Synthesis and Antimicrobial Characterization of Luotonin Derivatives

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Abstract
Twenty-one novel luotonin derivatives were synthesized from 2-aminobenzamide in excellent yields. The prepared compounds, when tested against 8 strains of bacteria and many plant pathogen fungi showed diverse and promising antimicrobial activities. Notably, compounds a5, a9, b4, and b5 demonstrated good activities and might be potential lead compounds for further development as antifungal agents. The relationship between structure and biological activity is also discussed.

Keywords
luotonin derivatives, synthesis, antimicrobial, agrochemicals, bioactivity

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In nature, tricyclic heteroaromatic skeletons are very important moieties that are widespread in a large family of natural products with a broad range of attractive bioactivities.1 Luotonin A (Figure 1) and derivatives, which possess a novel kind of tricyclic heteroaromatic scaffold, have displayed important biological activities such as anticancer, cytotoxic, and antiproliferative activity.2,3 Because of this wide scope of biological activities, a great deal of study toward the synthesis and antifungal activities of luotonin A has been reported.4-17 Therefore, we focused on structural modification of luotonin A in the hope that a lead compound could be found for the discovery of novel antifungal agents. Herein, we report the synthesis and bioactivity of a series of novel luotonin analogs using 2-aminobenzamide as the starting material.

To the best of our knowledge, the antimicrobial activities of the synthetic compounds are reported for the first time.

Materials and Methods

Instruments and Chemicals
All chemicals were purchased from commercial sources and purified based on standard procedures before use. Analytical thin-layer chromatography was performed with silica gel plates using silica gel 60 GF254 (Qingdao Haiyang Chemical Co., Ltd., China). The 1H nuclear magnetic resonance (NMR; 500 MHz) and 13C NMR (125 MHz) spectra were obtained on an AM-500 FT-NMR spectrometer (Bruker Corporation, Switzerland) with deuterated chloroform (CDCl3) as the solvent and trimethylsilane as the internal standard. Melting points were taken on an electrothermal digital apparatus (Beijing, China) and were uncorrected. Mass spectra (MS) were recorded under electrospray ionization (ESI) conditions using an LCQ Fleet instrument (Thermo Fisher, Waltham, MA, USA). The title compounds were synthesized under a nitrogen atmosphere. Yields were not optimized.

Synthesis
The general synthetic methods for luotonin derivatives are depicted in Scheme 1.

Synthesis of compound 3. A mixture of 2-aminobenzamide (2.72 g, 20 mmol) and diethyl oxalate (20 mL) was refluxed at 185.5 °C for 5 hours. The reaction mixture was then allowed to cool to room temperature (r.t). The precipitate was collected by filtration using a Hirsch funnel to give compound 3 as a white solid (3.55 g, 16.4 mmol, 82%).
Synthesis of compound 4. Compound 3 (3.5 g, 1.60 mmol) was added to ammonium hydroxide (30 mL) and stirred overnight at r.t. The precipitate was filtered and dried in a low-temperature drying oven to give compound 4 (2.76 g, 14.6 mmol, 91%).

Synthesis of compound 5. To a stirred solution of compound 4 (2.70 g, 1.43 mmol) and potassium carbonate (1.97 g, 2.86 mmol) in N,N-dimethylformamide (20 mL) was added propargyl bromide (2.15 mL, 2.86 mmol) at r.t. for 30 minutes. The solution was allowed to stir for 1 hour at r.t. and then extracted with dichloromethane (3 × 40 mL). The combined organic extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure yielding compound 5 as a white solid (2.64 g, 11.6 mmol, 83%).

Synthesis of compound 7. A mixture of crude residue 5 (4.5 g) and mercuric acetate as a catalyst in formic acid (30 mL) was heated to 50-55 °C with magnetic stirring for 1 hour. The reaction mixture was extracted with dichloromethane. The combined extracts were washed with brine and then dried with sodium sulfate. All the crude residues were purified by silica-gel column chromatography and evaporated under reduced pressure to provide the desired compound 7 as a yellow solid (2.52 g, 11.7 mmol, 82%).

Synthesis of compounds a1-a11. Compound 7 (0.20 g, 0.9 mmol) and potassium carbonate (0.25 g, 1.8 mmol) in N,N-dimethylformamide (10 mL) was stirred at 0 °C for 0.5 hours. The solution was allowed to stir for 1 hour at r.t. and then extracted with dichloromethane (3 × 40 mL). The combined organic extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure yielding compound 5 as a white solid (2.64 g, 11.6 mmol, 83%).

Scheme 1. Synthetic route for the title compounds.
to stir for 2-3 hours at r.t. The corresponding desired reagent was added to the flask and then extracted with dichloromethane (3 × 15 mL). The combined organic extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure yielding a white solid. All the crude residues were purified by silica-gel column chromatography and evaporated under reduced pressure to provide the desired compounds a1-a11.

Synthesis of compounds b1-b5. A solution of compound 7 (0.20 g, 0.9 mmol) and triethylamine (0.20 mL) in dichloromethane was added dropwise over 30 minutes to the corresponding desired reagent solution in an ice-salt bath. The mixture was stirred for 1 hour. Then the reaction mixture was allowed to warm up to r.t., and water was added. The mixture was extracted with dichloromethane (3 × 20 mL) and the combined organic extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure yielding a white solid. The crude residues were purified by silica-gel column chromatography and evaporated under reduced pressure to provide the desired compounds b1-b5.

Synthesis of compounds c1-c5. A solution of compound 7 (0.20 g, 0.9 mmol) and potassium carbonate (0.25 g, 1.8 mmol) in N,N-dimethylformamide (10 mL) was stirred at 0 °C for 0.5 hours. The solution was allowed to stir for 2-3 hours at r.t. The corresponding desired reagent was added to the flask and then extracted with dichloromethane (3 × 15 mL). The combined organic extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure yielding a white solid. All the crude residues were purified by silica-gel column chromatography and evaporated under reduced pressure to provide the desired compounds c1-c5.

3-Methyl-2-(4-methylbenzyl)-2H-pyrazino[2,1-b]quinazoline-1,6-dione (a1).

Yellow solid, m.p., 231-233 °C, Formula: C22H17N3O2 (81% yield), 1H NMR (500 MHz, CDCl3), δ (ppm): 8.38 (dd, J = 1.0 Hz, 8.0 Hz, 1 H, 7 H), 8.07 (d, J = 9.0 Hz, 1 H, 10 H), 7.86 (m, 1 H, 9 H), 7.60 (m, 1 H, 8 H), 7.52 (m, 1 H, 4 H), 7.18 (m, 2 H, 15-CH × 2), 7.12 (m, 2 H, 14-CH × 2), 5.27 (s, 2 H, 12-CH2), 2.31 (s, 3 H, 17-CH3), 2.28 (s, 3 H, 3-CH3). 13C NMR (125 MHz, CDCl3), δ (ppm): 157.98 (C = O), 157.06 (C = O), 146.91 (10a-C), 139.83 (11a-C), 137.70 (13 C), 134.94 (9-CH), 132.75 (16 C), 129.62 (15-CH × 2), 129.51 (8-CH, 128.28 (10-CH), 127.02 (3 C), 126.99 (14-CH × 2), 125.53 (7-CH), 119.77 (6a-C), 101.60 (4-CH), 47.45 (12-CH2), 21.07 (17-CH3), 17.45 (3-CH3). ESI-MS: [M + H]+ 332.1399; found 332.1387.

The data of the other compounds and their NMR spectral details can be found in the Supplemental Material.

Biological Activity
The antimicrobial activity of the N-protected luotonin derivatives was measured according to the previously reported method.18,19

Minimal inhibitory concentration. Antibacterial activities were evaluated by the micro broth dilution method in 96-well culture plates using Mueller-Hinton broth, according to the National Committee for Clinical Laboratory Standards.20,21 All compounds were tested in triplicate at each concentration.

Inhibition of spore germination method. All experiments were conducted in triplicate. The half-maximal effective concentration values were determined by performing the bioassay as described above with concentrations of 100, 75, 50, 37.5, and 25 µg/mL, respectively.

Results and Discussion
Design and Synthesis of Luotonin Analogs
The synthetic route is outlined in Scheme 1. Twenty-one luotonin analogs, including the previously prepared c1-c5, have been synthesized by this efficient method. The luotonin analogs were synthesized using 2-aminobenzamide as the starting material via acylation or alkylation at the N-position. The structures of the synthesized compounds were confirmed by 1H NMR and 13C NMR spectroscopy, and high-resolution mass spectrometry.

Antimicrobial Activity
All the target compounds were screened for antibacterial activities against 5 Gram-negative (Pseudomonas solanacearum, Pseudomonas aeruginosa, Escherichia coli, Ralstonia solanacearum, and Pseudomonas syringae pv. actinidiae) and 3 Gram-positive bacteria (Staphylococcus aureus, Bacillus subtilis, and B. cereus). The minimum inhibitory concentrations (MICs) were determined by the double-dilution method using penicillin sodium and fosfomycin sodium as the positive controls. The MIC values are summarized in Table 1. The target compounds showed moderate antibacterial activities against S. aureus, E. coli, P. aeruginosa, B. cereus, P. syringae pv. actinidiae, P. solanacearum, and R. solanacearum. Compounds a1, a3, a6, a9, b2, and b5 manifested greater activity against P. solanacearum than fosfomycin sodium. Compound a6 showed a similar degree of activity as fosfomycin sodium against P. aeruginosa. Compounds a5 and b5 showed similar
degrees of activity as fosfomycin sodium and penicillin sodium against \textit{P. syringae pv. actinidiae}.

The activity of the synthesized compounds toward plant pathogen fungi was also screened, and the results are listed in Table 2. A mycelial growth inhibition assay was utilized, with carbendazim as the positive control, to evaluate the activity of the 21 synthesized \textit{N}-protected luotonin derivatives against \textit{Alternaria alternata}, \textit{Fusarium oxysporum f. sp. niveum}, \textit{Colletotrichum gloesporioides}, \textit{Sclerotinia sclerotiorum}, \textit{Gibberella zeae}, \textit{Botrytis cinerea}, and \textit{Curvularia lunata} (Walk) Boedijn at a concentration of 100 µg/mL. Among the synthesized compounds, \textit{a5} displayed great activity against \textit{C. gloesporioides}, with 61.0% inhibition, and compound \textit{b5} showed 60.2% inhibition against \textit{G. zeae} (Table 2). Compound \textit{a9} produced 57.3% inhibition of \textit{C. gloesporioides}, compound \textit{b4} showed 52.4% inhibition of \textit{S. sclerotiorum}, and compound \textit{a5} showed 50.5% against \textit{B. cinerea}.

Although it is difficult to extract apparent structure-activity relationships from the bioassay results, some conclusions can still be obtained. First, \textit{N}-substituents (\textit{a5-a10}, \textit{b2}, and \textit{b5}) with a halogen-substituted aryl displayed more antibacterial activities than the compounds (\textit{c1-c5}) with alkyl groups. Third, compounds with the halogen atom on the aryl positions of the \textit{N}-substituents (\textit{a5}, \textit{a6}, \textit{a8-a10}, \textit{b2}, and \textit{b5}) showed better antibacterial activities than compounds with the halogen atom attached to the aliphatic chain on the aryl substituent (\textit{a7}).

### Conclusions

Twenty-one luotonin derivatives were synthesized using 2-aminobenzamide as the starting material via either alkylation or acylation at the \textit{N}-position, and their antimicrobial activities have been evaluated against 8 strains of bacteria and many plant pathogen fungi. The majority of the synthesized compounds showed potent activities. Notably, compounds \textit{a5}, \textit{a9}, \textit{b4}, and \textit{b5} demonstrated remarkably activities and might be novel potential lead compounds for further development of antifungal agents.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

| Compounds | B. c. | B. s. | S. a. | E. c. | P. a. | P. s. a. | P. s. | R. s. |
|-----------|-------|-------|-------|-------|-------|--------|-------|------|
|           | 2MIC (µg/mL) |       |       |       |       |        |       |      |
Table 2. Inhibitory Effects of Luotonin Derivatives Against A.a., F.O.N., C.g., S.s., G., B.c., and C.l.b.

| Compounds  | A.a.   | F.O.N. | C.g.   | S.s.   | G.    | B.c.   | C.l.b. |
|------------|--------|--------|--------|--------|-------|--------|--------|
| Carbendazim| 98.9   | 98.9   | 97.5   | 99.8   | 98.3 | 99.4   | 98.7   |
| a1         | 9.8    | 18.5   | 22.4   | 10.7   | 31.0 | 23.0   | 20.1   |
| a2         | 21.2   | 14.1   | 34.6   | 7.6    | 16.2 | 23.1   | 31.4   |
| a3         | 26.3   | 15.8   | 17.3   | 30.9   | 10.0 | 14.5   | 25.4   |
| a4         | 37.7   | 9.2    | 15.2   | 27.5   | 30.4 | 12.1   | 22.7   |
| a5         | 37.2   | 57.1   | 61.1   | 38.5   | 47.4 | 50.5   | 29.7   |
| a6         | 26.9   | 29.9   | 18.7   | 15.7   | 17.4 | 26.9   | 29.4   |
| a7         | 18.9   | 28.5   | 10.9   | 9.8    | 29.3 | 17.8   | 21.2   |
| a8         | 35.1   | 26.6   | 27.8   | 20.5   | 26.3 | 32.3   | 13.9   |
| a9         | 28.5   | 46.6   | 57.3   | 40.2   | 31.2 | 26.6   | 18.3   |
| a10        | 21.1   | 12.7   | 9.2    | 21.0   | 38.0 | 18.1   | 10.2   |
| a11        | 10.6   | 14.1   | 9.7    | 25.4   | 9.2  | 20.5   | 17.7   |
| b1         | 33.5   | 37.2   | 15.9   | 28.4   | 37.4 | 29.2   | 17.2   |
| b2         | 43.6   | 28.1   | 38.5   | 16.0   | 27.8 | 34.1   | 26.6   |
| b3         | 25.8   | 19.5   | 38.2   | 25.7   | 25.9 | 38.1   | 17.5   |
| b4         | 19.7   | 11.9   | 37.9   | 52.4   | 9.2  | 19.1   | 24.4   |
| b5         | 17.3   | 37.9   | 10.7   | 21.6   | 60.2 | 10.7   | 17.1   |
| c1         | 18.6   | 24.4   | 34.8   | 25.6   | 34.0 | 13.2   | 36.3   |
| c2         | 16.7   | 11.3   | 33.9   | 24.4   | 18.5 | 28.4   | 25.8   |
| c3         | 47.6   | 29.6   | 18.3   | 22.7   | 23.2 | 45.9   | 26.9   |
| c4         | 26.1   | 16.3   | 34.8   | 33.5   | 25.1 | 44.5   | 27.9   |
| c5         | 42.8   | 28.6   | 37.6   | 18.2   | 20.3 | 22.5   | 25.5   |

Abbreviations: A.a., Alternaria alternata; B.c., Botrytis cinerea; C.g., Colletotrichum gloeosporioides; C.l.b., Curvularia lunata (Walk) Boedijn; EC50, half maximal effective concentration; FON, Fusarium oxysporum f. sp. niveum; G, Gibberella zeae; S.s., Sclerotinia sclerotiorum.

Note. The carbendazim was used as the positive control.

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Supplemental Material
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