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The Secreted Aspartic Proteinases of *Candida albicans*

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor
of Philosophy in the Institute of Molecular BioSciences at
Massey University, New Zealand

Michelle E. Sullivan

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To my parents

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ABSTRACT

The common human fungal pathogen, *Candida albicans*, possesses at least nine genes encoding secreted aspartic proteinases (*SAPs*). Saps are widely regarded as virulence factors, despite historical controversy surrounding their actual roles in the onset and development of candidosis. While Sap1, Sap2 and Sap3 had been previously studied at the biochemical level, Sap4 and Sap5 had not been detected, purified or characterised. To facilitate analysis of the proteins, *SAP4* and *SAP5* were amplified by PCR and cloned. The nucleotide changes in *SAP4* were silent, and *SAP5* contained one conserved amino acid substitution, compared with the published sequences. The methylotrophic yeast *Pichia pastoris* was used as a host for heterologous expression of *SAP4* and *SAP5*, and the respective proteins were purified to homogeneity. Purification of Sap4 involved Mono Q anion exchange chromatography at pH 7.0, while Sap5 was purified by cation exchange chromatography on Resource S at pH 7.0. *C. albicans SAP1* was also over-expressed in *P. pastoris* as a control. Biochemical analysis of the recombinant proteins revealed that Sap4 and Sap5 were optimally active at pH 4.5, 1-2 pH units higher than the optima of Saps1, 2 and 3. At optimum pH, the specific activities of Sap4 and Sap5 were 239 and 33 μg tyrosine equivalents/min, respectively. These isoenzymes also retained significant activity at pH 7.0, which suggested roles in the disease process at host sites of neutral pH. Sap4 and Sap5 showed decreased specific activity towards denatured globin, and increased specific activity towards a fluorocasein substrate, when compared with Saps1-3. Substrate specificity analyses (performed using a peptide substrate, glucagon, and MALDI-TOF of the purified peptide fragments), showed that Sap4 and Sap5 hydrolysed the glucagon at the same sites, but the analysis did not reveal a consensus cleavage sequence. The deduced masses of Sap1, Sap4 and Sap5 were 36,179, 36,995 and 37,256, respectively. However, ES-MS indicated the masses of the recombinant Sap1 and Sap4 were larger than expected, by 2232 and 2041 respectively. Glycopeptide fragment ion analysis suggested the additional mass was due to attached sugar residues. Carbohydrate chromatography confirmed the presence of mannose and N-acetyl glucosamine. The presence of N-acetyl glucosamine species, and the lack of consensus *N*-linked glycosylation sites in the Sap1, Sap4 and Sap5 proteins suggests a novel pattern of *O*-linked glycosylation in *P. pastoris*. The purified enzymes were subjected to crystallisation trials and some promising crystals were produced. Previous studies showed that *SAP4, 5* and *6* are expressed during serum-induced germ-tube formation, but this was not confirmed in this study.

TABLE OF CONTENTS

Abstract i

Table of contents iii

List of figures ix

List of tables xii

Amino acid abbreviations and colour coding xiii

Abbreviations xiv

Chapter 1.0 Introduction 1

1.1 *Candida albicans* 1

1.2 Pathogenicity 3

1.2.1 Adhesion 4

1.2.2 Cell wall 4

1.2.3 Polymorphism 5

1.2.4 Phenotypic variation 6

1.2.5 Secreted hydrolases 6

1.2.6 Hydrophobicity 7

1.2.7 Signal transduction networks 8

1.3 Other *Candida* species 9

1.4 *C. albicans* and host immunity 9

1.5 Antifungal therapies 10

1.6 Phenotypic switching 12

1.7 Molecular mechanisms of phenotypic switching 14

1.8 Molecular mechanisms of polymorphism 17

1.9 Aspartic proteinases 21

1.9.1 Evolution 21

1.9.2 Substrate specificity 22

1.9.3 Structural features 23

1.9.4 Catalytic mechanism 23

1.10 Retroviral aspartic proteinases 24

1.11 Fungal aspartic proteinases 25

1.12 Secreted aspartic proteinases 26

1.13 Secretion of Sap 27

1.14 Regulation of Sap 28

1.15 Sap and pathogenicity 32

1.16 Sap4, Sap5 and Sap6 35

1.17 Sap structure 38

1.18 Project aims 39

2.0 Materials and Methods 40
2.1 Enzymes 40
2.2 Chemicals 40
2.3 Miscellaneous products 41
2.4 *Escherichia coli* phenotypes 41
2.5 Yeast strains 42
2.6 Plasmids utilised during this study 42
2.7 Plasmids developed during this study 42
2.8 Growth media 43
  2.8.1 Yeast media 43
  2.8.2 Bacterial media 43
  2.8.3 General buffers 44
2.9 General methods 44
  2.9.1 Restriction endonuclease DNA digestion 44
  2.9.2 Agarose gel electrophoresis of DNA 44
  2.9.3 DNA ligations 44
  2.9.4 Creating blunt-ended DNA 45
  2.9.5 Preparation and transformation of *E. coli* competent cells 45
    2.9.5.1 Preparation 45
    2.9.5.2 Transformation 45
  2.9.6 Selection of transformants 46
  2.9.7 Plasmid DNA preparation – small scale 46
  2.9.8 Plasmid DNA preparation – large scale 46
  2.9.9 DNA purification from a TAE agarose gel 46
  2.9.10 Genomic DNA purification from *C. albicans* 47
  2.9.11 PCR 47
  2.9.12 Southern blotting and hybridisation analyses 47
  2.9.13 Radiolabelling DNA 48
  2.9.14 Total RNA extraction from *C. albicans* 48
  2.9.15 Agarose gel electrophoresis of RNA 48
  2.9.16 Northern blotting and hybridisation analyses 49
  2.9.17 Oligonucleotide synthesis 49
  2.9.18 Dideoxy chain termination sequencing 49
2.10 Yeast and protein methods 49
  2.10.1 Preparation and transformation of yeast spheroplasts 49
  2.10.2 Screening yeast transformants 50
  2.10.3 *P. pastoris* growth and induction 50
  2.10.4 TCA precipitation of protein samples 51
  2.10.5 SDS-PAGE analysis 51
  2.10.6 Native PAGE analysis 51

2.10.7 Western blotting of proteins 51
2.10.8 N-terminal amino acid sequence analysis of proteins 52
2.10.9 Sap enzyme assays 53
2.10.9.1 Azocollagen assay 53
2.10.9.2 BSA-based assay 53
2.10.9.3 Globin-based assay 53
2.10.9.4 Fluorocasein-based assay 54
2.10.10 Substrate specificity of Sap4 and Sap5 54
2.10.11 Column chromatography 55
2.10.12 Carbohydrate cleavage 55
2.10.12.1 β-elimination reaction 55
2.10.12.2 Acid hydrolysis 56
2.10.13 DIG Glycan/protein analysis 56
2.10.13.1 Labelling 56
2.10.13.2 Detection 56
2.11 Antibody production 57
2.12 Crystallisation trials 57
2.13 Microscopy 58
2.14 Computing programs 58

3.0 Cloning and Vector Construction 59
3.1 Introduction 59
3.2 PCR amplification of *SAP4*, *SAP5* and *SAP6* 60
3.3 Optimising PCR amplification of *SAPs* 61
3.4 Restriction endonuclease analysis of PCR products 61
3.5 Analysis of *SAP4* and *SAP5* clones 66
3.6 DNA sequence analysis of *SAP4* and *SAP5* clones 70
3.7 Construction of heterologous expression vectors for Sap4 and Sap5 75
3.8 Construction of a Sap1 heterologous expression vector 78

4.0 Transformation and Expression 80
4.1 Introduction 80
4.2 Transformation of KM71 with heterologous expression constructs 81
4.3 Selection of KM71 transformants 81
4.4 Confirmation of plasmid integration 83
4.5 Preliminary expression of recombinant Sap proteins 83
4.6 Identification of the recombinant proteins 90
4.6.1 Identification by western blotting 90
4.6.2 Identification by enzyme assay 90
4.7 Large scale expression of Sap1, Sap4 and Sap5 92

4.7.1 Optimising strain storage conditions 92
4.7.2 Optimising starter culture conditions for Sap expression 95
4.7.3 Effect of methanol concentration on Sap expression 95
4.7.4 Effect of added buffer on Sap expression 95
4.7.5 Effect of induction culture size on Sap expression 97
4.7.6 Effect of culture aeration on Sap expression 97
4.8 Fermentation scale induction of Sap 99
4.9 Development of a new system for Sap expression 100
4.10 *E. coli* expression of Sap4 102
4.10.1 PCR primer design 102
4.10.2 Transformation of host strains 107
4.10.3 Expression of recombinant Sap4 107

5.0 Purification of Sap 108
5.1 Introduction 108
5.2 Purification of recombinant Sap1 109
5.3 Purification of recombinant Sap4 110
5.3.1 Anion exchange chromatography at pH 6.0 110
5.3.2 Cation exchange chromatography at pH 6.0 113
5.3.3 Anion exchange chromatography at pH 7.0 113
5.3.4 Anion exchange chromatography at pH 7.0, low ionic strength 114
5.3.5 Optimising the anion exchange gradient 114
5.3.6 Batchwise purification trials with DEAE sepharose 116
5.3.7 DEAE sepharose column chromatography 117
5.4 Large-scale purification of mature Sap4 118
5.5 Purification of recombinant Sap5 124
5.5.1 Anion exchange chromatography 124
5.5.2 Cation exchange chromatography 124
5.5.3 Gel filtration of Sap5 125
5.6 Sap 5 purification protocol 125

6.0 Biochemical Characterisation of Recombinant Sap Isoenzymes 126
6.1 Introduction 126
6.2 Sap enzyme activity assays 127
6.2.1 Sap4 substrate preference 127
6.2.2 Enzyme kinetics 127
6.2.2.1 Globin assay 127
6.2.2.2 Fluorocasein assay 129
6.3 pH optima of the Sap isoenzymes 131
6.4 Specific activity of the Sap isoenzymes 133

6.5 Substrate specificity of Sap 135
6.6 Thermal stability of the Sap isoenzymes 143
6.7 SDS-PAGE analysis of the Saps1-5 144
6.8 Native PAGE analysis of Saps1-5 144
6.9 Autodegradation of Sap isoenzymes 146
6.10 Pepstatin inhibition of Sap 146
6.11 Glycosylation of Sap 149
  6.11.1 Electrospray mass spectroscopy 149
  6.11.2 FACE analysis of Sap 153
  6.11.3 Carbohydrate chromatography 155
6.12 Interaction of recombinant Saps with SQAPI 162
6.13 Antibody production 164
6.14 Production of anti-Sap4 164
6.15 Purification of the IgG fraction 165
6.16 Titre of anti-Sap4 165
  6.16.1 Comparison of anti-Sap2 and anti-Sap4 165
  6.16.2 Anti-Sap4 titre 167
6.17 Specificity of anti-Sap4 167

7.0 Expression of Sap 171
7.1 Introduction 171
7.2 Expression of *SAPs 4, 5* and *6* 172
7.3 Northern analysis of *SAP4-6* expression: strains ATCC 10231 and A72 176
7.4 Northern analysis of *SAP4-6* expression: strain ATCC 10261 179
7.5 *In vitro* expression analyses 181

8.0 Crystallisation trials 183
8.1 Introduction 183
8.2 Crystallisation of Sap1 184
8.3 Crystallisation of Sap4 188
8.4 Crystallisation of Sap5 189
8.5 Homology modelling of Saps 1, 4 and 5 193

9.0 Discussion 198
9.1 Future work 208

10.0 Appendix 210
10.1 Sequence alignment of SAP1-SAP9 of *C. albicans* 211
10.2 Dionex analysis of Sap isoenzymes 214

10.2.1 Dionex calculations 214

10.2.2 Dionex analysis of Sap5 carbohydrate moieties 215

10.3 Purification and analysis of Sap5 216

10.3.1 Purification of Sap5 216

10.3.2 Anti Sap5 antiserum 218

10.4 ESMS data 219

10.5 MALDI-TOF analysis of glucagon peptide fragments 220

11.0 References 224

LIST OF FIGURES

1.1 Alternative morphologies of *C. albicans* 2
1.2 Organisation of *C. albicans* cell wall 5
1.3 Variant colony phenotypes in the switching repertoire of WO-1 14
1.4 A model for the regulation of white- and opaque-phase specific genes 16
1.5 A model for the regulation of pseudohyphal growth in *C. albicans* 20
1.6 Schematic representation of the substrate binding cleft of an aspartic proteinase 22
1.7 Expression of the *SAP* gene family in *C. albicans* 31
3.1 Protocol for generating *SAP* clones 62
3.2 Optimised PCR amplification of *SAP4, SAP5* and *SAP6* 64
3.3 Schematic of *SAP4, 5 & 6* showing relative positions of PCR primers and restriction sites .. 65
3.4 Restriction endonuclease analysis of *SAPs 4, 5* and *6* PCR products 67
3.5 Restriction digest analysis of *SAP4* and *SAP5* sequences cloned into pBluescript 69
3.6 Nucleotide sequence of *SAP4* from *C. albicans* strain ATCC 10261 71
3.7 Nucleotide sequence of *SAP5* from *C. albicans* strain ATCC 10261 72
3.8 Alignment of the sequence containing the amino acid substitutions in Sap5 with other members of the *C. albicans* Sap family 74
3.9 Schematic of expression vector construction 76
3.10 Screening pPIC3K clones for the correct orientation of *SAP4* and *SAP5* genes 77
3.11 Schematic of the *SAP1* cloning strategy 79
4.1 G418 screen of KM71 transformants 84
4.2 Schematic representation of pMS1 and pMS5 plasmids integrated into the KM71 genome .. 85
4.3 Southern blot of *P. pastoris* (KM71) transformants MS4A and MS5A, containing pMS4 and pMS5 86
4.4 SDS-PAGE of preliminary Sap1, Sap4 and Sap5 expression trials 88
4.5 N-terminal sequence of recombinant Sap proteins 89
4.6 Western analysis of Sap expression 91
4.7 Comparative induction of Sap4 and Bgl2 93
4.8 Effect of storage medium on Sap expression 94
4.9 Effect of starter culture medium on Sap expression 94
4.10 Effect of methanol concentration on Sap expression 96
4.11 Effect of buffer on Sap expression 96
4.12 Effect of induction culture volume on Sap expression 98
4.13 Effect of culture aeration on Sap expression 98
4.14 Sparger system for induction of recombinant Sap 101
4.15 PCR primers for *E. coli* expression 103
4.16 Strategy for cloning *SAP4* into pRSETa, an *E. coli* expression vector 104

4.17 SDS-PAGE and western blot analysis of *E. coli* expression of Sap4 107
5.1 Purification of Sap1 using ion-exchange chromatography 111
5.2 SDS-PAGE of Sap1 fractions from ion exchange chromatography 112
5.3 SDS-PAGE of Sap4 fractions from ion exchange chromatography (pH 7.0) 112
5.4 SDS-PAGE of Sap4 fractions from anion exchange chromatography at low ionic strength (pH 7.0) 115
5.5 Separation of the misprocessed species of Sap4 using Resource Q ion exchange chromatography 115
5.6 SDS-PAGE of Sap4 fractions from optimised anion exchange gradient 116
5.7 Batchwise DEAE Sepharose purification trial results 117
5.8 DEAE Sepharose chromatography at pH 7.0 119
5.9 Graph showing protein and Sap activity fractions from the DEAE Sepharose column 121
5.10 SDS-PAGE analysis of fractions from the DEAE Sepharose column 121
5.11 h.p.l.c. trace of Mono Q purification of Sap4 122
5.12 Sap4 at each stage of purification 123
5.13 Purified recombinant Sap4 123
6.1 Relative Sap4 activity toward various protein substrates 128
6.2 Time course of Sap activity in the globin assay 128
6.3 Plot of reaction velocity vs. substrate concentration 130
6.4 Hanes plot for Sap4 activity using the globin assay 130
6.5 The fluorocasein Sap assay 132
6.6 pH activity profiles of the Sap isoenzymes 132
6.7 Specific activities of Sap isoenzymes in the globin (A) and fluorocasein (B) assays 134
6.8 Reversed phase h.p.l.c of glucagon hydrolysates with Sap4 136
6.9 Reversed phase h.p.l.c of glucagon hydrolysis by Sap5 137
6.10 Model of cleavage of glucagon by Saps 1-5 140
6.11 Thermal stability profiles of Sap isoenzymes 143
6.12 SDS-PAGE analysis of Saps 1-5 145
6.13 Native PAGE analysis of Saps 1-5 145
6.14 Sap autodegradation 147
6.15 Pepstatin inhibition of Sap 148
6.16 ES mass spectra of Sap1, Sap4 and Sap5 149
6.17 Glycopeptide fragment ion analysis 150
6.18 DIG glycan/protein analysis of Saps 1, 2, 3 and 4 154
6.19 Sap Dionex analysis 158
6.20 Standard curve for quantitation of carbohydrate species 159
6.21 *O*-linked oligosaccharides of *S. cerevisiae* and *P. pastoris* 161
6.22 Biacore analysis of Sap interactions with SQAPI 163
6.23 Antibody titre after second booster 166
6.24 Western analysis of anti-Sap4 vs. *P. pastoris* supernatant 166

6.25 Comparison of the sensitivity of anti-Sap2 and anti-Sap4 168
6.26 Anti-Sap4 titre 168
6.27 Comparison of the specificity of anti-Sap2 and anti-Sap4 169
7.1 Time course of serum-induced hyphal formation 173, 174
7.2 Clumped *C. albicans* strain ATCC 10231 hyphae 175
7.3 RNA extracted from strain ATCC 10231 177
7.4 Northern hybridisations for *SAP4-6* and *ACT* 178
7.5 Northern hybridisations for *SAP4-6* and *LEU2* 180
8.1 Crystals of native Sap5 from the sparse matrix screen and crystal screen II 190
8.2 Crystals of Sap4 complexed with A70450 inhibitor 191
8.3 Crystals of Sap4 complexed with SQAPI inhibitor 192
8.4 Homology models using the Sap2 structure as a template 195
8.5 Active site models of Saps 1, 4 and 5 196
8.6 Sap2 active site structure (in complex with A70450) overlaid with the Sap4 model 197
9.1 Model of proposed roles of Sap isoenzymes in the onset and development of candidosis ...206
10.1 Alignment of the amino acid sequences of Sap1-Sap9 of *C. albicans* 211
10.2 Dionex chromatograms of Sap5 215
10.3 Purification of Sap5 using ion-exchange chromatography 216
10.4 SDS-PAGE of Sap5 following ion-exchange chromatography 217
10.5 Analysis of the specificity of anti-Sap5 218

LIST OF TABLES

1.1 Antifungal agents currently used for treatment of *Candida* infections 12
1.2 Table of identity between *SAP* sequences from *C. albicans* 27
3.1 Summary of the PCR conditions for *SAP4*, *SAP5* and *SAP6* PCR amplification reactions 63
3.2 Comparison of the expected and actual band sizes from restriction enzyme analysis of *SAP4*, *SAP5* and *SAP6* PCR products 68
5.1 Purification of recombinant Sap1 111
5.2 Purification of mature Sap4 122
6.1 Glucagon fragments produced by Sap4 and Sap5 139
6.2 Consensus sequences of Saps 1-5 cleavage sites in glucagon 141
6.3 Amino acid side chains which form subsites S_1 and S_1' 142
6.4 Predicted and experimental masses of Saps 152
6.5 Carbohydrate destruction during acid hydrolysis 156
8.1 Sparse matrix crystal screen 185
8.2 Crystal screen II 186
8.3 Additional crystallisation conditions for Sap1 and Sap4 187

AMINO ACID ABBREVIATIONS & COLOUR

Amino acid	Abbreviation	Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or Aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or Glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Grey	General non-polar	A,M,I,L,V
Green	General polar	S,T,N,Q
Blue	Positively charged	H,K,R
Red	Negatively charged	D,E
Purple	Aromatic	F,W,Y
Brown	“Structure breakers”	G,P
Yellow	Cysteine	C

ABBREVIATIONS

Amp	Ampicillin
BCIG	5'-bromo-4-chloro-3-indolyl-γ-D-galactopyranoside
BCIP	5'-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	Bovine serum albumin
CAPS	3-cyclohexylamino-1-propanesulphonic acid
cDNA	Complementary DNA
C-terminal	Carboxy-terminal
Da	Dalton
DEAE	Diethylaminoethyl
DIG	Digoxigenin
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
ES-MS	Electrospray mass spectrometry
G418	Geneticin – disulphide salt
GalNAc	N-acetyl galactosamine
GlcNAc	N-acetyl glucosamine
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid
h.p.l.c.	High pressure liquid chromatography
IPTG	Isopropyl-γ-D-thiogalactopyranoside
kb	kilobase
kDa	kiloDalton
MOPS	3-[N-morpholino]propanesulphonic acid

NBT	Nitro blue tetrazolium
mRNA	messenger RNA
N-terminal	Amino-terminal
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pI	Isoelectric point
PIPES	Piperazine-n,n'-bis(2-ethanesulphonic acid)
PVDF	Polyvinylidenedifluoride
RNA	Ribonucleic acid
SAP	SAP gene
Sap	Sap protein
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene-sorbitan monolaurate
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight

C. albicans GENETIC CODE

	SECOND POSITION				
FIRST POSITION	T	C	A	G	THIRD POSITION
T	TTT Phe	TCT Ser	TAT Tyr	TGT Cys	**T**
	TTC Phe	TCC Ser	TAC Tyr	TGC Cys	**C**
	TTA Leu	TCA Ser	TAA *	TGA *	**A**
	TTG Leu	TCG Ser	TAG *	TGG Trp	**G**
C	CTT Leu	CCT Pro	CAT His	CGT Arg	**T**
	CTC Leu	CCC Pro	CAC His	CGC Arg	**C**
	CTA Leu	CCA Pro	CAA Gln	CGA Arg	**A**
	CTG Ser	CCG Pro	CAG Gln	CGG Arg	**G**
A	ATT Ile	ACT Thr	AAT Asn	AGT Ser	**T**
	ATC Ile	ACC Thr	AAC Asn	AGC Ser	**C**
	ATA Ile	ACA Thr	AAA Lys	AGA Arg	**A**
	ATG Met	ACG Thr	AAG Lys	AGG Arg	**G**
G	GTT Val	GCT Ala	GAT Asp	GGT Gly	**T**
	GTC Val	GCC Ala	GAC Asp	GGC Gly	**C**
	GTA Val	GCA Ala	GAA Glu	GGA Gly	**A**
	GTG Val	GCG Ala	GAG Glu	GGG Gly	**G**

Codons are as they appear in the coding strand of DNA, reading 5’→3’. Termination codons are represnted by *. Note the unusual decoding of CTG as a Serine in *C. albicans*, rather than the standard amino acid Leucine.