Dihydrochalcones from the Leaves of *Pieris japonica*

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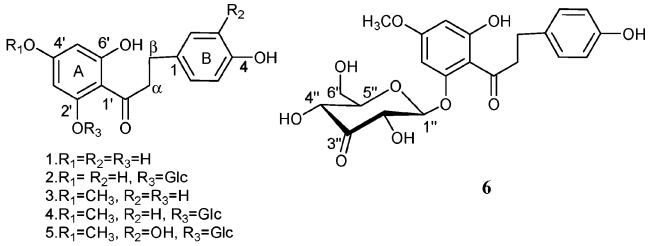
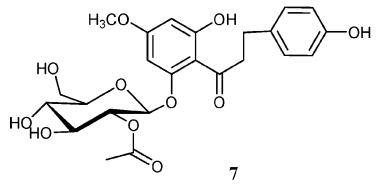
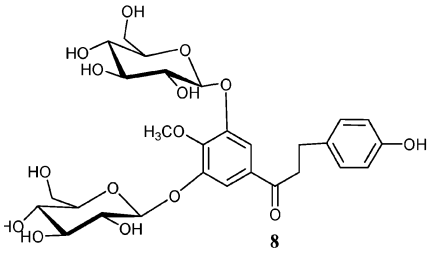
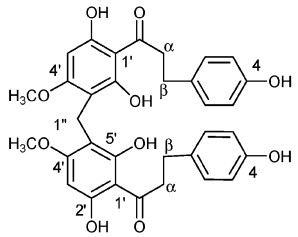
Six new dihydrochalcones, 3-hydroxyasebotin (**5**), asebogenin 2′-*O*-*β*-D-ribohexo-3-ulopyranoside (**6**), 2″-acetylasebotin (**7**), 3′,4,5′-trihydroxy-4′-methoxydihydrochalcone 3′,5′-di-*O*-*β*-D-glucopyranoside (**8**), and pierotins A (**9**) and B (**10**), along with four known dihydrochalcones, phloretin (**1**), phlorizin (**2**), asebogenin (**3**), and asebotin (**4**), were isolated from the leaves of *Pieris japonica*. Their structures were elucidated on the basis of spectroscopic analysis including HMQC, HMBC, NOESY, and X-ray crystal diffraction. Compounds **1**, **3**−**5**, and **7**−**10** inhibited the proliferation of murine B cells and compounds **5** and **10** inhibited the proliferation of murine T cells *in vitro* significantly.

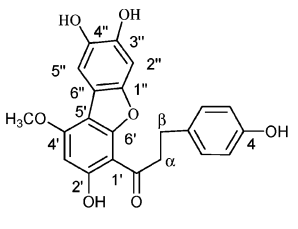
Pieris japonica (Wall) D. Don (Ericaceae) is a well-known poisonous plant, distributed mainly in hilly and valley regions of south and southwest China. The roots and leaves of the plant are used as folk medicine to cure vomiting and diarrhea caused by sunstroke. The leaves and stems of the plant are used as insecticide and as lotion for treatment of ring worm and scabies.[1] Previous chemical investigations on this plant resulted in the isolation of diterpenoids,[2] triterpenoids,[3] and flavonoids.[4] Recently, in the course of our search for bioactive substances from Chinese herb medicine, we have studied the leaves of *P. japonica* (Wall) D. Don and isolated six new dihydrochalcones, **5**−**10**, along with four known dihydrochalcones, **1**−**4**. In this paper we report the isolation and structure elucidation of new dihydrochalcones **5**−**10** as well as the immunomodulatory activities of these 10 dihydrochalcones.











Results and Discussion

The air-dried and powdered leaves of *P. japonica* were extracted with 95% EtOH. The EtOH extracts were evaporated under reduced pressure to give a residue, which was suspended in distilled water and partitioned successively with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH. Column chromatography of the EtOAc and *n*-BuOH fractions led to the isolation of 10 dihydrochalcones. Four known dihydrochalcones, **1**−**4**, were identified by comparison of their physical, MS, and NMR data with literature values as 2′,4,4′,6′-tetrahydroxydihydrochalcone (phloretin) **1**,[5] 2′,4,4′,6′-tetrahydroxydihydrochalcone 2′-*O*-*β*-D-glucopyranoside (phlorizin) **2**,[5,6] 2′,4,6′-trihydroxy-4′-methoxydihydrochalcone (asebogenin) **3**,[7] and 2′,4,6′-trihydroxy-4′-methoxydihydrochalcone 2′-*O*-*β*-D-glucopyranoside (asebotin) **4**,[8] respectively.

Compound **5**, obtained as colorless needles, had the molecular formula $C_{22}H_{26}O_{11}$, determined by HRESIMS (*m/z* 489.1429 $[M + Na]^+$, calc 489.1373). Hydrolysis of **5** with *β*-glucosidase afforded glucose as detected by co-TLC, suggesting that **5** is a glucoside. The IR spectrum of **5** showed the presence of a hydroxyl (3408 cm^{-1}), a conjugated ketone (1630 cm^{-1}), and an aromatic ring (1593, 1529 cm^{-1}). Its ^1H NMR spectrum (Table 1) is very similar to that of compound **4**, a known 4,2′,6′-trihydroxy-4′-methoxydihydrochalcone 2′-*O*-*β*-D-glucopyranoside, except the protons from ring B, which showed an ABX system (*δ* 6.59, d, *J* = 2.0 Hz, 1H; 6.58, d, *J* = 8.0 Hz, 1H; 6.46, dd, *J* = 2.0 and 8.0 Hz, 1H) instead of the A_2B_2 system in **4**,

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Table 1. ^{1}H NMR Data of Compounds **5**−**10** (DMSO-d_6)[a]

no.	5	6	7	8	9	10
2	6.59 d (2.0)	7.03 d (8.2)	6.99 d (8.4)	7.03 d (8.4)	6.99 d (8.3)	7.10 d (8.3)
3		6.64 d (8.2)	6.62 d (8.4)	6.63 d (8.4)	6.64 d (8.3)	6.69 d (8.3)
5	6.58 d (8.0)	6.64 d (8.2)	6.62 d (8.4)	6.63 d (8.4)	6.64 d (8.3)	6.69 d (8.3)
6	6.46 dd (2.0, 8.0)	7.03 d (8.2)	6.99 d (8.4)	7.03 d (8.4)	6.99 d (8.3)	7.10 d (8.3)
α	3.35 m	3.34 m	3.03 t (7.1)	3.05 m	3.22 t (7.6)	3.47 t (7.6)
		3.42 m	3.11 t (7.1)			
β	2.72 t (6.5)	2.81 t (7.2)	2.73 t (7.1)	2.72 m	2.75 t (7.6)	2.88 t (7.6)
2′				6.48 s		
3′	6.27 d (2.4)	6.33 d (1.5)	6.27 d (2.3)		5.99 s	6.46 s
5′	6.11 d (2.4)	6.17 d (1.5)	6.12 d (2.3)			
6′				6.48 s		
1″	4.98 d (7.5)	5.16 d (7.9)	5.35 d (7.1)	4.86 d (7.7)	3.60 s	
2″	3.28 m	4.30 d (7.8)	4.82 t (8.8)	3.18 m		7.02 s
3″	3.38 m		3.50 m	3.27 m		
4″	3.13 m	4.19 d (10.0)	3.24 m	3.11 m		
5″	3.28 m	3.60 m	3.48 m	3.29 m		7.27 s
6″	3.44 dd (6.0, 12.0)	3.61 m	3.45 dd (5.5, 10.6)	3.40 dd (6.0, 12.0)		
	3.70 dd (5.4, 10.2)	3.78 d (10.2)	3.70 dd, (6.0, 11.7)	3.70 dd (5.1, 10.5)		
3-OH	8.57 s					
4-OH	8.64 s	9.07 s	9.10 s	9.06 s	9.12 s	9.18 s
2′-OH					10.88 s	13.48 s
4′-OCH_3	3.77 s	3.79 s	3.76 s	3.75 s	3.67 s	4.02 s
6′-OH	13.35 s	13.23 s	12.56 s		13.48 s	
*CH_3*CO			1.97 s			
3″-OH						9.17 s
4″-OH						9.07 s

[a] *J* values (Hz) are showed in parentheses.

Table 2. ^{13}C NMR Data of Compounds **5**−**10** (in DMSO-d_6)

no.	5	6	7	8	9	10
1	132.3	131.2	131.4	132.3	131.6	131.2
2	115.5	129.1	129.3	129.0	129.3	129.3
3	150.0	115.2	115.1	114.9	115.2	115.3
4	143.2	155.2	155.4	155.1	155.5	155.6
5	115.9	115.2	115.1	114.9	115.2	115.3
6	118.9	129.1	129.3	129.0	129.3	129.3
α	45.2	44.9	45.3	49.3	45.9	44.4
β	29.1	28.8	28.6	28.3	29.6	29.2
1′	106.3	106.4	107.6	115.4	104.1	101.7
2′	160.3	159.4	158.4	95.8	160.6	164.5
3′	93.6	93.8	93.5	155.2	90.4	94.5
4′	165.2	165.0	164.4	164.1	163.8	160.1
5′	95.3	95.6	95.6	155.2	106.7	106.6
6′	165.0	164.6	163.1	95.8	162.4	155.8
C=O	205.2	204.7	204.2	202.3	205.0	202.7
1″	100.9	101.5	97.2	101.1	15.4	148.6
2″	73.2	76.2	73.4	73.3		98.5
3″	76.9	205.4	74.2	76.7		143.0
4″	69.8	72.0	69.8	69.9		144.9
5″	77.5	76.8	77.5	77.3		106.4
6″	60.8	60.6	60.7	60.8		113.1
4′-OCH_3	55.7	55.6	55.6	55.3	55.5	56.6
CH_3*CO*			169.5			
*CH_3*CO			20.9			

indicating that **5** should have one more hydroxyl group at C-3 of ring B. The ^{13}C NMR data (Table 2) showed the presence of a 2′,3,4, 4′,6′-pentaoxygenated dihydrochalcone and a glucose unit. In the HMBC spectrum of **5**, the correlation of *O*-methyl protons (*δ* 3.77, s, 3H), H-3′ (*δ* 6.27, d, *J* = 2.4 Hz, 1H), and H-5′ (*δ* 6.11, d, *J* = 2.4 Hz, 1H) with C-4′ (*δ* 165.2) was observed, suggested that the *O*-methyl group was located at C-4′. This was supported by the NOESY correlation of the *O*-methyl protons with H-3′ and H-5′. Furthermore, the HMBC correlation of H-3′ and H-1″ of glucose (*δ* 4.98, d, *J* = 7.5 Hz, 1H) with C-2′ (*δ* 160.3, s) revealed that the glucose unit was located at C-2′. The *β*-glucosyl linkage was deduced from the coupling constant of the anomeric proton (*δ* 4.98, d, *J* = 7.5 Hz, 1H, H-1″). On the basis of the above evidence, **5** was determined as 2′,3,4,6′-tetrahydroxy-4′-methoxydihydrochalcone 2′-*O*-*β*-D-glucopyranoside, named 3-hydroxyasebotin. The structure was confirmed by X-ray crystal diffraction (Figure 1).

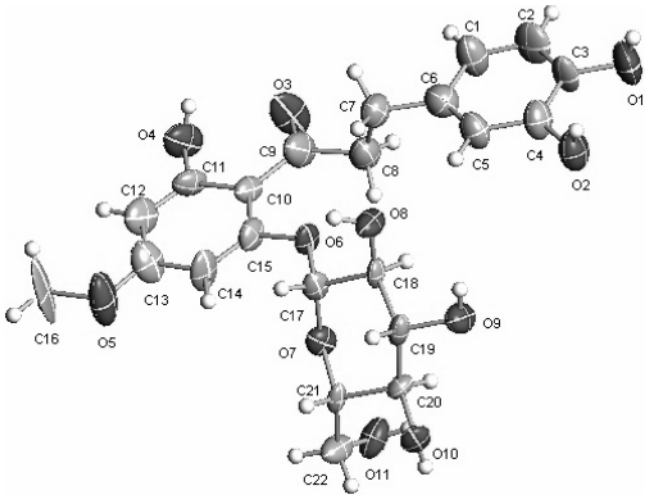

Figure 1. Computer-generated perspective view and X-ray numbering system for 3-hydroxyasebotin (**5**).

Compound **6**, colorless needles, had the molecular formula $C_{22}H_{24}O_{10}$, determined by HRESIMS (*m/z* 471.1273 $[M + Na]^+$, calcd for $C_{22}H_{24}O_{10}Na$, 471.1267). The ^{1}H NMR and ^{13}C NMR spectra were very similar to those of **4** except for their sugar moieties. The mass of **6** was 2 amu less than that of **4**, implying that the sugar unit of **6** was a didehydrohexose. The ^{13}C NMR signal at *δ* 205.2 and IR absorption at 1735 cm^{-1} suggested the presence of a keto-hexose, in which the carbonyl group was located at C-3″ by HMBC correlation between the carbonyl (*δ* 205.2) and two deshielded protons attributable to H-2″ (*δ* 4.30, d, *J* = 7.8 Hz, 1H) and H-4″ (*δ* 4.19, d, *J* = 10.0 Hz, 1H). Further investigation revealed the sugar moiety was ribohexo-3-ulopyranose due to good agreement of ^{13}C NMR data of **6** with those of literature values.[9,10] The *β*-configuration of the keto-hexose was deduced from the coupling constant (*J* = 7.9 Hz) of the anomeric proton (*δ* 5.16, d, *J* = 7.9 Hz, 1H). The large coupling constants of $^3J_{H-1'',H-2''}$ (7.8 Hz)

and $^3J_{H-4'',H-5''}$ (10.0 Hz) revealed that the protons at 1″, 2″, 4″, and 5″ must be close to trans diaxial, indicating the keto-hexose must have a chairlike conformation.[9] Thus, compound **6** was elucidated as 2′,4,6′-trihydroxy-4′-methoxydihydrochalcone 2′-*O*-*β*-D-ribohexo-3-ulopyranoside. A literature survey showed that only five iridoid keto-glycosides[9] and one acyclic monoterpene keto-glycoside[10] have been reported from natural sources. To our knowledge, **6** is the first dihydrochalcone containing a *β*-D-*ribo*-hexo-3-ulose moiety.

Compound **7**, colorless needles, had the molecular formula $C_{24}H_{28}O_{11}$, determined by HRESIMS (*m/z* 515.1568 $[M + Na]^+$, calcd for $C_{22}H_{24}O_{11}Na$, 515.1529). Comparison of the ^1H and ^{13}C NMR data of **7** with those of **4** showed that **7** had an acetyl group (*δ* 1.97, s, 3H; *δ* 169.5, s and 20.9, q) connected with the sugar moiety. The IR absorption at 1732 cm^{-1} further supported the presence of an ester carbonyl. Acidic hydrolysis of **7** yielded glucose as detected by co-TLC. The HMBC correlation of the anomeric carbon (*δ* 97.2, C-1″)/H-2″ of glucose (*δ* 4.82, 1H, t, *J* = 8.8 Hz) and the carbonyl carbon (*δ*169.5) of acetate/H-2″ of glucose suggested that the C-2″ hydroxyl group was acetylated. On the basis of the above evidence compound **7** was determined as the 2″-acetate of 2′,4,6′-trihydroxy-4′-methoxydihydrochalcone 2′-*O*-*β*-D-glucopyranoside, i.e., asebotin 2″-acetate.

Compound **8**, obtained as colorless needles, gave the molecular formula $C_{28}H_{36}O_{15}$ by HRESIMS (*m/z* 635.1930 $[M + Na]^+$, calcd for $C_{28}H_{36}O_{15}Na$, 635.1953). Hydrolysis of **8** with *β*-glucosidase afforded glucose as detected by co-TLC, suggesting that **8** was a glucoside. The ^1H NMR spectrum (Table 1) showed two doublets (*δ* 7.03, 2H, d, *J* = 8.4 Hz and 6.63, 2H, d, *J* = 8.4 Hz) for 1,4-disubstituted phenyl protons and a two-proton singlet (*δ* 6.48, 2H, s) for symmetrical *meta* aromatic protons. The presence of two methylene groups in **8** was deduced by ^1H NMR (Table 1) (*δ* 3.05 and 2.72, each 2H) and ^{13}C NMR (Table 2) data (*δ* 49.3 and 28.3). These evidences suggested **8** as a dihydrochalcone with 4-oxygenated ring B and 3′,4′,5′-trioxygenated ring A. The ^{13}C NMR data (*δ* 115.4, 1C; 95.8, 2C; 155.2, 2C; 164.4, 1C) supported a symmetrical substitution of ring A.

All ^1H NMR signals ascribable to glucose moieties showed double intensity, suggesting two equivalent glucose moieties existed, which was in agreement with the molecular formula. The large coupling constant of the anomeric proton (*δ* 4.86, *J* = 7.7 Hz, H-1″) indicated a *β*-configuration. The HMBC correlation between the carbonyl carbon (*δ* 202.3) and H-2′ and H-6′ (*δ* 6.48, 2H, s,); C-3′ and C-5′ (*δ* 155.2) with H-2′, H-6′, and anomeric protons (*δ* 4.86, H-1″ of glucose); and C-4′ (*δ* 164.1) with methoxyl protons (*δ* 3.75) established a symmetrical 3′,5′-diglucosyloxyl-4′-methoxybenzyl structure. When H-2′ and H-6′ were irradiated, a strong NOE of H″-1 of glucose was observed, further confirming the constitution of the above proposed ring A. Thus, compound **8** was determined to be 3′,4,5′-trihydroxy-4′-methoxydihydrochalcone 3′,5′-di-*O*-*β*-D-glucopyranoside.

Compound **9** was obtained as yellowish amorphous powder. Its molecular formula was determined to be $C_{33}H_{32}O_{10}$ from the $[M + Na]^+$ ion peak at *m/z* 611.1935 (calcd for $C_{33}H_{32}O_{10}$, 611.1893) in the HRESIMS. The ^1H NMR (Table 1) and ^{13}C NMR (Table 2) data of **9** were very similar to those of compound **3**. The main differences were in ring A of dihydrochalcone as follows: (a) the H-3′ signal changed from a doublet to a singlet at *δ* 5.99, (b) the H-5′ signal disappeared and the C-5′ signal changed from a methine to quaternary carbon at *δ* 106.7, and (c) an additional methylene signal appeared (*δ* 3.60, 2H, s; 15.4, t). Further study revealed that all ^1H NMR signals of dihydrochalcone showed double integrating intensity except for the additional methylene, suggesting that **9** was a dimer of **3**, connected at C-5′ via a methylene bridge (C-1″). The HMBC correlation of H-1″ (*δ* 3.60, 2H, s) to C-5′ (*δ* 106.7, s) and C-6′ (*δ* 162.4, s) and H-6′-OH (*δ* 13.48, 1H, s) to C-6′ (*δ* 162.4, s) confirmed that the methylene group should be attached to C-5′. The NOESY spectrum showed cross-peaks of H-3′ to *O*-methyl protons and H-2′-OH, confirming the proposed structure. Accordingly, compound **9** was determined to be bis[2′,4,6′-trihydroxy-4′-methoxydihydrochalcone-5′-yl]methane, named pierotin A.

Since the first dimeric dihydrochalcone was isolated from *Brackenridgea zanguebarica* in 1983,[11] only six dimeric dihydrochalcones have been reported.[12−16] Among them, piperaduncin C was the first dihydrochalcone dimer with a methylene bridge.[16]

Compound **10** was obtained as an amorphous powder. Its molecular formula was determined to be $C_{22}H_{24}O_{10}$ by HRESIMS (*m/z* 417.0942 $[M + Na]^+$, calcd for $C_{22}H_{18}O_7Na$, 417.0950). The UV spectrum exhibited absorption maxima at 220, 256, 264, 295, and 360 nm. Its ^1H NMR spectrum (Table 1) showed the signals for a dihydrochalcone moiety similar to that in **9**: two doublets (*δ* 7.10, 2H, d, *J* = 8.3 Hz and 6.69, 2H, d, *J* = 8.3 Hz) for a *p*-substituted ring B; two triplets (*δ* 3.47, 2H, t, *J* = 7.6 Hz and 2.88, 2H, t, *J* = 7.6 Hz) for the disubstitued ethane functionality, a three-proton singlet (*δ* 4.02, 3H, s) for an *O*-methyl group, and a singlet (*δ* 6.46, s, H-3′) of ring A. Two additional *para* aromatic protons appeared at *δ* 7.02 (1H, s) and 7.27 (1H, s), implying another aromatic ring combined with the dihydrochalcone moiety. Further ^1H NMR studies revealed the presence of four phenolic hydroxyl protons at *δ* 9.07, 9.17, 9.18, and 13.48 (exchangeable with D_2O). On the basis of the above evidence and molecular formula, **10** was considered as a benzofuran-containing dihydrochalcone, in which the benzofuran was formed by connection of C-5′ and C-6′-OH. The ^{13}C NMR spectrum (Table 2) contained 22 carbon signals, 16 of them representing a dihydrochalcone moiety, and the remaining 6 carbons ascribed to a benzofuran moiety including two non-oxygenated methines (*δ* 98.5, 106.4) and four quaternary carbons (three oxygenated at *δ* 148.6, 143.0, 144.9, and one non-oxygenated at *δ* 113.1).

The HMBC correlation between H-3′ (*δ* 6.46, 1H, s)/C-1′, C-2′, C-4′, C-5′, and 2′-OH (*δ*13.48,1H, s)/C-1′, C-2′, C-3′, C-4′, along with the NOESY correlation between H-3′/2′-OH, 4′-OCH_3 suggested that the *O*-methyl group was at C-4′ and the hydroxyl group at C-2′. The HMBC correlation between H-2″ (*δ* 7.02, 1H, s)/C-1″, 3″, C-4″, C-6″; H-5″ (*δ* 7.27, 1H, s)/C-3″, C-4″, C-5′; 4″-OH (*δ* 9.07, 1H, s)/C-4″, C-5″; and 3″-OH (*δ* 9.17, 1H, s)/C-2″, C-3″ established the substitution pattern of the dibenzofuran unit. In combination with the analysis of the NOESY, HMQC, and HMBC spectra, the structure of compound **10** was established as 1-methoxy-3,7,8-trihydroxy-4-[3-(4-hydroxyphenyl)-1-propanoyl]dibenzofuran, named pierotin B. This is the first report of a dihydrochalcone containing a dibenzofuran moiety.

Compounds **1**−**10** were subjected for evaluation of their immunomodulatory activity *in vitro*. It was found (Table 3) that **1**, **3**−**5**, and **7**−**10** inhibited the proliferation of murine B cells and **5** and **10** inhibited the proliferation of murine T cells in vitro, significantly. The MTT assay demonstrated that the antiproliferative activities of these compounds were not involved with the general cytotoxity.

Table 3. Effect of Compounds **1**−**10** on Murine Lymphocyte Proliferation Induced by Concanavalin A (ConA) (5 mg/mL) or Lipopolysaccharide (LPS) (10 mg/mL)[a]

		[^{3}H] TdR incorporation × 10^{-3} (cpm)			
compound	concentration (M)	ConA-induced T cell proliferation		LPS-induce B cell proliferation	
negative control		5.031 ± 0.643		4.555 ± 0.003	
positive control (ConA or LPS)		76.530 ± 4.361		47.483 ± 4.059	
1	1× 10^{-7}	63.858 ± 32.467	↓	32.656 ± 3.544	↓
	1× 10^{-6}	75.735 ± 4.892		29.253 ± 0.634	↓↓
	1× 10^{-5}	69.362 ± 3.516		30.656 ± 5.753	↓
3	1× 10^{-7}	79.283 ± 5.590		32.464 ± 1.798	↓↓
	1× 10^{-6}	80.258 ± 1.277		28.341 ± 2.144	↓
	1× 10^{-5}	58.431 ± 11.803		34.129 ± 12.284	
9	1× 10^{-7}	75.440 ± 3.340		38.125 ± 0.793	↓↓
	1× 10^{-6}	74.983 ± 3.297		32.696 ± 1.360	↓
	1× 10^{-5}	22.624 ± 6.058	↓	36.497 ± 3.964	↓
negative control		2.294 ± 0.400		2.985 ± 0.386	
positive control (ConA or LPS)		39.102 ± 4.298		3.6064 ± 0.700	
2	1× 10^{-7}	36.137 ± 2.125		33.017 ± 2.116	
	1× 10^{-6}	35.510 ± 3.862		32.423 ± 5.203	
	1× 10^{-5}	33.021 ± 1.462		30.478 ± 4.905	
6	1× 10^{-7}	38.281 ± 1.718		34.213 ± 1.934	
	1× 10^{-6}	26.730 ± 1.561	↓	35.003 ± 5.150	
	1× 10^{-5}	32.040 ± 1.813		16.413 ± 6.100	
negative control		6.272 ± 0.181		4.772 ± 0.684	
positive control (ConA or LPS)		80.704 ± 2.692		74.008 ± 0.798	
4	1× 10^{-7}	89.776 ± 4.134		47.422 ± 5.657	↓↓
	1× 10^{-6}	87.186 ± 35.39		40.734 ± 1.049	↓↓↓
	1× 10^{-5}	79.572 ± 2.063		35.159 ± 0.436	↓↓↓
5	1× 10^{-7}	66.721 ± 9.804	↓↓	22.160 ± 3.310	↓↓
	1× 10^{-6}	71.450 ± 2.282		22.098 ± 0.797	↓
	1× 10^{-5}	33.029 ± 8.526	↓↓	23.954 ± 4.0958	↓↓
7	1× 10^{-7}	83.283 ± 5.815		46.640 ± 2.198	↓↓
	1× 10^{-6}	81.638 ± 7.786		44.989 ± 5.970	↓
	1× 10^{-5}	70.144 ± 2.954		29.750 ± 1.724	
8	1× 10^{-7}	84.764 ± 2.289		30.863 ± 1.845	↓↓
	1× 10^{-6}	74.077 ± 1.921		22.772 ± 3.131	
	1× 10^{-5}	74.123 ± 4.877		24.556 ± 2.652	↓↓
10	1× 10^{-7}	76.194 ± 9.124		22.821 ± 1.467	↓↓↓
	1× 10^{-6}	58.950 ± 15.559	↓↓	28.639 ± 3.675	↓↓
	1× 10^{-5}	10.751 ± 3.680		3.944 ± 3.137	

[a] Results are represented as mean ± SD based on three independent experiments ($n = 3$; ↓ $P < 0.05$; ↓↓ $P < 0.01$; ↓↓↓ $P < 0.001$ compared with control group).

Experimental Section

General Experimental Procedures. Melting points were determined on a digital SGW X-4 apparatus and were uncorrected. Optical rotations were measured on a Perkin-Elmer Model 341 polarimeter. UV spectra were recorded on a Shimadzu UV-2550 UV−visible spectrophotometer. The FT-IR spectra were recorded on an IR-514 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Brüker AM-400 spectrometer with TMS as the internal standard and DMSO-d_6 as solvent. 2D-NMR spectra were recorded on a Brüker DRX-500 spectrometer. HRESIMS were carried out on a JEOL SX-102, using PEG-400 as calibration matrix.

Plant Material. The leaves of *P. japonica* were collected in the Huangshan Mountain of Anhui Province, China, and identified by one of the authors (B.-Y.D.). A voucher specimen (No. 20020715) is deposited in the herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Extraction and Isolation. The air-dried and powdered leaves of *P. japonica* (10 kg) were extracted with 95% EtOH, and the extract was evaporated under reduced pressure to give a residue (2 kg) that was then suspened in distilled H_2O and partitioned successively with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH. The EtOAc extract was evaporated to give a red mass (600 g), which was applied to a silica gel column, eluting with $CHCl_3$ containing increasing amounts of MeOH. Repeated column chromatography yielded **1** (2 g), **2** (1355 mg), **3** (1 g), **4** (2 g), **5** (75 mg), **6** (300 mg), **7** (857 mg), **9** (15 mg), and **10** (34 mg). Chromatography of the *n*-BuOH phase (800 g) led to the isolation of **8** (20 mg).

3-Hydroxyasebotin (2′,3,4,6′-tetrahydroxy-4′-methoxydihydrochalcone 2′-*O*-*β*-D-glucopyranoside, 5): colorless needles from $CHCl_3$ and MeOH; mp 155−156 °C; $[\alpha]_D^{20}$ −37° (*c* 0.095; MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.67), 222 (4.34), 284 (4.30) nm; IR (KBr) ν_{max} 3408, 3275 (br, OH), 2922, 1630 (chelated C=O), 1593, 1529 (aromatic ring), 1441, 1269, 1205, 1074, 808, 758 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 2; HRESIMS *m/z* 489.1429 for $[M + Na]^+$, calcd for $C_{22}H_{26}O_{11}Na$, 489.1373.

Asebogenin 2′-*O*-*β*-D-ribohexo-3-ulopyranoside (2′,4,6′-trihydroxy-4′-methoxydihydrochalcone 2′-*O*-*β*-D-ribohexo-3-ulopyranoside, 6): colorless needles from $CHCl_3$ and MeOH; mp 104−106 °C; $[\alpha]_D^{20}$ −75° (*c* 0.20; MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (4.69), 283 (4.60) nm; IR (KBr) ν_{max} 3419 (br, OH), 2929, 1735 (C=O), 1626 (chelated C=O), 1599, 1516 (aromatic ring), 1431, 1207, 1162, 1101, 1076, 1049, 825, 619 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 2; HRESIMS *m/z* 471.1273 for $[M + Na]^+$, calcd for $C_{22}H_{24}O_{10}Na$, 471.1267.

2″-Acetyl abosetin (2′,4,6′-trihydroxy-4′-methoxydihydrochalcone 2′-*O*-*β*-D-glucopyranoside 2″-acetate, 7): colorless needles from $CHCl_3$ and MeOH; mp 168−170 °C; $[\alpha]_D^{20}$ −55° (*c* 0.14; MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (4.26), 284 (4.18) nm; IR (KBr) ν_{max} 3388 (br, OH), 2922, 1732(C=O), 1626 (chelated C=O), 1599 and 1516 (aromatic ring), 1431, 1371, 1211, 1167, 1076, 1041, 820, 619 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 2; HRESIMS *m/z* 515.1568 for $[M + Na]^+$), calcd for $C_{24}H_{28}O_{11}Na$, 515.1529.

3′,4,5′-Trihydroxy-4′-methoxydihydrochalcone 3′,5′-di-*O*-*β*-D-glucopyranoside (8): colorless needles from $CHCl_3$ and

MeOH; mp 138−140 °C; $[\alpha]_D^{20}$ −50° (*c* 0.06; MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (4.30), 271 (3.78) nm; IR (KBr) ν_{max} 3421 (br, OH), 2920, 1672, 1610 (conjugated C=O), 1516 (aromatic rings), 1435, 1163, 1069, 1032, 831, 643 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 2; HRESIMS *m/z* 635.1930 for $[M + Na]^+$, calcd for $C_{28}H_{36}O_{15}Na$, 635.1952.

Pierotin A (bis[α,β-dihydro-2′,4,6′-trihydroxy-4′-methoxychalcone-5′-yl] methane, 9): yellowish amorphous powder; $[\alpha]_D^{20}$ −9° (*c* 0.055; MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (4.99), 287 (4.77) nm; IR (KBr) ν_{max} 3423 (br, OH), 2922, 1630 (conjugated C=O), 1587, 1514 (aromatic ring), 1425, 1218, 1143, 804, 602 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 2; HRESIMS *m/z* 611.1935 for $[M + Na]^+$, calcd for $C_{33}H_{32}O_{10}Na$, 611.1893.

Pierotin B (1-methoxy-3,7,8-trihydroxy-4-[3-(4-hydroxyphenyl)-1-propanoyl]dibenzofuran, 10): yellowish-brown amorphous powder; $[\alpha]_D^{20}$ −108° (*c* 0.06; MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (4.77), 256 (4.67), 264 (4.71), 295 (4.63), 360 (3.92) nm; IR (KBr) ν_{max} 3425 (br, OH), 2922, 1622 (conjugated C=O), 1514 (aromatic ring), 1441, 13867, 1203,1165, 1120, 1086, 555 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 2; HRESIMS *m/z* 417.0942 for $[M + Na]^+$, calcd for $C_{22}H_{18}O_7Na$, 417.0950.

X-ray Crystal Structure Determination of 3-Hydroxyasebotin (5). A colorless needle of 3-hydroxyasebotin **5**, obtained via slow evaporation of a $CHCl_3$−CH_3OH solution, with approximate dimensions of 0.307 × 0.221 × 0.048 mm was selected for data collection on a Brüker SMART 1000 diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). A total of 14 437 reflections were collected in the range 1.92° < θ < 26.00° by using the ω scan technique at 293(2) K. A total of 8045 observable reflections with $I > 2\sigma(I)$ were used in the succeeding refinements [R(int) = 0.1515]. *Lp* corrections were applied to the data.

Crystal Data: $C_{22}H_{26}O_{11}\cdot CHCl_3$, monoclinic with space group $P2(1)$, with a = 7.4935(13) Å, b = 31.081(5) Å, c = 11.3063(19) Å, α = 90°, β = 93.794(3)°, γ = 90°, V = 2627.6(8) Å^3, ρ_c = 1.481 Mg/m^3 for Z = 4 and M_r = 585.80, F(000) = 1216. All non-hydrogen atoms were refined anisotropically in a full-matrix least-squares refinement process with the SHELXL-97 software package. The final standard residual R_1 value was 0.0728 for observed data (8045 reflections) and 0.2023 for all data (14 437 reflections). The corresponding Sheldrick R values were wR2 of 0.1603 and 0.2097, respectively. A final difference Fourier map showed significant residual electron density, the largest difference peak and hole being 0.384 and −0.367 e Å^{-3}, respectively. Final bond distances and angels were within acceptable limits. The final structure of 3-hydroxyasebotin (**5**) is shown in Figure 1.

Enzymatic Hydrolysis of Dihydrochalcone Glucosides. Compound **5** (3 mg) was dissolved in H_2O (8 mL) and incubated with almond β-glucosidase (20 mg) for 24 h at 37 °C. The mixture was filtered, and the water phase was analyzed by silica gel HPTLC (Merck) developed with Me_2CO−2 mM NaOAc (17:3, v/v). The sugar was detected by spraying with 0.2% naphthoresorcinol in Me_2CO−3 N H_3PO_4 (5:1, v/v) and then heated at 105 °C for 5 min. d-Glucopyranose (Chemprosa Holding AG) was used as standard. The same method to identify the sugar moiety was used for compound **8**.

Acidic Hydrolysis of Compound 7. A mixture of compound **7** (15 mg) in 50% MeOH (7 mL) containing 5% HCl was heated in a boiling water-bath for 5 h. After cooling, the reaction mixture was poured into H_2O. The mixture was extracted with EtOAc. The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated to yield compound **3** (8 mg). The water phase was analyzed by silica gel HPTLC (Merck) developed with Me_2CO−2 mM NaOAc (17:3, v/v). The sugar was detected by spraying with 0.2% naphthoresorcinol in Me_2CO−3 N H_3PO_4 (5:1, v/v) and then heated at 105 °C for 5 min. D-Glucopyranose (Chemprosa Holding AG) was used as standard.

Lymphocyte Proliferation Test. The prepared spleen cells of mice (4×10^6) were seeded into each well of a 96-well microplate, and various concentrations of compounds **1**−**10** and 5 μg/mL of concanavalin A (Con A, from *Canavalia ensiformis* Type III, Sigma), selective stimuli on T cells, or lipopolysaccharide (LPS, from *Escherichia coli*, Sigma), selective stimuli on B cells, were added. The plates were cultured at 37 °C with 5% CO_2 in a humidified atmosphere for 48 h. For the last 6 h, each well was pulsed with 0.25 μCi/well ^3H-TdR (thymidine, [methyl-^3H], ICN Pharmaceuticals, Inc., Irvine, CA). The cells were harvested, and the radioactivity incorporated was counted by a liquid scintillation counter. All counts/min values shown are the mean of triplicate sample ± SD. Statistical analysis was carried out by the Student *t*-test. ConA or LPS was used as a positive control.[17] MTT assay was performed to evaluate the cytotoxicity of the compounds. Splenocytes were cultured in a 96-well plate at 4×10^5 cells/180 μL/well in a humidified CO_2 incubator at 37 °C for 48 h in the presence or absence of various concentrations of test compounds. An 18 μL portion of 5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to each well for the final 5 h. Then 90 μL of lysis buffer (10% SDS, 50% DMF, pH 7.2) was added to each well for 6−7 h, and the OD_{570} values were read by microplate reader (Bio-Rad, Model 550).

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