Immunosuppressive *ent*-Kaurene Diterpenoids from *Isodon serra*

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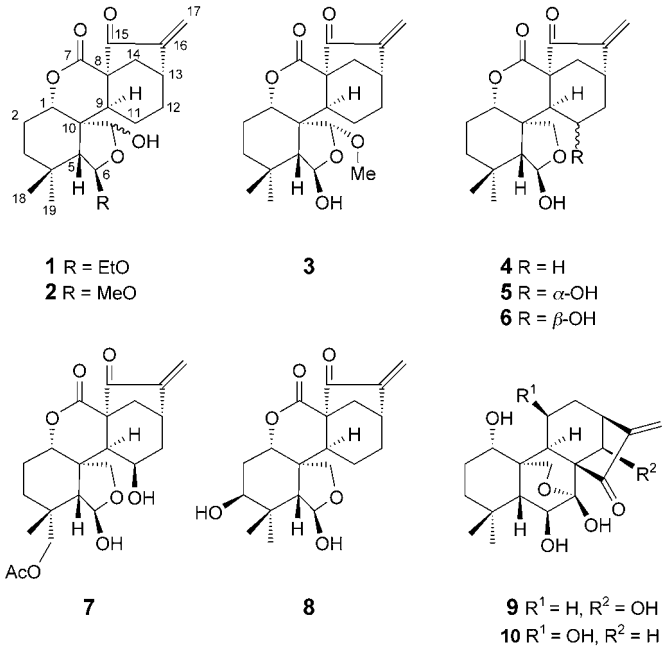
Three new enmein-type *ent*-kaurenoids, *i.e.*, the two pairs **1** and **2** of 20-epimers and the (20*R*)-isomer **3**, besides the seven known diterpenoids **4**–**10**, were isolated from the aerial parts of *Isodon serra*. Their structures were elucidated by spectroscopic techniques and X-ray diffraction. The immunosuppressive effect for T-lymphocytes proliferation induced by Con A in BALB/c mouse was evaluated for the isolates **1**–**10**. They all displayed a remarkable inhibitory effect, with multi-glycosides of *Tripterygium wilfordii* as positive reference substance (*Table 3*).

Introduction. – The genus *Isodon* is well known to abound in *ent*-kaurenoids, which have diverse bioactivities, especially antibacterial, anti-inflammatory, and antitumor activities [1–3]. But *ent*-kaurene diterpenoids used as immunosuppressors have not been reported so far. In the course of our search for immunosuppressive Chinese herbs, we reinvestigated the phytochemistry of *Isodon serra* (MAXIM.) HARA, a herb native to Jiangsu province, East China, which has been popularly used for acute jaundice hepatitis and acute cholecystitis in folk medicine [4]. As a result, the two new *ent*-kaurenoid pairs **1** and **2** of 20-epimers and the new (20*R*)-*ent*-kaurenoid **3** were obtained and characterized, along with five known analogues, *i.e.*, isodocarpin (**4**) [5–6], epinodosin (**5**) [7], nodosin (**6**) [8], carpalasionin (**7**) [9], and enmein (**8**) [10], and two diterpenoids devoid of oxygenation at C(20), *i.e.*, oridonin (**9**) [10][11] and lasiodonin (**10**) [9][12]. All isolates **1**–**10** were tested for their immunosuppressive effect for T-lymphocytes proliferation *in vivo*, and **1**–**10** displayed remarkable inhibitions to the tested cell induced by Con A in BALB/c mouse.

Results and Discussion. – Compound **1**, named serrin A, showed a single spot on TLC (silica gel) in several solvent systems and its purity was confirmed by its 1D ^{1}H- and ^{13}C-NMR spectra (*Tables 1* and *2*), measured immediately after dissolution in (D_5)pyridine. However, the NMR solution displayed two sets of very similar signals in the ratio 1:1 after subsequent 2D NMR experiments, indicating that the initially pure compound was transformed in solution to a pair of epimers. The structure of these epimers were determined by a combination of 1D and 2D NMR data (including HMQC, HMBC, and ROESY) and comparison with the literature data of similar structures. Although there were two sets of complex similar signals in the 2D NMR spectra, the data assignment of the two epimers could be achieved by means of the initially obtained well-resolved 1D NMR data of one epimer.



In the 1D NMR spectra of **1** (*Tables 1* and *2*), 2 Me groups at δ(C) 23.5 (q) and 33.2 (*q*) and δ(H) 1.05 and 1.06 (each *s*), a characteristic exocyclic methylene group conjugated with a ketone group at δ(C) 115.4 (*t*), 152.7 (*s*), and 197.5 (*s*) and δ(H) 5.41 and 6.11 (each *s*), and a typical lactone carbonyl group at 172.5 (*s*) were displayed, along with 5 CH_2 and 6 CH groups (including three oxygenated ones), 3 quaternary C-atoms, and an EtO group. Considering the compounds previously isolated from this plant [13–15], **1** was tentatively assumed to have an enmein-type *ent*-kaurenoid skeleton. Comparison of the ^{1}H-NMR and EI-MS data of **1** with those of compound **4** revealed the presence of a differently positioned OH and an additional EtO group in **1**. An *AB* pattern attributable to CH_2(20) in **4** was absent in **1** and replaced by a sharp *s* at δ(H) 6.31, indicating that C(20) of **1** was O-substituted. This was confirmed by a second hemiacetal C-Atom at δ(C) 101.1 (*d*) and the loss of 1 CH_2 group in the DEPT of **1** as compared to the data of **4**. Additionally, the HMBC cross-peak of the hemiacetal C(20) with H–C(5) and H–C(6), and of the EtO signal at δ(C) 62.3 (*t*) with H–C(6) verified the presence of an OH group at C(20) and of an EtO group at C(6). Careful analysis of the two sets of 2D NMR signals of the epimer pair allowed to establish full agreement with the above deductions.

Based on the ROESY correlation H–C(6)/Me(19), the β-configuration of substituents at C(6) of diterpenoids of this type has been established by our work and relevant publications [16], and, therefore, we concluded that two 20-hydroxy epimers were present rather than two 6-epimers.

According to the literature, the configuration at C(20) can be elucidated by ROESY correlations, *i.e.*, by H–C(20) (*s*)/Me(19) in the case of (20*S*) and by H–C(20) (*d*)/H–C(9) in the case of (20*R*) configuration, which was confirmed by X-ray diffraction. As expected, the *s* at δ(H) 6.31 (H–C(20)) in the 1D ^{1}H-NMR of **1** displayed a ROESY correlation with Me(19) but simultaneously also with H–C(9) (*d* at 6.24, J(20,OH) = 5.1 Hz) (see *Figs. 1* and *2*). These data confirmed that the mixture was a pair of 20-epimers. Moreover, H–C(20) gave rise to a *s* in the initial 1D ^{1}H-NMR, suggesting that the epimerization proceeded from (20*S*)-**1** to (20*R*)-**1** until a 1:1 equilibrium mixture was obtained.

Compound **2**, named serrin B, was also a pair of 20-epimers in pyridine solution under the conditions used for **1**. Careful inspection of the 1D and 2D NMR spectra of **2** and serrin A (**1**) revealed that the only difference between **1** and **2** was a MeO group at C(6) of **2** rather than an EtO group in **1** (see *Tables 1* and *2*).

Table 1. *^{1}H-NMR Data* ((D$_5$)pyridine) *of Compounds* **1**, **2**, *and* **3**. δ in ppm, J in Hz.

	1		**2**		**3**
	(20*S*)	(20*R*)	(20*S*)	(20*R*)	
H_β–C(1)	4.74 (t, J = 8.6)	4.74 (t, J = 8.6)	4.74 (t, J = 8.6)	4.74 (t, J = 8.6)	4.47 (dd, J = 10.8, 6.9)
CH_2(2)	1.62 (m)				1.98 (m)
CH_2(3)	1.29 (m), 1.43 (dt, J = 13.7, 3.5)				1.43 (t, J = 4.4), 1.31 (dt, J = 12.6, 4.4)
H_β–C(5)	2.24 (br. s)	2.16 (br. s)	2.25 (br. s)	2.14 (br. s)	2.33 (br. s)
H_α–C(6)	4.98 (br. s)	5.41 (br. s)	4.84 (br. s)	5.29 (br. s)	5.66 (br. s)
H_α–C(9)	3.32 (m)	2.98 (overlap)	3.28 (overlap)	2.46 (dd, J = 13.1, 5.0)	3.49 (dd, J = 13.1, 5.2)
CH_2(11)	1.63, 1.35 (2m)				2.25, 1.66 (2m)
CH_2C(12)	1.59, 1.32 (2m)				2.27 (overlap), 1.48 (m)
H_β–C(13)	2.99 (dd, J = 9.6, 4.3)	3.02 (dd, J = 8.7, 4.4)	2.97 (dd, J = 9.2, 4.0)	3.02 (dd, J = 9.0, 4.1)	2.90 (dd, J = 9.3, 4.4)
CH_2(14)	2.58 (d, J = 11.9), 2.05 (dd, J = 12.1, 4.4)	2.66 (d, J = 11.8)	2.57 (d, J = 11.8)	2.65 (d, J = 11.9)	2.53 (d, J = 11.9), 2.02 (overlap)
CH_2(17)	6.11, 5.41 (2s)	6.08, 5.43 (2s)	6.04, 5.31 (2s)	6.04, 5.38 (2s)	6.01, 5.26 (2s)
Me(18)	1.06 (s)	1.08 (s)	1.07 (s)	1.07 (s)	1.09 (s)
Me(19)	1.05 (s)	1.07 (s)	1.06 (s)	1.06 (s)	1.08 (s)
H–C(20)	6.31 (s)	6.24 (d, J = 5.1)	6.30 (s)	6.24 (d, J = 5.1)	5.56 (br. s)
$MeCH_2O$	3.87 (q, J = 7.0), 3.42 (q, J = 7.0), 1.09 (t, J = 7.0)	3.72 (q, J = 7.1), 3.42 (q, J = 7.1), 1.07 (t, J = 7.1)			
MeO			3.35 (s)	3.30 (s)	3.77 (s)

Table 2. *^{13}C-NMR Data* ((D$_5$)pyridine, 500 MHz) *of* **1**, **2**, *and* **3**. δ in ppm.

	1		**2**		**3**
	(20*S*)	(20*R*)	(20*S*)	(20*R*)	
H–C(1)	76.1	77.2	76.1	77.1	75.8
CH_2(2)	24.1	25.2	24.0	25.1	24.0
CH_2(3)	37.5	37.5	37.5	38.2	37.5
C(4)	31.3	31.4	31.5	31.3	31.3
H–C(5)	54.6	53.9	53.8	55.0	55.1
H–C(6)	103.8	106.1	105.7	108.0	99.6
C(7)	172.5	172.0	172.4	171.8	171.9
C(8)	56.1	57.0	56.1	57.0	56.1
H–C(9)	38.4	38.4	45.4	38.4	39.0
C(10)	50.7	51.9	51.9	55.0	51.6
CH_2(11)	20.7	19.4	20.7	19.3	20.7
CH_2(12)	30.2	30.8	29.9	29.9	30.0
H–C(13)	32.8	32.8	34.7	35.4	34.6
CH_2(14)	34.7	35.4	33.3	33.2	33.0
C(15)	197.5	201.1	197.5	201.1	197.5
C(16)	152.7	151.8	152.5	151.6	152.3
CH_2(17)	115.4	117.5	115.5	115.5	115.8
Me(18)	33.2	33.1	32.7	31.4	32.7
Me(19)	23.5	23.1	23.5	23.1	23.5
H–C(20)	101.1	105.0	101.3	105.2	108.7
$MeCH_2O$	62.3, 15.5	62.6, 15.6			
MeO			54.5	54.7	57.0

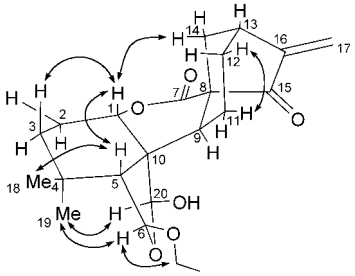


Fig. 1. *Key ROESY correlations of (20*S*)-serrin A* ((20*S*)-**1**)

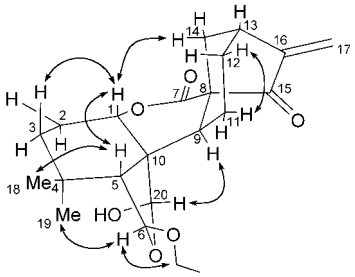


Fig. 2. *Key ROESY correlations of (20*R*)-serrin A* ((20*R*)-**1**)

The HMBC cross-peaks of C(6) of the two epimers of **2** at δ(C) 105.7 and 108.0 with the MeO groups at δ(H) 3.35 (*s*, 3 H) and 3.30 (*s*, 3 H), resp., verified the above deduction. Furthermore, the ROESY correlations of H–C(20) at δ(H) 6.30 (*s*) with Me(19) (*s*) and of H–C(20) at δ(H) 6.24 (*d*, J(20,OH) = 5.1 Hz) with H–C(9) at 2.46 (*dd*, J = 13.1, 5.0 Hz) again indicated the presence of 20-epimers, *i.e.*, of (20*R*)- and (20S)-**2**.

Compound **3**, named serrin C, had a quasi-molecular-ion peak at *m*/*z* 399.1778 (calc. 399.1778) in the HR-ESI-MS, consistent with the molecular formula $C_{21}H_{28}O_6$, as confirmed by its ^{1}H- and ^{13}C-NMR spectra (*Tables 1* and *2*, *Fig. 3*). Comparison of its NMR, especially 2D NMR spectra and those of serrin B (**2**), showed that **2** and **3** have very similar structures, except for two oxy-substituted positions. The structure of **3** established by the NMR data was confirmed by X-ray diffraction (*Fig. 4*).

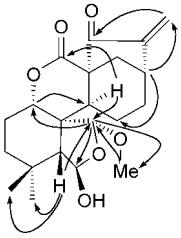


Fig. 3. *Key HMBC cross-peaks of* **3**

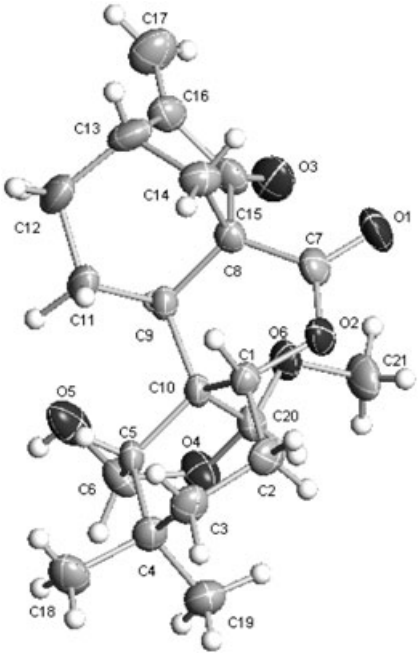


Fig. 4. *X-Ray crystal structure of* **3**

In the HMBC spectrum of **3** (*Fig. 3*), the MeO group at δ(H) 3.77 (*s*) showed a cross-peak with C(20) at δ(C) 108.7 (*d*) instead of the cross-peak MeO/C(6) of serrin B (**2**), suggesting a MeO group located at C(20) in **3**. Moreover, the presence of a different hemiacetal C-atom at δ(C) 99.6 (*d*) indicated another O-substituent (OH group) at C(6) of **3**. The configurations at C(6) and C(20) were established as β-orientation of OH and

(20*R*), respectively, by the ROESY correlations of H–C(6) at δ(H) 5.66 (br. *s*) with Me(19) at δ(H) 1.08 (*s*) and of H–C(20) at δ(H) 5.56 (*s*) with H_α–C(9) at 3.49 (*dd*, $J = 13.1, 5.2$), resp.

Bioactivity Evaluation. Compounds **1**–**10** were tested for their inhibition of T-lymphocytes proliferation induced by Con A in BALB/c mouse, by using multi-glycosides of *Tripterygium wilfordii* (MTW) as the positive reference substance with MTT methods. The results (*Table 3*) established that all tested compounds displayed remarkable inhibitory effects.

Table 3. *Immunosuppressive Activity of Compounds* **1**–**10** *for T-Lymphocytes Proliferation Induced by Con A in BALB/c Mouse*

	Concentration [mg/l]	*OD*	Livility [%]		Concentration [mg/l]	*OD*	Livility [%]
Blank		0.4775		**6**	10	0.0605	13.51
MTW	10	0.4490	94.49		20	0.0463	9.69
	20	0.3390	71.22		30	0.0420	8.80
	30	0.0730	15.39		40	0.0445	9.32
1	10	0.0203	4.24	**7**	10	0.2360	49.42
	20	0.0195	4.08		20	0.1405	29.42
	30	0.0230	4.82		30	0.0758	15.86
	40	0.0238	4.97		40	0.0778	16.20
2	10	0.0397	8.33	**8**	10	0.1880	39.37
	20	0.0377	7.91		20	0.1045	21.88
	30	0.0340	7.14		30	0.0663	13.87
	40	0.0393	8.12		40	0.0583	12.20
3	10	0.0727	15.27	**9**	10	0.0670	14.03
	20	0.0340	7.14		20	0.0415	8.69
	30	0.0343	7.21		30	0.0388	8.12
	40	0.0357	7.47		40	0.0640	9.01
4	10	0.0243	5.08	**10**	10	0.2350	49.21
	20	0.0243	5.08		20	0.0928	19.42
	30	0.0220	4.61		30	0.0660	13.82
	40	0.0220	4.61		40	0.0565	11.83
5	10	0.0770	16.18				
	20	0.0375	7.88				
	30	0.0327	6.86				
	40	0.0323	6.79				

Experimental Part

General. Column chromatography (CC) and TLC: silica gel (200–300 mesh) from *Qingdao Marine Chemical Factory*, Quingdao, People's Republic of China. Melting points: uncorrected; *SGW-X-4* apparatus. Optical rotations: *P-1030* digital polarimeter. UV Spectra: *Unico 2102-PCS* spectrometer; λ_{max} (log ε) in mn. IR spectra: *Bio-Rad-FTS-185* spectrometer; KBr pellets; $\tilde{\nu}$ in cm^{-1}. 1D and 2D NMR Spectra: *Bruker AM-400* and *DRX-500* instruments; (D_5)pyridine solns. with $SiMe_4$ as internal standard; δ in ppm, *J* in Hz. MS: *Aglient-5973* spectrometer.

Plant Material. The aerial parts of *Isodon serra* were purchased from the *Qunli Drugstore* in Shanghai, People's Republic of China, in March 2004. The authenticity of the sample was validated by Dr. *Zhao-hui Xu* of the Pharmacognosy Laboratory, and the voucher specimen (SJTU 04–03–01) has been deposited in the Herbarium of the School of Pharmacy, Shanghai Jiao Tong University.

Extraction and Isolation. The dried and powdered aerial plants (6 kg) were extracted with 75% acetone at r.t. for 5×3 h. The extract was evaporated and partitioned between AcOEt and H_2O. The AcOEt extract (85 g)

was subjected to CC (silica gel (200–300 mesh), petroleum ether/Me_2CO 1:0 → 0:1); *Fractions I–X*. *Fr. V* (28 g) and *VI* (17 g) were subjected to repeated CC (silica gel, $CHCl_3$/Me_2CO 15:1, 12:1, 9:1, and 4:2, $CHCl_3$/MeOH 500:1 and 300:1, and cyclohexane/Me_2CO 10:1, 9:1, and 4:1): **1** (38 mg), **2** (12 mg), **3** (16 mg), **4** (100 mg), **6** (3.7 g), **10** (27 mg), and **8** (240 mg). *Fr. III* (18 g) was purified by CC (silica gel, cyclohexane/acetone 10:1): **5** (14 mg), **7** (71 g), and **9** (210 mg).

Serrin A (= *(1α,6β,20*R*/20*S*)-1,7:6,20-Diepoxy-6-ethoxy-20-hydroxy-6,7-seco*-ent-*kaur-16-ene-7,15-dione*; **1**). Colorless needles. M.p. 214–216°. $[\alpha]_D^{25} = -158.5$ ($c = 0.26$, MeOH). UV (MeOH): 253 (2.13). IR (KBr): 3305, 2946, 2862, 1753, 1708, 1647, 1378, 1346, 1322, 1149, 1101, 1044, 999, 983, 951, 929. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*. EI-MS: 345 (13, $[M - EtO]^+$), 298 (25), 270 (26), 255 (25), 239 (14), 227 (14), 205 (16), 159 (15), 133 (22), 113 (100), 105 (45), 91 (62). HR-ESI-MS: 413.1935 ($[M + Na]^+$, $C_{22}H_{30}O_6^+$; calc. 413.1940).

Serrin B (= *(1α,6β,20*R*/20*S*)-1,7:6,20-Diepoxy-20-hydroxy-6-methoxy-6,7-seco*-ent-*kaur-16-ene-7,15-dione*; **2**). Colorless needles. M.p. 197–200°. $[\alpha]_D^{25} = -140.9$ ($c = 0.10$, MeOH). UV (MeOH): 252 (2.29). IR (KBr): 3507, 2962, 2941, 2870, 1755, 1710, 1646, 1454, 1317, 1280, 1168, 1139, 1085, 1047, 998, 985, 917. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*. ESI-MS: 377.2 ($[M + H]^+$). HR-ESI-MS: 399.1778 (M^+, $C_{21}H_{28}O_6^+$; calc. 399.1780).

Serrin C (= *(1α,6β,20*R*)-1,7:6,20-Diepoxy-6-hydroxy-20-methoxy-6,7-seco*-ent-*kaur-16-ene-7,15-dione*; **3**). Colorless needles. M.p. 229–232°. $[\alpha]_D^{25} = -190.1$ ($c = 0.10$, MeOH). UV (MeOH): 258 (1.83). IR (KBr): 3404, 3088, 2961, 2937, 1756, 1717, 1637, 1446, 1315, 1139, 1077, 1044, 1026, 947, 915. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*. EI-MS: 345 (5, $[M - MeO]^+$), 298 (41), 288 (32), 270 (100), 255 (58), 245 (36), 227 (36), 214 (17), 199 (14), 176 (26), 161 (21), 148 (28), 133 (32), 121 (22), 109 (36), 105 (45), 91 (52). HR-ESI-MS: 399.17781 (M^+, $C_{21}H_{28}O_6^+$; calc. 399.17787).

X-Ray Crystal Structure of **3**[1]). Colorless, transparent needles ($0.515 \times 0.208 \times 0.045$ mm); formula $C_{21}H_{28}O_6$, M_r 376.43; crystal system: orthorhombic; space group: $P2(1)2(1)2(1)$; Unit cell dimensions: $a = 6.7581(6)$ Å, $b = 15.0230(14)$ Å, $c = 19.0967(18)$ Å; volume: 1938.8(3) Å^3; $Z = 4$, $d = 1.290$ mg/m^3, max. and min. transmission: 1.00000 and 0.70821.

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[1]) Supplementary crystallographic data for this paper can be obtained free of charge *via* http://www.ccdc.cam.ac.uk/conts/retrieving.htlm (or from the *Cambridge Crystallographic Data Centre*, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).