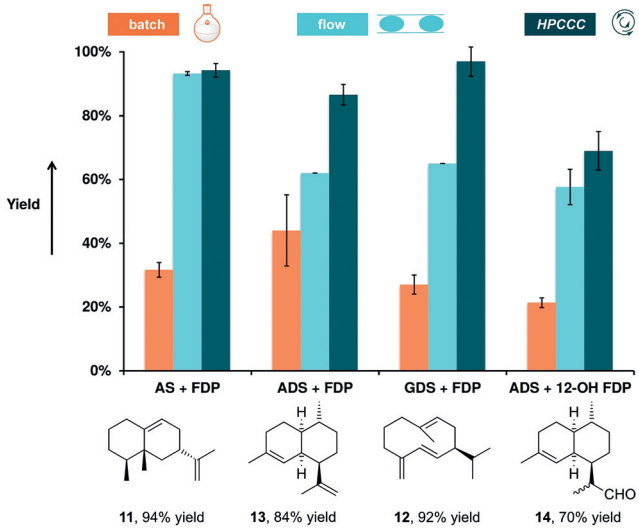


Figure 3. Comparison of batch, segmented flow and HPCCC methods for conversions of FDP (**10**) with aristolochene synthase (AS), amorphadiene synthase (ADS) and (*S*)-germacrene D synthase (GDS) and for 12-OH-FDP with ADS. Yields were determined by GC and calculated by using a calibration curve with α-humulene as standard. Isolated yields of the products have been obtained using the preparative scale (135 mL) HPCCC column with optimized reaction conditions (pentane flow rate: 5 mL min^{-1}).

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biocatalysis · biphasic reaction · HPCCC · phase-transfer catalysis · terpenoids

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[32] One reaction performed in the large HPCCC column uses 0.0455 mmol (19.7 mg) **10**, the reaction is performed 4 times.

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