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Potent Synergy between Spirocyclic Pyrrolidinoindolinones and Fluconazole against *Candida albicans*

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A spiroindolinone, (1*S*,3*R*,3a*R*,6a*S*)-1-benzyl-6′-chloro-5-(4-fluorophenyl)-7′-methylspiro[1,2,3*a*,6*a*-tetrahydropyrrolo[3,4-*c*]pyrrole-3,3′-1*H*-indole]-2′,4,6-trione, was previously reported to enhance the antifungal effect of fluconazole against *Candida albicans*. A diastereomer of this compound was synthesized, along with various analogues. Many of the compounds were shown to enhance the antifungal effect of fluconazole against *C. albicans*, some with exquisite potency. One spirocyclic piperazine derivative, which we have named synazo-1, was found to enhance the effect of fluconazole with an EC_{50} value of 300 pM against a susceptible strain of *C. albicans* and going as low as 2 nM against some resistant strains. Synazo-1 exhibits true synergy with fluconazole, with an FIC index below 0.5 in the strains tested. Synazo-1 exhibited low toxicity in mammalian cells relative to the concentrations required for antifungal synergy.

Introduction

Candida spp. account for 80% of major systemic fungal infections.[1] The mortality rate for invasive candidiasis has been estimated at around 40%.[2] Fluconazole has high bioavailability and is well tolerated;[3–7] it is the first-line antifungal treatment for candidiasis and is now available in generic form. However, there are reports of side effects, including hepatitis, leukopenia, thrombocytopenia, gastrointestinal distress, headache, anaphylaxis, and rash.[8–13] There is a need for drugs that can potently enhance the efficacy of azoles. Moreover, resistance to fluconazole and other azole drugs remains a major problem,[14] particularly among immunocompromised patients.[15]

Compounds that synergize with fluconazole at low concentrations (e.g., $MIC_{90} < 0.1\ \mu g\,mL^{-1}$) are unusual. The antifungal agents flucytosine[16–19] and fenpropimorph[20] have been shown to potently synergize with fluconazole against various strains of *C. albicans*. Micafungin[21–23] and caspofungin[20] are highly potent, but not synergistic with fluconazole, with FIC indices above 1.0. A number of drugs commonly used against non-fungal human diseases have also been shown to synergize with azoles against *C. albicans*. The calcineurin-inhibiting drug tacrolimus[24,25] potently enhances the activity of fluconazole against *C. albicans*. Quite a few other compounds have been reported to inhibit the growth of *Candida* in synergy with fluconazole, with MIC_{90} values below $1\ \mu g\,mL^{-1}$, but not below $0.1\ \mu g\,mL^{-1}$: e.g., *T. broussonetii* extract,[26] terbinafine,[27] amlodarone,[28] catechin, quercetin, epigallocatechin,[29] simvastatin,[30] tunicamycin,[20] cationic peptides IJ3, IJ4[31] and VS3,[32] ketorolac,[33] cyclosporin A,[24,34] nystatin,[35] sanguinarine,[36] allicin,[37] declofenac,[33] leaf extracts of *Lippia alba*,[38] diphenyldiselenide,[39] balcalein,[40] geldanamycin,[36] pseudolaric acid B,[41] and doxycycline.[42] Hundreds of other compounds have been reported to exhibit antifungal activity in concert with azoles but not below 1 μM. Chemical synthesis can be used to improve the potency of lead molecules; in a recent study, several analogues of the azole synergizer berberine (MIC_{80}: $1.0\ \mu g\,mL^{-1}$) were identified with up to eightfold greater potency.[43,44]

Several groups have screened large libraries of compounds in search for small molecules that synergize with azoles. In 2011, Spitzer et al. screened the Prestwick library of off-patent drugs[45] and demonstrated that sertraline and thiethylperazine synergize with fluconazole. The MIC for sertraline was as low as $0.5\ \mu g\,mL^{-1}$ in the presence of fluconazole. Also in 2011, LaFleur and co-workers screened a library of 120000 compounds in search of molecules that can act in synergy with clotrimazole against *C. albicans* biofilms, but none were active below 1 μM.[46] Starting in 2010 Lindquist, Schreiber, and others at the Broad Institute reported a massive screening campaign to identify small molecules capable of inhibiting the growth of *C. albicans* in synergy with fluconazole with a particular interest in Hsp90 and calcineurin pathways.[47,48] After an initial screen of over 300000 compounds and a subsequent rescreening, 296 compounds were found to have fluconazole-dependent potency against a partially resistant clinical strain CaCi-8 as well as a lack of cytotoxicity against mammalian fibroblasts. Three of those compounds[48–50] were selected for further opti-

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mization, but none of the resulting compounds (ML189, ML212, and ML229) were active below 0.7 μM against CaCi-8. We speculated that some of the other 296 compounds identified in the Broad screen might merit further study as potentiators of fluconazole activity in *C. albicans*. We set out to identify promising candidates from the Broad assay and to design analogues capable of potently inhibiting the growth of *C. albicans* in the presence of fluconazole. We were intrigued by the structurally interesting spirocyclic compound (1*S*,3*R*,3a*R*,6a*S*)-1-benzyl-6′-chloro-5-(4-fluorophenyl)-7′-methylspiro[1,2,3a,6a-tetrahydropyrrolo[3,4-*c*]pyrrole-3,3′-1*H*-indole]-2′,4,6-trione (PubChem CID 6584729) and the potential activity of synthetically accessible diastereomer **1** and related analogues (Figure 1).

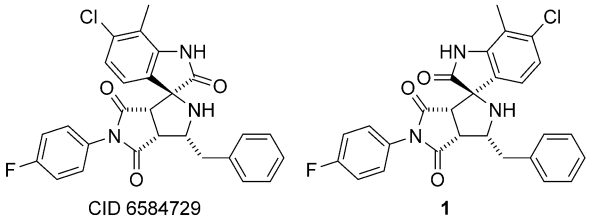


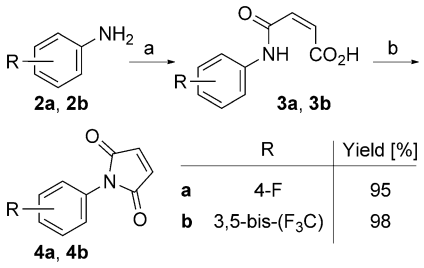
Figure 1. Fluconazole synergizer CID 6584729 and diastereomer **1**.

Results and Discussion

Chemistry

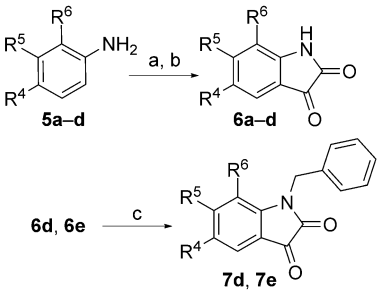
As shown in Scheme 1, *N*-phenylmaleimides **4a** and **4b** were synthesized through a two-step condensation of substituted anilines with maleic anhydride.[51] Anilines **2a** and **2b** were condensed with maleic anhydride to form the corresponding *N*-phenylmaleamic acids **3a** and **3b**, which were cyclized with acetic anhydride in the presence of sodium acetate to afford the corresponding maleimides.

Various substituted isatins were prepared from the corresponding anilines using the two-step Sandmeyer synthesis (Scheme 2).[52] Anilines were reacted with the oxime of chloral, generated in situ, to afford isonitrosoacetanilides, which were pure, as determined by TLC. The isonitrosoacetanilides were cyclized, without purification, through an intramolecular Friedel–Crafts reaction to afford the corresponding isatins **6a**–**6d** in good yield. *N*-Benzylisatins **7d** and **7e** were prepared



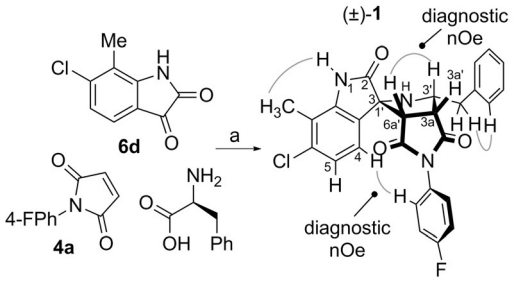
	R	Yield [%]
a	4-F	95
b	3,5-bis-(F_3C)	98

Scheme 1. Synthesis of substituted *N*-phenylmaleimides. *Reagents and conditions*: a) maleic anhydride (1.0 equiv), Et_2O, 23 °C, 15 min; b) NaOAc (0.7 equiv), $(CH_3CO)_2O$, 70 °C, 30 min.



	R^4	R^5	R^6	Yield [%]
6a	OMe	H	H	65
6b	H	Me	H	53
6c	H	Me	Me	55
6d	H	Cl	Me	72
7d	H	Cl	Me	81
7e	H	H	H	67

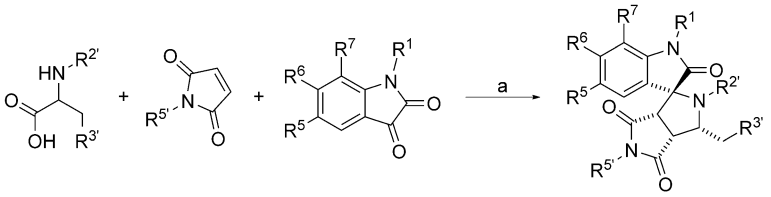
Scheme 2. Synthesis of substituted isatins. *Reagents and conditions*: a) chloral hydrate (1.1 equiv), $NH_2OH{\cdot}HCl$ (3.0 equiv), Na_2SO_4 (9.0 equiv), HCl (1.1 equiv), H_2O, 70 °C, 1 h; b) H_2SO_4, 23 °C, 5 min; c) BnBr (1.1 equiv), NaH (1.1 equiv), DMF, 23 °C, 2 h.



Scheme 3. Synthesis of compound **1** and nOes used in the assignment of relative configuration. *Reagents and conditions*: a) MeOH/H_2O (3:1), 65 °C, 16 h.

by alkylation with benzyl bromide using sodium hydride as a base.[53]

Spiroindolinones are readily accessible through one-pot three-component coupling reactions of isatins, amino acids, and maleimides.[54–56] The reaction of isatin **6d**, L-phenylalanine, and maleimide **4a** generated compound **1** as a single diastereomer in 74% yield (Scheme 3). The optically pure amino acid undergoes decarboxylation during the reaction; unless otherwise stated, all spiroindolinones were isolated and tested as racemates. The relative stereochemistry of compound **1** was secured through a NOESY experiment (Scheme 3) and shown to match that of related spiroindolinones prepared from isatins and maleimides under the same reaction conditions.[56] In particular, the strong nuclear Overhauser effect (nOe) between protons on C3′ and C6a′ of the pyrrolidine ring indicate that they are on the same face, and conversely that the benzyl group and succinimide ring are both on the opposing face. Furthermore, the strong nOe between the fluorophenyl proton and the proton on C4 of the indolone ring is consistent with the stereochemistry of compound **1**. Interest-



Compd	$R^{5'}$	$R^{2'}$	$R^{3'}$	R^5	R^6	R^7	R^1	Yield [%]
1	4-fluorophenyl	H	Ph	H	Cl	CH_3	H	74
8	Bn	H	Ph	H	H	H	H	60
9	Bn	H	3-indolyl	H	H	H	H	70
10	Bn	H	$(CH_2)_3NHBoc$	H	H	H	H	33
11	Bn	H	Ph	H	H	H	Bn	46
12	Ph	H	Ph	H	H	H	Bn	57
13	Ph	H	Ph	H	Cl	CH_3	H	26
14	Ph	$CH_2CH_2N(Boc)$		H	Cl	CH_3	H	60
15	4-fluorophenyl	$CH_2CH_2N(Boc)$		H	Cl	CH_3	H	49
16	3,5-bis(F_3C)phenyl	$CH_2CH_2N(Boc)$		H	Cl	CH_3	H	12
17	Ph	$CH_2CH_2N(Boc)$		H	Cl	CH_3	Bn	82
18	Bn	$CH_2CH_2N(Boc)$		H	H	H	Bn	56
19	Bn	$CH_2CH_2N(Boc)$		H	H	H	H	53
20	Ph	$CH_2CH_2N(Boc)$		H	H	H	Bn	71
21	Bn	$CH_2CH_2N(Boc)$		MeO	H	H	H	53
22	Ph	$CH_2CH_2N(Boc)$		H	H	CH_3	H	60
23	Ph	$CH_2CH_2N(Boc)$		H	CH_3	CH_3	H	61

Scheme 4. Synthesis of spirocyclic pyrrolidines via three-component, one-pot [1,3]-dipolar cycloaddition with amino acids. *Reagents and conditions*: a) MeOH/H_2O (3:1), 65 °C, 4–16 h.

ingly, the ^1H and ^{13}C NMR spectra and nOes for the compound sold as CID 6584729 (by Vitas-M) were indistinguishable from those of compound **1**. Thus, commercial STK 580951 is, in fact, the same as our synthetic compound **1** and does not match PubChem CID 6584729.

Other analogues of spirocycle **1** were synthesized (Scheme 4) using phenylalanine, tryptophan, and N_ε-Boc-lysine. In all cases, the limiting reagent, isatin **6** or **7**, was completely consumed, and the reaction gave the desired cycloadduct as a single diastereomer. The reactions of these amino acids were highly stereoselective, affording products with a relative configuration analogous to spiroindolinone **1**. We did not observe or isolate other diastereomers of the spiroindolinones **8**–**13**.

The formation of diastereomer **1**, and **8**–**13** was anticipated based on the work of Pavlovskaya et al.,[55] but is best explained by examining a larger body of work involving reactions of amino acids, enones, and either isatins or phenylglyoxalate derivatives, which are essentially acyclic analogues of isatins. All known reactions of amino acids, isatins, and enones react to give products consistent with *syn–anti* azomethine ylides (Figure 2, configuration A).[57–60] However, in the corresponding reactions with phenylglyoxylate, the reactive configuration of the azomethine ylide seems to depend on the type of amino acid: proline gives products consistent with *syn–anti* azomethine ylides (Figure 2, configuration A),[61] whereas acyclic amino acids give products consistent with *anti–anti* azomethine ylides (Figure 2, configuration B).[62–64]

The 1,3-dipolar cycloaddition can proceed through either an *endo* or *exo* transition state. The products derived from all known reactions of amino acids, acyclic enones, and either isatins or phenylglyoxalates can be rationalized to arise through *endo* transition states (Scheme 5, path A).[58–62,65] In contrast, reactions of amino acids, maleimides, and either isatins or phenyl-

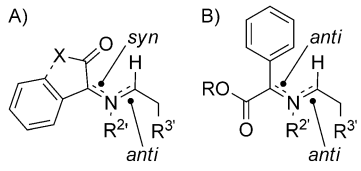
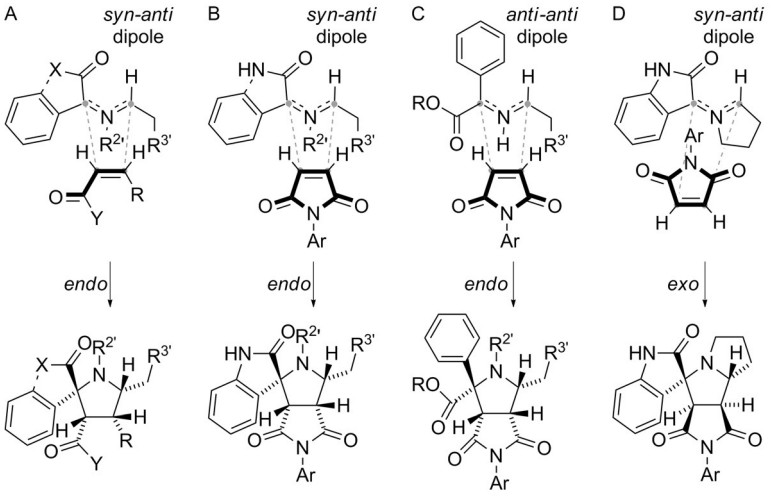


Figure 2. Stereochemical configurations of azomethine ylides.



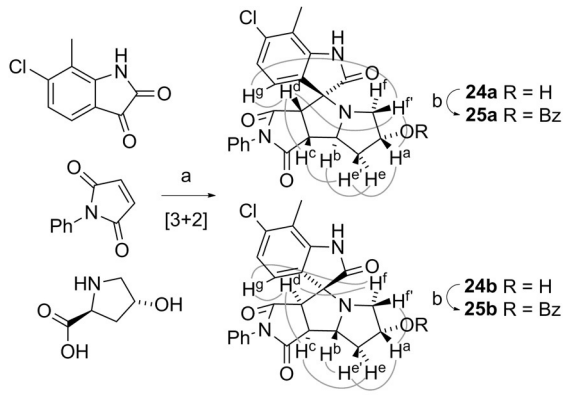
Scheme 5. *Endo*/*exo* selectivity in dipolar cycloadditions of azomethine ylides.

glyoxalates can proceed through *endo* or *exo* transition states, depending on the structure of the amino acid. Acyclic amino acids give products consistent with *endo* transition states (Scheme 5, paths B and C).[55,58,62–65] The only known reaction of a cyclic six-membered ring amino acid, pipecolic acid, with isatin and an acyclic dipolarophile also gives products consistent with an *endo* transition state (Scheme 5, path B);[59] however, the stereochemical outcome in the reaction of azomethine ylides with acyclic dipolarophiles cannot be extrapolated to reactions with maleimides.[56,60a] In contrast, the cyclic five-membered ring amino acid proline gives products consistent with an *exo* transition state (Scheme 5, path D).[56]

Similar ylides can be accessed from three-component reactions with amines instead amino acids, but there are cases in which the trends in ylide configuration[66] and *endo*/*exo* selectivity[67] no longer hold. Notably, Ardill et al. showed that 1,3-dipoles derived from *N*-methylpiperazine aminals and related compounds favor *exo* adducts over *endo* adducts—sometimes exclusively *exo*—in toluene at reflux.[67]

The assumption that isatins would react through path C (Scheme 5) may have led to the mis-assignment of the compound CID 6584729 by the commercial supplier along with over 100 spiroindolinones in the PubChem database. We cannot be sure of the stereochemistry of the compound CID 6584729 that was tested by Lindquist and co-workers, as the experimental data for compounds in PubChem assay IDs 1979, 2467, and 2423 were never reported.[68] One cannot rule out the possibility that the compound CID 6584729 tested in those assays was correctly assigned and generated through a more lengthy synthetic route than the one-pot reaction used in this and related work.

To provide access to diastereomeric spiroindolinones with defined absolute stereochemistries, we carried out a three-component coupling with isatin **6d**, *N*-phenylmaleimide, and (2*S*,4*R*)-4-hydroxyproline (Scheme 6). After 16 h the reaction generated an inseparable mixture of two spiroindolinones **24a** and **24b** in 30% yield along with unreacted isatin. The two optically pure stereoisomers were readily separated by silica gel chromatography after benzoylation of the hydroxy groups to afford esters **25a** and **25b**. The relative stereochemistry was assigned on the basis of diagnostic nOes. In particular, in both spiroindolinones **25a** and **25b**, there is an nOe between the bridgehead proton H^d and the arene proton H^g on the indolone ring. In spiroindolinone **25a**, protons $H^{f'}$, H^a, and $H^{e'}$ on the β-face of the proline ring exhibit vicinal nOes between each other. Protons $H^{e'}$ and $H^{f'}$ on the β-face of the proline ring exhibit long-range nOes with protons H^c and H^d on the succinimide ring, respectively; proton $H^{f'}$ exhibits highly diagnostic long-range nOes to the indolone aryl proton H^g. In spiroindolinone **25b**, protons $H^{f'}$, H^a, $H^{e'}$, and H^b on the β-face of the proline ring exhibit vicinal nOes between each other. Protons H^e and H^f on the α-face of the proline ring exhibit long-range nOes with protons H^c and H^d on the succinimide ring, respectively; proton H^f exhibits a highly diagnostic long-range nOe to the indolone aryl proton H^g. Thus, the [3+2] cycloaddition of *trans*-hydroxyproline proceeds via *exo* addition of maleimide to an azomethine ylide with a *syn*–*anti* configuration (Scheme 5, path D).



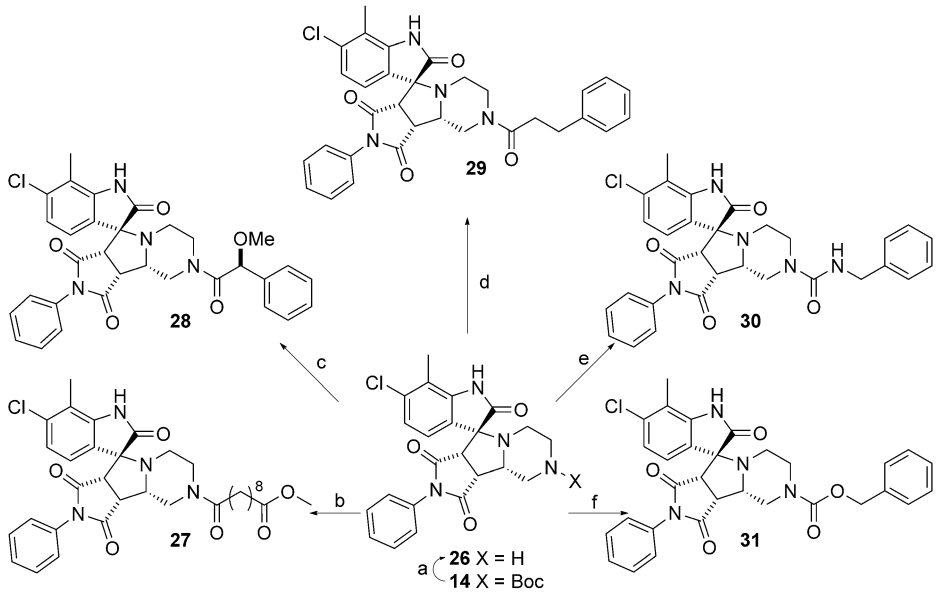
Scheme 6. Stereoselectivity in the three-component, one-pot [1,3]-dipolar cycloaddition with (2*S*,4*R*)-4-hydroxyproline. Stereochemistry was established by nOes (grey lines). *Reagents and conditions*: a) MeOH/H_2O (3:1), 90 °C, 16 h; b) BzCl (1.1 equiv), Et_3N (1.2 equiv), DMF, 23 °C, 20 h.

The reaction of the six-membered ring amino acid N_ε-Boc-piperazine-2-carboxylic acid proceeds in manner analogous with pipecolic acid (Scheme 5, path B) to afford spirocyclic indolinone **14** as a single diastereomer. The relative stereochemistry of spirocyclic piperazine **14** was secured with nOes after removal of the Boc group and shown to match that of compound **1**. Related spiroindolinones **15**–**23** were also prepared stereoselctively from N_ε-Boc-piperazine-2-carboxylic acid (Scheme 4).

The Boc group was removed from the spirocyclic piperazine **14** using trifluoroacetic acid to give piperazine **26** in 90% yield (Scheme 7). Piperazine **26** served as the precursor for various *N*-acyl derivatives **27**–**31** in the following reactions (Scheme 7). Compound **27** was synthesized by acylating **26** with methyl 10-chloro-10-oxodecanoate in the presence of sodium carbonate. Carbodiimide-mediated coupling of (*S*)-2-methoxy-2-phenylacetic acid with the racemic piperazine **26** in the presence of triethylamine resulted in a mixture of diastereomers; only one diastereomer **28** was readily purified, but the relative stereochemistry was not assigned. Acylation of **26** with hydrocinnamoyl chloride in the presence of triethylamine afforded amide **29**. The reaction of piperazine **26** with benzyl isocyanate generated the urea **30**. Carbamate **31** was synthesized by acylation of piperazine **26** with benzyl chloroformate.

Structure–activity relationships

CID 6584729 was reported to enhance the effect of fluconazole against the partially resistant clinical isolate of *C. albicans* CaCi-8 at an EC_{50} value of 0.12 μM.[68] We determined the antifungal potency of the new spiroindolinones in combination with fluconazole against a susceptible strain (HLY4123) derived from a commonly used laboratory strain of *C. albicans*. The activity of compound **1** was promising, with an EC_{50} value of 0.011 μM. We then compared the activity of compound **1**, derived from phenylalanine, with the activity of spiroindolinones derived from other amino acids (Table 1; compounds **1**, **8**, **9**, and **10**).



Scheme 7. Synthesis of pentacyclic pyrrolidines by further substitutions to compound **26**. *Reagents and conditions*: a) TFA/CH_2Cl_2 (1:1), 23 °C, 15 min, 90%; b) methyl 10-chloro-10-oxodecanoate (1.04 equiv), Na_2CO_3 (1.0 equiv), CH_2Cl_2, 23 °C, 30 min, 53%; c) (*S*)-2-methoxy-2-phenylacetic acid (1.3 equiv), EDC (2.1 equiv), Et_3N (3.5 equiv), CH_2Cl_2, 23 °C, 20 min, 45%; d) 3-phenylpropanoyl chloride (1.05 equiv), Et_3N (1.1 equiv), CH_2Cl_2, 23 °C, 3 h, 64%; e) (isocyanatomethyl)benzene (1.1 equiv), CH_2Cl_2, 23 °C, 4 h, 75%; f) benzyl chloroformate (1.1 equiv), DIPEA (2.1 equiv), CH_2Cl_2, 0–23 °C, 2.5 h, 54%.

Neither tryptophan nor N_ε-Boc-lysine derivatives were better than the parent compound **1** derived from phenylalanine. Regardless of the maleimide substituent, *N*-benzylisatin derivatives exhibited relatively low activity (compounds **11** and **12**). Compound **13**, derived from 6-chloro-7-methylisatin, but lacking the 4-fluoro substituent, was exceedingly potent with an EC_{50} value of 1 nM.

When we used the non-natural amino acid N_ε-Boc-piperazine-2-carboxylic acid, the resulting spirocyclic piperazine **14** was still highly active with an EC_{50} value of 5.6 nM. *N*-Benzylsuccinimide derivatives **18**, **19**, and **21** were not highly active. The substituents on the indolone ring were still important, even with the pentacyclic piperazine core (compounds **17**, **20**, **22**, and **23**). We removed the Boc group from the piperazine ring of compound **14**, leading to a loss of potency (compound **26**). Surprisingly, the carboxyl oxygen atom of the carbamate moiety appears to be essential for high potency, because carbamates **14** and **31** were more potent than amides **27** and **28**. Even more revealing, the isosteric amide **29** and urea **30** were two and three orders of magnitude less active, respectively, than benzyloxycarbamate **31**. Ultimately, benzyloxycarbamate **31** proved to be exquisitely active in improving the efficacy of fluconazole, with an EC_{50} value of 300 pM. The two hydroxyproline adducts **25a** and **25b** exhibited almost no activity under the assay conditions; these results are not surprising, given that the relative stereochemistry of those compounds is different from all the other compounds that were tested.

In general, substitution of small hydrophobic groups at the 6- and 7-positions of the indolone ring and phenyl substitution at the 3′-position of the central pyrrolidine improves antifungal activity. Moreover, a phenyl moiety—not benzyl—in the succinamide ring enhances antifungal activity. When the piperazine is present in the polycyclic pyrrolidine, a carbamoyl moiety, but not the acyl groups, on the piperazine ring significantly improves antifungal activity. On the other hand, fluorinat-

Table 1. Structure–activity relationships for polycyclic pyrrolidines against the susceptible strain HLY4123 of *C. albicans* in the presence of fluconazole (0.25 μg mL^{-1}).

Compd[a]	$R^{5'}$	$R^{2'}$	$R^{3'}$	R^5	R^6	R^7	R^1	EC_{50} [μM][b]
1	4-fluorophenyl	H	Ph	H	Cl	CH_3	H	0.011 ± 0.004
8	Bn	H	Ph	H	H	H	H	~10 ± 1.3
9	Bn	H	3-indolyl	H	H	H	H	~10 ± 1.8
10	Bn	H	$(CH_2)_3N(Boc)$	H	H	H	H	> 100
11	Bn	H	Ph	H	H	H	Bn	> 10
12	Bn	H	Ph	H	H	H	Bn	> 100
13	Bn	H	Ph	H	Cl	CH_3	H	0.001 ± 0.0005
14	Bn	$CH_2CH_2N(Boc)$		H	Cl	CH_3	H	0.0056 ± 0.003
15	4-fluorophenyl	$CH_2CH_2N(Boc)$		H	Cl	CH_3	H	0.037 ± 0.001
16	3,5-bis(F_3C)phenyl	$CH_2CH_2N(Boc)$		H	Cl	CH_3	H	0.0237 ± 0.01
17	Ph	$CH_2CH_2N(Boc)$		H	Cl	CH_3	Bn	> 100
18	Bn	$CH_2CH_2N(Boc)$		H	H	H	Bn	0.0318 ± 0.4
19	Bn	$CH_2CH_2N(Boc)$		H	H	H	H	> 100
20	Ph	$CH_2CH_2N(Boc)$		H	H	H	Bn	> 100
21	Bn	$CH_2CH_2N(Boc)$		MeO	H	H	H	230 ± 5.7
22	Ph	$CH_2CH_2N(Boc)$		H	H	CH_3	H	0.213 ± 0.08
23	Ph	$CH_2CH_2N(Boc)$		H	CH_3	CH_3	H	0.0057 ± 0.006
26	Ph	CH_2CH_2NH		H	Cl	CH_3	H	~10 ± 1.5
27	Ph	$CH_2CH_2N[CO(CH_2)_8CO_2Me]$		H	Cl	CH_3	H	0.0379 ± 0.009
28	Ph	$CH_2CH_2N[COCH(OCH_3)C_6H_5]$		H	Cl	CH_3	H	0.035 ± 0.007
29	Ph	$CH_2CH_2N[CO(CH_2)_2Ph]$		H	Cl	CH_3	H	0.0181 ± 0.004
30	Ph	$CH_2CH_2N[CONHCH_2Ph]$		H	Cl	CH_3	H	0.256 ± 0.1
31	Ph	$CH_2CH_2N(Cbz)$		H	Cl	CH_3	H	0.0003 ± 0.00001

[a] See Schemes 4 and 7 for structures. [b] Each value is the arithmetic mean ± SD of three independent experiments.

ed substituents at the *para* and *meta* positions of the phenyl group in the succinamide ring as well as N-substitution of the indolone moiety diminish the activity of the spiroindolinones against *C. albicans*.

Activity against resistant cell lines

A variety of resistant clinical isolates of *C. albicans* were screened with 64 μg mL^{-1} fluconazole and compound **31** at a single dose (3 μM) in a broth microdilution assay (Table 2).[69]

Table 2. Effect of compound **31** on the growth of resistant clinical strains in the presence of fluconazole.

Isolate[a]	MIC_{flc}[a]	Growth (OD_{600})[b]		Potency
		+ Fluc[c]	+ Compd **31**[d] + Fluc[c]	+ Compd **31** EC_{50} [nM][c,e]
17	32	3.96	0.76	5 ± 0.011
23	32	6.77	0.60	53 ± 0.005
26	32	6.44	0.83	2 ± 0.002
33	64	7.38	0.88	11 ± 0.008
36	64	5.08	1.07	5 ± 0.251
45	128	9.17	1.03	16 ± 0.002

[a] From ref. [69]. [b] Growth measured after 16 h in SC at 30 °C. [c] [fluconazole] = 64 μg mL^{-1}. [d] [compound **31**] = 3 μM. [e] Each value is the arithmetic mean ± SD of three independent experiments.

The strains grow at dramatically different rates. The published fluconazole minimum inhibitory concentrations (MICs) for these isolates convey the level of resistance. Strains for which growth in the presence of compound **31** and fluconazole was less than 25% of growth in the presence of fluconazole alone (isolates 17, 23, 26, 33, 36, and 45) were selected for determination of EC_{50} values. Compound **31** was particularly active against clinical isolates 17, 26, and 36 and exhibited good activity against the highly resistant isolate 45.

A checkerboard assay was used to determine the fractional inhibitory concentrations (FICs) for compound **31** and fluconazole against the fluconazole-susceptible strain (HLY4123) and two fluconazole-resistant clinical isolates 26 and 45 (Table 3).[69] In all of the strains tested, the FIC index was below 0.5, fitting the classical definition of synergy.[70] Compound **31** alone did not have measurable toxicity against any of the strains at the solubility limit (between 30 and 300 μM). In the strains tested,

Table 3. FIC indices for compound **31** and fluconazole in different strains of *C. albicans*.[a]

Strain	MIC_{cpd}	MIC_{cpd}(+ Flc)	MIC_{flc}	MIC_{flc}(+ Cpd)	FICI
HLY4123	> 300	0.03	0.5	0.125	0.25
26	> 30	0.3	836	105	0.14
45	> 30	0.03	836	105	0.13

[a] MIC_{90} measured in μM; MIC data are derived from single replicates of two independent experiments, and values were consistent between experiments.

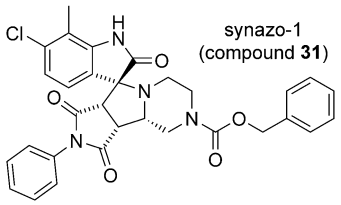


Figure 3. Structure of compound **31**, renamed synazo-1.

fluconazole dramatically enhances the activity of compound **31** against *C. albicans*. Conversely, compound **31** makes fluconazole more potent against those same strains, but the effect is less dramatic. We have named compound **31** 'synazo-1' (Figure 3).

Greater than 90% inhibition of *C. albicans* sterol α-demethylase (a.k.a. Erg11 or CYP51) would be expected at ten times the K_i value for fluconazole, which has been determined to be 0.03 μM.[71,72] When synazo-1 is present at 300 nM in the susceptible strain, the MIC_{90} for fluconazole is decreased from 0.5 to 0.125 μM, consistent with the theoretical limit for fluconazole potency of ~0.3 μM.

Cytotoxicity of synazo-1 against mammalian cells

We compared the cytotoxicity of spiroindoline **1** and synazo-1 against NIH 3T3 cells at higher concentrations, up to 1.5 mM (Figure 4). Both compounds exhibited only weak cytotoxicity. Synazo-1 was slightly more cytotoxic, but not at the concentrations required for antifungal synergy. According to PubChem, CID 6584729 was previously tested for cytotoxicity against NIH 3T3 cells (PubChem AID 2387), but the EC_{50} value was ≥ 160 μM, the limit of the assay. It is unclear how the biological activities reported for CID 6584729 should relate to that of the diastereomer **1**, or whether they were even distinct compounds.

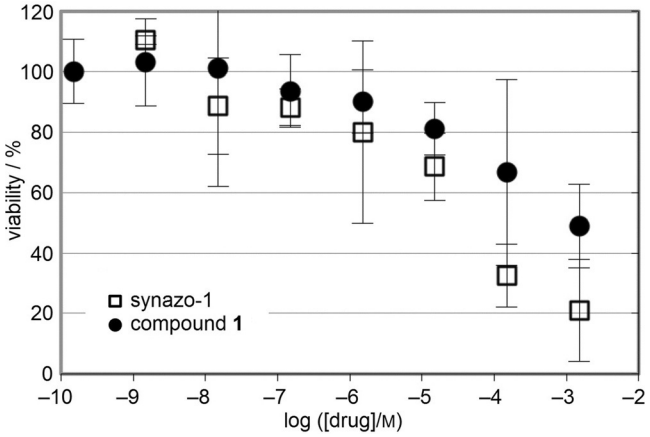


Figure 4. Cytotoxicity of synazo-1 and compounds **1** against NIH 3T3 cells; data are the arithmetic means ± SD of three independent experiments.

Drug-like parameters for synazo-1

A wide range of readily calculated properties are often used as indicators of oral bioavailability. The calculated physicochemical properties of synazo-1 were compared with typical ranges for lead-like molecules.[73–75] Synazo-1 flags just one of the common warnings for drug lead-like properties (Table 4): molecular weight. For comparison, the orally available azole posoconazole is outside the range on four of the parameters. It is widely recognized that the average molecular weight and complexity of newly approved oral drugs has been increasing with each year.[76,77] Both the carbamate and imide moieties are potential liabilities for metabolism, yet when the stability of synazo-1 was tested in 10% FBS/phosphate-buffered saline at 37 °C, no decomposition was observed over 16 h.

Table 4. Calculated physicochemical properties of synazo-1.[a]

Property	Range	Synazo-1
log *P*	−4.0 to 4.2	4.173
TPSA [Å^2]	≤120	99.3
M_r [Da]	≤460	571
N + O	≥1	9
H-bond donors	≤5	1
Rotatable bonds	≤10	4
Halogens	≤7	1
Fraction sp^3	0.15–0.80	0.20
H-bond acceptors	≤9	5

[a] Calculated with the Molinspiration property calculation service: www.molinspiration.com/cgi-bin/properties.

Conclusions

In summary, we have designed, synthesized, and studied spiroindolinones inspired by CID 6584729, which was previously reported to exhibit activity against *C. albicans* in combination with fluconazole. The relative stereochemistry of compound **1** and analogues was secured through 2D NMR experiments. The three-component, one-pot [3 + 2] dipolar cycloaddition of isatins, amino acids, and maleimides was found to proceed through *endo* addition of maleimides to a *syn–anti* azomethine ylide in all cases except for a proline derivative. A number of the new spiroindolinones were substantially more potent against *C. albicans* than the original lead compound **1** when used in combination with fluconazole. In particular, synazo-1 was exquisitely potent, with an EC_{50} value of 300 pM against a susceptible strain. Synazo-1 also exhibited low nanomolar activity against a number of resistant isolates of *Candida*. When tested in both susceptible and resistant strains of *C. albicans*, synazo-1 was a true synergizer with an FIC index below 0.5. Synazo-1 has many of the calculable parameters associated with orally available drug molecules and represents a promising candidate for development as an antifungal synergizer.

Experimental Section

Chemistry

General procedures: NMR spectral data were recorded at room temperature using a Bruker 500 or 600 MHz spectrometer, unless stated otherwise. The NMR data are reported as follows: chemical shifts in ppm from an internal tetramethylsilane standard on the δ scale, multiplicity (br = broad, app = apparent, s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet), coupling constants (Hz), and integration. Analytical thin-layer chromatography (TLC) was performed using EMD Reagents 0.25 mm silica gel 60-F plates. "Flash" chromatography on silica gel was performed with Silicycle silica gel (40-63 μm). All reactions were carried out under an atmosphere of nitrogen in glassware that was evacuated and backfilled with nitrogen three times. Reactions were carried out at room temperature unless otherwise indicated. Unless otherwise noted, all reagents were commercially obtained and, where appropriate, purified prior to use. THF, Et_2O, DMF, and CH_2Cl_2 were dried by filtration through alumina according to the procedure of Grubbs and co-workers.[78] For final compounds the purity was determined by HPLC (Agilent Technologies series 1200). The HPLC instrument consisted of an Agilent Technologies series 1200 autosampler, series 1200 UV/Vis detector, series 1100 pump, using ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The analytical column was a reversed-phase Waters Nova-pak C_{18} 150 mm × 3.9 mm column. A gradient elution was used (flow rate: 0.2 mL min^{-1}), starting with 80% H_2O and progressing to 100% CH_3CN over a period of 1 h, with both solvents containing 0.1% formic acid. All compounds have purity ≥95% (λ = 254 nm) by HPLC.

Synthesis of pyrrolidines by three-component dipolar cycloaddition (1, 8–23, 24a, and 24b):[54] A 100 mL round-bottom flask was charged with substituted isatin (1.0 equiv), N-substituted maleimide (1.1 equiv), the amino acid (1.1 equiv), and a stir bar. A 3:1 (*v*/*v*) mixture of H_2O and MeOH was added to the reaction flask such that the concentration of isatin was 0.25 M. The reaction was heated at reflux by immersing the reaction flask in a hot oil bath at 90 °C up to the level of the flask's contents. Initially a clear solution was obtained, and CO_2 evolution was observed. However, after a few hours the reaction mixture became cloudy. The reaction was monitored for consumption of the substituted isatin by TLC (EtOAc/hexanes). Upon consumption of the substituted isatin, the reaction was cooled to room temperature. Next, the reaction mixture was quenched by pouring it into a mixture of ice and saturated aqueous $NaHCO_3$. The resulting solid was washed thoroughly with H_2O in Büchner funnel to afford a grey solid. The solid was then dissolved in a minimum amount of CH_2Cl_2 and purified by flash chromatography with EtOAc/hexanes (1:1) to afford the racemic substituted pyrrolidine.

(±)-(3*R*,3′*R*,3a′*R*,6a′*S*)-3′-Benzyl-6-chloro-5′-(4-fluorophenyl)-7-methyl-2′,3′,3a′,6a′-tetrahydro-4′*H*-spiro[indoline-3,1′-pyrrolo[3,4-*c*]pyrrole]-2,4′,6′(5′*H*)-trione (1): A 100 mL round-bottom flask was charged with 1-(4-fluorophenyl)-1*H*-pyrrole-2,5-dione (0.50 g, 2.55 mmol, 1.0 equiv), *p*-fluoro-*N*-phenylmaleimide (0.53 g, 2.8 mmol, 1.1 equiv), L-phenylalanine (0.46 g, 2.8 mmol, 1.1 equiv), and a stir bar. A 3:1 mixture of H_2O and MeOH (11 mL) was added to the reaction flask. The content of the reaction flask was heated at reflux by immersing the reaction flask in a hot oil bath up to the level of the flask's contents. Initially a clear solution was obtained, and CO_2 was expelled. After few hours a cloudy solution was observed. Upon consumption of the substituted isatin (16 h), the reaction was allowed to cool to room temperature. Next, the reac-

tion mixture was quenched by pouring it into a mixture of ice and saturated aqueous $NaHCO_3$. The resulting solid was washed thoroughly with H_2O in Büchner funnel to afford a grey solid. The solid was then dissolved in a minimum amount of CH_2Cl_2 and purified by flash chromatography with various combinations of EtOAc/hexanes to afford pyrrolidine **1** as a white solid (0.93 mg, 1.9 mmol, 74%): $R_f = 0.35$ (1:1 EtOAc/hexanes); mp: 212–214 °C. The ^{1}H NMR chemical shifts were concentration-dependent in $CDCl_3$, particularly within the range 0.5–2 mM. ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 8.00$ (s, 1H), 7.41–7.38 (m, 2H), 7.27–7.16 (m, 6H), 7.02 (d, $J = 8.0$ Hz, 2H), 6.81 (d, $J = 8.5$ Hz, 1H), 4.72–4.71 (m, 1H), 3.75–3.69 (m, 1H), 3.45 (dd, $J = 14.0$, 4.0 Hz, 1H), 2.73 (dd, $J = 13.8$, 10.5 Hz, 1H), 2.16 (s, 1H), 1.99 ppm (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 180.4$, 175.1, 174.4, 163.4, 161.4, 140.5, 139.1, 136.2, 128.9, 128.8, 128.3, 127.6, 126.7, 124.6, 124.5, 123.5, 118.2, 116.6, 116.4, 68.2, 58.9, 51.6, 47.6, 37.9, 13.5 ppm; IR (thin film): $\tilde{\nu} = 3201$, 3065, 1710, 1696, 1623, 1601, 1510 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{28}H_{29}ClN_4O_5Na$ $[M+Na]^+$ 559.1724, found 559.1743; HPLC purity: 95.76%, $t_R =$ 21.73 min.

(±)-(3*R*,3′*R*,3a′*R*,6a′*S*)-3′,5′-Dibenzyl-2′,3′,3a′,6a′-tetrahydro-4′*H*-spiro[indoline-3,1′-pyrrolo[3,4-*c*]pyrrole]-2,4′,6′(5′*H*)-trione (8): Using the general procedure for the synthesis of pyrrolidines outlined above, indoline-2,3-dione (0.37 g, 2.5 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **8** as a white solid (0.65 mg, 1.5 mmol, 60%): $R_f = 0.50$ (1:1 EtOAc/hexanes); mp: 138–140 °C; ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.50$–7.49 (m, 2H), 7.40–7.38 (m, 3H), 7.29 (br s, 1H), 7.26–7.25 (m, 1H), 7.24–7.22 (m, 3H), 7.18–7.16 (m, 2H), 6.80 (t, $J = 7.5$ Hz, 1H), 6.69 (d, $J = 7.8$ Hz, 1H), 6.50 (d, $J = 7.5$ Hz, 1H), 4.83 (d, $J = 14.0$ Hz, 1H), 4.69 (d, $J = 14.0$ Hz, 1H), 4.68–4.67 (m, 1H), 3.58–3.56 (m, 1H), 3.42 (d, $J = 7.6$ Hz, 2H), 2.60 (dd, $J = 13.8$, 10.4 Hz, 1H), 2.01 ppm (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 313 K): $\delta = 180.1$, 175.8, 174.5, 140.3, 139.3, 135.8, 129.8, 129.0, 128.9, 128.8, 128.7, 128.1, 126.9, 126.5, 126.4, 122.6, 109.7, 67.6, 58.5, 51.5, 47.7, 42.7, 38.2 ppm; IR (thin film): $\tilde{\nu} = 3850$, 3646, 2971, 2843, 1697, 1619, 1054, 1032 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{27}H_{23}N_3O_3Na$ $[M+Na]^+$ 460.1637, found 460.1620; HPLC purity: 100%, $t_R = 20.04$ min.

(±)-(3*R*,3′*R*,3a′*R*,6a′*S*)-3′-((1*H*-Indol-3-yl)methyl)-5′-benzyl-2′,3′,3a′,6a′-tetrahydro-4′*H*-spiro[indoline-3,1′-pyrrolo[3,4-*c*]pyrrole]-2,4′,6′(5′*H*)-trione (9): Using the general procedure for the synthesis of pyrrolidines outlined above, indoline-2,3-dione (0.37 g, 2.5 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **9** as a yellow solid (0.83 mg, 1.7 mmol, 70%): $R_f = 0.35$ (1:1 EtOAc/hexanes); mp: 130–132 °C; ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.96$ (br s, 1H), 7.60 (d, $J = 7.9$ Hz, 1H), 7.52 (d, $J = 7.3$ Hz, 2H), 7.42–7.39 (m, 2H), 7.36–7.33 (m, 1H), 7.30–7.29 (m, 1H), 7.17–7.11 (m, 2H), 7.09–7.06 (m, 2H), 6.80–6.77 (m, 1H), 6.62 (d, $J = 7.7$ Hz, 1H), 6.49 (d, $J = 7.5$ Hz, 1H), 4.86 (d, $J = 14.0$ Hz, 1H), 4.80–4.76 (m, 1H), 4.70 (d, $J = 14.0$ Hz, 1H), 3.60 (t, $J = 7.7$ Hz, 1H), 3.49 (dd, $J = 14.6$, 3.7 Hz, 1H), 3.41 (d, $J = 7.7$ Hz, 1H), 2.82 (dd, $J = 14.6$, 10.3 Hz, 1H), 2.13 ppm (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 313 K): $\delta = 180.3$, 176.0, 174.8, 140.4, 136.2, 135.9, 129.6, 129.0, 128.8, 128.2, 127.5, 126.8, 126.5, 122.5, 122.4, 122.1, 119.5, 119.1, 113.6, 111.1, 109.7, 67.7, 58.1, 51.6, 47.7, 42.6, 27.7 ppm; IR (thin film): $\tilde{\nu} = 3679$, 2971, 2864, 2843, 1695, 1619, 1054, 1032 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{29}H_{24}N_4O_3Na$ $[M+Na]^+$ 499.1746, found 499.1739; HPLC purity: 98.81%, $t_R = 21.98$ min.

(±)-*tert*-Butyl (4-((3*R*,3′*R*,3a′*R*,6a′*S*)-5′-benzyl-2,4′,6′-trioxo-3′,3a′,4′,5′,6′,6a′-hexahydro-2′*H*-spiro[indoline-3,1′-pyrrolo[3,4-*c*]pyrrol]-3′-yl)butyl)carbamate (10): Using the general procedure for the synthesis of pyrrolidines outlined above, indoline-2,3-dione (0.37 g, 2.5 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **10** as a white solid (0.42 mg, 0.82 mmol, 33%): $R_f = 0.35$ (4:6 EtOAc/hexanes); mp: 109–111 °C; ^{1}H NMR (600 MHz, $CDCl_3$): $\delta =$ 7.45–7.44 (m, 2H), 7.38–7.33 (m, 3H), 7.29 (br s, 1H), 7.20–7.17 (m, 1H), 6.79–6.76 (m, 2H), 6.36 (d, $J = 7.4$ Hz, 1H), 4.78 (d, $J = 14.0$ Hz, 1H), 4.70–4.63 (m, 1H), 4.61 (d, $J = 14.0$ Hz, 1H), 4.34–4.32 (m, 1H), 3.50 (t, $J = 7.7$ Hz, 1H), 3.42 (d, $J = 7.7$ Hz, 1H), 3.18–3.08 (m, 2H), 2.06–1.91 (m, 2H), 1.57–1.53 (m, 2H), 1.43 ppm (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$, 313 K): $\delta = 180.15$, 175.8, 174.5, 140.3, 135.8, 129.8, 129.2, 128.8, 128.2, 126.5, 126.4, 122.7, 109.8, 68.1, 58.3, 51.7, 48.1, 42.7, 40.1, 31.2, 30.0, 28.5, 24.6, 14.3 ppm; IR (thin film): $\tilde{\nu} =$ 3707, 2971, 2843, 1345, 1054, 1032 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{29}H_{34}N_4O_5Na$ $[M+Na]^+$ 541.2427, found 541.2411; HPLC purity: 97.81%, $t_R = 20.74$ min.

(±)-(3*R*,3′*R*,3a′*R*,6a′*S*)-1,3′,5′-Tribenzyl-2′,3′,3a′,6a′-tetrahydro-4′*H*-spiro[indoline-3,1′-pyrrolo[3,4-*c*]pyrrole]-2,4′,6′(5′*H*)-trione (11): Using the general procedure for the synthesis of pyrrolidines outlined above, 1-benzylindoline-2,3-dione (0.07 g, 0.32 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **11** as a white solid (0.08 mg, 0.15 mmol, 46%): $R_f = 0.60$ (1:1 EtOAc/hexanes); mp: 173–175 °C; ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.50$–7.49 (m, 2H), 7.39–7.12 (m, 14H), 6.82–6.79 (m, 1H), 6.64 (d, $J = 9.3$ Hz, 1H), 6.54 (d, $J = 8.7$ Hz, 1H), 4.88–4.81 (m, 2H), 4.80–4.72 (m, 1H), 4.71–4.65 (m, 2H), 3.64–3.60 (m, 1H), 3.44–3.39 (m, 2H), 2.61 (dd, $J = 16.6$, 12.3 Hz, 1H), 2.01 ppm (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 313 K): $\delta = 178.7$, 175.9, 174.4, 142.6, 139.3, 135.8, 135.4, 129.8, 129.0, 128.9, 128.9, 128.8, 128.6, 128.1, 127.8, 127.3, 126.5, 126.4, 125.8, 122.6, 109.1, 67.5, 58.5, 51.8, 47.7, 43.6, 42.7, 38.2 ppm; IR (thin film): $\tilde{\nu} = 3850$, 3708, 2971, 2864, 1453, 1054, 1032 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{34}H_{29}N_3O_3Na$ $[M+Na]^+$ 550.2106, found 550.2108; HPLC purity: 98.45%, $t_R = 23.19$ min.

(±)-(3*R*,3′*R*,3a′*R*,6a′*S*)-1,3′-Dibenzyl-5′-phenyl-2′,3′,3a′,6a′-tetrahydro-4′*H*-spiro[indoline-3,1′-pyrrolo[3,4-*c*]pyrrole]-2,4′,6′(5′*H*)-trione (12): Using the general procedure for the synthesis of pyrrolidines outlined above, 1-benzylindoline-2,3-dione (0.30 g, 1.3 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **12** as a yellow solid (0.38 mg, 0.74 mmol, 57%): $R_f = 0.60$ (1:1 EtOAc/hexanes); mp: 183–185 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO): $\delta = 7.57$–7.52 (m, 2H), 7.46–7.44 (m, 1H), 7.40–7.37 (m, 4H), 7.34–7.33 (m, 3H), 7.31–7.25 (m, 3H), 7.21–7.17 (m, 2H), 7.14 (dd, $J = 7.4$, 1.5 Hz, 1H), 6.97 (t, $J = 7.5$ Hz, 1H), 6.84 (d, $J = 7.8$ Hz, 1H), 4.90 (d, $J =$ 15.6 Hz, 1H), 4.76 (d, $J = 15.6$ Hz, 1H), 4.52–4.50 (m, 1H), 3.73 (dd, $J = 7.5$, 1.8 Hz, 1H), 3.63 (dd, $J = 7.8$, 2.7 Hz, 1H), 2.61 (dd, $J = 16.6$, 12.3 Hz, 1H), 3.32 (br s, 1H), 2.82–2.78 ppm (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 313 K): $\delta = 178.7$, 175.9, 174.4, 142.6, 139.3, 135.8, 135.4, 129.8, 129.0, 128.9, 128.9, 128.8, 128.6, 128.1, 127.8, 127.3, 126.5, 126.4, 125.8, 122.6, 109.1, 67.5, 58.5, 51.8, 47.7, 43.6, 42.7, 38.2 ppm; IR (thin film): $\tilde{\nu} = 3680$, 2966, 2865, 1706, 1054, 1032 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{34}H_{29}N_3O_3Na$ $[M+Na]^+$ 550.2106, found 550.2108; HPLC purity: 95.07%, $t_R = 23.09$ min.

(±)-(3*R*,3′*R*,3a′*R*,6a′*S*)-3′-Benzyl-6-chloro-7-methyl-5′-phenyl-2′,3′,3a′,6a′-tetrahydro-4′*H*-spiro[indoline-3,1′-pyrrolo[3,4-*c*]pyrrole]-2,4′,6′(5′*H*)-trione (13): Using the general procedure for the synthesis of pyrrolidines outlined above, 6-chloro-7-methylindoline-2,3-dione (0.30 g, 1.7 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **13** as a white solid (0.21 g, 0.44 mmol, 26%): $R_f =$ 0.60 (1:1 EtOAc/hexanes); mp: 168–170 °C; ^{1}H NMR (600 MHz,

[D$_6$]DMSO): δ = 10.12 (s, 1H), 7.54–7.51 (m, 2H), 7.45–7.43 (m, 1H), 7.39–7.35 (m, 4H), 7.29–7.27 (m, 2H), 7.19–7.17 (m, 1H), 6.97 (d, J = 8.0 Hz, 1H), 6.90 (d, J = 8.0 Hz, 1H), 4.55 (br s, 1H), 3.72 (t, J = 7.5 Hz, 1H), 3.53 (d, J = 7.8 Hz, 1H), 3.38–3.35 (m, 2H), 2.27 ppm (s, 3H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 142.8, 140.6, 135.1, 134.5, 132.9, 129.4, 129.3, 128.7, 127.6, 127.3, 126.4, 125.0, 121.9, 117.3 ppm; IR (thin film): $\tilde{\nu}$ = 3850, 3626, 2971, 2864, 1710, 1693, 1014, 1032 cm^{-1}; HRMS (ESI): m/z calculated for $C_{27}H_{22}ClN_3O_3Na$ $[M+Na]^+$ 494.1247, found 494.1255; HPLC purity: 95.00%, t_R = 23.41 min.

(±)-*tert*-Butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-6-chloro-7-methyl-1′,2,3′-trioxo-2′-phenyl-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (14): Using the general procedure for the synthesis of pyrrolidines outlined above, 6-chloro-7-methylindoline-2,3-dione (0.81 g, 4.1 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **14** as a light-pink solid (1.3 g, 2.5 mmol, 60%): R_f = 0.35 (4:6 EtOAc/hexanes); mp: 201–203 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 400 K): δ = 10.90 (s, 1H), 7.54–7.51 (m, 2H), 7.47–7.46 (m, 1H), 7.31 (d, J = 7.8 Hz, 2H), 7.06 (d, J = 7.8 Hz, 1H), 6.73 (d, J = 7.8 Hz, 1H), 4.31 (br s, 1H), 3.85 (t, J = 7.5 Hz, 2H), 3.64–3.65 (m, 1H), 3.55 (d, J = 7.8 Hz, 1H), 2.74–2.58 (m, 2H) 2.25 (app s, 4H), 2.13–2.08 (m, 1H), 1.41 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 313 K): δ = 177.9, 174.7, 173.4, 153.6, 142.8, 134.6, 132.1, 128.9, 128.5, 126.9, 124.6, 123.2, 122.8, 117.3, 79.1, 71.7, 62.3, 58.7, 57.6, 50.4, 45.9, 44.9, 27.9, 13.7 ppm; IR (thin film): $\tilde{\nu}$ = 3840, 3708, 3626, 2971, 2843, 1713, 1695, 1032 cm^{-1}; HRMS (ESI): m/z calculated for $C_{28}H_{29}ClN_4O_5Na$ $[M+Na]^+$ 559.1724, found 559.1743; HPLC purity: 96.96%, t_R = 22.18 min.

(±)-*tert*-Butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-6-chloro-2′-(4-fluorophenyl)-7-methyl-1′,2,3′-trioxo-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (15): Using the general procedure for the synthesis of pyrrolidines outlined above, 6-chloro-7-methylindoline-2,3-dione (0.12 g, 0.63 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **15** as a white solid (0.17 mg, 0.31 mmol, 49%): R_f = 0.35 (4:6 EtOAc/hexanes); mp: 246–248 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 400 K): δ = 10.42 (s, 1H), 7.39–7.35 (m, 2H), 7.32–7.29 (m, 2H), 7.02 (d, J = 7.8 Hz, 2H), 6.75 (d, J = 7.8 Hz, 1H), 4.35 (dd, J = 12.6, 1.8 Hz, 1H), 3.89–3.83 (m, 2H), 3.74–3.71 (m, 1H), 3.56 (d, J = 7.8 Hz, 1H), 2.74–2.58 (m, 2H) 2.77–2.73 (m, 1H), 2.74–2.54 (m, 1H), 2.50 (s, 3H), 2.29–2.09 (M, 1H), 1.44 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 313 K): δ = 178.5, 175.3, 173.9, 161.0, 154.2, 143.3, 135.1, 129.7, 128.8, 125.1, 123.7, 122.6, 117.9, 116.6, 116.4, 79.6, 72.2, 58.1, 51.0, 46.6, 45.6, 45.9, 28.5, 14.3 ppm; IR (thin film): $\tilde{\nu}$ = 3850, 3708, 2965, 2866, 1706, 1689, 1032 cm^{-1}; HRMS (ESI): m/z calculated for $C_{28}H_{28}ClFN_4O_5Na$ $[M+Na]^+$ 577.1630, found 577.1644; HPLC purity: 100%, t_R = 22.20 min.

(±)-*tert*-Butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-2′-(3,5-bis(trifluoromethyl)phenyl)-6-chloro-7-methyl-1′,2,3′-trioxo-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (16): Using the general procedure for the synthesis of pyrrolidines outlined above, 6-chloro-7-methylindoline-2,3-dione (0.50 g, 2.6 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **16** as a white solid (0.13 mg, 0.30 mmol, 12%): R_f = 0.5 (2:3 EtOAc/hexanes); mp: 199–201 °C; ^{1}H NMR (600 MHz, $CDCl_3$, 320 K): δ = 8.51 (s, 1H), 7.93–7.92 (m, 3H), 7.12 (d, J = 8.1 Hz, 1H), 6.70 (d, J = 8.0 Hz, 1H), 4.59 (br s, 1H), 4.11 (br s, 1H), 3.80–3.82 (m, 1H), 3.81–3.78 (m, 1H), 3.74–3.72 (m, 1H), 2.82–2.89 (m, 1H), 2.65–2.71 (m, 1H), 2.33–2.34 (m, 2H), 2.17 (s, 3H), 1.47 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 177.6, 174.2, 173.0, 154.4, 141.2, 136.8, 133.0, 132.7, 126.2, 124.5, 123.8, 123.7, 122.5, 118.4, 81.0, 72.9, 62.3, 58.3, 57.6, 50.5, 46.17, 45.6, 28.4, 13.8 ppm; IR (thin film): $\tilde{\nu}$ = 3187, 1724, 1706, 1680, 1599, 1276, 1132 cm^{-1}; HRMS (ESI): m/z calculated for $C_{30}H_{27}ClF_6N_4O_5Na$ $[M+Na]^+$ 695.1472, found 695.1450; HPLC purity: 98.46%, t_R = 24.08 min.

(±)-*tert*-Butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-1,2′-dibenzyl-1′,2,3′-trioxo-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]-pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (18): Using the general procedure for the synthesis of pyrrolidines outlined above, 1-benzylindoline-2,3-dione (0.30 g, 1.3 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **18** as a white solid (0.39 mg, 0.66 mmol, 56%): R_f = 0.8 (1:1 EtOAc/hexanes); mp: 191–192 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 7.38–7.37 (m, 4H), 7.36–7.32 (m, 5H), 7.31–7.27 (m, 1H), 7.22–7.20 (m, 1H), 6.87 (d, J = 7.8 Hz, 1H), 6.82–6.79 (m, 1H), 6.50 (d, J = 7.2 Hz, 1H), 4.87 (s, 2H), 4.65 (AB q, J = 14.7 Hz, 2H), 4.36–4.34 (m, 1H), 3.85–3.83 (m, 1H), 3.79–3.76 (m, 1H), 3.72–3.71 (m, 1H), 3.49 (d, J = 7.8 Hz, 1H), 2.68–2.64 (m, 1H), 2.51–2.53 (m, 1H), 2.12–2.09 (m, 1H), 1.44 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 313 K): δ = 176.1, 175.8, 174.5, 155.2, 143.7, 136.6, 136.4, 130.1, 129.2, 129.0, 128.2, 128.1, 127.9, 127.7, 126.7, 124.2, 122.7, 109.7, 79.7, 71.7, 58.2, 50.8, 46.3, 45.5, 43.1, 42.2, 28.5 ppm; IR (thin film): $\tilde{\nu}$ = 3850, 3626, 2971, 2862, 1707, 1693, 1678, 1032 cm^{-1}; HRMS (ESI): m/z calculated for $C_{35}H_{36}N_4O_5Na$ $[M+Na]^+$ 615.2584, found 615.2572; HPLC purity: 100%, t_R = 24.32 min.

(±)-*tert*-Butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-2′-benzyl-1′,2,3′-trioxo-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]-pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (19): Using the general procedure for the synthesis of pyrrolidines outlined above, indoline-2,3-dione (0.37 g, 2.5 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **19** as a white solid (0.66 mg, 1.3 mmol, 53%): R_f = 0.45 (1:1 EtOAc/hexanes); mp: 207–208 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.46–7.43 (m, 3H), 7.36–7.35 (m, 3H), 7.23–7.20 (m, 1H), 6.83–6.81 (m, 1H), 6.77 (d, J = 7.7 Hz, 1H), 6.32 (d, J = 6.8 Hz, 1H), 4.80 (d, J = 14.1 Hz, 1H), 4.63 (d, J = 14.1 Hz, 1H), 4.22–4.12 (m, 1H), 3.80–3.78 (m, 1H), 3.60–3.58 (m, 1H), 3.41 (d, J = 7.9 Hz, 1H), 2.79–2.45 (m, 2H), 2.27–2.18 (m, 2H), 1.46 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 310 K): δ = 180.4, 175.8, 174.7, 156.1, 140.5, 135.8, 129.7, 129.2, 128.9, 128.8, 128.2, 126.5, 122.6, 109.9, 68.1, 58.3, 51.6, 48.1, 42.6, 40.1, 31.1, 30.0, 28.5, 24.6 ppm; IR (thin film): $\tilde{\nu}$ = 3679, 2971, 2864, 1706, 1642, 1054, 1032, 1012 cm^{-1}; HRMS (ESI): m/z calculated for $C_{28}H_{30}N_4O_5Na$ $[M+Na]^+$ 525.2114, found 525.2106; HPLC purity: 100%, t_R = 22.09 min.

(±)-*tert*-Butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-2′-benzyl-1′,2,3′-trioxo-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]-pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (20): Using the general procedure for the synthesis of pyrrolidines outlined above, 1-benzylindoline-2,3-dione (0.30 g, 1.3 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **20** as a yellow solid (0.43 mg, 0.75 mmol, 71%): R_f = 0.75 (1:1 EtOAc/hexanes); mp: 210–212 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 400 K): δ = 7.54–7.51 (m, 2H), 7.46–7.44 (m, 1H), 7.35–7.34 (m, 6H), 7.29–7.26 (m, 2H), 7.03–7.02 (m, 2H), 6.92 (d, J = 8.4 Hz, 1H), 4.91 (s, 2H), 4.40–4.38 (m, 1H), 3.92–3.89 (m, 2H), 3.81–3.77 (m, 1H), 3.61 (d, J = 7.8 Hz, 1H), 2.84–2.77 (m, 1H), 2.68–2.63 (m, 1H), 2.24–2.16 (m, 2H), 1.45 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 310 K): δ = 176.7, 175.7, 174.3, 155.2, 144.2, 137.1, 133.1, 130.7, 130.0, 129.7, 129.5, 128.4, 128.1, 127.9, 126.9, 124.8, 123.4, 110.2, 80.1, 72.4, 58.8, 51.7, 47.0, 46.8, 43.6, 28.9 ppm; IR (thin film): $\tilde{\nu}$ = 3850, 3671, 2972, 2843, 1712, 1690, 1135,

1032 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{34}H_{34}N_4O_5Na$ $[M+Na]^+$ 601.2427, found 601.2415; HPLC purity: 100%, $t_R = 23.46$ min.

(±)-*tert*-Butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-2′-benzyl-5-methoxy-1′,2,3′-trioxo-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (21): Using the general procedure for the synthesis of pyrrolidines outlined above, indoline-2,3-dione (0.45 g, 2.5 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **21** as a white solid (0.66 mg, 1.3 mmol, 53%): $R_f = 0.35$ (1:1 EtOAc/hexanes); mp: 218–220 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO): $\delta = 10.43$ (s, 1H), 7.38–7.36 (m, 2H), 7.33–7.32 (m, 2H), 7.29–7.27 (m, 1H), 6.77 (dd, $J = 8.7$, 2.1 Hz, 1H), 6.73–6.71 (m, 1H), 6.07 (s, 1H), 4.64 (AB q, $J = 15$ Hz, 2H), 4.31 (br s, 1H), 3.87–3.83 (m, 1H), 3.75–3.73 (m, 1H), 3.68–3.64 (m, 1H), 3.45 (d, $J = 7.8$ Hz, 1H), 2.61–2.59 (m, 2H), 2.18–2.16 (m, 1H), 2.04 (td, $J = 11.2$, 3.0 Hz, 1H), 1.40 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 310 K): $\delta = 177.7$, 176.1, 174.7, 154.9, 153.8, 136.4, 129.1, 127.9, 127.5, 126.0, 115.4, 113.4, 110.5, 79.7, 72.3, 61.2, 57.9, 55.5, 50.6, 46.3, 45.2, 42.0, 28.5 ppm; IR (thin film): $\tilde{\nu} = 3850$, 3648, 2967, 1710, 1641, 1130, 1032 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{28}H_{30}N_4O_5Na$ $[M+Na]^+$ 555.2222, found 555.2239; HPLC purity: 96.22%, $t_R = 23.24$ min.

(±)-*tert*-Butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-7-methyl-1′,2,3′-trioxo-2′-phenyl-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (22): Using the general procedure for the synthesis of pyrrolidines outlined above, 7-methylindoline-2,3-dione (1.0 g, 6.2 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **22** as a white solid (2.0 g, 4.0 mmol, 60%): $R_f = 0.3$ (1:1 EtOAc/hexanes); mp: 212–214 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 398 K): $\delta = 10.1$ (s, 1H), 7.53–7.51 (m, 2H), 7.45–7.42 (m, 1H), 7.34-7.33 (m, 2H), 7.05 (d, $J = 7.8$ Hz, 1H), 6.88–6.86 (m, 1H), 6.76 (d, $J = 7.2$ Hz, 1H), 4.37–4.35 (m, 1H), 3.87–3.82 (m, 2H), 3.75–3.73 (m, 1H), 3.55–3.54 (m, 1H), 2.79–2.75 (m, 1H), 2.65–2.61 (m, 1H), 2.31–2.22 (m, 4H), 2.21–2.19 (m, 1H), 1.44 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 178.6$, 175.5, 174.1, 154.5, 141.8, 132.7, 131.5, 129.6, 129.0, 127.5, 124.8, 123.9, 122.2, 119.5, 79.6, 72.3, 58.1, 50.9, 49.1, 46.7, 28.5, 16.7, 14.6 ppm; IR (thin film): $\tilde{\nu} = 3229$, 2973, 2360, 2340, 1714, 1696, 1391 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{28}H_{30}N_4O_5Na$ $[M+Na]^+$ 525.2114, found 525.2108; HPLC purity: 99.18%, $t_R = 20.85$ min.

(±)-*tert*-Butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-6,7-dimethyl-1′,2,3′-trioxo-2′-phenyl-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (23): Using the general procedure for the synthesis of pyrrolidines outlined above, 6,7-dimethylindoline-2,3-dione (0.38 g, 2.2 mmol, 1.0 equiv) was used, and the product was purified by flash chromatography with EtOAc/hexanes to afford pyrrolidine **23** as a white solid (0.60 g, 1.20 mmol, 61%): $R_f = 0.3$ (1:1 EtOAc/hexanes); mp: 215–217 °C; ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 10.6$ (s, 1H), 7.53–7.52 (m, 2H), 7.50–7.44 (m, 1H), 7.29–7.28 (m, 2H), 6.77 (d, $J = 7.5$ Hz, 1H), 6.60 (d, $J = 7.5$ Hz, 1H), 4.29 (br s, 1H), 3.82–3.79 (m, 2H), 3.64–3.65 (m, 1H), 3.47–3.46 (m, 1H), 2.62 (br s, 2H), 2.19–2.17 (m, 4H), 2.09–2.07 (m, 4H), 1.39 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 320 K): $\delta = 178.8$, 175.5, 174.1, 141.8, 138.7, 132.7, 129.6, 129.0, 127.4, 123.6, 123.5, 122.4, 118.2, 79.6, 72.4, 58.1, 50.9, 46.7, 28.5, 20.1, 13.5 ppm; IR (thin film): $\tilde{\nu} = 3739$, 3244, 2976, 1712, 1696, 1417, 1390 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{29}H_{32}N_4O_5Na$ $[M+Na]^+$ 539.2271, found 539.2268; HPLC purity: 98.24%, $t_R = 21.47$ min.

Synthesis of (+)-(3S,3a′S,7′R,8a′S,8b′R)-6-chloro-7-methyl-1′,2,3′-trioxo-2′-phenyl-2′,3′,3a′,6′,7′,8′,8a′,8b′-octahydro-1′H-spiro[indoline-3,4′-pyrrolo[3,4-a]pyrrolizin]-7′-yl benzoate (25a) and (−)-(3R,3a′R,7′R,8a′R,8b′S)-6-chloro-7-methyl-1′,2,3′-trioxo-2′-phenyl-2′,3′,3a′,6′,7′,8′,8a′,8b′-octahydro-1′H-spiro[indoline-3,4′-pyrrolo[3,4-a]pyrrolizin]-7′-yl benzoate (25b): Using the general procedure for the synthesis of pyrrolidines outlined above, 6-chloro-7-methylindoline-2,3-dione (0.50 g, 2.6 mmol, 1.0 equiv) was used. The resulting crude reaction mixture was dissolved in cold CH_2Cl_2, and the products precipitated as a yellow solid. The solid was then collected by vacuum filtration and washed with cold CH_2Cl_2 until the solid became white. The pyrrolidine products were collected as a mixture of two diastereomers. The resulting products were highly insoluble in most of the organic solvents, and this made it very hard to separate the two diastereomers by flash column chromatography.

The mixture of two stereoisomers (0.030 g, 0.07 mmol, 1 equiv) from the above procedure was added to a 5 mL oven-dried round-bottom flask. The flask was fitted with a septum and purged with nitrogen. Next, 1.4 mL of DMF was added to the reaction flask such that the concentration of the mixture of two diastereomers was 0.05 M. Next, benzoyl chloride (9.0 μL, 0.075 mmol, 1.1 equiv) and Et_3N (0.01 mL, 0.08 mmol, 1.2 equiv) were added to the reaction flask, and the reaction was allowed to stir at room temperature until the consumption of the alcohols was confirmed by TLC (9:1 CH_2Cl_2/MeOH). Upon consumption of the alcohols, the reaction mixture was concentrated under vacuum to give a yellow solid. The solid was then purified by flash chromatography with CH_2Cl_2/Et_2O (20:1) to afford esters **25a** and **25b**.

The first stereoisomer **25a** (0.012 g, 0.02 mmol, 65%) was isolated as a white solid. $R_f = 0.55$ (20:1 CH_2Cl_2/Et_2O); mp: 236–238 °C; $[\alpha]_D^{20} = 54.5$ ($c = 0.13$ in MeOH); ^{1}H NMR (600 MHz, [D$_6$]acetone): $\delta = 9.67$ (s, 1H), 7.97 (dd, $J = 8.4$, 1.2 Hz, 2H), 7.61–7.58 (m, 1H), 7.51 (d, $J = 7.8$ Hz, 1H), 7.48–7.37 (m, 5H), 7.28 (d, $J = 7.8$ Hz, 2H), 7.16 (d, $J = 7.8$ Hz, 1H), 5.63–5.61 (m, 1H), 4.71–4.69 (m, 1H), 4.35 (d, $J = 9.6$ Hz, 1H), 3.82 (dd, $J = 10.2$, 6.6 Hz, 1H), 3.52 (dd, $J = 11.1$, 5.1 Hz, 1H), 3.01 (d, $J = 10.8$ Hz, 1H), 2.76–2.72 (m, 1H), 2.56–2.52 (m, 1H), 2.33 ppm (s, 3H); ^{13}C NMR (125 MHz, [D$_6$]acetone): $\delta = 177.4$, 176.6, 174.9, 165.6, 135.6, 133.0, 130.2, 129.3, 128.6, 128.4, 128.1, 127.2, 125.6, 124.4, 122.4, 118.1, 74.7, 65.1, 55.3, 53.4, 53.1, 37.7, 13.3 ppm; IR (thin film): $\tilde{\nu} = 3685$, 2978, 2851, 1721, 1054, 1032, 1012 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{30}H_{24}ClN_3O_5Na$ $[M+Na]^+$ 564.1302, found 564.1302; HPLC purity: 96.63%, $t_R = 21.95$ min.

The second stereoisomer **25b** (0.015 g, 0.03 mmol, 81%) was also isolated as a white solid. $R_f = 0.48$ (20:1 CH_2Cl_2/Et_2O); mp: 221–223 °C; $[\alpha]_D^{20} = -80.2$ ($c = 0.23$ in MeOH); ^{1}H NMR (600 MHz, [D$_6$]acetone): $\delta = 9.69$ (s, 1H), 8.09 (dd, $J = 8.4$, 1.2 Hz, 2H), 7.67 (t, $J = 7.5$ Hz, 1H), 7.54 (t, $J = 7.8$ Hz, 1H), 7.48–7.37 (m, 3H), 7.28 (d, $J = 7.8$ Hz, 2H), 7.13 (d, $J = 8.4$ Hz, 1H), 5.44–5.42 (m, 1H), 4.55–4.52 (m, 1H), 4.39 (d, $J = 10.2$ Hz, 1H), 4.00 (dd, $J = 9.9$, 6.9 Hz, 1H), 3.38 (dd, $J = 10.2$, 6.0 Hz, 1H), 3.28 (dd, $J = 9.9$, 6.3 Hz, 1H), 2.83–2.78 (m, 1H), 2.39–2.36 (m, 1H), 2.35 ppm (s, 3H); ^{13}C NMR (125 MHz, [D$_6$]acetone): $\delta = 177.4$, 176.5, 174.8, 165.6, 135.6, 133.3, 133.0, 130.1, 129.5, 128.6, 128.2, 127.3, 125.5, 125.2, 124.6, 122.4, 74.7, 64.9, 54.4, 53.5, 53.3, 36.6, 13.3 ppm; IR (thin film): $\tilde{\nu} = 3686$, 2980, 2481, 1720, 1050, 1030, 1014 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{30}H_{24}ClN_3O_5Na$ $[M+Na]^+$ 564.1302, found 564.1305; HPLC purity: 95.36%, $t_R = 21.68$ min.

Synthesis of (±)-*tert*-butyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-1-benzyl-6-chloro-7-methyl-1′,2,3′-trioxo-2′-phenyl-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahy-

drospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (17):[53] A 5 mL oven-dried round-bottom flask was charged with NaH (60 wt% in mineral oil, 0.01 g, 0.37 mmol, 1.1 equiv) and the mineral oil dispersed in NaH was removed by washing with hexanes (3×2 mL). The resulting white solid of NaH was suspended in 0.2 mL of DMF. The suspension was cooled to 0 °C in an ice bath, after which pyrrolidine **14** (0.18 g, 0.34 mmol, 1.0 equiv) was added as a solid over the course of 15 min. After the addition was complete the ice bath was removed, and the solution was stirred for 1 h at room temperature. Next, benzyl bromide (48 μL, 0.40 mmol, 1.2 equiv) was added to the reaction flask, and the reaction was allowed to stir at room temperature until the consumption of **15** was confirmed by TLC (2:3 EtOAc/hexanes). Upon consumption of **14**, the reaction mixture was concentrated under vacuum to give a yellow solid. The solid was then purified by flash chromatography with EtOAc/hexanes (4:1) to afford pyrrolidine **17** as a white solid (0.17 g, 0.27 mmol, 82%): R_f = 0.65 (2:3 EtOAc/hexanes); mp: 145–146 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 400 K): δ = 7.51–7.48 (m, 2H), 7.42–7.41 (m, 1H), 7.34–7.31 (m, 4H), 7.26–7.24 (m, 1H), 7.18–7.17 (m, 2H), 7.13 (d, J = 8.1 Hz, 1H), 6.86 (d, J = 8.1 Hz, 1H), 5.21–5.16 (m, 2H), 4.37–4.35 (m, 1H), 3.87–3.86 (m, 2H), 3.74–3.72 (m, 1H), 3.60 (d, J = 8.1 Hz, 1H), 2.80–2.78 (m, 1H), 2.67–2.65 (m, 1H), 2.34–2.32 (m, 1H), 2.27 (s, 3H), 2.19–2.17 (m, 1H), 1.42 ppm (s, 9H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 310 K): δ = 177.6, 175.2, 173.8, 154.2, 143.4, 137.8, 136.6, 132.6, 129.6, 129.5, 129.0, 127.7, 127.5, 125.8, 125.1, 124.5, 124.0, 118.6, 79.7, 71.0, 63.9, 58.6, 51.9, 46.5, 45.8, 45.1, 30.7, 28.5, 14.7 ppm; IR (thin film): $\tilde{\nu}$ = 3680, 2971, 2843, 1713, 1054, 1032, 1012 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{35}H_{35}ClN_4O_5Na$ $[M+Na]^+$ 649.2194, found 649.2194; HPLC purity: 100%, t_R = 23.13 min.

Synthesis of (±)-(3*R*,3a′*R*,3b′*S*,9a′*S*)-6-chloro-7-methyl-2′-phenyl-3a′,4′,5′,6′,7′,9a′-hexahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]-pyrrolo[1,2-*a*]pyrazine]-1′,2,3′(2′*H*,3b′*H*)-trione (26):[79] A 10 mL round-bottom flask was charged with pyrrolidine **14** (0.57 g, 1.1 mmol, 1 equiv) and a stir bar. The flask was fitted with a septum and purged with nitrogen. Next, 3.1 mL CH_2Cl_2 was added to the reaction flask such that the concentration of **14** was 0.34 M and stirred for 10 min. A pink solution resulted. Then 3.1 mL trifluoroacetic acid was slowly added to the reaction mixture by syringe. A dark-brown solution resulted. The deprotection reaction was stirred until TLC indicated complete consumption of pyrrolidine **14** (15 min). Upon consumption of **14**, the reaction mixture was concentrated under vacuum to give a brown oil. Saturated aqueous $NaHCO_3$ (5 mL) was added to the oil, and the aqueous layer was then extracted with CH_2Cl_2 (3×5 mL). The combined organic layers were then washed with brine and dried over Na_2SO_4. The resulting organic solution was concentrated under vacuum to afford **26** as a pink solid (0.42 g, 0.96 mmol, 87%): R_f = 0.81 (20:80:5 EtOAc/hexanes/Et$_3$N); mp: 196–199 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 10.89 (s, 1H), 7.58–7.53 (m, 2H), 7.47–7.45 (m, 1H), 7.30 (d, J = 7.4 Hz, 2H), 7.06 (d, J = 8.0 Hz, 1H), 6.71 (d, J = 8.0 Hz, 1H), 3.77–3.76 (m, 2H), 3.51–3.50 (m, 1H), 3.42 (br s, 1H), 3.30 (d, J = 11.9 Hz, 1H), 2.79 (app d, J = 12.1 Hz, 1H), 2.43–2.41 (m, 1H), 2.25 (s, 3H), 2.24–2.21 ppm (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, 310 K): δ = 178.8, 175.5, 174.2, 143.4, 134.9, 132.7, 129.6, 128.9, 127.4, 125.2, 124.1, 122.5, 117.7, 72.6, 58.7, 50.7, 48.7, 47.1, 46.7, 44.9, 14.3 ppm; IR (thin film): $\tilde{\nu}$ = 3850, 3671, 2971, 2864, 1709, 1054, 1032, 1013 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{23}H_{21}ClN_4O_3Na$ $[M+Na]^+$ 459.1200, found 459.1190; HPLC purity: 100%, t_R = 17.25 min.

Synthesis of (±)-methyl 10-((3*R*,3a′*R*,3b′*S*,9a′*S*)-6-chloro-7-methyl-1′,2,3′-trioxo-2′-phenyl-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazin]-5′(1′*H*)-yl)-10-oxodecanoate (27):[80] A 15 mL round-bottom flask was charged with pyrrolidine **26** (0.13 g, 0.30 mmol, 1 equiv), Na_2CO_3 (0.03 g, 0.30 mmol, 1.0 equiv) and a stir bar. The flask was fitted with a septum and purged with nitrogen. Then CH_2Cl_2 (6 mL) was added to the reaction flask such that the concentration of **26** was 0.05 M. Next, methyl 10-chloro-10-oxodecanoate (0.07 mL, 0.31 mmol, 1.04 equiv) was added dropwise to the reaction mixture by syringe. The reaction was allowed to stir at room temperature until the consumption of **26** was confirmed by TLC (20:80:5 EtOAc/hexanes/Et$_3$N). Upon consumption of **26**, the reaction mixture was concentrated under vacuum to give a yellow oil. The oil was then purified by flash chromatography with EtOAc/hexanes/Et$_3$N (80:20:5) to afford pyrrolidine **27** as a yellow solid (0.10 g, 0.16 mmol, 53%): R_f = 0.75 (20:80:5 EtOAc/hexanes/Et$_3$N); mp: 110–111 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 400 K): δ = 10.42 (s, 1H), 7.53–7.51 (m, 2H), 7.45–7.43 (m, 1H), 7.33 (d, J = 7.8 Hz, 2H), 7.03 (d, J = 8.4 Hz, 1H), 6.78 (d, J = 7.8 Hz, 1H), 4.58 (br s, 1H), 4.08 (br s, 1H), 3.86 (t, J = 7.8 Hz, 1H), 3.73–3.72 (m, 1H), 3.61–3.58 (m, 4H), 2.61–2.82 (m, 4H), 2.34–2.27 (m, 5H), 2.22–2.18 (m, 1H), 1.59–1.53 (m, 4H), 1.22–1.42 ppm (m, 9H); ^{13}C NMR (125 MHz, $CDCl_3$, 313 K): δ = 178.2, 174.6, 174.3, 172.2, 171.7, 141.8, 136.6, 131.7, 129.4, 128.9, 126.3, 124.4, 123.5, 122.3, 118.7, 72.7, 58.7, 51.4, 50.4, 48.7, 46.2, 45.3, 44.5, 41.1, 34.1, 33.4, 29.7, 29.3, 29.2, 25.3, 24.9, 13.6 ppm; IR (thin film): $\tilde{\nu}$ = 3817, 3671, 2921, 2863, 1710, 1617, 1054, 1032 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{34}H_{39}ClN_4O_6Na$ $[M+Na]^+$ 657.2456, found 657.2435; HPLC purity: 97.45%, t_R = 23.69 min.

Synthesis of (±)-(3*R*,3a′*R*,3b′*S*,9a′*S*)-6-chloro-5′-((*S*)-2-methoxy-2-phenylacetyl)-7-methyl-2′-phenyl-3a′,4′,5′,6′,7′,9a′-hexahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-1′,2,3′(2′*H*,3b′*H*)-trione (28):[81] A 15 mL round-bottom flask was charged with pyrrolidine **26** (0.20 g, 0.46 mmol, 1.0 equiv), (*S*)-2-methoxy-2-phenylacetic acid (0.10 g, 0.60 mmol, 1.3 equiv), *N*-(3-*di*-methylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.18 g, 0.96 mmol, 2.1 equiv), and a stir bar. The flask was fitted with a septum and purged with nitrogen. Then CH_2Cl_2 (6 mL) was added to the reaction flask such that the concentration of **26** was 0.07 M. Next Et$_3$N (0.22 mL, 1.6 mmol, 3.5 equiv) was added to the reaction mixture, and the reaction was allowed to stir at room temperature until the consumption of **26** was confirmed by TLC (20:80:5 EtOAc/hexanes/Et$_3$N). Upon consumption of **26**, the reaction mixture was quenched with 5 mL of 1 N aqueous HCl, and the aqueous layer was then extracted with CH_2Cl_2 (3×5 mL). The combined organic layers were then washed with brine and dried over Na_2SO_4. The resulting organic solution was concentrated under vacuum to give a yellow oil. The oil was then purified by flash chromatography with EtOAc/hexanes/Et$_3$N (80:20:5) to afford pyrrolidine **28** as a white solid (0.12 g, 0.21 mmol, 45%): R_f = 0.65 (1:1 EtOAc/hexanes); mp: 127–129 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 400 K): δ = 10.42 (s, 1H), 7.51–7.49 (m, 2H), 7.44–7.41 (m, 1H), 7.39–7.35 (m, 4H), 7.32–7.31 (m, 3H), 7.01 (d, J = 8.0 Hz, 1H), 6.74 (d, J = 8.0 Hz, 1H), 5.17–5.16 (m, 1H), 4.71–4.70 (m, 1H), 4.19–4.17 (m, 1H), 3.79–3.75 (m, 1H), 3.64–3.60 (m, 1H), 3.54 (app dd, J = 8.0, 2.9 Hz, 1H), 3.39–3.37 (m, 3H), 2.73–2.64 (m, 2H), 2.27–2.22 (m, 4H), 2.07–2.05 ppm (m, 1H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 310 K): (two rotamers observed) δ = 178.5, 175.3, 173.9, 154.9, 137.4, 135.2, 132.6, 129.5, 129.0, 128.9, 128.6, 127.6, 127.5, 127.3, 125.2, 123.6, 122.6, 117.8, 81.8, 81.0, 72.1, 68.9, 63.9, 58.6, 58.2, 57.3, 56.3, 50.9, 46.5, 45.3, 44.5, 41.7, 32.5, 30.6, 30.1, 21.8, 19.0, 17.1 ppm; IR (thin film): $\tilde{\nu}$ = 3850, 3708, 2971, 2921, 1712, 1643, 1054, 1032, 1012 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{32}H_{29}ClN_4O_5Na$ $[M+Na]^+$ 607.1724, found 607.1736; HPLC purity: 97.04%, t_R = 20.02 min.

Synthesis of (±)-(3*R*,3a′*R*,3b′*S*,9a′*S*)-6-chloro-7-methyl-2′-phenyl-5′-(3-phenylpropanoyl)-3a′,4′,5′,6′,7′,9a′-hexahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-1′,2,3′(2′*H*,3b′*H*)-trione (29): A 10 mL round-bottom flask was charged with pyrrolidine **26** (0.05 g, 0.11 mmol, 1.0 equiv), Et_3N (18.0 μL, 0.13 mmol, 1.1 equiv), and a stir bar. The flask was fitted with a septum and purged with nitrogen. Then CH_2Cl_2 (2.0 mL) was added to the round-bottom flask by syringe. Hydrocinnamoyl chloride (18.0 μL, 0.12 mmol, 1.05 equiv) was then added to the reaction flask with a syringe. The reaction mixture was allowed to stir for 3 h at room temperature. The reaction was monitored for consumption of **26** by TLC (100:5 EtOAc/Et_3N). Upon consumption of **26**, the reaction mixture was quenched with 5 mL of saturated aqueous $NaHCO_3$, and the aqueous layer was then extracted with CH_2Cl_2 (3×5 mL). The combined organic layers were then washed with brine and dried over Na_2SO_4. The resulting organic solution was concentrated under vacuum to give a yellow oil. The oil was then purified by flash chromatography with EtOAc/hexanes/Et_3N (60:40:5) to afford pyrrolidine **29** as a white solid (0.04 g, 0.07 mmol, 64%): R_f = 0.2 (60:40:5 EtOAc/hexanes/Et_3N); mp: 236–239 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 400 K): δ = 10.40 (s, 1H), 7.53–7.51 (m, 2H), 7.45–7.42 (m, 1H), 7.34–7.33 (m, 2H), 7.27–7.23 (m, 4H), 7.17–7.15 (m, 1H), 7.03 (d, J = 7.8 Hz, 1H), 6.78 (d, J = 8.4 Hz, 1H), 4.59 (s, 1H), 7.09–7.06 (m, 1H), 3.87–3.84 (m, 1H), 3.73–3.69 (m, 1H), 3.59–3.57 (m, 1H), 2.90–2.88 (m, 2H), 2.84–2.82 (m, 2H), 2.78–2.74 (m, 2H), 2.31–2.28 (m, 4H), 2.19–2.15 ppm (m, 1H); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 310 K): δ = 178.6, 178.5, 175.4, 175.3, 174.0, 170.6, 143.4, 141.9, 135.2, 132.7, 129.6, 129.0, 128.8, 128.7, 127.5, 126.3, 125.2, 123.9, 122.6, 117.9, 72.2, 60.2, 58.9, 51.2, 48.5, 45.5, 44.9, 34.5, 31.2, 14.3 ppm; IR (thin film): $\tilde{\nu}$ = 3423, 2917, 1708, 1633, 1620, 1384, 1204 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{32}H_{29}ClN_4O_4Na$ $[M+Na]^+$ 591.1775, found 591.1763; HPLC purity: 98.20%, t_R = 21.15 min.

Synthesis of (3R,3a′R,3b′S,9a′S)-*N*-benzyl-6-chloro-7-methyl-1′,2,3′-trioxo-2′-phenyl-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-a]pyrazine]-5′(1′H)-carboxamide (30): A 10 mL round-bottom flask was charged with pyrrolidine **26** (0.06 g, 0.15 mmol, 1.0 equiv), and a stir bar. The flask was fitted with a septum and purged with nitrogen. Then CH_2Cl_2 (3.0 mL) was added to the round-bottom flask by syringe. Next, (isocyanatomethyl)benzene (0.02 mL, 0.16 mmol, 1.1 equiv) was added to the reaction flask by syringe. Then the reaction mixture was allowed to stir for 4 h at room temperature. The reaction was monitored for consumption of **26** by TLC (50:50:5 EtOAc/Hex/Et_3N). Upon consumption of **26**, the resulting organic solution was concentrated under vacuum to give a yellow oil. The oil was then purified by flash chromatography with EtOAc/hexanes/Et_3N (60:40:5) to afford pyrrolidine **30** as a white solid (0.63 g, 0.11 mmol, 75%): R_f = 0.24 (50:50:5 EtOAc/Hex/Et_3N); mp: 258–259 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 400 K): δ = 10.44 (s, 1H), 7.53–7.51 (m, 2H), 7.45–7.44 (m, 1H), 7.33–7.32 (m, 2H), 7.29–7.28 (m, 4H), 7.20 (m, 1H), 7.03 (d, J = 8.0 Hz, 1H), 6.76 (d, J = 8.0 Hz, 1H), 4.47–4.45 (m, 1H), 4.29–4.27 (m, 2H), 3.95 (d, J = 14.1 Hz, 1H), 3.82–3.77 (m, 2H), 3.56 (d, J = 7.9 Hz, 1H), 2.75–2.71 (m, 1H), 2.64–2.58 (m, 1H), 2.28 (s, 3H), 2.25–2.23 ppm (m, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 178.7, 175.4, 174.1, 157.6, 143.4, 141.4, 135.1, 132.7, 129.6, 129.1, 128.6, 127.5, 126.9, 125.1, 123.9, 122.6, 117.9, 72.3, 58.3, 51.1, 47.5, 46.7, 45.7, 44.1, 43.6 ppm; IR (thin film): $\tilde{\nu}$ = 3618, 2922, 2360, 2340, 1712, 1616, 1536, 1387 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{31}H_{28}ClN_5O_4Na$ $[M+Na]^+$ 592.1727, found 592.1724; HPLC purity: 97.18%, t_R = 19.94 min.

Synthesis of (±)-benzyl (3*R*,3a′*R*,3b′*S*,9a′*S*)-6-chloro-7-methyl-1′,2,3′-trioxo-2′-phenyl-2′,3′,3a′,3b′,4′,6′,7′,9a′-octahydrospiro[indoline-3,9′-pyrrolo[3′,4′:3,4]pyrrolo[1,2-*a*]pyrazine]-5′(1′*H*)-carboxylate (31):[82] A 5 mL round-bottom flask was charged with pyrrolidine **26** (0.20 g, 0.45 mmol, 1.0 equiv), *N*,*N*-diisopropylethylamine (0.17 mL, 0.96 mmol, 2.1 equiv), and a stir bar. The flask was fitted with a septum and purged with nitrogen. Then CH_2Cl_2 (0.92 mL) was added to the round-bottom flask by syringe. The reaction mixture was cooled to 0 °C with an ice bath. Meanwhile another 5 mL pear-shaped flask was charged with benzyl chloroformate (0.07 mL, 0.50 mmol, 1.1 equiv), and the flask was fitted with a septum and purged with nitrogen. Then CH_2Cl_2 (0.34 mL) was added to the pear-shaped vial containing the benzyl chloroformate and stirred for 10 min. Next the contents of the 5 mL pear-shaped vial were slowly added to the 5 mL round-bottom flask containing **26**. The ice bath was removed, and the reaction mixture was allowed to stir for 2.5 h at room temperature. The reaction was monitored for consumption of **26** by TLC (50:50:5 EtOAc/Hex/Et_3N). Upon consumption of **26**, the reaction mixture was quenched with 5 mL of saturated aqueous $NaHCO_3$, and the aqueous layer was then extracted with CH_2Cl_2 (3×5 mL). The combined organic layers were then washed with brine and dried over Na_2SO_4. The resulting organic solution was concentrated under vacuum to give a yellow oil. The oil was then purified by flash chromatography with EtOAc/hexanes/Et_3N (60:40:5) to afford pyrrolidine **31** as a white solid (0.14 g, 0.25 mmol, 54%): R_f = 0.81 (1:1 EtOAc/hexanes); mp: 209–211 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO, 400 K): δ = 10.40 (s, 1H), 7.53–7.51 (m, 2H), 7.45–7.42 (m, 1H), 7.37–7.35 (m, 6H), 7.32–7.31 (m, 1H), 7.03 (d, J = 8.0 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 5.16 (s, 2H), 4.48 (d, J = 12.7 Hz, 1H), 3.99 (d, J = 13.1 Hz, 1H), 3.89–3.87 (m, 1H), 3.82–3.79 (m, 1H), 3.61 (d, J = 8.0 Hz, 1H), 2.91–2.86 (m, 2H), 2.78–2.75 (m, 1H), 2.34–2.25 ppm (m, 5H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 178.5, 175.3, 173.9, 154.9, 143.4, 137.3, 135.2, 132.6, 129.5, 129.0, 128.9, 128.3, 128.1, 127.4, 72.3, 66.9, 58.2, 51.0, 47.2, 43.5, 30.9, 21.1, 14.3 ppm; IR (thin film): $\tilde{\nu}$ = 3850, 3678, 3262, 2966, 2864, 1715, 1694, 1032 cm^{-1}; HRMS (ESI): *m/z* calculated for $C_{31}H_{27}ClN_4O_5Na$ $[M+Na]^+$ 593.1567, found 593.1584; HPLC purity: 97.60%, t_R = 21.96 min.

Biological evaluations

Strains, media, and compounds: The *C. albicans* strain HLY4123 was used as the susceptible laboratory strain for the antifungal evaluation in this study. HLY4123 carries a GFP reporter for *ERG3* expression and was constructed by plasmid transformation of the commonly used laboratory *C. albicans* strain CAI4. Selected resistant *C. albicans* strains with different mechanisms of becoming drug resistant were obtained from Dr. David Rogers (Department of Clinical Pharmacy, University of Tennessee Health Sciences Center, Memphis, Tennessee, USA). The strains were cultured at 30 °C under constant shaking (200 rpm) in synthetic complete (SC) medium containing 2% glucose. The stock solution of fluconazole (Sigma–Aldrich, USA) was prepared in sterile H_2O (0.1 mg mL^{-1}), whereas the other test compounds were prepared in DMSO. The commercial sample sold as CID 6584729 was obtained from Vitas-M (supplier number STK 580951).

Dose–response curves for test compounds against C. albicans with and without fluconazole: *C. albicans* was grown in SC medium overnight and then diluted to an effective OD_{600} of 0.0625. Serial tenfold dilutions of the test compounds (0.15–1500 μM) were prepared in DMSO in 1.5 mL Eppendorf tubes. To each well in columns B–D (triplicate analysis) of a 24-well Palcon plate was added 2.5 μL of fluconazole solution. To each well in all four columns of the

plate was added 1 mL of cells in SC medium such that column A served as a control to assess the EC_{50} value of the compound in the absence of fluconazole. Then to each well in rows 2–5 was added a solution of the compound in DMSO (2 μL each) such that the final fluconazole concentration in columns 2–4 was 0.25 μg mL^{-1}, and the concentration of compound in each row varied from 0.003 to 30 μM. The plates were incubated in a rotary shaker/incubator at 30 °C for 16 h. The contents of each well were re-suspended with a micropipettor and a 20 μL aliquot was added to a polystyrene cuvette and diluted with 680 μL deionized water. The suspension was triturated again immediately before measuring the absorbance at $\lambda = 600$ nm (OD_{600}) for cell densities. EC_{50} values were determined by fitting to a standard curve using the Excel-based tool ED50PLUS v. 1.0 (Mario H. Vargas).

Determination of MIC_{90} values by checkerboard assay: Checkerboard assays were carried out using four 24-well plates. The results on each plate were normalized by duplication of one row and one column with a row and column on another plate. *C. albicans* was grown in SC medium overnight and then diluted to an effective OD_{600} of 0.0625. Serial tenfold dilutions of the test compounds (150 mM) were prepared in DMSO in 1.5 mL Eppendorf tubes. Serial twofold dilutions of fluconazole (6.53 μM) were prepared in sterile H_2O in 1.5 mL Eppendorf tubes. To each well was added 2 μL of a stock solution of compound in DMSO and 2.5 μL of a stock solution of fluconazole in H_2O. Concentrations of compound in rows 1–11 varied from 300 μM to 0.0003 nM; the last row 12 contained no compound. Concentrations of fluconazole in each column B–H varied from 0.0625 to 2 μM; the first column A contained no fluconazole. The plates were incubated in a rotary shaker/incubator at 30 °C for 16 h. The contents of each well were re-suspended with a micropipettor, and a 20 μL aliquot was added to a polystyrene cuvette and diluted with 680 μL deionized H_2O. The suspension was triturated again immediately before measuring the absorbance at $\lambda = 600$ nm. MIC_{90} values were determined as the lowest drug concentrations (alone or in combination) that inhibited fungal growth by 90% compared with that of the drug-free wells.

Molecular properties

Physicochemical properties were calculated from the SMILES representation of synazo-1 using the Molinspiration Property Calculation Service at www.molinspiration.com.

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Keywords: antifungal agents · *Candida albicans* · fluconazole · spiro compounds · synergy

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