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Repetitive Batch as an Efficient Method for Preparative Scale Enzymic Synthesis of 5-Azido-Neuraminic Acid and ^{15}N-L-Glutamic Acid

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Abstract: Syntheses of both title compounds have been achieved on a multigram scale by enzymic synthesis. For multiple use the enzymes were recovered by means of ultrafiltration. 5-Azido-neuraminic acid was obtained by enzymic aldol condensation starting from 2-azido-2-deoxy-D-mannose and pyruvic acid with *N*-acetylneuraminic acid aldolase. ^{15}N-L-glutamic acid was obtained by reductive amination with glutamate dehydrogenase and regeneration of the cofactor. For both systems optimization of reaction conditions led to simplified downstream processing. HPLC-analysis was used to follow the reactions and to verify optical purity.

Introduction

Biotechnology offers a great potential for the synthesis of chiral compounds as drugs and pharmaceutical intermediates.[1] Whole microorganisms as well as purified enzymes are used for the production of valuable products, even in industrial scale synthesis.[2] Both types of biocatalysts show specific advantages and disadvantages as discussed elsewhere.[2,3] According to the high costs of some biocatalysts, their separation from products and reactants and subsequent reuse is a major goal. For isolated enzymes there are two main methods: immobilization on an insoluble support by adsorption or covalent coupling,[4] and membrane ultrafiltration of the medium with retention of the soluble biocatalyst, e.g. in an enzyme membrane reactor.[3] Both methods are used from bench scale up to large scale industrial syntheses. Enzymes immobilized on a support often show better stability than the soluble form and may be used in batch- or continuously operating processes. The major disadvantage is a loss of activity during the immobilization step and, in some cases, low volumetric activity of the immobilized biocatalyst. If soluble enzymes show sufficient operational stability in this form their application is advantageous as there are no mass transfer limitations. Recovery of the biocatalysts may be achieved by ultrafiltration membranes. For bench scale synthesis enclosure of enzymes in dialysis membranes has been described.[5] In this case mass transport across the membrane becomes rate limiting.

Here we describe a method for multigram scale syntheses using soluble enzymes. They were recovered by means of ultrafiltration. The technique of repetitive batch synthesis has been proven to be very effective and easy to handle. Commercially available stirred ultrafiltration cells were used for this purpose. These cells and membranes are available from many suppliers e.g. Amicon, Filtron, Sartorius or Millipore, with volumina from 3 mL up to several litres.[6] Fig. 1 shows the principle of this technique, which is described in the following:

- filling of the cell with substrate and enzyme solution
- reaction
- pressurizing the cell with argon or nitrogen to remove the product solution by filtration; the retentate is concentrated to 5% to 10% of the initial volume; the enzymes are retained within the cell by an ultrafiltration membrane
- addition of fresh substrate solution and repeating the cycle.

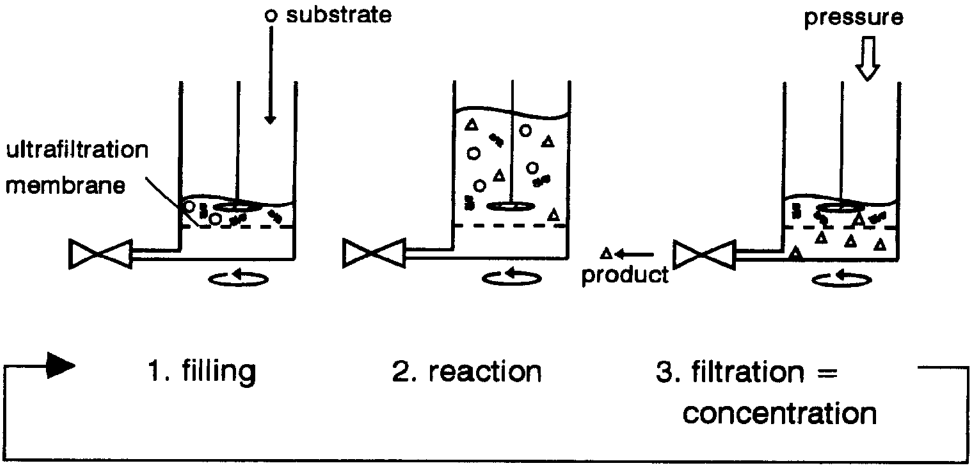


Figure 1: Principle of repetitive batch technique

The major advantages of the repetitive batch technique are:

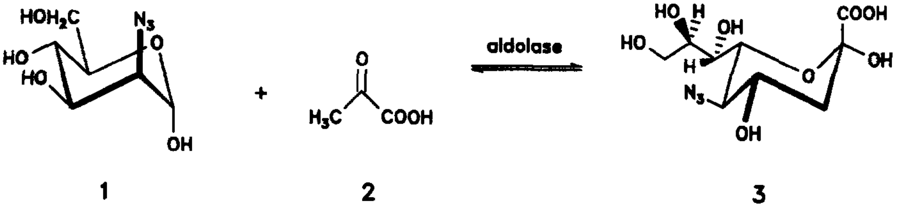
- no enzyme immobilization with loss of activity
- soluble enzymes cause no mass transfer limitations
- repeated use of enzymes reduces costs
- high volumetric activity possible to convert poor substrates at reasonable rates
- easy supply with fresh enzyme
- protein-free product solution

After synthesis is finished the enzyme may be washed several times with an appropriate buffer and stored in a refrigerator or deep freezer for further use.

Synthesis of 5-azido-neuraminic acid

Derivatives of *N*-acetylneuraminic acid (Neu5Ac), the most common compound of a class of carbohydrates known as sialic acids, are valuable building blocks for the synthesis of non-natural oligosaccharides.[7] They may act as enzyme inhibitors or influence biological recognition processes. A suitable access to Neu5Ac and its derivatives is via enzymatic synthesis using *N*-acetylneuraminic acid aldolase (E.C. 4.1.3.3) henceforth abbreviated as 'aldolase'. The aldolase catalyses the reversible condensation of pyruvate with *N*-acetylmannosamine. Variation of the carbohydrate gives an easy acces to Neu5Ac-derivatives.[8]

Here we describe the multigram synthesis of 5-azido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid **3** (5-azido-neuraminic acid) using the repetitive batch technique (scheme 1). The azido-group may be easily converted to the free amine and is therefore a valuable intermediate functionality. The first synthesis using immobilized aldolase has been described by Augé et al.[9]



Scheme 1

2-Azido-2-deoxy-D-mannose **1** was synthesized in high yield by treatment of *tert*-butyldimethylsilyl-3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-mannopyranoside[10] with sodium methoxide followed by neutralisation with cation exchange resin. The silyl ether was cleaved by treatment with HF/pyridine in THF.[11] The product was purified by flash-chromatography and was free of corresponding epimeric D-*gluco* derivative.

Following the results of a detailed investigation of thermodynamic and kinetic properties of the reaction system for the synthesis of Neu5Ac the conditions for the synthesis of **3** were chosen accordingly.[12] To determine conditions for achieving high conversion different concentrations of **1** and **2** were incubated together with aldolase. The change of concentrations during the reaction was followed by HPLC. In Fig. 2 conversion is shown as a function of reaction time.

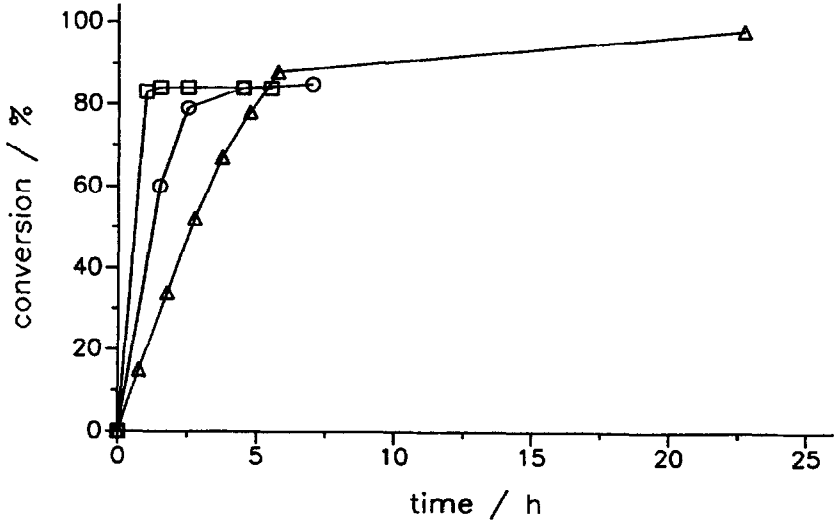


Figure 2: Synthesis of **3** at different substrate concentrations. □ 100 mM **1**, 400 mM **2**; ○ 300 mM **1**, 600 mM **2**; △ 300 mM **1**, 1200 mM **2**; aldolase 4 g/L, pH 7.5, 25 °C.

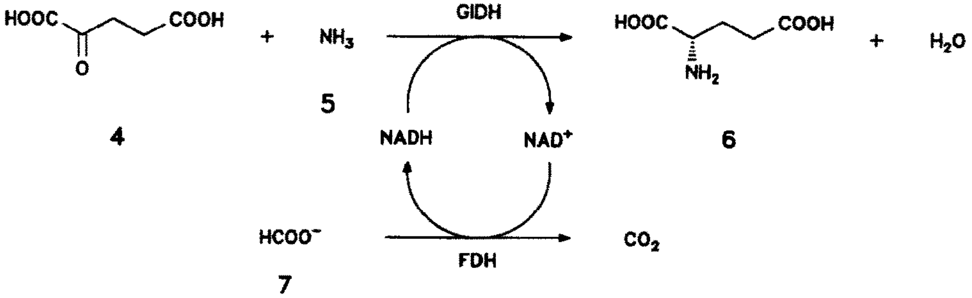
As in other experiments using Neu5Ac-aldolase no buffer, stabilizers or antibacterial agents were added. Especially buffer salts may be avoided as there is no pH-shift during the reaction. Furthermore, by omitting these compounds product isolation becomes easier. Increasing the overall substrate concentration the maximum conversion of 95% was obtained at only four-fold excess of pyruvate at reaction times of about one day.

For repetitive batch synthesis the highest substrate concentrations depicted in Fig. 2 were used. The reaction was performed in an ultrafiltration cell (max. volume 200 mL). After one day equilibrium conversion was achieved. The product solution was filtered off and fresh substrate solution was added. Seven batches were performed with the same enzyme yielding 700 mL product solution containing 60 g **3**. The product was recovered by ion-exchange chromatography on Dowex 1x2, formate form, by isocratic elution with 1 M formic acid. Fractions containing **3** were collected and lyophilised giving an overall yield of 80%. The product was identified by ^{1}H-NMR and mass spectrometry. The IR-spectrum shows the characteristic N_3 absorption.

Synthesis of ^{15}N-glutamic acid

Glutamic acid is an important amino acid residue in proteins and peptides and an important compound within the cell energy metabolism.[13] Isotope labelled glutamic acid may be useful for *in vivo* NMR studies or studying reaction centers of biologically active proteins. Recently, methods for the syntheses of ^{13}C- and ^{15}N-labelled L-glutamic acid have been developed.[14]

Here we decribe the improved enzymic synthesis of ^{15}N-L-glutamic acid **6** by reductive amination of 2-oxoglutaric acid **4** (2-oxopentanedioic acid, α-ketoglutaric acid) using glutamate dehydrogenase (GlDH, E.C. 1.4.1.3) applying the repetitive batch technique. Modifying the method used before, cofactor regeneration is done by formate dehydrogenase (FDH, E.C. 1.2.1.2)[15] (scheme 2), a well established method even for industrial scale synthesis.[3,16] By applying a high concentration of formate **7** the equilibrium of the reduction reaction is shifted towards the product side.



Scheme 2

For optimization of the reaction conditions small batch experiments were performed with non labelled material. Owing to contradictonary data about the enzyme, reaction conditions were simplified by omitting buffer substances, ADP and Zn^{2+}-ions. During the reaction no pH-shift was observed due to the buffer capacity caused by the reactants. Therefore there is no need for additional buffer substances. ADP is reported to be an activator for glutamate oxidation.[17] Inhibition and activation are reported for Zn^{2+}.[17] No significant loss of activity for the repeated use of GlDH lead to the conclusion, that zinc must be bound firmly to the protein, if it is required for enzymatic activity.

Some amino acids show quite low solubilities in water, especially at isoelectric pH. For glutamic acid, water solubility is given to be about 0.8 g/100 mL at 25 °C (equivalent to 54 mM), the isoelectric pH to be 3.2.[18] By increasing the concentration of **4** from 40 mM up to 200 mM the downstream processing is simplified to a large extent. The enzymes are removed by ultrafiltration of the solution. By adjusting the pH to 3.2 with concentrated hydrochloric acid and cooling 60% of formed **6** may be cristallized in a first step. Repeating this procedure after concentration of the remaining solution, most of the product is easily isolated in high purity. Ion-exchange work-up after the last cristallisation step is used to recover remaining **6**.

The concentrations finally used are summarized in table 1. For the labelled material a high conversion should be reached. Therefore no excess of ammonia **5** was used.

Table 1: Concentrations used for the synthesis of ^{15}N-L-glutamic acid **6**; the reaction was performed at pH 8.0 and 25 °C.

Compound	Concentration
2-oxoglutaric acid **4**	200 mM
$^{15}NH_4Cl$ '5'	200 mM
Na-formate '7'	400 mM
NAD^+	1 mM
GlDH	20 U/mL[a]
FDH	1 U/mL[a]

[a] units as defined in the data sheet

Concentrations of **4** and **6** were determined by HPLC. Under these conditions equilibrium conversion of **4** was found to be more than 95%; it was obtained after a reaction time of about four hours. Preparative scale synthesis was done in two series of batch experiments using two ultrafiltration cells with a maximum volume of 200 mL and 50 mL, respectively. Altogether six large batches and seven small batches were performed with the same enzymes yielding 1400 mL product solution containing 37 g **6**. To follow the reaction a small amount of the reaction mixture was put into a polarimeter and the change in optical rotation at 435 nm was recorded as depicted in Fig. 3 for five batches in the 200 mL cell.

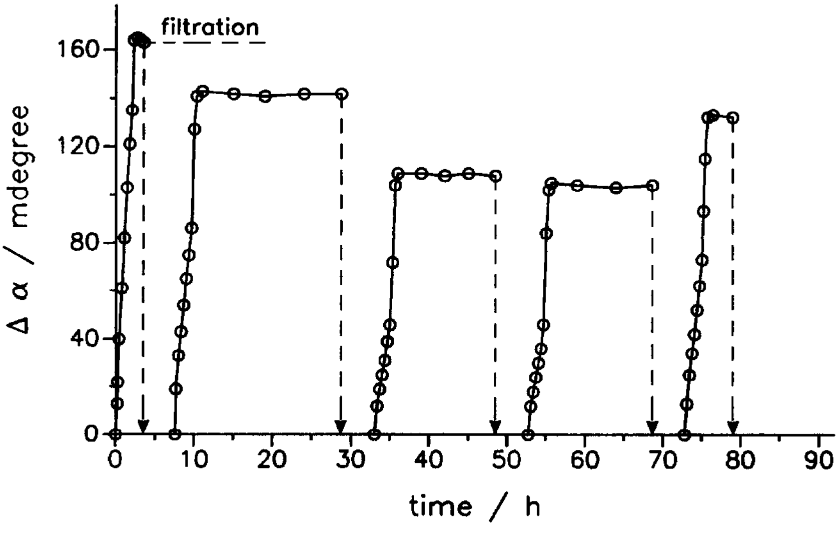


Figure 3: Change in optical rotation during repeated synthesis of **6**; measurement at 435 nm, 25 °C; reaction conditions as given in table 1.

As the optical rotation of amino acids is sensitive towards small variations in the pH of the solution the final value varies. The maximum value is reached after three to four hours. The filtration step requires the same time concentrating the solution to 5% to 10% of the initial volume. The figure also shows that the productivity of the reaction may be increased to a large extent if filtration and supplying with fresh substrate solution would be done as soon as possible.

The slope of the curves increases after two hours indicating a substrate surplus inhibition of GlDH. Due to the quite large inhibition constant of 24 mM[19], batch experiments are possible using high substrate concentrations. With smaller inhibition constants low reaction velocities at high substrate concentrations would be obtained. In this case continuously operating systems like the enzyme membrane reactor turn out to be advantageous as they are operating at high conversion corresponding to low stationary substrate concentrations.[20]

Crystallisation of the product at isoelectric pH yielded 33.7 g of **6** in three subsequent crops. From the remaining solution additional 2.4 g were obtained by ion-exchange chromatography, meaning an overall yield of 85% based on the labelled starting material. According to mass spectrometry the enrichment of nitrogen was 98%. The optical purity was checked by HPLC (Fig.4) using precolumn derivatization with *ortho*-phthalaldehyde and *N*-acetyl-L-cysteine. The optical purity of the glutamic acid derivative was found to be ›99.5%.

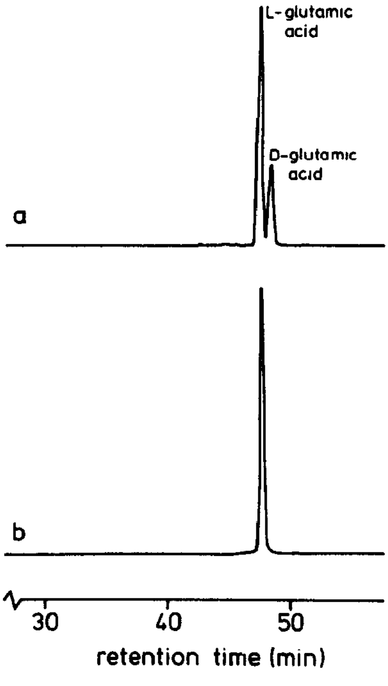


Figure 4: Optical purity determination of OPA-NAC-derivative of glutamic acid **6** by HPLC; a. analysis of a 70/30 mixture of L- and D-glutamic acid; b. optical purity of synthesized ^{15}N-glutamic acid (›99.5%); conditions are given in experimental section.

Discussion

As shown with these two examples, repetitive batch technique seems to be a feasible method for multigram enzymic syntheses avoiding additional expenditure on immobilizing the enzymes. The required apparatus is reasonably cheap and easy to handle. The method may be scaled up, limited only by the amount of enzyme or substrate available. Larger volumina may be handled using membrane stacks in ultrafiltration moduls possesing a large filtration area.

Furthermore some simple aspects for the optimization of reaction conditions were demonstrated. The major goal in each case was to simplify downstream processing. For **3** this was reached by reducing the required excess of **2** and by increasing the overall substrate concentration. Increasing the substrate concentration above product solubility at isoelectric pH, the major part of formed **6** has simply been isolated by precipitation. Buffers are often used in enzymic synthesis, but may be omitted to simplify product isolation if they have no stabilizing effect on the enzyme.

The biocatalyst consumption per unit weight of product, most often given as units (U) per kg product formed, is important for the evaluation of the process. By detailed thermodynamic and kinetic investigations this figure may be reduced to a large extent. For the synthesis of **3** 180000 U aldolase per kg were consumed, **6** required 27200 U GlDH and 1400 U FDH per kg product formed. Especially for the latter process these values may be reduced by applying lower enzyme concentrations or higher substrate concentration.

For the synthesis of **6** it is also shown that cofactor regeneration is not a problem preventing cost reduction for reactions catalyzed by dehydrogenases[21]. Applying a relatively high cofactor concentration of 1 mM a low cycle number of 200 was reached. Table 2 shows estimated costs for the synthesis of one mol **6** based on the conditions used here, which are not optimized to achieve lowest costs. By optimization of reaction conditions cycle numbers up to 600000 and enzyme consumptions less than 1000 U per kg product are possible.[3]

Table 2: Estimated costs (chemicals and enzymes) for synthesis of 1 mol ^{15}N-L-glutamic acid **6** (148 g/mol) based on 85% yield

Compound	Costs	
2-oxoglutaric acid **4** (1 mol)	62	DM[a]
$^{14}NH_4Cl$ '5' (1 mol)	6	DM[a]
$^{15}NH_4Cl$ '5' (1 mol)	5460	DM[b]
Na-formate '7' (2 mol)	4	DM[a]
NAD$^+$ (5 mmol, cycle number 200)	145	DM[a]
GlDH (4000 U)	94	DM[a]
FDH (200 U)	241	DM[c]
NADH (1 mol, no regeneration)	86060	DM[a]

[a] SIGMA, [b] ICON, [c] Boehringer

Experimental

General. Chemicals were purchased from FLUKA, Buchs, or SIGMA, Deisenhofen, otherwise indicated. $^{15}NH_4Cl$ (98%) was obtained from ICON (Services Inc.). NAD$^+$, free acid, was obtained from Merck, Darmstadt. All chemicals used were of the highest purity available.

Concentrations were determined by HPLC. Conditions for **1**, **2**, **3**, **4** and **7**: BioRad Aminex HPX-87H column, eluent 6 mM sulfuric acid, 65 °C, 0.8 mL/min, photometric detection at 205 nm. Conditions for **6**: Superspher RP 18 column, CS, Langerwehe, buffer A: 11 mM phosphate, pH 7.2, 0.5% THF, buffer B: 50% 11 mM phosphate pH 7.2, 35% methanol, 15% acetonitrile, gradient 50% A to 100% B within 10 min., 0.9 mL/min, 40 °C, precolumn derivatisation with *ortho*-phthalaldehyde, exitation/emission wavelengths 330/450 nm.

Conditions for determination of optical purity of **6**: RP C 18 Spherisorb ODS-2 (5 µm) Pharmacia column (250x4mm), buffer A: 30 mM sodium acetate pH 4, buffer B: 50% 30 mM sodium acetate pH 7.6, 50% acetonitrile, gradient 5% B to 20% B within 60 min, 0.4 mL/min, 25 °C, precolumn derivatisation with *ortho*-phthalaldehyde and *N*-acetyl-L-cysteine, exitation/emission wavelengths 360/405 nm.

NADH was estimated photometrically at 340 nm using 0.1 or 1 cm cuvettes (ϵ 6220 mol/(L*cm)).

Isotopic enrichment of the di-*n*-butyl-*N*-trifluoroacetyl derivative of **6** was measured with a Finnigan MAT 900 mass spectrometer (EI, 70 eV).

Enzymes. Neu5Ac-aldolase (E.C. 4.1.3.3, *E. coli*) was purchased from Toyobo, Osaka, Japan, respectively from the European distributor Seppim, Sees, France. Glutamate deydrogenase (GlDH, E.C. 1.4.1.3, bovine liver) was purchased from SIGMA as solution in 50% glycerol, lyophilized formate dehydrogenase (FDH, E.C. 1.2.1.2, *Candida boidinii*) was from Boehringer, Mannheim.

*Synthesis of 2-azido-2-deoxy-*D*-mannose* **1**. *tert*-Butyldimethylsilyl-3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-mannopyranoside[10] was treated with sodium methoxide followed by neutralisation with acidic ion exchange resin (Dowex 50Wx8). The anomeric silyl ether was cleaved by treatment with HF/pyridine in THF at 0 °C (85% yield). **1** was purified by flash-chromatography using chloroform/methanol (7:1 v/v) as eluent. The product was free of corresponding epimeric D-*gluco* derivative. The ^{1}H-NMR data (250 MHz) agreed with those reported in reference.[22] $[\alpha]_D^{20}$ = -32.0 (c = 0.5, methanol), lit. -36.4 (c = 1.1, methanol).[22] The IR-spectrum shows the characteristic N_3 absorption at 2105 cm^{-1}.

Synthesis of 5-azido-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid **3** *(5-azido-neuraminic acid)*. 90 mL of a solution containing 6.15 g **1** and 10.56 g **2**, sodium salt, is placed in an stirred ultrafiltration cell (Amicon, Model 202 respectively 8200, equipped with a membrane YM 5, cutoff 5,000 g/mol) after having the pH adjusted to 7.5 with diluted NaOH. 400 mg of aldolase (spezific activtity 23 U/mg)[23] are solubilised in 10 mL deionized water and added to the reaction solution (final concentrations: enzyme 4 g/L, **1** 300 mM, **2** 1200 mM). To follow the reaction samples are withdrawn at appropriate time intervals and analysed by HPLC. After 24 hours at 25 °C equilibrium conversion is reached (95% based on **1**). The solution containing product and non-reacted substrates is separated from the enzyme by pressurizing the filtration cell with argon or nitrogen. The retentate is concentrated to 10 mL. 90 mL fresh substrate solution containing **1** and **2** in the same concentrations is added to the remaining solution in the filtration cell and treated as the first batch. Seven batches yielding 700 mL product solution containing 290 mM **3** were performed. To recover the product 100 mL of the solution are applied to an column (5x50 cm) with Dowex 1x2, 200-400 mesh, formate form. Remaining **1** is removed by washing with water. **3** and **2** are eluted isocratically with 1 M formic acid. Fractions containing **3** are collected and lyophilised. Traces of formic acid are removed by dissolving **3** in a small amount of water and lyophilising a second time to give a white powder. After purification 85% of the product are recovered, giving an overall yield of 80%. The ^{1}H-NMR data (500 MHz, D_2O) agreed with those reported in reference.[9] From the signal intensity the anomeric ratio α:β was estimated to be 1:9. The IR-spectrum (nujol) shows the characteristic N_3 absorption at 2100 cm^{-1}. $[M-H]^+$ was found at 292 m/z (FAB-MS). $[\alpha]_D^{20}$ = -62.2 (c = 0.66, H_2O), lit. -54.8 (c = 5).[24]

Synthesis of ^{15}N-L-glutamic acid **6**. Only the procedure for the synthesis using the large ultrafiltration cell (Amicon, Model 202 respectiviely 8200, equipped with a membrane YM 5, cutoff 5,000 g/mol) is described, which is the same as for the smaller cell (Amicon, Model 8050, equipped with a membrane YM 5, cutoff 5,000 g/mol). 1450 mL substrate solution containing 200 mM **4** (free acid), 200 mM $^{15}NH_4Cl$ ('**5**') and 400 mM Na-formate ('**7**') were prepared. The pH was adjusted to 8.0 using NaOH. 190 mL of the solution were placed into the stirred ultrafiltration cell. 133 mg NAD$^+$, free acid, 300 mg FDH (0.6 U/mg) and 1 mL GlDH solution (3900 U/mL) were added. After mixing 1 mL of this solution was placed into a polarimeter cuvette and the change of the optical rotation was recorded using a Perkin-Elmer 241 polarimeter. The ultrafiltration cell was kept at 25 °C. When a steady state for the optical rotation was reached, samples were withdrawn for HPLC analysis. By pressurizing the ultrafiltration cell with argon the solution containing product and non-reacted substrates is separated from the enzymes. The retentate is concentrated to 10 mL. 180 mL fresh substrate solution and 133 mg NAD$^+$ are added to the remaining solution in the filtration cell and treated as the first batch. Six large batches and seven small batches were performed yielding 1400 mL product solution containing 37 g **6** according to HPLC analysis. Each of the seperate batches were acidified with concentrated hydrochloric acid and cooled overnight at 4 °C. The crystalline precipitates were filtered off and washed with cold water yielding 22.7 g of **6**. The residual filtrates were combined and concentrated until some precipitation occurs. The solids were redissolved by adding some water and the procedure of acidification was repeated. A second and third crop of 8.9 g and 2.7 g, respectively, were obtained. The remaining liquid was passed through a column (4x25 cm) packed with Dowex 1x8 ion-exchange resin, formate form. After washing with water, **6** was eluted with 1 M formic acid. Repetitive lyophilisation yielded another 2.4 g, resulting in a total quantity of 36.7 g **6** (86% yield). ^{1}H-NMR (200 MHz,NaOD/D_2O) and ^{13}C-NMR (50.1 MHz, NaOD/D_2O) data were in agreement with the literature.[14] The presence of ^{15}N-isotope is shown by the splitting of C2-signal (56.5 ppm) in the ^{13}C-NMR spectrum ($^1J(^{15}N-^{13}C)$ 4 Hz). MS analysis (EI, 70 eV) of the di-*n*-butyl-*N*-trifluoroacetyl derivative of **6** gives the characteristic signals[14], each one mass unit more than in the spectrum of natural abundant **6**: *m/z* 283 (20%), 255 (100%), 199 (90%), 181 (80%), 153 (65%). The enrichment of nitrogen was determined as 98%. The optical purity was determined by HPLC as being >99.5%.

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