5-Pyrrolidinylsulfonyl Isatins as a Potential Tool for the Molecular Imaging of Caspases in Apoptosis

Klaus Kopka,*[,‡,†,||] Andreas Faust,[‡,†,||] Petra Keul,[||,⊥] Stefan Wagner,[†] Hans-Jörg Breyholz,[†] Carsten Höltke,[†] Otmar Schober,[†] Michael Schäfers,[†,||] and Bodo Levkau[⊥]

Department of Nuclear Medicine, University Hospital of the Westfälische Wilhelms-Universität, Münster, Germany, Interdisciplinary Center of Clinical Research (IZKF), Münster, Germany, and Institute of Pathophysiology, Center of Internal Medicine, University Hospital, Essen, Germany

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Caspases are the unique enzymes responsible for the execution of the cell death program and may represent an exclusive target for the specific molecular imaging of apoptosis in vivo. 5-Pyrrolidinylsulfonyl isatins represent potent nonpeptidyl caspase inhibitors that may be suitable for the development of *c*aspase *b*inding *r*adioligands (CBRs). (*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]isatin (**7**) served as a lead compound for modification of its *N*-1-position. Corresponding pairs of *N*-1-substituted 2-methoxymethyl- and 2-phenoxymethylpyrrolidinyl derivatives were examined in vitro by biochemical caspase inhibition assays. All target compounds possess high in vitro caspase inhibtion potencies in the nanomolar to subnanomolar range for caspase-3 (K_i = 0.2−56.1 nM). As shown for compound (*S*)-1-(4-(2-fluoroethoxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (**35**), the class of *N*-1-substituted 5-pyrrolidinylsulfonyl isatins competitively inhibits caspase-3. All caspase inhibitors show selectivity for the effector caspases-3 and -7 in vitro. The 2-methoxymethylpyrrolidinyl versions of the isatins appear to possess superior caspase inhibition potencies in cellular apoptosis inhibition assays compared with the 2-phenoxymethylpyrrolidinyl inhibitors.

Introduction

Apoptosis is an energy-dependent and genetically controlled mechanism of cell disposal without inflammatory response that serves to maintain homeostasis in multicellular organisms.[1] Its dysregulation is linked both to increased cell proliferation or enhanced cell death, resulting in a variety of diseases, such as acute myocardial infarction,[2] atherosclerosis,[3,4] allograft rejection,[5−8] stroke,[9−11] neurodegenerative disorders,[12−14] and tumorigenesis.[15−18] Both the diagnosis and treatment of these diseases require in many cases the option of noninvasive and serial imaging of apoptosis in vivo, e.g. as a surrogate marker of successful chemotherapy of tumors, making it a tool of utmost importance in disease management and, at the same time, one of the current challenges in modern medicine.

Currently, radiolabeled[19−22] or fluorochrome[23]-labeled annexin V is suggested for imaging of apoptotic processes in animal models and patients in vivo. Furthermore, annexin V cross-linked with iron oxide particles (annexin V−CLIO) is currently in development for magnetic resonance imaging (MRI).[24] The 35.8-kDa protein annexin V binds in a calcium-dependent manner to anionic phospholipids, especially to phosphatidylserine (PS), which externalizes to the outer cell membrane during the early apoptotic signaling cascade. After an apoptotic trigger, e.g. an increase of the intracellular calcium concentration $[Ca^{2+}]_i$, the ATP-dependent enzymes translocase and floppase are deactivated while the membrane scramblase activity is enhanced, both leading to PS externalization. Until now the majority of apoptosis-imaging agents is derived from annexin V-based radiotracers.[19] However, there are several caveats that preclude the use of annexin V ligands as exclusive reporter probes for apoptosis in molecular imaging techniques: annexin V binds not only to apoptotic cells but also to PS contained in necrotic cell membrane fragments as well as to the PS molecules exposed during complement-mediated cell lysis or platelet activation.[25] Furthermore, it seems that annexin V targets not only PS but rather intracellularly accumulates in nonapoptotic lymphocytes by a PS-targeted, pinocytosis-like mechanism.[26] Due to these properties of PS, its detection in pathologies using annexin V has been defined as *phosphatidyl serinopathy* rather than apoptosis, thus allowing a better account for the difficulty in distinguishing borderline situations such as apoptosis, necrosis, and reversible damage by annexin V.[26,27] Accordingly, more specific targets for the exclusive and noninvasive apoptosis imaging in vivo are required.

One such molecular target may be represented by the family of intracellular death enzymes, the caspases (*c*ysteinyl *asp*artate-specific prote*ases*), which execute the common final path of the programmed cell death process. Recently, the peptide-based irreversible pan-caspase inhibitor Z-VAD-fmk was successfully radioiodinated and suggested as a caspase imaging agent. However, poor cell permeability hindered the intracellular targeting of activated caspases and prevented an in vivo application.[29] Instead of peptide-based caspase inhibitors, we propose nonpeptidyl caspase inhibitors of the 5-pyrrolidinylsulfonyl isatin-type as *c*aspase *b*inding *r*adioligands (CBRs) that may be capable of directly targeting apoptosis in vivo. 5-Pyrrolidinylsulfonyl isatins have been shown to selectively inhibit the effector (or downstream) caspases-3 and -7 in vitro.[30−32] Radiolabeled 5-pyrrolidinylsulfonyl isatins are expected to form intracellular enzyme−inhibitor complexes by covalent binding to the enzyme active site of the activated caspases. The dicarbonyl functionality of the isatins binds to the Cys residue of the active site. A thiohemiketal is formed via the electrophilic C-3 carbonyl carbon of the isatin and the nucleophilic Cys

* To whom correspondence should be addressed. Phone: +49−251−83−47362. Fax: +49−251−83−47363. E-mail: kopka@uni-muenster.de.
‡ These authors contributed equally to this work.
† Department of Nuclear Medicine, University Hospital of the Westfälische Wilhelms-Universität, Münster, Germany.
|| Interdisciplinary Center of Clinical Research (IZKF), Münster, Germany.
⊥ Institute of Pathophysiology, Center of Internal Medicine, University Hospital, Essen, Germany.

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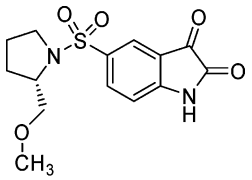

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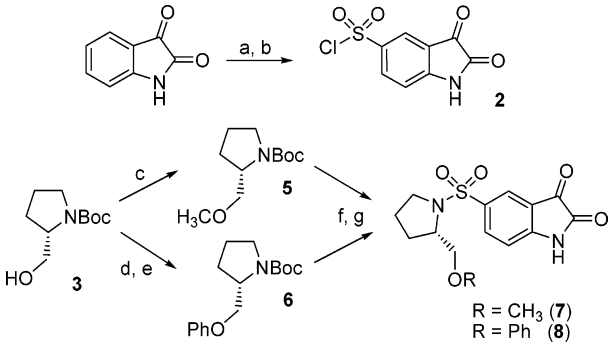
Figure 1. (*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]isatin (**7**) as lead structure for the development of *c*aspase *b*inding *r*adioligands (CBRs).

Scheme 1[a]



[a] (a) H_2SO_4/SO_3; (b) $POCl_3$, tetramethylene sulfone; (c) NaH, MeI, THF; (d) TosCl, pyridine, CH_2Cl_2; (e) NaH, phenol, THF; (f) TFA, CH_2Cl_2; (g) **2**, Hünig's base, $CHCl_3$/THF.

thiolate function. Recently, a 2.8-Å resolution X-ray cocrystal structure of an enzyme−inhibitor complex between recombinant human caspase-3 and (*S*)-1-methyl-5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]isatin (compound **10** in this paper) has been determined.[33] Consequently, the ability of the caspases to cleave substrates possessing a P1 Asp residue that reaches into the primary S1 pocket is blocked (reversible inhibitory effect).[33] (The S1 pocket itself is composed of Arg, Gln, Arg, and Ser.)[34] Therefore, the 5-pyrrolidinylsulfonyl isatins should be potentially applicable in noninvasive nuclear medicine diagnosis with high clinical impact to differentiate between balanced (physiological) and unbalanced (pathological) apoptosis using single photon emission computed tomography (SPECT) or positron emission tomography (PET).

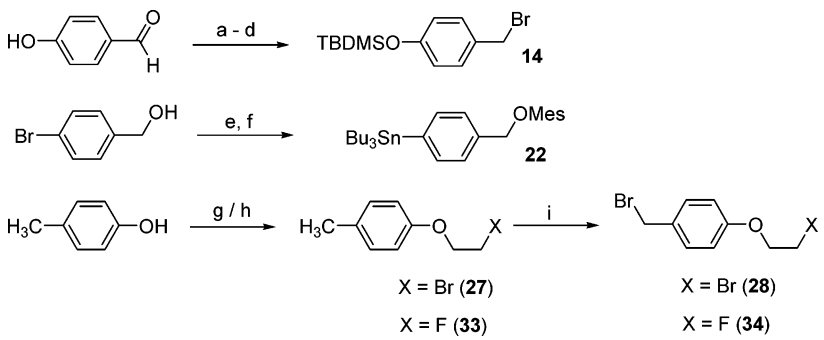
We have chosen the caspase inhibitor (*S*)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (**7**) (log $D = 0.23$, calculated by the ACD/LogD suite) (Figure 1), which possesses cardioprotective potential in isolated rabbit hearts after ischemic injury, as lead structure for the development of CBRs.[35] The *N*-1-position offers the feasibility to refine the caspase inhibition potency of the isatins. Here, the 2-methoxymethylpyrrolidinyl (**9**, **15**, **17**, **19**, **23**, **25**, **29**, **31**, and **35**, Scheme 3) as well as the corresponding 2-phenoxymethylpyrrolidinyl versions (**10**, **16**, **18**, **20**, **24**, **26**, **30**, **32**, and **36**, Scheme 3) of *N*-1-substituted isatins were synthesized to evaluate their caspase inhibition potency for caspase-3. The new compound (*S*)-1-(4-(2-fluoroethoxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]-isatin (**35**) with moderate lipophilicity was selected for enzyme kinetic studies in order to determine competitive or noncompetitive binding characteristics of the 5-pyrrolidinylsulfonyl isatins. In addition, the in vitro caspase-3 and -7 selectivity of the 5-pyrrolidinylsulfonyl isatins was proven by screening the caspase inhibition potencies of all compounds for different classes of caspases (here caspase-1, -3, -6, -7, and -8). Furthermore, cellular apoptosis assays with human endothelial cells were performed to determine the biological activity of eight nonradioactive counterparts of potential CBRs (**9**/**10**, **19**/**20**, **25**/**26**, and **35**/**36**). Finally, one model CBR was radiolabeled with iodine-125 ([^{125}I]**26**).

Results

Chemistry. The chosen 5-pyrrolidinylsulfonyl isatin lead compound **7** as well as the structurally related analogs **8**−**10**, **15**−**20**, **23**−**26**, **29**−**32**, and **35**−**36** were synthesized. Compounds **7**, **8**, and **10** were previously described as caspase inhibitors and suggested for the therapeutic inhibition of apoptosis.[31,33,35] These compounds served as lead structures for chemical modification. We decided to modify the isatins at the *N*-1-position, which does not seem to interact with the S1 pocket of the caspases usually incorporating an Asp residue to be cleaved in substrates containing Asp at the P1 position.[31] Ten radiolabeling precursor compounds (^{123}I-precursors, **17**, **18**, **23**, **24**; ^{11}C-precursors, **7**, **8**, **17**, **18**; ^{18}F-precursors, **29**, **30**, **31**, **32**) and eight corresponding nonradioactive counterparts of feasible SPECT- (**25**, **26**) and PET-compatible (**9**, **10**, **19**, **20**, **35**, **36**) CBRs were synthesized.

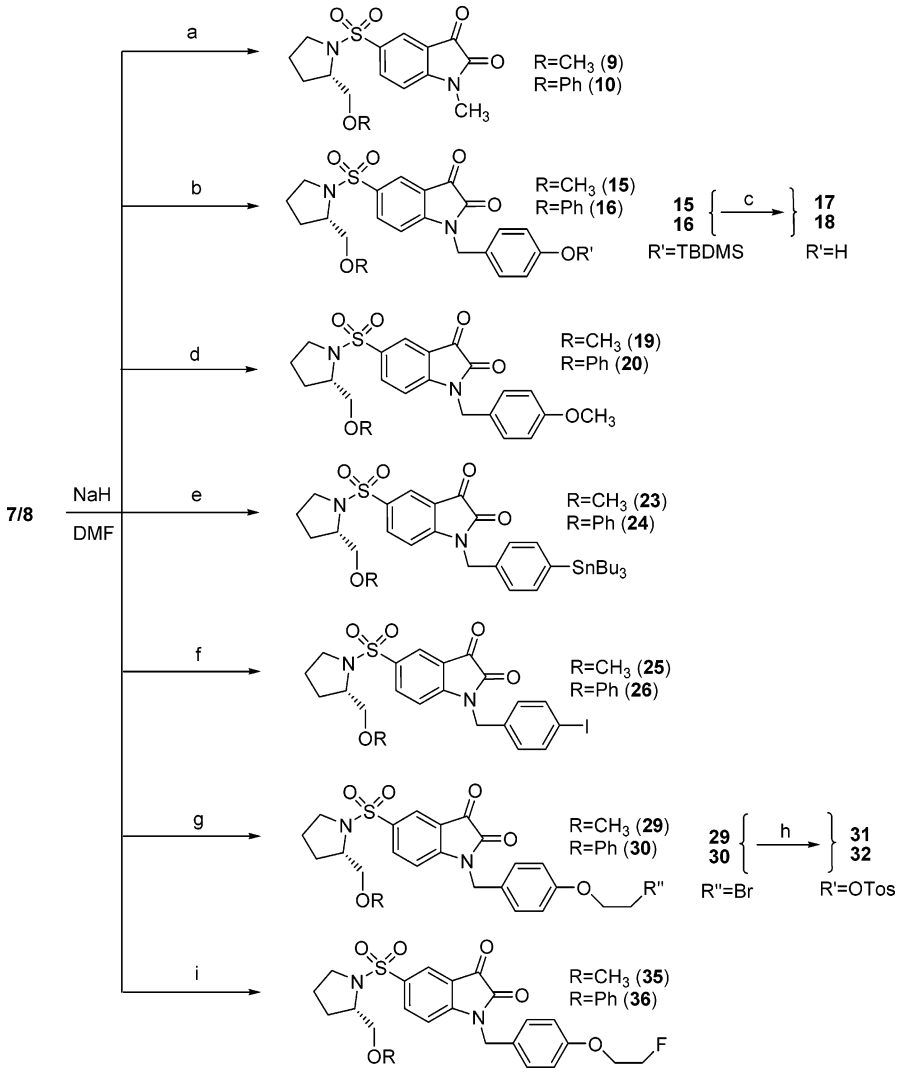
Lead compound **7** (Scheme 1) and the corresponding 2-phenoxymethylpyrrolidinyl compound **8** were obtained over seven- and eight-step syntheses, respectively, starting from isatin and L-proline as described elsewhere.[31] The new isatin compounds were prepared using a similar nine- or ten-step synthesis route, finishing with the *N*-1-alkylation step (Scheme 3). Some of the alkylation reagents were not commercially available and were prepared according to Scheme 2. Because of the low nucleophilicity of the isatin, nitrogen reaction partners were needed with high reactivity such as benzyl mesylates, bromides or chlorides. In the case of benzyl bromides, the alkylation step proceeded at ambient temperature within 3 h while the reaction mixtures were heated to 80 °C for benzyl mesylates or chlorides. Compounds **9** and **10** were synthesized by conversion of compounds **7** and **8**, respectively, in the presence of methyl

Scheme 2[a]



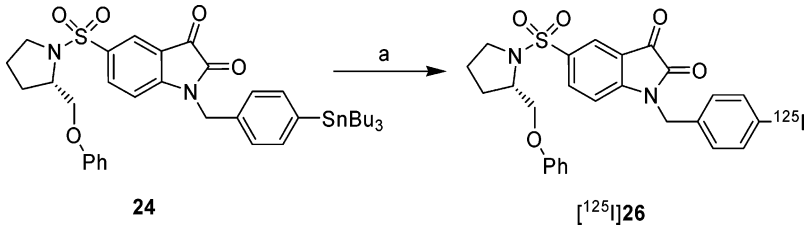
[a] (a) TBDMSCl, imidazole, THF; (b) $LiAlH_4$, THF; (c) TFA anhydride, THF; (d) LiBr, THF; (e) BuLi, Bu_3SnCl, THF; (f) MesCl, NEt_3, CH_2Cl_2; (g) (X = Br) dibromoethane, phase transfer catalyst, NaOH, water; (h) (X = F) fluoroethyl tosylate, Cs_2CO_3, DMF; (i) NBS, AIBN, CCl_4.

Scheme 3[a]



[a] (a) MeI; (b) **14**; (c) HCl, MeOH; (d) *p*-methoxybenzyl bromide; (e) **22**; (f) *p*-iodobenzyl bromide; (g) **28**; (h) silver *p*-toluenesulfonate, CH_3CN; (i) **34**.

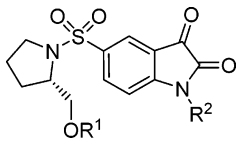
Scheme 4[a]



[a] (a) [^{125}I]NaI, chloramine-T, buffer.

iodide at ambient temperature. The chemical yields were 46 and 57%, respectively. To achieve the precursor compounds **17** and **18** for the potential radiosynthesis of corresponding [*O-methoxy*-^{11}C]-CBRs we prepared the *O*-protected alkylation agent **14** in a four-step synthesis starting from *p*-hydroxybenzaldehyde with overall yields of 40% (Scheme 2).[37] The *N*-alkylation step yielded the corresponding silyl-protected isatin derivatives **15** and **16**, and the deprotection resulted in the desired precursors **17** and **18** in nearly quantitative yields. The nonradioactive counterparts of [*O-methoxy*-^{11}C]-CBRs, **19** and **20**, were obtained via alkylation of compounds **7** and **8** with *p*-methoxybenzyl chloride in yields not less than 68%. The iodinated compounds **25** and **26** represent references of radioiodinated CBRs, the stannylated derivatives **23** and **24** the corresponding radioiodination precursors. The alkylation reagent *p*-tributylstannylbenzyl mesylate **22** was synthesized starting from *p*-bromobenzyl alcohol in two steps with an overall yield of 40% (Scheme 2).[38] The following *N*-alkylation of the isatin derivatives **7** and **8** yielded the precursors **23** and **24**, ready for radioiodination experiments. The nonradioactive reference isatins **25** and **26** were obtained with *p*-iodobenzyl bromide in acceptable yields (66%). For future ^{18}F-labeling experiments, we subsequently prepared the precursors **31** and **32** by nucleophilic substitution of the bromides **29** and **30** with silver tosylate in acetonitrile. The conversion was almost quantitative after 24 h. To synthesize the necessary bromides **29** and **30** and the fluorinated isatins **35** and **36**, we prepared the corresponding benzyl bromides **28** and **34** from the toluene derivatives **27** and

Table 1. Caspase-3 Binding Potencies of the 5-Pyrrolidinylsulfonyl isatins Expressed as K_i Values



compd	R^1	R^2	$K_{i,caspase-3}$,[a] nM	log *D* values[b]
7[c]	CH_3	H	41.8 ± 12.6 (60[33])	0.23
8[c]	Ph	H	8.3 ± 3.8 (IC_{50}: 44[31])	2.23
9[d]	CH_3	CH_3	1.2 ± 0.2	0.28
10[d]	Ph	CH_3	56.1 ± 5.3 (15[33])	2.27
15	CH_3	*p*-TBDMSO-C_6H_4-CH_2	1.9 ± 0.1	0.45
16	Ph	*p*-TBDMSO-C_6H_4-CH_2	9.8 ± 2.2	2.44
17[c]	CH_3	*p*-HO-C_6H_4-CH_2	23.5 ± 4.1	1.32
18[c]	Ph	*p*-HO-C_6H_4-CH_2	4.0 ± 2.8	3.31
19[d]	CH_3	*p*-CH_3O-C_6H_4-CH_2	9.0 ± 1.1	1.97
20[d]	Ph	*p*-CH_3O-C_6H_4-CH_2	4.8 ± 0.4	3.96
23[c]	CH_3	*p*-*n*Bu_3Sn-C_6H_4-CH_2	12.6 ± 2.0	9.86
24[c]	Ph	*p*-*n*Bu_3Sn-C_6H_4-CH_2	>5000	11.85
25[e]	CH_3	*p*-I-C_6H_4-CH_2	5.9 ± 0.7	3.09
26[e]	Ph	*p*-I-C_6H_4-CH_2	1.2 ± 0.4	5.08
29[c]	CH_3	*p*-Br-$(CH_2)_2$-O-C_6H_4-CH_2	8.0 ± 4.3	2.73
30[c]	Ph	*p*-Br-$(CH_2)_2$-O-C_6H_4-CH_2	12.6 ± 0.3	4.73
31[c]	CH_3	*p*-TosO-$(CH_2)_2$-O-C_6H_4-CH_2	0.8 ± 0.1	3.05
32[c]	Ph	*p*-TosO-$(CH_2)_2$-O-C_6H_4-CH_2	8.6 ± 1.8	5.05
35[d]	CH_3	*p*-F-$(CH_2)_2$-O-C_6H_4-CH_2	17.9 ± 2.7	2.20
36[d]	Ph	*p*-F-$(CH_2)_2$-O-C_6H_4-CH_2	0.2 ± 0.1	4.19

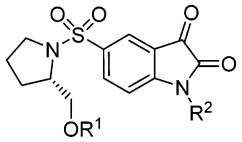
[a] $K_i = IC_{50}/(1 + [S]/K_M)$ with [S] = 10 μM, K_M = 9.7 μM ± 1.0 μM; S = Ac-DEVD-AMC; values are the mean ± SD of three assays. [b] log *D* values calculated with ACD/Chemsketch Labs 6.00 (log *D* = log *P* at physiological pH). [c] Precursor compounds ready for direct radiolabeling. [d] Nonradioactive counterparts of PET-compatible CBRs. [e] Nonradioactive counterparts of SPECT-compatible CBRs.

33 by radical bromination (Scheme 2). The resulting benzyl bromides were found to be very unstable and, thus, were used immediately for the *N*-alkylation step.

Radiochemistry. The ^{125}I-labeled model CBR [^{125}I]**26** was obtained by an iododemetalation reaction using no-carrier-added [^{125}I]NaI and the tributylstannylated precursor **24** with chloramine-T hydrate as the oxidant (Scheme 4).[39] Compound **26** was used as the nonradioactive reference compound of [^{125}I]**26** to prove its identity. Compound [^{125}I]**26** was isolated in 90% average radiochemical yield. The chemical and radiochemical purities were >95%. The calculated specific activity of [^{125}I]-**26** was 0.134 GBq/μg (80.5 GBq/μmol) at the end-of-synthesis (EOS).

Enzyme Assays. The binding potencies of the modified 5-pyrrolidinylsulfonyl isatin analogs which represent potential radiolabeling precursors (**7**, **8**, **17**, **18**, **23**, **24**, **29**−**32**) or nonradioactive counterparts of potential CBRs (**9**, **10**, **19**, **20, 25**, **26, 35**, **36**) were measured by fluorogenic in vitro caspase inhibition assays using recombinant full-length human caspase-3 and its substrate Ac-DEVD-AMC. The resulting caspase-3 inhibitions were obtained as IC_{50} values by a nonlinear regression fit of the concentration-dependent reaction rates and converted into the corresponding K_i values. The in vitro results are summarized in Table 1. Table 1 also contains the calculated log *D* values of the synthesized 5-pyrrolidinylsulfonyl isatin derivatives to consider the variations of lipophilicities, caused by the systematically attached 2-methoxymethyl- or 2-phenoxyphenylpyrrolidinyl residues. According to Lee et al.[31,33] compounds **7**, **8**, and **10** are competitive caspase inhibitors selective for the effector caspases-3 and -7. For comparison and to confirm our results, the K_i or IC_{50} values for caspase-3 inhibition found by Lee et al. were added in Table 1. With the exception of compound **24**, which has lost its binding potency in the chosen 2-phenoxymethyl/*N*-*p*-(tri-*n*-butylstannyl)benzyl substitution pattern of the 5-pyrrolidinylsulfonyl isatin, all *N*-1-alkylated analogs display similar or even better caspase-3 binding potencies compared with the *N*-desalkyl compounds.

Table 2. Caspase Inhibition Potencies of the 5-Pyrrolidinylsulfonyl isatin Analogs for Caspases-1, -3, -6, -7, and -8 Expressed as IC_{50} Values



			IC_{50}, nM[a]					
compd	R^1	R^2	caspase-1	caspase-3	caspase-6	caspase-7	caspase-8	log D values[b]
7[c]	CH_3	H	>15 000	84.9 ± 25.6	>500 000	1290 ± 466	>500 000	0.23
8[c]	Ph	H	>10 000	16.9 ± 7.7	>500 000	294 ± 178	>500 000	2.23
9[d]	CH_3	CH_3	>25 000	2.4 ± 0.3	>500 000	304 ± 73	>500 000	0.28
10[d]	Ph	CH_3	>500 000	114 ± 10.7	>500 000	190 ± 156	>500 000	2.27
15	CH_3	*p*-TBDMSO-C_6H_4-CH_2	>7 000	3.8 ± 0.2	>500 000	69.8 ± 39.2	>500 000	0.45
16	Ph	*p*-TBDMSO-C_6H_4-CH_2	>500 000	20.0 ± 4.5	>500 000	366 ± 111	>500 000	2.44
17[c]	CH_3	*p*-HO-C_6H_4-CH_2	>60 000	47.8 ± 8.3	>500 000	32.3 ± 7.6	>500 000	1.32
18[c]	Ph	*p*-HO-C_6H_4-CH_2	>25 000	8.2 ± 5.6	>500 000	52.7 ± 20.4	>500 000	3.31
19[d]	CH_3	*p*-CH_3O-C_6H_4-CH_2	>10 000	18.4 ± 2.3	>500 000	4.0 ± 3.0	>500 000	1.97
20[d]	Ph	*p*-CH_3O-C_6H_4-CH_2	>500 000	9.7 ± 0.8	>500 000	31.2 ± 16.5	>500 000	3.96
23[c]	CH_3	*p*-*n*Bu_3Sn-C_6H_4-CH_2	>500 000	25.5 ± 4.1	>500 000	29.2 ± 19.4	>500 000	9.86
24[c]	Ph	*p*-*n*Bu_3Sn-C_6H_4-CH_2	>500 000	>10 000	>500 000	42.8 ± 6.4	>500 000	11.85
25[e]	CH_3	*p*-I-C_6H_4-CH_2	>10 000	12.0 ± 1.4	>500 000	1.8 ± 0.3	>500 000	3.09
26[e]	Ph	*p*-I-C_6H_4-CH_2	>500 000	2.4 ± 0.9	>500 000	18.6 ± 14.9	>500 000	5.08
29[c]	CH_3	*p*-Br-$(CH_2)_2$-O-C_6H_4-CH_2	>15 000	16.3 ± 8.7	>500 000	25.7 ± 3.6	>500 000	2.73
30[c]	Ph	*p*-Br-$(CH_2)_2$-O-C_6H_4-CH_2	>500 000	25.6 ± 0.6	>500 000	42.1 ± 53.1	>500 000	4.73
31[c]	CH_3	*p*-TosO-$(CH_2)_2$-O-C_6H_4-CH_2	>500 000	1.7 ± 0.2	>500 000	13.8 ± 3.0	>500 000	3.05
32[c]	Ph	*p*-TosO-$(CH_2)_2$-O-C_6H_4-CH_2	>500 000	17.5 ± 3.6	>500 000	50.9 ± 11.6	>500 000	5.05
35[d]	CH_3	*p*-F-$(CH_2)_2$-O-C_6H_4-CH_2	>25 000	36.4 ± 5.5	>25 000	93.3 ± 1.0	>25 000	2.20
36[d]	Ph	*p*-F-$(CH_2)_2$-O-C_6H_4-CH_2	>500 000	0.3 ± 0.1	>500 000	22.4 ± 7.8	>500 000	4.19

[a] Values are the mean ± SD of three assays. [b] log *D* values calculated with ACD/Chemsketch Labs 6.00 (log *D* = log *P* at physiological pH). [c] Precursor compounds ready for direct radiolabeling. [d] Nonradioactive counterparts of PET-compatible CBRs. [e] Nonradioactive counterparts of SPECT-compatible CBRs.

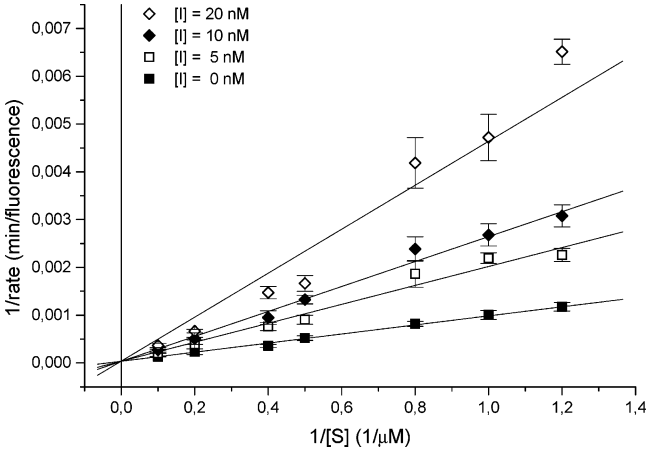


Figure 2. Competitive inhibition of caspase-3 by compound **35**. The concentrations of compound **35** were 0 nM (■), 5 nM (□), 10 nM (◆), and 20 nM (◇).

Interestingly, attaching the even more bulky *N*-benzyl as well as the *p*-substituted *N*-benzyl residues (*p*-methoxy, **19**, **20**; *p*-iodo, **25**, **26**; *p*-2-fluoroethyloxy, **35**, **36**) does not influence the binding potency of the model isatins in vitro. Therefore, the *N*-1-position of the 5-pyrrolidinylsulfonylisatins turns out as a possible position of choice for the modification and development of the isatins as CBRs. Lately, Chu et al. have also suggested the modification of the isatins i.a. at the *N*-1-position.[36] The caspase inhibition potencies of the 5-pyrrolidinylsulfonyl isatin derivatives were subsequently assayed for caspase-1, -3, -6, -7, and -8. The caspase inhibition potencies of all compounds expressed in IC_{50} values correspond to those recently determined for *N*-1-benzylated isatin analogs[31,33,36] and confirm the in vitro caspase-3 and -7 selectivity of the here described 5-pyrrolidinylsulfonyl isatins (Table 2). Exemplary enzyme kinetic studies were also performed using the model compound (*S*)-1-(4-(2-fluoroethoxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (**35**) to determine the mechanism of inhibition of caspase-3 activity. The Lineweaver−Burk diagram (Figure 2) displays competitive inhibiton for compound **35** versus Ac-DEVD-AMC. These data are consistent with previous studies demonstrating that the isatins bind to the cysteinyl active site of the activated caspase.[31,36]

Cellular Apoptosis Assays. Since the caspases represent an intracellular in vivo target, a set of pairs was generated, each containing the 2-methoxymethylpyrrolidinyl or the corresponding 2-phenoxymethylpyrrolidinyl building block (**7/8**, **9/10**, **15/16**, **17/18**, **19/20**, **23/24**, **25/26**, **29/30**, **31/32**, **35/36**), and examined for their potency of apoptosis inhibition in cellular assays. The 2-methoxymethyl or 2-phenoxyphenyl residues putatively occupy the shallow S3 pocket of the caspases.[33] Recently, a difference has been observed between the in vitro caspase-3 inhibition potency (IC_{50} = 30 nM) and the cellular caspase inhibition potency ($IC_{50} \approx 10$ μM in wild-type neutrophils) of the 2-phenoxymethyl compound **10**,[33] which suggests a limited cell penetration efficiency of inhibitor **10** and/or the influence of a reversible protein binding of **10** to cytosolic constituents. Therefore, we hypothesized that attaching the methoxy instead of the phenoxy group may affect the cellular caspase inhibition potency of the 5-pyrrolidinylsulfonyl isatins. Consequently, the pairs of the nonradioactive model isatin candidates **9/10**, **19/20**, **25/26**, and **35/36** were evaluated. Human umbilical vein endothelial cells (HUVEC) undergoing apoptosis after growth factor withdrawal were incubated with different concentrations of the nonradioactive isatins for 8 h. Western blot analysis with an antibody against the active p17- und p12-fragments of caspase-3 indicates that caspase processing is inhibited by the 2-methoxymethyl-substituted 5-pyrrolidinylsulfonyl isatins **9**, **19**, **25**, and **35** at concentrations of 10 μM (Figure 3). In contrast, the corresponding 2-phenoxyphenyl analogs **10**, **20**, **26**, and **36** were less effective in inhibiting caspase-3 processing.

Subsequently, we concentrated on the most promising 2-methoxymethyl compounds **9**, **19**, and **35** and tested their efficiency in the same system. We observed that all three compounds inhibited caspase-3 processing to its p17- and p12-subunits as well as the processing of caspase-8 and caspase-9 (Figure 4). In addition, the cleavage of the general caspase substrate poly(ADP-ribose)polymerase (PARP) known to be cleaved by several caspases as well as the cleavage of caspase substrates specific for unique caspases (lamin A/C for caspase-6 and α-fodrin for caspase-3) was inhibited (Figure 4 and Figure 5), suggesting that the activity of these caspases toward their endogenous substrates was also efficiently inhibited. Within the 2-methoxymethyl series of the model isatins, the cellular caspase

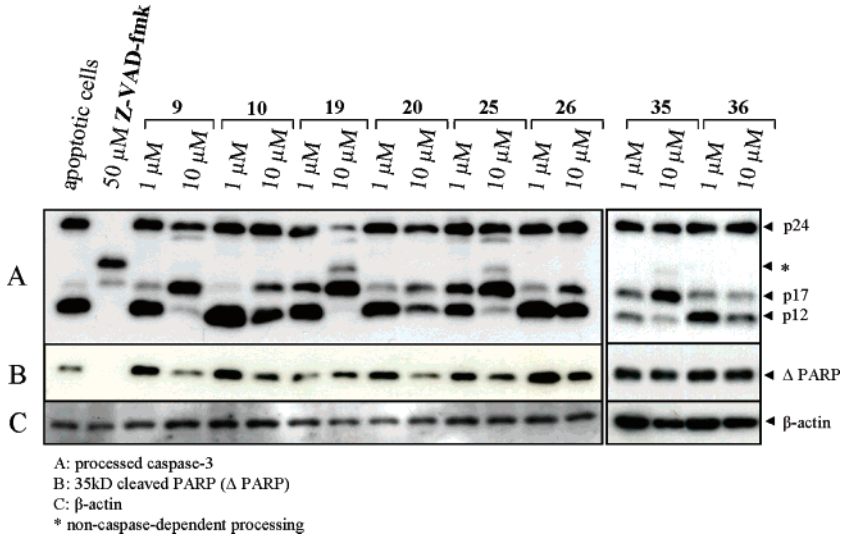


Figure 3. Western blot analysis of active, processed caspase-3 and the 35-kDa fragment of cleaved PARP in apoptotically dying human endothelial cells in the presence of different concentrations (c = 1 μM/c = 10 μM) of PET-compatible (**9**, **10**, **19**, **20**, **35**, **36**) and SPECT-compatible (**25**, **26**) nonradioactive conterparts of the CBRs. Z-VAD-fmk was used as a control for full inhibition of caspase processing and β-actin as a loading control.

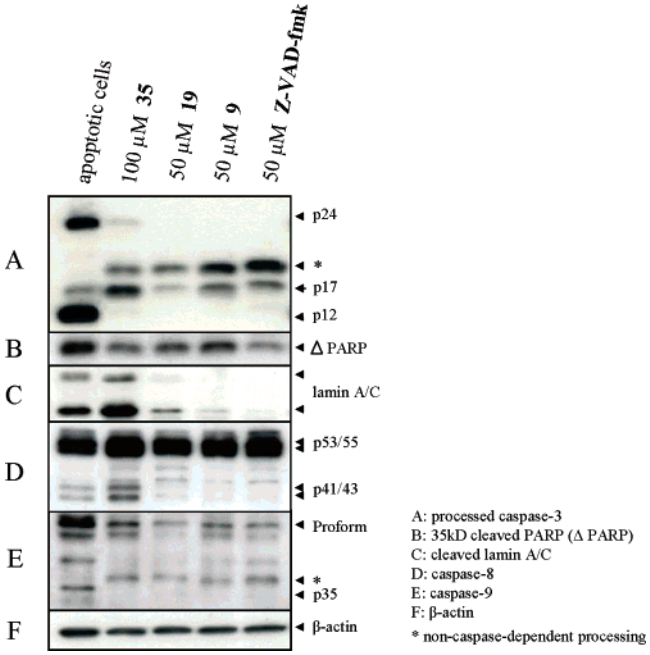


Figure 4. The caspase-3 inhibition efficiencies of the nonradioactive methoxymethyl isatins in apoptotically dying endothelial cells increase in the order **35** ($c = 100$ μM) < **19** ($c = 50$ μM) = **9** ($c = 50$ μM). The inhibition efficacy of 50 μM **9** and **19** is comparable with that of 50 μM Z-VAD-fmk. Similar values are observed with respect to the inhibition of cleavage of PARP and lamin A/C as well as the activation of caspase-8 and -9. β-Actin was used as a loading control.

inhibition potencies reflected by caspase processing to their active subunits decreased in the order **9** ($c = 50$ μM) = **19** ($c = 50$ μM) > **35** ($c = 100$ μM) (Figure 4). This inhibition correlated closely with the inhibition of the cleavage of the endogenous caspase substrates, confirming that inhibition of the caspases processing mirrors inhibition of their intracellular activity (Figures 4 and 5). Under the same conditions, the 2-phenoxymethyl compounds such as isatin **36** showed no comparable inhibitory effect at concentrations up to 300 μM (data not shown). Therefore, the 2-phenoxymethyl variants of the model isatins seem to be biologically less potent in our cellular apoptosis model. In contrast, when the 2-methoxymethyl substitution pattern of the 5-pyrrolidinylsulfonyl isatins **9**, **19**, and **35** were used in therapeutic rather than imaging-based doses, they showed a better cellular caspase inhibition potency against activated caspases in intact cells compared with the 2-phenoxymethyl substituted analogs such as compound **36**. Thus, the compounds **9**, **19**, and **35** from the established library of eight nonradioactive model isatins possessed the most efficient caspase inhibition potential.

Discussion

From our data, we would suggest that the nonpeptidyl caspase inhibitiors of the 5-pyrrolidinylsulfonyl isatin-type are promising compounds for the development as CBRs, thus constituting a potential class of apoptosis-imaging agents based on the imaging of activated caspases in vivo. The 5-pyrrolidinylsulfonyl isatins are the first example of potent nonpeptidyl caspase inhibitors that selectively bind to the effector caspases-3 and -7 in vitro.[31−33,36] We here present a library of eight corresponding nonradioactive counterparts of PET- and SPECT-compatible 5-pyrrolidinylsulfonyl isatin analogs. Our preliminary radiolabeling attempts resulted in the radiolabeled 5-pyrrolidinylsulfonyl isatin derivative [^{125}I]**26**. In future studies, the synthesized precursors **7**, **8**, **17**, **18**, **23**, **24**, **31**, and **32** will be radiolabeled with different radionuclides, including iodine-123, fluorine-18, or carbon-11, which are typically used for noninvasive scintigraphic imaging using SPECT and PET.[39−42] This should result in lead candidate CBRs capable of intracellular targeting early

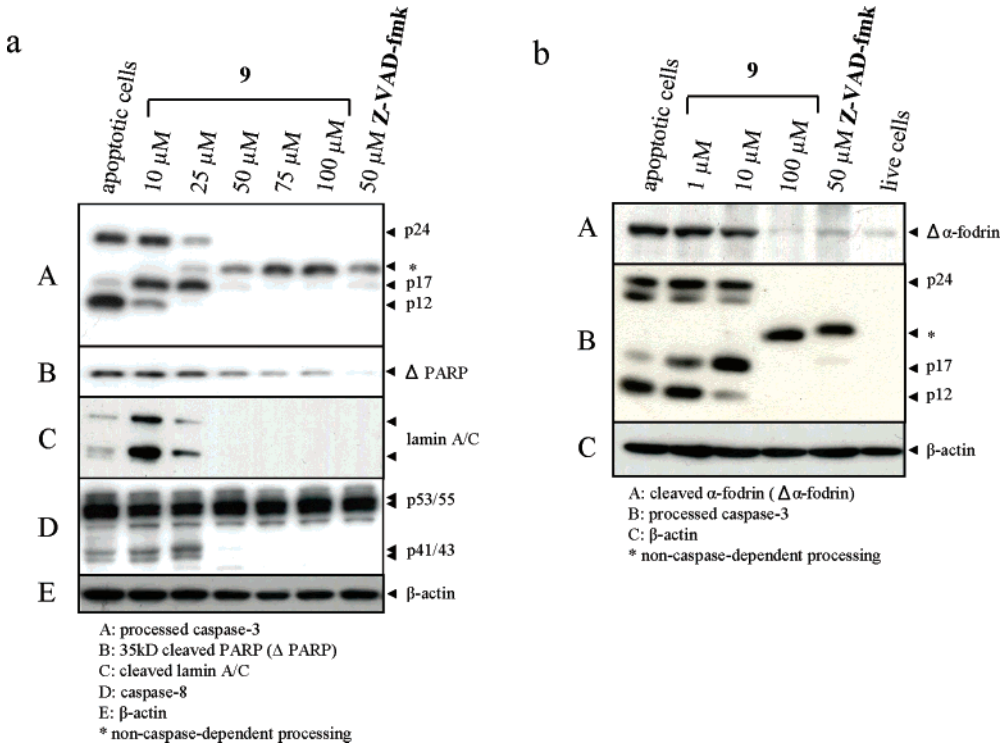


Figure 5. (a) Concentration kinetic of the inhibitory potential of compound **9** toward caspase-3, caspase-6 (lamin cleavage), and caspase-8 as well as cleavage of PARP (35 kDa cleaved PARP fragment). (b) Concentration kinetic of the inhibitory potential of compound **9** toward caspase-3 and its specific substrate α-fodrin. Z-VAD-fmk was used as a control for full inhibition of caspase processing, live cells as a nonapoptotic, negative control, and β-actin as a loading control.

stages of the apoptotic process. Caspase activation occurs in all settings of clinically relevant apoptosis, such as that in acute myocardial infarction, chronic heart failure, allograft rejection, stroke, neurodegenerative disorders, induction of apoptosis by chemotherapy or irradiation, and in other pathological conditions.[2−18] If successfully applied in clinical diagnostic algorithms in the future, the CBRs could represent the first synthetic nonpeptidyl biomarkers (radiotracers) that can exclusively and noninvasively detect apoptotic tissues in vivo. As the 5-pyrrolidinylsulfonyl isatins are potent nonpeptidyl caspase inhibitors that selectively bind to the caspases in vitro,[31−33,36] their in vivo application may substantially extend our knowledge of the final common path of apoptosis in vivo.

In a first step, a set of potential nonradioactive counterparts of SPECT-compatible CBRs (Table 1, **25**, **26**) as well as PET-compatible CBRs (Table 1, **9**, **10**, **19**, **20**, **35**, **36**) were synthesized without loss of in vitro caspase-3 inhibition potencies, which range between 56.1 and 0.2 nM [$K_{i,caspase-3}$(**10**) = 56.1 nM, $K_{i,caspase-3}$(**35**) = 17.9 nM, $K_{i,caspase-3}$(**19**) = 9.0 nM, $K_{i,caspase-3}$(**25**) = 5.9 nM, $K_i$caspase-3(**20**) = 4.8 nM, $K_{i,caspase-3}$(**26**) = 1.2 nM, $K_{i,caspase-3}$(**9**) = 1.2 nM, $K_{i,caspase-3}$(**36**) = 0.2 nM]. All isatin analogs were additionally assayed for caspase-1, -3, -6, -7, and -8 to prove the in vitro caspase-3 and -7 selectivity of the 5-pyrrolidinylsulfonyl derivatives (Table 2).[36] As already described elsewhere, the 5-pyrrolidinylsulfonyl isatins are competitive inhibitors.[31,33,36] The kinetic analysis of caspase-3 inhibition by compound **35** [(*S*)-1-(*p*-(2-fluoroethoxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin] confirms the competitive inhibition characteristics of the 5-pyrrolidinylsulfonyl isatins described here (Figure 2).

Subsequently, the caspase inhibition potential of the synthesized isatin derivatives **9**, **10**, **19**, **20**, **25**, **26**, **35**, and **36** that represent the nonradioactive authentic counterparts of potential CBR radiotracers was investigated for intracellular activity toward caspases-3, -6, -8, and -9. A potential CBR radiotracer must at least detect one or more of the activated caspases in vivo to be considered as an apoptosis-imaging agent. In the context of the performed cellular apoptosis assays, concentration- and time-dependent kinetics of the nonradioactive 2-methoxymethyl/2-phenoxymethyl-modified pairs of isatins **9**/**10**, **19**/**20**, **25**/**26**, and **35**/**36** were examined to evaluate the pharmacological caspase inhibition. A pharmacological caspase inhibition with the nonradioactive PET- or SPECT-compatible isatins similar to the one achieved in our study requires micromolar concentrations of the inhibitor. This is several orders of magnitude higher than the one needed for molecular imaging purposes, where — as one aspect — "mere" binding to the target and not saturation is required. Of course, successful binding of a radioligand to its in vivo target also depends on fundamental properties of the radiotracer, such as low nonspecific binding to plasma proteins, potent cell permeability, adequate radioligand retention in target tissues, in vivo stability, and others. Anyway, an interesting insight has emerged from the cellular caspase inhibition studies: the methoxymethyl-substituted isatins **9**, **19**, and **35** seem to possess a higher biological caspase inhibition potency in cells in comparison with the phenoxymethyl-modified compounds such as compound **36**. This outcome may (in part) depend on the lipophilicities of the compounds that increase in the order of log *D*(**9**) = 0.28, log *D*(**19**) = 1.97, log *D* (**35**) = 2.20 (for the 2-methoxymethyl-substitution pattern), and log *D*(**36**) = 4.19 (as example for the 2-phenoxyphenyl-substitution pattern). For all examined 2-methoxymethyl-substituted isatins **9**, **19**, **25**, and **35**, a commencing caspase inhibition in cells can be observed at concentrations of 10 μM. Despite promising in vitro caspase inhibition potencies of the corresponding 2-phenoxymethyl-substituted isatin derivatives **10**, **20**, **26**, and **36** toward caspase-3 [$K_{i,caspase-3}$(**10**) = 56.1 nM, $K_{i,caspase-3}$(**20**) = 4.8 nM, $K_{i,c}$aspase-3(**26**) = 1.2 nM, $K_{i,caspase-3}$(**36**) = 0.2 nM], these model compounds appear to be biologically less potent in cells, even at concentrations between 100 and 300 μM (e.g., for compound **10**, 100 μM, or for compound **36**, 300 μM; data not shown).

Significance. The 5-pyrrolidinylsulfonyl isatins are competitive nonpeptidyl caspase inhibitors showing in vitro selectivity for the caspases-3 and -7. The isatins are suitable for the development of potential CBRs, as shown by the radiosynthesis of compound [^{125}I]**26**. In addition, we have shown that the 2-methoxymethylpyrrolidinyl versions of the isatins possess an improved inhibition potential for caspases in intact cells if the *N*-1-position of the isatin is alkylated by a methyl (**9**) or a *p*-substituted benzyl group (**19**, **35**). Using pharmacological doses of compounds **9**, **19**, and **35** in cellular apoptosis assays, inhibition of caspases-6, -8, and -9 was also differentially observed. A potential CBR radiotracer must at least detect one or more of the activated caspases in vivo to be considered as an exclusive apoptosis-imaging agent. As a consequence for future studies, the 2-methoxymethyl-substituted isatins **9**, **19**, and **35** will be synthesized in their corresponding radiolabeled forms, reverting to the already prepared precursors, compounds **7** and **17**, resulting in [^{11}C]**9** and [^{11}C]**19**, respectively, by ^{11}C-methylation,[40] and compound **31**, resulting in [^{18}F]**35** by fluorodetosylation.[41] The final goal is to develop an exclusive apoptosis-imaging agent through visualization of activated caspases in vivo using scintigraphic imaging techniques.

Experimental Section

General Methods. Chemistry and Radiochemistry. All chemicals, reagents, and solvents for the synthesis of the compounds were analytical grade, purchased from commercial sources, and used without further purification, unless otherwise specified. Melting points were determined in capillary tubes on a Stuart Scientific SMP3 capillary melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker ARX 300 and AMX 400 spectrometer. Two-dimensional NMR spectra were determined on a Varian 600 MHz unity plus instrument. $CDCl_3$ contained tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on a Varian MAT 212 (EI = 70 eV) spectrometer and a Bruker MALDI-TOF-MS Reflex IV (matrix: DHB) instrument. Exact mass analyses were conducted on a Waters Quattro LC and a Bruker MicroTof apparatus. Elemental analysis was realized by a Vario EL III analyzer.

The separation of radioiodinated compound [^{125}I]**26** from unlabeled byproducts, analyses of the radiochemical yields, and the determination of the radiochemical purity were performed by gradient radio-HPLC each with a RP-HPLC Nucleosil column 100−5 C-18 (250 × 4.6 mm^2), a corresponding precolumn (20 × 4.0 mm^2), using a Knauer K-500 and a Latek P 402 pump, a Knauer K-2000 UV-detector (wavelength 254 nm), and a Crismatec NaI-(Tl) Scintibloc 51 SP51 gamma detector. Sample injection was carried out using a Rheodyne injector block (type 7125 incl 200 μL loop). The recorded data were processed by the NINA radio-HPLC software (GE Functional Imaging GmbH).

General Procedure for the Synthesis of the *N*-1-Alkylated Isatin Derivatives. 5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]-isatin (**7**) or 5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]isatin (**8**) was placed in a round-bottom flask and dissolved in 50 mL of dry dimethylformamide. Under an argon atmosphere, 0.8−1.5 equiv of sodium hydride were added. During stirring for 30 min at ambient temperature the solution became dark red. An excess of methyl iodide or the corresponding benzyl bromides was added and the reaction mixture was stirred for a further 2 or 3 h at ambient

temperature. In the case of benzyl chlorides and mesylates, the reaction mixture was heated to 80 °C. Removal of the solvent in vacuo afforded the crude products, which were purified by silica gel chromatography.

(*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]-1-methylisatin (9). (*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]isatin (**7**)-(485 mg, 1.25 mmol) was converted with sodium hydride (50 mg, 1 mmol, 60% in mineral oil) and methyl iodide (248 mg, 1.5 mmol, 0.1 mL) as described in the general procedure and stirred for 2 h at ambient temperature. The crude dark orange product was purified by silica gel chromatography (diisopropyl ether:acetone 4:1) to yield an orange solid. Yield: 175 mg (0.58 mmol, 46%). Mp: 143−144 °C. MS (MALDI-TOF) *m*/*e*: 361 $(M + Na)^+$. Anal. ($C_{15}H_{18}N_2O_5S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.61, 1.82, 3.18, 3.51, 3.52, and 3.68 (m, 9H, pyrrolidine-C*H*/H_2 and OCH_2); 3.24 (s, 3H, NCH_3); 3.28 (s, 3H, OCH_3); 6.94−6.98 (m, 1H, isatin-*H*); 7.96 (m, 1H, isatin-*H*); 8.02−8.04 (m, 1H, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 26.1, 28.6, 51.3, 61.0 (pyrrolidine-*C*H and *C*H_2) 30.8 (N*C*H_3), 61.2 (O*C*H_3), 76.8 (O*C*H_2), 112.2 (q-Ar*C*(CO)), 119.2, 126.3, 136.0 (Ar*C*H), 139.4 (q-Ar*C*SO_2), 156.0 (q-Ar*C*N), 159.8 ((CO)(*C*O)N), 183.8 ((*C*O)(CO)N).

(*S*)-5-[1-(2-Phenoxymethylpyrrolidinyl)sulfonyl]-1-methylisatin (10). (*S*)-5-[1-(2-Phenoxymethylpyrrolidinyl)sulfonyl]isatin (**8**) (500 mg, 1.3 mmol) was reacted with sodium hydride (52 mg, 1.3 mmol, 60% in mineral oil) and methyl iodide (553 mg, 3.9 mmol, 0.24 mL) as described in the general procedure and stirred for 5 h at ambient temperature. The crude orange product was purified by silica gel chromatography (diisopropyl ether:acetone 6:1) to yield an orange solid. Yield: 280 mg (0.7 mmol, 54%). Mp: 96−98 °C. MS (EI): *m*/*e* (intensity %) 400 (M+, 28), 293 (100), 224 (76), 160 (48). Anal. ($C_{20}H_{20}N_2O_5S$) C, H, N. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 1.58−1.67, 1.83−1.93, 3.20−3.37, 3.39−3.43, 3.89−4.11 (m, 9H, pyrrolidine-C*H*/H_2, OCH_2), 3.17 (s, 3H, NCH_3), 6.90−6.93 (m, 3H, Ar-*H*), 7.28 (d, 1H, $^3J_{H,H}$ = 8.1 Hz, isatin-*H*), 7.25−7.31 (m, 2H, Ar*H*), 7.81 (d, 1H, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*), 8.12 (dd, 1H, $^3J_{H,H}$ = 8.1 Hz, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 24.0, 28.8 (pyrrolidine-*C*H_2), 26.7 (N*C*H_3), 49.6, 58.7 (pyrrolidine-N*C*H_2), 69.9 (O*C*H_2), 111.6, 114.8 (Ar*C*H), 118.1 (q-Ar*C*CO), 121.2, 122.8, 129.9, 131.8 (Ar*C*H), 137.1 (q-Ar*C*SO_2), 154.6 (q-Ar*C*NH), 158.5 (q-Ar*C*), 158.8 (CO*C*ONH), 187.5 (*C*OCONH).

(*S*)-1-(4-(*tert*-Butyldimethylsilyloxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (15). (*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]isatin (**7**) (648 mg, 2 mmol) was reacted with sodium hydride (88 mg, 2.2 mmol, 60% in mineral oil) and 4-(*tert*-butyldimethylsilyloxy)benzyl bromide (**14**) (1.81 g, 6 mmol) as described in the general procedure. The crude orange product was purified by silica gel chromatography (cyclohexane:ethyl acetate 9:1 to 3:2) and yielded **15** as a yellow sticky oil. Yield: 510 mg (0.94 mmol, 47%). MS (MALDI-TOF) *m*/*e*: 567 $(M + Na)^+$, 545 $(M + H)^+$. Anal. ($C_{27}H_{36}N_2O_6SSi$) C, H, N. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.18 (s, 6H, SiCH_3); 0.97 (s, 9H, Si*t*Bu); 1.65−1.67, 1.88−1.90, 3.09−3.12, 3.40−3.42, 3.54−3.57, 3.71−3.73 (m, 9H, pyrrolidine-C*H*/H_2, CH_2O); 3.33 (s, 3H, OCH_3); 4.89 (s, 2H, NCH_2Ar); 6.83 (d, 2H, $^3J_{H,H}$ = 8.4 Hz, Ar-*H*), 6.93 (d, 1H, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 7.20 (d, 2H, $^3J_{H,H}$ = 8.4 Hz, Ar-*H*); 7.97 (dd, 1H, $^3J_{H,H}$ = 8.4 Hz, $^4J_{H,H}$ = 1.6 Hz, isatin-*H*); 8.04 (d, 1H, $^4J_{H,H}$ = 1.6 Hz, isatin-*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) −4.6 (Si*C*H_3), 18.1 (Si*C*CH_3), 25.5 (C(*C*H_3)$_3$), 24.0, 28.7, 49.2, 58.9 (pyrrolidine-*C*), 43.8 (N*C*H_2Ar), 59.1 (O*C*H_3), 74.7 (O*C*H_2), 117.2 (q-Ar*C*(CO)), 120.2, 122.7, 122.8, 124.3 (Ar-*C*), 129.5 (q-*C*CH_2N), 134.1 (isatin-*C*H), 137.1 (q-*C*SO_2), 153.3 (q-*C*N(CO)), 155.8 (q-*C*OSi), 157.7 (isatin-N(*C*O)), 181.8 (N(CO)-*C*O).

(*S*)-1-(4-(*tert*-Butyldimethylsilyloxy)benzyl)-5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]isatin (16). (*S*)-5-[1-(2-Phenoxymethylpyrrolidinyl)sulfonyl]isatin (**8**) (750 mg, 2 mmol) was reacted with sodium hydride (88 mg, 2.2 mmol, 60% in mineral oil) and 4-(*tert*-butyldimethylsilyloxy)benzyl bromide (**14**) (1.81 g, 6 mmol) as described in the general procedure. The crude orange product was purified by silica gel chromatography (cyclohexane:ethyl acetate 9:1 to 4:1) to yield a yellow sticky oil. Yield: 630 mg (1.01 mmol, 52%). MS (MALDI-TOF) *m*/*e*: 629 $(M + Na)^+$, 607 $(M + H)^+$. Anal. ($C_{32}H_{38}N_2O_6SSi$•EtOAc) C, H, N. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.18 (s, 6H, SiCH_3); 0.97 (s, 9H, Si*t*Bu); 1.77−1.80, 1.99−2.05, 3.22−3.24, 3.48−3.51, 3.89−3.97, 4.14−4.17 (m, 9H, pyrrolidine-C*H*/H_2, CH_2O); 4.84 (s, 2H, NCH_2Ar); 6.80−6.94 (m, 6H, Ar-*H*, isatin-*H*), 7.17−7.24 (m, 4H, Ar-*H*); 7.94 (dd, 1H, $^3J_{H,H}$ = 8.4 Hz, $^4J_{H,H}$ = 1.6 Hz, isatin-*H*); 8.07 (d, 1H, $^4J_{H,H}$ = 1.6 Hz, isatin-*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) −4.6 (Si*C*H_3), 18.1 (Si*C*CH_3), 26.8 (C(*C*H_3)$_3$), 24.0, 28.9, 49.4, 58.5 (pyrrolidine-*C*), 43.9 (C*C*H_2Ar), 69.0 (O*C*H_2), 117.0 (q-Ar*C*(CO)), 114.3, 120.2, 122.7, 122.8, 124.1, 126.2, 128.9 (Ar*C*), 129.4 (q-*C*CH_2N), 134.1 (isatin-*C*H), 136.9 (q-*C*SO_2), 153.3 (q-*C*N(CO)), 155.9 (q-*C*OSi), 157.6 (isatin-N(*C*O)), 158.1 (q-*C*OCH_2), 181.6 (N(CO)*C*O).

(*S*)-1-(4-Hydroxybenzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (17). (*S*)-1-(4-(*tert*-Butyldimethylsilyloxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (**15**) (500 mg, 0.92 mmol) was dissolved in methanol (15 mL) and concentrated HCl (1 mL) was added. The resulting mixture was stirred for 2 h at ambient temperature and then diluted with ethyl acetate (100 mL). The organic layer was washed with $NaHCO_3$, water, and brine and dried with magnesium sulfate. After removal of the solvent, the residue was purified by silica gel chromatography (cyclohexane: ethyl acetate 3:2 to 1:1) to yield a yellow sticky oil. Yield: 350 mg (0.81 mmol, 88%). MS (MALDI-TOF) *m*/*e*: 453 $(M + Na)^+$, 431 $(M + H)^+$. Anal. ($C_{21}H_{22}N_2O_6S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.65−1.71, 1.85−1.92, 3.10−3.13, 3.41−3.44, 3.53−3.57, 3.71−3.73 (m, 9H, pyrrolidine-C*H*/H_2, CH_2O); 3.33 (s, 3H, OCH_3); 4.61 (m, 1H, ArO*H*), 4.87 (s, 2H, NCH_2Ar); 6.83 (d, 2H, $^3J_{H,H}$ = 8.4 Hz, Ar-*H*), 6.94 (d, 1H, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 7.18 (d, 2H, $^3J_{H,H}$ = 8.4 Hz, Ar-*H*); 7.96 (dd, 1H, $^3J_{H,H}$ = 8.4 Hz, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*); 8.02 (d, 1H, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.1, 28.8, 49.3, 59.1 (pyrrolidine-*C*), 44.1 (C*C*H_2), 59.2 (O*C*H_3), 74.8 (O*C*H_2), 117.5 (q-Ar*C*(CO)), 116.2, 122.8, 124.4, 124.9 (Ar*C*), 129.5 (q-*C*CH_2N), 134.1 (isatin-*C*H), 137.3 (q-*C*SO_2), 153.5 (q-*C*N(CO)), 156.9 (q-*C*OH), 157.9 (isatin-N(*C*O)), 182.0 (N(CO)*C*O)).

(*S*)-1-(4-Hydroxybenzyl)-5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]isatin (18). (*S*)-1-(4-(*tert*-Butyldimethylsilyloxy)benzyl)-5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]isatin (**16**) (400 mg, 0.66 mmol) was dissolved in methanol (15 mL) and concentrated HCl (1 mL) was added. The resulting mixture was stirred for 2 h at ambient temperature and then diluted with ethyl acetate (100 mL). The organic layer was washed with $NaHCO_3$, water, and brine and dried with magnesium sulfate. After removal of the solvent, the yellow residue was purified by silica gel chromatography (cyclohexane:ethyl acetate 2:1 to 3:2) to yield a yellow sticky oil. Yield: 210 mg (0.43 mmol, 65%). MS (MALDI-TOF): *m*/*e*: 516 $(M + Na)^+$, 494 $(M + H)^+$. Anal. ($C_{26}H_{25}N_2O_6S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.71−1.82, 1.91−2.05, 3.19−3.26, 3.43−3.51, 3.60−3.71, 4.12−4.16 (m, 9H, pyrrolidine-C*H*/H_2, CH_2O); 4.82 (s, 2H, NCH_2Ar); 5.58 (m, 1H, ArO*H*); 6.79−6.94 (m, 6H, Ar-*H*, isatin-*H*), 7.17−7.31 (m, 4H, Ar-*H*); 7.95 (dd, 1H, $^3J_{H,H}$ = 8.4 Hz, $^4J_{H,H}$ = 1.6 Hz, isatin-*H*); 7.99 (d, 1H, $^4J_{H,H}$ = 1.6 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.5, 29.4, 49.9, 59.1 (pyrrolidine-*C*), 44.4 (N*C*H_2Ar), 59.2 (O*C*H_3), 72.7 (O*C*H_2), 117.9 (q-Ar*C*(CO)), 116.5, 124.7, 126.1, 127.6, 127.7, 129.1, (Ar-*C*), 129.6 (q-*C*CH_2N), 134.6 (isatin-*C*H), 137.5 (q-*C*SO_2), 153.7 (q-*C*N(CO)), 156.5 (q-*C*OH), 158.2, 158.6 (isatin-N(*C*O), q-*C*OCH_2), 182.2 (N(CO)*C*O)).

(*S*)-1-(4-Methoxybenzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (19). (*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]isatin (**7**) (500 mg, 1.54 mmol) was reacted with sodium hydride (61 mg, 1.54 mmol, 60% in mineral oil) and *p*-methoxybenzyl chloride (723 mg, 0.65 mL, 4.62 mmol) as described in the general procedure. The crude dark orange product was purified by silica gel chromatography (petroleum ether:ethyl acetate 3:1 to 1:1) to yield an orange foam. Yield: 462 mg (1.04 mmol, 68%). Mp: 123 °C. MS (EI): *m*/*e* (intensity %) 444 (90) $(M)^+$, 399 (100). Anal. ($C_{22}H_{24}N_2O_6S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.65−1.69, 1.85−1.89, 3.10−3.13, 3.35−3.41 (m, 7H,

pyrrolidine-C*H*/*H*$_2$); 3.33 (s, 3H, OC*H*$_3$); 3.52−3.54 and 3.72−3.75 (m, 2H, OC*H*$_2$); 3.79 (s, 3H, ArOC*H*$_3$); 4.90 (s, 2H, NC*H*$_2$-Ar); 6.87 (d, 1H, $^3J_{H,H}$ = 8.1 Hz, isatin-*H*); 6.94 (d, 2H, $^3J_{H,H}$ = 8.4 Hz, Ar*H*); 7.25 (d, 2H, $^3J_{H,H}$ = 8.4 Hz, Ar*H*); 7.98 (dd, 1H, $^4J_{H,H}$ = 1.8 Hz, $^3J_{H,H}$ = 8.1 Hz, isatin-*H*); 8.02 (d, 1H, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.1, 28.9 (pyrrolidine-*C*H$_2$), 44.0 (N*C*H$_2$Ar), 49.3, 55.4 (pyrrolidine-*C*H/*C*H$_2$), 59.1 (O*C*H$_3$), 74.9 (O*C*H$_2$), 111.2, 114.6 (Ar-*C*), 117.6 (q-Ar*C*(CO)), 124.4, 125.8, 129.1 (Ar-*C*), 134.1 (q-Ar*C*CH$_2$), 137.3 (q-Ar*C*SO$_2$), 153.4 (q-Ar*C*N(CO)), 157.8 (q-Ar*C*O), 159.8 (Ar(CO)-(*C*O)), 181.9 (Ar(*C*O)(CO)).

(*S*)-1-(4-Methoxybenzyl)-5-[1-(2-phenoxymethylpyrrolidinyl)-sulfonyl]isatin (20). (*S*)-5-[1-(2-Phenoxymethylpyrrolidinyl)sulfonyl]isatin (**8**) (386 mg, 1 mmol) was converted with sodium hydride (40 mg, 1 mmol, 60% in mineral oil) and *p*-methoxybenzyl chloride (670 mg, 3 mmol) as described in the general procedure. The crude dark orange product was purified by silica gel chromatography (diisopropyl ether:acetone 8:1) to yield an orange solid. Yield: 310 mg (0.61 mmol; 61%). Mp: 152 °C. MS (EI): *m*/*e* (intensity %) 506 (17) $(M)^+$, 399 (100). Anal. ($C_{27}H_{26}N_2O_6S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.77−1.81, 2.00−2.04, 3.22−3.26, 3.47−3.51, 4.15−4.19 (m, 7H, pyrrolidine-C*H*/*H*$_2$); 3.80 (s, 3H, OC*H*$_3$); 3.88−3.98 (m, 2H, ArOC*H*$_2$); 4.86 (s, 2H, NC*H*$_2$Ar); 6.81−6.98 (m, 6H, Ar*H*, isatin-*H*); 7.21−7.28 (m, 4H, Ar*H*); 7.95 (dd, 1H, $^4J_{H,H}$ = 1.5 Hz, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 8.01 (d, 1H, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 26.8, 28.7 (pyrrolidine-*C*H$_2$), 43.6 (N*C*H$_2$Ar), 49.2, 55.0 (pyrrolidine-*C*H/*C*H$_2$), 58.3, 58.4 (*C*H$_2$O and O*C*H$_3$), 110.9, 114.0, 114.3 (Ar-*C*), 117.1 (q-Ar*C*(CO)), 120.7, 123.9, 125.1, 128.7, 129.2 (Ar*C*), 133.8 (q-Ar*C*CH$_2$), 136.7 (q-Ar*C*SO$_2$), 153.0 (q-Ar*C*N(CO)), 157.4, 157.9 (q-Ar*C*O), 159.4 (Ar(CO)(*C*O)), 181.4 (Ar(*C*O)(CO)).

(*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]-1-(4-(tributylstannyl)benzyl)isatin (23). (*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]isatin (**7**) (324 mg, 1 mmol) was reacted with sodium hydride (60 mg, 1.5 mmol, 60% in mineral oil) and *p*-tributylstannylbenzyl methanesulfonate (**22**) (680 mg, 1.4 mmol) as described in the general procedure. The crude orange product was purified by silica gel chromatography (petroleum ether:ethyl acetate 4:1) to yield an orange oil. Yield: 378 mg (0.54 mmol, 54%). HRMS (ESI-EM) *m*/*e*: 759.2460 $(M + Na + CH_3OH)^+$ calcd for $C_{34}H_{52}N_2NaO_6SSn$ 759.2466. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.80 (t, 9H, $^3J_{H,H}$ = 7.5 Hz, SnBu-C*H*$_3$); 0.94−1.00, 1.16−1.28, 1.39−1.48 (m, 18H, SnC*H*$_2$), 1.58−1.61, 1.78−1.83, 3.02−3.09, 3.45−3.50, 3.64−3.69 (m, 7H, pyrrolidine-C*H*/*H*$_2$); 3.25 (s, 3H, C*H*$_3$OCH$_2$); 3.27−3.35 (m, 2H, OC*H*$_2$); 4.87 (s, 2H, NC*H*$_2$Ar); 6.86 (d, 2H, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 7.20 (d, 2H, $^3J_{H,H}$ = 7.2 Hz, SnBu$_3$Ar*H*); 7.38 (d, 2H, $^3J_{H,H}$ = 7.2 Hz, SnBu$_3$-Ar*H*); 7.91 (dd, 1H, $^4J_{H,H}$ = 1.8 Hz, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 7.97 (d, 1H, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 9.6 (Sn*C*H$_2$), 13.6 (SnCH$_2$CH$_2$CH$_2$*C*H$_3$), 27.3 (SnCH$_2$CH$_2$*C*H$_2$), 28.8 (SnCH$_2$*C*H$_2$), 24.1, 29.0, 49.3, 59.2 (pyrrolidine-*C*H/*C*H$_2$), 44.4 N*C*H$_2$Ar, 60.3 (O*C*H$_3$), 74.8 (*C*H$_2$O), 117.5 (q-Ar*C*(CO)), 111.2, 124.4, 126.9, 133.2, 143.0 (q-Ar*C*, Ar*C*H), 137.3 (q-*C*SO$_2$), 153.4 (q-*C*N(CO)), 157.8, 158.1 (q-Ar*C*OH, isatin-N(*C*O)), 180.0 (N(CO)(*C*O)).

(*S*)-5-[1-(2-Phenoxymethylpyrrolidinyl)sulfonyl]-1-(4-(tributylstannyl)benzyl)isatin (24). (*S*)-5-[1-(2-Phenoxymethylpyrrolidinyl)sulfonyl]isatin (**8**) (385 mg, 1 mmol) was reacted with sodium hydride (60 mg, 1.5 mmol, 60% in mineral oil) and *p*-tributylstannylbenzyl methanesulfonate (**22**) (1.42 g, 3 mmol) as described in the general procedure. The crude orange product was purified by silica gel chromatography (petroleum ether:ethyl acetate 6:1) to yield an orange oil. Yield: 410 mg (0.53 mmol, 53%). HRMS (ESI-EM) *m*/*e*: 821.2592 $(M + Na + CH_3OH)^+$ calcd for $C_{39}H_{54}N_2NaO_6SSn$ 821.2624. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.92 (t, 9H, $^3J_{H,H}$ = 7.5 Hz, SnBu-C*H*$_3$); 1.07−1.13, 1.31−1.43, 1.53−1.60 (m, 18H, SnC*H*$_2$), 1.81−1.89, 2.04−2.11, 3.28−3.34, 3.50−3.56 (m, 7H, pyrrolidine-C*H*/*H*$_2$); 3.94−4.04, 4.19−4.23 (m, 2H, ArOC*H*$_2$); 4.94 (s, 2H, NC*H*$_2$Ar); 6.86 (d, 2H, $^3J_{H,H}$ = 8.1 Hz, SnBu$_3$Ar*H*); 6.91−6.99 (m, 2H, Ar*H*); 7.23−7.34 (m, 4H, Ar*H*); 7.51 (d, 2H, $^3J_{H,H}$ = 8.1 Hz, SnBu$_3$Ar*H*); 8.00 (dd, 1H, $^4J_{H,H}$ = 1.8 Hz, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 8.07 (d, 1H, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 10.0 (Sn*C*H$_2$), 14.0 (SnCH$_2$CH$_2$CH$_2$*C*H$_3$), 27.7 (SnCH$_2$CH$_2$*C*H$_2$), 28.2 (SnCH$_2$*C*H$_2$), 24.6, 29.4, 49.9, 59.1 (pyrrolidine-*C*H/*C*H$_2$), 44.8 (N*C*H$_2$Ar), 69.6 (*C*H$_2$O), 117.9 (q-Ar*C*(CO)), 114.8, 121.5, 124.6, 127.4, 127.5, 129.9, 133.7, 134.7, 143.4 (q-Ar-*C*, Ar-*C*H), 137.5 (q-*C*SO$_2$), 153.8 (q-*C*N(CO)), 158.2, 158.6 (q-Ar*C*OH, isatin-N(*C*O)), 182.1 (N(CO)-(*C*O)).

(*S*)-1-(4-Iodobenzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (25). (*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]-isatin (**7**) (750 mg, 2.3 mmol) was reacted with sodium hydride (92 mg, 2.3 mmol, 60% in mineral oil) and *p*-iodobenzyl bromide (1.02 g, 3.45 mmol) as described in the general procedure. The crude dark orange product was purified by silica gel chromatography (diisopropyl ether:acetone 8:1) to yield an orange solid. Yield: 820 mg (1.52 mmol, 66%). Mp: 129−130 °C. MS (EI): *m*/*e* (intensity %) 540 (2) $(M)^+$, 495 (100). Anal. ($C_{21}H_{21}N_2IO_5S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.64−1.69, 1.86−1.91, 3.11−3.13, 3.35−3.41 (m, 7H, pyrrolidine-C*H*/*H*$_2$); 3.30 (s, 3H, OC*H*$_3$); 3.52−3.57, 3.72−3.74 (m, 2H, OC*H*$_2$); 4.91 (s, 2H, NC*H*$_2$Ar); 6.87 (d, 1H, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 7.08 (d, 2H, $^3J_{H,H}$ = 8.7 Hz, Ar*H*); 7.69 (d, 2H, $^3J_{H,H}$ = 8.7 Hz, Ar*H*); 7.97 (dd, 1H, $^4J_{H,H}$ = 1.8 Hz, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 8.04 (d, 1H, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.1, 28.9, 49.2, 59.1, 59.2 (pyrrolidine-*C*H/*C*H$_2$, O*C*H$_3$), 44.0 (N*C*H$_2$Ar), 74.8 (*C*H$_2$O), 94.1 (q-Ar*C*I), 117.6 (q-Ar*C*(CO)), 111.0, 124.6, 129.4, 133.5, 134.5 (Ar-*C*H and isatin-*C*H), 137.4 (q*C*SO$_2$), 138.4 (q-Ar*C*CH$_2$), 153.0 (q-*C*N(CO)), 157.8 (isatin-N(*C*O)), 181.5 (N(CO)-*C*O)).

(*S*)-1-(4-Iodobenzyl)-5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]isatin (26). (*S*)-5-[1-(2-Phenoxymethylpyrrolidinyl)sulfonyl]-isatin (**8**) (385 mg, 1 mmol) was reacted with sodium hydride (40 mg, 1 mmol, 60% in mineral oil) and *p*-iodobenzyl bromide (445 mg, 1.5 mmol) as described in the general procedure. The crude dark orange product was purified by silica gel chromatography (diisopropyl ether:acetone 4:1) to yield an orange solid. Yield: 400 mg (0.66 mmol; 66%). Mp: 88−90 °C. MS (ESI): *m*/*e* (intensity %) 657 (100) $(M + MeOH + Na)^+$, 625 (25) $(M + Na)^+$, 603 (10) $(M + H)^+$. Anal. ($C_{26}H_{23}IN_2O_5S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.71−1.74, 1.91−1.98, 3.13−3.18, 3.39−3.43, 4.05−4.09 (m, 7H, pyrrolidine-C*H*/*H*$_2$); 3.81−3.91 (m, 2H, ArOC*H*$_2$); 4.78 (s, 2H, NC*H*$_2$Ar); 6.72−6.76 (m, 3H, isatin-*H* and Ar*H*); 6.83−6.87 (m, 1H, Ar*H*); 6.98−7.01 (m, 2H, Ar*H*); 7.13−7.19 (m, 2H, Ar*H*); 7.61−7.63 (m, 2H, Ar*H*); 7.86 (dd, 1H, $^4J_{H,H}$ = 1.5 Hz, $^3J_{H,H}$ = 7.8 Hz, isatin-*H*); 7.93 (d, 1H, $^4J_{H,H}$ = 1.5 Hz, isatin-*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 24.1, 28.9, 49.4, 58.6 (pyrrolidine-*C*H and *C*H$_2$), 43.8 (N*C*H$_2$Ar), 69.1 (O*C*H$_2$), 94.0 (q-Ar*C*I), 117.4 (q-Ar*C*(CO)), 114.3, 120.9, 124.3, 124.4, 129.2, 129.4, 133.4, 134.4 (Ar*C*H, isatin-*C*H), 137.0 (q*C*SO$_2$), 138.3 (q-Ar*C*CH$_2$), 152.8 (q-*C*N(CO)), 158.1 (isatin-N(*C*O)), 181.3 (N(CO)-*C*O)).

(*S*)-1-(4-(2-Bromoethoxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (29). (*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]isatin (**7**) (800 mg, 2.46 mmol) was reacted with sodium hydride (98 mg, 2.46 mmol, 60% in mineral oil) and *p*-(2-bromoethoxy)bromomethylbenzene (**28**) (1.4 g, 4.92 mmol) as described in the general procedure. The crude orange product was purified by silica gel chromatography (petroleum ether:ethyl acetate 3:1 to 1:2) and yielded **29** as a yellow foam. Yield: 1.02 g (1.9 mmol, 77%). Mp: 61−62 °C. MS (EI): *m*/*e* (intensity %) 538 (42), 536 (42) $(M)^+$, 493 (100), 491 (100). Anal. ($C_{23}H_{25}BrN_2O_6S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.66−1.70, 1.86−1.90, 3.10−3.13, 3.53−3.57, 3.71−3.73 (m, 7H, pyrrolidine-C*H*/*H*$_2$); 3.33 (s, 3H, OC*H*$_3$); 3.35−3.39 (m, 2H, CH$_3$OC*H*$_2$); 3.63 (t, 2H, $^3J_{H,H}$ = 5.7 Hz, ArCH$_2$C*H*$_2$Br); 4.28 (t, 2H, $^3J_{H,H}$ = 5.7 Hz, ArC*H*$_2$CH$_2$Br); 4.91 (s, 2H, NC*H*$_2$Ar); 6.89−6.97 (m, 3H, Ar*H* and isatin-*H*); 7.27−7.30 (m, 2H, Ar*H*); 7.97 (dd, 1H, $^4J_{H,H}$ = 1.8 Hz, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 8.02 (d, 1H, $^4J_{H,H}$ = 1.5 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.5, 29.2, 29.4, 49.7, 59.4 (pyrrolidine-*C*H/ *C*H$_2$, CH$_2$*C*H$_2$Br), 44.3 (N*C*H$_2$Ar), 59.3 (O*C*H$_3$),

68.4 (CH_2CH_2Br), 75.2 (CH_2O), 111.6, 115.8, 117.9, 124.8, 127.0, 129.6, 134.7, 137.7 (q-Ar*C* and Ar*C*H), 153.7 (q-Ar*C*N(CO)), 158.2, 158.6 (q-Ar*C*O, Ar(CO)(*C*O)), 182.3 (Ar(*C*O)(CO)).

(*S*)-1-(4-(2-Bromoethoxy)benzyl)-5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]isatin (30). (*S*)-5-[1-(2-Phenoxymethylpyrrolidinyl)sulfonyl]isatin (**8**) (730 mg, 1.90 mmol) was reacted with sodium hydride (80 mg, 1.90 mmol, 60% in mineral oil) and *p*-(2-bromoethoxy)bromomethylbenzene (**28**) (882 mg, 3 mmol) as described in the general procedure. The crude orange product was purified by silica gel chromatography (petroleum ether:ethyl acetate 3:1 to 1:1) to yield a yellow solid. Yield: 910 mg (1.52 mmol, 80%). Mp: 162−163 °C. MS (EI): *m*/*e* (intensity %) 600 (3), 598 (3) $(M)^+$, 493 (100), 491 (100). Anal. ($C_{28}H_{27}BrN_2O_6S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.68−1.75, 1.91−1.97, 3.13−3.17, 3.39−3.42 (m, 6H, pyrrolidine-C*H*/H_2); 3.54 (t, 2H, $^3J_{H,H}$ = 6.0 Hz, ArCH$_2$CH_2Br); 3.80−3.90 (m, 2H, ArOCH_2); 4.05−4.09 (m, 1H, pyrrolidine-C*H*); 4.19 (t, 2H, $^3J_{H,H}$ = 6.0 Hz, ArCH_2-CH$_2$Br); 4.77 (s, 2H, NCH_2Ar); 6.71−6.87 (m, 6H, Ar*H* and isatin-*H*); 7.11−7.20 (m, 4H, Ar*H*); 7.86 (dd, 1H, $^4J_{H,H}$ = 1.8 Hz, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 7.91 (d, 1H, $^4J_{H,H}$ = 1.5 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.5, 29.3, 29.4 (pyrrolidine-*C*H$_2$, CH$_2$*C*H$_2$Br), 44.3 (N*C*H$_2$Ar), 49.9, 59.1 (pyrrolidine-*C*H/*C*H$_2$), 68.4 (*C*H$_2$O), 69.6 (*C*H$_2$CH$_2$Br), 111.6, 114.8, 115.8, 117.9, 121.5, 124.7, 127.0, 129.6, 129.9 (Ar-*C*), 134.6 (q-Ar*C*CH$_2$), 137.5 (q-Ar*C*SO$_2$), 153.7 (q-Ar*C*N(CO)), 158.2 (q-Ar(*C*O)), 158.6 (Ar(CO)-(*C*O)), 182.1 (Ar(CO)(*C*O)).

(*S*)-1-(4-(2-(4-Methylphenylsulfonyloxy)ethoxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (31). (*S*)-1-(4-(2-Bromoethoxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]-isatin (**29**) (500 mg, 0.93 mmol) was dissolved in 20 mL of dry acetonitrile under an argon atmosphere. Silver tosylate (1.26 g, 4 mmol) was added and the reaction mixture was heated to reflux for 24 h. During the reaction a gray precipitate was formed. The solvent was removed in vacuo and the crude orange product was purified by silica gel chromatography (toluene:ethyl acetate 2:1) to yield an orange solid. Yield: 540 mg (0.88 mmol, 94%). Mp: 61−62 °C. MS (EI): *m*/*e* (intensity %) 628 (1.5) $(M)^+$, 583 (100). Anal. ($C_{30}H_{32}N_2O_9S_2$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.63−1.68, 1.85−1.89, 3.09−3.13, 3.52−3.57, 3.70−3.74 (m, 7H, pyrrolidine-C*H*/H_2); 2.44 (s, 3H, ArCH_3); 3.33 (s, 3H, OCH_3); 3.33−3.39 (m, 2H, OCH_2); 4.12−4.15 (m, 2H, ArCH$_2$CH_2-OTos); 4.33−4.36 (m, 2H, ArCH_2CH$_2$OTos); 4.89 (s, 2H, NCH_2-Ar); 6.78−6.81 (m, 2H, Ar*H*) 6.91 (d, 1H, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 7.15−7.35 (m, 4H, Ar*H*); 7.78−7.82 (m, 2H, Ar*H*), 7.97 (dd, 1H, $^4J_{H,H}$ = 1.8 Hz, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 8.03 (d, 1H, $^4J_{H,H}$ = 1.8 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 22.0 (Ar-*C*H$_3$), 24.5, 29.2, 49.7, 59.6 (pyrrolidine-*C*H/*C*H$_2$), 44.3 (N*C*H$_2$-Ar), 60.7 (O*C*H$_3$), 66.0 (ArOCH$_2$*C*H$_2$O), 68.3 (ArO*C*H$_2$CH$_2$O), 75.2 (*C*H$_2$O), 117.9 (q-Ar*C*(CO)), 111.5, 115.7, 124.8, 126.9, 128.4, 129.4, 130.3, 133.3, 134.7, 137.7, 145.4 (q-Ar*C*, Ar*C*H), 153.7 (q-Ar*C*N(CO)), 158.2, 158.6 (q-Ar*C*O, Ar(CO)(*C*O)), 182.3 (Ar(*C*O)-(CO)).

(*S*)-1-(4-(2-(4-Methylphenylsulfonyloxy)ethoxy)benzyl)-5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]isatin (32). (*S*)-1-(4-(2-Bromoethoxy)benzyl)-5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]-isatin (**30**) (500 mg, 0.83 mmol) was dissolved in 20 mL of dry acetonitrile under an argon atmosphere. Silver tosylate (1.26 g, 4 mmol) was added and the reaction mixture was heated to reflux for 24 h. During the reaction a gray precipitate was formed. The solvent was removed in vacuo and the crude orange product was purified by silica gel chromatography (toluene:ethyl acetate 2:1) to yield an orange solid. Yield: 510 mg (0.75 mmol, 90%). Mp: 83−84 °C. MS (EI): *m*/*e* (intensity %) 583 (10) $(M\text{-}ArOCH_2)^+$, 385 (39), 91 (100). Anal. ($C_{35}H_{34}N_2O_9S_2$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.74−1.85, 1.95−2.05, 3.22−3.27, and 3.46−3.53 (m each, 6H, pyrrolidine-CH_2 and C*H*); 2.45 (s, 3H, ArCH_3); 3.96−3.99 (m, 2H, ArOCH_2); 4.12−4.18 (m, 3H, ArCH$_2$CH_2OTos and pyrrolidine-C*H*); 4.34−4.37 (m, 2H, ArCH_2-CH$_2$OTos); 4.85 (s, 2H, NCH_2Ar); 6.79−6.94 (m, 6H, Ar*H* and isatin-*H*); 7.21−7.36 (m, 6H, Ar*H*); 7.79−7.82 (m, 2H, Ar*H*), 7.96 (dd, 1H, *J* = 1.8 Hz, 8.4 Hz, isatin-*H*); 8.00 (d, 1H, *J* = 1.8 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 22.0 (Ar*C*H$_3$), 24.5, 29.4, 49.9, 59.1 (pyrrolidine-*C*H and *C*H$_2$), 44.3 (N*C*H$_2$Ar), 66.1 (*C*H$_2$O), 68.3 (ArOCH$_2$*C*H$_2$O), 69.6 (ArO*C*H$_2$CH$_2$O), 117.9 (q-Ar*C*(CO)), 111.6, 114.8, 115.7, 121.4, 124.6, 126.9, 128.4, 128.6, 129.5, 129.9, 130.3, 133.3, 134.6, 137.5, 145.4 (q-Ar*C*, Ar*C*H), 153.7 (q-Ar*C*N(CO)), 158.2, 158.6 (q-Ar*C*O, Ar(CO)(*C*O)), 182.1 (Ar(*C*O)(CO)).

(*S*)-1-(4-(2-Fluoroethoxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (35). (*S*)-5-[1-(2-Methoxymethylpyrrolidinyl)sulfonyl]isatin (**7**) (324 mg, 1.00 mmol) was reacted with sodium hydride (60 mg, 1.5 mmol, 60% in mineral oil) and *p*-(2-fluoroethoxy)bromomethylbenzene (**34**) (1.18 g, 5.16 mmol) as described in the general procedure. The crude orange product was purified by silica gel chromatography (petroleum ether:ethyl acetate 3:1) to yield a yellow powder. Yield: 313 mg (0.66 mmol, 66%). Mp: 68−69 °C. MS (EI): *m*/*e* (intensity %) 476 (8) $(M)^+$, 431 (100). Anal. ($C_{23}H_{25}N_2FO_6S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.54−1.64, 1.78−1.84, 3.00−3.07, 3.45−3.61, 3.60−3.65 (m, 7H, pyrrolidine-C*H*/H_2); 3.25 (s, 3H, OCH_3); 3.25−3.35 (m, 2H, OCH_2); 4.07−4.09, 4.16−4.18, 4.56−4.59, 4.72−7.75 (m, 4H, ArCH_2CH_2F); 4.83 (s, 2H, NCH_2Ar); 6.81−6.87 (m, 3H, Ar*H* and isatin-*H*); 7.19−7.23 (m, 2H, Ar*H*); 7.89 (dd, 1H, $^4J_{H,H}$ = 1.5 Hz, $^3J_{H,H}$ = 8.1 Hz, isatin-*H*); 7.95 (d, 1H, $^4J_{H,H}$ = 1.5 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.5, 29.2, 44.3, 44.7, 49.7 (pyrrolidine-*C*H/ *C*H$_2$, N*C*H$_2$Ar), 59.4 (O*C*H$_3$), 67.8 (*C*H$_2$O), 75.2 (*C*H$_2$CH$_2$F), 83.3 (CH$_2$*C*H$_2$F), 111.6, 115.7 (Ar*C*), 117.9 (q-Ar*C*(CO)), 124.8, 126.8, 129.5 (Ar*C*), 134.6 (q-Ar*C*CH$_2$), 137.7 (q-Ar*C*SO$_2$), 153.7 (q-Ar*C*N(CO)), 158.2 (q-Ar(*C*O)), 159.0 (Ar(CO)(*C*O)), 182.3 (Ar(*C*O)(CO)). ^{19}F NMR (282 MHz, $CDCl_3$): δ (ppm) −224.0.

(*S*)-1-(4-(2-Fluoroethoxy)benzyl)-5-[1-(2-phenoxymethylpyrrolidinyl)sulfonyl]isatin (36). (*S*)-5-[1-(2-Phenoxymethylpyrrolidinyl)sulfonyl]isatin (**8**) (374 mg, 1 mmol) was reacted with sodium hydride (60 mg, 1.5 mmol, 60% in mineral oil) and *p*-(2-fluoroethoxy)bromomethylbenzene **34** (1.18 g, 5 mmol) as described in the general procedure. The crude dark orange product was purified by silica gel chromatography (cyclohexane:ethyl acetate 1:1) to yield an orange foam. Yield: 170 mg (0.32 mmol, 32%). Mp: 145−146 °C. MS (EI): *m*/*e* (intensity %) 538 (8) $(M)^+$, 431 (100). Anal. ($C_{28}H_{27}N_2FO_6S$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.73−1.84, 1.94−2.06, 3.18−3.26, 3.45−3.51, 4.13−4.14 (m, 7H, pyrrolidine-CH_2/H_2); 3.94−3.97 (m, 2H, ArOCH_2); 4.14−4.16, 4.22−4.25, 4.63−4.66, 4.79−4.82 (m, 4H, ArCH_2CH_2F); 4.85 (s, 2H, NCH_2Ar); 6.79−6.92 (m, 6H, Ar*H* and isatin-*H*); 7.15−7.27 (m, 4H, Ar*H*); 7.93 (dd, 1H, $^4J_{H,H}$ = 1.5 Hz, $^3J_{H,H}$ = 8.4 Hz, isatin-*H*); 7.98 (d, 1H, $^4J_{H,H}$ = 1.5 Hz, isatin-*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.2, 29.1 (pyrrolidine-*C*H$_2$), 43.9 (N*C*H$_2$Ar), 49.5, 55.0 (pyrrolidine-*C*H/*C*H$_2$), 58.7 (*C*H$_2$O), 69.2 (*C*H$_2$CH$_2$F), 80.7 (CH$_2$*C*H$_2$F), 111.2, 114.4, 115.4 (Ar*C*), 117.5 (q-Ar*C*(CO)), 121.1, 124.3, 126.5, 128.2, 129.2 (Ar*C*), 134.2 (q-Ar*C*CH$_2$), 137.1 (q-Ar*C*N(CO)), 153.3, 157.8 (q-Ar*C*O), 158.6 (Ar(CO)(*C*O)), 181.8 (Ar(*C*O)(CO)). ^{19}F NMR (282 MHz, $CDCl_3$): δ (ppm) −224.0.

Radiosynthesis of (*S*)-1-(4-[^{125}I]Iodobenzyl)-5-(1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl)isatin ([^{125}I]26). In a conical glas vial (*S*)-5-(1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl)-1-(4-(tributylstannyl)benzyl)isatin (**24**) (0.56 mg, 0.725 μmol) in ethanol (100 μL) was added to a solution of [^{125}I]NaI (4 μl, approximately 14 MBq) in 0.05 N NaOH and 0.05 M H_3PO_4 (4 μL). The radiosynthesis was started by adding chloramine-T hydrate (CAT) (0.25 mg, 1.095 μmol) in 0.1 M K_2HPO_4 (25 μL, pH 7.36). The reaction mixture was vortexed and allowed to stand for 5 min at room temperature. The resulting reaction suspension was diluted with ethanol (50 μL) and injected onto a gradient radio-RP-HPLC-chromatograph (Nucleosil 100-5 C18 precolumn, 20 × 4.0 mm^2, Nucleosil 100-5 C18 column, 250 × 4.6 mm^2) to isolate the fraction of radiolabeled product [^{125}I]**26** [HPLC conditions: eluent A, CH_3CN/H_2O/TFA 950/50/1; eluent B, CH_3CN/H_2O/TFA 50/950/1; time program, isocratic run with 37% of eluent B; flow, 2.5 mL/min; λ = 254 nm; t_R (fraction) = 15.5−17.5 min]. The radiochemical yield was 90% and the radiochemical purity, determined via radio-HPLC

(HPLC conditions: eluent A, CH_3CN/H_2O/TFA 950/50/1; eluent B, CH_3CN/H_2O/TFA 50/950/1; time program, eluent B from 50% to 20% within 20 min, halt for 10 min, eluent B from 20% to 50% within 10 min; flow, 2.5 mL/min; $\lambda = 254$ nm; $t_R = 17.2$ min), was >95% with a calculated specific activity of 0.134 GBq/μg (80.5 GBq/μmol). The identity of the radioactive product [^{125}I]**26** was verified by HPLC using the nonradioactive reference **26**.

In Vitro Enzyme Inhibition Assays. Recombinant full-length human caspase-3 was purified as described previously.[43] The caspase-3 substrate Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-AMC, $K_M = 9.7 \pm 1$ mM) was purchased from Alexis Biochemicals and dissolved in a buffer consisting of 140 mM NaCl, 2.7 mM KCl, and 10 mM KH_2PO_4. Enzyme assays were performed in a 200 μL volume at 37 °C in reaction buffer containing 0.1% CHAPS, 50 mM KCl, 5 mM β-mercaptoethanol, 25 mM HEPES (pH 7.5), and nonradioactive isatins in DMSO (compounds **7**−**10**, **19**, **20**, **23**−**26**, **29**-**32**, **35**, **36**) each in single doses (end concentrations 500, 50, 5 μM; 500, 50, 5 nM; 500, 50, or 5 pM). Recombinant caspase-3 was diluted into the appropriate buffer to a concentration of 1 unit per assay (=0.5 pM, i.e., 100 pmol substrate conversion per minute). After a 10-min incubation time, Ac-DEVD-AMC (end concentration 10 μM) was added and reacted for a further 10 min. Reaction rates showing the inhibitory activity of the nonradioactive model inhibitor were measured with a Fusion universal microplate analyzer (PerkinElmer) at excitation and emission wavelengths of 360 and 460 nm, respectively. The IC_{50} values were determined by nonlinear regression analysis using the XMGRACE program (Linux software) and converted into the corresponding K_i values by the equation $K_i = IC_{50}/(1 + [S]/K_M)$ assuming competitive inhibition by the isatin derivatives, where [S] is the concentration and K_M is the Michaelis constant of substrate Ac-DEVD-AMC.

In a similar manner, the binding potencies of all isatin analogs were additionally assayed for recombinant human caspases-1, -3, -6, -7, and -8 (Alexis Biochemicals) using their peptide-specific substrates (Alexis Biochemicals) Ac-YVAD-AMC (Ac-Tyr-Val-Ala-Asp-AMC, caspase-1), Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-AMC, caspase-3), Ac-VEID-AMC (Ac-Val-Glu-Ile-Asp-AMC, caspase-6), Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-AMC, caspase-7), and Ac-IETD-AMC (Ac-Ile-Glu-Thr-Asp-AMC, caspase-8). The enzymatic activity of the caspases was again determined by measuring the accumulation of the cleaved fluorogenic product AMC (7-amino-4-methylcoumarin). All assays were performed at a volume of 200 μL at 37 °C in reaction buffer containing 0.1% CHAPS, 100 mM NaCl, 5 mM β-mercaptoethanol, 100 mM HEPES (pH 7.4), 2 mM EDTA, 10% sucrose (caspase-1); 0.1% CHAPS, 100 mM NaCl, 20 mM HEPES (pH 7.4), 2 mM EDTA, 10% sucrose (caspase-3); 0.1% CHAPS, 100 mM NaCl, 20 mM HEPES (pH 7.4), 2 mM EDTA, 10% sucrose (caspase-6); 0.1% CHAPS, 100 mM NaCl, 5 mM β-mercaptoethanol, 20 mM HEPES (pH 7.4), 2 mM EDTA, 10% sucrose (caspase-7); or 0.1% CHAPS, 100 mM NaCl, 20 mM HEPES (pH 7.4), 2 mM EDTA, 10% sucrose (caspase-8). Buffers contained nonradioactive isatins in DMSO each in single doses (end concentrations 500, 50, 5 μM; 500, 50, 5 nM; 500, 50, or 5 pM). Recombinant caspases were diluted into the appropriate buffer to a concentration of 0.5 units per assay (=500 pmol substrate conversion after 60 min). After a 10-min incubation time, the peptide substrate (end concentration 10 μM) was added and reacted for a further 10 min. The IC_{50} values were determined by nonlinear regression analysis using the XMGRACE program (Linux software).

Enzyme Kinetic Studies. The kinetic inhibition profile of the fluorinated compound **35** was examined for caspase-3. The concentration of the substrate Ac-DEVD-AMC was varied from 0.83 to 10 μM. Compound **35** was assayed at concentrations between 0 and 20 nM. The kinetic parameters of compound **35** were obtained by fitting the initial-rate data to

$$1/V = (1/V_{max}) + (K_M/V_{max})(1 + [I]/K_i)(1/[S])$$

where V is the observed velocity, [S] is the substrate concentration, V_{max} is the velocity at saturating substrate, K_M is the Michaelis constant of the substrate, [I] is the inhibitor concentration, and K_i is the dissociation constant of the inhibitor from the enzyme−inhibitor complex. The data were analyzed using Microcal Origin, version 5.0.

Cellular Apoptosis Assays. HUVEC (human umbilical vein endothelial cells) were cultivated on gelatin (2%)-coated dishes in RPMI-1640 containing 15% bovine calf serum, 1% Pen/Strep/Amph, 1% heparin, and 0.05 mg/mL bovine pituitary extract at 37 °C in 5% CO_2. Apoptosis was induced by growth factor withdrawal as previously described.[44] For caspase inhibition experiments, cells were preincubated with the compounds in the indicated concentrations for 30 min. The medium was then removed and replaced with RPMI-1640 alone, and the cells were incubated in the presence or absence of the various inhibitor concentrations for 8 h. All cells were then harvested in lysis buffer and incubated for 10 min on ice, and cell debris was removed by centrifugation at 14 000 rpm at 4 °C for 10 min. Protein concentration was determined by the Pierce protein assay, and 30 μg of cell lysate was loaded on 15% SDS−PAGE gels and transferred to Immobilon PVDF membranes. Western blots were performed with antibodies active to caspase-3 (only the active/processed forms p12/p17 are recognized and not the pro-form), caspase-9, cleaved lamin A/C (Cell Signaling), α-fodrin (anti-spectrin, Chemicon), caspase-8 (a gift from R. Jänicke, University of Düsseldorf), PARP (Transduction Labs; recognizes the 116-kDa native protein and the 35-kDa caspase cleavage product), and β-actin (Sigma) as previously described for endothelial cell apoptosis[45] and developed using ECL (Amersham).

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Supporting Information Available: Experimental procedures and analytical data of intermediates and alkylation agents. This material is available free of charge via the Internet at http://pubs.acs.org.

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