UNIVERSITA' DEGLI STUDI DI NAPOLI

FEDERICO II

PhD School in Chemical Sciences

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Design and chemical synthesis of heterocyclic alkaloid compounds isolated from marine organisms

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Sommario

Abstract

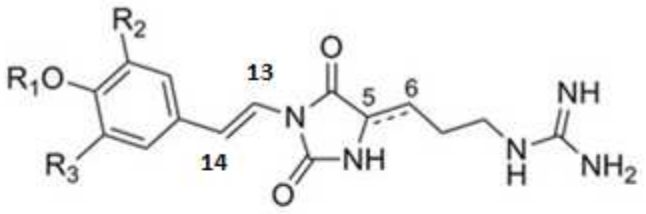
Alkaloids are naturally occurring nitrogen containing biologically active heterocyclic compounds. Over the last years, a large number of biologically alkaloids with antiviral, antibacterial, anti-inflammatory, antimalarial, antioxidant and anticancer activities have been isolated from marine source.

In this frame parazoanthines are a group of unique naturally-occurring marine alkaloids reported to date only from the Mediterranean sea anemone *Parazoanthus axinellae*. The chemical framework characteristic of these molecules is a 3,5-alkyl disubstituted hydantoin core bearing a terminal guanidine and an aromatic ring. Hydantoins and derivatives have been widely used in biomedical studies including novel therapeutic agents of interest as anti-convulsants and antimuscarinics,antiulcers and antiarrythmics, antivirals, antidiabetics, and inhibitors of antagonist of serotonin and fibrinogen receptors, inhibitors of glycine binding site of the NMDA receptor and antagonists of leukocyte cell adhesion.

Due to the biological potential of this class of molecules, a synthetic strategy was planed and developed to prepare natural and not natural analogs of parazoanthines; the molecular docking tests have described a promising antagonist activity on co-chemokine receptor CXCR4, involved in many tumors (breast cancer, melanoma, leukemia multiple myeloma, small cell lung cancer (SCLC), malignant melanoma and pancreatic cancer), rheumatoid arthritis, stem cell mobilization and HIV-1.

The preliminary tests in vitro have confirmed the antagonist effect of parazoanthine-A with the co-receptor CXCR4, describing a promising

pharmacological action for the treatment of diseases involved by the activation of this receptor.



	R_1	R_2	R_3	
Parazoanthine A	H	H	H	
Parazoanthine B	H	H	H	$\Delta^{5,6}$
Parazoanthine C	CH_3	H	H	$\Delta^{5,6}$
Parazoanthine D	CH_3	Br	H	
Parazoanthine E	CH_3	Br	H	$\Delta^{5,6}$
Parazoanthine F	CH_3	H	H	
Parazoanthine I	CH_3	Br	Br	
Parazoanthine J	CH_3	Br	Br	$\Delta^{5,6}$

Parazoanthines

Chapter I

1 Alkaloids

Alkaloids are a group of naturally occurring chemical compounds that mostly contain basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties. Some synthetic compounds of similar structure are also termed alkaloids.[1] In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulfur and, more rarely, other elements such as chlorine, bromine, and phosphorus. They are produced by a large variety of organisms including bacteria, fungi, plants, and animals. Millions of people around the Globe use purine alkaloids every day as well as starting the day with a cup of coffee or drinking a cup of tea in the afternoon. Alkaloids are molecules participating in both producer and consumer chains in nature and they are vital in feeding, and enjoy servations, agressivity and defence for all living species. [1]

The alkaloids content in plants is usually within a few percent and is inhomogeneous over the plant tissues. Depending on the type of plants, the maximum concentration is observed in the leaves (black henbane), fruits or seeds (Strychnine tree), root (*Rauwolfia serpentina*) or

[1] Aniszewski, Tadeusz *Alkaloids – secrets of life*. **2007** Amsterdam: Elsevier p.1

bark (cinchona).[2] Furthermore, different tissues of the same plants may contain different alkaloids.[3]

Beside plants, alkaloids are found in certain types of fungi, such as psilocybin in the fungus of the genus *Psilocybe*, and in animals, such as bufotenin in the skin of some toads. Some amines, such as adrenaline and serotonin, which play an important role in higher animals, are similar to alkaloids in their structure and biosynthesis and are sometimes called alkaloids.[4] Alkaloids were usually found from marine organisms also.[1]

1.1 *Alkaloids classification*

Compared with most other classes of natural compounds, alkaloids are characterized by a great structural diversity and there is no uniform classification of alkaloids. For the biologist, they are a pure and perfect natural products.

From the biological point of view, the alkaloid is any biologically active and heterocyclic chemical compound which contains nitrogen and could have some pharmacological activity and, in many cases, medicinal or ecological use. [5] For the medical scientist, the term "alkaloids" means any group of nitrogenous substances of vegetable origin, often of complex structure and high molecular mass. Medicine focuses on physiological action of alkaloids used as curative drugs. Some of these compounds can also be

[2] Grinkevich NI Safronich LN. *The chemical analysis of medicinal plants: Proc. allowance for pharmaceutical universities*. **1983** p.122-123
[3] Orekhov, AP. *Chemistry alkaloids* (Acad. 2 ed.). **1955** p.12
[4] Aniszewski, Tadeusz. *Alkaloids – secrets of life*. **2007** Amsterdam: Elsevier P.110-111
[5] Aniszewski, T.. *The biological basis of quinolizidine alkaloids. Science of Legumes*, **1994** 1: 1–24

highly toxic, even in very small doses. [6] Alkaloids can be classified in the terms of their: biological and ecological activity; chemical structures and biosynthetic pathway, but they are generally classified by their common molecular precursors, based on the biological pathway used by nature to build the molecule. From a structural point of view, alkaloids are divided according to their shapes and origins. There are three main types of alkaloids:

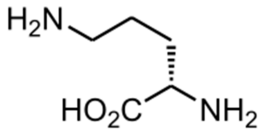
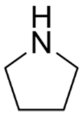
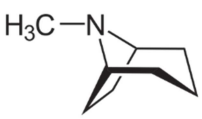
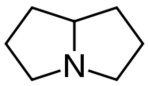
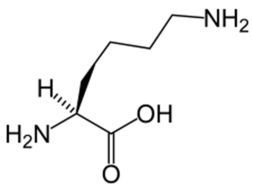
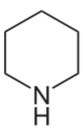
I. true alkaloids

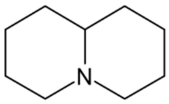
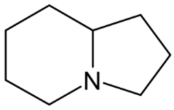
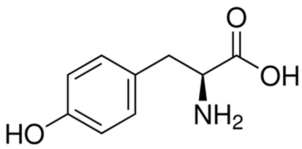
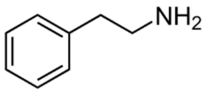
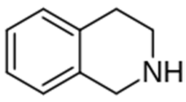
II. protoalkaloids

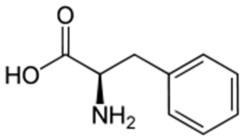
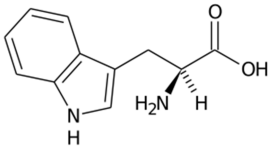
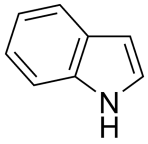
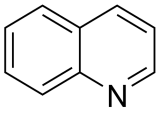
III. pseudoalkaloids.

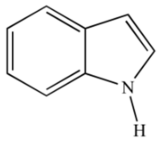
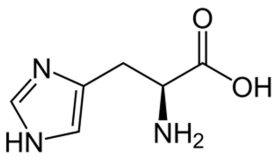
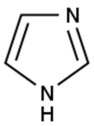
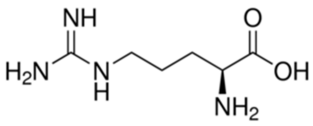
True alkaloids and protoalkaloids are derived from amino acids, whereas pseudoalkaloids are not (*Table 1*). [7]

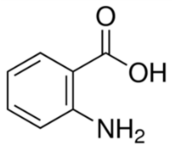
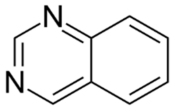
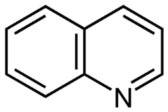
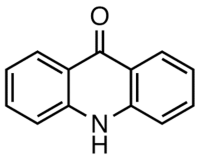
[6] Lovell Becker, E., Butterfield, W. J. H., McGehee Harvey, A., Heptinstall, R. H. and Lewis, T. (eds). *International Dictionary of Medicine and Biology*. **1986** New York: John Wiley & Sons.
[7] Aniszewski, Tadeusz. *Alkaloids – secrets of life*. **2007** Amsterdam: Elsevier P.6-10

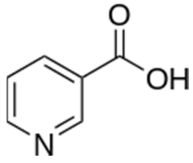
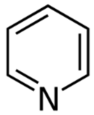
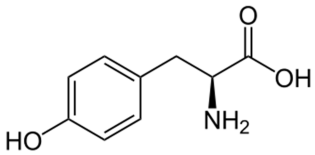
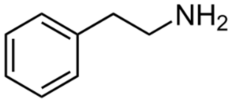
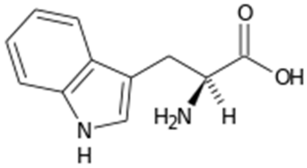
ALKALOID TYPE	PRECURSOR COMPOUND	CHEMICAL GROUP OF ALKALOIDS	EXAMPLES OF ALKALOIDS
True Alkaloids	*L-ornithine*		
		Pyrrolidine alkaloids 	Hygrine Cuscohygrine
		Tropane alkaloids 	Atropine Cocaine Hyoscyamine Scopolamine/ hyoscine
		Pyrrolizidine alkaloids 	Acetyllycopsamine Acetyl-intermedine Europine Homospermidine Ilamine Indicine-N-oxide Meteloidine Retronecine
	L-lysine		
		Piperidine alkaloids 	Anaferine Lobelanine Lobeline N-methyl pelletierine Pelletierine Piperidine Piperine Pseudopelletierine Sedamine

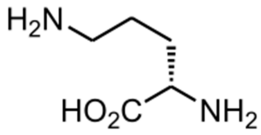
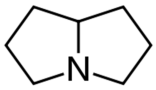
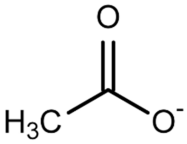
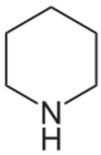
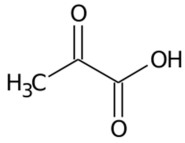
		Quinolizidine alkaloids 	Cytisine Lupanine Sparteine
		Indolizidine alkaloids 	Castanospermine Swansonine
	L-tyrosine		
		Phenylethylamino alkaloids 	Adrenaline Anhalamine Dopamine Noradrealine Tyramine
		Simple tetrahydroisoquinoline alkaloids 	Codeine Morphine Norcoclaurine Papaverine Tetrandrine Thebaine Tubocurarine

	L-tyrosine *or* *L-phenylanine*		
		Phenethylisoquinoline alkaloids	Autumnaline Crinine Floramultine Galanthamine Galanthine Haemanthamine Lycorine Lycorenine Maritidine Oxomaritidine Vittatine
	L-tryptophan		
		Indole alkaloids 	Arundacine Arundamine Psilocin Serotonin Tryptamine Zolmitriptan Harmine Elaeagnine Ajmalicine Catharanthine Secologanin Tabersonine
		Quinoline alkaloids 	Chloroquinine Cinchonidine Quinine Quinidine

		Pyrroloindole alkaloids 	A-yohimbine Chimonantheine Chimonantheine Corynantheine Corynantheidine Dihydrocorynantheine Corynanthine
		Ergot alkaloids	Ergobine Ergotamine Ergocryptine
	L-histidine		
		Imidazole alkaloids 	Histamine Pilocarpine Pilosine
		Manzamine alkaloids	Xestomanzamine A Xestomanzamine B
	L-arginine		
		Marine alkaloids	Saxitoxin Tetrodotoxin Parazoanthine

	Antranilic acid		
		Quinazinoline alkaloids 	Peganine
		Quinoline alkaloids 	Acetylfolidine Acutine Bucharine Dictamnine Dubunidine Flindersine Foliosidine Glycoperine Haplophyllidine Haplopine Helietidine Kokusaginine Maculosine Perfamine Perforine Polifidine Skimmianine
		Acridone alkaloids 	Acronycine Rutacridone

	Nicotinic Acid		
		Pyridine alkaloids 	Anabasine Cassinine Celapanin Evoline Evonoline Evorine Maymyrsine Nicotine Regelidine Wilforine
Proto-alkaloids	*L-tyrosine*		
		Phenylethylamino alkaloids 	Hordenine Mescaline
	L-tryptophan		
		Terpenoid indole alkaloids	Yohimbine

	L-ornithine		
		Pyrrolizidine alkaloids 	4-hydroxystachydrine Stachydrine
Pseudo-alkaloids	*Acetate*		
		Piperidine alkaloids 	Coniine Coniceine Pinidine
		Sesquiterpene alkaloids	Cassinine Celapanin Evonine Evonoline Evorine Maymyrsine Regelidine Wilforine
	Pyruvic acid		
		Ephedra alkaloids	Cathine Cathinone Ephedrine Norephedrine

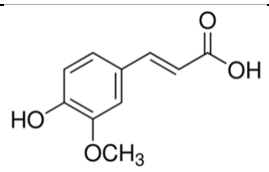
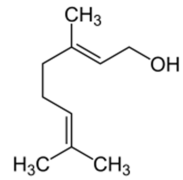
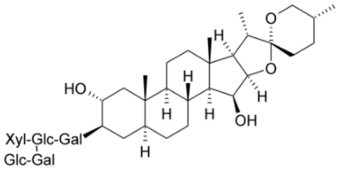
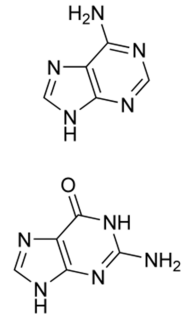
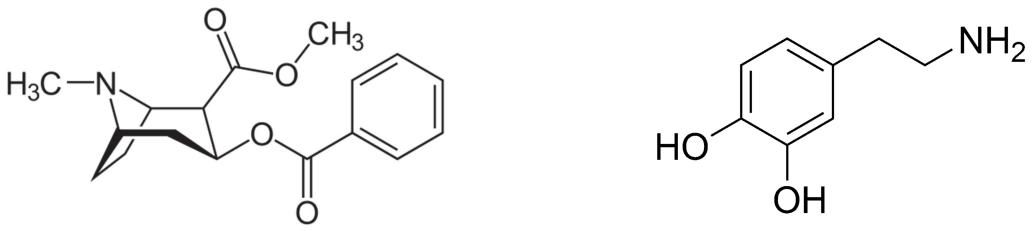
	Ferulic acid		
		Aromatic alkaloid	Capsaicin
	Geraniol		
		Terpenoid alkaloid	Aconitine Actinidine Atisine Gentianine
	Saponins		
	 (es. digitonin)	Steroid alkaloids	Cholestane Conessine Cyclopamine Jervine Pregnenolone Protoveratrine A Protoveratrine B Solanidine Solasodine Squalamine Tomatidine
	Adenine/ Guanine		
		Purine alkaloids	Caffeine Theobromine Theophylline

Table 1. Main types of alkaloids and their chemical groups

1.1.1 *True alkaloids*

True alkaloids derive from aminoacids and they share an heterocyclic ring with nitrogen. The non-nitrogen containing rings or side chains are derived from terpene units and / or acetate, while methionine is responsible for the addition of methyl groups to nitrogen atoms. These alkaloids are highly reactive substances with biological activity even in low doses. All true alkaloids have a bitter taste and appear as a white solid, with the exception of nicotine which has a brown color. True alkaloids form water-soluble salts. Moreover, most of them are well-defined crystalline substances which reacts with acids to form salts. True alkaloids may occur in plants in the free state, as salts and as N-oxides. These alkaloids occur in a limited number of species and families, and are those compounds in which decarboxylated amino acids are condensed with a non-nitrogenous structural moiety. The primary precursors of true alkaloids are such amino acids as L-ornithine, L-lysine, L-phenylalanine/L-tyrosine, L-tryptophan and L-histidine.[8] Examples of true alkaloids include such biologically active alkaloids as cocaine (*Figure 1A*), quinine, dopamine (*Figure 1B*), morphine and usambarensine. More examples appears in *Table 1.*



(A) (B)

Figure 1. Cocaine (A), Dopamine (B)

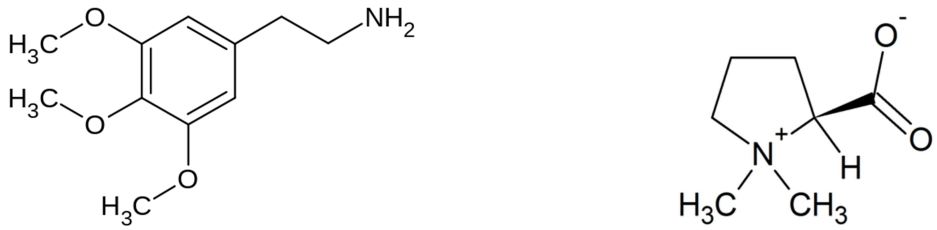
[8] Dewick, P. M.. *Medicinal Natural Products. A Biosynthetic Approach*. Second Edition. **2002** Chichester – New York: John Wiley & Sons Ltd

1.1.2 Protoalkaloids

Protoalkaloids are compounds, in which the N atom derived from an amino acid is not a part of the heterocycle. [9]

Such kinds of alkaloid include compounds derived from L-tyrosine and L-tryptophan (see *Table 1*). They form a minority of all alkaloids. Hordenine, mescaline (*Figure 2A*) and yohimbine are examples of these kinds of alkaloid. [10]

Chini *et al*.[11] have found new alkaloids, stachydrine (*Figure 2B*) and 4-hydroxystachydrine, derived from *Boscia angustifolia*, a plant belonging to the Capparidacea family. These alkaloids have a pyrroline nucleus and are basic alkaloids in the genus Boscia. The species from this genus have been used in folk medicine in East and South Africa. Boscia angustifolia is used for the treatment of mental illness, and occasionally to combat pain and neuralgia.



(A) (B)

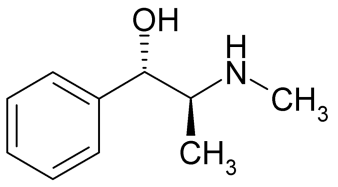
Figure 2. Mescaline (A), Stachydrine (B)

[9] Jakubke, H.-D., Jeschkeit, H. and Eagleson, M. *Concise Encyklopedia Chemistry*. **1994** Berlin – New York: Walter de Gruyter

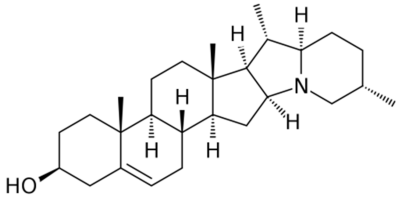
[10] Chini, C., Bilia, A. R., Keita, A. and Morelli, I. *Planta Medica*, **1992** 58: 476

1.1.3 Pseudoalkaloids

In this class of compounds the basic carbon skeletons are not derived from aminoacids. [11] Furthermore, pseudoalkaloids are connected with aminoacids pathways. They are derived from the precursors or postcursors (derivatives from degradation processes) of aminoacids. They can also result from the amination and transamination reactions[12] of the different pathways connected with precursors or postcursors of aminoacids. These alkaloids can also be derived from non-aminoacid precursors. The N atom is inserted into the molecule at a relatively late stage as in the case of steroidal or terpenoid skeletons. The N atom can also be donated by an aminoacidic source across a transamination reaction, if there is a suitable aldehyde or ketone. Pseudoalkaloids can be acetate and phenylalanine derived or terpenoid, as well as steroidal alkaloids. Examples of pseudoalkaloids include such compounds as coniine, capsaicin, ephedrine (*Figure 3A*), solanidine (*Figure 3B*), caffeine, theobromine and pinidine. More examples appear in *Table 1*.



(A)



(B)

[11] Jakubke, H.-D., Jeschkeit, H. and Eagleson, M. *Concise Encyklopedia Chemistry*. **1994** Berlin – New York: Walter de Gruyter

[12] Dewick, P. M. *Medicinal Natural Products. A Biosynthetic Approach*. Second Edition. **2002** Chichester – New York: John Wiley & Sons Ltd

Figure 3. Ephedrine (A), Solanidine (B)

1.2 *Biogenesis of alkaloids*

The synthesis and structural analysis of alkaloids leads to the following basic questions: why are alkaloids synthesized in an organism? It is known that alkaloids have a genetic nature[13] and the alkaloid content is diverse inside and between the species.[14] In nature the same species of plants may have both high and low alkaloid content. [15, 16] Natural hybridization has been successfully used in plant breeding for the development of the so-called "sweetcultivars" in crop production. In "Sweet cultivars", however, alkaloids are present and their total removal is not possible. "Sweet cultivars" are therefore plants, in which alkaloids are present at a very low level and the bioactivity of which is not of any significant or observable level. However, alkaloid decrease by hybridization is an undirect but strong argument for the case that alkaloids have an natural heredity and that their presence in plants has an evolutionary meaning. This is fundamental in doing the first question connected with the biogenesis of alkaloids. Alkaloids have a strong genetic–physiological function in the organisms which produce them. The biogenesis of alkaloids is therefore a part of the total genetic-functional strategy of such metabolisms.

[13] Nowacki, E. "Inheritance and biosynthesis of alkaloids in lupin." *Genetica Polonica* 4.2 **1963**: 161-202.
[14] Waller, G. R., and E. K. Nowacki. "Alkaloid biology and metabolism in plants Plenum Press." *New York* **1978**: 294.
[15] Aniszewski, T. *Lupine/a potential crop in Finland/studies on the ecology, productivity, and quality of Lupinus spp*. **1993** University of Joensuu: p.148
[16] Aniszewski, T. *Lupine/a potential crop in Finland/studies on the ecology, productivity, and quality of Lupinus spp*. **1993** University of Joensuu, 29: 1–50

1.3 *Chemistry models*

Since the year 1805, when alkaloid chemical research started, the topic regarding the biogenesis of alkaloids proved central for chemists. The background to this argument was the fact that chemical compounds are synthesized by plants, used by plants and degradaded by plants. In the case of alkaloids, it was still difficult in the middle of the 20th century to truly ascertain the purpose of alkaloids in plants. Certainly, the use of these compounds in many applications outside of the organisms producing them was well recognized but their role within the plants, especially in the metabolism, was not known. The general thinking was that alkaloids were "the waste" product of metabolisms and had no active role to play. Therefore, chemical cycle of alkaloid production were explained as chemical reactions, the "technical" process of life. Later, especially since the late 70s of the 20th century, the theory of "wastes" was debated and corrected.[17] However, chemical research has now extensively proved the existence of new alkaloids, the pathways of their biosynthesis and structural modification. Three directions in this research have been followed, one purely chemical, the second, biochemical, and the third purely biomolecular, or the molbiological direction. The chemical explanation of alkaloid biogenesis is based on the consideration that all reactions are of a chemical nature and that the energy needed for life is produced by chemical reactions.

[17] Waller, G. R., and E. K. Nowacki. "Alkaloid biology and metabolism in plants Plenum Press." *New York* **1978**, 294.

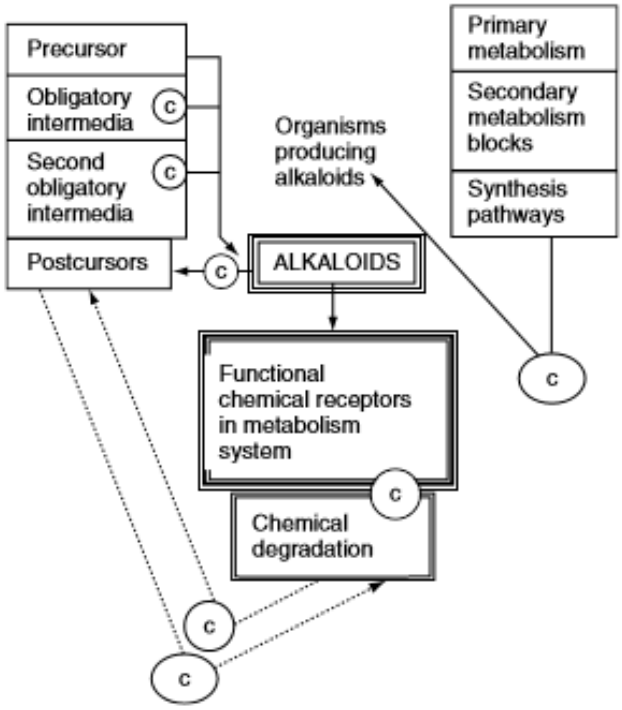


Figure 4. Chemical explanation for alkaloid biogenesis in organisms (c=catalysers).

Figure 4 shows a diagram of the chemical explanations for alkaloid biogenesis, from which it's clear that alkaloids are some of metabolic objects in the living organisms. It has a long chemical cycle, which includes synthesis before and degradation after its functional activity in the metabolism. Biogenesis is, therefore, considered by chemistry to be the chain of the reactions between molecules favoured by particular conditions and catalysers of special importance. Different alkaloids have their own biogenesis that are inspiration source for biochemical models and for developing new methods for synthetic reactions and structural modifications. Moreover, these models are also used in biotechnology. [18,19]

[18] Robins, R. J., Parr, A. J. and Walton, N. J. *Planta*, **1991** 183: 196–201
[19] Robins, R. J., Parr, A. J. and Walton, N. J. *Planta*, **1991** 183: 185–195

1.4 *Biochemistry models*

The description of single enzyme activity in chemical reactions, together with the activity of other biomolecules, is typical for biochemical models of alkaloids biogenesis. There is no contradiction between chemical and biochemical, which enrich each other. In many cases, typical chemical and biochemical models are unified in many papers nowadays.[19,20] Biochemical reactions are basically the same as other chemical organic reactions with their thermodynamic and mechanistic characteristics, but they have the enzymatic step. Thermodynamic laws, standard energy status and standard free energy change, reduction–oxidation (redox) and electrochemical potential equations are applicable to these reactions. Enzymes catalyse reactions and induce them to be much faster. [20,21]

The biochemical models are subject to both qualitative and quantitative alkaloid analysis. Not all enzymes participating in alkaloid synthesis and degradation are yet known. Alkaloid enzymatology is, therefore, a growing research area

1.5 *Molecular biology models*

Alkaloid research and bioanalysis of central-processing molecules (DNA and RNA) led to the important concept of the natural heredity of alkaloids metabolism. Recent investigations have proved empirically that alkaloids

[20] Torssell, K. B. G. *Natural Product Chemistry. A Mechanistic and Biosynthetic Approach to Secondary Metabolism.* **1983** Chichester – New York – Brisbane – Toronto – Singapore: John Wiley & Sons Limited
[21] Wilson, Keith, and John Walker. *Principles and techniques of practical biochemistry*. Cambridge University Press, **2000**, pp. 357–402

have a genetic background and that all their biogenesis is genetically determined. [22, 23, 24, 25, 26]

According to Tudzynski *et al*.[27], cpd1 gene coding for dimethylallyltryptophan syntase (DMATS) catalyses the first step in the biosynthesis of ergot alkaloids from Claviceps purpurea.

This means that detailed molecular genetic analysis of the alkaloid pathway is possible.[27] These results were confirmed by the research of Haarmann *et al*.[24] Moreover, Huang and Kutchan[26] found three genes (cyp80b1, bbe1 and cor1) which encode the enzymes needed for sanguinarine synthesis.

Molecular biology research on alkaloids is very revealing and its results can be used in the construction of alkaloid biogenetic models. At present, only a few alkaloid metabolism genes are known.

1.6 *Biosynthesis and metabolism*

Alkaloids are derived from the aminoacid in *L*-configuration (protein aminoacids) and from non-protein amino acids such as ornithine. However, it is important to note that alkaloids should be derived directly from the precursors of aminoacids as, for example, in the case of anthranilic acid (the precursor of trypthophan from the shikimate

[22] Sheppard, Donald C., et al. "The Aspergillus fumigatus StuA protein governs the up-regulation of a discrete transcriptional program during the acquisition of developmental competence." *Molecular biology of the cell* **2005,** 16(12): 5866-5879.
[23] Haarmann, Thomas, et al. "The ergot alkaloid gene cluster in Claviceps purpurea: extension of the cluster sequence and intra species evolution." *Phytochemistry* **2005,** 66(11): 1312-1320.
[24] Grothe,T.,Lenz,R.andKutchan,T.M. *Journal of Biological Chemistry*, **2001**, 276(33): 30717–30723
[25] Huang, F. C. and Kutchan, T. M. 2000. *Phytochemistry*, **2000**, 53(5): 555–564
[26] Tudzynski, P., Holter, K., Correia, T., Arntz, C., Grammel, N. and Keller, U. *Molecular and General Genetics*, **1999** , 261: 133–141

pathway) (*Figure 5*) or acetate (the precursor of lysine via ketoadipic acid and transamination in some algae and fungi). (*Figure 6*)

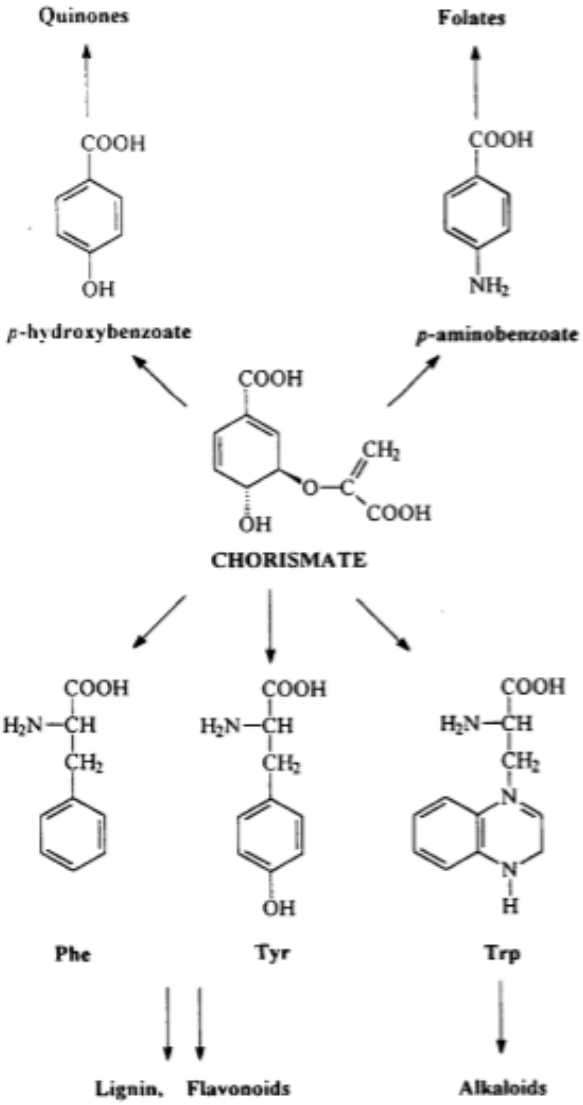


Figure 5. Chorismate it's the final product of Shikimate pathway, as a precursor for primary and secondary metabolites.

Each biomolecule in living organisms has its own synthesizing, transformational and interconverting processes. Therefore, the formation of the ring of the alkaloid molecule, and the flow of the nitrogen atom into this molecule, is the basic point for understanding alkaloid synthesis and its metabolism.

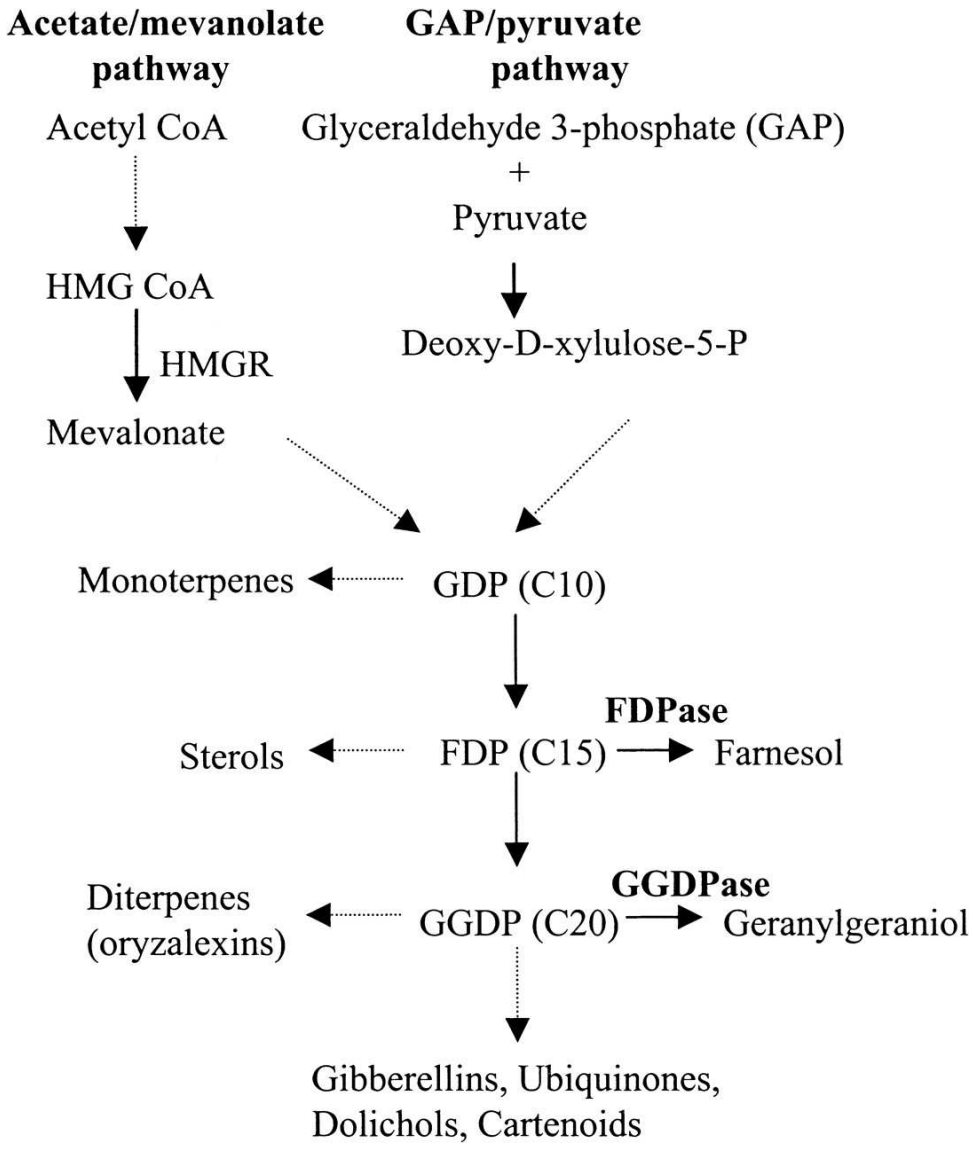


Figure 6. Acetate/Mevanolate and Deoxyxylulose pathway

Alkaloid biosynthesis needs the substrate. Substrates are derivatives of the secondary metabolism building blocks: the acetyl coenzyme A (acetyl-CoA), shikimic acid, mevalonic acid and 1-deoxyxylulose 5-phosphate. The synthesis of alkaloids starts from the acetate, shikimate (*Figure 5*), mevalonate and deoxyxylulose pathways (*Figure 6*). The acetyl coenzyme A pathway (acetate pathway) is the source of some alkaloids and their precursors (e.g., piperidine alkaloids or anthranilic acid as aromatized CoA ester (antraniloyl-CoA)). Shikimic acid is a product of the glycolytic and pentose phosphate pathways, a construction facilitated by parts of

phosphoenolpyruvate and erythrose 4-phosphate. The shikimic acid pathway is the source of such alkaloids as quinazoline, quinoline and acridine. The mevalonate pathway is based on mevalonic acid (three molecules of acetyl-CoA) which is closely related to the acetate pathway, while the deoxyxylulose phosphate pathway is based on a combination of pyruvic acid and glyceraldehyde 3-phosphate (both from the glycolytic pathway). Together, mevalonate and deoxyxylulose phosphate pathways produce terpenoid and steroid compounds. However, it is important to note that the Krebs cycle pathway is also key to many precursors of alkaloids. Ornithine, a postcursor of L-arginine in animals and of L-glutamate in plants, and, for example, L-lysine, a principal protein amino acid, deriving from the Krebs cycle pathway compound, are useful examples of the role of the Krebs cycle for alkaloid precursors (*Figure 7*).

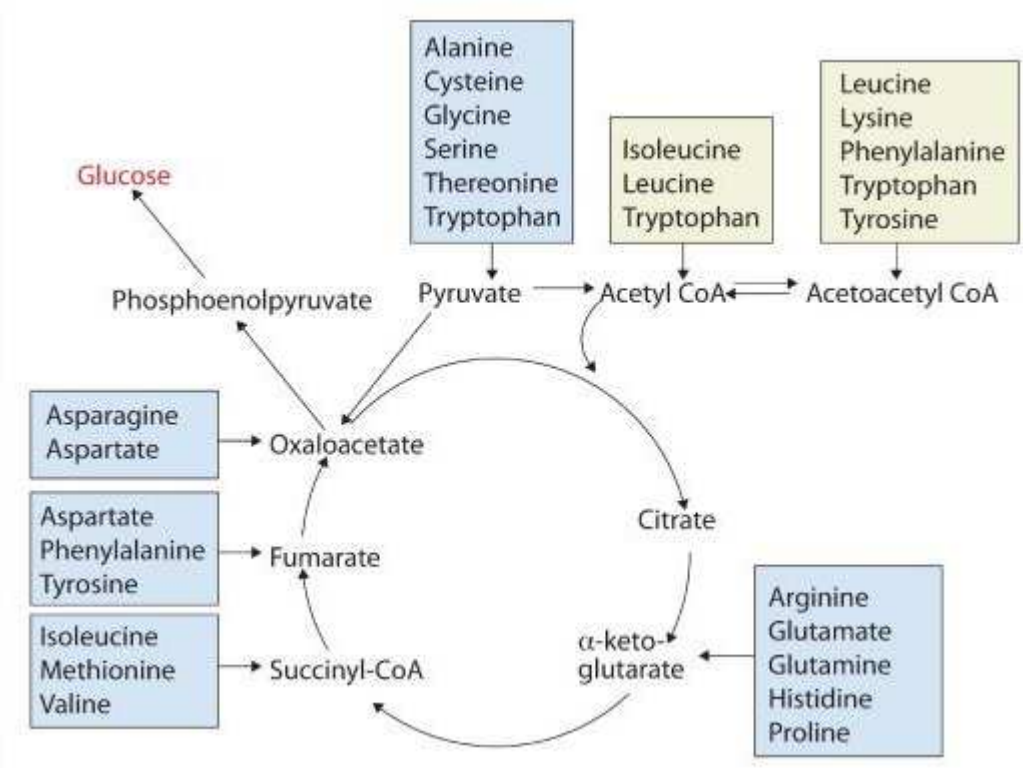


Figure 7. Krebs cycle pathway[27]

[27] Bruice. P.Y., *Essential Organic Chemistry* 2nd Ed. **2014** , Pearson.

Moreover, there are other sources of alkaloid substrates, particularly in purine alkaloids. Figure 8 represents the general scope of alkaloid synthesis in the metabolic system of organisms and their energy production. Enzymatic activity is very important in the primary metabolism of glycolysis and the Krebs cycle. Pyruvic acid and CoA are key compounds in the synthesis of alkaloid precursors. Moreover, these precursors (aminoacids) can be derived from different points in the glycolysis and Krebs cycles. Consequently, the synthesis of alkaloids as a secondary metabolic activity is a very challenging research subject. Generally, it is recognized in the literature that alkaloid metabolism in animals, and especially in mammals, is closely related to that of plants; [28,29] however, some exceptions exist. *Figure 8* shows two ways of L-ornithine synthesis. In plants, this non-protein aminoacid is derived from L-glutamate and in animals from L-arginine. Moreover, *Figure 8* demonstrates that synthesis of alkaloids is complicated by the ability of the same aminoacid to synthesize many different alkaloids.

[28] Brossi, A. Mammalian alkaloids: Conversion of tetrahydroisoquinoline-1carboxylic acids derived from Dopamine. *Planta Medica*, **1991** 57: S93–S100

[29] Xe, X. S., Tadic, D., Brzostowska, M., Brossi, A., Bell, M. and Creveling, C. *Helvetica Chimica Acta*, **1991** 74: 1399–1411

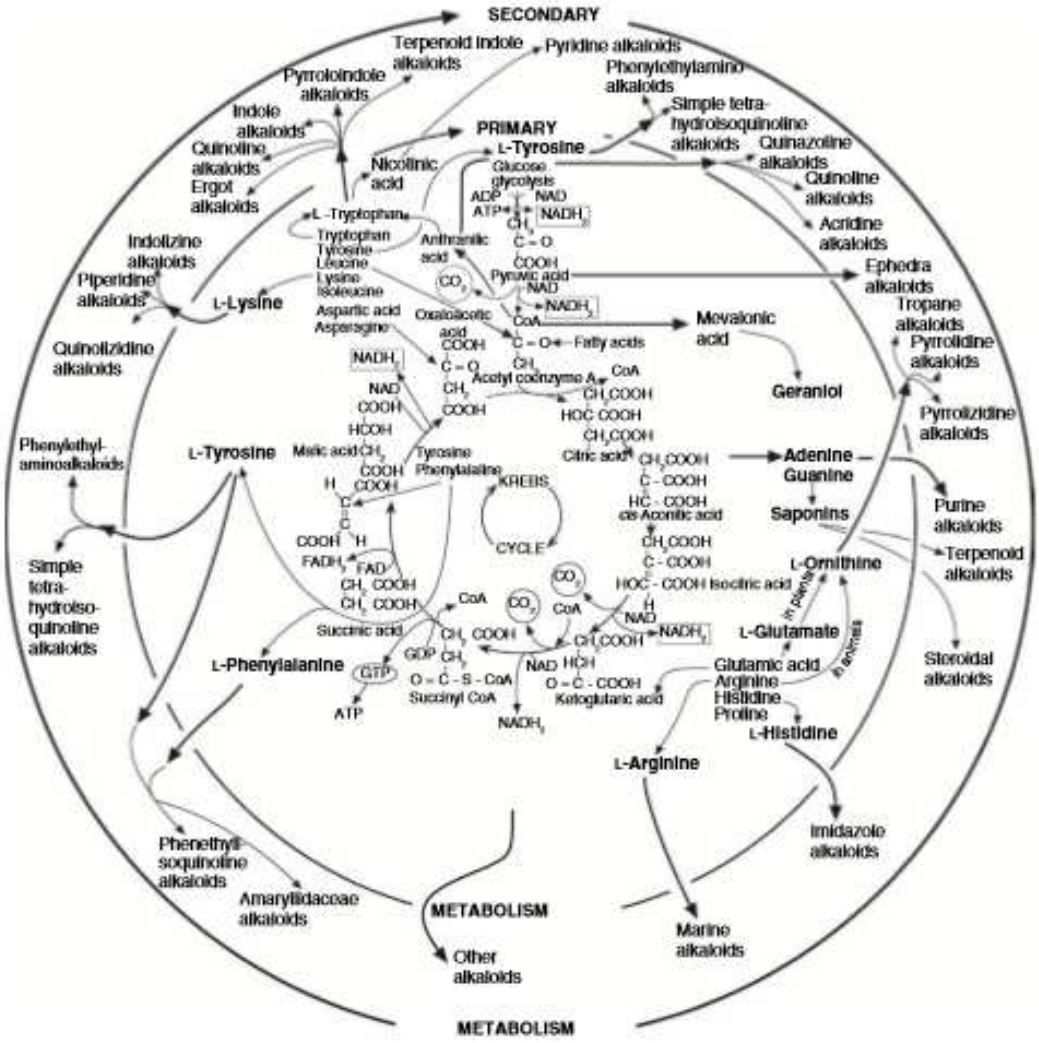


Figure 8. General scheme of alkaloid synthesis.

1.7 *Historical Application*

Alkaloidal applications can be found in different areas of the economy, industry, trade and services. The applicable characteristics of alkaloids are both chemical ones and the ability to be isolated as pure molecules or to be modified. The specific activity and utilization is a basis for the applications. Alkaloids have been used throughout history in folk medicine in different regions around the world. They have been a plants constituent part used in phytotherapy. Many of the plants containing alkaloids are just

medicinal plants and have been used as herbs. Since the days of Hippocrates (460–377 BCE), herbs were known in Europe as a very important way of improving health. In ancient China, herbs were known and used even since 770 BCE, and in Mesopotamia approximately since 2000 BCE. In particular in Mesopotamia plants such as Papaver somniferum and Atropa belladonna have served to many purpose (especially religious), and the use of Datura metel, Cannabis sativa and the mushroom Amanita muscaria can be traced to ancient India. Moreover, plants containing alkaloids have been historically used for other purposes. Hunters, priests, medicine men, witches and magicians have all been known to use alkaloidal plants. Humans have used alkaloids as poisons in weapons.[30] The most poisonous alkaloids such as aconitine and tubocarine were used in ancient times as poisons for arrows. Especially in Africa, these weapons have been used in tribal warfare, where the poisons (alkaloids) were generally prepared from plants but also from animal sources as toads, snakes and frogs. [31,32] Poisoned arrows have also been used in Asia, especially in the large region including Indonesia, Burma, Thailand and Cambodia. Three methods were used in preparing poisons.[32, 33] The first involved boiling arrows in water with a ground up plant. The second method used pounded fresh ingredients with glutinous sap added (especially in the case of oil-rich plants). The third method involved applying freshly squeezed plant material onto wooden-tipped arrows. Literature also refers to the fact that different alkaloid groups have been used as arrow poisons in different parts of the world. People in Africa and

[30] Mann, J. Murder, *Magic and Medicine*. **1992** London: Oxford University Press
[31] Neuwinger, H. D. *African Ethnobotany: Poisons and Drugs, Chemistry, Pharmacology, Toxicology*. **1996,** London: Chapman and Hall
[32] Bisset, N. G. Arrow and dart poisons. *Journal of Ethnopharmacology*, **1989** 25: 1–41
[33] Neuwinger, H. D. Alkaloids in arrow poisons. In: *Alkaloids. Biochemistry, Ecology, and Medicinal Applications* (Roberts, M. F. and Wink, M., eds), **1998** pp. 45–84. New York – London: Academic Press.

Asia predominantly used cardiac poisons, while South Americans almost exclusively preferred muscle-paralyzing (curarizing) poisons.[34] Alkaloids and especially plants containing alkaloids were also used in the Middle Ages as a basic and practical human and animal cure for various ailments. Some cases of using alkaloids in executions are also known.[34, 35, 36, 37, 38] Some alkaloids that have played an important role in this sense include aconitine, atropine, colchicine, coniine, ephedrine, ergotamine, mescaline, morphine, strychnine, psilocin and psilocybin. Although alkaloids have been used throughout history, their isolation from plants as relatively pure compounds occurred only in the beginning of the 1800s, and their exact molecule structures were not determined until the 1900s.

1.8 *Modern Application*

Some alkaloids are still used in medicine today.[35, 39, 40, 41, 42] Alkaloids generally exert pharmacological activity particularly in mammals such as humans. Even today many of our most commonly used drugs are alkaloids from natural sources and new alkaloid drugs are still being developed for clinical use (e.g., taxol froma *Taxus baccata).* Most of these compounds with biological activity in humans affect the nervous system, particularly the action of the chemical trasmitters, e.g. acetylcholine, epinephrine, norepinephrine, γ-aminobutyric acid (GABA), dopamine, and serotonin.

[34] Bellamy, D. and Pfister, A. *Word Medicine. Plants, Patients and People*. **1992** Oxford: Blackwell
[35] Schultes, R. A. and Hofmann, A. *The Botany and Chemistry of Hallucinegens*. **1980** Thomas: Springfield.
[36] Bisset, N. G. Arrow and dart poisons. *Journal of Ethnopharmacology*, **1989** 25: 1–41
[37] Mann, J. Murder, *Magic and Medicine*. **1992** London: Oxford University Press
[38] Wink, M. *Alkaloids. Biochemistry, Ecology, and Medicinal Applications* (Roberts, M. F. and Wink, M., eds.), **1998** pp. 11–44. New York – London: Plenum Press.
[39] O'Neil, M. J., Badavari, S., Heckelman, P. E., Merck and Co., Smith, A., D'Arecca, M. A., Gallipeau, J. A. R. and Obenchain, J. R. *The Merck Index* Thirteenth Edition. **2001** New York: John Wiley & Sons
[40] Smeller, T. and Wink, M. *Alkaloids. Biochemistry, Ecology, and Medicinal Applications* (Roberts, M. F. and Wink, M., eds), **1998** pp. 435–459. New York – London: Academic Press
[41] Harborne, J. B. and Baxter, H. *Phytochemical Dictionary: A Handbook of Bioactive Compounds from Plants.* **1993** London: Taylor & Francis
[42] Reynolds, J. E. F. (Ed.. *Martindale – The Extra Pharmacopoeia*. **1993** London: Pharmaceutical Press

Many alkaloids serve as models for the chemical synthesis of analogues with better properties. Important exemples are hyoscyamine and scopolamine (*Atropa belladonna* and *Datura* species) as models for synthetic parasympatholytic agents[43]; physostigmine (*Physostigma venenosum*) for synthetic parasympathomimetic agents[44]; tubocurarine (*Chondodendron tomentosum*) for skeletal muscle relaxants; cocaine (*Erythroxylum coca*) for local anesthetic [45]; morphine (*P. somniferum*) for analgesics;[46] and codeine (*P. somniferum*) for antitussive agents. These molecules have many other pharmacological activities including antihypertensive effects (many indole alkaloids)[47], antiarrhythmic effects (quinidine, ajmaline, sparteine)[48], antimalarial activity (quinine)[49]and anticancer effects (dimeric indoles, vincristine, vinblastine)[50]. These are just a few examples illustrating the great economic importance of this group of plants constituents. Antibiotic activities are common for alkaloids and some are even used as antiseptics in medicine, e.g., berberine in ophthalmics and sanguinarine in toothpastes; however, it is difficult to know the extent to which alkaloids give antimicrobial protection in the plant.[51]

[43] Hofmann, Albert; Schultes, Richard Evans, *Plants of the Gods: Origins of Hallucinogenic Use*, New York, Van der Marck Editions, **1987** pp. 88
[44] Roberto Michele Suozzi, *Le piante medicinali* Newton&Compton, **1994**, pag.35
[45] Francesco Capasso, R. De Pasquale e G. Grandolini, *Farmacognosia: Farmaci Naturali, Loro Preparazioni Ed Impiego Terapeutico,* **2000**, Springer Science & Business Media
[46] Beard Jr, Edward L. The American Society of Health System Pharmacists. *JONA'S healthcare law, ethics and regulation*, **2001**, 3.3: 78-79.
[47] Horie S, Yano S, Aimi N, Sakai S, Watanabe K. *Life Sci.* **1992**;50(7):491-8
[48] Sneader, Walter, *Drug Discovery: A History*. **2005**. John Wiley and Sons. p. 95
[49] Dorndorp A, Nosten F, Stepniewska K, et al. *Lancet*. **2005** 366 (9487): 717–25
[50] Takimoto, C. H.; Calvo, E. "Chapter 3: Principles of Oncologic Pharmacotherapy". **2008** In Pazdur, R.; Wagman, L. D.; Camphausen, K. A.; Hoskins, W. J. *Cancer Management: A Multidisciplinary Approach* (11th ed.)
[51] Margaret F. Roberts and Michael Wink – *Alkaloids: biochemistry, ecology, and medicinal apllications* **1998** Springer Science+Business Media New York

Chapter II

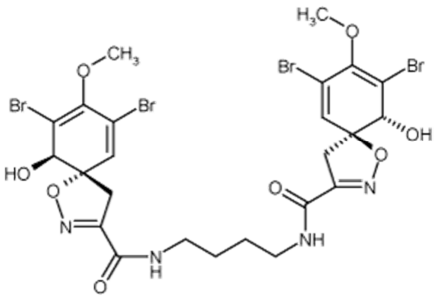
2 Marine alkaloids

Marine natural products chemistry is a dynamic field of research which had explosive growth in the last decades and is continuing to evolve. However, the biological and ecological functions of marine secondary metabolites are still poorly understood. Being the result of long evolutionary processes of biosynthetic pathway refinement, secondary metabolites are considered as products of natural selection and their diversity has been tentatively used in chemotaxonomy, complementary to morphological characters and/or genetic markers. Therefore, an increasing number of integrative taxonomical works on Porifera now successfully consider biochemical datasets in parallel to molecular or morphological ones.[52]

For a long time the man felt that the plants were the only medicinal resources at its disposal but the knowledge thirst to other natural substances that could improve their quality of life prompted him to look elsewhere. His interest, therefore, was addressed to the sea, a truly hidden world. Almost with an area twice that land, the sea is home to most of the world's flora and fauna. In the deep blue depths of our planet, nature seems to have played with shapes and colors to impress every time the men. Only since the end of the years ' 60, thanks to the development and dissemination of technologies needed for the discovering of the

[52] N. Cachet, G. Genta-Jouve, J. Ivanisevic, P. Chevaldonné, F. Sinniger, G. Culioli, T. Pérez, O. P. Thomas, *Sci. Rep*. **2015**, 5, 8282

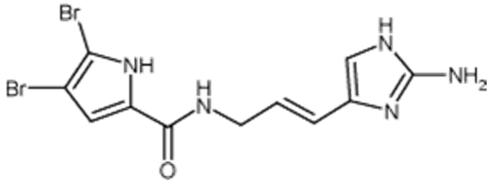
marine environment, the chemical study of marine flora and fauna has become systematic. A very small part of the organisms that inhabit our seas is represented by fish, shells, corals and porifera. In recent years the attention of researchers focused mainly on Porifera, commonly known as sponges. From these organisms discrete amounts of bioactive metabolites were isolated and this was made possible thanks to the development of advanced techniques of purification and structural characterization. The particular molecular architectures, most unusual and more complex than those identified in terrestrial organisms, so as to speak of a separate "chemistry of the sea", were thus put in highlights. Some examples of secondary metabolites, particularly alkaloids, isolated from sponges, were shown (*Figures 9-11*).



AEROTHIONINE [53]

from Aplysina aerophoba sponge (1970)

Figure 9. First marine alkaloid discovered

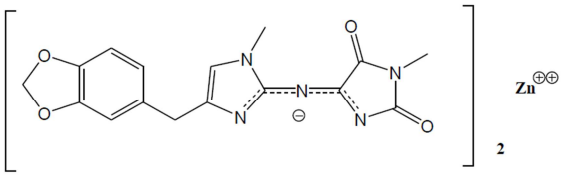


OROIDINS [54]

From Agelas oroides sponge (1971)

Figura 10. First bromoalkaloid discovered

[53] E. Fattorusso, L. Minale,G. Sodano, K. Moody, R.H. Thomson, *J. Chem. Soc. Chem.Comm.* **1970**, 752.
[54] S. Forenza, L. Minale, R. Riccio, E. Fattoruso, *J. Chem. Soc. Chem. Comm.* **1971**, *1129.*



CLATHRIDINE [55, 56]

from Clathrina clathrus sponge (1990)

Figure 11. First marine alkaloid complexed with zinc

However still limited known, the chemical diversity of the sea appears to be immense and this is partly due to the fact that, in addition to vegetable organisms, in the oceans huge multitude and variety of animal organisms live fixed to the seabed or in any case with a very low mobility. The coexistence of such a large number of species that interact with each other and with the environment, each in a different way, has led to the development of life forms capable of accumulating and / or producing a wide variety of chemically different compounds with an equally wide diversity of possible ecological roles.

These include:

A) Toxins, which can reduce predation, the larval settlement and overgrowth of neighboring organisms.

B) Compounds capable of reducing the palatability and / or the absorption of nutrients in the predators.

C) Compounds for direct larval settlement and reproduction.

[55] P. Ciminiello, E. F attorusso, A. Mangoni, B. DiBlasio, V. Pavone, *Tetrahedron Lett.* ***1990*** *,46, 4387*

[56] P. Ciminiello, E. Fattorusso, S. Magno, A. Mangoni, *Tetrahedron Lett.* ***1989*** *45,3873*

Status	Compound name	Trademark	Marine organism	Chemical class	Disease area
Approved	Cytarabine, Ara-C Vidarabine, Ara-A Ziconotide Trabectedin (ET-743) (EU Registered only)	Cytosar-U® Vira- A® Prialt® Yondelis®	Sponge Sponge Conesnail Tunicate	Nucleoside Nucleoside Peptide Alkaloid	Cancer Antiviral Pain Cancer
Phase III	EribulinMesylate(E7389) Soblidotin (TZT 1027)	NA NA	Sponge Bacterium	Macrolide Peptide	Cancer Cancer
Phase II	DMXBA (GTS-21) Plinabulin (NPI-2358) Plitidepsin Elisidepsin PM1004 Tasidotin (ILX-651) Pseudopterosins	NA NA Aplidin® Irvalec® Zalypsis® NA NA	Worm Fungus Tunicate Mollusc Nudibranch Bacterium Soft coral	Alkaloid Diketopiperazine Depsipeptide Depsipeptide Alkaloid Peptide Diterpene glycoside	Cognition Schizophrenia Cancer Cancer Cancer Cancer Cancer Wound healing
Phase I	Bryostatin 1 Hemiasterlin (E7974) Marizomib(Salinosporamide A; NPI-0052)	NA NA NA	Bryozoa Sponge Bacterium	Polyketide Tripeptide Beta-lactone-gamma lactam	Cancer Cancer Cancer

***Table 2*.** The odyssey of marine pharmaceuticals a current perspectiv [57]

In *Table 2* we can see the many marine-derived drugs already approved for clinical use and under approval. Focusing our attention to the alkaloids, we find, in Phase II, DMXBA, potential drug for the treatment of schizophrenia, currently being approved for Phase III and among those already approved for clinical use, the trabectedin (*Figure 12*), alkaloid extracted from tunicate Yondelis [58] used for the treatment of ovarian cancer.

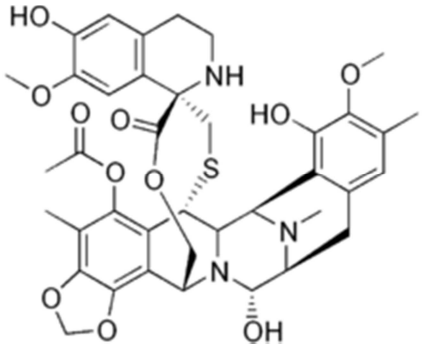


Figure 12. Trabectedin isolated by tunicate *Yondelis*

[57] Alejandro M.S. Mayer *et al*., TRENDS in *Pharmaceuical Sciences 31,* **2010**, 255-265
[58] D'Incalci, M., CM. Galmarini- *Mol Cancer Ther*- **2010**, 9(8), 2157-63

2.1 *Marine guanidine alkaloids*

The sponges are characterized by the presence inside of bioactive secondary metabolites pharmacologicaly interesting from different points of view, both structural and biosynthetic. Among these, the sponges belonging to the family Microcionidae and being part of the order Poecilosclerida, are particularly known as they contain polycyclic guanidine alkaloids used in therapy.[59,60,61] Three main families of this type of alkaloids is representative of the pentacyclic alkaloids Poecilosclerida order: crambescidine ureas as, those antidepressants such as batzelladine, and those compounds such as mono-and/or crambine.

2.2 *Guanidine pentacyclic alkaloids*

The first metabolite of this family, isolated in 1989 from the Caribbean sponge *Batzella sp*. (identified as *Ptilocaulis spiculifer*) is the Ptilomycalina A [62] (*Figure 13*).

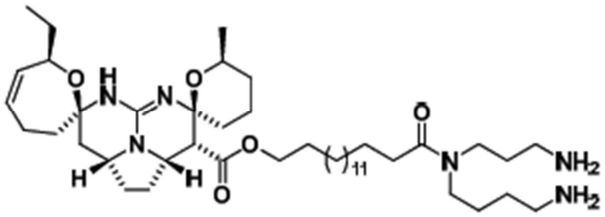


Figure 13. Ptilomycalina A isolated by caribbean sponge *Batzella* sp.

This compound is characterized by cytotoxic and antiviral activities.

[59] R.G.S. Berlinck, M.H. Kossuga*, Nat. Prod. Rep.,* **2005**, 22, 526-550
[60] R.G.S. Berlinck, *Nat. Prod. Rep.,* **2002**, 19, 617-649
[61] R.G.S. Berlinck, *Nat. Prod. Rep*., **1999**, 16, 339-365
[62] Y. Kashman, S. Hirsh, O.J. McConnell, T. Ohtani, H. Kusumi, H. Kakisawa, *J. Am. Chem. Soc.,* **1989**, 111, 8925-8926.

In 1991, Jares-Erijman et al.[63] first isolated the Crambescidine (*6-9 in Figure 14*) from a sponge (*Crambe crambe)* in the Mediterranean.

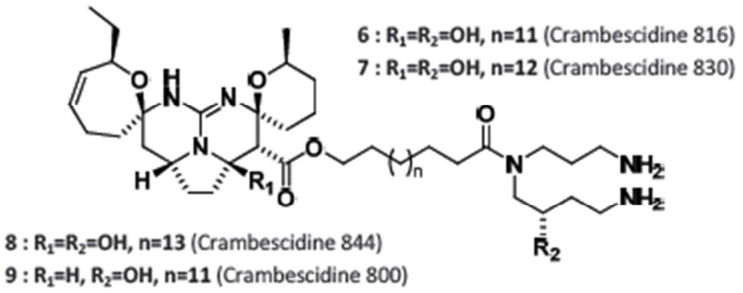


Figura 14*.* Crambescidine isolated from sponge *Crambe crambe*

Since then, different guanidine alkaloids have been isolated from sponges, the vast majority of which has pharmacological activity as antimicrobial, antiviral, antifungal, antiparasitic and/or chemotherapy.

2.3 *Guanidine tricyclic alkaloids*

There are two major subfamilies of this type of alkaloids which differ by the location of the tricyclic guanidine pattern.

I) The first subfamily consists of alkaloids that have the tricyclic guanidine moiety in the out position overall.

The ptilocaulina (10) and the isoptilocaulina (11), in *Figure 15*, isolated in 1981 by *Ptilaucaulis spiculifer* are the first representatives of this

[63] E.A. Jares-Erijman, R. Sakai, K.L. Rinehart, *J. Org. Chem.,* **1991**, 56, 5712-5715.

subfamily.[64]

To date, the final metabolite entered this subfamily is mirabilina G (*12 in Figure 15*), isolated in 2001 from the australian sponge Clathria.[65]

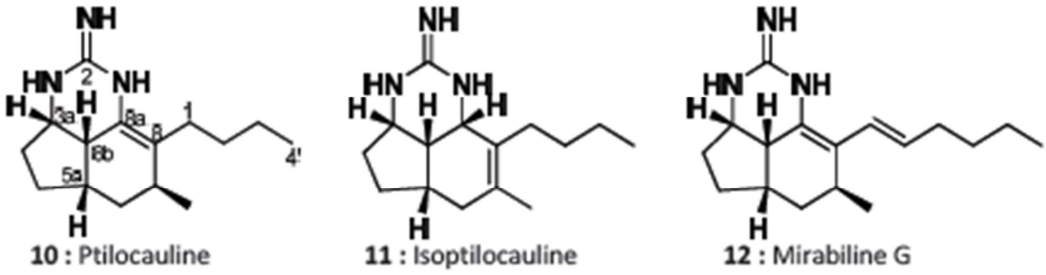


Figura 15. Ptilocauline (10) Isoptilocauline (11) Mirabiline G (12)

II) The second subfamily is composed of alkaloids which have the central tricyclic guanidine pattern.

The first belonging to this second subfamily are the Batzelladine A, D and F (*Figure 16*) discovered for the first time in 1995 by Batzella sponge in the Bahamas.[66]

[64] G.C. Harbour, A.A. Tymiak, K.L. Rinehart, P.D. Shaw R. Hughes, S.A. Mizsak, J.H. Coats, G.E. Zurenko L.H. Li, S.L. Kuentzel, *J. Am. Chem. Soc.,* **1981**, 103, 5604-5606.

[65] Capon, R.J. ; Miller, M. ; Rooney, F. *J. Nat. Prod., **2001**, 64, 643-644.*

[66] Patil, A.D. ; Kumar, N.V. ; Kokke, W.C. ; Bean, M.F. ; Freyer, A.J. ; Brosse, C.D. ; Mai, S. ; Truneh, A. ; Carte, B. *J. Org. Chem.,**1995**, 60, 1182-1188.*

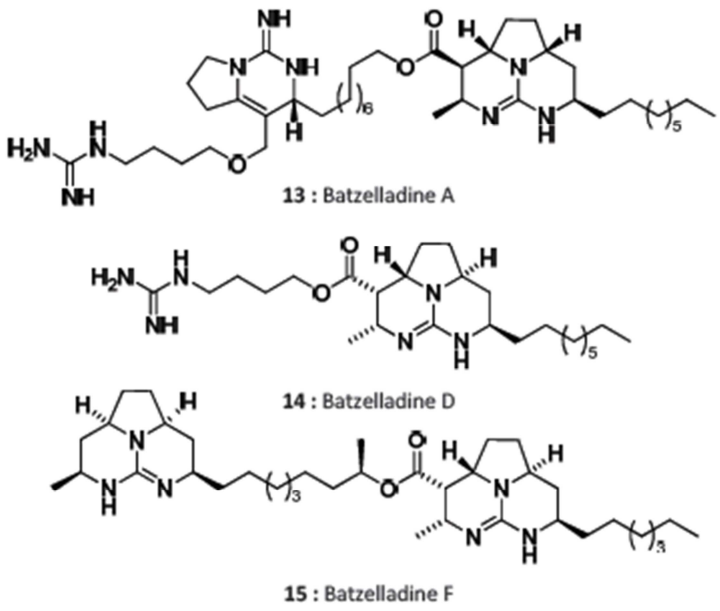


***Figure 16*.** Batzelladine A (13) Batzelladine B (14) Batzelladine F (15)

Compared to ptilocaulina (*10* in *Figure 14*), batzelladine (*13-15* in *Figure 16*) are characterized not only by the central location of the Guanidine in tricycle but batzelladine A (13) and F (15) have in addition a bicyclic or tricyclic Guanidine pattern tied with an alkyl chain on the first tricycle. The batzelladine have been tested as anti-HIV agents: a cheering activity was detected for batzelladina A (13), while moderate to batzelladina F (15) and no activity for batzelladina D (14); from this, the presence of the bicycle is indispensable for anti-HIV activity. The last metabolites related to this second subfamily have been recently isolated from a sponge of the genus Monanchora; the merobatzelladine A and B (*16-17 – Figure 17*) that exhibit antibacterial activity.[67]

[67] Takishima, S. ; Ishiyama, A. ; Iwatsuki, M. ; Otoguro, K. ; Yamada, H. ; Omura, S. ; Kobayashi, H. ; Van Soest, R.W.M. ;Matsunaga*, S. Org. Lett., **2009**, 11, 2655-2658*

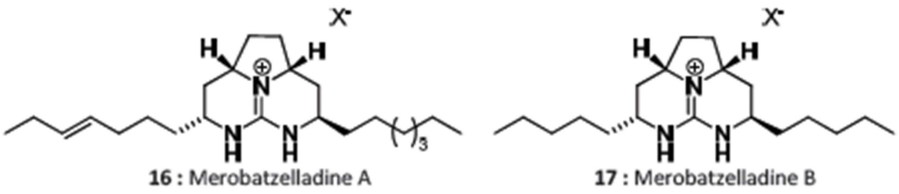


Figure 17. Merobatzelladine A and B isolated by sponge *Monanchora*

2.4 *Guanidine mono- and bi-cyclic alkaloids*

The first representatives of the family of mono- and bi-cyclic guanidine alkaloids are the Crambine A and B (*18-19 in Figure 18*), isolated from Mediterranean sponge crambe crambe in 1990.[68]

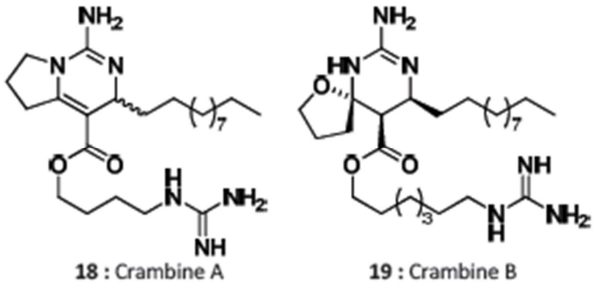


***Figure 18*.** Crambine A and B isolated by sponge *Crambe Crambe*

Jares-Erijman *et al*. and then Snider *et al.* later overhauled the structure of Crambine B (19) which has been renamed Crambescina B, and have also defined the stereochemistry as well as shown in *Figure 18*.[69,70] The

[68] Berlinck, R. G. S. ; Braekman, J. C. ; Daloze, D. ; Hallenga, K. ; Ottinger, R. ; Bruno, I. ; Riccio, R. *TetrahedronLett., **1990**, 31,6531-6534*

[69] Jares-Erijman, E. A. ; Ingrum, A. A. ; Sun, F. ; Rinehart, K. L. *J. Nat. Prod., **1993**, 56, 2186-2188*

crambine have no interesting biological activity.[71] After the study on the Crambine A (18) and on the difference of activity compared with the batzelladine A (13), with an extra bicycle, and the batzelladine D (14), it was concluded that the task is not only due to the presence of the bicycle but also to the synergy of two bi-and tri-cyclic reasons.

[70] Snider, B. B. ; Shi, Z *J. Org. Chem., **1992**, 57, 2526-2528*

[71] Patil, A.D. ; Kumar, N.V. ; Kokke, W.C. ; Bean, M.F. ; Freyer, A.J. ; Brosse, C.D. ; Mai, S. ; Truneh, A. ; Carte, B. *J. Org. Chem.,***1995**, 60, 1182-1188

Chapter III

3 Parazoanthus axinellae

Although sponges are the paramount source of marine bioactive metabolites, cnidarians, and especially anthozoans, display high biological and chemical diversity and as such they have been the focus of many promising researches on natural products.[72,73]

Figure 19. Sea Daisy *Parazoanthus axinellae* – photo L. Capurro.

[72] Behenna, D. C., Stockdill, J. L. & Stoltz, B. M. The Biology and Chemistry of the Zoanthamine Alkaloids. *Angew. Chem., Int. Ed.* **2008**, 47, 2365–2386.
[73] Rocha, J., Peixe, L., Gomes, N. C. M. & Calado, R. Cnidarians as a Source of New Marine Bioactive Compounds—An Overview of the Last Decade and Future Steps for Bioprospecting. *Mar. Drugs* 9, **2011,** 1860–1886.

Among them, relatively little is known about zoanthids (Cnidaria, Hexacorallia, Zoantharia) despite the fact that they are common in most shallow and deep marine environments. The phylum Cnidaria, containing more than 9,000 species, get a particularly interesting example.

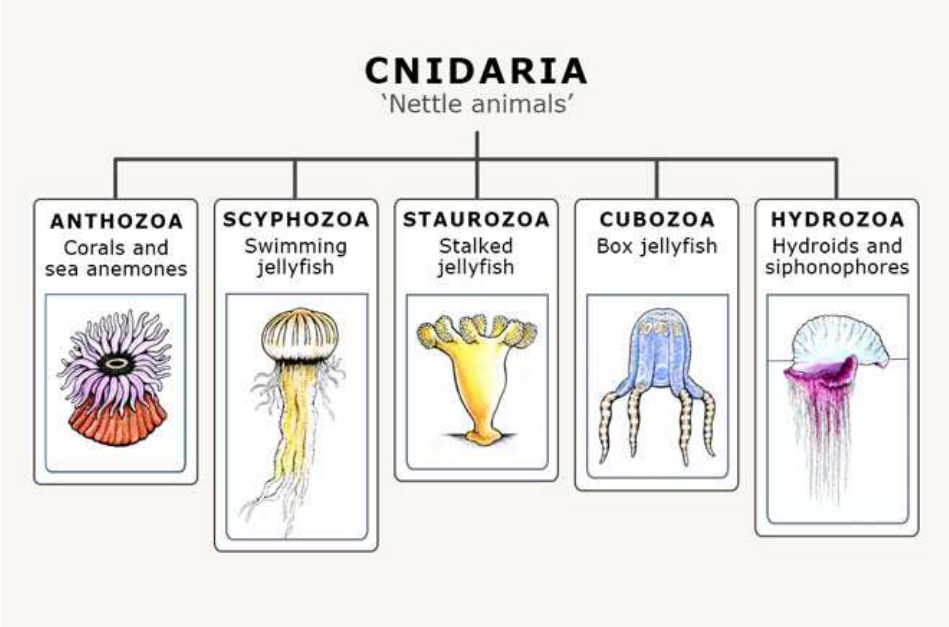


Figure 20. *Cnidaria* animals

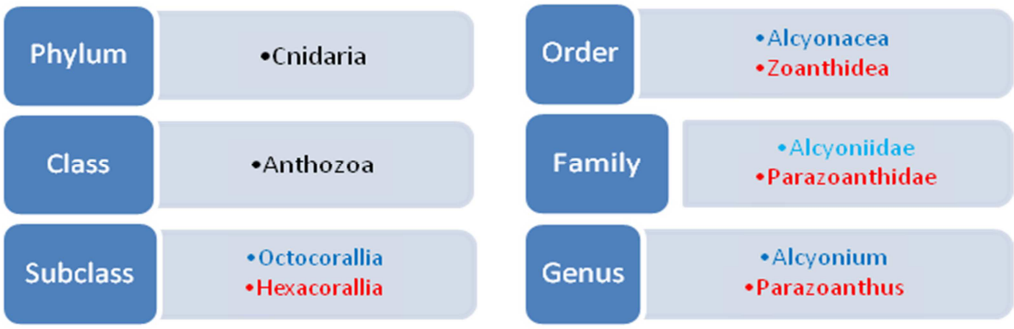
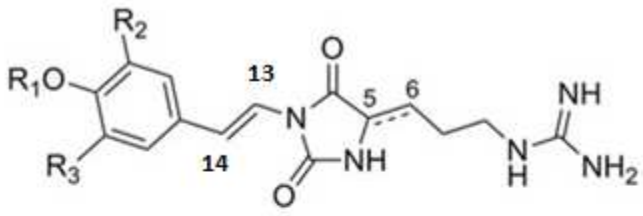


Figure 21. *Cnidaria* family

In particular, in the class of the Anthozoans, subclass Hexacorallia, there is a species of sea anemone, *Parazoanthus axinellae* (*Figure 19*), widespread in the Mediterranean and Eastern Atlantic Ocean; it is often associated with sponges of the genus *Axinella* or sea squirts like *Microcosmus*. *Parazoanthus axinellae* is a common organism in sublittoral rocky

communities, especially in habitats with low light irradiance, on shaded vertical cliffs, overhangs and at cave entrances. A new family of guanidine alkaloids was found from this anemone: parazoanthine A-J (*Figure 22*).[74,75]



	R_1	R_2	R_3	
Parazoanthine A	H	H	H	
Parazoanthine B	H	H	H	$\Delta^{5,6}$
Parazoanthine C	CH_3	H	H	$\Delta^{5,6}$
Parazoanthine D	CH_3	Br	H	
Parazoanthine E	CH_3	Br	H	$\Delta^{5,6}$
Parazoanthine F	CH_3	H	H	
Parazoanthine I	CH_3	Br	Br	
Parazoanthine J	CH_3	Br	Br	$\Delta^{5,6}$

Parazoanthines

Figure 22*.* Parazoanthine A-J isolated from *Parazoanthus axinellae* sea anemone

The secondary metabolome of *P. axinellae* was first studied in the 1970s with the isolation and structure elucidation of polyaromatic alkaloids named zoanthoxanthins and parazoanthoxanthins[76,77,78] Recently, a second original family of alkaloids, named parazoanthines,

[74] Nadja Cachet, Gregory Genta-Jouve, Erik L. Regalado, RedouaneMokrini, Philippe Amade, Gerald Culioli, and Olivier P. Thomas. *J.Nat.Prod.* ***2009****, 72, 1612-1615.*

[75] Audoin, C. *et al.* Metabolome Consistency: Additional Parazoanthines from the Mediterranean Zoanthid Parazoanthus Axinellae. *Metabolites* **4**, 421–432 (2014)

[76] Cariello, L., Crescenzi, S., Prota, G., Giordano, F. & Mazzarella, L. *J. Chem. Soc., Chem. Commun.*, **1973**, 99–100.

[77] Cariello, L. *et al. Tetrahedron,* **1974,** 30, 3281–3287.

[78] Cariello, L., Crescenzi, S., Prota, G. & Zanetti, L. *Experientia,* **1974,** 30, 849–850.

was recovered from the same species.[79] This new family of compounds was found to be very interesting as a biosynthetic standpoint and is the first example of natural products in which the hydantoinic core is disubstituted in N-3 and C-5; in fact the hydantoins known natural nowadays are not replaced in N-3 and in rare cases there is only one methyl substitution. [72]

From the point of biosynthetic view the hydantoins are considered the connection key for the formation of peptides derived from purine.[80]

The key reaction, in the first hypothesis proposed for the biosynthetic scheme (*Figure 23*), is the mono carbonylation taking place after the condensation of two aminoacids; in the second proposed hypothesis we have the contraction of a diketopiperazinic ring obtained from double condensing of two amminoacids.

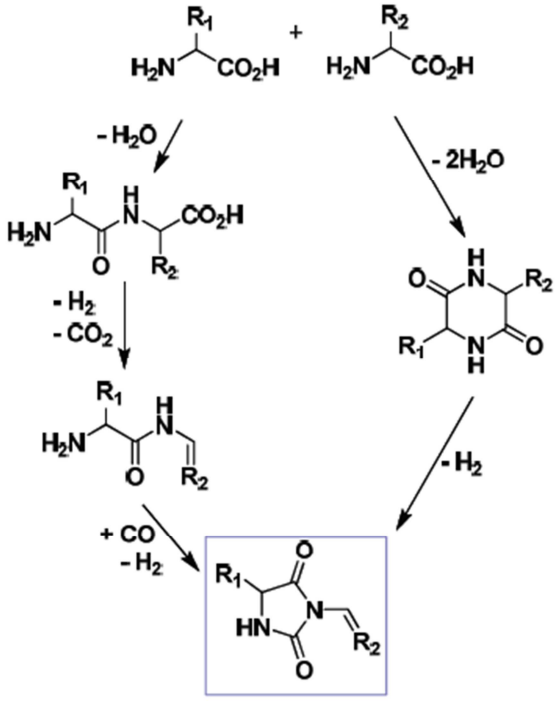


Figura 23. Biosynthetic scheme of hydantoinic ring

[79] Cachet, N. *et al. J. Nat. Prod.* **2009**, 72, 1612–1615.
[80] Huber, C. ; Eisenreich, W. ; Hecht, S. ; Waechtershaeuser, G. *Science*, **2003***,* 301, 938–940.

3.1 *In silico screening of parazoanthines as potential CXCR4 ligands*

In a previous work[81], a minimalist pharmacophoric model was developed for CXCR4 ligands that led to the identification of phidianidine A (*Figure 24*) , an alkaloid compound from marine source, as CXCR4 inhibitor endowed with low micromolar activity.

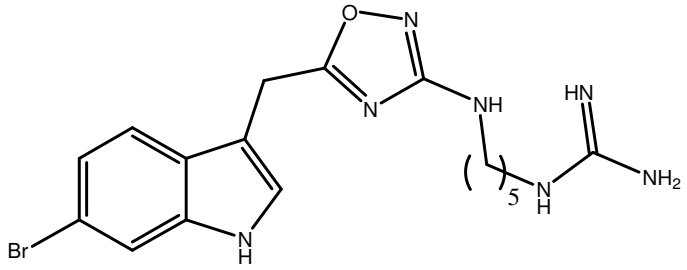


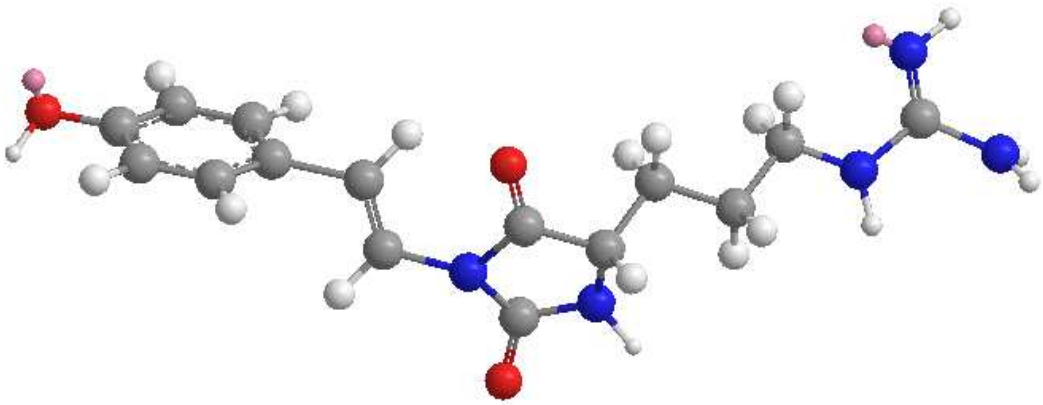
Figure 24. Phidianidine A

In this pharmacophoric model, the essential anchoring points for this receptor consist in a single pair of properly spaced aromatic and guanidinic functional groups, with the an upper limit of 18 Å for the distance between the two centers of mass. The search for new naturally-occurring molecules featuring the aforementioned requisites in our ICB collection gave parazoanthines (PARA) compounds, hydanthoin alkaloids from Mediterranean Sea Anemone Parazoanthus axinellae, as potential new CXCR4 ligands. The compounds within this family, named A-C, differ each other for the presence of an hydroxyl (A-B) or methoxy (C) group on the phenyl ring, and for the number of double bonds in the structure: two (Δ5,6 and Δ13,14) for B-C and only one (Δ13,14) for A. While sharing a

[81] Vitale RM, Gatti M, Carbone M, Barbieri F, Felicità V, Gavagnin M, Florio T, Amodeo P. Minimalist hybrid ligand/receptor-based pharmacophore model for CXCR4 applied to a small-library of marine natural products led to the identification of phidianidine a as a new CXCR4 ligand exhibiting antagonist activity. ACS *Chem Biol*. **2013** Dec 20;8(12):2762-70.

common guanidinium group, they differ from phidianidine in both the aromatic portion (phenyl vs indole ring) and in the length and nature of spacer, since they bring an hydanthoin group in place of an oxadiazole ring and double in place of single bond(s) in the carbon chain, as well as in the distance between the two anchoring groups (~14Å), smaller than that found in phidianidine. Since, in spite of the agreement with the (rather loose) pharmacophoric model, these substantial differences make the activity of the new compounds on CXCR4 not completely anticipatable, they underwent a cycle of computational and experimental validation.

Docking calculations (done by Dr. Piero Amodeo and Dr. Rosamaria Vitale of ICB) of PARA A-C into CXCR4 structure (PDB entry 3OE0) give as best poses for each compound a similar arrangement in the binding site: all compounds form bidentate H-bonds reinforced by ionic interactions with Asp97 and Asp187, H-bonds with the hydanthoin group and Asp187 and the NH backbone atom of Arg188, whereas the aromatic ring is sandwiched between Arg188 and Tyr116, with the hydroxyl group engaging an H-bond with Thr117. This last H-bond does not occur in PARA-C, due to the presence of the methoxy group, since Thr117 acts as H-bond acceptor in PARA-A and PARA-B ligands, being its polar hydrogen in turn involved in H-bond with backbone CO of His113. To best explore the potential role of both such H-bond, and the nature of the aromatic group, two non natural derivatives were also considered: one bearing a phenyl group the 18-deoxy-parazoanthine (**5**) and another bearing a naphthalene group (PARA-N). All energy minimized complexes are reported in *Figure 25A-D.*



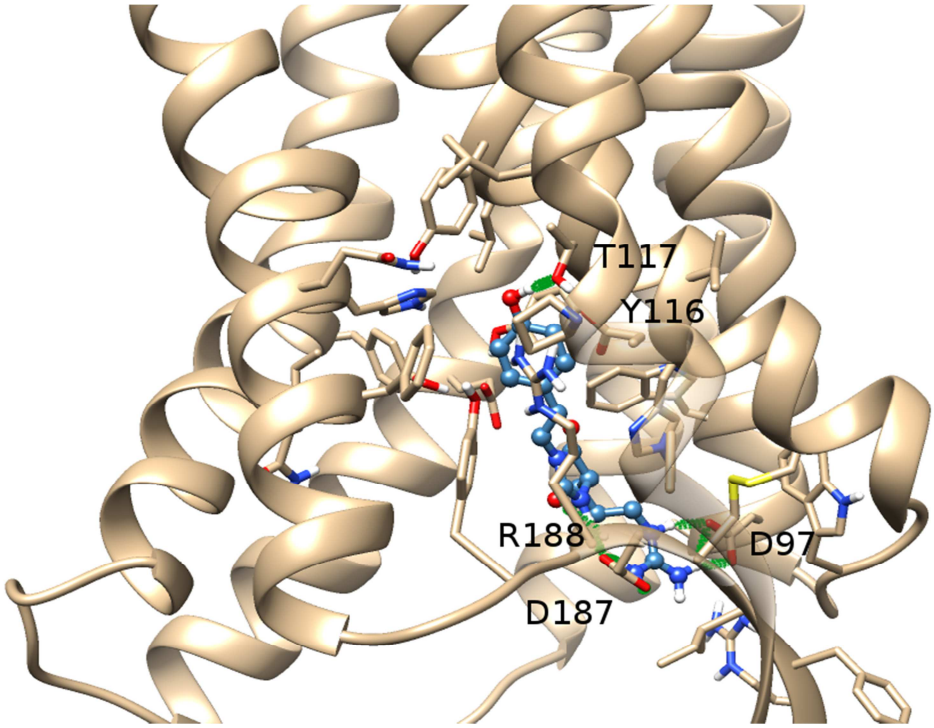
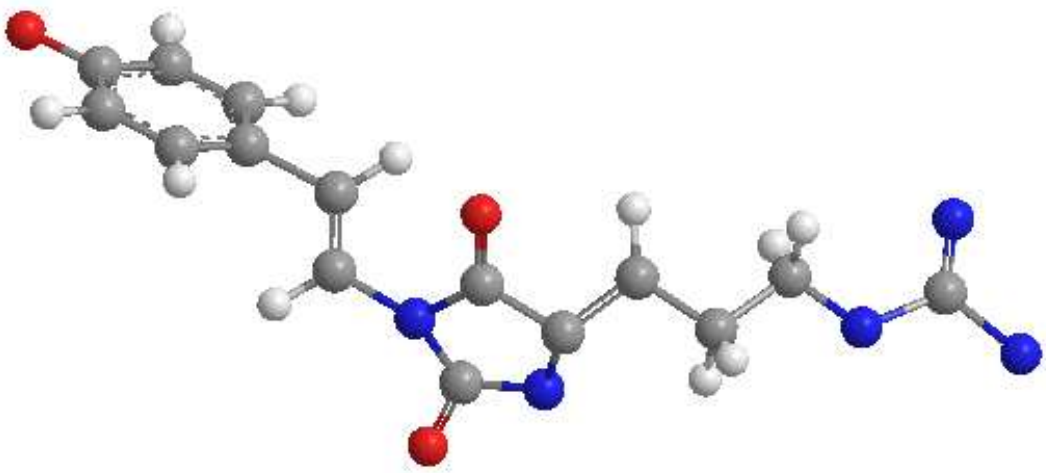


Figure 25A. Parazoanthine A



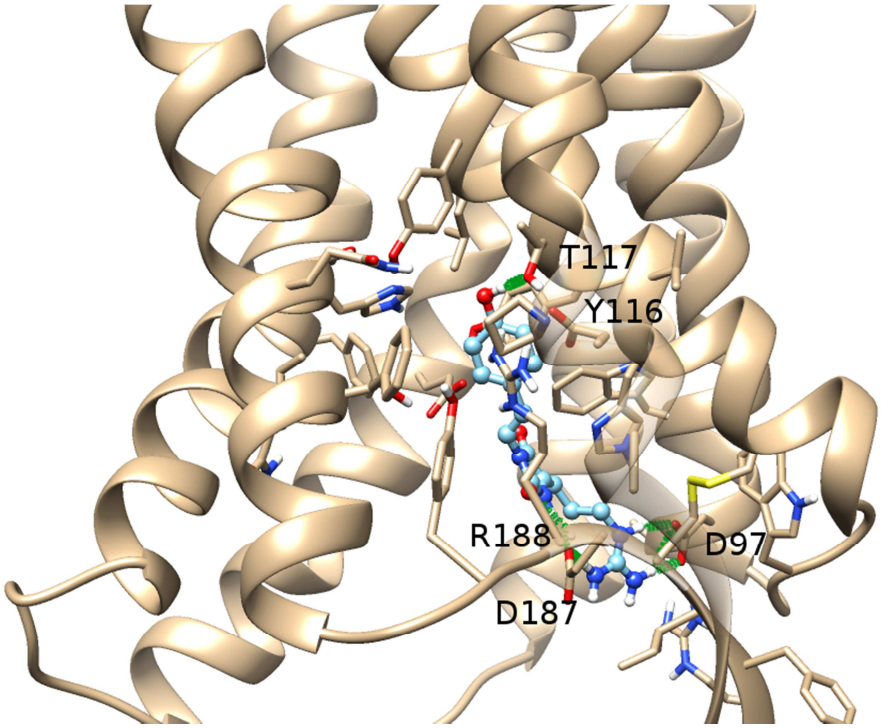
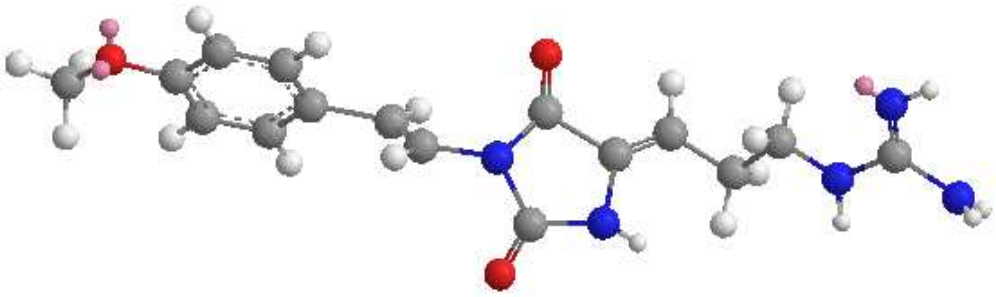


Figure 25B. Parazoanthine B



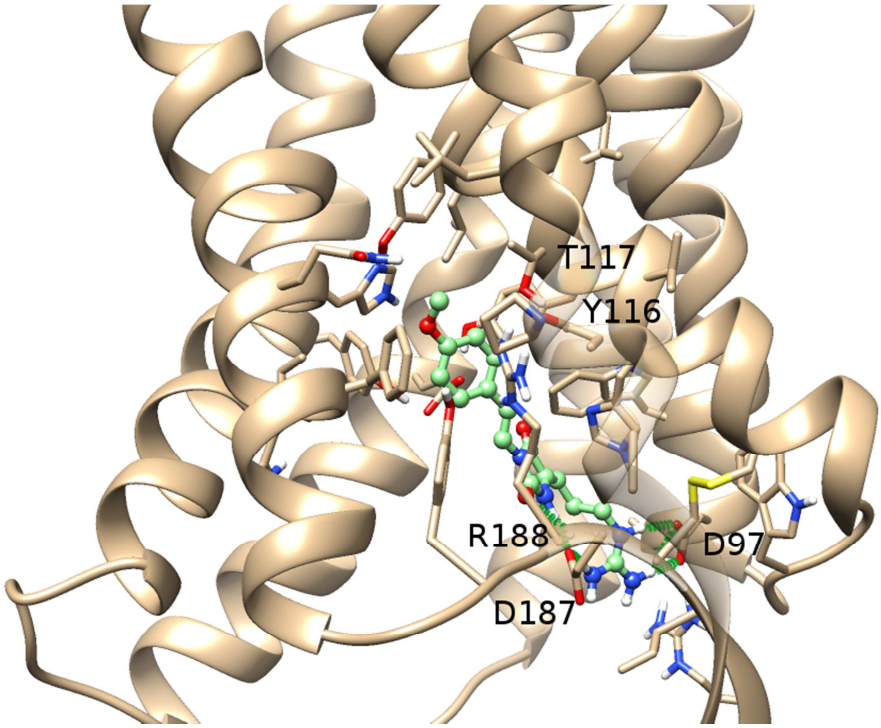
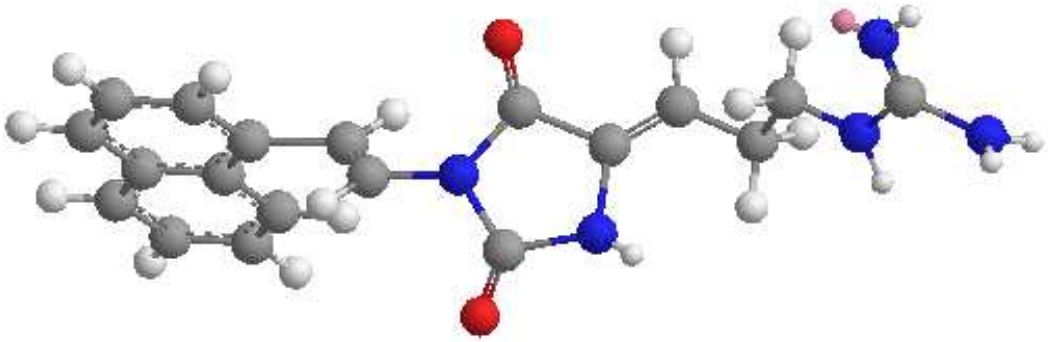


Figure 25C. Parazoanthine C



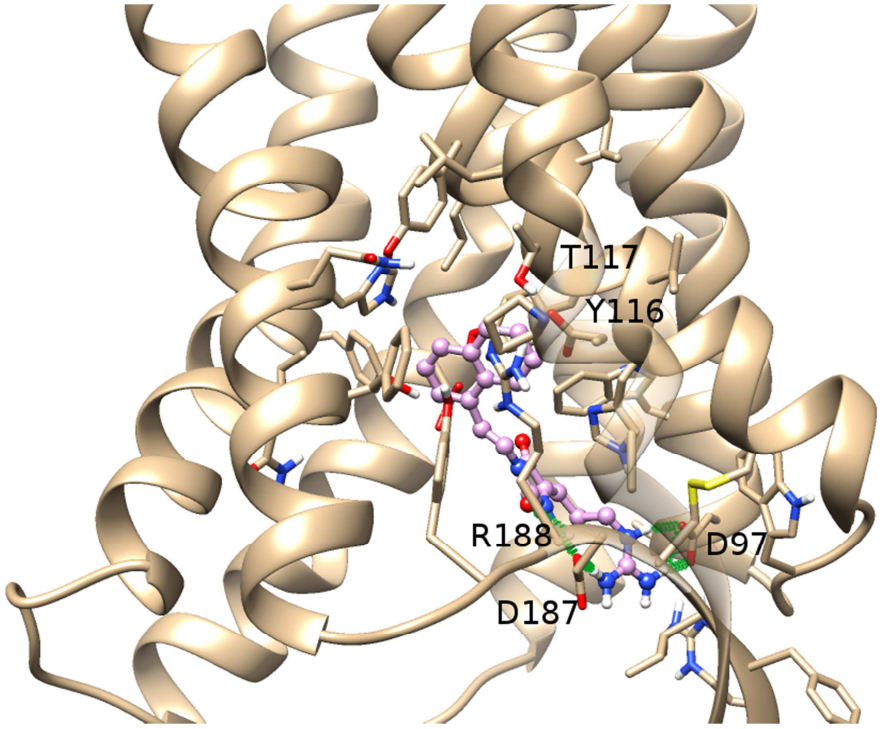


Figure 25D. Parazoanthine N

Figures 25A-D. CXCR4 complexes with PARA-A, -B, -C, -N ligands are shown adopting a partially-transparent tan ribbon representation for protein backbone, sticks for protein side chains within 5 Å from the ligand and ball and sticks for ligand. Only polar hydrogen atoms are shown. Atoms are colored according to the following scheme: O=red, N=blue, H=white, S=yellow. PARA-A, -B, -C, -N carbon atoms are colored in steel blue, sky blue, green and plum, respectively. Ligand-protein H-bonds are depicted with a green spring. All figures are plotted with Chimera program.

3.2 *CXCR4 receptor*

CXC chemokine ligand (CXCL)12 (also known as stromal cell-derived factor [SDF]-1 or pre-B-cell-growth-stimulating factor [PBSF]) is a member of a large family of structurally related chemoattractive cytokines and was first characterized as a growth-stimulating factor for the B cell precursor clone.[82] The primary physiologic receptor for CXCL12 is CXCR4, a hepta helical receptor coupled to heterotrimeric guanosine triphosphate (GTP) binding proteins, which also functions as an entry receptor for the HIV-1 virus. [83,84,85]

Studies of mutant mice with targeted gene disruption have revealed that CXCL12-CXCR4 signaling is essential for hematopoiesis, including B cell development and colonization of bone marrow by hematopoietic progenitors, including HSCs (Hematopoietic stem cells), during ontogeny as well as cardiovascular formation and neurogenesis. [82,83,84,86,87]

Lethality caused by deficiencies of CXCL12 and CXCR4 prevents immediate analysis of their role in adult hematopoiesis. Treatment with CXCR4-selective antagonist induces increase in HSCs in the peripheral blood, suggesting a role for CXCL12 in retaining HSCs in hematopoietic organs.[88]

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[82] T. Nagasawa, H. Kikutani, T. Kishimoto *Proc. Natl. Acad. Sci*. USA, 91, **1994**, pp. 2305–2309

[83] T. Nagasawa, S. Hirota, K. Tachibana, N. Takakura, S. Nishikawa, Y. Kitamura, N. Yoshida, H. Kikutani, T. Kishimoto *Nature*, **1996,** 382, pp. 635–638

[84] K. Tachibana, S. Hirota, H. Iizasa, H. Yoshida, K. Kawabata, Y. Kataoka, Y. Kitamura, K. Matsushima, N. Yoshida, S. Nishikawa, *et al. Nature*, **1998,** 393, pp. 591–594

[85] Y.R. Zou, A.H. Kottmann, M. Kuroda, I. Taniuchi, D.R. Littman, *Nature*, **1998**, 393, pp. 595–599

[86] T. Ara, K. Tokoyoda, T. Sugiyama, T. Egawa, K. Kawabata, T. Nagasawa *Immunity*, **2003,** 19, pp. 257–267

[87] T. Nagasawa Nat. Rev. *Immunol*., **2006,** 6, pp. 107–116

[88] H.E. Broxmeyer, C.M. Orschell, D.W. Clapp, G. Hangoc, S. Cooper, P.A. Plett, W.C. Liles, X. Li, B. Graham-Evans, T.B. Campbell, *et al. J. Exp. Med*., **2005,** 201, pp. 1307–1318

CXCL12-CXCR4 signaling is essential in adult bone marrow to maintain the HSC pool and suggest that many HSCs are in contact with a small population of reticular cells expressing high amounts of CXCL12.[89]

In addition, almost all HSCs near the sinusoidal endothelium appear to be in contact with these reticular cells surrounding endothelial cells in the extravascular spaces, suggesting that these cells are the key cellular components of HSC vascular niches.[90]

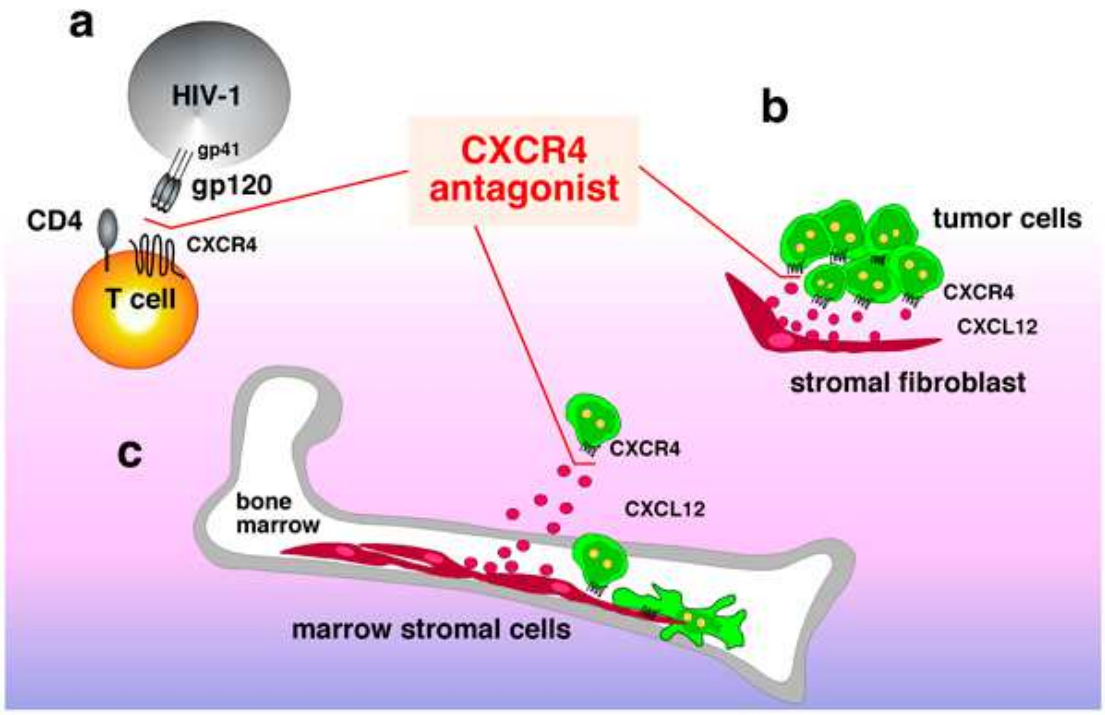

Figure 26. CXCR4 antagonists in human immunodeficiency (HIV-1) and cancer.

CXCR4 is the co-receptor used along with CD4 by T cell-tropic (X4) HIV-1 strains for cellular entry into T cells. A trimeric unit of viral envelope glycoproteins (gp120) that are anchored by gp41 binds CD4 on the surface

[89] K. Tokoyoda, T. Egawa, T. Sugiyama, B.I. Choi, T. Nagasawa *Immunity*, **2004,** 20, pp. 707–718
[90] T Sugiyama, H Kohara, M Noda, T Nagasawa - *Immunity*, **2006,** 25, pages 977-988

of T cells, inducing a conformational change of gp120, allowing it to interact with CXCR4 through the V3 loop of gp120. CXCR4 antagonists block the CXCR4-binding site for X4 HIV-1, and thereby prevent fusion of HIV-1 with T cells.

Stromal fibroblasts within the tumor microenvironment secrete CXCL12 and thereby attract and retain tumor cells in contact with the stroma. Adhesion of tumor cells to stromal cells confers survival, growth and drug resistance signals (cell adhesion-mediated drug resistance (CAM-DR)) that are, at least in part, mediated by activation of CXCR4 on the tumor cells. Stromal cell-mediated activation of CXCR4 is also called a 'paracrine' activation of tumor cells through CXCL12.[91] CXCR4 antagonists can disrupt the adhesive interactions between tumor cells and tumoral fibroblasts, mobilizing them from the tumor microenvironment, and making the tumor cells more accessible to cytotoxic drugs.

Tumor cells (hematopoietic and non-hematopoietic) also utilize the CXCR4-CXCL12 axis to migrate and home to target organs, such as the marrow. CXCL12 is constitutively secreted by marrow stromal cells retains leukemia cells in protective marrow niches and attracts circulating tumor cells for directional homing/metastasis. CXCR4 antagonists can inhibit this mechanism of tumor cell homing by blocking CXCR4 receptors responsible for migration to CXCL12-secreting stromal cells, thereby mobilizing tumor cells from tissue sites, such as the marrow.[92]

[91] Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R et al. *Cell* **2005**; 121: 335–348.

[92] J A Burger, A Peled *Leukemia*, **2009,** 23**,** 43–52

In summary, the rationale for targeting CXCR4 with CXCR4 antagonists in leukemia and other cancers is as follows:

1. disrupting the adhesive stromal interactions that confer survival and drug resistance signals to leukemia and other cancer cells;
2. mobilizing tumor cells from tissue sites, such as the marrow, and thereby making them better accessible to conventional therapy;
3. blocking of migration and dissemination of tumor cells in the process of tumor cell metastasis;
4. blocking of paracrine growth and survival signals through activation of the CXCR4-CXCL12 axis and
5. blocking pro-angiogenesis effects of CXCL12.

3.3 *CXCR4 antagonists*

CXCR4 antagonists were initially developed as new drugs for the treatment of HIV-1 infection. At the time of their discovery in the early 1990s, the mechanism of anti-HIV activity of the most prominent CXCR4 antagonists, T140 and its analogs,[93, 94] AMD3100 [95, 96] and ALX-4C,65 was unknown. After the discovery of the co-receptor function of CXCR4 for T tropic HIV-1, the specific CXCR4-blocking function of the different CXCR4 antagonists was rapidly demonstrated.[97, 98]

[93] Nakashima H, Masuda M, Murakami T, Koyanagi Y, Matsumoto A, Fujii N et al. *Antimicrob Agents Chemother* **1992**; 36: 1249–1255.
[94] Masuda M, Nakashima H, Ueda T, Naba H, Ikoma R, Otaka A *et al. Biochem Biophys Res Commun* **1992**; 189: 845–850.
[95] De Clercq E, Yamamoto N, Pauwels R, Balzarini J, Witvrouw M, De Vreese K *et al Antimicrob Agents Chemother* **1994**; 38: 668–674
[96] De Clercq E, Yamamoto N, Pauwels R, Baba M, Schols D, Nakashima H *et al. Proc Natl Acad Sci USA* **1992**; 89: 5286–5290
[97] Doranz BJ, Grovit-Ferbas K, Sharron MP, Mao SH, Goetz MB, Daar ES et al.. *J Exp Med* **1997**; 186: 1395–1400
[98] Murakami T, Nakajima T, Koyanagi Y, Tachibana K, Fujii N, Tamamura H et al. A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. *J Exp Med* **1997**; 186: 1389–1393.

Cancer type	*In vitro studies*	*In vivo studies*
Solid tumors		
Breast cancer	AMD3100: blocks CXCL12-induced HER2-neu activation	T140: reduced metastasis in murine model; AMD3100: prolongs survival in murine model
Small cell lung cancer (SCLC)	T140 and its analogs block adhesion and survival pathways	
Pancreatic cancer	AMD3100 inhibits tumor cell migration and growth	
Cholangiocarcinoma	AMD3100 inhibits tumor cell migration	
Gastric cancer		AMD3100 reduced tumor growth in a murine model
Colorectal cancer	AMD3100 inhibits tumor cell growth	
Malignant melanoma	AMD3100 inhibits tumor cell activation and proliferation	T140 analog inhibits metastatic melanoma, T22 increases efficacy of immunotherapy in metastatic melanoma
Glioma	AMD3100 inhibits tumor cell invasion	
Other CNS tumors		AMD3100 inhibits glioblastoma and medulloblastoma growth in xenograft model
Ovarian cancer	AMD3100 inhibits cancer cell migration and activation	
Rhabdomyosarcoma	T140 blocked *in vitro* responses to CXCL12	
Prostate cancer	T140 blocks tumor cell invasion and signaling	
Leukemia/lymphoma		
Chronic lymphocytic leukemia (CLL)	T140, TC14012 and TN14003 block migration, adhesion and stromal protection; AMD3100 blocks actin polymerization in CLL cells	
Acute myelogenous leukemia (AML)	RCP168 and AMD3465 block migration and CXCR4 signaling; AMD3100 reduced	

Cancer type	*In vitro studies*	*In vivo studies*
	AML cell survival	
Acute lymphoblastic leukemia (ALL)	T140 and its analogs and AMD3100 inhibit ALL cell migration and adhesion	T140 analogs, AMD3100 and AMD3465 mobilize ALL cells
Multiple myeloma	T140 analogs block CXCL12-induced osteoclast activity	AMD3100 inhibits *in vivo* homing of myeloma cells
Non-Hodgkin's lymphoma		CXCR4 neutralization inhibited lymphoma growth

Table 3. In vitro and in vivo efficacy of CXCR4 antagonists in solid tumors and leukemia/lymphoma [99]

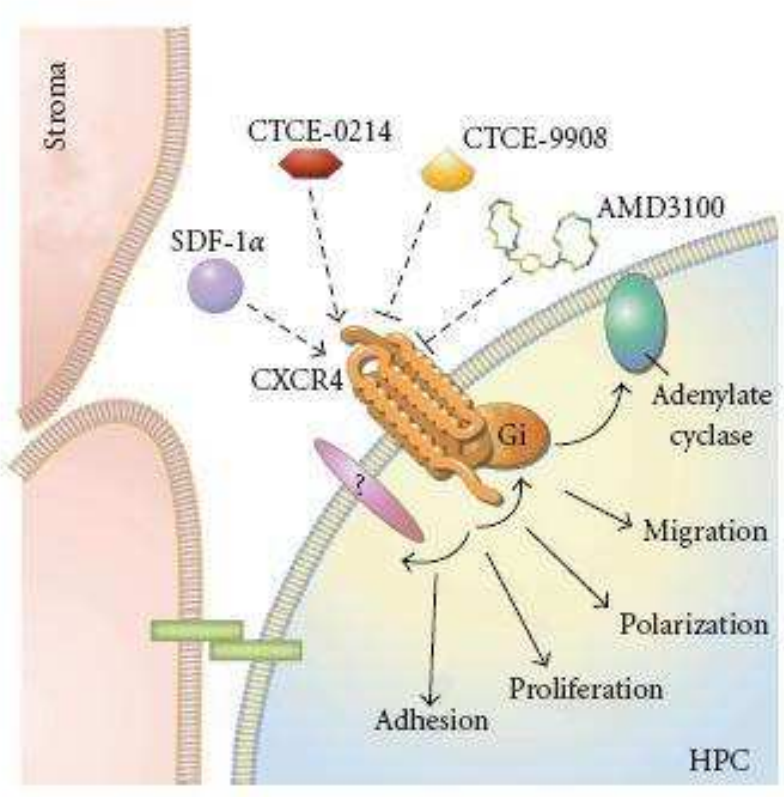

Figure 27. Effects on cell migration and podia formation.The effect of the SDF-1a, CXCR4 agonist, and CXCR4 antagonists on migration of human CD34+ cells was assessed in transwell migration experiments.[100]

[99] J A Burger, A Peled *Leukemia*, **2009**, 43–52

In general, four major classes of CXCR4 antagonists and agonists can be distinguished:

(a) ***small peptide CXCR4 antagonists***, such as T140 and its analogs (TN14003 and others). However the precise mechanism of anti-HIV activity remained unclear until the discovery that T tropic HIV-1 (X4-HIV-1) utilizes CXCR4 as a co-receptor for cellular entry into CD4-positive T cells. Soon after this, it was demonstrated the T22 specifically binds to CXCR4 and blocks CXCR4 receptor regions that are critical for HIV-1 viral entry and for activation by its natural ligand, CXCL12. The efficacy of T140 and its analogs for blocking CXCR4 in vitro and in vivo has been documented in numerous preclinical studies, including in vivo models for breast cancer and melanoma,[101, 102] rheumatoid arthritis [103] and stem cell mobilization.[104] Other studies explored the activity of these agents in acute[105, 106] and chronic leukemias,[107] multiple myeloma,[108] small cell lung cancer (SCLC),[109] malignant melanoma[92] and pancreatic cancer.[110]

(b) ***non-peptide CXCR4 antagonists***, such as the bicyclam AMD3100. This is a specific antagonist of CXCL12 binding to CXCR4, inhibiting

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[100] A. Faber, C. Roderburg, F. Wein, R. Saffrich, A. Seckinger, K. Horsch, A. Diehlmann, D. Wong, G. Bridger, V. Eckstein, A. D. Ho, W. Wagner *Journal of Biomedicine and Biotechnology* **2007**.

[101] Takenaga M, Tamamura H, Hiramatsu K, Nakamura N, Yamaguchi Y, Kitagawa A *et al. Biochem Biophys Res Commun* **2004**; 320: 226–232

[102] Tamamura H, Hori A, Kanzaki N, Hiramatsu K, Mizumoto M, Nakashima H *et al. FEBS Lett* **2003**; 550: 79–83.

[103] Tamamura H, Fujisawa M, Hiramatsu K, Mizumoto M, Nakashima H, Yamamoto N *et al. FEBS Lett* **2004**; 569: 99–104.

[104] Abraham M, Biyder K, Begin M, Wald H, Weiss ID, Galun E *et al. Stem Cells* **2007**; 25: 2158–2166.

[105] Juarez J, Bradstock KF, Gottlieb DJ, Bendall LJ. *Leukemia* **2003**; 17: 1294–1300.

[106] Juarez J, Dela Pena A, Baraz R, Hewson J, Khoo M, Cisterne A *et al. Leukemia* **2007**; 21: 1249–1257.

[107] Burger M, Hartmann T, Krome M, Rawluk J, Tamamura H, Fujii N *et al. Blood* **2005**; 106: 1824–1830.

[108] Zannettino AC, Farrugia AN, Kortesidis A, Manavis J, To LB, Martin SK *et al. Cancer Res* **2005**; 65: 1700–1709.

[109] Burger M, Glodek A, Hartmann T, Schmitt-Graff A, Silberstein LE, Fujii N *et al. Oncogene* **2003**; 22: 8093–8101.

[110] Mori T, Doi R, Koizumi M, Toyoda E, Ito D, Kami K *et al. Mol Cancer Ther* **2004**; 3: 29–37.

CXCL12mediated calcium mobilization, chemotaxis and GTP binding, and does not cross-react with other chemokine receptors. [111]

(c) ***antibodies to CXCR4.*** Neutralizing the interaction between CXCL12, the ligand for CXCR4, and CXCR4 by using anti-CXCR4 antibodies significantly inhibit HIV infection and tumor cell migration in vitro. Furthermore, anti-human CXCR4 or CXCL12 antibodies also significantly impair metastasis and progression of non-Hodgkin's lymphoma, breast, lung and prostate tumors in animal models.[112, 113]

(d) ***modified agonists and antagonists for SDF-1*** such as CTCE-9908 and CTCE-0214 are peptide analogs of CXCL12 with inhibitory and agonist activity, respectively. CTCE-9908 that has received orphan drug status by the Food and Drug Administration for the treatment of osteogenic sarcoma. CTCE-9908 decreases growth and adhesion of osteosarcoma cells and the metastatic dissemination of cancer cells in two murine models.[114]

Product name	***Company***	***Structure***	***Administration***	***Indication***	***Study phase***
Plerixafor (AMD3100)	Genzyme	Bicyclam	s.c.	Stem cell mobilizatio n	Phase III
AMD070	Genzyme	Bicyclam derived	Oral	HIV	Phase I/II
CTCE-9908 antagonist	Chemokine Therapeutics Corp.	Modified SDF-1	s.c./i.v.	Solid tumors	Phase I/II
CTCE-0214-	Chemokine	Modified	s.c./i.v.	Mobilizatio	Phase I/II

[111] Fricker SP, Anastassov V, Cox J, Darkes MC, Grujic O, Idzan SR *et al. Biochem Pharmacol* **2006**; 72: 588–596.
[112] Bertolini F, Dell'Agnola C, Mancuso P, Rabascio C, Burlini A, Monestiroli S *et al. Cancer Res* **2002**; 62: 3106–3112.
[113] Engl T, Relja B, Marian D, Blumenberg C, Muller I, Beecken WD *et al. Neoplasia* **2006**; 8: 290–301.
[114] Kim SY, Lee CH, Midura BV, Yeung C, Mendoza A, Hong SH *et al. Clin Exp Metastasis* **2008**; 25: 201–211.

Product name	*Company*	*Structure*	*Administration*	*Indication*	*Study phase*
agonist	Therapeutics Corp.	SDF-1		n BM recovery	
No name	Northwest Biotherapeutics, Bethesda, MD, USA	Antibody	s.c./i.v.	Cancer	Preclinical
TG-0054	TaiGen Biotechnology Co., Taipei, Taiwan	?	?	Stem mobilization for regeneration	Phase I/II
BKT140	Biokine Therapeutics	Modified peptide	s.c./oral	MM and leukemia	Phase I

Table 4. CXCR4 antagonists that are currently in preclinical and clinical development.[115]

[115] J A Burger, A Peled *Leukemia*, **2009**; **23,** 43–52

Chapter IV

4 Parazoanthines synthesis

The chemical preparation of natural metabolites and their analogs is of paramount importance for the biological and pharmacological studies of natural bioactive molecules.

Generally for the success of this kind of project is necessary to choose appropriately reactants and reaction conditions. The purpose of each synthesis is getting production of the desired product with the highest yields and purity possible, through a sequence of chemical reactions, which provides for the formation of a series of intermediates. The identification of synthetic strategy involves a preliminary *phase of research in literature* to identify any previously used methods for the production of intermediates of interest for synthesis. During the synthetic strategy the intermediates are compounds, to obtain which, methods are developed by following general rules, and theswe methods are called methodologies. Such methods should possibly give high yields and be available for a large number of substrates. Methodology research usually takes place in three stages: discovery, optimization, and studio applications and limitations. The discovery may provide a targeted method, when you search for substances in plant or animal compounds, through extractive methods and characterization at the highest technological level; or alternatively we rely on randomness of discovery (Serendipity), starting from the observation of an unexpected, unusual or unexpected data that has allowed to discover molecules with high

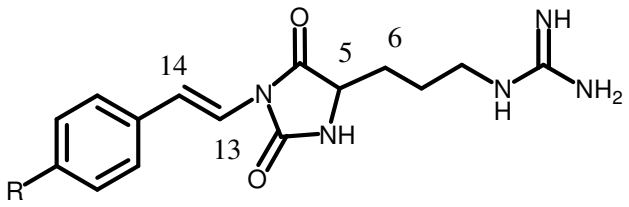
pharmacological activity (eg. Fleming with penicillin). Optimization involves a study aimed at improving the reaction conditions, as the variation in temperature, the duration of the reaction, the suitable selection of the solvent, the relative amounts of reagents, until optimum conditions indeed enhance the achievement of the desired molecule in maximum yield and purity. The third stage instead provides this program to a wide class of substrates, to verify its applicability.

During my PhD at the Institute of Biomolecular Chemistry, CNR, Pozzuoli (NA), Italy, I was involved in continuing the project started during the period of my thesis.

My project was focused on the chemical synthesis of most representative natural parazoanthines and no natural analogs leading to the preparation of discrete amount of these compounds to test in pharmacological assays to develop their pharmaceutical potential eventually confirming the in silico screening of parazoanthines compounds as potential CXCR4 ligands.

The synthesis of alkaloids and particularly of parazoanthines was immediately very interesting because they have unique structural chemical characteristics. The hydantoinic disubstituted ring gives flatness and rigidity to the molecule, that presents a guanidine linker bound to the ring at C-5 with a double or single bond depending on the parazonathine. The presence of the double bond (parazoanthine B eg) led to design synthetic strategy different from that developed for derivatives without the double bond (parazoanthine A eg.).

4.1 *Parazoanthine-A and its O-Me derivative synthesis*[116]



1 : R = OH
2 : R = OCH_3

Figure 28. Parazoanthine A (1) and O-Me (2)

Inspired by nature the knowledge and the hypothesis of biosynthetic mechanisms, by which these molecules are naturally produced, could help us to design a strategy of chemical synthesis.

In fact parazoanthine A and its *O*-methyl derivative (*Figure 28)* were prepared by a concise biomimetic synthesis based on the coupling reaction of *L*-arginine methyl ester dihydrochloride with isocyanate derivatives of *p*-coumaric acid and 4-methoxy-cinnamic acid, respectively. The synthetic approach is designed to obtain a wider class of parazoanthine analogs, with single bond on C5-C6.

To this aim, we found a literature method for the synthesis of hydantoin cores based on hypothesized biosynthetic mechanisms (*Figure 23*).

In Stilz et al. procedure[117] the coupling between *L*-arginine *O*-methyl ester with isocyanate derivative by use of *N*-ethylmorpholine in *N,N*-

[116] Manzo, E.; Pagano, D.; Nuzzo, G.; Gavagnin, M.; Ciavatta, M.L. *Tetrahedron Lett*. **2012**, *53(52),* 7083-7084

[117] Stilz, H.U.; Guba, W.; Jablonka, B.; Just, M.; Klingler, O.; Konig, W.; Wehner, V.; Zoller, G. *J. Med. Chem.* ***2001****, 44, 1158-1176*

dimethylformamide (DMF) led to the formation of the hydantoin framework by the subsequent treatment with 6 N HCl under reflux (*Figure 29*).

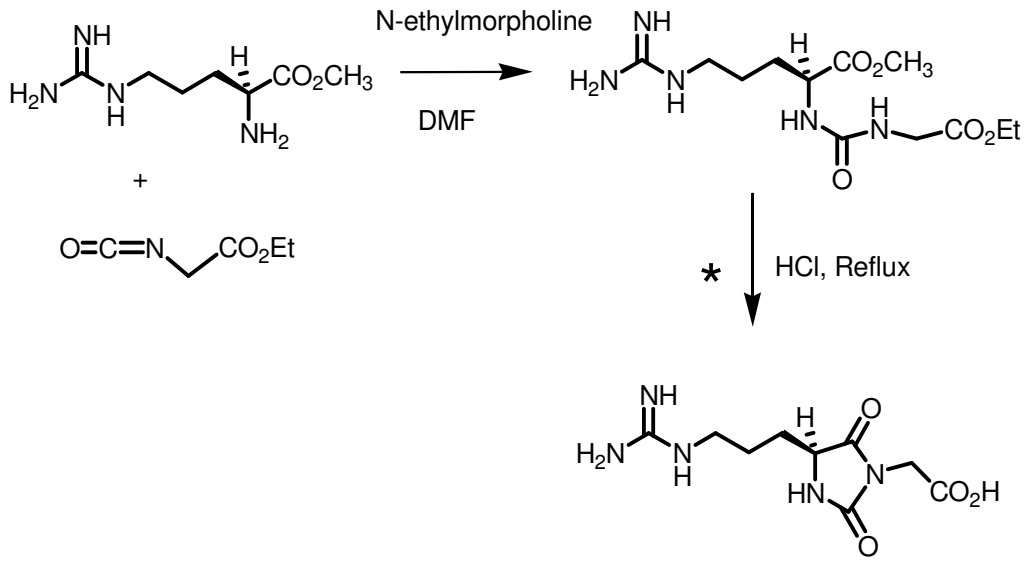


Figure 29. Stilz et al methodology [117] to obtain hydantoinic ring

According to this procedure, we planed the synthetic strategy in which the key-step was the coupling (*Figure 30*) between the commercially available *L*-arginine methyl ester dihydrochloride and the opportunely prepared isocyanates **A** or **A'**, by using *N*-ethylmorpholine in DMF. We observed that in our case, differently from literature data, the formation of the hydantoin moiety just occurred after the treatment with *N*-ethylmorpholine in DMF whereas the following use of 6 N HCl revealed to be disadvantageous because of the formation of a complex mixture of products. Thus, this step was not considered in our synthetic scheme.

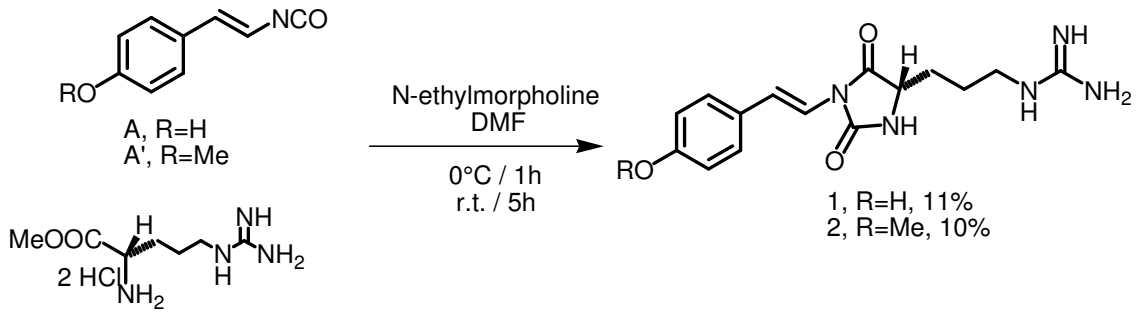


Figure 30. Coupling reaction to obtain the parazoanthine A (1) and its O-methyl derivative (2)

In *Figure 31* the mechanism, relating to the coupling between the isocyanate (**A** or **A'**) and the *L*-arginine methyl ester dihydrochloride, is represented to obtain respectively the parazoanthina A (**1**) or its O-Me derivative (**2**).

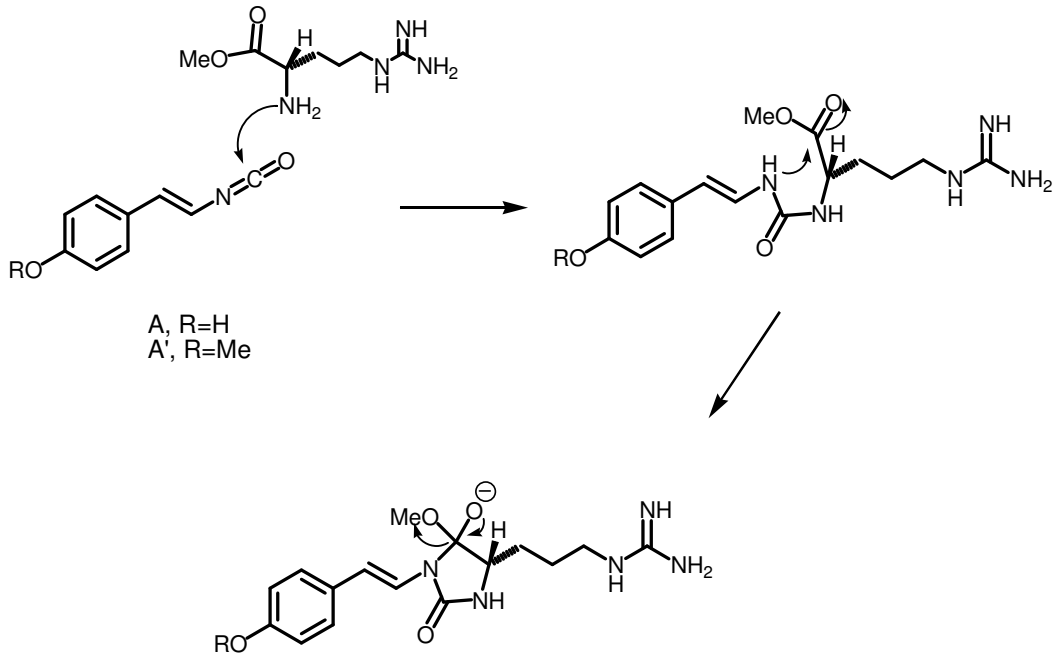


Figure 31. Coupling mechanism to obtain Parazoanthine A or its *O*Me-derivative.

The amino group of *L*-arginine methyl ester attacks the electrophilic carbon of the isocyanate by an addition mechanism to form a *N*-

substituted guanidine derivative; subsequently the amide nitrogen attacks the carboxylic carbon of the methyl ester by a nucleophilic acyl substitution mechanism eliminating methanol and thus leading to the hydantoinic ring formation.

The preparation of precursors **A** or **A'** is illustrated in *Figure 32.* Commercially available *p*-coumaric acid (**B**) or 4-methoxy-cinnamic acid (**B'**) were treated with sodium azide, triphenylphosphine, and trichloroacetonitrile in acetonitrile[118] to give the corresponding intermediate acyl azide derivatives **C** or **C'**.

The subsequent Curtius rearrangement of **C** or **C'** in toluene at 68°C overnight[119] led to the isocyanates **A** or **A'**, respectively, which were freshly used, due to their unstability.

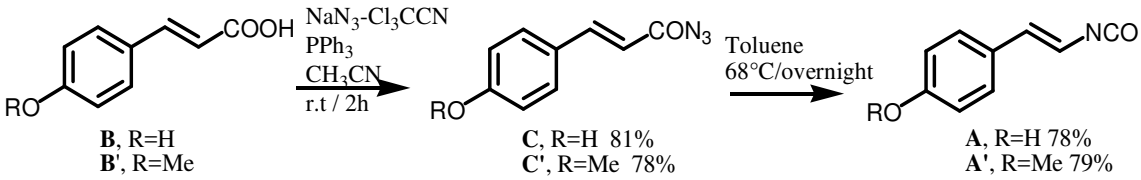


Figure 32. Synthesis of Isocyanates precursors parazoanthine A and its O-Me derivative.

In the coupling reaction (*Figure 30*) the obtained yields were quite low if compared to those reported by Stilz *et al*.[117] This was probably due to the less nucleophilic reactivity of the isocyanate nitrogen atom being conjugated to the double bond. However, with the aim at improving the yields, different experimental conditions in the coupling step were used; in particular, the temperature was varied in the range of 20–60°C, distinct

[118] Kim, J.G.& Jang, D.O., *Synlett*, **2008**, p.2072-2074

[119] Marinescu, L.& Thinggaard, J.& Thomsen, I.B.& Bols, M., *J. Org. Chem*., vol. 68, **2003**, p.9453-9455

solvents including DMF, DMSO, and THF were used, and *N*-ethylmorpholine was added in the range of 1–3 equiv.

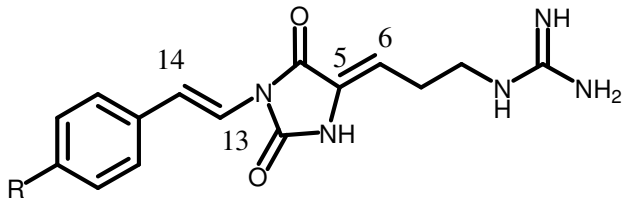
The compounds **1** and **2** were thus obtained in the best experimental conditions with a yield of 11% and 10%, carrying out the reaction at room temperature, in DMF, and with 1.2 equivalents of *N*-ethylmorpholine. Finally, under these reaction conditions the chirality of the C-5 was preserved.

By an accurate spectral analysis (^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-COSY, HSQC, HMBC and HRESIMS) of synthetic parazoanthine A (**1**), it has highlighted an exact match with the data reported in the literature related to those of the natural compound.[120]

The *O*-methyl ether derivative of parazoanthine A (**2**) showed spectroscopic data (^{1}H-NMR and ^{13}C-NMR) similar to parazoanthine A with the only difference due to the presence of the methoxyl on the aromatic ring.

[120] Nadja Cachet, Gregory Genta-Jouve, Erik L. Regalado, RedouaneMokrini, Philippe Amade, Gerald Culioli, and Olivier P. Thomas. *J.Nat.Prod.* ***2009,*** 72, 1612-1615**.**

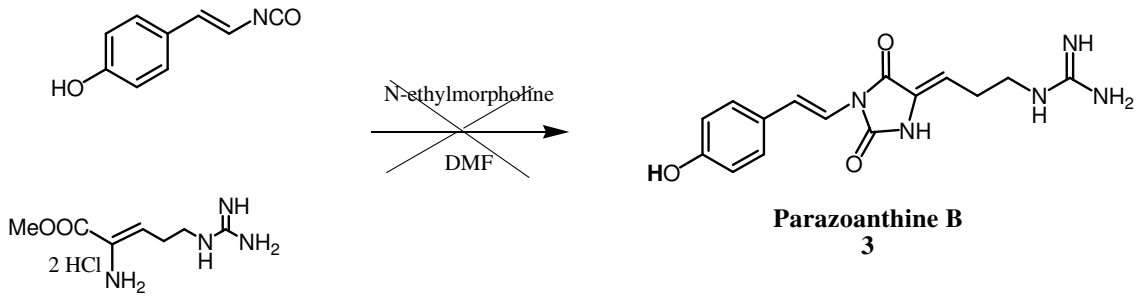
4.2 *Parazoanthine-B, Parazoanthine-C and 18-deoxy-parazoanthine B synthesis* [121]



3: R = OH
4: R = OCH_3
5: R = H

Figure 33. Structure of parazoanthine B (3), parazoanthine C (4) and 18-deoxy-parazoanthine B (5).

Within the natural class of parazoanthines other members, characterized by the presence of a *Z*-5,6 double bond (*Figure 33*) as the parazoanthine B (**3**) and C (**4**), are present; this double bond significantly complicates the synthetic strategy deviating from that used for the preparation of parazoanthine A.



Parazoanthine B
3

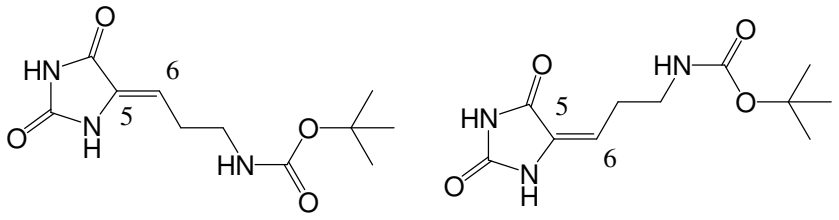
Figure 34. Unsuitable synthetic approach for parazoanthine B.

[121] Tinto Francesco; Dario Pagano; Emiliano Manzo. *Tetrahedron* **2015**, 71, 4379–4384

In fact the coupling between the 2,3 dehydro-arginine methyl ester and isocyanate with *N*-ethylmorpholine in DMF (*Figure 34*), has not led to the desired parazoanthine B even varying the temperature, solvents and other experimental conditions, probably due to the lower nucleophilicity of the nitrogen of the dehydro-arginine methyl ester.

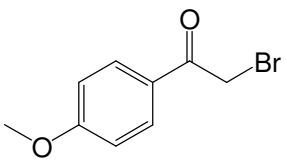
The various attempts to introduce unsaturation on the parazoanthine A in positions 5 and 6 using common literature strategies didn't lead significant results for the realization of parazoanthines characterized by the presence of the *Z*-5,6 double bond. All this has led us to the development of a different synthetic strategy for the preparation of these last parazoanthine derivatives.

The key step of this new procedure is the coupling of a hydantoinic precursor **Da/Db** (*Z/E* stereoisomers mixture) with α-bromo-4'-methoxy-acetophenone (**E**) (*Figure 35*), both prepared as illustrated in S*cheme 1* and *2*, respectively.



Da (*Z- isomer*)

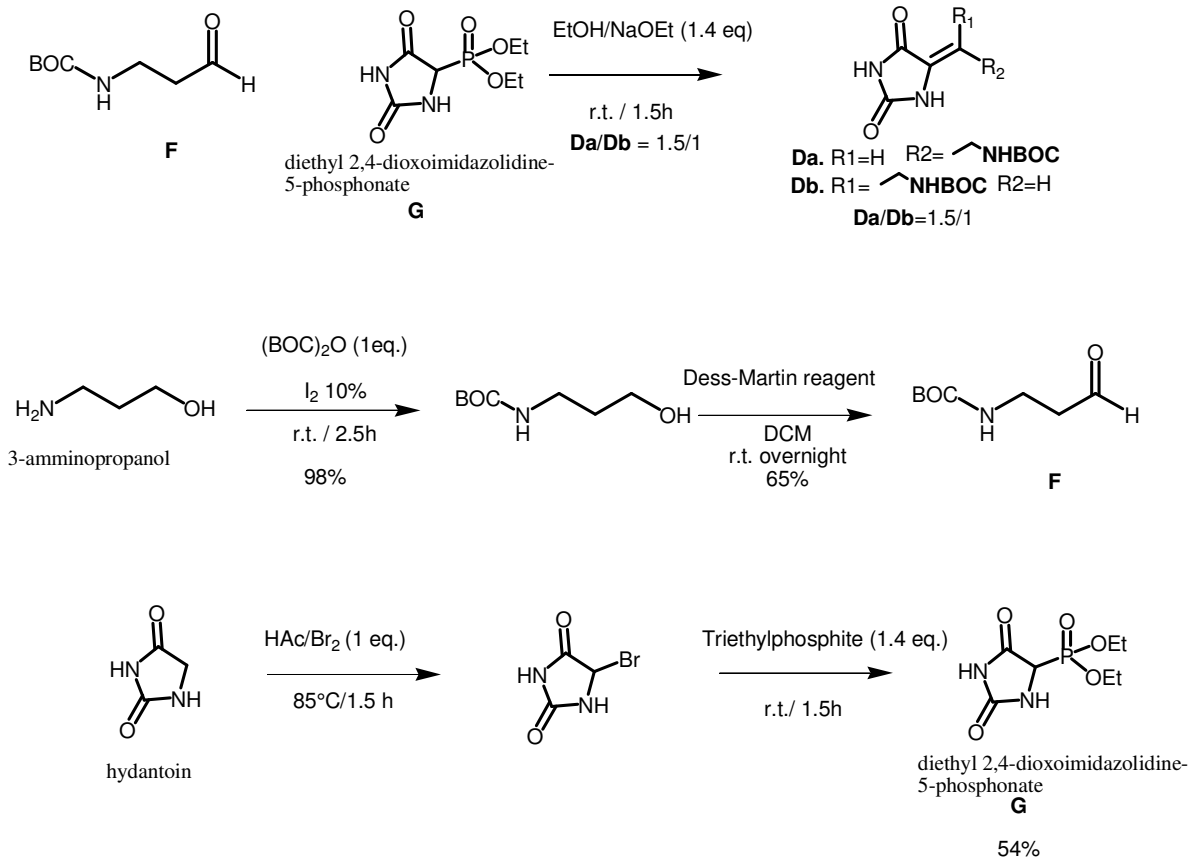
Db (*E- isomer*)



E

Figure 35. Hydantoinic derivative **Da** and **Db** stereoisomers and α-bromo-4'-methoxy-acetophenone (**E**).

In particular, the mixture of Z/E isomers **Da/Db**, was prepared by Horner – Wadsworth - Emmons reaction of the aldehyde (**F**), derived from of 3-amino-1-propanol, with diethyl 2,4-dioxoimidazolidine-5-phosphonate (**G**), in turn prepared by bromination and subsequent substitution with triethylphosphite, according to literature conditions[122] (*Scheme 1*).



Scheme 1. Preparation of synthetic precursors **Da/Db.**

We obtained a mixture of Z/E isomers **Da/Db** in the ratio of 1.5/1 that was not easily detectable on the basis of NMR chemical shifts, but only by an

[122] Meanwell, N.A.; Roth, H.R.; Smith, E.C.R.; Wedding, D.L.; Wright, J.J.K. *J. Org. Chem*. **1991**, 56, 6897-6904.

accurate analysis of the long-range ($^3J_{C-H}$) heteronuclear ^{13}C-^1H coupling constants that could distinguish them.[123, 124] In particular for **Da/Db** the different configurations of the exocyclic double bond were evident by measuring $^3J_{C4-H6}$ values (δH-6 5.73 and 5.56, δC-4 164.2 and 164.4, $^3J_{C4-H6}$ 4.7 and 9.8 Hz for **Da** and **Db** respectively) by non-proton-decoupled *J*-HMBC experiments (*Figure 36*) [115,116c]. Compound **Da** revealed *Z*-geometry with the olefinic H-6 and the carbonyl C-4 in a cis-orientation, while **Db** showed *E*-geometry with H-6 and C-4 in a trans-orientation.

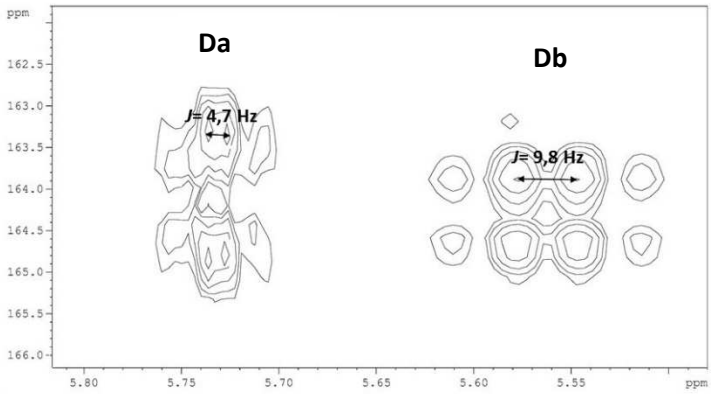
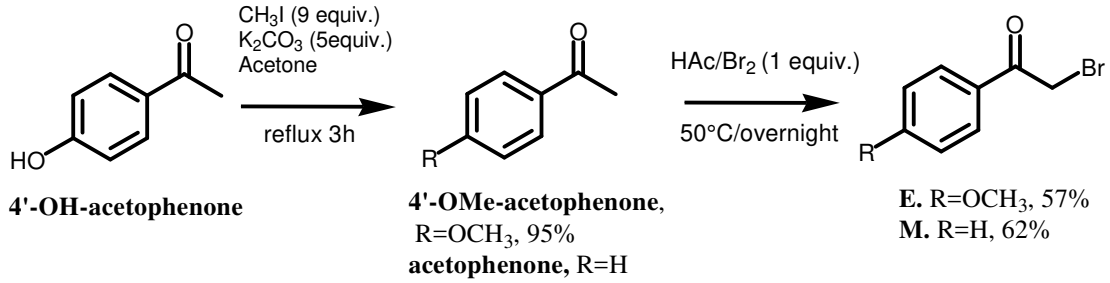


Figure 36. Correlation H-6/C-4 *J*-HMBC for **Da** and **Db**

On the other hand the precursor (**E**) was prepared by *O*-methylation and subsequent α-bromination of the commercially available 4'-hydroxy-acetophenone with iodomethane in refluxing acetone and bromine in acetic acid (*Scheme 2*).

[123] Cachet, N.; Genta-Jouve, G.; Regalado, E.L.; Mokrini, R.; Amade, P.; Culioli, G.; Thomas, O.P. *J. Nat. Prod*. **2009**, *72*, 1612-1615.
[124] (a) Jakše, R.; Rečnik, S.; Svete, J.; Golobič, A.; Golič, L.; Stanovnik, B. *Tetrahedron* **2001**, 8395-8403; (b) Vergne, C.; Appenzeller, J.; Ratinaud, C.; Martin, M.-T.; Debitus, C.; Zaparucha, A.; Al-Mourabit, A. *Org. Lett*. **2008**, *10*, 493-496; (c) Meissner, A.; Sørensen, W. *Magn. Reson. Chem*. **2001**, *39*, 49-52.



Scheme 2. Preparation of synthetic precursors **E** and **M**.

The coupling reaction between the prepared precursors **Da/Db** and **E** (*Figure 37* and *Scheme 3*) consisted of the use of potassium carbonate and tetrabutylammonium bromide (TBAB) at 50 °C in anhydrous *N,N*-dimethylformamide (DMF) addressing the *N*-imide bromine substitution to form compound **H**. (*Figure 37*)

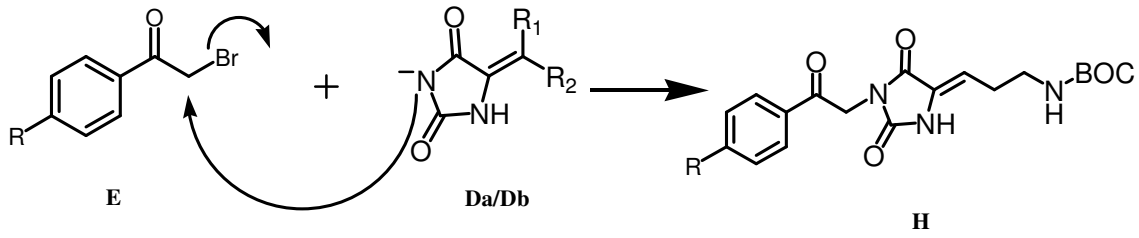


Figure 37. Coupling mechanism between **Da/Db** and **E**

Compound **H** presented the 3,5-alkyl disubstituted hydantoinic moiety typical of parazoanthines with an exocyclic 5,6-double bond with the desired *Z* geometry. The *E* isomer product was revealed only in trace probably due to the thermal isomerization of the double bond as indicated by an analytical test in which a small amount of *Z/E* mixture **Da/Db** was stirred at 50 °C in DMF, showing a significant increase of the portion of the *Z*-isomer (**Da**). (*Figure 38*)

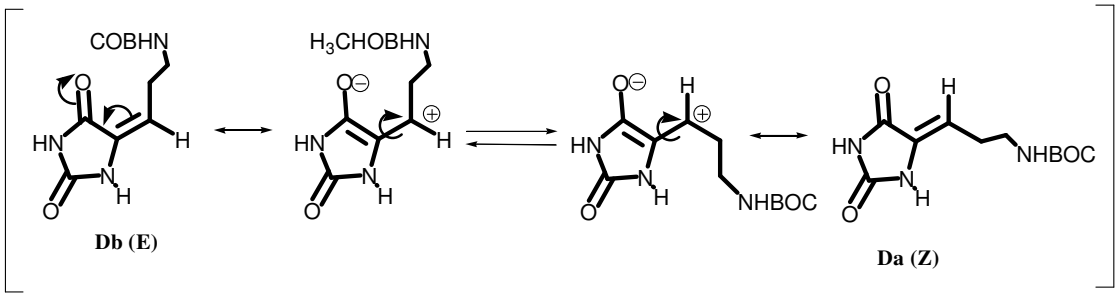
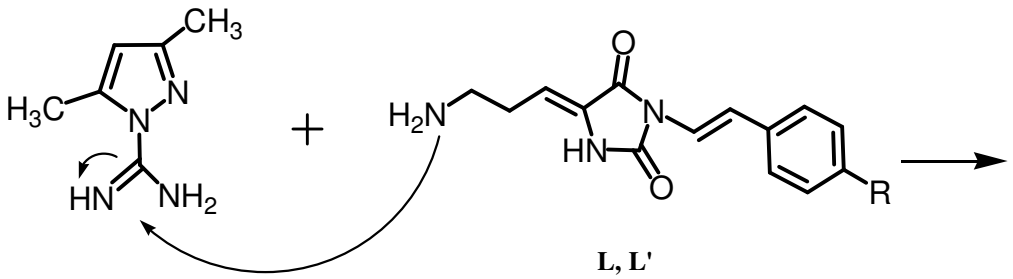


Figure 38. Equilibrium between Da/Db stirred at 50 °C in DMF

Reduction with sodium borohydride of the coupling adduct **H** gave compound **I**, that was then converted into **L** via dehydration and Boc-cleavage by two equivalents of *p*-toluenesulfonic acid (*p*-TsOH) at 110 °C in toluene (*Scheme 3*).

Compound **L** displayed a trans-double bond ($J_{H13\text{-}H14}$= 15.4 Hz) between carbon 13 and 14 of the parazoanthine skeleton and was subsequently guanylated by 3,5-dimethyl-1-pyrazolylformaminidium nitrate[125] and *N,N*-diisopropylethylamine (DIPEA) in anhydrous DMF to give parazoanthine C (**4**) and 18-deoxy-parazoanthine B (**5**) (*Figure 39 and scheme 3*).



[125] (a) Bernatowicz, M.S.; Wu, Y.; Matsueda, G.R. *J. Org. Chem*. **1992**, *57*, 2497-2502; (b) Manzo, E.; Pagano, D.; Carbone, M.; Ciavatta, M.L.; Gavagnin, M. *Arkivoc* **2012**, *IX*, 220-228.

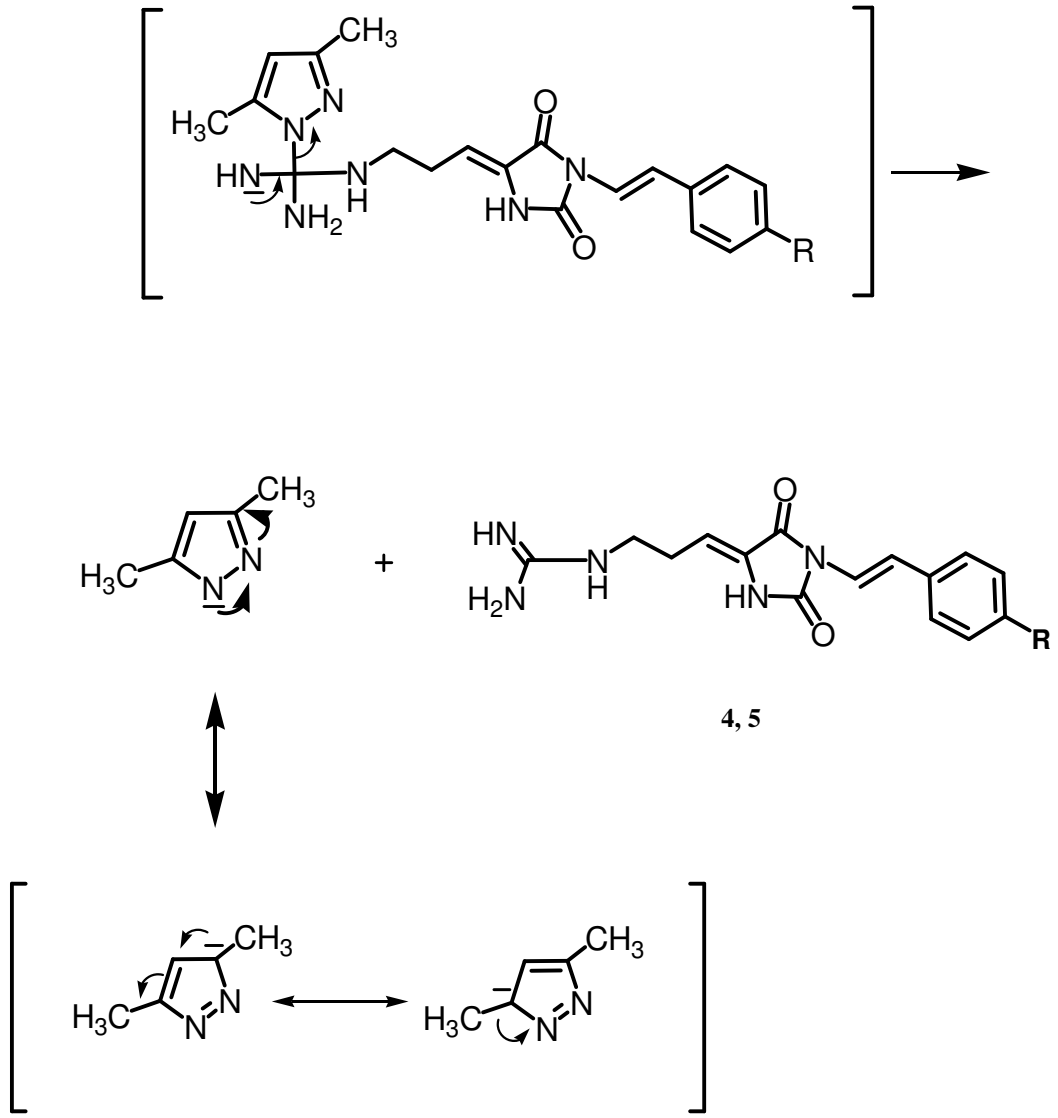
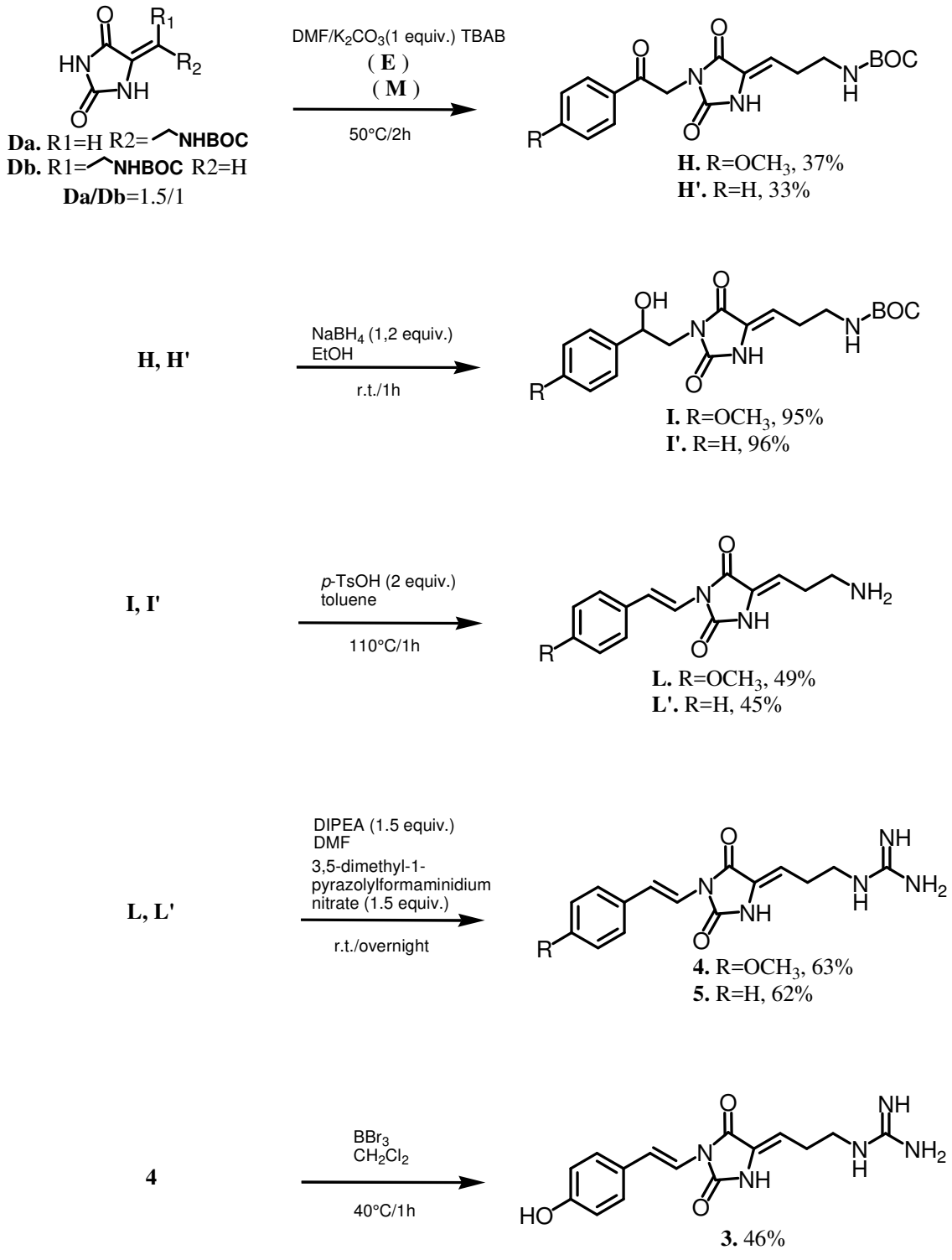


Figure 39. Guanidination mechanism

Synthetic parazoanthine C showed spectroscopic data identical with those of the natural molecule[126] and was also demethylated by boron tribromide in dichloromethane, to give parazoanthine B (**3**) (*scheme 3*).

[126] Cachet, N.; Genta-Jouve, G.; Regalado, E.L.; Mokrini, R.; Amade, P.; Culioli, G.; Thomas, O.P. *J. Nat. Prod*. **2009**, *72*, 1612-1615.

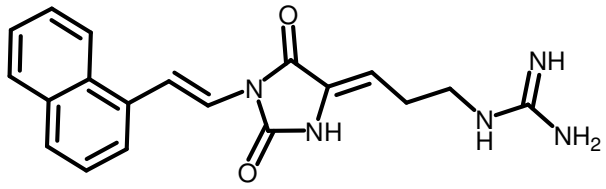


Scheme 3. Synthetic approach to get parazoanthine B (3), parazoanthine C (4) and 18-deoxy-parazoanthine B (5).

4.3 *Synthetic analog of natural parazoanthines (Para-N)*

The docking wise have directed to the synthesis of no-natural analogs that would have a greater steric hindrance in the aromatic moiety, and they left unchanged the guanidinic portion.

For this reason it was decided to synthetize a modified parazoanthine B in which the terminal phenolic ring is replaced with a bulkier naphthalene. This change was made because the pocket of the CXCR4 receptor is able to accommodate large molecules, and the aromatic moiety is capable of binding with the π-stacking interaction to the receptor that are definitely more with a naphthalene. (*Figure 40)*



(6)

Figure 40. Para-N (6)

To synthesize this parazoanthine analog (para-N), we used the method designed for the parazoanthine B; even in this case the key step of the procedure is the coupling of the hydantoinic derivatives **Da/Db** with α-bromo-1-acetonaphthone (**N**), prepared as illustrated in *Figure 41.*

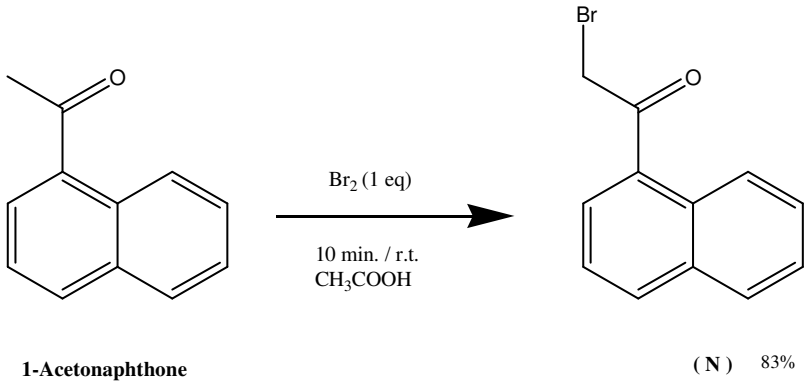
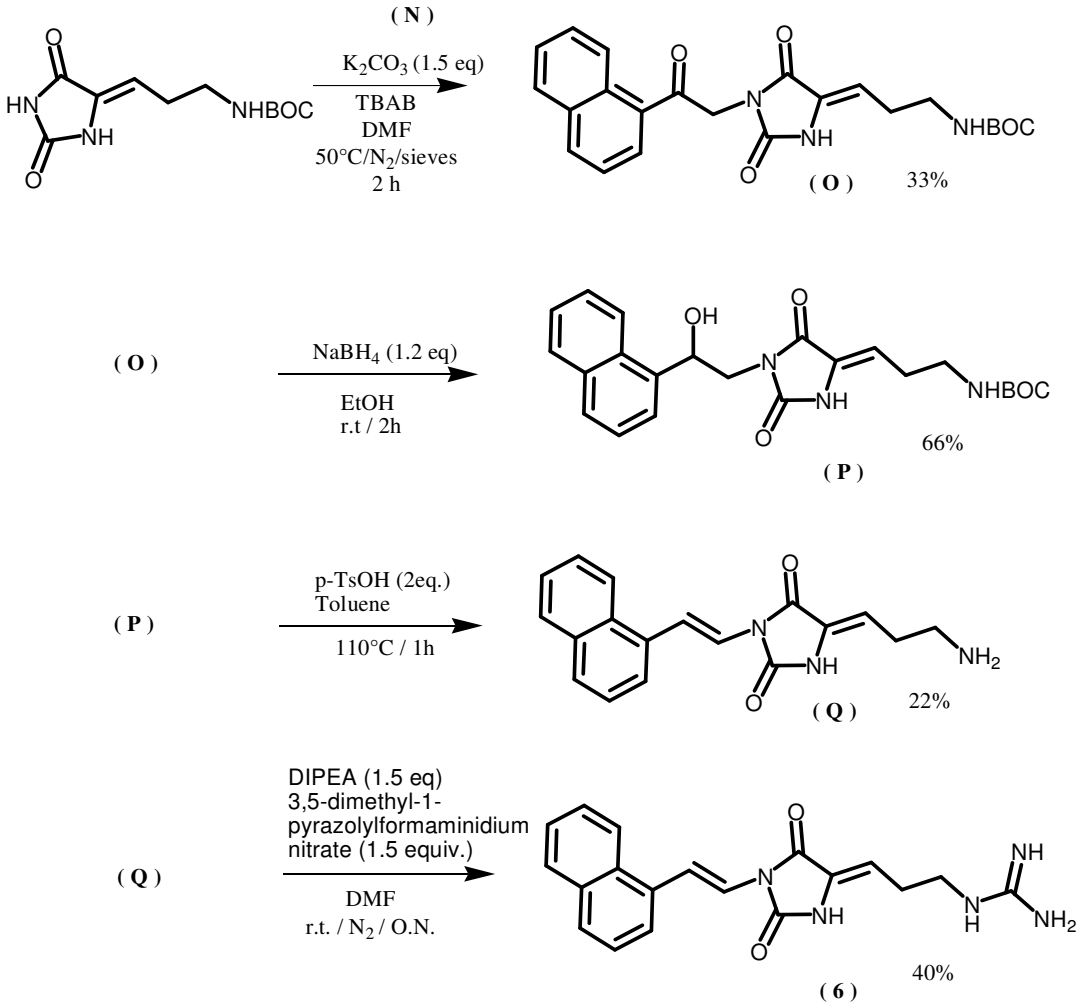


Figure 41. Alfa bromination of 1-acetonaphthone to obtain **N**

The precursor **N** was synthesized starting from '1-acetonaphthone, reacted with bromine in acetic acid for 10 minutes at room temperature. The compound **N** reacted with the synthon **Da/Db**, previously synthesized (according to the method described in *Scheme 1*) to obtain the intermediate **O**. The reduction with $NaBH_4$, and subsequent dehydration with *p*-TsOH in toluene at 110 ° C / 1h led to the intermediate **Q**. Finally we got the PARA-N (**6**), by the guanidination of **Q**, using the guanidinating reagent 3,5-dimethyl-1-pyrazolylformaminidium nitrate in basic conditions (*Scheme 4*).



Scheme 4. Synthetic approach to get PARA-N (6)

Chapter V

5 Pharmacological activity tests in vitro

Pharmacological tests *in vitro* were performed using only synthetic parazoanthine A.

5.1 *Parazoanthine A*

a) Proliferation Tests

Preliminarily, used to validate the model, we have demonstrated that CXCL12 (25nm) induces in cells GH4C1 a significant increase of DNA synthesis after 24 hours of treatment, in conditions of prolonged deprivation of growth factors contained in FBS (72h), using the BrdU incorporation test. This prolonged serum deprivation condition is necessary to suppress any residual activation of the CXCR4 receptor by CXCL12 present in the serum. (*Figure 42*)

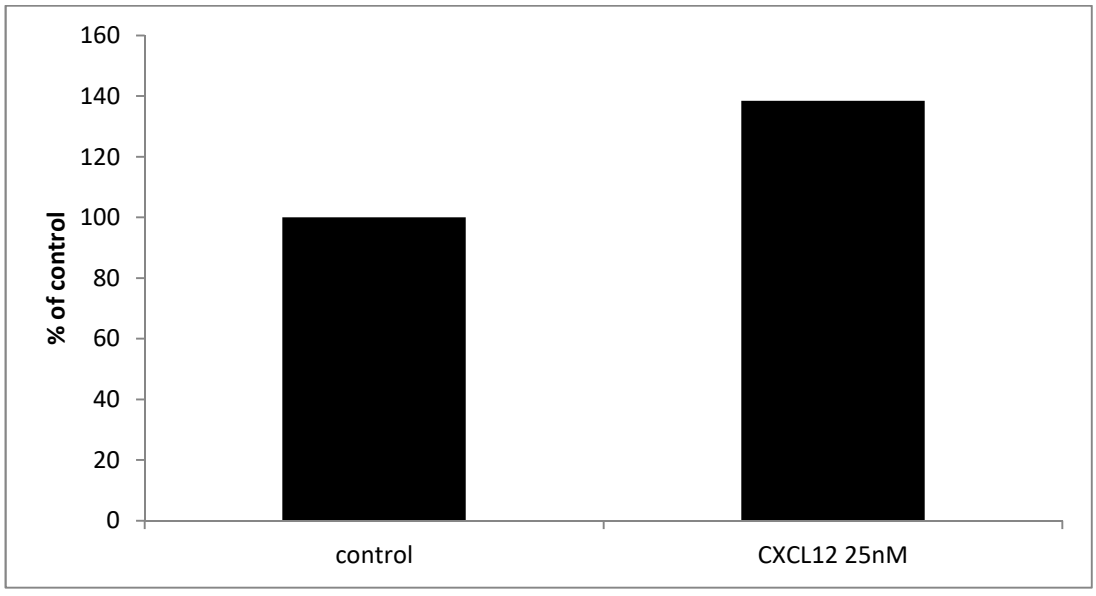


***Figure 42**. Control test CXCR4/CXCL12*

Always using BrdU incorporation test, we tested the ability of Parazoantina A to reduce the proliferation induced by CXCL12; the GH4 cells were pre-treated for 15 minutes with the test compound at various concentrations (5µM, 1 micron, 500nm, 100nM and 50nM) before adding CXCL12 at a concentration of 25nm. As is evident from the graph, the pretreatment with Parazoantina A is able to inhibit in a dose-dependent manner the proliferation induced by CXCL12 that reaches a statistically significant value to the dose of 1 µM (about -30%). The effect obtained shows a typical plot "U" with lower efficacy at the 5 µM dose. (*Figure 43*)

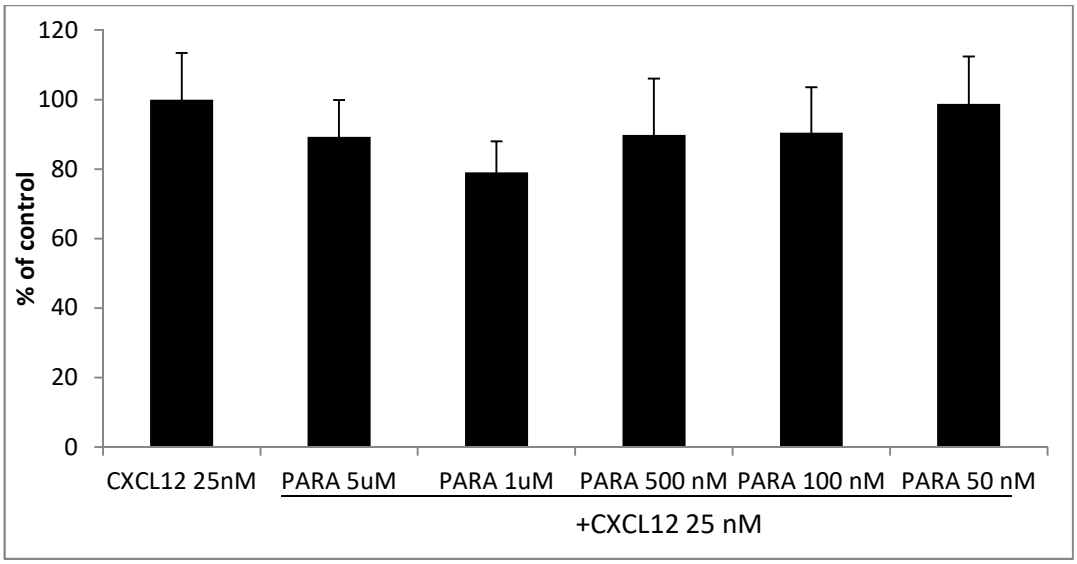
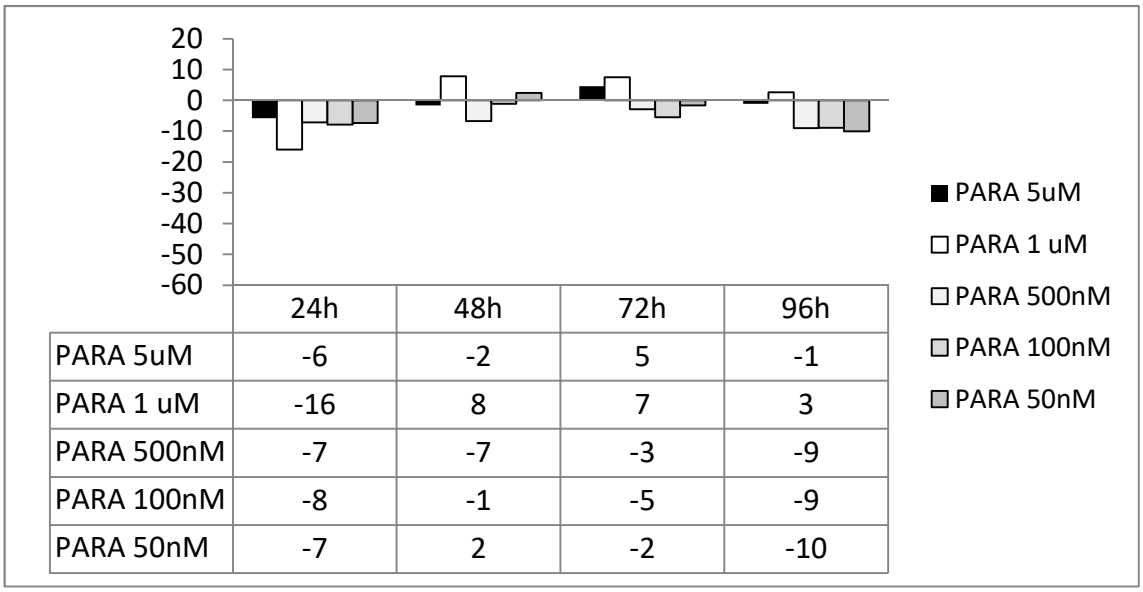


Figure 43. Proliferation Test CXCL12/Parazonathine A

b) Cytotoxicity MTT assay

To exclude non-specific cytotoxic effects of Parazoantina A, we performed a series of experiments of direct cytotoxicity by MTT assay in the absence of stimulation by CXCL12. None of the tested doses and the various treatment times are not significant toxic effects. This confirms that the inhibitory effect seen in the cell proliferation test was specifically

dependent of the antagonistic action of the molecule on CXCL12 activity and therefore by acting at the level of CXCR4. (*Figure 44*)



	24h	48h	72h	96h
PARA 5uM	-6	-2	5	-1
PARA 1 uM	-16	8	7	3
PARA 500nM	-7	-7	-3	-9
PARA 100nM	-8	-1	-5	-9
PARA 50nM	-7	2	-2	-10

Figure 44. Results of MTT tests; parazoanthine A haven't non-specific cytotoxicity effect in all doses tested.

Then we evaluated the effects Parazoanthine A on one of the intracellular pathways activated by CXCR4; in particular, we have considered the effects on the phosphorylation / activation of ERK1 / 2 induced by CXCL12 by Western Blot. (*Figure 46*) The cells were pretreated with the compound at various concentrations (5uM, 1uM, 500nm, 100nM and 50nM) for 15 minutes and subsequently stimulated with the chemokine 10 minutes at the concentration of 25nm. As can be observed, Parazoanthine A is able to inhibit the phosphorylation of ERK1 / 2 in the presence of CXCL12 at all concentrations tested.

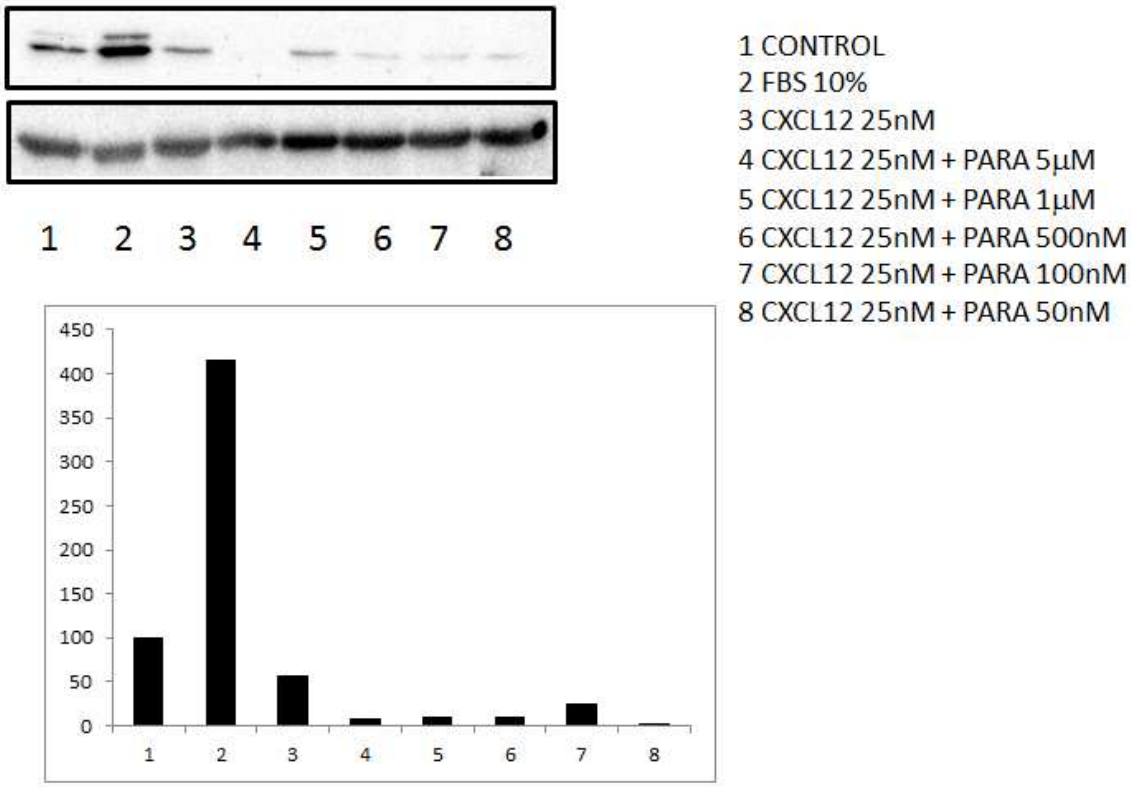


Figure 45. *Western Blot*

Preliminary biological assays on CXCR4 carried on Parazoanthine-A confirm the *in silico* results, whereas in vitro experiments are currently ongoing on Parazoanthine-B and its derivatives, in order to evaluate the effects of either substituents on aryl group or the bulkiness of the aromatic system on the biological activity for this class of molecules.

Chapter VI

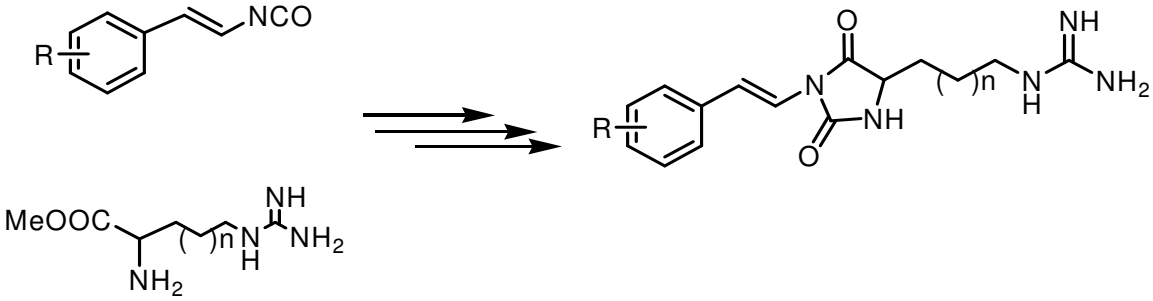
6 Conclusion

The natural alkaloids characterized by the presence of the hydantoinic heterocycles have always awakened great interest in the scientific community not only for the interesting pharmacological activities, but also for their enormous applicative potential. In this context, the possibility of obtaining, by means of chemical synthesis methodologies, pure amounts of natural and no-natural analogs, opens the way to more extensive studies of structure-activity relationship, as well as the possibility to undertake any *in vivo* experimentation which in fact require amounts of substances not usually found in nature. In this regard we have been synthesized some representative guanidine alkaloids with hydantoinic core of the parazoanthines family, isolated and characterized previously by the anemone Parazoanthus axinellae.

In particular the first synthesis of parazoanthina A (**1**) and its analogue-*O*-methyl derivative[127] (**2**), was carried out according to the known method of Stilz et al[128], finalized to the preparation of variously substituted hydantoinic nucleus; the key reaction resulted to be the coupling between the *L*-arginine methyl ester dihydrochloride and a feniletilen-isocyanate (**A** or **A'**); common sequence of reactions, including a keto-reduction with sodium borohydride, dehydration with *p*-TsOH and

[127] Manzo E., Pagano D., Nuzzo G., Gavagnin M. and Ciavatta M.L:, *TetrahedronLetters* **2012,** 53(52),7083–7084.
[128] Stilz, H.U.; Guba, W.; Jablonka, B.; Just, M.; Klingler, O.; Konig, W.; Wehner, V.; Zoller, G. *J. Med. Chem.* ***2001****, 44, 1158-1176.*

final guanidination with an appropriate guanidination reagent led to target compound. This synthetic strategy might open the way for the preparation of other structural analogues (*Scheme 5*) and therefore permitting further structure-activity relationship studies aimed to increase the pharmacological potential of parazoanthines.

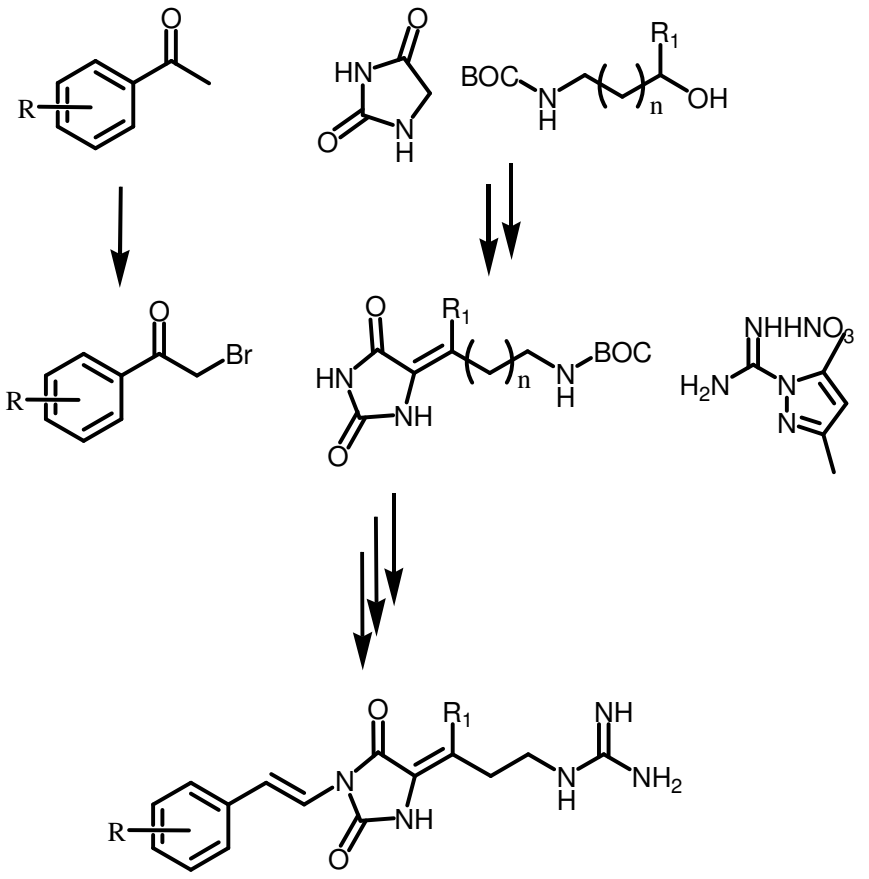


Scheme 5. General synthetic strategy for derivatives parazoanthines

We have also successfully synthesized the parazoanthine B,[129] natural representative of parazoanthine with exocyclic *Z*-5,6-double bond. In this case the synthesis includes a first coupling reaction between a hydantoinic derivative (isomers mixture **Da/Db**) and the α-bromo-acetophenone, obtaining the 3,5-alkyl disubstituted hydantoinic moiety typical of parazoanthines with an exocyclic 5,6-double bond characterized by the desired *Z* geometry (**H**), which by subsequent reduction with sodium borohydride, dehydration, BOC-deprotection and final guanidination, led to desired parazoanthines B and C. Even in this case this synthetic approach may open the way to the realization of natural and no-natural analogues of parazoanthines with *Z*-5,6 double bond and other derivatives

[129] Tinto Francesco; Dario Pagano; Emiliano Manzo. *Tetrahedron* **2015**, *71,* 4379–4384

that may differ in the length of the alkyl chain and / or in the type of substitution on the phenyl (*Scheme 6*).



***Schema 6*.** General scheme for the preparation of analogues of parazoanthine with the *Z*-5,6 double bond.

Chapter VII

7 General experimental procedures

All synthetic products and intermediates structures were assigned using ^{1}H, ^{13}C, COSY, HSQC, HMBC NMR spectra.

^{1}H, ^{13}C and 2D NMR spectra were acquired by the NMR Service of the Institute of Biomolecular Chemistry of the National Council of Research (ICB-CNR) and recorded on a Bruker DRX-600 spectrometer, equipped with a TCI CryoProbe™, fitted with a gradient along the Z-axis and on Bruker instruments at 400 and/or 300 MHz.

Samples for NMR spectroscopic analysis were dissolved in the appropriate solvent; spectra in $CDCl_3$ (^{1}H,^{13}C): 7.26, 77.0 ppm; CD_3OD (^{1}H,^{13}C): 3.34, 49.0 ppm. HRESIMS were performed on a Micromass Q-TOF Micro™ coupled with a HPLC Waters Alliance 2695. TLC plates (Kieselgel 60 F_{254}) and silica gel powder (Kieselgel 60, 0.063-0.200 mm) were from Merck.

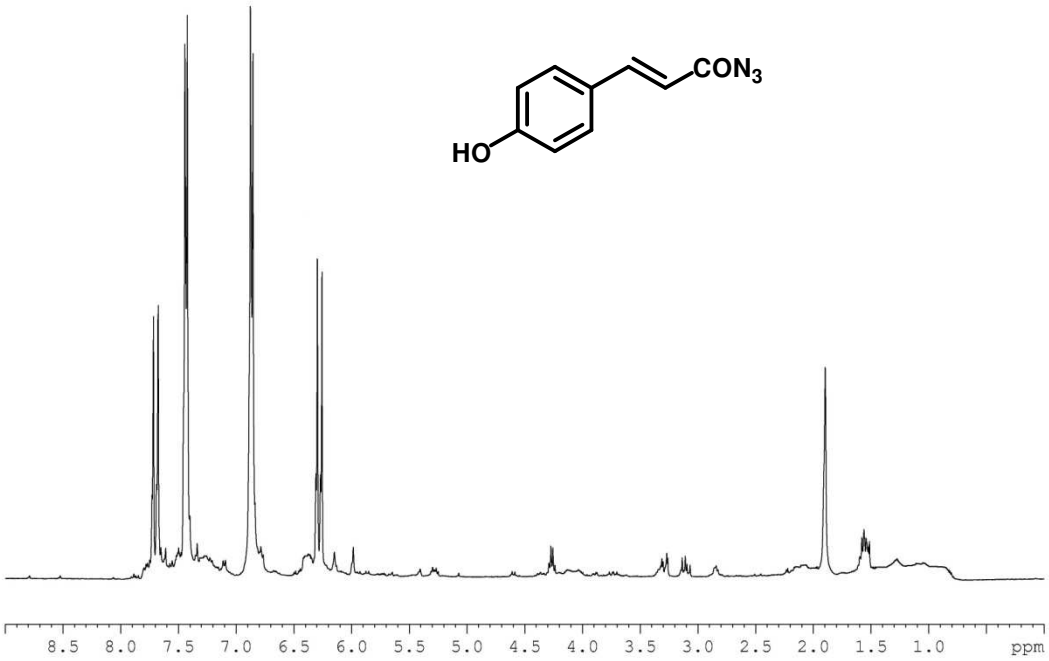
All the reagents and solvents were purchased from Sigma-Aldrich and used without any further purification.

7.1 *Procedures for the synthesis of parazoanthine A (1) and its O-Me-analog (2) and spectral data of the synthetic intermediates.*

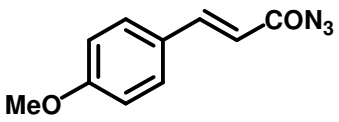
Compound C: *p*-coumaric acid (**B**) (0.5 g, 3.10 mmol), triphenylphosphine (1.62 g, 6.20 mmol) and sodium azide (0.24 g, 3.70 mmol) were dissolved in anhydrous acetonitrile (6 mL) under argon; trichloroacetonitrile (0.886 g, 6.20 mmol) was added dropwise at room temperature; the reaction

mixture was stirred for 1.5 h and evaporated under reduced pressure; the residue was purified by silica gel chromatography using a gradient of petroleum ether/ethyl acetate to give compound **C** (0.48 g, 2.98 mmol, 81%) as a pale yellow oil; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.71 (1H, d, *J*= 16.1 Hz), 7.43 (2H, d, *J*= 8.42 Hz), 6.87 (2H, d, *J*= 8.42 Hz), 6.28 (1H, d, *J*=16.1 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 173.0 (C), 158.8 (C), 146.8 (CH), 130.6 (CH), 116.3 (CH), 116.1 (CH); HRESIMS *m/z* 212.0431 $[M+Na]^+$ (calcd for $C_9H_7N_3O_2Na$, 212.0436).

^{1}H-NMR compound C, $CDCl_3$, 400 MHz



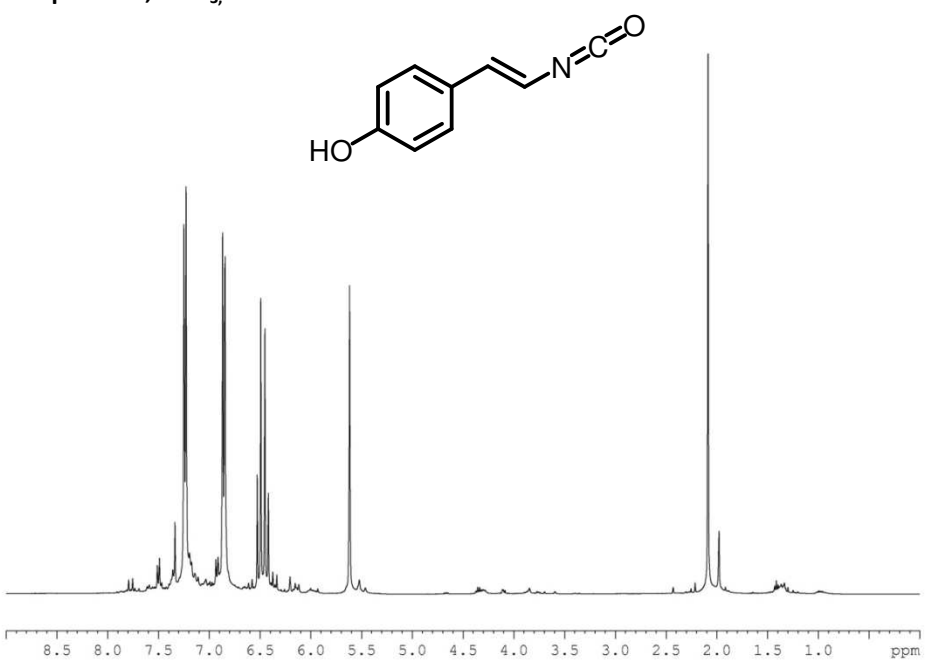
Compound C': 4-Methoxy-cinnamic acid (**B'**) (0.6 g, 3.37 mmol), triphenylphosphine (1.62 g, 6.74 mmol) and sodium azide (0.24 g, 4.04 mmol) were dissolved in anhydrous acetonitrile (6 mL) under argon; trichloroacetonitrile (0.964 g, 6.74 mmol) was added dropwise at room temperature; the reaction mixture was stirred for 1.5 h and was evaporated under reduced pressure; the residue was purified by silica gel chromatography using a gradient of petroleum ether/ethyl acetate to give compound **C'** (0.46 g, 2.63 mmol, 78%) as a pale yellow oil; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.73 (1H, d, *J*= 16.1 Hz), 7.46 (2H, d, *J*= 8.42 Hz), 6.90 (2H, d, *J*= 8.42 Hz), 6.31 (1H, d, *J*=16.1 Hz), 3.78 (3H, s, OCH_3); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 173.1 (C), .160.1 (C), 146.9 (CH), 130.8 (CH), 116.3 (CH), 116.1 (CH), 56.8 (OCH_3); HRESIMS *m/z* 226.0596 $[M+Na]^+$ (calcd for $C_{10}H_9N_3O_2Na$, 226.0592).



C'

***Compound A*:** Compound **C** (0.48 g, 2.98 mmol) was dissolved in anhydrous toluene (5 mL) and added dropwise to hot anhydrous toluene (10 mL) and heated to 68°C overnight; afterward the toluene was evaporated under reduced pressure obtaining compound **A** (0.374 g, 2.32 mmol, 78%) as a pale yellow oil; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.24 (2H, d, *J*= 8.7 Hz), 6.51 (1H, d, *J*=15.7 Hz), 6.86 (2H, d, *J*= 8.7 Hz), 6.44 (1H, d, *J*= 15.7 Hz).

^{1}H-NMR compound A, $CDCl_3$, 400 MHz

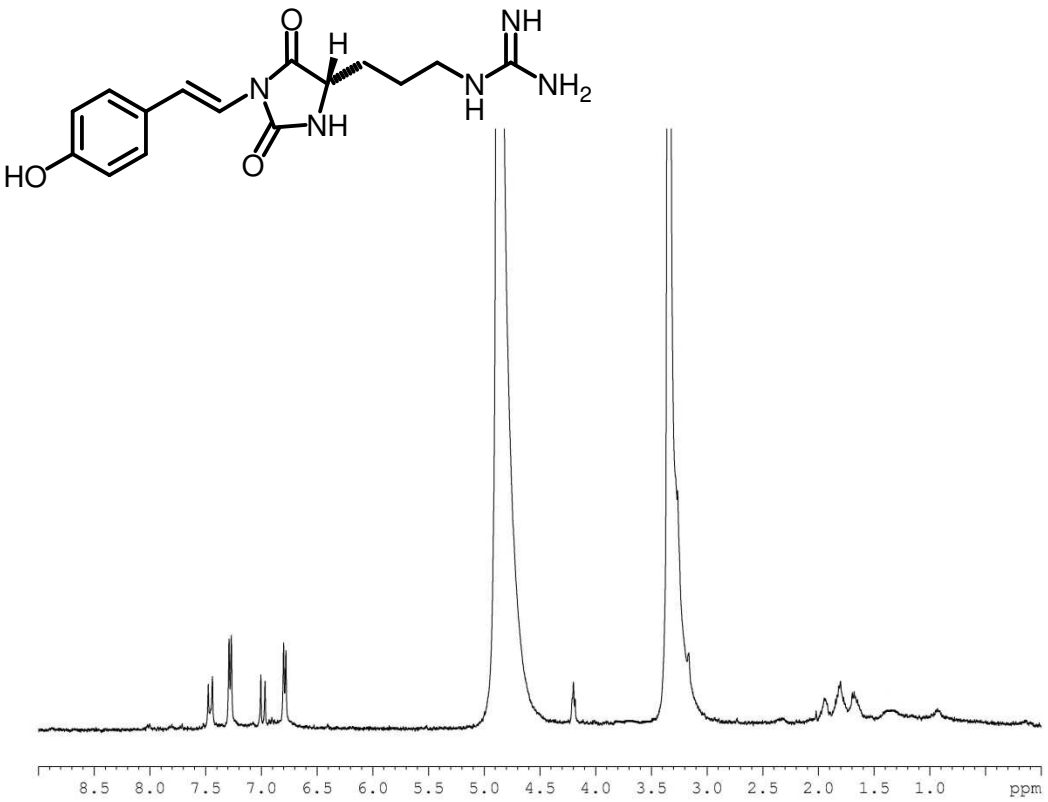


Compound A': Compound **C'** (0.46 g, 2.63 mmol) was dissolved in anhydrous toluene (5 mL) and added dropwise to hot anhydrous toluene (10 mL) and heated to 68°C overnight; afterward the toluene was evaporated under reduced pressure obtaining compound **A'** (0.368 g, 2.10 mmol, 79%) as a pale yellow oil; ^{1}H-NMR (CD_3CN, 400 MHz): δ 7.74 (1H, d, *J*=15.8 Hz), 7.59 (2H, d, *J*= 8.6 Hz), 6.92 (2H, d, *J*= 8.6 Hz), 6.43 (1H, d, *J*= 15.8 Hz), 3.79 (3H, s, OCH_3).

Compound 1: at 0°C, *N*-ethylmorpholine (67 mL, 0.522 mmol) and compound **A** (70 mg, 0.435 mmol) were added to a solution of *L*-arginine methyl ester dihydrochloride (114 mg, 0.435 mmol) in DMF (2 mL). The reaction mixture was stirred at 0°C for 1h and at r.t. for 5h; under a stream of nitrogen, the residue was purified by silica gel chromatography using a chloroform/methanol gradient and further purified by HPLC RP-phase

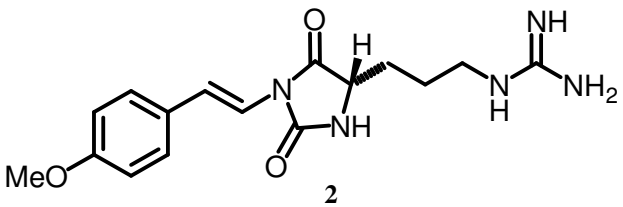
(H_2O-TFA 0.1%/MeOH, from 60/40 to 50/50 in 20 minutes, RP-amide semipreparative column, flow rate 2 mL/min) to give compound **1** (15 mg, 0.0521 mmol, 11%) as a pale yellow oil; $[\alpha]_D$ - 7.4 (*c* 0.2, CH_3OH); UV (MeOH) λ_{max} (log *e*) 267 (3.85); IR (film) ν_{max} 3362, 2948, 2920, 2853, 1716, 1660, 160 cm^{-1}; ^1H NMR and ^{13}C NMR of compound **1** were identical to those of natural parazoanthine A;[130] HRMS-ESI: *m/z* $[M + H]^+$ calcd for $C_{15}H_{20}N_5O_3$: 318.1561; found: 318.1564.

^{1}H-NMR compound 1, CD_3OD, 400 MHz



[130] Nadja Cachet, Gregory Genta-Jouve, Erik L. Regalado, RedouaneMokrini, Philippe Amade, Gerald Culioli, and Olivier P. Thomas. *J.Nat.Prod.* ***2009,*** 72, 1612-1615.

Compound 2**:** at 0°C, *N*-ethylmorpholine (62 μL, 0.480 mmol) and compound **A'** (70 mg, 0.400 mmol) were added to a solution of *L*-arginine methyl ester dihydrochloride (105 mg, 0.435 mmol) in DMF (2 mL). The reaction mixture was stirred at 0°C for 1h and at r.t. for 5h; after evaporation under a stream of nitrogen, the residue was purified by silica gel chromatography using a chloroform/methanol gradient and further purified by HPLC RP-phase (H_2O-TFA 0.1%/MeOH, from 60/40 to 50/50 in 20 minutes, RP-amide semipreparative column, flow rate 2 mL/min) to give compound **2** (14 mg, 0.0439 mmol, 10%) as a pale yellow oil; UV (MeOH) λ_{max} (log *e*) 261 (4.50); IR (film) ν_{max} 3359, 2945, 2929, 1714, 1668, 1623 cm^{-1}; ^1H NMR (CD_3OD, 400 and 600 MHz): δ 7.48 (1H, d, *J*= 15.0 Hz, H-13), 7.37 (2H, d, *J* = 8.0 Hz, H-16 and H-20), 7.03 (1H, d, *J*=15.0 Hz, H-14), 6.92 (2H, d, *J*= 8.0 Hz, H-17 and H-19), 3.78 (3H, s, OMe), 4.20 (1H, t, *J* = 5.0 Hz, H-5), 3.26 (2H, t, *J*= 7.0 Hz, H_2-8), 1.96-1.88 (1H, m, H-6a), 1.84-1.78 (1H, m, H-6b), 1.79-1.71 (1H, m, H-7a), 1.69-1.62 (1H, m, H-7b); ^{13}C NMR (CD_3OD, 75 MHz): δ 174.2 (C, C-4), 163.1 (C, C-18), 162.0 (C, C-10), 157.2 (C, C-2), 129.6 (C, C-15), 128.2 (CH, C-16 and C-20), 121.4 (CH, C-14), 117.1 (CH, C-13), 115.1 (CH, C-17 and C-19), 56.9 (CH_3, OMe), 55.7 (CH, C-5), 41.9 (CH_2, C-8), 29.7 (CH_2, C-6), 24.9 (CH_2, C-7); HRMS-ESI: *m/z* $[M + H]^+$ calcd for $C_{16}H_{22}N_5O_3$: 332.3696; found: 332.3691.



2

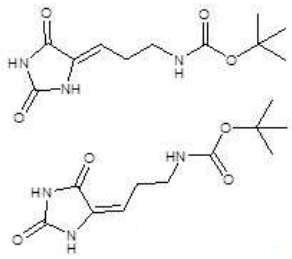
7.2 Synthetic procedure Parazoanthina B

Compound F: $(Boc)_2O$ (0.873 g, 4.0 mmol) and iodine (0.102 g, 0.4 mmol) were added to 3-amino-1-propanol (313 mL, 4.0 mmol) and the mixture was stirred for 2.5 h at room temperature; after portioning between sodium thiosulfate (6.0 g) and sodium bicarbonate (10.0 g) water solution (100 mL) and diethylether (200 mL), the organic phase was evaporated to give *N*-tert-butoxycarbonyl-amino-1-propanol (0.644 g, 3.9 mmol, 98%) that was oxidized with Dess-Martin reagent (2.80 g, 6.6 mmol) in dichloromethane (34 mL) overnight at room temperature; the mixture was partitioned between sodium thiosulfate (9.0 g) and sodium biocarbonate (10.0 g) water solution (100 mL) and dichloromethane (100 mL); the water was extracted with dichloromethane (100 mL) three times; the organic phase was evaporated and purified by silica gel chromatography using a gradient of *n*-hexane and ethyl acetate to give **F** (0.433 g, 2.5 mmol, 65%): colorless oil; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.65 (1H, bs, H-1), 3.27 (2H, bq, *J*= 6.2 Hz, H_2-3), 2.55 (2H, t, *J*= 5.9 Hz, H_2-2), 1.40-1.22 (9H, s, Boc methyls); HRESIMS *m/z* 196.0953 $[M+Na]^+$ (calcd for $C_8H_{15}NNaO_3$, 196.0950).[13]

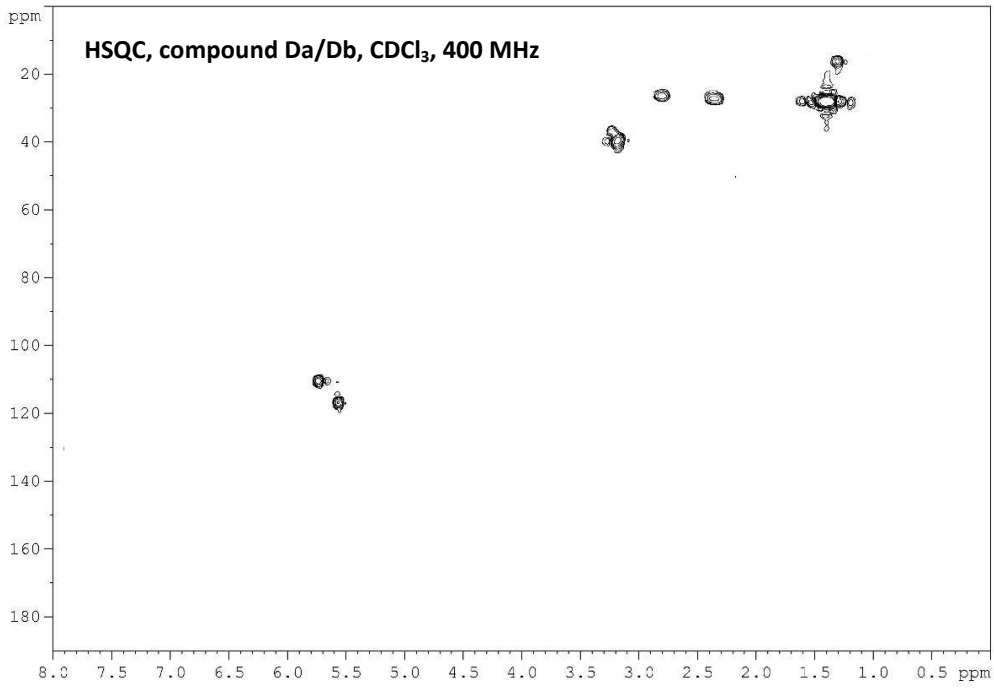
Compound G: Hydantoin (2.000 g, 20.0 mmol), was dissolved in glacial acetic acid (8 mL) and heated at 85 °C; a small amount of bromine (0.120 mL) was added and after 1 h and when the red color disappeared more bromine (1.08 mL) was added dropwise. After 30 min the mixture was cooled to 0 °C and triethyl phosphite (3.300 g, 28.0 mmol) was added; the mixture was heated at room temperature, stirred for 1.5 h and evaporated. The residue was crystallized in diethylether to give **G** (2.55 g,

10.8 mmol, 54%): white solid; NMR data was identical to those reported in the literature.[10]

Compound (Da/Db): Compound **G** (0.212 g, 0.9 mmol) was dissolved in an ethanolic solution (10 mL) of sodium ethoxide (74 mg, 1.08 mmol); compound **F** (0.130 g, 0.75 mmol) was added and the reaction was stirred at room temperature for 1.5 h; the mixture was evaporated and purified by silica gel chromatography using a chloroform/methanol gradient to give compound **Da/Db** as *Z*/*E* isomers mixture in the ratio 1.5/1.0 (0.161 g, 0.63 mmol, 84%): pale yellow oil; R_f (chloroform/methanol 8/2) = 0.72; IR (film) ν_{max} 3311, 2921, 2901, 2845, 1680, 1620 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.73 (1H, t, *J*= 8.4 Hz, H-3, *Z* form), 5.56 (1H, t, *J*= 8.1 Hz, H-3, *E* form), 3.23-3.14 (2H, m, H_2-1, *Z* and *E* forms), 2.79 (2H, m, H_2-2, *E* form), 2.35 (2H, m, H_2-2, *Z* form), 1.52-1.23 (9H, s, Boc methyls); ^{13}C NMR ($CDCl_3$): δ 164.4 (C, C-4', *E* form), 164.2 (C, C-4', *Z* form), 156.4 (C, Boc), 131.4 (C, C-5', *Z* form), 129.8 (C, C-5', *E* form), 116.7 (CH, C-3, *E* form), 110.5 (CH, C-3, *Z* form), 79.7 (C, Boc), 39.7 (CH_2, C-1, *Z* and *E* form), 28.4 (CH_3, Boc), 27.2 (CH_2, C-2, *Z* form), 26.5 (CH_2, C-2, *E* form); HRESIMS *m/z* 278.1112 $[M+Na]^+$ (calcd for $C_{11}H_{17}N_3NaO_4$, 278.1117).



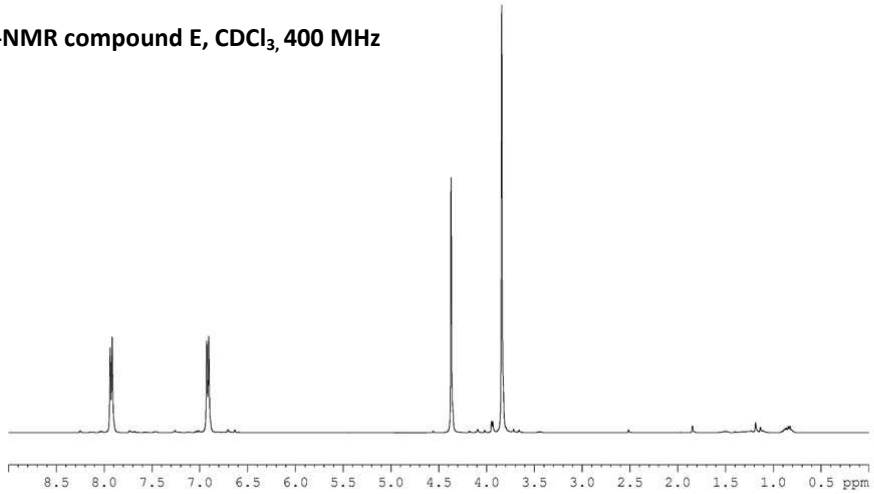
^{1}H-NMR compound Da/Db, $CDCl_3$, 400 MHz



4'-OMe-acetophenone: 4'-OH-acetophenone (1 g, 0.0074 mol) was dissolved in acetone (750 mL) with potassium carbonate (5.1 g, 0.037 mol); the mixture was refluxed for 15 min and iodomethane (9.6 g, 0.066 mol) was added; the reaction was refluxed for 3 h, filtered, evaporated and purified by silica gel chromatography using a gradient of petroleum ether and diethylether to give 4'-OMe-acetophenone (1.6 g, 0.007 mmol, 95%):colorless oil; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.94 (2H, d, *J*= 8.1 Hz, H-2', H-6'), 6.94 (2H, d, *J*= 8.1 Hz, H-3', H-5'), 3.86 (3H, OMe), 2.54 (3H, s, H_3-1); ^{13}C NMR ($CDCl_3$): δ 198.4 (C), 165.0 (C), 129.4 (2 CH), 127.4 (C), 112.3 (2 CH), 55.9 (OCH_3), 23.4 (CH_3).

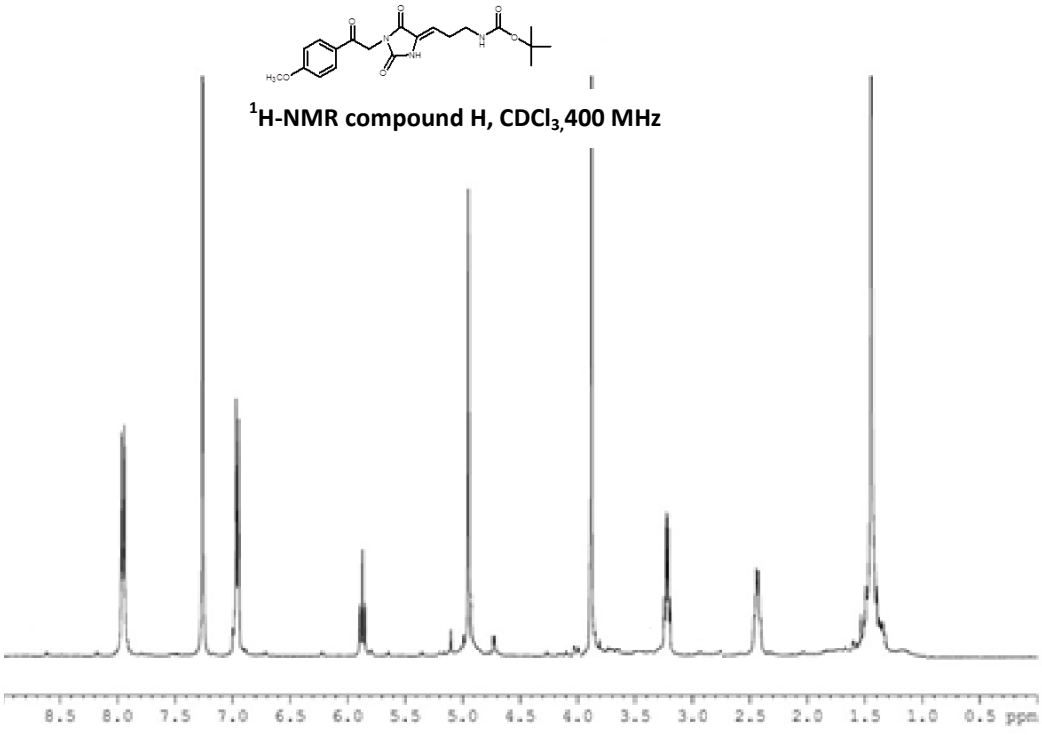
Compound E: 4'-OMe-acetophenone (1.04 g, 0.0066 mol) was dissolved in glacial acetic acid (7 mL); the temperature was increased at 50 °C and bromine (0.344 mL, 0.0066 mol) was added; the reaction was stirred overnight at 50 °C, evaporated and partitioned between a saturated sodium bicarbonate water solution (100 mL) and diethylether (100 mL); the organic phase was purified by silica gel chromatography using a gradient of petroleum ether and diethylether to give **E** (0.866 g, 0.0038 mol, 57%): colorless oil; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.95 (2H, d, *J* = 8.7 Hz, H-2', H-6'), 6.94 (2H, d, *J* = 8.7 Hz, H-3', H-5'), 4.38 (2H, bs, H_2-1), 3.86 (3H, OMe).

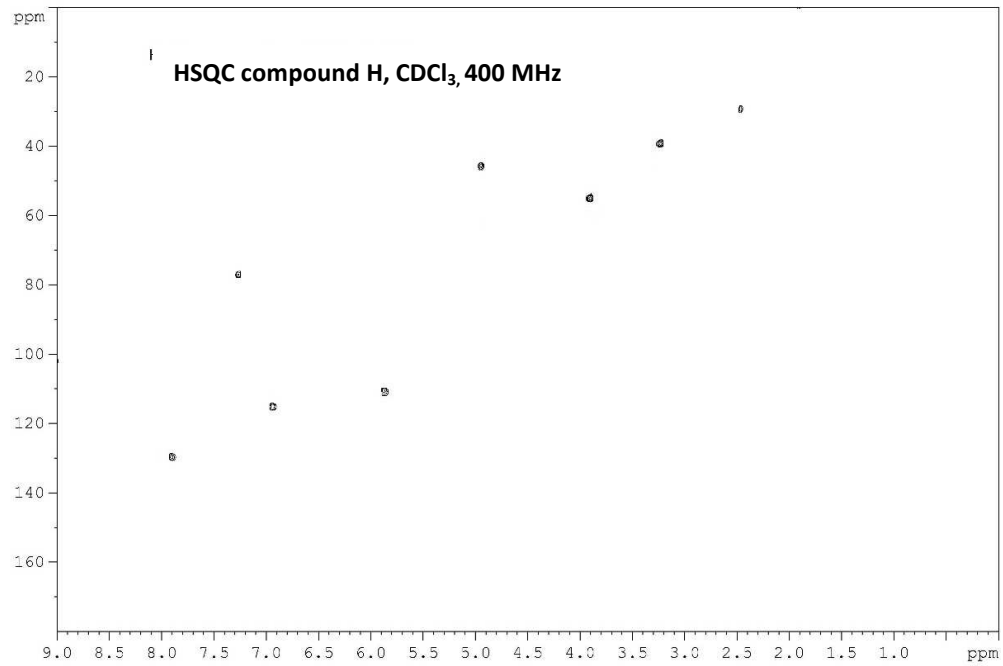
^{1}H-NMR compound E, $CDCl_3$, 400 MHz



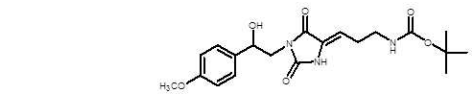
Compound H: Compound **Da/Db** (61 mg, 0.239 mmol) was dissolved in anhydrous DMF (2.5 mL); potassium carbonate (34 mg, 0.239 mmol) and TBAB (2 mg) were added; the mixture was heated at 50 °C under argon atmosphere and compound **E** (65 mg, 0.287 mmol) was added after 15-30 min; after 2 h the reaction was evaporated and purified by silica gel chromatography using a gradient of *n*-hexane and dichloromethane to give compound **H** (35 mg, 0.088 mmol, 37%): pale yellow oil; R_f (chloroform/methanol 95/5) = 0.35; IR (film) ν_{max} 3315, 2921, 2901, 2845, 1719, 1626 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.95 (2H, d, *J*= 8.7 Hz, H-16, H-20), 6.96 (2H, d, *J*= 8.7 Hz, H-17, H-19), 5.87 (1H, bt, *J*= 8.0 Hz, H-6), 4.95 (2H, bs, H_2-13), 3.90 (3H, s, OCH_3), 3.22 (2H, bq, *J*= 6.6 Hz, H_2-8), 2.44 (2H, m, H_2-7), 1.57-1.36 (9H, s, Boc methyls); ^{13}C NMR ($CDCl_3$): *δ* 189.0 (C, C-14), 162.4 (C, C-4), 164.1 (C, C-18), 156.2 (C, Boc), 153.8 (C, C-2), 131.2 (C, C-5), 130.0 (CH, C-16 and C-20), 129.7 (C, C-15), 115.2 (CH, C-17 and C-19), 111.0 (CH, C-6), 80.2 (C, Boc), 54.9 (CH_3, OMe), 43.4 (CH, C-13), 39.9 (CH_2,

C-8), 29.4, 29.0, 28.6 (CH_3, Boc methyls), 29.1 (CH_2, C-7); HRESIMS *m/z* 426.1644 $[M+Na]^+$ (calcd for $C_{20}H_{25}N_3NaO_6$, 426.1641).

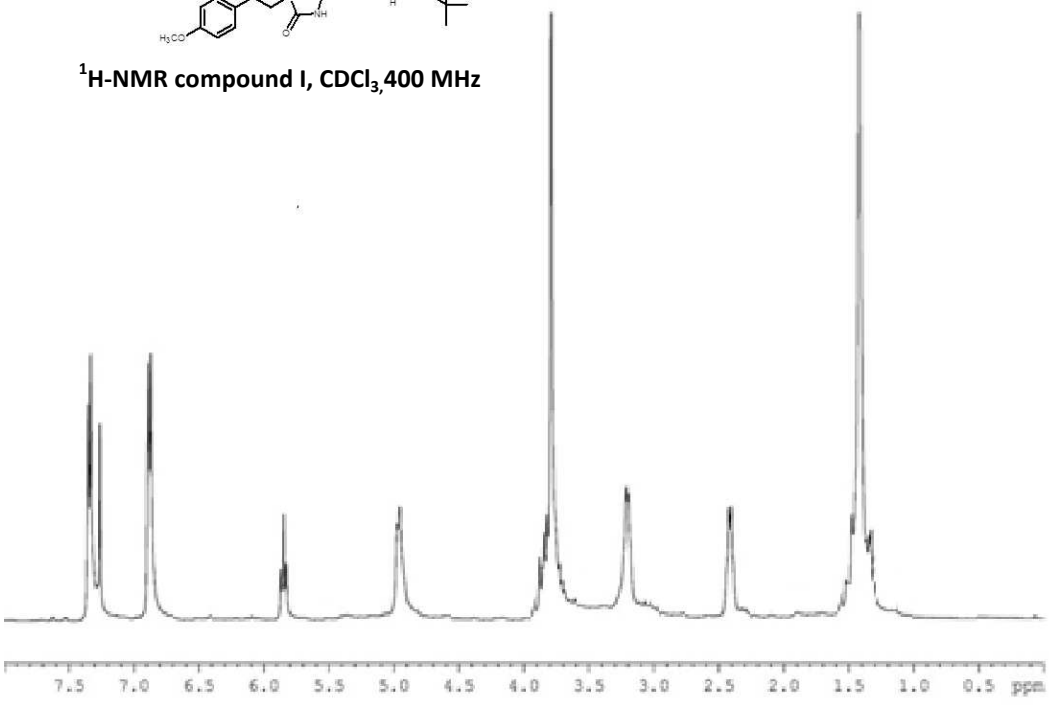


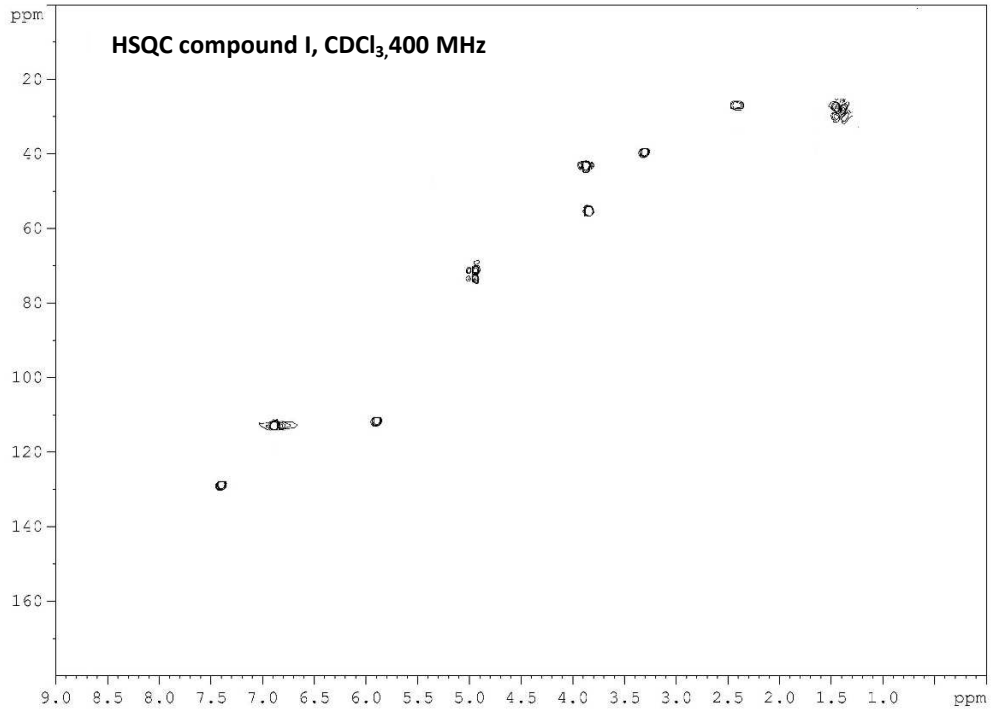


Compound I: Compound **H** (35 mg, 0.088 mmol) was dissolved in ethanol (3 mL) and sodium borohydride (4 mg, 0.105 mmol) was added; the mixture was stirred for 1 h at room temperature and after addition of some drops of water was partitioned between water (20 mL) and ethylacetate (20 mL); the organic phase was evaporated to get **I** (34 mg, 0.084 mmol, 95%): pale yellow oil; R_f (chloroform/methanol 95/5) = 0.30; IR (film) ν_{max} 3331, 2920, 2915, 2855, 1715, 1611 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.34 (2H, d, *J*= 7.7 Hz, H-16, H-20), 6.88 (2H, d, *J*= 7.7 Hz, H-17, H-19), 5.85 (1H, t, *J*= 7.3 Hz, H-6), 4.97 (1H, bd, *J*= 6.5 Hz, H-14), 3.82 (2H, m, H_2-13), 3.81 (3H, s, OCH_3), 3.20 (2H, m, H_2-8), 2.42 (2H, m, H_2-7), 1.47-1.34 (9H, s, Boc methyls); ^{13}C NMR ($CDCl_3$): *δ* 162.9 (C, C-4), 159.1 (C, C-18), 156.3 (C, Boc), 156.1 (C, C-2), 132.8 (C, C-15), 130.1 (C, C-5), 127.5 (CH, C-16 and C-20), 115.2 (CH, C-17 and C-19), 111.8 (CH, C-6), 80.1 (C, Boc), 73.4 (CH, C-14), 54.9 (CH_3, OMe), 44.5 (CH, C-13), 39.8 (CH_2, C-8), 29.8, 29.2, 28.6 (CH_3, Boc methyls), 29.0 (CH_2, C-7); HRESIMS *m/z* 428.1794 $[M+Na]^+$ (calcd for $C_{20}H_{27}N_3NaO_6$, 428.1798).



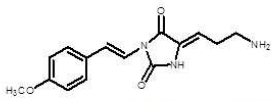
^{1}H-NMR compound I, $CDCl_3$, 400 MHz



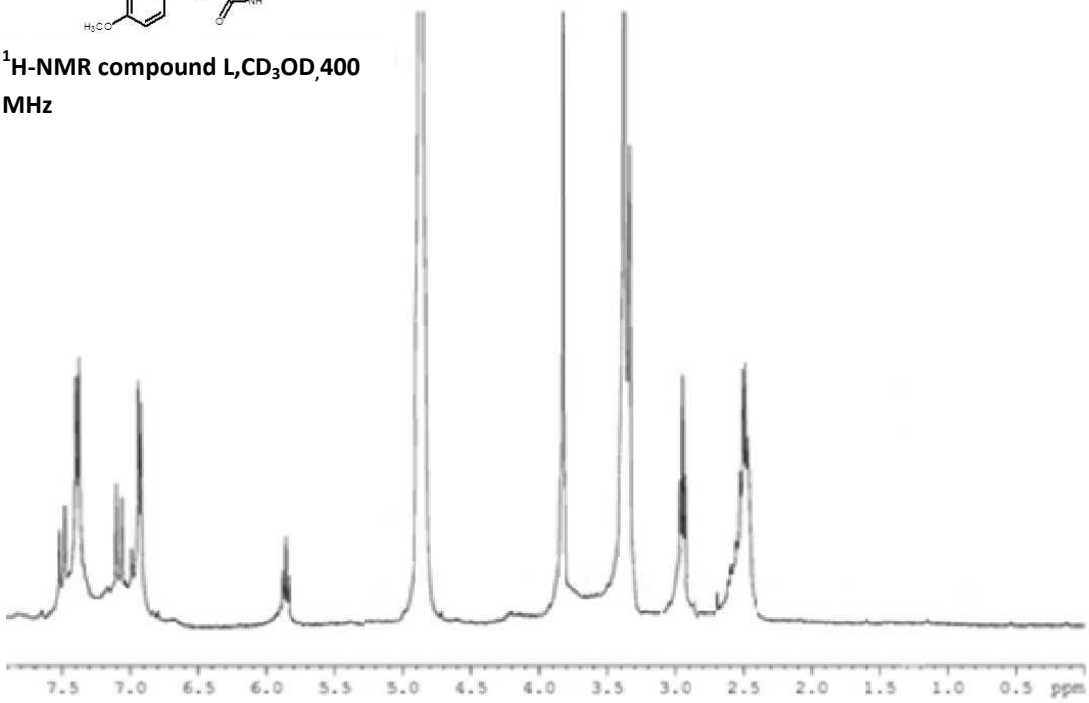


Compound L: Compound **I** (34 mg, 0.084 mmol) was dissolved in toluene (3 mL) with monohydrated *p*-toluensulfonic acid (32 mg, 0.168 mmol); the mixture was heated at 110 °C for 1 h, evaporated and purified twice by silica gel chromatography using a gradient of chloroform and methanol to give **L** (11.7 mg, 0.041 mmol, 49%): pale yellow oil; R_f (chloroform/methanol 6/4) = 0.50; IR (film) ν_{max} 3319, 2955, 2928, 2881, 1719, 1626 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.50 (1H, d, *J*= 15.4 Hz, H-14), 7.39 (2H, bd, *J*= 8.4 Hz, H-16, H-20), 7.08 (1H, d, *J*= 15.4 Hz, H-13), 6.93 (2H, d, *J*= 8.4 Hz, H-17, H-19), 5.86 (1H, t, *J*= 7.6 Hz, H-6), 3.86 (3H, s, OCH_3), 2.95 (2H, bt, *J*= 7.3 Hz, H_2-8), 2.50 (2H, bq, *J*= 7.6 Hz, H_2-7); ^{13}C NMR (CD_3OD): *δ* 160.6 (C, C-4), 159.2 (CH, C-18), 153.6 (C, C-2), 131.7 (C, C-5), 127.7 (CH, C-16 and C-20), 126.4 (C, C-15), 120.4 (CH, C-14), 116.0 (CH, C-13), 114.4 (CH, C-17, C-19), 110.5 (CH, C-6), 54.9 (CH_3, OMe), 40.2 (CH_2, C-

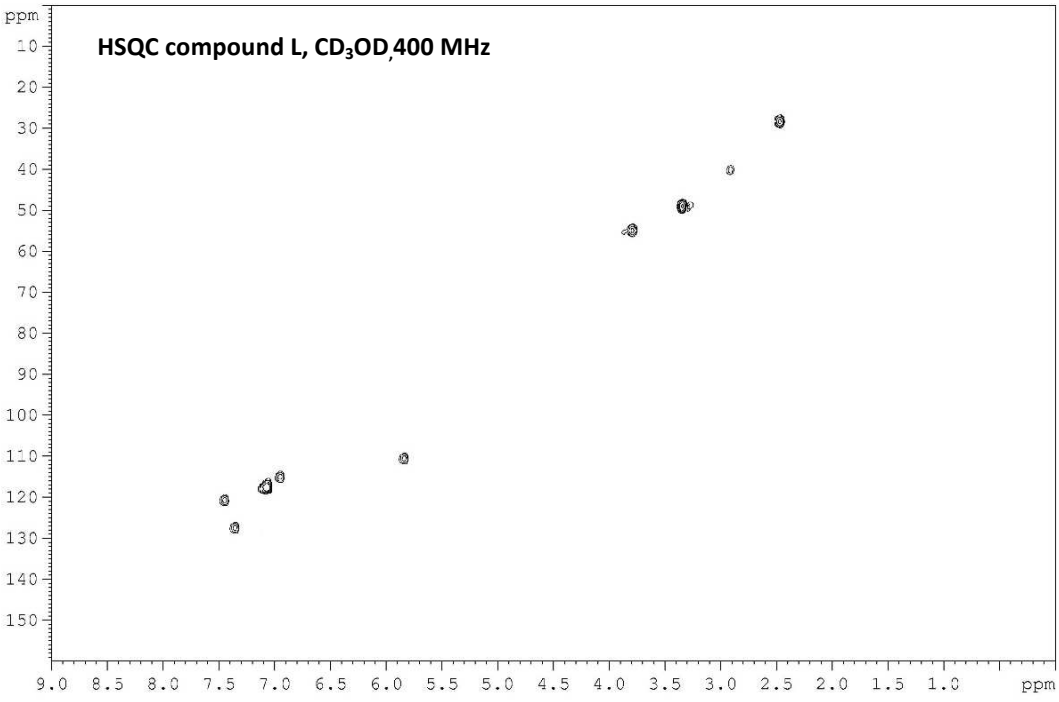
8), 28.3 (CH_2, C-7); HRESIMS *m/z* 287.1275 $[M+H]^+$ (calcd for $C_{15}H_{18}N_3O_3$, 287.1270).



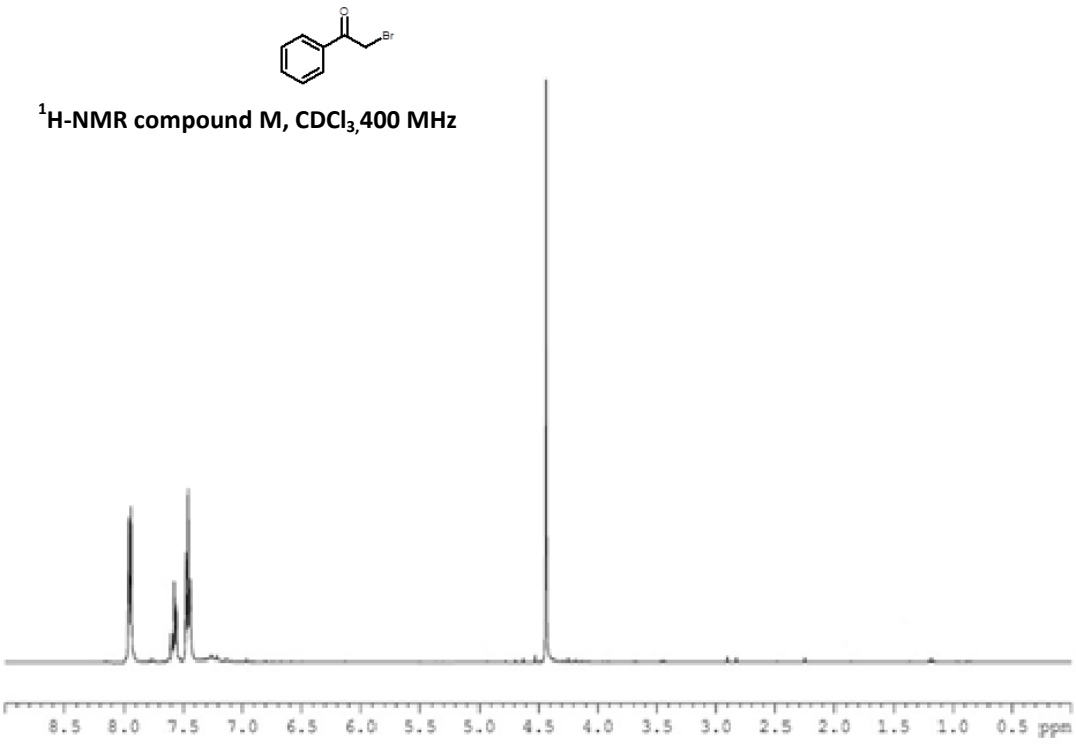
^{1}H-NMR compound L,CD_3OD,400 MHz



HSQC compound L, CD_3OD,400 MHz

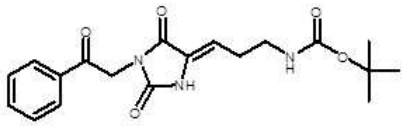


Compound M: Acetophenone (0.466 mL, 0.004 mol) was dissolved in glacial acetic acid (4 mL) and heated at 50 °C; a small amount of bromine (0.040 mL) was added and after 1 h the rest of bromine (0.166 mL) was added; after overnight of stirring the mixture was evaporated and partitioned between a saturated sodium bicarbonate water solution (20 mL) and diethylether (20 mL) that was evaporated and purified by silica gel chromatography using a gradient of light petroleum ether/diethylether to give compound **M** (0.494 g, 2.48 mmol, 62%): pale yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.97 (2H, d, *J*= 8.1 Hz, H-2', H-6'), 7.59 (1H, bt, *J*= 7.6 Hz, H-4'), 7.48 (2H, t, *J*= 8.1 Hz, H-3', H-5'), 4.45 (2H, bs, H_2-1).

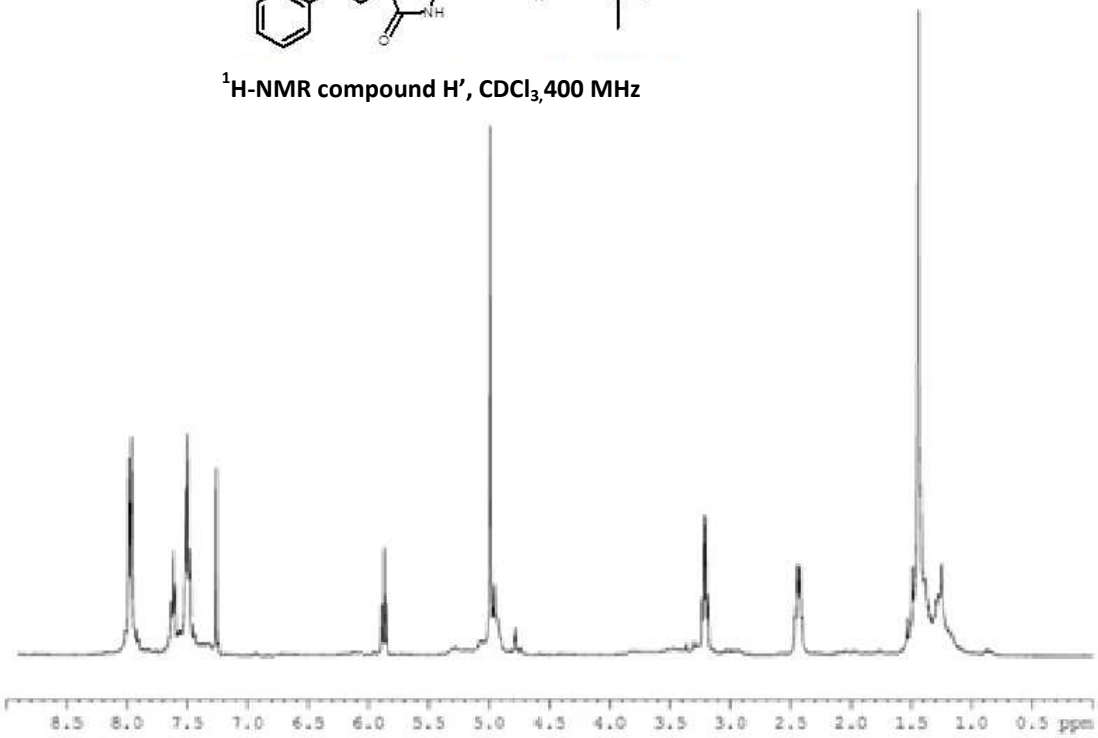


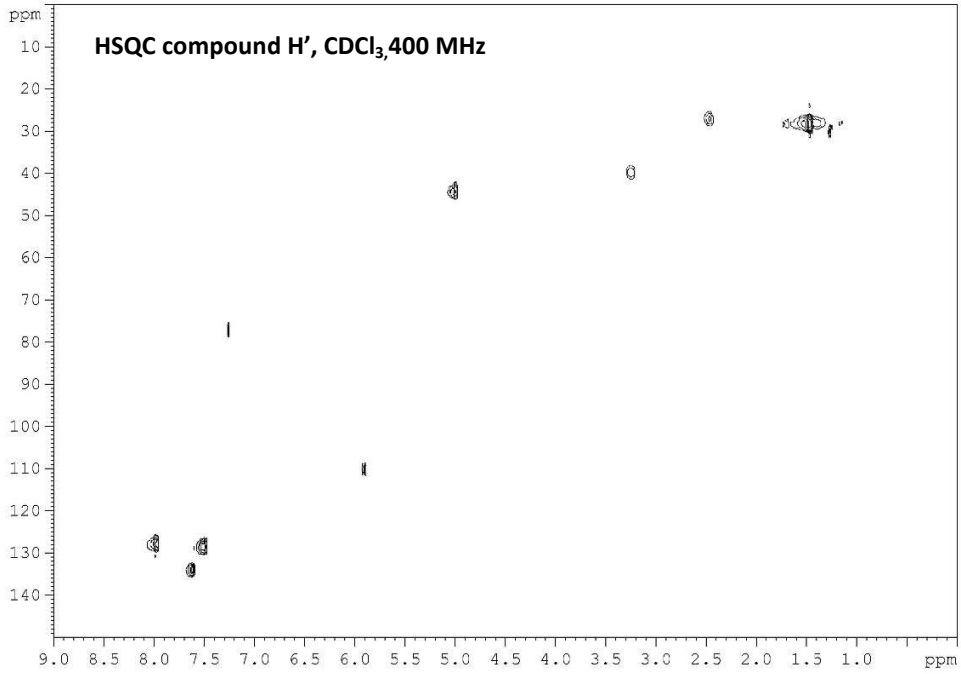
^{1}H-NMR compound M, $CDCl_3$, 400 MHz

Compound H': Compound **Da/Db** (61 mg, 0.239 mmol) was dissolved in anhydrous DMF (2.5 mL), potassium carbonate (34 mg, 0.239 mmol) and TBAB (2 mg) were added; the mixture was heated at 50 °C and compound **M** (58 mg, 0.287 mmol) was added; after 2h the reaction was evaporated and purified by silica gel chromatography using a gradient of *n*-hexane and dichloromethane to give compound **H'** (30 mg, 0.079 mmol, 33%): pale yellow oil; R_f (chloroform/methanol 95/5) = 0.80; IR (film) ν_{max} 3350, 2918, 2939, 2840, 1728, 1620 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.97 (2H, d, *J*= 7.5 Hz, H-16, H-20), 7.62 (1H, bt, *J* = 7.7 Hz, H-18), 7.50 (2H, t, *J* = 7.5 Hz, H-17, H-19), 5.88 (1H, t, *J* = 7.9 Hz, H-6), 4.99 (2H, bs, H_2-13), 3.22 (2H, bq, *J* = 6.4 Hz, H_2-8), 2.44 (2H, m, H_2-7), 1.53-1.35 (9H, s, Boc methyls); ^{13}C NMR ($CDCl_3$): *δ* 190.5 (C, C-14), 162.8 (C, C-4), 156.3 (C, Boc), 153.4 (C, C-2), 131.1 (C, C-5), 134.1, 128.7, 127.8 (CH, aromatic), 110.3 (CH, C-6), 80.4 (C, Boc), 44.3 (CH, C-13), 39.8 (CH_2, C-8), 28.7, 28.2, 27.9 (CH_3, Boc methyls), 27.1 (CH_2, C-7); HRESIMS *m/z* 396.1531 $[M+Na]^+$ (calcd for $C_{19}H_{23}N_3NaO_5$, 396.1535).

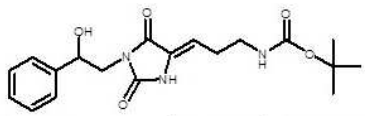


^{1}H-NMR compound H', $CDCl_3$, 400 MHz

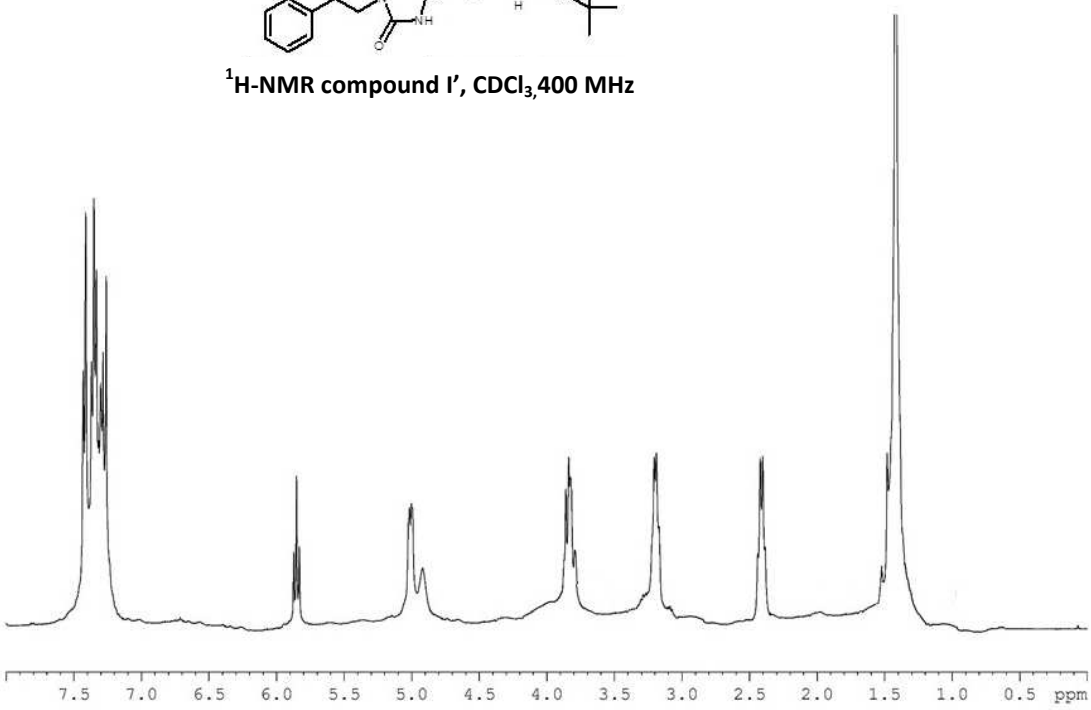




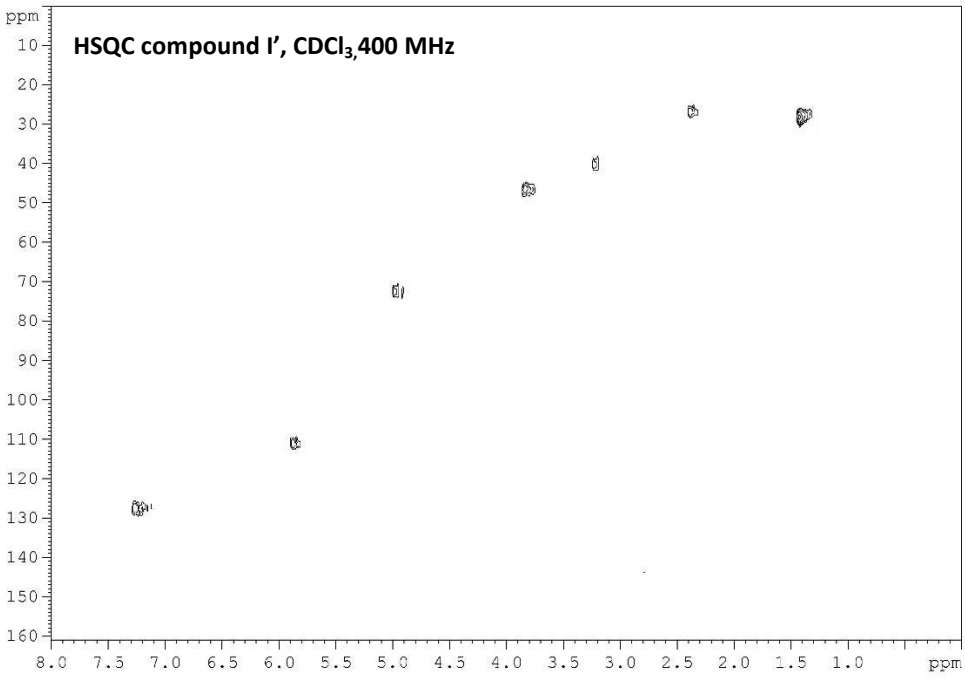
Compound I': Compound **H'** (30 mg, 0.079 mmol) was dissolved in ethanol (3 mL) and sodium borohydride (3.6 mg, 0.095 mmol) was added; the mixture was stirred for 1 h at room temperature and after addition of some drops of water was partitioned between water (20 mL) and ethylacetate (20 mL); the organic phase was evaporated to give **I'** (28 mg, 0.076 mmol, 96%): pale yellow oil; R_f (chloroform/methanol 95/5) = 0.45; IR (film) ν_{max} 3350, 2929, 2909, 2863, 1735, 1618 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.42 (2H, bd, *J* = 7.8 Hz, H-16, H-20), 7.35 (2H, bt, *J* = 7.8 Hz, H-17, H-19), 7.30 (1H, bt, *J* = 7.6 Hz, H-18), 5.85 (1H, t, *J*= 8.4 Hz, H-6), 5.01 (1H, dd, *J* = 2.7, 7.8 Hz, H-14), 3.86 (2H, m, H_2-13), 3.20 (2H, bq, *J* = 6.1 Hz, H_2-8), 2.41 (2H, bq, *J* = 6.9 Hz, H_2-7), 1.50-1.38 (9H, s, Boc methyls); ^{13}C NMR ($CDCl_3$): *δ* 162.8 (C, C-4), 156.8 (C, Boc), 156.2 (C, C-2), 140.8 (C, C-15), 130.3 (C, C-5), 128.2, 127.5, 127.1 (CH, aromatic), 110.6 (CH, C-6), 80.5 (C, Boc), 73.3 (CH, C-14), 47.2 (CH, C-13), 40.4 (CH_2, C-8), 28.7, 28.4, 27.5 (CH_3, Boc methyls), 27.9 (CH_2, C-7); HRESIMS *m/z* 398.1696 $[M+Na]^+$ (calcd for $C_{19}H_{25}N_3NaO_5$, 398.1692).



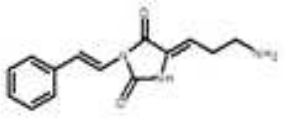
^{1}H-NMR compound I', $CDCl_3$,400 MHz



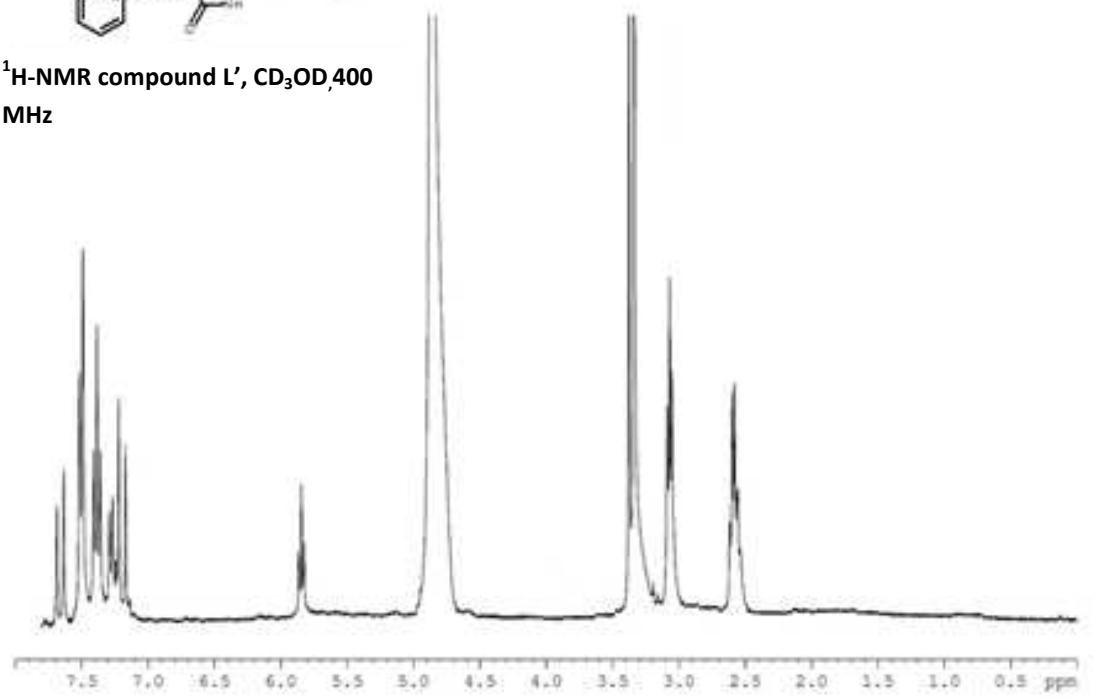
HSQC compound I', $CDCl_3$,400 MHz

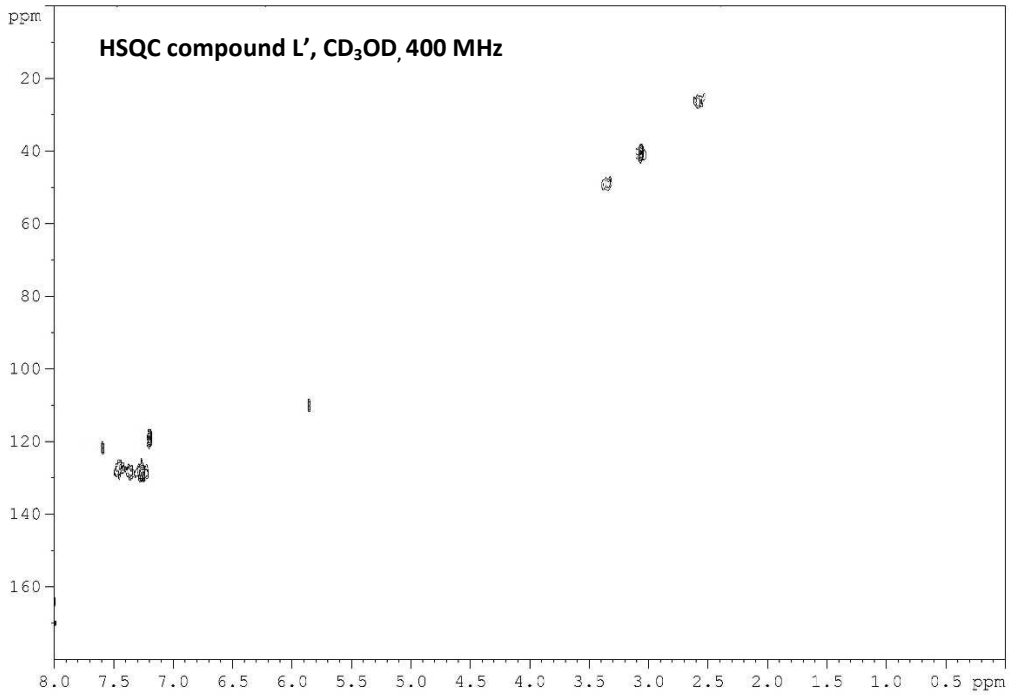


Compound L': Compound **I'** (28 mg, 0.076 mmol) was dissolved in toluene (3 mL) with monohydrated *p*-toluensulfonic acid (26 mg, 0.152 mmol); the mixture was heated at 110 °C for 1 h, evaporated and purified twice by silica gel chromatography using a gradient of dichloromethane and methanol to give **L'** (8.7 mg, 0.034 mmol, 45%): pale yellow oil; R_f (chloroform/methanol 7/3) = 0.35; IR (film) ν_{max} 3320, 2935, 2909, 2872, 1716, 1620 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.67 (1H, d, *J*= 15.1 Hz, H-14), 7.50 (2H, bd, *J* = 7.5 Hz, H-16, H-20), 7.38 (2H, bt, *J* = 7.5 Hz, H-17, H-19), 7.27 (1H, bt, *J* = 7.6 Hz, H-18), 7.23 (1H, d, *J*= 15.1 Hz, H-13), 5.87 (1H, t, *J* = 7.6 Hz, H-6), 3.11 (2H, bt, *J* = 7.3 Hz, H_2-8), 2.58 (2H, bq, *J* = 7.3 Hz, H_2-7); ^{13}C NMR (CD_3OD): δ 162.4 (C, C-4), 153.3 (C, C-2), 137.1 (C, C-15), 131.8 (C, C-5), 128.5, 128.0, 127.3 (CH, aromatic), 120.7 (CH, C-14), 118.4 (CH, C-13), 109.8 (CH, C-6), 40.1 (CH_2, C-8), 26.3 (CH_2, C-7); HRESIMS *m/z* 257.1205 $[M+H]^+$ (calcd for $C_{14}H_{16}N_3O_2$, 257.1200); HRESIMS *m/z* 280.2773 $[M+Na]^+$ (calcd for $C_{14}H_{15}N_2NaO_2$ 280.2776)



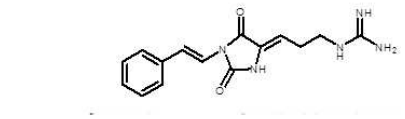
^{1}H-NMR compound L', CD_3OD, 400 MHz



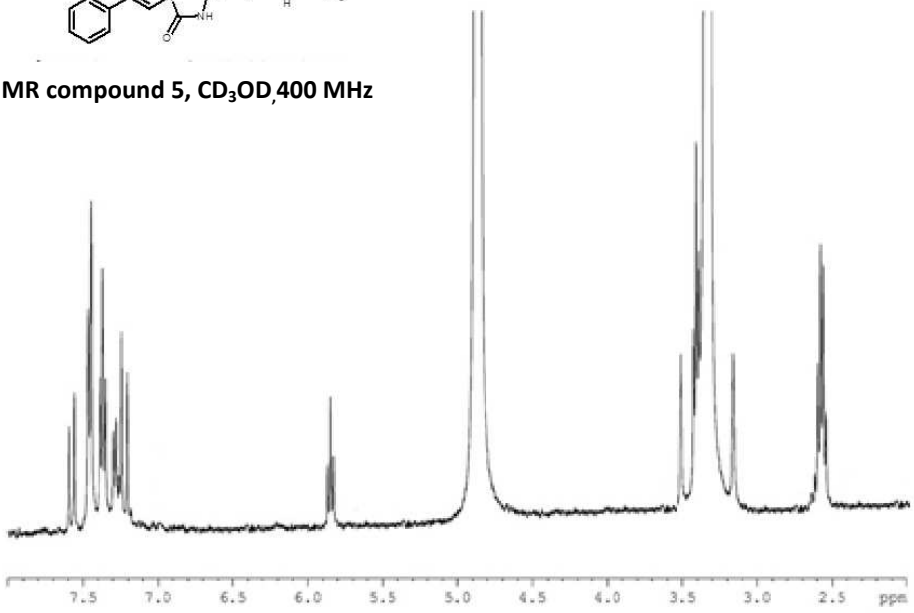


18-deoxy-parazoanthine B (5): Compound **L'** (8.7 mg, 0.034 mmol) was dissolved in anhydrous DMF (0.5 mL) with DIPEA (0.051 mmol) and 3,5-dimethyl-1-pyrazolylformaminidium nitrate (0.010 g, 0.051 mmol); the mixture was stirred overnight at room temperature under argon atmosphere, evaporated and fractioned by silica gel chromatography using a gradient of chloroform and methanol and further purified by HPLC RP-phase (H_2O-TFA 0.1%/MeOH, from 60/40 to 50/50 in 20 minutes, RP-amide analytical column, flow rate 1 mL/min) to give **5** (6.3 mg, 0.021 mmol, 62%): pale yellow oil; R_f (chloroform/methanol 6/4) = 0.30; IR (film) ν_{max} 3340, 2948, 2929, 2852, 1723, 1648 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.58 (1H, d, *J*= 15.0 Hz, H-14), 7.46 (2H, d, *J* = 7.4 Hz, H-16, H-20), 7.37 (2H, bt, *J* = 7.5 Hz, H-17, H-19), 7.28 (1H, bt, *J* = 7.4 Hz, H-18), 7.22 (1H, d, *J* = 15.0 Hz, H-13), 5.85 (1H, bt, *J* = 7.7 Hz, H-6), 3.41 (2H, bt, *J* = 7.0 Hz, H_2-

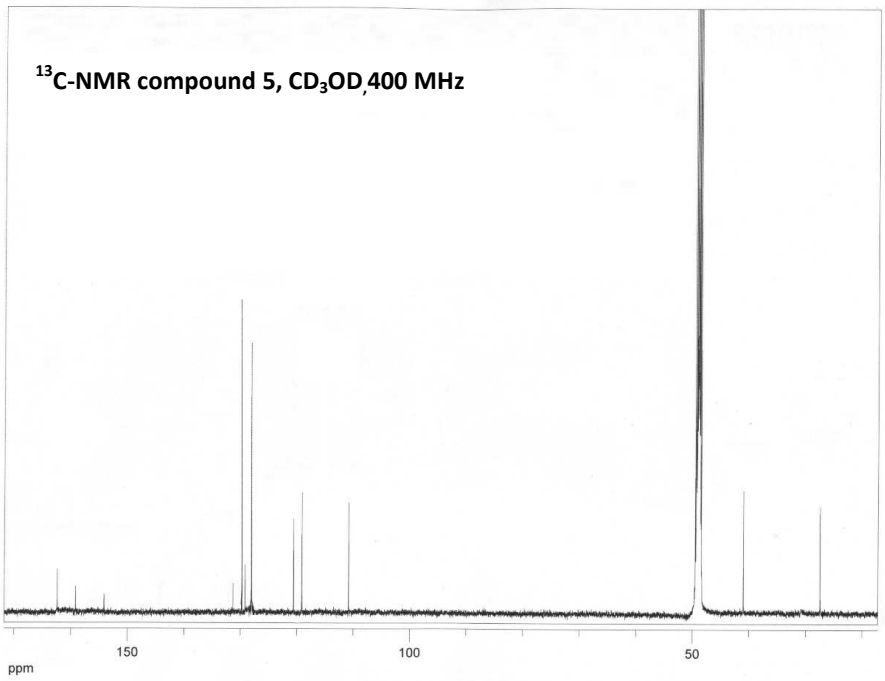
8), 2.58 (2H, bq, *J* =7.0, H_2-7); ^{13}C NMR (CD_3OD): *δ* 162.4 (C, C-4), 158.7 (C, C-10), 153.9 (C, C-2), 137.2 (C, C-15), 131.6 (C, C-5), 129.5 (CH, C-17 and C-19), 129.0 (CH, C-18), 127.2 (CH, C-16 and C-20), 120.8 (CH, C-14), 119.0 (CH, C-13), 110.8 (CH, C-6), 40.9 (CH_2, C-8), 26.4 (CH_2, C-7); HRESIMS *m/z* 300.1465 $[M+H]^+$ (calcd for $C_{15}H_{18}N_5O_2$, 300.1460).



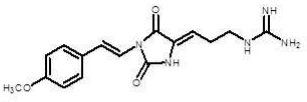
^{1}H-NMR compound 5, CD_3OD, 400 MHz



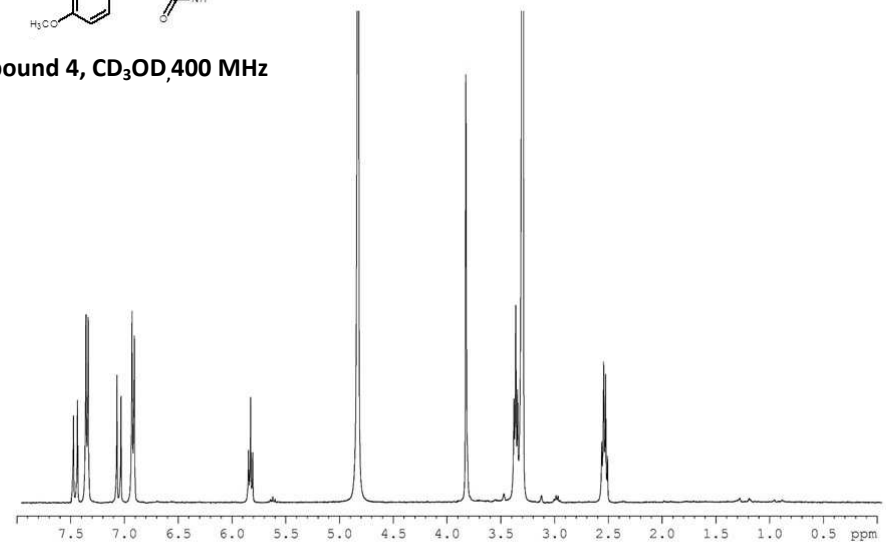
^{13}C-NMR compound 5, CD_3OD, 400 MHz



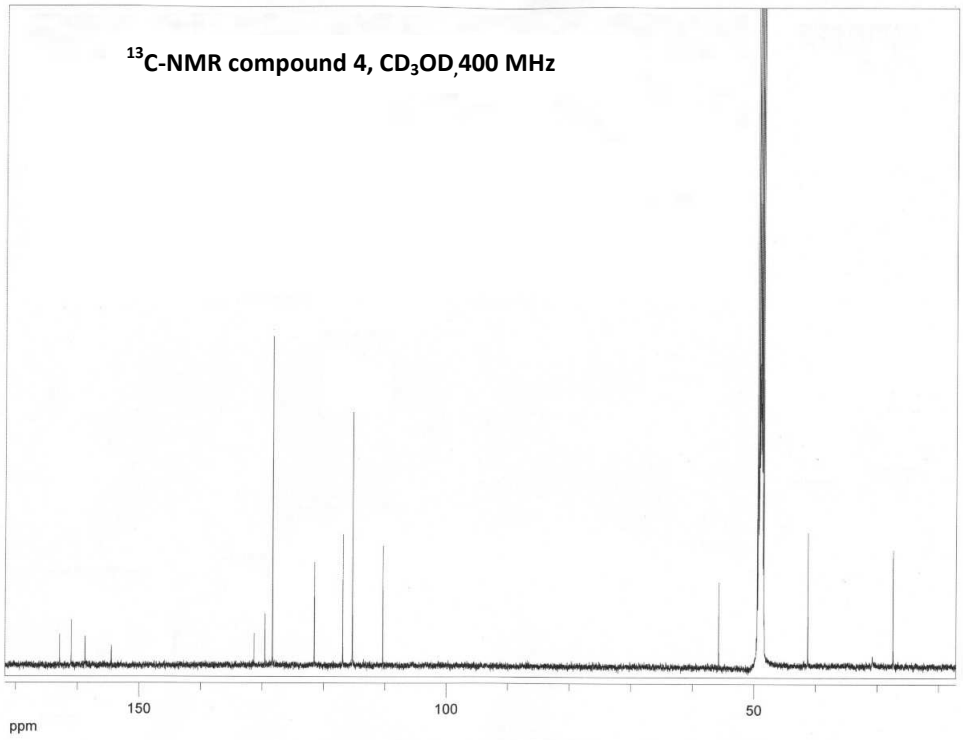
Parazoanthine C (4): Compound **L** (11.7 mg, 0.041 mmol) was dissolved in anhydrous DMF (0.5 mL) with DIPEA (0.062 mmol) and 3,5-dimethyl-1-pyrazolylformaminidium nitrate (0.012 g, 0.062 mmol); the mixture was stirred overnight at room temperature under argon atmosphere, evaporated, fractioned by silica gel chromatography using a gradient of chloroform and methanol and further purified by HPLC RP-phase (H_2O-TFA 0.1%/MeOH, from 60/40 to 50/50 in 20 minutes, RP-amide analytical column, flow rate 1 mL/min) to give parazoanthine C **(4)** (8.6 mg, 0.026 mmol, 63%): pale yellow oil; R_f (chloroform/methanol 6/4) = 0.25; IR (film) ν_{max} 3368, 2951, 2925, 2852, 1720, 1656 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.48 (1H, d, *J* = 14.8 Hz, H-14), 7.36 (2H, d, *J* = 8.3 Hz, H-16, H-20), 7.06 (1H, d, *J* = 14.8 Hz, H-13), 6.91 (2H, d, *J* = 8.3 Hz, H-17, H-19), 5.83 (1H, bt, *J* = 7.8 Hz, H-6), 3.80 (3H, s, OMe), 3.38 (2H, bt, *J* = 7.0 Hz, H_2-8), 2.54 (2H, bq, *J* =7.0, H_2-7); ^{13}C NMR (CD_3OD): δ 163.0 (C, C-4), 161.0 (C, C-18), 158.9 (C, C-10), 154.6 (C, C-2), 131.3 (C, C-5), 129.6 (C, C-15), 128.5 (CH, C-16 and C-20), 121.5 (CH, C-14), 117.0 (CH, C-13), 115.4 (CH, C-17 and C-19), 110.2 (CH, C-6), 55.8 (CH_3, OMe), 41.3 (CH_2, C-8), 27.5 (CH_2, C-7); HRESIMS *m/z* 330.1571 $[M+H]^+$ (calcd for $C_{16}H_{20}N_5O_3$, 330.1566).



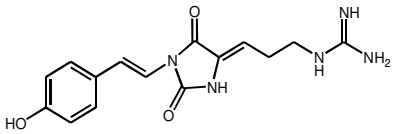
^{1}H-NMR compound 4, CD_3OD, 400 MHz



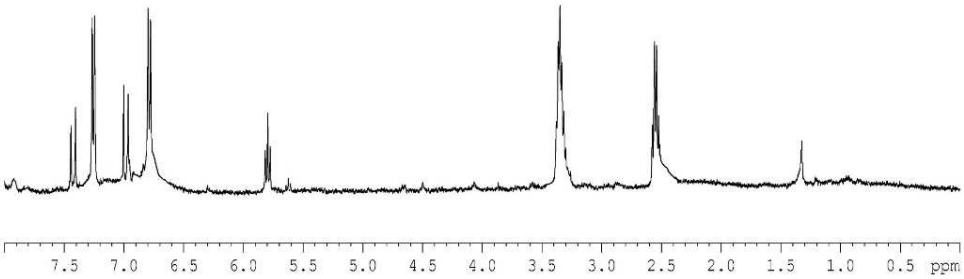
^{13}C-NMR compound 4, CD_3OD, 400 MHz



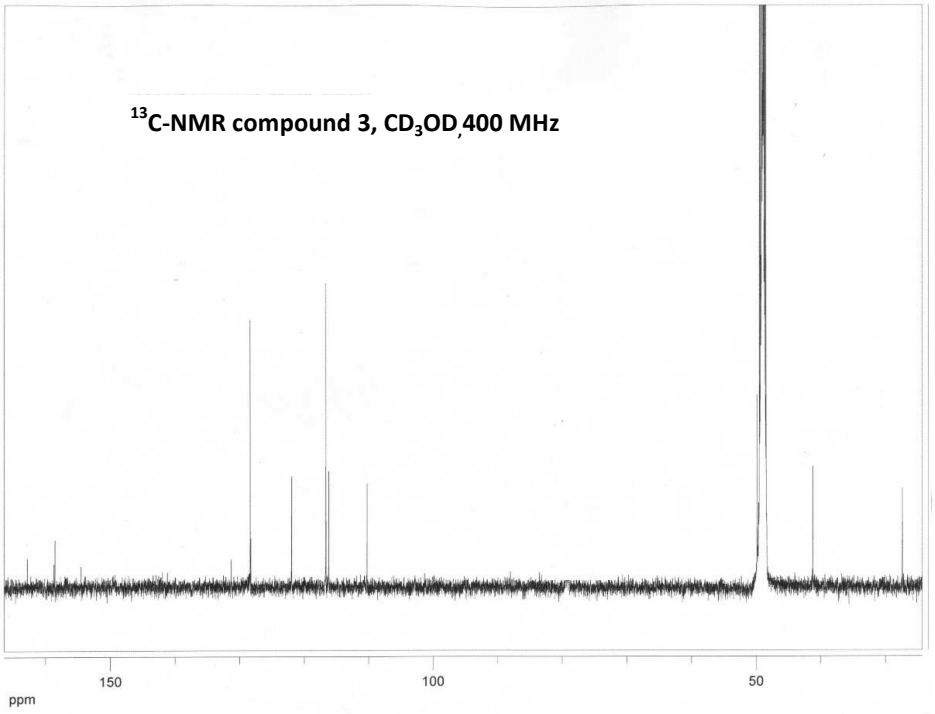
Parazoanthine B (3): Parazoanthine C (**4**) (8.6 mg, 0.026 mmol) was dissolved in 1 mL of anhydrous dichloromethane boron tribromide solution (0.3 M), prepared by diluting a more concentrated dichloromethane boron tribromide solution (1M); the mixture was stirred for 1 h at 40 °C under argon atmosphere, evaporated, stirred with methanol/water solution (1/1, 1.5 mL) for ten minutes, dried and purified twice by silica gel chromatography using a gradient of dichloromethane and methanol to give **3** (3.8 mg, 0.012 mmol, 46 %): pale yellow oil; IR (film) ν_{max} 3347, 2956, 2928, 2857, 1716, 1671 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.43 (1H, d, *J* = 14.7 Hz, H-14), 7.27 (2H, d, *J* = 8.2 Hz, H-16, H-20), 6.98 (1H, d, *J* = 14.7 Hz, H-13), 6.78 (2H, d, *J* = 8.2 Hz, H-17, H-19), 5.80 (1H, bt, *J* = 7.8 Hz, H-6), 3.35 (2H, bt, *J* = 7.1 Hz, H_2-8), 2.54 (2H, bq, *J* = 7.0, H_2-7); ^{13}C NMR (CD_3OD): *δ* 162.9 (C, C-4), 158.9 (C, C-10), 157.7 (C, C-18), 154.5 (C, C-2), 131.3 (C, C-5), 128.5 (CH, C-16 and C-20), 128.4 (C, C-15), 121.9 (CH, C-14), 116.7 (CH, C-17 and C-19), 116.2 (CH, C-13), 110.2 (CH, C-6), 41.1 (CH_2, C-8), 27.4 (CH_2, C-7); HRESIMS *m/z* 316.1405 $[M+H]^+$ (calcd for $C_{15}H_{18}N_5O_3$, 316.1410).



^{1}H-NMR compound 3, CD_3OD, 400 MHz



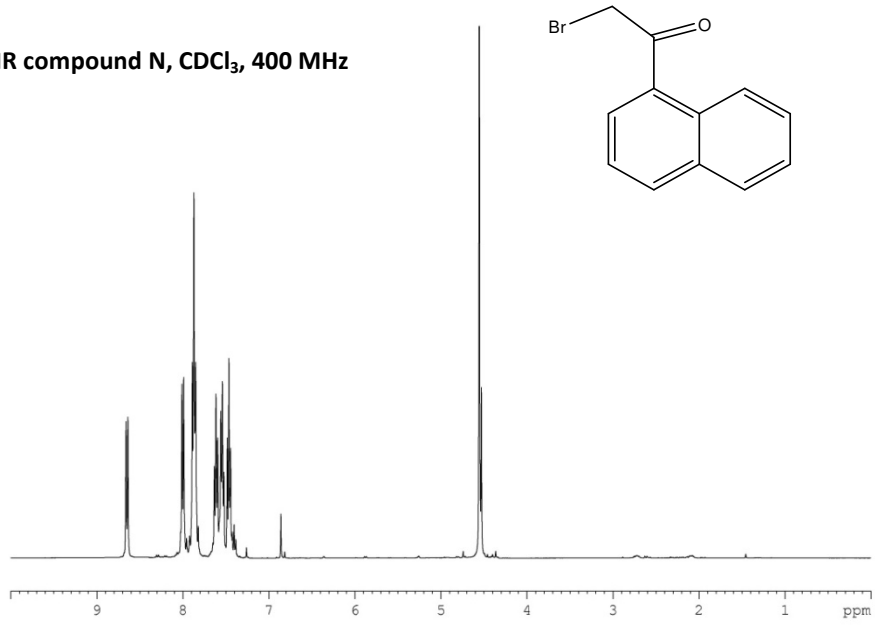
^{13}C-NMR compound 3, CD_3OD, 400 MHz



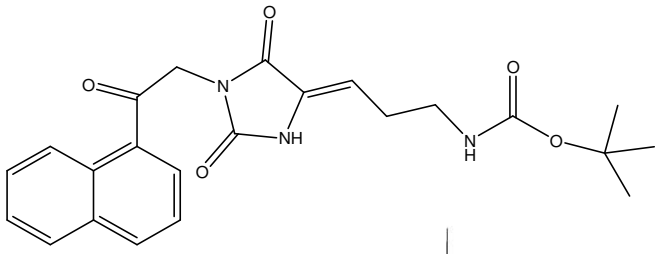
7.3 *Synthesis of Para-N (6)*

***Compound N*:** 1-acetophenone (3.318 g, 0.0194 mol) was dissolved in glacial acetic acid (30 mL) at room temperature and bromine (1 mL, 0.0390 mol) was added; the reaction was stirred 10 minutes at room temperature, evaporated and partitioned between a saturated sodium bicarbonate water solution (100 mL) and diethylether (100 mL); diethylether was dry with sodium sulfate to give **N.** (4 g, 0.0161 mol, 83%); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.65 (1H, d, *J* = 8.7 Hz), 8.01 (1H, d, *J* = 8.2 Hz), 7.88 (1H, d, *J* = 7.04 Hz partially overlapped), 7.85 (1H, d, *J* = 8.05 Hz partially overlapped), 7.62 (1H, t, *J* = 7.95 Hz), 7.55 (1H, t, *J* = 7.52 Hz), 7.47 (1H, t, *J* = 7.62 Hz), 4.55 (2H, s); ^{13}C NMR ($CDCl_3$): *δ* 194.4 (C=O), 128.1 (C), 133.6 (CH), 128.2 (CH), 127.8 (2CH), 125.5 (CH), 125.1 (CH), 123.1 (CH), 32.0 (CH_2); HRESIMS *m/z* 249.2701 $[M+H]^+$ (calcd for $C_{12}H_9BrO$, 247.9837).

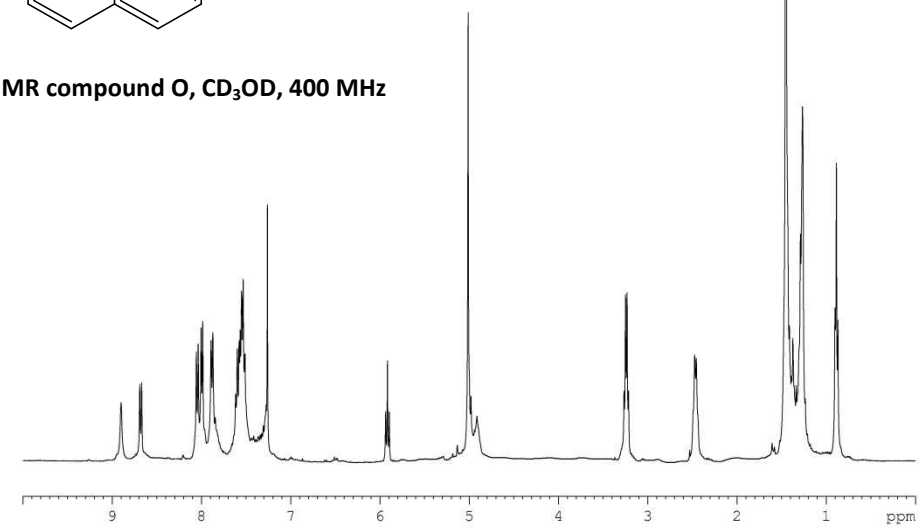
^{1}H-NMR compound N, $CDCl_3$, 400 MHz



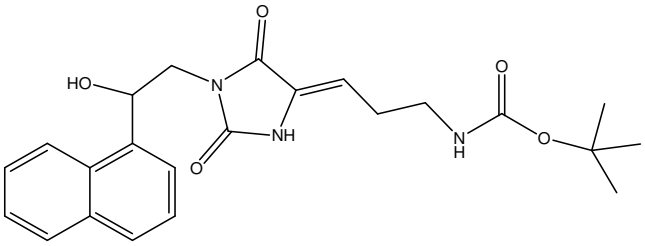
Compound O: Compound **Da/Db** (150 mg, 0.587 mmol) was dissolved in anhydrous DMF (6.0 mL); potassium carbonate (121 mg, 0.876 mmol) and TBAB (14 mg) were added; the mixture was heated at 50 °C under argon atmosphere with molecular sieves and compound **N** (176 mg, 0.416 mmol) was added after 15-30 min; after 2 h the reaction was evaporated and purified by silica gel chromatography using a gradient of chloroform and methanol to give compound **O** (58 mg, 0.137 mmol, 33%); ^{1}H-NMR (400 MHz, CD_3OD): δ 8.89 (1H, bs, NH), 8.67 (1H, d, *J*= 8.37 Hz), 8.04 (1H, d, *J*= 8.2 Hz), 7.99 (1H, d, *J*= 7.27 Hz), 7.88 (1H, d, *J*= 7.95), 7.60-7.50 (2H, m, overlapped), 5.91 (1H, t, *J*= 8.23 Hz) 5.06 (2H, s), 3.22 (2H, m), 2.44 (2H, m) 1.44 (9H, s, Boc methyls); ^{13}C NMR (CD_3OD): *δ* 194.4 (C), 162.4 (C), 156.2 (C, Boc), 153.8 (C), 132.7 (CH), 127.6 (CH), 127.1 (CH) 124.9 (CH), 109.9 (CH), 80.2 (C, Boc), 45.1 (CH_2), 38.7 (CH_2), 26.8 (CH_2), 29.4, 29.0, 28.6 (CH_3, Boc methyls),; HRESIMS *m/z* 423.1798 $[M+Na]^+$ (calcd for $C_{23}H_{25}N_3O_5$, 423.1794).



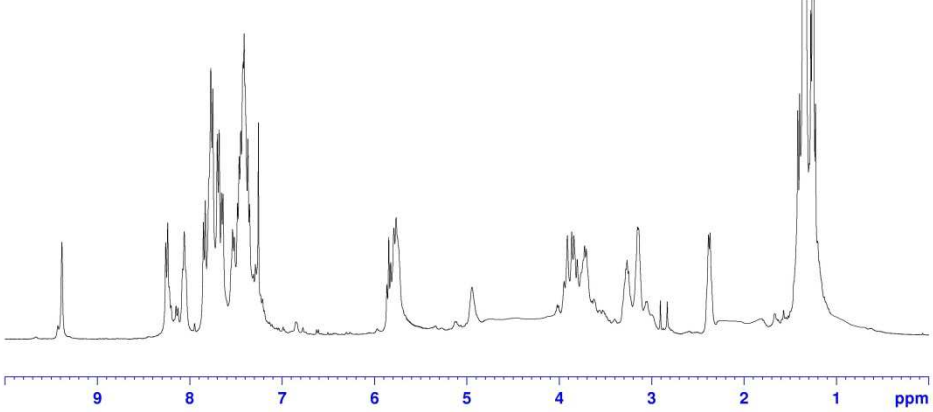
^{1}H-NMR compound O, CD_3OD, 400 MHz



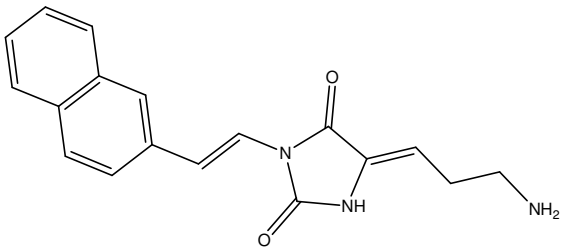
Compound P: Compound **O** (58 mg, 0.137 mmol) was dissolved in ethanol (4 mL) and sodium borohydride (6 mg, 0.156 mmol) was added; the mixture was stirred for 2 h at room temperature and after addition of some drops of water was partitioned between water (20 mL) and ethylacetate (20 mL); the organic phase was evaporated to give **P** (39 mg, 0.091 mmol, 66%); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.25 (1H,m), 8.06 (1H, m), 7.88 – 7.70 (2H, m, overlapped), 7.58-7.32 (3H, m, overlapped), 5.86 (1H, m, overlapped) 5.79 (1H, epimer, m, overlapped), 5.62 (1H, epimer, m, overlapped), 3.98 – 3.64 (4H, m, overlapped) 1.44 (9H, s, Boc methyls); ^{13}C NMR (CD_3OD): *δ* 170.7 (C), 164.7 (C), 155.3 (C, Boc), 127.7 (CH), 127.0 (CH) 124.7 (CH), 122.1 (CH), 110.1 (CH), 80.1 (C, Boc), 70.3 (CH, epimer), 68.2 (CH, epimer) 59.1 (CH_2), 47.6 (CH_2), 44. (CH_2), 27.7 (CH_3, Boc methyls),; HRESIMS *m/z* 425.1948 $[M+Na]^+$ (calcd for $C_{23}H_{27}N_3O_5$, 425.1951).



^{1}H-NMR compound P, $CDCl_3$, 400 MHz



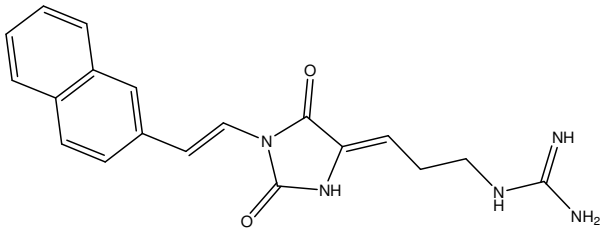
Compound Q: Compound **P** (39 mg, 0.091 mmol) was dissolved in toluene (5 mL) with monohydrated *p*-toluensulfonic acid (70 mg, 0.37 mmol); the mixture was heated at 110 °C for 1 h, evaporated and partitioned between a saturated sodium bicarbonate water solution (100 mL) and chloroform (100 mL); chloroform was dry with sodium sulfate and purified further with silica gel chromatography using a gradient of chloroform and methanol to give **Q** (6 mg, 0.02 mmol, 22%); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.33 (1H, d, *J* = 14.8 Hz), 8.14 (1H, d, *J* = 8.6 Hz), 7.90 (1H, d, *J* = 6.6 Hz), 7.90 (1H, d, *J* = 8.3 Hz), 7.83 (1H, d, *J* = 7.0 Hz), 7.76 (1H, d, *J* = 7.9 Hz), 7.65 – 7.41 (3H, m), 7.18 (1H, d, *J* = 14.5 Hz), 5.79 (1H, bt, *J* = 6.8 Hz), 2.87 (2H, m), 2.44 (2H, bq, *J* = 6.1 Hz); HRESIMS *m/z* 308,1325 $[M+H]^+$ (calcd for $C_{18}H_{17}N_3O_2$, 307,1321).



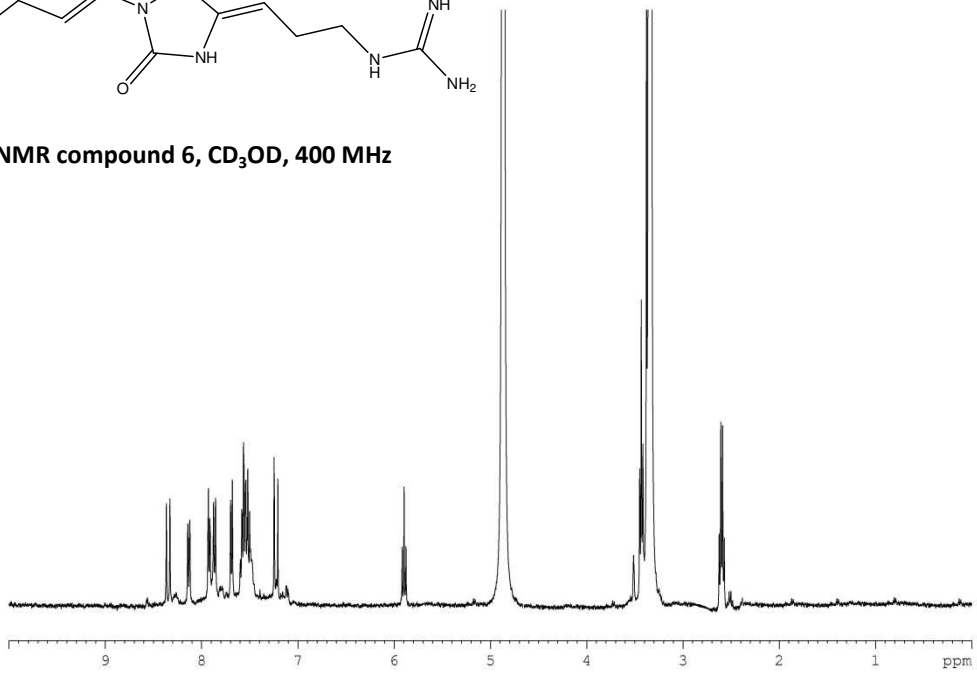
Q

Compound 6: Compound **Q** (8.6 mg, 0.02 mmol) was dissolved in anhydrous DMF (1.0 mL) with DIPEA (6 µL, 0.03 mmol) and 3,5-dimethyl-1-pyrazolylformaminidium nitrate (6 mg, 0.03 mmol); the mixture was stirred overnight at room temperature under argon atmosphere, evaporated, fractioned by silica gel chromatography using a gradient of chloroform and methanol and further purified by HPLC RP-phase (H_2O-TFA 0.1%/MeOH, from 60/40 to 50/50 in 20 minutes, RP-amide analytical column, flow rate 1 mL/min) to give compound **6** (3 mg, 0.008 mmol,

40%); ^{1}H-NMR (400 MHz, CD_3OD): δ 8.34 (1H, d, *J* = 14.2 Hz), 8.13 (1H, d, *J* = 8.5 Hz), 7.92 (1H, d, *J* = 6.6 Hz), 7.86 (1H, d, *J* = 8.3 Hz), 7.68 (1H, d, *J* = 7.0 Hz), 7.61 – 7.44 (3H, m), 7.22 (1H, d, *J* = 14.6 Hz), 5.89 (1H, bt, *J* = 7.8 Hz), 3.42 (2H, bt, *J* = 6.8 Hz), 2.60 (2H, bq, *J* = 7.0); ^{13}C NMR (CD_3OD): *δ* 162.5 (C), 157.2 (C=NH), 153.8 (C), 125.4 (C), 129.4 (CH), 128.9 (CH), 126.7 (CH), 124.5 (CH), 124.0 (CH), 119.9 (CH), 118.3 (CH), 110.1 (CH), 40.9 (CH_2), 27.3 (CH_2); HRESIMS *m/z* 350,3277 $[M+H]^+$ (calcd for $C_{19}H_{19}N_5O_2$, 349,1539).



^{1}H-NMR compound 6, CD_3OD, 400 MHz



7.4 *Pharmacological assay*

Cell line: In these experiments we used the GH4C1 cell line, immortalized cells derived from rat pituitary adenoma; we have previously shown that these cells express the receptor CXCR4 (but not CXCR7) and are sensitive to the proliferative chemokine CXCL12. These cells grow in terrain F10 supplemented with 10% FBS.

BrdU incorporation test: To study the modifications of the proliferative cell following treatment with Parazoantina, we used a quantitative colorimetric assay (BrdU ELISA kit, Roche) which is based on the bromodeoxyuridine (BrdU) ability to incorporate into the DNA, during the replicative phase. We seeded the cells in the basal soil and after 24h were put in a plot of serum free for a period of 72h. After this time, we pre-treated cells 15 'with the test compound at various concentrations (5µM, 1µM, 500nm, 100 nM and 50 nM) and later added CXCL12 25 nM for 24h. About twelve hours before the experimental time we BrdU added to each well to a final concentration of 10 µM. Subsequently the cells were fixed by exposure for 30 minutes to the alcoholic fixative (FixDenat) contained in the kit. After three washes of 5 minutes each with a wash solution provided in the kit, we incubated the cells for three minutes at room temperature, with Lumino / 4-Iodophenol, substrate of the enzyme peroxidase. The chemiluminescence was read in a spectrophotometer.

Cytotoxicity MTT assay: This currency methodical cell vitality by measuring the activity of the mitochondrial dehydrogenase. We plating cells in medium containing serum and treated with the drug at different concentrations. The cells, and after 24,48,72 and 96 h of appropriate treatments, they were incubated 1h at 37 ° C with 3- (4,5-dimethylthiazol-

2-yl) -2,5-diphenyltetrazolium (0.25mg / ml). The mitochondria of viable cells convert the tetrazolium salt (water soluble and yellow) into blue insoluble formazan crystals; the crystals are then solubilized in dimethyl-sulfoxide (DMSO) and the color intensity is quantified using a spectrophotometer (570 nm). The quantity of formazan and thus the intensity of the blue color is proportional to the viability of cells in culture.

Western Blot: The cells deprived of serum for 72h have been pre-treated with the various concentrations Parazoantina (5µM, 1µM, 500nM, 100nM and 50nM) and subsequently stimulated with CXCL12 for 10 '. Subsequently, we washed the cells with PBS and lysed at 4 ° C in a solution containing 20mM Tris-HCL pH 7.4, 140mm NaCl, 2mM EDTA, 10% glycerol, 1% NP-40, 1mM sodium ortovanadato, 1mM phenyl-methyl- sulfonyl-fluoride (PMSF), 10mM NaF and a mixture of protease inhibitors ("Complete", Roche). After centrifugation (10 'at 5000 rpm at 4 ° C) carried out to remove the nuclei, we calculated the amount of protein by the Bradford method (BioRad). Appropriate amount of protein (20 ug) of each sample were resuspended in a reducing buffer (2% SDS, 62.5 mM Tris pH 6.8, 0:01% of Bromophenol Blue, 1:43 mM B-mercaptoethanol and 0.1% glycerol), denatured by boiling, separated by electrophoresis on polyacrylamide gel (10% SDS-PAGE) and transferred via the membrane of polyvinylidene difluoride (PVDF). The membranes were then incubated with the antibody directed against the phosphorylated form of ERK 1/2. Immunoreactivity was detected by a subsequent incubation with secondary antibody linked to peroxidase, followed by reaction of chemiluminescence. The same cell lysates were analyzed for the total expression by the tubulin protein, to check that each treatment contained the same amount of protein.

7.5 *Computational Methods*

Docking studies were performed with AutoDock 4.2[131]. Both the crystallographic structure of CXCR4 (PDB entry 3OE0) and the investigated ligands were processed with AutoDock Tools (ADT) package to merge non polar hydrogens, calculate Gasteiger charges and desolvation parameters, and select all rotatable ligand bonds as flexible. Grids for docking evaluation with a spacing of 0.375 Å and 70x70x50 points, centered in the ligand binding pocket, were generated using the program AutoGrid 4.2 included in Autodock 4.2 distribution. Lamarckian Genetic Algorithm (LGA) was adopted to perform 100 docking runs with the following parameters: 100 individuals in a population with a maximum of 15 million energy evaluations and a maximum of 37000 generations, followed by 300 iterations of Solis and Wets local search. PARAZOANTHINE-CXCR4 complexes were selected on the basis of binding energy and cluster population. The x-ray structure was cleaned of the fusion protein at the intracellular loop 3 (IL3) and completed in its not-resolved loop regions using MODELLER program version 9.15[132] and the resulting complexes, after addition of all hydrogen atoms, underwent energy minimization with SANDER module of Amber14 package[133] using the ff14SB version of AMBER force field for the protein and GAFF for the ligand. Ligand atomic

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charges were obtained with the RESP methodology[134], according to the prescription for molecule parametrization in the GAFF force field. This latter requires an *ab initio* full geometry optimization at the Hartree-Fock level using the STO-3G basis set, followed by a single-point energy calculations on the optimized molecule at the RHF/6-31G* level. *Ab initio* calculations were performed with GAMESS program[135].

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