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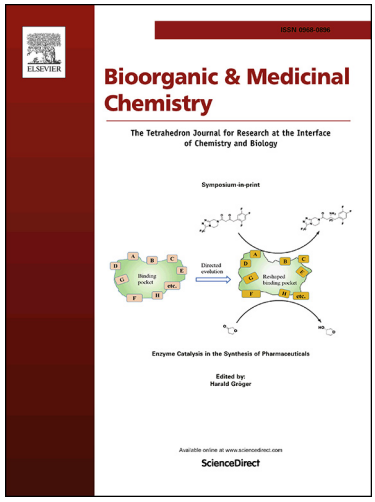

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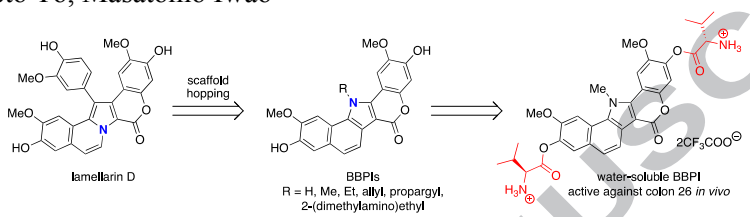

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Lamellarin-inspired potent topoisomerase I inhibitors with the unprecedented benzo[*g*][1]benzopyrano[4,3-*b*]indol-6(13*H*)-one scaffold

Tsutomu Fukuda [a], Yusuke Nanjo [a], Masahiro Fujimoto [a], Kenyu Yoshida [a], Yuko Natsui [a], Fumito Ishibashi [b], Fumiyasu Okazaki [c], Hideto To [c], Masatomo Iwao [a,*]

[a]*Division of Chemistry and Materials Science, Graduate School of Engineering, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*
[b]*Division of Marine Life Science and Biochemistry, Graduate School of Fisheries and Environmental Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*
[c]*Department of Medical Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*

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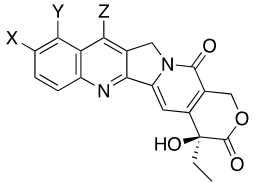
ABSTRACT

A new class of topoisomerase I inhibitors containing the unprecedented benzo[*g*][1]benzopyrano[4,3-*b*]indol-6(13*H*)-one (abbreviated as BBPI) ring system have been developed based on structure-activity relationship studies of the cytotoxic marine alkaloid lamellarin D. The pentacyclic BBPI scaffold was constructed from *N*-*tert*-butoxycarbonylpyrrole by sequential and regioselective functionalization of the pyrrole core using directed lithiation, conventional electrophilic substitution, and palladium-catalyzed cross-coupling reactions. Further *N*-alkylation of the scaffold followed by selective deprotection of the *O*-isopropyl group produced a range of *N*-substituted BBPI derivatives. The BBPIs thus prepared exhibited potent topoisomerase I inhibitory activity in DNA relaxation assays. The activities of BBPIs were higher than those of lamellarin D and camptothecin; they showed potent and selective antiproliferative activity in the panel of 39 human cancer cell lines established by Japanese Foundation for Cancer Research. COMPARE analyses indicated that the inhibition patterns of the BBPIs correlated well with those of the known topoisomerase I inhibitors such as SN-38 and TAS-103. The water-soluble valine ester derivative exhibited antitumor activity *in vivo* against murine colon carcinoma colon 26. The activity was comparable to that of the approved anticancer agent irinotecan.

1. Introduction

Topoisomerase I is a nuclear enzyme that mediates the relaxation of supercoiled DNA during replication, transcription, and other nuclear processes via reversible DNA single strand cleavage and religation.[1–3] Mechanistically, this process involves a simple transesterification between the phosphodiester bond of DNA and the tyrosine 723 hydroxyl group of topoisomerase I. Topoisomerase I inhibitors stabilize the transient topoisomerase I–DNA covalent complex and inhibit the subsequent religation step. This action of the inhibitors causes single-strand DNA breakages that are then transformed into double-strand breakages lethal for growing cells. Therefore, topoisomerase I inhibitors can be regarded as effective anticancer agents. In fact, the plant alkaloid camptothecin (**1**)-derived topoisomerase I inhibitors such as topotecan (**2**) and irinotecan (**3**) have been approved for the treatment of a wide range of cancers.[4] Compound **3** is a water-soluble prodrug that is used to facilitate the administration of the parental potent inhibitor SN-38 (**4**) (Fig. 1). After the success of these drugs, structurally unique non-camptothecin-type topoisomerase I inhibitors such as indolocarbazoles, indenoisoquinolines, benzophenanthridines, dibenzonaphthylidines have been developed[5] and some of them are even being clinically tested.



camptothecin (**1**): X = Y = Z = H

topotecan (**2**): X = OH, Y = CH_2NMe_2, Z = H

irinotecan (**3**): X = OCO–N⟨⟩–N⟨⟩, Y = H, Z = Et

SN-38 (**4**): X = OH, Y = H, Z = Et

Fig. 1. Camptothecin family of topoisomerase I inhibitors.

* Corresponding author.
E-mail address: iwao@nagasaki-u.ac.jp (M. Iwao).

Lamellarins are biologically active marine alkaloids that contain a common 14-phenyl-6*H*-[1]benzopyrano[4′,3′:4,5]pyrrolo[2,1-*a*]isoquinoline scaffold.[6–8] The anticancer activity of lamellarins was first discovered by Quesada *et al.* in 1996.[9] They showed that the triacetates of lamellarins D (**5**), K, and N exhibit potent cytotoxicity against a range of cancer cell lines including the multidrug-resistant (MDR) phenotype. In 1997, we achieved the first total synthesis of **5** via *N*-ylide-mediated cyclization as a key reaction.[10] Subsequently, we prepared several non-natural analogues of **5** and conducted a structure–activity relationship (SAR) study.[11] It was confirmed that **5** is quite cytotoxic (IC_{50} = 10.5 nM in colony formation assay using HeLa cell line) and its activity is dependent on the substitution pattern of the oxygen functionalities on the lamellarin scaffold. Namely, the hydroxy groups at C8 and C20 are essential for realizing potent cytotoxicity, whereas the hydroxy group at C14 is less important. Later, Ploypradith and coworkers performed a more precise SAR study using more lamellarins and various cancer cell lines, and confirmed our results.[12]

In 2003, Bailly and coworkers reported that lamellarin D (**5**) was a potent inhibitor of topoisomerase I.[13] The good correlation between the cytotoxicity and topoisomerase I inhibitory activities of lamellarin D analogues suggested that the principal cellular target of **5** is topoisomerase I.[14] Moreover, Bailly et al. proposed a theoretical model of lamellarin D (**5**)–DNA–topoisomerase I ternary complex that explained the results of the SAR studies.[13,14] According to this model, **5** intercalates at the +1 (C·G) and −1 (A·T) cleavage site of the DNA–topoisomerase I covalent complex and is stabilized by hydrogen bonding interactions with three specific amino acid residues of topoisomerase I. The hydroxy groups at C8 and C20 and the carbonyl oxygen of the lactone ring are at a hydrogen bonding distance from Asn722, Glu356, and Arg364, respectively. On the other hand, the aryl ring at C1 (F-ring) is directed toward the major groove cavity and does not have any direct interaction with the enzyme. This model suggests that the F-ring is probably not essential for topoisomerase I inhibition and it may be possible to replace the F-ring with other groups without affecting the biological activity. We confirmed this speculation by synthesizing a series of F-ring-defected lamellarin D analogues **6** (X = H, CH_3, CH_2NMe_2, CHO, F, Cl, Br) (Fig. 2).[15] Antiproliferative activities of **6** were found to be as potent as that of the parent compound **5** in the panel of 39 human cancer cell lines established by the Japanese Foundation for Cancer Research (JFCR39).[16] The COMPARE analyses[17] of the inhibition patterns suggested that the molecular target of these analogues was also topoisomerase I.

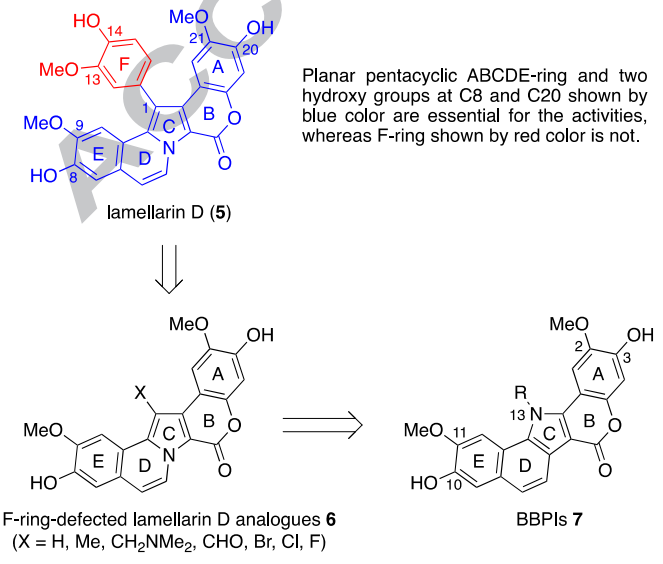


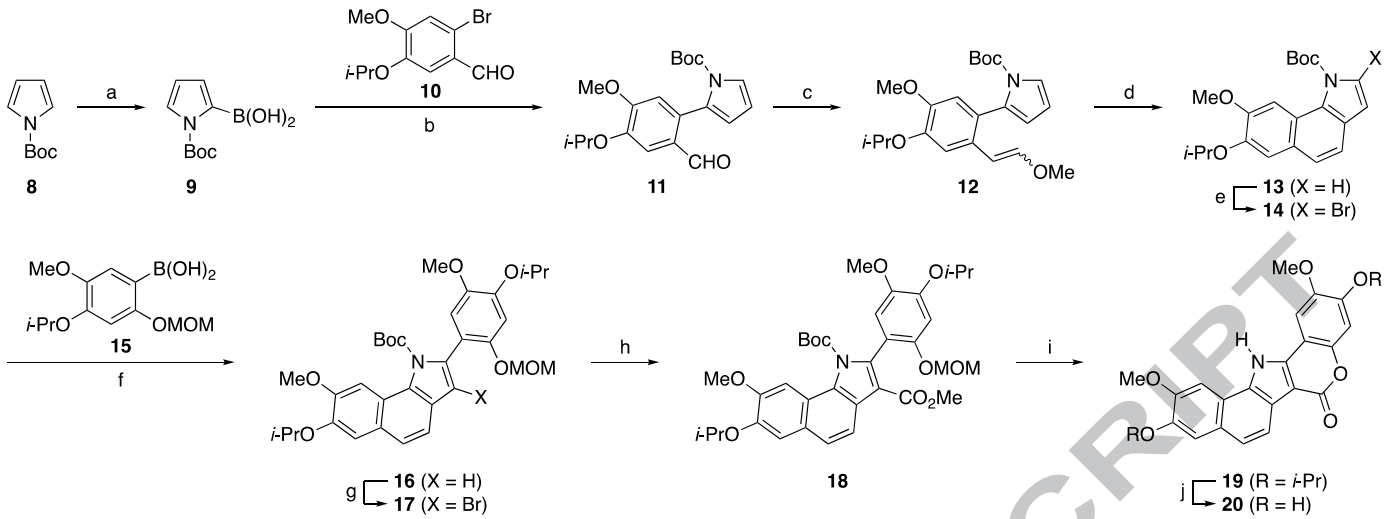
Fig. 2. Design of a new class of topoisomerase I inhibitors BBPIs **7** by structural modification of lamellarin D (**5**).

Based on these fundamental studies, we initiated a project to generate a new class of potent topoisomerase I inhibitors via scaffold hopping.[18] Among several potential candidates, we selected the benzo[*g*][1]benzopyrano[4,3-*b*]indol-6(13*H*)-one (BPPI) scaffold. This scaffold can be regarded as a regioisomer of the pentacyclic core (ABCDE-ring) of lamellarin D (**5**) with respect to the position of the ring-nitrogen (Fig. 2). Therefore, it was presumed that the BBPI derivatives **7** having two hydroxy groups at C3 and C10 and two methoxy groups at C2 and C11, respectively, could maintain the potent activity of **5**. In addition, it was anticipated that a variety of R groups could be readily introduced to the ring-nitrogen (N13) by simple alkylation and they could modulate the physical and biological properties of **7**. Based upon these assumptions, the synthesis and biological evaluation of BBPI derivatives were investigated.

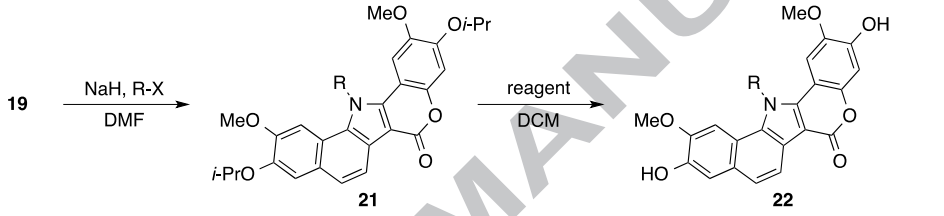
2. Results and discussion

2.1. Synthesis of BBPIs

The synthesis of *N*-unsubstituted BBPI **20** is shown in Scheme 1. The basic strategy to construct the unprecedented BBPI ring system comprises sequential and regioselective functionalization of the central pyrrole core corresponding to the C-ring via directed lithiation, conventional electrophilic substitution, and palladium-catalyzed cross-coupling reactions. Thus, directed lithiation of *N*-Boc-pyrrole (**8**) with LDA followed by the reaction with trimethyl borate afforded pyrrole-2-boronic acid **9** in 75% yield.[19] This compound was easily isolated on a preparative scale (>15 g) after a simple purification step of hexane washing of the crude product. Suzuki–Miyaura coupling of **9** with the known bromide **10**[20] produced **11** in 94% yield. The D-ring moiety of the BBPI scaffold was constructed in two steps from **11** by applying the method utilized for the synthesis of polyaromatic hydrocarbons.[21] Thus, Wittig reaction of **11** with commercially available (methoxymethyl)triphenylphosphonium chloride in the presence of *t*-BuOK produced the enol ether **12** in 93% yield as a mixture of *E*- and *Z*-isomers. Acid-catalyzed cyclization of **12** led to D-ring formation and the tricyclic benzo[*g*]indole compound **13** was obtained in 92% yield. Conventional electrophilic bromination of **13** with *N*-bromosuccinimide (NBS) in DMF afforded an approximately 1:1 mixture of **14** and the unwanted 3-bromo analogue. On the other hand, directed lithiation of **13** with *t*-BuLi followed by a reaction with 1,2-dibromo-1,1,2,2-tetrafluoroethane provided the 2-bromo compound **14** selectively in 95% yield. Suzuki–Miyaura coupling of **14** with the known boronic acid **15**[22] afforded **16** in 97% yield. Subsequent electrophilic bromination of **16** with NBS gave **17** regioselectively in 99% yield. Bromine–lithium exchange of **17** with 2 equiv. of *t*-BuLi followed by a reaction with methyl chloroformate produced the methoxycarbonylated compound **18** in 81% yield. Simultaneous deprotection of the *tert*-butoxycarbonyl (Boc) and methoxymethyl (MOM) protecting groups with hydrochloric acid led to concomitant lactonization and the formation of the key BBPI intermediate **19** in 99% yield. Final deprotection of the *O*-isopropyl groups with BCl_3 afforded *N*-unsubstituted BBPI **20**. The overall yield of **20** from commercially available **8** was 39% in ten steps. The modular nature of this synthesis warrants easy access to a wide range of BBPIs with different substitution patterns at the A- and E-rings by simple modification of the aromatic building blocks corresponding to **10** and **15**.



Scheme 1. Synthesis of *N*-unsubstituted BBPI **20**. *Reagents and conditions:* (a) (1) LDA, THF, −78 °C, 1 h, (2) $B(OMe)_3$, −78 °C, 1 h, then rt, 15 h (75%); (b) **10**, $Pd(PPh_3)_4$, Na_2CO_3, THF, water, reflux, 18 h (94%); (c) $MeOCH_2P^+Ph_3{\cdot}Cl^-$, *t*-BuOK, THF, 0 °C, 3 h (93%); (d) CH_3SO_3H, CH_2Cl_2, 0 °C, 23 h (92%); (e) (1) *t*-BuLi, THF, −78 °C, 1 h, (2) $BrCF_2CF_2Br$, −78 °C, 1 h (95%); (f) **15**, $Pd(PPh_3)_4$, Na_2CO_3, DME, water, reflux, 23 h (97%); (g) NBS, DMF, 0 °C, 6.5 h (99%); (h) (1) *t*-BuLi, THF, −78 °C, 1 h, (2) $ClCO_2Me$, −78 °C, 1 h (81%); (i) HCl, MeOH, $CHCl_3$, 50 °C, 24.5 h (99%); (j) BCl_3, DCM, −78 °C to rt (88%).



21	R	R-X	yield
21a	Me	MeI	88%
21b	Et	EtI	58%
21c	$CH_2CH{=}CH_2$	$H_2C{=}CHCH_2Br$	74%
21d	$CH_2C{\equiv}CH$	$HC{\equiv}CCH_2Br$	65%
21e	$CH_2CH_2NMe_2$	$Me_2NCH_2CH_2Cl$	47%

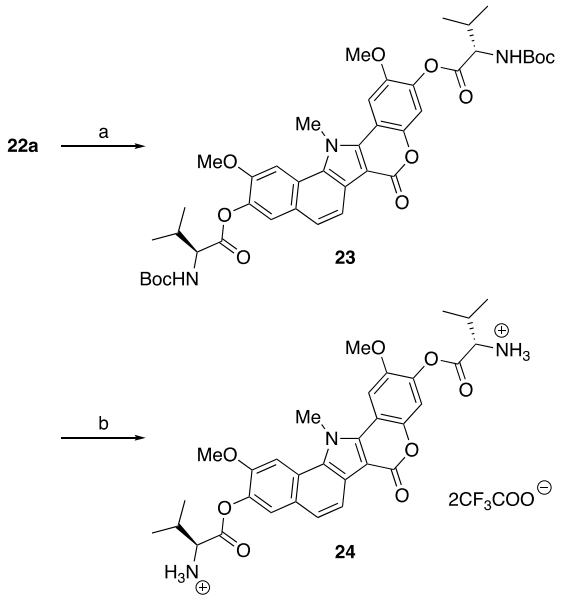
22	R	reagent	yield
22a	Me	BCl_3	94%
22b	Et	BCl_3	94%
22c	$CH_2CH{=}CH_2$	BCl_3	50%
22d	$CH_2C{\equiv}CH$	BCl_3	63%
22e[a]	$CH_2CH_2NMe_2$	$AlCl_3$	86%

[a]isolated as a TFA salt

Scheme 2. Synthesis of *N*-substituted BBPIs **22a**–**e**.

The synthesis of *N*-substituted BBPIs **22a**–**e** is shown in Scheme 2. *N*-Alkylation of **19** with a range of commercially available alkylating agents such as methyl iodide, ethyl iodide, allyl bromide, propargyl bromide, and 2-(dimethylamino)ethyl chloride in the presence of sodium hydride in DMF afforded **21a**–**e** in good to modest yields. Care must be taken in the synthesis of **21e** to preclude water from the hygroscopic 2-(dimethylamino)ethyl chloride hydrochloride. The yield was considerably decreased when the wet reagent was employed probably because of hydrolytic ring opening of the lactone moiety of the starting material. The *O*-isopropyl groups of **21a**–**d** were selectively deprotected by BCl_3 to give **22a**–**d**, respectively. An attempt to deprotect **21e** with BCl_3 produced **22e** in very low yield (15%) together with an unidentified poorly soluble solid. Fortunately, however, **22e** was obtained in good yield by deprotection with $AlCl_3$.

The BBPIs **20** and **22** thus synthesized were essentially insoluble in water, except for the trifluoroacetic acid (TFA) salt **22e**. The aqueous insolubility is a major concern for *in vivo* biological evaluations of BBPIs. Bailly and coworkers have previously reported that the cationic amino acid ester derivatives of lamellarin D (**5**) maintain the topoisomerase I inhibitory and cytotoxic activities.[23] Especially, the activities of the valine ester are equally potent as those of the parent compound **5**. Based on these important findings, we prepared the valine ester **24** in two steps (Scheme 3). Condensation of **22a** and *N*-Boc-*L*-valine with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) in the presence of 4-(*N,N*-dimethylamino)pyridine



Scheme 3. Synthesis of the water-soluble valine ester derivative **24**. *Reagents and conditions:* (a) *N*-Boc-*L*-valine, EDC·HCl, DMAP, rt, 24 h (66%); (b) TFA, rt, 1 h (quant).

(DMAP) produced bis-*O*-valinate **23** in 66% yield. Subsequent acid-promoted cleavage of the *N*-Boc groups with TFA gave the desired compound **24** in quantitative yield. This cationic species was readily soluble in water and suitable for *in vivo* biological evaluations.

Finally, we performed the X-ray crystallographic analysis of the *N*-methylated BBPI **21a**. The ORTEP drawing shows that the fused pentacyclic core of **21a** is essentially planar as expected (Fig. 3). However, it is interesting to note that the methyl group at N13 is slightly bent (*ca.* 9.5°) from the plane of the BBPI core. This unexpected structure may be a result of severe steric hindrance between the methyl group at N13 and the *peri*-hydrogens at C1 and C12.

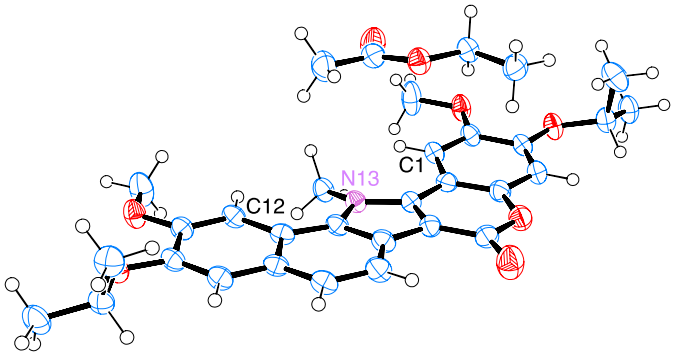


Fig. 3. ORTEP drawing of **21a**·AcOEt. Thermal ellipsoids are drawn at 50% probability.

2.2. Topoisomerase I inhibitory activity

Topoisomerase I inhibitory activities of the selected BBPIs **20**, **22a**, **22e**, and **24** were evaluated in DNA relaxation assay[24] using supercoiled pBR322 plasmid DNA and topoisomerase I isolated from calf thymus (Fig. 4). Camptothecin (**1**) and lamellarin D (**5**) were used for the positive controls. The experiments were performed at 10 μM concentration of the inhibitors. The supercoiled DNA changed to the relaxed DNA in the absence of an inhibitor, which appeared as multiple bands corresponding to the topoisomers of the different linking numbers in the agarose gel electrophoresis, while the treatment with topoisomerase I

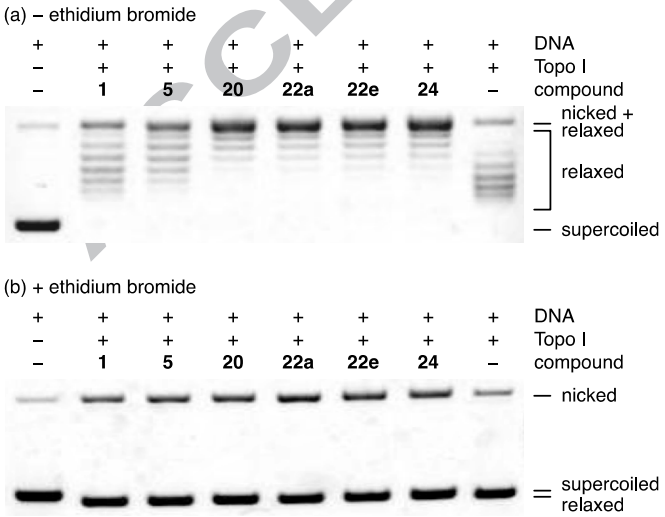


Fig. 4. DNA relaxation assay of the BBPIs **20**, **22a**, **22e**, and **24**. All inhibitors including camptothecin (**1**) and lamellarin D (**5**) were used at 10 μM. Agarose gel electrophoresis was performed in the absence or presence of ethidium bromide.

inhibitors resulted in accumulation of the nicked DNA. The tested BBPIs induced the intense bands corresponding to the nicked + relaxed DNA and the reduced number of the bands corresponding to the relaxed topoisomers (Fig. 4a). The enzyme inhibitory activities of the BBPIs appear to be higher than those of camptothecin (**1**) and lamellarin D (**5**).

Next, we performed the agarose gel electrophoresis in the presence of ethidium bromide in order to exclude the possibility that the BBPIs act as non-specific inhibitors of the enzyme.[25] It has been established that the bands corresponding to the nicked DNA and the relaxed DNA can be clearly separated in the presence of ethidium bromide.[24] As shown in Fig. 4b, the intense nicked DNA bands were induced by all BBPIs as well as by the reference compounds. The result indicates that the BBPIs are the specific inhibitors, like camptothecin (**1**) and lamellarin D (**5**), to stabilize the DNA–topoisomerase I cleavable complex and induce single-strand DNA breakage.

2.3. Docking study

In order to speculate how BBPIs interfere with the enzymatic action of topoisomerase I, we performed *in silico* docking simulations using single X-ray crystallographic data of the topotecan (**2**)–DNA–topoisomerase I ternary complex.[26] The interfacial inhibitor **2** was replaced by docking with BBPI **22a** and the resulting complex was minimized by the Molecular Operating Environment (MOE) program.[27] The two most plausible models (poses A and B) are shown in Fig. 5. In the case of pose A, the pentacyclic core (ABCDE-ring) of BBPI **22a** intercalated at the DNA cleavage site in such a way that the methyl group at N13 was directed toward the DNA major groove cavity. The A-ring pointed in toward the backbone of the DNA non-scissile strand whereas the E-ring was oriented toward the DNA cleaved strand. The pentacyclic core was stabilized by both +1 (C·G) and −1 (A·T) base pairs by forming stacking interactions. The hydroxy groups at C3 and C10 and the carbonyl oxygen of the lactone ring were hydrogen-bonded with Asn722, Glu356, and Arg364, respectively. The docking score of pose A was estimated to be −9.58 kcal/mol. In the case of pose B, as compared to pose A, the pentacyclic core was turned around and the orientations of the A- and E-rings were reversed. Similar hydrogen bonding interactions were observed between the hydroxy group at C10 and Asn722 and between the carbonyl oxygen and Arg364 while the hydroxy group at C3 was not hydrogen-bonded to Glu356 owing to the longer distance between O3 and the carboxylate oxygen of Glu356 (ca. 4.0 Å). Despite the lack of the hydrogen bonding interaction, the docking score of pose B was scarcely affected (−9.47 kcal/mol) because the additional stacking interactions stabilized this orientation. The small difference of the docking scores (0.11 kcal/mol) may indicate that both poses A and B are possible.

2.4. In vitro antiproliferative activity

The antiproliferative activities of all synthesized BBPIs, i.e., **20**, **22a**–**e**, and **24**, were evaluated on a panel of JFCR39.[17] The 50% growth-inhibitory concentration (GI_{50}) values of the selected nine cell lines and the mean-graph midpoint (MG-MID) of the average GI_{50} values across the entire panel of JFCR39 are shown in Table 1. For comparison, the activities of SN-38 (**4**) and lamellarin D (**5**) have also been included. All the *N*-substituted BBPIs **22a**–**e** and **24** exhibited potent activities at low nanomolar concentrations (MG-MID = 22.9–89.1 nM). Among them, the activities of **22a** (R = Me), **22b** (R = Et), **22e** (R = $CH_2CH_2NMe_2$), and **24** (valine ester) were shown to be higher than that of SN-38 (**4**) by comparison of their MG-MID values. The BBPIs **22c** and **22d**, which had an unsaturated double or triple bond in the *N*-substituent, were somewhat less potent. In

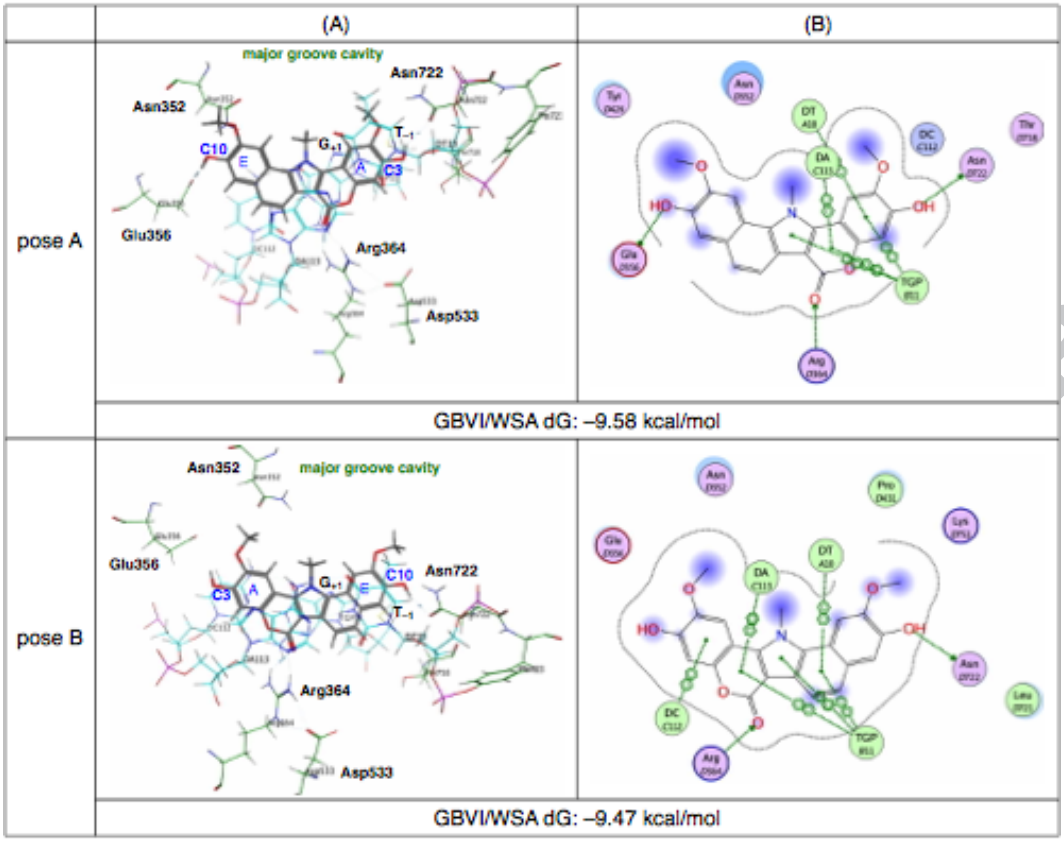


Fig. 5. Plausible docking models of the BBPI **22a**–DNA–topoisomerase I ternary complex. (A) View along the axis of the DNA double helix; (B) ligand–receptor interaction diagram.

Table 1

In vitro antiproliferative activities of BBPIs **20**, **22a–e**, and **24** against selected human cancer cell lines.

Human tumor cell line		Antiproliferative activity (GI_{50} in nM)[a]								
		20[e]	**22a**[f]	**22b**[e]	**22c**[g]	**22d**[e]	**22e**[g]	**24**[g]	SN-38 (**4**)[f]	lamellarin D (**5**)[e]
Breast	MCF-7	68	9.6	<10	14.0	18	2.6	6.1	3.0	<10
CNS	U251	<10	1.3	<10	7.6	<10	4.0	3.4	2.8	<10
Colon	HCT-116	88	8.8	<10	25	34	9.7	15	23	<10
Lung	NCI-H522	<10	1.8	<10	9.5	<10	3.9	3.4	3.7	<10
Melanoma	LOX-IMVI	21	2.6	<10	6.4	<10	2.7	2.5	5.3	<10
Ovarian	SK-OV-3	330	9.4	<10	29	56	26	20	23	38
Renal	ACHN	110	<1.0	<10	4.2	<10	1.9	2.9	5.9	<10
Stomach	MKN28	1600	33	<10	82	66	67	41	110	50
Prostate	DU-145	51	2.5	<10	6.5	27	2.0	3.2	4.3	<10
MG-MID[b]		660.7	22.9	24.5	58.9	89.1	30.9	27.5	40.7	41.7
Delta[c]		1.82	1.36	0.39	1.15	0.95	1.26	1.04	1.26	0.62
Range[d]		4.00	4.00	3.11	2.37	3.11	2.77	2.60	2.91	2.30

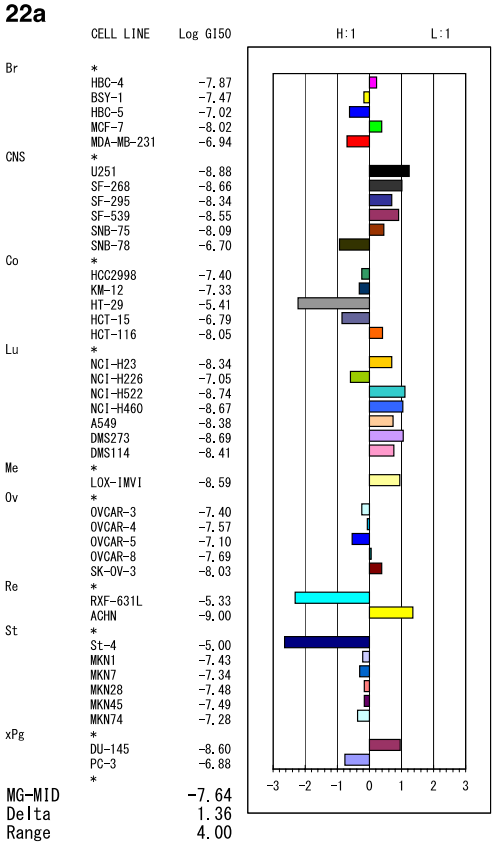
[a] Concentration for 50% inhibition of cell growth relative to control. Cell growth was determined according to sulforhodamine B assay.
[b] Mean GI_{50} value in all cell lines tested.
[c] Difference in log GI_{50} value between the most sensitive cells and the MG-MID value.
[d] Difference in log GI_{50} value between the most and least sensitive cells.
[e] The GI_{50} value was obtained from the dose-response curve in the test range between 10^{-4}–10^{-8} M.
[f] The GI_{50} value was obtained from the dose-response curve in the test range between 10^{-5}–10^{-9} M.
[g] The GI_{50} value was obtained from the dose-response curve in the test range between 10^{-6}–10^{-10} M.

contrast to the high activities of the *N*-substituted BBPIs, the activity of *N*-unsubstituted BBPI **20** was much lower (MG-MID = 660.7 nM). The diminished activity may be accounted for by considering the factors unique to living cells such as cell membrane permeability, since **20** showed good topoisomerase I inhibitory activity like the *N*-substituted BBPIs in the DNA relaxation assay.

The COMPARE analyses[17] of the chemosensitivity patterns of the BBPIs against a panel of JFCR39 suggested that the major cellular target of these compounds was topoisomerase I. For typical examples, the mean graph patterns, also called fingerprints, of **22a** and **24** were derived from their log GI_{50} values against JFCR39 and are shown in Fig. 6. The patterns of these compounds showed good correlation to those of the known topoisomerase I inhibitors such as SN-38 (**4**) and TAS-103[28] (r = 0.726–0.793).

2.5. In vivo antitumor activity

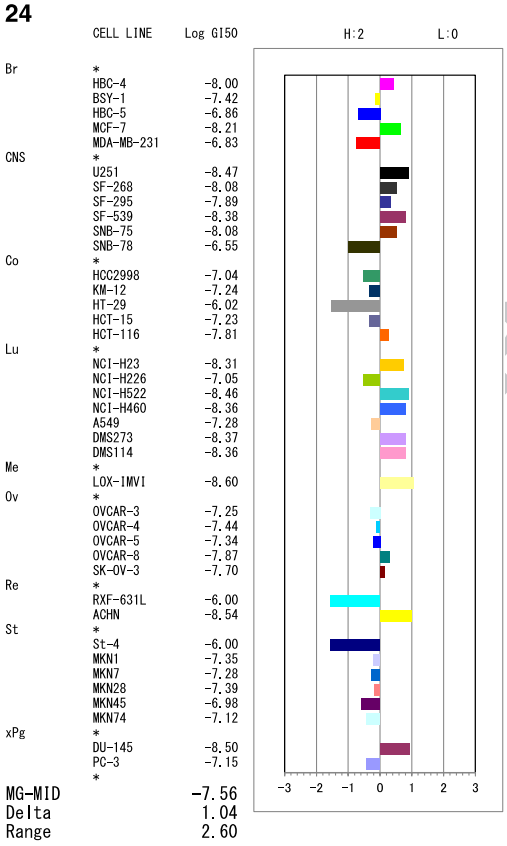
22a



Results of COMPARE:

Rank	Compounds	r	Molecular Targets / Drug Type
1	TAS-103	0.741	DNA Topoisomerase I, II Inhibitors DNA-Intercalating Drugs topoisomerase (I, II)
2	KW2170	0.735	DNA Topoisomerase Inhibitors DNA intercalater, Mitoxantron - like
3	TAS-103	0.726	DNA Topoisomerase I, II Inhibitors DNA-Intercalating Drugs topoisomerase (I , II)

24



Results of COMPARE:

Rank	Compounds	r	Molecular Targets / Drug Type
1	SN-38	0.793	DNA Topoisomerase I Inhibitors topoisomerase (I, II)
2	SN-38	0.777	DNA Topoisomerase I Inhibitors topoisomerase (I, II)
3	SN-38	0.747	DNA Topoisomerase I Inhibitors topoisomerase (I, II)

Fig. 6. Chemosensitivity patterns of BBPIs **22a** and **24** against a panel of JFCR39 and the results of COMPARE analyses. The mean graph shows the deviation of log GI_{50} value of each cell line from MG-MID. The correlation coefficient r (r = 0–1) shows the similarity of the chemosensitivity pattern of the tested compound to that of the known antitumor agent in the database.

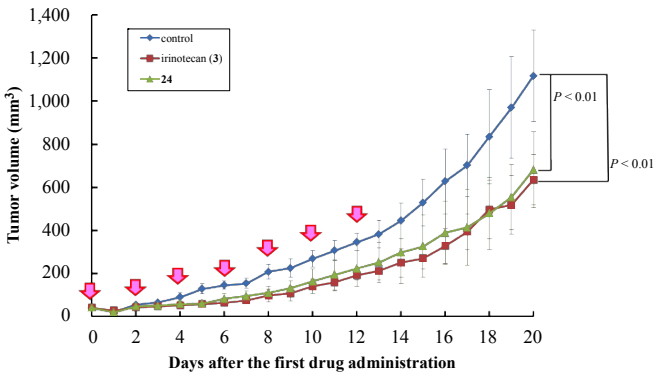


Fig. 7. *In vivo* antitumor activity of **24** in comparison with irinotecan (**3**) toward murine colon carcinoma colon 26 implanted in BALB/c mice.

Finally, we evaluated the *in vivo* antitumor activity of the water-soluble BBPI **24** against colon 26 (murine colon carcinoma cell line) implanted on the BALB/c mice (Fig. 7).[29] For comparison, the antitumor activity of irinotecan (**3**) was also estimated. One day after implantation, 50 mg/kg dose of **24** or **3** was administered intravenously every two days seven times and the tumor volumes were recorded daily until day 20. As shown in Fig. 7, **24** showed antitumor activity comparable to that of **3**. Body weight loss in the treated mice was compared with that of controls and found to be at an acceptable level, suggesting that **24** caused no serious damage to the mice at doses less than 50 mg/kg (Supplementary data).

3. Conclusion

In this study, a new class of topoisomerase I inhibitors with the benzo[*g*][1]benzopyrano[4,3-*b*]indol-6(13*H*)-one (BBPI) scaffold were designed and synthesized by structural modification of the marine natural product lamellarin D (**5**). The topoisomerase I inhibitory activities of the selected BBPIs were found to be higher than those of the reference compounds, camptothecin (**1**) and the parental **5**, in DNA relaxation assay. Although the antiproliferative activity of the *N*-unsubstituted BBPI **20** was modest, those of the *N*-substituted BBPIs **22a**, **b**, **e**, and **24** were higher than that of SN-38 (**4**) against the JFCR39 panel. Furthermore, the water-soluble BBPI **24** exhibited antitumor activity against colon 26. The activity was comparable to the approved anticancer agent irinotecan (**3**). Unlike

lamellarins, a variety of BBPIs can be produced by introducing diverse substituents on the ring nitrogen. This provides an excellent opportunity to discover more effective topoisomerase I inhibitors having improved antitumor activity and such investigations are currently in progress in our laboratories.

4. Experimental section

4.1. Synthesis–general

Melting points were determined using the Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were obtained with a Thermo Nicolet Nexus 670 NT FT-IR instrument and are reported in terms of absorption frequency (cm^{-1}). NMR spectra were recorded on a JEOL JNM-AL400 instrument (400 MHz for ^1H and 100 MHz for ^{13}C) or a Varian NMR System 500PS SN instrument (500 MHz for ^1H and 126 MHz for ^{13}C). Chemical shifts for ^1H NMR are expressed in parts per million (ppm) relative to the following internal standards: $CDCl_3$ (tetramethylsilane, δ 0.0 ppm); acetone-d_6 (tetramethylsilane, δ 0.0 ppm); DMSO-d_6 (DMSO, δ 2.50 ppm); methanol-d_4 (tetramethylsilane, δ 0.0 ppm). Data for ^1H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, dd = double doublet, t = triplet, sext = sextet, sep = septet, m = multiplet, br s = broad singlet), coupling constant (Hz), and integration. Chemical shifts for ^{13}C NMR are expressed in ppm relative to the following internal standards: $CDCl_3$ (tetramethylsilane, δ 0.0 ppm); acetone-d_6 (tetramethylsilane, δ 0.0 ppm); DMSO-d_6 (DMSO-d_6, δ 39.52 ppm). High-resolution mass spectra (HRMS) were recorded on a JEOL JMS-T100TD (direct analysis in real-time mass spectrometry, DARTMS) instrument or a JEOL JMS-700N (fast atom bombardment mass spectrometry, FABMS or electron ionized mass spectrometry, EIMS) instrument. Column chromatography was conducted using silica gel 60N, 63–210 µm (Kanto Chemical Co., Inc.) or Chromatorex NH-DM1020 (Fuji Silysia Chemical Ltd.). Flash chromatography was conducted using silica gel 60N, 40–50 µm (Kanto Chemical Co., Inc.).

*4.2. Synthesis of N-unsubstituted BBPI **20***

*4.2.1. 1-(tert-Butoxycarbonyl)-1H-pyrrole (**8**)*. To a solution of pyrrole (30.9 g, 460 mmol), Et_3N (31.9 mL, 229 mmol) and DMAP (2.81 g, 23.0 mmol) in THF (250 mL), was added dropwise via syringe a solution of Boc_2O (100 g, 458 mmol) in THF (30 mL) at 0 °C under Ar atmosphere. After the mixture had been stirred at rt for 18 h, the solvent was distilled off *in vacuo*. Distillation of the crude product yielded **8** (58.5 g, 76%) as a colorless oil, bp 80 °C/47 mmHg. ^1H NMR (500 MHz, $CDCl_3$): δ 1.59 (s, 9H), 6.21 (t, J = 2.3 Hz, 2H), 7.23 (t, J = 2.3 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 28.0, 83.6, 111.8, 120.0, 148.9. These physical and spectroscopic data are in good agreement with those previously reported.[30]

*4.2.2. [1-(tert-Butoxycarbonyl)-1H-pyrrol-2-yl]boronic acid (**9**)*. To a solution of diisopropylamine (18.2 mL, 130 mmol) in THF (450 mL), was added dropwise a hexane solution of BuLi (1.61 M, 74.8 mL, 120 mmol) at −78 °C under Ar atmosphere. The mixture was stirred at −78 °C for 5 min, gradually warmed up to 0 °C, and kept at the same temperature for 10 min. The whole was again cooled to −78 °C and a solution of **8** (16.7 g, 100 mmol) in THF (30 mL) was added dropwise. After 1 h at −78 °C, trimethyl borate (16.7 mL, 150 mmol) was added dropwise. After 1 h, the mixture was gradually warmed up to room temperature and stirred for 15 h. The reaction was then quenched by adding saturated aqueous NH_4Cl and the THF was removed *in vacuo*. The pH of the residual liquid was made 3 with AcOH and the whole was extracted 3 times with ether. The ethereal extracts were combined, washed with brine, dried (Na_2SO_4), and concentrated *in vacuo*. The residual solid was triturated with hexane and filtered to give **9** (15.6 g, 75%) as white powder. ^1H NMR (500 MHz, $CDCl_3$): δ 1.62 (s, 9H), 6.26 (t, J = 3.2 Hz, 1H), 7.10 (dd, J = 1.6 and 3.2 Hz, 1H), 7.18 (br s, 2H), 7.45 (dd, J = 1.6 and 3.2 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 28.0, 85.6, 112.0, 127.1, 128.7, 152.2. These physical and spectroscopic data are in good agreement with those previously reported.[19]

*4.2.3. 2-Bromo-5-isopropoxy-4-methoxybenzaldehyde (**10**)*. A mixture of isovanillin (25.0 g, 164 mmol), K_2CO_3 (45.1 g, 329 mmol), 2-bromopropane (23.1 mL, 247 mmol) in DMSO (300 mL) was heated at 55 °C for 2 h. After cooling, the mixture was poured into water and extracted 3 times with ether. The ethereal extracts were combined, washed successively with water, 10% NaOH solution and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The oily residue was distilled to give 3-isopropoxy-4-methoxybenzaldehyde (**10′**) (29.0 g, 91%) as a colorless oil, bp 85–87 °C/0.2 mmHg. ^1H NMR (500 MHz, $CDCl_3$): δ 1.40 (d, J = 6.1 Hz, 6H), 3.94 (s, 3H), 4.65 (sep, J = 6.1 Hz, 1H), 6.98 (d, J = 8.2 Hz, 1H), 7.42 (d, J = 1.7 Hz, 1H), 7.45 (dd, J = 1.7 and 8.2 Hz, 1H), 9.84 (s, 1H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 21.9, 56.2, 71.3, 110.9, 112.7, 126.5, 130.1, 147.9, 155.7, 191.0. These physical and spectroscopic data are in good agreement with those previously reported.[31]

To a solution of **10′** (19.4 g, 99.9 mmol) in DMF (50 mL), was added dropwise a solution of NBS (35.6 g, 199 mmol) in DMF (100 mL) at room temperature. After 19.5 h, the mixture was diluted with water, extracted with ether, washed successively with Na_2SO_3 solution, water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The crude product was purified by recrystallization from ether–hexane to give **10** (17.0 g, 62%) as pale brown needles. The mother liquor was evaporated and the residue was chromatographed on silica gel using hexane–EtOAc (10:1) as an eluent to give an additional **10** (3.36 g, 12%) as pale brown solid. IR (KBr): 1681, 1588, 1508, 1269, 1217, 1158, 1021 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.38 (d, J = 6.1 Hz, 6H), 3.94 (s, 3H), 4.63 (sep, J = 6.1 Hz, 1H), 7.05 (s, 1H), 7.43 (s, 1H), 10.18 (s, 1H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 21.8, 56.5, 71.5, 113.6, 115.9, 120.1, 126.5, 147.2, 155.7, 190.9. Anal. Calcd for $C_{11}H_{13}BrO_3$: C, 48.37; H, 4.80. Found: C, 48.23; H, 4.72. These physical and spectroscopic data are in good agreement with those previously reported.[32]

*4.2.4. tert-Butyl 2-(2-formyl-4-isopropoxy-5-methoxyphenyl)-1H-pyrrole-1-carboxylate (**11**)*. Under Ar atmosphere, a mixture of **10** (5.49 g, 20.1 mmol), **9** (5.06 g, 24.0 mmol), $Pd(PPh_3)_4$ (2.42 g, 2.10 mmol), and Na_2CO_3 (12.9 g, 126 mmol), THF (420 mL), and degassed water (38 mL) was refluxed for 18 h. After cooling to room temperature, the solvent was removed *in vacuo* and the residue was diluted with water and extracted 3 times with DCM. The organic extracts were combined, washed successively with water (twice) and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The oily residue was chromatographed on silica gel eluted with hexane–EtOAc (10:1) to give **11** (6.78 g, 94%) as a reddish viscous oil. IR (KBr): 1743, 1683, 1511, 1332, 1155, 1124 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.29 (s, 9H), 1.41 (d, J = 6.1 Hz, 6H), 3.92 (s, 3H), 4.70 (sep, J = 6.1 Hz, 1H), 6.24 (dd, J = 1.8 and 3.3 Hz, 1H), 6.29 (t, J = 3.3 Hz, 1H), 6.82 (s, 1H), 7.44 (dd, J = 1.8 and 3.3 Hz, 1H), 7.43 (s, 1H), 9.74 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.9, 27.6, 56.2, 71.2, 84.0, 110.6, 111.2, 113.9, 116.9, 122.6, 128.7, 129.3, 132.8, 147.4, 148.9, 154.0, 190.7. HRFABMS *m/z*. Calcd for $C_{20}H_{25}NO_5$ (M^+): 359.1733. Found: 359.1738.

*4.2.5. tert-Butyl 2-[2-(2-methoxyethenyl)-4-isopropoxy-5-methoxyphenyl]-1H-pyrrole-1-carboxylate (**12**)*. To a mixture of (methoxymethyl)triphenylphosphonium chloride (2.68 g, 7.82 mmol) in THF (39 mL) cooled to 0 °C under Ar atmosphere, was added dropwise a suspension of *t*-BuOK (1.05 g, 9.36 mmol) in THF (9.4 ml). After 10 min at 0 °C, a solution of **11** (2.31 g, 6.26 mmol) in THF (27 mL) was added dropwise. After 3 h at 0 °C, the reaction was quenched by adding water (100 mL). The THF was removed *in vacuo* and the residue was extracted 3 times with ether. The combined ethereal layers were washed successively with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The oily residue was chromatographed on silica gel using hexane–EtOAc (10:1) as an eluent to give **12** (2.26 g, 93%) as an *E*/*Z* mixture (*E*:*Z* = 64:36, based on NMR) as a reddish viscous oil. IR (KBr): 1736, 1509, 1336, 1158, 1125 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.22 (s, 3.28H), 1.26 (s, 5.72H), 1.38 (d, *J* = 6.1 Hz, 3.82H), 1.40 (d, *J* = 6.2 Hz, 2.18H), 3.50 (s, 1.91H), 3.71 (s, 1.09H), 3.82 (s, 1.91H), 3.82 (s, 1.09H), 4.50–4.61 (m, 1H), 4.84 (d, *J* = 7.2 Hz, 0.364H), 5.47 (d, *J* = 12.9 Hz, 0.636H), 5.95 (d, *J* = 7.2 Hz, 0.364H), 6.11 (dd, *J* = 1.8 and 3.4 Hz, 0.364H), 6.12 (dd, *J* = 1.9 and 3.3 Hz, 0.636H), 6.24 (t, *J* = 3.4 Hz, 0.364H), 6.24 (t, *J* = 3.3 Hz, 0.636H), 6.72 (d, *J* = 12.9 Hz, 0.636H), 6.74 (s, 0.364H), 6.76 (s, 0.636H), 6.87 (s, 0.636H), 7.37 (dd, *J* = 1.9 and 3.3 Hz, 0.636H), 7.38 (dd, *J* = 1.8 and 3.4 Hz, 0.364H), 7.70 (s, 0.364H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.2, 27.3, 27.5, 56.0, 56.1, 56.3, 60.4, 71.4, 71.7, 76.7, 77.0, 77.4, 83.1, 83.1, 103.3, 103.4, 110.3, 110.4, 112.1, 114.0, 114.2, 114.3, 114.6, 116.1, 121.3, 121.4, 125.8, 128.8, 129.0, 132.9, 133.1, 146.4, 146.5, 147.0, 147.7, 148.1, 148.3, 149.4, 149.6. HRFABMS *m/z* Calcd for $C_{22}H_{29}NO_5$ (M^+): 387.2046. Found: 387.2053.

*4.2.6. tert-Butyl 7-isopropoxy-8-methoxy-1H-benzo[g]indole-1-carboxylate (**13**)*. A solution of **12** (1.49 g, 3.85 mmol) in DCM (25 mL) was cooled to 0 °C and methanesulfonic acid (0.025 mL, 0.365 mmol) was added. After 23 h at 0 °C, Na_2CO_3 (103.7 mg, 0.948 mmol) and $MgSO_4$ (101.8 mg, 0.846 mmol) were added as solids and the mixture was stirred for a while and filtered. The filtrate was concentrated and the residue was purified by a column chromatography on silica gel using hexane–EtOAc (10:1) as an eluent to give **13** (1.26 g, 92%) as white crystals, mp 102.5–103 °C (DCM/hexane). IR (KBr): 1740, 1491, 1389, 1308, 1250, 1165, 1112 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.45 (d, *J* = 6.1 Hz, 6H), 1.68 (s, 9H), 4.02 (s, 3H), 4.72 (sep, *J* = 6.1 Hz, 1H), 6.61 (d, *J* = 3.7 Hz, 1H), 7.25 (s, 1H), 7.46 (d, *J* = 8.4 Hz, 1H), 7.52 (d, *J* = 8.4 Hz, 1H), 7.59 (d, *J* = 3.7 Hz, 1H), 8.43 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.0, 28.1, 55.8, 70.9, 83.6, 105.8, 108.0, 111.6, 111.6, 118.0, 118.8, 123.8, 126.9, 127.8, 128.2, 130.5, 146.0, 149.2, 150.5. Anal. Calcd for $C_{21}H_{25}NO_4$: C, 70.96; H, 7.09; N, 3.94. Found: C, 71.06; H, 7.26; N, 3.76.

*4.2.7. tert-Butyl 2-bromo-7-isopropoxy-8-methoxy-1H-benzo[g]indole-1-carboxylate (**14**)*. To a cooled (−78 °C) solution of **12** (491 mg, 1.38 mmol) in THF (50 mL), was added dropwise a pentane solution of *t*-BuLi (1.46 M, 1.14 mL, 1.66 mmol) under Ar atmosphere. After 1 h at this temperature, 1,2-dibromo-1,1,2,2-tetrafluoroethane (0.246 mL, 2.07 mmol) was added and the mixture was stirred for 1 h. The reaction was quenched by adding water and the THF was removed *in vacuo*. The residue was extracted 3 times with DCM and the combined organic layers were washed successively with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The oily residue was chromatographed on silica gel using hexane–EtOAc (10:1) as an eluent to give **14** (567 mg, 95%) as white solid, mp 108.5–109.0 °C. IR (KBr): 1749, 1493, 1299, 1247, 1168, 1106 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.45 (d, *J* = 6.1 Hz, 6H), 1.75 (s, 9H), 3.96 (s, 3H), 4.70 (sep, *J* = 6.1 Hz, 1H), 6.70 (s, 1H), 7.23 (s, 1H), 7.38 (d, *J* = 8.5 Hz, 1H), 7.45 (d, *J* = 8.5 Hz, 1H), 7.49 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.0, 27.9, 55.8, 71.0, 85.7, 102.5, 107.4, 111.3, 111.9, 116.9, 117.2, 123.1, 125.6, 127.4, 131.0, 146.1, 149.8, 151.0. HRFABMS *m/z* Calcd for $C_{21}H_{24}BrNO_4$ (M^+): 433.0889. Found: 433.0903.

*4.2.8. tert-Butyl 7-isopropoxy-2-[4-isopropoxy-5-methoxy-2-(methoxymethoxy)phenyl]-8-methoxy-1H-benzo[g]indole-1-carboxylate (**16**)*. In a Schlenk tube, **14** (454 mg, 1.05 mmol), arylboronic acid **15**[22] (421 mg, 1.56 mmol), Na_2CO_3 (727 mg, 6.86 mmol) and $Pd(PPh_3)_4$ (116 mg, 0.100 mmol) were placed and the whole purged with Ar. To this mixture, DME (15 mL) and degassed (Ar) water (2.0 mL) were added and the whole was heated under reflux under Ar atmosphere for 23 h. After cooling to room temperature, the solvent was removed *in vacuo* and the residue was diluted with water and extracted 3 times with DCM. The combined organic layers were washed successively with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The oily residue was chromatographed on silica gel using hexane–EtOAc (5:1) as an eluent to give **16** (589 mg, 97%) as white crystals, mp 102.5–103.0 °C (DCM/hexane). IR (KBr): 1738, 1494, 1391, 1308, 1150, 1022 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.33 (s, 9H), 1.42 (d, *J* = 6.1 Hz, 6H), 1.46 (d, *J* = 6.1 Hz, 6H), 3.29 (s, 3H), 3.84 (s, 3H), 3.97 (s, 3H), 4.59 (sep, *J* = 6.1 Hz, 1H), 4.72 (sep, *J* = 6.1 Hz, 1H), 4.94 (s, 2H), 6.59 (s, 1H), 6.82 (s, 1H), 6.93 (s, 1H), 7.27 (s, 1H), 7.49 (s, 2H), 7.95 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.0, 22.0, 27.3, 55.7, 56.3, 56.6, 71.0, 71.9, 83.6, 96.1, 103.6, 106.2, 108.9, 112.2, 115.2, 117.8, 118.1, 118.1, 122.8, 125.7, 127.7, 130.5, 135.5, 145.5, 145.7, 147.9, 149.3, 149.5, 152.0. Anal. Calcd for $C_{33}H_{41}NO_8$: C, 68.37; H, 7.13; N, 2.42. Found: C, 68.36; H, 7.28; N, 2.26.

*4.2.9. tert-Butyl 3-bromo-7-isopropoxy-2-(4-isopropoxy-5-methoxy-2-methoxymethoxyphenyl)-8-methoxy-1H-benzo[g]indole-1-carboxylate (**17**)*. To a cooled (0 °C) solution of **16** (2.21 g, 3.81 mmol) in DMF (40 mL), was added dropwise a solution of NBS (746 mg, 4.19 mmol) in DMF (5.0 mL). After 6.5 h at 0 °C, the reaction was quenched by adding water. The mixture was extracted 3 times with ether and the combined extracts were washed successively with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The crude product was purified by a column chromatography on silica gel using hexane–EtOAc (3:1) as an eluent to give **17** (2.50 g, 99%) as pale yellow crystals, mp 64.5–66.0 °C. IR (KBr): 1743, 1494, 1304, 1250, 1219, 1152 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.32 (s, 9H), 1.42 (d, *J* = 6.1 Hz, 3H), 1.44 (d, *J* = 6.1 Hz, 3H), 1.46 (d, *J* = 6.1 Hz, 6H), 3.30 (s, 3H), 3.84 (s, 3H), 3.96 (s, 3H), 4.62 (sep, *J* = 6.1 Hz, 1H), 4.74 (sep, *J* = 6.1 Hz, 1H), 4.94 (d, *J* = 6.7 Hz, 1H), 5.00 (d, *J* = 6.7 Hz, 1H), 6.87 (s, 1H), 6.90 (s, 1H), 7.28 (s, 1H), 7.50 (d, *J* = 8.6 Hz, 1H), 7.57 (d, *J* = 8.6 Hz, 1H), 7.79 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8, 22.0, 22.0, 22.2, 27.2, 55.7, 56.1, 56.6, 71.0, 71.7, 84.1, 96.0, 99.7, 103.4, 105.3, 111.9, 115.0, 115.7, 116.3, 117.6, 123.5, 124.9, 128.3, 129.7, 132.6, 145.3, 146.2, 148.6, 149.7, 150.0, 151.3. HRFABMS *m/z* Calcd for $C_{33}H_{40}BrNO_8$ (M^+): 657.1937. Found: 657.1925.

*4.2.10. 1-tert-Butyl 3-methyl 7-isopropoxy-2-(4-isopropoxy-5-methoxy-2-methoxymethoxyphenyl)-8-methoxy-1H-benzo[g]indole-1,3-dicarboxylate (**18**)*. To a cooled (−78 °C) solution of **17** (2.50 g, 3.80 mmol) in THF (100 mL), was added dropwise a 1.59 M solution of *t*-BuLi in pentane (1.43 M, 5.85 mL, 8.36 mmol) under Ar atmosphere. After 1 h at −78 °C, a solution of methyl chloroformate (0.883 mL, 11.4 mmol) in THF (8 mL) was dropwise added. After 1 h at −78 °C, the reaction was quenched by adding saturated aqueous NH_4Cl. The THF was removed *in vacuo* and the residue was extracted 3 times with ether. The ethereal extracts were combined, washed

successively with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The crude product was purified by a column chromatography on silica gel using with hexane–EtOAc (3:1) as an eluent to give **18** (1.95 g, 81%) as reddish crystals, mp 147–149 °C (DCM/hexane). IR (KBr): 1754, 1700, 1503, 1251, 1217, 1149 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.33 (s, 9H), 1.42 (d, *J* = 6.1 Hz, 3H), 1.44 (d, *J* = 6.1 Hz, 3H), 1.47 (d, *J* = 6.1 Hz, 6H), 3.32 (s, 3H), 3.75 (s, 3H), 3.81 (s, 3H), 3.94 (s, 3H), 4.63 (sep, *J* = 6.1 Hz, 1H), 4.74 (sep, *J* = 6.1 Hz, 1H), 4.94 (d, *J* = 6.8 Hz, 1H), 4.99 (d, *J* = 6.8 Hz, 1H), 6.86 (s, 1H), 6.90 (s, 1H), 7.31 (s, 1H), 7.56 (s, 1H), 7.60 (d, *J* = 8.7 Hz, 1H), 8.17 (d, *J* = 8.7 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8, 22.0, 22.0, 22.2, 27.1, 51.0, 55.7, 55.9, 56.5, 71.0, 71.7, 85.1, 95.9, 102.3, 104.9, 110.2, 111.9, 114.6, 115.3, 116.8, 118.4, 123.6, 123.6, 127.7, 129.4, 139.8, 144.9, 146.2, 148.6, 149.8, 150.3, 151.6, 165.1. Anal. Calcd for $C_{35}H_{43}NO_{10}$: C, 65.92; H, 6.80; N, 2.20. Found: C, 65.92; H, 6.89; N, 2.02.

*4.2.11. 3,10-Diisopropoxy-2,11-dimethoxybenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**19**)*. A solution of **18** (2.02 g, 3.17 mmol) in a mixture of $CHCl_3$ (40 mL) and MeOH (40 mL) containing conc. HCl (8.0 mL) was stirred at 50 °C for 24.5 h. After cooling to room temperature, the solvents were removed *in vacuo*, and the residue was diluted with water and extracted 3 times with DCM. The combined organic extracts was washed successively with saturated aqueous $NaHCO_3$, water, and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The crude product was purified by a column chromatography on silica gel using $CHCl_3$–EtOAc (1:1) as an eluent to give **19** (1.45 g, 99%) as white crystals, mp 186–187 °C (EtOAc). IR (KBr): 1680, 1515, 1470, 1257, 1216, 1108 cm^{-1}. ^1H NMR (400 MHz, acetone-d_6) δ 1.40 (d, *J* = 6.0 Hz, 12H), 3.89 (s, 3H), 3.94 (s, 3H), 4.76 (sep, *J* = 6.0 Hz, 1H), 4.77 (sep, *J* = 6.0 Hz, 1H), 7.05 (s, 1H), 7.48 (s, 1H), 7.51 (s, 1H), 7.62 (d, *J* = 8.5 Hz, 1H), 7.66 (s, 1H), 8.04 (d, *J* = 8.5 Hz, 1H), 12.03 (br s, 1H). ^{13}C NMR (100 MHz, acetone-d_6): δ 22.2, 22.3, 56.1, 56.7, 71.4, 72.0, 101.9, 104.4, 104.7, 106.5, 113.0, 117.6, 118.1, 121.3, 122.8, 128.0, 133.7, 141.3, 148.0, 148.3, 149.2, 150.7, 152.0, 159.5. HREIMS *m/z* Calcd for $C_{27}H_{27}NO_6$ (M^+): 461.1838. Found: 461.1833.

*4.2.12. 3,10-Dihydroxy-2,11-dimethoxybenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**20**)*. To a cooled (−78 °C) solution of **19** (99.1 mg, 0.215 mmol) in DCM (37 mL), was added dropwise a solution of BCl_3 in heptane (1.0 M, 2.58 mL, 2.58 mmol) under Ar atmosphere. The mixture was stirred at −78 °C for 30 min, 0 °C for 1 h and room temperature for 22 h. The reaction was quenched by adding saturated aqueous $NaHCO_3$ and the DCM was removed *in vacuo*. The precipitated solid was filtered, washed successively washed with water and 2 M HCl solution, and dried overnight *in vacuo* to give **1a** (71.3 mg, 88%) as white crystals, mp 254–255 °C (decomp). IR (KBr): 3503, 3237, 1653, 1500, 1288, 1209, 1145 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 3.97 (s, 3H), 4.04 (s, 3H), 6.94 (s, 1H), 7.30 (s, 1H), 7.48 (d, *J* = 8.6 Hz, 1H), 7.85 (d, *J* = 8.6 Hz, 1H), 8.32 (s, 1H), 8.36 (s, 1H), 13.70 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.2, 56.6, 99.4, 102.6, 103.7, 104.9, 105.6, 111.4, 116.2, 116.7, 118.9, 121.0, 126.7, 133.1, 140.9, 145.3, 146.2, 147.7, 149.1, 149.2, 158.8. HRFABMS *m/z* Calcd for $C_{21}H_{15}NO_6$ (M^+): 377.0899. Found: 377.0892.

*4.3. Synthesis of N-substituted BBPIs **22a–e***

*4.3.1. 3,10-Diisopropoxy-2,11-dimethoxy-13-methylbenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**21a**)*. To a cooled (0 °C) and stirred slurry of NaH (22.4 mg, 0.560 mmol of 60% NaH in mineral oil which had been freed from mineral oil by washing with hexane) in DMF (0.5 mL), was added dropwise a solution of **19** (32.0 mg, 69.3 μmol) in DMF (1.0 mL) under Ar atmosphere. After 40 min, 18-crown-6 (32.8 mg, 0.124 mmol) dissolved in DMF (1.0 mL) was added. After 40 min, iodomethane (18.0 μL, 0.289 mmol) was added and the mixture was stirred for 3 h. The reaction was then quenched by adding saturated aqueous NH_4Cl. The mixture was diluted with EtOAc and the two layers were separated. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was chromatographed on silica gel using hexane–EtOAc (2:1–0:1) as an eluent to give **21a** (29.0 mg, 88%) as pale yellow solid, mp 231.5–232 °C (EtOAc). IR (KBr): 1709, 1519, 1427, 1266, 1227, 1110 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.45 (d, *J* = 6.1 Hz, 6H), 1.48 (d, *J* = 6.1 Hz, 6H), 3.99 (s, 3H), 4.04 (s, 3H), 4.48 (s, 3H), 4.60 (sep, *J* = 6.1 Hz, 1H), 4.72 (sep, *J* = 6.1 Hz, 1H), 6.94 (s, 1H), 7.27 (s, 1H), 7.41 (s, 1H), 7.51 (d, *J* = 8.4 Hz, 1H), 7.66 (s, 1H), 8.20 (d, *J* = 8.4 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 21.8, 22.0, 37.7, 56.0, 56.9, 71.1, 71.5, 101.6, 101.8, 103.8, 105.0, 105.8, 112.3, 116.8, 117.9, 121.0, 123.1, 128.3, 134.4, 141.5, 146.3, 146.4, 148.7, 149.3, 149.9, 159.2. HREIMS *m/z* Calcd for $C_{28}H_{29}NO_6$ (M^+): 475.1995. Found: 475.1991.

*4.3.2. 13-Ethyl-3,10-diisopropoxy-2,11-dimethoxybenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**21b**)*. To a cooled (0 °C) and stirred slurry of NaH (31.3 mg, 0.783 mmol of 60% NaH in mineral oil which had been freed from mineral oil by washing with hexane) in DMF (2.0 mL), was added dropwise a solution of **19** (82.6 mg, 0.179 mmol) in DMF (3.0 mL) under Ar atmosphere. After 30 min, iodoethane (56.0 μL, 0.716 mmol) was added. After 30 min, the mixture was allowed to warm to room temperature and stirred for 23.5 h. The reaction was then quenched by adding saturated aqueous NH_4Cl and 28% aqueous ammonia. The mixture was diluted with EtOAc and the two layers were separated. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was chromatographed on silica gel using hexane–EtOAc (1:2) as an eluent to give **21b** (50.9 mg, 58%) as colorless powder, mp 219.2–220.0 °C (EtOAc). IR (KBr): 1711, 1520, 1438, 1256, 1228, 1112 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.46 (d, *J* = 6.1 Hz, 6H), 1.49 (d, *J* = 6.1 Hz, 6H), 1.98 (t, *J* = 7.2 Hz, 3H), 4.00 (s, 3H), 4.06 (s, 3H), 4.61 (sep, *J* = 6.1 Hz, 1H), 4.74 (sep, *J* = 6.1 Hz, 1H), 4.94 (q, *J* = 7.2 Hz, 2H), 6.97 (s, 1H), 7.30 (s, 1H), 7.45 (s, 1H), 7.54 (d, *J* = 8.5 Hz, 1H), 7.69 (s, 1H), 8.26 (d, *J* = 8.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 15.9, 21.8, 22.0, 42.9, 55.9, 56.8, 71.0, 71.5, 101.4, 101.4, 104.0, 104.6, 105.7, 112.6, 116.4, 118.1, 121.3, 123.1, 128.5, 132.7, 139.6, 146.2, 146.6, 148.7, 149.2, 150.2, 159.2. Anal. Calcd for $C_{29}H_{31}NO_6$: C, 71.15; H, 6.38; N, 2.86. Found: C, 71.06; H, 6.45; N, 2.79.

*4.3.3. 13-Allyl-3,10-diisopropoxy-2,11-dimethoxybenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**21c**)*. To a cooled (0 °C) and stirred slurry of NaH (12.1 mg, 0.303 mmol of 60% NaH in mineral oil which had been freed from mineral oil by washing with hexane) in DMF (2.0 mL), was added dropwise a solution of **19** (43.4 mg, 94.0 μmol) in DMF (2.0 mL) under Ar atmosphere. After 30 min, allyl bromide (32.0 μL, 0.370 mmol) was added. After 30 min, the mixture was allowed to warm to room temperature and stirred for 22 h. The reaction was then quenched by adding saturated aqueous NH_4Cl and 28% aqueous ammonia. The mixture was diluted with EtOAc and the two layers were separated. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was chromatographed on silica gel using hexane–EtOAc (1:2) as an eluent to give **21c** (35.1 mg, 74%) as pale yellow solid, mp 219.2–220.0 °C (ether/hexane). IR (KBr): 1704, 1516, 1436, 1258, 1226, 1039 cm^{-1}. ^1H NMR (400 MHz,

$CDCl_3$): δ 1.45 (d, *J* = 6.1 Hz, 6H), 1.48 (d, *J* = 6.1 Hz, 6H), 3.92 (s, 3H), 3.97 (s, 3H), 4.61 (sep, *J* = 6.1 Hz, 1H), 4.74 (sep, *J* = 6.1 Hz, 1H), 5.45 (br s, 1H), 5.55–5.63 (m, 1H), 5.71–5.77 (m, 1H), 6.56–6.67 (m, 1H), 6.98 (s, 1H), 7.32 (s, 1H), 7.43 (s, 1H), 7.57 (d, *J* = 8.5 Hz, 1H), 7.67 (s, 1H), 8.24 (d, *J* = 8.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8, 22.0, 51.4, 56.0, 56.6, 71.1, 71.6, 101.8, 102.1, 103.8, 104.6, 105.5, 112.3, 116.4, 117.9, 119.8, 121.3, 123.2, 128.3, 133.0, 134.5, 142.0, 146.4, 146.6, 148.7, 149.3, 150.2, 159.3. HRFABMS *m/z* Calcd for $C_{30}H_{31}NO_6$ (M^+): 501.2151. Found: 501.2139.

*4.3.4. 3,10-Diisopropoxy-2,11-dimethoxy-13-propargylbenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**21d**)*. To a cooled (0 °C) and stirred slurry of NaH (45.3 mg, 1.13 mmol of 60% NaH in mineral oil which had been freed from mineral oil by washing with hexane) in DMF (2.0 mL), was added dropwise a solution of **19** (96.7 mg, 0.210 mmol) in DMF (2.0 mL) under Ar atmosphere. After 30 min, propargyl bromide (63.0 µL, 0.836 mmol) was added. After 30 min, the mixture was allowed to warm to room temperature and stirred for 3.5 h. The reaction was then quenched by adding saturated aqueous NH_4Cl and 28% aqueous ammonia. The mixture was diluted with EtOAc and the two layers were separated. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was chromatographed on silica gel using hexane–EtOAc (1:1) as an eluent to give **21d** (68.6 mg, 65%) as pale yellow solid, mp 196–197 °C (ether/hexane). IR (KBr): 1711, 1514, 1434, 1257, 1227, 1163 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.45 (d, *J* = 6.0 Hz, 6H), 1.48 (d, *J* = 6.0 Hz, 6H), 3.96 (s, 3H), 4.01 (s, 3H), 4.59 (sep, *J* = 6.0 Hz, 1H), 4.70 (sep, *J* = 6.0 Hz, 1H), 5.35 (d, *J* = 6.2 Hz, 2H), 6.89 (s, 1H), 7.20 (s, 1H), 7.32 (t, *J* = 6.2 Hz, 1H), 7.50 (d, *J* = 8.5 Hz, 1H), 7.75 (s, 1H), 8.04 (s, 1H), 8.20 (d, *J* = 8.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8, 22.0, 55.8, 56.5, 71.0, 71.5, 83.6, 98.1, 101.4, 101.8, 103.3, 104.3, 105.8, 111.8, 116.9, 117.6, 120.7, 123.2, 128.2, 132.8, 139.7, 146.2, 146.3, 148.6, 149.2, 149.9, 159.3, 209.7. HRFABMS *m/z* Calcd for $C_{30}H_{30}NO_6$ [$(M+H)^+$]: 500.2073. Found: 500.2076.

*4.3.5. 13-[2-(Dimethylamino)ethyl]-3,10-diisopropoxy-2,11-dimethoxybenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**21e**)*. To a cooled (0 °C) and stirred slurry of NaH (8.6 mg, 0.215 mmol of 60% NaH in mineral oil which had been freed from mineral oil by washing with hexane) in DMF (2.0 mL), was added dropwise a solution of **19** (32.0 mg, 69.3 µmol) in DMF (2.0 mL) under Ar atmosphere. After 20 min, 2-(dimethylamino)ethyl chloride hydrochloride (23.2 mg, 0.161 mmol) was added and the mixture was warmed to 75 °C. After 8 h, the mixture was cooled to 0 °C. Sodium hydride (60% dispersion in mineral oil, 3.8 mg, ca 95 µmol) and 2-(dimethylamino)ethyl chloride hydrochloride (23.2 mg, 0.161 mmol) was added to the mixture, and then warmed again to 75 °C. After 13 h, the mixture was cooled to 0 °C. Sodium hydride (60% dispersion in mineral oil, 3.9 mg, ca 98 µmol) and 2-(dimethylamino)ethyl chloride hydrochloride (23.2 mg, 0.161 mmol) was added to the mixture, and then warmed again to 75 °C. After 4 h, the mixture was cooled to room temperature. The reaction was quenched by adding saturated aqueous NH_4Cl. The mixture was diluted with EtOAc and the two layers were separated. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was chromatographed on silica gel using hexane–EtOAc (1:1) as an eluent to give **21e** (13.4 mg, 47%) as pale yellow solid, mp 226.5–227.5 °C (EtOAc). IR (KBr): 1727, 1521, 1437, 1245, 1228, 1110, 1035 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.46 (d, *J* = 6.1 Hz, 6H), 1.49 (d, *J* = 6.1 Hz, 6H), 2.38 (s, 6H), 2.94–3.14 (m, 2H), 3.99 (s, 3H), 4.02 (s, 3H), 4.60 (sep, *J* = 6.1 Hz, 1H), 4.60–4.72 (m, 2H), 4.71 (sep, *J* = 6.1 Hz, 1H), 6.92 (s, 1H), 7.24 (s, 1H), 7.45 (s, 1H), 7.49 (d, *J* = 8.5 Hz, 1H), 7.61 (s, 1H), 8.22 (d, *J* = 8.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8, 22.0, 46.0, 47.4, 56.1, 56.6, 58.8, 71.0, 71.5, 100.9, 101.3, 103.7, 103.8, 105.4, 112.4, 116.4, 117.9, 121.2, 123.2, 128.3, 132.4, 139.6, 146.1, 146.8, 148.4, 148.9, 150.4, 159.1. Anal. Calcd for $C_{31}H_{36}N_2O_6$: C, 69.91; H, 6.81; N, 5.26. Found: C, 69.62; H, 6.97; N, 5.16.

*4.3.6. 3,10-Dihydroxy-2,11-dimethoxy-13-methylbenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**22a**)*. The title compound was obtained in a similar manner as described for the synthesis of **20**. Yellow crystals, mp 188–192 °C (decomp). Yield, 94%. IR (KBr): 3227, 1670, 1502, 1424, 1271, 1212, 1159 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.96 (s, 3H), 4.01 (s, 3H), 4.56 (s, 3H), 6.95 (s,1H), 7.34 (s, 1H), 7.53 (d, *J* = 8.5 Hz, 1H), 7.61 (s, 1H), 7.81 (s, 1H), 7.94 (d, *J* = 8.5 Hz, 1H), 9.69 (br s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 37.9, 55.4, 56.1, 99.8, 101.9, 104.3, 104.6, 105.3, 112.2, 116.1, 116.6, 119.7, 122.3, 128.0, 134.2, 141.5, 144.8, 145.9, 148.1, 148.5, 149.2, 158.2. HRFABMS *m/z* Calcd for $C_{22}H_{17}NO_6$ (M^+): 391.1056. Found: 391.1062.

*4.3.7. 13-Ethyl-3,10-dihydroxy-2,11-dimethoxybenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**22b**)*. The title compound was obtained in a similar manner as described for the synthesis of **20**. Deep green crystals, mp 185–186 °C (decomp). Yield, 94%. IR (KBr): 3370, 1683, 1504, 1440, 1296, 1208, 1159 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.93 (t, *J* = 7.1 Hz, 3H), 3.96 (s, 3H), 4.02 (s, 3H), 5.07 (q, *J* = 7.1 Hz, 2H), 6.99 (s, 1H), 7.37 (s, 1H), 7.57 (d, *J* = 8.5 Hz, 1H), 7.59 (s, 1H), 7.79 (s, 1H), 8.02 (d, *J* = 8.5 Hz, 1H), 9.73 (br s, 1H), 10.36 (br s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.7, 43.1, 55.5, 56.2, 100.0, 101.5, 104.6, 104.7, 105.0, 112.7, 115.9, 117.0, 120.1, 122.8, 128.4, 132.7, 139.8, 145.3, 145.9, 148.2, 149.0, 149.3, 158.5. HRFABMS *m/z* Calcd for $C_{23}H_{19}NO_6$ (M^+): 405.1212. Found: 405.1237.

*4.3.8. 13-Allyl-3,10-dihydroxy-2,11-dimethoxybenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**22c**)*. The title compound was obtained in a similar manner as described for the synthesis of **20**. Yellow crystals, mp 170–172 °C (decomp). Yield, 50%. IR (KBr): 3453, 1677, 1504, 1438, 1296, 1244, 1169 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.88 (s, 3H), 3.94 (s, 3H), 5.38 (d, *J* = 17.3 Hz, 1H), 5.61 (br s, 2H), 5.66 (d, *J* = 11.0 Hz, 1H), 6.70–6.82 (m, 1H), 7.01 (s, 1H), 7.37 (s, 1H), 7.50 (s, 1H), 7.60 (d, *J* = 8.5 Hz, 1H), 7.70 (s, 1H), 8.02 (d, *J* = 8.5 Hz, 1H), 9.65 (s, 1H), 10.30 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 51.4, 55.6, 56.2, 100.5, 102.0, 104.4, 104.5, 105.2, 112.3, 115.8, 116.9, 118.3, 120.0, 122.8, 128.2, 134.2, 134.2, 141.8, 145.1, 146.1, 148.3, 148.8, 149.4, 158.6. HRFABMS *m/z* Calcd for $C_{24}H_{19}NO_6$ (M^+): 417.1212. Found: 417.1227.

*4.3.9. 3,10-Dihydroxy-2,11-dimethoxy-13-propargylbenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**22d**)*. The title compound was obtained in a similar manner as described for the synthesis of **20**. Yellow crystals, mp 247–250 °C (decomp). Yield, 63%. IR (KBr): 3216, 1708, 1437, 1245, 1210, 1162 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.92 (s, 3H), 3.97 (s, 3H), 5.70 (d, *J* = 6.3 Hz, 2H), 6.95 (s, 1H), 7.33 (s, 1H), 7.57 (d, *J* = 8.6 Hz, 1H), 7.95 (d, *J* = 8.6 Hz, 1H), 7.96 (s, 1H), 7.97 (t, *J* = 6.3 Hz, 1H), 8.23 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.5, 56.1, 84.4, 97.9, 100.3, 101.5, 104.3, 104.6, 104.7, 112.1, 116.4, 116.8, 119.6, 122.8, 128.2, 132.8, 139.8, 145.0, 146.2, 148.3, 148.7, 149.4, 158.5, 209.0. HRFABMS *m/z* Calcd for $C_{24}H_{18}NO_6$ [$(M+H)^+$]: 416.1134. Found: 416.1113.

*4.3.10. Trifluoroacetic acid salt of 13-[2-(Dimethylamino)ethyl]-3,10-dihydroxy-2,11-dimethoxybenzo[g][1]benzopyrano[4,3-b]indol-6(13H)-one (**22e**)*. To a solution of **21e** (100 mg, 0.188 mmol) in DCM (6.0 mL), was added dropwise a 1.0 M solution of $AlCl_3$ in nitrobenzene (1.01 mL, 1.01 mmol) under Ar atmosphere. The mixture was stirred at room temperature for 48 h. The reaction was quenched by adding a solution of $NaHCO_3$ (255 mg, 3.04 mmol) and Rochelle salt (857 mg, 3.04 mmol) in water (6.0 mL). The mixture was stirred for 1 h and the DCM was removed *in vacuo*. The nitrobenzene was removed azeotropically with water under reduced pressure. The precipitated solid was filtered, washed successively washed with water, and dried overnight *in vacuo* to give 13-[2-(dimethylamino)ethyl]-3,10-dihydroxy-2,11-dimethoxybenzo[*g*][1]benzopyrano[4,3-*b*]indol-6(13*H*)-one (**22e′**) (78.9 mg, 94%) as pale yellow solid, mp >300 °C. IR (KBr): 3535, 3207, 1682, 1440, 1298, 1207, 1154 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 2.29 (s, 6H), 2.93 (br s, 2H), 3.91 (s, 3H), 3.92 (s, 3H), 4.57 (br s, 2H), 6.88 (s, 1H), 7.30 (s, 1H), 7.42 (s, 1H), 7.48 (d, J = 8.5 Hz, 1H), 7.57 (s, 1H), 7.93 (d, J = 8.5 Hz, 1H), 9.57 (br s, 1H), 10.18 (br s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 45.6, 47.0, 55.3, 55.9, 58.1, 99.8, 100.8, 104.0, 104.0, 104.4, 112.4, 115.5, 116.6, 119.8, 122.5, 128.1, 132.2, 139.4, 145.0, 145.7, 147.9, 148.8, 148.9, 158.0. HRFABMS *m/z* Calcd for $C_{25}H_{25}N_2O_6$ [(M+H)$^+$]: 449.1713. Found: 449.1734.

To a solution of **22e′** (100 mg, 0.223 mmol) in DCM (2.0 mL) was added trifluoroacetic acid (2.0 mL) at room temperature. After stirring for 5 min, the mixture was evaporated. The residue was purified by column chromatography over Sephadex LH-20 (MeOH) to give **22e** (115 mg, 91%) as a brown powder, mp 263–264 °C (decomp) (sealed capillary). IR (KBr): 3265, 1684, 1519, 1439, 1298, 1205 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 2.78 (s, 6H), 3.46 (t, J = 6.9 Hz, 2H), 4.01 (s, 3H), 4.08 (s, 3H), 5.55 (br s, 2H), 7.02 (s, 1H), 7.40 (s, 1H), 7.60 (s, 1H), 7.63 (d, J = 8.6 Hz, 1H), 7.69 (s, 1H), 8.04 (d, J = 8.6 Hz, 1H), 9.70 (br s, 1H), 10.41 (br s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 42.9, 44.1, 54.8, 55.9, 56.6, 101.6, 102.1, 104.4, 104.6, 105.6, 112.5, 116.0, 116.7, 121.0, 123.4, 128.4, 133.0, 141.4, 145.2, 146.2, 148.3, 149.1, 149.7, 158.0. HRFABMS *m/z*. Calcd for $C_{25}H_{25}N_2O_6$ [(M−CF_3COO)$^+$]: 449.1713. Found: 449.1721.

*4.4. Synthesis of the water-soluble valine ester derivative **24***

*4.4.1. 2,11-Dimethoxy-13-methyl-6-oxo-6,13-dihydrobenzo[g][1]benzopyrano[4,3-b]indole-3,10-diyl bis{(2S)-2-[(tert-butoxycarbonyl)amino]-3-methylbutanoate} (**23**)*. A solution of *N*-(*tert*-butoxycarbonyl)-*L*-valine (150 mg, 0.690 mmol), EDCI·HCl (132 mg, 0.690 mmol) and DMAP (6.2 mg, 51 μmol) in DMF (7.0 mL) was stirred at room temperature for 10 min under Ar atmosphere. To the mixture, **22a** (50.0 mg, 0.128 mmol) was added and stirred for 24 h. The reaction was quenched by adding water. The mixture was diluted with DCM and the two layers were separated. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was chromatographed on silica gel using hexane–EtOAc (1:1) as an eluent to give **23** (66.9 mg, 66%) as white crystals, mp 127–128 °C (ether/hexane). IR (KBr): 1766, 1717, 1497, 1367, 1258, 1160 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.06–1.20 (m, 12H), 1.51 (s, 18H), 2.30–2.56 (m, 2H), 3.89 (s, 3H), 3.92 (s, 3H), 4.18 (s, 3H), 4.53–4.66 (m, 2H), 5.22–5.32 (m, 2H), 7.02 (s, 1H), 7.27 (s, 1H), 7.35 (d, J = 8.5 Hz, 1H), 7.43 (s, 2H), 8.01 (d, J = 8.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.3, 17.3, 19.2, 19.2, 28.4, 31.3, 31.4, 37.5, 55.6, 56.3, 58.6, 79.9, 80.0, 101.8, 102.5, 104.7, 111.5, 112.9, 117.8, 120.3, 121.9, 122.2, 123.8, 127.3, 133.8, 138.6, 140.1, 140.6, 146.9, 147.5, 149.8, 155.7, 155.7, 158.3, 170.2, 170.8. HRFABMS m/z. Calcd for $C_{42}H_{51}N_3O_{12}$ (M^+): 789.3473. Found: 789.3456.

*4.4.2. Trifluoroacetic acid salt of 2,11-dimethoxy-13-methyl-6-oxo-6,13-dihydrobenzo[g][1]benzopyrano[4,3-b]indole-3,10-diyl bis[(2S)-2-amino-3-methylbutanoate] (**24**)*. To the compound **23** (20.5 mg, 26.0 μmol) was added trifluoroacetic acid (1.0 mL) at room temperature. After stirring for 1 h at room temperature, the mixture was evaporated. The residue was chromatographed on Sephadex LH-20 using methanol as an eluent to give **24** (25.4 mg, quant) as a pale yellow powder, mp 158.5–160 °C. IR (KBr): 3435, 1771, 1678, 1520, 1428, 1260, 1204 cm^{-1}. ^1H NMR (400 MHz, methanol-d_4): δ 1.25–1.32 (m, 12H), 2.52–2.62 (m, 2H), 3.95 (s, 3H), 4.00 (s, 3H), 4.30 (s, 3H), 4.34 (d, J = 4.3 Hz, 2H), 7.18 (s, 1H), 7.48 (d, J = 8.6 Hz, 1H), 7.55 (s, 1H), 7.64 (s, 1H), 7.67 (s, 1H), 7.89 (d, J = 8.6 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 17.4, 17.4, 17.9, 17.9, 29.0, 29.7, 38.2, 55.8, 56.3, 57.3, 101.8, 103.2, 106.4, 112.2, 112.4, 117.5, 120.8, 121.7, 122.0, 123.8, 126.7, 134.1, 137.7, 139.4, 140.5, 146.5, 147.1, 149.3, 157.5, 167.0, 167.5. HRFABMS (*m/z*) Calcd for $C_{32}H_{36}N_3O_8$ [(M−2CF_3COO−H)$^+$]: 590.2502. Found: 590.2527.

*4.5. X-ray crystallographic analysis of **21a***

Crystallographic data for **21a** can be summarized as follows: compound formula $C_{32}H_{37}NO_8$, Mw = 563.63, Triclinic, P–1, a = 9.221(5) Å, b = 10.578(4) Å, c = 16.355(7) Å, α = 74.248(13)°, β = 79.774(18)°, γ = 69.424(17)°, V = 1431.5(11) Å^3, Z = 2, D_{calc} = 1.308 g/cm^3, monochromatized radiation λ(Mo Kα) = 0.71075 Å, μ = 0.094 mm^{-1}, F(000) = 600, T = 123(2) K. Data were collected on an Rigaku FR-E SuperBright diffractometer. There were 6500 unique reflections with 3657 being observed at the 2σ level. The structure was solved by direct methods (SHELXS-97)[33] and the non-hydrogen atoms were refined anisotropically by full-matrix least-squares procedures on F^2 using SHELXL Version 2018/3 software.[33] Hydrogen atoms were refined using the riding model. All calculations were performed using the CrystalStructure[34] crystallographic software package except for refinement, which was performed using SHELXL Version 2018/3. The final model was refined using 382 parameters and data from all 6500 reflections. The final agreement statistics are as follows: R = 0.0767 (based on 3657 reflections with $I > 2\sigma(I)$), wR = 0.2505, S = 1.040. The maximum peak height in the final difference Fourier map was 1.005 eÅ^3, which is without chemical significance. The program ORTEP-3 for Windows was used to generate the X-ray structural diagrams.[35] CCDC 1857984 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

4.6. Biological assays–general

The purity of all tested compounds [**5**, **20**, **22a**–**e**, and **24**] was determined to be >95% using HPLC with a GL Science Inertsil diol column (4.6 mm i.d. × 250 mm). The detection wavelength was fixed at 305 nm. The HPLC profiles of **5**, **20**, **22a**–**e**, and **24** are shown in Supplementary data (Figure S53).

4.7. Topoisomerase I inhibitory assay

The topoisomerase I relaxation assay was performed according to previous reports.[24,25] In brief, 2U of DNA topoisomerase I from calf thymus (TaKaRa Bio) was mixed with 250 ng of supercoiled DNA pBR322 (TaKaRa Bio) in 20 μL of a reaction buffer (35 mM Tris–HCl, pH 8, 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% BSA) in the presence or absence of the test drugs previously dissolved in

DMSO. The mixture was incubated at 37 °C for 30 min and then the reaction was terminated by adding 2 μL of 10% SDS solution. After digestion of the enzyme by adding 2 μL of 2 mg/mL of proteinase K and incubating at 37 °C for 30 min, excess compounds were removed by extraction with $CHCl_3$/isoamyl alcohol (24:1). The reaction product was subjected to 1% agarose gel electrophoresis in the absence or presence of ethidium bromide (0.5 μg/mL). When the agarose gel electrophoresis was performed in the absence of ethidium bromide, the gel was stained with 0.5 μg/mL ethidium bromide after electrophoresis.

4.8. Docking simulation

Docking studies were performed using MOE 2014.0901.[27] Crystal structure of the topotecan (**2**)–DNA–topoisomerase I ternary complex (PDB ID: 1K4T)[26] was obtained from the Protein Data Bank. The protein structures for the docking process were prepared according to the following sequence: (i) The topoisomerase I–DNA–topotecan ternary complex was loaded. (ii) Hydrogen atoms and electric charge were added to the complex by Protonate 3D (default settings). (iii) The hydrogen atoms were optimized by MMFF94x force field (heavy atoms were fixed during the optimization). (iv) The dummy atoms were disposed in the docking site by Site Finder (default settings). On the other hand, the conformers of **22a** were obtained by Conformational Search using the LowModeMD search method with default parameters except for the following: The check box for the hydrogens was selected in order to include both hydrogen and heavy atoms in the RMSD calculation for duplication detection and the value of energy window was set to 10 kcal/mol. Finally, the ligands were docked into the binding site of the kinases by using the Dock docking program according to the following sequence: (i) Initial poses were obtained using the Triangle Matcher placement (timeout: 3000 s; No. of return poses: 10000), London dG rescoring 1 (maximum number of poses: 500), GridMin refinement (default settings), and GBVI/WSA dG rescoring 2 (maximum number of poses: 100). (ii) The poses obtained in (i) were refined by Forcefield refinement (default settings) and rescored by GBVI/WSA dG scoring function (maximum number of poses: 100). (iii) The poses obtained in (ii) were refined by Forcefield refinement (sidechain: tether, the tether value was set to 10) and rescored by GBVI/WSA dG scoring function (maximum number of poses: 100). (iv) The poses obtained in (iii) were refined by Forcefield refinement (sidechain: free) and rescored by GBVI/WSA dG scoring function (maximum number of poses: 100). The obtained poses were evaluated using the GBVI/WSA dG scoring function and the two most plausible models (pose A and B) are shown in Fig. 5.

4.9. Antiproliferative activity against 39 human cancer cell lines (JFCR39)

This experiment was carried out at the Screening Committee of Anticancer Drugs according to the standard protocol used by the Committee. Inhibition of cell growth was assessed by measuring the changes in the total cellular protein levels following 48 h treatment with a given test compound, using the sulforhodamine B colorimetric assay. The molar concentration of a test compound required for 50% growth inhibition (GI_{50}) of cells was calculated as reported previously.[17]

4.10. Evaluation of in vivo antitumor activity

4.10.1. Animals

Six-week-old male BALB/c mice were purchased from Japan SLC Ltd, Sizuoka, Japan. The mice were maintained, six per cage, under standardized 12 h light–dark cycle conditions in a specific pathogen-free room for three days. During the acclimatization period, food and water were freely available.

4.10.2. Preparation of dosing drugs

Irinotecan hydrochloride trihydrate (**3**) was dissolved in distilled water (final concentration of 6.25 mg/mL). Compound **24** was dissolved in 5% glucose solution (final concentration of 6.25 mg/mL). The drugs (50 mg/kg) were administered by tail vein injection. Controls were given the vehicle (saline solution).

4.10.3. Cell culture

Colon 26 cell line was purchased from Cell Bank Riken BioResource Center. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 0.5% penicillin G/streptomycin (penicillin G: 25 units/mL, streptomycin: 25 μg/mL) at 37 °C in a humidified 5% CO_2 atmosphere. Before use, the concentration of colon 26 was adjusted to 2×10^7 cells/mL.

4.10.4. Determination of antitumor effect

Colon 26 cells (5×10^5 cells) were subcutaneously injected into the footpad of each mouse. One day after the injection of colon 26 cells, the drug was administered to the animal at two day intervals. A total of seven doses were administered and the mice were weighted daily. Tumor dimensions was measured using a Vernier caliper and the tumor volume was calculated daily according to the following equation: tumor volume (mm^3) = $4/3 \times 3.14 \times (A/2) \times (B/2) \times (C/2)$, where A is the longer dimension, B is the shorter dimension, and C is the thickness (mm).

4.10.5. Statistical analysis

Data are shown as the mean ± s.d. Groups were compared by one-way analysis of variance (ANOVA) and repeated ANOVA, and the differences between the groups were determined by Scheffe's test. All *P* values less than 0.05 were considered to be significant.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2018.00.000.

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