Differential Anti-angiogenic Activities of Acetal and Ketal Andrographolide Derivatives in *in vitro* HUVEC and in *in vivo* Zebrafish

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[†] These authors contributed equally to this paper.

Contents:

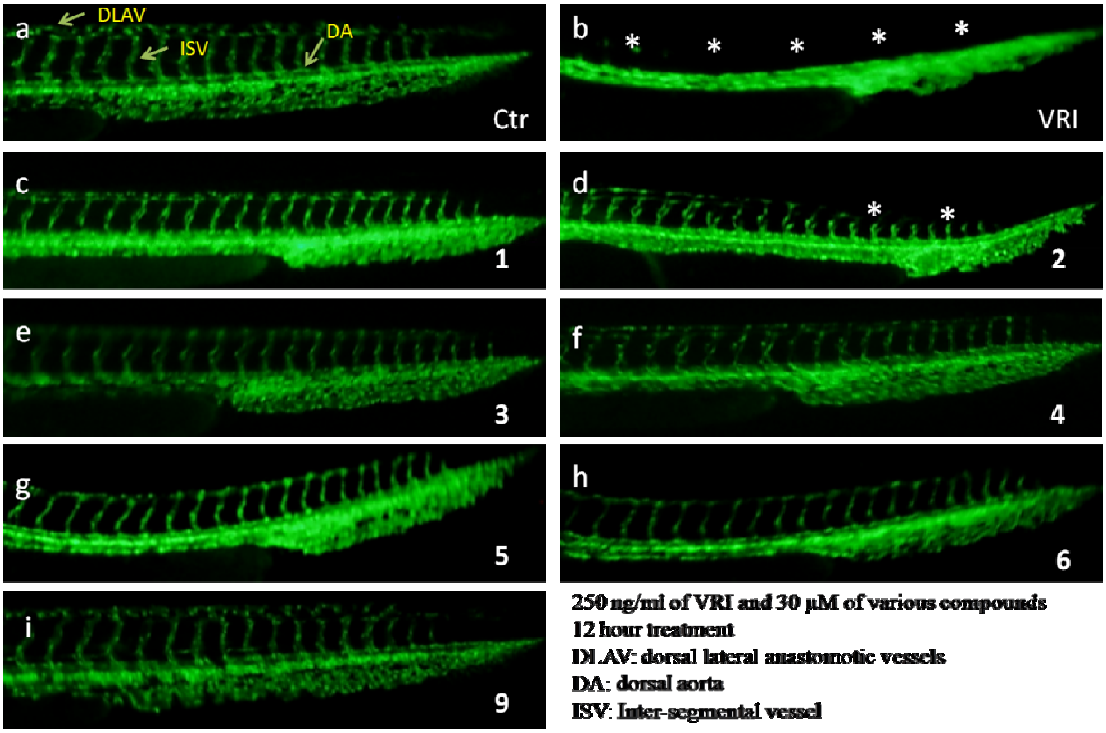


Figure s1. *In vivo* Anti-angiogenic effect of ketal **2** on zebrafish blood vessel formation compared with andrographolide (**1**) and various derivatives at 30 μM.

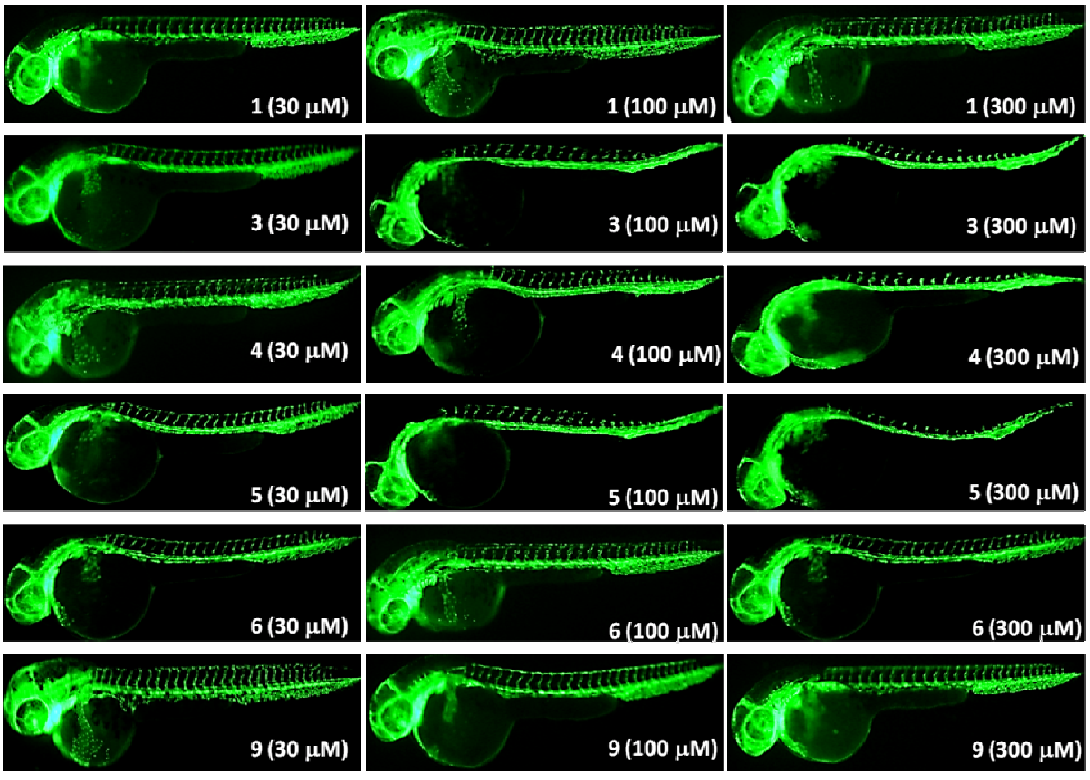


Figure s2. *In vivo* Anti-angiogenic effect of **1**, **3**, **4**, **5**, **6** and **9** on zebrafish blood vessel formation.

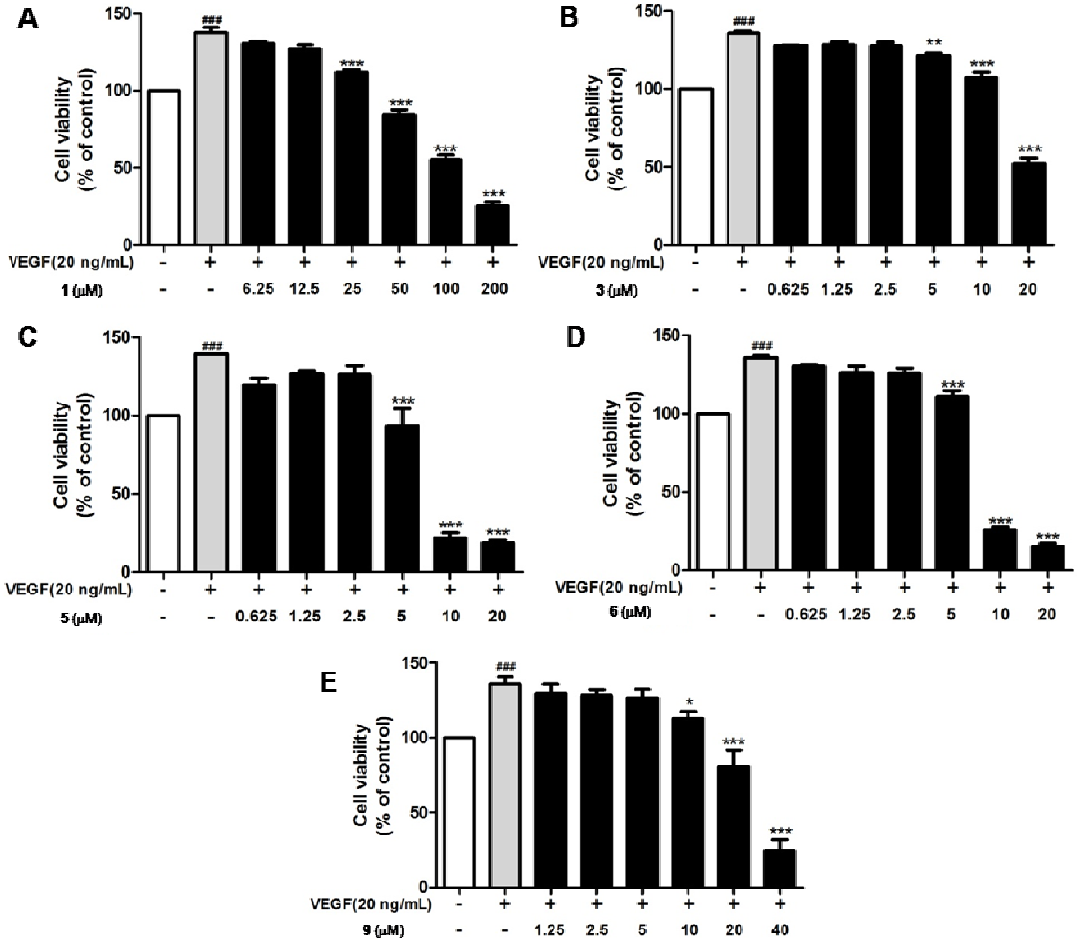


Figure s3. Effects of **1** (A), **3** (B), **5** (C), **6** (D) and **9** (E) on VEGF-induced HUVECs Proliferation Assays.

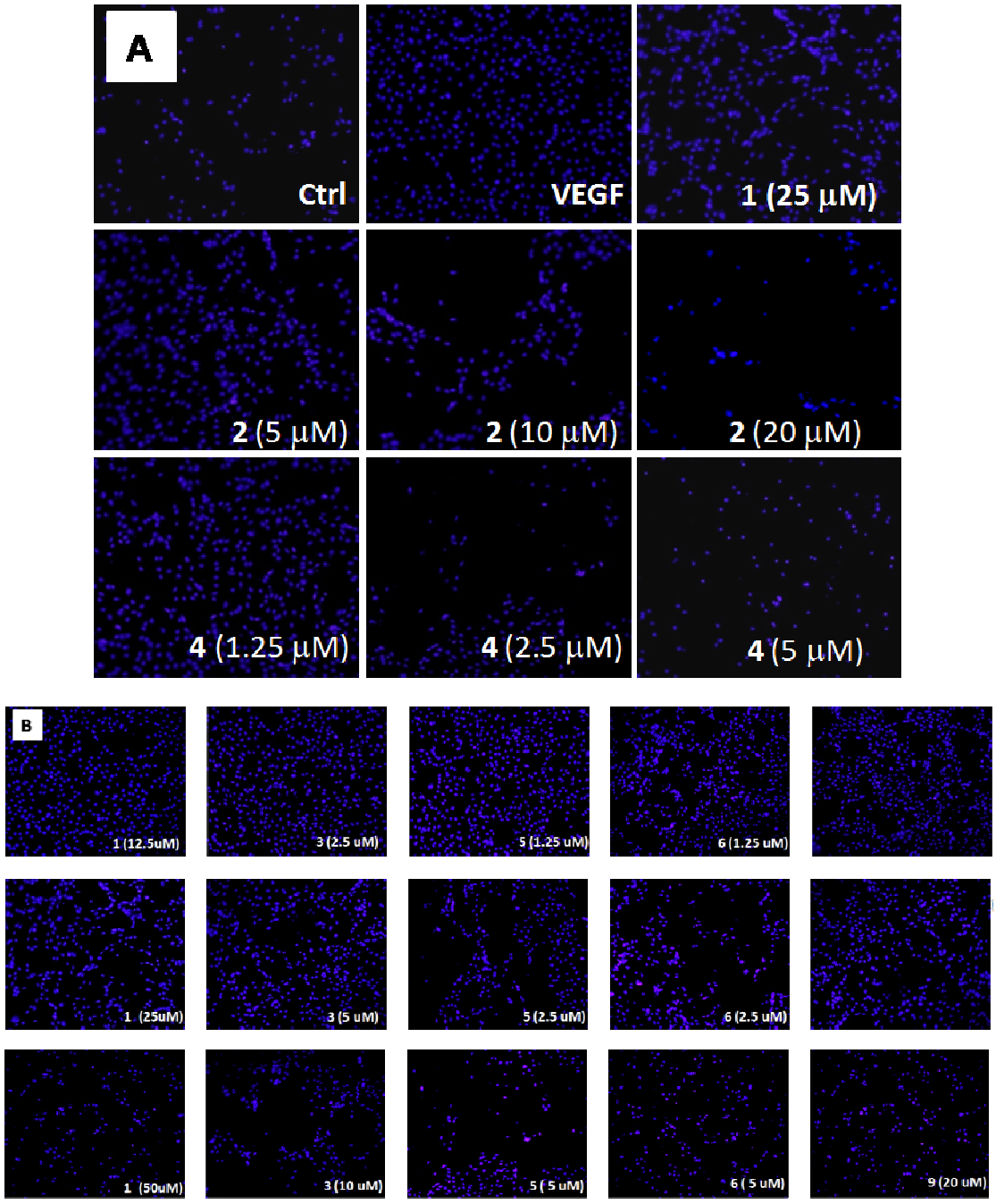


Figure s4. Effects of **2** and **4** (A), **1**, **3**, **5**, **6** and **9** (B) on VEGF-induced HUVECs transwell migration assay.

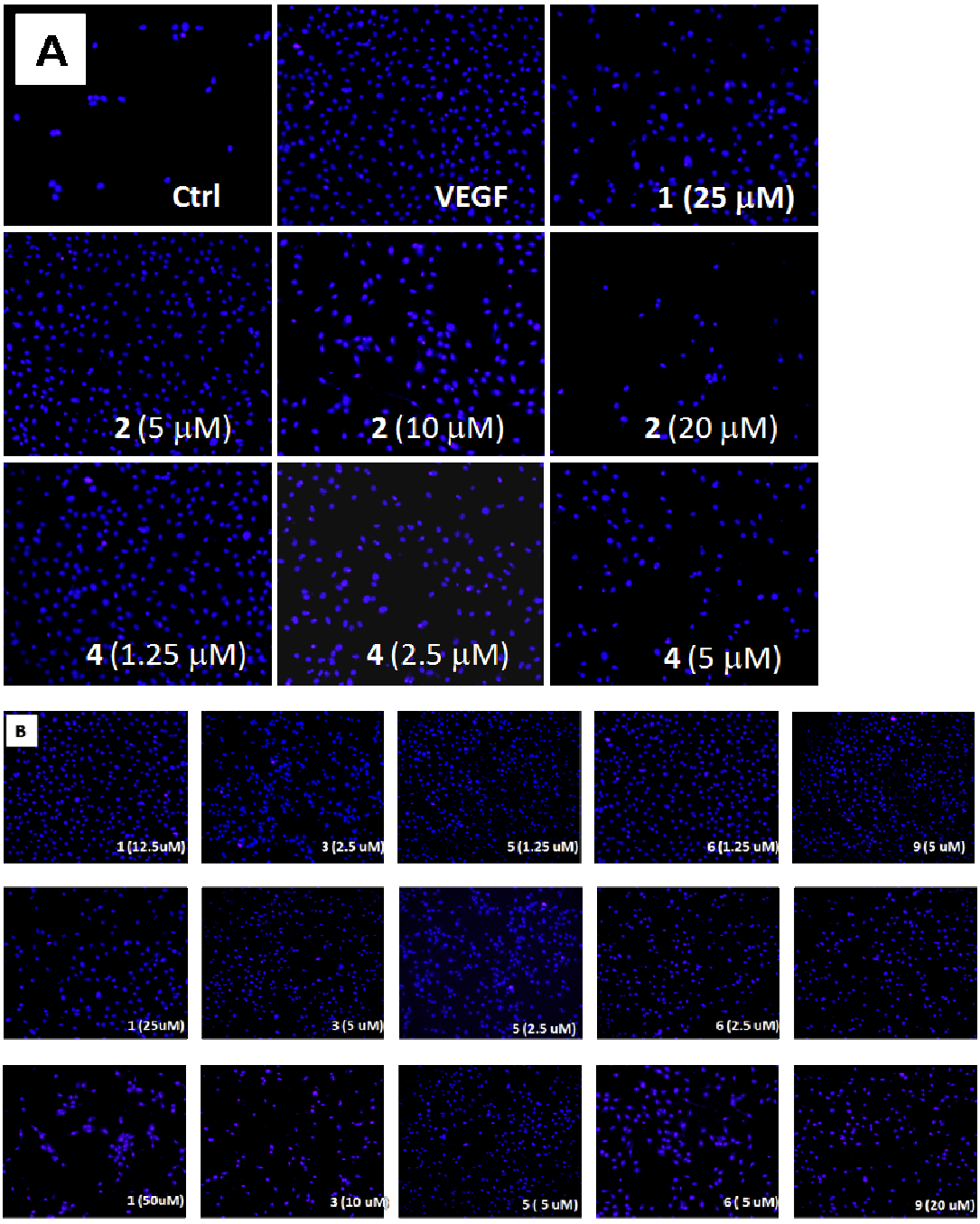


Figure s5. Effects of **2** and **4** (A), **1**, **3**, **5**, **6** and **9** (B) on VEGF-induced HUVECs transwell invasion assays.

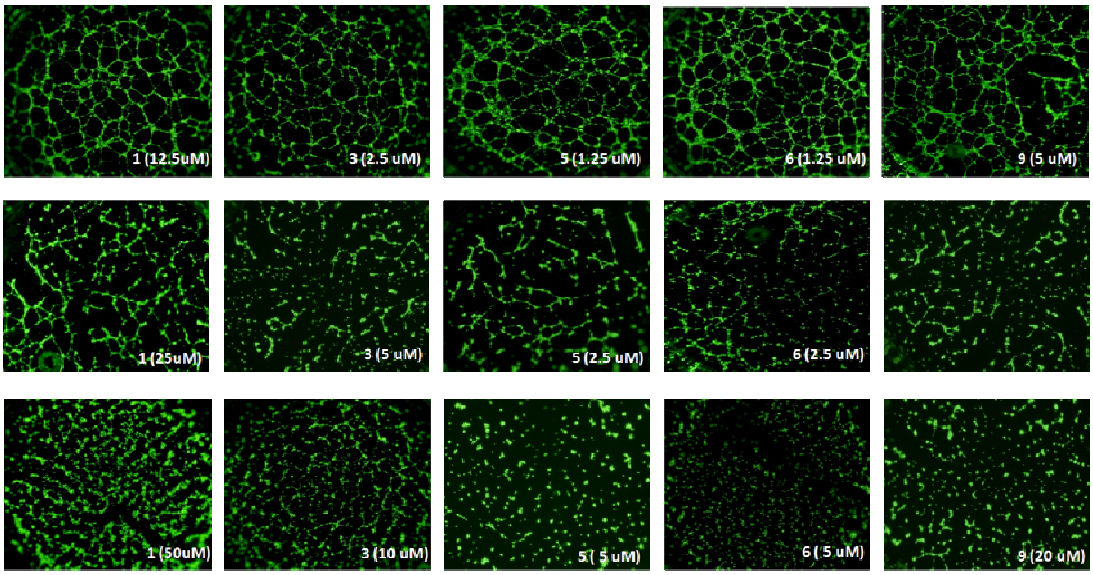


Figure s6. Effect of **1**, **3**, **5**, **6** and **9** on VEGF-induced HUVECs tube formation assays.

HPLC analysis of 2 and 4 in HUVEC media and zebrafish media by C18 Phenomenex Gemini 5 µm, 250 x 4.6 mm, 80% methanol and 20% H_2O, rate = 0.8mL/min

HUVEC Cell culture medium formulation: F-12 K medium, 100 ug/ml Heparin, 30 ug/ml ECGS (E-2759 Sigma), 10% FBS, 1% P/S, (PH 7.2).

Zebrafish embryo medium formulation: 0.58 g/L NaCl, 0.027 g/L KCl, 0.097 g/L $CaCl_2 \cdot H_2O$, 0.163 g/l $MgCl_2 \cdot 6H_2O$, (PH 7.2).

References of 1, 2, 3 and 4 and media controls (Figure s7)

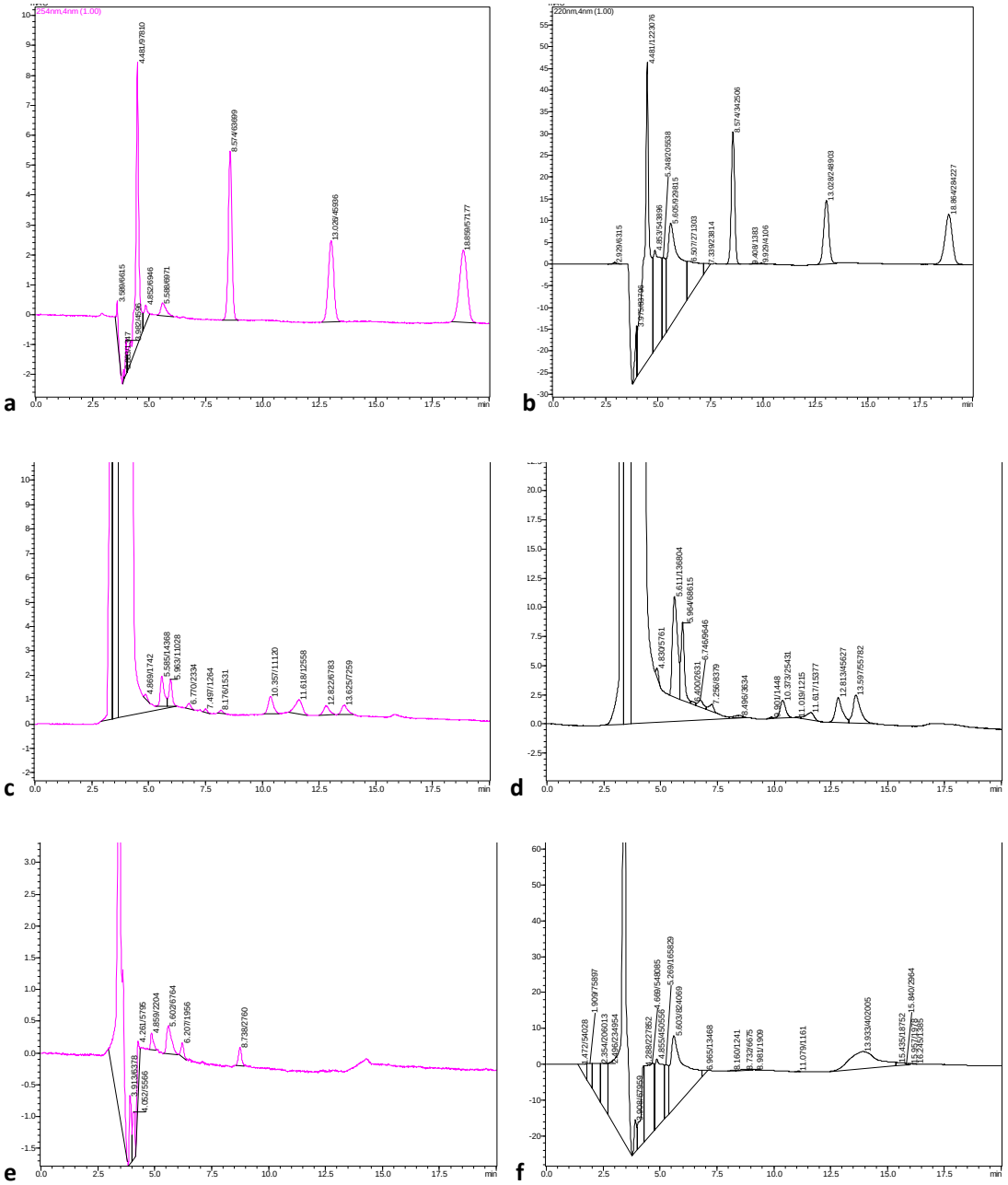
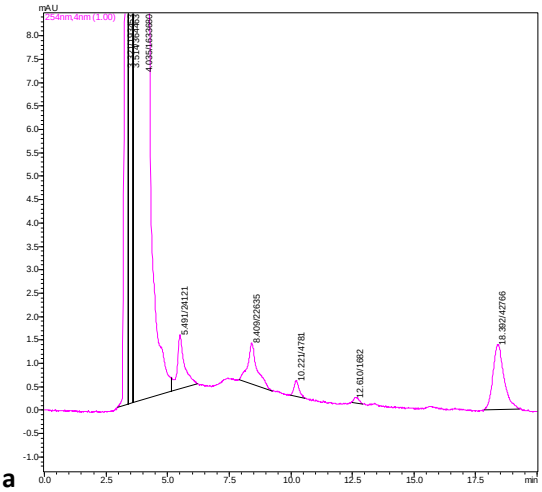


Figure s7. HPLC analysis of references of **1**, **2**, **3** and **4** and controls of HUVEC media and zebrafish media. The mixture (~10 µM for **1** and 20 µM for **2**, **3** and **4**) of **1** (4.481 min), **3** (8.574 min), **4** (13.02 min) and **2** (18.859 imn) at 254 (a) and 220 nm (b). HUVEC cell culture medium control at 254 (c) and 220 nm (d). Zebrafish embryo medium control at 254 (e) and 220 nm (f).

2 in HUVEC media (Figure s8): initial final concentration of 2 is ~ 20 µM



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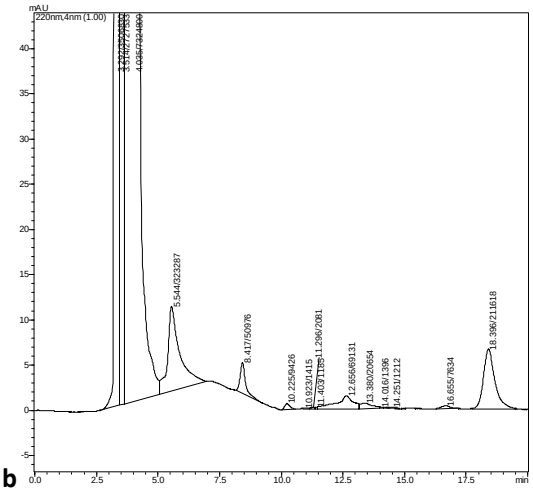

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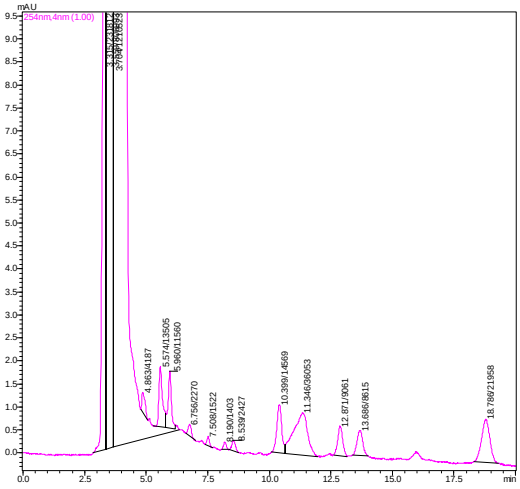

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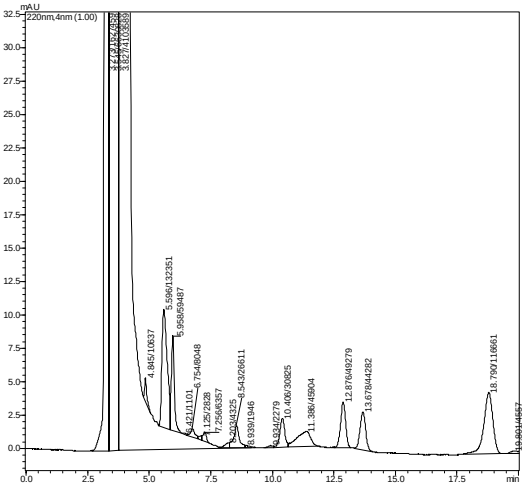

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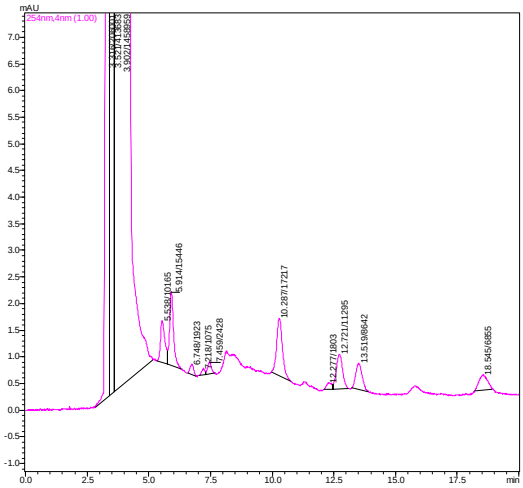

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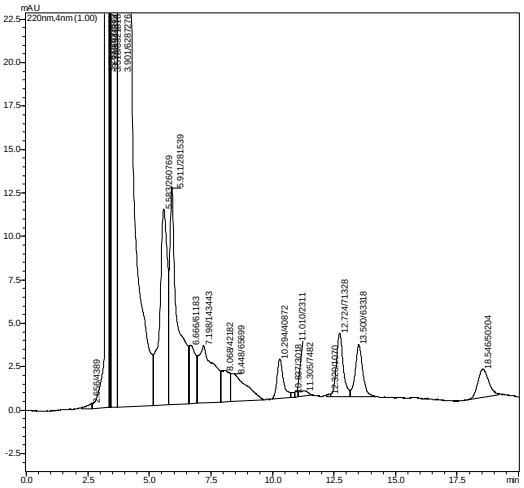

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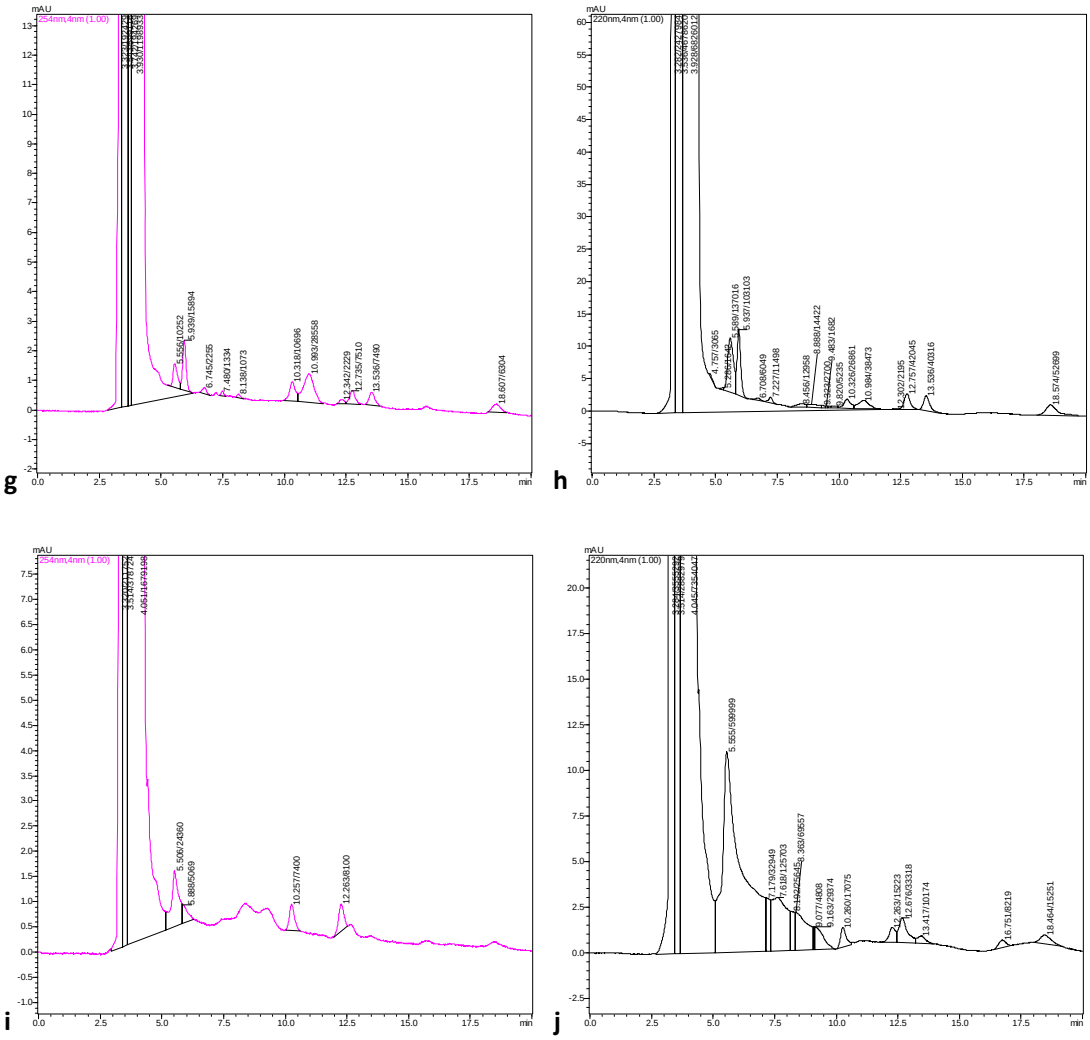
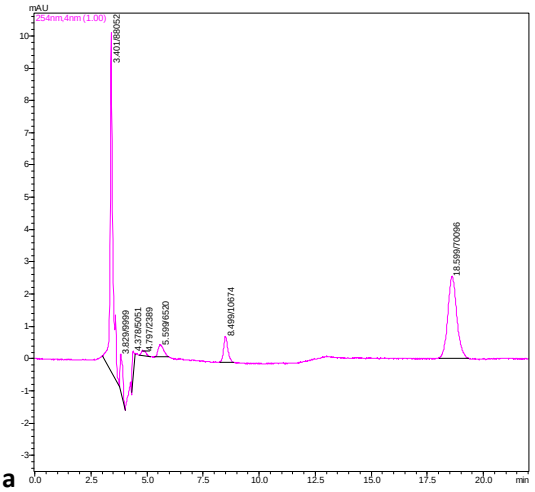


Figure s8. 2 in HUVEC media for 0.5 h at 254 (a) and 220 nm (b). **2** in HUVEC media for 3 h at 254 (c) and 220 nm (d). **2** in HUVEC media for 8 h at 254 (e) and 220 nm (f). **2** in HUVEC media for 16 h at 254 (g) and 220 nm (h). **2** in HUVEC media for 22 h at 254 (i) and 220 nm (j).

Conclusion: 2 (RT ~18.5 min) was not stable for long time in HUVEC media but it was not decomposed completely in 22 h (Figures s8i and s8j). Since none of **3** (RT ~8.5 min) was observed in HUVEC media (Figure s8), **1** should be formed but **1** (RT ~4.5 min) was overlapped with component/s of HUVEC media (Figure s8).

2 in zebrafish media (Figure s9): initial final concentration of 2 is ~ 20 µM



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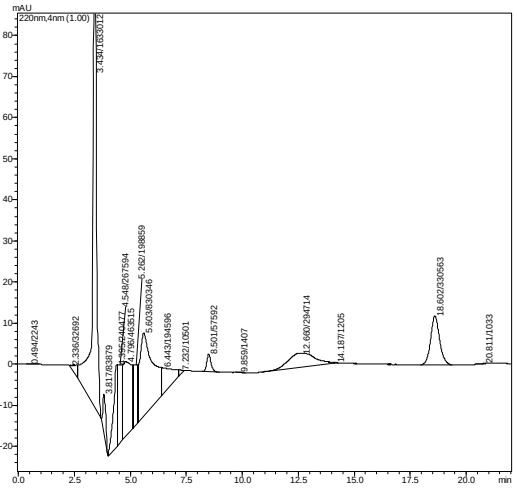

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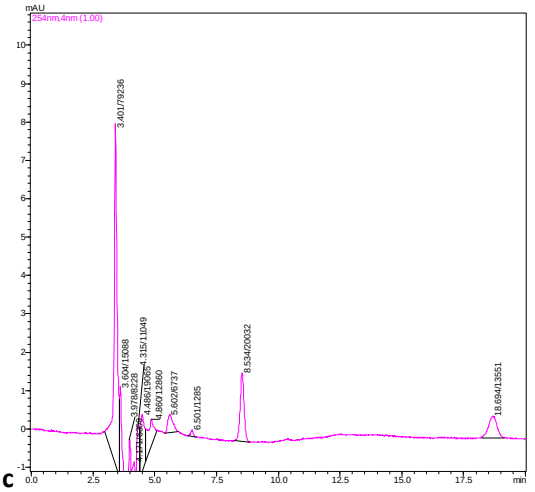

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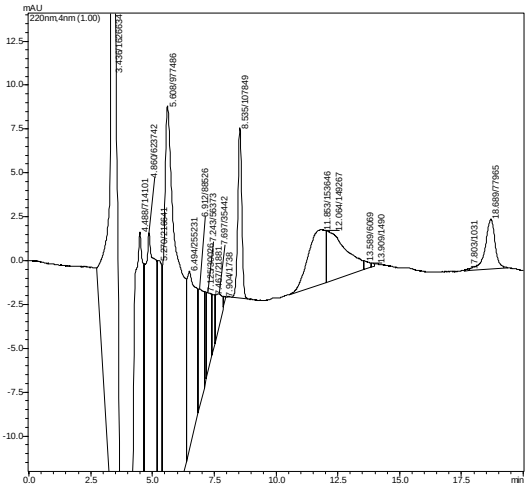

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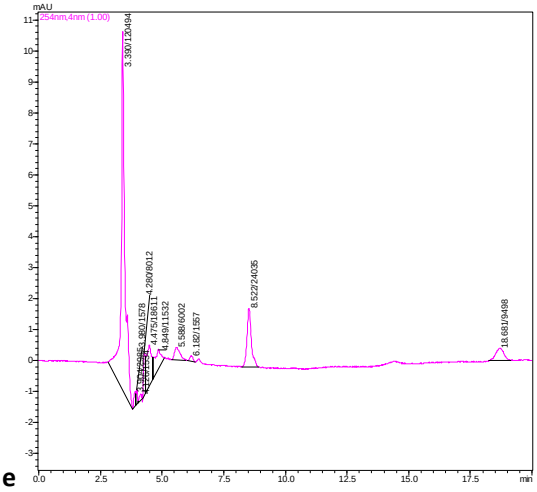

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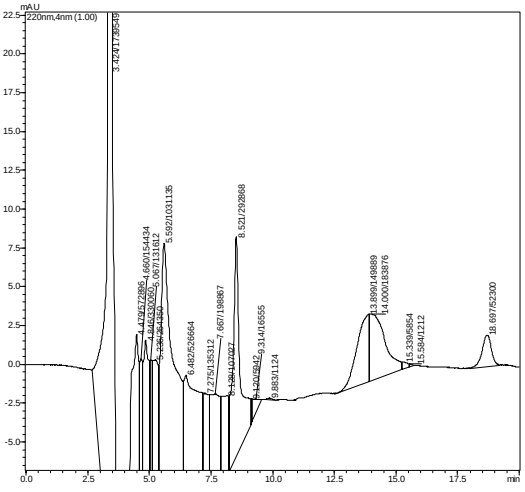

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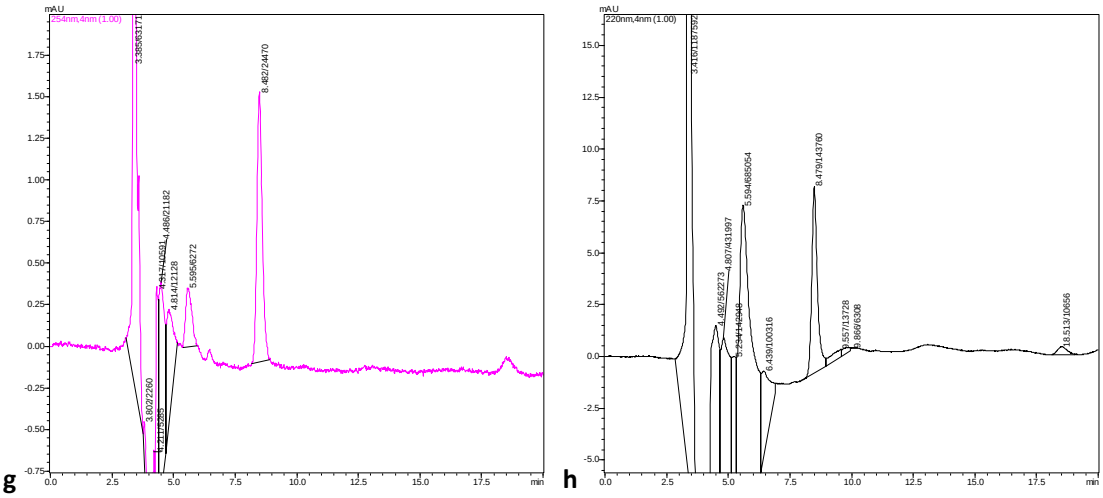
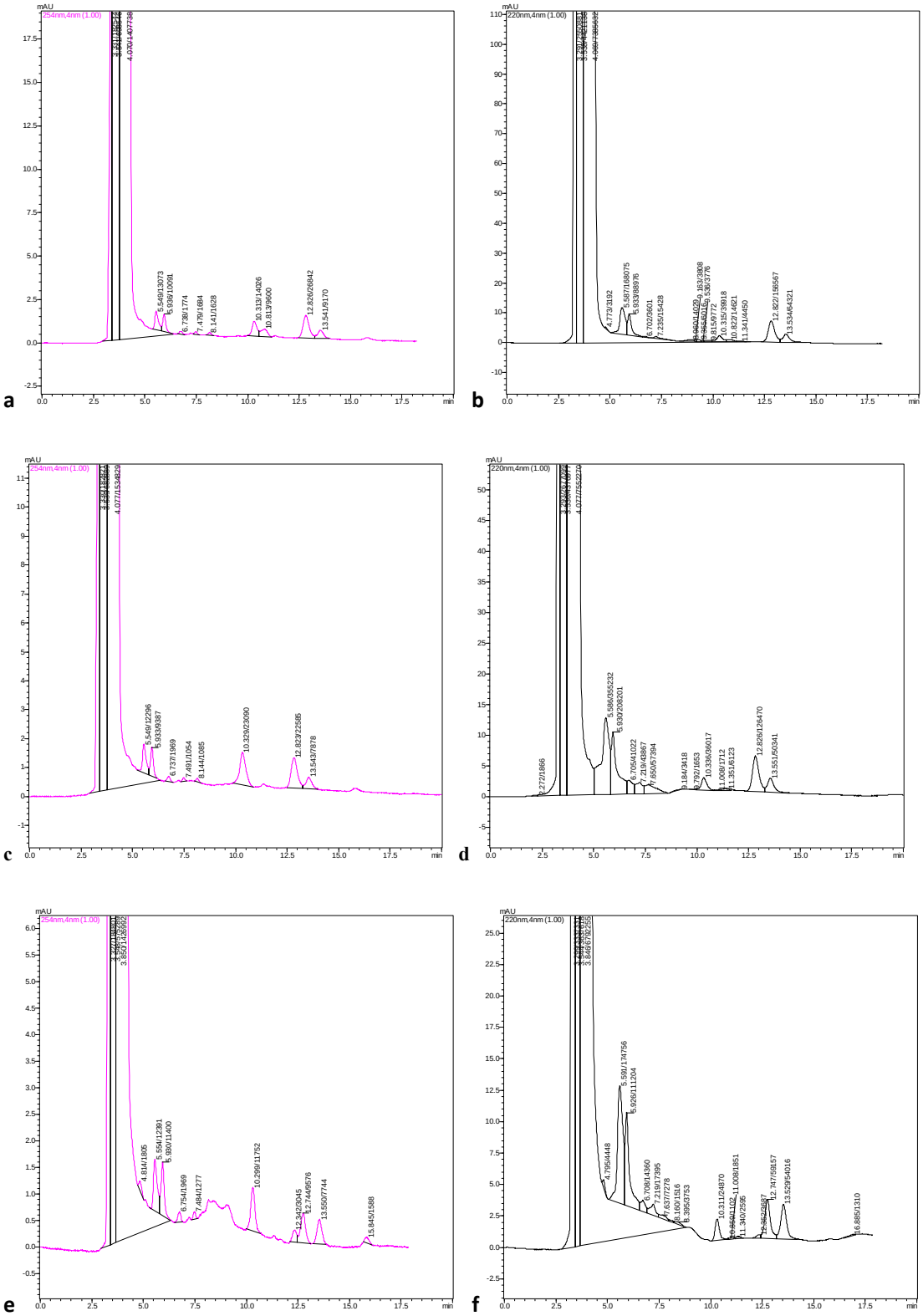
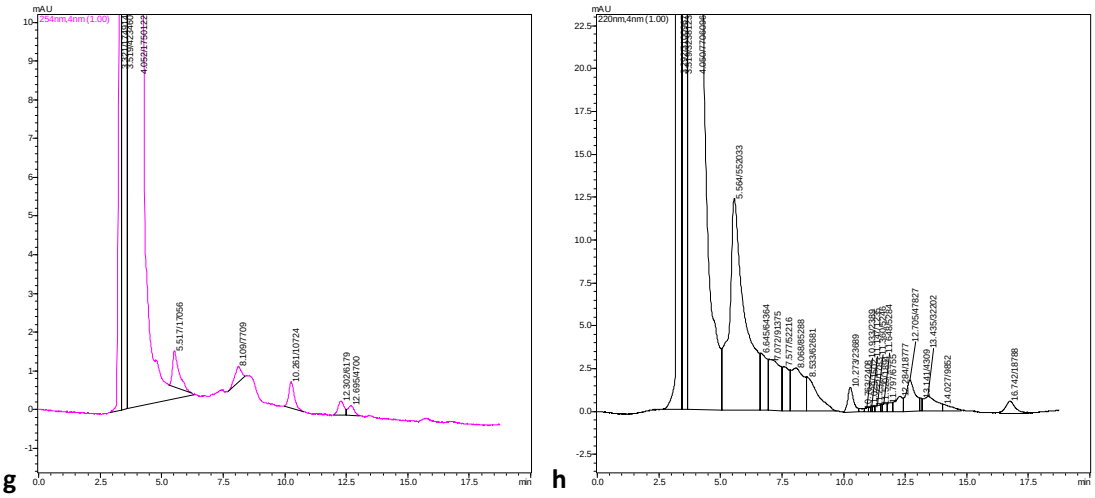


Figure s9. 2 in zebrafish media for 1 h at 254 (a) and 220 nm (b). **2** in zebrafish media for 6 h at 254 (c) and 220 nm (d). **2** in zebrafish media for 10 h at 254 (e) and 220 nm (f). **2** in zebrafish media for 21 h at 254 (g) and 220 nm (h).

Conclusion: 2 (RT ~18.5 min) was decomposed gradually in zebrafish media (Figure s9) and a small amount of **2** was left in 21 h (Figures s9g and s9h); whereas, none of **1** was obviously observed around 4.5 min (Figure s9) but **2** was quantitatively transformed into **3** in zebrafish media (Figure s9).

4 in HUVEC media (Figure s10): initial final concentration of 4 is ~ 20µM





g h

Figure s10. **4** in HUVEC media for 1 h at 254 (a) and 220 nm (b). **4** in HUVEC media for 5 h at 254 (c) and 220 nm (d). **4** in HUVEC media for 12 h at 254 (e) and 220 nm (f). **4** in HUVEC media for 22 h at 254 (g) and 220 nm (h).

Conclusion: Since the peak of **4** (RT ~12.8 min) was totally overlapped with component/s from HUVEC media, it is difficult to distinguish **4** from media but it is referable from the integrated area comparing Figure s10 with Figures s7c and s7d. **4** was also unstable for long time in HUVEC media while none of **3** was obviously observed and **1** should be formed and overlapped with component/s of HUVEC media, which are coincident with **2** in HUVEC media.

4 in zebrafish media (Figure s11): initial final concentration of 4 is ~ 20µM

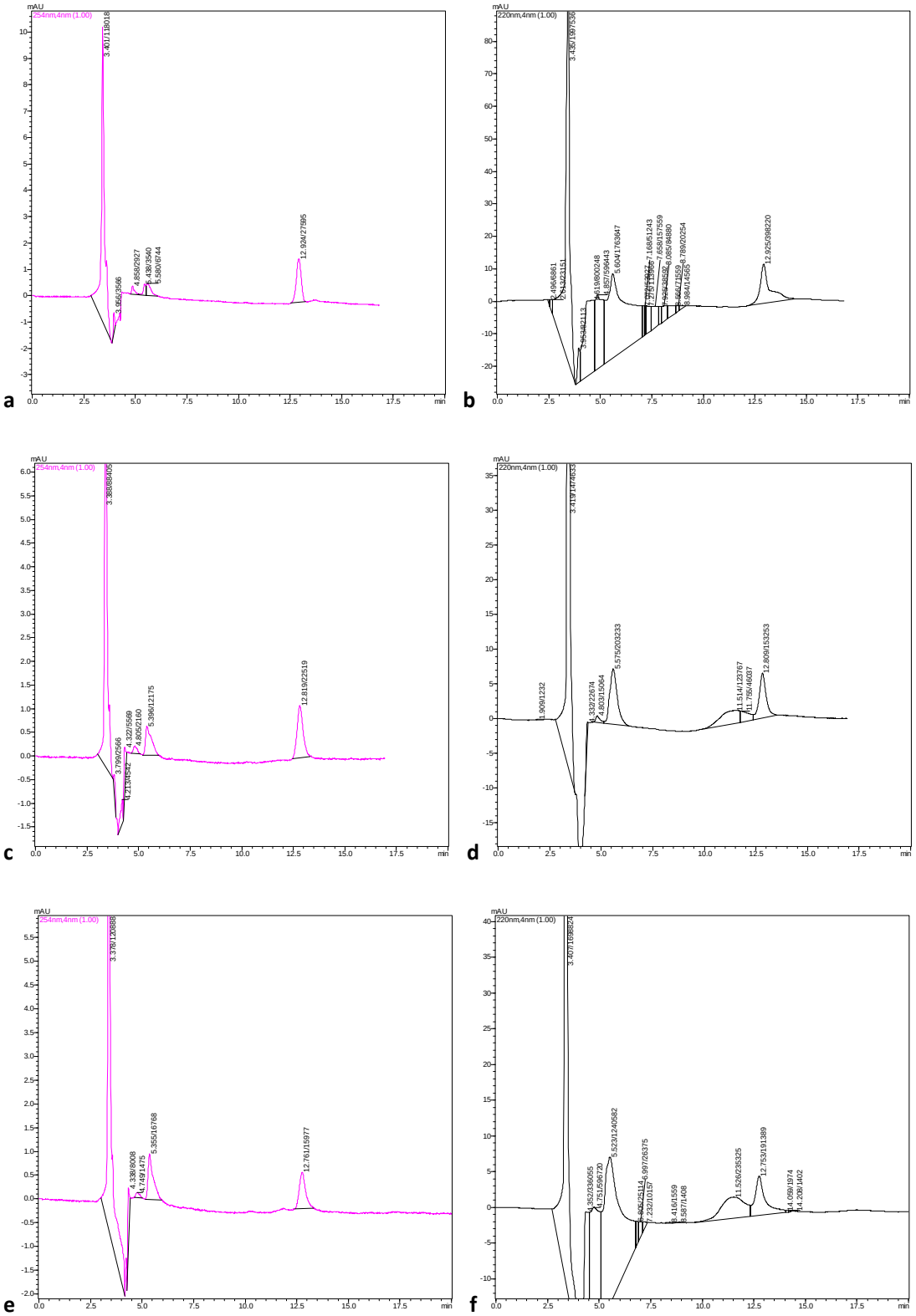


Figure s11. 4 in zebrafish media for 1.5 h at 254 nm (a) and 220 nm (b). **4** in zebrafish media for 8 h at 254 (c) and 220 nm (d). **4** in zebrafish media for 23 h at 254 (e) and 220 nm (f).

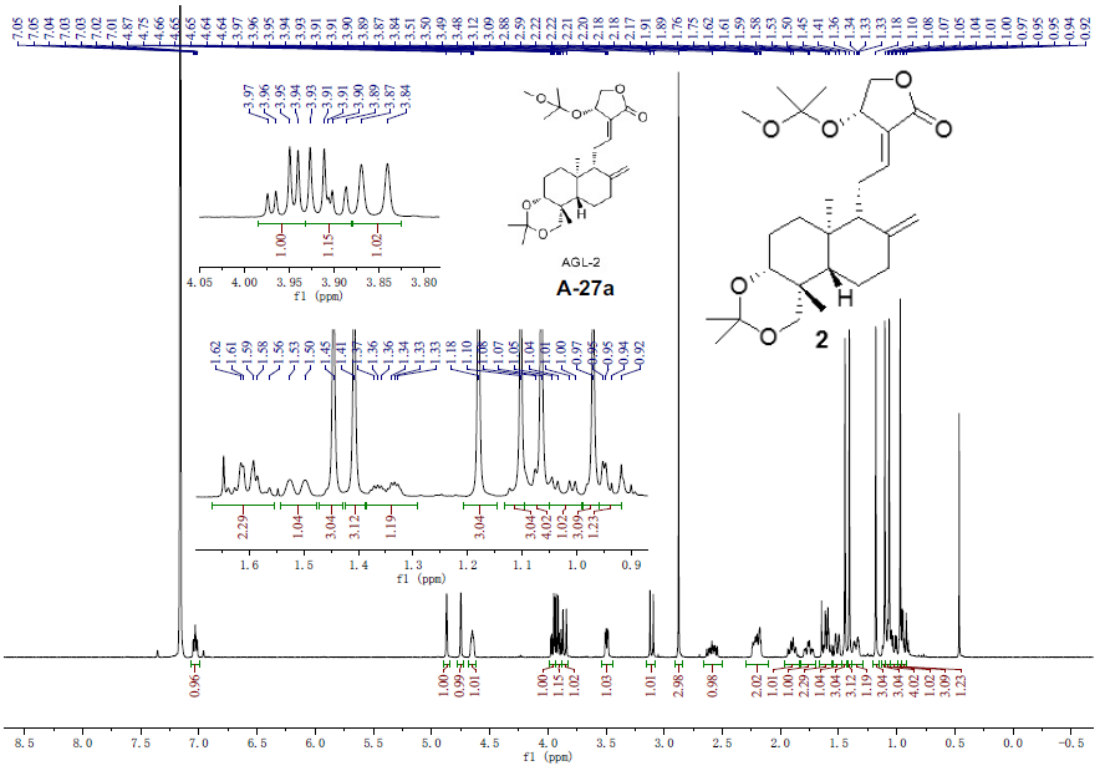
Conclusion: 4 was stable in zebrafish media and none of **1** and **3** was observed.

Table s1. Remaining percentages (%) of migrated or invasive HUVECs or branched chords to VEGF control group

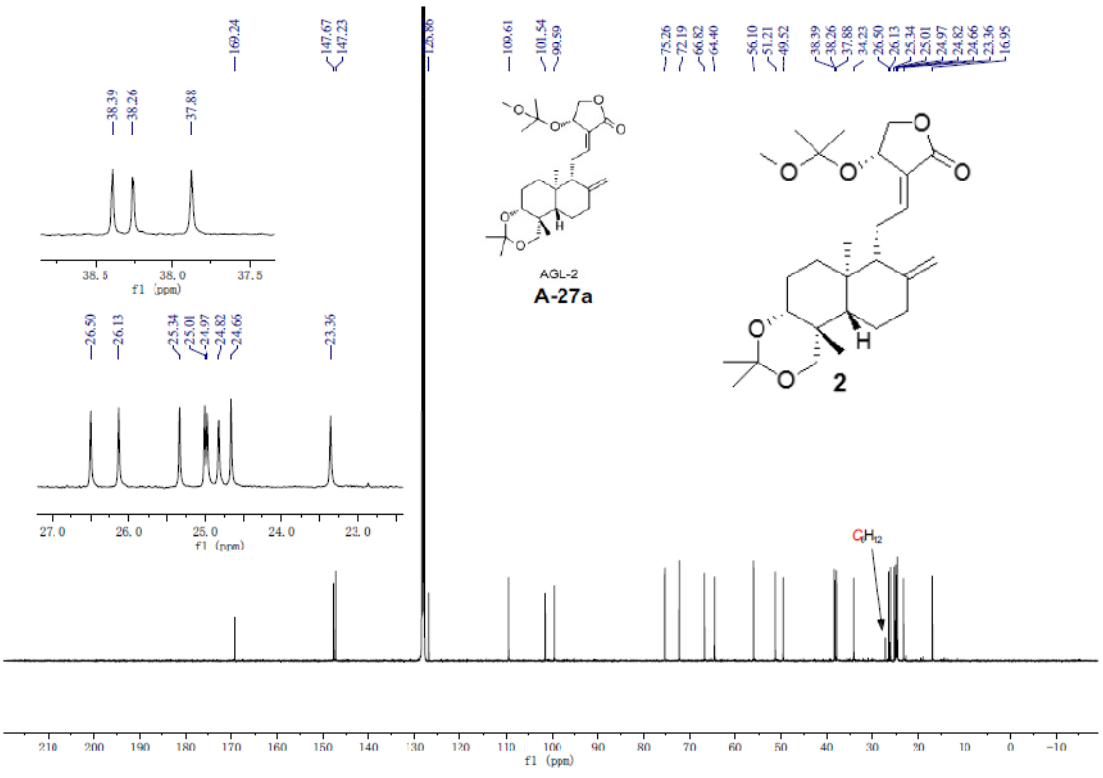
effect	entry	cmpd	concentration (μM)					
			1.25	2.5	5.0	10	20	25
migration	1	**1**	/	/	/	/	/	81.2
	2	**2**	/	/	98.3	65.7	17.4	/
	3	**3**	/	95.4	85.6	40.0	/	/
	4	**4**	96.4	46.2	9.0	/	/	/
	5	**5**	98.5	51.8	12.4	/	/	/
	6	**6**	96.5	45.0	29.6	/	/	/
	7	**9**	/	/	98.2	39.1	31.4	/
invasion	8	**1**	/	/	/	/	/	44.6
	9	**2**	/	/	99.2	44.5	9.7	/
	10	**3**	/	96.5	45.9	14.3	/	/
	11	**4**	94.1	46.2	20.1	/	/	/
	12	**5**	99.1	87.4	44.1	/	/	/
	13	**6**	97.3	93.7	40.8	/	/	/
	14	**9**	/	/	99.5	60.3	32.3	/
tube formation	15	**1**	/	/	/	/	/	31.3
	16	**2**	/	/	98.8	38.7	3.3	/
	17	**3**	/	98.8	6.6	1.2	/	/
	18	**4**	99.0	49.5	1.1	/	/	/
	19	**5**	98.3	45.5	1.3	/	/	/
	20	**6**	96.7	47.6	1.5	/	/	/
	21	**9**	/	/	99.1	5.3	1.2	/

Compound 2 (^{1}H NMR, ^{13}C NMR, HPLC)

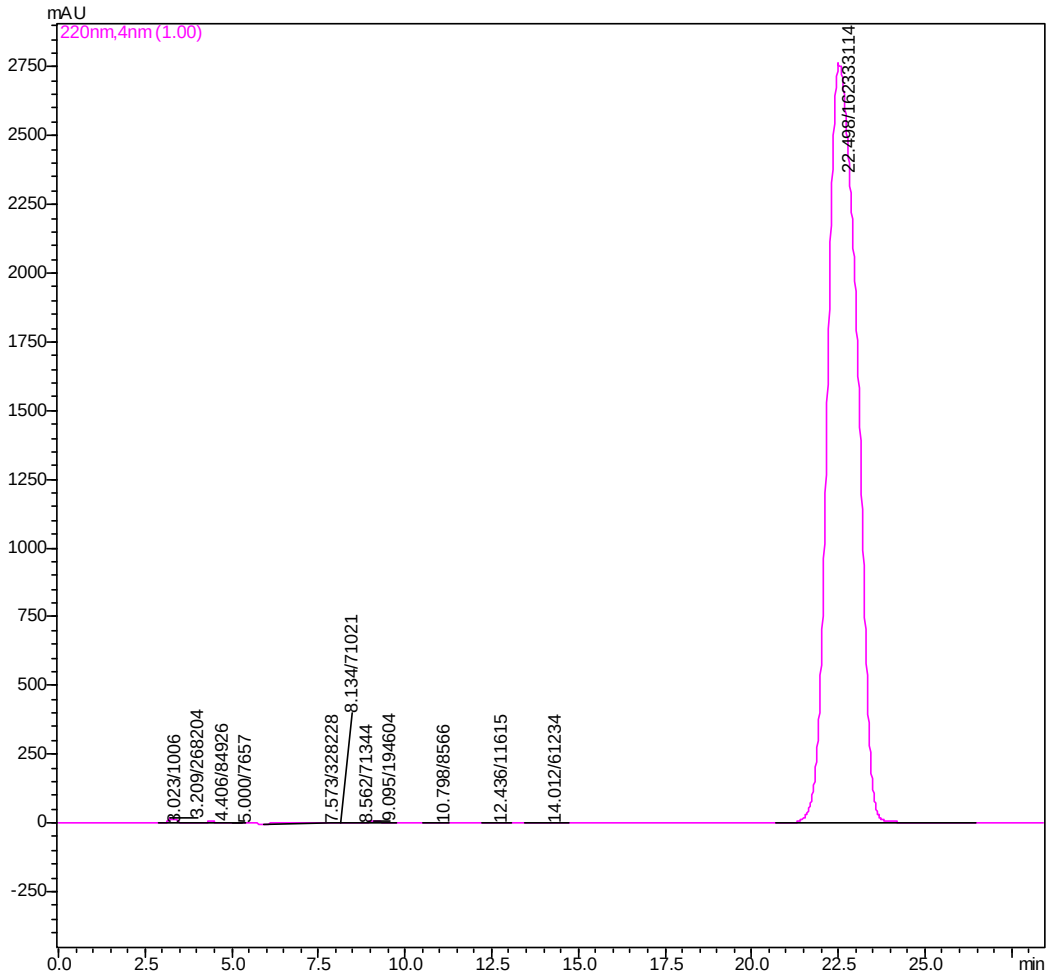
^{1}H NMR of 2:



^{13}C NMR of 2:

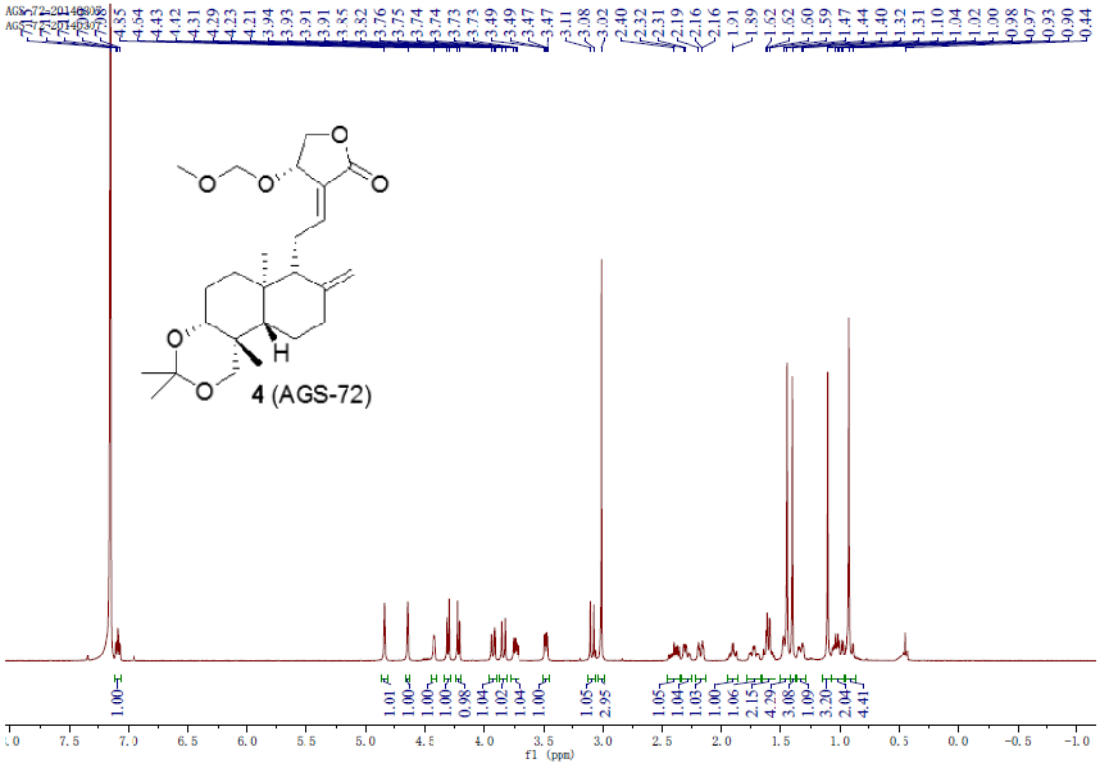


HPLC of 2 (HPLC purity ≧98%)**:**

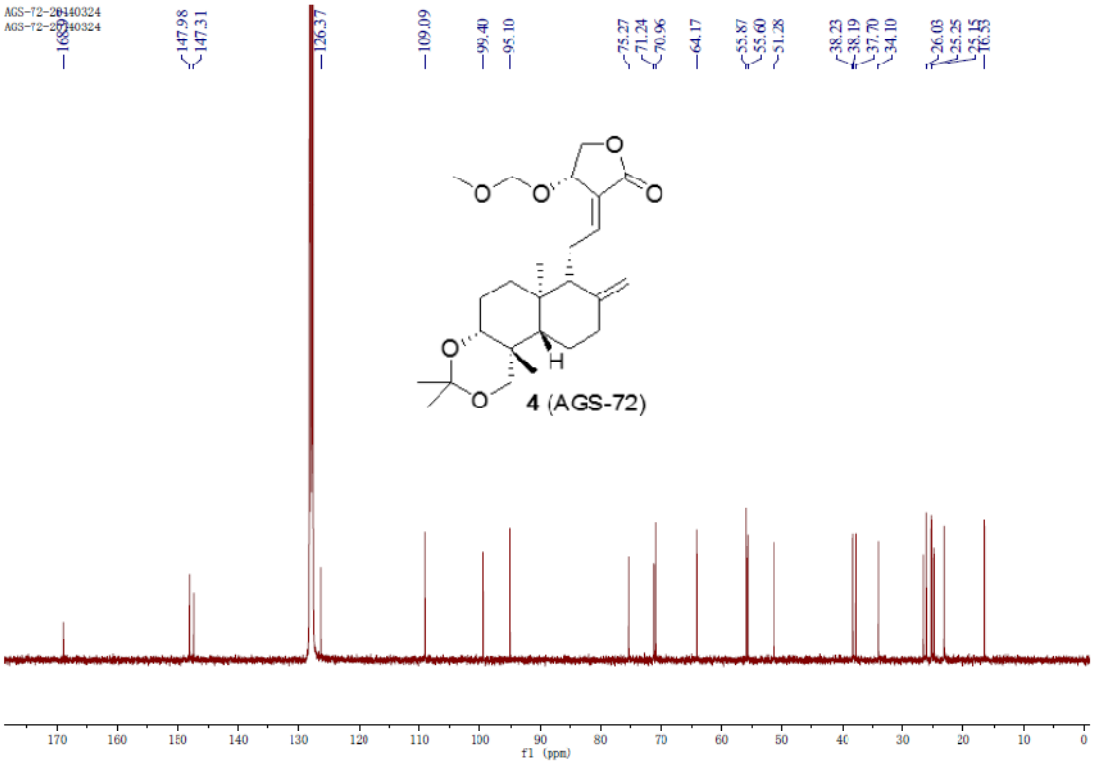


Compound 4 (^{1}H NMR, ^{13}C NMR, HPLC)

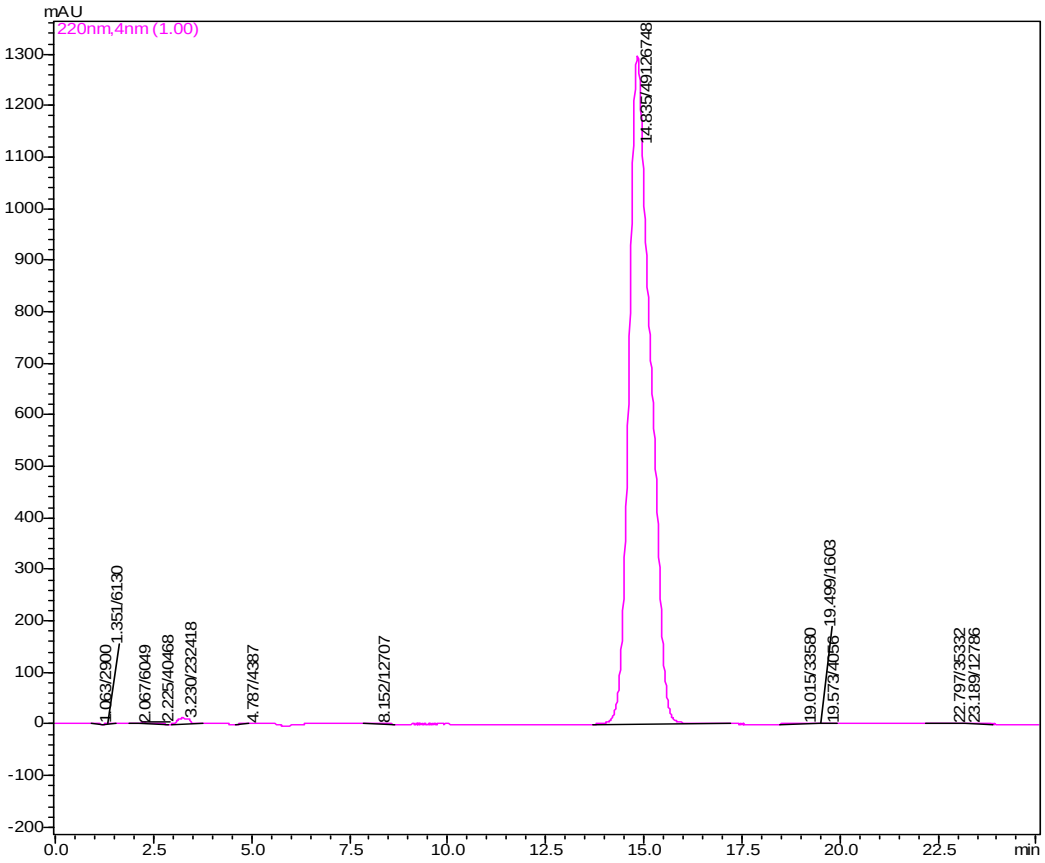
^{1}H NMR of 4:



^{13}C NMR of 4:



HPLC of 4 (HPLC purity ≧98%)**:**



Compound 5 (^{1}H NMR, ^{13}C NMR, HPLC)

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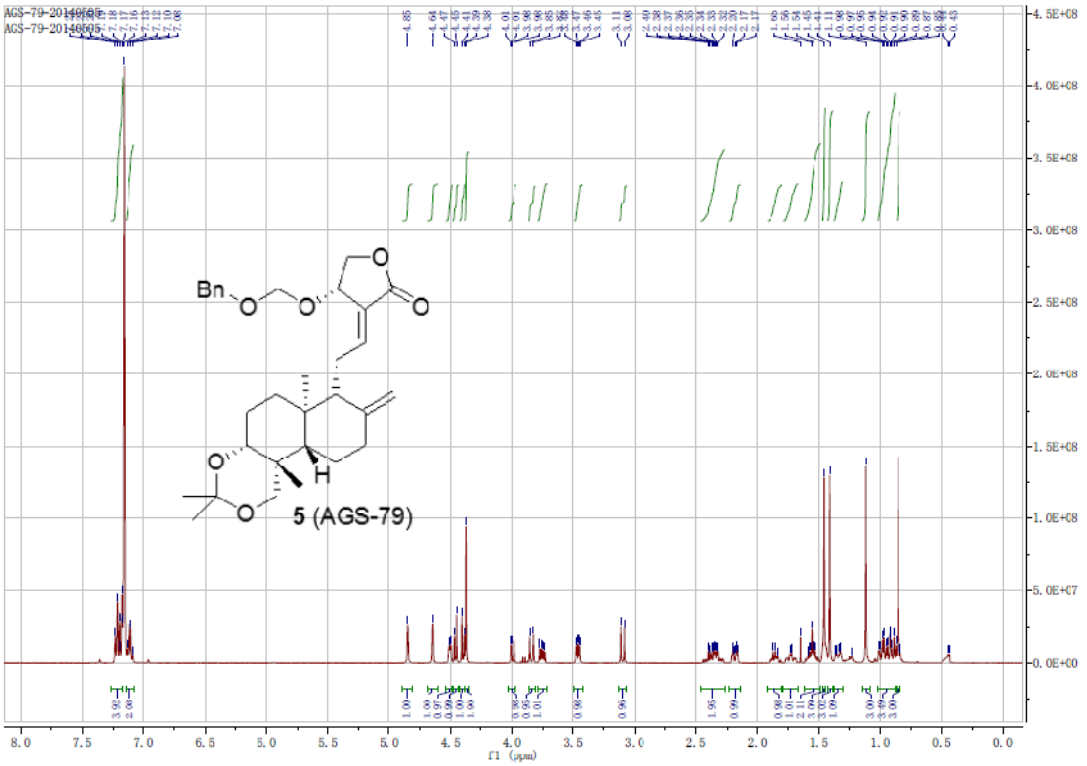
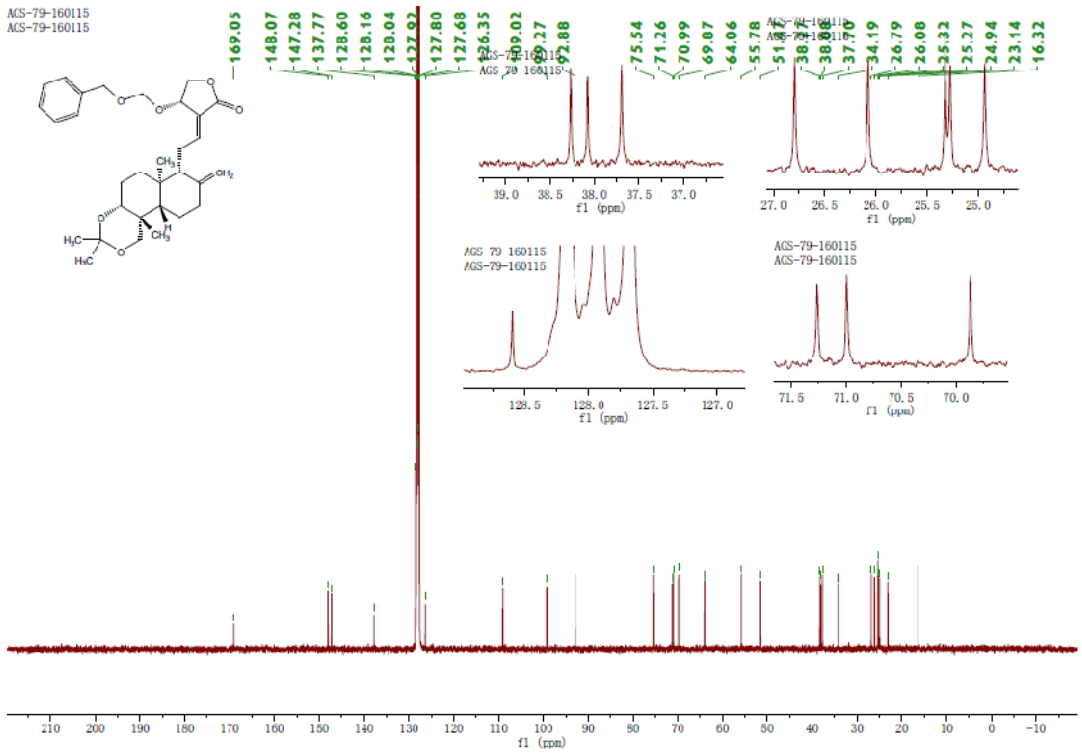
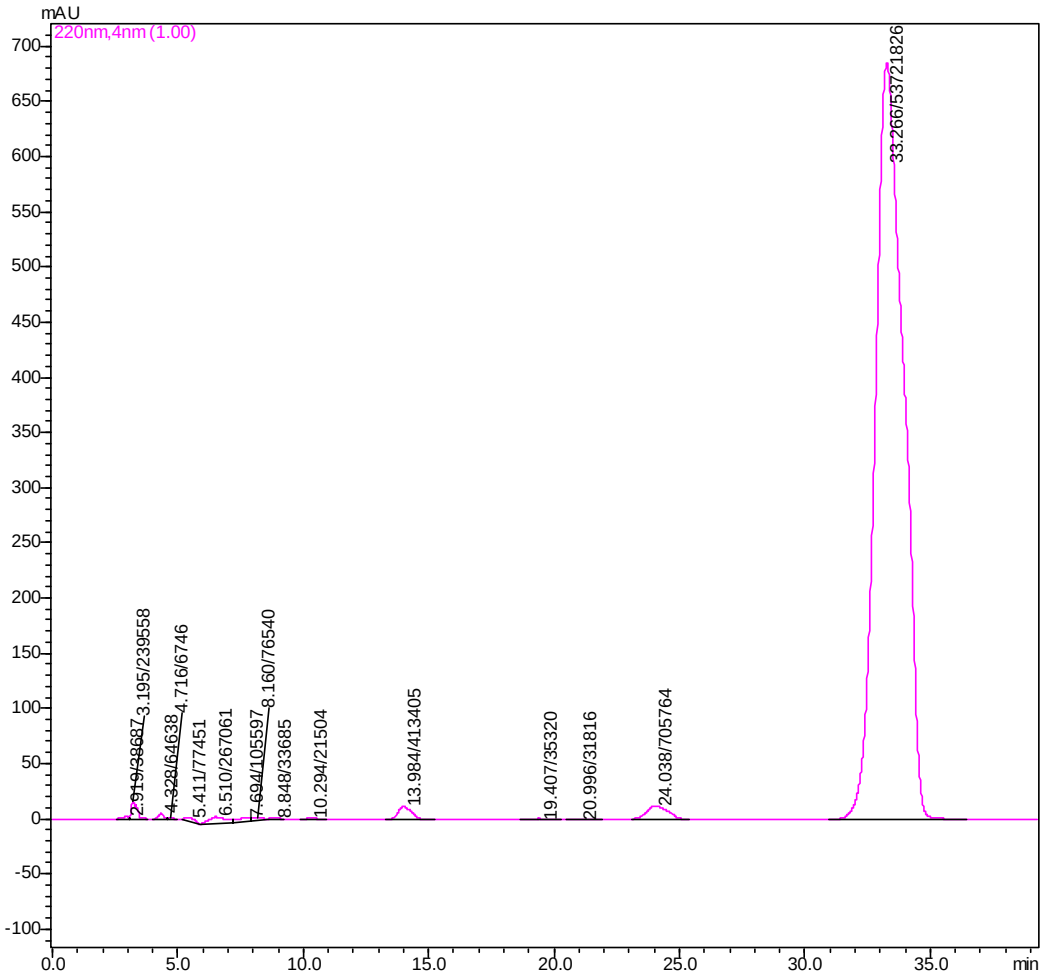


^{13}C NMR of 5:

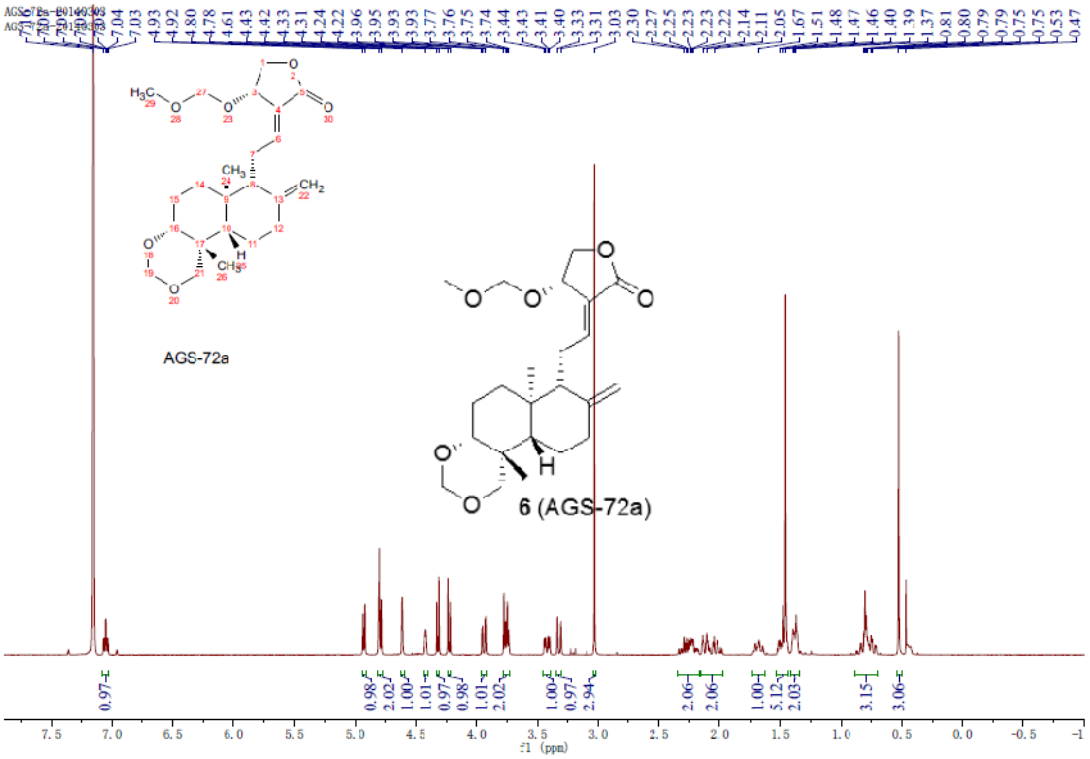


HPLC of 5 (HPLC purity ≧98%)**:**

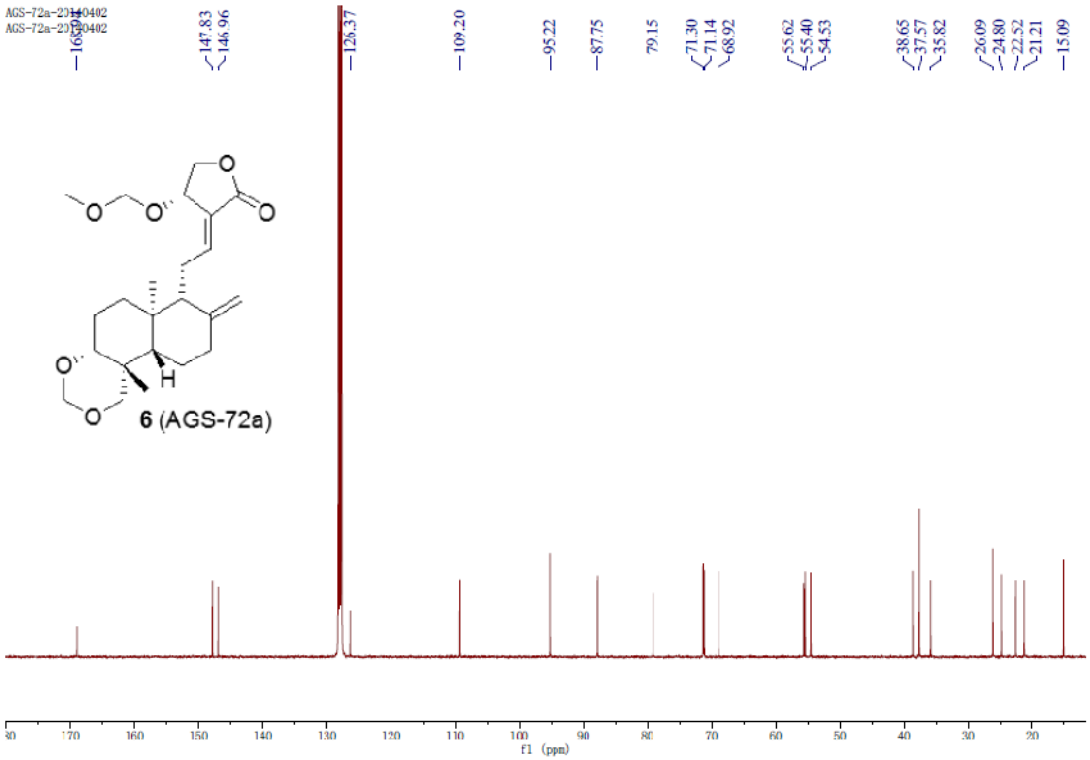


Compound 6 (^{1}H NMR, ^{13}C NMR, HPLC)

^{1}H NMR of 6:



^{13}C NMR of 6:



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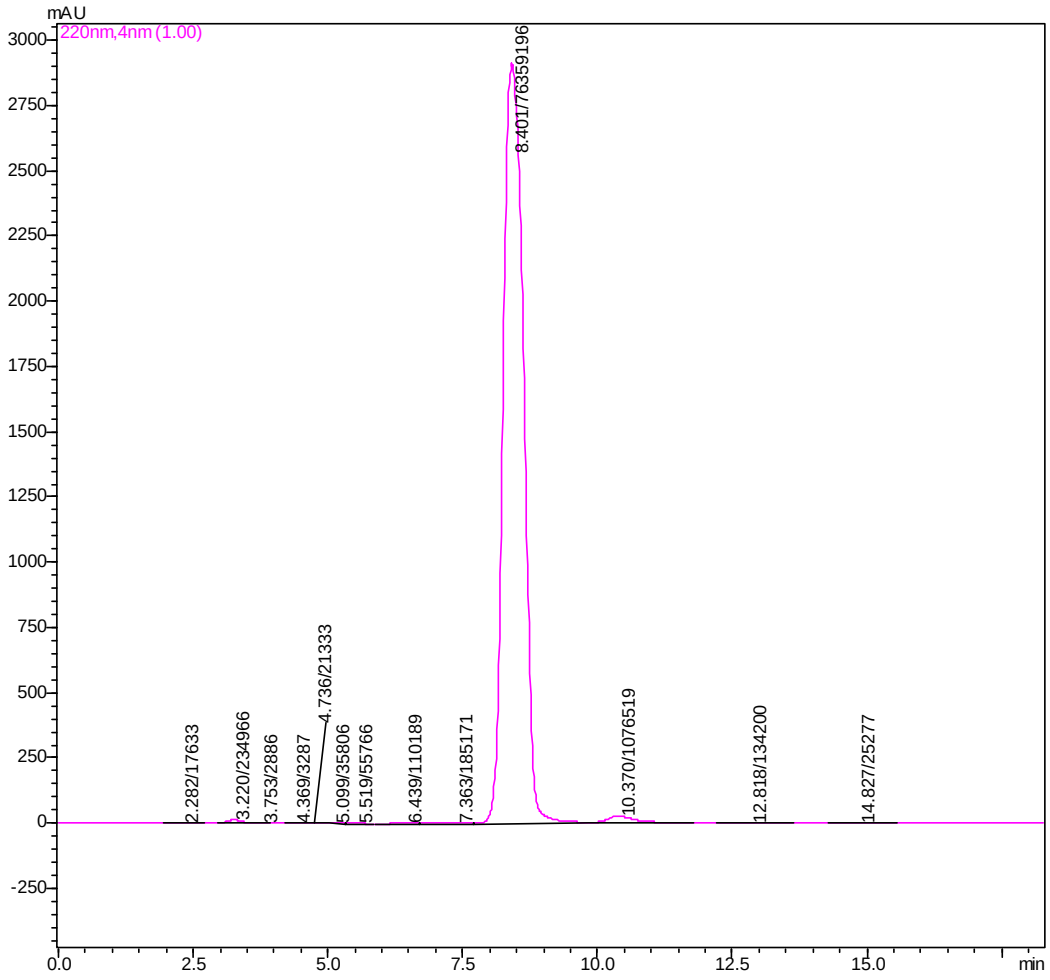
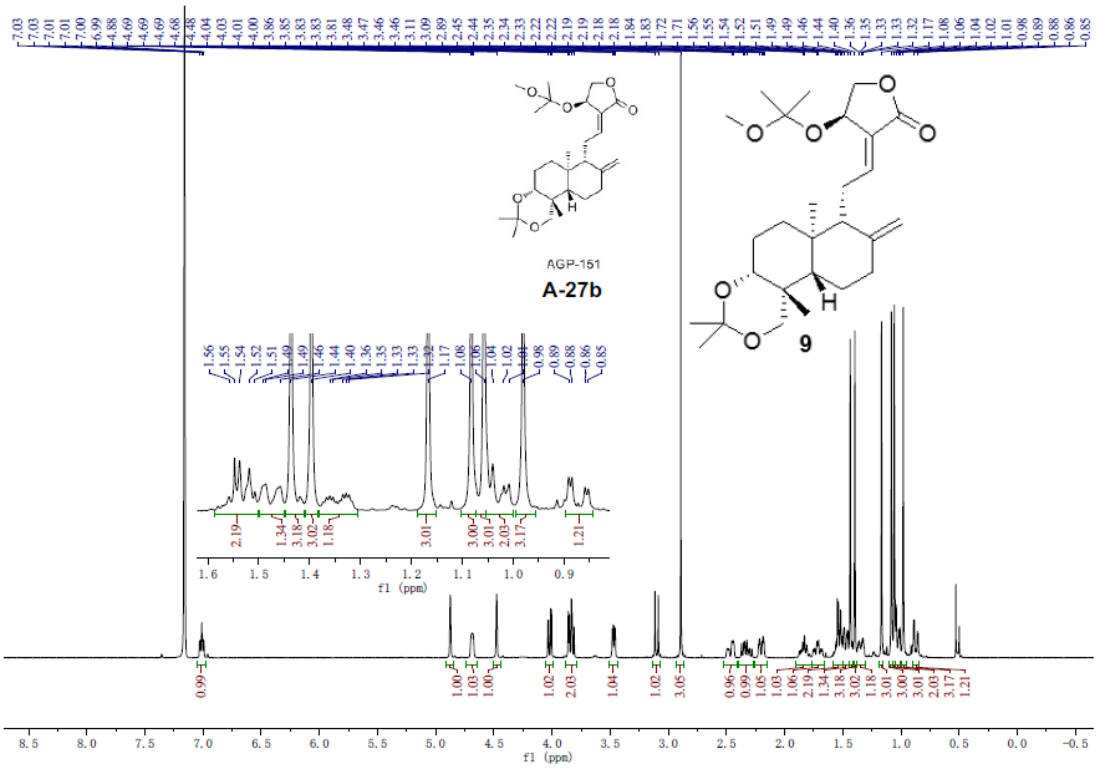
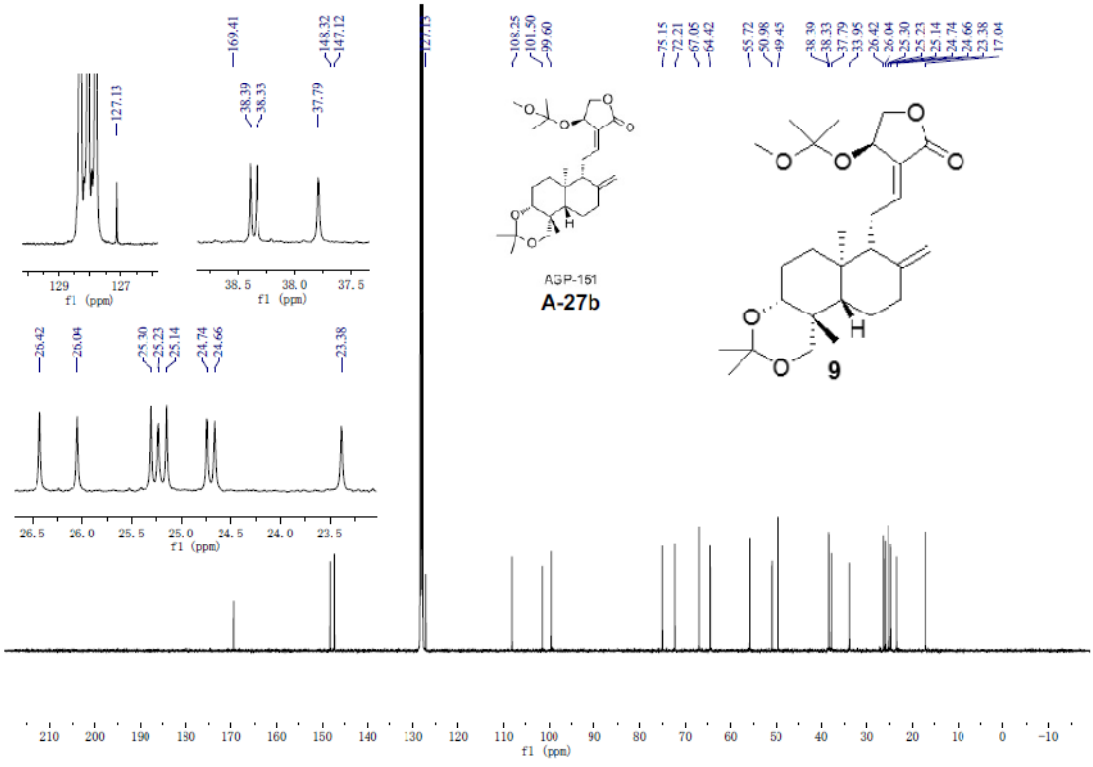


Compound 9 (^{1}H NMR, ^{13}C NMR, HPLC)

^{1}H NMR of 9:



^{13}C NMR of 9:



HPLC of 9 (HPLC purity ≧95%)**:**