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Design and Syntheses of Permethyl Ningalin B Analogues: Potent Multidrug Resistance (MDR) Reversal Agents of Cancer Cells

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A series of novel *N*-arylalkyl-3,4-diaryl-substituted pyrrole-2,5-diones were synthesized. They exhibited promising P-gp modulating activity in a P-gp overexpressing breast cancer cell line (LCC6MDR). Compound **6** (with three methoxy groups at D-ring) displayed the highest P-gp modulating activity. **6** at 1 μM can sensitize LCC6MDR cells toward paclitaxel by 18.2-fold. Interestingly, a synergy on modulating P-gp was noted when **6** and **25** were used together (fractional inhibitory concentration index FICI = 0.42). Combination of **6** (0.5 μM) and **25** (0.5 μM) resulted in a 66-fold sensitization of LCC6MDR cells toward paclitaxel. They also reversed P-gp mediated doxorubicin (DOX) and vincristine resistance. Kinetic characterization suggests that permethyl ningalin B analogues likely act as a noncompetitive inhibitor of P-gp-mediated DOX transport (K_i = 5.4−5.8 μM). The present study demonstrates that synthetic analogues of permethyl ningalin B can be employed as effective and safe modulators of P-gp-mediated drug resistance in cancer cells.

Introduction

Chemotherapy is one of the most important treatment methods for cancers. Several anticancer drugs including paclitaxel, doxorubicin, and vinblastine have been used clinically to treat breast, lung, stomach, prostate, colon, and pancreatic tumor and other solid tumors. Extensive multidrug resistance (MDR[a]) in cancer cells has been a major obstacle to successful cancer chemotherapy.[1] The major resistance mechanism is through increased expression of the ABCB1 (also known as mdr1) gene which encodes for a membrane permeability glycoprotein (P-gp).[2,3] P-gp belongs to a family of ATP-binding cassette (ABC) transporters.[4−8] Most ABC transporters consist of two transmembrane domains and two highly conserved nucleotide binding domains. P-gp is probably the best characterized ABC transporter. It pumps a variety of hydrophobic anticancer drugs out of the cell, resulting in a lowered intracellular drug accumulation.[4−8] Recent determination of three-dimensional structure of P-gp by electron crystallography[9] could allow a better understanding of molecular mechanism of P-gp and the development of MDR inhibitors.

Great efforts have been made to discover effective and safe MDR inhibitors over the past 2 decades. Verapamil, a calcium channel blocker, was the first MDR reversal agent that was found to inhibit P-gp-mediated drug efflux.[10,11] Verapamil and other more effective P-gp inhibitors including calcium channel blockers,[12] calmodulin antagonists,[13] indole alkaloids,[14] cyclosporine A,[15] quinoline,[16] and steroid[17] are classified as first-generation MDR modulators.[2,18] However, their unacceptable toxicity precluded them from clinical use. Although second-generation MDR modulators including 6-[(2*S*,4*R*,6*E*)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]-7-L-valine-cyclosporin A (valspodar PSC833),[19] 1,7-di(pyridin-3-yl)heptan-4-yl-1-[2-oxo-2-(3,4,5-trimethoxyphenyl)acetyl]piperidine 2-carboxylate (biricodar, VX-710),[20] dexverapamil,[21] and dexniguldipine[22] have lower toxicity and higher MDR-reversal activity, their uses were limited by their unpredictable pharmacokinetic interactions with the coadministered anticancer drug.[2,18] Third-generation MDR modulators,[2,23,24] developed by structure−activity relationship studies and combinatorial chemistry approaches, included (2*R*)-1-{4-[(1a*R*,6r,10b*S*)-1,1-difluoro-1,1a,6,10b-tetrahydrodibenzo[*a*,*e*]cyclopropa[*c*]cyclohepten-6-yl]pipera- zin-1-yl}-3-(quinolin-5-yloxy)propan-2-ol (zosuquidar, LY-335979), *N*-[2-[[4-[2-(6,7-dimethoxy-3,4-dihydro-1*H*-isoquinolin-2-yl)-ethyl]phenyl]carbamoyl]-4,5-dimethoxyphenyl]quinoline-3-carboxamide (tariquidar, XR-9576), and *N*-[4-[2-[4-[3-ethoxy-1-(*E*)-propenyl]phenyl]-4-[4-(isopropylamino)phenyl]-1*H*-imidazol-5-yl]phenyl]-*N*-isopropylamine (OC144-093). Tariquidar, one of the most potent P-gp inhibitors, is in phase III trial in breast cancer.

Marine natural products are important sources for development of new MDR modulators. Some pyrrole-derived alkaloids including permethyl ningalin B (Figure 1), permethyl ningalin D, permethyl lamallarine D, lamallarine K, lamallarine I, lamallarine O, lukianol A, and permethyl storniamide A have been reported to act as nontoxic inhibitors of MDR in various cancer cell lines.[25−33]

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[a] Abbreviations: MDR, multidrug resistance; ABC, ATP-binding cassette; P-gp, P-glycoprotein; RF, relative fold; DOX, doxorubicin.

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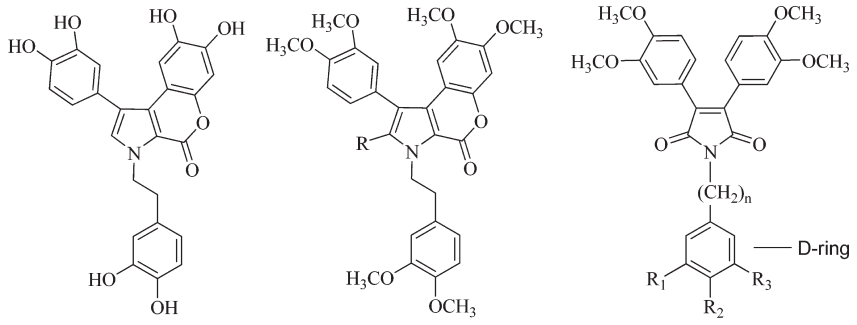


Figure 1. Structures of ningalin B compounds and target compounds.

Ningalin B (Figure 1) is the second member of this newly described family of marine natural products. It was first isolated by Fenical's group from an ascidian of the genus *Didemnum* collected in Western Australia near Ningaloo Reef in 1997.[30] Total synthesis of ningalin B, permethyl ningalin B (**1**), and their derivatives including **2** and **3** (Figure 1) were performed by Boger and Gupton.[28,32,33] These compounds, containing a common 3,4-diaryl-substituted pyrrole nucleus bearing 2- or 2,5-carboxylates, can reverse MDR more effectively than verapamil by inhibiting the P-gp-mediated drug efflux at a noncytotoxic concentration. We are interested in improving the MDR-reversing activity of permethyl ningalin B by modifying its pyrrole nucleus. In this study, we have replaced the nucleus of pyrrole 2- or 2,5-carboxylate of Boger's compounds **2** and **3** with pyrrole-2,5-dione. 3,4-Diaryl-substituted pyrrole-2,5-dione has been reported to be more stable and easier to prepare than 3,4-diaryl-substituted pyrrole-2,5-carboxylates.[31] The target molecules were formed by linking 3,4-diaryl-substituted pyrrole-2,5-dione with a benzene ring (D-ring) containing various alkyl linkers. These novel permethyl ningalin B analogues were assessed for their MDR-reversing activity in P-gp overexpressing cancer cell line.

Results

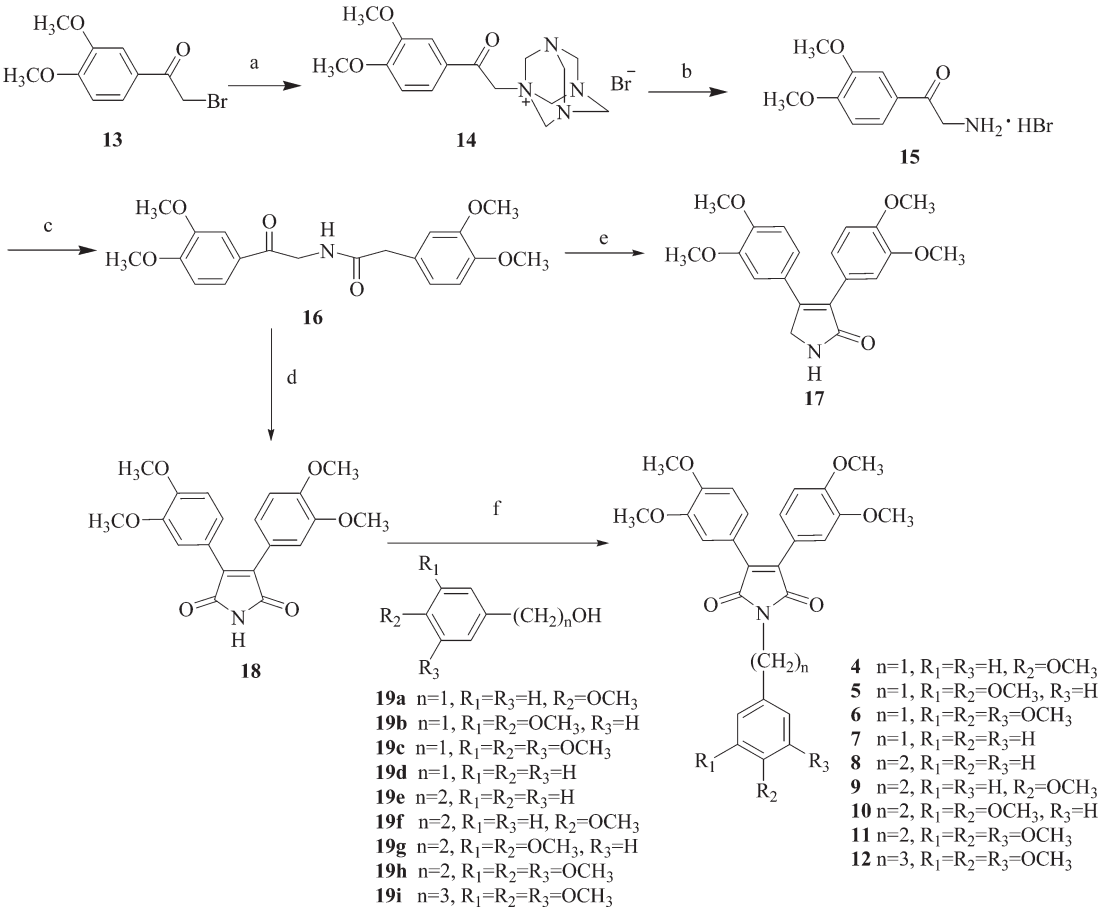
Synthesis of Analogues of Permethyl Ningalin B. A concise synthetic method was used in the preparation of key intermediate 3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (**18**) (Scheme 1).[34] Ammoniation of 2-bromo-1-(3,4-dimethoxyphenyl)ethanone (**13**) with hexamethylenetetramine, followed by addition of concentrated hydrobromic acid in absolute methanol, provided 2-amino-1-(3,4-dimethoxyphenyl)ethanone hydrobromide (**15**). Coupling of compound **15** with 2-(3,4-dimethoxyphenyl)acetic acid, catalyzed by EDCI and HOBt, resulted in compound **16**. Subsequent cyclization of **16**, catalyzed by *t*-BuOK in *t*-BuOH under nitrogen protection, yielded 3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2-one (**17**). The key intermediate 3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (**18**) was obtained from cyclization of **16** under air with a yield of 45%. Following reported methods,[35] trace amounts of **17** and **18** were derived from the cyclization of **16** catalyzed by K_2CO_3 in aqueous ethanol or DBU in acetonitrile. The Mitsunobu method, used in the preparation of ningalin B derivative **29**, was also adopted in the synthesis of target compound **10** because of the NH acidity of **18**. Intermediate **18** was coupled with 2-(3,4-dimethoxyphenyl)-ethanol in the presence of PPh_3 and DIAD to produce compound **10** with a yield of 66%. With the same synthesis strategy, compounds **4**, **5**, **6**, **7**, **8**, **9**, **11**, and **12** with one to three carbon linkers and various numbers of methoxy groups at the D-ring were obtained.

Coupling of **18** with (*E*)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-ol under Mitsunobu reaction conditions provided compound **21** (Scheme 2). Catalyzed by K_2CO_3 and in DMF, compounds **24**, **25**, and **26** were obtained by N-alkylation of **18** with 2-bromo-1-phenylethanone, 2-bromo-1-(4-methoxyphenyl)ethanone, and 2-bromo-1-(3,4-dimethoxyphenyl)ethanone, respectively (Scheme 2). No products were obtained when intermediate **17** reacted with (3,4,5-trimethoxyphenyl)methanol (**27**) using the above two coupling methods. When compounds **17** and **27** were treated with NaH in DMF, byproduct **28** was isolated from the reaction mixture (Scheme 2). Compound **28** was characterized by ^{1}H NMR and ^{13}C NMR. The two methylene groups of compound **28** were further confirmed by ^{13}C NMR-DEPT and HMQC.

Dimers **30**, **31**, and **32** were prepared respectively by treating intermediate **18** with dibromomethane, 1,2-dibromoethane, and 1,3-dibromopropane catalyzed by K_2CO_3 in DMF (Scheme 3). The Mitsunobu reaction was tried but found to be inefficient in the synthesis of dimers. Compound **29** was obtained when intermediate **18** was treated with ethane-1,2-diol under Mitsunobu reaction conditions (Scheme 3). Additionally, compounds **7** and **11** were refluxed with Lawesson's reagent in toluene to provide compounds **33** and **34**, respectively (Scheme 3).

Biological Studies. Effect of Permethyl Ningalin B Analogues on Reversing Paclitaxel Resistance of MDA435/LCC6MDR Cells. We employed two cell lines in this study, namely, MDA435/LCC6 cell line (an estrogen-independent breast cancer cell line) and its ABCB1-transfected derivative (MDA435/LCC6MDR).[36] Paclitaxel is one of the drugs for treating breast cancer,[37] and it has been demonstrated that paclitaxel resistance is mediated by overexpression of P-gp.[38−40] We found that LCC6MDR cells were about 70.5-fold more resistant to paclitaxel (IC_{50} = 183.2 ± 6.3 nM) than its parental LCC6 cells (IC_{50} = 2.6 ± 0.5 nM) (Table 1). None of the permethyl ningalin B analogues showed any cytotoxicity toward LCC6, LCC6MDR, or normal mouse connective tissue fibroblast (L929) even when used at a very high concentration (100 μM) except for compound **9** (Table 2). MDR reversal activity of permethyl ningalin B analogues was compared by measuring the relative fold (RF), defined as a ratio of IC_{50} without modulator to IC_{50} with modulator. A relatively low

Scheme 1. Synthesis of Compounds **4**–**12**[a]



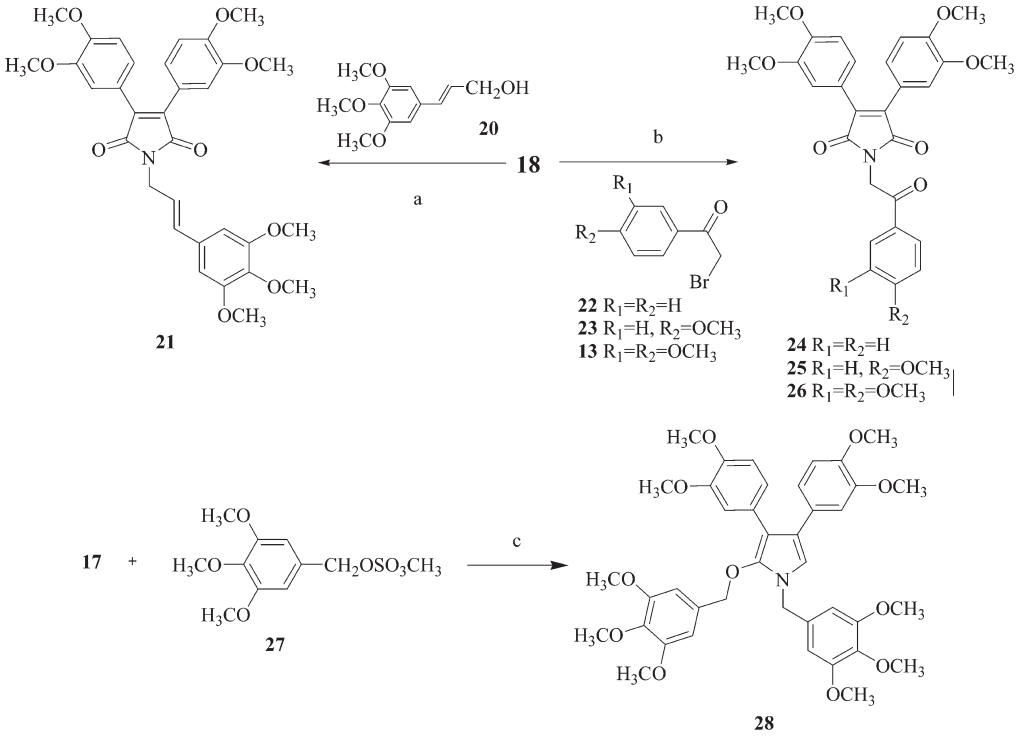
[a] Reagents and conditions: (a) hexamethylenetetramine, $CHCl_3$, room temp, 4 h; (b) HBr, CH_3OH, 0 °C to room temp, 12 h; (c) 2-(3,4-dimethoxyphenyl)acetic acid, HOBt, DCC, Et_3N, CH_2Cl_2, 0 °C to room temp, N_2, 18 h; (d) *t*-BuOK, *t*-BuOH, 30 °C, air, 2 h; (e) *t*-BuOK, *t*-BuOH, 30 °C, N_2, 2 h; (f) PPh_3, DIAD, THF, 0 °C to room temp.

and noncytotoxic concentration of permethyl ningalin B analogues (1 μM) was used in this study. Permethyl ningalin B analogues are grouped into seven series according to types of substituents and linkers used. Among all permethyl ningalin B analogues, compound **6** displayed the highest modulating activity with a relative fold (RF) of 18.2 (Table 1). Well-known P-gp modulators including verapamil, cyclosporine A, and PSC833 were employed as positive controls. Verapamil displayed a moderate activity with a RF of 5.6 (Table 1). In contrast, both cyclosporine A and PSC833 exhibited the strongest reversal activity with a RF of 91.6 and 101.8, respectively (Table 1).

Among series 1, 2, and 5, it was found that permethyl ningalin B analogues containing methoxy substituent(s) on the benzyl group gave higher RF values than one without methoxy group(s) (Table 1), suggesting that the presence of methoxy substituent(s) is needed for inhibiting the functionality of P-gp. Moreover, the number of methoxy substituents on the benzyl group is also an important factor for determining the P-gp modulating activity. In series 1, compound **6** with trimethoxy substituents displayed better activity than the di- and monomethoxy analogues (compounds **4** and **5**) (Table 1). In series 2, the analogue with a monomethoxy substituent (compound **9**) showed stronger activity than the analogues with di- and trimethoxy substituents (compounds **10** and **11**). On the contrary, analogues with either mono- or dimethoxy substituent(s) (compounds **25** and **26**) displayed similar activity in series 5. In addition to the methoxy substitution, the length and the nature of linker also play an important role in resensitizing LCC6MDR to paclitaxel. The analogue with the shortest linker displayed a stronger P-gp modulating activity than other analogues with longer linker (Table 1). Compound **7** with methylene linker (RF = 7.9) displayed about 2-fold higher modulating activity than compound **8** with bismethylene (RF = 3.6) and compound **24** with carbonylmethylene (RF = 3.9), suggesting that shorter linker in permethyl ningalin B analogues is crucial for modulating P-gp. When these two requirements are put together, the permethyl ningalin B analogue **6** containing the shortest linker and three methoxy substituents displayed the highest P-gp modulating activity with a RF of 18.2 (Table 1). Among the dimeric analogues, only compound **30** (RF = 8.0) and compound **32** (RF = 5.2) gave moderate reversal activities, indicating that the dimeric analogues have poorer reversal activities than monomeric analogues.

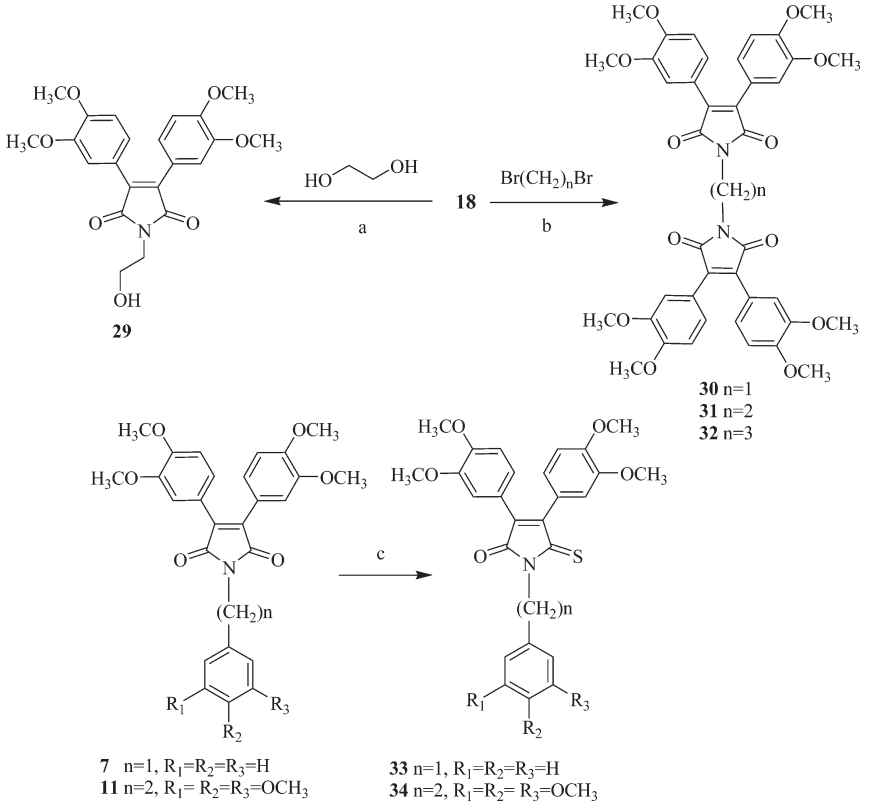
Synergistic Effect of Ningalin B Analogues on Reversing Paclitaxel Resistance of MDA435/LCC6MDR Cells. Compound **6** (RF = 18.2) is the strongest P-gp modulator among all permethyl ningalin B analogues (Table 1). We mixed compound **6** with equimolar concentrations of other moderate modulators including **5**, **9**, **25**, or **26** and tested for their P-gp modulating activity. We found that a combination of 0.5 μM **6** and 0.5 μM **25** resulted in a 66-fold resensitization of LCC6MDR toward paclitaxel (IC_{50} = 2.8 nM, Table 3).

Scheme 2. Synthesis of Compounds **21**, **24**−**26**, and **28**[a]



[a] Reagents and conditions: (a) PPh_3, DIAD, THF, 0 °C to room temp, N_2; (b) K_2CO_3, DMF, room temp, N_2; (c) NaH, DMF, 0 °C to room temp, −80 °C, 12 h, N_2.

Scheme 3. Synthesis of Compounds **29**−**34**[a]



[a] Reagents and conditions: (a) PPh_3, DIAD, THF, 0 °C to room tempt, N_2; (b) K_2CO_3, DMF, 90 °C, N_2; (c) Lawesson's reagent, toluene, reflux, 8 h, N_2.

This level is similar to that of parental LCC6 (IC_{50} = 2.6 nM, Table 1). Moreover, this combination almost displayed comparable paclitaxel resistance reversal activity as the standard P-gp modulators such as cyclosporine A (IC_{50} = 2.0 nM) and PSC833 (IC_{50} = 1.8 nM) (Table 1). On the basis of the RF values, the combination of **6** and **25** is about 3.6-fold

Table 1. P-gp Modulating Activity of Permethyl Ningalin B Analogues[a]

series	compd (1 μM)	no. of methoxy on benzyl group	types of linker	mean IC_{50} of paclitaxel (nM)	RF
1	**7**		methylene	23.1 ± 4.2	7.9
	4	mono	methylene	19.6 ± 2.5	9.3
	5	di	methylene	14.7 ± 1.9	12.4
	6	tri	methylene	10.1 ± 0.6	18.2
2	**8**		bismethylene	51.1 ± 7.3	3.6
	9	mono	bismethylene	17.1 ± 1.3	10.7
	10	di	bismethylene	26.6 ± 4.3	6.9
	11	tri	bismethylene	40.7 ± 4.5	4.5
3	**12**	tri	trismethylene	39.0 ± 2.7	4.7
4	**21**	tri	vinylmethylene	29.4 ± 1.2	6.2
5	**24**		carbonylmethylene	47.3 ± 12.3	3.9
	25	mono	carbonylmethylene	18.4 ± 1.1	9.9
	26	di	carbonylmethylene	22.4 ± 1.6	8.2
6	**29**		hydroxyethyl	99.0 ± 24.1	1.8
	28	tri	methylene	115.9 ± 10.1	1.6
	30	di	methylene	23.0 ± 1.5	8.0
	31	di	bismethylene	131.3 ± 2.3	1.4
	32	di	trismethylene	35.0 ± 3.8	5.2
7	**33**		methylene	54.4 ± 11.5	3.4
	34	tri	bismethylene	66.5 ± 5.1	2.8
	verapamil			32.9 ± 3.5	5.6
	cyclosporine A			2.0 ± 0.2	91.6
	PSC833			1.8 ± 0.3	101.8
	LCC6MDR[b]			183.2 ± 6.3	1.0
	LCC6[c]			2.6 ± 0.5	70.5

[a] IC_{50} values were determined for LCC6MDR cells after they were treated with a series of concentrations of paclitaxel in the presence of 1 μM of different permethyl ningalin B analogues, verapamil, cyclosporine A, and PSC833. Relative fold (RF) represents fold-change in drug sensitivity. RF = (IC_{50} without modulator)/(IC_{50} with modulator). N = 2−18 independent experiments were performed, and averaged IC_{50} values are presented as the mean ± standard error of mean. [b] No modulator was used in LCC6MDR cells. IC_{50} value from this control is used for normalization (RF = 1.0). [c] LCC6 cells were used without addition of modulator.

Table 2. In Vitro Cytotoxicity of Permethyl Ningalin B Analogues to LCC6, LCC6MDR, and L929 Cells[a]

	IC_{50} (μM)		
compd	LCC6	LCC6MDR	L929
4	> 100	> 100	> 100
5	> 100	> 100	> 100
6	> 100	> 100	> 100
7	> 100	> 100	> 100
8	ND	> 100	> 100
9	32.5 ± 2.5	18.0 ± 2.1	21.6 ± 5.9
10	> 100	> 100	> 100
11	> 100	> 100	> 100
12	> 100	> 100	> 100
21	> 100	> 100	> 100
24	ND	> 100	> 100
25	> 100	90.0 ± 0.0	> 100
26	> 100	> 100	> 100
28	ND	84	> 100
29	> 100	> 100	> 100
30	> 100	> 100	> 100
31	ND	> 100	> 100
32	> 100	> 100	> 100
33	ND	> 100	> 100
34	ND	ND	ND

[a] The IC_{50} values were determined after exposure to a series of concentrations of permethyl ningalin B analogues using LCC6, LCC6MDR, and L929. L929 is a mouse connective tissue fibroblast cell line. Each experiment has been repeated one to three times with the data presented as the mean ± standard error of mean. Many of permethyl ningalin B analogues did not show cytotoxicity at the highest concentration used (100 μM). ND = not determined.

($P < 0.0001$) and 6.7-fold ($P < 0.0001$) better than **6** and **25** alone, respectively, suggesting that **6** and **25** can act synergistically to modulate P-gp. On the contrary, other combinations including **6** and **5** (RF = 13.8), **6** and **9** (RF = 18.0), and **6** and **26** (RF = 12.9) did not display any synergy (Table 3).

To quantitatively measure the synergistic effect among different combinations of modulators, we used the fractional inhibitory concentration index (FICI) approach modified from Chou and Talalay[41] (see Experimental Section for details). We can determine the fractional IC_{50} (FIC) for each combination of modulators. The sum of two FICs will give the FICI value for that combination. A FICI value lower than or equal to 0.5 indicates synergy. As shown in Table 3, the synergistic effect was observed only when 0.5 μM of **6** was used in combination with 0.5 μM **25** (FICI = 0.42). Other combinations were found to give an additive effect because their FICI values were between 1.6 and 2.2. A different approach using a plot of different fractions affected (F_a) against FICI also demonstrated that there was a synergy between **6** and **25** but not in **6** and **5** or **6** and **9** or **6** and **26** combinations in modulating paclitaxel resistance (see Supporting Information).

Effect of Permethyl Ningalin B Analogues on Reversing DOX and Vincristine Resistance of MDA435/LCC6MDR Cells. We found that permethyl ningalin B analogues also potentiated the action of other anticancer drugs including DOX and vincristine in LCC6MDR cells. Both DOX and vincristine are known P-gp substrates. For DOX cytotoxicity, compound **6** (RF = 9.5) exhibited a higher modulating activity than **25** (RF = 5.2) (Table 4). The combination of **6** and **25** (RF = 12.0) displayed a similar modulating activity as compound **6** alone, and the FICI value was greater than 0.5 (FICI = 1.26) (Table 4), suggesting that there was no synergy in modulating P-gp. For vincristine cytotoxicity, compound **6** (RF = 14.6) was better than compound **25** (RF = 9.3) in resensitizing LCC6MDR (Table 4). Interestingly, the combination of them (RF = 26.9)

Table 3. In Vitro Synergistic Interaction of Permethyl Ningalin B Analogues on Reversing Paclitaxel Resistance of LCC6MDR Cells[a]

compd	mean IC_{50} of paclitaxel (nM)	RF	FIC_6	FIC_5	FIC_9	FIC_{25}	FIC_{26}	FICI	drug interaction
	183.2 ± 6.3	1.0							
0.5 μM **6** + 0.5 μM **5**	13.3 ± 3.5	13.8	1.31	0.90				2.21	additive[c]
0.5 μM **6** + 0.5 μM **9**	10.2 ± 1.4	18.0	1.01		0.60			1.61	additive[d]
0.5 μM **6** + 0.5 μM **25**	2.8 ± 0.5[b]	66.0	0.27			0.15		0.42	synergistic[e]
0.5 μM **6** + 0.5 μM **26**	14.3 ± 2.5	12.9	1.41				0.64	2.05	additive[f]
1 μM **6**	10.1 ± 0.6[b]	18.2	1.00					1.00	
1 μM **5**	14.7 ± 1.9	12.4		1.00				1.00	
1 μM **9**	17.1 ± 1.3	10.7			1.00			1.00	
1 μM **25**	18.4 ± 1.1[b]	9.9				1.00		1.00	
1 μM **26**	22.4 ± 1.6	8.2					1.00	1.00	

[a] The IC_{50} values were displayed asthe mean ± standard error of mean, and the IC_{50} values of the combination of two modulators were from two to four independent experiments. The *P* value of IC_{50} of the combination of 0.5 μM **6** and 0.5 μM **25** compared to **6** alone or **25** alone was determined by Student's *t* test. [b] $P < 0.0001$. FIC values were calculated using IC_{50} values. Interaction is classified as synergistic (FICI ≤ 0.5), additive (FICI between 0.5 and 4), or antagonistic (FICI > 4). [c] Drug interaction was relative to 1 μM **6** alone or 1 μM **5** alone. [d] Drug interaction was relative to 1 μM **6** alone or 1 μM **9** alone. [e] Drug interaction was relative to 1 μM **6** alone or 1 μM **25** alone. [f] Drug interaction was relative to 1 μM of **6** alone or 1 μM of **26** alone.

Table 4. In Vitro Additive Interaction of Permethyl Ningalin B Analogues on Reversing DOX and Vincristine Resistance of LCC6MDR Cells[a]

anticancer drugs	compd	RF	FICI	drug interaction
DOX	1 μM **6**	9.5 ± 0.8	1.00	
	1 μM **25**	5.2 ± 0.6	1.00	
	0.5 μM **6** + 0.5 μM **25**	12.0 ± 1.5	1.26	additive[b]
vincristine	1 μM **6**	14.6 ± 3.3	1.00	
	1 μM **25**	9.3 ± 0.1	1.00	
	0.5 μM **6** + 0.5 μM **25**	26.9 ± 4.8	0.89	additive[b]

[a] Relative fold (RF) represents fold-change in drug sensitivity. RF = (IC_{50} without modulator)/(IC_{50} with modulator). RF values were presented as the mean ± standard error of mean. $N = 2-5$ independent experiments. The IC_{50} values were used to calculate the FICI value. Interaction is classified as synergistic (FICI ≤ 0.5), additive (FICI between 0.5 and 4), or antagonistic (FICI > 4). [b] Drug interaction was relative to 1 μM **6** alone or 1 μM **25** alone.

resulted in a higher P-gp modulating activity, which was about 1.8-fold and 2.9-fold higher than compound **6** alone (RF = 14.6) and compound **25** alone (RF = 9.3) (Table 4), respectively. However, there was no synergistic activity of **6** and **25** on reversing vincristine resistance of LCC6MDR because the FICI value was greater than 0.5 (FICI = 0.89) (Table 4).

Dosage Effect of Permethyl Ningalin B Analogues on Intracellular Accumulation of DOX in MDA435/LCC6MDR Cells. The above results showed that the permethyl ningalin B analogues are promising P-gp modulators. We are interested in studying whether modulation of P-gp-mediated drug resistance is associated with an increase in drug accumulation. DOX is a fluorescent P-gp substrate that can be used to monitor intracellular drug accumulation. As shown in Figure 2A, the level of DOX accumulation in parental LCC6 cells was about 3.3-fold higher than that of LCC6MDR cells in the absence of modulators. This is because P-gp can pump DOX out of the cells, thereby lowering intracellular DOX level. Treatment of LCC6 cells with 3 μM **6**, **25**, or combination of **6** and **25** did not increase DOX accumulation. In contrast, treatment of LCC6MDR cells with 3 μM **6**, **25**, or combination of **6** and **25** resulted in 3.0-, 2.1-, or 2.8-fold increase in intracellular DOX accumulation, respectively (Figure 2A). This result suggests that the mechanism of reversal activity of permethyl ningalin B analogues is by inhibiting the transport activity of P-gp. The combination of **6** and **25** (2.8-fold higher than the LCC6MDR's control) restored the DOX accumulation to a level similar to that treated with **6** alone (3.0-fold higher than the LCC6MDR's control) (Figure 2A).

We determined the dosage effect of **6** and **25** on increasing DOX accumulation. We found that restoration of DOX accumulation in LCC6MDR cells by **6** or **25** was dosage-dependent up to 3 μM (Figure 2B and Figure 2C). Further increase in concentration of **6** or **25** resulted in a slight drop in DOX accumulation (Figure 2B and Figure 2C). Intracellular DOX accumulation in LCC6MDR, however, cannot be completely restored to LCC6's level (Figure 2A). Surprisingly, the combination of **6** and **25** displayed a dosage-dependent restoration of DOX accumulation in LCC6MDR (Figure 2D). Intracellular DOX accumulation can be restored to the parental LCC6 level when a combination of 8 μM **6** and 8 μM **25** was applied (Figure 2D).

Dixon Plot Suggests a Noncompetitive Relationship between Permethyl Ningalin B Analogues and DOX in Binding P-gp. We have employed Dixon analysis to further characterize the inhibition mechanism of permethyl ningalin B analogues on P-gp's DOX transport activity. The increase in DOX retention in LCC6MDR cells was generally dependent on the concentration of permethyl ningalin B analogues. The Dixon plot is a common method to determine inhibition constants (K_i) of competitive inhibitors in which the reciprocal of the velocity ($1/v$) is plotted against the inhibitor concentration at two or more substrate concentrations.[42] The intersection point in the Dixon plot provides a measure of K_i. Linear regression line obtained from the replot of slopes of Dixon plot versus the reciprocal of substrate concentration can distinguish between a competitive and noncompetitive or partially competitive relationship. A competitive inhibitor will give a regression line passing through the origin, whereas a noncompetitive inhibitor would not.[43,44]

The K_i value was determined to be 5.5 μM for **6** (Figure 3A), 5.4 μM for **25** (Figure 3B), and 5.8 μM for the combination of **6** and **25** (Figure 3C), respectively. Linear regression line does not coincide with the origin for **6** (inset of Figure 3A), **25** (inset of Figure 3B), and the combination of **6** and **25** (inset of Figure 3C), suggesting that permethyl ningalin B analogues likely act as a noncompetitive inhibitor of P-gp-mediated DOX transport. The K_i value of the combination of **6** and **25** was similar to that of compound **6** or **25** alone, indicating that there is no synergy for **6** and **25** in inhibiting the P-gp-mediated DOX transport and that these data are in good agreement with the in vitro DOX cytotoxicity assay in which an additive effect was found when combining **6** and **25** (Table 4).

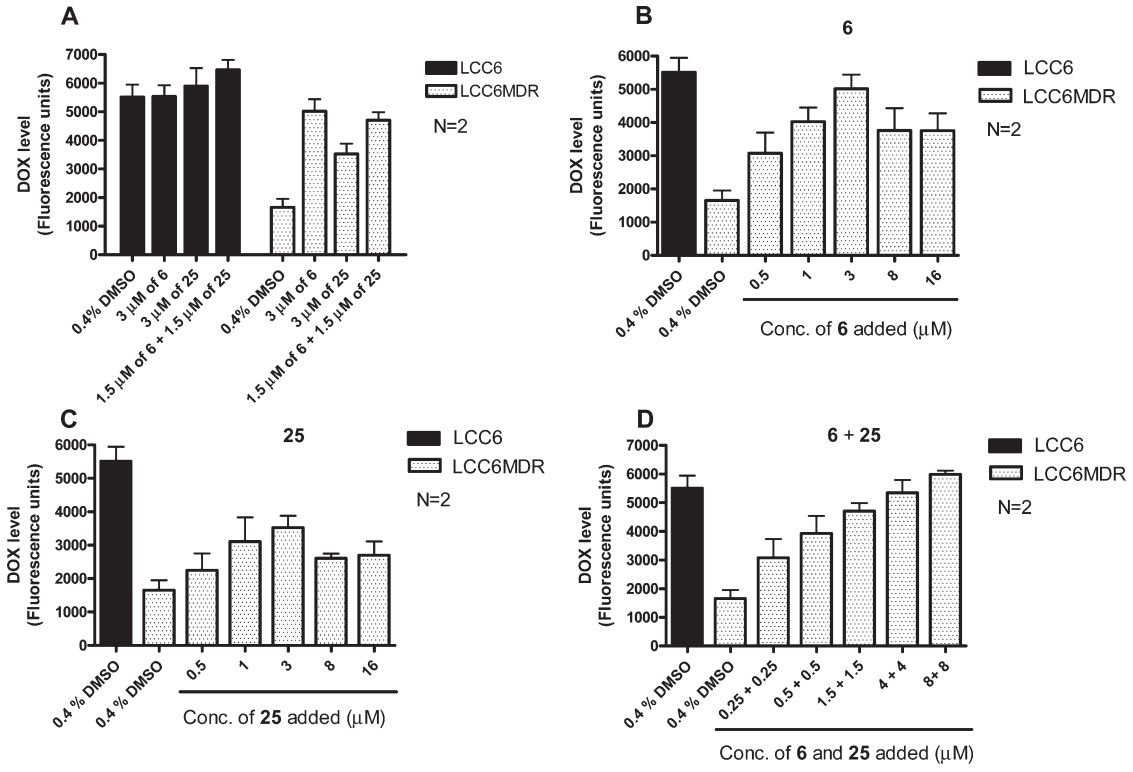


Figure 2. Effect of permethyl ningalin B analogues added at different concentrations on intracellular DOX accumulation in LCC6 and LCC6MDR cells. (A) LCC6 or LCC6MDR cells were incubated with 20 μM DOX for 150 min at 37 °C with or without **6**, **25**, or combination of **6** and **25**. After the incubation period, we lysed the cells and used the supernatant to measure the DOX level by spectrofluorometry. Experiments were performed in duplicate and repeated twice. The results were presented as the mean ± standard error of mean. The dosage effect of permethyl ningalin B analogues on intracellular DOX accumulation was also studied by incubating LCC6MDR cells with different concentrations of modulators including **6** (B), **25** (C), combination of **6** and **25** (D). DMSO (0.4%) was used as negative control.

Discussion

Overexpression of P-gp is one of the major obstacles for successful treatment of cancer. Numerous agents have been developed to reverse P-gp-mediated MDR; however, none of them could be used clinically because of lack of potency or high cytotoxicity at an effective concentration. Herein, synthetic permethyl ningalin B analogues were found to be noncytotoxic (IC_{50} > 100 μM) to L929 mouse fibroblast cell lines and cancer cell lines even when used at a very high concentration (Table 2). While lacking inherent cytotoxicity, synthetic permethyl ningalin B analogues exhibited a promising P-gp modulating activity at a low concentration (1 μM) and were found to be more effective than other traditional modulators like verapamil (Table 1). As all permethyl ningalin B analogues exerted no cytotoxicity toward fibroblast even at 100 μM (except **9**), we estimate that the therapeutic index for permethyl ningalin B analogues to be used as P-gp modulator will be at least 100 (IC_{50} of permethyl ningalin B analogues toward fibroblast/concentration of permethyl ningalin B analogues used).

We found that methoxy substituents on benzyl group and the type of linkers used are two key structural features for determining P-gp modulating activity of synthetic permethyl ningalin B analogues. Compound **6** with trimethoxy substituents and the shortest linker displayed the strongest P-gp modulating activity, and it caused about 18.2-fold resensitization of LCC6MDR toward paclitaxel (Table 1). It is possible that permethyl ningalin B with methoxy substituents and the shortest linker would bind more specifically to P-gp than other analogues with longer linker and nonmethoxy substituents. Intracellular DOX accumulation data shown in Figure 2 suggested that the mechanism of synthetic permethyl ningalin B analogues for reversing MDR is by increasing the intracellular drug concentration, thereby sensitizing LCC6MDR cells toward anticancer drugs.

Surprisingly, a synergistic activity on reversing paclitaxel resistance was noted when the combination of compounds **6** and **25** was used. This synergy resulted in a 66-fold (IC_{50} = 2.8 nM) sensitization of LCC6MDR to paclitaxel. This potent modulating activity can completely reverse the IC_{50} of LCC6MDR (IC_{50} = 183.2 nM, Tables 1 and 3) to that of parental LCC6 (IC_{50} = 2.6 nM, Table 1), and its potency is comparable to other standard P-gp modulators such as cyclosporine A (IC_{50} = 2.0 nM) and PSC833 (IC_{50} = 1.8 nM) (Table 1). The above data suggest that a cocktail chemotherapy including two modulators acting synergistically and an anticancer drug could likely be a novel, safe, and highly potent way to treat MDR cancer when other traditional treatment methods fail.

Synergism of **6** and **25** on modulating P-gp suggests that they might have slightly different mechanisms of action, possibly by binding to different regions of P-gp, although there is no experimental proof at this point. No synergy was noted in DOX and vincristine cytotoxicity when combining **6** and **25**, suggesting that the synergism by **6** and **25** on reversing drug resistance is drug/substrate specific. It is possible that **6** and **25** could work more cooperatively to prevent paclitaxel from being pumped out from the cell by P-gp than DOX and vincristine. One possible mechanism is that **6** and **25** could share more overlapping binding sites with paclitaxel on P-gp than DOX or vincristine, thereby inhibiting paclitaxel from binding onto P-gp to a larger extent.

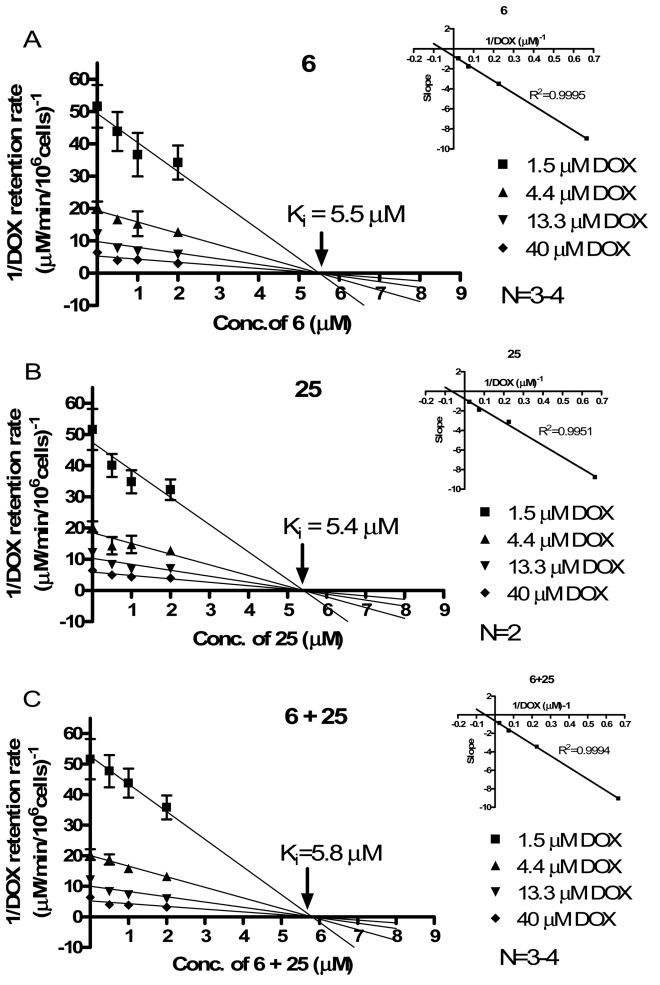


Figure 3. Kinetic study of relationship between permethyl ningalin B analogues and DOX using Dixon plot. LCC6MDR cells were incubated with different concentration of DOX (1.5, 4.4, 13.3, and 40 μM) in the presence of four concentrations (0, 0.5, 1, and 2 μM) of compound **6** (A), **25** (B), or combination of **6** and **25** (C), respectively. The reciprocal of DOX retention rate is plotted against the concentration of modulator. The intersection point represents the K_i value. The slope of the Dixon plot is plotted against reciprocal of DOX concentration to determine their relationship (insets of parts A, B, and C). Each data point represents two to four independent experiments and is presented as the mean ± standard error mean.

Kinetic analysis suggests that permethyl ningalin B analogues likely act as a noncompetitive inhibitor of DOX transport with K_i values ranging from 5.4 to 5.8 μM (Figure 3). The permethyl ningalin B analogues probably bind to P-gp at a binding site(s) different from the binding site(s) of DOX. Binding of permethyl ningalin B analogues to P-gp would reduce the affinity of DOX for its substrate binding site, diminish the amount of DOX being pumped out by P-gp, and eventually chemosensitize LCC6MDR cells to DOX.

In summary, we demonstrated that synthetic permethyl ningalin B analogues exhibited promising MDR reversal activity without any inherent cytotoxicity to cancer cell lines or normal mouse fibroblast cell lines. The methoxy substituents and types of linker used are two key structural features for determining MDR reversal potency of permethyl ningalin B analogues. Compound **6** containing trimethoxy substituents and the shortest linker displayed the strongest modulating activity and caused about 18.2-fold hypersensitization of LCC6MDR toward paclitaxel. The mechanism of synthetic permethyl ningalin B analogues for modulating MDR is by virtue of increasing intracellular drug accumulation. Surprisingly, a synergy (FICI = 0.42) on reversing paclitaxel resistance was noted for the combination of **6** and **25**, which caused about 66-fold resensitization of LCC6MDR toward paclitaxel and completely reversed the IC_{50} of LCC6MDR to the parental LCC6's level. Kinetic analysis suggests that permethyl ningalin B analogues likely act as a noncompetitive inhibitor of DOX transport. The present study demonstrates that the synthetic permethyl ningalin B analogues can be employed as safe and effective modulators of P-gp-mediated drug resistance in cancer cells.

Experimental Section

General. The starting materials and reagents, purchased from commercial suppliers, were used without further purification. Literature procedures were used for the preparation of 2-bromo-1-(3,4-dimethoxyphenyl)ethanone (**13**),[45] 2-bromo-1-phenylethanone (**22**),[45] 2-bromo-1-(4-methoxyphenyl)ethanone (**23**),[45] (4-methoxyphenyl)methanol (**19a**),[46,47] (3,4-dimethoxyphenyl)methanol (**19b**),[46,47] (3,4,5-trimethoxyphenyl)methanol (**19c**),[46,47] 2-(4-methoxyphenyl)ethanol (**19f**),[48,49] 2-(3,4-dimethoxyphenyl)ethanol (**19g**),[48,49] 2-(3,4,5-trimethoxyphenyl)ethanol (**19h**),[48,49] 3-(3,4,5-trimethoxyphenyl)propan-1-ol (**19i**),[48,50] (*E*)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-ol (**20**),[51] 3,4,5-trimethoxybenzylmethyl sulfate (**27**).[28]

All reactions were monitored by thin-layer chromatography (TLC) on aluminum sheets (silica gel 60-F254, E. Merck). Compounds were visualized by UV light. Anhydrous THF was distilled under nitrogen from sodium benzophenone ketyl. Anhydrous methylene chloride was distilled under nitrogen from CaH_2. Anhydrous DMF was distilled under vacuum from CaH_2. Reaction flasks were flame-dried under a stream of N_2. Flash chromatography was carried out using silica gel 60 (200−300 mesh). Silica gel chromatography solvents were of analytical grade. Melting points were recorded on a micromelting point apparatus MP-500D and were uncorrected. ^{1}H NMR and ^{13}C NMR (600 or 400 MHz) spectra were measured with TMS as an internal standard when $CDCl_3$ was used as a solvent. Chemical shifts are expressed in δ (ppm) and coupling constants (J) in Hz. High-resolution (ESI) mass spectra were recorded using a QTOF-2 Micromass spectrometer.

2-Hexamethylenetetramino-1-(3,4-dimethoxyphenyl)ethanone-2-ium Bromide (14). To a stirred solution of hexamethylenetetramine (3.57 g, 25.5 mmol) in chloroform (60 mL) at room temperature, a solution of **13** (5.5 g, 21.2 mmol) in chloroform (5 mL) was added quickly. After 4 h, the resulting reaction suspension was filtered, washed with chloroform, dried in vacuo, and recrystallized in methanol to afford the desired compound **14** (8.5 g, 99% yield): mp 110−112 °C.

2-Amino-1-(3,4-dimethoxyphenyl)ethanone Hydrobromic Acid (15). A solution of **14** (15.5 g, 37.5 mmol) in absolute methanol (20 mL) was added to a stirring solution of concentrated hydrobromic acid (40 mL) in absolute methanol (200 mL) at 0 °C. The clear reaction solution gradually changed into a thick suspension. The above mixture was allowed to warm to room temperature naturally. After 12 h, the resulting suspension was filtered, washed with ethyl ether and acetone, and dried in vacuo to afford the desired compound **15** (9.8 g, 95% yield): mp 236−238 °C; m/z $[M + H]^+$ 196.1.

2-(3,4-Dimethoxyphenyl)-*N*-(2-(3,4-dimethoxyphenyl)-2-ethanone)acetamide (16). To a stirred solution of 2-(3,4-dimethoxyphenyl)acetic acid (3.75 g, 19 mmol) and HOBt (2.56 g, 19 mmol) in dry CH_2Cl_2 (30 mL) at 0 °C under N_2 atmosphere was added DCC (3.9 g, 19 mmol) in batch over 10 min. After 30 min, to the above reaction solution was added **15** (3.3 g, 12 mmol) and triethylamine (1.57 g, 15.5 mmol). The reaction mixture was stirred for 18 h while the reaction temperature rose

to room temperature naturally. Solid dicyclohexylurea in the above reaction mixture was removed by filtration. The filter cake was washed with CH_2Cl_2. The filtrate was evaporated under reduced pressure and purified by flash chromatography on silica gel (EtOAc/PE = 3/1, v/v) to afford the desired compound **16** (5.45 g, 77% yield): mp 138−140 °C; ESI-MS; *m*/*z* $[M + H]^+$ 374.2; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.59−7.57 (dd, J = 8.4, 1.8 Hz, 1 H), 7.46 (d, J = 1.8 Hz, 1 H), 6.91−6.85 (m, 4 H), 6.65 (s, 1 H), 4.70−4.69 (d, J = 4.3 Hz, 2 H), 3.95−3.89 (m, 12H), 3.62 (s, 2 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 192.38, 171.5, 154.0, 149.2, 149.1, 148.3, 127.3, 126.8, 122.5, 121.5, 112.3, 111.4, 110.2, 109.7, 56.1, 55.8, 45.9, 43.1.

3,4-Bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (18). *t*-BuOK (250 mg, 2.25 mmol) was added to a stirred solution of **16** (280 mg, 0.75 mmol) in *t*-BuOH (20 mL) at 35 °C under air. After 2 h, the resulting reaction solution was poured into ice-cold water (100 mL), and then the pH was adjusted to 7−8 by adding 2 N hydrochloric acid to give a yellow thick suspension. The above yellow thick suspension was filtered and purified by flash chromatography on silica gel (EtOAc/PE = 2/1, v/v) to afford the desired compound **18** (120 mg, 45% yield): mp 238−240 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.63 (s, 1 H), 7.22−7.20 (dd, J = 8.4, 2.2 Hz, 2 H), 7.03−7.02 (d, J = 2.2 Hz, 2 H), 6.88−6.86 (d, J = 8.4 Hz, 2 H), 3.91 (s, 6 H), 3.71 (s, 6 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 170.7, 150.4, 148.7, 134.9, 123.5, 121.1, 112.4, 110.9, 55.8, 55.7; HRMS calcd for $(C_{20}H_{19}NO_6 + H)^+$ 370.1290, found 370.1284.

3,4-Bis(3,4-dimethoxyphenyl)-1*H*-pyrrol-2(5*H*)-one (17). *t*-BuOK (250 mg, 2.25 mmol) was added to a stirring solution of **16** (280 mg, 0.75 mmol) in *t*-BuOH (20 mL) at 35 °C under N_2 atmosphere. After 2 h, the pH of the resulting reaction solution was adjusted to 7−8 by adding 2 N hydrochloric acid under N_2 atmosphere. Then 80 mL of water was poured into the above solution to give yellow thick suspension. The suspension was then filtered and purified by flash chromatography on silica gel (EtOAc/PE = 1/1, v/v) to yield the desired compound **17** (140 mg, 50% yield): mp 170−172 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.70 (brs, 1 H), 7.01 (dd, J = 2.2, 8.2 Hz, 1H), 6.95−6.89 (m, 3 H), 6.83 (d, J = 2.2 Hz, 1H), 6.80 (d, J = 8.3 Hz, 1 H), 4.35 (s, 2 H), 3.87 (d, J = 5.5 Hz, 6 H), 3.78 (s, 3 H), 3.56 (s, 3 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 174.9, 150.1, 149.5, 149.0, 148.7, 130.7,125.9, 124.8, 122.4, 120.3, 112.7, 111.5, 111.0, 55.9, 5.7, 48.1; HRMS calcd for $(C_{20}H_{21}NO_5 + H)^+$ 356.1498, found 356.1491.

3,4-Bis(3,4-dimethoxyphenyl)-1-(2-hydroxyethyl)-1*H*-pyrrole-2,5-dione (29). DIAD (548 mg, 2.71 mmol) was added dropwise to a stirring solution of glycol (1 mL), PPh_3 (710 mg, 2.71 mmol), and **18** (200 mg, 0.54 mmol) in dry THF (40 mL) at 0 °C under N_2 atmosphere. The reaction mixture was allowed to warm to room temperature and stirred for 12 h until TLC showed the reaction has completed. The resulting reaction mixture was poured into water (60 mL), extracted with EtOAc, washed with brine, dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel (EtOAc/PE = 1/2, v/v) to yield the desired compound **29** (158 mg, 71% yield): mp 152−154 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.21 (dd, J = 1.9, 8.4 Hz, 2 H), 7.05 (d, J = 1.9 Hz, 2 H), 6.86 (d, J = 8.4 Hz, 2 H), 3.90 (s, 6 H), 3.86 (m, 4 H), 3.71 (s, 6 H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 172.5, 151.3, 149.5, 135.1, 124.4, 122.1, 113.4, 111.8, 62.1, 56.7, 56.6, 42.0; HRMS calcd for $(C_{22}H_{23}NO_7 + H)^+$ 414.1553, found 414.1561.

General Procedure for Compounds 30−32. An appropriate dibromide (0.5 mmol) was added to a suspension of **18** (1 mmol) and K_2CO_3 (2 mmol) in DMF (10 mL) at 90 °C under N_2 atmosphere. The suspension was stirred for 1 h until TLC showed the reaction has completed. The resulting solution was cooled to room temperature and poured into water (40 mL), extracted with EtOAc, washed with brine, dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel (EtOAc/PE = 1/1, v/v) to afford the desired compounds **30−32** (yield, 77−81%). The physical and spectral data for **30−32** are listed below.

1-((3,4-Bis(3,4-dimethoxyphenyl)-2,5-dioxo-2*H*-pyrrol-1(5*H*)-yl)methyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (30). Yield 81%; mp 187−188 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.21 (dd, J = 1.8, 8.7 Hz, 4 H), 7.05 (d, J = 1.8 Hz, 4H), 6.82 (d, J = 8.7 Hz, 4 H), 5.53 (s, 2 H), 3.89 (s, 12 H), 3.70 (s, 12 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 169.8, 150.6, 148.8, 134.3, 123.8, 121.2, 112.7, 111.0, 56.0, 55.9, 40.7; HRMS calcd for $(C_{41}H_{38}N_2O_{12} + Na)^+$ 773.2322, found 773.2311.

1-(2-(3,4-Bis(3,4-dimethoxyphenyl)-2,5-dioxo-2*H*-pyrrol-1(5*H*)-yl)ethyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (31). Yield 80%; mp 232−234 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.10 (dd, J = 1.8, 8.3 Hz, 4 H), 6.96 (d, J = 1.8 Hz, 4 H), 6.78 (d, J = 8.3 Hz, 4 H), 3.94 (s, 4 H), 3.88 (s, 12 H), 3.63 (s, 12 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 171.2, 150.4, 148.7, 134.5, 123.6, 121.5, 112.6, 110.9, 55.9, 55.8, 37.1; HRMS calcd for $(C_{42}H_{40}N_2O_{12} + H)^+$ 765.2659, found 765.2652.

1-(3-(3,4-Bis(3,4-dimethoxyphenyl)-2,5-dioxo-2*H*-pyrrol-1(5*H*)-yl)propyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (32). Yield 77%; mp 76−79 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.21−7.16 (m, 4 H), 7.03 (m, 4 H), 6.82 (m, 4 H), 3.89 (d, J = 3.6 Hz, 12 H), 3.73−3.69 (m, 16 H), 2.02 (m, 2 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 171.2, 150.5, 148.7, 134.2, 123.7, 121.5, 112.7, 111.0, 55.9, 55.8, 36.1, 31.5; HRMS calcd for $(C_{43}H_{42}N_2O_{12} + H)^+$ 779.2816, found 779.2825.

1-(2,3,4,5-Tetramethoxybenzyl)-2-(3,4,5-trimethoxybenzyloxy)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole (28). NaH (300 mg, 50−65%, moistened in oil) was added to dry DMF (20 mL) at 0 °C under N_2 atmosphere. To the above stirring suspension, **17** (200 mg, 0.56 mmol) in DMF (20 mL) was added drop by drop. The mixture was stirred at room temperature for 30 min, followed by the addition of a solution of **27** (200 mg, 0.72 mmol) in DMF (5 mL). The reaction mixture was heated at 80 °C for 12 h until TLC showed the reaction has completed. The reaction mixture was then cooled to room temperature and quenched with methanol (2 mL). The resulting solution was poured into water (100 mL), extracted with EtOAc, washed with brine, dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel (EtOAc/PE = 1/1, v/v) to yield the desired compound **28** (75% yield): mp 78−79 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.99 (dd, J = 1.8, 8.2 Hz, 1 H), 6.87 (s, 1 H), 6.79 (d, J = 8.7 Hz, 1 H), 6.65 (d, J = 8.2 Hz, 1 H), 6.51 (dd, J = 1.8, 8.2 Hz, 1 H), 6.48 (s, 1 H), 6.46 (s, 1 H), 6.28 (s, 2 H), 6.03 (s, 2 H), 4.88 (d, J = 14.6 Hz, 1 H), 3.87 (d, J = 14.6 Hz, 1 H), 3.81 (s, 3 H), 3.78 (d, J = 12.8 Hz, 1 H), 3.75 (s, 3 H), 3.73 (s, 3 H), 3.72 (s, 3 H), 3.69 (s, 3 H), 3.66 (s, 6 H), 3.60 (s, 3 H), 3.51 (s, 6 H), 3.23 (d, J = 12.8 Hz, 1 H); ^{13}C NMR-DEPT ($CDCl_3$, 600 MHz) δ 153.5, 152.3, 149.4, 148.9, 148.7, 147.9, 137.5, 136.3, 132.1, 131.8, 126.5, 126.1, 125.1, 118.9, 118.1, 111.5, 111.3, 110.0, 108.5, 106.5, 104.8, 60.8, 60.7, 56.1, 55.9, 55.7, 45.8 (CH_2), 39.9 (CH_2); HRMS calcd for $(C_{40}H_{45}NO_{11} + H)^+$ 716.3071, found 716.3076.

General Procedure for Compounds 24−26. Appropriate substituted 2-bromo-1-phenylethanone **13**, **22**, or **23** (1 mmol) was added to a suspension of **18** (1 mmol) and K_2CO_3 (2 mmol) in DMF (10 mL) at room temperature under an N_2 atmosphere. The suspension was stirred for 1 h until TLC showed the reaction has completed. The resulting solution was poured into water (40 mL), extracted with EtOAc, washed with brine, dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel (EtOAc/PE = 1/1, v/v) to afford the desired compounds **24−26** (yields 88−91%). The physical and spectral data for **24−26** are listed below.

3,4-Bis(3,4-dimethoxyphenyl)-1-[2-phenyl-2-oxoethyl]-1*H*-pyrrole-2,5-dione (24). Yield 88%; mp 175−177 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.00 (dd, J = 1.3, 7.8 Hz, 2 H), 7.63 (m, 1 H), 7.51 (t, J = 7.8 Hz, 2 H), 7.23 (dd, J = 1.8, 8.7 Hz, 2 H), 7.09 (d, J = 1.8 Hz, 2 H), 6.86 (d, J = 8.7 Hz, 2 H), 5.08 (s, 2H), 3.90

(s, 6 H), 3.71 (s, 6 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 191.6, 170.8, 150.5, 148.7, 134.7, 134.5, 134.1, 129.0, 128.2, 123.7, 121.5, 112.7, 111.0, 56.0, 55.9, 44.5; HRMS calcd for ($C_{28}H_{25}NO_7 + H)^+$ 488.1709, found 488.1704.

1-[2-(4-Methoxyphenyl)-2-oxoethyl]-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (25). Yield 90%; mp 170−172 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.98 (d, J = 8.7 Hz, 2 H), 7.24 (dd, J = 1.8, 8.2 Hz, 2 H), 7.09 (d, J = 1.8 Hz, 2 H), 6.98 (d, J = 8.7 Hz, 2 H), 6.86 (d, J = 8.7 Hz,2 H), 5.03 (s, 2 H), 3.90 (s, 6 H), 3.88 (s, 3 H), 3.71 (s, 6 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 189.9, 170.9, 164.2, 150.4, 148.7, 134.7, 130.5, 127.5, 123.7, 121.6, 114.2, 112.7, 111.0, 56.0, 55.9, 55.6, 44.2; HRMS calcd for ($C_{29}H_{27}NO_8 + H)^+$ 518.1815, found 518.1823.

1-[2-(3,4-Dimethoxyphenethyl)-2-oxoethyl]-3,4-bis(3,4-diethoxyphenyl)-1*H*-pyrrole-2,5-dione (26). Yield 91%; mp 91−94 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.65 (dd, J = 1.8, 8.7 Hz, 1 H), 7.52 (d, J = 1.4 Hz, 1 H), 7.24 (dd, J = 1.8, 8.7 Hz, 2 H), 7.09 (s, 2 H), 6.93 (d, J = 8.7 Hz, 1 H), 6.86 (d, J = 8.7 Hz, 2 H), 5.05 (s, 2 H), 3.96 (s, 3 H), 3.92 (s, 3 H), 3.89 (s, 6 H), 3.71 (s, 6 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 190.1, 170.9, 154.1, 150.5, 149.3, 148.7, 134.7, 127.7, 123.7, 122.8, 121.5, 112.7, 111.0, 110.3, 56.2, 56.1, 56.0, 55.9, 44.2; HRMS calcd for ($C_{30}H_{29}NO_9 + H)^+$ 548.1920, found 548.1912.

General Procedure for Compounds 4−12. DIAD (5 mmol) was added dropwise to a stirred solution of the appropriate alcohol **19a−i** (1.05 mmol), PPh_3 (5 mmol), and **18** (1 mmol) in dry THF (40 mL) at 0 °C under N_2 atmosphere. The reaction mixture was allowed to warm to room temperature and stirred for 12 h until TLC showed that the reaction has completed. The resulting reaction mixture was poured into water (100 mL), extracted with EtOAc, washed with brine, dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel (EtOAc/PE = 1/4 to 1/1, v/v) to afford the desired compounds **4−12** (yield 61−71%). The physical and spectral data for **4−12** are listed below.

1-(4-Methoxybenzyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (4). Yield 71%; mp 125−126 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.40 (dd, J = 7.8, 2.2 Hz, 2 H), 7.19 (dd, J = 8.2, 2.2 Hz, 2 H), 7.03 (d, J = 2.2 Hz, 2 H), 6.86−6.83 (m, 4 H), 4.72 (s, 2 H), 3.89 (s, 6 H), 3.78 (s, 3 H), 3.70 (s, 6 H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 171.6, 160.0, 151.1, 149.4, 134.9, 131.0, 129.7, 124.4, 122.3, 114.6, 113.4, 111.7, 56.7, 56.6, 56.0, 42.1; HRMS calcd for ($C_{28}H_{27}NO_7 + H)^+$ 490.1866, found 490.1861.

1-(3,4-Dimethoxybenzyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (5). Yield 68%; mp 122−124 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.18 (dd, J = 8.2, 1.6 Hz, 2 H), 7.03−7.01 (m, 4 H), 6.84 (d, J = 8.2 Hz, 2 H), 6.81 (d, J = 8.2 Hz, 1 H), 4.71 (s, 2 H), 3.88 (s, 6 H), 3.87 (s, 3 H), 3.84 (s, 3 H), 3.69 (s, 6 H) ; ^{13}C NMR ($CDCl_3$, 400 MHz) δ 171.7, 151.2, 149.7, 149.5, 135.0, 130.0, 124.4, 122.2, 113.4, 113.0, 111.8, 56.7, 56.6, 42.5; HRMS calcd for ($C_{29}H_{29}NO_8 + H)^+$ 520.1971, found 520.1965.

1-(3,4,5-Trimethoxybenzyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (6). Yield 67%; mp 154−155 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.20 (dd, J = 8.2, 2.2 Hz, 2 H), 7.03 (d, J = 2.2 Hz, 2 H), 6.86 (d, J = 8.2 Hz, 2 H), 6.71 (s, 2 H), 4.71 (s, 2 H), 3.89 (s, 6 H), 3.86 (s, 6 H), 3.82 (s, 3 H), 3.69 (s, 6 H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 171.6, 154.1, 151.2, 149.5, 138.4, 135.0, 133.0, 124.4, 122.2, 113.4, 111.8, 107.0, 61.6, 57.0, 56.7, 56.6, 43.0; HRMS calcd for ($C_{30}H_{31}NO_9 + H)^+$ 550.2077, found 550.2072.

3,4-Bis(3,4-dimethoxyphenyl)-1-phenethyl-1*H*-pyrrole-2,5-dione (8). Yield 67%; mp 128−131 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.31−7.27 (m, 5 H), 7.17 (dd, J = 1.8, 8.2 Hz, 2 H), 7.03 (d, J = 1.8 Hz, 2 H), 6.85 (d, J = 8.2 Hz, 2 H), 3.9 (s, 6 H), 3.85 (t, J = 7.8 Hz, 2 H), 3.72 (s, 6 H), 2.97 (t, J = 7.8 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 171.1, 150.4, 148.8, 138.3, 134.2, 128.9, 128.6, 123.6, 121.5, 112.7, 111.1, 56.0, 55.9, 39.7, 34.9; HRMS calcd for ($C_{28}H_{27}NO_6 + H)^+$ 474.1916, found 474.1922.

1-(4-Methoxyphenethyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (9). Yield 65%; mp 108−110 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.19−7.17 (m, 4 H), 7.03 (d, J = 1.8 Hz, 2 H), 6.86−6.84 (m, 4 H), 3.89 (s, 6 H), 3.83 (t, J = 7.7 Hz, 2 H), 3.78 (s, 3 H), 3.72 (s, 6 H), 2.90 (t, J = 7.7 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 171.1, 158.4, 150.4, 148.8, 134.2, 130.3, 129.9, 123.6, 121.6, 114.1, 112.6, 111.0, 56.0, 55.9, 55.3, 39.9, 33.9; HRMS calcd for ($C_{29}H_{29}NO_7 + H)^+$ 504.2022, found 504.2028.

1-(3,4-Dimethoxyphenethyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (10). Yield 66%; mp 178−180 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.18 (dd, J = 8.2, 2.2 Hz, 2 H), 7.03 (d, J = 2.2 Hz, 2 H), 6.86 (d, J = 8.2 Hz, 2 H), 6.82 (s, 2 H), 6.77 (s, 1H), 3.91 (s, 6 H), 3.87−3.84 (m, 8 H), 3.72 (s, 6 H), 2.93 (t, J = 7.6 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 171.8, 151.2, 149.7, 149.5, 148.5, 134.9, 131.5, 124.3, 122.2, 121.7, 113.4, 112.8, 112.1, 111.8, 56.7, 56.6, 40.5, 35.0; HRMS calcd for ($C_{30}H_{31}NO_8 + H)^+$ 534.2128, found 534.2133.

3,4-Bis(3,4-dimethoxyphenyl)-1-(3-(3,4,5-trimethoxyphenyl)-propyl)-1*H*-pyrrole-2,5-dione (12). Yield 61%; mp 59−60 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.16 (dd, J = 1.8, 8.3 Hz, 2 H), 7.00 (d, J = 1.8 Hz, 2 H), 6.84 (d, J = 8.3 Hz, 2 H), 6.41 (s, 2 H), 3.89 (s, 6 H), 3.79 (s, 6 H), 3.77 (s, 3 H), 3.71−3.69 (m, 8 H), 2.64 (t, J = 7.3 Hz, 2 H), 2.03 (quintet, J = 7.3 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 171.3, 153.1, 150.4, 148.8, 136.9, 134.1, 123.6, 121.5, 112.7, 111.1, 105.2, 60.8, 56.0, 55.9, 38.2, 33.6, 29.4; HRMS calcd for ($C_{32}H_{35}NO_9 + H)^+$ 578.2390, found 578.2381.

1-Benzyl-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (7). Yield 70%; mp 162−165 °C; ESI-MS (m/z) $[M + H]^+$ 460.1; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.46 (m, 2 H), 7.33 (m, 2 H), 7.28 (m, 1 H), 7.20 (dd, J = 8.2, 2.2 Hz, 2 H), 7.04 (d, J = 2.2 Hz, 2 H), 6.84 (d, J = 8.2 Hz, 2 H), 4.79 (s, 2 H), 3.89 (s, 6 H), 3.70 (s, 6 H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 171.6, 151.2, 149.4, 137.4, 134.9, 129.5, 128.6, 124.4, 122.2, 113.4, 111.7, 56.7, 56.6, 42.6; HRMS calcd for ($C_{27}H_{25}NO_6 + H)^+$ 460.1760, found 460.1768.

1-(3,4,5-Trimethoxyphenethyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (11). Yield 63%; mp 126−127 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.16 (d, J = 7.7 Hz, 2 H), 7.01 (s, 2 H), 6.84 (d, J = 7.7 Hz, 2 H), 6.46 (s, 2 H), 3.89 (s, 6 H), 3.85 (t, J = 7.7 Hz, 2 H), 3.83 (s, 6 H), 3.79 (s, 3 H), 3.70 (s, 6 H), 2.92 (t, J = 7.7 Hz, 2H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 171.1, 153.3, 150.5, 148.8, 136.7, 134.2, 133.8, 123.6, 121.4, 112.6, 111.1, 105.8, 60.9, 56.1, 56.0, 55.9, 39.5, 35.0; HRMS calcd for ($C_{31}H_{33}NO_9 + H)^+$ 564.2233, found 564.2225.

(*E*)-1-(3,4,5-Trimethoxycinnamyl)-3,4-bis(3,4-dimethoxyphenyl)-1*H*-pyrrole-2,5-dione (21). Yield 61%; mp 129−130 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.18 (dd, J = 1.8, 8.7 Hz, 2 H), 7.02 (d, J = 1.8 Hz, 2 H), 6.83 (d, J = 8.7 Hz, 2 H), 6.75 (s, 2 H), 6.61 (dt, J = 7.2, 10.1, 17.5 Hz, 1 H), 5.78 (d, J = 7.2 Hz, 1 H), 5.34 (dd, J = 10.1, 17.8 Hz, 2 H), 3.89 (s, 6 H), 3.85 (s, 6 H), 3.81 (s, 3 H), 3.69 (s, 6 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 170.6, 153.3, 150.5, 148.7, 137.6, 134.6, 134.5, 134.1, 128.7, 123.7, 121.4, 112.7, 111.0, 105.5, 60.9, 57.5, 56.3, 56.0, 55.9; HRMS calcd for ($C_{32}H_{33}NO_9 + H)^+$ 576.2233, found 576.2239.

General Procedure for Compounds 33 and 34. 2,4-Bis-(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane 2,4-disulfide (Lawesson reagent) (1.05 mmol) was added to a stirring solution of **7** or **11** (1 mmol) in dehydrated toluene (30 mL) at room temperature under an N_2 atmosphere. The mixture was refluxed for 8 h until TLC showed that the reaction has completed. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel (AcOEt/*n*-hexane1 = 1/3, v/v) to afford the desired compounds **33** and **34**. The physical and spectral data for **33** and **34** are listed below.

1-Benzyl-3,4-bis(3,4-dimethoxyphenyl)-5-thioxo-1*H*-pyrrol-2(5*H*)-one (33). Yield 68%; mp 177−178 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.46 (d, J = 7.3 Hz, 2 H), 7.32 (m, 2 H), 7.29−7.24 (m, 2 H), 7.04 (s, 1 H), 6.90 (d, J = 8.2 Hz, 1 H), 6.87 (d, J = 8.2 Hz, 1 H), 6.83 (s, 1 H), 6.80 (d, J = 8.2 Hz, 1 H), 5.19 (s, 2 H), 3.90 (s, 3 H), 3.88 (s, 3 H), 3.74 (s, 3 H), 3.63 (s, 3 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 199.9, 173.1, 150.4, 149.8, 148.6, 139.0, 136.5, 129.8, 128.9, 128.6, 127.8, 124.2, 123.9, 123.6, 121.5,

113.8, 112.8, 110.9, 110.8, 56.0, 55.9, 55.7, 44.8; HRMS calcd for $(C_{27}H_{25}NO_5S + H)^+$ 476.1531, found 476.1541.

1-(3,4,5-Trimethoxyphenethyl)-3,4-bis(3,4-dimethoxyphenyl)-5-thioxo-1*H*-pyrrol-2(5*H*)-one (34). Yield 60%; mp 60−62 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.19 (d, J = 8.7 Hz, 1 H), 7.01 (s, 1 H), 6.89 (s, 2 H), 6.81−6.80 (m, 2 H), 6.48 (s, 2 H), 4.21 (t, J = 7.8 Hz, 2 H), 3.91 (s, 3 H), 3.88 (s, 3 H), 3.85 (s, 6 H), 3.82 (s, 3 H), 3.75 (s, 3 H), 3.64 (s, 3 H), 2.95 (t, J = 7.8 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 600 MHz) δ 200.1, 172.9, 153.3, 150.4, 149.8, 148.7, 148.6, 138.9, 136.6, 133.9, 129.8, 124.1, 123.8, 123.5, 121.5, 113.8, 112.7, 111.0, 110.9, 105.9, 60.9, 56.2, 55.9, 55.7, 42.7, 34.6; HRMS calcd for $(C_{31}H_{33}NO_8S + H)^+$ 580.2005, found 580.2013.

Materials for Biological Studies. DMSO, verapamil, cyclosporine A, doxorubicin (DOX), paclitaxel, and vincristine were purchased from Sigma-Aldrich. PSC833 is a generous gift of Novartis pharmaceutical company. Dulbecco's modified Eagle's medium (DMEM), trypsin−ethylenediaminetetracetic acid (EDTA), and penicillin/streptomycin were from Gibco BRL. Fetal bovine serum (FBS) was from Hyclone Laboratories. 2-(4,5-Dimethylthiazol-2-yl)-5-[3-(carboxymethoxy)phenyl]-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) and phenazine methosulfate (PMS) were purchased from Promega. Human breast cancer cell lines MDA435/LCC6 and MDA435/LCC6MDR were kindly provided by Dr. Robert Clarke (Georgetown University, Washington, DC). L929, a mouse connective tissue fibroblast, was purchased from ATCC.

Cell Culture. MDA435/LCC6, MDA435/LCC6MDR, and L929 cells were maintained in DMEM media supplemented with 10% (v/v) heat-inactivated FBS and 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. When the cells reached confluence, they were detached by 0.05% trypsin−EDTA.[18]

P-gp Modulating Activity. Six-thousand cells of LCC6 or LCC6MDR and anticancer drug paclitaxel, DOX, or vincristine were mixed with or without modulators to a final volume of 200 μL in each well of 96-well plates. The plates were then incubated for 5 days at 37 °C. The CellTiter 96 AQ_{ueous} assay (Promega) was used to measure the cell proliferation according to the manufacturer's instructions. MTS (2 mg/mL) and PMS (0.92 mg/mL) were mixed in a ratio of 20:1. An aliquot (10 μL) of the freshly prepared MTS/PMS mixture was added to each well, and the plate was incubated for 2 h at 37 °C. Optical absorbance at 490 nm was then recorded with a microplate absorbance reader (Bio-Rad). IC_{50} values were calculated from the dose−response curves of MTS assays (Prism 4.0). All experiments were performed in triplicate and repeated at least thrice, and the results were represented as mean ± standard error of mean.[52]

Cytotoxicity Assay. Ten-thousand cells of LCC6 or LCC6-MDR or L929 were mixed with a series of concentrations of ningalin B analogues to a final volume of 100 μL in each well of 96-well plates. The plates were then incubated for 3 days at 37 °C. Then 50% inhibitory concentration (IC_{50}) of ningalin B analogues was determined using MTS proliferation assay as described previously.

Determination of in Vitro Synergistic Effect. In vitro drug interaction between ningalin B analogues on reversing paclitaxel, DOX, or vincristine resistance was assessed using fractional inhibitory concentration index (FICI) method modified from Chou and Talalay.[41] LCC6MDR was seeded into 96-well flat bottom microtiter plate at 6000 cells per well in a final volume of 200 μL of medium. A combination of two modulators, 0.5 μM each, was added to each well with various concentrations of paclitaxel, DOX, or vincristine. The 50% inhibitory concentration (IC_{50}) of paclitaxel, DOX, or vincristine at different combinations was determined using MTS proliferation assay as described above. The IC_{50} values allow the calculation of the fractional inhibitory concentration (FIC) and fractional inhibitory concentration index (FICI). For example, (FICI = $[FIC_{\mathbf{6}} + FIC_{\mathbf{25}}]$; $FIC_{\mathbf{6}}$ = IC_{50} of paclitaxel with the combination of **6** and **25** divided by IC_{50} of paclitaxel with **6** alone; $FIC_{\mathbf{25}}$ = IC_{50} of paclitaxel with the combination of **6** and **25** divided by IC_{50} of paclitaxel with **25** alone). FICI values were used to classify the overall nature of drug interaction. A FICI greater than 0.5 to 4.0 represents additivity; less than or equal to 0.5 represents synergy or potentiation, and greater than 4.0 means antagonism.[53] The modulator interaction on reversing drug resistance is further confirmed by a F_a−FICI plot[54] which is a plot with FICI against F_a value.[53]

Intracellular DOX Accumulation. The 1×10^6 cells of LCC6 or LCC6MDR cells were added to an Eppendorf tube containing 20 μM DOX and different concentrations of ningalin B analogues at 37 °C for 150 min. Then 0.4% DMSO in place of ningalin B analogues was used as a negative control. After incubation, the cells were spinned down and washed with cold PBS, pH7.4, and lysed with lysis buffer (0.75 M HCl, 0.2% Triton-X100 in isopropanol). The lysate was spinned down and the supernatant was saved. The fluorescence level of DOX was determined by fluorescence spectrophotometer (BMG Technologies) using an excitation and an emission wavelength of 460 and 610 nm.[52]

Kinetic Characterization of P-gp Inhibition by Modulator. Kinetic parameters for P-gp inhibition by modulators were determined by incubating 1×10^6 LCC6MDR cells with various concentrations of DOX (1.5, 4.4, 13.3, and 40 μM) in the presence of four concentrations (0, 0.5, 1, and 2 μM) of compounds **6** and **25** or the combination of **6** and **25** for 150 min at 37 °C. The cell lysis and fluorescence intensity were carried out as described previously. The relationship between DOX and ningalin analogue for P-gp inhibition was analyzed by Dixon plot. In the Dixon plot, the K_i was estimated from the linear regression of reciprocal plot of DOX retention rate versus modulator concentration. The slope of Dixon plot was plotted against the reciprocal of DOX concentration to define the relationship between the modulator and DOX.

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Supporting Information Available: ^{1}H NMR and ^{13}C NMR spectra for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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