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Disparate SAR Data of Griseofulvin Analogues for the Dermatophytes *Trichophyton mentagrophytes*, *T. rubrum*, and MDA-MB-231 Cancer Cells

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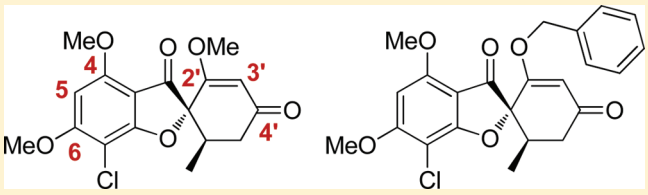
Supporting Information

ABSTRACT: Griseofulvin and 53 analogues of this compound have been tested against the pathogenic dermatophytes *Trichophyton rubrum* and *Trichophyton mentagrophytes* as well as against the breast cancer cell line MDA-MB-231. The modifications to griseofulvin include the 4, 5, 6, 2′, 3′, and 4′ positions. The SAR of the griseofulvin analogues toward the two fungi followed the same trend with the majority being less active than griseofulvin and none had more than twice the potency of the parent compound. A comparison of the antifungal and the anticancer SAR revealed distinct differences, as the majority of analogues showed increased activity against the cancer cell line MDA-MB-231, highlighted by 2′-benzyloxy-2′-demethoxy-griseofulvin, which showed low activity against both fungi but was among the most potent compounds against MDA-MB-231 cancer cells. Tubulin has been proposed as the target of griseofulvin in both fungal and mammalian cells, but the differences revealed by this SAR study strongly suggest that the mode-of-action of the compound class toward fungi and mammalian cancer cells is different.



INTRODUCTION

Griseofulvin (**1**, see Figure 1) was one of the first antifungal natural products isolated[1] from filamentous fungi and has been known as an antifungal agent for decades.[2,3] The compound was, until the approval of Terbinafine[4] by the US Food and Drug Administration[5] in 2007, the only drug available for treatment of tinea capitis,[6] a superficial fungal skin infection caused by dermatophytes, which predominantly affects children.[7] Upon administration to man, griseofulvin accumulates in the skin (stratum corneum), where it presumably binds to keratin.[8] The mode of action is still not determined, but tubulin binding has been proposed.[9,10]

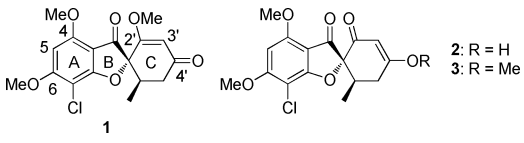


Figure 1. The structure of griseofulvin (**1**), griseofulvic acid (**2**), and isogriseofulvin (**3**). The rings A, B, and C as well as the positions modified in this study in **1** are shown.

More than 400 griseofulvin analogues have been reported since its discovery, and the activity of over 300 of these have been compared with griseofulvin (**1**) against six dermatophytes in a study by Crosse et al.[11] The curling of hyphae in *Botrytis allii* were also tested, but this phenotype did not correlate with the growth inhibitory effect of the analogues.[11] Griseofulvin analogues with modifications at positions 4, 6, 2′, and 3′ as well as isogriseofulvin analogues with modifications at the 4, 6, 3′, and 4′ positions were tested. No 2′ analogues excelled in growth inhibition of the dermatophytes,[11] but elongation did increase the curling of hyphae with the optimal analogues being 2′-propoxy (**46**) and 2′-butoxy analogues.[12] Most griseofulvin analogues tested showed increased potency against some dermatophytes but exhibiting lower activity against others.

Position 5 has been functionalized with nitro (**9**) and amine (**10**) groups, rendering the analogues inactive at relevant concentrations against four dermatophytes.[13] An ethoxycarbonyl group has also been introduced in this position and the analogue found to be inactive.[14] The 4′ position has also been

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examined with the 4′ alcohol analogue (**50**) being inactive and the 4′ oxime (**36**) being 7-fold less potent compared to **1**, with both analogues tested against *Microsporum gypseum*.[15]

Although the initial isolation of **1** was completed in 1939,[1] both anticancer[16−19] and antiviral[20] properties of griseofulvin have been discovered recently. Three analogues tested for the former by Oda et al.[21] against chinese hamster V79 cells showed increased cytotoxicity, with 2′-propoxy-2′demethoxy-griseofulvin being the most potent (**46**, IC_{50} 0.7 μM; **1**, 8 μM), and it was proposed that additional structural modifications at the 2′ position could enhance activity further. This was supported by ourselves in a whole-cell phenotype-based anticancer assay for spindle multipolarity induction, where increased activity was seen for analogues with modifications in the 2′ position, with the 2′-benzyloxy-2′-demethoxy-griseofulvin analogue (**17**) being the most potent compound tested.[22] Multiple papers state that griseofulvin arrests several cancer cell lines in G_2/M phase of the cell cycle.[17−19] Several investigators have proposed tubulin as the main target for griseofulvin, although for mammalian cells this suggestion is not undisputed.[9,17,18,20,21,23,24] Recently, Panda et al.[25] proposed two griseofulvin binding sites on tubulin using molecular docking studies and similar to Oda[21] reported that microtubule dynamics were disrupted by **1**. Using the hepatitis C virus-1b cell culture system Huh7/Rep-Feo, Jin et al. reported that G_2/M phase arrest in infected cells was induced by griseofulvin (**1**).[20] It was speculated that the effect was due to interaction with microtubule polymerization.[20]

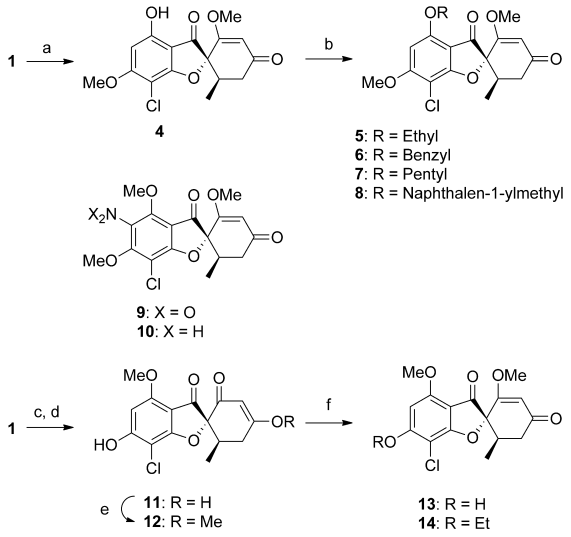
Griseofulvin exhibits activity against fungi and mammalian cancer cells as well as suppressing RNA replication by the hepatitis C virus, with tubulin having been proposed to be involved in all three cases. Tubulins are very conserved within different eukaryotic cell types,[24] and most of the variation among different tubulin isoforms is found in the amino acids near the C-terminus, which form a ridge on the surface of microtubules. Therefore, variations among different isoforms are expected to affect primarily the association of accessory proteins with the surface of microtubules rather than microtubule polymerization per se. In case tubulin is the sole target of griseofulvin in both fungi and mammalian cells, the activity profile of an array of analogues against these cell types should be similar. To test this hypothesis, we decided to carry out an SAR study of griseofulvin analogues. This is, to the best of our knowledge, the first study of griseofulvin (**1**) and analogues thereof which compares antifungal and anticancer SAR from growth inhibition assays.

Griseofulvin (**1**) and 54 griseofulvin analogues (11 reported for the first time here) have been tested in an antifungal assay against *Trichophyton mentagrophytes* and *Trichophyton rubrum*, two dermatophytes causing tinea capitis.[7] All compounds were also tested in an anticancer assay against the human cell line MDA-MB-231 (breast adenocarcinoma), which was chosen because this cell line represents a common cancer type and is known to harbor supernumerary centrosomes which are regularly clustered into a bipolar mitotic spindle array in a high percentage of cells.[26] All analogues, having alterations at the 4, 5, 6, 2′, 3′, and 4′ positions, have been synthesized from commercially available griseofulvin in one to five synthetic steps, as described here or following published procedures.

RESULTS AND DISCUSSION

Chemistry. The 4-phenol (**4**) was synthesized from **1** by treatment with freshly prepared MgI_2, procured by sonication of Mg and I_2 in Et_2O/toluene, affording **4** in 99% yield, an improvement on prior methods (Scheme 1).[22,27] Alkylation of **4** to synthesize **5** and **6** has previously been described.[22,27]

Scheme 1[a]

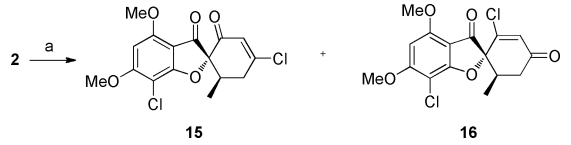


[a](a) MgI_2, toluene, Et_2O; (b) RBr, Ag_2O, dioxane; (c) HOAc, 2 M H_2SO_4; (d) 0.66 M NaOH; (e) MeOH, CSA; (f) MeOH, CSA (**13**) or EtBr, Ag_2O, dioxane, then MeOH, CSA (**14**).

Position 4 analogues **7** and **8** were prepared from **4** with Ag_2O and the appropriate alkyl bromide in dioxane as solvent.[27] The syntheses of **9** and **10** have already been described.[22,28]

For the preparation of **11**, **12**, and **13**, see Rønnest et al.[29] and Arkley et al.[27] Compound **12** was alkylated using Ag_2O and EtBr followed by repeated solvolysis in MeOH with camphor sulfonic acid (CSA) to afford **14**. The dichloro analogues **15** and **16** were synthesized using **2**,[30] $POCl_3$, LiCl, and dioxane, a modification of a known method (Scheme 2).[31] The

Scheme 2[a]



[a](a) LiCl, $POCl_3$, dioxane.

compounds **17**−**33** were synthesized by 1,4-addition of the corresponding alcohol to **16** using either NaH or 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) as base (Scheme 3).[31] The isogriseofulvin analogues **3** and **35** were synthesized in the same manner but from **15**. Compound **3** has previously been prepared by treating **2** with excess diazomethane, yielding both **1** and **3**.[32] Compounds **26** and **38** have been reported in a patent,[33] which is also the case for **19**, **21**, and **22**.[34] The dimer **34**, which was conveniently synthesized together with **22** by a second 1,4-addition of **22** to **16**, has previously been published in a Japanese patent.[35]

The analogues with an oxime functionality **36**, **37**, and **38** were synthesized from the corresponding ketones (**1**, **17**, and **26**) using hydroxylamine hydrochloride in ethanol and DMSO.[13,22] Isogriseofulvin (**39**−**44**) and griseofulvin analogues (**45**−**49** and **17**) were synthesized simultaneously in pairs by solvolysis

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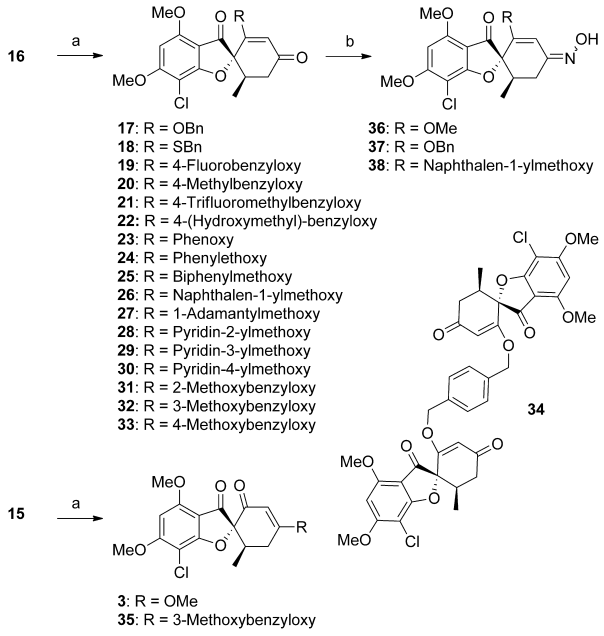
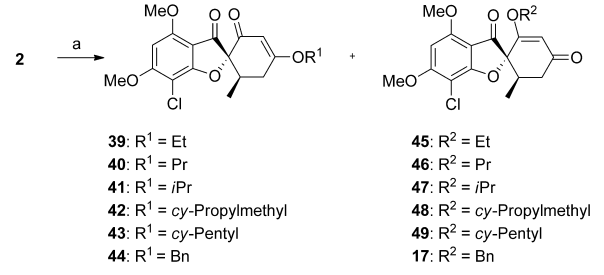
Scheme 3[a]



[a](a) RH, DBU, THF, or dioxane; (b) hydroxylamine, NaOAc, EtOH, DMSO.

with the respective alcohols and catalytic CSA and subsequently separated by chromatography (Scheme 4).

Scheme 4[a]



[a](a) ROH, CSA, dioxane (**17**, **42**–**44**, **48**, and **49**).

Analogues **50**–**55** were synthesized according to published procedures (Figure 2).[22] The fungal secondary metabolite geodin (**56**) was recently isolated from *Aspergillus terreus*.[36] Please see the Supporting Information for experimental details and spectral data.

Assays. Griseofulvin (**1**), geodin (**56**), and 53 griseofulvin analogues covering variations on six positions (see Figure 1) were tested against two dermatophytes (*T. rubrum* and *T. mentagrophytes*) and against the breast cancer cell line MDA-MB-231 in a cytotoxicity assay. For all the test results the following definitions were used: if no activity was observed at 50 μM, a given compound was deemed *inactive*; if activity was observed but 50% inhibition was not reached at 50 μM, a given compound is described as having low activity and no IC_{50} value is calculated (see Supporting Information for examples). The IC_{50} of griseofulvin (**1**) was determined to be 0.38 ± 0.048 μM against the *T. rubrum* isolate and 0.058 ± 0.018 μM against the *T. mentagrophytes* isolate. All IC_{50} values and 95% confidence intervals are given in Table 1. All dilution series were made starting with 50 μM of the tested compound in order to ensure

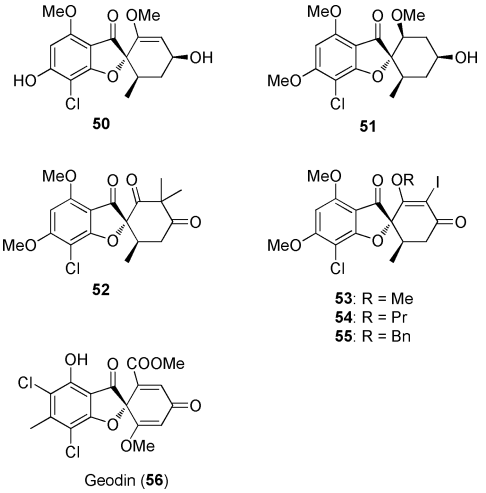


Figure 2. The analogues **50**–**55** were prepared according to literature procedures.[22] Geodin (**56**) was isolated as previously described.[36]

that all compounds were soluble in the assay media, which for some compounds were not the case at higher concentrations.

Antifungal SAR for *T. rubrum* and *T. mentagrophytes*. All compounds in the position 4 series of **4**–**8** were less active than griseofulvin (**1**) against the two fungi and **8** was completely inactive. For *T. rubrum*, **4** and **6** were inactive, **7** had low activity, and **5** was, with an IC_{50} of 2.0 μM, approximately five times less potent than griseofulvin. For *T. mentagrophytes*, analogue **4** showed low activity and the rest were less potent than **1**: **5** (0.29 μM), **6** (0.25 μM), and **7** (0.17 μM). The activity of **6** has previously been reported against a number of dermatophytes (*Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton interdigitale*, *Trichophyton persicolor*, *T. mentagrophytes*, and *T. rubrum*) and found to be less active than **1** against all of them.[11]

The nitro (**9**) and amine (**10**) position 5 analogues were inactive against both fungi. Compound **10** has also been reported as inactive, but **9** has been reported as weakly active with inhibition for both fungi starting at around 25 μM.[13] Other position 5 analogues (ethoxycarbonyl,[14] methoxy, methyl, or chloro[37]) have been reported to have lower activity than **1** against dermatophytes.

Whereas the position 6 phenols **13** and **11** were inactive against both fungi, the 6-ethyl analogue (**14**) had an IC_{50} of 1.3 μM against *T. rubrum* and was as active as griseofulvin (**1**) against *T. mentagrophytes* with an IC_{50} of 0.062 μM, which is in accordance with the literature.[11]

The 2′ series is the most extensively studied due to the increased activity observed in the anticancer phenotype-based assay[19] by these analogues. For *T. rubrum*, elongation to 2′-ethyloxy (**45**, 0.69 μM) and 2′-propyloxy (**46**, 0.62 μM) lowered the activity 2-fold, confirming the findings of Crosse et al.[11] The bulkier 2′-isopropyloxy (**47**, 3.1 μM) was 10 times less potent. Increasing bulkiness through 2′-cyclopropylmethoxy (**48**, 1.5 μM), cyclopentyloxy (**49**, 2.5 μM), and benzyloxy (**17**, 1.1 μM) enol ethers did not reveal a trend, but all three were less active toward *T. rubrum* than **1**. No correlation between the size of the 2′ substituent and the activity was seen for *T. mentagrophytes* with **46** (0.050 μM) retaining the activity of **1** and IC_{50} values of 0.25 μM and 0.11 μM for **45** and **47**, respectively. Both **45** and **46** have been reported to retain the activity of **1** against *T. mentagrophytes* by Crosse et al.[11]

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Table 1. All Available IC_{50} Values from the Screen[a]

Cmp. no.	*T. rubrum* (μM)	± STD IC_{50} (μM)	*T. mentagrophytes* (μM)	± STD IC_{50} (μM)	cytotox (μM)	± STD IC_{50} (μM)
1	0.38	0.05	0.058	0.02	18	4
2	*inactive*	*inactive*	low activity		*inactive*	*inactive*
3	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*
4	*inactive*	*inactive*	low activity		*inactive*	*inactive*
5	2.0	0.7	0.29	0.1	*inactive*	*inactive*
6	*inactive*	*inactive*	0.25	0.009	20	4
7	low activity		0.17	0.02	low activity	
8	*inactive*	*inactive*	*inactive*	*inactive*	17	2
9	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*
10	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*
11	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*
13	*inactive*	*inactive*	*inactive*	*inactive*	low activity	
14	1.3	0.1	0.062	0.02	36	3
15	*inactive*	*inactive*	*inactive*	*inactive*	1.0	0.1
16	low activity		low activity		3.2	0.3
17	1.1	0.2	0.26	0.1	2.1	0.2
18	1.3	0.4	0.058	0.02	4.7	0.7
19	0.83	very wide	0.041	0.01	6.5	1
20	1.0	0.2	0.24	0.1	1.8	0.3
21	1.5	0.2	0.15	0.1	7.0	0.9
22	low activity		1.7	1	2.8	0.6
23	6.1	7	1.6	3	7.2	1
24	low activity		0.28	0.2	17	2
25	*inactive*	*inactive*	inactive	*inactive*	5.8	1
26	2.6	0.3	0.25	0.09	13	2
27	*inactive*	*inactive*	*inactive*	*inactive*	4.7	0.5
28	low activity		1.3	3	32	6
29	low activity		0.35	0.2	10	2
30	low activity		low activity		19	2
31	low activity		0.70	0.6	11	3
32	0.96	0.4	0.14	0.07	8.1	2
33	*inactive*	*inactive*	*inactive*	*inactive*	low activity	-
34	*inactive*	*inactive*	*inactive*	*inactive*	8.5	2
35	*inactive*	*inactive*	low activity		25	5
36	1.3	0.5	0.19	0.1	12	2
37	7.9	0.9	0.30	0.2	1.4	0.3
38	*inactive*	*inactive*	*inactive*	*inactive*	8.3	0.8
39	*inactive*	*inactive*	low activity		low activity	
40	*inactive*	*inactive*	low activity		43	6
41	*inactive*	*inactive*	low activity		48	10
42	*inactive*	*inactive*	low activity		52	10
43	*inactive*	*inactive*	low activity		27	4
44	*inactive*	*inactive*	low activity		low activity	
45	0.69	0.2	0.25	0.08	13	3
46	0.62	0.09	0.050	0.02	16	5
47	3.1	1	0.11	0.05	11	3
48	1.5	0.3	0.060	0.02	9.4	2
49	2.5	0.8	0.24	0.09	3.2	0.5
50	low activity		low activity		*inactive*	*inactive*
51	low activity		3.0	1	*inactive*	*inactive*
52	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*	*inactive*
53	*inactive*	*inactive*	low activity		22	1
54	1.6	0.4	0.23	0.08	8.0	1
55	*inactive*	*inactive*	1.1	0.5	low activity	
56	*inactive*	*inactive*	*inactive*	*inactive*	9.9	1

[a]If no activity was observed at 50 μM, a given compound is deemed *inactive*. If activity was observed, but 50% inhibition was not reached at 50 μM, a given compound is described as having low activity and the IC_{50} value is not calculated.

Analogue **48** (0.060 μM) had the same activity as **1**, but **17** (0.26 μM) and **49** (0.24 μM) were about four times less potent.

The activities of **17**, **45**, and **46** have been reported against *T. mentagrophytes* and *T. rubrum*, and all showed lower activity when tested against the latter.[11] Lower activity than **1** toward *T. mentagrophytes* was also reported for **17**, but **45** and **46** had similar potency.[11] The three compounds were also tested against *E. floccosum*, *M. canis*, *T. interdigitale*, and *T. persicolor* displaying the same trends, with **17** having lower activity and **45** and **46** retaining the activity of **1**.[11]

The rest of the 2′ series includes bulkier analogues (**25**−**27**), *para*-substituted variations of **17** (**19**−**22**), two sets of *ortho*-, *meta*-, and *para*-analogues (**28**−**30** and **31**−**33**), modification of the linker part (**23** and **24**), and a vinyl sulfide analogue **18**.

Of the bulkier 2′ analogues, the naphthalen-1-ylmethoxy **26** was the only active analogue and **25** and **27** were inactive against both fungi. With IC_{50} values of 2.6 μM (*T. rubrum*) and 0.25 μM (*T. mentagrophytes*), compound **26** is three and four times less potent than **1**, respectively.

The three 2′ pyridine analogues (**28**−**30**) had low activity toward *T. rubrum*, and **30** also showed low activity toward *T. mentagrophytes*. Compounds **28** and **29** had IC_{50} values of 1.3 and 0.35 μM, meaning they were approximately 22 and 6 times less potent toward *T. rubrum* compared to **1**, respectively. The series with *ortho*-, *meta*-, and *para*-methoxy groups revealed **33** as inactive against both fungi, **31** with low activity toward *T. rubrum* and an IC_{50} of 0.70 μM against *T. mentagrophytes*. The *meta*-substituted analogue **32** (0.96 μM) was 3-fold less active against *T. rubrum* compared to **1** and displayed a 2-fold decrease in activity against *T. mentagrophytes* with an IC_{50} of 0.14 μM.

The 2′-phenoxy analogue (**23**) had IC_{50} values of 6.1 μM against *T. rubrum* and 1.6 μM against *T. mentagrophytes*, while the phenylethoxy analogue (**24**) had low activity against *T. rubrum* and an IC_{50} of 0.28 μM against *T. mentagrophytes*. Lower activity for **23** has previously been reported.[11] The vinyl sulfide analogue **18** (1.3 μM) was as active as **17** against *T. rubrum*, but against *T. mentagrophytes* the IC_{50} (0.058 μM) was equal to that of griseofulvin (**1**).

The 3′-dimethyl analogue **52** was inactive against both fungi. The series of 3′ analogues include three 3′-iodo analogues with 2′ modifications, 2′-methoxy (**53**), 2′-propoxy (**54**), and 2′-benzyloxy (**55**). The 3′-iodo griseofulvin (**53**) was inactive against *T. rubrum* and had low activity toward *T. mentagrophytes* in accordance with Crosse et al.[11] The 2′-benzyloxy (**55**) analogue was inactive against *T. rubrum* and close to 20 times less potent (1.1 μM) than **1** against *T. mentagrophytes*. Analogue **55** was also four times less potent than **17**, which has no 3′ iodo group. The propoxy (**54**) analogue showed activity against both fungi (*T. rubrum* 1.6 μM and *T. mentagrophytes* 0.23 μM) but was less potent than both **1** and the analogue **46**, which does not contain iodide. Analogue **54** has been reported to be the most potent at inducing curling of hyphae but less active against all dermatophytes spare one.[11]

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The introduction of an oxime functionality on the parent compounds **1**, **17**, and **26** afforded the analogues **36**, **37**, and **38** and lowered the activity toward both fungi compared to the parent ketones (**38** was inactive against both fungi altogether). The IC_{50} values for **36** were 1.3 μM against *T. rubrum* and 0.19 μM against *T. mentagrophytes*, which is in accordance with previously published data.[13] Analogue **36** has also been reported active against *M. gypseum*.[15] Compound **37** had IC_{50} values of 7.9 μM against *T. rubrum* and of 0.30 μM against *T. mentagrophytes*, 20 and 3 times less potent than **1**, respectively. The 4′ alcohol **50** exhibited low activity toward both fungi, which was not in agreement with prior work, as it has been reported that **50** causes 100% growth inhibition of *T. rubrum* and visible inhibition against *T. mentagrophytes* at 28 μM.[13] Compound **50** has however been tested inactive against *M. gypseum*.[15]

The series including isogriseofulvin (**3**) and isogriseofulvin analogues **35** and **39**−**44** was inactive against *T. rubrum* and showed low activity against *T. mentagrophytes*. This is in accordance with the literature, where **3** and **39** have been reported to be less active than griseofulvin against a number of dermatophytes (*E. floccosum*, *M. canis*, *T. interdigitale*, *T. persicolor*, *T. mentagrophytes*, and *T. rubrum*).[11]

Griseofulvic acid (**2**) was inactive against *T. rubrum* and had low activity against *T. mentagrophytes* contrary to prior work, which reported **2** to be inactive toward *T. mentagrophytes* but having visible growth inhibition at 30 μM against *T. rubrum*.[13] The griseofulvin dimer **34** was inactive against both fungi. The dichloro analogue **16** showed low activity toward both fungi, while **15** was inactive against both. The reduced analogue **51** had low activity against *T. rubrum* and an IC_{50} of 3.0 μM against *T. mentagrophytes*, which is 50 times less potent compared to **1**. Geodin (**56**) was inactive against both fungi.

Of the 55 compounds tested, 27 analogues were inactive or exhibited low activity for both fungi (see Table 1). Nine of the compounds were active against both fungi within the same order of magnitude compared to griseofulvin (**17**, **20**, **21**, **23**, **26**, **32**, **36**, **49**, and **54**).

There were however some compounds that fared differently against the two fungi. The compounds **6**, **7**, **22**, **24**, **28**, **29**, **31**, **51**, and **55** were all inactive or had low activity toward *T. rubrum* but had IC_{50} values in the range of 0.25−3.0 μM against *T. mentagrophytes*. For other analogues (**14**, **18**, **19**, **37**, **47**, and **48**) the activity against *T. mentagrophytes* was similar to that of **1** but against *T. rubrum* it was significantly lower. This is in accordance with earlier observations by Crosse et al., demonstrating that a given analogue was more active against some fungi but less active against others.[11]

Anticancer SAR. Looking at the IC_{50} values for the 16 analogues (**1**, **17**, **18**, **20**, **23**−**25**, **27**, **36**, **37**, **45**−**49**, and **54**) tested active in both the multipolarity assay[19] and the cytotoxicity-based assay there was good correlation between the data with an R^2 of 0.70 (see Supporting Information).

The IC_{50} of griseofulvin (**1**) was determined to be 18 ± 4 μM (20 ± 1 μM in the phenotype-based spindle multipolarity assay[22]), while an IC_{50} of 25 ± 4 μM against HeLa cells was found by Panda et al.[18] In the position 4 series, **4**, **5**, and **7** were all inactive but **6** and **8** harboring aromatic moieties had activities similar to griseofulvin (**1**), with IC_{50} values of 20 (**6**) and 17 μM (**8**). This indicates a mode of action for cytotoxicity that does not involve induction of multipolar mitosis, as **6** was inactive in the multipolarity assay. Both the position 5 analogues **9** and **10** were inactive in the cytotoxicity assay as they were in the assay for multipolarity induction.

The 6-phenol griseofulvic acid analogue **11** was inactive, and the 6-phenol griseofulvin analogue (**13**) showed low activity. The 6-ethyl griseofulvin (**14**) analogue was approximately 50% less active than griseofulvin. The two dichloro analogues **15** and **16** were some of the most cytotoxic among the tested analogues, with IC_{50} values of 1.0 and 3.2 μM, respectively. 2′-Chloro analogue **16** has previously been observed by us to have a different phenotype from all other analogues in the cell-based assay for multipolarity induction.[22] The highly electrophilic nature of **16** prompted an investigation of the ability of this analogue, together with **1** and **17**, to act as Michael acceptors. Briefly, the three compounds were incubated in buffer with two different potential nucleophiles, 2-aminoethanol and 2-mercaptoethanol, for up to seven days (see Supporting Information for details). The experiments showed that neither **1** nor **17** underwent addition of the nucleophiles, whereas **16** did react with 2-mercaptoethanol to form the Michael addition/elimination product, which was the major constituent after 72 h of incubation. This confirms that the highly electron withdrawing properties of the chlorine atom enable **16** to react as a general alkylating agent and one could imagine the formation of a covalent bond to reactive cysteine residues in proteins. The experiment also shows that in the absence of the chlorine atom, this type of reactivity is not present for the griseofulvin analogues. Furthermore, the three representative compounds were found to be stable in the absence of nucleophiles for 1 week of incubation, showing only a slight decrease in purity.

When elongating the 2′ position from the parent methoxy (**1**) to ethoxy (**45**) and propoxy (**46**) and then further increasing the bulkiness with isopropoxy (**47**), cyclopentoxy (**49**), and benzyloxy (**17**), the activity increased through the series (see Figure 3), plateauing with **17** and **49** at 2.1 and 3.2 μM, respectively. The same trend was seen when these compounds were tested in the phenotype-based multipolarity assay. Moving from the benzyloxy derived analogues to even bulkier groups like the naphthalen-1-ylmethoxy (**26**, 13 μM), biphenylmethoxy (**25**, 5.8 μM), and 1-adamantylmethoxy (**27**, 4.7 μM) analogues, the activity did not increase further. The three compounds were still more potent than griseofulvin though, which was also observed for **25** and **27** in the phenotype-based assay. The trend seen for substituents of increasing size

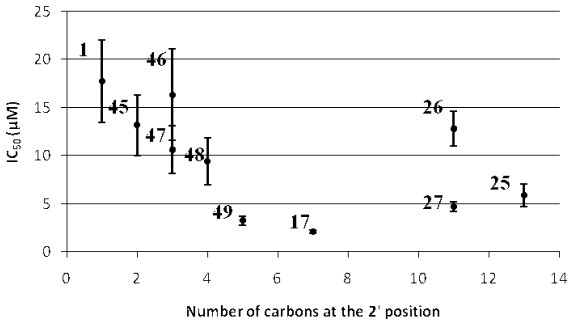

Figure 3. The activity increases with the number of carbon atoms at the 2′ position from griseofulvin itself to the 2′ ethoxy (**45**), propoxy (**46**), isopropoxy (**47**), and cyclopropylmethoxy (**48**) before leveling out with the cyclopentoxy (**49**) and benzyloxy (**17**) analogues. The bulkier analogues **25**, **27**, and **26** were less active than the benzyloxy analogue (**17**).

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does not indicate that increasing lipophilicity also leads to increasing potency, which would be the case if transport over the cell membrane was a determining parameter for activity. This is further illustrated by the analogues **17**, **23**, and **24**, where the former is the most active (Table 1), indicating that a binding event is responsible for the activity.

A number of variations of **17** have been tested (**18**−**24**, **28**−**30**, and **31**−**33**), and although all except **33** (low activity), **28** (32 μM), and (**24**, 17 μM; **30**, 19 μM) were more active than **1**, only **20** was as active as **17** with an IC_{50} of 1.8 μM. The difference in activity between the phenoxy (**23**, 7.2 μM) and phenylethoxy (**24**, 17 μM) analogues was less pronounced in the cytotoxicity assay than in the multipolarity assay with two and a half orders of magnitude compared to the approximately 7-fold difference in the phenotype-based assay. The dimer (**34**) was about twice as active as **1** with an IC_{50} value of 8.5 μM.

The introduction of an oxime functionality at the 4′ position (**36**) increased potency 2-fold to 12 μM, an increase in activity that was also seen in the phenotype-based assay. Introducing the oxime to analogues **17** and **26** affording **37** and **38** improved the activity further for both compounds. Apart from **15**, **37** is the most active analogue in the cytotoxicity assay with an IC_{50} of 1.4 μM. The stability of the oximes in PBS buffer (pH 7.4) was tested, and less than 5% hydrolysis to the parent ketones could be detected after 48 h (data not shown).

The isogriseofulvin analogues tested in the multipolarity assay (**3** and **39**−**44**) were all inactive. In the cytotoxicity assay, **35** retained the activity of **1**, while the rest were either inactive (**3**), showed low activity (**39**, **44**), or were less potent (**40**, 43 μM; **41**, 48 μM; **42**, 52 μM; **43**, 27 μM).

Geodin (**56**) did not induce multipolar mitoses and was in that respect deemed inactive.[36] Geodin (**56**) is however twice as cytotoxic as griseofulvin when tested against the MDA-MB-231 cell line, with an IC_{50} of 9.9 μM opposed to 18 μM for **1**.

The reduced analogues **50** and **51** as well as the 3′-dimethyl analogue **52** were inactive in both anticancer assays. In the 3′ iodo series, **53** (22 μM) retained activity, the 2′ propoxy analogue (**54**, 8.0 μM) had increased activity, and **55** exhibited low activity. **53** and **55** were inactive in the phenotype-based assay, but **54** was more potent than **1**.

Anticancer SAR versus Antifungal SAR. The difference between the antifungal and anticancer data is illustrated in Figure 4, demonstrating that most of the analogues had increased potency against the cancer cell line but against the two fungal strains the activity was lower than for griseofulvin (**1**). Looking at the 4 position, there were inconsistencies throughout all three cell types. The ethyl analogue **5** was active against both fungi but inactive toward the cancer cells, while the bulkier naphtyl analogue **8** was inactive against both fungi but more potent than griseofulvin against the MDA-MB-231 cells. Analogues **6** and **7** were more potent than **5** against *T. mentagrophytes*, but both compounds were inactive or showed low activity toward *T. rubrum*. The most potent analogue toward the cancer cells (**15**) was inactive against both fungi and the isomer (**16**), which was among the most active compounds, only had low activity toward the two fungi. The two bulky analogues **25** and **27** as well as the dimer (**34**) were inactive in the antifungal assays but were all more potent than griseofulvin against the cancer cell line. It is also worth noticing that geodin (**56**) was inactive against both fungi but twice as potent as **1** against the cancer cell line.

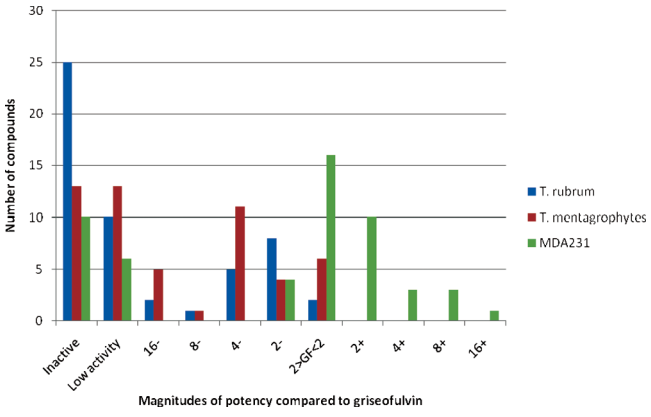

Figure 4. The IC_{50} values for each cell line have been normalized defining the activity of griseofulvin as having a value of 1. It is seen that of the 53 analogues the majority was less active against the two fungi opposed to the anticancer activity where most analogues were more active than griseofulvin.

The introduction of an oxime at the 4′ position (**36**, **37**, and **38**) decreased the potency compared to the parent compounds (**1**, **17**, and **26**) against both fungi. The opposite effect was seen for the cancer cell line, where the introduction of this moiety increased the activity for all three compounds.

There were however some similar trends for all three cell types. A number of analogues were inactive or had low activity toward both fungi and cancer cells. Among these were the position five analogues (**9**, **10**), isogriseofulvin (**3**), and the series of isogriseofulvin analogues (**39**−**44**), with **35** as the sole exception. The two 6 phenols (**11**, **13**) were virtually inactive against all cell types, which was the same for **50**−**52**.

CONCLUSION

The first comparison of antifungal and anticancer SAR for griseofulvin analogues is presented in this work covering 53 analogues of griseofulvin as well as the natural product geodin (**56**). All compounds have been tested against *T. rubrum*, *T. mentagrophytes*, and in a cytotoxicity assay against MDA-MB-231 breast cancer cells.

Even though there were similarities between the SAR of the two fungi, with some compounds showing no activity against both fungi and some active against both, there were also some differences. Analogues **46** and **48** retain the activity of griseofulvin against *T. mentagrophytes* but were 2- and 4-fold less active against *T. rubrum* than **1**, respectively. This is in accordance with reported observations by Crosse et al. that analogues differ in activity against different dermatophytes.[11]

We show that there is a good correlation between the IC_{50} values from analogues that were active in both the phenotype-based assay for spindle multipolarity[22] and the cytotoxicity assay used in this work with an R^2 of 0.70. Comparing the two fungal SARs with the anticancer cytotoxicity SAR afforded distinct differences. The analogues **8**, **25**, **27**, and **38** were inactive or had low potency against both fungi but either retained the activity (**8**) or were at least 2-fold more active against the cancer cells compared to **1**. The 2′-benzyloxy analogue (**17**) was less active against both fungi in this study and against six dermatophytes in the study by Crosse et al.[11] This analogue is however nine times more potent than **1** against the cancer cell line MDA-MB-231. It is noteworthy that the two dichloro compounds **15** and **16** were inactive against both fungi but featured as some of the most cytotoxic agents against the cancer cell line, possibly due to their electrophilic nature, which is unique among the tested analogues.

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The differences in activity observed for the two fungi and the MDA-MB-231 cell line could rise from a number of factors, such as transport over the cell membrane, efflux pumps, or different modes of action. We find it unlikely that transport or efflux pumps could explain that the majority of analogues were more active against MDA-MB-231 cells and less active against both fungi. Our conclusion therefore is that the mode-of-action of griseofulvin(s) toward fungal and mammalian cells is different, making it unlikely that tubulin itself constitutes the main cellular target in both fungi and mammalian cells. Because it has been shown that griseofulvin leads to mitotic arrest in both fungal and mammalian cells, an alternative explanation is that griseofulvin disrupts microtubule dynamics without directly interacting with tubulin, e.g., through interaction with microtubule-associated proteins (MAPs).

EXPERIMENTAL SECTION

General. Starting materials, reagents, and solvents were purchased from Sigma-Aldrich and used without further purification. ^{1}H NMR spectra were recorded using either a Varian Unity Inova 500 MHz spectrometer or a Varian Mercury 300 MHz spectrometer both from Agilent (Santa Clara, CA, US). ^{13}C NMR spectra were recorded using either a Varian Mercury 300 MHz or a Bruker AC 200 MHz from Bruker Optics (Ettlingen, Germany). Chemical shifts were measured in ppm and coupling constants in Hz. When $CDCl_3$ was used as solvent, the residual peak was used as internal reference at δ 7.27 for ^{1}H NMR and δ 77.00 for ^{13}C NMR spectra. IR spectra were recorded using a Bruker Alpha ATR and measured in cm^{-1}. All melting points are uncorrected. TLC was performed on aluminum sheets precoated with silica gel 60 F254 (Merck 1.05554.0001). Compounds were visualized by charring after dipping in a solution of 1% $KMnO_4$, 6.7% K_2CO_3, and 0.08% NaOH in water. UV visualization was done using a model UVGL-25 Mineralight lamp. High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray electrospray ionization (ESI) source and a LockSpray probe (M + H 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from m/z 100−900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetonitrile (50 ppm TFA) in water gradient starting from 15% to 100% over 25 min with a flow rate of 0.3 mL/min. Purity of all compounds was found to be greater than 95% by LC-DAD. Microanalyses were obtained from H. Kolbe Mikroanalytisches Laboratorium in Mülheim an der Ruhr, Germany. The purity of all tested compounds was found to be >95% by HPLC.

Antifungal Assay. The fungal micro broth dilution assay was performed in sterile flat bottomed microplates (cat. no. 655101) and lids (cat. no. 656161) from Greiner Bio-One GmbH (Frickenhausen, Germany). Each microplate accommodated five dilution series starting at 50 μM of a given analogue. Each plate was also fitted with four negative and four positive control wells. The fungi *T. rubrum* (IBT29284) and *T. mentagrophytes* (IBT2724) were kept in the IBT fungal collection at the Department of Systems Biology at the Technical University of Denmark. The identity of the fungal cultures was verified by microscopy after the last test round in the antifungal assay. For testing against *T. rubrum*, a 4-fold dilution series was used, resulting in 10 concentrations with the lowest at 0.2 μM. For *T. mentagrophytes*, an 8-fold dilution series was used, resulting in 10 concentrations with the last one at 0.4 pM.

Inoculum preparation was performed in accordance with the document M38-A2[38] from the "Committee for Clinical Laboratory Standards". Inoculum was adjusted to 0.90−1.5 OD at 530 nM on a Genesys 10uv from Thermo Electron Corporation (Waltham, MA, US). The inoculums were diluted 50-fold with RPMI media (with L-glutamine, without $NaHCO_3$, cat. no. R6504 in MOPS buffer (cat. no.69947)), both from Sigma-Aldrich (St Louis, MO, USA). The pH was adjusted to 7.00 with 1 M NaOH, and the volume with Milli-Q water from a Milli-Q gradient fitted with a Millipore 0.22 μm filter (cat. no. MPGL04001), both from Millipore (Billerica, MA, USA).

Each well contained 99 μL of RPMI media with inoculum, 99 μL of Milli-Q water, and 2 μL of DMSO. Microplates were incubated for 7 days at 37 °C in a WTB binder incubator from VWR (Radnor, PA, US), at which point the OD of the wells were measured at 530 μM on a BioTek Synergy 2 (Brøndby, Denmark) using Gen5TM 1.10.8 software. The half-maximal inhibitory concentration (IC_{50}) values were obtained with GraphPad Prism5 (ver. 5.04) from GraphPad Software (La Jolla, CA, USA) using the "nonlinear regression, sigmoidal dose−response (variable slope)" function. The fitting method used was "automatic outlier elimination",[39] which eliminated less than 1.1% of data points.

Cytotoxicity Assay. MDA-MBA-231 human adenocarcinoma cells were cultivated in Leibovitz's L-15 medium in the presence of 10% fetal calf serum at 37 °C. Cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma Chemical, St. Louis, MO) colorimetric assay, as previously described.[40] Briefly, cells were plated in 96-well microtiter plates at a density of (2−3) × 104 cells per well, and each plate was incubated for 24 and/or 48 h, with MTT added to each well for at least 4 h. The absorbance of each well was measured at 570/630 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA). Each condition was analyzed in at least three replicates, and the results are presented as the mean ± standard deviation of replicates of a representative experiment that was repeated at least three times.

General Procedure for the Synthesis of Position 4 Ethers (7 and 8). The appropriate alkyl bromide (1.2 mmol, 3 equiv) was added to a solution of **4** (0.4 mmol, 1 equiv), Ag_2O (1.2 mmol, 3 equiv), and 1,4-dioxane (5 mL). The mixture was stirred at 50 °C for 18 h and then cooled to 20 °C. EtOAc (10 mL) was added to the solution, and the mixture was washed with brine (15 mL). The aqueous phase was extracted with EtOAc (2 × 10 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:3) to afford the desired product.

(2*S*,6′*R*)-(7-Chloro-6-ethoxy-4-methoxy-benzofuran-3-one)-2-spiro-1′-(2′-methoxy-6′-methyl-cyclohex-2′-ene-4′-one) 14. Ethyl bromide (0.9 mmol, 3 equiv) was added to a solution of **12** (0.3 mmol, 1 equiv), Ag_2O (0.9 mmol, 3 equiv), and 1,4-dioxane (3 mL). The mixture was stirred at 50 °C for 18 h and then cooled to 20 °C. EtOAc (10 mL) was added to the solution, and the mixture was washed with brine (15 mL). The aqueous phase was extracted with EtOAc (2 × 10 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated. The following process was repeated three times: The residue was dissolved in MeOH (10 mL), CSA (0.1 mmol) was added, and the mixture was heated to 65 °C for 18 h. EtOAc (10 mL) was added to the solution, and the mixture was washed with satd aq $NaHCO_3$ (15 mL). The aqueous phase was extracted with EtOAc (2 × 10 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:3) to afford the desired product **14**. Yield, 5 mg (5%) (yellow oil); R_f value (EtOAc/heptane, 5:1), 0.43. IR (neat, cm^{-1}): 1709, 1666, 1613, 1587 (lit.[41]). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.13 (1H, s), 5.56 (1H, s), 4.25 (2H, q, J = 7.0 Hz), 3.98 (3H, s), 3.63 (3H, s), 3.05 (1H, dd, J = 16.7, 13.4 Hz), 2.86 (1H,), 2.45 (1H, dd, J = 16.7, 4.7 Hz), 1.56 (3H, t, J = 7.0 Hz), 0.98 (3H, d, J = 6.7 Hz). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 197.0, 192.4, 170.8, 169.6, 164.1, 157.7, 104.9 (2C), 93.4, 90.7, 90.2, 65.8, 56.6, 56.3, 40.1, 36.4, 14.6, 14.2; $[\alpha]^{20}_D$ = +252° (c = 0.5 in CCl_3) (Lit.[27]). HRMS (ESI$^+$) calcd for $[M + H]^+$ $[C_{18}H_{20}ClNO_6]^+$ 367.0948, found 367.0949.

General Procedure for the Synthesis of 2′ Enol Ethers (19, 21, 22, 26, 28−33). To a solution of **16** (0.65 mmol, 1 equiv) in 1,4-dioxane (3 mL, 0.2 M) was added the desired alcohol (1.30 mmol, 2 equiv) and DBU (1.63 mmol, 2.5 equiv). The mixture was heated to 100 °C and stirred for 12 h. The mixture was then cooled to 20 °C, and excess reagent was quenched with satd aq NH_4Cl (30 mL). The aqueous phase was extracted with EtOAc (3 × 30 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated. The

residue was purified by column chromatography (EtOAc:heptane: 1:3) affording the product. When possible, the product was recrystallized from EtOAc/heptane.

(2*S*,6′*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1′-(2′-(naphthalen-1-ylmethoxy)-6′-methylcyclohex-2′-ene-4′-one-4′-oxime) 38. To a solution of **26** (0.08 mmol, 1.0 equiv) in EtOH (2 mL) and DMSO (1 mL) was added hydroxylamine hydrochloride (0.30 mmol, 3.5 equiv) and sodium acetate (0.37 mmol, 4.3 equiv). The mixture was stirred at 75 °C for 18 h, allowed to reach 20 °C, and diluted with EtOAc (5 mL). The mixture was washed with brine (3 × 4 mL). The organic phase was dried ($MgSO_4$) and concentrated. The crude mixture was purified by column chromatography (EtOAc:heptane 1:1) to afford **38** (40 mg, 96%) as a yellow oil. R_f values (EtOAc/heptane, 5:1), 0.60 and 0.51. IR (neat, cm^{-1}): 1704, 1613, 1590. ^1H NMR (500 MHz, $CDCl_3$): δ 7.82−7.73 (3H, m), 7.50−7.42 (2H, m), 7.37−7.32 (2H, m), 6.54 (0.4H, s), 5.99 (0.6H, s), 5.96 (0.4H, s), 5.84 (0.6H, s), 5.31 (0.4H, d, J = 12.0 Hz), 5.29 (0.6H, d, J = 12.0 Hz), 5.26 (0.4H, d, J = 11.9 Hz), 5.23 (0.6H, d, J = 11.9 Hz), 3.95 (1.8H, s), 3.93 (1.2H, s), 3.91 (1.8H, s), 3.90 (1.2H, s), 3.15 (0.6 H, dd, J = 16.8, 4.9 Hz), 3.06 (0.4 H, dd, J = 15.0, 13.3 Hz), 2.75 (0.6 H, dd, J = 16.8, 13.1 Hz), 2.70−2.57 (1 H, m), 2.44 (0.4 H, dd, J = 15.0, 4.1 Hz), 0.98 (1.8H, d, J = 6.7 Hz), 0.98 (1.2H, d, 6.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 194.0 (0.5C, s), 193.9 (0.5C, s), 169.3 (0.5C, s), 164.0 (0.5C, s), 160.0 (0.5C, s), 157.3 (0.5C, s), 157.3 (0.5C, s), 157.3 (0.5C, s), 155.0 (0.5C, s), 151.8 (0.5C, s), 133.3 (0.5C, s), 131.2 (0.5C, s), 131.1 (0.5C, s), 130.8 (0.5C, s), 130.6 (0.5C, s), 128.9 (0.5C, s), 128.9 (0.5C, s), 128.8 (0.5C, s), 128.3 (0.5C, s), 128.2 (0.5C, s), 128.1 (0.5C, s), 126.3 (0.5C, s), 126.3 (0.5C, s), 126.2 (0.5C, s), 126.0 (0.5C, s), 125.7 (0.5C, s), 125.6 (0.5C, s), 125.2 (0.5C, s), 125.0 (0.5C, s), 124.9 (0.5C, s), 123.6 (0.5C, s), 123.5 (0.5C, s), 105.6 (0.5C, s), 105.5 (0.5C, s), 100.5 (0.5C, s), 96.9 (0.5C, s), 96.8 (0.5C, s), 93.9 (0.5C, s), 91.5 (0.5C, s), 91.4 (0.5C, s), 89.0 (0.5C, s), 89.0 (0.5C, s), 69.4 (0.5C, s), 69.0 (0.5C, s), 56.7 (1 C, s), 56.1 (0.5C, s), 56.1 (0.5C, s), 36.4 (0.5C, s), 35.2 (0.5C, s), 30.9 (0.5C, s), 25.5 (0.5C, s), 14.4 (0.5 C, s), 14.3 (0.5 C, s); $[\alpha]^{20}_D$ = +112° (c = 1.0 in $CDCl_3$). HRMS (ESI+) calcd for M + H $[C_{27}H_{25}ClNO_6]^+$ 494.1370, found 494.1371.

The analogue **35** was synthesized according to the general procedure for 2′ enol ethers with the starting material being **15** instead of **16**.

(2*S*,6′*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1′-(4′-(3-methoxybenzyl)oxy-6′-methyl-cyclohex-3′-en-2′-one] 35. Yield: 83 mg (32%) (white crystals); R_f value (EtOAc/heptane, 5:1): 0.65; mp 173−175 °C. IR (neat, cm^{-1}): 1691, 1653, 1605, 1584. ^1H NMR ($CDCl_3$, 300 MHz): δ 7.37−7.27 (1H, m), 7.02−6.84 (3H, m), 6.09 (1H, s), 5.54 (1H, d, J = 1.0 Hz), 5.01−4.85 (2H, m), 4.01 (3H, s), 3.93 (3H, s), 3.84 (3H, s), 3.26 (1H, ddd, J = 17.5, 11.9, 1.0 Hz), 2.96−2.80 (1H, m), 2.54 (1H, dd, J = 17.5, 5.6 Hz), 1.03 (3H, d, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 191.7, 188.8, 177.7, 169.7, 164.5, 159.9, 157.7, 136.0, 129.7, 120.0, 114.2, 113.2, 105.1, 100.7, 97.3, 95.1, 89.5, 71.0, 57.0, 56.2, 55.3, 35.2, 33.2 14.5; $[\alpha]^{20}_D$ = +179° (c = 0.6 in CCl_3). HRMS (ESI^+) calcd for $[M + H]^+$ $[C_{24}H_{24}ClO_7]^+$ 459,1211, found 459.1216.

General Procedure for the Synthesis of Enol Ethers by Solvolysis (42 and 43). CSA (0.1 mmol, 0.1 equiv) was added to a solution of griseofulvic acid (0.6 mmol, 1 equiv) in the appropriate alcohol (6 mL, 0.1 M). The mixture was stirred at 100 °C for 6 h and then cooled to 20 °C. EtOAc (20 mL) was added to the solution, and the mixture was washed with satd aq NaH_2PO_4 (20 mL) and water (20 mL). The combined aqueous phases were extracted with EtOAc (3 × 20 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (toluene:CH_2Cl_2:EtOAc 7:7:1) to afford the desired products. When possible, the products were recrystallized from EtOAc/heptane.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental data for compounds **3**, **7**, **8**, **19**, **21**, **22**, **26**, **28**−**34**, and **39**−**44**, as well as copies of NMR spectra for compounds **3**, **7**, **8**, **15**, **19**, **21**, **22**, **26**, **28**−**35**, and **38**−**44**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CSA, camphor sulfonic acid; DBU, 1,8-diazabicyclo[5.4.0]-undec-7-ene; MAP, microtubule-associated protein; PBS, phosphate-buffered saline; SAR, structure−activity relationship

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