Supplementary Information

Stable Isotope and Mass Spectrometry-based Metabolomics as Tools in Drug Metabolism: A Study Expanding Tempol Pharmacology

Fei Li,[†,¶] Xiaoyan Pang,[†,¶] Kristopher W. Krausz,[†] Changtao Jiang,[†] Chi Chen,[†,‡] John A. Cook,[§] Murali C. Krishna,[§] James B. Mitchell,[§] Frank J. Gonzalez,[†] and Andrew D. Patterson [*,†,□]

[†]Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

[‡]Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55108

[§]Radiation Biology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

[□]Department of Veterinary and Biomedical Sciences and the Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, PA 16802

[¶]These authors contributed equally to this manuscript

[*]Corresponding author: Andrew D. Patterson, Department of Veterinary and Biomedical Sciences and the Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, PA 16802. Tel: (814) 867-4565; Fax: (814) 863-1696; Email: adp117@psu.edu

Structural elucidation of tempol metabolites

Metabolites **T1** and **T2** were calculated as $C_9H_{19}NO_2$ and $C_9H_{19}NO$ with a mass error of 0.6 and -1.3 ppm based on their accurate mass measurement, *m/z* 174.1495^+ (RT = 1.58 min) and 158.1543^+ (RT = 0.45 min), respectively. Compared with the chemical composition of tempol ($C_9H_{18}NO_2$), metabolite **T1** was the reduced form of tempol, the hydroxylamine, generated by the reduction of N-oxide to the hydroxyl group. Generally, there is an equilibrium between the three tempol oxidation forms, nitroxide, hydroxylamine, and oxoammonium cation. However, the ions of nitroxide and oxommonium cation were not detected in this study. Compared with chemical composition of **T1**, metabolite **T2** was its dehydroxylation metabolite. Further analysis indicated that the retention time and MS/MS spectra of **T2** were different from that of reduced tempo (RT = 2.58), suggesting that 1-OH was eliminated from **T2**. **T2** was identified as the amine form. The characteristic fragment ions of *m/z* 74.0597^+ and 58.0658^+ were derived from **T1** and **T2** by McLafferty rearrangement, respectively (Figures S-2A and 4A). Metabolites **T1** and **T2** have been found in the incubation of tempol and liver microsome or keratinocytes. Compare with the chemical composition of **T2** ($C_9H_{19}NO$), **T6** ($C_{15}H_{27}NO_7$) was its glucuronideconjugated. In its MS/MS spectrum (Figure S-4B), the elimination of 176 Da gave the ion *m/z* 158.1510^+, suggesting that **T6** was generated by the conjugation of glucuronic acid with **T2**. The fragment ions *m/z* 58.0674^+ was generated by McLafferty rearrangement. In addition, **T6** was degraded from urine following treatment with 3M HCl for 1 hr (Figures S-8A), verifying that **T6** was a conjugated metabolite. Therefore, **T6** was identified as 1-dehydroxytempol 4-*O*-glucuronide.

Metabolites **T3** and **T5** were calculated as $C_9H_{17}NO$ and $C_{15}H_{25}NO_7$ with a mass error of -5.8 and -1.5 ppm based on their accurate mass measurement, *m/z* 156.1329^+ (RT = 2.21 min) and 332.1704^+ (RT = 1.84 min), respectively. Compared with the chemical composition of **T1** ($C_9H_{19}NO_2$), metabolite **T3** eliminated one water molecule by desaturation. This desaturation effect happened in the 4-OH. Two of its fragment ions *m/z* 138.1281^+ and 100.0770^+ confirmed that **T3** was the desaturation metabolite. Compared with chemical composition of **T3**, **T5** was the glucuronide-conjugated metabolite of **T3**. In its

MS/MS spectrum (Figures S-3C), the elimination of 176 Da gave the ion *m/z* 156.1389$^+$, suggesting that **T5** was generated by the conjugation of glucuronic acid with **T3**. The other fragment ions of **T5** were similar to those of **T3** (Figures S-3A and C). In addition, **T5** was degraded from urine following treatment with 3M HCl for 1 hr (Figures S-8A), verifying that **T5** was a conjugated metabolite. Therefore, **T3** and **T5** were identified as 4-dehydroxytempol and 4-dehydroxytempol 1-*O*-glucuronide.

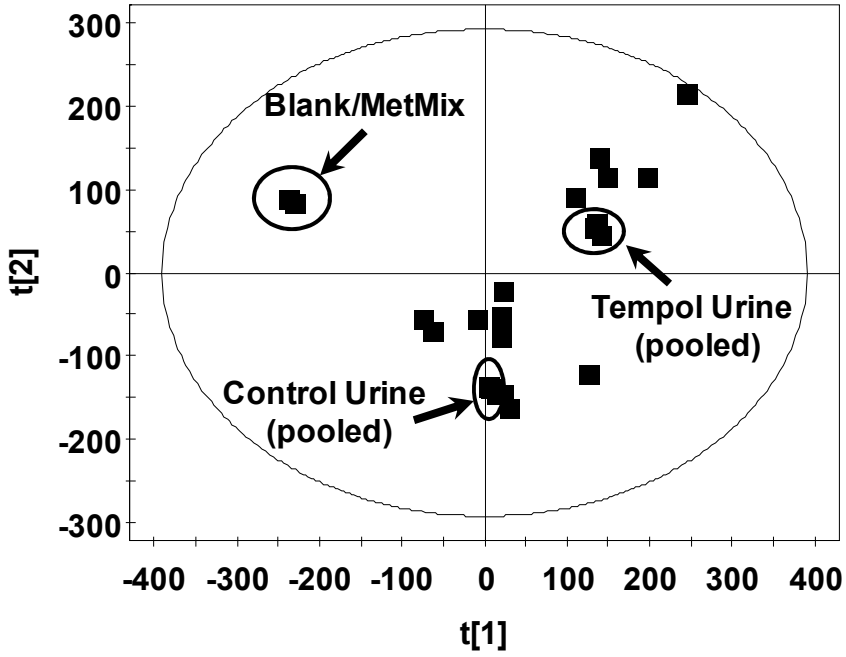
Metabolite **T4** was calculated as $C_9H_{19}NO_3$ with a mass error of -0.5 based on its accurate mass measurement, *m/z* 190.1442$^+$ (RT = 1.21 min). Compared with the chemical composition of **T1** ($C_9H_{19}NO_2$), metabolite **T4** was a hydroxylated metabolite of **T1**. The fragment ions of *m/z* 74.0599$^+$ were generated from **T4** by McLafferty rearrangement, suggesting that it was a substituent of the -OH in the six-membered ring. The elimination of 18 Da and 36 Da gave the fragment ions *m/z* 172.1356$^+$ and 154.1236$^+$ (Figures S-3B), confirming another two hydroxyl groups in the ring. Therefore, **T4** was identified as 3-hydroxytempol.

Metabolites **T7** and **T8** were calculated as $C_{15}H_{29}NO_7$ and $C_{15}H_{27}NO_8$ with a mass error of -1.2 and 5.4 ppm based on their accurate mass measurement, *m/z* 336.2018$^+$ (RT = 1.48 min) and 350.1834$^+$ (RT = 1.31 min), respectively. Compared with the chemical composition of **T1** ($C_9H_{19}NO_2$), **T7** and **T8** were the glucoside and glucuronide-conjugated metabolites of **T1**, respectively. In the MS/MS spectrum of **T7** (Figures S-3D), the elimination of 162 Da gave the parent ions *m/z* 174.1490$^+$, suggesting that **T7** was generated by the conjugation of glucose with **T1**. The other fragment ions of **T7** were similar to those of **T1**. In the MS/MS spectrum of **T8** (Figures S-2C), the elimination of 176 Da gave the parent ions *m/z* 174.1488$^+$, suggesting that **T8** was generated by the conjugation of glucuronic acid with **T1**. The other fragment ions of **T8** were similar to those of **T1**. As described the above, the retention time of 2,2,6,6-tetramethylpiperidine 4-OH was less than 1.0 min (such as **T2**, RT = 0.45), whereas that of 2,2,6,6-tetramethylpiperidine 1-OH was more than 1.0 min (such as reduced tempo, RT = 2.58). This suggested that 4-OH of **T8** was conjugated with glucuronic acid. Similarly, 4-OH of **T7** was conjugated with glucose. The high peak intensity of 4-glucuronide (**T8**) and glucoside (**T7**) can be detected in urine,

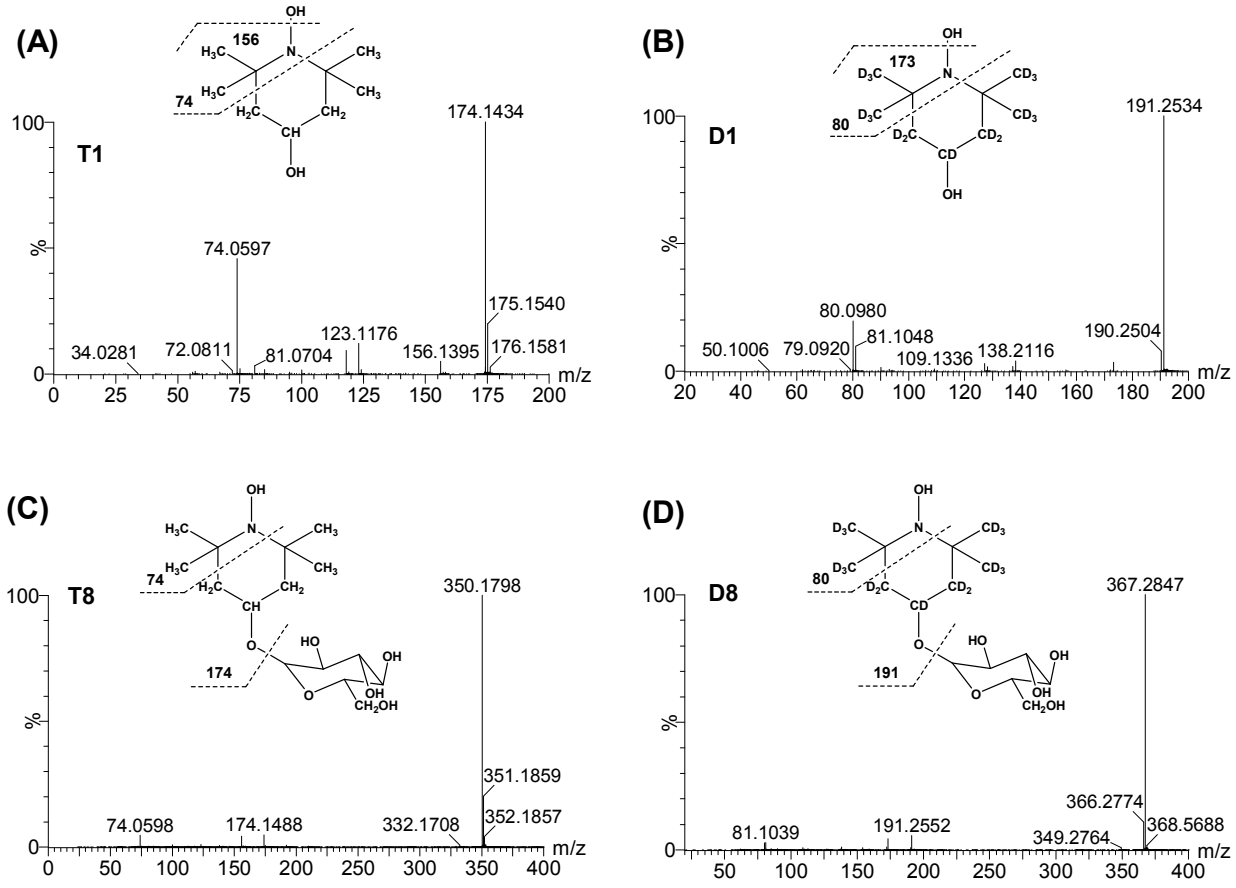
however, 1-glucuronide and glucoside cannot be detected, suggesting that 4-OH of tempol is more active than 1-OH during phase II metabolism (Figures S-2C and 3D). In addition, **T7** and **T8** were degraded from urine following treatment with 3M HCl for 1 hr (Figures S-8A), verifying that two metabolites were phase II metabolites. **T8** also was verified by the in vitro glucuronidation assay. Tempol glucuronide was synthesized in the incubation of tempol and mouse liver microsome containing UDP-glucuronic acid. The MS/MS spectrum of **T8** (Figures S-8A) was matched with the synthesized glucuronide conjugate (Figures S-8B). Therefore, **T7** and **T8** were identified as tempol 4-*O*-glucoside and tempol 4-*O*-glucuronide, respectively.

Metabolites **T9** and **T10** were calculated as $C_{21}H_{37}NO_{13}$ with a mass error of -5.4 and 1.8 ppm based on their accurate mass measurement, *m/z* 512.2334^+ (RT = 1.69 min) and 512.2349^+ (RT = 1.41 min), respectively. Compared with the chemical composition of **T7** ($C_{15}H_{29}NO_7$) and **T8** ($C_{15}H_{27}NO_8$), both OH groups in **T9** and **T10** were conjugated with glucuronic acid or glucose. In the mass spectrum of **T9** and **T10** (Figures S-4C and D), the elimination of 176 Da and 162 Da was the representative fragment ions of glucuronide and glucoside. In addition, **T9** and **T10** were degraded from urine following treatment with 3M HCl for 1 hr (Figures S-8A), verifying that both metabolites were phase II metabolites. The relative intensity of **T9** was higher than that of **T10** (Figures S-7B), suggesting that the 4-OH of **T9** was conjugated with glucuronic acid. Therefore, **T9** and **T10** were identified as tempol 1-*O*-glucoside and 4-*O*-glucuronide, and tempol 1-*O*-glucuronide and 4-*O*-glucoside, respectively.

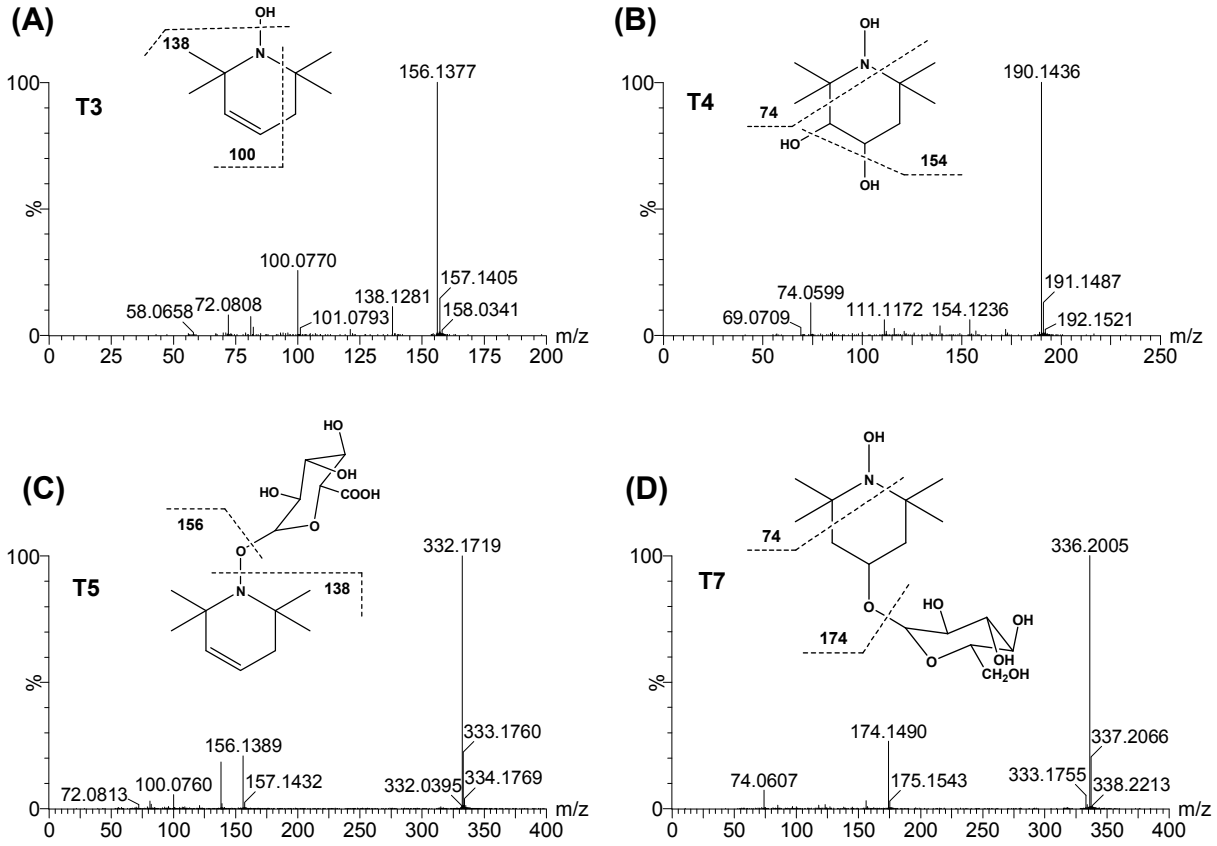
Supplementary Figure 1. PCA model of QC samples including Blank/MetMix, Control Urine (pooled), and Tempol urine (pooled). N=5 for each group.



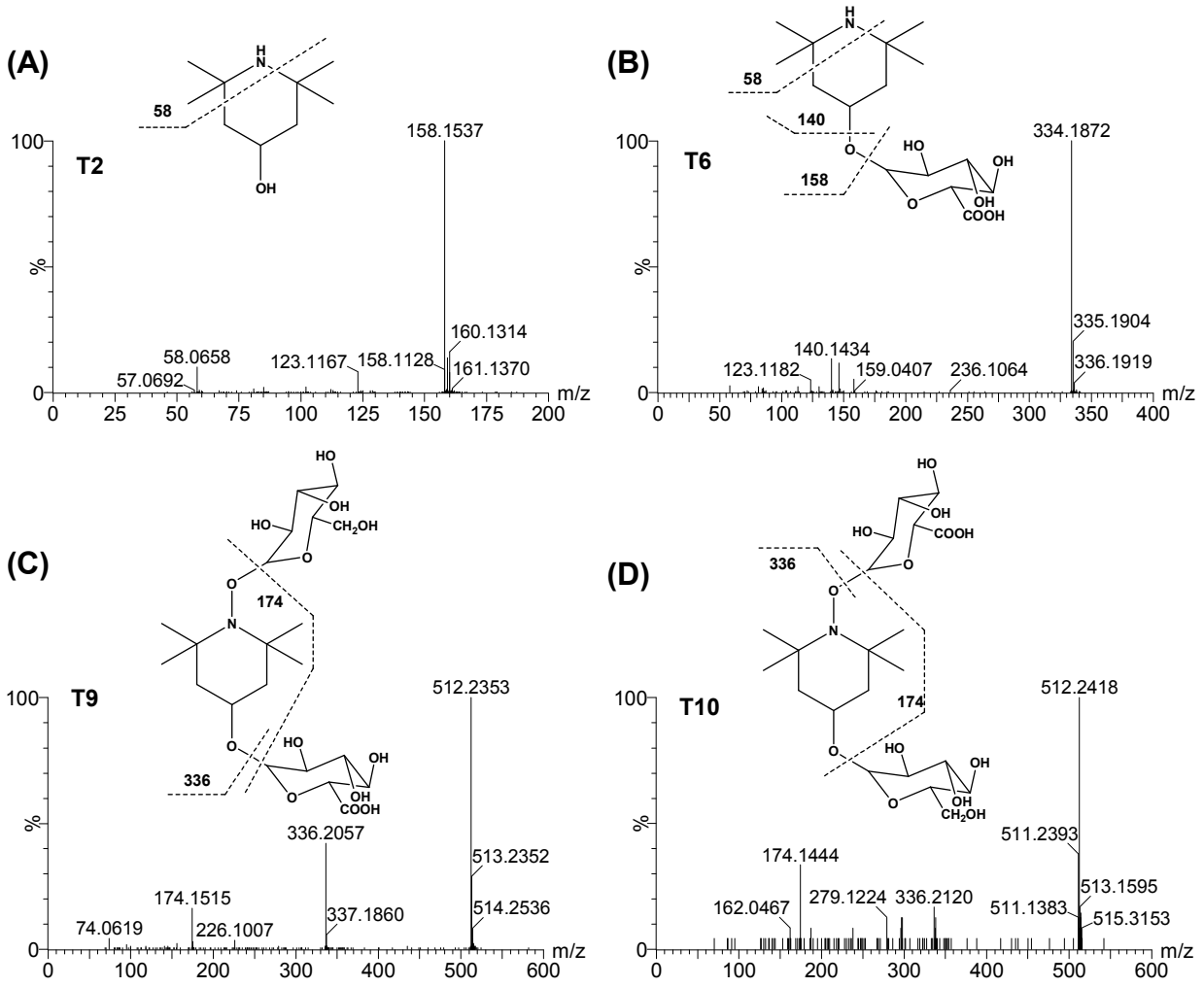
Supplementary Figure 2. Representative MS/MS fragmentography of metabolites of tempol and D-tempol. (A) Tempol hydroxylamine (T1), (B) D-tempol hydroxylamine (D1), (C) Tempol 4-*O*-glucuronide (T8), and (D) D-tempol 4-*O*-glucuronide (D8).



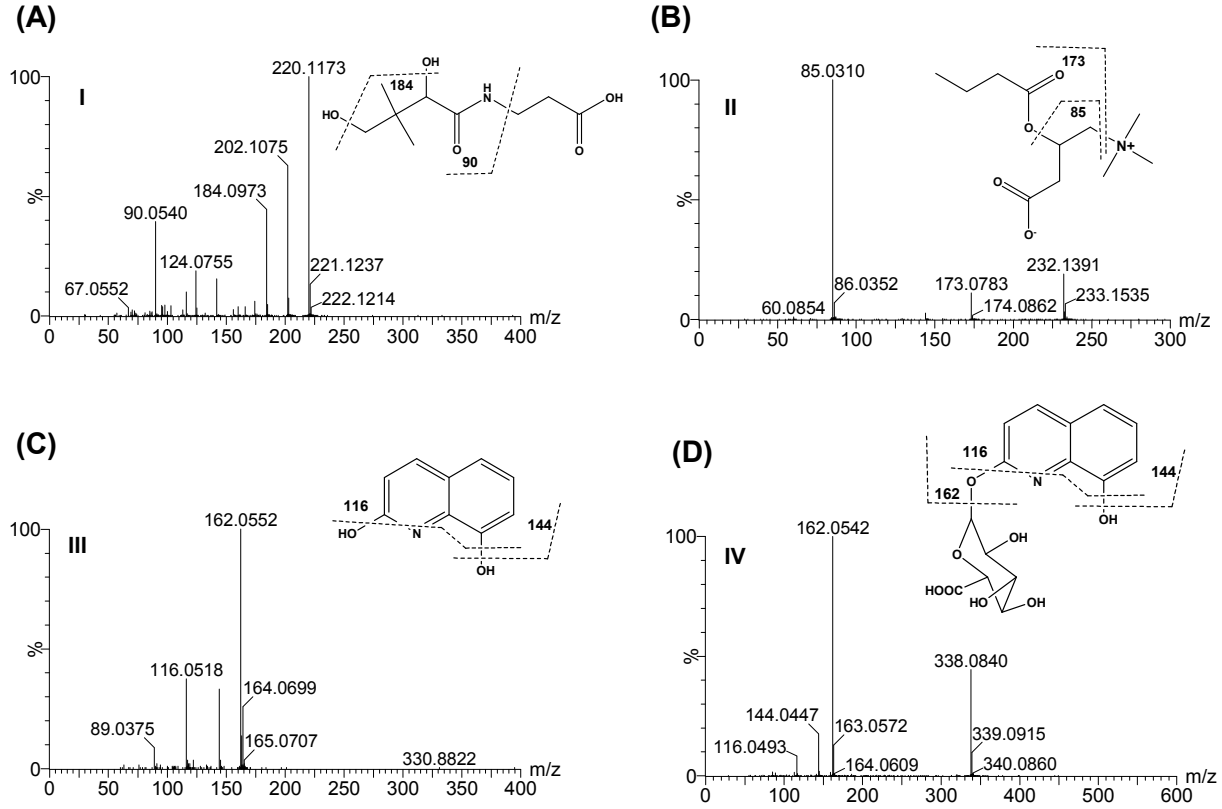
Supplementary Figure 3. Tandem MS and chemical structures of tempol metabolites T3, T4, T5, and T7. (A) 4-Dehydroxytempol (**T3**), (B) 3-Hydroxytempol (**T4**), (C) 4-Dehydroxytempol 1-*O*-glucuronide (**T5**), and (D) Tempol 4-*O*-glucoside (**T7**).



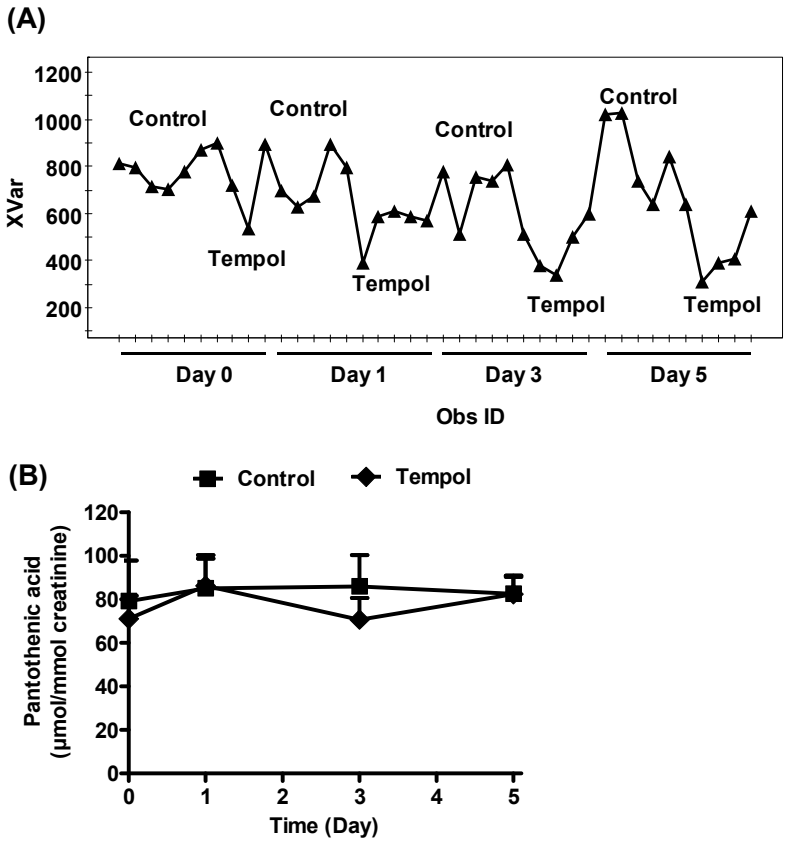
Supplementary Figure 4. Tandem MS and chemical structures of tempol metabolites T2, T6, T9, and T10. (A) Amine (T2), (B) 1-Dehydroxytempol 4-*O*-glucuronide (**T6**), (C) Tempol 1-*O*-glucoside and 4-*O*-glucuronide (**T9**), and (D) Tempol 1-*O*-glucuronide and 4-*O*-glucoside (**T10**).



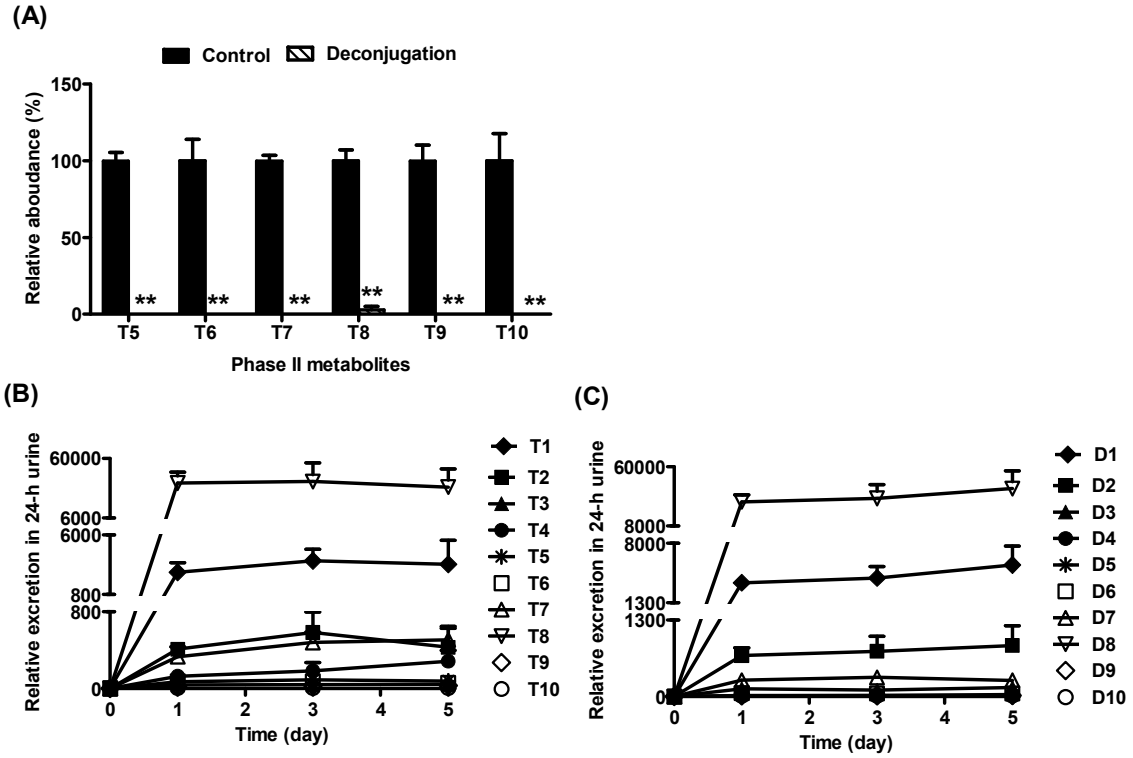
Supplementary Figure 5. Tandem MS and chemical structures of endogenous metabolites I, II, III, and IV. (A) Pantothenic acid (**I**), (B) Isobutrylcarntine (**II**), (C) 2,8-Dihydroxylquinoline (**III**), and (D) 2,8-Dihydroxylquinoline glucuronide (**IV**).



Supplementary Figure 6. The level of urinary pantothenic acid. (A) Trend plots of pantothenic acid in the low dose tempol (50 mg/kg) treated and control mice from day 0 to 5. (B) Quantitation of pantothenic acid by LC-MS in low dose tempol treated and control mice from day 0 to 5. There are no significant differences between the tempol treated group and control group from day 0 to 5.



Supplementary Figure 7. Validation of phase II metabolites and the relative excretion of tempol, D-tempol, and their metabolites. (A) Urine samples from mice treated with tempol were hydrolized with 3M HCl for 1 hr. Compared with the control, the phase II metabolites of tempol, **T5** to **T10**, were dramatically degraded following the hydrolysis. (B) The relative excretion of tempol and its metabolites in 24 hr urine from day 1 to 5. (C) The relative excretion of D-tempol and its metabolites in 24 hr urine from day 1 to 5. *P*-values were calculated using an unpaired *t*-test. **significant differences ($P < 0.01$).



Supplementary Figure 8. Validation of tempol 4-*O*-glucuronide by in vitro glucuronidation assay. (A) Tandem MS of tempol 4-*O*-glucuronide **(T8)** in urine. (B) Tandem MS of synthesized tempol 4-*O*-glucuronide**.**