N-Linked Glycopeptide Mimetics as Tools in Kinetic, Mechanistic and Structural Studies of Peptide *N*:Glycanase F

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by

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CERTIFICATE OF REGULATORY COMPLIANCE

This is to certify that the research carried out in the Doctoral Thesis entitled "N-linked Glycopeptide Mimetics as Tools in Kinetic, Mechanistic and Structural Studies of Peptide N:Glycanase F" in the Institute of Molecular BioSciences at Massey University, New Zealand, and at IRL, Lower Hutt (with the approval of the Head of Institute of IMBS):

(a) is the original work of the candidate, except as indicated by appropriate attribution in the text and/or in the acknowledgements;

(b) that the text, excluding appendices/annexes, does not exceed 100,000 words;

(c) all the ethical requirements applicable to this study have been complied with as required by Massey University, other organizations and/or committees which had a particular association with this study, and relevant legislation.

Please insert Ethical Authorisation code(s) here (if applicable): GMO 00/MU/22

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Date: 22 March 2004

Supervisor's Name: Gillian E. Norris

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CANDIDATE'S DECLARATION

This is to state that the research carried out for my Doctoral thesis entitled "N-linked Glycopeptide Mimetics as Tools in Kinetic, Mechanistic and Structural Studies of Peptide N:Glycanase F" under the auspices of IMBS at IRL with the approval of the Head of Institute and in the Institute of Molecular BioSciences at Massey University, Palmerston North, New Zealand is all my own work. This is also to certify that thesis material has not been used for any other degree.

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SUPERVISOR'S DECLARATION

This is to state that the research carried out for the Doctoral thesis entitled "N-linked Glycopeptide Mimetics as Tools in Kinetic, Mechanistic and Structural Studies of Peptide N:Glycanase F" was done by Mr Dirk Henning Lenz in the Institute of Molecular BioSciences at Massey University, Palmerston North, New Zealand and at IRL, Lower Hutt (with the approval of the Head of Institute, IMBS). This thesis material has not been used in part or in whole for any other qualification, and I confirm that the candidate has pursued the course of study in accordance with the requirements of the Massey University regulations.

Supervisor's Name: Gillian E. Norris

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ABSTRACT

PNGases (Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidases (E.C. 3.5.1.52) cleave the carbohydrate chains from the asparagine side chains of glycoproteins. They are widely used to deglycosylate *N*-linked glycoproteins and glycopeptides for analytical purposes. PNGase F from *Flavobacterium meningosepticum* is the best characterised of this class of enzymes but little is known so far about the biological significance or the catalytic mechanism of these intriguing enzymes.

The substrate binding and cleavage mechanism of PNGase F has now been investigated.

The first part of this work describes the synthesis of various novel *N*-linked glycopeptide mimetics which were then used in kinetic investigations with PNGase F. To facilitate kinetic studies at low substrate concentrations, a discontinuous HPLC based assay using a fluorescently labelled ovalbumin glycopeptide had to be developed. These experiments led to a better understanding of the structural requirements for substrate binding which will aid the future development of potent PNGase F inhibitors.

In the second part of the thesis, a virtual *N*-linked glycopeptide from human lactoferrin was modelled into the active site region of PNGase F using molecular modelling techniques. This model has resulted in the proposal of a mechanism for catalysis that predicts an important role for Arginine 248, a residue that had previously not been considered part of the catalytic machinery. The model also provides a basis for explaining the substrate specificity of the enzyme. The mechanism is supported by kinetic studies with targeted PNGase F mutants. As a result of this study, new PNGase F mutants have been designed to test the current findings.

To my Family

To be consistent [...]: no penicillin, no lightning rods, no eyeglasses, no DDT, no radar and so on. We live technologically, with man as the master of nature, man as the engineer, and let anyone who raises his voice against it stop using bridges not built by nature. To be consistent, they would have to reject any kind of operation; that would mean people dying every time they had appendicitis. What an outlook! No electric-light bulbs, no engines, no atomic energy, no adding machines, no anesthetics-back to jungle!

From "Homo Faber", A Report by Max Frisch (1959)

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This investigation would not have been possible without the valuable input from many people:

Natalie for her support and understanding during a truly difficult and exciting phase of my life. I wish you all the best for the final phase of your own PhD. Thumbs up.

My parents for their support of my scientific spirit.

Prof. Dr. Joachim Thiem for introducing me into the world of carbohydrates.

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George Slim for his witty spirit, exquisite taste in music and enthusiasm for classic cars. I fully agree that a car (in its conceptual context) is a piece of art and not designed to simply get from A to B.

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Posthumous **André Citroën, Pierre Boulanger, André Lefèbvre, Paul Mages and Flaminio Bertoni** the names of which are inseparably connected with a pinnacle of automobile engineering, "la déesse (DS)" (the goddess) [1].

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LIST OF ABBREVIATIONS

Boc	*t*-butyl oxycarbonyl
BTP	(1,3-bis[tris(hydroxymethyl)-methylamino]propane)
CNBr	cyanogen bromide
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	dicyclohexylcarbodiimide
DDQ	2,3-dichloro-5,6-dicyano-*p*-benzoquinone
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DTPA	diethylenetriamine pentaacetate
EA	ethylacetate
eq.	equivalent
ER	endoplasmatic reticulum
ES-MS	Electrospray Mass Spectrometry
Eu	europium
FITC	fluoresceine isothiocyanate
Fmoc	9-fluorenyl methoxycarbonyl
FPLC	Fast Performance Liquid Chromatography
Fuc	fucose
Glc	glucose
GlcNAc	*N*-acetyl glucosamine
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HPLC	High Pressure/Performance Liquid Chromatography
Man	mannose
NMR	Nuclear Magnetic Resonance
PE	petroleum ether
Phth	phthalimido
PMB	*p*-methoxybenzyl
PTSA	*p*-toluenesulphonic acid
r.t.	room temperature
RP-HPLC	Reverse Phase-HPLC

sat.	saturated
SDS	sodium dodecyl sulfate
SDS/PAGE	sodium dodecyl sulfate/Polyacrylamide Gel Electrophoresis
SPPS	Solid Phase Peptide Synthesis
TBDMS	*tert*-butyl dimethylsilyl
TFA	trifluoroacetic acid
TLC	Thin Layer Chromatography
TMSCN	trimethylsilyl cyanide
TMSOTf	trimethylsilyl trifluoromethanesulfonate
Tol	toluene