Electronic Supplementary Material (ESI) for Green Chemistry.


**Supporting Information**

# Synthesis of *N*-heterocycles from diamines *via* $H_2$-driven NADPH recycling in the presence of $O_2$

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## 1. Experimental section

### 1.1. Chemicals and materials

Except otherwise noted, all solvents, buffer components and chemicals were obtained from Sigma-Aldrich, Merck and Fluka (Steinheim, Germany), Carl Roth GmbH (Karlsruhe, Germany) and Alfa Aesar (Karlsruhe, Germany). Putrescine and 1,5-diaminopentane were obtained from Acros Organics (Geel, Belgium). 1,5-Diamino-2-methyl-pentane was purchased from Sigma Aldrich (Steinheim, Germany) as well as the standards pyrrolidine and (*S*)-2-methylpiperidine. Piperidine was from Fluka (Steinheim, Germany). Diethylentriamine, 3-methylpiperidine and 2-methylpiperidine were obtained from Alfa Aesar (Karlsruhe, Germany). 1,5-Diaminohexane was obtained from FCH Group. Glucose-6-phosphate dehydrogenase (1000 U/mL) from *Leuconostoc mesenteroides* was purchased from Sigma Aldrich (Steinheim, Germany) and acrylamide was from Serva (Heidelberg, Germany). Restriction enzymes were from NEB (Frankfurt am Main, Germany), *Pfu* polymerase from GeneOn (Ludwigshafen, Germany). Kits for PCR product purification, gel extraction of DNA and mini-preparations were from Zymo Research (Freiburg, Germany). Gene synthesis was done by GeneArt (Life Technologies GmbH, Darmstadt, Germany). DNA sequencing was performed by Seqlab (Göttingen, Germany). Oxygen adhesive spot sensors were purchased from PreSens (Regensburg, Germany)

### 1.2. Gene cloning

Sequence of the synthetic DNA for PuO-*Re* (Met start codon in green, *N*-terminal region of $His_6$ tag in blue and stop codons in red):

CACTATAGGGCGAATTGAAGGAAGGCCGTCAAGGCCGCATGAATTCTTTTTTGGGCTAGCAGGAGGATTAACCATGGGATCGCATCACCATC
ACCATCACGGATCCCCTACTCTCCAGAGAGATGTTGCAATCGTCGGCGCCGGCCCCTCTGGCCTGGCAGCGGCAACCGCGCTGCGCAAGGCC
GGCTTGTCCGTCGCCGTGATCGAAGCACGCGATCGTGTCGGAGGCCGCACGTGGACCGACACCATCGACGGCGCAGTCCTGGAGATCGGCG
GCCAGTGGGTCTCCCCCGACCAGACTGCTCTGATCTCCTTGCTCGACGAACTCGGCCTGAAGACTTTCGAGCGCTACCGCGAGGGCGAGTCC
GTCTACATCTCGTCAGCAGGCGAGCGGACTCGATACACGGGCGATTCCTTCCCCACGAACGAGACCACCAAGAAGGAGATGGACCGTCTCAT
CGACGAGATGGACGATCTCGCAGCGCAGATCGGCGCCGAGGAGCCGTGGGCACATCCCCTCGCCCGCGATCTCGACACAGTCTCCTTCAAGC
AGTGGCTGATCAATCAGTCCGACGACGCCGAGGCCCGTGACAACATCGGCCTCTTCATCGCGGGTGGTATGCTCACCAAGCCCGCCCACTCG
TTCTCCGCCCTACAGGCCGTACTCATGGCCGCTTCCGCAGGCTCGTTCTCCCACCTCGTGGACGAGGACTTCATCCTCGACAAGCGAGTGATC
GGCGGAATGCAGCAGGTATCTATCCGCATGGCGGAGGCCCTCGGTGACGACGTCTTCCTCAACGCACCCGTGCGTACGGTGAAGTGGAACG
AATCCGGTGCAACGGTGTTGGCGGACGGCGACATTCGCGTCGAGGCAAGCCGAGTGATCCTGGCCGTACCACCCAACCTCTACTCCCGGATC
TCCTACGATCCCCCGCTGCCGCGTCGTCAGCACCAGATGCATCAGCATCAGTCTCTCGGCCTCGTCATCAAGGTGCACGCCGTGTACGAGACG
CCTTTCTGGCGCGAAGACGGCCTCTCCGGCACCGGCTTCGGCGCGTCCGAGGTAGTGCAGGAGGTGTACGACAACACCAACCACGAGGACG
ATCGCGGCACCCTGGTCGCTTTTGTCTCCGACGAGAAGGCCGACGCGATGTTCGAGCTTTCCGCCGAGGAGCGTAAGGCCACGATTCTGGCC
TCACTCGCCCGCTACCTGGGCCCGAAGGCCGAAGAGCCGGTTGTGTACTACGAATCCGACTGGGGCTCGGAGGAATGGACCCGCGGTGCGT
ACGCGGCGAGCTTCGATCTCGGCGGCCTGCACCGCTACGGCGCGGATTCCCGCACGCCCGTCGGACCGATCCACTTCTCGTGCTCCGACATC
GCAGCCGAGGGATACCAGCACGTGGACGGTGCCGTTCGGATGGGTCAGCGCACCGCCGCCGACATCATCGCCCGCAGCAAGGCCTGATAAC
CCGGGTCTAGAGTCGACAAGCTTCTGGGCCTCATGGGCCTTCCTTTCACTGCCCGCTTTCCAG

The start codon was modified from valine (GTG) to methionine (ATG) and a glycine as second amino acid was inserted which led to an additional *Nco*I restriction site (CCATGG). The gene synthesis was performed by GeneArt™ (Life Technologies GmbH, Darmstadt, Germany) and DNA sequencing was performed by GATC Biotech (Konstanz, Germany). The synthetic gene of PuO was cloned into the multiple cloning site of pBAD18.

### 1.3. Random mutagenesis by epPCR and preparation of mutant libraries with degenerated codon primer of PuO

For the generation of random mutant libraries of PuO error prone PCR (epPCR) was used like described by Cirino *et al.* The condition of the epPCR varies to a usual PCR by the dNTP mixture, which consists of final 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP and 1 mM dTTP. Furthermore, $MgCl_2$ was increased, $MnCl_2$ was included (1-10µM), the DNA template was reduced to 10 ng and the *Taq* polymerase from *Thermus aquaticus* was applied. The epPCR template DNA was digested with *Dpn*I and the libraries were cloned with *EcoR*I and *Hind*III into pBAD18 and transformed into electrocompetent cells of arabinose deficient *E. coli* JW5510.

### 1.4. High-throughput solid phase assay and activity assay of PuO

The solid phase assay screening for the putrescine oxidase was based on the high-throughput assay of monoamine oxidase by the detection of the co-product $H_2O_2$.

Therefore, the cells were plated on nylon membranes in a sufficient amount. The membranes were incubated over night at 25°C on LB-Amp-Ara plates for cell growth as well as expression of PuO mutants. After the expression the membranes were frozen at -20°C for at least 1 h for partial cell lysis. After thawing the membranes, a background reduction of 1 h was performed by placing the membranes on a 90 mm diameter filter paper in a glass petri dish (SCHOTT AG, Mainz, Germany), which was soaked with 1.8 mL 50 mM Tris-HCl buffer pH 8.0 with 0.1 mg/mL horseradish peroxidase (HRP). Afterwards the nylon membranes were transformed into a new glass petri dish containing a filter paper soaked with the assay solution 50 mM Tris-HCl buffer pH 8.0, 0.01 mg/mL HRP, 0.5 mg/mL diaminobenzidine and 10 mM of substrate **3** or **4** and incubated at room temperature until active colonies developed a dark brown color. The positive colonies were transformed to a new nylon membrane and re-screened in order to eliminate false positives. The active colonies of the second screening round were transferred to a LB-Amp plate and then grown in 96 well plates for a lysate activity assay.

### 1.5. Protein production and purification

For protein purification by affinity chromatography, the genes of $PuO^{E203G}$ variant were cut using *Nco*I and *Hind*III from pBAD18 into corresponding digested pASK-iba65b-plus (Life Science, Göttingen, Germany), which contains an encoded N-terminal *Strep-tag* II. The IRED-*Sr* gene was already cloned into pET28a-(+) and provided by Dr. Murray Brown von GlaxoSmithKline (UK). For the protein production both plasmids were transformed into *Rosetta™(DE3)* (NEB). The expression was performed in TB-media (12 g/L trypton, 24 g/L yeast extract and 1% glycerol) containing 100 µg/mL ampicillin plus 35 µg/mL chloramphenicol for PuO and 50 µg/mL kanamycin plus 35 µg/mL chloramphenicol for IRED-*Sr* . The cells were grown at 37°C and 150 rpm to an $OD_{600}$ of about 1 and then induced with 200 µg/L anhydrotetracycline for PuO and with 0.3 mM IPTG for IRED. Afterwards the temperature was shifted to 18°C and the cultivation was performed overnight. The cells were harvested by centrifugation for 30 min at 4°C and 9.000 x g. PuO and IRED purifications were performed at 4°C. The cell pellet was directly resuspended with 3 mL/mg in buffer A (50 mM Tris-HCl pH 8.0, 150 mM KCl and 5% glycerol) for PuO and (50 mM Tris-HCl pH 7.5 with 10 mM imidazol, 5% glycerol) for IRED and then disrupted by passing twice through French press 18 kPa (G.

HEINEMANN Ultraschall und Labortechnik, Schwäbisch Gemünd). The lysate was cleared by ultra-centrifugation (Ultra-Beckman coulter optima-XE-90, Krefeld, Germany) for 50 min at 4°C and 36.000 rpm. The soluble extract containing PuO and IRED were applied to Strep-Tactin® and Ni-NTA columns (Iba Life Science, Göttingen, Germany), respectively. Subsequently, the columns were washed with 10 column volumes (CV) of buffer A. The enzymes were eluted with 5 CV buffer B (50 mM Tris-HCl pH 8.0, 150 mM KCl, 5 mM desthiobiotin (Sigma) and 5% glycerol) for PuO and (50 mM Tris-HCl pH 7.5 with 300 mM imidazole) for IRED. The fractions containing highly purified PuO and IRED were concentrated using an Amicon spin column (10 kDa MWCO; Merck, Darmstadt, Germany). The activity of IRED towards methylpyrroline was determined as described by Borlinghaus *et al*.[1] $SH^{E341A,S342R}$ encoded on pGE771 was produced in *Ralstonia eutropha* HF903 and the activity of SH ($H_2$ driven $NADP^+$ reduction) was determined as described by Lauterbach *et al*.[2] Protein concentrations were determined by BCA assay (Pierce, Thermo Scientific, Schwerte, Germany) according to the manufacturer's instruction.

### 1.6. Determination Kinetic parameters of $PuO^{native}$ and $PuO^{E203G}$

The kinetics parameters of PuO were measured by following the formation of $H_2O_2$ in a spectrophotometric assay with 4 U/mL horseradish peroxidase (HRP) and 0.5 mM 4-aminoantipyrine (AAP) plus 5 mM dichlorohydroxybenzenesulfonic acid (DCHBS) similar as previously described by Federico *et al*.[3] For determination of the $K_M$ values for $PuO^{native}$ and $PuO^{E203G}$ towards diamines, a 3 mL gas sealed cuvette was filled with 2 mL 50 mM Tris-HCl buffer pH 8.0 containing a mixture of AAP, DCHBS and HRP that was saturated with $O_2$. The reaction was started by adding PuO and analyzed at 30 °C. The oxidation of AAP/ DCHBS with $H_2O_2$ resulted in the formation of a quinoneimine dye, which formation was followed at 515 nm ($\varepsilon_{515}$ = 26000 $M^{-1}$ $cm^{-1}$) using a Cary 50 (Varian) spectrophotometer. For determination of the $K_M$ for $O_2$, the same mixture containing 1 mM putrescine was added to a gas sealed cuvette filled with buffer containing different $O_2$ concentrations. The $O_2$ concentration was adjusted by mixing a 100% $O_2$ saturated buffer with 100% $N_2$ saturated buffers. For example for a 40% $O_2$ solution, 0.8 mL 100% $O_2$ saturated buffer was mixed with 1.2 mL 100% $N_2$ saturated buffer. The precise $O_2$ concentration was determined at 30° C using PreSens adhesive spot sensors. Unfortunately, the PuO activity determination by following the decrease of $O_2$ concentration failed without added HRP or catalase through protein precipitation caused by $H_2O_2$. Therefore, the PuO activity was followed by the HRP coupled system described above. The specific activity in [U/mg] was calculated and plotted against the concentration of the substrate. U is defined as the enzyme amount required to form 1 µmol $H_2O_2$ per min. The data were fitted by Origin 2017 using the Michaelis-Menten equation for enzyme kinetics:

$$v = \frac{v_{max} \cdot [S]}{K_M + [S]}$$

$v$ is the rateof the reaction and ***S*** is the substrate concentration. The turnover rate ($k_{cat}$) was calculated by using the PuO molecular weight of 51.95 kDa.

**Determination of the flavin content**

In order to determine the FAD content of isolated PuO, 20 µL of 20% TCA (trichloroacetic acid) was added to an equal volume of the protein. After protein precipitation, the supernatant was neutralized by adding 10 µL of 5 M $K_2HPO_4$. The absorption at 450 nm was measured using a plate reader (Molecular devices). The calibration was done by measuring the absorption at 450 nm of defined FAD concentrations.

### 1.7. $H_2$-driven enzymatic cascade for the biosynthesis of *N*-heterocycles

Biotransformation were performed in small explosive secured and enclosed vessels with 1 mL reaction mixture and excess of headspace (7 mL), which contains 50% $H_2$ and 50% $O_2$ gas mixtures. 500 µL of 50 mM Tris-HCl buffer pH 7.5 buffer was gassed with $H_2$ for 1.5 h. Then 500 µL of $O_2$-saturated buffer was added. The oxygen content was monitored via PreSens spot sensor; in all samples the concentration was around 47% ± 2. The 1 mL reaction mixture contained 10 mM diamine substrate, 1 µM FMN, 1.5 mM TCEP and 2.5 mM of $NADP^+$ in final concentration. Both TCEP and FMN were added to increase the SH activity. 1200 U/mL of catalase was added to the mixture to remove $H_2O_2$ formed during the oxidation. The biotransformation was started with adding the enzyme mixture of ($PuO^{E203G}$, *R*-IRED_*Sr* and $SH^{E340A,S341}$). Concentrations of $PuO^{E203G}$ and *R*-IRED_*Sr* were adapted to substrate related activities: (PuO: 0.05-1.5 mg/mL; IRED-*Sr*: 1-2 mg/mL). 1.5 U/mL of $SH^{E340A,S341}$ was added to recycle the NADPH cofactor. The biotransformation was conducted at 25 °C with shaking at 180 rpm. Samples of 50 µL were taken at 0, 5, 10, 20, 30, 60, 120, 180, 240, and 960 min and added to 50 µL of 5 M NaOH to terminate the reaction. For the negative controls, the buffer was gassed only with $N_2$. For product analysis, samples were extracted with MTBE after adding 1 µmol of 2-methylpyrrolidine as internal standard and derivatized with acetic anhydride as described by Dobos *et al*.[4]

### 1.8. GC-Analytics

GC-FID analyses of performed biotransformations were carried out using a Shimadzu GC-2010 equipped with an AOC-20i auto injector. The injector temperature was 250°C and compounds were detected via flame ionization detector (FID) at 330°C or 255°C. Identification of compounds was performed by comparing the retention time of the produced compounds with commercial available reference materials pyrrolidine, piperidine, 3-methylpiperidine, and 2-methylpiperidine. A DB-5 capillary column (Restek 5 MS, length 30 m × 0.25 mm × 0.25 µm) was used with $H_2$ as carrier gas (51.3 mL/min, linear velocity 30cm/s) and samples were injected in split mode (1:50). The temperature program was as following: 1 min at 70°C, 15°C/min to 90°C, 20°C/min to 185°C, hold 1 min, 25°C/min to 300°C and hold for 3 min.
The chiral analysis for determining the enantiomeric excess of 2-methylpiperidine and 3-methylpiperidine was performed with the Supelco β-Dex 225 column (Sigma Aldrich, Steinheim, Germany, length 30 m × 0.25 mm × 0.25 µm) with $H_2$ as carrier gas (26 mL/min, linear velocity 30 cm/s) and the samples were injected in split mode (1:25). The temperature program was as following: 2 min at 100°C, 50°C/min to 130 °C, 1.5°C/min to 150 °C, 75°C/min to 220°C and hold for 1 min.

## 2. Additional results

### 2.1. Protein purification and activity measurements

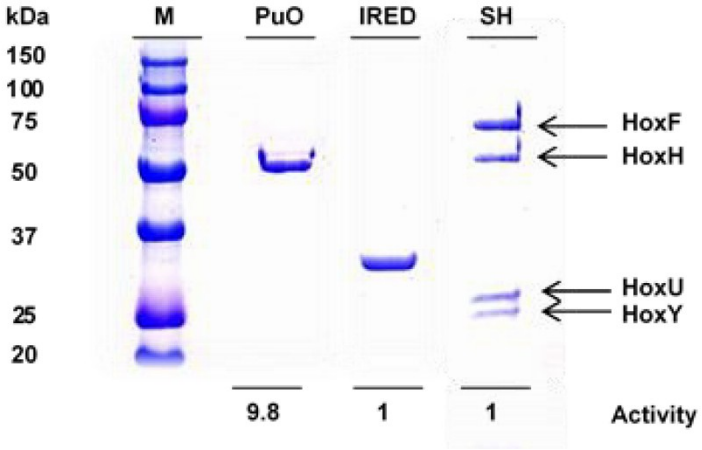



Figure S1: Purification of the PuO, IRED and SH proteins. A protein amount of 6 µg of purified proteins by affinity chromatography separated on a 12% SDS-PAGE gel and subsequently stained with Coomassie brilliant blue. Lane M contains marker proteins and their corresponding molecular weights are given on the left-hand side. The specific activity of each enzyme (U/mg of protein) is shown below. The specific activities were determined for putrescine, methylpyrroline and $NADP^+$ for PuO, IRED and $SH^{E341A,S342R}$,

### 2.3 Spectral properties of PuO$^{native}$ and PuO$^{E203G}$

Native PuO showed a dark orange color compared to a bright yellow color shown by the variant. The spectral analysis of both enzymes showed a typical flavoenzyme spectrum with maxima at 377 nm and 455 nm (Figure S2). However, for the wild type enzyme the anionic red flavin semiquinone at 410 nm was observed, which disappeared after incubation overnight at room temperature while the intensity at 455 nm increased. The same spectral feature was observed by glycolate oxidase reported by Pennati *et al*. [5]

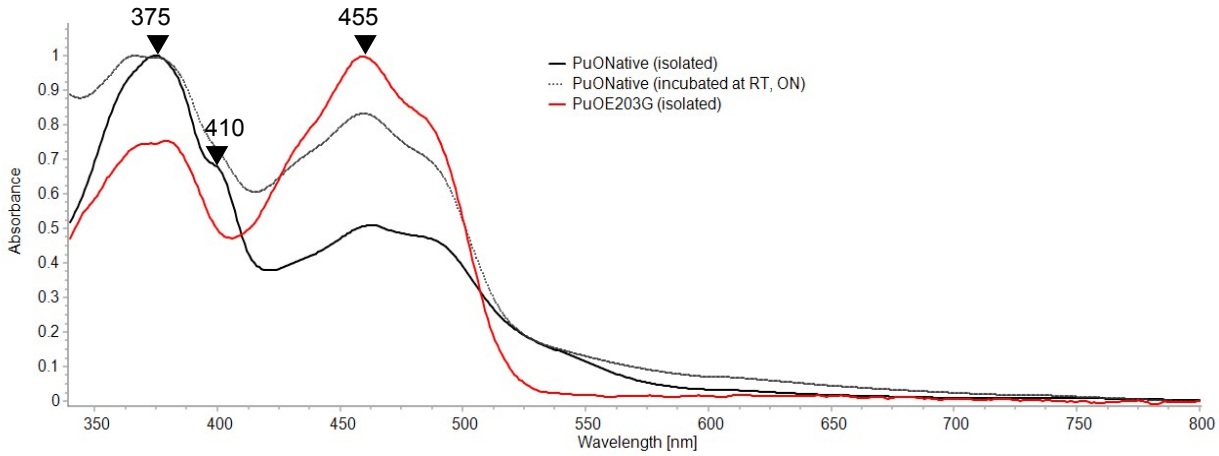
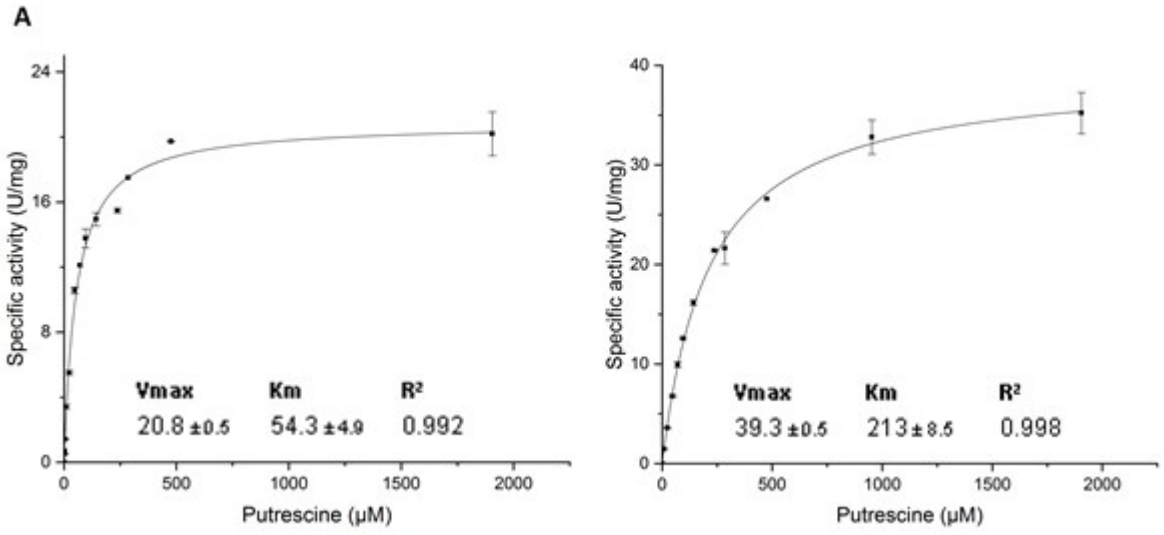
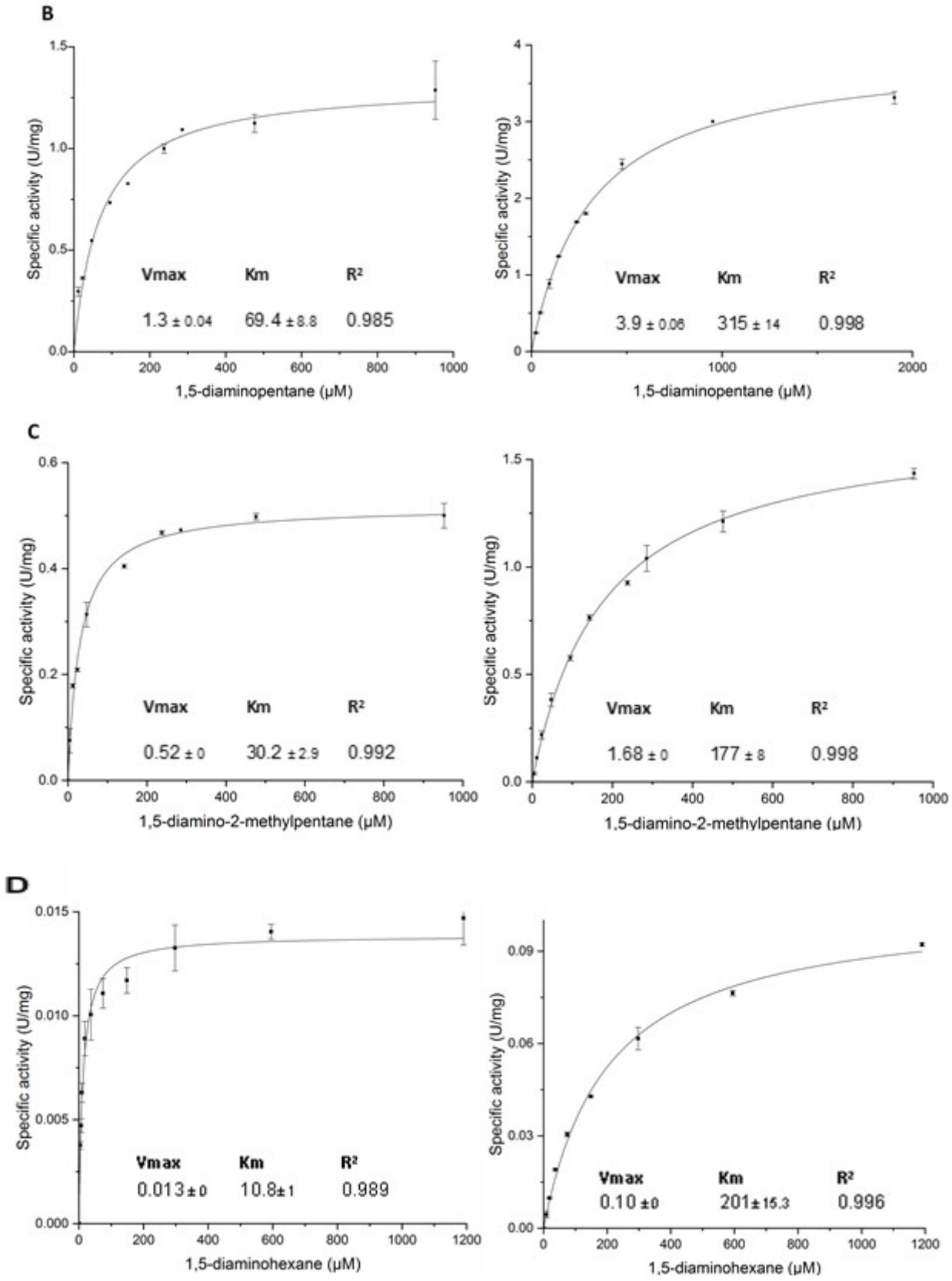



Figure S2: Deconvoluted absorption spectra of PuO$^{native}$ , PuO$^{native}$ after incubation overnight at room temperature and variant PuO$^{E203G}$. The graphs were normalized to PuO$^{native}$ using spectragraph. The enzyme concentrations were 9 mg/mL and 3.5 mg/mL for PuO$^{native}$ and PuO$^{E203G}$ respectively.

### 2.4. Kinetics




B
Specific activity (U/mg)
1,5-diaminopentane (μM)
Vmax
Km
R²
1.3 ± 0.04
69.4 ± 8.8
0.985
3.9 ± 0.06
315 ± 14
0.998
C
1,5-diamino-2-methylpentane (μM)
0.52 ± 0
30.2 ± 2.9
0.992
1.68 ± 0
177 ± 8
0.998
D
1,5-diaminohexane (μM)
0.013 ± 0
10.8 ± 1
0.989
0.10 ± 0
201 ± 15.3
0.996

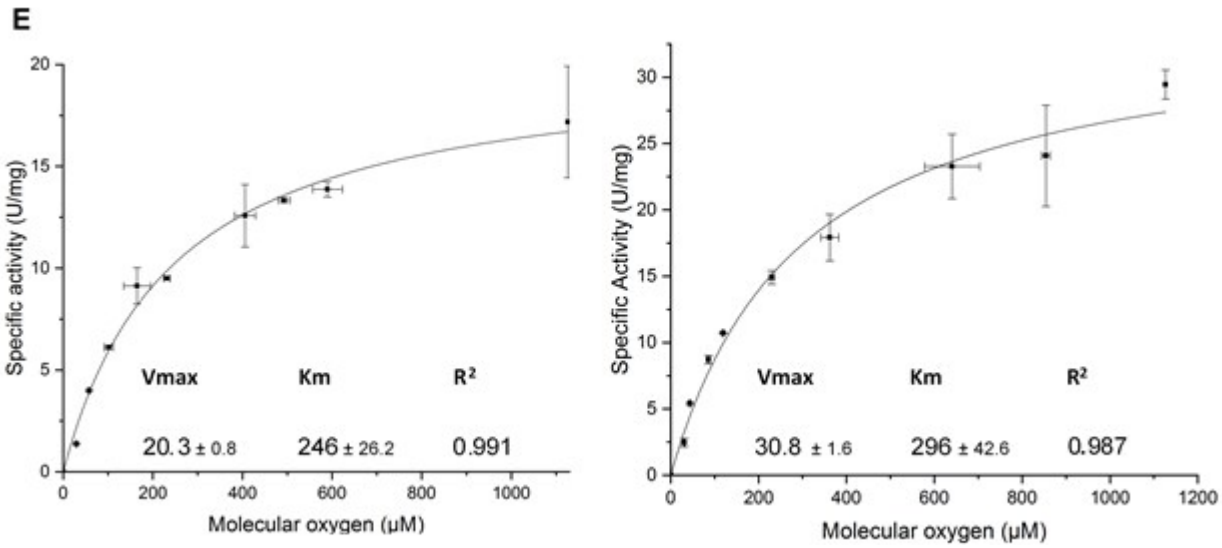
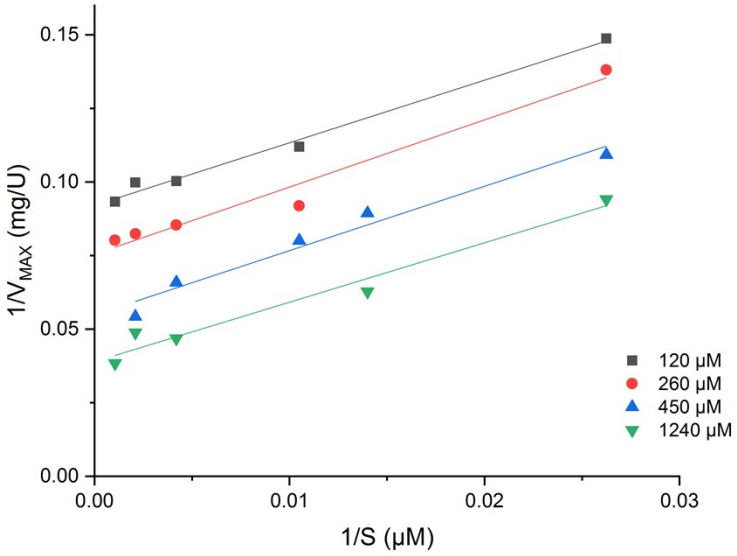



**Figure S3:** Activities of both $PuO^{E203G}$ and $PuO^{native}$ at different substrate concentrations towards substrates putrescine **1** (A), 1,5-diaminopentane **2** (B), 1,5-diamino-2-methylpentane **3** (C) and 1,5-diaminohexane **4** (D) and molecular oxygen (E). The kinetic parameters towards diamines were determined at 100 % $O_2$. The kinetic parameters towards $O_2$ were determined at 1 mM putrescine. All measurements were conducted at 30°C and pH 8.0. Individual data points of the activity measurements for different substrates used for the determination of the Michaelis-Menten constants by non-linear regression. The left panel shows the kinetics for $PuO^{native}$ and the right panel for $PuO^{E203G}$.

## 2.5. Steady state kinetics

Steady-state kinetics for putrescine as a function of $O_2$ for native putrescine oxidase was studied. All measurements were conducted at 30°C in 50 mM Tris-HCl buffer pH 8.0 at different concentrations of putrescine **1**. The concentrations of $O_2$ were 120, 260, 450 and 1240 µM. The data were fitted in a Linewaver-Burk plot. The parallel plots in the figure below indicates a ping pong reaction mechanism without ternary complex of enzyme- substrate-oxygen.




| $O_2$ [µM] | Km (1) [µM] |
|---|---|
| 120 | 23 ± 1.3 |
| 260 | 30 ± 3.2 |
| 450 | 39.8 ± 4.2 |
| 1240 | 51.8 ± 5.5 |

Figure S4: Steady-state kinetics for $PuO^{native}$ for putrescine (1) as a function of $O_2$.

## 2.6. Enzymatic cascade for the biosynthesis of *N*-heterocycles

Table S1 shows the production of *N*-heterocycles (**11**-**14**) from diamines (**1**-**4**) at three different $O_2$ concentrations (260, 620 and 1240 µM) after 5 and 16 hours. Only the yield of **14** was increased at higher $O_2$ concentrations. Due to the low activity of PuO towards **4**, higher $O_2$ concentrations enhanced significantly the oxidation rate of **4** and thus the production yield of **14**. Since SH has no $H_2$ available anymore at saturated $O_2$ conditions, glucose-6-phosphate dehydrogenase system (GDH) was used for regeneration of NADPH in this study. Detailed information on product formations and selectivities in the synthesis of methylpiperidines after 30 min reaction time is given in table S2.

**Table S1:** Cascade reaction in vitro with PuO$^{E203G}$ combined with *R*-IRED-*Sr* at different $O_2$ concentrations. The conversion of the substrate into the corresponded *N*-heterocycle is shown in %.

| | **11** | | **12** | | **13** | | **14** | |
|---|---|---|---|---|---|---|---|---|
| $O_2$ [µM] | **5 h** | **16 h** | **5 h** | **16 h** | **5 h** | **16 h** | **5 h** | **16 h** |
| **260** | 23 ± 1 | 35 ± 2 | 57 ± 3 | 63 ± 8 | 56 ± 1 | 54 ± 1 | 6 ± 1 | 8 ± 1 |
| **620** | 22 ± 2 | 33 ± 0 | 56 ± 6 | 53 ± 0 | 64 ± 7 | 49 ± 3 | 7 ± 1 | 15 ± 0 |
| **1240** | 25 ± 2 | 36 ± 1 | 58 ± 5 | 57 ± 5 | 54 ± 1 | 55 ± 0 | 9 ± 1 | 18 ± 1 |

Reaction conditions: 1 mL in 8 mL gas sealed glass bottles, horizontal shaking 180 rpm. 50 mM Tris-HCl pH 7.5 buffer contains: 10 mM substrate, 2 µM of each (PuO$^{E203G}$, IRED, Catalase), 5 U/mL glucose-6-phosphate dehydrogenase, 2 mM NADPH and 20 mM glucose-6-phosphate, 25°C. Conversion in % was measured by GC-FID.

**Table S2:** Product formation and selectivity in the formation of 3-methylpiperidine (**13**) and 2-methylpiperidine (**14**) after 30 minutes with different ratios of PuO$^{E203G}$/ *R*-IRED_*Sr.*

| | | | | | |
|---|---|---|---|---|---|
| **3-Methylpiperidine (13)** | | | | | |
| PuO$^{E203G}$ [mg/ml]: | 0.1 | 0.2 | 1 | 5 | 5 |
| *R*-IRED_*Sr* [mg/ml]: | 1 | 1 | 1 | 1 | 1 |
| Product formation [%]: | 22 ± 3 | 33 ± 1 | 72 ± 1 | 90 ± 1 | 99[a] ± 1 |
| *ee* [%]: | 93 (*R*) | 87 (*R*) | 43 (*R*) | *rac.* | *rac.*[a] |
| **2-Methylpiperidine (14)** | | | | | |
| PuO$^{E203G}$ [mg/ml]: | 0.1 | 0.5 | 1 | 5 | 5 |
| *S*-IRED_*Pe* [mg/ml]: | 1 | 1 | 1 | 1 | 1 |
| Product formation [%]: | 2 ± 0.1 | 12 ± 3 | 16 ± 4 | 47 ± 3 | 86[a] ± 3 |
| *ee* [%]: | 57 (*S*) | 55 (*S*) | 56 (*S*) | 47 (*S*) | 40[a] (*S*) |

Reaction conditions: 400 µl in 2ml glass-vials, horizontal shaking 180 rpm, 50 mM Tris-HCl pH = 8.0, 5 mM diamine, 0.1-5 mg/ml PuO, 1 mg/ml IRED, 2.5 mM NADPH, 1U/ml G6PDH, 20 mM G6P, 25°C, 0.5 h.
*R*-IRED_*Sr*: *R*-selective IRED from *Streptosporangium roseum*
*S*-IRED_*Pe*: *S*-selective IRED from *Paenibacillus elgii*
[a] values after 2h

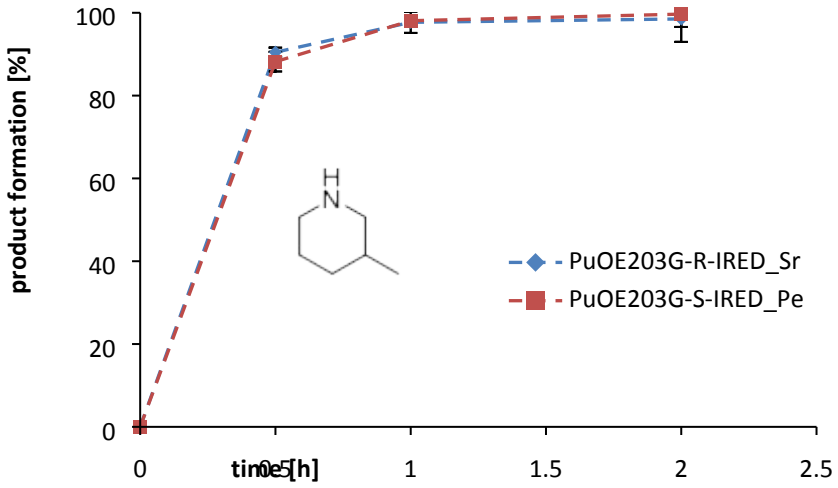



**Figure S5:** Product formation of 3-methylpiperidine (**13**) in the first two hours under optimized reaction conditions: 400 µl in 2ml glass-vial, horizontal shaking 180 rpm, 50 mM Tris-HCl pH = 8.0, 5 mM 2-methylpentane-1,5-diamine (**3**), 5 mg/ml PuO, 1 mg/ml IRED, 2.5 mM NADPH, 1U/ml G6PDH, 20 mM G6P, 25°C, 0-2h. For this reaction enantiocomplementary IREDs (*R*-selective IRED from *Streptosporangium roseum* and *S*-selective IRED from *Paenibacillus elgii*) were combined with PuO$^{E203G}$. Similar activities were observed for both IREDs. Optimized reaction conditions were also used for the formation of 34 % **15** (Table 3, column 4 in the manuscript).

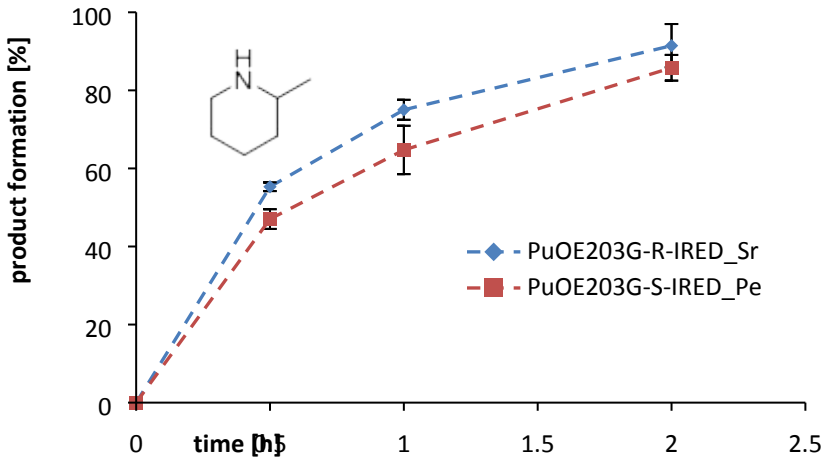



**Figure S6:** Product formation of 2-methylpiperidine (**14**) in the first two hours under optimized reaction conditions: 400 µl in 2ml glass-vials, horizontal shaking 180 rpm, 50 mM Tris-HCl pH = 8.0, 5 mM 1,5-diaminohexane (**4**), 5 mg/ml PuO, 1 mg/ml IRED, 2.5 mM NADPH, 1U/ml G6PDH, 20 mM G6P, 25°C, 0-2h. For this reaction enantiocomplementary IREDs (*R*-selective IRED from *Streptosporangium roseum* and *S*-selective IRED from *Paenibacillus elgii*) were combined with PuO$^{E203G}$. Activity was slightly reduced for the setup with *S*-IRED_*Pe*.

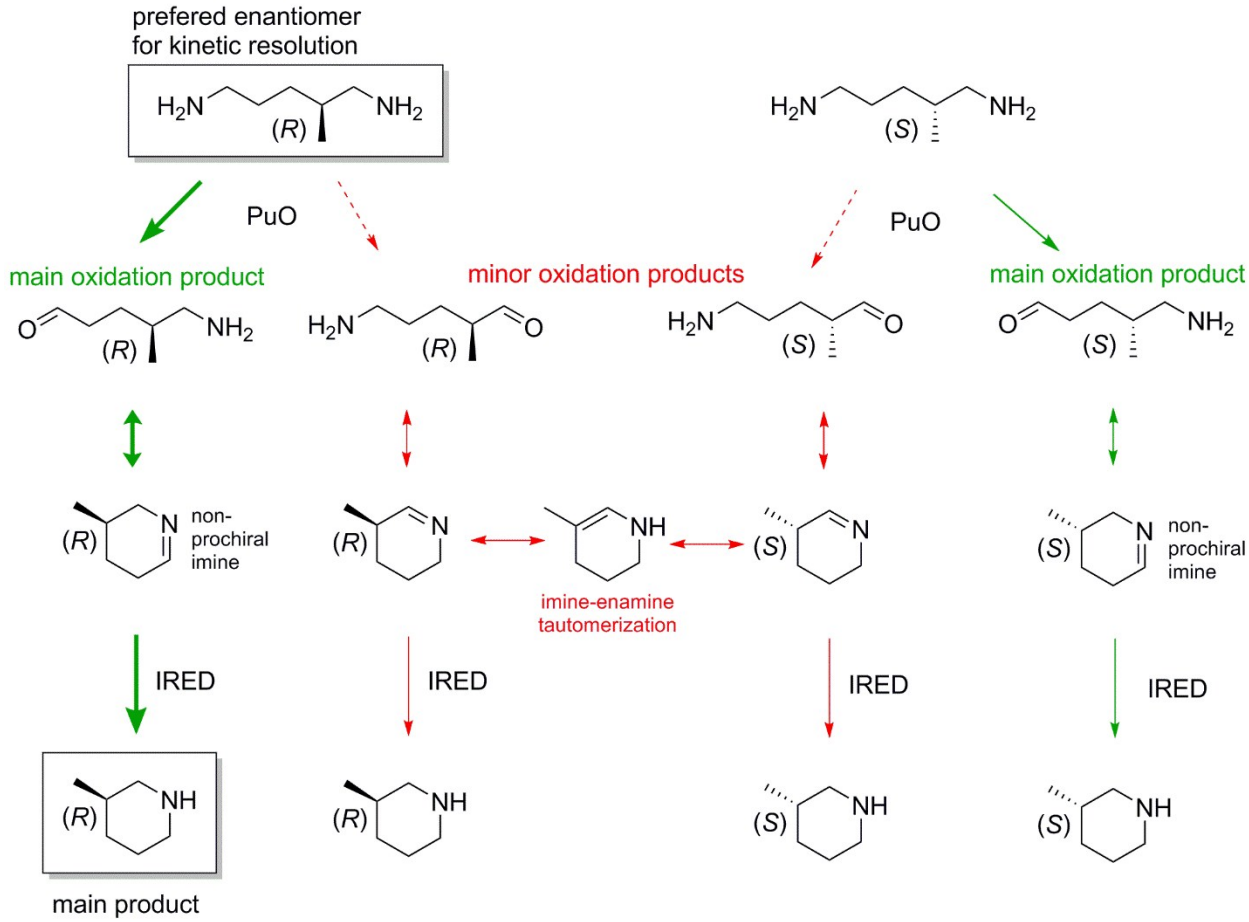



**Figure S7:** Possible reaction pathways for the formation of *R*- and *S*-3-methylpiperidine (**13**). PuO prefers the *R*-enantiomer resulting in the formation of *R*-3-methylpiperidine as main product. For both enantiomers we expect the oxidation mainly at the sterically less-hindered amine group. For all pathways no prochiral imine intermediate is formed.

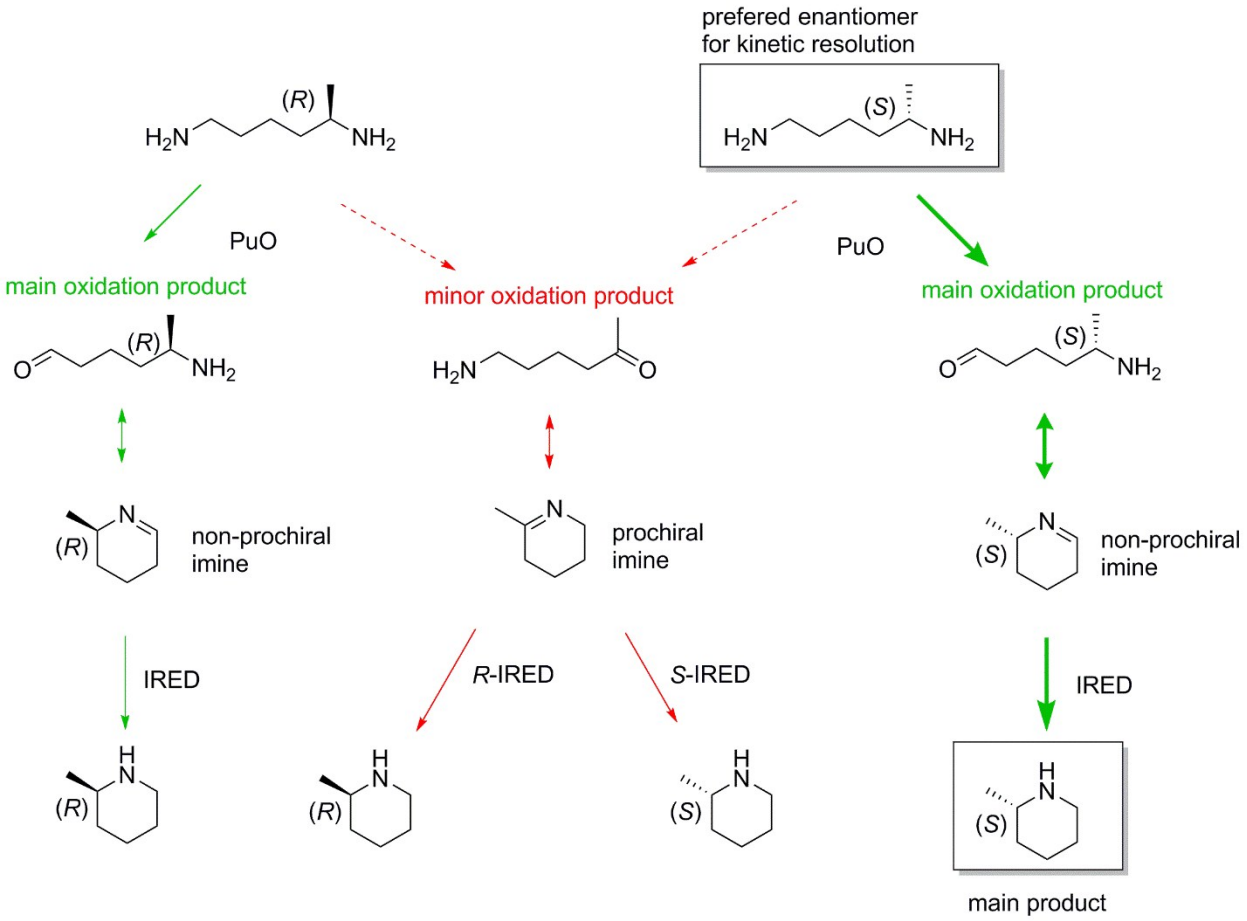



**Figure S8:** Possible reaction pathways for the formation of *R*- and *S*-2-methylpiperidine (**14**). PuO prefers the *S*-enantiomer leading to the formation of *S*-2-methylpiperidine as main product. For both enantiomers we expect the oxidation mainly at the sterically less-hindered amine group. Only small amounts of prochiral imine intermediate are formed, which can be selectively reduced by either *R*- or *S*-selective IREDs. The enantiomeric excess in the formation of *S*-2-methylpiperidine can be further increased by using a *S*-selective IRED.

### 2.7. Comparison of NADPH regeneration systems in the enzymatic cascade

Table S3 shows the production of piperidine **(12)** from 1,5-diaminopentane **(2)** after 4 and 16 hours using two NADPH regeneration systems. The first one is based on glucose-6-phosphate dehydrogenase and the other one on $O_2$ tolerant $NADP^+$ reducing hydrogenase.

**Table S3:** Comparison of two different NADPH regeneration systems for the *N*-heterocycles production cascade. The conversion of 1,5-diaminopentane **(2)** into piperidine **(12)** is shown in %

| regeneration system | Conversion [%] | |
|---|---|---|
| | **4 h** | **16 h** |
| **SH[1]** | 74 ± 2 | 97 ± 2 |
| **G6P-DH[2]** | 91 ± 1 | 91 ± 6 |

Reaction conditions: 1mL in 8 mL gas sealed glass bottles, horizontal shaking 180 rpm. 50 mM Tris-HCl pH 8.0 contains: 10 mM 1,5-diaminopentane, 1 U/mL of each ($PuO^{E203G}$, IRED and catalase), 2 mM $NADP^+$ and $O_2$:$H_2$ (1:1), 25°C.
[1)] 1 mM TCEP, 2µM of FMN and 1 U/mL $SH^{E341A,S342R}$ were added.
[2)]20 mM glucose-6-phosphate and 1 U/mL glucose-6-phosphate dehydrogenase were added.

### 2.8. Reaction of diamines with glucose-6-phosphate

The effect of the glucose-6-phosphate on the activity of the PuO was tested. A decrease in the activity was only observed at low putrescine concentrations. The reaction with glucose-6-phosphate at this low substrate concentration is decreasing the affinity of PuO towards the substrate and thus the activity.

**Table S4:** The influence of glucose-6-phosphate on the activity of $PuO^{E203G}$.

| **putrescine** mM | **Without G6P** TOF / $s^{-1}$ | **With G6P**[a] TOF / $s^{-1}$ | **With G6P**[a] **+ preincubation**[b] TOF / $s^{-1}$ |
|---|---|---|---|
| 0.05 | 14.4 ± 0.7 | 12.7 ± 0.28 | 11.8 ± 0.35 |
| 1 | 14.6 ± 1.3 | 16.7 ± 0.37 | 17.2 ± 0.18 |
| 10 | 18.8 ± 0.78 | 21 ± 0.48 | 20.1 ± 0.23 |

**Reaction conditions:** Volume 200 µl, buffer 50 mM Tris-HCl pH 8.0, 0.05-10 mM diamine, 0.01 µM $PuO^{E203G}$, 2 mM ABTS, 8 U mL-1 HRP, 30°C. The oxidation of ABTS was followed photometrically for 15 min at λ=405 nm.
[a] addition of glucose-6-phosphate to a final concentration of 20 mM
[b] Glucose-6-phosphate and putrescine were pre-incubated together for 1h at 25°C.

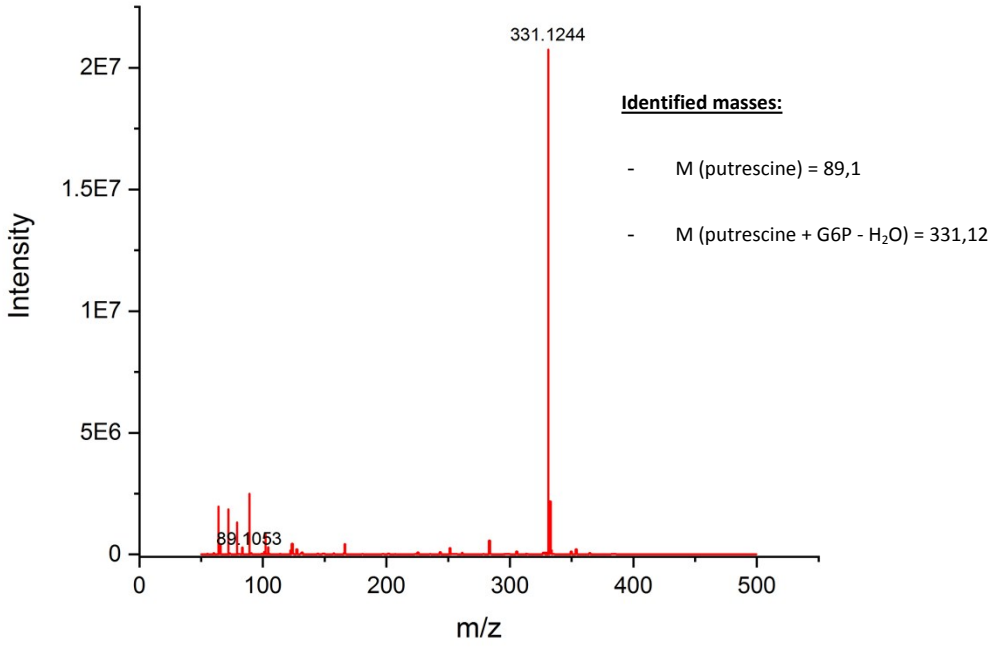
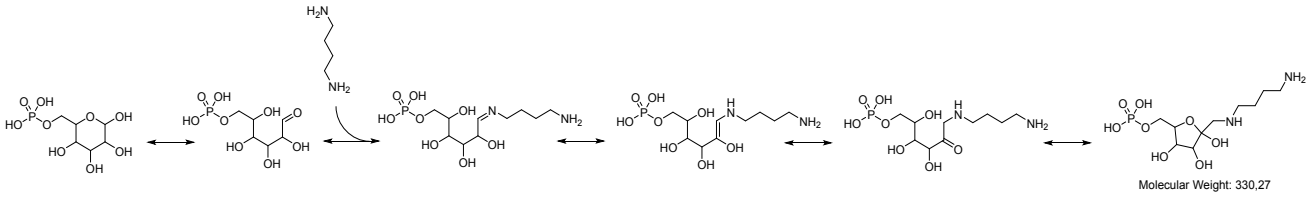



**Figure S9:** ESI-MS spectrum of the reaction between putrescine and glucose-6-phosphate. 200 mM of putrescine was mixed with 400 mM glucose-6-phosphate in Tris-HCl buffer pH 8.0, incubated over night at room temperature and analyzed by LC-MS.




**Scheme S1:** Proposed mechanism of the reaction of putrescine with glucose-6-phosphate

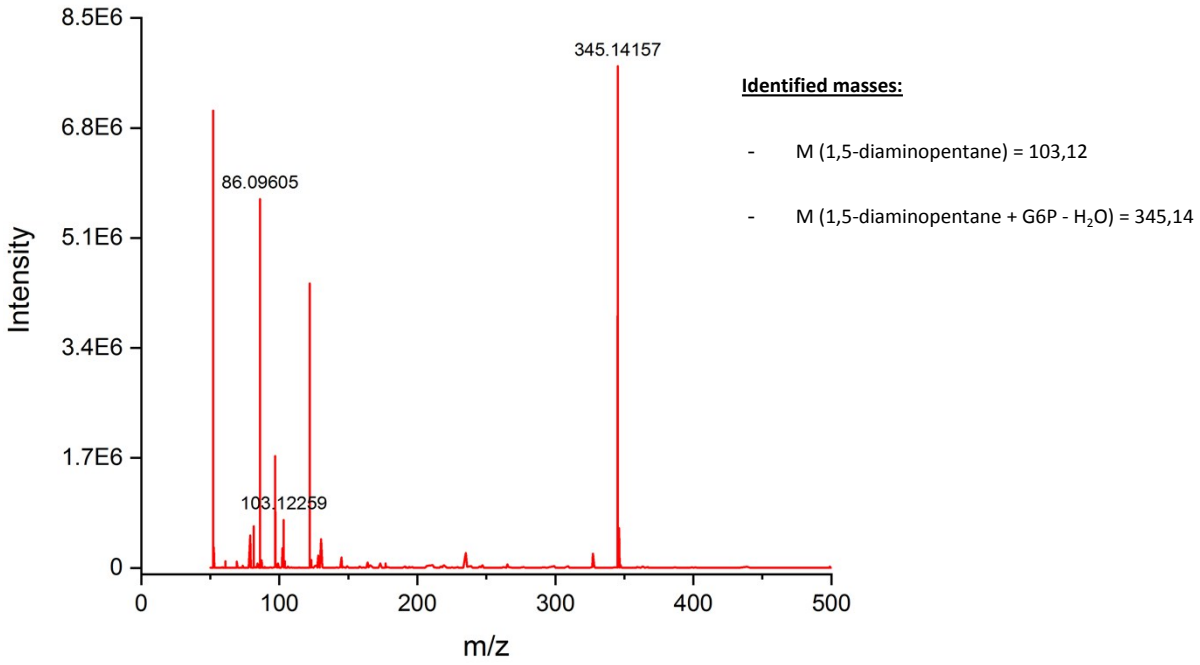
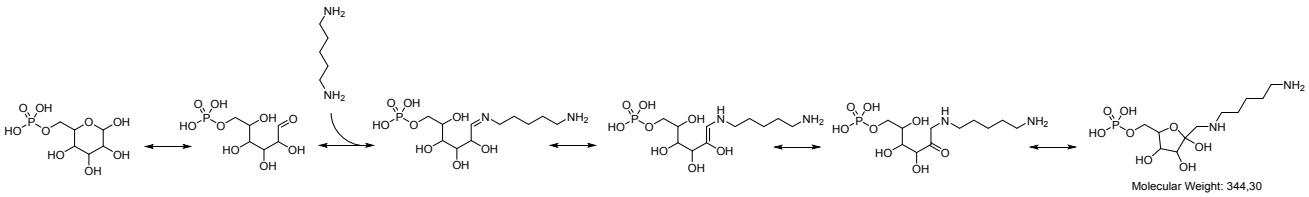



**Figure S10:** ESI-MS spectrum of the reaction between 1,5 diaminopentane and glucose-6-phosphate. 200 mM of 1,5 diaminopentane was mixed with 400 mM glucose-6-phosphate in Tris-HCl buffer pH 8.0, incubated over night at room temperature and analyzed by LC-MS.




**Scheme S2:** Proposed mechanism of the reaction of 1,5-diaminopentane with glucose-6-phosphate

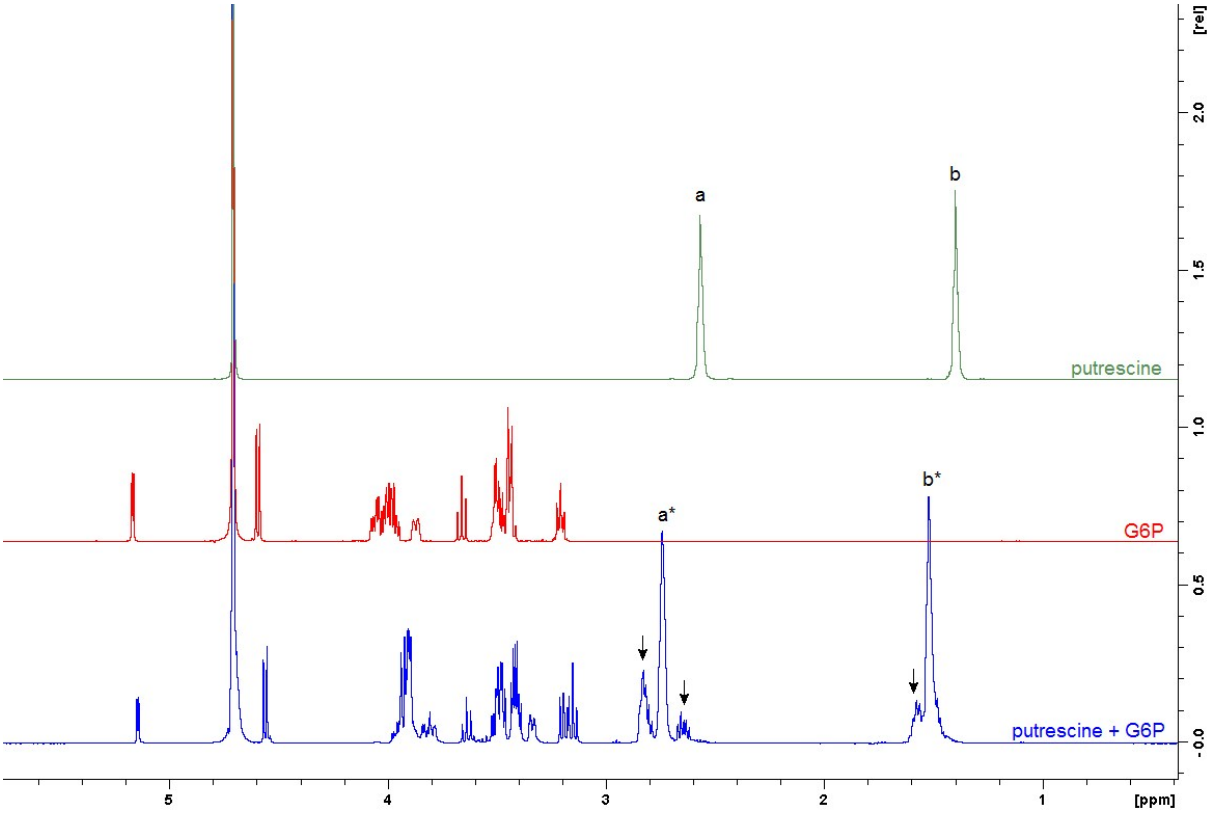



**Figure S11:** $^{1}$H NMR-spectra (500 MHz, $D_2O$) of putrescine (green), glucose-6-phosphate (G6P, red) and a mixture of both, putrescine and G6P (blue) are compared with each other. Signals of putrescine (a, b) are slightly shifted, when it is mixed with G6P (a*, b*). A difference in pH could be the reason for this observation. Interestingly, in the mixture, some additional peaks appear (marked with arrows). These proton signals are related to carbon atoms of putrescine (see HSQC Figure S13). Furthermore, some shifted peaks and additional peaks occur in the mixture, which are related to the signals of glucose.

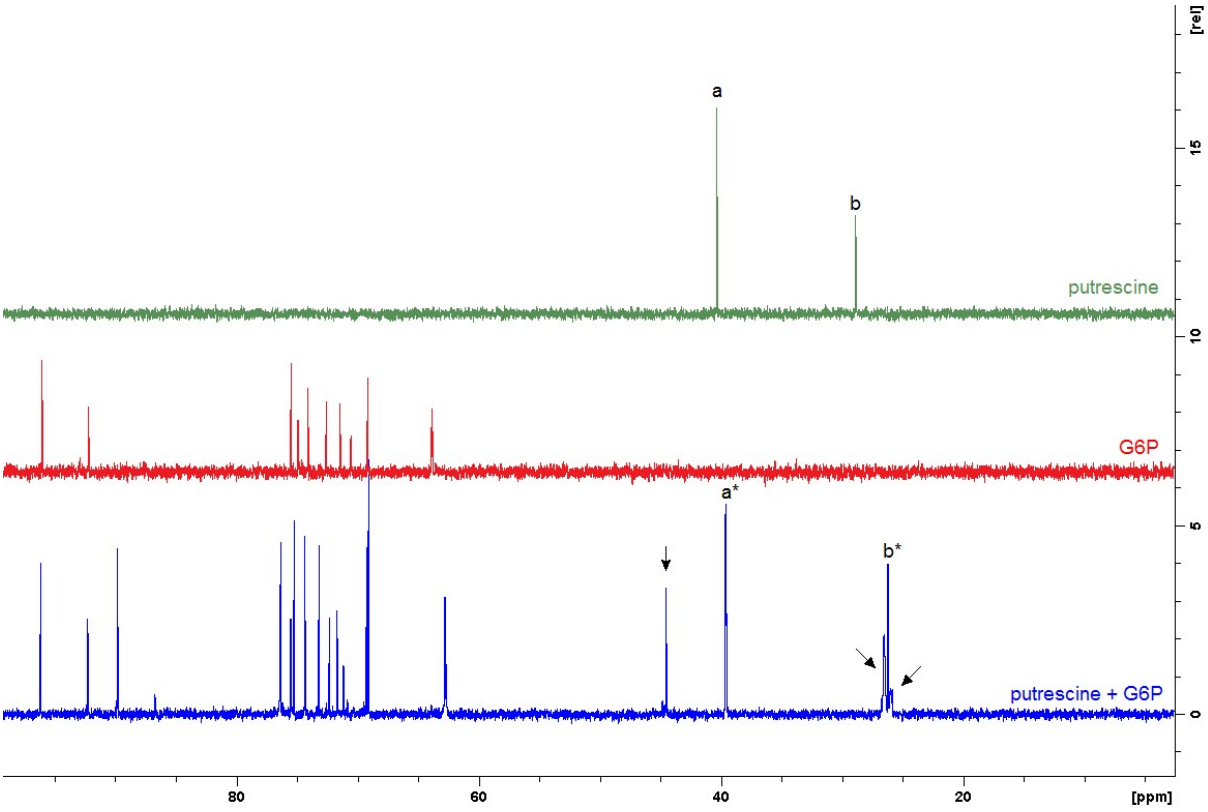



**Figure S12:** $^{13}C$ NMR-spectra (500 MHz, $D_2O$) of putrescine (green), glucose-6-phosphate (G6P, red) and a mixture of both, putrescine and glucose-6-phosphate (blue) are compared with each other. Signals of putrescine (a, b) are slightly shifted, when it is mixed with G6P (a*, b*). A difference in pH could be the reason for this observation.

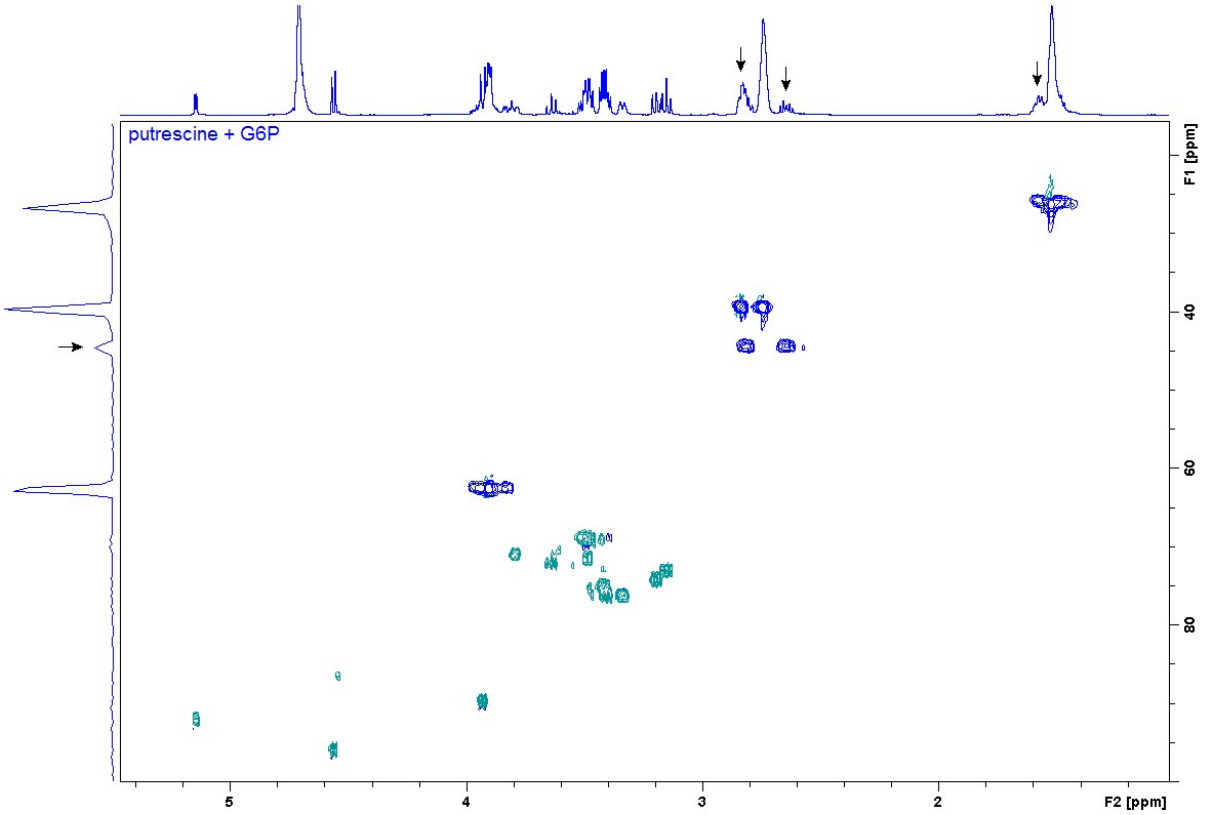



**Figure S13:** $^{1}H^{13}C$-HSQC spectrum (500 MHz, $D_2O$) of the mixture of putrescine and glucose-6-phosphate. Some peaks appear when both molecules react with each other, like it is observed for some changes in the putrescine signals (marked with arrows). This spectrum shows that those peaks are related to putrescine. The multitude and uncertainty of novel occurring signals indicates that putrescine react with glucose-6-phosphate in different ways leading to different adducts. This could be explained by Maillard-like reactions between putrescine and glucose-6-phosphate.[6]

## 3. References

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