

## Supplementary Methods

### Materials:

The following reagents were purchased and used as received: oxaloacetic acid ( $\geq 97\%$ , Sigma-Aldrich O4126), DL-malic acid ( $\geq 99\%$ , Sigma-Aldrich 240176), sodium malonate dibasic monohydrate (Sigma-Aldrich M4795), 50 wt% in H<sub>2</sub>O glyoxylic acid solution, 30 wt% in H<sub>2</sub>O hydrogen peroxide solution (contains inhibitor, assayed 29.0 to 32.0%, Sigma-Aldrich 216763), sodium pyruvate ( $\geq 99\%$ , Sigma-Aldrich P2256), malonic acid ( $\geq 99\%$ , Sigma-Aldrich M1296). (1,2-<sup>13</sup>C<sub>2</sub>)glyoxylic acid (contaminated with 3% glycolic acid, and 1.5% oxalic acid), and (2-<sup>13</sup>C<sub>1</sub>)malonic acid (99%) were purchased from Cambridge Isotope Laboratories, Inc., and used as received.

### Procedures:

#### HKG cycle

*Oxaloacetate to HKG*: 119 mg of oxaloacetic acid (0.90 mmol, 90 mM) was dissolved in 9.9 ml of a 1.0 M aq. pH 7 sodium phosphate buffer in a 20-ml vial (Chemglass, CG-4912-05) with a small stirbar. 119  $\mu$ l (160 mg) of 50 wt% glyoxylic acid in H<sub>2</sub>O (1.2 eq.) was added by micropipette. The reaction pH was adjusted to 7.0 with 4.0 M aq. NaOH as measured by a calibrated Mettler Toledo InLab Expert Pro probe. The reaction vial was placed on a magnetic stir plate (Ika, RCT basic) fitted with a vial reaction block heater (ChemGlass, CG-1991-01) and stirred at 23 °C for 3 h to give >98% HKG. *oxalomalate (OXM)*: <sup>1</sup>H NMR, 400 Mz, D<sub>2</sub>O – *diastereomer A*:  $\delta$  4.27 ppm (1H, d),  $\delta$  4.32 ppm (1H, d), *diastereomer B* -  $\delta$  4.28 ppm (1H, d),  $\delta$  4.34 ppm (1H, d). *4-hydroxy-2-ketoglutarate (HKG)*: <sup>1</sup>H NMR, 400 Mz, D<sub>2</sub>O –  $\delta$  2.90 ppm (1H, dd),  $\delta$  3.05 ppm (1H, dd),  $\delta$  4.25 ppm (1H, dd)

*HKG to malate*: To the HKG-containing reaction described above, was added 184  $\mu$ l (203 mg) of 30 wt% hydrogen peroxide in water (2 eq. H<sub>2</sub>O<sub>2</sub>). The reaction was stirred at 23 °C for 30 min to give >98% malate. *malate*: <sup>1</sup>H NMR, 400 Mz, D<sub>2</sub>O –  $\delta$  2.20 ppm (1H, dd),  $\delta$  2.50 ppm (1H, dd),  $\delta$  4.15 ppm (1H, dd)

*Malate to malonate*: To the malate-containing reaction described above, was added 1.38 ml (1.53 g) of 30 wt% hydrogen peroxide in water (15 eq. H<sub>2</sub>O<sub>2</sub>). The reaction was stirred at 50 °C for 24 h to give 55% malate. *malonate*: <sup>1</sup>H NMR, 400 Mz, D<sub>2</sub>O –  $\delta$  2.93 ppm (2H, s), <sup>13</sup>C NMR, 400 Mz, D<sub>2</sub>O –  $\delta$  42 ppm

*Additional Notes*: The aldol addition (to generate OXM), the decarboxylation (to generate HKG), and the oxidation (to generate malonate) are tolerant to buffer type (phosphate and carbonate were employed) and pH values (5.5 to 10.5 were employed), see Supplementary Figs. 4–6. The oxidation reaction of malate to malonate is accelerated by the inclusion of iron-based Lewis acids (ferrous sulfate, ferric sulfate, iron disulfide), see Supplementary Table S1.

## Malonate cycle

*Malonate to 3-carboxymalonate (3-CM)*: 149 mg of sodium malonate dibasic monohydrate (0.90 mmol, 90 mM) was dissolved in 9.7 ml of a 1.0 M aq. sodium carbonate buffer in a 20-ml vial. 298  $\mu$ l (400 mg) of 50 wt% glyoxylic acid in H<sub>2</sub>O (3.0 eq.) was added by micropipette. The reaction pH was measured (8.4), and the vial was placed in a heat block and stirred at 50 °C for 3 h to give >98% 3-CM. Lower pH values (7.0, phosphate buffer) produced an equilibrium mixture of the aldol addition product (3-CM) and the aldol condensation product (3-carboxyfumarate, 3-CF, see Table S1). *3-carboxymalate (3-CM)*: <sup>1</sup>H NMR, 400 Mz, D<sub>2</sub>O –  $\delta$  3.45 ppm (1H, d),  $\delta$  4.22 ppm (1H, d), *3-carboxyfumarate (3-CF)*: <sup>1</sup>H NMR, 400 Mz, D<sub>2</sub>O –  $\delta$  6.17 ppm (1H, s)

*3-carboxymalonate (3-CM) to malonate*: To the 3-CM-containing reaction described above, was added 1.38 ml (1.53 g) of 30 wt% hydrogen peroxide in water (15 eq. H<sub>2</sub>O<sub>2</sub>). The reaction was stirred at 50 °C for 24 h. An additional 15 eq. of hydrogen peroxide was added and the reaction was stirred for another 24 h at 50 °C to give 51% malonate.

## Malonate Cycle Turnover

*1<sup>st</sup> iteration (<sup>13</sup>C<sub>1</sub> malonic acid to <sup>13</sup>C<sub>2</sub> malonic acid)*: 42 mg of (2-<sup>13</sup>C<sub>1</sub>)malonic acid (0.40 mmol, 200 mM) was dissolved in 2.0 ml of 1.0 M aqueous NaHCO<sub>3</sub> in a 1-dram vial. 61 mg of (1,2-<sup>13</sup>C<sub>2</sub>)glyoxylic acid (2 eq.) were added and the reaction pH was measured (8.0). The reaction was stirred at 50 °C for 3 h. 30 wt% aqueous H<sub>2</sub>O<sub>2</sub> was subsequently added at a rate of 40 eq/h, and the reaction was stirred at 50 °C for 10 h to produce (1,2-<sup>13</sup>C<sub>2</sub>)malonic acid (Calcd. [M-H] m/z: 105.0098, Found: 105.0020).

*2<sup>nd</sup> iteration (<sup>13</sup>C<sub>2</sub> malonic acid to <sup>13</sup>C<sub>3</sub> malonic acid)*: The reaction volume was reduced back to 2.0 ml under vacuum to maintain a consistent reaction concentration between iterations. 61 mg of (1,2-<sup>13</sup>C<sub>2</sub>)glyoxylic acid (2 eq.) were added and the reaction pH was measured (8.0). 30 wt% aqueous H<sub>2</sub>O<sub>2</sub> was added to the solution at a rate of 40 eq/h, and the reaction was stirred at 50 °C for 10 h to produce a 1:1 mixture of (1,2-<sup>13</sup>C<sub>2</sub>)malonic acid and (1,2,3-<sup>13</sup>C<sub>3</sub>) malonic acid (Calcd. [M-H] m/z: 106.0132, Found: 106.0033).

## Pyruvate to HKG

149 mg of sodium pyruvate (0.90 mmol, 90 mM) was dissolved in 9.7 ml of 1.0 M aq. sodium bicarbonate buffer in a 20-ml vial. 149  $\mu$ l (200 mg) of 50 wt% glyoxylic acid in H<sub>2</sub>O (1.5 eq.) was added by micropipette. The reaction pH was measured at 7.9. The reaction was then placed in a heat block and stirred at 50 °C for 1 h.

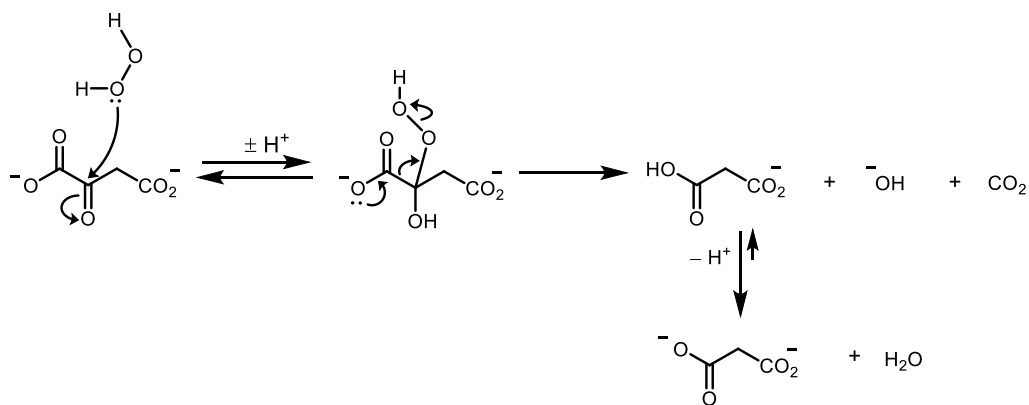
## Aspartate Synthesis

*glyoxylate to  $\alpha$ -hydroxyglycine*: 5.0 g of ammonium acetate (65 mmol) in 5.0 ml of deionized water was chilled to 0 °C. 3.7 ml of chilled 50 wt% glyoxylic acid in H<sub>2</sub>O (0.52 eq, 2.5 g) was added and the reaction was stirred at 0 °C for 45 min. The product was collected as a white precipitate and washed twice with 5 ml of cold deionized water to yield >95%  $\alpha$ -hydroxyglycine (15).  *$\alpha$ -hydroxyglycine*: <sup>13</sup>C NMR, 400 Mz, D<sub>2</sub>O –  $\delta$  87 ppm, 176 ppm.

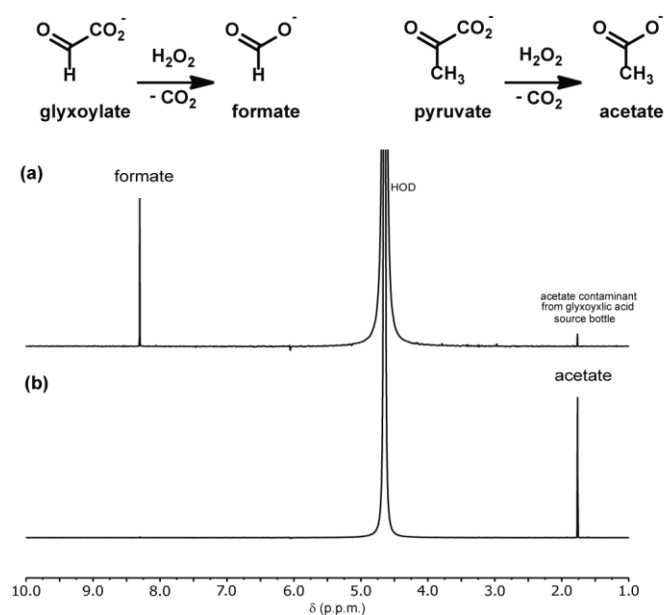
*α*-hydroxyglycine and malonic acid to *β*-carboxyaspargate: 100 mg of malonic acid (0.96 mmol, 480 mM) was dissolved in 2.0 ml of 1.0 M aq. sodium bicarbonate buffer in a 1-dram vial. 170 mg (2 eq.) of *α*-hydroxyglycine was added. The reaction was stirred at 23 °C for 5 d; the pH drifted from 7.2 to 6.8 over the reaction course. *β*-carboxyaspargate: <sup>13</sup>C NMR, 400 Mz, D<sub>2</sub>O – δ 59 ppm, 74 ppm, 173 ppm, 173 ppm, 174 ppm. Mass Spec. MH<sup>+</sup> 178.03

*β*-carboxyaspargate to aspartate: To the *β*-carboxyaspargate-containing reaction described above, was added 195 mg of magnesium chloride hexahydrate (1 eq.), and the reaction was stirred at 60 °C for 5 h to give a two-step yield of 50% for the malonic acid to aspartate transformation. *aspartate*: <sup>1</sup>H NMR, 400 Mz, D<sub>2</sub>O – δ 2.55 ppm (1H, dd), δ 2.68 ppm (1H, dd), δ 3.76 ppm (1H, dd). Mass Spec. MH<sup>+</sup> 134.04

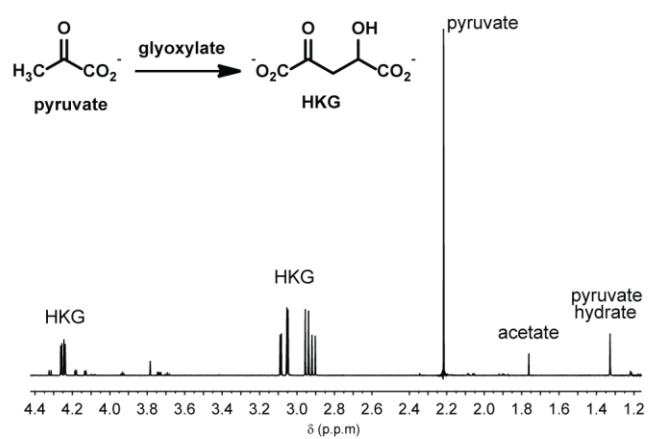
## Supplementary Figures



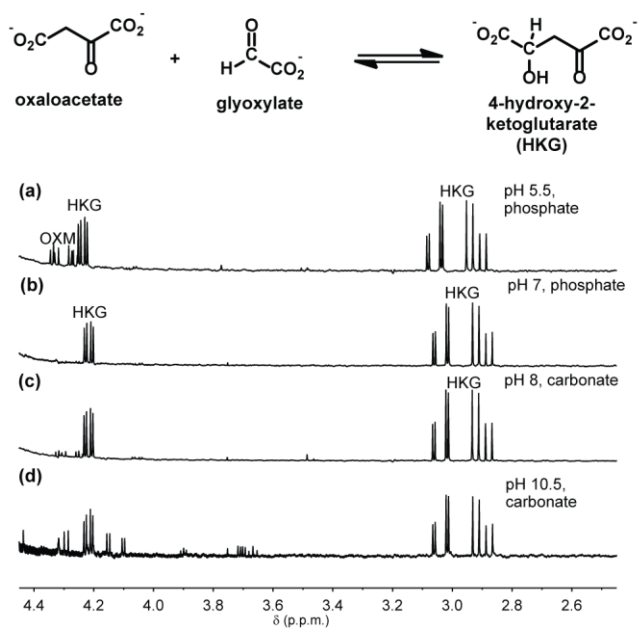
**Supplementary Figure 1.** The electron-moving mechanism of oxidative decarboxylation of oxaloacetate to produce malonate.



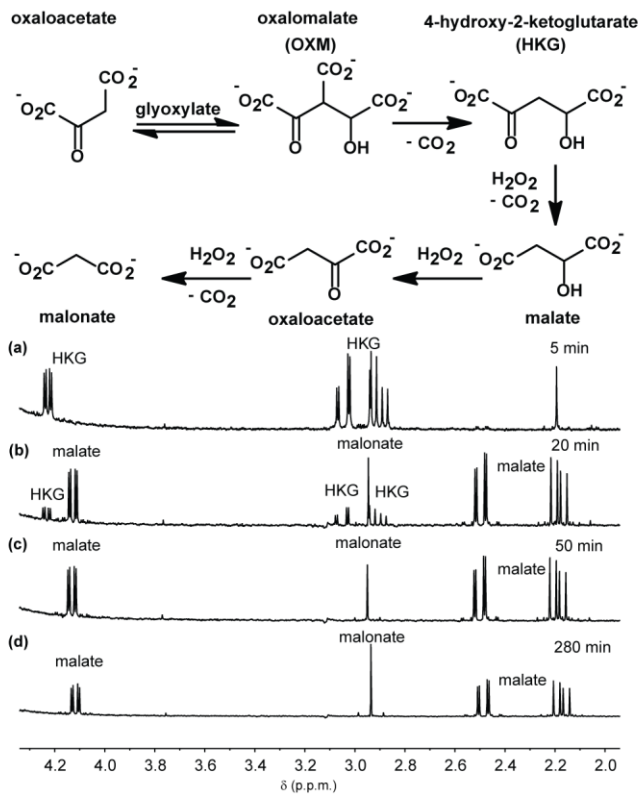
**Supplementary Figure 2.** Oxidation of glyoxylate and pyruvate by H<sub>2</sub>O<sub>2</sub> to formate and acetate respectively. (a) 100 mM glyoxylate, 2 eq. H<sub>2</sub>O<sub>2</sub>, pH 7, 1 M phosphate buffer, 15 min, 23 °C, (b) 100 mM pyruvate, 2 eq. H<sub>2</sub>O<sub>2</sub>, pH 7, 1 M phosphate buffer, 15 min, 23 °C.



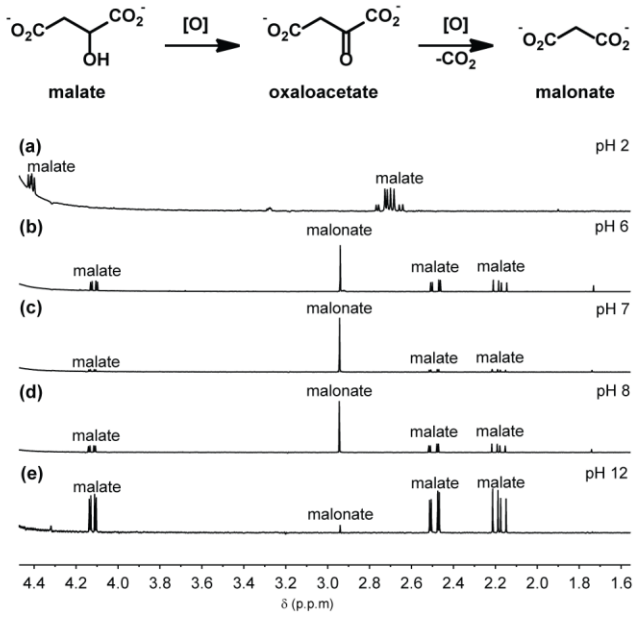
**Supplementary Figure 3.** Entry into the protometabolic cycles from pyruvate. 90 mM pyruvate, 1.5 eq. glyoxylate, in 10 ml of 1.0 M aq. sodium bicarbonate at pH 7.9, was heated at 50 °C for 1 h to produce HKG in 65% yield.



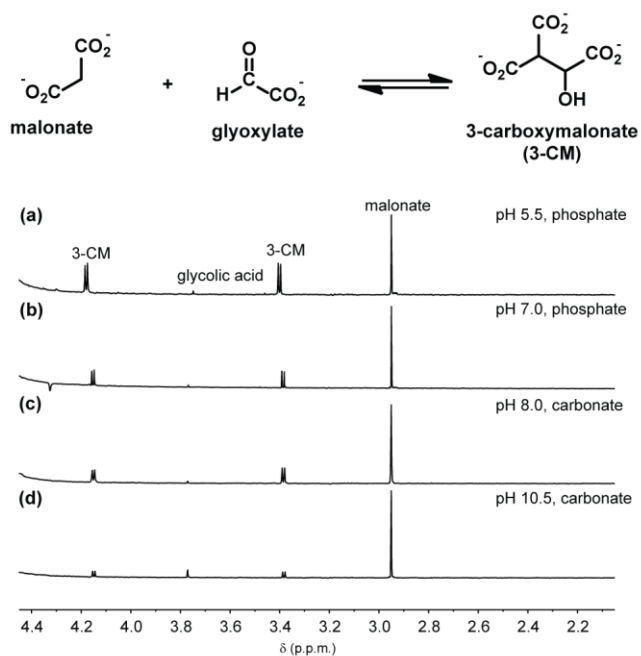
**Supplementary Figure 4. The pH and buffer dependence of the aldol addition of oxaloacetate to glyoxylate in the HKG cycle.** 90 mM oxaloacetate with 1.5 eq. of glyoxylic acid at 23 °C for 100 min, (a) pH 5.5 in 1.0 M sodium phosphate, (b) pH 7.0 in 1.0 M sodium phosphate, (c) pH 8.0 in 1.0 M sodium bicarbonate, (d) pH 10.5 in 1.0 M sodium carbonate.



**Supplementary Figure 5.** Progression of the HKG cycle from oxaloacetate to malate, and then on to malonate in carbonate buffer. 90 mM oxaloacetate in 1.0 M  $\text{NaHCO}_3$ , 1.5 eq. of glyoxylic acid, pH 7.2 to 8.2, 52 °C. 30 wt% hydrogen peroxide was added at a rate of 5 eq./h.  $^1\text{H}$  NMR at (a) 5 min, (b) 20 min, (c) 50 min, (d) 280 min.

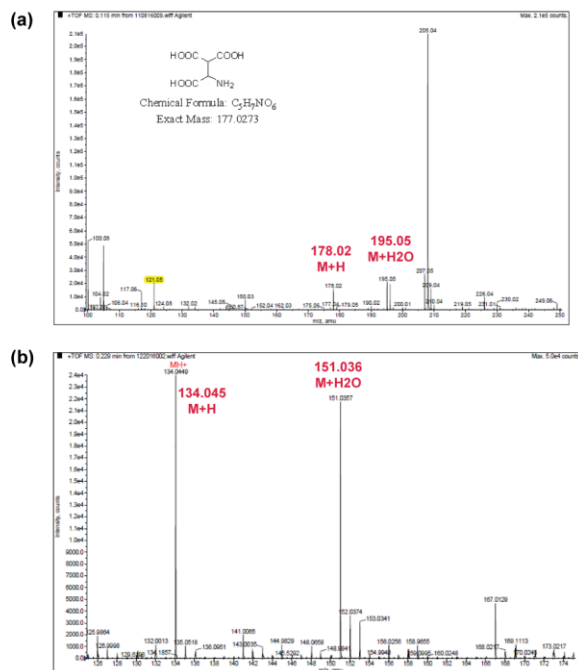


**Supplementary Figure 6.** The pH dependence of the oxidation of malate to malonate. 90 mM malate in 1.0 M sodium phosphate, 30 eq.  $\text{H}_2\text{O}_2$  at once, 50 °C for 24 h at (a) pH 2, (b) pH 6, (c) pH 7, (d) pH 8, (e) pH 12.



**Supplementary Figure 7.** The pH and buffer dependence of the aldol addition of malonate to glyoxylate in the Malonate cycle. 90 mM sodium malonate with 3 eq. of glyoxylic acid at 23 °C for 48 h. (a) pH 5.5 in 1.0 M sodium phosphate (b) pH 7.0 in 1.0 M sodium phosphate, (c) pH 8.0 in 1.0 M sodium bicarbonate, (d) pH 10.5 in 1.0 M sodium carbonate. All reactions adjusted to initial pH with 2 M NaOH. Final reaction pH values at 48 h were 5.4, 7.0, 8.3, and 10.7 respectively.



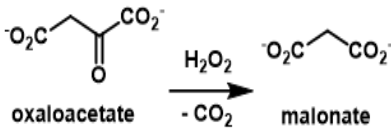
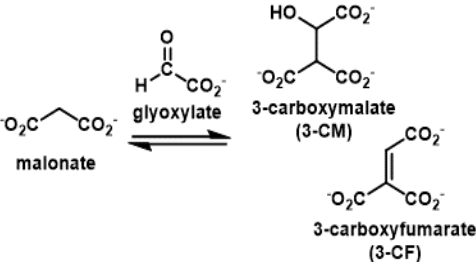
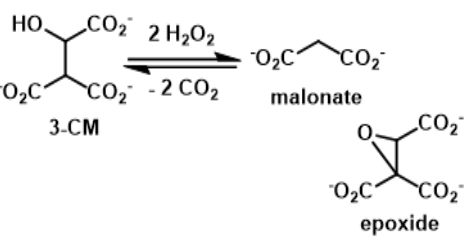


**Supplementary Figure 8.** Aspartate synthesis from malonate and  $\alpha$ -hydroxyglycine. (a) +TOF MS of 480 mM malonic acid, with 2 eq. of  $\alpha$ -hydroxyglycine in 1.0 M sodium bicarbonate buffer at 23 °C for 5 d generates  $\beta$ -carboxy aspartate (178.03). (b) Subsequent addition of 1 eq. of  $MgCl_2$  and heating at 60 °C for 5 h produces aspartate (134.04).

**Supplementary Table 1:** Reaction conditions and yields for HKG and Malonate cycle reactions.

Reaction Step	Reaction Scheme	Product Yield <sup>i</sup>	SM	Reaction Conditions
<b>HKG Cycle</b>				
oxaloacetate to HKG	<p>oxaloacetate <math>\xrightarrow[\text{-CO}_2]{\text{glyoxylate}}</math> HKG</p>	>98%	<2%	90 mM oxaloacetic acid, 1.2 eq. glyoxylate, pH 7 in 1 M phosphate buffer for 3 h at 23 °C
		>98%	<2%	90 mM oxaloacetic acid, 1.2 eq. glyoxylate, pH 8 in 1 M carbonate buffer for 3 h at 23 °C
HKG to malate	<p>HKG <math>\xrightarrow[\text{-CO}_2]{\text{H}_2\text{O}_2}</math> malate</p>	>98%	<2%	90 mM HKG, 2 eq. H <sub>2</sub> O <sub>2</sub> , pH 7, 1 M phosphate buffer for 30 min at 23 °C
		>98%	<2%	90 mM HKG, 2 eq. H <sub>2</sub> O <sub>2</sub> , pH 8 1 M carbonate buffer for 30 min at 23 °C
malate to malonate	<p>malate <math>\xrightarrow[\text{-CO}_2]{2 \text{ H}_2\text{O}_2}</math> malonate</p>	55%	14%	90 mM malic acid, 15 eq. H <sub>2</sub> O <sub>2</sub> , pH 7 in 1 M phosphate buffer for 24 h at 50 °C
		57%	4%	90 mM malic acid, 15 eq. H <sub>2</sub> O <sub>2</sub> , pH 7 in 1 M phosphate buffer for 3 h at 50 °C, with 0.25 eq. of ferrous sulfate
		52%	16%	90 mM malic acid, 15 eq. H <sub>2</sub> O <sub>2</sub> , pH 8 in 1 M carbonate buffer for 3 h at 50 °C, with 0.25 eq. of ferrous sulfate
<b>pyruvate to HKG</b>	<p>pyruvate <math>\xrightarrow[\text{-CO}_2]{\text{glyoxylate}}</math> HKG</p>	65%	30%	90 mM pyruvate, 1.5 eq. of glyoxylic acid, pH 7.9 in 1 M bicarbonate buffer for 1 h at 50 °C.
<b>malonate to aspartate</b>	<p>malonate <math>\xrightarrow[\text{-CO}_2]{\text{H}_2\text{N}-\text{CH}(\text{OH})-\text{CO}_2^-}</math> <math>\beta</math>-carboxy-aspartate <math>\xrightarrow{\text{Mg}^{2+}}</math> aspartate</p>	50%		480 mM malonic acid, 2 eq. $\alpha$ -hydroxyglycine, pH 7 in 1 M bicarbonate buffer for 5 days at 23 °C. Subsequently, 1 eq. MgCl <sub>2</sub> was added and the reaction was heated for 5 h at 60 °C

Supplementary Table 1 continued

Reaction Step	Reaction Scheme	Product Yield <sup>i</sup>	SM <sup>i</sup>	Reaction Conditions
<b>Malonate Cycle</b>				
oxaloacetate to malonate	 <p>oxaloacetate <math>\xrightarrow[\text{-CO}_2]{\text{H}_2\text{O}_2}</math> malonate</p>	>98%	<2%	90 mM oxaloacetic acid, 1.2 eq. H <sub>2</sub> O <sub>2</sub> , pH 7 in 1 M phosphate buffer for 30 min at 23 °C
		>98%	<2%	90 mM oxaloacetic acid, 1.2 eq. H <sub>2</sub> O <sub>2</sub> , pH 8 in 1 M phosphate buffer for 30 min at 23 °C
malonate to 3-CM	 <p>malonate <math>\xrightleftharpoons[\text{glyoxylate}]{} 3\text{-carboxymalate (3-CM)}</math>  <math>\rightleftharpoons 3\text{-carboxyfumarate (3-CF)}</math></p>	71% (+ 26% 3-CF)	3%	90 mM malonate, 3 eq. of glyoxylic acid, pH 7 in 1 M phosphate buffer for 8 h at 50 °C
		>98%	<2%	90 mM malonate, 3 eq. of glyoxylic acid, pH 8.4 in 1 M bicarbonate buffer for 24 h at 50 °C
3-CM to malonate	 <p>3-CM <math>\xrightleftharpoons[\text{-2 CO}_2]{2 \text{ H}_2\text{O}_2}</math> malonate</p> <p>epoxide</p>	21% (+ 74% epoxide)	<1%	90 mM malic acid, 15 eq. H <sub>2</sub> O <sub>2</sub> , pH 7, in 1 M phosphate buffer for 24 h at 50 °C
		36%	48%	90 mM malic acid, 15 eq. H <sub>2</sub> O <sub>2</sub> , pH 8, in 1 M bicarbonate buffer for 48 h at 50 °C
		51%	49%	90 mM malic acid, 15 eq. H <sub>2</sub> O <sub>2</sub> , + 15 eq. H <sub>2</sub> O <sub>2</sub> at 24 h, pH 8 in 1 M bicarbonate buffer for 48 h total at 50 °C

i. Product yields and remaining starting material (SM) were measured by <sup>1</sup>H NMR integration compared with an internal standard (*t*-butanol) in the D<sub>2</sub>O NMR solvent. Yields are calculated as the percent of the theoretical yield from limiting reactant (non-isolated).