Supporting Information

Dual-Acting Small Molecules: Subtype-Selective Cannabinoid Receptor 2 Agonist/Butyrylcholinesterase Inhibitor Hybrids Show Neuroprotection in an Alzheimer's Disease Mouse Model

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1. Calculation of the combination index

Table S-1. Calculation of combination index (CI) for the **2** + **6** mix.

Treatment (mg/kg IP)	*PP (%)*	$C_{x,UW\text{-}MD\text{-}95}$	$C_{x,UW\text{-}MD\text{-}131}$	*CI*
(a) *Spontaneous alternation (Fig. 8A)*				
2 (0)	0.0 ± 11.2			
2 (0.1)	51.3 ± 14.2			
2 (0.3)	65.3 ± 14.8 [1]			
6 (0)	0.0 ± 11.2			
6 (0.1)	45.6 ± 15.7			
6 (0.3)	51.3 ± 24.3 [2]			
2 (0.1) + **6** (0.1)	82.9 ± 12.2	0.36 ± 0.05	0.47 ± 0.08	**0.49 ±0.08**
(b) *Passive avoidance (Fig. 8C)*				
2 (0)	0.0 ± 34.9			
2 (0.1)	11.6 ± 38.1			
2 (0.3)	32.5 ± 33.9			
2 (1)	104.5 ± 51.6 [3]			
6 (0)	0.0 ± 34.9			
6 (0.1)	20.7 ± 31.8			
6 (0.3)	80.1 ± 39.3 [4]			
2 (0.1) + **6** (0.1)	56.0 ± 37.0	0.54 ± 0.21	0.22 ± 0.08	**0.65 ± 0.25**

Percent protection (PP) was calculated using 100% for V1/V2-treated animals and 0% for A$ß_{25\text{-}35}$/V2/V2-treated animals. CI: combination index. $C_{x,Drug}$ was calculated using the linear regression from responses with the drug alone: [1] y = 196.4x +12.68; [2] y = 150.7x + 12.217; [3] y = 103.9x +0.314; [4] y = 271.2x - 2.56. CI in bold shows synergy.

2. Neuroprotection and Neurotoxicity of compounds **14** and **15d**

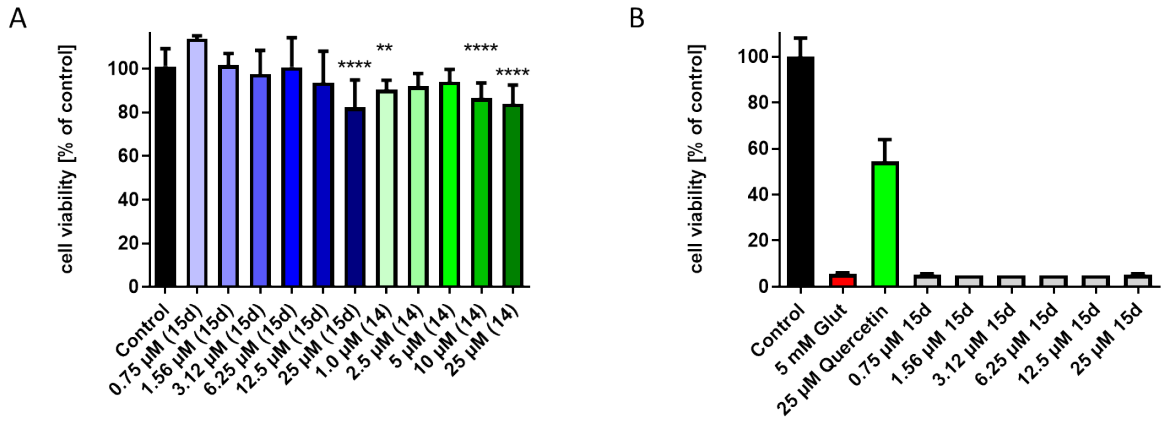

Figure S-1. Neurotoxicity studies on neuronal HT-22 cells for compounds **15d** and **14** (A) and neuroprotection against glutamate-induced oxidative stress of compound **15d** (B). Results of the modified MTT test are presented as means ± SD of three independent experiments, each performed in sextuplicate, and refer to untreated control cells, which were set as 100% values. Statistical analysis was achieved by applying one-way ANOVA followed by Dunnett's multiple comparison post-test. Levels of significance: ** $p < 0.01$; **** $p < 0.0001$. Treated cells were compared to (A) untreated cells and (B) cells treated with glutamate only.

3. Time-dependent inhibition of *h*BChE by compounds **15d** and **21d**

To determine K_m and v_{max}, residual enzyme activity was plotted against time for several concentrations.

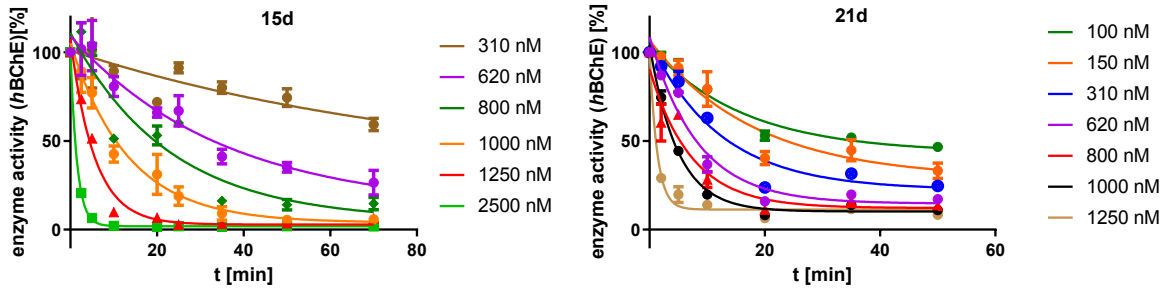

Figure S-2. Time-dependent inhibition of hBChE by compounds **15d** and **21d**.

4. βarr2 screening of the complete library

Figure S-3. Functional activity screening of compounds **4**, **14**, **15a-j**, **20** and **21a-e** by NanoBiT® *h*CB_2R βarr2 recruitment assay. Receptor activation was monitored at 10 μM and normalized to the maximum response of compound **4**; n=3.

5. Pharmacokinetic measurements

Table S-2. Plasma concentrations of compound **15d** in male CD-1 mice following IV (2 mg/kg) administration.

Sample collection time point, min	**Plasma concentration (ng/ml)**					
	Mouse A	**Mouse B**	**Mouse C**	**Mean**	**SD**	**SE**
0	BQL			**BQL**	ND	ND
5	1912	1857	1645	**1805**	141	81
15	794	638*	791	**793**	2	2
60	20	19	13	**18**	4	2
120	1	1	BQL	**1**	1	0
240	BQL	BQL	BQL	**BQL**	ND	ND

BQL - Below the lower limit of quantitation (LLOQ)

ND - Not determined

*Grubbs' outlier test: Significant outlier. $P < 0.05$

Table S-3. Brain concentrations of compound **15d** in male CD-1 mice following IV (2 mg/kg) administration.

Sample collection time point, min	**Brain concentration (ng/g)**					
	Mouse A	**Mouse B**	**Mouse C**	**Mean**	**SD**	**SE**
0	BQL			**BQL**	ND	ND
5	178	171	232	**194**	33	19
15	38	30	39	**36**	5	3
60	1	1	1	**1**	0	0
120	BQL	BQL	BQL	**BQL**	ND	ND
240	BQL	BQL	BQL	**BQL**	ND	ND

BQL - Below the lower limit of quantitation (LLOQ)

ND - Not determined

Table S-4. Plasma concentrations of metabolite **14** in male CD-1 mice following IV administration of compound **15d** (2 mg/kg).

Sample collection time point, min	**Plasma concentration (ng/ml)**					
	Mouse A	**Mouse B**	**Mouse C**	**Mean**	**SD**	**SE**
0	BQL			**BQL**	ND	ND
5	114	109	86	**103**	15	9
15	51	42	43	**45**	5	3
60	6	4	2	**4**	2	1
120	2	1	1	**1**	1	0
240	BQL	1*	BQL	**BQL**	ND	ND

BQL - Below the lower limit of quantitation (LLOQ)

ND - Not determined

*Grubbs' outlier test: Significant outlier. $P < 0.05$

Table S-5. Brain concentrations of metabolite **14** in male CD-1 mice following IV administration of compound **15d** (2 mg/kg).

Sample collection time point, min	**Brain concentration (ng/g)**					
	Mouse A	**Mouse B**	**Mouse C**	**Mean**	**SD**	**SE**
0	BQL			**BQL**	ND	ND
5	3	3	3	**3**	0	0
15	1	1	2	**2**	0	0
60	BQL	BQL	BQL	**BQL**	ND	ND
120	BQL	BQL	BQL	**BQL**	ND	ND
240	BQL	BQL	BQL	**BQL**	ND	ND

BQL - Below the lower limit of quantitation (LLOQ)

ND - Not determined

Table S-6. Plasma concentrations of compound **21d** in male CD-1 mice following IV administration (2 mg/kg).

Sample collection time point, min	**Plasma concentration (ng/ml)**					
	Mouse A	**Mouse B**	**Mouse C**	**Mean**	**SD**	**SE**
0	BQL			**BQL**	ND	ND
5	133	117	122	**124**	8	5
15	109	89	86	**95**	13	7
60	18	25	37	**27**	10	6
120	7	7	6	**7**	1	0
240	1	1	4	**2**	1	1

BQL - Below the lower limit of quantitation (LLOQ)

Table S-7. Brain concentrations of compound **21d** in male CD-1 mice following IV administration (2 mg/kg).

Sample collection time point, min	**Brain concentration (ng/g)**					
	Mouse A	**Mouse B**	**Mouse C**	**Mean**	**SD**	**SE**
0	BQL			**BQL**	ND	ND
5	32	38	44	**38**	6	4
15	18	23	21	**21**	2	1
60	9	8	11	**9**	2	1
120	5	4	3	**4**	1	0
240	2	1	2	**2**	0	0

BQL - Below the lower limit of quantitation (LLOQ)

ND - Not determined

Table S-8. Plasma concentrations of metabolite **20** in male CD-1 mice following IV administration of compound **21d** (2 mg/kg).

Sample collection time point, min	**Plasma concentration (ng/ml)**					
	Mouse A	**Mouse B**	**Mouse C**	**Mean**	**SD**	**SE**
0	BQL			**BQL**	ND	ND
5	6	6	17	**10**	7	4
15	7	6	7	**6**	0	0
60	4	4	9	**6**	3	2
120	2	1	1	**1**	1	0
240	BQL	1	1	**1**	0	0

BQL - Below the lower limit of quantitation (LLOQ)

ND - Not determined

Table S-9. Brain concentrations of metabolite **20** in male CD-1 mice following IV administration of compound **21d** (2 mg/kg).

Sample collection time point, min	**Brain concentration (ng/g)**					
	Mouse A	**Mouse B**	**Mouse C**	**Mean**	**SD**	**SE**
0	BQL			**BQL**	ND	ND
5	7	7	14	**9**	4	2
15	13	13	15	**14**	1	0
60	13	14	14	**14**	1	0
120	6	4	3	**4**	2	1
240	1	1	1	**1**	0	0

BQL - Below the lower limit of quantitation (LLOQ)

ND - Not determined

6. HPLC chromatograms of target compounds

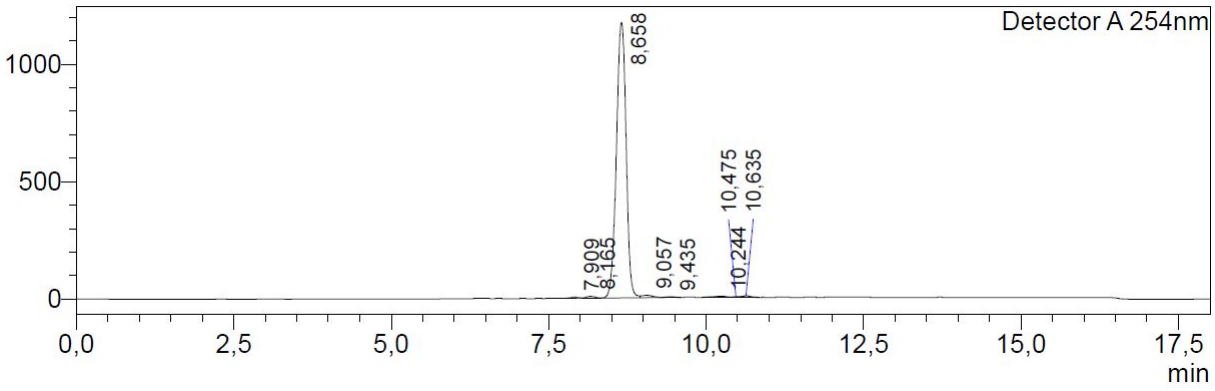

Figure S-4. HPLC chromatogram of **14**.

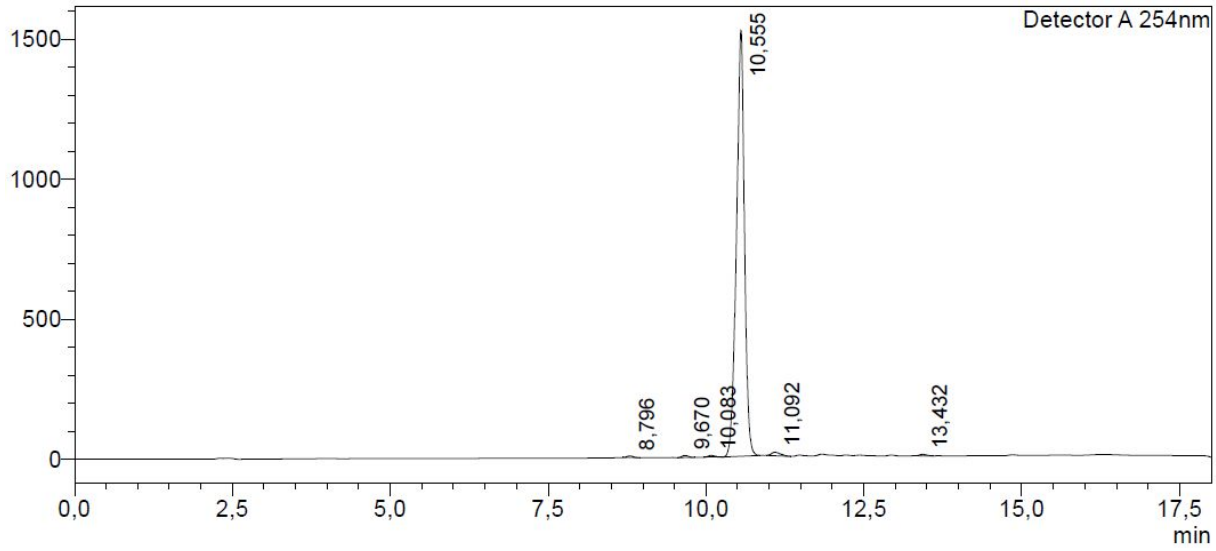

Figure S-5. HPLC chromatogram of **15a**.

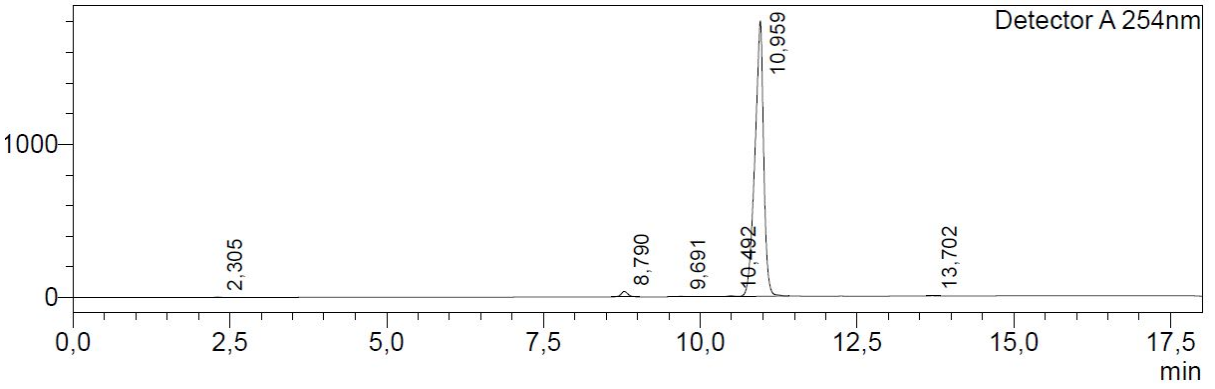

Figure S-6. HPLC chromatogram of **15b**.

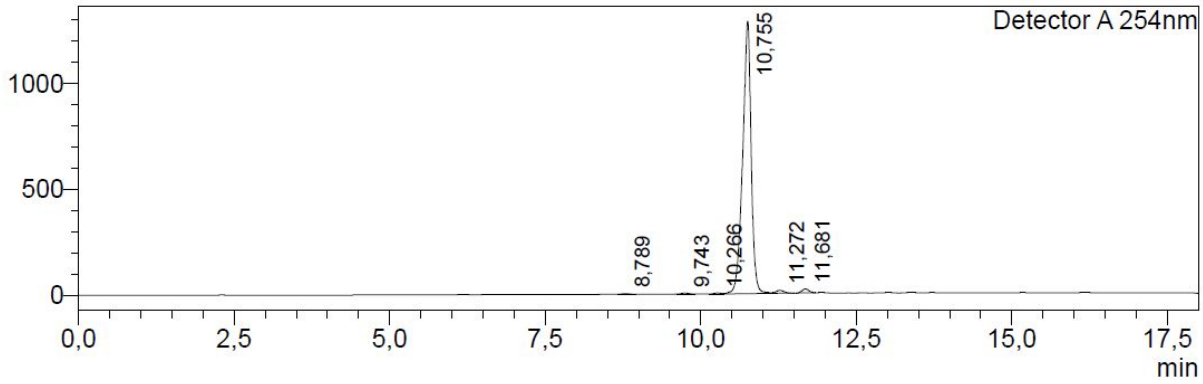

Figure S-7. HPLC chromatogram of **15c**.

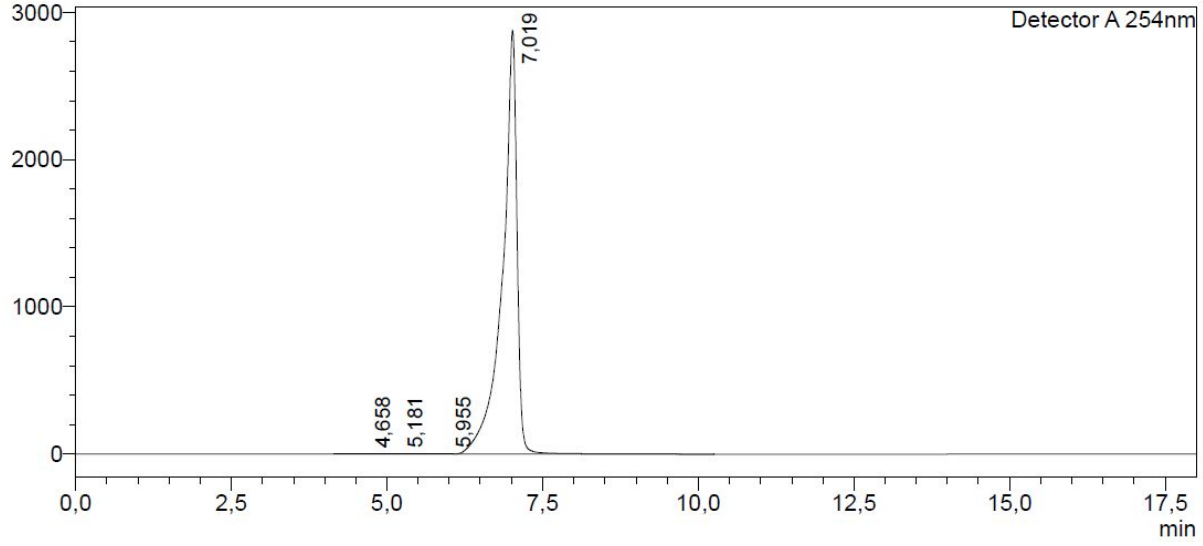

Figure S-8. HPLC chromatogram of **15d**.

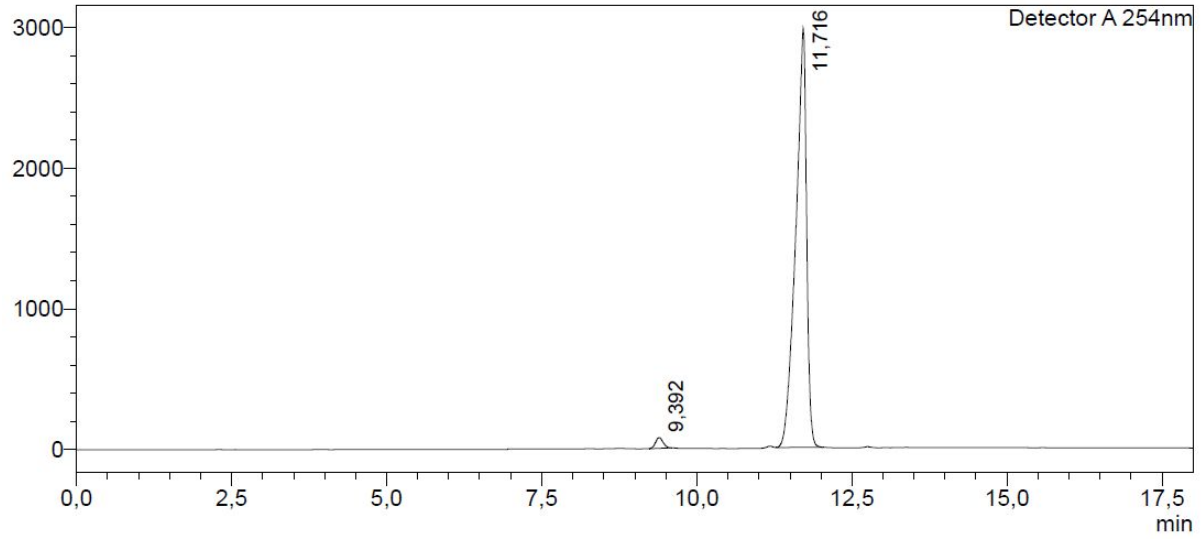

Figure S-9. HPLC chromatogram of **15e**.

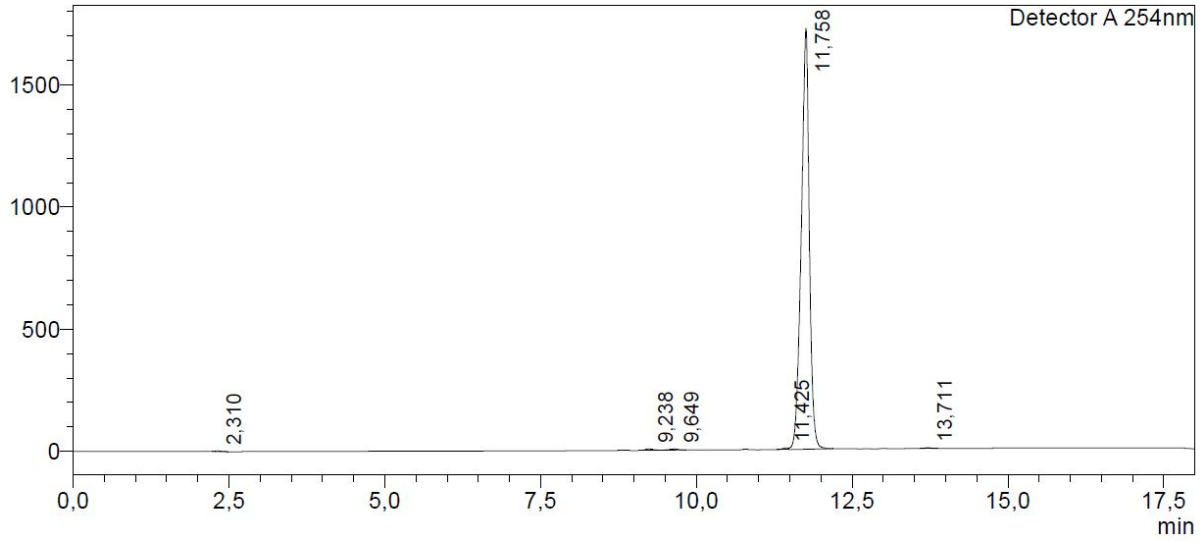

Figure S-10. HPLC chromatogram of **15f**.

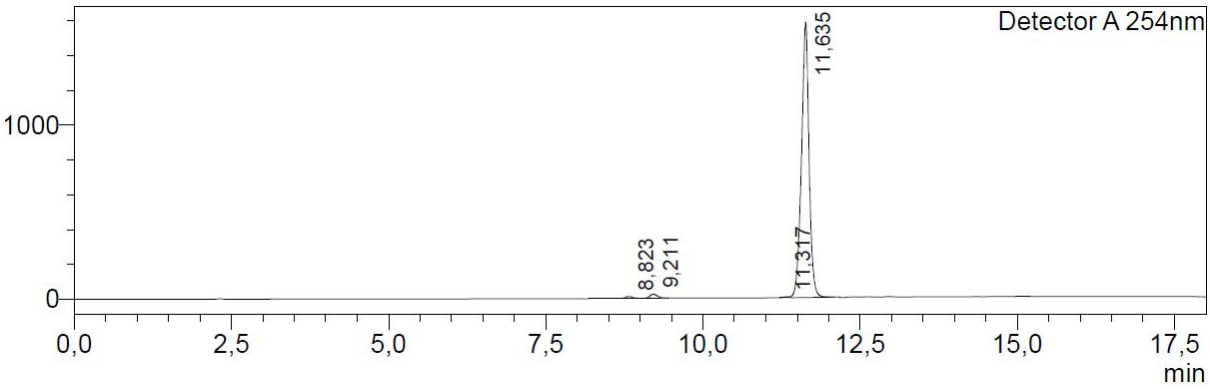

Figure S-11. HPLC chromatogram of **15g**.

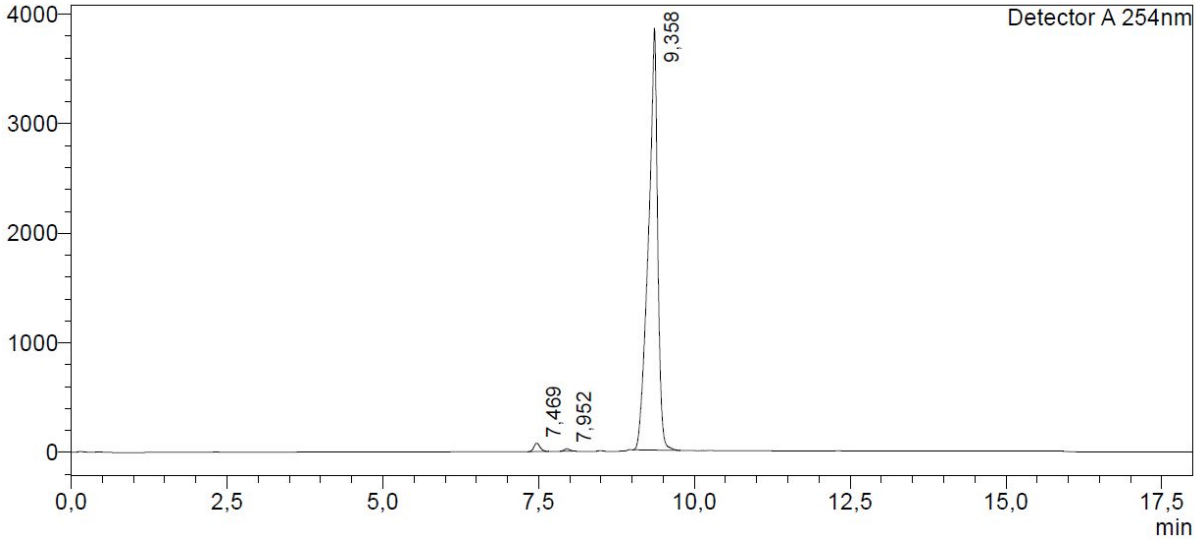

Figure S-12. HPLC chromatogram of **15h**.

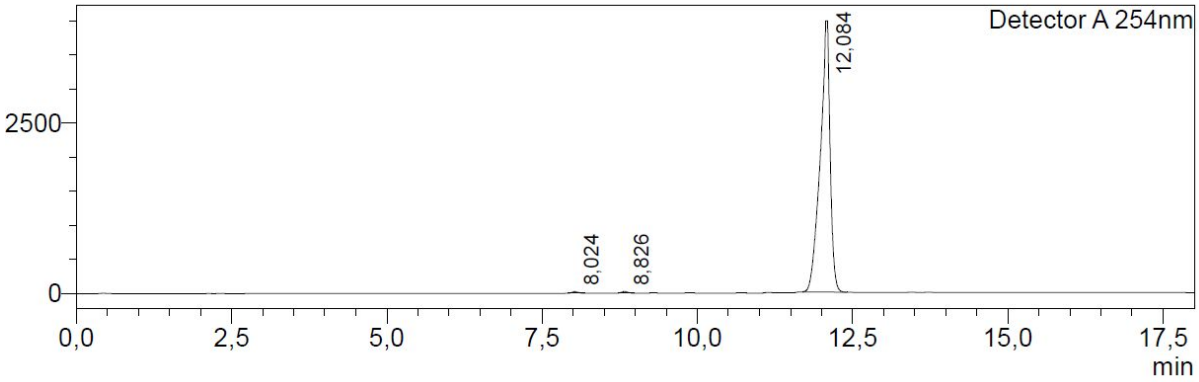

Figure S-13. HPLC chromatogram of **15i**.

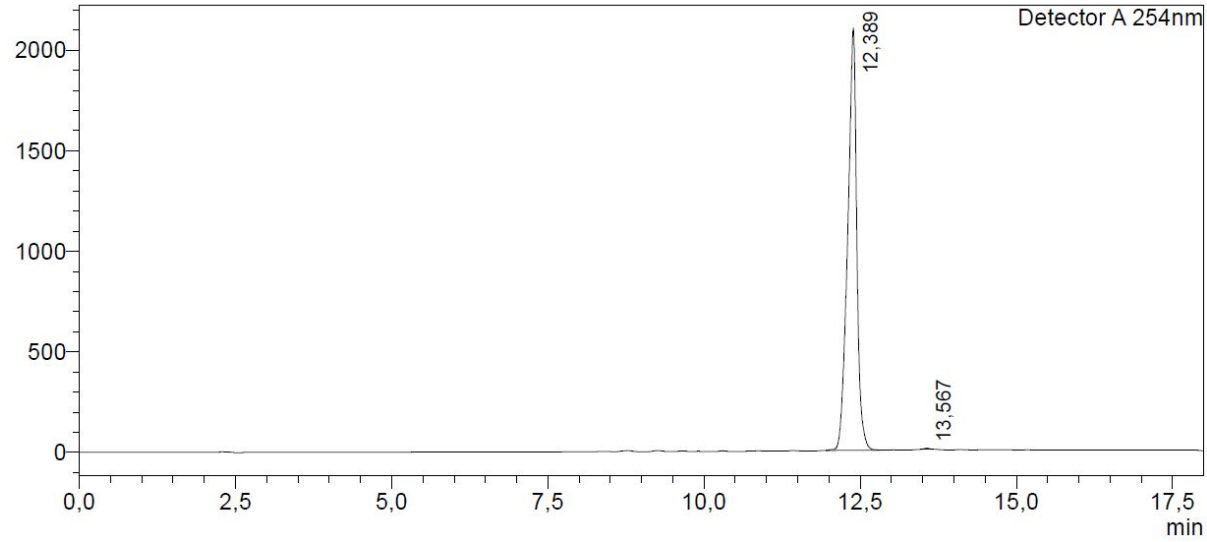

Figure S-14. HPLC chromatogram of **15j**.

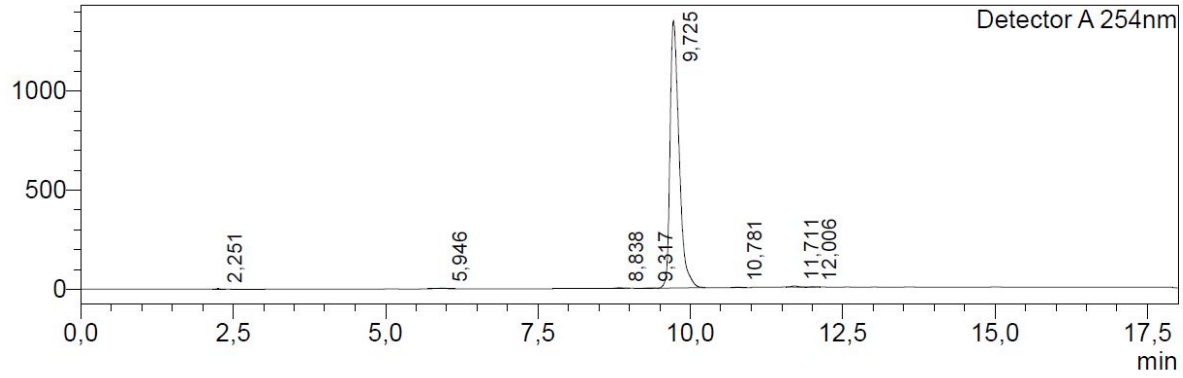

Figure S-15. HPLC chromatogram of **20**.

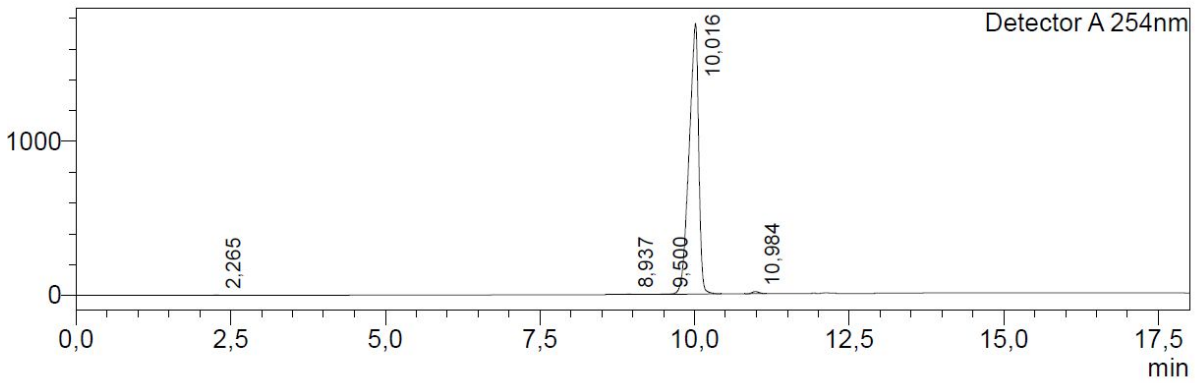

Figure S-16. HPLC chromatogram of **21a**.

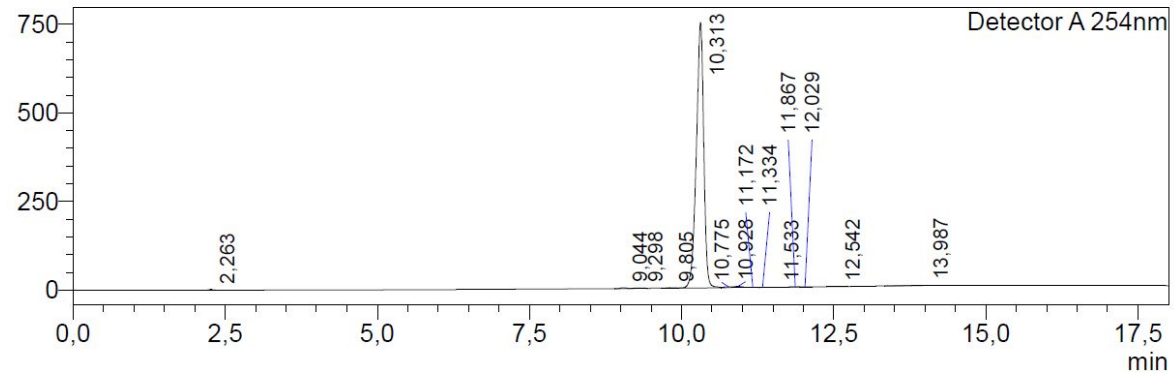

Figure S-17. HPLC chromatogram of **21b**.

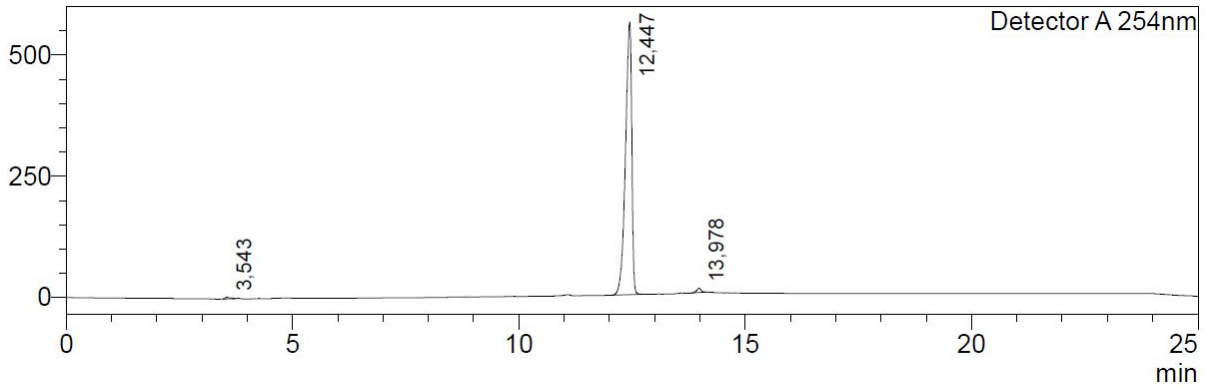

Figure S-18. HPLC chromatogram of **21c**.

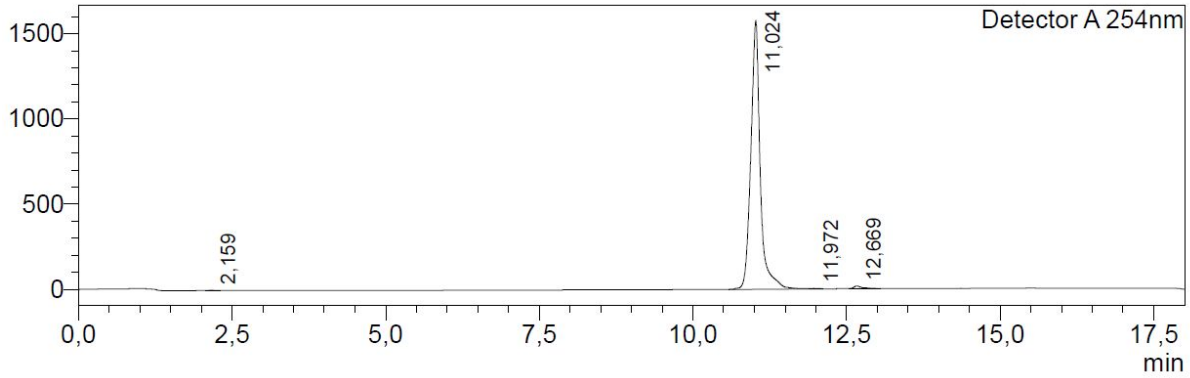

Figure S-19. HPLC chromatogram of **21d**.

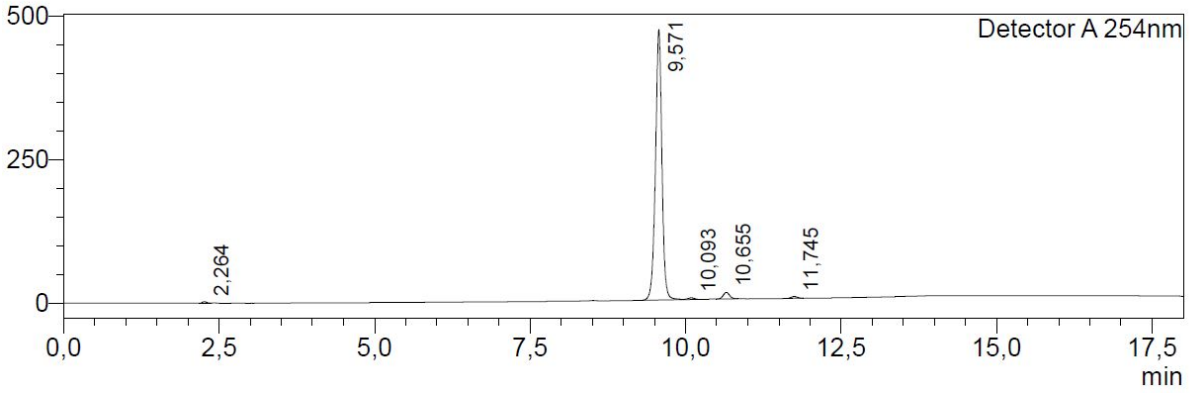

Figure S-20. HPLC chromatogram of **21e**.

7. Melting point measurement

Melting point were measured with OptiMelt MPA 100, an automated meltingpoint system from Stanford Research Systems. The device records a sigmoidal melting curve, wherein the inflection point is reported as the melting point.