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PREPARATION AND USE OF BIOTINYLATED LIGANDS FOR LHRH RECEPTOR PURIFICATION.

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Summary: The synthesis of biotinylated analogues of LHRH is described in which the peptides simultaneously combine biotin and either a photolabile or an amino substituent. In rat anterior pituitary membranes the conjugate [biotinyl-aminoethylglycyl-D-Lys^6, des Gly^{10}]-LHRH ethylamide showed approximately 50% specific binding and could be covalently crosslinked to the LHRH receptor site with ethylene glycolbis(succinimidylsuccinate).

Photoaffinity labelling has developed into a regular technique for the investigation of neuropeptide-receptor interactions. In this procedure a ligand containing a photolabile species, normally an azido group, is activated by photolysis and the resulting nitrene covalently binds to the receptor[1,2]. This technique combined with affinity chromatography based on the biotin-avidin/streptavidin interaction[3-5] provides a method for the identification, localisation and purification of hormone-receptor complexes[6]. We have adopted and extended this approach for the purification of the luteinizing hormone releasing hormone (LHRH) receptor. In addition to investigating the photoaffinity approach to receptor labelling we have developed an alternative method in which an amino group can be covalently attached to the receptor with bifunctional crosslinkers[7]. LHRH(1), a hypothalamic decapeptide, plays a key role in the mammalian reproductive cycle and knowledge of the structure of the receptor binding site would further our understanding of the mechanism of cellular response. In this report we describe the synthesis of two novel biotinylated reagents, their conjugation to LHRH analogues and preliminary biological studies of the peptide derivatives in rat anterior pituitary membranes.

Two active LHRH analogues, D-Lys^6-LHRH(2) and [D-Lys^6, des Gly^{10}]-LHRH ethylamide(3) were selected for this study. The ε-amino moiety of the D-Lys^6 residue provides a convenient group on which to couple reagents. The latter peptide was chosen as previous reports[8] indicate that better specific:non-specific binding ratios are observed with the des Gly^{10}-ethylamide C-terminus. D-Lys^6-LHRH was obtained commercially (Peninsula) and [D-Lys^6, des Gly^{10}]-LHRH ethylamide was synthesised by the solid-phase method using Sheppard's Fmoc-t-butyl-polyamide chemistry[9]. After purification by ion-exchange chromatography this peptide showed the expected amino acid composition and molecular weight (Glu_1 1.02, His_1 0.96, Ser_1 0.89, Tyr_1 0.97, D-Lys_1 1.00, Leu_1 1.00, Arg_1 1.00, Pro_1 0.95; FAB MS: found MH^+ 1225, calc. MH^+ $C_{59}H_{86}N_{17}O_{12}$ 1225).

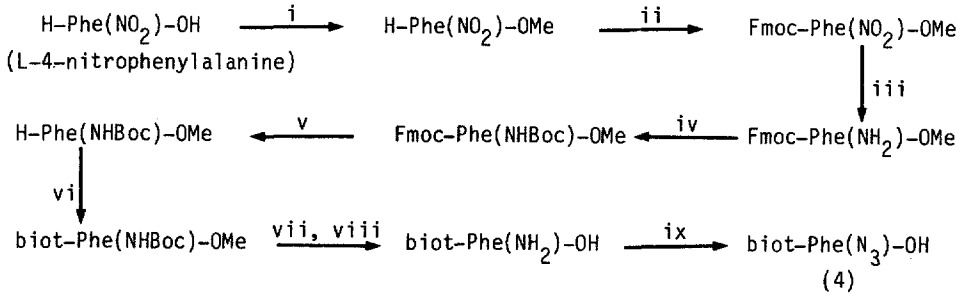
(1) pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

(2) pGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2

(3) pGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-NHEt

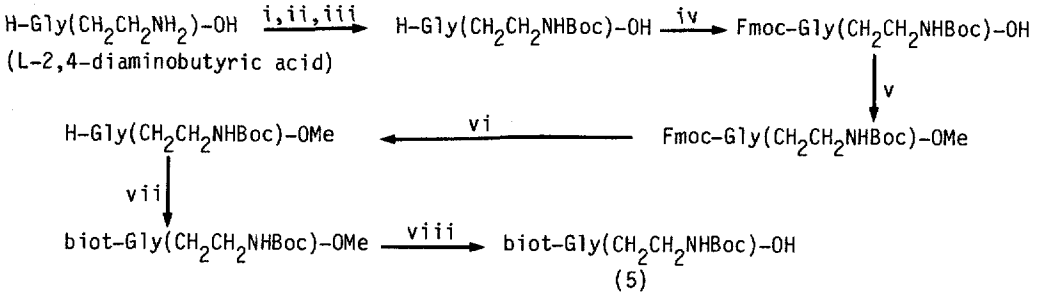
The reagents biotinyl-p-azidophenylalanine (4) and biotinyl-t-butoxycarbonylaminoethylglycine(5)[10] were prepared from L-p-nitrophenylalanine and L-2,4-diaminobutyric acid respectively and the syntheses are outlined in schemes 1 and 2. Both reagents were converted to their N-hydroxysuccinimide esters for coupling to the peptide backbone[11].

Scheme 1, Synthesis of biotinyl-p-azidophenylalanine (4)



Conditions: i, $SOCl_2$-MeOH; ii, FmocCl-K_2CO_3-H_2O-dioxan; iii, H_2-5%Pd-C-AcOH; iv, 10%$NaHCO_3$-$(Boc)_2O$-H_2O-dioxan; v, 20% piperidine in DMF; vi, biotin-pentafluorophenyl ester-HOBT-DMF; vii, NaOH-H_2O-MeOH; viii, aq TFA; ix, $NaNO_2$-NaN_3-1MHCl.

Scheme 2, Synthesis of biotinyl-t-butoxycarbonylaminoethylglycine (5)



Conditions: i, $CuCO_3.Cu(OH)_2$-H_2O; ii, MgO-$(Boc)_2O$-MeOH; iii, H_2S-H_2O-NH_4OH; iv, FmocCl-K_2CO_3-H_2O-dioxan, v, CH_2N_2-Et_2O; vi, 20% piperidine in DMF; vii, biotin-pentafluorophenyl ester-HOBT-DMF; viii, NaOH-H_2O-MeOH

Three conjugates were prepared, [biotinyl-p-azidophenylalanyl-D-Lys^6]-LHRH (6) [biotinyl-p-azidophenylalanyl-D-Lys^6, des Gly^{10}]-LHRH ethylamide (7) and [biotinyl-aminoethylglycyl-D-Lys^6, des Gly^{10}]-LHRH ethylamide (8), and the coupling procedure was similar in each case. For example, D-Lys^6-LHRH (2mg, 1.6μmol) was dissolved in freshly distilled DMF (2ml) and triethylamine (2 drops) was added. Biotinyl-p-azidophenylalanine-N-hydroxysuccinimide ester (5mg, 9.45μmols) in DMF (1ml) was added and the resulting mixture was left standing in the dark[12] for 1 h. The DMF was evaporated under high vacuum and the residue was triturated with ethyl acetate (3 x 2ml) to remove excess reagent. The crude product was purified by semi-preparative reverse-phase hplc[13] to yield 1.84mg (69%)[14] of pure (single peak on analytical hplc) conjugate. The amino acid compositions of the three conjugates are given in Table 1.

pGlu-His-Trp-Ser-Tyr-D-Lys-[N^{ε}-biot-Phe(N_3)]-Leu-Arg-Pro-Gly-NH_2

(6)

pGlu-His-Trp-Ser-Tyr-D-Lys-[N^{ε}-biot-Phe(N_3)]-Leu-Arg-Pro-NHEt

(7)

pGlu-His-Trp-Ser-Tyr-D-Lys-[N^{ε}-biot-Gly($CH_2CH_2NH_2$)]-Leu-Arg-Pro-NHEt

(8)

Table 1, Amino acid analysis of LHRH conjugates[15]

No.	PGlu	His	Ser	Tyr	D-Lys	Leu	Arg	Pro	Gly	Phe(N_3)*	Gly($CH_2CH_2NH_2$)
6	1.07	1.00	0.81	1.07	1.01	1	1.00	0.96	1.04	0.30	-
7	1.05	0.96	0.83	0.95	1.02	1	0.89	0.91	-	0.20	-
8	1.09	1.01	0.91	1.01	1.07	1	0.96	0.99	-	-	0.94

* largely decomposed on hydrolysis

Of the three ligands prepared, two contain a photolabile moiety (6,7) whilst the third (8) contains an amino substituent suitable for reaction with bifunctional chemical crosslinkers. The ligands were iodinated and the specificity of their binding to anterior pituitary membranes of male rats was examined by displacement with 1μM LHRH (see Table 2). Their affinity and maximal number of specific binding sites were determined (where appropriate) by saturation analysis and affinity values for the uniodinated forms were confirmed to be similar by displacement of iodinated buserelin ([D-Ser(Bu^t)^6, des Gly^{10}]-LHRH ethylamide). A proportion of the specific binding was rendered resistant to dissociation (4 hrs, 23°C in the presence of excess LHRH) either by illumination for 10 min on ice using a 4 Watt Hg lamp (ligands 6, 7) or by incubation for 30 min on ice with 5mM ethylene glycol<u>bis</u>(succinimidylsuccinate) in the case of ligand (8). Preliminary experiments have shown that membrane protein covalently labelled with (8) will subsequently bind to streptavidin-agarose columns and can be efficiently eluted with 2mM biotin.

Table 2, Biological experiments with iodinated ligands[16]

ligand No	specific binding: as % of total binding	specific binding: affinity (K_D) pM	specific binding: number of sites (Bmax) fmol/pituitary	covalent crosslinking of specific binding: as % of initial specific binding	covalent crosslinking of specific binding: overall yield as % of total ligand binding
buserelin	78 ± 4(8)	280 ± 20	92 ± 3	-	-
6	12 ± 4(4)	-	-	< 4(4)	< 0.5
7	10 ± 3(3)	-	-	28 ± 5(3)	3 ± 1
8	44 ± 6(3)	131 ± 16	63 ± 5	18 ± 3(5)	8 ± 1

values are means ± SEM; numbers of experiments in parentheses

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10. The amino group of this reagent was protected with the t-butoxycarbonyl group during coupling to the peptide and was removed, with TFA, from the conjugate.
11. All new compounds gave satisfactory spectral analyses.
12. Reactors involving the p-azido reagent were performed in the dark and all handling of the p-azido-conjugates was carried out in subdued lighting.
13. Conditions: semi-preparative Aquapore RP300 column; eluant A, 0.1% aqueous TFA; B, 90% CH_3CN 10% A. The column was eluted isocratically for 2 min with 10% B and then with a linear gradient of 10-70% B over 30 min, flow rate 3ml/min. Optical density was monitored at 230nm. During purification of the azido-conjugates (6,7) the detector was switched off whilst the product peak was collected.
14. Yield calculated from amino acid analysis.
15. Hydrolysis conditions: constant boiling HCl containing phenol in a sealed tube for 18 h at 110°C.
16. Full details of the biological experiments will be reported elsewhere, Biochem. J. (in press).

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