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(57) Abstract: Provided herein are cyclic polypeptide compounds that can, e.g., bind specifically to human proprotein convertase subtilisin/kexin type 9 (PCSK9) and optionally also inhibit interaction between human PCSK9 and human low density lipoprotein receptor (LDLR), and pharmaceutical compositions comprising one or more of these compounds. Also provided are methods of reducing LDL cholesterol level in a subject in need thereof that include administering to the subject one or more of the cyclic polypeptide compounds or a pharmaceutical composition provided herein.

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CYCLIC POLYPEPTIDES FOR PCSK9 INHIBITION

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Application No. 62/688,037, filed June 21, 2018, which is incorporated herein by reference in its entirety.

BACKGROUND

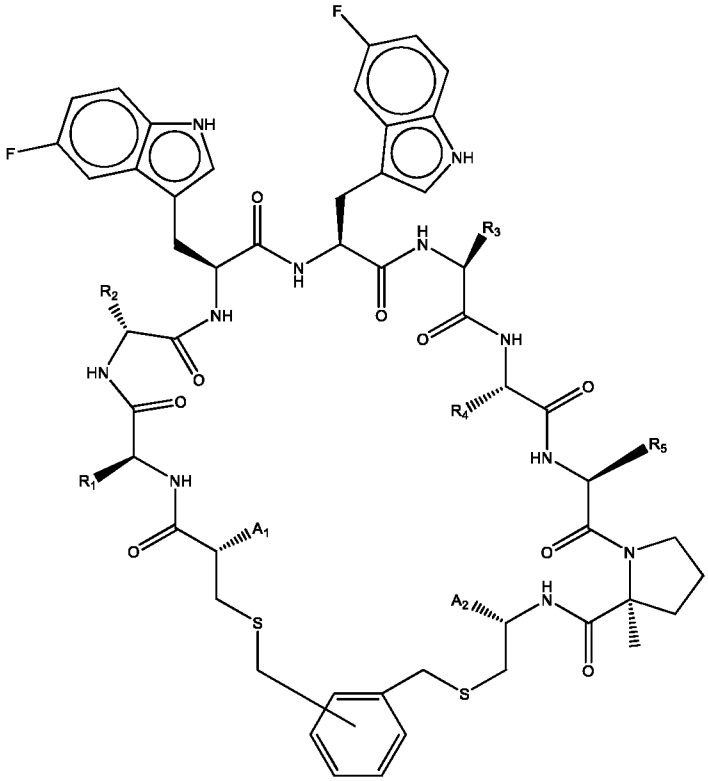
Hypercholesterolemia is characterized by the presence of high levels of low-density lipoprotein (LDL) cholesterol in the blood, which can lead to the development of atherosclerosis and associated ischemic cardiovascular disease (e.g., myocardial infarction and stroke) (Lambert et al., *J. Lipid Res.* 53:2515-2524, 2012). More than 34 million American adults have hypercholesterolemia (NIH Genetics Home Reference Website, 2014). Proprotein convertase subtilisin/kexin type 9 (PCSK9) has been proposed as a target for treatment of hypercholesterolemia.

PCSK9 is a proprotein convertase belonging to the proteinase K subfamily of the secretory subtilase family. PCSK9 binds to the epidermal growth factor-like-repeat A (EGF-A) domain of the low-density lipoprotein receptor (LDLR) and directs LDLR to lysosomes, where it is degraded. Inhibition of PCSK9 leads to increased LDLRs at the cell surface, resulting in increased removal of LDL from the extracellular fluid. Therefore, the inhibition of PCSK9 results in lower LDL cholesterol concentrations in the blood.

There remains a need in the art for compounds that inhibit the binding of PCSK9 to LDLR, and treat or prevent hypercholesterolemia. Administration of these compounds can lead to significantly improved prognosis, diminished progression of hypercholesterolemia, and a decrease in diseases related to hypercholesterolemia.

SUMMARY

Provided herein are cyclic polypeptides useful for the inhibition of PCSK9 in a subject in need thereof. In particular, provided herein are cyclic polypeptide compounds having the structure of Formula (I):



(I)

or a pharmaceutically acceptable salt thereof.

Also provided herein is a cyclic polypeptide compound selected from compound 001, 003, 004, 005, 006, 007, 008, 009, 010, 011, 012, 013, 014, 015, 016, 017, 018, 019, 020, 021, 022, 023, 024, 025, 026, 027, 028, 042, 043, 044, 045, 046, 049, 055, 056, 059, 060, 067, 072, 073, 074, 075, 076, 077, 078, 079, 080, 090, 096, 097, 098, 099, 100, 101, 102, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 118, 119, or 120.

Also provided herein is a cyclic polypeptide compound selected from compound 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, or 131.

In an embodiment, provided herein is a pharmaceutical composition comprising one of the compounds of Formula (I) or compounds 001-131, together with a pharmaceutically acceptable carrier.

Also provided herein is a method of reducing low density lipoprotein (LDL) cholesterol level in a subject in need thereof comprising administering to the subject a therapeutically effective amount of one or more of cyclic polypeptide compounds of Formula (I) or compounds 001-131, or a pharmaceutical composition comprising one or more of cyclic polypeptide compounds of Formula (I) or compounds 001-131.

In addition, provided herein is a method of treating hypercholesterolemia in a subject in need thereof comprising administering to the subject a therapeutically effective amount of one or more cyclic polypeptide compounds of Formula (I) or compounds 001-131, or a

pharmaceutical composition comprising one or more of cyclic polypeptide compounds of Formula (I) or compounds 001-131.

Additionally, provided herein is a method of treating a disease that shows comorbidity with hypercholesterolemia in a subject in need thereof comprising administering to the subject a therapeutically effective amount of one or more cyclic polypeptide compounds of Formula (I) or compounds 001-131, or a pharmaceutical composition comprising one or more of cyclic polypeptide compounds of Formula (I) or compounds 001-131. For example, the disease that shows comorbidity can be one or more of nephrotic syndrome, kidney failure, coronary artery disease, atherosclerosis, stroke, peripheral vascular disease, diabetes, and high blood pressure.

Also provided herein is a method of inhibiting PCSK9 activity comprising administering to a subject in need thereof a therapeutically effective amount of one or more cyclic polypeptide compounds of Formula (I) or compounds 001-131, or a pharmaceutical composition comprising one or more of cyclic polypeptide compounds of Formula (I) or compounds 001-131.

In addition, provided herein is a method of inhibiting the interaction between PCSK9 and the EGF-A domain of LDLR in a subject in need thereof comprising administering a therapeutically effective amount of one or more cyclic polypeptide compounds of Formula (I) or compounds 001-131, or a pharmaceutical composition comprising one or more of cyclic polypeptide compounds of Formula (I) or compounds 001-131.

Also provided herein is a method of inhibiting PCSK9 activity in a cell comprising contacting the cell with one or more cyclic polypeptide compounds of Formula (I) or compounds 001-131, or a pharmaceutical composition comprising one or more of cyclic polypeptide compounds of Formula (I) or compounds 001-131.

DETAILED DESCRIPTION OF THE DISCLOSURE

Provided herein are cyclic polypeptide compounds, and pharmaceutical compositions comprising such compounds, that are useful for the inhibition of PCSK9. For example, the compounds are useful in inhibiting the interaction between human PCSK9 and the EGF-A domain of human LDLR in subject. These compounds and pharmaceutical compositions are also useful for the treatment of hypercholesterolemia, as well as diseases that show comorbidity with hypercholesterolemia.

Definitions

Listed below are definitions of various terms used to describe this invention. These definitions apply to the terms as they are used throughout this specification and claims, unless otherwise limited in specific instances, either individually or as part of a larger group.

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry, and peptide chemistry are those well-known and commonly employed in the art.

As used herein, the articles "a" and "an" refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element. Furthermore, use of the term "including" as well as other forms, such as "include," "includes," and "included," is not limiting.

As used herein, the term "cyclic polypeptide compounds" means polypeptide chains in which the amino termini and carboxyl termini; amino termini and side chain; carboxyl termini and side chain; or side chain and side chain, are linked with a covalent bond that generates the ring.

As used herein, the term "disease that has comorbidity with hypercholesterolemia" means a disease in which elevated cholesterol levels (e.g., elevated LDL cholesterol levels) (e.g., as compared to a reference level) are associated with an elevated risk of developing the disease and/or an elevated risk of poor prognosis of the disease (e.g., increased risk of severe disease, increased risk of death, and/or an increased risk of cardiovascular event (such as stroke, myocardial infarction, or heart disease)) (e.g., as compared to a subject not having an elevated LDL cholesterol level, e.g., as compared to a reference level).

As used herein, the term "treatment" or "treating" is defined as the application or administration of a therapeutic agent, i.e., a compound of the invention (alone or in combination with another pharmaceutical agent), to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient (e.g., for diagnosis or ex vivo applications), who has hypercholesterolemia, or related diseases, a symptom of hypercholesterolemia, or related diseases, or the potential to develop hypercholesterolemia, or related diseases, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the hypercholesterolemia, or related diseases, the symptoms of hypercholesterolemia, or related diseases, or the potential to develop hypercholesterolemia, or related diseases. Such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. As used herein, to "treat" includes systemic amelioration of the symptoms associated with the pathology and/or a delay in onset of symptoms. Clinical and sub-clinical evidence of "treatment" will vary with the pathology, the individual and the treatment.

As used herein, the term "prevent" or "preventing" comprises the prevention of at least one symptom associated with or caused by the state, disease or disorder being prevented. As used herein, the term "prevent" or "prevention" also means no disorder or

disease development if none had occurred, or no further disorder or disease development if there had already been development of the disorder or disease. Also considered is the ability of one to prevent some or all of the symptoms associated with the disorder or disease.

As used herein, "inhibit" means to decrease an activity, such as the activity of PCSK9 or the activity of a molecule that binds PCSK9.

As used herein, the term "patient," "individual," or "subject" refers to a human or a non-human mammal. Non-human mammals include, for example, livestock and pets, such as ovine, bovine, porcine, canine, feline and marine mammals. Preferably, the patient, subject, or individual is human.

As used herein, the terms "effective amount," "pharmaceutically effective amount," and "therapeutically effective amount" refer to a nontoxic but sufficient amount of an agent to provide the desired biological result. That result may be reduction or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. An appropriate therapeutic amount in any individual case can be determined by one of ordinary skill in the art using routine experimentation.

As used herein, the term "pharmaceutically acceptable" refers to a material, such as a carrier or diluent, which does not abrogate the biological activity or properties of the compound, and is relatively non-toxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

As used herein, the term "composition" or "pharmaceutical composition" refers to a mixture of at least one compound useful within the invention with a pharmaceutically acceptable carrier. The pharmaceutical composition facilitates administration of the compound to a patient or subject. Multiple techniques of administering a compound exist in the art including, but not limited to, intravenous, oral, aerosol, parenteral, ophthalmic, pulmonary, and topical administration.

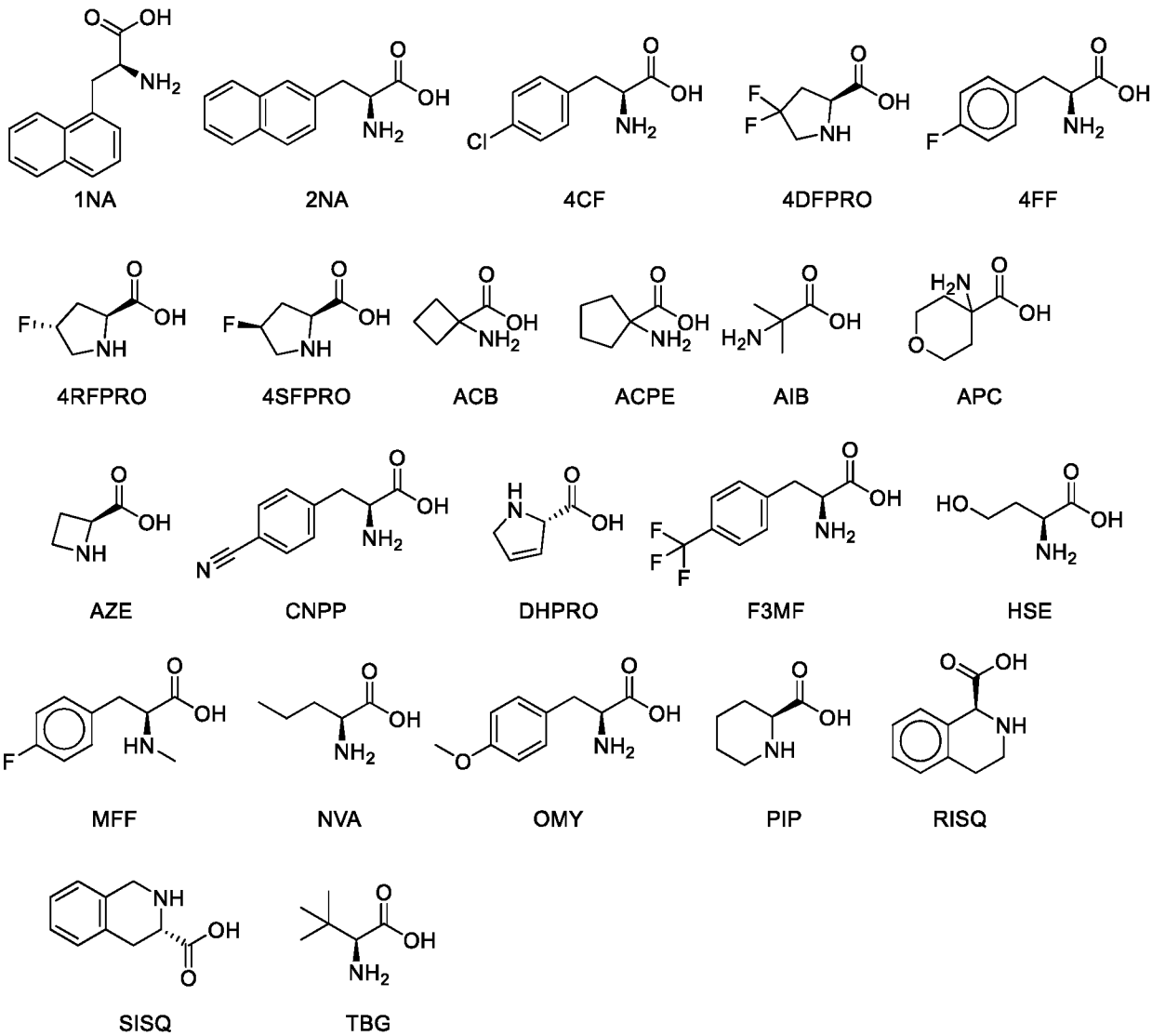
As used herein, the term "pharmaceutically acceptable carrier" means a pharmaceutically acceptable material, composition or carrier, such as a liquid or solid filler, stabilizer, dispersing agent, suspending agent, diluent, excipient, thickening agent, solvent or encapsulating material, involved in carrying or transporting a compound useful within the invention within or to the patient such that it may perform its intended function. Typically, such constructs are carried or transported from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation, including the compound useful within the invention, and not injurious to the patient. Some examples of materials that may serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and

sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; surface active agents; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

As used herein, "pharmaceutically acceptable carrier" also includes any and all coatings, antibacterial and antifungal agents, and absorption delaying agents, and the like that are compatible with the activity of the compound useful within the invention, and are physiologically acceptable to the patient. Supplementary active compounds may also be incorporated into the compositions. The "pharmaceutically acceptable carrier" may further include a pharmaceutically acceptable salt of the compound useful within the invention. Other additional ingredients that may be included in the pharmaceutical compositions used in the practice of the invention are known in the art and described, for example in Remington's Pharmaceutical Sciences (Genaro, Ed., Mack Publishing Co., 1985, Easton, PA), which is incorporated herein by reference.

A "pharmaceutically acceptable salt" is intended to mean a salt of a free acid or base of the compounds provided herein that is non-toxic, biologically tolerable, or otherwise biologically suitable for administration to the subject. It should possess the desired pharmacological activity of the parent compound. See, generally, G.S. Paulekuhn, et al., "Trends in Active Pharmaceutical Ingredient Salt Selection based on Analysis of the Orange Book Database", J. Med. Chem., 2007, 50:6665–72, S.M. Berge, et al., "Pharmaceutical Salts", J Pharm Sci., 1977, 66:1-19, and Handbook of Pharmaceutical Salts, Properties, Selection, and Use, Stahl and Wermuth, Eds., Wiley-VCH and VHCA, Zurich, 2002. Examples of pharmaceutically acceptable salts are those that are pharmacologically effective and suitable for contact with the tissues of patients without undue toxicity, irritation, or allergic response. The compounds provided herein may possess a sufficiently acidic group, a sufficiently basic group, or both types of functional groups, and accordingly react with a number of inorganic or organic bases, and inorganic and organic acids, to form a pharmaceutically acceptable salt.

As used herein, unnatural amino acids used in this invention have the following amino acid code and structure:

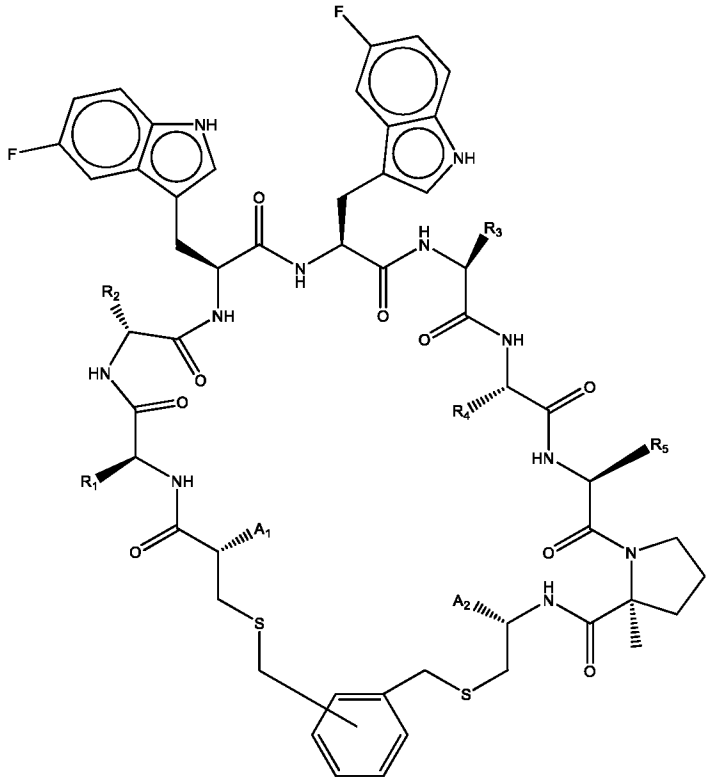


Compounds

Provided herein are cyclic polypeptide compounds (also referred herein as **"compounds of the invention") that are useful for inhibiting PCSK9 activity. In one aspect,** the compounds of the invention are useful for inhibiting the interaction between PCSK9 and EGF-A domain of LDLR in a subject. Additionally, the compounds of the invention are useful for reducing low density lipoprotein (LDL) cholesterol level in a subject. The subject whose LDL cholesterol is being lowered can suffer from a diagnosis of elevated LDL levels. The subject can also have hypercholesterolemia.

In another aspect, the compounds of the invention are useful for treating or preventing hypercholesterolemia. In addition, the compounds of the invention are useful for treating or preventing diseases that show comorbidity with hypercholesterolemia.

In another aspect, provided herein are cyclic peptides having the structure of Formula (I):



(I)

or a pharmaceutically acceptable salt thereof;

wherein:

A_1 is H or an acyl protected amine;

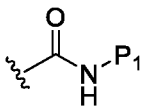
R_1 is selected from the group consisting of the amino acid side chains of ALA, GLY, ACB, ACPE, APC, LYS, NVA, PRO, and TBG;

R_2 is selected from the group consisting of the amino acid side chains of ALA and GLY;

R_3 is selected from the group consisting of the amino acid side chains of 4SFPRO, AIB, AZE, 4RFPRO, DHPRO, 4DFPRO, PIP, PRO, HSE, SISQ, and RISQ;

R_4 is selected from the group consisting of the amino acid side chains of ALA, HIS, GLY, and PRO;

R_5 is selected from the group consisting of the amino acid side chains of TYR, 4CF, 4FF, MFF, CNPP, 2NA, 1NA, F3MF, PHE, PRO, and OMY;

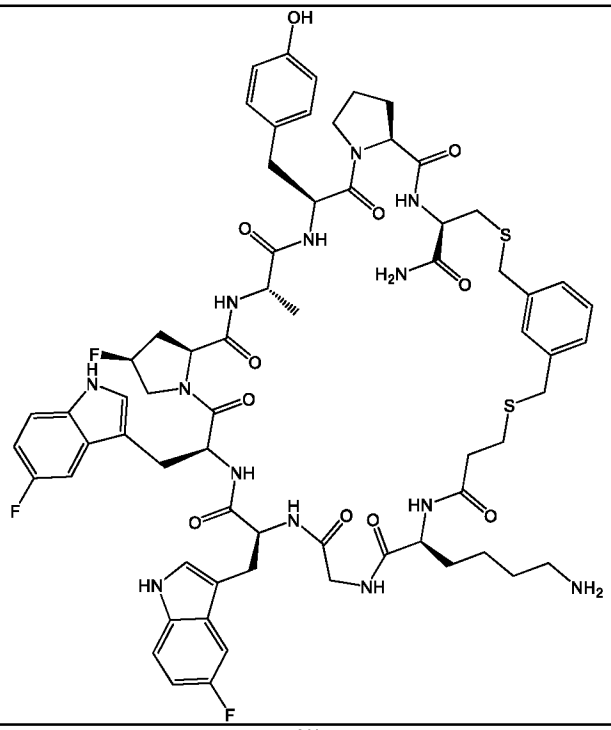
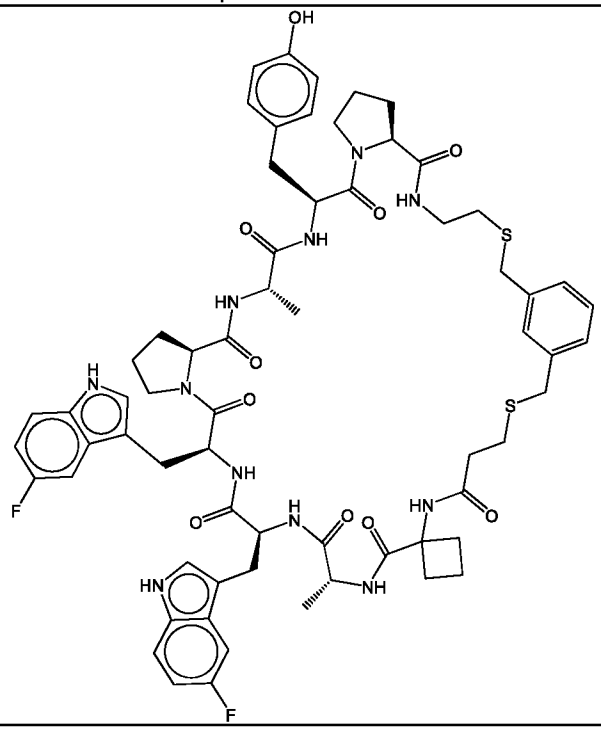
A_2 is absent or  ; and

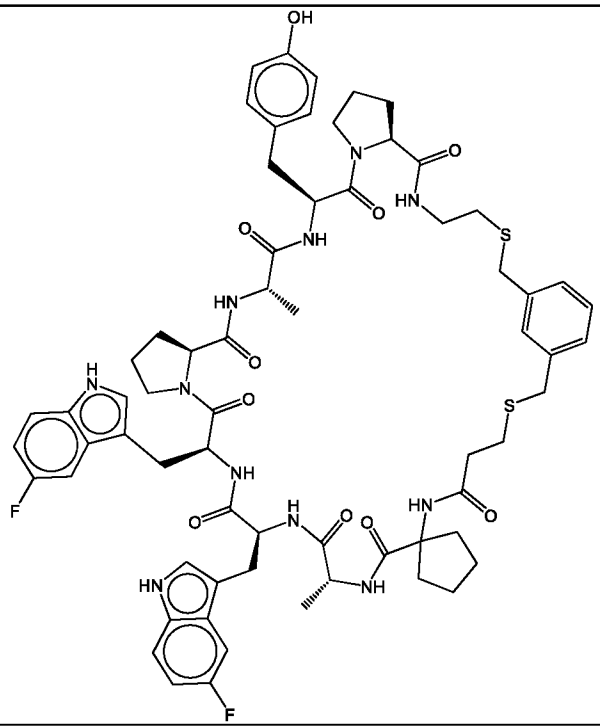
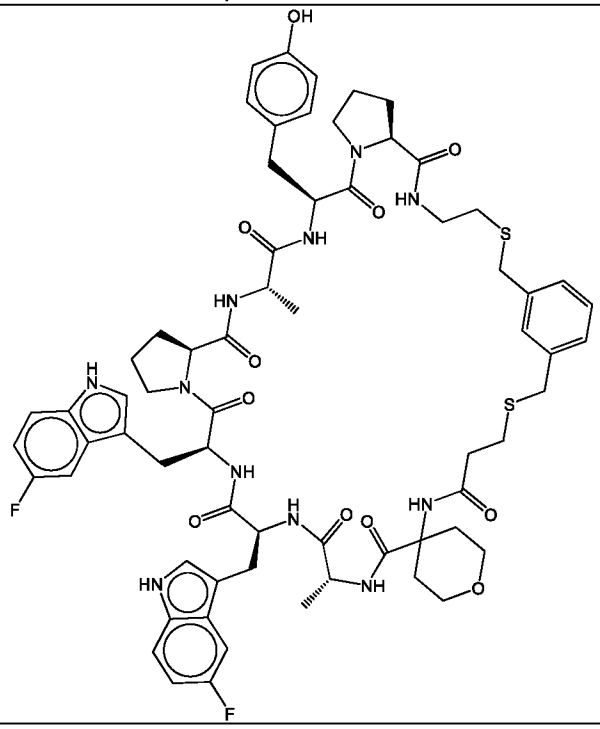
P_1 is selected from the group consisting of H and acetyl;

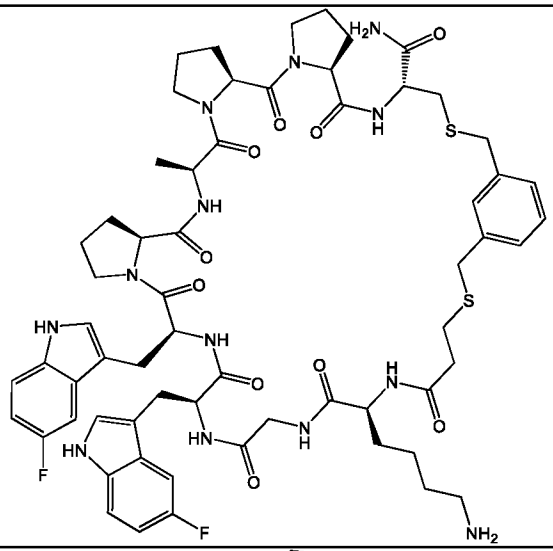
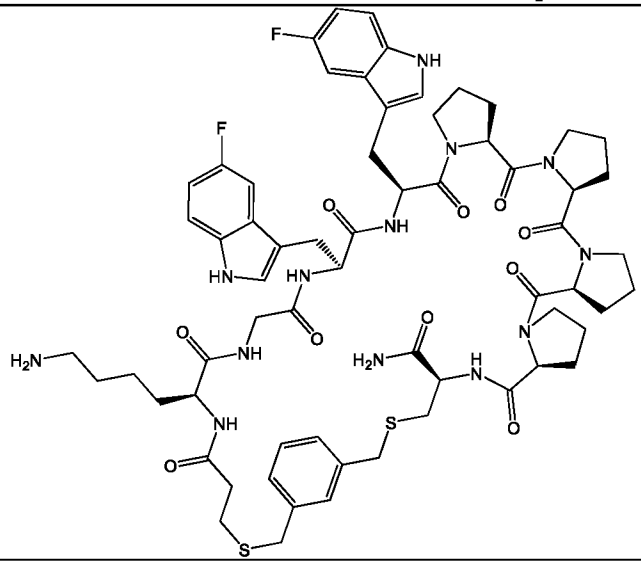
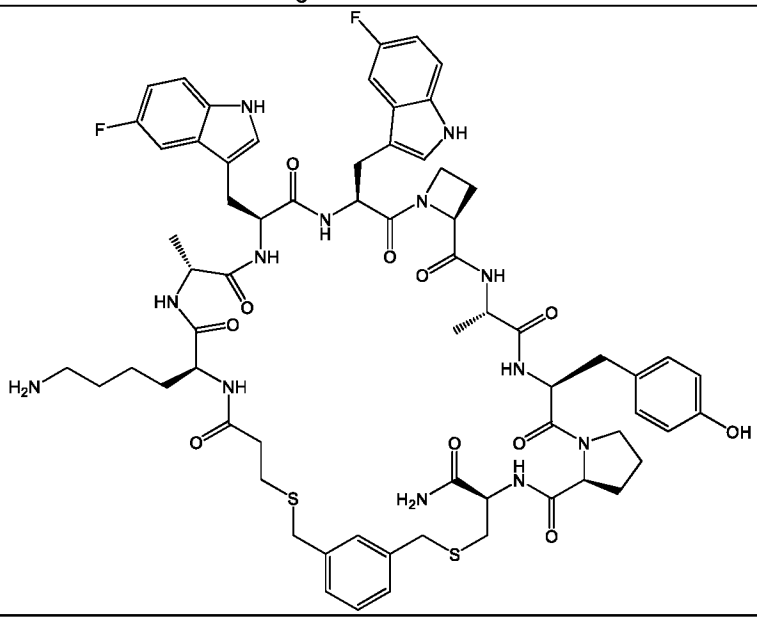
wherein each amino acid residue is optionally an N-methylated amino acid;

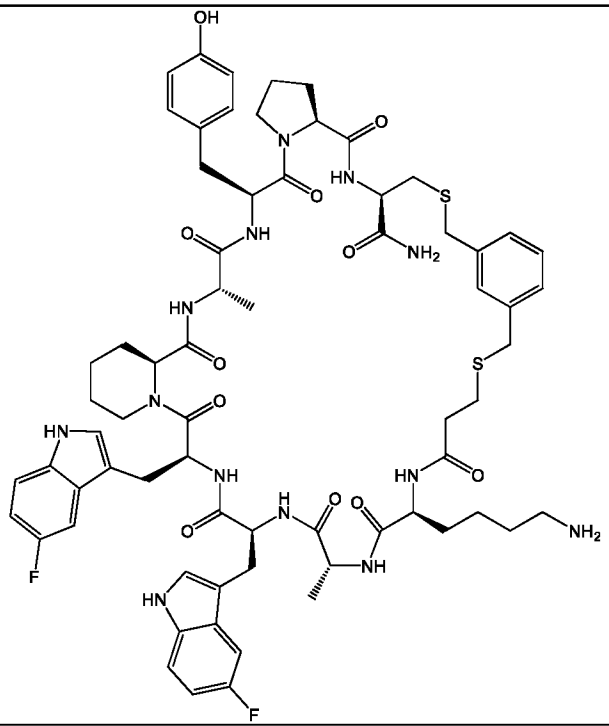
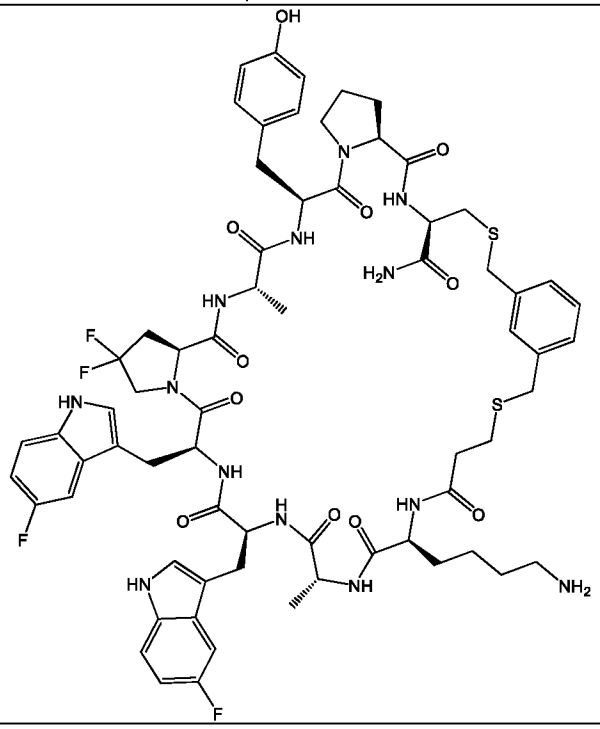
wherein each amino acid residue can be the R or S-enantiomer configuration.

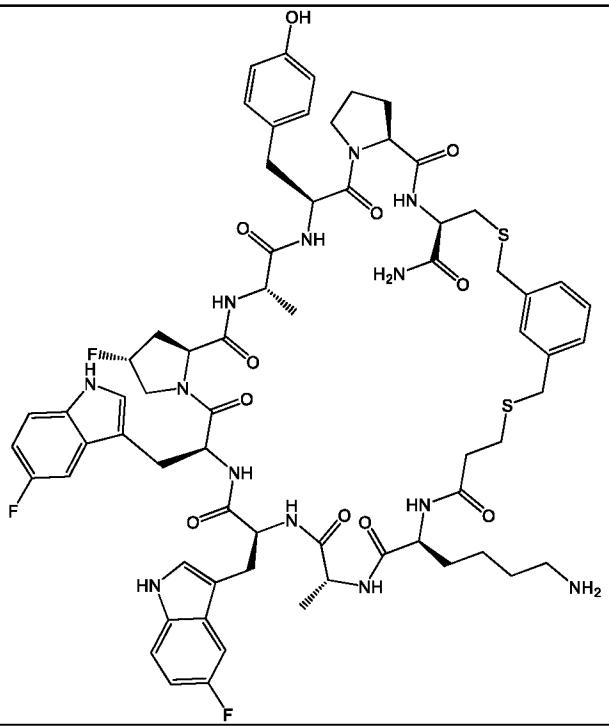
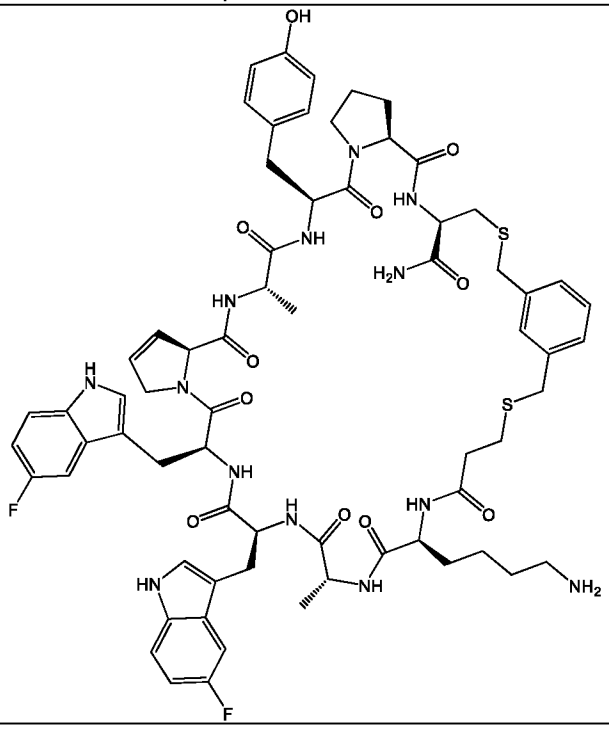
In an embodiment, the compound of Formula (I), or a pharmaceutically acceptable salt thereof, is selected from the following compounds of Table 1 below:

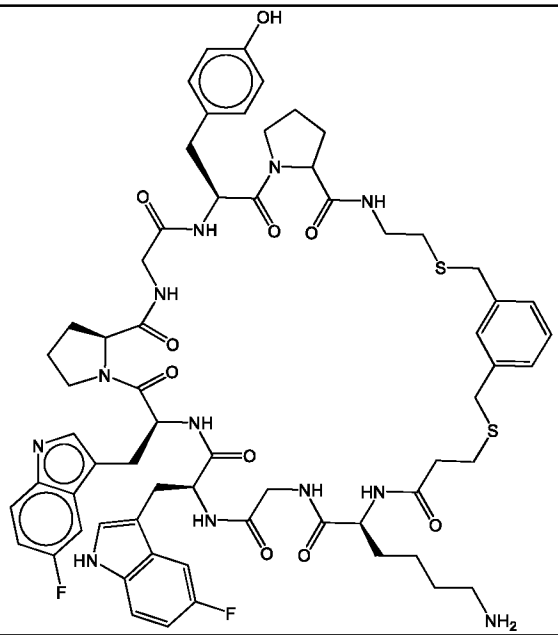
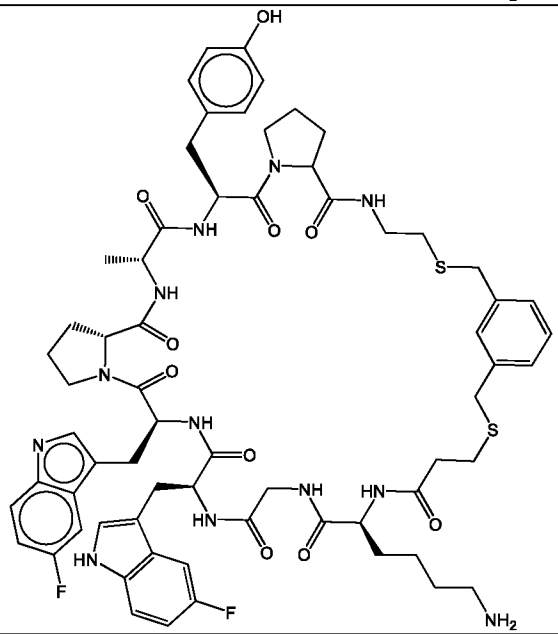
002	
029	

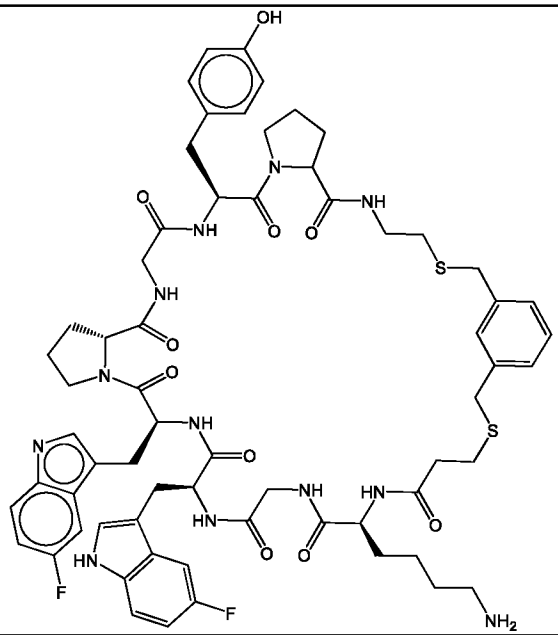
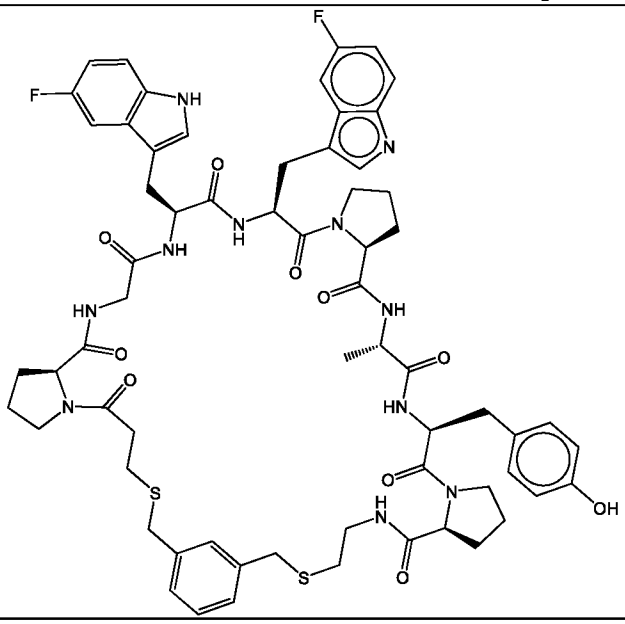
030	
031	

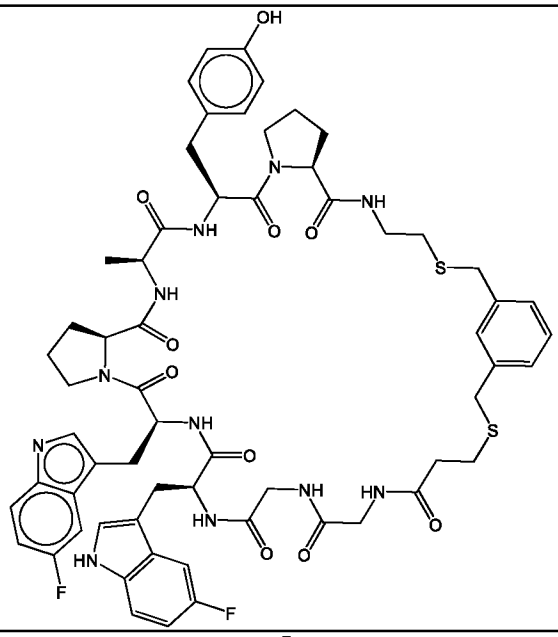
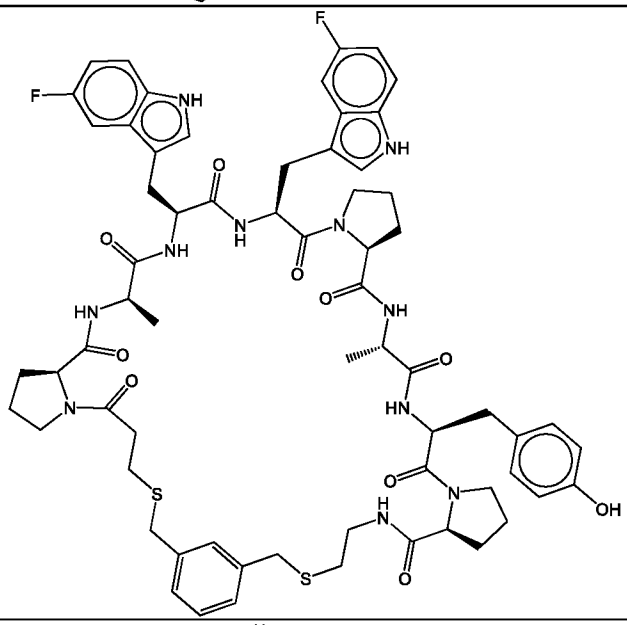
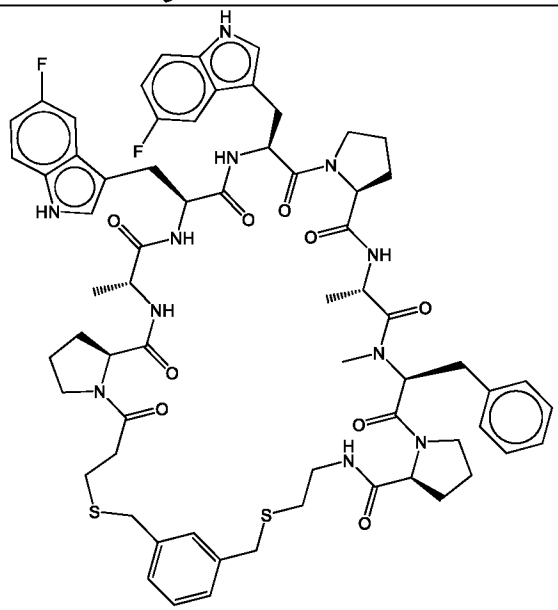
032	
033	
034	

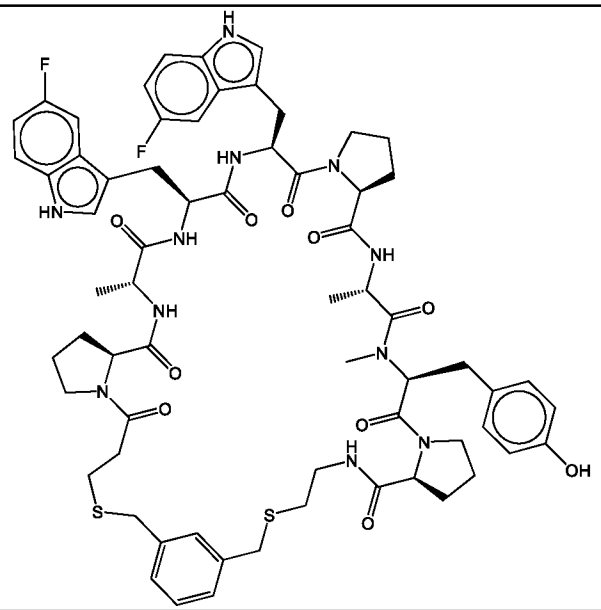
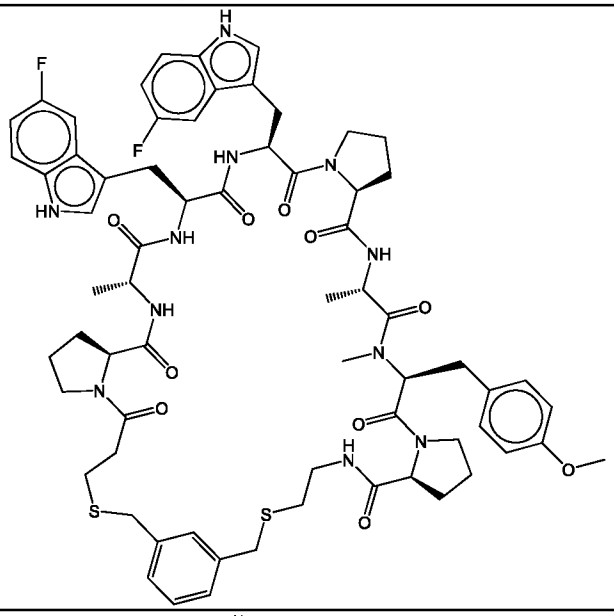
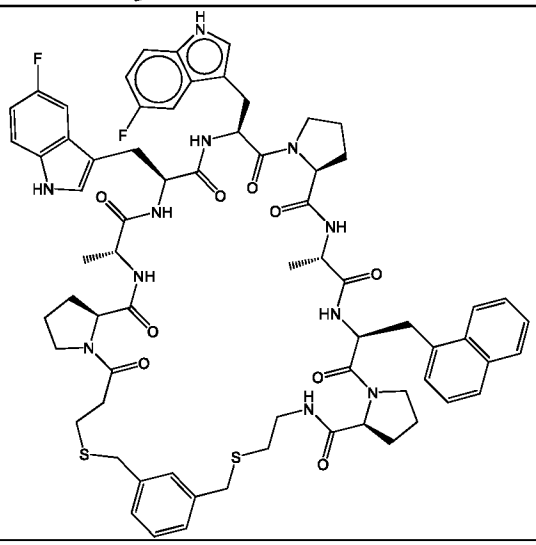
035	
036	

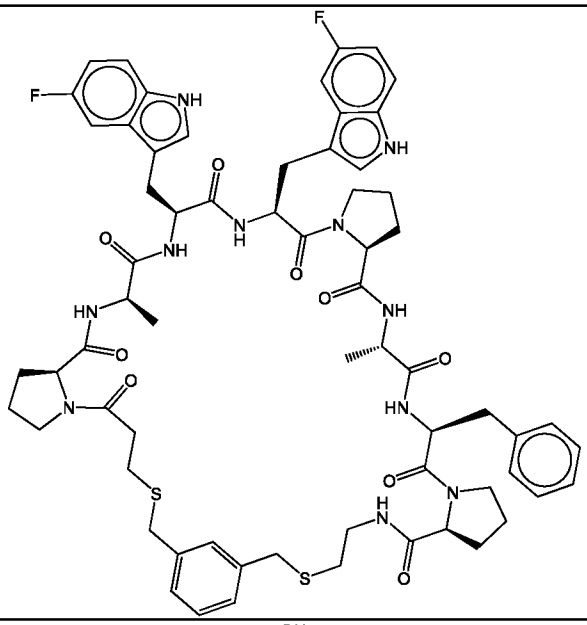
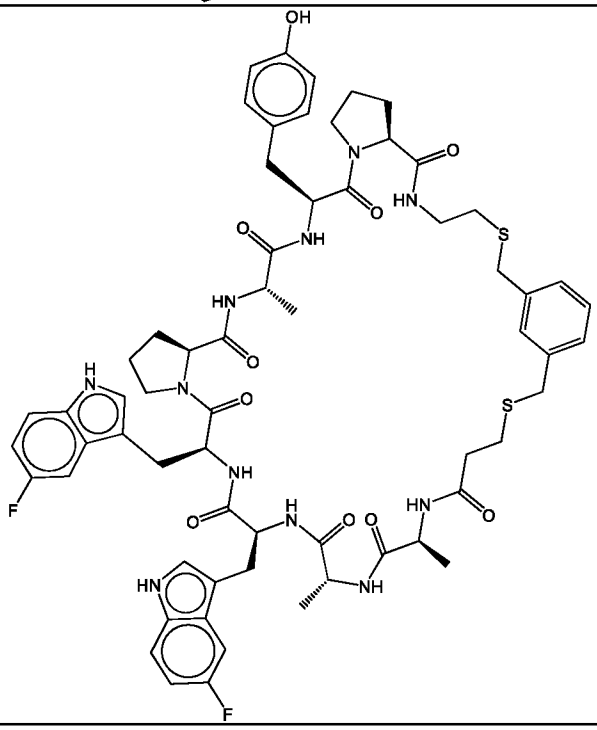
037	
038	

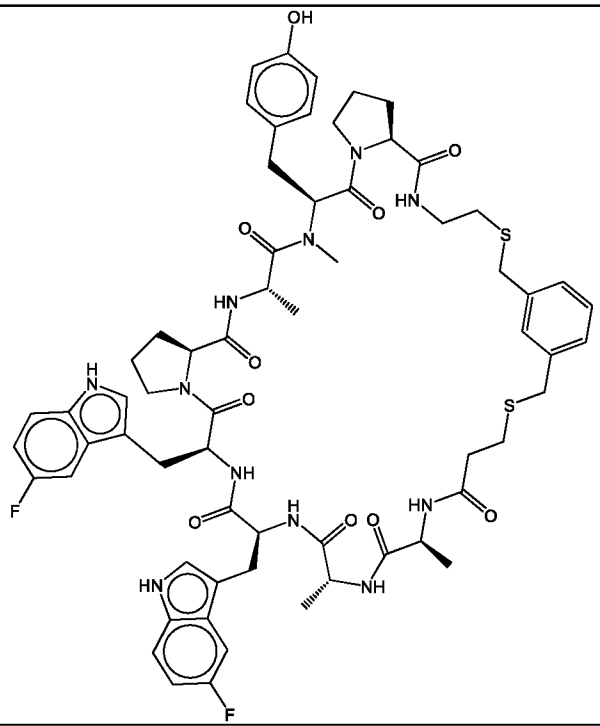
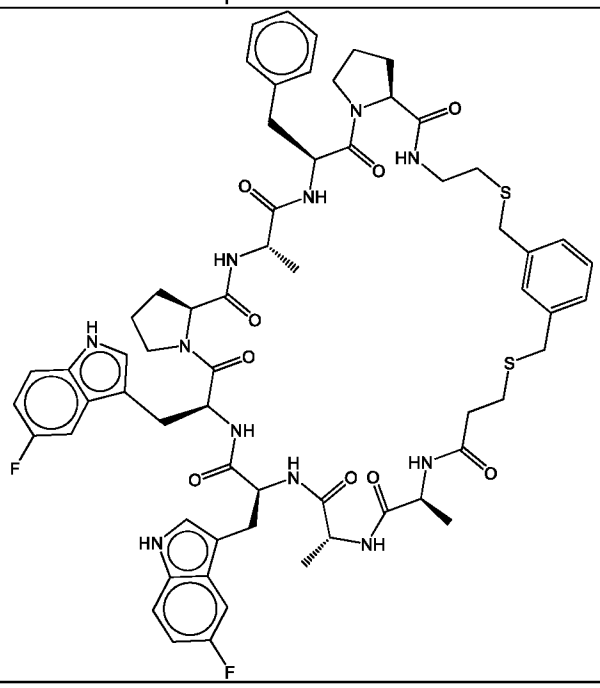
039	
040	

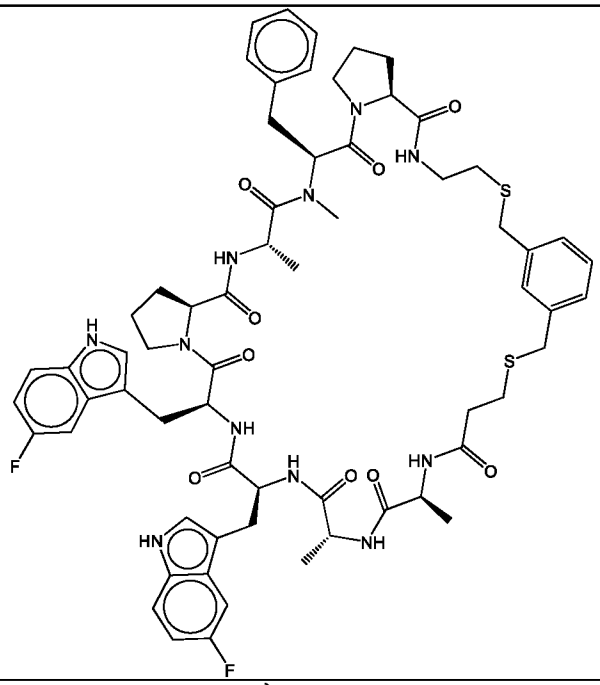
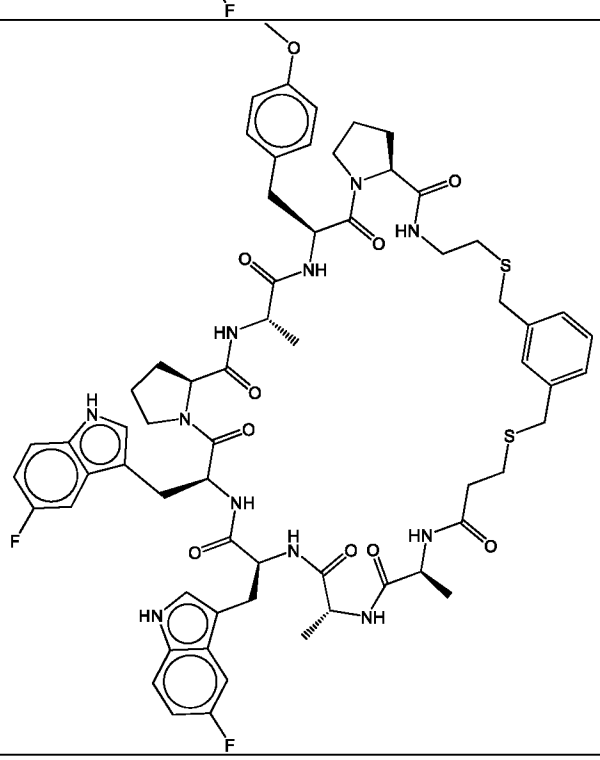
041	
047	

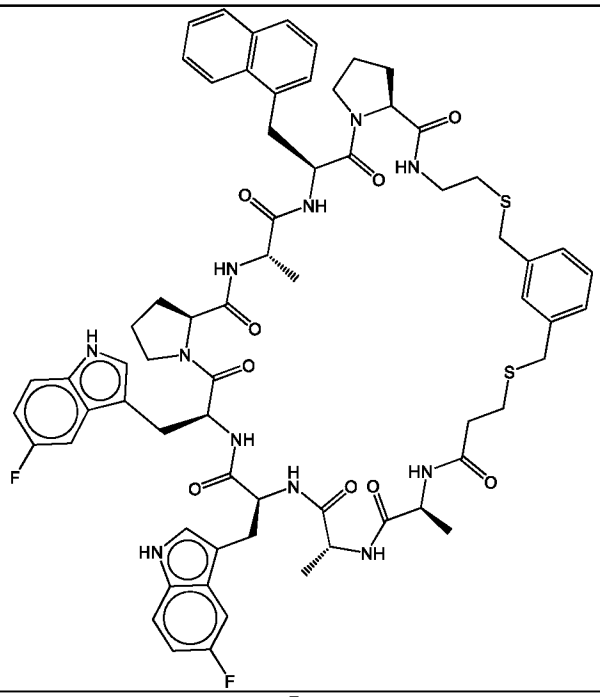
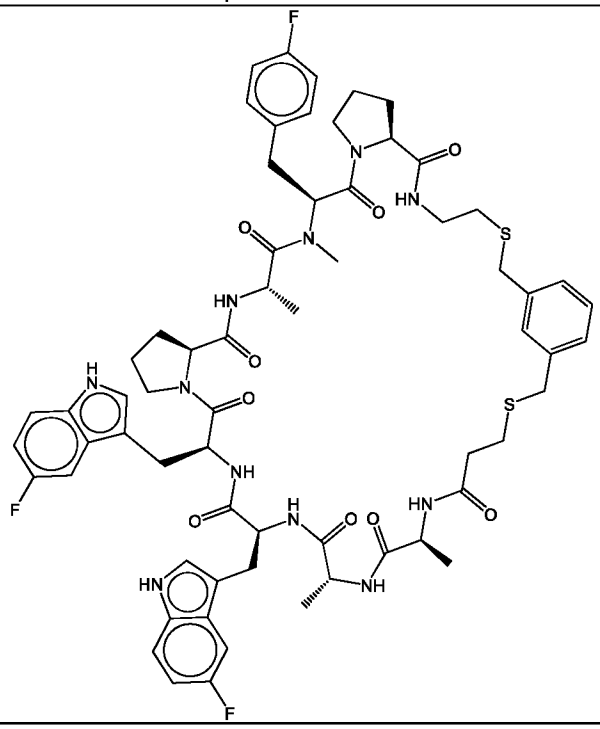
048	
050	
051	

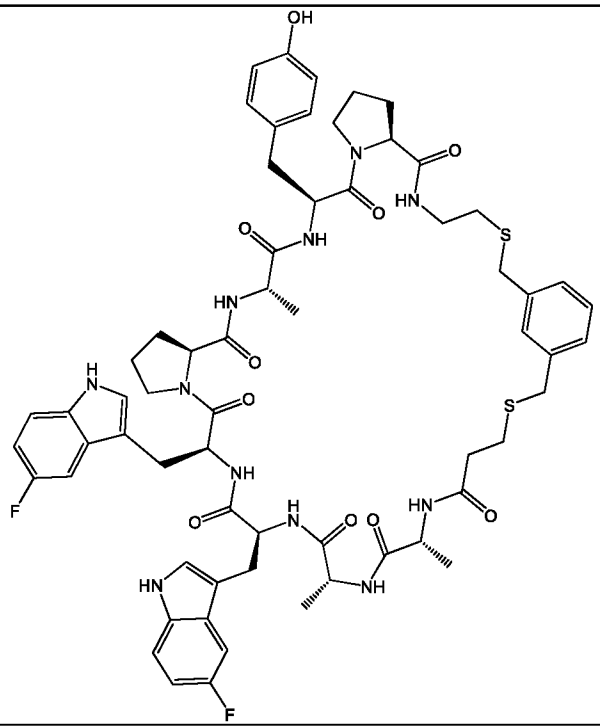
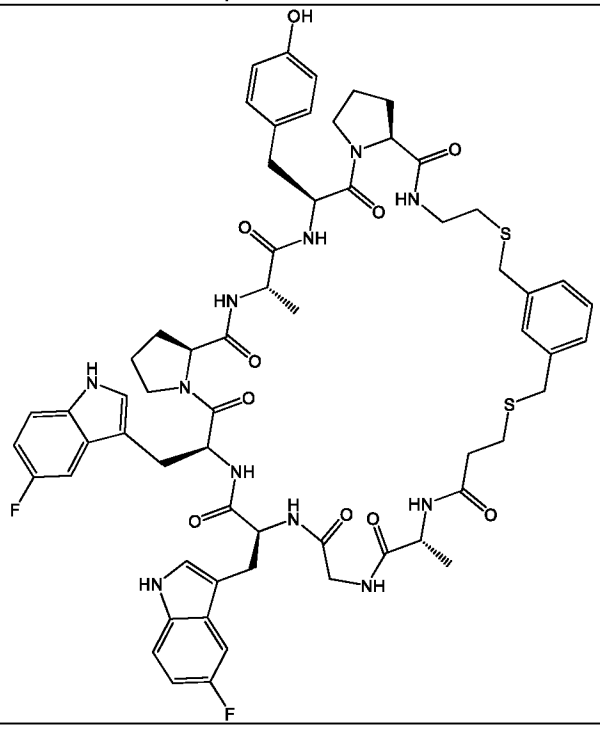
052	
053	
054	

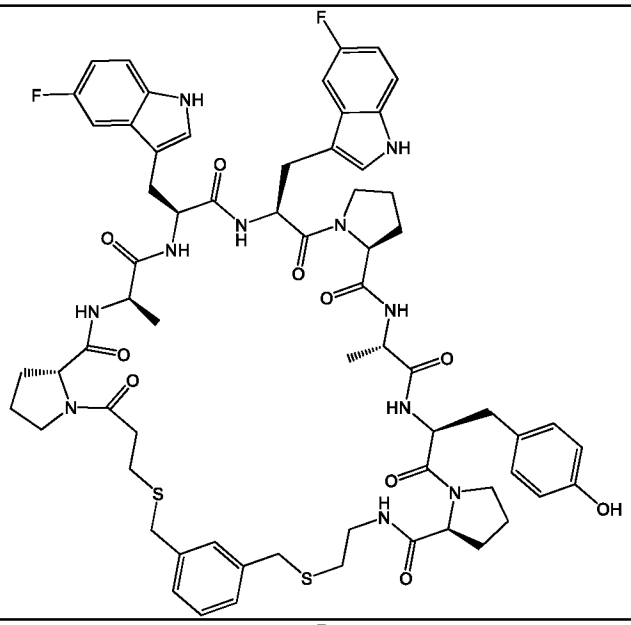
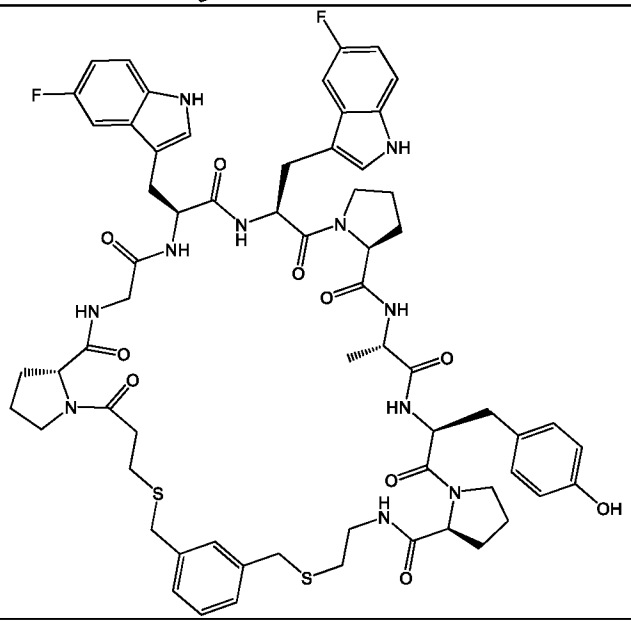
057	
058	

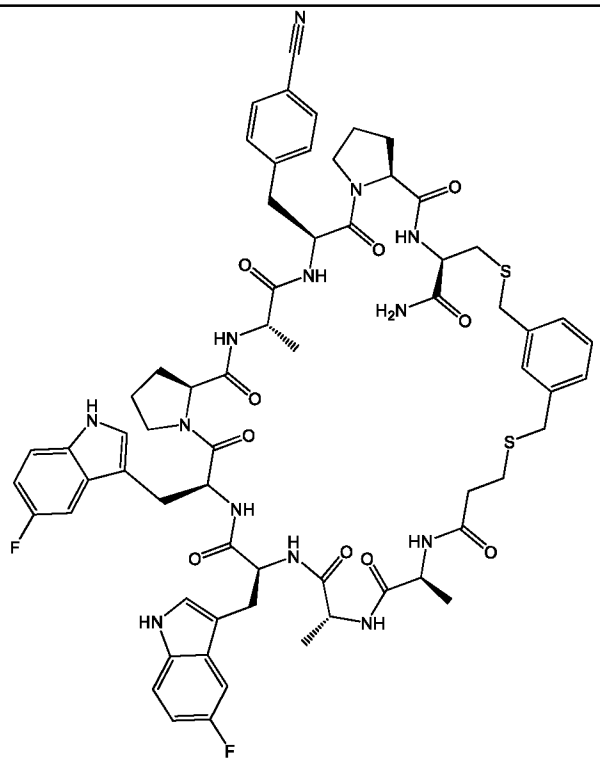
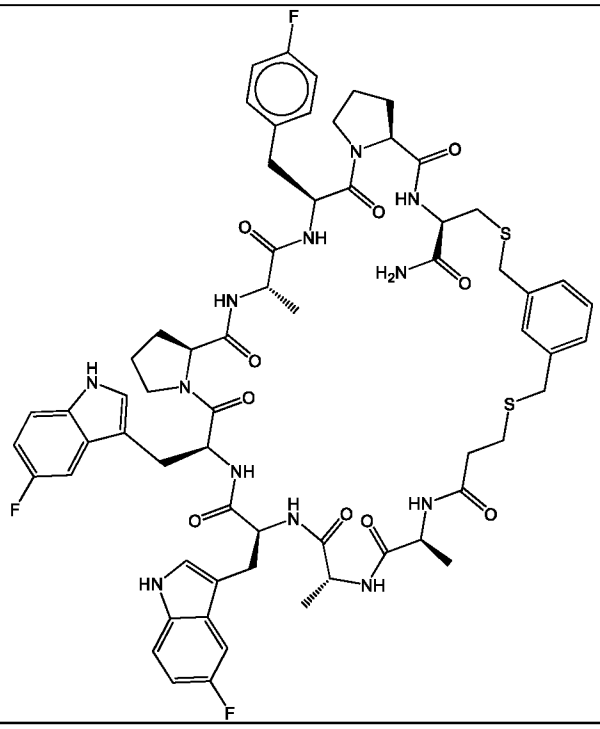
061	
062	

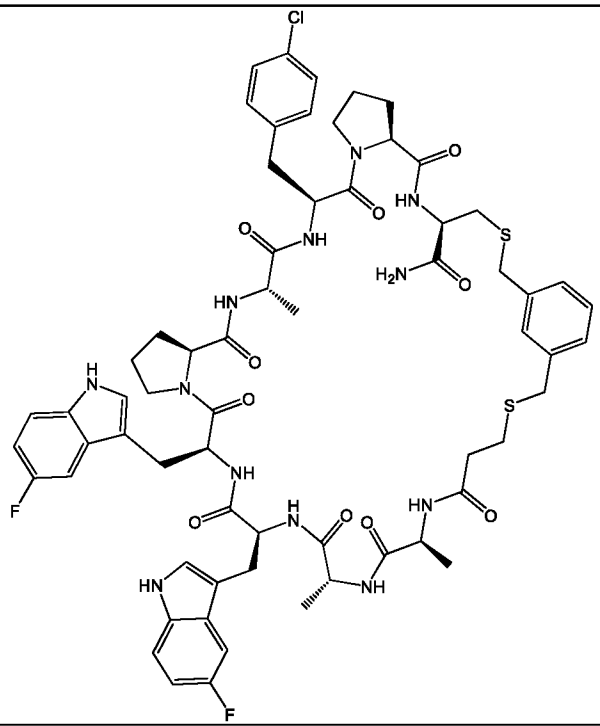
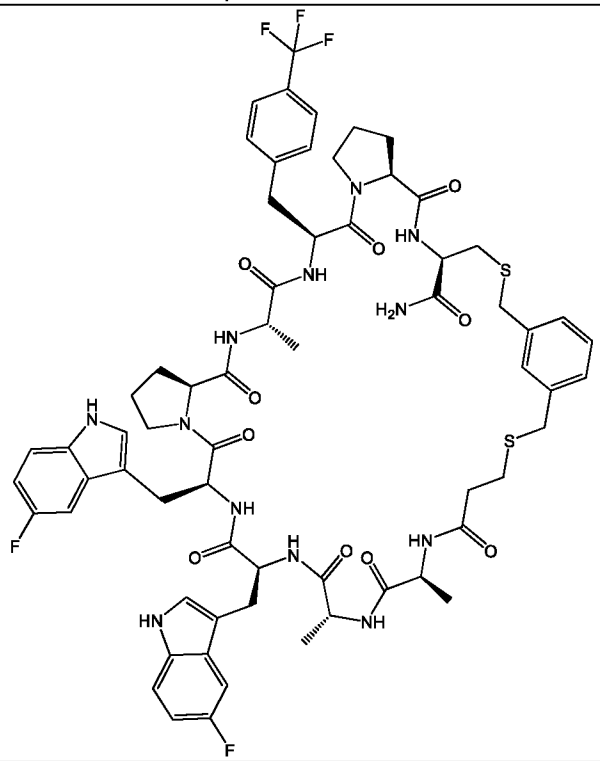
063	
064	

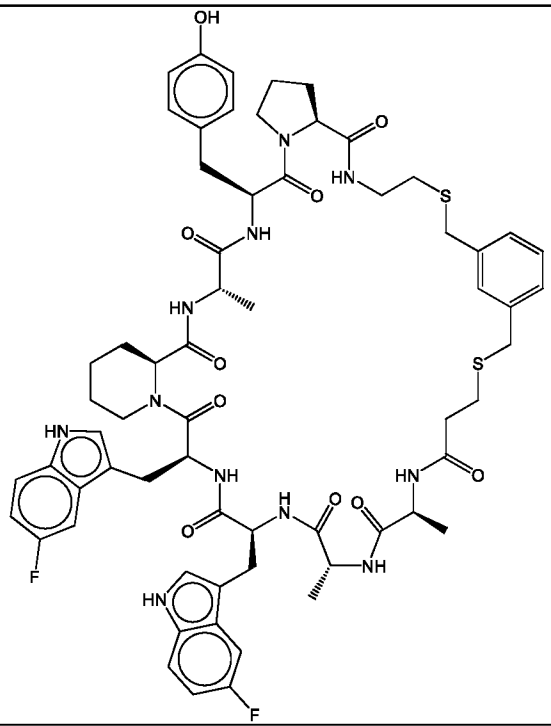
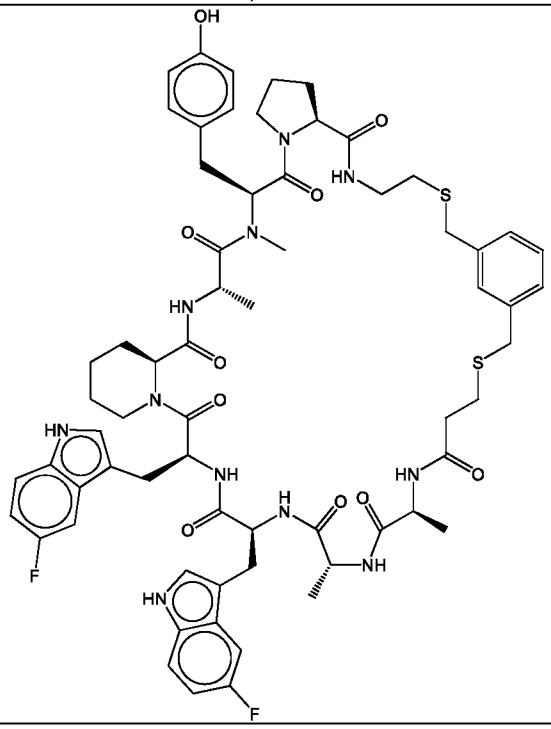
065	
066	

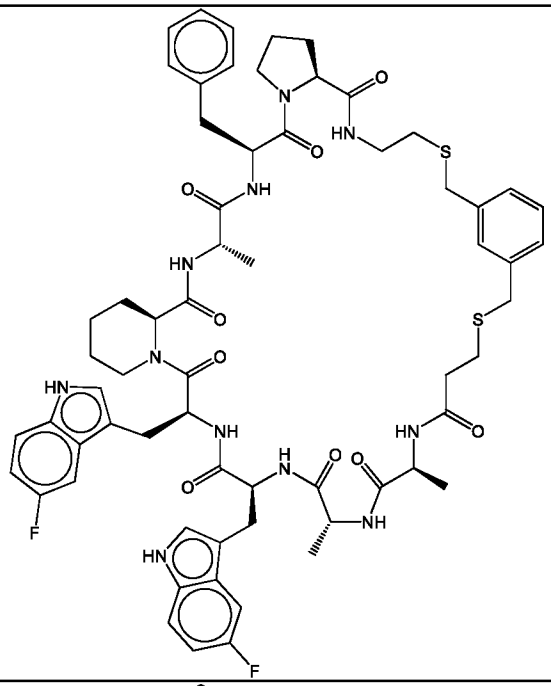
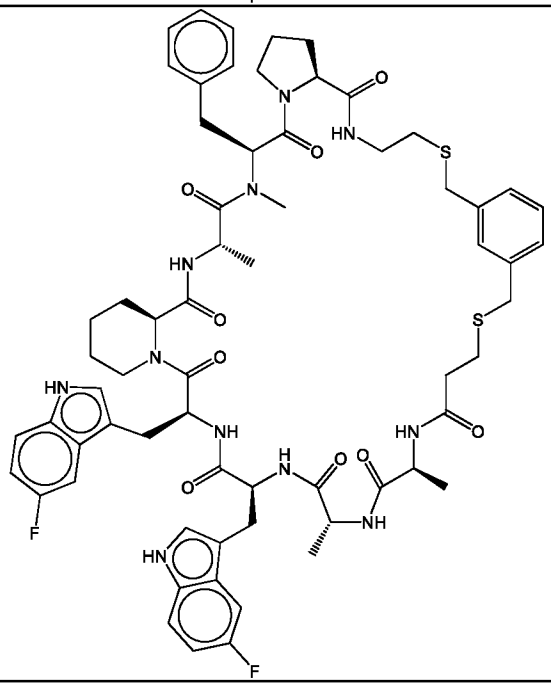
068	
069	

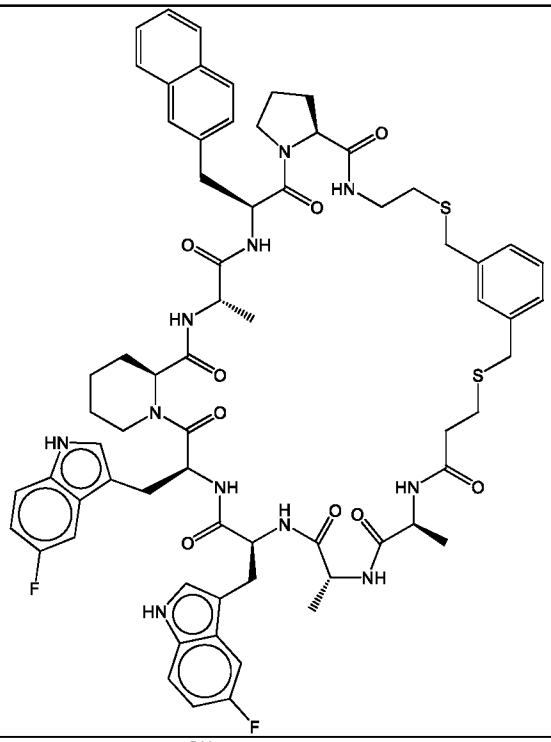
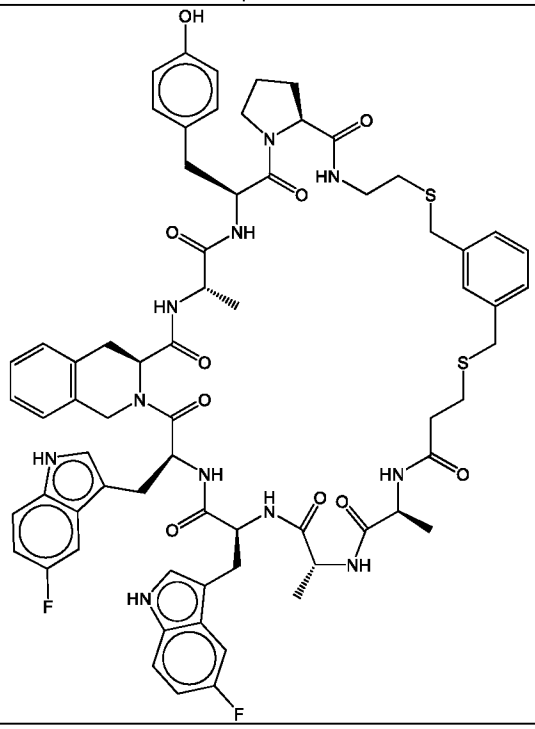
070	
071	

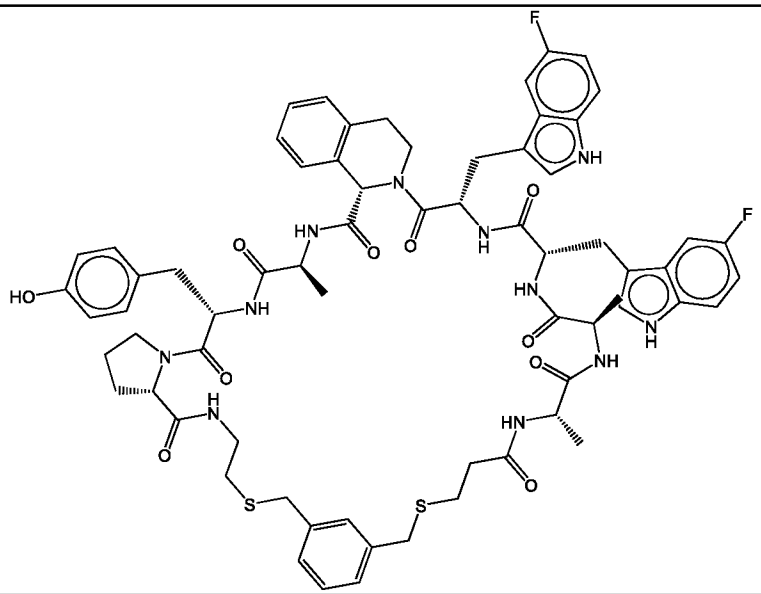
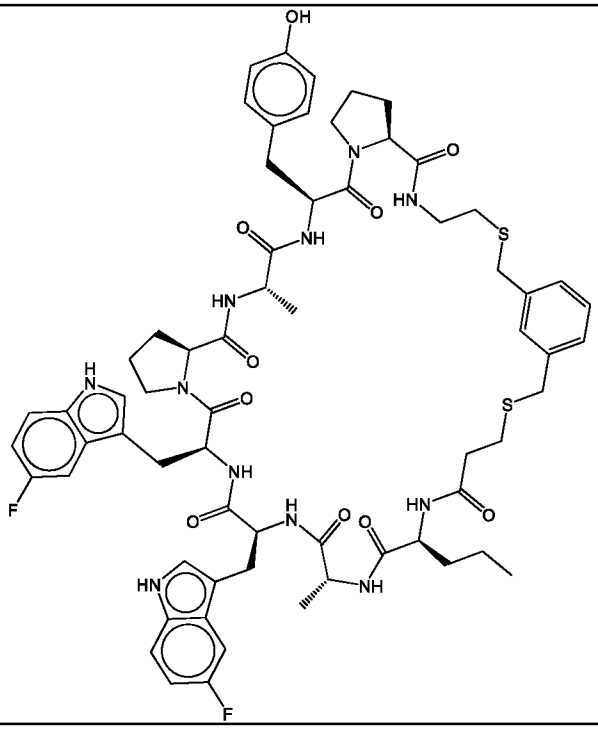
081	
082	

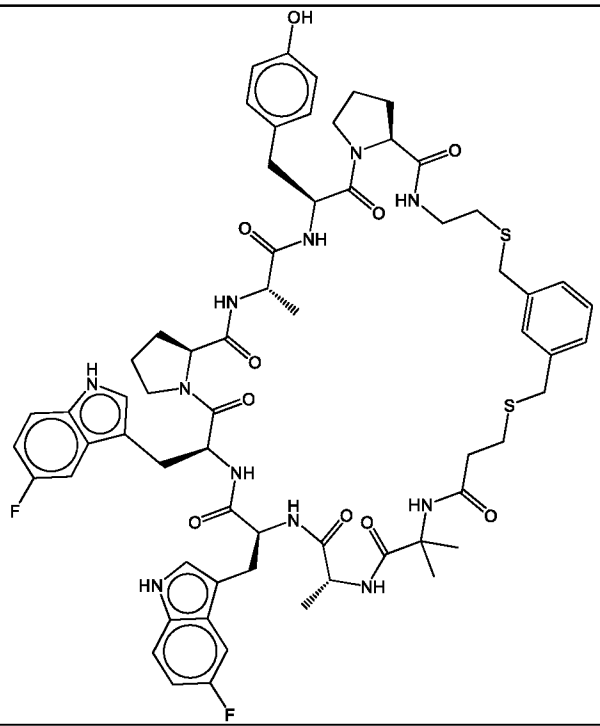
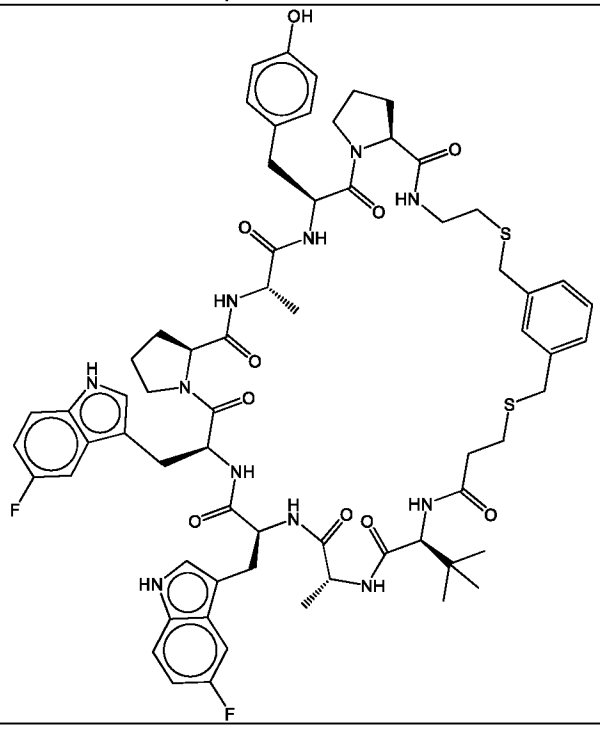
083	
084	

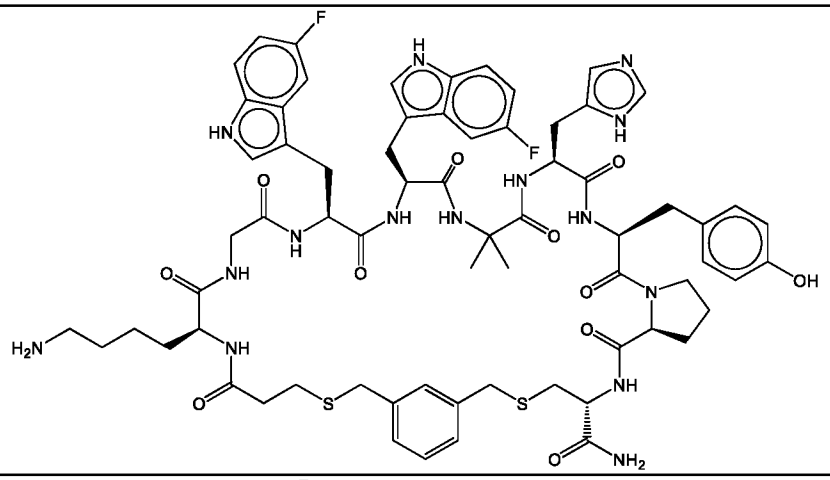
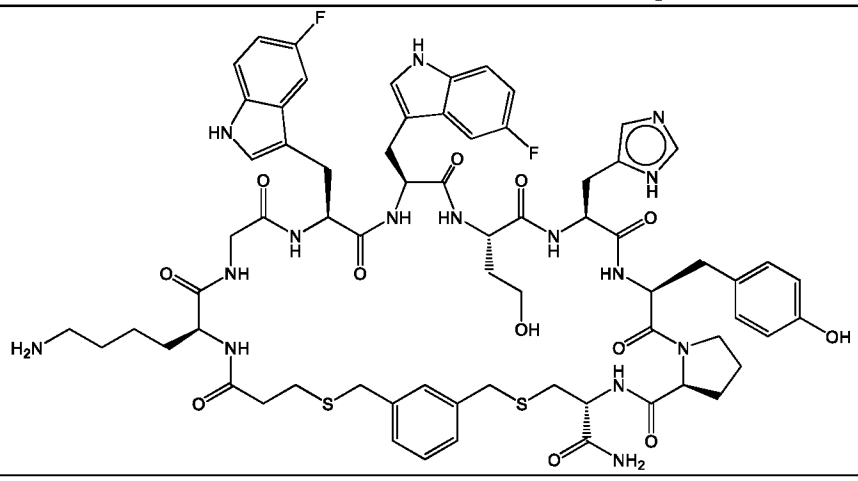
085	
086	

087	
088	

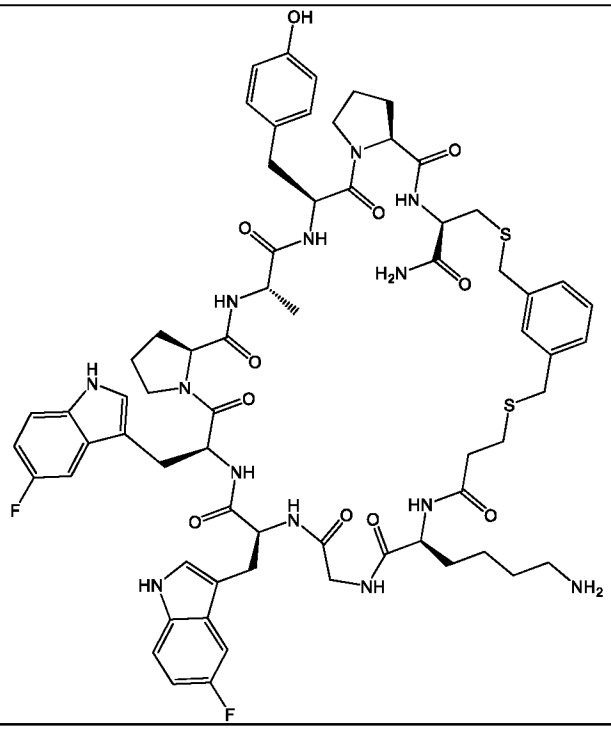
089	
091	

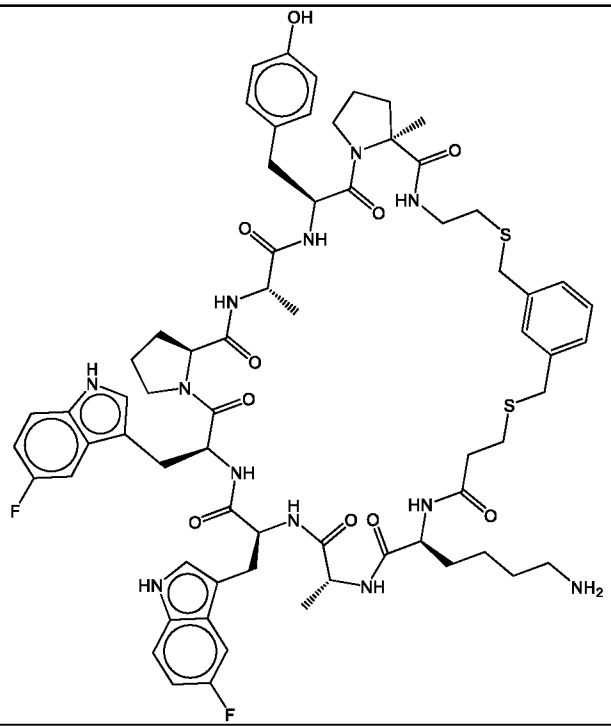
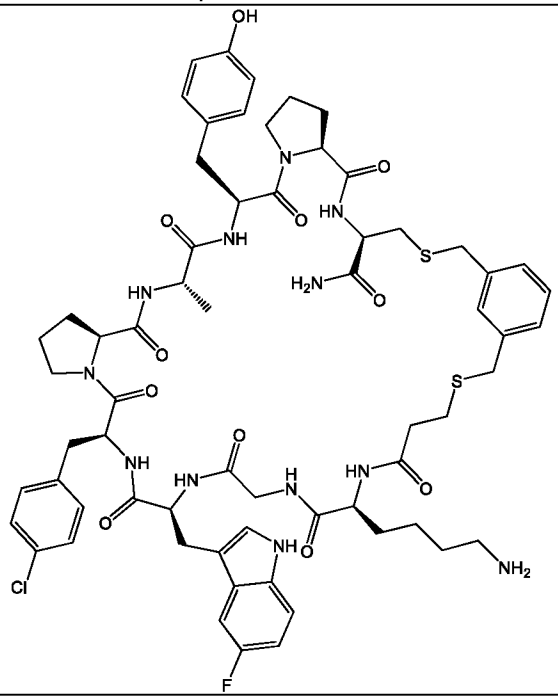
092	
093	

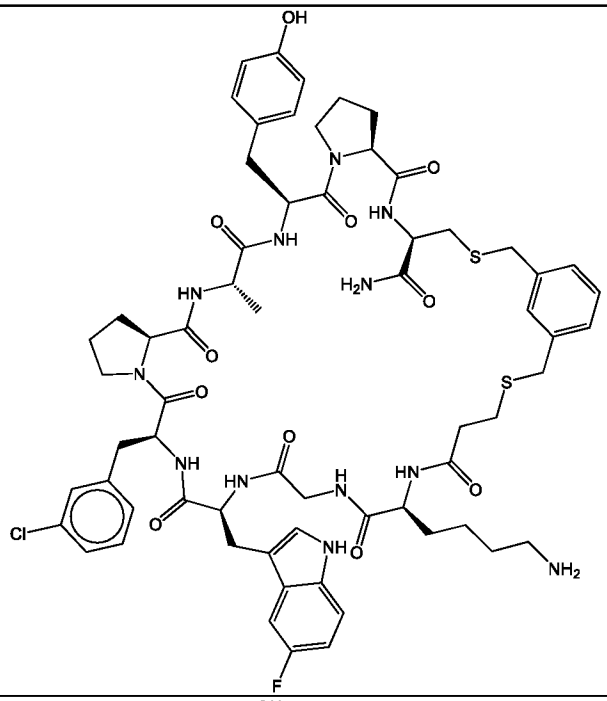
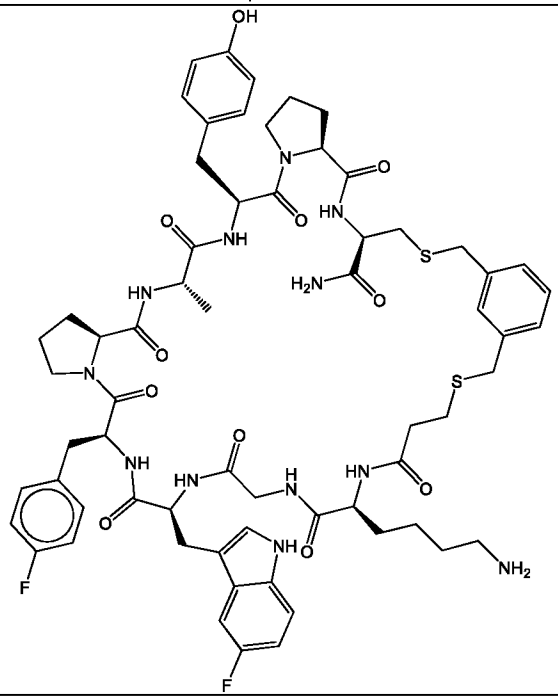
094	
095	

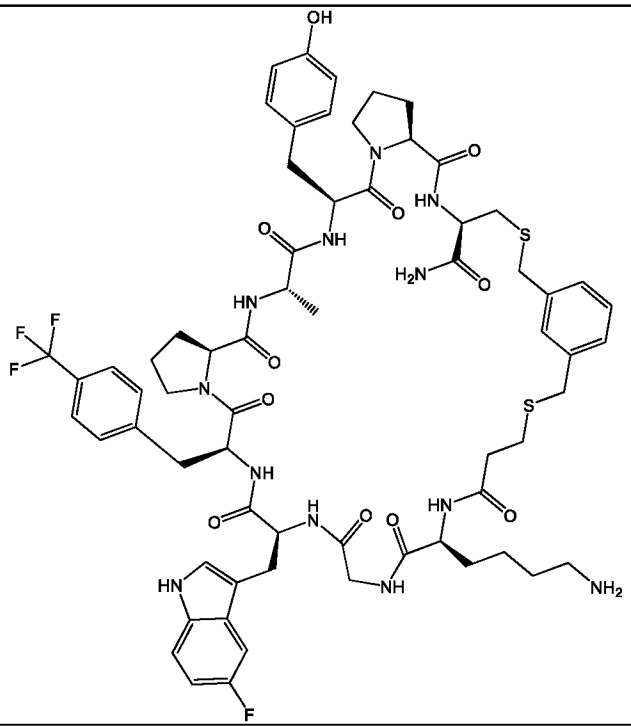
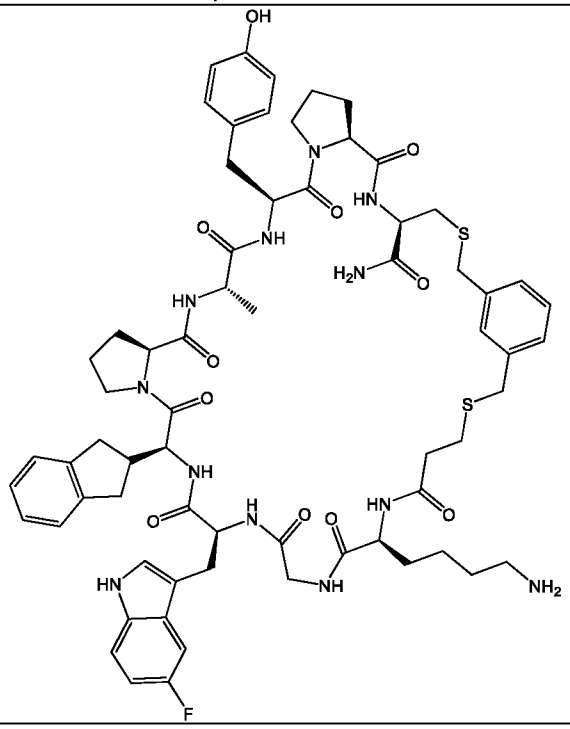
103	
117	

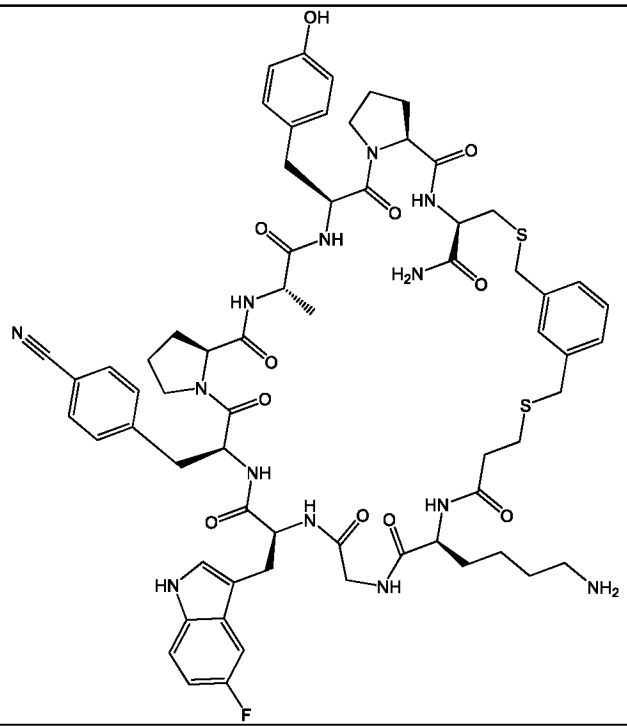
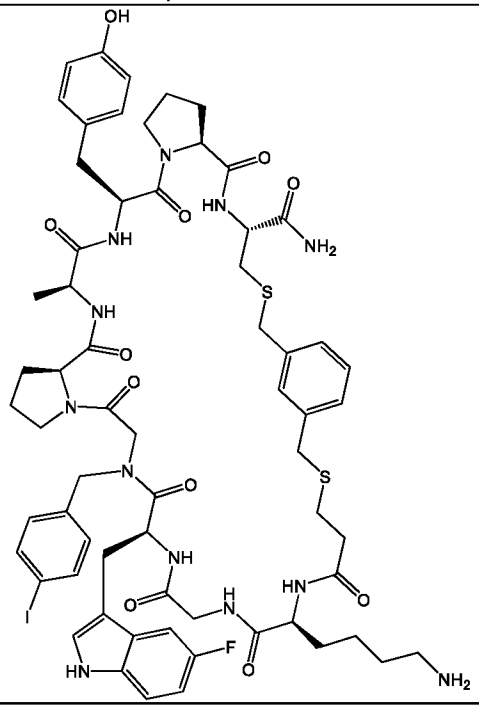
In another aspect, the compounds of the invention, or pharmaceutically acceptable salts thereof, are selected from the following compounds of Table 2 below:

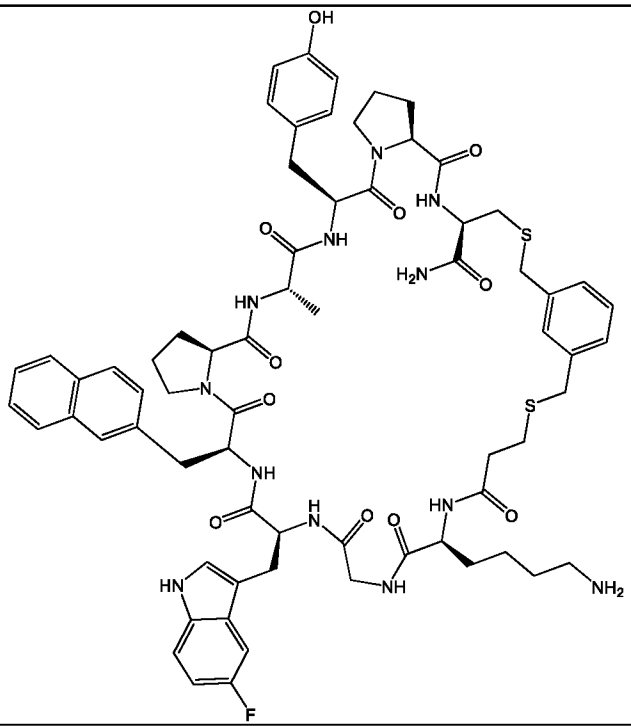
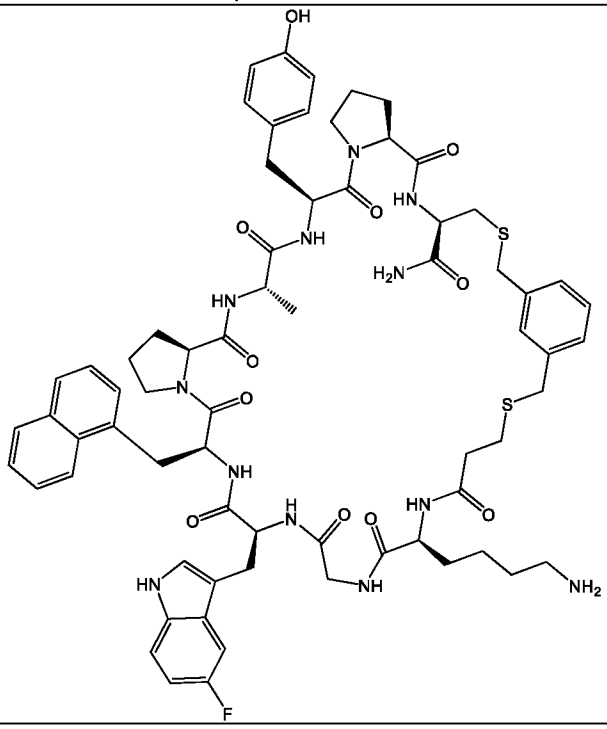
001	

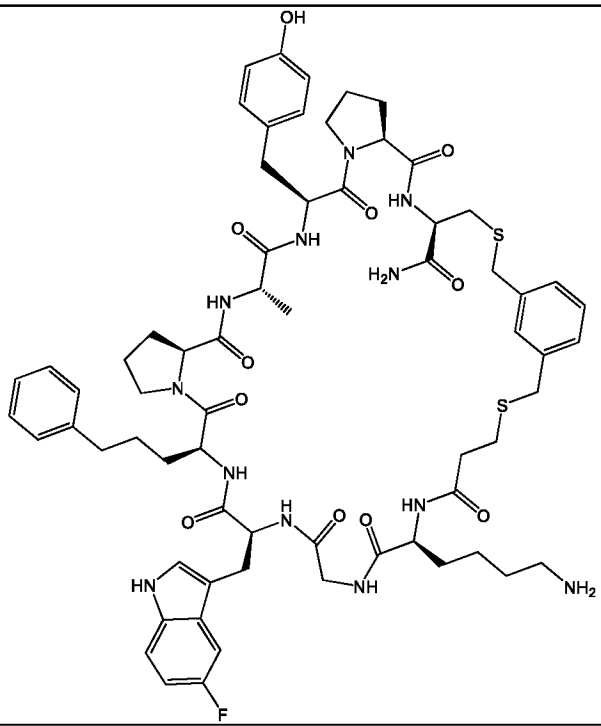
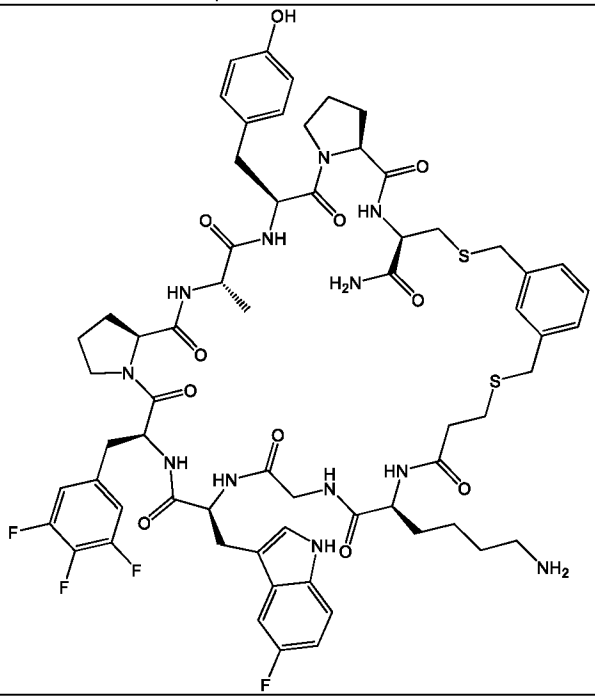
003	
004	

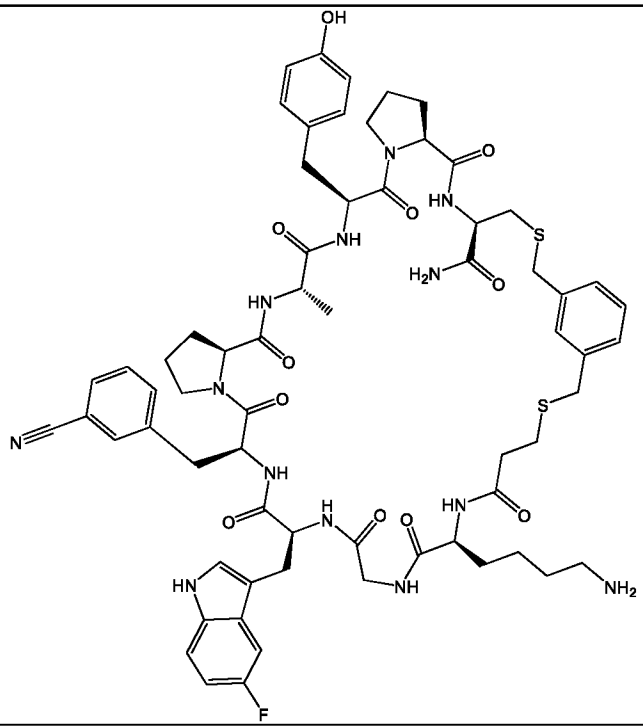
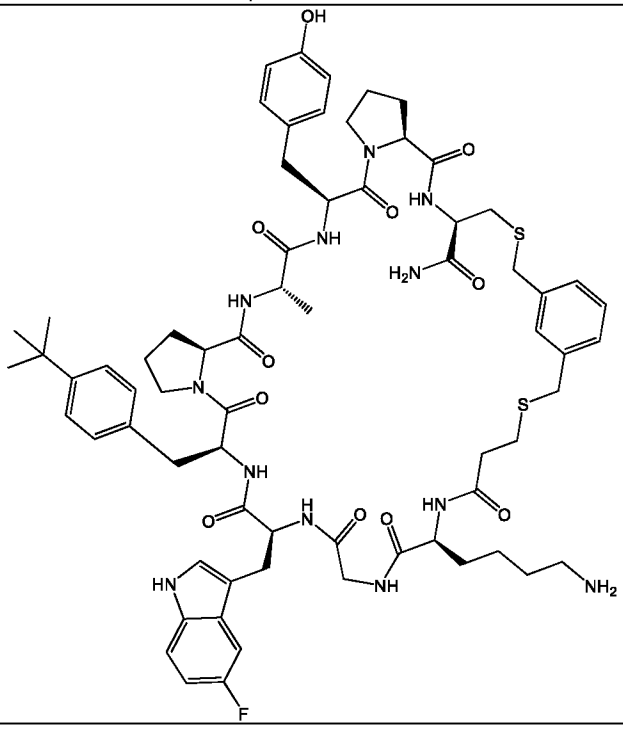
005	
006	

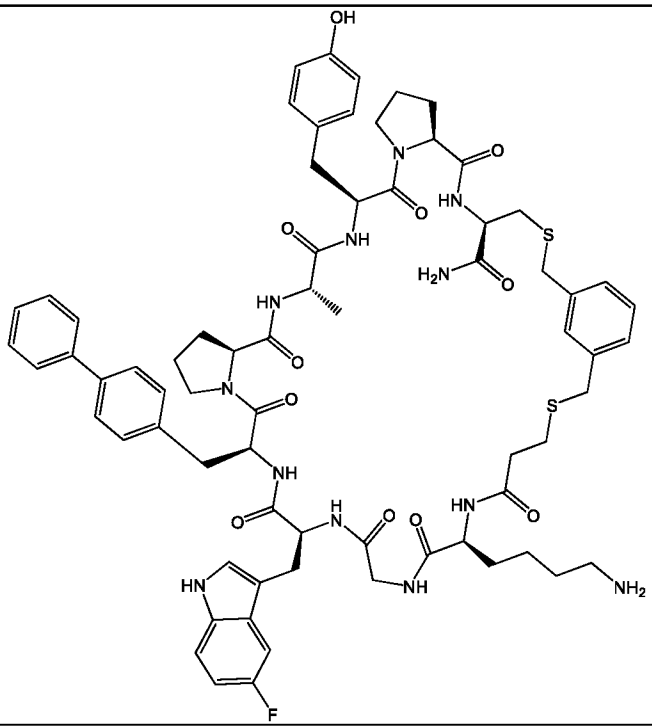
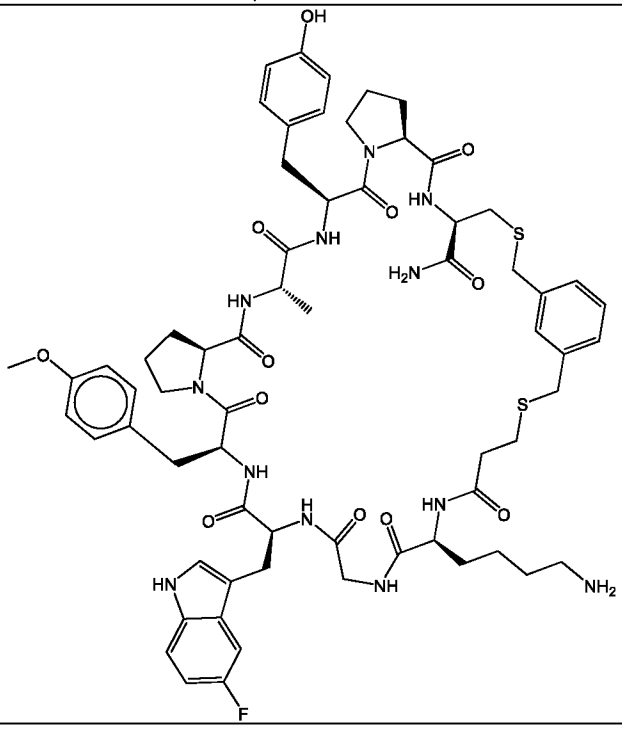
007	
008	

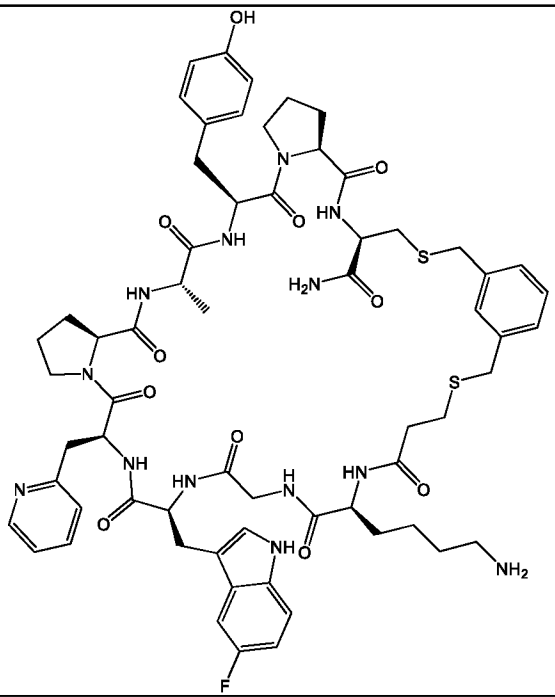
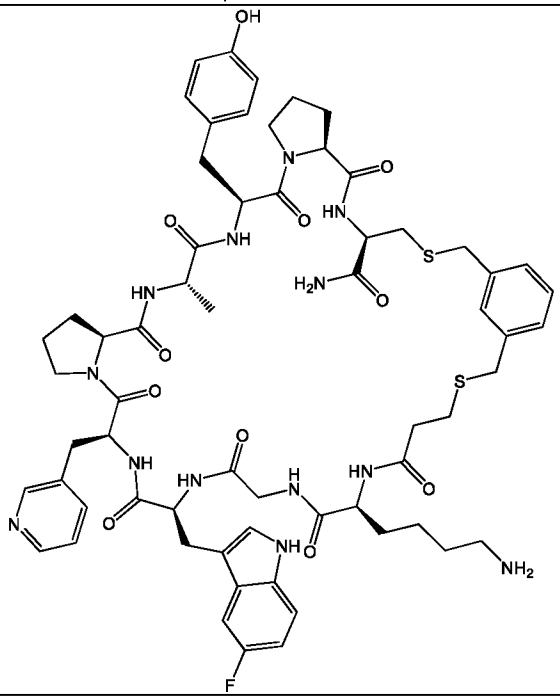
009	
010	

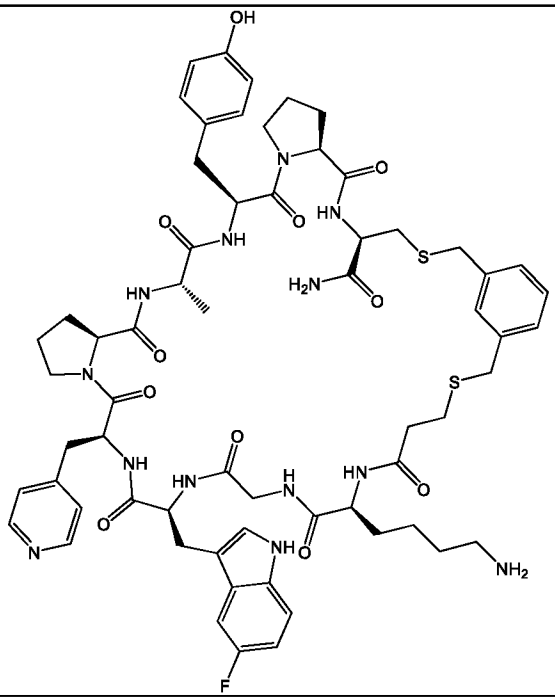
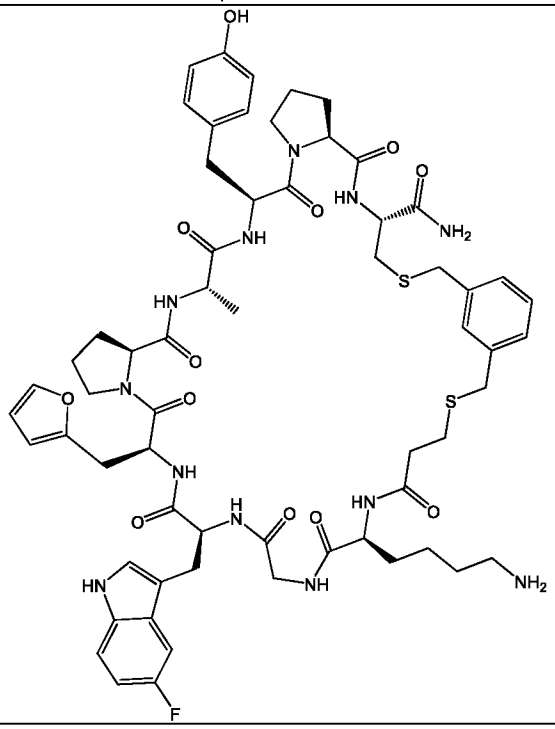
011	
012	

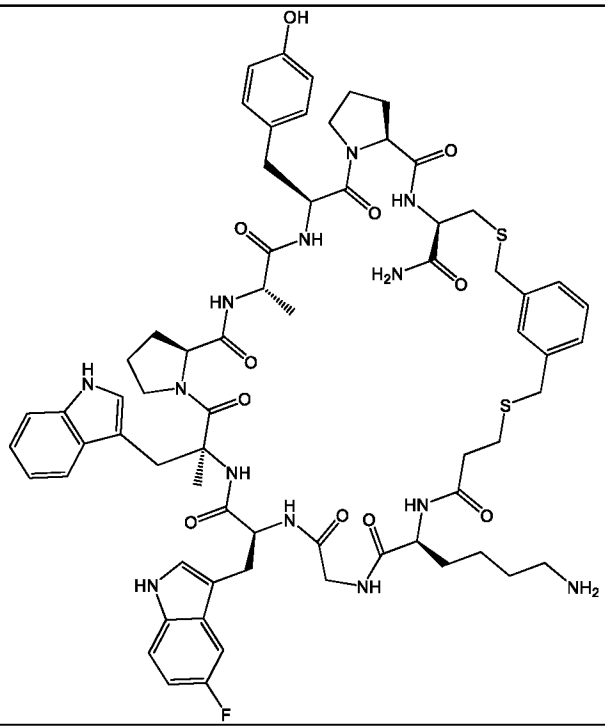
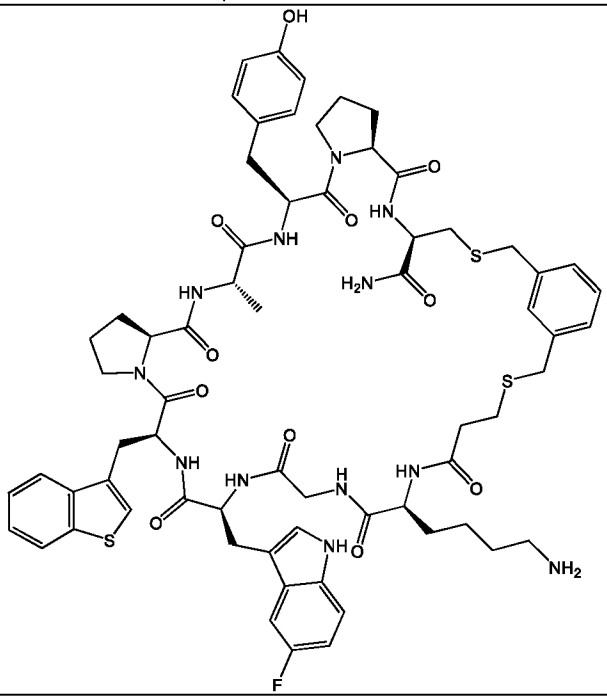
013	
014	

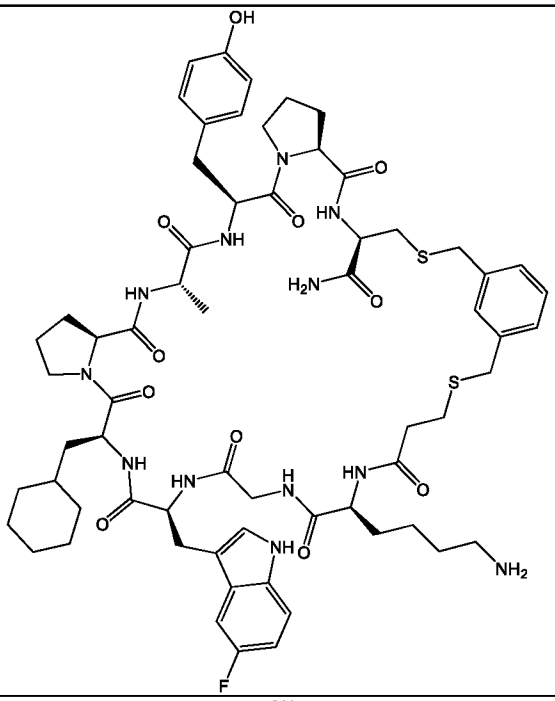
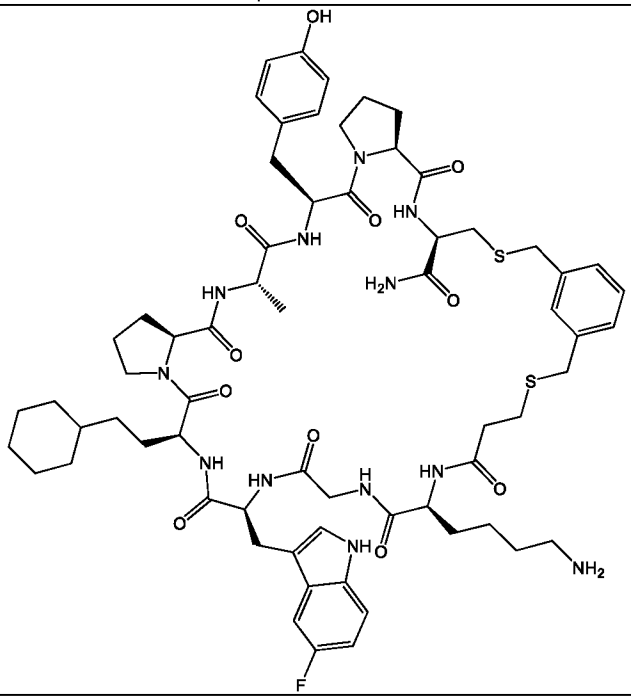
015	
016	

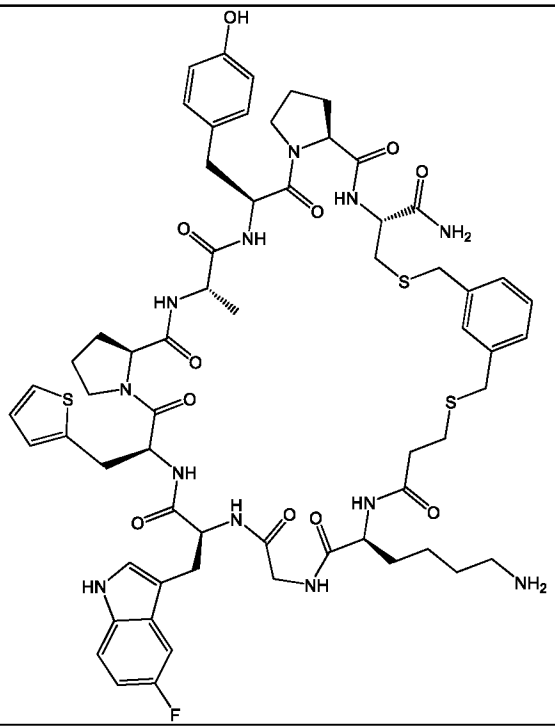
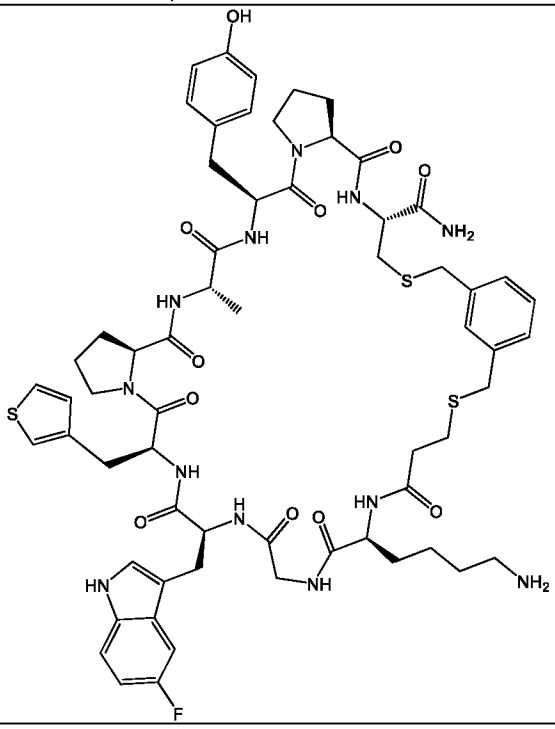
017	
018	

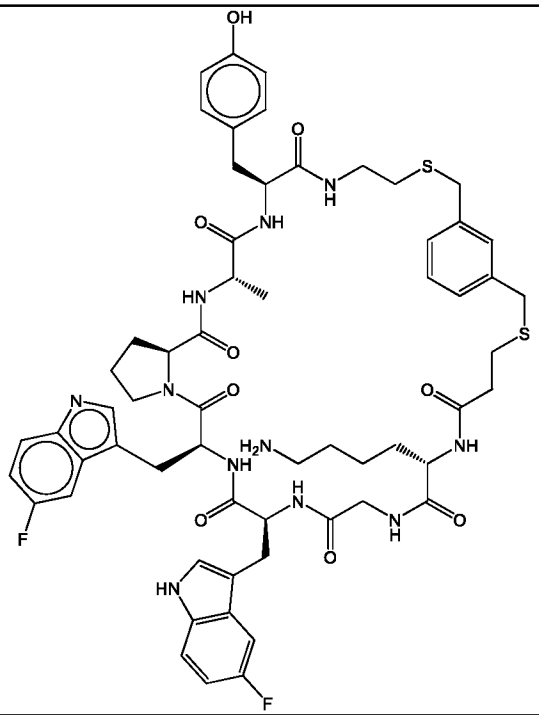
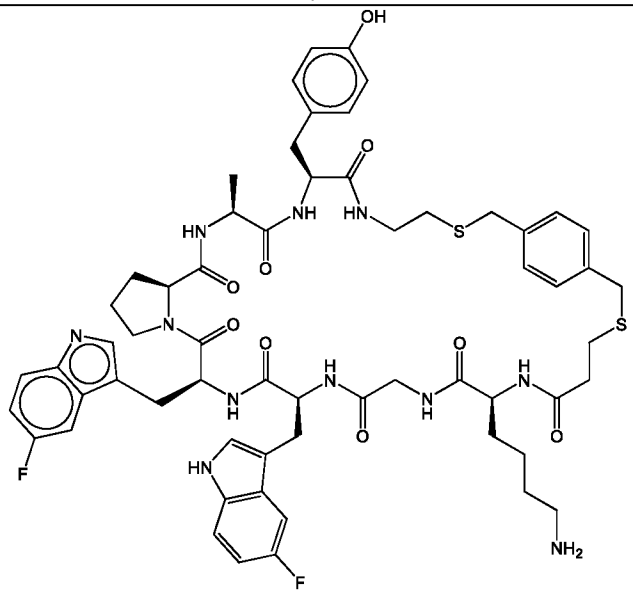
019	
020	

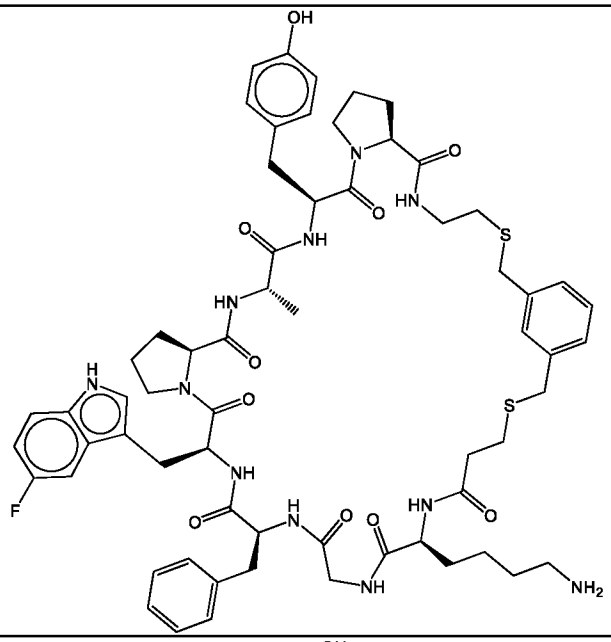
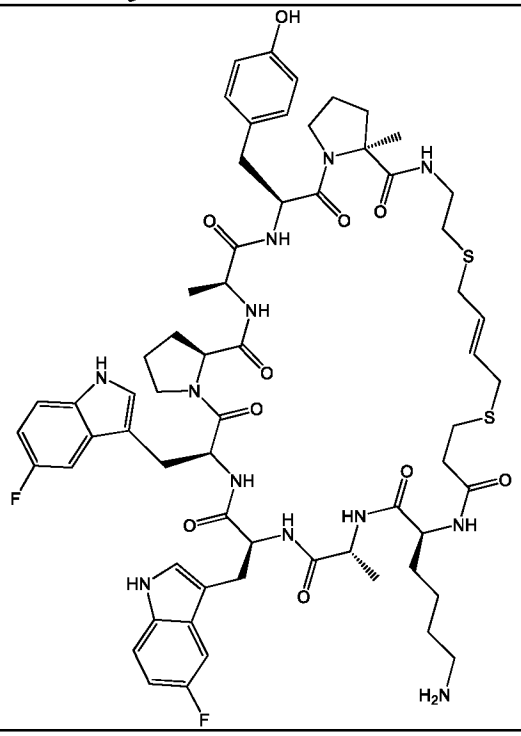
021	
022	

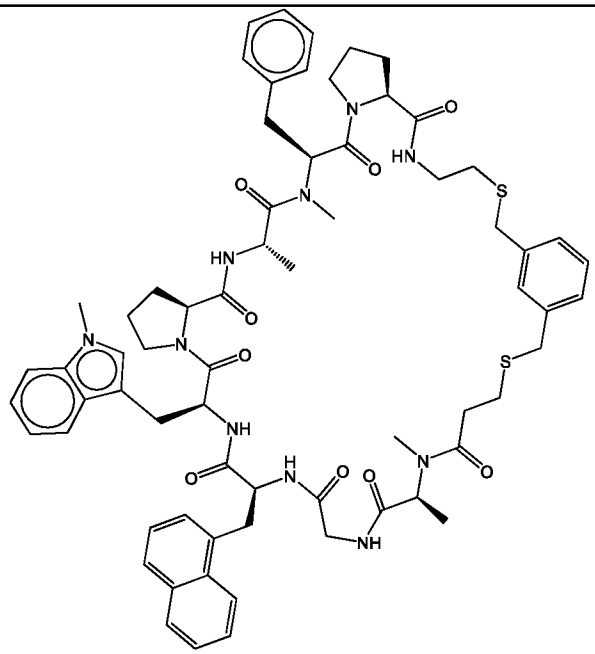
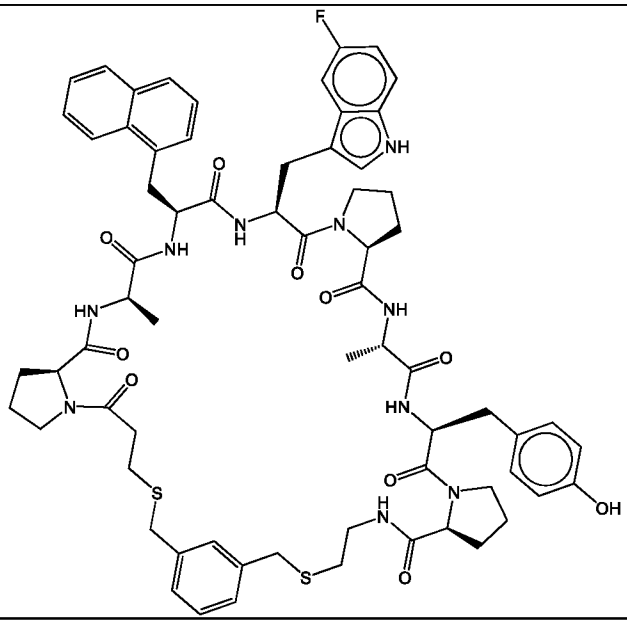
023	
024	

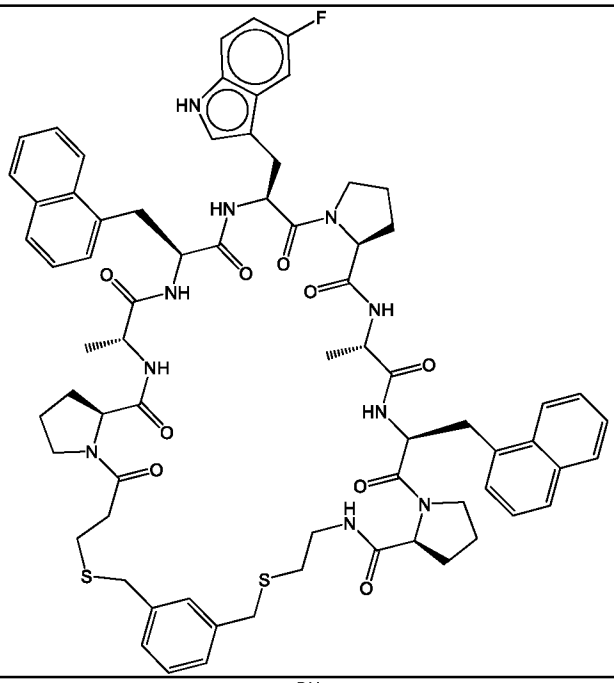
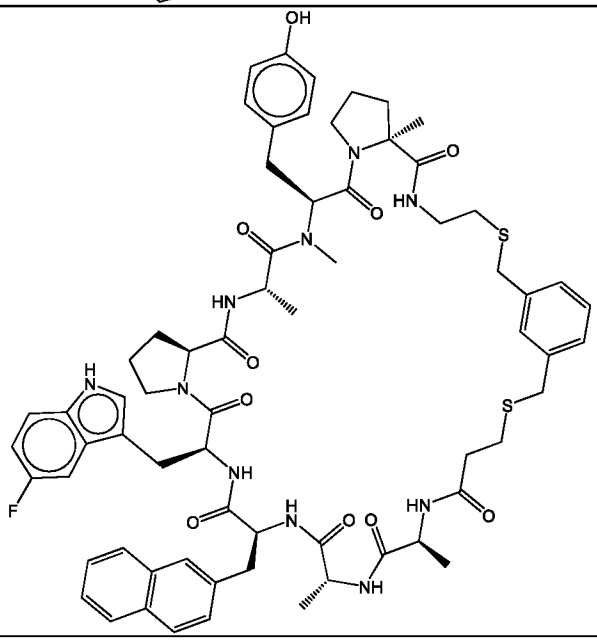
025	
026	

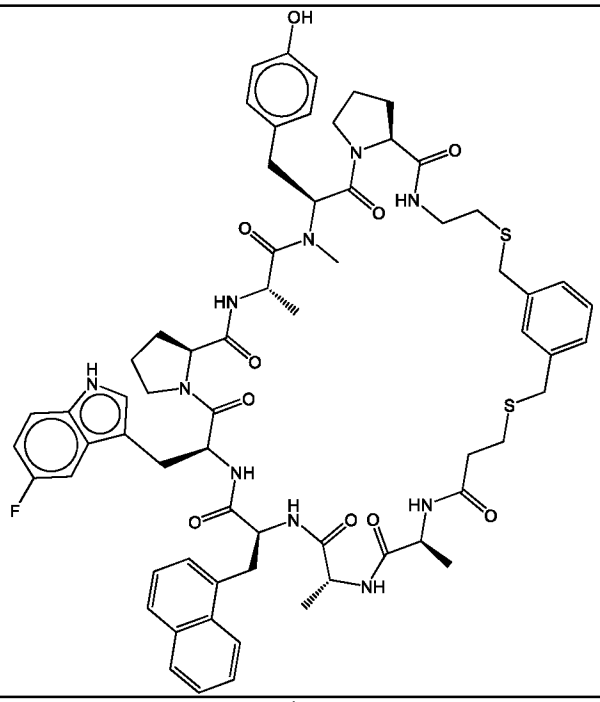
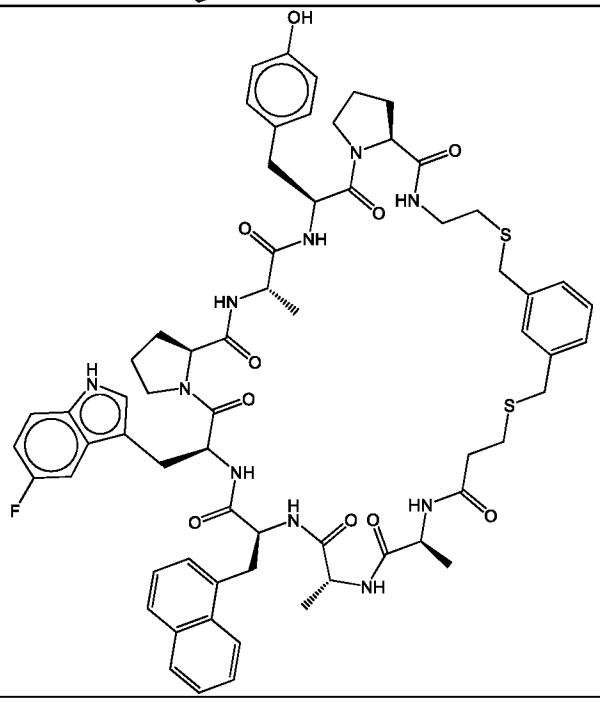
027	
028	

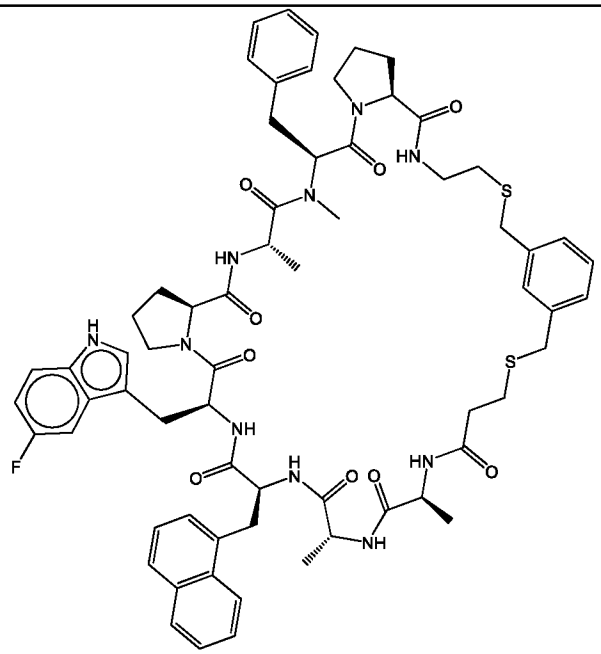
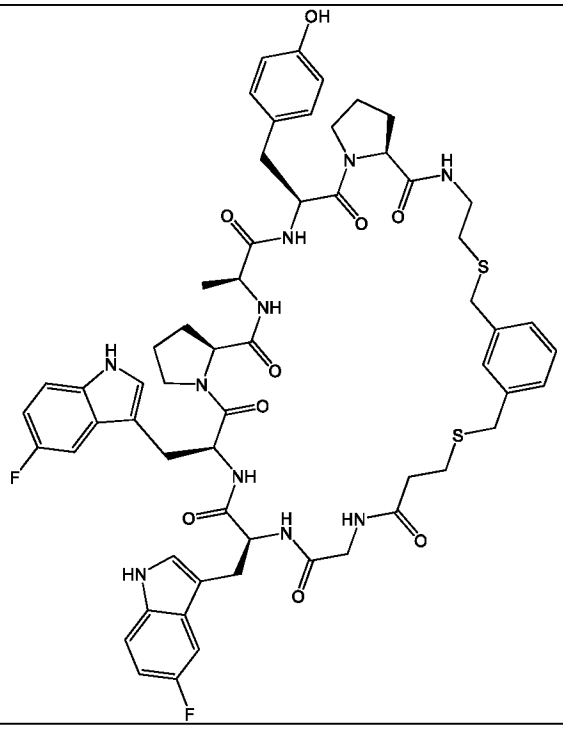
042	
043	

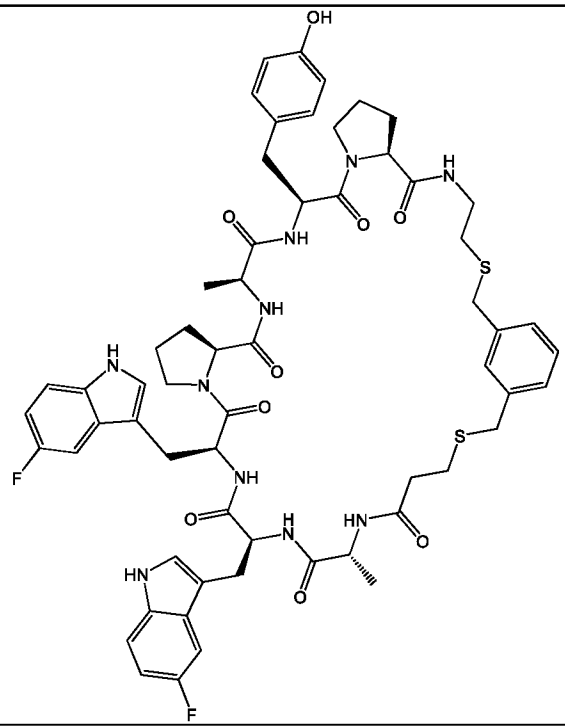
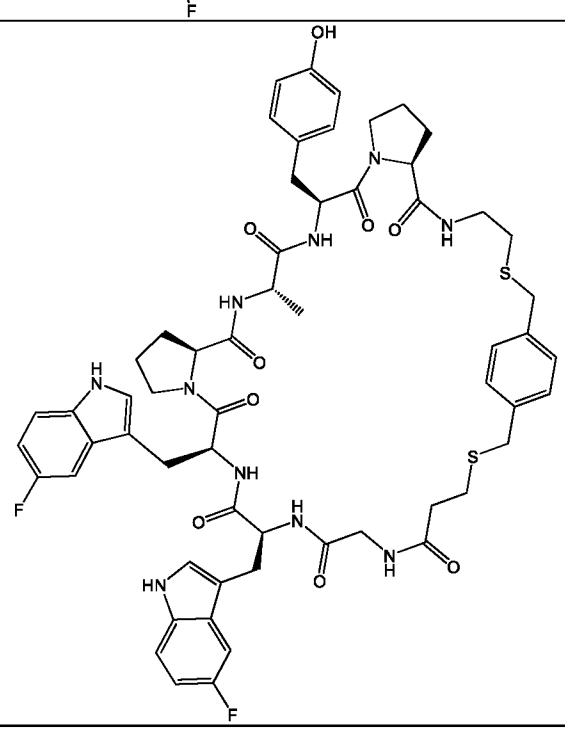
044	
045	

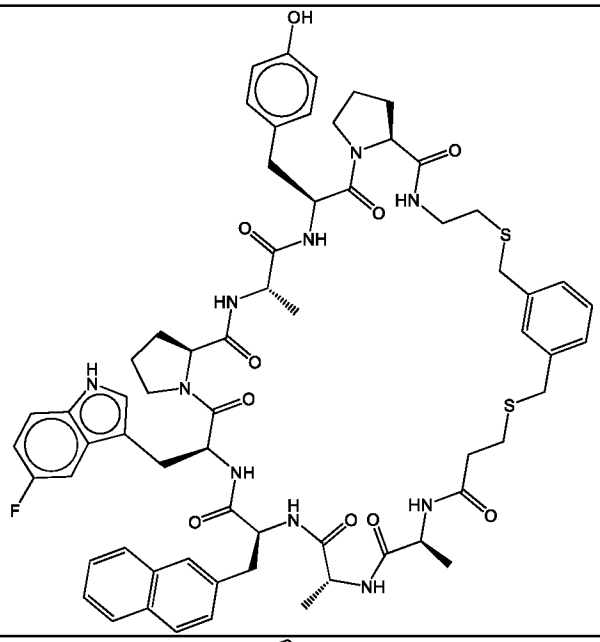
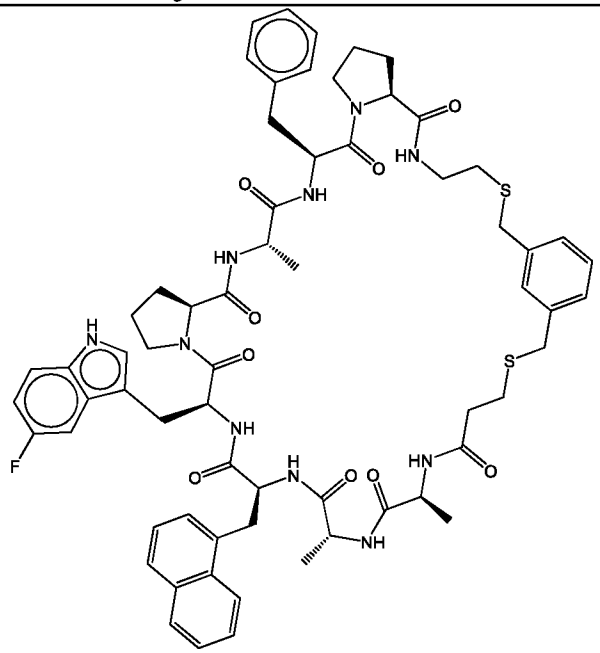
046	
049	

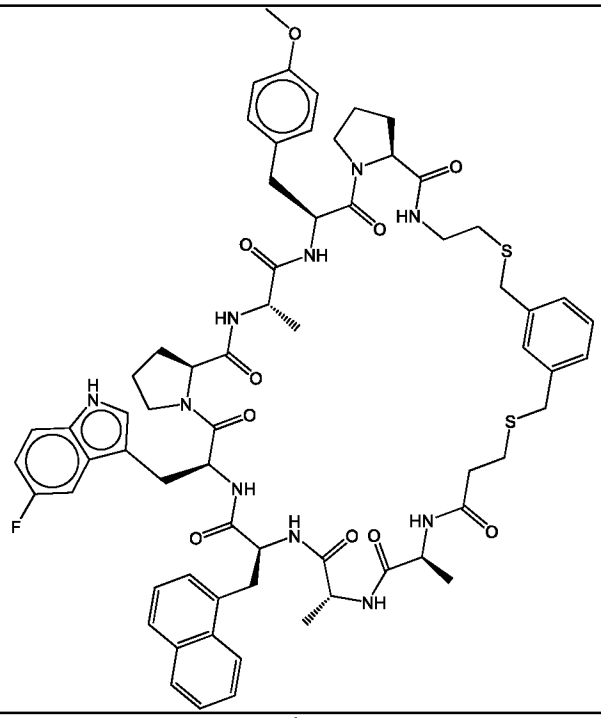
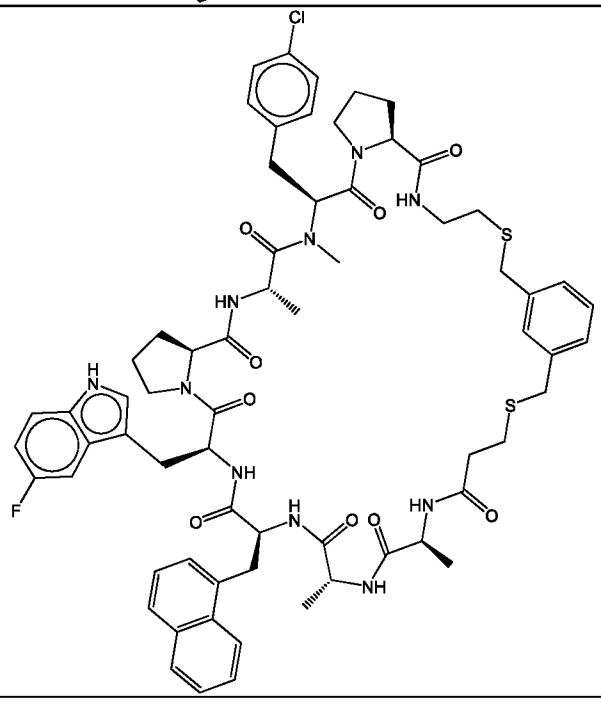
055	
056	

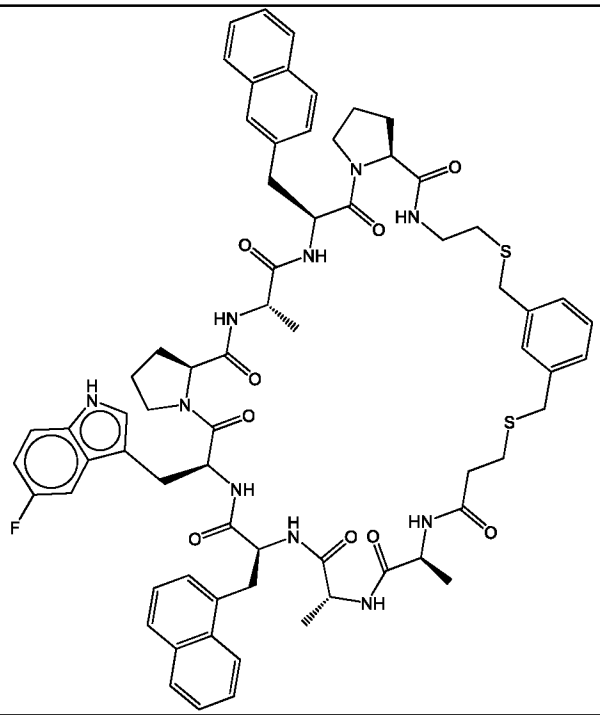
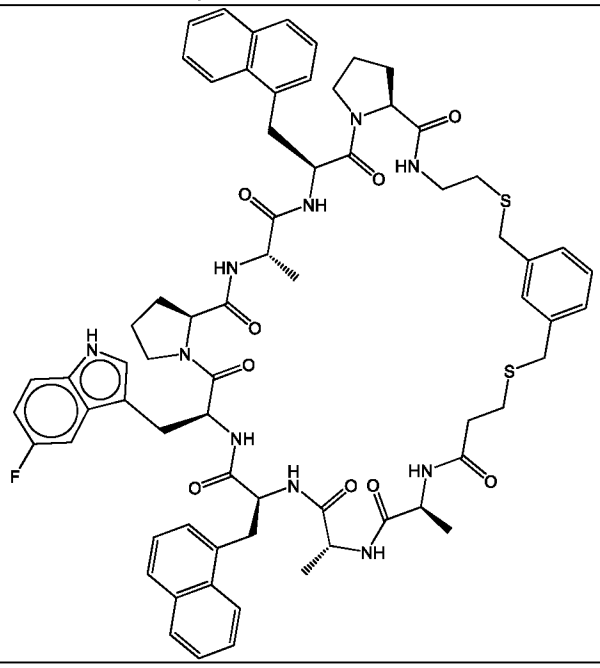
059	
060	

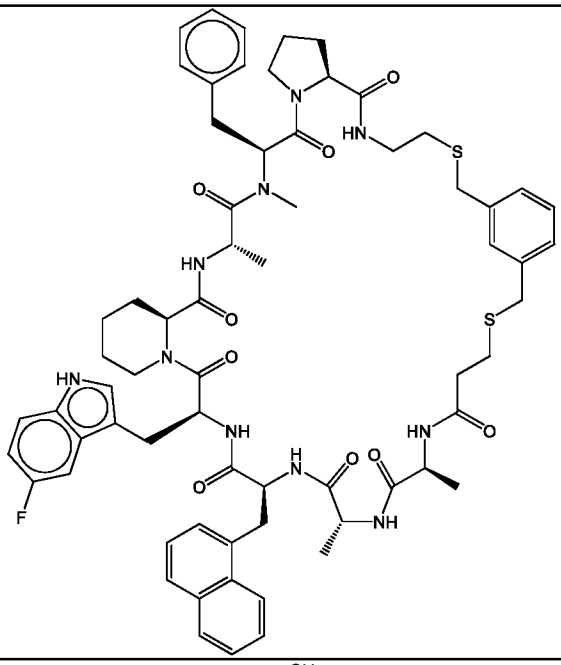
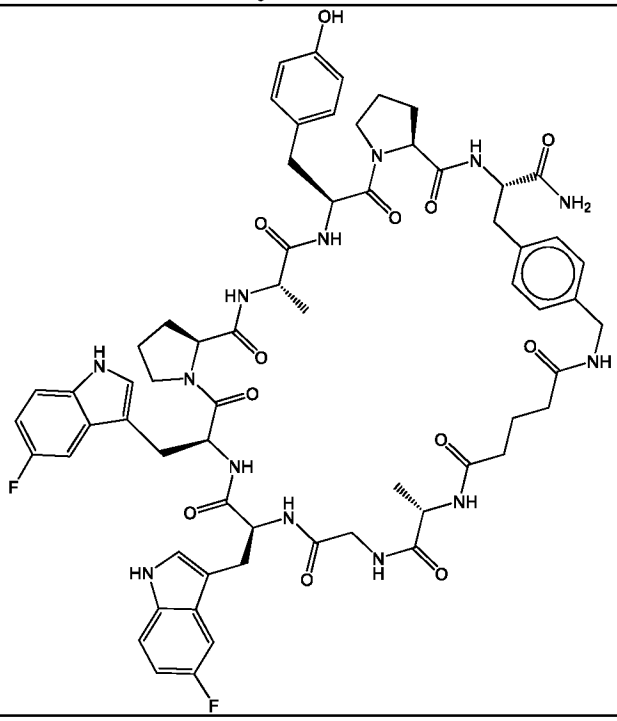
067	
072	

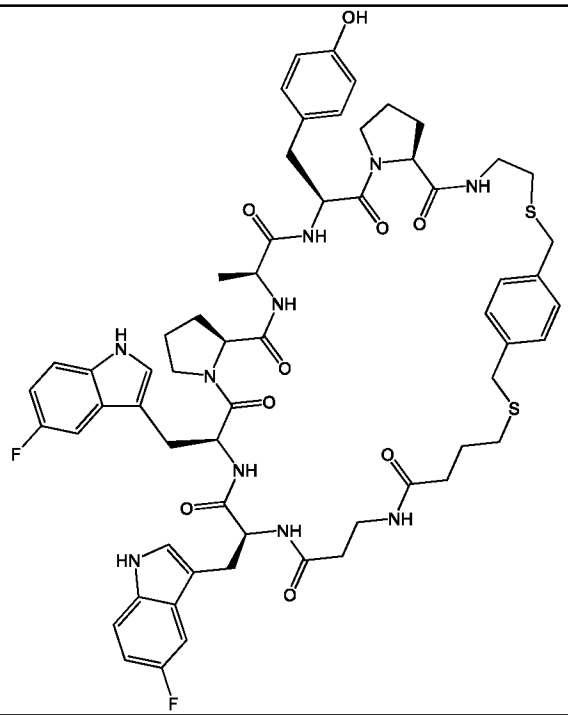
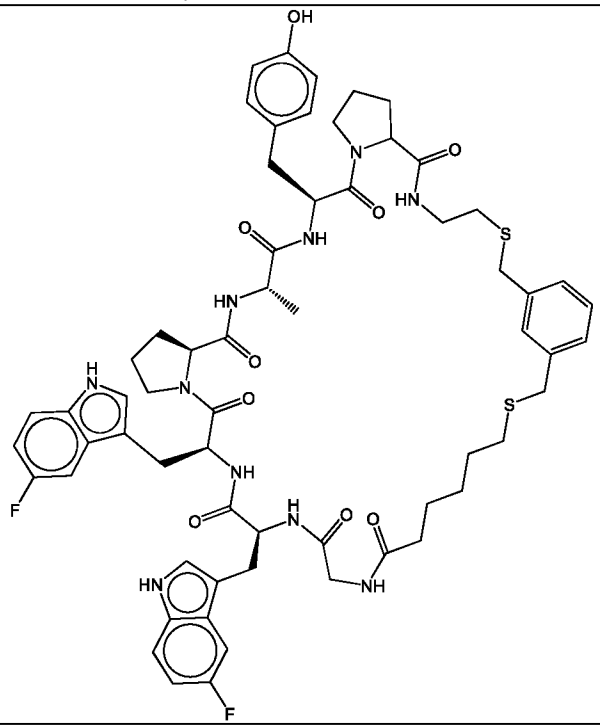
073	
074	

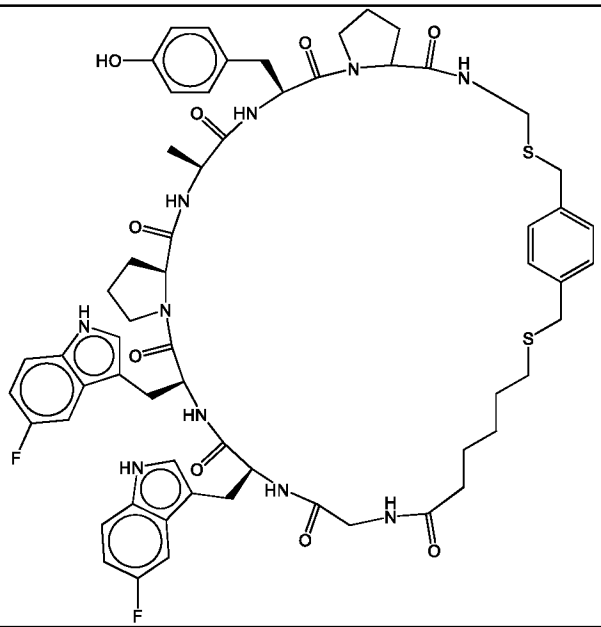
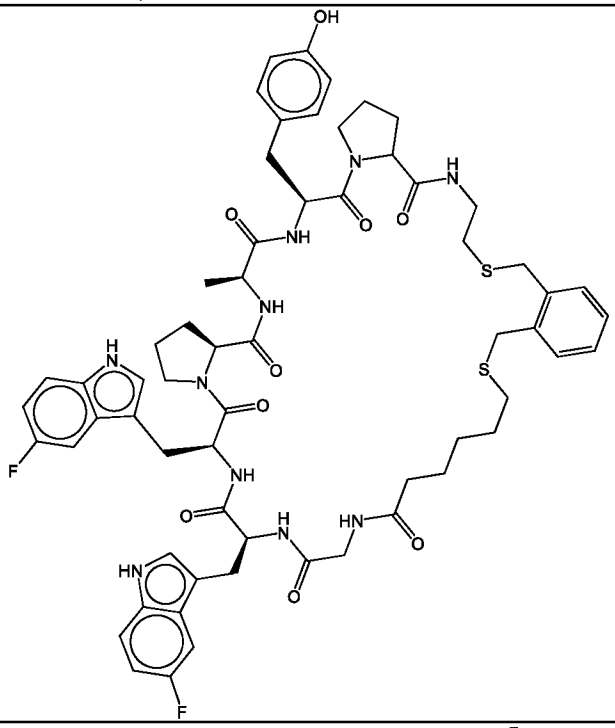
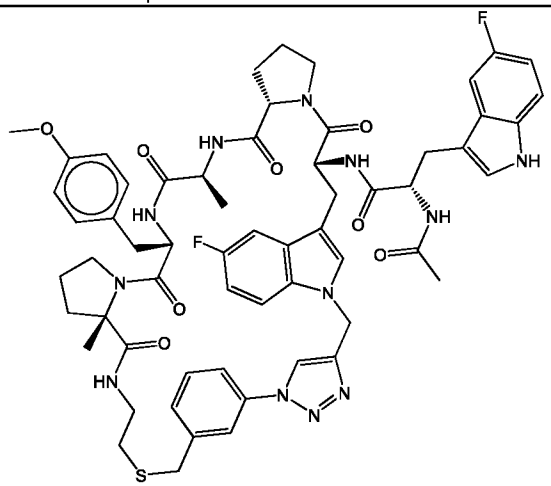
075	
076	

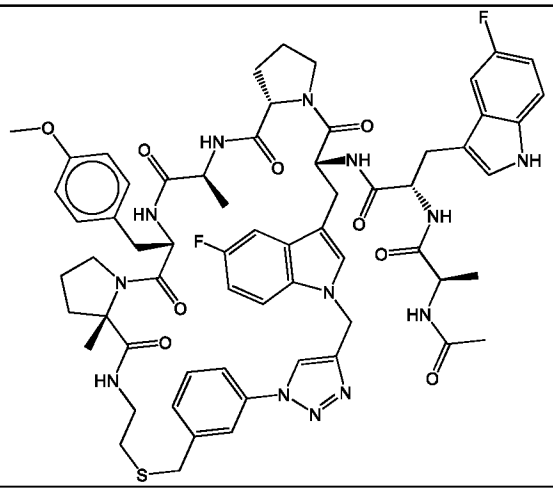
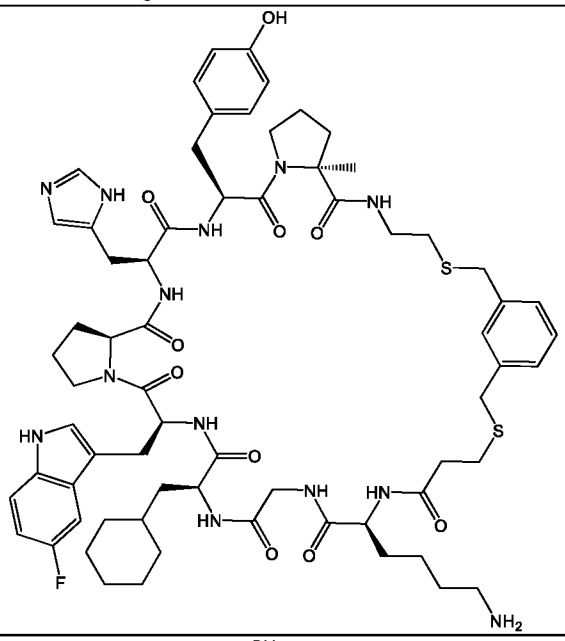
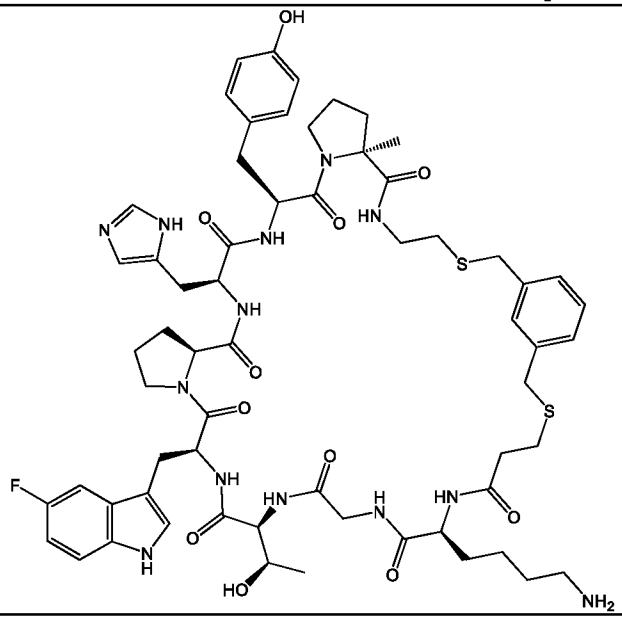
077	
078	

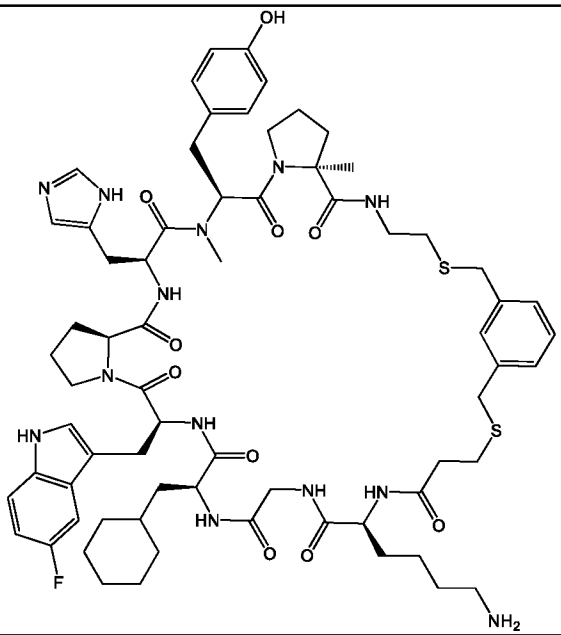
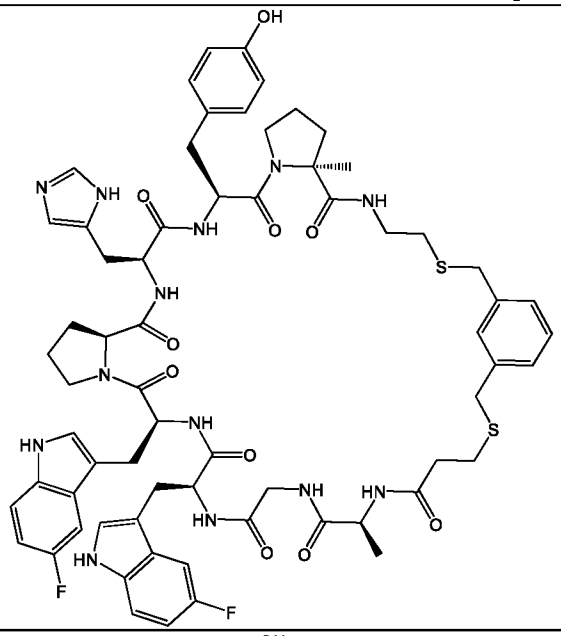
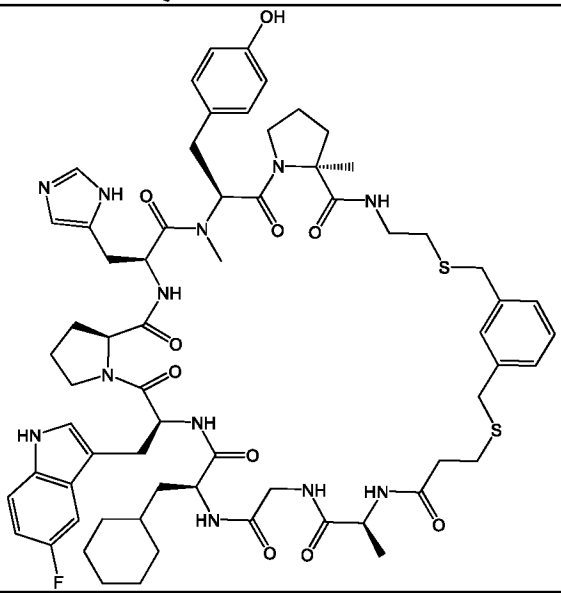
079	
080	

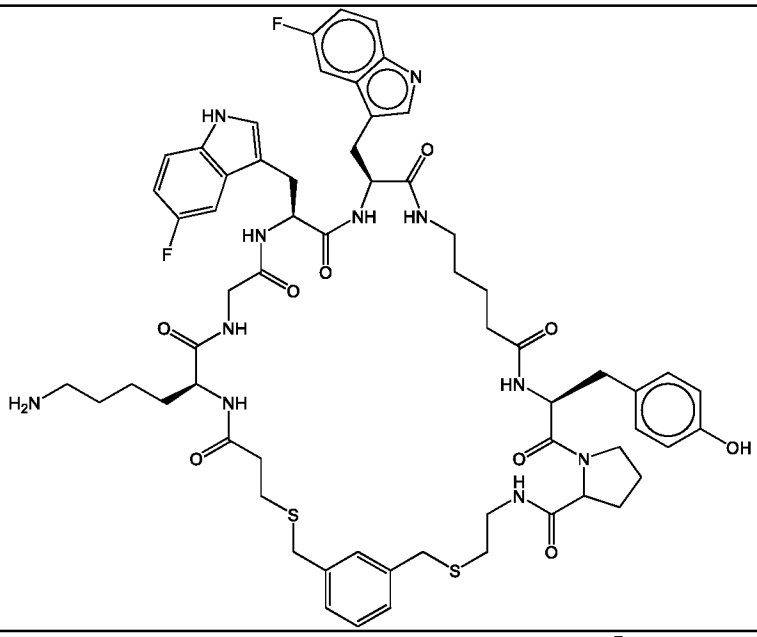
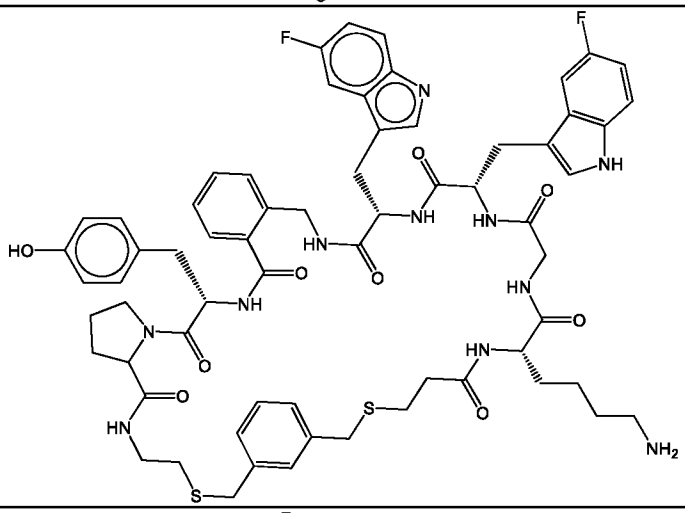
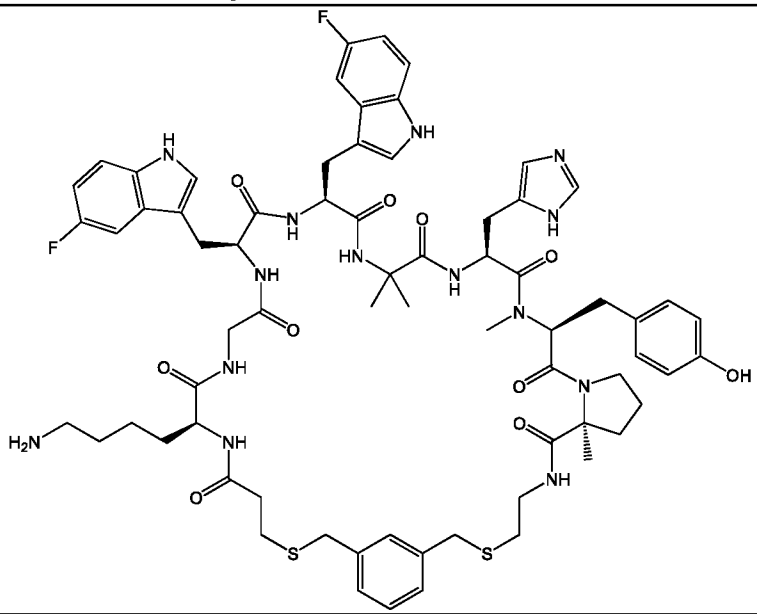
090	
096	

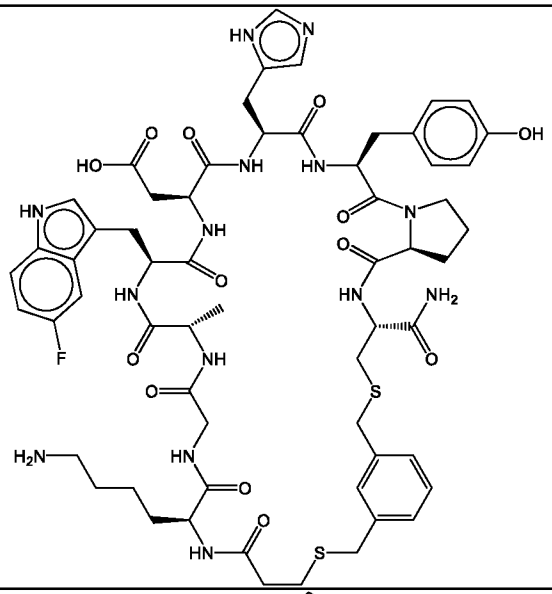
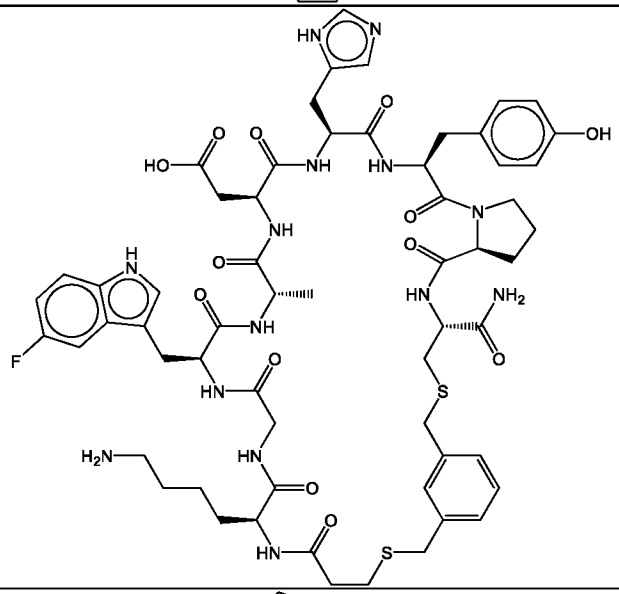
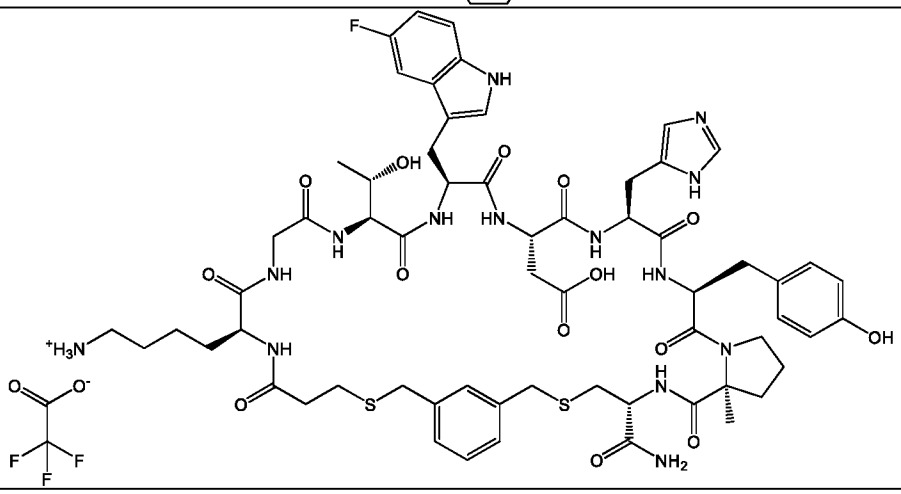
097	
098	

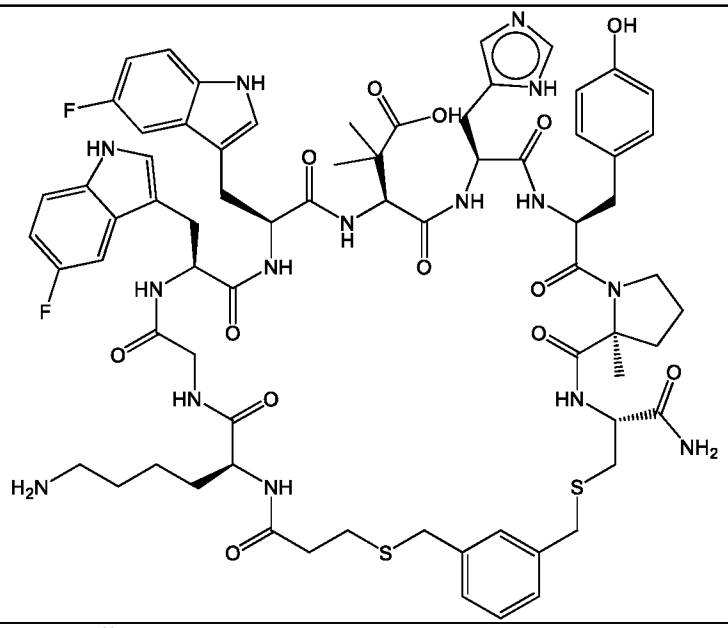
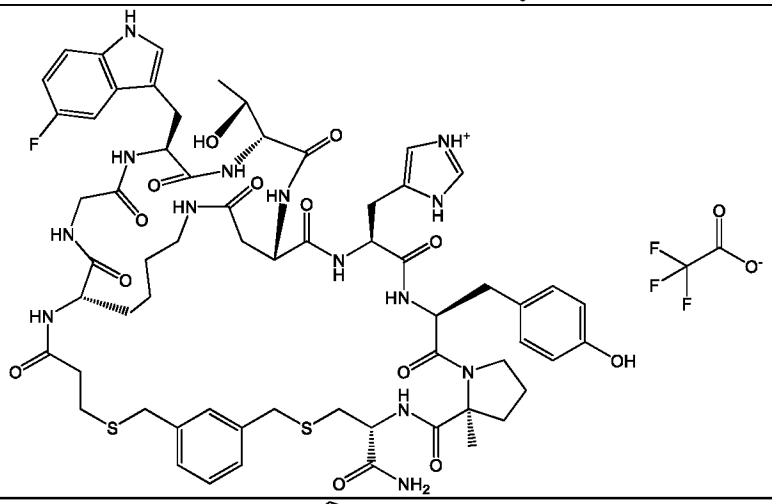
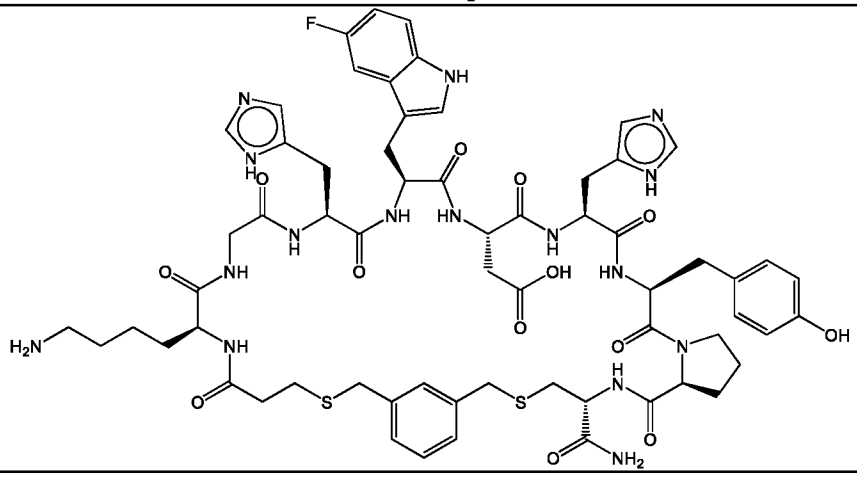
099	
100	
101	

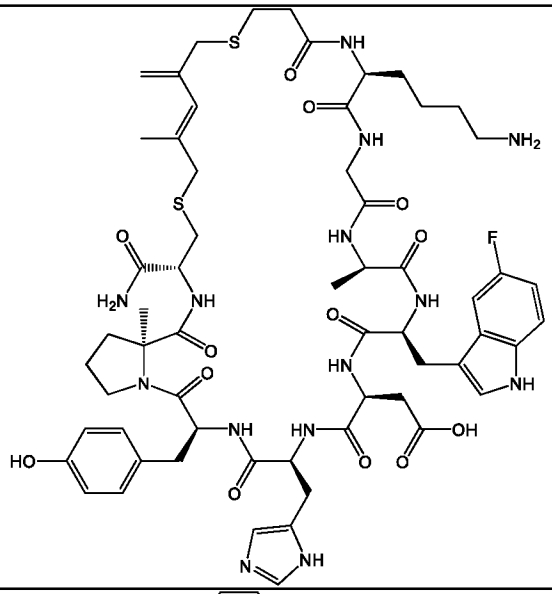
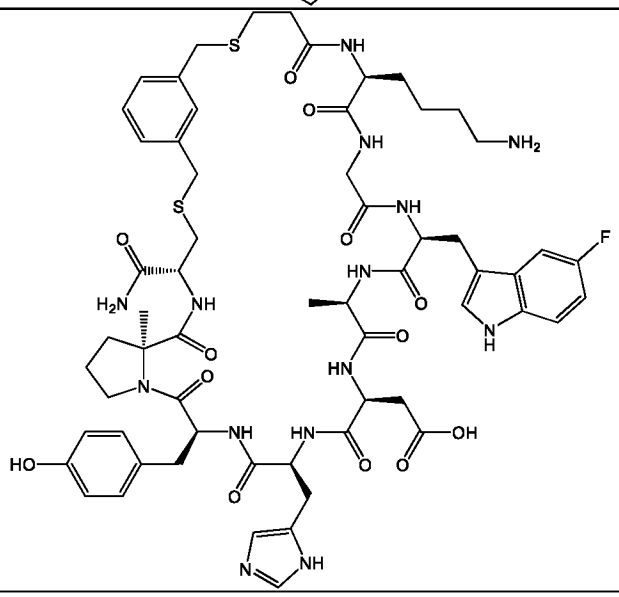
102	
104	
105	

106	
107	
108	

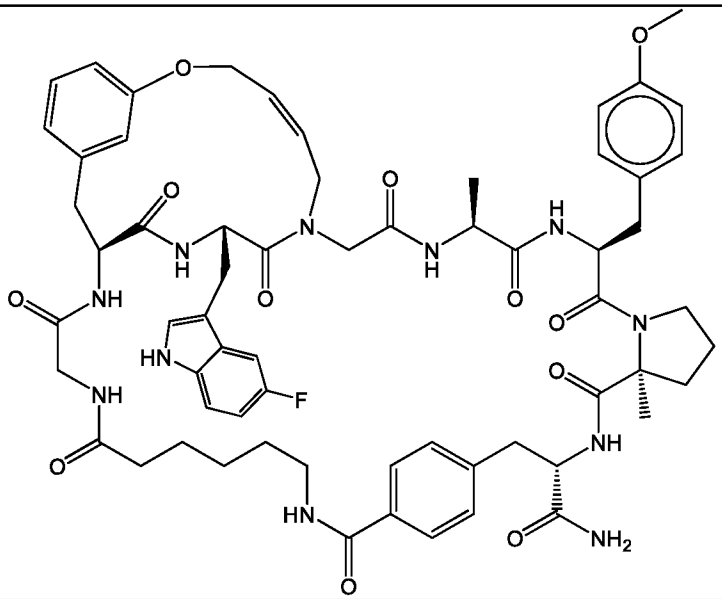
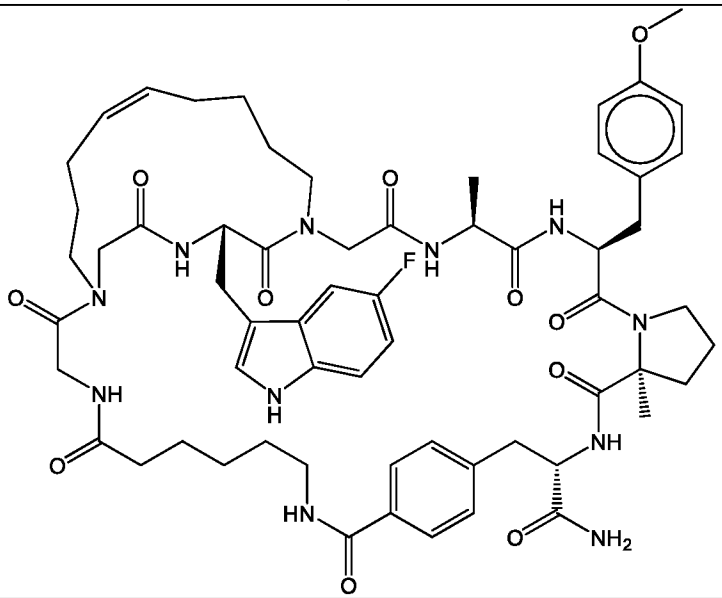
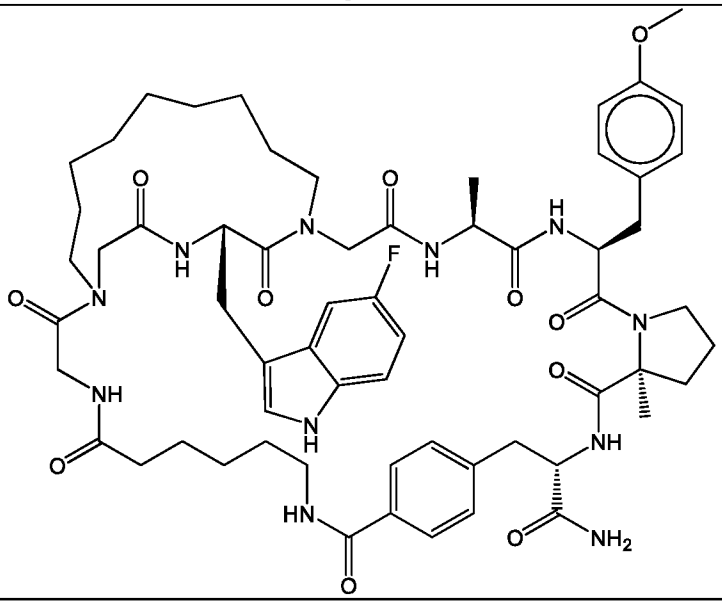
109	
110	
111	

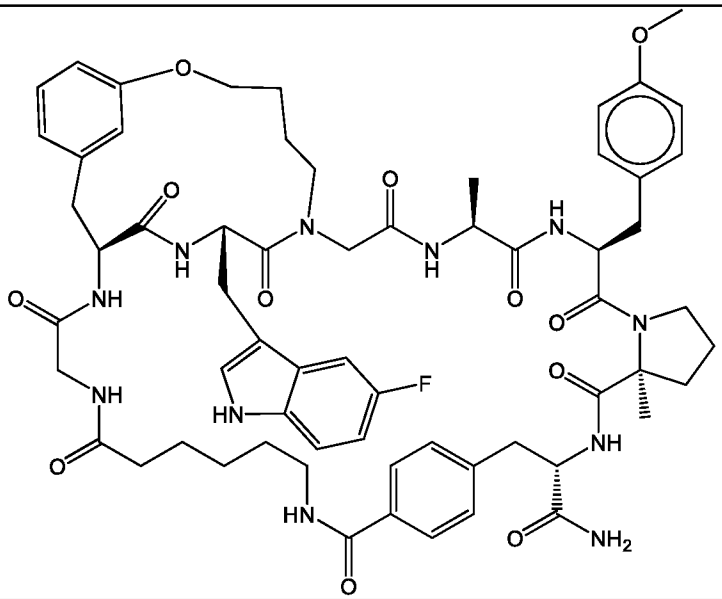
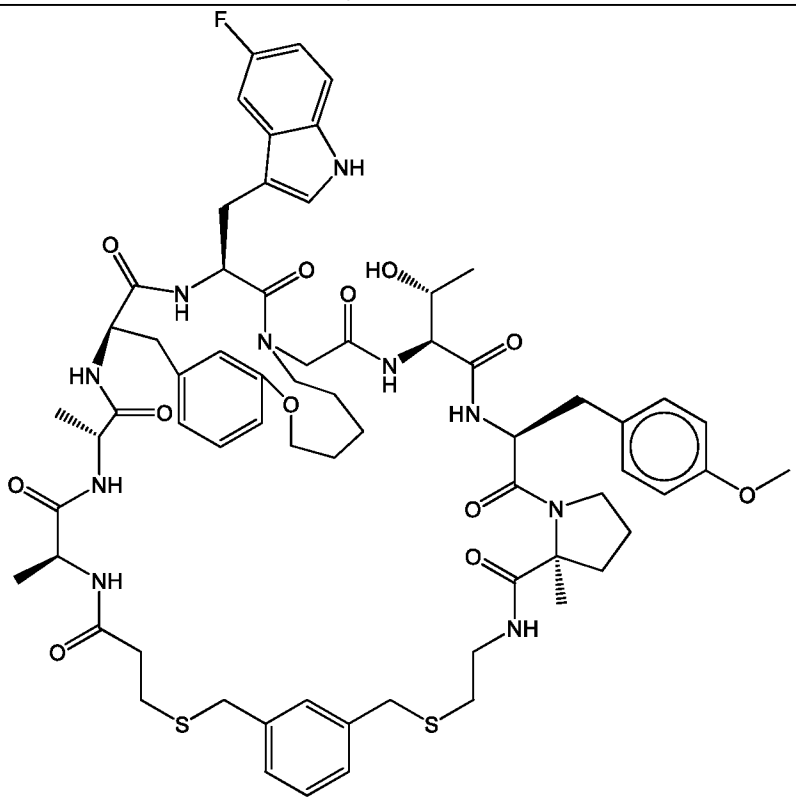
112	
113	
114	

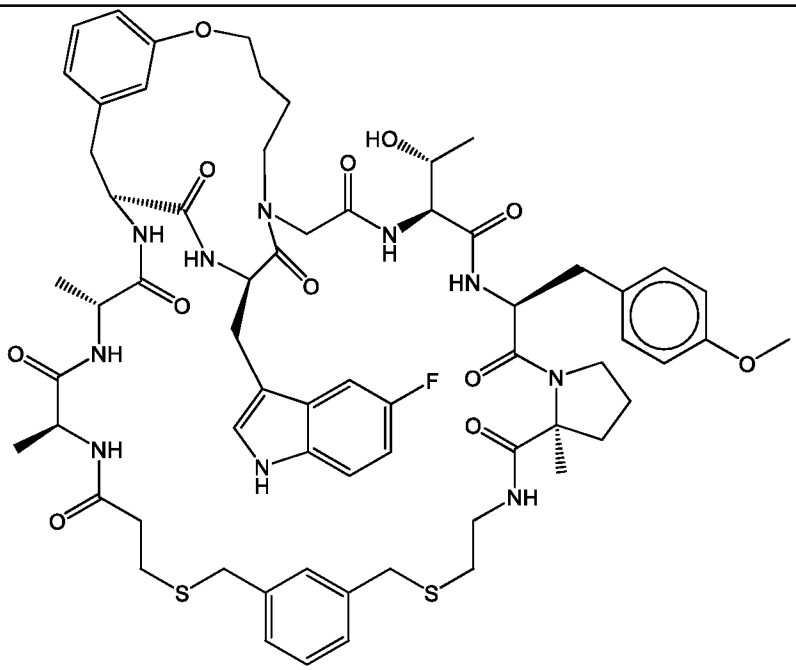
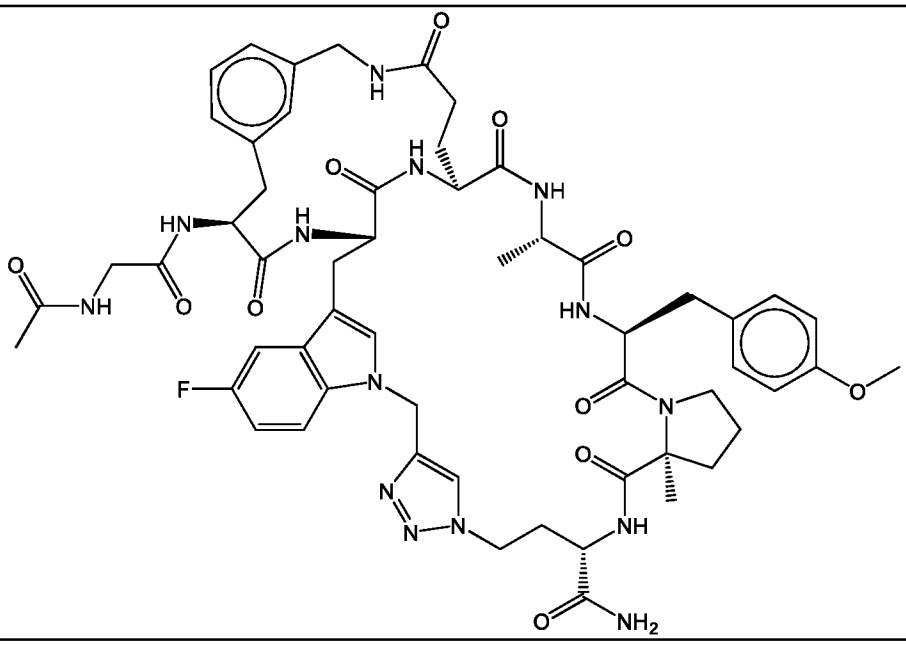
115	
116	
118	

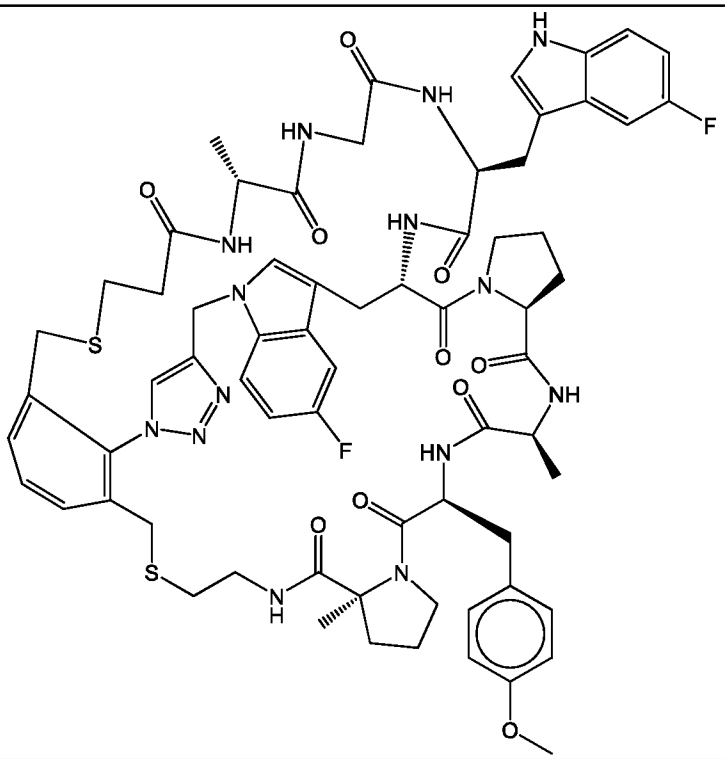
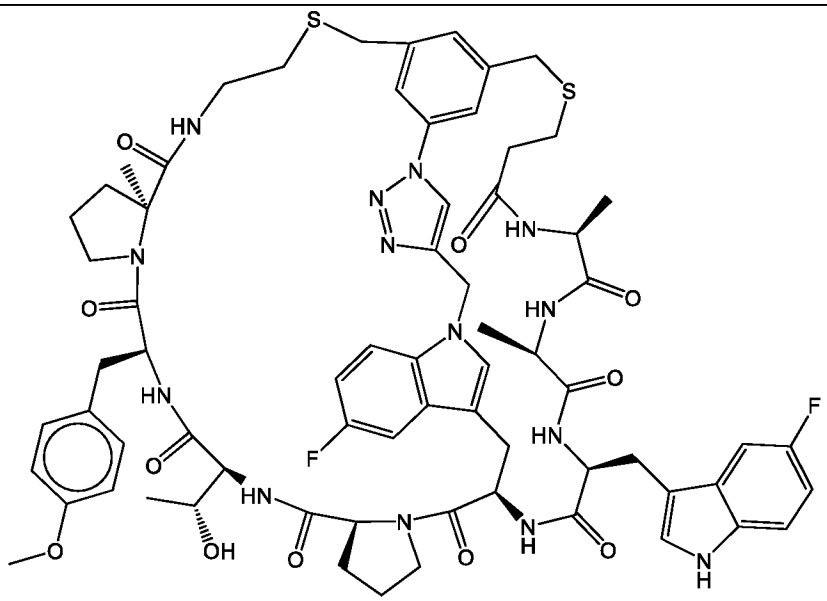
119	
120	

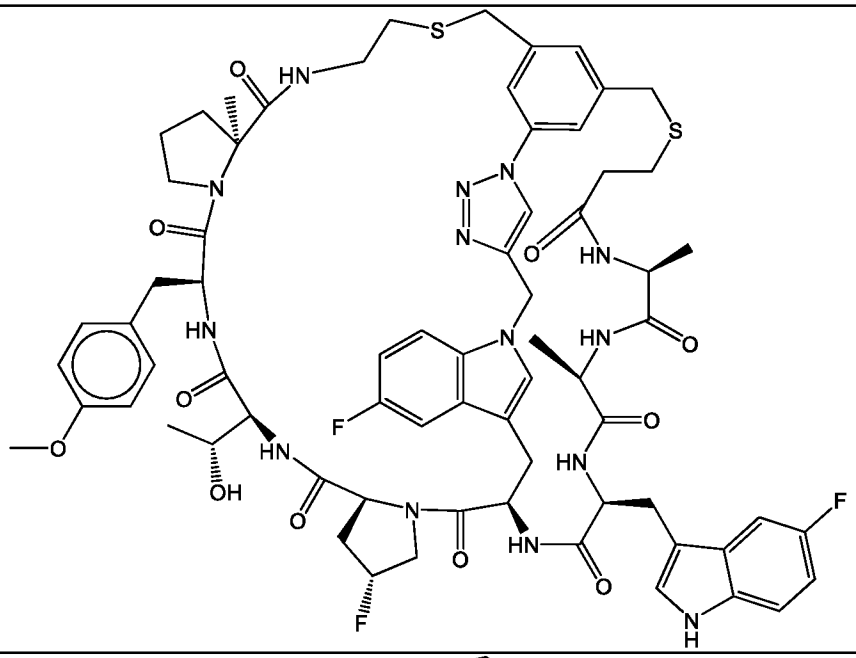
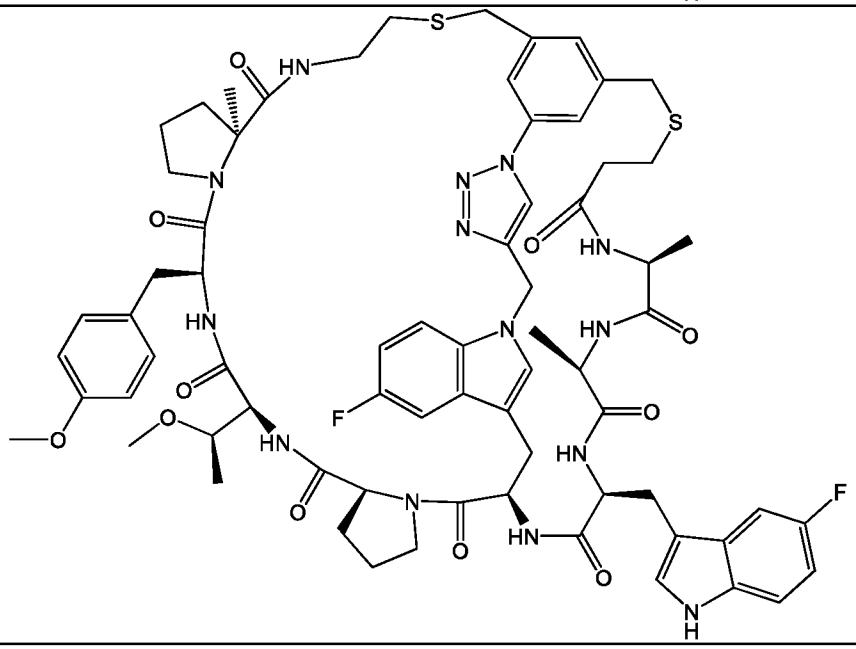
In another aspect, the compounds of the invention, or pharmaceutically acceptable salts thereof, are selected from the following compounds of Table 3 below:

121	
122	
123	

124	
125	

126	
127	

128	
129	

130	
131	

The compounds of Formula (I), as well as compounds 001-131, are referred to herein as "compounds of the invention."

It is to be understood that the compounds described herein encompass racemic, optically-active, regioisomeric and stereoisomeric forms, or combinations thereof that possess the therapeutically useful properties described herein.

The preparation of optically active forms is achieved in any suitable manner, including by way of non-limiting example, by resolution of the racemic form with recrystallization techniques, synthesis from optically-active starting materials, chiral synthesis, or chromatographic separation using a chiral stationary phase. In one embodiment, a mixture of one or more isomer is utilized as the compound of the invention

described herein. In another embodiment, compounds described herein contain one or more chiral centers. These compounds are prepared by any means, including stereoselective synthesis, enantioselective synthesis or separation of a mixture of enantiomers or diastereomers. Resolution of compounds and isomers thereof is achieved by any means including, by way of non-limiting example, chemical processes, enzymatic processes, fractional crystallization, distillation, and chromatography.

Within the present disclosure, any open valency appearing on a carbon, oxygen, or nitrogen atom in any structure described herein indicates the presence of a hydrogen atom. Where a chiral center exists in a structure, but no specific stereochemistry is shown for that center, both enantiomers, separately or as a mixture, are encompassed by that structure. The methods for the determination of stereochemistry and the separation of stereoisomers are well-known in the art.

In embodiments, the compounds of the invention may exist as tautomers. All tautomers are included within the scope of the compounds presented herein.

Compounds described herein also include isotopically-labeled compounds wherein one or more atoms is replaced by an atom having the same atomic number, but an atomic mass or mass number different from the atomic mass or mass number usually found in nature. Examples of isotopes suitable for inclusion in the compounds described herein include and are not limited to ^{2}H, ^{3}H, ^{11}C, ^{13}C, ^{14}C, ^{36}Cl, ^{18}F, ^{123}I, ^{125}I, ^{13}N, ^{15}N, ^{15}O, ^{17}O, ^{18}O, ^{32}P, and ^{35}S. Isotopically-labeled compounds are useful in drug or substrate tissue distribution studies. In another embodiment, substitution with heavier isotopes such as deuterium affords greater metabolic stability (for example, increased in vivo half-life or reduced dosage requirements).

In yet another embodiment, substitution with positron emitting isotopes, such as ^{11}C, ^{18}F, ^{15}O and ^{13}N, is useful in Positron Emission Topography (PET) studies for examining substrate receptor occupancy. Isotopically-labeled compounds are prepared by any suitable method or by processes using an appropriate isotopically-labeled reagent in place of the non-labeled reagent otherwise employed.

In one embodiment, the compounds described herein are labeled by other means, including, but not limited to, the use of chromophores or fluorescent moieties, bioluminescent labels, or chemiluminescent labels.

The compounds described herein, and other related compounds having different substituents are synthesized using techniques and materials described herein and as described, for example, in Fieser and Fieser's Reagents for Organic Synthesis, Volumes 1-17 (John Wiley and Sons, 1991); Rodd's Chemistry of Carbon Compounds, Volumes 1-5 and Supplementals (Elsevier Science Publishers, 1989); Organic Reactions, Volumes 1-40 (John Wiley and Sons, 1991), Larock's Comprehensive Organic Transformations (VCH

Publishers Inc., 1989), March, Advanced Organic Chemistry 4th Ed., (Wiley 1992); Carey and Sundberg, Advanced Organic Chemistry 4th Ed., Vols. A and B (Plenum 2000, 2001), and Green and Wuts, Protective Groups in Organic Synthesis 3rd Ed., (Wiley 1999) (all of which are incorporated by reference for such disclosure). General methods for the preparation of compound as described herein are modified by the use of appropriate reagents and conditions, for the introduction of the various moieties found in the formula as provided herein.

Compounds described herein are synthesized using any suitable procedures starting from compounds that are available from commercial sources, or are prepared using procedures described herein.

Without being bound to any particular mechanism of action, the cyclic polypeptide compounds provided herein can inhibit interaction between PCSK9 (e.g., human PCSK9) and LDLR (e.g., human LDLR) at an IC_{50} of less than 50 μM, less than 45 μM, less than 40 μM, less than 35 μM, less than 30 μM, less than 25 μM, less than 20 μM, less than 15 μM, less than 10 μM, less than 9 μM, less than 8 μM, less than 7 μM, less than 6 μM, less than 5 μM, less than 4 μM, less than 3 μM, less than 2 μM, less than 1 μM, less than 900 nM, less than 800 nM, less than 700 nM, less than 600 nM, or less than 500 nM (e.g., as determined using the assay described in the Examples). In any of these examples, the IC_{50} could be as low as 10 nM, 25 nM, 50 nM, or 100 nM (e.g., as determined using the assay described in the Examples). Additional methods for determining the IC_{50} of any of the compounds for inhibiting the interaction between PCSK9 (e.g., human PCSK9) and LDLR (e.g., human LDLR) are known in the art.

Pharmaceutical Compositions

Also provided are pharmaceutical compositions comprising at least one (e.g., one, two, three, or four) of the cyclic polypeptide compounds provided herein. Two or more (e.g., two, three, or four) of any of the cyclic polypeptide compounds can be present in a pharmaceutical composition in any combination. The pharmaceutical compositions may be formulated in any manner known in the art.

Pharmaceutical compositions for use in accordance with a method disclosed herein thus may be formulated in conventional manner using one or more pharmaceutically acceptable carriers including excipients and auxiliaries that facilitate processing of the compound into preparations that can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

In an embodiment, the administration of the pharmaceutical composition is selected from the group consisting of oral, intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, and intravitreal.

In an embodiment, the pharmaceutical composition comprises at least one of compounds of Formula (I) or compounds 001-131 and a pharmaceutically acceptable carrier.

A pharmaceutical composition as disclosed herein comprises at least one compound of the invention. Such a pharmaceutical composition may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

For intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, and intravitreal administration, the agents disclosed herein may be formulated in aqueous solutions, for instance in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compound can be formulated readily by combining the compound with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as a tablet, pills, capsule, liquid, gel, syrup, slurry or suspension, for oral ingestion by a subject to be treated.

Solid dosage forms can be prepared with coatings and shells, such as enteric coatings and others well-known in the art. They can contain pacifying agents and can be of such composition that they release the active compound or compounds in a certain part of the intestinal tract in a delayed manner. Examples of embedded compositions that can be used are polymeric substances and waxes. The active compounds also can be in microencapsulated form, if appropriate, with one or more of the above-mentioned excipients. For example, a pharmaceutical composition of a compound of the invention can be encapsulated by a pharmaceutical grade capsule in a dry powdered form.

Compound formulations include controlled duodenal release formulations, time release formulations, osmotic-controlled release delivery systems, microemulsions, microspheres, liposomes, nanoparticles, patches, pumps, drug depots, and the like. Specifically included herein are solid oral dosage forms, such as powders, softgels, gelcaps, capsules, pills, and tablets.

In one embodiment, the peptide is formulated as a sterile aqueous solution. In one embodiment, the peptide is formulated in a non-lipid formulation. In another embodiment, the peptide is formulated in a cationic or non-cationic lipid formulation. In either embodiment, the sterile aqueous solution may contain additional active or inactive components. Inactive components ("excipients") can include, but are not limited to, physiologically compatible salts, sugars, bulking agents, surfactants, or buffers.

Methods of Treatment

Provided herein is a method of reducing low density lipoprotein (LDL) cholesterol level in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a cyclic polypeptide compound, or a pharmaceutical composition comprising a cyclic polypeptide compound.

In an embodiment, the subject has hypercholesterolemia. Some subjects of the embodiment can be diagnosed as having a disease that shows comorbidity with hypercholesterolemia, such as nephrotic syndrome, kidney failure, coronary artery disease, atherosclerosis, stroke, peripheral vascular disease, diabetes, and high blood pressure.

In a further embodiment, the compound inhibits the interaction between human PCSK9 and epidermal growth factor-like repeat A (EGF-A) domain of human low density lipoprotein (LDLR) in a subject.

In another embodiment, the compound inhibits the interaction between human PCSK9 and epidermal growth factor-like repeat A (EGF-A) domain of human low density lipoprotein (LDLR) in a cell.

Also provided herein is a method of treating hypercholesterolemia in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a compound of the invention.

Hypercholesterolemia is a disease characterized by the presence of elevated levels of cholesterol (total cholesterol and/or LDL levels) in a sample obtained from a subject (e.g., as compared to a reference level that is a preselected threshold level). In some examples of hypercholesterolemia, the subject can also have a decreased level of high density lipoprotein (HDL) cholesterol as compared to a reference level, e.g., a preselected threshold level. Hypercholesterolemia is typically diagnosed by a medical professional by determining a cholesterol level(s) in a subject. In a general patient population, a preselected threshold level of total cholesterol (LDL plus HDL) can be, e.g., 180 mg/dL, 185 mg/dL, 190 mg/dL, 195 mg/dL, 200 mg/dL, 205 mg/dL, 210 mg/dL, 215 mg/dL, 220 mg/dL, 225 mg/dL, 230 mg/dL, 235 mg/dL, 240 mg/dL, 245 mg/dL, 250 mg/dL, 255 mg/dL, or 260 mg/dL. In a general patient population, a preselected threshold level of LDL cholesterol can be, e.g., 125 mg/dL, 130 mg/dL, 135 mg/dL, 140 mg/dL, 145 mg/dL, 150 mg/dL, 155 mg/dL, 160 mg/dL, 165 mg/dL, 170 mg/dL, 175 mg/dL, 180 mg/dL, 185 mg/dL, 190 mg/dL, 195 mg/dL, 200 mg/dL, 205 mg/mL, 210 mg/dL, 215 mg/dL, 220 mg/dL, 225 mg/dL, or 230 mg/dL. In a general patient population, a preselected threshold level of HDL cholesterol can be, e.g., 60 mg/dL, 58 mg/dL, 56 mg/dL, 54 mg/dL, 52 mg/dL, 50 mg/dL, 48 mg/dL, 46 mg/dL, 44 mg/dL, 42 mg/dL, 40 mg/dL, 38 mg/dL, 36 mg/dL, 34 mg/dL, 32 mg/dL, 30 mg/dL, 28 mg/dL, 26 mg/dL, or 24 mg/dL.

For subjects having a high risk of coronary disease (e.g., a subject having greater

than a 20% risk of myocardial infarction in 10 years, or a subject having coronary artery disease, diabetes, peripheral artery disease, carotid artery disease, or aortic aneurysm), the preselected threshold level of LDL cholesterol can be, e.g., 60 mg/dL, 65 mg/dL, 70 mg/dL, 75 mg/dL, 80 mg/dL, 85 mg/dL, 90 mg/dL, 95 mg/dL, or 100 mg/dL. For subjects having a moderately high risk of coronary disease (e.g., a subject having a 10% to 20% risk of myocardial infarction in 10 years and two or more risk factors of coronary disease), the preselected threshold level of LDL can be, e.g., 90 mg/dL, 95 mg/dL, 100 mg/dL, 105 mg/dL, 110 mg/dL, 115 mg/dL, 120 mg/dL, 125 mg/dL, 130 mg/dL, 135 mg/dL, 140 mg/dL, 145 mg/dL, or 150 mg/dL. For subjects having a moderate risk of coronary disease (e.g., a subject having a less than 10% risk of having a myocardial infarction in 10 years and having greater than one risk factor of coronary artery disease), the preselected threshold level of LDL cholesterol can be, e.g., 115 mg/dL, 120 mg/dL, 125 mg/L, 130 mg/dL, 135 mg/dL, 140 mg/L, 145 mg/dL, or 150 mg/dL. Risk factors of coronary disease include, but are not limited to, family history, smoking behavior, high blood pressure, diabetes, obesity, physical inactivity, and high stress.

In any of the methods, the subject can be a subject having, diagnosed, or identified as having hypercholesterolemia, that is, for example, caused by diet and/or a genetic mutation (e.g., familial hypercholesterolemia) (e.g., any of the genetic mutations described herein or known in the art to result in an increased LDL cholesterol level).

In some of examples of any of the methods, the subject can be a subject having, diagnosed, or identified as having a disease showing comorbidity with hypercholesterolemia. Non-limiting examples of diseases showing comorbidity with hypercholesterolemia include hypertension, heart disease, arthritis, diabetes, coronary artery disease, stroke, breast cancer, and myocardial infarction.

Accordingly, provided herein is a method of treating a disease that shows comorbidity with hypercholesterolemia such as nephrotic syndrome, kidney failure, coronary artery disease, atherosclerosis, stroke, peripheral vascular disease, diabetes, and high blood pressure, in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a compound of the invention.

Methods for diagnosing hypertension, heart disease, arthritis, diabetes, coronary artery disease, stroke, breast cancer, and myocardial infarction are well known in the art. For example, hypertension can be diagnosed in a subject by measuring the subject's blood pressure at one or more time points. Heart disease can be diagnosed in a subject, e.g., using blood tests, chest X-rays, electrocardiogram, echocardiography, an exercise stress test, radionuclide ventriculography, multiple-gated acquisition scanning, or cardiac catheterization. Diabetes (type I or type II) can be diagnosed, e.g., by measuring blood glucose level(s) in a subject (e.g., fasting blood glucose levels) and/or detecting ketones in

urine from the subject. Arthritis can be diagnosed in a subject, e.g., by physical examination for pain, stiffness, swelling, redness, and decreased range of motion in one or more joints, laboratory tests, X-rays, computerized tomography, magnetic resonance imaging, ultrasound, or athroscopy. Coronary artery disease can be diagnosed in a subject, e.g., by electrocardiogram, echocardiogram, stress test, cardiac catheterization, angiogram, heart scan (e.g., using computerized tomography), or magnetic resonance angiography. Stroke can be diagnosed, e.g., by examination, blood tests, computerized tomography, magnetic resonance imaging, carotid ultrasound, cerebral angiogram, or echocardiogram. Breast cancer can be diagnosed, e.g., by breast examination, mammogram, breast ultrasound, biopsy, or magnetic resonance imaging. Myocardial infarction is diagnosed, e.g., by electrocardiogram, blood tests, chest X-ray, echocardiogram, coronary catheterization, exercise stress test, cardiac computerized tomography, or magnetic resonance imaging. Provided are methods of treating a disease showing comorbidity with hypercholesterolemia, where the methods include administering to a subject in need thereof (e.g., a subject having hypercholesterolemia) a cyclic polypeptide compound or a pharmaceutical composition provided herein. The administration reduces the risk as compared to the risk of developing such a disease in a subject having similar cholesterol levels but administered a different treatment or administered no treatment. Also provided are methods of delaying the onset or delaying the worsening of a disease showing comorbidity with hypercholesterolemia, where the methods include administering to a subject in need thereof (e.g., a subject having hypercholesterolemia and optionally, one or more risk factors of developing a disease showing comorbidity with hypercholesterolemia, or a subject having, diagnosed as having, or identified as having a disease showing comorbidity with hypercholesterolemia) a compound or a pharmaceutical composition provided herein (e.g., as compared to a subject having the same disease showing co-morbidity with hypercholesterolemia but administered a different treatment or administered no treatment).

Also provided are methods of reducing LDL cholesterol level in a subject in need thereof that include administering to the subject a therapeutically effective amount of any of the compounds or pharmaceutical compositions described herein (the reducing being as compared to, for example, a level in a control subject or a baseline level in the subject prior to treatment with any of the compounds or any of the pharmaceutical compositions provided herein). Successful reduction in a LDL cholesterol level in a subject can be determined by a health professional (e.g., a nurse, a physician, or a physician's assistant). For example, successful treatment can result in at least a 2%, at least a 3%, at least a 4%, at least a 5%, at least a 6%, at least a 7%, at least a 8%, at least a 9%, at least a 10%, at least a 11%, at least a 12%, at least a 13%, at least a 14%, at least a 15%, at least a 16%, at least a 17%, at least a 18%, at least a 19%, at least a 20%, at least a 21%, at least a 22%, at least a

23%, at least a 24%, at least a 25%, at least a 26%, at least a 27%, at least a 28%, at least a 29%, or at least a 30% reduction in a subject's LDL cholesterol level (e.g., as compared to a control subject or as compared to a LDL cholesterol level in the subject prior to the administration of any of the compounds or any of the pharmaceutical compositions provided herein). In any of these examples, the reduction in a subject's LDL level can be as high as 40%, 45%, 50%, 55%, 60%, or 65% (e.g., as compared to a control subject or as compared to a LDL cholesterol level in the subject prior to the administration of any of the compounds or any of the pharmaceutical compositions provided herein). In addition, the methods can further provide for a reduction in the total cholesterol level of a subject, e.g., a reduction of at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, or at least 15% (e.g., as compared to a control subject or compared to a total cholesterol level in the subject prior to the administration of any of the compounds or any of the pharmaceutical compositions provided herein). In any of these examples, the reduction in a subject's total cholesterol level can be as high as 40%, 45%, 50%, 55%, 60%, or 65% (e.g., as compared to a control subject or compared to a total cholesterol level in the subject prior to the administration of any of the compounds or any of the pharmaceutical compositions provided herein).

Methods for determining levels of LDL cholesterol and total cholesterol are well known in the art. Non-limiting examples of kits for determining the levels of LDL cholesterol and total cholesterol are commercially available from Abcam (Cambridge, MA), Sigma Aldrich (St. Louis, MO), Cell Biolabs, Inc. (San Diego, CA), Genway Biotech (San Diego, CA), Biolabo (Lawrenceville, Georgia), BioAssay Systems (Hayward, CA), Medibena (Vienna, Austria), and Abnova (Taipei, Taiwan).

The subject in these methods can, e.g., be a subject having, diagnosed as having, or identified as having hypercholesterolemia (e.g., familial hypercholesterolemia) or a subject identified or previously identified as having an increased risk of developing hypercholesterolemia. In some examples, the subject in these methods can be, e.g., a subject having, diagnosed as having, or identified as having a disease showing comorbidity with hypercholesterolemia (e.g., any of the examples of diseases showing comorbidity with hypercholesterolemia described herein or known in the art). Also provided are methods of reducing the risk of developing a disease showing comorbidity with hypercholesterolemia (e.g., as compared to the risk in a control patient or patient population that has not received treatment with any of the compounds or has not received any of the pharmaceutical compositions provided herein) that include administering to a subject in need thereof a compound or a pharmaceutical composition provided herein. The subject in these methods can be a subject having, diagnosed as having, or identified as having hypercholesterolemia

or identified or previously identified as having an increased risk of developing hypercholesterolemia. In some examples, the subject can be identified or previously identified as having an increased risk of developing a disease showing comorbidity with hypercholesterolemia (e.g., a subject having one or more risk factors for developing a disease showing comorbidity with hypercholesterolemia or having one or more risk factors for developing hypercholesterolemia).

In some examples, the subject may already be receiving or have received a treatment for hypercholesterolemia (e.g., a statin, niacin, bile-acid resins, fibrates, and cholesterol adsorption inhibitors). In some examples, the prior treatment for hypercholesterolemia resulted in no significant reduction in LDL cholesterol level in the subject in need thereof (e.g., any of the subjects descried herein), and/or insufficient reduction in the LDL cholesterol level in the subject (e.g., any of the subjects described herein) (i.e., not sufficient to reduce LDL cholesterol level to below a reference level that is a preselected threshold level).

In some embodiments, the subject is administered a dose of between 1 mg to 400 mg of any of the compounds or any of the pharmaceutical compositions (e.g., between 1 mg and 300 mg, between 1 mg and 250 mg, between 1 mg and 200 mg, between 1 mg and 150 mg, between 1 mg and 100 mg, between 1 mg and 80 mg, between 1 mg and 70 mg, between 1 mg and 60 mg, between 1 mg and 50 mg, between 1 mg and 40 mg, between 1 mg and 30 mg, between 1 mg and 20 mg, between 1 mg and 10 mg, between 20 mg and 120 mg, between 30 mg and 90 mg, or between 40 mg and 80 mg). In some examples, the subject is administered a dose of the compound or pharmaceutical composition described herein of about 0.1 mg/kg to about 4.0 mg/kg (e.g., between about 0.1 mg/kg and about 3.5 mg/kg, between about 0.1 mg/kg and about 3.0 mg/kg, between about 0.1 mg/kg and about 2.5 mg/kg, between about 0.1 mg/kg and about 2.0 mg/kg, between about 0.1 mg/kg and about 1.5 mg/kg, between about 0.5 mg/kg and about 1.5 mg/kg, or between about 0.7 mg/kg and about 1.3 mg/kg).

Also provided are methods of inhibiting the interaction between PCSK9 (e.g., any of the exemplary mammalian PCSK9 described herein, such as human PCSK9) and LDLR (e.g., any of the exemplary mammalian LDLR described herein, such as human LDLR) that include contacting PCSK9 and LDLR with any of the compounds or pharmaceutical compositions provided herein (e.g., the inhibition being compared to, for example, a level of interaction between PCSK9 and LDLR in the absence of the compounds or pharmaceutical composition). In some examples, the contacting takes place *in vitro* (e.g., in a cell culture). In other examples, the contacting takes place *in vivo* (e.g., in any of the exemplary subjects described herein or any of the animal models described herein). In any of these methods, LDLR can be present in the plasma membrane of a mammalian cell (e.g., a hepatocyte, an

epithelial cell, or an endothelial cell), and oriented in the plasma membrane such that LDLR can interact with PCSK9 present in an extracellular medium (e.g., culture medium).

Non-limiting methods for detecting the interaction (or amount of interaction) between PCSK9 and LDLR are described herein. For example, LDLR can be attached to a solid substrate (e.g., a plate, film, or a bead) (e.g., in such a way that allows LDLR to interact with PCSK9) and contacted with PCSK9 in the presence or absence of any of the compounds or pharmaceutical compositions described herein, and the amount of PCSK9 bound to the LDLR in the presence of the compound or pharmaceutical composition can be compared to the amount bound in a control assay that does not include the compound or pharmaceutical composition. Detecting the amount of PCSK9 bound to LDLR in the presence or absence of the compound or pharmaceutical composition can be performed by immunoprecipitation and immunoblotting, surface plasmon resonance, or time-resolved fluorescence energy transfer (TR-FRET).

As is known in the art, an inhibition of the interaction between PCSK9 and LDLR can be measured as an increase in the K_D value and/or a decrease in K_A value for the interaction between PCSK9 (e.g., human PCSK9) and LDLR (e.g., human LDLR) (e.g., the EGF-A domain of LDLR) in the presence of any of the compounds or pharmaceutical compositions provided herein (e.g., as compared to levels of interaction in the absence of the compound or pharmaceutical composition).

The interaction between PCSK9 and LDLR can be indirectly detected when the interaction between PCSK9 and LDLR occurs *in vitro*. For example, interaction between PCSK9 and LDLR, where the LDLR is expressed in the plasma membrane of a hepatocyte, can be indirectly detected by determining the amount of LDLR remaining in the hepatocyte it has been contacted with PCSK9 in the presence or absence of any the compounds or pharmaceutical compositions provided herein (where a higher level of LDLR in the hepatocyte after incubation in the presence of the compound or pharmaceutical composition as compared to the level of LDLR in the hepatocyte after incubation in the absence of the compound or pharmaceutical composition indicates that the interaction between LDLR and PCSK9 has been inhibited).

The amount of inhibition of the interaction between LDLR and PCSK9 *in vivo* can also be indirectly determined by measuring the level of LDL cholesterol in a subject administered the compound or pharmaceutical composition as compared to a control level of LDL (e.g., a level of LDL cholesterol in the same subject prior to treatment with the compound or pharmaceutical composition). In such an assay, a decreased level of LDL cholesterol in the subject after treatment, as compared to the level of LDL cholesterol in the subject before treatment, indicates that the compound or pharmaceutical composition

administered to the subject inhibited the interaction between PCSK9 and LDLR in the subject.

In an embodiment of these methods, the administration is selected from the group consisting of oral, intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, and intravitreal.

In an embodiment of these methods, provided herein is a method of treating hypercholesterolemia in a subject in need thereof comprising administering to the subject at least one of compounds of Formula (I) or compounds 001-131or a pharmaceutically composition comprising at least one of compounds of Formula (I) or compounds 001-131.

It is to be understood that the methods described in this disclosure are not limited to particular methods and experimental conditions disclosed herein, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

Furthermore, the experiments described herein, unless otherwise indicated, use conventional molecular and cellular biological and immunological techniques within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature. See, e.g., Ausubel, et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y. (1987-2008), including all supplements, Molecular Cloning: A Laboratory Manual (Fourth Edition) by MR Green and J. Sambrook and Harlow et al., Antibodies: A Laboratory Manual, Chapter 14, Cold Spring Harbor Laboratory, Cold Spring Harbor (2013, 2nd edition).

Unless otherwise defined herein, scientific and technical terms used herein have the meanings that are commonly understood by those of ordinary skill in the art. In the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Unless otherwise required by context, singular terms shall include **pluralities and plural terms shall include the singular. The use of "or" means "and/or" unless stated otherwise. The use of the term "including," as well as other forms, such as "includes" and "included," is not limiting. As used herein, unless otherwise stated, the singular forms "a," "an," and "the" include plural reference. Thus, for example, a reference to "a protein"** includes a plurality of protein molecules.

Generally, nomenclatures used in connection with cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art. The methods and techniques provided herein are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. Enzymatic reactions and purification techniques are performed

according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclatures used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the methods described herein may be made using suitable equivalents without departing from the scope of the embodiments disclosed herein. Having now described certain embodiments in detail, the same will be more clearly understood by reference to the following examples, which are included for purposes of illustration only and are not intended to be limiting.

EXAMPLES

The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only, and the invention is not limited to these Examples, but rather encompasses all variations that are evident as a result of the teachings provided herein.

Compounds of the invention can be prepared according to the following examples.

Example 1: Synthesis of Cyclic Polypeptide Compounds

Procedure B

Step 1: Peptide Synthesis

The peptides were synthesized using Fmoc-protected amino acids on a solid-phase Rink Amide MBHA (NovaBiochem, ~0.5 meq/g, 100-200 mesh) or AM (NovaBiochem, ~0.73 meq/g, 100-200 mesh) or 2-aminoethanethiol-2-chlorotrityl resin (AnaSpec, ~0.9 meq/g, 200-400 mesh) with a CEM Liberty Blue automated microwave peptide synthesizer. Peptides were typically synthesized on a 0.1 mmol scale. Typical reaction conditions were as follows: Deprotection Conditions: 20% piperidine (v/v) in DMF (2 X 2 min at 75 °C); Residue Coupling Conditions: 5 eq (relative to resin) of activated amino acid (2.5 mL of a 0.2 M amino acid stock solution in DMF) was delivered to the resin, followed by 5 eq of DIC activator (1 mL of a 0.5 M solution in DMF), and 5 eq of Oxyma Pure (0.5 mL of a 1 M solution in DMF) and allowed to react for 5 min at 75 °C. The resultant product was used directly in the next step.

Step 2: Olefin Metathesis

For a 0.1 mmol scale: The linear peptide was synthesized on solid-support according to Step 1. The peptide was not cleaved from resin. A solution of Benzylidene-bis(tricyclohexylphosphine)-dichlororuthenium (Grubb's generation I catalyst) was prepared by dissolving 16 mg (0.2 equiv) (based on peptide resin of 0.1 mmol) in anhydrous 1,2-dichloroethane (5 mL). The solution was degassed by bubbling nitrogen through the solution for 10 minutes. The degassed solution was then added to the resin-bound peptide in a 10 mL fritted syringe. The reaction was allowed to proceed at 45 °C for two hours and then the catalyst was filtered off. The resin was then re-subjected to the metathesis conditions by adding an additional 5 mL of freshly-prepared catalyst solution (16 mg Grubb's generation I catalyst in 5 mL of degassed anhydrous 1,2-dichloroethane). The reaction was allowed to proceed for 2 hours at 45 °C. The resin-bound peptide was then washed with dichloromethane (5 X 10 mL). The reaction can be monitored for completion by removing a few beads, cleaving the peptide (95% TFA / 2.5% H20 / 2.5% triisopropylsilane) and analyzing by LCMS.

Step 3: Cleavage from Solid-Support

The resin from Step 2 was transferred to either a 50 mL polypropylene centrifuge tube or a 10 mL fritted syringe. The peptides were cleaved from their solid support using trifluoroacetic acid / triisopropylsilane / DL-dithiothreitol / water (92.5 / 2.5 / 2.5 (w/v) / 2.5) mixture. For 0.1 mmol scale of resin, ~10 mL cleavage solution was used. The suspended resin was rocked in cleavage solution for 3 hours at room temperature. The filtrate was collected in 50 mL polypropylene centrifuge tubes and precipitated with chilled diethyl ether (~50 mL per ~5 mL cleavage filtrate). The precipitated crude peptide was collected by centrifugation. The white pellet was then subsequently suspended in chilled diethyl ether and collected by centrifugation two additional times. The resulting solid was air-dried to afford the crude peptide.

Step 4: Cyclization

To a 250 mL round-bottomed flask fitted with a stir bar, was added PyAOP (210 mg, 0.4 mmol; (7-Azabenzotriazol-1-yloxy)tripyrro-lidinophosphonium hexafluorophosphate), HOAt (14 mg, 0.1 mmol; 1-Hydroxy-7-azabenzotriazole) and DIPEA (90 µL, 0.5 mmol) in DMF (80 mL). The precipitated solid crude peptide from Step 3 was dissolved in DMF (25 mL) and added dropwise to the activator solution using a syringe pump (addition over 1.5 h). The resulting yellow solution was stirred overnight at room temperature. After completion of the cyclization (monitored by LCMS), the reaction mixture was acidified by addition of TFA (100 µL). DMF solvent was evaporated under high vacuum and the resulting crude residue was purified by C18 reverse-phase HPLC.

Procedure I:

Step 1: Peptide Synthesis

The peptides were synthesized using Fmoc-protected amino acids on a solid-phase Rink Amide MBHA (NovaBiochem, ~0.5 meq/g, 100-200 mesh) or AM (NovaBiochem, ~0.73 meq/g, 100-200 mesh) or 2-Aminoethanethiol-2-chlorotrityl resin (AnaSpec, ~0.9 meq/g, 200-400 mesh) with a CEM Liberty Blue automated microwave peptide synthesizer. Peptides were typically synthesized on a 0.1 mmol scale. Typical reaction conditions were as follows: Deprotection Conditions: 20% piperidine (v/v) in DMF (2 X 2 min at 75 °C); Residue Coupling Conditions: 5 eq (relative to resin) of activated amino acid (2.5 mL of a 0.2 M amino acid stock solution in DMF) was delivered to the resin, followed by 5 eq of DIC activator (1 mL of a 0.5 M solution in DMF), and 5 eq of Oxyma Pure (0.5 mL of a 1 M solution in DMF) and allowed to react for 5 min at 75 °C. The resultant product was used directly in the next step.

Step 2: Cleavage from Solid-Support

The resin from Step 1 was transferred to either a 50 mL polypropylene centrifuge tube or a 10 mL fritted syringe. The peptides were cleaved from their solid support using trifluoroacetic acid / triisopropylsilane / DL-dithiothreitol / water (92.5 / 2.5 / 2.5 (w/v) / 2.5) mixture. For 0.1 mmol scale of resin, ~10 mL cleavage solution was used. The suspended resin was rocked in cleavage solution for 3 hours at room temperature. The filtrate was collected in 50 mL polypropylene centrifuge tubes and precipitated with chilled diethyl ether (~50 mL per ~5 mL cleavage filtrate). The precipitated crude peptide was collected by centrifugation. The white pellet was then subsequently suspended in chilled diethyl ether and collected by centrifugation two additional times. The resulting solid was air-dried to afford the crude peptide.

Step 3: Cyclization

To a 250 mL round-bottomed flask fitted with a stir bar, was added PyAOP (210 mg, 0.4 mmol; (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate), HOAt (14 mg, 0.1 mmol; 1-Hydroxy-7-azabenzotriazole) and DIPEA (90 µL, 0.5 mmol) in DMF (80 mL). The precipitated solid crude peptide from Step 2 was dissolved in DMF (25 mL) and added dropwise to the activator solution using a syringe pump (addition over 1.5 h). The resulting yellow solution was stirred overnight at room temperature. After completion of the cyclization (monitored by LCMS), the reaction mixture was acidified by addition of TFA (100 µL). The resultant product was used directly in the next step.

Step 4: Cyclization

The solid crude peptide (~0.1 mmol) was dissolved in 4 mL of a DMSO:H_2O (3:1) solution in a 50 mL polypropylene centrifuge tube equipped with stirbar. Sodium ascorbate (2 eq / 40 mg) and copper(II) sulfate pentahydrate (1 eq / 25 mg) were added and the

reaction was stirred at room temperature for 14 hours. The reaction was monitored for complete conversion by LCMS analysis of the crude reaction. The desired product was purified directly from the crude reaction mixture by C18 reverse phase chromatography.

5 Procedure J:

Step 1: Peptide Synthesis

The peptides were synthesized using Fmoc-protected amino acids on a solid-phase Rink Amide MBHA (NovaBiochem, ~0.5 meq/g, 100-200 mesh) or AM (NovaBiochem, ~0.73 meq/g, 100-200 mesh) or 2-Aminoethanethiol-2-chlorotrityl resin (AnaSpec, ~0.9 meq/g,
10 200-400 mesh) with a CEM Liberty Blue automated microwave peptide synthesizer. Peptides were typically synthesized on a 0.1 mmol scale. Typical reaction conditions were as follows: Deprotection Conditions: 20% piperidine (v/v) in DMF (2 X 2 min at 75 °C); Residue Coupling Conditions: 5 eq (relative to resin) of activated amino acid (2.5 mL of a 0.2 M amino acid stock solution in DMF) was delivered to the resin, followed by 5 eq of DIC
15 activator (1 mL of a 0.5 M solution in DMF), and 5 eq of Oxyma Pure (0.5 mL of a 1 M solution in DMF) and allowed to react for 5 min at 75 °C. The resultant product was used directly in the next step.

Step 2: Cleavage from Solid-Support

The resin from Step 1 was transferred to either a 50 mL polypropylene centrifuge
20 tube or a 10 mL fritted syringe. The peptides were cleaved from their solid support using trifluoroacetic acid / triisopropylsilane / DL-dithiothreitol / water (92.5 / 2.5 / 2.5 (w/v) / 2.5) mixture. For 0.1 mmol scale of resin, ~10 mL cleavage solution was used. The suspended resin was rocked in cleavage solution for 3 hours at room temperature. The filtrate was collected in 50 mL polypropylene centrifuge tubes and precipitated with chilled diethyl ether
25 (~50 mL per ~5 mL cleavage filtrate). The precipitated crude peptide was collected by centrifugation. The white pellet was then subsequently suspended in chilled diethyl ether and collected by centrifugation two additional times. The resulting solid was air-dried to afford the crude peptide.

Step 3: Cyclization

30 For a 0.1 mmol scale: The peptide was synthesized, cleaved from solid support, and isolated according to Steps 1-2. The solid crude peptide was dissolved with stirring in 15 mL of a degassed water:acetonitrile (1:1) solution in a 50 mL polypropylene centrifuge tube. Additional acetonitrile was added as needed to ensure complete solubility of the peptide. To this stirred solution, aqueous NH_4HCO_3 (200 mM, degassed) was added dropwise to bring
35 the pH to ~8. Either 1-azido-3-(bromomethyl)benzene (26 mg / 0.1 mmol / 1 eq) or 2-azido-1,3-bis(bromomethyl)benzene (26 mg / 0.1 mmol / 1 eq) was added neat to the reaction. After complete addition, the reaction was tested to ensure the pH was maintained at ~8. If

the pH was below ~8, additional aqueous ammonium bicarbonate (200 mM, degassed) was added dropwise to the reaction until the pH was ~8. The centrifuge tube was capped and the resulting reaction mixture was stirred at room temperature for 1 h. The reaction was then acidified to pH 1 with trifluoroacetic acid and lyophilized to afford the crude peptide as a powder.

The resulting crude peptide was suspended in diethyl ether (~40 mL), pelleted by centrifugation, and the diethyl ether layer was decanted off. This was repeated two additional times to remove residual unreacted linker. The resulting precipitated peptide was then air dried overnight to afford the solid crude peptide, which was used in subsequent steps without further purification.

The solid crude peptide (~0.1 mmol) was dissolved in 4 mL of a DMSO:H_2O (3:1) solution in a 50 mL polypropylene centrifuge tube equipped with stirbar. Sodium ascorbate (2 eq / 40 mg) and copper(II) sulfate pentahydrate (1 eq / 25 mg) were added and the reaction was stirred at room temperature for 14 hours. The reaction was monitored for complete conversion by LCMS analysis of the crude reaction. The desired product was purified directly from the crude reaction mixture by C18 reverse phase chromatography.

Procedure E:

Step 1: Peptide Synthesis

The peptides were synthesized using Fmoc-protected amino acids on a solid-phase Rink Amide MBHA (NovaBiochem, ~0.5 meq/g, 100-200 mesh) or AM (NovaBiochem, ~0.73 meq/g, 100-200 mesh) or 2-Aminoethanethiol-2-chlorotrityl resin (AnaSpec, ~0.9 meq/g, 200-400 mesh) with a CEM Liberty Blue automated microwave peptide synthesizer. Peptides were typically synthesized on a 0.1 mmol scale. Typical reaction conditions were as follows: Deprotection Conditions: 20% piperidine (v/v) in DMF (2 X 2 min at 75 °C); Residue Coupling Conditions: 5 eq (relative to resin) of activated amino acid (2.5 mL of a 0.2 M amino acid stock solution in DMF) was delivered to the resin, followed by 5 eq of DIC activator (1 mL of a 0.5 M solution in DMF), and 5 eq of Oxyma Pure (0.5 mL of a 1 M solution in DMF) and allowed to react for 5 min at 75 °C. The resultant product was used directly in the next step.

Step 2: Cleavage from Solid-Support

The resin from Step 1 was transferred to either a 50 mL polypropylene centrifuge tube or a 10 mL fritted syringe. The peptides were cleaved from their solid support using trifluoroacetic acid / triisopropylsilane / DL-dithiothreitol / water (92.5 / 2.5 / 2.5 (w/v) / 2.5) mixture. For 0.1 mmol scale of resin, ~10 mL cleavage solution was used. The suspended resin was rocked in cleavage solution for 3 hours at room temperature. The filtrate was collected in 50 mL polypropylene centrifuge tubes and precipitated with chilled diethyl ether

(~50 mL per ~5 mL cleavage filtrate). The precipitated crude peptide was collected by centrifugation. The white pellet was then subsequently suspended in chilled diethyl ether and collected by centrifugation two additional times. The resulting solid was air-dried to afford the crude peptide.

Step 3: Cyclization

For a 0.1 mmol scale: The linear peptide was synthesized, cleaved from solid support, and isolated according to Step 2. The solid crude peptide was dissolved with stirring in 30 mL of a degassed water:acetonitrile (1:1) solution in a 50 mL polypropylene centrifuge tube. Additional acetonitrile was added as needed to ensure complete dissolution of the peptide. To this stirred solution, aqueous ammonium bicarbonate (200 mM, degassed) was added dropwise to bring the pH to ~8. Alkylating reagent 1,3-bis(bromomethyl)benzene (0.1 mmol / 1 eq) in acetonitrile (1 mL) was added dropwise to the reaction over ~2 min. After complete addition, the reaction was tested to ensure the pH was maintained at ~8. If the pH was below ~8, additional aqueous ammonium bicarbonate (200 mM, degassed) was added dropwise to the reaction until the pH was ~8. The centrifuge tube was capped and the resulting reaction mixture was stirred at room temperature for 1 h. The reaction was then acidified to pH ~1 with trifluoroacetic acid and lyophilized to afford the crude peptide as a powder. The desired product was purified by C18 reverse phase chromatography.

Table 4 shows the synthetic procedure that was used to synthesize the compounds disclosed herein.

TABLE 4	
Compound	**Synthetic Procedure**
001	**E**
002	**E**
003	**E**
004	**E**
005	**E**
006	**E**
007	**E**
008	**E**
009	**E**
010	**E**
011	**E**
012	**E**
013	**E**
014	**E**
015	**E**

016	E
017	E
018	E
019	E
020	E
021	E
022	E
023	E
024	E
025	E
026	E
027	E
028	E
029	E
030	E
031	E
032	E
033	E
034	E
035	E
036	E
037	E
038	E
039	E
040	E
041	E
042	E
043	E
044	E
045	E
046	E
047	E
048	E
049	E
050	E
051	E
052	E
053	E
054	E
055	E
056	E
057	E

058	E
059	E
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063	E
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065	E
066	E
067	E
068	E
069	E
070	E
071	E
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073	E
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076	E
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078	E
079	E
080	E
081	E
082	E
083	E
084	E
085	E
086	E
087	E
088	E
089	E
090	E
091	E
092	E
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095	E
096	E
097	E
098	E
099	E

100	E
101	E
102	E
103	E
104	E
105	E
106	E
107	E
108	E
109	E
110	E
111	E
112	E
113	E
114	E
115	E
116	E
117	E
118	E
119	E
120	E
121	B
122	B
123	B
124	B
125	B
126	B
127	I
128	J
129	J
130	J
131	J

LC/MS analysis: $(M)^{+}$: Compound 005, expected: 1309.97, found: 1308.5 $(M+1)^{1+}$; Compound 009, expected: 1300.54, found: 1299.54 $(M+1)^{1+}$; Compound 012, expected: 1325.59, found: 1324.56 $(M+1)^{1+}$; Compound 013, expected: 1303.58, found: 1302.57 $(M+1)^{1+}$; Compound 021, expected: 1276.5, found: 1275.54 $(M+1)^{1+}$; Compound 025, expected: 1281.6, found: 1280.59 $(M+1)^{1+}$; Compound 029, expected: 1272.51, found: 1271.51 $(M+1)^{1+}$; Compound 039, expected: 1274.49, found: 1273.51 $(M+1)^{1+}$; Compound 040, expected: 1288.52, found: 1287.53 $(M+1)^{1+}$; Compound 044, expected: 1234.52, found: 1233.55 $(M+1)^{1+}$; Compound 046, expected: 1233.56, found: 1232.56 $(M+1)^{1+}$; Compound

050, expected: 1272.5, found: 1271.51 $(M+1)^{1+}$; Compound 052, expected: 1286.53, found: 1285.53 $(M+1)^{1+}$; Compound 058, expected: 1246.46, found: 1245.5 $(M+1)^{1+}$; Compound 063, expected: 1244.49, found: 1243.52 $(M+1)^{1+}$; Compound 069, expected: 1232.43, found: 1231.48 $(M+1)^{1+}$; Compound 071, expected: 1258.47, found: 1257.5 $(M+1)^{1+}$; Compound 072, expected: 1161.35, found: 1160.44 $(M+1)^{1+}$; Compound 073, expected: 1175.38, found: 1174.46 $(M+1)^{1+}$; Compound 077, expected: 1253.52, found: 1252.52 $(M+1)^{1+}$; Compound 080, expected: 1273.55, found: 1272.53 $(M+1)^{1+}$; Compound 083, expected: 1307.93, found: 1306.47 $(M+1)^{1+}$; Compound 094, expected: 1260.5, found: 1259.51 $(M+1)^{1+}$; Compound 099, expected: 1203.45, found: 1202.49 $(M+1)^{1+}$; Compound 114, expected: 1327.52, found: 1326.53 $(M+1)^{1+}$; Compound 115, expected: 1458.67, found: 1457.59 $(M+1)^{1+}$; Compound 121, expected: 1196.35, found: 1195.55 $(M+1)^{1+}$; Compound 122, expected: 1160.36, found: 1159.59 $(M+1)^{1+}$; Compound 127, expected: 1131.24, found: 1130.51 $(M+1)^{1+}$; Compound 129, expected: 1383.6, found: 1382.55 $(M+1)^{1+}$.

Example 2. Identification of Polypeptides that Bind Specifically to Human PCSK9 and Inhibit Interaction between Human PCSK9 and Human LDLR

A set of cyclic polypeptides were identified to inhibit the interaction between human PCSK9 and human LDLR. Each polypeptide has an N-terminal amino acid or moiety and a C-terminal amino acid or moiety that is linked by a covalent bond to the other.

A time-resolved fluorescence resonance energy (TR-FRET) assay was used to measure inhibition of the PCSK9-LDLR protein-protein interaction. Briefly, 20 nM avitag-biotinylated human PCSK9 was incubated with 20 nM his-tagged human LDLR EGFa domain in the presence of 5 nM LANCE Ulight Streptavidin (Perkin Elmer) and 5 nM europium-Anti-6xHis (Perkin Elmer) for 2 hours covered at room temperature in buffer containing 50 mM HEPES, 150 mM NaCl, 5 mM CaCl2, 0.01% BSA and 0.01% Surfactant P20. Compounds were tested in dose-response and concentrations giving half-maximal inhibition calculated using a 4-parameter fit equation.

Table 5 shows IC_{50} values obtained by the assay for a group of select compounds. In **Table 5**, **"A" represents 1 nM < IC_{50} ≤ 100 nM**; **"B" represents** 100 nM < IC_{50} ≤ 1000 nM; **"C" represents 1000 nM < IC_{50} < 12,100 nM; and "D" represents 21,000 nM < IC_{50}** < 50,000 nM.

TABLE 5	
Compound	**IC_{50}**
002	**A**
003	**C**
004	**C**

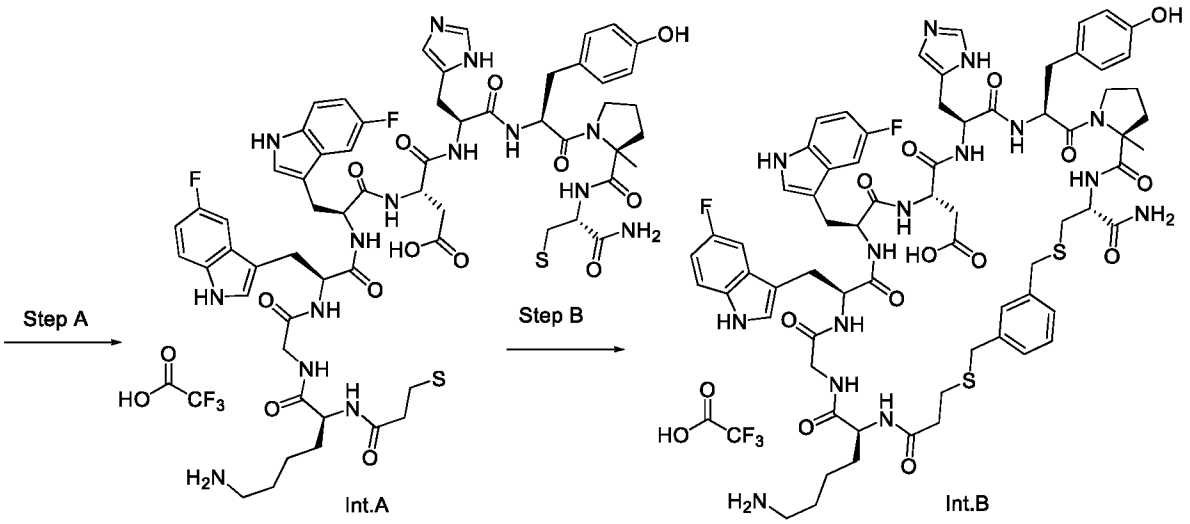
005	B
006	B
007	C
008	B
010	B
011	A
012	A
013	A
014	A
015	A
016	A
017	B
018	B
019	D
020	D
021	B
023	C
024	A
025	A
026	A
030	B
031	B
032	B
033	C
034	B
035	B
036	B
037	B
038	B
040	A
041	A
045	A
050	A
051	A
052	B
053	B
054	B
055	C
057	B
058	D
062	A
063	A

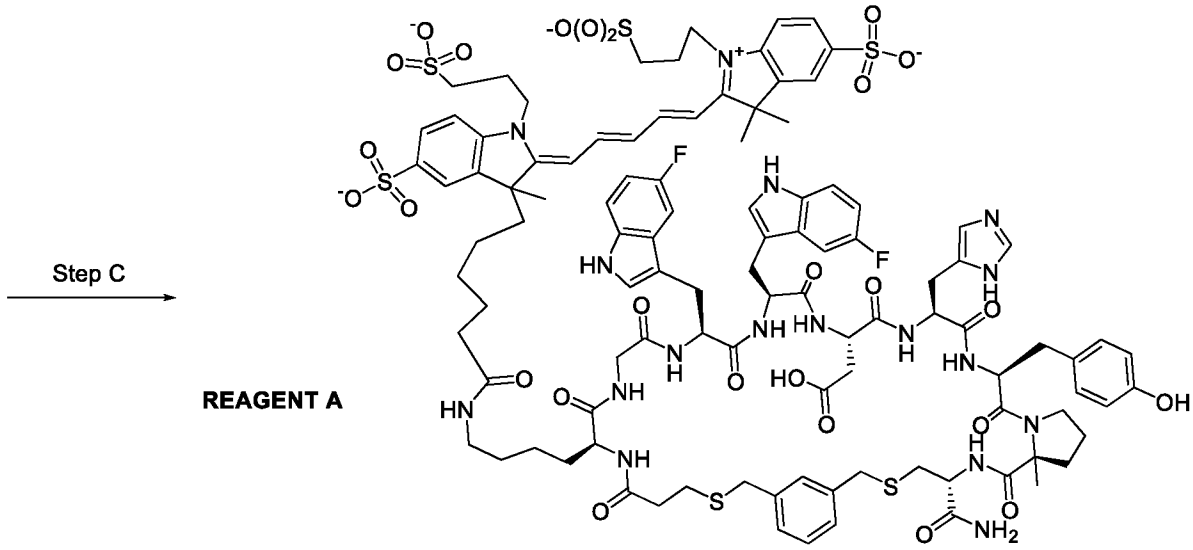
065	D
067	C
078	B
079	B
080	C
081	C
083	D
084	B
088	D
089	A
090	C
091	B
092	D
093	C
094	B
095	B
098	B
099	C
100	C
101	A
102	A
104	C
105	B
106	B
110	C
111	C
112	B
114	B
115	B
117	B
121	B
122	C
123	B
124	B
125	B
126	A
127	B
128	B
129	C

Example 3. ALEXA FRET

The PCSK9 Alexa FRET Standard assay measured the interaction between PCSK9 and an AlexaFluor647 (AF) tagged cyclic peptide, Reagent A (K_D = 83nM). A solution containing 1 nM biotinylated PCSK9 + 2.5 nM Lance Streptavidin Europium (Strep-Eu) was made in 50 mM HEPES pH 7.4, 0.15 M NaCl, 5 mM CaCl2, 0.01% BSA, and 0.01% Surfactant P20. A separate solution containing 40 nM of the AlexaFluor tagged cyclic peptide was made in the same buffer system. An Echo was used to transfer 0.750ul of compound to an assay plate followed by the addition of 15ul of PCSK9+Stept-Eu and 15ul of AF peptide. The final assay volume was 30.750ul containing 0.5nM PCSK9, 1.25nM Strep-Eu, and 20nM AF cyclic peptide. The reaction was incubated at room temperature for at least two hours prior to fluorescence measurements using an Envision Multilabel Reader. IC_{50} values were determined by fitting data to a sigmoidal dose-response curve using nonlinear regression. K_i was then calculated from the IC_{50} and the K_D of AF cyclic peptide. Counts (B-counts) of the europium-labeled PCSK9 were followed to observe if compounds were adversely PCSK9. A fall off of the B-counts likely indicated a false positive of inhibition. **Data from this procedure was reported as "A = 'numerical value' (nanomolar)"**

Reagent A was prepared in accordance with the following method:





Step A – Synthesis of Intermediate Compound ***Int-A***

The peptide was synthesized on a 0.250 mmol scale on CEM Liberty Blue, Microwave synthesizer using Fmoc/*t*Bu chemistry on PS Rink-Amide MBHA resin, 0.32 mmol g^{-1}. The assembly was performed using single-couplings using 4eq of Fmoc protected amino acid 0.2M in DMF, 4eq of 0.5M HATU in DMF, 4eq of 2M DIPEA (double coupling for Tyr). Fmoc deprotection cycles were performed using 20% (V/V) piperidine in DMF.

The sequence of Fmoc protected amino acids and building blocks used were:

1. N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-trityl-L-cysteine
2. (S)-1((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-methylpyrrolidine-2-carboxylic acid
3. (((9H-fluoren-9-yl)methoxy)carbonyl)-L-tyrosine
4. N-(((9H-fluoren-9-yl)methoxy)carbonyl)-N-trityl-L-histidine
5. (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(tert-butoxy)-4-oxobutanoic acid
6. (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(5-fluoro-1H-indol-3-yl)propanoic acid
7. (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(5-fluoro-1H-indol-3-yl)propanoic acid
8. (((9H-fluoren-9-yl)methoxy)carbonyl)glycine
9. N^2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N^6-(tert-butoxycarbonyl)-L-lysine
10. 3-(tritylthio)propanoic acid

At the end of the assembly, the resin was washed with DMF, MeOH, DCM, Et_2O. The peptide was cleaved from solid support using 50 ml of TFA solution (v/v) (91% TFA, 5% H_2O, 4% TIPS) for approximately 1.5 hours, at room temperature. The resin was filtered, washed with TFA and solution concentrated to dryness and lyophilized. Lyophilization afforded *Intermediate Compound* **Int. A** (399mg), which was used as crude in the next step. LCMS analysis was calculated for C61H75F2N15O13S2: 1328.48, found: 1328.2 $(M+1)^+$

Step B – Synthesis of Intermediate Compound ***Int-B***

Crude ***Int-A*** (0.22 mmol) was redissolved in 24ml of DMF. 6ml of 1M aqueous solution of sodium bicarbonate was added to raise the pH to 7. Then 0.26 mmol of **1,3-bis(bromomethyl)benzene** (0.1M in DMF) was added dropwise. The reaction was left under stirring at room temperature for 20 minutes, quenched with TFA (pH to 3-4) and then concentrated in vacuo to provide crude ***Int-B***, which was purified by RP-HPLC (Waters XBridge, C18, 50x150 mm, 5□m, 130A; 25% to 40% ACN/water + 0.1% TFA modifier over 20 min). Collected fractions were lyophilized to afford 35mg of *Intermediate Compound* ***Int-B.*** LCMS analysis was calculated. for C71H85F2N15O13S2: 1458.67; found: 1458.8 $(M+1)^+$.

Step C – Synthesis of Compound ***Reagent A:*** *Intermediate Compound* ***Int-B*** (15mg) was dissolved in 0.2ml of dry DMSO. Then 15mg of ALEXAFLUOR 647NHS Ester (A37566, Life technology) dissolved in 1.5ml of dry DMSO was added. 20uL of dry DIPEA was added. The reaction was left under stirring at room temperature for 12 hours under Nitrogen atmosphere in the dark. Quenched with TFA (pH to 3-4) and purified by RP-HPLC (Dr Maish, Reprosil Gold C18, 250x20 mm, 120 Å, 10μm; 20% to 35% of 0.1% TFA in ACN/0.1% TFA in H_2O, over 20min, then 35% to 40% over 5min at 20 mL/min flow rate). Collected fractions were lyophilized to afford 16.1 mg of *Compound* ***Reagent A.***
LCMS analysis was calculated for $C105H122F2N17O26S6^{3-}$: 2268.58; 1135.8 $(M+2)^{2+}$.

Table 6 shows IC_{50} values obtained by the assay for a group of select compounds. In **Table 6**, **"A" represents 1 nM < IC_{50} ≤ 100 nM**; **"B" represents 100** nM < IC_{50} ≤ 1000 nM; **"C" represents 1000 nM < IC_{50} < 12,100 nM; and "D" represents 21,000 nM < IC_{50}** < 50,000 nM.

TABLE 6	
Compound	**IC_{50}**
006	**B**
011	**A**
012	**A**
013	**A**

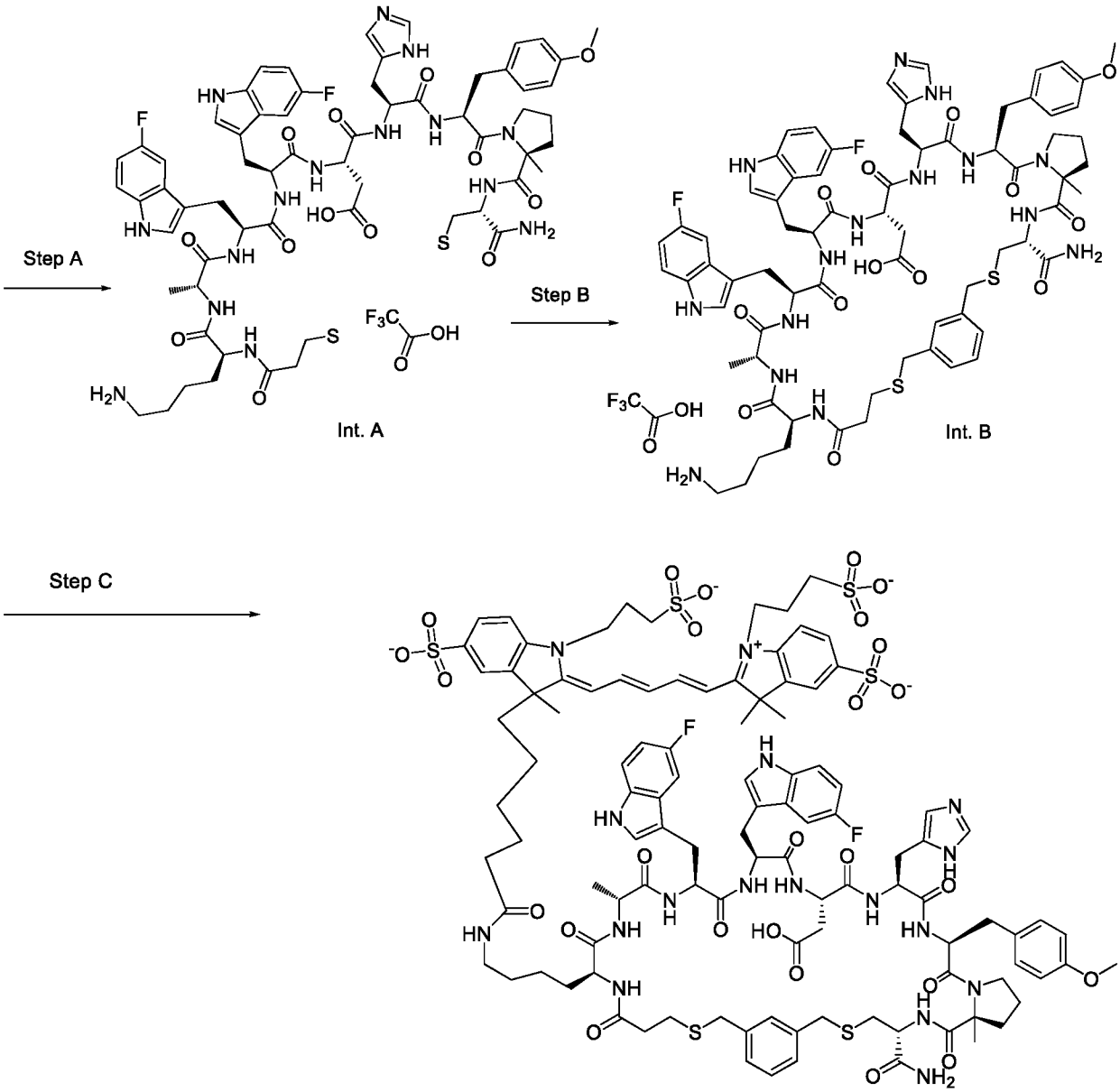
014	A
015	A
016	A
017	C
024	A
025	A
026	A
034	A
036	B
041	A
045	A
050	C
053	C
062	B
063	A
084	A
089	A
101	A
102	A
114	B
117	A
121	A
122	B
123	A
124	A
125	B
126	A
127	A
128	A
129	C
130	B
131	A

Example 4. ALEXA PLUS FRET

The PCSK9 Alexa FRET Plus assay measured the interaction between PCSK9 and an AlexaFluor647 (AF) tagged cyclic peptide, Reagent B (K_D = 35nM). A solution containing 1 nM biotinylated PCSK9 + 2.5 nM Lance Streptavidin Europium (Strep-Eu) was made in 50 mM HEPES pH 7.4, 0.15 M NaCl, 5 mM CaCl2, 0.01% BSA, and 0.01% Surfactant P20. A separate solution containing 1920 nM of the AlexaFluor tagged cyclic peptide was made in the same buffer system. An Echo was used to transfer 0.075ul of compound plus 0.675ul of DMSO to each well of an assay plate followed by the addition of 15ul of PCSK9+Stept-Eu and 15ul of AF peptide. The final assay volume was 30.750ul containing 0.5nM PCSK9,

1.25nM Strep-Eu, and 960nM AF cyclic peptide. The reaction was incubated at room temperature for at least two hours prior to fluorescence measurements using an Envision Multilabel Reader. IC_{50} values were determined by fitting data to a sigmoidal dose-response curve using nonlinear regression. K_i was then calculated from the IC_{50} and the K_D of AF cyclic peptide. Counts (B-counts) of the europium-labeled PCSK9 were followed to observe if compounds were adversely affecting PCSK9. A fall off of the B-counts likely indicated a false positive of inhibition. Data from this procedure was reported as "P= 'numerical value' (nanomolar)"

Reagent B was prepared by the following procedure.



Step A – Synthesis of Intermediate Compound ***Int-A***

The peptide was synthesized on a 0.250 mmol scale on CEM Liberty Blue, Microwave synthesizer using Fmoc/*t*Bu chemistry on PS Rink-Amide MBHA resin, 0.32 mmol g^{-1}. The assembly was performed using single-couplings using 4eq of Fmoc protected amino acid 0.2M in DMF, 4eq of 1M Oxyme in DMF, 4eq of 0.5M *N,N*-diisopropylcarbodiimide (DIC) (double coupling for Y01). Fmoc deprotection cycles were performed using 20% (V/V) piperidine in DMF.

The sequence of Fmoc protected amino acids and building blocks used were:

1. N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-trityl-L-cysteine
2. (S)-1((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-methylpyrrolidine-2-carboxylic acid
3. (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-methoxyphenyl)propanoic acid
4. N-(((9H-fluoren-9-yl)methoxy)carbonyl)-N-trityl-L-histidine
5. (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(tert-butoxy)-4-oxobutanoic acid
6. (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(5-fluoro-1H-indol-3-yl)propanoic acid
7. (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(5-fluoro-1H-indol-3-yl)propanoic acid
8. (((9H-fluoren-9-yl)methoxy)carbonyl)-D-alanine
9. N^2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N^6-(tert-butoxycarbonyl)-L-lysine
10. 3-(tritylthio)propanoic acid

At the end of the assembly, the resin was washed with DMF, MeOH, DCM, Et_2O. The peptide was cleaved from solid support using 50 ml of TFA solution (v/v) (91% TFA, 5% H_2O, 4% TIPS) for approximately 1.5 hours, at room temperature. The resin was filtered, washed with TFA and solution concentrated to dryness and lyophilized. Lyophilization afforded *Intermediate Compound* **Int. A** (300mg), which was used as crude in the next step. LCMS analysis was calculated for C63H79F2N15O13S2: 1356.53, found: 1356.9 $(M+1)^+$.

Step B – Synthesis of Intermediate Compound ***Int-B***

Crude ***Int-A*** (0.22 mmol) was redissolved in 24ml of DMF. 6ml of 1M aqueous solution of sodium bicarbonate was added to raise the pH to 7. Then 0.26 mmol of **1,3-bis(bromomethyl)benzene** (0.1M in DMF) was added dropwise. The reaction was left under stirring at room temperature for 20 minutes, quenched with TFA (pH to 3-4) and then concentrated in vacuo to provide crude ***Int-B***, which was purified by RP-HPLC (Waters

XBridge, C18, 50x150 mm, 5□m, 130A; 25% to 40% ACN/water + 0.1% TFA modifier over 20 min). Collected fractions were lyophilized to afford 35mg of *Intermediate Compound* ***Int-B.*** LCMS analysis was calculated. for C71H85F2N15O13S2: 1458.67; found: 1458.8 $(M+1)^+$.

Step C – Synthesis of Compound ***Reagent B***

Intermediate Compound ***Int-B*** (15mg) was dissolved in 0.2ml of dry DMSO. Then 15mg of ALEXAFLUOR 647NHS Ester (A37566, Life technology) dissolved in 1.5ml of dry DMSO was added. 20uL of dry DIPEA was added. The reaction was left under stirring at room temperature for 12 hours under Nitrogen atmosphere in the dark. Quenched with TFA (pH to 3-4) and purified by RP-HPLC (Dr Maish, Reprosil Gold C18, 250x20 mm, 120 Å, 10µm; 20% to 35% of 0.1% TFA in ACN/0.1% TFA in H_2O, over 20min, then 35% to 40% over 5min at 20 mL/min flow rate). Collected fractions were lyophilized to afford 16.1 mg of *Compound* ***Reagent B.*** LCMS analysis for $C107H126F2N17O26S6^{3-}$:2296.64; found: 1150.6 $(M+2)^{2+}$.

Activity data obtained by one or both of the above-described procedures was reported for selected example compounds of the invention in the following format:

Example **No.: A (standard TR Fret) = 'numerical value'; P (Alexa Fret plus standard TR Fret) = 'numerical value' /, note that all values reported are nanomolar.**

Table 7 shows IC_{50} values obtained by the assay for a group of select compounds. In **Table 7**, "A" represents 1nM < $IC_{50} \leq$ **100 nM; "B" represents 100 nM < $IC_{50} \leq$** 500 nM; **and "C" represents 500 nM < $IC_{50} \leq$** 5000 nM.

TABLE 7	
Compound	**IC_{50}**
016	**A**
024	**A**
025	**A**
026	**B**
062	**A**
089	**A**
130	**B**
131	**A**

* * * * *

The contents of the articles, patents, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicants

reserve the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other physical and electronic documents.

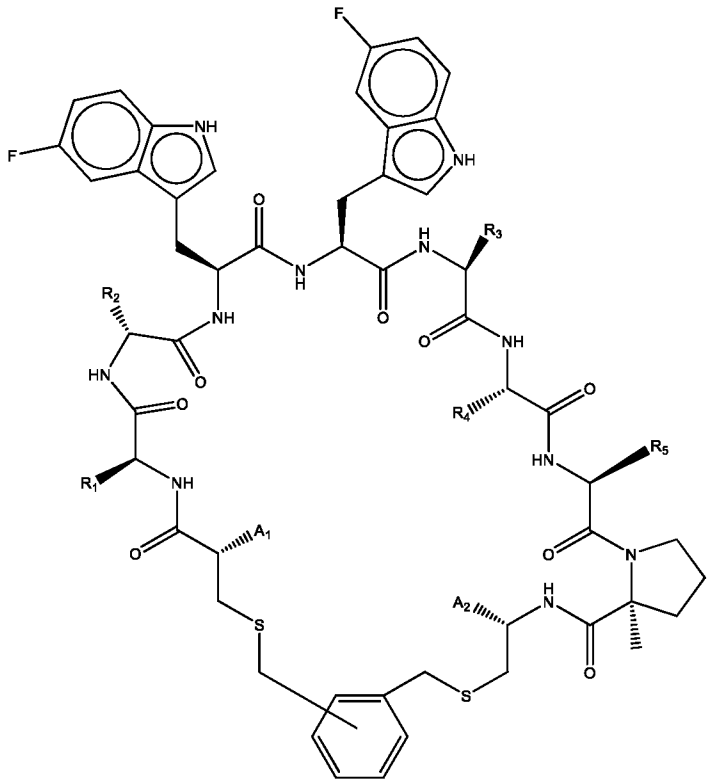
The methods illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising," "including," containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof. It is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the invention embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the methods. This includes the generic description of the methods with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

CLAIMS

What is claimed is:

1. A polycyclic peptide of Formula (I):



(I)

or a pharmaceutically acceptable salt thereof;

wherein:

A_1 is H or an acyl protected amine;

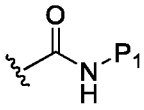
R_1 is selected from the group consisting of the amino acid side chains of ALA, GLY, ACB, ACPE, APC, LYS, NVA, PRO, and TBG;

R_2 is selected from the group consisting of the amino acid side chains of ALA and GLY;

R_3 is selected from the group consisting of the amino acid side chains of 4SFPRO, AIB, AZE, 4RFPRO, DHPRO, 4DFPRO, PIP, PRO, HSE, SISQ, and RISQ;

R_4 is selected from the group consisting of the amino acid side chains of ALA, HIS, GLY, and PRO;

R_5 is selected from the group consisting of the amino acid side chains of TYR, 4CF, 4FF, MFF, CNPP, 2NA, 1NA, F3MF, PHE, PRO, and OMY;

A_2 is absent or  ; and

P_1 is selected from the group consisting of H and acetyl;
wherein each amino acid residue is optionally an N-methylated amino acid;
wherein each amino acid residue can be the R or S-enantiomer configuration.

2. The compound of claim 1, wherein the compound is selected from:
002, 029, 030, 031,
032, 033, 034, 035, 036, 037, 038, 039, 040, 041, 047, 048, 050, 051, 052, 053, 054, 057, 058, 061, 062, 063, 064, 065, 066, 068, 069, 070, 071, 081, 082, 083, 084, 085, 086, 087, 088, 089, 091, 092, 093, 094, 095, 103, or 117.

3. The compound of claim 1, wherein the compound is selected from: 002, 040, 041, 050, 051, 062, 063, or 089.

4. A cyclic peptide compound:
001, 003, 004, 005, 006, 007, 008, 009, 010, 011, 012, 013,
014, 015, 016, 017, 018, 019, 020, 021, 022, 023, 024, 025, 026, 027, 028, 042, 043, 044, 045, 046, 049, 055, 056, 059, 060, 067, 072, 073, 074, 075, 076, 077, 078, 079, 080, 090, 096, 097, 098, 099, 100, 101, 102, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 118, 119, or 120.

5. The compound of claim 4, wherein the compound is selected from: 011, 012, 013, 014, 015, 016, 024, 025, 026, 045, 101, or 102.

6. The compound of claim 4, wherein the compound is selected from: 101 or 102.

7. A cyclic peptide compound: 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, or 131.

8. The compound of claim 7, wherein the compound is selected from: 126 or 127.

9. A pharmaceutical composition comprising a cyclic polypeptide compound of any one of claims 1-8 and a pharmaceutically acceptable carrier.

10. A method of reducing low density lipoprotein (LDL) cholesterol level in a subject in need thereof comprising administering to the subject a therapeutically effective amount of the cyclic polypeptide compound of any one of claims 1-8, or the pharmaceutical composition of claim 9.

11. The method of claim 10, wherein the subject has hypercholesterolemia.

12. The method of claim 11, wherein the cyclic polypeptide compound inhibits the interaction between human PCSK9 and epidermal growth factor-like repeat A (EGF-A) domain of human low density lipoprotein (LDLR).

13. A method of treating hypercholesterolemia in a subject in need thereof comprising administering to the subject a therapeutically effective amount of the cyclic polypeptide compound of any one of claims 1-8, or the pharmaceutical composition of claim 9.

14. The method of claim 13, wherein the subject further suffers from a disease that shows comorbidity with hypercholesterolemia.

15. The method of claim 14, wherein the disease that shows comorbidity with hypercholesterolemia is selected from the group consisting of nephrotic syndrome, kidney failure, coronary artery disease, atherosclerosis, stroke, peripheral vascular disease, diabetes, and high blood pressure.

16. A method of inhibiting PCSK9 activity in a subject in need thereof comprising administering to the subject a therapeutically effective amount of the cyclic polypeptide compound of claims 1-8, or the pharmaceutical composition of claim 9.

17. A method of inhibiting PCSK9 activity in a cell comprising contacting the cell with the cyclic polypeptide compound of claims 1-8, or the pharmaceutical composition of claim 9.

18. A method of inhibiting the interaction between PCSK9 and the EGF-A domain of LDLR in a subject in need thereof comprising administering to the subject a therapeutically effective amount of the cyclic polypeptide compound of claims 1-8, or the pharmaceutical composition of claim 9.

19. The method of any of claims 10-18, wherein the administration is selected from the group consisting of oral, intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, and intravitreal.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/38221

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/12, 31/175, 38/08 (2019.01)

CPC - A61K 38/12, 31/175, 38/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2017/0189470 A1 (RA PHARMACEUTICALS, INC.) 06 July 2017; paragraphs [0014], [0121], [0191]	1, 4-5, 91/, 9/4-5
A	WO 2012/040259 A2 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA, et al.) 29 March 2012; abstract; page 3, paragraph 4; claim 7	1, 4-5, 91/, 9/4-5
A	(ZHANG, Y et al.) Identification of a Small Peptide That Inhibits PCSK9 Protein Binding to the Low Density Lipoprotein Receptor. Journal of Biological Chemistry. 13 November 2013; Vol. 289, No. 2; pages 942-955; page 943, column 1, paragraphs 3, 5; DOI: 10.1074/jbc.M113.514067	1, 4-5, 91/, 9/4-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 October 2019 (28.10.2019)	18 NOV 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/38221

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2-3
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

-***- Please See Next Supplemental Page -***-

3. ☒ Claims Nos.: 10-19
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***- Please See Next Supplemental Page -***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Group I, Claims 1, 4-5 (each in-part), 9 (in-part)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

-***-Continued from Box No. II Observations where certain claims were found unsearchable-***-

Claims 2 and 3 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims 2 and 3 are indefinite for the following reason(s): the claims recite compounds inconsistent with the structural formula present in the claim from which they are indicated to depend (Claim 1). In particular, none of the recited compounds includes a required 2-methylproline residue in the peptide immediately following the amino acid containing the R5 group. As such, these claims have been considered improper dependent claims because the claims expand upon, rather than restrict, the claim from which they are indicated to depend. Therefore these claims have been considered unsearchable.

-***-Continued from Box III Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, Claims 1, 4-5 (each in-part), 9 (in-part) are directed toward cyclic peptides and pharmaceutical compositions comprising the peptides. [It is noted that compounds 003, 045, 107 and 111 are consistent with Formula I, and thus these compounds in Claims 4 and/or 5 constitute the portions of Claims 4 and/or 5 that apply to Group I].

Groups II+, Claims 4-6 (each in-part), 9 (in-part) and compound 001 (cyclic peptide compound) are directed toward cyclic peptides and pharmaceutical compositions comprising the peptides. [It is noted that compounds 003, 045, 107 and 111 are excluded from Groups II+ with regard to Claims 4 and/or 5].

The cyclic peptides and compositions can be searched to the extent they encompass compound 001 (first exemplary cyclic peptide compound). Applicant is invited to elect additional cyclic peptide(s), with specified compound number for each, and available as an option within at least one searchable claim, to be searched. Additional cyclic peptide(s) can be searched upon the payment of additional fees. It is believed that claims 4 (in-part), and 9 (in-part) encompass this first named invention of Groups II+ and thus these claims can be searched with payment of a fee for the search of Groups II+, to the extent that they encompass compound 001 (cyclic peptide compound). Applicants must specify the searchable claims that encompass any additionally elected cyclic peptide(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention of Groups II+, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) can result in only the first claimed invention of groups II+ to be searched/examined. An exemplary election would be compound 004 (cyclic peptide compound).

Groups III+, Claims 7, 8, 9 (in-part) and compound 121 (polycyclic peptide compound) are directed toward polycyclic peptides and pharmaceutical compositions comprising the peptides.

The polycyclic peptides and compositions can be searched to the extent they encompass compound 121 (first exemplary polycyclic peptide compound). Applicant is invited to elect additional polycyclic peptide(s), with specified compound number for each, and available as an option within at least one searchable claim, to be searched. Additional polycyclic peptide(s) can be searched upon the payment of additional fees. It is believed that claims 7 (in-part), and 9 (in-part) encompass this first named invention of Groups III+ and thus these claims can be searched with payment of a fee for the search of Groups III+, to the extent that they encompass compound 121 (polycyclic peptide compound). Applicants must specify the searchable claims that encompass any additionally elected polycyclic peptide(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention of Groups III+, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "III+" group(s) can result in only the first claimed invention of groups III+ to be searched/examined. An exemplary election would be compound 122 (polycyclic peptide compound).

The inventions listed as Groups I, II+ and III+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a compound of Formula I, not present in any of Groups II+ or III+; the special technical features of Groups II+ include compound 001, not present in either of Groups I or III+; the special technical features of Groups III+ include compound 121, not present in any of Groups I or II+.

Groups I, II+ and III+ share the technical features including: a cyclic peptide comprising at least one 1,4-bis[(methylsulfanyl)methyl] benzene bridging moiety; and a pharmaceutical composition comprising a cyclic polypeptide compound and a pharmaceutically acceptable carrier. Groups III+ share the technical features including: polycyclic peptides.

However, these shared technical features are previously disclosed by US 2017/0189470 A1 to Ra Pharmaceuticals, Inc. (hereinafter 'Ra').

Ra discloses a cyclic peptide (cyclic peptides; paragraph [0014]) comprising at least one 1,4-bis[(methylsulfanyl)methyl]benzene bridging moiety (wherein the peptide is cyclized with a bis(bromomethyl)benzene (comprising at least one bridged 1,4-bis[(methylsulfanyl)methyl] benzene bridging group); paragraph [0191]); a pharmaceutical composition comprising a cyclic polypeptide compound and a pharmaceutically acceptable carrier (a pharmaceutical composition comprising a cyclic polypeptide compound and a pharmaceutically acceptable carrier; paragraphs [0014], [0022]); and polycyclic peptides (bicyclic (polycyclic) peptides; paragraph [0121]).

No technical features are shared between the cyclic peptides of Groups II+ and, accordingly, these groups lack unity a priori.

No technical features are shared between the polycyclic peptides of Groups III+ and, accordingly, these groups lack unity a priori.

Since none of the special technical features of the Groups , II+ and III+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Ra references, unity of invention is lacking.