A New Class of HIV-1 Integrase Inhibitors: The 3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′,6,6′-tetrol Family

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Integration is a required step in HIV replication, but as yet no inhibitors of the integration step have been developed for clinical use. Many inhibitors have been identified that are active against purified viral-encoded integrase protein; of these, many contain a catechol moiety. Though this substructure contributes potency in inhibitors, it is associated with toxicity and so the utility of catechol-containing inhibitors has been questioned. We have synthesized and tested a systematic series of derivatives of a catechol-containing inhibitor (**1**) with the goal of identifying catechol isosteres that support inhibition. We find that different patterns of substitution on the aromatic ring suffice for inhibition when Mn^{2+} is used as a cofactor. Importantly, the efficiency is different when Mg^{2+}, the more likely in vivo cofactor, is used. These data emphasize the importance of assays with Mg^{2+} and offer new catechol isosteres for use in integrase inhibitors.

Introduction

HIV encodes three enzymes: reverse transcriptase (RT), protease (PR), and integrase (IN). Inhibitors of RT and PR have been extremely useful for treating HIV-infected people, particularly when used in combination.[1−3] IN is a promising target because integration is an essential step in retroviral replication cycle.[4−9] No cellular homologue of IN has been described, and so potential IN-selective inhibitors could be relatively nontoxic.[10,11]

IN protein carries out the initial DNA breaking and joining reactions responsible for the attachment of HIV cDNA to host DNA (Figure 1A).[12−18] Prior to integration, two nucleotides are removed from each 3′ end in the linear cDNA precursor (terminal cleavage).[19−24] This reaction may be important to the virus by preparing a defined substrate for subsequent reaction steps, because RT often adds nontemplated bases to the 3′ ends of unintegrated cDNAs.[25,26] The recessed 3′ ends are then joined to protruding 5′ ends of breaks made in the target DNA (strand transfer).[14−16] The remaining DNA strands are then attached, probably by the action of host DNA repair enzymes, to complete the formation of an integrated provirus.

The terminal cleavage and strand-transfer reactions can be modeled in vitro by using purified recombinant IN protein.[23,24,27] Under simple reactions conditions, purified IN can form a covalent bond between a double-stranded DNA substrate mimicking the viral long terminal repeat (LTR) and another DNA mimicking the integration target (Figure 1B).

Integration activity in vitro can also be provided by replication intermediates isolated from HIV-infected cells, called preintegration complexes (PICs).[28−31] These PICs, which contain IN and additional viral and cellular proteins,[26,32−35] can direct integration of the endogenously synthesized viral cDNA into an added target DNA in vitro.[36−38] Reactions with PICs mimic in vivo integration more closely than do reactions with purified IN.[24,28,29,36,39,40] PICs assays have not been widely studied due to the requirement for handling large amounts of infectious HIV.[41,42] Recently we described a method for producing PICs using HIV-based vectors,[43,44] biologically inactivated derivatives of HIV that greatly reduce the biohazard in such assays.[45] PIC assays have been shown to be more resistant to inhibition than assays with purified IN and display a response that more closely matches the response of HIV in vivo,[46] emphasizing their utility for inhibitor screens. Nevertheless, certain IN inhibitors may be missed in PIC assays, for instance compounds preventing the correct assembly of IN into a PIC.[47]

Systematic screening of potential inhibitors has been undertaken using mostly purified IN-based assays. From such screens several IN inhibitor classes have now been identified, including[48−54] hydroxylated aromatic compounds such as aurintricarboxylic acids,[55] bis-catechols,[56] caffeic acid phenethyl ester (CAPE),[57] flavones and flavanoids,[58] curcumin,[59,60] tyrphostins,[61] lignanolides,[62] coumarin derivatives,[63] cosalanes,[64] hydrazide derivatives,[65] depside and depsidones,[66] strylquinoline derivatives,[67] and lamellarins.[68] Also some peptides have been described as IN inhibitors.[69,70] Despite the fact that many IN inhibitors have been identified, to date no clinically useful inhibitors have been developed and only a handful display antiviral activity.[71−76] Structure-activity-based correlations and more rational studies[71,77−81] of inhibitors identified the catechol structure[59] as a possible pharmacophore. For reasons that are still not well understood, most of the catechol-containing inhibitors display a toxic effect on cell

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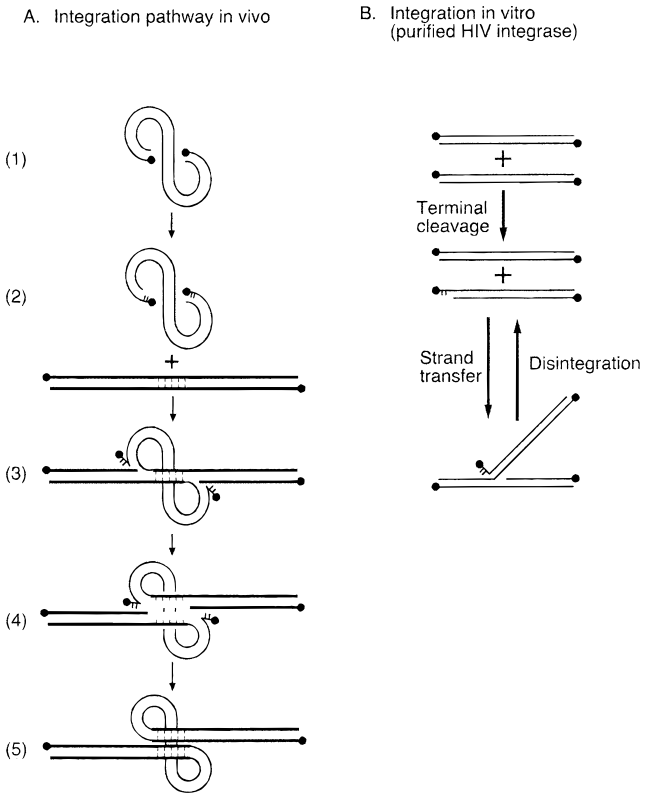


Figure 1. Pathways of integration. (A) Integration pathway in vivo. DNA 5′ ends are shown as balls. The linear viral cDNA (shown curved in 1) is first cleaved to remove two nucleotides from each 3′ end (2). The recessed 3′ ends are then joined to protruding 5′ ends of breaks made in target DNA (3). Gaps due to unpairing in this intermediate (4) are then filled in and joined (5), probably by host DNA repair enzymes. (B) Integration reactions in vitro using purified HIV-1 IN. The lines represent oligonucleotide DNAs in which the sequence matches one end of the viral cDNA. The terminal cleavage and strand-transfer reactions are as marked. The inverse reaction disintegration employs a Y-shaped molecule like that shown.

culture[46,58,73,82−86] which may be related to the cross-reactivity with other metal-requiring enzymes or covalent protein modification by the catechol unit.[86]

In this study, our initial goal was to elucidate new structural motifs that can substitute for catechol. In previous work we found 3,3,3′,3′-tetramethyl-1,1′-spirobi(indan)-5,5′,6,6′-tetrol (**1**) inhibited purified IN. Consequently, we set out to synthesize a series of derivatives of **1** with the aim of finding related inhibitors lacking the undesirable catechol moiety. Here we describe the synthesis of such compounds and their ability to selectively inhibit at micromolar concentrations HIV-IN, compared to MCV topoisomerase. At these concentrations some of these compounds show no cellular toxicity.[87]

Results and Discussion

Chemistry. Screening of catechol derivatives[86] yielded a molecule (**1**) which was found to be active and selective against IN but was highly toxic. Because very often the toxicity of IN inhibitors is attributed to the presence of the catechol moiety, we chose to synthesize a class of compounds structurally related to **1** (Table 1) with a different pattern of substitution on the aromatic ring hoping that they would retain or improve the activity and selectivity of **1** but exhibit less toxicity. The pattern of substitution on the analogues was chosen in order to modify the hydrogen-bonding ability and steric requirements of **1**.

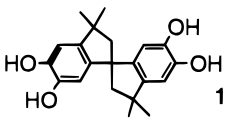
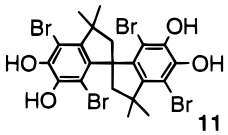
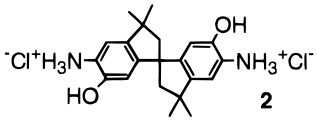
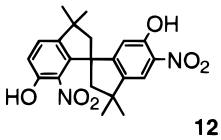
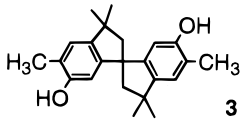
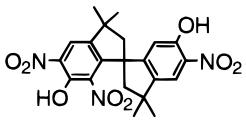
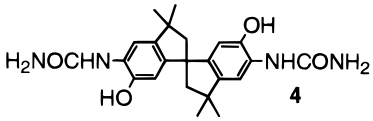
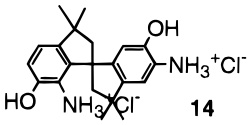
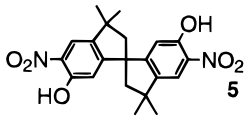
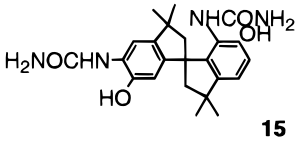
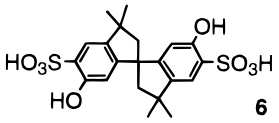
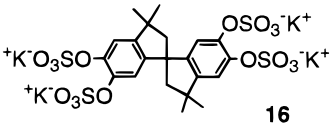
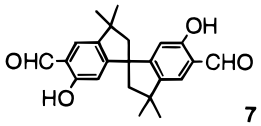
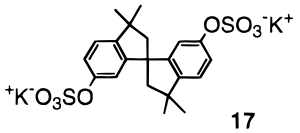
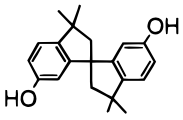
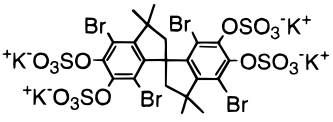
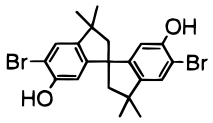
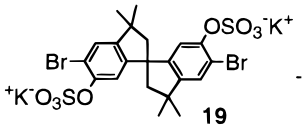
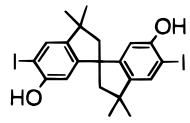
Three different synthetic strategies have been used to synthesize the derivatives: (I) synthesis of the spirobisindanol nucleus by condensation of phenols with acetone; (II) modification of the spirobisindanol nucleus by aromatic electrophilic substitution and further modifications; (III) sulfonation of the spirobisindanol nucleus (Scheme 1).

Strategy I was used to obtain the known compounds **3**[88] and **8**[89−91] from the acid-mediated condensation of acetone with *o*-cresol and phenol, respectively. Treatment of **8** with nitric acid (45%) produced the dinitro compounds **5** and **12** and the trinitro derivative **13** as a mixture, from which they were subsequently separated. The reduction of **5** and **12** with zinc in ethanol followed by acid treatment[92] led to the bisammonium salts **2** and **14**, respectively. These could then be converted into the respective bisureas **4** and **15** by reaction with sodium cyanate in acetic acid.[93] The independent reactions of **8** with chlorosulfonic acid, bromine,[94] iodine monochloride,[95] and dichloromethyl methyl ether in the presence of titanium tetrachloride[96] yielded the bissulfonic acid **6**, the dibromide **9**, the diiodide **10,** and the dialdehyde **7**, respectively. The tetrabromide **11** was obtained by reaction of **1** with excess bromine.[97] The bissulfates **17** and **19** were obtained from **8** and **9** and the tetrasulfates **16** and **18** from **1** and **11** by reaction of the appropriate precursor with chlorosulfonic acid in pyridine,[98] followed by crystallization.

Inhibitor Screening for HIV-1 IN in Vitro. All of the derivatives were tested first against 3′-processing (TC) and strand-transfer (ST) activities in the presence of Mn^{2+} as the cationic cofactor by gel assay. Compounds were titrated into assays containing purified HIV-1 IN proteins and DNAs mimicking one end of the unintegrated viral DNA and target DNA. Inhibition was revealed by a reduction in the accumulation of the TC and ST products. Audioradiograms were quantitated by phosphorimager. The IC_{50} values for the molecules tested are summarized in Table 1. Because it is generally accepted that Mg^{2+} is the more likely cofactor in vivo,[99] we retested all the compounds using Mg^{2+} (Table 1). For this we used a convenient microtiter plate assay[27] that monitors the ST reaction. Note that the test with either gel or microtiter plate assays in Mn^{2+} yielded essentially the same values for every compound, indicating that it is the nature of the metal present and not the assay procedure which causes the change in inhibition. Differential inhibition in the presence of Mn^{2+} versus Mg^{2+} has been observed in several previous studies.[41,86,100]

On the basis of their structure, the compounds can be roughly classified as symmetric derivatives of **1** (**2**−**11**), asymmetric derivatives (**12**−**15**), and symmetric sulfated derivatives (**16**−**19**). In the first class of compounds two of the four hydroxyl groups of **1** were substituted with groups differing in their hydrogen-bonding ability and size. When the hydroxyl groups were substituted with classical isosteres (NH_2 and CH_3),[101] the resulting compounds did not show any detectable activity at 200 μM. The same lack of potency was observed for the compound **4** in which the hydroxyl

Table 1. Inhibition of HIV-1 IN[a]

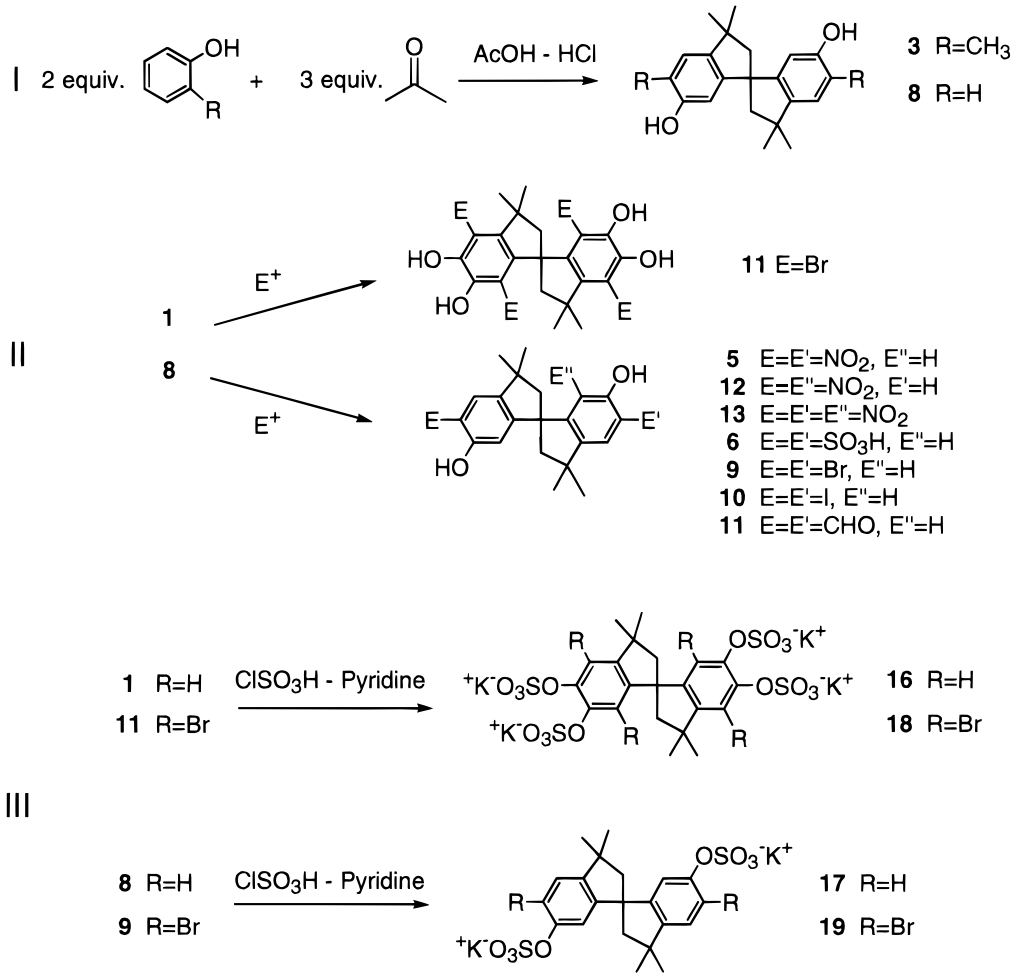
Compounds	IC_{50} (μM) Mn^{++}		IC_{50} (μM) Mg^{++}	Compounds	IC_{50} (μM) Mn^{++}		IC_{50} (μM) Mg^{++}
	T.C.	S.T.	S.T.		T.C.	S.T.	S.T.
 **1**	17	5	-@200	 **11**	3.14	1.78	15
 **2**	-@243	-@243	33	 **12**	47	53	100
 **3**	-@200	-@200	-@200	 **13**	44	25	47
 **4**	-@200	-@200	-@200	 **14**	-@200	-@200	-@200
 **5**	-@200	-@200	-@200	 **15**	420	350	-@200
 **6**	-@200	-@200	69	 **16**	2.8	2	-@200
 **7**	35	6	16	 **17**	32	12	54
 **8**	275	231	-@200	 **18**	1.4	0.35	4
 **9**	11	10	-@200	 **19**	-@200	-@200	-@200
 **10**	6.28	3.12	-@200				

[a] Compounds were also tested in PIC assays and were found to be inactive @80 μM.

groups were replaced with two $NHCONH_2$ units. These substituents are often found to produce a similar biological effect when hydrogen bonding is essential (nonclassical bioisosteres). Also compounds **5**, **6,** and **8**, which differ from **1** in their hydrogen-bonding properties, showed no activity.

High inhibitory activity was obtained for the molecules **9** and **10**, where the hydroxyl groups are replaced with halogen atoms, and for **7**, which contains two salicylic aldehyde units. This result shows once again[67] that the catechol fragment is not necessary for the inhibitory activity and moreover, at least in this case, that the second hydroxyl group is not important for its hydrogen-bonding properties. Interestingly, the catechol-containing tetrabromide **11** also showed a high level of activity.

Scheme 1



Among the asymmetric derivatives only the nitro derivatives **12** and **13** displayed detectable inhibitory activity. The amine **14** and the urea **15** were completely inactive as were their symmetric analogues (**2** and **4**).

Similar to previous work that identified a number of sulfated marine natural products[15,68,102] and sulfated synthetic molecules[11,45,54] as IN inhibitors, we observed that three of the four sulfated molecules (**16**−**18**) were inhibitors. Specifically, they were more active than the parent hydroxyl compounds (**1**, **8,** and **11**). However even if the promise of these compounds is unclear because in many cases negative charges impair permeation through cell membranes, these derivatives can be used as substrates for cocrystallization studies with IN because of their high water solubility.

For IN and several other metal-requiring enzymes, it has been found that the metal cofactor present in vitro influences the function of inhibitors. Many of the catechol-containing IN inhibitors reported previously were active only in the presence of Mn^{2+}, serving to reduce enthusiasm for this class of inhibitors. In contrast, the catechol isosteres **2**, **6**, **7**, **11**, **13**, and **18** presented here did not follow this trend. Compounds **2** and **6**, which were inactive at a concentration of 200 μM with Mn^{2+}, displayed a clear inhibitory activity in the presence of Mg^{2+}. Compounds **7**, **11**, **13**, and **18** showed only a slight decrease in inhibitory activity in Mg^{2+}, while compounds **1**, **9**, **10**, **12**, and **17** completely lost their potency. These results are difficult to rationalize, but they highlight that the metal cofactor plays an important role in the mechanism of inhibition.[103−105]

Compounds were tested for inhibition of PICs, replication intermediates isolated from infected cells. None of the compounds displayed inhibition over the range tested. In studies of L-chicoric acid, which also contains catechol moieties, it has been suggested that catechol can inhibit PIC formation.[86] None of the compounds have been tested for their antiviral activity yet.

Inhibitor Screening for MCV Topoisomerase in Vitro. All the compounds in the initial screen were also tested for inhibition of the type 1B topoisomerase of molluscum contagiosum virus (MCV).[87] In this context, the MCV assay serves as a counterscreen for candidate IN inhibitors, though inhibitors of this enzyme are also of interest in their own right. The DNA relaxation activity of MCV topoisomerase was monitored by gel electrophoresis, while DNA cleavage and religation activities were monitored using a microtiter assay. The values of IC_{50} are collected in Table 2. Most of the compounds were found to be inactive against topoisomerase in both assays, the exceptions being inhibitors **11** and **18** that inhibited religation with an IC_{50} of 50 and 75 μM, respectively, evidence of a low selectivity against IN.

Toxicity. The toxicity of the compounds was tested using the MTT cytotoxicity assay (Table 3). In this method, HeLa cells in culture are exposed to various

Table 2. Inhibition of MCV Topoisomerase

compd	IC_{50} DNA relaxation gel assay	IC_{50} DNA cleavage and religation plate assay
1	−@100	−@200
2	−@243	−@200
3	−@200	−@200
4	−@200	−@200
5	−@200	−@200
6	−@200	−@200
7	ND	−@200
8	−@500	−@200
9	−@200	−@200
10	−@200	−@200
11	−@200	50
12	−@200	−@200
13	+@200	−@200
14	−@200	−@200
15	−@200	−@200
16	123	−@200
17	ND	−@200
18	ND	75
19	ND	−@200

Table 3. Cytotoxicity

compd	LD_{50} (μM)	compd	LD_{50} (μM)
1	65	**11**	18
2	47	**12**	41
3	10	**13**	41
4	53	**14**	3
5	198	**15**	60
6	>600	**16**	134
7	4	**17**	>400
8	<20	**18**	207
9	4	**19**	50
10	<20		

concentrations of the cytotoxic agent under investigation. After 3 days, the culture supernatant is removed and the MTT dye added, which is metabolized by living cells to yield a blue color. Thus titration of the inhibitor under investigation can yield a LD_{50} (dose at which the signal is reduced 50% due to cell death). Most of the compounds were found to be cytotoxic, but the bissulfonic acid **6** and the bissulfonate **17** displayed a LD_{50} higher than 600 and 400 μM, respectively, indicative of little toxicity.

Conclusion

Catechol has been proposed to be undesirable in IN inhibitors, because inhibitors containing this fragment often inhibit other metal-requiring enzymes and some catechol derivatives may covalently modify proteins. We have synthesized and analyzed a series of catechol isosteres with the goal of replacing the catechol group in IN inhibitors. We did find that inhibitory activity differs depending on the divalent metal used in the assay, but we also found catechol isosteres that support inhibition in either metal tested. Inspection of the isosteres reveals no obvious pattern in the active groups. No member of this family inhibited integration by preassembled replication intermediates from infected cells (preintegration complexes). On the basis of this work, it would be possible to systematically replace catechol fragments in IN inhibitors with functional isosteres, potentially leading to more potent and less toxic compounds.

Experimental Section

Enzyme Assays. Assays of purified HIV-1 IN protein were carried out as described previously.[22] Assays using microtiter plates were carried out as described,[106] except that detection was carried out using a digoxygenin label in DNA. Assays of preintegration complexes were carried out using a microtiter assays as described.[45] Assays of MCV topoisomerase were analyzed using gels as described[87] or a microtiter assay.

General Data. ^{1}H and ^{13}C NMR spectra were recorded on Varian (Mercury 300 MHz/400 MHz) spectrometers with tetramethylsilane as the internal standard. High-resolution mass spectra were obtained from the University of California Riverside mass spectrometry facility in the FAB mode. Melting points were obtained on a Mel-Temp melting point apparatus and are reported uncorrected.

Techniques and Materials. Analytical thin-layer chromatography was performed on aluminum-backed silica gel 60 F_{254} plates from Alltech. All liquid chromatography separations were performed using silica gel (230−425 mesh) from Fisher Scientific Co. Commercial chemicals were used as supplied. Dichloromethane was distilled from calcium hydride, and pyridine was dried with 4 Å molecular sieves. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials, unless otherwise stated. Characterization of all new compounds was done by ^{1}H and ^{13}C NMR as well as high-resolution mass spectroscopy. Purity of the active compounds was determined by elemental analysis or HPLC. Reactions involving air- or water- sensitive compounds were conducted in glassware which was flame-dried and carried out under a positive pressure of argon.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′-diamino-6,6′-diol Dihydrochloric Salt (2). To a warm solution of **5** (96 mg, 0.23 mmol) in ethanol (7 mL) were added calcium chloride (105 mg, 0.94 mmol) and zinc dust (769 mg, 11.75 mmol). The mixture was refluxed for 1 h and filtered, after cooling, in concentrated hydrochloric acid (2 mL). Ethanol was partially evaporated. From the residue a gray precipitate was formed, filtered and desiccated (47.3 mg, 50%): mp 250 °C dec; ^{1}H NMR (400 MHz, D_2O) δ = 7.22 (s, 1H), 7.07 (d, J = 8.4 Hz, 1H), 6.90 (d, J = 8.4 Hz, 1H), 6.53 (s, 1H), 2.47 (d, J = 13.6 Hz, 1H), 2.44 (d, J = 13 Hz, 1H), 2.11 (d, J = 13.6 Hz, 1H), 2.09 (d, J = 13 Hz, 1H), 1.34 (s, 3H), 1.29 (s, 3H), 1.27 (s, 3H), 1.23 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 151.09, 150.22, 147.47, 145.61, 141.17, 122.27, 119.87, 119.49, 117.01, 114.44, 111.81, 109.69, 60.19, 57.80, 56.40, 44.25, 44.20, 32.26, 32.01, 30.32, 30.01; HR-MS calcd for $C_{21}H_{27}N_2O_2$ 339.2072, found 339.2057.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′-dimethyl-6,6′-diol (3). A mixture of *o*-cresol (10.4 g), acetic acid (26 mL), acetone (11 mL), and concentrated hydrochloric acid (21 mL) was heated under reflux for 2 days. The very thick black mass was poured into water and extracted with ether. The organic phase was dried on $MgSO_4$ and the solvent was evaporated. The residue was purified on silica gel using a gradient of hexanes/ether (start 98:2, finish 9:1). The material obtained was washed with hexanes giving a white solid (1.78 g, 11%): mp 242−246 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.90 (s, 1H), 6.09 (s, 1H), 4.26 (s, 1H), 2.29 (d, J = 13.2 Hz, 1H), 2.23 (s, 3H), 2.18 (d, J = 13.2 Hz, 1H), 1.36 (s, 3H), 1.29 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 152.94, 149.77, 144.34, 123.47, 122.75, 110.317, 59.45, 57.04, 43.14, 31.94, 30.29, 16.09; HR-MS calcd for $C_{23}H_{28}O_2$ 336.2089, found 336.2082.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-6,6′-dihydroxy-5,5′-bisurea (4). To a solution of **2** (20 mg, 0.048 mmol) in acetic acid (0.5 mL) and water (1 mL) at 35 °C was added dropwise a solution in water of sodium cyanate (12.64 mg, 0.194 mmol). After 10 min at 35 °C, the mixture was allowed to stir overnight at room temperature. The white precipitate that was formed was filtered, washed with water and dried (12 mg, 59%): mp 180 °C dec; ^{1}H NMR (400 MHz, DMSO) δ = 9.55 (s, 1H), 7.91 (s, 1H), 7.73 (s, 1H), 6.20 (s, 1H), 6. 17 (s, 1H), 2.27 (d, J = 15 Hz, 1H), 2.10 (d, J = 15 Hz, 1H), 1.35 (s, 3H), 1.29 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ = 156.15, 144.98, 144.50, 142.79, 131.25, 111.62, 109.21, 59.32, 56.20,

42.55, 31.59, 30.53; HR-MS calcd for $C_{23}H_{28}N_4O_4$ 424.2110, found 424.2116.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′dinitro-6,6′-diol (5), 3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,7′-dinitro-6,6′-diol (12), and 3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′,7-trinitro-6,6′-diol (13). HNO_3 (45%) (8 mL) was added dropwise to **8** (2 g, 6.48 mmol) at 0 °C. The semisolid yellow mixture was stirred overnight. Thereafter water was added and the solid was filtered, washed with a lot of water, filtered and desiccated. The crude material was cromatographed on silica gel using a gradient of hexanes/ether (start 90:10, finish 70:30). **5** (180 mg, 7%), **12** (232.4 mg, 9%), **8** (not reacted) and **13** (574 mg, 20%) were eluted in this order.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′-dinitro-6,6′-diol (5): mp 238−241 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 10.59 (s, 1H), 7.91 (s, 1H), 6.54 (s, 1H), 2.42 (d, J = 13.2 Hz, 1H), 2.29 (d, J = 13.2 Hz, 1H), 1.44 (s, 3H), 1.37 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 160.33, 155.146, 144.88, 133.41, 118.55, 114.76, 58.74, 58.23, 43.51, 31.75, 30.20; HR-MS calcd for $C_{21}H_{22}N_2O_6$ 398.1477, found 398.1490.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,7′-dinitro-6,6′-diol (12): mp 213−215 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 10.66 (s, 1H), 10.56 (s, 1H), 7.89 (s, 1H), 7.43 (d, J = 8.8 Hz, 1H), 7.22 (d, J = 8.8 Hz, 1H), 6.39 (s, 1H), 2.68 (d, J = 13 Hz, 1H), 2.48 (d, J = 13 Hz, 1H), 2.33 (d, J = 13 Hz, 1H), 2.18 (d, J = 13 Hz, 1H), 1.54 (s, 3H), 1.53 (s, 3H), 1.38 (s, 3H), 1.37 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 160.96, 155.54, 155.42, 147.39, 145.02, 142.03,132.86, 130.65, 120.66, 118.67, 111.107, 60.44, 59.99, 54.67, 43.54, 43.13, 33.07, 31.30, 30.08, 29.22; HR-MS calcd for $C_{21}H_{22}N_2O_6$ 398.1477, found 398.1485.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′,7-trinitro-6,6′-diol (13): mp 239−241 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 10.90 (s, 1H), 10.57 (s, 1H), 8.09 (s, 1H), 7.87 (s, 1H), 6.58 (s, 1H), 2.77 (d, J = 13.2 Hz, 1H), 2.51 (d, J = 13.2 Hz, 1H), 2.39 (d, J = 13.2 Hz, 1H), 2.27 (d, J = 13.2 Hz, 1H), 1.47 (s, 6H), 1.53 (s, 3H), 1.43 (s, 3H), 1.35 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 155.36, 154.84, 147.95, 147.44, 145.94, 144.82, 134.212, 133.76, 120.22, 118.78, 114.26, 59.51, 58.62, 55.55, 43.87, 43.644, 32.81, 31.25, 29.95, 29.90; HR-MS calcd for $C_{21}H_{20}N_3O_8$ 442.1250, found 442.1257.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-6,6′-dihydroxy-5,5′-disulfonic Acid (6). To a solution of **8** (1 g, 3.24 mmol) in chloroform (6 mL) at −10 °C was added dropwise chlorosulfonic acid (860 μL, 13.0 mmol). The mixture was kept at −10 °C for 2 h and then was stirred at room temperature for 16 h. The resulted pinkish suspension was diluted with chloroform and poured into ice. The phases were separated, the aqueous one was washed with more chloroform, and the water was then evaporated giving a white solid (909 mg, 60%): mp 220 °C dec; ^{1}H NMR (400 MHz, DMSO) δ = 7.26 (s, 1H), 6.04 (s, 1H), 2.26 (d, J = 13.2 Hz, 1H), 2.12 (d, J = 13.2 Hz, 1H), 1.33 (s, 3H), 1.26 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ = 152.80, 152.72, 141.69, 129.821, 119.99, 110.75, 58.90, 56.88, 42.52, 31.60, 30.34; HR-MS calcd for $C_{21}H_{23}S_2NaO_8$ 489.0653, found 489.0640.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-6,6′-dihydroxy-5,5′-biscarbaldehyde (7). To a solution of **8** (100 mg, 0.324 mmol) in dichloromethane (2 mL) was added titanium tetrachloride (143 μL, 1.28 mmol) followed by the dropwise addition of dichloromethyl methyl ether (88 μL, 0.972 mmol) under vigorous stirring. The mixture was stirred for 48 h. HCl (10%) was added, the organic phase was separated and the residual aqueous phase was repeatedly extracted with ether. The combined organic phases were washed, dried, and evaporated under vacuum and the residue was submitted to flash chromatography on silica gel (hexanes:ether = 9:1) (41.3 mg, 35%): mp 248−250 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 10.99 (s, 1H), 9.88 (s, 1H), 7.34 (s, 1H), 6.37 (s, 1H), 2.38 (d, J = 13.2 Hz, 1H), 2.27 (d, J = 13.2 Hz, 1H), 1.43 (s, 3H), 1.36 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 195.72, 161.44, 159.90, 144.90, 126.99, 120.26, 112.74, 58.83, 58.47, 43.17, 31.94, 30.37; HR-MS calcd for $C_{23}H_{24}O_4$ 364.1674, found 364.1670.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-6,6′-diol (8):[90] mp 184−186 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + 1% DMSO) δ = ^{1}H NMR (300 MHz, $CDCl_3$) = 8.16 (s, 1H), 6.84 (d, J = 8.2 Hz, 1H), 6.55 (dd, J = 8.2 Hz and J = 2.4 Hz, 1H), 6.15 (d, J = 2.4 Hz, 1H), 2.18 (d, J = 13.2 Hz, 1H), 2.09 (d, J = 13.2 Hz, 1H), 1.22 (s, 3H), 1.17 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$ + 1% DMSO) δ = 155.96, 151.57, 142.7, 121.85, 114.12, 110.38, 59.28, 42.53, 31.65, 30.35.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′-dibromo-6,6′-diol (9). To an ice-cooled solution of **8** (1 g, 3.24 mmol) in chloroform (6 mL) was slowly added a solution of bromine (346 μL, 6.74 mmol) in chloroform (1 mL). The reaction mixture was stirred for a further 2 h at 5 °C and then overnight at room temperature. After evaporation of the solvent the crude material was chromatographated on silica gel (hexanes:ether = 7:3) (1.45 g, 96%): mp 216−218 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.12 (s, 1H), 6.43 (s, 1H), 5.32 (s, 1H), 2.32 (d, J = 13.2 Hz, 1H), 2.21 (d, J = 13.2 Hz, 1H), 1.35 (s, 3H), 1.30 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 151.44, 151.27, 145.83, 125.04, 111.35, 109.15, 59.32, 57.29, 43.18, 31.78, 30.36; HR-MS calcd for $C_{21}H_{22}O_2Br_2$ 463.9986, found 464.0003.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′-diiodo-6,6′-diol (10). To a stirred solution of **8** (200 mg, 0.648 mmol) in 9.4 mL of acetic acid was added iodine monochloride (1 M) in dichloromethane (1.3 mL, 1.3 mmol). The reaction mixture was stirred at room temperature for 3 h and poured into water, and the resulting precipitate was filtered, washed with cold water until free of acid, and dried in vacuo to yield an orange solid (344 mg, 95%): mp 210−212 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.41 (s, 1H), 6.42 (s, 1H), 5.11 (s, 1H), 2.31 (d, J = 13.2 Hz, 1H), 2.20 (d, J = 13.2 Hz, 1H), 1.35 (s, 3H), 1.30 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 153.72, 152.78, 146.61, 131.25,110.37, 84.35, 59.37, 57.11, 43.04, 31.84, 30.42; HR-MS calcd for $C_{21}H_{22}O_2I_2$ 559.9709, found 559.9696.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-4,4′,7,7′-tetrabromo-5,5′,6,6′-tetrol (11). To an ice-cooled solution of **1** (1 g, 2.93 mmol) in chloroform (6 mL) was slowly added a solution of bromine (627 μL, 12 mmol) in chloroform (1 mL). The reaction mixture was stirred for a further 2 h at 5 °C and then 4 days at room temperature. After evaporation of the solvent the crude material was purified on silica gel (hexanes:ether = 1:1) and then crystallized from a mixture of hexanes:ether = 1:1 in the presence of some drops of ethanol giving nice deep red crystals scarcely soluble in every solvent (1.6 mg, 84%): mp 228−232 °C; ^{1}H NMR (400 MHz, DMSO) δ = 9.18 (bs, 2H), 2.35 (d, J = 12.8 Hz, 1H), 2.21 (d, J = 12.8 Hz, 1H), 1.54 (s, 3H), 1.44 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ = 143.59, 143.46, 140.64, 138.69, 108.35, 108.154, 59.49, 55.98, 45.39, 28.38, 27.87; HR-MS calcd for $C_{21}H_{20}O_4Br_4$ 651.8095, found 651.8066.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,7′-diamino-6,6′-diol Dihydrochloric Salt (14). To a warm solution of **12** (96 mg, 0.23 mmol) in ethanol (7 mL) were added calcium chloride (105 mg, 0.94 mmol) and zinc dust (769 mg, 11.8 mmol). The mixture was refluxed for 1 h and filtered, after cooling, in concentrated hydrochloric acid (2 mL). Ethanol was partially evaporated. From the residue a gray precipitated was formed, filtered and desiccated (47.3 mg, 50%): mp 240 °C dec; ^{1}H NMR (400 MHz, D_2O) δ = 7.22 (s, 1H), 7.07 (d, J = 8.4 Hz, 1H), 6.90 (d, J = 8.4 Hz, 1H), 6.53 (s, 1H), 2.47 (d, J = 13.6 Hz, 1H), 2.44 (d, J = 13 Hz, 1H), 2.11 (d, J = 13.6 Hz, 1H), 2.09 (d, J = 13 Hz, 1H), 1.34 (s, 3H), 1.29 (s, 3H), 1.27 (s, 3H), 1.23 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 151.09, 150.22, 147.47, 145.61, 141.17, 122.27, 119.87, 119.49, 117.01, 114.44, 111.81, 109.69, 60.19, 57.80, 56.40, 44.25, 44.20, 32.26, 32.01, 30.32, 30.01; HR-MS calcd for $C_{21}H_{27}N_2O_2$ 339.2072, found 339.2060.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-6,6′-dihydroxy-5,7′-bisurea (15). To a solution of **14** (24 mg, 0.058 mmol) in acetic acid (0.5 mL) and water (1 mL) at 35 °C was added dropwise a solution of sodium cyanate (15.1 mg, 0.23 mmol) in water. After 10 min at 35 °C, the mixture was allowed to stir overnight at room temperature. The white precipitated that was formed was filtered, washed with water and desiccated (15.6 mg, 63%): mp 194−200 °C dec; ^{1}H NMR (300 MHz, DMSO) δ = 9.60 (s, 1H), 8.94(s, 1H), 7.88 (s, 1H), 7.74 (s, 1H),

6.91 (d, $J = 8.1$ Hz, 1H), 6.77 (d, $J = 8.1$ Hz, 1H), 6.13 (s, 2H), 6.01 (s, 1H), 5.49 (s, 1H), 2.32 (d, $J = 12.7$ Hz, 1H), 2.27 (d, $J = 13.3$ Hz, 1H), 2.06 (d, $J = 13.3$ Hz, 1H), 1.97 (d, $J = 12.7$ Hz, 1H), 1.30 (s, 3H), 1.29 (s, 3H), 1.22 (s, 6H); ^{13}C NMR (100 MHz, DMSO) $\delta = 157.2$, 156.23, 150.54, 145.45, 143.99, 141.70, 140.98, 127.87, 122.30, 119.16, 117.31, 112.53, 107.70,60.02, 56.43, 55.15, 42.88, 42.31, 32.09, 31.99, 30.66, 30.07; HR-MS calcd for $C_{21}H_{28}N_4O_4$ 425.2188, found 425.2177.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′,6,6′-tetrol Tetrasulfate (16). Pyridine (1.3 mL) was cooled to −20 °C and chlorosulfonic acid (0.2 mL, 2.95 mmol) was added dropwise with a lot of caution and efficient stirring. Always at −20 °C, a solution of **1** (200 mg, 0.59 mmol) in pyridine (1 mL) was added and the mixture was stirred overnight at room temperature. Thereafter, excess of solvent was distilled in vacuo and the residue was redissolved in distilled water (2 mL) and the pH adjusted to 8.0 with KOH (1 M). After 15 min, the solvent was removed in vacuo. The residue was dissolved in boiling ethanol, filtered still warm, redissolved in very slightly basic (pH 8) methanol−water (4:1) and filtered hot before crystallization. After one night the solid that precipitated was filtered and desiccated (95.7 mg, 20%): mp >300 °C; ^{1}H NMR (400 MHz, DMSO) $\delta = 7.30$ (s, 1H), 6.88 (s, 1H), 2.21 (d, $J = 12.8$ Hz, 1H), 2.08 (d, $J = 12.8$ Hz, 1H), 1.23 (s, 3H), 1.24 (s, 3H); ^{13}C NMR (100 MHz, DMSO) $\delta =$ 145.13, 143.45, 142.84, 142.78, 115.58, 112.83, 59.37, 56.66, 42.43, 31.34, 30.44; HR-MS calcd for $C_{21}H_{20}S_4K_3O_{16}$ 772.8545, found 772.8546.

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-6,6′-diol Disulfate (17). This compound was synthesized with the same procedure described for the synthesis of **16**. **17** was obtained starting from **8** (50 mg, 0.16 mmol) in 50% yield (46.8 mg): mp >300 °C; ^{1}H NMR (400 MHz, DMSO) $\delta = 7.10$ (d, $J = 8.4$ Hz, 1H), 6.99 (dd, $J = 8.4$ Hz, $J = 1.6$ Hz, 1H), 6.46 (d, $J = 1.6$ Hz, 1H), 2.27 (d, $J = 12.8$ Hz, 1H), 2.14 (d, $J = 12.8$ Hz, 1H), 1.34 (s, 3H), 1.28 (s, 3H); ^{13}C NMR (100 MHz, DMSO) $\delta =$ 152.59, 150.07, 146.16, 121.58, 119.79, 115.80, 59.17, 56.95, 42.45, 31.46, 30.33; HR-MS calcd for $C_{21}H_{22}S_2KO_8$ 505.0393, found 505.0381

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-4,4′,7,7′-tetrabromo-5,5′,6,6′-tetrol Tetrasulfate (18). This compound was synthesized with the same procedure described for the synthesis of **16**. **18** was obtained starting from **1** (50 mg, 0.08 mmol) in 20% yield (18 mg): mp >300 °C; ^{1}H NMR (400 MHz, D_2O) $\delta = 2.42$ (d, $J = 13.2$ Hz, 1H), 2.15 (d, $J = 13.2$ Hz, 1H), 1.43 (s, 3H), 1.32 (s, 3H); ESI(+) m/z 969 (M^{4-}).

3,3,3′,3′-Tetramethyl-1,1′-spirobi(indan)-5,5′-dibromo-6,6′-diol Disulfate (19). This compound was synthesized with the same procedure described for the synthesis of **16**. **19** was obtained starting from **9** (50 mg, 0.11 mmol) in 25% yield (20 mg): mp >300 °C; ^{1}H NMR (400 MHz, D_2O) $\delta = 7.50$ (s, 1H), 6.84 (s, 1H), 2.34 (d, $J = 13.2$ Hz, 1H), 2.20 (d, $J = 13.2$ Hz, 1H), 1.29 (s, 3H), 1.23 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ = 149.42, 149.09, 147.61, 125.60, 116.76, 113.38, 58.89, 56.86, 42.63, 31.11, 30.08; FAB-MS m/z 663 ($M^{2-} + K^+$).

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