Chemical Synthesis of Arabidopsis CLV3 Glycopeptide Reveals the Impact of Hyp Arabinosylation on Peptide Conformation and Activity

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[Supplementary methods]

Synthesis of Fmoc-Hyp-OBn (2)

Benzyl alcohol (0.84 ml, 8.0 mmol), *N,N*-dimethyl-4-aminopyridine (DMAP) (89 mg, 0.73 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl) (1.53 g, 8.0 mmol) were added to a solution of Fmoc-Hyp(*t*Bu)-OH (3.0 g, 7.3 mmol, Watanabe Chemicals) dissolved in anhydrous CH_2Cl_2 (20 ml). After stirring for 2 h at 4ºC, the reaction mixture was washed with 20% aqueous citrate and concentrated *in vacuo*. The residue was further treated with 95% TFA (10 ml) for 10 min at room temperature to deprotect the *t*Bu group. The reaction mixture was partitioned between CH_2Cl_2 and aqueous $NaHCO_3$ and the organic phase was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography, eluting with *n*-hexane-EtOAc (1:1) to yield **2** (2.14 g, 66%).

Synthesis of Fmoc-[(3,5-Bn)Ara]Hyp-OBn (4)

Thioarabinoside **1** was synthesized from L-arabinose as previously described (Desire and Prandi 1999). To a solution of **1** (1.86 g, 3.40 mmol) and **2** (1.0 g, 2.30 mmol) in dry CH_2Cl_2 (100 ml) was added 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (1.03 g, 4.5 mmol) and 4 Å molecular sieves. The mixture was stirred for 20 min at room temperature and quenched with an aqueous solution of ascorbic acid (0.7%), citric acid (1.3%) and NaOH (0.9%) (20 ml). The organic phase was washed with aqueous $NaHCO_3$ and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography, eluting with *n*-hexane-EtOAc (2:1) to give the intermediate mixed acetal **3** (1.6 g, 74%). This acetal was taken up in dry CH_2Cl_2 (50 ml) and reacted with iodonium collidine perchlorate (IDCP) (2.41 g, 5.13 mmol) in the presence of 4 Å molecular sieves for 15

min at room temperature. The mixture was washed with saturated $Na_2S_2O_3$ and 20% citrate and the organic phase was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography, eluting with *n*-hexane-EtOAc (1:1) to yield **4** (1.06 g, 82%).

^{1}H NMR (500 MHz, $CDCl_3$) (a mixture of Hyp *cis*/*trans* rotational conformers): δ = 7.77-7.72 (m, 2 H), 7.58-7.49 (m, 2 H), 7.37-7.24 (m, 19 H), 5.21-5.02 (m, 2 H), 5.02 (d, J = 5.1 Hz, 0.4 H, H-1^{Ara} (isomer)), 4.99 (d, J = 4.7 Hz, 0.6 H, H-1^{Ara}), 4.76 (d, J = 12.0 Hz, 1 H), 4.60 (d, J = 11.7 Hz, 1 H), 4.53-4.33 (m, 5.6 H), 4.26-4.22 (m, 1.9 H), 4.11-4.08 (m, 1 H), 3.99-3.95 (m, 0.5 H), 3.88-3.83 (m, 1 H), 3.70-3.65 (m, 1.3 H), 3.52-3.43 (m, 2.6 H), 2.48-2.38 (m, 1 H), 2.11-2.01 (m, 1 H). ^{13}C NMR (125 MHz, $CDCl_3$) (a mixture of Hyp *cis*/*trans* rotational conformers): δ = 172.3, 155.0, 154.6, 144.4, 144.2, 144.0, 141.5, 138.1, 138.0, 135.7, 135.5, 128.8, 128.7, 128.6, 128.5, 128.2, 128.1, 128.0, 127.3, 125.4, 125.3, 125.2, 120.3, 120.2, 101.4, 101.3, 84.0, 81.1, 81.0, 77.9, 77.8, 76.5, 75.6, 73.6, 72.3, 71.5, 67.9, 67.8, 67.3, 67.2, 58.4, 58.1, 52.4, 52.0, 47.4, 37.9, 36.8. ESI-MS: *m*/*z* calcd for $[C_{46}H_{45}NO_9+Na]^+$: 778.3; found: 778.4 ($[M+Na]^+$).

Synthesis of Fmoc-[(3,5-Bn)Ara$_2$]Hyp-OBn (6)

To a solution of **4** (500 mg, 0.66 mmol) and **1** (1.0 g, 2.00 mmol) in dry CH_2Cl_2 (30 ml) was added DDQ (602 mg, 2.65 mmol) and 4 Å molecular sieves. The mixture was stirred for 20 min at room temperature and quenched with the above mentioned quenching solution (20 ml). The organic phase was washed with aqueous $NaHCO_3$ and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography, eluting with *n*-hexane-EtOAc (2:1) to give the intermediate mixed acetal **5** (379 mg, 46%). This acetal was taken up in dry CH_2Cl_2 (25 ml) and reacted with IDCP (429 mg, 0.91 mmol) in the presence of 4 Å molecular sieves for 15 min at room temperature. The mixture was washed with saturated $Na_2S_2O_3$ and 20% citrate and the organic phase was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography, eluting with *n*-hexane-EtOAc (3:2) to yield **6** (243 mg, 75%).

Synthesis of Fmoc-[(3,5-Bn)Ara$_3$]Hyp-OBn (8)

To a solution of **6** (243 mg, 0.23 mmol) and **1** (338 mg, 0.68 mmol) in dry CH_2Cl_2 (10 ml) was added DDQ (155 mg, 0.68 mmol) and 4 Å molecular sieves. The mixture was stirred for 20 min at room temperature and quenched with the above

mentioned quenching solution (10 ml). The organic phase was washed with aqueous $NaHCO_3$ and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography, eluting with *n*-hexane-EtOAc (2:1) to give the intermediate mixed acetal **7** (191 mg, 54%). This acetal was taken up in dry CH_2Cl_2 (2 ml) and reacted with IDCP (172 mg, 0.37 mmol) in the presence of 4 Å molecular sieves for 15 min at room temperature. The mixture was washed with saturated $Na_2S_2O_3$ and 20% citrate and the organic phase was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography, eluting with *n*-hexane-EtOAc (3:2) to yield **8** (109 mg, 65%).

Synthesis of Fmoc-[AcAra$_3$]Hyp-OH (9)

Compound **8** (300 mg, 0.23 mmol) was dissolved in CH_2Cl_2/methanol (1:1, v/v) (6 ml) with 5 drops of acetic acid and hydrogenolyzed in the presence of 10% $Pd(OH)_2$/C (50 mg) at room temperature for 2 days. Because the benzyl protecting groups of **8** were somewhat resistant to hydrogenolysis, $Pd(OH)_2$/C was removed by filtration and replaced by an equal amount of fresh $Pd(OH)_2$/C after 3 h and again after 24 h. Progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was filtered and concentrated *in vacuo*. Deprotected compound was dissolved in CH_3CN/water (1:1, v/v) (5 ml) and treated with Fmoc-OSu (155 mg, 0.46 mmol) in the presence of $NaHCO_3$ (10 mg, 12 mmol) for 16 h at room temperature. After lyophilization, the mixture was further reacted with Ac_2O/pyridine (1:1, v/v) (6 ml) for 16 h at room temperature to protect the free hydroxyl residues on the arabinosides. After quenching by addition of water, the reaction mixture was partitioned between water and EtOAc, and the organic phase was concentrated *in vacuo*. Reverse-phase HPLC purification gave compound **9** (75 mg, 31%, 3 steps).

^{1}H NMR (500 MHz, $CDCl_3$) (a mixture of Hyp *cis*/*trans* rotational conformers): δ = 7.77-7.65 (m, 2 H), 7.61-7.53 (m, 2 H), 7.42-7.28 (m, 4 H), 5.32-4.92 (m, 7 H), 4.72-3.57 (m, 18 H), 2.83-2.28 (m, 2 H), 2.11-1.82 (m, 21 H). ESI-MS: *m*/*z* calcd for $[C_{49}H_{57}NO_{24}+Na]^+$: 1066.3; found: 1066.4 ($[M+Na]^+$).

Synthesis of [Ara$_3$]CLV3 (10)

Asp(*t*Bu)-Pro-Leu-His(Trt)-His(Trt)-His(Trt)-resin was prepared by conventional solid-phase peptide synthesis using an ABI 431A peptide synthesizer. A mixture of Fmoc-[AcAra$_3$]Hyp-OH **9** (6.0 mg, 5.8 mmol), 1-hydroxybenzotriazole (HOBT) (2.7 mg, 20 mmol), *O*-benzotriazole-*N,N,N′,N′*-tetramethyluronium

hexafluorophosphate (HBTU) (7.6 mg, 20 mmol) and *N,N*-diisopropylethylamine (DIEA) dissolved in dry *N*-methylpyrrolidone (200 ml) was added to a peptide-resin (25 mmol) pre-swollen with dry *N*-methylpyrrolidone (50 ml). The mixture was stirred for 2 h at room temperature. Peptide-resin was recovered by filtration, and the remaining N-terminal amino acids were added to the peptide using the peptide synthesizer. The synthesized peptide was deprotected and cleaved from the resin by treating with a solution of trifluoroacetic acid/water (95:5 v/v) (1 ml) for 30 min. The peptide was precipitated by treating the solution with ether at -20 °C for 5 min. The precipitated peptide was washed twice with cold ether, dissolved in water and lyophilized. Crude peptide was dissolved in dry methanol (3 ml) and treated with sodium methoxide (28% solution, 60 ml) at room temperature for 1 h. The reaction was terminated by adding acetic acid (60 ml). HPLC purification using an amide column (TSK-gel amide-80, TOSOH) gave analytically pure [Ara_3]CLV3 **10** (2.5 mg).

ESI-MS: *m*/*z* calcd for $[C_{78}H_{120}N_{22}O_{32}+2H]^{2+}$: 939.4; found: 939.4 ($[M+2H]^{2+}$).

Synthesis of Fmoc-[$AcAra_2$]Hyp-OH (11) and Fmoc-[$AcAra_1$]Hyp-OH (12)

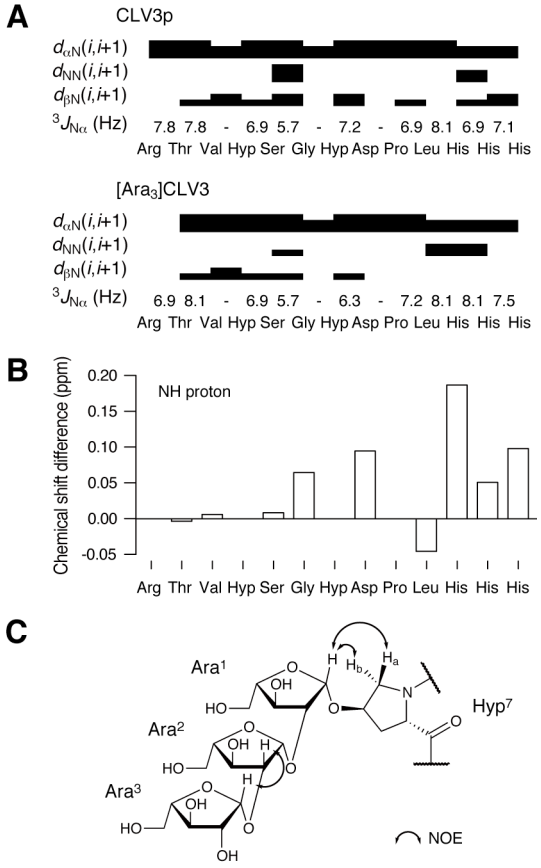
Fmoc-[$AcAra_2$]Hyp-OH **11** and Fmoc-[$AcAra_1$]Hyp-OH **12** were prepared from Compound **6** and Compound **4**, respectively, by the procedure used for the synthesis of Fmoc-[$AcAra_3$]Hyp-OH **9** described above.

ESI-MS: *m*/*z* calcd for **11** $[C_{40}H_{45}NO_{18} + Na]^+$: 850.3; found: 850.4 ($[M+Na]^+$); *m*/*z* calcd for **12** $[C_{31}H_{33}NO_{12}+Na]^+$: 634.2; found: 634.3 ($[M+Na]^+$).

References

Desire, J. and Prandi, J. (1999) Synthesis of methyl beta-D-arabinofuranoside 5-[1D (and L)-myo-inositol 1-phosphate], the capping motif of the lipoarabinomannan of *Mycobacterium smegmatis*. *Carbohydr Res* 317: 110-118.

[Supplementary figure]



Supplementary Fig. 1. Summary of NMR data.

(A) Schematic representation of the NOEs observed for [Ara_3]CLV3 and CLV3p. The intensities are indicated by the thickness of the line, and are grouped into strong, medium, and weak. In the case of proline, NH refers to the δH protons. (B) Chemical shift differences between CLV3p and [Ara_3]CLV3 amide protons. Chemical shift differences were obtained by subtracting the chemical shift value of CLV3p from that of [Ara_3]CLV3. (C) Schematic depiction of NOE connections observed between the peptide and the arabinose chain of [Ara_3]CLV3.

[Supplementary tables]

Supplementary Table 1. ^{1}H NMR chemical shifts and amide proton temperature coefficients (dδ/dT) of CLV3p.

Residue	NH (ppm)	αH (ppm)	βH (ppm)	γH (ppm)	δH (ppm)	Others (ppm)	dδ/dT (ppb/K)
Arg[1]		4.13	1.95	1.64	3.19	NH 7.30	
Thr[2]	8.90	4.42	4.11	1.21			–6.6
Val[3]	8.65	4.44	2.10	0.94, 0.98			–8.9
Hyp[4]		4.60	2.08, 2.40	4.63	3.86, 3.95		
Ser[5]	8.74	4.49	3.92				–8.4
Gly[6]	8.41	4.05, 4.20					–6.9
Hyp[7]		4.53	2.01, 2.31	4.60	3.65, 3.77		
Asp[8]	8.88	4.92	2.71, 2.87				–8.6
Pro[9]		4.39	1.93, 2.30	2.05	3.82		
Leu[10]	8.26	4.22	1.52, 1.58	1.35	0.83, 0.91		–6.7
His[11]	8.18	4.67	3.12, 3.25			2H 7.27, 4H 8.59	–4.9
His[12]	8.53	4.67	3.22			2H 7.27, 4H 8.59	–6.6
His[13]	8.56	4.53	3.15, 3.28			2H 7.27, 4H 8.59	–7.4

Supplementary Table 2. ^{1}H NMR chemical shifts and amide proton temperature coefficients (dδ/dT) of [Ara$_3$]CLV3.

Residue	NH (ppm)	αH (ppm)	βH (ppm)	γH (ppm)	δH (ppm)	Others (ppm)	dδ/dT (ppb/K)
Arg^{1}		4.13	1.95	1.64	3.18	NH 7.29	
Thr^{2}	8.90	4.42	4.11	1.21			ND
Val^{3}	8.65	4.44	2.10	0.94, 0.98			–9.0
Hyp^{4}		4.61	2.09, 2.41	4.63	3.85, 3.94		
Ser^{5}	8.73	4.51	3.92				–8.0
Gly^{6}	8.35	4.00, 4.25					–6.8
Hyp^{7}		4.52	2.02, 2.45	4.64	3.75, 3.87		
Asp^{8}	8.78	4.89	2.67, 2.81				–7.4
Pro^{9}		4.40	1.95, 2.32	2.05	3.89		
Leu^{10}	8.30	4.21	1.50, 1.60	1.33	0.82, 0.91		–5.8
His^{11}	7.99	4.66	3.09, 3.26			2H 7.26, 4H 8.57	–3.9
His^{12}	8.47	4.66	3.22			2H 7.26, 4H 8.57	–7.3
His^{13}	8.49	4.49	3.13, 3.27			2H 7.26, 4H 8.57	ND
Ara^{1}	1H 5.34	2H 4.37	3H 4.16	4H 3.92	5Ha 3.61	5Hb 3.79	
Ara^{2}	1H 5.11	2H 4.30	3H 4.23	4H 3.98	5Ha 3.72	5Hb 3.84	
Ara^{3}	1H 4.95	2H 4.12	3H 4.11	4H 3.90	5Ha 3.69	5Hb 3.81	