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Design, synthesis and biological evaluation of glutamic acid derivatives as anti-oxidant and anti-inflammatory agents

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ABSTRACT

A series of glutamic acid derivatives was synthesized and evaluated for their antioxidant activity and stability. We found several potent and stable glutamic acid derivatives. Among them, compound **12b** exhibited good in vitro activity, chemical stability and cytotoxicity. A prototype compound 12b showed an anti-inflammatory effect in LPS-stimulated RAW 264.7 cell lines and in a zebrafish model.

Reactive oxygen species (ROS) are produced in living organisms either due to normal cell metabolisms *in situ* or due to environmental factors. When ROS (free radicals) such as superoxide anions, hydroxyl radicals, hydrogen peroxide, and hypochlorous acid cannot be effectively eliminated, their accumulation in the body generates oxidative stress. This can cause chronic and degenerative illnesses such as inflammation, cardiovascular diseases, cancer, aging-related disorders, metabolic disorders, and atherosclerosis.[1] ROS attack unsaturated fatty acids resulting in membrane lipid peroxidation, reduced membrane fluidity, loss of enzymes and receptor activities and damage to membrane proteins, ultimately leading to cell inactivation.[2] Despite the existence of natural defense mechanisms, excessive production of ROS over the lifetime of a cell causes progressive oxidative damage and ultimately cell death.[3]

Many antioxidant compounds are extracted from plants and tested as the main ingredients of cosmetics. Ramalin, isolated from the Antarctic lichen, *Ramalina terebrata* (Ramalinaceae) has anti-oxidant and anti-inflammatory activities.[4-6] It has also been shown *in vivo* to inhibit melanogenesis.[7]

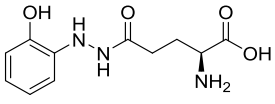


Figure 1. Structure of Ramalin.

Despite these properties, the use of Ramalin is limited because it is unstable, and spontaneously decomposes at room temperature.[8] To overcome its instability, a method for stabilizing Ramalin using vitamin C has been reported, however, the instability associated with the parent structure unsolved. This situation prompted us to structurally modify Ramalin to improve its stability as well as its potency. In the present study, we report the synthesis, structure activity relationships (SAR) and biological evaluation of glutamic acid derivatives as anti-oxidant and anti-inflammatory agents.

The Ramalin structure can be divided based on three functional units such as an ortho hydroxy phenyl group, hydrazine group and L-glutamic acid as shown in Figure 2. We tried to modify and optimize Ramalin to identify more stable and potent derivatives.

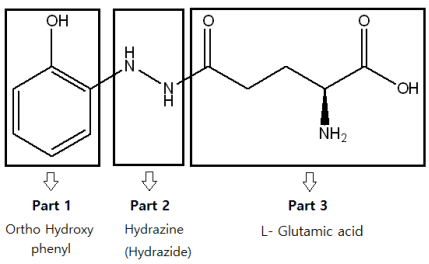
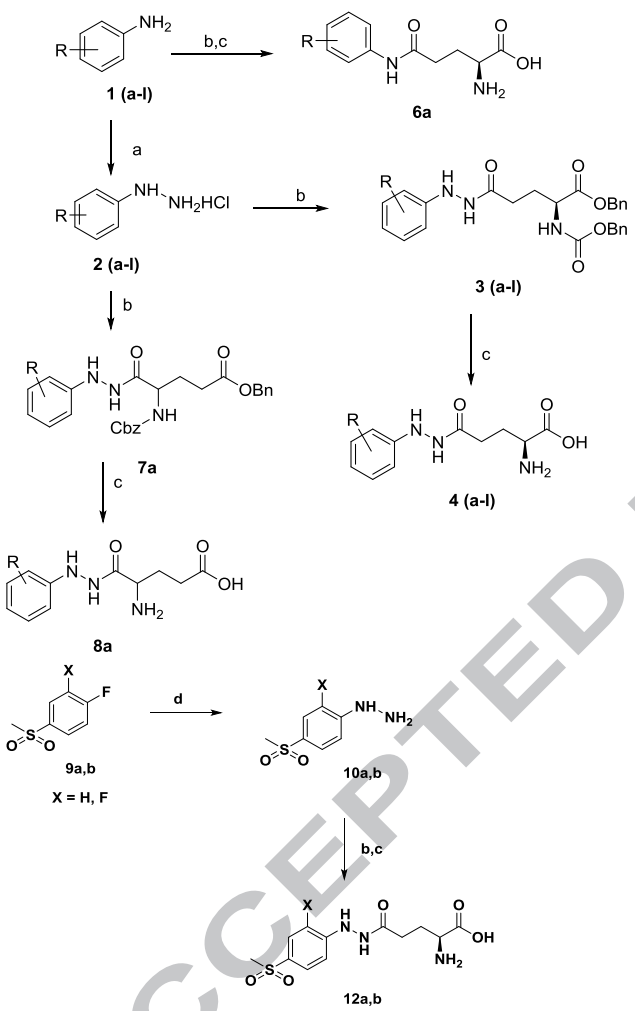


Figure 2. Structure of Ramalin divided into three functional units.

The synthesis of glutamic acid derivatives **4(a-l), 6a, 8a,** and **12a,b** is depicted in Scheme 1. A commercially available aniline derivative **1(a-l)** was reacted with sodium nitrite under acidic conditions to obtain the phenyl hydrazine hydrochloride **2(a-l)**. Compound **2(a-l)** reacted with 1-benzyl N-carbobenzoxy-L-glutamate **13** and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate to give Cbz protected glutamic acid derivatives **3(a-l)**. Benzyloxy carbamate and benzyl groups were deprotected with hydrogen gas and palladium on charcoal to obtain **4(a-l)**. 2-Hydroxy aniline **1a** coupled with 1-benzyl N-carbobenzoxy-L-glutamate **13** followed by hydrogenation to give **6a**.



Scheme 1. Reagents and conditions: (a) 1) Sodium nitrite, 6M HCl, H_2O, 0 °C; 2) Tin chloride, 6M HCl, 0 °C, 75% (over two steps); (b) 1-Benzyl N-carbobenzoxy-L-glutamate **13** or 5-(benzyloxy)-2-(((benzyloxy)carbonyl)-amino)-5-oxopentanoic acid **14**, o-(benzotriazol-1-yl)-N,N,N',N'-tetramethy-luroniumtetrafluoroborate, DIPEA, DMF, room temperature, 85%; (c) H_2, Pd/C, methanol, room temperature, 80-85%; (d) Hydrazine monohydrate, ethanol, reflux, 70%.

(2-(Benzyloxy)phenyl)hydrazine **2a** was coupled with 5-(benzyloxy)-2-(((benzyloxy)carbonyl)amino)-5-oxopentanoic acid **14** to afford **7a** followed by hydrogenation to give **8a**. 1-Fluoro-4-(methylsulfonyl)benzene **9a** and 1,2-difluoro-4-(methylsulfonyl)benzene **9b** were treated with hydrazine monohydrate afforded the hydrazine hydrochlorides **10a,b**. Subsequent condensation with 1-benzyl N-carbobenzoxy-L-glutamate **13** followed by hydrogenation was carried out to obtain **12a,b**.

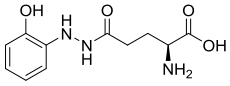
The *in vitro* antioxidant activity of the synthesized glutamic acid derivatives was evaluated using a DPPH assay. The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol.[9] This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to a colorless ethanol solution. The DPPH assay is an easy and rapid method to evaluate antioxidants by spectrophotometry. Thus it allows assessment of multiple products at a time. Sixteen different types of glutamic acid derivatives were screened by using the DPPH assay. Ascorbic acid was used as a reference compound.

Initially, the SARs of glutamic acid derivatives were investigated and the results are summarized in **Table 1**. Ramalin exhibited antioxidant activity with an IC_{50} value of 8.67 μg/ml, which is 2 fold higher than that of ascorbic acid. Replacement of hydrazine in Ramalin with an amide **(6a)** caused loss of *in vitro* activity. In addition, opposite glutamic acid **(8a)** and protected glutamic acid derivatives **(3a)** showed decreased antioxidant activities. On the basis of these data, we retained the hydrazine and glutamic acid moieties and further optimized the hydroxyphenyl moiety.

Diverse mono-substituted phenylhydrazine glutamic acid derivatives were synthesized and evaluated for their activity. As can be seen in **Table 2**, the o-methyl derivative **(4b)** showed better *in vitro* activity than Ramalin **(4a)** with an IC_{50} value of 6.24 μg/ml. The o-ethyl derivative **(4c)** exhibited decreased activity. Although several meta substituted compounds were synthesized and evaluated, however the activities were not effective than Ramalin. Para methyl **(4h)** and methysulfonyl **(12a)** derivatives were found to possess single digit IC_{50} value of 9.99 and 9.57 μg/ml, respectively. Based on these data, we further optimized the phenylhydrazine moiety with more substituents.

Several disubstituted phenylhydrazine glutamic acid derivatives were synthesized and evaluated. As shown in **Table 3**, 2,4-di-substituted glutamic acid derivative **4j** showed better in vitro activity than 3,5-di-substituted **4l**. Therefore, more 2,5-di-substitued compounds were evaluated. Compound **12b** showed good *in vitro* activity with an IC_{50} value of 8.72 μg/ml.

Table 1. Anti-oxidant activity of Ramalin derivatives

compound	Structure	IC_{50} (μg/ml)
4a(Ramalin)		8.67

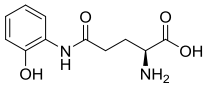
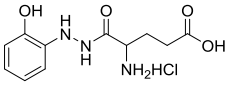
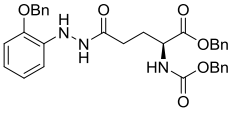
6a		162.42
8a		37.19
3a		44.98
Ascorbic acid		17.38

Table 2. Anti-oxidant activity of glutamic acid derivatives with monosubstitutents

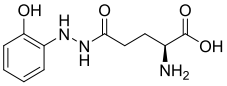
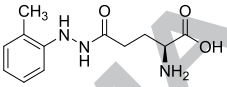
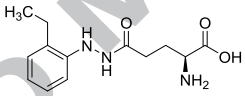
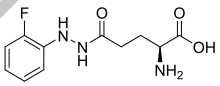
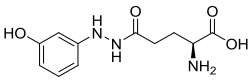
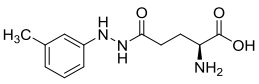
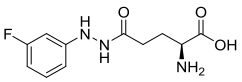
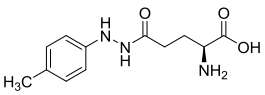
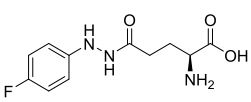
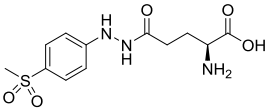
compound	Structure	IC_{50} (μg/ml)
4a(Ramalin)		8.67
4b		6.24
4c		17.14
4d		10.86
4e		10.67
4f		10.1
4g		11.44
4h		9.99
4i		13.77
12a		9.57

Table 3. Anti-oxidant activity of glutamic acid derivatives with disubstituents

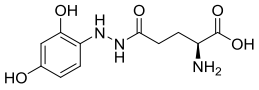
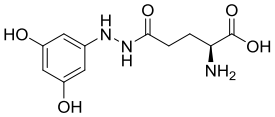
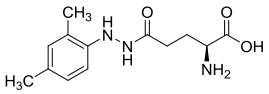
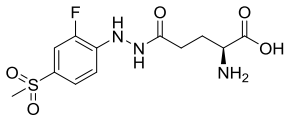
compound	Structure	IC_{50} (μg/ml)
4j		28.01
4l		316.86
4k		15.75
12b		8.72

Table 4. Chemical stability of Ramalin and glutamic acid derivatives

compound	initial	1day(45 ℃)	7day(45 ℃)	14day(45 ℃)	21day(45 ℃)	28day(45 ℃)
4a(Ramalin)	100%*	45.71%	-	-	-	-
4b	100%	82.33%	73.78%	56.74%		
4h	100%	85.1%	82.0%	72.2%	58.1%	50.4%
12a	100%	100.2%	94.6%	85.5%	79.2%	70.7%
12b	100%	99.4%	95.0%	87.0%	88.8%	87.2%

Conditions: Compound stability was analyzed by HPLC after storage at 45 °C; 1 mg of each sample was dissolved in 1mL DW. *Calculated HPLC AUC of day 0 as 100%

On the basis of the DPPH assay results, we chose **4b, 4h, 12a** and **12b** for chemical stability studies. Ramalin was unstable at 45 °C (**Table 4**) as well as room temperature.[8] Compounds**12a** and **12b** showed improved stability. Over ~ 95% of parent compound remained after 7 days at 45 °C.

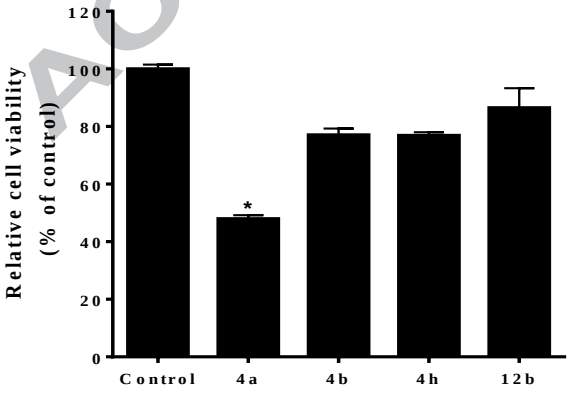


Figure 3. Effects of glutamic acid derivatives on cell viability in Raw 264.7 mouse macrophages. Cells were treated with glutamic acid derivatives (100 μg/mL) for 24h. Relative cell viability was evaluated using a modified WST-1 assay. All results are representative of 3 independent experiments. (means ± SD) *P<0.01 compared with the control group.

To evaluate cell viability (compound toxicity) *in vitro*, a modified WST-1 assay was performed.[10] WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay has been broadly used to measure toxin effects on mammalian cells.[11-13] As shown in Figure 3, glutamic acid derivatives **4b, 4h** and **12b** were less cytotoxic than Ramalin **4a** at 100 μg/mL.

On the basis of *in vitro* stability and toxicity test results, compound **12b** was selected as a prototype. Compound **12b** was evaluated for inhibition of nitric oxide (NO) production in LPS-stimulate RAW264.7 mouse macrophage cell lines.[14] NO is an important molecule for host defense immune system against various pathogens such as bacteria, fungi, viruses and parasites.[15] Over-expression of NO induces tissue damage related with acute and chronic inflammations.[16] To accomplish this study, cells were simultaneously treated with 0.3 μg/mL LPS and different concentrations of compound **12b**. The RAW 264.7 cells were activated and increased NO production by LPS, and NO production was measured as nitrite concentration in the culture medium after 24 h incubation. Compound **12b** significantly inhibited (p<0.01) the nitrite accumulation in LPS-activated RAW 264.7 cells in a concentration dependent manner.

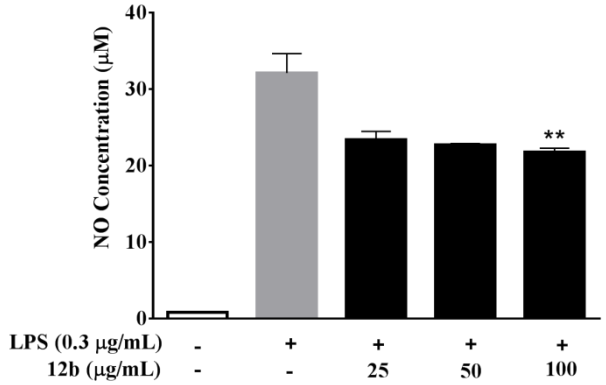


Figure 4. Inhibition of NO production in LPS-stimulated RAW 264.7 cell lines. NO production was measured using the Griess reagent. All results are representative of 3 independent experiments. Values are means ± SD. **P<0.01 compared with the LPS group.

To investigate the anti-inflammatory effects of **12b** *in vivo*, we used a zebrafish model. The *Tg(mpx:GFP)* zebrafish line is an *in vivo* model used for the analysis of inflammatory response, which expresses GFP under the control of the neutrophil-specific myeloperoxidase promoter.[17] At 3 dpf (3 days post fertilization), *Tg(mpx:GFP)* larvae were anesthetized with tricaine and amputated tail fin. After amputation of tail fin, GFP-expressing neutrophils migrated to the wounded region. At 4 hours post amputation, the embryonic medium containing wounded larvae was treated with **12b** for 6 hours. **12b** decreased neutrophil recruitment to wounded tail fin. However, un-amputated larvae did not recruit neutrophils recruitment in response to **12b** treatment. Dexamethasone was used as a positive control. This result confirms that **12b** suppresses neutrophil-mediated wound inflammation.

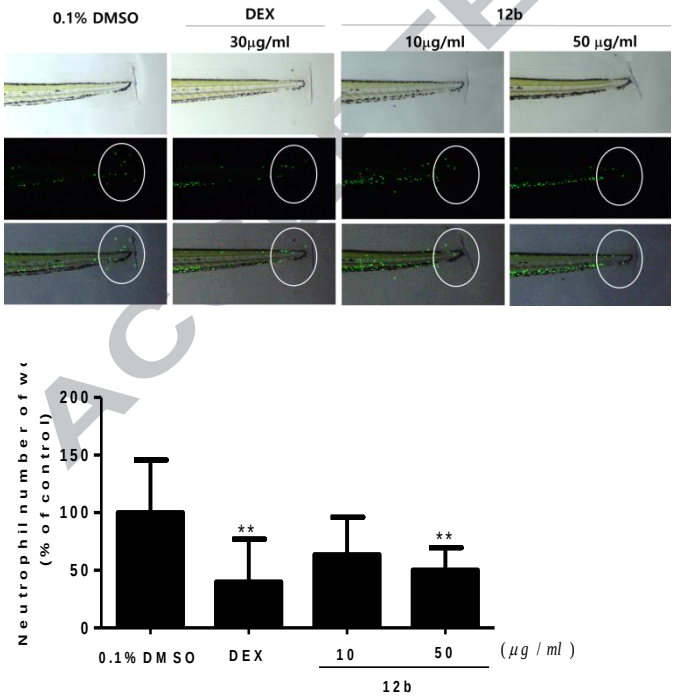


Figure 5. *In vivo* anti-inflammatory activity of compound **12b.**The compound reduced neutrophil numbers in wound sites in response to injury in Tg (mpx:GFP) zebrafish. **P<0.01

In conclusion, we investigated the antioxidant activity of a series of glutamic acid derivatives. Several glutamic acid derivatives with ortho and para substituents were found to be potent anti-oxidant agents. Among the synthesized glutamic acid derivatives, compounds **4b, 12a,** and **12b** showed better *in vitro* activity, chemical stability and cytotoxicity than Ramalin **4a**. Compound **12b**, which is a prototype, showed anti-inflammatory effects in LPS-stimulated RAW 264.7 cell lines and in a zebrafish model.

Acknowledgments

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[†]These authors contributed equally to this work

References and notes

1. Ames BN, Shigenaga MK, Hagen TM. *Proc. Natl. Acad. Sci. U.S.A.* 1993;90:7915.
2. Dean RT, Davies MJ. *Trends Biochem. Sci*. 1993;18:437.
3. Tseng TH, Kao ES, Chu CY, Chou FP, LinWu HW, Wang CJ. *Food Chem Toxicol*. 1997;35:1159.
4. Roullier C, Chollet-Krugler M, Weghe Pv, Devehat FL, Boustie J. *Bioorganic & Medicinal Chemistry Letters*. 2010;20: 4582.
5. Paudel B, Bhattarai HD, Koh HY, Lee SG, Han SJ, Lee HK, Oh H, Shin HW, Yim JH. *Phytomedicine*, 2011;18(14):1285.
6. Park B, Yim JH, Lee HK, Kim BO, Pyo S. *Biosci. Biotechnol.Biochem*. 2015;79(4):539.
7. Chang YH, Ryu JS, Lee SH, Park SG, Bhattarai HD, Yim JH, Jin MH. *J. Soc. Cosmet. Scientist Korea*. 2012;38:247.
8. Yim JH, Kim IC, Lee SG, et al., WO 2012008785, 2012. Yim JH, Kim IC, Kim DK, et al., WO2013141576, 2013.
9. Huang DJ, Ou BX, Prior RL. *J Agric Food Chem.* 2005;53:1841.
10. Ngamwongsatit P, Banada PP, Panbangred W, Bhunia AK. *J. Microbiol. Methods*. 2008;73:211.
11. Kau JH, Lin CG, Huang HH, Hsu HL, Chen KC, Wu YP, Lin HC. *Curr. Microbiol*. 2002;44:106.
12. Scobie HM, Rainey GJ, Bradley KA, Young JA. *Proc. Natl. Acad. Sci. U.S.A.* 2003;100:5170.
13. Moravek M, Dietrich R, Buerk C, Broussolle V, Guinebretiere MH, Granum PE, Nguyen-The C, Martlbauer E. *FEMS Microbiol. Lett*. 2006;257:293.
14. Sherman MP, Aeberhard EE, Wong VZ, Griscavage JM, Ignarro LJ. *Biochemical and Biophysical Research Communications*. 1993;191:1301.
15. Bogdan C, Röllinghoff M, Diefenbach A. Immunol Rev. 2000;173:17.
16. Taira J, Nanbu H, Ueda K. *Food Chem*. 2009;115:1221.
17. Renshaw SA, Loynes CA, Trushell DM, Elworthy S, Ingham PW, Whyte MK. *Blood*. 2006;108:3976.

Supplementary Material

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Graphical Abstract

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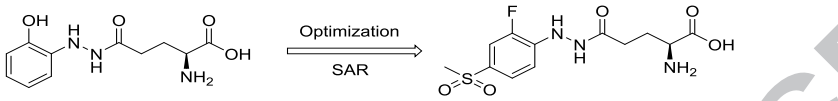

Ramalin
unstable at room temperature

Retained in vitro anti-oxidant activity
Improved stability and cell cytotoxicity
Showed anti-inflammatory effect in vitro and in vivo