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Polyoxypregnane Steroids from the Stems of *Marsdenia tenacissima*

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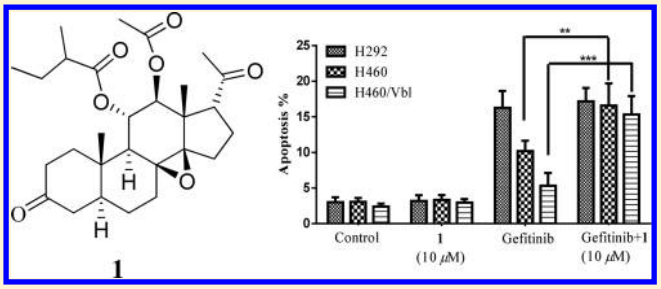
Supporting Information

ABSTRACT: A new polyoxypregnane aglycone, tenacigenin D (**1**), and seven new C_{21} steroid glycosides, tenacissimosides D−J (**2**−**8**), were isolated from the stems of *Marsdenia tenacissima*. Their structures were determined by interpretation of their 1D and 2D NMR and other spectroscopic data, as well as by comparison with published values for related known compounds. Compound **1** was found to circumvent P-glycoprotein (P-gp)-mediated multidrug resistance through an inhibitory effect on P-gp with a similar potency to verapamil. In addition, compound **1** potentiated the activity of erlotinib and gefitinib in epidermal growth factor receptor tyrosine kinase inhibitor (EGFR TKI)-resistant non-small-cell lung cancer cells.



Multidrug resistance (MDR), the ability of cancer cells to acquire resistance to anticancer drugs, severely hinders the effectiveness of different forms of anticancer chemotherapy. Many cellular mechanisms are known to cause MDR, including increased drug efflux, reduced drug uptake, activation of drug-metabolizing enzymes, activation of DNA repair, mutation of drug acting targets, and disruption in apoptotic signaling.[1,2] Among them, P-glycoprotein (P-gp), which belongs to a family of ATP-binding cassette (ABC) transporters, is known to play a crucial role in MDR by increasing drug efflux out of cancer cells.[1,2]

On the basis of the strategy of inhibiting drug efflux, three generations of P-gp inhibitors have been developed to enhance the effect of chemotherapeutic drugs in MDR cancer cells in vitro and in vivo during the past few decades.[3−6] Nevertheless, attempts to translate the inhibitors of P-gp transporters into useful drug targets have been unsuccessful, and no significant benefit of P-gp inhibition has been achieved yet.

Natural products and their derivatives are valuable therapeutic agents, including those obtained from traditional Chinese medicines (TCM).[7] Numerous classes of compounds isolated from TCM, including coumarins, terpenoids, and steroids, have been reported to exhibit MDR-modulating activity.[8−10] *Marsdenia tenacissima* (Roxb.) Wight et Arn., belonging to the family Asclepiadaceae, is distributed mainly in the southwest region of mainland China. Its rhizomes and roots have been recorded in medicinal texts as being useful for the treatment of cancer.[11] Chemical investigations have revealed that this plant is rich in polyoxypregnane glycosides. The polyoxypregnane glycosides isolated from *M. tenacissima* have been identified as tenacigenin B derivatives with an oligosaccharide chain at C-3, acylated groups such as acetyl, benzoyl, 2-methylbutyryl, and tigloyl esters at C-11 and/or C-12,[12−14] and an epoxide ring at C-8 and C-14. Several of the polyoxypregnane compounds isolated from this plant showed cytotoxic activity against cancer cell lines.[13,14c,14d] A recent study reported that tenacissoside C from *M. tenacissima* inhibited tumor growth in nude mice bearing K562 human erythroleukemia xenografts.[15] In addition to their cytotoxic activity for cancer cells, two derivatives of tenacigenin B isolated from this plant have also been reported to increase drug sensitivity in P-gp-overexpressing human hepatoma cells.[16] Furthermore, a recent in vitro study reported that an extract of *M. tenacissima* restored gefitinib sensitivity in resistant non-small-cell lung cancer (NSCLC) cells.[17]

In an in-depth search for potential constituents and MDR-reversal agents from natural sources, eight new polyoxypregnane compounds (**1**−**8**) were isolated and evaluated from *M. tenacissima*. Among these eight compounds, only compound **1** was found to significantly circumvent anticancer drug resistance by inhibiting the P-gp-mediated efflux of substrate anticancer

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drugs in the P-gp-overexpressing cancer cell lines. Compound **1** was found to potentiate the activity of two P-gp substrate epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), erlotinib and gefitinib, in two resistant NSCLC cell lines, via a dual action on reversal of both the P-gp-overexpression and K-ras-mutation-mediated MDR mechanisms.

RESULTS AND DISCUSSION

A 95% EtOH extract of air-dried *M. tenacissima* stems (23.0 kg) was suspended in water and partitioned successively with EtOAc and *n*-BuOH. The EtOAc fraction was subjected to column chromatography over silica gel, MCI gel, Sephadex LH-20 gel, and finally preparative HPLC, affording eight new polyoxypregnane compounds, **1**−**8** (Figure 1).

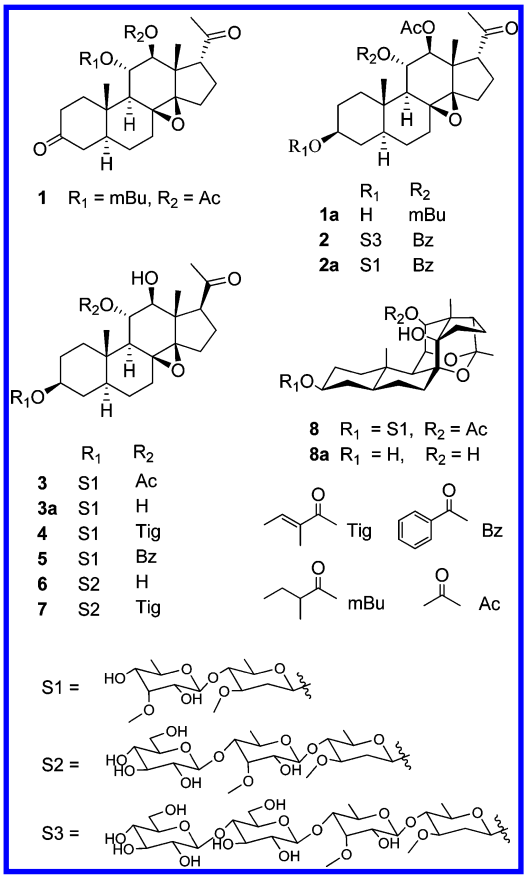

Figure 1. Structures of **1**−**8** isolated from *Marsdenia tenacissima*.

Tenacigenin D (**1**) was obtained as a colorless, amorphous powder. HREIMS (*m*/*z* 488.2763 $[M]^+$) analysis revealed a molecular formula of $C_{28}H_{40}O_7$ with nine degrees of unsaturation. IR absorptions at 1726 and 1708 cm^{-1} indicated the presence of carbonyl groups. In the ^1H NMR spectrum (Table 1), characteristic protons for three methyls at δ_H 1.08 (s), 1.28 (s), and 2.20 (s) and two oxygenated methines at δ_H 5.40 (t, *J* = 10.0 Hz) and 5.00 (d, *J* = 10.0 Hz) were observed. The proton signals at δ_H 1.97 (s) and [δ_H 2.20 (m), 1.42 (m), and 1.63 (m), 0.88 (t, *J* = 7.5 Hz), 1.04 (d, *J* = 7.0 Hz)], together with carbon resonances at δ_C 170.6 and 20.8 and at δ_C 175.5, 41.2, 26.1, 11.7, and 15.3, suggested the presence of acetyl and 2-methylbutyryl groups, respectively.

Analysis of the 1D NMR spectroscopic data of **1** showed this compound closely resembles 11α-*O*-2-methylbutyryl-12β-*O*-acetyltenacigenin B (**1a**), which was isolated from the same plant.[16] The significant differences found were that the signal at δ_H 3.48 (m) assigned to H-3 in **1a** and the signal at δ_C 70.4 were absent, but a resonance for a carbonyl group appeared at δ_C 210.5 in **1**. This suggested the hydroxy group at C-3 in **1a** was replaced by a carbonyl group in **1**. This deduction was supported by the cross-peaks of H-2/C-3 and H-4/C-3 in the HMBC spectrum of **1** (see Supporting Information Figure 1A). The 2-methylbutyryl and acetyl ester groups were located at C-11 and C-12, respectively, by interpretation of the HMBC correlations of H-11/C-1′ (δ_C 175.5) and H-12/C-1″ (δ_C 170.6). Therefore, compound **1** was determined to be 11α-*O*-2-methylbutyryl-12β-*O*-acetyl-3-oxo-17α-tenacigenin B.

Tenacissimoside D (**2**) gave a molecular formula of $C_{56}H_{82}O_{24}$ from the quasimolecular ion peak at *m*/*z* 1161.5045 $[M + Na]^+$ in its HRESIMS. The IR absorption bands at 3419, 1724, 1662, and 1452 cm^{-1} suggested the presence of hydroxy, carbonyl, and aromatic functional groups. The ^1H NMR spectrum of compound **2** (Tables 1 and 3) showed four anomeric proton signals at δ_H 4.37 (d, *J* = 7.7 Hz), 4.38 (d, *J* = 7.4 Hz), 4.60 (d, *J* = 8.5 Hz), and 4.66 (d, *J* = 7.8 Hz), with the corresponding carbon resonances occurring at δ_C 105.1, 106.6, 99.1, and 102.6, indicating four sugar moieties with a β-orientation. The signals for two methyls at δ_H 1.27 and 1.28 (d, *J* = 5.8 Hz) and two methoxy groups at δ_H 3.36 and 3.58 (s) in the ^1H NMR spectrum and the signals at δ_C 19.3, 18.6, 57.9, and 62.5 suggested that compound **2** possesses a 2,6-didexoy-3-*O*-methylpyranosyl moiety and a 6-deoxy-3-*O*-methylpyranosyl unit, as supported by TOCSY and HMBC correlations (see Supporting Information Figure 2A).

Enzymatic hydrolysis of **2** with β-cellulase yielded only one sugar unit in the aqueous extract, which was identified as β-D-glucose by comparing the R_f value on thin-layer chromatography (TLC) with an authentic sample, as well as with its specific rotation measurement. The sequence of the sugar chain of **2** was deduced as β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-3-*O*-methyl-6-deoxy-β-D-allopyranosyl-(1→4)-β-D-oleandropyranoside by HMBC correlations. Further analysis on the hydrolysate in the organic extract revealed a single product from enzymatic hydrolysis of **2**, which was identified as tenacissoside I (**2a**) from its 1D NMR spectra, a steroid glycoside isolated from the title plant in 1999.[14a] Therefore, the structure of compound **2** was established as 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1→4)-β-D-oleandropyranosyl 11α-*O*-benzoyl-12β-*O*-acetyl-17α-tenacigenin B.

Tenacissimoside E (**3**) gave a quasimolecular ion at *m*/*z* 733.3817 $[M + Na]^+$ in its HRESIMS, suggesting a molecular formula of $C_{37}H_{58}O_{13}$. In the ^1H NMR spectrum, two anomeric proton signals at δ_H 4.56 (d, *J* = 8.8 Hz) and 4.78 (d, *J* = 8.2 Hz) (Table 3), combined with resonances at δ_C 96.8 and 99.2 (Table 4) in the ^{13}C NMR spectrum, suggested two sugar units to be present with a β-linkage. Interpretation of the TOCSY and HSQC spectra of compound **3** revealed the presence of the partial moieties −C(6′)H_3−C(5′)H−C(4′)H−C(3′)H−C(2′)-H_2−C(1′)H− and −C(6″)H_3−C(5″)H−C(4″)H−C(3″)H−C(2″)H−C(1″)H−. HMBC correlations were observed from the methoxy group signal at δ_H 3.36 (s) to the oxygenated methine carbon at δ_C 78.7 (C-3′) and from the methoxy group signal at δ_H 3.64 (s) to the oxygenated methine carbon at δ_C 81.0 (C-3″), indicating the two methoxy groups to be attached

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Table 1. ^{1}H NMR Data of the Aglycones of Compounds 1−7 (400 MHz, δ in ppm, J in Hz)

position	1[a]	2[b]	3[a]	4[a]	5[a]	6[b]	7[b]
1	1.65, m	1.30, m	1.26, m	1.21, m	1.26, m	1.26, m	1.25, m
	1.88, m	1.56, m	1.54, m	1.53, m	1.54, m	2.27, m	1.46, m
2	2.20, m	1.31, m	1.47, m	1.45, m	1.44, m	1.46, m	1.38, m
	2.35, m	1.57, m	1.80, m	1.74, m	1.78, m	1.78, m	1.67, m
3		3.61, m	3.62, m	3.62, m	3.63, m	3.63, m	3.62, m
4	2.24, m	1.26, m	1.30, m	1.30, m	1.31, m	1.22, m	1.24, m
	2.38, m	1.72, m	1.66, m	1.67, m	1.66, m	1.72, m	1.68, m
5	1.79, m	1.43, m	1.29, m	1.29, m	1.30, m	1.30, m	1.35, m
6	1.46, m	1.44, m	1.40, m	1.40, m	1.40, m	1.38, m	1.40, m
	1.64, m	1.55, m	1.58, m	1.58, m	1.59, m	1.49, m	1.54, m
7	1.40, m	1.28, m	1.26, m	1.27, m	1.27, m	1.28, m	1.24, m
	1.90, m	1.84, m	1.68, m	1.70, m	1.69, m	1.68, m	1.80, m
9	2.10, d (10.0)	2.10, d (10.2)	1.76, d (9.8)	1.81, d (9.7)	1.96, d (8.6)	1.51, d (9.5)	1.90, d (9.7)
11	5.40, t (10.0)	5.55, t (10.2)	5.04, t (9.8)	5.15, t (9.7)	5.35, t (8.6)	3.44, t (9.5)	5.10, t (9.7)
12	5.00, d (10.0)	5.12, d (10.2)	3.40, m	3.42, d (9.7)	3.36, m	3.28, d (9.5)	3.49, d (9.7)
15	1.46, m	1.60, m	1.69, m	1.70, m	1.71, m	1.58, m	1.62, m
	1.65, m	2.00, m	1.98, m	1.98, m	1.99, m	1.96, m	2.00, m
16	1.62, m	1.66, m	2.01, m	2.01, m	2.01, m	1.78, m	1.82, m
	1.98, m	2.23, m	2.13, m	2.13, m	2.12, m	2.11, m	2.10, m
17	2.92, d (7.6)	3.02, d (7.3)	2.65, dd (5.9, 11.9)	2.65, dd (6.2, 11.8)	2.67, dd (5.5, 12.6)	2.92, dd (11.6, 6.1)	3.07, dd (11.7, 6.4)
18	1.08, s	1.12, s	0.70, s	0.99, s	1.04, s	0.82, s	0.90, s
19	1.28, s	1.06, s	1.03, s	1.03, s	1.08, s	1.02, s	1.00, s
21	2.20, s	2.14, s	2.24, s	2.23, s	2.22, s	2.30, s	2.24, s
C-11	mBu	Bz	Ac	Tig	Bz		Tig
2′	2.20, m		2.04, s				
3′	1.42, m	7.92, br d (7.4)		6.85, d (8.7)	8.04, d (7.4)		6.85, q (8.7)
	1.63, m						
4′	0.88, t (7.5)	7.47, br d (7.4)		1.84, t (8.7)	7.44, d (7.4)		1.82, d (8.7)
5′	1.04, d (7.0)	7.60, t (7.4)		1.78, d (8.7)	7.51, t (7.4)		1.79, s
6′		7.47, br d (7.4)			7.44, d (7.4)		
7′		7.92, br d (7.4)			8.04, d (7.4)		
C-12	Ac	Ac					
2″	1.97, s	1.55, s					

[a]In $CDCl_3$. [b]In methanol-d_4.

to C-3′ and C-3″ of a sugar moiety, respectively. Consequently, the oligosaccharide chain of compound **3** was deduced as pachybiose (2,6-dideoxy-4-*O*-(6-deoxy-3-*O*-methyl-β-D-allopyranosyl)-3-*O*-methyl-β-D-arabinohexopyranose), which was identical to that of tenacigenoside A (**3a**).[18] This deduction was confirmed by the fact that the mild acid hydrolysate of compounds **3** and **3a** gave the same sugar unit, which was identified as pachybiose by TLC comparison with the authentic sample. The ^{1}H and ^{13}C NMR spectroscopic data of the aglycone of compound **3** resembled closely those of compound **3a**, suggesting that they share the same carbon skeleton. The methyl protons at δ_H 2.04 (s) and the carbon signals at δ_C 21.6 and 171.4 supported the presence of an acetyl group, placed at the C-11 hydroxy group from the HMBC correlation from H-11 to the carbonyl of the acetyl unit. The β-orientation of H-11 was inferred from the ROESY correlation of H-11/H_3-19. The α-orientation of H-12 was deduced by the coupling constant of H-11/H-12. The signal of H-17 appeared at δ_H 2.65 (dd, J = 5.9 and 11.9 Hz), different from that in compound **1**, suggesting that H-17 possesses an α-orientation instead of a β-orientation, which was supported by the ROESY correlation of H_3-18/H_3-21. Therefore, compound **3** was established as 3-*O*-pachybiosyl-11-*O*-acetyl-17β-tenacigenin B.

The molecular formulas of tenacissimosides F (**4**) and G (**5**) were determined by HRESIMS as $C_{40}H_{62}O_{13}$ and $C_{42}H_{60}O_{13}$, respectively. Analysis of the NMR data (Tables 1−4) of compounds **4** and **5** indicated that their structures are similar to that of compound **3**. The observed differences revealed that the acetyl group in compound **3** is replaced by a tigloyl unit in compound **4** and by a benzoyl unit in compound **5**, respectively. The location of the acylated groups was ascertained by HMBC correlations from δ_H 5.15 (H-11 of the aglycone) to δ_C 168.2 (C═O of the tigloyl unit) in compound **4** and from δ_H 5.35 (H-11 of the aglycone) to δ_C 166.7 (C═O of the benzoyl moiety) in compound **5**. Thus, the aglycones of compounds **4** and **5** were determined to be 11-*O*-tigloyl-17β-tenacigenin B and 11-*O*-benzoyl-17β-tenacigenin B, respectively. The sugar moiety of compounds **4** and **5** was identical to that of compound **3**, which was confirmed by co-TLC and the specific rotations of the products after mild acid hydrolysis of compounds **3**−**5**. Further, the same glycosidation shift of C-3 was observed for compounds **4** and **5** when comparing the ^{13}C NMR data with compound **3a**, indicating the same linkage of the sugar moiety. Therefore, the structures of compounds **4** and **5** were established as shown.

Tenacissimoside H (**6**) gave a molecular formula of $C_{41}H_{66}O_{17}$ based on the quasimolecular ion at m/z 853.4233 $[M + Na]^+$ in its HRESIMS. The ^{1}H NMR spectrum (Tables 1 and 3) of **6** displayed three anomeric signals at δ_H 4.34 (d, J = 7.6 Hz), 4.64 (dd, J = 9.7, 1.3 Hz), and 4.69 (d, J = 8.3 Hz),

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Table 2. ^{13}C NMR Data (100 MHz) of the Aglycones of Compounds 1−7 (δ in ppm)

carbon	1[a]	2[b]	3[a]	4[a]	5[a]	6[b]	7[b]
1	38.9, CH_2	39.3, CH_2	36.9, CH_2	37.3, CH_2	37.4, CH_2	39.9, CH_2	39.1, CH_2
2	44.7, CH_2	28.4, CH_2	28.9, CH_2	28.9, CH_2	28.8, CH_2	30.7, CH_2	30.6, CH_2
3	210.5, C	78.4, CH	76.2, CH	76.0, CH	76.1, CH	78.9, CH	78.4, CH
4	37.4, CH_2	36.4, CH_2	34.3, CH_2	34.3, CH_2	34.4, CH_2	36.3, CH_2	36.2, CH_2
5	46.2, CH	45.6, CH	44.2, CH	44.3, CH	44.4, CH	46.3, CH	45.8, CH
6	26.8, CH_2	30.7, CH_2	26.9, CH_2	27.0, CH_2	27.0, CH_2	28.7, CH_2	28.6, CH_2
7	31.2, CH_2	33.4, CH_2	32.3, CH_2	32.5, CH_2	32.5, CH_2	34.0, CH_2	33.9, CH_2
8	66.4, C	68.7, C	60.8, C	65.8, C	65.9, C	68.2, C	68.0, C
9	50.6, CH	53.3, CH	51.8, CH	52.1, CH	52.1, CH	55.4, CH	53.9, CH
10	38.9, C	40.7, C	39.2, C	39.3, C	39.4, C	40.8, C	40.8, C
11	68.4, CH	71.3, CH	70.2, CH	70.2, CH	71.0, CH	69.7, CH	72.0, CH
12	74.9, CH	76.5, CH	78.8, CH	79.3, CH	79.0, CH	82.0, CH	80.4, CH
13	45.7, C	47.6, C	47.1, C	47.4, C	47.3, C	49.4, C	49.5, C
14	71.5, C	73.6, C	70.5, C	70.7, C	70.6, C	73.4, C	73.3, C
15	26.5, CH_2	28.2, CH_2	27.9, CH_2	28.0, CH_2	28.0, CH_2	29.2, CH_2	29.3, CH_2
16	24.8, CH_2	26.5, CH_2	25.4, CH_2	25.5, CH_2	25.5, CH_2	26.6, CH_2	26.8, CH_2
17	60.1, CH	61.4, CH	63.5, CH	63.9, CH	63.5, CH	64.5, CH	64.1, CH
18	16.7, CH_3	17.4, CH_3	10.2, CH_3	10.3, CH_3	10.3, CH_3	11.5, CH_3	11.4, CH_3
19	12.0, CH_3	13.7, CH_3	12.8, CH_3	12.8, CH_3	12.9, CH_3	13.8, CH_3	13.8, CH_3
20	210.6, C	213.6, C	212.1, C	211.9, C	211.9, C	214.5, C	214.2, C
21	29.6, CH_3	30.8, CH_3	30.6, CH_3	30.8, CH_3	30.6, CH_3	32.6, CH_3	32.4, CH_3
C-11	mBu	Bz	Ac	Tig	Bz		Tig
1′	175.5, C	167.9, C	171.4, C	168.2, C	166.7, C		169.9, C
2′	41.2, CH	131.8, C	21.6, CH_3	128.8, C	130.4, C		130.6, CH
3′	26.1, CH_2	131.0, CH		138.0, CH	129.7, CH		139.4, CH
4′	11.7, CH_3	130.3, CH		12.2, CH_3	128.3, CH		12.7, CH_3
5′	15.3, CH_3	135.1, CH		14.6, CH_3	132.9, CH		14.9, CH_3
6′		130.3, CH			128.3, CH		
7′		131.0, CH			129.8, CH		
C-12	Ac	Ac					
1″	170.6, C	172.6, C					
2″	20.8, CH_3	20.8, CH_3					

[a]Measured in $CDCl_3$. [b]Measured in methanol-d_4.

together with the corresponding ^{13}C NMR signals at δ_C 106.7, 99.1, and 102.6 (Table 4), indicating the presence of three sugar moieties with β-linkages. By cellulase hydrolysis, compound **6** afforded a sugar unit and a steroidal glycoside. The sugar unit was established as β-D-glucose by comparison with the authentic sample on TLC, as well as the specific rotation. This steroidal glycoside was deduced to share the same skeleton with tenacissoside A (**3a**) from their same R_f value on TLC and similar NMR data.[18] The sugar sequence was determined to be β-D-glucopyranosyl-(1→4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1→4)-β-D- oleandropyranosyl by an HMBC experiment. The oligosaccharide chain could be located at C-3 via the HMBC correlation from the anomeric proton at δ_H 4.64 to C-3. Therefore, the structure of compound **6** was constructed as shown.

Tenacissimoside I (**7**) was obtained as a colorless, amorphous powder. The molecular formula was determined to be $C_{46}H_{72}O_{18}$ by HRESIMS. The ^{1}H and ^{13}C NMR data (Tables 1−4) closely resembled those of compound **6**, indicating that they share the same nucleus and sugar moiety. By comparison of its ^{1}H NMR data with those of compound **6**, a set of additional signals at δ_H 6.85 (q, J = 8.7 Hz), 1.82 (d, J = 8.7 Hz), and 1.79 (s), ascribed to a tigloyl moiety, suggested the presence of this ester group in compound **7**. The HMBC correlation from H-11 at δ_H 5.10 (t, J = 9.7 Hz) to the carbonyl carbon at δ_C 169.9 revealed the tigloyl group to be linked to the hydroxy group at C-11. Therefore, the structure of compound **7** was established as 3-*O*-β-D-glucopyranosyl-(1→4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1→4)-β-D-oleandropyranosyl 11α-*O*-tigloyl-17β-tenacigenin B.

The molecular formula of tenacissimoside J (**8**) was deduced as $C_{37}H_{58}O_{13}$ from the HRESIMS (733.3764 $[M + Na]^+$). Two anomeric protons at δ_H 4.56 (d, J = 8.8 Hz) and 4.78 (d, J = 8.2 Hz) in the ^{1}H NMR spectrum and the corresponding carbons at δ_C 97.1 and 99.1 suggested the presence of two sugar units with a β-linkage. The oligosaccharide chain of compound **8** was identified as pachybiose from the NMR data and confirmed by comparing the hydrolysis products of compound **8** with those of compound **3** under mild acidic conditions. The ^{1}H and ^{13}C NMR data (Table 5) of the aglycone of compound **8** compared closely with those of tenacigenin A (**8a**),[19] indicating the same aglycone skeleton in these two compounds. The methyl protons at δ_H 2.10 (s) and the carbon resonances at δ_C 21.4 and 170.2 supported the occurrence of an acetyl group. The HMBC correlation from H-12 [δ_H 5.26 (d, J = 4.0 Hz)] to the carbonyl carbon of the acetyl unit suggested the acetyl group to be located at C-12. The oligosaccharide chain was assigned at C-3 on the basis of the long-range correlation from the anomeric proton at δ_H 4.56 to C-3 in the HMBC spectrum. Consequently, the structure of compound **8** was established as shown.

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Table 3. ^{1}H NMR Data of Sugar Moieties of Compounds 2−7 (400 MHz, δ in ppm, J in Hz)

position	2[a]	3[b]	4[b]	5[b]	6[a]	7[a]
I	D-ole	D-ole	D-ole	D-ole	D-ole	D-ole
1	4.60, d (8.5)	4.56, d (8.8)	4.56, d (9.7)	4.54 d (8.8)	4.64 dd (1.3, 9.7)	4.62, d (9.3)
2	1.32, m	1.48, m	1.30, m	1.32, m	1.33, m	1.34, m
	2.19, m	2.29, m	2.29, m	2.29, m	2.23, m	2.22, m
3	3.34, m	3.39, m	3.38, m	3.39, m	3.37, m	3.36, m
4	3.12, m	3.33, m	3.34, m	3.34, m	3.16, m	3.15, m
5	3.32, m	3.34, m	3.31, m	3.32, m	3.33, m	3.34, m
6	1.28, d (5.8)	1.36, d (5.8)	1.34, d (5.4)	1.35, d (5.2)	1.34, d (6.2)	1.34, d (6.2)
OMe-3	3.36, s	3.36, s	3.36, s	3.36, s	3.38, s	3.39, s
II	D-allo	D-allo	D-allo	D-allo	D-allo	D-allo
1	4.66, d (7.8)	4.78, d (8.2)	4.78, d (8.3)	4.77, d (8.3)	4.69, d (8.3)	4.70, d (7.8)
2	3.30, m	3.46, m	3.46, m	3.45, m	3.30, m	3.28, m
3	3.90, m	3.79, t (2.8)	3.78, t (3.1)	3.78, t (2.8)	3.92, m	3.93, m
4	3.30, m	3.17, dd (2.8, 9.5)	3.17, dd (9.3, 3.1)	3.16, dd (2.8, 9.5)	3.31, m	3.32, m
5	3.82, m	3.55, m	3.52, m	3.53, m	3.80, m	3.80, m
6	1.27, d (6.0)	1.25, d (6.2)	1.24, d (6.2)	1.24, d (6.2)	1.28, d (6.0)	1.28, d (6.0)
OMe-3	3.58, s	3.64, s	3.65, s	3.64, s	3.57, s	3.57, s
III	D-glc				D-glc	D-glc
1	4.37, d (7.7)				4.34, d (7.6)	4.32, d (7.8)
2	3.24, m				3.18, m	3.16, m
3	3.50, m				3.34, m	3.32, m
4	3.51, m				3.24, m	3.26, m
5	3.42, m				3.21, m	3.22, m
6	3.83, m				3.63, m	3.63, m
	3.91, m				3.89, m	3.88, m
IV	D-glc					
1	4.38, d (7.4)					
2	3.21, m					
3	3.33, m					
4	3.32, m					
5	3.28, m					
6	3.64, m					
	3.84, m					

[a]In methanol-d_4. [b]In $CDCl_3$.

The isolated compounds were screened initially for potential reversal of P-gp-mediated MDR using parental SW620 and P-gp overexpressing resistant SW620 Ad300 cells. The SW620 Ad300 cells have been fully characterized and proven to be suitable for evaluating P-pg-mediated MDR reversal activity.[21] Among the major transporters responsible for MDR, only P-gp is overexpressed in SW620 Ad300. As demonstrated in Table 6, resistant SW620 Ad300 cells are remarkably resistant to doxorubicin (166-fold), a commonly used P-gp substrate anticancer drug in the clinic. Among all compounds tested, only compound **1** (tested at 2 or 10 μM) was found to significantly potentiate the cytotoxicity of doxorubicin in SW620 Ad300 cells (Table 6). Its effect was found to be specific because compound **1** did not affect the cytotoxic activity of doxorubicin in the parental SW620 cells. Moreover, compound **1** also did not affect the cytotoxic effect of cisplatin, a non-P-gp substrate anticancer drug, in both parental SW620 and the P-gp overexpressing resistant SW620 Ad300 cells (Table 6).

Since P-gp mediates MDR by actively effluxing substrate anticancer drugs out of the cells, the inhibition of P-gp efflux activity by compound **1** was evaluated subsequently. As indicated in Figure 2, compound **1** was found to inhibit the efflux of Rh123 (a fluorescent P-gp probe substrate) in the P-gp overexpressing resistant SW620 Ad300 cells in a concentration-dependent manner. Importantly, compound **1** was shown to exhibit a P-gp inhibitory effect similar to verapamil, a known P-gp inhibitor, at the same concentration (50 μM).

EGFR tyrosine kinase inhibitors are promising molecular-targeted agents used for the treatment of lung cancer. However, their usefulness is affected greatly by acquired resistance due to various mechanisms, including secondary mutations in EGFR, mutation of the oncogene, K-ras, and overexpression of P-gp. A panel of three NSCLC cell lines, H292 (sensitive), H460 (resistant; K-ras-mutated), and H460 Vbl (resistant; K-ras-mutated and P-gp overexpression), was used to investigate the potentiation effect of compound **1** on the activity of erlotinib and gefitinib.

In the sensitive NSCLC cell line H292, the concurrent treatment of compound **1** (10 or 20 μM) did not affect the activity of either erlotinib or gefitinib (Table 7). On the other hand, in the resistant NSCLC cells, H460 (with a K-ras mutation), compound **1** significantly potentiated the growth inhibitory activity of both erlotinib and gefitinib in a concentration-dependent manner (Table 7). Since compound **1** was previously found to inhibit P-gp, it was reasonable to speculate that P-gp inhibition was also involved in this potentiation. Verapamil, a proven P-gp inhibitor, was also tested against the H460 cells, but no potentiation of activity of the EGFR TKIs was observed (Table 7).

Table 4. ^{13}C NMR Data (100 MHz) of Sugar Moieties of Compounds 2−7 (δ in ppm)

carbon	2[a]	3[b]	4[b]	5[b]	6[a]	7[a]
I	D-ole	D-ole	D-ole	D-ole	D-ole	D-ole
1	99.1, CH	96.8, CH	96.6, CH	96.7, CH	99.1, CH	99.0, CH
2	38.4, CH_2	36.0, CH_2	36.0, CH_2	36.0, CH_2	38.4, CH_2	38.4, CH_2
3	81.0, CH	78.7, CH	78.8, CH	78.8, CH	81.0, CH	81.0, CH
4	84.4, CH	79.1, CH	79.0, CH	79.2, CH	84.5, CH	84.4, CH
5	73.0, CH	71.2, CH	71.3, CH	71.3, CH	73.0, CH	73.0, CH
6	19.3, CH_3	18.5, CH_3	18.6, CH_3	18.6, CH_3	19.4, CH_3	19.3, CH_3
OMe-3	57.9, CH_3	55.6, CH_3	55.6, CH_3	55.6, CH_3	58.0, CH_3	57.9, CH_3
II	D-allo	D-allo	D-allo	D-allo	D-allo	D-allo
1	102.6, CH	99.2, CH	99.2, CH	99.1, CH	102.6, CH	102.6, CH
2	73.5, CH	71.7, CH	71.7, CH	71.7, CH	73.4, CH	73.4, CH
3	83.7, CH	81.0, CH	78.8, CH	81.0, CH	83.7, CH	83.7, CH
4	84.5, CH	72.8, CH	72.8, CH	72.8, CH	84.4, CH	84.4, CH
5	70.6, CH	71.2, CH	71.3, CH	71.3, CH	70.6, CH	70.6, CH
6	18.6, CH_3	17.8, CH_3	17.9, CH_3	17.8, CH_3	18.6, CH_3	18.6, CH_3
OMe-3	62.5, CH_3	61.9, CH_3	62.0, CH_3	61.9, CH_3	62.5, CH_3	62.4, CH_3
III	D-glc				D-glc	D-glc
1	105.1, CH				106.7, CH	106.7, CH
2	75.7, CH				76.0, CH	76.0, CH
3	76.8, CH				78.4, CH	78.4, CH
4	81.6, CH				72.3, CH	72.4, CH
5	77.0, CH				78.5, CH	78.5, CH
6	62.8, CH_2				63.5, CH_2	63.5, CH_2
IV	D-glc					
1	106.6, CH					
2	75.4, CH					
3	78.4, CH					
4	71.9, CH					
5	78.6, CH					
6	62.9, CH_2					

[a]In methanol-d_4. [b]In $CDCl_3$.

Table 5. ^1H (400 MHz) and ^{13}C NMR (100 MHz) Data of Compound 8 in $CDCl_3$

position	δ_H (J in Hz)	δ_C, type	position	δ_H (J in Hz)	δ_C, type
1	1.19, m	38.5, CH_2	18	1.03 s	16.2, CH_3
	1.66, m		19	1.03 s	15.7, CH_3
2	1.48, m	28.5, CH_2	20		99.7, C
	1.87, m		21	1.19 s	24.2, CH_3
3	3.65, m	77.2, CH	1′		170.2, C
4	1.27, m	33.5, CH_2	2′	2.10 s	21.4, CH_3
	1.65, m		D-ole		
5	1.30, m	45.8, CH	1	4.56 d (8.8)	97.1, CH
6	1.28, m	27.7, CH_2	2	1.48 m	36.1, CH_2
7	1.31, m	34.1, CH_2		2.29 m	
	2.28, m		3	3.39 m	78.8, CH
8		77.8, C	4	3.33 m	79.2, CH
9	2.38 m	57.4, CH	5	3.34 m	71.3, CH
10		35.3, C	6	1.36 d (5.8)	18.6, CH_3
11	4.29, dd (4.0, 1.8)	68.3, CH	OMe-3	3.36 s	55.6, CH_3
			D-allo		
12	5.26, d (4.0)	71.8, CH	1	4.78 d (8.2)	99.1, CH
13		44.0, C	2	3.46 m	71.8, CH
14		81.0, C	3	3.79 t (2.8)	81.0, CH
15	1.58, m	34.3, CH_2	4	3.17 dd (2.8, 9.5)	72.8, CH
	2.38, m		5	3.55 m	71.4, CH
16	1.46, m	22.9, CH_2	6	1.25 d (6.2)	17.8, CH_3
	1.86, m		OMe-3	3.64 s	61.9, CH_3
17	1.97, d (6.0)	55.1, CH			

Table 6. Effects of Compounds 1−8 on Reversal of P-gp-Mediated Resistance to Doxorubicin

	IC_{50} ± SD[a] (μM)		
treatment	SW620	SW620 Ad300	fold resistance[b]
doxorubicin alone	0.17 ± 0.05	28.2 ± 0.52	166
+ PSC833 (400 nM)	0.10 ± 0.06	0.22 ± 0.03**	2
+ **1** (2 μM)	0.11 ± 0.06	2.1 ± 0.21**	19
+ **1** (10 μM)	0.22 ± 0.09	0.85 ± 0.11**	4
+ **2** (10 μM)	0.16 ± 0.06	26.8 ± 4.16	168
+ **3** (10 μM)	0.18 ± 0.06	23.4 ± 3.41	130
+ **4** (10 μM)	0.12 ± 0.05	25.6 ± 3.25	213
+ **5** (10 μM)	0.17 ± 0.04	23.4 ± 4.19	138
+ **6** (10 μM)	0.15 ± 0.08	24.8 ± 4.38	165
+ **7** (10 μM)	0.18 ± 0.10	23.9 ± 3.98	133
+ **8** (10 μM)	0.14 ± 0.03	27.7 ± 5.16	198
cisplatin alone	10.2 ± 1.32	11.8 ± 1.53	1.2
+ **1** (2 μM)	11.5 ± 0.98	12.0 ± 0.59	1.0
+ **1** (10 μM)	9.4 ± 0.76	10.2 ± 0.72	1.1

[a]Values represent the mean ± SD of three independent experiments performed in quadruplicate. **$p < 0.01$, compared with doxorubicin alone in SW620 Ad300 cells. [b]Fold resistance was calculated by the IC_{50} values of doxorubicin/cisplatin in resistant SW620 Ad300 cells divided by the IC_{50} values of doxorubicin/cisplatin in SW620 cells in the presence or absence of PSC833 or individual compounds tested.

Furthermore, by prolonged exposure to vinblastine, H460/Vbl cells were derived from H460, and the cells were known to overexpress P-gp to mediate MDR. Compared with H292 (sensitive) and resistant H460 (K-ras mutation), H460/Vbl was found to be much less responsive to both erlotinib and gefitinib (>20-fold) (Table 7), indicating that such additional resistance was due to its P-gp overexpression. Consistent with the P-gp inhibitory effect, compound **1** was found to reverse the resistance of both EGFR TKIs in the H460/Vbl cells, and its activity at 20 μM was similar or even higher than that of the known P-gp inhibitor verapamil at 50 μM for the two TKIs tested (Table 7). Collectively, the results in H460 (K-ras mutated) and H460/Vbl (K-ras mutated and P-gp overexpressing) suggest that compound **1** has a dual action and is

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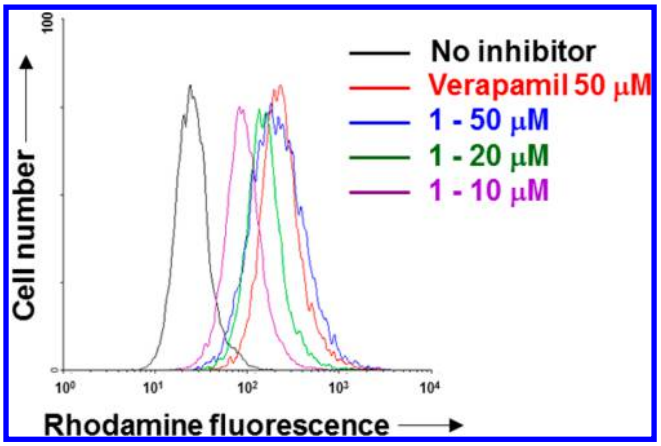

Figure 2. Reduced efflux of the P-gp substrate rhodamine 123 by compound **1** (10, 20, and 50 μM) in P-gp overexpressing resistant SW620 Ad300 cells.

able to circumvent EGFR TKI resistance mediated by both K-ras mutation and P-gp overexpression.

In order to confirm the effects on EGFR TKI, an apoptosis assay was performed in the three NSCLC cell lines after treatment with gefitinib. Consistent with the responsiveness of the NSCLC cells to the EGFR TKIs, the induction of apoptosis by gefitinib (1 μM) was found as follows: H292 > H460 > H460/Vbl (Figure 3A). While compound **1** alone did not exhibit any appreciable apoptotic effect, the concurrent treatment of gefitinib with compound **1** (10 μM) was found to increase the percentage of apoptotic cells in H460 and H460/Vbl cells to a considerable extent and to almost completely circumvent the resistance of gefitinib in both resistant cell lines (Figure 3B).

In summary, the new polyoxypregnane compound **1** from *M. tenacissima* exhibited reversal activity on P-gp-mediated MDR by inhibiting P-gp. The results also revealed that compound **1** produces sensitization effects on two P-gp substrate anticancer TKIs, gefitinib and erlotinib, in two resistant NSCLC cell lines via a dual action on reversal of both P-gp overexpression and K-ras-mutation mediated MDR mechanisms. The results obtained have demonstrated that compound **1** generates a promising MDR-reversing effect via inhibition of different resistant mechanisms and suggest that further investigation of its concurrent use with anticancer drugs for MDR cancer therapy may be worthwhile.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a PerkinElmer 341 MC polarimeter. UV spectra were recorded with a Beckman DU-7 spectrometer. IR spectra were recorded on a Nicolet Magna FT-IR 750 spectrophotometer using KBr disks. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AM-300 or AM-400 and INVOR-600 NMR spectrometers. The chemical shift (δ) values are given in ppm with TMS as internal standard, and coupling constants (J) in Hz. EIMS and HREIMS were recorded on a Finnigan MAT-95 mass spectrometer, while ESIMS and HRESIMS were recorded on a Micromass Q-Tof Global mass spectrometer. All solvents used were of analytical grade (Shanghai Chemical Plant). Column chromatographic separations were carried out using silica gel (200−300 mesh; Qingdao Marine Chemical Ltd.), Sephadex LH-20 (25−100 μm; Pharmacia Fine Chemicals), and MCI gel CHP 20P (high porous polymer, 75−150 μm; Mitsubishi Chemical Industrials) as packing materials. TLC was carried out on precoated silica gel GF_{254} plates (Yantai Chemical Industrials). Analytical HPLC was performed on a Waters 2690 instrument with a 996 PAD (photodiode array detector) and an Alltech ELSD 2000 detector. Preparative HPLC was preformed on a Varian Pro-star solvent delivery module with a Varian Pro-star UV−vis detector. Chromatographic separations were carried out on C_{18} columns (220 × 25 mm, 10 μm, Waters), using a gradient solvent system comprising H_2O and MeCN, with a flow rate of 15.0 mL/min, respectively. β-D-Glucose (anhydrous, 98%), α-L-glucose (99%), and cellulase from *Aspergillus niger* (powder, ≥ 0.3 units/mg solid) were purchased from Sigma-Aldrich.

Plant Material. The stems of *M. tenacissima* were collected in March 2005 from Simao County, Yunnan Province, People's Republic of China. A voucher specimen (no. 200503) was deposited at the Shanghai Research Center for Modernization of Traditional Chinese Medicine, Shanghai Institute of Materia Medica, Shanghai.

Extraction and Isolation. The dried stems of *M. tenacissima* (20.3 kg) were extracted three times with 95% EtOH at room temperature for 3 days each, which afforded a dark residue (3.4 kg) after evaporation. The residue was suspended in water and then partitioned with EtOAc and *n*-butanol, successively, to give EtOAc (460 g) and butanol (500 g) fractions, respectively. A portion of EtOAc fraction (270 g) was subjected to CC (silica gel, petroleum ether−EtOAc, 3:1 to 1:2, 100% EtOAc, and then EtOAc−MeOH, 20:1 to 4:1) to give Frs. A−H. Compound **1** (23 mg) was recrystallized from Fr. C in a petroleum ether−EtOAc mixture. Fr. D was subjected to CC over MCI gel (gradient MeOH−H_2O, 65% to 100%, then acetone) and then silica gel ($CHCl_3$−MeOH, 100:1) to afford compound **8** (38 mg). Fr. E was separated over a MCI gel column (MeOH−H_2O, 60% to 100%, then acetone) and then by preparative HPLC (MeCN−H_2O, 20% to 50%, 15 mL/min, 0 to 192 min), yielding compounds **4** (78 mg) and **5** (25 mg). Fr. F was subjected to CC (silica gel, $CHCl_3$−MeOH, 10:1), then purified by preparative HPLC (MeCN−H_2O, 15%

Table 7. Sensitization of Cytotoxicity of Erlotinib and Gefitinib by Compound 1 in Human NSCLC Cell Lines[a]

cell line				IC_{50} (μM) of erlotinib with/without compound **1**			
	EGFR status	K-ras status	P-gp expression	erlotinib alone	erlotinib + **1** (10 μM)	erlotinib + **1** (20 μM)	erlotinib + verapamil (50 μM)
H292	WT	WT	low	1.0 ± 0.19	0.84 ± 0.14	0.60 ± 0.20	1.2 ± 0.15
H460	WT	Mut	low	9.5 ± 1.96**	5.4 ± 0.65$^{\#}$	3.1 ± 0.42$^{\#\#}$	10.3 ± 1.78
H460/Vbl	WT	Mut	high	21.5 ± 2.57**	11.1 ± 2.16$^{\#\#}$	6.5 ± 0.80$^{\#\#}$	13.3 ± 1.96$^{\#}$
cell line				IC_{50} (μM) of gefitinib with/without compound **1**			
	EGFR status	K-ras status	P-gp expression	gefitinib alone	gefitinib + **1** (10 μM)	gefitinib + **1** (20 μM)	gefitinib + verapamil (50 μM)
H292	WT	WT	low	0.19 ± 0.19	0.14 ± 0.14	0.16 ± 0.20	0.18 ± 0.15
H460	WT	Mut	low	21.0 ± 2.67**	11.3 ± 2.19$^{\#}$	7.2 ± 1.63$^{\#\#}$	19.6 ± 1.87
H460/Vbl	WT	Mut	high	ND[b]	32.2 ± 3.56	21.8 ± 2.54	25.7 ± 2.76

[a]Data represent mean ± SD from four independent and reproducible experiments. WT: wild type; Mut = mutant (K-ras Q61H). **$p < 0.01$, compared with TKI alone in H292 cells, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with TKI alone in the same cell line. [b]ND: not accurately determined due to the limitation of gefitinib solubility; its highest concentration tested did not reach 50% eradication of cells.

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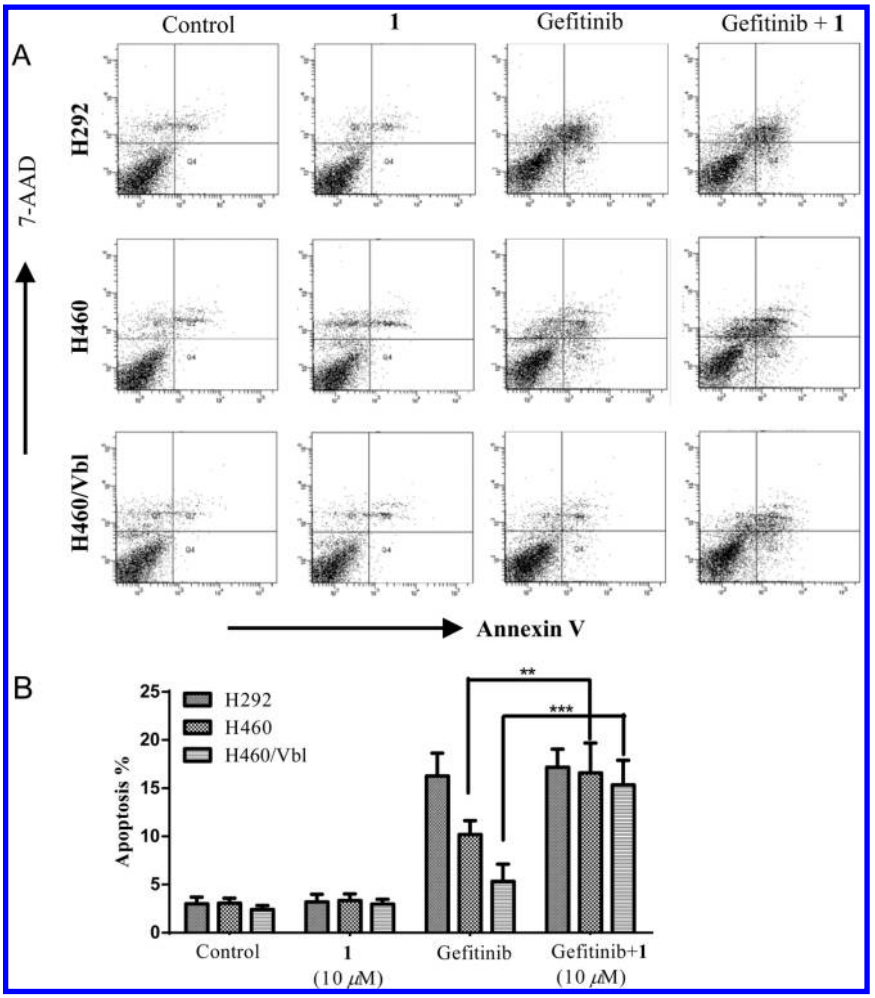

Figure 3. Restoration of gefitinib-mediated apoptosis by compound **1** in gefitinib-resistant cell lines. (A) Representative set of data obtained from the annexin V-7-AAD double staining apoptosis assay. Abscissa: cells positive for annexin-V; ordinate: cells positive for 7-AAD uptake. Lower left quadrant shows viable cells; lower right, annexin-V positive cells (early apoptotic); upper right, cells positive for both annexin-V and 7-AAD (late apoptotic). (B) Percentage of apoptosis determined from three independent experiments presented as mean ± SD (a confidence level of $p < 0.05$ was considered to be statistically significant).

to 40%, 15 mL/min, 0 to 192 min), to give compounds **3** (38 mg) and **3a** (21 mg). Fr. G (2.0 g) was separated by CC (silica gel, $CHCl_3$–MeOH, 10:1) and then preparative HPLC (MeCN–H_2O, 15% to 54%, 15 mL/min, 0 to 192 min) to yield compounds **6** (14 mg) and **7** (30 mg). Fr. H (3.2 g) was separated by CC over silica gel (EtOAc–MeOH, 5:1), then MCI gel (gradient MeOH–H_2O, 40% to 100%, then acetone), and finally preparative HPLC (MeCN–H_2O, 30% to 55%, 15 mL/min, 0 to 192 min), to give compound **2** (38 mg).

*Tenacigenin D (**1**):* colorless, amorphous powder; $[\alpha]^{20}_D$ +32 (*c* 0.32, $CHCl_3$); IR (KBr) ν_{max} 2934, 1726, 1708, 1452, 1277, 1161, 1072 cm^{-1}; ^1H NMR, see Table 1, ^{13}C NMR, see Table 2; EIMS *m/z* 488 $[M]^+$ (10), 386 (24), 344 (78), 327 (16), 283 (100), 230 (57), 156 (46); HREIMS *m/z* 488.2763 $[M]^+$ (calcd for $C_{28}H_{40}O_7$, 488.2774).

*Tenacissimoside D (**2**):* colorless, amorphous powder; $[\alpha]^{20}_D$ −5 (*c* 0.25, MeOH); IR (KBr) ν_{max} 3419, 2953, 1724, 1662, 1452, 1375, 1277, 1161, 1072 cm^{-1}; ^1H NMR, see Tables 1 and 3, ^{13}C NMR, see Tables 2 and 4; ESIMS *m/z* 1161.6 $[M + Na]^+$, 1137.7 $[M - H]^-$; HRESIMS *m/z* 1161.5045 $[M + Na]^+$ (calcd for $C_{56}H_{82}O_{24}Na$, 1161.5094).

*Tenacissimoside E (**3**):* colorless, amorphous powder; $[\alpha]^{20}_D$ −4 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3425, 2929, 1705, 1628, 1735, 1163, 1082 cm^{-1}; ^1H NMR, see Tables 1 and 3; ^{13}C NMR, see Tables 2 and 4; ESIMS *m/z* 733.5 $[M + Na]^+$, 709.6 $[M - H]^-$; HRESIMS *m/z* 733.3817 $[M + Na]^+$ (calcd for $C_{37}H_{58}O_{13}Na$, 733.3775).

*Tenacissimoside F (**4**):* colorless, amorphous powder; $[\alpha]^{20}_D$ −16 (*c* 0.21, $CHCl_3$); IR (KBr) ν_{max} 3448, 2933, 1700, 1648, 1450, 1269, 1163, 1082 cm^{-1}; ^1H NMR, see Tables 1 and 3; ^{13}C NMR, see Tables 2 and 4; ESIMS *m/z* 773.3 $[M + Na]^+$, 749.4 $[M - H]^-$; HRESIMS *m/z* 773.4056 $[M + Na]^+$ (calcd for $C_{40}H_{62}O_{13}Na$, 773.4088).

*Tenacissimoside G (**5**):* colorless, amorphous powder; $[\alpha]^{20}_D$ +14 (*c* 0.10, $CHCl_3$); IR (KBr) ν_{max} 3434, 2933, 1714, 1627, 1452, 1276, 1068 cm^{-1}; ^1H NMR, see Tables 1 and 3; ^{13}C NMR, see Tables 2 and 4; ESIMS *m/z* 795.3 $[M + Na]^+$, 771.4 $[M - H]^-$; HRESIMS *m/z* 795.3943 $[M + Na]^+$ (calcd for $C_{42}H_{60}O_{13}Na$, 795.3932).

*Tenacissimoside H (**6**):* colorless, amorphous powder; $[\alpha]^{20}_D$ +2 (*c* 0.40, MeOH); IR (KBr) ν_{max} 3423, 2931, 1691, 1637, 1452, 1383, 1163, 1078, 617 cm^{-1}; ^1H NMR, see Tables 1 and 3; ^{13}C NMR, see Tables 2 and 4; ESIMS *m/z* 853.4 $[M + Na]^+$, 829.4 $[M - H]^-$; HRESIMS *m/z* 853.4233 $[M + Na]^+$ (calcd for $C_{41}H_{66}O_{17}Na$, 853.4198).

*Tenacissimoside I (**7**):* colorless, amorphous powder; $[\alpha]^{20}_D$ 0 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.12) nm; IR (KBr) ν_{max} 3423, 2933, 1651, 1452, 1383, 1267, 1161, 1078, 989 cm^{-1}; ^1H NMR, see Tables 1 and 3; ^{13}C NMR, see Tables 2 and 4; ESIMS *m/z* 935.4 $[M + Na]^+$, 911.6 $[M - H]^-$; HRESIMS *m/z* 935.4586 $[M + Na]^+$ (calcd for $C_{46}H_{72}O_{18}Na$, 935.4661).

*Tenacissimoside J (**8**):* colorless, amorphous powder; $[\alpha]^{20}_D$ −2 (*c* 0.30, $CHCl_3$); IR (KBr) ν_{max} 3457, 2937, 1739, 1637, 1454, 1384, 1236, 1062 cm^{-1}; ^1H and ^{13}C NMR, see Table 5; ESIMS *m/z* 773.4

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$[M + Na]^+$, 709.3 $[M - H]^-$; HRESIMS m/z 733.3764 $[M + H]^+$ (calcd for $C_{37}H_{58}O_{13}Na$, 733.3775).

Mild Acid Hydrolysis of Compounds 3, 3a, 4, 5, and 8. MeOH (15 mL) and 0.1 M H_2SO_4 (5 mL) were added to compound **3** (15 mg), and the mixture was kept at 60 °C for 30 min. After evaporation of MeOH under reduced pressure, H_2O (15 mL) was added and the mixture was kept around 60 °C for another 30 min, then cooled to room temperature. The solution was then extracted with Et_2O (10 mL × 3), and the Et_2O layer was washed with water (5 mL × 4), dried (Na_2SO_4), and evaporated to dryness. The residue was dissolved in EtOAc, and compound **3** was crystallized from a petroleum ether−EtOAc mixture. The aqueous acidic layer of the hydrolysate solution was neutralized with 5% $Ba(OH)_2$ aqueous solution. The sediment was filtered and the solution was evaporated. The residue was identified as pachybiose by TLC comparison with an authentic sample [chromatography conditions: upper layer of *n*-BuOH−AcOH−H_2O (4:1:5, v/v), R_f 0.78; $CHCl_3$−MeOH (9:1, v/v), R_f 0.35], and its specific optical rotation {$[\alpha]^{20}_D$ −6.8 (*c* 0.23, H_2O)} was comparable with the published value in the literature {$[\alpha]^{20}_D$ −10 (*c* 0.47, H_2O)}.[20] The same mild acid hydrolysis procedure was applied to compounds **3a** (4 mg), **4** (5 mg), **5** (8 mg), and **8** (5 mg), and the aqueous acidic layer of their hydrolysates all contained pachybiose, as determined by TLC comparison with an authentic sample.

Cellulase Treatment of Compounds 2 and 6. Compounds **2** (5 mg) and **6** (7 mg) were treated with cellulase (20 mg) (Sigma-Aldrich, C1184) in a 0.1 M AcOH−NaOAc (pH 5.0) buffer solution (1 mL) at 37 °C for 7 days. Each reaction mixture was then extracted with $CHCl_3$. Tenacissoside I (**2a**, 2 mg) and tenacigenoside A (**3a**, 3 mg) were both obtained from the organic layer by preparative TLC. The β-D-glucose was identified by TLC comparison with the authentic sample and the specific optical rotation: $[\alpha]^{20}_D$ +52 (*c* 0.10, H_2O) [β-D-glucose: $[\alpha]^{20}_D$ +52.7 (*c* 0.16, H_2O); α-L-glucose: $[\alpha]^{20}_D$ −56.6 (*c* 0.11, H_2O)].

Cell Culture. All cell lines used (except H292) were generous gifts provided by Dr. Susan Bates (National Cancer Institute, NIH, Bethesda, MD, USA). A human colon parental SW620 cell line and a drug selected resistant SW620 Ad300 (P-gp overexpressing) subline were used to screen for the reversal effect of P-gp-mediated multidrug resistance. The SW620 Ad300 subline was developed from parental SW620 cells by stepwise selection in increasing concentrations of doxorubicin.[21] These cells have been fully characterized and proven to be appropriate for studying MDR-mediated resistance and its reversal. Among the most common drug transporters, only P-gp is overexpressed in SW620 Ad300 cells. The human NSCLC cell lines H292 (ATCC, Manassas, VA, USA), H460, and H460/Vbl were used to test for potentiation of EGFR TKI activity. H460/Vbl is a resistant subline developed from H460 by prolonged selection in vinblastine.[22] H460/Vbl is highly resistant to vinblastine (>2000-fold), and it was found to overexpress only P-gp among all major MDR transporters. All cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/mL streptomycin sulfate, and 100 units/mL penicillin G sulfate and were incubated at 37 °C in 5% CO_2.

Growth Inhibition Assay. The growth inhibitory effects of various standard anticancer drugs (including cisplatin, doxorubicin, erlotinib, and gefitinib), with or without the concomitant treatment of the individual test compounds (**1**−**8**), were evaluated using a sulforhodamine B assay.[23] Briefly, cells were seeded into 96-well plates in 100 μL at a seeding density of 3000−5000 cells/well and allowed to incubate overnight. The cells were then treated with the anticancer drugs at a range of concentrations in the presence or absence of a fixed concentration of individual compound tested (2, 10, or 20 μM) and allowed to incubate at 37 °C in 5% CO_2 for 72 h. Each drug concentration was evaluated in quadruplicate, and controls were tested in replicates of eight. Each experiment was carried out independently at least three times. To determine whether differences between IC_{50} values were significant, Student's *t*-test was performed, with $p < 0.05$ being considered statistically significant.

Flow Cytometry-Based Substrate Efflux Assay. A flow cytometry-based assay was employed to study the inhibition of P-gp efflux activity by compound **1**, as described previously.[24] Briefly, cells were allowed to incubate with the fluorescent P-gp substrate probe rhodamine 123 in the presence or absence of compound **1**. The retention of a greater fluorescence signal inside the cells was used as the readout to measure the inhibition of P-gp efflux activity. The known P-gp inhibitor verapamil (50 μM) was used as the positive control in a parallel study.

Apoptosis Assay. H292, H460, and H460/Vbl cells were grown on a 60 mm tissue culture dish at a density of about 2.0×10^5 cells/well. They were treated for 48 h with 1 μM gefitinib in the presence or absence of compound **1** (10 μM). At the end of the treatment, both floating and attached cells were collected and washed twice with ice-cold phosphate buffer solution. The extent of apoptosis was determined by using the APC annexin V apoptosis kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. Cells positive for both annexin V and 7-AAD were considered apoptotic. One-way analysis of variance (one-way ANOVA) with Bonferroni's multiple comparison test was used to compare the differences among various treatment groups. A confidence level of $p < 0.05$ was considered significant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Key HMBC and ROESY correlations of **1** (Figure 1A), key TOCSY, HMBC, and ROSEY correlations of compound **2** (Figure 2A), and available copies of ^1H and ^{13}C NMR, $^1H-^1H$ COSY, HSQC, HMBC, and ROESY spectra for compounds **1**−**8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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