

Identification, Synthesis, and Biological Evaluation of Metabolites of the Experimental Cancer Treatment Drugs Indotecan (LMP400) and Indimitecan (LMP776) and Investigation of Isomerically Hydroxylated Indenoisoquinoline Analogues as Topoisomerase I Poisons

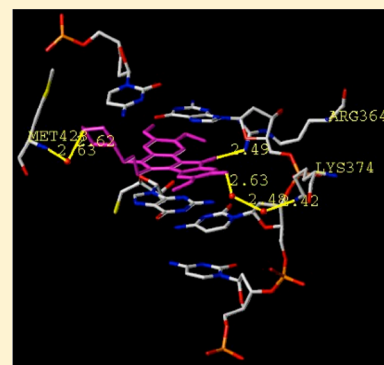
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ABSTRACT: Hydroxylated analogues of the anticancer topoisomerase I (Top1) inhibitors indotecan (LMP400) and indimitecan (LMP776) have been prepared because (1) a variety of potent Top1 poisons are known that contain strategically placed hydroxyl groups, which provides a clear rationale for incorporating them in the present case, and (2) the hydroxylated compounds could conceivably serve as synthetic standards for the identification of metabolites. Indeed, incubating LMP400 and LMP776 with human liver microsomes resulted in two major metabolites of each drug, which had HPLC retention times and mass fragmentation patterns identical to those of the synthetic standards. The hydroxylated indotecan and indimitecan metabolites and analogues were tested as Top1 poisons and for antiproliferative activity in a variety of human cancer cell cultures and in general were found to be very potent. Differences in activity resulting from the placement of the hydroxyl group are explained by molecular modeling analyses.



■ INTRODUCTION

Topoisomerases are nature's ubiquitous solution for managing the topology and torsional states of DNA. Topoisomerase I (Top1) is an essential enzyme that relaxes supercoiled DNA so that it may be replicated, transcribed, and repaired.^{1–4} The enzyme acts through a nucleophilic tyrosine residue (Tyr723), which nicks the phosphodiester backbone of double-stranded, supercoiled DNA and forms a transient "cleavage complex" in which the 3' end of the broken DNA strand is covalently linked to the enzyme. Within this "cleavage complex", the scissile (broken) strand undergoes "controlled rotation" around the unbroken strand, a process that relaxes the DNA. The catalytic cycle ends when the 5' end of the scissile strand religates the DNA and the enzyme is released. If this cycle is inhibited, DNA damage ensues, which in turn activates DNA damage responses, leading to cell cycle arrest or the eventual triggering of proapoptotic cascades.^{1,5–9}

As Top1 is overexpressed and DNA damage responses are defective in some human tumors, several Top1 inhibitors have been developed as chemotherapeutic agents.^{4,10} Representative examples are shown in Figure 1. The alkaloid camptothecin (1)¹¹ is not used clinically, but its semisynthetic derivatives

topotecan (2) and irinotecan (3) are FDA-approved.^{1,10,12} These compounds act by intercalating between the base pairs in the cleavage complex and binding at the Top1–DNA interface,¹³ where they "poison" the complex (prevent DNA religation), resulting in persistent, covalent Top1–DNA lesions that are then converted into irreversible double-strand breaks when they collide with the advancing replication machinery, resulting in apoptosis.^{6–9} Although potent, camptothecin derivatives suffer from many shortcomings, including poor solubility, dose-limiting toxicity, pharmacokinetic limitations resulting from the instability of the E-ring lactone under physiological pH, and binding of the lactone hydrolysis product to plasma proteins.^{10,14–16}

The indenoisoquinolines were therefore developed as therapeutic alternatives. In 1998 a COMPARE analysis^{17,18} was performed on NSC 314622 (4), which indicated that it may act in a manner similar to that of camptothecin and derivatives. Indeed, this compound was found to be a Top1 inhibitor.¹⁹ Since then, many optimization and SAR studies

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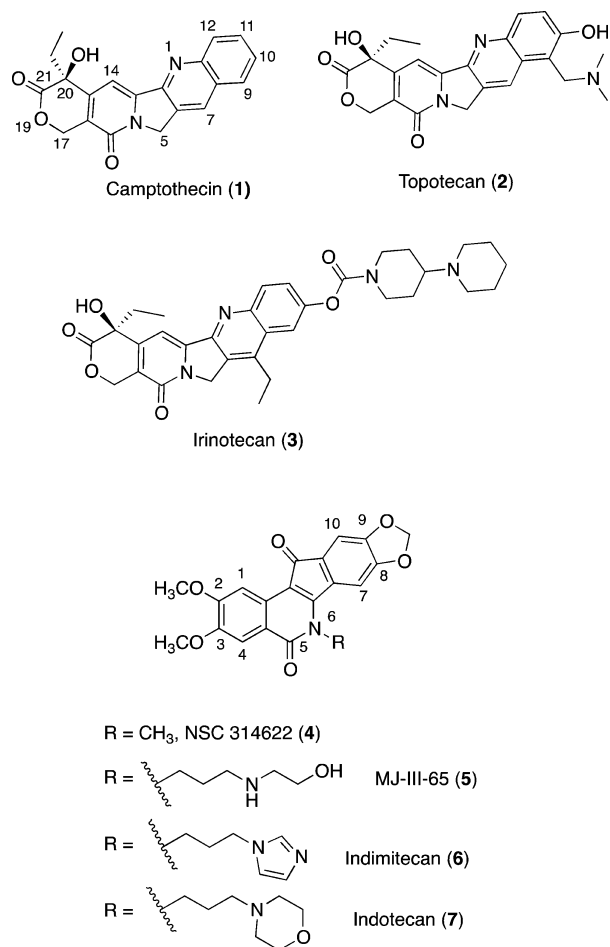


Figure 1. Representative Top1 inhibitors.

have produced potent indenoisoquinolines such as MJ-III-65 (5),^{20–24} which inhibit Top1 through an intercalation and interfacial mechanism similar to that of compound 1. Two of these compounds, indimitecan (LMP776, 6) and indotecan (LMP400, 7) were promoted into phase I clinical trials at the National Cancer Institute.²⁵ These compounds appear to be stable and are powerful, cytotoxic Top1 poisons that induce long-lasting DNA breaks and overcome the drug resistance issues associated with the camptothecins.^{20,26,27}

The metabolism of 6 and 7 is currently under investigation, which has led to the synthesis of potential metabolites to be used as synthetic standards for metabolism studies. As part of this study, structural analogues of the proposed metabolites are also being prepared and investigated for Top1 inhibitory activity. It was proposed that the indenoisoquinolines 6 and 7 could be metabolically labile at several positions (Figure 2). The methoxy groups of 6 and 7 are likely to be cleaved *in vivo*. O-Dealkylation, catalyzed chiefly by hepatic cytochrome P450 enzymes, is a common and well-precedented metabolic process that, for example, plays a significant role in human metabolism of the chemotherapeutic agents etoposide and teniposide,²⁸ opiates and opiate antagonists,^{29,30} and the topoisomerase inhibitors berberine and Genz-644282.^{31,32}

Methylenedioxy rings are also possible sites of metabolism (likely via oxidation). Both demethylation (to yield catechols) and demethylation/alkylation processes (to yield *o*-methoxyphenols) are observed in rodent and human metabolism of berberine,³¹ safrole and piperonal deriva-

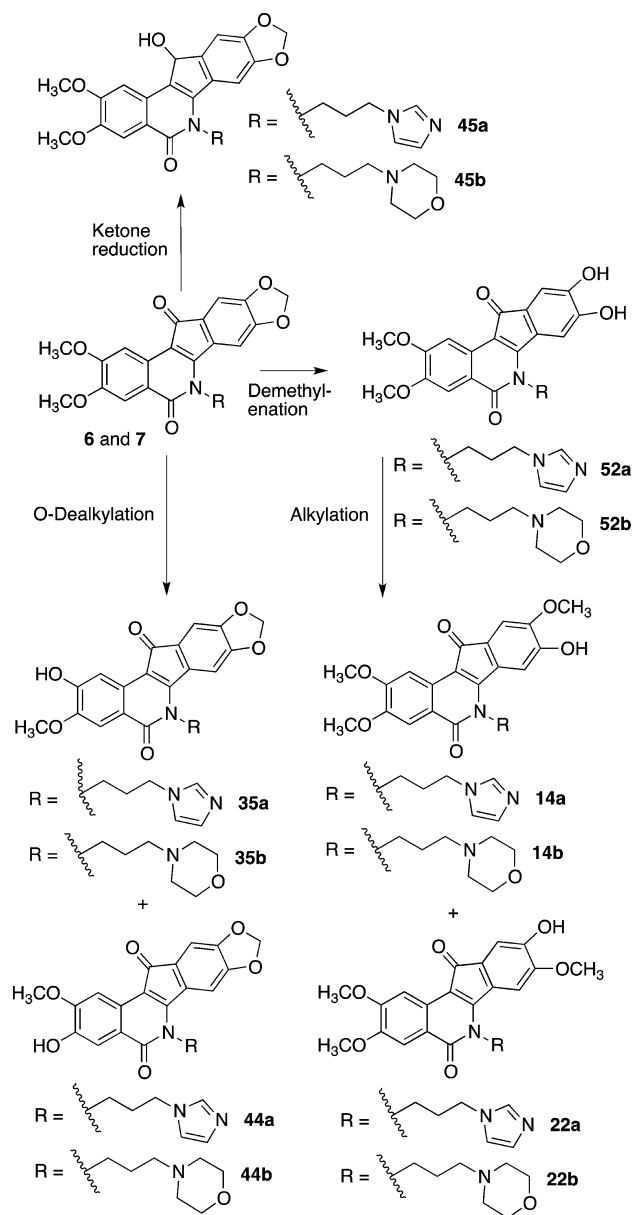


Figure 2. Proposed metabolic pathways and potential metabolites of 6 and 7.

tives,^{33,34} MDMA (“ecstasy”),³⁵ and the designer drug MDPPA.³⁶

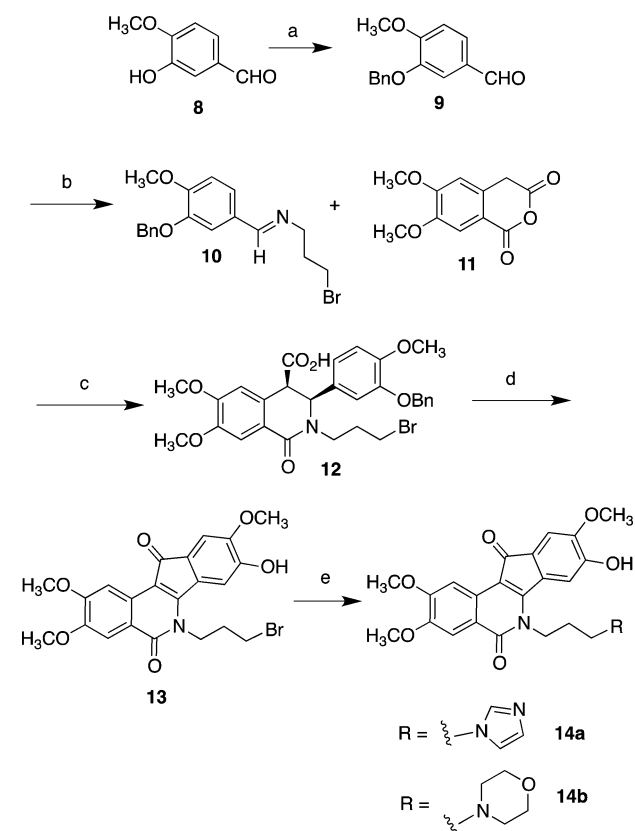
Compounds 6 and 7 could also be substrates for metabolic ketone reductases. This phase I process is observed in mice for the related indenoisoquinoline oracin³⁷ as well as for the indenoisoquinoline rexinoid AM6-36 in human hepatocytes.³⁸ The synthesis of hydroxy and reduced analogues is also justified by literature that is rich with potent Top1 inhibitors bearing phenolic hydroxyls, including the active metabolite of 3 (SN-38), the alkaloid fagaronine,¹⁰ various other 10- and 11-hydroxycamptothecins,^{39,40} indolocarbazoles,⁴¹ and the dual Top1/Top2 inhibitor TAS-103.⁴² Additionally, some 11-hydroxy (reduced) indenoisoquinolines have been synthesized and tested against Top1 with promising results,^{43,44} and 9-hydroxyindenoisoquinolines have been assayed against Top2,⁴⁵ but to our knowledge, no A- or D-ring hydroxyindenoisoquinolines have been assayed against Top1. To this end, the demethylated/alkylated analogues 14a,b (Scheme 1) and

regioisomers **22a,b** (Scheme 2), the demethylated analogues **35a,b** (Scheme 4) and regioisomers **44a,b** (Scheme 5), the 11-hydroxyindenoisoquinolines **45a,b** (Scheme 6), and the catechols **52a,b** (Scheme 7) were synthesized and evaluated for Top1 inhibitory and antiproliferative activity and used as standards for metabolism studies.

CHEMISTRY

Indenoisoquinolines have been prepared by many routes, including the condensation of primary amines with the appropriately substituted isochromenones,⁴⁶ Suzuki–Miyaura cross-coupling followed by ring-closing metathesis,⁴⁷ and the oxidative cyclization of the cis acid condensation products formed by the reaction of homophthalic anhydrides and benzylidene Schiff bases.⁴⁸ The last route was envisioned for the synthesis of hydroxyindenoisoquinolines, as it has a high functional group tolerance and was previously used to synthesize **6** and **7**.²² Because of the presence of harsh and oxidative intermediary steps, the phenol would have to be unmasked at a late stage in the synthesis, and preparation of the CD ring (indene) system of 8-hydroxy-9-methoxyindenoisoquinolines (Scheme 1) thus began with benzylation of isovanillin (**8**) to yield the ether **9**. Condensation of this aldehyde with 3-bromopropylamine afforded the Schiff base **10** in excellent yield. The isoquinolone (AB) ring system **12** was synthesized from the condensation of **10** with 4,5-dimethoxyhomophthalic anhydride (**11**),⁴⁹ prepared by established

Scheme 1^a

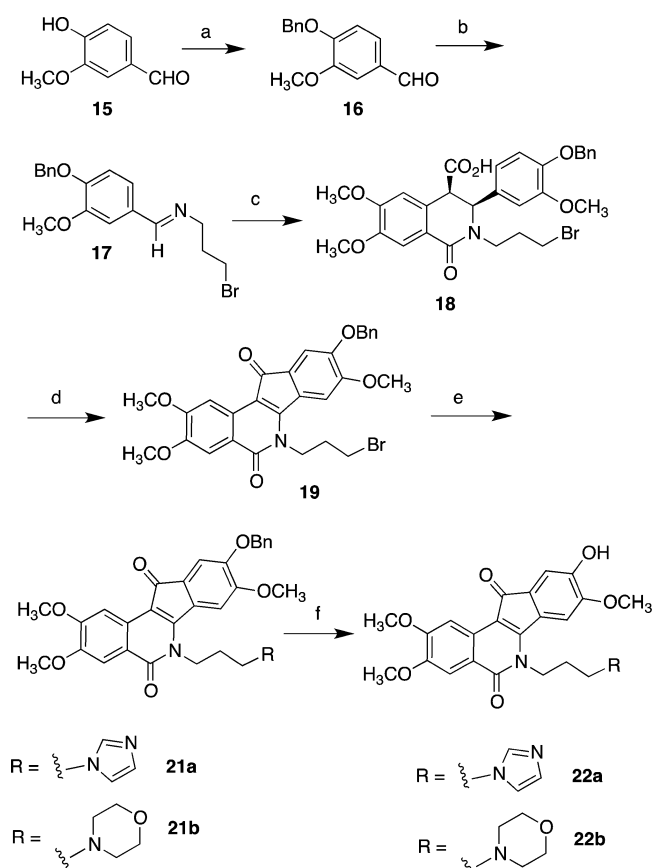


^aReagents and conditions: (a) BnBr, DMF, K₂CO₃, rt; (b) 3-bromopropylamine HBr, Et₃N, Na₂SO₄, CHCl₃; (c) **11**, CHCl₃, 10 °C to rt; (d) (i) SOCl₂, rt, (ii) AlCl₃ (2 equiv), 1,2-dichloroethane, rt; (e) imidazole or morpholine, NaI, DMF, 70 °C.

literature procedures.^{50,51} When conducted at low temperature, the reaction provided the thermodynamically less stable cis diastereomer **12** as a solid precipitate in good yield. Treatment of **12** with thionyl chloride at room temperature, followed by AlCl₃ in dichloroethane, resulted in four reactions (acid chloride formation, Friedel–Crafts cyclization, dehydrogenation, and debenylation) to yield the crude phenol **13**. Reaction of **13** with imidazole or morpholine in the presence of sodium iodide in DMF yielded, respectively, the imidazolyl compound **14a** or the morpholinyl compound **14b**.

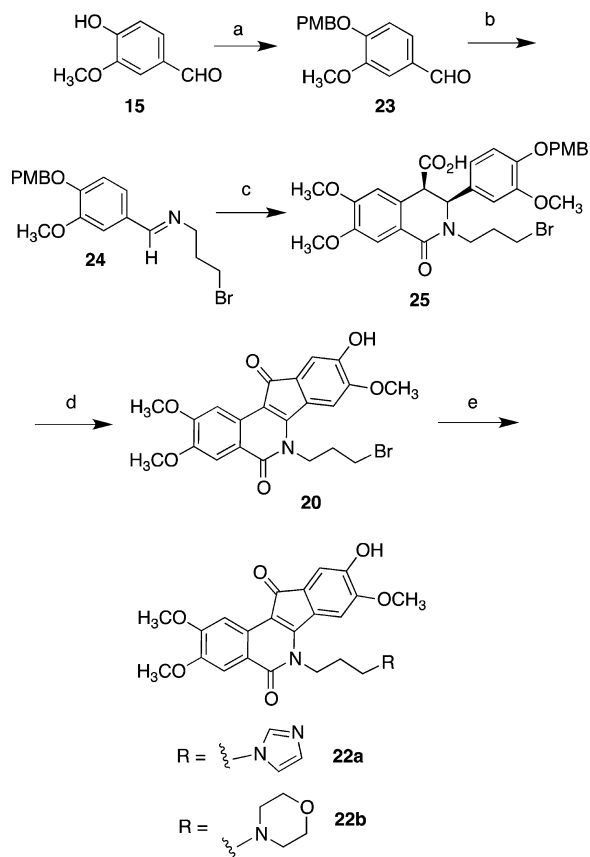
Analogously, 9-hydroxy-8-methoxyindenoisoquinolines were prepared from vanillin (**15**, Scheme 2). Benzylation (to yield

Scheme 2^a



^aReagents and conditions: (a) BnBr, DMF, K₂CO₃, rt; (b) 3-bromopropylamine HBr, Et₃N, Na₂SO₄, CHCl₃, rt; (c) **11**, CHCl₃, 10 °C to rt; (d) SOCl₂, rt; (e) imidazole or morpholine, NaI, DMF, 70 °C; (f) HBr, AcOH, H₂O, 55–70 °C.

16) and treatment with 3-bromopropylamine afforded the Schiff base **17**. Condensation with **11** likewise yielded the cis acid **18**. Treatment with thionyl chloride led to the benzyl-protected phenol **19**. Interestingly, adding 2 equiv of AlCl₃ did not produce a clean debenylation as observed for the regioisomer and gave only very low yields of the phenol **20** (Scheme 3). Compound **19** was therefore elaborated with imidazole or morpholine to obtain the benzyl-protected phenols **21a** and **21b**, respectively. Unfortunately, many standard conditions for debenylation failed, yielding either unchanged starting material or decomposition products. Aqueous hydrobromic acid in AcOH afforded the free phenols **22a** and **22b**, but the poor solubility of the starting materials

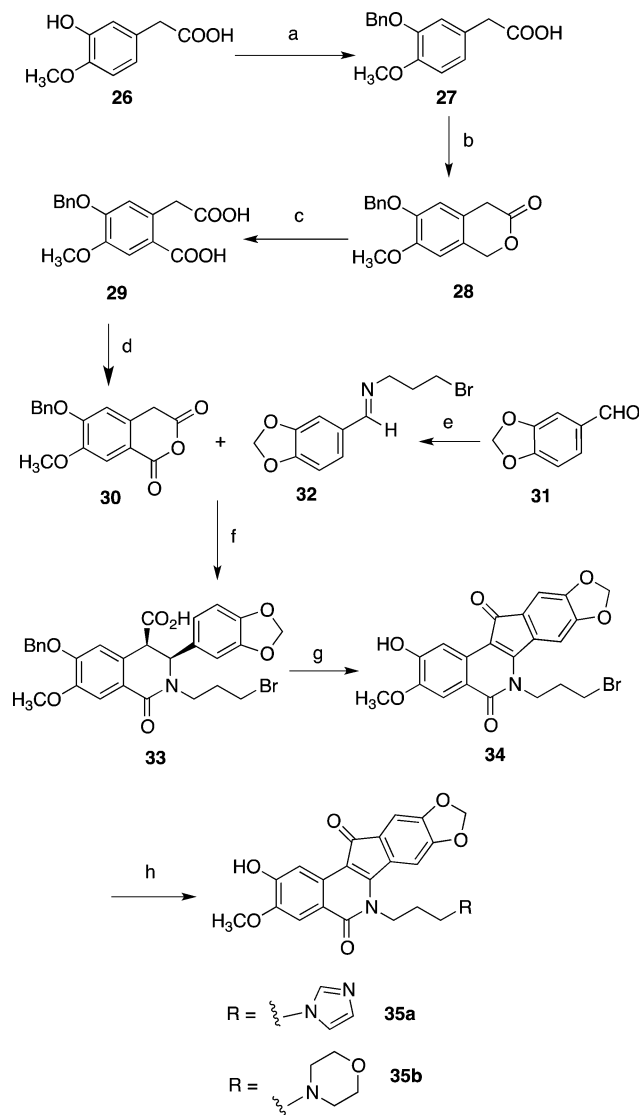
Scheme 3^a

^aReagents and conditions: (a) PMB-Cl, DMF, K₂CO₃, 70 °C; (b) 3-bromopropylamine HBr, Et₃N, Na₂SO₄, CHCl₃, rt; (c) **11**, CHCl₃, 0 °C to rt; (d) SOCl₂, rt; (e) imidazole or morpholine, NaI, DMF, 70 °C or dioxane, reflux.

often resulted in competing demethylation and oxidation processes, and this method was low-yielding and hard to reproduce.

An alternative route (Scheme 3) was therefore devised. From compound **15**, O-PMB-vanillin (**23**) was prepared and converted into the Schiff base **24**. Condensation with compound **11** afforded the cis acid **25**. Treatment of compound **25** with SOCl₂ again resulted in four reactions (acid chloride formation, Friedel–Crafts acylation, dehydrogenation, and PMB cleavage) to yield **20**, which could be obtained on a multigram scale. Reaction of **20** with imidazole or morpholine and sodium iodide in DMF (the use of dioxane under argon resulted in improved yields) led to **22a** and **22b** in acceptable yields.

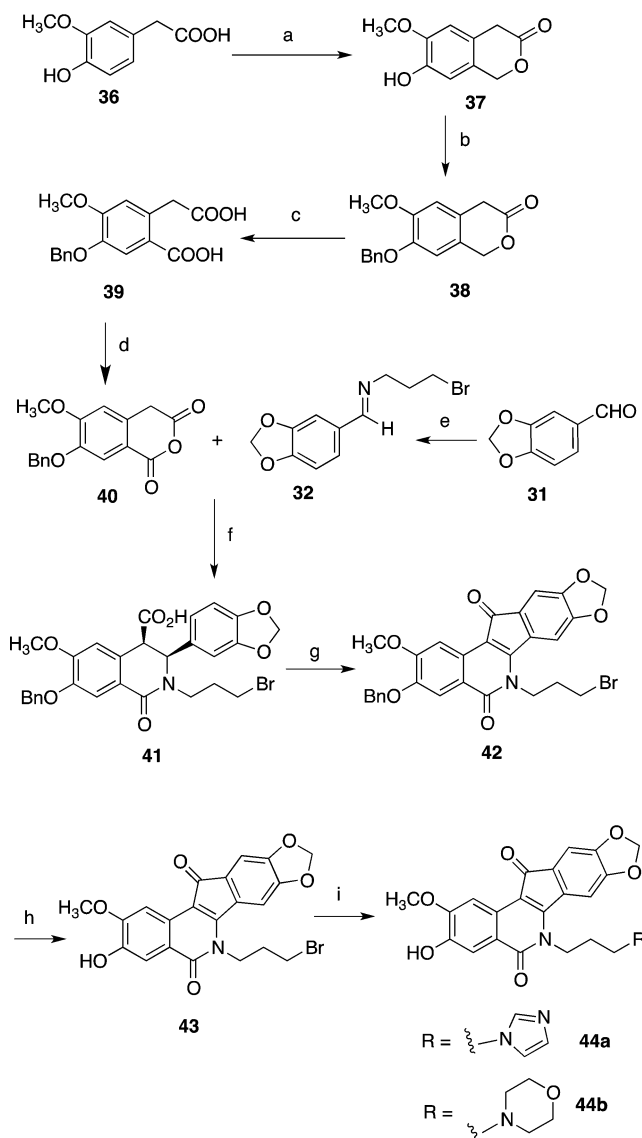
Several procedures were used to prepare A-ring demethylated analogues. A similar benzyl protecting group strategy was employed to prepare homophthalic anhydrides containing the desired protected phenols, which was necessary in order to avoid colored byproducts and other unidentified impurities formed from the unprotected phenols in the presence of the harsh acidic, basic, and oxidative conditions (Schemes 4 and 5). Therefore, to prepare 2-hydroxy-3-methoxyindenoisoquinolines, homoisovanillic acid (**26**, Scheme 4) was first benzylated to yield compound **27**.⁵² Ortho-hydroxymethylation afforded the homophthalide **28**,⁵³ which was saponified and oxidized with KMnO₄ to yield the diacid **29**. The anhydride **30** was prepared by heating the diacid in refluxing acetyl chloride.

Scheme 4^a

^aReagents and conditions: (a) (i) KOH, BnCl, EtOH, reflux, (ii) KOH, H₂O, reflux; (b) H₂CO, H₂O, HCl, AcOH, 45 °C; (c) (i) KOH, H₂O, rt, (ii) KMnO₄, H₂O, 0 °C to rt, (iii) EtOH, reflux; (d) AcCl, reflux; (e) 3-bromopropylamine HBr, Et₃N, Na₂SO₄, CHCl₃, rt; (f) CHCl₃, 0 °C to rt; (g) (i) SOCl₂, rt, (ii) AlCl₃ (2 equiv) DCE, 0 °C; (h) imidazole or morpholine, NaI, DMF, 60 °C.

Compound **30** was condensed with the Schiff base **32** derived from piperonal (**31**) to yield the cis acid **33** in excellent yield.²¹ The use of aluminum chloride resulted in debenzoylation following the cyclization, but yields of the indenoisoquinoline **34** were very low (10–15%). A large amount of grayish insoluble decomposition product formed, presumably via competing demethylation, oxidation, and complexation processes. Nonetheless, **34** could be treated with sodium iodide and imidazole or morpholine, and the analogues **35a** and **35b** were eventually obtained in pure form.

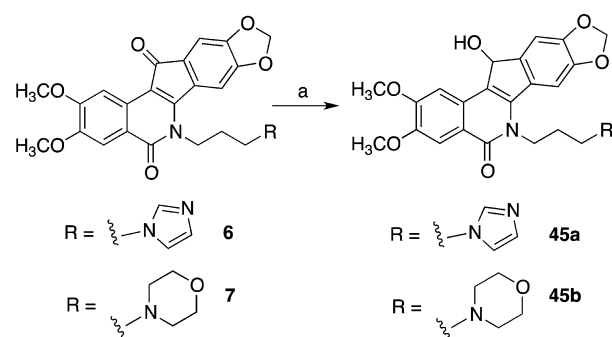
A similar route was used to prepare 3-hydroxy-2-methoxyindenoisoquinolines. Homovanillic acid (**36**, Scheme 5) was hydroxymethylated to yield compound **37**.⁵⁴ Benzoylation yielded **38**, and a saponification/oxidation sequence was employed to prepare the homophthalic acid **39**, which was then converted into the anhydride **40**. Condensation with **32**

Scheme 5^a

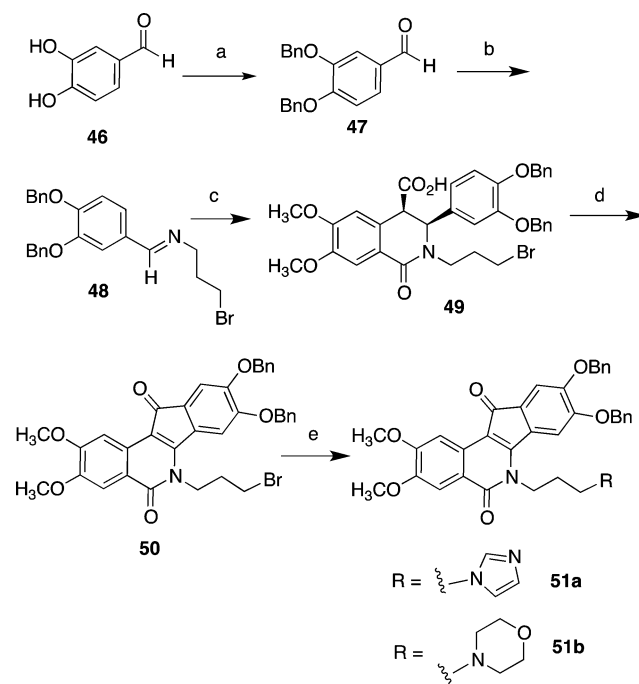
^aReagents and conditions: (a) H_2CO , H_2O , HCl , AcOH , 120°C to rt; (b) BnBr , K_2CO_3 , acetone, 56°C ; (c) (i) KOH , H_2O , rt, (ii) KMnO_4 , H_2O , 0°C to rt, (iii) EtOH , reflux; (d) AcCl , reflux; (e) 3-bromopropylamine HBr , Et_3N , Na_2SO_4 , CHCl_3 , 0°C to rt; (f) CHCl_3 , 0°C to rt; (g) SOCl_2 , rt; (h) AlCl_3 (2 equiv), nitrobenzene, 90°C ; (i) imidazole or morpholine, NaI , DMF , 70°C .

afforded the cis acid **41**. Treatment of **41** with SOCl_2 produced the benzyl intermediate **42**, but deprotection (here performed with AlCl_3 in nitrobenzene) was also low yielding ($\sim 10\%$), as observed for the regioisomer (vide supra). The resulting phenol **43** was elaborated as earlier described to yield the analogues **44a** and **44b**.

11-Hydroxy (keto-reduced) indenoisoquinoline analogues **45a** and **45b** were prepared via sodium borohydride reduction of **6** and **7**, respectively (Scheme 6). Finally, the catechols **52a** and **52b** (Scheme 7) were also prepared using the benzyl protecting group strategy. 3,4-Dihydroxybenzaldehyde (**46**) was dibenzylated to yield compound **47** and converted into the Schiff base **48**. Condensation with anhydride **32** afforded the cis acid **49**, which was cyclized in cold SOCl_2 to furnish the intermediate indenoisoquinoline **50**. Treatment with imidazole

Scheme 6^a

^aReagents and conditions: (a) NaBH_4 , MeOH , 0°C .

Scheme 7^a

^aReagents and conditions: (a) BnBr , DMF , rt to 70°C ; (b) 3-bromopropylamine HBr , Et_3N , Na_2SO_4 , rt; (c) **11**, CHCl_3 , 0°C to rt; (d) SOCl_2 , -4°C to rt; (e) imidazole or morpholine, NaI , dioxane, 65°C ; (f) 48% $\text{HBr}-\text{H}_2\text{O}$, 70°C (for **51a**); (g) H_2 , Pd/C , $\text{MeOH}-\text{THF}$ (for **51b**).

or morpholine as described earlier yielded the protected compounds **51a** and **51b**, respectively. Heating with aqueous hydrobromic acid affected the debenzoylation of **51a** (yielding the catechol **52a**), and atmospheric pressure hydrogenation of **51b** afforded the analogous compound **52b**.

METABOLISM STUDIES

Compounds **6** and **7** were incubated at 37 °C with pooled human liver microsomes in the presence of NADPH (full details in Experimental Section). After chilling and termination of the reactions, the samples were centrifuged, and the supernatants were analyzed by LC–MS and LC–tandem electrospray mass spectrometry (LC–MS/MS). Indenoisoquinolines **14a,b**, **22a,b**, **35a,b**, **44a,b**, **45a,b**, and **52a,b** were used as synthetic standards.

In the case of both **6** and **7**, positive-ion electrospray LC–MS detected two major metabolites in their protonated forms. The formation of these species required both liver microsomes and NADPH, since omission of either produced no detectable metabolites. In the case of **6**, the most abundant metabolite M1 (Figure 3) was detected at a retention time of 12.8 min.

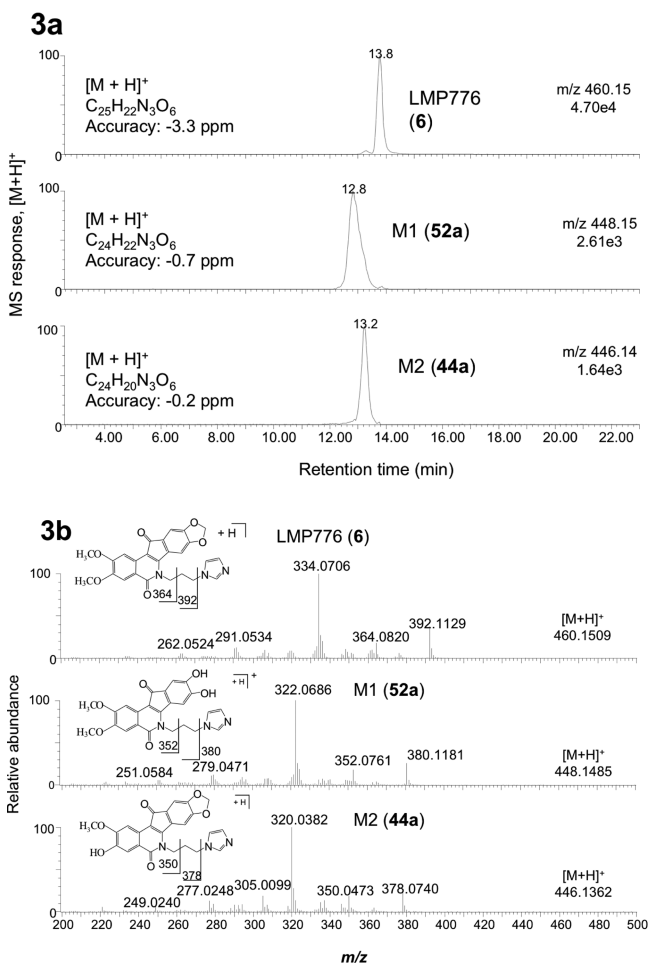


Figure 3. LC–MS retention times (a) and positive ion electrospray ion tandem mass spectra fragmentation patterns (b) for metabolites obtained upon incubation of **6** with human liver microsomes.

Accurate mass measurement provided an m/z of 448.1508, which was within -0.2 ppm of the theoretical formula of C₂₄H₂₂N₃O₆. This formula was consistent with the loss of a methylene group, and LC–MS/MS comparison with the synthetic standards confirmed this metabolite as catechol **52a**. A second metabolite, M2, eluted at 13.2 min and produced an m/z of 446.1361, which corresponded to the elemental composition C₂₄H₂₀N₃O₆ (2.0 ppm). This formula suggested

the loss of a methyl group, and comparison with the standards identified this metabolite as the 3-desmethyl compound **44a**.

An identical analysis performed for compound **7** (Figure 4) yielded similar results; the most abundant metabolite M1 [12.3

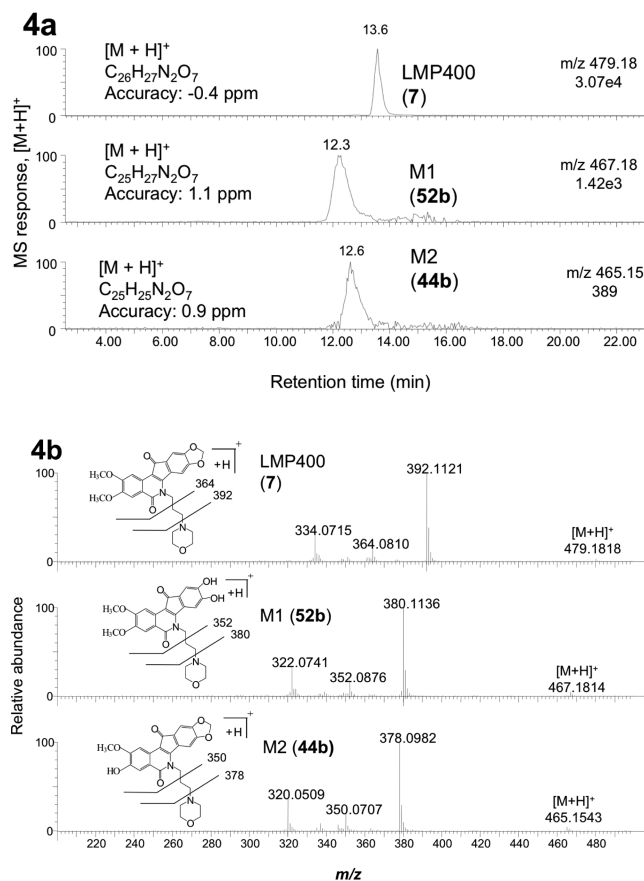


Figure 4. LC–MS retention times (a) and positive ion electrospray ion tandem mass spectra fragmentation patterns (b) for metabolites obtained upon incubation of **7** with human liver microsomes.

min, m/z 467.1823, C₂₅H₂₇N₂O₇ (1.1 ppm)] matched a synthetic standard of catechol **52b**. Likewise, the structure of metabolite M2 [13.6 min, m/z 465.1654, C₂₅H₂₅N₂O₇ (-1.7 ppm)] was confirmed as 3-desmethyl-LMP400 (**44b**) by comparison of the retention time and fragmentation pattern of the metabolite with those of the synthetic standard.

In both cases, these were the only metabolites detected, and no matches were found with the other standards (**14a,b**, **22a,b**, and the regiomeric 2-desmethyl compounds **35a,b** or **45a,b**), indicating that the predominant routes of human hepatic metabolism for **6** and **7** are likely demethylation and 3-O-demethylation, as predicted by both the apparent metabolic lability of these sites (Figure 2) and extensive literature precedent. Catechol methylation, 2-O-demethylation, and ketone reduction do not occur.

BIOLOGICAL EVALUATION OF HYDROXYINDENOISOQUINOLINES

The indenoisoquinolines **14a,b**, **22a,b**, **35a,b**, **44a,b**, **45a,b**, and **52a,b** were tested for antiproliferative activity in the National Cancer Institute's developmental therapeutics assay (the "NCI-60") against cell lines derived from a variety of human tumors (approximately 60 lines were used).^{55,56} After an initial one-

Table 1. Antiproliferative Potencies and Topoisomerase I Inhibitory Activities of Hydroxyindenoisoquinolines

compd	cytotoxicity (GI ₅₀ in μM) ^a										Top 1 cleavage ^c
	lung HOP-62	colon HCT-116	CNS SF-539	melanoma UACC-62	Ovarian OVCAR-3	renal SN12C	prostate DU-145	breast MCF-7	MGM ^b		
1 ⁵⁷	0.01	0.03	0.01	0.01	0.22	0.02	0.01	0.01	0.040 ± 0.0187 ^d	++++	
4 ⁵⁷	1.3	35	41	4.2	73	68	37	1.58	20.0 ± 14	++	
5 ⁴⁶	0.02	0.10	0.04	0.03	0.5	<0.01	<0.01	<0.01	0.21 ± 0.19	++++	
6 ⁴⁶	<0.01	<0.01	0.04	<0.01	0.08	<0.01	<0.01	0.01	0.079 ± 0.023	+++++	
7 ⁴⁶	1.78	1.15	0.04	0.03	74.1	0.813	0.155	0.37	4.64 ± 1.25	+++++	
14a	>100	>100		0.331	>100		>100	0.05	41.8 ± 7.15	++(+)	
14b	0.180	2.34	0.282	0.07	8.32	0.191	0.204	0.03	3.07 ± 0.32	+++	
22a	<0.01	<0.01	<0.01	<0.01	0.02	<0.01	<0.01	<0.01	0.055 ± 0.003	++++(+)	
22b	0.02	0.03	0.02	0.02	0.03	0.02	0.02	0.02	0.087 ± 0.063	+++++	
35a	<0.01	0.02	<0.01	0.03	0.07	<0.01	<0.01		0.049	+++++	
35b	0.257	0.279	0.335	0.282	0.871	0.195	0.257	0.144	0.412 ± 0.005	++(+)	
44a	<0.01	<0.01	<0.01	<0.01	0.03	<0.01	<0.01	<0.01	0.043	++++	
44b	0.01	0.02	<0.01	<0.01	0.03	<0.01	<0.01	<0.01	0.056	+++(+)	
45a	0.549	1.35	0.479	0.372	1.95	0.501	0.407	0.427	1.66	++	
45b	2.09	2.40	2.40	0.776	4.79	3.39	2.63	0.646	3.16	++	
52a	0.04	0.229	0.01	<0.01	0.407	0.03	0.100	0.02	0.224	++	
52b	0.371	0.407	0.148	0.078	0.501	0.065	0.490	0.050	0.602	++++	

^aThe cytotoxicity GI₅₀ values are the concentrations corresponding to 50% growth inhibition. ^bMean-graph midpoint for growth inhibition of all human cancer cell lines successfully tested, ranging from 10⁻⁸ to 10⁻⁴ M. ^cCompound-induced DNA cleavage due to Top1 inhibition is graded by the following rubric relative to 1 μM camptothecin: 0, no inhibitory activity; +, between 20% and 50% activity; ++, between 50% and 75% activity; +++, between 75% and 95% activity; +++++, equipotent; ++++++, more potent. ^dFor MGM GI₅₀ values in which a standard error appears, the GI₅₀ values for individual cell lines are the average of two determinations; values without standard error are from one determination. The values for 1, 4, 5, 6, and 7 are from many determinations.

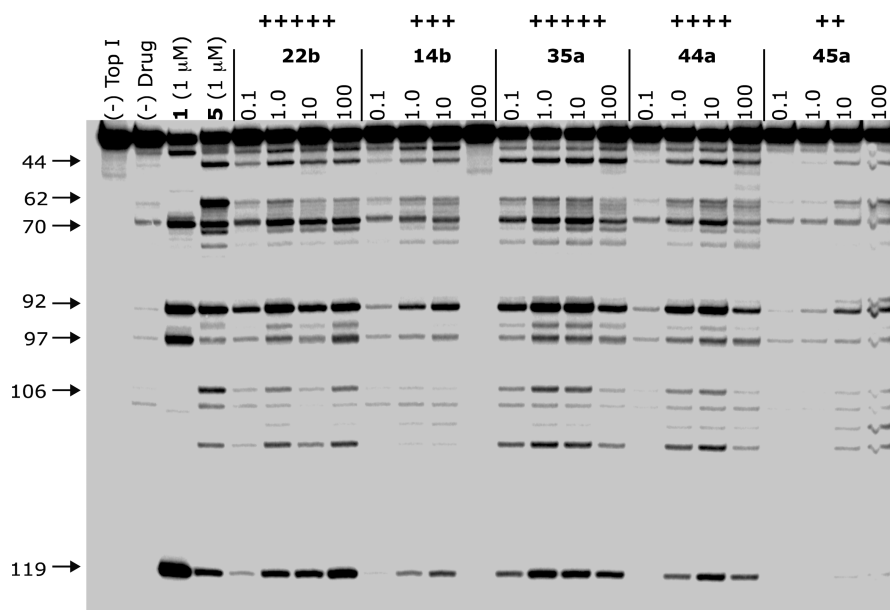


Figure 5. Top1-mediated DNA cleavage induced by indenoisoquinolines 22b, 14b, 35a, 44a, and 45a: lane 1, DNA alone; lane 2, Top1 + DNA; lane 3, 1, 1 μM; lane 4, 5, 1 μM; lanes 5–24, 22b, 14b, 35a, 44a, and 45a at 0.1, 1, 10, and 100 μM, respectively, from left to right. Numbers and arrows on the left indicate arbitrary cleavage site positions.

dose prescreening assay at moderately high concentration (10⁻⁵ M), selected compounds were tested at five concentrations ranging from 10⁻⁸ to 10⁻⁴ M. Overall antiproliferative potential is quantified as a mean-graph midpoint (MGM). This value can be interpreted as a rough average GI₅₀ across the whole assay, where values that fall outside the tested concentration range (<10⁻⁸ or >10⁻⁴ M) are respectively assigned either 10⁻⁸ or 10⁻⁴ M. These MGM values, along with GI₅₀ values from

selected cell lines, are reported in Table 1. For completeness, the MGM values for 1, 4, 5, 6, and 7 are also given.

Top1 inhibition was graded by the ability of a compound to induce enzyme-linked DNA breakage and is reported on a semiquantitative scale relative to 1 μM camptothecin: 0, no inhibitory activity; +, between 20% and 50% activity; ++, between 50% and 75% activity; +++, between 75% and 95% activity; +++++, equipotent; ++++++, more potent. Ambiguous scores (e.g., between two values) are designated with

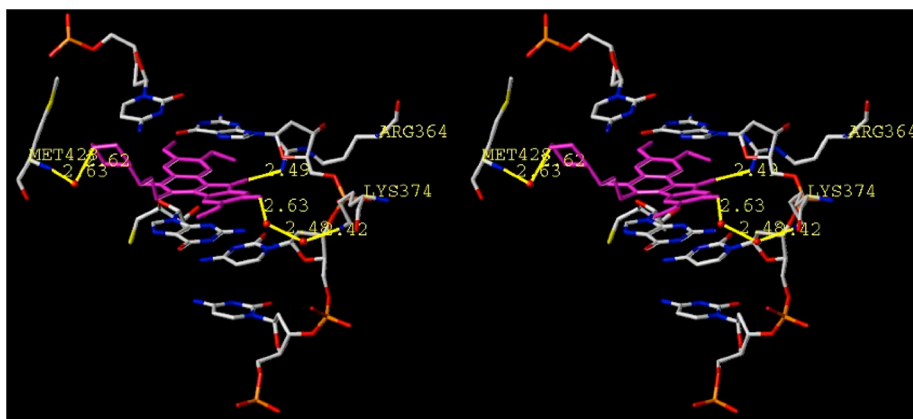


Figure 6. Minimized, top-ranked GOLD pose of compound **22b** in ternary complex with DNA and Top1, constructed in SYBYL. The ligand is colored in purple. Surrounding structures are colored by atom. Water molecules are depicted as red spheres, and hydrogens have been omitted. All distances are measured from heavy atom to heavy atom. The diagram is programmed for wall-eyed (relaxed) viewing.

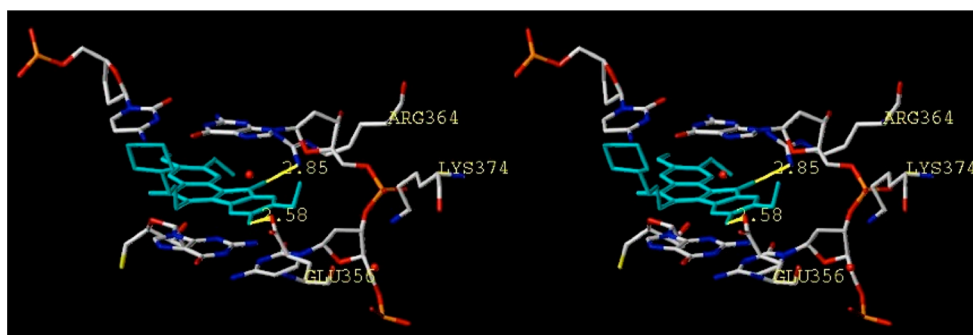


Figure 7. Minimized, top-ranked GOLD pose of compound **14b** in ternary complex with DNA and Top1 (constructed in SYBYL). The ligand is colored in cyan, surrounding structures are colored by element, and hydrogens have been omitted. All distances are measured from heavy atom to heavy atom. The same water molecules from Figure 4 are shown as red spheres.

parentheses (e.g., ++(+)) would be between ++ and +++). These values are also reported in Table 1.

A representative example of Top1-linked DNA cleavage by hydroxyindenoisoquinolines is shown in Figure 5. As observed in the figure and listed in Table 1, all compounds tested exhibited at least some Top1 inhibitory activity, with compounds **22a**, **22b**, and **35a** possessing excellent inhibitory activity comparable to or greater than the clinical candidates **6** and **7**. These compounds also have submicromolar antiproliferative potency (MGM values of 55 nM for **22a**, 87 nM for **22b**, and 49 nM for **35a**), comparable to that of **6** (79 nM), and although they were weaker Top1 poisons, the confirmed metabolites **44a** and **44b** had MGM values of 43 and 56 nM, respectively, which could serve to prolong the antitumor effects of **6** and **7** in vivo. A standard COMPARE analysis^{17,18} seeded with the GI₅₀ data for **22a**, **22b**, and **35a** displayed moderate to high correlations (Pearson coefficients of 0.65–0.8) with the cytotoxicity profile of topotecan (**2**), indicating that it is likely these compounds exert their cytotoxic or antiproliferative effects predominantly via Top1 inhibition. Generally, anti-Top1 activity and antiproliferative activity correlate well, with those compounds that are better Top1 inhibitors displaying higher potency in the NCI-60 screen.

Interestingly, the D-ring hydroxyindenoisoquinolines display a striking correlation between regiochemistry, Top1 inhibition, and cytotoxicity. While the 9-hydroxy-8-methoxyindenoisoquinolines **22a** and **22b** are extremely potent, the regiomer 8-hydroxy-9-methoxyindenoisoquinolines **14a** and **14b** possess

approximately half the anti-Top1 activity and their MGM values are orders of magnitude greater than their isomeric counterparts. Similar regiochemical effects are reported for other classes of Top1 inhibitors (indenoisoquinolines, camptothecins, and aramathecins, among others).

What could be responsible for such a drastic effect? A molecular modeling and docking study was performed in an attempt to rationalize this disparity. By use of GOLD, the minimized structures of morpholine **22b** and its regioisomer **14b** were docked into the cleavage site of a mutant, solvated Top1/topotecan crystal structure (modified from PDB code 1K4T) using the procedure previously described by Peterson et al.⁴⁶ The minimized, highest-ranked GOLD pose for compound **22b** is shown in Figure 6.

A major commonality among Top1 poisons is that a large amount of steric bulk (e.g., multiple methoxy groups, halogens) projecting toward the non-scissile strand often has deleterious effects on potency in general.^{39,40,57} Indeed, crystal structures of several Top1 inhibitors⁵⁸ reveal this region to be very “tight” sterically, in agreement with the observation of a low functional group tolerance. However, the 9-hydroxyl group of **22b** appears to be quite well accommodated here. The indenoisoquinoline is calculated to bind in an intercalative mode identical to that observed in the crystal structure of a related Top1–DNA–indenoisoquinoline ternary complex,⁵⁸ and the hydroxyl group is involved in water-mediated hydrogen bonding with Lys374. It is possible that with induced fit, this hydroxyl could also make contact with the nearby deoxyfuranose oxygen (here, 4.0

Å away), while the 8-methoxy group projects toward the major groove of the complex.

The highest ranked, energy minimized GOLD pose for compound **14b** is shown in Figure 7. The non-scissile side appears to be significantly more crowded, as the 9-methoxy group now projects back toward the phosphodiester backbone, and the network of water-mediated contacts is absent. The 8-hydroxy group projects toward the solvent, where it hydrogen-bonds with a nearby glutamate residue (Glu356). It is conceivable that this contact could stabilize the binding of **14b**, as its activity is not abolished completely relative to **22b**, and Glu356 is proposed to aid the binding of indolocarbazoles and compound **2**.⁵⁸ Nonetheless, there is a sizable difference in the GoldScores (67.9 for **14b** vs 81.6 for **22b**), and the average difference in GoldScores between the top four poses for **22b** and **14b** is approximately 16 points. As predicted by the model, the difference in the hydrogen bonding term of the GoldScore between the regioisomers is negligible (average of 10.3 for the top four poses of **14b**; 12.1 for the top four of **22b**), but the difference in the external van der Waals terms (a component of the fitness score that reflects a simple attractive/repulsive potential)^{59,60} is significant, 56 (average 53.3) for **14b** versus 65 (average 62.6) for **22b**, likely symbolizing a “less favorable” or “more repulsive” fit for the 9-methoxy isomer. Interestingly, some 9-methoxyindenoisoquinolines are quite active against Top1, although most of these compounds also contain a 3-nitro group (which is well-documented to improve potency),^{23,57} indicating that favorable A-ring electronics may somehow be enough to offset a negative steric effect.

Additionally, perhaps the placement of 9-hydroxyl group is important as well. In a similar study of camptothecins performed by Wall et al.,⁴⁰ most substitutions at position 11 of the camptothecin system attenuate the Top1 inhibitory activity (Morrell and others hypothesize that this position is roughly analogous to the indenoisoquinoline position 9^{57,61}). One notable exception to this SAR is the active 11-hydroxycamptothecin (**53**, Figure 8), which would point its

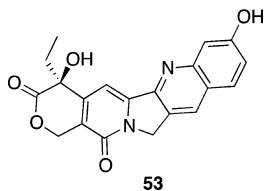


Figure 8. 11-Hydroxycamptothecin.

phenolic hydroxyl toward the non-scissile strand as well. Further support comes from a study of the regioisomers of hydroxylated indolocarbazoles performed by Zembower et al.⁶² Those compounds possessing a hydroxyl at position 9 (also roughly analogous to the numerically identical position of indenoisoquinolines) are the most potent!⁶²

Interestingly, the regiochemical dependence is not observed on the A-ring side, and activity here is variable. The 2-hydroxyindenoisoquinoline **35a** and 3-hydroxyindenoisoquinoline **44b** both possess significant anti-Top1 activity, although some differences between the morpholines and imidazoles are observed. Assuming these compounds bind in ternary complex as **22b** and **14b** are proposed to, the indenoisoquinoline A-ring fits into the scissile-strand cavity. The steric tolerance of this spacious region is much higher, and it may render regiochemical differences minimal to irrelevant. Docking

supports this hypothesis as well: there is no difference (~0.03 points) between the GoldScores for the top-ranked poses of **35b** and **44b** (and the average scores for the top four poses are only ~3 points apart), and the van der Waals and hydrogen-bond contributions are approximately equal in both cases, indicating that the difference in Top1 activity between these compounds (lower than that observed between series **22** and series **14**) may not be due to placement of the hydroxyl group. The literature supports this assumption as well: the benzonaphthridine Top1 poison Genz-644282 is very similar in structure to **6** and **7**, and its A-ring desmethyl metabolites do not display any correlation between regiochemistry and Top1 inhibition.³²

The lowest activity observed was from the 11-hydroxy (reduced keto) analogues **45a** and **45b**. Although some potent 11-hydroxy and 11-alkoxyindenoisoquinolines have been reported, the molecular modeling studies indicated that they bind differently in the ternary cleavage complex, with an *N*-(*p*-methoxybenzyl) group instead of the indenoisoquinoline ring system intercalating between the base pairs.^{43,44} The fact that the indenoisoquinoline oracin is deactivated by ketone reduction is also consistent with the low potency of **45a** and **45b**.³⁷ Overall, the evidence indicates that the ketone is important for optimal binding of the indenoisoquinoline in the DNA intercalative mode. The crystal structure of an indenoisoquinoline–DNA–Top1 ternary complex (PDB code 1SC7)⁵⁸ has demonstrated that the ketone hydrogen bonds to the minor groove residue Arg364 (proposed to aid in the stabilization of other bound inhibitors). The hydroxyl groups of **45a** and **45b** still can behave as hydrogen bond acceptors in our models (not shown), so lack of hydrogen-bond-accepting capability per se cannot be responsible for the decrease in activity. Perhaps the reduction of the ketone changes the electronics of the system, interfering with π – π stacking, or the introduction of a tetrahedral carbon introduces nonplanarity that could disrupt intercalation.

The catechols **52a** and **52b** are both also active against Top1, although the imidazole **52a** has only half the potency of the morpholine **52b**. Significant differences between imidazole and morpholine substituents were observed for other pairs in this study (cf. **35a** and **35b**), although in the case of **35b** vs **35a** the substituent effects on potency are opposite those observed with **52a** vs **52b**.

CONCLUSIONS

A series of 8-, 9-, 2-, and 3-hydroxyindenoisoquinolines, 8,9-dihydroxyindenoisoquinolines, and reduced keto analogues of the clinical candidates **6** and **7** were designed based on the rationale that (a) they are potential metabolites of indotecan and indimitecan and could be useful as synthetic standards to compare with the actual metabolites and (b) there are many examples of potent hydroxylated/phenolic Top1 poisons in the literature, so the hydroxyindenoisoquinolines might be of value as anticancer drugs. To this end, the indenoisoquinolines were prepared by the homophthalic anhydride/Schiff base condensation method⁴⁹ and were assayed against human Top1 and a panel of human cancer cells. Interestingly, many of these hydroxylated indenoisoquinolines are extremely potent Top1 poisons, especially those bearing the 9-hydroxy-8-methoxy substitution pattern on the D ring. Molecular modeling supports the observed activity, especially when compared to the much less active 8-hydroxy-9-methoxyindenoisoquinolines. A less significant dependence of Top1 inhibitory potency on

regiochemistry was observed for A-ring hydroxyindenoisoquinolines, a fact that is in agreement with our docking study. Additionally, a metabolism study indicates that several of the prepared hydroxyindenoisoquinolines are produced upon incubation of the parent drugs with human liver microsomes and NADPH. These data indicate that the primary routes of metabolism are 3-demethylation and cleavage of the 8,9-methylenedioxy group. Three additional points can be made about the potential future significance of this study: (1) the fact that the metabolites are biologically active can be expected to prolong the duration of antitumor activity expressed after drug administration; (2) the biologically active hydroxylated indenoisoquinolines provide convenient starting points for further drug development, especially with regard to prodrugs, since the phenolic hydroxyl groups provide convenient points for attachment of prodrug modules; (3) the altered solubilities of the hydroxylated derivatives may offer advantages for iv formulation.

EXPERIMENTAL SECTION

General Procedures. Reagents and solvents were purchased from commercial vendors and were used without further purification. Melting points were determined in capillary tubes using a Mel-Temp apparatus and are not corrected. Infrared spectra were obtained as films on salt plates unless otherwise specified, using a Perkin-Elmer Spectrum One FT-IR spectrometer, and are baseline-corrected. ^1H NMR spectra were obtained at 300 or 500 MHz, using a Bruker ARX300 and Bruker Avance 500 (QNP probe or TXI 5 mm/BBO probe). ^{13}C NMR spectra were obtained at 125 or 75 MHz. Mass spectral analyses were performed at the Purdue University Campus-Wide Mass Spectrometry Center. ESIMS was performed using a FinniganMAT LCQ Classic mass spectrometer system. The electrospray ionization high resolution mass measurements were obtained in the peak matching mode using a FinniganMAT XL95 (FinniganMAT Corp., Bremen, Germany) mass spectrometer. The instrument was calibrated to a resolution of 10 000 with a 10% valley between peaks using the appropriate polypropylene glycol standards. EI/CIMS was performed using a Hewlett-Packard Engine or GCQ FinniganMAT mass spectrometer system. APCI-MS was performed using an Agilent 6320 trap mass spectrometer. Combustion microanalyses were performed by Midwest Microlab LLC (Indianapolis, IN). Reported values are within 0.4% of calculated values. HPLC was performed using a Waters 1525 binary HPLC pump with a Waters 2487 dual absorbance detector and an injection volume of 10 μL . A Sunrise C18 5 μm 100 \AA reverse-phase column, with dimensions of 15 cm \times 4.6 mm (ES Industries), was used for all HPLC experiments. The intensity of the major peak in the analytical HPLC trace of each target compound was $\geq 95\%$ that of the combined intensities of all of the peaks detected at 254 nm, and two different solvent systems were used to ascertain purity. Analytical thin-layer chromatography was performed on Baker-flex silica gel IB2-F plastic-backed TLC plates. Compounds were visualized with both short and long wavelength UV light. Silica gel flash chromatography was performed using 40–63 μm flash silica gel.

Benzylisovanillin (9).⁶³ Isovanillin (8, 3.00 g, 19.7 mmol) was diluted with anhydrous DMF (50 mL). Benzyl bromide (3.47 g, 20.3 mmol) was added slowly, followed by K_2CO_3 (6.54 g, 47.3 mmol). The yellow mixture was rapidly stirred at room temperature for 2 h and 15 min. The mixture was partitioned into an ether–water mixture (1:1, 100 mL of each) and stirred. The organic and aqueous layers were separated, and the aqueous layer was extracted with ether (1 \times 50 mL, 2 \times 25 mL). The combined organic layers were washed with H_2O (2 \times 50 mL) and saturated aqueous NaCl (50 mL), dried over anhydrous sodium sulfate, and concentrated to yield a white microcrystalline solid (4.43 g, 93%) after washing with hexanes (50 mL) and refiltering the filtrate: mp 55–58 $^\circ\text{C}$ (lit.⁶³ mp 61–62 $^\circ\text{C}$).

^1H NMR (CDCl_3) δ 9.82 (s, 1 H), 7.49–7.32 (m, 6 H), 7.02 (d, J = 8.2 Hz, 1 H), 5.20 (s, 2 H), 3.97 (s, 3 H).

N-[3'-(Benzyloxy)-4'-methoxybenzylidene]-3-bromopropan-1-amine (10). 3-Bromopropylamine hydrobromide (2.08 g, 9.49 mmol) was diluted with CHCl_3 (10 mL). Compound 9 (2.00 g, 8.26 mmol) was added slowly as a solution in CHCl_3 (10 mL) and quantitatively transferred with 2 mL of the same solvent. Et_3N (0.917 g, 9.08 mmol) was added slowly, upon which the solution became clear and colorless. Na_2SO_4 (1.00 g, 5.21 mmol) was added, and the mixture was stirred at room temperature for 24 h. Additional 3-bromopropylamine salt (0.905 g, 4.13 mmol), Et_3N (0.500 g, 4.96 mmol), and approximately 0.500 g of Na_2SO_4 were added. After a total of 48 h, the mixture was washed with H_2O (3 \times 70 mL) and saturated aqueous NaCl (70 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated to yield a dark yellow syrup (2.86 g, 96%). IR (film) 3291, 2932, 2837, 1645, 1601, 1511, 1431, 1265, 1137, 1024, 741, 697 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.21 (s, 1 H), 7.49–7.31 (m, 6 H), 7.21 (dd, J = 2.1, 8.3 Hz, 1 H), 6.92 (d, J = 8.3 Hz, 1 H), 5.19 (s, 2 H), 3.93 (s, 3 H), 3.92 (s, 3 H), 3.73 (td, J = 1.0, 6.2 Hz, 2 H), 3.51 (t, J = 6.5 Hz, 2 H), 2.30–2.20 (m, 2 H); ESIMS m/z (rel intensity) 362/364 (MH^+ , 97/100).

cis-[3-(Bromopropylamino)]-4-carboxy-3,4-dihydro-6,7-dimethoxy-3-(3-benzyloxy-4-methoxyphenyl)-1(2H)-isoquinoline (12). Anhydride 11 (1.40 g, 6.30 mmol) was dissolved in CHCl_3 (35 mL), and the resulting solution was cooled to 0 $^\circ\text{C}$. A solution of compound 10 (2.40 g, 6.63 mmol) in cold CHCl_3 (15 mL) was added slowly over 5 min and quantitatively transferred with CHCl_3 (5 mL). Precipitate began forming after 30 min. The mixture was stirred for 2 h at 0 $^\circ\text{C}$ and then warmed to room temperature and stirred for an additional 2 h. The precipitate was filtered out and washed with CHCl_3 (50 mL) to afford the title compound as a white solid (2.81 g, 77%) after drying: mp 193–195 $^\circ\text{C}$ (dec). IR (film) 2933, 1733, 1619, 1597, 1573, 1515, 1169, 1027, 696 cm^{-1} ; ^1H NMR (CDCl_3 - $\text{DMSO}-d_6$) δ 7.53 (s, 1 H), 7.19–7.13 (m, 5 H), 7.05 (s, 1 H), 6.53 (s, 3 H), 4.83 (d, J = 6.4 Hz, 1 H), 4.79 (s, 2 H), 4.44 (d, J = 6.2 Hz, 1 H), 3.81 (s, 3 H), 3.81–3.70 (m, 1 H), 3.73 (s, 3 H), 3.64 (s, 3 H), 3.30–3.20 (m, 2 H), 2.95–2.90 (m, 1 H), 2.01–1.80 (m, 2 H); APCI-MS m/z (rel intensity) 540 ($\text{MH}^+ - \text{CO}_2$, 100). Anal. Calcd for $\text{C}_{29}\text{H}_{30}\text{BrNO}_7 \cdot \text{H}_2\text{O}$: C, 57.81; H, 5.35; N, 2.32. Found: C, 57.57; H, 4.99; N, 2.32.

6-(3-Bromopropyl)-5,6-dihydro-8-hydroxy-2,3,9-trimethoxy-5,11-dioxo-11H-indeno[1,2-c]isoquinoline (13). The cis acid 12 (1.00 g, 1.71 mmol) was diluted with SOCl_2 (25 mL). The dark reddish-brown mixture was stirred at room temperature for 2 h and 20 min and was concentrated. The residue was dissolved in 1,2-dichloroethane (40 mL), and aluminum chloride (0.479 g, 3.6 mmol) was added. The mixture was stirred at room temperature for 20 h and was quenched slowly by the addition of saturated aqueous NaHCO_3 (40 mL) and H_2O (50 mL). The aqueous and organic layers were separated, and the aqueous phase was extracted with CHCl_3 (2 \times 50 mL). The organic phase was washed with H_2O (2 \times 100 mL) and saturated aqueous NaCl (100 mL), dried over anhydrous sodium sulfate, and concentrated. The obtained residue was adsorbed onto SiO_2 (~ 7 g) and purified by flash column chromatography (SiO_2 , ~ 50 g), eluting with a gradient of 0.25% MeOH in CHCl_3 to 1% MeOH in CHCl_3 to afford the crude product as a pinkish solid (0.414 g, 51%) after washing with 10% CHCl_3 in ether (2 \times 50 mL) and ether (50 mL) and drying: mp 248–252 $^\circ\text{C}$. IR (film) 3296, 1635, 1554, 1471, 1396, 1396, 1030, 871, 762, 761 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.04 (s, 1 H), 7.64 (s, 1 H), 7.31 (s, 1 H), 7.16 (s, 1 H), 7.95 (s, 1 H), 4.62 (t, J = 7.5 Hz, 2 H), 4.06 (s, 3 H), 3.99 (s, 6 H), 3.65 (t, J = 6.5 Hz, 1 H), 2.50–2.40 (m, 2 H); EIMS m/z (rel intensity) 475 (M^+ , 100).

5,6-Dihydro-8-hydroxy-6-[3-(1H-imidazol-1-yl)propyl]-2,3,9-trimethoxy-5,11-dioxo-11H-indeno[1,2-c]isoquinoline (14a). Compound 13 (0.300 g, 0.632 mmol), imidazole (0.517 g, 7.59 mmol), and NaI (1.14 g, 0.759 mmol) were diluted with anhydrous DMF (90 mL), and the mixture was heated to 60 $^\circ\text{C}$ for 18 h. The mixture was cooled and poured into H_2O (200 mL), and the solution was extracted with CHCl_3 (2 \times 100, 1 \times 50 mL). The burgundy-colored organic layer was washed with H_2O (5 \times 200 mL) and

saturated aqueous NH_4Cl (200 mL). The organic phase was dried over anhydrous sodium sulfate, concentrated, and the residue was washed with ether (100 mL) and filtered. The precipitate was dissolved in CHCl_3 (50 mL), and the solution was concentrated and adsorbed onto SiO_2 (~7 g) and purified by flash column chromatography (SiO_2 , 50 g), eluting with a gradient of 1% MeOH in CHCl_3 to 4% MeOH in CHCl_3 , to yield a fuchsia-colored solid (0.112 g, 38%) after suspending in DMF (10 mL) and precipitating with ether (150 mL) and washing with ether (100 mL) and drying: mp 265–266 °C (dec). IR (film) 3306, 2917, 1659, 1622, 1554, 1472, 1394, 1335, 1265, 1085, 1032, 762, 688 cm^{-1} ; ^1H NMR ($\text{DMSO}-d_6$) δ 10.10 (br s, 1 H), 7.84 (s, 1 H), 7.73 (s, 1 H), 7.44 (s, 1 H), 7.27 (s, 1 H), 7.04 (s, 1 H), 6.99 (s, 1 H), 6.92 (s, 1 H), 4.37 (t, $J = 7.2$ Hz, 2 H), 4.20 (t, $J = 7.0$ Hz, 2 H), 3.88 (s, 3 H), 3.85 (s, 3 H), 3.84 (s, 3 H), 2.25–2.20 (m, 2 H); ESIMS m/z (rel intensity) 462 (MH^+ , 100). Purity was estimated to be ~100% by HPLC in both 90% MeOH– H_2O and 85% MeOH– H_2O . Anal. Calcd for $\text{C}_{25}\text{H}_{23}\text{N}_3\text{O}_6\cdot\text{H}_2\text{O}$: C, 62.62; H, 5.26; N, 8.76. Found: C, 62.40; H, 4.85; N, 8.57.

5,6-Dihydro-8-hydroxy-2,3,9-trimethoxy-6-[3-(*N*-morpholino)propyl]-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (14b). Compound 13 (0.090 g, 0.190 mmol) and NaI (0.200 g, 1.32 mmol) were diluted with anhydrous DMF (20 mL). Morpholine (0.115 g, 1.32 mmol) was added, and the mixture was heated to 70 °C for 18 h. The mixture was cooled and poured into H_2O (100 mL), and the solution was extracted with CHCl_3 (3 \times 50 mL). The burgundy-colored organic layer was washed with H_2O (4 \times 100 mL). The organic layer was dried over anhydrous sodium sulfate, concentrated, and the resulting residue was adsorbed onto SiO_2 (~3 g) and purified by flash column chromatography (SiO_2 , 26.0 g), eluting with a gradient of 1% MeOH in CHCl_3 to 2.5% MeOH in CHCl_3 , to yield a mauve-colored solid (0.033 g, 36%) after dissolving in CHCl_3 (5 mL) and precipitating with ether (40 mL) and drying: mp 282–285 °C. IR (film) 2917, 1687, 1635, 1553, 1495, 1472, 1395, 1326, 1255, 1211, 1020, 867, 788, 757 cm^{-1} ; ^1H NMR ($\text{DMSO}-d_6$) δ 10.06 (br s, 1 H), 7.97 (s, 1 H), 7.46 (s, 1 H), 7.18 (s, 1 H), 7.06 (s, 1 H), 4.40–4.30 (m, 2 H), 3.89 (s, 3 H), 3.86 (s, 3 H), 3.84 (s, 3 H), 3.54 (s, 4 H), 2.50–2.44 (m, 2 H, partially obscured by solvent peak), 2.36 (s, 4 H), 2.00–2.90 (m, 2 H); ESIMS m/z (rel intensity) 481 (MH^+ , 100). Purity was estimated to be 98.3% by HPLC in 95% MeOH–5% H_2O and 99% in 90% MeOH–10% H_2O . Anal. Calcd for $\text{C}_{26}\text{H}_{28}\text{N}_2\text{O}_7\cdot 0.5 \text{H}_2\text{O}$: C, 63.79; H, 5.97; N, 5.72. Found: C, 63.88; H, 5.78; N, 5.69.

Benzylvanillin (16).⁶⁴ Vanillin (15, 3.00 g, 19.7 mmol) was diluted with anhydrous DMF (50 mL). Benzyl bromide (3.47 g, 20.3 mmol) was added slowly, followed by K_2CO_3 (6.54 g, 47.3 mmol). The yellow mixture was rapidly stirred at room temperature for 2 h 20 min. The mixture was partitioned into an ether/water mixture (1:1, 100 mL of each) and stirred. The organic and aqueous layers were separated, and the aqueous layer was extracted with ether (2 \times 25 mL, 1 \times 50 mL). The combined organic layers were washed with H_2O (1 \times 50 mL, 1 \times 30 mL) and saturated aqueous NaCl (50 mL), dried over anhydrous sodium sulfate, and concentrated to yield a white microcrystalline solid (4.51 g, 95%) after washing with hexanes (50 mL) and refiltering the filtrate: mp 51–54 °C (lit.⁶³ mp 60–61 °C). ^1H NMR (CDCl_3) δ 9.84 (s, 1 H), 7.46–7.35 (m, 7 H), 7.00 (d, $J = 8.2$ Hz, 1 H), 5.25 (s, 2 H), 3.95 (s, 3 H).

***N*-[4'-(Benzylloxy)-3'-methoxybenzylidene]-3-bromopropan-1-amine (17).** 3-Bromopropylamine hydrobromide (2.08 g, 9.49 mmol) was diluted with CHCl_3 (10 mL). A solution of aldehyde 16 (2.00 g, 8.26 mmol) was added slowly as a solution in CHCl_3 (12 mL) and transferred quantitatively with the same solvent (3 mL). Et_3N (0.917 g, 9.08 mmol) was added, upon which the suspension became clear. Na_2SO_4 (anhydrous, approximately 2.00 g) was added, and the mixture was stirred at room temperature for 23 h. Additional 3-bromopropylamine hydrobromide (0.904 g, 4.13 mmol) and Et_3N (0.500 g, 4.96 mmol) were added, and the mixture was stirred for a total of 44 h. The reaction mixture was diluted to a volume of 50 mL with CHCl_3 and was washed with H_2O (3 \times 70 mL) and saturated aqueous NaCl (50 mL). The organic phase was dried over anhydrous sodium sulfate and concentrated to yield a yellow syrup (3.06 g, 100% with residual solvent). IR (film) 2935, 2840, 1645, 1600, 1585, 1511,

1455, 1419, 1267, 1231, 1138, 1033, 743, 697 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.22 (s, 1 H), 7.45–7.31 (m, 6 H), 7.10 (dd, $J = 8.2, 1.8$ Hz, 1 H), 6.90 (d, $J = 8.2$ Hz, 1 H), 5.21 (s, 2 H), 3.95 (s, 3 H), 3.74 (t, $J = 6.2$ Hz, 2 H), 3.51 (t, $J = 6.3$ Hz, 2 H), 2.30–2.20 (m, 2 H); ESIMS m/z (rel intensity) 362/364 (MH^+ , 100/95).

***cis*-3-(4-Benzylloxy-3-methoxyphenyl)-*N*-(3-bromopropyl)-4-carboxy-3,4-dihydro-6,7-dimethoxy-1(2*H*)-isoquinoline (18).** Compound 11 (1.93 g, 8.71 mmol) was diluted with CHCl_3 (40 mL), and the mixture was cooled to 10 °C. The Schiff base 17 (3.00 g, 8.28 mmol) was diluted in CHCl_3 (20 mL, cooled to 10 °C), and this solution was added slowly to the anhydride solution and transferred with 5 mL of the same solvent. The mixture was stirred at 10 °C for 30 min and was then allowed to slowly warm to room temperature. After 3 h and 20 min, a precipitate had formed. This precipitate was collected and washed with CHCl_3 (30 mL) to yield a white solid (2.86 g, 59%) after drying: mp 172–175 °C. IR (film) 2933, 1743, 1621, 1595, 1575, 1464, 1289, 1259, 1174, 1103, 1028, 908, 730, 697 cm^{-1} ; ^1H NMR (CDCl_3) δ 7.72 (s, 1 H), 7.38–7.29 (m, 5 H), 7.09 (s, 1 H), 6.71 (d, $J = 8.9$ Hz, 1 H), 6.60–6.57 (m, 2 H), 5.04 (s, 2 H), 5.00 (d, $J = 6.2$ Hz, 1 H), 4.70 (d, $J = 6.2$ Hz, 1 H), 4.03–3.98 (m, 1 H), 3.96 (s, 3 H), 3.88 (s, 3 H), 3.63 (s, 3 H), 3.22–3.41 (m, 2 H), 3.22–3.15 (m, 2 H), 2.28–2.21 (m, 1 H), 2.18–2.08 (m, 1 H); ESIMS m/z (rel intensity) 584/585 (MH^+ , 100/94). Anal. Calcd for $\text{C}_{29}\text{H}_{30}\text{BrN}_2\text{O}_7$: C, 59.60; H, 5.17; N, 2.40. Found: C, 59.49; H, 5.13; N, 2.34.

9-Benzylloxy-6-3-(bromopropyl)-5,6-dihydro-2,3,8-trimethoxy-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (19). Compound 18 (1.00 g, 1.71 mmol) was diluted with SOCl_2 (40 mL), and the reaction mixture was stirred at room temperature, upon which it became a bright reddish-purple color. TLC indicated completion after 4.5 h. The SOCl_2 was evaporated, and the residue was dissolved in CHCl_3 (40 mL) and quenched by the slow addition of saturated aqueous NaHCO_3 (50 mL). The layers were separated, and the aqueous layer was extracted with CHCl_3 (30 mL). The organic layers were washed with H_2O (50 mL), and the aqueous layers were extracted with CHCl_3 (20 mL). The organic layers were finally washed with saturated aqueous NaCl (50 mL), and the aqueous layers were again extracted with CHCl_3 (20 mL). The combined organic layers were dried over anhydrous sodium sulfate, concentrated, and adsorbed onto SiO_2 (~3.0 g). The residue was purified by flash column chromatography (SiO_2 , 37.2 g), eluting with CHCl_3 to yield a purple solid (0.448 g, 45%) after dissolving in CHCl_3 (30 mL) and precipitating with ether (200 mL). The precipitate was filtered and washed with ether (40 mL): mp 223–226 °C (dec). The ^1H NMR indicated that the product contained some minor impurities, and no further purification or characterization was performed. This crude material was used to prepare analogues. ^1H NMR (CDCl_3) δ 8.04 (s, 1 H), 7.62 (s, 1 H), 7.47–7.19 (m, 7 H), 5.23 (s, 2 H), 4.64 (t, $J = 7.6$ Hz, 2 H), 4.05 (s, 3 H), 4.03 (s, 3 H), 3.98 (s, 3 H), 3.71 (t, $J = 6.0$ Hz, 2 H), 2.40–2.40 (m, 2 H).

9-Benzylloxy-5,6-dihydro-6-[3-(1*H*-imidazol-1-yl)propyl]-2,3,8-trimethoxy-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (21a). Compound 19 (0.200 g, 0.354 mmol), NaI (0.360 g, 2.40 mmol), and imidazole (0.163 g, 2.40 mmol) were diluted with anhydrous DMF (40 mL). The mixture was heated to 70 °C for 19 h and cooled. The mixture was poured into H_2O (100 mL) and extracted with CHCl_3 (3 \times 50 mL). The solution was washed with H_2O (1 \times 200 mL, 3 \times 300 mL), and the aqueous layer was extracted with CHCl_3 (30 mL). The combined organic layers were dried over anhydrous sodium sulfate, concentrated, and adsorbed onto SiO_2 (~4.00 g). The residue was purified by flash column chromatography (SiO_2 , ~26.0 g), eluting with a gradient of 0.7% MeOH in CHCl_3 to 2% MeOH in CHCl_3 to yield a bright purple solid (0.103 g, 53%) after washing with 1% CHCl_3 in ether (60 mL) and ether (20 mL) and drying: mp 236–240 °C. IR (film) 2918, 1690, 1546, 1554, 1495, 1303, 1260, 1212, 1025, 863, 786 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.03 (s, 1 H), 7.64 (s, 1 H), 7.62 (s, 1 H), 7.46–7.33 (m, 5 H), 7.19 (s, 1 H), 7.11 (s, 1 H), 7.06 (s, 1 H), 6.79 (s, 1 H), 5.21 (s, 2 H), 4.58 (t, $J = 6.4$ Hz, 2 H), 4.24 (t, $J = 6.7$ Hz, 2 H), 4.05 (s, 3 H), 4.00 (s, 3 H), 3.86 (s, 3 H), 2.40–2.35 (m, 2 H); ESIMS m/z (rel intensity) 552 (MH^+ , 100).

9-Benzyloxy-5,6-dihydro-2,3,8-trimethoxy-6-[3-(*N*-morpholino)propyl]-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (21b). Compound 19 (0.100 g, 0.177 mmol) and NaI (0.178 g, 1.19 mmol) were diluted with anhydrous DMF (25 mL). Morpholine (0.104 g, 1.19 mmol) was added, and the mixture was heated to 70 °C for 2 h and then stirred for 17 h at room temperature. The mixture was poured into H₂O (100 mL) and extracted with CHCl₃ (3 × 50 mL). The organic phase was washed with H₂O (4 × 200 mL), dried over anhydrous sodium sulfate, and adsorbed onto SiO₂ (~3.0 g). The residue was purified by flash column chromatography (SiO₂, ~30 g), eluting with a gradient of 0.2% MeOH in CHCl₃ to 1% MeOH in CHCl₃ to yield a purple-brown solid (0.070 g, 69%) after washing with 2% CHCl₃ in ether (50 mL) and drying: mp 252–254 °C. IR (film) 2944, 1690, 1652, 1553, 1496, 1303, 1211, 1118, 1014, 918, 870, 786, 730 cm⁻¹; ¹H NMR (CDCl₃) δ 8.04 (s, 1 H), 7.65 (s, 1 H), 7.47–7.34 (m, 5 H), 7.21 (s, 2 H), 7.11 (s, 1 H), 5.23 (s, 2 H), 4.58 (d, *J* = 7.1 Hz, 2 H), 4.05 (s, 1 H), 3.99 (s, 3 H), 3.98 (s, 3 H), 3.70 (t, *J* = 4.6 Hz, 4 H), 2.59 (t, *J* = 6.8 Hz, 2 H), 2.55 (br s, 4 H), 2.13–2.07 (m, 2 H); ESIMS *m/z* (rel intensity) 571 (MH⁺, 100). Anal. Calcd for C₃₃H₃₄N₂O₆·H₂O: C, 67.33; H, 6.16; N, 4.76. Found: C, 67.28; H, 5.81; N, 4.80.

5,6-Dihydro-9-hydroxy-6-[3-(1*H*-imidazol-1-yl)propyl]-2,3,8-trimethoxy-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (22a, Method 1). Compound 21a (0.090 g, 0.163 mmol) was diluted in H₂O (30 mL), and the mixture was sonicated to ensure a homogeneous suspension. A solution of HBr in AcOH (33%, 32 mL) was added slowly, and the mixture was heated at 65 °C for 2.5 h. The mixture was a clear, reddish-purple color. The mixture was concentrated, and H₂O (30 mL) was added to the residue. Saturated aqueous NaHCO₃ was added until the pH was neutral (an amount of approximately 6 mL was needed). The suspension was extracted with CHCl₃ (3 × 50 mL), and the organic layers were washed with H₂O (4 × 200 mL) and dried over anhydrous sodium sulfate. The solution was concentrated, adsorbed onto SiO₂ (~4.0 g), and the residue was purified by flash column chromatography (SiO₂, ~27 g), eluting with a gradient of 1.5% MeOH in CHCl₃ to 4.5% MeOH in CHCl₃ to yield the title compound as a chalky, mauve-colored solid (0.023 g, 30%) after washing with ether (30 mL) and drying: mp 254–257 °C (dec). IR (film) 2537, 1689, 1634, 1557, 1512, 1462, 1432, 1307, 1223, 1082, 1020, 872, 786 cm⁻¹; ¹H NMR (DMSO-*d*₆) δ 10.00 (br s, 1 H), 7.89 (s, 1 H), 7.72 (s, 1 H), 7.49 (s, 1 H), 7.29 (s, 1 H), 7.00 (s, 1 H), 6.94 (s, 1 H), 6.90 (s, 1 H), 4.48 (t, *J* = 6.7 Hz, 2 H), 4.18 (t, *J* = 6.8 Hz, 2 H), 3.89 (s, 3 H), 3.87 (s, 3 H), 3.85 (s, 3 H), 2.27–2.23 (m, 2 H); ESIMS *m/z* (rel intensity) 462 (MH⁺, 100). Purity was estimated to be 100% by HPLC in MeOH and 99.3% in 90% MeOH–10% H₂O. Anal. Calcd for C₂₅H₂₃N₃O₆·0.5 H₂O: C, 63.82; H, 5.14; N, 8.93. Found: C, 63.70; H, 4.96; N, 8.56.

5,6-Dihydro-9-hydroxy-2,3,8-trimethoxy-6-[3-(*N*-morpholino)propyl]-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (22b, Method 1). Compound 21b (0.074 g, 0.130 mmol) was diluted in H₂O (60 mL), and the mixture was sonicated to ensure a homogeneous suspension. A solution of HBr in AcOH (33%, 60 mL) was added slowly, and the mixture was heated at 70 °C for 4 h. The reddish-brown solution was concentrated, and the residue was diluted with H₂O (100 mL). Saturated aqueous NaHCO₃ was added until the gas evolution ceased (approximately 30 mL was required). The suspension was extracted with CHCl₃ (2 × 50 mL, 1 × 25 mL), and the organic layers were washed with H₂O (2 × 150 mL) and saturated aqueous NaCl (150 mL) and dried over anhydrous sodium sulfate. The solution was concentrated, adsorbed onto SiO₂ (~4 g) and the residue was purified by flash column chromatography (SiO₂, ~25 g), eluting with a gradient of 1% MeOH in CHCl₃ to 2.5% MeOH in CHCl₃ to yield the title compound as a brown solid (0.035 g, 56%) after washing with 2% CHCl₃ in ether (50 mL) and ether (10 mL) and drying: mp 258–260 °C (dec). Combining this with several reactions run in parallel and repurifying the resulting material twice by flash column chromatography (SiO₂, ~25 g), eluting with a gradient of 0.5% MeOH in CHCl₃ to 2.5% MeOH in CHCl₃, afforded an analytically pure sample (0.040 g) after dissolving in tetrahydrofuran (10 mL) and precipitating with ether (50 mL). IR (film) 2917, 1692, 1650, 1552,

1496, 1394, 1261, 1210, 1116, 870, 786, 757 cm⁻¹; ¹H NMR (CDCl₃) δ 8.05 (s, 1 H), 7.64 (s, 1 H), 7.16 (s, 1 H), 7.02 (s, 1 H), 6.10 (br s, 1 H), 4.57 (t, *J* = 7.2 Hz, 2 H), 4.05 (s, 3 H), 4.01 (s, 3 H), 4.99 (s, 3 H), 3.70 (t, *J* = 4.5 Hz, 2 H), 2.60 (t, *J* = 7.1 Hz, 2 H), 2.40–2.50 (m, 4 H), 2.14–2.09 (m, 2 H); ESIMS *m/z* (rel intensity) 481 (MH⁺, 100). Purity was estimated to be 97.5% by HPLC in 95% MeOH–5% H₂O and 97.7% in 90% MeOH–10% H₂O. Anal. Calcd for C₂₆H₂₈N₂O₇: C, 64.99; H, 5.87; N, 5.83. Found: C, 64.92; H, 5.86; N, 5.75.

O-(4-Methoxybenzyl)vanillin (23).⁶⁵ Vanillin (15, 3.00 g, 19.7 mmol) was diluted in dry DMF (55 mL). PMB-Cl (3.18 g, 20.9 mmol) was added, followed by anhydrous potassium carbonate (6.53 g, 47.3 mmol). The yellow mixture was heated to 70 °C for 3 h and was then cooled and partitioned between ether and water (200 mL of each). The layers were separated, and the aqueous layer was extracted with ether (100 mL) and CHCl₃ (1 × 100 mL, 2 × 50 mL). The combined organic extracts were washed with H₂O (3 × 200 mL) and saturated aqueous NaCl (100 mL). The organic phase was dried over anhydrous sodium sulfate, filtered to remove particulate matter, and concentrated to yield a yellow residue. This residue was dissolved in CHCl₃ (20 mL), and addition of hexanes (175 mL) resulted in the precipitation of a pale-yellow solid (4.82 g, 90%) which was washed with hexanes (25 mL) and dried: mp 101–104 °C (lit.⁶⁵ mp 100–102 °C). ¹H NMR (CDCl₃) δ 9.39 (s, 1 H), 7.42–7.36 (m, 4 H), 7.02 (d, *J* = 8.0 Hz, 1 H), 6.93 (dd, *J* = 2.6, 6.7 Hz, 2 H), 5.18 (s, 2 H), 3.94 (s, 3 H), 3.81 (s, 3 H).

***N*-[4'-(4'-Methoxybenzyloxy)-3'-methoxybenzylidene]-3-bromopropan-1-amine (24).** 3-Bromopropylamine HBr (1.85 g, 8.44 mmol) was diluted with CHCl₃ (12 mL). Compound 23 (2.00 g, 7.34 mmol) was added as a solution in CHCl₃ (15 mL) and was quantitatively transferred with CHCl₃ (3 mL). Et₃N (0.811 g, 8.7 mmol) was added slowly, which discharged the cloudiness of the reaction mixture, followed by anhydrous Na₂SO₄ (2.00 g, 14.1 mmol). The mixture was stirred at room temperature for 24 h and diluted to 70 mL with CHCl₃. The mixture was washed with H₂O (70 mL), and this aqueous phase was extracted with CHCl₃ (50 mL). The combined organic layers were washed with H₂O (2 × 50 mL) and saturated aqueous NaCl (70 mL), dried over anhydrous sodium sulfate, and concentrated to yield an orange oil (2.84 g, 99%) that solidified upon standing: mp 78–80 °C. IR (film) 2934, 2837, 1644, 1613, 1585, 1515, 1267, 1251, 1138, 1033 cm⁻¹; ¹H NMR (CDCl₃) δ 8.22 (s, 1 H), 7.41–7.35 (m, 3 H), 7.11 (d, *J* = 8.0 Hz, 1 H), 6.92 (d, *J* = 8.1 Hz, 3 H), 5.13 (s, 2 H), 3.94 (s, 3 H), 3.81 (s, 3 H), 3.74 (t, *J* = 6.2 Hz, 2 H), 3.51 (d, *J* = 6.5 Hz, 2 H), 2.30–2.20 (m, 2 H); ESIMS *m/z* (rel intensity) 392/394 (MH⁺, 100/97).

***cis*-3-(4-Methoxybenzyloxy-3-methoxyphenyl)-*N*-(3-bromopropyl)-4-carboxy-3,4-dihydro-6,7-dimethoxy-1(2*H*)-isoquinoline (25).** Anhydride 11 (1.03 g, 4.64 mmol) was diluted with CHCl₃ (30 mL), and the solution was cooled to 0 °C. The Schiff base 24 (1.19 g, 4.88 mmol) was added slowly as a precooled solution in CHCl₃ (20 mL) and quantitatively transferred with CHCl₃ (3 mL). The mixture was stirred at 0 °C for 2 h, followed by 2 h at room temperature, upon which a precipitate had formed. Hexanes (12 mL) were added, and the precipitate was collected, washed with 20% hexanes in CHCl₃ (50 mL), and dried to yield the title compound as a white amorphous solid (2.22 g, 78%): mp 139–141 °C (dec). IR (film) 1996, 1722, 1619, 1592, 1573, 1251, 1172, 1138 cm⁻¹; ¹H NMR (CDCl₃/DMSO-*d*₆) δ 7.63 (s, 1 H), 7.24 (d, *J* = 8.6 Hz, 2 H), 7.15 (s, 1 H), 6.81 (d, *J* = 8.6 Hz, 2 H), 6.63–6.51 (m, 3 H), 4.95–4.89 (m, 3 H), 4.54 (d, *J* = 6.4 Hz, 1 H), 4.00–3.88 (m, 1 H), 3.88 (s, 3 H), 3.81 (s, 3 H), 3.72 (s, 3 H), 3.60 (s, 3 H), 3.41–3.34 (m, 2 H), 3.10–3.00 (m, 1 H), 2.20–2.00 (m, 2 H); ESIMS *m/z* (rel intensity) 614/616 (MH⁺, 4.04/3.68), 534 (MH⁺ – HBr, 100).

3-(Bromopropyl)-5,6-dihydro-9-hydroxy-2,3,8-trimethoxy-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (20). The *cis* acid 25 (4.71 g, 7.67 mmol) was diluted with SOCl₂ (80 mL). The mixture was stirred at room temperature for 5 h. The dark burgundy mixture was then concentrated, and the residue was diluted with CHCl₃ (40 mL). Ether (250 mL) was added, and the precipitate was collected, washed with ether (100 mL), subjected to a second, identical precipitation (20 mL of CHCl₃, 200 mL of ether, followed by washing

with 100 mL of ether), and dried to afford the crude product as a reddish-brown solid (2.48 g, 68%). This material was used without any further purification. ^1H NMR (DMSO- d_6) δ 10.00 (br s, 1 H), 7.86 (s, 1 H), 7.46 (s, 1 H), 7.10 (s, 1 H), 6.93 (s, 1 H), 4.52 (t, J = 8.5 Hz, 2 H), 3.91 (s, 3 H), 3.88 (s, 3 H), 3.84 (s, 3 H), 3.74 (t, J = 3.6 Hz, 2 H), 2.35–2.25 (m, 2 H).

5,6-Dihydro-9-hydroxy-6-[3-(1H-imidazol-1-yl)propyl]-2,3,8-trimethoxy-5,11-dioxo-11H-indeno[1,2-c]isoquinoline (22a, Method 2). Compound 20 (1.12 g, 2.35 mmol), imidazole (1.60 g, 23.5 mmol), and NaI (3.53 g, 23.5 mmol) were diluted with dioxane (300 mL). The mixture was heated under argon to reflux for 29 h. The mixture was cooled, concentrated, and the residue was dissolved in 5% MeOH in CHCl_3 (200 mL). This solution was washed with H_2O (300 mL), and the aqueous phase was exhaustively extracted with CHCl_3 (~500 mL). The organic phase was washed with H_2O (approximately 2×600 mL) and dried over anhydrous sodium sulfate. The solution was concentrated, adsorbed onto SiO_2 (~10 g), and purified by flash column chromatography (SiO_2 , ~50 g), eluting with a gradient of 1.5% MeOH in CHCl_3 to 5% MeOH in CHCl_3 to yield a mauve-colored solid (0.474 g, 44%) after dissolving in CHCl_3 (10 mL), precipitating with ether (300 mL), and washing with ether (50 mL). The analytical data for this compound are identical to that prepared by method 1.

5,6-Dihydro-9-hydroxy-2,3,8-trimethoxy-6-[3-(N-morpholino)propyl]-5,11-dioxo-11H-indeno[1,2-c]isoquinoline (22b, Method 2). Compound 20 (0.100 g, 0.211 mmol) and NaI (0.189 g, 1.27 mmol) were diluted with dry DMF (50 mL). The mixture was heated to 65 °C, and morpholine (0.111 g, 1.27 mmol) was added. After 18 h at 65 °C, the mixture was cooled and poured into H_2O (120 mL). The mixture was extracted with CHCl_3 (3×75 mL) and the organic phase was washed with H_2O (5×250 mL) and saturated aqueous NH_4Cl (250 mL). The extract was dried over anhydrous sodium sulfate. The solution was concentrated, adsorbed onto SiO_2 (~3 g), and the product was purified by flash column chromatography (SiO_2 , ~20 g), eluting with a gradient of 0.5% MeOH in CHCl_3 to 2% MeOH in CHCl_3 . The product was suspended in CHCl_3 (3 mL), precipitated with ether (100 mL), and washed with ether (20 mL) to yield a brown solid (0.045 g, 44%). The analytical data for this compound are identical to that prepared by method 1.

O-Benzylhomoisovanillic Acid (27).⁵² KOH (3.93 g, 70.0 mmol) was diluted with absolute EtOH (100 mL), and the mixture was warmed to 55 °C, upon which the mixture became a clear solution. Homoisovanillic acid (26, 5.00 g, 27.4 mmol) was added in small portions, and the mixture was heated to gentle reflux under a continuous argon flow. Benzyl chloride (7.49 g, 60.0 mmol) was added as a solution in absolute EtOH (10 mL) dropwise over 15 min and was quantitatively transferred with EtOH (3 mL). The mixture was heated at reflux for 2 h following addition, upon which a cloudy precipitate formed. A solution of KOH (1 g) in H_2O (20 mL) was added to the mixture, and reflux was continued for 20 min. The solution was cooled to room temperature and stirred for 1 h before it was concentrated to $1/3$ of its original volume. The resulting residue was diluted with H_2O (400 mL) and made acidic (pH 1) by the addition of HCl (concentrated, 5 mL). The resulting precipitate was collected, dried, and recrystallized from 40% acetone in ether to yield the product as an off-white solid (5.68 g, 76%) after drying: mp 111–114 °C (lit.⁶³ mp 124–125 °C). ^1H NMR (DMSO- d_6) δ 12.4 (br s, 1 H), 7.45–7.32 (m, 5 H), 6.96 (s, 1 H), 6.91 (d, J = 9.6 Hz, 1 H), 6.80–6.79 (m, 1 H), 5.01 (s, 2 H), 3.72 (s, 3 H), 3.45 (s, 2 H).

6-(Benzyloxy)-7-methoxy-1H-isochromen-3(4H)-one (28).⁵⁴ Compound 27 (7.00 g, 25.7 mmol) was diluted with glacial AcOH (90 mL). Formalin (37% formaldehyde in H_2O , 24 mL) was added, followed by HCl (concd, 6 mL). The mixture was heated at 45 °C for 22 h, cooled, and poured into H_2O (300 mL). The cloudy suspension was extracted with CHCl_3 (3×100 mL). The organic layers were washed with saturated aqueous NaHCO_3 (3×150 mL) and H_2O (3×200 mL). The combined aqueous layers were extracted with CHCl_3 (100 mL). The organic layers were dried over anhydrous sodium sulfate and concentrated. The resulting pale yellow oil was dissolved in CHCl_3 (10 mL), and hexanes (100 mL) were added slowly to precipitate a white amorphous solid, which was collected by vacuum

filtration. Refiltration of the filtrate afforded additional product. A total of 5.00 g (69%) was obtained, containing a small amount of impurities: mp 105–110 °C (lit.⁵³ mp 122 °C). ^1H NMR (CDCl_3) δ 7.42–7.34 (m, 5 H), 6.76 (s, 1 H), 6.72 (s, 1 H), 5.25 (s, 2 H), 5.15 (s, 2 H), 3.89 (s, 3 H), 3.58 (s, 2 H).

4-(Benzyloxy)-2-(carboxymethyl)-5-methoxybenzoic Acid (29). Compound 28 (5.00 g, 17.6 mmol) was diluted with a solution of KOH (2.47 g, 44.0 mmol) in H_2O (250 mL). The mixture was stirred at room temperature for 17 h. The clear orange solution was cooled to 0 °C, and KMnO_4 (6.21 g, 40.0 mmol) was added in portions over 10 min. The mixture was stirred at 0 °C for 3 h and then at room temperature for 48 h. Absolute EtOH (60 mL) was added, and the mixture was heated at 75 °C for 30 min. The mixture was filtered hot to remove inorganic materials, and the filter cake was washed with H_2O (100 mL). The filtrate was concentrated to half its volume and was washed with EtOAc (2×150 mL). The aqueous layer was diluted with H_2O to a volume of 300 mL and, while stirring, was made acidic (pH 1) by the addition of HCl (concentrated, 12 mL). The mixture was chilled to 0 °C for 3 h, and the resultant white precipitate was collected by vacuum filtration, dried, triturated with EtOAc–hexanes (70:30, 75 mL), and washed with EtOAc (30 mL) to afford an off-white solid (3.11 g, 56%): mp 199–200 °C. IR (neat) 3583, 3408, 2917, 1682, 1598, 1575, 1276, 1218, 1173, 1074, 666 cm^{-1} ; ^1H NMR (DMSO- d_6) δ 12.3 (br s, 1 H), 7.47–7.36 (m, 6 H), 7.07 (s, 1 H), 5.11 (s, 2 H), 3.86 (s, 2 H), 3.77 (s, 3 H). One of the carboxyl protons is not visible because of exchange with residual water. ^{13}C NMR (DMSO d_6 , 125 MHz) δ 172.9, 168.0, 150.7, 147.4, 136.8, 131.3, 128.8, 128.4, 128.4, 122.5, 117.2, 114.1, 70.2, 55.9; ESIMS m/z (rel intensity) 339.2 (MNa^+ , 100); negative ion 315.1 [$(\text{M} - \text{H})^-$, 100]. Anal. Calcd for $\text{C}_{17}\text{H}_{16}\text{O}_6 \cdot 0.5\text{H}_2\text{O}$: C, 62.77; H, 5.27. Found: C, 62.60; H, 4.99.

6-(Benzyloxy)-7-methoxy-4H-isochromene-1,3-dione (30). Compound 29 (2.25 g, 7.11 mmol) was diluted with acetyl chloride (40 mL), and the mixture was heated at reflux for 2.5 h. The mixture was then concentrated to yield a yellow solid (2.12 g, 100%): mp 160–165 °C. IR (neat) 2939, 1741, 1515, 1277, 1224, 1028 cm^{-1} ; ^1H NMR (CDCl_3) δ 7.60 (s, 1 H), 7.42–7.26 (s, 5 H), 6.71 (s, 1 H), 5.24 (s, 2 H), 4.00 (s, 2 H), 3.96 (s, 3 H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 164.9, 160.6, 154.4, 149.7, 135.2, 128.8, 128.7, 128.4, 127.1, 113.6, 111.5, 110.3, 71.0, 56.2, 33.9; CIMS m/z (rel intensity) 299 (MH^+ , 40), 209 (100), 181 (100).

N-[3',4'-(Methylenedioxy)benzylidene]-3-bromopropan-1-amine (32).²¹ 3-Bromopropylamine hydrobromide (3.35 g, 15.3 mmol) was diluted with CHCl_3 (15 mL). Piperonal (31, 2.0 g, 13.3 mmol) was added slowly as a solution in CHCl_3 (15 mL) and quantitatively transferred with 5 mL of the same solvent. Et_3N (1.6 g, 16.0 mmol) was added slowly, upon which the solution became clear and colorless. Na_2SO_4 (3.00 g, 21.1 mmol) was added, and the mixture was stirred at room temperature for 27 h. The mixture was diluted with CHCl_3 to a volume of 70 mL and washed with H_2O (70 mL). The aqueous layer was extracted with CHCl_3 (50 mL), and the combined organic layers were washed with H_2O (2×70 mL) and saturated aqueous NaCl (70 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated to yield a yellow oil (3.44 g, 96%). ^1H NMR (CDCl_3) δ 8.21 (s, 1 H), 7.34 (d, J = 0.9 Hz, 1 H), 7.13 (dd, J = 0.9, 7.9 Hz, 1 H), 6.85 (d, J = 7.9 Hz, 1 H), 6.01 (s, 2 H), 3.73 (t, J = 6.3 Hz, 2 H), 3.52 (t, J = 6.4 Hz, 2 H), 2.30–2.20 (m, 2 H).

cis-6-(Benzyloxy)-N-(3-bromopropyl)-4-carboxy-3,4-dihydro-7-methoxy-3-(3,4-methylenedioxyphenyl)-1(2H)-isoquinolone (33). Compound 30 (2.09 g, 7.03 mmol) was dissolved in CHCl_3 (65 mL), and the solution was cooled to 0 °C. A solution of Schiff base 32 (2.00 g, 7.40 mmol) in CHCl_3 (15 mL) was cooled to 0 °C and added slowly via addition funnel to the anhydride. Within minutes, a yellow precipitate began to form. The mixture was stirred for 2 h at 0 °C and then warmed to room temperature, where it was stirred for 2 h. The precipitate was collected via vacuum filtration and washed with CHCl_3 (100 mL) to yield the desired product as a cream-colored solid (2.63 g, 66%) after drying: mp 198–199 °C (dec). IR (film) 2972, 2937, 1742, 1592, 1570, 1259, 1226, 1168 cm^{-1} ; ^1H NMR (DMSO- d_6) δ 12.96 (br s, 1 H), 8.31 (s, 1 H), 7.53–7.35 (s, 5 H),

7.26 (s, 1 H), 6.80 (d, $J = 8$ Hz, 1 H), 6.57 (dd, $J = 1.4, 8.1$ Hz, 2 H), 6.46 (s, 1 H), 5.95 (s, 2 H), 5.03–5.00 (m, 3 H), 4.69 (d, $J = 6$ Hz, 1 H), 3.88–3.82 (s, 1 H), 3.82 (s, 3 H), 3.56–3.50 (s, 2 H), 2.95–2.88 (m, 1 H), 2.14–1.96 (m, 2 H); ^{13}C NMR (DMSO, 75 MHz) δ 171.5, 163.6, 151.4, 148.8, 148.0, 147.8, 137.4, 132.0, 129.4, 129.1, 127.9, 122.6, 122.4, 113.2, 110.9, 108.9, 108.6, 102.1, 71.0, 62.4, 56.4, 48.3, 45.5, 33.4, 31.9; ESIMS m/z (rel intensity) 488.5 ($\text{MH}^+ - \text{HBr}$, 100). Anal. Calcd for $\text{C}_{28}\text{H}_{26}\text{BrN}_7\text{H}_2\text{O}$: C, 57.35; H, 4.81; N, 2.39. Found: C, 57.34; H, 4.46; N, 2.42.

6-(3-Bromopropyl)-5,6-dihydro-2-hydroxy-3-methoxy-8,9-methylenedioxy-5,11-dioxo-11H-indeno[1,2-c]isoquinoline (34). Compound 33 (1.35 g, 2.38 mmol) was diluted with SOCl_2 (30 mL). The mixture was stirred at room temperature for 3 h and 40 min. The dark red mixture was concentrated, and the residue was suspended in 1,2-dichloroethane (200 mL). Aluminum chloride (0.475 g, 0.357 mmol) was added, and the mixture was stirred at 0 °C for 10 min. Additional AlCl_3 (0.237 g, 1.78 mmol) was then added, and the mixture was sonicated for 1.5 h at 0 °C. The mixture was poured into a mixture of ice and saturated aqueous NaHCO_3 (200 mL). The mixture was extracted with CHCl_3 (4 \times 150 mL), and the organic layer was filtered to remove aluminum salts. The organic layer was washed with H_2O (3 \times 200 mL), dried over anhydrous sodium sulfate, concentrated, and adsorbed onto SiO_2 (~5 g). The residue was purified by flash column chromatography (SiO_2 , ~40 g), eluting with CHCl_3 to yield a purple-gray solid (0.108 g, 10%) after suspending in CHCl_3 (10 mL), precipitating with ether (100 mL), and washing with ether (20 mL): mp 276–278 °C. IR (film) 3368, 2918, 1681, 1652, 1615, 1491, 1432, 1389, 1304, 1267, 1033 cm^{-1} ; ^1H NMR (DMSO- d_6) δ 10.44 (s, 1 H), 7.81 (s, 1 H), 7.47 (s, 1 H), 7.35 (s, 1 H), 7.05 (s, 1 H), 6.17 (s, 2 H), 4.50 (t, $J = 6.9$ Hz, 2 H), 3.84 (s, 3 H), 3.73 (t, $J = 6.5$ Hz, 2 H), 2.30–2.20 (m, 2 H); ESIMS m/z (rel intensity) 458/460 (MH^+ , 9/8.5), 378 ($\text{MH}^+ - \text{HBr}$, 100).

5,6-Dihydro-2-hydroxy-6-(3-(1H-imidazol-1-yl)propyl)-3-methoxy-8,9-methylenedioxy-5,11-dioxo-11H-indeno[1,2-c]isoquinoline (35a). Compound 34 (0.212 g, 0.463 mmol), imidazole (0.315 g, 4.63 mmol), and NaI (0.693 g, 4.63 mmol) were diluted with dry DMF (60 mL), and the mixture was heated at 60 °C for 20 h. Additional imidazole (0.200 g, 2.94 mmol) was added, and the mixture was heated for an additional 40 min. The mixture was cooled and poured into H_2O (200 mL). CHCl_3 (200 mL) was added, and an insoluble suspension formed. This suspension was saved, and the remaining aqueous layer was extracted with CHCl_3 (2 \times 200 mL). These layers were washed with H_2O (4 \times 400 mL), saturated aqueous NH_4Cl (200 mL), dried over anhydrous sodium sulfate, and concentrated. The residue was washed with ether (50 mL), dissolved in CHCl_3 (50 mL), concentrated, and adsorbed onto SiO_2 (3 g). This residue was purified by flash column chromatography (SiO_2 , 60 g), eluting with a gradient of 0.5% MeOH in CHCl_3 to 7% MeOH in CHCl_3 to afford a purple solid. Additional product was obtained by drying the CHCl_3 -insoluble suspension over anhydrous sodium sulfate and concentrating the mixture. This solid was washed with H_2O (80 mL) and purified by dissolving in DMF (5 mL) and precipitating with ether (50 mL). The solid (0.054 g, 26%) was washed with 10% CHCl_3 in ether (50 mL) and ether (20 mL) and dried. To produce an analytically pure sample (0.034 g), the solid was adsorbed onto SiO_2 (3 g) and purified by flash column chromatography (SiO_2 , 20 g), eluting with a gradient of 2% MeOH in CHCl_3 to 5% MeOH in CHCl_3 and then washing the product with 10% CHCl_3 in ether (20 mL) and ether (20 mL): mp 269–272 °C (dec). IR (film) 3391, 3117, 2916, 1691, 1637, 1605, 1488, 1304, 1225, 1091, 1028 cm^{-1} ; ^1H NMR (DMSO- d_6) δ 10.46 (br s, 1 H), 7.81 (s, 1 H), 7.76 (s, 1 H), 7.47 (s, 1 H), 7.27 (s, 1 H), 7.04 (s, 1 H), 6.98 (s, 1 H), 6.92 (s, 1 H), 6.16 (s, 2 H), 4.40–4.30 (m, 2 H), 4.29 (t, $J = 6.1$ Hz, 2 H), 2.20–2.10 (m, 2 H); ESIMS m/z (rel intensity) 446.3 (MH^+ , 100); HRESIMS m/z 446.1347 (MH^+), calcd for $\text{C}_{24}\text{H}_{20}\text{N}_3\text{O}_6$ 446.1352. Anal. Calcd for $\text{C}_{24}\text{H}_{19}\text{N}_3\text{O}_4 \cdot 1.25\text{H}_2\text{O}$: C, 61.60; H, 4.63; N, 8.98. Found: C, 61.67; H, 4.27; N, 8.79.

5,6-Dihydro-2-hydroxy-3-methoxy-8,9-methylenedioxy-6-(3-(morpholino)propyl)-5,11-dioxo-11H-indeno[1,2-c]isoquinoline (35b). Compound 34 (0.080 g, 0.17 mmol) was

dissolved in anhydrous DMF (10 mL), and NaI (0.314 g, 2.09 mmol) and morpholine (0.18 mL, 2.06 mmol) were added. The mixture was heated to 80 °C for 26 h under argon. After the evaporation of the solvent, the residue was partitioned between chloroform (60 mL) and water (30 mL). The organic layer was washed with water (30 mL) and purified by silica gel column chromatography (chloroform–MeOH, 10:0.5), which afforded the product (0.020 g, 25%): mp 228–230 °C. IR (neat) 3583, 2918, 2852, 1701, 1634, 1610, 1470, 1389, 1270, 1115, 1033, 666 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) δ 7.90 (s, 1 H), 7.46 (s, 1 H), 7.16 (s, 1 H), 6.97 (s, 1 H), 6.05 (s, 2 H), 4.61 (t, $J = 7.2$ Hz, 2 H), 4.05 (s, 3 H), 3.70–3.63 (m, 5 H), 2.51 (t, $J = 6.8$ Hz, 2 H), 2.43 (br, 4 H), 1.96–1.91 (m, 2 H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 187.3, 160.7, 152.5, 150.5, 148.5, 146.2, 138.5, 131.7, 126.9, 124.9, 124.6, 122.2, 109.3, 107.3, 105.8, 103.9, 102.1, 66.8, 56.2, 53.4, 41.0, 25.5; ESIMS m/z (rel intensity) 465.1 (MH^+ , 100); negative ion 463.2 [$(\text{M} - \text{H}^+)^-$, 100]; HRESIMS m/z 465.1659 (MH^+), calcd for $\text{C}_{25}\text{H}_{25}\text{N}_2\text{O}_7$: 465.1662. Anal. Calcd for $\text{C}_{25}\text{H}_{24}\text{N}_2\text{O}_7$: C, 64.65; H, 5.21; N, 6.03. Found: C, 64.69; H, 5.22; N, 6.18.

7-Hydroxy-6-methoxyisochroman-3-one (37).⁵⁴ Glacial AcOH (25 mL) was added to 36 (0.96 g, 5.3 mmol). The solution was heated to 90 °C, and concentrated HCl (0.9 mL) and formalin (37% formaldehyde in H_2O , 0.9 mL) were added rapidly, and then heating was stopped immediately. After 1.5 h at room temperature, ice (20 g) and H_2O (20 mL) were added to the solution. The organic material was extracted with CHCl_3 (4 \times 50 mL), washed with saturated aqueous NaHCO_3 (2 \times 40 mL) and saturated aqueous NaCl (50 mL), and the solvent was evaporated to yield the crude product (0.81 g). Recrystallization from CH_2Cl_2 –ether gave pure 37 as a white powder (0.36 g, 35%): mp 178–179 °C (lit.⁵⁴ 174–177 °C). ^1H NMR (DMSO- d_6 , 300 MHz) δ 9.05 (s, 1 H), 6.88 (s, 1 H), 6.75 (s, 1 H), 5.18 (s, 2 H), 3.74 (s, 3 H), 3.62 (s, 2 H).

7-(Benzoyloxy)-6-methoxyisochroman-3-one (38). A mixture of 37 (0.160 g, 0.82 mmol), anhydrous K_2CO_3 (500 mg, 3.62 mmol), and benzyl bromide (0.25 mL, 2.08 mmol) in dry acetone was heated at 56 °C for 4 h. The inorganic compounds were filtered off and washed with acetone. Evaporation of the filtrate afforded a residue that was partitioned between CHCl_3 (30 mL) and H_2O (30 mL). The organic layer was dried over anhydrous sodium sulfate, concentrated, and the residue was purified by flash column chromatography (SiO_2 , ~25 g), eluting with a gradient of 10% EtOAc–hexanes to 25% EtOAc–hexanes to yield the product (0.132 g, 57%) as a white powder: mp 129–130 °C (lit.⁵⁴ 137–138 °C). ^1H NMR (CDCl_3 , 500 MHz) δ 7.31–7.43 (m, 5 H), 6.74 (s, 1 H), 6.73 (s, 1 H), 5.19 (s, 2 H), 5.14 (s, 2 H), 3.89 (s, 3 H), 3.62 (s, 2 H).

5-(Benzoyloxy)-2-(carboxymethyl)-4-methoxybenzoic Acid (39). A solution of compound 38 (2.00 g, 7.00 mmol) and KOH (2 g, 36.0 mmol) in H_2O (60 mL) was stirred at 0 °C, and KMnO_4 (3.30 g, 20.1 mmol) was added slowly. After the mixture was allowed to stand for 24 h at room temperature, excess KMnO_4 was destroyed by addition of EtOH (20 mL) and by heating at 60–70 °C for 0.5 h. The solution was filtered and concentrated to a small volume under reduced pressure. Acidification with concentrated HCl (to pH 2) gave a precipitate that was collected. Recrystallization from acetone gave the pure product (0.675 g, 31%) as a yellow powder: mp 236–237 °C (lit.⁶⁶ 238 °C). ^1H NMR (DMSO- d_6 , 300 MHz) δ 12.35 (s, 2 H), 7.50 (s, 1 H), 7.33–7.42 (m, 5 H), 6.93 (s, 1 H), 5.08 (s, 2 H), 3.83 (s, 2 H), 3.80 (s, 3 H).

7-(Benzoyloxy)-6-methoxyisochroman-1,3-dione (40). Acetyl chloride (4.0 mL) was added to 39 (0.175 g, 0.550 mmol). The solution was heated at reflux for 2 h and was concentrated to yield an oily residue. Recrystallization from CHCl_3 –ether gave pure 40 as a yellow powder (0.157 g, 91%): mp 107–109 °C (dec). IR (film) 2940, 1710, 1601, 1517, 1272, 1221, 1015 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) δ 7.63 (s, 1 H), 7.26–7.47 (m, 5 H), 6.70 (s, 1 H), 5.18 (s, 2 H), 4.05 (s, 2 H), 3.96 (s, 3 H); CIMS m/z (rel intensity) 299 (MH^+ , 100).

cis-7-(Benzoyloxy)-N-(3-bromopropyl)-4-carboxy-3,4-dihydro-6-methoxy-3-(3,4-methylenedioxyphenyl)-1(2H)-isoquinolone (41). A solution of the anhydride 40 (0.276 g, 0.92 mmol) dissolved in CHCl_3 (3 mL) was added dropwise to a solution of the

Schiff base 32 (0.191 g, 0.92 mmol) in CHCl_3 (3 mL). After 24 h, hexanes (5 mL) were added to the mixture, and the precipitate that formed was filtered off and washed with a mixture of ethyl acetate (5 mL) and CHCl_3 (5 mL) to afford an impure mixture containing the desired product. This mixture was purified by flash column chromatography (SiO_2 , ~25 g), eluting with a gradient of 10% MeOH in CHCl_3 to yield the product as a yellow powder (0.188 g, 36%): mp 195–197 °C (dec). IR (film) 2942, 1712, 1677, 1601, 1578, 1526, 1220, 1178 cm^{-1} ; ^1H NMR (DMSO, 300 MHz) δ 7.78 (s, 1 H), 7.32–7.50 (m, 5 H), 7.08 (s, 1 H), 6.60–6.65 (m, 2 H), 6.48 (s, 1 H), 5.90 (s, 2 H), 5.22 (s, 2 H), 4.97–4.99 (d, J = 6.00 Hz, 1 H), 4.68–4.71 (d, J = 6.48 Hz, 1 H), 3.95–3.97 (m, 1 H), 3.88 (s, 3 H), 3.45 (m, 2 H), 3.11–3.13 (m, 1 H), 2.13–2.24 (m, 2 H); ESIMS m/z (rel intensity) 488.3 (MH^+ – HBr, 100).

6-(3-Bromopropyl)-5,6-dihydro-3-benzyloxy-2-methoxy-8,9-methylenedioxy-5,11-dioxo-11H-indeno[1,2-c]-isoquinoline (42). The cis acid 41 (1.00 g, 1.76 mmol) was diluted in SOCl_2 (45 mL), and the mixture was stirred at room temperature for 20 h. The solvent was removed under reduced pressure, and the organic material was extracted with chloroform (3 \times 20 mL), washed with saturated aqueous NaHCO_3 (2 \times 20 mL) and saturated aqueous NaCl (20 mL), and then concentrated. The obtained residue was adsorbed onto SiO_2 (~10 g) and purified by flash column chromatography (SiO_2 , ~30 g), eluting with 1.25% MeOH in CHCl_3 to yield the solid product (0.404 g, 42%): mp 234–236 °C. IR (film) 3421, 1698, 1644, 1483, 1309, 1282, 1036, 698 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) δ 8.04 (s, 1 H), 7.70 (s, 1 H), 7.29–7.50 (m, 5 H), 7.27 (s, 1 H), 7.07 (s, 1 H), 6.09 (s, 2 H), 5.24 (s, 2 H), 4.55–4.60 (m, 2 H), 4.04 (s, 3 H), 3.78–3.82 (t, J = 6.0 Hz, 2 H), 2.33–2.38 (m, 2 H); ESIMS m/z (rel intensity) 468.5 (MH^+ – HBr, 100).

6-(3-Bromopropyl)-5,6-dihydro-3-hydroxy-2-methoxy-8,9-methylenedioxy-5,11-dioxo-11H-indeno[1,2-c]isoquinoline (43). The benzylated indenoisoquinoline 42 (1.73 g, 3.16 mmol) was dissolved in nitrobenzene (30 mL), and AlCl_3 (0.839 g, 6.32 mmol) was added. The mixture was stirred at room temperature for 15 min and then heated at 90 °C for 45 min. The mixture was cooled and poured into a mixture of ice and saturated aqueous NaHCO_3 (80 mL). The mixture was extracted with CHCl_3 (4 \times 100 mL) and the organic layer was filtered to remove aluminum salts. The organic layer was washed with H_2O (3 \times 100 mL), dried over anhydrous sodium sulfate, concentrated, and adsorbed onto SiO_2 (~5 g). The residue was purified by flash column chromatography (SiO_2 , ~50 g), eluting with a gradient of 0.25% MeOH in CHCl_3 to 1% MeOH in CHCl_3 to afford the crude product as a pinkish solid (0.230 g, 16%): mp 226–228 °C (dec). IR (film) 3446, 2346, 1698, 1638, 1483, 1394, 1310, 1215, 1034 cm^{-1} ; ^1H NMR (DMSO, 300 MHz) δ 9.86 (s, 1 H), 7.85 (s, 1 H), 7.46 (s, 1 H), 7.35 (s, 1 H), 7.05 (s, 1 H), 6.17 (s, 2 H), 4.48 (m, 2 H), 3.89 (s, 3 H), 3.82–3.87 (t, J = 6.18 Hz, 2 H), 2.19 (m, 2 H); ESIMS m/z (rel intensity) 458/460 (MH^+ , 28/27).

5,6-Dihydro-3-hydroxy-6-(3-(1H-imidazol-1-yl)propyl)-2-methoxy-8,9-methylenedioxy-5,11-dioxo-11H-indeno[1,2-c]-isoquinoline (44a). Compound 43 (0.210 g, 0.46 mmol) and NaI (0.82 g, 5.52 mmol) were diluted with anhydrous DMF (15 mL). Imidazole (0.375 g, 5.52 mmol) was added, and the mixture was heated to 70 °C for 24 h under an argon atmosphere. Ice (30 g) was added, and the solution was extracted with CHCl_3 (5 \times 50 mL). The organic layer was washed with saturated NaHCO_3 (3 \times 30 mL), water (3 \times 30 mL) and dried over anhydrous sodium sulfate. The solution was concentrated, adsorbed onto SiO_2 (~5 g), and purified by flash column chromatography (SiO_2 , ~60 g), eluting with a gradient of 1% MeOH in CHCl_3 to 5% MeOH in CHCl_3 to yield the product as a dark brown solid (0.045 g, 28%): mp >350 °C. IR (film) 3446, 1693, 1642, 1484, 1431, 1394, 1309, 1225, 1033, 659 cm^{-1} ; ^1H NMR (DMSO, 300 MHz) 9.88 (s, 1 H), 7.88 (m, 2 H), 7.48 (s, 1 H), 7.32 (s, 1 H), 7.09 (s, 1 H), 7.05 (s, 1 H), 6.99 (s, 1 H), 6.17 (s, 2 H), 4.38 (m, 2 H), 4.17 (m, 2 H), 3.89 (s, 1 H), 2.18 (m, 2 H); ESIMS m/z (rel intensity) 446.5 (MH^+ , 100); HRESIMS m/z 446.1355 (MH^+), calcd for $\text{C}_{24}\text{H}_{19}\text{N}_3\text{O}_6$ 446.1352. Purity was estimated to be 95.2% by HPLC in both 95% MeOH– H_2O and 85% MeOH– H_2O .

5,6-Dihydro-3-hydroxy-2-methoxy-8,9-methylenedioxy-6-(3-((morpholino)propyl))-5,11-dioxo-11H-indeno[1,2-c]-isoquinoline (44b). Compound 43 (1.23 g, 2.74 mmol) and NaI (4.93 g, 32.88 mmol) were diluted with anhydrous DMF (40 mL). Morpholine (2.87 mL, 32.88 mmol) was added, and the mixture was heated to 65 °C for 22 h under an argon atmosphere. The mixture was cooled, poured into H_2O (200 mL), and extracted with CHCl_3 (4 \times 100 mL). The organic phase was washed with H_2O (200 mL) and saturated aqueous NaHCO_3 (200 mL) and was dried over anhydrous sodium sulfate. The solution was concentrated, adsorbed onto SiO_2 (~5 g), and purified by flash column chromatography (SiO_2 , ~60 g), eluting with a gradient of 1% MeOH in CHCl_3 to 5% MeOH in CHCl_3 to yield the title compound as a purple solid (0.481 g, 38%): mp 321–323 °C (dec). IR (film) 3244, 2962, 2811, 1687, 1655, 1503, 1286, 1111, 1035, 861 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) δ 8.05 (s, 1 H), 7.75 (s, 1 H), 7.44 (s, 1 H), 6.91 (s, 1 H), 6.09 (s, 2 H), 4.47–4.52 (m, 2 H), 4.09 (s, 3 H), 3.74–3.77 (m, 4 H), 2.52–2.56 (m, 6 H), 2.00–2.04 (m, 2 H); ESIMS m/z (rel intensity) 465.2 (MH^+ , 100); HRESIMS m/z 465.1665 (MH^+), calcd for $\text{C}_{25}\text{H}_{24}\text{N}_2\text{O}_7$ 465.1662. Anal. Calcd for $\text{C}_{25}\text{H}_{24}\text{N}_2\text{O}_7 \cdot \text{H}_2\text{O}$: C, 62.23; H, 5.43; N, 5.81. Found: C, 62.14; H, 5.00; N, 5.70.

5,6-Dihydro-11-hydroxy-6-(3-imidazolylpropyl)-2,3-methylenedioxy-5-oxo-11H-indeno[1,2-c]isoquinoline (45a). Indenoisoquinoline 6 (0.170 g, 0.370 mmol) was dissolved in MeOH (50 mL). NaBH_4 (0.025 g, 0.740 mmol) was added at 0 °C, and the reaction mixture was stirred at room temperature for 3 h. The mixture was concentrated. CHCl_3 (150 mL) was added, and the mixture was washed with water (2 \times 100 mL). The CHCl_3 was removed on a rotary evaporator and the crude mixture was purified by flash column chromatography (SiO_2), eluting with 10% MeOH in CHCl_3 to yield the title compound (0.110 g, 64%) as white solid: mp >280 °C. IR (KBr) 3583, 1635, 1610, 1481, 1292, 1258, 1034, 750, 666 cm^{-1} ; ^1H NMR (CDCl_3 + CD_3OD , 300 MHz) δ 7.49 (s, 1 H), 7.43 (s, 1 H), 7.24 (s, 1 H), 7.03 (s, 1 H), 6.91 (s, 1 H), 6.87 (s, 1 H), 6.55 (s, 1 H), 5.90 (d, J = 1.6 Hz, 2 H), 5.25 (s, 1 H), 4.40 (m, 1 H), 4.21 (m, 1 H), 4.04 (m, 2 H), 3.89 (s, 3 H), 3.80 (s, 3 H), 2.13 (m, 2 H); ^{13}C NMR (CDCl_3 + CD_3OD , 75 MHz) δ 162.7, 153.7, 148.6, 148.2, 147.5, 142.4, 138.0, 136.6, 129.8, 128.6, 128.1, 120.2, 118.8, 116.8, 107.8, 106.2, 103.0, 101.78, 101.72, 71.1, 55.8, 55.6, 44.5, 40.8, 30.9; ESIMS (m/z , relative intensity) 462 (MH^+ , 100); HRESIMS m/z 462.1670 (MH^+), calcd for $\text{C}_{25}\text{H}_{23}\text{N}_3\text{O}_6$ 462.1665. Purity by HPLC was estimated to be 99.4% in 85% MeOH– H_2O and 99.5% in 90% MeOH– H_2O .

5,6-Dihydro-11-hydroxy-2,3-methylenedioxy-6-(3-morpholinylpropyl)-5-oxo-11H-indeno[1,2-c]isoquinoline (45b). Indenoisoquinoline 7 (0.1 g, 0.209 mmol) was dissolved in MeOH (30 mL). NaBH_4 (0.014 g, 0.418 mmol) was added at 0 °C, and the reaction mixture was stirred at room temperature for 3 h. The mixture was concentrated. CHCl_3 was added (100 mL), and the solution was washed with water (2 \times 50 mL). The CHCl_3 was removed on a rotary evaporator and the crude mixture was purified by flash column chromatography (SiO_2), eluting with 5% MeOH in CHCl_3 to give the title compound (0.55 g, 55%) as white solid: mp 228–229 °C. IR (KBr) 3325, 2956, 1610, 1629, 1553, 1483, 1294, 1257, 1037, 665 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) δ 7.33 (s, 1 H), 7.23 (s, 1 H), 7.12 (s, 1 H), 7.09 (s, 1 H), 6.06 (s, 1 H), 6.02 (s, 1 H), 5.29 (s, 1 H), 4.31 (m, 1 H), 4.05 (s, 3 H), 3.72 (m, 4 H), 3.63 (s, 3 H), 3.42 (m, 1 H), 2.40 (m, 6 H), 1.76 (m, 2 H); ^{13}C NMR (CDCl_3 , 75 MHz) δ 162.3, 153.5, 148.2, 147.4, 142.9, 138.2, 129.3, 128.7, 119.6, 116.9, 107.9, 106.5, 102.9, 102.8, 101.7, 77.2, 71.6, 66.7, 56.2, 56.1, 55.4, 54.0, 42.5, 25.9; ESIMS (m/z , relative intensity) 481 (MH^+ , 100); HRESIMS m/z 481.1972 (MH^+), calcd for $\text{C}_{26}\text{H}_{28}\text{N}_2\text{O}_7$ 481.1975. Purity by HPLC was estimated to be 98.3% in 95% MeOH– H_2O and 97.2% in 40% MeOH– H_2O .

3,4-Dibenzoyloxybenzaldehyde (47).⁶⁷ Compound 46 (3.00 g, 21.7 mmol) was diluted with dry DMF (50 mL). Benzyl bromide (7.65 g, 44.7 mmol) was added slowly, followed by anhydrous K_2CO_3 (9.60 g, 69.4 mmol). The mixture was stirred at room temperature for 2 h. Additional K_2CO_3 (2.4 g, 17.3 mmol) was added, and the mixture was heated to 70 °C for 30 min and then cooled to room temperature.

The mixture was partitioned between H₂O and ether (120 mL each). The organic layer was separated, and the water layer was extracted with ether (3 × 50 mL). The pooled organic layers were washed with H₂O (2 × 50 mL) and saturated aqueous NaCl (50 mL). The pale, straw-colored extracts were dried over anhydrous sodium sulfate and concentrated to yield a cream-colored solid (6.57 g, 95%) after washing with hexanes (75 mL) and drying: mp 87–88.5 °C (lit⁶⁷ mp 85–87 °C). ¹H NMR (CDCl₃) δ 9.81 (s, 1 H), 7.49–7.31 (m, 12 H), 7.04 (d, *J* = 8.3 Hz, 1 H), 5.27 (s, 2 H), 5.22 (s, 2 H).

***N*-[3',4'-(Dibenzoyloxy)benzylidene]-3-bromopropan-1-amine (48).** 3-Bromopropylamine hydrobromide (0.791 g, 3.61 mmol) was diluted with CHCl₃ (5 mL). Compound 47 (1.00 g, 3.14 mmol) was added slowly as a solution in CHCl₃ (6 mL) and quantitatively transferred with 3 mL of the same solvent. Et₃N (0.348 g, 3.45 mmol) was added slowly, upon which the solution became clear and colorless. Na₂SO₄ (1.20 g, 6.24 mmol) was added, and the mixture was stirred at room temperature for 22 h. The mixture was diluted to a volume of 30 mL with CHCl₃ and was washed with H₂O (3 × 40 mL) and saturated aqueous NaCl (40 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated to yield a dark yellow syrup (1.36 g, 99%). IR (film) 3031, 2840, 1344, 1600, 1582, 1509, 1454, 1432, 1383, 1268, 1135, 1022, 735, 691 cm⁻¹; ¹H NMR (CDCl₃) δ 8.20 (s, 1 H), 7.50–7.33 (m, 11 H), 7.20 (dd, *J* = 1.6, 8.3 Hz, 1 H), 6.96 (d, *J* = 8.3 Hz, 1 H), 5.21 (s, 4 H), 3.73 (td, *J* = 1.2, 6.2 Hz, 2 H), 3.51 (t, *J* = 6.6 Hz, 2 H), 2.30–2.22 (m, 2 H); ESIMS *m/z* (rel intensity) 438/440 (MH⁺, 100/92).

***cis*-[3',4'-(Dibenzoyloxy)phenyl]-*N*-3-(bromopropyl)-4-carboxy-3,4-dihydro-6,7-dimethoxy-3-1(2*H*)-isoquinolone (49).** Anhydride 11 (1.0 g, 4.50 mmol) was diluted with CHCl₃ (30 mL), and the solution was cooled to 0 °C. The Schiff base 48 (1.97 g, 4.50 mmol) was added slowly as a precooled solution in CHCl₃ (20 mL) and quantitatively transferred with CHCl₃ (3 mL). The mixture was stirred at 0 °C for 2 h, followed by 10 h at room temperature, upon which a precipitate had formed. Hexanes (50 mL) were added, and the precipitate was collected, washed with 20% hexanes in CHCl₃ (50 mL), and dried to yield the title compound as a light yellow amorphous solid (2.34 g, 79%): mp 168–169 °C (dec). IR (KBr) 3583, 2937, 1736, 1597, 1513, 1426, 1286, 1265, 1138, 1023, 665 cm⁻¹; ¹H NMR (CDCl₃ + DMSO-*d*₆, 300 MHz) δ 7.53 (s, 1 H), 7.30–7.20 (m, 10 H), 7.10 (s, 1 H), 6.70 (s, 1 H), 6.63 (s, 1 H), 4.94 (s, 2 H), 4.90 (d, *J* = 7.2 Hz, 1 H), 4.83 (s, 2 H), 4.50 (d, *J* = 7.4 Hz, 1 H), 3.83 (s, 3 H), 3.76 (s, 3 H), 3.33 (m, 2 H), 2.93 (m, 2 H), 2.00 (m, 2 H); ESIMS *m/z* (rel intensity) 660/662 (MH⁺, 100/97). Anal. Calcd for C₃₅H₃₄BrNO: C, 63.64; H, 5.19; N, 2.12. Found: C, 63.46; H, 5.21; N, 2.12.

8,9-Dibenzoyloxy-6-(3-bromopropyl)-5,6-dihydro-2,3-dimethoxy-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (50). Compound 49 (1.00 g, 1.51 mmol) was cooled to –4 °C and then diluted with SOCl₂ (25 mL) and stirred for 3 h. After that the reaction mixture was stirred at room temperature for an additional 4 h, upon which it had become a bright reddish-purple color. The SOCl₂ was evaporated. The residue was dissolved in CHCl₃ (40 mL), and the solution was neutralized by the slow addition of saturated aqueous NaHCO₃ (40 mL). The layers were separated, and the aqueous layer was extracted with CHCl₃ (30 mL). The organic layers were washed with H₂O (50 mL) and saturated aqueous NaCl (50 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated. The residue was purified by flash column chromatography (SiO₂), eluting with CHCl₃ to yield a reddish-purple solid (0.610 g, 63%): mp 214–215 °C. IR (KBr) 2939, 1688, 1643, 1613, 1591, 1552, 1494, 1432, 1301, 1265, 1207, 1015, 737, 665 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz) δ 7.99 (s, 1 H), 7.58 (s, 1 H), 7.46–7.29 (m, 10 H), 7.20 (s, 1 H), 7.18 (s, 1 H), 5.26 (s, 2 H), 5.22 (s, 2 H), 4.51 (t, *J* = 7.3 Hz, 2 H), 4.02 (s, 3 H), 3.95 (s, 3 H), 3.58 (t, *J* = 6.2 Hz, 2 H), 2.33 (m, 2 H); ESIMS *m/z* (rel intensity) 639/641 (M⁺, 2/2).

8,9-Dibenzoyloxy-5,6-dihydro-6-[3-(1*H*-imidazol-1-yl)propyl]-2,3-dimethoxy-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (51a). Compound 50 (0.500 g, 0.782 mmol) was dissolved in dioxane (75 mL). NaI (0.971 g, 6.52 mmol) and imidazole (0.443 g, 6.52 mmol) were added at room temperature, and the reaction mixture was

heated at 70 °C for 20 h. The reaction mixture was cooled to room temperature and concentrated. H₂O (75 mL) was added, and the mixture was extracted with CHCl₃ (2 × 100 mL). The combined organic layer was washed with saturated aqueous NaCl (50 mL). The organic layer was concentrated and the crude mixture was purified by silica gel flash column chromatography, eluting with 2% MeOH in CHCl₃ to yield the product (0.320 g, 67%) as a purple solid: mp 235–236 °C. IR (KBr) 2961, 1689, 1648, 1589, 1553, 1493, 1433, 1398, 1300, 1206, 1021, 747 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz) δ 7.97 (s, 1 H), 7.57 (s, 1 H), 7.51 (s, 1 H), 7.47–7.33 (m, 10 H), 7.20 (s, 1 H), 7.06 (s, 1 H), 6.95 (s, 1 H), 6.72 (s, 1 H), 5.23 (s, 2 H), 5.19 (s, 2 H), 4.35 (t, *J* = 6.3 Hz, 2 H), 4.02 (s, 3 H), 3.96 (m, 2 H), 3.94 (s, 3 H), 1.98 (m, 2 H); ESIMS *m/z* (rel intensity) 628 (MH⁺, 100), 1255 (2MH⁺, 26). Anal. Calcd for C₃₈H₃₃N₃O₆: C, 72.71; H, 5.30; N, 6.69. Found: C, 72.30; H, 5.29; N, 6.62.

8,9-Dibenzoyloxy-5,6-dihydro-2,3-dimethoxy-6-[3-(*N*-morpholino)propyl]-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline (51b). Compound 50 (0.250 g, 0.392 mmol) was dissolved in dioxane (50 mL). NaI (0.466 g, 3.92 mmol) and morpholine (0.271 g, 3.12 mmol) were added at room temperature, and the reaction mixture was heated at 65 °C for 20 h. The reaction mixture was cooled to room temperature and concentrated. H₂O (75 mL) was added, and the mixture was extracted with CHCl₃ (2 × 100 mL). The combined organic layer was washed with saturated aqueous NaCl (50 mL). The organic layer was concentrated and the crude mixture was purified by flash column chromatography (SiO₂), eluting with 1% MeOH in CHCl₃ to yield the product (0.150 g, 60%) as a brown solid: mp 208–210 °C. IR (KBr) 2827, 1676, 1649, 1612, 1591, 1552, 1496, 1380, 1300, 1254, 1206, 1117, 870, 697 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz) δ 8.01 (s, 1 H), 7.60 (s, 1 H), 7.46 (m, 10 H), 7.22 (s, 1 H), 7.03 (s, 1 H), 5.24 (s, 1 H), 5.23 (s, 1 H), 4.39 (m, 2 H), 4.03 (s, 3 H), 3.95 (s, 3 H), 3.62 (m, 4 H), 2.41 (m, 6 H), 1.87 (m, 2 H); ESIMS *m/z* (rel intensity) 647 (MH⁺, 100). Anal. Calcd for C₃₉H₃₈N₂O₇·0.75H₂O: C, 70.95; H, 6.03; N, 4.24. Found: C, 71.04; H, 5.86; N, 4.23.

6-(3-(1*H*-imidazol-1-yl)propyl)-8,9-dihydroxy-2,3-dimethoxy-5*H*-indeno[1,2-*c*]isoquinoline-5,11(6*H*)-dione (52a). Compound 51a (0.100 g, 0.159 mmol) was diluted in 48% aqueous HBr (25 mL), and the solution was heated at 70 °C for 15 h. After the mixture was cooled to room temperature, chloroform and acetone (10 mL each) were added to the reaction mixture and removed on the rotary evaporator three times. The leftover solid in water was cooled to 0 °C and then filtered off. The solid was washed with 10% MeOH in chloroform (25 mL) and with acetone (50 mL) to afford catechol 52a (0.068 g, 81%) as a light brown solid: mp 273–274 °C. IR (KBr) 3230, 1688, 1628, 1577, 1557, 1425, 1303, 1247, 865, 782, 665 cm⁻¹; ¹H NMR (DMSO-*d*₆, 300 MHz) δ 9.14 (s, 1 H), 7.91 (s, 1 H), 7.84 (s, 1 H), 7.67 (s, 1 H), 7.47 (s, 1 H), 7.07 (s, 1 H), 6.94 (s, 1 H), 4.42 (m, 4 H), 3.88 (s, 3 H), 3.84 (s, 3 H), 2.33 (m, 2 H); ¹³C NMR (DMSO-*d*₆, 125 MHz) δ 190.1, 161.6, 154.4, 154.3, 149.0, 148.3, 146.5, 135.5, 128.3, 127.8, 126.6, 121.9, 119.8, 115.7, 112.8, 111.6, 107.7, 106.2, 102.1, 55.6, 55.4, 46.5, 40.8, 29.9; ESIMS *m/z* (rel intensity) 448 (MH⁺, 100); HRESIMS *m/z* 448.1514 (MH⁺), calcd for C₂₄H₂₂N₂O₆ 448.1509. Purity was estimated to be 98.1% by HPLC in 85% MeOH/1% TFA–15% H₂O and 98.3% in 70% MeOH/1% TFA–30% H₂O.

8,9-Dihydroxy-2,3-dimethoxy-6-(3-morpholinopropyl)-5*H*-indeno[1,2-*c*]isoquinoline-5,11(6*H*)-dione (52b). Compound 51b (0.125 g, 0.195 mmol) was dissolved in a mixture of MeOH and THF (20 and 10 mL) and hydrogenated (with a balloon) in the presence of 10% Pd–C (10 mg) for 24 h. The Pd–C was filtered off and the filtrate was concentrated and purified by flash column chromatography (SiO₂), eluting with 7% MeOH in CHCl₃ to provide the product 52b (0.041 g, 47%) as brown solid: mp 285–287 °C. IR (KBr) 3217, 2927, 1645, 1614, 1591, 1550, 1520, 1471, 1354, 1322, 1253, 1216, 870, 785, 657 cm⁻¹; ¹H NMR (CDCl₃ + CD₃OD, 300 MHz) δ 7.88 (s, 1 H), 7.49 (s, 1 H), 7.01 (s, 1 H), 6.90 (s, 1 H), 4.36 (m, 2 H), 3.94 (s, 1 H), 3.88 (s, 1 H), 3.68 (t, *J* = 4.4 Hz, 2 H), 2.51 (m, 6 H), 2.02 (m, 2 H); ¹³C NMR (DMSO-*d*₆, 125 MHz) δ 190.2, 162.0, 154.8, 154.5, 149.4, 148.7, 146.8, 128.6, 128.2, 126.9, 116.1, 113.1, 111.9, 108.1, 106.5, 102.4, 63.7, 55.9, 55.8, 53.7, 51.4, 41.1, 23.9; ESIMS *m/z* (rel intensity) 467 (MH⁺, 100); HRESIMS *m/z* 467.1824 (MH⁺), calcd

for $C_{25}H_{27}N_4O_7$, 467.1818. Purity was estimated to be 97.7% by HPLC in 85% MeOH/1% TFA–15% H_2O and 98.8% in 70% MeOH/1% TFA–30% H_2O .

Metabolism of LMP400 and LMP776 by Human Liver Microsomes.³⁸ Pooled human liver microsomes were purchased from Life Technologies (Grand Island, NY). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All organic solvents were HPLC grade or higher and were purchased from Fisher Scientific (Hanover Park, IL).

Compound 7 (LMP400) or 6 (LMP776) (10 μM) was incubated at 37 °C with pooled human liver microsomes (15 donors, mixed gender) containing 1 mg/mL of microsomal protein and 50 mM phosphate buffer at pH 7.4 in a total volume of 200 μL . After a 5 min preincubation, 1 mM NADPH was added to initiate reaction, and the mixture was incubated for an additional 60 min. The reaction was stopped by chilling the mixture on ice and by addition of 20 μL of ice-cold acetonitrile/water/formic acid (86:10:4, v/v/v) to precipitate proteins. Samples were centrifuged. Supernatants were removed, evaporated to dryness under nitrogen, and the residues were dissolved in the mobile phase prior to analysis using liquid chromatography–mass spectrometry (LC–MS) and LC–tandem mass spectrometry (LC–MS/MS). Control incubations were identical except for the elimination of microsomal protein or NADPH.

LC–MS and LC–MS/MS. Analyses of LMP400 or LMP776 and their metabolites were carried out using a Waters (Milford, MA) 2690 HPLC system equipped with a Waters Xterra 2.1 mm \times 100 mm C_{18} column. The solvent system consisted of a linear gradient from 0.1% formic acid in water to methanol as follows: 20–45% methanol over 10 min, 45–90% methanol over 1 min, and isocratic 90% methanol for another 2 min. The column was equilibrated with 20% methanol for at least 10 min between analyses. The flow rate was 0.2 mL/min, and the column temperature was 33 °C. The HPLC was interfaced with a high resolution Waters Q-TOF Synapt hybrid quadrupole/time-of-flight mass spectrometer, and positive ion electrospray was used for sample ionization. For accurate mass measurements, leucine enkephalin ($[M + H]^+$ of m/z 556.2771) was introduced postcolumn as a lock mass. The mass accuracy obtained was <5 ppm. Data were acquired from m/z 100–700. Tandem mass spectra were acquired at a collision energy of 20 eV using argon as the collision gas at a pressure of 2.0×10^{-5} mbar.

Topoisomerase I Mediated DNA Cleavage Reactions. Human recombinant Top1 was purified from baculovirus as previously described.⁶⁸ DNA cleavage reactions were prepared as previously reported²⁶ with the exception of the DNA substrate.⁶⁹ Briefly, a 117 bp DNA oligonucleotide (Integrated DNA Technologies) encompassing the previously identified Top1 cleavage sites in the 161 bp fragment from pBluescript SK(–) phagemid DNA was employed. This 117 bp oligonucleotide contains a single 5'-cytosine overhang, which was 3'-end-labeled by fill-in reaction with [α -³²P]dGTP in React 2 buffer (50 mM Tris-HCl, pH 8.0, 100 mM MgCl₂, 50 mM NaCl) with 0.5 units of DNA polymerase I (Klenow fragment, New England BioLabs). Unincorporated ³²P-dGTP was removed using mini Quick Spin DNA columns (Roche, Indianapolis, IN), and the eluate containing the 3'-end-labeled DNA substrate was collected. Approximately 2 nM radiolabeled DNA substrate was incubated with recombinant Top1 in 10 μL of reaction buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, and 15 $\mu g/mL$ BSA] at room temperature for 20 min in the presence of various concentrations of compounds. The reactions were terminated by adding SDS (0.5% final concentration) followed by the addition of two volumes of loading dye (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). Aliquots of each reaction were subjected to 20% denaturing PAGE. Gels were dried and visualized by using a phosphorimager and ImageQuant software (Molecular Dynamics). For simplicity, cleavage sites were numbered as previously described in the 161 bp fragment.⁶⁸

Docking and Modeling Studies. The Top1 crystal structure for docking was prepared, and the docking protocol was validated as previously described.⁴⁶ The ternary complex centroid coordinates for docking were defined using the ligand in the Top1/topotecan crystal structure (PDB code 1K4T) as the center of the binding pocket ($x =$

21.3419, $y = -3.9888$, $z = 28.2163$). The ligand was then deleted. Indenoisoquinolines to be modeled were constructed in SYBYL. Atom types were assigned using SYBYL atom typing. Hydrogens were added, and the ligands were minimized by conjugate gradient method using the MMFF94s force field with MMFF94 charges, a distance-dependent dielectric function, and a 0.01 kcal mol⁻¹ Å⁻¹ energy gradient convergence criterion. Each ligand was docked into the mutant crystal structure using GOLD 3.2 using default parameters, and the coordinates were defined by the crystal structure as described above. The top four poses for each ligand were examined (because lower-ranked poses with much lower GoldScores were sometimes “flipped” or rotated binding modes). The highest-ranked poses for these ligands were merged into the crystal structure, and the entire complex was subsequently subjected to minimization using a standard Powell method, the MMFF94s force field and MMFF94 charges, a distance-dependent dielectric function, and a 0.05 kcal mol⁻¹ Å⁻¹ energy gradient convergence criterion. During the minimization, the ligand and a 7 Å sphere surrounding the ligands were allowed to move while the structures outside this sphere were frozen in an aggregate.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; GOLD, Genetic Optimisation for Ligand Docking (program); MDMA, 3,4-methylenedioxy-*N*-methylamphetamine; MDPPA, 3',4'-methylenedioxy- α -pyrrolidinopropiophenone; MGM, mean-graph midpoint; MMFF94, Merck Molecular Force Field 94; NCI-60, National Cancer Institute 60 cell line screening assay; Top1, topoisomerase 1

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