An immunogen synthesis strategy for the development of specific anti-deoxynivalenol monoclonal antibodies

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Online Supplementary Material

The cyclic peptide was synthesized using manual Fmoc solid phase peptide synthesis (SPPS) according to the following procedure:

Coupling of first Amino acid:

Chlorotriyl chloride resin (100 mg, 0.16 mmol, loading = 1.6 mmol/g) was swollen in dichloromethane (dry, 10 mL/g) prior to the loading of the first amino acid. Fmoc-Gly-OH (0.190 g, 0.64 mmol) was dissolved in dry DCM with N,N'-diisopropylethylamine (DIPEA) (0.22 mL, 1.28 mmol) and the reaction was shaken for 3 h. The resin was then washed with 3 x DCM, 3 x DMF, 3 x DCM and was dried under vacuum for 2 h. The loading was determined via Fmoc determination and was calculated to be 0.9 mmol/g (82% yield). The resin was then capped with methanol/DIPEA/DCM = 2:1:17 by shaking for 2 h.

Fmoc deprotection for the first amino acid:

The resin was swollen in DMF prior to Fmoc deprotection. A solution of 20% piperidine in DMF was added to the resin and the mixture was shaken for 30 min. The solution was then washed with 3 x DMF, 3 x methanol, 3 x DMF. This protocol was repeated to ensure complete Fmoc removal.

Coupling for the remaining amino acids:

Fmoc protected glycine (38.9 mg, 0.131 mmol) along with benzotriazol-1-yl oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (273.2 mg, 0.525 mmol) were dissolved in dry DMF. DIPEA (0.183 mL, 1.048 mmol) was added to it and the mixture was shaken for 30 s. The mixture was then added to resin pre-swollen in DMF (dry, 10 mL/g) and the reactor was shaken for 3 h. The solution was filtered followed by washing with 3 x DMF, 3 x methanol, 3 x DMF. Using this protocol, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-D-Phe-OH and Fmoc-L-Asp-OH were coupled consecutively.

Fmoc deprotection for the remaining amino acids:

The resin was swollen in DMF prior to Fmoc deprotection. A solution of 20% piperidine in DMF was added to the resin and the mixture was shaken for 2 min, 5 min and 15 min. The solution was washed with 3 x DMF, 3 x methanol, 3 x DMF after each deprotection step.

Mild acid resin cleavage:

After assembly of the linear peptide, the resin pre-swollen in DCM (dry, 10 mL/g) was treated with trifluoroethanol (TFE)/acetic acid (AcOH)/DCM (1/1/3, v/v/v) for 2 h to ensure complete cleavage of the protected peptide from the resin. The solvents were evaporated under vacuum and the residual material was analysed using reversed phase (RP)-HPLC/MS (Phenomenex Luna C18 column (250 x 4.6 mm, 5 µm at 35 °C) connected to an ESI mass spectrometer. A mobile phase consisting of 5 mM ammonium acetate (A) and acetonitrile (B) was used at a flow rate of 1 mL/min. The gradient elution

programme started with 100% mobile phase A for 2 min with a linear decrease to 0% mobile phase A in 15 min. An isocratic gradient of 100% mobile phase B was continued for 5 min.

HPLC trace of the protected linear peptide ($C_{49}H_{75}N_9O_{13}S$): eluting at 15.68 min with calculated m/z : 1029.52 and observed m/z (ESI⁺): 1030.4

Peptide Cyclisation:

The crude linear peptide (77.5 mg, 0.077 mmol) was dissolved in 5 mL dry DCM, followed by the addition of 1-hydroxybenzotriazole (HOBt) (10.4 mg, 0.077 mmol), PyBOP (40.1 mg, 0.077 mmol) and DIPEA (30.5 mL, 0.175 mmol). The mixture was stirred for 24 h at room temperature. Only the cyclic peptide was observed as the main peptide product on HPLC/MS (HPLC/MS conditions same as above).

HPLC trace of the protected cyclic peptide ($C_{49}H_{73}N_9O_{12}S$): eluting at 17.66 min with calculated m/z : 1011.53 and observed m/z (ESI⁺): 1012.4

Removal of protecting groups:

The reaction mixture was dissolved in 5 mL TFA/TIS/H₂O (95/2.5/2.5, v/v/v) to remove the protecting groups in solution. The peptide was then precipitated in MTBE/Hexane (1/1, v/v) and the reaction mixture was analysed using RP-HPLC/MS (Phenomenex Kinetex C18 100Å column (150 x 4.6 mm, 5 μm at 35 °C) connected to an ESI mass spectrometer. A mobile phase consisting of 5 mM ammonium acetate (A) and acetonitrile (B) was used at a flow rate of 1 mL/min. The gradient elution programme started with 100% mobile phase A for 1.5 min with a linear decrease to 0% mobile phase A in 6 min. An isocratic gradient of 100% mobile phase B was continued for 2.5 min.

HPLC trace of the deprotected cyclic peptide ($C_{27}H_{41}N_9O_7$): eluting at 3.10 min with calculated m/z : 603.31 and observed m/z (ESI^+): 604.2

Diazo transfer:

A diazo transfer was performed on 50.5 mg of the deprotected cyclic peptide using the imidazole-1-sulfonylazide hydrochloride reagent as described by Goddard-Borger and Stick (2007). The conversion was complete and no starting material was observed when analysed with HPLC/MS. The reaction mixture was redissolved in methanol and purified using RP-HPLC.

HPLC trace of the deprotected crude cyclic peptide after diazo transfer ($C_{27}H_{39}N_{11}O_7$): eluting at 4.10 min with calculated m/z : 629.30 and observed m/z (ESI^+): 630.2

HPLC purification of crude peptide:

The crude peptide was purified using RP-HPLC on an Agilent 1100 Series using a Phenomenex semiprep Luna C18 (250 x 10 mm) column. A mobile phase consisting of 0.1% TFA in H_2O (A) and acetonitrile (B) was used at a flow rate of 4.5 mL/min at 35°C. The gradient elution programme started with 100% mobile phase A for 2 min with a linear decrease to 0% mobile phase A in 15 min. An isocratic gradient of 100% mobile phase B was continued for 5 min.

HPLC trace of the final cyclic peptide after HPLC purification eluting at a retention time of 10.95 min.

References:

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