

Synthesis, Antimicrobial and Cytotoxicity Studies of Some Novel Modified Strobilurin Derivatives

Ajjanna M. Sridhara,^{a,b} Kallam R. Venugopala Reddy,^{*,b} Jathi Keshavayya,^c
Dasannana Malige S. Ambika,^e Vadiraj S. Gopinath,^a Prosenjit Bose,^a
Sanath Kumar Goud^a and Sanenahalli K. Peethambar^d

^aAdvinus Therapeutics Pvt. Ltd, 21 & 22, Peenya II phase, Bangalore-560 058, India

^bDepartment of Studies in Industrial Chemistry, ^cDepartment of Studies in Chemistry,

^dDepartment of Biochemistry, Kuvempu University, Jnana Sahyadri,
Shankaragatta-577 451, Karnataka, India

^eDepartment of Plant Pathology, G.K.V.K, UAS, Bangalore-65 India

Uma série de novas 3-isoxazolinias substituídas, derivadas do metil-3-metóxi-2-(4-oxo-3,4-diidroftalazin-1-il)prop-2-enoato foram estudadas e sintetizadas a partir do metil-(4-oxo-3,4-diidroftalazin-1-il)acetato, o qual foi preparado a partir do anidrido ftálico. As estruturas dos novos compostos sintetizados foram caracterizadas por dados espectrais e suas atividades antimicrobianas e citotóxicas estudadas. Vários desses compostos mostraram boa atividade antimicrobiana.

A series of some new 3-isoxazoline substituted methyl-3-methoxy-2-(4-oxo-3,4-dihydrophthalazin-1-yl)prop-2-enoate derivatives were designed and synthesized from methyl-(4-oxo-3,4-dihydrophthalazin-1-yl)acetate, which in turn was prepared from phthalic anhydride. The structures of synthesized new compounds were characterized by spectral data and studied for their antimicrobial activities and cytotoxicity. Several of these compounds showed good antimicrobial activity.

Keywords: phthalazin-1(2H)-one, β -methoxyacrylate, strobilurins, isoxazoline

Introduction

Nitrogen-containing heterocyclic molecules constitute the largest portion of chemical entities, which are part of many natural products, fine chemicals, and biologically active pharmaceuticals. Strobilurins and their analogues constitute a large group of compounds that represent a new class of plant-protecting agents¹ that meet all the demands that are made nowadays for pesticides. They exhibit efficacy against a broad-spectrum of fungal diseases, possess significant post-infective activity, and have a unique mode of action.² Intensive studies aimed at a search for novel biologically active methoxyacrylate fungicides are currently under way by different manufacturers.³ Studies on the structure of natural methoxyacrylates have made possible to create a novel class of synthetic fungicides with enhanced stability, high activity, and a

broad spectrum of action. β -Methoxyacrylate which is a critical and common structural element of strobilurins, find use in agrochemical agents,⁴ antivirals,⁵ antimalarials,⁶ and fungicides.⁷ There has been extensive industrial development of these compounds and their analogues.^{8,9} In finding new β -methoxyacrylate derivatives, we observed that, phthalazines attached to these pharmacophore were not studied. Phthalazin-1(2H)-one derivatives are of considerable interest due to their antidiabetic,¹⁰ antiallergic,¹¹ vasorelaxant,¹² PDE4 inhibitor,¹³ VEGF (vascular endothelial growth factor) receptor tyrosine kinase (for the treatment of cancer),¹⁴ antiasthmatic,¹⁵ and herbicidal¹⁶ activities. A number of established drug molecules like Hydralazine,^{17,18} Budralazine,^{19,20} Azelastine,^{21,22} Ponalrestat,²³ and Zopolrestat,²⁴ were prepared from the corresponding phthalazinones.

Similarly, isoxazolines represents an important class of heterocyclic compounds with broad spectrum of biological activities. Substituted isoxazolines have revealed anti-

*e-mail: venurashmi30@rediffmail.com

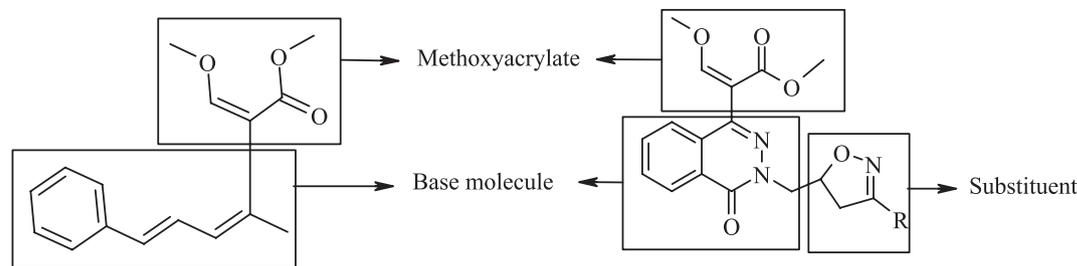


Figure 1. Strobilurin A and modified strobilurin derivatives.

influenza virus,²⁵ antifungal,²⁶ antitubercular,²⁷ spermicidal and anti-HIV,²⁸ β -adrenergic receptor antagonist,²⁹ analgesic and anti-inflammatory³⁰ properties.

The diverse biological activities of β -methoxyacrylate, phthalazin-1(2*H*)-one, and isoxazoline pharmacophores envisaged us to plan a new lead compounds that may exhibit wide pharmacological activities. By combining these pharmacophore components in a molecule to give a compact system, we designed and synthesized a series of phthalazin-1(2*H*)-one derivatives containing β -methoxyacrylate and isoxazoline moieties. Figure 1 reveals a framework of these three important biological active pharmacophore component systems.

The synthesized new isoxazoline substituted phthalazine-methoxyacrylate derivatives were characterized by mass, IR and NMR studies. Purity of compounds was assessed by analytical HPLC and monitored by photodiode array (PDA) detector.

Results and Discussion

Chemistry

The core intermediate in the synthesis of targeted compounds **7a-1** is methyl 2-(4-oxo-3,4-dihydrophthalazin-1-yl)acetate³¹ (**4**) which was prepared from phthalic anhydride (Scheme 1).

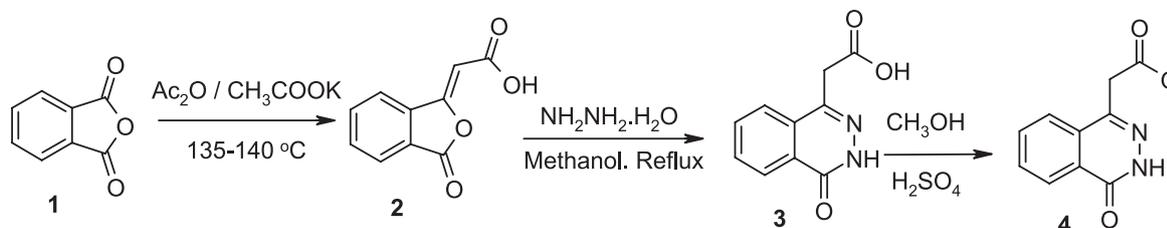
N-allylation of compound **4** with allyl bromide in presence of potassium carbonate as base gave methyl 2-(3-allyl-4-oxo-3,4-dihydrophthalazin-1-yl)acetate (**5**). *N*-allylation was confirmed by mass, IR, and NMR spectra. The active methylene part of phthalazin-1-yl acetate (**5**) was formylated

with methylformate in presence of sodium hydride as base giving hydroxyacrylate intermediate, which then alkylated with dimethyl sulfate in presence of potassium carbonate to afford compound (**6**). The allyl function of compound **6** underwent 1,3 dipolar cyclization³² with aldoximes via nitrile oxide in presence of *N*-chlorosuccinimide, dimethylformamide and potassium carbonate to give the desired compounds **7(a-1)** (Scheme 2). The desired aldoximes were prepared by the condensation of hydroxyl amine hydrochloride with the corresponding aldehydes.³³

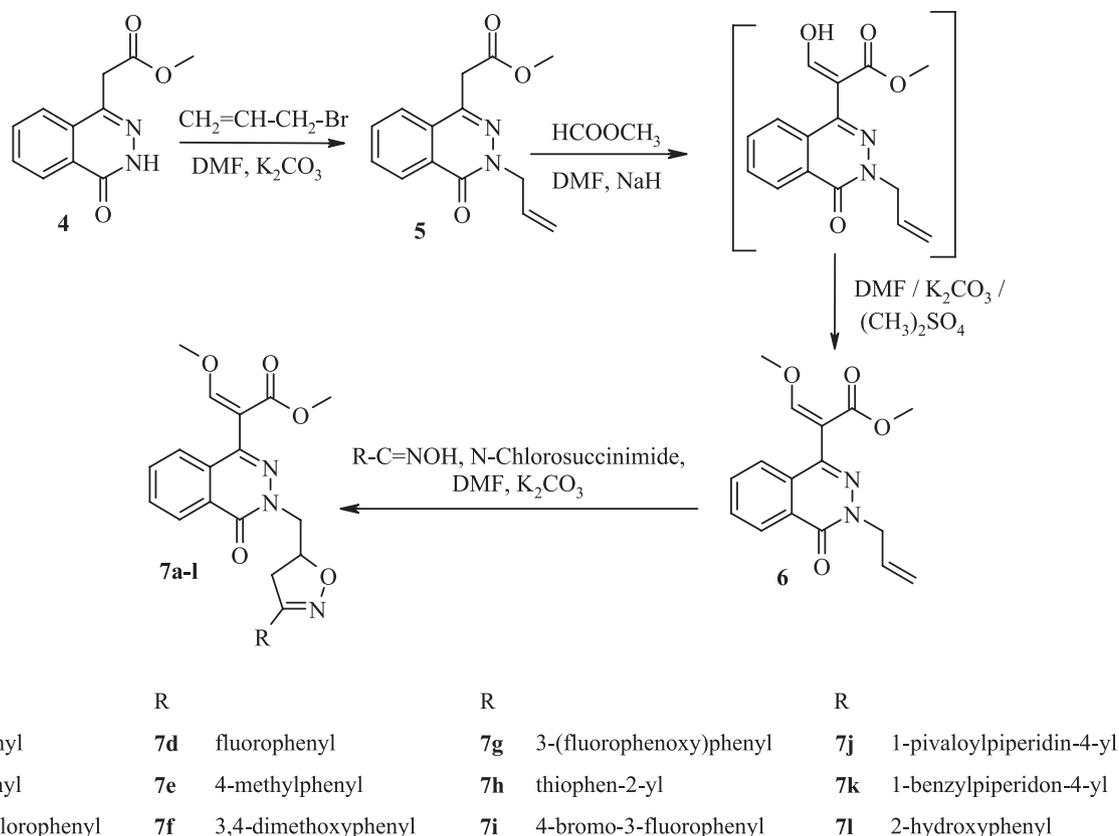
Biological study

All the synthesized compounds were screened for their antibacterial activity against *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), *Salmonella typhi* (*S. typhi*), and *Escherichia coli* (*E. coli*) and antifungal activity against *Aspergillums niger* (*A. niger*) and *Candida albicans* (*C. albicans*). Minimum inhibitory concentration (MIC) of all compounds was determined, which is defined as the lowest concentration of inhibitor at which bacterial growth was not visually apparent.

Investigation on antibacterial screening data (Table 1) showed some of the compounds were active against four human pathogenic bacteria. Compounds **7a**, **7b**, **7c**, **7k** and **7l** exhibited good activity against *S. aureus*. Similarly compounds **7e**, **7h** and **7i** showed activity against *B. subtilis*. The compounds **7e** and **7h** showed activity against *S. typhi*. Also the compounds **7e**, **7h** and **7k** showed activity against *Escherichia coli*. From these results it could be generalized that the thiophene substituted isoxazoline derivatives shows higher activity compared to other analogues.



Scheme 1. Synthesis of methyl 2-(4-oxo-3,4-dihydrophthalazin-1-yl)acetate.



Scheme 2. Synthesis of 3-isoxazoline substituted phthalazine-methoxyacrylate derivatives **7(a-l)**.

The antifungal results (Table 1) revealed that the synthesized compounds showed variable degree of inhibition against the tested fungi. Compounds **7c**, **7d**, **7g** and **7h** possessed the good antifungal activity against *A. niger* and *C. albicans*. From the results it was concluded that the thiophene substituted isoxazoline compounds showed better activity.

Cytotoxicity assay

MTT cell viability assay³⁴ was performed to study the cytotoxicity effect of newly synthesized compounds **7(a-l)** against Calu-6 cells at 50 μ M concentration using 96-well tissue culture plates. As observed in the results (Table 2), the *in vitro* cytotoxicity assay did not show any reduction in the growth rate. This is reflected by the cell number and the degree of inhibition of growth, thus the assay provided indication that the tested compounds are not cytotoxic.

Conclusions

In this article we report the synthesis of new 3-isoxazoline substituted modified strobilurin phthalazine-methoxyacrylate derivatives (**7a-l**), starting from

commercially available phthalic anhydride. Investigation of their antimicrobial activity revealed that phthalazine with a thiophene substitution (**7h**) was the most active compound although it was significantly less than that of positive control. Also the cytotoxicity assay showed the compounds were non toxic.

Experimental

All chemicals used for the synthesis were of analytical grade and were procured from Sigma Aldrich Chemical Co, Bangalore, India and the intermediates were prepared as per known literature procedures. ¹H NMR spectra were recorded on a 400 MHz Varian-AS NMR spectrometer using TMS as an internal standard. IR spectra were recorded by using Perkin Elmer 100 Series FT-IR spectrometer. Mass spectra were recorded on an Agilent 1200 Series LC/MSD VL system. Melting points were determined by using Buchi melting point B-545 instrument and are uncorrected. All the reactions were monitored by thin layer chromatography (TLC) using Merck pre coated TLC silica gel plates. The crude compounds were purified by using CombiFlash[®] Companion Flash Chromatography, Teledyne, ISCO, USA.

HPLC method for compounds 7(a-l): The purity of compounds was assessed by HPLC methods using an Agilent 1200 series LC system. The mobile phase was 10 mmol ammonium acetate adjusted to pH 3.0 with acetic acid (A) and acetonitrile (B). The column was an Inertsil ODS-3V (250 × 4.6 mm, 5 μ particle size) and a PDA Detector was employed in the UV range 190-490 nm. Gradient elution was performed, according to the following protocol: 0-2 min: 50% B; 2-10 min: 50-90% B; 10-20 min: 90% B; 20-25 min: 90-50% B and 25-30 min: 50% B.

Preparation of methyl 2-(3-allyl-4-oxo-3,4-dihydro-phthalazin-1-yl)acetate (5)

Allyl bromide (60.5 g, 0.5 mol) was added to a stirred solution of methyl 2-(4-oxo-3,4-dihydrophthalazin-1-yl)acetate (**4**) (21.8 g, 0.10 mol) in dimethylformamide (250 mL) and potassium carbonate (69.0 g, 0.5 mol) at room temperature and heated to 60-65 °C for 6 h. After completion of reaction, filtered the inorganics, the filtrate obtained was distilled completely under reduced pressure at 60-65 °C. The residue obtained was diluted with ice water (500 mL) and stirred for 30 min. The precipitated product was filtered, dried and recrystallised using isopropyl alcohol to yield **5** as white solid. Yield 86%; mp 90.2-94.8 °C; MS (ESI) *m/z*: 259.1 (M+H)⁺; Purity 98.67%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.64 (s, 3H, CH₃), 4.11 (s, 2H, CH₂), 4.73 (d, *J* 5.6 Hz, CH₂), 5.08-5.19 (m, 2H, CH₂), 5.94-6.04 (m, 1H, CH), 7.87-7.92 (m, 3H, Ar-H), 8.30-8.32 (m, 1H, Ar-H); IR (KBr) *v*_{max}/cm⁻¹: 3012, 1708, 1675, 1599.

Preparation of methyl 2-(3-allyl-4-oxo-3,4-dihydro-phthalazin-1-yl)-3-methoxy acrylate (6)

A solution of compound **5** (20.0 g, 0.077 mol) in methylformate (100 g, 1.66 mol) and dimethylformamide (40 mL) was added slowly to the slurry of sodium hydride (60% paste in paraffin oil) (6.0 g, 0.15 mol) in dimethylformamide (200 mL) at 0-5 °C in 1 h under nitrogen atmosphere. After complete addition, the reaction mixture was stirred for 6 h at 0-10 °C. After of the reaction, it was quenched by addition of saturated solution of ammonium chloride, ethyl acetate (250 mL) was added and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (3×100 mL). The combined organic layers were washed with sat. sodium chloride, and dried with anhydrous sodium sulfate. After filtration, the solvent was completely evaporated under reduced pressure and the residue was in dimethylformamide (200 mL) and cooled to 5-10 °C. Potassium carbonate (21.2 g, 0.154 mol), and dimethyl sulfate (19.7 g, 0.153 mol) were added to

the solution at 0-10 °C, and stirred for 8 h at ambient temperature. After completion of reaction, the reaction mass was filtered and the filtrate was evaporated under reduced pressure. The residue was diluted with water, extracted with ethyl acetate (3×100 mL), washed with water, dried with anhydrous sodium sulfate. After filtration the solvent was evaporated under reduced pressure to afford the crude product, which was purified by column chromatography with ethyl acetate/hexane (1: 5) to get 15.2 g of compound (**6**) as a white solid. Yield 65%; mp 129.0-132.2 °C; MS (ESI) *m/z*: 301.1 (M+H)⁺; Purity 98.99%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.60 (s, 3H, CH₃), 3.85 (s, 3H, CH₃), 4.74 (d, *J* 4.8 Hz, 2H, CH₂), 5.08-5.19 (m, 2H, CH₂), 5.94-6.03 (m, 1H, CH), 7.62-7.64 (m, 1H, Ar-H), 7.85-7.94 (m, 2H, Ar-H), 7.97 (s, 1H, =CH), 8.29-8.31 (m, 1H, Ar-H); IR (KBr) *v*_{max}/cm⁻¹: 2956, 1747, 1658.

General method for the preparation of 3-isoxazoline substituted phthalazine-methoxyacrylate derivatives 7(a-l)

A mixture of aldoxime (10.0 mmol), *N*-chlorosuccinimide (11.0 mmol) and potassium carbonate (15.0 mmol) were slurred in dimethylformamide (10 mL) and heated to 60 °C for 30 min. The compound **6** (8.0 mmol) was added, and continued the heating for 4 h. After completion of reaction, cooled, diluted with water (50 mL) and extracted with ethyl acetate (3×50 mL). After drying with anhydrous sodium sulfate and filtration, the solvent was evaporated to provide the crude product, which was purified by using flash chromatography eluting with ethyl acetate / hexane (1: 5) to get the corresponding phthalazine derivatives **7a-l**.

Methyl 3-methoxy-2-(3-((3-methyl-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)acrylate (7a). Yield 64%; Purity 93.7%; White solid; mp 125.1-129.2 °C; MS (ESI) *m/z*: 358.1 (M+H)⁺; ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.92 (s, 3H, -C-CH₃), 3.07-3.35 (2m, 2H, CH₂), 3.67 (s, 3H, COOCH₃), 3.97 (s, 3H, OCH₃), 4.02-4.40 (2m, 2H, CH₂), 4.83-4.95 (m, 1H, oxazole CH), 7.61-7.63 (m, 1H, Ar-H), 7.85-7.93 (m, 2H, Ar-H), 7.94 (s, 1H, =CH), 8.29-8.31 (m, 1H, Ar-H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 177.8, 166.7, 160.8, 159.7, 140.5, 133.1, 131.7, 131.5, 129.8, 127.8, 126.9, 125.7, 62.5, 53.3, 51.8, 38.2, 29.6; IR (KBr) *v*_{max}/cm⁻¹: 2950, 1761, 1654.

Methyl 3-methoxy-2-(4-oxo-3-((3-phenyl-4,5-dihydroisoxazol-5-yl)methyl)-3,4-dihydrophthalazin-1-yl)acrylate (7b). Yield 71%; Purity 96.6%; White solid; mp 179.2-181.4 °C; MS (ESI) *m/z*: 420.1 (M+H)⁺; ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.34-3.55 (2m, 2H, CH₂), 3.59 (s, 3H, CH₃), 3.82 (s, 3H, CH₃), 4.10-4.48 (2m, 2H, CH₂),

5.08-5.15 (m, 1H, oxazole-CH), 7.42-7.44 (m, 3H, Ar-H), 7.59-7.64 (m, 3H, Ar-H), 7.83-7.91 (m, 2H, Ar-H), 7.94 (s, 1H, =CH), 8.27-8.30 (m, 1H, Ar-H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 177.9, 166.7, 164.9, 163.3, 162.4, 159.6, 155.5, 140.5, 133.0, 131.5, 129.8, 128.9, 127.8, 126.8, 125.7, 125.6, 105.6, 62.4, 53.3, 51.8, 38.2, 29.5; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 2951, 1762, 1654.

Methyl 2-(3-((3-(4-chlorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (7c). Yield 72%; Purity 97.1%; White solid; mp 162.0-164.7 °C; MS (ESI) m/z : 454.1 (M+H) $^+$; ^1H NMR (400 MHz, DMSO- d_6) δ 3.35-3.56 (2m, 2H, CH $_2$), 3.57 (s, 3H, CH $_3$), 3.85 (s, 3H, CH $_3$), 4.15-4.51 (2m, 2H, CH $_2$), 5.14-5.19 (m, 1H, oxazole-CH) 7.54 (d, J 8.4 Hz, 2H, Ar-H), 7.63-7.68 (m, 3H, Ar-H), 7.89 (d, J 8.4 Hz, 2H, Ar-H), 7.94 (s, 1H, C=CH), 8.31-8.33 (m, 1H, Ar-H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 177.4, 166.6, 164.6, 163.7, 162.9, 159.2, 155.5, 140.4, 133.1, 131.0, 129.6, 128.8, 127.9, 126.8, 125.7, 125.5, 105.6, 62.3, 53.7, 51.2, 38.4, 29.7; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 2989, 1760, 1681, 1542.

Methyl 2-(3-((3-(4-fluorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (7d). Yield 58%; Purity 96.4%; White solid; mp 129.3-131.9 °C; MS (ESI) m/z : 438.1 (M+H) $^+$; ^1H NMR (400 MHz, DMSO- d_6) δ 3.36-3.42 (2m, 2H, CH $_2$), 3.71 (s, 3H, CH $_3$), 3.87 (s, 3H, CH $_3$), 4.39-4.60 (2m, 2H, CH $_2$), 5.28-5.32 (m, 1H, oxazole-CH) 7.05-7.14 (m, 2H, Ar-H), 7.54-7.56 (m, 1H, Ar-H), 7.64-7.68 (m, 2H, Ar-H), 7.76-7.78 (m, 2H, Ar-H), 7.82 (s, 1H, C=CH), 8.45-8.47 (m, 1H, Ar-H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 177.8, 166.8, 164.9, 163.4, 162.4, 159.7, 155.6, 140.6, 133.1, 129.7, 128.8, 127.8, 126.9, 125.7, 125.6, 115.8, 105.6, 62.5, 53.3, 51.8, 38.2, 29.6; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 2956, 1761, 1654.

Methyl 3-methoxy-2-(3-((3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)acrylate (7e). Yield 45%; Purity 96.7%; White solid; mp 126-129 °C; MS (ESI) m/z : 450.1 (M+H) $^+$; ^1H NMR (400 MHz, DMSO- d_6) δ 3.36-3.37 and 3.66-3.72 (2m, 2H, CH $_2$), 3.63 (s, 3H, CH $_3$), 3.80 (s, 3H, CH $_3$), 3.95 (s, 3H, CH $_3$), 4.13-4.46 (2m, 2H, CH $_2$), 5.08-5.14 (m, 1H, oxazole-CH), 7.01 (d, J 8.4 Hz, 2H, Ar-H), 7.59 (d, J 8.4 Hz, 2H, Ar-H), 7.64-7.65 (m, 1H, Ar-H), 7.87-7.94 (m, 2H, Ar-H), 7.95 (s, 1H, C=CH), 8.31-8.33 (m, 1H, Ar-H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 177.9, 166.8, 164.9, 163.4, 162.4, 159.7, 155.7, 140.3, 133.5, 131.7, 129.6, 128.8, 127.8, 126.8, 125.9, 125.5, 105.5, 62.5, 55.7, 53.3, 51.8, 38.2, 29.6; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 2979, 2815, 1673.

Methyl 2-(3-((3-(3,4-dimethoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (7f). Yield 45%; Purity 95.3%; Off-white solid; mp 120.1-125.2 °C; MS (ESI) m/z : 480.1 (M+H) $^+$; ^1H NMR (400 MHz, DMSO- d_6) δ 3.45-3.56 (2m, 2H, CH $_2$), 3.63 (s, 3H, CH $_3$), 3.80 (s, 9H, 3 \times CH $_3$), 3.97-4.44 (2m, 2H, CH $_2$), 5.10-5.12 (m, 1H, oxazole-CH), 7.09-7.12 (m, 2H, Ar-H), 7.50-7.55 (m, 4H, Ar-H), 7.94 (s, 1H, C=CH), 8.30-8.34 (m, 1H, Ar-H); ^{13}C NMR (400 MHz, DMSO- d_6) δ 177.7, 166.6, 164.7, 163.5, 162.3, 159.7, 155.7, 140.2, 133.6, 131.6, 129.8, 128.9, 127.7, 126.8, 125.9, 125.5, 105.4, 62.5, 55.6, 55.5, 53.3, 51.8, 38.2, 29.5; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 2979, 2815, 1760, 1673.

Methyl 2-(3-((3-(3-(4-fluorophenoxy)phenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (7g). Yield 53%; Purity 94.2%; Brown solid; mp 137.5-140.2 °C; MS (ESI) m/z : 530.1 (M+H) $^+$; ^1H NMR (400 MHz, DMSO- d_6) δ 3.31-3.53 (2m, 2H, CH $_2$), 3.57 (s, 3H, CH $_3$), 3.86 (s, 3H, CH $_3$), 4.15-4.46 (2m, 2H, CH $_2$), 5.15-5.16 (m, 1H, oxazole-CH), 7.02-7.07 (m, 3H, Ar-H), 7.15-7.20 (m, 1H, Ar-H), 7.43-7.93 (m, 5H, Ar-H), 7.64-7.93 (m, 2H, Ar-H), 7.94 (s, 1H, C=CH), 8.29-8.32 (m, 1H, Ar-H); ^{13}C NMR (100 MHz, CDCl $_3$) δ 177.7, 166.6, 164.7, 163.9, 162.3, 159.5, 156.7, 156.6, 155.2, 153.2, 144.2, 140.2, 139.9, 133.7, 132.0, 129.8, 128.5, 127.8, 127.5, 126.4, 124.0, 125.5, 105.4, 62.5, 53.3, 51.8, 38.2, 29.6; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 2970, 2830, 1761, 1673.

Methyl 3-methoxy-2-(4-oxo-3-((3-(thiophen-2-yl)-4,5-dihydroisoxazol-5-yl)methyl)-3,4-dihydrophthalazin-1-yl)acrylate (7h). Yield 70%; Purity 94.6%; Off-white solid; mp 143.2-148.6 °C; MS (ESI) m/z : 426.1 (M+H) $^+$; ^1H NMR (400 MHz, DMSO- d_6) δ 3.36-3.56 (2m, 2H, CH $_2$), 3.63 (s, 3H, CH $_3$), 3.86 (s, 3H, CH $_3$), 4.16-4.48 (2m, 2H, CH $_2$), 5.12-5.19 (m, 1H, oxazole-CH) 7.15-7.17 (m, 1H, Ar-H), 7.36 (d, J 3.6 Hz, 1H, Ar-H), 7.63-7.65 (m, 1H, Ar-H), 7.70 (d, J 5.2 Hz, 1H, Ar-H), 7.74-7.94 (m, 2H, Ar-H), 7.95 (s, 1H, C=CH), 8.31-8.33 (m, 1H, Ar-H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 177.4, 166.6, 164.6, 163.7, 162.9, 159.2, 140.4, 133.1, 131.0, 129.6, 127.9, 126.8, 125.7, 125.5, 105.6, 62.3, 53.7, 51.2, 38.4, 29.7; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 2972, 2835, 1768, 1675.

Methyl 2-(3-((3-(4-bromo-3-fluorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (7i). Yield 59%; Purity 95.4%; White solid; mp 133.5-137.2 °C; MS (ESI) m/z : 516.1 (M+H) $^+$; ^1H NMR (400 MHz, DMSO- d_6) δ 3.31-3.55 (2m, 2H, CH $_2$), 3.62 (s, 3H, CH $_3$), 3.85 (s, 3H, CH $_3$),

4.19-4.49 (2m, 2H, CH₂), 5.28-5.32 (m, 1H, oxazole-CH) 7.32-7.37 (m, 1H, Ar-H), 7.63-7.69 (m, 2H, Ar-H), 7.82-7.92 (m, 3H, Ar-H), 7.95 (s, 1H, C=CH), 8.31-8.33 (m, 1H, Ar-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 177.3, 166.6, 164.8, 163.4, 162.3, 159.6, 155.6, 140.5, 133.2, 129.6, 128.9, 127.8, 126.9, 125.7, 125.5, 115.9, 105.5, 62.4, 53.2, 51.7, 38.2, 29.4; IR (KBr) ν_{\max} /cm⁻¹: 2989, 1763, 1652.

Methyl 3-methoxy-2-(4-oxo-3-((3-(1-pivaloyl-piperidin-4-yl)-4,5-dihydro isoxazol-5-yl)methyl)-3,4-dihydrophthalazin-1-yl)acrylate (7j). Yield 56%; Purity 95.6%; White solid; mp 163.3-167.9 °C. MS (ESI) *m/z*: 511.2 (M+H)⁺; ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.27 (s, 9H, 3CH₃), 1.51-1.61 (m, 2H, piperidine CH₂), 1.82-1.91 (m, 2H, piperidine CH₂), 2.63-2.70 (m, 1H, piperidine CH), 2.89-2.98 (m, 2H, Piperidine N-CH₂), 2.99-3.05 (m, 2H, CH₂); 3.71 (s, 3H, CH₃); 3.87 (s, 3H, CH₃); 4.28-4.39 (m, 2H, piperidine N-CH₂), 4.40-4.48 (m, 2H, CH₂); 5.07-5.14 (m, 1H, oxazole-CH), 7.52-7.56 (m, 1H, Ar-H), 7.74-7.79 (m, 2H, Ar-H), 7.82 (s, 1H, C=CH), 8.44-8.45 (m, 1H, Ar-H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 176.2, 166.7, 163.3, 160.8, 159.7, 140.5, 133.1, 131.6, 131.5, 129.6, 127.8, 126.9, 125.7, 62.5, 53.0, 51.8, 44.7, 38.6, 35.6, 29.4, 28.3; IR (KBr) ν_{\max} /cm⁻¹: 2948, 1710, 1651.

Methyl 2-(3-((3-(1-benzylpiperidin-4-yl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (7k). Yield 45%; Purity 95.9%; White solid; mp 158.3-161.9 °C; MS (ESI) *m/z*: 517.1 (M+H)⁺; ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.49-1.56 (m, 2H, piperidine CH₂), 1.73-1.76 (m, 2H, piperidine CH₂), 2.02 (m, 1H, piperidine CH), 2.33(s, 2H, CH₂), 2.36-2.41 (m, 2H, piperidine N-CH₂), 2.81-2.87 (m, 2H, piperidine CH₂), 2.88-3.10 (2m, 2H, CH₂), 3.63 (s, 3H, -OCH₃), 3.86 (s, 3H, -CH₃), 3.95-4.39 (2m, 2H, CH₂), 4.89-4.93 (m, 1H, oxazole-CH), 7.11-7.34 (m, 5H, Ar-H), 7.62-7.64 (m, 1H, Ar-H), 7.86-7.94 (m, 2H, Ar-H), 7.95 (s, 1H, C=CH), 8.29-8.31 (m, 1H, Ar-H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 176.8, 166.8, 164.9, 163.3, 162.4, 159.6, 155.5, 140.5, 133.0, 131.5, 129.8, 128.9, 127.9, 127.8, 126.8, 125.7, 125.6, 105.6, 62.4, 53.1, 51.9, 51.7, 44.6, 38.5, 35.3, 29.4, 28.2; IR (KBr) ν_{\max} /cm⁻¹: 2949, 1720, 1652.

Methyl 2-(3-((3-(2-hydroxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (7l). Yield 46%; Purity 97.6%; White solid; mp 144.9-146.8 °C. MS (ESI) *m/z*: 436.1 (M+H)⁺; ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.31-3.55 (2m, 2H, CH₂), 3.61 (s, 3H, CH₃), 3.84 (s, 3H, CH₃), 4.16-4.50 (2m, 2H, CH₂), 5.09-5.13 (m, 1H, oxazole-CH), 6.89-6.96 (m, 2H,

Ar-H), 7.29-7.34 (m, 1H, Ar-H), 7.42-7.45 (m, 1H, Ar-H), 7.62-7.64 (m, 1H, Ar-H), 7.86-7.94 (m, 3H, Ar-H), 7.95 (s, 1H, C=CH), 9.85 (brs, 1H, OH); ¹³C NMR (400 MHz, DMSO-*d*₆) δ 177.8, 166.6, 164.8, 163.2, 162.3, 159.6, 155.6, 140.4, 133.1, 131.4, 129.8, 128.8, 127.6, 126.2, 125.7, 125.1, 105.6, 78.4, 62.4, 53.3, 51.8, 38.2, 29.5; IR (KBr) ν_{\max} /cm⁻¹: 3320, 2950, 1710, 1657.

Antimicrobial assay

All the synthesised 3-isoxazoline substituted phthalazine-methoxyacrylate compounds **7(a-I)** were evaluated for their *in vitro* antimicrobial activity against *E.coli*, *S. aureus*, *B. subtilis*, *S. typhi*, bacterial stains and *A. niger*, *C. albicans*, fungal strains by disc diffusion method. Amoxicillin and Ketoconazole were used as standard drugs for bacteria and fungi respectively. Preliminary screening of 3-isoxazoline substituted phthalazine-methoxyacrylates and standard drugs were performed at fixed concentrations of 500 µg/mL. Inhibition was recorded by measuring the diameter of the inhibition zone at the end of 24 h for bacteria and 72 h for fungi. Each experiment was repeated twice.

Based on the results of zone of inhibition, the minimum inhibitory concentration (MIC) of compounds (**7a-I**) against all bacterial and fungal strains was determined by liquid dilution method. Stock solutions of tested compounds with 200, 100, 50, 25, 12.5 and 6.25 µg mL⁻¹ concentrations were prepared with DMSO solvent. The solutions of standard drugs, Amoxicillin and Ketoconazole were prepared in the same concentrations. Inoculums of the bacterial and fungal culture were also prepared. To a series of tubes containing 1 mL each of phthalazine compound solution with different concentrations and 0.2 mL of the inoculums was added. Further 3.8 mL of the sterile water was added to each of the test tubes. These test tubes were incubated for 24 h at 37 °C and observed for the presence of turbidity. This method was repeated by changing phthalazine compounds with standard drugs Amoxicillin and Ketoconazole for comparison. The minimum inhibitory concentration at which no growth was observed was taken as the MIC values (Table 1). The comparison of the MICs (in µg/mL) of potent compounds and standard drugs against tested strains are presented in Table 1.

In-vitro Cytotoxicity Assay Using Calu-6 Cells

Materials. MTT reagent, CO₂ incubator, plate reader, Calu-6 cell line, 96 well plate, and solubilization solution.

Method. *In vitro* cytotoxicity assay was carried out at G7 Synergion Pvt. Ltd., Bangalore-560 092, India. The Calu-6 cells were initially purchased from ATCC at passage no.16 were grown in MEM with 10% FBS. The 70-80% confluent

Table 1. Antimicrobial activity of compounds **7(a-l)**

Compounds	Minimum inhibitory concentration (MIC) in µg/mL					
	Bacterial strains				Fungal strains	
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>S. typhi</i>	<i>E. coli</i>	<i>A. niger</i>	<i>C. albicans</i>
7a	25	200	50	100	250	500
7b	25	50	50	50	125	125
7c	25	50	50	50	62.5	62.5
7d	50	100	50	100	62.5	62.5
7e	12.5	12.5	12.5	25	125	250
7f	50	50	100	50	250	250
7g	200	200	100	100	62.5	125
7h	12.5	12.5	25	12.5	31.25	31.25
7i	50	100	100	100	125	125
7j	50	50	100	50	250	125
7k	25	50	50	25	125	250
7l	25	25	50	100	250	250
Amoxicillin	6.25	6.25	6.25	6.25	–	–
Ketoconazole	–	–	–	–	31.25	31.25

cells were trypsinized, centrifuged and seeded in a 96 well plate (50,000 cells/well). The cells were incubated at 37 °C for overnight. The confluent cells were washed with HBSS buffer & further incubation with 50 µM concentration of the compounds **7a-l** for 24 h at 37 °C. After 24 h incubation, the MTT reagent (15 µL/well) was added and incubated for 4 h at 37 °C. Following this, stop solution was added and incubated for 1 h, mixed using multi channel pipette. Measured the absorbance at 570 nm in Tecan plate reader

Table 2. Cytotoxicity assay for compounds **7(a-l)** on Calu-6 cells

Compounds	Average OD at 570 nm ± SD	Viability %
Control	1.96 ± 0.11	100
Vehicle control (1% DMSO)	1.71 ± 0.16	100
7a	1.78 ± 0.06	104
7b	1.84 ± 0.16	108
7c	1.70 ± 0.25	99
7d	1.92 ± 0.18	113
7e	1.93 ± 0.04	115
7f	1.58 ± 0.09	92
7g	1.82 ± 0.04	107
7h	1.73 ± 0.05	101
7i	1.69 ± 0.12	117
7j	1.41 ± 0.06	102
7k	1.68 ± 0.08	102
7l	1.60 ± 0.18	99

and repeated the entire experiment twice and the ± SD was calculated and reported in Table 2.

Supplementary Information

Supplementary information (Figure S1-S24) is available free of charge at <http://jbc.ssbq.org.br> as a PDF file.

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Synthesis, Antimicrobial and Cytotoxicity Studies of Some Novel Modified Strobilurin Derivatives

Ajjanna M. Sridhara,^{a,b} Kallam R. Venugopala Reddy,^{*,b} Jathi Keshavayya,^c
Dasannana Malige S. Ambika,^e Vadiraj S. Gopinath,^a Prosenjit Bose,^a
Sanath Kumar Goud^a and Sanenahalli K. Peethambar^d

^aAdvinus Therapeutics Pvt. Ltd, 21 & 22, Peenya II phase, Bangalore-560 058, India

^bDepartment of Studies in Industrial Chemistry, ^cDepartment of Studies in Chemistry and
^dDepartment of Biochemistry, Kuvempu University, Jnana Sahyadri, Shankaragatta-577 451,
Karnataka, India

^eDepartment of Plant Pathology, G.K.V.K, UAS, Bangalore-65 India

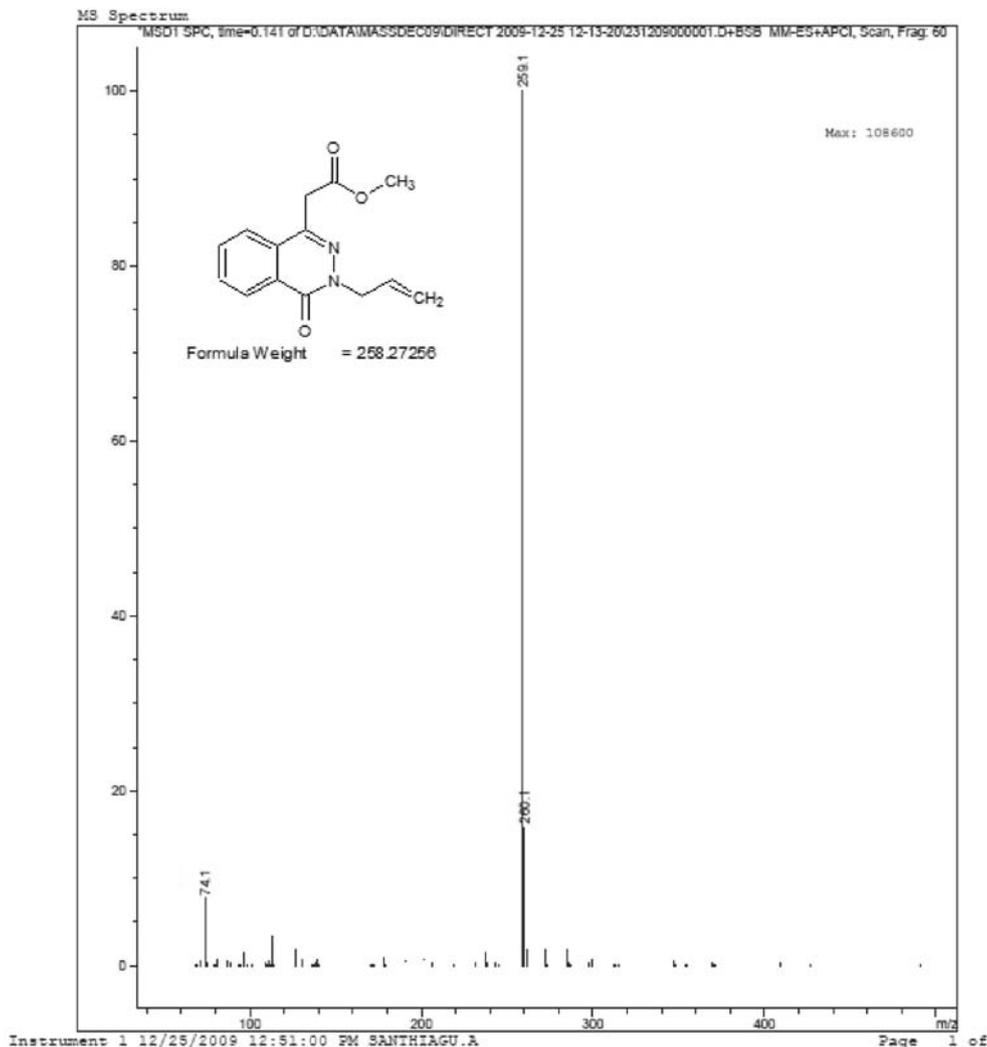


Figure S1. Mass spectrum of methyl 2-(3-allyl-4-oxo-3,4-dihydrophthalazin-1-yl)acetate (5).

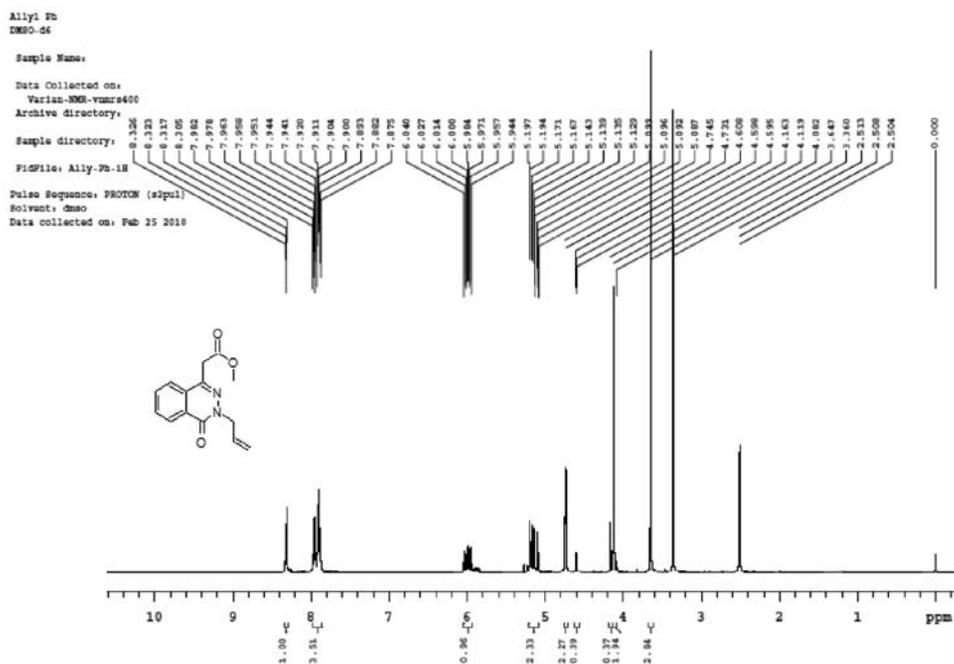


Figure S2. ^1H NMR spectrum of methyl 2-(3-allyl-4-oxo-3,4-dihydrophthalazin-1-yl)acetate (5).

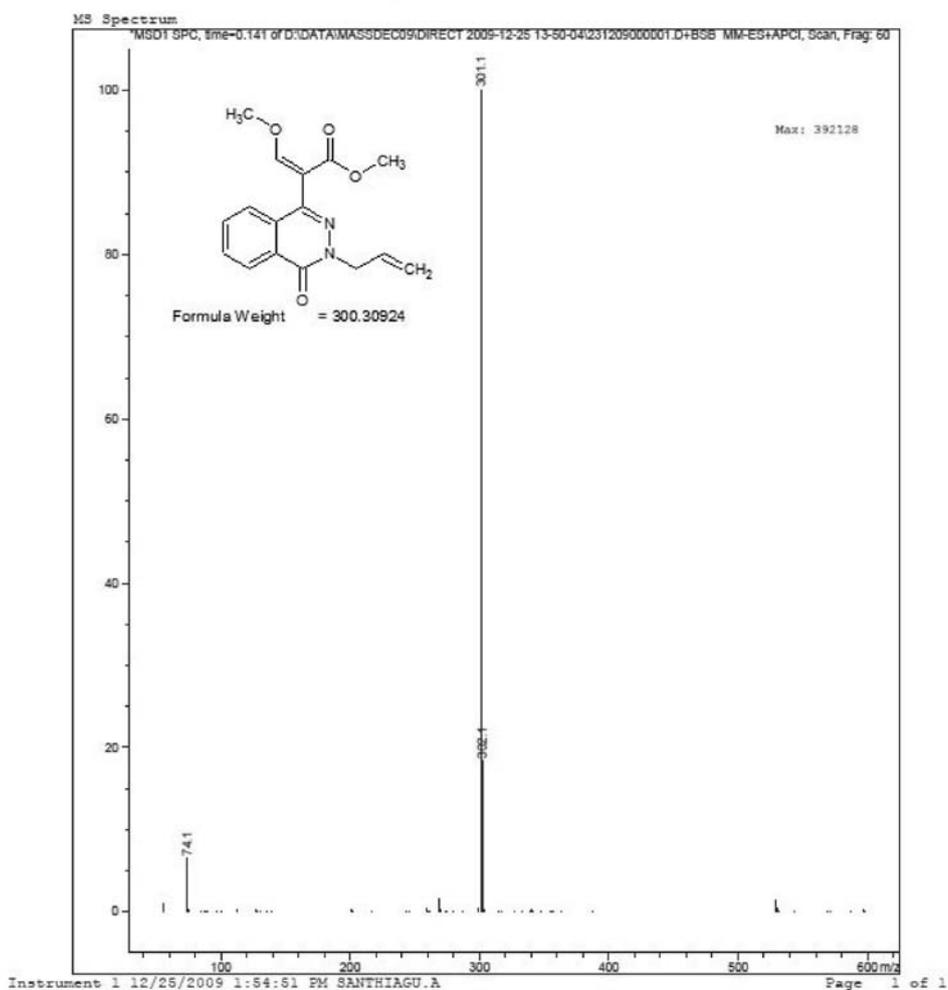


Figure S3. Mass spectrum of methyl 2-(3-allyl-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxy acrylate (6).

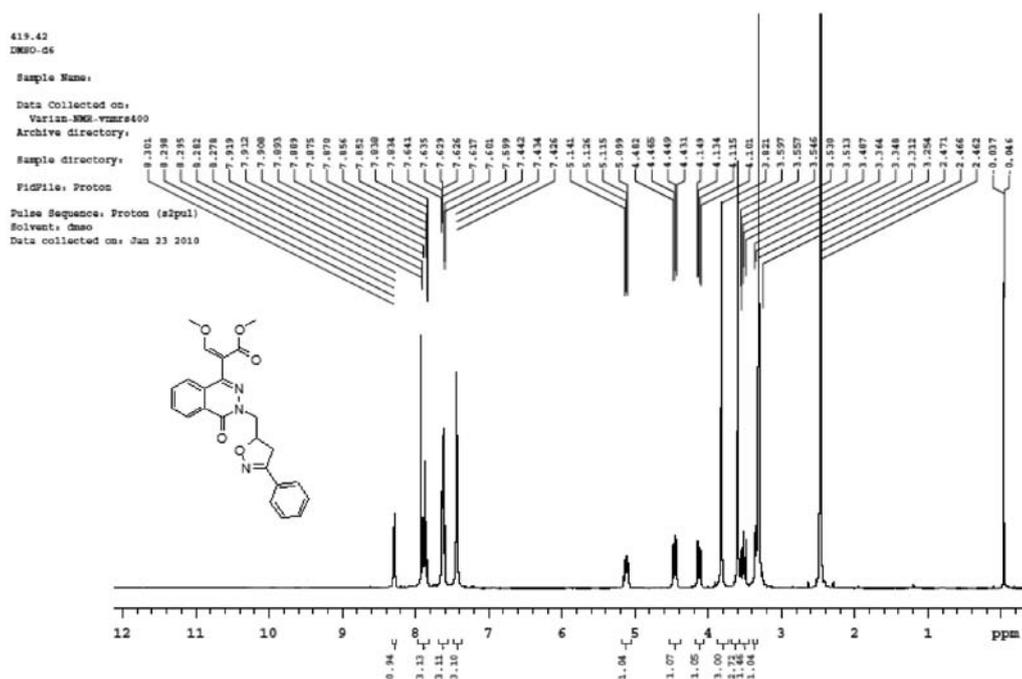


Figure S8. ^1H NMR spectrum of methyl 3-methoxy-2-(4-oxo-3-((3-phenyl-4,5-dihydroisoxazol-5-yl)methyl)-3,4-dihydrophthalazin-1-yl)acrylate (**7b**).

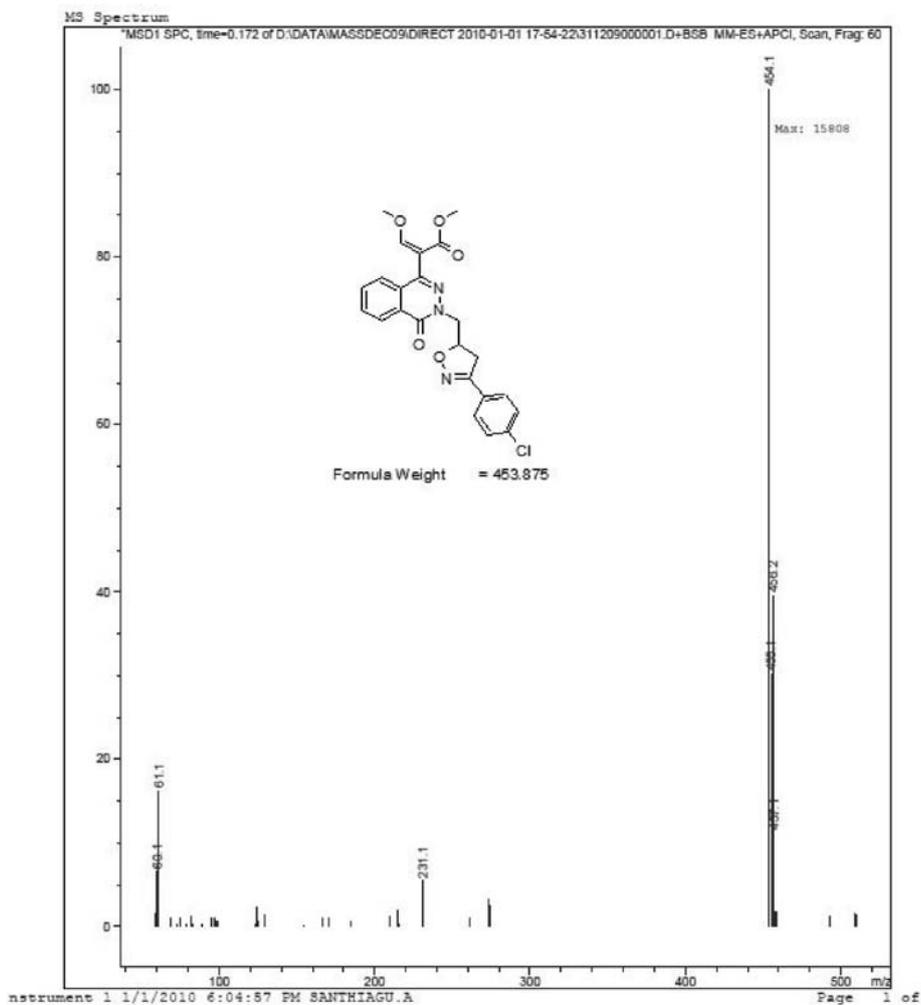


Figure S9. Mass spectrum of methyl 2-(3-((4-chlorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (**7c**).

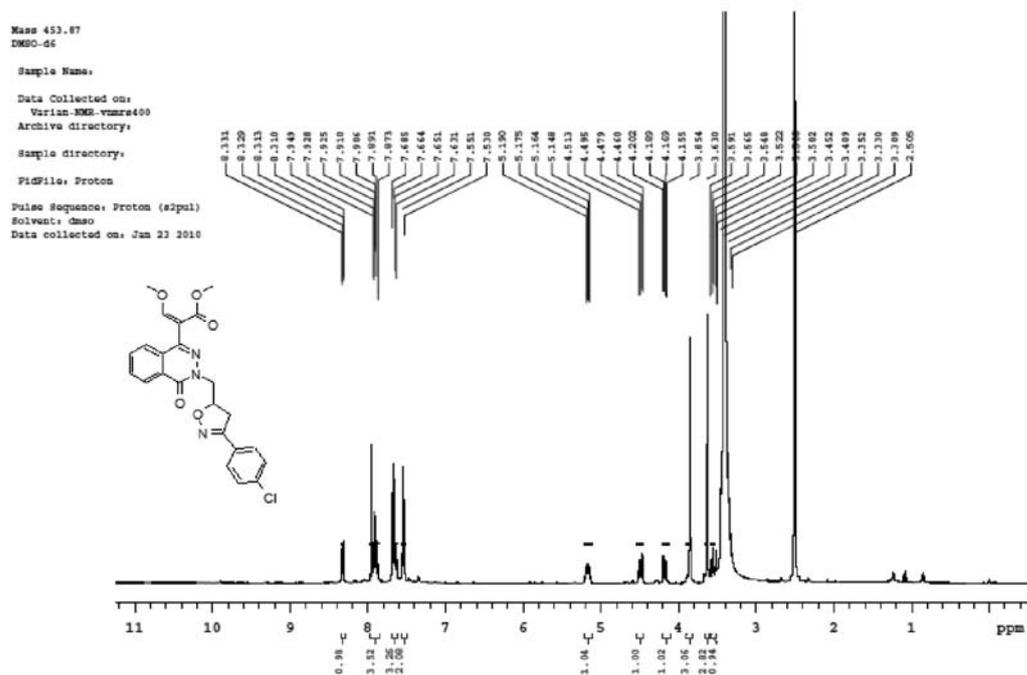


Figure S10. ^1H NMR spectrum of methyl 2-(3-((3-(4-chlorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (**7c**).

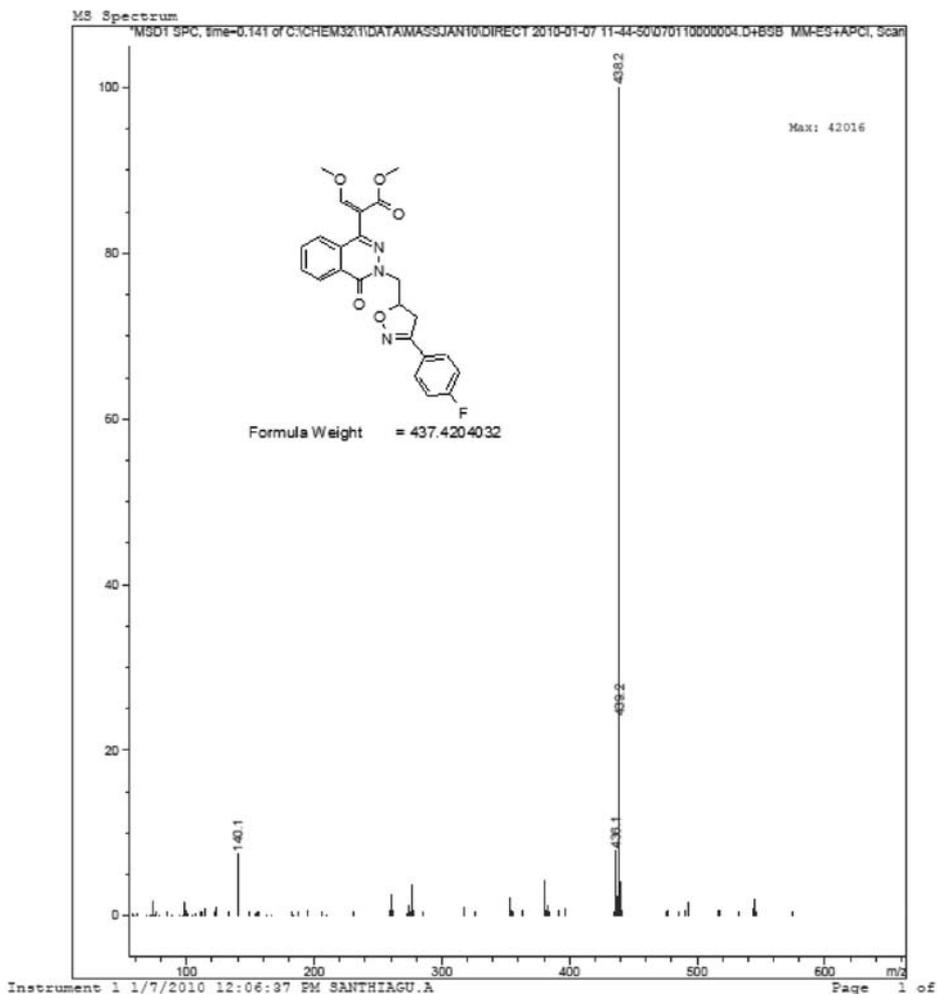


Figure S11. Mass spectrum of methyl 2-(3-((3-(4-fluorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (**7d**).

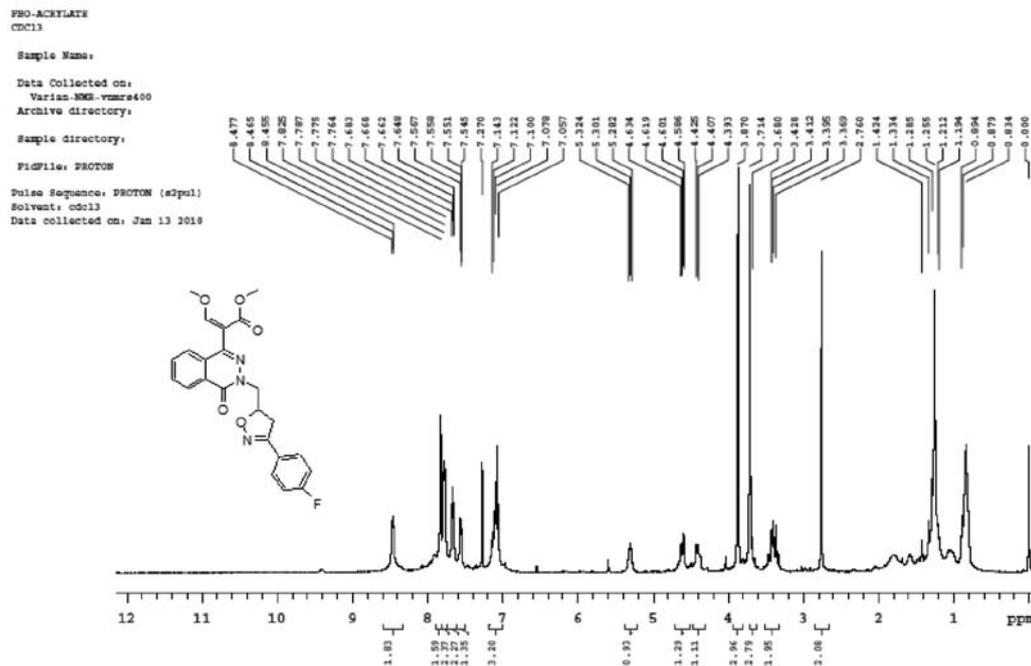


Figure S12. ¹H NMR spectrum of methyl 2-(3-((3-(4-fluorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (7d).

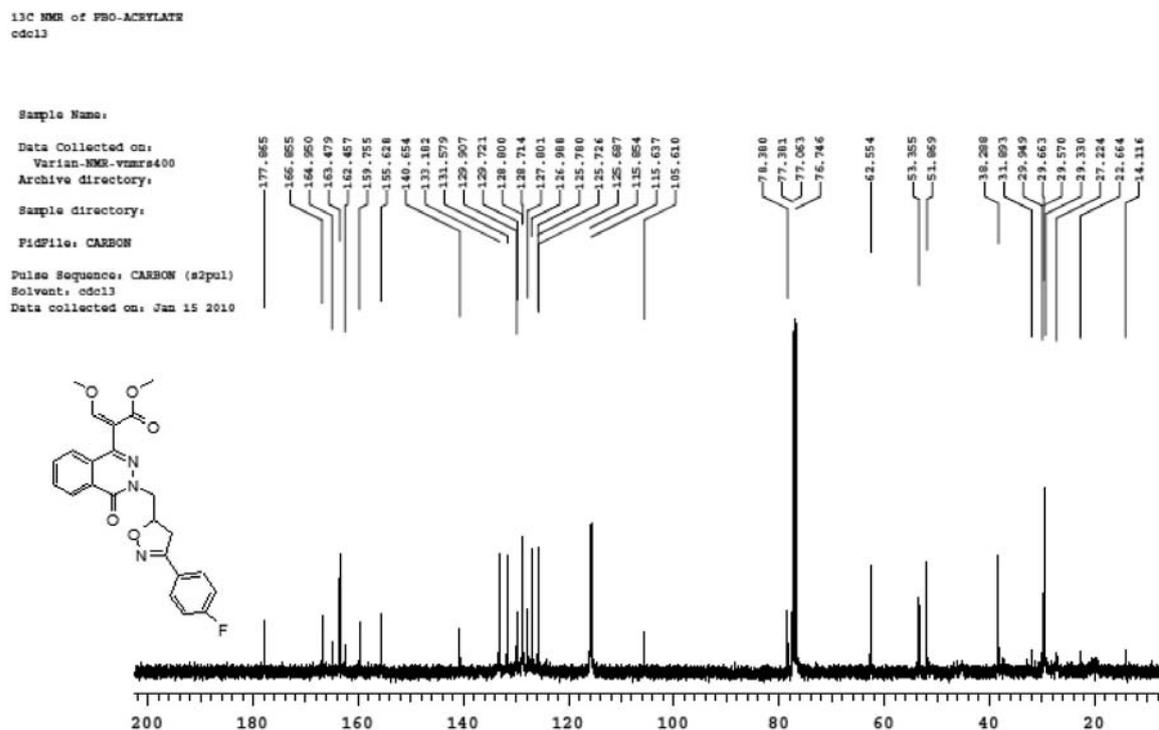


Figure S13. ¹³C NMR spectrum of methyl 2-(3-((3-(4-fluorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (7d).

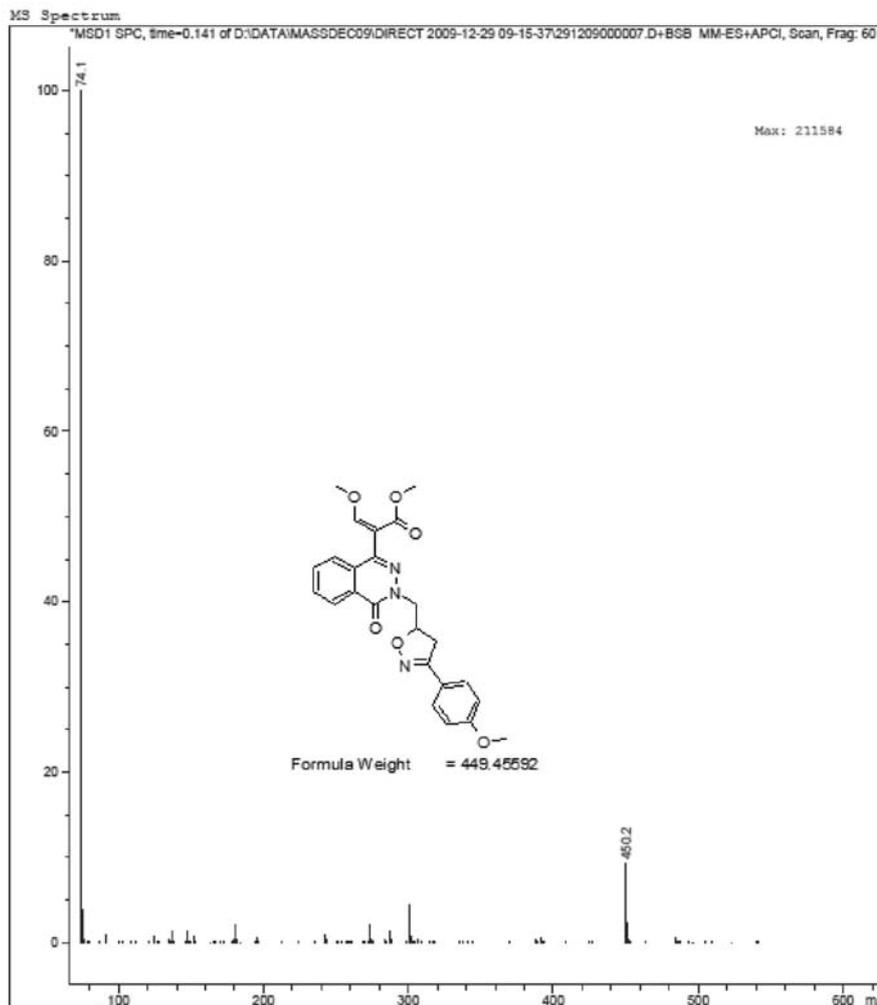


Figure S14. Mass spectrum of methyl 2-(3-((3-(3,4-dimethoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (**7e**).

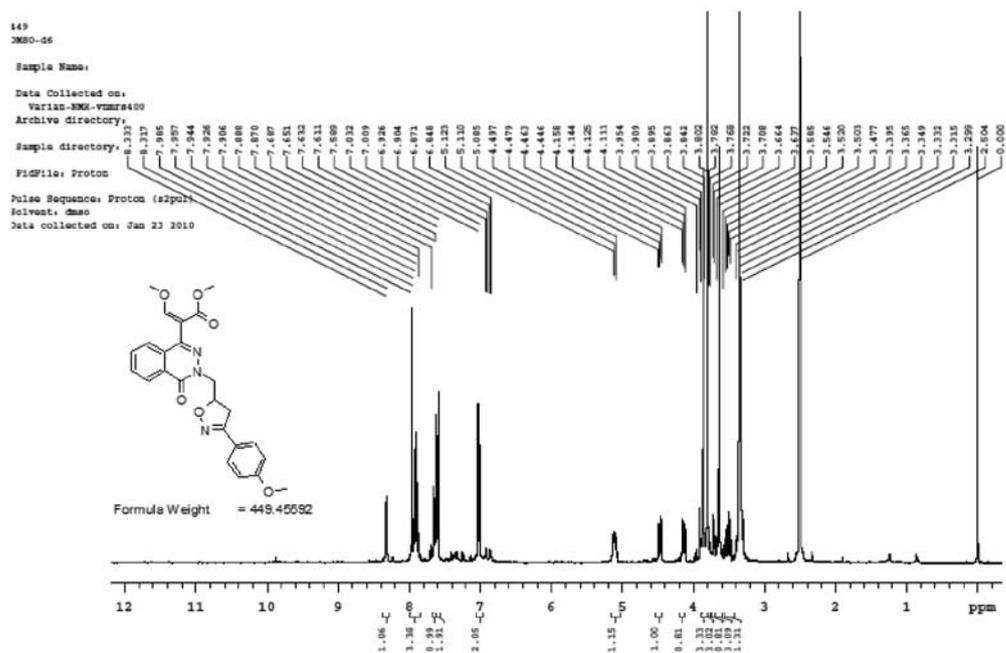


Figure S15. ^1H NMR spectrum of methyl 2-(3-((3-(3,4-dimethoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (**7e**).

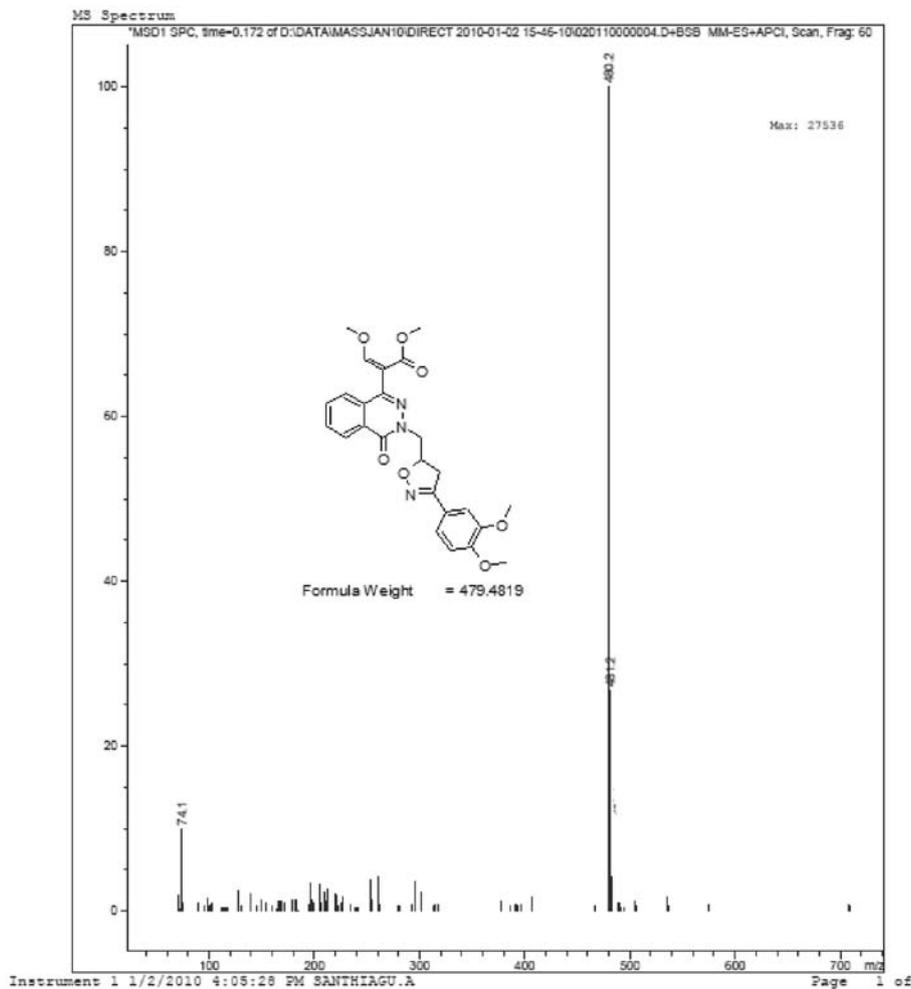


Figure S16. Mass spectrum of methyl 2-(3-((3,4-dimethoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (**7f**).

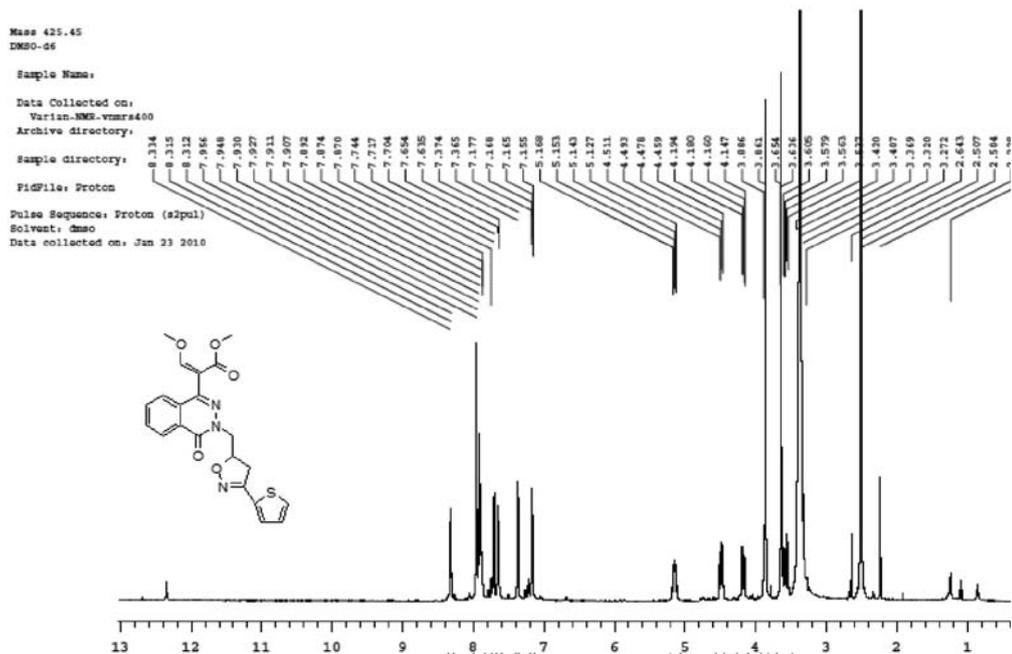


Figure S17. ^1H NMR spectrum of methyl 3-methoxy-2-(4-oxo-3-((3-(thiophen-2-yl)-4,5-dihydroisoxazol-5-yl) methyl)-3,4-dihydrophthalazin-1-yl) acrylate (**7h**).

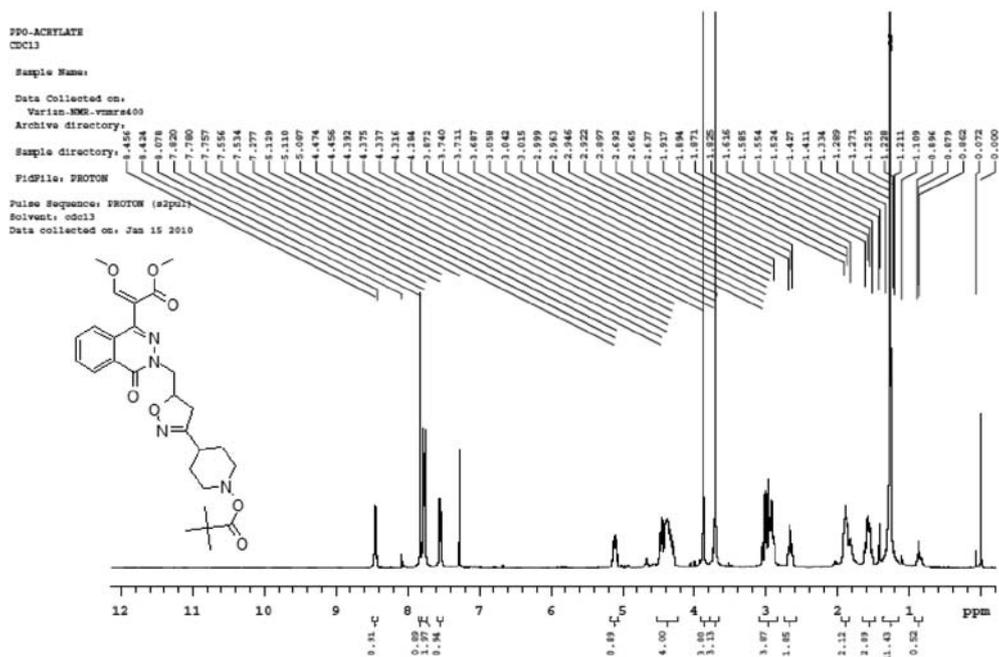


Figure S20. ¹H NMR spectrum of methyl 3-methoxy-2-(4-oxo-3-((3-(1-pivaloylpiperidin-4-yl)-4,5-dihydroisoxazol-5-yl)methyl)-3,4-dihydrophthalazin-1-yl)acrylate (**7j**).

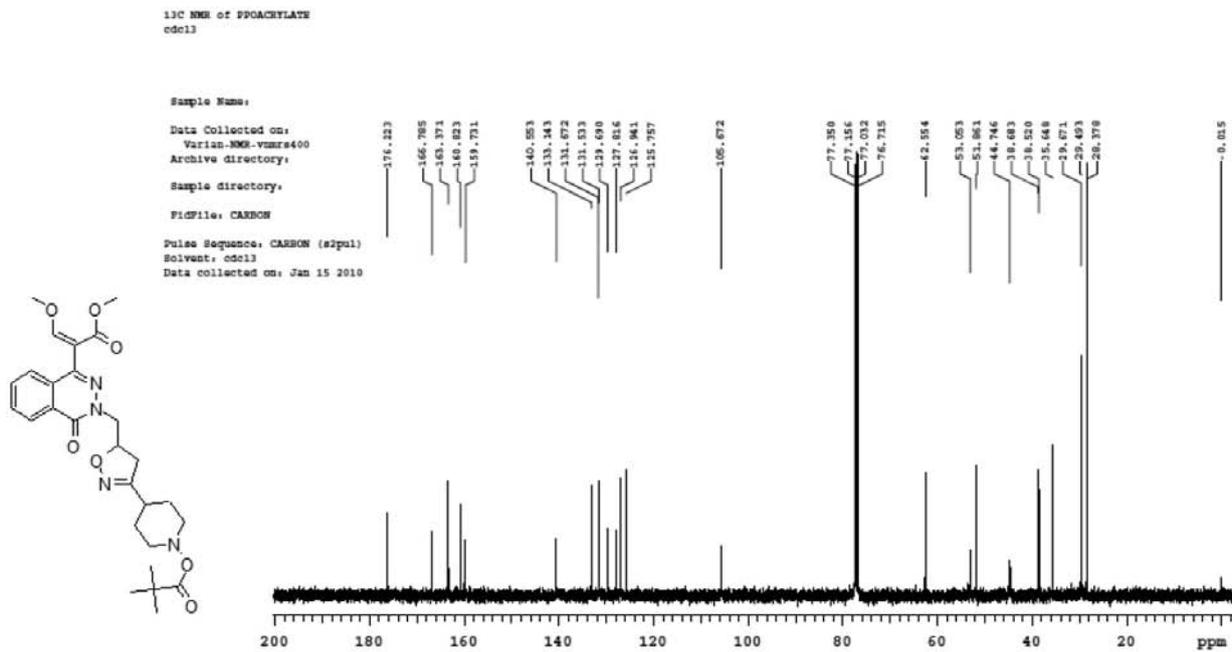


Figure S21. ¹³C NMR spectrum of methyl 3-methoxy-2-(4-oxo-3-((3-(1-pivaloylpiperidin-4-yl)-4,5-dihydroisoxazol-5-yl)methyl)-3,4-dihydrophthalazin-1-yl)acrylate (**7j**).

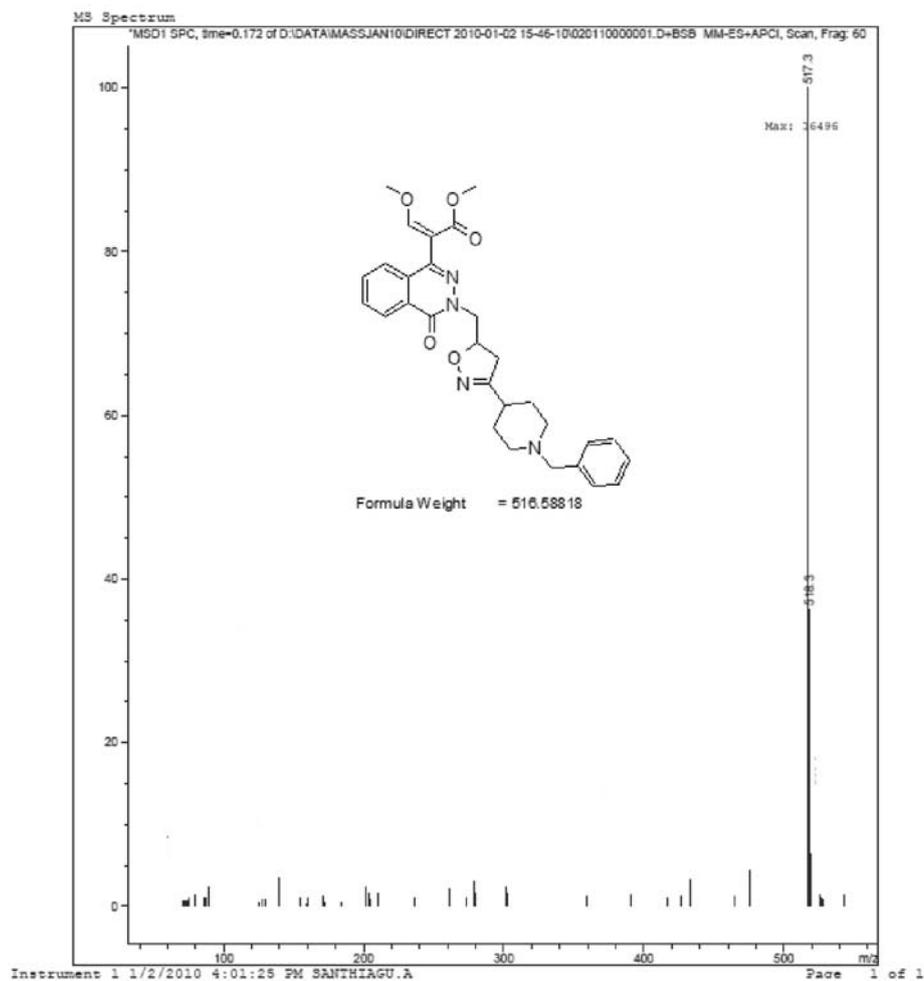


Figure S22. Mass spectrum of methyl 2-(3-((3-(1-benzylpiperidin-4-yl)-4,5-dihydroisoxazol-5-yl)methyl)-4-oxo-3,4-dihydrophthalazin-1-yl)-3-methoxyacrylate (**7k**).

