Supporting material for

Dimerization of the immunoactive “danger” peptide derived from mycobacterial hsp65 using *N*-hydroxysuccinimide based bifunctional reagents is critical for their immunoactivating and antitumor properties

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Supporting Figure 1

Supporting Figure 1. Example chromatogram for HPLC separation of the entire reaction (peptide dimerization) mixture on C18 reverse phase column. Peak 1 corresponds to the nonreacted peptide, peak 2 to the dimerized peptide, peak 3 to peptide reacted with cross-linker with the second reactive group hydrolyzed, and peak 4 to an unknown contaminant. Peaks at the beginning of the chromatogram are nonspecified injection peaks. Individual traces are teste separations from an initial optimization of synthesis of compound **8**.

Supporting Table 1: ^{13}C NMR data of peptides **3**, and **5** (600.23 MHz for ^1H , and 150.93 MHz for ^{13}C , DMSO- d_6 , 30 °C).

		3		5*	
Amino acid residue	Atom	δ_c	m.	δ_c	m.
Tyr-7	COOH	172,81	S	n.d.	-
	C- α	53,85	D	55.2	D
	C- β	36.17	T	37.6	T
	C- <i>ipso</i>	127.38	S	n.d.	-
	C- <i>ortho</i>	130.04	D	131.3	D
	C- <i>meta</i>	115.04	D	116.3	D
	C- <i>para</i>	155.95	S	n.d.	-
Gly-6	CO	168.40	S	n.d.	-
	C- α	41.57	T	43.0	T
Glu-5	CO	171.12	S	n.d.	-
	C- α	51.96	D	53.2	D
	C- β	27.41	T	28.8	T
	C- γ	29.96	T	31.4	T
	COOH	173.93	S	n.d.	-
Thr-4	CO	169.86	S	n.d.	-
	C- α	57.98	D	59.5	D
	C- β	66.50	D	67.9	D
	C- γ	19.52	Q	20.9	Q
Leu-3	CO	172.05	S	n.d.	-
	C- α	51.04	D	52.5	D
	C- β	40.53	T	41.9	T
	C- γ	24.08	D	25.4	D
	C- δ_1	23.12	Q	24.4	Q
	C- δ_2	21.57	Q	23.0	Q
Glu-2	CO	170.78	S	n.d.	-
	C- α	51.73	D	53.2	D
	C- β	27.15	T	28.5	T
	C- γ	30.15	T	31.5	T
	COOH	173.98	S	n.d.	-
Leu-1	CO	171.77	S	n.d.	-
	C- α	50.68	D	53.2	D
	C- β	41.84	T	41.9	T
	C- γ	23.82	D	25.6	D
	C- δ_1	23.09	Q	24.4	Q
	C- δ_2	22.03	Q	23.0	Q
Linker	CO	171.45	S	n.d.	-
	CH ₂	-		35.6	T
	CH ₂	-		23.1	
	CH ₂ OH	72.64	T		

* gHSQC readouts

Supporting Table 2 :¹H NMR data of peptides **3**, and **5** (600.23 MHz for ¹H, DMSO-*d*₆, 30 °C). *TOCSY readouts

		3			5		
Amino acid residue	Atom	δ _H	m.	³ J _{HH} [Hz]	δ _H	m.	³ J _{HH} [Hz]
Tyr-7	NH	8.039	d	8.0	8.032	br s	-
	C-α	4.341	ddd	5.4, 8.0, 8.3	4.333	m	-
	C-β	2.904	dd	5.4, 13.9	2.903	dd	5.3, 13.8
		2.765	dd	8.3, 13.9	2.764	dd	8.2, 13.8
	<i>C-ortho</i>	6.989	m	-	6.986	m	-
	<i>C-meta</i>	6.649	m	-	6.646	m	-
	<i>para</i> -OH	9.143	s	-	9.143	br s	-
Gly-6	NH	8.036	d	5.8, 6.9	8.043	m	-
	C-α	3.711	dd	6.9, 16.8	3.687	m	-
		3.671	dd	5.8, 16.8			
Glu-5	NH	7.807	d	7.6	7.802	d	7.4
	C-α	4.274	ddd	5.5, 7.6, 8.9	4.270	m	-
	C-β	1.925	m	-	1.92*	m	-
		1.764	m	-	1.76*	m	-
	C-γ	2.284	m	-	2.258	m	-
		2.239	m	-			
Thr-4	NH	7.781	d	8.3	7.782	m	-
	C-α	4.219	dd	4.2, 8.3	4.217	dd	3.8, 8.2
	C-β	3.999	dq	4.2, 6.4	4.000	m	-
	C-γ	1.016	d	6.4	1.014	d	6.2
	β-OH	n.d.	-	-	n.d.	-	-
Leu-3	NH	7.915	d	8.0	7.870	brd	7.4
	C-α	4.376	m	-	4.373	m	-
	C-β	1.468	m	-	1.468	m	-
	C-γ	1.595	m	-	1.575	m	-
	C-δ ₁	0.875	d	6.7	0.867	d	6.4
	C-δ ₂	0.828	d	6.5	0.825	d	6.4
Glu-2	NH	8.136	d	8.0	8.102	m	-
	C-α	4.285	m	-	4.270	m	-
	C-β	1.886	m	-	1.89*	m	-
		1.744	m	-	1.75*	m	-
	C-γ	2.251	m	-	2.251	m	-
		2.198	m	-	2.203	m	-
Leu-1	NH	7.582	d	8.6	8.102	m	-
	C-α	4.403	ddd	5.6, 8.6, 9.4	4.270	m	-
	C-β	1.479	m	-	1.417	m	-
		1.431	m				
	C-γ	1.646	m	-	1.615	m	-
	C-δ ₁	0.862	d	6.7	0.862	d	6.4
	C-δ ₂	0.855	d	6.5	0.831	d	6.4
Linker	CH ₂	-	-		2.076	m	-
	CH ₂	-	-		1.720	m	-
	CHOH	4.232	s	-	-		
	OH	n.d.	-	-	-		

Supporting Table 3 : ^{13}C NMR data of peptides **4**, and **6** (600.23 MHz for ^1H , and 150.93 MHz for ^{13}C , DMSO- d_6 , 30 °C).

		4		6	
Amino acid residue	Atom	δ_{C}	m.	δ_{C}	m.
Tyr-7	COOH	172.93	S	173.05	S
	C- α	53.93	D	54.07	D
	C- β	36.21	T	36.29	T
	C- <i>ipso</i>	127.47	S	127.60	S
	C- <i>ortho</i>	130.12	D	130.24	D
	C- <i>meta</i>	115.07	D	115.20	D
	C- <i>para</i>	156.00	S	156.08	S
Gly-6	CO	168.50	S	168.67	S
	C- α	41.59	T	41.72	T
Glu-5	CO	171.17	S	171.34	S
	C- α	51.92	D	52.08	D
	C- β	27.49	T	27.54	T
	C- γ	30.02	T	30.23	T
	COOH	174.05	S	174.19	S
Leu-4	CO	171.94	S	172.13	S
	C- α	50.94	D	51.09	D
	C- β	40.38	T	40.42	T
	C- γ	24.15	D	24.21	D
	C- δ_1	23.16	Q	23.24	Q
	C- δ_2	21.47	Q	21.53	Q
Leu-3	CO	171.86	S	172.01	S
	C- α	50.94	D	51.12	D
	C- β	40.61	T	40.69	T
	C- γ	24.11	D	24.29	D
	C- δ_1	23.20	Q	23.11	Q
	C- δ_2	21.73	Q	21.88	Q
Glu-2	CO	170.79	S	171.03	S
	C- α	51.76	D	51.98	D
	C- β	27.30	T	27.34	T
	C- γ	30.20	T	30.12	T
	COOH	174.05	S	174.26	S
Leu-1	CO	171.84	S	173.39	S
	C- α	50.72	D	51.54	D
	C- β	41.89	T	40.60	T
	C- γ	23.86	D	24.38	D
	C- δ_1	23.07	Q	23.26	Q
	C- δ_2	22.09	Q	21.65	Q
Linker	CO	171.53	S	172.48	S
	CH ₂	-		34.18	T
	CH ₂	-		22.03	T
	CH ₂ OH	72.68	D	-	

Supporting Table 4 :¹H NMR data of peptides **4**, and **6** (600.23 MHz for ¹H, DMSO-*d*₆, 30 °C).

Amino acid residue	Atom	4			6		
		δ_{H}	m.	$^3J_{\text{HH}}$ [Hz]	δ_{H}	m.	$^3J_{\text{HH}}$ [Hz]
Tyr-7	NH	8.074	d	8.0	8.065	d	7.8
	C- α	4.331	ddd	5.2, 8.0, 8.7	4.322	ddd	5.3, 7.8, 8.6
	C- β	2.903	dd	5.2, 13.9	2.896	dd	5.3, 13.9
		2.761	dd	8.7, 13.9	2.755	dd	8.6, 13.9
	<i>C-ortho</i>	6.988	m	-	6.984	m	-
	<i>C-meta</i>	6.644	m	-	6.640	m	-
	<i>para</i> -OH	9.186	s	-	n.d.	s	-
Gly-6	NH	8.038	d	5.8	8.033	t	5.9
	C- α	3.683	d	5.8	3.675	d	5.9
Glu-5	NH	7.846	d	7.6	7.836	d	7.8
	C- α	4.222	m	-	4.205	ddd	5.6, 7.8, 8.0
	C- β	1.883	m	-	1.868	m	-
		1.736	m	-	1.732	m	-
	C- γ	2.240	m	-	2.227	m	-
Leu-4	NH	7.987	d	8.1	7.982	d	7.8
	C- α	4.286	m	-	4.278	m	-
	C- β	1.455	m	-	1.442	m	-
	C- γ	1.586	m	-	1.583	m	-
	C- δ_1	0.856	d	6.6	0.846	d	6.5
	C- δ_2	0.804	d	6.7	0.791	d	6.3
Leu-3	NH	7.917	d	8.3	7.856	d	8.4
	C- α	4.264	m	-	4.266	m	-
	C- β	1.424	m	-	1.424	m	-
	C- γ	1.563	m	-	1.540	m	-
	C- δ_1	0.865	d	6.5	0.846	d	6.5
	C- δ_2	0.815	d	6.7	0.801	d	6.3
Glu-2	NH	8.156	d	7.7	8.168	d	7.8
	C- α	4.264	m	-	4.244	m	-
	C- β	1.849	m	-	1.855	m	-
		1.729	m	-	1.725	m	-
	C- γ	2.211	m	-	2.219	m	-
Leu-1	NH	7.602	d	8.5	8.201	d	7.2
	C- α	4.392	ddd	6.0, 8.2, 8.5	4.252	m	-
	C- β	1.446	m	-	1.409	m	-
	C- γ	1.639	m	-	1.620	m	-
	C- δ_1	0.856	d	6.6	0.846	d	6.5
	C- δ_2	0.849	d	6.4	0.820	d	6.6
Linker	CH ₂	-			2.052	m	-
	CH ₂	-			1.698	m	-
	CHOH	4.222	d	7.2	-		
	OH	5.776	d	7.2	-		

Supporting Figure 2

Supporting Figure 2. MALDI MS analysis of fraction 1 to 4 from HPLC separation shown in Supporting Figure 2.