Syntheses and Characterization of Steroid Glucuronides for the Preparation of Horseradish Peroxidase Conjugates *via* Hemin Modification

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Abstract

Steroid glucuronides including estrone glucuronide **12**, estriol 3-, 16α- and 17β-monoglucuronides **13-15** and pregnanediol glucuronide **16** have been successfully synthesised. In particular, a new scheme for the synthesis of estriol monoglucuronides **13-15** from estriol provides a simple procedure and good yields of pure products based on the protection and deprotection of hydroxyl groups of estriol, glucuronidation, and hydrolysis. The new synthetic route retains the original stereochemical integrity of the estriol, and thus produced the estriol monoglucuronides with the correct stereochemistry. The steroid glucuronides **12-16** were characterised by ^{1}H-^{1}H 2D-COSY, 2D-NOESY and ^{1}H-^{13}C HETCOR spectra and the results unambiguously showed the β-linkage of the glucuronide ring with the steroid moiety in all of the steroid glucuronides. The conjugation positions of the glucuronic acid to estriol, as in estriol 16- or 17-glucuronide **14-15**, were distinguished from their ^{13}C chemical shift values and the proton 2D-NOESY spectra. The crystal structure analysis of one estriol 17-glucuronide derivative **112** also confirmed that the absolute configuration at all stereocentres was maintained during synthesis.

Subsequently some α-amino acids (DL- or L-phenylalanine, L-tryptophan and L-arginine), estrone glucuronide (E1G) **12** and pregnanediol glucuronide (PdG) **16** have been successfully linked to hemin IX **227** (the prosthetic group of horseradish peroxidase) either by selective mono-acylation of protoporphyrin IX **216** followed by insertion of Fe^{2+} or by direct mono-conjugation of hemin IX **227** with α-amino acids or steroids. The mono-coupling reactions provided good yields and simlple reaction conditions, which have established the feasibility of this new methodology. The mono-conjugated structures and the high purities of both hemin-phenylalanine mono-conjugate **230** and the hemin-estrone glucuronide mono-conjugate **232** were confirmed by their ^{1}H NMR and mass spectra. Both purified conjugates (**230**, **232**) showed no contamination by unreacted hemin IX **227** by HPLC analyses.

The reconstitution of hemin-estrone glucuronide mono-conjugate **232** with apo-horseradish peroxidase has been successfully achieved to form a new enzyme. The new enzyme (estrone glucuronide-horseradish peroxidase conjugate) retains good peroxidase activity (76% relative to reconstituted horseradish peroxidase), which is sufficient for exploitation in immunoassays. A suitable molecular linker (L-lysine) between the hemin propionate side chain and the estrone glucuronide moiety is crucial for retaining good peroxidase activity. Without a molecular linker, reconstitution of hemin-phenylalanine monoconjugate **230** with the apo-horseradish peroxidase showed a very poor reconstitution yield and activity. The extra carboxyl group, introduced by L-lysine, probably also made a great contribution in retaining a high activity of the new enzyme. Therefore, this thesis has exploited a new methodology in the preparation of horseradish peroxidase-hapten conjugates *via* hemin-modification. The new methodology is generic and it can be extended to the synthesis of horseradish peroxidase-conjugates with any analytes of interest. It will be very useful, not only as markers of fertility in home assays, but also in many other areas such as food, agriculture, medicine and the environmental monitoring. Hence, wide commercial applications for this new technology are expected in the future.

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Abbreviations

A	alanine
A_{278}	absorbance at 278 nm
A_{403}	absorbance at 403 nm
Arg	arginine
Asn	asparagine
BSA	bovine serum Aabumin
CcP	cytochrome *c* peroxidase
CLIA	chemiluminescence immunoassay
DCC	dicyclohexylcarbodiimide
2D-COSY	two-dimensional homonuclear correlation spectroscopy
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
2D-NOESY	two-dimensional nuclear overhause effect spectroscopy
DtBP	ditertiary butyl peroxide
E (Glu)	glutamic acid
E1G	estrone 3-glucuronide or estrone glucuronide
E1G-hemin	estrone glucuronide-hemin mono-conjugate
E1G-HRP	estrone glucuronide-horseradish peroxidase conjugate
E3-3G	estriol 3-glucuronide
E3-16G	estriol 16α-glucuronide or estriol 16-glucuronide
E3-17G	estriol 17β-glucuronide or estriol 17-glucuronide
EIA	enzyme immunoassay
FAD	flavin adenine dinucleotide
FIA	fluoroimmunoassay
FPLC	fast protein liquid chromatography
Gln	glutamin
H (His)	histidine
HETCOR	heteronuclear correlation spectroscopy
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
Hz	hertz
Ile	isoleucine
K	lysine
Lip	lignin perosidase

Lit	literature
NMR	nuclear magnetic resonance
P-450scc	cytochrome P-450 enzyme for side chain cleavage
PdG	pregnanediol 3-glucuronide or pregnanediol glucuronide
PGLIA	prosthetic group-labelled immunoassay
ppm	part per million
Pro	proline
ref	reference
RIA	radioimmunoassay
R_F	rate of flow
rt	room temperature
RZ	reinheitszahl
Ser	serine
ΔT	change in transmission
THF	tetrahydrofuran
TLC	thin layer chromatography
Tyr	tyrosine
Val	valine