

# Topochemically-Equivalent Peptidomimetics and the Design of Postprandial Serum Triglyceride Level-Reducing Compounds

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## Abstract

The alarming prevalence of obesity and cardiovascular diseases worldwide and the limited success in alleviating them continue to call for new strategies in terms of cellular pathways and pharmacotherapeutic agent design. With a focus on the reduction of serum triglyceride levels, a series of low molecular-weight retro-inverso-peptidomimetics has been developed and their biological activities tested with animal experiments involving oral administration. The results exhibit a remarkable reduction of postprandial serum triglyceride concentration, potentially providing a novel avenue of addressing these health problems. Due to the small size and incorporation of D-amino acids, the peptides exhibit excellent solubility and bioavailability.

**Keywords:** Serum triglyceride; Oligopeptide; Retro-inverso modification; Peptidomimetic; Postprandial; Cardiovascular disease; Obesity

## Introduction

It has long been the aspiration of biomolecular scientists - from structural biologists and synthetic organic chemists to computational chemists and medicinal/pharmaceutical chemists - to design bioactive compounds that mimic and/or improve upon the function and selectivity of naturally-occurring molecules. Particularly with peptide hormones and neurotransmitters, peptidomimetics has served as a very fruitful avenue of endeavour, resulting in the development of a myriad of pharmacologically active compounds [1]. Broadly viewed, two philosophies of molecular design and analyses exist: the empirical, data-based structure-activity relationship (SAR) methodology and the atomic-structure-based, conformational/topographical/topochemical methodology. It goes without saying that both are mutually-complementing and continuously interacting, and is never to be considered in isolation." - Change to "hence are effectively never considered separate in practice.

The scientific scope of our group's research lies in the understanding of the protein folding/misfolding/alternative folding problem through the utilization of various spectroscopic techniques including our mainstay, biomolecular NMR spectroscopy [2-5]. While work on the larger proteins provide the satisfaction (or perhaps frustration) of dealing with complex problems [6], the smaller peptide-size cases are no less interesting [7], in part due to the in-depth reckoning of the atoms involved in the biological interactions. As a means to humbly engage in such "quest to achieve biological equivalence/improvement", we have decided to apply retro-inverso technology [8-12], which involves the design of synthesized oligopeptides similar to naturally occurring oligopeptides but with mirror image amino acids put in reverse sequence order. By utilizing non-natural D-amino acids instead of L-amino acids, this methodology provides an advantage in bioavailability due to inherent resistance against various natural proteases *in vivo*, but there is no expectation *a priori* for its use to effectively mimic or better the biological action of naturally occurring peptides. Here we show that peptide analogues generated using this technology exhibit significantly improved biological activity, and hence may suggest promising candidates for the case of postprandial triglyceride (TG) level control.

High serum triglyceride level, independent of the well-known risk factor of serum cholesterol, has been regarded as an additional risk factor for developing cardiovascular diseases, including coronary heart disease [13-15] and atherosclerosis [16,17]. A number of pharmaceutical developments have been made to restrict the elevation of serum triglyceride levels to prevent such cardiovascular ailments [18].

More significantly, the excessive intake of lipid with respect to energy expenditure leads to obesity [19], which is currently being regarded as one of the prime health concerns in the Western World. Obesity is a complex medical disorder with implications for diabetes, high cholesterol, cardiovascular conditions, some forms of cancer, and is a major cause of premature mortality. Dietary restriction and behavioral changes are key to prevent obesity; however it is now becoming evident that the success in preventing or treating obesity can be increased with pharmaco-therapy. Several drugs have been developed to combat obesity, however most of these are central-nervous system (CNS)-active, and hence have high abuse potential. Therefore, it would be desirable to have a pharmaceutical agent that would not have these dependency complications [20].

Recently, a group of low molecular weight peptides which were originally obtained and purified from a non-specific enzymatic proteolysate preparation of bovine reticulocyte protein has been shown to inhibit the elevation of serum triglyceride levels [21,22]. The peptides isolated are low molecular weight, i.e., 3-4 residues in length, and are comprised solely of natural amino acids. The detailed mechanism of action by these peptides is yet to be described, however, with the

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advent of numerous food-derived peptides possessing biological activity [23,24], further investigation using the aforementioned atomic-structure-based, topographical/topochemical philosophy has proven to be quite intriguing.

## Experimental

### Chemical synthesis and purification of peptides

Peptides of D-Pro-D-Tyr-D-Val-D-Val-NH<sub>2</sub>, D-Pro-D-Tyr-D-Val-NH<sub>2</sub> and D-Leu-D-Thr-D-Val-NH<sub>2</sub> were synthesized on an Applied Biosystems/Perkin-Elmer 432A Synergy Peptide Synthesizer using FastMoc cycles. The synthesis chemistry involves 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/piperidine activation, and uses dimethylformamide (DMF)/N-methylpyrrolidine (NMP)/dimethylsulfoxide (DMSO) as the coupling solvent. Synergy Fmoc-Amide resin (Applied Biosystems/Perkin-Elmer) or Rink amide methylbenzhydrylamine (MBHA) resin (NovaBiochem) was used for the solid-phase support. The constituting N-, $\alpha$ -9-fluorenylmethoxycarbonyl (Fmoc) protected D-amino acids (N- $\alpha$ -Fmoc-D-proline, N- $\alpha$ -Fmoc-O-t-butyl-D-tyrosine, N- $\alpha$ -Fmoc-D-valine, N- $\alpha$ -Fmoc-D-leucine, N- $\alpha$ -Fmoc-O-t-butyl-D-threonine) were from NovaBiochem. The peptides were cleaved by adding 1.8 ml of trifluoroacetic acid (TFA) with 0.1 ml of 1,2-ethanedithiol (EDT) and 0.1 ml of thioanisole as scavengers for 1 hour, then precipitated with 15 ml of methyl tert-butyl ether (MTBE) at 4°C and centrifugation at 2000 x g. The MTBE washing was repeated three more times, and the peptides were solubilized with 20% acetic acid. To use as reference compounds, L-Val-L-Val-L-Tyr-L-Pro, L-Val-L-Tyr-L-Pro, and L-Val-L-Thr-L-Leu peptides were prepared using the same methodology. To supplement the quantity of peptides required for the animal experiments, alternatively, the six peptides described above were ordered and synthesized from CS Bio Co. (San Carlos, CA, USA). All purchased peptides were analyzed by the vendor to be of purity above 98% using reverse-phase HPLC and the molecular weights were verified using MALDI-TOF mass spectrometry.

When necessary, a purification of the peptides was performed using preparative reversed-phase HPLC. A Kromasil KR-100-10-C8 (10 mmx250 mm, C8, 10  $\mu$ m, 100 Å, Akzo Nobel) Column was used, with a linear gradient of 5 to 20% acetonitrile (CH<sub>3</sub>CN) in 0.1% TFA over 20 column volumes. The fractionated peak was checked for purity using a Vydac 218TP52 RP-HPLC column (2.1 mmx250 mm, C8, 5  $\mu$ m, 300 Å) with a linear gradient of 1 to 25% CH<sub>3</sub>CN in 0.1% TFA. The

final purity of each peptide was greater than 97%. MALDI-TOF mass spectrometry analyses using cinnapinic acid as matrix on a Kompact Research MALDI IV instrument (Kratos Analytical) confirmed the identities of the peptides.

### Oral Administration of Chemically Synthesized Peptides

Olive oil (Extra virgin; 250 mg; experimentally determined volume 286  $\mu$ l) was administered via gastric intubation to male ICR or CD1 mice (6-week old, body weight: 20 g) that were acclimated in the laboratory for at least 3 days and fasted overnight. The peptides are dissolved in 0.1 ml saline solution and administered orally one hour after lipid administration. The animals are allowed to freely access water but not food until blood collection. After two hours, blood was collected using submandibular bleeding with sterilized animal lancets (MEDI point Goldenrod, 4 mm point), which requires no anesthesia, surgical procedures or restraint apparatus. A safe single bleed volume of 0.2 ml is withdrawn from each mouse, and the serum separated by centrifugation (3000 rpm, 30 min, 4°C). Serum triglyceride levels were assayed with two different diagnostic kits (EnzyChrom Triglyceride Assay Kit, BioAssay Systems, Hayward, CA, USA or Serum Triglyceride Determination Kit, Sigma-Aldrich). Because the animals were relatively unaffected with the administration of olive oil and the peptidic compound as well as the bleeding procedure, subsequent compound trials were possible by alternating cheeks after a suitable recovery period. The results were compared with L-Val-L-Val-L-Tyr-L-Pro (Reference Peptide 1), L-Val-L-Tyr-L-Pro (Reference Peptide 2), and L-Val-L-Thr-L-Leu (Reference Peptide 3). Two controls were used for comparison. (i) 286  $\mu$ l of distilled water instead of the olive oil with no peptide administration, and (ii) olive oil only with no peptide administration. All live animal experiments were approved by the BioResources Ethics Review Committee of Trinity College Dublin (Ref 290909; 16th Nov 2009) with licence granted by the Environmental Health Unit, Department of Health & Children of Ireland (Ref B100/4302; 5th May 2010).

## Results and Discussion

As shown in Table 1, Retro-inverso Peptide 2 and Retro-inverso Peptide 3 displayed higher activities in lowering elevated serum triglyceride levels than Reference Peptides 2 and 3, respectively. Retro-inverso Peptide 1, although less active than Reference Peptide 1, nevertheless exhibited demonstrable serum triglyceride lowering activity, its activity being about half of Reference Peptide 1. In general, it

	Peptide Dosage (mg/mouse)	Number of animals (n)	Serum Triglyceride (mg/100 ml)	% Decrease
Control 1 (Distilled Water)	-	5	92.2 $\pm$ 15.7	-
Control 2 (Olive Oil)	-	24	376.2 $\pm$ 23.8	-
Retro-inverso Peptide 1 (D-Pro-D-Tyr-D-Val-D-Val-NH <sub>2</sub> )	1.0	14	313.7 $\pm$ 45.9	22.0 %
Ref. Peptide 1 (L-Val-L-Val-L-Tyr-L-Pro)	1.0	7	244.7 $\pm$ 25.5	46.3 %
Retro-inverso Peptide 2 (D-Pro-D-Tyr-D-Val-NH <sub>2</sub> )	1.0	7	231.8 $\pm$ 27.6	50.8 %
Ref. Peptide 2 (L-Val-L-Tyr-L-Pro)	1.0	14	407.7 $\pm$ 42.0	-
Retro-inverso Peptide 3 (D-Leu-D-Thr-D-Val-NH <sub>2</sub> )	1.0	7	294.6 $\pm$ 33.8	28.7 %
Ref. Peptide 3 (L-Val-L-Thr-L-Leu)	1.0	14	352.7 $\pm$ 35.6	8.3 %

**Table 1:** Serum triglyceride levels of mice after oral administration of peptides.

can be shown that for the three cases, there exist at least 20% or greater statistically significant decrease in serum triglyceride levels, and for the cases of tripeptides, the retro-inverso compounds substantially exceed the activity of the corresponding natural oligo-peptides.

These results indicate that the retro-inverso modification of the compounds yielded superior biological activity compared to the natural oligopeptides originally identified to reduce postprandial serum triglyceride levels [21]. We note an additional significance in that our results have been obtained through oral administration of peptides with excellent solubility properties, thereby showing promise in the ease of drug delivery. This by no means precludes other methods – intravenous, subcutaneous, intramuscular or intraperitoneal – for administration, and hence ending potential flexibility with regards to the pharmaceuticals.

In addition, further functional group modifications that allow for greater specificity and/or selectivity are readily possible at this stage. Although the retro-inverso operation hypothetically provides topochemical equivalence in terms of the R groups of each amino acid residue [10,12], the so-called “end-group” problem (where the negative charge on the C-terminus and/or the positive charge on the N-terminus cannot be reverted) persists [11]. Routes of modification for these termini involve either (i) a C-2 substituted malonyl (or malonamyl) residue substitution for the N-terminal retro-inverso peptide residue, or (ii) a gem-diamino alkyl residue substitution for the C-terminal retro-inverso peptide residue [11]. Both synthetic procedures are generally straightforward and are currently being carried out in our laboratory along with an exploration of a wider range of chemical modifications to increase the diversity of structures.

Opinions have varied on whether serum TG levels can serve as biomarkers for various cardiovascular diseases. Whereas the Helsinki Heart Study [25] and the Lipid Research Clinics Studies [26] concluded that TG levels are not closely relevant to coronary heart disease risk, the PROCAM (Prospective Cardiovascular Munster) study [27] – a significantly larger trial of 25,000 people over a period of 8 years – and a recent systematic review of population-based cohort studies have all shown that TG levels do correlate with major coronary events, even independent of high-density lipoprotein cholesterol levels [28]. On the other hand, the correlation of free fatty acid metabolism with obesity and its adverse health consequences is proving to be undeniable [19]. Hence, it appears that further development of peptidomimetics based on the compounds tested here will provide a novel avenue to control or treat cardiovascular and obesity-related health problems.

## Conclusions

A series of postprandial serum triglyceride level-reducing peptidomimetics has been developed based upon results obtained from animal experiments involving oral administration. The results exhibit a striking, statistically-significant reduction of serum triglyceride concentration. Due to these designed compounds possessing excellent solubility and presumed bioavailability properties, novel avenues of treatment of cardiovascular and obesity-related health problems appear to be a possibility.

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