C500 Report
A Diabetic Panacea

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Contents

Protein Medicinal Chemistry

Optimizing Peptide Hormones Used in the Treatment of Diabetes

Diabetes: Diabetes is a sizable public health problem that continues to grow in prevalence largely as the result of urbanization in the world's population. The growing prevalence of adult onset diabetes (Type II) has been described as an epidemic with as much as 30% of adults in select populations such as the Pima Indians being diagnosed with disease. There are 18.2 million people in the United States, or 6.3% of the population, who have diabetes. While an estimated 13 million have been diagnosed with diabetes, an additional 5.2 million people (or nearly one-third) are unaware that they have the disease[1]. Poorly managed diabetes can lead to a host of long-term complications — among these are heart attacks, strokes, blindness, kidney failure, blood vessel disease that may require an amputation, nerve damage, and impotence in men. The morbidity and mortality associated with diabetes is high and the financial burden on society is huge.

Diabetes is a disease in which the body is unable to properly use and store glucose (a form of sugar). Glucose accumulates in the bloodstream, causing blood glucose to rise to unacceptably high concentrations[2]. When glucose builds up in the blood instead of going into cells, it can cause two problems. Cells are immediately starved for energy and over time the elevated glucose concentration can harm the eyes, kidneys, nerves and heart. There are three major types of diabetes.

1) Type 1 diabetes is usually diagnosed in children and young adults, and was previously known as juvenile diabetes. In type 1 diabetes, the body does not produce insulin. Insulin is necessary for the body to properly use glucose. In type 1 (also called juvenile-onset or insulin-dependent) diabetes, the body produces insufficient insulin, a hormone that enables the body to use glucose. People with type 1 diabetes must take daily insulin injections to survive.

2) Type 2 diabetes (also called adult-onset or non insulin-dependent) diabetes results when the body doesn't produce enough insulin and/or is unable to use insulin properly (insulin resistance). This form of diabetes usually occurs in people who are over 40 years of age, overweight, and have a family history of diabetes. It is increasingly common today for Type 2 diabetes to occur in younger people, even adolescents. Type 2 diabetes is the most common form of diabetes. In type 2 diabetes, the body does not produce enough insulin and the insulin target cells respond poorly to insulin. Insulin is necessary for the body to be able to use sugar.

3) Pregnant women who have never had diabetes before but who have high blood sugar (glucose) levels during pregnancy are said to have gestational diabetes. Gestational diabetes affects about 4% of all pregnant women - about 135,000 cases of gestational diabetes in the United States each year. The cause of gestational diabetes is not known but seems similar to Type II diabetes where pregnancy induces a level of insulin resistance.

Insulin: Diabetes is mainly caused by the absolute or relative deficiency of insulin. Insulin is a rather small protein, with a molecular weight of about 6,000 daltons. It is composed of two peptide chains referred to as the A and B chains. A and B chains are linked together by two disulfide bonds, and an additional disulfide bond is formed within the A chain. In most species, the A chain consists of 21 amino acids and the B chain of 30 amino acids.
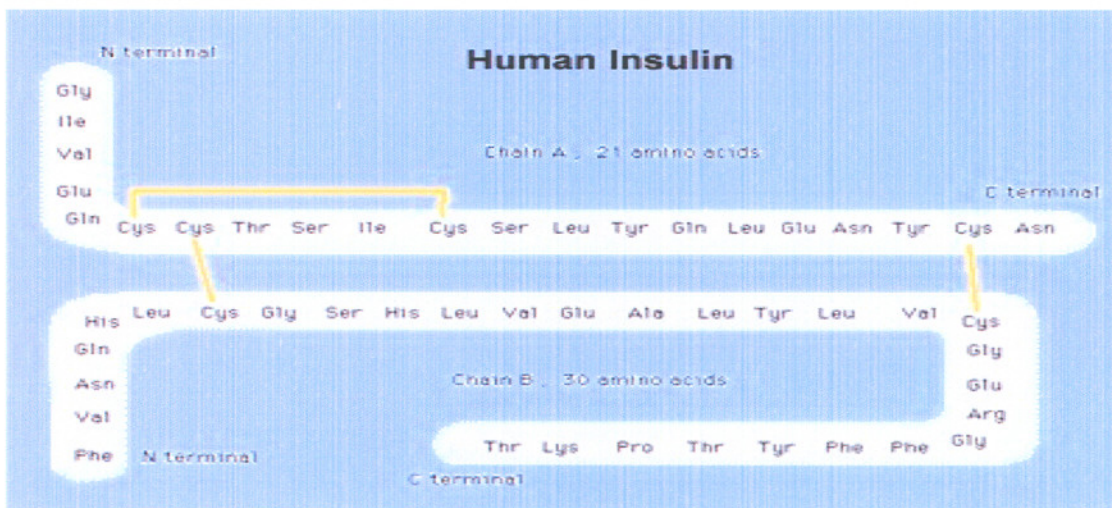

There are several types of insulin. They are classified by duration of action (pharmacodynamics)

a) Very fast

b) Fast

c) Slow

d) very slow acting.

The figure below shows the examples of common insulin regimens with the action curves for representative human insulins. A new type of insulin called Lantus® glargine has just

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been made available for patients. Lantus® is a very long acting insulin, with no peak over 24 hours. It is usually used in combination with a fast acting insulin such as Humalog® or regular insulin.
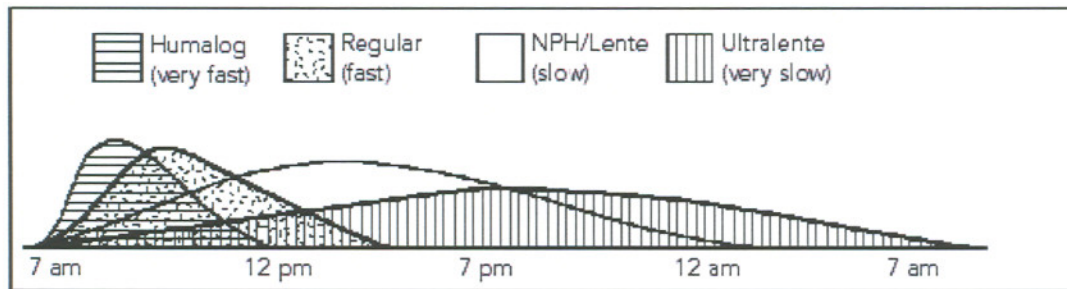
	Onset	Peak Action	Duration
Humalog	15 min.	1/2 -1 1/2 hrs	3-5 hrs
Regular	1/2 hr	2-4 hrs	6-8 hrs
NPH, Lente	1-3 hrs	6-12 hrs	18-24 hrs
Ultralente	approx. 4-8 hrs	12-18 hrs	approx. 24-28 hrs

Insulin is thus indeed one proven medicine that can normalize blood glucose in virtually all forms of diabetes. However, while insulin is a miraculous substance it is challenging medicine. Insulin has a relatively narrow therapeutic index and the propensity for life-threatening hypoglycemia significantly limits proper use of the drug. What is most

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needed is a medicine that is capable of normalizing blood glucose without the risk of hypoglycemia.

Insulin dimer   **Insulin hexamer**

Glucagon Incretin Peptide 1 (GLP 1): Glucagon-like peptide 1 is a hormone that offers the promise of revolutionizing the treatment of Type II diabetes since early clinical experiences suggest it to have an attractive pharmacologic signature[7].

Glucagon-like peptide 1 (7-36) amide (GLP-1) is an endogenous peptide that is secreted from the gut in response to the presence of food. It exists in two principal major molecular forms, as GLP-1(7-36) amide and GLP-1(7-37) acid. The peptide was first identified in the early 1980s in the course of cloning the genes for proglucagon[5].

Initial studies of GLP-1 biological activity in the mid 1980s utilized the full length amino-terminal extended forms of GLP-1 (1-37 and 1-36 amide). These larger GLP-1 molecules were generally devoid of biological activity. In 1987, three independent

research groups demonstrated that removal of the first six amino acids resulted in a shorter version of the GLP-1 molecule with substantially enhanced biological activity. The majority of circulating biologically active GLP-1 is found in the GLP-1(7-36) amide form), with lesser amounts of the bioactive GLP-1(7-37) acid form also detectable[5].
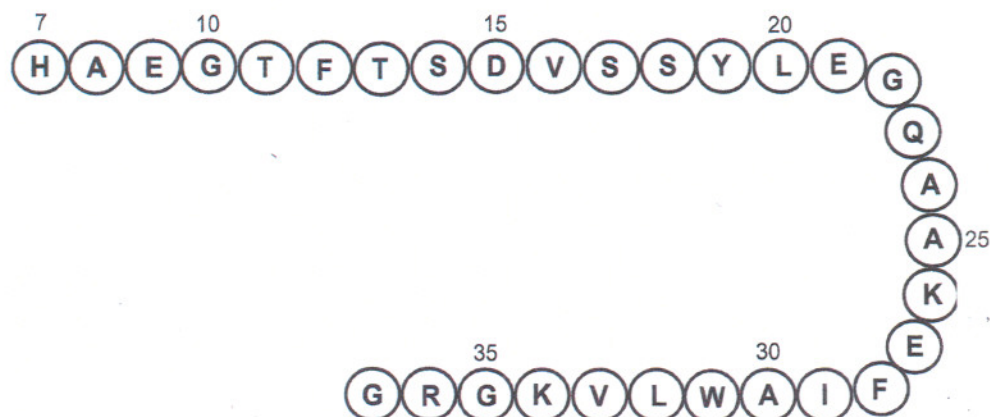
Recent studies have established that GLP-1 and its longer-acting analog exendin-4 have multiple synergistic effects on glucose dependent, insulin secretion pathways in the pancreatic β cells[3,4]. While the initial clinical experiences with native forms of GLP-1 and exendin-4 have yield sizable efficacy without overt hypoglycemia these peptide drug candidates are less than ideal medicines. Most notably these peptides have appreciably short biological half-lives and physical properties that render them prone to denaturation through aggregation. Lastly, exendin-4 being of non-human origin is reported to induce an immune response that compromises clinical efficacy.

An important locus for regulation of GLP-1 biological activity is the N-terminal degradation of the peptide by Dipeptidyl Peptidase (DPP-IV) -mediated cleavage at the position 2 alanine[4,5]. Rapid removal of the N-terminal dipeptide, His[7]-Ala[8], by the ubiquitous enzyme dipeptidyl peptidase IV (DPP IV) curtails the biological activity of GLP-1[6,7]. This is shown in the diagram below:

GLP-1　　　　　　　　　　　　　　GLP-2

HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR HADGSFSDEMNTILDNLAARDFINWLIQTKITD

↑　　　　　　　　　　　　　　　↑
DP IV　　　　　　　　　　　DP IV

GLP-1 and GLP-2 are co-secreted from enteroendocrine cells and rapidly degraded by DP IV

Endocrine cell

DP IV in blood vessels

The biological activities of GLP-1 include stimulation of glucose-dependent insulin secretion and insulin biosynthesis, inhibition of glucagon secretion and gastric emptying, and inhibition of food intake. The N-terminal histidine of glucagon-like peptide (GLP-1) and of exendin-4 have been shown to be critical for the activation of the pancreatic receptor (GLP-1R). Previous amino acid substitutions of this histidine with lysine and with alanine have been reported to result in a loss of activity in the peptide[3,4,8]. Hence it is interpreted that the imidazole ring of the histidine is necessary for the potency of the molecule.

The finding that GLP-1 lowers blood glucose in patients with diabetes, taken together with suggestions that GLP-1 may restore β cell sensitivity to exogenous secretagogues, suggests that augmenting GLP-1 signaling is a useful strategy for treatment of diabetic patients.

Human Glucagon Like Incretin Peptide (7-37)-OH

Oxyntomodulin: Oxyntomodulin is a 37 amino acid peptide that contains the 29 amino acid sequence of glucagons followed by an 8 amino acid carboxy-terminal extension[6].

Prodrug: A precursor (forerunner) of a drug is a prodrug. A prodrug must undergo chemical conversion by metabolic processes before becoming an active pharmacological agent. Many drugs are administered at sites that are remote from their site of action. The optimal physicochemical properties to allow high transcellular absorption following administration are well established and include a limit on molecular size, hydrogen

bonding potential and adequate lipophilicity. The prodrug that we are trying to make however has certain additional characteristics. By modifying our peptide, we shall appropriately extend its biological half life and thus improve its pharmacodynamics.

For many drug targets, synthetic strategies can be devised to balance the physicochemical properties required for high transcellular absorption and the SAR for the drug target. However, there are drug targets where the SAR requires properties at odds with good membrane permeability. These requirements include a requirement for significant polarity and groups that exhibit high hydrogen bonding potential such as carboxylic acids and alcohols. In such cases, prodrug strategies have been employed.

The rationale behind the small molecule prodrug strategy is to introduce lipophilicity and mask hydrogen bonding groups of an active compound by the addition of another moiety, most commonly an ester. An ideal ester prodrug for purposes of oral bioavailability should exhibit the following properties[9]:

1)   Weak (or no) activity against any pharmacological target,

2)   Chemical stability across a pH range.

3)   High aqueous solubility,

4)   Good transcellular absorption,

5)   Resistance to hydrolysis during the absorption phase,

6)   Rapid and quantitative breakdown to yield high circulating concentrations of the active component post absorption.

Our prodrug concept is not focused on oral bioavailability as these conventional small drug approaches but upon extended biological half life and thus many of the stringent necessities of conventional prodrugs are not relevant.

This is happening in the body.
(In Vivo)

TI/2 EXTENDED: HYPOTHESIS
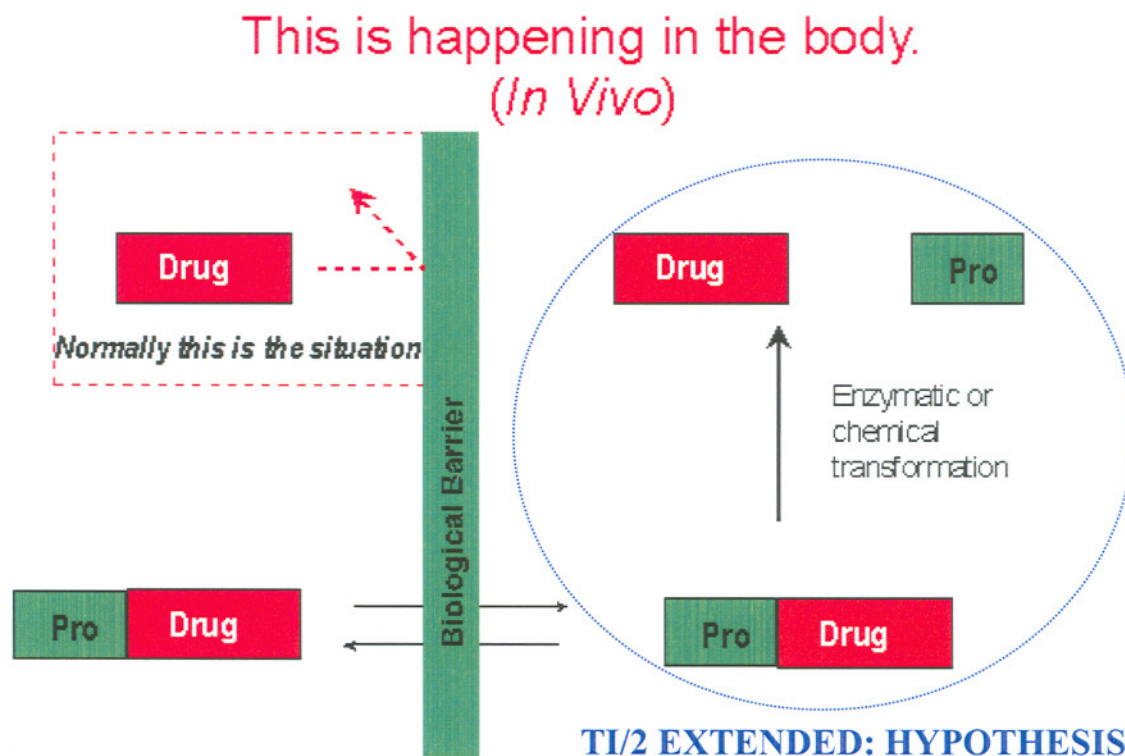
Prodrugs helps by:

1. **Improving physicochemical properties**

2. **Extending the biological half life**

Hypothesis (A Diabetic Panacea): We believe that by making appropriate chemical changes in the terminal ends of GLP-1 it should be possible to improve the pharmacodynamics and thus the pharmacology of the peptide hormone.

We have set our objectives in this C500 project as the identification of a GLP-1 analog that is physically and chemically stable, while being pharmacologically sustained in its duration of action. Furthermore, we desire the analog to possess minimal non-native amino acid sequence as a means to eliminate adverse immune response.

Our first approach is to establish a core GLP-1 sequence with appropriate stability by exploring alternatives to the C-terminal peptide extension commonly found in exendin-4. Additionally, we will simultaneously pursue the optimization of the C-exendin sequence as a C-terminal hybrid to GLP-1. The third goal stands largely independent of the C-terminal synthetic studies and pertains to the development of N-terminally modified GLP-1 analogs. The purpose of this latter work is focused on the development of a GLP-1 prodrug.

The N-terminal histidine is required for biological activity. We intend to explore chemical modifications that reversibly alter this single amino acid such that it remains inactive until the point of reversible to the native structure. We are particularly interested in diketopiperazine formation as a possible means to achieve the reversal of dipeptide extended GLP-1 derivatives. This chemistry is reasonably straightforward and allows a number of points where structure can be appropriately altered to refine the rate of

formation with release of the native hormone. It is possible that alternatives to the native N-terminal histidine will be required to achieve proper reversibility to a fully active GLP-1.

In simple words, we propose to make a prodrug that will slowly convert to the parent drug at body pH 7.2. Thus the pharmacodynamics of the drug will be much improved. We rely on the pH for this as then we will not have to be dependent on any specific enzyme. We also propose to increase the half life of the drug so that it is present for a longer time in vivo. Lastly, we need to slow the rate of in vivo clearance of an otherwise optimized pro-drug (pegylation).[10,11,12]

Getting down to the specifics, as described before oxyntomodulin is a 37 amino acid peptide that contains the 29 amino acid sequence of glucagon followed by an 8 amino acid carboxy-terminal extension. Oxyntomodulin also mimics the effects of GLP-1 and GLP-2 on gastric acid secretion and gut motility. Glucagon is largely homologous with GLP-1. On closer observation, one finds that the amino acids largely responsible for the potency of these two peptides are similar. The table below provides reference for structural comparison[3,4,13].

Structure-Activity of GLP-1

Peptide	7	10	15	20	25	30	35	40	45	50

GLP-1 H A E G T F T S D V S S Y L E G Q A A K E F I A W L V K G R amide

Exendin-4 H G E G T F T S D L S K Q M E E E A V R L F I E W L K N G G P S S G A P P P S amide

GLP-2 H A D G S F S D E M N T I L D N L A A R D F I N W L I Q T K I T D R

Glucagon H S Q G T F T S D Y S K Y L D S R R A Q D F V Q W L M N T

GIP Y A E G T F I S D Y S I A M D K I H Q Q D F V N W L L A Q K G K K N D W K H N I T Q

VIP H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N amide

Secretin H S D G T F T S E L S R L R D S A R L Q R L L Q G L V amide

PACAP-38 H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L G K R Y K Q R V K N K amide

PHI H A D G V F T S Q F S R L L G Q L S A K K Y L E S L I amide

GRF Y A D A I F T N S Y R K V L G Q L S A R K L L Q D I M S R Q Q G E S N Q E R G A R A R L amide

FIG. 1. **Comparison of the GLP-1 sequence to the sequence of other peptides in the GRF superfamily and the sequence of Exendin-4.** *Dark areas* represents regions of homology. Position numbers refer to the nomenclature used for GLP-1 (position 7 corresponds to position 1 of the other peptides).

It seems reasonable to hypothesize that the last eight amino acids of oxyntomodulin can be added to the carboxyl end of GLP-1 (since it is so similar to glucagon) to form a new bioactive peptide (GLP-oxyntomodulin). Additionally, this chimeric peptide will provide alternative sites for carboxy-terminal optimization of GLIP-1 performance. Dipeptides can be added to the amino-terminus to investigate differential tendencies for intramolecular cyclization (diketopiperazine formation) and release of the parent drug.

In such an investigation reported with cyclosporine-A prodrugs[14,15], it has been seen that thropugh modulating the chemical nature of dipeptide esters it was possible to get conversion rates at physiological conditions ranging from minutes to several days. Hence, it is appears worthy to investigate a similar prodrug approach with GLP-1.

For this we proposed to investigate four chemical functions attached to the amino-terminual histidine of the GLP-oxyntomodulin peptide:

- GlyPro, GLP-oxyntomodulin peptide

- ProPro, GLP-oxyntomodulin peptide

- γGlu, GLP-oxyntomodulin peptide

- Tetrahydrophthalic(H_4Pht), GLP-oxyntomodulin peptide

However, since we are initially interested only in testing the dissociation chemistry of the DKPs, we made a smaller model peptide GLP (7-15). This approach should save time and is expected to be less cumbersome. The four chemical functions were attached to this smaller peptide.

Methods of Synthesis: *Synthesis of GLP analogues*

The following GLP analogues were synthesized:

1. GLP 7-37 Oxyntomodulin chimeric peptide

2. ProPro,GLP (7-15) extended peptide

3. GlyPro,GLP (7-15) extended peptide

4. γ –Glu,GLP (7-15) extended peptide

5. H₄Pht,GLP (7-15) extended peptide

Synthesis of these analogues was performed on the Applied BiosystemsModel 430 A Peptide Synthesizer. Synthetic peptides were constructed by sequential addition of amino acids, and activated esters of each amino acid were generated by the addition of 3.8 ml of 0.5 M O-Benzotriazol-1-yl-N,N,N',N'–tetramethyl uranium hexafluorophosphate (HBTU) in DMF to a cartridge containing 2mmol of Boc protected residue. The amino acids were dissolved by bubbling Nitrogen gas through the cartridge. 1 ml of N,N-Diisopropylethylamine was then added to the cartridge to effect ester formation. This mixture was then transferred to the reaction vessel containing the solid phase resin upon which the peptide was synthesized.

The amino acid residue was then added to the vessel, vortexed several times, and the residue was allowed to couple to the resin for 10 minutes. Reaction vessel at the end of the synthesis contained approximately 300 mg of Boc amino acid PAM resin (PAM resin= OCH_2 phenylacetamidomethyl –copoly (styrene- 1 % divinyl benzene). The resin was washed numerous times with dimethylformamide (DMF), and treated with trifluoroacetic acid to remove the Boc protecting group and then washed again with DMF. Finally, the resin was washed several times with DMF.

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- After Gln residues various steps were implemented to prevent internal cyclization of this amino acid to pyrollidone carboxylic acid (PCA or pGlu). When mixed DMF and TFA produce heat. This small amount of energy is sufficient to induce Gln cyclization. Therefore, washes of Dichloromethane were implemented between these steps to prevent this exothermic reaction.

The resin with the attached peptide was placed in the hydrogen fluoride (HF) reactor vessel for cleavage. 500μL of p-cresol was added to the vessel, and the vessel was submerged in the methanol/dry ice mixture and added to the closed HF system. The vacuum pump was turned on and the closed system was monitored for any changes in pressure. 10ml of HF was then distilled to the reaction vessel and isolated. This vessel was allowed to react for one hour at 0°C. After this, the pump was turned on and the HF was slowly evacuated (10-15 min). The vessel was removed carefully and filled with approximately 35 ml of ether. This mixture was then filtered utilizing a teflan filter and repeated twice to remove all excess cresol. This solution was disposed. The filter was then washed with roughly 20ml of 10% Acetic Acid in distilled water and filtered three times. The filtrate, which contained the desired peptide, was collected and lyophilized.

Peptides were purified using high performance liquid chromatography (HPLC). The instruments used were: Waters Associates model 600 pump, Injector model 717, and UV detector model 486. A wavelength of 220 nm was used for all samples. Solvent A normally contained 10% CH_3CN /0.1% TFA in distilled water, and solvent B contained 0.1% TFA in CH_3CN. A linear gradient was employed in increasing percentage of B,

normally a 0 to 100%B in 2 hours. A silica based C 4 or C18 column (5μ particle size, 300' pore size) was used for all purifications. The mass spectrometer was used to identify peptides produced and also to check purity. The instruments and conditions were: Electrospray Sciex API III triple quad MS, Ion spray voltage= 4500 V, Orifice potential= 90V, and Sample flow rate= 5 μl/ min.

Experimental Results

The GLP-oxyntomodulin chimeric peptide was synthesized. The last eight amino acids derive from oxyntomodulin and are shown in red.

HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRGKRNRNNIA

After synthesizing this peptide, its molecular mass was checked my mass spectrometry. It showed a mass of 4322.5 Daltons. In this way, we confirmed that we have synthesized the GLP-Oxyntomodulin. After this we checked for the receptor binding and potency of GLP-Oxyntomodulin in Luciferase assay. The mass spec and the assay results are shown below.

+Profile Q1SCAN
Charge agent: 1, Fenn method
Glip-oxynto Fr14-19 - 11/10/2004 - 16:53
No Title
1 peak

Glip-oxynto fr 14-19 rec

2,370,157

4322.8

DI	50
ISV	4200
IN	650
OR	60
R0	30
M1	1000
RE1	121.2
DM1	0.150
R1	27
L7	-40
R2	-45
M3	1000
RE3	120
DM3	0
RX	-10
R3	-70
L9	-250
FP	-250
MU	-5200
CC	10
CG	Off
DI μA	7.5
CGT	7.9
IS v	4001.0
UV	8334.1

LA111604

Vassy Gelfanov

GLP-1 Receptor Luciferase Assay

Objective To GLP-1 receptor-mediated cAMP induction by oxyntomodulin and GLP-1-oxyntomodulin

Design:

LA111604

Peptides:
GLP-1	(A-B)(2-12)	0.005 μM 3 fold dilutions	Highest at 12			
GLP-1-o (C-D)(2-12)	0.1 μM	3 fold dilutions	Highest at 12	Starving: A-H	O/N	DMEM-0.25% BGS
Oxyntom (E-F)(2-12)	0.1 μM	3 fold dilutions	Highest at 12			
Glucagon (G-H)(2-12)	0.1 μM	3 fold dilutions	Highest at 12	Incubation:	A-H	5 h

LCPS: 10 min   A30_21

	1	2	3	4	5	6	7	8	9	10	11	12
A	566.9	504.3	548	573.1	1107.2	2689	5817.2	10786.4	16082.6	14318.7	9903.3	7957.3
B	411.7	414.8	505.1	577.9	1092.4	2287.1	5221.4	9396.6	13706.9	11840.6	8510.1	8400.6
C	504.3	615.8	1185.7	2893.3	8080.5	11890.2	16862.2	16236.3	13725.4	15549.4	11853.4	13905.2
D	523.9	786.8	1166.1	3023.6	6689.8	13903	21266.6	22922.3	21782.5	18436.8	17636.7	16192.1
E	559.5	578.8	687.4	718.3	1147.4	2931	5740.9	12552.3	17034	21255.8	18819.2	15841.5
F	601.8	573	622.2	626.1	974.8	2378.9	5560.4	11122.1	19948.3	16217.2	16539.7	15575.5
G	530.8	570.7	604.9	695.7	706.7	905	1214	2830.8	5109.7	9155.4	14933.4	16643.5
H	526	565.8	623	690.5	680.5	772.7	1275.5	2201.8	4922.6	9022.6	15724.7	13757.2

[Peptide]	[Peptide]	[Peptide]	[Peptide]	GLP-1		GLP-1-oxynto		Oxyntomodulin		Glucagon	
nM	nM	nM	nM	CPM 1	CPM 2	CPM 1	CPM 2	CPM 1	CPM 2	CPM 1	CPM 2
2.8E-05	0.00055	2E-05	0.05645	566.9	411.7	504.3	523.9	560	602	526
8.5E-05	0.00169	0.0001	0.16935	504	415	616	787	579	573	571	566
0.00025	0.00505	0.00051	0.50805	548	505	1186	1166	687	622	605	623
0.00076	0.01524	0.00256	1.52416	573	578	2893	3024	718	628	690	691
0.00229	0.04572	0.0128	4.57247	1107	1092	8081	6680	1147	675	707	681
0.00686	0.13717	0.064	13.7174	2689	2287	11890	13903	2931	2379	905	773
0.02058	0.41152	0.32	41.1523	5817	5221	16862	21266	5741	5560	1214	1276
0.06173	1.23457	1.6	123.457	10786	9397	16235	22922	12552	11122	2831	2202
0.18519	3.7037	8	370.37	16083	13707	13725	21783	17034	19948	5110	4923
0.55556	11.1111	40	1111.11	14319	11841	15549	18437	21256	16217	9155	9023
1.66667	33.3333	200	3333.33	9903	8510	11853	17637	18819	16540	14933	15725
5	100	1000	10000	7957	8401	13905	16192	15842	15576	16644	13757
			CPS$_{1c}$	8293	7059	8683	11723	10906	10261	8587	8125
			EC$_{50}$	0.06	0.06	0.10	0.14	1.54	1.33	12.53	11.11

[Peptide]	[Peptide]	[Peptide]	[Peptide]	GLP-1		GLP-1-oxynto		Oxyntomodulin		Glucagon	
nM	nM	nM	nM	AVER.	STDEV	AVER.	STDEV	AVER.	STDEV	AVER.	STDEV
2.8E-05	0.00055	2E-05	0.05645	489	109.74	514	13.86	581	29.91	526	3.39
8.5E-05	0.00169	0.0001	0.16935	460	63.29	701	120.92	576	4.10	568	3.46
0.00025	0.00505	0.00051	0.50805	527	30.33	1176	13.86	655	46.10	614	12.80
0.00076	0.01524	0.00256	1.52416	626	67.32	2958	92.14	673	63.78	693	3.68
0.00229	0.04572	0.0128	4.57247	1100	10.47	6385	430.84	1061	122.05	694	16.53
0.00686	0.13717	0.064	13.7174	2488	284.19	12897	1423.26	2655	390.39	839	93.55
0.02058	0.41152	0.32	41.1523	5519	421.29	19065	3115.80	5651	127.63	1245	43.49
0.06173	1.23457	1.6	123.457	10092	982.74	19979	4728.42	11837	1011.30	2516	444.77
0.18519	3.7037	8	370.37	14896	1679.87	17754	5607.23	18491	2060.72	5016	132.30
0.55556	11.1111	40	1111.11	13080	1752.28	16993	2041.70	18737	3562.83	9089	93.90
1.66667	33.3333	200	3333.33	9207	985.14	14745	4089.41	17679	1611.85	15329	569.53
5	100	1000	10000	8179	313.46	15049	1617.08	15709	189.09	15200	2040.92
			EC$_{50}$	0.06	0.00	0.12	0.030	1.44	0.16	11.82	1.003

GLP-1 Receptor-Transfected Cells

- GLP-1
- GLP-1-oxynto
- Oxyntomodulin
- Glucagon

The GLP-oxyntomodulin was found to be at least as active as the native GLIP-1 peptide in the luciferase assay. This signifies that the first portion of the project is successful and we focus on an additional element, specifically the amino-terminal prodrug design.

As discussed previously the initial prodrugs were prepared on a shortened peptide resemebling the GLP (7-15) peptide sequence. The structure of this peptide is
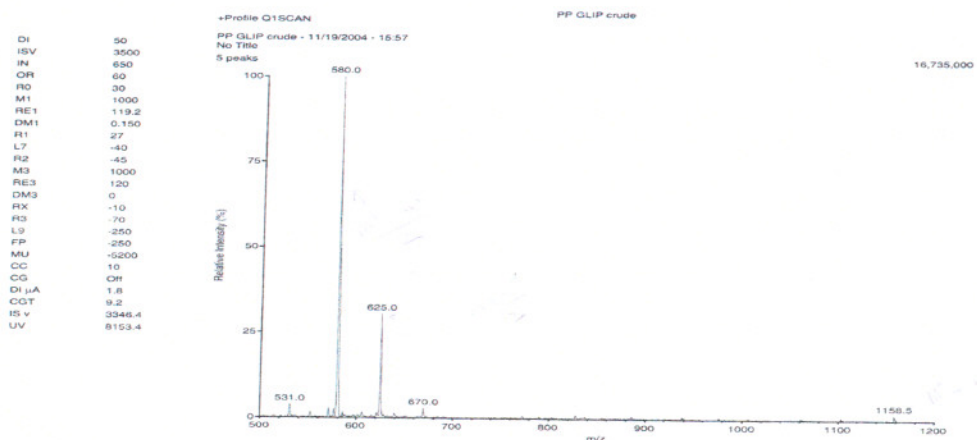
PPHAEGTFTSD.

The GLP (7-15) peptide was prepared synthetically by solid phase synthesis. The MALDI MS analysis yielded a peak at 965 units.
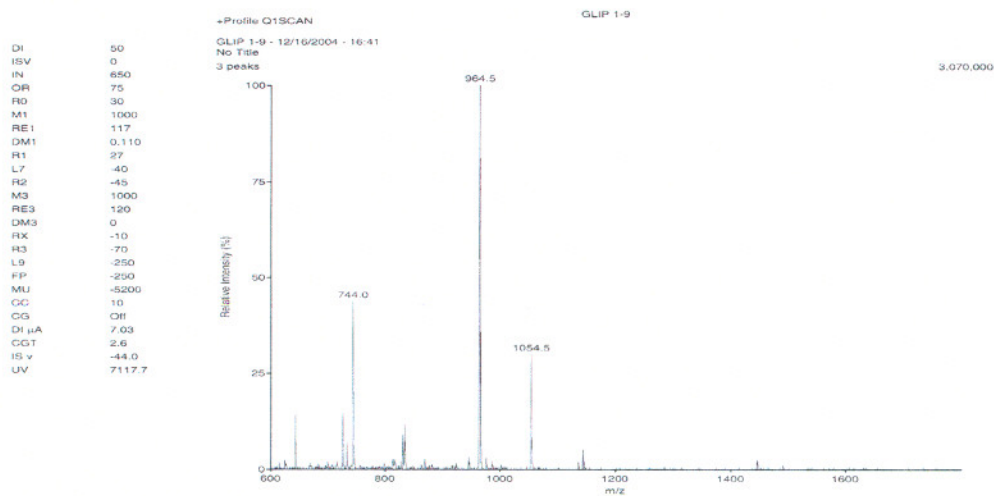
This represents the desired nine residue GLP peptide. To this peptide we couple the fours different functional groups individually

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The first synthesis yielded **PPHAEGTFTSD**

The peak at 1158 represents the desired peptide. We also observed the +2 charged peptide at 580 units.

To explore the possible formation of DKP formation and regeneration of the GLP nonapeptide, the PP-extended peptide was stored in PBS buffer for approximately two days. No apparent cleavage was seen.

The second synthesis yielded **GPHAEGTFTSD**

We synthesized this peptide in the same way. The MALDI is attached below.
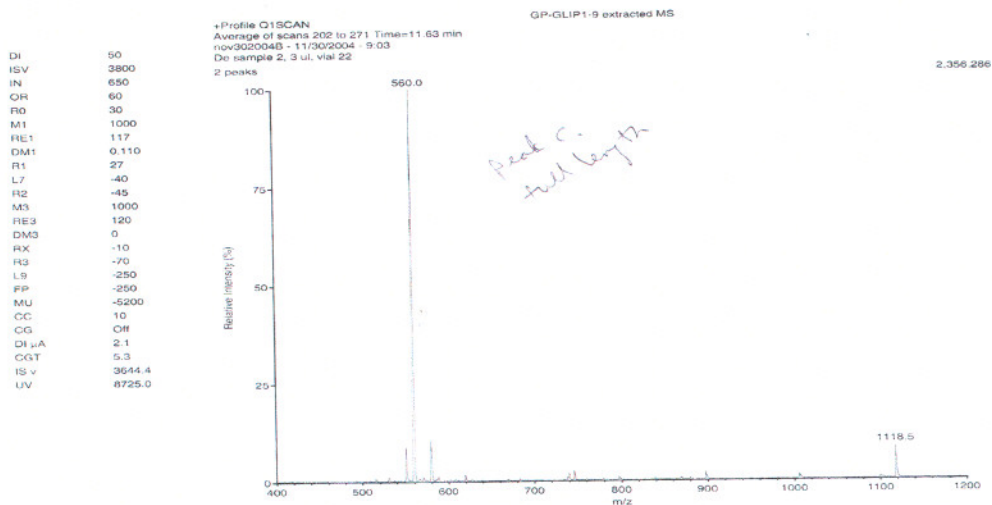
The peak at 1118 units represents the correct peptide. To explore the possible formation

of DKP formation and regeneration of the GLP nonapeptide, the GP-extended peptide

was stored in PBS buffer for approximately two days. As reported previously with the

PP-extended peptide no apparent cleavage was seen.

The third synthesis yielded **γ** **Glu** GLP 7-15 peptide. The nature of the chemistry in

cyclization differs from DKP.

The MALDI analysis of this synthesis is attached below.

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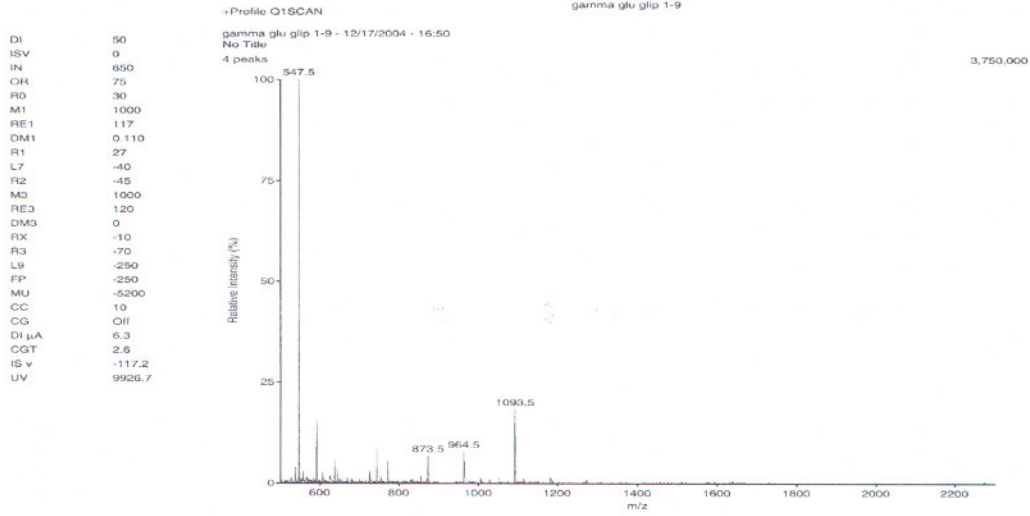
The peak at 1093.5 units represents the correct peptide.

We explored the purity of the peptide by reverse phase HPLC. The results are shown below.

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In the left diagram is the chromatogram for GLP (7-15) with the peak at 9.987 minutes. In the right diagram is the chromatogram for γGlu GLP (7-15) with the peak at 11.047 minutes. In both the cases we observe another peak at 14 min for cresol. This was proven in a control experiment. We verified the integrity of the results by running both peptides together and observing the two respective peaks at 9.3 min and 11.12 min. This conclusively proves that we have been able to separate the peptides.

As discussed previously we explored the regeneration of the parent GLP-1 (7-15) peptide with γGlu, GLP (7-15) by HPLC. We focused on the two peaks of interest at 9 and 11 min to determine the level of cleavage. As the dissociation increases, the GLP (7-15) should increase and correspondingly the first peak at 11 min should grow relative to the one at 12 min. We do note that the peaks at 9 and 11 later shifted to 11 and 12 for the same compounds respectively. The results are shown below:

a) Thirty-six hours at 37° C
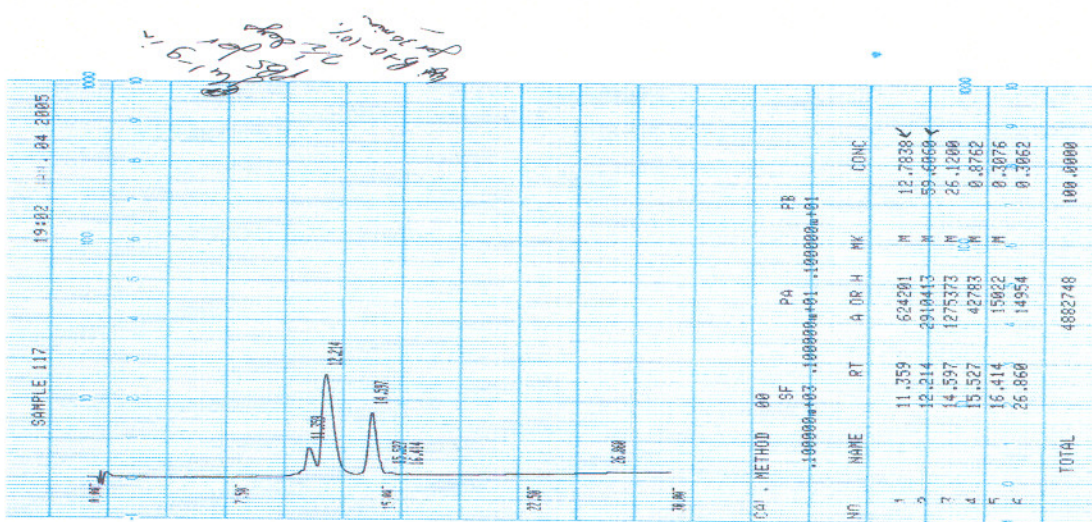
b) One hundred and sixty-eight hours at 37° C

c) two hours at 100° C

25
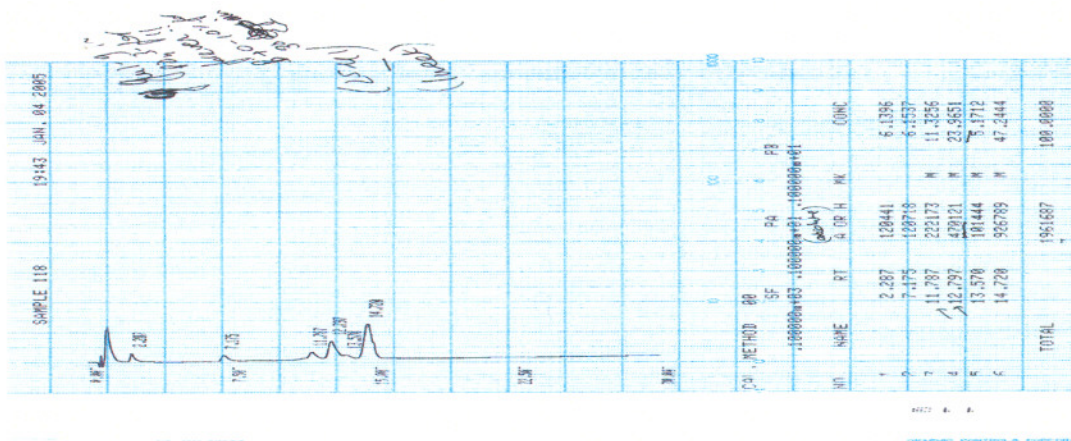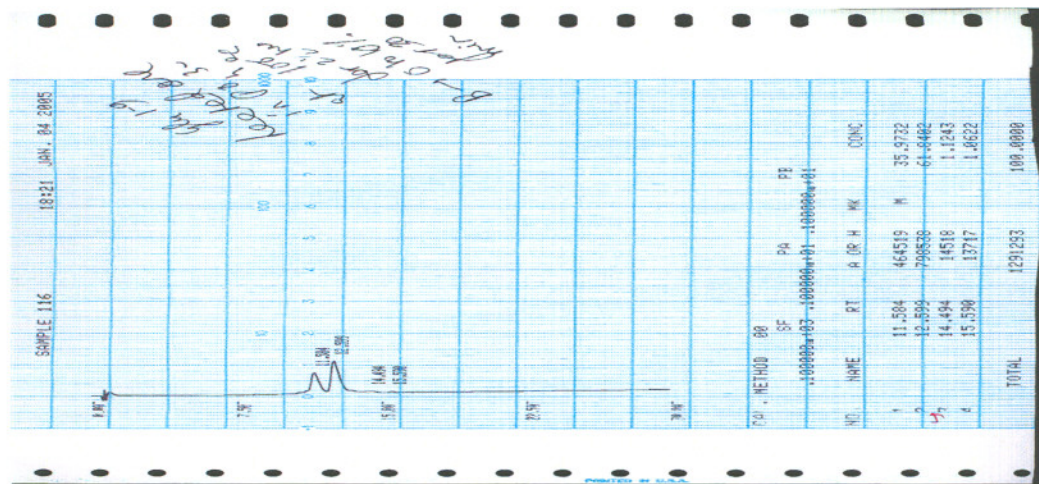
The fourth synthesis yielded **H₄Pht** GLP 7-15 peptide. The nature of the chemistry in adduct formation differs from DKP and γ Glu.
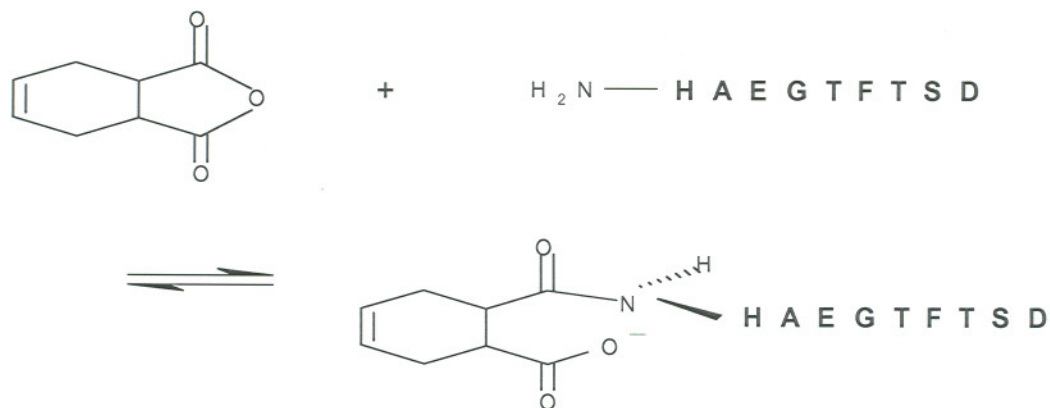
The GLP (7-15) was synthesized and the HPLC analysis was performed as shown previously. The H₄Pht was added as follows; one mmole of GLP 7-15 (1mg/ml) was treated with ten mmoles of H₄Pht (TetrahydroPhthalic Anhydride) (1.52mg/ml). Care was taken to maintain the pH close to 8 during throughout the reaction[16]. The reaction was monitored for the formation of the adduct H₄Pht,GLP (7-15) peptide by sequential HPLC analysis. Analytical chromatography shown below was conducted in alkaline buffers. The A buffer was 0.1M NH_4HCO_3 and the B buffer was 0.1M NH_4HCO_3 and 50%acetonitrile.

Shown below is the chromatogram of GLP 7-15 and the adduct H₄Pht, GLP 7-15. It shows that we have been clearly able to separate the two.
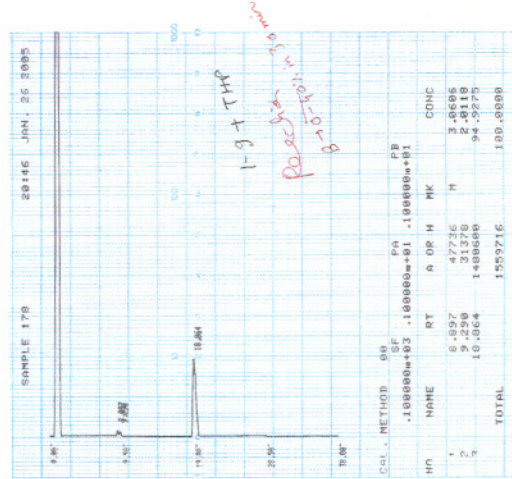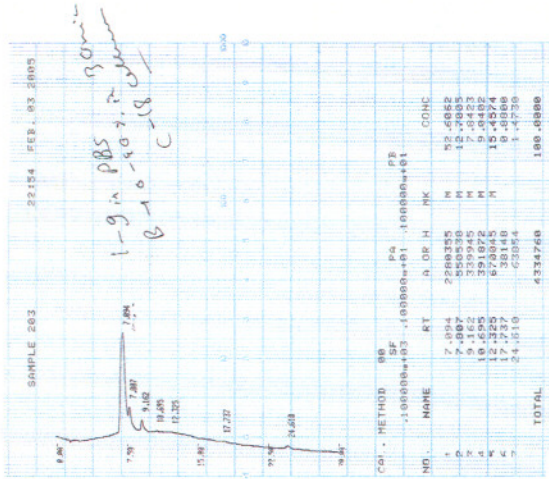
The H$_4$Pht adduct is unstable in acid, consequently we would expect the peak in the right hand chromatogram at 18 min to disappear. After exposure to 1N HCl the adduct is removed and the chromatogram changes as expected. The left hand side chromatogram is the MALDI spectra that shows that the adduct is formed at 1117.6 mass units.

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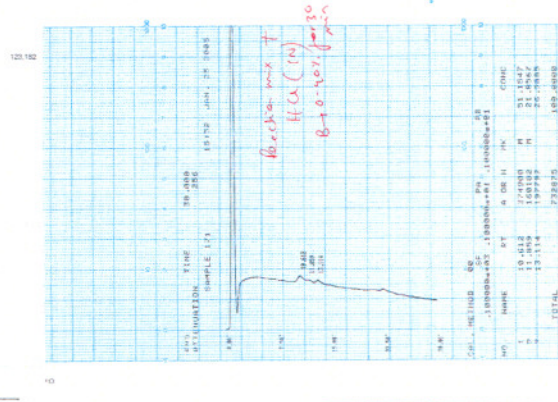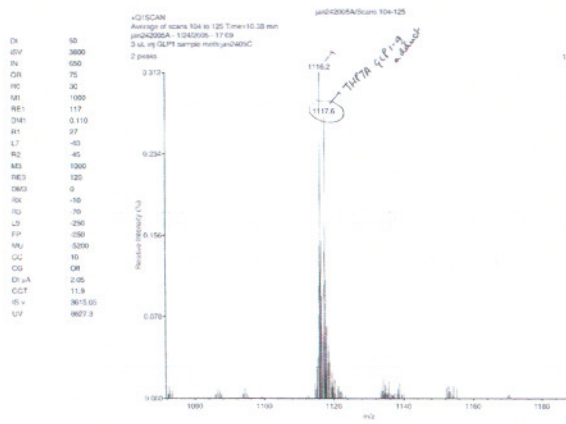
Cleavage of H₄Pht GLP 7-15: The H_4Pht, GLP (7-15) was stored in PBS buffer for approximately seventy-two hours at 37 degrees to examine H_4Pht removal. We should essentially get two peaks at 6 and 8 min respectively. As the dissociation increases, the GLP 7-15 will increase and correspondingly the first peak at 6 min should grow relative to the one at 8 min. They are shown below:

a) After 0 minutes: Only 1 peak observed at 6 min

b) After 4.5 hours

c) After 13.5 hours

d) After 17.5 hours

e) After 1 day :

f) After 3 days at 37°C: The GLP 7-15 peak at 6 min is actually bigger than the peak at 8 min. It implies that the cleavage has definitely been more than 50%.

g) After 2 hours in PBS buffer at 100°C: The peak at 8 min (adduct peak) has almost disappeared. We deduce that the reaction here has been nearly complete.

The cleavage reaction is showed below:
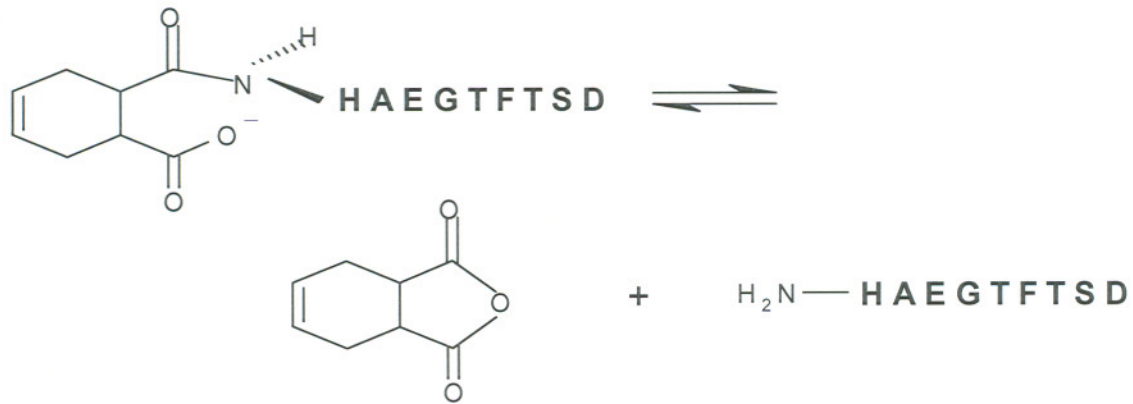
The HPLC chromatograms are shown on the next pages.
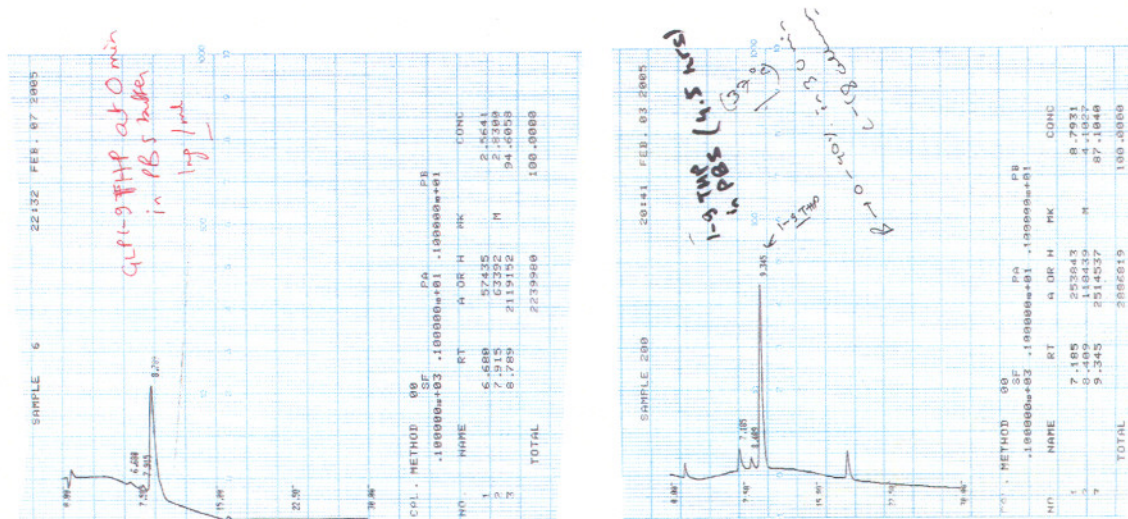
0 min at 37°C

4.5 hours at 37°C

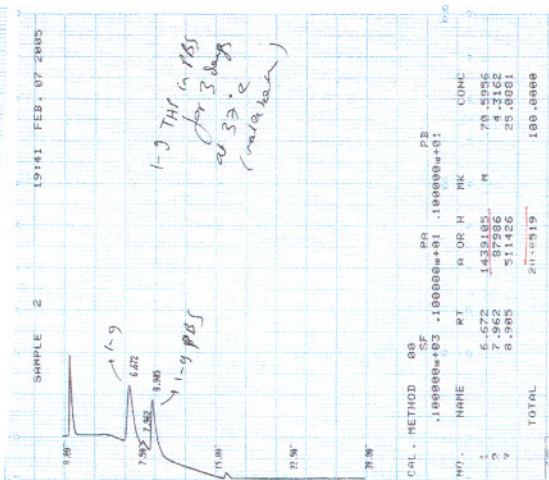After 4.5 hours, we already begin to see the small peak of GLP 7-15 appearing at 6 min.
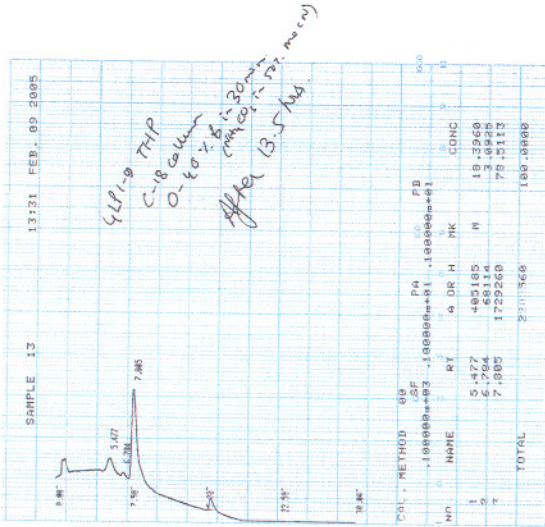
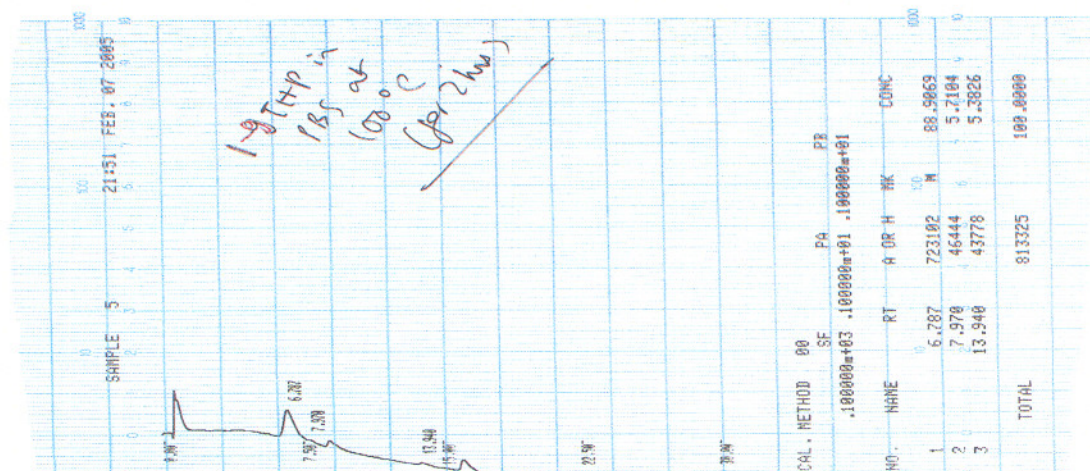13.5 hours at 37°C
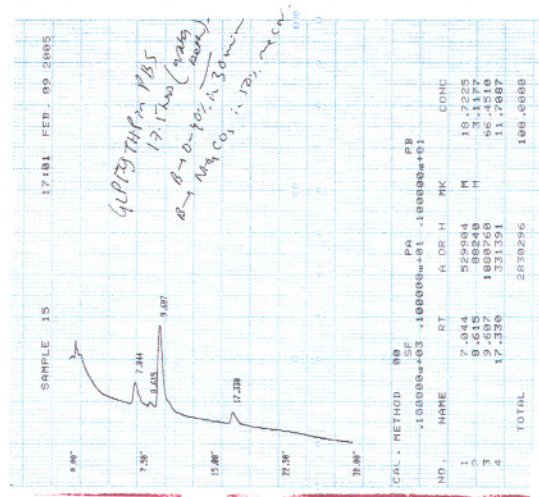
17.5 hours at 37°C
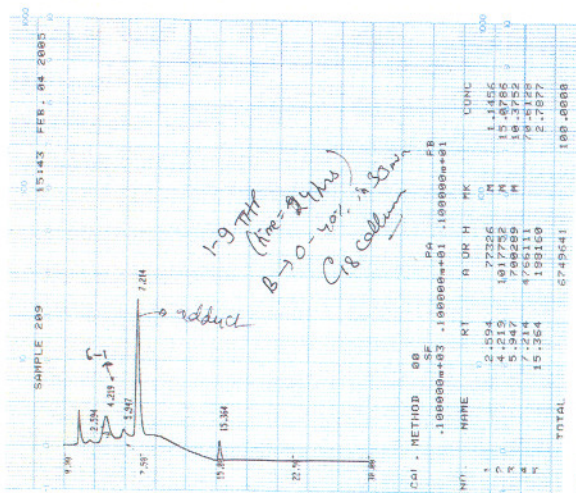
24 hours at 37°C

72 hours at 37°C

SAMPLE 5   21:51 FEB. 07 2005

19THP in 1BS at (100°C (for 2hrs)

NO.	NAME	RT	A OR H	AR H	MK	CONC
1		6.787	723102		M	88.9869
2		7.970	46444			5.7104
3		13.940	43778			5.3826
TOTAL			813325			100.0000

CAL. METHOD 00
SF .100000æ+03 .100000æ+01 .100000æ+01
PA .100000æ+01 PR .100000æ+01

2 hours at 100°C

Discussion of the Results: We found that the PPHAEGTFTSD and the GPHAEGTFTSD yielded no cleavage of the first two residues as DKP. The γGluHAEGTFTSD peptide exhibited some cleavage to pyroglu. Cleavage in the H4Pht,HAEGTFTSD peptide was appreciable and worthy of closer inspection.

To begin with we tested the stereochemistry of proline attached to the histidine in a syn configuration bringing the attacking and the leaving group closer to each other The other advantage is that we are using natural amino acids here. But the cleavage does not happen. This reaction does not readily occur at pH 7.2 as at this pH the primary amine is partially protonated and its nucleophilic character would be reduced.

In the next step, we invesitagted the γGlu GLP 7-15 peptide. Here by introducing the γ carboxyl group, a five membered ring cyclization is compared to the six represented by the DKP. As expected we find more cleavage in this case. If the concentration of the GLP

7-15 is designated by 'a' and that of an adduct is designated by 'b', then the cleavage at a certain time is determined by using the formula:

$$\% \text{ of cleavage} = a/a+b$$

In this formula, it is assumed that the concentration of GLP 7-15 when the reaction is complete will be the same as the concentration of the adduct at the beginning of the reaction. This assumption, though not necessarily correct is probably reasonable.

By using this formula, it was determined that % of cleavage for the γGlu GLP (7-15) peptide, was as follows:

a) after 36 hours @ 37°C: 20% (17% and 27% in two different sets)

b) after 168 hours @ 37°C: 31% (30% and 32% in two different sets)

c) after 2 hours @ 100°C: 36.7%

The data does reflect that the percent of cleavage is progressively increasing. So in the next approach with H_4Pht-GLP (7-15), we maintained the syn geometry (the reason that we use Tetrahydro Phthalic anhydride and not Phthalic anhydride). Additionally, there is, a better nucleophile in this case (COO^- carboxylate), though the leaving group is still the same. Thus, we can test the importance of the attacking nucleophile and the reformation of a five-membered anhydride rine. We expected the percent of cleavage to increase and that is what happens as described below.

a) At 0 min @ 37°C :   0% cleavage

b) At 4.5 hours @ 37°C: 9% cleavage

c) At 13.5 hours @ 37°C: 18% cleavage

d) At 17.5 hours @ 37°C: 20% cleavage

e) After 24 hours @ 37°C: 23% cleavage

f) After 72 hours @ 37°C: 86-96% cleavage (2 sets done on the same day).

g) After 2 hours @ 100°C: 85-93% dissociation (2 sets done on 2 different

    days).

Cleavage with Time (hr)
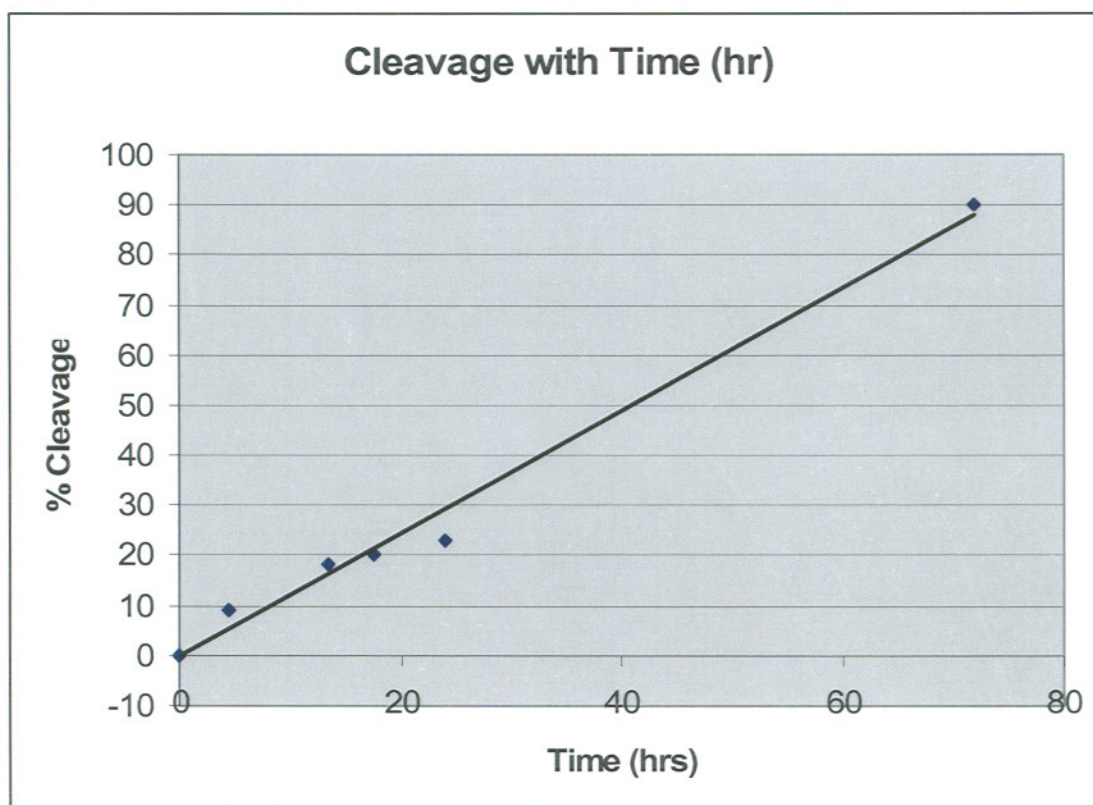
We observe a steadily progressing cleavage with time at constant temperature.

Additionally observational points in the mid region would be needed to calculate a rate

constant. However, we could not do this as we ran into a technical problem. The

analytical C18 column that we were using was adversely affected by the reaction mixture. The columns were steadily fouled with each run. It probably has something to do with the H$_4$Pht but we were not sure. The results are promising and worthy of additional and closer inspection.

The results looked apparently anomalous at first sight. It was remarkable that the same reaction (the making of the adduct) that had been quite successful on the preparative scale in just over two hours could revert back to an extent reflected in the figures.
Now I would think that the making of the adduct was a kinetically controlled process, while the disassociation reaction has been thermodynamically controlled. The time scale of the two reactions will bear first testimony to that. If there was any backward reaction that would immediately be neutralized by the very high concentration (ten times) of the THP that was being used in the starting material. The cleavage is slightly more complicated. It is thermodynamically controlled. That is why the rate is limited at the beginning (At 4.5 hours - 9% dissociation), but the percentage improves dramatically with time to an extent that after 3 days, the peak of the adduct almost vanishes.

At a pH of 7.4 there exists a negative charge on the carboxylate ion (and this COO⁻ is a strong nucleophile), so there is an intramolecular displacement reaction which is favored because of proximity between the O⁻ and the carbonyl carbon. Any such intramolecular reaction is entropically favorable as there are two products coming from one reactant. Also the bond strength of the products should be greater that the reactants, if compared. Hence both the reactions occur to a great extent under different conditions.

It might be useful to ponder for a moment why the same thing did not happen with γ Glu GLP 7-15 peptide last time earlier. Probably the deciding factor was the attacking atom in the dissociation reaction, although the 5 membered rings also differ. For the THP GLP 7-15, it is a naked carboxylate anion (100%). In the former case it was an amine (that is approximately 50% protonated). I think that can sum up the reason for the reluctance of the γ Glu GLP 7-15 peptide to cyclize. Although, it might be worth noting that even in that case the reaction had gone to about 37% thermodynamically.

Future Plans: The cleavage seen in H$_4$Pht,HAEGTFTSD is promising. However, we need an alternative analytical approach to study of the cleavage in the C18 columns are being destroyed, as noted previously. Hence, we have made a more anionic GLP-1 peptide by making subtle changes (without disturbing the amino acids responsible for the potency) for use with a anion-exchange Mono Q column. Then we can add the THP to this peptide and carry out the reaction. In this way, additional time points can be obtained using a full length GLP-1 peptide. The new peptide synthesized is HAEGTFTSDVSSYLEGQAAREFIAWLVEGEG with changes from the normal GLP 7-37 peptide noted in red.

As a means to explore methods to increase the rate of DKP, and pyroglu, cyclizations we will explore ester linkages as replacement for the previously studied amides. We are focused on this specific GLP-1 analog.

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