Supplemental Material for

Structural and Biochemical Basis for the Chemo- and Regioselectivity of the Nitro-forming *N*-Oxygenase AurF

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Material and Methods

Protein production and purification

Native or mutated AurF was produced as MalE fusion protein with the plasmid pMR1 in E. *coli* BL21(DE3) cells.[1] 1 L LB medium with ampicillin (100 µg mL^{-1}) was inoculated with 10 ml overnight culture LB medium (ampicillin 100 µg mL^{-1}) containing *E. coli* transformed with the appropriate plasmid. The cells were grown at 28 °C with orbital shaking (200 rpm) to an OD_{600}~0.4 and induced with IPTG (0.5 mM final concentration) for 12 h at 28 °C. Subsequently, the cells were harvest by centrifugation at 10543 g at 4 °C for 25 min. The pellet was resuspended in 100 mL buffer A (20 mM Tris/500 mM NaCl/pH 7.5) and the cells were disrupted by sonication. The crude extract was centrifuged (12000 x g, 4°C, 40 min), passed through a 1.2 µm filter (Millipore RAWPO4700) and loaded on a 4 mL amylose column (NEB amylose FF, XK16 Amersham). The column was washed with 5 CV and eluted with buffer B (20 mM Tris, 500 mM NaCl, 10 mM Maltose at pH 7.5).

Mutagenesis

Mutagenesis was performed using the QuikChange II XL site-directed mutagenesis kit according to the manufacturer's recommendation. The template plasmids were pMR1 and pMR1-L202F.[2] To introduce the second mutation, Thr100Ala, primer pairs GCG CGT CAT CGC CGC CGA GCA GCT CAT CGC CG and CGG CGA TGA GCT GCT CGG CGG CGA TGA CGC GC were used. To introduce the second and third mutation, Arg96Glu/Thr100Ala, we used GGC TAC AAC GAG GAA GTC ATC GCC GCC GAG CAG CTC ATC G and CGA TGA GCT GCT CGG CGG CGA TGA CTT CCT CGT TGT AGC C. The correctness of the mutants was verified by forward and reverse sequencing.

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aurF                    268 cggctacaacgagcgcgtcatcgccaccgagcagct
aurF-R96E-T100A-L202F   268 ............gaa.........g..........
aurF-T100A-L202F        268 ........................g..........

aurF                    582 ggccgagacctgcatcaacgccctgctggcgctgct
aurF-R96E-T100A-L202F   582 ......................t.t...........
aurF-T100A-L202F        582 ......................t.t...........
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Figure S1. Alignment of the nucleotide sequences of *aurF* with mutated versions (displayed: 261-302 bp and 575-616 bp).

In vitro enzyme assays

Sample preparation and activity assay were carried out as reported,[2] using enzyme concentration of 0.8 mg mL^{-1} and 2.4 mg mL^{-1}, respectively. A reaction mixture with heat-inactivated enzyme served as a negative control.

In vivo enzyme assays

The *in vivo* assays were performed as described previously.[3]

Synthesis of p-aminophenylguanidine sulfate[4]

p-phenylenediamine (1.68 g) and *S*-methylisothiourea (2.75 g) were dissolved in H_2O (7 mL) and heated under stirring in a boiling water bath for 2 h. After cooling to ambient temperature, methanol (7 mL) was added and the mixture was chilled in an ice-bath. The precipitate was harvested using a frit filters, dried and re-precipitated three times adding H_2O (5 mL) and methanol (7 mL). Yield: 0.47 g, 20 % (after several recrystallisation steps). The identity of the product was confirmed by ESI-MS.

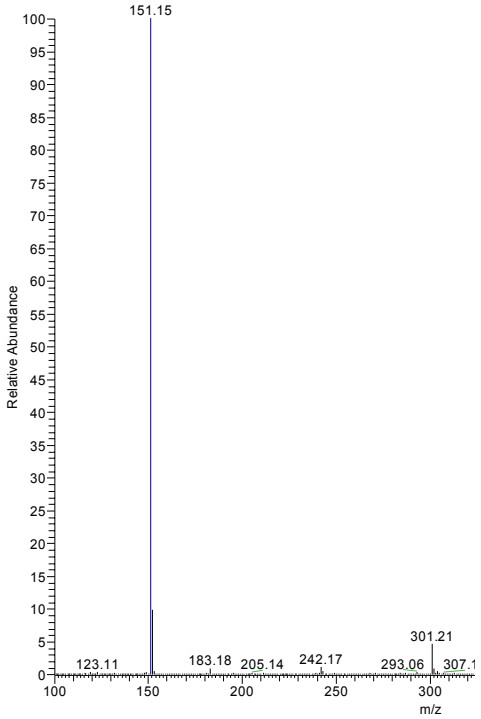

Figure S2. ESI-MS of *p*-aminophenylguanidine (**3**), $[M+H]^+$ = 151.

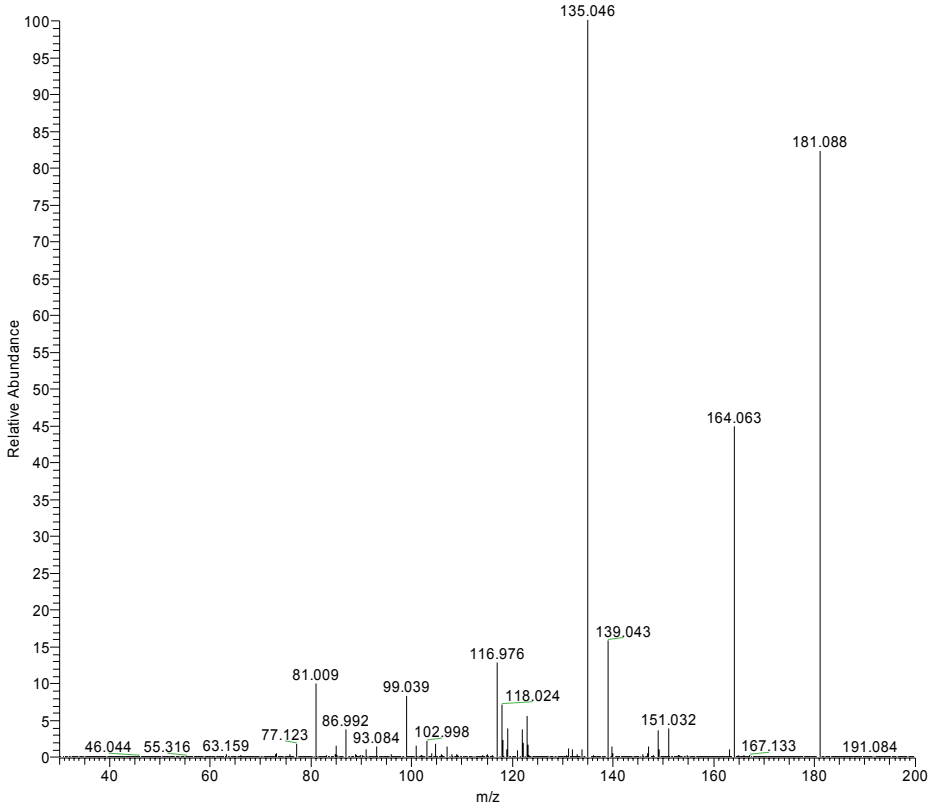

Figure S3. MS/MS spectrum of *m/z* = 181 (*p*-nitrophenylguanidine, **4**) from the AurF *in vitro* enzyme assay. $[M+H]^+$ = 181. M-46 corresponds to the loss of the nitro group, M-17 corresponds to the loss of an amino group.

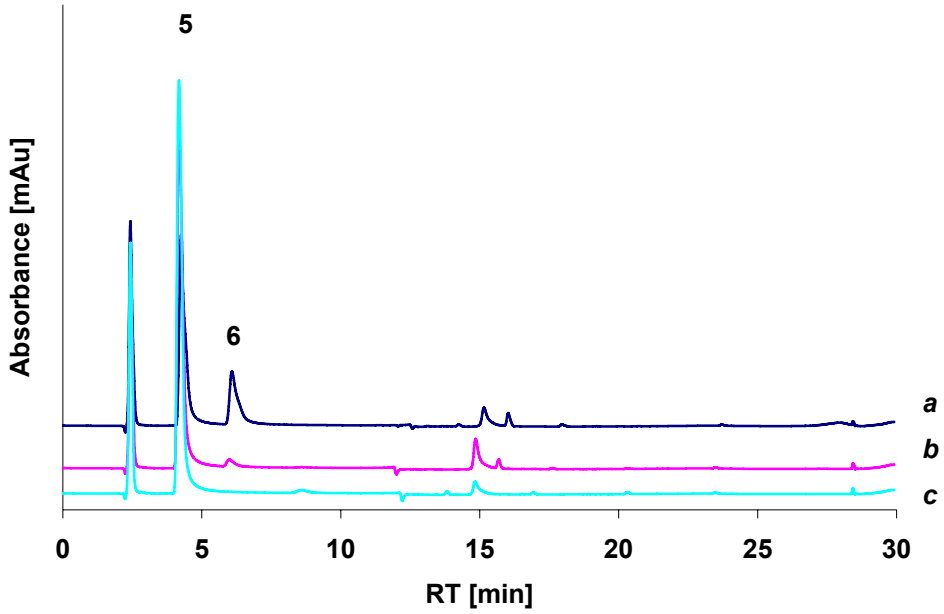

Figure S4. LC-MS-profile of *in vitro* biotransformation of *p*-aminobenzamidine (**5**) after 24 h using a) AurF-R96E/T100A/L202F or b) AurF. c) AurF-R96E/T100A/L202F$^-$ (heat activated enzyme) as a negative control; detection at 280 nm;

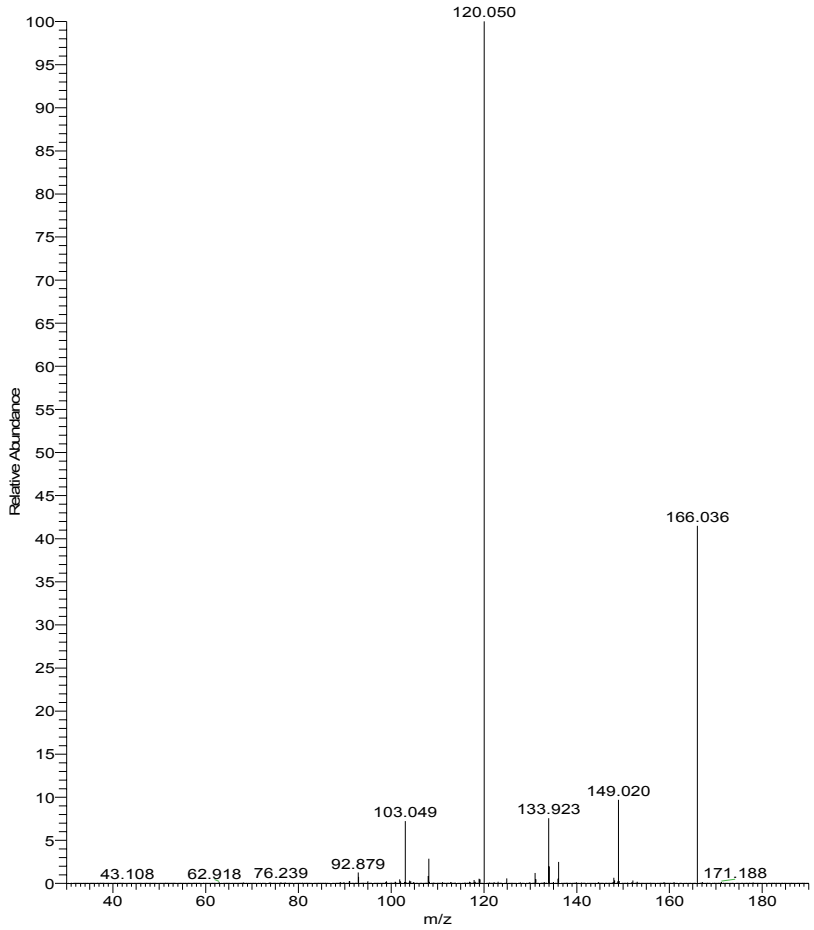

Figure S5. MS/MS spectrum of *m/z* = 166 (*p*-nitrobenzamidine, **6**) from the AurF *in vitro* enzyme assay. $[M+H]^+$ = 166. M-46 corresponds to the loss of the nitro group, M-17 corresponds to the loss of an amino group.

Supplemental References

[1] R. Winkler, M. E. Richter, U. Knupfer, D. Merten, C. Hertweck, *Angew. Chem. Int. Ed. Engl.* 2006, **45**, 8016.
[2] G. Zocher, R. Winkler, C. Hertweck, G. E. Schulz, *J. Mol. Biol.* 2007, **373**, 65.
[3] R. Winkler, G. Zocher, I. Richter, T. Friedrich, G. E. Schulz, C. Hertweck, *Angew. Chem. Int. Ed. Engl.* 2007, **46**, 8605.
[4] C. E. Braun, *J. Am. Chem. Soc.* 1932, **54**, 1511.