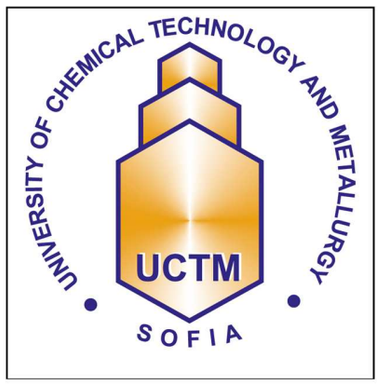

SCIENTIFIC CONTRIBUTIONS

of Assoc. Prof. Dr. Eng. Dancho Lyubenov Danalev

for participation in the contest for the academic position of "**Professor**" in the field of higher education Technical Sciences, professional field 5.11. Biotechnology, scientific specialty Technology of biologically active substances, announced in the State Gazette issue 3 / 10/01/2020

Sofia, February 2020

Reference for scientific contributions was made on the basis of **47** publications submitted for participation in the competition for academic position, of which **31** were in journals referenced in the *Web of Science* and *Scopus* data bases with Impact Factor or Scientific Journal Ranking, and 16 were in non-refereed peer-reviewed journals or in peer-reviewed conference volume. In addition to the competition, I participate with **36** citations noted beyond those for the acquisition of previous academic degrees and academic positions. The required developments were presented at **25** conferences in the form of **61** posters and **12** oral presentations.

All applications submitted for participation in the competition are in the field of biotechnology, in particular synthesis, characterization, isolation, study of biological activity, analysis and structure-activity relationship of biologically active molecules, as well as development of new methods for training and evaluation in chemistry and environmental protection and biotechnology, which are the base for establishing of good specialists in the technical and natural sciences.

Scientific contributions can be summarized in four main areas:

1. Synthesis, biological studies and some structure-activity relationships on biomolecules with diverse biological activity and membranes with incorporated biomolecules with potential application in biotechnology
2. Kinetic studies on enzymes, biologically active amino acids and peptides
3. Development of bioanalytical techniques for the determination of biologically active substances in the analysis and control of food and medical drugs and the detection of pollutants
4. Research on modern approaches to training in chemistry and engineering disciplines in the field of biotechnology

1. *Synthesis, biological studies and derivation of some structure-activity dependencies on biomolecules with diverse biological activity and membranes with incorporated biomolecules with potential applications in biotechnology* (articles A1 to A8, A11, A12, A14, A15, A19, A20, A27 to A29, B2, B3, B4, B6, B10 and B11).

Many peptide hormones and other peptide-protein regulators are involved in carrying out much of the biological and biochemical processes in the living organism. They can be neurotransmitters, neuromodulators, hormones, inhibitors and activators of various enzyme systems etc. They have been found to have a specific structure and exhibit high biological activity over a wide concentration range. Many of them have found application in the treatment and diagnosis of various diseases in human and veterinary medicine. The advantages of peptide and amino acid drugs over all other molecules are that they do not accumulate in the body and in their pure form usually do not cause undesired side effects. The products of their metabolism are amino acids, which are also formed by the metabolism of proteins contained in food. Therefore, a large number of peptide-based drugs with different biological activity (immunosuppressant, anti-inflammatory, analgesic, antitumor, antibacterial, etc.) have been successfully synthesized and applied in medical practice through the approaches of synthetic bio-organic chemistry and biotechnology. Carbohydrates and glycopeptides have also been widely used in medical practice. One of the most commonly used substances with a carbohydrate structure is the anticoagulant agent heparin,

hyaluronic acid, which has been widely used in the treatment of pain syndrome in adult degenerative diseases and in diseases and traumas that damage the articular cartilage in young people, the chitosan is included in nutritional supplements to lower the level of bad cholesterol in the body and many others. Apart from medical practice and diagnostics, peptides have been widely used in the food industry (aspartame, glutamic acid salts, etc.). The incorporation of biologically active peptides, amino acids and glycopeptides into membranes of different composition can serve as a basis for the development of new carriers with diverse applications including for biofilm formation for ecological application, biofuel production, bioreactor design, and biosensor design, developing protective coatings with antibacterial, anticorrosive and other activities. In the context of the importance mentioned above of these biomolecules, the following scientific contributions have been made:

- Thioglycoside bonds are crucial for biomolecules as their incorporation results in more stable glycomimetics with potential application as medical drugs. So far, only chemical methods for their introduction in glycofuranosyl conjugates have been reported in the literature. For the first time, a wide variety of thioaryl derivatives have been synthesized in moderate to excellent yields using mutant furanothioglycoligase. Of great interest is the fact that stable 1-thioimidoyl arabinofuranose, classically used in the chemical glycosylation process, is able to effectively act as a donor through an original enzyme activation mechanism in distance. The study of the chemical structure as well as the nucleophilicity of the thiol allowed us to optimize this biocatalyzed process. As a result, the creation of such a mutant enzyme is an original, soft and environmentally friendly method of thioligation. Model reactions using different thiols have made it possible to optimize the biocatalytic thioligation process (article A1).
- Current review summarize information available to date in the scientific literature as well as own studies on serine proteinase inhibitors from the blood coagulation cascade used or potential candidates for new drugs for the prevention and treatment of patients with haemostatic disorders (article A3).
- New hybrid molecules containing conjugated peptide fragment and medical drug have been synthesized in order to investigate the vectorization ability of the peptide carrier with a potential application for drug delivery system in medical practice. An original strategy was used with specifically protected COOH groups Asp and Glu as allyl esters, through which to introduce the drug molecule (articles A4 and B2).
- The anticoagulant activity of peptide amide analogs of isoforms 2 and 3 of antistasis has been synthesized and investigated. Our study reveals that replacement of the carboxyl with amide function at the C-terminus of the peptides leads to a significant increase in anticoagulant activity. Some kinetic studies of the same analogues have also been performed. The results show that both free acids and amide analogues have a mixed type of inhibition of serine proteinases of the blood coagulation cascade. The calculated Ki values for the model serine proteinases examined indicate some selectivity of the Phe-Ile-Arg-Pro-Lys-Arg-NH_2 analogue. (articles A6 and B1).

- A specific design of a series of 6 peptides, potential β-secretase inhibitors, analogues of the β-secretase inhibitor OM99-2 was done. The planned compounds were synthesized in order to investigate the possibility of influencing enzymatic disorders in Alzheimer's disease. All newly synthesized compounds were obtained in very high yields (article A8).
- New galantamine derivatives containing peptide fragments with β-secretase inhibitory activity have been synthesized. All new peptides were tested in mice for acute toxicity and showed low toxicity (LD50> 1000 mg / kg) after i.p. Their influence on the conditioned reflexes related to the improvement of learning and memory processes has been investigated. The compound 11-N-demethyl-11-N-N- [Boc-Asp (Asp-Leu-Ala-Val-NH-Bz1)] -galantamine was tested by a two-way active avoidance method. It showed a good influence on the conditioned reflexes, which improves the learning and memory processes. The inhibitory activity of the newly synthesized compounds was tested against the enzyme BuChE and IC_{50} values were determined. All compounds exhibit activity in micromolar concentration. Compounds 5 and 6 have about 10 times higher activity than galanthamine. Compounds 4 and 9 also show good activity. All newly synthesized compounds exhibit low acute toxicity (articles A11 and B10).
- Extracts of natural products used in Bulgarian traditional cuisine with different extraction systems and under different conditions have been made and their antibacterial activity against model strains of *Arthrobacter oxydans 1388* (model Gram + microorganism) and *Pseudomonas fuorescens 1442* (model Gram-microorganism) has been investigated. Our studies show that *Arthrobacter oxydans* are sensitive to the antimicrobial activity of honey, while *Pseudomonas fuorescens* are resistant. Garlic extracts showed good activity against both *Arthrobacter oxydans* and *Pseudomonas fuorescens* (article A12).
- Two novel compounds hybrid molecules between a specifically substituted pyrrole (Pyr) and analogues of the Tyr-MIF-1 peptide have been synthesized. Their analgesic activity in mice was investigated. All analgesic activity studies showed better activity at the same dose than the natural peptide Tyr-MIF-1 for the Pyr-Tyr-Phe-Leu-Ala-OH analogue. The Pyr-Ala-Leu-Phe-Tyr-OH compound had no better effect than the parent peptide. The results clearly indicate that it is important that the Tyr residue occupies the N-terminal position of the MIF-1 analogue. The lack of better activity of the Pyr-Ala-Leu-Phe-Tyr-OH analogue indicates that the Pyr residue does not affect the analgesic activity. In addition, we found that the amide function of the C-terminus, typically represented in native MIF-1, is not absolutely required for activity (articles A14, A19 and B3)
- A series of amino acid derivatives of pyrrole were synthesized and their antibacterial activity against model Gram positive (*Bacillus cerreus 1085*), Gram negative (*Pseudomonas fluorescens*) microorganisms and fungi (*Candida lipolytica*) was investigated using a standard disk-diffusion method. The highest activity against the model Gram positive strain of microorganisms (*Bacillus cereus 1085*) was demonstrated by the Pyr-Ile and Pyr-β-Phe. The best activity against model Gram negative microorganisms (*Pseudomonas fluorescens 1442*) was found for Pyr-Met and Pyr-β-Phe. All tested compounds did not show activity against the *Yarrowia*

lipolytica 3344 model strain. The Pyr-β-Phe showed a strong bacteriostatic effect against the *Bacillus cereus 1085* model strain (article A15).

- New galantamine-based hybrid molecules containing a peptide fragment with potential antiplatelet and anticholinesterase activity have been synthesized. The pentapeptide Leu-Pro-Tyr-Phe-Asp was included in the positions 6 or 11 of galantamine molecule using a suitably designed synthetic scheme by peptide synthesis in solution. Through the obtained molecules, we expect to combine two pharmacological effects in one molecule: an anticholinesterase effect and anti-aggregating properties, which will be demonstrated in subsequent studies on the biological activity of the newly synthesized compounds and to derive important structure-activity relationships (article A27)
- Antitumor activity and docking of a series of novel analogs of the biologically active peptide BIM-23052 containing spatially bulky non-natural amino acids have been synthesized and investigated. The peptides were synthesized by standard methods of solid phase peptide synthesis, Fmoc strategy. The cytotoxic effects of the compounds were tested *in vitro* against a panel of tumor cell lines: HT-29, MDA-MB-23, Hep-G2, HeLa, and the normal human diploid Lep-3 cell line. All somatostatin receptor subtypes were modeled and docking was performed to determine the rate of binding of the analogues. The new peptides exhibit a different concentration-dependent antiproliferative effect on tumor cell lines after 24 hours of treatment. Compound 3B (Aib6) exhibited the most pronounced antiproliferative effects on HepG-2 cells with IC_{50} = 0.01349 nM. Docking confirmed that all compounds bind well to the somatostatin receptors with preference for sstr3 and sstr5, which perfect correlate to the observed biological effects (article A28)
- Analogues of the adamantan derivatives amantadine and rimantadine were synthesized containing a thiazole motif, and their antiviral activity against influenza strain H1N1 and antibacterial activity against model strains gram positive (*Bacillus cereus*), gram negative (*Escherichia coli*) microorganism and fungi strain *Yarrowia lipolytica 3344* were studied. Cytotoxicity assay was performed to determine CC_{50} and IC_{50} values were calculated. The Gly-Thz-rimantadine thiazole analog of rimantadine shows good activity against influenza A/Hongkong/68 with IC_{50} = 0.11 μg/mL and CC_{50} = 50 μg/mL. The Gly-Thz-rimantadine compound has good antiviral activity and also exhibits very good antifungal activity at two different concentrations (article A29).
- Trinucleotides with potential applications in pharmacy and medicine have been synthesized and proven through novel approaches to activate methyl oxirane and H-phosphonate chemistry (article B4).
- RALLKAL matrix heptapeptide has been synthesized to serve as the basis for the creation of biosensor with enhanced biocompatibility, chemical stability, simplicity of structural prediction and modifications, as well as improved sensitivity due to the amplification of the catalytic signal. This peptide matrix will be used in the combinatorial synthesis of a library of short peptides, including as cofactors in a novel selection procedure (SELEX strategy) for the design of deoxyribozymes - for the production of deoxyribozymes that cleave the RNA phosphodiester linkage with a catalytic rate greater than that of known deoxyrobozymes (article B6).

- Different synthetic conditions were experimented and a new potential ribavirin-based lipoxygenase inhibitor was obtained by replacing the triazine base with thiourea in order to investigate its inhibitory potential at a later stage (article B11).
- New hybrid organo-inorganic membranes based on cellulose acetate butyrate/copolymer polyacrolonitrile acrylic amide/TiO_2 have been synthesized. Tests have revealed that when we use our polymers as carriers, there is a limit on the titanium concentration of 5%. Further increase of Ti concentration leads to precipitation processes. QCM analyzes show that the low $Ti(OBu)_4$ concentration does not affect the viscosity of the resulting matrices, but their elasticity changes significantly. The resulting membranes have been successfully applied to form a biofilm from the yeast strain *Saccharomyces cerevisiae* (article A2).
- New biocompatible hybrid materials containing amino acids have been synthesized for their use to produce an active biofilm of *Pseudomonas species 1625* gram-positive bacteria for application in aniline biodegradation (article A5).
- Hybrid polymer matrices containing acryloylglycine and poly(ethylene glycol) dimethacrylate have been synthesized. The rheological properties of the resulting gels and the formed biofilms were investigated using a quartz-crystal micro balance. The experimental results showed that the resulting matrices were suitable for the formation of biofilms from *Escherichia coli* and *Pseudomonas fluorescents* strains (article A7).
- The potential of immobilized onto hybrid matrix laccase for degradation of several dyes in water was investigated. The structure of the resulting hybrids was studied by X-ray diffraction, Fourier transform infrared spectroscopy, scanning electron microscopy and other analysis. The results show that the interaction between the SiO_2 network and the polysaccharide by H-linkage formation is successful. The laccase is immobilized by crosslinking and the degradation ability is tested against rhodamine B dyes, methyl orange and malachite green. The most effective decolourisation was achieved for malachite green and methyl orange. (Article A20).

2. *Kinetic studies on enzymes, biologically active amino acids and peptides* (A6, A13, A16, A17, A18, A23, B1, B5, B7, B8 and B9)

The mechanism by which a biomolecule acts on a particular enzyme system, and the strength and duration of binding depends on the type of interaction of that substrate with the active site of the enzyme(s). In this context, the kinetics of various enzymatic reactions in the presence of newly synthesized biomolecules or amino acids were investigated:

- The kinetics and the type of inhibition of enzymes from the blood coagulation cascade from peptide amide analogues of isoforms 2 and 3 of antistasin were investigated. The results show that both C-terminal free acids and amide analogues have a mixed type of inhibition of serine proteinases of the blood coagulation cascade. The calculated Ki values for the model serine proteinases examined indicate some selectivity of the Phe-Ile-Arg-Pro-Lys-Arg-NH_2 analogue (articles A6 and B1).
- The kinetics of inhibition of lipoxygenase isolated from avocados from the natural amino acids L- and D-serine were investigated. Inhibition constants and the type of inhibition of the

enzyme inhibitors used were determined. Our studies have shown that the affinity of lipoxygenase is higher for L-Ser compared to D-Ser. This means that the natural form of the amino acid is more compatible with the enzyme, but at the same time Ki values indicate that D-Ser has a more potent inhibitory effect. L-Ser shows a competitive inhibition type, whereas for D-Ser it is a mixed one (article A16).

- The inhibitory activity of two peptide analogues of galantamine Boc-Asp(norGal)-Asp-Leu-Ala-Val-NH-Bzl and Boc-Asp(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl was investigated агаинст free and immobilized tyrosinase using a newly constructed biosensor. As a result of the study, IC_{50} values for both peptides in the micromolar range were established. In addition, both conjugates were found to be non-competitive tyrosinase inhibitors (articles A17 and B9).
- The inhibitory activity of the peptide analogs of galantamine Boc-Val-Asn-Leu-Ala-Gly-OGal, Boc-Val-Asn-Leu-Ala-Val-Gly-OGal, Boc-Asp-(norGal)-Asp-Leu-Ala-Val-NH-Bzl, Boc-Asp-(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl and Boc-Asp-(norGal)-Val-Asn-Leu-β-Ala-Val-NH-Bzl toward acetylcholinesterase AChE using a newly constructed biosensor was study. The calculated IC_{50} values for both peptides are in the micromolar range, but these were 7-8 times lower than those of free galantamine. Further studies on the kinetics of enzyme inhibition indicate that all four analogues are competitive inhibitors of the AChE (article A18).
- The fermentation parameters for the production of *Candida Antarctica* lipase have been investigated and optimized, and its kinetic parameters have been determined as well as its activity. The strain is cultured by observing different parameters during the proliferation process. Several cultivation procedures were performed to find optimal conditions. Furthermore, crude lipase activity, its temperature and pH optimum were determined using a potentiometric method. We found that the temperature optimum of our isolated lipase was at 60°C and the pH optimum was at pH 8.0. The specific activity of crude lipase was determined to be 97.2 U/mg. The activity and protein content of the 'crude' liquid after cultivation were also determined (article A13).
- The fermentation method for the synthesis of lipase B from *Pseudozyma Antarctica* in a laboratory bioreactor has been optimized. A three-step method for isolating and purifying lipase B produced by *Pseudozyma Antarctica* has been developed. Highly purified lipase B has been obtained and characterized with potential application in the process of regioselective synthesis of modified amino acids and biologically active peptides (article A23).
- Designs and synthesis of nucleoside and 2'-deoxynucleoside substrates were realized. They all are suitable for kinetic studies on the intramolecular cyclization (lactamization) reaction in order to clarify the role of the acyl (aminoacyl) residue in the process of acceleration of catalytic reaction of the peptidyltransferase (article B5).
- Two model substrates 2'/3'-O-[Bz(NO_2)-Lys(Boc)]-5'-O-pivaloyladenosine and its 2'-deoxy analogue 3'-O-[Bz(NO_2)-Lys(Boc)]-5'-O-pivaloyl-2'-deoxyadenosine were synthesized by two different approaches. Further they will be used for kinetic studies on the intramolecular aminolysis reaction to demonstrate the major role of 2'-OH in ribose ring and the exceptional role of substrate-mediated peptidyl-mRNA catalysis during protein biosynthesis on the ribosome (Articles B7 and B8).

3. *Development of bioanalytical techniques for the determination of biologically active substances in the analysis and control of foods and medical drugs and the detection of pollutants* (A9, A21, A25, A26, A30, A31, B12 and B14)

The rapid development of the capabilities of synthetic chemistry and biotechnology poses new challenges to the analysis of substances contained in various matrices for the needs of the medical, food and cosmetic industries. New functionalized foods, new medical drugs, nutritional supplements and cosmetics are created, and often the processes of preparation and / or isolation of some of the constituent substances, as well as the processes of their storage, are related to technologies and conditions that enable penetration into them of various undesirable by-products, some of which with a possible or proven carcinogenic or other undesired effect on the body. Although the sensitivity of the detection techniques used has increased significantly, the matrices to be analyzed are becoming increasingly complex, and sometimes the target substances are difficult to detect due to the interfering effects of the matrix and the difficulties related to selective extraction of the target compounds. Therefore, it requires the creation of new, more sensitive and refined methods for analyzing and controlling substances entering the market for consumers. All this is related to the development and implementation of various national and European control regulations, part of which is the introduction of new methods of analysis in the laboratory and industrial practice. The following contributions can be listed in this aspect:

- A modified QuEChERS method has been developed, followed by selective analysis using high performance liquid chromatography with a fluorescence detector to determine N-methyl carbamates in a liver matrix (article A9).

- A modified EN 16619:2015 procedure for the determination of four polycyclic aromatic hydrocarbons (PAHs) has been developed. The method has been developed with a new internal standard and modifications have been made to the sample preparation procedure for two low-fat food matrices (wheat flour and smoked pork leg) (article A21).

- An optical biosensor was designed to detect N-methylcarbamates in liver samples. A suitable method for sample pre-treatment has been developed and the biosensor is validated according to Regulation SANTE/11945/2015. The kinetic parameters of the enzymes immobilized on the biosensor have also been determined (article A25).

- An optical biosensor has been designed to detect organophosphate pesticides in liver samples. A suitable method for sample preparation has been developed and the biosensor is validated according to Regulation SANTE/11945/2015. The kinetic parameters of the enzymes immobilized on the biosensor have also been determined (article A26).

- A laboratory method for determination of the bioactive substances yohimbine, vardenafil, sildenafil, tadalafil, dapoxetine and sibutramine in products offered for sexual enhancement,

weight loss or bodybuilding purposes has been developed and validated. The method is validated according to the requirements of the European and Bulgarian legislation (article A30).

- A laboratory method for the determination of 16 polycyclic aromatic hydrocarbons under the control of European legislation due to their proven toxic, carcinogenic and mutagenic properties in the porc matrix has been developed and validated. The method is validated according to European and International legislation (article A31).

- The effect of several different combinations of polar and nonpolar solvents on the analytical yield of organochlorine pesticide lindane in chicken eggs has been investigated. The extraction was performed using an Accelerate Solvent Extractor, followed by treatment on a florisil column as the next purification step. Gas chromatography with electron capture detector was used to quantify the pesticide content. The results obtained show that a mixture of 20/80 (v/v) polar / non-polar solvent (ethanol / toluene) is the best ratio for both the determination of fat and the analytical yield of lindane (article B12).

- Optimization and calibration of a method for determination of phthalates using GC / MS is done. Dicyclohexylphthalate (DICHP) was selected as the internal standard. Hexane was used as the solvent to obtain the required standard phthalate solutions (article B14).

4. *Research on modern approaches to training in chemistry and engineering disciplines in biotechnology (A10, A24, B14, B15 and B16)*

All the achievements in the field of dynamically developing science and in particular interdisciplinary area of biotechnology would not be possible without the training and the creation of personnel with sufficient competence, technological literacy and the ability to make adequate decisions, which is based on training in chemistry, biotechnology and special engineering and technology disciplines. The development of modern on-line and internet technologies makes it impossible to study chemistry and engineering disciplines, in particular biotechnology, without the use of information technology tools. On the other hand, the process of globalization is coming at a rapid rate, and the mobility opportunities for students have increased dramatically with the opening of European frontiers, making it almost unthinkable for professional development without be familiar with foreign languages. Creating appropriate methods, tools and training platforms is a key element in science education and teaching, in particular chemistry and engineering, as well as biotechnology. The following significant contributions have been made in this area:

- A new model for bilingual engineering training has been created and this model has been successfully implemented in biotechnology training. Interdisciplinary modules have been found to enable students to become aware of the meanings and connections between relevant subjects and to present a number of perspectives on how to complete the tasks (article A10).

- The student-centered (SCL) educational approach is applied to develop engineering and communication skills of students studying biotechnology. A new educational model has been developed. It is based on: taking into account students' needs, using authentic professional assignments and materials in a bilingual form; collaboration and teamwork, learning activities that take into account the professional responsibilities of professionals at their workplace; specific assessment of achievement (academic knowledge, skills and communication competences) (article A24).

- Professional practice requires the development of competences that combine both professional (technical) and communication skills, including English. In order to support the development of the two areas of competence, an integrated technology course (Pharmaceutical Biotechnology) and English for Specific Purposes (ESP) was developed and implemented based on an analysis of students' language competency needs. Students' self-assessment data are analyzed in terms of measured communication needs in English. The statistical analysis verifies the effectiveness of the new approach based on the assessment of student needs (article B14).

- Enigmatics is considered as an innovative tool for raising interest in the subject in teaching chemistry. During this study we proved increasing among students of both the motivation to learn chemistry and the interest in the subject by using specific enigmatic tasks in the learning process. Conclusions have been done regarding students' attitudes and utility in using enigmatic tasks in chemistry training (article B15).

- In the context of the emerging digital technologies, a study has been realized on the attitudes of students in education of chemistry and environmental protection in high school course to use information technology tools as a tool for learning and controlling as well as evaluating academic achievement. Some conclusions have also been done about the degree of student achievement in the use of electronic forms of control and assessment (article B16).

PERSPECTIVE FOR FUTURE WORK AND DEVELOPMENT

1. Synthesis and studies of novel biomolecules based on modified amino acids, peptides and glycopeptides with potential anti-tumor properties.
2. Creating conjugates of bioactive molecules with antitumor properties and peptides with suitable design for attacking the specific targets. Model studies on cell penetration ability of newly synthesized biomolecules including peptide moiety.
3. Synthesis and study of new molecules based on modified amino acids, peptides and glycopeptides with potential antibacterial properties.
4. Incorporation of antibacterial biomolecules by various methods into hybrid matrices.
5. Investigations on the kinetic of enzymatic reactions in presence of newly synthesized biomolecules with activating or inhibiting properties against enzymatic systems related to different diseases.

Sofia, February 2020

Prepared by:

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