RAMALIN, A NOVEL PHENYL HYDRAZIDE FROM THE LICHEN *RAMALINA TEREBRATA*; ISOLATION, TOTAL SYNTHESIS AND BIOLOGICAL ACTIVITIES

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ABSTRACT

Ramalin, a new L-glutamic acid derivative of phenylhydrazide (γ-glutamyl-N'-(2-hydroxyphenyl) hydrazide, 1) was isolated from the Antarctic lichen, Ramalina terebrata after a series of bioactivity guided fractionation of crude aqueous methanolic extract. Ramalin showed stronger antioxidant activities than commercially available standards, ascorbic acid, trolox, BHA, kojic acid in both, in vitro and in vivo test systems. In addition, ramalin showed no/less toxicity effects against two human cell lines; fibroblast (CCD-986SK) cells and keratinocyte (HaCaT). Thus, ramalin merits for cosmetic application and industrial scale production were needed. We developed a cost effective total synthesis of ramalin with 71.5% yield and described here.

Key Words: Antioxidant, ramalin, *Ramalina terebrata*, total synthesis.

INTRODUCTION

In our previous study[1-3], the Antarctic lichen *Ramalina terebrata* showed potent antibacterial and antioxidant activity. The bioactivity guided isolation of secondary metabolites from *R. terebrata* yielded ramalin (γ-glutamyl-N'-(2-hydroxyphenyl) hydrazide) as a potential antibacterial[4] and antioxidant constituent *in vitro*[5,6] and *in vivo*[7]. Ramalin did not show any toxicity within its working dose in two human cell lines: human fibroblast (CCD-986SK) cells and keratinocyte (HaCaT) cells[7]. Ramalin was stronger than kojic acid to inhibit tyrosinase activity which is related to skin whitening effects leading to cosmetic importance.

Later, a similar structure was reported as pygmeine from the marine lichen *Lichina pygmaea*. The synthesis of pygmeine with the final yield of 48% was reported by the same authors by coupling of benzoic protected L-glutamic acid with benzyloxy phenyl hydrazine followed by deprotection with Pd/C. These starting compounds were either not commercially available in the market or with high price increasing the cost of large-scale production. As this natural product deserves future

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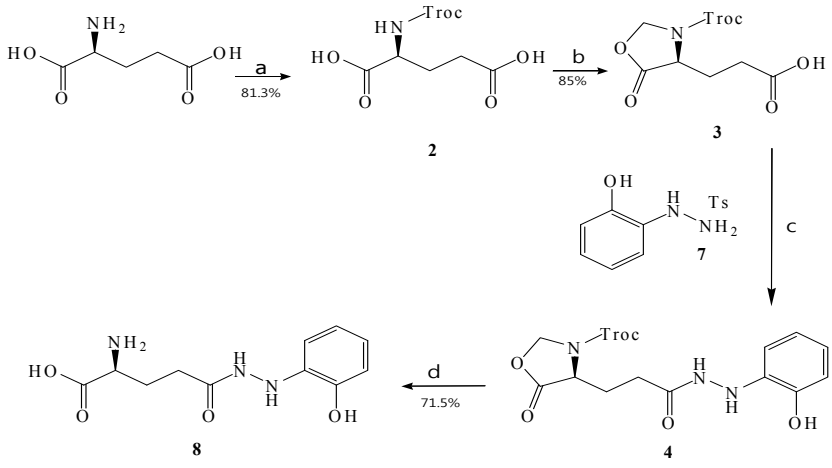
commercial production especially with skin cosmetic applications, we developed a cost effective total synthesis of ramalin and presented here to facilitate its commercialization.

RESULTS AND DISCUSSION

ISOLATION OF RAMALIN AND STRUCTURE ELUCIDATION

Ramalin was isolated as amorphous powder (mp, 136.64 °C) which exhibited $[\alpha]^{25}_{D}$= +14 (c 0.1, EtOH) suggesting the 2-position stereochemistry as 'S' type. The molecular formula of ramalin [**1**, γ-glutamyl-N'-(2-hydroxyphenyl) hydrazide, Fig.-1] was determined as $C_{11}H_{15}N_3O_4$ by analysis of its HRESIMS data [*m/z* 254.1141 $(M + H)^+$; Δ 0.0 mmu], indicating six degrees of unsaturation. This formula was supported by ^1H and ^{13}C NMR data (Table-1). We described the structure elucidation with 1D and 2D NMR data analysis for the first time[5] from the Antarctic lichen *Ramalina terebrata* and later similar structure was reported as pygmeine[8] from the marine lichen *Lichina pygmaea.*

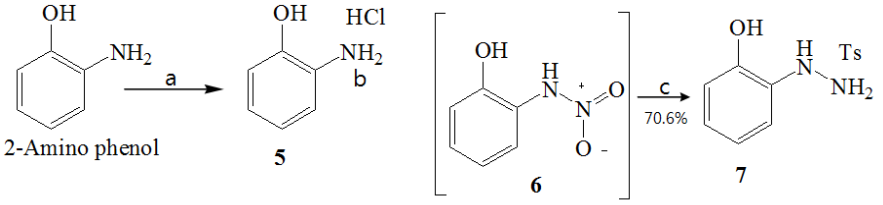
TOTAL SYNTHESIS OR RAMALIN



Scheme 1. Reagents and condition: (a) Troc Cl, $NaHCO_3$, 16h. (b) $(CH_2O)_n$, TsOH, Toluene, reflux, 3h. (c) DCC, HOBt, MC, Overnight. (d) Zn, Acetic acid/H_2O

The cost effective total synthesis of ramalin was designed as shown in scheme 1 with cheap L-glutamic acid and 2-amino phenol as starting materials. L-glutamic acid which has the same chiral center as of ramalin, was subjected to amine protection with 2,2,2-trichloro

ethylchloroformate (Troc)[9] and resulted in the troc-protected di acid **2**. We even tried with other amine protecting group: benzyl group[10], Boc group[11], Cbz group[12]. These three groups could easily protect the amine part of L-glutamic acid but low yield of **4** was obtained during coupling step. Therefore, the troc-procted di acid **2** was further transformed to mono acid **3**. On the other side, the 2-aminophenol was converted to 2-hydroxy phenyl hydrazine toluene sulfonic acid salt **7** (Scheme 2). The monoacid **3** and hydrazine sulfonic acid salt **7** were coupled resulting in N-benzyloxycarbonyl-L-glutamic acid lactone phenyl hydrazine **4**. The final deproctection of **4** yielded ramalin **8** with similar spectroscopic data (1D, 2D NMR and optical rotation) with that of natural ramalin (Table 1).



Scheme 2. Reagents and conditions: (a) HCl gas, MeOH. (b) Isopentyl nitrite, EtOH. (c) $SnCl_2$, TsOH, EtOH.

BIOLOGICAL ACTIVITIES

Ramalin showed antibacterial activity against human pathogenic gram positive bacteria[4]. It also showed stronger *in vitro* and *in vivo* antioxidant activity[7] than the commercial standards ascorbic acid, kojic acid, trolox and BHA. Moreover, it did not show cytotoxic effects to two human cell lines: keratinocyte and fibroblast cells at antioxidant working dose[7] showing its strong candidacy for future cosmetic application.

EXPERIMENTAL

GENERAL INFORMATION

All reagents and solvents were purchased from Sigma-Aldrich. Optical rotation was measured in a polarimeter (Autopol III, Rudolph, USA). Melting point was measured using DSC Q-1000 (TA Instrument, USA). ESIMS data were obtained by using a Mariner ESI-MS instrument (Perceptive Biosystem, USA). NMR spectra (1D and 2D) were recorded in D_2O using a JEOL JNM ECP-400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C), and chemical shifts were referenced relative to tetramethylsilane (δ_H/δ_C=0). HMQC and HMBC experiments were optimized for $^1J_{CH}$ = 140 Hz and $^nJ_{CH}$ = 8 Hz, respectively. Flash column

chromatography was carried out using Aldrich octadecyl-functionalized silica gel (C_{18}). HPLC separations were performed on a Shiseido Capcell Pak® C_{18} column (10 × 250 mm; 5 μm particle size) with a flow rate of 2 mL/min. Compounds were detected by UV absorption at 280 nm.

ISOLATION OF RAMALIN

Ramalin was isolated as a result of bioactivity guided fractionation of methanol-water extract of *Ramalina terebrata*.

SYNTHESIS OF RAMALIN

N-TRICHLOROETHYLOXYCARBONYL-L-GLUTAMIC ACID (2)

A solution of 31.5 g of $NaHCO_3$ in water 125 ml (0.375 mol) was poured in 500 ml two necked round bottom flask equipped with a reflux condenser and an isobar dropping funnel. To this solution, 14.7g (0.1mol) of L-glutamic acid was slowly added at room temperature with moderate stirring. The reaction was carried out with 25.4g of 2,2,2-trichloroethylchloroformate (0.12 mol) which was added drop wise and the temperature was increased to 35°C. The resulting stirred solution was heated at 40-45°C for 6 h, and then was maintained at 20°C for 15 h. The aqueous phase was washed with ether (30 ml), then acidified drop wise by a solution of 5M HCl 15ml (pH 2) and extracted by ethyl acetate (3 X 50 ml). The combined extracts were dried with $MgSO_4$. After evaporation of the solvents in vacuum, the product was obtained as yellow oil (26.2g). ^{1}H NMR (acetone d_6): δ 1.55-2.75 (m, 4H, CH_2CH_2); 4.15-4.55 (m, 1H, CH); 4.70 (s, 2H, CH_2CCl_3); 6.65 (d, J = 8 Hz, 1H, NH); 10.6 (s, 2H, OH). ^{13}C NMR (CDCl3, 100 MHz): δ 26.7, 29.3, 53.0, 60.5, 74.7, 154.2, 176.4, 178.4

N-TRICHLOROETHYLOXY CARBONYL-L-GLUTAMIC ACID LACTONE (3)

A solution of N-troc glutamic acid 12.34 g (0.04mol) in 200 ml toluene was poured in 500 ml round bottom flask fitted with a dean stark apparatus and a reflux condenser. A portion of 2.39g of paraformaldehyde (0.08mol) and 0.46 g of p-TsOH (0.0024mol) were added. The mixture was refluxed for 3 h, until the end of the azeotropic separation. After cooling, ethyl acetate (100 ml) was added. The organic phase was separated, washed with an aqueous solution of K_2CO_3 0.3 M (4 ml) followed with water (3 X 100 ml). After drying with $MgSO_4$, the solvent was partially evaporated in vacuum. The obtained amorphous (24.2 g) was filtered and dried over vacuum.

^{1}H NMR ($CDCl_3$, 400MHz): δ 2.15-2.65 (m, 4H, CH_2CH_2); 4.51 (m, 1H, CH); 4.82-5.00 (m, 2H, CH_2CCl_3); 5.37-5.59 (m, 2H, NCH_2O); 10.32 (s, 1H, OH). ^{13}C NMR ($CDCl_3$, 100MHz): δ 25.7, 29.0, 54.0, 61.0, 75.1, 94.6, 151.3, 171.1, 177.8

2-AMINOPHENOL HYDROGEN CHLORIDE (5)

2-Aminophenol (10 g) was dissolved in MeOH (100 ml) at RT. HCl gas was bubbled until the reaction mixture's pH reached to 2~5. After stirring the mixture for 15 h (keeping pH 2~5), nitrogen gas was purged for 30 min and concentrated with rotary evaporation. Crude aminophenol salt **5** was washed with hexane/ ethyl acetate (3:7, v/v) mixture and vacuum dried. This salt was used for next step without purification.

2-HYDROXY PHENYL HYDRAZINE TOLUENE SULFONIC ACID SALT (7)

2-Aminophenol hydrogen chloride **5** (10 g) was dissolved in EtOH 50 ml and cooled to -5~0°C. Isopentyl nitrite 9.2 g (0.068mol) in EtOH 30 ml was added slowly and stirred for 30 min. Reaction temperature was maintained to -5~0°C during the reaction period. 26.05 g of tinchloride (0.136 mol) and 13.17 g of p-TsOH (0.068 mol) was dissolved in EtOH 80 ml in another round bottom flask and cooled to -5~0°C. Aminophenol mixture was added drop wise into tinchloride mixture in 30 min and stirred for 1 h. Then ethyl ether (100 ml) was added and kept stirred for 30 min. Thus, produced hydrazine Ts salt was filtered and washed with mixture of hexane/ ethyl acetate (2:1, v/v). The vacuum dried 18 g of solid **7** was obtained.

^{1}H NMR (δppm, CD_3OD): δ 2.37 (s, 3H); 6.85 (m, 2H); 7.00 (m, 2H); 7.24 (d, *J* = 10, 2H); 7.71 (d, *J* = 10, 2H). ^{13}C NMR (Acetone D6, 100MHz): δ 21.2, 116.8, 117.6, 120.6, 124.5, 126.8, 129.4, 131.8, 140.4, 144.3, 147.3

N-BENZYLOXYCARBONYL-L-GLUTAMIC ACID LACTONE PHENYL HYDRAZINE (4)

A total of 5.65 g of 2-hydroxyl phenylhydrazine toluene sulfonic acid salt **7** (0.02 mol) was added at 0°C to a solution of 5.75 g (0.017 mol) of N-trichloroethyloxy carbonyl-L-glutamic acid lactone **2**, 4.75 g (0.023 mol) of DCC (dicyclohexylcarbodiimide) (1.35 equivalent), and 3.51 g (0.026 mol) of HOBt (hydroxybenzotriazole) (1.5 equivalent) in 100ml of CH_2Cl_2. After 1 h, the reaction mixture was warmed to room temperature and stirred for 15h. The reaction mixture was washed three times with each 1N HCl 50 ml, saturated $NaHCO_3$ 50 ml, and brine 50 ml, respectively. The organic layer was dried over $MgSO_4$ and the solvent was removed in vacuum. The dried crude product **4** (9 g) was used without further purification.

RAMALIN (8)

A solution of 5 g (0.011 mol) of compound **4** in 35 ml of glacial acetic acid was placed in a 50 ml flask. The solution was stirred with a magnetic stirrer. 40 ml water was added followed by zinc powder 5.5 g. When the suspension of zinc was homogenous (in 2 min), 5 ml water was slowly added and the stirred continueously at room temperature for 5 min. The mixture was filtered immediately and washed with MC (2 X 100 ml). The aqueous phase was concentrated and amorphous solid appeared. The obtained solid product was filtered and washed with hexane 100 ml, dried in vacuum (dry weight 1.94g). The optical rotation, melting point and NMR data were obtained as same with natural ramalin. The NMR data are listed in Table-1.

CONCLUSION

In summary, ramalin showed high antioxidant activity than several other commercial standard ascorbic acid and kojic acid without any toxicity effects to human cell lines. This work demonstrated the cost-effective total synthesis of ramalin with far better final yield than the previously published report[8]. Thus, this research work has facilitated to produce the ramalin at industrial level for future commercial application.

WORKS CITED

Bhattarai, H.D, Paudel B., Hong S.G., Lee H.K. & Yim, J.H. (2008). *J Nat Med,* 62, 481.

Elena, C., Lucia, R.L., Manuela, R. & Maurizio, T. (2009). *Tetrahedron*. 65, 844.

Jean-Francois, C., Léone, M., Jérôme, R. & Jean-Louis B. (1993). *Synthetic communications,* 23(15), 2101.

Luzzio, F.A., Duveau, D.Y., Lepper E.R. & Figg, W.D. (2005). *J Org Chem*. 70, 10117.

Padrón, J.M., Kokotos, G., Martín, T., Markidis, T., Gibbons, W.A. & Martín, V.S. (998). *Tetrahedron: Asymmetry*. 9, 3381.

Paudel, B, Bhattarai, H.D., Lee, H.K., Oh, H., Shin, H.W. & Yim, J.H.*Z.* (2010). *Naturforsch*. 65c, 34.

Paudel, B., Bhattarai, H.D., Koh, H.Y., Lee, S.G., Han, S.J., Lee, H.K., Oh, H., Shin, H.W. & Yim, J.H. (2011). *Phytomedicine*. 18, 1285.

Paudel, B., Bhattarai, H.D., Lee, J.S., Hong, S.G., Shin, H.W. & Yim, J.H. (2008). *Polar biol,* 31, 605.

--- (2008). *Phytother Res*, 22, 1269.

Roullier, C., Chollet-Krugler M., van de Weghe P. & Lohézic-Le, D. (2010). *Bioorg Med Chem Lett*., 20, 4582.

Yim, J.H., Lee, H.K., Bhattarai, H.D., Paudel, B., Kim, I.C., Hong, S.G., Kim, D.K., Lee, S.G. & Oh, H.C. (2010). International patent. WO/2010.053327.

Annex

Table-1: ^{1}H and ^{13}C NMR spectroscopic data for natural and synthetic ramlin in D_2O.

1[d]				**8**[e]	
No	δ_H[a] (int., mult., *J* in Hz)[a]	δ_C[b]	HMBC (H → C#)	δ_H[a] (int., mult., *J* in Hz)[a]	δ_C[b]
1	--	173.9		--	173.9
2	3. 80 (1H, t, 6.2)	54.3	1, 3, 4	3. 80 (1H, t, 6.2)	54.3
3	2.19 (2H, m)	26.3	1, 2, 4	2.19 (2H, m)	26.3
4	2.53 (2H, m)	29.7	2, 3, 5	2.53 (2H, m)	29.7
5		174.7			174.7
1'		135.7			135.7
2'		144.0			144.0
3'		115.6[c]			115.6[c]
4'	6.89 – 6.83 (4H, m)	121.2[c]		6.89 – 6.83 (4H, m)	121.2[c]
5'		121.9[c]			121.9[c]
6'		114.0[c]			114.0[c]

[a]Recorded at 400 MHz. [b]Recorded at 400 MHz. [c]assignments interchangeable, [d]isolated ramalin, [e]synthetic ramalin