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# Characterisation of Lysozyme-Steroid Glucuronide Conjugates

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## Abstract

The steroid glucuronides estrone glucuronide and pregnanediol glucuronide were synthesised using the O-glycosylation reaction of a glycosyl donor with the appropriate steroid under standard Koenigs-Knorr conditions. X-ray crystallographic studies showed that the synthetic estrone glucuronide molecule had the correct stereochemistry. Estrone glucuronide and pregnanediol glucuronide conjugates of hen egg white lysozyme were prepared by both the mixed anhydride and active ester coupling procedures. Both methods gave good yields of conjugate but the active ester procedure gave a more diverse range of products. Unreacted lysozyme, which was present in all cases, was removed by a combination of cation-exchange and hydrophobic-interaction chromatography to give purified conjugate material whose lytic activity was inhibited by over 90% in the presence of excess anti-steroid glucuronide antibody. Steroid glucuronide-lysozyme conjugates purified in this way could be used in a homogeneous enzyme immunoassay system to measure the levels of urinary estrone and pregnanediol glucuronide encountered in a normal menstrual cycle. Chromatography of the conjugation reaction mixtures on an S-Sepharose (fast flow) column in the presence of 7 M urea allowed the isolation of the different conjugate products. Conjugation of lysozyme with estrone glucuronide by the mixed anhydride method gave one major derivative exclusively acylated at lysine residue 33 while acylation with pregnanediol glucuronide gave two major derivatives exclusively acylated at lysine residues 33 and 97 respectively. On the other hand, conjugation of lysozyme with the two steroid glucuronides by the active ester method gave six derivatives which were acylated at combinations of one or more of three lysine residues, 33, 97, and 116. The correlation of the protein environments of the lysine amino groups in the crystal structure of lysozyme with the acylation positions in the conjugate families suggested that these positions were determined not only by the surface accessibility and nucleophilicity of the lysine residues but also by the steroid glucuronide and the acylating reagent. Computer derived three dimensional structures of the estrone glucuronide-lysozyme and pregnanediol glucuronide-lysozyme conjugates suggested that the enzyme conjugate may be inactivated by the antibody in the immune complex by either providing a physical barrier to approach by the large bacterial substrate or by disrupting the binding of the bacterial cell wall polymer into the active site cleft. The lytic activity of the estrone glucuronide-lysozyme E3 conjugate was not inhibited in the presence of excess antibody when the small chitohexaose substrate was used, implying that the substrate could access the active site even when the conjugate was bound by antibody. The detailed characterisation of the mixed anhydride estrone glucuronide-lysozyme conjugate coupled with the current knowledge of the antigenic determinants of hen egg white lysozyme has made it possible to design, in principle, a novel sandwich solid phase immunoassay format for the measurement of estrone glucuronide levels.

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## Table of Contents

	<b>Page</b>
<b>Abstract</b>	ii
<b>Acknowledgements</b>	iii
<b>Acknowledgement of Published Work</b>	iv
<b>Table of Contents</b>	v
<b>List of Figures</b>	xv
<b>List of Tables</b>	xxv
<b>List of Schemes</b>	xxvii
<b>Abbreviations</b>	xxviii
<b>Amino Acid Abbreviations</b>	xxx

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### Chapter One

#### Introductory Chapter

The Framework for the Study Discussed in the Following Chapters

<b>1.1</b>	The Physiological Mechanisms and Control of the Menstrual Cycle	1
1.1.1	The Menstrual Cycle and the Hypothalamic-Pituitary-Ovarian Axis	1
1.1.2	Recruitment, Selection and Emergence of the Dominant Follicle	3
1.1.2.1	The Scheele and Schoemaker model [10] for the selection of the dominant follicle	4
1.1.3	Continued Growth of the Dominant Follicle and the Excretion of Ovarian Steroid Hormones	6
1.1.3.1	The follicular phase of the menstrual cycle	6
1.1.3.2	The luteal phase of the menstrual cycle	8
<b>1.2</b>	Biosynthesis of the Ovarian Steroid Hormones	11
1.2.1	The Synthesis of Progesterone	11
1.2.2	The Synthesis of Androgens and Estrogens	12
1.2.3	Metabolism and Excretion of Ovarian Steroids	14
<b>1.3</b>	Markers used for Definition of the Fertile Period	17
1.3.1	Natural Family Planning (NFP)	17

1.3.2	Steroid Hormone Metabolites as Markers of the Fertile Period	18
1.3.2.1	Methods for the recognition of first estrogen rises to define the beginning of the fertile period	19
1.3.2.2	Definition of the end of the fertile period	21
1.4	Methods for the Measurement of Urinary Estrone Glucuronide and Pregnanediol Glucuronide as Markers of the Fertile Period	23
1.4.1	Early Measurements of Steroid Glucuronide Levels	23
1.4.2	Immunoassays	24
1.4.2.1	Radioimmunoassay	24
1.4.2.2	Enzyme immunoassay	25
1.5	The Ovarian Monitor	28
1.6	New Systems for the Definition of the Fertile Period	34
1.6.1	The Problems Associated with the Development of New Colour Assay Systems	34
1.6.2	The Unipath Personal Contraceptive System	34
1.6.3	Difficulties with Horseradish Peroxidase as an Enzyme for Producing a Colour Test	35
1.7	Aims of the Current Study	37

---

## Chapter Two

### The Preparation and Purification of Estrone Glucuronide- and Pregnanediol Glucuronide-Lysozyme Conjugates

Conjugates Suitable for Use in a Homogeneous Enzyme Immunoassay System for the Measurement of Menstrual Cycle Levels of Urinary Estrone Glucuronide and Pregnanediol Glucuronide

2.1	Introduction	38
2.2	Experimental	42
2.2.1	Apparatus	42
2.2.2	Reagents	42
2.2.3	Methods	43
2.2.3.1	Synthesis of methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl- $\alpha$ -D-glucopyranuronate	43
2.2.3.2	Synthesis of 17-oxoestra-1,3,5(10)-triene-3-yl- $\beta$ -D-glucopyranosiduronic acid	43

2.2.3.3	Synthesis of 5 $\beta$ -pregnane-3 $\alpha$ ,20S-diol-3-yl- $\beta$ -D-glucopyranosiduronic acid	45
2.2.3.4	Purification of hen egg white lysozyme	46
2.2.3.5	Preparation of steroid glucuronide-lysozyme conjugates by the active ester method	47
2.2.3.6	Purification of steroid glucuronide-lysozyme conjugates	48
2.2.3.7	Lysine titrations	48
2.2.3.8	Preparation of estrone glucuronide-thyroglobulin and pregnanediol-3 $\alpha$ -glucuronide-thyroglobulin immunogen conjugates	49
2.2.3.9	Production of estrone glucuronide and pregnanediol-3 $\alpha$ -glucuronide antisera	49
2.2.3.10	Standard curves for estrone glucuronide and pregnanediol-3 $\alpha$ -glucuronide using lytic assays	49
<b>2.3</b>	<b>Results</b>	<b>50</b>
2.3.1	The Synthesis of Estrone Glucuronide (E1G[H]) and Pregnanediol Glucuronide (PdG[H])	50
2.3.2	The Preparation and Purification of Estrone Glucuronide-Lysozyme Conjugates	50
2.3.3	The Preparation and Purification of Pregnanediol Glucuronide-Lysozyme Conjugates	58
<b>2.4</b>	<b>Discussion</b>	<b>64</b>
2.4.1	The Synthesis of Estrone Glucuronide and Pregnanediol Glucuronide	64
2.4.2	The Synthesis and Purification of Estrone Glucuronide-Lysozyme and Pregnanediol Glucuronide-Lysozyme Conjugates	66
2.4.2.1	The preparation of steroid glucuronide-lysozyme conjugates	66
2.4.2.2	Analytical Mono-S cation-exchange chromatography in 7 M urea of conjugation reaction mixtures	66
2.4.2.3	The purification of steroid glucuronide-lysozyme conjugates by Mono-S cation-exchange chromatography under non-denaturing conditions	69
2.4.2.4	The chromatography of steroid glucuronide-lysozyme conjugates on hydrophobic Alkyl Superose columns	71

2.4.2.5	Possible explanation for the lower than expected levels of inhibition of the lytic activity of conjugates separated from unreacted lysozyme by strong cation-exchange chromatography on a Mono-S column	72
2.4.2.6	Standard curves for estrone glucuronide and pregnanediol glucuronide using lytic assays	73
2.5	Conclusions	74

---

## Chapter Three

### The Characterisation of Estrone Glucuronide- and Pregnanediol Glucuronide-Lysozyme Conjugates

3.1	Introduction	75
3.2	Experimental	77
3.2.1	Apparatus	77
3.2.2	Reagents	77
3.2.3	Methods	78
3.2.3.1	Preparation of estrone glucuronide-lysozyme and pregnanediol glucuronide-lysozyme conjugates by the mixed anhydride method	78
3.2.3.2	Preparation of estrone glucuronide-lysozyme and pregnanediol glucuronide-lysozyme conjugates by the active ester method	79
3.2.3.3	Purification of individual estrone glucuronide-lysozyme and pregnanediol glucuronide-lysozyme conjugates	80
3.2.3.4	Enzyme assays for lytic activity of chromatography column fractions	80
3.2.3.5	Reduction and alkylation of estrone glucuronide-lysozyme and pregnanediol glucuronide-lysozyme conjugates with iodoacetic acid	81
3.2.3.6	Tryptic digestion	81
3.2.3.7	Separation of trypsin fragments	81
3.2.3.8	Manual Edman sequencing	82
3.2.3.9	Analysis of tryptic peptides	82
3.2.3.10	Immunochemical analysis of estrone glucuronide-lysozyme E1 tryptic peptides	82

3.2.3.11	Analysis of estrone glucuronide-lysozyme conjugates by acid polyacrylamide gel electrophoresis	83
<b>3.3</b>	<b>Results</b>	<b>84</b>
3.3.1	Preparation and Purification of Individual Estrone Glucuronide-Lysozyme Conjugates	84
3.3.2	Preparation and Purification of Individual Pregnanediol Glucuronide-Lysozyme Conjugates	88
3.3.3	Identification of Native, Unmodified Lysozyme Tryptic Digest Peptides	92
3.3.4	Characterisation of E1G-Lysozyme Conjugate Families	93
3.3.4.1	Tryptic digestion of estrone glucuronide-lysozyme conjugate families E1 and E2	94
3.3.4.2	Tryptic digestion of estrone glucuronide-lysozyme conjugate family E3	98
3.3.4.3	Tryptic digestion of estrone glucuronide-lysozyme conjugate families E4, E5 and E6	98
3.3.4.4	Mass spectral data for the estrone glucuronide acylated peptides	100
3.3.5	Characterisation of PdG-Lysozyme Conjugate Families	102
3.3.5.1	Tryptic digestion of pregnanediol glucuronide-lysozyme conjugate families P1 and P2	103
3.3.5.2	Tryptic digestion of pregnanediol glucuronide-lysozyme conjugate families P3 and P4	106
3.3.5.3	Tryptic digestion of pregnanediol glucuronide-lysozyme conjugate families P5 and P6	108
3.3.5.4	Mass spectral data for the pregnanediol glucuronide acylated peptides	109
3.3.6	Analysis of Estrone Glucuronide-Lysozyme Conjugates by Acid Polyacrylamide Gel Electrophoresis	110
<b>3.4</b>	<b>Discussion</b>	<b>113</b>
3.4.1	The Effect of the Acylating Agent on the Preparation of Estrone Glucuronide-Lysozyme and Pregnanediol Glucuronide-Lysozyme Conjugates	113
3.4.2	The Purification of Individual Estrone Glucuronide-Lysozyme and Pregnanediol Glucuronide-Lysozyme Conjugates	114
3.4.2.1	Isolation and purification of individual estrone glucuronide-lysozyme conjugate families	114

3.4.2.2	Isolation and purification of individual pregnanediol glucuronide-lysozyme conjugate families	116
3.4.3	Characterisation of Individual E1G-Lysozyme Conjugate Families	117
3.4.4	Analysis of Estrone Glucuronide-Lysozyme Conjugates by Acid Polyacrylamide Gel Electrophoresis	121
3.4.5	Characterisation of Individual PdG-Lysozyme Conjugate Families	124
3.5	Conclusions	127

---

## Chapter Four

### Structural Aspects of the Steroid Glucuronide-Lysozyme Conjugate-Anti-Hapten Antibody Immune Complexes

4.1	Introduction	130
4.2	Experimental	132
4.3	Results and Discussion	133
4.3.1	Structural Modelling of the Estrone Glucuronide and Pregnanediol Glucuronide Moieties	133
4.3.2	The Importance of the Chemical Bridge Linking Hapten and Protein in Enzyme Immunoassay	136
4.3.3	Computer Models of the Tertiary Structures of the Estrone Glucuronide-Lysozyme Conjugate Family E6	138
4.3.4	The Molecular Basis for the Immune Recognition of an Antigen by Anti-Antigen Antibodies	142
4.3.5	Modelling of the Tertiary Structures of the Steroid Glucuronide-Lysozyme-Anti-Steroid Glucuronide Antibody Immune Complexes	146
4.4	Conclusions	153

---

## Chapter Five

### The Kinetics and Mechanism of the Estrone Glucuronide-Lysozyme-Anti-Estrone Glucuronide Antibody Binding Reaction

<b>5.1</b>	Introduction	154
<b>5.1.1</b>	The Mechanism of Lysozyme Action	154
<b>5.1.2</b>	Measurement of Lysozyme Activity	159
<b>5.1.3</b>	Mechanistic Detail of the Ovarian Monitor Assay System	160
<b>5.2</b>	Experimental	161
<b>5.2.1</b>	Apparatus	161
<b>5.2.2</b>	Reagents	161
<b>5.2.3</b>	Methods	161
5.2.3.1	Preparation of estrone glucuronide monoclonal antibody Fab fragments	161
5.2.3.2	Purification of Fab fragments	162
5.2.3.3	SDS-PAGE analysis of the fragmentation products during the preparation of Fab fragments from the intact IgG estrone glucuronide monoclonal antibody	163
5.2.3.4	Measurement of the displacement off-rate constants of the estrone glucuronide polyclonal and monoclonal antibodies, and the monoclonal Fab fragment, from the conjugate-antibody (or conjugate-Fab) immune complexes	164
5.2.3.5	Measurement of the apparent dissociation constant ( $K_d$ ) for the E3 conjugate-monoclonal antibody immune complex	167
5.2.3.6	Lytic activity of the mixed anhydride E1G-lysozyme E3 conjugate as measured by the hydrolysis of the non-bacterial hexa-N-acetyl-chitohexaose substrate	168
<b>5.3</b>	Results	170
<b>5.3.1</b>	Preparation of Estrone Glucuronide Monoclonal Antibody Fab Fragments	170
<b>5.3.2</b>	Displacement Off-Rate Constants of the Estrone Glucuronide Polyclonal and Monoclonal Antibodies and the Monoclonal Fab Fragment from the Conjugate-Antibody (or Conjugate-Fab) Immune Complexes	172
<b>5.3.3</b>	Apparent Dissociation Constant ( $K_d$ ) Measurements for the Conjugate-Monoclonal Antibody Immune Complex	177

5.3.4	Lytic Activity of the Mixed Anhydride Estrone Glucuronide E3 Conjugate as Measured by the Hydrolysis of the Non-Bacterial Hexa-N-Acetyl-Chitohexaose Substrate, (GlcNAc) <sub>6</sub>	184
5.4	Discussion	189
5.4.1	Preparation of Fab Fragments from the Estrone Glucuronide Monoclonal Antibody	189
5.4.2	Displacement Off-Rate Constants for Displacement of E1G-Lysozyme Conjugate E3 from the Estrone Glucuronide Polyclonal and Monoclonal Antibodies, and the Monoclonal Fab Fragment	191
5.4.3	Apparent Dissociation Constant ( $K_d$ ) Measurements for the Conjugate-Monoclonal Antibody Immune Complex	195
5.4.4	Lytic Activity of the Mixed Anhydride Estrone Glucuronide E3 Conjugate as Measured by the Hydrolysis of the Non-Bacterial Hexa-N-Acetyl-Chitohexaose Substrate, (GlcNAc) <sub>6</sub>	197
5.5	Conclusions	201

## Chapter Six

### Studies Toward the Development of a Colour Test Immunoassay For the Detection of the Fertile Period

6.1	Introduction	202
6.2	Colour Test Formats for the Measurement of Urinary E1G and PdG Levels using Steroid Glucuronide-Horseradish Peroxidase Conjugates	207
6.2.1	Introduction	207
6.2.2	Apparatus, Reagents, and Methods	207
6.2.2.1	Apparatus	207
6.2.2.2	Reagents	208
6.2.2.3	Preparation of an estrone glucuronide-horseradish peroxidase conjugate	209
6.2.2.4	Ion-exchange and hydrophobic interaction chromatography analysis of the estrone glucuronide-horseradish peroxidase conjugation reaction mixture	209
6.2.2.5	Purification of the estrone glucuronide polyclonal antibody from other serum proteins	209

6.2.2.6	Preparation of nylon membranes for use in, and the procedures involved in, immunofiltration experiments for the detection of estrone glucuronide levels	210
6.2.2.7	Preparation of nitrocellulose membranes for use in, and the procedures involved in, colloidal gold systems for the detection of estrone glucuronide levels	212
<b>6.2.3</b>	<b>Results and Discussion</b>	<b>214</b>
6.2.3.1	The preparation and analysis of an estrone glucuronide-horseradish peroxidase conjugate	214
6.2.3.2	The purification of the estrone glucuronide polyclonal antibody from other serum proteins	217
6.2.3.3	Immunofiltration colour tests for the measurement of estrone glucuronide levels	218
6.2.3.4	Colloidal gold based colour tests for the measurement of estrone glucuronide levels	221
<b>6.3</b>	<b>Development of a Lysozyme Colloidal Gold Based Colour Test for the Measurement of Estrone Glucuronide Levels</b>	<b>226</b>
<b>6.3.1</b>	<b>Introduction</b>	<b>226</b>
<b>6.3.2</b>	<b>Apparatus, Reagents, and Methods</b>	<b>226</b>
<b>6.3.3</b>	<b>Results and Discussion</b>	<b>226</b>
<b>6.4</b>	<b>The Design of a Possible Solid Phase Sandwich Immunoassay for the Measurement of Estrone Glucuronide Levels using a Lysozyme-Estrone Glucuronide Conjugate</b>	<b>232</b>
<b>6.5</b>	<b>Conclusions</b>	<b>237</b>

## Chapter Seven

### Crystallographic Studies

<b>7.1</b>	<b>Introduction</b>	<b>238</b>
<b>7.1.1</b>	<b>The Principles of X-ray Crystallographic Analysis</b>	<b>238</b>
7.1.1.1	Crystal lattices, the unit cell, and the symmetry in crystal systems	238
7.1.1.2	The process of X-ray diffraction	241
<b>7.1.2</b>	<b>The Rationale for Attempting to Obtain the Three Dimensional Structures of the Components which Constitute the Ovarian Monitor Immunoassay System for Estrone Glucuronide</b>	<b>245</b>

<b>7.2</b>	<b>The X-ray, Three Dimensional Structural Analysis of Estrone Glucuronide (E1G)</b>	<b>248</b>
7.2.1	Experimental	248
7.2.2	Results and Discussion	249
<b>7.3</b>	<b>Crystallisation and X-ray Studies of an Estrone Glucuronide-Lysozyme Conjugate</b>	<b>255</b>
7.3.1	Initial Crystallisation Studies	255
7.3.2	Further Crystallisation Studies	258
7.3.3	Data Collection, Processing and Structure Determination from an Estrone Glucuronide-Lysozyme Conjugate Crystal	259
7.3.3.1	Data collection	259
7.3.3.2	Data processing	259
7.3.3.3	Structure determination	261
7.3.3.4	Molecular replacement	261
7.3.3.5	X-PLOR	263
7.3.3.6	Results and discussion of the structure determination	263
<b>7.4</b>	<b>Crystallisation Studies of Estrone Glucuronide-Lysozyme Conjugate-Fab Immune Complexes</b>	<b>267</b>
7.4.1	Initial Crystallisation Attempts	267
7.4.2	Future Crystallisation Attempts	269
<b>7.5</b>	<b>Conclusions</b>	<b>270</b>
<hr/>		
	<b>Summary</b>	<b>271</b>
	<b>Bibliography</b>	<b>273</b>

## List of Figures

Figure		Page
1.1.1	A schematic representation of the three-pillar cornet model for follicle selection in the normal menstrual cycle as proposed by Scheele and Schoemaker [10]	5
1.1.2	A schematic representation of pre-ovulatory follicular development in the menstrual cycle in relation to LH, FSH, and estradiol levels throughout the follicular phase of the menstrual cycle	7
1.1.3	The mean values of serum luteinising hormone, follicle stimulating hormone, estradiol, and progesterone as seen in a normal human menstrual cycle	9
1.1.4	The mean serum estradiol and progesterone concentrations measured every two hours for five days during the midcycle of the menstrual cycle in seven cycles as published by Hoff <i>et al.</i> [25]	10
1.2.1	The conventional representation of the steroid glucuronide molecules	15
1.2.2	The daily mean serum estradiol and urinary estrone conjugates concentrations in ten ovulatory menstrual cycles	16
1.3.1	The most common urinary total estrogen and pregnanediol value patterns observed in a normal menstrual cycle	19
1.3.2	An idealised profile of the daily levels of urinary estrone glucuronide (E1G) and pregnanediol glucuronide (PdG) excreted during a normal menstrual cycle	22
1.4.1	The principle reactions involved in radioimmunoassay	25
1.4.2	The principles behind the homogeneous enzyme immunoassay technique	27
1.5.1	The components of the Ovarian Monitor assay system	29
1.5.2	The three principle reactions involved in the measurement of the ovarian steroid metabolites estrone glucuronide and pregnanediol glucuronide by the homogeneous enzyme immunoassay system in the Ovarian Monitor	30
1.5.3	A standard curve generated for the measurement of urinary estrone glucuronide levels	32

1.5.4	A normal menstrual cycle profile for the levels of urinary estrone glucuronide and pregnanediol glucuronide obtained using the Ovarian Monitor	33
2.1.1	CPK space-filling representation of the three dimensional structure of hen egg white lysozyme as revealed by the crystallographic studies of Ramanadham <i>et al.</i> [118] with the active site cleft orientated to the right hand side of the molecule	39
2.1.2	CPK space-filling representation of the three dimensional structure of hen egg white lysozyme as revealed by the crystallographic studies of Ramanadham <i>et al.</i> [118] with the active site cleft orientated to the left hand side of the molecule	39
2.2.1	The structure of methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl- $\alpha$ -D-glucopyranuronate (bromosugar)	43
2.2.2	The structure of 17-oxoestra-1,3,5(10)-triene-3-yl- $\beta$ -D-glucopyranosiduronic acid (estrone glucuronide) and numbering system	45
2.2.3	The structure of 5 $\beta$ -pregnane-3 $\alpha$ ,20S-diol-3-yl- $\beta$ -D-glucopyranosiduronic acid (pregnanediol glucuronide) and numbering system	46
2.3.1	ElG-lysozyme active ester conjugation reaction mixture in 7 M urea on a Mono-S cation-exchange HR 5/5 column	51
2.3.2	ElG-lysozyme active ester conjugation reaction mixture on a Mono-S cation-exchange HR 5/5 column	53
2.3.3	Fractions a, c, and e from Fig. 2.3.2 in 7 M urea on a Mono-S cation-exchange HR 5/5 column	53
2.3.4	ElG-lysozyme conjugate reaction mixture on a Mono-S cation-exchange HR 5/5 column in the presence of 2-propanol	55
2.3.5	Fraction e from Fig. 2.3.2 in 1.4 M ammonium sulfate on an Alkyl Superose hydrophobic interaction HR 5/5 column	55
2.3.6	Fraction f and g from Fig. 2.3.5 in 7 M urea on a Mono-S cation-exchange HR 5/5 column	56
2.3.7	An ElG standard curve using (1) conjugate fraction g from Fig. 2.3.5 and (2) the mixed anhydride conjugate from the Ovarian Monitor	56
2.3.8	A typical example of a menstrual cycle pattern obtained using the standard curves described in Fig. 2.3.7	57
2.3.9	PdG-lysozyme active ester conjugation reaction mixture in 7 M urea on a Mono-S cation-exchange HR 5/5 column	58

2.3.10	PdG-lysozyme active ester conjugation reaction mixture on a Mono-S cation-exchange HR 5/5 column	59
2.3.11	Fractions h, i, j, and k from Fig. 2.3.10 in 7 M urea on a Mono-S cation-exchange HR 5/5 column	60
2.3.12	Fraction k from Fig. 2.3.10 in 1.4 M ammonium sulfate on an Alkyl Superose hydrophobic interaction HR 5/5 column	61
2.3.13	Fraction m from Fig. 2.3.12 in 7 M urea on a Mono-S cation-exchange HR 5/5 column	62
2.3.14	A PdG standard curve using (1) conjugate fraction m from Fig. 2.3.12 and (2) the mixed anhydride conjugate from the Ovarian Monitor	63
3.3.1	E1G-lysozyme conjugate reaction mixtures (mixed anhydride and active ester methods) in 7 M urea on a Mono-S cation-exchange HR 5/5 column	84
3.3.2	E1G-lysozyme conjugate reaction mixtures (active ester and mixed anhydride methods) in 7 M urea on an S-Sepharose (fast flow) column	85
3.3.3	The A <sub>280</sub> profile of E1G-lysozyme mixed anhydride conjugate family E3 on a Butyl Sepharose hydrophobic interaction column	88
3.3.4	PdG-lysozyme conjugate reaction mixtures (mixed anhydride and active ester methods) in 7 M urea on a Mono-S cation-exchange HR 5/5 column	89
3.3.5	PdG-lysozyme conjugate reaction mixtures (mixed anhydride and active ester methods) in 7 M urea on an S-Sepharose (fast flow) column	90
3.3.6	The HPLC profile from the amino acid sequencer when a lysine residue acylated with estrone glucuronide was sequenced	94
3.3.7	Reversed-phase HPLC separation of the tryptic peptides from tryptic digests of unmodified lysozyme, and active ester E1G-lysozyme conjugates E1, and E2	96
3.3.8	The immune reactivities of the tryptic peptides from unmodified lysozyme and active ester E1G-lysozyme conjugate E1 as measured using an Ovarian Monitor	97
3.3.9	Reversed-phase HPLC separation of the tryptic peptides from tryptic digests of active ester E1G-lysozyme conjugate E3 and mixed anhydride E1G-lysozyme conjugate E3	99

3.3.10	Reversed-phase HPLC separation of the tryptic peptides from tryptic digests of active ester E1G-lysozyme conjugates E4, E5, and E6	100
3.3.11	A typical electrospray mass spectrometry analysis as recorded for peptide peak m derived from the tryptic digestion of E1G-lysozyme conjugates	101
3.3.12	Reversed-phase HPLC separation of the tryptic peptides from tryptic digests of unmodified lysozyme, mixed anhydride PdG-lysozyme conjugate P1, and active ester PdG-lysozyme conjugates P1, and P2	105
3.3.13	Reversed-phase HPLC separation of the tryptic peptides from the tryptic digests of mixed anhydride PdG-lysozyme conjugates P3, and P4, and active ester PdG-lysozyme conjugates P3 and P4	107
3.3.14	Reversed-phase HPLC separation of the tryptic peptides from the tryptic digests of active ester PdG-lysozyme conjugates P5, and P6, and mixed anhydride PdG-lysozyme conjugate P5	108
3.3.15	A typical electrospray mass spectrometry analysis recorded for peptide peak f derived from the tryptic digestion of PdG-lysozyme conjugates	109
3.3.16	Analysis of E1G-lysozyme active ester conjugates by acid-PAGE	111
4.1.1	The conventional schematic representation of the basic Y-shaped immunoglobulin molecule	131
4.3.1	The basic steroid skeleton structure, numbering system and ring designations	134
4.3.2	Computer generated three dimensional structures of the estrone glucuronide moiety after energy minimisation	135
4.3.3	Computer generated three dimensional structures of the pregnanediol glucuronide moiety after energy minimisation	135
4.3.4	CPK space-filling representation of a computer generated estrone glucuronide-lysozyme conjugate tertiary structure showing the positions of acylation and orientation of the E1G moiety relative to the lysozyme molecule and the active site cleft when acylation occurs at lysine residues 33, 97 and 116 as in the active ester E6 conjugate family with the active site cleft oriented to the left hand side of the molecule	139

- 4.3.5 CPK space-filling representation of the computer generated estrone glucuronide-lysozyme conjugate E6 tertiary structure described in Fig. 4.3.4 with the active site cleft oriented to the right hand side of the molecule 139
- 4.3.6 Ribbon diagram representation of Fig. 4.3.5 which clearly shows the dog leg formed between the acylated lysine residue and the steroid glucuronide moiety as seen above the active site cleft when acylation occurs at lysine residue 116 140
- 4.3.7 CPK space-filling representation of the computer generated E6 conjugate family tertiary structure when looking directly into the active site cleft 140
- 4.3.8 Three dimensional structures of four lysozyme-Fab complexes solved by X-ray crystallographic studies 143
- 4.3.9 Ribbon diagram representation of the three dimensional structure of a progesterone-Fab complex 143
- 4.3.10 A schematic representation of the differences in Fab-hapten and Fab-protein antigen binding sites 145
- 4.3.11 The structures of the progesterone steroid moiety, pregnanediol glucuronide, and estrone glucuronide showing the carbon atoms of the steroid glucuronides used in the least squares fit to the progesterone structure 147
- 4.3.12 CPK space-filling representation of the computer generated three dimensional structure of the PdG-lysozyme anti-hapten antibody immune complex when the PdG moiety is attached at lysine residue 116 and the active site cleft is oriented to the left hand side 148
- 4.3.13 CPK space-filling representation of the computer generated three dimensional structure of the PdG-lysozyme anti-hapten antibody immune complex when the PdG moiety is attached at lysine residue 116 and the active site cleft is oriented to the right hand side 148
- 4.3.14 CPK space-filling representation of the computer generated three dimensional structure of the PdG-lysozyme anti-hapten antibody immune complex when the steroid glucuronide moiety is attached at lysine residue 97 150
- 4.3.15 CPK space-filling representation of the computer generated three dimensional structure of the PdG-lysozyme anti-hapten antibody immune complex when the PdG moiety is attached at lysine residue 33 150

- 4.3.16 CPK space-filling representation of the computer generated three dimensional structure of the E1G-lysozyme anti-hapten antibody immune complex when the E1G moiety is attached to lysine residue 33 151
- 4.3.17 CPK space-filling representation of the computer generated three dimensional structure of the PdG-lysozyme anti-hapten antibody immune complex when the E1G moiety is attached at lysine residue 13 151
- 5.1.1 The structure of the polysaccharide component of the *Micrococcus lysodeikticus* bacteria cell wall 155
- 5.1.2 The site of hydrolysis of the chitin derived hexa N-acetylglucosamine oligosaccharide moiety by lysozyme 156
- 5.1.3 The steps in deducing that it is the glycosidic bond between the sugar residues in subsites D and E is that cleaved by lysozyme 157
- 5.1.4 The hydrolysis of substrate by lysozyme 158
- 5.3.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the peptic digestion of IgG anti-estrone glucuronide monoclonal antibody 170
- 5.3.2 The elution profile resulting from the purification of Fab fragments on a Superdex G-75 gel filtration column 171
- 5.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of IgG anti-estrone glucuronide monoclonal antibodies, F(ab')<sub>2</sub> fragments, and purified Fab fragments 171
- 5.3.4 A typical twenty minute clearing curve of a *Micrococcus lysodeikticus* solution by a mixed anhydride estrone glucuronide-lysozyme E3 conjugate solution in the presence and absence of excess polyclonal anti-estrone glucuronide antibody 173
- 5.3.5 A typical initial rate plot (over 180 seconds) for the clearing of a *Micrococcus lysodeikticus* solution by an E1G-lysozyme mixed anhydride E3 conjugate solution in the presence and absence of excess polyclonal anti-E1G antibody 173
- 5.3.6 The effect on the initial rate of clearing of a *Micrococcus lysodeikticus* solution by an E1G-lysozyme mixed anhydride E3 conjugate solution in the presence of excess polyclonal anti-E1G antibody upon the addition of excess free estrone glucuronide to the assay system 174

- 5.3.7** Single exponential fit to the initial rate data obtained for the clearing of a *Micrococcus lysodeikticus* solution by an EIG-lysozyme conjugate solution in the presence of excess polyclonal anti-estrone glucuronide antibody after the addition of excess, free estrone glucuronide 174
- 5.3.8** Two phase exponential fit to the initial rate data obtained for the clearing of a *Micrococcus lysodeikticus* solution by an EIG-lysozyme conjugate solution in the presence of excess polyclonal anti-estrone glucuronide antibody after the addition of excess, free estrone glucuronide 175
- 5.3.9** Single exponential fit to the initial rate data obtained for the clearing of a *Micrococcus lysodeikticus* solution by an EIG-lysozyme conjugate solution in the presence of excess monoclonal anti-estrone glucuronide antibody after the addition of excess, free estrone glucuronide 175
- 5.3.10** Single exponential fit to the initial rate data obtained for the clearing of a *Micrococcus lysodeikticus* solution by an EIG-lysozyme conjugate solution in the presence of excess anti-estrone glucuronide monoclonal antibody Fab fragments after the addition of excess, free estrone glucuronide 176
- 5.3.11** Method one for determining  $C_T$  and  $C_{T1}$  giving the same lytic rate when calculating the concentration of conjugate bound in the presence of a fixed amount of monoclonal antibody 177
- 5.3.12** Binding curve for the binding of lysozyme-estrone glucuronide mixed anhydride E3 conjugate by monoclonal anti-EIG antibody with increasing conjugate concentrations and a constant antibody concentration when the concentration of conjugate bound and conjugate free are calculated by method 1 180
- 5.3.13** Scatchard plot for the binding of lysozyme-EIG E3 mixed anhydride conjugate by monoclonal anti-EIG antibody with increasing conjugate concentrations and a constant antibody concentration when the concentration of conjugate bound and conjugate free are calculated by method 1 180
- 5.3.14** Method two for determining the concentration of bound conjugate in the presence of a fixed amount of monoclonal antibody 181

5.3.15	Binding curve for the reaction of lysozyme-estrone glucuronide mixed anhydride E3 conjugate with monoclonal anti-E1G antibody at increasing conjugate concentrations and a constant antibody concentration when the concentration of conjugate bound and conjugate free are calculated by method 2	183
5.3.16	Scatchard plot for the binding of lysozyme-E1G E3 mixed anhydride conjugate by monoclonal anti-E1G antibody with increasing conjugate concentrations and a constant antibody concentration when the concentration of conjugate bound and conjugate free are calculated by method 2	183
5.3.17	A typical elution profile of (GlcNAc) <sub>6</sub> when analysed by HPLC on a YMC-Pack NH <sub>2</sub> column	184
5.3.18	A (GlcNAc) <sub>6</sub> standard curve generated from HPLC data showing the relationship between the amount of chitohexaose injected onto the column and the area under the eluted (GlcNAc) <sub>6</sub> peak	185
5.3.19	Time series showing the rate of hydrolysis of the (GlcNAc) <sub>6</sub> substrate by an E1G-lysozyme conjugate solution (E3)	186
5.3.20	A plot showing the rate of hydrolysis of (GlcNAc) <sub>6</sub> by an E1G-lysozyme conjugate solution as a function of time	187
5.4.1	The preparation of Fab fragments from IgG estrone glucuronide monoclonal antibody as described in the text	189
5.4.2	The implications of the slow displacement off-rate constants on the Ovarian Monitor assay system	194
6.2.1	The immunofiltration apparatus	208
6.2.2	A schematic diagram of a nylon immunofiltration membrane with immobilised anti-estrone glucuronide antibody	211
6.2.3	The make-up of nitrocellulose solid phase test strips for immunogold chromatography immunoassay	214
6.2.4	Native horseradish peroxidase and an estrone glucuronide-horseradish peroxidase mixed anhydride conjugation reaction mixture in 7 M urea on a Mono-S cation-exchange HR 5/5 column	215
6.2.5	Native horseradish peroxidase and an estrone glucuronide-horseradish peroxidase mixed anhydride conjugation reaction mixture in 1.4 M ammonium sulfate on an Alkyl Superose hydrophobic interaction HR 5/5 column	216

<b>6.2.6</b>	SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of unpurified anti-estrone glucuronide antiserum and purified anti-estrone glucuronide antibody	218
<b>6.2.7</b>	A nylon immunofiltration test strip run using various concentrations of estrone glucuronide-horseradish peroxidase conjugate reaction mixture	219
<b>6.2.8</b>	A nylon immunofiltration test strip run using 1/10 000 diluted estrone glucuronide-horseradish peroxidase conjugate reaction mixture and various E1G standards of known concentrations	219
<b>6.2.9</b>	A nylon immunofiltration test strip run using 1/10 000 diluted estrone glucuronide-horseradish peroxidase conjugate reaction mixture and various E1G standards of known concentrations	220
<b>6.2.10</b>	Nitrocellulose immunogold E1G-HRP test strips run and prepared with various concentrations of immunogold	222
<b>6.2.11</b>	Nitrocellulose E1G-HRP test strips run using 5 $\mu$ L of 1/20 diluted antibody prepared immunogold, 35 $\mu$ L of 2% BSA in PBS and 10 $\mu$ L of various estrone glucuronide standard concentrations	222
<b>6.2.12</b>	Nitrocellulose immunogold E1G-HRP test strips run using (1) 45 $\mu$ L of 1/40 diluted antibody prepared immunogold which was further diluted by 1/4 with 2% BSA in PBS and (2) various E1G standard concentrations	224
<b>6.2.13</b>	Nitrocellulose immunogold E1G-HRP test strips run using the conditions described in Fig. 6.2.12 and 5 $\mu$ L of sample urine	224
<b>6.3.1</b>	Nitrocellulose immunogold E1G-lysozyme test strips run and prepared as described in the text	227
<b>6.3.2</b>	Nitrocellulose immunogold E1G-lysozyme test strip	227
<b>6.3.3</b>	Nitrocellulose immunogold E1G-lysozyme test strips run and prepared with purified anti-E1G antibody	229
<b>6.3.4</b>	Nitrocellulose immunogold E1G-lysozyme test strips run using 10 $\mu$ L of immunogold and 40 $\mu$ L of a 20 mg/mL lysozyme solution (strip 1) and 40 $\mu$ L of a 10 mg/mL lysozyme solution (strip 2)	229
<b>6.4.1</b>	The principles behind the proposed estrone glucuronide-hen egg white lysozyme conjugate sandwich immunoassay colour test	235
<b>7.1.1</b>	The three dimensional unit cell	239
<b>7.1.2</b>	The simple (primitive), body-centred and face-centred lattices	240

7.1.3	Bragg's law shows that the waves from different planes of atoms are only in phase when the equation $2d \sin\theta = n\lambda$ is obeyed	242
7.2.1	The estrone glucuronide moiety and its amphipathic nature	248
7.2.2	A crystal of estrone glucuronide	249
7.2.3	ZORTEP [278] drawing of $C_{24}H_{30}O_8$ (estrone glucuronide hydrate) showing thermal ellipsoids drawn at the 50% probability level	251
7.3.1	Cubic crystals of the mixed anhydride estrone glucuronide-lysozyme E3 conjugate	257
7.3.2	Diamond shaped crystals of the mixed anhydride estrone glucuronide-lysozyme E3 conjugate	262
7.3.3	Final electron density map (contoured at a level of $0.9\sigma$ ) of the estrone glucuronide-lysozyme E3 conjugate showing lysine residue 33 and the surrounding environment	262
7.3.4	A two dimensional representation of the estrone glucuronide-lysine conjugation site	266
7.4.1	The chunky type crystals grown under conditions of 0.1 M EPPS (pH 7.0) and 12% PEG 6000 in attempts to obtain crystals of the estrone glucuronide-lysozyme-anti-estrone glucuronide Fab fragment immune complex	269

## List of Tables

<b>Table</b>	<b>Page</b>	
2.3.1	A comparison of the peak areas of the estrone glucuronide-lysozyme conjugates in the active ester reaction mixture relative to the unreacted lysozyme peak before and after dialysis against hydroxylamine	52
2.3.2	Retention of E1G-lysozyme conjugate fraction e on a Mono-S column relative to unreacted lysozyme and the corresponding ionic strength at elution	54
3.3.1	Summary of yields and specific activities for estrone glucuronide-lysozyme conjugates purified by cation-exchange in 7 M urea on Mono-S and S-Sepharose (fast flow) columns	86
3.3.2	Summary of yields and specific activities for pregnanediol glucuronide-lysozyme conjugates purified by cation-exchange in 7 M urea on Mono-S and S-Sepharose (fast flow) columns	91
3.3.3	The native lysozyme peptides containing lysine (K) residues	92
3.3.4	The acylation positions of lysozyme with estrone glucuronide in the lysozyme-estrone glucuronide conjugates characterised as described in the text	95
3.3.5	Sequence and mass spectral data for the estrone glucuronide acylated lysozyme tryptic peptides	102
3.3.6	The positions of acylation of lysozyme with pregnanediol glucuronide in the lysozyme-pregnanediol glucuronide conjugates characterised as described in the text	104
3.3.7	Sequence and mass spectral data for the pregnanediol glucuronide acylated lysozyme tryptic peptides	110
3.3.8	Retention of the E1G-lysozyme conjugates prepared by the active ester method on a Mono-S column in 7 M urea and the distance migrated by the E1G-lysozyme conjugates by acid-PAGE relative to unreacted lysozyme	112
5.3.1	Calculated displacement off-rate constants for the various E1G antibodies from the mixed anhydride E1G-lysozyme E3 conjugate-antibody immune complex	176

5.3.2	The reaction rates for the hydrolysis of (GlcNAc) <sub>6</sub> by the mixed anhydride estrone glucuronide-lysozyme conjugate (E3) in the absence and presence of various amounts of the polyclonal estrone glucuronide antibody	187
6.1.1	Chromatographic strip and sandwich colour tests for various analytes using microsphere dyed latex particles for the measurement and visualisation of bound label	205
7.1.1	Properties of the unit cells of the seven crystal systems	239
7.2.1	Crystal data, data collection and refinement details for estrone glucuronide	250
7.2.2	Fractional atomic coordinates and equivalent isotropic displacement parameters (Å <sup>2</sup> ) for 17-oxoestra-1,3,5(10)-triene-3-yl-β-D-glucopyranosiduronic acid hydrate, C <sub>24</sub> H <sub>30</sub> O <sub>8</sub>	252
7.2.3	Selected bond distances (Å) for 17-oxoestra-1,3,5(10)-triene-3-yl-β-D-glucopyranosiduronic acid hydrate, C <sub>24</sub> H <sub>30</sub> O <sub>8</sub>	253
7.2.4	Selected bond angles (°) for 17-oxoestra-1,3,5(10)-triene-3-yl-β-D-glucopyranosiduronic acid hydrate, C <sub>24</sub> H <sub>30</sub> O <sub>8</sub>	254
7.3.1	Data processing statistics for the estrone glucuronide-lysozyme E3 conjugate data	260
7.3.2	Statistics for the scaled R-Axis estrone glucuronide-lysozyme E3 conjugate data as a function of resolution	260
7.3.3	Statistics for the unrefined test set of R-Axis estrone glucuronide-lysozyme reflections as a function of resolution	264
7.3.4	Statistics for the refined working set of R-Axis estrone glucuronide-lysozyme reflections as a function of resolution	264

## List of Schemes

<b>Scheme</b>		<b>Page</b>
<b>1.2.1</b>	The synthesis of progesterone	12
<b>1.2.2</b>	The synthesis of the estrogens from progesterone	13
<b>2.2.1</b>	The synthesis of estrone glucuronide	44
<b>2.2.2</b>	The synthesis of steroid glucuronide-lysozyme conjugates by the active ester method	47
<b>2.4.1</b>	The proposed reaction pathway for the formation of 1,2-orthoester intermediates leading to the thermodynamically favoured $\beta$ -glycosidic product under Koenigs-Knorr reaction conditions	65
<b>3.2.1</b>	Synthesis of steroid glucuronide-lysozyme conjugates by the mixed anhydride method	78
<b>5.2.1</b>	The reactions occurring in the assay reaction mixture during displacement off-rate constant ( $k_{1\text{off}}$ ) measurements	166

## Abbreviations

A <sub>280</sub>	Absorbance at 280 nm
AB	Antibody
AE	Active ester
BBT	Basal body temperature
$\alpha$ -bromosugar	Methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl- $\alpha$ -D-glucopyran-uronate
BSA	Bovine serum albumin
C <sub>B</sub>	Concentration of bound conjugate
C <sub>F</sub>	Concentration of free conjugate
C <sub>T</sub>	Total conjugate concentration
CDR	Complementary determining region
Conj	Lysozyme-steroid glucuronide conjugate
DCC	Dicyclohexylcarbodiimide
DMF	Dimethylformamide
DMPTU	N,N-dimethyl-N'-phenylthiourea
E1G	Estrone glucuronide
E1G-AB	Anti-estrone glucuronide antibody
E1G[H]	Estrone glucuronide (acid form)
E1G-HEWL	Estrone glucuronide-hen egg white lysozyme conjugate
E1G-HRP	Estrone glucuronide-horseradish peroxidase conjugate
E2-17 $\beta$ -3G	Estradiol-17 $\beta$ -3-glucuronide
E3-3G	Estriol-3-glucuronide
E3-16G	Estriol-16 $\alpha$ -glucuronide
E3-17G	Estriol-17 $\beta$ -glucuronide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediamine tetra-acetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assays
EMIT	Enzyme-multiplied immunoassay technique
EPPS	N-(2-hydroxyethyl)piperazine-N'-(3-propane sulfonic acid)
Fab	Antigen binding fragment
FPLC	Fast protein liquid chromatography
FSH	Follicle stimulating hormone
GlcNAc	N-acetylglucosamine
(GlcNAc) <sub>2</sub>	Di-N-acetyl-chitobiose
(GlcNAc) <sub>3</sub>	Tri-N-acetyl-chitotriose
(GlcNAc) <sub>4</sub>	Tetra-N-acetyl-chitotetraose
(GlcNAc) <sub>5</sub>	Penta-N-acetyl-chitopentaose

(GlcNAc) <sub>6</sub>	Hexa-N-acetyl-chitohexaose
GnRH	Gonadotropin releasing hormone
HCG	Human chorionic gonadotrophin
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid)
HEWL	Hen egg white lysozyme
HEWL-AB	Anti-hen egg white lysozyme antibody
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HRP-E1G	Horseradish peroxidase-estrone glucuronide conjugate
I.D.	Internal diameter
IgG	Immunoglobulin G class of antibody
LH	Luteinizing hormone
MA	Mixed anhydride
MCAB	Estrone glucuronide monoclonal antibody
m.p.	Melting point
MurNAc	N-acetylmuramic acid
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NFP	Natural Family Planning
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PdG	Pregnanediol glucuronide
PdG[H]	Pregnanediol glucuronide (acid form)
PEG	Polyethylene glycol
PIPES	Piperazine-N,N'-bis-(2-ethane sulphonic acid)
PITC	Phenylisothiocyanate
RIA	Radioimmunoassay
r.m.s.	Root mean square
μs	Microsphere
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ΔT	Change in transmission
TFA	Trifluoroacetic acid
TMB	Tetramethylbenzidine
TNBS	2,4,6-trinitrobenzene 1-sulfonic acid

tris Tris(hydroxymethyl)aminomethane  
WHO World Health Organisation

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### Amino Acid Abbreviations

A	Ala	Alanine
C <sup>†</sup>		S-carboxymethylated cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
I	Ile	Isoleucine
K	Lys	Lysine
K*		Steroid glucuronide acylated lysine residue
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

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