Supporting Information

Cross-Regulation of an Artificial Metalloenzyme

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Supporting Information
Materials and Instruments
The Sav K121R variant was produced, purified and characterized as previously reported.\cite{1} Urease from *Canavalia ensiformis* (Jack bean) and esterase from porcine liver were obtained from Sigma-aldrich. \cite{2} The synthesis of the reduced enrofloxacin 2 is described below. Other reagents, substrates and materials were purchased from Sigma-Aldrich, Alfa Aesar and Umicore. UV–Vis measurements were conducted on a Varian 50 Scan UV-vis spectrophotometer or Tecan Infinite M1000 Pro. NMR experiments were performed on a Bruker Avance III NMR spectrometer operating at 400 MHz proton frequency. Chemical shifts are referenced vs. residual CHDCl₂ for ¹H spectra (5.32 ppm) and ¹³C spectra (77.16 ppm). High-resolution MS analyses were carried out on a Bruker maXis 4G.

Buffers and stock solutions
MES (pH 5.5 and 6.0), MOPS (pH 6.5, 7.0, 7.5 and 7.9) and CHES (pH 8.5 and pH 9.0) were used. The pH of all buffers and stock solutions including HCOONa and urea in buffers was adjusted with NaOH (5 M).
Synthesis of reduced enrofloxacin 2

Enrofloxacin 1 (500 mg, 1.4 mmol) was suspended in MeOH (20 ml). NaBH₄ (210 mg, 5.57 mmol) was added portionwise. The reaction mixture was stirred for 30 min at room temperature. A catalytic amount of p-toluenesulfonic acid was added and stirred at 65 °C for 1 hour. After evaporation, the residue was dissolved in CHCl₃ and washed with water. After evaporation, 311 mg (70 %) of the reduced enrofloxacin 2 was obtained as a yellow solid.

¹H NMR (400.1 MHz, CDCl₃) δ 7.52 (d, 1H, J = 13.2 Hz), 6.72 (d, 1H, J = 7.4 Hz), 3.48 (t, 2H, J = 8.0 Hz), 3.26 (t, 4H, J = 6.0 Hz), 2.62 (t, 4H, J = 6.0 Hz), 2.58 (t, 2H, J = 8.0 Hz), 2.48 (q, 2H, J = 7.2 Hz), 2.31 (tt, 1H, J = 7.2, 3.6 Hz), 1.12 (t, 3H, J = 7.2 Hz), 0.89 (m, 2H), 0.71 (m, 2H).

¹³C NMR (100.6 MHz, CDCl₃) δ 151.11, 146.35, 141.03, 113.81, 102.77, 52.66, 52.33, 50.07, 49.77, 49.72, 40.99, 38.55, 32.31, 12.00, 8.49.

HRMS [ESI(+)]TOF: calculated for C₁₈H₂₄FN₃O [M+H]+ 318.1976; found 318.1980

Figure S1. (a) ¹H-NMR of the reduced enrofloxacin 2 and (b) UV-vis absorption spectra of 0.1 mM enrofloxacin 1 (red line) and its reduced form 2 (blue line) in 0.3 M MOPS pH 7.9. The molar absorption coefficient of reduced enrofloxacin 2 at 430 nm was determined as 1333 M⁻¹cm⁻¹
Determination of the TON of the ATHase [(Biot-Cp*)IrN^N] 3 · Sav K121R toward the reduction of enrofloxacin 1

Experiments were performed in a 96-well plate using the conditions listed in Tables S1 and S2. The reaction mixtures were incubated at 37 °C for 8 h. Absorption at 430 nm was determined with a Tecan Infinite® M1000 Pro plate reader.

Table S1. ATHase stock solution (200 µM, 300 µl)

| Ingredient | Stock solutions | Reaction mixture |
|------------|----------------|-----------------|
|            | Conc. | Solvent | Vol. | Order of addition | Final Conc. |
| [Biot-Cp*IrCl]2Cl | 5 | 2.5 mM | N^N^N (5.5 mM in DMSO) | 12 µl | 1 | 100 µM |
| Sav K121R | 1.28 mM | MOPS (100 mM, pH 7.0) | 93.8 µl | 3<sup>c</sup> | 400 µM |
| MOPS (100 mM, pH 7.0) | | Up to 300 µl | 2 |

* 4,7-dihydroxy-1,10-phenanthroline  * Total free biotin-binding sites. <sup>c</sup> incubated at room temperature for 30 min prior to addition.

Table S2. Composition of the ATHase reaction mixture for the reduction of enrofloxacin 1

| Ingredient | Reaction mixture |
|------------|----------------|
|            | Conc. of stock solution | Solvent | Vol. | Order of addition |
| MOPS (100 mM, pH 7.0) | 88 µl | 1 |
| [(Biot-Cp*)Ir(N^N)] 3 · Sav K121R | 200 µM | See Table S1 | 10 µl | 2 |
| Enrofloxacin 1 | 200 mM | NaOH (300 mM) | 2 µl | 3 |
| HCOONa | 4 M | MOPS (100 mM, pH 7.0) | 100 µl | 4 |
pH dependence of ATHase \([(\text{Biot-Cp}^*)\text{IrN}^N]\) 3 · Sav K121R and \([(\text{Cp}^*)\text{IrN}^N]\) 4 toward the reduction of enrofloxacin 1 (Scheme 2b)

Experiments were performed in a 96-well plate using the conditions listed in Table S3 and S4. The reaction mixtures were incubated at 37 °C. Absorption at 430 nm was recorded by means of a Tecan Infinite® M1000 Pro plate reader. The relative activity was calculated by comparison of the slope of the absorption increase in the initial linear phase.

**Table S3. ATHase stock solution (200 µM, 300 µl)**

| Ingredient | Stock solutions | Reaction mixture |
|------------|-----------------|-----------------|
| \([(\text{Biot-Cp}^*)\text{IrCl}_2]\text{Cl}_2\) 5 | | |
| or 2.5 mM N^N (5.5 mM in DMSO) 12 µl 1 200 µM | |
| \([(\text{Cp}^*)\text{IrCl}_2]\text{Cl}_2\) 6 | | |
| Sav K121Rb or Water 1.28 mMc Water 93.8 µl 3c 400 µM | |
| Water | | Up to 300 µl 2 |

* 4,7-dihydroxy-1,10-phenanthroline. b Sav K121R was used with \([(\text{Biot-Cp}^*)\text{IrCl}_2]\text{Cl}_2\) to prepare ATHase. c Total free biotin-binding sites. d incubated at room temperature for 30 min prior to addition.

**Table S4. Composition of the reaction mixture of the ATHase for the reduction of enrofloxacin 1**

| Ingredient | Reaction mixture |
|------------|-----------------|
| Conc. of stock solution | Solvent | Vol. | Order of addition |
| Buffera (100 mM, pH 5.5 – 9.0) | 89 µl 1 | |
| \([(\text{Cp}^*)\text{Ir(N}^N\text{N})]\) 4 or \([(\text{Biot-Cp}^*)\text{Ir(N}^N\text{N})]\) 3 · Sav K121R | 200 µM See Table S3 10 µl 2 | |
| Enrofloxacin 1 | 200 mM NaOH (300 mM) 1 µl 3 | |
| HCOONa | 4 M Buffer (100 mM, pH 5.5 – 9.0) 100 µl 4 | |

*a See SI p. 2 for the buffer composition*
Switching catalytic activity of the ATHase [(Biot-Cp*)IrN^N] 3·Sav K121R by HCl and NaOH (Scheme 2c)

Experiments were performed in a glass-vial with a magnetic stirrer at ambient temperature using the conditions listed in Table S5 and S6. HCl_aq (5 M) with Enrofloxacin 1 (5 µl, 200 mM in 250 mM NaOH) and NaOH_aq (5 M) that were sequentially added to switch the pH between 5 and 8, respectively. An aliquot (200 µl) of the reaction mixture was transferred in a 96 well plate and the absorption at 430 nm was determined by means of a Tecan Infinite®, M1000 Pro plate reader. After the UV-vis measurement, the aliquot was re-transferred to the reaction mixture.

Table S5. ATHase stock solution (200 µM, 1300 µl)

| Ingredient | Stock solutions | Reaction mixture |
|------------|-----------------|------------------|
|            | Conc. | Solvent | Vol. | Order of addition | Final Conc. |
| [(Biot-Cp*)IrCl]_2Cl | 2.5 mM | N^N^a (5.5 mM in DMSO) | 52 µl | 1 | 200 µM |
| Sav K121R | 1.28 mM^b | CHES (10 mM, pH 9.0) | 406.3 µl | 3^c | 400 µM |
| CHES (10 mM, pH 9.0) | | | Up to 1300 µl | 2 | |

^a 4,7-dihydroxy-1,10-phenanthroline. ^b Total free biotin-binding sites. ^c incubated at room temperature for 30 min prior to addition.

Table S6. Composition of the reaction mixture of the ATHase for the reduction of enrofloxacin 1

| Ingredient | Reaction mixture |
|------------|------------------|
|            | Conc. of stock solution | Solvent | Vol. | Order of addition |
| Buffer^a (100 mM, pH 5.5 – 9.0) | 3700 µl | | 1 |
| [(Biot-Cp*)Ir(N^N)J]·Sav K121R | 200 µM | See Table S3 | 1250 µl | 2 |
| Enrofloxacin 1 | 200 mM | NaOH (250 mM) | 50 µl | 3 |
| HCOONa | 4 M | CHES (10 mM, 9.0) | 5000 µl | 4 |

Figure S2. Relative ATHase activities for each cycle presented in Scheme 2c.
Bromothymol Blue solutions used to estimate the pH of catalysis solutions by UV-vis spectroscopy

Stock solutions of 0.04 wt % bromothymol blue (BTB) were prepared in EtOH. The resulting BTB stock solution (10 µl) was added to a buffer solution (10 mM, 200 µl, see SI p. 2) set at different pHs.

**Figure S3.** (a) Structures of BTB in acidic (top) and in basic conditions (bottom). (b) UV-vis absorption spectra of bromothymol blue (BTB) at different pHs. (c) Plots of the ratio of absorptions at 618 nm and 501 nm at different pHs.
pH-variation caused by the decomposition of urea by urease in the presence of iridium complexes or ATHase

To investigate the influence of the iridium complexes and the corresponding ATHase on the activity of urease, the pH-variation caused by the decomposition of urea was evaluated by monitoring absorption of bromothylmol blue at 618 nm and 501 nm, used as a semi-quantitative reporter for the pH (See Figure S2).

The experiments were performed in a 96-well plate using the conditions listed in Tables S7 and S8. The reaction mixtures were incubated at 37 °C. Absorption was recorded using a Tecan Infinite® M1000 Pro plate reader.

**Table S7.** Composition of the ATHase and iridium stock solutions (200 µM, 300 µl)

| Ingredient | Stock solutions | Reaction mixture |
|------------|-----------------|------------------|
|            | Conc. | Solvent | Vol. | Order of addition | Final Conc. |
| [(Biot-Cp*)IrCl]_2Cl_2 or [(Cp*)IrCl]_2Cl_2 | 2.5 mM | N^N^N° (5.5 mM in DMSO) | 12 µl | 1 | 200 µM |
| Sav K121R or CHES (10 mM, pH 9.0) | 1.28 mM° | CHES (10 mM, pH 9.0) | 93.8 µl | 3° | 400 µM |
| CHES (10 mM, pH 9.0) | Up to 300 µl | 2 |

° 4,7-dihydroxy-1,10-phenanthroline. ° Sav K121R was used with [(Biot-Cp*)IrCl]_2Cl_2 to prepare ATHase. °° Total free biotin-binding sites. °" incubated at room temperature for 30 min prior to addition.

**Table S8.** Composition of the reaction mixtures used to determine the urease activity in the presence of iridium complexes (See Scheme S1)

| Ingredient | Reaction mixture |
|------------|------------------|
|            | Conc. of stock solution | Solvent | Vol. | Order of addition |
| CHES (10 mM, pH 9.0) | 200 µM | See Table S5 | 25 µl | 2 |
| Iridium complexes or ATHase | 4 mg/ml | CHES (10 mM, pH 9.0) | 10 µl | 3 |
| Urease | 4 M / 0.2-0.6M | CHES (10 mM, pH 9.0) | 100 µl | 4 |
| HCOONa / Urea | 0.04 wt% | EtOH | 10 µl | 5 |
| Bromothylmol blue | 5 M | water | 3.2 µl | 6 |
Scheme S1. Time-course evolution of the pH reflecting the urease activity in the presence and absence of iridium complexes and ATHase. As can be appreciated, the ATHase has a very modest effect on the urease activity. In stark contrast, the iridium complexes \([\text{(Biot-Cp}^\ast\text{)Ir(N}^\ast\text{N)}]_3\) and \([\text{(Cp}^\ast\text{)Ir(N}^\ast\text{N)}]_4\) inhibit the urease highlighting the inhibitory effect of the organometallic complexes on urease.
Effect of urea, urease and ammonia on the ATHase activity

Experiments were performed in a 96-well plate using the conditions listed in Tables S7 and S9. The reaction mixtures were incubated at 37 °C. Absorption at 430 nm was recorded using a Tecan Infinite® M1000 Pro plate reader.

**Table S9.** Composition of the reaction mixture used in Scheme S2.

| Ingredient                        | Reaction mixture          | Conc. of stock solution | Solvent            | Vol. | Order of addition |
|----------------------------------|---------------------------|-------------------------|--------------------|------|------------------|
| CHES (10 mM, pH 9.0)             |                           |                         |                    | 64 µl| 1                |
| [(Biot-Cp*)Ir(N^N)] 3·Sav K121R  |                           | 200 µM                  | See Table S5       | 25 µl| 2                |
| Urease                           |                           | 0-8 mg/ml               | CHES (10 mM, pH 9.0)| 10 µl| 3                |
| Enrofloxacin 1                   |                           | 200 mM                  | NaOH (250 mM)      | 1 µl | 4                |
| HCOONa / Urea                    |                           | 4 M / 0-0.6M            | CHES (10 mM, pH 9.0)| 100 µl| 5                |
| HCl                              |                           | 5 M                     | water              | 3.2 µl| 6                |

**Scheme S2.** Time-course determination of the ATHase activity in the presence of either urea (left) or urease (right).
Reactivation of urease by addition HCl.

To a reaction mixture (10 ml) containing HCOONa (2M), urea (300 mM) and urease (0.4 mg/ml) in CHES buffer (10 mM, pH 9.0), HCl\text{aq} (5 M) was added dropwise to reach pH 5.0. The reaction mixture was incubated at room temperature (22 °C). In the presence of urea and urease, the pH slowly rises back to pH > 8.0. Renewed addition of HCl\text{aq} (5 M) and urea (100 µl, 8 M in 10 mM CHES, pH 9.0) allows to lower the pH down to 5.0. Five consecutive HCl\text{aq} additions highlight the stability of the system under the experimental conditions used in this study, Scheme S3. The longer time required to bring the pH > 8.0 in the latter cycles is attributed to the gradual build-up of NH₄⁺HCOO⁻ resulting from the urease activity which buffers the solution.

Scheme S3. Time course evolution of the pH of a reaction mixture containing urease, urea and HCOONa. The pH was monitored with pH meter.
ATHase activity cross-regulated by urease activity (Scheme 3b)

Experiments were performed in a 96-well plate using the conditions listed in Tables S7 and S10. The reaction mixtures were incubated at 37 °C. Absorption at 430 nm was recorded using a Tecan Infinite® M1000 Pro plate reader.

Table S10. Reaction conditions used to determine the influence of urease and urea on the ATHase activity (See Scheme 3b)

| Ingredient | Reaction mixture | Conc. of stock solution | Solvent | Vol. | Order of addition |
|------------|------------------|-------------------------|---------|------|------------------|
| CHES (10 mM, pH 9.0) | | | | 54 µl | 1 |
| [(Biot-Cp")Ir(N^N)] 3 · Sav K121R | 200 µM | See Table S5 | 25 µl | 2 |
| Urease | 1-4 mg/ml | CHES (10 mM, pH 9.0) | 20 µl | 3 |
| Enrofloxacin 1 | 200 mM | NaOH (250 mM) | 1 µl | 4 |
| HCOONa / Urea | 4 M / 0.3-0.6 M | CHES (10 mM, pH 9.0) | 100 µl | 5 |
| HCl | 5 M | water | 3.2 µl | 6 |
Re-activation of ATHase /Urease system (Scheme 3c and Figure S3)

Experiments were performed in a 96-well plate using the conditions listed in Tables S7, S11, S12 and S13. The reaction mixtures were incubated at 37 °C. Absorption at 430 nm was recorded using a Tecan Infinite® M1000 Pro plate reader. Absorption displayed in Scheme 3c was corrected to a normalized a volume of 200 µl. The pH variation was monitored using the ratio of absorption of bromothylmol blue at 618 nm and 501 nm.

**Table S11. Composition of the reaction mixture prior to the 1st HCl addition**

| Ingredient                                | Reaction mixture                  | Conc. of stock solution | Solvent                  | Vol.  | Order of addition |
|-------------------------------------------|-----------------------------------|-------------------------|--------------------------|-------|-------------------|
| CHES (10 mM, pH 9.0)                      |                                   |                         |                          | 96 µl | 1                 |
| [(Biot-Cp*)Ir(N^N)] 3 · Sav K121R         |                                   | 200 µM                  | See Table S5             | 37.5 µl | 2                |
| Urease                                    |                                   | 8 mg/ml                 | CHES (10 mM, pH 9.0)     | 15 µl  | 3                 |
| Enrofloxacin 1                            |                                   | 200 mM                  | NaOH (250 mM)            | 1.5 µl | 4                 |
| HCOONa / Urea                             |                                   | 4 M / 0.6 M             | CHES (10 mM, pH 9.0)     | 150 µl | 5                 |

**Table S12. Composition of the reaction mixture after 1st addition of HCl**

| Ingredient | Reaction mixture                  | Conc. of stock solution | Solvent      | Vol.  | Order of addition |
|------------|-----------------------------------|-------------------------|--------------|-------|-------------------|
| HCl        |                                   | 5 M                     | water        | 4 µl  | 1                 |
|            | Reaction mixture of Table S9      |                         | 250 µl       | 2     |                   |

**Table S13. Composition of the reaction mixture after 2nd HCl addition**

| Ingredient | Reaction mixture                  | Conc. of stock solution | Solvent      | Vol.  | Order of addition |
|------------|-----------------------------------|-------------------------|--------------|-------|-------------------|
| HCl        |                                   | 5 M                     | water        | 5 µl  | 1                 |
|            | Reaction mixture of Table S10     |                         | 200 µl       | 2     |                   |
Figure S4. Time-course determination of the activity (left, i.e absorbance vs. time) and rate (right, ΔAbs/Δt vs. time) of the ATHase combined with the urease by addition of HCl.
Re-activation cycles of the of ATHase /Urease system

Experiments were performed in a 96-well plate using the conditions listed in Tables S5 and S14. The reaction mixtures were incubated at 37 °C. An aliquot (200 µl) of the reaction mixture was transferred to a 96-well plate and mixed with CHES buffer (1 M, pH 9.6) to quench the reaction every 5 min. Aliquots (65.6, 72.5 and 42.5 µl) of HClaq (5 M) were added to the reaction mixture for the first, second and third activation respectively. Absorption at 430 nm was recorded using a Tecan Infinite® M1000 Pro plate reader.

Table S14. Composition of the reaction mixture prior to the 1st HCl addition

| Ingredient                           | Reaction mixture | Conc. of stock solution | Solvent               | Vol.  | Order of addition |
|--------------------------------------|------------------|--------------------------|-----------------------|-------|-------------------|
| CHES (10 mM, pH 9.0)                  |                  |                          |                       | 1440 µl| 1                 |
| [(Biot-Cp*)Ir(N^N)] 3 · Sav K121R    |                  | 200 µM                   | See Table S5          | 562.5 µl| 2                 |
| Urease                               |                  | 8 mg/ml                  | CHES (10 mM, pH 9.0)  | 225 µl | 3                 |
| Enrofloxacin 1                       |                  | 200 mM                   | NaOH (250 mM)         | 22.5 µl| 4                 |
| HCOONa / Urea                        |                  | 4 M / 0.8 M              | CHES (10 mM, pH 9.0)  | 2250 µl| 5                 |

Scheme S4. Re-activation of the ATHase and urease by addition of acid. The relative conversions for the three cycles are 100%, 86% and 83%.
ATHase/Esterase/Urease system (Scheme 4b)
Experiments were performed in a 96-well plate using the conditions listed in Tables S7 and S15. The reaction mixtures were incubated at 37 °C. Absorption at 430 nm was recorded using a Tecan Infinite® M1000 Pro plate reader. The pH variation was evaluated by monitoring the ratio of absorption of bromothylmol blue at 618 nm and 501 nm.

Table S15. Composition of the reaction mixture for the Esterase / ATHase / Urease system

| Ingredient | Reaction mixture | Conc. of stock solution | Solvent | Vol. | Order of addition |
|------------|------------------|-------------------------|---------|------|------------------|
| CHES (10 mM, pH 9.0) | | 54 µl | 1 |
| [(Biot-Cp*)Ir(N^N)]3·Sav K121R | 200 µM | See Table S5 | 25 µl | 2 |
| Urease | 4 mg/ml | CHES (10 mM, pH 9.0) | 10 µl | 3 |
| Esterase | 10 mg/ml | CHES (10 mM, pH 9.0) | 10 µl | 4 |
| Enrofloxacin 1 | 200 mM | NaOH (250 mM) | 1 µl | 5 |
| HCOONa / Urea | 4 M / 0.3 M | CHES (10 mM, pH 9.0) | 100 µl | 6 |
| Ethyl butyrate | 5 M | DMSO | 3.2 µl | 7 |

References
[1] V. Köhler, J. Mao, T. Heinisch, A. Pordea, A. Sardo, Y. M. Wilson, L. Knorr, M. Creus, J. C. Prost, T. Schirmer, T. R. Ward, Angew. Chem., Int. Ed. 2011, 50, 10863-10866; Angew. Chem. 2011, 123, 11055-11058.
[2] J. M. Zimbron, T. Heinisch, M. Schmid, D. Hamels, E. S. Nogueira, T. Schirmer, T. R. Ward, J. Am. Chem. Soc. 2013, 135, 5384-5388.