Isatin Sulfonamide Analogs Containing a Michael Addition Acceptor: A New Class of Caspase 3/7 Inhibitors

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A series of isatin sulfonamide analogs having a Michael acceptor were prepared and their potencies for inhibiting caspase-1, -3, -6, -7, and -8 were evaluated. These compounds have nanomolar potency for inhibiting the executioner caspases, caspase-3 and caspase-7, and have a low potency for inhibiting caspase-1, caspase-6, and caspase-8. The inhibition mechanism was investigated through NMR studies of the reaction between **11d** and benzylmercaptan as a model for Cys-285 in the active site of caspase-3.

Introduction

Apoptosis, or programmed cell death, is a conserved process that is mediated by the activation of a series of cysteine aspartyl-specific proteases called caspases. Apoptosis plays an important role in a wide variety of normal cellular processes including fetal development, tissue homeostasis, and maintenance of the immune system.[1] However, abnormal apoptosis has been observed in a large number of pathological conditions, including ischemia-reperfusion injury (stroke and myocardial infarction), cardiomyopathy, neurodegeneration (Alzheimer's disease, Parkinson's disease, Huntington's disease, and ALS), sepsis, type I diabetes, and allograft rejection.[2−6] Therefore, the development of drugs that can halt the process of apoptosis has been an active area of research in the pharmaceutical industry.[2,7] In addition, the benefits of many drugs, especially antitumor drugs, can be attributed to their activation of the apoptotic process.[8−13] Therefore, the development of a noninvasive imaging procedure that can study the process of apoptosis in a variety of disease states and monitor the ability of a drug to either induce or halt apoptosis would be of tremendous value to the research and clinical community.

The caspase family of cysteine proteases has two different classes of caspases involved in apoptosis, the initiator caspases and the executioner caspases.[14] The initiator caspases, which include caspase-2, -8, -9, and -10, are located at the top of the signaling cascade; their primary function is to activate the executioner caspases, caspase-3, -6, and -7. The executioner caspases are responsible for the physiological (e.g., cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase-1, nuclear laminins, and cytoskeleton proteins) and morphological changes (DNA strand breaks, nuclear membrane damage, and membrane blebbing) that occur in apoptosis.[2] A third class of caspases, caspases-1, -4, -5, and -13, are involved in cytokine maturation and are not believed to play an active role in apoptosis.

Because of their roles in inflammation and apoptosis, the caspase proteases have received enormous research interest, and a diverse range of caspase inhibitors have been developed over the years.[15] There are two different classes of caspase inhibitors, reversible and irreversible, depending on the interaction of the "warhead" group with Cys-285 in the active site of the enzyme. Although many irreversible inhibitors with a Michael acceptor group have been reported as cysteine protease inhibitors, only a few compounds with a Michael acceptor group have been reported as caspase-3 inhibitors. Ekici et al. have reported that aza-peptide Michael acceptors with an aza-Asp residue at P1, for example, Cbz-Asp-Glu-Val-AAsp-CH═CH-COOBzl, **1** (Figure 1), are potent inhibitors of caspase-3.[16,17]

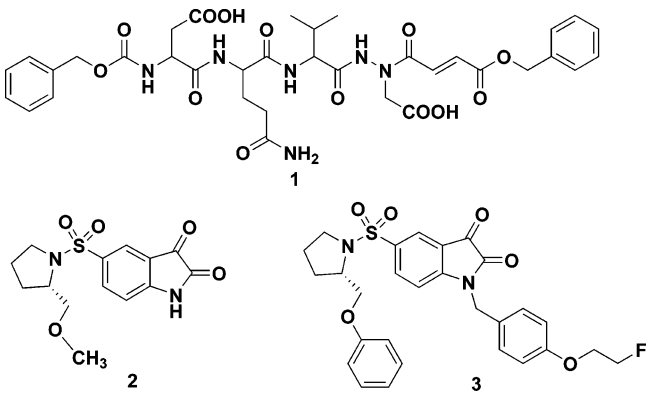

Figure 1.

Recently, a number of isatin-based inhibitors of caspase-3 and caspase-7 have been reported.[18,19] One compound, (*S*)-(+)-5-[1-(2-methoxymethyl-pyrrolidine)sulfonyl]isatin, **2** (Figure 1), has been shown to reduce tissue damage in an isolated rabbit heart model of ischemic injury.[20] Our research interest is centered around the development of nonpeptide small molecule inhibitors of executioner caspases for use as therapeutic drugs and radiotracer imaging of apoptosis. We have reported that the replacement of the 2-methoxymethyl group in **2** with a phenoxymethyl or 2-pyridin-3-yl-oxymethyl moiety, the replacement of the pyrrolidine ring with an azetidine ring, and the substitution of the isatin nitrogen atom with an alkyl group, resulted in a dramatic improvement in potency for inhibiting caspase-3 activity.[21] One isatin sulfonamide analog, 1-[4-(2-fluoroethoxy)-benzyl]-5-(2-phenoxymethyl-pyrrolidine-1-sulfonyl)-1*H*-indole-2,3-dione, **3** (**WC-II-89**, Figure 1), has been labeled with ^{18}F and is currently being examined as a potential radiotracer for imaging caspase-3 activation in tissues undergo-

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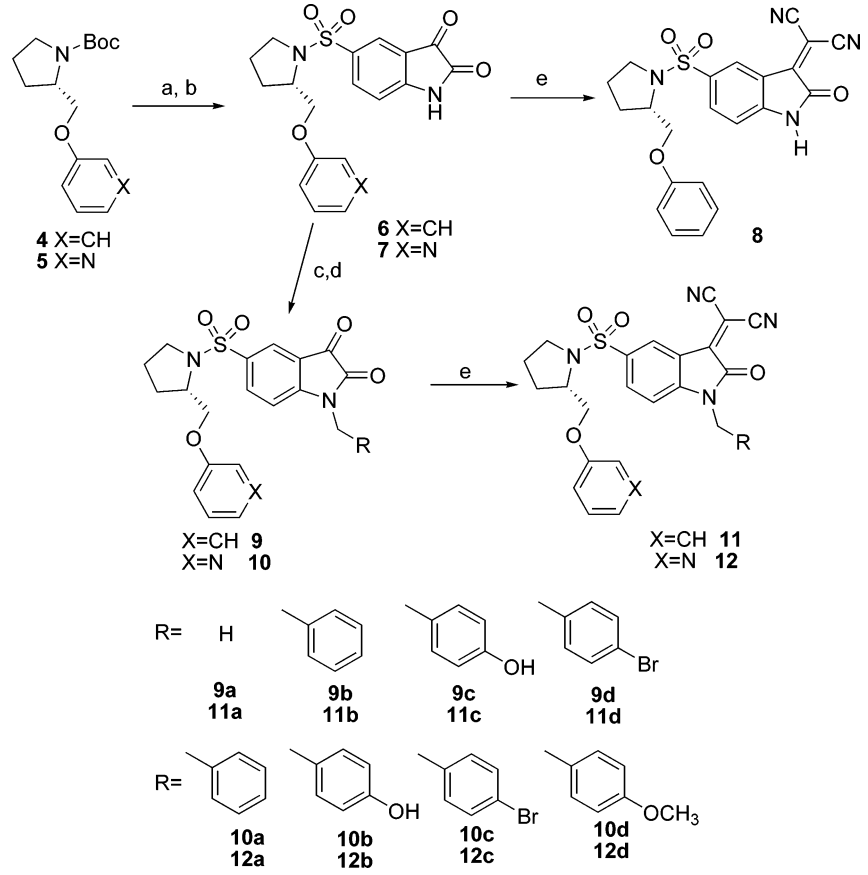
Scheme 1[a]



[a] Reagents: (a) TFA, CH_2Cl_2; (b) 5-sulfonylisatin chloride, Et_3N; (c) NaH, DMF; (d) R-C_6H_4-CH_2Br; (e) malononitrile, MeOH.

ing apoptosis.[22] On the basis of our result showing isatin derivatives as potent inhibitors of caspase-3/7, here we report a new series of isatin derivatives containing a Michael acceptor as caspase-3/7 inhibitors. We refer to these new compounds as isatin Michael acceptor (IMA[a]) caspase-3/7 inhibitors.

Results and Discussion

The synthesis of 5-(2-phenoxymethyl-pyrrolidine-1-sulfonyl)-isatin and its IMA analogs are shown in Scheme 1. The isatin analogs **6**, **9a**−**c**,[21] and **9d** were reacted with malononitrile in methanol to give the IMA analogs, **8** and **11a**−**d**, respectively. The 5-(2-pyridin-3-yl-oxymethyl)pyrrolidine-1-sulfonyl)isatin analogs **10a**, **10c**, and **10d** were prepared by using the same sequence of reactions described in the synthesis of **9d** (Scheme 1). For the compound **10b**, the isatin nitrogen of **7** was first alkylated with (4-bromomethyl-phenoxy)-*tert*-butyl-diphenyl-silane, then the protecting group *tert*-butyl-diphenyl-silane was removed with *n*-Bu_4NF in THF to afford **10b**. The IMA analogs of the 5-(2-pyridin-3-yl-oxymethyl)pyrrolidine-1-sulfonyl)isatin, **12a**−**d**, were prepared with the same methods of **11a**−**d**.

The IC_{50} values from the enzyme assays are summarized in Table 1. The results show that the phenoxymethyl and pyridin-3-yl-oxymethyl isatin analogs, **9d**, **10b**, and **10c**, are potent and selective inhibitors for caspase-3/7 relative to caspases-1, -6, and -8. The IMA analogs of phenoxymethyl isatin compounds **8** and **11a**, where the isatin nitrogen of the indol ring is not alkylated or instead possesses a methyl group, have low potency for caspase-3 and -7 inhibition; these IC_{50} values are 272 and 119.3 nM for caspase-3 and 1585 and 785 nM for caspase-7. When the isatin nitrogen of the indol ring was alkylated with an aromatic group, the potency of IMA analogs **11b**, **11c**, and **11d** improved drastically for caspase-3/7, with IC_{50} values of 27.8, 31.8, and 20.1 nM for caspase-3 and 51.7, 126.0, and 92.2 nM for caspase-7, while retaining their high selectivity. Also, all of these compounds have less activity for inhibition of caspase-1 (IC_{50}: 2300−6200 nM), caspase-6 (IC_{50} 744−926 nM), and caspase-8 (IC_{50} > 50 000 nM) upon addition of the aromatic group. Similarly, the IMA analogs of pyridin-3-yl-oxymethyl isatin, **12a**, **12b**, **12c**, and **11d**, are potent and selective inhibitors for caspase-3 (IC_{50}: 7.6, 7.8, 5.1, and 7.8 nM) and caspase-7 (IC_{50}: 32.8, 28.6, 26.3, and 15.1 nM) and show weak inhibition of caspase-1 (IC_{50}: 2700−3200 nM), caspase-6 (IC_{50}: 515−770 nM), and caspase-8 (IC_{50}: >50 000 nM). The IMA analogs of pyridin-3-yl-oxymethyl isatin also display improved potency for inhibiting caspases-3/7 than the corresponding IMA analogs of phenoxymethyl isatin (Table 1,

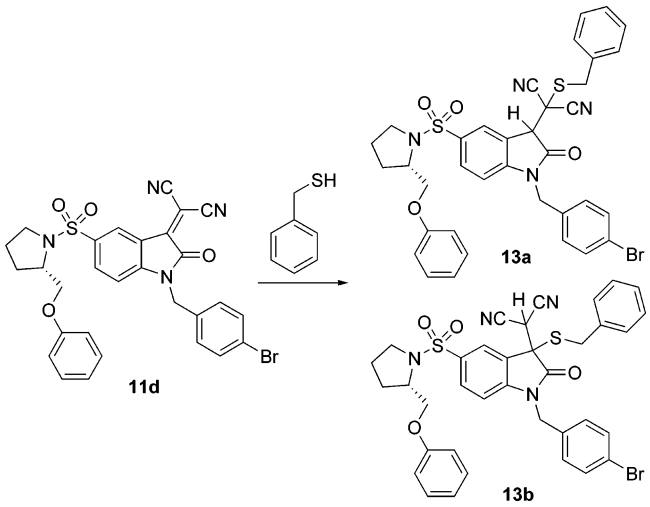
Table 1

	IC_{50} (nM)					
#	casp-1	casp-3	casp-6	casp-7	casp-8	Log P
9d	>15 000	9.85 ± 0.9	8900 ± 424	34.8 ± 1.4	>50 000	4.82
10b	>15 000	3.9 ± 0.6	9550 ± 354	11.7 ± 1.0	>50 000	2.25
10c	>15 000	3.6 ± 0.5	5025 ± 318	6.6 ± 0.1	>50 000	3.76
8	1830 ± 128	272 ± 24.7	407 ± 15	1585 ± 163	>50 000	1.07
11a	2825 ± 248	119.3 ± 4.0	698 ± 94	785 ± 276	>50 000	1.71
11b	6220 ± 1250	27.8 ± 2.5	918 ± 151	51.7 ± 6.2	>50 000	3.50
11c	2300 ± 250	31.8 ± 6.2	744 ± 48	126.0 ± 19.3	>50 000	2.77
11d	5700 ± 850	20.1 ± 1.3	840 ± 125	92.2 ± 11.8	>50 000	4.28
12a	3250 ± 450	7.6 ± 1.1	823 ± 86	32.8 ± 4.9	>50 000	2.45
12b	2720 ± 580	7.8 ± 1.9	650 ± 22	28.3 ± 5.4	>50 000	1.77
12c	3400 ± 0	5.1 ± 0.7	515 ± 77	26.3 ± 0.8	>50 000	3.22
12d	3900 ± 530	7.8 ± 1.5	610 ± 113	29.6 ± 1.4	>50 000	2.36
3[a]	>50 000	9.7 ± 1.3	3700 ± 390	23.5 ± 3.5	>50 000	4.19

[a] Reference 22.

[a] Abbreviation: IMA, isatin Michael acceptor.

Scheme 2



11b, **11c**, and **11d** compare with **12a**, **12b**, and **12c**, respectively). It is interesting to note that all the IMA analogs have an increased potency of roughly 10-fold for caspase-6 when compared to their complementary isatin analogs (see Table 1).

The inhibition mechanism was further investigated by using **11d** and its reaction with benzylmercaptan as a model. There are two possible Michael addition products (**13a** or **13b**) produced by attack of the thiol nucleophile of benzylmercaptan to **11d**. The products depend on the position of attack of the thiol group of benzylmercaptan on the carbon−carbon double bond of **11d (**Scheme 2**)**. Initially, we hoped to purify the Michael addition product in order to obtain a crystal structure by X-ray diffraction. Therefore, benzylmercaptan was reacted with **11d** in CH_2Cl_2, and a white solid was obtained following evaporation of the CH_2Cl_2 and excess benzylmercaptan in vacuo. However, when the white solid was recrystallized from ethyl acetate, a purple solid was produced and NMR structural analysis revealed it was the starting material, **11d**. This result shows that the Michael addition product is easily reversible and leads to the formation of the starting material. Hence, **11d** is a reversible Michael acceptor inhibitor. This result is consistent with our inhibition studies of human caspase-3 with IMA inhibitors. Human caspase-3 activity is inhibited when incubated with caspase-3 and the IMA inhibitor, yet caspase-3 activity can be recovered when the IMA inhibitor is removed by gel filtration and washed with water (Supporting Information, Figure 2). In an effort to better understand the chemical structure of the Michael addition product, a series of detailed NMR studies were carried out. The proton and carbon chemical shifts for the Michael addition product were assigned through two-dimensional correlation spectroscopy (COSY, HMQC, and HMBC, see Supporting Information, Figures 2−6). The results show that the structure of the Michael addition product is **13b** instead of **13a**, thereby demonstrating that the thiol group of benzylmercaptan prefers to attack the indol ring carbon versus the exocyclic methylene group of **11d**.

The current study is focused on the development of caspase-3 inhibitors as radiotracers that are specific for imaging apoptosis using PET, as well as drugs for therapy of the numerous diseases associated with unregulated apoptosis. We have reported that biodistribution studies using [^{18}F]**3**, a reversible isatin-based caspase-3 inhibitor, revealed higher uptake in liver and spleen of cycloheximide-treated rats, an animal model of apoptosis, relative to control animals.[22] MicroPET imaging studies also showed a high uptake of the radiotracer in the liver of cycloheximide-treated rats relative to the untreated control.[22] At this moment, it is not clear which is better, reversible or irreversible caspase-3 inhibitors, for imaging apoptosis in vivo. Nevertheless, it is our opinion that IMA-based radiotracers are capable of producing similar if not better imaging results compared to their isatin-based counterparts. Furthermore, the Log P value of the IMA analogs are lower than the corresponding isatin analogs value (e.g., **9d** to **11d**, Log P 4.82 to 4.28, **10b** to **12b**, 2.25 to 1.77, and **10c** to **12c**, 3.76 to 3.22, respectively (Table 1)). This lower Log P value of the IMA caspase-3 inhibitor increases the drug's ability to penetrate the cell in vivo and label the target.

Conclusions

We have completed the synthesis and in vitro evaluation of a series of IMA analogs having a high potency for inhibiting caspase-3 and caspase-7 and a low potency for inhibiting caspase-1, caspase-6, and caspase-8. The inhibition mechanism was further investigated through the reaction of **11d** and benzylmercaptan. This reaction serves as a model for the inhibition mechanism of the IMA inhibitors. The results of the current study reveal a new class of nonpeptide-based caspase inhibitors possessing a Michael acceptor, which may be useful in assessing the role of executioner caspase inhibitors, minimizing tissue damage in diseases characterized by unregulated apoptosis, and in vivo imaging of apoptosis.

Experimental Section

1-(4-Bromo-benzyl)-5-(2-Phenoxymethyl-pyrrolidine-1-sulfonyl)-1*H*-indole-2,3-dione (9d). NaH (60%, 10 mg, 0.25 mmol) was added to a solution of **6**[19,21] (97 mg, 0.25 mmol) in DMF (3 mL) at 0 °C. The mixture was stirred 15 min at 0 °C and then 4-bromobenzyl bromide (125 mg, 0.5 mmol) was added. The mixture was stirred 1 h at room temperature, ethyl acetate (50 mL) was added, washed with water (30 mL) and saturated NaCl (30 mL), and dried over Na_2SO_4. After evaporation of the solvent, the crude product was purified with hexane−CH_2Cl_2−ether (1:1:1) to afford 108 mg (78%) of **9d** as a yellow solid, mp 112.1−113.4 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.02 (s, 1H), 7.96 (d, J = 8.1 Hz, 1H), 7.50 (d, J = 8.4 Hz, 2H), 7.20 (m, 5H), 6.92 (t, J = 7.8 Hz, 1H), 6.80 (d, J = 8.1 Hz, 2H), 4.87 (s, 2H), 4.15 (m, 1H), 3.93 (m, 2H), 3.49 (m, 1H), 3.23 (m, 1H), 2.02 (m, 2H), 1.79 (m, 2H).

1-(4-Hydroxy-benzyl)-5-[2-(pyridin-3-*yl*-oxymethyl)-pyrrolidine-1-sulfonyl]-1*H*-indole-2,3-dione (10b). 1-[4-(*tert*-Butyl-diphenyl-silanyloxy)-benzyl]-5-[2-(pyridin-3-yloxymethyl)-pyrrolidine-1-sulfonyl]-1*H*-indole-2,3-dione (150 mg, 0.2 mmol) and *n*-Bu_4NF (53 mg, 0.2 mmol) in THF (6 mL) and water (2 mL) was stirred for 2 h, ethyl acetate (50 mL) was added, and the mixture was washed with water (30 mL) and saturated NaCl (30 mL) and dried over Na_2SO_4. The crude product was purified with ether−ethyl acetate (1:1) to afford 65 mg (66%) of **10b** as a yellow solid, mp 126.7−128.8 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.21 (m, 2H), 8.01 (s, 1H), 7.95 (d, J = 8.1 Hz, 1H), 7.26 (m, 2H), 7.19 (d, J = 8.4 Hz, 2H), 6.90 (d, J = 8.7 Hz, 1H), 6.84 (d, J = 8.4 Hz, 2H), 4.87 (s, 2H), 4.19 (m, 1H), 3.95 (m, 2H), 3.48 (m, 2H), 3.19 (m, 1H), 2.00 (m, 2H), 1.79 (m, 2H).

1-(4-Bromo-benzyl)-5-[2-(pyridin-3-*yl*-oxymethyl)-pyrrolidine-1-sulfonyl]-1*H*-indole-2,3-dione (10c). Compound **10c** was prepared according to the same procedure for compound **9d**, except using **7** and 4-bromobenzyl bromide, purified with CH_2Cl_2−ethyl acetate (1:1) to afford 53 mg (38%) of **10c** as a yellow solid, mp 92.1−93.3 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (m, 2H), 8.04 (s, 1H), 7.98 (d, J = 8.1 Hz, 1H), 7.51 (d, J = 8.1 Hz, 2H), 7.23 (m, 4H), 6.86 (d, J = 8.4 Hz, 1H), 4.90 (s, 2H), 4.23 (m, 1H), 3.97 (m, 2H), 3.50 (m, 1H), 3.17 (m, 1H), 2.02 (m, 2H), 1.78 (m, 2H).

2-[2-Oxo-5-(2-phenoxymethyl-pyrrolidine-1-sulfonyl)-1,2-dihydro-indol-3-*yl*-idene]-malononitrile (8). A solution of **6** (97 mg, 0.25 mmol) and malononitrile (18 mg, 0.27 mmol) in methanol (4

mL) was heated to reflux for 1 h, then cooled to room temperature. The solid was filtered out and dried in vacuum to afford 93 mg (86%) of **8** as a red solid, mp 245.7−248.4 °C. ^{1}H NMR (300 MHz, DMSO) δ 11.66 (s, 1H), 8.23 (s, 1H), 8.02 (d, J = 8.7 Hz, 1H), 7.25 (t, J = 8.7 Hz, 2H), 7.09 (d, J = 8.7 Hz, 1H), 6.90 (m, 3H), 4.05 (m, 1H), 3.92 (m, 2H), 3.39 (m, 1H), 3.15 (m, 1H), 1.90 (m, 2H), 1.72 (m, 2H).

2-[1-Methyl-2-oxo-5-(2-phenoxymethyl-pyrrolidine-1-sulfonyl)-1,2-dihydro-indol-3-*yl*-idene]-malononitrile (11a). Compound **11a** was prepared according to the same procedure for compound **8**, except using **9a**,[19,21] to afford 39 mg (87%) of **11a** as a red solid, mp 217.5 °C (dec). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.47 (s, 1H), 8.06 (dd, J = 8.6 Hz, J = 1.8 Hz, 1H), 7.21 (t, J = 7.8 Hz, 2H), 6.93 (t, J = 7.5 Hz, 1H), 6.89 (d, J = 8.7 Hz, 1H), 6.73 (d, J = 7.8 Hz, 2H), 4.11 (m, 1H), 4.08 (m, 1H), 4.00 (m, 1H), 3.49 (m, 2H), 3.26 (s, 3H), 2.11−1.88 (m, 4H).

2-[1-Benzyl-2-oxo-5-(2-phenoxymethyl-pyrrolidine-1-sulfonyl)-1,2-dihydroindol-3-*yl*-idene]-malononitrile (11b). Compound **11b** was prepared according to the same procedure for compound **8**, except using **9b**,[21] to afford 92 mg (88%) of **11b** as a purple solid, mp 196.6 °C (dec). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (s, 1H), 7.93 (dd, J = 8.6 Hz, J = 1.8 Hz, 1H), 7.39−7.29 (m, 5H), 7.14 (t, J = 7.2 Hz, 2H), 6.89 (t, J = 7.2 Hz, 1H), 6.83 (d, J = 8.7 Hz, 1H), 6.68 (d, J = 7.8 Hz, 2H), 4.90 (s, 2H), 4.05 (m, 2H), 3.97 (m, 1H), 3.45 (m, 2H), 2.07−1.85 (m, 4H).

2-[1-(Hydroxy-benzyl-2-oxo-5-(2-phenoxymethyl-pyrrolidine-1-sulfonyl)-1,2-dihydro-indol-3-*yl*-idene]-malononitrile (11c). Compound **11c** was prepared according to the same procedure for compound **8**, except using **9c**,[21] to afford 68 mg (84%) of **11c** as a purple solid, mp 174.9 °C (dec). ^{1}H NMR (300 MHz, DMSO) δ 9.51 (s, 1H), 8.30 (s, 1H), 8.10 (dd, J = 8.6 Hz, J = 1.8 Hz, 1H), 7.32−7.20 (m, 5H), 6.95−6.84 (m, 3H), 6.76 (d, J = 8.7 Hz, 2H), 4.87 (s, 2H), 4.09 (m, 1H), 3.97 (m, 2H), 3.40 (m, 1H), 3.20 (m, 1H), 1.90 (m, 2H), 1.71 (m, 2H).

2-[1-(4-Bromo-benzyl)-2-oxo-5-(2-phenoxymethyl-pyrrolidine-1-sulfonyl)-1,2-dihydro-indol-3-*yl*-idene]-malononitrile (11d). Compound **11d** was prepared according to the same procedure for compound **8**, except using **9d**, to afford 52 mg (86%) of **11d** as a purple solid, mp 237.0 °C (dec). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.46 (s, 1H), 7.95 (d, J = 8.1 Hz, 1H), 7.51 (d, J = 8.4 Hz, 2H), 7.20−7.13 (m, 4H), 6.90 (t, J = 7.5 Hz, 1H), 6.79 (d, J = 8.4 Hz, 1H), 6.68 (d, J = 7.8 Hz, 2H), 4.84 (m, 2H), 4.06 (m, 2H), 3.97 (m, 1H), 3.46 (m, 2H), 2.08−1.86 (m, 4H).

2-{1-Benzyl-2-oxo-5-[2-(pyridine-3-yloxymethyl)-pyrrolidine-1-sulfonyl]-1,2-dihydro-indol-3-*yl*-idene}-malononitrile (12a). Compound **12a** was prepared according to the same procedure for compound **8**, except using **10a**,[21] to afford 59 mg (75%) of **12a** as a purple solid, mp 216.5 °C (dec). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (s, 1H), 8.23 (m, 2H), 7.98 (dd, J = 8.4 Hz, J = 1.8 Hz, 1H), 7.42−7.35 (m, 5H), 7.20 (m, 2H), 6.91 (d, J = 8.7 Hz, 1H), 4.98 (s, 2H), 4.23 (m, 1H), 4.05 (m, 2H), 3.55 (m, 1H), 3.33 (m, 1H), 2.08 (m, 2H), 1.90 (m, 2H).

2-{1-(4-Hydroxy-benzyl)-2-oxo-5-[2-(pyridine-3-yloxymethyl)-pyrrolidine-1-sulfonyl]-1,2-dihydro-indol-3-*yl*-idene}-malononitrile (12b). Compound **12b** was prepared according to the same procedure for compound **8**, except using **10b**, to afford 46 mg (85%) of **12b** as a purple solid, mp 203.3 °C (dec). ^{1}H NMR (300 MHz, DMSO) δ 9.50 (s, 1H), 8.31 (s, 1H), 8.25 (d, J = 2.7 Hz, 1H), 8.17 (dd, J = 4.5 Hz, J = 1.5 Hz, 1H), 8.10 (d, J = 8.1 Hz, 1H), 7.34 (m, 3H), 7.24 (d, J = 8.4 Hz, 2H), 6.76 (d, J = 8.7 Hz, 2H), 4.88 (s, 2H), 4.16 (m, 1H), 4.08 (m, 1H), 3.96 (m, 1H), 3.41 (m, 1H), 3.18 (m, 1H), 1.91 (m, 2H), 1.71 (m, 2H).

2-{1-(4-Bromo-benzyl)-2-oxo-5-[2-(pyridine-3-yloxymethyl)-pyrrolidine-1-sulfonyl]-1,2-dihydro-indol-3-*yl*-idene}-malononitrile (12c). Compound **12c** was prepared according to the same procedure for compound **8**, except using **10c**, to afford 16 mg (45%) of **12c** as a purple solid, mp 232.3 °C (dec). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.50 (s, 1H), 8.21 (m, 1H), 8.16 (s, 1H), 7.96 (d, J = 8.4 Hz, 1H), 7.51 (d, J = 8.1 Hz, 2H), 7.20 (m, 4H), 6.84 (d, J = 8.7 Hz, 1H), 4.89 (m, 2H), 4.20 (m, 1H), 4.02 (m, 2H), 2.05−1.78 (m, 4H).

2-{1-(4-Methoxy-benzyl)-2-oxo-5-[2-(pyridin-3-yloxymethyl)-pyrrolidine-1-sulfonyl]-1,2-dihydro-indol-3-*yl*-idene}-malononitrile (12d). Compound **12d** was prepared according to the same procedure for compound **8**, except using **10d**,[21] to afford 91 mg (82%) of **12d** as a purple solid, mp 132.4 °C (dec). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.47 (s, 1H), 8.20 (m, 2H), 7.95 (dd, J = 8.4 Hz, J = 1.8 Hz, 1H), 7.26 (d, J = 8.7 Hz, 2H), 7.19 (m, 2H), 6.92 (d, J = 8.4 Hz, 1H), 6.89 (d, J = 8.7 Hz, 2H), 4.88 (s, 2H), 4.21 (m, 1H), 4.02 (m, 2H), 3.80 (s, 3H), 3.50 (m, 1H), 3.29 (m, 1H), 2.05 (m, 2H), 1.86 (m, 2H).

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Supporting Information Available: Experimental details corresponding to the synthesis of the compounds described in this paper, elemental analysis (C, H, N) results, enzyme inhibition assays, reversibility assay, and two-dimensional NMR study. This material is available free of charge via the Internet at http://pubs.acs.org.

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