Enzymatic Inhibitory Activity of Hydroxycinnamates (HCs) *In silico* studies

MASTER DISSERTATION

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MASTER IN APPLIED BIOCHEMISTRY

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Abstract

Diabetes is a worldwide health issue that has been expanding mainly in developed countries. It is characterized by abnormal levels of blood sugar due to several factors. The most common are resistance to insulin and the production of defective insulin which exerts little or no effect. Its most common symptoms include tissue damage to several systems due to elevated levels of blood sugar. One of the key enzymes in hydrocarbon metabolism is α-glucosidase (EC 3.2.1.20). It catalyzes the breakdown of complex carbohydrates into their respective monomers (glucose) which allows them to be absorbed. In this work, caffeoyl quinic acids and their metabolites were analyzed as potential inhibitors for α-glucosidase. The search for the best inhibitor was conducted using molecular docking. The affinity of each compound was compared to the inhibitor present in the crystal structure of the protein. As no inhibitor with a similar affinity was found, a new approach was used, *in situ* drug design. It was not possible to achieve an inhibitor capable of competing with the one present in the crystal structure of the enzyme, which is also its current commercial inhibitor. It is possible to draw some conclusions as to which functional groups interact best with certain residues of the active site.

This work was divided into three main sections. The first section, Diabetes, serves as an introduction to what is Diabetes, its symptoms and/or side effects and how caffeoyl quinic acids could be used as a treatment. The second section, Caffeoylquinic acids and their metabolites as inhibitors for Alfa-glucosidase, corresponds to the search through molecular docking of caffeoyl quinic acids as inhibitors for α-glucosidase and what was possible to draw from this search. The last section, *In situ* design of an inhibitor for α-glucosidase (EC 3.2.1.20), corresponds to the *in situ* drug design study and what it achieved. The representation of each of the molecules used as a ligand can be found in the Annexes.

Keywords:

Diabetes; α-glucosidase; caffeoyl quinic acid; molecular docking; inhibitor; in situ drug design

Abbreviations and Acronyms

AD - Alzheimer's Disease, ADT - AutoDock Tools, AMPK - Adenosine Monophosphate-activated Protein Kinase, AR - Aldose Reductase, CPT - Carnitine Palmitoyltransferase, DPP-4 - Dipeptidyl Peptidase-4, G6P - Glucose-6-phosphatase, GA - Genetic Algorithms, GIP - Gastric Inhibitory Polypetide, GLP-1 - Glucagon-like Peptide 1, HBV - Hepatitis B Virus, HDL - High Density Lipoprotein, HIV - Human Immunodeficiency Virus, IC - Incremental Construction, IGT - Impaired Glucose Tolerance, LDL - Low Density Lipoprotein, MC - Monte Carlo, MCSS - Multiple Copy Simultaneous Search, MD - Molecular Dynamics, QSAR - Quantitative Structure-Activity Relationship

Diabetes

According to the World Health Organization (WHO) [1], 346 million people suffer from Diabetes. Although death due to Diabetes occurs mainly in countries with low or medium income, highly developed countries have a larger number of citizens who suffer from Diabetes or Impaired Glucose Tolerance (IGT).

The most common types of Diabetes are: Diabetes type 1, Diabetes type 2 and Gestational Diabetes [2]. Type 1 Diabetes is the result of an autoimmune disease in which the beta cells of the pancreas (insulin producing cells) are destroyed. In type 2 Diabetes, the insulin production is generally unhindered but, the insulin that is produced is either defective (mutated) or the individual has developed a resistance to it, making it less effective. Gestational Diabetes is a type of Diabetes that affects women only during pregnancy. It also increases the risk of developing type 2 Diabetes [2]. Alzheimer's disease is considered in the work of Monte *et al*. [3–5] as type 3 Diabetes. Their work also indicates that the use of Diabetes prescribed medication in Alzheimer's patients reversed its effects.

Approximately 21 million people suffer from type 2 Diabetes and 42 million suffer from IGT in the USA [6]. In Japan, approximately 13,5% of the population is affected by either type 2 Diabetes or IGT [7]. In sub-Saharan Africa, Diabetes causes a severe burden on the populations [8]. The most affected, are the inhabitants of urban Kenya with 12% suffering from type 2 Diabetes and the less affected population is from rural Uganda with only 1% suffering from this form of Diabetes.

Diabetes is characterized by high levels of glucose in the blood stream due to either low production of insulin or ineffective insulin. The elevated glucose levels cause damage to several organs and systems and have a higher impact in the nervous and circulatory systems [1].

Kolluru *et al.* [9], describe the several body systems affected by Diabetes (Figure 1). Diabetes makes the host more susceptible to infections, such as tuberculosis and pneumonia. Some retro-viral treatments for HIV cause insulin resistance and thus increase the chances of developing Diabetes [8].

Treatments and Therapeutic targets in Diabetes Treatment

There are several treatments and therapeutic targets in the treatment of Diabetes. These vary according to the type of Diabetes and, if the treatment is intended only for Diabetes or if it is also intended for any of the associated medical conditions. The most common and cost effective control in Diabetes is monitoring blood pressure and glucose and cholesterol levels [7,10,11].

Adenosine monophosphate-activated protein kinase (AMPK)

AMPK is vital in regulating energy metabolism [12,13]. It affects lipid and carbohydrate metabolisms by regulating the fatty acid oxidation, cholesterol synthesis, glucose uptake by muscle cells and insulin secretion among other processes. AMPK is present in several organs such as the liver, brain and skeletal muscle.

Activating AMPK on the liver will inhibit the production of fatty acids and sterols and inhibits lipogenesis in adipose tissue [12,13]. The levels of activated AMPK vary according to physical activity and, when activated, AMPK increases the glucose uptake of skeletal muscle cells [13,14]. AMPK activation also increases nitrous oxide (NO) bioactivity, lowers insulin resistance, suppresses the production of radicals and reduces inflammation [12].

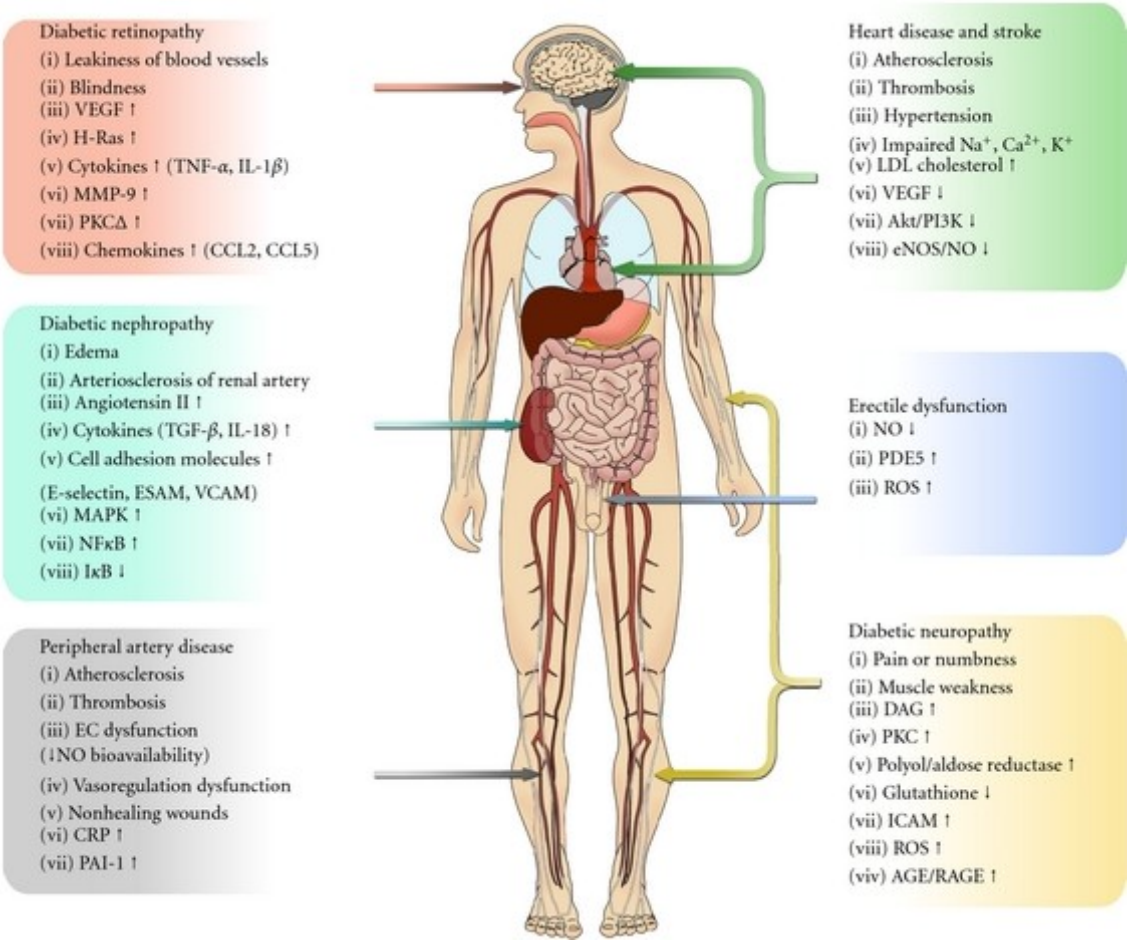


Figure 1 - Effects of Diabetes as described by Kolluru *et al.* [9]

Glucagon-like Peptide 1(GLP-1) and Gastric Inhibitory Polypetide (GIP)

Both GLP-1 and GIP are released in response to a meal in the intestines and are commonly referred to as incretin hormones. They are responsible for up to 70% of the release of insulin after a meal [15–17]. In type 2 Diabetes, the production of GIP does not significantly change but its insulin secretion effect is compromised. On the other

hand, GLP-1 secretion is impaired with type 2 Diabetes but its functions are not compromised [15].

GLP-1 inhibits glucagon secretion, reduces glucose levels in the blood and reduces hunger [6,15–19]. Due to its high therapeutic value, GLP-1 receptor agonists or GLP-1 analogues are used in the treatment of type 2 Diabetes. They generate the same response as GLP-1 in patients including weight loss and reduction of accumulated fat [15,16,18]. Individuals with lower body weight are less likely to develop type 2 Diabetes [17].

GIP stimulates insulin secretion, reduces body weight and induces the proliferation of pancreatic β-cells and prevents their apoptosis [17,20]. A higher number of β-cells has a higher chance of producing more insulin. However, the protection from apoptosis will lead to the preservation of non functional cells leading to a reduction in insulin production.

The effects of GIP, GLP-1 and GLP-1 receptor agonists or analogues are glucose dependant [6,15,21]. They are great therapeutic venues due to their effects and low chance of inducing hypoglycemia and are more practical than GLP-1 infusions [19]. These are designed to resist inactivation and metabolism through Dipetidyl peptisade-4 (DPP-4) [15].

The common side effects of GLP-1 receptor agonists are: nausea, diarrhea, headaches and dizziness [21,22]. The nausea these cause has been linked to the slower gastric emptying [21]. No gastrointestinal side effects were observed by Zander *et al.* [16] when administering a 6 week GLP-1 therapy. Mundil *et al.* [23] recently reviewed the side effects of GLP-1 receptor agonists on cardiovascular health. They found that these drugs had no adverse effects on cardiovascular health and additionally they reduced the risk factors for cardiovascular disease by reducing body weight, systolic blood pressure and reduced several biomarkers of cardiovascular disease.

Marathe *et al.* [19] suggest the use of GLP-1 receptor agonists in combination with insulin. As insulin affects the regulation of blood glucose before meals and GLP-1 agonists help regulate blood glucose after meals, Marathe *et al.* [19] stress that this would provide a better control of blood glucose levels without aggravated risk for hypoglycemia.

Regarding the safety of GLP-1 therapies, GLP-1 therapies my increase the risk of pancreatitis, pancreatic and thyroid cancer [23,24]. A more recent review by Marathe *et al.* [19] indicates that safety studies conducted in humans and monkeys did not provide a definite link between pancreatitis or pancreatic and thyroid cancers while of GLP-1 therapies. The authors indicated that the different levels of expression of the GLP-1 receptors in rats increased their odds of developing thyroid cancer.

Dipeptidyl peptidase-4(DPP-4)

DPP-4 is responsible for the metabolism and inactivation of incretin hormones such as GLP-1 and GIP [15,22]. Inhibitors for DPP-4 are used in the treatment of Diabetes as an

alternative or in conjunction with other drugs [25]. DPP-4 inhibitors are less effective than other therapies at lowering blood glucose levels and do not induce weight loss [19,21,25]. However, these have a very low chance of inducing hypoglycemia as, their effect is glucose dependant [21,22,25]. Their common side effects are: upper respiratory tract infections, headaches and inflammation of the nasal passages and upper pharynx [21,22]. The higher incidence of infections in patients undergoing DPP-4 inhibitor therapy has been associated with inhibition of DPP-4 in T cells [22]. Cases of pancreatitis have been reported in DPP-4 inhibitor therapy only after the marketing of the drug was initiated. Similar cases were not detected during clinical trials [21,22]. Reid [21] also indicates that these cases were resolved even with continued therapy of DPP-4 inhibitors and concludes that the pancreatitis is unlikely to be a side effect of the DPP-4 inhibitor therapy.

A new candidate for the inhibition of DDP-4 is presented by Parmar *et al.* [26]. Naringin, a flavonoid glucoside, successfully inhibited DPP-4 *in silico*, *in vitro* and *in vivo*. Although Parmar *et al.* [26] indicate that narigin is more effective than sitaglipin (a commercial inhibitor of DPP-4) at lowering blood glucose, pancreatic lipid peroxidation and of nitrate levels in the pancreas, tests for increased incidence of pancreatitis, pancreatic and thyroid cancer (all reported side effects of inhibiting DPP-4 [27,28]) were not conducted. Parmar *et al.* [26] conclude their work by stressing that despite their encouraging results, drug safety and toxicity tests should be performed with naringin before any clinical trial or use of the compound.

Busek *et al.* [27] indicate that several DPP-4 substrates promote malignant carcinogenesis in the colon and brain. Sun *et al.* [28] suggest that inhibiting DPP-4 may trigger prostate cancer metastasis as the invasive capacity of prostate cancer cells becomes enhanced.

Glucosidases/Glycosidases

Glucosidases or Glycosidases are enzymes under the category EC 3.2.1. Two common targets in this class of enzymes are alpha-amylase (EC 3.2.1.1) and alpha-glucosidase (EC 3.2.1.20). Alpha-Amylase catalyzes the hydrolysis of internal alpha-1,4-glucan links in polysaccharides containing 3 or more alpha-1,4-linked D-glucose units (endohydrolysis) while alpha-glucosidase hydrolyzes terminal non-reducing 1-4 linked alpha-glucose residues to release a single alpha-glucose molecule (exohydrolysis). Inhibiting either of these enzymes will delay the digestion and absorption of carbohydrates [2,29,30] but will generally cause flatulence and diarrhea as the carbohydrates that are not absorbed are later fermented [2]. The control over carbohydrate digestion and absorption will lead to a lower risk of Diabetes and obesity [30]. Inhibitors of these enzymes make up for the first line of defense against Diabetes [31] and they may also help in the fight against notorious diseases such as Human immunodeficiency virus (HIV), hepatitis B virus (HBV) and cancer [31–33]. In anti-viral therapy, inhibiting alpha-glucosidase (EC 3.2.1.20) prevents proper protein folding of some glycoproteins [32] and this prevents the proper formation of the viral envelope [31,32,34].

Hakamata *et al.* [31] stress that when designing inhibitors for these enzymes, like all other enzymes, researchers must consider substrate specificity and what is target organism. Each organism may have different sequences for the same enzyme and, as such, inhibitors that are effective in one species may not function in another.

Despite the existence of several synthetic inhibitors for alpha-amylase and alpha-glucosidase, the use of functional foods and their components as inhibitors for these enzymes is a heavily studied topic in several pieces of literature [2,30,35–39].

Davies *et al.* [40] reviewed the differences in structure and reaction mechanisms on glycosyl hydrolases. Their work is critical when attempting to inhibit or design an inhibitor for alpha-amylase and alpha-glucosidase as it describes the structural diferences between the active sites of these enzymes. While alpha-amylase has an active site with a cleft shape, the active site of alpha-glucosidase is shaped as a crater.

Although an inhibitor for these two enzymes can be designed under the same principles, binding to the active site or binding near to the active site and preventing proper interaction with the substrate, there are some key differences in how these inhibitions are achieved in each enzyme. An inhibitor for alpha-amylase that binds to the active site can bind to part of the cleft or to its entire extension while an inhibitor for alpha-glucosidase that is intended to bind to the active site will have a more limited space. An inhibitor that prevents the proper interaction of these enzymes by disrupting the approach of the substrate to them also has differences between alpha-amylase and alpha-glucosidase. While the inhibitor of alpha-amylase can bind to the sides or on top of the cleft, the inhibitor of alpha-glucosidase will inevitably have to bind to the general area above the pocket. In addition to these types of inhibitors, a third kind can be considered, an inhibitor that bind to any other part of the enzyme and consequently alters its conformation and thus disrupts the catalytic process.

The inhibitors were tested and designed in this work under the principle of binding to the crater/pocket of the active site of alpha-glucosidase.

Functional foods

Foodstuffs are often used to treat Diabetes in folk medicine and with the development of new scientific techniques and methodologies their efficiency is put to the test, leading to the production of the so-called functional foods. Dembinska-Kiec *et al.* [41] review the risk factors for type 2 Diabetes that can be treated by phytochemicals: reduction of oxidative stress, arthrosclerosis, enhancement of glucose and lipid metabolism, cytoprotection of β-cells, inhibition of aldose reductase (EC 1.1.1.21), improvement of cardiovascular health by promoting vasorelaxation and reduction of blood vessel thinness and the control of angiogenesis.

Souza [37] wrote a thesis on the inhibitory activity of several vegetable species and found 29 extracts that inhibited alpha-glucosidase by over 80%. Several studies focused on only one or a few plant species have also yielded encouraging results in the use of functional foods [42–46]. Marathe *et al.* [19] indicate that ingesting whey protein 30 to 60 minutes before a meal will enhance the release of GLP-1 and insulin.

However, promising as they are, so far the studies of functional foods in the treatment of Diabetes have been conducted not using human subjects or human enzymes [36,38,42,47].

Food rich in caffeoylquinic acids and other Hydroxycinnamates, such as coffee, has gained significant importance as these compounds have several mechanisms of action all relevant for the treatment of Diabetes. These inhibit alpha-glucosidase (EC 3.2.1.20) [38,47], stimulate the release of GLP-1, and inhibit the release of GIP [17,35,48]. Caffeoylquinic acids inhibit glucose-6-phosphatase (EC 3.1.3.9) [35,39,49–52]. Welsche *et al.* [53] were able to document their blockage of glucose uptake. These compounds will also reduce the accumulation of fat and stimulate lipolysis [17,52,54]. Additionally, they induce a loss of body weight [52,55] and improve cardiovascular health [56–59].

Physical Exercise and Diet

Engaging in mild physical exercise is a common recommendation in the treatment of Diabetes [7,10,11,60]. Despite being highly recommended and used in combination with other treatment [15], many diabetics or individuals at risk of developing Diabetes still dismiss it [61].

One of the major benefits of physical exercise is the activation of AMPK [12,14]. It will also reduce risk of developing type 2 Diabetes, obesity and will improve insulin sensitivity [6].

The purpose of dieting in Diabetic patients is to control energy intake [62]. As the reduction of energy intake has to be quite significant, very few patients achieve normal values of plasma glucose through dieting alone. The main limitation in dieting is the lack of meaningful and lasting results [63]. The success of dieting is limited by patients overeating and inability to restrain [64]. On the other hand, the researched performed by Clark [63] indicates that restraint will negatively impact quality of life and lead to weight fluctuations.

The control of body weight through exercise is far more effective at providing a higher quality of life than attempting to reduce body weight [65]. The best approach to treat Diabetes is to diet, exercise and take medicine simultaneously, as patients had higher quality of live and enjoyed the benefits of all three approaches simultaneously [66].

High blood pressure, cholesterol, glucose and obesity

Elevated blood pressure, cholesterol, glucose levels and obesity occur very often in people suffering from Diabetes. Obesity is quite often present in situations of elevated cholesterol and hyperglycemia.

High blood pressure

High blood pressure results from a combination of several factors. One of these is an elevated oxidative stress value in the blood stream [10,56]. Ochiai *et al.* [56], found that when the oxidative stress is reduced with anti-oxidants, such as chlorogenic acid (Figure 2) and its metabolites, the amount of radicals that react with NO decreases and thus enables it to exert its vasodilation effect.

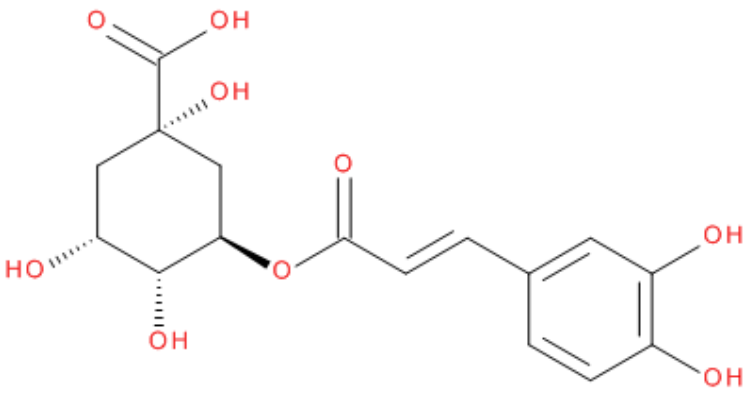


Figure 2 - Chlorogenic acid

A rigorous blood pressure control has been is effective in preventing Diabetes cardio-vascular complications, including renal failure, and also reduces the financial burden regarding Diabetes treatment [11,58,67]. According to Ochiai *et al.* [56], ferulic acid (Figure 3), one of the metabolites of chlorogenic acid, has a significant hypotensive effect (up to 1,4% with 50mg/kg of body weight) when administered alone and an enhanced effect when combined with medication to reduce blood pressure, such as captopril Although drugs such as captopril and other regulators for blood pressure increase the cost of treating patients, the possibility of combining them with chlorogenic acid and/or its metabolites offers many theraputic venues. These are ingested daily in several foods and beverages such as coffee or apples, making this approach very appealing from a therapeutic point of view [46,68]. When blood pressure is monitored, heart health is also analyzed. This is usually done using a bio-marker for heart health, homocysteine. Homocysteine levels rise with high doses (2g/day) of chlorogenic acid [60], twice what is usually ingested [68]. Despite proving that homocysteine levels increase, it was not proven that this increase was due to changes in heart health [60]. Ingesting a low amount of chlorogenic acid significantly lowered homocysteine levels (approximately less 2nmol/ml after 4 months on 140mg per day) [10].

A recent study on the cost of hypertension and obesity on US diabetic patients [69], indicates that an obese diabetic has 14% higher health care costs while a diabetic with hypertension has 26% more medical costs. Condliffe *et al.* [69] further explain that

diabetics with both hypertension and obesity have 40% higher medical costs and that the amount of diabetics with both hypertension and obesity is higher than the amount of diabetics with one or neither of these conditions.

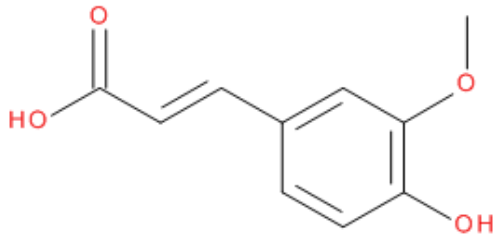


Figure 3 - Ferulic Acid

Elevated Cholesterol, Body fat

High levels of cholesterol in the blood lead to increased blood pressure and high risk of cardio-vascular diseases. Additionally, monitoring cholesterol levels in the blood stream is also recommended for diabetics as a way to reduce costs of treatment and chances of complications and thus improving quality of life [1,11,52,58].

Another useful approach is to reduce fat absorption and/or to enhance lipid metabolism. This also leads to a reduction in body weight. One of the ways to enhance lipid metabolism is by increasing carnitine palmitoyltransferase (CPT) activity. This enzyme is responsible for transporting lipids into mitochondria to be oxidized. Green coffee extract which contains, among other compounds, chlorogenic acid and other caffeoylquinic acids, was proven to reduce fat accumulation around the liver of mice and enhance CPT activity [55]. Two other investigations [10, 67], provide contradictory results regarding the changes in cholesterol levels. Those studies had very different durations and number of participants. While Tucker *et al.* [10] analysed only 20 individuals, they are observed over a period of four months. On the other hand, Crozier *et al.* [67] studies 117 individuals but for only 28 day. Despite its small sample, the results presented by Tucker *et al.* [10] clearly show that there is a significant reduction in the levels of total cholesterol, high density lipoprotein (HDL) and phospholipids. The results from Crozier *et al.* [67] have a major flaw, they are not statistically significant because the *p* value for the levels of triglycerides, total cholesterol, HDL cholesterol and LDL cholesterol are all smaller than 0,9. This is due to an error in the measurements. The triglycerides values have an error margin of nearly ± 50%. Johnston *et al.* [48] present results that concur with the ones obtained by Tucker *et al.* [10]. There is a reduction of body fat accumulation over an extended period of time.

Li *et al.* [70] have found that a predisposition for obesity increases the risk for type 2 Diabetes. They were able to establish a link between eight of 12 single-nucleotide polymorphisms associated with obesity and risk for type 2 Diabetes, concluding that

for each of the Body Mass Index (BMI) increasing allele present, the risk for type 2 Diabetes increases by 4%.

Hyperglycemia

Glucose levels should be monitored in diabetics because, when elevated, they lead to most of the complications associated with this disease, specially nerve, kidney and eye damage [1]. Monitoring the blood glucose levels has been proven to be cost reducing and provide diabetics with better quality of life [1,11,52,58]. There are several ways to lower blood glucose levels. The simplest way is through a controlled diet low in carbohydrates. Other common approaches are insulin injections and/or through medication that controls glucose level by inhibiting its absorption or the catabolism of carbohydrates. The insulin therapy proves to be a problem in cases where the patient has defective insulin receptors or has insulin resistance. Therefore, it is highly useful to have a drug that can regulate blood glucose through several mechanisms.

Chlorogenic acid is capable of producing changes in the release of intestinal hormones [50]. One of the key enzymes in glucose metabolism is glucose-6-phosphatase (G6P). It is simultaneously responsible for the first step of the glycolysis and the last step of gluconeogenisis by either adding or removing the phosphate group from a glucose molecule respectively. As such, inhibiting this enzyme is a great approach to control glucose levels. *In vitro* tests conducted by Salazar-Martinez *et al.* [71], indicated that chlorogenic acid and its isomer (4-caffeoylquinic acid) successfully inhibited G6P by 9,2±1,4 and 14,4±1,2% respectively. By combining all the analyzed caffeoylquinic acids and di-caffeoylquinic acids the authors achieved 34,8±4,0% inhibition. In coffee, it is possible to find both caffeoylquinic and di-caffeoylquinic acids (ie: 3,5-dicaffeoylquinic acid,
Figure 4). Long term coffee consumption significantly lowers the risk for type 2 Diabetes [17]. This was observed in both decaffeinated and caffeinated coffee consumers, despite short term studies indicating that caffeine decreases insulin effectiveness. Chlorogenic acid alters glucose absorption in the intestines by inhibiting it's uptake [20,50]. Chlorogenic acid reduced the release of Glucose-dependent insulinotropic polypeptide (GIP) and increased the release of Glucagon-like peptide 1 (GLP-1) [50]. GIP induces β-cell proliferation and protects them from apoptosis. Although it is useful that β-cells proliferate, as they are protected from apoptosis they waste resources and do not produce insulin as expected [16,17,20].

GLP-1 has many functions [6,15–18]. The work of Araki *et al.* [72] analyzes the effects of GLP-1 over six weeks on type 2 diabetics. They found that GLP-1 was capable of reducing glucose levels (both fasting and average), glycated hemoglobin and free fatty acids (fasting and average as well), increased insulin sensitivity and β-cell function. GLP-1 also caused a reduction in appetite and caused a weight loss of almost 2kg.

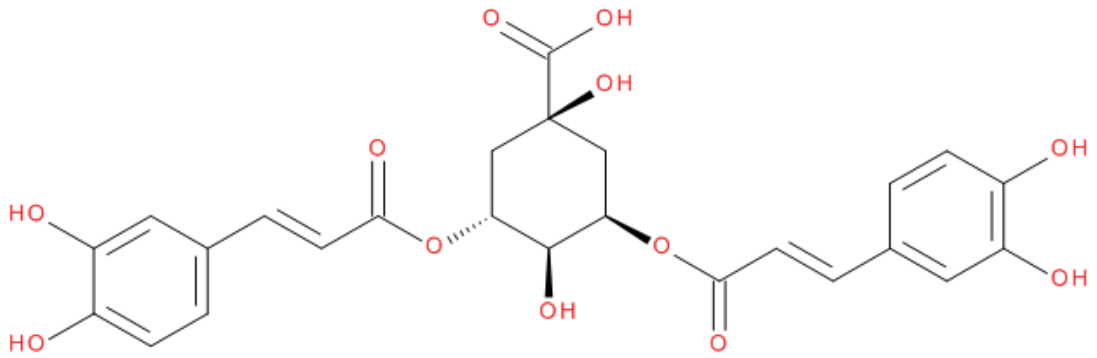


Figure 4 - 3,5-Dicaffeoylquinic Acid

Diabetic Nephropathy

Between 10 to 20% of diabetics die from renal failure [1]. As such, it is imperative to monitor the risk for nephropathy in all diabetics [1,11,52,58]. This may prevent renal damage, reduce odds of complications and will cut treatment costs. The risk factors for nephropathy are usually exhibited by all diabetics [73–75]. These include: hypertension, poor control of blood glucose and aging. Retinopathy is also one of the symptoms for diabetic kidney failure [75].

Although there are very few studies that analyze the effects of chlorogenic acid on diabetic nephropathy, the fact that this compound can control and reduce the risk factors for it, make it relevant in the treatment of diabetic kidney damage. Furthermore, the fact that there is an international patent, submitted by Alexiadou *et al.* [76], that uses chlorogenic acid as a treatment for diabetic nephropathy demonstrates its potential application as a therapy for Diabetes. Age is also one of the key risk factors for kidney failure in diabetics [73–75]. Since the aging process is sped up by increased oxidative stress, which can also be one of the causes for Diabetes, the anti-oxidant properties of chlorogenic acid make it an even greater asset in treating this condition by regulating the oxidative processes.

Ulcers and lower limb damage

Diabetic foot ulcers occur due to a reduced blood flow and neuropathy [1,77]. Despite receiving appropriate treatment, quite often diabetic ulcers fail to heal [78]. These ulcers are the result of a persistent bacterial infection that, with reduced blood flow and sensitivity, is sheltered from self examination and from the immune system.

The most common genera and species of bacteria found in diabetic ulcers, as described by Almeida *et al.*l [79], include the *Streptococcus, Staphylococcus, Pseudomonas* and *Eschericia* genera. These are some of the bacteria genera that are vulnerable to chlorogenic acid [80]. Additionally, quinic acid (Figure 5), which results from the first step of the catabolism of chlorogenic acid, enhances DNA repair

mechanisms and boosts the immune system of its consumers [81]. Almeida *et al.* [79] recommend the use of antimicrobial films to dress wounds as a viable treatment for diabetic ulcers. Diabetic patients should be screened and treated for depression, if applicable, as diabetics with depression are twice as likely to develop ulcers and have a higher chance of complication [82]. As diabetic ulcers result from the combination of reduced blood flow and neuropathy, cholorogenic acid is clearly useful. In addition to its anti-bacterial and immune system boosting capabilities, it also affects blood flow and has neurological effects. The effects of chlorogenic acid on blood flow were previously described on the topic entitled "High blood pressure, cholesterol, glucose and obesity" and its neurological effects are described further ahead in the chapter "Diabetic neuropathy".

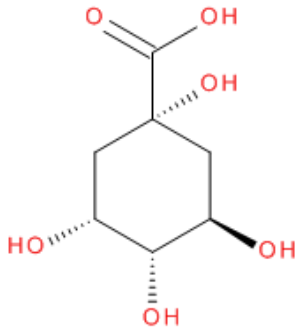


Figure 5 - Quinic Acid

Diabetic retinopathy

After having Diabetes for 15 years, 2% of patients become blind and 10% are visually impaired [1]. These numbers are consistent to those reported by Hall *et al.* [8], where retinopathy ranged from 7 to 66% depending on the country. South Africa is the country with the highest number of studies for retinopathy [8] and in all the analyzed study cases, the prevalence of retinopathy is extremely high, >32%. Monitoring of retinopathy is recommended as a way to reduce costs and improve quality of life of diabetics [1]. Frequent monitoring of retinopathy (at least once a year) has been shown to be cost saving by several model studies. [58] Diabetic retinopathy, is the result of an elevated oxidative stress that results from the reduction of glucose to sorbitol through aldose reductase (AR) (EC 1.1.1.21) [83,84]. The accumulation of sorbitol in the retina also leads to diabetic cataracts [83].

There are several inhibitors for AR from natural products and one of these is chlorogenic acid. It is capable of inhibiting AR function and, its anti-oxidant properties counter the increased oxidative stress produced [82]. Therefore, chlorogenic acid is capable of treating and preventing damage to the eyes that results from excessive AR activity which will usually lead to diabetic cataracts [83].

In addition to the oxidative stress that is a result from elevated AR activity, diabetics with retinopathy also exhibit elevated lipid peroxidation, protein oxidation and DNA damage due to oxidative stress [84]. The most important risk factors for type 2

diabetics, associated with developing diabetic retinopathy, are elevated glycated hemoglobin, elevated blood pressure, high body mass index and length of time that the patients suffer from Diabetes [85,86]. Type 1 Diabetes patients, retinopathy has almost 100% prevalence and as such what is analyzed are the risk factors for proliferative retinopathy. The risk factors are elevated levels of glycated hemoglobin and the presence of non-proliferative retinopathy. Blood pressure, body mass index and duration of Diabetes are not considered risk factors for type 1 diabetics [87].

Diabetic neuropathy

Up to 50% of diabetics suffer from diabetic neuropathy [1]. In Sub Saharan Africa, neuropathy affects 27 to 66% of diabetics [8] and in a 12 year follow-up study conducted in India it was found that neuropathy affected between 35,3% of the subjects with normal renal function and 68,7% of individuals with compromised renal function. [75] In Sweden, peripheral neuropathy affected 67% of diabetics [88]. In Singapore, neuropathy rates vary with duration of Diabetes from 34,1 to 72,4% for less than five years and over 10 years of Diabetes, respectively [3]. The best diagnostics method for peripheral neuropathy is using a neurometer as opposed to the more usual methods, the pinprick test and the Semmes-Weinstein Monofilament Testing (SWMT). However, despite being less accurate, the pinprick test is still a low cost and reliable diagnostics method for peripheral neuropathy [3].

Alzheimer's disease (AD) is a result of a large number of factors and over the past few years it has been proposed and coined as type 3 Diabetes [3–5]. A large number of factors lead to neural degeneration and the most important are: elevated oxidative stress, DNA damage, lipid peroxidation, and insulin resistance [4,5,89]. DNA damage and lipid peroxidation are both present in diabetics due to elevated glucose levels [83]. The degeneration caused by AD can be reversed through medication used in type 2 Diabetes due to an increase in insulin efficiency and reduced oxidative stress [4]. Chlorogenic acid is highly neuroprotective against elevated oxidative stress and amnesia [90,91]. Both these conditions are symptoms of AD. Some researchers claim that AD is a form of Diabetes that affects the brain specifically and an increase of insulin production and/or efficiency would be able to treat and even reverse the damage that occurs with AD [4,5,89].

In this manner, chlorogenic acid proves once again to be extremely useful as it is capable of both improving insulin production and sensitivity [17,20,50,71]. Chlorogenic acid reversed the impairments that were induced in mice and also improved their short term memory [90]. It has been proposed that the increased incidence of Diabetes type 1, 2 and 3 is due to an increasing exposure to nitrates, nitrites and nitrosamines through several sources but mainly through the elevated consumption of processed foods in which these compounds are used as preservatives. This also helps to explain the elevated prevalence of Diabetes in developed countries [91].

Diabetes compendium

Considering all the literature reviewed so far, we show that chlorogenic acid and other caffeoylquinic acids are highly bioactive in the treatment of Diabetes. It is also shown that their exerted effects are highly useful while treating Diabetes and/or conditions associated with it. As such, we believe that the use of chlorogenic acid and other caffeoylquinic acids or green coffee bean extract, very rich in these, should be considered while treating Diabetes and associated diseases, even if just as a supplement to regular treatments.

Caffeoylquinic acids and their metabolites as inhibitors for Alfa-glucosidase

Literature on the metabolism of Caffeoylquinic and Dicaffeoylquinic acids has increased in recent years. Although still limited, the development of novel techniques and methods in analytical chemistry has jump started the amount of information available. The production of caffeoylquinic acids and dicaffeoylquinic acids can be induced or enhanced in artichoke by irradiation with UV-c radiation [92]. In other plant species, wounding or irradiation with UV-b increased the production of chlorogenic acid (5-O-caffeoylquinic acid, 5-CQA).

Although a higher concentration of caffeoylquinic acids would benefit the consumer, it is necessary to consider that understanding their bioavailability and absorption are crucial for their use in medicinal applications.

Most of the literature that studies the effects of caffeoylquinic acids is performed *in vivo* and the effects produced are not compared to the effects caused by the administration of the known metabolites of these compounds.

As such, the hypothesis that caffeoylquinic acids might be used as enzymatic inhibitors has been tested. The hypothesis that the effects often observed *in vivo* could be generated by the presence of the metabolites and not the caffeoylquinic acid itself was also tested. Considering this, the literature was analyzed for information on caffeoylquinic acids and dicaffeoylquinic acids' metabolism and absorption. Then, these compounds and some designed derivatives along with their metabolites were tested as inhibitors of α-glucosidase (EC 3.2.1.20) through molecular docking using the crystal structure resolved by Ren *et al.* [93] for the enzyme.

Absorption

The absorption of caffeoylquinic acids has been established to occur in the intestines. This was shown mainly by the recovery of undigested caffeoylquinic acids from the stomach, even *post-mortem* [94]. A study simulating the digestive process on several polyphenols and also reached the same conclusion by recovering the caffeoylquinic and caffeic acid (Figure 6) portions nearly intact from the simulated gastric juice [95]. The metabolism of caffeoylquinic acids starts at the duodenum with the cleavage of the ester bond between caffeic and quinic acids accounting for 37% of the initial amount of caffeoylquinic acids [95]. Lafay *et al.* [96], found that, in the small intestine, a small amount of 5-CQA (approximately 10%) is metabolized to caffeic acid and its conjugates. In an older study, Azuma *et al.* [97] state that the small intestine may not be the place where the ester bond is cleaved. According to Crozier *et al.* [67], the small intestine still plays an important part in the absorption of chlorogenic acids. These are absorbed in a significant amount (30%) in the small intestine and the remaining acids are absorbed in the colon. This is also confirmed by the difference between the amount of chlorogenic acids excreted in the urine, with ileostomists excreting nearly four times less than subjects with a colon [67].

Caffeoyl and dicaffeoylquinic acids appear to have different absorption mechanisms, as the ratio in which both were detected was different from the ratio in which they had been ingested [98]. This difference may be due to the increased hydrophobicity of dicaffeoylquinic acids allowing them to cross the cellular membranes through diffusion.

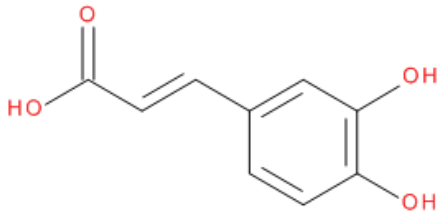


Figure 6 - Caffeic Acid

Metabolism

The colon is not only the prime site for the absorption of chlorogenic acids but also for their metabolism as the main reactions occur in this area [67,99]. Part of the metabolism of these acids also occurs in the liver. This was confirmed with *in vitro* tests, even if in a less extensive manner [95].

Metabolism of chlorogenic acids consists of the breaking of the ester bond between the quinic acid and the hydroxycinnamic acid, followed by methylation, the conjugation of the hydroxycinnamic acid with sulfates, glucuronides and or the saturation of the double bond in the C3 chain of the acid [67,98–100].

The main reactions, the places where they occur, the intervening enzymes and their products are described in detail in Figure 7. It is the metabolism for chlorogenic acids proposed by Crozier *et al.* [67]. It was designed considering the experimental results from the digestion of coffee by human volunteers.

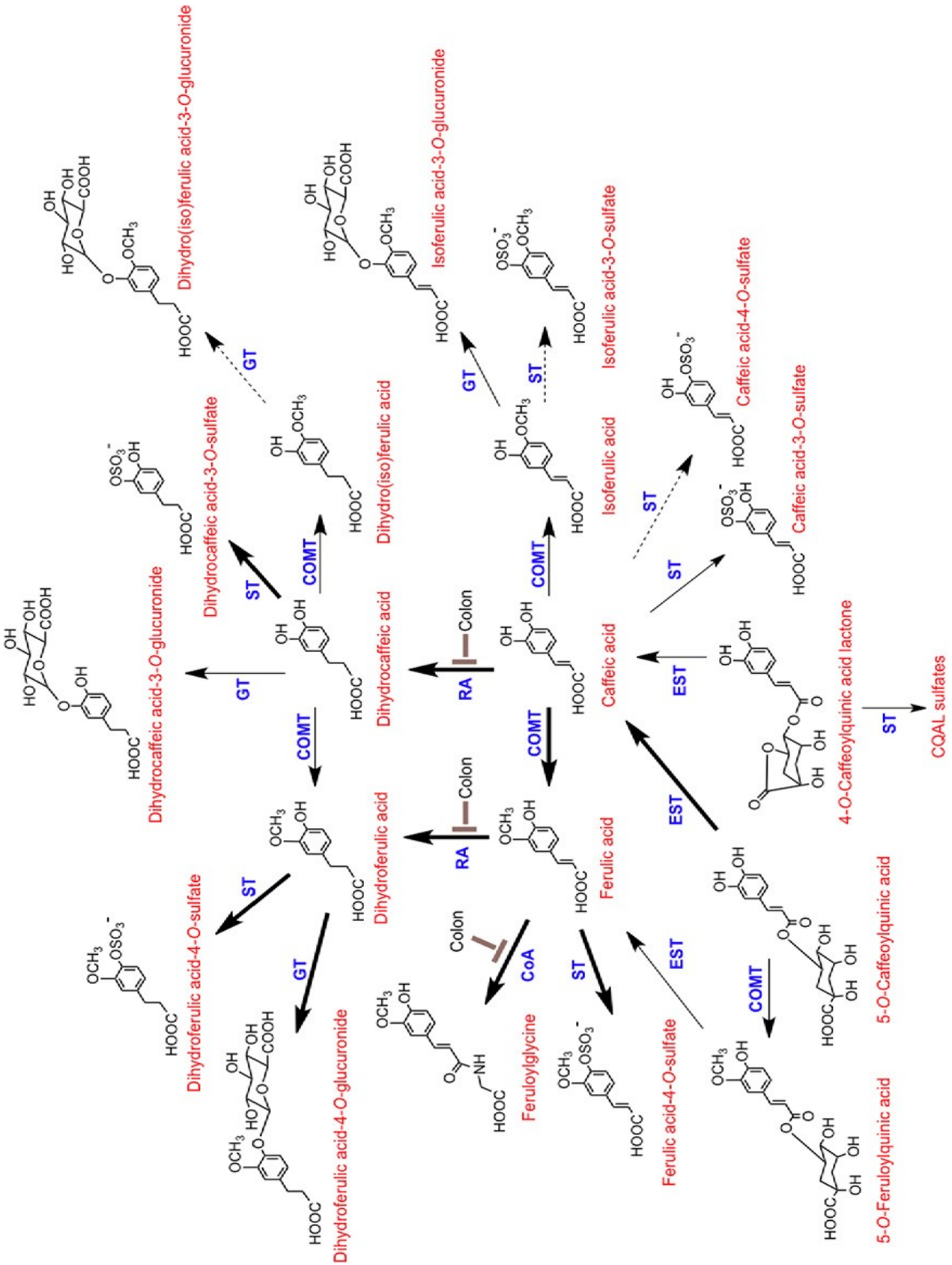


Figure 7 - Metabolism for chlorogenic acid proposed by Crozier *et al.* [67]; COMT, catechol-O- methyltransferase; ET, esterase; RA, reductase; GT, UDP-glucuronyltransferase; ST, sulfuryltransferase; Co-A, co-enzyme A. Bold arrows indicate major routes, dotted arrows minor pathways

Molecular docking

Molecular docking was created in the early 1980's by Kuntz *et al.* [101]. As it is explained by Meng *et al.* [102], the aim of molecular docking is to predict the interaction between two molecules, the ligand and the receptor. Its ultimate goal is to find the most stable conformation between the two molecules [103]. Molecular docking has been successful to identify binding mechanisms, it has explained experimental results and has been able to identify binding sites of new molecular targets [104].

Molecular docking is performed in two steps. Firstly, conformations of the ligand interacting with the receptor are generated resorting to one of several algorithms. These resort to empirical force fields [103]. Meng *et al.* [102], refer that they use different search methods. Once the conformations are generated, they are then scored and ranked using Scoring Functions [102,104].

Molecular docking algorithms

As said by Meng *et al.* [102], matching algorithms (MA) represent both the ligand and the receptor as pharmacophores. These are described by Wermuth *et al.* [105] as *"an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response"*. As such, pharmacophores do not represent molecules but instead the features that are present in a group of compounds and that allow them to interact with their target. MA are fast and most useful when improving a set of compounds with an already established effect [102].

Incremental construction (IC) and Multiple Copy Simultaneous Search (MCSS) are fragment-based methods. These divide the ligand into small fragments that are placed in the active site (or docking target) in an incremental fashion. Although both methods are fragment-based, their approach is quite distinct. IC fits the biggest fragment of the ligand in the docking site and adds the remaining fragments one by one and orientations are generated for each fragment to best describe the ligand flexibility and interaction in the docking site. MCSS, on the other hand, generates thousands of copies of each functional group and randomly places them in the target site. These are then subjected to energy minimization and limited by force fields. To obtain a new molecule that is complementary to the target site, all that is required is to link the best ranking functional groups in their proper conformation [102].

Stochastic methods randomize the ligand's conformation in the docking site [102]. Two common algorithms that employ stochastic methods are Monte Carlo (MC) and Genetic Algorithms (GA). MC algorithms generate conformations of the ligand by rotating bonds and by rotating and translating the ligand as a rigid body. Once a conformation is generated, it is then compared to the criteria established and, if it fits these criteria, it is saved and improved until the number of desired conformations is achieved. The fact that MC results are not deterministic, is both its best and worse

feature. This may generate a set of results that only meet the minimum requirements established or it may generate outstanding results that otherwise would be very hard to achieve. GA are based on theory of evolution. Degrees of freedom are considered as genes that make up a chromosome, the ligand's conformation. Like regular genes, these can be affected by mutation (that randomly change the genes) and by crossover (that exchanges "genes" between conformations). After mutation and crossover each conformation is subjected to a fitness test (scoring function) and, if they survive (overcome an energy threshold) they will be used on the next generation. Although GA are non deterministic like MC methods, GA benefit from the mutation and crossover mechanisms.

Molecular Dynamics (MD) simulates the movement of each atom individually. It is limited by the fact that it advances in small steps but it is quite efficient at local optimization. As such, it is most effective when used after a random search has been conducted. While the random search will find a local energy minimum, MD will then optimize it and provide even better results [102].

Molecular docking: Scoring and validation of results

The success of docking is determined by the ability to discriminate between correct and incorrect conformations and by the ability to properly rank the produced conformations [102–104].

There are several kinds of scoring functions such as: Empirical functions [102,106], Molecular/Quantum mechanics functions [102,103,106], Knowledge based functions [102,107] and Consensus scoring [102,103,106,107]. Some functions also consider important factors in molecular mechanics like Entropy and Solvation [103,106].

Entropy is a key factor in binding energy. Due to its high difficulty to calculate, it is only accounted for in specialized scoring functions and functions with extension of force-fields [102,103,106].

Solvation is another factor that is often omitted or simplified in the scoring process. This is due to the many degrees of freedom that it implies. It is mainly considered by knowledge based functions but still most models do not consider the effects that solvent molecules would exert on the receptor, ligand and their interaction in the binding site [103,106].

Empirical functions are created by combining experimental data and parts of other already established functions. Their greatest feature is simplicity but it often limits the accuracy of the results that these functions provide [102,106,107].

Molecular/Quantum mechanics functions resort to force fields and quantum mechanics to describe the binding of the ligand to the receptor. These consider hydrogen bonds, solvation, entropy and Van der Waals interactions [102,103,106,107].

Knowledge based functions are based on statistical methods. These are based on the consideration that the best interactions are the most statistically common. Despite

being able to provide insight into uncommon interactions, they are very limited by their training set that often does not properly represent all interactions [102,107].

Consensus functions are usually used to improve docking results. With consensus functions, several scoring functions are combined [102,103,106]. These functions operate under a similar principal to knowledge based functions. As such, they assume that the best ligands will have consistent performances with several methods and thus the ligands that score better through the array of tests will likely have a better experimental affinity. Although consensus functions are thought to provide one of the best solutions to achieve better results, they are still very limited by the scoring functions at their core [102,103,106,107].

In a review about the accuracy of docking results from seven popular docking programs and their respective experimental results, Augustyniak *et al.* [107] ranked Autodock (created by Morris *et al.* [108]) at the bottom of the table. This was based on the RMSD value of the docked ligands . Despite this, Autodock still achieved a 50% of correct pose prediction. Autodock is still one of the most popular programs due to its simplicity and ease of use, mainly due to its Graphical user interface [104]. Despite having been published recently, these two studies do not take Autodock Vina (Vina - created by Trott *et al.*[109]) into account. Vina can be considered as the second generation of Autodock and it offers great increases in speed, accuracy and supports multi-core computing.

A review comparing the results obtained with Autodock4 and with Vina authenticates the improvements of Vina relating to Autodock4 [110]. As such it is established that not only does Autodock Vina execute tasks faster, it is also produces more accurate results when compared with Autodock4. Despite the fact that both programs suffer from size bias, Vina is better at accurately ranking larger molecules.

As such, considering the vast improvements of Autodock Vina over Autodock 4, its similarities in use and, previous experience with Autodock 4, Vina was chosen to perform all the docking operations in this work.

Molecules used on the docking

As it was referenced previously, on the initial stage of this work, (di)caffeoylquinic acids, generated derivatives and metabolites were used as enzymatic inhibitors. The metabolites are the molecules identified as Inib22 up to and including Inib35 (Annex 22 to Annex 35 respectively). The naturally occurring compounds are the molecules identified as: Inib1, Inib2, Inib4, Inib5, Inib8 and Inib10 (Annex 1, Annex 2, Annex 4, Annex 5, Annex 8 and Annex 10 respectively). The derivate molecules are the molecules identified as: Inib3, Inib6, Inib7, Inib9 (Annex 3, Annex 6, Annex 7, Annex 9) and, Inib11 up to and including Inib21 (Annex 11 to Annex 21 respectively). The Molecules identified as "Opt" are also considered derivative molecules (Annex 36 to Annex 56).

Methods

The chlorogenic acids and metabolites where designed in HyperChem 8.0 [111], optimized using MM+ force field, until the RMS gradient was 0,01 kcal/mol or lower, and saved as .hin. All the files were then converted to .pbd through OpenBabel [112] and opened with AutoDock Tools (ADT) [113]. After opening the files on ADT, the Gasteiger charge was computed ("Edit -> Charges -> Compute Gasteiger") and the atoms where assigned as AD4 type ("Edit -> Atoms -> Assign AD4 type"). After these two steps each molecule was saved as .pdbqt. After being saved as .pdbqt, each inhibitor file was then imported to ADT in the Ligand menu ("Ligand -> Input -> Open"). Then, the rotatable bonds where defined ("Ligand -> Torsion Tree -> Detect Root"), ("Ligand -> Torsion Tree -> Choose Torsions -> Done") and ("Ligand -> Torsion Tree -> Set Number of Torsions -> Fewest atoms -> Dismiss").

As for the receptor, human alpha-glucosidase (EC 3.2.1.20), the .pdb file was downloaded from the pdb database (PDB ID: 3TOP). As the receptor is a symmetric dimmer, the B chain of the receptor was deleted and only chain A was used in the dockings. The water molecules in the ligand in the file were then deleted and the receptor file was saved to .pdbqt in the same manner as the chlorogenic acids. Unlike the inhibitors, however, hydrogen atoms were added ("Edit -> Hydrogens -> Add -> All Hydrogens -> noBondOrder -> yes -> OK") to the receptor as these are lacking in .pdb files of proteins.

The Molecular Docking was performed using Vina [109]. The Vina box was set at -31,426; 35,7; 26.325 (x; y; z), with size 35 Å in all axes (x; y; z) and the exhaustiveness value was set to "20".

The docking results output by Vina were then analyzed both through ADT and PyMOL [114]. In ADT, to analyze a docking result the Vina output was opened ("Analyze -> Dockings -> Open AutoDock vina result -> Single Molecule with multiple conformations -> OK"), the receptor file was opened ("Analyze -> Macromolecule -> Open") and the interactions between both were shown ("Analyze -> Dockings -> Show Interactions"). On Windows, to ensure memory is not an issue, all atoms further than 10Å from the ligand were deleted. Such action was not necessary while working on linux OS (Ubuntu 12.04 distribution). To display the interactions between ligand and receptor through PyMOL both the receptor and the Vina output were opened in PyMOL ("File -> Open"). Then the polar interactions were displayed ("Action -> preset -> ligands") and the residues that established the interactions were identified.

Results from *in silico* study

Some Hydrogen bonds between the ligand and the receptor couldn't be detected in ADT but where detected in PyMOL. On the other hand, ADT was capable of detecting close contacts and π – π stacking that PyMOL could not. This may be due to the way Vina treats hydroxyl groups in docking. The hydroxyl groups in some cases formed an angle that was not recognized as an Hydrogen bond that could be established between the donor and acceptor of the bond. PyMOL's detection of polar interaction was not as constrained, unlike ADT's, and as such detected all the possible H-bonds and polar interactions.

Of the total 35 inhibitors tested, the top five regarding the affinity towards the target were Inhibitors 5, 7, 2, 1 and 8 (Annex 5, Annex 7, Annex 2, Annex 1 and Annex 8 respectively), the last two having an equal value of affinity (Graph 1).

Inhibitor 5 (best docked conformation in Figure 8) was then analyzed to see what reactions would be possible to perform in order to reduce polarity but maintaining a similar structure. This transformation was intended to enhance the amount of inhibitor that is absorbed through passive transport in the intestinal wall without significantly altering the function and or metabolism of the compound. This would prevent the compounds from reaching the intestinal esterases that would break the ester bond between the quinic acid and the hydroxycinnamic acid moieties.

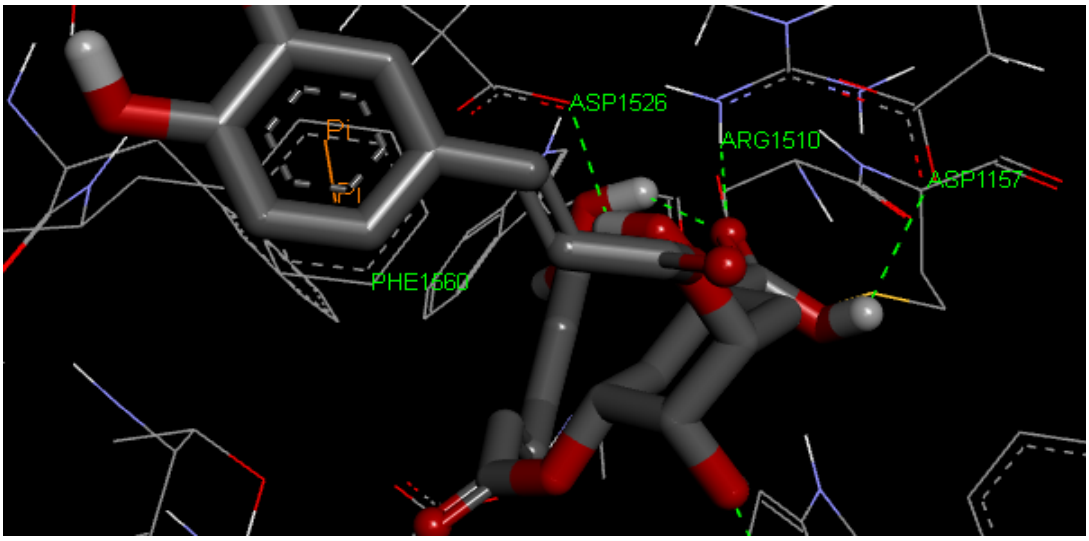


Figure 8 - Inhibitor 5's best docked conformation (orange line indicates π-π stacking and dashed green lines indicate H-bonds and or electrostatic interactions) the aromatic ring that is stacked with Phe1560 corresponds to the branch from position 3' of Quinic Acid

The optimized inhibitors (Annex 36 to Annex 56) were: an ester at the carboxylic acid functional group of the quinic acid moiety (aliphatic chain with one to eight carbon atoms in length), an inhibitor in which the double bond between the carboxylic acid group and the aromatic ring was converted to a single bond (verified for each caffeic acid moiety), and the transformation of the hydroxyl groups of each aromatic ring to dioxolanes (verified for each caffeic acid moiety and for both simultaneously). The

affinity of these optimizations can be found in Graph 2. Out of this set of inhibitors the best performing was the inhibitor in which both diphenols had been converted to dioxolanes (Figure 9), its affinity was 0,1 kcal/mol better than inhibitor 5. The optimized inhibitors are composed by two generations, the first that includes Optimization 1 to 13 and the second generation that encompasses Optimization 14 to 21. The goal of the first generation was to establish what modifications were the most beneficial while on the second generation the goal was to see the effect of extending the lipophilicity on the best optimization from generation one.

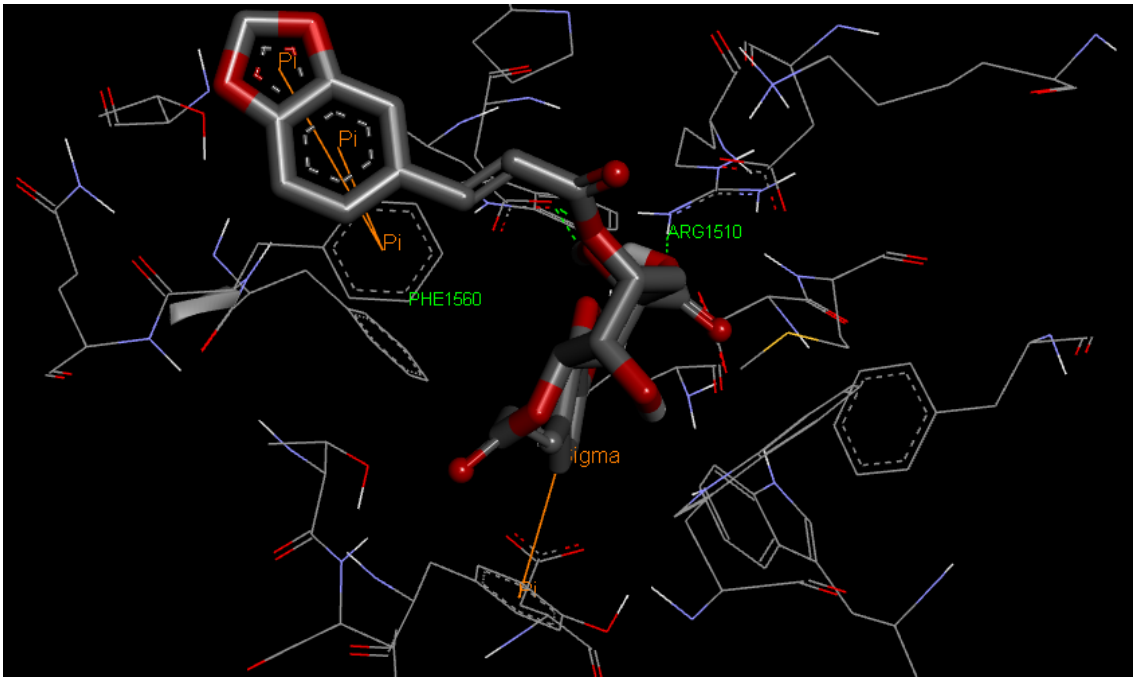
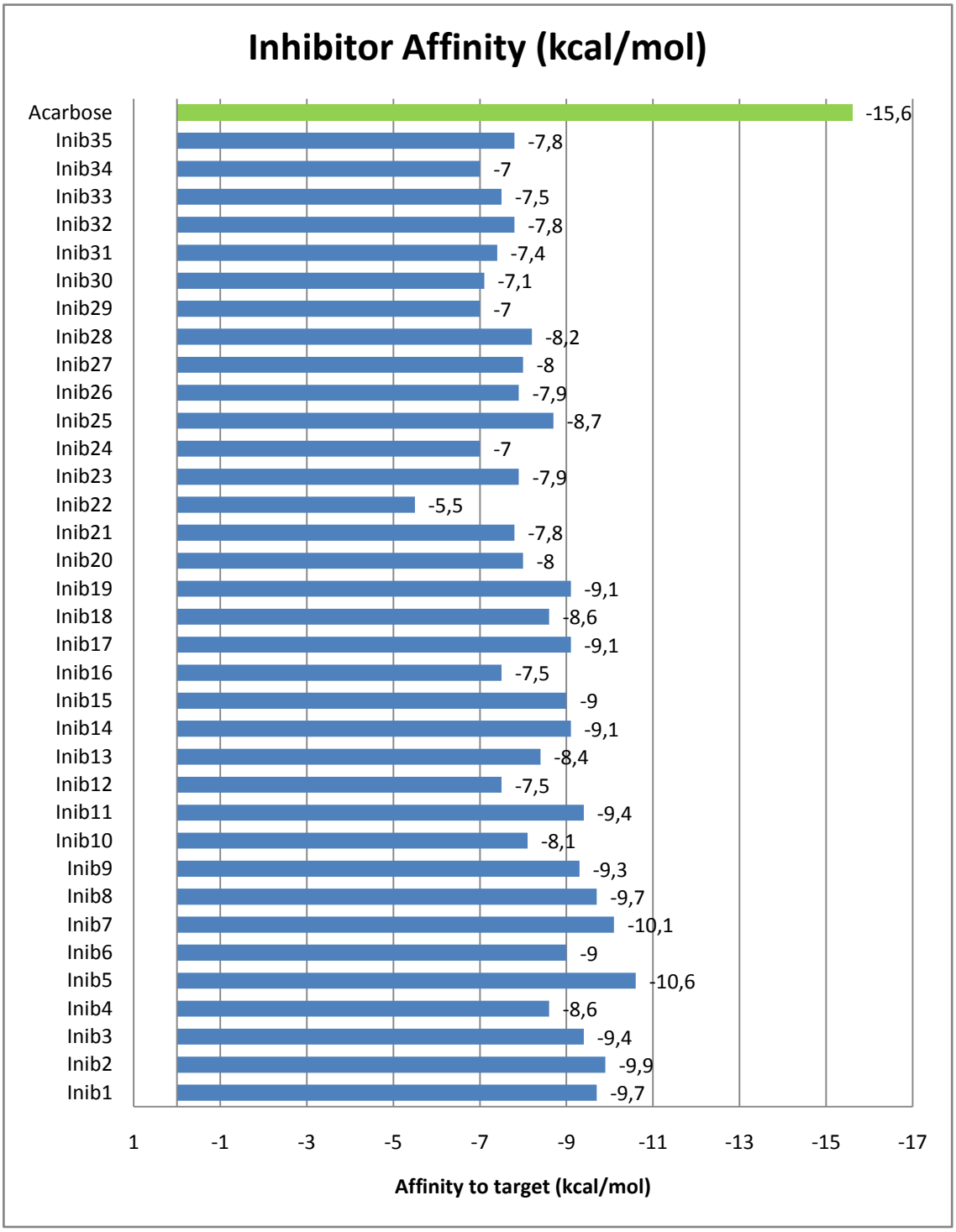


Figure 9 - Optimization 11's best docked conformation (orange line indicates π-π stacking or π-σ interactions and dashed green lines indicate H-bonds) the aromatic ring that is stacked with Phe1560 corresponds to the branch from position 5 of Quinic Acid

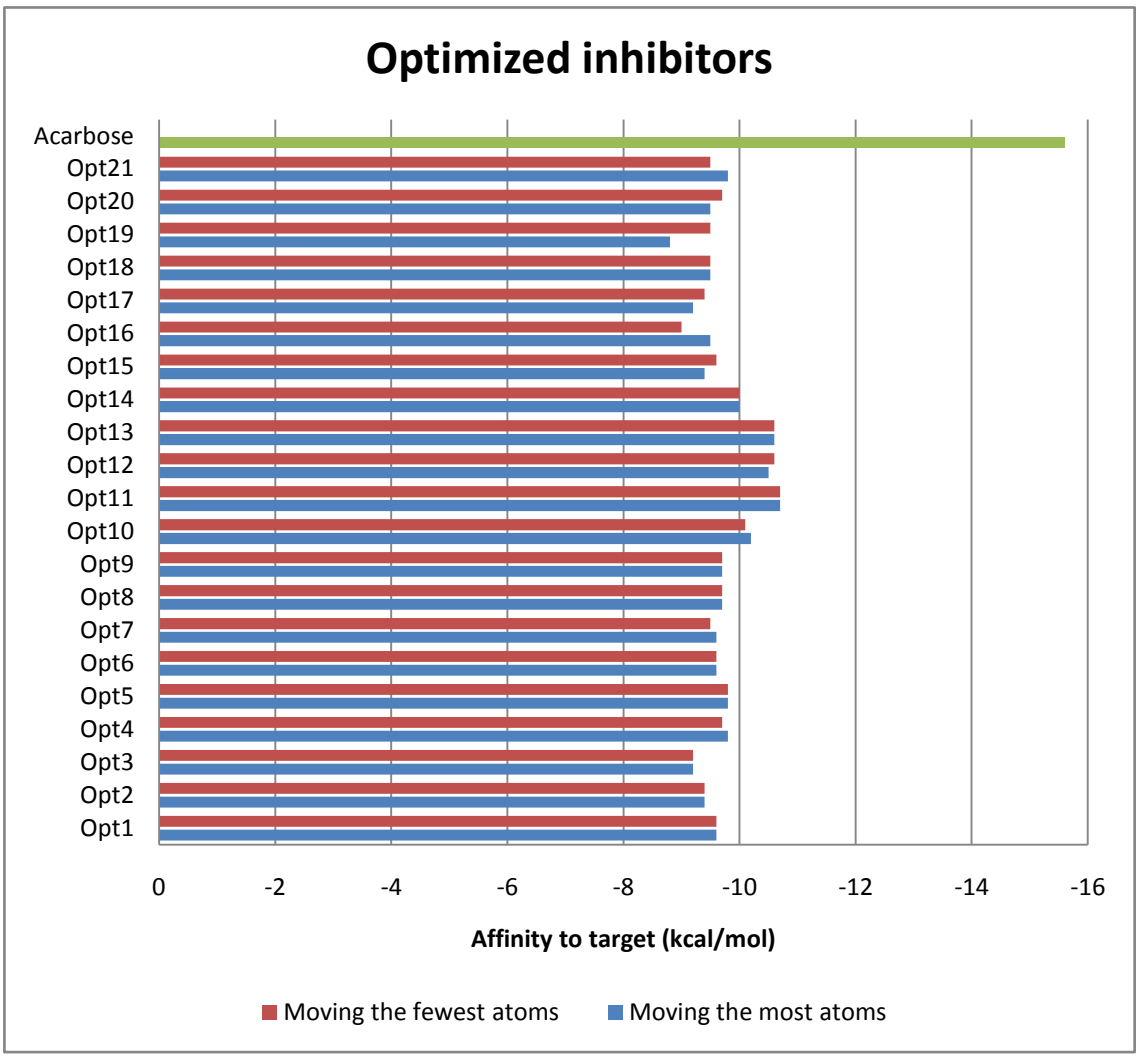
The docking for each optimized ligand was ran while moving the most and the fewest possible atoms (defined through the file preparation in ADT). In both cases the affinity calculated by Vina was the same but when observing the conformations produced in the output the conformations where slightly different. The positions of the atoms were close in both conformations but they varied on the rotation of the hydroxyl groups.



Graph 1 - Affinity of caffeoyl quinic acids and their metabolites and affinity of Acarbose (in green)

To determine if this variation in the conformation was due to the different amount of movable atoms or due to the different algorithm seeds, a *cross-over* test was conducted. In this test, the docking was ran on an inhibitor while moving the fewest amount of atoms possible and using the seed for the same inhibitor used to run the docking with the most movable atoms. The results from the test indicate that a variation in either the algorithm seed or the number of movable atoms produces

identical results. The geometrical placement of the atoms was always very similar and the variation was mostly noticeable in the rotation of the hydroxyl groups.



Graph 2 - Affinity of each optimized inhibitor, 1st generation: Opt1 to Opt13; 2nd generation: Opt14 to Opt21 and affinity of Acarbose (in green)

The docking of Acarbose was also performed as a control. The docking of acarbose was done in four different methods: using the same conformation that it exhibits in the crystal structure (with a search box of the same size used for all the inhibitors and a search box in which the x size was set to “20”, ran with fewest and most movable atoms) and with a random conformation of acarbose produced in HyperChem. None of these dockings provided a conformation of Acarbose similar to the one present in the crystal structure. The affinity values for these conformations of Acarbose were: -6,8 kcal/mol for the docking of the random conformation, -8,8 kca/mol for the docking with the search box the same size as the other inhibitors and -8,5 kcal/mol for the docking with the smaller search box, for both fewest and most movable atoms. These ligands were named as "acarb" (ligand with search box of the same size), "acarb2" and "acarb3" (ligands with smaller search box) and "control4" (ligand with random conformation) (Figure 10).

Figure 10 - Conformations of "acarb" (red), "acarb2" (green), "acarb3" (blue), "control4" (cyan) and crystal structure conformation (magenta)

As none of these dockings resulted in the same conformation as the one in the crystal structure, a final control docking was performed where the search box was the same size as it was for the inhibitors but the ligand had no rotatable bonds (named "acarb4"). The conformation produced by this docking (Figure 11) had an elevated affinity (-15,6kcal/mol) being -5 kcal/mol better than inhibitor 5 and -4,9 kcal/mol better than the best optimization of inhibitor 5 which consisted on the conversion of both diphenols to dioxanes. Figure 12 is a molecular representation of Acarbose. It provides information on functional groups that is not represented in Figure 10 or Figure 11 in order to simplify the interpretation of the docking results.

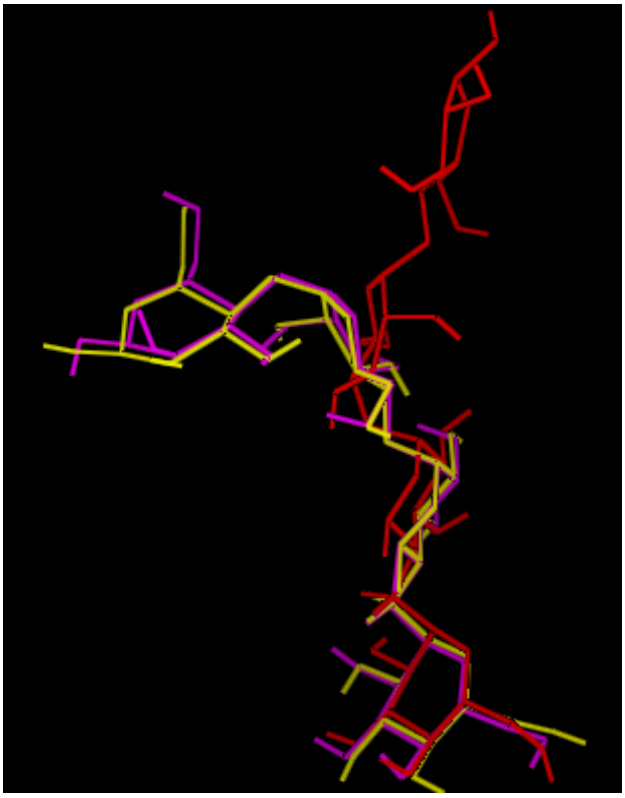


Figure 11 - Conformation of "acarb" (red), crystal structure conformation (magenta) and "acarb4" (yellow)

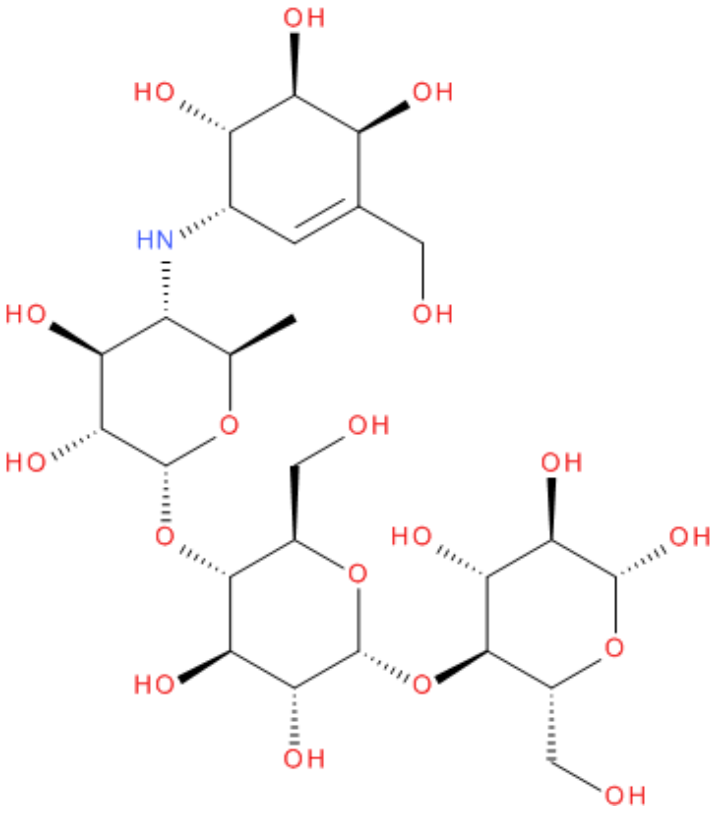


Figure 12 - Acarbose

Results from *in vitro* tests

There are a few studies that analyze the effects of caffeoylquinic acids on α-glucosidase through *in vitro* essays. These provide simultaneously insights into the inhibition mechanism and a way to confirm and compare the results obtained through the *in silico* study.

When analyzing the effects of flower buds of *Tussilago farfara* and their main components, as inhibitors for α-glucosidase, Gao *et al.* [115] reached a few key conclusions. They were able to determine that 3,4-Dicaffeoylquinic acid along with 3,5-Dicaffeoylquinic acid and 4,5-Dicaffeoylquinic acid inhibited α-glucosidase but did not inhibit sucrase, isomaltase or a-amylase. In addition, their results show that quinic acid, caffeic acid and chlorogenic acids perform poorly as inhibitors for α-glucosidase and that the 3,4; 3,5 and 4,5 Dicaffeoylquinic acids moderately inhibit α-glucosidase (62-65%) . These results corroborate the results obtained by molecular docking.

Ooi *et al.* [47], also analyzed the inhibition of α-glucosidase using 3,4-Dicaffeoylquinic acid. Their results indicate that 3,4-Dicaffeoylquinic acid is 30 times less effective as a specific α-glucosiadase inhibitor than acarbose. As such they conclude that the inhibitory effect is most like caused by its antioxidant properties.

Ma *et al.* [39], compared the effectiveness of several ketal and diketal derivates of chlorogenic acid shows that long alkyl chains provide better inhibition than the compounds with shorter alkyl chains. Despite the fact that these inhibitors did not perform as well as Acarbose, it is still possible to draw the conclusion that a lower polarity will provide a better inhibition of α-glucosidase. When compared to Acarbose, these had one third of its inhibition efficiency. A more recent study, used the results of Ma *et al.* [39] and performed a QSAR study [116]. The analysis performed by Yuriev *et al.* [106] identified a few key requirements for these α-glucosidase inhibitors such as: having equal hydrophobic and hydrophilic features at the center of mass and being hydrophobic on the molecular surface. Yuriev *et al.* [116] also conclude that the presence of the hydrophobic chains reduces the amount of energy to interact as an inhibitor. This occurs since the interaction with water molecules is reduced.

Sakulnarmrat *et al.* [117], studied the inhibitory effects of several Australian plant extracts on α-glucosidase. The extract of Tasmannia pepper leaf is mainly composed of chlorogenic acid and did not perform as well as Anise myrtle or Lemon myrtle. Anise myrtle extract was found to contain myricetin, quercetin and catechin while the Lemon myrtle extract contained hesperetin, myricetin, and quercetin. The main compounds in the extracts of these two plants were Ellagic acid and its derivatives. Only the extracts of plants were analyzed and not their individual or main compounds.

Discussion

Considering the results obtained, it's clear that hydroxycinnamic acids namely caffeoylquinic and dicaffeoylquinic acids have the potential to bind to the active site of human α-glucosidase's (EC 3.2.1.20) C terminal chain in a similar way as Acarbose, its commercial inhibitor. It is important to notice, however, that the affinity of the

compounds studied in this work is lower than acarbose by approximately 30%. We also established that the metabolites of caffeoyl quinic acids are not suitable inhibitors for α-glucosidase.

Despite having a lower affinity *in silico*, *in vitro* tests should be ran in order to establish if the difference in affinity is as big as the dockings indicate. Also the other effects that (di)caffeoyl quinic acids can exert in the human organism should be considered and weighted against its lower affinity, as these "side effects" could in fact help achieve a way to treat Diabetes and or its symptoms.

The results of this study also provide insight in order to develop a new inhibitor of α-glucosidase considering the structures of the inhibitors tested and the nature of the interactions that they establish with the active site of the enzyme. The most suitable method to achieve this goal might be *in situ* design of the drug.

In situ design of an inhibitor for α-glucosidase (EC 3.2.1.20)

The objective was always to create a ligand that had an affinity value close to the one that was calculated for acarbose (-15,6kcal/mol). To achieve this, interactions between opposing charges with charged functional groups ($[R\text{-}NH_3]^+$; $[R{=}NH_2]^+$ and $[COO]^-$) and favoring π-π orbital stacking from aromatic rings was imperative. The affinity of all the inhibitors designed *in situ* can be found in Graph 3 and a representation of each molecule can be found in the Annexes from Annex 57 to Annex 143.

The first inhibitor designed was very bulky and was expected to interact with a lot of residues which was not what resulted from the docking. So, the idea of starting from a big ligand was abandoned and the design process focused on starting with smaller ligands and optimizing them to increase the number of interactions and improving overall affinity. The smaller ligands initially proved difficult to improve as due to their small size the best conformations usually were docked outside of the active site. As there was always at least one conformation within the active site it was still possible to introduce modifications intended to improve the binding of the ligand. The will to achieve a better ligand led to the modification of several functional groups from one ligand to the other. This led sometimes to ligands whose binding was far from the expected and in some cases with an affinity far lower than the originating molecule.

Throughout the development of the several ligands, the residues Asp1157, Arg1510 and Asp1526 were regarded as guiding residues. Their unique positions (Figure 13) and the fact that they possess alternating opposite charges makes it possible to "easily" fashion a complementary branch in the ligand and ensure the binding of it to the active site.

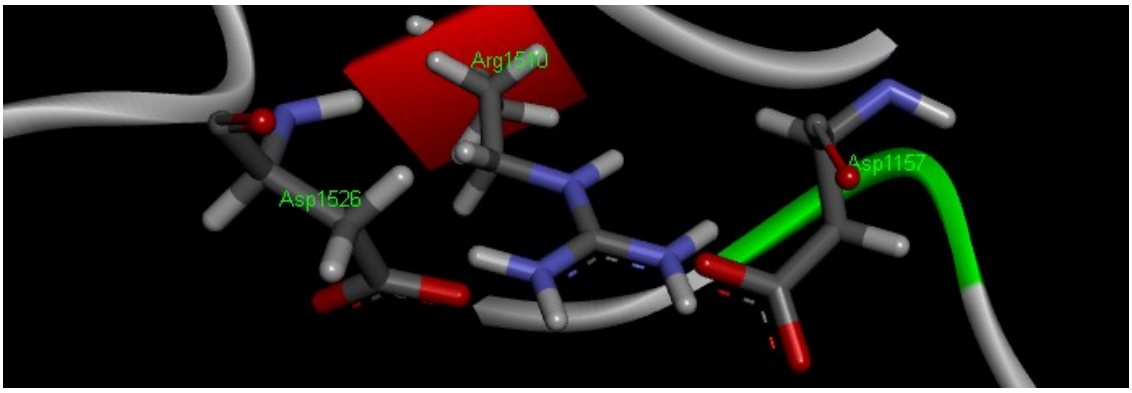
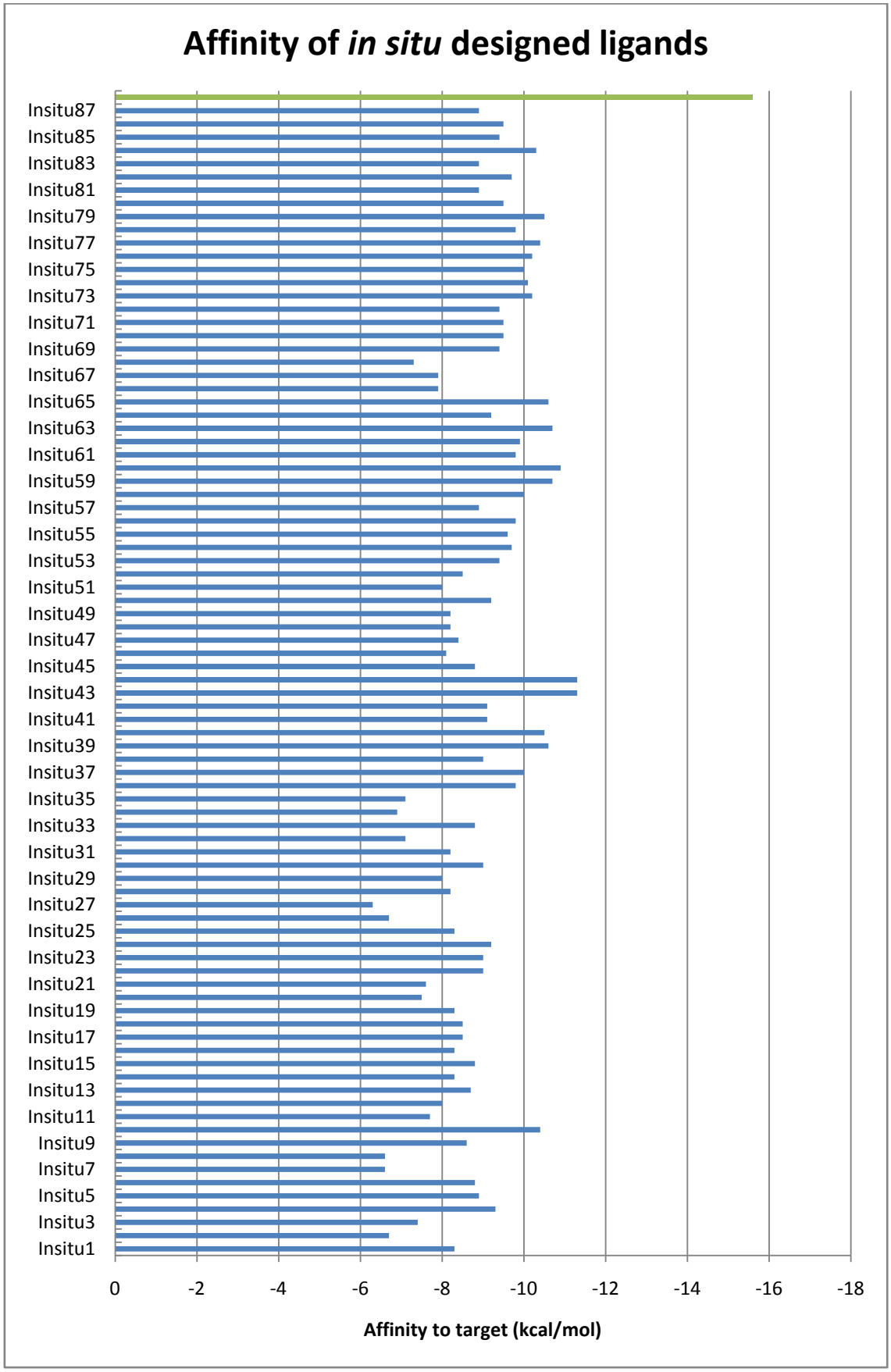


Figure 13 - Charged residues used as targeting guides

The residue Phe1560 was also used as a guiding residue. However, using aromatic residues as a guide significantly increased the size of ligand. The π-π stacking between the ligand and Phe1560 sometimes did not occur because the stacking occurred between the ligand and Trp1369.



Graph 3 - Affinity of each inhibitor designed *in situ* and affinity of Acarbose (in green)

In ligands with more than one aromatic ring, the binding was sometimes similar with the binding of the dicaffeoyl quinic acids with one aromatic ring inside the pocket and the other stacking with Phe1560. Alternatively, some ligands stacked with both Phe1560 and Trp1369. The spatial placement of these residues within the active site is represented in Figure 14.

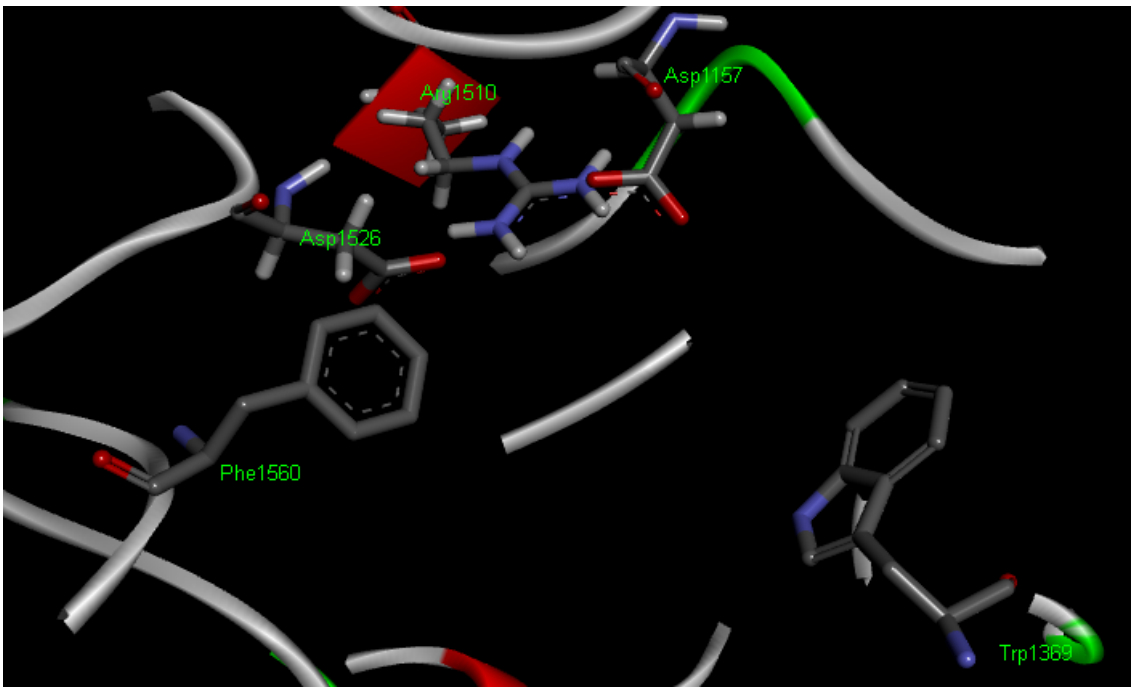


Figure 14 - Residues Asp1157, Trp1369, Asp1526 and Phe1560 within the active site

The aromatic ring also proved to be an extraordinary way to interact with residues that were close to each other but also with residues that are extremely far apart. The carbon atom of the carboxyl side chain groups from Asp1279 and Asp1526 are 10.3 Å apart but with a ligand with a 1,2-diamine benzene ring the ligand interacts simultaneously with both residues (Figure 15).

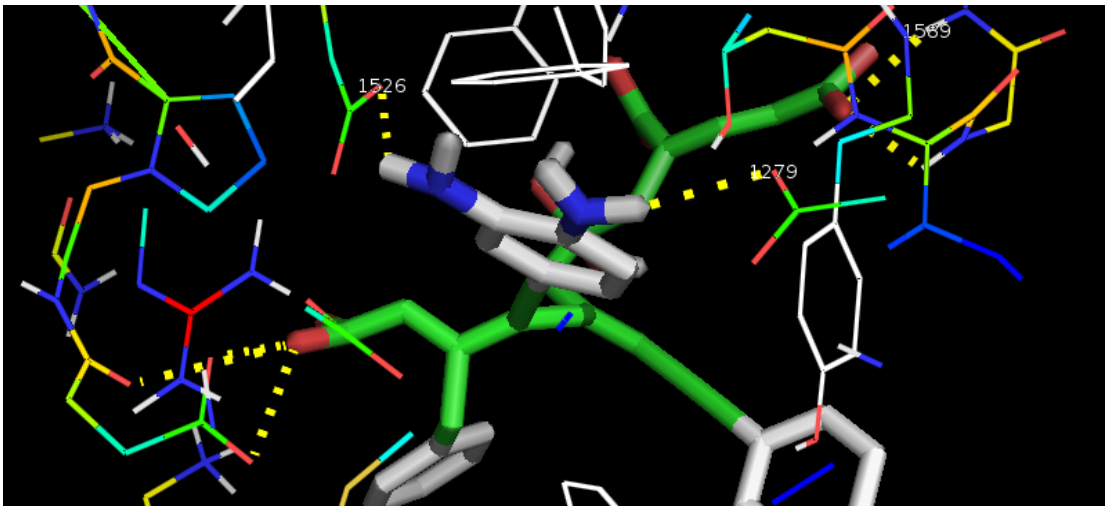


Figure 15 - Best conformation of inhibitor designed *in situ* 56 interacting with Asp1526 and Asp1279 through a 1,2-diamine functional group

Another way to achieve interaction between the ligand and both Asp1279 and 1526 is by using 2-(aminoethyl)-aniline group (Figure 16). As the ethyl group allows the amine group to be closer to Asp1279 without constraining the docking conformation as much as when using the 1,2-diamine benzene ring.

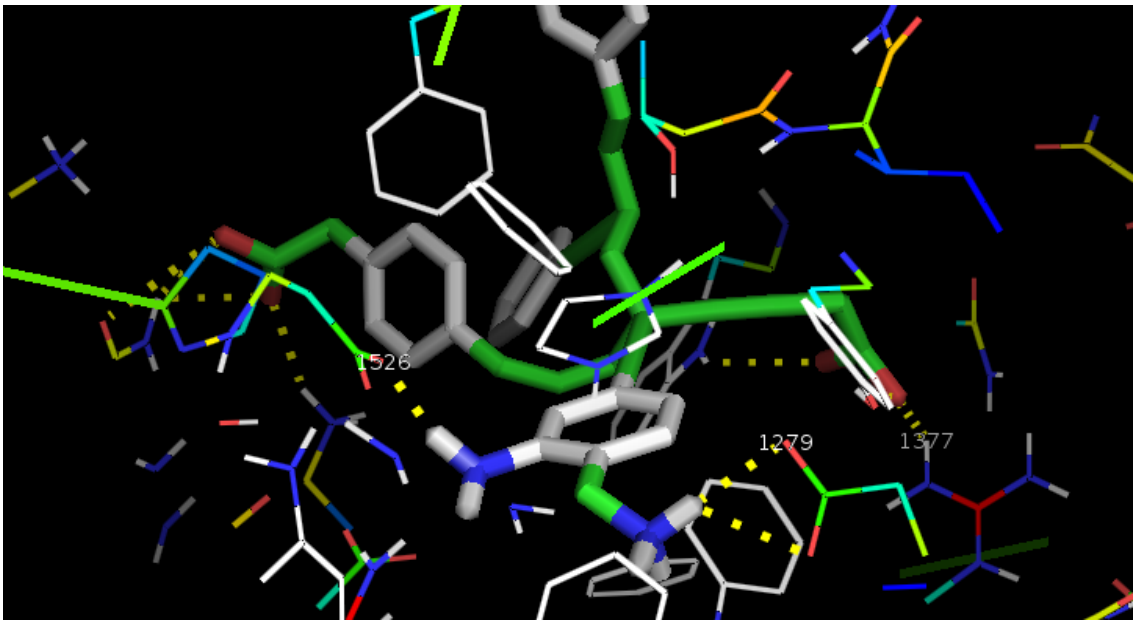


Figure 16 - Ligand designed *in situ* 73 with a 2-(aminoethyl)-aniline group interacting with both Asp 1279 and 1526

The 1,2-diamine benzene ring also presented complementary interaction with residues Asp1279 and Asp1420 in numerous times. This interaction (Figure 17) was seen more often than the one depicted in Figure 15.

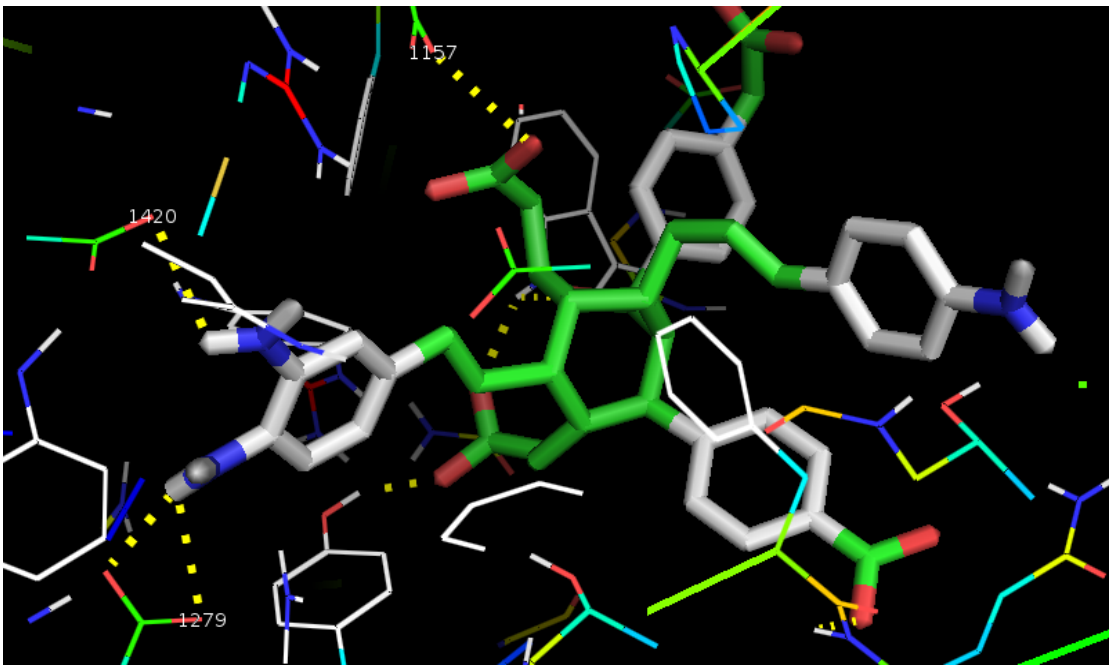


Figure 17 - Best conformation of inhibitor designed *in situ* 43 interacting with Asp1420 and Asp1279 through a 1,2-diamine functional group

There is also a set of three residues that can be used as a guide for the docking of the ligand. However, establishing a simultaneous interaction with all the residues is difficult, and the most common result is the interaction between two of these residues. The residues referred here are: Tyr1251, Trp1369 and Arg1377 (Figure 18).

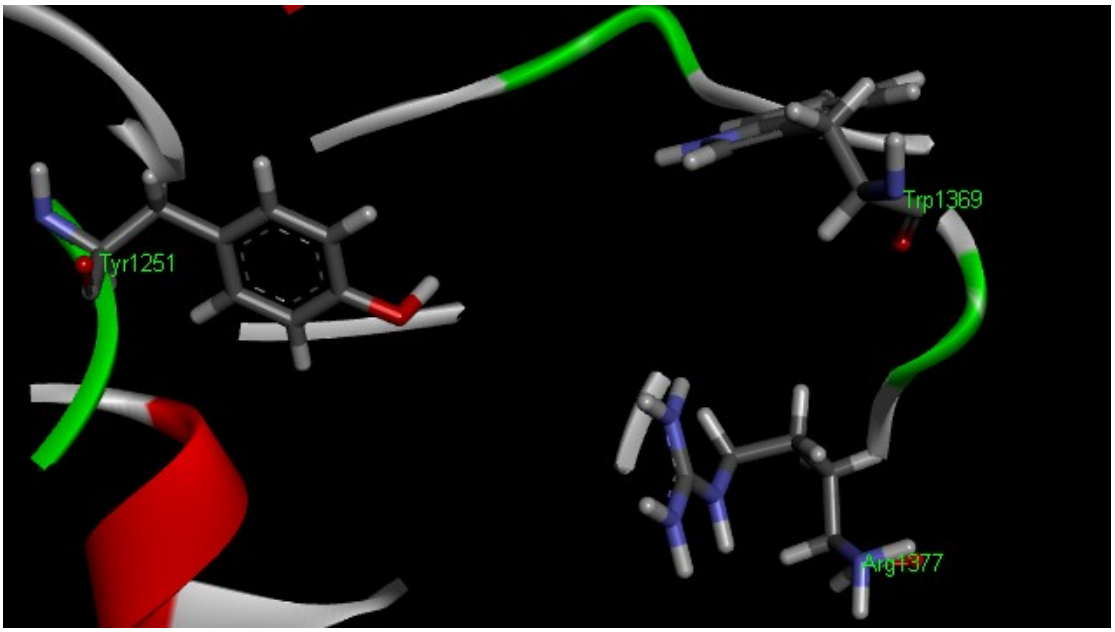


Figure 18 - Position of the residues Tyr1251, Trp1369 and Arg1377 within the active site

The inhibitor designed *in situ* number 73 is an example of an inhibitor interacting with all the residues through a carboxylic acid functional group (Figure 19).

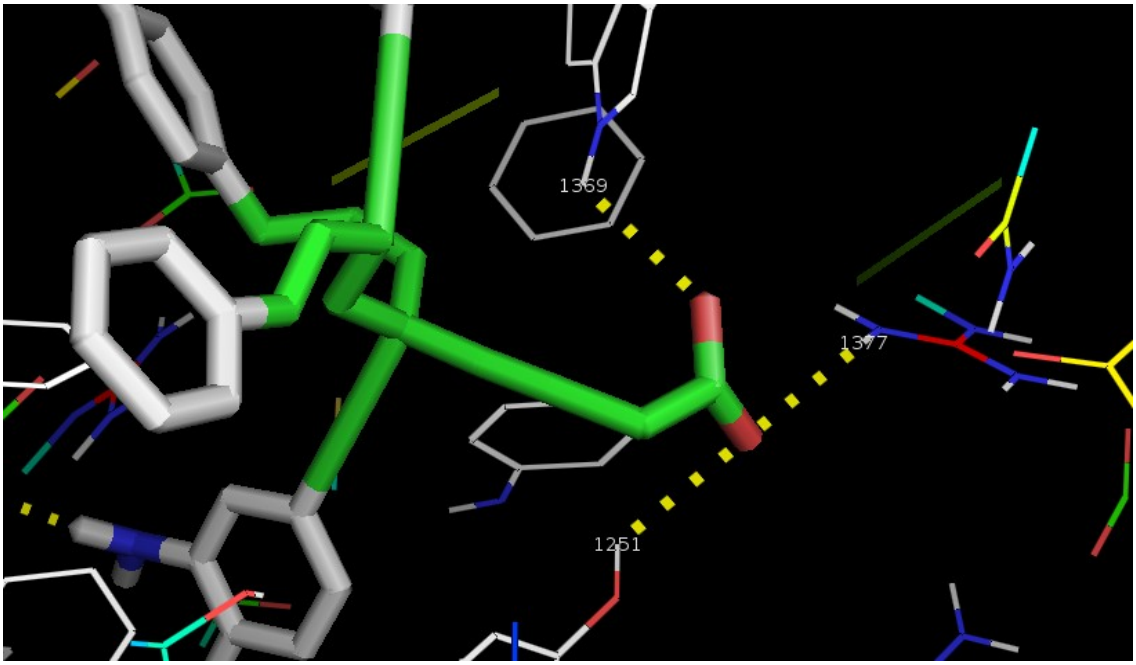


Figure 19 - Inhibitor designed in situ number 73 interacting with Try1251, Trp1369 and Arg1377

The interaction between Tyr1251 and Trp1369 is presented in Figure 20. Ligands that can establish interactions with the residues from Figure 18 usually interact with Tyr1251 and Trp1369 with an alternative conformation.

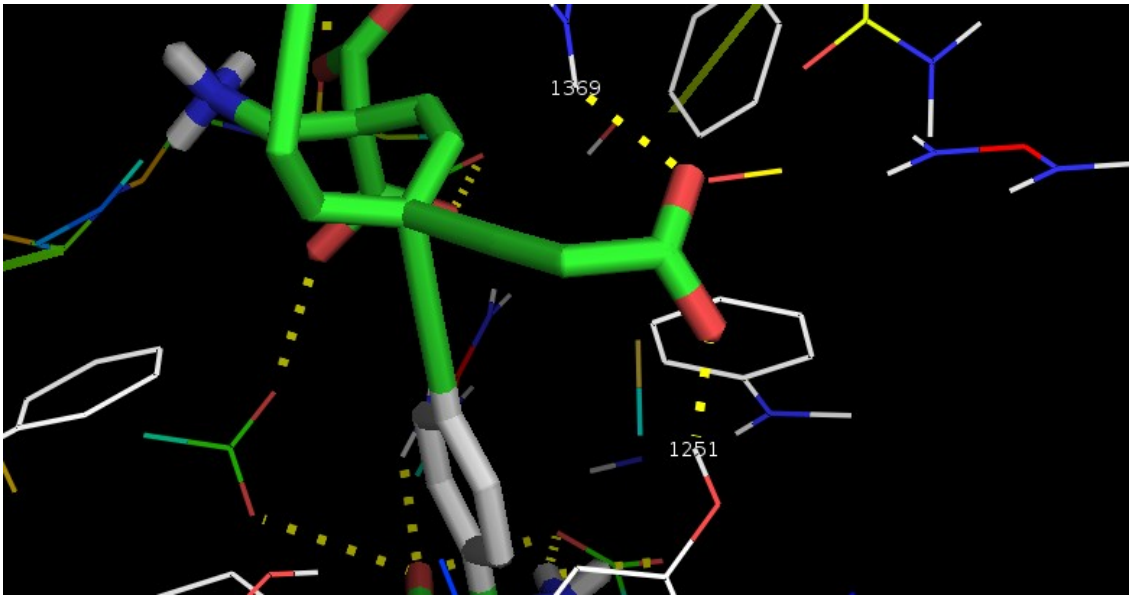


Figure 20 - Inhibitor designed in situ number 81 interacting with residues Tyr1251 and Trp1369

The carboxylic acid group in a different angle also interacts with Trp1369 and Arg1377 (Figure 21).

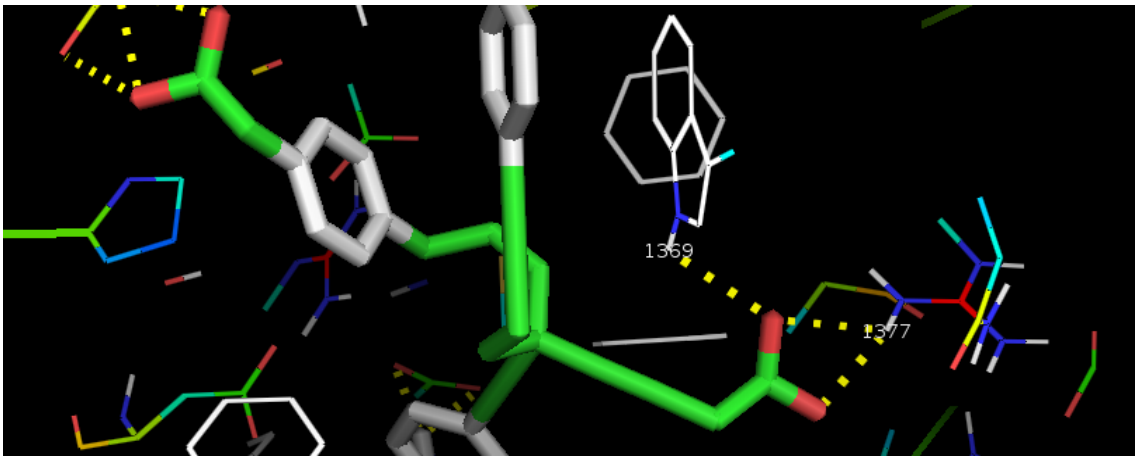


Figure 21 - Inhibitor designed in situ number 72 interacting with residues Trp1369 and Arg1377

The representation style of Figure 15 to Figure 17 and Figure 19 to Figure 21 is different from the other molecular representations as Discovery Studio 3.5 [118] was not capable of correctly opening/interpreting the Vina output files. As such the representations were created using PyMol [114].

Conclusion

At the end of this work it is possible to reach several conclusions. First, we were able to establish that caffeoyl quinic acids and their respective metabolites do not demonstrate a proper inhibitory activity towards α-gllucosidase (EC 3.2.1.20). This also leads to the conclusion that the positive effects that these compounds exert in diabetic patients is therefore reached through one or more alternative mechanisms of action. One of such mechanisms may be the interaction with glucose-6-phospate and its inhibition [49–51]. The fact that these compounds may also serve as inhibitors for other enzymes in the several pathways of the carbohydrate metabolism cannot be dismissed. A third mechanism of action is the blockage of the Na^+ dependent D-glucose transporter. This has been verified in mice with an effect up to 80% with 1mM of chlorogenic acid by Welsch *et al.* [53]. In humans, the effect has been established to occur but its extend has not been determined yet, to the best of our knowledge. As anti-oxidants, it is quite possible that caffeoylquinic acids alter the cellular chemical environment and prevent the naturally occurring reactions. If we consider an hydrolysis reaction, it is an oxidation or reduction reaction depending on which reactant is considered. It is quite possible that caffeoylquinic acids undergo the oxidation process instead of the intended reactant and thus prevent the formation of the reaction's regular products. It is also quite possible that all of these mechanisms coexist simultaneously and thus provide the outcomes observed in patients treated with caffeoylquinic acids/green coffee extract.

Secondly, the design of and enzymatic inhibitor is extremely difficult. Being able to create a ligand that will bind to the required site and interact specifically with the intended residues in the way it was designed to requires a lot of trial and error and experience. The design of a new ligand from an existing known enzymatic inhibitor is a process that should be considered when developing a new inhibitor. Having an inhibitor that has known interactions with the target under research makes the initial development time of candidates smaller. It also makes it easier to establish connections with certain residues if the structural change to the ligand is not very extensive. However, it poses a few problems during the design process. First the added functionality may completely alter the interactions between the ligand and the receptor. The best way to avoid this, as it was discovered throughout this study, is to introduce a single functional group change at a time. Another disadvantage of optimizing an existing ligand is that the optimization process may improve affinity but jeopardizes the drug's usability as it often becomes quite large and stops complying with the Lipinski's rules of five.

Thirdly it is possible to conclude that molecular docking programs, algorithms and functions can still be vastly improved to provide more accurate results. Considering the results from the acarbose control dockings this becomes even more evident. Despite this, their value in providing research guides or priority lists cannot be dismissed. The fact that hundreds of ligands can be tested and compared in a very small amount of time make computational methods invaluable. In addition, having an experimental

result to reproduce through computational methods provides simultaneously a control experiment and reduces the limitations in the accuracy of the results obtained.

On a future work, the interaction between caffeoylquinic acids and all the enzymes in the Carbohydrate metabolism should be determined. It is possible that studying the inhibition of all these enzymes by caffeoylquinic acids resorting to computational methods would help to confirm or exclude and shed some light on currently accepted mechanisms of action. It would also provide new insights into the molecular mechanisms of the effects that these compounds produce.

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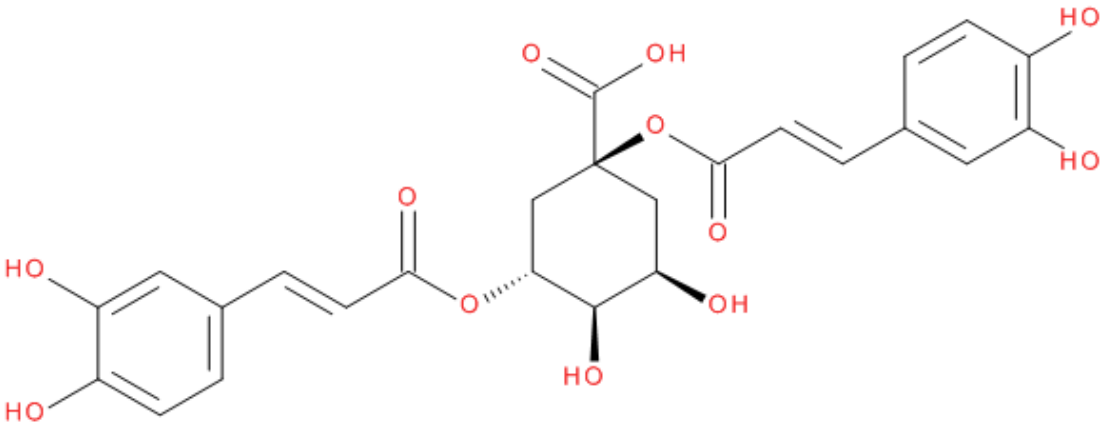
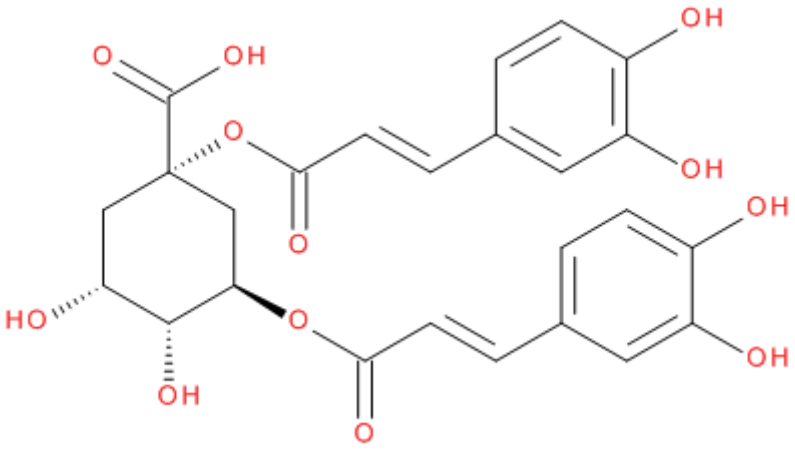
Annexes

Naturally occurring Caffeoylquinic acids, derivates and metabolites



Annex 1 - Representation of Inib1



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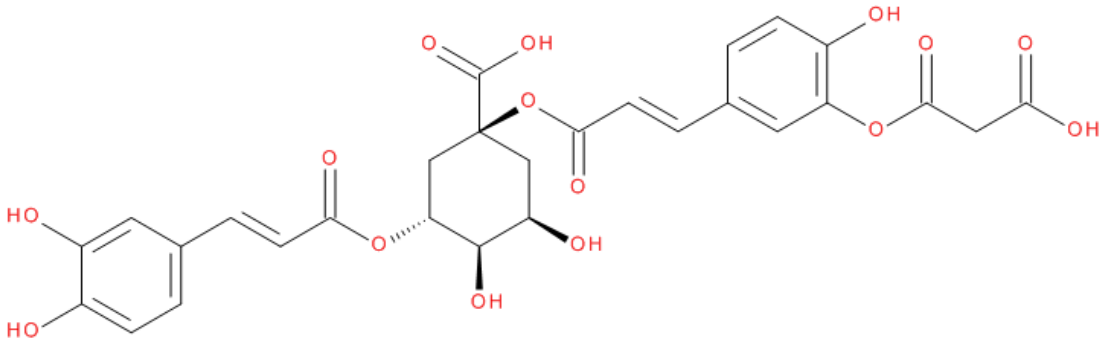

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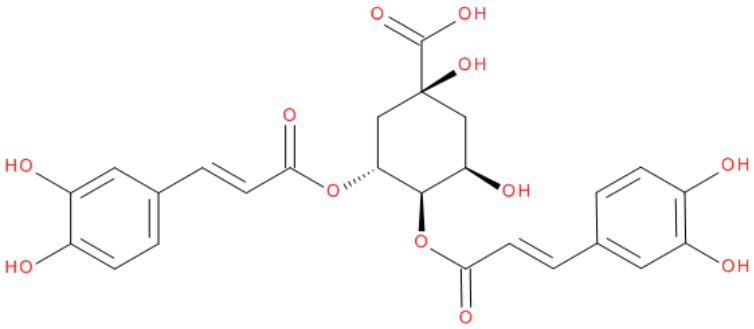

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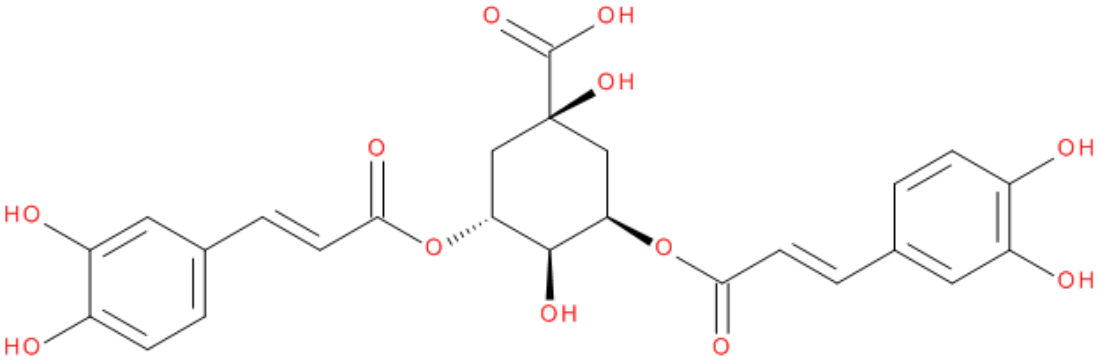

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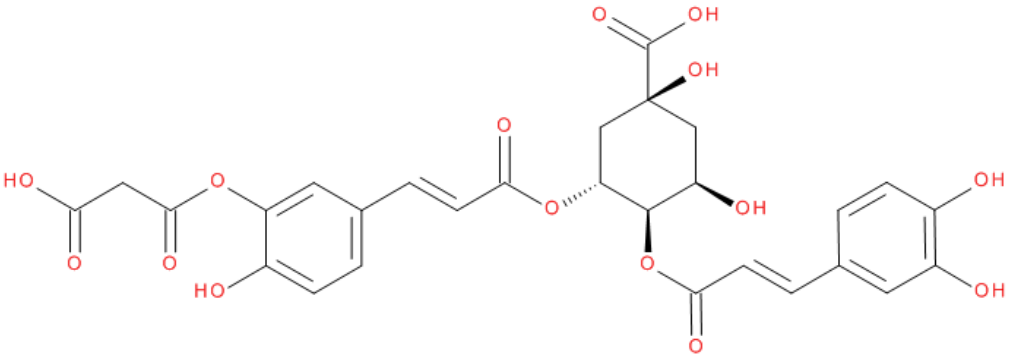

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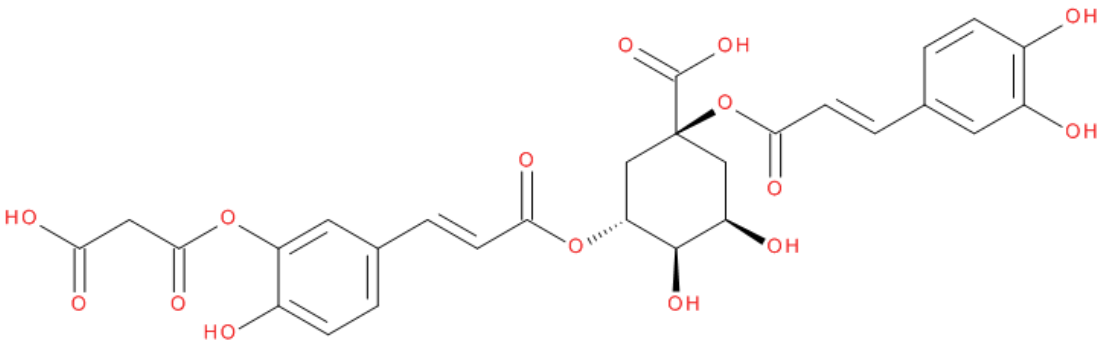

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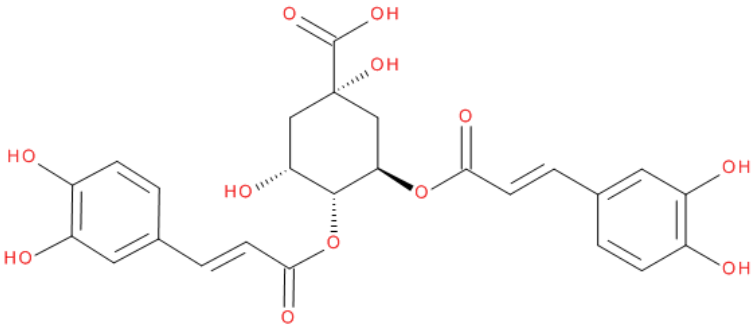

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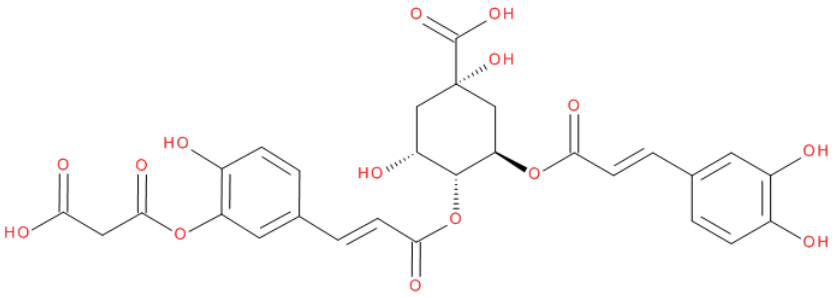

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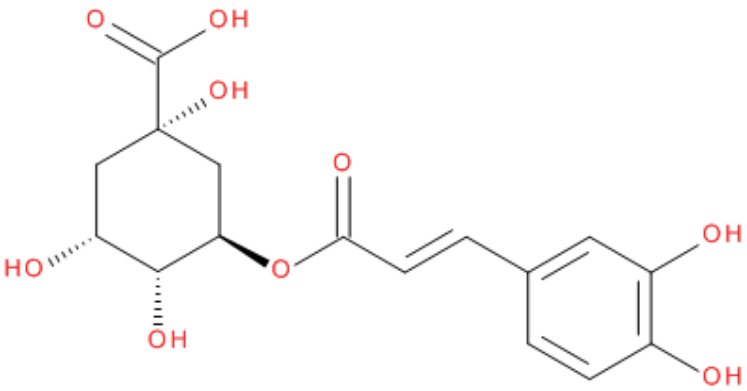

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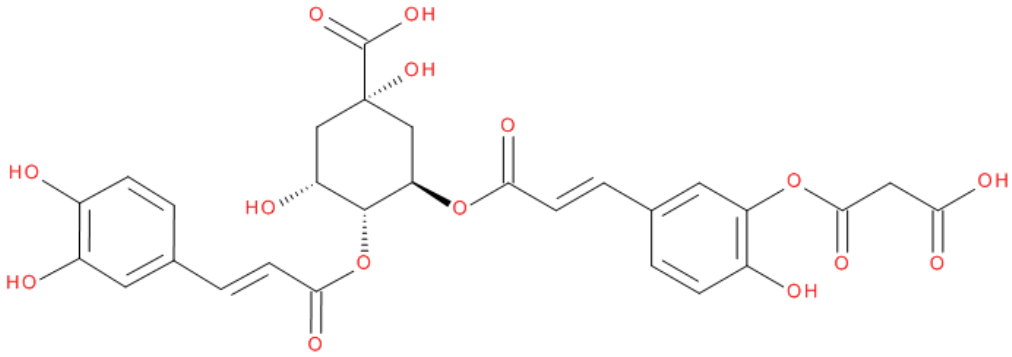

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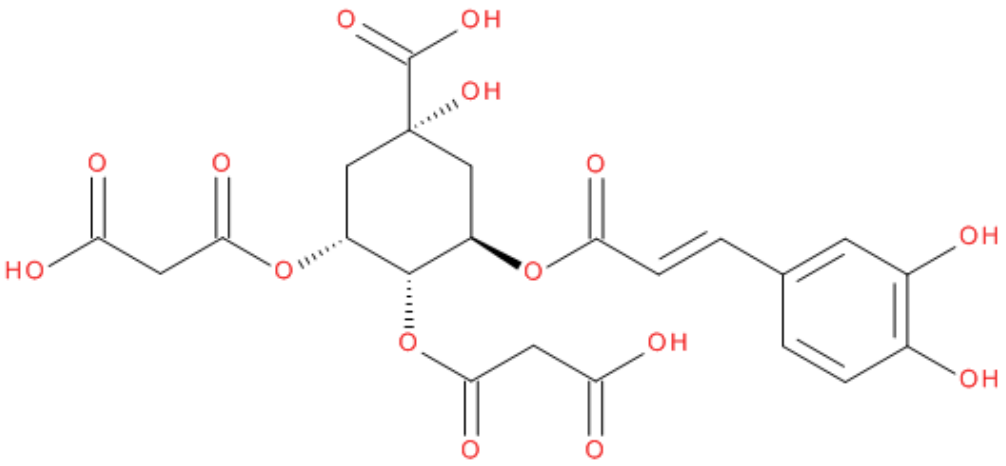

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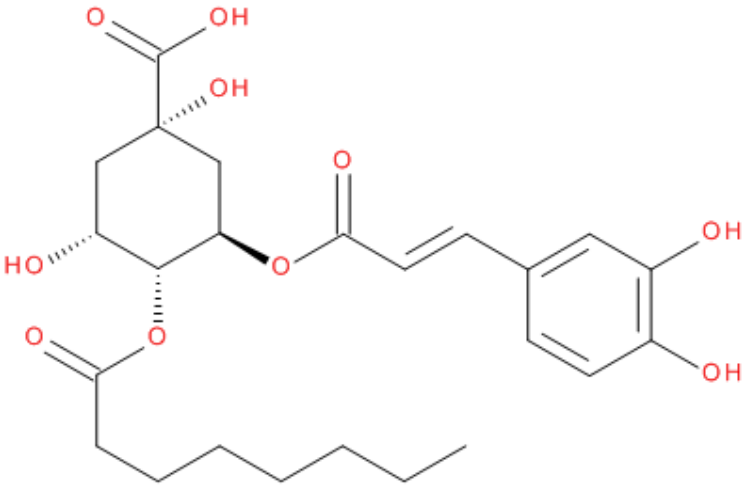

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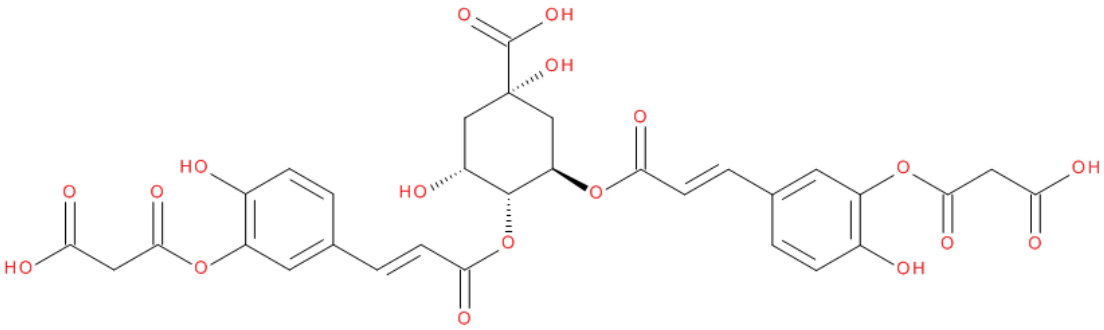

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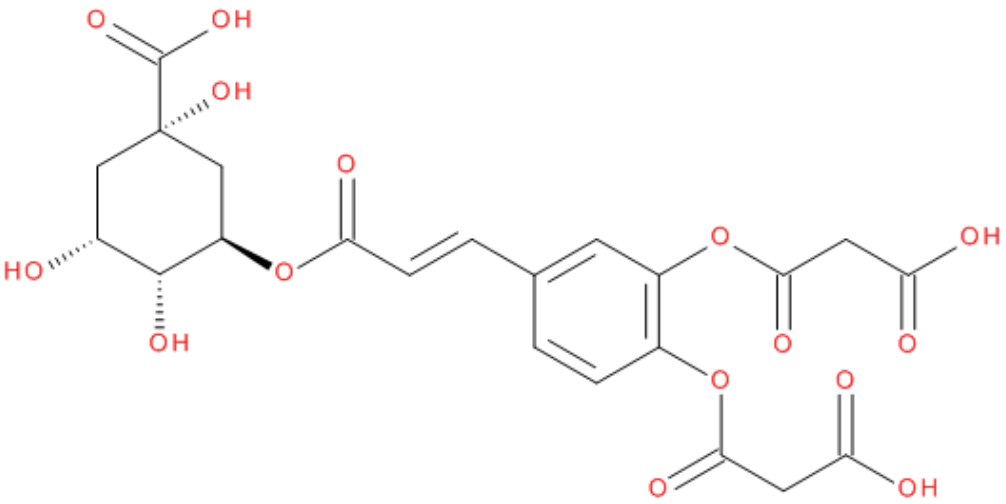

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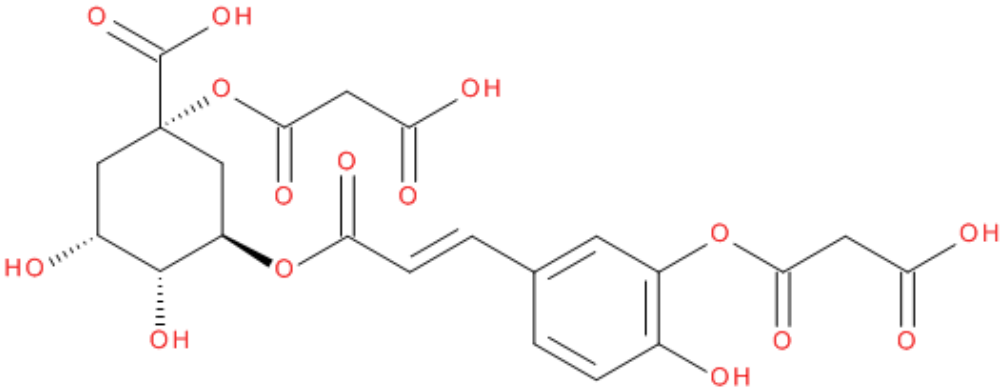

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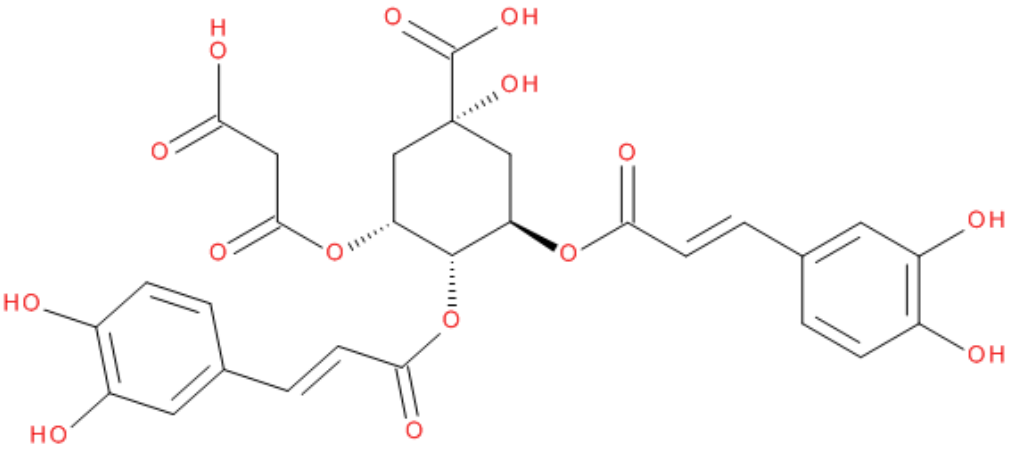

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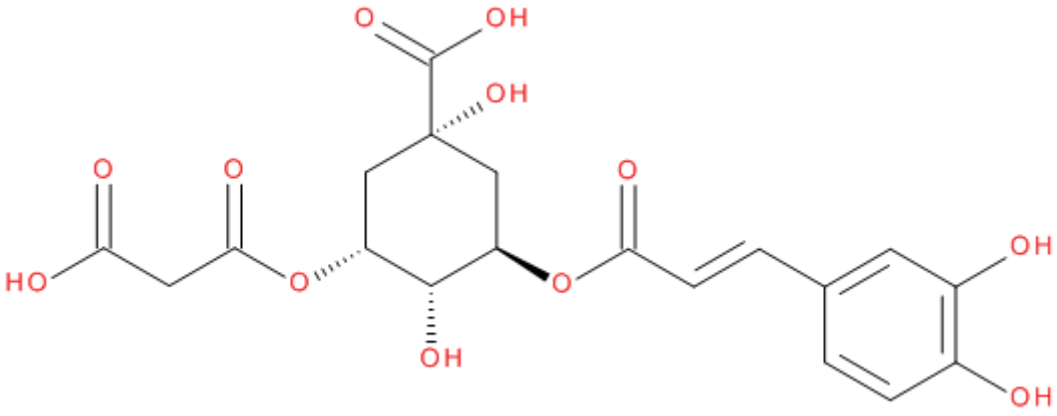

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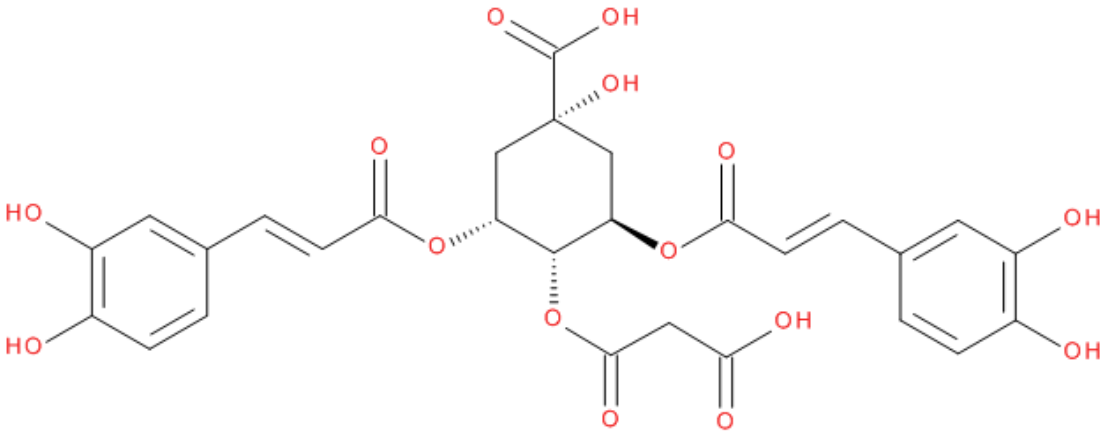

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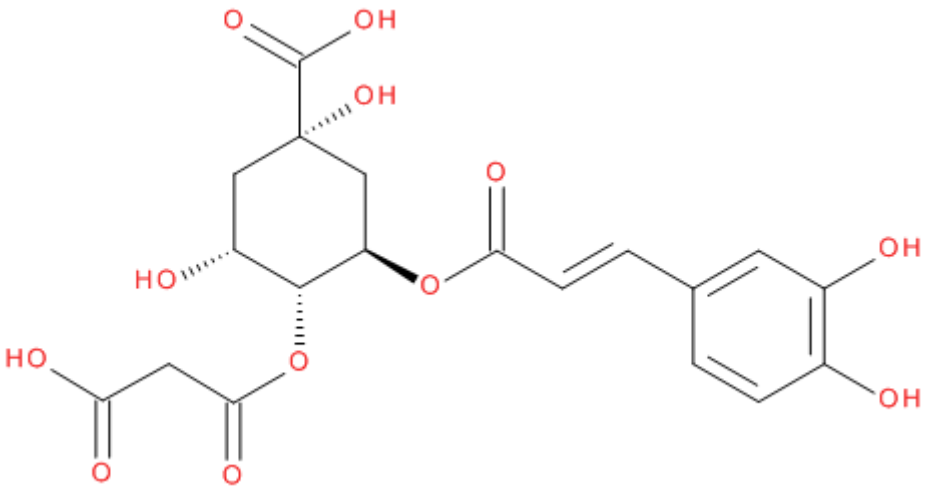

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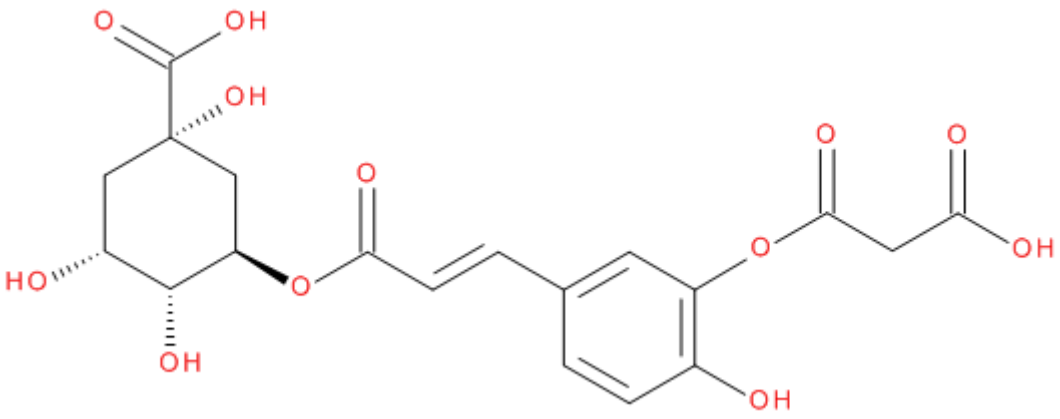

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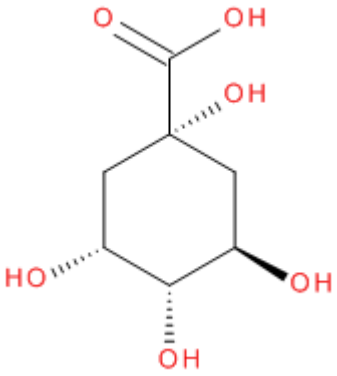

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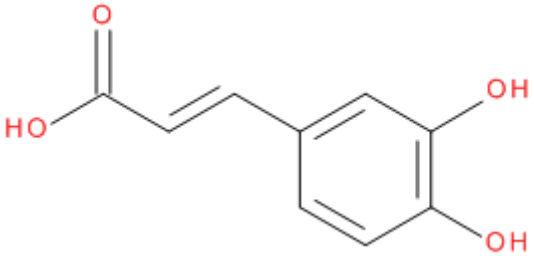

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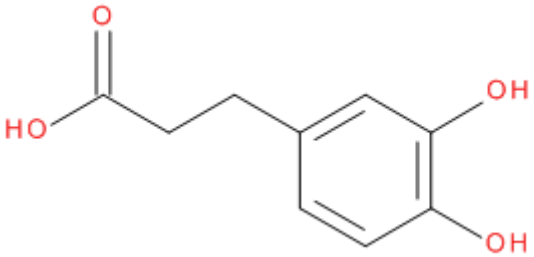

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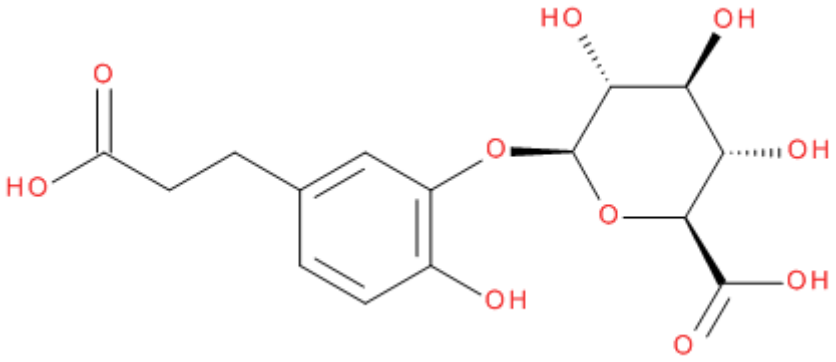

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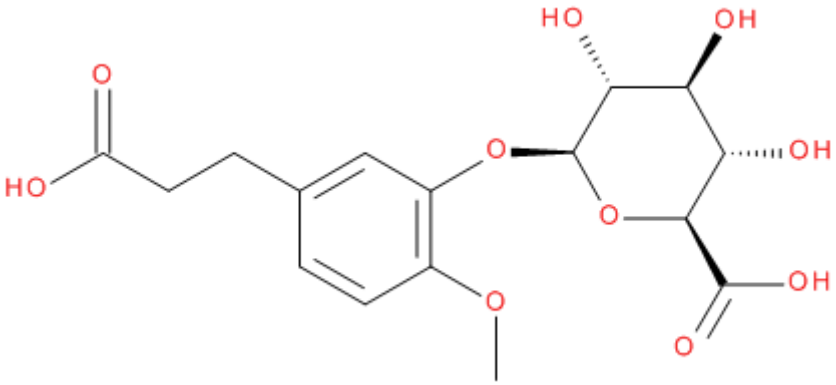

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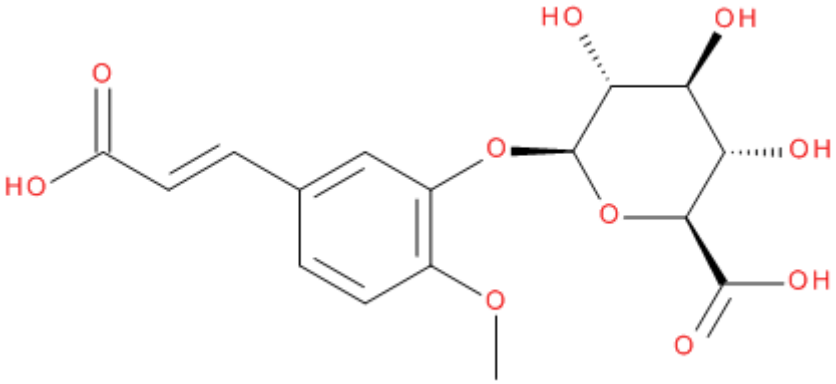

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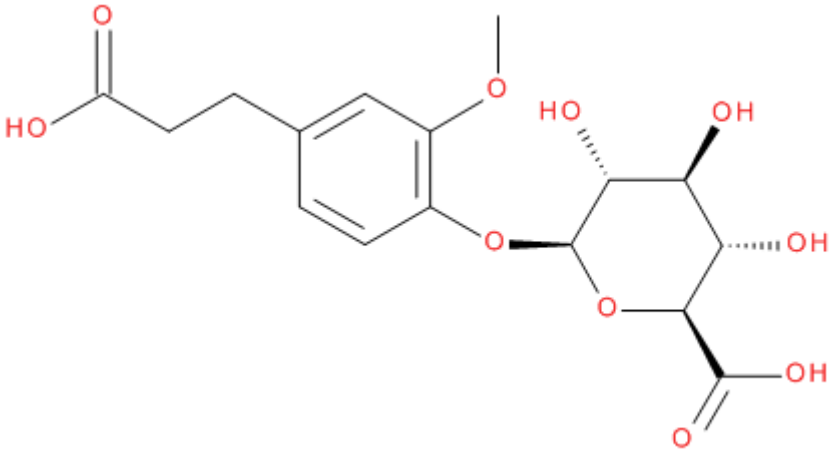

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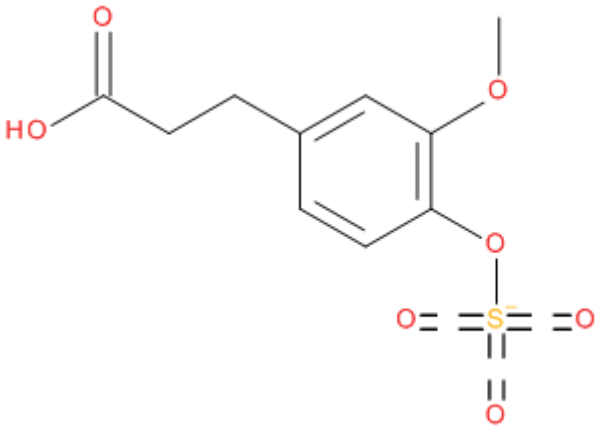

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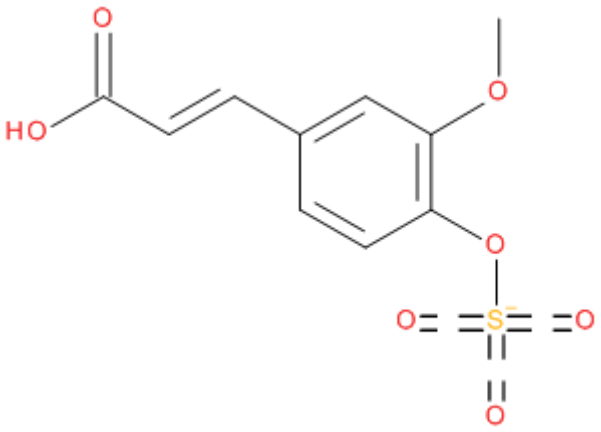

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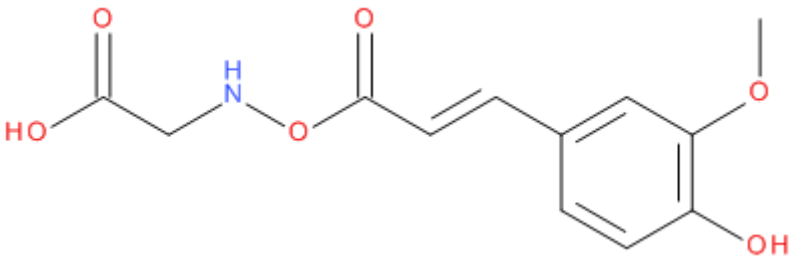

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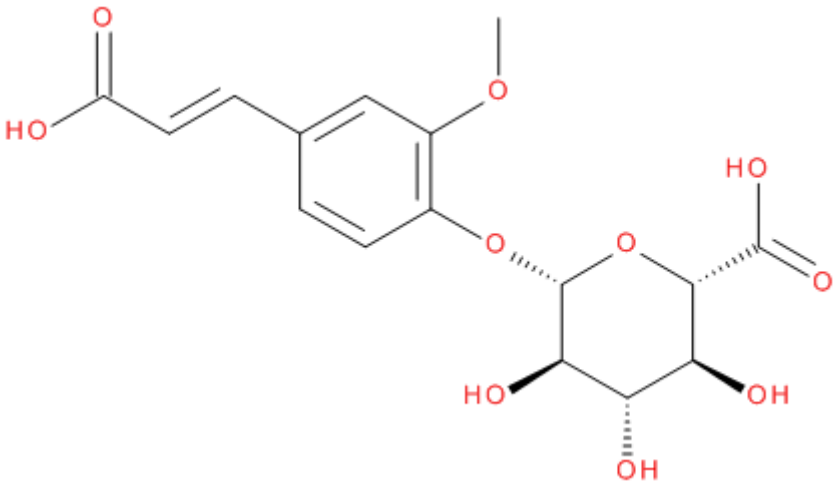

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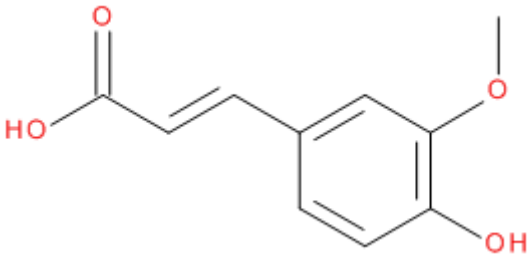

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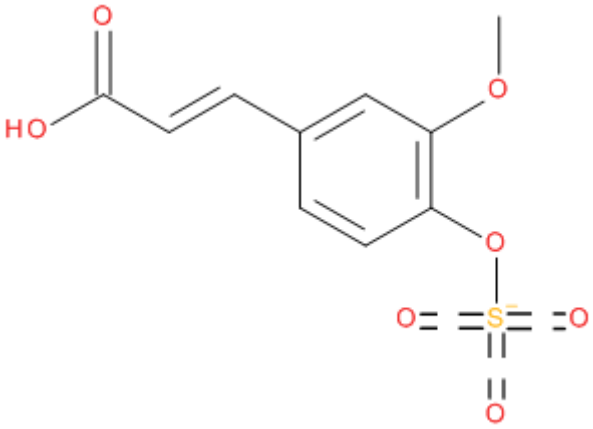

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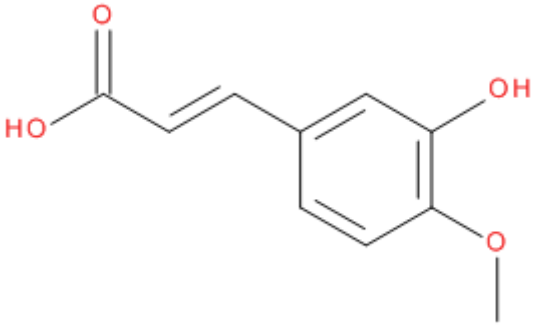

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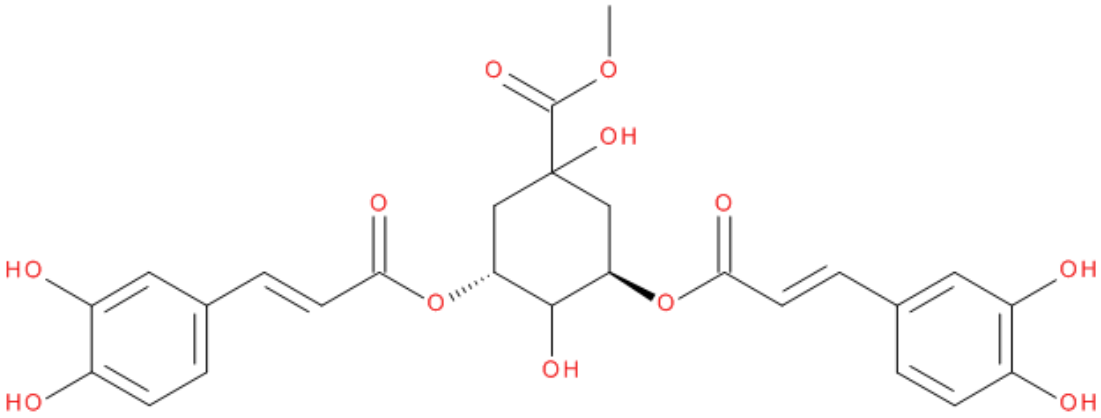
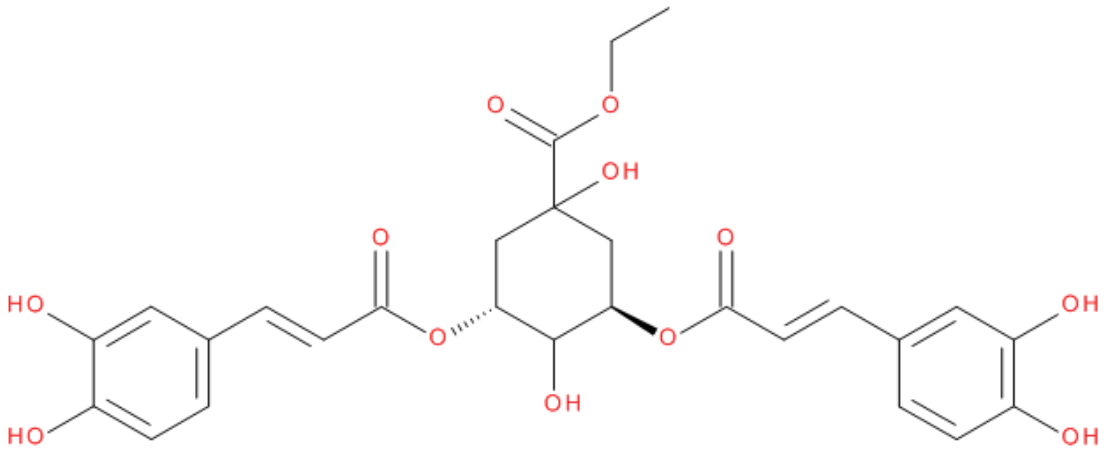


Annex 36 - Representation of Opt1



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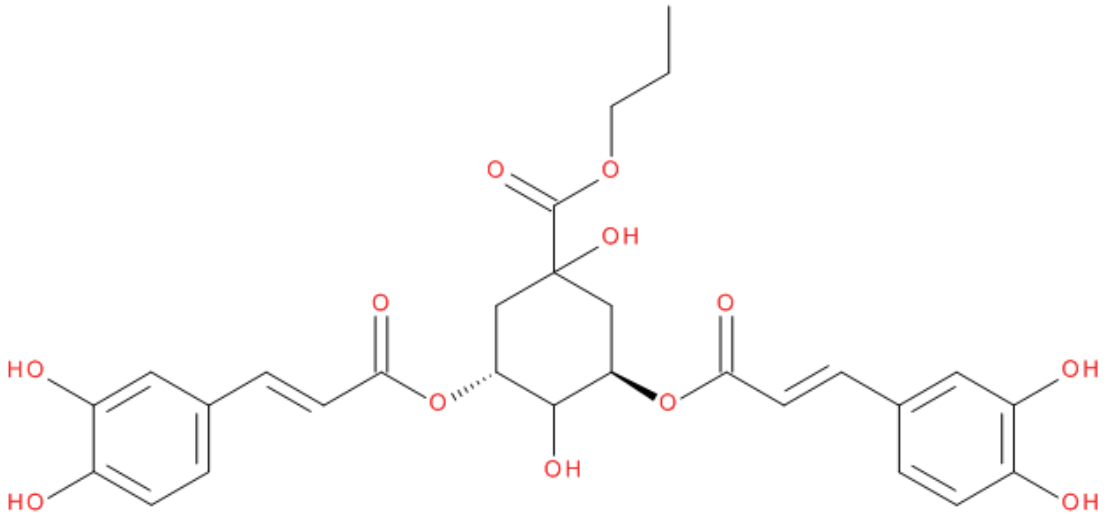
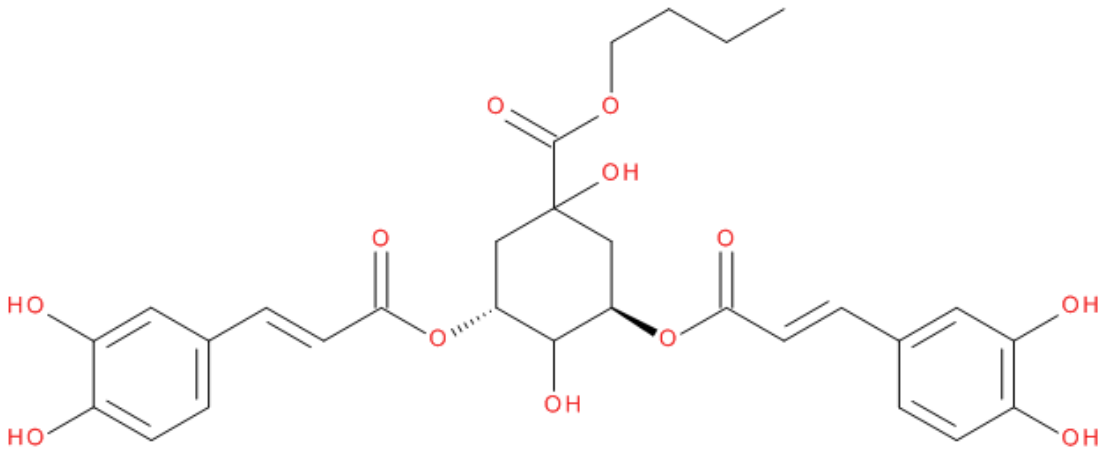
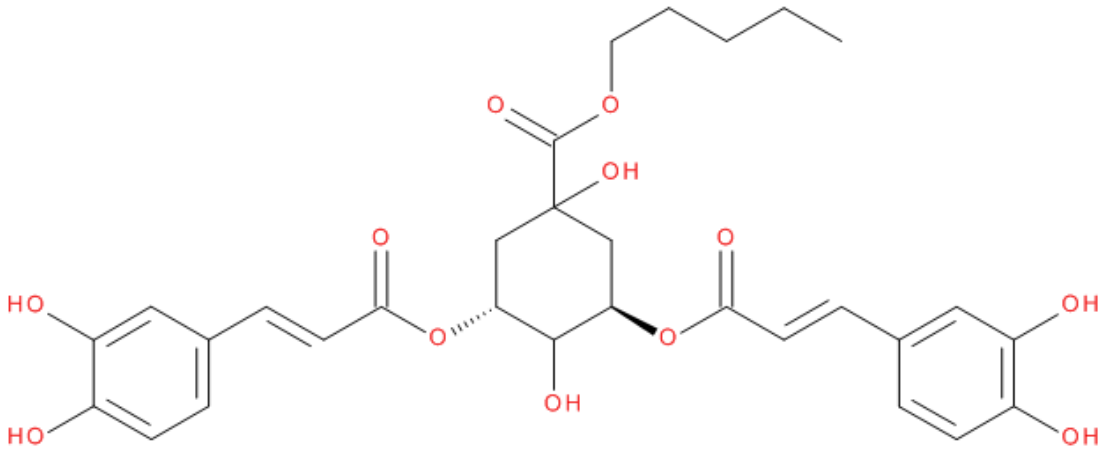


Annex 38 - Representation of Opt3



Annex 39 - Representation of Opt4



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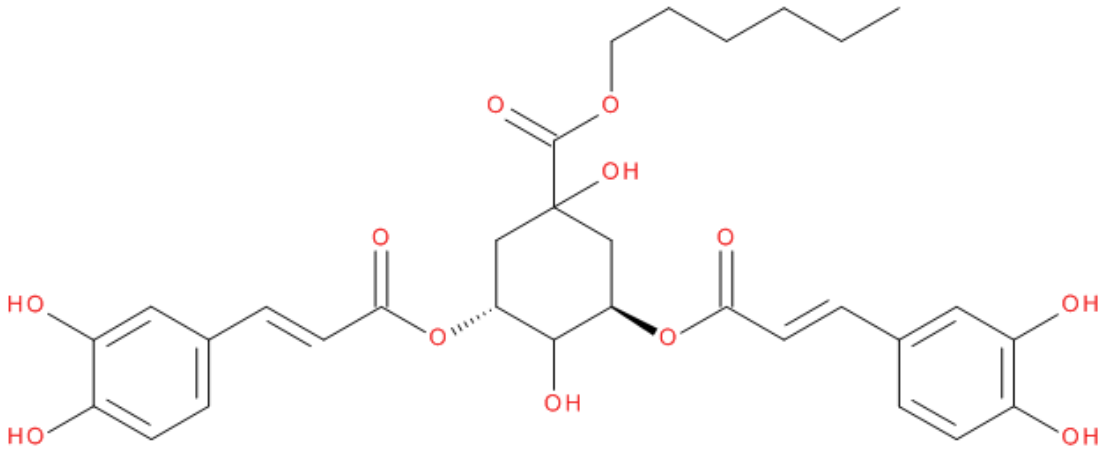

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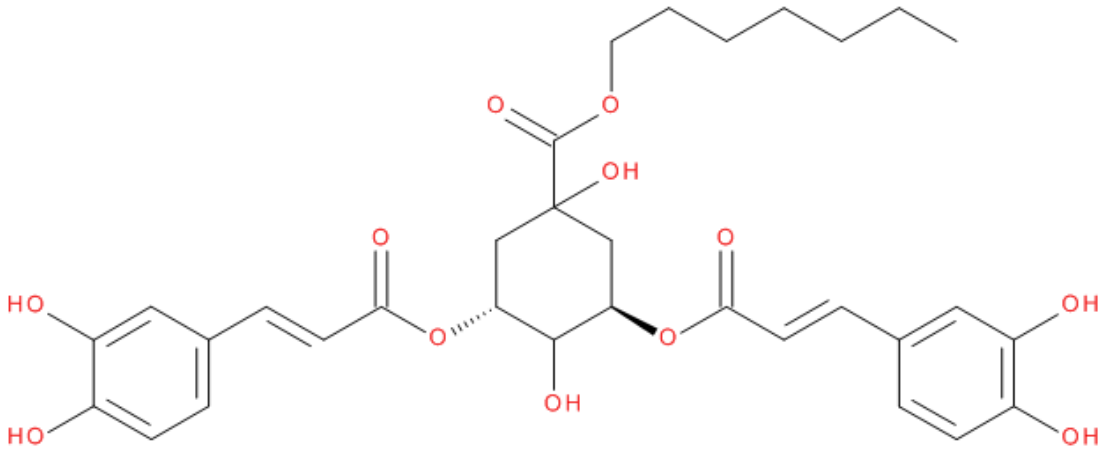

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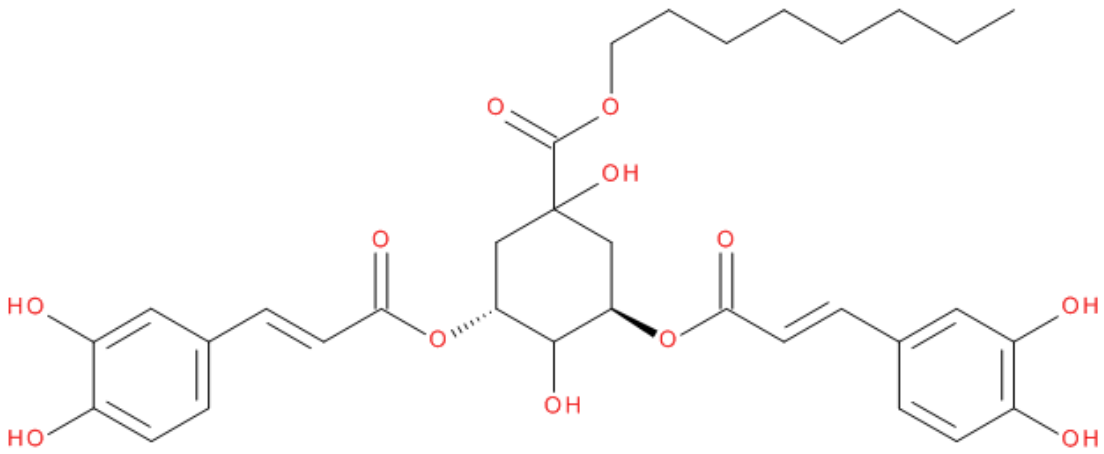

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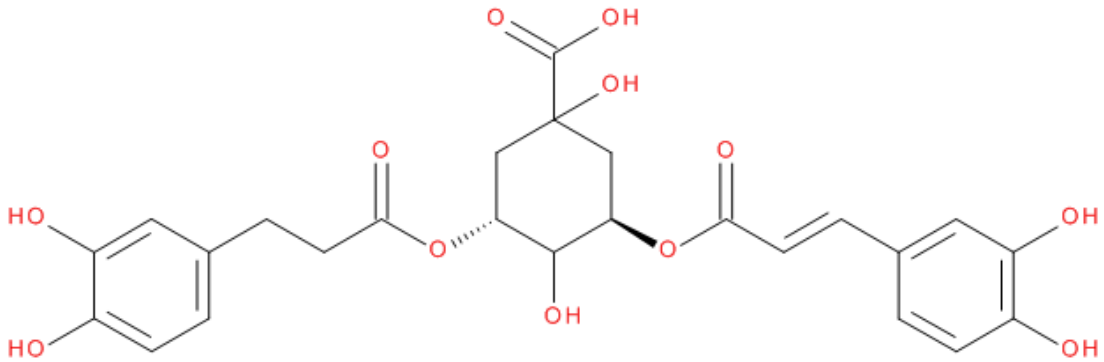

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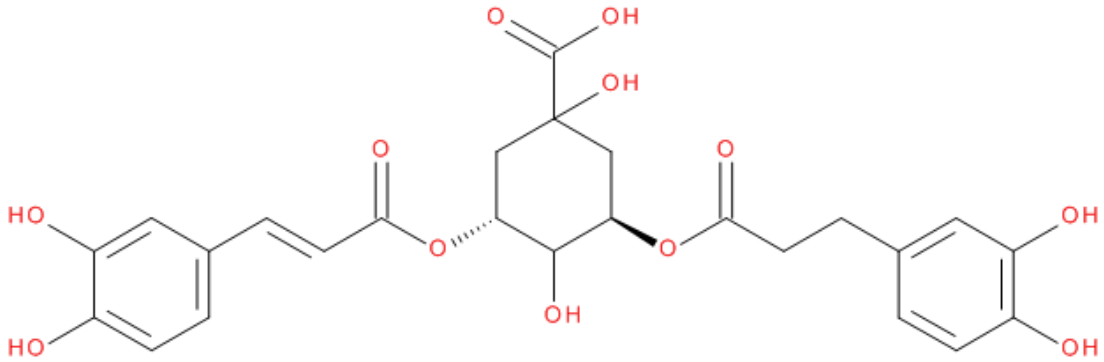
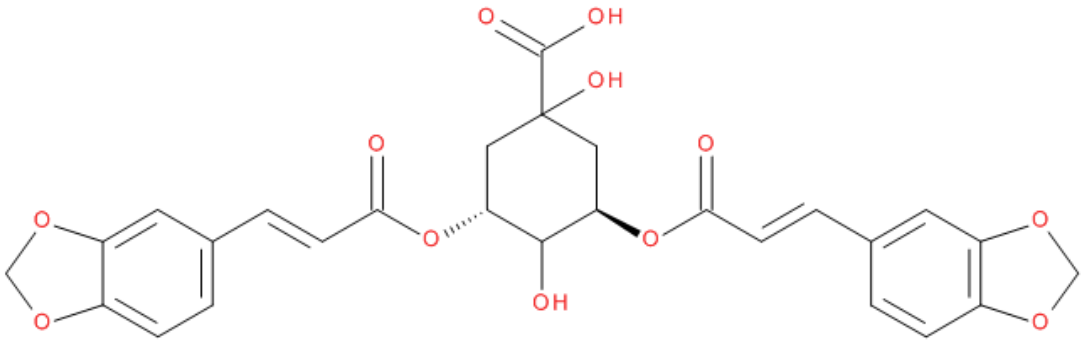
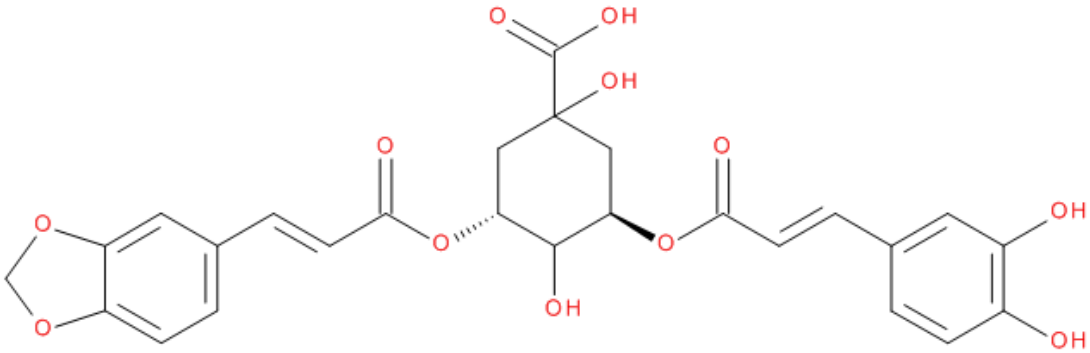


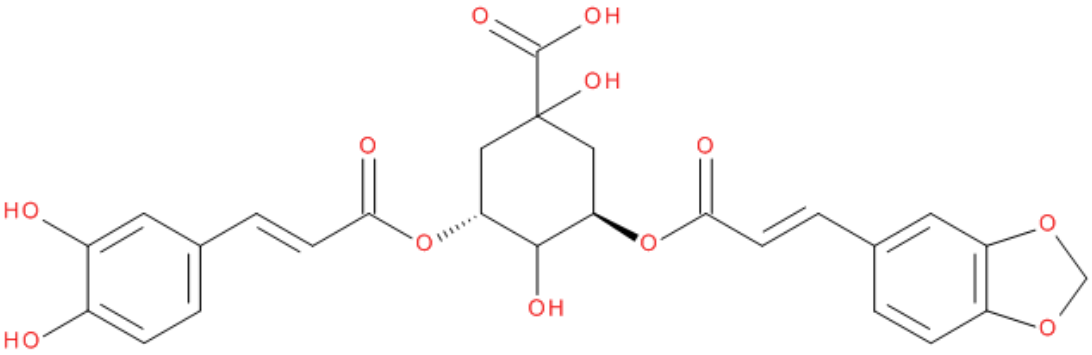
Annex 45 - Representation of Opt10



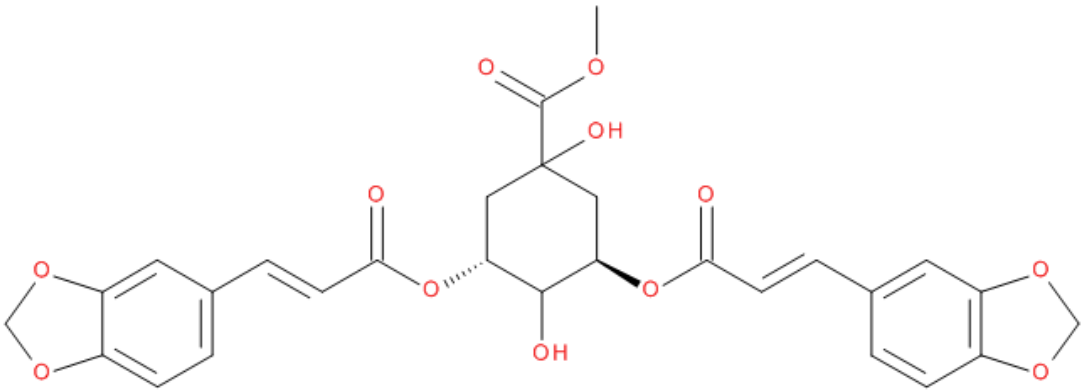
Annex 46 - Representation of Opt11



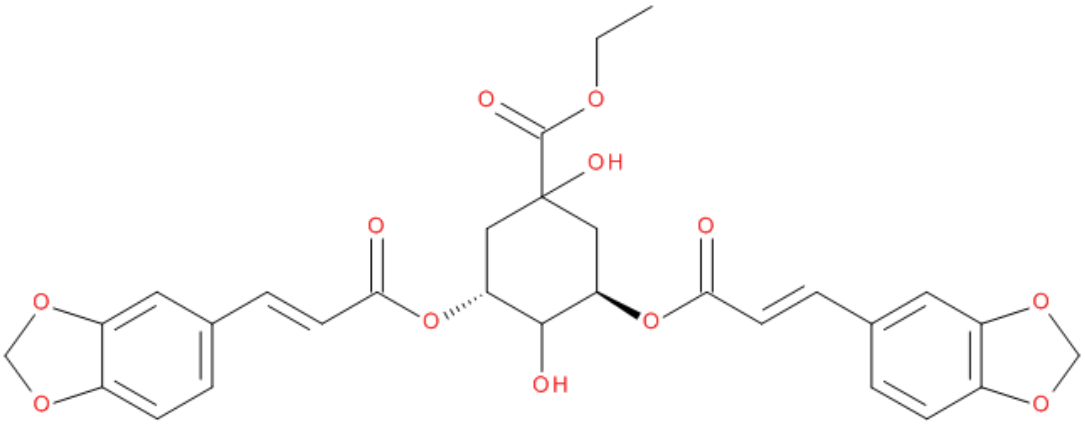
Annex 47 - Representation of Opt12



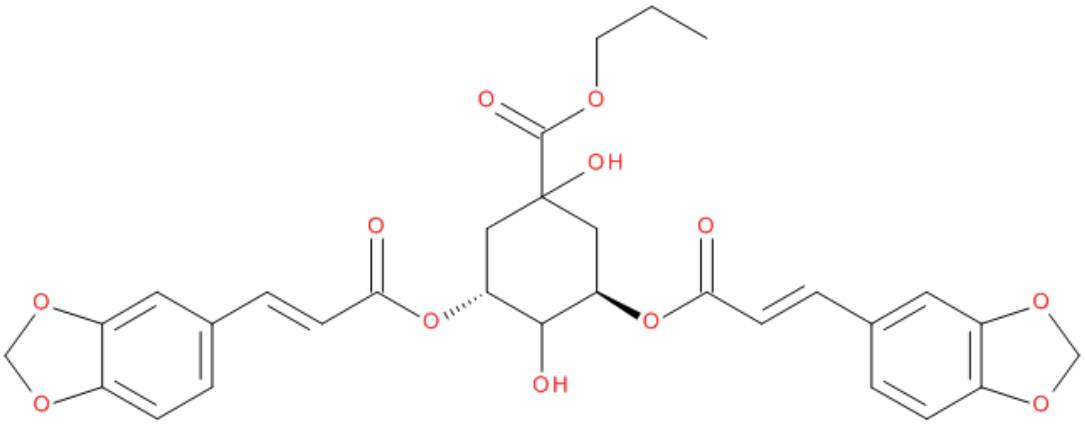
Annex 48 - Representation of Opt13



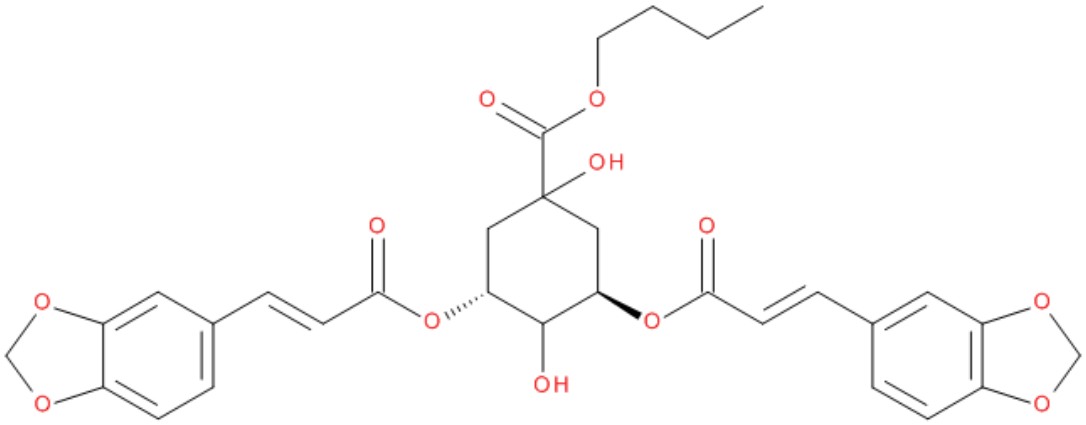
Annex 49 - Representation of Opt14



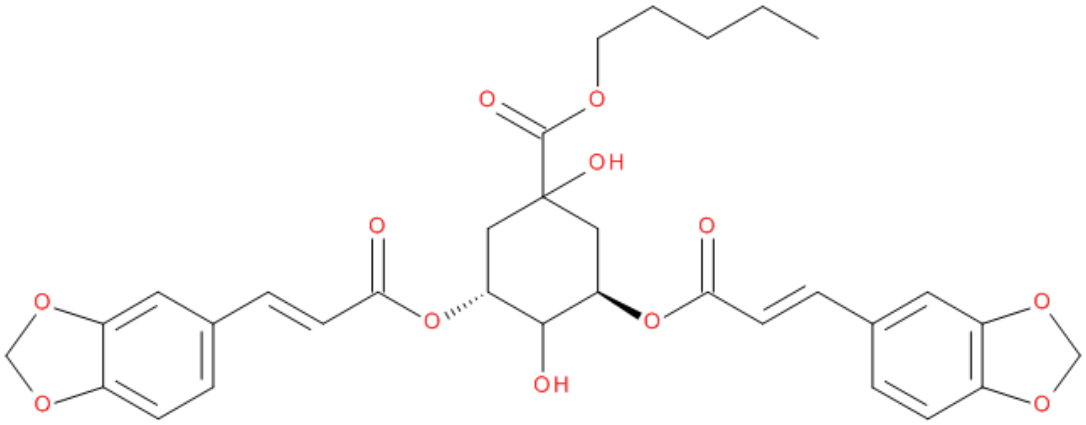
Annex 50 - Representation of Opt15



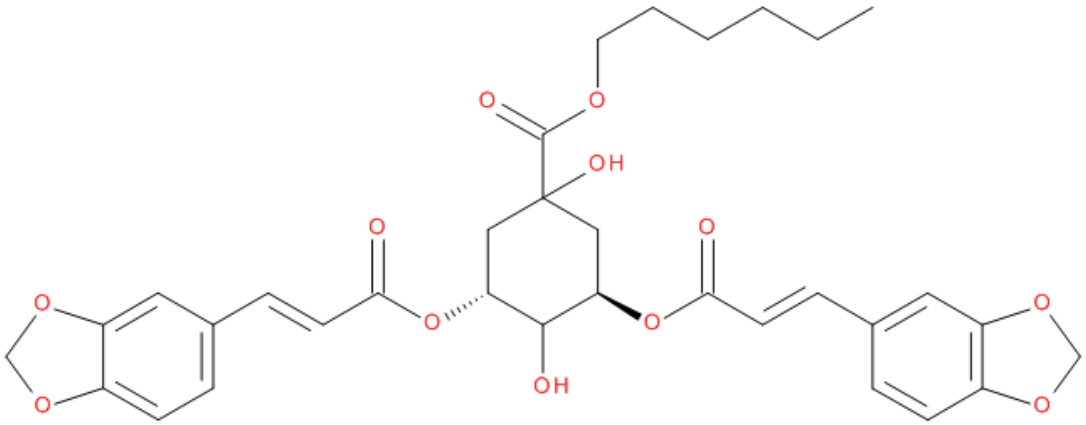
Annex 51 - Representation of Opt16



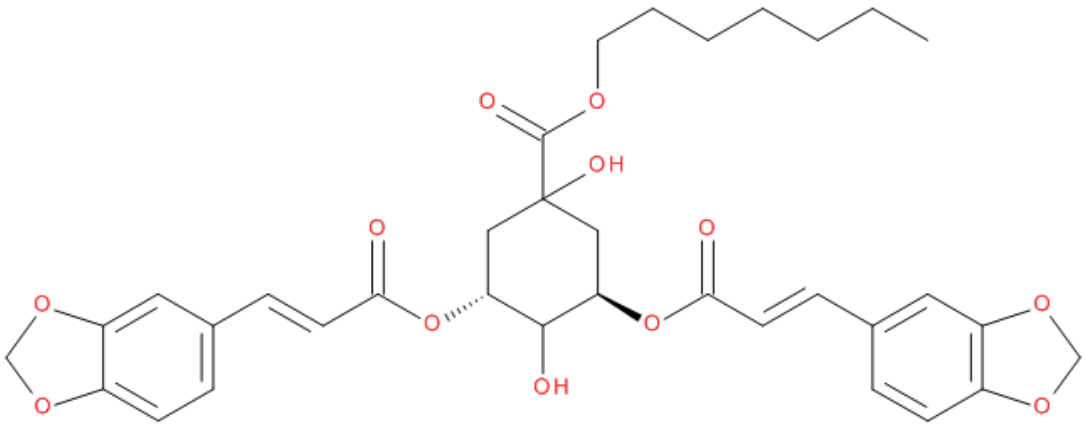
Annex 52 - Representation of Opt17



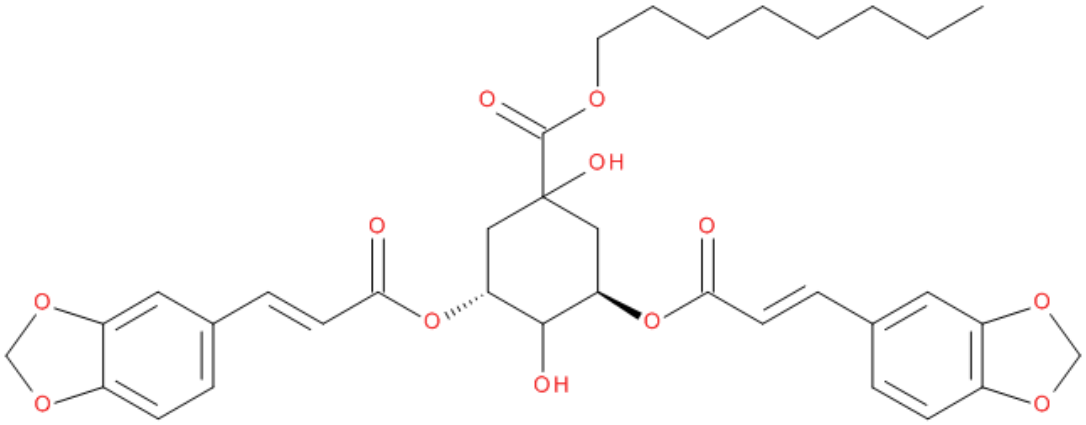
Annex 53 - Representation of Opt18



Annex 54 - Representation of Opt19

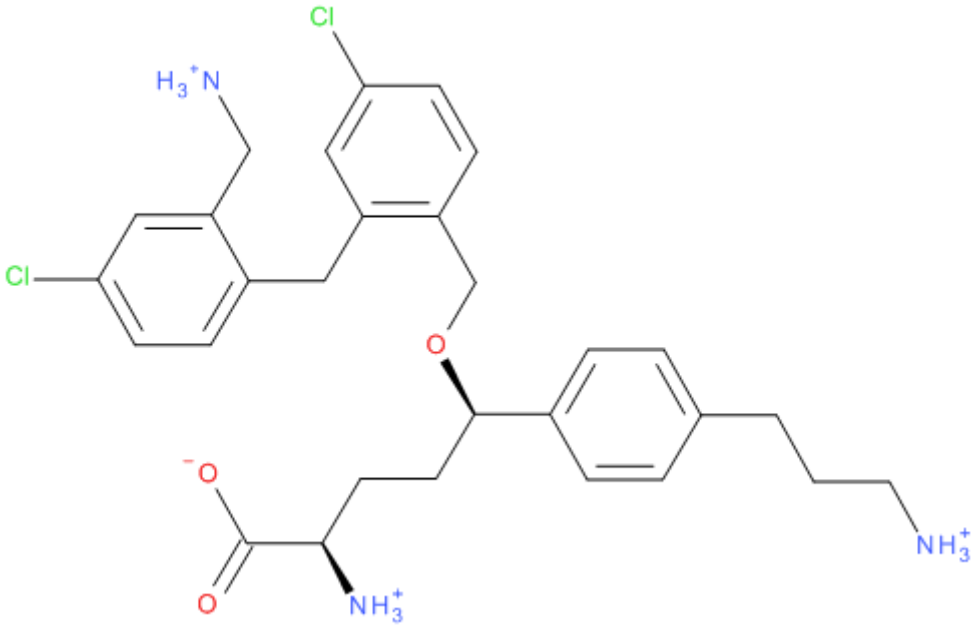


Annex 55 - Representation of Opt20

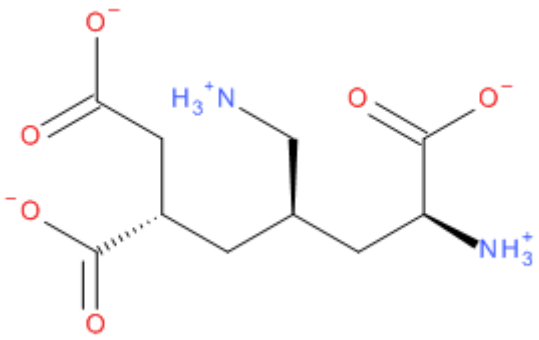


Annex 56 - Representation of Opt21

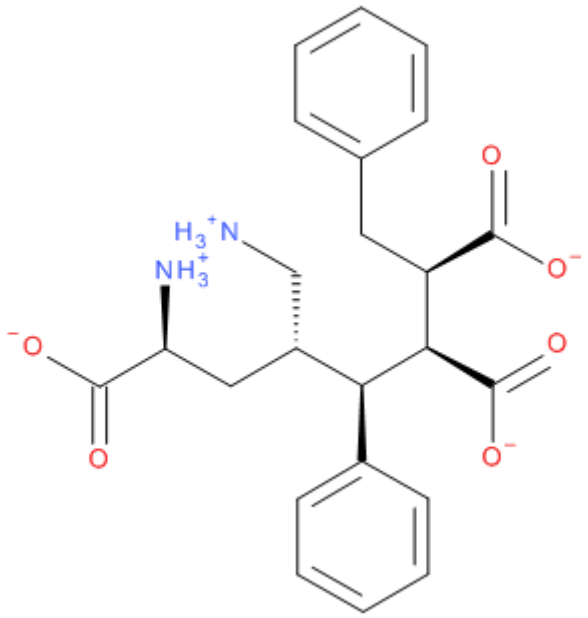
Ligands designed *in situ*



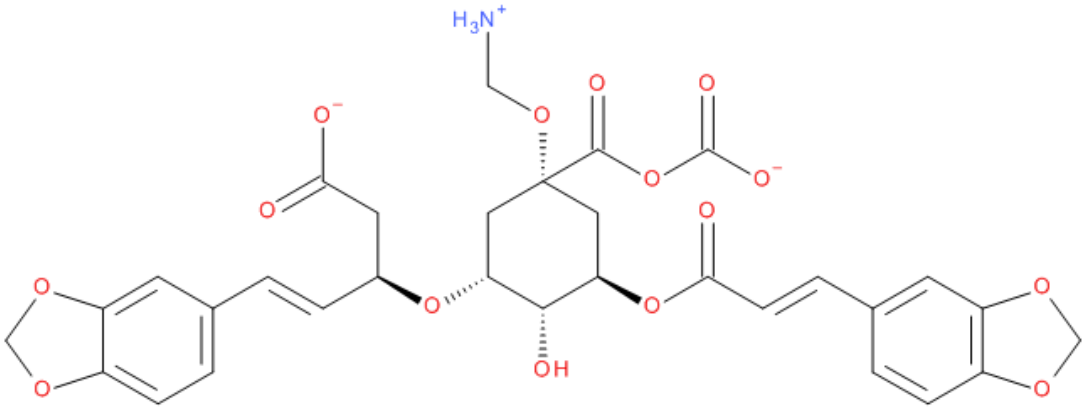
Annex 57 - Representation of Insitu1



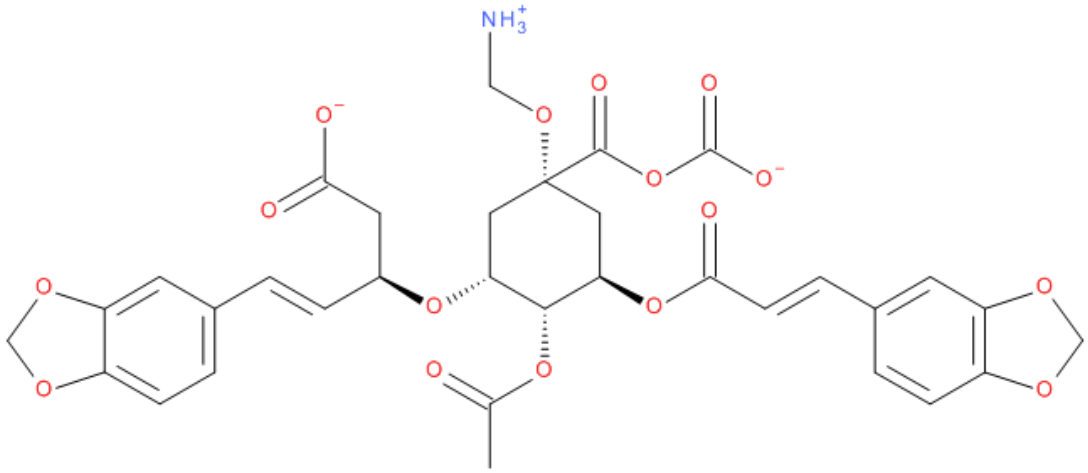
Annex 58 - Representation of Insitu2



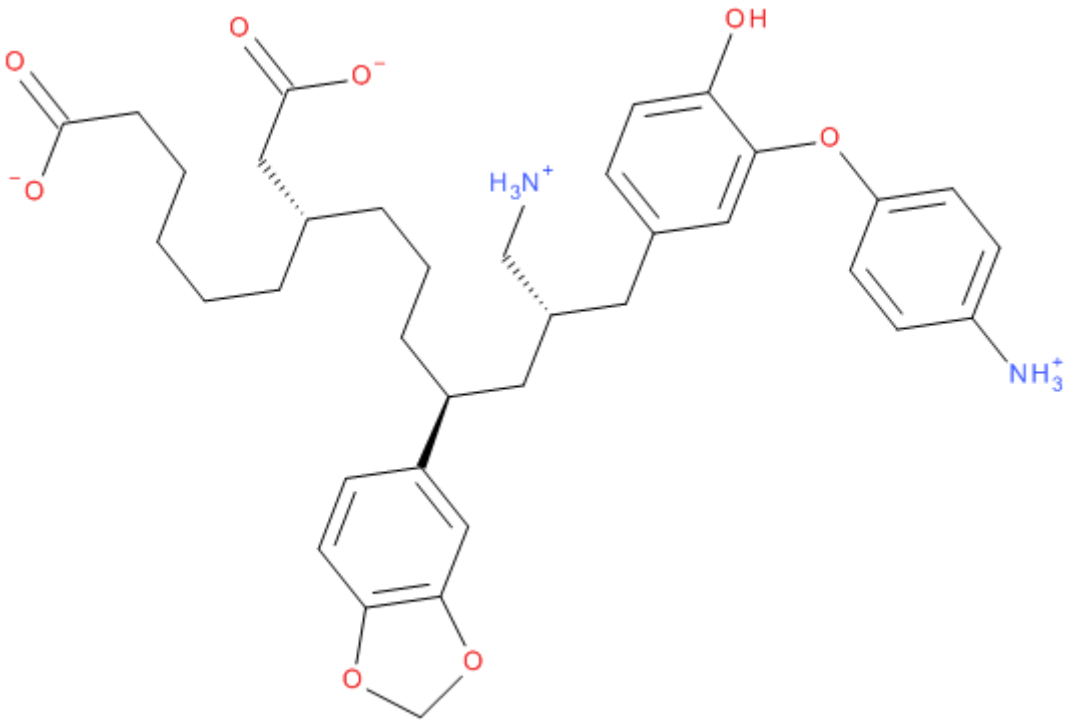
Annex 59 - Representation of Insitu3



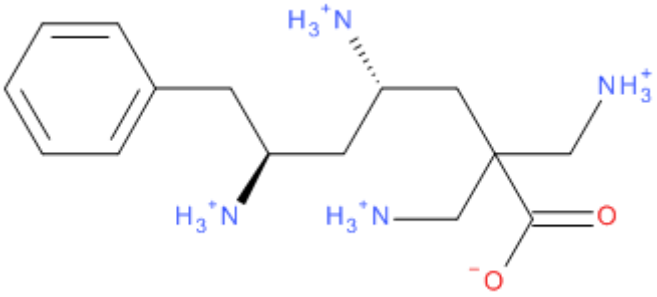
Annex 60 - Representation of Insitu4



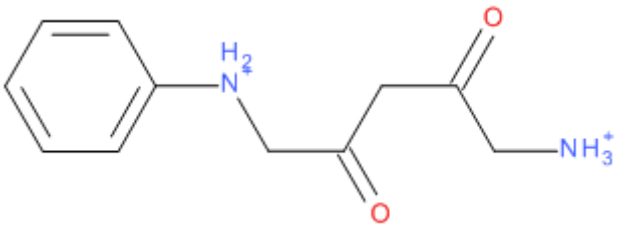
Annex 61 - Representation of Insitu5



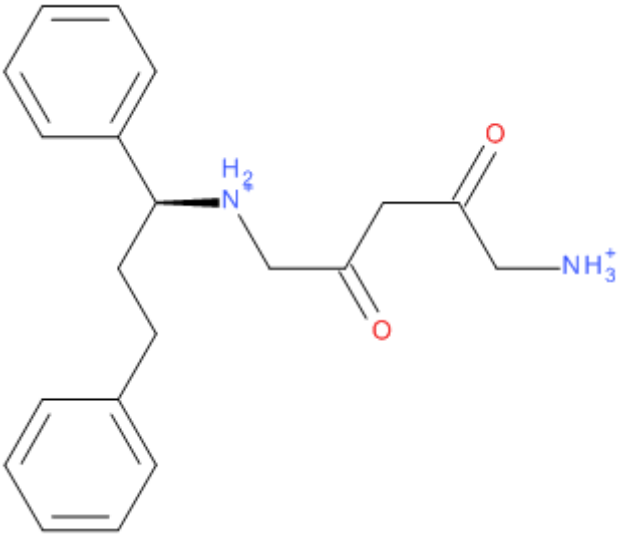
Annex 62 - Representation of Insitu6



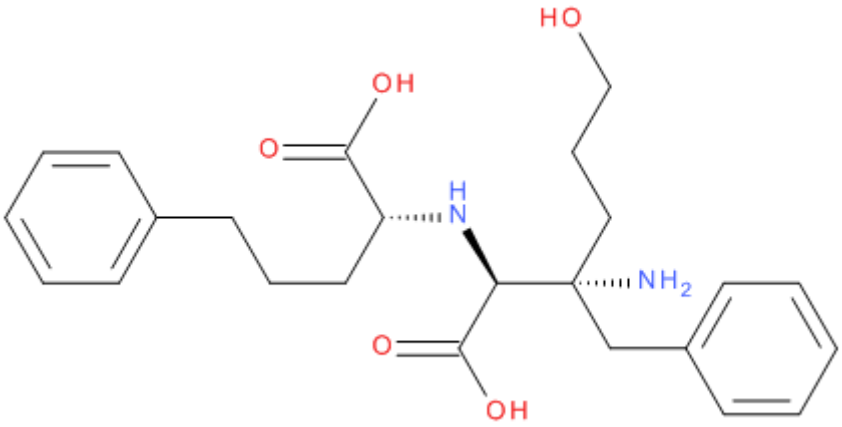
Annex 63 - Representation of Insitu7



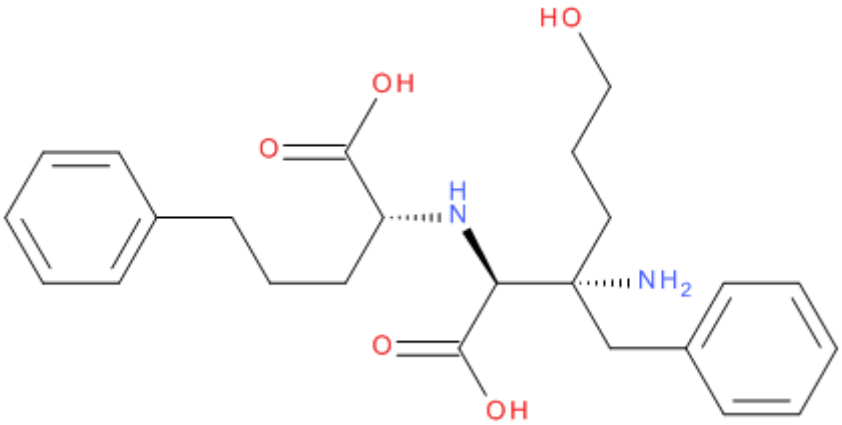
Annex 64 - Representation of Insitu8



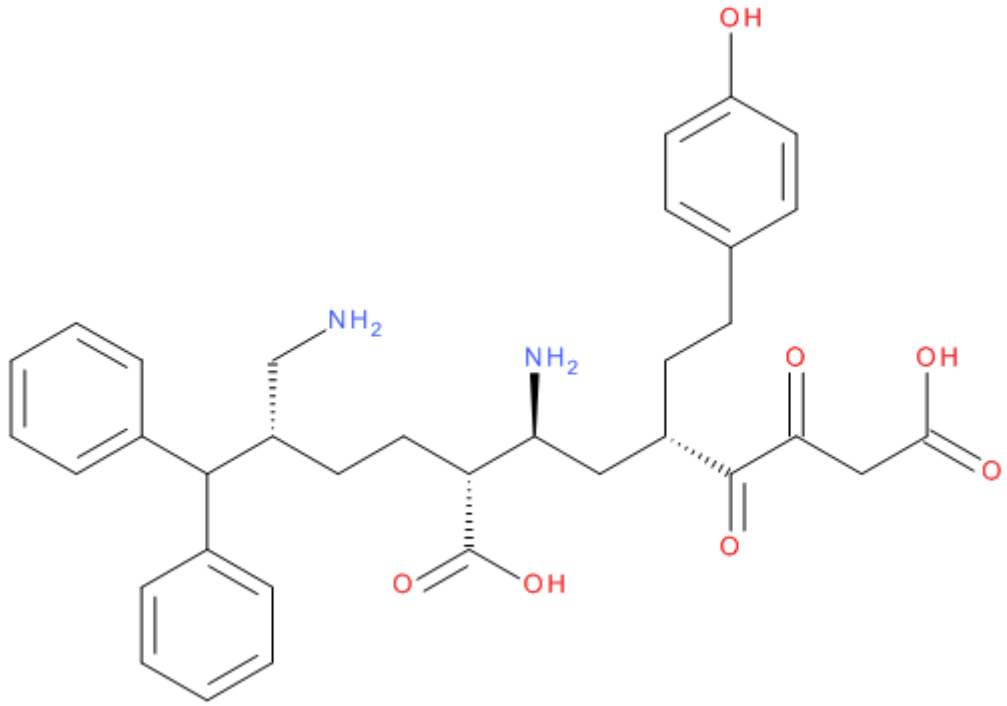
Annex 65 - Representation of Insitu9



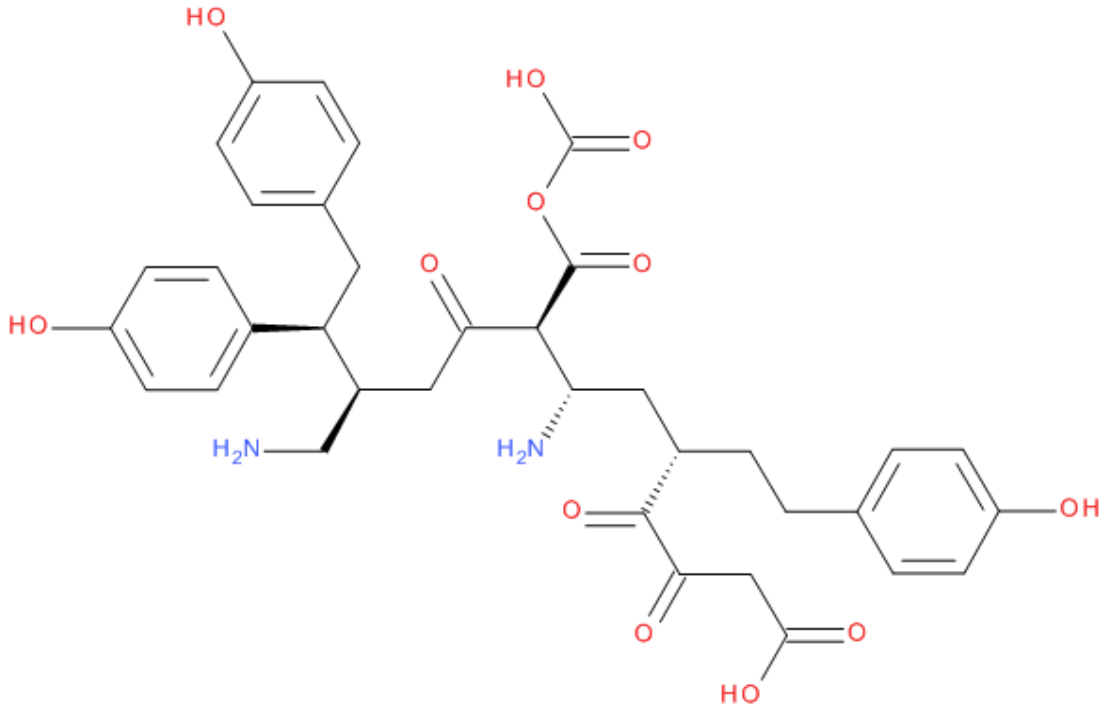
Annex 66 - Representation of Insitu10 (the three dimensional structure of this ligand had no rotatable bonds)



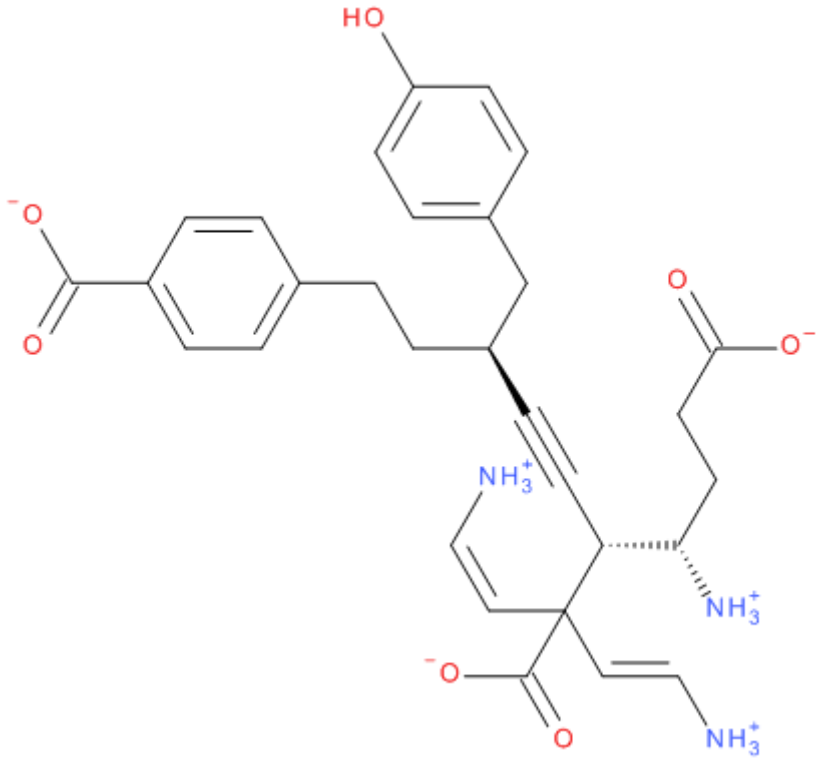
Annex 67 - Representation of Insitu11 (same molecule as Insitu10 but with all bonds being rotatable)



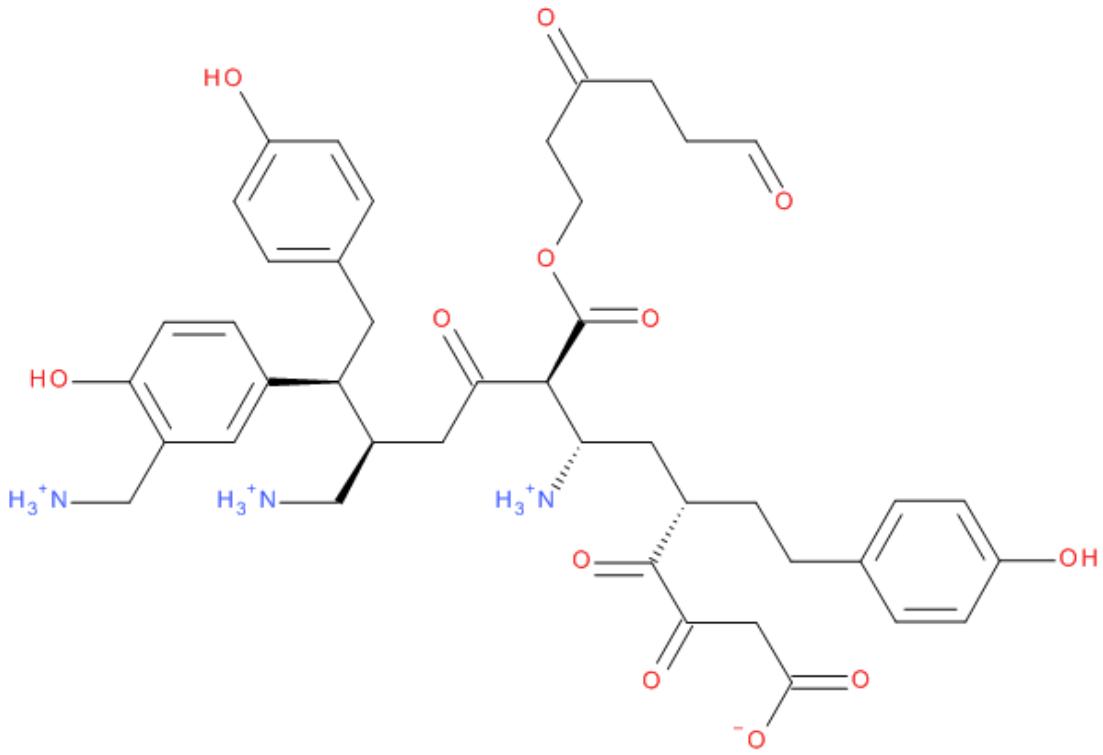
Annex 68 - Representation of Insitu12



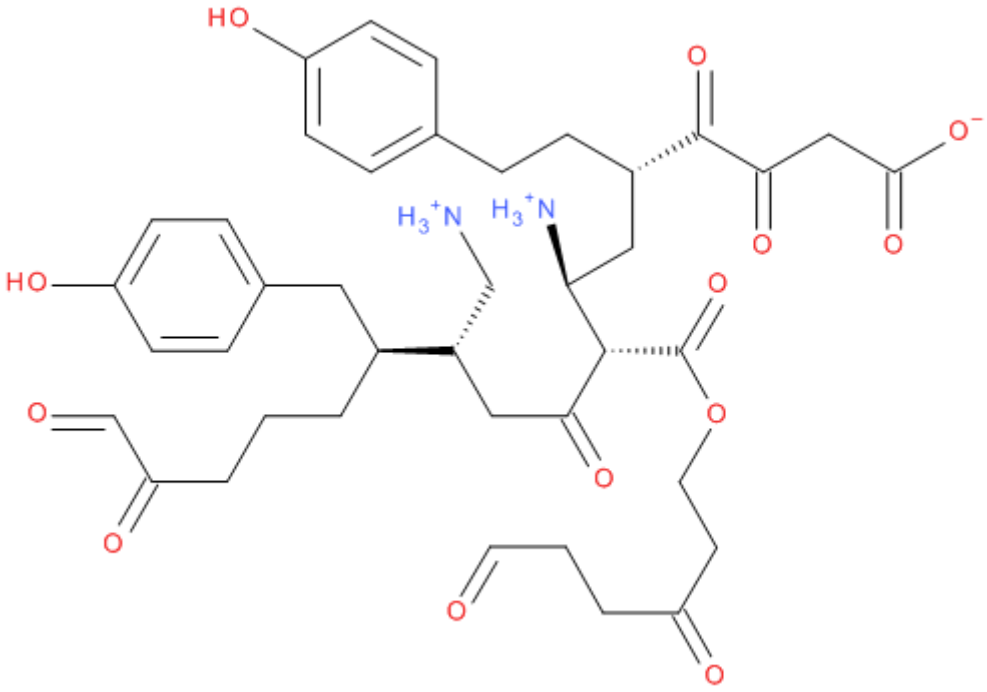
Annex 69 - Representation of Insitu13



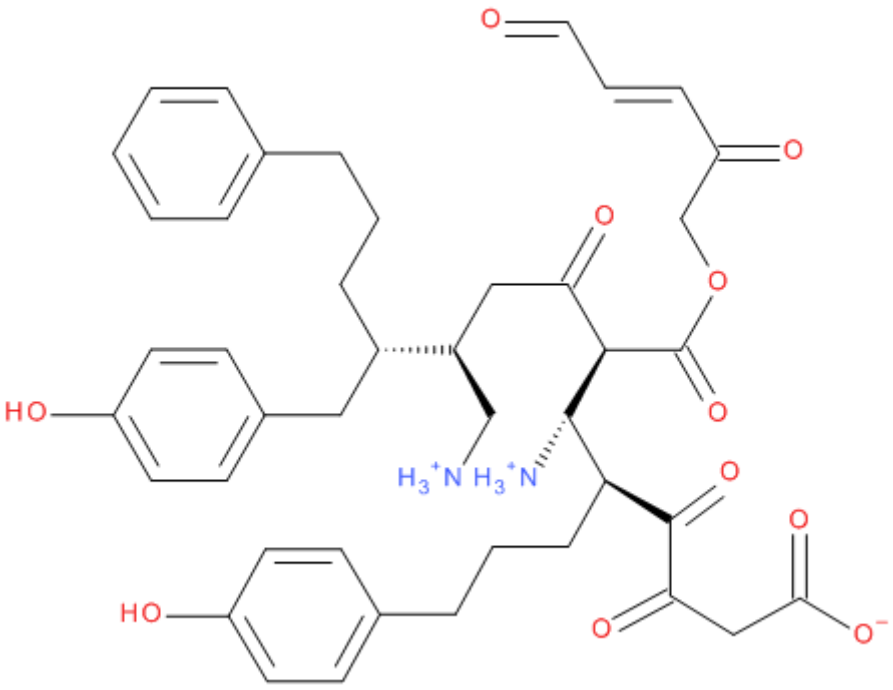
Annex 70 - Representation of Insitu14



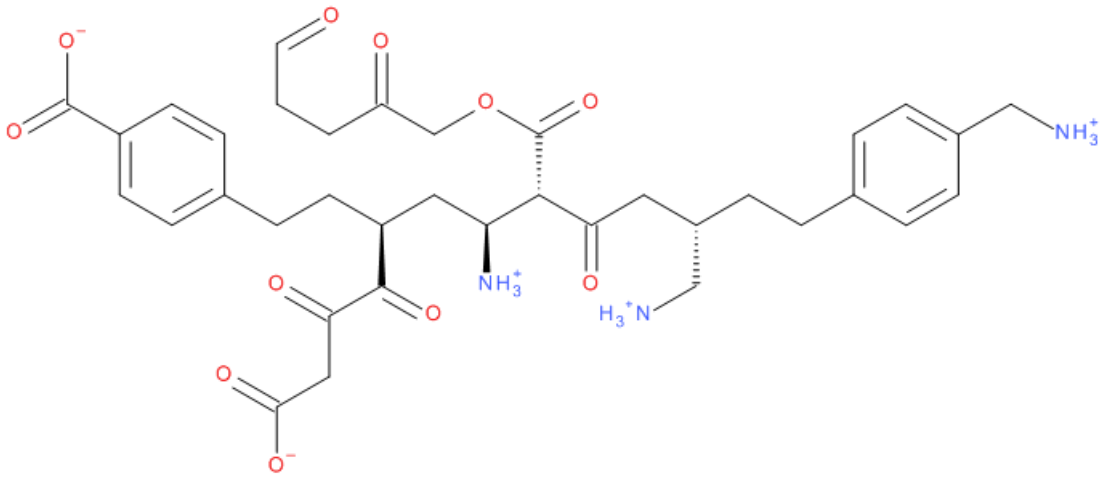
Annex 71 - Representation of Insitu15



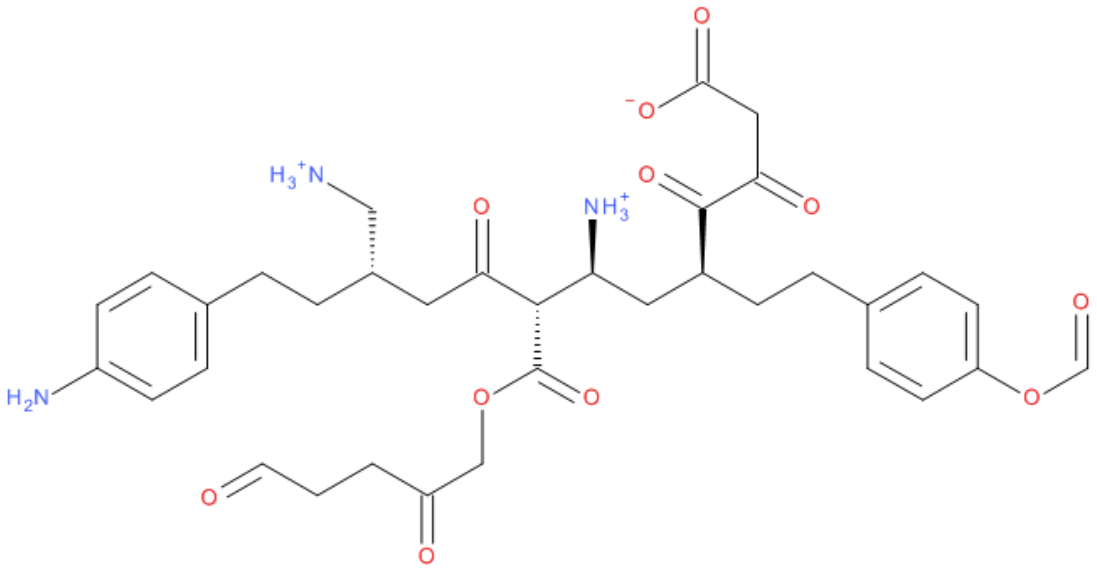
Annex 72 - Representation of Insitu16



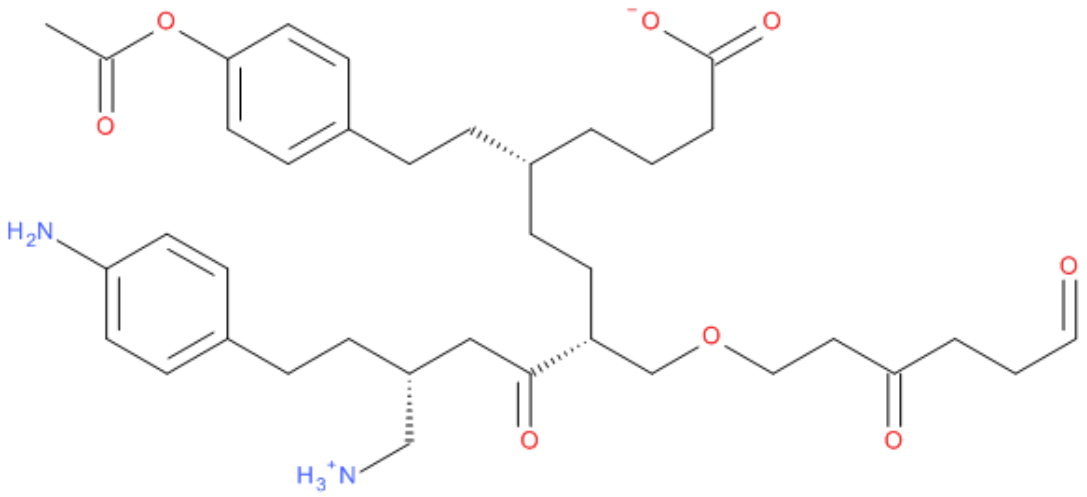
Annex 73 - Representation of Insitu17



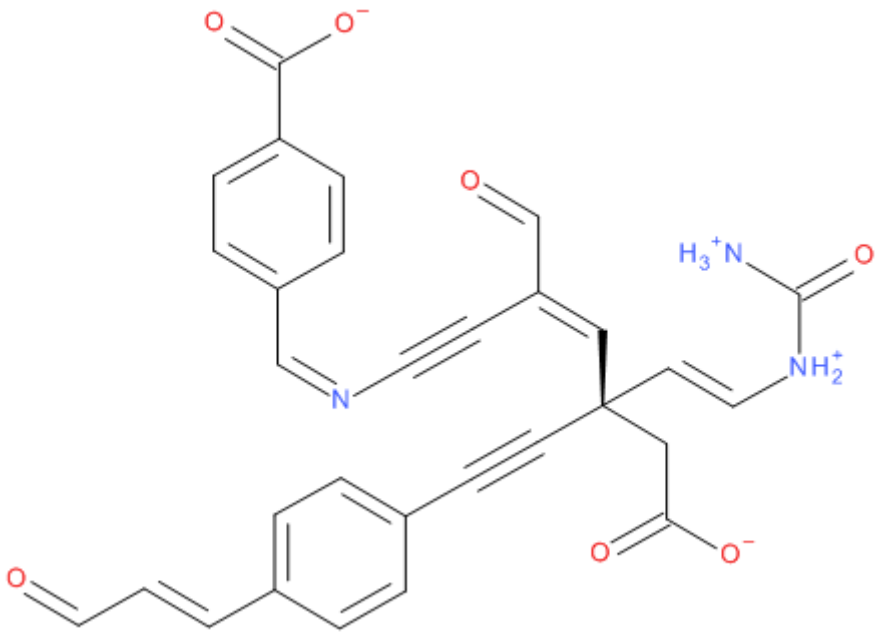
Annex 74 - Representation of Insitu18



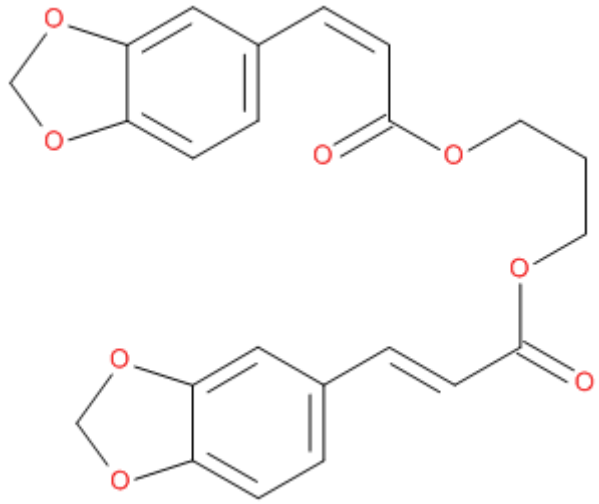
Annex 75 - Representation of Insitu19



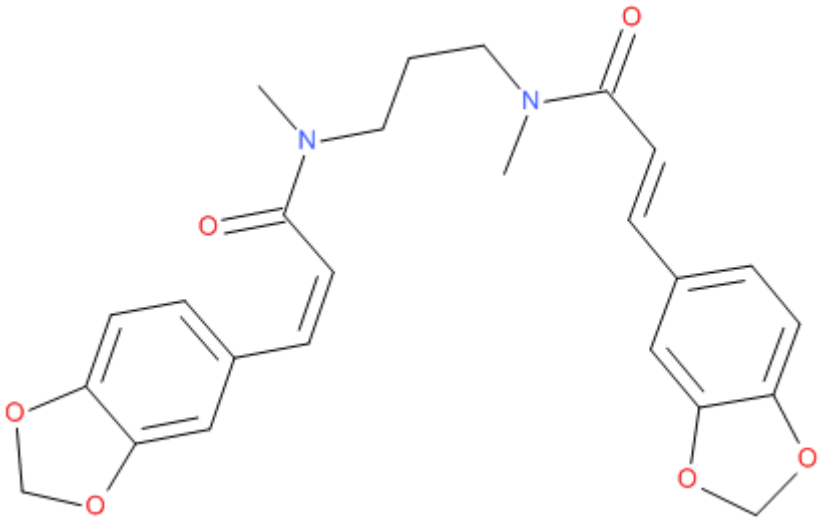
Annex 76 - Representation of Insitu20



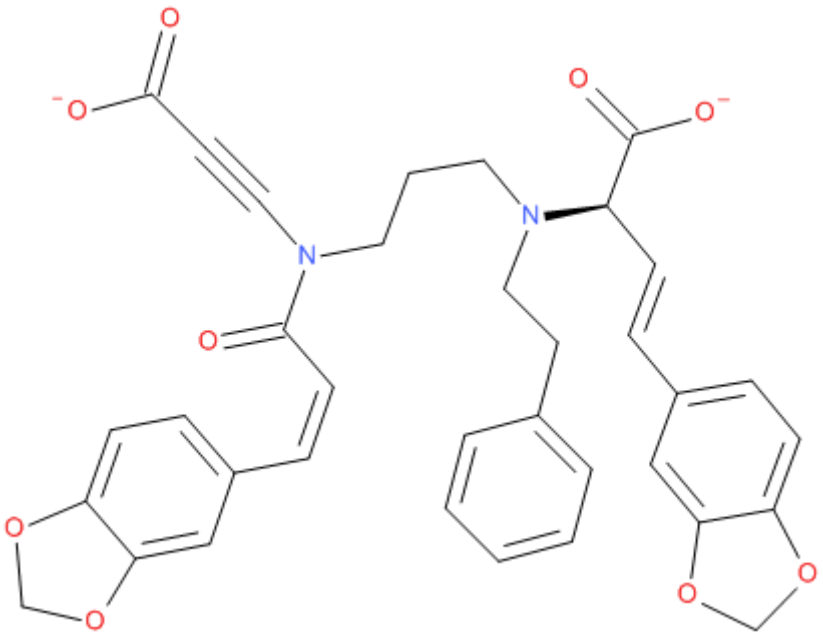
Annex 77 - Representation of Insitu21



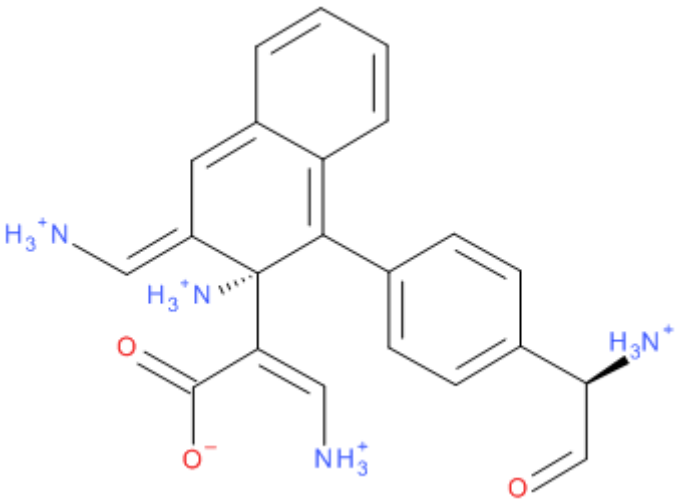
Annex 78 - Representation of Insitu22



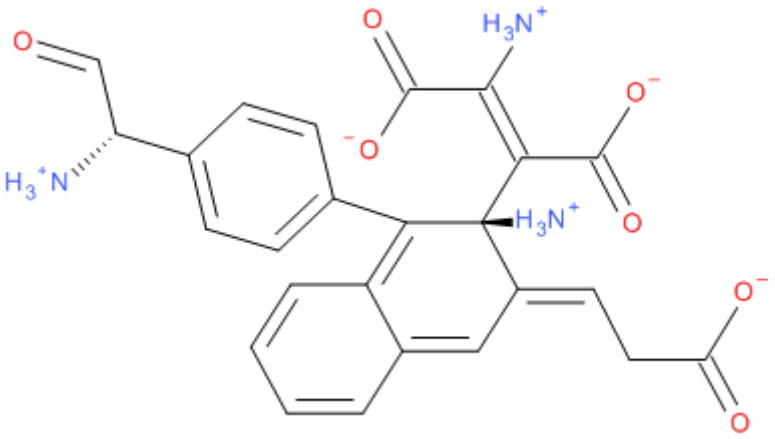
Annex 79 - Representation of Insitu23



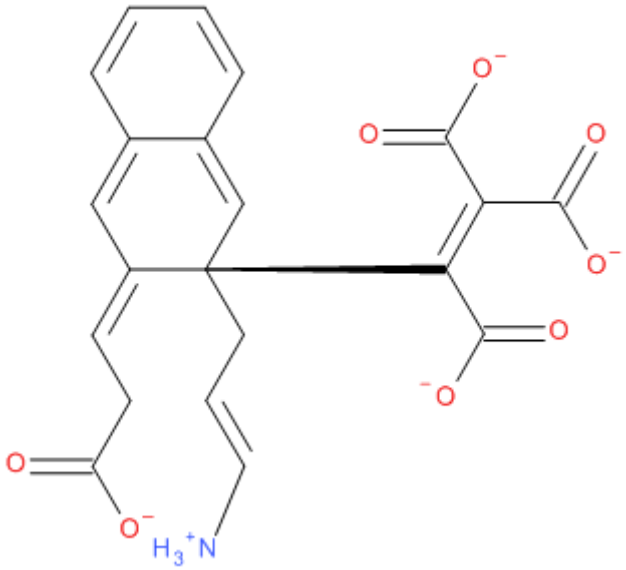
Annex 80 - Representation of Insitu24



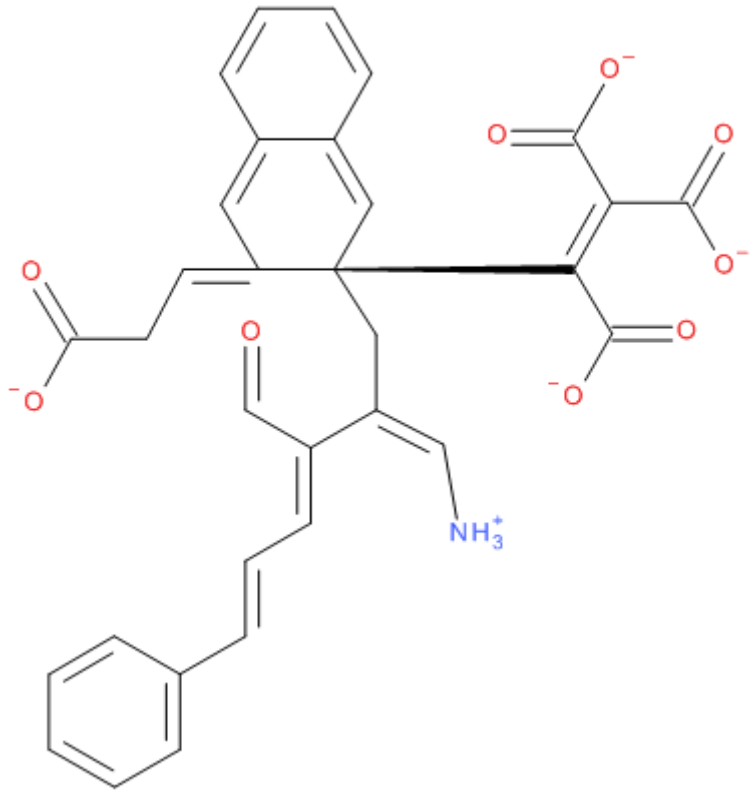
Annex 81 - Representation of Insitu25



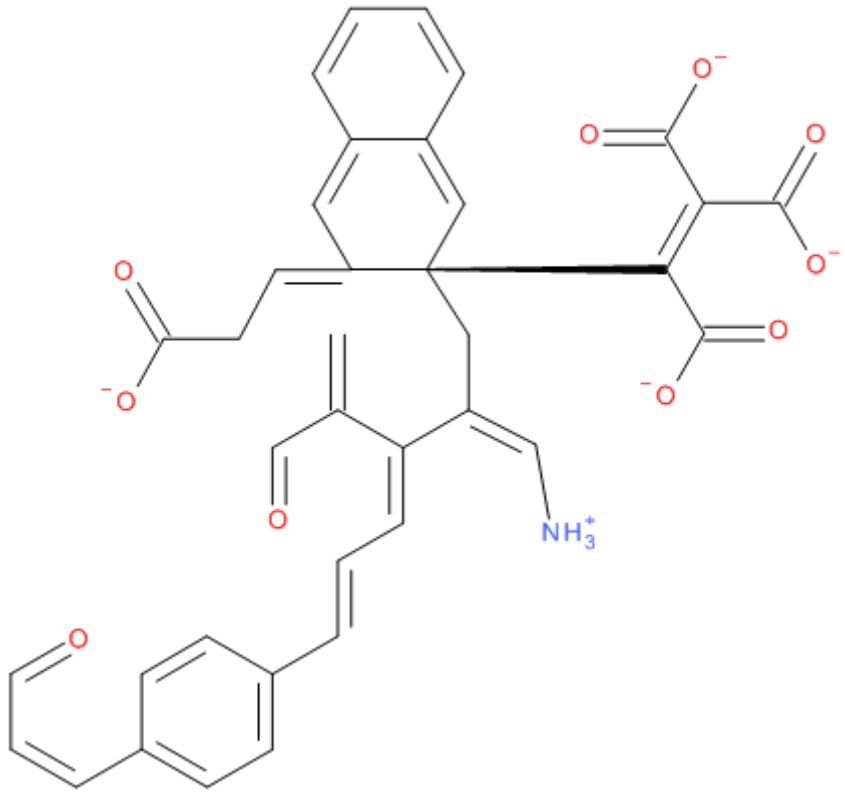
Annex 82 - Representation of Insitu26



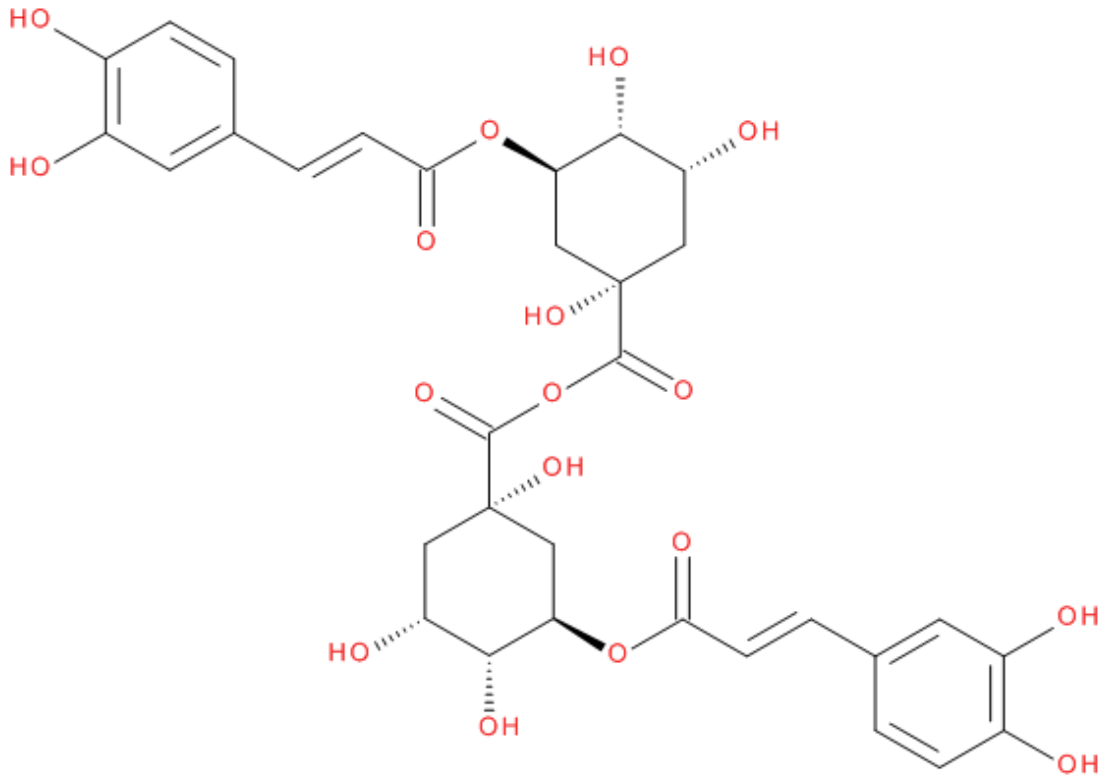
Annex 83 - Representation of Insitu27



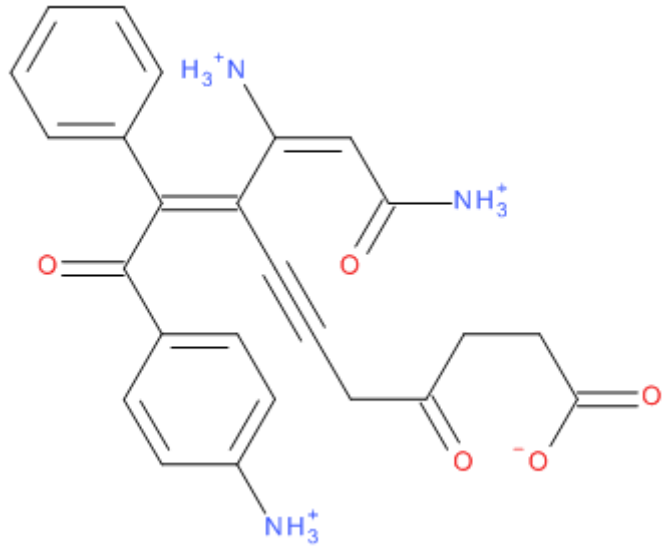
Annex 84 - Representation of Insitu28



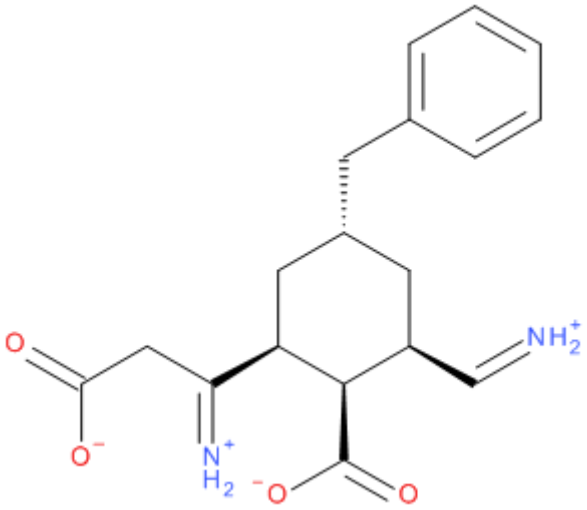
Annex 85 - Representation of Insitu29



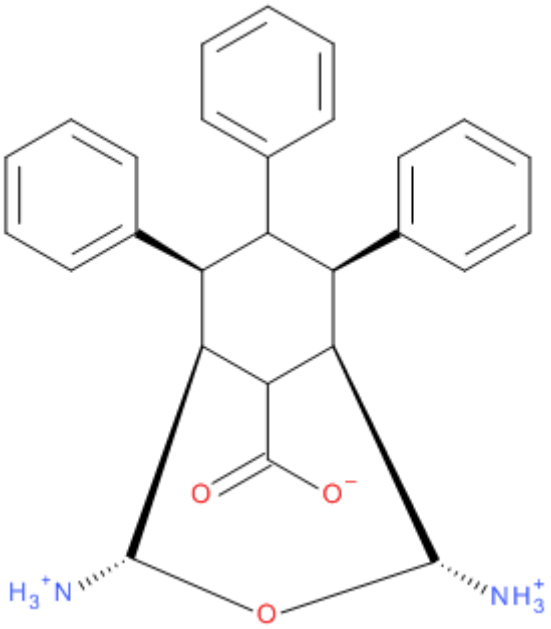
Annex 86 - Representation of Insitu30



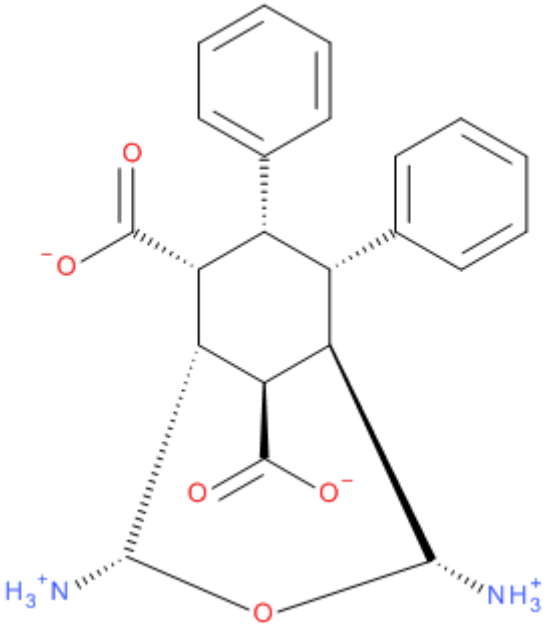
Annex 87 - Representation of Insitu31



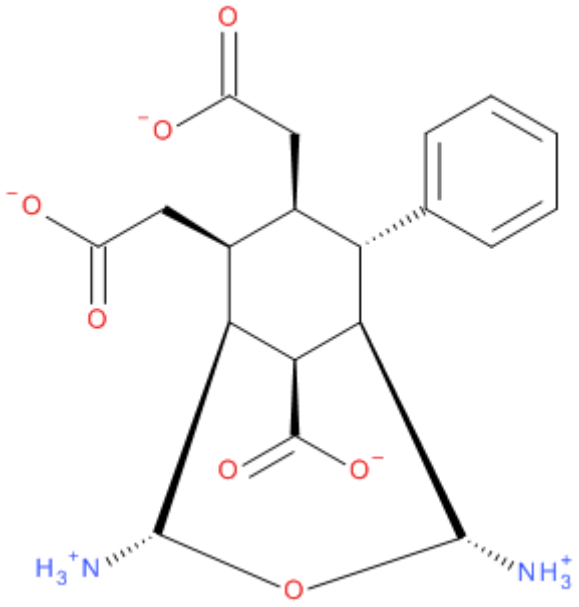
Annex 88 - Representation of Insitu32



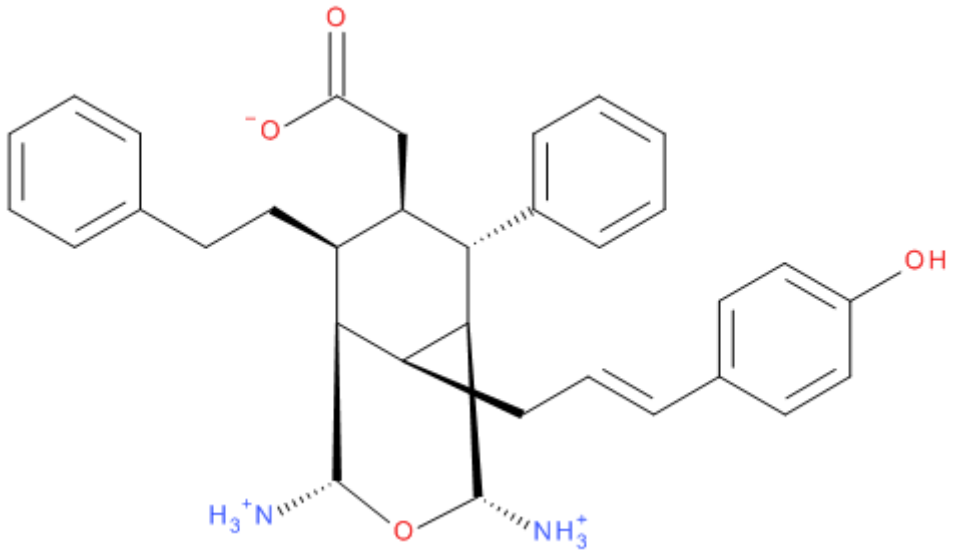
Annex 89 - Representation of Insitu33



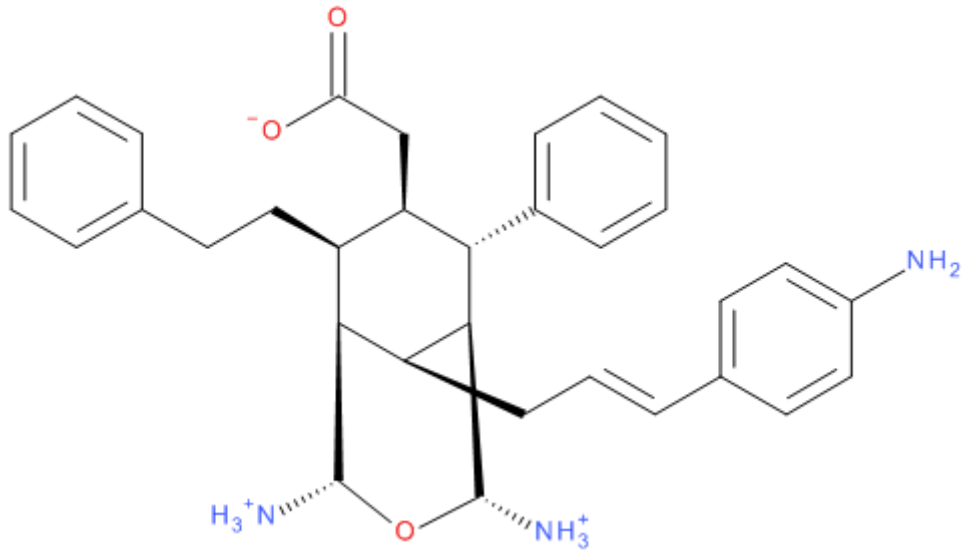
Annex 90 - Representation of Insitu34



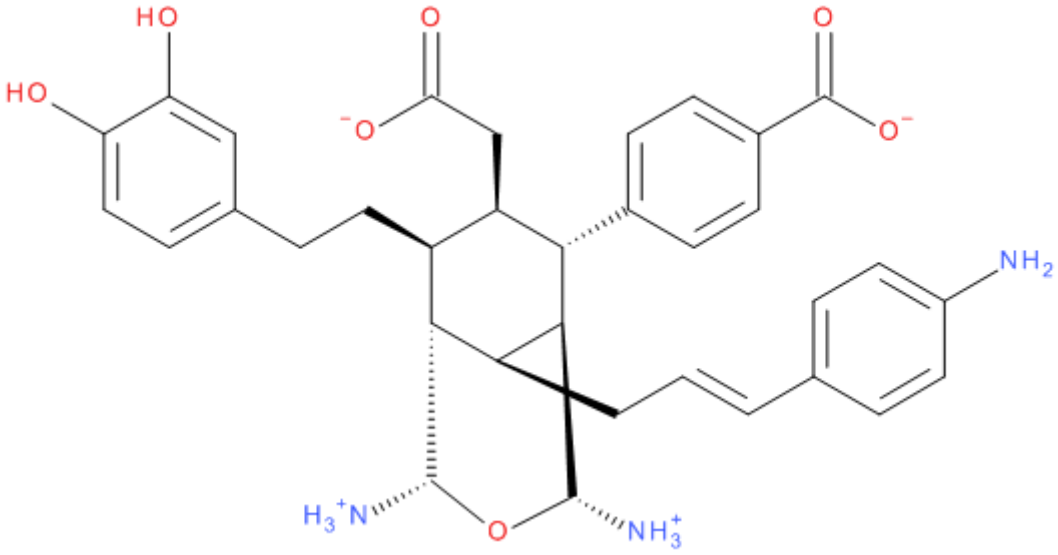
Annex 91 - Representation of Insitu35



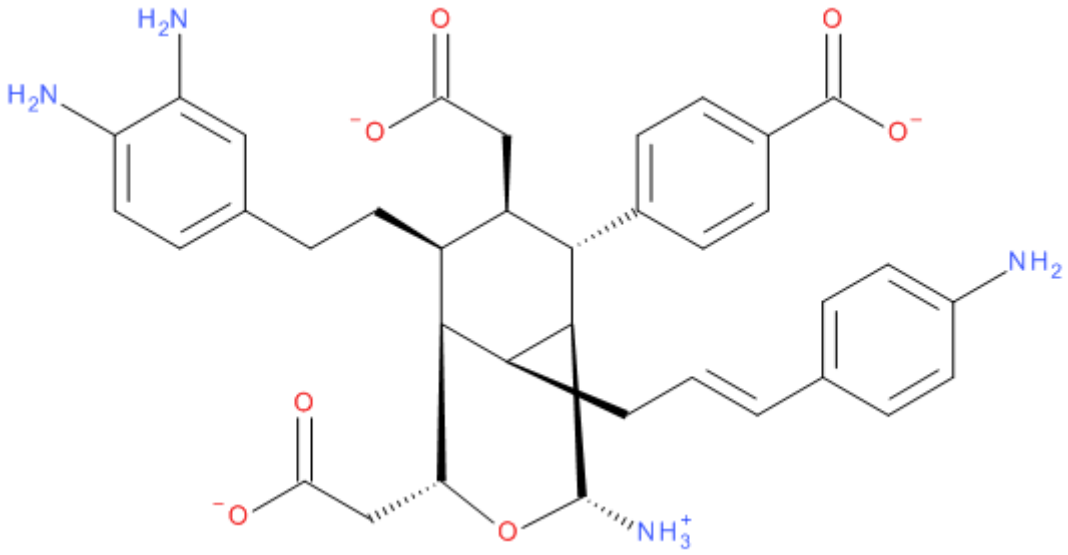
Annex 92 - Representation of Insitu36



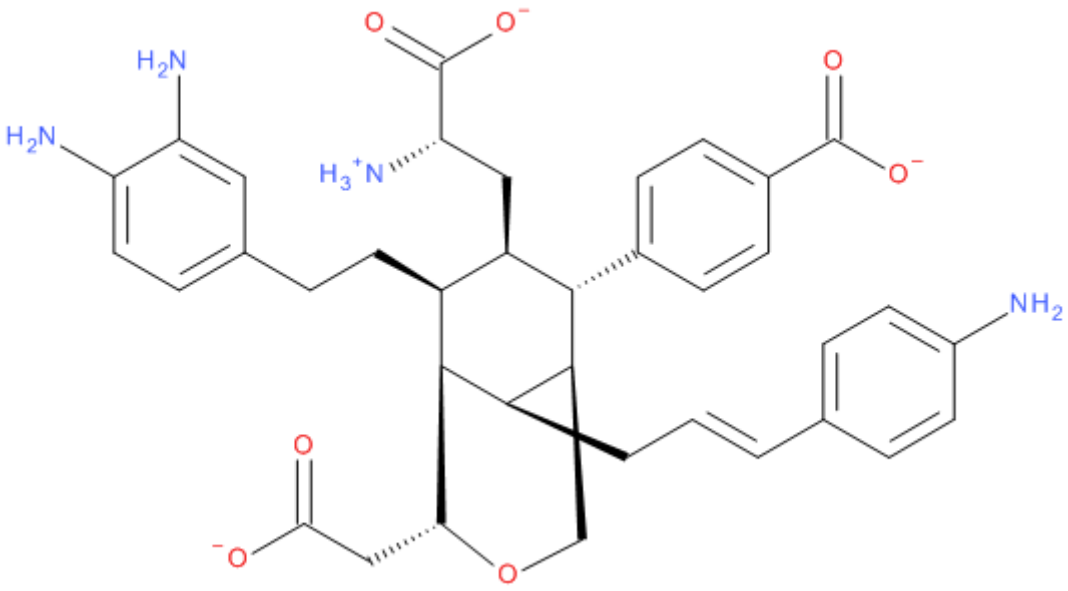
Annex 93 - Representation of Insitu37



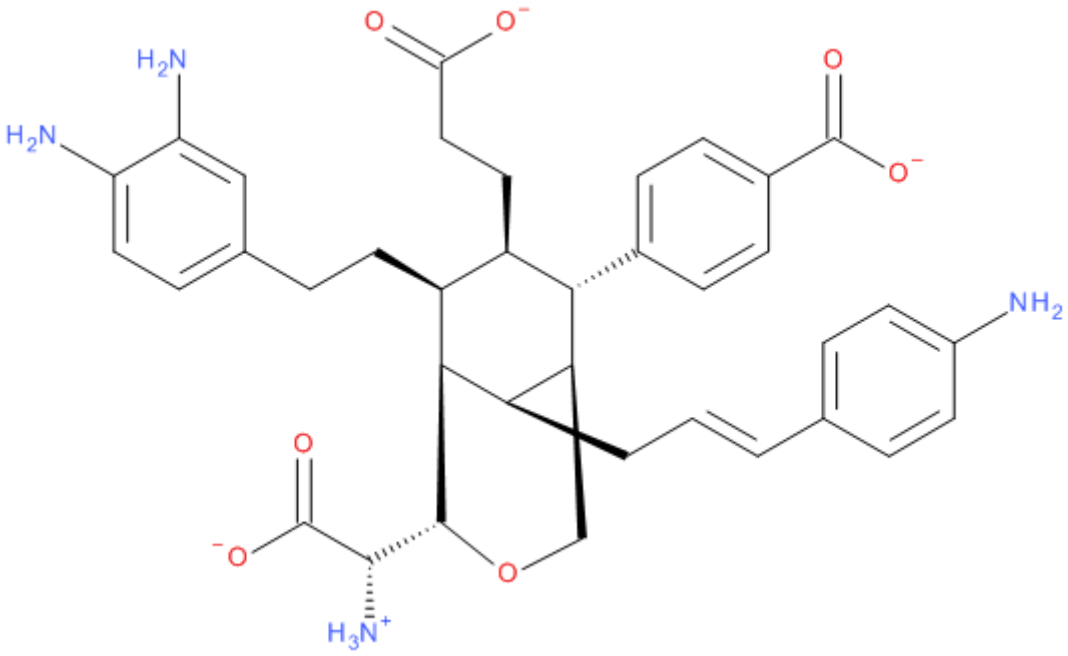
Annex 94 - Representation of Insitu38



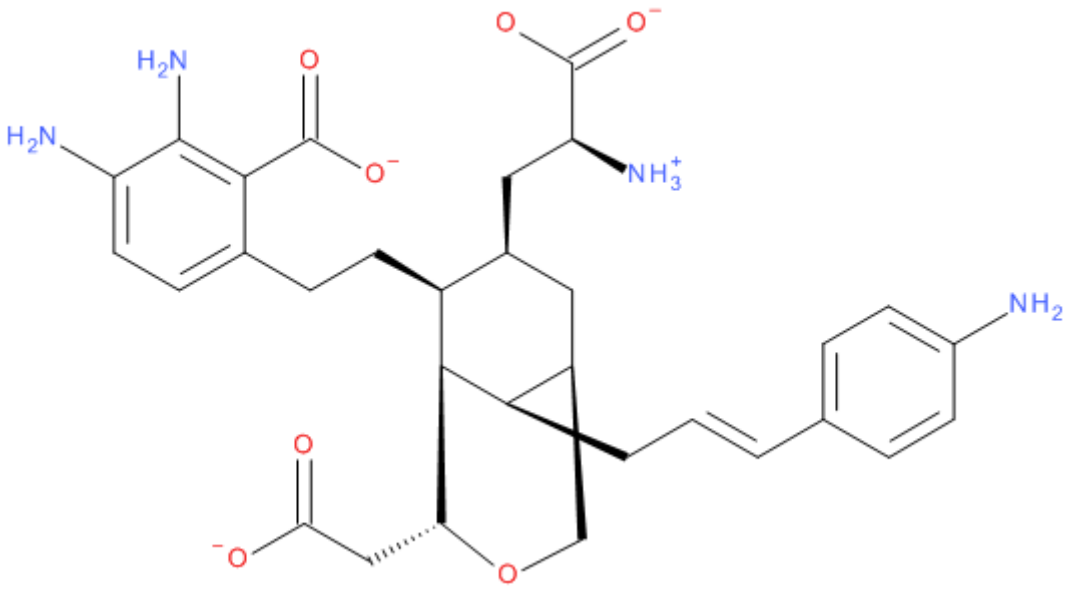
Annex 95 - Representation of Insitu39



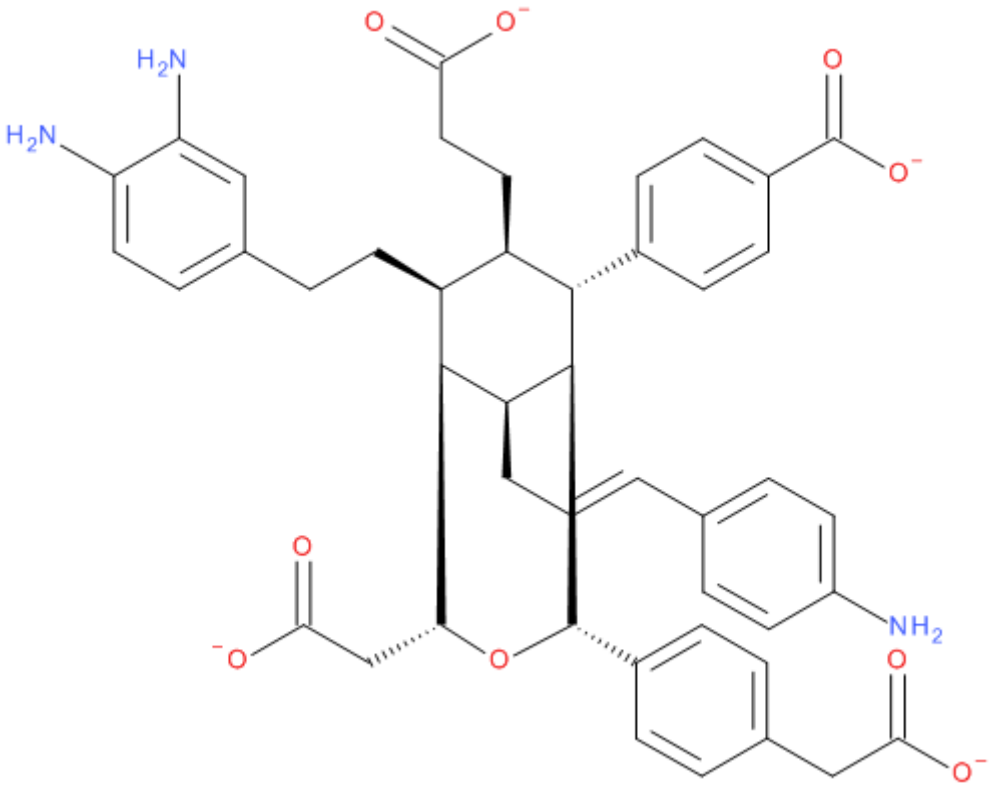
Annex 96 - Representation of Insitu40



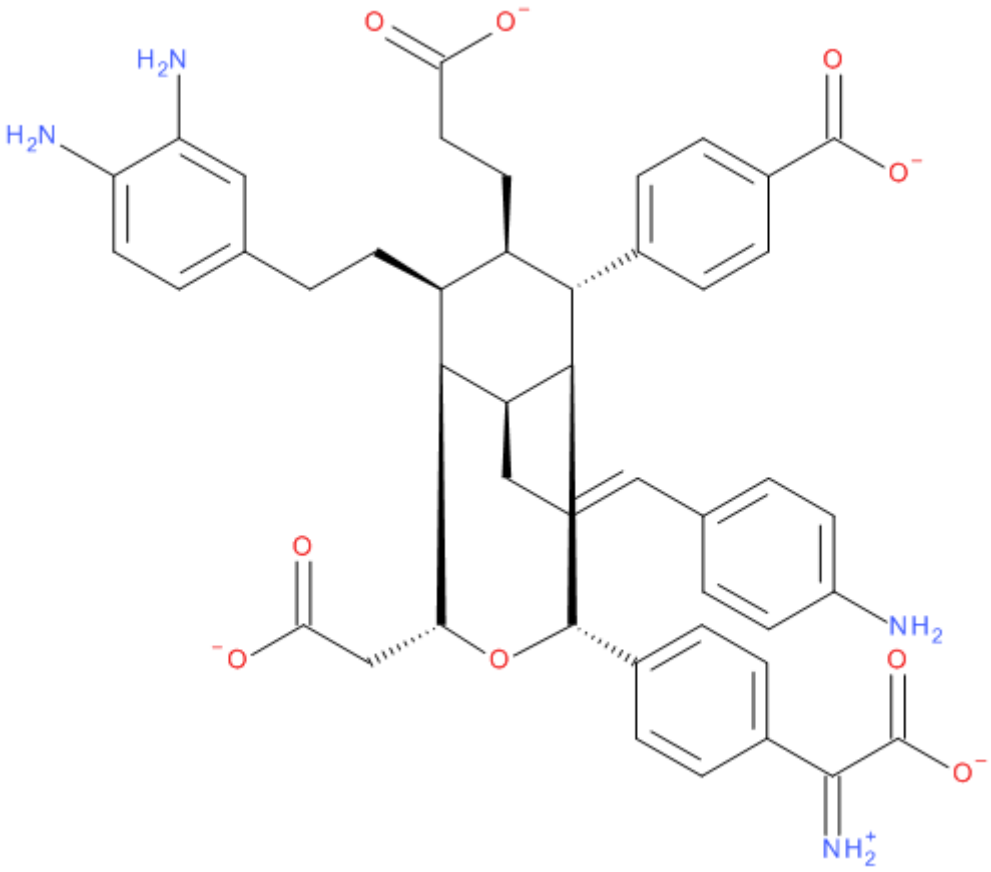
Annex 97 - Representation of Insitu41



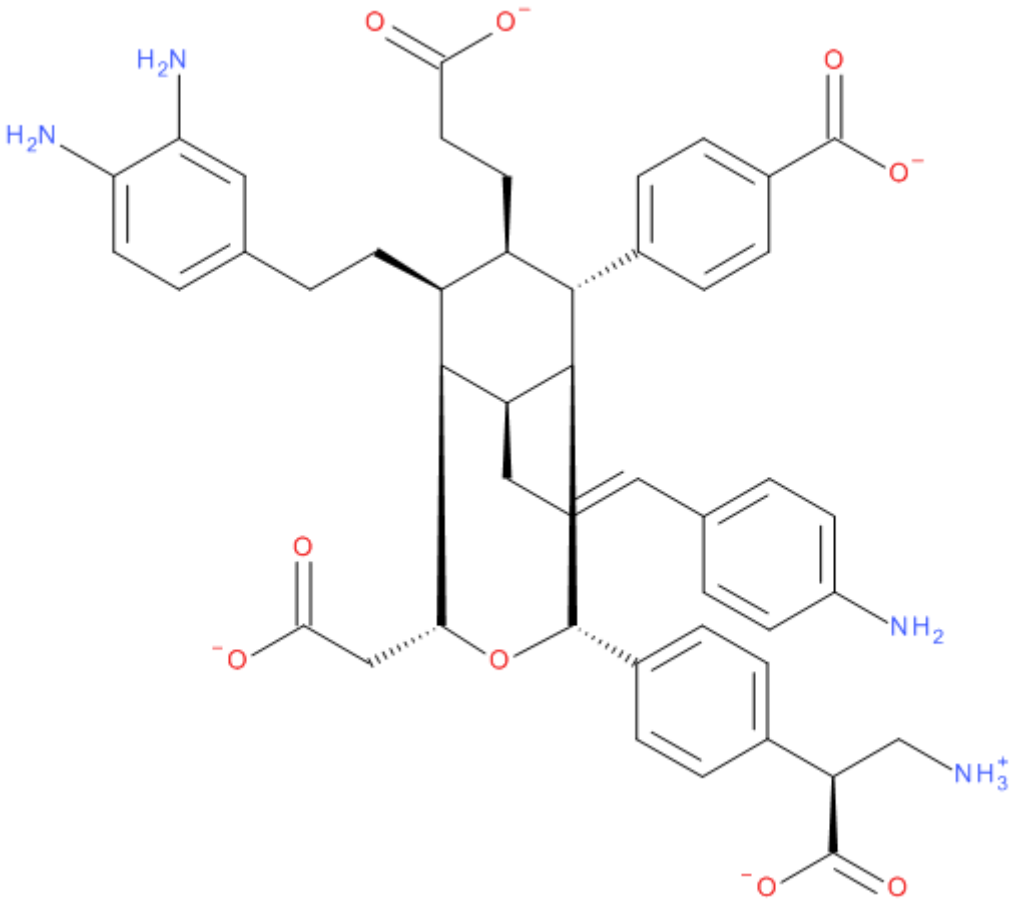
Annex 98 - Representation of Insitu42



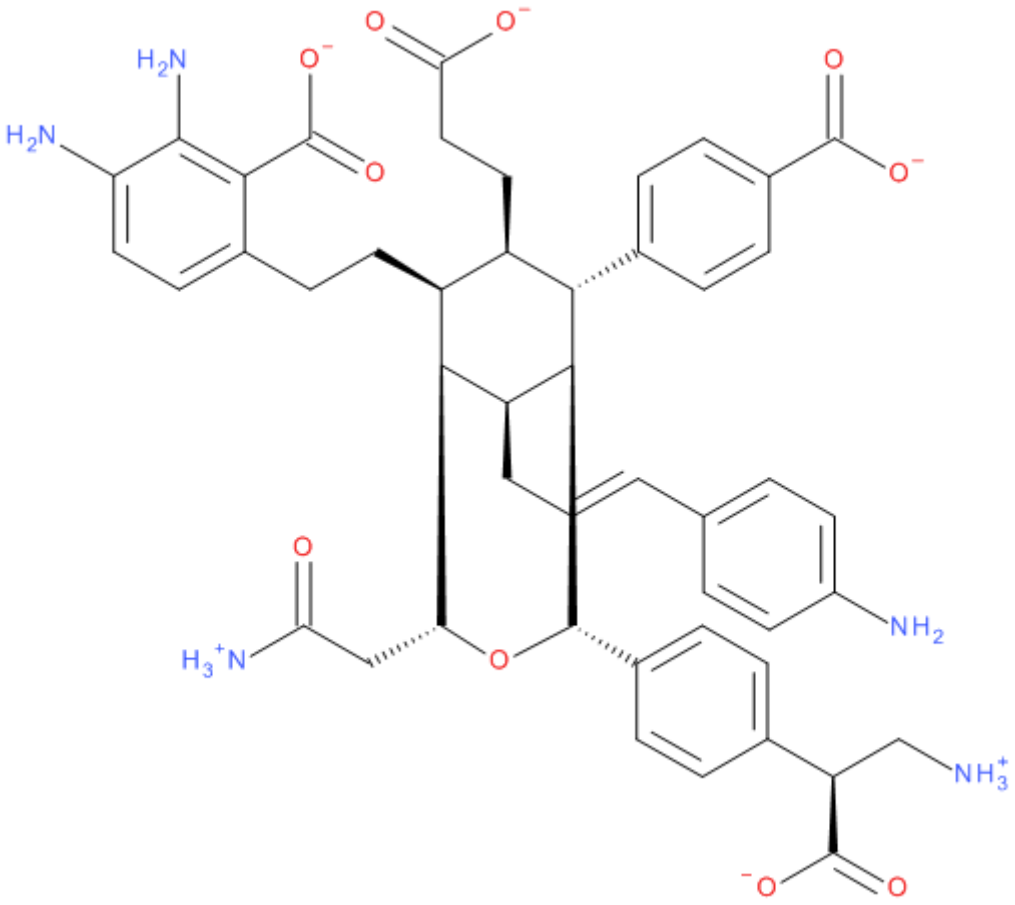
Annex 99 - Representation of Insitu43



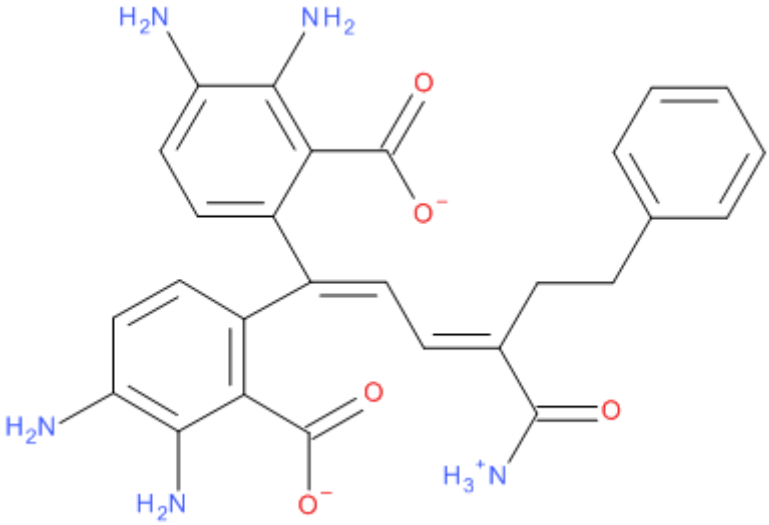
Annex 100 - Representation of Insitu44



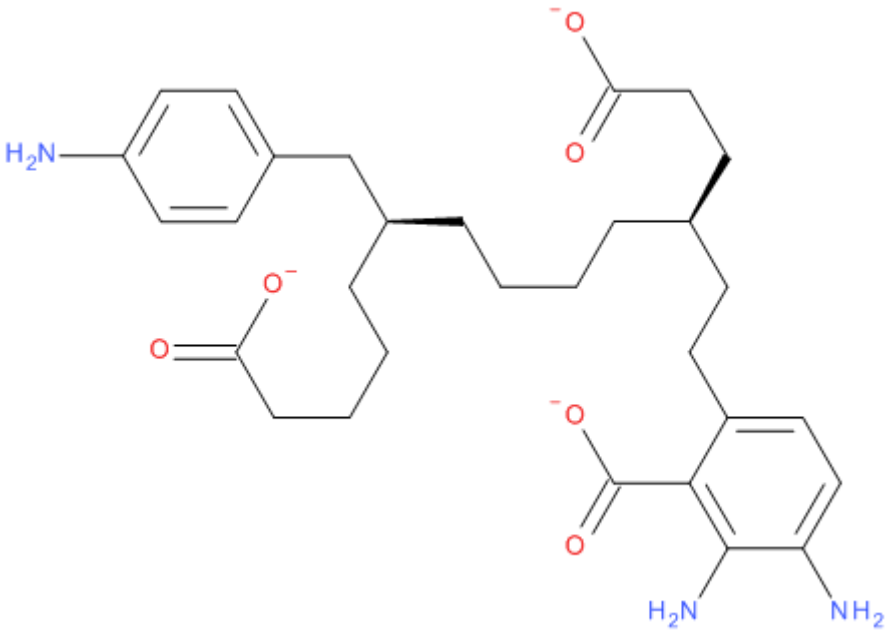
Annex 101 - Representation of Insitu45



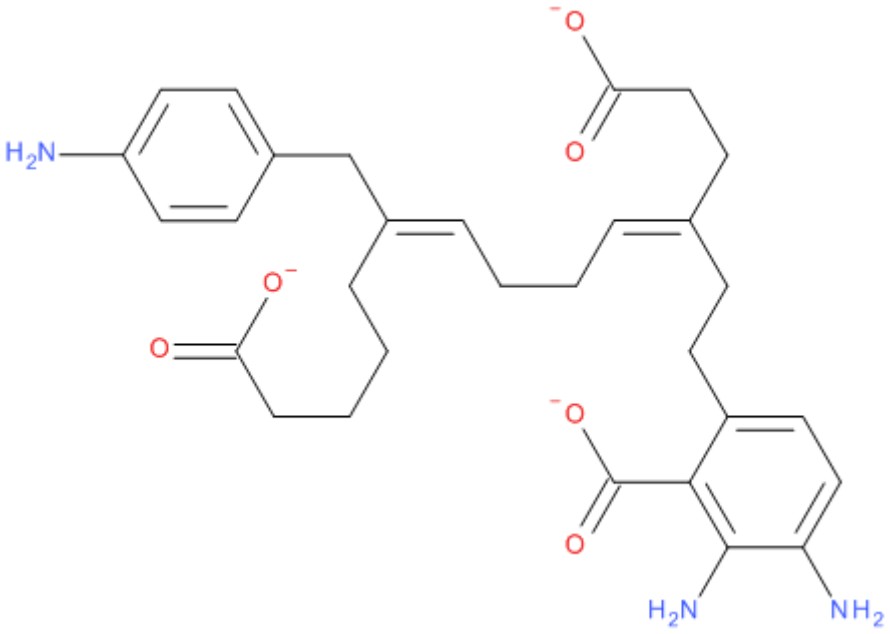
Annex 102 - Representation of Insitu46



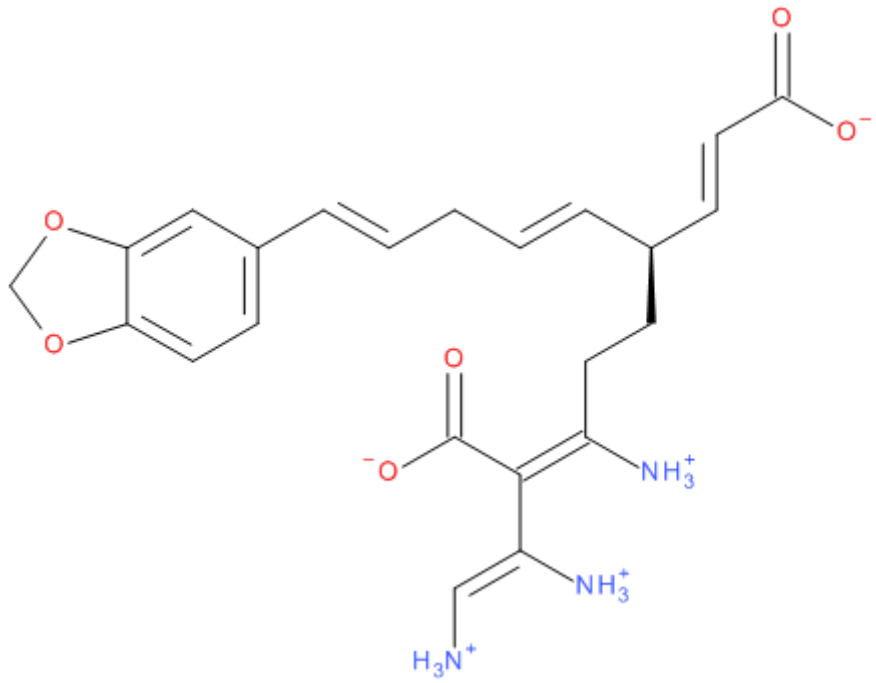
Annex 103 - Representation of Insitu47



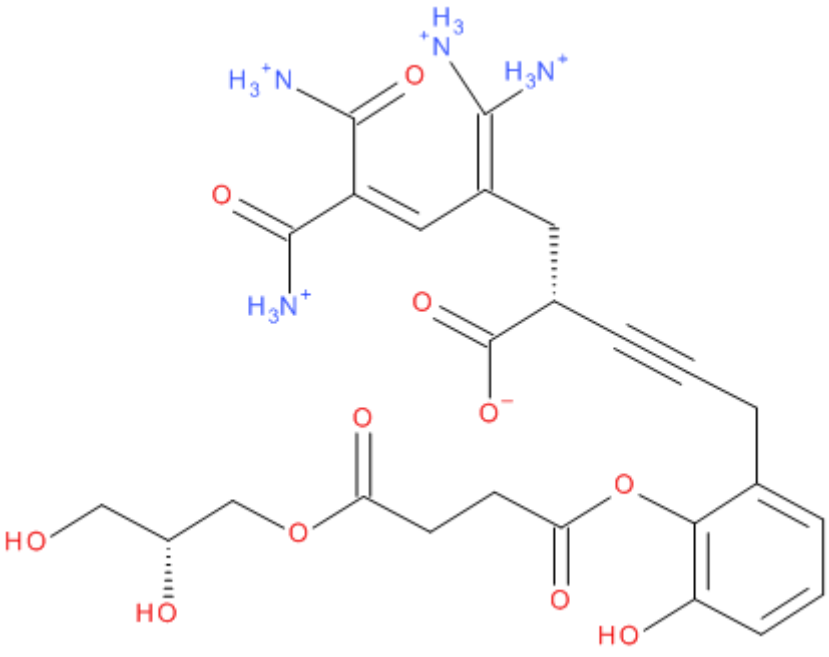
Annex 104 - Representation of Insitu48



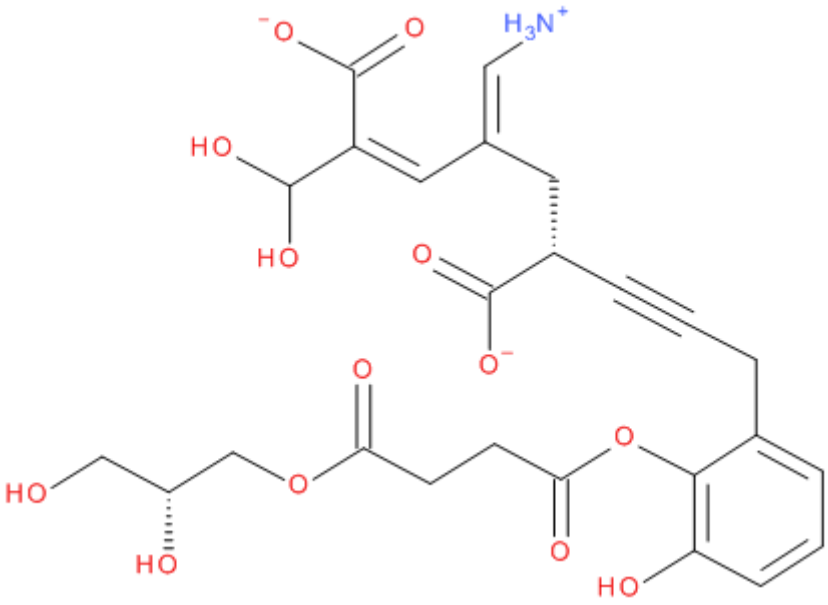
Annex 105 - Representation of Insitu49



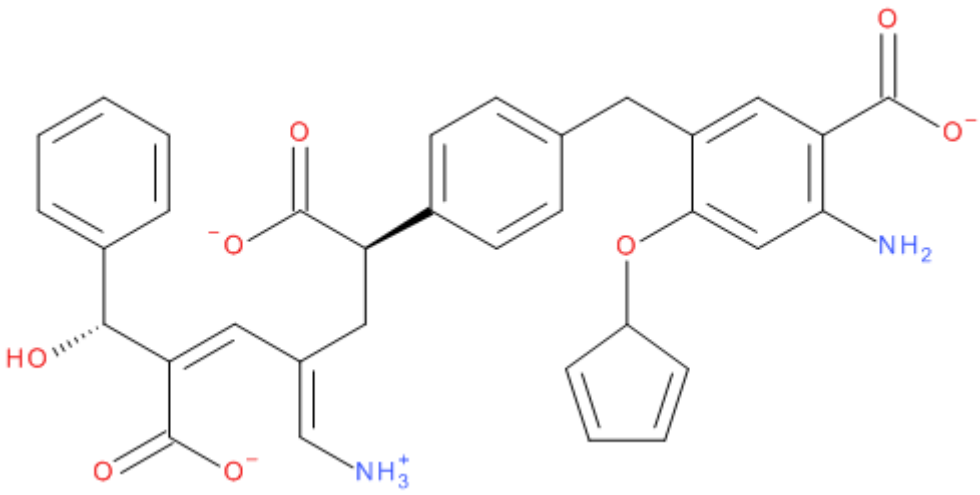
Annex 106 - Representation of Insitu50



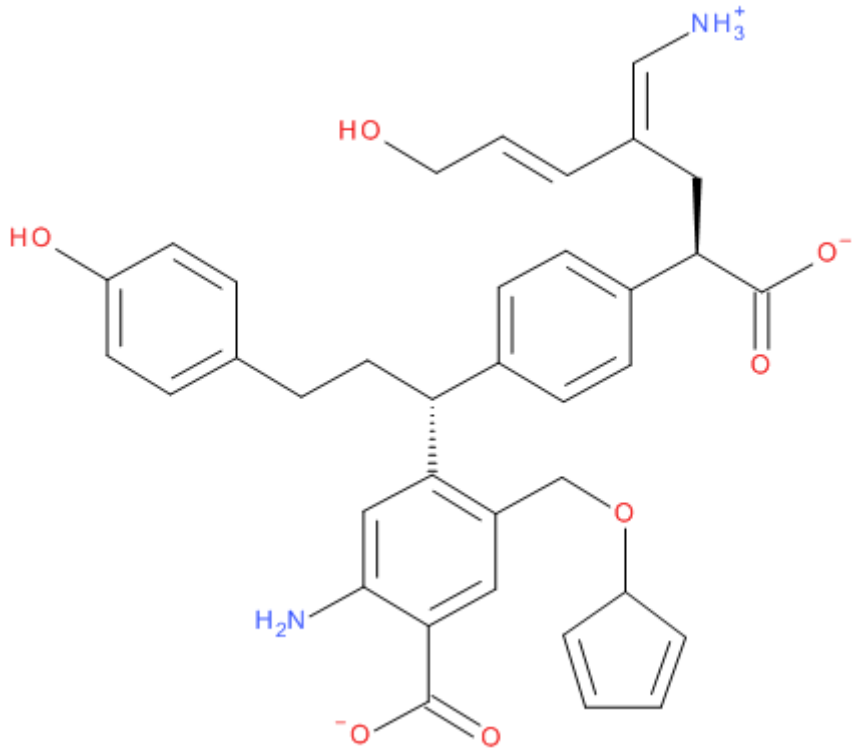
Annex 107 - Representation of Insitu51



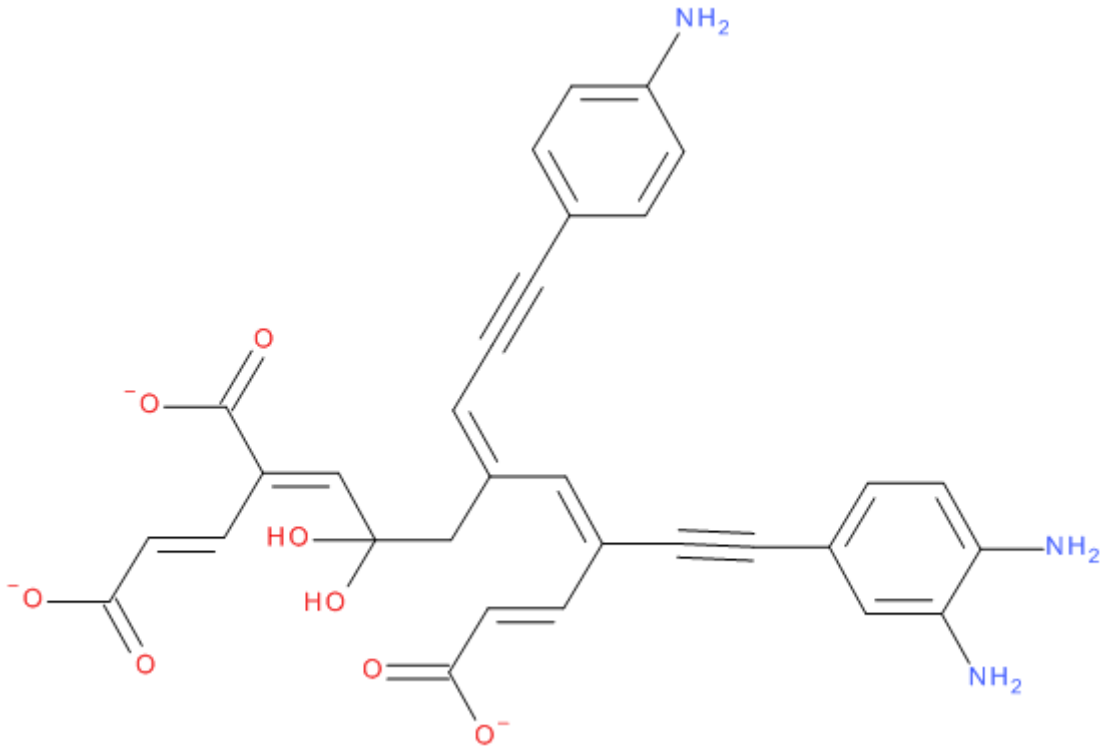
Annex 108 - Representation of Insitu52



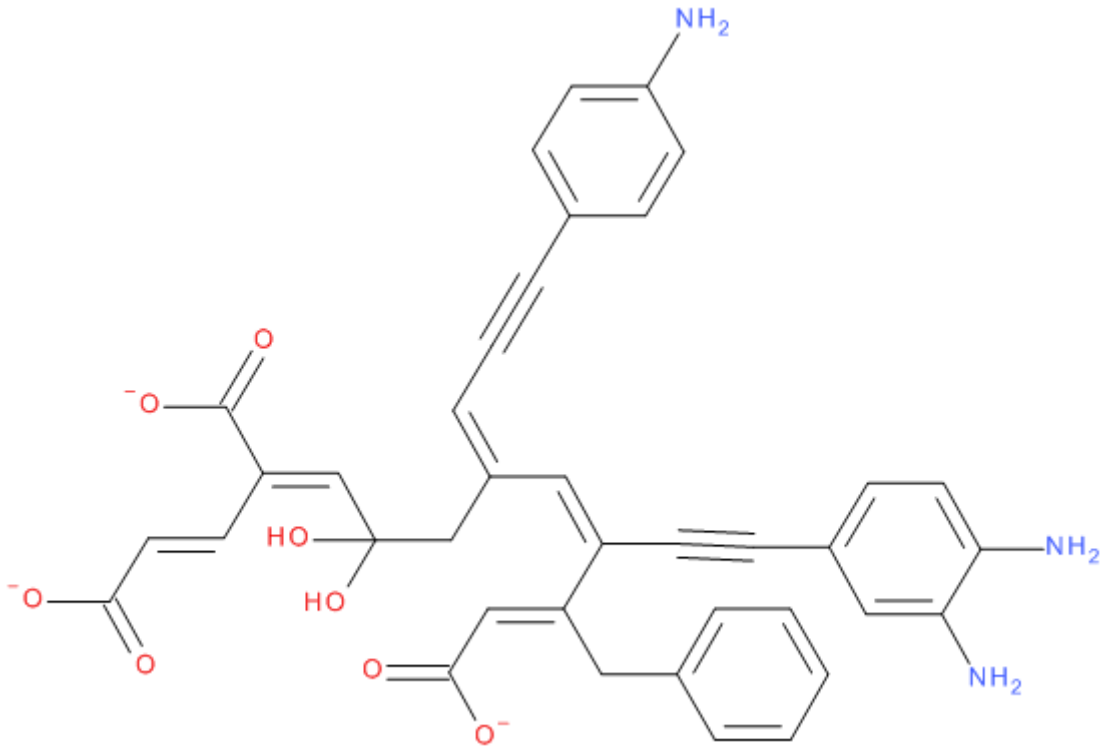
Annex 109 - Representation of Insitu53



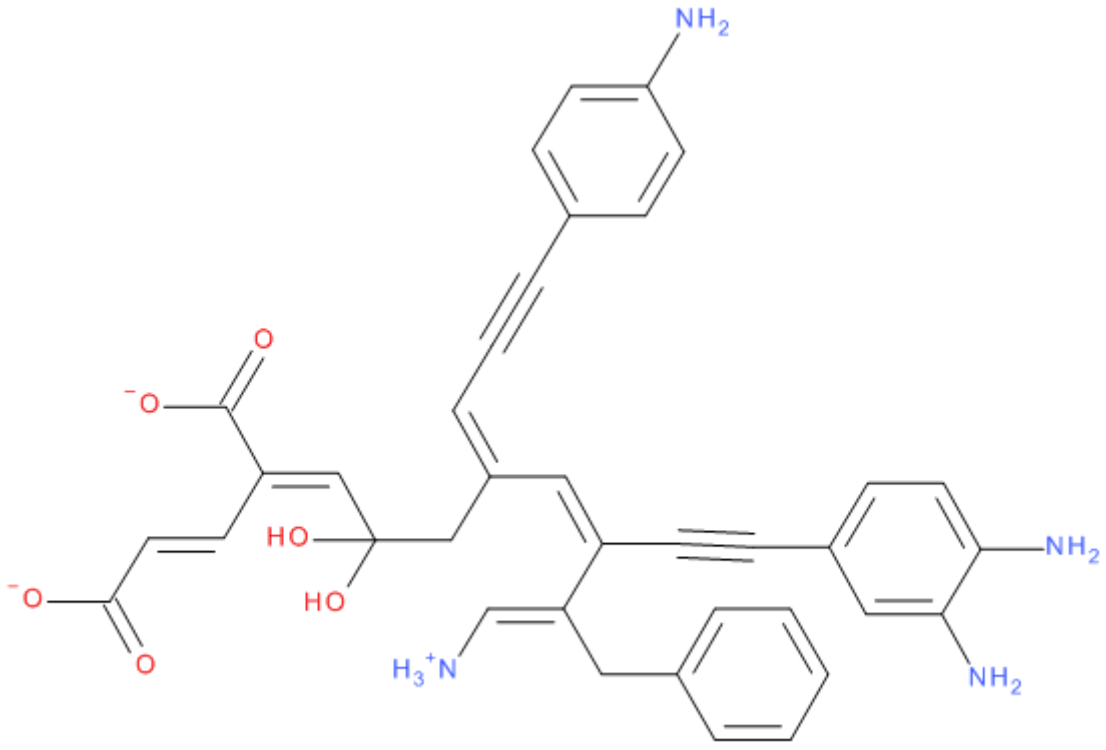
Annex 110 - Representation of Insitu54



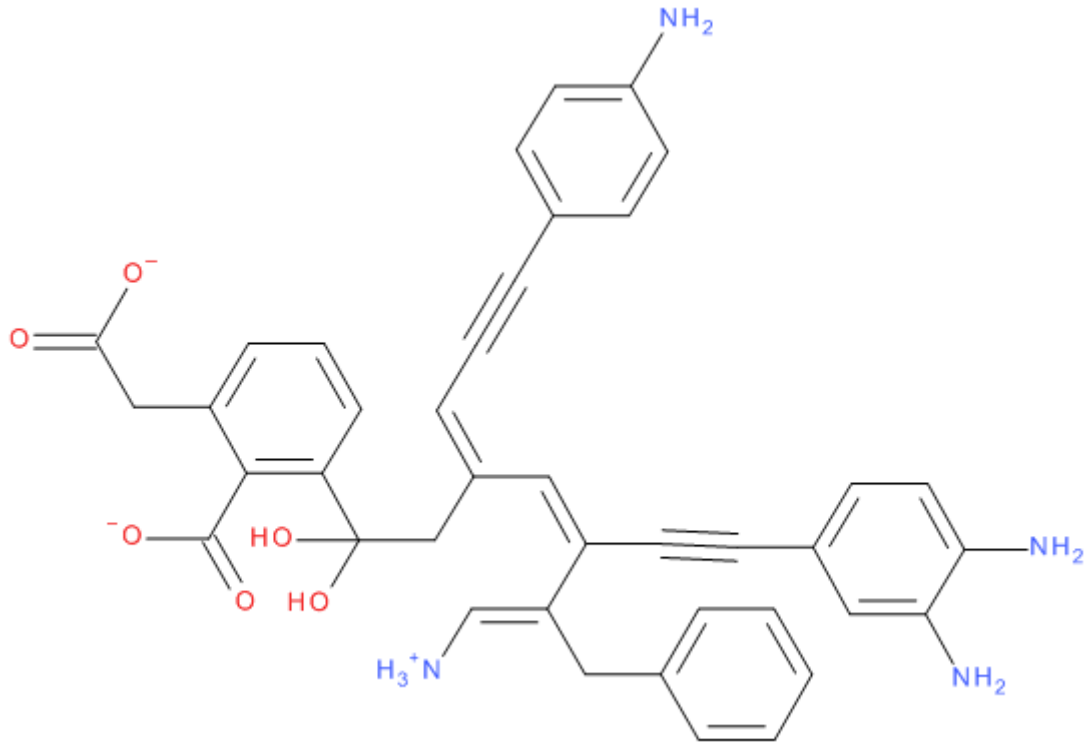
Annex 111 - Representation of Insitu55



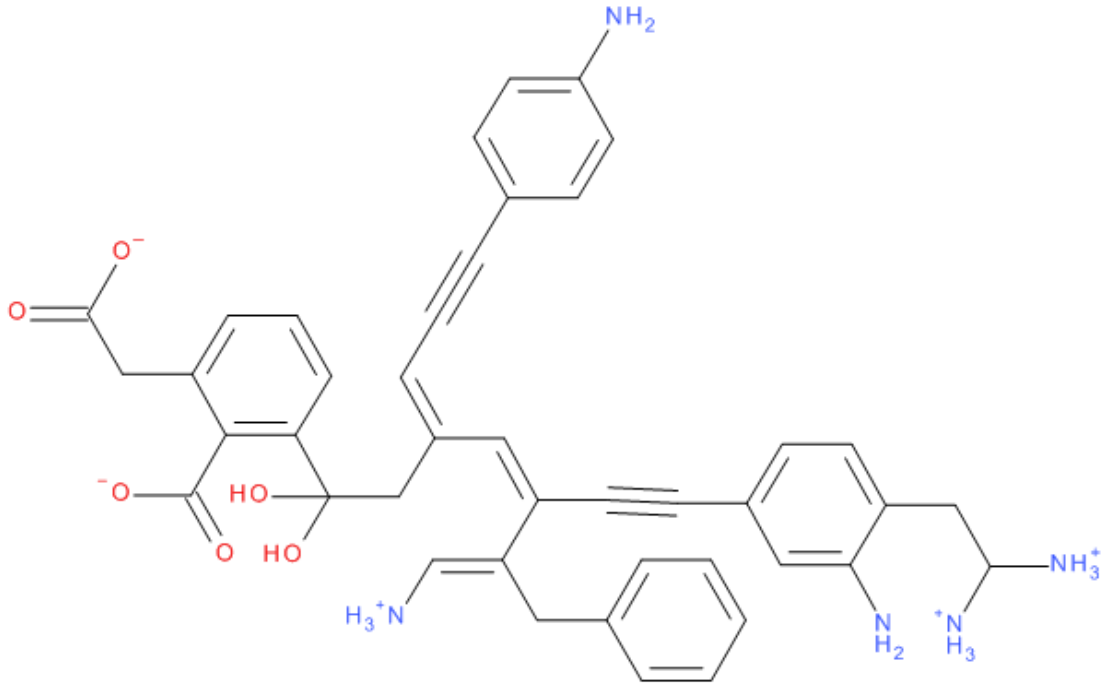
Annex 112 - Representation of Insitu56



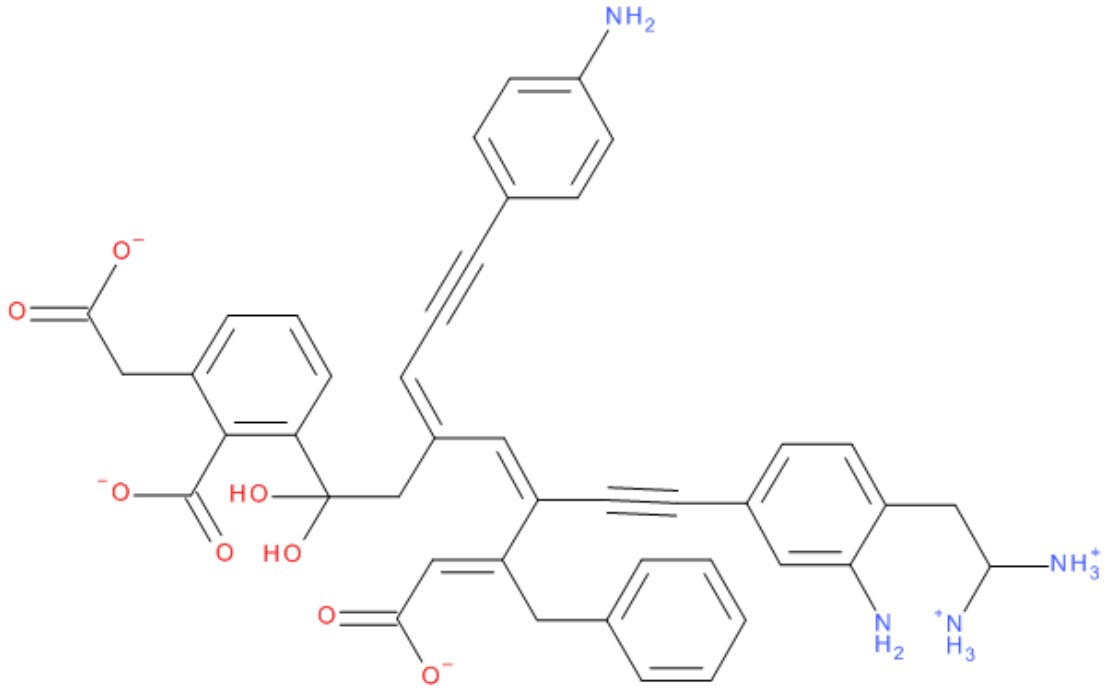
Annex 113 - Representation of Insitu57



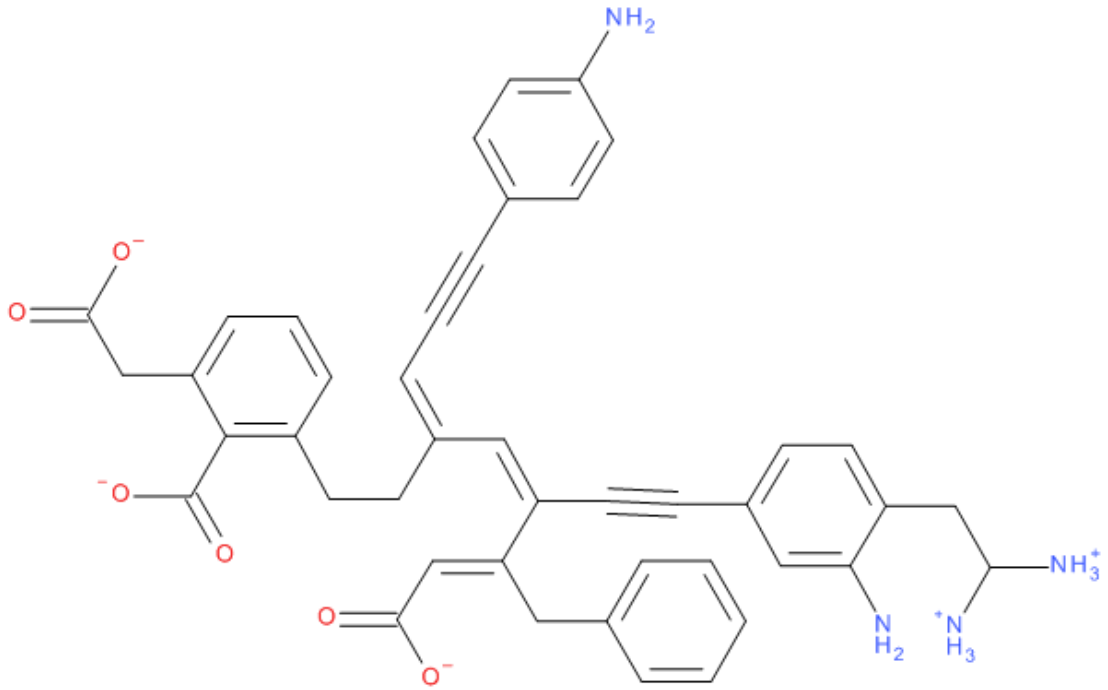
Annex 114 - Representation of Insitu58



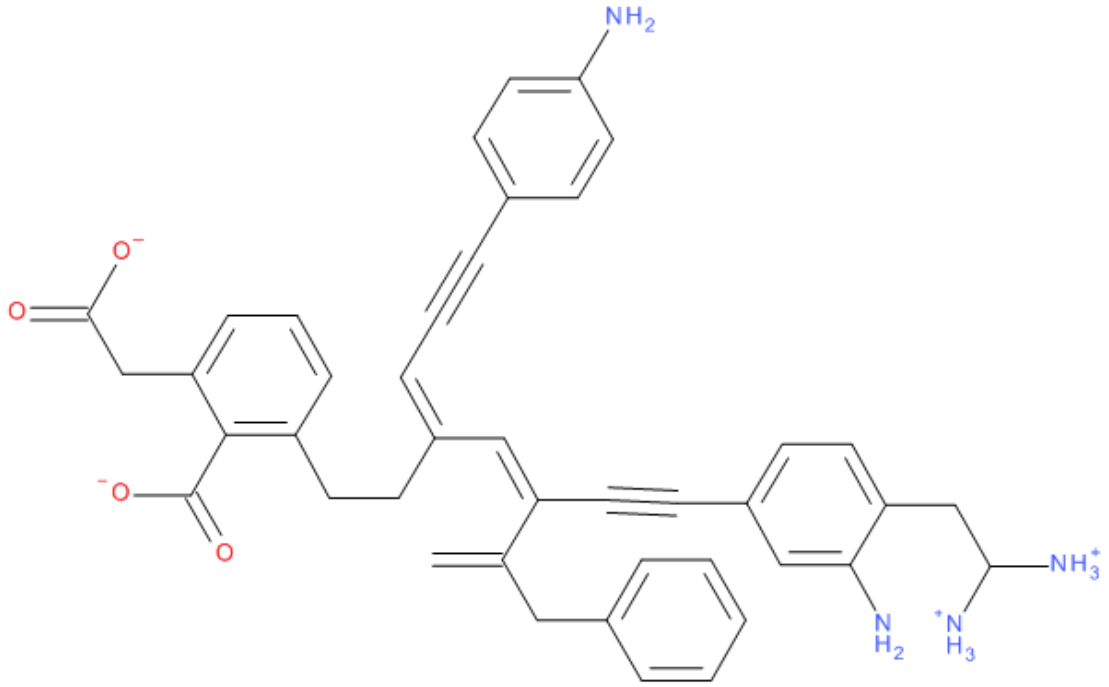
Annex 115 - Representation of Insitu59



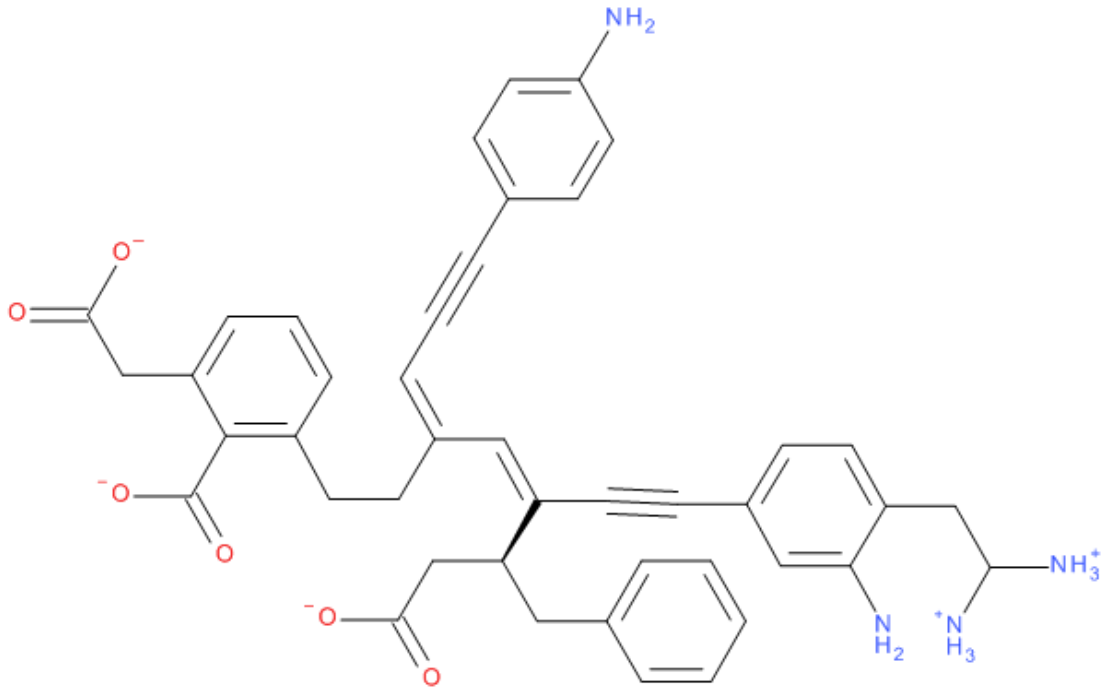
Annex 116 - Representation of Insitu60



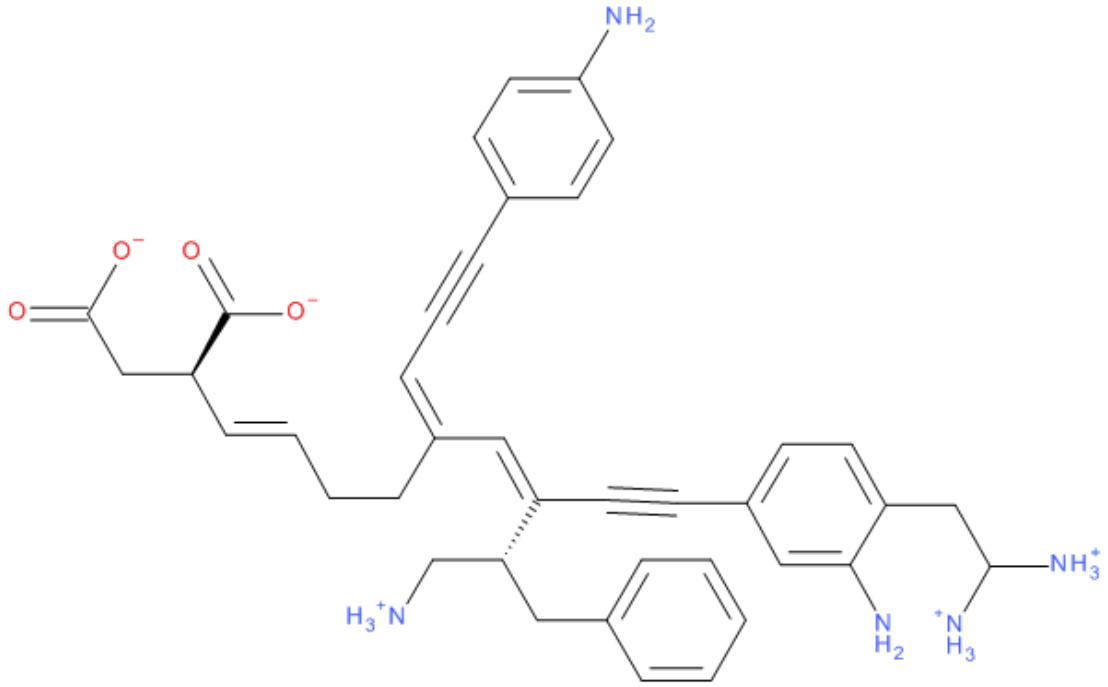
Annex 117 - Representation of Insitu61



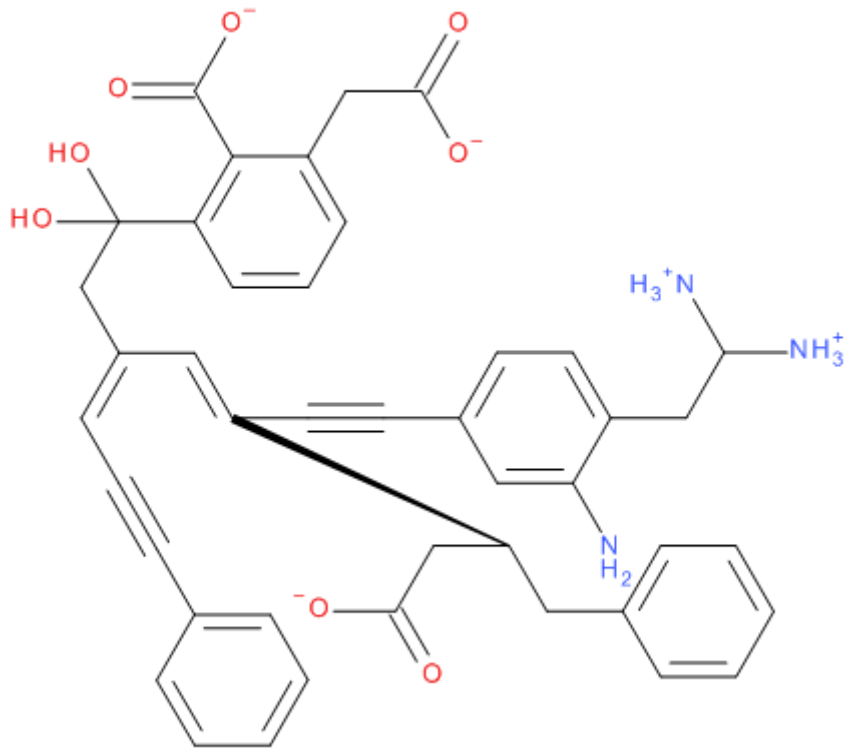
Annex 118 - Representation of Insitu62



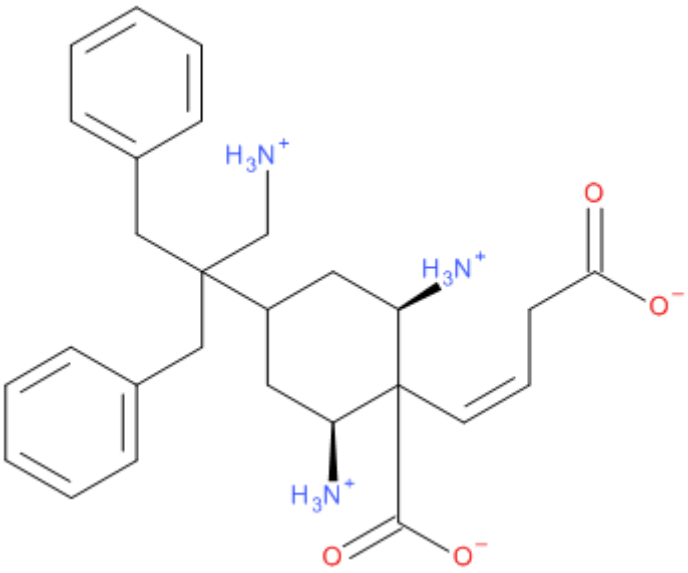
Annex 119 - Representation of Insitu63



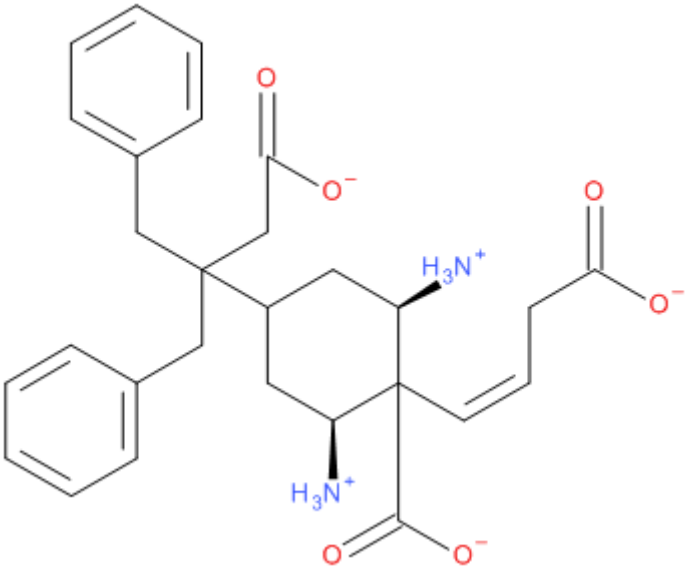
Annex 120 - Representation of Insitu64



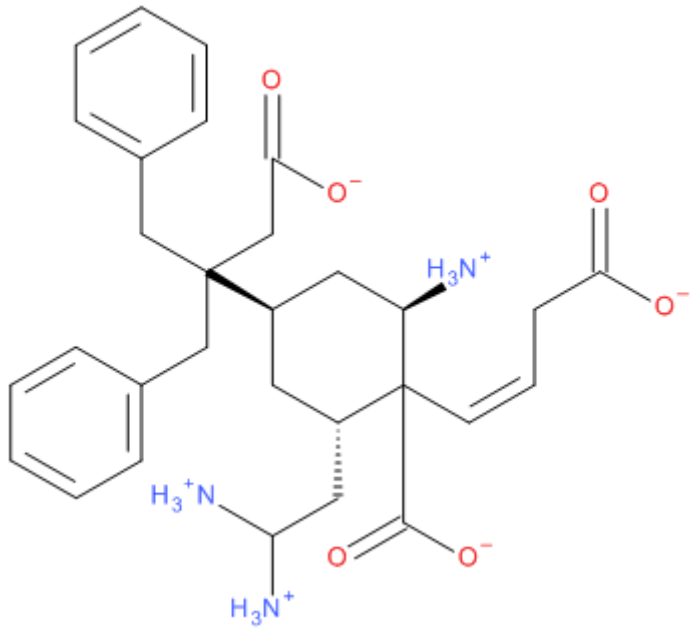
Annex 121 - Representation of Insitu65



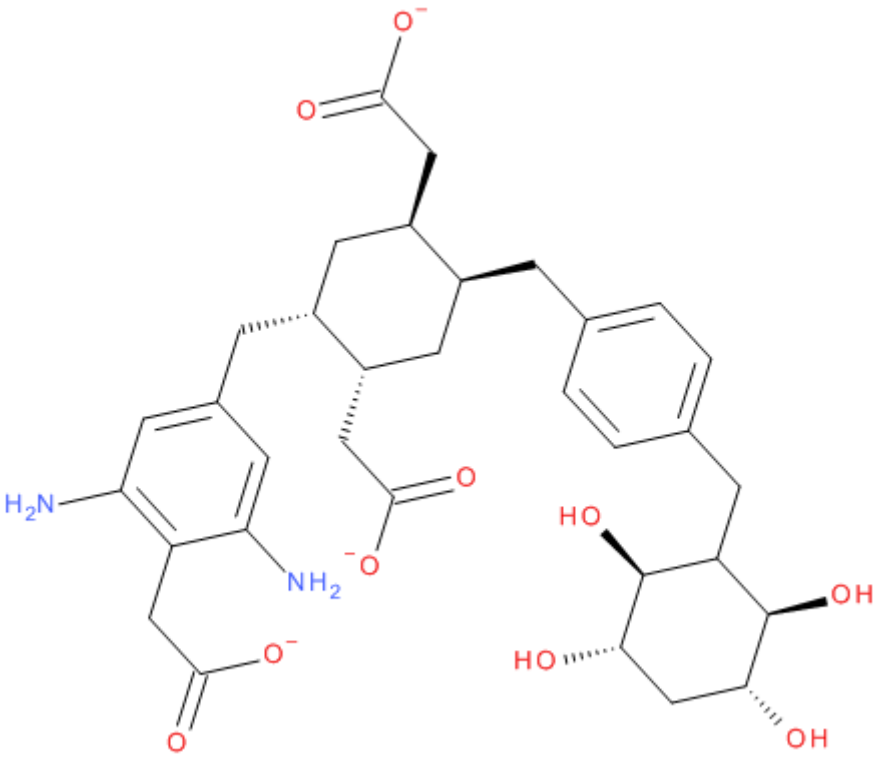
Annex 122 - Representation of Insitu66



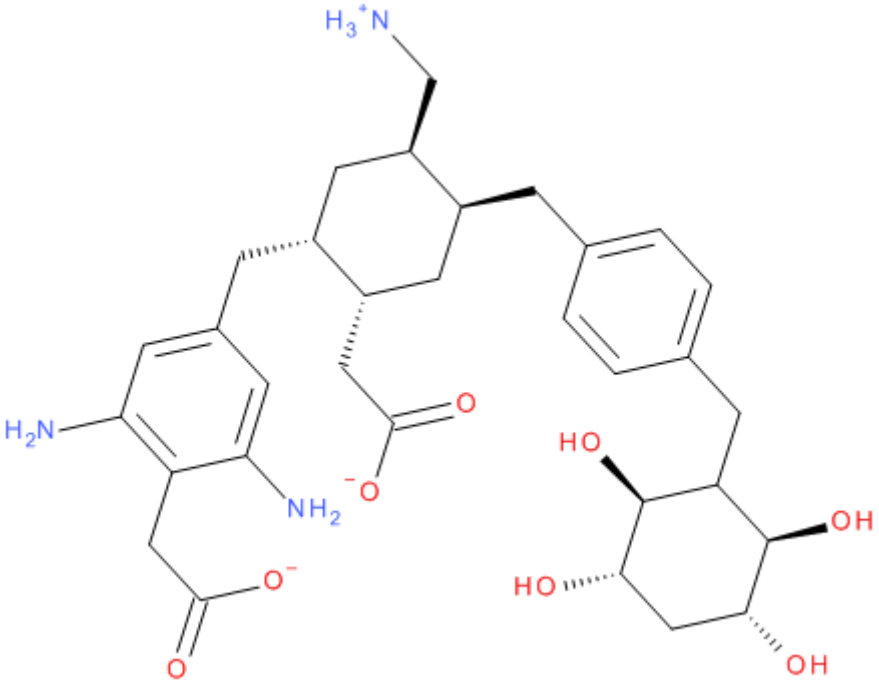
Annex 123 - Representation of Insitu67



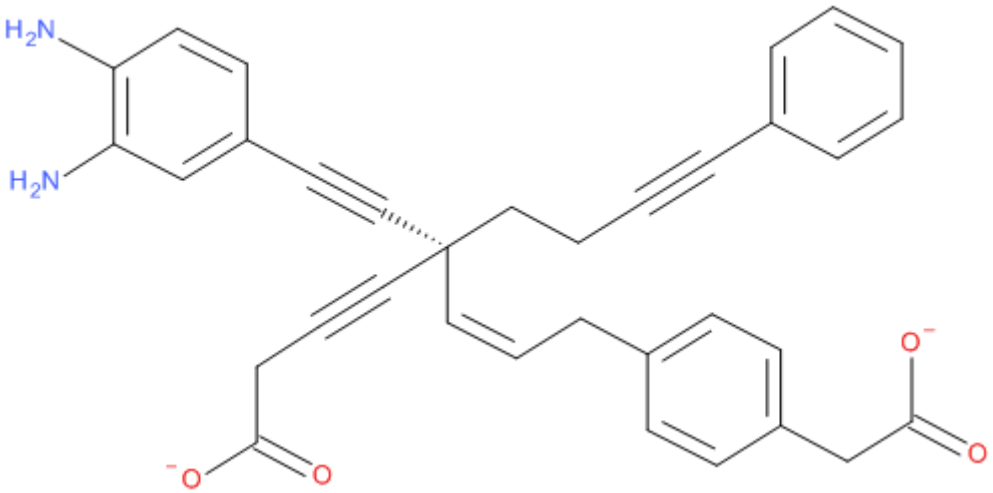
Annex 124 - Representation of Insitu68



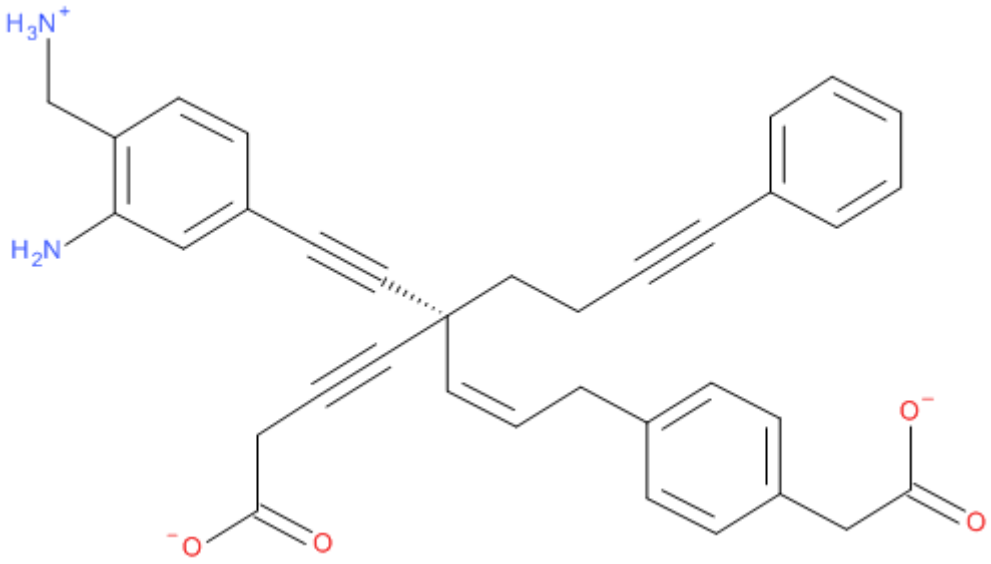
Annex 125 - Representation of Insitu69



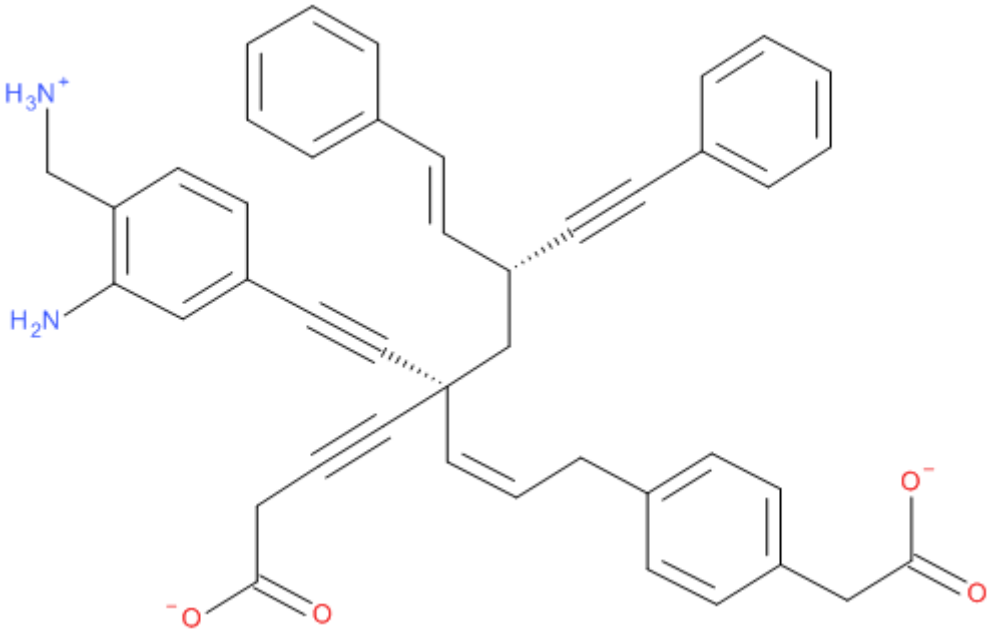
Annex 126 - Representation of Insitu70



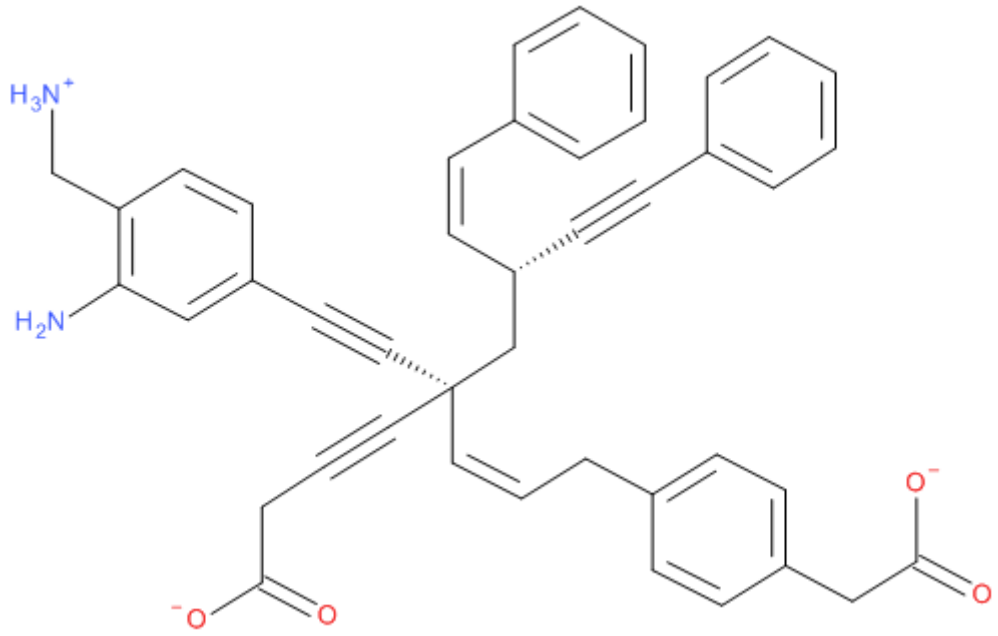
Annex 127 - Representation of Insitu71



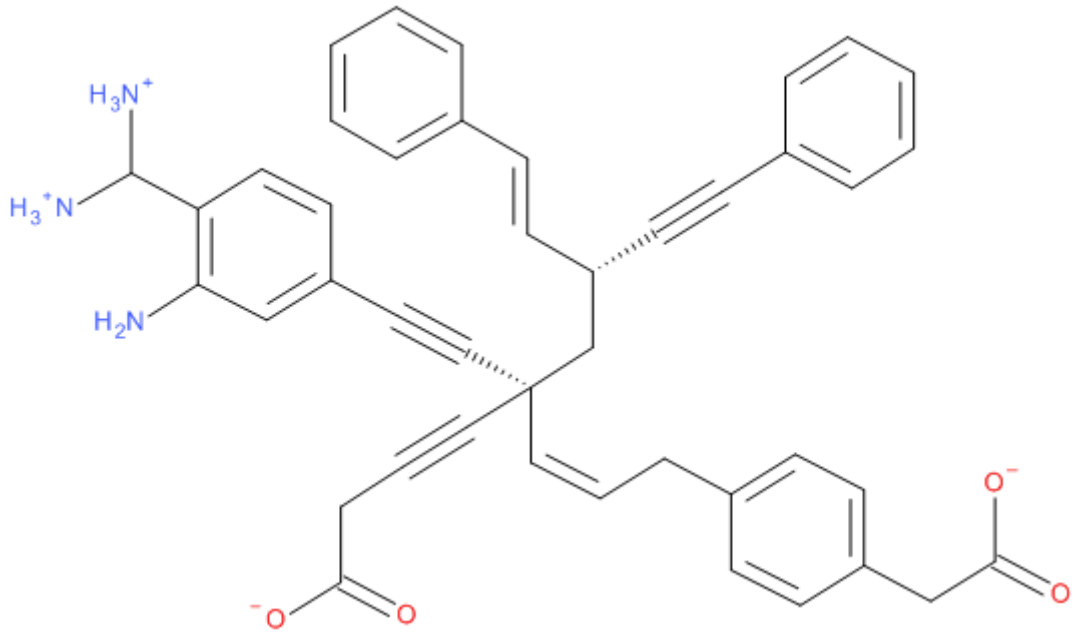
Annex 128 - Representation of Insitu72



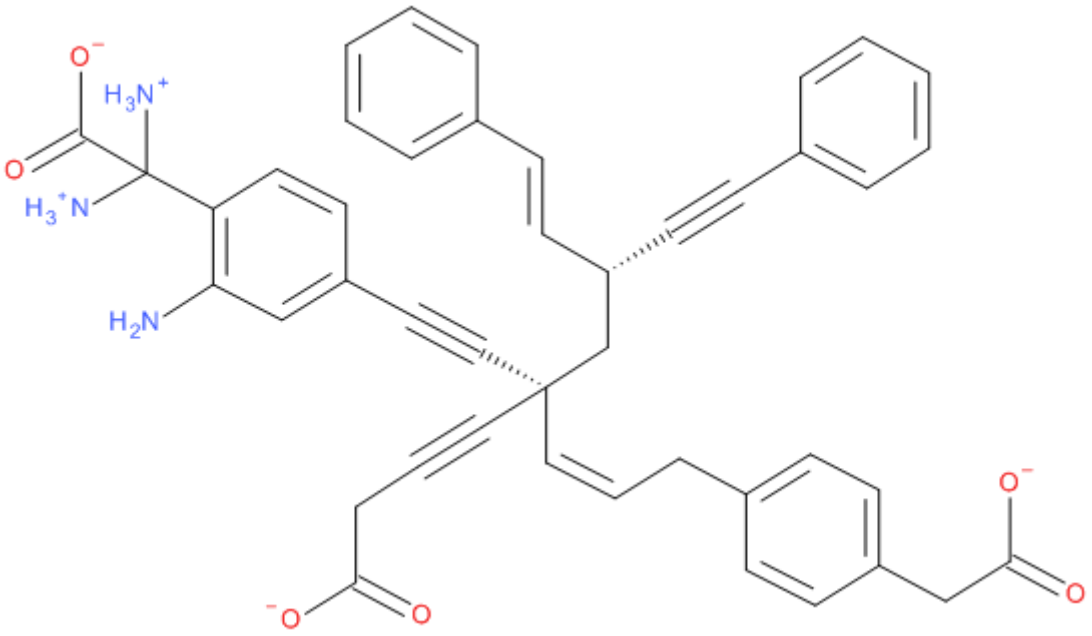
Annex 129 - Representation of Insitu73



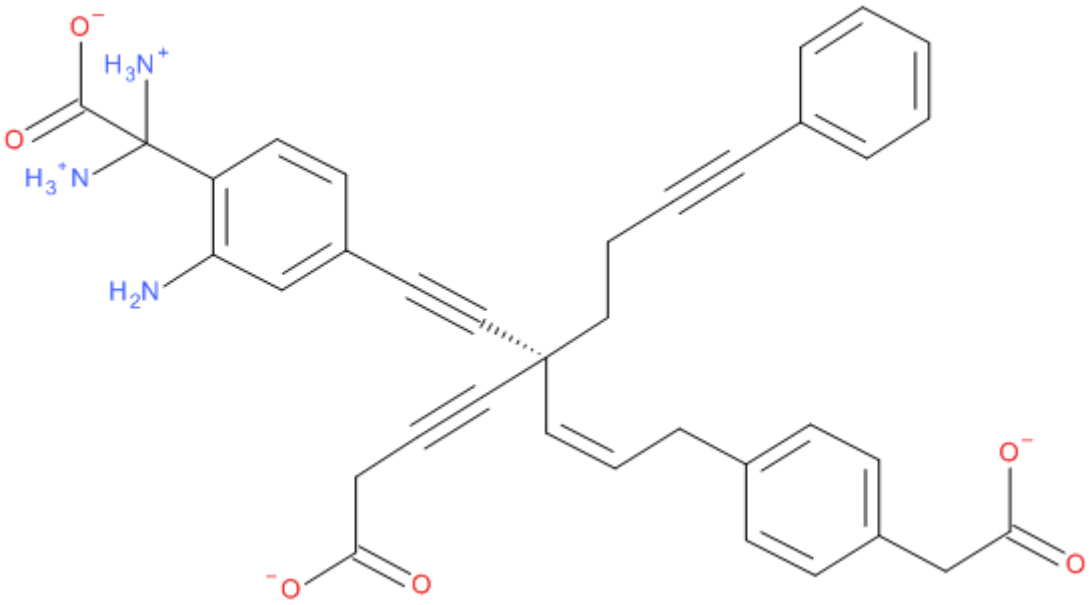
Annex 130 - Representation of Insitu74



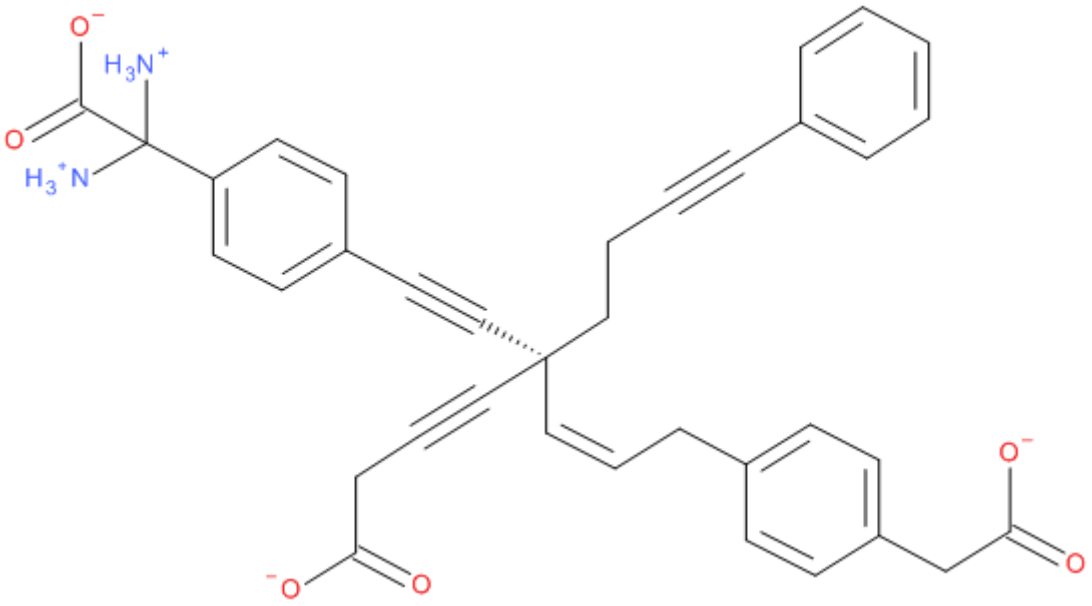
Annex 131 - Representation of Insitu75



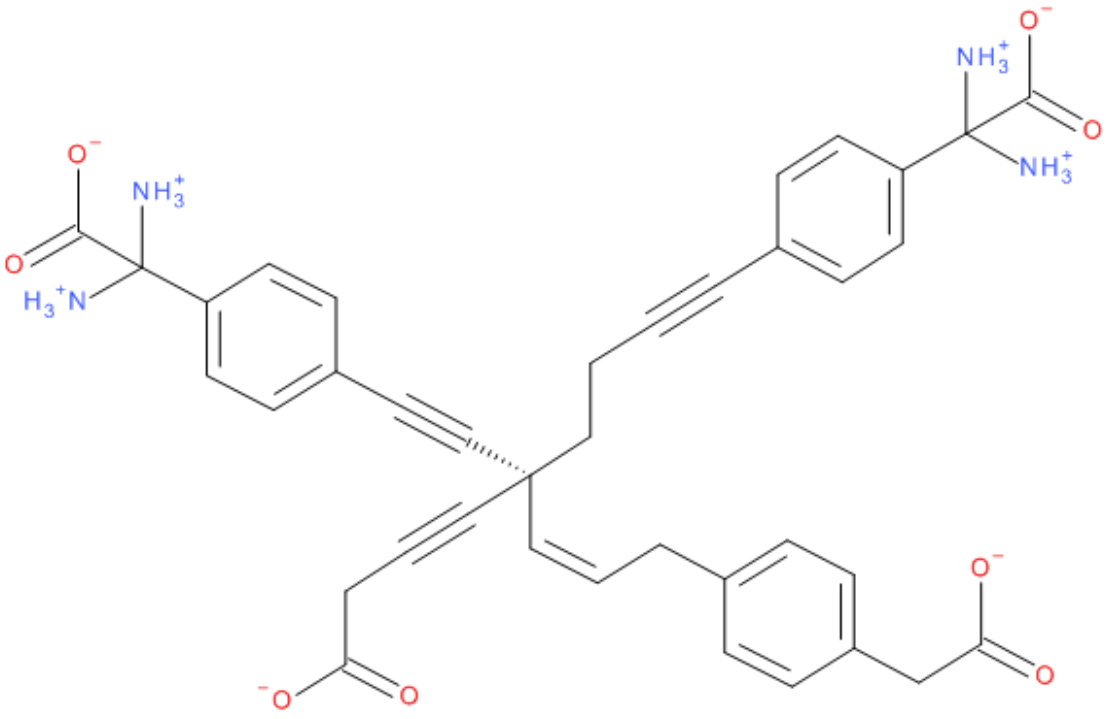
Annex 132 - Representation of Insitu76



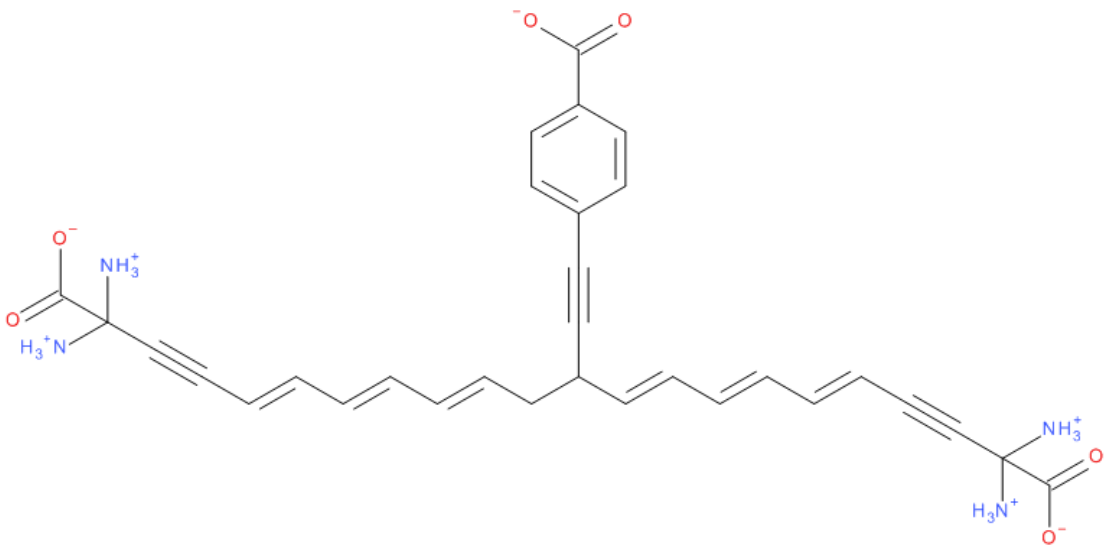
Annex 133 - Representation of Insitu77



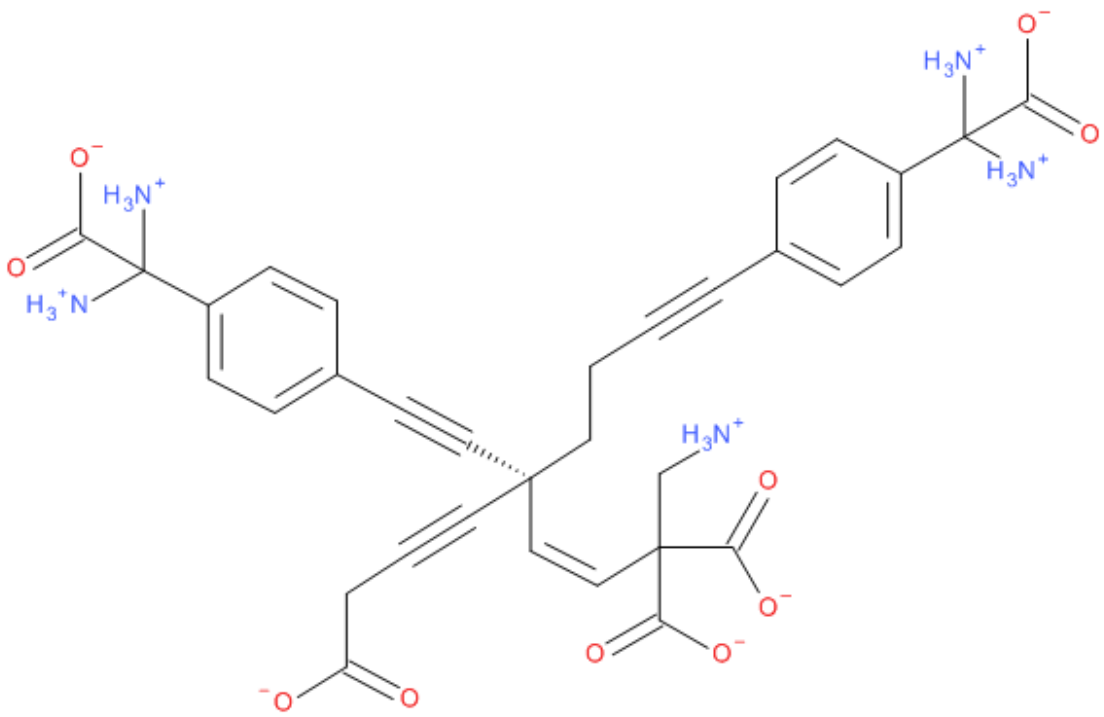
Annex 134 - Representation of Insitu78



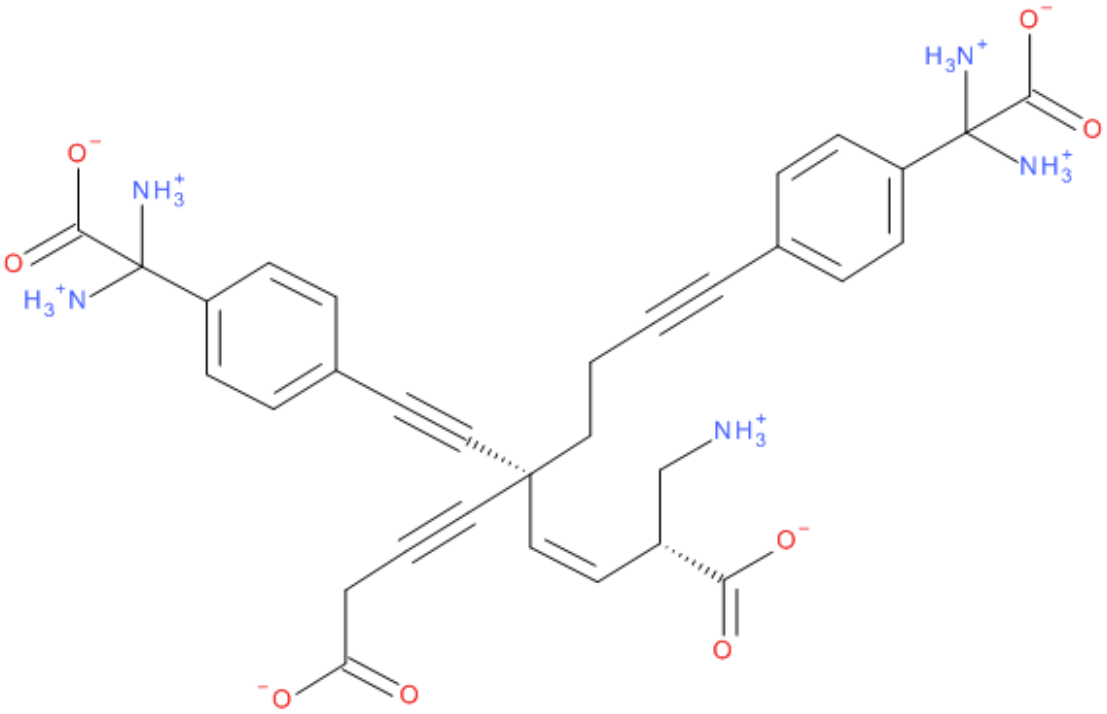
Annex 135 - Representation of Insitu79



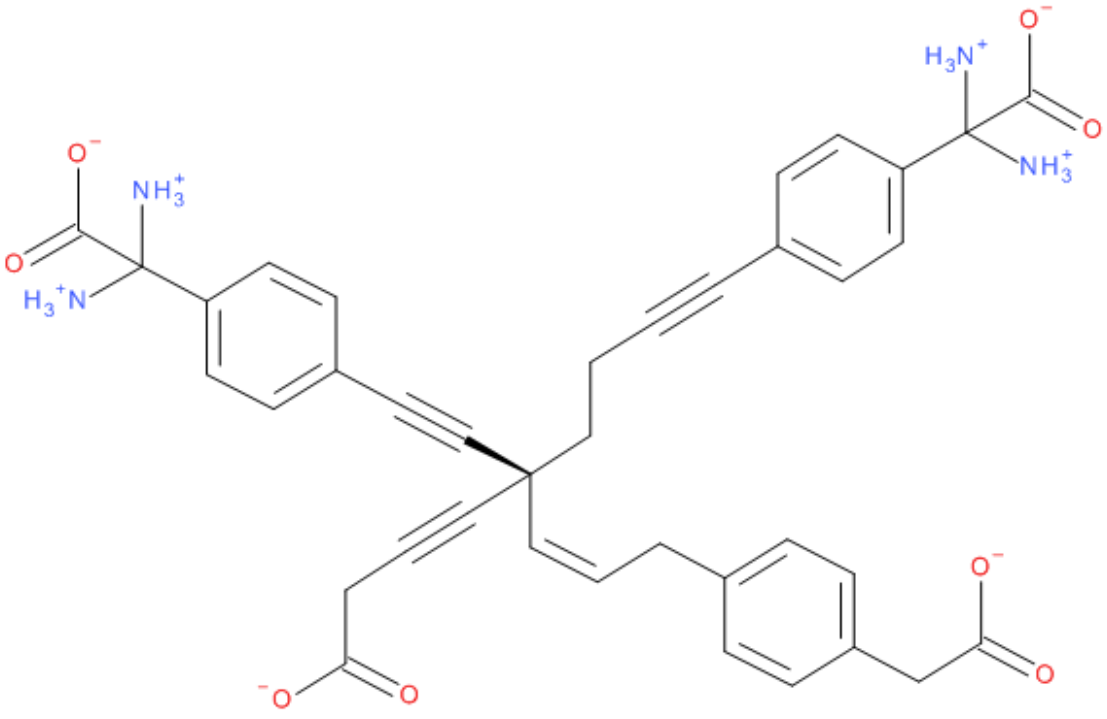
Annex 136 - Representation of Insitu80



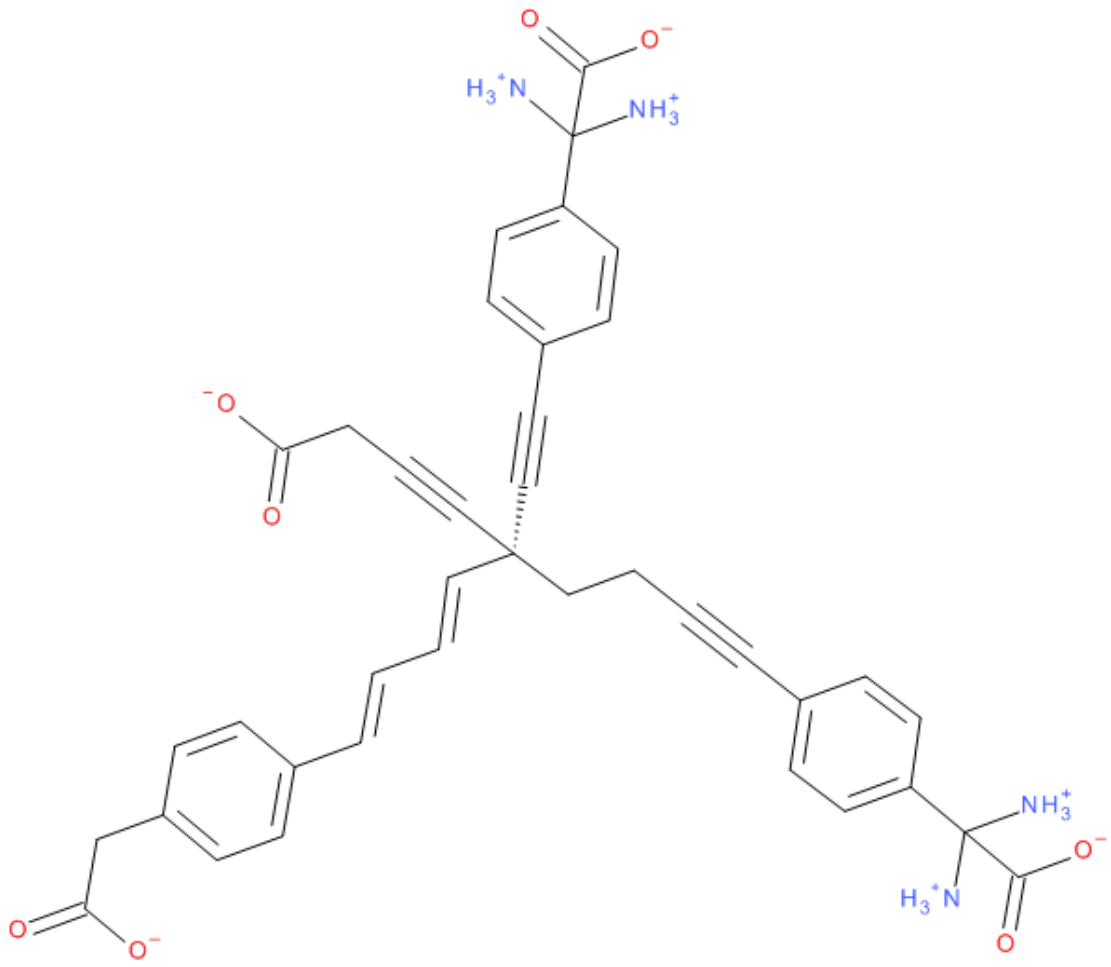
Annex 137 - Representation of Insitu81



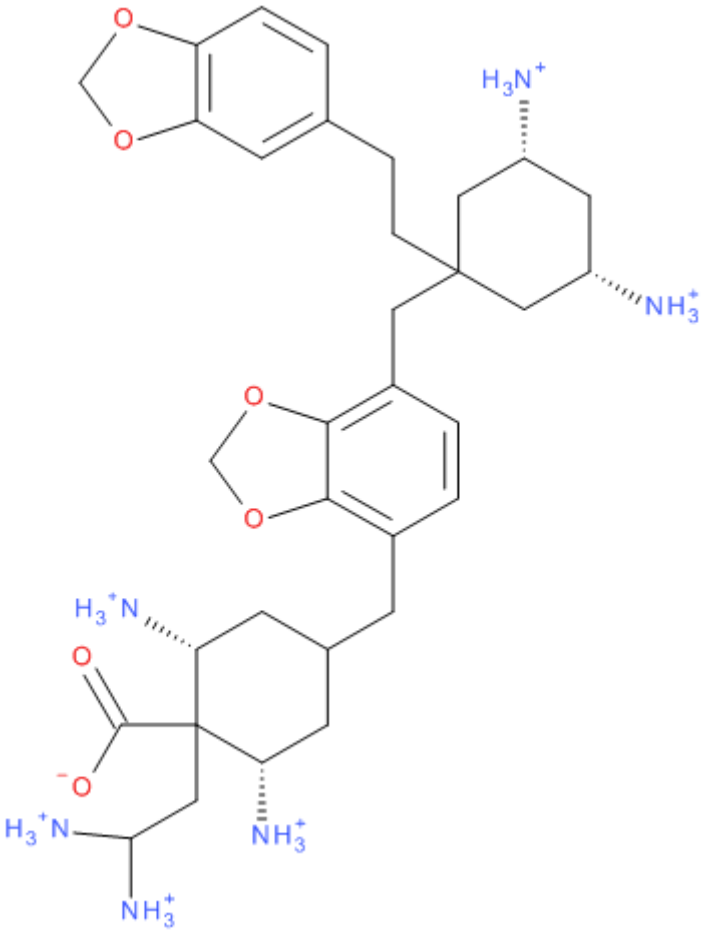
Annex 138 - Representation of Insitu82



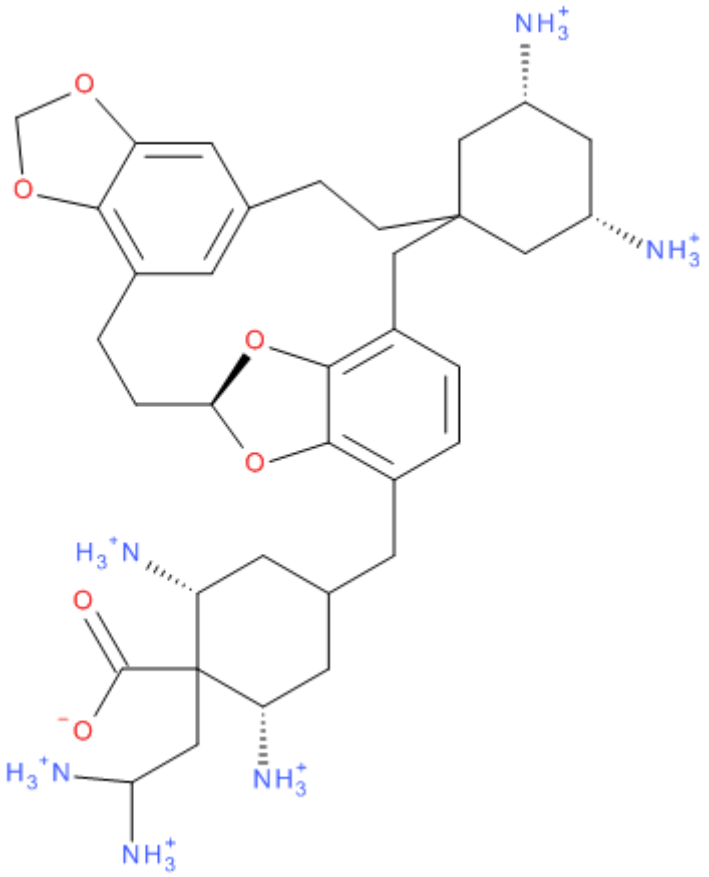
Annex 139 - Representation of Insitu83



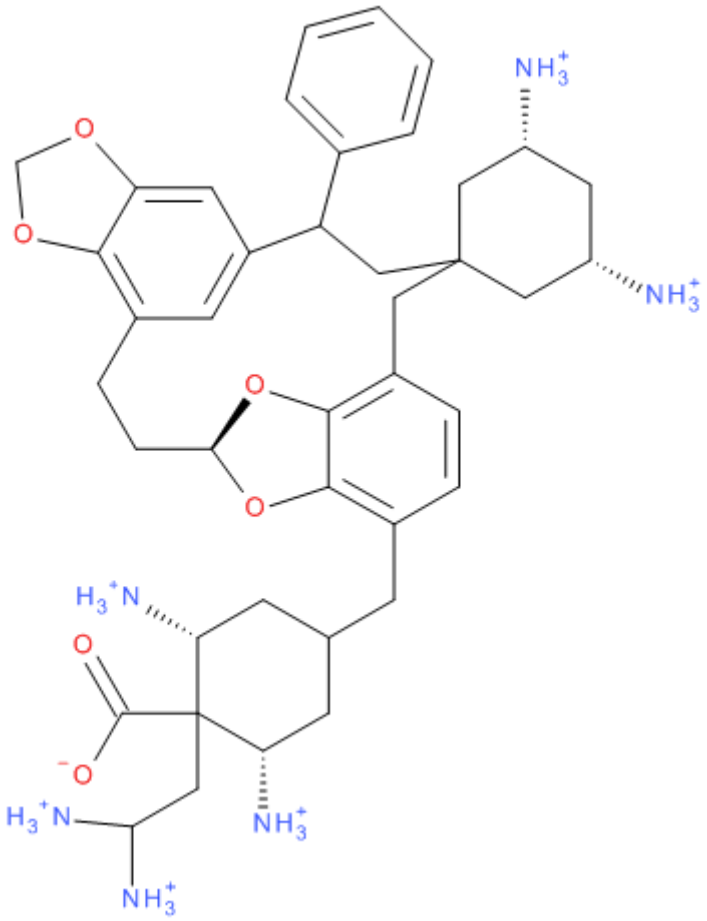
Annex 140 - Representation of Insitu84



Annex 141 - Representation of Insitu85



Annex 142 - Representation of Insitu86



Annex 143 - Representation of Insitu87