GLYCATION OF PEPTIDES WITH CHITOSAN OLIGOSACCHARIDES ON THE SOLID SUPPORT

W.A. Neugebauer*, R. Brzezinski[1], S. Bélanger, A. Fortier, D. Barbaz, F. Gobeil Jr.

Département de Pharmacologie, Université de Sherbrooke, Sherbrooke (Québec) Canada

[1]Département de Biologie, Université de Sherbrooke, Sherbrooke (Québec) Canada

*witold.neugebauer@usherbrooke.ca

Abstract

Glycopeptides are polyfunctional molecules widely distributed in biological systems. The significant influence of the glycation on the peptide hormones and regulatory peptides has been reported [1]. Hormonal peptide (bradykinin) synthesis was performed on solid phase by the Fmoc strategy. Glucosamine oligosaccharide was obtained by enzymatic hydrolysis of highly deacetylated chitosan with endochitosanase [2] and then coupled on solid phase support by the initial step of Maillard reaction [3]. The anchoring of glucosamine oligosaccharides on N-terminal side of the peptide via the anomeric link to peptide was obtained in DMF at 60° C while the peptide was maintained on the solid phase resin. In the same manner, FITC-labeled glucosamine oligosaccharides were coupled to bradykinin side-chain protected on solid support. Final cleavage of the glycopeptides and protecting groups from the resin was achieved with 90% TFA reagent in conditions, which do not damage the oligosaccharide integrity. These glycopeptides were tested in various aspects of their biological activity and their fluorescent analogues resulted to be useful as labels for confocal microscopy.

Introduction

Natural non-enzymatic glycation of proteins and peptides occurring mainly by Maillard reaction has been implicated in number of pathologies as well in food chemistry, having some impact on the flavour and appearance of cooked food. Recently more attention is directed to significance of glycated peptide hormones [1] since the final products of Maillard cascade reaction [Schiff base-Amadori products-advanced glycation end products (AGE)] are known to accumulate with age and to result in the acceleration of protein ageing disorders. Maillard classical cascade reaction to complete requires sugar molecule and not 2-amino sugar to be completed. 2-Amino sugar can complete only Schiff base formation step in Maillard cascade showed in **Figure 1**. In our study we intent to couple single amino sugar moiety as well oligo amino sugar to biologically active hormone peptide (bradykinin). Then we wanted to examine the pharmacological impact of such hydrophilic element onto dynamic interaction of bradykinin. We modeled this reaction with monomeric 2-glucosamine or N-acetyl glucosamine with $NaBH_3CN$ reductive amination procedure. In both procedures we also coupled in the same manner glucosamine oligosaccharides obtained by enzymatic hydrolysis of highly deacetylated chitosan with endochitosanase [3]. The enzymatically hydrolyzed chitosan was a mixture of glucosamine oligomers of 4 to 7 units in length and was used in synthesis without further modification. All sugar couplings were performed on peptide amino terminus while protected peptide is still on solid phase synthesis stage. Coupling reaction was kept for 24-72 hours at 60°C in DMF to complete slow Schiff base formation. Reductive amination in presence of $NaBH_3CN$ during sugar coupling was performed within 24 hours. 10-fold excess of coupling sugar component was used to ensure complete coupling. Excess of reagents was washed

off and the final product was cleaved from resin with 95% TFA. Purified glycopeptides were tested with human umbilical vein for bradykinin agonistic activity.

Figure 1 : Maillard reaction pathway to Amadori product for glucose and reductive amination for glucosamine.
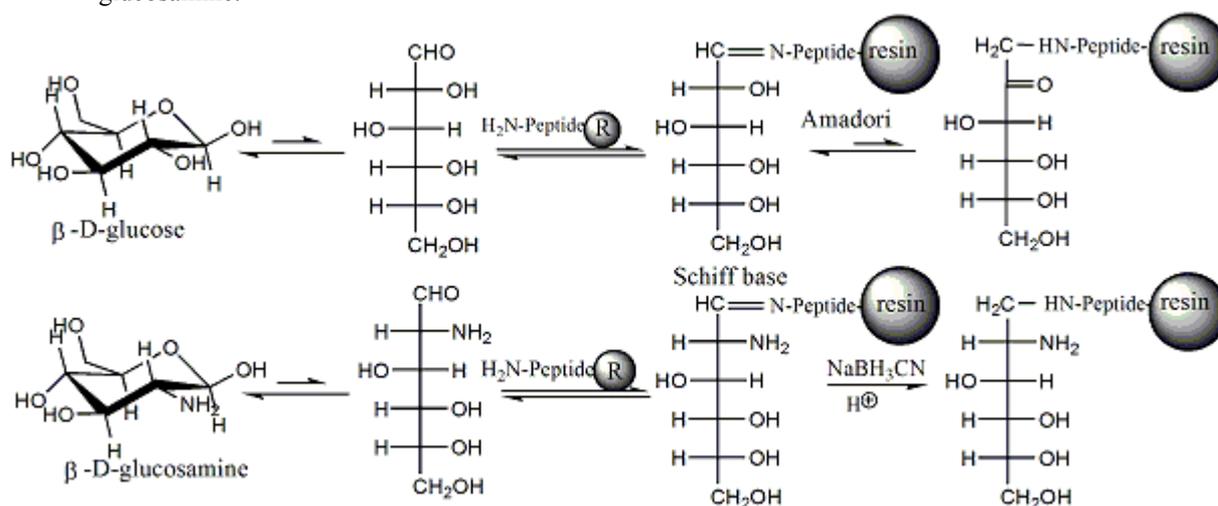
Materials and Methods

Oligochitosan-fluorescein

Glucosamine oligosaccharides were obtained by enzymatic hydrolysis of highly deacetylated chitosan with endochitosanase from *Streptomyces* sp. N174 [2]. Molecular weight of hydrolyzed chitosan determined by MALDI mass spectrometry ranged from majority of 4 units to minority of 10 units with corresponding masses $[M+Na^+]$ from 697 to 1656 accordingly. In amber glass vessel 500 mg of glucosamine oligosaccharide was suspended in 50 mL of dichloromethane (DCM) and 100 μL diisopropylethylamine was added. While it was vigorously stirred, 2-fold excess of fluorescein isothiocyanate was added and was stirred overnight. DCM was evaporated and remaining oligosaccharide dissolved in 0.1 M acetic acid and filtered through small C18 cartridge to trap fluorescein excess. Filtrate was lyophilized to yield yellow randomly coupled fluorescein on oligochitosan, which was used in further couplings to bradykinin.

Peptide synthesis.

Bradykinin (BK) synthesis was assembled on solid phase resin [TentaGel (TGA) Rapp Polymere (Germany)]. Continuous flow peptide synthesis was performed on Pioneer Perspetive Peptide Synthesizer with Fmoc strategy using TBTU as coupling agent. Fmoc protected amino acids were purchased from NovaBiochem (USA) and Chem-Impex (USA). Two fold amino acids excess was used for coupling at every synthetic step. The final protected peptide on the resin H_2N-Arg(Pbf)-Pro-Pro-Gly-Phe-Ser(OBut)-Pro-Phe-Arg(Pbf)-**RESIN** was used in glycation procedure.

Glycation

H_2N-Arg(Pbf)-Pro-Pro-Gly-Phe-Ser(OBut)-Pro-Phe-Arg(Pbf)-[TGA-**RESIN**] was divided into 100 mg portions and mixed with 10 fold excess of sugar component (N-acetyl-glucosamine, oligochitosan and fluorescein-oligochitosan) each to separate resin-peptide portion. Dry DMF with 2% acetic acid was added in the vial as a solvent and shacked at 60^oC for 3 days. The same procedure was repeated replacing N-acetyl-glucosamine with glucosamine hydrochloride. To all three reactions, DMF with 2% acetic acid solution was added and completed with $NaBH_3CN$ in quantity equimolar to sugar component. These reacting mixtures were stirred for 3 days at room temperature. Then resin was filtered off and washed with water, isopropanol, DCM and dried in vacuum. Glycated peptides were cleaved and deprotected with TFA 95%-water 2.5%-triisoptopylsilane 2.5% mixture while stirring for 2 hours at room temperature. Glycated peptides

were filtered off into diethyl ether where peptide product precipitated. The product was then centrifuged, dissolved in water and lyophilized. The final product was then purified on C18 column and its purity verified by HPLC and identified by MALDI mass spectrometry.

Bioassay.

Experiments on human tissue were reviewed and approved by the Ethics Committee of the Medical School of the Sherbrooke University, Quebec, Canada. The kinin surrogates were tested in vasoconstriction bioassays as agonist using the human umbilical vein (hUV) [4]. Human umbilical cords (middle segments) from healthy women were collected after spontaneous delivery at term and placed in cold (4°C) Krebs's solution. Tissues were dissected free of surrounding tissue and cut into spiral strips. The endothelium was rubbed off with a cotton swab. Tissues were then suspended in 10 mL-organ baths containing warm (37°C), oxygenated (95% O_2/5% CO_2) Krebs's solution of the following composition (mM): NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, and D-glucose 5.5. The hUV were stretched to a resting tension of 2 g. Changes of tension were measured isometrically with force transducers (FT03, Grass Instruments) and recorded on multichannel chart recorders (Grass model 7D). In all experiments, the kininase II inhibitor captopril was added to the Krebs's solution at a concentration of 10 μM to prevent enzymatic degradation of bradykinin and analogs that may occur in some tissues [4]. Before any agent was tested, the human tissues were allowed to equilibrate for 90 to 120 minutes. Cumulative concentration-response curves to bradykinin and surrogates (ranging from 0.1 nM to 1 μM) were constructed in order to determine their apparent affinity expressed in terms of pEC_{50} values (-log of the concentration (M) of the agonist that produces 50% of the maximum effect) [4].

Confocal microscopy.

MDA cells were grown on coverslips until quasi confluency in Dulbecco's modified Eagle's Medium (DMEM) prior to beginning of experiments. Cells were incubated for 30 min at 37 °C with FITC conjugated-bradykinin analogs (30 μM), fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and washed once with PBS. Fixed cells were examined using an Olympus laser scan inverted microscope with an argon-krypton laser with fluorescein. A single optical mid-section in the z axis (1.0 μm) was analyzed. No cell autofluorescence was observed in the absence of the FITC conjugated-bradykinin analogs (not shown). Phase contrast optical micrographs were taken in parallel to illustrate cell position and morphological structure.

Results and Discussion

Procedure of peptide glycations on the solid support was tested with few glyco components. Glycation of peptide on solid support with glucosamine in competed Maillard reaction and Amadori rearrangement was recently described [5]. Herein we reported the possibilities of direct glycation of solid support-attached peptide by glucosamine derivatives. Unlike in previously described work [4], initial step of Maillard cascade with glucosamine due to its amine substitution in position 2, cannot progress to Amadori rearrangement. Schiff base formation at 60°C seems to be quite stable to survive peptide isolation procedure. In this manner we synthesized on bradykinin at solid phase adduct of N-acetyl-glucosamine [I] and oligochitosan [IV] shown in **Figure 2** and **Figure 4**. In order to decrease fragileness of Schiff linkage we tried reductive amination procedure, which was the same procedure as Schiff base formation with $NaBH_3CN$ additive. This reaction proceeded at room temperature. This way we coupled this way glucosamine [II] and oligochitosan [III] to bradykinin in solid phase as is shown in **Figure 2** and **Figure 3**. Our intention was to study the impact of such hydrophilic moiety (amino sugar or oligochitosan) on the pharmacological property of the potent peptide hormone bradykinin. We were also interested in finding whether oligosaccharide or just a single amino sugar might drive active hormone from its biological destination and/or increase its biological activity. Agonistic activities of glyco-bradykinin [I], [II], [III] and [IV] along with bradykinin were tested on the isolated human umbilical vein, an established bioassay for the human form of the bradykinin B_2 receptors [4]. Vasoconstrictive power

of [I] and [II] with a single amino sugar substitution its pEC$_{50}$ remained in the range of original bradykinin activity (see **Table 1**). Glyco-bradykinins [III] and [IV] showed less potency, however the values reported for their activity are not exact because their concentration was calculated as it was pure tetramer saccharide while it was only one component of 4 to 10 mixture. The same pEC$_{50}$ value calculated on the basis of the minor 10-mer component would be much higher. As the exact composition of synthesized oligochitosan-bradykinins remains undetermined, we should considered those values as minimal.

Table 1. Agonistic BK-B$_2$ receptor activity of glycolated peptides.

Peptide/Glycopeptide	pEC50	EC$_{50}$ (nM)
Bradykinin [BK]	8.60	2.5
N-acetyl-glucosamine-bradykinin [I]	8.30	5.0
Glucosamine-bradykinin [II]	8.35	4.4
Oligochitosan-bradykinin [III]	7.79	16.0
Oligochitosan-bradykinin [IV]	7.28	53.0

EC$_{50}$, is a concentration of the agonist that produces 50% of the maximum response.

Figure 2 : Bradykinin glycations products with glucosamine [II] and N-acetyl-glucosamine [I] to form Schiff base and NaBH$_3$CN reduced Schiff base form [II].
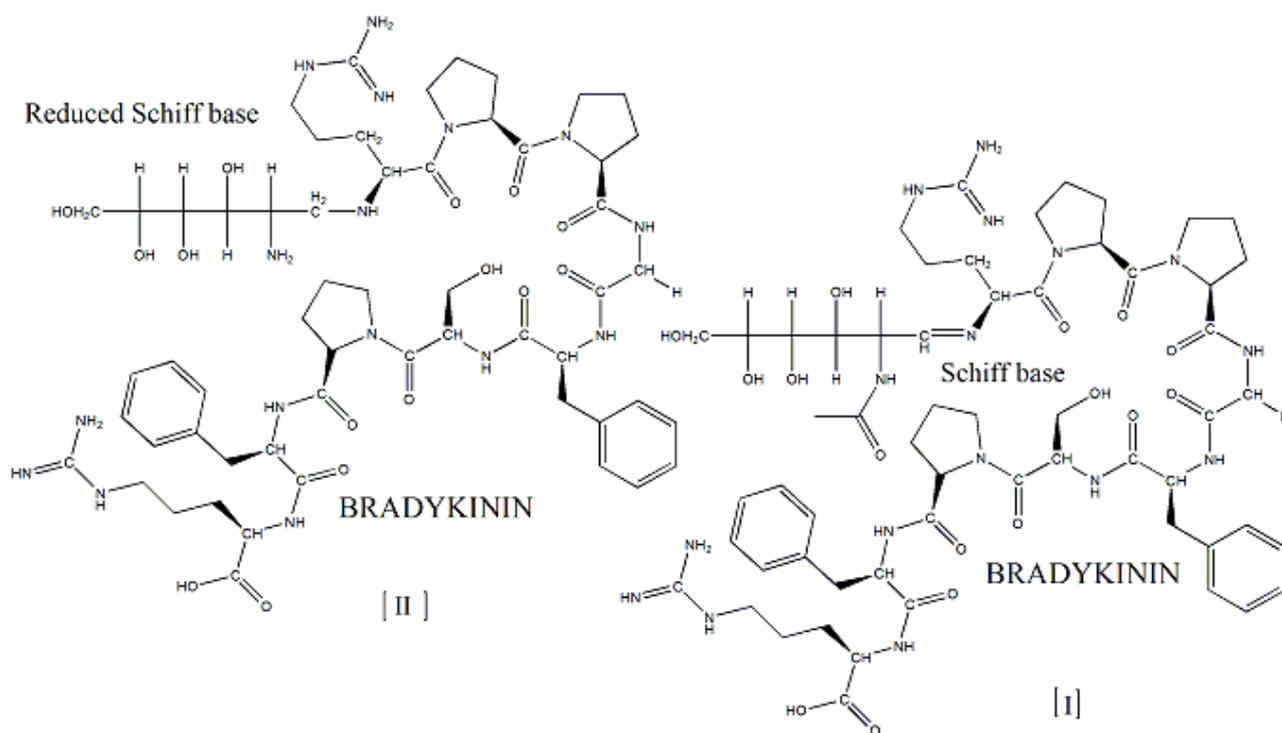
Glyco-bradykinins [III] and [IV] were also synthesized with randomly coupled fluorescein isothiocyanate to oligochitosan. Their synthesis was performed as for [III] and [IV] derivatives. Those analogs, as well as oligochitosan-fluorescein were tested on MDA cells (human breast cancer cells), purportedly expressing bradykinin B$_2$ receptors, by means of confocal microscopy. Oligochitosan-fluorescein alone exhibited staining on cell surface and notably; inside the cells (see **Figure 5**). Moreover, the fluorescein-oligochitosan linked to bradykinin (**Figure 6**), either via a Schiff base or in a reduced double bond form, displayed scarce, sporadic unspecific intracellular localization. Experiments with FITC alone or coupled to BK produced no specific labeling (**Figure 5**). It is possible that the coupling of FITC with BK ligand renders the latter molecule inapt for

interaction with its cognate B_2 receptor. This warrants further investigations. Thus, it appears that the conjugation of oligochitosan with bradykinin affords intracellular delivery, independently from a B_2 receptor-mediated process.

Figure 3 :   Bradykinin glycation products with glucosamine pentamer to form $NaBH_3CN$ reduced Schiff base form [III].
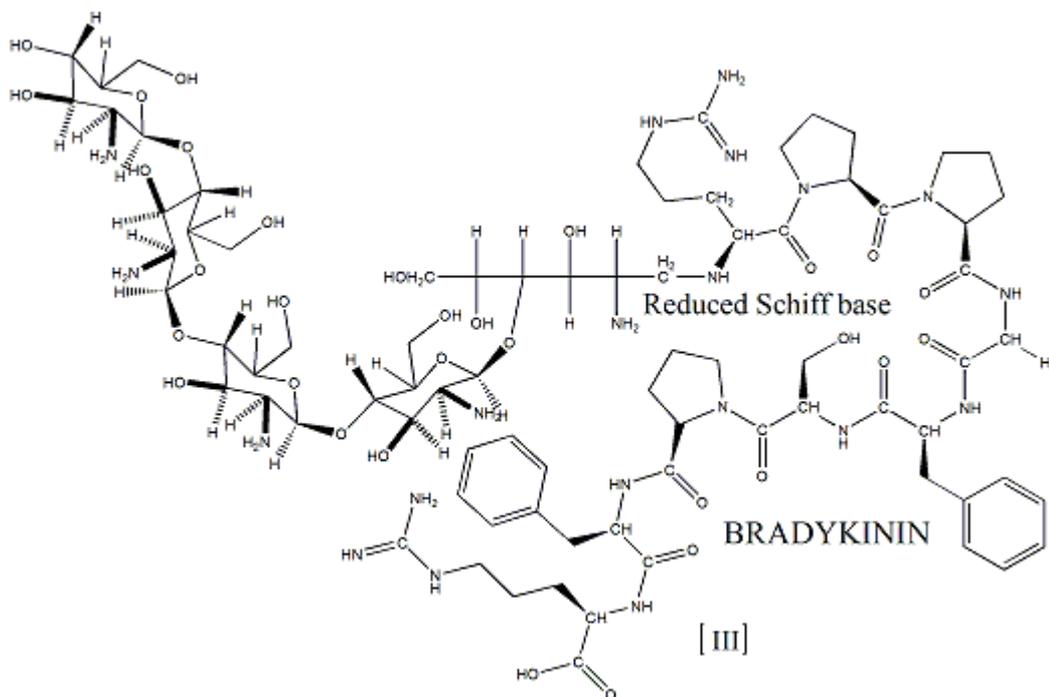
Figure 4 :   Bradykinin glycation products with glucosamine pentamer to Schiff base form [IV].
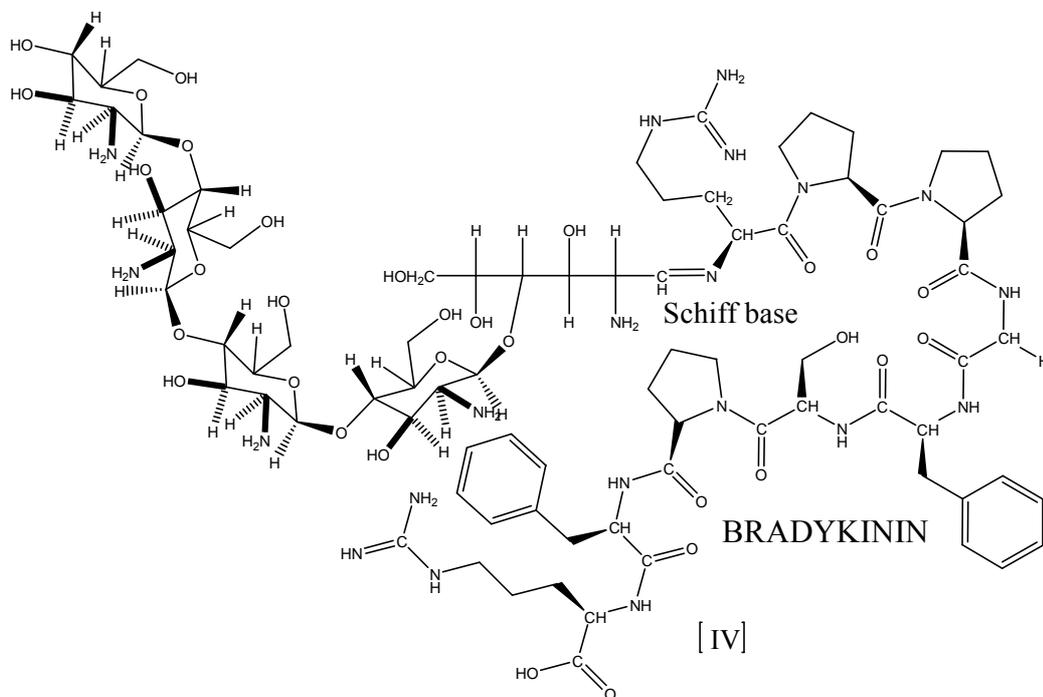
Figure 5 :   Confocal microscopy image fluorescein-oligochitosans-bradykinin on MDA cancer cells
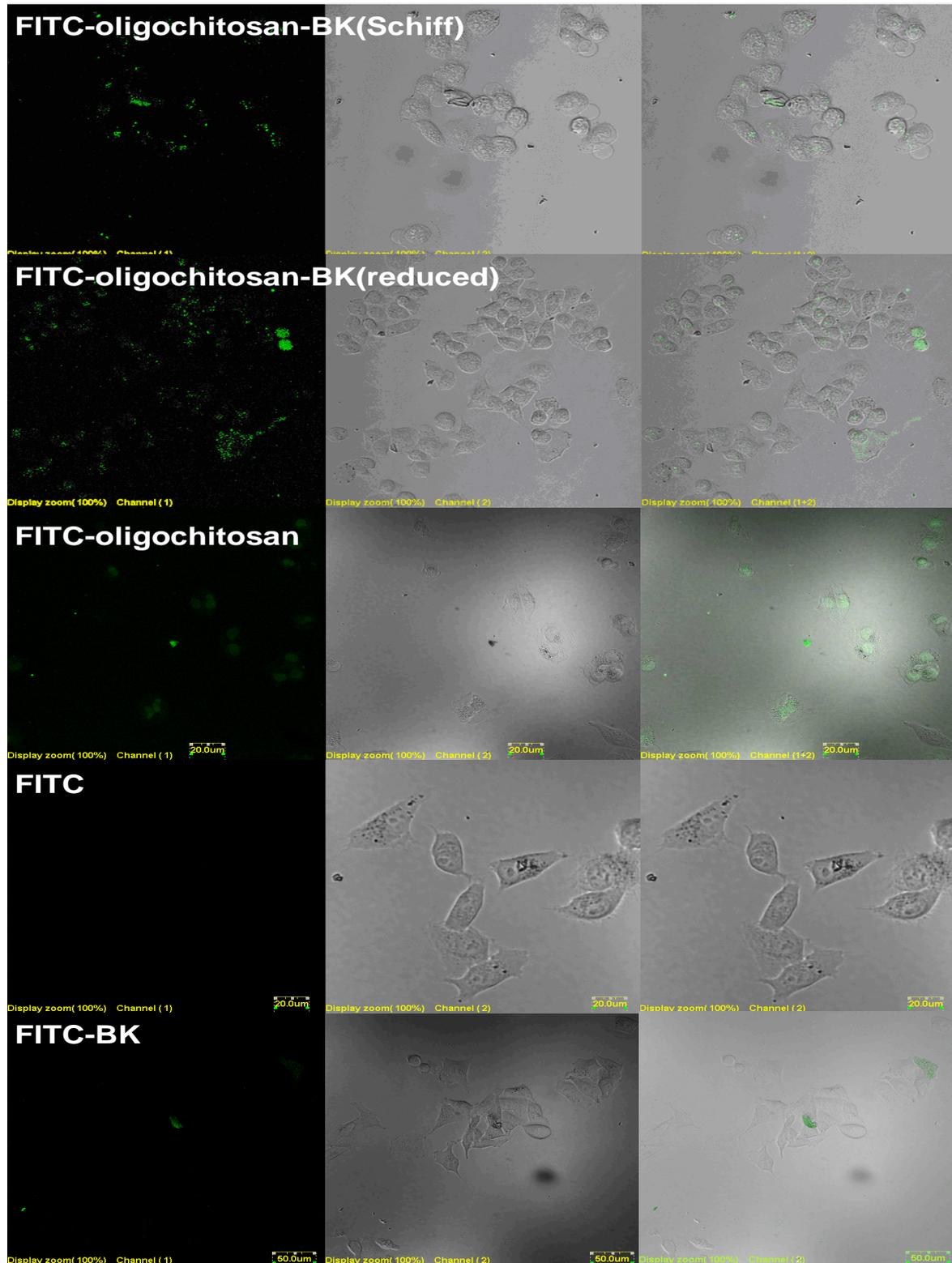
**Figure 6 :**   Structure of fluorescein labeled (at random position) oligochitosan-bradykinin.
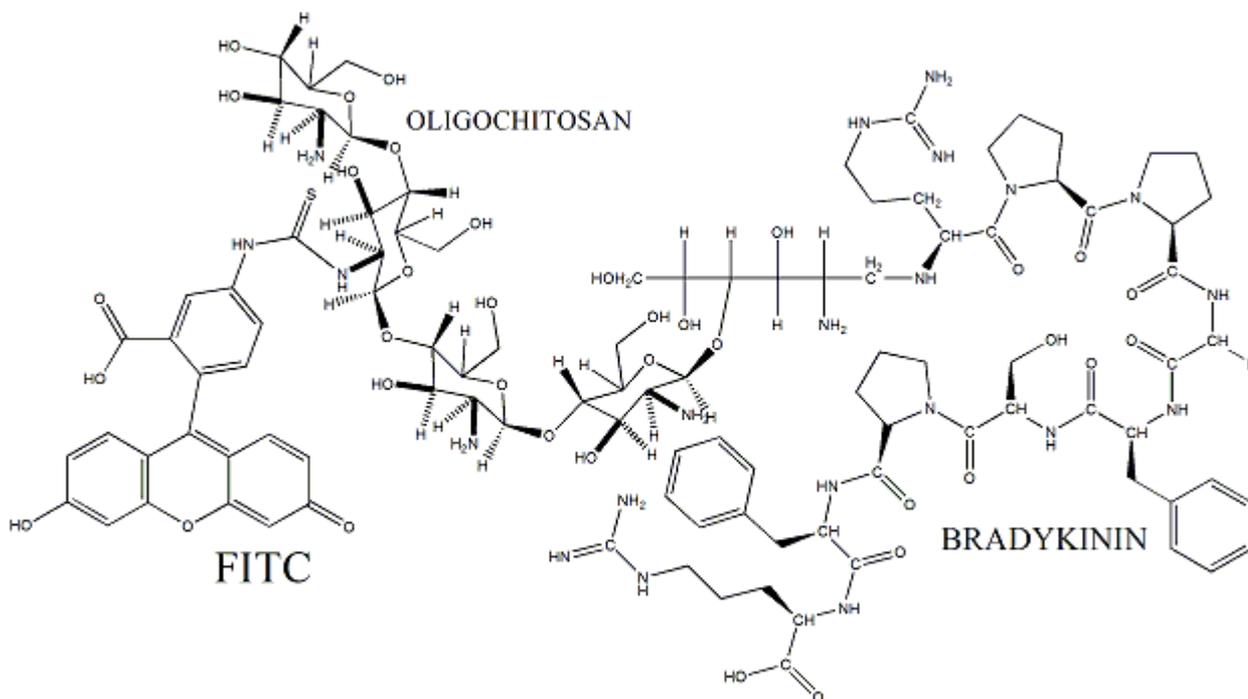
References

[1]   A.M. Killop, Y.H. Abdel-Wahab, M.H. Mooney, F.P. O'Harte, P.R. Flatt, Regulatory Peptides, 113(2003) 1-8.

[2]   I. Boucher, A. Dupuy, P. Vidal, W.A. Neugebauer, R. Brzezinski, Appl. Microb. Biotechnol., 38(1992)188-193.

[3]   S. Horvat, A. Jakas J.Peptide Sci., 10(2004) 119-137.

[4]   F. Gobeil, L.H. Pheng, I. Badini, X.K. Nguyen-Le, A. Pizard, A. Rizzi, D. Blouin, D. Regoli, Brit. J. Pharmacol., 118(1996) 289-294.

[5]   P. Stefanowicz, Z. Szewczuk, Peptides 2004, Proc. of the 3rd International and 28th European Peptide Symposium, Eds: M.Flegel, M. Fridkin, C. Gilon, J. Slaninova, Prague,(2004), pp 605-606.