



EDGEWOOD CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1178

METABOLOMIC ANALYSIS OF THE SECRETOME OF HUMAN EMBRYONIC STEM CELLS FOLLOWING METHYL PARATHION AND METHYL PARAOXON EXPOSURE

PHASE II: METABOLITE DOWNSELECTION FOR STRUCTURAL CONFIRMATION

Janna S. Madren-Whalley
Jennifer W. Sekowski

RESEARCH AND TECHNOLOGY DIRECTORATE

Paul R. West
Elizabeth L. R. Donley
Robert E. Burrier

STEMINA BIOMARKER DISCOVERY, INC.
Madison, WI 53719-1256

December 2013

Approved for public release; distribution is unlimited.



U.S. ARMY
RDECOM

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 h per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) XX-12-2013		2. REPORT TYPE Final		3. DATES COVERED (From - To) Oct 2011 – Jan 2012	
4. TITLE AND SUBTITLE: Metabolomic Analysis of the Secretome of Human Embryonic Stem Cells Following Methyl Parathion and Methyl Paraaxon Exposure, Phase II: Metabolite Downselection for Structural Confirmation				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S): Madren-Whalley, Janna S.; Sekowski, Jennifer W. (ECBC); West, Paul R.; Donley, Elizabeth L. R.; Burrier, Robert E. (Stemina)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Director, ECBC, ATTN: RDCB-DRB, APG, MD 21010-5424 Stemina Biomarker Discovery, Inc., 504 South Rosa Road, Suite 150, Madison, WI 53719-1256				8. PERFORMING ORGANIZATION REPORT NUMBER ECBC-TR-1178	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Edgewood Biological Chemical Center (ECBC), In-House Laboratory Independent Research (ILIR) Program, Aberdeen Proving Ground, MD 21010-5424				10. SPONSOR/MONITOR'S ACRONYM(S) ECBC ILIR	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: This technical report is the second of three reports from the U.S. Army Edgewood Chemical Biological Center (ECBC) In-House Laboratory Independent Research funded project, Molecular Toxicology of toxic industrial chemicals (TICs) in Human Embryonic Stem Cells (hESC). One of the goals of the study was to identify metabolites from the secretome of hESC exposed to the TICs methyl parathion and methyl paraoxon. A nontargeted liquid chromatography followed by mass spectrometry (LC-MS) approach was used in the Phase I work (Sekowski, J.W., et al. <i>Metabolomic Analysis of the Secretome of WA09 Human Embryonic Stem Cells following Methyl Parathion and Paraaxon Exposure, Phase I: Initial Nontargeted LC-MS</i> ; ECBC-TR-1177; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2013). In the Phase I work, hundreds of human metabolites and multiple metabolic pathways altered as a result of exposure to both chemicals were putatively identified. The purpose of this Phase II study was to downselect putatively identified metabolites for structural confirmation analysis by liquid chromatography followed by tandem mass spectrometry (LC-MS-MS). This report describes the subset of mass features that were downselected based on the following criteria: (1) impact on the arginine-proline metabolism pathway, (2) involvement with reactive oxygen species, and (3) good quality mass features suitable for structure confirmation by LC-MS-MS.					
15. SUBJECT TERMS Human embryonic stem cells (hESC) Methyl parathion (MP) LC-MS Metabolomics Methyl paraoxon (MPO) Reactive oxygen species (ROS)					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Renu B. Rastogi
U	U	U	UU	32	19b. TELEPHONE NUMBER (include area code) (410) 436-7545

Blank

PREFACE

The work described in this report was authorized under a U.S. Army Edgewood Chemical Biological Center (ECBC) In-House Laboratory Independent Research (ILIR) Program during the 2012 fiscal year. The work was started in October 2011 and completed in January 2012.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

Acknowledgments

The authors acknowledge the following individuals for their hard work and assistance with the execution of this technical program:

- Way Fountain (ECBC) and Laura Borland (Booz Allen Hamilton, Aberdeen, MD) for their support of this project under the ECBC ILIR program.
- Kelley Betts (ECBC) for her technical assistance in the preparation of this manuscript.

CONTENTS

1.	INTRODUCTION	1
2.	EXPERIMENTAL OVERVIEW.....	2
3.	RESULTS.....	2
4.	KEGG PATHWAYS	4
5.	CONCLUSIONS.....	18
	LITERATURE CITED	19
	ACRONYMS AND ABBREVIATIONS	21

FIGURES

1.	Krypto Encyclopedia at Genes and Genomes (KEGG) pathway diagram of arginine and proline metabolism.	4
2.	KEGG pathway diagram of D-arginine and D-ornithine metabolism	5
3.	KEGG pathway diagram of glycine, serine and threonine metabolism.....	6
4.	KEGG pathway diagram of valine, leucine and isoleucine degradation.	7
5.	KEGG pathway diagram of valine, leucine and isoleucine biosynthesis.....	8
6.	KEGG pathway diagram of lysine degradation	9
7.	KEGG pathway diagram of pyruvate metabolism.....	10
8.	KEGG pathway diagram of purine metabolism.....	11
9.	KEGG pathway diagram of pantothenate and CoA biosynthesis	12
10.	KEGG pathway diagram of glyoxylate and dicarboxylate metabolism.....	13
11.	KEGG pathway diagram of glycerophospholipid metabolism	14
12.	KEGG pathway diagram of glutathione metabolism.....	15
13.	KEGG pathway diagram of cysteine and methionine metabolism	16
14.	KEGG pathway diagram of citrate cycle (TCA cycle).....	17
15.	KEGG pathway diagram of propanoate metabolism.	18

TABLES

Putative Metabolites Passing Criteria for Potential LC-MS-MS Confirmation.....	3
--	---

Blank

METABOLOMIC ANALYSIS OF THE SECRETOME OF HUMAN EMBRYONIC STEM CELLS FOLLOWING METHYL PARATHION AND METHYL PARAOXON EXPOSURE, PHASE II: METABOLITE DOWNSELECTION FOR STRUCTURAL CONFIRMATION

1. INTRODUCTION

This technical report is the second of three reports from the U.S. Army Edgewood Chemical Biological Center (ECBC) In-House Laboratory Independent Research (ILIR) “Molecular Toxicology of TICs in Human Embryonic Stem Cells”. The overarching goal of the study was to provide a better understanding of the basic biological and toxicological mechanisms in human embryonic stem cells (hESC) exposed to an organophosphate toxic industrial chemical (TIC). This work has broad implications toward many U.S. Army goals from developing better toxicant screening tools, including organ-on-a-chip type applications, to medical applications, including regenerative medicine applications and stem cell based therapies. It also supports U.S. Army Training and Doctrine Command (TRADOC) Pamphlet (Pam) 525-66 (March 08) *Maneuver Sustainment*, Force Operating Capabilities (FOC)-09-07 – “Casualty Prevention”, which states the importance of protecting the warfighter from TICs and toxic industrial materials (TIM)s in general.

Chemicals that have certain known effects in adults can have dramatically different toxic effects during embryonic and prenatal development. For example, the successful adult anti-epileptic drug, valproate, has a dramatic toxic effect on embryonic development, leading to neural tube deficits, autism, and cognitive dysfunction. In their 2007 study, Cezar et al. used liquid chromatography followed by mass spectrometry (LC-MS) to examine the metabolites in spent medium from pluripotent hESC exposed to valproate and found a blockage of the serotonin production pathway. Therefore, an important part of any complete toxicological evaluation must include examination of the compound’s effect on human embryonic development. The use of hESC to explore human embryonic molecular toxicological endpoints is a promising development in the field of toxicology. Since pluripotent hESC contain the ability to differentiate into any somatic cell in the body, they provide a unique window into the influence of toxicants on the entire early human development process.

To address the aim of this study, we used nontargeted LC-MS based metabolomic analysis of the secretome of hESC exposed to methyl parathion (MP) and methyl paraoxon (MPO), the active metabolite of MP. In the course of this work, we also confirmed that hESC are not able to convert parathion to paraoxon, thus in order to understand the effect of both the parent compound and its metabolite, the testing of both compounds was necessary. This nontargeted LC-MS approach was able to identify hundreds of human metabolites and multiple metabolic pathways that were altered as a result of exposure to both chemicals that would be present in vivo.

The purpose of this Phase II study was to refine parts of the analysis and down-select metabolites of interest from the previous initial Phase I work described in Sekowski et al. (2013). That technical report describes the results from initial LC-MS based untargeted metabolomic analysis that was performed on hESC exposed to MP or MPO. The goal of this

work was to identify metabolites secreted from those cells that were altered by exposure to MP or MPO. Based on the initial results, further literature-based investigation revealed that several specific metabolic processes, including the arginine and proline metabolism pathway and the involvement of reactive oxygen species (ROS) should be included in subsequent analysis. This report delineates a set of statistically significant mass features that were putatively annotated and determined to be involved with these and other key metabolic processes. Of these, those mass features best suited for structural confirmation by liquid chromatography followed by tandem mass spectrometry (LC-MS-MS) were chosen. That work is reported in the Phase III Technical Report (Madren-Whalley et al., 2013).

2. EXPERIMENTAL OVERVIEW

Statistically significant mass features from the Phase I study were re-analyzed by hand to determine the quality of the features for LC-MS-MS analysis. In order to determine the features which had the best potential for structural confirmation, the following criteria were used: (1) sufficient abundance (1×10^3), (2) fold change (comparing dosed to controls), and (3) peak shape. After this review and putative annotation of these features, the raw LC-MS data files were re-analyzed using the “Find by Formula” routine in the Agilent (Santa Clara, CA) MassHunter software. This semi-automated analysis determines if the mass spectra for a feature (i.e., the specific molecular formula for the annotation) is a reasonable match with the formula. This is much faster than human manual data interpretation. The algorithm takes into account several factors, including parts per million (ppm) mass error, adducts and isotopic peak abundances, isotope spacing, and mass per charge (m/z) values. It outputs a score, where 100 is perfect, for each feature. Any features with a score of less than 70 were removed. Other factors also considered were potential for ionization for a given polarity [e.g., a quaternary cation such as choline will not be detected in negative ion electrospray ionization (ESI)] and previously confirmed compound retention time matches. All of these factors were taken into account resulting in a list of good quality features for consideration for inclusion in the Table. All sample preparation and LC-MS methods are described in detail in the Phase I Technical Report (Sekowski et al., 2013).

3. RESULTS

The table shows the 20 putative metabolites which passed criteria for structural confirmation analysis by LC-MS-MS. This table includes the pathways revealed in the Phase I work described in ECBC TR-1177, as well those involved with ROS and pathways with known biomarkers of developmental toxicity such as arginine and proline metabolism, revealed in this Phase II work. All of these putative metabolites have passed criteria for good likelihood of structural confirmation whether they were detected via the automated data analysis pipeline or by manual inspection.

Table. Putative Metabolites Passing Criteria for Potential LC-MS-MS Confirmation

Metabolic Pathway	No. of Features in Pathway	Putative Metabolite	Study Phase
Arginine and proline metabolism	8	Asymmetric dimethylarginine (ADMA)	II
		Ornithine	II
		L-proline	II
		2-oxo-5-aminovalerate	II
		Trans-4-hydroxy-L-proline	II
		L-glutamic-gamma-semialdehyde	II
		4-acetamidobutanoic acid	II
		cis-4-hydroxy-D-proline	II
D-arginine and D-ornithine metabolism	4	Ornithine	II
		5-amino-2-oxopentanoic acid	II
		2-amino-4-oxo-pentanoic acid	II
		(2R,4S)-2,4-diaminopentanoate	II
Gly, Ser, and Thr metabolism	3	L-cystathionine	II
		Choline	II
		5-aminolevulinic acid	II
Val, Leu, and Ile degradation	2	Alpha-ketoisovaleric acid	II
		Valine	II
Val, Leu, and Ile biosynthesis	2	Alpha-ketoisovaleric acid	II
		Valine	II
Lys degradation	2	Pipecolic acid	II
		2-keto-6-aminocaproate	II
Pyruvate metabolism	1	Malic acid	I
Purine metabolism	1	Guanine	I
Propanoate metabolism	1	Valine	II
Pantothenate and CoA biosynthesis	1	Alpha-ketoisovaleric acid	I
Glyoxylate/dicarboxylate metabolism	1	Malic acid	I
Glycerophospholipid metabolism	1	Choline	II
Glutathione metabolism	1	Ornithine	II
Cys and Met metabolism	1	L-cystathionine	I
Citrate cycle (TCA cycle)	1	Malic acid	I

ADMA, asymmetric dimethylarginine

CoA, coenzyme A

Cys, cysteine

Gly, glycine

Ile, isoleucine

Leu, leucine

Lys, lysine

Met, methionine

Ser, serine

TCA, trichloroacetic

Thr, threonine

Val, valine

All of the pathways in the table have been mapped or remapped to reflect the 20 putative metabolites which pass the criteria for LC-MS-MS analysis described above. The location of the metabolites in the pathways are illustrated below in Figures 1–15. The arginine and proline metabolism pathway (Figure 1) had the most perturbation with the most metabolites that passed the criteria set for best prospect of successful LC-MS-MS based structural confirmation.

4. KEGG Pathways

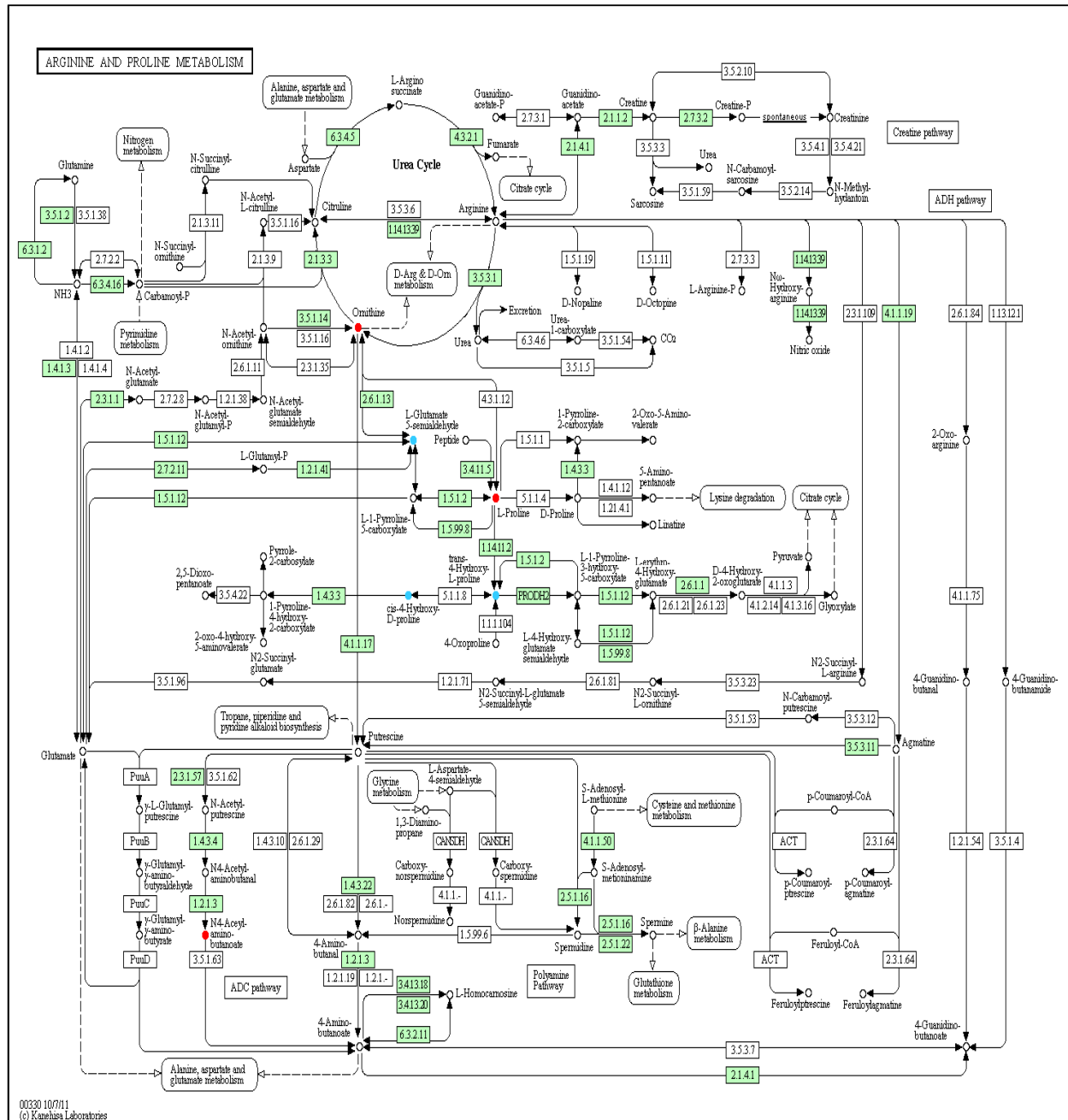


Figure 1. Krypto Encyclopedia at Genes and Genomes (KEGG) pathway diagram of arginine and proline metabolism.

KEGG pathway diagram of the putatively annotated metabolites ornithine, L-proline, 2-oxo-5-aminovalerate, 4-acetamidobutanoic acid (red dots) and L-glutamic-gamma semialdehyde, trans-4-hydroxy-L-proline and cis-4-hydroxy-D-proline (blue dots indicating isomers) and ADMA, an inhibitor of nitric oxide synthase (NOS), in the arginine and proline metabolism pathway that were determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

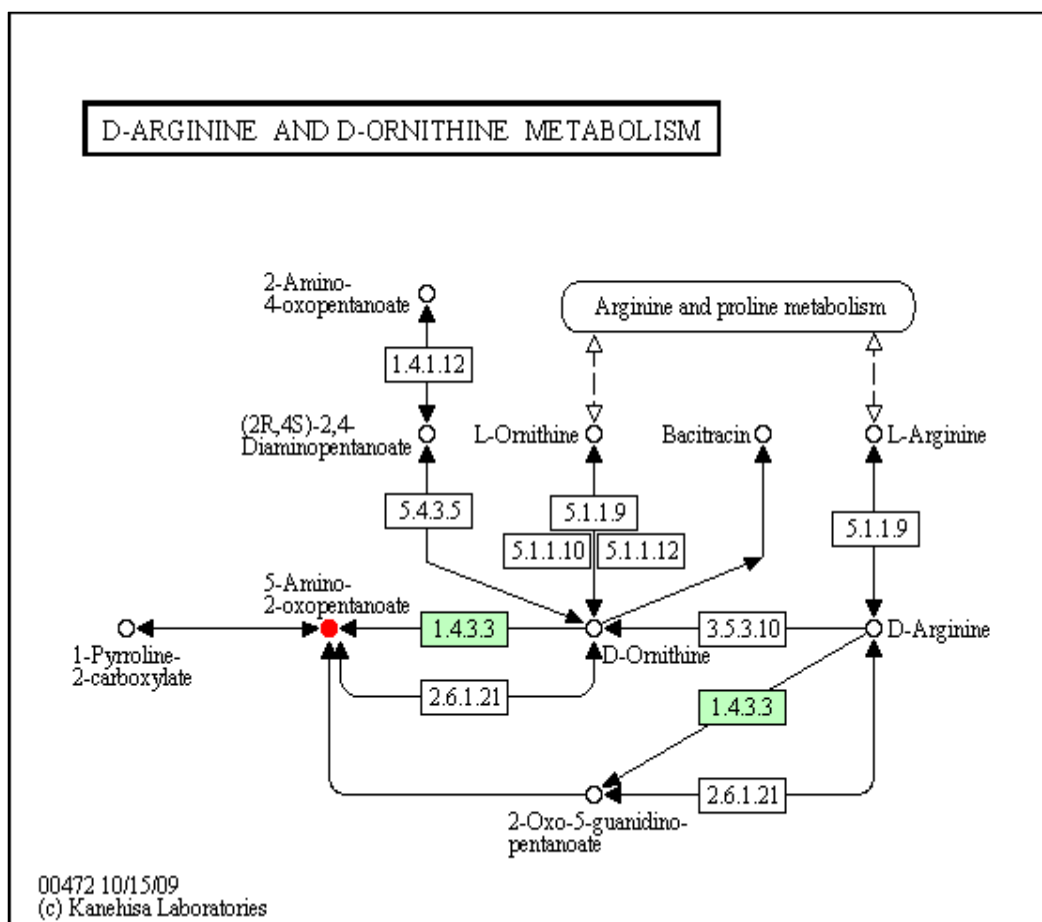


Figure 2. KEGG pathway diagram of D-arginine and D-ornithine metabolism.

KEGG pathway diagram of the putatively annotated metabolite 5-amino-2-oxopentanoate (red dot) in the D-arginine and D-ornithine metabolism pathway that was determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are colored green colored boxes. Putative metabolites observed in this study are represented with colored circles. Arrows represent direction of enzymatic activity.

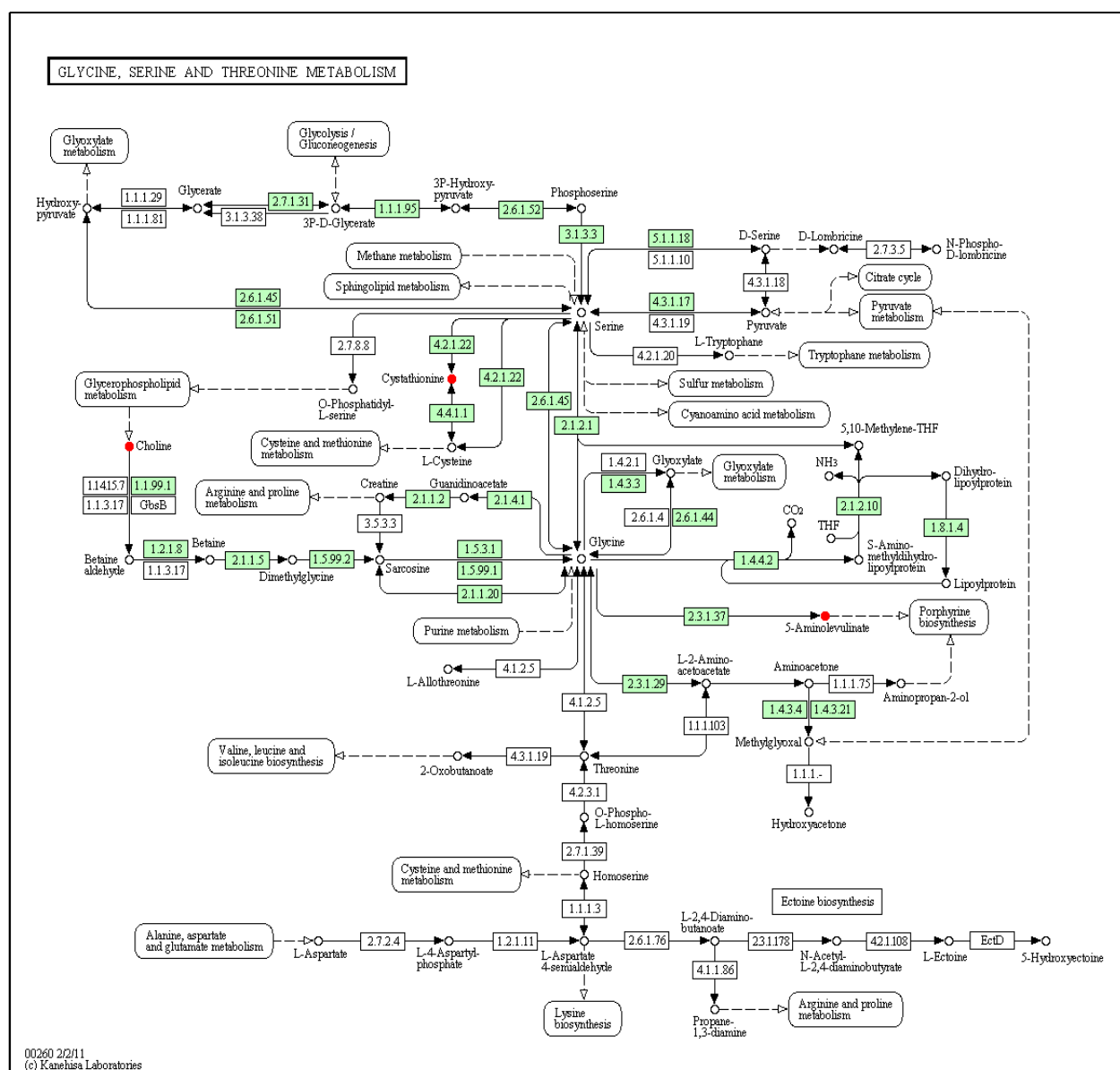


Figure 3. KEGG pathway diagram of glycine, serine and threonine metabolism. KEGG pathway diagram of the putative metabolites L-cystathionine, choline, and 5-aminolevulinic acid (red dots) in the glycine, serine, and threonine metabolism pathway that were determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

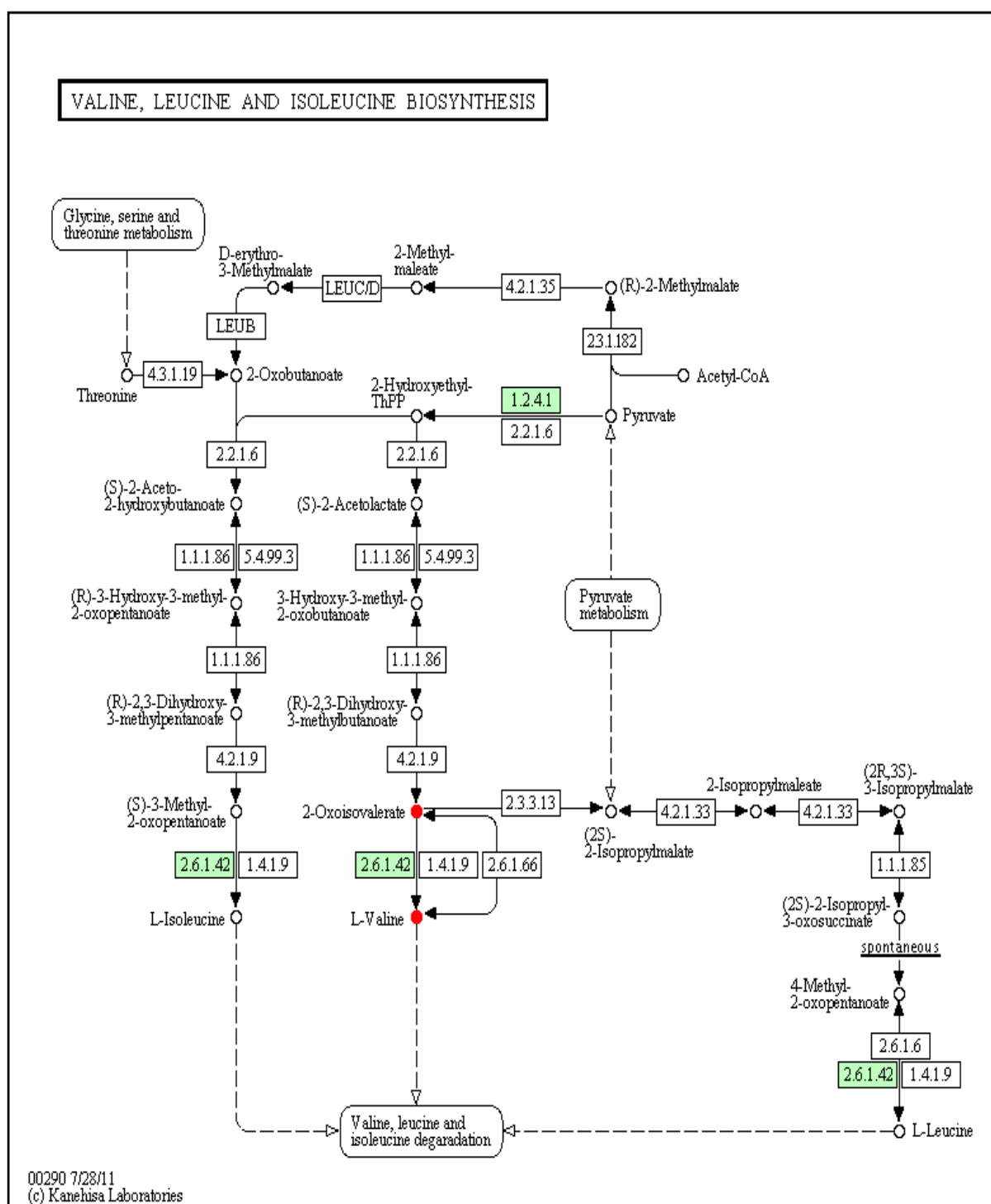


Figure 5. KEGG pathway diagram of valine, leucine and isoleucine biosynthesis.
KEGG pathway diagram of the putatively annotated metabolites valine and 3-methy-2-oxobutanoate (alpha-ketoisovaleric acid) (red dots) in the valine, leucine and isoleucine biosynthesis pathway that were determined to show sufficient quality spectra to be candidates for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

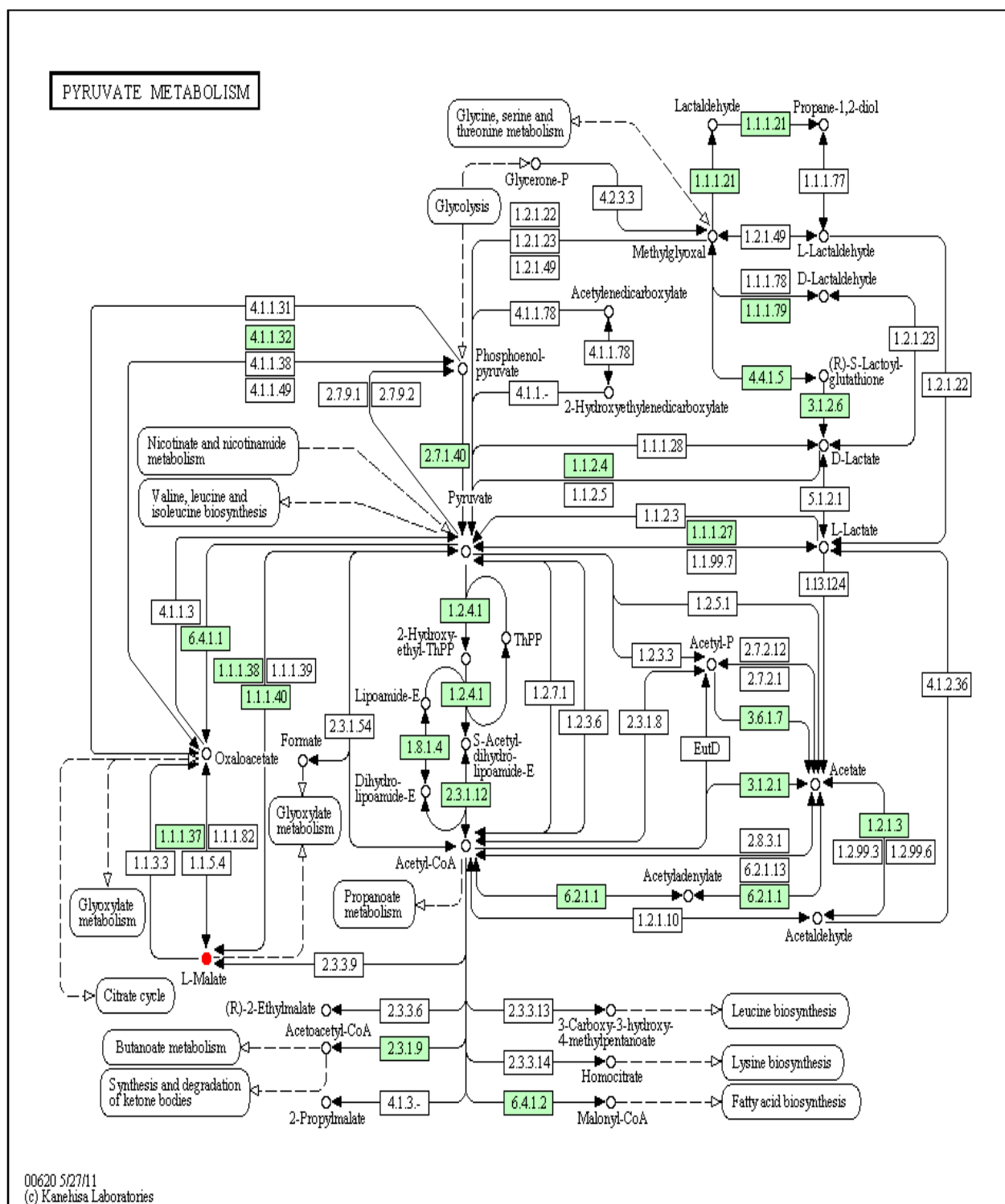


Figure 7. KEGG pathway diagram of pyruvate metabolism.

KEGG pathway diagram of the putatively annotated metabolite L-malate (red dot) in the pyruvate metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

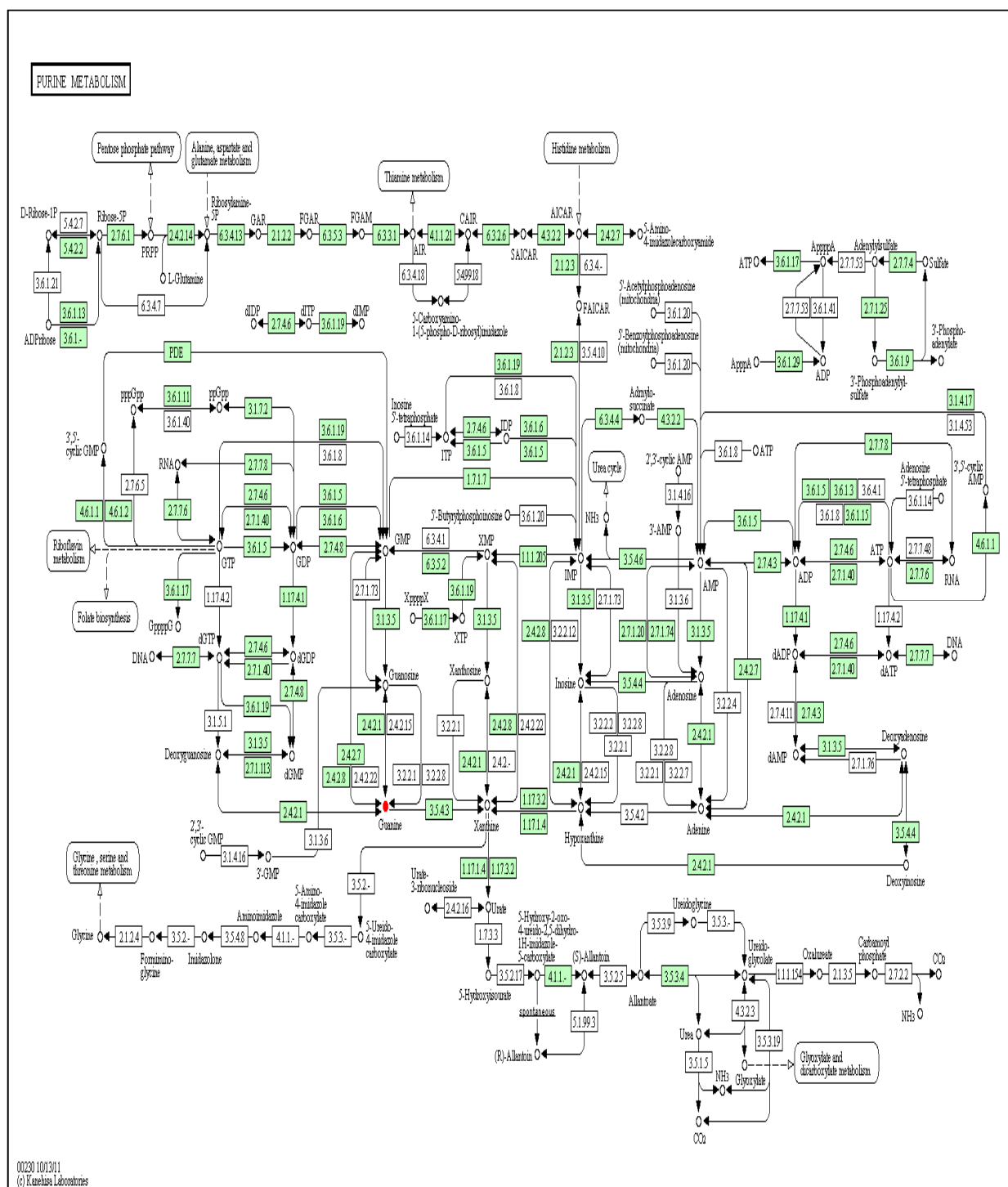


Figure 8. KEGG pathway diagram of purine metabolism.

KEGG pathway diagram of the putatively annotated metabolite guanine (red dot) in the purine metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

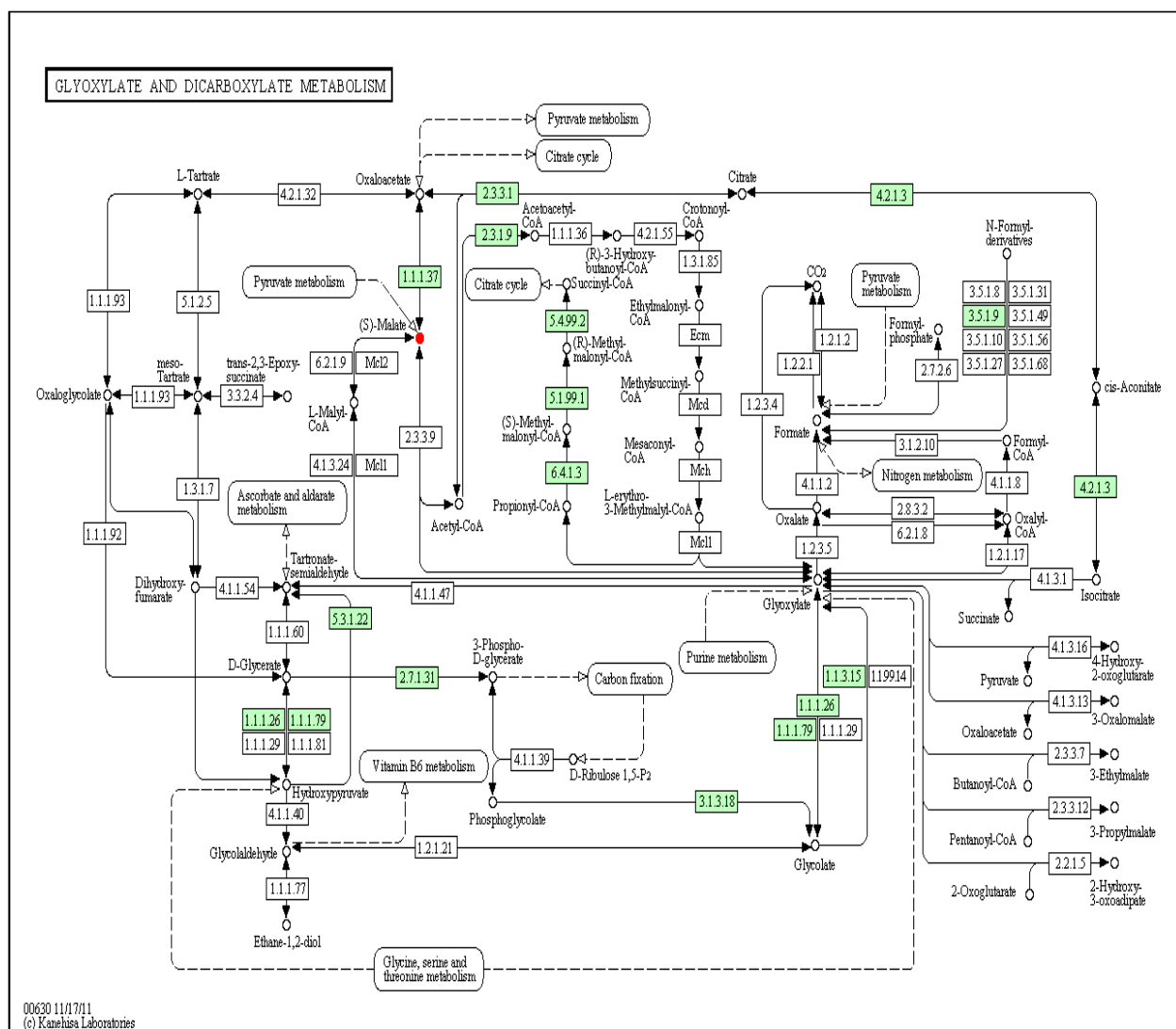


Figure 10. KEGG pathway diagram of glyoxylate and dicarboxylate metabolism.
KEGG pathway diagram of the putatively annotated metabolite S-malate (red dot) in the glyoxylate and dicarboxylate metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. Arrows represent direction of enzymatic activity.

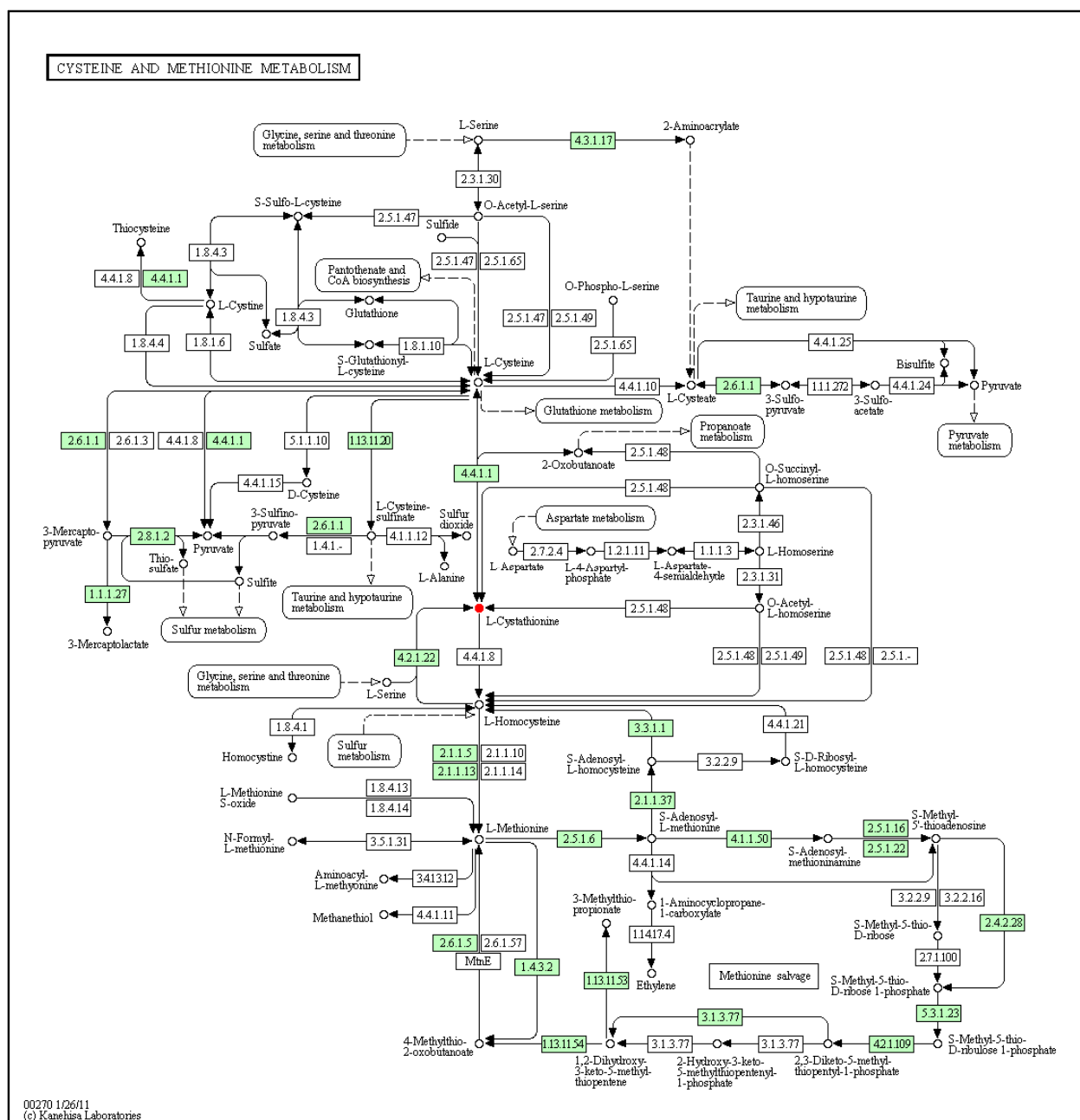
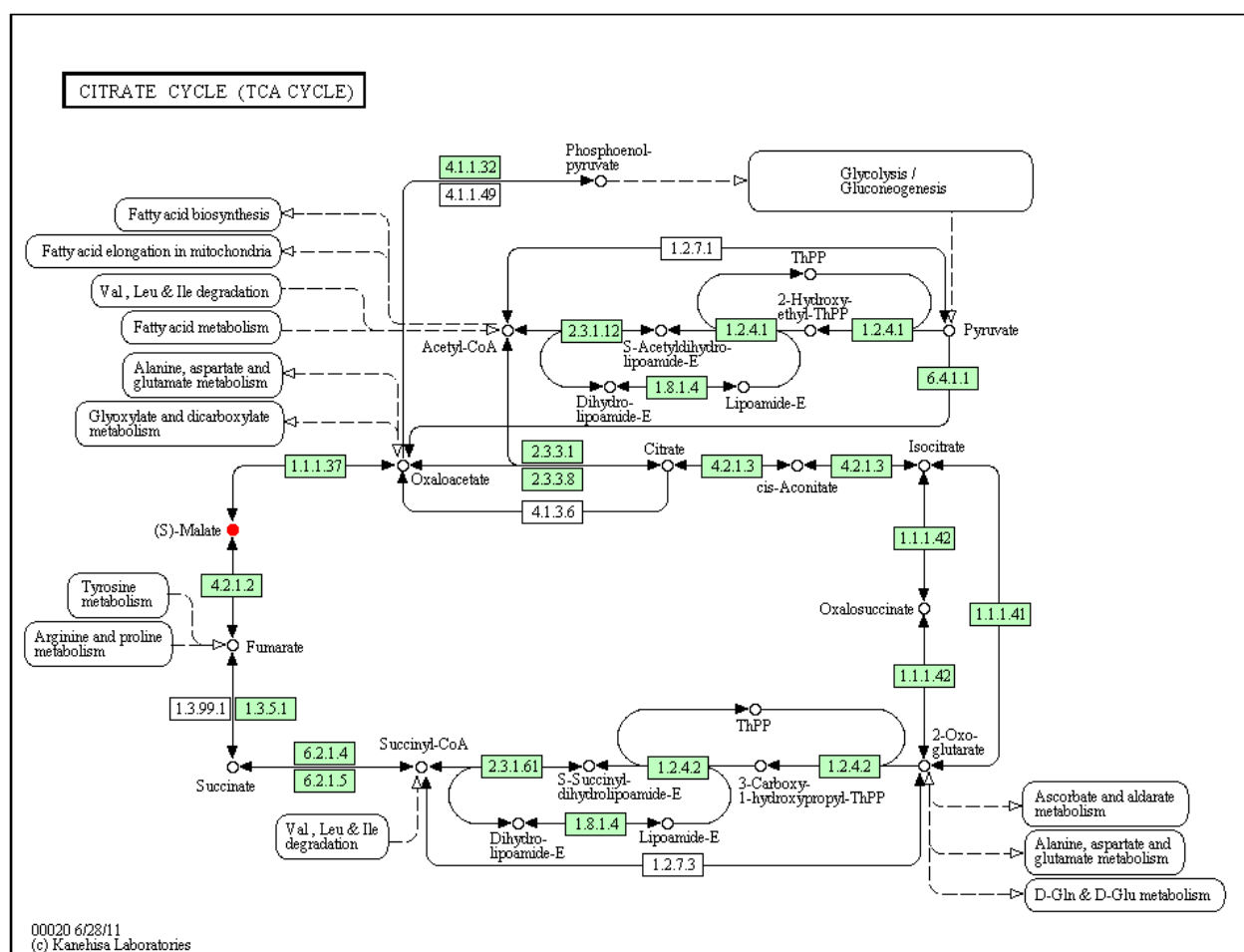


Figure 13. KEGG pathway diagram of cysteine and methionine metabolism.

KEGG pathway diagram of the putatively annotated metabolite L-cystathionine (red dot) in the cysteine and methionine metabolism pathway that was determined to show sufficient quality spectra to be a candidate for chemical structure confirmation by LC-MS-MS. Enzymes or enzymatic activity observed in humans are green colored boxes. represent direction of enzymatic activity.



LITERATURE CITED

- Cezar, G.G.; Quam, J.A.; Smith, A.M.; Guilherme, J.M.R.; Piekarczyk, M.S.; Brown, J.F.; Gag, F.H.; Muotri, A.R. Identification of Small Molecules from Human Embryonic Stem Cells Using Metabolomics. *Stem Cell Dev.* **2007**, *16*(6), 869–892.
- Madren-Whalley, J.S.; Palmer, J.S.; Donley, E.L.R.; West, P.; Conard, K.; Sekowski, J.W. *Metabolomic Analysis of the Secretome of Human Embryonic Stem Cells following Methyl Parathion and Methyl Paraoxon Exposure, Phase III: LC-MS-MS Structural Confirmation*; ECBC-TR-1161; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2013; UNCLASSIFIED Report.
- Sekowski, J.W.; Bevilacqua, V.L.H.; Palmer, J.A.; Donley, E.L.R.; Burrier, R.E.; Madren-Whalley, J.S. *Metabolomic Analysis of the Secretome of WA09 Human Embryonic Stem Cells following Methyl Parathion and Paraoxon Exposure, Phase I: Initial Nontargeted LC-MS*; ECBC-TR-1177; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2013; UNCLASSIFIED Report.

Blank

ACRONYMS AND ABBREVIATIONS

ADMA	asymmetric dimethylarginine
APG	Aberdeen Proving Ground
CoA	coenzyme A
Cys	cysteine
ECBC	U.S. Army Edgewood Chemical Biological Center
ESI	electrospray ionization
FOC	Force Operating Capabilities
Gly	glycine
hESC	human embryonic stem cell
Ile	isoleucine
ILIR	in-house laboratory independent research
KEGG	Krypto Encyclopedia at Genes and Genomes
LC-MS	liquid chromatography followed by mass spectrometry
LC-MS-MS	liquid chromatography followed by tandem mass spectrometry
Leu	leucine
Lys	lysine
Met	methionine
MP	methyl parathion
MPO	methyl paraoxon
m/z	mass over charge ratio
NOS	nitric oxide synthase
Pam	pamphlet
ppm	parts per million
ROS	reactive oxygen species
Ser	serine
TCA	trichloroacetic cycle, also known as citrate cycle or Krebs cycle
Thr	threonine
TIC	toxic industrial chemical
TIM	toxic industrial material
Val	valine

