Enzyme-purification and catalytic transformations in a microstructured PASS*flow* reactor using a new tyrosine-based Ni-NTA linker system attached to a polyvinylpyrrolidinone-based matrix†

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Received 20th August 2007, Accepted 18th September 2007
First published as an Advance Article on the web 5th October 2007
DOI: 10.1039/b712804e

The synthesis of a Ni-nitrilotriacetic acid (Ni-NTA) attached *via* a new tyrosine-based linker matrix on monolithic crosslinked poly(vinyl benzyl chloride)/poly(vinylpyrrolidinone) is described. This matrix is incorporated inside a microstructured PASS*flow* reactor which was used for automatic purification and immobilisation of His_6-tagged proteins. These could be used as stable and highly active biocatalysts for the synthesis of (*R*)-benzoin (**6**), (*R*)-2-hydroxy-1-phenylpropan-1-one (**7**) and 6-*O*-acetyl-D-glucal (**17**) in a flow-through mode.

Introduction

Continuous flow reactors using immobilised enzymes have been widely used in industrial applications.[1] Commonly, enzymes are immobilised onto a solid support by either physical adsorption, membrane entrapment, polymeric gel entrapment or covalent bonding. Immobilisation has been achieved on different carrier materials such as controlled pore glass, microporous silica, nanoporous aluminium oxide, polyaminostyrene, nylon, poly(2-hydroxyethylmethacrylate), polyurethane, and sepharose, just to name the most important ones.[2] Additionally, crosslinked enzyme membranes have been described.[3] A major advantage of immobilised enzymes is their insolubility, allowing easy product separation and reuse. Often they show increased stability compared to their homogeneous counterparts.

So far little work has been devoted to the development of an immobilisation strategy within a continuous flow reactor system that allows purification of the target enzymes by means of Ni-NTA-based immobilisation as well as synthetic use of the enzyme probe under continuous flow conditions. In order to achieve this goal, the solid phase, the linker system, as well as the reactor, have to be optimised to form an ideal enabling technology platform.[4]

In this report we disclose a microreactor concept for immobilised enzymes, which is based on our recently developed PASS*flow* (polymer-assisted solution phase synthesis under flow conditions) system.[5,6] The reactor concept is based on a pressure-resistant reactor housing filled with a porous, monolithic glass/polymer composite material which is appropriately functionalised for immobilising different chemical species. The composite is set up of a continuous polymeric phase (interconnected beads 1–10 μm) and a porous glass body (irregular microchannels; 10–100 μm diameter), which leads to a forced convective flow of the solution along the polymeric matrix. The reactor itself is integrated into an HPLC-based solvent-system, allowing automated reactor loading, reaction control and reactor regeneration (Fig. 1).

For the present purposes we had to find the best polymeric phase and linker system for functionalising the interior of the reactor with Ni-NTA groups. The immobilised nitrilotriacetic acid (NTA) is a phase suitable for metal ion affinity chromatography (IMAC). IMAC matrices can be used for purification of His_6-tagged proteins from crude cell extracts and have served as carriers for tagged enzymes in biocatalysis.[7] Besides the known lysine linker, we planned to develop a new linker system based on tyrosine.

Technically, we first tested the new, functionalised polymers in form of a powder (obtained by precipitation polymerization)[6] and then transferred the conditions to a monolithic glass/polymer composite material shaped in the form of Raschig rings (Fig. 2). Finally, we incorporated the new IMAC-phases inside a PASS*flow* microreactor which contains the same composite material.

Results and discussion

Preparation and immobilisation of lysine- and tyrosine-based Ni-NTAs on optimised polymer

First, we prepared two NTA-linker systems. While one linker is based on an established literature procedure[8] using lysine, the other NTA-linker utilises tyrosine as central linker element. Besides optimising the linker for the chosen polymer phase, the latter approach allows us to circumvent the current patent situation. The preparation of both linker systems is described in Scheme 1 and Scheme 2. Thus, Cbz-protected L-lysine (**1**) was alkylated with bromoacetic acid to yield the NTA functionality. Protection of the three carboxylic acids as methyl esters was necessary to enhance the solubility in *N*-methylpyrrolidinone (NMP) required for the solid phase attachment. Finally, Cbz cleavage was accomplished by hydrogenation using Pd/C.

Immobilisation was first addressed using poly(vinyl benzyl chloride) (VBC) crosslinked with 5% divinyl benzene (DVB). Amino ester **2** was reacted with Merrifield resin and sodium

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† Electronic supplementary information (ESI) available: IR spectra of functionalised polymers and SDS-Page. See DOI: 10.1039/b712804e

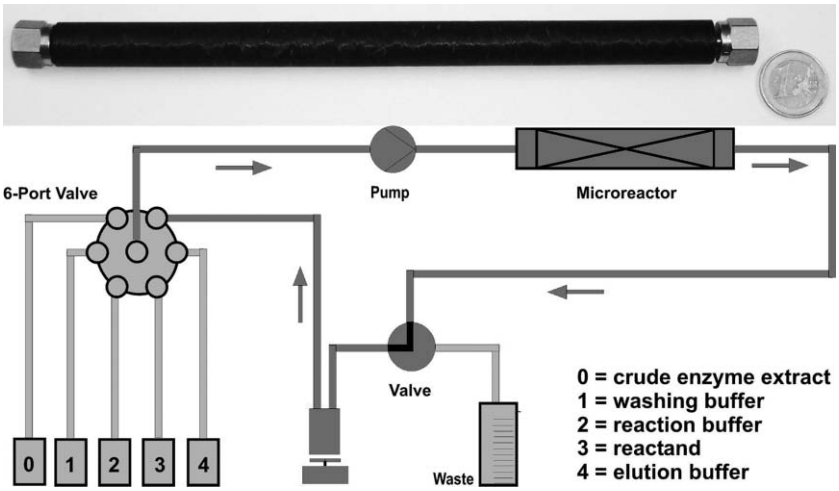
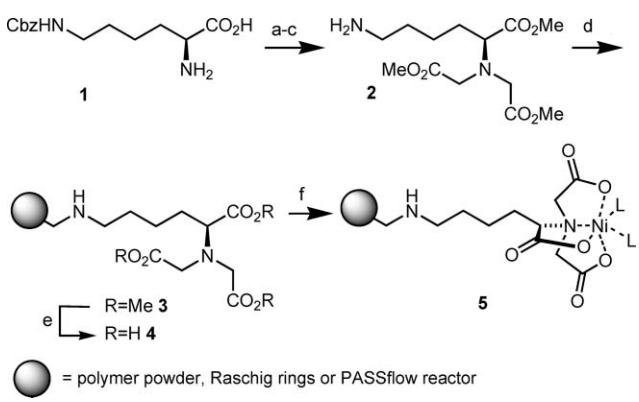
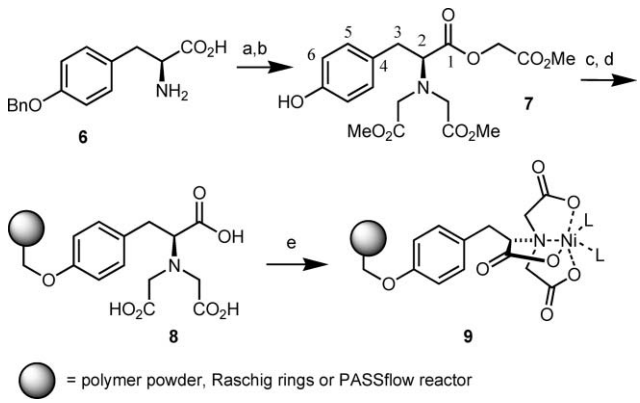


Fig. 1 Typical PASS*flow* reactor used for this study, and schematic view of the PASS*flow*-NTA reactor setup.

Fig. 2 Types of solid phases used.



Scheme 1 Synthesis of lysine-based NTA linker on several samples of crosslinked polymers. *Reagents and conditions*: a) $BrCH_2CO_2H$, NaOH, H_2O, 40 °C, 12 h (87%); b) H_2SO_4, MeOH, reflux, 12 h; c) Pd/C, H_2, MeOH, rt, 12 h (98% for two steps); d) polymer with $ArCH_2Cl$ groups, NaO*t*Bu, NMP, 60 °C, 72 h [64% for Merrifield resin (5% DVB) determined by gravimetry]; e) LiOH, MeOH–H_2O (1 : 1), 50 °C, 60 h; f) $NiCl_2$, H_2O, DMSO. Cbz = benzyloxycarbonyl; NMP = *N*-methylpyrrolidinone.



Scheme 2 Synthesis of tyrosine-based NTA linker on crosslinked poly(vinylpyrrolidinone)/poly(vinyl benzyl chloride) polymer. *Reagents and conditions*: a) K_2CO_3, TBAI, $BrCH_2CO_2Me$, 3-pentanone, 108 °C (55%); b) Pd/C, H_2, MeOH, rt (99.5%); c) polymer with $ArCH_2Cl$ groups in Pass*flow* reactor, CsI (0.5 eq.), DMF, 60 °C, 72 h (~30% of theoretical loading, 1.7 mmol g^{-1}); d) LiOH, MeOH–H_2O (1 : 1), 50 °C, 60 h; e) $NiCl_2$, H_2O, DMSO, rt, 10 min.

tert-butylate in *N*-methylpyrrolidinone, affording resin **3**. Completion of loading was judged by IR-spectroscopy. Mild ester hydrolysis was accomplished (LiOH, MeOH–H_2O 1 : 1), yielding resin **4**.[9] Finally, treatment with an aqueous solution of $NiCl_2$ yielded Ni-NTA on the polystyrene-derived matrix **5**, which could be visualized by the colour change of the polymer from light yellow to light blue. Several experiments failed to purify or immobilise His_6-tagged proteins on this polymer, which led us to conclude that the polarity of the polymeric phase prevented exposure of the Ni-NTA to the aqueous phase.

In order to increase the polarity of the solid phase, we prepared several crosslinked polymeric samples composed of DVB, VBC and *N*-vinylpyrrolidinone (10: 70: 20 vol %).[10] This resin was prepared by precipitation polymerisation in the pore volume of highly porous glass rods or Raschig rings to yield a polymeric matrix inside the glass pores, as well as an excess of the corresponding polymeric powder. The rods were embedded in a solvent- and pressure-resistant PASS*flow* housing.[6] The glass-supported polymer was then treated with amino ester **2** under the conditions described above, followed by ester cleavage (LiOH, MeOH–THF). This was followed by treatment with a solution of Cl_2SiMe_2 in CH_2Cl_2 for silyl capping of silanol groups on the glass. This step prevents non-specific binding of proteins to the glass inside the Raschig rings and PASS*flow* reactor, respectively. After Ni^{2+} complexation we accomplished the formation of a Ni-NTA phase **5** attached to a novel monolithic, sufficiently polar polymeric backbone.

In the same manner we prepared samples of tyrosine-based NTA-linker **7** (prepared from *O*-benzyltyrosine **6** by a standard three-step sequence). Triester **7** was attached to the crosslinked copolymer composed of DVB, VBC and vinyl pyrrolidinone as

described for the established linker **2** (Scheme 2). The best results were achieved for a monomeric composition of DVB/VCB/*N*-vinyl pyrrolidinone (10 : 70 : 20 vol %), leading to maximum loading of 30% for **8** (analysed by IR and gravimetrically).[10]

Loading of benzaldehyde lyase (BAL) and optimisation of benzoin reaction

Studies on the protein loading and optimisation of the reaction conditions were first conducted with the polymer powder obtained from the precipitation polymerisation protocol. Thus, the Ni-NTA-functionalised polymers were employed for the stepwise purification and immobilisation of His_6-tagged proteins, and in the following could directly be used in enzymatic transformations. As a model enzyme we settled on recombinant benzaldehyde lyase (BAL, EC 4.1.2.38)[11] a thiamine pyrophosphate-dependent enzyme which utilises acyl anion equivalents as intermediates.[12] Both optimised Ni-NTA phases proved to be suitable for immobilising His_6-tag-modified BAL from a cell lysate (from *E. coli*; recombinant expressed BAL with His_6-tag). The tyrosine-based polymer showed improved capacities compared to the lysine-based phase (Fig. 3). Quantification of the immobilised protein was done with the Bradford assay[13] after excessive washing (phosphate buffer, pH 7 and imidazole buffer, 10 mM) followed by protein elution with an imidazole buffer solution (250 mM). The purity of the protein fractions were determined to be >98% by SDS-Page.[14]

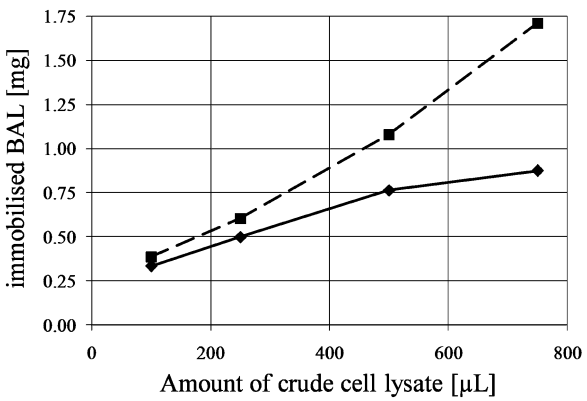
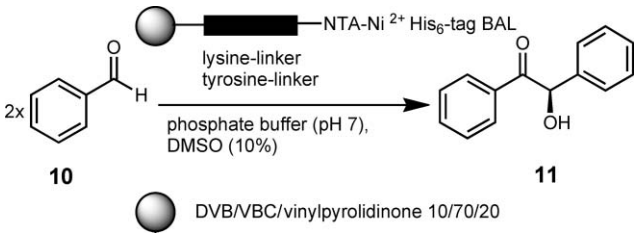


Fig. 3 Amount of immobilised BAL for different volumes of cell lysate (solid line = lysine-linker; dashed line = tyrosine linker).

For the benzoin reaction with benzaldehyde **10**, it turned out that addition of 10% of DMSO to the reaction buffer (phosphate buffer, pH 7)[15] gave best results (Scheme 3). Although BAL usually operates best at pH 9.5, we had to lower the pH to 7 because we encountered formation of nickel hydroxide at pH > 8.0. Under these optimised reaction conditions the leaching of protein was not detectable with the Bradford assay.[16]

The lysine-based polymer loaded with His_6-tagged BAL rapidly transformed 15 and 30 µL (0.15 and 0.3 mmol) of benzaldehyde (Fig. 4), while larger amounts (100 and 200 µL) required prolonged reaction times. At these higher concentrations partial enzyme inhibition exerted by the product was observed. In fact, we noted that it was difficult to completely remove the product from the polymeric phase.

In contrast, the tyrosine-based polymer showed better properties under the standardised reaction conditions. All benzaldehyde



Scheme 3 Optimised conditions for benzoin reaction of benzaldehyde (**10**) on polymeric powder.

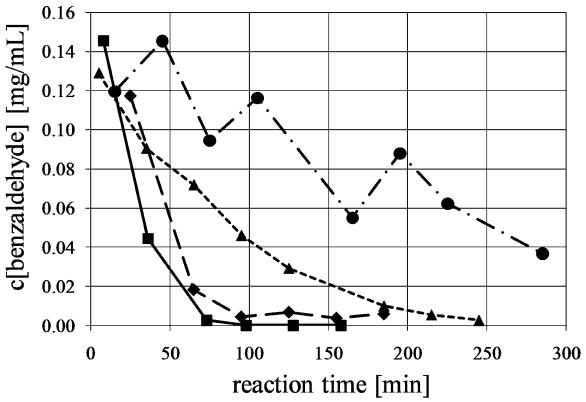


Fig. 4 Benzoin reaction (according to Scheme 3) with lysine-based linker employing different amounts of benzaldehyde **10** (■ = 15 µL; ◆ = 30 µL; ▲ = 100 µL; ● = 200 µL); all reactions were monitored by GC.

samples (15, 30, 100 and 200 µL) were quantitatively consumed within a reasonable time (Fig. 5). When 500 µL benzaldehyde (**10**) was added the reaction did not lead to completion (only 74%) within 300 min.

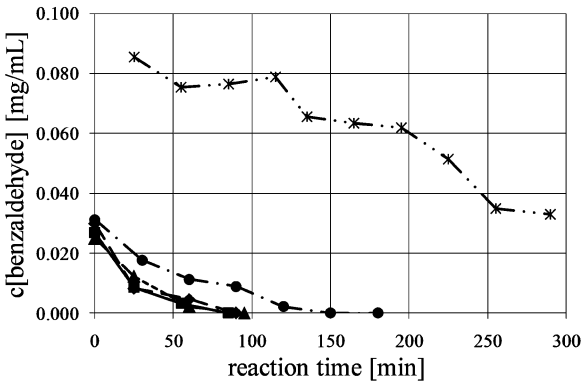


Fig. 5 Benzoin reaction (according to Scheme 3) with tyrosine-based linker employing different amounts of benzaldehyde **10** (■ = 15 µL; ◆ = 30 µL; ▲ = 100 µL; ● = 200 µL; ✱ = 500 µL); all reactions were monitored by GC.

The immobilized BAL could be reused without loss of activity after washing the polymer either with MTB-ether or ethyl acetate after each run and reactivation with phosphate buffer. In this way four consecutive batch transformations were conducted (Fig. 6).

Benzoin reaction under continuous flow conditions

Immobilising the His_6-tagged BAL inside a PASS*flow* reactor loaded with functionalized polymer in principle allows one to

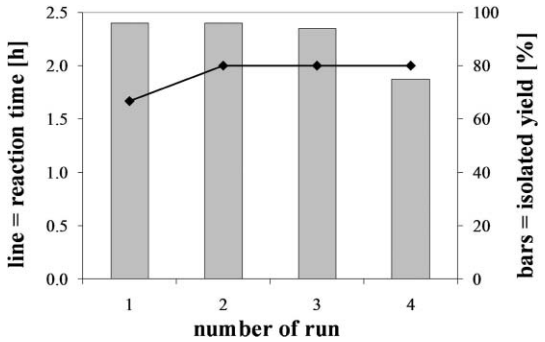
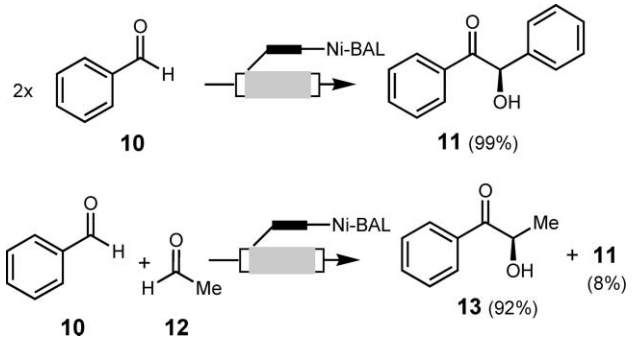


Fig. 6 Repeated benzoin reactions (according to Scheme 3) with tyrosine-based linker; all reactions were monitored by GC.

conduct the benzoin reaction in a circular mode (using the reactor in a batch mode) or in one run in a continuous flow manner. Attachment of the lysine- and tyrosine-based NTA-linker to the polymeric phase (DVB/VBC/*N*-vinylpyrrolidinone = 10 : 70 : 20) inside the PASS*flow* reactor (which contained approx. 200 mg of polymer), silylation of the glass matrix[17] and the trapping of Ni^{2+} were carried out according to the optimised protocols described above for the powder. The reaction mixtures were commonly circulated at flow rates of 1 mL min^{-1}.

Next, we studied the cross-benzoin reaction between benzaldehyde **10** and acetaldehyde **12** which yielded (*R*)-benzoin (**11**) and (*R*)-2-hydroxy-1-phenylpropan-1-one (**13**)[18] in very good yield under flow-through conditions (Scheme 4).



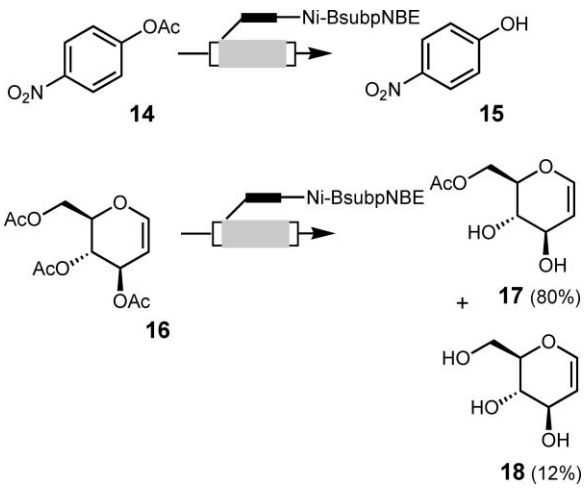
Scheme 4 Flow-through synthesis of (*R*)-benzoin (**11**) and (*R*)-2-hydroxy-1-phenylpropan-1-one (**13**) with His_6-tag BAL attached to polymeric phase *via* tyrosine-linked Ni-NTA.

In a similar manner the reactor was loaded with His_6-tagged BAL and the eluted protein was analysed with the Bradford assay and SDS Page (see ESI†). When a solution of benzaldehyde (30 μL) in phosphate buffer (5 mL) was pumped through the PASS*flow* reactor (flow rate = 1 mL min^{-1}) at 37 °C, full consumption of benzaldehyde was observed when analysing the solution that left the reactor (Scheme 4). After extraction with ethyl acetate (*R*)-benzoin (**11**) was isolated in 99% yield. This reaction was repeated with the same reactor another three times, giving the same results.

Regioselective ester hydrolysis under continuous flow conditions

Esterase-catalyzed deacetylation was studied using *p*-nitrobenzyl esterase from *Bacillus subtilis* (BsubpNBE[19]). This highly active enzyme is commonly used to cleave carboxyl protecting groups such as *tert*-butyl, methyl, benzyl, allyl and chloroethyl esters.[20] After immobilization of His_6-tagged BsubpNBE inside the PASS*flow* reactor, enzyme leaching was studied using *p*-nitrophenyl acetate (**14**), which was readily cleaved to yield **15** (Scheme 5). After having passed through the reactor, a sample of the reaction mixture was analyzed by HPLC and stored at 37 °C for 1 h to eventually allow further enzymatic reaction catalyzed by cleaved protein. After this time, the sample was again analyzed and compared in order to evaluate enzyme leaching. It turned out that the co-solvent used for dissolving the reactant is crucial for binding; DMSO leads to detectable leaching but methyl *tert*-butyl ether was fully compatible, and no transfer of enzymatic activity to the reaction medium was observed.

Next, we used tri-*O*-acetyl-D-glucal (**16**) as substrate. In solution, **16** is completely deacetylated within minutes by BsubpNBE. Under flow-through conditions with immobilized enzyme this reaction is much slower. After complete consumption of **16** (60 h), 6-*O*-acetyl-D-glucal (**17**) could be isolated in good yields along with traces of **18** (Scheme 5).



Scheme 5 Flow-through acetate cleavage with His_6-tag BsubpNBE attached to polymeric phase *via* tyrosine-linked Ni-NTA.

Conclusions

In summary, we have presented a mild and rapid method for simultaneous purification and immobilization of His_6-tagged proteins allowing facile biocatalysis in a flow-through mode. For this purpose we optimised the solid phase as well as introduced an improved linker system for IMAC. This microstructured reactor system should be broadly applicable for the purification of His_6-tagged proteins as well as their synthetic use.

Experimental

General remarks

IR spectra were recorded with a Bruker Vektor 22 FT-IR spectrophotometer (GoldenGate ATR unit). Optical rotations were measured with a Perkin Elmer 341 polarimeter. NMR

spectra were recorded on a Bruker ARX-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer. All spectra were measured using standard Bruker pulse sequences. Multiplicities are described using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. ESI mass spectra were recorded on a LCT mass spectrometer (Micromass) with Lock-Spray dual ion source. The LCT-spectrometer was coupled with a Waters Alliance 2695 HPLC unit. All solvents used were of reagent grade and were further dried. Reactions were monitored by thin layer chromatography (TLC) on silica gel 60 F_{254} (E. Merck, Darmstadt) and spots were detected either by UV-absorption or by charring with $KMnO_4$/NaOH in water. Preparative column chromatography was performed on silica gel 60 (E. Merck, Darmstadt). Reagents were purified and dried by standard techniques.

Methyl 6-amino-2-(bis(methoxycarbonylmethyl)amino)hexanoate (2)

Bromoacetic acid (1.4 g, 10.0 mmol) was dissolved in NaOH (10 mL, 2 M). A solution of ω-*N*-Cbz L-lysine (981 mg, 3.50 mmol) in NaOH (15 mL, 2 M) was added dropwise at 0 °C and stirred for 2 h. Stirring was continued for 12 h at r.t. The mixture was adjusted to pH 1.0 with aqueous HCl (10%). The crude product was filtered, purified by recrystallisation in HCl at pH 1.0 and dried under vacuum to yield bisalkylated ω-*N*-Cbz lysine as a white solid (1.21 g, 3.05 mmol, 87%).

To a solution of the alkylated lysine (1.2 g, 3.03 mmol) in MeOH (10 mL) was added H_2SO_4 (1 mL), and the mixture was heated under reflux for 12 h. After cooling to r.t. the reaction mixture was neutralised with saturated $NaHCO_3$ and extracted with dichloromethane (3×). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. Without further purification the product was dissolved in MeOH. After addition of Pd/C (400 mg) the mixture was stirred at r.t. under an H_2 atmosphere. After 12 h the solution was filtered through a pad of Celite™ and the filtrate was concentrated under reduced pressure to yield the title compound **2** (903 mg, 2.97 mmol, 84%).

^{1}H NMR (400 MHz, d_6-DMSO, d_5-DMSO = 2.50 ppm): δ = 3.71 (d, J = 5.3 Hz, 2 H, NC*H*H′CO_2CH_3), 3.69 (s, 3 H, CO_2CH_3) 3.68 (s, 6 H, $NCH_2CO_2CH_3$), 3.66 (d, J = 5.3 Hz, 2 H, NCH*H*′CO_2CH_3), 3.47 (t, J = 7.5 Hz, 1 H, H-2), 2.95–2.90 (m, 2 H, H-6), 1.65–1.47 (m, 2 H, H-3), 1.44–1.21 (m, 4 H, H-4, H-5) ppm. ^{13}C NMR (100 MHz, d_6-DMSO, d_6-DMSO = 39.5 ppm): δ = 174.4, 173.7, 65.4, 53.5, 52.1, 52.1, 52.0, 40.7, 30.5, 28.2, 23.7 ppm. HRMS (ESI): m/z calcd for $C_{13}H_{25}N_2O_6$: 305.1115, found: 305.1110.

Preparation of polar Merrifield Ni-NTA phase (5)

1,4-Divinylbenzene (3.41 g, 26.2 mmol), *p*-vinyl benzyl chloride (48.09 g, 315.1 mmol) and *N*-vinylpyrrolidinone (20.42 g, 183.8 mmol) were mixed and the volume was made up to 630 mL with C_{14}–C_{17} n-paraffin. After dissolution of azo-bis(isobutyronitrile) (788 mg, 4.8 mmol), porous glass rods (5.3 mm diameter; 110 mm length) and Raschig rings (6 mm inner diameter, 9 mm outer diameter, 9 mm length) were immersed in this solution. To remove air bubbles from the pore volume, a vacuum was applied for several minutes. Then, the mixture was heated to 80 °C overnight. The solidified mass was rinsed with cyclohexane using a Soxhlet-extractor. At this point the monolithic materials were separated from the polymer powder, which was formed as a by-product. Typically, 200 mg of polymer could be incorporated into one glass rod. The preparation of the casing for the glass rods has been described elsewhere,[6c] as well as the morphology of the polymer phase formed by precipitation polymerisation.

IR (Golden Gate/ATR): 2919, 2324, 1681, 1487, 1445, 1421, 1265, 833, 797, 708 cm^{-1}.

Amine **2** (184 mg, 0.6 mmol) and sodium *tert*-butylate (73 mg, 0.74 mmol) were dissolved in *N*-methylpyrrolidinone (15 mL) and pumped at 60 °C in cycles through a PASS*flow* reactor containing the resin (approx. 200 mg, 1 mmol chloride) described above. After 12 h the reactor was washed (H_2O, MeOH, CH_2Cl_2, MeOH, H_2O, MeOH and CH_2Cl_2; 10 mL each) and dried under reduced pressure to yield resin **3**. The loading was determined gravimetrically and by IR-spectroscopy (45 mg, 0.15 mmol).

IR (Golden Gate/ATR): 2921, 1733, 1661, 1445, 1224, 1154, 1021, 797, 704 cm^{-1}.

For ester saponification, lithium hydroxide (99 mg, 4.1 mmol) in MeOH–water (1 : 1, 20 mL) was pumped through the reactor at 50 °C for 60 h, after which time the reactor was washed with HCl (1 M), H_2O, MeOH, CH_2Cl_2, MeOH, H_2O, HCl (1 M), H_2O, MeOH, CH_2Cl_2 (10 mL each). Loading of **4** was determined gravimetrically and by IR spectroscopy (33 mg, 0.14 mmol).

IR (Golden Gate/ATR): 2918, 2359, 1729, 1667, 1607, 1443, 1269, 1153, 1017, 800, 707 cm^{-1}.

Treatment with dichlorodimethylsilane (10 mL, 10% in dichloromethane, 12 h at 20 °C) followed by $NiCl_2$ (10 mL, saturated, in water–DMSO 10 : 1) and water (10 mL) yielded Ni-NTA PASS*flow* reactor **5**.

(Methoxycarbonyl)methyl 2-(bis(methoxycarbonylmethyl)-L-amino)-3-(4-hydroxyphenyl)propionate (7). A solution of *O*-benzyl-L-tyrosine (2.0 g, 7.4 mmol) in 3-pentanone (15 mL) was treated with methyl bromoacetate (8.5 g, 55.5 mmol), K_2CO_3 (5.5 g, 55.5 mmol) and tetra-*N*-butylammonium iodide (20 mg, 54 μmol), and was stirred for 16 h at 108 °C. After cooling the suspension to r.t., water was added until all the K_2CO_3 was dissolved. The aqueous layer was extracted with ethyl acetate and the combined organic phases were dried ($MgSO_4$). After concentration under reduced pressure, the crude product was purified by flash column chromatography (petroleum ether–EtOAc 5 : 1) to yield (methoxycarbonyl)-methyl 2-(bis(methoxycarbonylmethyl)-L-amino)-3-(4-(benzyloxy)-phenyl)propionate (1.99 g, 4.08 mmol, 55%).

^{1}H NMR (400 MHz, $CDCl_3$, $CHCl_3$ = 7.26 ppm): δ = 7.42–7.30 (m, 5 H, OBn), 7.15 (d, J = 8.5 Hz, 2 H, H-5), 6.88 (d, J = 8.5 Hz, 2 H, H-6), 5.01 (s, 2 H, OBn), 4.59 (d, J = 15.8 Hz, 2 H, OC*H*H′CO_2CH_3), 4.50 (d, J = 15.8 Hz, 2 H, OCH*H*′CO_2CH_3), 3.80 (dd, J = 8.7 and 6.3 Hz, 1 H, H-2), 3.73 (s, 4 H, N$CH_2CO_2CH_3$), 3.70 (s, 3 H, $OCH_2CO_2CH_3$), 3.67 (s, 6 H, $NCH_2CO_2CH_3$), 3.05 (dd, J = 13.6 and 8.7 Hz, 1H, H-3a), 3.00 (dd, J = 13.6 and 6.3 Hz, 1H, H-3b) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, $CDCl_3$ = 77.0 ppm): δ = 171.5, 170.8, 167.9, 137.0, 130.2, 129.4, 128.4, 127.8, 127.3, 114.6, 69.8, 66.4, 60.4, 52.5, 52.0, 51.6, 35.9 ppm. HRMS-ESI: m/z $[M + H]^+$ calcd for $C_{25}H_{30}NO_9$: 488.1921, found: 488.1925.

Pd/C (10%, 600 mg) was added to a solution of (methoxycarbonyl)methyl 2-(bis(methoxycarbonylmethyl-L-amino)-3-(4-benzyloxyphenyl)propionate (1.99 g, 4.08 mmol) in MeOH, and the mixture was stirred at r.t. under an H_2 atmosphere. After 12 h the solution was filtered through a short pad of Celite™ and the eluant was concentrated under reduced pressure to yield the title compound **7** (1.61 g, 4.06 mmol, 99%).

IR (Golden Gate/ATR): 2954, 1733, 1516, 1437, 1205, 1142, 1012, 829 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$, $CHCl_3 = 7.26$ ppm): $\delta = 7.07$ (d, $J = 8.5$ Hz, 2 H, H-5), 6.73 (d, $J = 8.5$ Hz, 2 H, H-6), 4.60 (d, $J = 15.7$ Hz, 2 H, $OCHH'CO_2CH_3$), 4.53 (d, $J = 15.7$ Hz, 2 H, $OCHH'CO_2CH_3$), 3.80 (dd, $J = 8.6$ and 6.6 Hz, 1 H, H-2), 3.73 (s, 7 H, $NCH_2CO_2CH_3$, $OCH_2CO_2CH_3$), 3.69 (s, 6 H, $NCH_2CO_2CH_3$), 3.03 (dd, $J = 13.6$ and 8.6 Hz, 1H, H-3a), 2.98 (dd, $J = 13.6$ and 6.6 Hz, 1H, H-3b) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, $CDCl_3 = 77.0$): $\delta = 171.8, 171.1, 168.1, 154.7, 130.4, 128.7, 115.3, 66.6, 60.6, 52.7, 52.3, 51.7, 36.0$ ppm. HRMS-ESI: m/z $[M + Na]^+$ calcd for $C_{18}H_{23}NO_9Na$: 420.1271; found: 420.1271.

Preparation of a polar Merrifield Ni-NTA phase (9)

Tyrosine-based NTA-linker **7** (227 mg, 0.60 mmol) was dissolved in anhydrous DMF (15 mL). K_2CO_3 (166 mg, 1.2 mmol) and CsI (130 mg, 0.5 mmol) were added and the mixture was pumped at 60 °C in a circular mode through a PASS*flow* reactor containing the resin described above (approx. 200 mg; 1 mmol chloride). After 72 h the reactor was washed with H_2O, MeOH, CH_2Cl_2, MeOH, H_2O, MeOH and CH_2Cl_2 (10 mL each), and dried under reduced pressure to yield resin **8**. The loading was verified gravimetrically and by IR spectroscopy (110 mg, 0.30 mmol, 30% of theoretical binding sites).

IR (Golden Gate/ATR): 2919, 2324, 1741, 1680, 1512, 1441, 1266, 1215, 1155, 1017, 796, 709 cm^{-1}.

For ester saponification, lithium hydroxide (99 mg, 4.1 mmol) in MeOH–water (1 : 1, 20 mL) was pumped through the reactor at 50 °C for 60 h, after which time the reactor was washed with HCl (1 N), H_2O, MeOH, CH_2Cl_2, MeOH, H_2O, HCl (1 N), H_2O, MeOH, dichloromethane (10 mL each). The loading of **9** was determined gravimetrically and by IR-spectroscopy (110 mg, 0.29 mmol, 97%).

IR (Golden Gate/ATR): 2918, 2324, 1731, 1679, 1608, 1511, 1442, 1220, 1176, 1016, 797, 707 cm^{-1}.

Treatment with dichlorodimethylsilane (10 mL, 10% in dichloromethane, 12 h at 20 °C in cycles) followed by $NiCl_2$ (10 mL, saturated in water–DMSO 10 :1) and water (10 mL) yielded Ni-NTA PASS*flow* reactor **9**.

Synthesis of (*R*)-benzoin (11) using BAL on Ni-NTA resin (5)

Centrifuged lysate (250 µL, 50× concentrated) of *E. coli* (BAL, EC 4.1.2.38) was diluted with one aliquot lysis buffer [sodium phosphate (50 mM, pH 8.0), NaCl (300 mM), imidazole (10 mM), glycerol (10%)], and 100 mg of Ni^{2+} NTA-resin **5** was added. After one minute the resin was filtered and washed with lysis buffer (2 × 1 mL), washing buffer (4 × 1 mL) [sodium phosphate (50 mM, pH 8.0), NaCl (300 mM), imidazole (20 mM), glycerol (10%)] and reaction buffer (2 × 1 mL) [sodium phosphate (125 mM, pH 7.0), $MgSO_4$ (2.5 mM), thiamine diphosphate (0.25 mM), DMSO (25%)]. Then, benzaldehyde (15 µL to 200 µL in 5 mL reaction buffer) was added. The mixture was shaken (80 rpm) at 30 °C. For kinetic analysis (Fig. 4), a sample (200 µL) was taken every 30 min, extracted with EtOAc (200 µL) and analyzed by GC. For measuring the overall yield of benzoin production, the reaction was repeated under identical conditions, with the results described in Table 1.

Table 1

Amount of benzaldehyde	Reaction time	Isolated yield of (*R*)-benzoin
15 µL, 16 mg, 0.15 mmol	95 min	15 mg, 0.07 mmol, 93.0%
30 µL, 29 mg, 0.27 mmol	158 min	29 mg, 0.13 mmol, 99.9%
100 µL, 104 mg, 0.98 mmol	245 min	102 mg, 0.48 mmol, 98.0%
200 µL, 209 mg, 1.97 mmol	405 min	205 mg, 0.97 mmol, 98.0%

Synthesis of (*R*)-benzoin (11) using BAL on Ni-NTA resin (9)

Centrifuged lysate (250 µL, 50× concentrated) of *E. coli* (BAL, EC 4.1.2.38) was diluted with one aliquot lysis buffer [sodium phosphate (50 mM, pH 8.0), NaCl (300 mM), imidazole (10 mM), glycerol (10%)], and 100 mg of Ni^{2+} NTA-resin **9** was added. After one minute the resin was filtered and washed with lysis buffer (2 × 1 mL), washing buffer (4 × 1 mL) [sodium phosphate (50 mM, pH 8.0), NaCl (300 mM), imidazole (20 mM), glycerol (10%)] and reaction buffer (2 × 1 mL) [sodium phosphate (125 mM, pH 7.0), $MgSO_4$ (2.5 mM), thiamine diphosphate (0.25 mM), DMSO (25%)]. Then, benzaldehyde (15 µL to 200 µL in 5 mL reaction buffer) was added. The mixture was shaken (80 rpm) at 30 °C. For kinetic analysis (Fig. 5), a sample (200 µL) was taken every 30 min, extracted with EtOAc (200 µL) and analyzed by GC. For measuring the overall yield of benzoin production, the reaction was repeated under identical conditions, with the results described in Table 2.

Flow-through synthesis of (*R*)-benzoin (11)

BAL (EC 4.1.2.38)[11] containing cell lysate [750 µL, 25× concentrated in 50 mM potassium phosphate pH 8.0, *E. coli* SG13009 transformed with pKK233-2/BALHis$_6$ and induced with IPTG (0.1 mmol); cell lysis by ultrasonification on ice] was centrifuged and diluted with the same volume of lysis buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol) and pumped through the Ni^{2+}-NTA PASS*flow* reactor **9** at 0.5 mL min^{-1}. The column was washed with 3 mL lysis buffer and with 8 mL washing buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 20 mM imidazole, 10% glycerol). The reactor was equilibrated with 5 mL reaction buffer [125 mM sodium phosphate pH 7.0, containing DMSO (25%), $MgSO_4$ (2.5 mM) and thiamine diphosphate (0.25 mM)]. Benzaldehyde (30 µL, 0.3 mmol) in reaction buffer (5 mL, see above) was pumped through the reactor (37 °C, 1 mL min^{-1}) followed by

Table 2

Amount of benzaldehyde	Reaction time	Isolated yield of (*R*)-benzoin
15 µL, 16 mg, 0.15 mmol	85 min	14 mg, 0.07 mmol, 93.0%
30 µL, 29 mg, 0.27 mmol	90 min	29 mg, 0.13 mmol, 99.9%
100 µL, 104 mg, 0.98 mmol	90 min	98 mg, 0.46 mmol, 94.0%
200 µL, 209 mg, 1.97 mmol	150 min	200 mg, 0.94 mmol, 96.0%
500 µL, 522 mg, 4.97 mmol	540 min	386 mg, 1.82 mmol, 74.0%

reaction buffer (5 mL). No benzaldehyde could be detected in the eluate. The column was washed with EtOAc (20 mL) and the aqueous buffer was extracted with EtOAc. The combined extracts were dried ($MgSO_4$). After concentration under reduced pressure, the crude product was purified by flash column chromatography (petroleum ether–EtOAc 5 : 1) to yield the title compound **11** (31.5 mg, 0.15 mmol, 99%). The NMR data were found to be identical with those reported in the literature.[15]

$[\alpha]_D^{20}$ −141.2 (*c* 0.99 in acetone) 98% ee; R_f: 0.35 (CH_2Cl_2). HRMS-ESI: *m*/*z* [M + Na]$^+$ calcd for $C_{14}H_{11}O_2Na$: 235.0784, found: 235.0779.

Flow-through synthesis of (*R*)-2-hydroxy-1-phenylpropan-1-one (13)

BAL (EC 4.1.2.38)[11] containing cell lysate [750 μL, 25× concentrated in 50 mM potassium phosphate pH 8.0, *E. coli* SG13009 transformed with pKK233-2/BALHis$_6$ and induced with IPTG (0.1 mmol); cell lysis by ultrasonification on ice] was centrifuged and diluted with the same volume of lysis buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol) and pumped through the Ni^{2+}-NTA PASS*flow* reactor **9** at 0.5 mL min^{-1}. The column was washed with 3 mL lysis buffer and with 8 mL washing buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 20 mM imidazole, 10% glycerol). The reactor was equilibrated with 5 mL reaction buffer [125 mM sodium phosphate pH 7.0, containing DMSO (25%), $MgSO_4$ (2.5 mM) and thiamine diphosphate (0.25 mM)]. A mixture of benzaldehyde (30 μL, 0.3 mmol) and acetaldehyde (85 μL, 1.5 mmol) in reaction buffer (6 mL, see above) was pumped through the reactor in a circular mode. After 3 h at 37 °C no benzaldehyde could be detected in the eluate by GC, and the reaction was stopped. The column was washed with ethyl acetate (20 mL) and the aqueous buffer was extracted with ethyl acetate. The combined extracts were dried ($MgSO_4$). After concentration under reduced pressure, the crude product was purified by flash column chromatography (petroleum ether–EtOAc 5 : 1) to yield the title compound **13** (42 mg, 0.28 mmol, 92%). The NMR data were found to be identical with those reported in the literature.[15]

Flow-through synthesis of 6-*O*-acetyl-D-glucal (17)

BsubpNBE (5 mg) in loading buffer (1 mL, 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10% glycerol) was pumped though the Ni^{2+}-NTA PASS*flow* reactor **9** at 0.5 mL min^{-1}. The column was washed with 3 mL loading buffer and 7 mL reaction buffer (50 mM sodium phosphate pH 7.0). A solution of tri-*O*-acetyl-D-glucal (**16**, 100 mg, 0.38 mmol) in reaction buffer (45 mL) and MTBE (5 mL) was pumped through the reactor in a circular mode. After 60 h at 37 °C no starting material could be detected by TLC, and the column was washed with MTBE (20 mL). The combined aqueous and organic extracts were dried and purified by flash column chromatography (EtOAc) to yield the title compound **17** (57 mg, 0.30 mmol, 80%) and D-glucal **18** (7 mg 0.05 mmol, 12%).

^{1}H NMR (400 MHz, d_6-acetone, d_5-acetone = 2.05 ppm): δ = 6.31 (dd, J = 6.0 and 1.5 Hz, 1 H, H-1), 4.72 (dd, J = 6.0 and 2.2 Hz, 1 H, H-2), 4.58 (br d, J = 4.4 Hz, 1 H, OH), 4.43 (dd, J = 12.0 and 2.4 Hz, 1 H, H-6a), 4.32 (dd, J = 12.0 and 5.8 Hz, 1 H, H-6b), 4.16 (br m, 1H, H-3), 3.93 (ddd, J = 10.2, 5.8 and 2.4 Hz, 1 H, H-5), 3.62 (br m, 1 H, H-4), 2.94 (s, 1 H, -OH), 2.08 (s, 3 H, OAc) ppm. ^{13}C NMR (200 MHz, d_6-acetone, d_6-acetone = 29.8 ppm): δ = 171.0, 143.7, 105.2, 77.2, 70.6, 69.9, 63.8, 20.6 ppm.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG; grants Ki 397/8-1 and excellence cluster REBIRTH) and the Fonds der Chemischen Industrie. We thank Prof. Martina Pohl (University of Düsseldorf) for the expression vector pKK233-2/BALHis$_6$ and Prof. Uwe T. Bornscheuer (University of Greifswald) for a sample of BsubpNBE.

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