Supplementary Information

Direct Assay to Evaluate Phosphoenolpyruvate Carboxykinase Activity

Bruno S. do Amaral,[a] Katia Celina S. Correa,[a] Arlene G. Corrêa,[a] Dulce H. F. de Souza*[,a] and Quezia B. Cass*[,a]

[a]Departamento de Química, Universidade Federal de São Carlos, Rod. Washington Luís, km 235, 13565-905 São Carlos-SP, Brazil

Synthesis of coumarin derivatives

Unless otherwise noted, all commercially available reagents were purchased from Aldrich Chemical Co. (Milwaukee, USA) and used without purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX-400 (Rheinstetten, Germany) (400 and 100 MHz respectively). The IR spectra refer to films and were measured on a Bomem M102 spectrometer (Zurich, Switzerland). Analytical thin-layer chromatography was performed on a 0.25 mm film of silica gel containing fluorescent indicator UV_{254} supported on an aluminum sheet (Sigma-Aldrich, St. Louis, USA). Flash column chromatography was performed using silica gel (Kieselgel 60, 230-400 mesh, E. Merck, Darmstadt, Germany).

Ethyl 2-oxo-7-(2-(piperidin-1-yl)ethoxy)-2*H*-chromene-3-carboxylate [LSPN223] (**10**)

In round bottom flask containing, a solution of coumarin-3-carboxylic acid (61.2 mg, 0.261 mmol) in *N*,*N*-dimethylformamide (DMF) (4 mL) was added K_2CO_3 (80 mg, 0.574 mmol) and the resulting mixture was stirred for 10 min. Then, 3-chloro-ethyl piperidine (58 mg, 0.313 mmol) and KI (2 mg, 0.012 mmol) were added and the mixture stirred at 70 ºC for 15 h. The solvent was evaporated by using rotaevaporator and the product purified on a flash column chromatography using dichloromethane/methanol 95:5 v/v as eluent. Compound **10** was obtained as a yellow solid in 62% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (s, 1H), 7.49 (d, 1H, *J* 8.0), 6.90 (dd, 1H, *J* 2.0, 8.0), 6.82 (d, 1H, *J* 2.0), 4.40 (q, 2H), 4.18 (t, 2H), 2.80 (t, 2H), 2.52-2.50 (m, 4H), 1.64-1.58 (m, 4H), 1.48-1.44 (m, 2H), 1.40 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.2, 24.1, 25.9, 55.0, 57.4, 61.6, 66.9, 101.1, 111.6, 114.0, 114.1, 130.6, 148.9, 157.1, 157.5, 163.4, 164.4.

3-(Piperidine-1-carbonyl)-2*H*-chromen-2-one [LSPN224] (**11**)

In a round bottom flask under nitrogen atmosphere, equipped with a magnetic stirrer and reflux condenser, coumarin-3-carboxylic acid (0.203 g; 1.06 mmol) was dissolved in $SOCl_2$ (6 mL, 82 mmol), and then the system was heated to approximately 70 ºC. After 7 h, anhydrous hexane (10 mL) was added and the reflux system was replaced by a distillation system in order to remove excess of thionyl chloride. This procedure was repeated twice. The acid chloride obtained was dissolved in dichloromethane (5 mL) under a nitrogen atmosphere and ice bath, then piperidine (0.172 g, 2.02 mmol) was slowly added. The reaction mixture was heated to 50 °C, and after 15 h of the reaction, a saturated solution of $NaHCO_3$ (15 mL) was added, and then organic phase was extracted with dichloromethane (4 × 15 mL). The

*e-mail: qcass@ufscar.br, dulce@ufscar.br

organic phase was dried with anhydrous sodium sulfate and the solvent evaporated on a rotaevaporator. The product was purified on a flash column chromatography in silica gel and ethyl acetate and hexane (7:3) as eluent affording compound **11** in 56% yield (0.152 g); IR (KBr) ν / cm^{-1} 2935, 2853, 1717, 1625, 1252; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (s, 1H), 7.63-7.51 (m, 2H), 7.37-7.28 (m, 2H), 3.72 (bs, 2H), 3.34 (bs, 2H), 1.67 (bs, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.3, 25.6, 26.3, 42.4, 47.8, 114.1, 118.8, 125.2, 125.7, 129.3, 133.0, 141.9, 153.8, 163.0.

2-Oxo-*N*-(2-(piperidin-1-yl)ethyl)-2*H*-chromene-3-carboxamide [LSPN272] (**13**)

Coumarin-3-carboxylic acid (0.15 g, 0.77 mmol) was dissolved in anhydrous dichloromethane (5 mL) in a round bottom flask under the N_2 atmosphere and continuous magnetic stirring. Oxalyl chloride (2.07 mmol, 0.26 g) and DMF (1 drop) were added and allowed to stir for 12 h at 0 ºC using an ice bath. Then, triethylamine (2.07 mmol, 0.209 g) and 1-(2-aminoethyl)piperidine (2.07 mmol, 0.26 g) were added. After 12 h, a saturated solution of $NaHCO_3$ (55 mL) was added and the mixture was extracted with dichloromethane (2 × 10 mL). The organic layer was washed with H_2O (2 × 10 mL) and brine (10 mL), and dried with anhydrous Na_2SO_4. Then, solvent was evaporated using a rotaevaporator to afford the oily liquid, which was further purified to afford the compound **13** through flash chromatography in silica gel and ethyl acetate/methanol 9:1 as eluent. Compound **13** was obtained as a yellow solid in 78% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43-1.50 (m, 2H), 1.58-1.69 (m, 4H), 2.47 (t, 4H, *J* 8.0), 2.57 (t, 2H, *J* 12.0), 3.56 (q, 2H, *J* 12.0, 16.0), 7.34-7.42 (m, 2H), 7.61-7.71 (m, 2H), 8.90 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.40, 26.01, 37.12, 54.37, 59.99, 116.57, 118.67, 125.13, 129.70, 133.81, 147.94, 154.43, 161.36.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method qualification

Before initiating the method qualification, through injection of phosphoenolpyruvate (PEP) standard solutions in the presence and absence of oxaloacetate (OAA), it was verified that OAA has an effect on PEP ionization. Adenosine 5′-triphosphate (ATP) and adenosine-5'-diphosphate (ADP) did not interfere in the PEP ionization. This was carried out due to the co-elution of substrates and products in the chromatographic analysis to monitor *Tc*PEPCK activity, in which PEP was selectively monitored by MS. Another observation was that *Tc*PEPCK causes matrix effect. In order to eliminate the matrix effects, a constant concentration of OAA was used, and the enzyme was added at the same concentration used for the activity reactions.

Linearity was evaluated using external calibration curves to phosphoenolpyruvate (PEP). Stock solution of PEP at 5 mmol L^{-1} was prepared in ammonium acetate buffer (15 mmol L^{-1}, pH 8.50), and from that, working solutions for calibration and quality controls (QC) were prepared at the following concentrations: 5.0, 10.0, 15.0, 25.0, 45.0, 65.0, 85.0, 100.0, 150.0, 200.0 μmol L^{-1} and 12.5, 120.0, and 180.0 μmol L^{-1}, respectively.

Calibration standards and quality control samples were prepared as follows: to aliquots of 5 μL of the appropriated PEP, standard working solutions were added 10 μL of *Tc*PEPCK-II and 85 μL of buffer A (50 mmol L^{-1} ammonium acetate buffer pH 9.00, containing 1 mmol L^{-1} DTT and 1 mmol L^{-1} $MnCl_2$), maintaining a constant concentration of 100 μmol L^{-1} OAA. The solutions were vortex-mixed during 10 s, followed by addition of 100 μL MeOH, vortex-mixed during 10 s, centrifugation at 9,300 × g, 10 min and 4 °C. Aliquots of 200 μL were transferred to auto-sampler vials. Samples of 10 μL were injected in LC-MS/MS system.

The samples were prepared in triplicate for the calibration curves that were constructed from a linear regression by plotting the peak area of ion fragment from PEP $[PO_4]^-$ 79 *m/z* against the concentration of PEP (Figure S1). Intra and inter-batch precisions and accuracies of the method were determined by the analysis of QC samples. Five samples

of each concentration were prepared as described above. Accuracy was evaluated by back-calculation and expressed as percentage of deviation between the amount found and the amount added at the three concentrations examined. Selectivity of the method was evaluated by blank samples, in which no ion of the same ratio *m/z* was observed.

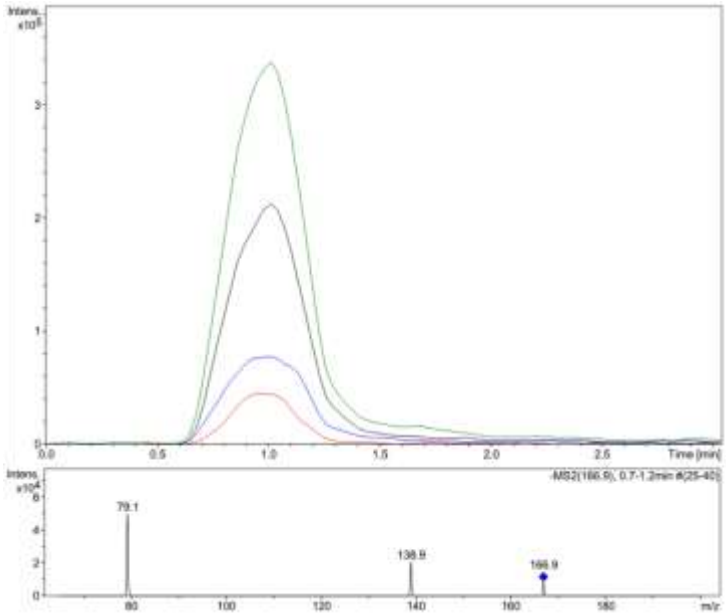

Figure S1. Typical LC-MS/MS chromatograms using the multiple reaction-monitoring (MRM) mode: Luna® C18(2) column (30.0 × 2.00 mm, 3 µm) with ammonium acetate buffer (15 mmol L^{-1}, pH 8.5)/MeOH 1:1 v/v as mobile phase. Monitoring PEP precursors ion, $[M - H]^-$ 167 *m/z* (red) and its fragments $[M - CO]^-$ 139 *m/z* (blue) as qualifier ion and $[PO_3]^-$ 79 *m/z* (black) for the quantification.

The regression equation obtained for the calibration curve was $y = 741.1054x - 5065.1000$, with a determination coefficient (R^2) greater than 0.99. The values result of precision for the low (12.5 µmol L^{-1}), mid (120 µmol L^{-1}) and high (180 µmol L^{-1}) quality controls were in the range from 5.33 to 9.01%. Accuracies furnished values ranging from 87.0 to 115%.

Ligand screening

As the compounds screened were of small molecules, PEP co-eluted with the screened compounds. To overcome this problem the influence on PEP ionization was examined in two different ways. At first, the selectivity was examined for each screened compound, as negative controls samples, and the ions 167 → 79 *m/z* were not observed.

Co-eluting compounds might affect the ion intensity of the target analyte by interfering in the ionization process.[1] Based on the procedure described by Kruve *et al.*,[2] two sets of samples were prepared: (*i*) sample set 1 was composed of 150 µmol L^{-1} OAA, 100 µmol L^{-1} PEP and 10 µL of the purified enzymatic fraction. The LC-MS/MS analysis furnished a peak area $A_{standard}$; (*ii*) sample set 2 was prepared at the same concentrations and conditions of the sample set 1, but now containing 50 µmol L^{-1} of the screened compounds to furnish area A_{sample}. In both set of samples, the final volume was adjusted to 100 µL with buffer A, the solutions were vortex-mixed for 10 s, followed by addition of 100 µL of MeOH, vortex-mixed for 10 s centrifugation at 9,300 × g, 10 min and 4 °C. The aliquots of 200 µL were transferred to autosampler vials. Samples of 10 µL were analyzed.

The ratio $A_{sample}/A_{standard}$ was calculated with the MS/MS areas and defined as the matrix effect. A value of 1

indicates that there is no effect. Signal enhancement if the value is > 1 and signal suppression if the value is < 1.

For correcting the matrix effect on PEP ionization ($A_{estimated}$), equation S1 was used, where the $A_{screening}$ is the PEP area obtained in the screening assay.

$$A_{estimated} = A_{screening} + \left\{A_{screening} \times \left[1 - \left(\frac{A_{sample}}{A_{standard}}\right)\right]\right\} \quad \text{(S1)}$$

The percentage of inhibition was then calculated using equation S2, where A_{PC} is the positive control area of the screening assay (in "Ligand screening" sub-section).

$$\text{Inhibition}\,(\%) = 100 - \left(\frac{A_{estimated}}{A_{PC}} \times 100\right) \quad \text{(S2)}$$

References

1. Petrosino, T.; Petrosino, T.; *J. Chromatogr. B* **2014**, *965*, 100.
2. Kruve, A.; Rebane, R.; Kipper, K.; Oldekop, M. L.; Evard, H.; Herodes, K.; Ravio, P.; Leito, I.; *Anal. Chim. Acta* **2015**, *870*, 8.