Supplementary Information

Biocatalytic access to betazole by one-pot multienzymatic system in continuous flow

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Batch reaction of biocatalytic synthesis of betazole drug from 2-(1H-pyrazol-3-yl)ethanol with and without the presence of LpNOX

Batch reactions with pure soluble enzymes were performed at 30º C in 5 mL of reaction mixture containing 10 mM 2-(1H-pyrazol-3-yl)ethanol, 10 mM NAD^+(without LpNOX) or 1 mM NAD^+(with LpNOX), 10 mM (S)-(−)-α-methylbenzylamine, 0.1 mM PLP, 0.1 mM FAD (only in reaction with LpNOX), 50 mM potassium phosphate buffer pH 8, HLADH (0.8 mg mL^{-1}), HEWT (0.2 mg mL^{-1}), and LpNOX (0.2 mg mL^{-1}). The reactions were monitored by HPLC on a Waters XBridge C18 column (3.5µm, 2.1 × 150mm) with a gradient method of phase A (0.1% TFA in water) and B (0.1% TFA in acetonitrile). Gradient: 0 min 95% A 5% B; 1 min 95% A 5% B; 5min 5% A 95% B; 5.10 min 0% A 100% B; 6.60 min 0% A 100% B; 7min 95% A 5% B; 10 min 95% A 5% B. Injection volume 2 µL, at 45º C with a flow rate of 0.8 mL/min. Retention time of acetophenone: 3.85 min. Molar conversions were calculated through a standard curve of acetophenone.

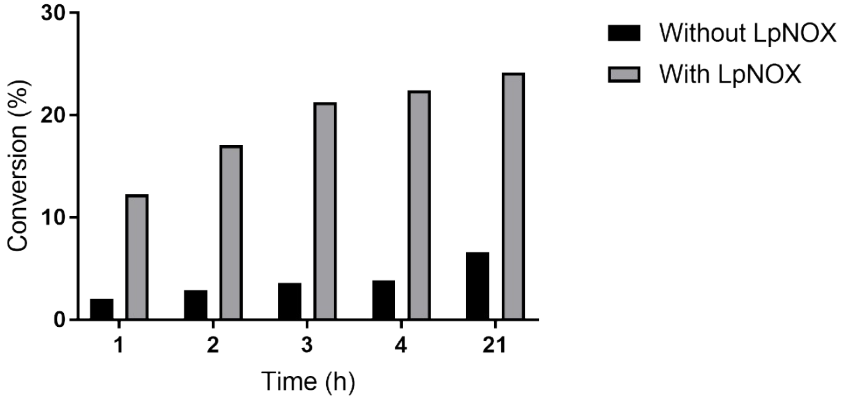

Fig. S1 Synthesis of betazole drug in batch reactions catalysed by soluble HLADH and HEWT (black bars) or soluble HLADH, HEWT and LpNOX (grey bars), monitoring the formation of acetophenone by HPLC. Reaction conditions: 10 mM 2-(1H-pyrazol-3-yl)ethanol in phosphate buffer (0.05 M, pH 8), 10 mM NAD^+(without LpNOX) or 1 mM NAD^+(with LpNOX), 10 mM (S)-(−)-α-methylbenzylamine, 0.1 mM FAD, 0.1 mM PLP. T = 30º C.

Covalent immobilisation of HLADH

A) On glyoxyl-functionalysed carrier: Covalent immobilisation of HLADH on methacrylate-based porous carriers functionalised with glyoxyl groups was developed using different loadings of this enzyme per gram of carrier. 1 g of Relisorb®EP40OSS functionalised with 100 $\mu mol_{glyoxyl}\ g_{carrier}^{-1}$, as described in *Experimental section*, were

resuspended in 10 mL solution containing 1 mg, 5 mg, 10 mg, or 20 mg of pure HLADH, respectively, in 100 mM $NaHCO_3$ pH 10. An aliquot of the same enzyme solutions was taken before resuspending the functionalised carrier and was used as control. After 1 h incubation at 4ºC under mild agitation, 10 mg of $NaBH_4$ were added and further incubated for 30 min at 4º C under mild agitation. The resulting HLADH immobilised biocatalyst was filtered and washed with H_2O. The HLADH activity in the remaining liquid phase before $NaBH_4$ addition and in the control was measured. 2 samples of 20 mg of the immobilised HLADH biocatalyst were taken to determine the specific activity of the immobilised HLADH enzyme (in duplicate). 100% immobilisation yield was obtained for all the HLADH loadings tested.

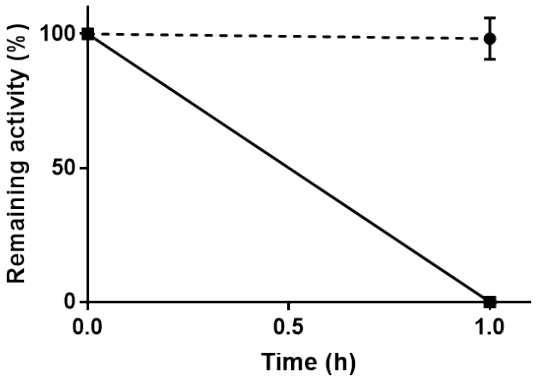

Fig. S2 Remaining activity of the control (dashed line) and the supernatant (solid line) from the suspension on the covalent immobilisation of HLADH on glyoxyl-functionalised methacrylate carrier (at 20 $mg_{HLADH}\ g_{carrier}^{-1}$).

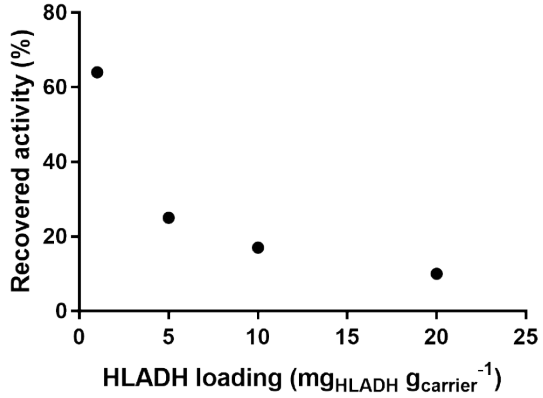

Fig. S3 Recovered activity (%) obtained from covalent immobilisation of HLADH on glyoxyl-functionalised methacrylate carrier at different HLADH loadings per gram of carrier.

B) On epoxy-functionalised carrier: Covalent immobilisation of HLADH on methacrylate-based porous carriers functionalised with epoxy groups was also attempted. 1 g of Relisorb®EP40OSS were resuspended in 10 mL solution containing 20 mg of pure HLADH in 100 mM $NaHCO_3$ pH 10. An aliquot of the same enzyme solutions was taken before resuspending the functionalised carrier and was used as control. After 1 h incubation at 4ºC under mild agitation, the HLADH activity in the remaining liquid phase before and in the control was measured.

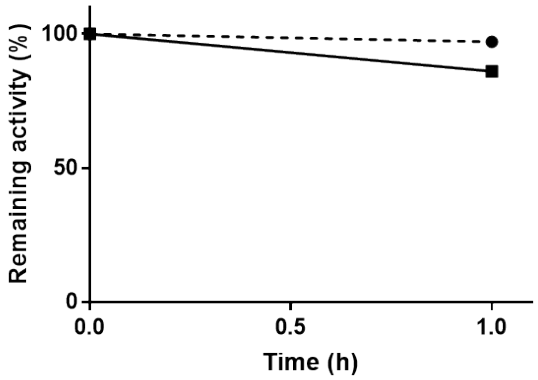

Fig. S4 Remaining activity of the control (dashed line) and the supernatant (solid line) from the suspension on the covalent immobilisation of HLADH on epoxy-functionalised methacrylate carrier (at 20 mg_{HLADH} $g_{carrier}^{-1}$).

Covalent immobilisation of LpNOX

Covalent immobilisation of LpNOX based on Co^{2+} - epoxy chemistry, was developed by the previously described procedure.[2] In short, 0.5 g of Relizyme®EP403/S were resuspended in 1 mL of 0.1 M sodium borate, 2 M iminodiacetic acid in 50 mM phosphate buffer pH 8.5, and incubated for 2 h at room temperature under mild agitation. The carrier was filtered, washed with H_2O, and added to 2.5 mL of 1 M NaCl 5 mg mL^{-1} $CoCl_2$ in 50 mM phosphate buffer pH 6. After 2 h incubation at room temperature under mild agitation, the carrier was filtered and washed with H_2O. The resulting Co^{2+} - epoxy carrier was used to immobilise LpNOX. A 5 mL solution containing 2.5 mg of pure LpNOX in 50 mM potassium phosphate buffer pH 7 – 8, was added to the carrier. The suspensions were incubated for different times at 4º C under mild agitation. The resulting LpNOX immobilised catalysts were filtered, washed with 50 mM EDTA 0.5 M NaCl in 20 mM phosphate buffer pH 7.2 and finally washed with H_2O.

In case of covalent immobilisation based on glyoxyl reactive groups, 0.5 g of Relizyme®HG403/S or Relisorb®HG400SS were resuspended in 5 mL of 10 mM $NaIO_4$. After 2 h incubation at room temperature under mild agitation, the resulting carrier, was filtered and washed with H_2O. The resulting glyoxyl group density was 100 µmol $g_{carrier}^{-1}$. In case of 50 µmol $g_{carrier}^{-1}$ glyoxyl group density (a), 0.5 g of carrier were resuspended in 5 mL of 5 mM $NaIO_4$, and the same procedure was followed. In case of Relizyme®HFA403/S (b), prior to oxidation of diol groups, 0.5 g of this carrier were resuspended in 5 mL of 0.5 M H_2SO_4. The suspension was incubated for 30 min at room temperature under mild agitation. The hydrolysed carrier was filtered and washed with H_2O. Resulting glyoxyl groups were used to immobilise LpNOX. A 5 mL solution containing 2.5 mg of pure LpNOX in 100 mM $NaHCO_3$ pH 9, or 50 mM potassium phosphate buffer pH 7 – 8, was added to the resin. In case of LpNOX immobilisation in the presence of ethylendiamine (EDA) (c), different amounts of EDA were added to this mixture to yield 2 mM – 20 mM EDA. The suspensions were incubated for different times at 4º C under mild agitation, and 30 mg of $NaBH_4$ or 30 mg of $NaBH_4$ resuspended in 100 mM $NaHCO_3$ pH 8.5, were then added and further incubated for 30 min at 4º C under mild agitation. In one immobilisation condition (d), prior to reduction, 20% oxidized dextran (Dex20, prepared as previously described[3]) was added to the suspension at a ratio of 10 mg Dex20 mg_{LpNOX}^{-1}. After reduction, the resulting LpNOX immobilised catalysts were filtered and washed with H_2O. LpNOX activity in the remaining liquid phase before $NaBH_4$ addition was measured. The immobilised LpNOX biocatalysts were filtered and washed with H_2O.

In each case, 2 samples of 20 mg of the immobilised LpNOX biocatalyst were taken to determine the specific activity of the immobilised LpNOX enzyme (in duplicate).

Table S1 Immobilisation yield (%) and recovered activity (%) obtained from covalent immobilisation of LpNOX at different conditions. LpNOX offered to the carrier 5 mg_{LpNOX} $g_{carrier}^{-1}$. Immobilisation temperature 4ºC.

Reactive group	Matrix	Immobilisation time	pH	Immobilisation yield (%)	Recovered activity (%)
$Co2^{+}$- Epoxy	Relizyme® EP403/S	4 h	8	100	5

$Co2^{+}$- Epoxy	Relizyme® EP403/S	4 h	7	98	8
$Co2^{+}$- Epoxy	Relizyme® EP403/S	1 h	8	100	1
Glyoxyl	Relizyme® HG403/S	3 h	8	58	10
Glyoxyl	Relizyme® HG403/S	3 h	9	51	7
Glyoxyl	Relizyme® HG403/S	1 h	8	66	2
Glyoxyl	Relizyme® HG403/S	3 h	7 - 8.5	75	10
Glyoxyl (50 µmol g^{-1})[a]	Relizyme® HG403/S	24 h	7 - 8.5	37	4
Glyoxyl[b]	Relizyme® HFA403/S	2 h	7 - 8.5	100	3
Glyoxyl (50 µmol g^{-1})[a,b]	Relizyme® HFA403/S	2 h	7 - 8.5	100	3
Glyoxyl, dextran[d]	Relizyme® HG403/S	3 h	7 – 8.5	92	5
Glyoxyl	Relisorb® HG400SS	3 h	7 - 8.5	89	16
Glyoxyl, 2 mM EDA[c]	Relisorb® HG400SS	3 h	7 - 8.5	78	18
Glyoxyl, 10 mM EDA[c]	Relisorb® HG400SS	3 h	7 - 8.5	88	24
Glyoxyl, 20 mM EDA[c]	Relisorb® HG400SS	3 h	7 - 8.5	77	15

Quantification of initial diol and epoxy groups density in Relisorb® EP400SS

Quantification of initial diol groups density: 0.3 g of Relisorb® EP400SS were resuspended in 3 mL of of 10 mM $NaIO_4$. After 2 h incubation at room temperature under mild agitation, the resulting carrier, was filtered and washed with H_2O. Resulting glyoxyl groups density in $\mu mol_{glyoxyl}\ g_{carrier}^{-1}$, were quantified as previously described.[1] The initial diol groups density in $\mu mol_{glyoxyl}\ g_{carrier}^{-1}$, was calculated as follows:

$$Initial\ diol\ groups\ density\ \left(\mu mol_{glyoxyl}\ g_{carrier}^{-1}\right) = Resulting\ glyoxyl\ groups\ density\ \left(\mu mol_{glyoxyl}\ g_{carrier}^{-1}\right)$$

Quantification of initial epoxy groups density: 0.3 g of Relisorb® EP400SS were resuspended in 3 mL of 0.5 M H_2SO_4, and the suspension was incubated for 2 h at room temperature under mild agitation. The hydrolysed carrier was filtered, washed with H_2O and resuspended in 30 mL of 40 mM $NaIO_4$. After 2 h incubation at room temperature under mild agitation, the resulting carrier, was filtered and washed with H_2O. Resulting glyoxyl groups density after total hydrolysis in $\mu mol_{glyoxyl}\ g_{carrier}^{-1}$, were quantified as previously described.[1] The initial epoxy groups density in $\mu mol_{glyoxyl}\ g_{carrier}^{-1}$, was calculated as follows:

$$Initial\ epoxy\ groups\ density\ \left(\mu mol_{glyoxyl}\ g_{carrier}^{-1}\right) = Resulting\ glyoxyl\ groups\ density\ after\ total\ hydrolysis\ \left(\mu mol_{glyoxyl}\ g_{carrier}^{-1}\right) - Initial\ diol\ groups\ density\ \left(\mu mol_{glyoxyl}\ g_{carrier}^{-1}\right)$$

Table S2 Estimated values of initial diol groups density and initial epoxy group density of Relisorb® EP400SS.

	$\mu mol_{glyoxyl}\ g_{carrier}^{-1}$
Initial diol groups density	67
Initial epoxy groups density	94

Stability of co-immobilised HLADH, HEWT and LpNOX

The co-immobilised biocatalyst resulting from immobilisation of HLADH, HEWT and LpNOX, was evaluated for stability at reaction conditions, 30ºC and pH 8.

0.5 g of the co-immobilised HLADH and HEWT biocatalyst after coating with PEI were fabricated, and the specific activities of the immobilised HLADH and HEWT were determined as described in *Methods* (initial specific activity). 4 aliquots of 0.1 g of this catalyst resuspended in 1 mL 0.1 mM PLP 50 mM phosphate buffer pH 8 were prepared and respectively incubated for 1 h, 2 h, 24 h or 48 h at 30º C under constant shaking. At these time points, the catalyst was filtered and the specific activities of the immobilised HLADH and HEWT were determined (in duplicate). The relative activities at different time points were calculated as the percentage of the specific activities of immobilised HLADH and HEWT at a time point with respect to the initial specific activities.

0.5 g of the co-immobilised HLADH, HEWT and LpNOX biocatalyst were fabricated, and the specific activity of the immobilised LpNOX was determined as described in *Methods* (initial specific activity). 4 aliquots of 0.1 g of this catalyst resuspended in 1 mL 1 mM FAD 50 mM phosphate buffer pH 8 were prepared and respectively incubated for 1 h, 2 h, 24 h or 48 h at 30º C under constant shaking. The same procedure was followed.

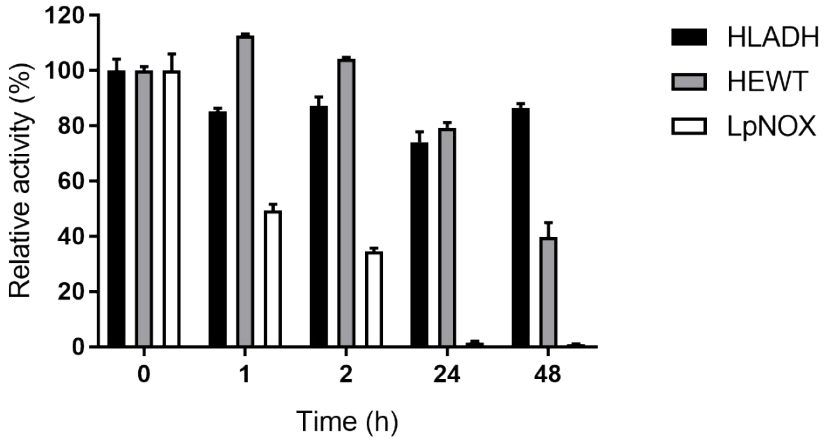

Fig. S5 Stability of co-immobilised HLADH, HEWT and LpNOX biocatalyst at 30º C pH 8.

Desorption and re-immobilisation of LpNOX

The co-immobilised biocatalyst resulting from immobilisation of HLADH, HEWT and LpNOX, was packed in a column and used to catalyse the synthesis of betazole from 2-(1H-pyrazol-3-yl)ethanol in continuous flow. The reaction was developed as follows: a single liquid phase containing the substrate at 10 mM, 1 mM NAD^+, 20 mM IPA, 0.1 mM PLP, 0.1 mM FAD, a residence time of 30 min and 30º C. The reaction was running for 2 h, and then, the co-immobilised catalyst was unpacked, and 2 samples were taken to determine the specific activity of the immobilised LpNOX as described in *Methods*. The 3 g co-immobilised biocatalyst were resuspended in 30 mL 0.2 M NaCl and incubated for 30 min at room temperature and mild agitation to desorb the immobilised LpNOX enzyme. The co-immobilised biocatalyst was filtered, washed with water and the specific activities of the immobilised HLADH and HEWT were determined as described in *Methods*. Fresh LpNOX was immobilised on the same catalyst and the specific activity of the immobilised LpNOX was determined as described in *Methods*. The relative activities after different steps were calculated as the percentage of the initial specific activities.

Table S3 Relative specific activities of immobilised HLADH, HEWT and LpNOX after flow reaction, LpNOX desorption and fresh LpNOX immobilisation. Flow reaction conditions: a single liquid phase containing 10 mM 2-(1H-pyrazol-3-yl)ethanol, 1 mM NAD+, 20 mM IPA, 0.1 mM PLP, 0.1 mM FAD, in phosphate buffer at pH 8; 30 min residence time; 30º C; and 2 h reaction time. Desorption conditions: 2 M NaCl (10 mL $g_{catalyst}^{-1}$) for 30 min at room temperature.

	Relative activity (%)		
	HLADH	HEWT	LpNOX
Initial	100	100	100
After flow reaction	-	-	1.0
After desorption	84.3	134.6	-
LpNOX immobilisation[a]	-	-	35.3

[a]Immobilisation yield was 65.4%

Evaluation of HLADH activity to 2-(1H-pyrazol-3-yl)ethanol

HLADH activity assays were performed in triplicate in 96-well microplates using Epoch 2 Microplate Spectrophotometer (Biotek). The activity assays were performed at 25º C and pH 8 (potassium phosphate buffer), following the formation of NADH at 340 nm using. One unit of enzymatic activity was defined as the formation of 1 µmol of NADH per minute. The assay conditions were 1 mM – 50 mM 2-(1H-pyrazol-3-yl)ethanol, 1 mM NAD^+, 2 equivalents IPA (2 mM – 100 mM), 0.1 mM PLP, 71 µg/mL HEWT and 31.4 µg/mL – 62.8 µg/mL HLADH.

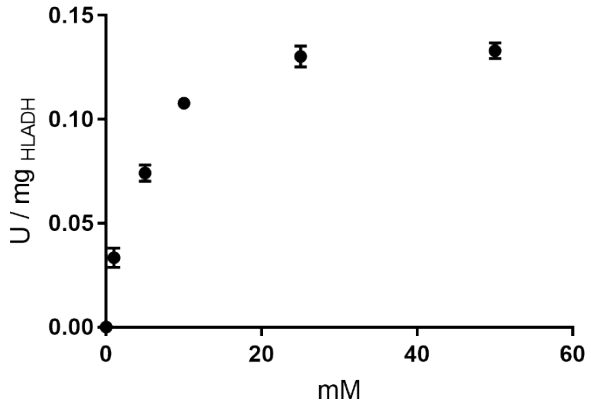

Fig. S6 Activity HLADH to different concentrations of 2-(1H-pyrazol-3-yl)ethanol monitoring the formation of NADH by UV (340 nm) in a 96-well microplate. Assay conditions: 1 mM – 50 mM 2-(1H-pyrazol-3-yl)ethanol in phosphate buffer (0.05 M, pH 8), 1 mM PLP, 71 µg $_{HEWT}$ mL^{-1} and 31.4 µg/mL – 62.8 µg $_{HLADH}$ mL^{-1}. T = 25º C.

Calculation of space time yield and catalyst productivity

The space time yield (STY) was calculated as follows:

$$STY\left(g_{betazole}\,L^{-1}\,h^{-1}\right) = \frac{Substrate\ concentration_{inlet}\,(M)\ \times\ Conversion_{outlet}\,(\%)\times\ Flow\ rate\left(L\,h^{-1}\right)\times MW}{Reactor\ volume\,(L)\ \times\ 100}$$

The flow rate was 0.0072 L h^{-1}, 0.00732 L h^{-1}, and 0.00738 L h^{-1}, for the 10 mM, 30 mM or 50 mM scale reactions, respectively. The reactor volume is 0.0036 L, 0.00366 L, and 0.00369 L, for the 10 mM, 30 mM or 50 mM scale reactions, respectively. Molecular weight of betazole is 111.145 g mol^{-1}.

The catalyst productivity was calculated as follows:

$$Catalyst\ productivity\left(\mu mol_{betazole}\ h^{-1}\,mg_{enzyme}{}^{-1}\right) = \frac{STY\,(g_{betazole}\,L^{-1}\,h^{-1})}{\frac{Total\ enzyme\ load\ (mg)}{Reactor\ volume\,(L)}}$$

The total enzyme load was calculated as follows:

$$Total\ enzyme\ load\ (mg) = Packed-bed\,(g)\times\left[HLADH\ load\left(mg\ g_{carrier}{}^{-1}\right)+HEWT\ load\left(mg\ g_{carrier}{}^{-1}\right)+LpNOX\ load\left(mg\right.\right.$$

The amount of packed-bed is 3 g and the HLADH, HEWT and LpNOX loads were 20 mg $g_{carrier}^{-1}$, 5 mg $g_{carrier}^{-1}$ and 5 mg $g_{carrier}^{-1}$, respectively.

SDS-page electrophoresis at the exit of flow reaction

A sample was taken at the exit of the 10 mM flow reaction (after 20 x 15 min residence time) in the synthesis of betazole and analysed by SDS-page electrophoresis.

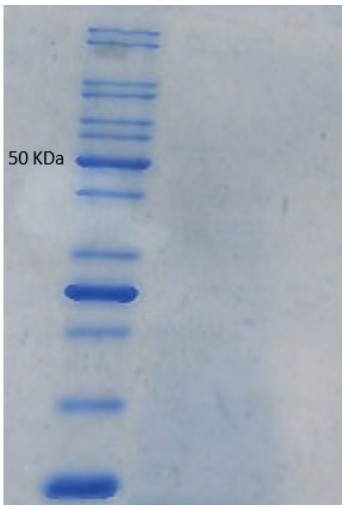

Fig. S7 SDS-page electrophoresis of a sample taken at the exit of a 10 mM flow reaction (after 20 x 15 min residence time) in the synthesis of betazole.

Downstream recovery of betazole drug

8.5 mL of product mixture resulting from the 30 mM flow reaction (implemented with segmented flow and recirculation, 20 x 15 min) yielding 85% m. c., was flowed into a column packed with tailored aldehyde-resin to trap primary amines-containing molecules, betazole and IPA. The flow rate was set up to 122 µL min^{-1} to allow a residence time of 30 min (temperature off). 4 column volumes of 50 mM phosphate buffer pH 8 were flowed to the system for washing. The elution of the trapped primary amines by the aldehyde groups was carried out with 4 column volumes of 0.2% HCl. All the fractions were collected and betazole concentration was determine by HPLC, as described in *Analytical methods* section.

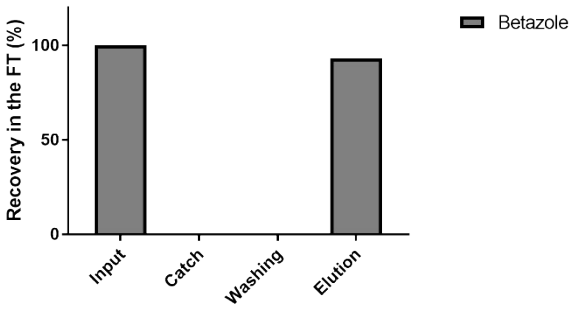

Fig. S8 In-line recovery of betazole and IPA resulting in flow using a column packed with tailored aldehyde-resin. Column volume 3.66 mL. 30 min residence time. Washing: 50 mM potassium phosphate buffer pH 8, 4 column volumes. Elution: 0.2% (v/v) HCl, 4 column volumes.

The second and third elution fractions containing betazole (summing up 93% of initial betazole) were pooled and freeze-dried for 90 h to remove H_2O. The resulting solid fraction was resuspended in deuterium oxide and analysed by ^{1}H NMR, ^{13}C NMR, and ^{13}P NMR.

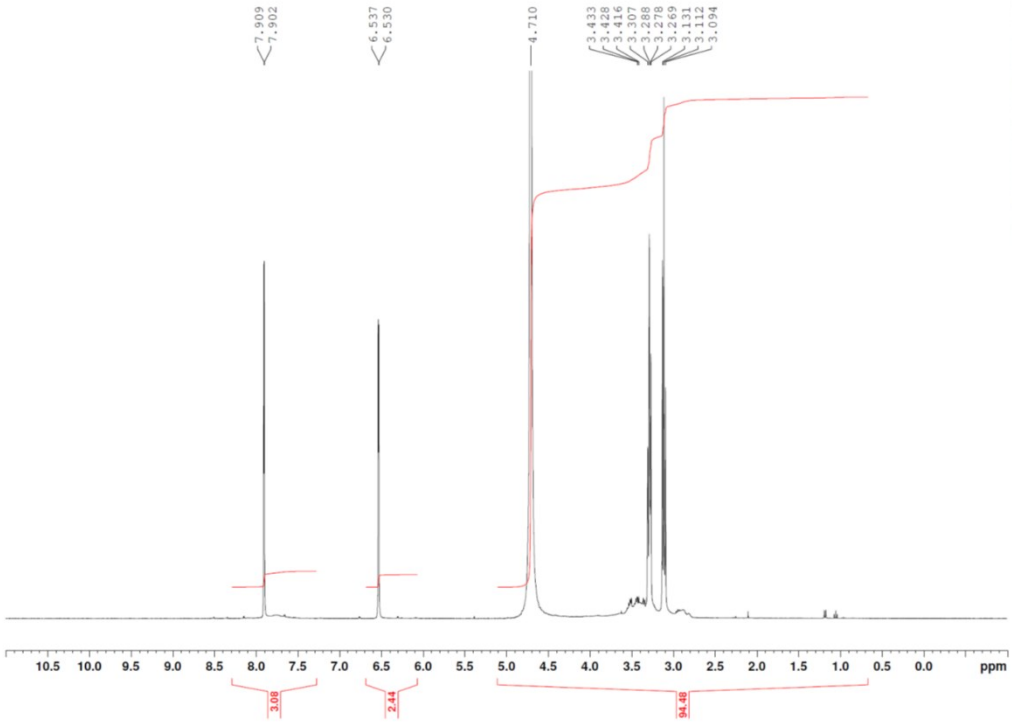

Fig. S9 ^{1}H NMR spectra of produced betazole after downstream recovery and freeze-drying.

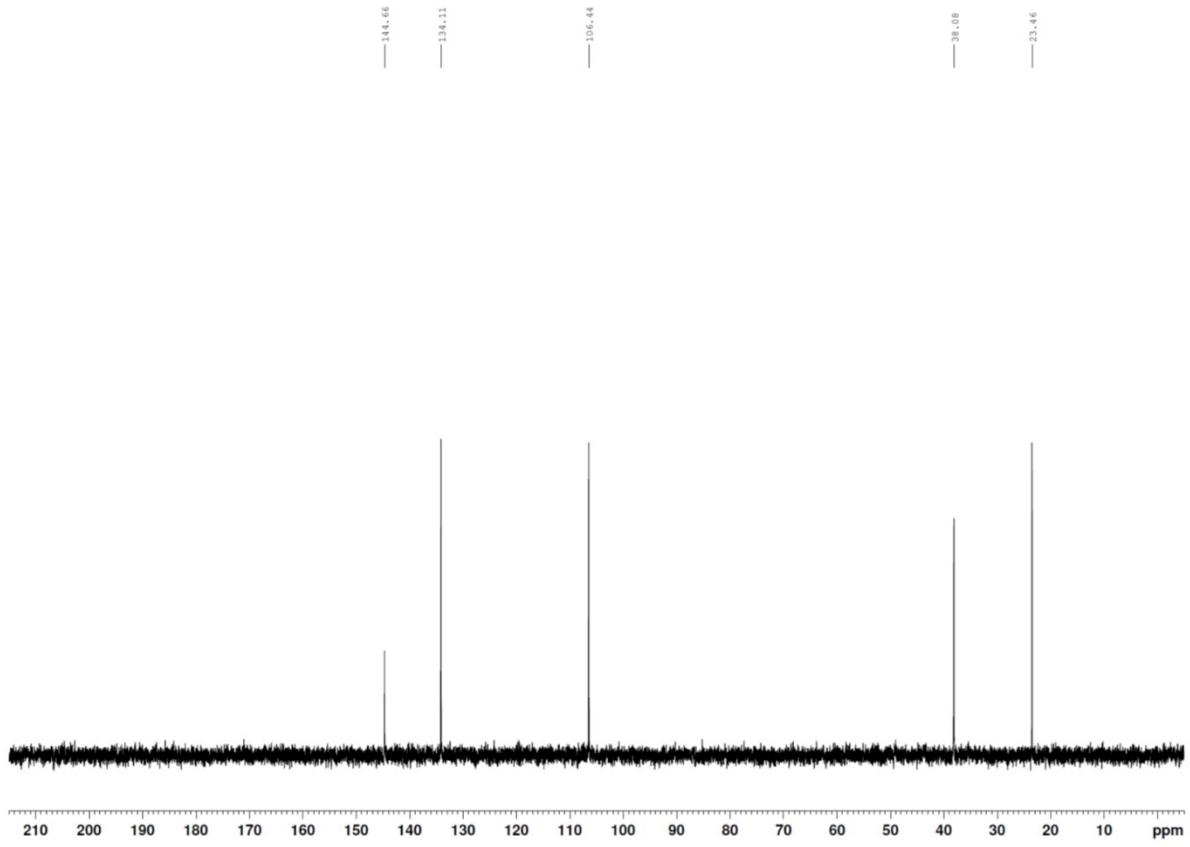

Fig. S10 ^{13}C NMR spectra of produced betazole after downstream recovery and freeze-drying.

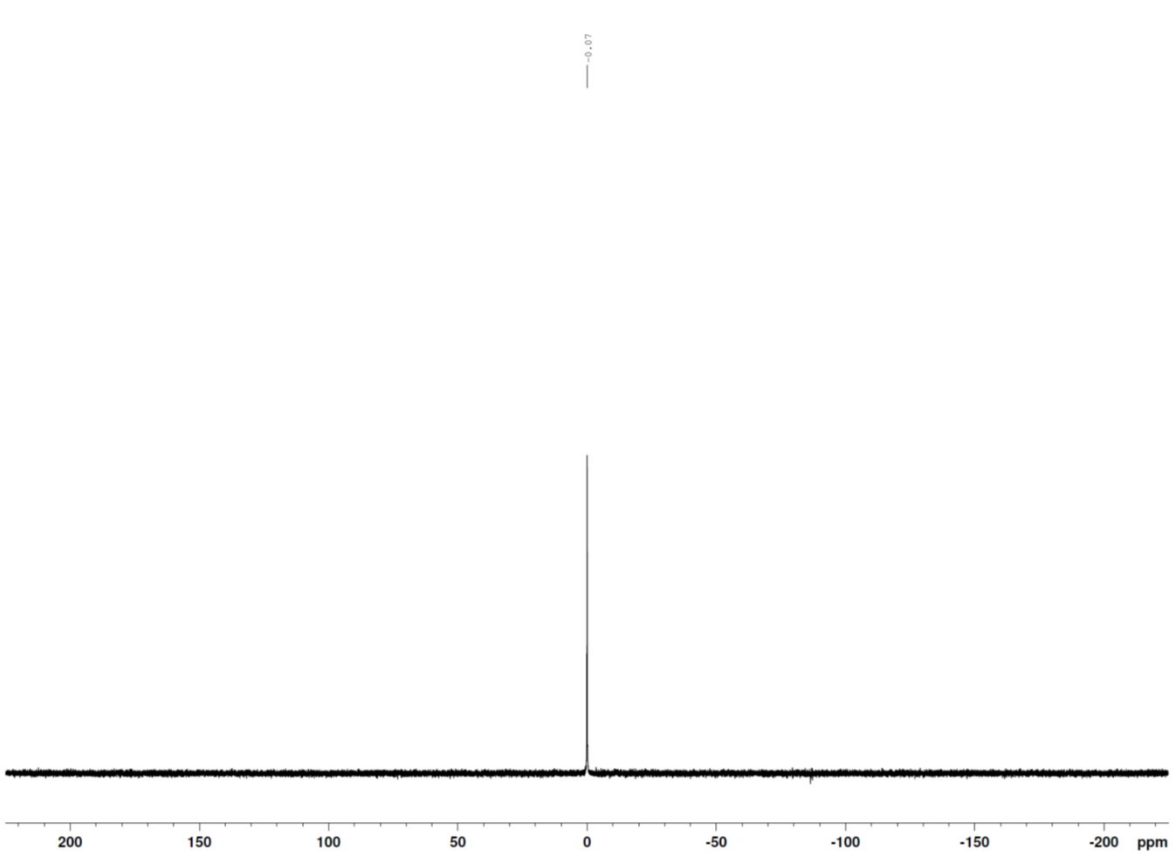

Fig. S11 ^{31}PNMR spectra of produced betazole after downstream recovery and freeze-drying.

Supplementary references

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