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Synthetic hFSH Peptide Constructs in the Evaluation of Previous Studies on the hFSH Receptor Interaction

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Abstract: The human follicle-stimulating hormone (hFSH) belongs to a family of glycoprotein hormones which contains two non-identical subunits. This paper describes the design and synthesis of a series of synthetic hFSH constructs as putative ligands for the receptor. The design of these constructs is based on the crystal structure of hCG and molecular modelling using the program package Insight II/Discover. The designed constructs contain peptides ranging from 7 to 48 amino acid residues, disulphide bridges and glycan residues. All the synthetic peptides were synthesized by the stepwise solid-phase method using Fmoc chemistry. Two of the synthetic peptides contain the glycosylated amino acid, Asn(GlcNAc-GlcNAc) and both were prepared using fully protected glycosylated building blocks in the solid-phase peptide synthesis. The disulphide bridges were formed from acetamidomethyl-protected glycopeptides and peptides by a direct deprotection/oxidation method using thallium(III) trifluoroacetate. Mass spectroscopy and amino acid analysis were used for characterization of the synthetic hFSH glycopeptides and peptides. The synthetic hFSH constructs were tested for binding activity on FSH receptor assays but none showed improved binding properties compared with the naturally occurring hormone. It was finally demonstrated that non-related peptides showed non-specific binding at the same level as reported for specific peptides. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

The human follicle-stimulating hormone (hFSH) is a heterodimeric glycoprotein hormone which is a member of the same family as hLH (luteinizing hormone), hTSH (thyroid-stimulating hormone) and hCG (chorionic gonadotropin) [1, 2]. This family of hormones contains two subunits, α and β chains, which both are glycosylated at certain amino acid residues and internally cross-linked by disulphide bridges. The α-chain is identical for all the glycoprotein hormones whereas the β-chain is hormone-specific. FSH is essential for the growth stimulation of ovarian follicles and secretion of oestradiol as well

Abbreviations: Acm, acetamidomethyl; DCM, dichloromethane; Dhbt-OH, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; DIEA, *N,N'*-diisopropylethylamine; hCG, human chorionic gonadotropin; hFSH, follitropin, human follicle-stimulating hormone; HMBA, 4-hydroxymethylbenzoic acid; LH, luteinizing hormone; MALDI-MS, matrix-assisted laser desorption mass spectroscopy; MSNT, 2,4,6-mesitylene-sulphonyl-3-nitro-1,2,4-triazine; NEM, *N*-ethylmorpholine; PEGA, polyethylene glycol dimethyl acrylamide; Pmc, 2,2,5,7,8-pentamethyl chroman-6-sulphonyl; $Tl(tfa)_3$, thallium(III)trifluoroacetate; TSH, thyroid-stimulating hormone.

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as in the stimulation of spermatogenesis in testes [3]. Regions that have been identified as being important in the hFSH receptor binding are the α(26–46) [4, 5], α(52–57) [6] and α(88–92) [7] sequences of the α-chain and the βhFSH(32–51) [8, 9] and βhFSH(87–94) [10]. Most of these fragments have been demonstrated to inhibit receptor binding with 10^{-3}–10^{-4} M binding constants in radioreceptor binding assays. It could therefore be argued that a combination of these parts of the hFSH molecule with linkers of correct spacing would enable them to interact additively with the receptor by induced fit to generate high-affinity ligands. However, the previous investigations all suffer from insufficient control experiments to test the validity of the fragments as true ligands and one report even claims affinity of a peptide fragment which is completely buried in the interior of the hFSH molecule [5]. To evaluate the binding model resulting from previous study, these receptor binding regions have been combined in single constructs to develop synthetic peptides with potential FSH agonistic and antagonistic activity [11, 12]. However, recently the 3D X-ray crystallographic structure of hCG has been solved [13, 14]. The highly disulphide bonded backbones of hCG and hFSH are very similar and it was possible to combine the most important receptor binding sites in hFSH in constructs based on this 3D structure of hCG in order to develop synthetic for FSH constructs.

The present report describes several peptide constructs generated by modelling using the program package Insight II/Discover. The constructs were expected to mimic the active site of the native hFSH structure. Also their syntheses with or without glycosylated amino acid residues are described. The synthetic peptides and glycopeptides were prepared by the stepwise solid-phase peptide synthesis [15, 16] and intramolecular disulphide bridges were formed by using acetamidomethyl protected cysteine pairs [17] which were selectively deprotected and oxidized with thallium(III) trifluoroacetate in TFA [18]. The glycosylated amino acid residue, Asn(GlcNAc-GlcNAc), was selected to mimic the more complex Asp($Man_3GlcNAc_2$) moiety which has been shown to be important for the biological activity of hFSH [19]. The FSH–receptor interactions of these synthetic peptides and glycopeptides were evaluated in two different FSH–receptor binding assays.

MATERIALS AND METHODS

General Methods

Molecular modelling was performed using the program package Insight II/Discover from Biosym (San Diego, California) on an Indigo 2 work station from Silicon Graphics. TLC was performed on Merck Silica Gel 60 F254 with detection either by charring with sulphuric acid or by UV light when applicable. Medium-pressure chromatography was performed, using a Sephadex LH 20 column on a Buchi B-680 chromatographic system, with DMF as the eluent and UV detection at 320 nm. All organic solvents were purchased from Labscan Ltd (Dublin, Ireland). Concentrations were performed under reduced pressure at temperatures 40 °C. Suitable protected N^{α}-Fmoc-amino acids, *p*-((α-Fmoc-amino)-2,4-dimethoxybenzyl)-phenoxy-acetic acid (Rink-amide-linker) and HMBA were purchased from Nova Biochem (Switzerland), TBTU and Dhbt-OH from Fluka (Switzerland), NEM from Merck (Germany), Sephadex LH 20 from Pharmacia and thallium trifluoroacetate from Aldrich. The molecular weights of the peptide and glycopeptide compounds were determined, using matrix-assisted laser desorption time-of-flight mass spectroscopy (MALDI-MS), recorded on a Finnigan Mat Lasermat 2000. Quantitative amino acid analyses were performed on a Pharmacia LKB Alpha Plus amino acid analysis following hydrolysis with 6 M HCl at 110 °C for 24 h. Asn and Gln were determined as Asp and Glu and Cys was determined as cysteic acid after performic acid oxidation. Analytical HPLC was performed using a Waters RCM 8 × 10 module and with a Deltapak C-18 column (19 × 300 mm). Preparative HPLC was performed on a Hitachi L-6250 Preparative Intelligent Pump using a Delta Pak C-18 column (25 × 200 mm). The solvent system for both analytical and preparative HPLC was buffer A; 0.1% TFA in water and buffer B; 0.1% TFA in 90% acetonitrile–10% water and UV detection was at 215 or 280 nm for analytical and only 215 nm for preparative. The gradient for analytical HPLC (1 ml/min); a linear gradient of 0–100% buffer B over 50 min and preparative HPLC (10 ml/min); a linear gradient of 0–100% buffer B over 85 min unless otherwise indicated. Purified hFSH (7000 IU/mg) was obtained from Sigma (USA, MO).

Solid-phase Synthesis, General

The synthesis of peptides and glycopeptides using the PEGA-resin [20] were performed by the plastic syringe technique [21] or a custom-made fully automated, continuous flow peptide synthesizer [22, 23] with a solid-phase photometer. Fully protected N^{α}-Fmoc amino acid OPfp esters (3 mol equiv.) were coupled in DMF with the addition of Dhbt-OH (1 mol equiv.) as an acylation catalyst and an indicator of the end-point of the acylation reaction. The side-chain amino acid protecting groups were ^{t}Bu for Ser, Thr and Tyr, trityl for Asn, Gln, and His, *O*-^{t}Bu for Asp and Glu, Acm or Trt for Cys, *tert*-butyloxycarbonyl (Boc) for Lys and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. The N^{α}-Fmoc group was removed by 20% piperidine in DMF. After completion of the syntheses, the Fmoc group was cleaved and the terminal amino group was capped with 20% acetic anhydride in DMF for 20 min. The resin was then washed with DMF (5×1 min) and DCM (5×1 min) and dried before deprotection and cleavage. The peptides and glycopeptides were side-chain-deprotected and cleaved simultaneously from the Rink-linker by treatment with 95% TFA for 2 h and the resin rinsed with 95% acetic acid (4×2 min). For peptides containing a single –SH group, the cleavage was mediated by a mixture of 95% TFA and triisopropylsilane (3 mol equiv.) to scavenge the long-lived trityl carbonium ion. TFA and acetic acid were removed under reduced pressure and after precipitation with diethyl ether the crude product was

Table 1 The Amino Acid Sequences for Compounds **1–8b**

Compound	Structure
1	Ac-YTRDLVYKDPARPKIQKT-NH_2
2a	Ac-KC(Acm)DSDSTDC(Acm)T-NH_2
2b	Ac-KCDSDSTDCT-NH_2 (C–C)
2c	Ac-KC(Acm)DSDNTDC(Acm)T-NH_2
2d	Ac-KCDSDNTDCT-NH_2 (C–C)
2e	Ac-KC(Acm)DSDN(Ac_5GlcNAc-GlcNAc)TDC(Acm)T-NH_2
2f	Ac-KC(Acm)DSDN(GlcNAc-GlcNAc)TDC(Acm)T-NH_2
2g	Ac-KCDSDNGlcNAc-GlcNAc)TDCT-NH_2 (C–C)
3a	Ac-LVQKNVTSEST-NH_2
3b	Ac-LVQKN(Ac_5GlcNAc-GlcNAc)VTSEST-NH_2
3c	Ac-LVQKN(GlcNAc-GlcNAc)VTSEST-NH_2
4	Ac-TCYYHKS-NH_2
5	YTRDLVYKDPARPKIQKTCTF-NH_2
6	Ac-YTRDLVYKDPARPKIQGPKC(Acm)DSDSTDC(Acm)T-NH_2
6b	Ac-YTRDLVYKDPARPKIQGPKCDSDSTDCT-NH_2 (C–C)
7a	Ac-YTRDLVYKDPARPKIQGPKC(Acm)DSDSTDC(Acm)GPGYYHKS-OH
7b	Ac-YTRDLVYKDPARPKIQGPKCDSDSTDCGPGYYHKS-OH (C–C)
8a	Ac-LVQKNVTSESTGPGRDLVYKDPARPKIQGPKC(Acm)DSDSTDC(Acm)GPGYYHKS-OH
8b	Ac-LVQKNVTSESTGPGRDLVYKDPARPKIQGPKCDSDSTDCGPGYYHKS-OH (C–C)

purified by preparative HPLC. Peptides coupled to the HMBA-linker were first treated with 95% TFA for 2 h for removing the side-chain protecting groups, followed by rinsing the resin with 95% acetic acid (4 × 2 min), neutralization of the resin with 5% DIEA in DMF (2 × 2 min), rinsing with DMF (2 × 2 min) and DCM (5 × 2 min) and drying of the resin by air-flow for 1.5 h. Cleavage from the resin was performed by treatment with 0.1 M NaOH for 2 h and the resin was rinsed with water (7 × 2 min). Finally, the basic solution was neutralized with 0.1 HCl and the crude product was purified by preparative HPLC. The amino acid sequences are given in Table 1. All peptides were analysed by analytical reverse-phase HPLC, MALDI-MS (Table 2) and amino acid analysis (Table 3).

Table 2 Analysis of the Compounds **1–8b** by MALDI-MS

Compounds	Molecular formula	Relative molecular mass	MALDI-MS[a]
1	$C_{101}H_{164}N_{29}O_{28}$	2232.59	2234.0[b]
2a	$C_{46}H_{75}N_{14}O_{23}$	1256.30	1257.3[b]
2b	$C_{40}H_{65}N_{12}O_{21}S_2$	1114.14	1114.1[b]
2c	$C_{47}H_{76}N_{15}O_{23}S_2$	1283.33	1284.9[b]
2d	$C_{41}H_{66}N_{13}O_{21}S_2$	1141.17	1142.8[b]
2e	$C_{73}H_{112}N_{17}O_{37}S_2$	1883.90	1883.2[c]
2f	$C_{63}H_{102}N_{17}O_{32}S_2$	1673.72	1696.3[d]
2g	$C_{57}H_{92}N_{15}O_{30}S_2$	1531.56	1554.7[d]
3a	$C_{52}H_{90}N_{15}O_{20}$	1245.38	1246.5[b]
3b	$C_{78}H_{126}N_{17}O_{34}$	1845.95	1868.4[d]
3c	$C_{68}H_{116}N_{17}O_{29}$	1635.77	1659.2[d]
4	$C_{42}H_{58}N_{11}O_{12}S_1$	941.05	942.7[b]
5	$C_{115}H_{184}N_{33}O_{30}S_1$	2540.99	2542.1[b]
6a	$C_{142}H_{226}N_{41}O_{49}S_2$	3355.72	3356.7[b]
6b	$C_{136}H_{216}N_{39}O_{47}S_2$	3213.56	3213.9[b]
7a	$C_{180}H_{273}N_{50}O_{59}S_2$	4145.57	4214.7[e,f]
7b	$C_{174}H_{263}N_{48}O_{57}S_2$	4003.41	4074.0[e,f]
8a	$C_{226}H_{356}N_{65}O_{77}S_2$	5279.82	5349.3[e,f]
8b	$C_{220}H_{346}N_{63}O_{75}S_2$	5137.66	5206.8[e,f]

[a] 10 mg of α-cyano-4-hydroxy-cinnamic acid in 1 ml 70% acetonitrile was used as matrix.
[b] Peptides were detected as $[peptideH]^+$ ions.
[c] Average values of $[glycopeptideH]^+$, $[glycopeptideNa]^+$ and $[glycopeptide2Na]^+$ ions.
[d] Glycopeptides were detected as $[glycopeptideNa]^+$ ions.
[e] Peptides were detected as $[peptide3Na]^+$ ions.
[f] 10 mg of *trans*-3,5-dimethoxy-4-hydroxy-cinnamic acid in 1 ml 70% acetonitrile was used as matrix.

Anchoring of the HMBA-linker and the First Amino Acid

HMBA [24] (3 mol equiv.) was dissolved in DMF and cooled to 0 °C, and TBTU (3 mol equiv.) and NEM (6 mol equiv.) were added. After 10 min, the solution was added to the PEGA-resin (1 mol equiv.) which had been swelling in DMF (1 h). The flask was gently shaken for 2 h and the resin was filtered, rinsed with DMF (6 × 1 min), DCM (5 × 1 min) and dried under high vacuum for one day. A solution of N^{α}-Fmoc-Ser(tBu)-OH (3.1 mol equiv., 24 mM) in dry DCM was added to *N*-methyl imidazole (MeIm) (2.37 mol equiv.) under argon. This solution was then added to a flask containing 1-mesityl-sulphonyl-3-nitro-1,2,4-triazole (MSNT) (3.1 mol equiv.). The dried resin was mixed with the above mixture for 30 min, filtered and rinsed with DCM (5 × 1 min), DMF (5 × 1 min) and DCM (5 × 1 min). This procedure was repeated and the resin was lyophilized. The level of the incorporation of the first amino acid was determined by measuring the Fmoc-piperidine absorbance at 290 nm and comparing with a standard curve.

Anchoring of the Rink-linker and the First Amino Acid

p-((Fmoc-amino)-2,4-dimethoxybenzyl)phenoxyacetic acid (Rink-amide-linker) [25] (3 mol equiv.) was attached to the PEGA-resin using the TBTU method as described above. The unreacted amino groups were capped by addition of 10% acetic anhydride (20 min). The first amino acid (3 mol equiv.) was coupled to the resin as Fmoc–amino acid–OPfp ester in the presence of Dhbt-OH (1 mol equiv.) and in DMF as the solvent.

Automated Solid-phase Synthesis

The syntheses of compounds **1**, **5**, **6a**, **7a** and **8a** were carried out on the fully automatic, continuous flow peptide synthesizer with a 1.44 ml/min flow. Each cycle consisted of the following steps: coupling of the Fmoc–amino acid–OPfp ester (3 mol equiv.) with Dhbt-OH (1 mol equiv.), rinsing of the resin using DMF (10 min), removing of the Fmoc group by treatment with 20% piperidine in DMF (20 min) and successive rinsing of the resin with DMF (28 min). The acylation reactions were monitored by a solid-phase photometer operating at 440 nm and the acylation time for Pfp esters were at least 30 min determined by the photometer. The cleavage of the

Table 3 Amino Acid Analysis of Selected Compounds (Theoretical Values in Parentheses)

Compounds	Ala	Arg	Asp	Cys[a]	Glu	Gly	His	Ile	Leu	Lys	Phe	Pro	Ser	Thr	Tyr	Val
1	1.00(1)	1.98(2)	2.04(2)		1.20(1)			0.94(1)	1.07(1)	2.97(3)		1.97(2)		1.88(2)	1.80(2)	0.97(1)
2b			2.98(3)	1.91(2)						0.98(1)			1.85(2)	2.00(2)		
2d			4.03(4)	1.98(2)						1.00(1)			0.96(1)	1.94(2)		
2g			4.05(4)	1.97(2)						1.00(1)			0.98(1)	1.98(2)		
3a			0.99(1)		2.06(2)				1.04(1)	1.01(1)			1.91(2)	1.94(2)		1.94(2)
3c			1.03(1)		1.94(2)				0.94(1)	1.00(1)			1.94(2)	1.99(2)		1.75(2)
4				1.08(1)			0.95(1)			0.95(1)			0.97(1)	1.01(1)	1.77(2)	
5	1.14(1)	1.95(2)	2.02(2)	1.07(1)	1.01(1)			1.00(1)	1.04(1)	3.00(3)	1.01(1)	1.83(2)		2.84(3)	1.86(2)	0.87(1)
6b	1.08(1)	2.02(2)	5.06(5)	1.81(2)	1.15(1)	1.11(1)	1.18(1)	0.91(1)	1.05(1)	3.00(3)		3.20(3)	1.95(2)	2.99(3)	2.00(2)	0.94(1)
7b	1.11(1)	1.83(2)	5.12(5)	2.04(2)	0.89(1)	3.28(3)	0.93(1)	1.13(1)	1.02(1)	3.94(4)		4.09(4)	3.03(3)	1.93(2)	3.71(4)	1.02(1)
8b	1.03(1)	1.85(2)	6.07(6)	2.11(2)	3.18(3)	5.18(5)	0.90(1)	1.33(1)	2.31(2)	4.98(5)		5.06(5)	5.04(5)	2.88(3)	2.88(3)	3.06(3)

[a] Cys was determinated as cysteic acid after performic acid oxidation.

Fmoc group was followed by an UV spectrophotometer at 320 nm.

Solid-phase Peptide Synthesis: Plastic Syringe Technique

The syntheses of compounds **2a**, **2c**, **2g**, **3a**, **3c** and **4** were performed using a disposable plastic syringe (without piston) fitted with a sintered Teflon filter (pore size 70 μm) and connected to a vacuum waste bottle through a two way Teflon valve. After the first amino acid was attached to the linker, the Fmoc group was removed with 20% piperidine (1 × 20 min) and the resin was rinsed with DMF (6 × 1 min). The next amino acid was coupled in DMF and the acylation time was visually followed by a decrease in the yellow colour of the resin (for 30–35 min). Finally, the resin was rinsed with DMF (10 × 1 min) and the rest of the amino acids were coupled as described above. For the synthesis of glycopeptides, Fmoc-Asn(Ac_3GlcNAc-Ac_2GlcNAc)-OH (3 mol equiv.) was coupled with TBTU (2.8 mol equiv.) and NEM (2.8 mol equiv.) for 24 h, followed by treatment with 20% acetic anhydride in DMF (1 × 20 min).

O-deacylation of the Glycopeptides

The fully protected glycopeptide was dissolved in dry methanol (1 mg/ml) and sodium methoxide in methanol was added until a wetted pH paper indicated pH 9. The solution was kept at room temperature and the progress of the reaction was monitored by analytical HPLC. The solution was neutralized by the addition of solid CO_2, followed by concentration under reduced pressure. The crude product was dissolved in water and purified by preparative HPLC using a linear gradient of 0–100% buffer B over 85 min.

Disulphide Formation Using Thallium(III) Trifluoroacetate as Oxidative Reagent

Compounds **2b**, **2d**, **2g**, **6b**, **7b** and **8b** contain disulphide bridges and were synthesized according to the following method. To a solution of peptide or glycopeptide (1 mol equiv.) in TFA (0.8 mM) was added anisole (38.3 mol equiv.) and the solution was cooled to 0 °C. Thallium(III) trifluoroacetate (1.5 mol equiv.) in TFA (15.5 mM) was added and the resulting mixture was stirred for 1.5 h at 0 °C. TFA was removed under reduced pressure and after precipitation with diethyl ether (three times), the crude product was purified by preparative HPLC.

Disulphide Formation Using I_2 as Oxidative Reagent

Compound **2a** (17 mg) was dissolved in HOAc/H_2O (4 : 1) (30 ml). Then I_2 (10 mM) was added under argon until the colour of the solution turned yellow. The reaction was kept in the dark and under argon and stirred for 1 h. The progress of the reaction was monitored by analytical RP-HPLC using a linear gradient of 0–100% buffer B over 50 min. The solution was diluted with water to a total volume of 60 ml. Finally, excess of I_2 was removed by extraction with CCl_4 (4 × 15 ml) and the aqueous solution was lyophilized. The crude product was purified by preparative HPLC.

2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl Amine

Penta *O*-acetyl chitobiosyl azide (201 mg, 0.303 mmol) [32] was dissolved in EtOAc : MeOH (1 : 1, v/v) (12 ml) and hydrogenated at room temperature for 2 h in presence of 5% Pd/C. The catalyst was filtered off and the solution was concentrated. The product was found to be pure according to TLC ($CHCl_3$: MeOH (5 : 1, v/v); R_F, 0.58. Yield: 96% (185.2 mg, 0.202 mmol).

N^{γ}-(2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-asparagine

To a solution of N^{α}-Fmoc-Asp(Cl)-OPfp (176.7 mg, 0.329 mmol) in THF (10 ml) at −40 °C penta *O*-acetyl chitobiosyl amine (185.2 mg, 0.292 mmol) dissolved in THF (150 ml) was added, followed by NEM (41.1 μl, 0.329 mmol). The reaction was stirred overnight under argon and the progress of the reaction was followed by TLC ($CHCl_3$: MeOH, (20 : 1, v/v); R_F, 0.56; R_F, 0.30(amine)). The precipitate was removed by filtration and the solution was concentrated (at 40 °C). The compound was purified by medium-pressure chromatography using Sephadex LH 20 and DMF as solvent. It was then used directly for glycopeptide synthesis. Yield: 81.4% (270 mg, 0.238 mmol). Found: MALDI-MS *m/z*, 994.8 ($M+Na^+$) and ($M+K^+$), $C_{45}H_{54}N_4O_{20}$, requires *M*; 970.94.

Synthesis of Compound 1

Solid-phase peptide synthesis of compound **1** was carried out as described above by automated solid-

phase synthesis using a PEGA-resin (250 mg, 0.44 mmol/g) and a Rink-linker. The crude product (70.5 mg, 50%) was purified by semi-preparative RP-HPLC. Compound **1** was pure according to analytical HPLC (a linear gradient of 10–60% buffer B over 25 min, t_R 14.4 min), obtained as a solid (32.5 mg, 23%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 2a

Compound **2a** was prepared as described above by solid-phase synthesis using the plastic syringe technique and was performed on a PEGA-resin (146 mg, 0.45 mmol/g) using the Rink-linker. The crude product was purified by semi-preparative RP-HPLC. Compound **2a** was pure according to analytical HPLC (t_R 12.9 min), obtained as a solid (35.1 mg, 46%) and characterized by MALDI-MS (Table 2).

Synthesis of Compound 2b using I_2

Pure compound **2a** (17 mg) was treated as described above with I_2 as oxidative reagent. The crude material was purified by semi-preparative RP-HPLC to give the compound **2b** (2.5 mg, 16%) and its reduced form 2b(r) (3.7 mg, 24%). Both compounds were pure according to analytical HPLC ((**2b**) t_R 12.4 min and **2b**(r) t_R 12.9 min) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 2b Using Thallium(III) Trifluoroacetate

Crude compound **2a** (18 mg) was treated with thallium(III) trifluoroacetate as described above. The crude disulphide product (18.4 mg, 96%) was purified by semi-preparative RP-HPLC. Compound **2b** was pure according to analytical HPLC (t_R 12.3 min), obtained as a solid (5.1 mg, 33%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 2c

Compound **2c** was prepared above as described in solid-phase synthesis using the plastic syringe technique and performed on a PEGA-resin (146 mg, 0.45 mmol/g) and the Rink-linker. The crude compound **2c** (t_R 12.7 min) was obtained as a solid (38.4 mg, quantitative) and characterized by MALDI-MS (Table 2).

Synthesis of Compound 2d

Crude compound **2c** (25.9 mg) was treated with thallium(III) trifluoroacetate. The crude product (25.2 mg, quantitative) was purified by semi-preparative RP-HPLC. Compound **2d** was pure according to analytical HPLC (t_R 12.2 min), obtained as a solid (7 mg, 31%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 2e

Compound **2e** was prepared as described above by solid-phase synthesis using the plastic syringe technique and performed on a PEGA resin (200 mg, 0.14 mmol/g) and the Rink-linker. The crude compound **2e** (t_R 19.1 min) was obtained as a solid (44.9 mg, quantitative) and characterized by MALDI-MS (Table 2).

Synthesis of Compound 2f

Crude compound **2e** (3.7 mg) was deprotected as described in *O*-deacylation of the glycopeptides for 8 h and purified by semi-preparative RP-HPLC. Compound **2f** was pure according to analytical HPLC (t_R 12.9 mm), obtained as a solid (1.3 mg, 42%) and characterized by MALDI-MS (Table 2).

Synthesis of Compound 2g

Pure compound **2f** (4.2 mg) was treated as described above with thallium(III) trifluoroacetate and the disulphide was purified by semi-preparative RP-HPLC. Compound **2g** was pure according to analytical HPLC (t_R 12.9 min), obtained as a solid (2.9 mg, 76%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 3a

Compound **3a** was prepared as described above by solid-phase synthesis using the plastic syringe technique and performed on a PEGA-resin (250 mg, 0.36 mmol/g) using the Rink-linker. The product was purified by semi-preparative RP-HPLC. Compound **3a** was pure according to analytical HPLC (linear gradient of 0–45% buffer B over 35 min; t_R 22.1 min), obtained as a solid (28.5 mg, 33%) and

characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 3b

Compound **3b** was prepared as described above by solid-phase synthesis using the plastic syringe technique and performed on a PEGA resin (400 mg, 0.14 mmol/g) and the Rink-linker. The crude compound **3b** (t_R 21.9 min) was obtained as a solid (25.1 mg, 94%) and characterized by MALDI-MS (Table 2).

Synthesis of Compound 3c

Crude compound **3b** (5 mg) was deprotected as described in *O*-deacylation of the glycopeptides for 24 h and purified by semi-preparative RP-HPLC. Compound **3c** was pure according to analytical HPLC (t_R 16.7 min) obtained as a solid (2.1 mg, 48%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 4

Compound **4** was prepared as described above by solid-phase synthesis using the plastic syringe technique on a PEGA-resin (138 mg, 0.40 mmol/g) using the Rink-linker. The peptide was cleaved with 95% TFA in the presence of triisopropylsilane (3 mol equiv.). The crude product (26 mg, 100%) was purified by semi-preparative RP-HPLC. Compound **4** was pure according to analytical HPLC (t_R 16.4 min), obtained as a solid (12.4 mg, 51%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 5

Compound **5** was prepared as described above by automated solid-phase synthesis on a PEGA-resin (200 mg, 0.44 mmol/g) using the Rink-linker. The peptide was cleaved with 95% TFA in the presence of triisopropylsilane (3 mol equiv.). The peptide was purified by semi-preparative RP-HPLC. Compound **5** was pure according to analytical HPLC (t_R 19.8 min), obtained as a solid (13.2 mg, 17%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 6a

The synthesis of compound **6a** was carried out as described above by automated solid-phase synthesis using a PEGA-resin (500 mg, 0.14 mmol/g) and a Rink-linker. Compound **6a** was purified by semi-preparative RP-HPLC and was pure according to analytical RP-HPLC (t_R 19.8 min), obtained as a solid (15.6 mg, 26%) and characterized by MALDI-MS (Table 2).

Synthesis of Compound 6b

Crude compound **6a** (6.5 mg) was treated with thallium(III) trifluoroacetate as described above. The crude disulphide was purified by semi-preparative RP-HPLC and was pure according to analytical RP-HPLC (t_R 20.1 min), obtained as a solid (1.8 mg, 32%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 7a

The synthesis of compound **7a** was carried out as described above by automated solid-phase synthesis using a PEGA-resin (350 mg, 0.14 mmol/g) and an HMBA-linker. The crude compound **7a** (t_R 20.4 min) was obtained as a solid (82.6 mg, 81%) and characterized by MALDI-MS (Table 2).

Synthesis of Compound 7b

Crude compound **7a** (50 mg) was treated with thallium(III) trifluoroacetate as described above. The crude disulphide (19.7 mg, 41%) was purified by semi-preparative RP-HPLC (a linear gradient of 0–20% buffer B over 15 min followed by a linear gradient of 20–45% over 60 min). Compound **7b** was pure according to analytical HPLC (t_R 20.8 min) (Figure 7), obtained as a solid (3.9 mg, 8.1%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Synthesis of Compound 8a

Solid-phase peptide synthesis of compound **8a** was carried out as described above by automated solid-phase synthesis using a PEGA-resin (350 mg, 0.14 mmol/g) with an HMBA-linker. Compound **8a** (t_R 20.5 min) eluted as a single peak in analytical HPLC was obtained as a solid (104 mg, 90%) and characterized by MALDI-MS (Table 2).

Synthesis of Compound 8b

Crude compound **8a** (50 mg) was treated with thallium(III) trifluoroacetate as described above. The crude disulphide (51 mg, 100%) was purified by semi-preparative RP-HPLC (a linear gradient of 0–20% buffer B over 15 min followed by a linear gradient of 20–45% over 60 min). Compound **8b** was pure according to analytical HPLC (t_R 20.9 min) (Figure 8), obtained as a solid (6.7 mg, 14%) and characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3).

Preparation of Calf Testis Membranes

Calf testis were decapsulated, weighed and minced in a blender with membrane buffer A (10 mM Tris–HCl, 1 mM $MgCl_2$, 10 μM *p*-hydroxymercuribenzoate Na, 0.001% mercaptoethanol and 15% sucrose (pH 7.4). The ratio of tissue to buffer was 0.25 g/ml. After blending, the suspension was further homogenized in 30 ml aliquots followed by centrifugation for 45 min at 7000 g_{av}. The pellet was discarded and the supernatant was centrifuged at 30,000 g_{av} for 30 min. The pellet was weighed and resuspended in half volume buffer A and half volume buffer B (10 mM Tris–HCl, 1 mM $MgCl_2$, 0.001% NaN_3, pH 7.2) with a ratio of membranes to buffer of 150 mg/ml. Membranes were stored at −80 °C in aliquots for further use.

Iodination of Follicle-stimulating Hormone

The hFSH was solubilized in phosphate buffered saline (PBS, pH 7.4, 0.5 mg/ml). The hFSH solution (10 μl) was mixed with lactoperoxidase (10 μl) in PBS (1.0 unit/ml). $Na^{125}I$ (500 μCi) were added followed by five sequential additions of 1 μl hydrogen peroxidase (30% H_2O_2 diluted 20,000 times in PBS) at intervals of 1 min. The reaction was stopped by addition of 25 μl lactoperoxidase stop buffer (10 mg/ml tyrosine, 10% glycerol, 0.1% xylene cylanol in PBS) and the solution was chromatographed on a column with G-10 Sephadex (Pharmacia). The fraction containing the labelled hFSH was stored in aliquots at −20 °C. The specific activity was approximately 100,000 c.p.m./ng of hFSH.

Radio Receptor Binding Assay on Bovine Testis Membranes

The K_d value was determined by incubating dilutions of the radioligand with calf testis membranes. Unspecific binding was determined by addition of cold FSH in excess. Scathard plots showed a K_d of hFSH of 6×10^{-10} M. Displacement curves for FSH were generated with increasing concentrations of unlabelled pure hFSH in a concentration range from 10^{-7} to 10^{-12} M and the ability of the peptides to inhibit hFSH-binding was tested in a concentration range from approximately 10^{-3} to 10^{-7} M. The assays were performed as follows: membranes were thawed, centrifuged at 30,000 g_{av} and resuspended in incubation buffer (50 mM HEPES, 5 mg $MgCl_2$, 100 mM sucrose, 0.1% ovalbumin, pH 7.5). Membranes (3 mg, wet weight) were preincubated with hFSH or peptides for 2 h in incubation buffer and a fixed amount of [^{125}I]hFSH (approximately 2.5 ng) was added to the vials. The tubes were incubated for 16 h at room temperature. Membranes were harvested on low protein-binding Durapore filters (Millipore) and washed once with 3 ml cold incubation buffer without ovalbumin. The filters were counted in a Cobra γ-counter (Packard).

Radio Receptor Binding Assay on hFSH Expressing 293 Cells

The human fetal kidney cell line 293 stably transfected with the gene for the human FSH receptor was obtained from Dr A. Hsueh, Stanford University School of Medicine, Stanford, California. The procedure was essentially the same as for the membranes. K_d was determined to be 1×10^{-9} M. Briefly described, 2×10^5 cells were mixed with a fixed concentration of iodinated hFSH and varying concentrations of peptides. Tubes were incubated for 16 h at room temperature and cells were harvested by centrifugation at 600 *g* and washed once in incubation buffer using 10,000 *g* to separate cells and supernatant. The pellet was counted in a gamma counter.

RESULTS AND DISCUSSION

General Considerations in the Design of a Synthetic hFSH Antagonist

The hormone hFSH is a heterodimeric glycoprotein hormone that belongs to the same family as hCG, hLH and hTSH [1, 2]. While the 92 amino acid long α-chain is identical for hFSH, hLH, hCG and hTSH, the β-chain is different and determines the specificity for each hormone. All the hormones also contain *N*-linked complex carbohydrate structures. The struc-

ture of hFSH is unknown but, recently, the three-dimensional structure of hCG has been published [12, 13]. In the present investigation various constructs for a suitable hFSH antagonist have therefore been designed based on this structure. The focus has been directed to four important regions of hCG that affect the biological activity and these have been linked together in our attempt to mimic the native structure of the active site of the hFSH hormone. The four regions are:

(I) the region βhCG(38–57) (corresponding to βhFSH(32–51)), for hCG known as the Keutman loop and which inhibits binding of hCG to the receptor [8, 9].
(II) the region βhCG(93–100) (corresponding to βhFSH(87–94)), called the determinant loop which is close to both the Keutman loop and the carbohydrate moiety on α-Asn52 [10]. The residue βThr97 (corresponding to βSer91 in hFSH) is quite close to this carbohydrate and the activity is not significantly affected by mutagenesis of this residue [10].
(III) the C-terminal α(88–92) of the α-chain which is also close to the determinant loop [7]. The binding of hCG to the receptor is abolished when this region is removed by carboxypeptidase.
(IV) the region α(52–57) which contains the glycosylated αAsn52 [6]. Removal of this carbohydrate moiety on αAsn52 abolishes the activity without affecting the receptor binding affinity, whereas removing the carbohydrates from the β-unit does not change the biological activity [6].

Modelling

The two structures of hCG that have recently been published [13, 14] were retrieved from the Brookhaven Protein Data Bank. The two structures are similar except for αTyr94 which either is buried in [13] or points away [14] from the hormone molecule. However, the hCG structure with the buried tyrosine [13] was exploited and all the amino acid residues in the β chain were replaced with the corresponding amino acids from hFSH. The amino acid residues included in the four above-mentioned regions and exposed on the surface of hFSH were labelled as red (I), yellow (II), orange (III) and blue (IV), respectively (Figure 1). All the other amino acid residues were then deleted. Each of the four regions was subjected to constrained MD calculations. During the dynamic simulation, selected distances were constrained within 1 Å using an appropriate small penalty force (5 kcal/mol/Å^2). Initially, a minimization of 200 steps was performed for each of the four regions using the Discover/Amber force field at 500 K *in vacuo*, followed by a dynamic simulation (10,000 steps of 1 fs). The resulting structures for the four regions deviated less than 2 kcal/Å from their initial values (Table 1, compounds **1**, **2b**, **2d**, **2g**, **3b**, **3c**, **4**). Second, because the hFSH hormone binding sites only consist of a few peptide fragments, we were interested to combine them in several constructs. Three different constructs were modelled and the connecting loops that link the peptide fragments together to mimic the hFSH structure were designed using the Insight/Discover program package. In the first construct, the regions βhFSH(39–54) and βhFSH(92–101) were linked since in hFSH they are connected through hydrogen bonds. The linker Gly-Pro (GP) which would facilitate the formation of a β-turn was found to combine the two peptide fragments (Figure 2) (Table 1, compound **6b**). Furthermore, it has been demonstrated that in FSH it is important for the binding that the C-terminus of the α-chain can approach the determinant loop. It was found that GPG could form a suitable loop in the second construct, βhFSH(39–54)-GP-βhFSH(92–101)-GPG-α(94–98) (Table 1, compound **7b**), allowing the fragments to maintain their inter-residue distances during weakly constrained MD calculation. Finally, a construct that contains all the four important regions was designed using once again a GPG linker; α(45–55)-GPG-βhFSH(41–54)-GP-βhFSH(92–101)-GPG-α(94–98) (Figure 3) (Table 1, compound **8b**). Other linkers were also incorporated for connection of the fragments but were less favourable and discarded. Minimization and dynamic simulations for the three peptide constructions were performed *in vacuo* at 500 K using two to three intra-fragment and three to four inter-fragment constraints with a 5 kcal/mol penalty function for each compound. A satisfactory fit between these constructions and the native hFSH structure were obtained. The four regions from the second hCG structure [14] and the three combined constructs were analysed using the same protocol as described above. However, these sequences did not deviate significantly from the above sequences after minimization and dynamic simulation. All the calculated constructs were selected as synthetic targets in the attempt to develop hFSH antagonists (Table 1). For binding studies, the sequence βhFSH(33–53) (Table 1, compound **5**) was selected as reference since it has previously been found to bind to the FSH receptor with a $K_d = (5.5 \pm 1.4) \times 10^{-5}$ M [26].

Preparation of Peptides, 1, 2b, 2d, 3a, 4 and 5

Peptides **1**, **2a**, **2c**, **3a**, **4** and **5** were synthesized by the stepwise solid-phase synthesis [15] using Fmoc chemistry [27, 28] on a PEGA-resin [20]. The couplings were performed with Fmoc–amino acid–Pfp esters with the addition of Dhbt-OH as an acetylation catalyst and a Rink-linker [25] was used to generate an α-carboxamide group upon the TFA cleavage. The plastic syringe technique [21] was utilized for peptides containing 11 amino acid residues or less while a custom-made automated continuous flow peptide synthesizer [22, 23] was used for peptides longer than 11 amino acid residues. All functional side chains were protected, including Asn and Gln which were protected with trityl groups. In peptides **2a** and **2c**, the side chains of the two Cys intended to form the disulphide bond of the determinant loop were protected with acetamido methyl groups [17] to protect them from oxidation while the single Cys in peptides **4** and **5** was protected with trityl. Peptides **1**, **2a** and **2c** were side-chain deprotected and cleaved from the resin support by treatment with 95% TFA, providing a compound that eluted as one major peak in analytical HPLC and afforded an overall average yield of 73% of the crude product. The identity of the peptides were confirmed by MALDI-MS (Table 2) and amino acid analysis (Table 3). However, when peptide **4** was cleaved by 95% TFA, the expected peptide with the Cys residue protected (the trityl group, $M+243$) was isolated as shown by MALDI-MS. This was not unexpected since TFA deprotection of Cys(Trt) is reversible in the absence of suitable scavengers [29]. Therefore, two different methods to cleave peptide **4** were investigated: (a) TFA : thioanisole : EDT : H_2O (87.5 : 2.5 : 5 : 5, v/v) [29] and (b) 95% TFA containing 4 equivalent of triisopropylsilane (TIS) [30]. The scavenger-rich TFA mixture gave peptide **4** with an additional mass of 243 units (the trityl group) whereas the presence of TIS in TFA led to complete detritylation and afforded the expected product in 51% yield after purification as confirmed by MALDI-MS (Table 2) and amino acid analysis (Table 3). In the amino acid analysis, Cys was determined as cysteic acid after oxidation with performic acid [31]. Peptide **5** which also contained a Cys residue was cleaved as well with 4 equivalent of TIS in TFA. MALDI-MS (Table 2) confirmed the molecular mass, and amino acid analysis (Table 3) gave the expected composition for peptide **5**.

To prepare the cyclic peptide, **2a** was treated with I_2 in $HOAc/H_2O$ (4 : 1) [32] to remove the Acm groups on the two Cys residues and concurrently form an intramolecular disulphide bond. Unfortunately, this method provided two peaks (2 : 3 ratio) in analytical HPLC and characterization of these two peaks by MALDI-MS and amino acid analysis showed that the cyclic peptide **2b** was formed in 16% yield whereas its reduced form (free –SH groups) was formed in 24% yield. Alternatively peptide **2b** was then prepared by direct deprotection/oxidation using thallium(III)trifluoroacetate ($Tl(tfa)_3$) in TFA [18] and the cyclic peptide was obtained as the major product in a yield of 33%. Similar, peptide **2c** was cyclized to **2d** in 31% overall yield. The cyclic nature of compounds **2b** and **2c** was determined by MALDI-MS (Table 2).

Preparation of the Glycopeptides, 2g and 3c

Both glycopeptides **2g** and **3c** contained the disaccharide β-D-GlcNAc–(1->4)β-D-GlcNAc (*N*,*N*-Diacetylchitobiose) (GlcNAc-GlcNAc) to mimic the activity of the more complex $Man_3GlcNAc_2$ structure [19]. The chitobiose moiety was attached to Asn and incorporated as a building block during the stepwise solid-phase peptide synthesis using Fmoc chemistry [16]. The building block, Fmoc-Asn(Ac_3GlcNAc-Ac_2GlcNAc)-OH, was prepared by two different methods [33–35]. The glycosylated Asn derivative was incorporated into the peptide sequence as its free carboxylic acid using the TBTU method [36] (Figure 4). The coupling time for Fmoc-Asn(Ac_3GlcNAc-Ac_2GlcNAc)-OH was 24 h and the peptide resin was then capped using 20% acetic anhydride to minimize purification problems. Both *N*-glycopeptides were cleaved from the resin by 95% TFA after the synthesis and gave only one major peak by HPLC analysis (see Figures 5(a) and 6(a)) and afforded crude **2e** and **3b** in 100% and 94% yield, respectively. The partially deprotected glycopeptides **2e** and **3b** were characterized by MALDI-MS (Table 2). The acetyl group of **2e** and **3b** could be removed by sodium methoxide in methanol at pH 9–10 (Figure 4). Thus, the sample was suspended in methanol and pH was adjusted to 9–10 by addition of sodium methoxide. The progress of the reaction as monitored by analytical RP-HPLC was completed within 24 h for both glycopeptides (Figures 5(b) and 6(b)). An excessively long deacylation time was required owing to the low solubility of the glycopeptides. Glycopeptides **2f** and **3c** were isolated by preparative RP-HPLC in 42% and 48% yield, respectively. Glycopeptide **3c** was characterized by MALDI-MS (Table 2) and amino acid analysis (Table 3), while **2f** was identified by MALDI-MS (Table 2). However, the

remaining Acm group on the two Cys moieties of **2f** were removed by $Tl(tfa)_3$ to give the cyclic product (Figure 5(c)). Glycopeptide **2g** was purified by preparative RP-HPLC in 76% yield and its cyclic nature was confirmed by MALDI-MS (Table 2).

Preparation of the Constructs, 6b, 7b and 8b

The syntheses of peptides **6a**, **7a** and **8a** were all carried out by Fmoc chemistry using a fully automatic custom-made continuous flow peptide synthesizer [22, 23]. For preparation of **6a**, a Rink-linker was used to provide an α-carboxamide whereas the acid-stable HMBA-linker [23] was utilized for the synthesis of **7a** and **8a** to generate a free carboxylic acid at the C-terminal. Deprotection and cleavage of **6a** was mediated by 95% TFA and afforded the pure product in 26% yield (Table 2) after preparative HPLC purification. Peptides **7a** and **8a** were first side-chain deprotected by 95% TFA, followed by neutralization and cleavage from the resin using 0.1 M NaOH. Analytical HPLC of crude **7a** and **8a** provided only one peak (see Figures 7(a) and 8(a)) and the overall yield of the crude products were 81% and 90%, respectively. However, characterization of the purified peptides **7a** and **8a** by MALDI-MS showed the correct mass (Table 2) as well as an additional mass (*M*-134*u*), corresponding to a deletion peptide lacking a His residue. The crude peptides were purified after their final cyclization by disulphide bond formation. The cyclic peptides **6b**, **7b** and **8b** were prepared using $Tl(tfa)_3$ in TFA [18] (Figures 7(b) and 8(b)). Peptide **6a** was purified with no difficulty by preparative HPLC and gave 32% yield. However, purification of **7b** and **8b** could be a complicated task because the only major peak contained in addition to the main product was a deletion side product. This serious problem of purification could partly be solved by using a slow gradient in HPLC and indeed, using the gradient 0–20% buffer B over 15 min, followed by a linear gradient of 20–45% of buffer B over 60 min afforded pure fractions of peptides **7b** and **8b** however only in 8.1% and 14% yield, respectively. The cyclizations of the three peptides were confirmed by MALDI-MS (Table 2).

Binding Studies

The binding capability of the peptides to the FSH receptor was tested in a competitive radio receptor assay. All the unglycosylated peptides were tested in

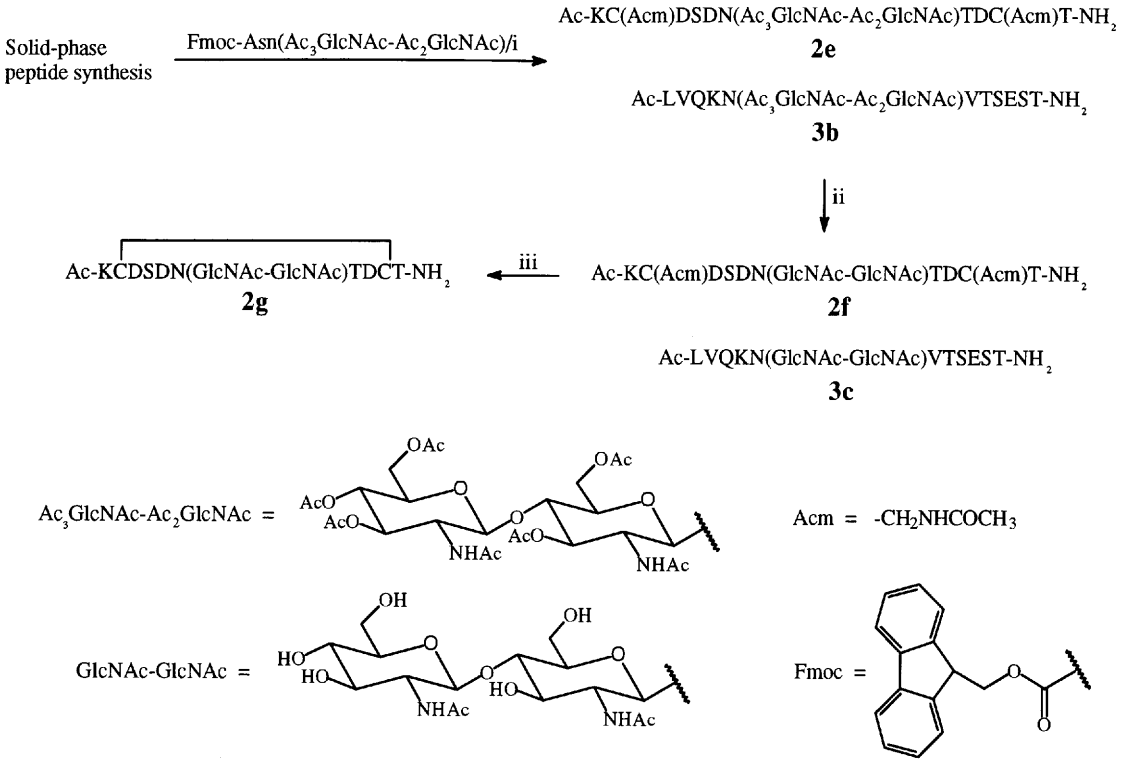

Figure 4 Reagents and conditions: (i) TBTU, NEM (3 molar equiv. each); (ii) NaOMe, MeOH; (iii) $Tl(tfa)_3$, TFA.

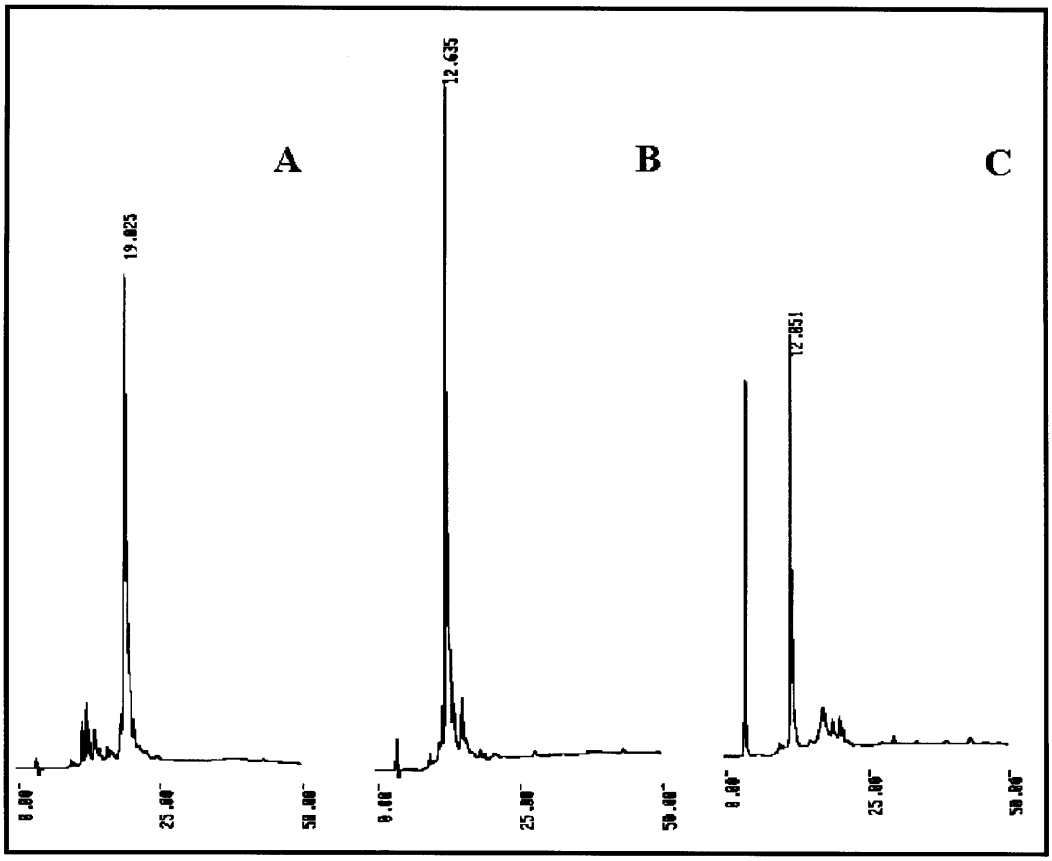

Figure 5 C-18 reverse-phase HPLC profile of crude glycopeptide **2e** after TFA cleavage (a), crude glycopeptide **2f** after deacylation with sodium methoxide in methanol (b) and the crude glycopeptide **2g** after intramolecular oxidation using $Tl(tfa)_3$ (c).

addition to the glycosylated sequence of peptide **2** and **3**. The peptides were tested in concentration ranges from approximately 10^{-3} to 10^{-4} M for the short peptides (**1**, **2b**, **2d**, **2g**, **3a**, **3c**, **4**, **5**) and from approximately 10^{-4} to 10^{-7} M for the longer peptides (**6b**, **7b**, **8b**). The hormone hFSH was included in all experiments. IC_{50} values for hFSH only varied from 1.0 to 0.7 nM between independent experiments and a clear dose–response relationship for cold hFSH was observed in all experiments, demonstrating that the assays were functional. The group of peptides constituting the determinant loop (**2b**, **2d** and **2g**) inhibited the binding of iodinated FSH in a concentration-dependent manner (Figure 9) with IC_{50} values of 5.4×10^{-4} M (**2b**), 1.1×10^{-4} M (**2d**) and 9.5×10^{-5} M (**2g**). Compound **5** did also show this inhibition in the range from 10^{-3} to 10^{-4} (Figure 10. However, a randomly selected control peptide also inhibited the binding of hFSH in the same range. Therefore, three additional control peptides were randomly selected and included to elucidate the nature of the binding (Figure 11). These peptides also inhibited the binding of iodinated hFSH in the interval from 10^{-3} to 10^{-4} M. Conclusively all the binding constants determined for peptides were considerably lower than the binding of hFSH and close to the binding affinities often observed for non-specific binding. Therefore, it could not be concluded that the observed inhibition for the group of determinant loop peptides and compound **5** was due to specific binding to the hFSH receptor binding site. The remaining peptides including the longer construct had no effect on the binding of iodinated FSH. Compound **5** is known from the literature to inhibit the binding of iodinated FSH to bovine testis membranes [25, 36]. The assay used in these reports is very similar to the present assay, the sensitivity is the same and the present data match the ones observed previously. However, the present inclusion of control peptides leaves the question as to which extent this peptide inhibits the FSH in a specific manner, since the unrelated control peptides also

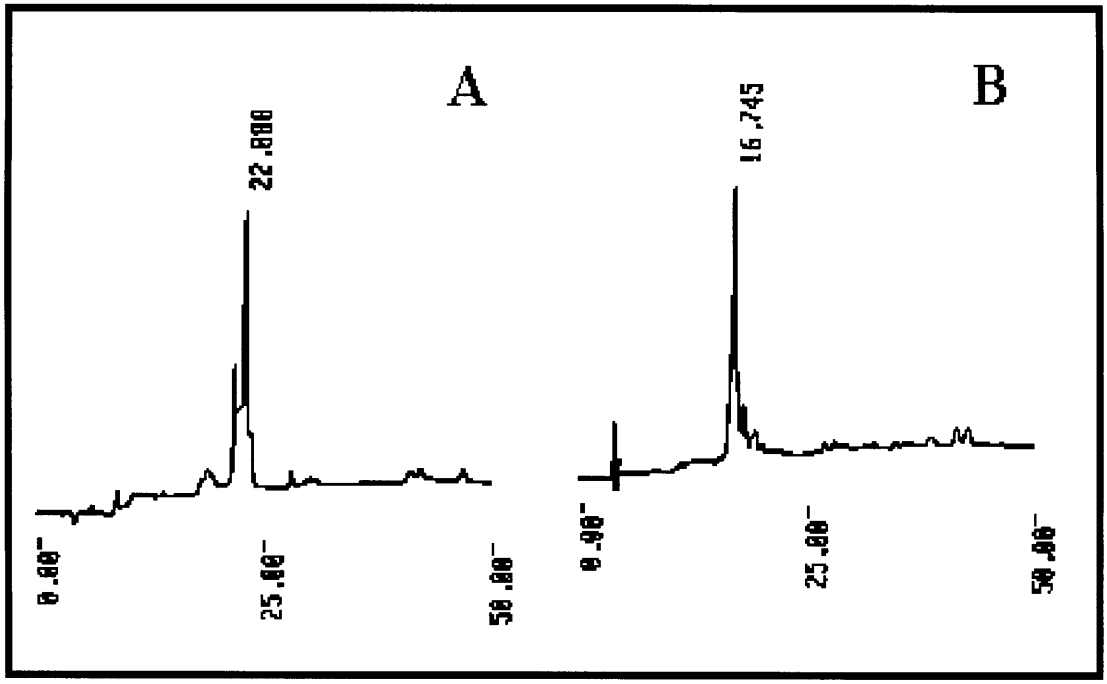

Figure 6 C-18 reverse-phase HPLC profile of crude glycopeptide **3b** after TFA-cleavage (a) and crude glycopeptide **3c** after deacylation with sodium methoxide in methanol (b).

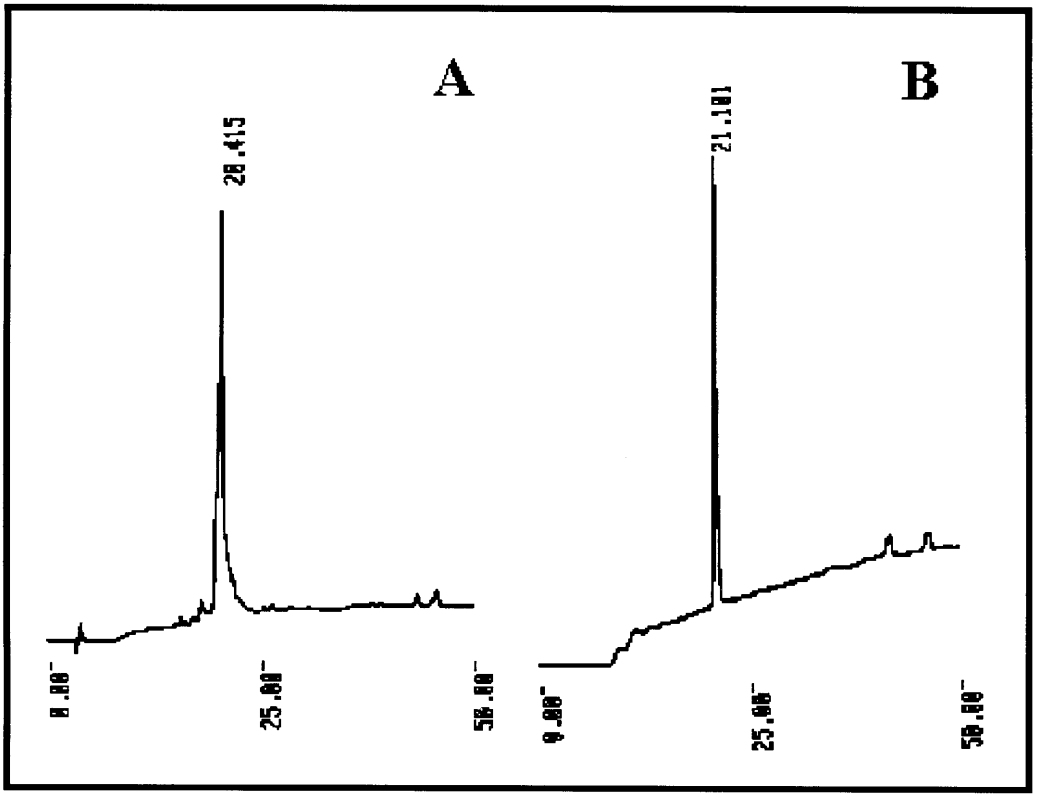

Figure 7 C-18 reverse-phase HPLC profile of crude peptide **7a** after TFA deprotection and NaOH cleavage (a) and treatment with $Tl(tfa)_3$ to form **7b** (b).

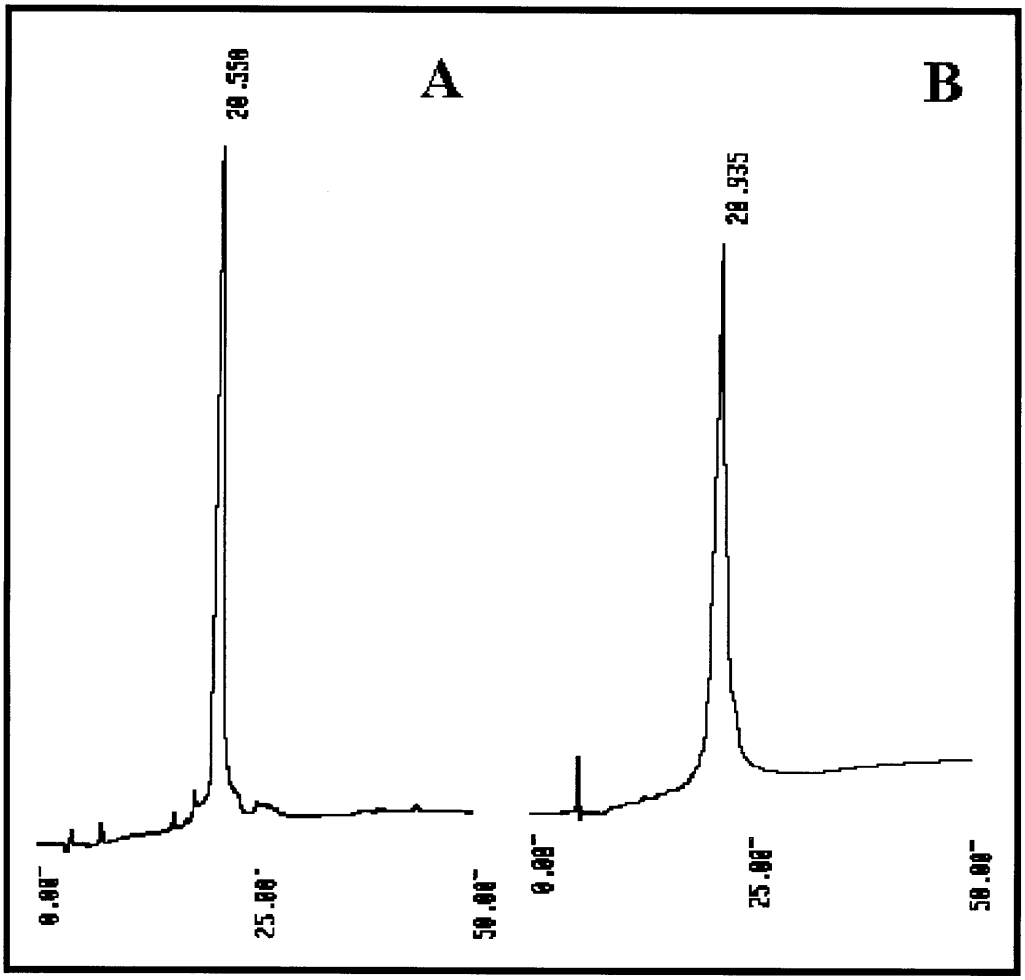

Figure 8 C-18 reverse-phase HPLC profile of crude peptide **8a** after TFA deprotection and NaOH cleavage (a) and treatment with $Tl(tfa)_3$ to form **8b** (b).

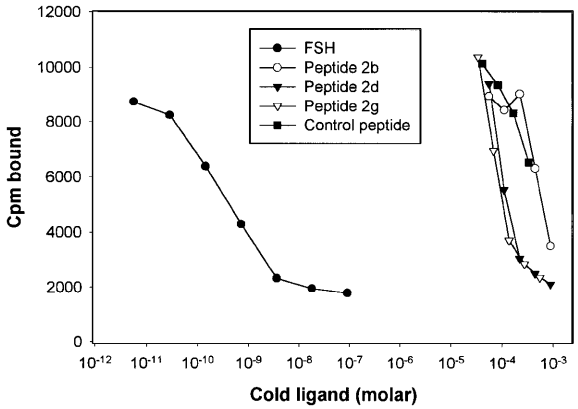

Figure 9 Inhibition of bound iodinated FSH to calf testis membranes by cold FSH and three peptides from the determinant loop; **2b**, **2d** and **2g**.

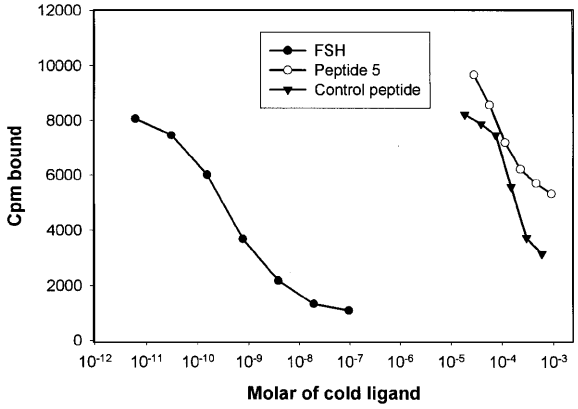

Figure 10 Inhibition of bound iodinated FSH to calf testis membranes by cold FSH and peptide **5**.

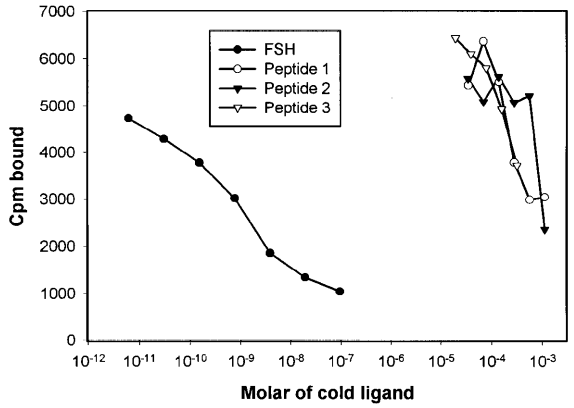

Figure 11 Inhibition of bound iodinated FSH by cold FSH and three different control peptides.

inhibit FSH-binding in a dose-dependent way and to the same degree as **5**. The determinant loop sequence (peptide **2b**), the S to N substituted peptide (**2d**) and the glycosylated sequence (**2g**) also showed inhibition in the same range as **5**. The substituted and glycosylated sequence inhibited slightly better than the natural sequence. However, the affinities for the receptor of all these peptides are very close to the range of unspecific binding as shown by the addition of control peptides. The failure of the longer peptide constructs to inhibit the binding of FSH stresses the problems in mimicking the structure of complex peptide hormones. More detailed information on the exact structure of FSH and the interaction with the receptor are desired for further attempts to develop FSH agonists.

CONCLUSIONS

In the present work three constructs in the development of synthetic hFSH antagonists were designed based on the 3D structure of hCG using a combination of molecular dynamic calculations and energy minimization. Furthermore, the synthesis of an entire series consisting of the three constructs and the four regions which are included in these constructs with or without the glycosylated amino acid, Asn(GlcNAc-GlcNAc), is achieved by the stepwise solid-phase method. However, in the synthesis of the two designed peptide constructs **7b** and **8b** containing 35 and 48 amino acid residues, respectively, the major peak that was isolated contained the expected product including a deletion side product. The purification problem of these two peptides was solved, although the yields were low. The other peptides and glycopeptides in the entire series provided only the expected product as the major product and were purified with no difficulty. The building block Fmoc-Asn(Ac_3GlcNAc-Ac_2GlcNAc) was synthesized and used in the synthesis of a linear and cyclic glycopeptide, respectively. The cyclic peptides and glycopeptide were prepared from their acetamidomethyl protected linear form by treatment with $Tl(tfa)_3$ in TFA and which proved to be more efficient than cyclization promoted by iodine.

All the synthetic peptides and glycopeptides were tested for binding activity in FSH receptor assays using radio receptor assays on bovine testis membranes and on hFSH receptor expressing human 293 cells. None of the synthetic peptides and glycopeptides showed improved inhibition FSH binding. The peptides derived from the determinant loop which according to the literature is known to inhibit binding of FSH showed the strongest binding activities. The determinant loop peptide (**2b**) was only able to inhibit the binding of the FSH with a IC_{50} value of 5.5×10^{-4} M. The S to N substituted peptide (**2d**) inhibited approximated five times better than the native sequence (1.1×10^{-4} M), whereas the glycosylated peptide (**2g**) had a little effect on the inhibition (9.5×10^{-5} M). Peptide **5**, which was used as a reference and was known from the literature [26], was shown to inhibit the FSH binding with a IC_{50} value of 6×10^{-4} M. However, inhibition in this range was also seen with the three different unrelated control peptides, which leaves the conclusion that no specific binding is actually seen. For further attempts to develop synthetic FSH constructs more detailed information on the interaction with the receptor is desired. It is difficult to mimic complex protein protein interactions with synthetic peptide constructions using a rational design-based approach.

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