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THE SYNTHESIS AND ANTIMICROBIAL EVALUATION OF SOME SPIRO-PHTHALIDYL BENZOXAZINONES

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Abstract – Five *N*-substituted spiro-phthalidyl benzoxazinones (**5a-e**) were prepared from anthranilic acid (**1**) and the appropriate alkyl or aryl halides (**2**), followed by condensation of the *N*-substituted anthranilic acids (**3**) with symmetrical phthaloyl dichloride (**4**). These compounds were then evaluated for antimicrobial activity against: *E. coli, S. aureus, B. subtilis, P. aeruginosa, S. cerevisiae,* and *A. nidulans*. Compound **5a** was active against all the microbes tested, especially the *A. nidulans*. Compound **5c** was active against all the bacteria, except the fungus *A. nidulans*. The *N*-benzyl (**5b**) and *N*-phenyl (**5d**) derivatives were not active at all. The *N*-4-fluorophenyl analog **5e** showed activity against *S. aureus* and *B. subtilis*.

We became interested in *N*-alkyl and *N*-aryl-substituted spiro-phthalidyl benzoxazinones (5) as potential antimicrobial agents, in view of their similar structure to Griseofulvin (**6**), Figure 1.

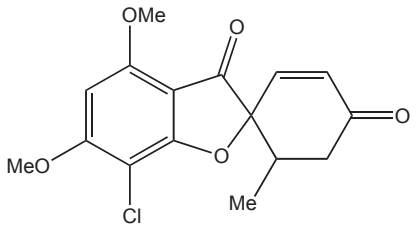

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Figure 1. Griseofulvin

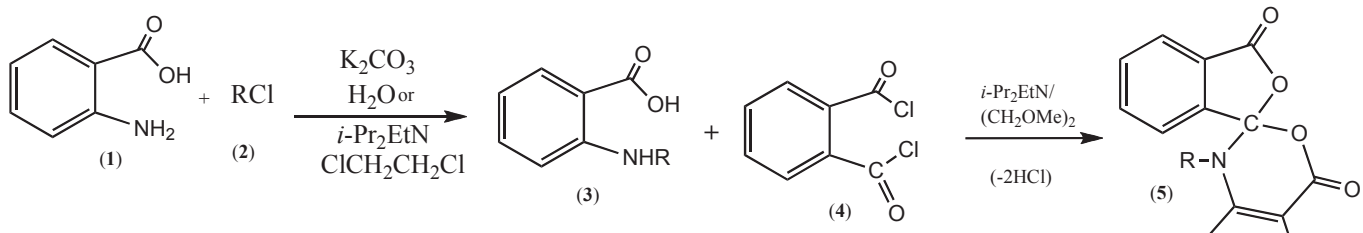
Griseofulvin a well-known antifungal agent, was first isolated from the fungus *Penicillium griseofulvum* in 1939.[1] It inhibits fungal mitosis by disrupting the mitotic spindle through interaction with polymerizedmicrotubules.[2] Griseofulvin is mainly effective against a specific group of fungi known as

dermatophytes,[3] which cause skin disease in humans and animals.

We chose to determine antimicrobial activity of our benzoxazinones, **5a-e** by the method developed by Bauer and Kirby.[4] The principle of this method is to measure the zone of inhibition of microbial growth around the antimicrobial agent. The diameter (in mm) of the zone of inhibition around the disk is an indication of the susceptibility or resistance of a microorganism to the microbial agent.

1. Synthesis

Five *N*-substituted spiro-phthalidyl benzoxazinediones (**5a-e**) were prepared from anthranilic acid (**1**) and the appropriate alkyl or aryl halide (**2**), followed by condensation of the *N*-substituted anthranilic acids (**3**) with symmetrical phthaloyl dichloride (**4**) (Scheme 1).



Compound	R
5a	Me
5b	$C_6H_5CH_2$-
5c	$MeOCH_2$
5d	C_6H_5-
5e	*p*-FC_6H_4-

Scheme 1. Synthesis of five spiro-phthalidyl benzoxazinediones (**5a-e**)

2. Antimicrobial Testing

Mueller Hinton Agar (MHA) plates were prepared according to Difco™. The powder (38 grams) was suspended in 1 liter of purified water and mixed thoroughly, then heated with frequent agitation and boiled for one minute to obtain complete solution. It was autoclaved at 121 °C for 15 minutes, then approximately 25 mL portions of MHA were poured into sterile Petri dishes.

Each test compound was dissolved in DMSO, at four different concentrations (100, 50, 25, and 12.5 mg/mL) and 50 μL was applied to 5 ½ inch sterile paper discs (diameter 12.5 mm). A control disc containing only DMSO was also prepared. The disks were uniformly placed in a circle on the Mueller Hinton Agar plates seeded with the microorganisms. Plates were prepared in triplicate. The plates were then incubated at 37 °C for 24 hours to allow the microbial species to grow. Antibiotic effects of the test compounds were quantified by measuring (millimeters) the average of the three zones of growth-inhibition. *N*-Phenyl and *N*-4-fluorophenyl-spiro-[1,2-dihydro-4*H*-3,1-benzoxazin-2,1'-phthalan]-4,3'-

diones were not readily soluble in DMSO. These concentrations were made by suspending the 200 mg of the compound in 2 mL of DMSO and then treated in an ultrasonic homogenizer to ensure a uniform suspension of the compound. Serial dilutions were then prepared at concentrations of 50, 25, and 12.5 mg/mL, and pipetted onto the discs. The diameter of the zone of inhibition of each compound is reported as a measure of growth inhibition.

The results of the antimicrobial screening we performed show (Table 1) that these compounds have potential antimicrobial activity. The *N*-methyl derivative **5a** showed significant activity against all the microbes tested, especially the *A. nidulans*. The *N*-methoxymethyl analog **5c** showed activity against all the bacteria, as well as strong activity against the yeast, but was inactive against the fungus *A. nidulans*. The *N*-4-fluorophenyl derivative **5e** showed activity against *B. subtilis*.

Intended future work to modify these structures by introducing polar groups on the rings, can potentially increase water solubility, and perhaps enhance the antimicrobial activity.

Table 1. Antimicrobial test results of phthalidyl spiro compounds of growth inhibition

Compound	R	Conc. (mg/mL)	Zone of Inhibition (mm)*					
			E. coli	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*S. cerevisiae*	*A. nidulans*
5a	Me-	25	0	0	0	0	0	0
		50	14.3	14.5	16.0	14.2	17.7	19.0
		100	15.9	16.5	19.0	16.6	22.2	24.2
5b	$C_6H_5CH_2$-	100	0	0	0	0	0	0
5c	$MeOCH_2$-	12.5	0	0	0	0	0	0
		25	0	1.8*	0	0	-	0
		50	0	17.5	12.8	0	23.3	0
		100	16.5	22.7	23.3	16.5	29.2	0
5d	C_6H_5-	100	0	0	0	0	0	0
5e	4-F-C_6H_4-	12.5	0	0	0	0	0	0
		25	0	0	14.7	0	0	0
		50	0	1.7*	16.0	0	0	0
		100	0	15.4	18.5	0	0	0

EXPERIMENTAL

Melting points are uncorrected and were measured on either a Thomas-Hoover® or Mel-Temp® capillary melting point apparatus. Thin layer chromatography (TLC) was preformed with Analtech® silica gel glass backed plates (250 microns) and R_f values were recorded. Infrared spectra (IR) were recorded using

a Perkin Elmer® Fourier Transform (FTIR) Spectrum 1000 Spectrophotometer. NMR Spectra (^{1}H, ^{13}C, and ^{13}C APT) were recorded on a 400 MHz Bruker Spectrometer. NMR spectra are presented using TMS as the internal standard and chemical shifts are given in ppm as δ values. Atlantic Microlab, Inc (Norcross, Georgia) performed elemental analyses. Gas chromatography-mass spectrometry (GC-MS) studies were performed on either a Hewlett Packard® G1800A GCD System or a Shimadzu model QP5050A.

Anthranilic acid, *N*-methylanthranilic acid, and symmetrical phthaloyl dichloride were purchased from Alfa Aesar (Ward Hill, MA). 4-Fluorophenylanthranilic acid, benzyl bromide, chloromethyl methyl ether, *N,N*-diisopropylethylamine, and triethylamine were purchased from Aldrich (Milwaukee, WI). *N*-Phenylanthranilic acid was purchased from Fluka (Milwaukee, WI).

The microorganisms, obtained from Ward's (Rochester, New York), were *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli* MG1655*, Saccharomyces cerevisiae* W303*,* and *Aspergillus nidulans*. Mueller Hinton Agar and Yeast Peptone Dextrose Agar were purchased from Difco. Media used was Tryptic Soy Broth for bacteria, YPD liquid media for yeast, and Yeast Glucose media with Ampicillin for fungus. Antibiotic discs were purchased from VWR (Bristol, CT).

***N*-Benzylanthranilic acid (3b).** Anthranilic acid, **1**, (6.86 g, 60 mmol) and benzyl bromide (10.2 g 60 mmol) were added to an aqueous solution of K_2CO_3 (3.46 g) (62 mL water) with stirring. The reaction mixture was refluxed for 2.5 h, then allowed to cool to rt, and 50 mL of EtOAc was added. The organic layer was washed with water and dried over $MgSO_4$. The EtOAc was removed under reduced pressure to obtain the crude product, yield 10.4 g, 76%. This was recrystallized from heptane/EtOAc (30/20 mL) to give 9.8 g (72%), mp 171-172 ^{o}C (lit.[6] mp 174-176 ^{o}C); TLC (90:10 hexane/*i*-PrOH alcohol): R_f = 0.46; IR (CCl_4 cm^{-1}): 3540 (OH), 3380 (N-H), 3066 and 3030 (aromatic C-H), 2891 (aliphatic C-H), and 1662 with a shoulder at 1698 cm^{-1} (aromatic α-electronegative-substituted COOH); MS: m/z 227 (M^+, $C_{14}H_{13}NO_2$); 209 (M – H_2O); 208 ($C_{14}H_{10}NO$); 180 ($C_{13}H_{10}N$); 152 ($C_{12}H_8$); 132 ($C_8H_4O_2$); 106 (C_7H_8N); 91 base peak ($C_7H_7^+$), 77 ($C_6H_5^+$), 65 ($C_5H_5^+$); 51 ($C_4H_3^+$).

***N*-Methoxymethylanthranilic acid (3c).** Diisopropylethylamine (25.0 g, 194 mmol) was slowly added to a mixture of anthranilic acid, **1,** (10.97 g, 80 mmol) and 1,2-dichloroethane (125 mL) with stirring. Chloromethyl methyl ether (7.08 g, 88 mmol) was added to the reaction mixture and stirred for 30 min. at rt, then washed with water and the organic layer was dried over $MgSO_4$. After filtration the 1,2-dichloroethane was removed under reduced pressure yielding 24.5 g of solid crude product which was crystallized upon treatment with aqueous MeOH; Recrystallization from MeOH gave 1.6 g (11%) of pure *N*-methoxymethylanthranilic acid, mp 89-91 ^{o}C; TLC (90/10 hexane/*i*-PrOH): R_f = 0.52; IR (CCl_4, cm^{-1}): 3365 (N-H) and 1690 (aromatic carboxylic acid); ^{1}H-NMR ($CDCl_3$): δ = a 3.51 (s, 3H), b 4.81 (t, 1H), c 5.42 (s, 2H), d 6.67 (t, 1H), e 6.86 (d, 1H), f 7.40 (t, 1H), g 7.97 (d, 1H), and h 8.27 (bs, 1H) ppm. ^{13}C-

NMR ($CDCl_3$): δ = C_1: 57.73, C_2: 90.48, C_3: 110.52, C_4:111.85, C_5: 115.98, C_6: 132.01, C_7: 135.24, C_8: 150.26, C_9: 168.04 ppm; MS: 181 (M^+, $C_9H_{11}NO_3$); 150 ($M - OCH_3$)$^+$; 132 base peak (C_8H_6NO)$^+$, 120 ($C_7H_4O_2$)$^+$; 105 (C_7H_5O)$^+$; 92 (C_6H_6N)$^+$; and 65 (C_5H_5)$^+$. Anal. Calcd for $C_9H_{11}NO_3$: C 59.67; H 10.26; N 12.97. Found: C59.99; H 10.25; N 12.917.

***N*-Methyl-spiro-[1,2-dihydro-4*H*-3,1-benzoxazin-2,1'-phthalan]-4,3'-dione (5a)**. The method used by Butula and Otting[5] was modified as follows. Symmetrical phthaloyl dichloride (2.03 g, 10 mmol) was dissolved in 105 mL of 1,2-dimethoxyethane. The solution was stirred at rt. Triethylamine (2.02 g, 20 mmol) was added slowly as the flask was immersed in an ice-bath. A solution of *N*-methylanthranilic acid, 1.51 g, 10 mmol, in 40 mL of dimethoxyethane was added dropwise while stirring was continued at rt. The precipitate ($Et_3NH^+Cl^-$) was filtered, and the solvent was removed under reduced pressure. The solid residue was recrystallized from aqueous MeOH to yield 2.2 g (78%) of colorless crystals, mp 212-214 °C (lit.[5] mp 210-212 °C); TLC (90/10 hexane/*i*-ProH); R_f = 0.30; IR, cm^{-1} (NaCl plate): 1770 (γ-lactone); 1759 (δ-lactone); ^{1}H-NMR ($CDCl_3$): δ = 2.77 (s, 3H); 6.97 (d, 1H); 7.12; 7.74 (d, 1H); 7.76 (t, 1H); 7.86 (t, 1H); 7.95 (d, 1H); 8.08 (d, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ = C_1: 33.04, C_2: 112.24, C_3: 113.90, C_4: 114.80, C_5: 121.63, C_6: 123.80, C_7: 126.36, C_8: 127.74, C_9: 130.96, C_{10}: 132.87, C_{11}: 135.83, C_{12}: 137.06, C_{13}: 142.89; C_{14}: 146.54; C_{15}: 160.02; C_{16}: 166.31 ppm; MS: m/z 281 (M^+, $C_{16}H_{11}NO_4$); 236 (M-45, M-CH_3NO, $C_{15}H_8O_3$); 152 ($C_{12}H_8$); 133 (C_8H_7NO); 132 (C_8H_6NO); 105 base peak, (C_7H_7N); 104 ($C_7H_7O_{12}$); 77 (C_6H_5)$^+$and 50 (C_4H_2); 76 (C_6H_4).

***N*-Benzyl-spiro-[1,2-dihydro-4*H*-3,1-benzoxazin-2,1'-phthalan]-4,3'-dione (5b).** Symmetrical phthaloyl chloride (1.48 g, 7.27 mmol) was stirred at rt in 20 mL $CHCl_3$. Diisopropylethylamine (2.07 g, 16.0 mmol) was added slowly as the flask was cooled in an ice-bath. A suspension of *N*-benzylanthranilic acid (1.5 g, 6.61 mmol) in 30 mL $CHCl_3$ was then added dropwise while stirring at rt. The $CHCl_3$-layer was washed with water and dried over $MgSO_4$. The solvent was removed under reduced pressure. The solid residue crystallized upon treatment with MeOH. It was recrystallized from aqueous MeOH to give 2.1 g (89%) of pure product, mp 180-183 °C; TLC (90/10 hexane/*i*-ProH): R_f = 0.51; IR (CCl_4): 1797 cm^{-1} (γ-lactone); 1766 cm^{-1} (δ-lactone); ^{1}H-NMR ($CDCl_3$): δ = a: 4.31 (d, 1H), b: 4.44 (d, 1H), c: 6.75 (d, 1H), d: 7.08 (t, 1H), e: 7.21-7.33 (m, 5H), f 7.47 (d, 1H), g: 7.65-7.71 (m, 3H), h: 7.91 (d, 1H), i: 8.10 (d, 1H) ppm. ^{13}C-NMR ($CDCl_3$): δ = C_1: 50.26, C_2: 112.28, C_3: 114.63, C_4: 114.87, C_5: 121.52, C_6: 123.35, C_7: 125.89, C_8: 126.18, C_9: 127.71, C_{10}: 127.91, C_{11}: 129.22, C_{12}: 130.89, C_{13}: 132.74; C_{14}: 135.49; C_{15}: 135.55; C_{16}: 136.67; C_{17}: 142.31; C_{18}: 145.66; C_{19}: 159.92; and C_{20}: 165.95 ppm; MS: 357 (M^+, $C_{22}H_{15}NO_4$); 209 ($C_{14}H_{11}NO$); 180 base peak ($C_{13}H_{10}N$); 152 ($C_{12}H_8$); 105 (C_7H_5O); 91 ($C_7H_7^+$); and 77 ($C_6H_5^+$); Anal. Calcd for $C_{22}H_{15}NO_4$: C 73.94; H 4.23; N 3.92. Found: C 73.99; H 4.25; N 3.97.

***N*-Methoxymethyl-spiro-[1,2-dihydro-4*H*-3,1-benzoxazin-2,1'-phthalan]-4,3'-dione (5c).** A mixture of symmetrical phthaloyl dichloride (6.91 g, 34 mmol) and 1,2-dimethoxyethane (75 mL) was stirred at rt. *N,N*-Diisopropylethylamine (9.68 g, 75 mmol) was added. *N*-Methoxymethyl anthranilic acid (**3c**, 5.6 g, 31 mmol) in of 1,2-dimethoxyethane, 25 mL was added dropwise to the flask while stirring at rt. Stirring was continued for 48 h after the addition was completed. The white precipitate, consisting of *N,N*-diisopropylethylamine hydrochloride, was collected. The filtrate was evaporated to dryness to give an oil, 8.29 g, 86%; TLC (90/10 hexane/*i*-PrOH): R_f = 0.33; IR (cm^{-1} NaCl plate): 1811 (γ-lactone); 1725 (δ-lactone); ^{1}H-NMR ($CDCl_3$): δ = a: 3.43 (s, 3H); b: 5.35 (s, 2H); c: 7.09 (d, 1H); d: 7.26 (t, 1H); e: 7.55 (t, 1H); f: 7.78 (t, 1H); g: 7.86 (t, 1H); h: 7.96 (d, 1H); i: 8.06 (d, 1H); j: 8.13 (d, 1H); ^{13}C-NMR ($CDCl_3$): δ = C_1: 57.77, C_2: 91.06, C_3: 121.34, C_4: 122.62, C_5: 124.10, C_6: 124.83, C_7: 125.59, C_8: 128.48, C_9: 130.28, C_{10}: 131.28, C_{11}: 133.39, C_{12}: 133.69, C_{13}: 135.67; C_{14}: 146.56; C_{15}: 148.66; C_{16}: 164.43; and C_{17}: 165.25 ppm; MS: m/z 311 (M^+ $C_{17}H_{13}NO_5$); 280 $(M\text{-}OCH_3)^+$; 250 (M-61, unassigned); 223 ($M\text{-}2CO_2$, $C_{15}H_{13}NO$); 179 ($C_9H_9NO_3$); 163 ($C_9H_9NO_2$); 148 (base peak, $C_8H_4O_3$); 120 (C_7H_4O); 104 (C_7H_4O); 76 $(C_6H_4)^+$; 45 $(CH_3OCH_2)^+$. Anal. Calcd for $C_{17}H_{13}NO_5$: C 65.65; H 4.21; N 4.52. Found: C 65.49; H 4.29; N 4.58.

***N*-Phenyl-spiro-[1,2-dihydro-4*H*-3,1-benzoxazin-2,1'-phthalan]-4,3'-dione (5d).** A mixture of symmetrical phthaloyl dichloride (5.24 g, 26 mmol), $CHCl_3$ (75 mL), and *N,N*-diisopropylethylamine (7.33 g, 57 mmol) was stirred at rt. *N*-Phenylanthranilic acid (5.0 g, 23 mmol) was suspended in $CHCl_3$ (75 mL) and added dropwise to the flask. After 15 h of stirring at rt, the mixture was washed with water and the organic layer was dried over $MgSO_4$. The $CHCl_3$ was removed on a rotary evaporator, giving 6.1 g of crude product. This was triturated with EtOAc and the resulting crystals were collected on a Büchner funnel, and recrystallized from aqueous MeOH to give pure **5d**, 4.8 g (61%), mp 241-242 °C; TLC (90/10 EtOAc/MeOH): R_f = 0.65; IR (cm^{-1} NaCl plate): 1769 (γ-lactone); 1758 (δ-lactone); ^{1}H-NMR (DMSO): δ = a: 6.65-6.80 (m, 1H); b: 6.95-7.10 (m, 2H); c: 7.12-7.30 (m,6H); d: 7.31-7.40 (m, 3H); e: 7.80-7.96 (dd, 1H) ppm; ^{13}C-NMR (DMSO-d_6): δ = C_1: 125.71, C_2: 126.39, C_3: 126.46, C_4: 127.03, C_5: 127.13, C_6: 127.61, C_7: 127.83, C_8: 128.07, C_9: 128.21, C_{10}: 128.94, C_{11}: 129.88, C_{12}: 130.37, C_{13}: 130.83; C_{14}: 131.18; C_{15}: 132.21; C_{16}: 132.93; C_{17}: 136.48; C_{18}: 142.03; C_{19}: 143.65; C_{20}: 167.23; and C_{21}: 168.92 ppm; MS: m/z 343 (M^+ $C_{21}H_{13}NO_4$); 298 (unassigned); 195 (base peak, $C_{13}H_9NO$); 167 ($C_{12}H_9N$); 152 ($C_{12}H_8$); 119 ($C_6H_5N{=}C{=}O$) and 77 ($C_6H_5^+$). Anal. Calcd for $C_{21}H_{13}NO_4$: C 73.53; H 3.81; N 4.10. Found: C 73.49; H 3.92; N 4.08.

***N*-4-Fluorophenyl-spiro-[1,2-dihydro-4*H*-3,1-benzoxazin-2,1'-phthalan]-4,3'-dione (5e).** A mixture of symmetrical phthaloyl dichloride (4.83 g, 24 mmol), $CHCl_3$ (75 mL), and *N,N*-diisopropylethylamine

(6.76 g, 52 mmol) was stirred at rt. *N*-4-Fluorophenylanthranilic acid (5.0 g, 22 mmol) was suspended in $CHCl_3$ (75 mL) and added dropwise to the flask. After 15 h of stirring at rt, the reaction mixture was extracted with water and the organic layer was dried over $MgSO_4$. The filtrate was evaporated to dryness on a rotary evaporator, giving 5.9 g of a solid residue. This was triturated with EtOAc until it turned crystalline, and collected on a Büchner funnel. The product was recrystallized from aqueous MeOH affording 4.4 g (55%) of pure **5e**, mp 205-207 ^oC; TLC (90/10 EtOAc/MeOH): IR (NaCl plate): 1769 cm^{-1} (γ-lactone); 1758 cm^{-1} (δ-lactone); ^{1}H-NMR (DMSO-d_6): δ = a: 6.99 (m, 1H); b: 7.07 (t, 2H); c: 7.10 (d, 1H); d: 7.16 (t, 1H); e: 7.18 (t, 1H); f: 7.44 (d, 1H); g: 7.47 (t, 2H); h: 7.54 (t, 2H); i: 7.95 (d, 1H) ppm; ^{13}C-NMR (DMSO-d_6): δ = C_1: 116.27, C_2: 116.49, C_3: 123.66, C_4: 127.72, C_5: 127.99, C_6: 128.81, C_7: 129.45, C_8: 129.66, C_9: 130.27, C_{10}: 130.98, C_{11}: 131.51, C_{12}: 132.12, C_{13}: 132.83; C_{14}: 133.55; C_{15}: 136.71; C_{16}: 140.46; C_{17}: 142.24; C_{18}: 159.50; C_{19}: 161.92; C_{20}: 167.71; and C_{21}: 169.36 ppm; MS: m/z 361 (M^+ $C_{21}H_{12}NO_4F$); 316 (M-45); 213 (base peak, $C_{13}H_8NOF$); 185 ($C_{12}H_8NF$); 119 (C_6H_5NCO); 92 (C_6H_6N); 76 (C_6H_4); and 50 (C_4H_2). Anal. Calcd for $C_{21}H_{12}NO_4F$: C .69.86; H 3.82; N 4.45. Found: C 69.59; H 3.69; N 4.58.

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