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Synthesis of gallic acid amide derivatives containing 1,3,4-thiadiazole and their inhibitory activity on *Vibrio harveyi*

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Abstract: Using Gallic acid as raw material, 1-(substituted aromatic acyl)-4-(3,4,5-trihydroxybenzoyl) thiosemicarbazone was prepared by a two-step reaction and a series of brand-new gallic acid amide derivatives that contained 1,3,4-thiadiazole were synthesized by cyclic reaction. The newly prepared compounds' *Vibrio harveyi* inhibition activities were evaluated. The results indicated that all compounds showed different degree of inhibitory activity on *Vibrio harveyi*. Among them, the best inhibition effect was shown by compound 5b and its minimum inhibitory concentration (MIC) was 0.0313mg/mL.

Keywords: 1,3,4-thiadiazole; *Vibrio harveyi* inhibitory activity; gallic acid

INTRODUCTION

Gallic acid is a natural polyphenol compound with the simplest chemical structure, which is mainly derived from Chinese herbal plants such as gallnut, cornus officinalis, pomegranate and rhubarb (Al Zahrani *et al*., 2020, BenSaad *et al*., 2017, Tan *et al*., 2022, Watrelot *et al*., 2020). In recent years, the synthesis of Gallic acid derivatives by structural modification has become a hot area of research to improve their biological activities. Plenty of researches have revealed that Gallic acid derivatives have antibacterial, anti-inflammatory, antioxidant and other biological activities (Choińska *et al*., 2021, Tuli *et al*., 2022, Liu *et al*., 2020, Fei *et al*., 2017).

1,3,4-thiadiazole compounds are a class of common nitrogen-containing and sulfur-containing heterocyclic compounds with rich biological activities, such as bacteriostasis, weeding, antiviral, insecticidal and plant growth regulation, etc., which are widely used in materials, medicine, pesticides and chemical fields(Serban *et al*., 2018, Hu *et al*., 2014, Chen *et al*., 2021). In recent decades, a variety of pesticides containing 1,3,4-thiadiazole have been developed at home and abroad, such as the fungicides Bismerthiazol and Thiodiazole copper and the herbicide Buthidazole (Han *et al.*, 2021). Similarly, amide structures are also widely found in compounds with biological activity, especially in bactericidal and bacteriostatic aspects (Żołnowska *et al*., 2019) and many derivatives containing amide structures are widely used.

Vibrio harveyi belongs to Vibrio genus, Vibriaceae, is a gram-negative, luminous Marine bacteria and is a common opportunistic pathogen in mariculture. With the increase of breeding density and the deterioration of breeding environment, the morbidity of *Vibrio harveyi* is increasing, which poses a serious threat to mariculture production (Plágaro *et al*., 2019). At present, streptomycin sulfate, gentamicin and other antibiotics are mainly used to control vibriosis in aquaculture, but the long-term continuous application of antibiotics will cause drug resistance of pathogens (Kang *et al*., 2014, Miller and Harbottle *et al*., 2018). Therefore, finding new lead compounds for structural modification to develop new aquatic antimicrobial drugs has become a research focus in this field.

Based on the above situation, we propose to introduce the amide bond and 1,3,4-thiadiazole with antibacterial activity into Gallic acid compounds and design and synthesize a series of brand-new Gallic acid derivatives. Using the Oxford cup method to test the anti-*V. harveyi* activity of the synthesized compounds. Finally, we obtained Gallic acid derivatives with good *V. harveyi* inhibitory activity.

MATERIALS AND METHODS

Materials and equipment

The melting point was measured by X-4B digital melting-point apparatus (Shanghai Yi Electrophysical Optics Co., LTD, China). Infrared data were obtained using THE Cary630 Fourier infrared spectrometer (Bruker, Germany). ^{1}H NMR data were obtained using AVANCE hydrogen magnetic resonance spectrometer (Bruker, Germany). HRMS (ESI) data were obtained using Agilent 6230 Mass Spectrometer (Agilent, USA). *V. harveyi* was purchased from China General Microbial Strain Preservation and Management Center.

General procedure for the synthesis of 3,4,5-trihydroxybenzoyl isothiocyanate 2

Take a 50mL round-bottom flask, take an ice bath, add 9mL acetonitrile, triphenylphosphine (TPP) (0.58g,

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2.2mmol), trichloroisocyanuric acid (TCCA) (0.16g, 0.7mmol), dissolve the solid and precipitate again, then add Gallic acid (0.3g, 1.8mmol), After stirring for 15 min, potassium thiocyanate (KSCN) (0.26g, 2.6mmol) was added and the solution gradually turned light yellow. The solution was reacted overnight at 20℃ and examined by TLC. After the reaction, rapid extraction filtration, organic phase concentration, yellow oil-like coarse product 2.

General procedure for the synthesis of 1-(substituted aromatic acyl)-4-(3,4,5-trihydroxybenzoyl) thiosemicarbazone 4a-i

5 mL dioxane, compound 2 (0.3 g, 1.4 mL) substituted benzoyl hydrazine (0.15 g, 0.7 mmol) were successively added into a dry 50 mL round-bottom flask and reacted at 60℃ for 6 h. After the reaction, cool to room temperature, add 32 mL dichloromethane into the reaction solution to precipitate a large amount of solid, filter, filter cake washed with dichloromethane, get crude product 4a-j.

General procedure for the synthesis of N-(5-substituted-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide 5a-i

Take 50mL round-bottom flask, ice bath, add compound 4a-j (0.35mmol), 4mL anhydrous ethanol, drop concentrated sulfuric acid to PH=2~3, reflux reaction, solid gradually dissolved, TLC detection. Then pour the reaction solution into 10mL ice water to precipitate solids and filter. The filter cake was washed with ethanol and distilled water and recrystallized with anhydrous ethanol to obtain pure compound 5a-j.

In vitro V. harveyi activity assay

Under aseptic environment, pour about 20mL of Mueller-Hinton Broth into the petri dish. After solidification, 200μL of Vibrio harrier suspension was added, the coating rod was evenly coated and let it stand for 10 min. Place required Oxford cup vertically and evenly on plate solid medium and add 200μL solutions of the compounds to Oxford cup. After being placed in 37°C constant temperature incubator for 18h, the diameter of bacteriostatic zone was determined. All samples were assayed in triplicate.

STATISTICAL ANALYSIS

The data of inhibition circle diameter was expressed as mean ± standard deviation, n = 3. All analysis were done my using Microsoft Office 2019(Excel).

RESULTS

Synthesis study

This study has successfully synthesized Gallic acid amide derivatives containing 1,3,4-thiadiazole with good yield. Furthermore, structural characterizations have concluded the validity of the synthesized compounds.

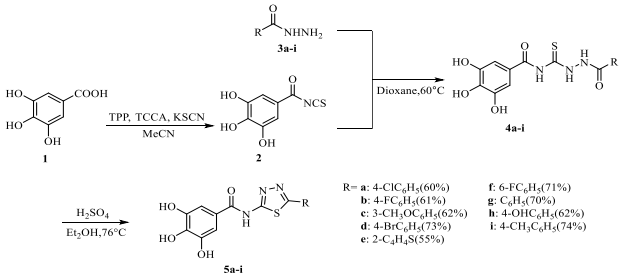
Anti-vibrio activity study

The result of *in vitro* inhibitory activity on *V. harveyi* shown in table 1. The minimum inhibitory concentration (MIC) of *N*-(5-(4-fluorophenyl)-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (compound 5b) is 0.0313mg/mL.

DISCUSSION

Chemistry

In order to develop a simple synthesis pathway of 1,3,4-thiadiazole containing gallic amide derivatives, 3,4,5-trihydroxybenzoyl isothiocyanate (2) was used as the key intermediate. In this study, the carboxyl sites of Gallic acid were modified by trichloroisocyanuric acid (TCCA), triphenylphosphine (TPP) and potassium thiocyanate (KSCN) without the protection of hydroxyl groups and the key intermediate of 3,4,5-trihydroxybenzoyl isothiocyanate (2) was obtained. In addition, compound 4 was obtained by the reaction of substituted Benzoyl hydrazine with intermediate 2 in the solvent of dioxane. Finally, *N*-(5-substituted-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide derivatives 5a-i (Scheme 1) were obtained through cyclization reaction catalyzed by concentrated sulfuric acid.



Scheme 1: Synthesis of the Gallic acid Amide derivatives Containing 1,3,4-thiadiazole

In the infrared (IR) spectrum, The peaks of compound 5f at 3625 cm^{-1} and 3602 cm^{-1} are characteristic absorption peaks of Phenolic hydroxyl(-OH). The absorption peak at 1682 cm^{-1} was the C=O unit on the amide bond. The absorption peak at 1608cm^{-1} indicates the presence of -C=N- unit of thiadiazole. The absorption peaks at 1542 cm^{-1} and is C=C unit. The absorption peak at 1027 cm^{-1} indicates the presence of the C-S-C unit of thiadiazole. In the ^{1}H nuclear magnetic resonance (NMR) spectrum, compound 5f shows a single peak (1H) at δ 12.47, which is the N-H proton peak at the amide bond. The two single peaks (3H) in the range of δ 9.31~9.12 are proton peaks of three hydroxyl groups in the benzene ring. The peaks in the δ 8.26~7.40 range are fluorine replacing hydrogen on the benzene ring. A single peak (2H) at δ 7.15 is hydrogen in the benzene ring of Gallic acid. In the mass spectra (electrospray-ionization [ESI]), the compounds 5a-i all showed molecular ion peaks for $[M+H]^+$. The detailed data of IR, ^{1}H NMR and HRMS (ESI) of compounds 5a-i are shown below.

N-(5-(4-chlorophenyl)-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (5a)

White solid; m.p. > 310 °C; IR (KBr), *v*/cm^{-1}: 3625, 3601, 3425, 3365, 2976, 1661, 1609, 1517, 1202, 1042, 840, 745; ^{1}H NMR(500 MHz, DMSO-d_6), δ = 12.77 (s, 1H, NH),

9.31 (s, 2H, OH), 9.12 (s, 1H, OH), 7.98 (d, J = 8.5 Hz, 2H, Ar-H), 7.60 (d, J = 8.5 Hz, 2H, Ar-H), 7.14 (s, 2H, Ar-H); HRMS (ESI): m/z [M-H]$^-$ calcd for: $C_{15}H_9ClN_3O_4S^-$: 362.0002; Found: 362.0013.

N-(5-(4-fluorophenyl)-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (5b)

Yellow solid; m.p. > 310 °C; IR (KBr), *v*/cm^{-1}: 3625, 3599, 3425, 3216, 2979, 1679, 1610, 1526, 1194, 1042, 840, 744; ^{1}H NMR(500 MHz, DMSO-d_6), δ = 12.74 (s, 1H, NH), 9.30 (s, 2H, OH), 9.14 (s, 1H, OH), 8.02 (dd, J = 8.8, 5.4 Hz, 2H, Ar-H), 7.42 – 7.34 (m, 2H, Ar-H), 7.14 (s, 2H, Ar-H). HRMS (ESI): m/z [M-H]$^-$ calcd for: $C_{15}H_9FN_3O_4S^-$: 346.0298; Found: 346.0288.

N-(5-(3-methoxyphenyl)-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (5c)

Yellow solid; m.p. 301-303 °C; IR (KBr), *v*/cm^{-1}: 3626, 3600, 3411, 3202, 2979, 1681, 1610, 1529, 1458, 1198, 1039, 831, 779; ^{1}H NMR(500 MHz, DMSO-d_6), δ = 12.73 (s, 1H, NH), 9.29 (s, 2H, OH), 9.11 (s, 1H, OH), 7.50 (d, J = 7.7 Hz, 2H, Ar-H), 7.44 (t, J = 7.9 Hz, 1H, Ar-H), 7.14 (s, 2H, Ar-H),7.09 (dd, J = 7.0, 1.2 Hz, 1H, Ar-H), 3.85 (s, 3H, OCH_3); HRMS (ESI): m/z [M-H]$^-$ calcd for: $C_{16}H_{12}N_3O_5S^-$: 358.0498; Found: 358.0487.

N-(5-(4-bromophenyl)-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (5d)

White solid; m.p. > 310 °C; IR (KBr), *v*/cm^{-1}: 3625, 3600, 3426, 3367, 2976, 1663, 1613, 1518, 1461, 1202, 1039, 847, 750; ^{1}H NMR(500 MHz, DMSO-d_6), δ = 12.77 (s, 1H, NH), 9.30 (s, 2H, OH), 9.11 (s, 1H, OH), 7.91 (d, J = 8.5 Hz, 2H, Ar-H), 7.73 (d, J = 8.5 Hz, 1H, Ar-H), 7.14 (s, 2H, Ar-H); HRMS (ESI): m/z [M-H]$^-$ calcd for: $C_{15}H_9BrN_3O_4S$: 405.9497; Found: 405.9425.

N-(5-thiophene-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (5e)

Yellow solid; m.p. > 310 °C; IR (KBr), *v*/cm^{-1}: 3626, 3599, 3428, 3263, 2978, 1677, 1612, 1525, 1199, 1038, 850, 726; ^{1}H NMR(500 MHz, DMSO-d_6), δ = 12.75 (s, 1H, NH), 9.28 (s, 2H, OH), 9.13 (s, 1H, OH), 7.75 (d, J = 5.1 Hz, 1H, Ar-H), 7.71 (d, J = 2.7 Hz, 1H, Ar-H), 7.20 (dd, J = 5.0, 3.7 Hz, 1H, Ar-H), 7.13 (s, 2H, Ar-H); HRMS (ESI): m/z [M-H]$^-$ calcd for: $C_{13}H_8N_3O_4S_2^-$: 333.9956; Found: 333.9945.

N-(5-(2-fluorophenyl)-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (5f)

Pale yellow solid; m.p. > 310 °C; IR (KBr), *v*/cm^{-1}: 3625, 3602, 3402, 3289, 2982, 1682, 1608, 1542, 1028, 781; ^{1}H NMR(500 MHz, DMSO-d_6), δ = 12.79 (s, 1H, NH), 9.31 (s, 2H, OH), 9.12 (s, 1H, OH), 8.26 (t, J = 7.0 Hz, 1H, Ar-H), 7.60 (td, J = 7.3, 1.7 Hz, 1H, Ar-H), 7.47 (dd, J = 11.2, 8.4 Hz, 1H, Ar-H), 7.40 (t, J = 7.6 Hz, 1H, Ar-H), 7.15 (s, 2H, Ar-H); HRMS (ESI): m/z [M-H]$^-$ calcd for: $C_{15}H_9FN_3O_4S^-$: 346.0298; Found: 346.0294.

N-(5-phenyl-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (5g)

White solid; m.p. > 310 °C; IR (KBr), *v*/cm^{-1}: 3625, 3600, 3419, 3284, 2977, 1677, 1609, 1541, 1196, 1028, 851, 759; ^{1}H NMR(500 MHz, DMSO-d_6), δ = 12.72 (s, 1H, NH), 9.30 (s, 2H, OH), 9.11 (s, 1H, OH), 7.96 (d, J = 5.5 Hz, 2H, Ar-H), 7.54 (s, 1H, Ar-H), 7.52 (d, J = 1.3 Hz, 2H, Ar-H), 7.14 (s, 2H, Ar-H); HRMS (ESI): m/z [M-H]$^-$ calcd for: $C_{15}H_{10}N_3O_4S^-$: 328.0392; Found: 328.0399.

N-(5-(4-hydroxyphenyl)-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (5h)

Yellow solid; m.p. > 310 °C; IR (KBr), *v*/cm^{-1}: 3625, 3601, 3406, 3258, 2976, 1662, 1610, 1520, 1202, 1043, 840, 748; ^{1}H NMR(500 MHz, DMSO-d_6), δ = 12.59 (s, 1H, NH), 10.05 (s, 1H, OH), 9.28 (s, 2H, OH), 9.08 (s, 1H, OH), 7.77 (d, J = 8.5 Hz, 2H, Ar-H), 7.12 (s, 2H, Ar-H), 6.89 (d, J = 8.6 Hz, 2H, Ar-H); HRMS (ESI): m/z [M-H]$^-$ calcd for: $C_{15}H_{10}N_3O_5S^-$: 344.0341; Found: 344.0356.

N-(5-(4-methylphenyl)-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (5i)

Yellow solid; m.p. > 310 °C; IR (KBr), *v*/cm^{-1}: 3625, 3601, 3409, 3285, 2977, 1674, 1607, 1538, 1190, 1029, 847, 815; ^{1}H NMR(500 MHz, DMSO-d_6), δ = 12.68 (s, 1H, NH), 9.29 (s, 2H, OH), 9.10 (s, 1H, OH), 7.84 (d, J = 8.1 Hz, 2H, Ar-H), 7.34 (d, J = 8.1 Hz, 2H, Ar-H), 7.13 (s, 2H, Ar-H), 2.37 (s, 3H, CH_3); HRMS (ESI): m/z [M-H]$^-$ calcd for: $C_{16}H_{12}N_3O_4S^-$: 342.0549; Found: 342.0582.

Biological activity

The inhibitory activity of the newly synthesized compound 5a-i of *V. harveyi* was determined by the Oxford Cup method which results are shown in table 1. Using Streptomycin sulfate as the reference drug, if the inhibition zone of the compounds is greater than 13 mm that should test the MIC of the compounds.

Table 1: Results of *in vitro* inhibitory activity of 1 and 5a-5i on *Vibrio harveyi*

Compound	Anti-Vibrio *harveyi* activity	
	Diameter of bacteriostatic circle[a] (mm)	MIC (mg/mL)
1	7.80±0.05	–
5a	13.11±0.12	0.0625
5b	14.78±0.27	0.0313
5c	13.67±0.21	0.0625
5d	11.56±0.09	–
5e	14.46±0.18	0.0625
5f	13.01±0.13	0.0625
5g	13.65±0.31	0.0625
5h	11.91±0.07	–
5i	11.72±0.25	–
Streptomycin sulfate	–	0.0160
DMSO	10.76±0.06	–

[a]The data are the inhibition circle diameter of the compound against Vibrio *harveyi* at the concentration of 1mg/mL, expressed as mean±standard deviation, n=3.

As shown above, all the prepared compounds have good anti-*V. harveyi* activity and the MIC of *N*-(5-(4-fluorophenyl) -1, 3, 4- thiadiazole- 2- yl) -3, 4, 5-trihydroxybenzamide (compound 5b) is 0.0313 mg/mL, which has the best inhibitory effect on *V. harveyi*. In

addition, the results showed that the inhibitory activity of 1,3,4-thiadiazole containing Gallic acid amide derivatives varied with their types of substituents on thiadiazole. The compounds with halosubstituted benzene ring(5a,5b,5f), methoxy substituted benzene ring(5c) and thiophene ring(5e) have better activity than those with methyl and hydroxyl substituted benzene ring(5h,5i), among which, the para fluorine substituted benzene ring (5b) has the best activity. In conclusion, the introduction of 1,3,4-thiadiazole into the structure of Gallic acid can greatly improve its antibacterial activity. These data provide guidance and ideas for us to find new inhibitors of *Vibrio harveyi*.

CONCLUSION

Nine gallic amide derivatives which contained 1,3,4-thiadiazole were designed and synthesized by a simple and efficient method and all structures of nine compounds were confirmed by HRMS, IR and ^{1}H NMR. Compounds 5a-i showed inhibitory activity against *V. harveyi*. Among them, *N*-(5-(4-fluorophenyl)-1,3,4-thiadiazole-2-yl)-3,4,5-trihydroxybenzamide (compound 5b) had the best activity and its minimum inhibitory concentration (MIC) was 0.0313mg/mL.

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