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Bioactive C_{21} Steroidal Glycosides from the Roots of *Cynanchum otophyllum* That Suppress the Seizure-like Locomotor Activity of Zebrafish Caused by Pentylenetetrazole

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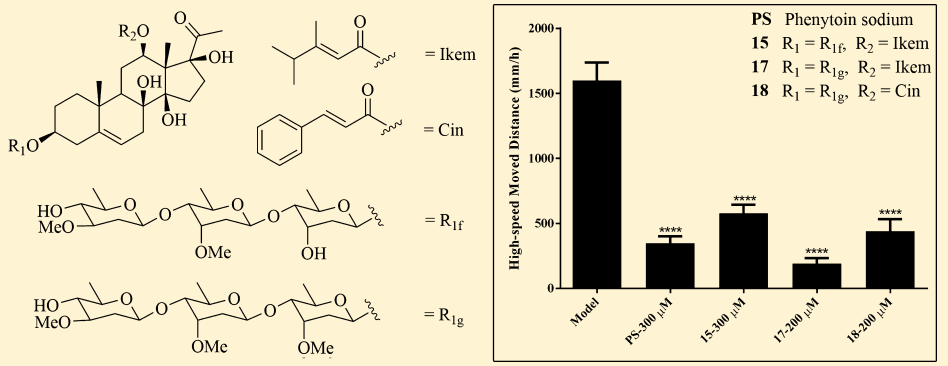
Supporting Information



ABSTRACT: Six new C_{21} steroidal glycosides, cynotophyllosides A–F (**1**–**6**), together with 16 known compounds, were isolated from the roots of *Cynanchum otophyllum*. The structures of the new compounds were elucidated by spectroscopic analysis and chemical methods. The three major components, otophylloside F (**15**), otophylloside B (**17**), and rostratamine 3-*O*-*β*-D-oleandropyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-cymaropyranoside (**18**), suppressed the seizure-like locomotor activity caused by pentylenetetrazole in zebrafish. Preliminary structure–activity relation studies revealed that a pregnene skeleton with a C-12 ester group (ikemaoyl > cinnamoyl > hydroxy > *p*-hydroxybenzoyl) and a C-3 sugar chain consisting of three 2,6-dideoxysaccharide units is essential for this suppressive activity.

Epilepsy is a brain disorder that is characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition.[1] The WHO estimates that approximately 50 million people worldwide have epilepsy. The prevalence and incidence of epilepsy differ by demographic factors including age, gender, race, and socioeconomic status.[2,3] A variety of epilepsy syndrome types affect the nervous system in distinct ways; however, seizures are prominently phenotypic. Symptoms of epilepsy, whether induced or spontaneous, are the results of excessive electrical discharges in a group of brain cells. Although some people consider epilepsy to be the result of brain damage, stroke, certain genetic syndromes, or brain tumors, in most cases it has no identifiable causes,[4] and the process by which a normal brain becomes epileptic is a complex mechanism that is still not completely understood. Thus, suppressing the seizure-like locomotor activity is an efficient way to control epilepsy. After almost 100 years in clinical practice, antiepileptic drugs (AEDs) remain the primary means of epilepsy treatment, but their adverse effects on mood and behavior are inevitable for most of these drugs.[5] In addition to synthesized AEDs, antiepileptic herbs and their extracts have also occupied a share in the market of the People's Republic of China. Different places of origin and harvest dates could affect the amount of active ingredients in those herbs. Therefore, the identification of antiepileptic natural products for the production of safe and quality-controllable herbal medicines and for the discovery of lead structures of AEDs has been attracting considerable interest.

Zebrafish (*Danio rerio*), a small tropical freshwater fish with more than 71% orthologous human protein-coding genes, has been suggested as an alternative animal model for studying mechanisms of epilepsy and for screening AEDs.[6−11] After being induced by pentylenetetrazole (PTZ), a central nervous system convulsant, zebrafish larvae showed small-amplitude discharges and up-regulated *c-fos* mRNA expression, which are

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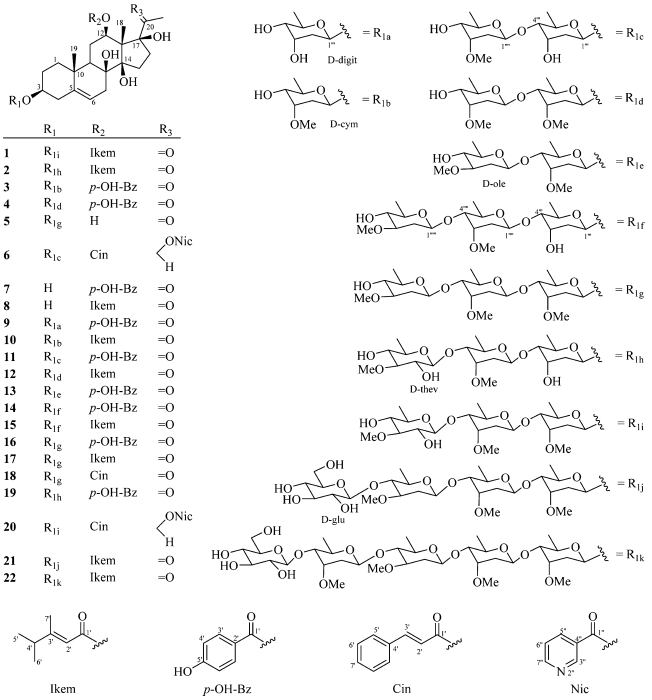
actions that are characteristic of epileptic rodents or humans.[12,13] In addition, biochemical studies have revealed that hormones, cytokines, and peripheral blood transcriptomes, which are implicated in human and rodent epilepsy, may also be tested in zebrafish epilepsy models.[10] Most FDA-approved AEDs (e.g., carbamazepine, phenytoin, sodium valproate, diazepam, gabapentin, levetiracetam) were also shown to be efficient in zebrafish assays.[9,13,14] Zebrafish were also used to screen therapeutic agents against genetic epilepsies, such as Dravet syndrome, a disease that is insensitive to almost all AEDs.[15] Compared with mammalian models, the zebrafish can be used to screen more compounds economically, rapidly, and conveniently.[16] Zebrafish are permeable to drugs that are placed in the bathing medium, so the drugs can be delivered to freely swimming zebrafish, and the locomotion and electrophysiology features of seizure activity can be readily monitored quantitatively.[11] Therefore, the utility of the zebrafish model for epilepsy research is an effective approach to AED discovery.

The genus *Cynanchum* is a natural source of C_{21} steroidal glycosides, which reportedly possess antidepressant,[17] immunosuppressive,[18] antitumor,[19,20] and MDR-reversing[21] activities. Recently, we reported on C_{21} steroidal glycosides from the roots of *C. auriculatum*, a local species of Binhai County, Jiangsu Province, People's Republic of China, as a natural appetite suppressant.[22] *Cynanchum otophyllum* C.K. Schneid (Asclepiadaceae), an endemic species in the People's Republic of China, is distributed primarily in Southwest China, and its roots have been used for the treatment of epilepsy, rheumatic pain, kidney weakness, and muscle injury in Chinese folk medicine.[23] In our continuous effort to search for novel and



interesting biologically active natural products, six new C_{21} steroidal glycosides (**1**–**6**), named cynotophyllosides A–F, together with 16 known compounds (**7**–**22**), were isolated from the roots of *C. otophyllum*. The structures were identified by spectroscopic analysis, chemical methods, and comparison of observed and reported spectroscopic data. The abundant

Table 1. ^{1}H NMR Spectroscopic Data (500 MHz) for the Aglycone Part of Compounds 1–6

position	1[a]	2[a]	3[a]	4[a]	5[a]	6[b]
1	1.07, m; 1.88, m	1.10, m; 1.90, m	1.14, m; 1.90, m	1.11, m; 1.90, m	1.07, m; 1.86, m	1.04, m; 1.74, m
2	1.60, m; 1.91, m	1.60, m; 1.88, m	1.62, m; 1.93, m	1.62, m; 1.88, m	1.65, m; 1.94, m	1.78, m; 2.07, m
3	3.55, m	3.57, m	3.59, m	3.56, m	3.54, m	3.88, m
4	2.29, m; 2.37, m	2.32, m; 2.38, m	2.33, m; 2.42, m	2.31, m; 2.40, m	2.30, m; 2.39, m	2.10, m; 2.59, m
6	5.36, brs	5.37, brs	5.38, brs	5.37, brs	5.34, brs	5.32, m
7	2.20, m	2.20, m	2.23, m	2.22, m	2.28, m	2.08, m; 2.49, m
9	1.50, m	1.51, m	1.59, m	1.58, m	1.44, m	1.75, m
11	1.81, m	1.82, m	1.94, m	1.92, m	1.86, m	2.00, m; 2.38, m
12	4.54, dd (5.4, 7.4)	4.54, dd (6.2, 7.9)	4.79, m	4.79, m	3.67, m	5.34, m
15	1.95, m	1.97, m	2.03, m	2.02, m	1.93, m; 2.05, m	2.37, m; 1.75, m
16	1.85, m; 2.84, m	1.88, m; 2.85, m	1.92, m; 2.84, m	1.91, m; 2.84, m	1.97, m; 2.75, m	2.10, m; 2.17, m
18	1.40, s	1.40, s	1.51, s	1.51, s	1.25, s	2.13, s
19	1.15, s	1.12, s	1.11, s	1.10, s	1.14, s	1.30, s
20						5.30, m
21	2.16, s	2.17, s	2.08, s	2.08, s	2.33, s	1.57, d (6.1)
2′	5.50, s	5.51, s				6.56, d (16.0)
3′			7.79, d (8.6)	7.80, d (8.8)		7.88, d (16.0)
4′	2.33, m	2.35, m	6.82, d (8.6)	6.83, d (8.8)		
5′	1.05, d (6.9)	1.06, d (6.9)				7.45, m
6′	1.05, d (6.9)	1.06, d (6.9)				7.35, m
7′	2.11, s	2.12, s				7.35, m
3″						9.54, d (2.1)
5″						8.31, dt (2.1, 8.0)
6″						7.20, dd (4.8, 8.0)
7″						8.83, dd (1.8, 4.8)
14-OH			4.32, s		4.14, s	
17-OH			4.48, s		4.62, s	

[a]Spectra were recorded in $CDCl_3$. [b]Spectrum was recorded in pyridine-d_5.

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Table 2. ^{1}H NMR Spectroscopic Data (500 MHz) for the Sugar Moiety of Compounds 1−6

position	1[a]	2[a]	3[a]	4[a]	5[a]	6[b]
	Cym	Digit	Cym	Cym	Cym	Digit
1‴	4.84, dd (2.1, 9.8)	4.92, dd (2.1, 9.8)	4.80, dd (1.9, 9.6)	4.85, dd (2.1, 9.7)	4.83, dd (2.2, 9.8)	5.51, dd (2.1, 9.6)
2‴	1.57, m; 2.09, m	1.76, m; 2.11, m	1.59, m; 2.17, m	1.59, m; 2.02, m	1.60, m; 2.07, m	2.08, m; 2.42, m
3‴	3.80, dd (2.8, 6.9)	4.25, dd (3.2, 6.4)	3.63, dd (3.1, 6.1)	4.83, dd (3.3, 6.3)	3.81, m	4.68, dd (2.9, 6.1)
4‴	3.23, dd (2.8, 9.5)	3.21, dd (3.3, 9.3)	3.23, dd (3.4, 10.2)	3.23, dd (3.2, 9.9)	3.23, m	3.54, dd (2.9, 9.4)
5‴	3.83, dq (6.1, 9.5)	3.79, dq (6.2, 9.2)	3.60, m	3.79, dq (6.3, 9.8)	3.85, m	4.35, dq (6.2, 9.4)
6‴	1.21, d (6.1)	1.24, d (6.3)	1.29, d (6.3)	1.23, d (6.3)	1.23, d (6.3)	1.48, d (6.2)
3‴-OMe	3.44, s		3.43, s	3.44, s	3.44, s	
	Cym	Cym		Cym	Cym	Cym
1⁗	4.74, dd (2.2, 9.9)	4.82, dd (2.1, 9.7)		4.68, dd (2.1, 9.8)	4.74, dd (1.9, 9.5)	5.15, dd (2.1, 9.7)
2⁗	1.65, m; 2.17, m	1.64, m; 2.17, m		1.62, m; 2.24, m	1.64, m; 2.12, m	1.70, m; 2.35, m
3⁗	3.78, dd (2.7, 7.2)	3.80, dd (3.0, 9.3)		3.63, dd (3.4, 9.6)	3.87, m	3.74, dd (3.1, 6.1)
4⁗	3.25, dd (2.7, 9.8)	3.25, dd (3.0, 9.7)		3.21, dd (3.6, 9.6)	3.21, m	3.50, dd (3.1, 9.5)
5⁗	3.90, dq (6.3, 9.8)	3.96, dq (6.1, 9.6)		3.57, m	3.87, m	4.12, dq (6.2, 9.5)
6⁗	1.26, d (6.1)	1.27, d (6.1)		1.27, d (6.2)	1.22, d (6.3)	1.47, d (6.2)
3⁗-OMe	3.42, s	3.44, s		3.41, s	3.44, s	3.46, s
	The	The			Ole	
1′′′′′	4.30, d (7.7)	4.31, d (8.1)			4.49, dd (1.8, 9.8)	
2′′′′′	3.50, dd (7.7, 9.1)	3.50, t (8.7)			1.49, m; 2.32, m	
3′′′′′	3.10, t (9.1)	3.10, t (9.0)			3.15, dd (4.6, 11.4)	
4′′′′′	3.18, t (9.1)	3.19, t (9.1)			3.13, dd (7.3, 9.1)	
5′′′′′	3.36, dq (6.1, 9.1)	3.38, dq (6.2, 9.2)			3.28, dq (6.2, 8.8)	
6′′′′′	1.30, d (6.1)	1.31, d (6.1)			1.31, d (6.2)	
3′′′′′-OMe	3.64, s	3.65, s			3.38, s	

[a]Spectra were recorded in $CDCl_3$. [b]Spectrum was recorded in pyridine-d_5.

compounds **1**, **3**−**5**, **7**−**19**, **21**, and **22** were quantitatively assessed for their antiseizure-like locomotor activity in the zebrafish model. Compounds **15**, **17**, and **18** exhibited significant therapeutic effects, suppressing zebrafish epilepsy as induced by PTZ. Herein, their isolation, structure elucidation, and activity evaluation are reported.

RESULTS AND DISCUSSION

Compound **1** was isolated as a white, amorphous powder, and its molecular formula was deduced as $C_{49}H_{78}O_{17}$ by a sodium adduct ion at m/z 961.5168 in the HRTOFMS and ^{13}C NMR data. The IR spectrum of **1** showed absorption bands for hydroxy (3471 cm^{-1}), ketocarbonyl (1712 cm^{-1}), ester carbonyl (1652 cm^{-1}), and olefinic (1450, 1165 cm^{-1}) functions. The ^{1}H NMR spectrum of the aglycone portion exhibited two olefinic proton signals at δ_H 5.50 (1H, brs) and 5.36 (1H, brs), six methyl resonances at δ_H 1.05 (6H, d, J = 6.9 Hz), 1.15, 1.40, 2.11, and 2.16 (each 3H, s) (Table 1), as well as three oxygenated tertiary carbon signals at δ_C 74.4, 88.1, and 91.6, four olefinic carbons at δ_C 166.0, 140.8, 117.7, and 113.1, one ketocarbonyl at δ_C 209.0, and one ester carbonyl at δ_C 167.0 in its ^{13}C NMR data (Table 3). An analysis of the ^{1}H−^{1}H COSY spectrum of **1** revealed fragments C1−C2−C3-C4, C6−C7, C9−C11−C12, C15−C16, and C5′−C4′−C6′ in its structure (Figure 1). An observation of ^{1}H−^{13}C long-range correlations from δ_H 5.36 (H-6) to δ_C 37.3 (C-10), 38.9 (C-4), and 74.4 (C-8); from δ_H 4.54 (H-12) to δ_C 9.5 (C-18) and 91.6 (C-17); from δ_H 2.16 (H-21) to δ_C 91.6 (C-17); from δ_H 1.40 (H-18) to δ_C 88.1 (C-14) and 91.6 (C-17); and from δ_H 1.15 (H-19) to δ_C 38.9 (C-1), 43.8 (C-9), and 140.8 (C-5) enabled the establishment of the 3,8,12,14,17-pentahydroxypregn-5-en-20-one skeleton. Further HMBC correlations from H-4′ (δ_H 2.33) to C-2′ (δ_C 113.1); from H-7′ (δ_H 2.11) to C-4′ (δ_C 38.3) and C-2′ (δ_C 113.1); from H-5′, 6′ (δ_H 1.05) to C-3′ (δ_C 166.0); and from H-12 (δ_H 4.54) to C-1′ (δ_C 167.0) revealed that an ikemaoyl group was connected to C-12 (Figure 1), and caudatin was thus identified as the aglycone of **1**.[24−28] Three anomeric proton signals were evident at δ_H 4.84 (1H, dd, J = 2.1, 9.8 Hz), 4.74 (1H, dd, J = 2.2, 9.9 Hz), and 4.30 (1H, d, J = 7.7 Hz), corresponding to carbon signals at δ_C 96.2, 99.7, and 104.5 in its HSQC spectrum, respectively, which indicated that **1** contained three saccharide moieties with β glycosidic linkages. The three glycosyl units were defined as two cymaroses and one thevetose, upon analyzing the spin systems of the protons of each sugar unit [δ_H 3.80 (dd, J = 2.8, 6.9 Hz, H-3‴), 3.23 (dd, J = 2.8, 9.5 Hz, H-4‴), 3.83 (dq, J = 6.1, 9.5 Hz, H-5‴); 3.78 (dd, J = 2.7, 7.2 Hz, H-3⁗), 3.25 (dd, J = 2.7, 9.8 Hz, H-4⁗), 3.90 (dq, J = 6.3, 9.8 Hz, H-5⁗); and 3.50 (dd, J = 7.7, 9.1 Hz, H-2′′′′′), 3.10 (t, J = 9.1 Hz, H-3′′′′′), 3.18 (t, J = 9.1 Hz, H-4′′′′′), and 3.36 (dq, J = 6.1, 9.1 Hz, H-5′′′′′)] with the aid of ^{1}H−^{1}H COSY (Figure 1) and selective 1D-TOCSY spectra (Figure S1H, Supporting Information). ^{1}H−^{13}C long-range correlation (Figure 1) signals from H-1‴ (δ_H 4.84) to C-3 (δ_C 78.0); from H-1⁗ (δ_H 4.74) to C-4‴ (δ_C 82.7); and from H-1′′′′′ (δ_H 4.30) to C-4⁗ (δ_C 82.8) revealed the structure of the sugar chain and its location at C-3 of the aglycone moiety. The ^{13}C NMR spectroscopic data of the sugar moiety of **1** matched well with those of P57 isolated from *Hoodia gordonii*,[29] which also supported the above deduction. Acid hydrolysis of **1** followed by column chromatography yielded purified cymarose and thevetose. Their specific rotation values ($[\alpha]_D$ = +46.2 and +15.5, respectively) were obtained from an aqueous solution after a 24 h equilibration period. The sugar units were D-configured based on comparison of experimental and reported $[\alpha]_D$ values.[30,31] Thus, the structure of **1** was defined as caudatin 3-*O*-β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside and was named cynotophylloside A.

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Table 3. ^{13}C NMR Spectroscopic Data (125 MHz) for the Aglycone Part of Compounds 1−6

position	1[a]	2[a]	3[a]	4[a]	5[a]	6[b]
1	38.9	38.8	38.8	38.9	38.9	39.2
2	29.0	29.0	28.9	29.0	29.1	29.9
3	78.0	78.0	77.9	78.1	78.0	77.6
4	38.9	38.9	38.9	38.9	39.0	39.2
5	140.8	140.8	140.5	140.6	140.6	139.2
6	117.7	117.7	117.9	117.9	117.9	119.3
7	34.3	34.3	34.4	34.4	34.3	34.2
8	74.4	74.4	74.3	74.4	74.4	74.1
9	43.8	43.8	43.7	43.7	44.2	44.0
10	37.3	37.3	37.2	37.2	37.2	37.3
11	24.4	24.4	24.3	24.4	28.1	25.7
12	71.7	71.7	73.0	73.0	69.6	74.3
13	58.0	58.0	58.6	58.6	61.0	57.1
14	88.1	88.1	88.1	88.1	87.9	89.0
15	33.2	33.2	33.4	33.4	33.4	34.8
16	32.0	32.0	32.0	32.0	32.6	33.7
17	91.6	91.6	91.6	91.6	92.0	87.4
18	9.5	9.5	9.6	9.7	7.9	11.5
19	18.7	18.8	18.6	18.7	18.8	18.0
20	209.0	209.0	210.1	210.0	214.0	76.4
21	27.3	27.3	27.6	27.6	28.4	15.4
1′	167.0	167.0	165.4	165.4		166.8
2′	113.1	113.1	121.8	121.9		120.3
3′	166.0	166.1	131.9	131.9		144.0
4′	38.3	38.3	115.5	115.5		134.9
5′	21.1	21.1	161.0	160.9		128.5
6′	21.0	21.0				129.3
7′	16.6	16.7				130.6
1″						164.7
3″						151.4
4″						126.9
5″						137.3
6″						123.7
7″						153.8

[a]Spectra were recorded in $CDCl_3$. [b]Spectrum was recorded in pyridine-d_5.

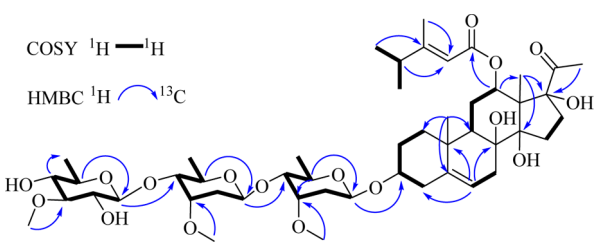

Figure 1. ^{1}H−^{1}H COSY and key HMBC correlations of compound 1.

The molecular formula of 2 was determined as $C_{48}H_{76}O_{17}$ based on the HRTOFMS (*m/z* 947.5013 $[M + Na]^+$) and ^{13}C NMR data. By comparing its NMR data with those of 1 (Tables 1 and 3), compound 2 was deduced to share the same aglycone moiety as 1. Three anomeric carbon signals were observed at δ_C 104.5, 98.4, and 95.9, corresponding to the anomeric proton resonances at δ_H 4.31 (d, *J* = 8.1 Hz), 4.82 (dd, *J* = 2.1, 9.7 Hz), and 4.92 (dd, *J* = 2.1, 9.8 Hz), respectively. An analysis of ^{1}H−^{1}H COSY, HSQC, HMBC, and 1D-TOCSY spectra enabled the identification of these three saccharide units as thevetose, cymarose, and digitoxose, respectively, and their D absolute configurations were confirmed by the specific rotation values of purified saccharides ($[\alpha]_D$ = +10.5, +50.6, and +46.8, respectively) that were obtained from the hydrolysate of 2.[30−32] The connection of these glycosyl units was determined by the HMBC correlations from H-1‴ (δ_H 4.92) to C-3 (δ_C 78.0); from H-1⁗ (δ_H 4.82) to C-4‴ (δ_C 82.6); and from H-1′′′′′ (δ_H 4.31) to C-4⁗ (δ_C 82.7). Therefore, 2 was identified as caudatin 3-*O*-*β*-D-thevetopyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-digitoxopyranoside, and it was named cynotophylloside B.

The negative mode HRTOFMS of 3 showed an $[M - H]^-$ peak at *m/z* 643.3119, revealing its molecular formula to be $C_{35}H_{48}O_{11}$. A pair of *ortho*-coupled aromatic proton signals were observed at δ_H 7.79 (2H, d, *J* = 8.6 Hz) and 6.82 (2H, d, *J* = 8.6 Hz) in its ^{1}H NMR data (Table 1), which indicated the presence of a *para*-disubstituted benzene ring. Analysis of its ^{13}C NMR, ^{1}H−^{1}H COSY, HSQC, and HMBC spectra showed the aglycone of 3 to be qingyangshengenin.[26,28,33] The hydrolysis of 3 yielded only D-cymarose as its sugar component, which was defined by comparing its observed and reported ^{1}H NMR data and the positive specific rotation value ($[\alpha]_D$ = +48.9).[33,34] An HMBC correlation from H-1‴ (δ_H 4.80) to C-3 (δ_C 77.9) revealed that the cymarosyl unit was connected to C-3 of the aglycone. Thus, compound 3 was identified as qingyangshengenin 3-*O*-*β*-D-cymaropyranoside, and it was named cynotophylloside C.

Compounds 3 and 4 possessed an identical aglycone moiety according to their NMR data (Tables 1 and 3). The molecular formula of cynotophylloside D (4) was deduced as $C_{42}H_{60}O_{14}$ on the basis of an $[M + Na]^+$ ion at *m/z* 811.3890 in the HRTOFMS and ^{13}C NMR data. Two anomeric proton signals were observed at δ_H 4.85 (1H, dd, *J* = 2.1, 9.7 Hz) and 4.68 (1H, dd, *J* = 2.0, 9.8 Hz) in its ^{1}H NMR data (Table 3). The acid hydrolysate of 4 was identical to that of 3, indicating the presence of two cymaropyranosyl moieties. The 1→4 connection of the two saccharide units was determined by using an HMBC correlation signal from H-1⁗ (δ_H 4.68) to C-4‴ (δ_C 82.6), and the HMBC correlation from H-1‴ (δ_H 4.85) to C-3 (δ_C 78.1) enabled the location of the saccharide chain at C-3. Therefore, 4 was characterized as qingyangshengenin 3-*O*-*β*-D-cymaropyranosyl-(1→4)-*β*-D-cymaropyranoside.

Compound 5 gave a sodium adduct ion at *m/z* 835.4471 in the HRTOFMS, revealing its molecular formula to be $C_{42}H_{68}O_{15}$. The aglycone of 5 was defined as deacetylmetaplexigenin,[22] based on the characteristic NMR signals of C_{21}-glycosides isolated from *Cynanchum*. Three glycosyl units should be present in the structure of 5 according to three anomeric proton signals at δ_H 4.83 (1H, dd, *J* = 2.2, 9.8 Hz), 4.74 (1H, dd, *J* = 1.9, 9.5 Hz), and 4.49 (1H, dd, *J* = 1.8, 9.8 Hz), and they were determined to be two cymaropyranosyl moieties and one oleandropyranosyl unit by analyzing the coupling constants of each sugar proton (Table 2). The D configurations of cymarose and oleandrose that were isolated from the hydrolysate of 5 were identified according to their specific rotation values ($[\alpha]_D$ = +50.4 and −11.7, respectively).[30] The HMBC correlations from H-1‴ (δ_H 4.83) to C-3 (δ_C 78.0); from H-1⁗ (δ_H 4.74) to C-4‴ (δ_C 82.7); and from H-1′′′′′ (δ_H 4.49) to C-4⁗ (δ_C 82.6) revealed the connectivity within the sugar chain. Thus, the structure of 5 was established as deacetylmetaplexigenin 3-*O*-*β*-D-oleandropyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-cymaropyranoside, and it was named cynotophylloside E.

Cynotophylloside F (6) had a molecular formula of $C_{49}H_{65}O_{14}N$ as determined by HRTOFMS (*m/z* 892.4512

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[M + H]$^+$). The aglycone of **6** possessed ^{1}H and ^{13}C NMR signals that were characteristic of 3,8,12,14,17,20-hexahydroxypregn-5-ene, a cinnamoyl group, and a nicotinoyl group (Tables 1 and 2).[35,36] The HMBC correlations from H-12 (δ_H 5.34) to C-1′ (δ_C 166.8) and from H-20 (δ_H 5.30) to C-1″ (δ_C 164.7) indicated that the cinnamoyl group was attached to C-12, and the nicotinoyl group was connected to C-20 of the aglycone. The C-20 stereogenic center was determined to be *S*-configured by the NOEs between H-21 (δ_H 1.57) and H-16β (δ_H 2.10); between H-20 (δ_H 5.30) and H-16α (δ_H 2.17); and between H-18 (δ_H 2.13) and H-20 (δ_H 5.30), H-3″ (δ_H 9.54), and H-5″ (δ_H 8.31) (Figure 2).[37] On the basis of the above

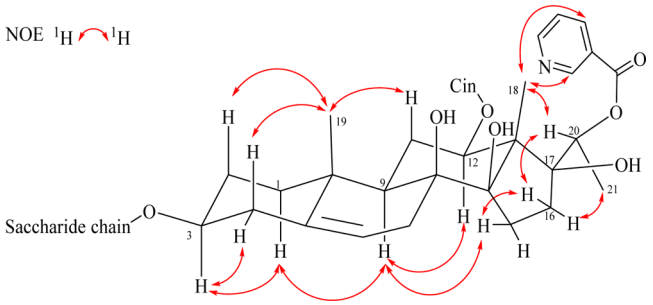

Figure 2. Key NOE correlations of the aglycone part of compound **6**.

evidence, the aglycone was identified as gagaminin. β-Cymaropyranosyl and β-digitoxopyranosyl moieties were deduced to exist in the structure of **6** according to two anomeric proton signals at δ_H 5.15 (1H, dd, J = 2.1, 9.7 Hz) and 5.51 (1H, dd, J = 2.1, 9.6 Hz) and NOE correlations between H-1‴ (δ_H 5.51) and H-2‴$_{eq}$ (δ_H 2.42), H-5‴ (δ_H 4.35); between H-3‴ (δ_H 4.68) and H-2‴$_{ax}$ (δ_H 2.08), H-2‴$_{eq}$ (δ_H 2.42); between H-4‴ (δ_H 3.54) and H-2‴$_{ax}$ (δ_H 2.08), H-3‴ (δ_H 4.68), H-6‴ (δ_H 1.48); between H-1⁗ (δ_H 5.15) and H-2⁗$_{eq}$ (δ_H 2.35), H-5⁗ (δ_H 4.12); between H-3⁗ (δ_H 3.74) and H-2⁗$_{ax}$ (δ_H 1.70), H-2⁗$_{eq}$ (δ_H 2.35); and between H-4⁗ (δ_H 3.50) and H-2⁗$_{ax}$ (δ_H 1.70), H-3⁗ (δ_H 3.74), H-6⁗ (δ_H 1.47). The connection of the sugar chain was established by the HMBC correlation from H-1⁗ (δ_H 5.15) to C-4‴ (δ_C 83.5) and from H-1‴ (δ_H 5.51) to C-3 (δ_C 77.6). The D absolute configurations of the two monosaccharides were determined on the basis of their positive specific rotation values ([α]$_D$ = +49.4 and +48.6, respectively).[30,32] Thus, **6** was characterized as gagaminin 3-*O*-β-D-cymaropyranosyl-(1→4)-β-D-digitoxopyranoside.

Sixteen known compounds were identified as qingyangshengenin (**7**),[23] caudatin (**8**),[23] otophylloside C (**9**),[24] caudatin 3-*O*-β-cymaropyranoside (**10**),[25] qingyangshengenin 3-*O*-β-D-cymaropyranosyl-(1→4)-β-D-digitoxopyranoside (**11**),[26] caudatin 3-*O*-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside (**12**),[27] qingyangshengenin 3-*O*-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside (**13**),[19] qingyangshengenin 3-*O*-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-digitoxopyranoside (**14**),[26] otophylloside F (**15**),[24] otophylloside A (**16**),[28] otophylloside B (**17**),[28] rostratamine 3-*O*-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside (**18**),[26] otophylloside T (**19**),[33] stephanoside H (**20**),[35] caudatin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside (**21**),[36] and otophylloside M (**22**)[23] by comparing their experimental and reported spectroscopic and spectrometric data.

The abundant compounds **1**, **3**–**5**, **7**–**19**, **21**, and **22** (>50 mg) were selected to evaluate their antiseizure-like locomotor activity in the zebrafish bioassay model. The preliminary testing of these pure isolates at their maximum-tolerated concentrations revealed that **1**, **3**, **9**, **10**, **12**, **13**, and **16** tended to

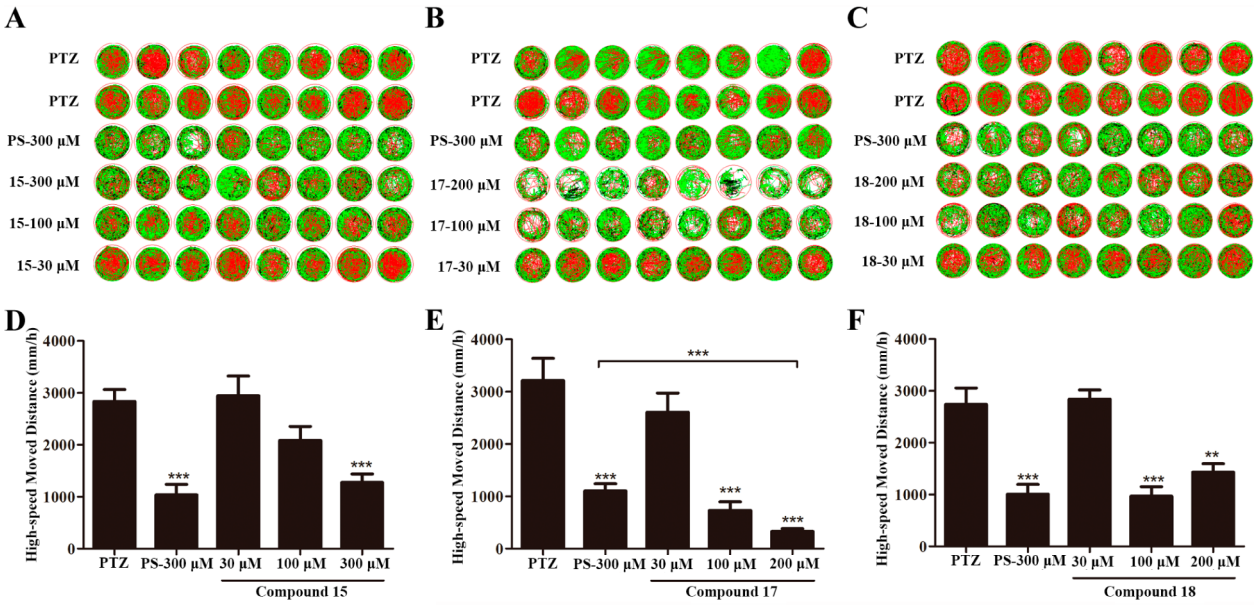

Figure 3. (A–C) Effects of phenytoin sodium (PS, 300 μM) and compounds **15** (30, 100, and 300 μM), **17** (30, 100, and 200 μM), and **18** (30, 100, and 200 μM) on seizure-like activity induced by PTZ. The effects were measured by using movement-tracking software. (D) Treating with **15** at a 300 μM concentration showed a therapeutic efficacy of 55%, whereas the efficacy of PS was 63% at 300 μM. (E) Treating with **17** at concentrations of 100 and 200 μM showed therapeutic efficacies of 77% and 90%, respectively, and the efficacy of PS was 66% at 300 μM. (F) Treating with **18** at 100 and 200 μM concentrations showed therapeutic efficacies of 65% and 52%, respectively, and the efficacy of PS was 63% at 300 μM (**p < 0.01 and ***p < 0.001).

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reduce or stop epilepsy, and **15**, **17**, and **18** exhibited significant therapeutic effects against epilepsy (Figure S8, Supporting Information). Further evaluation of the efficacy of **15**, **17**, and **18** at lower concentrations indicated that treating with **15** at a 300 μM concentration showed a therapeutic efficacy of 55%; treating with **17** at 100 and 200 μM concentrations showed therapeutic efficacies of 77% and 90%, respectively; and treating with **18** at 100 and 200 μM concentrations showed treatment efficacies of 65% and 52%. As a comparison, the positive control drug phenytoin sodium showed a therapeutic efficacy of 66% at 300 μM (Figure 3). We also ran a parallel assay (only tested compounds) in an attempt to identify whether compounds **15**, **17**, and **18** had nonspecific neurotoxic or sedative effects or if they were drugs with general effects on locomotor activity. The result showed that these three compounds do not have any nonspecific neurotoxic or sedative effects, nor do they have effects on locomotor activity (Figure S11, Supporting Information).

A preliminary SAR analysis suggested the following: (1) the C-12 substituent is essential for antiseizure-like locomotor activity, and ikemaoyl is preferable to the cinnamoyl group (**17** > **18** > **5** > **16**). Compounds with a *p*-hydroxybenzoyl substituent at C-12 (**3**, **4**, **7**, **9**, **11**, **13**, **14**, **16**, and **19**) showed weak activity or even pro-epileptic activities. (2) The presence of a sugar chain at C-3 is important to the activity, and a sugar chain consisting of three saccharide moieties is preferable (**17** > **12** >**10** > **8**). A sugar chain consisting of 2,6-dideoxysaccharides exhibits better inhibitory activity than those containing 6-deoxysaccharides or glucose (**17** > **1** > **22**).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a PerkinElmer 341 polarimeter (PerkinElmer, Waltham, MA, USA). HRTOFMS analyses were performed on an LCT Premier XE mass spectrometer (Waters, Milford, MA, USA). IR spectra were recorded on a PerkinElmer 577 spectrometer (PerkinElmer) by using KBr disks. NMR experiments were performed on a Varian-MERCURY Plus-400 (Varian, Palo Alto, CA, USA) or Bruker Advance III 500 (Bruker, Ettlingen, Germany) spectrometer in $CDCl_3$ or pyridine-d_5 with TMS as an internal standard. Preparative HPLC was performed on a Unimicro EasySep-1010 binary pump system (Unimicro, Shanghai, People's Republic of China) with a Unimicro EasySep-1010 detector (Unimicro) by using a YMC-Pack ODS-A (250 × 20 mm, 5 μm; YMC Co., Ltd., Kyoto, Japan) column. Silica gel (300−400 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, People's Republic of China) and C_{18} reversed-phase (RP-18) silica gel (150−200 mesh; Merck, Whitehouse Station, NJ, USA) were used for column chromatography (CC). Precoated silica gel GF254 plates (Qingdao Haiyang Chemical Co., Ltd.) were used for TLC detection.

Plant Material. The roots of *Cynanchum otophyllum* Schneid were collected in October 2013 from Hunan Province, People's Republic of China, and identified by Prof. Jin-Gui Shen, Shanghai Institute of Materia Medica. A voucher specimen (no. WMZ-20131106-QYS) has been deposited at Shanghai Institute of Materia Medica.

Extraction and Isolation. Air-dried roots of *C. otophyllum* (5.0 kg) were powdered and percolated with 95% EtOH (3 × 10 L) at room temperature to afford the crude extract, which was suspended in water and extracted with EtOAc (3 × 1 L). The EtOAc extract was applied to a silica gel column, which was eluted with petroleum ether−acetone (from 5:1 to 0:1, v/v) to yield four fractions (Fr.1−4). Fr.3 was subjected to an RP-18 column that was eluted with MeOH−H_2O (from 40% to 100% v/v) to obtain five subfractions (Fr.31−35). Fr.31 was separated using the RP-18 column (from 30% to 50% MeOH) to yield **7** (2.0 g) and **9** (0.646 g); compounds **3** (0.195 g) and **5** (0.689 g) were isolated from Fr.32 by using preparative HPLC (35% CH_3CN); Fr.33 was subjected to silica gel CC (petroleum ether−acetone, 5:1 to 3:1, v/v) and further purified by preparative HPLC to yield compounds **4** (0.213 g), **8** (3.475 g), **11** (0.557 g), **13** (0.064 g), **14** (1.189 g), **16** (1.261 g), and **19** (0.176 g); Fr.34 was chromatographed over silica gel CC (petroleum ether−acetone, 3:1 to 0:1, v/v) and purified by preparative HPLC to yield **1** (0.115 g), **2** (0.032 g), **6** (0.012 g), **10** (0.995 g), **12** (0.965 g), **15** (1.035 g), **17** (2.079 g), **18** (0.066 g), **20** (0.016 g), **21** (0.337 g), and **22** (0.065 g).

*Cynotophylloside A (**1**):* white, amorphous powder; $[\alpha]_D^{25}$ +17 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3471, 2967, 2932, 1712, 1652, 1450, 1165, 1086, 1003, and 990 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1−4; HRTOFMS *m*/*z* 961.5168 $[M + Na]^+$ (calcd for $C_{49}H_{78}O_{17}Na$, 961.5137).

Table 4. ^{13}C NMR Spectroscopic Data (125 MHz) for the Sugar Moiety of Compounds 1−6

position	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[b]
	Cym	Digit	Cym	Cym	Cym	Digit
1‴	96.2	95.9	95.8	96.2	96.2	96.3
2‴	35.7	37.2	34.1	35.6	35.7	38.8
3‴	77.2	66.5	77.4	77.2	77.2	67.5
4‴	82.7	82.6	72.6	82.6	82.7	83.5
5‴	68.7	68.2	70.9	68.7	68.7	68.6
6‴	18.5	18.4	18.4	18.5	18.4	18.9
3‴-OMe	58.2		57.4	58.1	58.1	
	Cym	Cym		Cym	Cym	Cym
1⁗	99.7	98.4		99.6	99.5	99.8
2⁗	35.3	35.3		33.8	35.6	35.6
3⁗	77.1	77.0		77.5	77.1	78.8
4⁗	82.8	82.7		72.5	82.6	74.6
5⁗	68.4	68.7		70.8	68.3	71.0
6⁗	18.3	18.5		18.3	18.3	18.7
3⁗-OMe	58.1	58.3		57.4	58.3	58.1
	The	The			Ole	
1′′′′′	104.5	104.5			101.6	
2′′′′′	74.8	74.8			35.4	
3′′′′′	85.4	85.3			80.7	
4′′′′′	74.7	74.7			75.5	
5′′′′′	71.7	71.8			71.7	
6′′′′′	17.9	18.0			18.1	
3′′′′′-OMe	60.8	60.9			56.4	

[a]Spectra were recorded in $CDCl_3$. [b]Spectrum was recorded in pyridine-d_5.

*Cynotophylloside B (**2**):* white, amorphous powder, $[\alpha]_D^{25}$ +13 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3462, 2969, 2934, 1712, 1642, 1450, 1384, 1224, 1166, 1085, 1005, and 998 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1−4; HRTOFMS *m*/*z* 947.5013 $[M + Na]^+$ (calcd for $C_{48}H_{76}O_{17}Na$, 947.4980).

*Cynotophylloside C (**3**):* white, amorphous powder; $[\alpha]_D^{25}$ +22 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3459, 2957, 2939, 1711, 1609, 1515, 1450, 1384, 1278, 1164, 1089, 1059, and 983 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1−4; HRTOFMS *m*/*z* 643.3119 $[M - H]^-$ (calcd for $C_{35}H_{47}O_{11}$, 643.3118).

*Cynotophylloside D (**4**):* white, amorphous powder; $[\alpha]_D^{24}$ +30 (*c* 0.3, MeOH); IR (KBr) ν_{max} 3462, 2969, 2934, 1711, 1609, 1593, 1515, 1450, 1278, 1164, 1093, 1060, and 982 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1−4; HRTOFMS *m*/*z* 811.3890 $[M + Na]^+$ (calcd for $C_{42}H_{60}O_{14}Na$, 811.3881).

*Cynotophylloside E (**5**):* white, amorphous powder; $[\alpha]_D^{25}$ +26 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3552, 3427, 2968, 2936, 1702, 1405, 1382, 1365, 1192, 1155, 1103, 1056, 1003, and 986 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1−4; HRTOFMS *m*/*z* 835.4471 $[M + Na]^+$ (calcd for $C_{42}H_{68}O_{15}Na$, 835.4456).

*Cynotophylloside F (**6**):* white, amorphous powder; $[\alpha]_D^{26}$ +125 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3450, 2971, 2933, 1716, 1637, 1589, 1448,

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1382, 1286, 1163, 1085, and 991 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1−4; HRTOFMS m/z 892.4512 $[M + H]^+$ (calcd for $C_{49}H_{66}O_{14}N$, 892.4483).

Acid Hydrolysis of Compounds 1−6. One drop of 2 N HCl was added to a solution of **1** (10 mg) in dioxane (3 mL), and the reaction mixture was stirred at 60 °C for 1 h; after cooling, 10 mL of water was added, and the mixture was extracted with EtOAc (10 mL). The aqueous phase was dried to afford a crude sugar fraction. The crude sugar fraction was subjected to silica gel CC, with successive eluting with DCM−MeOH to afford cymarose (70:1, v/v), oleandrose (50:1, v/v), thevetose (20:1, v/v), and digitoxose (10:1, v/v). Compounds **2−6** were hydrolyzed by using the same method.

D-Digitoxose: colorless gum; $[\alpha]_D^{18}$ +46.8 to +48.6 (ref $[\alpha]_D^{25}$ = +48.0, H_2O);[32] ^1H NMR (β-anomer, major, D_2O) δ_H 1.22 (3H, d, J = 6.2 Hz), 1.70 (1H, ddd, J = 2.8, 9.8, 13.4 Hz), 2.04 (1H, dt, J = 3.0, 13.4 Hz), 3.30 (1H, dd, J = 3.0, 9.8 Hz), 3.82 (1H, dd, J = 6.0, 9.8 Hz), 4.10 (1H, dd, J = 3.0, 6.2 Hz), and 5.09 (1H, brd, J = 9.8 Hz); ^1H NMR (α-anomer, minor, D_2O) δ_H 1.17 (3H, d, J = 6.2 Hz), 1.86 (1H, m), 1.95 (1H, m), 3.38 (1H, dd, J = 3.2, 8.6 Hz), 3.80 (1H, m), 4.12 (1H, m), and 5.15 (1H, t, J = 3.3 Hz). The data were consistent with those of D-digitoxose.[38]

D-Thevetose: colorless gum; $[\alpha]_D^{18}$ +10.5 to +15.5 (ref $[\alpha]_D^{20}$ +23.9, H_2O);[30] ^1H NMR (β-anomer, major, D_2O) δ_H 1.25 (3H, d, J = 6.4 Hz), 3.19 (2H, m), 3.37 (1H, m), 3.45 (1H, m), 3.58 (3H, s), and 4.47 (1H, d, J = 7.6 Hz). The data were consistent with those of D-thevetose.[30]

D-Cymarose: colorless gum; $[\alpha]_D^{18}$ +46.2 to +50.6 (ref $[\alpha]_D^{25}$ +47.6, H_2O);[30] ^1H NMR [α-anomer, D_2O (α:$\beta \approx$ 1:1)] δ_H 1.21 (3H, d, J = 6.0 Hz), 2.01 (1H, brd, J = 12.4 Hz), 2.28 (1H, ddd, J = 2.1, 4.4, 12.4 Hz), 3.40 (3H, s), 3.42 (1H, m), 3.74 (1H, m), 4.12 (1H, m), and 5.56 (1H, d, J = 3.6 Hz); ^1H NMR (β-anomer, D_2O) δ_H 1.17 (3H, d, J = 6.6 Hz), 1.90 (1H, m), 2.15 (1H, m), 3.32 (3H, s), 3.38 (1H, m), 3.72 (1H, m), 4.04 (1H, brs), and 5.00 (1H, brd, J = 9.5 Hz). These data were consistent with those for D-cymarose.[34]

D-Oleandrose: colorless gum; $[\alpha]_D^{17}$ −11.7 (ref $[\alpha]_D^{25}$ = −11.3, H_2O);[32] ^1H NMR [α-anomer, D_2O (α:$\beta \approx$ 1:1)] δ_H 1.22 (3H, d, J = 6.1 Hz), 1.52 (1H, ddd, J = 3.9, 11.5, 12.2 Hz), 2.42 (1H, dd, J = 4.8, 12.2 Hz), 3.12 (1H, t, J = 9.3 Hz), 3.35 (1H, m), 3.37 (3H, s), 3.84 (1H, dq, J = 6.4, 9.7 Hz), and 5.30 (1H, brs); ^1H NMR (β-anomer, D_2O) δ_H 1.20 (3H, d, J = 6.5 Hz), 1.31 (1H, dd, J = 9.7, 13.4 Hz), 2.28 (1H, dd, J = 5.1, 13.4 Hz), 3.06 (1H, t, J = 9.3 Hz), 3.35 (1H, m), 3.36 (3H, s), 3.55 (1H, dq, J = 6.5, 9.3 Hz), and 4.84 (1H, d, J = 9.7 Hz). The data were consistent with those of D-oleandrose.[39]

Zebrafish Assay. Adult AB strain zebrafish were housed in a light- and temperature-controlled aquaculture facility under a standard 14:10 h light/dark photoperiod and fed with live brine shrimp twice daily and dry flake once a day. Zebrafish embryos were obtained from natural crosses. Embryos were maintained at 28.5 °C in fish water (0.2% Instant Ocean Salt in deionized water, pH 6.9−7.2, conductivity 480−510 mS/cm, and hardness 53.7−71.6 mg/L $CaCO_3$). For all measurements described here, 6-day-postfertilization (dpf) zebrafish larvae were used. After the completion of the experiment, the zebrafish were sacrificed under anesthesia by overexposure to tricaine. The execution steps were performed in accordance with the American Veterinary Medical Association's (AVMA) animal anesthesia specifications. The zebrafish facility at Hunter Biotechnology, Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAA LAC) International, and the assay protocol number is 001458. As described above, exposing larvae zebrafish to PTZ elicited locomotor activity, and we analyzed the behavior of 6-dpf larvae that were exposed to PTZ concentrations ranging from 1.25 to 30 mM. Concentration-dependent increases in locomotor activity (as measured by assessing the seizure-like locomotor distance moved) were observed in response to treatments with PTZ at concentrations from 1.25 to 10 mM, but the fish move less at high doses, perhaps as a result of neural damage, and then eventually die (Figure S9, Supporting Information). A 10 mM PTZ concentration was selected for all subsequent experiments. Different DMSO concentrations were also evaluated, and as a result, the 1.0% DMSO concentration was selected for the following experiments (Figure S10, Supporting Information). Phenytoin sodium (PS), a first-line treatment for epilepsy, also suppressed the seizure-like locomotor activity of zebrafish caused by PTZ.[9] Thus, PS was selected as the positive control in this assay. The selected compounds were dissolved in 100% DMSO (stock solutions) and diluted in fish water to achieve a final DMSO concentration of 1% w/v. Zebrafish larvae were incubated with these compounds at 28.5 °C in the dark. After 90 min, each zebrafish was checked under a stereomicroscope for signs of acute locomotor reduction (hypoactivity, decreased or no touch/escape in response to acupuncture, loss of posture, body deformation, exophthalmos, slow or absent heartbeat, and death).[13] The maximum-tolerated concentration (MTC) was defined as the maximum concentration that did not cause death, and no more than 2/12 larvae exhibited any sign of locomotor activity. The MTCs of those compounds are presented in Table S1 (Supporting Information). Zebrafish larvae were transferred to 48-well microplates, with one larva per well. The microplates were incubated at 28.5 °C in the dark during the experimental procedure. The zebrafish were first pretreated with test compounds or the positive control drug PS for 1 h. After the preincubation, a 40 mM PTZ solution was added to obtain a final concentration of 10 mM. The zebrafish exhibited signs of agitation within seconds of contact with PTZ. The zebrafish were allowed to habituate for 10 min in the chamber of an automated tracking device called a ZebraBox (Viewpoint, Lyon, France). The locomotor activity was tracked, recorded for 1 h, and then quantified with ZebraLab software (Viewpoint, Lyon, France). Here, the definition of "locomotor activity" in this assay was the moment when the velocity of a PTZ-treated larva reached at least 20 mm/s. All tracking experiments were performed at least in triplicate. A quantitative analysis of the seizure-like locomotor distance was performed to evaluate the efficacy of the test compounds. The efficacy was calculated by using the following equation: Efficacy = $(D_{PTZ} - D_{compounds})/D_{PTZ} \times 100\%$, where D represents the seizure-like locomotor distance.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra of all new compounds are provided together with bioassay results of tested compounds. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/np501058b.

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Notes

The authors declare no competing financial interest.

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